ABSTRACT

RUNA, FARHANA. Ploidy and Nuclear Condition in *Aspergillus flavus* and their Effect on its Ecology. (Under the direction of Dr. Gary A. Payne and Dr. Ignazio Carbone).

Aspergillus flavus is a saprophyte that infects a wide range of hosts as an opportunistic pathogen. Some isolates produce aflatoxins, toxic and carcinogen mycotoxins that pose health concerns for both animals and humans. Factors driving genetic diversity within the strains of *A. flavus* remain poorly understood. Mycelia and conidia of *Aspergillus flavus* are predominately multinucleate and presumably haploid. But careful characterization of ploidy or nuclear condition in this species has not been reported. The overall objective of our study was to determine if nuclear condition and ploidy contribute to diversity and ecology of *A. flavus*. Nuclear condition was observed by labeling nuclei in different strains with two nuclear fluorescent markers (yellow, EYFP or cyan, ECFP Fluorescent Protein). Fusion of nuclei labeled with different fluorescent markers resulted in mycelia and conidia of three types: expressing only EYFP; expressing only ECFP; or expressing both EYFP+ECFP. Conidia containing nuclei expressing EYFP+ECFP were separated by Fluorescence–Activated Cell Sorting (FACS) to assess heterokaryosis and ploidy. A few conidia were determined to contain one nucleus expressing yellow and cyan fluorescent markers in the same nucleus. Our findings suggest that although conidial are predominantly homokaryotic, a small percentage (below 10%) of the population could be heterokaryotic or diploid. Since the diploids formed in our study were stable, we hypothesized that populations of the fungus may have haploid nuclei, diploid nuclei or both. To address this hypothesis, we obtained putative diploid strains isolated from field studies and assessed the stability of ploidy under stress. To further study ploidy and nuclear condition in *A. flavus,* we exposed the putative

diploid of natural isolates to high temperature (40^0C) and examined the resulting colony sectors. We found that when grown under high temperature, the natural isolates formed two different sectors. Conidia from sectors were analyzed for ploidy by flow cytometry and found to be diploid in one sector. Whereas conidia from the other sector appeared as haploids. Haploid and the diploid cultures showed diversity of growth, nutrient utilization and pathogenicity on an insect model *Galleria mellonella*. The putative diploids (field isolates) were more aggressive than the stress induced diploids. Further, evidence of dikaryons was obtained in another species, *Aspergillus nomius*. We found that conidia of *A. nomius* IC1516 to be multinucleate, but the majority had two nuclei (dikaryons). This strain showed similarity to *A. flavus* in carbon utilization and virulence. Our results provide insight into the nuclear conditions and ploidy of *A. flavus* and their effects on fungal ecology.

Ploidy and Nuclear Condition in *Aspergillus flavus* and their Effect on its Ecology

by
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DEDICATION

To my daughter Rumaysa Radeela Ahmad and my husband Dr. Rais Ahmad.

BIOGRAPHY

Farhana Runa was born in Dhaka, Bangladesh. She obtained BS and MS in Microbiology from the University of Dhaka, Bangladesh working on molecular characterization of *Mycobacterium tuberculosis* and evaluating methods for diagnosis of tuberculosis. She obtained her MS in Plant Pathology in 2007 from the University of Arizona (U of A). During her Masters, she had the privilege of working with filamentous fungi named *Alternaria* and *Ulocladium.* She started her Ph.D. in 2009 at Plant pathology Department, North Carolina State University (NCSU). For the last five years, Farhana has been working on an interesting fungus named *Aspergillus flavus* to explore its nuclear condition. During those years at U of A & NCSU, Farhana has met many fellow students, professors and coworkers that have had a profound effect on his professional life and person life. She will be always grateful to them. Farhana likes to travel and spend time with her family.

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CHAPTER 1

Introduction

1.1 Introduction

Recent research suggests that differences in the nuclei of filamentous fungi contribute to fungal diversity, evolution and emergence of new pathogenic strains (Roper et al, 2011; Roper et al., 2013). Similar to many other filamentous fungi, the mycelium of *Aspergillus flavus* is multinucleate. The conidia of *A. flavus* are also multinucleate. Research on nuclear condition and its significance on *A. flavus* biology have received little attention. Research presented in this dissertation is focused on the nuclear condition and ploidy of *A. flavus* with the goal of achieving a better understanding the effects of nuclei on fungal ecology.

1.2 Current Research Challenge

A. flavus is a major producer of aflatoxin among the species of *Aspergillus* Section *Flavi. A. flavus* commonly infects and contaminates major seed crops such as maize, peanut and cotton (Diener et al., 1987) with aflatoxin, a polyketide derived secondary metabolite. Because aflatoxin is carcinogenic, mutagenic and highly toxic to humans and animals, its content in food and feed is highly regulated in the USA and many other countries. More than 20 ppb of total aflatoxin is unacceptable for human consumption in the United States (Park and Liang, 1993). Concentrations of aflatoxin exceeding 10^6 ppb have been reported (Lee et al., 1990). In addition to aflatoxin, *A. flavus* can produce other mycotoxins including cylopiazonic acid

(CPA), aspertoxins, and aflatrem, which also contaminate agriculture products and are detrimental to animal health when consumed. This fungus is regarded as a serious agricultural risk because it causes losses in the US exceeding several million dollars. Genetic diversity of *A. flavus* and environmental stressors play a key role in the frequency and severity of aflatoxin contamination in crops. Under environmental stress, such as high temperature or drought, contamination of crops with *A. flavus* can be quite extensive. In order to control aflatoxin contamination, it is important to know the factors that contribute to genetic diversity within the strains of *A. flavus* and their adaptation under stress.

1.3 Biology of *Aspergillus flavus*

Aspergillus flavus is cosmopolitan in nature and adapted as a saprophyte and as an opportunistic pathogen for plants, animals (St. Leger et al., 1997), insects (Gupta & Gopal, 2002) and also humans (Hedayati, 2007). As a saprophytic fungus, *A. flavus* predominantly exists in the soil. But as an opportunist pathogen, it can readily colonize a wide variety of living cells and utilize their carbon and nitrogen sources. In soil, the fungus colonizes organic materials and persists as either mycelia or as sclerotia (heavily melanized survival structures) (Ashworth et al, 1969; Horn, 2003). Sclerotia within the organic debris may germinate under suitable environmental conditions (e.g. elevated temperature) to form mycelium or they may support the direct production of conidia (asexual reproductive structures). Conidia can disperse in the air and settle on plant tissues transported by wind or be moved by insects. Germination of conidia followed by colonization and infection of plant tissues results in

aflatoxin production. Conidia produced on plant surfaces are considered as the primary sources of inoculum for secondary infection of plants. In a single growing season, several cycles of primary and secondary infection may occur in plants (Bock et al, 2004; Diener et al, 1987). The prevalence of phenotypically and genotypically diverse strains of *A. flavus* at each phase of disease and life cycle can extensively affect aflatoxin contamination in crops.

1. 4 Phenotypic and Genotypic Diversity

Phenotypes of *A. flavus* vary extensively and are commonly categorized with two morphological types; S and L. S strains make several small (average diameter < 400 μm) sclerotia and relatively few conidia. Isolates of L strains produce large numbers of conidia with fewer, large sclerotia (average diameter > 400 μm) (Cotty, 1989). Aflatoxin production varies within the phenotypically diverse isolates. *Aspergillus flavus* S strain isolates produce relatively more aflatoxin than L strains (Probst et al., 2010; Cotty, 1989). Atoxigenic isolates of L strains have been found in several regions (Probst et al., 2011, Cotty, 1997, Atehnkeng, et al., 2008, Cotty, 1990) of Africa and the US, but there is no report of atoxigenic isolates of S strains. It is unclear why morphologically different strains diverged in their ability to produce different aflatoxin chemotypes, nor why aflatoxin production is not stable in L strains

Phenotypic variations in *A. flavus* populations are attributed to differences among Vegetative Compatibility Groups (VCGs) (Horn et al., 1996). Vegetative or heterokaryon

incompatibility is a self/nonself recognition system whereby compatible hypha cells, having identical alleles of vegetable compatibility, can fuse and form heterokaryons. Nuclei in heterokaryons can occasionally fuse shuffle or exchange genetic material through parasexuality (Leslie, 1993). Parasexuality in *A. flavus* has been described (Leaich and Papa 1975, Papa, 1973) in controlled laboratory conditions but its prevalence in nature is unknown. Further, the sexual state of *A. flavus* has been observed within compatible strains having opposite mating type genes (Horn et al., 2009). *Aspergillus flavus* is a heterothallic fungus having two mating type genes, *MAT1-1* and *MAT1-2* (Ramirez-Prado et al., 2008). Genetic studies and sexual crosses in the laboratory show that populations of *Aspergillus* are able to undergo genetic exchange and recombination (Moore et al., 2009; Olarte et al., 2012). Recombination can also occur by fusion of heterokaryons through parasexuality to make diploid nuclei (Leaich and Papa 1975, Papa 1973). Evidence from previous studies (mentioned above) suggests that nuclei of *A. flavus* can exist as heterokaryons, or recombinant haploids or diploids. We hypothesize that the nuclear condition (whether nuclei are homo or heterokaryons) and ploidy of *A. flavus* could be sources of fungal genetic diversity.

1.5 Multinucleate Nuclear Condition, Ploidy and Fungal Ecology

A single mycelial colony of filamentous fungi can harbor genetically different nuclei. Wild isolates of *Fusarium moniliforme* can contain up 26% genetically diverse nuclei (Sidhu,

1993). Genetically diverse nuclei in mycelium have been shown to contribute to phenotypic plasticity (Jinks, 1952) and virulence (Caten, 1966: Rep et al., 2010: Ma et al., 2010).

Like mycelia, *A. flavus* conidia are multinucleate and predominantly haploid (n). A conidium is an asexual reproductive structure that can survive over a long period of time in extreme adverse environment. Under suitable environmental conditions, *A. nidulans* conidia germinate and differentiate in the following sequential order to form: mycelia, foot cells, stalks (conidiophores), vesicles, metulae, phialides and conidia (Adams et al., 1998). During conidiation nuclei can migrate through all the differentiated structures from mycelia to conidia. Conidia of *A. nidulans* have one nucleus whereas conidia *A. flavus* have more than one nucleus per conidium (Yuill, 1950). Because there is evidence of multiple nuclei in conidia (Yuill, 1950) and cryptic heterokaryosis (Olarte et al., 2012) in *A. flavus*, we hypothesize that heterokaryotic nuclei (haploid) or diploid nuclei could be packaged during conidiation.

Although most filamentous fungi are haploids, diploids are also observed in yeast and some other fungi (Roper, 1952; Holliday, 1961; Ishitani et al., 1956, Nga, et al., 1981, Cogliati et al., 2001 and Ezov et al., 2006). Ploidy of fungi is known to affect morphology (Hickman et al., 2013) and virulence (Lin et al., 2008 & 2009). Ploidy variations in human pathogenic fungi are thought to represent an adaptive mechanism that allows them to survive in hostile host environments (Marrow et al., 2013). The diversity within *A. flavus* and its ubiquitous (soil, living and non-living things) distribution allows the fungus to persist over a wide temperature range and survive under adverse conditions. Changes of climate affect the

density, population structure, and the severity of aflatoxin contamination (Jaime-Garcia and Cotty, 2010, Cotty and Jaime-Garcia, 2007). Contamination of crops with aflatoxin is more pronounced during drought and high temperatures (Diener et al., 1987, Cole et al., 1985, Payne, et al., 1988). Our hypothesis is that environmental stresses (high temperature, drought) can affect the ploidy of the fungus and allow it to adapt under certain environmental conditions.

Many recent studies support the idea that ploidy variation affects the ecological roles of fungi (Sudova et al., 2014) and yeast (Zörgö et al., 2013). The ecological roles of *A. flavus* are diverse. *A. flavus* can utilize complex protein and carbohydrate substrates for their growth and obtain nutrition both as a saprobe and pathogen from different living and non-living sources (St. Leger et al., 1997). Diverse adaptive strategies may allow populations of *A. flavus* to survive under adverse host environments. It is important to understand whether nuclear condition or ploidy of *A. flavus* can affect the fungal ecology in its saprophytic or parasitic stages of its life cycle.

1.6 Current Technology

Recent advances in nuclear labeling with fluorescent marker proteins and high-speed imaging technology allow the observation of nuclei and their migration in mycelia. Heterokaryons also have been detected in several filamentous fungi by labeling the nuclei with two different fluorescent markers (Rech et al., 2007, Ruiz-Roldán et al., 2010 Ishikawa

et al., 2012). Flow cytometry has been used for ploidy analysis of fungi (Hara et al., 2011 and 2002). Further integrated approaches with cell biology and phylogenomics can identify evolutionary and ecological traces of gene transfer in nuclei. In our study, current technologies were utilized to observe nuclear condition and ploidy of *A. flavus.*

1.7 Research Objectives

The overall goal of our study was to understand nuclear condition and ploidy of *A. flavus,* and characterize their effects on fungal ecology. The first objective of our studies was to observe nuclear heterogeneity in conidial populations of *A. flavus*. Two strains of *Aspergillus flavus* were labeled with different nuclear fluorescent marker proteins. Protoplasts prepared from these strains were fused, and regenerated cultures from these fusants were used to determine the number of nuclei within the conidia and whether the nuclei were homokaryotic or heterokaryotic. Further analysis of conidia by flowcytometry determined the ploidy of the conidia.

The second objective was to understand ploidy shifting and its effect on *A. flavus* ecology. Natural isolates of putative diploid strains of *A. flavus* were assessed under elevated temperature to generate sectors. These sectors were examined for ploidy, and conidia from haploid and diploid sectors were compared for their carbon utilization profiles, growth and pathogenicity on an insect model *Galleria mellonella.* Finally, we included another aflatoxin

producing species of *Aspergillus* section *Flavi, A. nomius*, and compared it with *A. flavus* to better understand *A. nomius* nuclear condition and its effects on ecology.

1.8 Application of the Research

Several efforts are underway to reduce aflatoxin contamination. Fungicides have not been effective. The development of resistant hybrids is a robust technology for controlling plant disease, but no commercial hybrids with adequate resistance to aflatoxin contamination are currently available. Currently, application of biological control preparations consisting of non-aflatoxin producing strains of *A. flavus* is considered to be the most promising approach for reducing aflatoxin accumulation. To better understand how non-toxigenic strains compete with toxin producing strains, it is important to develop a comprehensive knowledge of *A. flavus* biology. Understanding the nuclear condition and ploidy will provide insight on *A. flavus* biology and ecology. Knowledge gained on *A. flavus* biology will guide us to identify the exact underlying mechanism of interaction between toxin and non-toxin producing strains in reducing aflatoxin contamination.

1.9 Concluding Remarks

The study is based on investigation of nuclei in *A. flavus* isolates synthesized in the laboratory and collected from natural populations. The implications of our research are

broad. They will facilitate, 1) the design of effective control strategies for mycotoxin contamination and 2) provide a better understanding of the biology of fungi and the factors that impact genetic diversity, secondary metabolism and pathogenicity of fungi.

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CHAPTER 2

Nuclear Heterogeneity in Conidial Populations of *Aspergillus flavus*

ABSTRACT

Aspergillus flavus is a major producer of aflatoxin and an opportunistic pathogen for a wide range of hosts. Understanding genotypic and phenotypic variations within strains of *A. flavus* is important for controlling disease and reducing aflatoxin contamination. *A. flavus* is multinucleate and predominantly haploid (n) and homokaryotic. Although cryptic heterokaryosis may occur in nature, it is unclear how nuclei in *A. flavus* influence genetic heterogeneity and if nuclear condition plays a role in fungal ecology. *A. flavus* mainly reproduces asexually by producing conidia. In order to observe whether the nuclei in conidia are homokaryotic or heterokaryotic, we labeled nuclei of *Aspergillus flavus* using two different nuclear localized fluorescent reporters (Christine et al., 2007). The reporter constructs (pYH2A, pCH2B), encode histones HH2A and HH2B fused at the C terminus with either yellow (EYFP) or cyan (ECFP) fluorescent proteins respectively. The constructs were transformed into the double auxotrophic strain AFC-1 (-*pyrG, -argD*) to generate a strain containing each reporter construct. Taking advantage of the nutritional requirement for each strain we were able to generated fusants between FR36 (*-argD)* expressing yellow fluorescence, and FR46 (-*pyr4*) expressing cyan fluorescence. Conidia from the fusants between FR36 and FR46 showed three types of fluorescence, only EYFP, only ECFP or both EYFP+ECFP. Conidia containing nuclei expressing EYFP+ECFP were separated by Fluorescence–Activated Cell Sorting (FACS) and were found to contain both yellow and

cyan fluorescent markers in the same nucleus. Further characterization of conidia having only one nucleus but expressing both EYFP+ECFP confirmed that they were diploid (2n). Our findings suggest that *A. flavus* maintains nuclear heterogeneity in conidial populations.

2.1 Introduction

Aspergillus flavus is a soil saprobe that can be pathogenic on a diverse array of plant hosts and animals. It commonly infects and contaminates major seed crops such as maize, peanut and cottonseed with aflatoxin, which is highly toxic to humans and animals (Bayman et al., 1993, Klich et al., 2007, Probst et al., Schroeder et al., 1973). In addition, *A. flavus* is the second leading cause for Invasive Aspergellosis (IA), which can be fatal in immunocompromised patients (Hedayati et al., 2007). Phenotypic and genotypic variations are common within strains of *A. flavus*, and studies (Atehnkeng et al., 2008, Cotty et al., 2007, Jaima-Garcia et al., 2006) have shown that genetic variation within *A. flavus* populations is correlated with the frequency and severity of aflatoxin contamination in crops.

Much of the phenotypic variation in *A. flavus* populations, including morphology and mycotoxin production, is due to differences between Vegetative Compatibility Groups (VCGs) (Horn et al., 1996). Vegetative or heterokaryon incompatibility is a self/nonself recognition system that occurs within genotypically diverse *A. flavus* isolates. This recognition system largely prevents nuclear exchange between strains in different VCG groups. In contrast, individual isolates having identical alleles at the loci that govern vegetable compatibility can undergo hyphal fusion, exchange nuclei, and form a stable

heterokaryon. In a heterokaryotic cell, nuclei with different genotypes share common cytoplasm and can occasionally fuse, shuffle or exchange genetic material through parasexuality (Leslie, 1993). Parasexuality in *A. flavus* has not been demonstrated in nature, but it is observed under controlled laboratory conditions (Leaich and Papa 1975, Papa, 1973). Although not common in *A. flavus*, sexual reproduction has been observed within strains of *A. flavus* (Horn et al. 2009). *Aspergillus flavus* is a heterothallic fungus having two mating type genes, *MAT1-1* and *MAT1-2* (Ramirez-Prado et al. 2008). Results from genetic analyses (Moore et al., 2009) and sexual crosses in the laboratory (Olarte et al., 2012) have shown that *A. flavus is* able to undergo genetic exchange and recombination. Because this fungus can undergo sexual and parasexual recombination events, we assume that at least in certain stages of its life cycle nuclei may be heterokaryotic, recombinant homokaryotic, haploid, or diploid. Therefore, nuclear condition and ploidy may drive genetic variations within the strains and influence the phenotypic and genotypic characteristics of *A. flavus*.

Several molecular genetic tools have been applied to better understand genetic diversity of *A. flavus.* Detection of microsatellites markers (Grubisha and Cotty, 2009 and 2010, Wang et al., 2012), chromosomal karyotyping (Keller et al., 1992), nucleotide sequence data (Ehrlich, et al, 2007; Geiser et al, 1998) and genetic characterization of aflatoxin cluster genes (Chang et al., 2005, Payne et al., 2006) have confirmed the existence of genetic diversity within strains of *A. flavus.* To our knowledge, we are unaware of any study, however, that has used fluorescently labeled nuclei to investigate the nuclear condition in *A. flavus*.

Like many filamentous fungi, the nuclear condition of *A. flavus* is predominantly homokaryotic and haploid. In contrast, mycelia and conidia of *A. flavus* are multinucleate (Yuill, 1950). Nuclei of *Aspergillus* mycelium migrate through the phialides into the conidia during conidiogenesis. Nuclear number in conidia and among conidia with a conidial chain of *Aspergillus* may vary based on nuclear migration. *Aspergillus nidulans* makes conidia with only one nucleus, whereas *A. oryzae* have multinucleate conidia (Ishi et al., 2005). Foundin *et al*. (1980) showed that if a phialide of *A. flavus* contains two nuclei, conidia within the conidial chain originating from the same phialide have 2 nuclei, indicating that the phialide has some control over nuclear number in the conidium. Since there is evidence of multiple nuclei in conidia (Yuill, 1950) and cryptic heterokaryosis (Olarte et al., 2012) in *A. flavus*, we hypothesized that heterokaryotic nuclei (haploid) or diploid nuclei could be packaged during conidiation.

Our hypothesis was that there are heterokaryotic nuclei in conidial populations of *A. flavus* that may serve as a potential source of variability for the fungus. The objectives of this study were to determine nuclear number, type of nuclei (such as homo or heterokaryon), and ploidy of nuclei in conidial populations of *A. flavus*. The research addressed three questions: 1) what is the predominant number of nuclei in conidial populations? 2) Are conidia predominantly homogeneous or heterogeneous with respect to nuclear type? 3) Are conidia always haploid? To conduct the research we developed two strains, one (FR36) strain having nuclei labeled with yellow fluorescence protein (EYFP) and the other (FR46) having nuclei labeled with cyan fluorescence protein (ECFP). Protoplasts of these two strains were fused to incorporate the two differently labeled nuclei into the same cell. Conidia produced by

regenerated colonies of the fused protoplasts were examined to determine the distribution of the reporter constructs with the conidia and thus the nuclear condition of the conidia.

2.2 Materials and Methods

2.2.1 Strains and Culture Conditions

Aspergillus flavus strains used in this study (Table 2.1) were grown at 30^0C and maintained on culture media. PDA (Potato Dextrose Agar, Difco), PDAU (PDA plus 1.12 g/L uracil), PDB (Potato Dextrose Broth, Difco), PDBU (PDB plus 1.12 g/L uracil), MM (Czapeck-Dox Broth, Difco), MMU (MM plus 1.12 g/L uracil), MMA (MM plus 0.262g/L arginine), MLS (MM plus 0.4 M $(NH4)_2SO_4$ and 1% agar), MLSU (MLS plus 1.12 g/L uracil), MLSA (MLS plus 0.262g/L arginine) and MLSAU (MLS plus 0.262g/L arginine plus 1.12 g/L uracil) were used for growing the cultures.

2.2.2 Plasmids and DNA Constructs

Two recombinant plasmids, pYH2A and pCH2B developed by Christine et al., 2007 were used in our study. These two plasmids were used for labeling the nuclei of *A. flavus*. Plasmids pYH2A and pCH2B encode histone HH2A or HH2B, which are fused at their C terminus with either the yellow (EYFP) or cyan (ECFP) fluorescent protein. Two DNA constructs pBSK-*pyr4* (He et al., 2007) and Topo-*argD* (Georgianna et al., 2010) were used

for co-transformation. Plasmids and DNA constructs were maintained in *Escherichia coli* strain DH5a and purified with Qiagen DNA miniprep columns (Qiagen, Hilden, Germany).

2.2.3 Labeling of *A. flavus* Nuclei

Two different co-transformations were performed to label the nuclei in *A. flavus.* We used AFC-1 (Table 2.1), which was grown for 7 days on PDAU. Conidia were harvested from 7 days-old cultures using 0.05% Triton-X 100. Protoplasts were obtained from the harvested conidia and co-transformations were performed using the protocol described by He et al. (2007). Briefly, one milliliter of a conidial suspension containing 10^6spores/mL was transferred into 100mL PDBU. The cultures were grown at 30°C with 200 rpm for 10 hours. The cultures were centrifuged and growth medium was removed. The following steps were followed according to the previous protocol (He et al., 2007): addition of cell wall lysing enzymes; removal of the cell debris and collection of the protoplast by washing with STC buffer. A hemocytometer was used to count protoplasts. Protoplasts were diluted to 10^7/mL per ml. 100-µL aliquots of protoplasts (10^7 protoplasts/ml) in STC buffer (1.2 M sorbitol, 10 mM Tris-HCl -pH 7.5, and 50 mM CaCl2) at a concentration of 10^7 protoplasts/mL were transferred into 1.5 mL tubes and kept on ice for 20 minutes. 1-3 µg of pYH2A and pBSK-*pyr4* DNA were added to each tube and mixed by tapping. One of the tubes was used as a negative control (no DNA). After incubation on ice for 20 minutes, 1 mL of a 50% PEG solution (50g PEG, Mr. 4, 000), 1 mL of 1 M Tris-HCl (pH 8) and 1 mL of 1 M CaCl2 (in a final volume of 100 mL) were added, mixed and incubated at room temperature for 20

minutes. 100–300 µl of each transformation was plated on the surface of regeneration MLSA medium. The plates were incubated at 37°C for 3-7 days. Each putative transformant originating from a single colony was designated as AFC (+*pyr4-argD).* Conidia from each of the transformants were observed under a fluorescence microscope (Zeiss Axioskop 2 plus, Germany) with Zeiss filter set 46 (excitation BP 500/26, beam splitter FT4515 and emission BP 535/30) for EYFP. The strain that carries pYH2A and pBSK-*pyr4* requires arginine and expresses HH2A-EYFP. The strain was grown on MLSA and was designated as FR36. Using the same protocol, an *A. flavus* strain expressing ECFP was constructed. Co-transformation was performed by adding 1-3 µg of each pCH2B and Topo-*argD* plasmid DNA to AFC-1 protoplasts. The putative transformants were selected based on their growth on MLSU and were designated as AFC (-*pyr4+argD).* Conidia from each of the transformants were observed under a fluorescence microscope with Zeiss filter set 47 (excitation BP 436/20, beam splitter FT455 and emission BP 480/40) for ECFP. The strain that carries pCH2B and Topo-*argD* requires uracil and expresses ECFP. This strain was designated FR46.

2.2.4 Generation of Fusants

Protoplasts from the two auxotrophs, FR36 expressing EYFP (requiring arginine) and FR46 expressing ECFP (requiring uracil), were isolated as described by He et al., (2007). The Co-transformation protocol is described briefly. One hundred microliter (100 µL) of protoplast (2.5×10^7 protoplasts /mL) from each strain were mixed with 1 mL of 50% PEG in 1.5 mL tubes and were incubated at 30^oC for 33 minutes. 100 –300 µL protoplast in PEG was plated

on the surface of regeneration MLS medium. In addition, 100-300 µL of protoplasts from parental strains was also plated on MLS, MLSA and MLSU plates. All plates were incubated at 37°C for 3-7 days. Fusant colonies grown on MLS medium were transferred again to MLS medium and incubated for 7 days at 30^{o}C. Each putative fusant, originating from a single colony, was designated as fusant 1, 2, 3 etc. Mycelia and conidia from each fusant were observed under a fluorescence microscope using both Zeiss filter sets 46 and 47 for EYFP and ECFP.

2.2.5 DAPI Staining

Conidia were harvested with 0.05% Triton X-100 and centrifuged at 3000 rpm for 5 minutes. After removing the supernatant, the conidia were washed twice with 1XPBS buffer. The pellet was re-suspended with 200-µL DAPI stain solution. We prepared DAPI stain solution with some modifications of the protocol from Nichols et al. (Nichols et al., 2004). DAPI stain solution was prepared by adding 5µl of 10-mg/ml stocks DAPI (4', 6'-diamino-2-phenylinodole, Sigma-Aldrich, USA) plus 100-µL antifade PPD (P-phenylenediamine, Sigma-Aldrich, USA, stock solution 1ng/µL) plus 900 µL of 70% glycerol. Conidia were incubated at room temperature in the dark for 20-30 minutes. Stained conidia were washed twice with 1XPBS buffer and then twice with water. The conidia were observed under a fluorescence microscope (Zeiss Axioskop 2 plus, Germany) using a DAPI filter set (excitation 359 and emission 461).

2.2.6 Light and Fluorescence Microscopy

Fluorescence and light microscopy were performed using a fluorescence microscope (Zeiss Axioskop 2 plus, Germany) with HBO100 Hg or XBO 75 xenon lamp for fluorescence excitation. Fluorescence was observed using Chroma filter set 46 (excitation BP 500/26, beam splitter FT515 and emission BP 535/30) and 47 (excitation BP 436/20, beam splitter FT455 and emission BP 480/40) (Chroma Technology Crop. USA). Images were captured with a QImaging camera (Software Q Capture Suite 2.98.2.) and processed in ImageJ1.46r (NIH, USA) software.

2.2.7 Sorting of Conidia by FACS

Parental strain, FR36 was grown on MLSA and FR46 was grown and MLSU media for 7 days. Three fusants (FU10-2, FU11-2 and FU14-2) were grown on MLS. Conidia were harvested with 0.05% Triton X-100 and centrifuged at 3000 rpm for 5 minutes. Conidia were washed twice with PBS buffer, resuspended in PBS buffer and adjusted to 10^7 conidia per mL. Two to four milliliters of a conidial suspension were used to sort the conidia in a Becton-Dickinson FACSCalibur. ECFP was excited with a 405 nm laser and emissions were collected with a photomultiplier tube, PMT with a 450/65 nm filter. EYFP was excited with a 488 nm laser and emissions were collected with a photomultiplier tube, PMT with a 530/40 filter. Summit and FlowJo software were used for data analysis and for counting sorted conidia. FACS sorted conidia were collected in 10 mL round–bottom (BD Falcon™) tubes.

Sorted conidia were washed twice with PBS buffer and then washed twice with liquid minimal medium (MM) supplemented with ampicillin (50 μg/mL) before growing on media.

2.2.8 PI Staining and Flow Cytometry

Propidium Iodide (PI) staining was as described for conidia of *A. oryzae* (Hara et al., 2011 and 2002). We used the same protocol with some modification. Strains of *A. flavus* were grown on PDA or PDAU for 7 days. The conidia were harvested with 0.01% Tween 80 (0.01% Tween 80 + 1M NaCl) and were poured through Miracloth (Calbiochem, USA). The conidial suspension was fixed with 70% ethanol at 4^{o}C for 30 minutes. Conidia were collected by centrifugation at 3000 rpm for 5 minutes and washed twice with 0.01% Tween 80. Conidia were suspended in TE buffer (1M Tris.Cl, pH 8.0+ 0.5M EDTA) and incubated at 37^{o}C in the presence of RNase (1mg/mL) for 2 hours. Conidia were washed twice with 0.01% Tween 80 and stained with 25 μg/mL Propidium Iodide (PI, Sigma-Aldrich, USA, P4170) at room temperature for 30 minutes. Stained conidia were analyzed on the FL1 channel by a flow cytometer (Becton–Dickinson FACscan). Data were analyzed by Cell Quest Pro software.

2.3 Results

2.3.1 *Aspergillus flavus* Conidia have Predominately One or Two Nuclei

To observe nuclei in mycelia and conidia the nuclei in two strains were labeled with different fluorescent markers. AFC-1 (*-pyrG, -argD)* was co-transformed with *pyr4* and pYH2A (HH2A-EYFP) and uracil prototrophs were selected to examine EYFP expression in nuclei. One strain, FR 36, was selected for further study. Similarly, *argD* and pCH2B (HH2B-ECFP) containing plamids were transformed into AFC-1 and selected for arginine prototrophs and examined for ECFP expression. One strain FR 46 was selected for further study. The co-transformation efficiency was 0.5 transformants μg-1 DNA. EYFP and ECFP expression was observed from mycelia and conidia from an individual transformant colony by fluorescence microscopy (Zeiss, Axioskop 2 plus). Mycelia were multinucleate whereas conidia from the transformants had predominately one or two nuclei (Fig. 2.1& 2.2).

We verified that the fluorescent markers were associated with nuclei in conidia by measuring the correspondence between fluorescent and DAPI staining of nuclei. In all cases, DAPI staining co-localized with EYFP and ECFP fluorescence (Fig. 2.2A) confirming the power of these reporters to tag nuclei. Through the use of DAPI staining we also determined the frequency of nuclei from *A. flavus* strains 3357, A270, and AF36 (a biocontrol strain), in which nuclei were not labeled with any fluorescent markers. Similar to the transformed strains, these strains also had predominantly one or two nuclei per conidium (Fig. 2.2), indicating that the nuclear number per conidium in our experimental strains was similar to

that found in strains used in other studies. We conclude from these studies that strains of *A. flavus* contain one to two nuclei per conidium.

2.3.2 Nuclear Heterogeneity in Mycelia and Conidia

Our second objective was to determine if conidia of *A. flavus* are predominantly homogeneous or heterogeneous with respect to nuclear type. The two strains described above, FR 36 and FR 46, allowed us to determine if two different nuclei, each with a different fluorescent marker (EYFP or ECFP) could exist in the same conidium. To address this question we fused protoplasts of FR36 and FR46 and regenerated mycelia on minimal medium. Protoplasts that regenerated on minimal medium (MM, Czapek Dox Agar medium) were assumed to be complemented for the auxotrophic mutations of each strain. We observed protoplast fusion efficiency of 1.43 fusants μg^{-1} DNA. No protoplasts from FR36 or FR46 regenerated on minimal medium.

When colonies were formed on minimal medium, mycelia were taken from 5 different spots of an individual fusant colony. After 3-5 days the mycelia were observed under the fluorescence microscope and found that the fusant colonies showed heterogeneity with nuclei expressing EYFP or ECFP or expressing EYFP+ECFP in mycelia (Fig. 2.3A). Further, spores were collected from the edge of the growing colonies of the fusants and observed under the fluorescence microscope. We were able to detect conidia expressing only ECFP, only EYFP or merged fluorescence of EYFP and ECFP (Fig. 2.3B). Most of the

conidia within a conidial chain had one or two nuclei; occasionally more than two nuclei were observed (Fig. 2.3C).

To determine the percentages of conidia having each of the three fluorescent phenotypes we detected the percentages of conidia having each type of nuclear fluorescent marker by fluorescence microscopy and FACS (Fluorescence Activating Cell Sorting). FACS allowed us to quantify a large number (Thousands) of conidia. After assessing the reliability of FACS for sorting the fluorescence phenotypes, we examined the conidial populations from three fusants (FU10-2, FU 11-2 and FU14-2) by FACS. A bivariate dot plot for fluorescent markers of conidia having EYFP, ECFP and EYFP+ECFP is shown in (Fig. 2.4A). Fluorescence microscopy and FACS data revealed that the relative frequency of the three nuclear fluorescent markers in conidial populations was not equal (Fig. 2.4B). The majority of conidia contained nuclei expressing either EYFP or ECFP in three different fusants (Fig. 2.4B). A small percentage of conidia (below 10%) had nuclei with merged fluorescence (expressing EYFP+ECFP). Our results suggest that nuclei in conidia are predominantly homokaryotic having either EYFP or ECFP. But a small percentage of nuclei (below 10%) in conidia were chimeric representing merged fluorescence of EYFP and ECFP. Because neither of the parental strains (FR36, FR46) could grow on MM, we expected the conidia with merged fluorescence of EYFP and ECFP to be either heterokaryotic or diploid. We hypothesized that conidia with the merged fluorescence nuclei could exist in one of two conditions: (1) as heterokaryons, in which two different fluorescent proteins fused in nuclei; or (2) as diploids where two different haploid nuclei (with different fluorescent markers) had fused.

2.3.3 A Conidium of a Fusant is: (1) Heterokaryon or (2) Diploid

To address the hypothesis that conidia having both fluorescent markers EYFP+ECFP could be either heterokaryons or diploids, conidia from fusant 11-2 (FU11-2) were sorted by FACS. A bivariate dot plot (Fig. 2.4A) showed three sub-populations (sub-populations 1, 2 and 3) of conidial populations of the fusant 11-2. Conidia from each of sub-populations 1, 2 and 3 (Fig. 2.4A) were further examined using a fluorescence microscope. The majority of the conidia in sub-populaton 3 had nuclei expressing both EYFP and ECFP (Fig. 2.4C and 2.4D). A few conidia from this population had nuclei expressing only EYFP or only ECFP (Fig. 2.4D). Two hundred FACS sorted conidia from sub-populations 1, 2, 3 were cultured on MLSA, MLSU and MLS media respectively. We found that conidia from sub-population 1 produced mycelia and conidia only expressing only EYFP in their nuclei. We also observed only ECFP fluorescence in mycelia and conidia of colonies grown on MLSU from subpopulation 2. Thus, we confirmed that the majority of conidia had homokaryotic nuclei and retained EYFP or ECFP after regeneration. In addition, we regenerated 200 FACS - sorted conidia from sub-population 3 on MLS medium.

When conidia with merged fluorescence were FASC-sorted and then used to regenerate new fungal colonies, the majority of the conidia from these colonies expressed either EYFP or ECFP, but a few retained both EYFP and ECFP. We counted the number of conidia having EYFP, ECFP and EYFP+ECFP by fluorescence microscopy from several colonies. We observed that the number of conidia with EYFP, ECFP and EYFP+ECFP were not equally distributed. We observed that two colonies predominantly maintained EYFP expressing conidia (67% and 56%), whereas another colony retained ECFP expressing

conidia (52%) (Fig. 2.5A). Our results suggest that conidial populations of each colony are heterogeneous. Conidia not only maintained each parental type nuclei (EYFP or ECFP) but also a small percentage of chimeric nuclei, which are heterokaryotic. Further, we observed that a few colonies (1%) grown on MLS contained conidia having only one nucleus with both nuclear fluorescent markers. We used DAPI staining to confirm if fluorescence of DAPI and both yellow and cyan (EYFP+ECFP) could overlap in the same nucleus. We observed that DAPI co-localized with the nuclei that had both nuclear markers (EYFP+ECFP) and showed merged fluorescence of EYFP+ECFP (Fig. 2.5B). We found that uninucleate conidia having EYFP+ECFP appeared to be diploid in which both fluorescent markers were maintained during conidiation (Fig. 2.5C). The diploid strains, designated D1 and D2, originated from a single conidium having a nucleus with merged fluorescence (EYFP +ECFP). In summary, our findings demonstrate that when conidia having EYFP+ECFP were regenerated on MLS media, few colonies (1%) were diploids, but most of the colonies were heterokaryons.

To recover parental type nuclei from putative heterokaryons or diploids, mycelia from colonies were transferred onto MMA, MMU and MMAU media with benomyl (Fig. 6A). Benomyl was used to induce haploidization in growing colonies of diploids (Morpurgo et al., 1979). We observed white sectors of mycelia in colonies on MMA+benomyl and MMU + benomyl (Fig. 2.6A, top). We observed nuclei in the sectors of mycelia under the fluorescence microscope and found that mycelia of white sectors on MMA had nuclei expressing only EYFP. On the other hand, white sectors of mycelia grown on MMU had nuclei expressing only ECFP. To recover the parental type haploid, we also transferred

mycelia from a diploid (D2-2) colony to plates containing MMAU plus benomyl (Fig. 2 6A, top). We observed white sectors where mycelia had nuclei expressing only EYFP and only ECFP (Fig. 2.6B). Our data suggests that parental type nuclei can be recovered from diploids.

2.3.4 Confirmation of Diploid Conidia by Flow Cytometry

To determine whether uninucleate conidia expressing both fluorescent markers are diploid, we stained the conidia from putative diploid D2-2 with PI staining and analyzed them by flow cytometry. FL histograms of the parental strain showed two major peaks, whereas FL histograms for the diploid D2-2 showed only one major peak (Fig. 2.7A). The one major peak in the D2-2 FL histogram showed that the quantity of chromosomal DNA per conidium was doubled in D2-2 compared with the parental haploids. We also observed nuclei of conidia from the parental haploids (FR36 and FR46) and the diploid D2-2 using DAPI staining. We found that the parental strains had conidia with one nucleus or two nuclei, but the diploid D2-2 had conidia mostly with one nucleus (Fig. 2.7B). Thereby it was confirmed that D2-2 was a diploid and was formed from the fusion of two haploid nuclei.

Further, we confirmed that conidial populations from a heterokaryon did not show the typical FL peaks in the histogram like a FL peak of a diploid. We used conidia stained with PI from a colony of heterokaryons (fusant 11-2) and compared with PI stained conidia of the strain *A. flavus* 3357. The multiple peaks of the FL histogram of 3357 and the heterokaryon demonstrated that the both were haploids. Our results indicate that conidia having more than

one nucleus are predominately haploid and heterokaryotic, whereas diploid conidia have predominantly only one nucleus.

2.3.5 Heterokaryosis and Diploidy in natural and Laboratory Synthesized Isolates of *A. flavus*

We investigated the conidial populations from several natural and laboratory isolates of *A. flavus* to obtain evidence for diploidy. Isolate IC5236 was used as a haploid control and IC4761 was used as a diploid control. We observed two major peaks in the FL histogram for the haploid IC5236. But the FL histogram of the diploid IC4761 showed only one peak. We observed that the FL histograms of most strains from natural isolates showed two major peaks. Figure 2.8A showed the FL histogram of *A. flavus* IC244. The peaks of the FL histogram of IC244 suggested that this strain was haploid. But the FL histogram of IC4050 (Fig. 2.8A) had one major peak (with other minor peaks) and thus suggested this strain was a putative diploid.

To investigate whether conidia of natural isolates have diploids, we grew conidia from several isolates on both PDA and PDAU with and without benomyl. We observed sectors on colonies of natural isolates growing on PDA plus benomyl and PDAU with benomyl (Fig. 2.8B). We found sectors on colonies of *A. flavus* isolates IC244, IC5236, IC4761, IC5040 (Fig. 2.8B). Sector formation with benomyl supports the idea that nuclei of *A. flavus* could harbor heterkaryotic or diploid nuclei within conidial populations.

2.4 Discussion

Our hypothesis was that not all conidia within the conidial population of *A. flavus* are homogeneous. To test this hypothesis, we: 1) examined the predominant number of nuclei in conidia; 2) observed whether conidia were predominantly homokaryotic or heterokaryotic; and 3) analyzed whether diploids could exist in conidial populations of *A. flavus*

To visualize nuclei in conidia, we tagged nuclei with the fluorescent proteins EYFP (yellow) or ECFP (cyan). Expression of these proteins was stable through repeated generations, and we confirmed that the fluorescent proteins (HH2A-EYFP, HH2B-ECFP) were reliable reporters as the fluorescence of the reporters and the DAPI staining co-localized in the same nuclei of *A. flavus* (Fig. 2.2A). Fluorescence and DAPI staining of the reporter strains, the aflatoxin producing strains (NRRL335, A270 FR36, FR46), and the non-aflatoxin producing biological control strain AF36 showed conidia of *A. flavus* contain one or two nuclei, with a greater tendency to harbor two nuclei (Fig 2.2B). Our results are similar to those found in other species of *Aspergillus*. The frequency of conidia with two nuclei has been reported for *A. oryzae* (Maruyama et al., 2001), a phylogenetically closely related species to *A. flavus* in *Aspergillus* section *Flavi.* Yuill (1950) first reported that *Aspergillus flavus* conidia had 1-3 nuclei, but did not report the frequency of conidia having one, two or three nuclei.

We also found that *A. flavus* can harbor genetically different nuclei, and have diploid and haploid nuclei. By fusing protoplasts of FR36 expressing EYFP with protoplast FR46 expressing ECFP we generated fusants whose mycelium produced conidia that expressed

only EYFP (shown green in Fig. 2.3), only ECFP (shown blue in Fig. 2.3) or both EYFP+ECFP (shown cyan, merged fluorescence of green and blue in Fig. 2. 3). Analysis of the phenotype of the conidia from the fusants revealed that conidia were heterogeneous for nuclear fluorescent markers. The frequency of conidia having nuclei expressed either EYFP or ECFP was higher than that for conidia having nuclei expressed both yellow and cyan fluorescence proteins (EYFP+ECFP) (Fig. 2.4B). Our results provided evidence of heterogeneity where the relative frequency of homokaryotic nuclei (either EYFP or ECFP) is higher than the heterokaryotic nuclei (merged fluorescence of cyan and yellow, EYFP+ECFP). The underlying mechanism controlling the frequency of homo or heterokaryons in conidial population is unknown. Foundin et al. (1980) reported that nuclear number in conidia of *A. flavus* depends on the number of nuclei in phialide. Perhaps phialides control nuclear number and thereby control the homokaryotic and heterokaryotic nuclei in conidia during conidiogenesis, but our studies did not address the possibility.

To address whether conidia expressing both fluorescent markers (EYFP+ECFP) were heterokaryotic or diploid, we further examined conidial populations of the fusant 11-2 by FACS. Two hundred FACS sorted conidia expressing EYFP+ECFP were grown on MLS media to examine the stability of this phenotype. We found that conidia from this one phenotype gave rise to conidia having with all three phenotypes previously observed (Fig, 2.5A). Those conidia expressing EYFP+ECFP were infrequent. These data suggest that nuclear ratio is not balanced in conidial populations. Imbalance in nuclear ratio has been demonstrated in some filamentous fungi (James et al., 2008, Pitchaimani 2003 and Ramsdale 1993). The study by James et al., (2008) showed that the nuclear ratio in mycelia and conidia

varied and competition of nuclei during germination, nuclear migration in mycelia, genotype of nuclei, epigenetic mechanism or environmental conditions could control the nuclear disproportion in heterokaryons. To understand the mechanism of nuclear heterogeneity and imbalanced nuclear ratios in conidial populations, we observed the germination of multinucleate conidia and found that multinucleate conidia sometimes germinated with more than one germ tube. In our study, conidia having both fluorescent markers (EYFP+ECFP) were regenerated on MLS (minimal medium). We hypothesize that genetically different nuclei could compete in germ tube formation and or migration in mycelia. Nuclei expressing EYFP or EYFP could compete for migration during colony formation.

We found only two putative diploid colonies from the 200 colonies regenerated from FACS sorted conidia. Conidia from the two colonies had only one nucleus per conidium and both fluorescent markers (EYFP+ECFP). Our results showed that conidia observed from the fusants were predominantly homokaryotic but a small percentage (1-9%) of conidia could be heterokaryotic or diploid.

To further address if diploid conidia can exist in *A. flavus* conidial populations we used flow cytometry to examine isolate (D2-2) which had uninucleate conidia expressing both fluorescent markers. The FL histogram of D2-2 showed predominantly one major peak (Fig. 2.7) suggested that the diploid had predominantly uninicleate conidia. The previous study (Leaich and Papa 1975) demonstrated that the majority of conidia of a diploid are uninucleate. Further, the FL histogram indicated that the DNA content per conidium of the diploid to be twice than that of the parental haploid strains (Fig. 2.7). Similar findings have

been observed for ploidy of *A. oryzae* (Hara et al., 2011 and 2002). A typical FL histogram for *A. oryzae* RIB40 haploid strain usually has 4 major peaks. But the FL histogram of *A. oryzae* diploid has an even number of nuclear peaks (2 and 4 nuclei). The diploid strain of RIB40 (Hara et al., 2011 and 2002) was found to have double the DNA of the haploid strain. It is known that *A. flavus* diploid conidia have predominantly one nucleus per conidium, but haploid conidia have multiple nuclei (Leaich and Papa, 1975). In our study we found that diploid conidia had predominantly one nucleus and the quantity of total DNA content of a diploid conidium was twice that of the parental haploids. Our results argue that isolate D2-2 is a stable diploid. The diploid D2-2 was originally recovered from the regeneration of FACS sorted fusant conidia, which had nuclei with both fluorescent markers (EYFP+ECFP) (sub-population 3, Fig. 2.4). This provides evidence that diploid conidia can exist in *A. flavus* conidial populations.

Diploid conidia are found in natural isolates of *A. nidulans* (Upshall 1981) and *A. niger* (NGA et al., 1975). Although *A. flavus* can undergo sexual and parasexual reproduction under laboratory conditions, no evidence of diploidy has been reported in natural isolates. We investigated whether natural isolates of *A. flavus* have diploid conidia in conidial populations. We found that FL histograms for most of the isolates from natural and some lab synthesized isolates had two major peaks which represented the typical FL histogram of the haploids (Fig. 2.8). We hypothesized the frequency of diploids (2n) could be low in conidial populations or they could mix with haploids (n+n). For the isolate, IC4050, we observed predominantly one major peak (with some minor peaks) (Fig. 2.8) in the FL histogram. We

hypothesized that some of the isolates in our investigations had putative diploids in conidial populations.

To better understand whether laboratory synthesized and natural isolates could harbor diploids, the isolates were grown on different type of media MMAU, MMA, and MMU with benomyl (Fig. 2.6) and PDAU, PDAU with benomyl (2.8). Most of the isolates made frequently sectors (Fig. 2.6 and 2.8) in the colonies. We showed that parental type nuclei (either EYFP or ECFP) were recovered from the heterokaryon of a fusant (11-2) and the diploid (D2-2). Sectoring was also observed in other isolates of haploid, diploid and putative diploid isolates (Fig. 2.8). The prevalence of sector formation in several isolates indicates that natural or lab synthesized isolates may harbor diploids in mycelia and conidia.

2.5 Conclusion

Our findings suggest that conidia of *A. flavus* have predominately one or two nuclei. Although the majority of conidia are homokaryotic, a small percentage of conidia can harbor two genetically dissimilar nuclei (heterokaryon) or diploid nuclei. The nuclear system of *A. flavus* is complex where heterogeneous nuclei and ploidy levels of conidia could contribute to fungal genetic diversity. We have demonstrated that strains constructed in which nuclei were tagged with two different fluorescent markers; EYFP and ECFP are stable and useful to observe the nuclei in subsequent analyses. Protoplast fusion of the strains labeled with different fluorescent markers is a potential system to better understand the nuclear condition

of *A. flavus*. Our experimental results also suggest that FACS is a powerful tool to detect and sort homokaryons and heterokaryons from conidial populations. Further, we found that diploids and haploids synthesized in the laboratory or natural isolates of *A. flavus* can be readily and consistently detected by flow cytometry analysis.

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Figures

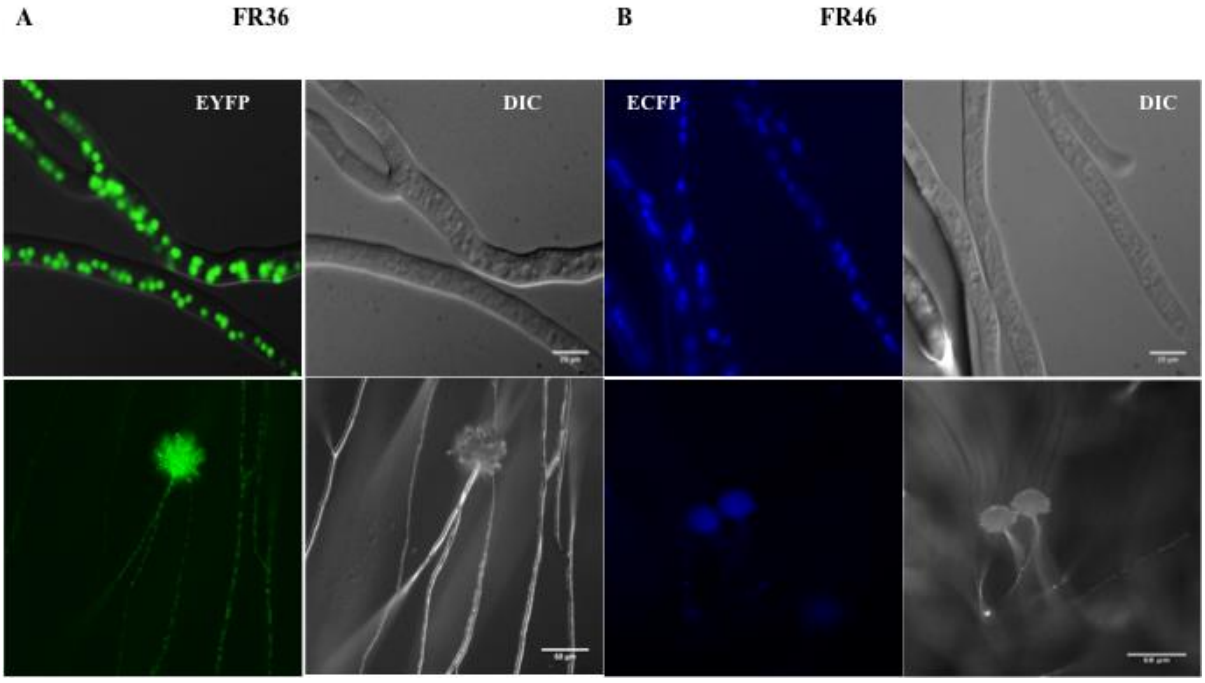

Fig. 2.1. Observation of EYFP and ECFP fluorescence in *Aspergillus flavus* mycelia and conidiophores. (A) Expression of HH2A-EYFP in strains of *Aspergillus flavus* FR36 in mycelia (top) and a conidiophore with conidia (bottom). (B) Expression of HH2B-ECFP in strains of *A. flavus* FR46 mycelia (top) and a conidiophore with conidia (bottom). Hyphae and conidiophore from FR36 and FR46 were observed on PDA and PDAU after 36 and 72 hours at 28^0C. The pictures were observed with DIC and fluorescence microscopy with specific filters for EYFP and ECFP at 63X for mycelia and 20X magnification for conidiophore. The pictures were opened and processed by Image J.

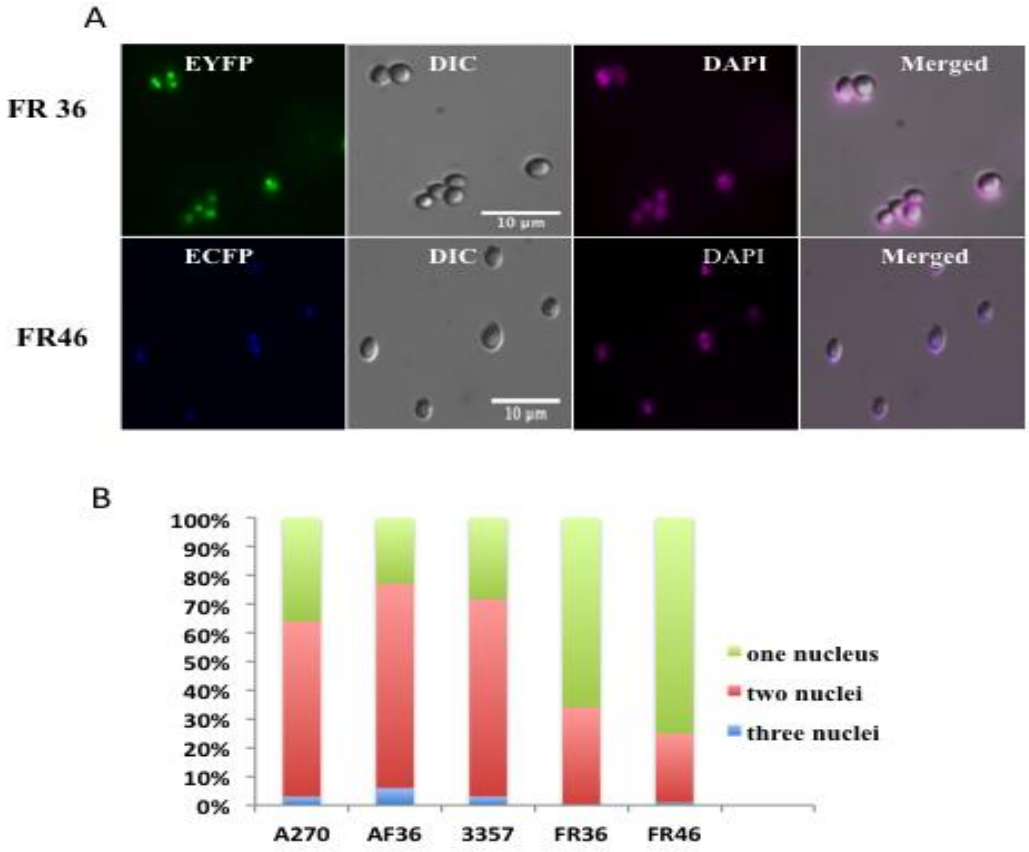

Fig. 2.2. Strains of *Aspergillus flavus* conidia have predominantly one or two nuclei. (A) Expression of HH2A-EYFP and HH2B-ECFP in conidia of *A. flavus* FR36 and FR46. Spores were collected from the cultures grown on PDA and PDAU, stained with DAPI and observed with DIC and fluorescence microscopy with specific filters for EYFP, ECFP and DAPI. (B) Percentage of conidia from different strains containing one or more than one nucleus. Conidia of *A. flavus* have predominately one or two nuclei per conidium. Spores were collected from the 7-day-old cultures and were observed under the filters of EYFP, ECFP and DAPI. Twenty fields were observed where each field contains 20-30 conidia. Three replicates were used for each strain and each replicate contains approximately 400-600 conidia. Percentages are shown from three replicates. Conidia were observed at 63X magnification. Scale bar 10 µm.

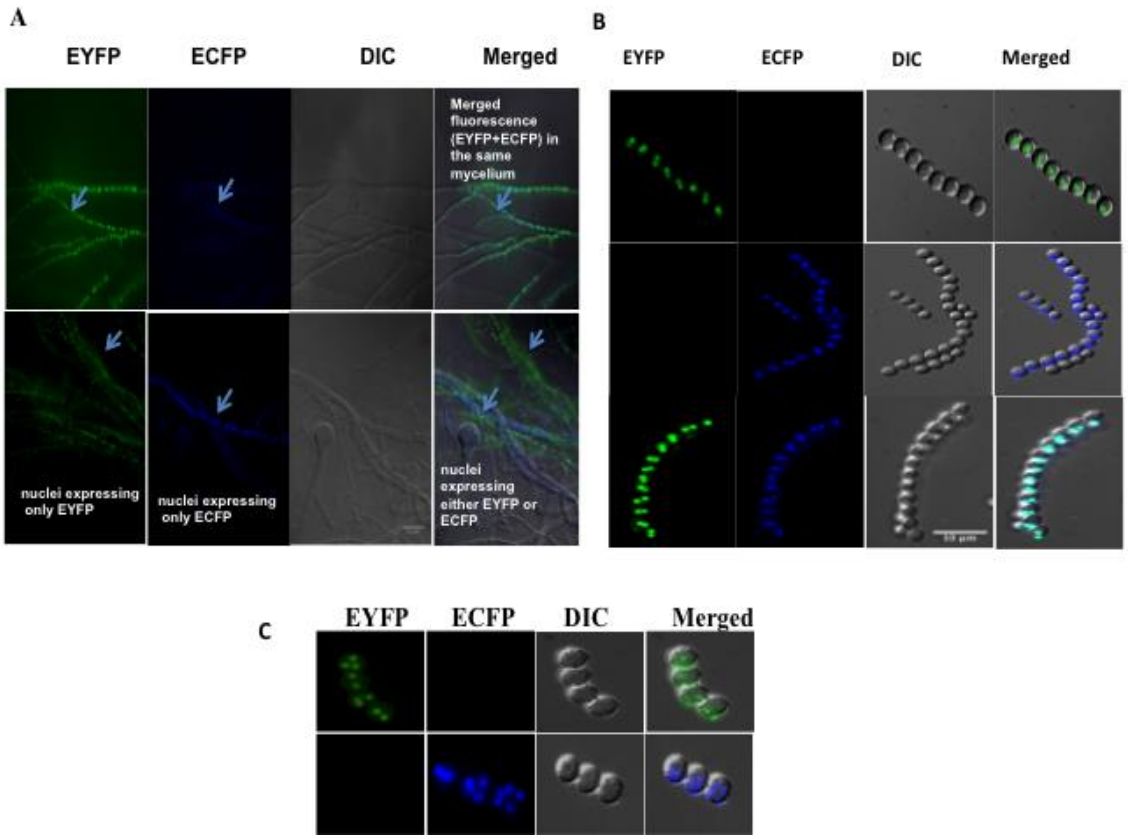

Fig. 2.3. Heterogeneous nuclei in mycelia and conidia of *A. flavus*. (A) Visualization of nuclear fluorescent markers in mycelia of a fusant (11-2) by fluorescence microscopy. Mycelia were observed under 40X magnification. Scale bar 25 µm. (B, C) Chains of conidia with nuclei expressing ECFP, EYFP and EYFP+ECFP (merged fluorescence) in fusant 11-2. Conidia were taken from the edge of the growing colony of the fusant and observed under EYFP, ECFP filters and DIC at 63X magnification. Scale bar 10 µm. Green color is used for EYFP, blue color is used for ECFP and cyan color is used for EYFP+ECFP.

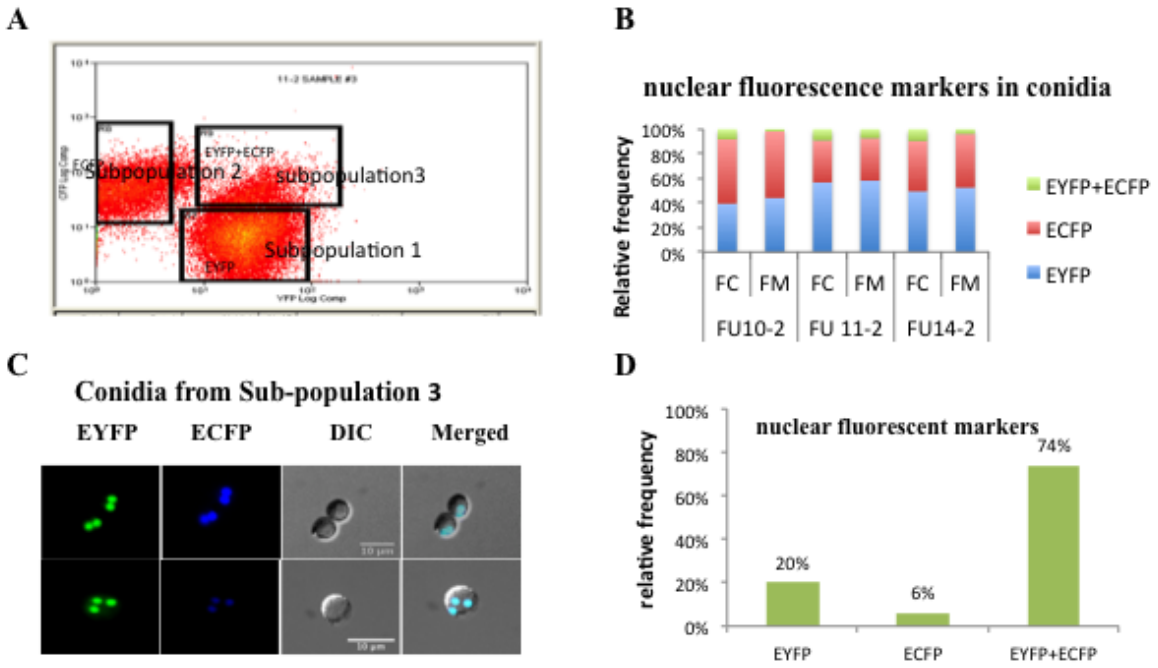

Fig. 2.4. Detection and sorting of conidia by FACS. (A) A bivariate dot plot showing nuclei in conidial populations of fusant 11-2 express EYFP, ECFP and both (EYFP+ECFP). In a flow cytometer, ECFP and EYFP were excited with 405 and 488 nm laser and emissions were collected by filters 450/65 and 530/40 nm. (B) Percentages of conidia having EYFP, ECFP and EYFP+ECFP from three fusants (10-2, 11-2 and 14-2). Twenty microscopic fields were observed under the filters of EYFP, ECFP and DIC. Each field contains 10-20 conidia and each replicate contains 100-200 conidia. Flow Cytometer counted 50000 conidia. FC represents Flowcytometry and FM represents fluorescence microscopy. (C) Conidia with merged fluorescence expressing EYFP+ECFP in subpopulation 3 (shown in Fig. 4A) were observed at 63X magnification by fluorescence microscope. Scale bar 10 µm. D. Percentages of conidia from FACS sorted subpopulation-3.

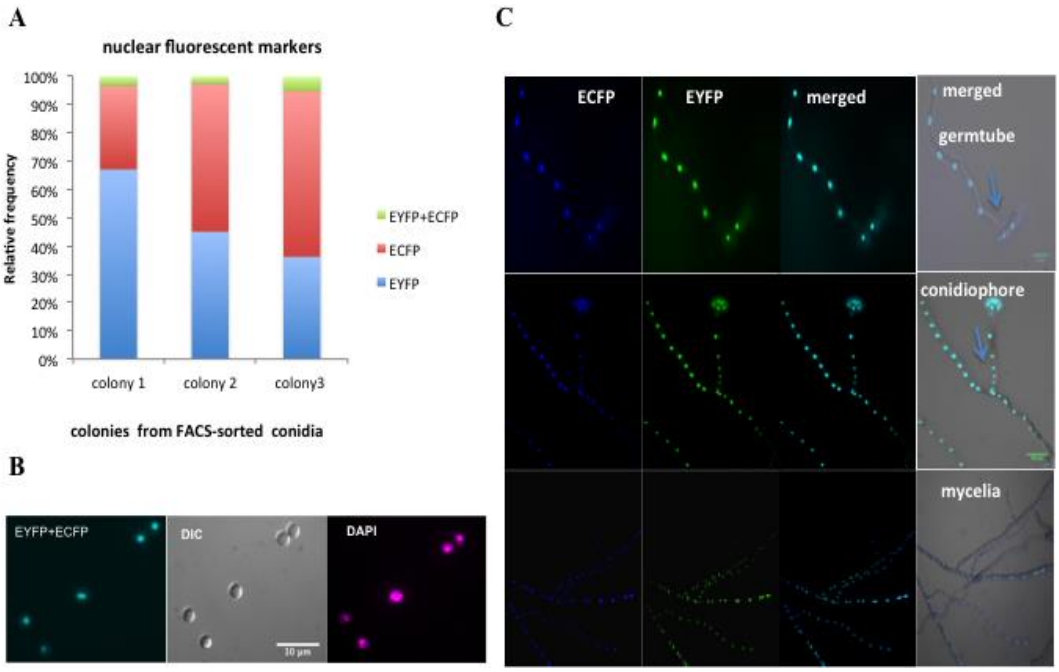

Fig. 2.5. Merged fluorescence in diploid nuclei. (A) Relative frequency of conidia with nuclei, which express EYFP, ECFP and EYFP+ECFP, was determined from three different colonies of fusant 11-2. Conidia from a 5-day-old culture were collected and were observed under the filters of EYFP, ECFP, and DIC. Twenty fields were observed and each field contains 20-30 conidia. Percentages are based on three replications and each replication contains approximately 400-500 conidia. (B) Expression of EYFP+ECFP in uninucleate conidia of a putative diploid (D2-2) was confirmed by DAPI staining. (C) Conidiation of a diploid D2-2 on MM medium. Conidia were grown on MM at 30^0C and observed under a fluorescence microscope for 5 days. Nuclei expressing both EYFP and ECFP were observed in a germ tube (top), a mycelium, a conidiophore and conidia (middle) and network of mycelia in a colony (bottom).

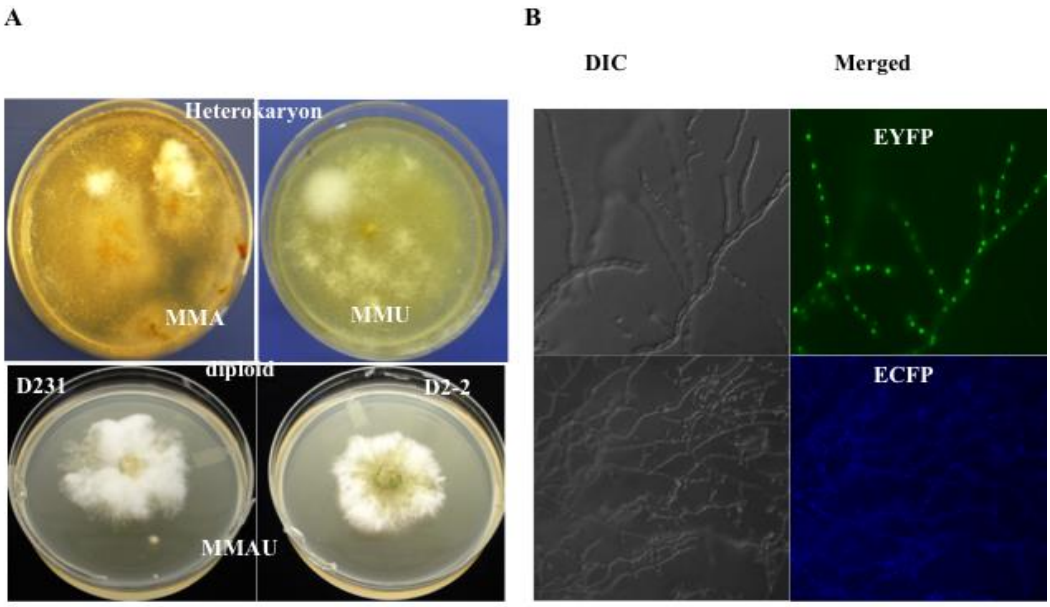

Fig. 2.6. Nuclei in white sectors of mycelia. (A) Observation of sectors within colonies grown on MMA, MMU and MMAU media with 1.5ug/mL benomyl. Growing mycelia were transferred from a heterkaryon and a diploid colony and incubated for several weeks. White sectors were formed when mycelia of a heterokaryon were grown on MMA and MMAU media (top) and mycelia from diploid D2-2 were grown on MMAU medium (bottom). A diploid strain D231 was used to observe the sectors on MMAU+1.5ug/mL benomyl. (B) Observation of only EYFP and only ECFP fluorescence in nuclei of white sectors of diploid (D2-2) colony.

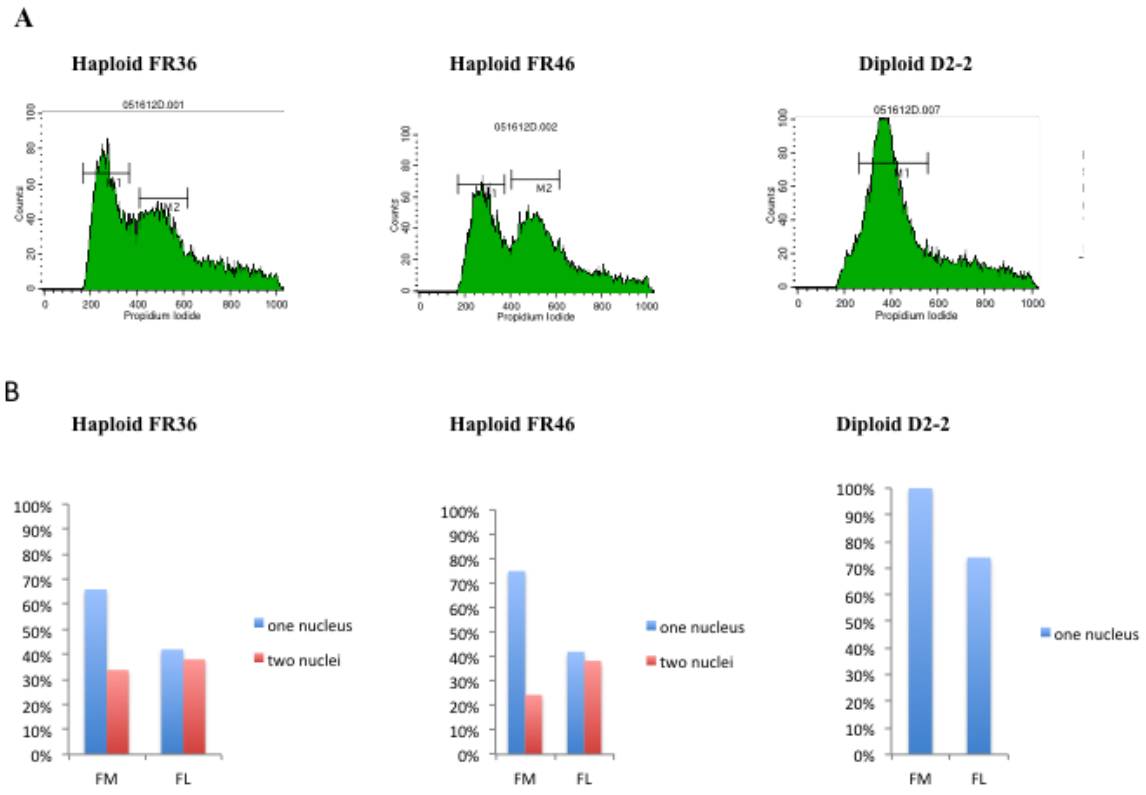

Fig. 2.7. Flowcytometry analysis of haploid and diploid conidia. (A) Comparison of conidia from the parental haploids (FR36, FR46) and the diploid (D2-2). Conidia were collected, fixed, and stained with Propidium Iodide (PI) .The FL (fluorescence) histograms of the parental strains with two peaks whereas The FL histogram of the diploid strain shows only one peak. The quantity of chromosomal DNA per nucleus of a conidium was doubled in the diploid compared to the haploids. X- axis represents PI intensity and Y-axis represents the number of conidia. The unit for the X-axis is arbitrary. (B) Percentages of conidia having one and two nuclei in haploid parental strains and one nucleus in a diploid strain. Five-six hundred DAPI stained conidia were observed under DAPI filter set by fluorescence microscopy. Five thousand PI stained conidia were analyzed by flowcytometry. Here, FC: Flowcytometery and FM: fluorescence microscopy.

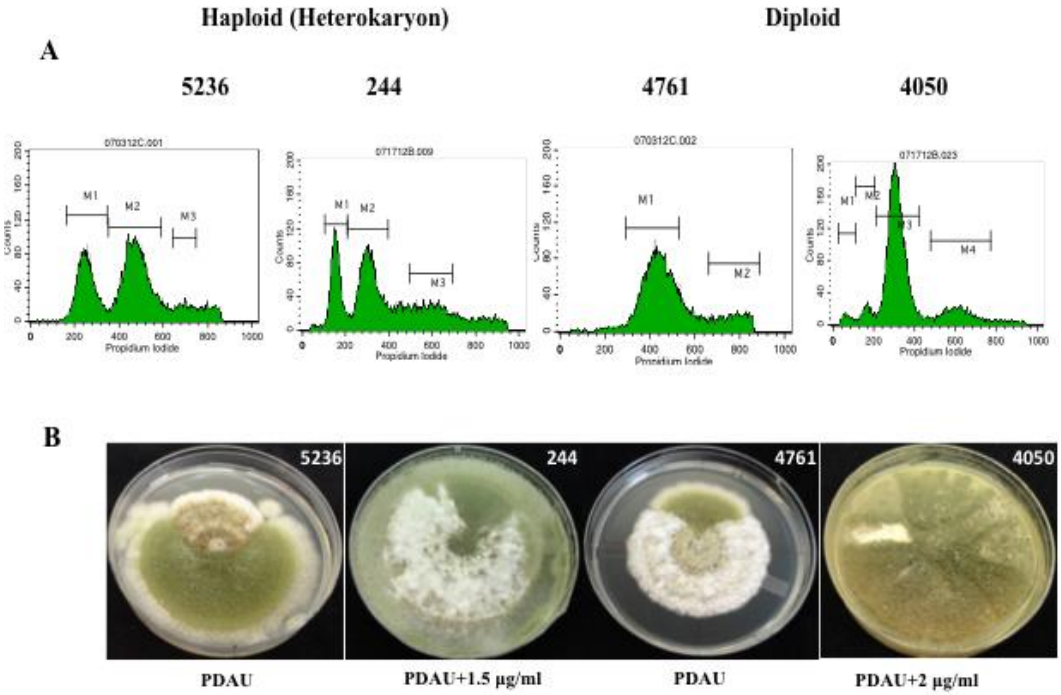

Fig. 2.8. Ploidy variation in conidial populations of *A. flavus.* (A) Detection of ploidy from natural and laboratory synthesized isolates of *A. flavus.* Conidia from haploid and diploid and natural isolates (Table 1) were collected, fixed, and stained with Propidium Iodide (PI). Two major peaks in the FL histogram were observed for a haploid IC 5236 and IC244. Only one major peak was observed in the FL histogram of a diploid 4761 and IC4050. X- axis represents PI fluorescence intensity and Y-axis represents the number of conidia. The unit for the X-axis is arbitrary. (B) Observation of sectors on colonies of *A. flavus*. Spores were collected from the 7-day-old culture. Five microliters spores from $1x10^6$ spores/ml were spotted on PDA and PDAU with or without benomyl. Concentration of benomyl was used as 1, 1.5, 2 and 2.5 µg/mL. All plates were incubated for 4-5 weeks at 30^0C. Pictures were taken by camera Sony Cyber-Shot16.1 Mega Pixels and were opened by Image J.

Tables

Table 2.1. *Aspergillus flavus* strains used in this study

Strain	Characteristics	References
3357	Wild type strain	NRRL
AFC-1	Mutant (*-pyrG-, -argD)* requires uracil and arginine	Georgianna et al., 2010
FR 36	*+Pyr4,-argD*-expressing yellow fluorescence (HH2A-EYFP) requires arginine	This study
FR46	*-pyr4, +argD* expressing cyan florescence (HH2B-ECFP) requires uracil	This study
FU 10-2, 11-2, 14-2	Fusants generated from protoplast fusion of FR36 and FR46	This study
D2-2	Diploid	This study
IC244, IC4050, A270, AF36	Natural Isolates	
IC5236	Haploid control	Papa et al., 1973
IC4761	Diploid control	Woloshuk et al., 1995

CHAPTER 3

Ploidy Shifting and Effects of Haploid and Diploid Conidia in Carbon Utilization and Pathogenicity of *Aspergillus flavus and Aspergillus nomius*

ABSTRACT

Aspergillus flavus persists in soil as a saprobe but infects a wide range of hosts as an opportunistic pathogen. Conidia of *A. flavus* are multinucleate and haploid (N). In our previous research (Runa, et al., 2014), we reported that conidial populations of *A. flavus* can exhibit a mixture of haploid (N, N + N) and diploid (2N) nuclear conditions. Because we found that a laboratory-synthesized diploid was stable under normal growth condition, we hypothesized that natural isolates of *A. flavus* may exist as diploids or that some strains may have mixed ploidy containing both haploid and diploid nuclei. To test our hypothesis, we obtained putative diploids from natural (field) isolates of *A. flavus* and assessed their stability under high and low temperature. We found that when grown under high temperature (40^0C temperature) or on benomyl plates, the natural putative diploid isolates formed two different sectors. Conidia from one sector were predominately diploid whereas conidia from the other sector were haploid. Further, we found that the haploid and the diploid conidia showed diversity in their growth, carbon utilization profiles and pathogenicity on an insect model, *Galleria mellonella*. These results suggest that ploidy level can be plastic and shifting of ploidy can affect growth, metabolism and pathogenicity of the fungus. We also included an isolate of *A. nomius,* a closely related species that is known to be heterokaryotic for mating type, in contrast to the predominantly homokaryotic *A. flavus*. We found that conidia of *A.*

nomius were multinucleate and predominantly dikaryotic. *Aspergillus nomius* showed higher metabolism on D-glucose compared to *A. flavus* and was similar to *A. flavus* in pathogenicity on *Galleria mellonella*

3.1 Introduction

A. flavus is commonly considered to be a haploid organism. Diploidy in natural isolates of *A. flavus* has long been overlooked, although their presence is not surprising as this fungus can undergo parasexual and sexual reproduction, both of which involve a transient diploid (Leaich & Papa 1975; Papa, 1973; Horn et al., 2009). In our previous study, we showed that the nuclear condition of *A. flavus* is heterogeneous and conidial populations of natural isolates can harbor diploid nuclei (Runa et al., 2014). But it is unknown whether the occurrence of diploidy or ploidy shifting during the life of the fungus is a common phenomenon in *A. flavus*. Additionally, the effect of ploidy on fungal biology and ecology has not been studied.

Although most fungi are haploids, diploids are found in species of yeasts and filamentous fungi (Roper, 1952; Holliday, 1961; Ishitani et al. 1956; Nga, et al., 1981, Cogliati et al., 2001; Ezov et al., 2006). Changes in ploidy are usually associated with phenotypic variations and have been observed in the filamentous fungi such as *Aspergillus nidulans* (Kafer and Upshall, 1973), *Penicillium (P. granulatum, P. martensii, P. crustosum*, Bridge et al., 1973) and *Neurospora crassa* (Threlkeld and Stephens, 1966). A recent study

(Hickman et al, 2013) demonstrated that the obligate diploid *Candida albicans* also harbors viable haploids. These haploids can undergo changes in morphology and virulence. Natural isolates of diploids also are found in the haploid pathogen, *Cryptococcus neoformans*, where diploids have a significant influence on fungal pathogenicity (Lin et al., 2008 & 2009). Adverse environmental conditions can affect ploidy in yeast (Iwaguch et al.; 2000) and cause plants to form diploid gametes (Pe´crix et al., 2011). Ultraviolet radiation can affect ploidy of Candida *albicans* and generate strains that are polyploids (Iwaguch et al., 2000).

The impact of ploidy in fungi and yeast on fungal ecology has been studied recently (Sudova et al., 2014; Zörgö et al., 2013), but we found no studies on *A. flavus*. Studies have shown that *A. flavus* occupies very diverse ecological niches. St. Leger et al., (1997, 2000) showed that *A. flavus* can utilize complex protein and carbohydrate substrates for their growth, likely due to their production of a broad spectrum of protein and polysaccharide hydrolyzing enzymes for exploiting available living and non-living organic resources. Reverberi et al., (2013) showed that *A. flavus* NRRL 3357 was able to utilize different carbon sources under different growth conditions. They stated the any adaptive changes in conidia of *A. flavus* under different growth conditions might affect the nutrient utilization of *A. flavus*. We hypothesized that nuclear content and ploidy level of conidia of *A. flavus* grown under certain condition could affect the metabolism of the fungus. To address whether haploid and diploid isolates grown under same growth conditions can vary in their metabolism of different carbon sources, we compared the carbon metabolism profiles of both haploids and diploids.

A. flavus is also an opportunistic pathogen and is commonly associated with plant, animal and insect pathogenicity. It is a well-known pathogen of silkworms (*Bombyx mori)* (Kumar et al., 2004), grasshoppers, houseflies and mealy bugs (Gupta & Gopal, 2002). The closely related species, *A. nomius,* is also frequently isolated from soil and silkworm excrement (Ito et al., 1998). Ploidy shifting in other fungi plays an important role in their virulence (Lin et al., 2008 & 2009; Hickman et al., 2013) and has been studied in insect and murine (rats and mice from Muraniae family) models. In this research, one of our goals is to determine whether *A. flavus* and *A. nomius* ploidy has any role in fungal pathogenicity.

Papa showed formation of diploids in heterokaryons and subsequent segregation during haploidization in *A. flavus* and *A. parasiticus* (Papa, 1973; Papa, 1977a; Papa, 1977b; Papa 1978). Whether formation of diploids occurs in non-forced mutants or in nature is unknown. The overall goal of this study was to examine diploidy in natural isolates and to understand the phenomenon of ploidy shifting under controlled environmental conditions. We addressed three research questions. 1) Does exposure to high and low temperatures and benomyl induce transitions in ploidy? 2) Do haploids and diploids show similar profiles of growth and carbon utilization? 3) Are diploids more pathogenic than haploids using the *Galleria mellonella* model system?

3.2 Materials and Methods

3.2.1 Strains and Culture Conditions

Aspergillus flavus was randomly sampled from soil and infected peanut seeds from a single field (Herod, Terrell County, Georgia, USA) (Horn and Greene, 1995). The sample consisted of 79 single-spore isolates grouped into 44 vegetative compatibility groups. An initial screening of all isolates revealed four putative diploids (IC274, IC275, IC295, IC302) based on restricted growth on benomyl (benlate) containing medium (Bruce Horn, unpublished data). Conidia of IC274, IC275, and IC295 were observed to be predominantly haploid (n and n+n), but a careful examination of their ploidy was not determined. Here we focused on these isolates as a possible representative sample of putative diploids from nature. Because the conidial populations in these strains could be a mix of n, n + n and 2n we wanted to further determine the nuclear condition in *A. nomius,* a closely related and sympatric species that is heterokaryotic for mating type and predominantly dikaryotic (n + n). Such an examination could allow us to examine differences in growth and pathogenicity in the haploid segregants that are potentially of different genetic backgrounds than the parental strain. We focused on *A. nomius* isolate IC1516 that was sampled from soil in a cotton field (Concordia Parish, Louisiana, USA) and previously shown to be capable of mating with both *MAT1-1* and *MAT1-2* strains (Horn et al 2011). The strains used in this study are presented in Table 3.1.

Strains were grown at 30^0C and maintained on PDA (Potato Dextrose Agar, Difco), PDAU (PDA plus 1.12 g/L uracil), PDB (Potato Dextrose Broth, Difco) or PDBU (PDB plus

1.12 g/L uracil). All cultures were grown at 40^0C. Cultures also were grown at 30^0C with PDA or PDAU + 0.2µg/mL benomyl to induce sectoring. Isolates from each sector were single-spored and kept at 4^0C for long time storage.

3.2.2 PI Staining and Flow Cytometry

We used the same protocol for PI (Propidium Iodide) that was used for staining of *A. oryzae* conidia as described by Hara et al. (2011 and 2002) with some modifications. Strains of *A. flavus* were grown on PDA or PDAU for 7 days. Conidia were harvested with 0.01% Tween 80 (0.01% Tween 80 + 1M NaCl) and were filtered through Miracloth (Calbiochem, USA). The conidial suspension was fixed with 70% ethanol at 4^0C for 30 minutes. Conidia were collected by centrifugation at 3000 rpm for 5 minutes and washed twice with 0.01% Tween 80. Conidia were resuspended in TE buffer (1M Tris.Cl, pH 8.0+ 0.5M EDTA) and incubated at 37^0C in the presence of RNase (1mg/mL) for 2 hours. Conidia were washed twice with 0.01% Tween 80, stained with 25 µg/mL Propidium Iodide (PI, Sigma-Aldrich, USA, P4170) and incubated at room temperature for 30 minutes. Stained conidia were analyzed on the FL1 channel by a flow cytometer (Becton–Dickinson FACscan). Data were analyzed by Cell Quest Pro software.

3.2.3 Biolog Phenotype Microarray

Carbon-utilization profiles were examined using FF MicroPlates (Biolog) following the protocol described by Reverberi et al. (2013) with modifications. Cultures (Table 3.1) were grown on PDA or PDAU for 7 days. In this assay, conidia (1 x 10^6 /mL) were suspended in sterile Phytagel solution [0.25% (w/v) Phytagel, 0.03% (v/v) Tween 40]. The suspension was adjusted to the optical density of 0.2 at 590 nm wavelength. One hundred microliter (100 µL) of spore suspension was inoculated into each well of the microplates. After inoculation, the microplates were incubated at 30^0C in the dark. FF Microplates have 96 wells in which 95 wells were coated with different carbon-containing compounds and one well contained water. Each well had pre-filled Iodonitrotetrazolium violet (INT), which is used as a redox dye. A red-colored formazen dye (peak absorbance at 490 nm) was formed when INT was reduced. Optical Density (OD) values were recorded after 0, 24, 48, 72, 96 hours at 490 nm using a microplate reader (BioTek Miroplate reader). The red color developed by fungal respiration indicates that the substrates were metabolized.

3.2.4 Statistical Analysis

Agglomerative Hierarchical Clustering (AHC) using Euclidean distance was performed by XLSTAT software (Addinsoft, Paris) after 24, 48, and 72 and 96 hours of incubation to observe the similarity of the haploids and the diploids. The AHC was constructed for the isolates (using Ward's method) and also for the carbon sources (using complete linkage)

One-way ANOVA analyses were performed to determine significant differences on substrate utilization by the haploid and diploid strains. Factor analysis was performed using Pearson Correlation and Principle Component Analysis (PCA) extraction method by XLSTAT (Addinsoft, Paris). One-way ANOVA analyses were performed to determine significant differences of substrate utilization by the haploid and diploid isolates of *A. flavus* and also for *A. nomius*.

3.2.5 Colony Diameter Measurement

Conidia collected from 7 days old cultures were suspended in 0.05% triton X100. Cultures were grown on PDA or PDAU with and without 2-ug/mL benomyl for 7 days. Five microliter of 1×10^6 conidia/mL was inoculated in the center of three 90 mm diameter Petri dishes containing 25 ml agar medium and incubated at 30°C. Colonies were observed after 24, 48, 72, 96 and 120 hours. For each colony, two diameter measurements were obtained. Three replicates were used for each strain. For each replicate three plates were used. A total of nine plates were used for colony diameter measurement. One-way ANOVA analysis was performed from the average colony diameter of three replicates.

3.2.6 Biomass Assay

Conidia were collected from 7 days old cultures grown on PDA or PDAU, suspended in 0.5% triton X100 and diluted to 1×10^6 conidia/mL. Five hundred microliters of 1×10^6 conidia/mL were added into 75 mL of liquid media, PDB or PDBU in a 125 mL flask. The isolates were grown at 30^0C, 200 rpm for 3 days. Mycelia from the cultures were filtered through Miracloth (Calbiochem, USA) and collected after 3 days (72 hour) incubation. Mycelia were collected into 15 mL tubes and were dried by lyophilization. The dry weight of mycelia for each isolate was recorded.

3.2.7 Pathogenicity Assay

Larvae of *Gallerria mellonella* (wax-moth) were injected with conidia following the protocol established for *Cryptococcus neoformans* (Mylonakis et al., 2005). A total of 12 wax-moth larvae (about 0.3-0.4 gram body weight) were selected from the final instar larvae stage (Vander host, Inc., St. Mary, Ohio) for each strain. Larvae were kept at room temperature in the dark and stored with wood shavings. Five-microliter of conidial suspension ($1x10^6$ spores/mL) was injected through the last left proleg into the hemocoel of each wax moth larva. The wax moth larvae were incubated in Petri dishes at room temperature (28^0C). Morbidity was detected when the caterpillars could not respond to touch and changed color. The survival rate of each larva was plotted against time. Statistical analyses were performed using the student t-test. Three independent experiments were done for each strain.

3.2.8 Light and Fluorescence Microscopy

Fluorescence and light microscopy were performed using a fluorescence microscope (Zeiss Axioskop 2 plus, Germany) with a HBO100 Hg or XBO 75 xenon lamp for fluorescence excitation. Conidia stained with PI were observed with a specific filter set for PI stain (41004 Texas Red, Chroma Technology Crop. USA, excitation (EX) wavelength 595nm and emission (EM) wavelength 620 nm). Images were captured with a QImaging camera (Software Q Capture Suite 2.98.2) and processed using ImageJ1.46r (NIH, USA) software.

3.3 Results

3.3.1 Sectors in Colonies of Natural Isolates of *A. flavus* Exposed to Stresses

Stress is considered one of the inducers of sectoring in filamentous fungi (Li et al., 2008). To determine whether stress can induce sectors with different phenotypes in natural isolates of *A. flavus,* we grew several isolates under high temperature and with an antimicrobial agent (benomyl). Several sectors appeared in cultures grown at 40^0C on PDA. Two types of sectors were observed in cultures of IC274 (Fig. 3.1A top). One sector maintained the wild type color of IC274 (green) and the other sector developed a tan color (Fig. 3.1A top). We also observed sector formation when isolates were grown at 30^0C on PDA amended with 2.0 µg /ml benomyl. Mycelium in these sectors appeared as either green or white (Fig. 3.1A, bottom). All sectors were transferred onto PDA and allowed to grow at 30^0C for production of conidia. Single conidium isolates were obtained from the different sectors of IC274 and

IC295 and designated as IC274S1, IC274S2, IC295S1 and IC295S2. We also collected a white sector from *A. flavus* putative diploid isolate IC4050 and designated it as IC4050S1 (Supplementary Fig. 3.1).

3.3.2 Ploidy Shifting at High Temperature and Benomyl

To characterize the sectors that arose due to temperature or benomyl treatment, we stained nuclei of conidia from the sector strains, the original culture strains, and those of the characterized diploid strain IC4761 (Woloshuk et al., 1995), with Propidium Iodide (PI) and analyzed them for ploidy using flow cytometry. In this analysis the natural isolates (IC274) and sector (IC274S2) showed two distinct peaks in the fluorescence (FL) histogram (Fig. 3.1B). Conidia of IC274 and IC274S2 had one or two nuclei per conidium (Table 3.2). The fluorescence peaks in the histogram (Fig.3.1B) and nuclear number in conidia suggested that IC274 and IC274S2 were haploids. In contrast, the peak in the fluorescence histogram of the other sector, IC274S1 (Fig. 3.2A) exactly overlapped with the peak of the fluorescence histogram of the diploid control (2n). These findings indicate that the DNA content of conidia of IC274S1 is similar to the DNA content of the diploid IC4761. The conidial populations of IC4761 and IC274S1 had predominantly one nucleus. The observation that diploid conidia contain predominately one nucleus has been reported (Leaich & Papa 1975, Runa et al, 2014). Taken together, these observations suggest that IC274 S1 is a diploid having conidia with predominantly one nucleus.

Similarly, the FL histogram of IC295S1 showed only one major peak that exactly overlapped with the peak of the fluorescence histogram of the diploid control (2n) (Fig. 3.2B). This indicates that the DNA content of conidia of IC295S1 is similar to the DNA content of the diploid IC4761. The nuclear number of conidia in IC295S1 is predominantly one and thus suggests that IC295S1 is a diploid. On the other hand, the parental strain (IC295) and its sector (IC295S2) showed two distinct peaks in the fluorescence (FL) histogram (Fig. 3.2B). Compared with the diploid IC4761, the two FL peaks for the histograms of IC295 and IC295S2 indicate that IC295 and IC295S2 are haploids. Thus, ploidy and nuclear number in conidia of IC295S2 suggests that although the sector IC295S2 originated from IC295, it had the ploidy of the original culture. But the other sector IC295S1 was diploid (2n) (Fig. 3.2B). Our findings suggest that high temperature could affect or select the ploidy of *A. flavus.* High temperature could induce ploidy shifting or favor the diploid from the putative diploid isolate IC274. As a result, both haploid and diploid were separated and obtained in the two different sectors (Figs. 3.1A & 3.2A). Benomyl also was found to induce ploidy shifting, which was observed in the putative diploid IC295 (Figs. 3.1 and 3.2B)

We also observed a single sector for isolate IC4050 and found that a diploid (IC4050S1) originated from IC4050 (Fig. 3.3A). Additionally, we compared the ploidy of the isolates, IC302 and NRRL3357 with the diploid control IC4761 (Fig. 3.3B) and found that the peaks of FL histograms were aligned with other haploids. In our study neither isolates IC302 nor IC3357 made any sectors at high temperature.

3.3.3 Ploidy Shifting at Low Temperature

To determine the stability of diploids at low temperature, 7-day-old cultures plates inoculated with diploids IC274S1 and IC295S1 were kept at 4^0C for several weeks. Conidia from plates were stained with PI and analyzed by flow cytometry. We found that fluorescence histograms from conidia held at 4^0C (IC274S1, IC295S1) were different from conidia of cultures grown at 30^0C (Fig. 3.4). The result suggested that the diploid isolates IC274S1 and IC295S1 kept at 4^0C for long time could have conidia with a mix of diploids (2N, 2N+2N) or polyploids (2N, 4N).

3.3.4 Clustering of Haploids and Diploids Based on Carbon Utilization Profiles

Our second objective was to determine whether the carbon utilization profiles of haploids and diploids differ significantly. Conidia from the 14 isolates (IC274, IC274S1, IC274S2, IC295, IC295S1, IC295S2, IC4050, IC4050S1, IC302, 3357, IC4761) assigned to the five different groups (Table 3.2) were collected and incubated in FF Microplates coated with 95 carbon sources. To compare carbon utilization profiles among the strains, average respiration values (OD_{490} nm) after 24, 48, 72 and 96 hours of incubation were measured and the data analyzed by Agglomerative Hierarchical Clustering (AHC) using Ward's method. Figure 3.5 describes the AHC of the 14 isolates (Table 3.2). The four dendrograms show progressive grouping of the data for the different incubation times and place isolates into clusters based on higher to lower similarities (Fig. 3.5). Less distance indicates to more similarity among

isolates, which was observed after a 24-hour incubation time (Fig. 3.5A). Greater distances and thus greater dissimilarity of carbon utilization were observed within 48-96 hour of incubation. At early incubation times (after 24 and 48 hour incubation) the diploids D2-2, IC4050 and control diploid IC4761 were similar to the sector diploids IC4050S1, IC274S1 and IC295S1, respectively.

At the 72-hour time point, two major clusters emerged and *A. flavus* 3357 grouped with the diploid D2-2 (Figure 3.5 B). Within the two major clusters, the haploid and diploid strains were further separated into different clusters based on their similarity for carbon utilization (Fig. 3.5 B). Isolate IC302 clustered with the diploids IC4050 and IC274S1 after 48 to 72 hour incubation. After 96 hours, the two diploids, D2-2 and IC4050S1, showed more similarity to the parental isolates and were grouped together with their respective haploid parents. Moreover, after 96 hours diploid D2-2 carbon utilization was similar to parental isolates FR36 and FR46. Isolates IC4050 and IC4050S1 were clustered together and existed within the same group after the 96-hour incubation time point (Fig. 3.5 A, 96-hour incubation).

We observed greater distances separating clusters using AHC after 72 hours. Overall carbon metabolism increased between 48-72 hours (Supplementary Fig. 3.2). Because dissimilarity of carbon utilization would be expected to be higher after 72-hour incubation, we performed AHC of 95 carbon sources after 72-hour incubation. Figure 3.5 shows AHC based on Euclidean distance by complete linkage analysis. Cluster-I was comprised of 25 readily utilizable carbon substrates. Metabolism of *A. flavus* isolates was highest for these 25

carbon substrates. The average respiration values (at 490 nm) of haploids and diploids were in the range of 0.5-4.0 (Supplementary Fig. 3.3). The carbon sources in this cluster were monosaccharaides (N-acetyl-D-Glucosamine, L-arabinose, D-Ribose, D-Xylose), disaccharides (D-cellulose, Palatinose, Turanose), hexoses (Rhamnose), sugar alcohols (Adonitol, i-Erythritol, Glycerol, D-Sorbitol) amino acids (L-Arginine, L-Asparagine, L-Proloine, L-Threonine), organic acids (D-Glucurinic acid, g-Amino butyric acid, L-Pyroglutamic acid and Quinic) and glycoside (Amygdalin).

Cluster-II included 35 carbon sources and metabolic profiles revealed moderate respiration values (the average OD_{490nm} values were between 0.4-3.5) (Supplementary Fig. 3.4, Appendix A). Cluster-II includes sugars, (D-Glucose, D-Mannose, D-Fructose, Sucrose, L-sorbose, D-Galactose, Maltose, Gentibiose, Maltrotiose, Glycogen, Dextrin), organic acids (Succinic acid, Fumaric acid, L-Mallic acid, Bromosuccinic acid, sebacic acid, Glycyl-Glutamic Acid, L-Aspartic Acid, a-keto-glutaric Acid, Saccharic acid, D-Malic acid, p-Hydroxyphenyl-acetic acid, Galactouronic acid, L-glutamic acid, 2-keto-D-Gluconic Acid) and sugar alcohols (Inositiol, D-Mannitol, D-Arabitol, Xylitol, Salicin). Other carbon sources included were glycosides (putrescine, Arbutin, amino acid L-Serine) and glucose -1-phosphate.

Cluster-III also included 35 carbon sources in which the OD values were less than the carbon sources of cluster I and II. The average OD_{490nm} values range from 0.3-2.5 and were recorded after 72-hour incubation. (Supplementary Fig. 3.5, Appendix A). Carbon sources were organic acids, (g-Hydroxy-butyric Acid, b-Hydroxy-butyric Acid, L-Lactic Acid,

Succinamic acid, N-Acetyl-L-Glutamic Acid), sugars (a-Clycodextrin, b-Cyclodextrin, D-glucosamine, D-Arabinose, D-Melezitose, D-Tagatose, D-Raffinose, Stachynose, Lactulose, D-Lactose, D-Psicose, D-Melibiose, L-Fucose, Sedoheptulosan), sugar alcohol (Maltilol) and others (Methyl-D- galactiside, Tween 80, Adenosine, (L-phenylalanine), Alaninamide, Succinic Acid Mono-Methyl Ester, a-methy-D-Glucoside, a-Methyl-D-galactoside, N-acetyl-D-Mannosamine, N-Acetyl-D-Galactosamine, Adenosine $5^{/-}$ Monophosphate, Glucuronamide, D-Lactic Acid Methyl Ester, Uridine).

3.3.5 Carbon Utilization by Haploids and Diploids

We analyzed carbon utilization profiles to determine differences between haploids and diploids. One-Way ANOVA analyses were performed on the respiration values (OD_{490} nm) of carbon substrates for all isolates after 24, 48, 72 and 96 hours of incubation. We found that at 72 hours, the diploid IC274S1 was significantly different from the original isolate IC274 and the haploid IC274S2 for 23 carbon sources of cluster I (Table 3.3); the isolates IC274 and IC274S2 had higher respiration values in utilizing sugars, sugar alcohol, amino acid, organic acids and glycoside. In Group 2, the isolates (IC295 and IC295S2) showed higher respiration values for sugars (L-Arabinose, D-cellobiose, Turanose), aminoacids (L-Asparagine, L-Threonine) and organic acids (D-glucuronic acid, L-Pyroglutamic Acid) than the diploid IC295S1 (Table 3.4). In Group 3, isolate IC4050 showed better utilization of sugars, sugar alcohols, amino acids, organic acids and glycoside (Table 3.5).

Table 3.6 shows that the metabolism of diploid D2-2 was higher on different carbon sources compared to haploid strains FR36 and FR46. In Group 5, the natural isolate IC302 was significantly different from 3357 and the control, lab-synthesized diploid strain 4761 in utilizing carbon sources. The natural isolate IC302 showed higher metabolism of all the carbon sources in Cluster I than 3357 and the diploid IC4761 (Table 3.7).

ANOVA was also performed on single substrates from Cluster II and Cluster III. In Cluster II, 14 isolates within the five groups showed no differences in utilization of the following carbon sources: Glycogen, L-Serine, Maltose, Fructose, Sucrose, 2-Keto-D-Gluconic Acid, Mannose and a-D-Glucose. For other carbon sources of Cluster II, the diploids showed significant differences from the haploids (Supplementary Table 1-5, Appendix A). In Cluster III, no significant differences were observed for the 12 carbon sources: Cyclodextrin, b-Methyl-D-Galactoside, Tween 80, L-Lactic Acid, D-Melezitose, D-Raffinose, D-Melibiose, a-Methyl-D-Glucoside, N-Acetyl-D-Mannosamine, L-Fucose, Glucuronamide, N-Acetyly-L-Glutamic Acid, D-Lactic Acid Methyl Ester and water. The haploids and the diploids from each group showed significant differences for other carbon sources from Cluster III (Supplementary Table 6-10, Appendix A). In summary, our results suggest that diploids originating from natural isolates were significantly different in carbon utilization than their parental isolates. On the other hand, diploid D2-2 was more similar to NRRL 3357 even though its metabolism was higher on different carbon sources than the parental haploids FR36 and FR46. The natural isolate IC302 also metabolized carbon sources more readily than NRRL 3357 and the diploid control IC4761.

3.3.6 Growth of Diploids and Haploids

To observe the growth of diploid and haploid strains, we measured the colony diameter of all isolates after 72-hour incubation (Fig. 3.6A). One-way ANOVA followed by Tukey's HCD t-test showed that colony diameters of isolates IC274, IC295 and IC4050 (within Groups 1, 2 and 3) were smaller than their sectors (Fig. 3.7 and Table 3.8). The colony diameters of diploid D2-2 and haploid FR36 were higher than haploid FR46. However, there were no significant differences in the growth of isolates IC302, 3357 and IC4761. Further, we analyzed the data for mycelia dry weight at the 72-hour incubation time point (Fig. 3.8). One-way ANOVA followed by Tukey's HCD t-test showed that sector biomass was significantly different from parental strains within Groups 1, 2 and 3 (Fig. 3.8 and Table 3.9). Diploid D2-2 yielded more biomass than haploids FR36 and FR46l. There were no significant differences in dry weights of isolates IC302, 3357 and 4761. The biomass production for diploid D2-2 was higher than in haploids FR36 and FR46. The biomass of diploid IC295S1 was higher than parental strain IC295. On the other hand, diploids IC274S1 and IC4050S1 made less biomass than parental isolates IC274 and IC4050 (Table 3.9). Finally, diploid D2-2 had more biomass than its parental haploid strain and diploid IC274S1 had less biomass than the parent isolate IC274.

3.3.7 Pathogenicity of Haploids and Diploids of *A. flavus*

We addressed the question of whether diploids were more pathogenic than haploids. To do this, we compared pathogenicity within isolates of each Group. Pathogenicity assay was performed at room temperature (28^0C). In Group 1, isolate IC274 and its haploid sector IC274S2 started to kill the larva of *G. mellonella* within 2-3 days post inoculation. Subsequently, all the larvae were killed and the percentage of larvae survival dropped to zero by day 14. Although diploid IC271S1 started to kill the larvae at the same time, it took a longer time (22 days) to kill all the larvae than the original isolate IC274 and the haploid IC274 and the haploid IC274S2. We also found that haploid IC272S2 was more virulent than diploid IC274S1 (Fig. 3.9A). In Group 2, isolate IC295 and diploid IC295S1 started to kill the larvae at the same time (2 days after post inoculation). But diploid IC295S1 killed all the larvae earlier (6 days) than the parental strain IC295 (11 days) and the haploid sector IC295S2 (8 days) (Fig. 3.9 B). The haploids IC295 and IC295S2 did not show a significant difference in virulence compared to diploid IC295S1. In Group 3, diploid IC4050 and its sector IC4050S1 did not kill all the larvae within 3 weeks and were similar in virulence (Fig. 3.9 C). In Group 4, the parental haploids FR36 and FR46 slowly killed the larvae and appeared less virulent than the diploid D2-2. The diploid D2-2 was significantly different in pathogenicity on *G. mellonella* compared to the parental haploid FR36 (Fig. 3.10 A). Further, we compared all the strains within Group 5 and found that haploid IC302, genomic strain NRRL 3357 and diploid IC4761 showed similar pathogenicity (Fig. 3.10B). In addition, comparing strain NRRL 3357 with all of the haploids and diploids revealed that only the

haploid auxotroph FR36 which requires arginine was significantly different in virulence compared to strain NRRL 3357 (Fig. 3.11).

3.3.8 Nuclear Condition of Conidia in *Aspergillus nomius*

To observe the nuclei in conidial populations of *A. nomius strain* IC1516, we collected conidia from a 5-day-old culture. Conidia were stained with DAPI and approximately 500 conidia were observed using fluorescence microscopy. We found that conidia of *A. nomius* have one, two or three nuclei (Fig. 3.12A); however, the majorities (70%) of conidia have two nuclei (Fig. 3.12B). To further investigate ploidy in *A. nomius*, conidia were stained with PI and flow cytometry was performed. Isolate IC4761 was used as a diploid control. The fluorescence histogram (FL) of *A. nomius* showed one major peak (Fig. 3.12C). Compared to the FL peak of the diploid control, *A*. nomius IC1516 could be diploid (2n); however, the nuclear frequency in conidial populations of *A. nomius* suggested that conidia had predominantly two nuclei (Fig. 3.12A and B). Thus, our results support that *A. nomius,* which is presumed to be heterokaryotic for mating type, is mostly dikaryotic in its nuclear condition.

3.3.9 Carbon utilization profiles of *A. nomius*

The carbon utilization profile of *A. nomius* IC1516 was investigated to explore if the strain has the capacity to utilize a broad spectrum of carbon sources. Biolog Phenotype data revealed that *A. nomius* could metabolize 95 different carbon sources (Supplementary Fig. 3.6). In this study, we performed Agglomerative Hierarchical Clustering (AHC) analysis of carbon utilization in *A. nomius* and compared carbon profiles to isolates of *A. flavus*. Our data showed that *A. nomius* was clustered within the diploid *A. flavus* isolates (IC4050, IC4761, IC295S1, D2-2) and *A. flavus* NRRL 3357 (Fig. 3.13) In order to observe any dissimilarity between carbon metabolism for *A. nomius* IC1516, and other diploids and haploids, we examined *A. nomius* IC1516, diploid *A. flavus* D2-2 and the haploid *A. flavus* NRRL 3357 using factor analysis. Factor analysis was performed on carbon profiles after 24, 48, 72 and 96-hour incubation time points. Figure 3.14 shows the dissimilarity within the three strains using a scatter plot. The plot was generated using Principal Component Analysis (PCA). After 24 hour, *A. nomius* IC1516 and the diploid *A. flavus* D2-2 were similar in their metabolism (placed in the same quadrant in the plot, Fig. 3.13, top left); however, *A. flavus* NRRL 3357 was dissimilar (left quadrant in the bottom) clustering with *A. nomius* and diploid *A. flavus* D2-2 after 24-hour incubation. After 48, 72 and 96-hour incubation, the metabolic behavior changed for those three strains. At early incubation (24-48 hour), *A. nomius* IC1516 was metabolically similar to the diploid D2-2. When the incubation time increased, *A. nomius* IC1516, *A. flavus* D2-2 and *A. flavus* 3357 diverged for carbon utilization. *Aspergillus nomius* IC1516 carbon utilization was higher than *A. flavus* 3357 and

diploid D2-2 after 72 and 96-hour incubation. Oneway ANOVA followed by Tukey's HCD test showed that there were no significant differences for most of the carbon sources utilized by *A. nomius* IC1516, diploid D2-2 and *A. flavus* NRRL 3357. For only a few carbon sources, *A. nomius* showed dissimilarity to either *A. flavus* NRRL 3357 or diploid D2-2 (Table 3.10). Table 3.10 shows that at early incubation (24 hour) *A. nomius* metabolism was similar to the diploid for 5 carbon sources (Bromosuccinic acid, Fumaric acid, N-Acetyl Glutamic acid, L-Ornithine and Uridine) but dissimilar to *A. flavus* NRRL 3357. After 48-hour incubation *A. nomius* showed similarity to *A. flavus* NRRL 3357 only for sucrose utilization but was different compared to diploid D2-2. After 72 and 96-hour incubation, *A. nomius* metabolisms for D-Xylitol- D-glucose were higher than *A. flavus* 3357 respectively. The data suggests that *A. nomius* metabolism may be higher for some carbon sources (especially sugar and sugar alcohol) than *A. flavus* NRRL 3357.

3.3.10 Pathogenicity of *A. nomius* IC1516 on *Galleria mellonella*

We compared the pathogenicity of *A. nomius* IC1516 with *A. flavus* NRRL 3357 and the diploid *A. flavus* D2-2. We found that *A. nomius* IC1516, *A. flavus* NRRL 3357 and the diploid D2-2 showed similar virulence to the insect *Galleria mellonella* (Fig. 3.15). The only difference was that the diploid D2-2 and *A. nomius* IC1516 killed larvae earlier than *A. flavus* NRRL 3357.

3.4 Discussion

Ploidy variations exist in some fungi but the reasons and the consequences of ploidy shifting are not well understood. Runa et al., (2014) reported that a small percentage of conidia could exist as diploids in conidial populations of *A. flavus.* Since, lab synthesized diploids were stable under normal growth conditions, we hypothesized that natural isolates of *A. flavus* also may exist as diploids or that some strains may have both haploid and diploid nuclei. To test our hypothesis we: 1) examined the growth of putative diploids exposed to high temperature or benomyl; 2) observed carbon utilization profiles and growth of putative diploids and the stress induced haploids and diploids; 3) determined the pathogenicity of haploid and diploid isolates of *A. flavus*

3.4.1 Influence of Temperature on Ploidy

To observe whether stress can change ploidy, we grew the putative diploid natural isolates of *A. flavus* under stress for several weeks. We observed that some sectors were formed in a colony of IC274 when conidia were grown at high temperature (Fig. 3.1A). Sectors were also found when the putative diploids were grown on medium amended with benomyl. We analyzed the ploidy of conidia by flow cytometry and observed the nuclei in conidia by fluorescence microscopy from the original isolates, IC274 and IC295 and their sectors. We found that the natural isolates IC274 and IC295 and their sectors IC274S2 and IC295S2 were predominantly haploid (Fig. 3.2 and Table 3.2); whereas, sectors IC274S1 and IC295S1

(isolates originating from IC274 and IC295) harbored predominantly diploid conidia. Our results showed that under stress conditions, both haploids and diploids were recovered from natural isolates. The exact mechanism underlying stress-induced ploidy shifting in putative diploids of *A. flavus* is unknown. High temperature could either induce ploidy shifting or select the diploid allowing to grow under stress of high temperature. The effects of high temperature on ploidy have been studied in plants (Pe´crix et al., 2011). High temperature can cause an increase in gamete ploidy level in Rosa sp. (Pe´crix et al., 2011). It has been proposed that heat-induced spindle mis-orientations in telophase II allow the presence of polyploidy in plant cells. Also defects in the spindle pole body leads to asymmetric DNA segregation in yeast cells (Snyder and Davis, 1988; Rose and Fink, 1987; Vallen et al., 1992). As a result of asymmetric DNA segregation, during mitosis in *S. cerevisiae* one daughter cell could have increased ploidy and another daughter cell become aneuploid. There is also evidence for auto-diploidization in haploid *Candidia albicans* induced by mitotic defects (Hickman et al., 2013). Evidence of auto-diploidization mediated by cell fusion or endo mitosis was reported for vertebrates (Yi et al., 2009, Fred et al., 1970) and stem cells (Elling et al., 2011). We hypothesized that auto-diploidization may also occur in *A. flavus*. Under normal condition, auto-diploidization could result in a small percentage of diploid conidia that may be undetectable by flow cytometry. Our hypothesis was that prolonged incubation under high temperature could induce the fusion of haploid nuclei. Fusion of haploids could increase the frequency of diploid nuclei. Under stress, the diploids may be selected and separated from haploids in the sectors. We also found that ploidy can shift during long time storage at 4^0C of diploid isolates IC274S1 and IC295S1 from their putative diploid parents

IC274 and IC295 (Fig. 3.4). Under low temperature auto-diploidization could induce fusion of diploids (2n) to generate higher ploidy in conidia (4n). Our results support a similar phenomenon of auto-diploidization that has been demonstrated in *Candida albicans* (Hickman et al., 2013). It was reported that long-term storage during the shipment of cultures of haploid *Candida albicans* allowed a transition to diploidy. Moreover, our results suggest that stress may contribute to ploidy shifts and thus allow the segregation of haploid and diploid nuclei in a single strain.

3.4.2 Effects of Ploidy on Carbon Utilization

Our second objective was to determine if diploids and haploids showed any significant difference in carbon utilization. We observed carbon utilization profiles for the 14 isolates (IC274, IC274S1, IC274S2, IC295, IC295S1, IC295S2, IC4050, IC4050S1, FR36, FR46, D2-2, IC302, 3357, IC4761). Conidia from the 14 isolates (mentioned above) of five different groups (Table 3.2) were grown in FF Microplates coated with 95 carbon sources. The average respiration values (OD_{490} nm) after 24, 48, 72 and 96 hours were measured and the data were analyzed by Agglomerative Hierarchical Clustering (AHC) and one-way ANOVA analysis. We found that after 72-hour incubation both the haploid and the diploid isolates could readily metabolize the carbon sources including monosaccharaides, disaccharides, hexoses, sugar alcohols, amino acids, organic acids and glycoside (Fig. 3.5 and 3.6). Our findings suggest that both the diploid and the haploid strains have the ability to metabolize all 95-carbon sources. For some categories of carbon sources, the OD values

varied between haploids and diploids. This phenomenon may depend on the rate that conidia germinate and begin to metabolize the carbon sources. We compared carbon utilization and growth of putative diploids (IC274, IC295, IC302, and IC4050) and their sectors (haploids IC274S2, IC295S2; diploids IC274S1, IC295S1, and IC4050S1). Our data suggest that there were significance differences in carbon utilization of cluster I carbon sources between the natural isolates and their haploid and diploid sectors (Table 3.3-3.5). The natural isolate IC274, haploid IC274S2 and the isolate IC302 can readily utilize the carbon sources than the other natural and laboratory synthesized haploids and diploids (Table 3.3-3.7). For cluster II and III carbon sources (Fig. 3.6), we observed both haploid and diploid isolates could utilize the carbon sources moderately (Supplementary Table 1-10).

The differences in single substrate utilization between haploids and diploids could depend not only on their ploidy state but also on their genetic background. In our study, we did not observe either only haploids or only the diploids were always superior in their utilization of carbon sources. For cluster I carbon sources, the natural isolates (putative diploids) were better than the other haploids. On the other hand, for cluster II and III carbon sources, the putative diploids (IC274, IC295, IC302) and their diploid sectors (274S1, 295S1), as well as the laboratory synthesized diploids (D2-2, IC4761) grew better. Differences in carbon utilization could be due to possible mutations in the putative diploids since the diploid and haploid sectors were generated under high temperature or in the presence of benomyl. We found that diploid IC274S1 was less efficient in utilizing some carbon sources and had less biomass than the original isolate IC274 and the haploid

IC274S2. The diploid IC274S1 also made less biomass than the diploids D2-2, IC295S1, IC4761 and haploids (IC295S2, FR36, FR46, 3357) and natural isolate IC302 (Fig. 3.8 and Table 3.9). It was expected that the diploid IC274S1 might have a lower fitness because the isolate could metabolize carbon sources slowly and make less biomass compared to the original isolate IC274. The variability of carbon utilization and biomass production often depends on the specific combination of alleles inherited by each haploid and diploid sector from the original natural isolates. Reverberi et al., (2013) reported that growth conditions of cultures prior to conidial production by *A. flavus* can affect the carbon utilization of conidia subsequently produced from the cultures. Since, we measured growth and observed carbon utilization profiles for the isolates of *A. flavus* only at 30^0C, it is not known whether the diploids could provide advantages over the haploids at higher or lower temperatures than 30^0C. One possibility is that the ecology of *A. flavus* could be modulated by its interaction of environmental conditions and the ploidy state of conidia.

3.4.3 Ploidy and Pathogenicity

Our third objective was to determine whether diploids are more than haploids. We found that diploids IC274S1, IC295S1 and IC4050S1 were less virulent than their respective parental isolates IC274, IC295 and IC4050 and that IC274S1 was less virulent than the haploid sector IC274S2 (Fig. 3.9 A). Since IC274S1 grew slowly *in vitro* and was less efficient in carbon utilization than the haploid sector IC274S2, we expected that IC274S1 would be less pathogenic than its haploid counterpart in *G. mellonella*. Similarly, haploids FR36 and FR46

both grew slowly *in vitro*, which might affect their virulence. In addition to growth, carbon utilization of conidia could affect their pathogenicity. We observed that the putative diploid IC274 and the haploid IC274S2 showed higher metabolism on different carbon sources and had more biomass compared to the diploid IC274S1. The isolates IC274 and IC274S2 also killed larvae earlier than the diploid IC274S1. Further, the auxotroph FR36 was less efficient in carbon utilization and was less pathogenic to *G. mellonella*. Thus, growth and the carbon utilization of both haploid and diploid conidia may substantially impact the pathogenicity of *A. flavus.*

3.4.4 Nuclear Condition, Carbon Utilization and Pathogenicity of *A. nomius*

One of our objectives was to examine the nuclear condition of *A. nomius* and its effect on carbon utilization and pathogenicity. Some strains of *A. nomius* have both mating type genes but they are not homothallic. Strain IC1516 has both mating type genes (*MAT1-1 and MAT1-2),* but the organization and location of these mating genes is not known. We observed the nuclear number in conidia by DAPI staining and found that *A. nomius* IC1516 conidia had one, two or three nuclei (Fig. 3.12), with most conidia having 2 nuclei (Fig. 3.12B). Further, ploidy analysis of the strain showed a single major peak in the fluorescence histogram (FL), which is typical for diploids (Fig. 3.12C). The unique FL histogram for *A. nomius* IC1516 suggests that it is either diploid (2n) or dikaryotic (n+n). Since the majority of conidia of *A. nomius* IC1516 have 2 nuclei, it appears that *A. nomius* IC1516 is dikaryotic (n+n).

To understand the role of the dikaryotic nuclear condition in fungal nutrient utilization, we investigated carbon utilization in *A. nomius*. For the majority of the carbon sources, there was no significant difference in carbon metabolism between *A. nomius* and *A. flavus*; however, *A. nomius* IC1516 did show higher metabolism on D-glucose than *A. flavus* NRRL 3357 and diploid D2-2 after 96-hour incubation (Fig.3.14 and Table 3.10). We speculate that *A. nomius* IC1616 could be better adapted to specific ecological niches due to its higher metabolism on monsaccharides such as D-glucose. We found that *A. nomius* was more pathogenic on *G. mellonella*. *A. nomius* is economically and medically important. The fungus can contaminate different crops with aflatoxin (Horn and Dorner, 1998, Ehrlich et al., 2007). The species has been isolated from various agricultural products including tree nuts (Olsen et al., 2008, Doster et al., 2009), sugarcane (Kumeda et al., 2003) and varities of seeds and grains (Kurtzman et al., 1987; Pitt et al., 1993; Kumeda et al., 2003). Further, it is recognized as an insect pathogen and is commonly associated with bees (Hesseltine et al., 1970, Kurtzman et al., 1987), termites (Rojas et al., 2001) and silkworm (Ito et al., 1998, Peterson et al. 2001). In addition, *A. nomius* can cause mycotic keratitis (Manikandan et al. 2009). Since monosocaharide such as D-glucose is abundant carbon source found in most of the hosts, utilization of D-Glucose, perhaps allow *A. nomius* to be opportunistic for a wide range of hosts.

3.5 Concluding Remarks

Although *A. flavus* is predominantly haploid, the ploidy state of natural isolates can shift under stress. High temperature stress can induce ploidy shifts and allow recovery of haploid and diploid conidia. Our results suggest that carbon utilization; growth and pathogenicity of diploids can vary from their parental strains. Our results also suggest that prolonged incubation at high temperature can induce or select for diploidy in sectors. It is clear that *A. flavus* ploidy is fluid and can shift under adverse conditions. Furthermore, ploidy differences may play a role in the ecology of *A. flavus* because variation in carbon utilization and growth directly impact *A. flavus* pathogenicity. It appears that the dikaryotic nuclear condition of *A. nomius* has allowed this fungus to be better adapted for nutrient utilization and pathogenicity on insects compared to *A. flavus*.

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Figures

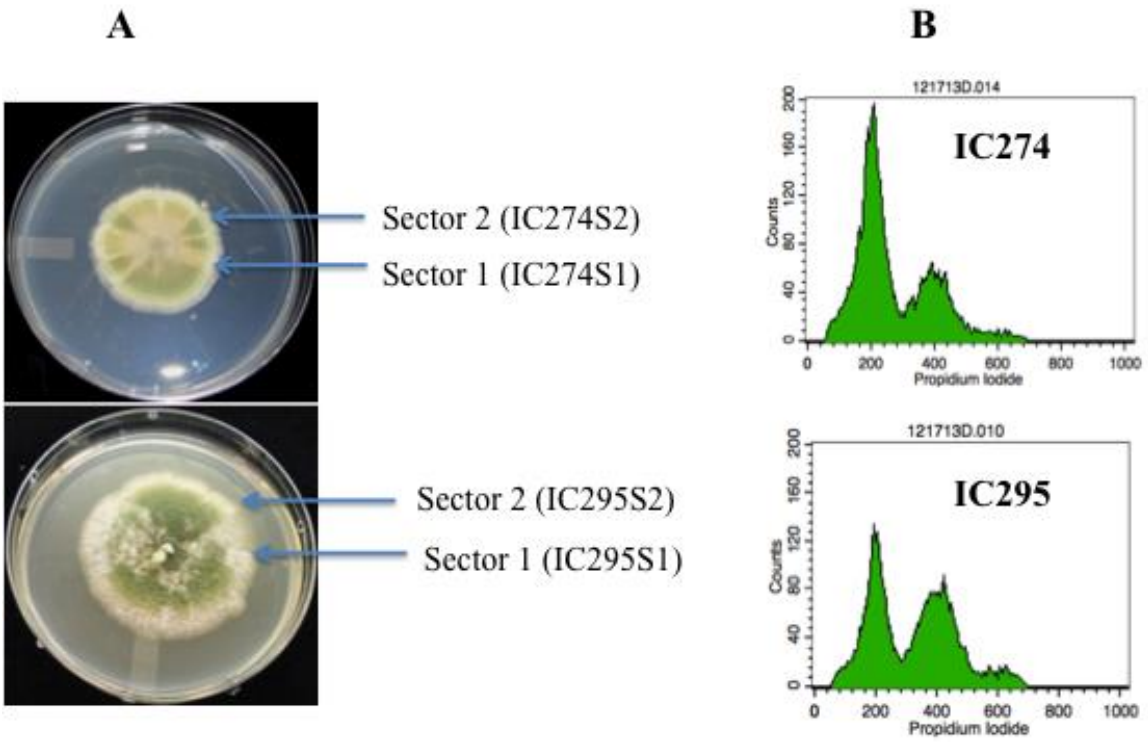


Fig. 3.1. Sectors in colonies of natural isolates of *Aspergillus flavus* under stress. (A) The natural isolate IC274 was grown on PDA at 40^0C. The natural isolate IC295 was grown on PDA+ 2.0 µg /ml benomyl at 30^0C. The isolates designated IC274S1 and IC274S2 originated from a single conidium of each sector of the isolate IC274. The isolates labeled IC295S1 and IC295S2 originated from a single conidium of each sector of the isolate IC295. (B) Flow cytometry analysis of the DNA content of conidia from the natural isolates of *A. flavus*. Conidia were collected, fixed and stained with Propidium Iodide (PI). The FL (fluorescence) histograms of the parental strains show two peaks. X- axis represents PI intensity and Y-axis represents the number of conidia.

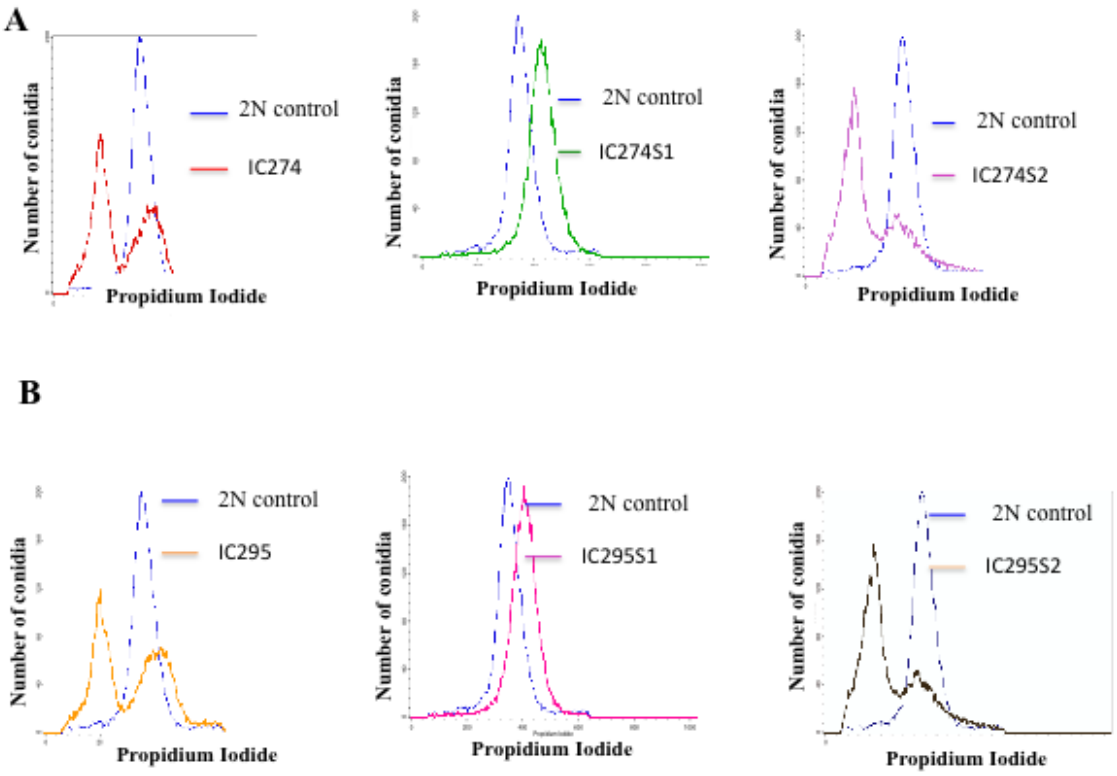


Fig. 3.2. Recovery of diploids and haploids from sectors of the natural isolates of *Aspergillus flavus*. (A) Flowcytometry analysis of DNA content of conidia from the natural isolate of *A. flavus* IC274 and its sectors (Group 1). The peaks of fluorescence (FL) histograms of isolates IC274 (red line), IC274S1 (green line) and IC274S2 (light pink line) were compared to the peak of the histogram of the diploid control IC4761 (blue line). (B) Flow cytometry analysis of the DNA content of conidia from the natural isolates of *A. flavus*, IC295 and its sectors (Group 2). Isolates IC295 (orange line), IC 295S1(magenta line) and IC295S2 (light orange line) were compared to the diploid control IC4761 (blue line). All isolates were grown on PDA at 30^0C for 7 days. Conidia from each isolate were stained with PI staining and analyzed by flow cytometry. The overlay pictures of fluorescence histograms were generated by Cell Quest Pro software.

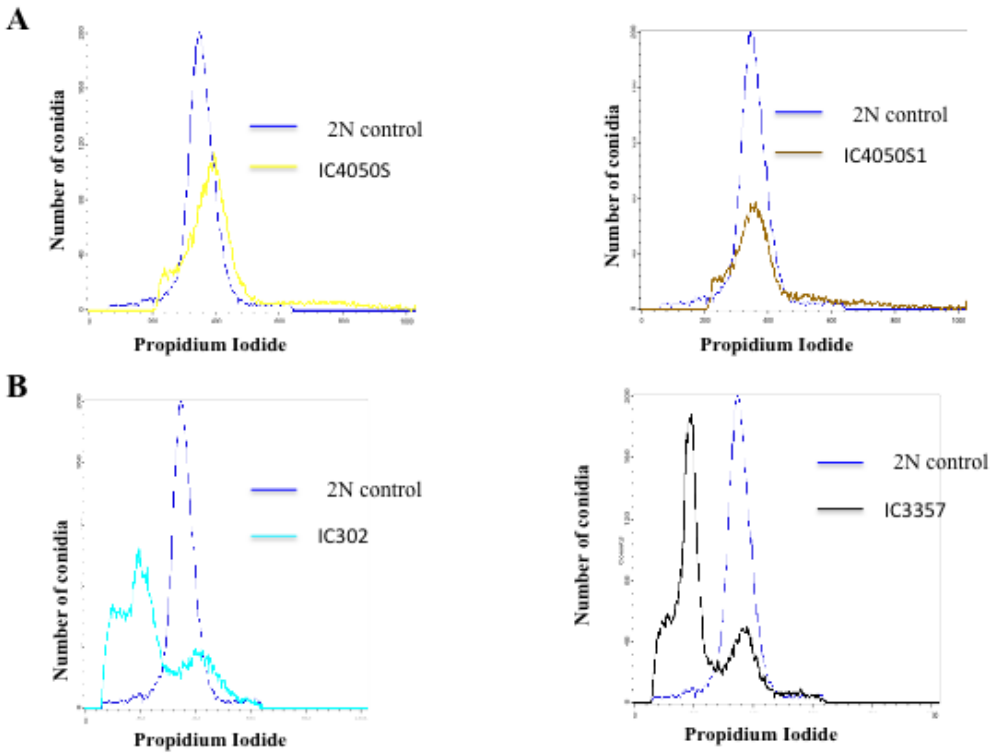


Fig. 3.3. Diploid and haploid conidia of *Aspergillus flavus* isolates. (A) Flowcytometry analysis of the DNA content of conidia of *A. flavus*, IC4050 (Group 3). The isolates, IC4050 (yellow line) and IC4050 S1 (brown line) were compared to the diploid control IC4761 (blue line). Isolate IC4050 was grown on PDA with 2.0 µg /ml benomyl to detect sectors on the growing colony. The isolate IC4050S1originated from a single conidium from the isolate IC4050. (B) Flow cytometry analysis of the DNA content of conidia from the natural isolate of *A. flavus* IC302 (sky blue line) and the genomic strain 3357 (black line). Conidia were collected from the 7-days old cultures grown on PDA at 30^0C for flow cytometry analysis.

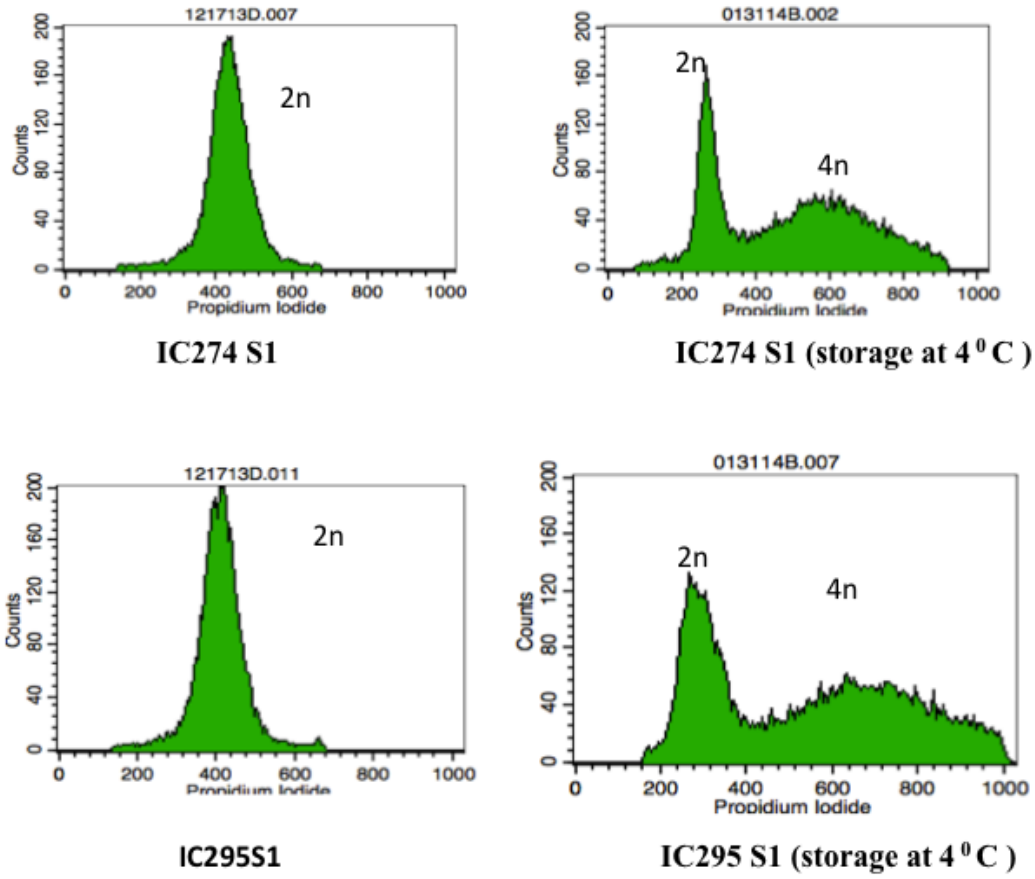
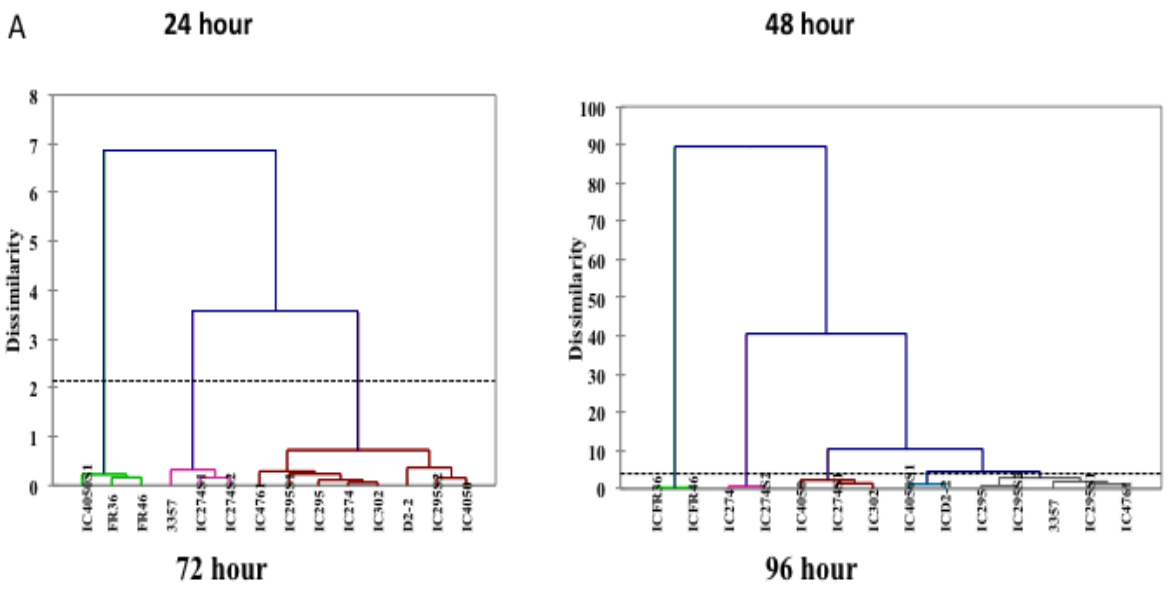
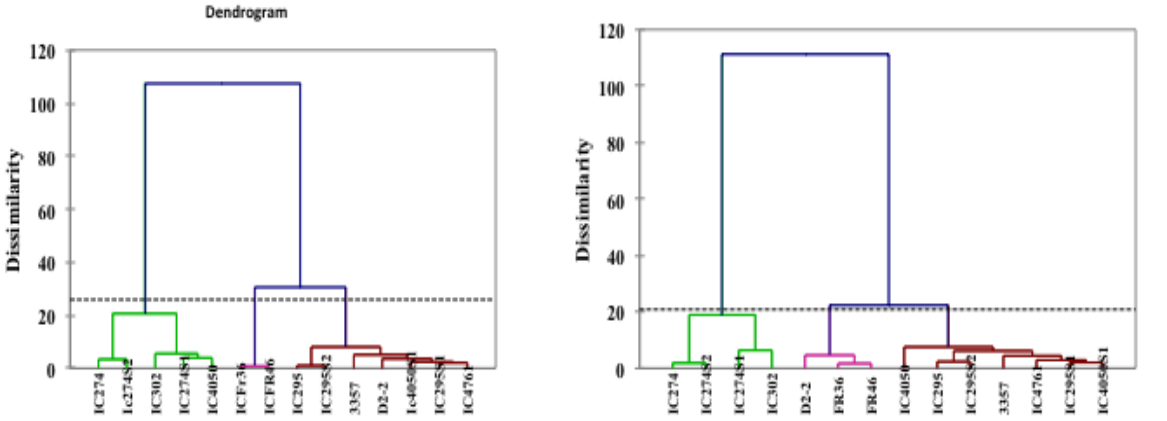


Fig. 3.4. Flow cytometry analysis of DNA content of conidia from the diploids of *A. flavus* after storage of low temperature. The diploid isolates were kept at 4^0C for 4-5 weeks and conidia were collected, fixed, and stained with Propidium Iodide (PI). The diploid Conidia 2n and 4n were observed after long time storage of low temperature. X- axis represents PI intensity and Y-axis represents the number of conidia.

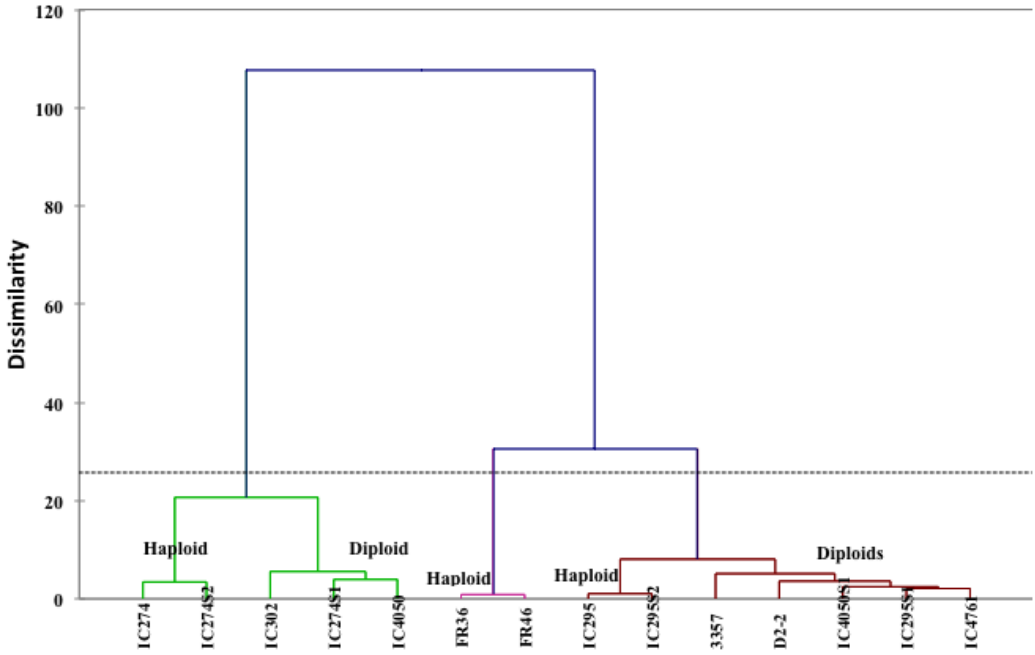
Fig. 3.5. Agglomerative Hierarchical Clustering (AHC) based on carbon source utilization profiles. (A) Agglomerative Hierarchical Clustering (AHC) for fourteen isolates grown on 95 carbon sources after incubation of 24, 48, 72 and 96 hours. Conidia collected from the 14 strains including the haploids and the diploids were grown on FF Micro-Plates (Biolog). Carbon utilization profiles were shown after 24, 48, 72 and 96-hour incubation. The dendrograms show the progressive grouping of the data among the different isolates of *A. flavus*. The gray horizontal lines show distance measures of the carbon source utilization profiles and metabolic similarity between the haploids and the diploids at different incubation times. (B) Clustering was shown only for the 72 hour incubation time point. The dendrogram shows the major two clusters in which the haploids and diploids are grouped based on metabolic similarity.

A


24 hour
48 hour
72 hour
96 hour
Dissimilarity
FR36
FR46
3357
D2-2


Dendrogram
Dissimilarity
IC274
IC302
IC4050
IC295
3357
D2-2
FR36
FR46

B


120
100
80
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40
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Dissimilarity
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Haploid
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Diploids
IC274
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FR46
IC295
IC29552
3357
D2-2
IC40506
IC2955
IC4761

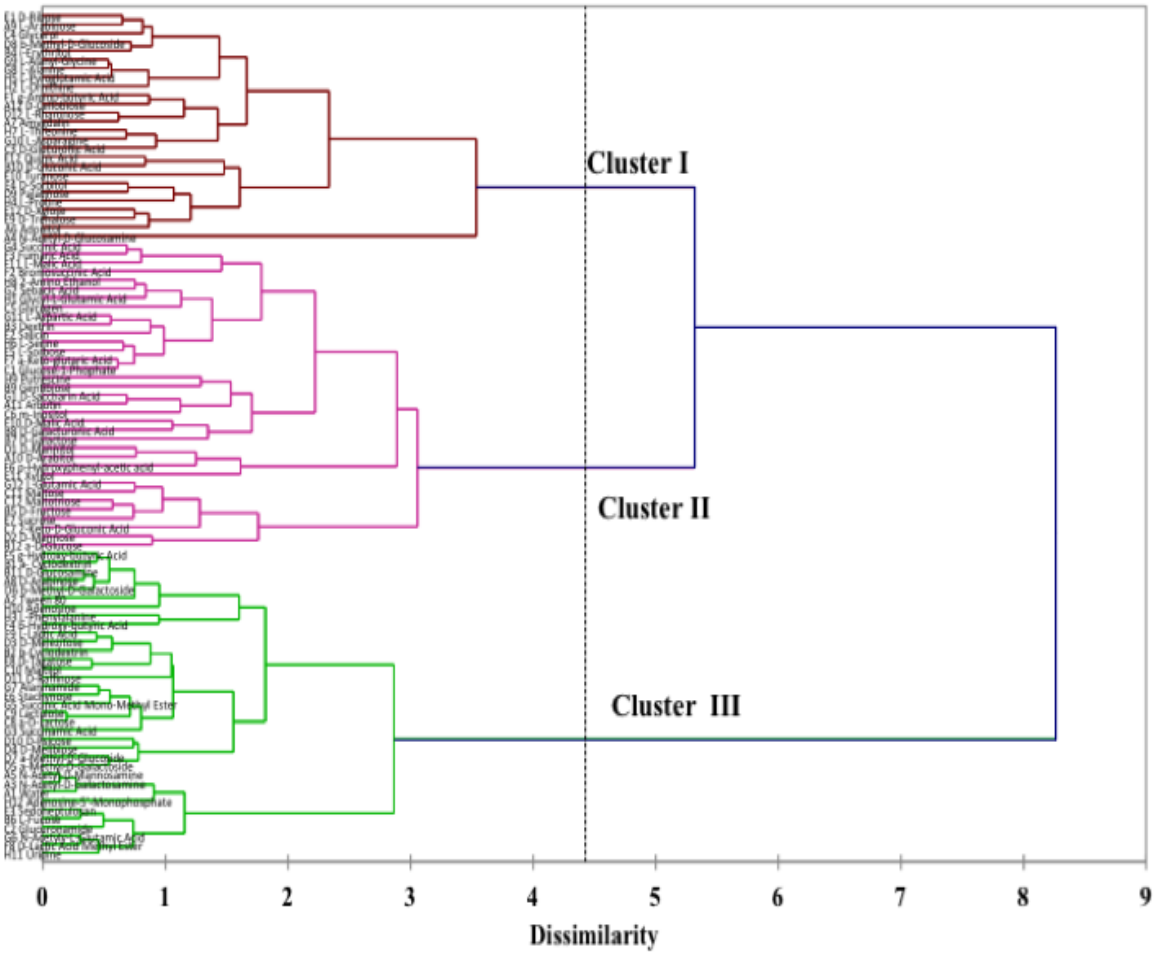


Fig. 3.6. Agglomerative Hierarchical Clustering (AHC) for carbon sources after 72-hour incubation. AHC was performed using Euclidean distance by complete linkage analysis. The dendogram shows three distinct clusters of the carbon sources utilized by the haploid and the diploid strains. Cluster I describes the 25 best-utilized carbon sources (25 carbon). Cluster II shows 35 carbon sources. Cluster III contains 34 carbon sources and water. The respiration values for the cluster carbon I carbon sources are higher than the cluster II and III carbon sources.

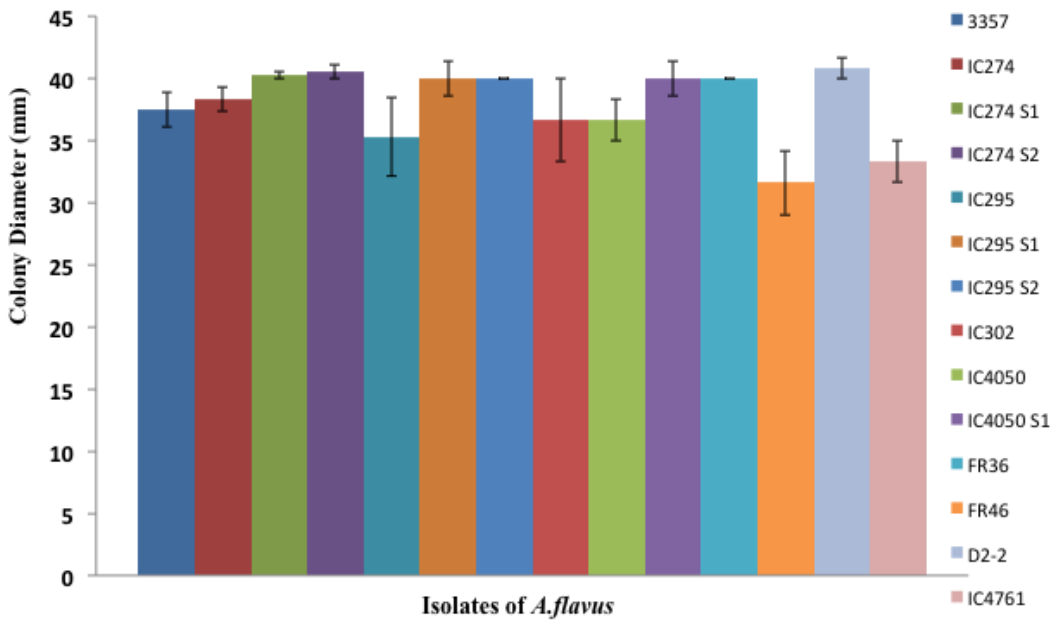


Fig. 3.7. Growth of the haploids and the diploids on solid media after 72 hours-incubation. Fourteen isolates of *A. flavus* were grown on PDA with or without uracil. The isolates were grown for seven days at 30°C and the diameter of each colony for each isolate were measured. Three replicates were used for each strain and each replicate contained three plates. Bars represent the standard error of three replicates.

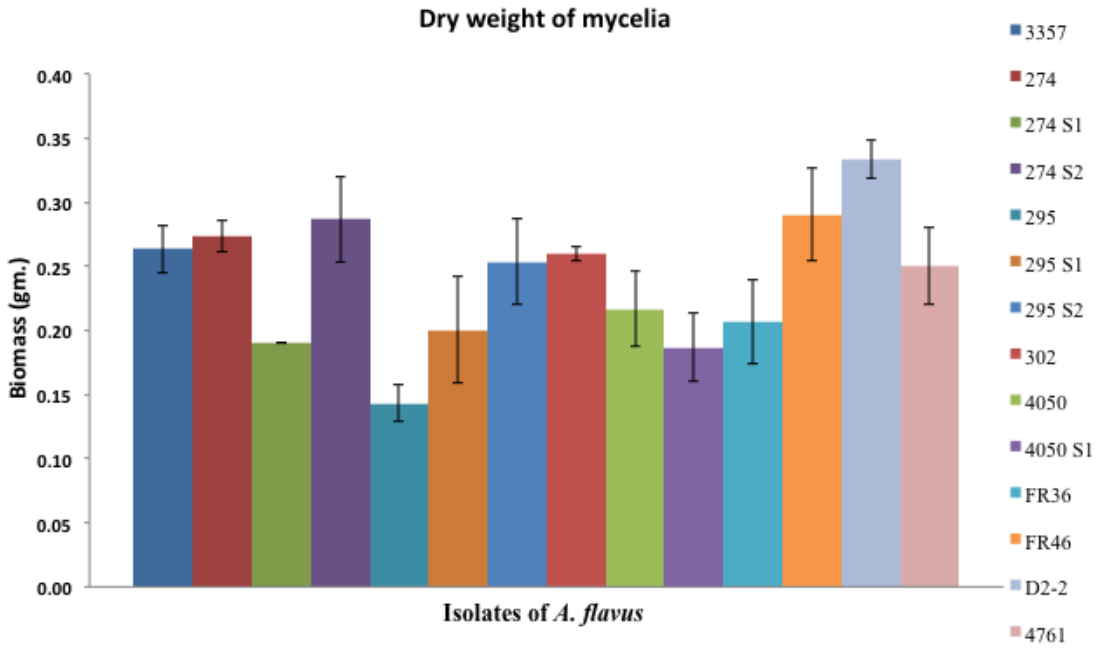


Fig. 3.8. Biomass (dry weight of mycelia) assay of the haploids and the diploids after 72 hours-incubation. Fourteen isolates of *A. flavus* were grown in PDB with or without uracil. Five hundred microliters of 10^6 spores/ml were added in 75 ml PBD and grown into 125 ml flasks. Isolates were grown at 200 rpm for 5 days at 30°C. Dry weight of mycelia was measured after filtration and lyophilization of mycelia. Three replicates were used for each isolate. Bars represent standard error among average of three replicates.

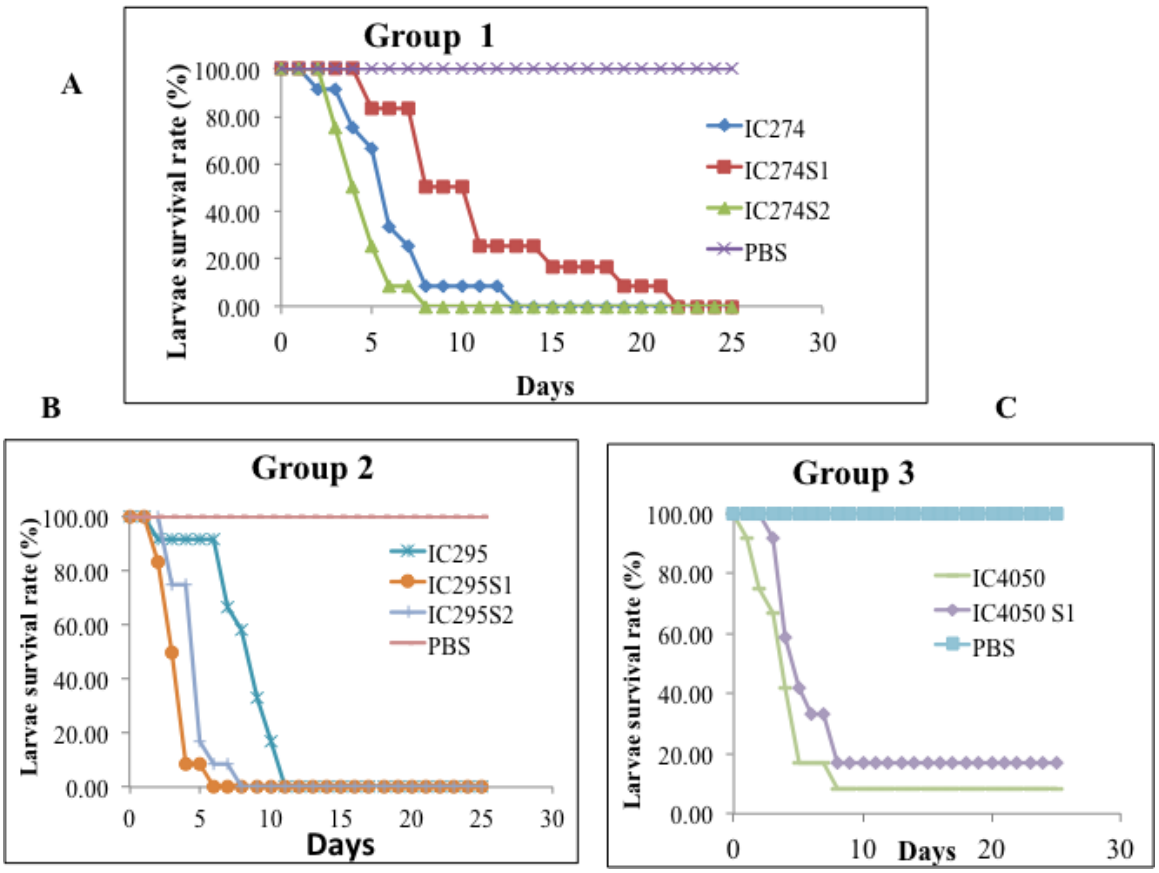


Fig. 3.9. Comparison of pathogenicity between the parental strains and the sectors on *Galleria mellonella* model. Five microliters of 10^6 spores/ml were injected per caterpillar. *G. mellonella* survival versus days (post inoculation) were plotted. (A) The diploid sector IC274S1 showed less virulence than the haploid sector IC274S2 ($p = 0.022$). The isolate IC274 and its haploid sector IC274S2 showed similar virulence ($p = 0.544$). (B) The isolate IC295 and its diploid sector IC295S1 ($p = 0.588$) and haploid sector IC295S2 ($p = 0.233$) showed similar virulence. (C) The isolate IC4050 and its sector IC4050S1 show no significance difference in pathogenicity ($p = 0.1813$).

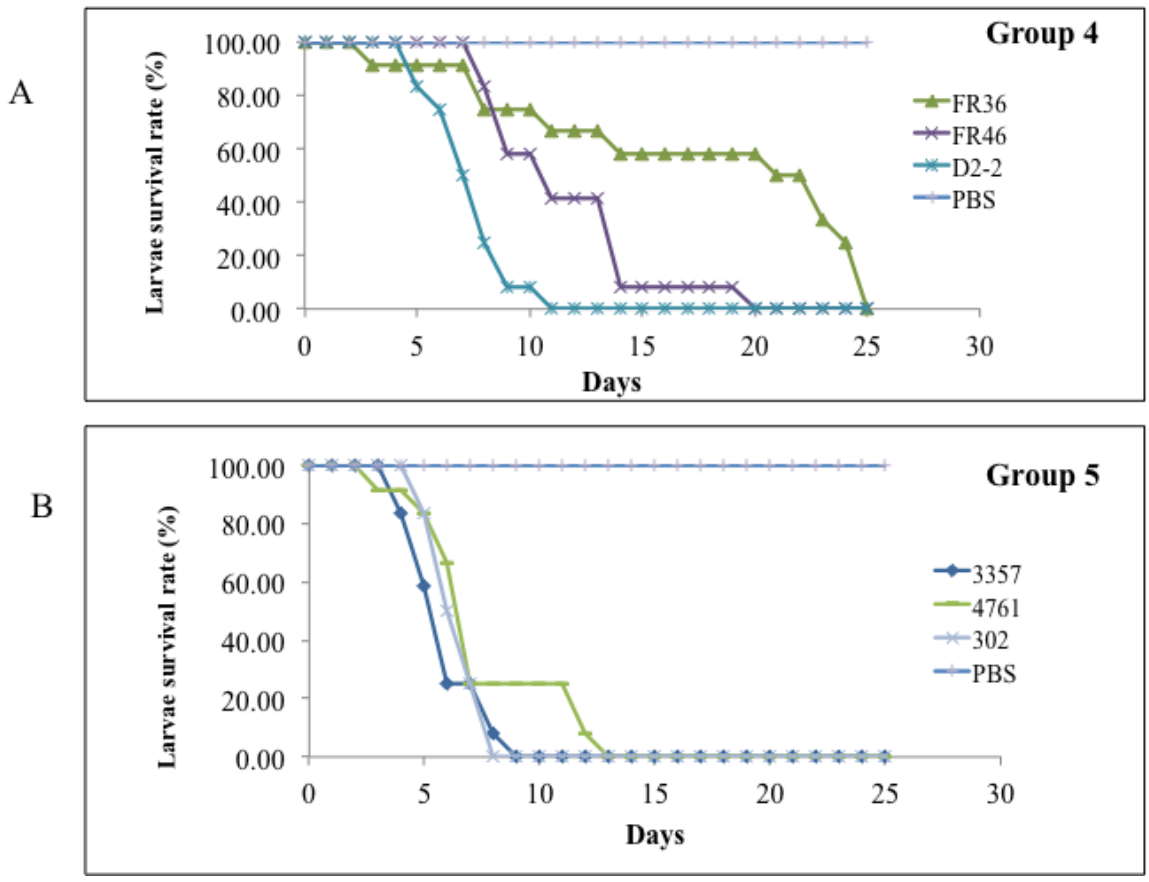


Fig. 3.10. Comparison of pathogenicity between the haploids and the diploids on *Galleria mellonella* model. Five microliter of 10^6 spores/ml were injected per caterpillar. *G. mellonella* survival versus days (post inoculation) were plotted. (A) The diploid D2-2 was more pathogenic than the haploid parental strain FR36 ($p = 0.00024102$) but not significantly different from the haploid FR46 ($p = 0.17204597$). (B) No significant difference was observed for virulence of the isolate IC302 ($p = 0.841744112$) and the diploid IC4761 ($p = 0.55752419$) compared to *A. flavus*. The natural isolate IC302 and the diploid IC4761 show no significant difference ($p = 0.712536674$).

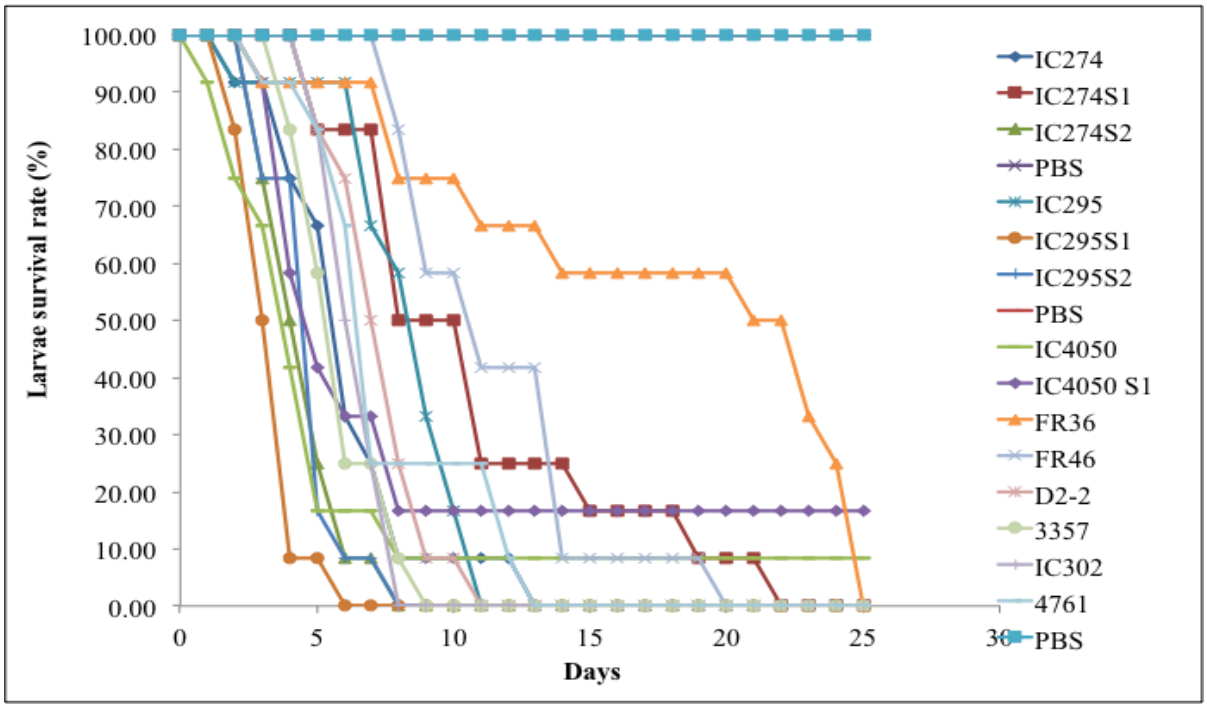


Fig. 3.11. Comparison of pathogenicity of the genomic strain 3357 with the diploids and the other haploids on *Galleria mellonella*. Five hundred microliter of 10^6 spores/ml were injected per caterpillar. The *G. mellonella* survival versus days (post inoculation) was plotted. The genomic strain 3357 was more virulent than the haploid FR36 (Arginine requiring auxotroph, p = 1.42982E-05). The diploids, D2-2 (p = 0.609814373), IC274S1 (p = 0.082715421), IC295S1 (p = 0.332456379), IC4050 (p = 0.918948968), IC4050 S1 (p = 0.307348169) and the haploids, IC274 (p = 0.9274492), IC274S2 (p = 0.619184301), IC295 (p = 0.4290959440), IC295S2 (p = 0.668436943) had similar virulence with the genomic strain 3357.

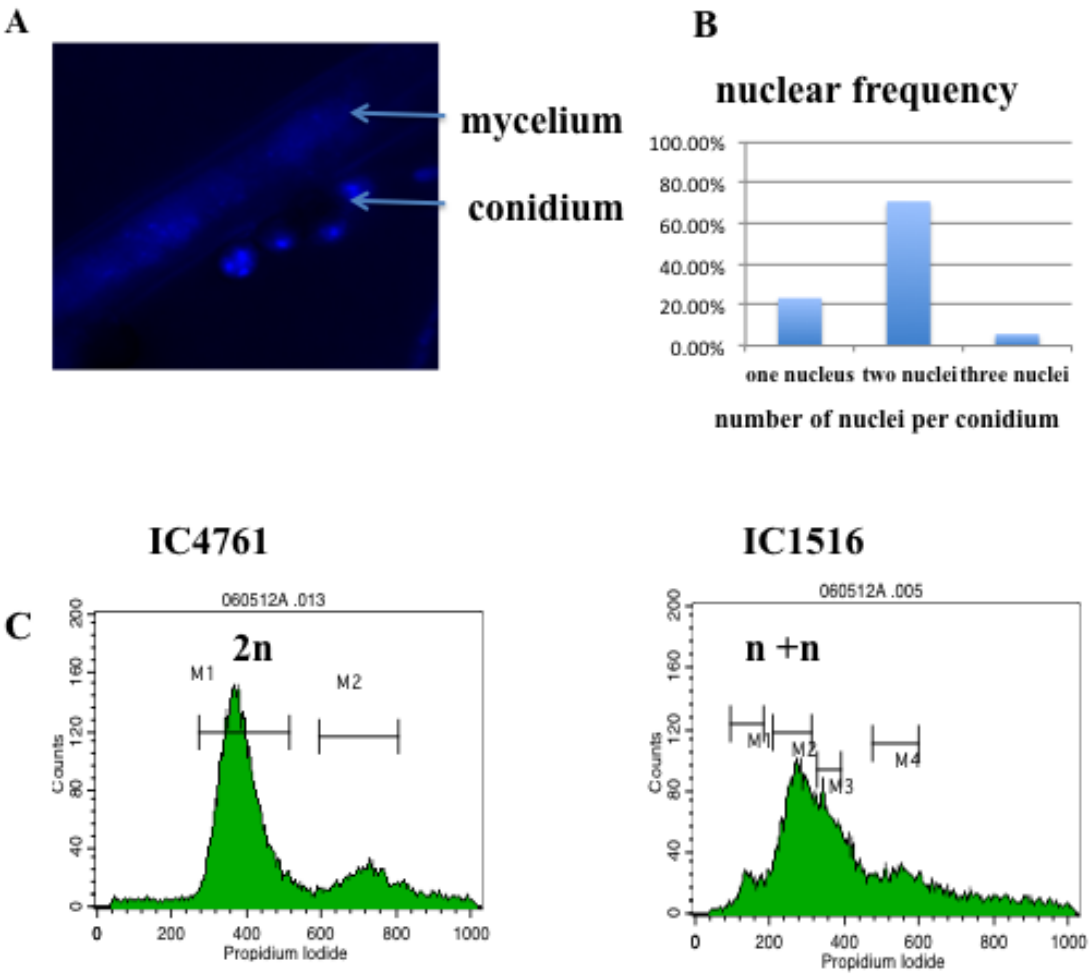


Fig. 3.12. Observation of nuclei and ploidy in *Aspergillus nomius* IC1516. (A) Multinucleate conidia of *A. nomius*. Conidia have one, two or three nuclei. (B) Percentage of conidia having one, two or three nuclei per conidium. Spores were collected from the 5-day-old cultures and were stained with DAPI. Approximately 500 conidia were observed under twenty fields using DAPI filter. Conidia were observed at 100X magnification. (C). Flow cytometry analysis of DNA content of conidia from the diploid control A. *flavus* IC4761 and *A. nomius* IC1516.

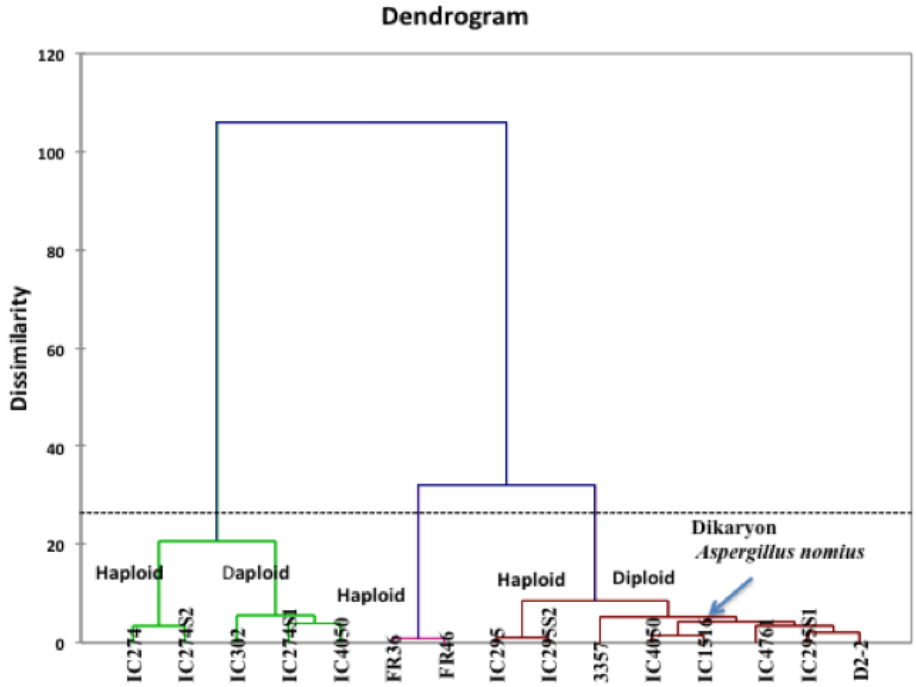


Fig. 3.13. Agglomerative Hierarchical Clustering (AHC) on the carbon source utilization profiles for the isolates of *A. flavus* and *A. nomius*. Clustering was shown only for the 72-hour incubation time point. The dendrogram shows that IC1516 was similar to the other diploids in the cluster. The carbon metabolism profiles for the isolates of IC274, IC274S1, IC274S2, IC295, IC295S1, IC295S2, FR-36, FR-46, IC4050, IC4050S1, IC302, IC4761 and 3357 were combined to carbon metabolism profile of *A. nomius* 1516

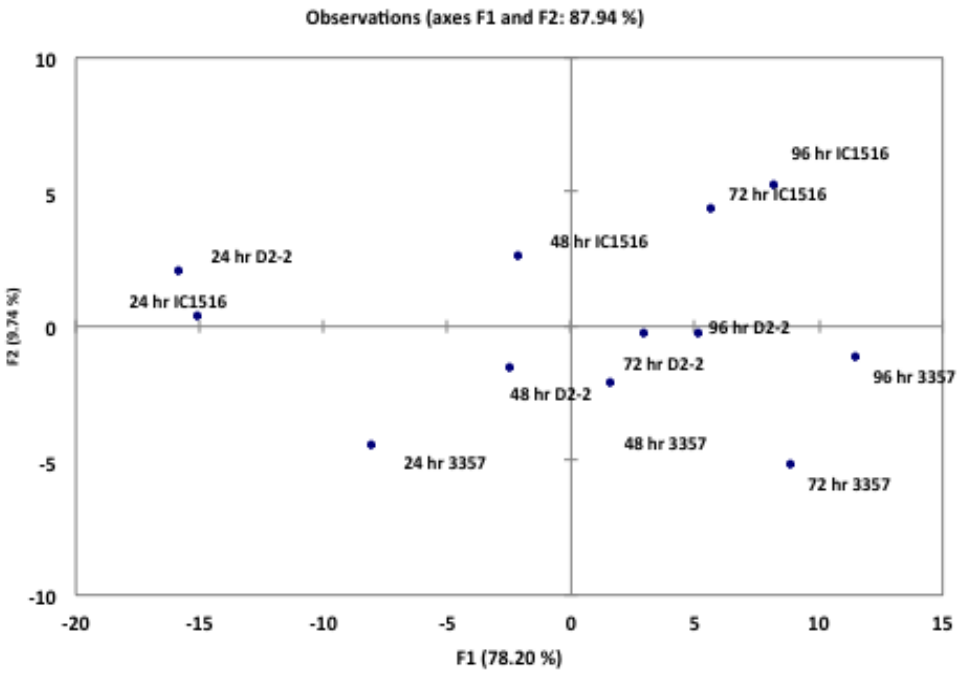


Fig. 3.14. A scatter plot from the Factor Analysis of the carbon utilization profiles of *A. flavus and A. nomius.* Carbon utilization profiles were investigated for *A. nomius* and *A. flavus* 3357, and the diploid isolate *A. flavus* D2-2 after 24, 48, 72 and 96 hour incubation. Factor analysis was performed using Pearson correlation and Principle Component Extraction (PCA) method.

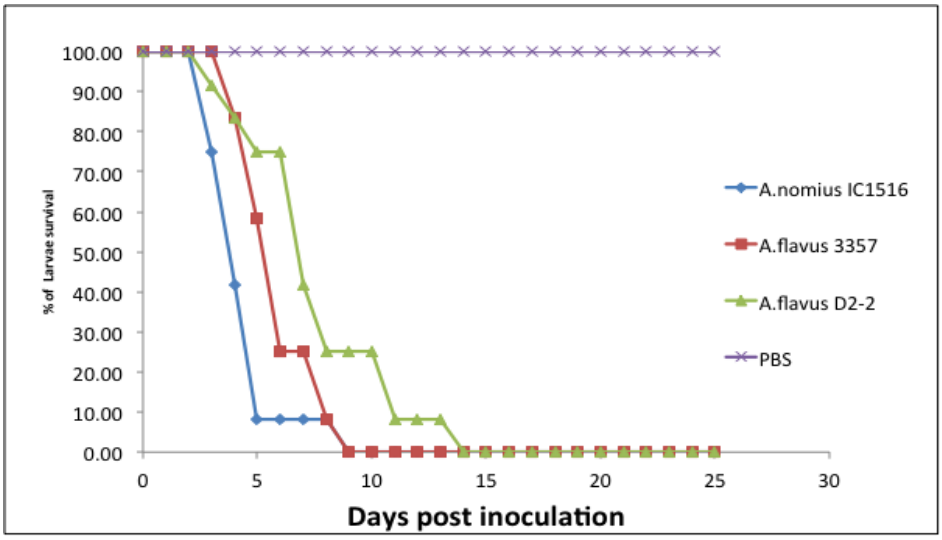


Fig. 3.15. Comparison of pathogenicity *A. nomius* IC1516 and *A. flavus* diploid D2-2 on *Galleria mellonella*. Isolates of *A. nomius* IC1516, *A. flavus* NRRL 3357 and *A. flavus* D2-2 were injected with 5ul of 10^6 spores/ml per caterpillar. The *G. mellonella* survival versus days (post inoculation) was plotted. No significant difference was observed in pathogenicity of *A. nomius* IC1516 (p=0.574) and *A. flavus*. *Aspergillus nomius* IC1516. *A. nomius* and *A. flavus* D2-2 showed similar virulence (p=0.238).

Tables

Table 3.1. *Aspergillus flavus and Aspergillus nomius* strains used in this study.

Strain	References
A. flavus 3357	NRRL
A. flavus IC274	Georgia, USA
A. flavus IC275	Georgia, USA
A. flavus IC295	Georgia, USA
A. flavus IC302	Georgia, USA
A. flavus IC4050	Georgia, USA
A. flavus IC4761	Woloshuk et al., 1995
A. flavus FR36	Runa, et al., 2014
A. flavus FR46	Runa, et al., 2014
A. flavus D2-2	Runa, et al., 2014
A. nomius IC1516	Concordia Parish, Louisiana, USA

Table 3.2. Ploidy and nuclear number of nuclei *Aspergillus flavus* strains

Group No.	Isolates	Nuclei in conidia	Ploidy Detected by flow cytometry
Group 1			
Natural isolate (putative diploid)	IC274	1-2	Haploid (N, N+N)
Sectors	IC274S1	1	Diploid (2N)
	IC274S2	1-2	Haploid (N, N+N)
Group 2			
Natural Isolate	IC295	1-2	Haploid (N, N+N)
Sectors	295S1	1	Diploid (2N)
	295S2	1-2	Haploid N, N+N)
Group 3			
Lab synthesized	IC4050	1-2	Putative diploid (N, 2N)
Sectors	IC4050S1	1, a very few 2	Diploid (2N)
Group 4			
Haploid Strains	FR36	1-2	Haploid (N, N+N)
	FR46	1-2	Haploid (N, N+N)
Lab synthesized	D2-2	1	Diploid (2N)
Group 5			
Natural Isolate (putative diploid)	NRRL3357	1-3	Haploid (N, N+N)
	IC302	1-3	Haploid (N, N+N)
Laboratory synthesized diploid (control)	IC4761	1	Diploid (2N)

Table 3.3. One -way ANOVA for Cluster I carbon sources after 72 hour for Group1 isolates. The ANOVA followed by Tuckey's HCD t-test was performed based on respiration values (OD_{490} readings) after 72-hour incubation. Statistically significant differences ($p<0.001$) in the substrate use between the strains are marked with different letters (A, B, C, D, E, F).

Carbon sources	IC274		IC274S1		IC274S2	
Monosaccharide						
N-Acetyl-D-Glucosamine	3.723	A	2.669	AB	3.536	A
L-Arabinose	2.696	A	2.220	ABC	2.904	A
D-Ribose	2.459	ABC	2.257	ABCD	2.785	A
D-Xylose	2.834	AB	2.720	ABCD	3.171	ABC D
Disaccharide						
D-Cellobiose	2.758	A	2.318	ABC	2.841	A
Palatinose	2.821	A	2.174	ABCD	2.717	A
Turanose	3.256	A	2.704	AB	3.385	A
Deoxy hexose						
L-Rhamnose	2.783	A	2.179	ABCDE	2.738	A
Sugar alcohol						
Adonitol	2.678	A	2.165	AB	2.587	A
i-Erythritol	2.743	A	2.480	ABC	2.358	ABC D
Glycerol	2.779	A	2.350	A	2.888	A
D-Sorbitol	2.798	A	2.626	ABCD	2.910	A
Amino acid						
L-Alanine	2.500	A	1.689	DEF	2.141	AB
L-Alanyl-Glycine	2.502	A	1.978	AB	2.306	A
L-Asparagine	2.314 2.285	A ABC	1.883	ABCDE	2.192	ABC D
L-Ornithine	2.055	A	1.778	A	2.088	A
L-Proline	2.635	AB	1.966	CDE	2.881 2.795	A AB
L-Threonine	2.367	ABC	1.874	ABCD	2.667 2.603 2.470	A AB ABC
Organic acid						
D-Gluconic	3.315	A	2.242	ABCD	3.002	AB
D-Glucuronic Acid	2.184	A	2.189	ABCD	2.832	A
g-Amino-butyric Acid	2.935	A	2.167	BCDEF	2.975	A
L-Pyroglutamic Acid	2.439 2.379	A AB	2.057	ABC	2.205	ABC

Table 3.3. (Continued)

Quinic Acid	2.981	A	2.027	BCDE	3.096	A
Glycoside						
b-Methyl-D-Glucoside	2.765	A	2.317	ABC	2.777	A
Amygdalin	2.690	A	2.327	ABCD	2.716	A

Table 3.4. One -way ANOVA for Cluster I carbon sources after 72 hour for Group 2 isolates.

Carbon sources	IC295		IC295S1		IC295S2	
Monosaccharide						
A4 N-Acetyl-D-Glucosamine	2.439	ABC	2.438	ABC	2.031	BC
L-Arabinose	1.959	ABCD	1.856	BCD	2.025	ABCD
D-Xylose)	1.776	F	1.994	DEF	1.862	EF
Disaccharide						
D-Cellobiose	1.540	BCDE	1.501	CDE	1.396	DE
Palatinose	1.717	CD	1.804	BCD	1.548	D
Turanose	1.838	BCD	1.780	BCDE	1.684	CDE
Deoxy hexose						
Rhamnose	1.669	EF	1.846	DEF	1.731	DEF
Sugar alcohol						
i-Erythritol	1.846	BCD	2.092	ABCD	1.837	CD
D-Sorbitol	1.938	DE	1.952	DE	1.821	D
Aminio acid						
L-Alanine	1.715	BC	1.948	AB	1.790	BC
L-Asparagine	1.787	CDE	1.578	EFG	1.834	BCDE
L-Proline	1.708	DE	2.159	BCD	1.617	DE
L-Threonine	1.720	ABCD	1.557	BCD	1.737	ABCD
Acid group compound						
D-Glucuronic Acid	1.639	ABCD	1.355	BCD	1.877	ABCD
g-Amino-butyric Acid	1.412	FGH	2.168	BCDE	1.426	FGH
L-Pyroglutamic Acid	1.769	ABCD	1.721	ABCDE	1.796	ABC
Quinic Acid	2.052	BCD	1.512	EF	1.473	EF
Glycoside						
Amygdalin	1.527	DE	1.604	CDE	1.580	CDE

Table 3.5. One-way ANOVA for Cluster I carbon sources after 72 hour for Group 3 isolates.

Carbon Sources	IC4050		IC4050S1	
Monosaccharide				
D-Ribose	1.817	DE	1.978	CDE
D-Xylose	2.340	BCDE	2.180	CDEF
Disaccharide				
D-Cellobiose	2.096	ABC	1.726	BC
Palatinose	2.038	BCD	2.278	ABC
Turanose	2.470	AB	1.966	BC
Deoxy hexose				
L-Rhamnose	1.541	EF	1.896	CDE
Sugar alcohol				
Adonitol	2.508	A	2.075	AB
i-Erythritol	2.046	ABCD	1.780	D
D-Sorbitol	2.129	BCD	2.342	ABC
Aminio acid				
L-Asparagine	1.630	EF	1.466	FG
L-Ornithine	1.823	A	1.739	AB
L-Proline	2.011	CD	1.876	CDE
L-Threonine	1.744	ABCD	1.461	CD
Organic acid				
D-Gluconic	1.885	BCD	1.548	CD
Quinic Acid	1.842	DE	1.842	CDE
L-Pyroglutamic Acid	1.853	ABC	1.679	ABCDE
Glycoside				
Amygdalin	1.838	BCD	1.621	CDE

Table 3.6. One -way ANOVA for Cluster I Carbon sources after 72 hours-incubation for Group 4 isolates.

Carbon sources	FR36		FR46		D2-2	
Monosaccharide						
N-Acetyl-D-Glucosamine	1.682	BC	1.401	C	2.065	BC
L-Arabinose	1.340	DE	0.892	E	1.610	CDE
D-Ribose	1.145	E	1.176	F	1.624	F
D-Xylose	1.573	F	1.583	F	1.852	EF
Disaccharide						
D-Cellobiose	1.067	EF	0.746	F	1.253	DEF
Palatinose	1.411	D	1.684	CD	1.576	D
Turanose	1.161	DE	1.045	E	1.460	CDE
Deoxy hexose						
L-Rhamnose	0.667	G	0.659	G	1.398	F
Sugar alcohol						
Adonitol	1.651	BC	1.442	C	1.802	BC
i-Erythritol	0.967	E	0.767	E	1.611	D
D-Sorbitol	1.893	CD	1.873	CD	1.705	D
Aminio acid						
L-Alanyl-Glycine	0.859	C	1.050	C	1.357	BC
L-Asparagine	0.882	I	1.059	HI	1.255	GH
L-Ornithine	1.377	AB	1.048	B	1.323	AB
L-Proline	1.557	DE	1.417	E	1.349	E
L-Threonine	0.901	DE	0.646	E	1.245	DE
Organic acid						
D-Gluconic Acid	1.116	D	1.089	D	1.418	CD
D-Glucuronic Acid	0.799	D	0.778	D	1.221	CD
g-Amino-butyric Acid	1.013	HI	0.769	I	1.251	GHI
L-Pyroglutamic Acid	1.050	E	1.089	DE	1.448	BCDE
Glycoside						
b-Methyl-D-Glucoside	1.087	C	1.034	C	1.542	BC
Amygdalin	0.802	F	0.669	F	1.441	E

Table 3.7. One -way ANOVA for Cluster I Carbon sources after 72 hour for Group 5 isolates.

Carbon sources	IC302		3357		IC4761	
Monosaccharide						
N-Acetyl-D-Glucosamine	3.448	A	1.993	BC	2.559	AB
L-Arabinose	2.637	AB	1.904	ABCD	1.759	BCD
D-Ribose	2.584	AB	2.046	BCDE	1.778	DE
D-Xylose	2.906	AB	1.848	EF	1.716	F
Disaccharide						
D-Cellobiose	2.410	A	1.351	DEF	1.499	CDE
Palatinose	2.630	AB	1.916	BCD	1.753	BCD
Turanose	2.708	AB	1.852	BCD	1.623	CDE
Deoxy hexose						
L-Rhamnose	2.338	ABCD	2.078	BCDE	1.574	EF
Sugar alcohol						
Adonitol	2.323	AB	1.750	BC	1.650	BC
i-Erythritol	2.582	ABC	1.775	D	1.731	D
Glycerol						
D-Sorbitol	2.665	A	1.990	BCD	1.851	D
Amino acid						
L-Alanine	2.074	AB	1.624	BC	1.673	BC
L-Alanyl-Glycine	2.172	A	1.726	AB	1.683	AB
L-Asparagine	1.731	DEF	1.462	FG	1.439	FG
L-Ornithine						
L-Proline	2.290	ABC	1.796	CDE	2.030	CD
L-Threonine	1.957	ABCD	1.693	ABCD	1.389	CDE
Organic acid						
D-Gluconic Acid	2.602	AB	1.334	CD	1.688	BCD
D-Glucuronic Acid	2.194	ABC	1.476	ABCD	1.043	D
g-Amino-butyric Acid	2.302	ABCD	1.608	EFG	1.529	FGH
L-Pyroglutamic Acid	2.140	ABC	1.379	CDE	1.457	BCD
Quinic Acid	2.631	AB	1.287	F	1.419	EF
Glycoside						
b-Methyl-D-Glucoside	2.447	A	1.817	ABC	1.684	ABC
Amygdalin	2.427	AB	1.808	BCDE	1.505	E

Table 3.8. One-way ANOVA for diameter of colonies after 72-hour incubation.

Isolates of *A. flavus*	LS Means (colony diameter, mm)	
Group 1		
IC274	36.667	AB
IC274S1	40.833	A
IC274S2	40.00	A
Group 2		
IC295	35.00	AB
IC295S1	40.00	A
IC295S2	40.00	A
Group 3		
IC4050	36.667	AB
IC4050S1	40.00	A
Group 4		
FR36	40.00	A
FR46	28.00	B
D2-2	40.00	A
Group 5		
IC3357	36.667	AB
IC302	36.667	AB
IC4761	33.33	AB

Table 3.9. One-way ANOVA for dry weight of mycelia after 72-hour incubation

Isolates of *A. flavus*	LS Means (dry weight of mycelia, gm)	
Group 1		
IC274	0.273	ABC
IC274S1	0.190	BC
IC274S2	0.287	AB
Group 2		
IC295	0.143	AB
IC295S1	0.200	A
IC295S2	0.253	ABC
Group 3		
IC4050	0.217	ABC
IC4050S1	0.187	BC
Group 4		
FR36	0.190	BC
FR46	0.290	AB
D2-2	0.333	A
Group 5		
IC3357	0.263	ABC
IC302	0.260	ABC
IC4761	0.250	ABC

Table. 3.10. One -way ANOVA for carbon source utilization of *A. nomius* and *A. flavus* after 24, 48, 72 and 96-hour incubation.

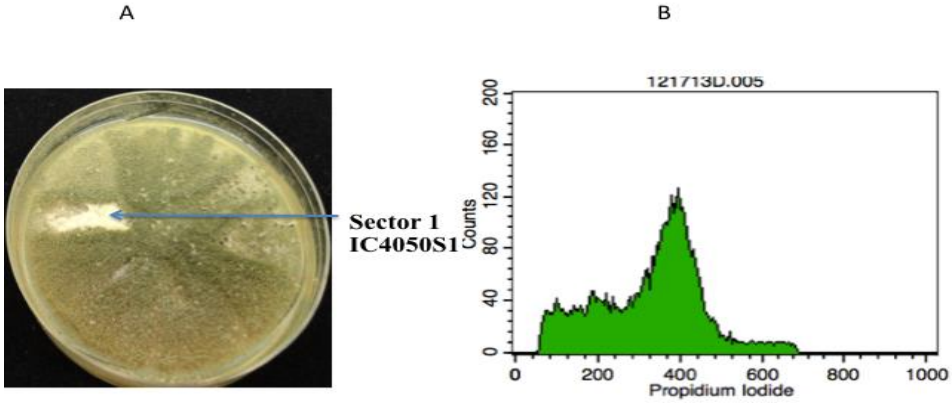
Carbon Sources	IC1516		3357		D2-2	
24 hour incubation						
Bromosuccinic Acid	0.427 0.295 0.224	AB B B	0.460	A	0.310	B
Fumaric Acid	0.507 0.347 0.288	AB AB B	0.531	A	0.344	B
N-Acetyly-L-Glutamic Acid	0.490 0.371 0.264	AB AB B	0.560	A	0.368	AB
L-Ornithine	0.528 0.440 0.322	AB B B	0.702	A	0.450	AB
Uridine	0.344 0.329 0.208	AB AB B	0.543	A	0.410	AB
48 hour incubation						
m-Inositol	0.859	AB	1.146	A	0.728	B
Sucrose	1.705	A	1.524	A	1.294	B
72 hour incubation						
L-Rhamnose	2.243	A	2.078	A	1.398	B
Xylitol	1.037 1.028 0.875	AB AB B	1.479	A	0.835	B

Table 3.10. (Continiued)

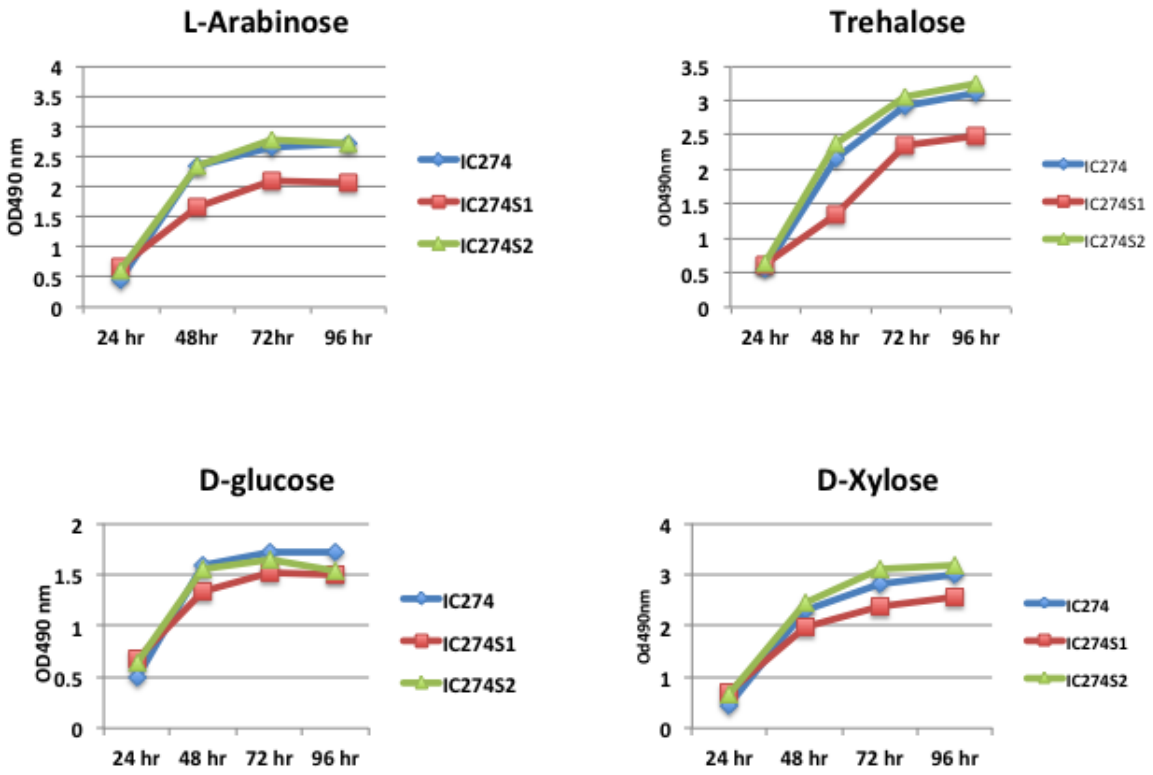
Carbon Sources	IC1516		3357		D2-2	
D-Xylose	2.246 2.244 1.894	A A B	1.852	B	1.848	B
Sebacic Acid	1.167 1.160 0.277	A A C	0.953	AB	0.809	B
96 hour incubation						
a-D-Glucose	1.811 1.650 1.623	A AB AB	1.406	B	1.421	AB
i-Erythritol	2.119 2.008 1.963	A AB AB	2.176	A	1.860	B
Salicin	1.638 1.595 1.525	A A A	1.6443	A	1.093	B
L-Sorbose	2.359 2.353 2.265	A A A	2.359	A	1.333	B
Xylitol	1.380 1.291 1.189	AB AB B	2.279	A	0.950	B

APPENDIX

Supplementary Figures

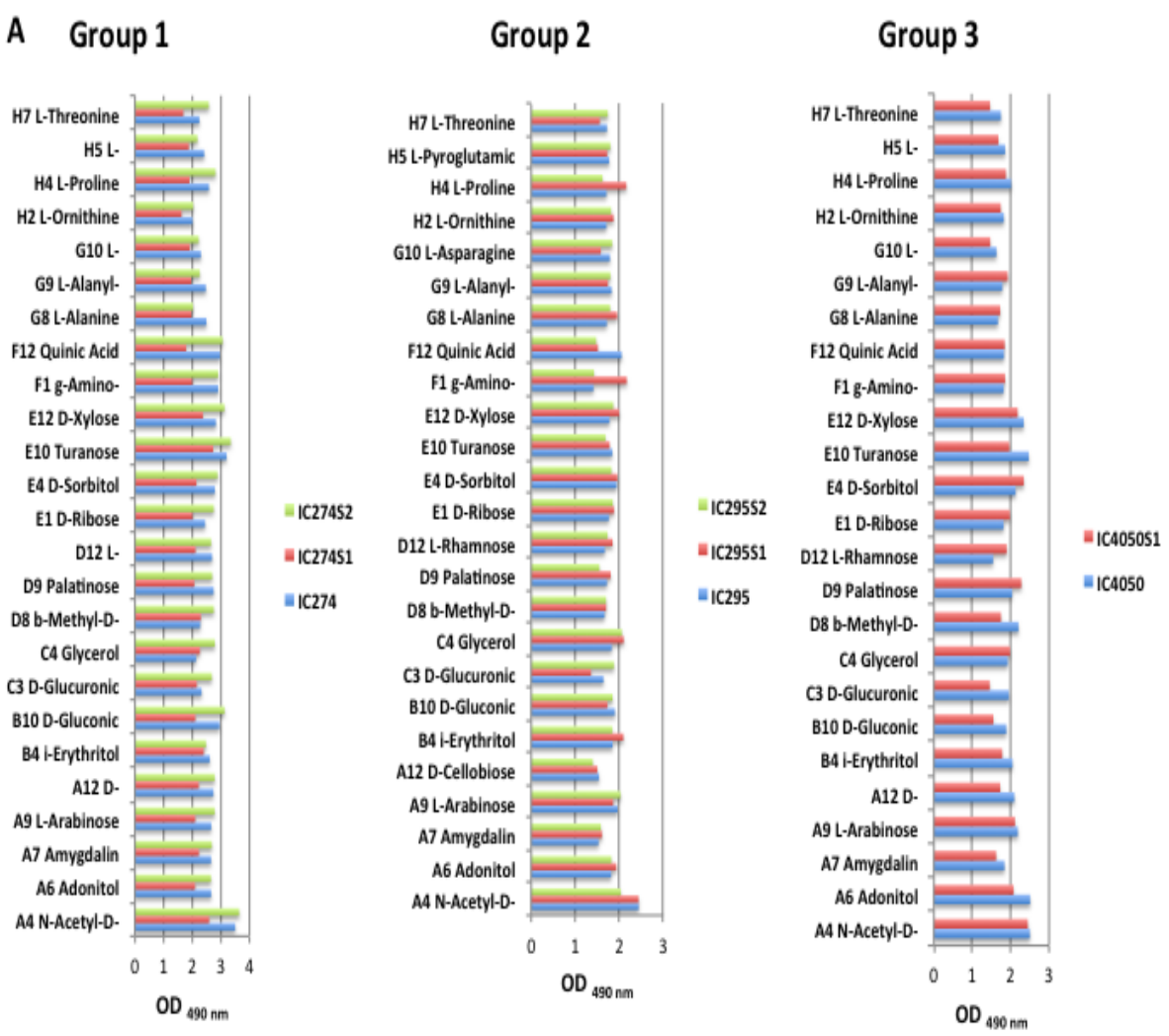


Supplementary Fig. 3.1. Sector formation in the colony of *A. flavus* isolate IC4050. (A) The isolate IC4050 was grown on PDA + 2.5 µg /ml benomyl at 30 ^{0} C. The isolate designated as IC4050S1 was originated from a single conidium of the sector of IC4050. (B) Flowcytometry analysis of DNA content of conidia from the isolate of *A. flavus* 4050. Conidia were collected, fixed, and stained with Propidium Iodide (PI). X- axis represents PI intensity and Y-axis represents the number of conidia.

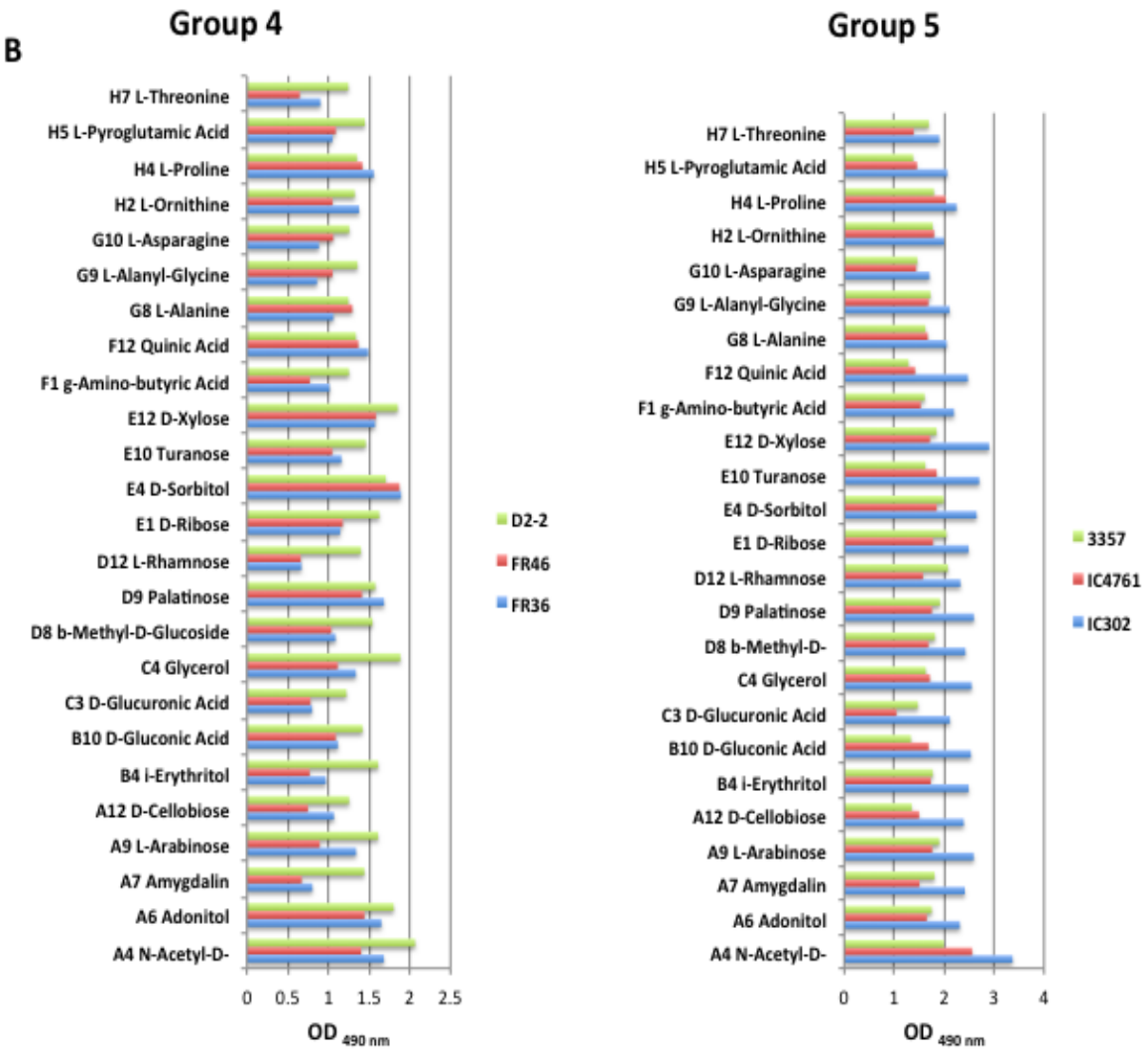


Supplementary Fig. 3.2. Metabolism of sugar by Group 1 isolates. Conidia collected from the 14 strains including the haploids and the diploids were grown on FF Micro-Plates (Biolog). Respiration values (OD_{490nm}) were recorded after 24, 48, 72 and 96-hour incubation. Carbon metabolism is higher between 48-72 hour incubation.

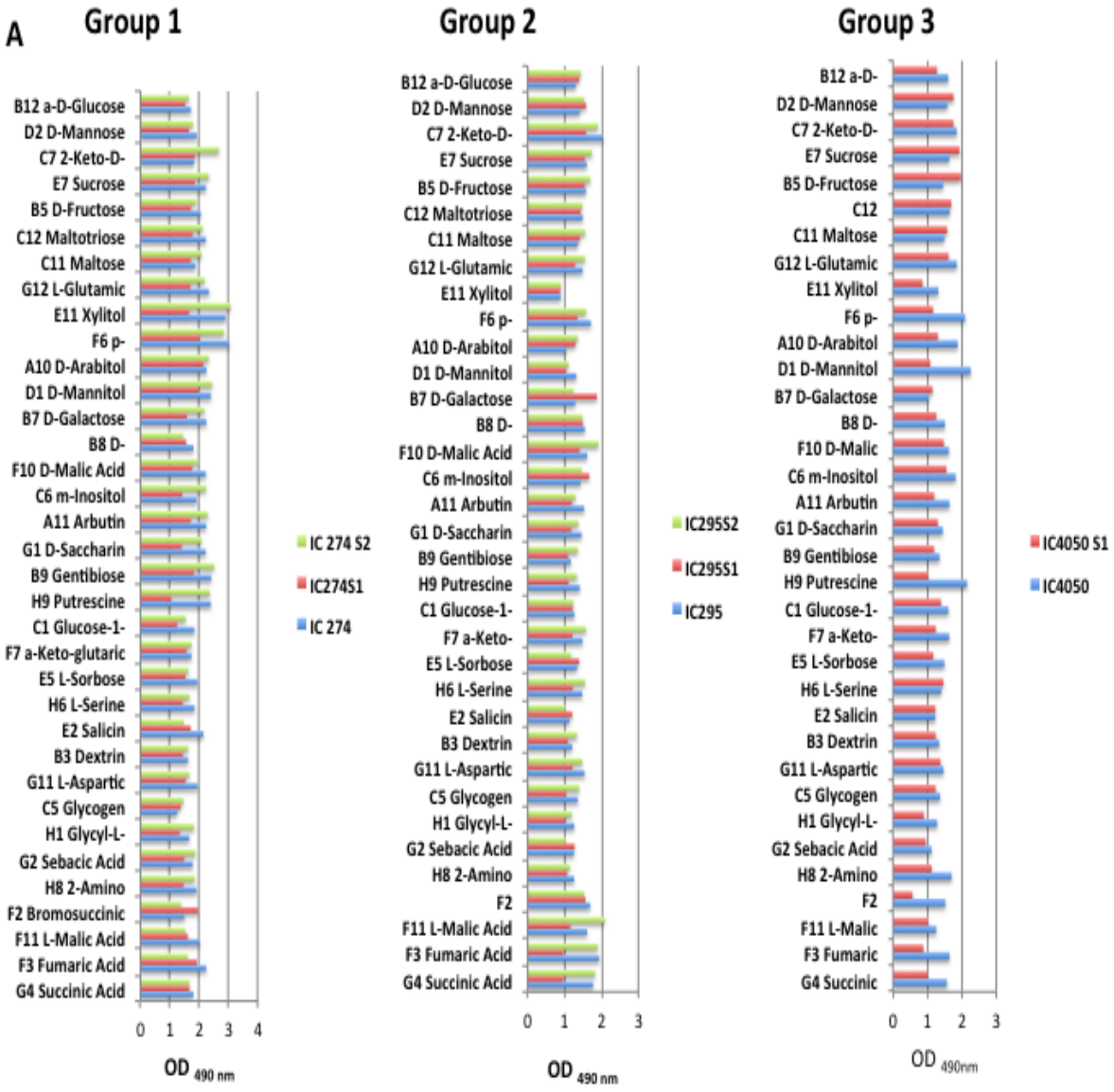
Supplementary Fig. 3.3. Metabolic profiles for carbon sources (Cluster I) after 72-hour incubation as inferred by Biolog Phenotype Microarray analysis. (A) Metabolic profiles of the carbon sources for the isolates of Groups 1-3. (B) Metabolic profiles of the carbon sources for the isolates of Groups 4-5. Fungal respiration plays important role in evaluation of metabolic reaction caused the formation of red color. The color was measured at absorbance 490 nm using a microplate reader (BioTek Miroplate reader). The average range of OD_{490} nm values were from 0.5 to 4.0.


A
Group 1
Group 2
Group 3
H7 L-Threonine
H5 L-
H4 L-Proline
H2 L-Ornithine
G10 L-
G9 L-Alanyl-
G8 L-Alanine
F12 Quinic Acid
F1 g-Amino-
E12 D-Xylose
E10 Turanose
E4 D-Sorbitol
E1 D-Ribose
D12 L-
D9 Palatinose
D8 b-Methyl-D-
C4 Glycerol
C3 D-Glucuronic
B10 D-Gluconic
B4 i-Erythritol
A12 D-
A9 L-Arabinose
A7 Amygdalin
A6 Adonitol
A4 N-Acetyl-D-
H5 L-Pyroglutamic
G10 L-Asparagine
D12 L-Rhamnose
A12 D-Cellobiose
IC274S2
IC274S1
IC274
IC295S2
IC295S1
IC295
IC4050S1
IC4050
0 1 2 3 4
0 1 2 3
OD 490 nm

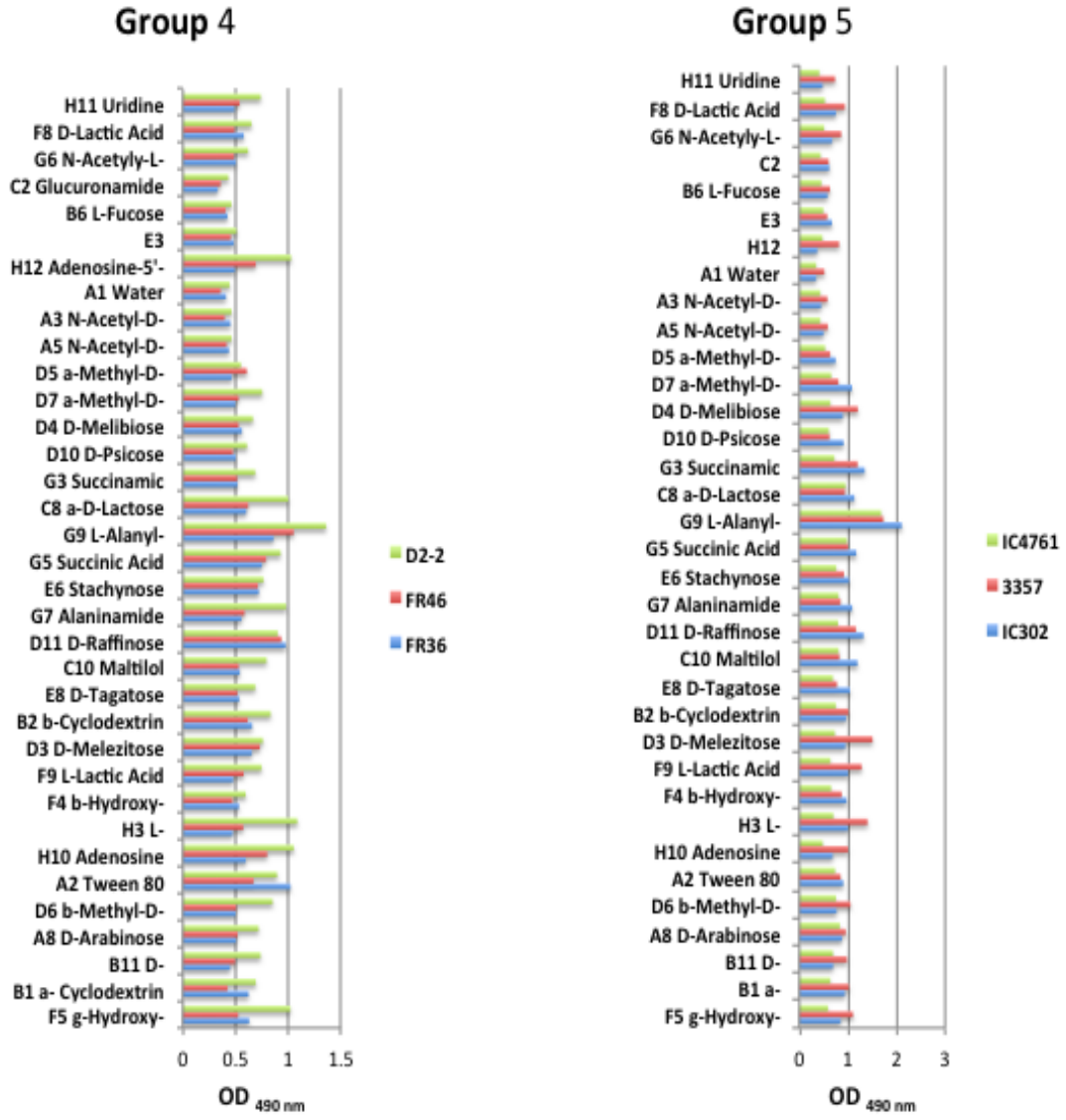
B


Group 4
H7 L-Threonine
H5 L-Pyroglutamic Acid
H4 L-Proline
H2 L-Ornithine
G10 L-Asparagine
G9 L-Alanyl-Glycine
G8 L-Alanine
F12 Quinic Acid
F1 g-Amino-butyric Acid
E12 D-Xylose
E10 Turanose
E4 D-Sorbitol
E1 D-Ribose
D12 L-Rhamnose
D9 Palatinose
D8 b-Methyl-D-Glucoside
C4 Glycerol
C3 D-Glucuronic Acid
B10 D-Gluconic Acid
B4 i-Erythritol
A12 D-Cellobiose
A9 L-Arabinose
A7 Amygdalin
A6 Adonitol
A4 N-Acetyl-D-
0
0.5
1
1.5
2
2.5
OD 490 nm
D2-2
FR46
FR36
Group 5
H7 L-Threonine
H5 L-Pyroglutamic Acid
H4 L-Proline
H2 L-Ornithine
G10 L-Asparagine
G9 L-Alanyl-Glycine
G8 L-Alanine
F12 Quinic Acid
F1 g-Amino-butyric Acid
E12 D-Xylose
E10 Turanose
E4 D-Sorbitol
E1 D-Ribose
D12 L-Rhamnose
D9 Palatinose
D8 b-Methyl-D-
C4 Glycerol
C3 D-Glucuronic Acid
B10 D-Gluconic Acid
B4 i-Erythritol
A12 D-Cellobiose
A9 L-Arabinose
A7 Amygdalin
A6 Adonitol
A4 N-Acetyl-D-
0
1
2
3
4
OD 490 nm
3357
IC4761
IC302

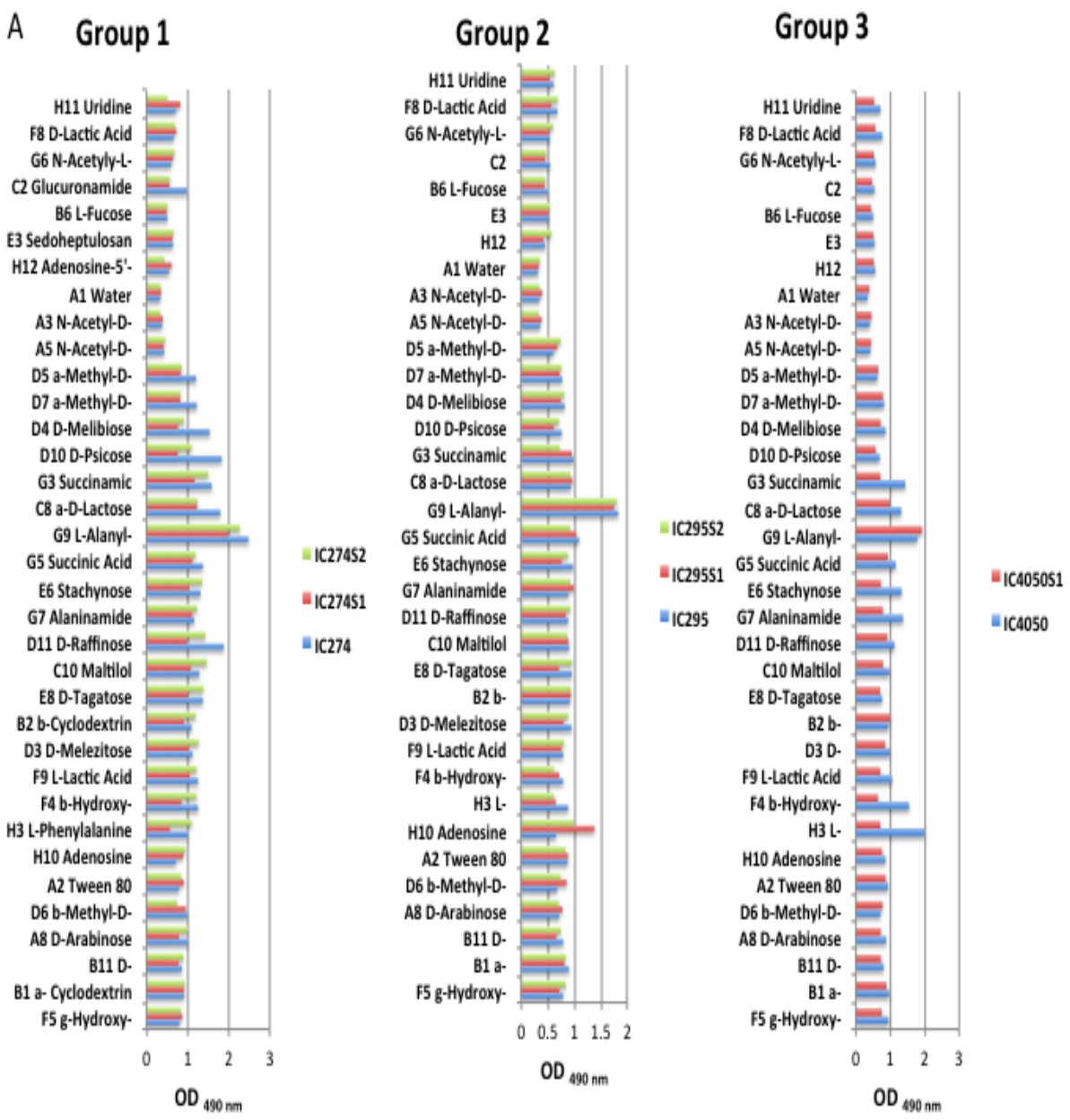
Supplementary Fig. 3.4. Metabolic profiles for carbon sources (Cluster II) after 72-hour incubation as inferred by Biolog Phenotype Microarray analysis. (A) Metabolic profiles of the carbon sources for the isolates of Groups 1-3. (B) Metabolic profiles of the carbon sources for the isolates of Groups 4-5. The color was measured at absorbance 490 nm using a microplate reader (BioTek Miroplate reader). The average range of $OD_{490\ nm}$ values were recorded from 0.4 to 3.0.


A
Group 1
B12 a-D-Glucose
D2 D-Mannose
C7 2-Keto-D-
E7 Sucrose
B5 D-Fructose
C12 Maltotriose
C11 Maltose
G12 L-Glutamic
E11 Xylitol
F6 p-
A10 D-Arabitol
D1 D-Mannitol
B7 D-Galactose
B8 D-
F10 D-Malic Acid
C6 m-Inositol
A11 Arbutin
G1 D-Saccharin
B9 Gentibiose
H9 Putrescine
C1 Glucose-1-
F7 a-Keto-glutaric
E5 L-Sorbose
H6 L-Serine
E2 Salicin
B3 Dextrin
G11 L-Aspartic
C5 Glycogen
H1 Glycyl-L-
G2 Sebacic Acid
H8 2-Amino
F2 Bromosuccinic
F11 L-Malic Acid
F3 Fumaric Acid
G4 Succinic Acid
0 1 2 3 4
OD 490 nm
IC 274 S2
IC274S1
IC 274
Group 2
B12 a-D-Glucose
D2 D-Mannose
C7 2-Keto-D-
E7 Sucrose
B5 D-Fructose
C12 Maltotriose
C11 Maltose
G12 L-Glutamic
E11 Xylitol
F6 p-
A10 D-Arabitol
D1 D-Mannitol
B7 D-Galactose
B8 D-
F10 D-Malic Acid
C6 m-Inositol
A11 Arbutin
G1 D-Saccharin
B9 Gentibiose
H9 Putrescine
C1 Glucose-1-
F7 a-Keto-
E5 L-Sorbose
H6 L-Serine
E2 Salicin
B3 Dextrin
G11 L-Aspartic
C5 Glycogen
H1 Glycyl-L-
G2 Sebacic Acid
H8 2-Amino
F2
F11 L-Malic Acid
F3 Fumaric Acid
G4 Succinic Acid
0 1 2 3
OD 490 nm
IC295S2
IC295S1
IC295
Group 3
B12 a-D-
D2 D-Mannose
C7 2-Keto-D-
E7 Sucrose
B5 D-Fructose
C12
C11 Maltose
G12 L-Glutamic
E11 Xylitol
F6 p-
A10 D-Arabitol
D1 D-Mannitol
B7 D-Galactose
B8 D-
F10 D-Malic
C6 m-Inositol
A11 Arbutin
G1 D-Saccharin
B9 Gentibiose
H9 Putrescine
C1 Glucose-1-
F7 a-Keto-
E5 L-Sorbose
H6 L-Serine
E2 Salicin
B3 Dextrin
G11 L-Aspartic
C5 Glycogen
H1 Glycyl-L-
G2 Sebacic Acid
H8 2-Amino
F2
F11 L-Malic
F3 Fumaric
G4 Succinic
0 1 2 3
OD 490nm
IC4050 S1
IC4050

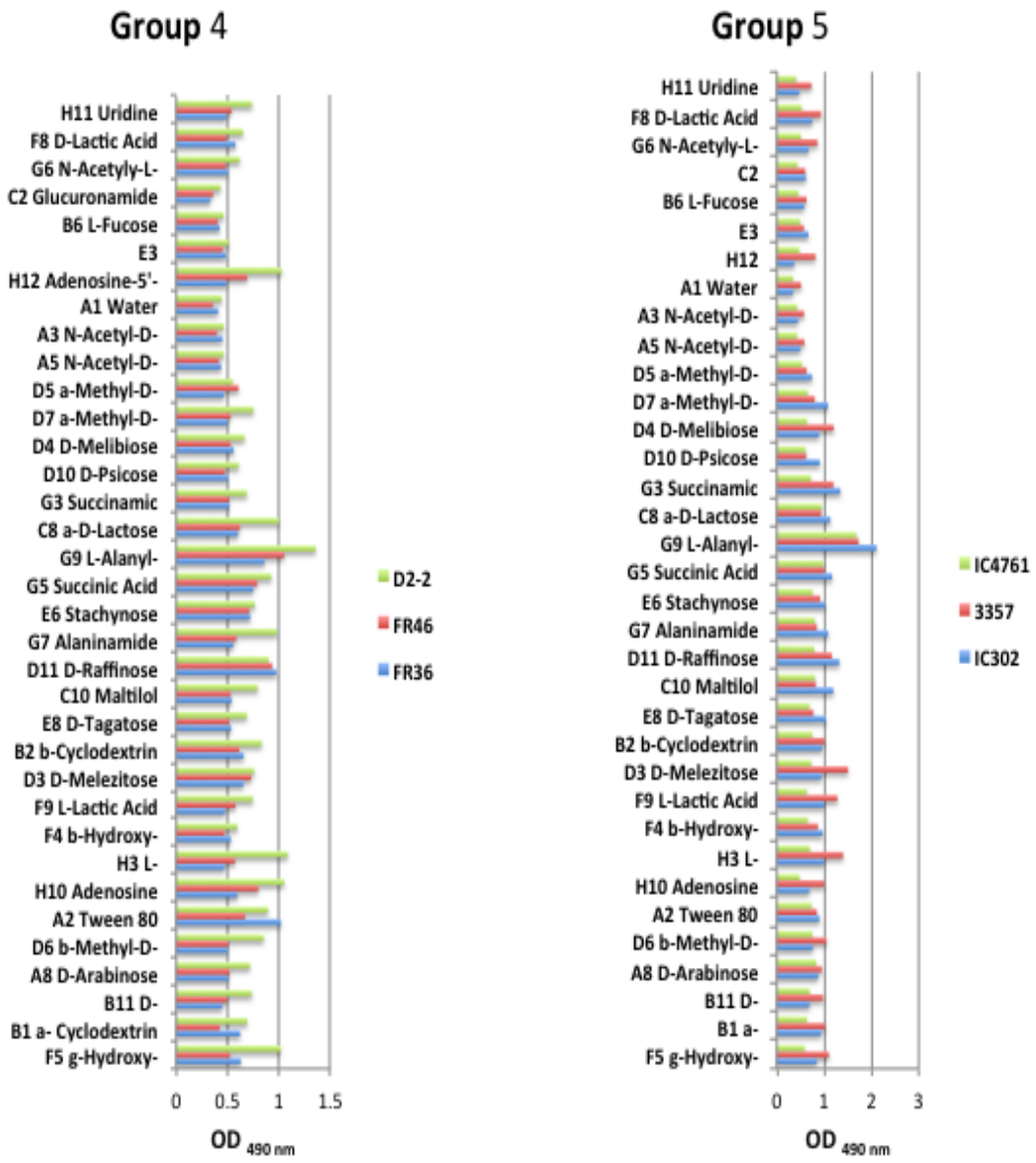
B

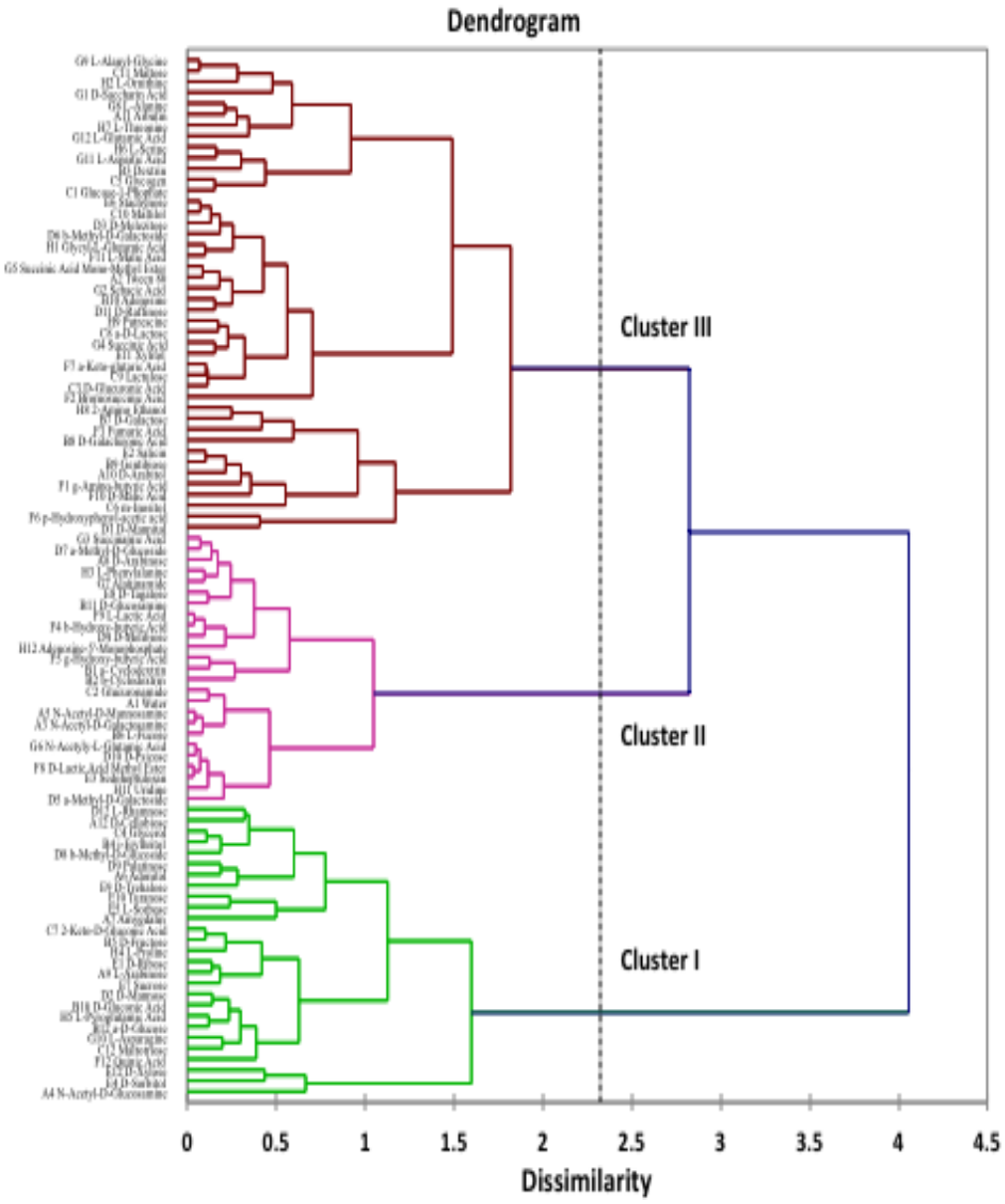

Group 4
H11 Uridine
F8 D-Lactic Acid
G6 N-Acetyly-L-
C2 Glucuronamide
B6 L-Fucose
E3
H12 Adenosine-5'-
A1 Water
A3 N-Acetyl-D-
A5 N-Acetyl-D-
D5 a-Methyl-D-
D7 a-Methyl-D-
D4 D-Melibiose
D10 D-Psicose
G3 Succinamic
C8 a-D-Lactose
G9 L-Alanyl-
G5 Succinic Acid
E6 Stachynose
G7 Alaninamide
D11 D-Raffinose
C10 Maltilol
E8 D-Tagatose
B2 b-Cyclodextrin
D3 D-Melezitose
F9 L-Lactic Acid
F4 b-Hydroxy-
H3 L-
H10 Adenosine
A2 Tween 80
D6 b-Methyl-D-
A8 D-Arabinose
B11 D-
B1 a- Cyclodextrin
F5 g-Hydroxy-
0
0.5
1
1.5
OD 490 nm
D2-2
FR46
FR36
Group 5
H11 Uridine
F8 D-Lactic Acid
G6 N-Acetyly-L-
C2
B6 L-Fucose
E3
H12
A1 Water
A3 N-Acetyl-D-
A5 N-Acetyl-D-
D5 a-Methyl-D-
D7 a-Methyl-D-
D4 D-Melibiose
D10 D-Psicose
G3 Succinamic
C8 a-D-Lactose
G9 L-Alanyl-
G5 Succinic Acid
E6 Stachynose
G7 Alaninamide
D11 D-Raffinose
C10 Maltilol
E8 D-Tagatose
B2 b-Cyclodextrin
D3 D-Melezitose
F9 L-Lactic Acid
F4 b-Hydroxy-
H3 L-
H10 Adenosine
A2 Tween 80
D6 b-Methyl-D-
A8 D-Arabinose
B11 D-
B1 a-
F5 g-Hydroxy-
0
1
2
3
OD 490 nm
IC4761
3357
IC302

Supplementary Fig. 3.5. Metabolic profiles for Cluster III carbon sources after 72-hour incubation as inferred by Biolog Phenotype Microarray analysis. (A) Metabolic profiles of the carbon sources for the isolates of Groups 1-3. (B) Metabolic profiles of the carbon sources for the isolates of Groups 4-5. The color was measured at absorbance 490 nm using a microplate reader (BioTek Miroplate reader). The OD values were recorded at 490 nm from 0.3 to 2.5.


A
Group 1
H11 Uridine
F8 D-Lactic Acid
G6 N-Acetyly-L-
C2 Glucuronamide
B6 L-Fucose
E3 Sedoheptulosan
H12 Adenosine-5'-
A1 Water
A3 N-Acetyl-D-
A5 N-Acetyl-D-
D5 a-Methyl-D-
D7 a-Methyl-D-
D4 D-Melibiose
D10 D-Psicose
G3 Succinamic
C8 a-D-Lactose
G9 L-Alanyl-
G5 Succinic Acid
E6 Stachynose
G7 Alaninamide
D11 D-Raffinose
C10 Maltilol
E8 D-Tagatose
B2 b-Cyclodextrin
D3 D-Melezitose
F9 L-Lactic Acid
F4 b-Hydroxy-
H3 L-Phenylalanine
H10 Adenosine
A2 Tween 80
D6 b-Methyl-D-
A8 D-Arabinose
B11 D-
B1 a- Cyclodextrin
F5 g-Hydroxy-
0 1 2 3
OD 490 nm
IC274S2
IC274S1
IC274
Group 2
H11 Uridine
F8 D-Lactic Acid
G6 N-Acetyly-L-
C2
B6 L-Fucose
E3
H12
A1 Water
A3 N-Acetyl-D-
A5 N-Acetyl-D-
D5 a-Methyl-D-
D7 a-Methyl-D-
D4 D-Melibiose
D10 D-Psicose
G3 Succinamic
C8 a-D-Lactose
G9 L-Alanyl-
G5 Succinic Acid
E6 Stachynose
G7 Alaninamide
D11 D-Raffinose
C10 Maltilol
E8 D-Tagatose
B2 b-
D3 D-Melezitose
F9 L-Lactic Acid
F4 b-Hydroxy-
H3 L-
H10 Adenosine
A2 Tween 80
D6 b-Methyl-D-
A8 D-Arabinose
B11 D-
B1 a-
F5 g-Hydroxy-
0 0.5 1 1.5 2
OD 490 nm
IC295S2
IC295S1
IC295
Group 3
H11 Uridine
F8 D-Lactic Acid
G6 N-Acetyly-L-
C2
B6 L-Fucose
E3
H12
A1 Water
A3 N-Acetyl-D-
A5 N-Acetyl-D-
D5 a-Methyl-D-
D7 a-Methyl-D-
D4 D-Melibiose
D10 D-Psicose
G3 Succinamic
C8 a-D-Lactose
G9 L-Alanyl-
G5 Succinic Acid
E6 Stachynose
G7 Alaninamide
D11 D-Raffinose
C10 Maltilol
E8 D-Tagatose
B2 b-
D3 D-
F9 L-Lactic Acid
F4 b-Hydroxy-
H3 L-
H10 Adenosine
A2 Tween 80
D6 b-Methyl-D-
A8 D-Arabinose
B11 D-
B1 a-
F5 g-Hydroxy-
0 1 2 3
OD 490 nm
IC4050S1
IC4050

B


Group 4
H11 Uridine
F8 D-Lactic Acid
G6 N-Acetyly-L-
C2 Glucuronamide
B6 L-Fucose
E3
H12 Adenosine-5'-
A1 Water
A3 N-Acetyl-D-
A5 N-Acetyl-D-
D5 a-Methyl-D-
D7 a-Methyl-D-
D4 D-Melibiose
D10 D-Psicose
G3 Succinamic
C8 a-D-Lactose
G9 L-Alanyl-
G5 Succinic Acid
E6 Stachynose
G7 Alaninamide
D11 D-Raffinose
C10 Maltilol
E8 D-Tagatose
B2 b-Cyclodextrin
D3 D-Melezitose
F9 L-Lactic Acid
F4 b-Hydroxy-
H3 L-
H10 Adenosine
A2 Tween 80
D6 b-Methyl-D-
A8 D-Arabinose
B11 D-
B1 a- Cyclodextrin
F5 g-Hydroxy-
0
0.5
1
1.5
OD 490 nm
D2-2
FR46
FR36
Group 5
H11 Uridine
F8 D-Lactic Acid
G6 N-Acetyly-L-
C2
B6 L-Fucose
E3
H12
A1 Water
A3 N-Acetyl-D-
A5 N-Acetyl-D-
D5 a-Methyl-D-
D7 a-Methyl-D-
D4 D-Melibiose
D10 D-Psicose
G3 Succinamic
C8 a-D-Lactose
G9 L-Alanyl-
G5 Succinic Acid
E6 Stachynose
G7 Alaninamide
D11 D-Raffinose
C10 Maltilol
E8 D-Tagatose
B2 b-Cyclodextrin
D3 D-Melezitose
F9 L-Lactic Acid
F4 b-Hydroxy-
H3 L-
H10 Adenosine
A2 Tween 80
D6 b-Methyl-D-
A8 D-Arabinose
B11 D-
B1 a-
F5 g-Hydroxy-
0
1
2
3
OD 490 nm
IC4761
3357
IC302



Supplementary Fig. 3.6. Agglomerative Hierarchical Clustering (AHC) for the carbon sources after 24, 48, 72 and 96-hour incubation in *A. nomius IC1516.* AHC was performed using Euclidean distance by complete linkage analysis. The dendrogram shows three distinct clusters of the carbon sources utilized by *Aspergillus nomius* IC1516. Cluster I describes the 26 best-utilized carbon sources (25 carbon). Cluster II shows 33 carbon sources and water that were metabolized very slowly. Cluster III contains 36 carbon sources and the isolate metabolize the carbon sources moderately.

Supplementary Tables

Supplementary Table 1. One-way ANOVA for metabolism of Cluster II Carbon sources by Group 1 isolates at 72 hours of incubation.

Carbon sources	IC274		IC274S1		IC274S2	
Fumaric Acid	2.379	A	1.992	ABC	1.678	BCDE
L-Malic Acid	2.100	A	1.664	ABC	1.578	ABCD
2-Amino Ethanol	1.913	A	1.664	ABC	1.873	AB
H1 Glycyl-L-Glutamic Acid	1.705	A	1.541	AB	1.853	A
G11 L-Aspartic Acid	1.962	A	1.635	ABCD	1.757	ABC
Dextrin	1.616	A	1.535	ABCD	1.631	A
Salicin	2.207	A	1.798	AB	1.727	ABC
a-Keto-glutaric Acid	1.802	A	1.586	AB	1.976	A
Putrescine	2.413	A	1.401	ABC	2.442	A
Gentibiose	2.406	A	2.891	AB	2.559	A
D-Saccharin Acid	2.129	AB	1.537	BCD	2.115	AB
A11 Arbutin	2.269	AB	1.839	BC	2.290	AB
C6 m-Inositol	2.495	A	1.912	ABC	2.285	A
D-Malic Acid	2.218	A	1.790	ABCD	2.211	AB
D-Galactose	2.269	A	1.666	ABC	2.342	A
D-Mannitol	2.418	A	2.173	AB	2.492	A
p-Hydroxyphenyl-acetic acid	3.024	A	1.971	DEF	2.894	AB
Xylitol	2.989	A	1.713	B	3.110	A
L-Glutamic Acid	2.341	A	1.874	ABCD	2.317	AB

Supplementary Table 2. One-Way ANOVA for metabolism of Cluster II Carbon sources by Group 2 isolates at 72 hours of incubation.

Carbon sources	IC295		IC295S1		IC295S2	
Succinic Acid	1.767	A	0.965	BC	1.819	A
Fumaric Acid	1.933	ABC	0.958	FG	1.897	ABC
L-Malic Acid	1.604	ABC	1.148	DEFG	2.018	A
Bromosuccinic Acid	1.686	A	1.560	AB	1.523	AB
2-Amino Ethanol	1.256	BCD	1.057	CD	1.138	CD
Sebacic Acid	1.247	AB	1.268	AB	1.020	BCD
Glycyl-L-Glutamic Acid	1.705	A	1.541	AB	1.853	A
L-Aspartic Acid	1.536	ABCD	1.210	CD	1.474	ABCD
Dextrin	1.203	CDE	1.087	E	1.325	ABCD
Salicin	1.123	CDE	1.205	BCDE	1.014	DE
a-Keto-glutaric Acid	1.482	AB	1.208	ABC	1.572	AB
Gentibiose	1.116	BCD	1.090	CD	1.351	BCD
D-Saccharin Acid	1.453	CD	1.178	D	1.369	CD
A11 Arbutin	1.525	CD	1.187	F	1.286	DEF
D-Malic Acid	1.606	BCD	1.399	DEF	1.906	ABC
D-Galactose	1.282	CD	1.870	AB	1.234	CD
D-Mannitol	1.311	CDE	1.037	EF	1.104	DEF
D-Arabitol	1.042	BC	1.285	ABC	1.352	ABC
F6 p-Hydroxyphenyl-acetic acid	1.709	EF	1.341	FG	1.580	EFG
L-Glutamic Acid	1.485	CDE	1.279	DE	1.543	BCDE

Supplementary Table 3. One-way ANOVA for metabolism of Cluster II Carbon sources by Group 3 isolates at 72 hours of incubation.

Carbon sources	IC4050		IC4050S1	
Succinic Acid	1.560	A	0.986	BC
Fumaric Acid	1.633	CDE	0.877	FGH
L-Malic Acid	1.248	CDEF	1.024	FGHI
Bromosuccinic Acid	1.513	AB	0.536	CDE
2-Amino Ethanol	1.696	AB	1.120	CD
Sebacic Acid	1.113	BC	0.945	BCD
a-Keto-glutaric Acid	1.624	A	1.237	ABC
Putrescine	2.138	A	1.020	BC
Arbutin	1.629	C	1.193	EF
D-Malic Acid	1.615	BCD	1.468	CDE
D-Galactose	1.035	DE	1.141	CD
D-Mannitol	2.248	A	1.076	DEF
D-Arabitol	1.870	AB	1.296	ABC
p-Hydroxyphenyl-acetic acid	2.084	DE	1.155	G
E11 Xylitol	1.306	B	0.852	C
L-Glutamic Acid	1.844	ABCD	1.609	BCDE

Supplementary Table 4. One-way ANOVA for metabolism of Cluster II Carbon sources by Group 4 isolates at 72 hours of incubation.

Carbon sources	FR36		FR46		D2-2	
Succinic Acid	0.614	C	0.692	BC	0.878	BC
Fumaric Acid	0.597	GH	0.555	H	0.739	GH
L-Malic Acid	0.666	HI	0.575	I	0.824	GHI
Bromosuccinic Acid	0.467	DE	0.410	E	0.540	CDE
2-Amino Ethanol	0.526	E	0.503	E	1.002	D
Sebacic Acid	0.481	D	0.561	CD	0.809	BCD
H1 Glycyl-L-Glutamic Acid	0.759	B	0.772	B	1.058	AB
G11 L-Aspartic Acid	1.10 2	D	1.145	CD	1.214	CD
Dextrin	1.140	DE	1.140	DE	1.209	CDE
Salicin	0.867	E	0.954	E	0.992	DE
L-Serine	0.892	B	1.041	B	1.155	AB
L-Sorbose	0.819	B	0.723	B	1.123	AB
a-Keto-glutaric Acid	0.696	C	0.775	BC	1.017	BC
Glucose-1-Phophate	0.788	B	0.791	B	1.188	B
Putrescine	0.720	BC	0.614	C	1.087	ABC
Gentibiose	0.852	D	0.845	D	1.116	CD
D-Saccharin Acid	1.265	CD	1.270	CD	1.208	D
A11 Arbutin	0.998	FG	0.831	G	1.134	F
C6 m-Inositol	0.857	BC	0.819	BC	1.502	ABC
D-Malic Acid	0.594	H	0.682	H	0.962	GH
D-Galacturonic Acid	0.435	C	0.379	C	1.128	B
D-Galactose	0.540	EF	0.470	F	0.995	DE
D-Mannitol	0.638	F	0.648	F	0.932	EF
D-Arabitol	0.770	BC	0.607	C	1.021	BC

Supplementary Table 4 (Continued)

Carbon sources	FR36		FR46		D2-2	
p-Hydroxyphenyl-acetic acid)	0.606	H	0.637	H	1.079	GH
L-Glutamic Acid	1.519	BCDE	1.316	DE	1.221	DE
D-Mannose	1.871	AB	1.924	A	1.529	AB

Supplementary Table 5. One-way ANOVA for metabolism of Cluster II Carbon sources by Group 5 isolates at 72 hours of incubation.

Carbon sources	IC302		3357		IC4761	
Succinic Acid	1.201	ABC	1.248	A	0.759	BC
Fumaric Acid	1.257	EF	0.908	FGH	0.663	GH
L-Malic Acid	0.922	FGHI	0.751	HI	0.605	I
Bromosuccinic Acid	1.708	A	1.104	ABCDE	0.903	BCDE
2-Amino Ethanol	1.586	ABC	1.107	D	1.122	CD
Sebacic Acid	1.429	AB	0.953	BCD	0.921	BCD
Glycyl-L-Glutamic Acid	1.738	A	1.461	AB	0.868	AB
G11 L-Aspartic Acid	1.546	ABCD	1.243	CD	1.153	CD
Dextrin	1.547	ABCD	1.188	CDE	1.065	E
Salicin	1.326	BCDE	1.305	BCDE	1.102	CDE
L-Sorbose	1.619	A	1.778	A	1.283	AB
a-Keto-glutaric Acid	1.490	AB	1.463	AB	0.960	BC
Glucose-1-Phophate	1.480	AB	1.445	AB	0.932	B
Putrescine	1.464	ABC	1.650	A	1.121	ABC
Gentibiose	1.815	AB	1.208	BCD	0.978	B
D-Saccharin Acid	1.935	AB	1.413	CD	1.136	D
Arbutin	1.878	ABC	1.482	CDE	1.207	EF
D-Malic Acid	1.776	ABCD	1.158	EFG	0.873	GH
D-Galactose	1.891	AB	1.090	CD	1.549	BC
D-Mannitol	2.286	A	1.472	BCDE	0.975	EF
D-Arabitol	2.199	A	1.257	ABC	1.227	ABC
Hydroxyphenyl-acetic acid	2.476	ABCD	1.477	EFG	1.325	FG
Xylitol	2.554	A	1.479	B	0.802	B

Supplementary Table 5 (Continued)

Carbon sources	IC302		3357		IC4761	
L-Glutamic Acid	2.005	ABCD	1.180	E	1.241	DE
Maltotriose	2.006	AB	1.774	AB	1.343	B

Supplementary Table 6.One-way ANOVA for metabolism of Cluster III Carbon sources by Group 1 isolates at 72 hours of incubation.

Carbon sources	IC274		IC274S1		IC274S2	
b-Hydroxy-butyric Acid	1.296	AB	0.854	BCDE	1.322	AB
b-Cyclodextrin	1.152	AB	0.926	ABCD	1.346	A
D-Tagatose	1.385	A	1.093	AB	1.486	A
Maltilol	1.404	ABC	1.072	ABCDE	1.441	AB
D-Raffinose	2.089	A	0.963	B	1.456	AB
Stachynose	1.314	A	1.074	ABC	1.431	A
Succinic Acid Mono-Methyl Ester	1.382	A	1.160	AB	1.222	AB
L-Alanyl-Glycine	2.502	A	1.978	AB	2.301	A
D-Psicose	1.125	AB	0.779	B	1.167	AB
Adenosine-5'-Monophosphate	0.545	BC	0.790	ABC	0.509	BC

Supplementary Table 7. One-way ANOVA for metabolism of Cluster III Carbon sources by Group 2 isolates at 72 hours of incubation.

Carbon sources	IC295		IC295S1		IC295S2	
L-Phenylalanine	0.881	A	0.648	C	0.614	C
b-Hydroxy-butyric Acid	0.788	BCDE	0.715	BCDE	0.613	CDE
D-Tagatose	0.953	BC	0.707	CDE	0.956	BC
Maltilol	0.900	CDEF	0.886	DEFG	0.867	DEFG
Stachynose	0.973	ABC	0.759	C	0.878	BC
Adenosine-5'-momophosphate	0.453	C	0.412	C	0.568	BC

Supplementary Table 8. One-way ANOVA for metabolism of Cluster III Carbon sources by Group 3 isolates at 72 hours of incubation.

Carbon sources	IC4050		IC4050S1	
D-Arabinose	0.877	A	0.721	AB
L-Phenylalanine	1.985	A	0.708	C
b-Hydroxy-butyric Acid	1.554	A	0.641	CDE
b-Cyclodextrin	0.947	ABCD	0.992	ABC
D-Tagatose	0.76	CD	0.704	CDE
Maltilol	0.970	BCDE	0.790	EFG
D-Raffinose	1.121	AB	0.914	B
Alaninamide	1.367	A	0.782	BC
Stachynose	1.328	B	0.725	C
a-D-Lactose	1.317	AB	0.976	B

Supplementary Table 9. One-way ANOVA for metabolism of Cluster III Carbon sources by Group 4 isolates at 72 hours of incubation.

Carbon sources	FR36		FR46		D2-2	
g-Hydroxy-butyric Acid	0.628	BC	0.523	C	1.108	AB
D-Glucosamine	0.447	B	0.493	B	0.736	AB
D-Arabinose	0.503	B	0.518	B	0.717	AB
L-Phenylalanine	0.471	C	0.571	C	1.084	BC
b-Hydroxy-butyric Acid	0.531	DE	0.469	E	0.594	CDE
b-Cyclodextrin	0.657	CD	0.612	D	0.831	BCD
D-Tagatose	0.533	DE	0.514	E	0.685	CDE
Maltilol	0.540	FG	0.528	G	0.793	EFG
Alaninamide	0.553	C	0.584	C	0.978	ABC
Succinic Acid Mono-Methyl Ester	0.749	B	0.788	B	0.925	AB
L-Alanyl-Glycine	1.050	C	0.859	C	1.357	BC
a-D-Lactose	0.599	B	0.619	B	0.999	B
a-Methyl-D-Glucoside	0.496	B	0.527	B	0.752	AB
Adenosine-5'-Monophosphate	0.490	BC	0.690	BC	1.025	A
Uridine	0.488	B	0.537	AB	0.737	AB

Supplementary Table 10. One-way ANOVA for metabolism of Cluster III Carbon sources by Group 5 isolates at 72 hours of incubation.

Carbon sources	IC302		3357		IC4761	
F5 g-Hydroxy-butyric Acid	0.877	ABC	1.104	A	0.589	BC
Adenosine	0.782	AB	1.000	A	0.478	B
H3 L-Phenylalanine	0.924	BC	1.403	AB	0.698	C
b-Hydroxy-butyric Acid	0.981	BCD	0.870	BCDE	0.652	CDE
b-Cyclodextrin	1.015	AB	1.011	AB	0.748	BCD
D-Tagatose	1.052	B	0.773	CD	0.689	CDE
Maltilol	1.199	ABCDE	0.820	DEFG	0.805	EFG
D-Raffinose	1.333	AB	1.162	AB	0.794	B
Alaninamide	1.104	AB	0.846	ABC	0.805	BC
Stachynose	1.046	ABC	0.917	ABC	0.758	C
a-D-Lactose	1.169	AB	0.936	B	0.947	B
N-Acetyl-D-Galactosamine	0.494	AB	0.575	A	0.429	AB
Adenosine-5'-Monophosphate	0.368	C	0.818	AB	0.473	C