

**DIVERSITY OF TOXIGENIC FUNGI AND THEIR MYCOTOXINS
FROM RICE MARKETED IN PENINSULAR MALAYSIA**

by

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LIST OF ABBREVIATIONS

%	percentage
&	and
°C	Degree of celcius
µg/kg	microgram per kilogram
µl	microliter
µm	micrometer
AF	Aflatoxin
AFB ₁	Aflatoxin B ₁
AFB ₂	Aflatoxin B ₂
AFG ₁	Aflatoxin G ₁
AFG ₂	Aflatoxin G ₂
BEA	Beauvericin
BEN	Balkan Endemic Nephropathy
CIT	Citrinin
CLA	Carnation leaf agar
cm	Centimeter
ELISA	Enzyme-linked Immunosorbent Assay
em	emission
EU	European Union
ex	excitation
f. sp.	formae speciales
FA ₁	Fumonisin A ₁
FA ₂	Fumonisin A ₂
FB ₁	Fumonisin B ₁
FB ₂	Fumonisin B ₂
FB ₃	Fumonisin B ₃
FB ₄	Fumonisin B ₄
g	gram
GC	Gas Chromatography
H ₂ O	Water
HPLC	High Performance Liquid Chromatography
IARC	International Agency for Research on Cancer
LC/ESI/MS	Liquid chromatography/electron spray ionization/mass spectrometry
LEM	leukoencephalomalacia
LLE	Liquid-liquid extraction
MeOH	methanol
mg	milligram
mg/kg	milligram per kilogram
min	minutes
ml	milliliter
ml/min	milliliter per minute
mm	millimeter
Mt	Million tons
nd	Not detected

ng	nanogram
nm	nanometer
No.	Number
OTA	Ochratoxin A
p.s.i.	pounds per square inch
PDA	Potato Dextrose Agar
pg	
PPA	Pentachloronitrobenzene agar
ppm	parts per million
SD	Standard deviation
SEA	Soil extract agar
sp.	Species
SPE	Solid Phase Extraction
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
UPLC	Ultra Performance Layer Chromatography
UV	Ultra violet
w/w	weight per weight
WA	Water agar
ZEN	Zearalenone

**KEPELBAGAIAN KULAT TOKSIGEN DAN MIKOTOKSIN DARIPADA
BERAS YANG DIPASARKAN DI SEMENANJUNG MALAYSIA**

ABSTRAK

Penyimpanan komoditi makanan termasuk beras kerap dicemari oleh kulat toksigen kerana keadaan cuaca di Malaysia yang sesuai bagi pertumbuhan kulat-kulat tersebut. Kajian ini dijalankan untuk menentukan kepelbagaian kulat toksigen dan kepekatan mikotoksin pada sampel beras yang dipasarkan di Semenanjung Malaysia. Keputusan penyelidikan ini diharapkan dapat menjadi panduan untuk mewujudkan undang-undang berkaitan dengan tahap maksimum yang dibenarkan bagi mikotoksin utama di dalam makanan ruji ini. Sembilan puluh enam sampel beras yang terdiri daripada basmati (2), beras pulut hitam (5), beras perang (7), beras wangi (12), produk beras (7), beras separa rebus (3), padi (4), beras putih (45) dan beras pulut putih (11) dikutip secara rawak dari kedai dan pasaraya di Semenanjung Malaysia untuk mengkaji tahap serangan kulat dan kandungan mikotoksinya iaitu aflatoxin (AFB₁, AFB₂, AFG₁ dan AFG₂) dan fumonisin (FB₁ dan FB₂). Dengan menggunakan kaedah pensterilan permukaan, sebanyak 1,969 pencilan kulat telah berjaya dipencilkan. Berdasarkan ciri-ciri morfologi, lima genus kulat toksigen telah dikenalpasti iaitu *Aspergillus*, *Alternaria*, *Curvularia*, *Fusarium* dan *Penicillium*. *Aspergillus* spp. merupakan kulat toksigen yang paling kerap dipencilkan daripada semua jenis sampel beras dan produk beras, diikuti oleh *Penicillium* spp., *Alternaria* spp., *Fusarium* spp. dan *Curvularia* spp.. Di antara kulat toksigen dalam genus *Aspergillus*, *A. niger* adalah spesies kulat yang paling banyak mencemari beras

diikuti oleh *A. flavus*, *A. fumigatus*, *A. tamarii*, *A. nidulans* dan *A. ochraceus*. Kewujudan AFB₁, AFB₂, AFG₁, AFG₂, FB₁ dan FB₂ dikesan dengan menggunakan Kromatografi Cecair Prestasi Tinggi (HPLC) dengan pengesan pendafluor. Lima sampel (5%) (dua beras pulut hitam, dua beras putih, dan satu produk beras) dicemari AFB₁ dengan purata kepekatan daripada 0.39 hingga 24.09 µg/kg dan dua sampel (2%) (beras pulut hitam dan produk beras) mengandungi AFB₂ dengan purata kepekatan daripada 0.04 hingga 2.45 µg/kg. Tiada sampel melebihi tahap yang ditetapkan oleh Malaysia (35 µg/kg bagi keseluruhan aflatoksin) manakala satu sampel (beras pulut hitam) melebihi tahap yang ditetapkan oleh Suruhanjaya Eropah bagi AFB₁ (4 µg/kg bagi keseluruhan aflatoksin) . Fumonisin B₁ dan B₂ dikesan di dalam 22 sampel (23%) beras dengan purata kepekatan daripada 10 hingga 120 µg/kg. Kewujudan bersama kedua-dua aflatoksin dan fumonisin hanya ditemui di dalam satu sampel (produk beras) dengan kepekatan AFB₁ dan AFB₂ ialah 1.59 µg/kg dan 0.04 µg/kg sementara FB₁ dan FB₂ ialah 30 µg/kg dan 100 µg/kg. Kajian ini merupakan yang pertama berkaitan dengan kulat toksigen dan pencemaran mikotoksin yang dihasilkannya di dalam beras yang dipasarkan di Malaysia untuk makanan manusia.

DIVERSITY OF TOXIGENIC FUNGI AND THEIR MYCOTOXINS FROM RICE MARKETED IN PENINSULAR MALAYSIA

ABSTRACT

Stored food commodities including rice are commonly contaminated with toxigenic fungi as climatic conditions in Malaysia are conducive for their growth. The present study was conducted to determine the diversity of toxigenic fungi and their mycotoxins in rice samples marketed in Peninsular Malaysia. The results hopefully will lead to public awareness and implementation of legislation regarding the maximum level allowed for key mycotoxins in this staple food. Ninety-six samples of rice consisted of basmati (2), black glutinous rice (5), brown rice (7), fragrant rice (12), rice products (7), parboiled rice (3), paddy (4), white rice (45) and white glutinous rice (11) were collected randomly from sundry shops and supermarkets in Peninsular Malaysia to study levels of fungal colonization and mycotoxin contents namely, aflatoxins (AFB₁, AFB₂, AFG₁ and AFG₂) and fumonisins (FB₁ and FB₂). By using surface-sterilization method, total 1,969 isolates of fungi were successfully isolated. Based on morphological characteristics, five genera of toxigenic fungi were identified as *Aspergillus*, *Alternaria*, *Curvularia*, *Fusarium* and *Penicillium*. *Aspergillus* spp. were the most common toxigenic fungi isolated from all types of rice and rice products in Malaysia, followed by *Penicillium* spp., *Alternaria* spp., *Fusarium* spp. and *Curvularia* spp.. Among the toxigenic *Aspergillus*, *A. niger* was the most prevalent species followed by *A. flavus*, *A. fumigatus*, *A. tamarii*, *A.*

nidulans and *A. ochraceus*. The presences of AFB₁, AFB₂, AFG₁, AFG₂, FB₁ and FB₂ in the samples were detected by using High Pressure Liquid Chromatography (HPLC) with a fluorescent detector. Five samples (5%) (two black glutinous rice, two white rice, and one rice product) were contaminated with AFB₁ ranging from 0.39-24.09 µg/kg and two samples (2%) (black glutinous rice and rice product) with AFB₂ ranging from 0.04 to 2.45 µg/kg. None of the samples tested exceeded the Malaysian permissible limits (35 µg/kg total aflatoxins) while one sample (black glutinous rice) exceeded the limit of AFB₁ by European Commission (4 µg/kg total aflatoxins). Fumonisin B₁ and B₂ were detected in 22 samples (23%) of rice analyzed with concentrations ranging from 10 to 120 µg/kg. Co-occurrence of both aflatoxins and fumonisins were detected in one sample (rice product) with concentrations of AFB₁ and AFB₂ were 1.59 µg/kg and 0.04 µg/kg while FB₁ and FB₂ were 30 µg/kg and 100 µg/kg. The present study is the first report of toxigenic fungi and their mycotoxins contamination of Malaysian rice marketed for human consumption.

CHAPTER 1

INTRODUCTION

Rice (*Oryza sativa*) is one of the important staple food crop and major food source for half of the world's population especially in Asia. Rice is widely cultivated and Food and Agriculture Organization (FAO) reported that about 700 million Metric tons (Mt) was produced globally in year 2010. The world rice production increased 3% more than in 2009 (FAO, 2011). Asia is the primary region of rice cultivation followed by Latin America and North America.

The main rice producing countries are China, Thailand, Vietnam, Indonesia, Myanmar, Bangladesh and India (Reiter *et al.*, 2010). For these countries, trading is only an option as their domestic need and adequate buffer stock are secured. In fact, only a small proportion of rice production has been traded which is around 5-7% of total rice production compared with 20% for wheat, 13% for maize and 30% for soybeans (International Rice Research Institute, 2008).

In many rice-consuming countries such as the Philippines, Bangladesh, Nigeria, Iran and Saudi Arabia, rice self-sufficiency has become a sensitive political issue, prompting policymakers to implement programs to reduce dependence on the global market (Mohanty, 2009). Malaysia is one of the top rice importers and world's ninth

biggest rice buyers. Annually, Malaysia produce 1.39 million Mt of rice, however a total of 2.2 million Mt is consumed. Therefore, Malaysia needs to import rice from other countries mainly from India, Pakistan, Thailand and Vietnam (FAO, 2008). In 2004, United Nation launched International Year of Rice with the purpose to increase the production of rice (US Department of State, 2003).

The structure and chemical composition of rice grain make it an ideal substrate for the establishment and growth of fungal species, especially toxigenic fungi. Moreover, Malaysia has tropical climate with high ambient temperature and high relative humidity that provide optimal condition for the growth of toxigenic fungi. Toxigenic fungi apparently damage the rice quality, decrease nutritional value, weight and palatability (Goswami & Kistler, 2005). Toxigenic fungi in food can be classified into field fungi and storage fungi based on their ecological requirements for growth (Bankole, 1994). Field fungi requires above 20% grain moisture to cause diseases and toxin production before harvest. The storage fungi usually grow in grains with moisture content in equilibrium with 70-90% relative humidity, which corresponds to less than 18% moisture content in cereals (Ayejuyo *et al.*, 2008).

Among the toxigenic fungi which can bring significant losses on rice is *Fusarium graminearum* causing a disease, known as scab (Goswami & Kistler, 2005). It was the first scab disease of rice that has been recorded in most of the rice growing regions in the world (Lee *et al.*, 1992; Goswami & Kistler, 2005) and there have been several reports of scab on rice in Asian countries (Desjardins *et al.*, 2000). Other field fungi that contaminate rice are *F. semitectum*, *Bipolaris oryzae*, *Curvularia*, *Phoma* and

Colletotrichum species (Pitt *et al.*, 1994). Another diseases caused by *Fusarium* species is bakanae disease and *F. fujikuroi* is commonly associated with the disease. Bakanae disease shows a complex of disease symptoms and gibberellins hormones produced by this pathogen will cause seedling blight, root and crown rot, stunting, and the classic symptoms of etiolation and abnormal growth of the stem (Nicholson *et al.*, 1998; Desjardins *et al.*, 2000; Nur Ain Izzati, 2007).

Besides cause diseases on plants, toxigenic fungi also produced a range of mycotoxins that could pose a serious threat to plant, animal and human health (Marasas *et al.*, 1984; Joffe, 1986, Salleh & Strange, 1988; Salleh, 1998). Stored commodities and products will be the main medium for the formation of mycotoxins when the fungus is viable and the conditions for their formation including moisture and temperature are conducive. Moreover, poorly developed processing and storage facilities are also in favour for fungal growth and subsequently mycotoxin contamination.

Rice samples that are naturally infected with toxigenic fungi is reported to have high potential to produce mycotoxins such as fumonisins (FUM), ochratoxin A (OTA), trichothecenes (TRI), and zearalenone (ZEN) (Abbas *et al.*, 1998; Kim *et al.*, 2002; Makun *et al.*, 2007; Ayejuyo *et al.*, 2008). In Republic of Korea, it was found that contamination of fumonisins in rice were due to *F. proliferatum* infection while ochratoxin A was caused by *Penicillium verrucosum* (Park *et al.*, 2005). A research conducted in Nigeria found that rice was commonly contaminated by *A. niger*, *Penicillium* spp., and *A. flavus* with aflatoxin B₁ (AFB₁) was found to be the common mycotoxin contaminated the rice samples (Makun *et al.*, 2007). In Vietnam, naturally

occurrence of *A. ochraceus* in rice samples was found positive with ochratoxin A and citrinin by *P. citrinum* (Trung *et al.*, 2001).

It was estimated that 25% of the world food crops are affected by mycotoxins (Charmley *et al.*, 1995). Mycotoxins are group of secondary metabolites produced by certain toxigenic fungi which mostly are saprophytic moulds that grow on a variety of foodstuffs (Moss, 1994). Hundreds of mycotoxins are known, but relatively only several of the mycotoxins are recognized as serious contaminants of food. Among the mycotoxins, aflatoxin B₁ (AFB₁), fumonisin B₁ (FB₁) and ochratoxin A (OTA) are reported as major rice contaminants (Abbas *et al.*, 1998; Kim *et al.*, 2002; Makun *et al.*, 2007; Ayejuyo *et al.*, 2008).

Aflatoxins are produced by *Aspergillus* species such as *A. flavus* and *A. parasiticus*. Aflatoxins are highly toxic, mutagenic, teratogenic and carcinogenic compounds (Massey *et al.*, 1995; Castegrano & Pfohl-Leszkowicz, 1999; Hussein & Brasel, 2001). AFB₁ is listed as a group 1 carcinogen by the International Agency for Research on Cancer (IARC) and it cause hepatic and extrahepatic carcinogenesis in human (IARC, 1993). Contamination of rice by aflatoxins affects the quality of rice grains and their derivatives used for human consumption.

Fumonisin are produced mainly by *Fusarium* spp. such as *F. verticillioides*, *F. proliferatum* and *F. nygamai* as well as *Alternaria alternata* (Rheeder *et al.*, 2002) and *A. niger* (Jens *et al.*, 2007; Reddy *et al.*, 2010). IARC has classified fumonisin as group 2B and it cause oesophageal cancer to humans and may cause allergic or carcinogenic

symptoms, in long term consumption of contaminated food (Bottalico, 1998). It is one of the common natural mycotoxins that were found in contaminated cereals such as rice.

Ochratoxin A is a teratogenic, potent renal carcinogen (IARC, 1993; Manderville & Pfohl-Leszkowicz, 2006) and affected certain nephropathies in animals and human (Pfohl-Leszkowicz *et al.*, 2002; Bennett & Klich, 2003; Castegnaro *et al.*, 2006). OTA is produced by *A. ochraceus*, *P. cyclopium*, *P. viridicatum*, *A. versicolor*, *A. glaucus*, *A. flavus* and *A. niger* (Makun *et al.*, 2007) and classified in group 2B which is possible carcinogenic compound to humans by IARC. It has been implicated in Balkan Endemic Nephropathy (BEN) and urinary tumors in human (Monaci & Palmisano, 2004).

Mycotoxicoses are diseases or disorders resulted from ingestion of foods or feeds contaminated by mycotoxins. Mycotoxin contamination is favoured by stress factors during plant growth, late harvesting crops, high ambient humidity which preventing drying, improper storage practices and lack of awareness on mycotoxin contamination (Bankole & Adebajo, 2003). Moreover, there are three important factors which enhanced the growth of toxigenic fungi and mycotoxin production namely oxygen, temperature and water activity (Frisvad *et al.*, 2007). Some toxigenic fungi are also able to grow under anaerobic conditions (Ayejuyo *et al.*, 2008).

Determination of mycotoxins in food-stuffs can be done through various methods such as thin-layer chromatography (TLC), enzyme-linked immunosorbent assay (ELISA) and high performance liquid chromatography (HPLC) combined mass spectroscopy (Valenta, 1998). HPLC with fluorescent detection is the most widely used

technique in determining mycotoxins in food since very low detection limit can be reached, due to the fact that most of the toxins in food have natural fluorescence (Zinedine *et al.*, 2006).

Although mycotoxins in food cause harmful effects on human health, it is impossible to completely eliminate mycotoxins in food. However, establishment of mycotoxins regulations by national and international organizations and agencies such as FAO of the United Nations and the U.S. Food and Drug Administration Committee on Additives and Contaminants can help to reduce the consumption of mycotoxin contaminated food. Until now, the European Union (EU) limit exists for 40 toxins and this number will further grow in the coming years (Van Egmond & Jonker, 2004).

Since rice is a staple food of Malaysia, daily consumption without knowing the negative effects of the toxigenic fungi and their mycotoxins can cause health-related problems among peoples. Regarding this issue, until now there is limited research on the toxigenic fungi and their mycotoxins in rice in Malaysia. Therefore, the studies were carried out to isolate, identify toxigenic fungi found on rice and analyze the natural occurrence of mycotoxins in various types of rice by HPLC. The specific objectives were:

1. To identify toxigenic fungi isolated from rice grain samples.
2. To study the fungal distribution on various types of rice.
3. To analyze mycotoxins level in various types of rice grain samples.

CHAPTER 2

LITERATURE REVIEW

2.1 Rice

2.1.1 Rice Variety

Rice is a member of the family Poaceae (true grass) with 23 species are known worldwide. It is classified into two species, *Oryza sativa* and *Oryza glaberrima* (Reiter *et al.*, 2010). The most widely cultivated species in Asia is *O. sativa*, which is characterized as an annual grass, with round, hollow, jointed culms, rather flat, sessile leaf blades, and a terminal panicle. It may grow more than one year under favourable conditions. In West Africa where *O. glaberrima* is originated, it is strictly annual and lacks of secondary branching on the primary branches of the panicle. The grain is small, pear-shaped and has a red bran and an olive-to-black seed coat (Linares, 2002). There are only minor differences between the two species which is related to pubescence on the lemmas and length of the ligule.

Rice plant become dominant species which was originated from wild rice variations, and this particular grain then spread throughout Asia. Today, the modern rice is a result of human invention after hundred of years of studies. Three main varieties of rice had developed in Asia based on the amylose content of the grain which were indica (high in amylose and cooking to fluffy grains to be eaten with the fingers), japonica (low in amylose and cooking to sticky masses suitable for eating as clumps with chopsticks),

and javanica (intermediate amylose content and stickiness). Indica rice is grown mostly in Southern Asia including India, Thailand, Vietnam, Southern China and Malaysia where it has hot and tropical climates. Japonica rice is planted in the temperate climates like Australia, California, Egypt and Japan (IRRI, 1993).

There are 140,000 rice varieties that have been spread all over the world (FAO, 2005). Rice varieties can be divided into long, medium and short-grained which are grown in different regions. The most well known variety is jasmine and basmati that also known as 'queen of fragrance'. Basmati belongs to long grain rice that mainly grown and exported by Pakistan and India (Reiter *et al.*, 2010). Jasmine rice is mainly grown in Thailand has a rich aroma after cooking and slightly sticky than basmati.

2.1.2 Rice as Staple Food

Rice is a predominant staple food for at least 17 countries in Asia including Malaysia and the Pacific, nine countries in North and South America and eight countries in Africa (FAO, 2004). In 2010/2011, 452.28 million Mt of rice were consumed which showed the importance of rice as source of food for almost half of the world' populations (WASDE, 2011). Three billion people which is one out of every two people on the earth consume an average 70 kg of rice a year. This includes the countries that are not major rice consumers (FAO, 2005). This number is even higher in the major rice-eating countries. Normally, two or three times of rice meals are taken by Asian for a day.

Rice is the Asian staple food for many reasons. It is convenience to cook especially for the poor because rice cooks quickly and requires less fuel than most other

staple foods. Rice forms an integral part of the culinary tradition for many Asian cultures as preferences of rice by each culture are based on the taste, texture, colour and stickiness of rice varieties (FAO, 2004). Most people in Asia can survive with a bowl of rice and small amount of side dishes. For example, Koreans can be satisfied with only rice and 'kimchi' and the Thais are content with just rice and fish sauce. For Japanese, they prefer fresh rice and the Indians favour stored grains (Zhou *et al.*, 2002; Reiter *et al.*, 2010).

2.1.3 Rice Production

The market price of rice has been declining since 1995 which result in the farmers are not in the position to increase their yields. The governments of rice-producing countries also want to protect their domestic rice market to ensure their national food security. This cause the farmers to grow rice for local consumption in order for them to be involved in international trade (FAO, 2005) and rice production begun to decline over the years.

Asia is the major world's rice producer which provides about 90% of the world's rice production. Many Asian fields have already converted to high-yielding varieties which restricted the Asian fields to continue increasing production. This becomes a problem since the rice production has been less than rice consumption. Due to a steady increased in population, the top rice producing countries are beginning to import rice from other countries. Indonesia for example, imports 14% and the other largest importers are Bangladesh (4%) and Brazil (3%). China and India consumed the majority

of the rice produce domestically although they are the largest two exporting countries. In 2005, FAO reported that the population in Asia is 3.5 billion and increasing to 50 million is a year, while the rice production is increasing at the rate less than 1% per year.

In 2009, world rice production was quite low due to several reasons mainly related to climatic reasons such as typhoon in the Philippines, drought and floods in India. However, the number increased in 2010 with FAO reported that China is the top rice-producing country which produced 166,417,000 Mt followed by India (132,013,000 Mt), Indonesia (52,078,832 Mt) and Bangladesh (38,060,000 Mt) (FAO, 2011).

2.1.4 Rice Sector in Malaysia

Commercial rice plantation system in Malaysia was introduced by British colonial government in the early 20th century. During that time, rubber, coconuts and peppers were planted for export purposes. However, after World War I, those crops had declining in price resulted in other food crops such as fruits and rice took over (IRRI, 1993). Contribution of agriculture sector towards Malaysian economy started to give a significant contribution prior to 1970s. The agricultural sector contributed one-third of the gross domestic product (GDP), provided employment to half of the workforce and accounted for 50% of the foreign exchange income (Elenita & Erna, 2005).

Rice is regarded as the most important crop in the food-sector in Malaysia in addition to coconuts, sugar-cane, maize, tea, coffee and sweet potato. In the 1960s, the Rice Self-Sufficient policy was introduced by Malaysian government to ensure food-security as rice is considered as a security crop and Malaysian government encouraged

sufficient domestic production. The government is focusing on automation, precision farming and implementing of various mechanisms to develop and modernize the rice industry. The policy also aim to generate income for producers mainly based on social, economic and political considerations and premised on increasing the economic status of rice farmers, who majority are Malays and living in rural areas (Elenita & Erna, 2005). An incentive schemes were drawn up by the Ministry of Agriculture and Agro-based Industries to attract private sector investments to boost rice production. The government also has established a subsidy scheme to encourage farmers to continue planting rice. The farmers are paid a certain amount of money according to their yields and were provided with fertilizers at special prices (IRRI, 1993).

Malaysia placed rice under highly sensitive list along with beverages, poultry, poultry egg, sugar, maize and tapioca. Thus, import of rice is needed for Malaysia is increasingly dependent on rice while protecting the interest of vulnerable segment of its population that remains reliant of rice production (Elenita & Erna, 2005). Most of rice exported to Malaysia are from Southeast Asia countries such as Thailand, Indonesia and Vietnam.

BERNAS is the sole importer of rice that handles approximately 700,000 to 900,000 imported rice tons per year (Suthida *et al.*, 2008). The New Strait Times (2004) reported that there are 275 licensed millers and 30% of them are under BERNAS. Polished rice that is marketed under BERNAS Group are Yew Heng Leong, Jasmine Food Corporation, Serbawangi, Era Bayam Kota, Fajar Jerlun, Dayabest and Sazarice. Jasmine, Saga, Sunwhite, Sakura and Jati are among the established brands distributed

by BERNAS Group. Since BERNAS is a government-interest company, the trades deal of the rice usually tied country-to-country (Elenita & Erna, 2005).

2.1.5 Importance of Rice

Rice is considered as source of energy for half of the people in the world. Compare to other cereal, rice produces 20% of the world's dietary energy supply while wheat supplies 19% and only 5% comes from maize (FAO, 2004). It provides more than 800 calories per person per day and it is rich in carbohydrates and has a moderate amount of protein, vitamin B, thiamine, riboflavin, niacin and dietary fibre but lacks of vitamin C and D and beta-carotene (FAO, 2005). Unmilled rice has a significant amount of dietary fibre (FAO, 2004).

There are over 40,000 varieties of rice and the varieties mainly grown worldwide are basmati, brown, jasmine, black, red and glutinous rice. Several types of black rice and red rice have a high content of antioxidant which can help to reduce cholesterol and the oil from some rice bran is claimed to be effective in fighting coronary heart disease and cancer (FAO, 2005).

Enriched rice is white containing ordinary milled rice and a small percentage of milled rice heavily fortified with thiamin, niacin, and iron phosphate to raise the vitamin and iron content slightly above the level present in brown rice (IRRI, 1965). When the yellow coloured riboflavin is added to the enriching agents, white pigments such as calcium oxide, talc, and titanium dioxide are also included in the enriching mixture to make the finished product appear white (IRRI, 1965).

Rice also provides income to the poor. For an example, rice is processed into products such as snack and breakfast food. In India, rice was applied in the arts and crafts which rice paste is used in creating patterns on cloth by employed it in the resist-dyeing technique. Rice plantation provides jobs to many people especially to those who live in rural areas (Sunthonvit *et al.*, 2003).

Rice is also used as decorative features frequently consist of wall paintings and floor patterns in traditional homes. In many parts of India, they used rice powder as part of daily ritual, ephemeral, abstract designs by paste it on domestic thresholds and floors (Ahuja, & Ahuja, 2010). These colourful decorations are known by various names such as alpana in Bengal, mandana in Rajasthan and kolam in South India. These decorations were drawn in front of the door that mostly can be seen during 'Deepavali' festivals which are meant to bring good luck to the home (Ahuja & Ahuja, 2010).

Straw, bran, hulls and husks can be used for animal feed and broken grains can be salvaged and mix with rice bran to make rice pellets to feed the fish or can be sell to aquaculturists. The straw can be woven into hats or shoes or used for mushroom culture (Makun *et al.*, 2007). It also can be fed directly to the animals or treated with urea or ammonia to make it easily digestible (FAO, 2005).

2.2 Pests and Diseases of Rice

In tropical areas, rice is usually planted throughout the year as the climate is hot and humid which can enhances the growth and development of pathogens that cause diseases on the plants. There are several rice pests such as weeds, birds, rodents, snails

and pathogens that can significantly reduce the yield and constrains for sustainable production of rice. Pathogens not only cause losses in rice yields but they have been associated to distribute toxigenic fungi. The fungi that infect crops on the field can persist and proliferate in storage resulting in increased fungal and mycotoxin contamination (Bainton *et al.*, 1980).

Rice fields in many rice-growing regions worldwide have problems regarding the growth of weeds (Guo *et al.*, 2005; Juan *et al.*, 2008). Weedy rice or also known as red rice because it has red pericarp has high competitive ability and persistence in rice fields. It causes significant losses of rice yields due to its high fecundity and seed dormancy (Federici *et al.*, 2001). Weedy rice occurs in all major rice growing areas in the tropics, being a particular problem in direct seeded rice agriculture of Latin and North America, the Caribbean, Africa and Southeast Asia (Oka, 1988). In Jiangsu Province, China, weedy rice commonly occurs in fields with a wheat-rice rotation, under no tillage and direct-seeded rice establishment (Juan *et al.*, 2008).

Rice blast caused by the fungus *Pyricularia oryzae*, (*Magnaporthe grisea*), is the most significant disease affecting rice cultivation all over the world and rendered serious losses to susceptible varieties under favourable conditions (Ou, 1985). In Japan, blast causes annual yield losses up to 275,000 tons of rice which is 25% of rice production (Ribot *et al.*, 2008). The disease is often called leaf blast, rotten neck, or panicle blast depends on the part that the infections occur. The infected rice crops show symptoms such as spots or lesions on leaves, nodes, panicles, and collar of the flag leaves. Blast causes longer lesions and develops more rapidly compare to brown leaf spot. The fungi frequently attack the node at the base of the panicle and the branches of the panicle. If

the panicle is attacked early in its development, the grains on the lower portion of the panicle may be blank, giving the head a bleached whitish colour, giving the term 'blasted' head or rice 'blast'. Panicle of rice may break if the node at the base of the panicle is infected causing the 'rotten neck' condition. The fungus may also attack the nodes or joints of the stem. The sheath tissue rots and the part of the stem above the point of infection often is killed when the node is infected (Ribot *et al.*, 2008).

Bakanae disease is one of the serious diseases that first identified on rice in Asia (Sun & Snyder, 1981) and cause up to 70% losses. It is caused by *Fusarium fujikuroi* which is an anamorph in the *Gibberella fujikuroi* species complex. The basic symptom of bakanae disease is that the infected rice will have an abnormal elongation growth, produce empty panicles, eventually topple and die. The infected seedlings are elongated, more slender and slightly chlorotic compared to healthy seedling that is green in colour. The heavily infected seedlings will die before they are transferred into the fields. The seedlings that manage to survive although have been infected will retarded and have abnormal growth (Pavgi & Singh, 1964). It has been reported that bakanae disease cause rice losses till it reached 3.1-14.7% area in North Thailand. In Malaysia, this disease was not considered as a serious disease but at the end of 1985, this disease started to develop seriously especially in a few areas of rice plantation in Perlis, Kedah, Perak and Kelantan (Saad, 1986).

2.3 Toxigenic Fungi

Toxigenic fungi can be divided into two groups which produce toxins before harvest and the fungi that produce toxins after harvest. The fungi that produce toxins before harvest is called field fungi such as *Alternaria* and *Fusarium* and the second group is known as storage fungi especially *Aspergillus*, *Penicillium*, *Rhizopus* and *Mucor* species infect grains after harvest and can grow on them during storage (Miller, 1995; Amadi & Adeniyi, 2009). Toxigenic fungi are well-known to infest rice grain under storage conditions and produce mycotoxins (Reddy *et al.*, 2009). Some fungi produce severe toxins only at specific level of moisture, temperature or oxygen. Toxigenic fungal contamination not only causes deterioration of food but also cause food borne intoxicant in humans and animals as they produce mycotoxins (Murthy *et al.*, 2009). The main genera of toxigenic fungi are *Aspergillus*, *Penicillium* and *Fusarium*, but *Trichoderma*, *Trichothecium* and *Alternaria* are also important as food contaminants or as plant pathogens (Smith, 1983). Toxigenic fungi can cause health hazards because inhalations of their spores can cause allergy especially to those suffering from hay fever and asthma as well as many other related diseases.

2.3.1 *Aspergillus*

Aspergillus is one of the major fungi that cause spoilage on stored cereals, spices, nuts, bread, pickles, jams and preserves (Lacey, 1994; Filterborg *et al.*, 2002). Naturally occurring *Aspergillus* toxins have proven to be a threat to the human and

animal health (Bhat *et al.*, 2010) and garner people interests due to the discovery of *Aspergillus* species worldwide on foods and feeds.

There are eight teleomorphic genera which have *Aspergillus* conidial state. They are *Chaetosartoya*, *Emericella*, *Eurotium*, *Fennelia*, *Hemicarpenales*, *Neosartoya*, *Petromyces* and *Sclerocleista*. Many *Aspergillus* species consist of *Eurotium* and *Emricella* in which members of *Eurotium* are responsible for the spoilage of foodstuffs although many *Aspergillus* species have no known sexual state (Pitt *et al.*, 2000; Webster & Weber, 2007). The ability to reproduce sexually has been lost on many separate occasions, because many strictly mitotic *Aspergillus* species have teleomorphic species as their closest relatives (Geiser *et al.*, 1996). The loss of teleomorph is indicated by the fact that many *Aspergillus* species still produces sterile structure (e.g. sclerotia) or cells which are similar to cleistothecia (Webster & Weber, 2007).

Aspergillus species has frequently reported as pathogen of human and animals which cause severe health problems mainly related with respiratory tract and lung. Infection by *Aspergillus* has caused the rising number of immunosuppressed people. At least, 40 species of *Aspergillus* are sources of immunosuppression and about 90% of human aspergillosis involved *A. fumigatus* followed by *A. flavus*, *A. terreus*, *A. niger*, *A. nidulans* and *A. ochraceus* (Bertout *et al.*, 2001). Aspergillosis is a major cause of death among cancer patients and is strongly on the increase among AIDS sufferers. Besides, *A. fumigatus* causes allergic bronchopulmonary aspergillosis which infects patients already suffering from chronic irritation of the lung due to asthma or cystic fibrosis. The disease can lead to fatal destruction of the lung tissues (Webster & Weber, 2007).

2.3.2 *Penicillium*

Penicillium is one of the most important fungi that is ubiquitous and can be found in soil, water, air, indoor and food as contaminants. The conidia are usually present in the air, therefore *Penicillium* colonies are frequent contaminants of cultures in the laboratory. *Penicillium* species is not as thermotolerant as *Aspergillus* with only a few species are able to grow at 37°C (Webster & Weber, 2007).

Some species of *Penicillium* have teleomorphs which can be assigned to *Eupenicillium* and *Talaromyces*. Members of the genus *Eupenicillium* produce conidiophores which may either be mono-, bi- or ter-verticillate. The terverticillate species is mostly relevant as food spoilage which are mainly *P. aurantiogriseum*, *P. digitatum*, *P. italicum* and *P. expansum* or as mycotoxin producers such as *P. expansum* (Webster & Weber, 2007).

Approximately, there are 100 species of *Penicillium* that are toxigenic and the range of the mycotoxins produce by these fungi is much broader than any other genus (Sweeney & Dobson, 1998). Pitt and Leistner (1991) have listed 27 mycotoxins produced by 32 species of *Penicillium* possess demonstrated toxicity. Luteoskyrin, erythskyrin, cyclochlorotine and islanditoxin are among the mycotoxins produced by *P. islandicum* found in yellow rice. Other commonly occurring *Penicillium* toxins are patulin and citrinin produced by *P. expansum* and *P. citrinum* (Pitt, 1991)

The toxicity of the *Penicillium* toxins is very diverse in which most of the toxins can be placed in one or other of two broad groups. It is those that affect liver and kidney function, and those that are neurotoxins (Pitt, 1991). *Penicillium verrucosum* produce

OTA that appears to be responsible for kidney degeneration and acute cardiac beriberi was a mycotoxicosis resulting from the growth of *P. citreonigrum* in rice (Pitt, 1991). Although *Penicillium* is a toxigenic fungi but clinical reports of *Penicillium* infections are uncommon except of *P. marneffe* which is a pathogenic species. *Penicillium marneffe* causes systemic and disseminated infections in Southeast Asia which have increased dramatically with the spread of AIDS (Webster & Weber, 2007). The species also causes immunosuppression in humans. Therefore, *P. marneffe* causes systemic and disseminated infections whereby disseminated infections often occur in lung, liver and skin but it can be treated with amphotericin B and itraconazole (Harrison & Levitz, 1996).

2.3.3 *Fusarium*

Fusarium is a genus of phytopathogenic fungi reported to have increased in the virulence and importance in causing plant disease in the tropics (Salleh, 2007). Some *Fusarium* species are wholly saprophytic while others, in addition to their saprophytic potential, also range from being widely to highly pathogenic and non-pathogenic; and, some are obligate parasites. Furthermore, they may be pathogenic in one environment and saprophytic in another.

Fusarium-type conidia are known in several species of *Nectria* and also in the related genus *Gibberella* (Samuels *et al.*, 2001; Webster & Weber, 2007). For many *Fusarium* species, a teleomorph has not yet been found. *Fusarium oxysporum* is commonly isolated species from soil has no sexual stage known but it is monophyletic

with the *Gibberella-Fusarium* complex and can be regarded as an asexual *Gibberella* (Samuels *et al.*, 2001; Webster & Weber, 2007). *Gibberella fujikuroi* (anamorph *F. fujikuroi*) is a *Fusarium* species that commonly cause bakanae disease in rice due to the production of a toxin known as gibberellic acid.

Toxigenic *Fusarium* commonly found in tropical areas where the temperature and relative humidity is high. It is also commonly found on seed and also as contaminants in human food and feed in West Malaysia (Salleh & Sulaiman, 1984; Salleh & Zunaidah, 1984; Salleh & Strange, 1988). There are 20 species of toxigenic *Fusarium* that have been examined by Marasas *et al.* (1985). Among the common toxigenic *Fusarium* are *F. equiseti*, *F. semitectum*, *F. proliferatum*, *F. solani* and *F. graminearum*. *Fusarium* toxins can be divided into several common biosynthetic categories of secondary metabolites such as polyketides (zearalenone), tetramic acid (tenuazonic acid) and the sesquiterpenes (trichothecenes) (Pitt, 1991).

Mycotoxins produce by *F. graminearum* cause oestrogenic, emetic and feed-refusal syndromes. It also causes vulvovaginitis and infertility in cattle and pigs (Joffe, 1986; Webster & Weber, 2007). Mycotoxins produce by *F. verticillioides* cause human oesophageal cancer and equine leukoencephalomalacia. Some species are also known to cause diseases of humans such as onychomycosis (nail infections), keratomycosis of the cornea, ulcers, necroses, skin infections and fatal infections of internal organs, especially in immunocompromised patients (Joffe, 1986; Webster & Weber, 2007). Toxic aleukia cause by several other *Fusarium* species reduces white blood cell count in animals and humans (Joffe, 1986).

2.4 Mycotoxins

Mycotoxins are secondary metabolites produced by certain fungi which grow on agricultural commodities under particular conditions. Mycotoxin-producing moulds are widely distributed in nature and they contaminate food, feeds, or raw materials. Major food contaminants occur on cereals, nuts, dried fruit, coffee, cocoa, spices, oil seeds, dried peas, beans and fruits (Turner *et al.*, 2009). Mycotoxins can occur in both temperate and tropical regions, depending on the fungal species. The production of mycotoxin is influenced by the organism genotype and its environment such as humidity, temperature, nutrients and competition from other organisms (Bosch *et al.*, 1989). The levels of mycotoxin become higher when the conditions are suitable and fungi proliferate into colonies.

Currently, there are more than 400 mycotoxins that have been identified worldwide (Zinedine & Manes, 2009). Mycotoxins usually enter the body via inhalation of toxigenic spores or ingestion of contaminated food. It may cause mycotoxicoses in humans and animals which is the result of ingesting toxins contaminated diets (Marasas & van Rensburg, 1979; Moss, 1994). Some mycotoxins are lethal and some can have health effects such as weakening of the immune system without producing symptoms specific to the mycotoxins, allergic or irritations.

The first outbreak of mycotoxicoses was ergotism, associated with the consumption of ergot caused by *Claviceps purpurea*. Other known outbreaks of mycotoxicoses were stachybotryotoxicosis in horses and alimentary toxic aleukia in humans in the USSR in the 1930's followed by an outbreak of aflatoxicosis of turkeys in

England in 1960 (Athapol & Thomas, 1989). Mycotoxins affect a variety of tissues and organs, such as liver, kidneys, nervous systems and gastrointestinal problems.

Consumption of fungal infected crops, either by eaten directly or by being used as livestock feed can cause mycotoxins to appear in the food chain. Mycotoxins can remain in the food chain in meat and dairy products because mycotoxins are resistant to decomposition and not broken down in digestion. Mycotoxins cannot be destroyed by temperature treatments such as cooking and freezing (Hong *et al.*, 2010). These substances are not only dangerous for human health but also deteriorate the marketable quality of the food product, thus could cause economic losses.

In the 20th century, food-based mycotoxins were extensively studied worldwide especially in Europe. European Directives and Commission Regulations is one of the responsible body which set the statutory levels of a range of mycotoxins permissible in food and animal feed. In 1995, Codex Alimentarius Commission agreed to adopt safety and quality criteria in rice for human consumption (Codex Standard for Rice). Other Codex work includes setting maximum limits for pesticide residues and discusses the possible limits for certain heavy metals such as cadmium and also mycotoxins. The standards set for rice are accepted by the World Trade Organization (WTO), FAO and the World Health Organization (WHO) to ensure that the limits are based on a sound scientific basis.

2.5 Major Groups of Rice Mycotoxins

2.5.1 Aflatoxin (AF)

Aflatoxin is a group of mycotoxins produced by *Aspergillus* species with aflatoxin B₁ (AFB₁) is the most toxic and potent carcinogen associated with liver cancer. The basic structure of aflatoxin is bis-furano-isocoumarin (Figure 2.1) and the most important is aflatoxin B₁ produced by *A. flavus*, aflatoxin G₁ and G₂ produced by *A. parasiticus*. At least eight aflatoxins are now generally recognized namely, B₁, B₂, G₁, G₂, M₁, M₂, B_{2a} and G_{2a}. Among these aflatoxins, B₁ and G₁ are the most frequently encountered in quantities sufficient to be toxic.

Aflatoxins is a great concern since it is carcinogenic, mutagenic, teratogenic and immunosuppressive effects which has detrimental effects on human and animal's health (Eaton & Gallagher, 1994). It causes liver damage and impaired productivity, including reduced growth rate and milk production. AFB₁ is the most hepatocarcinogen known in mammals and is classified as Group I carcinogen by IARC.

Aflatoxins has been found as a natural contaminant in many agricultural commodities including cassava, corn, cottonseed, cottonseed meal, peanuts, peanut meal and oats (Borker *et al.*, 1966). It is common in Latin America, Africa, Asia and Australia (Mabbett, 1999). Athapol and Thomas (1989) reported that aflatoxicosis in Brazil was caused by feeding aflatoxin-contaminated peanut meal to human. Over 123 deaths were reported in Kenya due to aflatoxins outbreaks in 2004 and 2005 which was due to maize contamination with aflatoxins, related to the local methods of harvesting, storing and preparing maize (Centers for Disease Control, 2004). AFB₁ is also associated with the

incidence of human hepatocellular carcinoma and frequent consumption of aflatoxins contaminated foods has been established in hepatic endemic areas of the world (Makun *et al.*, 2007). In Kenya, acute mycotoxicosis of aflatoxin has been reported by Marasas (1988). The mycotoxin is also probably occurred in South Africa but misdiagnosed (Marasas, 1988). In India, Malaysia and Kenya, there have been reports of acute liver disease following consumption of maize contaminated with aflatoxin (Bhat, 1991; Lye *et al.*, 1995; Shephard, 2004). Beriberi has been reported in the late 19th century due to the fungus-infected rice in Japan which could be due to contamination of aflatoxin (Uraguchi & Yamazaki, 1978). Consumption of contaminated cereals and nuts with aflatoxin also cause malabsorption syndrome and decrease bone strength (Nelson *et al.*, 1982; Osborne *et al.*, 1982).

Aflatoxin level of 20 ppb is permitted in feed grains and mixed feeds in the USA and for maize export, an acceptable aflatoxin level of 50 ppb during loads. In many Asian countries, the maximum tolerated levels for aflatoxins in all foods ranging between 10-30 ppb (Stoloff *et al.*, 1991).

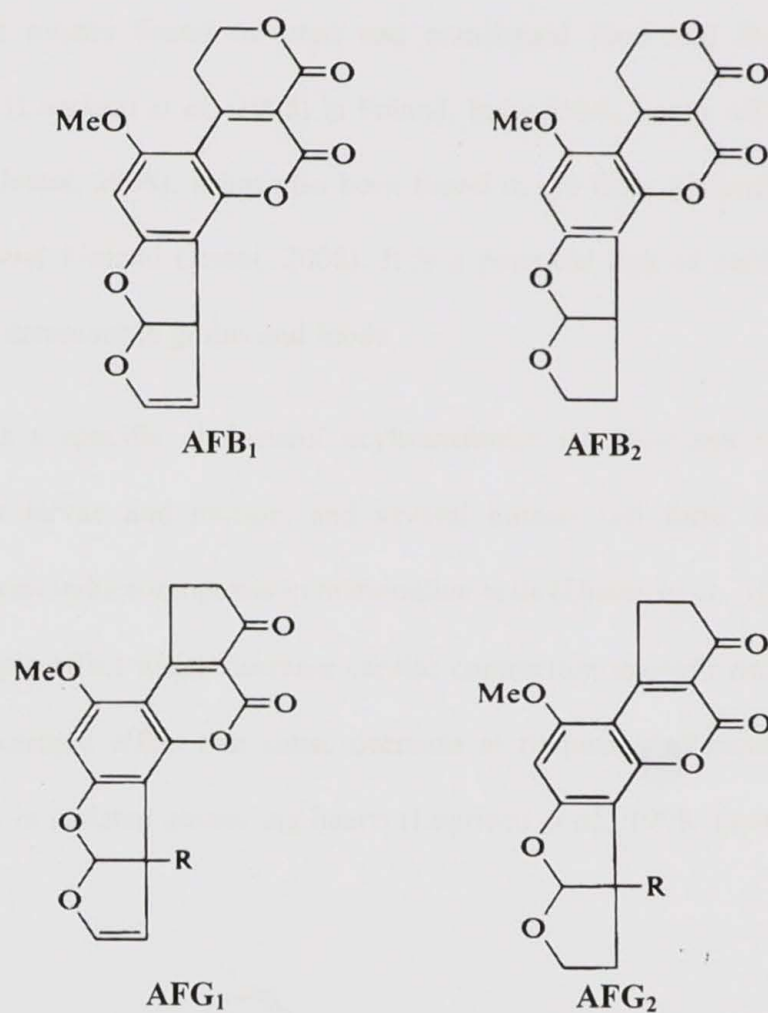


Figure 2.1: Structure of aflatoxins (Reddy *et al.*, 2010)

2.5.2 Beauvericin (BEA)

Beauvericin is a cyclic hexadepsipeptide mycotoxin produced by *Fusarium proliferatum*, *F. semitectum*, and *F. subglutinans*. BEA was first reported to be produced by entomopathogenic fungi such as *Beauveria bassiana* and *Paecilomyces fumosoroseus* (Logrieco *et al.*, 1998). The mycotoxin consists of three D-2-hydroxycarboxylic acid and N-methylamino acid residues linked alternately (Sørensen *et al.*, 2008) (Figure 2.2).