CHAPTER 8.0 GENERAL DISCUSSION

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Food fungi and mycotoxins problems have not by-passed tropical Asian countries including Malaysia. This is due to the high temperature and humidities prevailing and postharvest practices, which favour the growth of fungi including toxin-producing fungi. The frequency of contamination by mycotoxins in Malaysia is unknown. Much basic research and a more extensive and systematic screening program remain to be done to draw a clearer picture of the nature and extent of food fungi and mycotoxin problems in Malaysia with the aim of reducing contamination of food and feedstuffs. Hence, contributing to regional and global efforts in boosting domestic and international trade, and promoting improved human and animal health. Recent survey work has shown the occurrence of the fungus *A. flavus* to be widespread throughout the tropics and aflatoxin is frequently detected in a variety of feeds and agricultural commodity in Malaysia (Mat Isa and Tee, 1984).

Humans are exposed to mycotoxins by consuming foods contaminated with products of fungal growth. Such exposure is difficult to avoid because fungal growth in foods is not easy to prevent. Where foods are heavily contaminated, acute intoxication results but long-term exposure to low levels of mycotoxins is also a problem. Moss (1996) quoted that, in terms of acute toxicity, even the most poisonous of the mycotoxins commonly encountered in food are a factor of about a million times less toxic than the most virulent of the botulinum toxins and a factor of a thousand times less toxic than many algal toxins. The number of cases of confirmed acute mycotoxin poisoning in humans during the last few decades is very small compared with the numbers made ill by food poisoning bacteria such as

Salmonella and infectious diseases. However, the possibility of long-term chronic toxicity which is of special concern because several of these fungal metabolites are known to be teratogenic, carcinogenic, oestrogenic or immunosuppressive and their presence in foods may have more subtle effects on human health especially in the developing countries of the tropics where people may use badly stored plant products.

In Malaysia, Standard and Industrial Research Institute of Malaysia or SIRIM has issued a national code of practice to control aflatoxins in the processing of groundnuts to protect public health. Several other items awaiting approval by SIRIM includes fresh flowers and fruits, spices, condiments, coffee, palm oil, dairy products, sauces and food additives as they are essential items for consumers. There is no acceptance specifications of mycoflora count in raw materials used in the manufacture of food products including the starch-based food set by SIRIM or food manufacturers in Malaysia. The raw materials were used as they are obtained in the market with no microbiological tests being carried out. However, as indicated in Chapter 1.5, it was mentioned in the Food Act 1983 that the maximum permitted proportions of mycological contaminant in the proportions of mycological contaminant (all mycotoxins) is 35 μgkg^{-1}.

In this study, a survey carried out on 50 samples of starch-based food obtained at random from retail outlets indicate that mycoflora occurred on all samples of wheatflour, followed by 74% of glutinous rice grains and riceflour samples, 60% of ordinary rice grain samples, 48% of glutinous riceflour samples and 26% of cornflour samples. Aflatoxin producing colonies were also highest on wheatflour samples (20% positive) occurring at an average of 27 ± 73 $cfug^{-1}$. Halt

(1994) detected *A. flavus* in 9.9% samples of wheat and its resultant flour and flour samples had a mean value of 4.13 μgkg^{-1} aflatoxin B_1. Hence, in a survey of aflatoxins in ordinary rice grains and wheatflour, positive samples were mostly detected on wheatflour (21.7% positive) ranging from 11.25 - 296.88 μgkg^{-1}. Furthermore, samples collected from private homes are more susceptible to aflatoxin contamination where they are subjected to different environmental conditions during long-term storage. Aflatoxin contamination in ordinary rice grains occurred in a small fraction of the sample (6% positive) however their universal distribution and known effect on animals in tiny concentrations is sufficient justification for paying serious attention to them. Studies in Asia have shown positive correlations between long-term exposure to low levels of aflatoxin and the incidence of liver cell cancer (Chao *et al.* 1994; Ng *et al.*, 1994; Tseng, 1994). However, this link has not been clearly established in all countries or studies.

Although the fungal load in starch-based food obtained in the market in Malaysia were found to be low and acceptable according to the standards set by the International Commission on Microbiological Specifications for Foods, it is important to ensure that environmental changes are not conducive to the proliferation of the existing fungal inoculum in food. Studies on the effects of initial spore load by Sharma *et al.* (1980) and Karunaratne and Bullerman (1990), obtained comparable final dry mycelial weights irrespective of the size of inoculum. Furthermore, at the lowest inoculum level of *A. flavus*, germination of even one spore may result in a very high level of aflatoxins when environments are favourable for growth. However, Seiler (1986) showed that there is a steady

reduction in numbers of fungi with storage time at 25 °C in wheat and the resultant flour stored in bulk at moisture content of 12 - 13% i.e at an unfavourable condition for fungal growth.

The most important factors influencing fungal growth on an intrinsically susceptible substrate are water activity and temperature and mycotoxins may be formed as a result of such fungal growth. In many practical situations, water activity is the dominant environmental factor controlling growth of fungi and hence determining the stability of stored products. Water activity can be shown to influence each of the four main growth cycle phases by its effects upon the germination time or the length of the lag phase, the growth rate, the size of the stationary population, and the subsequent death rate.

Water vapour sorption isotherms are a fundamental characteristic of food materials, essential for the dehydration process and related to almost every aspect of the storage stability of dried products. It gives information about water activity, which is closely related to deteriorative reactions in foods. In this study, from the water vapour adsorption isotherms obtained for starch-based food, the critical moisture content (% dry basis) i.e the moisture content to be maintained at 25 °C that will not allow fungal growth was found to be 13.01% for ordinary rice grains, 12.87% for glutinous rice grains, 9.56% for riceflour, 10.61% for glutinous riceflour, 10.72% for wheatflour and 10.52% for cornflour. Correspondingly, in order to maintain these moisture content levels starch-based food must be stored at water activity level of not more than 0.65 equivalent to an equilibrium relative humidity of 65%. An increase of slightly more than 1% moisture content above the critical moisture content will result in premature spoilage. In accordance with

this study, research carried out by Dhiauddin and Ibni Hajar (1983) also suggested that paddy should be dried as soon as possible after harvest to a moisture content of 13% for long-term storage. A somewhat larger safety margin is advisable for long storage period and for large scale storage of raw materials for further processing. Rohani *et al.* (1985) reported that post-harvest losses of properly dried paddy due to bulk storage in the National Paddy Board ('Lembaga Padi Negara'-LPN) complexes in Malaysia was high due to hot spots developing in the grain bulk. Seventy per cent of the grains were damaged due to discolouration by fungi within three to four months of storage in 750-tonne bulk. This greatly reduced the market value of the final product.

For the quality control of food, the identification of the contaminating mycoflora is essential. The identification to species level should provide information about the possible production of mycotoxins. At present, the taxonomy of most food-borne fungal genera is known (Pitt and Hocking, 1985; Samson *et al.*, 1986; Samson and Van reenen-Hoekstra, 1988) so identification to species level is possible. Hence, in this study the descriptions of fungi isolated from starch-based food in Malaysia and known mycotoxins produced by each species was given together with their keys to aid in the identification. Storage of starch-based food at different levels of water activity resulted in the proliferation of 45 fungal species of which 12 species are from the genus *Aspergillus* and its teleomorphs, 22 species are from the genus *Penicillium* and its teleomorphs, six zygomycetes from five genera and five other species consisting of *Curvularia*, *Dreschlera*, *Monascus*, *Moniliella* and *Trichoderma*. Storage fungi are mainly of the genera *Aspergillus* and *Penicillium* since they have the ability to grow at lower

A_w levels than any other fungi. Of the 45 species isolated, 21 species are new records in Malaysia, 11 species have not been reported previously to occur on starch-based food including cereals and 26 species have been known to produce mycotoxins (Chapter 5). Hence, there are differences in the profile of fungal species isolated from the tropical regions compared to the temperate countries. It was also observed that the *Penicillium* species isolated had different colony colours and conidia ornamentation by SEM from those described in Ramirez (1982). Bridge *et al.* (1986) indicated that variations in morphological and biochemical characteristics in *Penicillium* species may be present in nature or could have arisen in preservation. They recommended that the extent of any variation present in single conidium isolates should be considered in future descriptions of conidial fungi in order to clarify taxonomically significant characters.

Different types of foods have characteristic mycofloras associated with them (Keen and Martin, 1971; Martin, Gilman and Keen, 1971). Water activity can determine the types of fungi most common on particular foods as the tolerance of water activity by different species of fungi are often sharply defined. Even though the starch-based food studied are intermediate moisture foods they harboured abundant viable fungi when plated on agar media. In this study, the dominant fungal species occurring on starch-based food stored at water activity ranging from 0.65 - 0.98 are *A. candidus, A. flavus, A. niger, R. arrhizus* and *R. microsporus* on ordinary rice, *A. flavus, A. niger, R. arrhizus* and *R. microsporus* on glutinous rice, *A. flavus, A. niger, A. terreus, P. chrysogenum, R. arrhizus* and *R. microsporus* on riceflour *and A. flavus, A. niger, P. chrysogenum, R. arrhizus*

and *R. microsporus* on glutinous wheatflour, *A. candidus*, *A. flavus* and *P. chrysogenum* on wheatflour and only *P. chrysogenum* was dominant on cornflour. All these dominant fungal species isolated from starch-based food have been known to produce mycotoxins and similarly with other improperly stored food as shown by Jimenez *et al.* (1991), Scudamore and Hetmanski (1995) and Mills *et al.* (1995), mycotoxins were produced which may constitue a potential hazard for the consumers. Moreover, fungi dominant on starch-based food were found to have high growth rates ranging from 2.4 mmday^{-1} colony diameter for *A. terreus* to 31.8 mmday^{-1} colony diameter for *R. microsporus*. *Rhizopus arrhizus* was most fast growing covering the petri dish in 2 days on starch extract agar at 25 °C. Most of the species studied also possess amylolytic activity i.e. they are capable of utilising starch for growth. Hence, they play an important role in the biodeterioration of starch-based food.

Regulation of mycotoxin-contaminated food demands constant monitoring. A screening method suitable for monitoring mycotoxins for food safety must be developed for raw and finished products susceptible to contamination. In this study, an extraction method and reversed-phase HPLC using C_{18} Econosil column and elution solvent consisting of water and 0.05% TFA in acetonitrile was able to detect and quantitate seven mycotoxins in 34 min. The reversed-phase HPLC used in this study gave highly reproducible retention times and was most sensitive for the detection of patulin being able to detect a minimum concentration of 31 ng followed by griseofulvin of 42 ng, aflatoxin G_1 of 125 ng, sterigmatocystin of 188 ng, aflatoxin B_1 of 200 ng and ochratoxin A of 250 ng. Cytochalasin E was least sensitive being able to detect a minimum concentration of 11.25 µg. The

extraction method used to extract mycotoxins from starch-based food gave recoveries > 90% for patulin, > 91% for aflatoxin G_1, > 92% for aflatoxin B_1, > 83% for griseofulvin, > 89% for cytochalasin E, > 88% for ochratoxin A and > 90% for sterigmatocystin.

Chemical analysis of mycotoxins lacks a genuine multi-mycotoxin assay, that is a single extraction and analytical procedure that will detect all the mycotoxins of major significance. Since food are often contaminated with various fungi and that some fungi possess the ability to simultaneously produce more than one toxin, therefore, a combination of both bioassay and chemical methods is necessary in an integrated chemico-biological approach for screening of potential toxicity in foods. Complete relience on chemical assay can prove misleading, particularly since mycotoxins not yet chemically characterized may also be present. Scudamore and Hetmanski (1995), on examining 50 samples of poorly stored cereals for mycotoxins, fungi and biological activity found that cereal extracts and fungal extracts were often toxic to brine shrimp larvae, but in some toxic extracts no known mycotoxins were identified. Undetected mycotoxins, even at concentrations that are apparently non-toxic, can interact with other factors to produce seriously detrimental effects. Furthermore, the presence of a toxigenic fungus on a substrate does not necessarily mean that some or all of the metabolites associated with it will be present, since the substrate and environmental condition appear to be crucial in affecting which mycotoxin is produced. This is supported by Jayaraman and Kalyanasundaram (1994) who observed a higher incidence of aflatoxin B_1 corresponding to a greater dominance of *A. candidus* instead of *A. flavus*. A factor which needs to be considered is the effects of competing fungi on

mycotoxin production. Both enhancement and inhibition of aflatoxin production in *A. parasiticus* by *P. rubrum* have been reported (Moss and Frank, 1987). Inhibition of aflatoxin production but not growth, of *A. parasiticus* by *A. candidus, A. chevalieri, A.niger*, and *E. chevalieri* have also been reported (Boller and Schroeder, 1973; Lacey, 1986). These interactions between fungi in turn depends on the environmental conditions, hence, *A. oryzae* and *A. niger* inhibited aflatoxin production at 0.98 A_w , had little effect at 0.95 A_w and stimulated it at 0.90 A_w.

The co-occurrence of several different mycotoxins by various fungi contaminating a food can affect both the level of mycotoxin production and the possibility of synergism in their toxicity (Miller, 1991). Such synergy has been demonstrated for pairs of mycotoxins, such as penicillic acid with ochratoxin and also aflatoxin with T-2 toxin (Ueno and Ueno,1972) and combinations of some *Fusarium* toxins (Coker, 1995). In the outbreak of acute hepatic encephalopathy in Malaysia, Lye *et al.* (1995) estimated a consumption of 3 mg of aflatoxins in 250 g (equivalent to one bowl) of noodles. However, none of the residual raw ingredients were found to be contaminated with aflatoxins. Contamination appeared likely in one batch of raw ingredients and of uneven distribution or from this study toxicity might be attributed to synergism of mycotoxins since proliferation of more than one fungal species took place and hence more than one mycotoxins were produced.

Food spoilage by fungi are organoleptically undesirable changes that effect the quality of foods. Of more concern is the production of mycotoxins by fungi at appreciable amount in food or feed that can cause mycotoxicoses before any signs

of spoilage is visible. The shelf-life of ordinary rice grains at the lowest A_w of 0.65 was 57 $\pm$ 2 days whereby visible appearance of fungi was observed. No mycotoxins were detected and the extracts showed < 50% mortality against brine shrimp at this water activity level even after 96 days. Among the mycotoxins screened, aflatoxin B_1 and sterigmatocystin were detected at the lowest A_w of 0.80 by 26 days and >50% mortality against the brine shrimp was exhibited by extracts stored at the lowest A_w of 0.80 by 26 days. The visible appearance of fungi at this water activity was earlier i.e. 13 $\pm$ 1 days.

The shelf-life of glutinous rice grains at the lowest A_w of 0.65 was 73 $\pm$ 1 days whereby visible appearance was observed. Similarly, no mycotoxins were detected at this A_w and the extracts showed < 50% mortality against the brine shrimp at this water activity level even after 96 days. Among the mycotoxins screened, ochratoxin A was detected at the lowest water activity of 0.80 by 26 days and > 50% mortality against the brine shrimp was exhibited by extracts stored at the lowest A_w of 0.80 by 26 days. The visible appearance of fungi at this water activity occurred earlier i.e. 17 $\pm$1 days.

At the lowest water activity of 0.65, no visible appearance of fungi was detected on riceflour, glutinous riceflour, and wheatflour at 6 months incubation. Also, no mycotoxins were detected at this water activity and the extracts showed < 50% mortality against the brine shrimp even after 96 days. Among the mycotoxins screened, aflatoxin B_1 was detected at the lowest A_w of 0.80 by 26 days for riceflour and 96 days for glutinous riceflour. More than 50% mortality against brine shrimp was exhibited by extracts stored at the lowest A_w of 0.80 by 26 days for riceflour and 54 days for glutinous riceflour. At this water activity

level, visible appearance of fungi was observed at 28 $\pm$ 3 days for riceflour and 64 $\pm$ 4 days for glutinous riceflour. For wheatflour, among the mycotoxins screened, aflatoxin B_1 and G_1 were detected at the lowest A_w of 0.80 by 54 days but > 50% mortality against brine shrimp was exhibited by wheatflour extracts at the lowest A_w of 0.75 by 54 days. The visible appearance of fungi on wheatflour at 0.75 A_w was observed by 27 $\pm$ 2 days while at 0.80 A_w visible appearance occurred by 17 $\pm$ 2 days. Cornflour can be stored at a higher water activity of 0.80 since no visible appearance of fungi was observed at 6 months of storage. Similarly, no mycotoxins were detected at this A_w and the extracts exhibited < 50 % mortality against the brine shrimp at this water activity level even after 96 days. Among the mycotoxins screened, aflatoxin B_1 was detected at the lowest A_w of 0.90 by 26 days but > 50% mortality was exhibited by extracts stored at the lowest activity of 0.85 by 54 days. This toxicity may be due to other toxins produced which are not screened for. The visible appearance of fungi at 0.85 and 0.90 A_w was observed later at 124 $\pm$ 4 days and 69 $\pm$ 2 days respectively. Hence, for ordinary rice grains, glutinous rice grains, riceflour, glutinous riceflour and wheatflour visible appearance of fungi occurred first than production of mycotoxins or extracts exhibiting toxicity against the brine shrimp. For cornflour, visible appearance of fungi occurred later than production of mycotoxins or extracts exhibiting toxicity against the brine shrimp.

Despite the large amount of information generated, there is still a lack of awareness among farmers, food processors, and consumers regarding mycotoxins and its potential risk to human and animal health. An information campaign is needed to alert consumers and it is imperative that farmers, traders, and

processors be informed and trained on the proper handling of commodities. Success in preventing mycotoxin contamination of raw materials for human consumption, however, must involve more than farmers and traders. Government participation is imperative if an environment conducive to the sustained implementation of a mycotoxin control program is to be created. In support of policies formulated, grades and standards must be established for each commodity and a pricing system must be developed so that incentives can be provided to the farmers and processors alike to encourage them to produce good quality raw materials.