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Research Article

Safer management practices for Aflatoxigenic fungi in nutmeg (*Myristica fragrans*)

SALLY K. MATHEW[1], K. SURENDRA GOPAL[2*], N. MINIRAJ[3], ANJALY VARGHESE[1] and R. JEEVA[2]

[1]*Department of Plant Pathology, College of Horticulture, Kerala Agricultural University, Vellanikkara, Thrissur, Kerala, India*
[2]*Department of Agricultural Microbiology, College of Horticulture, Kerala Agricultural University, Vellanikkara, Thrissur, Kerala, India*
[3]*Department of Plantation crops and Spices, College of Horticulture, Kerala Agricultural University, Vellanikkara, Thrissur, Kerala, India*
**Corresponding author Email:* ksurgopal@yahoo.co.in

ABSTRACT: A study was undertaken to determine the presence of aflatoxigenic fungi and other fungal contaminants in the kernel, mace and shell of ripened nutmeg tree fruits collected from nutmeg plots of Ernakulam and Thrissur districts of Kerala. Mace and nut were separated from fruits and washed in tap water to remove soil particles and other debris adhering to it. The kernel, mace and shell were subjected to different chemical/botanicals by four methods viz; dipping, decoction, samples in cloth bag and fumigation in order to prevent aflatoxigenic fungi and other fungal growth during storage. Based on the present studies, it was found that pre-treatment of kernel, mace and shell of nutmeg with either citrus leaf decoction (@100 g/litre) or curry leaf decoction (@ 100 g/litre) or *Anona* seed extract (5%) using decoction method were effective against aflatoxigenic fungi as well as fungal contamination. However, further studies are needed on pilot scale to confirm.

KEY WORDS: Aflatoxigenic fungi, botanicals, chemicals, nutmeg

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INTRODUCTION

The nutmeg tree **(***Myristica fragrans*) is a native of Moluccas islands in East Indonesia. The tree yields two spices namely the kernel of the seed and mace covering the shell of the seed. The two common problems with nutmeg quality are hidden mold inside the nutmeg and aflatoxin contamination.

Among the various fungal contamination of spices, aflatoxin producing *Aspergillus* spp. are widely distributed in nature. Since, these toxins are strongly carcinogenic in humans and domestic animals, it is important to analyze foodstuffs for their presence. Aflatoxins are toxins produced by *Aspergillus flavus* and *A. parasiticus* which are considered to be serious due to their association with various diseases in human and animals, such as aflatoxicoses and liver cancer. There are four naturally occurring aflatoxins in many stored commodities, i.e. aflatoxins B1, B2, G1 and G2. The most common and toxic aflatoxin is aflatoxin B (Basappa, 2009). A study conducted on the distribution of aflatoxigenic fungi in 25 imported Indonesian nutmeg reported high levels of aflatoxins B and G. The aflatoxigenic isolates were identified as *Aspergillus nomius* and *A. bombycis* and it was confirmed that these two species are mainly responsible for aflatoxin G contamination in nutmeg products (Kiyoshi Okano, *et. al.,* 2012). Nutmeg imported from India, Sri Lanka, Indonesia and Brazil were infected by *A. niger*, *A. flavus* and *Rhizopus stolonifer*. The dominant fungi in these samples were *A. flavus*.

Aspergillus niger and *Endomyces fibuliger* were the dominant fungi in nutmeg kernels from farmers and collectors, while *Endomyces repens* was dominant in nutmeg samples obtained from exporters in North Sulawesi province. Aflatoxin B and total aflatoxin contents in nutmeg samples collected from farmers and exporters were relatively high. Ezekiel et al. (2013) reported that *Aspergillus* was the most predominant (78.9%) genera. Of the three spices, calabash nutmeg showed the highest significant ($p<0.05$) fungal count (3.45 Log CFU_{10}), incidence of toxigenic *Aspergillus flavus* (50%) and AFB1 (50%). Spices like calabash nutmeg are prone to contamination by moulds including toxigenic *Aspergillus.* Based on the literature available, it is clear that aflatoxin contamination is a problem in spices and few reports indicated the presence of *Aspergillus* sp. in nutmeg.

The studies on incidence of aflatoxins in spices is scanty in Kerala particularly in nutmeg. Moreover, there are no eco-friendly or safer management practices in nutmeg to control fungal contamination as well as aflatoxin production. Hence, a study was undertaken to determine the fungal contamination and aflatoxin production in mace, kernel and shell of nutmeg during storage with an objective to prevent aflatoxigenic fungi through organic, ecofriendly and cost effective manner.

MATERIALS AND METHODS

Ripened tree split nutmeg fruits were collected from nutmeg plot in Ernakulam and Thrissur districts of Kerala. Mace and nut were separated from fruits and washed in tap water to remove soil particles and other debris adhering to it. The mace and kernel were subjected to different treatments such as chemical/botanicals by dipping, decoction, cloth bag and fumigation methods in order to prevent fungal growth during storage.

Chemicals/botanicals treatment

Desired concentrations of chemicals/botanicals were prepared. The mace and nuts were dipped separately in the solution for 10 minutes, and air dried followed by drying at 40° C in the hot air oven for 2-4 days and stored in a plastic containers. The treatments were T_1- potassium meta-bisulphate (KMS)1%, T_2- sodium chloride (salt)10%, T_3- baking soda 2%, T_4- sodium benzoate1%, T_5-turmeric powder + baking soda (5:1/1litre),T_6- turmeric oil 0.2%, T_7- asafoetida 0.1%, T_8- control.

Decoction treatment

Decoction of the leaves were prepared @ 100g / litre. Likewise, 10% tamarind extract and 5% *Anona* seed extract were also prepared. Mace and kernel were treated separately in the decoction supernatant /extract for 10 minutes, air dried followed by drying in the hot air oven for 2-4 days and stored them in a plastic containers. The treatments were T_1- Citrus leaves, T_2- Curry leaves, T_3- Black pepper leaves, T_4- Long pepper leaves(*Piper longum*), T_5- Tamarind extract, T_6- Anona seed extract (5%), T_7- control.

Cloth bag treatment

The dried leaves were kept in muslin cloth bag @ 2g/bag and kept along with properly dried mace and nut separately and stored in labeled polythene covers. The treatments were T_1- long pepper leaves (*Piper longum*), T_2- curry leaves, T_3- citrus leaves, T_4- neem leaves, T_5- *Vitex* leaves, T_6- *Ocimum* leaves, T_7- henna leaves, T_8- *Aeglemarmelos leaves* (Bael), T_9- cabbage leaves, T_{10}- turmeric rhizome bit, T_{11}- garlic segments, T_{12}- control.

Fumigation treatment

Properly dried nut and mace were fumigated for 2 h separately within a metal box and stored in a plastic container. The treatments were T_1- henna leaves, T_2- cabbage leaves, T_3- *Lantana camera* leaves, T_4- long pepper leaves, T_5- *Vitex* leaves, T_6- neem leaves, T_7- black pepper leaves, T_8- *Ocimum* leaves, T_9- *Leucas* leaves, T_{10}- garlic segments, T_{11}- control.

Treated samples were stored separately in plastic containers /polythene covers and enumeration of the fungi were done at 6 months and 12 months after storage by serial dilution technique (Johnson and Curl, 1952). The fungal cultures obtained with different morpho-types were identified using standard protocols and sent for confirmation to a reputed laboratory of National Centre for Fungal Taxonomy (NCFT), New Delhi.

RESULTS AND DISCUSSION

The fungal population and its identity at 6 and 12 months after storage are presented below:

Effect of chemicals / botanicals on the growth of fungi

The effect of different treatments on the growth of fungi at 6 months after storage revealed that sodium chloride (salt) @ 10%, baking soda@ 2% and sodium benzoate (1%) did not record any fungal growth in nut and mace indicating its effectiveness (Table 1). None of the treatments showed fungal growth on mace, while, potassium meta bisulphate (KMS)1%and turmeric powder + baking soda (5:1/1litre) showed single colony of *Aspergillus* sp. on shell and kernel respectively. Similarly, potassium meta-bisulphate (KMS) (1%), and turmeric oil (0.2%) recorded *Penicillium* sp. on kernel. However, kernel and mace of control samples showed *Aspergillus* spp. and 3 colonies of *Phytophthora* on shell. The highest fungal population was recorded in the case of control (3-28 x 10^3cfu/g). The highest population of fungi was in the case of kernel (28 x 10^3cfu/g) followed by mace (11 x 10^3cfu /g).

Sodium benzoate (1%) was the most effective among the treatments at 12 months after storage. There were no fungal growth on mace, kernel and shell. The highest fungal population was recorded in samples treated with 0.2% turmeric (T_6) and lowest number was found in samples treated with 1% potassium meta-bisulphate (T_1). The fungus identified were *Aspergillus niger*, *A. oryzae, A.ochraceous, A. flavus, Acremonium kiliense*, *Penicillium*

citrinum and *Syzgytes* sp. *novo.* However, among the treatments, sodium benzoate (1%) was the most effective in preventing the growth of fungi in nutmeg. Even though, *A. flavus* was recorded, but it does not produce aflatoxin in nutmeg. The aflatoxin in nutmeg is produced either by *Aspergillus nomius* and *A. bombycis*. (Kiyoshi Okano, *et. al.,* 2012) Since, such fungi were not found in the present studies, it may be presumed that there was no aflatoxin in nutmeg samples. Most of the fungi recorded in the samples were *A. ochraceous*.

Effect of different leaf extract on the growth of fungi

The effect of leaf decoction on the growth of fungi at 6 MAS revealed that all the treatments were effective as none of them recorded fungal growth on kernel, mace and shell (Table 2). The treatments did not show any fungal growth in kernel, shell and mace of the nutmeg except control indicating the effectiveness of leaf decoction. However, the highest fungal population was recorded in the case of control (T_8) with fungal population of 3 to 28 x 10^3cfu/g. The kernel (28 x 10^3cfu/g) recorded highest population followed by mace (11 x 10^3cfu/g) in control and *Aspergillus* sp. was predominant in kernel and mace, whereas, *Phytophthora* was noticed on shell. These results indicate that there were no aflatoxin producing fungi but only general fungi in nutmeg.

At 12 MAS, citrus leaves, curry leaves andanona seed extract (5%) were effective as there was no fungal growth. The highest fungal population were recorded in long pepper leaves and *Penicillium citrinum, Aspergillus oryzae* and *Alternaria alternata* were the major contaminants. However, black pepper leaves and tamarind extract (10%) showed less fungal population and the fungi identified were *A. oryzae and Al. alternate.* The citrus leaves, curry leaves and anona seed extract (5%) were effective in preventing the growth of fungus when compared with other treatments. In a similar study, Maruti, *et.al*. (2011) reported that citrus peel oils had strong antimicrobial activity against *Pseudomonas aeruginosa* (NCIM 2036) with methanol as solvent, *Salmonella typhimurium* (NCIM 5021) in the presence of acetone. It is well known that citrus flavonoids have a broad spectrum of biological activity including antibacterial, antifungal, anti-diabetic, anticancer and antiviral activities.

Table 1. Effect of chemicals and botanicals on the fungal growth in kernel, shell and mace of nutmeg at 6 and 12 months after storage (MAS)

Treatments	Fungal Population (x 10^3cfu/g)					
	6 MAS			12 MAS		
	Kernel	Shell	Mace	Kernel	Shell	Mace
T_1 (1% Potassium mono sulphate)	1 *(Penicillium* sp.*)*	1 *(Aspergillus* sp.*)*	0	0.5 *(Aspergillus oryzae)*	Absent	0.5 *(Aspergillus niger)*
T_2 (10% Sodium chloride Salt),	0	0	0	Absent	Absent	8.5 *(Aspergillus ochraceous* and *Penicillium citrinum)*
T_3 (2% Baking Soda)	0	0	0	Absent	1 *(Acremonium kiliense)*	3 *(Aspergillus flavus)*
T_4 (1% Sodium benzoate)	0	0	0	Absent	Nil Absent	Nil
T_5 (Turmeric Powder + Baking soda(5:1/1litre)	1 *(Aspergillus* sp.*)*	0	0	1.5 *(Aspergillus ochraceous)*	0.5 *(Aspergillus ochraceous)*	0.5 *(Aspergillus niger)*
T_6 (0.2% Turmeric oil)	2 *(Penicillium* sp*)*	0	0	95.5 *(Aspergillus ochraceous)*	127 *(Aspergillus ochraceous)*	0.5 *(Aspergillus ochraceous)*
T_7 (0.1% Asafoetida),	0	0	0	Absent	0.5	1 *(Syzgytes sp.novo)*
T_8 (Control)	28 (*Aspergillus* sp.*)*	3 *(Phytopthora* sp.*)*	11 *(Aspergillus* sp.*)*			

( ) Indicate the fungus present in the samples

Table 2. Effect of different leaf extract on the growth of fungi in kernel, shell and mace of nutmeg at 6 and 12 months after storage (MAS)

Treatments	Fungal Population (10^3cfu/g)					
	6 MAS			12 MAS		
	Kernel	Shell	Mace	Kernal	Shell	Mace
T_1 (Citrus leaves)	0	0	0	0	0	0
T_2 (Curry leaves	0	0	0	0	0	0
T_3 (Black Pepper Leaves	0	0	0	0	1.5 *(Aspergillus oryzae* and *Alternaria alternata)*	0
T_4 (Long pepper-leaves)	0	0	0	7 *(Penicillium citrinum)*	89 *(Aspergillus oryzae* and *Alternaria alternata)*	0
T_5 (Tamarind extract (10%)	0	0	0	16 (*Aspergillus oryzae)*	0	0
T_6 (Anona Seed extract (5%)	0	0	0	0	0	0
T_7 (Control)	28 (*Aspergillus* sp.)	3 *(Phytophthora* sp. *)*	11 *(Aspergillus* sp.*)*			0

( ) Indicate the fungus present in the samples

In the present studies, citrus leaf were used and it showed antifungal activity against stored nutmeg. In the case of curry leaves, it was found effective against aflatoxigenic fungi as no growth of such fungi were observed. Manisha *et al*. (2011) reported that curry leaf is commonly used against diarrhea, dysentery and to prevent vomiting, as it is a rich source of carbazole alkoids, carbohydrates, steroids and flavonoids It also showed some antimicrobial activity as well as antifungal activity. In the present studies, curry leaf was effective in preventing the growth of aflatoxin producing fungi in stored nutmeg. *Annona squamosa* was also effective against the fungal growth in present studies. Vidyasagar and Shivakumar (2012) reported that plant of *A. squamosa* are antimutagenic, anthelminthic, scavenging, antidiabetic, hepatoprotective, anti-thyroid, anti-genotoxic, antiplasmodial, molluscicidal, analgesic activities and antimicrobial activity which is in agreement with the present studies. In a similar studies, Koushik *et al.* (2017) also reported that the extracts of *A. squamosa* (L) and *Manilkara zapota* (L.) could be considered as potential sources of antifungal compounds for treating fungal infections. The extracts exhibited antifungal activity, even at very lower concentrations. In the present studies, *Annona* seed extract were effective against aflatoxin producing fungi on stored nutmeg.

Effect of leaves on the growth of fungi

All the treatments with leaves under fumigation were effective than control (Table 3). There were no *Aspergillus* in the nutmeg samples treated with long pepper leaves, curry leaves, neem leaves and vitex leaves. The curry leaves (T_2) and long pepper leaves ((T_1) recorded *Penicillium* and *Phytophthora*. No fungal growth was observed in kernel and shell treated with citrus leaves (T_3), neem leaves (T_4) and vitex leaves ((T_5), whereas *Phytophthora* sp. was recorded in the mace. However, control samples recorded highest number of *Aspergillus* sp. (7 x10^3 cfu/g) on mace followed by kernel and shell. Moreover, *Phytophthora* infection was also noticed on mace and kernel. Among the treatments, nutmeg treated with dried leaves of citrus, neem and vitex were effective at 6 MAS.

No fungal growth was found in nutmeg samples treated with citrus leaves (T_3), Vitex leaves (T_5), *Aegelmarmalos* leaves (bael) (T_8) and turmeric rhizome bit (T_{10}) at 12 MAS. Highest population of number of fungal colonies were found in samples treated with long pepper leaves (T_1), curry leaves (T_2), henna leaves (T_7) and garlic segments (T_{11}). At 12 months after storage, *Aspergillus niger, A. ochraceous, Penicillium citrinum, Acremonium alternatum, Eurotium amestaldomi, A. nidulans* were the major fungus identified. Eventhough, *Aspergillus ochraceous* was found

Table 3. Effect of different leaves in a cloth bag on the growth of fungi in kernel, shell and mace of nutmeg at 6 and 12 MAS

Treatments	Fungal Population (x 10^3cfu/g)					
	6 MAS			12 MAS		
	Kernel	Shell	Mace	Kernal	Shell	Mace
T1(Long pepper leaves)	3 (*Phytophthora* sp.)	1 (*Phytophthora* sp.)	0	4 *(Aspergillus niger)*	0.5 *(Aspergills niger)*	Nil
T_2 (Curry leaves)	2 (*Phytophthora* sp.)	0	3 (*Penicillium* sp.)	3 *(Aspergillus niger)*	Nil	1 *(Aspergills ochraceous)*
T_3 (Citrus leaves)	0	0	1 (*Phytophthor*a sp.)	Nil	Nil	Nil
T_4 (Neem leaves)	0	0	1 (*Phytophthora* sp.)	2 (*Acremonium strictum* and *Acremonium* sp. novo)	Nil	Nil
T_5 (*Vitex* leaves)	0	0	1 (*Phytophthora* sp.)	Nil	Nil	Nil
T_6(*Ocimum* leaves)	0	1 (*Aspergillus* sp.)	0	0.5 (*Aspergillus sclerotium*)	Nil	Nil
T_7 (Henna leaves)	2 (*Aspergillus* sp.)	0	0	1	0.5 (*Aspergillus niger*)	2.5 *(Penicillium-citrinum)*
T_8 (*Aeglemar-mlos*leaves (Bael)	2 (*Phytophthora* sp.)	2 (*Aspergillus* sp.)	3 (*Aspergillus* sp.)	Nil	Nil	Nil
T_9 (Cabbage leaves)	3 (*Phytophthora* sp.) 1 (*Aspergillus* sp.) 1 (*Penicillium* sp.)	3 (*Phytophthora* sp.) 1 (*Phytophthora* sp.)	1 (*Aspergillus* sp.)	0.5 (*Penicillium citrinum*)	Nil	Nil
T_{10} (Turmeric rhizome bit)	0`	1 (*Aspergillus* sp.)	2 (*Aspergillus* sp.)	Nil	Nil	Nil
T_{11} (Garlic segments)	2 (*Phytophthora* sp.)	1 (*Aspergillus* sp.) 1 (*Phytophthora* sp.)	2 (*Aspergillus* sp.)	Nil	Nil	13 *(Aspergills-nidulans* and *Penicillum citrinum)*
T_{12} Control	28 (*Aspergillus* sp.)	3 *(Phytophthora* sp.*)*	11 *(Aspergillus* sp.*)*			

( ) Indicate the fungus present in the samples

in the sample, it is not reported to be aflatoxigenic fungi in nutmeg. However, lowest number of colonies were seen in the case of neem leaves (T4) and the fungus found were *Acremonium strictum* and *Acremonium sp.novo*. In a similar study, Dharmaputra *et. al*., (2015) reported that the dominant fungi in nutmeg samples from farmers were *Penicillium citrinum*(81%) followed by *A. niger*(69%) and *Eurotium repens* (63%) whereas nutmegs collected from collectors were *Endomyces fibuliger* (76%), *A. niger* (76%) and *P. citrinum* (76%) which might have been due to high moisture content. In the present studies, similar fungi were recorded which is in agreement with the earlier studies.

Effect of fumigation of leaves on the growth of fungi

All the treatments were effective except for *ocimum* (Table 4.). There were no fungal growth in samples fumigated

Table 4. Effect of fumigation of leaves on the growth of fungi in kernel, shell and mace of nutmeg at 6 and 12 MAS

Treatments	Fungal Population (x 10^3cfu/g)					
	6 MAS			12 MAS		
	Kernel	Shell	Mace	Kernal	Shell	Mace
T_1 (Henna leaves)	2 *(Phytophthora* sp)	0	0	Nil	1 (*Alternaria alternata*)	Nil
T_2 (Cabbage leaves)	0	0	0	Nil	Nil	*(Alternaria alternate* and *Aspergillus oryzae)*
T_3 (*Lantana camera* leaves)	0	0	0	Nil	Nil	232.5 (*Aspergillus nidulans*)
T_4 (Long pepper thippali)	0	0	0	0.5 (*Alternaria alternata*)	Nil	Nil
T_5 (*Vitex* leaves)				1.5 (*Aspergillusclerotium)*	0.5 *(Aspergills sclerotium)*	2 *(Aspergillus niger)*
T_6 (Neem leaves)	0	0	0	Nil	Nil	1 *(Aspergillus sclerotium)*
T_7 (Black pepper leaves)	0	0	0	Nil	Nil	Nil
T_8 (*Ocimum* leaves)	2 (*Aspergillus* sp.) 1 *(Penicillium* sp.) 5 (*Phytophthora* sp.)	3 (*Aspergillus* sp.) 2 (*Penicillium* sp.) 2 (*Phytophthora* sp.)	1 *(Aspergillus* sp.) 8 (*Phytophthora* sp.)	3.5 *(Aspergillus sclerotium)*	Nil	Nil
T_9 (*Leucas* leaves)	0	0	0	Nil	Nil	Nil
T_{10} (Garlic segments)	0	0	0	Nil	0.5	2.5 *(Aspergillus sclerotium)*
T_{11} (Control)	28 (*Aspergillus* sp.)	3 *(Phytophthora sp.)*	11 *(Aspergillus sp.)*	Nil	0.5	1.5 (*Aspergillus niger*)

( ) Indicate the fungus present in the samples

with cabbage leaves, *Lantana camera* leaves, long pepper leaves, Vitex leaves, neem leaves, black pepper leaves, Leucas leaves and Garlic pieces. *Aspergillus* sp. were noticed in all the types of samples fumigated with *Ocimum*. Henna treated samples showed *Phytophthora* growth on kernel. . However, control samples showed maximum colonies of *Aspergillus* (7 x10^3cfu/g) on mace followed by kernel and shell. In addition, *Phytophthora* sp. infection was also noticed on mace and kernel. All the treatments except for *Ocimum* and henna leaves were most effective in the management of fungal growth after 6 MAS.

Highest fungal population at 12 MAS was in the samples treated with *Lantana camera* leaves and the fungus identified was *Aspergillus nidulans* from mace. Minimum fungal population was recorded in treatments with henna leaves, cabbage leaves, long pepper leaves, vitex leaves, neem leaves, ocimum leaves and garlic segments. Major fungus identified in these treatments were *Alternaria alternata, Aspergillus oryzae, A. sclerotium, and A nidulans.* Black pepper leaves (T_7) and Leucas leaves (T_9) were most effective among the treated nutmeg samples.

CONCLUSION

Based on the present studies, it may be concluded that pre-treatment of mace and nut of nutmeg with citrus leaf decoction (@100 g/litre) or curry leaf decoction (@ 100 g/litre) or *Anona*

seed extract (5%) using decoction method were effective against fungal contamination as well as aflatoxigenic fungi. However, further studies are needed on pilot scale to confirm.

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