Natural Occurrence of Toxigenic Fungi Species and Aflatoxin in Freshly Harvested Groundnut Kernels in Tigray, Northern Ethiopia

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A mycological survey was carried out, for the first time, on freshly harvested groundnut kernels from the Northern Ethiopia, in three major groundnut growing woredas or districts of the Tigray Region in the 2009 harvest season, to detect the occurrence, severity of infection and distribution of Aspergillus species and to quantify aflatoxin contamination level. A total of 168 groundnut kernel samples, collected from farmers and research center fields were analyzed for prevalence of the Aspergillus fungi; and 141 smashed and grinded groundnut samples were analyzed for aflatoxin contamination. All samples were found 100 percent positive for Aspergillus species; however, there was significant (P<0.05) variation in infection severity among locations. Species of the genus Aspergillus flavus and A. niger were the two most prevalent component of the groundnut mycoflor in the region. Across the surveyed areas on average, 41.5% (range: 6.7 to 96.7%) and 12.3% (range: 0 to 90%) of the groundnut kernels were found to be infected by A. flavus and A. niger, respectively. Despite variations in contamination level among location, Aflatoxin B1 type was detected in all the samples. The detected aflatoxin concentrations were ranging from 0.1 to 397.8 ppb (mean: 28.7 and median 5.2 ppb). The highest level of Aflatoxin was detected in groundnut samples from T. abergele area (55.3 ppb). By All standards, according to the EU and FAO food safety guidelines for direct human consumption, before processing and EU import limits, the qualities of the analyzed groundnut samples were very low, and significant amount of the samples were unsafe for human consumption as well as unfit for international market. The prevalence of the toxigenic fungi and associated extent of groundnut contamination in the region calls for urgent interventions of management practices to reduce the impact and awareness creation in the public.

Keywords: Aflatoxin, A. flavus, A. niger, Groundnut

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INTRODUCTION

Groundnut (Arachis hypogaea L.) plays an important role as a food as well as a cash crop in Ethiopia. Currently the crop is becoming one of the high value crops that are growing in the dryland areas of the Tigray region, Northern Ethiopia. However, toxigenic fungal pathogens are important constraints to the production of the crop, affecting the quality of the seeds through spoilage. Toxigenic fungi can attack groundnut crop prior to harvest and further decay the kernel during storage. As a result, fungal metabolites (mycotoxins) may form both during crop development and storage. Generally, there are several toxigenic fungi types, but the predominant field fungi genus that causes grain contamination are Fusarium, Alternaria and Aspergillus; these are known to produce Fumonisin, Alternaria and Aflatoxin mycotoxins, respectively. Worldwide Aspergillus and consequent aflatoxin contamination is the most important quality problem in groundnut with serious health consequences for human and livestock (Bhat and Vasanthi, 2003; D’Mello, 2003; Waliyar et al., 2003). Diverse species of Aspergillus exist and are highly variable in their ability to produce mycotoxins (Cole et al., 1982).

Mycotoxins induce multiple problems. Mycotoxins are poisonous and are known to cause chronic health risks. Prolonged exposure to mycotoxins through diet has been linked to cancer, immune-system diseases etc. in human beings (Bhat and Vasanthi, 2003). Thus, in developed countries, many governmental agencies such as the European Food Safety Authority (EFSA) in the European Union, the U.S. Food and Drug Administration (FDA) in the USA and the FAO, requires tests of food and feed products for mycotoxin and have established guidelines for a Maximum Tolerable Intake Level (MTL) for specific products. According to EU (2006), the MTIL of aflatoxin B$_1$ for direct human consumption is 2 ppb, and the EU has banned the import of groundnuts with AFB$_1$ content above 6 ppb. In the other side, FAO (2005) categorizes unprocessed samples with over 30 ppb as unfit for human consumption. Most countries have adopted and established the FAO limit as MTL for unprocessed groundnuts.

Worldwide various studies reveled that these mycotoxins cause extensive contamination of groundnut (ICRISAT and ICAR, 2005; Bhat and Vasanthi, 2003; Waliyar et al., 2003). Although, infection of groundnut by Aspergillus spp. occurs before and after harvest conditions, under semi-arid tropical environment the pre-harvest infection and subsequent aflatoxin concentration is more important, because the risk of aflatoxin concentration is more pronounced when drought occurs at the later stages of the crop (Cole et al., 1989; Sanders et al., 1993).

Among the various mycotoxins, aflatoxin in groundnuts pose major, but unappreciated risks to human residing in developing countries of tropical regions (Willliams et al., 2004). In the year 2004 and 2005 one of the largest aflatoxin outbreaks was reported causing deaths of 123 people in Kenya (Nyamongo and Okloma, 2005). In Eastern Ethiopia, aflatoxin levels’ ranging from 5 to 250 ppb was detected in groundnut samples (Amare et al., 1995). A preliminary survey made in 2008 for mycotoxin occurrence in Northern Ethiopia in groundnut and other crops also confirmed the incidence of aflatoxin contaminations ranging from low to high levels (Dereje unpublished). In developing countries, such as Ethiopia, the risk of human and animal exposure to consumption of mycotoxin contaminated food feed is very high. This is principally due to lack of awareness, regulations, management recommendations and grain-test check points and at times due to serious food shortage when people tend to consume moldy grains.

Therefore, this study was designed to examine the identity, prevalence and the distribution of most predominant Aspergillus field fungi species and to quantify aflatoxin contamination level from freshly harvested groundnut kernels grown in the Tigray region. Knowledge of the identity and distribution of the prime species potentially causing contamination in the region is helpful in devising mycotoxin control or reduction strategies in the future.

MATERIALS AND METHODS

Survey was conducted in major groundnut production areas of the Tigray region, i.e. potentially favorable areas for the development of toxigenic fungi. The survey area covers major groundnut growing woredas (districts) in the region: Merebleke and Tankua abergelle (Central Tigray) and Tahtay Adyabo (Western Tigray). In addition samples from the research plots of Ramma research center (Ramm RC, which is located at Merebleke woreda), were collected.

Sampling

Representative samples of freshly harvested groundnut kernels were collected in different zones and woredas of the region. From 2009 production season, freshly harvested groundnut pods were sampled (approximately one km radius between sampling points) from five locations in each of the three surveyed woreda. At each location, eight groundnut fields were identified and arbitrarily 100-150 pods were sampled from each field at the time of harvest. A
total of 168 groundnut samples (40 samples per woreda from farmers’ field and 48 samples from the Ramma research center groundnut fields) were examined for Aspergillus infection and of the total samples collected, 141 smashed and grinded groundnut samples were analyzed for aflatoxin contamination. To prevent post-harvest accumulation of moulds and aflatoxin prior to analysis, all the samples were stored at 4°C.

Kernel health testing
Sample seeds were assessed for occurrence of fungi by employing standard blotter technique (ISTA, 1985). Seeds were surface sterilized for 1 min in 2.5% NaOCl, rinsed in three changes of distilled water, and from each samples 100-seeds were tested. For the standard blotter technique, seeds were placed at an equal distance in sterile plastic trays (25 seeds per plastic tray), filled with three layers of moistened germination papers (blotter). In the deep-freezing method, trays were first kept for 24h at room temperature, and 24h in a freezer followed by 7-days incubation at 25 ± 2°C in a seed germinator fitted with florescent lights at 12 hr dark and 12 hr light cycles.

Isolation and identification of fungi species
The kernels were assayed for presence of mould fungi and internal infestation using direct plating technique. For the identification of fungi species, fungal spores or hypha from infected kernels were transferred directly onto the surface of ¼ strength Potato Dextrose Agar (PDA) containing 9.75 g/l Potato Dextrose Broth (Difco) and 20 g/l agar, amended with 2 ml/l of lactic acid to suppress bacterial contamination. Plated kernels were incubated at 31°C for 3 days. Finally, fungi identification was carried out based on macro morphological (reverse and surface coloration of colonies, presence of pigment, and colony texture) and micro morphological characteristics (conidia size, conidial head, shape of vesicle). Aspergillus fungi growing on seeds were identified after reference to Klich (2002). Fungal samples morphologically consistent with known toxigenic fungi species were recorded under microscopic observation. Mycotoxin contamination was determined using ELISA technique, in Mali ICRISAT laboratory.

Statistical analysis
Data on percentage of seed infection was transformed using a ‘natural logarithm’ method before subjected to analysis of variance. Data on fungal infection-severity and aflatoxin levels were summarized and analyzed using Minitab software (MINITAB 2010). One way analysis of variance was carried out using the general linear model procedure and test were performed at a probability level of P = 0.05. Comparison between means was done using Tukey’s, to determine significance differences among the samples obtained from the different woredas. A descriptive statistical method was executed to determine the proportion of fungal infected samples and samples with aflatoxin level above the safe limit.

RESULTS AND DISCUSSION
Incidence and identification of toxigenic fungi
All the 168 groundnut samples were found to be 100 percent positive for Aspergillus species, with various levels of severity across location. A total of 9036 Aspergillus isolates were detected from the 168 samples and of which two species, A. flavus and A. niger were identified. From these two species, A. flavus had the highest incidence (77.2%) than A. niger (22.8%).

Distribution and infection level of Aspergillus species
The relative proportions of the isolates of A. flavus and A. niger varied among the locations sampled. Geographically, from these two species, A. flavus had the highest preponderance in all the three major groundnut growing woredas as well as in the Ramma research center, ranging from 6.7 – 96.7%, with a total mean infection level of 41.5% (Table 1), while A. niger was found predominantly in T. abergele woreda, followed by M. leke with an infection level of 43.8% and 6.6%, respectively (Figure 1). In the other two locations, A. niger was isolated occasionally, but never at a frequency >1% of the total (Table 1).

Infection severity of A. flavus was significantly (P<0.05) higher in T. adiabo and T. abergele, while a marked variation (P<0.05) in A. niger infection was detected in the latter (T. abergele) (Table 2).
Aflatoxin contamination level in groundnut samples

A total of 141 smashed and grinded groundnut samples were analyzed for mycotoxin contamination, and despite the variation in contamination level the Aflatoxin B₁ type was detected in all the samples. A wide range of aflatoxin concentrations (0.1-397.8 ppb) was observed in the groundnut samples. The contamination in groundnut kernels obtained from farmers plot and research center field also varied among locations (Figure 2). The highest concentrations of aflatoxin were detected in samples collected from T. abergele. However, as seen in Table 2, the variation among the surveyed areas was not statistically significant ($P>0.05$), this is apparently because of the wide range of variation within locations, (Table 1). The highest average concentration of aflatoxin was in samples from T. abergele (53.3 ppb), followed by Rama research center (33.9 ppb) (Table 2).

As there are no standards set in Ethiopia, the aflatoxin concentration levels of the samples collected from the Tigray region were evaluated and compared against all the three limits, EFSA, FAD and FAO safety guidelines discussed above. Accordingly, the comparison reveals that from the total samples analyzed, 83.9% were unsafe for direct human consumption as per the EU MTIL and 46.6% were unfit for export to EU counties (as per the EU safe limit for import of groundnut); and on the basis of the FAO MTIL, 16.6% of the samples exceeded the 30 ppb limit (Table 3). By all standards, the qualities of groundnut samples were very low. The average concentration for the total samples had over 10-fold of the recommended maximum aflatoxin level, even in the worst case, samples from T. abergele were heavily contaminated, which had over 27-fold higher aflatoxin. This indicates that the likelihood of aflatoxin exposure to humans in the region and particularly in these woredas is high, where the frequency of groundnut consumption is relatively high.

Table 1. Prevalence and distribution of two *Aspergillus* spp. (per cent) isolated according to their location and aflatoxin concentrations (ppb)

<table>
<thead>
<tr>
<th>Location</th>
<th>A. flavus</th>
<th></th>
<th></th>
<th>A. niger</th>
<th></th>
<th></th>
<th>Aflatoxin level (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Median</td>
<td>Range</td>
<td>Mean</td>
<td>Median</td>
<td>Range</td>
<td>Mean</td>
</tr>
<tr>
<td><em>T. adiabo</em></td>
<td>50.6</td>
<td>45</td>
<td>26.7 - 96.7</td>
<td>0.67</td>
<td>0</td>
<td>0 - 10</td>
<td>19.3</td>
</tr>
<tr>
<td><em>T. abergele</em></td>
<td>43.9</td>
<td>50</td>
<td>6.7 - 86.7</td>
<td>43.8</td>
<td>38.3</td>
<td>10 - 90</td>
<td>55.3</td>
</tr>
<tr>
<td><em>M. leke</em></td>
<td>38.0</td>
<td>36.7</td>
<td>13.3 - 86.7</td>
<td>6.6</td>
<td>0</td>
<td>0 - 36.7</td>
<td>14.1</td>
</tr>
<tr>
<td><em>Ramma RC</em></td>
<td>34.9</td>
<td>33.3</td>
<td>13.3 - 70</td>
<td>0.5</td>
<td>0</td>
<td>0 - 10.0</td>
<td>33.9</td>
</tr>
<tr>
<td><em>Mean</em></td>
<td>41.5</td>
<td>36.7</td>
<td>6.7 – 96.7</td>
<td>12.3</td>
<td>0</td>
<td>0.0 - 90</td>
<td>28.7</td>
</tr>
</tbody>
</table>
Figure 2. Average level of aflatoxin (AfB₁) in ppb in freshly harvested groundnut samples collected from four locations of Tigray region in 2009

Table 2. Mean infection level by the two Aspergillus spp and aflatoxin contamination of groundnut samples collected from four sites (woredas) of Tigray

<table>
<thead>
<tr>
<th>Toxigenic fungi and toxin</th>
<th>Sample size (n)</th>
<th>T. adiabo</th>
<th>M. leke</th>
<th>T. abergele</th>
<th>Ramma RC</th>
<th>Pooled St. Dev.</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. flavus (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>168</td>
<td>50.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>A. niger (%)</td>
<td></td>
<td>168</td>
<td>0.72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.75&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.54&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.66</td>
</tr>
<tr>
<td>Aflatoxin (in ppb)</td>
<td></td>
<td>168</td>
<td>19.3</td>
<td>14.1</td>
<td>55.3</td>
<td>33.9</td>
<td>69.2</td>
</tr>
</tbody>
</table>

Means in the same row with different letters (a-c) are different at (P<0.05)

Table 3. Percent of contaminated groundnut samples above the safe limit as per the EU and FAO food safety guidelines for direct human consumption, before processing and EU import limit

<table>
<thead>
<tr>
<th>Location</th>
<th>Sample size (n)</th>
<th>% of samples above Aflatoxin-safe levels*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>≥2 ppb&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>M. leke</td>
<td>39</td>
<td>97.4</td>
</tr>
<tr>
<td>T. abergele</td>
<td>22</td>
<td>100.0</td>
</tr>
<tr>
<td>T. adiabo</td>
<td>37</td>
<td>89.2</td>
</tr>
<tr>
<td>Ramma RC</td>
<td>43</td>
<td>51.2</td>
</tr>
<tr>
<td>Total</td>
<td>141</td>
<td>83.9</td>
</tr>
</tbody>
</table>

*<sup>1</sup>EU Maximum Tolerated Intake Level (MTIL) for direct human consumption (<2ppb);<sup>2</sup>FAO unfit limit of unprocessed samples for human consumption (>30ppb);<sup>3</sup>EU countries’ import limit of groundnut (>6ppb)
This study provides the first comprehensive documentation of the occurrence and frequency of the toxigenic *Aspergillus* species in groundnut producing areas of the Tigray region. Although occurrence frequency of the *A. flavus* varied across locations, it was the most dominant species in all the four surveyed areas (77%). As reported elsewhere, *A. flavus* is the most predominant member of the toxigenic *Aspergillus* species in soils of Africa (Cardwell and Cutty, 2002). *A. flavus* produces Aflatoxin B₁, B₂, G₁, and G₂ types, which are carcinogenic and produce liver cancer (Purchase, 1974; Diener and Davis, 1969; Pesta and Bonday 1990). In this study, however, Aflatoxin B₁ type was only detected.

The study clearly shows that the groundnut production in the region is at high risk of contamination with aflatoxin. As compared to previous studies in Ethiopia and elsewhere in Africa, the aflatoxin values obtained in this study from freshly harvested groundnut samples are even likely to be not the highest, as aflatoxin contaminations usually increase during storage. Under a production system with no irrigation, dryer soil conditions associated with higher temperature particularly after the peg development stage of groundnut favor infection by *Aspergillus* and the development of aflatoxin prior to harvest (Waliyar et al., 2005). Thus higher aflatoxin in those major groundnut producing areas of Tigray is not unexpected, since all the groundnut producing woredas of the region are characterized by late season drought, at which the groundnut plants are often at their post-flowering stage, a stage which is sensitive for infection by the fungi.

**CONCLUSION**

The study has demonstrated a high prevalence of the toxin producing *Aspergillus* species *A. flavus* and *A. niger* from harvested groundnut seeds, however, their occurrence and level of infection significantly varied across locations.

Moreover, Aflatoxin B₁ was determined as a strong contaminant of groundnut producing areas of the region, ranging from low to high level contaminations; and based on international safety standards a significant amount of the samples were found unsafe for human consumption as well as unfit for international market, such as EU.

This study revealed the extent of the contamination of the groundnut products in the region in particular the high probability of aflatoxin exposure to human in domestic use too where the frequency of groundnut consumption is relatively high. Hence, the study suggests urgency for intervention, proper storage of groundnut seeds to minimize further fungal infestation and Aflatoxin production during storage. Further prospective studies are also desirable in major groundnut producing regions of the country and across the value chain actors to monitor the Aflatoxin status nationally and understand the extent of the problem in detail. In general, the prevalence of the toxigenic fungi and associated extent of groundnut contamination in the region calls for urgent interventions of management practices to reduce the impact and awareness creation in the public.

**ACKNOWLEDGMENT**

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