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Review

**Trichoderma: A scientific approach against soil borne pathogens**

Mukesh Srivastava*, Mohammad Shahid, Sonika Pandey, Vipul Kumar, Anuradha Singh, Shubha Trivedi, Y. K. Srivastava and Shivram

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Received 1 October, 2015; Accepted 3 November, 2015

The members of the genus *Trichoderma* are widely used as bioagent for the control of phytopathogenic fungi in agriculture sectors. The members of this genus are reproducing asexually by the formation of cyanide and chlamydospore, in wild habitats they reproduce by formation of ascospores. *Trichoderma* species are well known for the production of cell wall degrading enzymes (CWDEs). These CWDEs, play a major role in biocontrol mechanism. We all know that all living organisms are made up of genes that code for a particular function. Similarly, in *Trichoderma*, some genes are responsible for the secretion of these CWDEs. These genes, which aid in the biocontrol action, are called biocontrol genes. These bacterial genes code for a particular enzyme and protein that degrade the pathogen cell wall. These biocontrol genes can be isolated and cloned for large scale commercial production. It has also been found that some of the genes of *Trichoderma* are also helpful in the abiotic and biotic stress. The mechanisms which are employed by *Trichoderma* for the phytopathogenic action are generally included atbiosis, mycoparasistism, competition for nutrients, etc.

**Key words:** *Trichoderma*, biocontrol, phytopathogen, lytic enzymes, biocontrol mechanisms, biocontrol agent.

**INTRODUCTION**

In recent times, excessive use of chemical pesticides has posed a threat on the environment. *Trichoderma* based biocontrol agents have better ability to promote plant defense response, promote plant growth and soil remediation. *Trichoderma* species has gained wide acceptance as effective biocontrol agents against several phytopathogens. Micropropagules of *Trichoderma* spp. in the form of conidia are preferred over chlamydospores and mycelia biomass because of its viability and stability in field application (Rosane et al., 2008; Chet, 1987).

The genus *Trichoderma* are commonly found in soils and on decaying wood and vegetable matter. Strains of *Trichoderma* are rarely associated with diseases of living plants, although an aggressive strain of *Trichoderma* causes a significant disease of the commercial mushroom and soil borne pathogens. Samuels (1996) provides a comprehensive review of *Trichoderma* spp. for enzyme production and biological control mechanisms. In *Trichoderma* spp., sexual reproduction not present are believed to be mitotic and clonal. The main

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problem with the nomenclature of *Trichoderma* is pleomorphism present within the genus. In *Trichoderma*, there are two stages, the sexual stage is called Hypocrea (telomorphic) and the asexual stage is called *Trichoderma* (anamorphic). The genus is called Hypocrea/Trichoderma (Druzhinina et al., 2011). However, despite these significant advances in our knowledge about this genus, the full taxonomic history of *Trichoderma* is still not complete, and the detailed description of the taxonomic history of *Trichoderma* remains problematic. A refined classification and identification is necessary for predictive indications about ecology.

*Trichoderma* is widely used for the control of many soil borne plant pathogens. *Trichoderma* spp. are the efficient producer of cell wall degrading enzymes (Srivastava et al., 2014a); some of these enzymes are of commercial importance. Many research workers have proved that *Trichoderma* spp. possess some biocontrol genes that can be isolated and cloned for commercial large scale production (Massart and Jijakli, 2007).

The biocontrol ability of *Trichoderma* is of much importance as it does not accumulate in food chain and thus do not harm plants, animals and humans. The genes involved in the biocontrol mechanisms of *Trichoderma* are of great importance.

**TAXONOMY OF TRICHODERMA**

Although the genus *Trichoderma* has been known since the 19th century. Its association with Hypocrea Fr. was discovered by the Tulasne brothers in 1865; its taxonomy has remained obscure until recent decades. Bisby (1939) thought that the morphological variation could be ascribed to a single species, *Trichoderma viride*. The first serious attempt to morphologically distinguish species, or rather "species aggregate", was made by Rifai (1969). Some new species subsequently described were keyed out by Domsch et al. (1980). Teleomorph connections were established by means of ascospore isolates by Dingley (1957) and Webster and Rifai (1968). In Japan, Doi (1969a, 1972b) studied a number of telemorphs and described them with cultural and anamorph characters, but no cultures were preserved from that study. After this, no morphological differentiation was given. Bissett (1984, 1991b, c) gave a detailed description of the morphological studies, who distinguished about 21 taxa. These studies have shown that the delimitation of biological species is extremely difficult in this genus on morphological grounds alone.

Apart from morphological studies, there are many other methods that are used in the taxonomy, such as study of secondary metabolites; this has shown a great diversity in this genus (Okuda et al., 1982). Physiological features that are detected by the microtiter plate assay are the useful tools that are used for the identification. Isoenzyme profiles are also used for taxonomic classification (Samuels et al., 1994; Leuchtmann et al., 1996). In modern era molecular techniques, such as sequences of its region of ribosomal DNA and fingerprinting techniques provide the finest resolution of taxonomic entities (Meyer et al., 1992; Fujimori and Okuda, 1994; Muthumeenakshi et al., 1994).

**BIOCONTROL GENES OF TRICHODERMA**

*Trichoderma* is widely used for the control of many soil borne plant pathogens (Table 1). *Trichoderma* spp. Are efficient producers of cell wall degrading enzymes (Srivastava et al., 2014a), some of which are of commercial importance. Many research workers have proved that *Trichoderma* spp. possess some biocontrol genes that can be isolated and cloned for commercial large scale production (Massart and Jijakli, 2007).

Kuc (2001) has proved that some genes, providing resistance to abiotic and biotic stress are present in *Trichoderma* (Table 2). Mycoparasistism (Figure 1), antibiosis and competition for the nutrients are the main strategies employed by *Trichoderma* for the phytopathogenic action (Janisiewicz and Korsten, 2002). Among the different species tested, *Trichoderma harzianum* was found to be the most promising strain (Gao et al., 2002).

**GENOMICS OF TRICHODERMA**

The genome size of filamentous fungi is very small as it is about 25 to 50 Mb. However, with the advancement of pulse field gel electrophoresis, karyotyping of filamentous fungi is possible. Karyotyping is helpful in the detection, translocations and variations in chromosome numbers. Through pulsed-field gel electrophoresis (PFGE), chromosomal DNA was separated from *Trichoderma* (Gilly and Sands, 1991; Mäntylä et al., 1992; Hayes et al., 1993; Herrera-Estrella et al., 1993). The expected genome size and chromosome number of *Trichoderma* ranges from 31 to 39 Mb and from 3 to 7, respectively. From the data obtained through DNA homology, it was found that *T. harzianum* and *Trichoderma veins* are closely related, and it was concluded that they may have the same phylogenetic origin (Herrera-Estrella et al., 1993). On the other hand, Mäntylä et al. (1992) determined molecular karyotypes of various strains of *Trichoderma reesei* that had undergone mutagenesis and screening for the hyper production of cellulase enzyme. The authors found that extensive alteration in the genome organization of these strains occurred.

The first member of the genus sequenced was *T. reesei* (Table 3). This fungus is the first choice, because the genome size of this organism is very small (33 Mb) and has only seven chromosomes. Fungal genomics laboratory of NCSU has expressed sequence tag (EST),
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<th>Trichoderma spp. used</th>
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<td><em>Vigna mungo</em> (Black gram) (<em>Raguchander</em> et al., 1997; <em>Dubey</em> et al., 2012; <em>Mishra</em> et al., 2011)</td>
<td>Macrophomina phaseolina, Alternaria alternate</td>
<td><em>T. viride, T. harzianum</em></td>
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<tr>
<td><em>Cicer arietinum</em> (Chickpea) (<em>Mukherjee</em> et al., 1997; <em>Haware</em> et al., 1999; <em>Pandey</em> et al., 2003; <em>Poddar</em> et al., 2004)</td>
<td>Fusarium <em>oxysporum</em>, <em>Rhictonia solani</em>, <em>Aspergillus niger</em>, <em>Chaetomium</em> spp., <em>Sclerotium rolfsii</em>, <em>Penicillium</em> spp. <em>Macrophomina phaseolina</em></td>
<td><em>T. harzianum, T. viride</em></td>
</tr>
<tr>
<td><em>Capsicum annuum</em> L. (Chilli), (<em>Rini</em> and <em>Sulochana</em>, 2006; <em>Kapoor</em>, 2008; <em>Vasanthakumari</em> and <em>Shivanna</em>, 2013)</td>
<td><em>Sclerotium rolfsii</em>, <em>Fusarium oxysporum</em>, <em>Pythium</em> spp., <em>Rhizoctonia solani pseudokoningii</em> 2013</td>
<td><em>T. viride, T. harzianum</em></td>
</tr>
<tr>
<td><em>Cocos nucifera</em> L (Coconut) (<em>Karthikeyan</em> et al., 2006)</td>
<td><em>Ganoderma lucidum</em></td>
<td><em>T. harzianum, T. viride</em></td>
</tr>
<tr>
<td><em>Coffea arabica</em> L. (Coffee) (<em>Deb</em> et al., 1999)</td>
<td><em>Phomopsis theaeae</em>, <em>Glomerella cingulata</em></td>
<td><em>T. harzianum</em></td>
</tr>
<tr>
<td><em>Vigna sinensis</em> (Cowpea) (<em>Pan and Das</em>, 2011)</td>
<td><em>Rhictonia solani</em></td>
<td><em>T. harzianum</em></td>
</tr>
<tr>
<td><em>Arachis hypogaea</em> L. (Groundnut) (<em>Biswas and Sen</em>, 2010; <em>Kishore</em> et al., 2001; <em>Rakholiya</em> and <em>Jadeja</em>, 2010; <em>Bagwan</em>, 2011; <em>Sreedevi</em> et al., 2011)</td>
<td><em>Thievaliopsis basicola</em>, <em>Sclerotium rolfsii</em> Sacc., <em>Aspergillus niger</em>, <em>Rhictonia solani</em>, <em>Pythium aphanidermatum</em>, <em>Macrophomina phaseolina</em></td>
<td><em>T. harzianum, T. viride, T longibrachiatum</em></td>
</tr>
<tr>
<td><em>Agaricus bisporus</em> (Mushroom) (<em>Rawal</em> et al., 2013)</td>
<td><em>Rhizopus stolonifer</em>, <em>Coprinopsis kimuraee</em>, <em>Penicillium glabrum</em>, <em>Fusarium oxysporum</em></td>
<td><em>T. viride</em></td>
</tr>
<tr>
<td><em>Cajanus cajan</em> (Pigeon pea) (<em>Hukma and Pandey</em>, 2011)</td>
<td><em>Fusarium udum</em></td>
<td><em>T. viride, T. harzianum</em></td>
</tr>
<tr>
<td><em>Solanum lycopersicum</em> (Tomato) (<em>Sreenivasaprasad</em> and <em>Manibhushanrao</em>, 1990; <em>Dutta and Das</em>, 2002; <em>Jayaraj</em> et al., 2006)</td>
<td><em>Fusarium oxysporum</em> f. sp. lycopersici, <em>Pythium aphanidermatum</em>, <em>Rhictonia solani, Sclerotium rolfsii</em></td>
<td><em>T. harzianum, T. viride, T longibrachiatum, T. virens</em></td>
</tr>
<tr>
<td><em>Oryza sativa</em> (Rice) (<em>Chakravarthy</em> et al., 2011; <em>Bhramaramba</em> and <em>Nagamani</em>, 2013; <em>Biswas and Datta</em>, 2013; <em>Gangwar and Sharma</em>, 2013)</td>
<td><em>Rhictonia solani, Fusarium spp</em></td>
<td></td>
</tr>
<tr>
<td><em>Capsicum annuum</em> L. (Capsicum) (<em>Kapoor</em>, 2008)</td>
<td><em>Alternaria alternata</em></td>
<td><em>T. viride T. harzianum</em></td>
</tr>
</tbody>
</table>
Table 1. Contd.

<table>
<thead>
<tr>
<th>Plant Species</th>
<th>Pathogens and Microorganisms</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Brassica oleracea</em> (Cauliflower) (Sharma and Sain, 2004, 2005; Ahuja et al., 2012)</td>
<td><em>Rhictonia solani, Pythium aphanidermatum</em></td>
</tr>
<tr>
<td>Citrus (Kalita et al., 1996; Singh et al., 2000)</td>
<td><em>Fusarium solani</em></td>
</tr>
<tr>
<td><em>Gossypium hirsutum</em> (Cotton) (Sreenivasaprasad and Manibushanrao, 1990; Gaur et al., 2005)</td>
<td><em>Rhictonia solani, Sclerotium rolfsii, Pythium aphanidermatum</em></td>
</tr>
<tr>
<td><em>Zingiber officinale</em> (Ginger) (Gupta et al., 2010)</td>
<td><em>Pythium aphanidermatum</em></td>
</tr>
<tr>
<td><em>Sesamum indicum</em> L (Sesame) (Tamimi and Hadvan, 1985; Sankar and Jeyarajan, 1996; Jeyalakshmi et al., 2013)</td>
<td><em>Aspergillus flavus, Curvularia lunata, Pythium notatum, Pythium chrysogenum, Fusarium moniliforme, Fusarium oxysporum, Rhictonia nigricans, Macrophomia phaseolina</em></td>
</tr>
</tbody>
</table>

Table 2. Some biocontrol genes of *Trichoderma* and their function (Srivastava et al., 2014a, b).

<table>
<thead>
<tr>
<th>Name of gene</th>
<th>Source organism</th>
<th>Function</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Tvsp1</em></td>
<td><em>Trichoderma virens</em></td>
<td>This gene encodes for serine protease. <em>Rhizoctonia solani</em> which affects the cotton seedlings has been controlled biologically by serine protease.</td>
<td></td>
</tr>
<tr>
<td><em>tr5</em></td>
<td><em>Trichoderma harzianum</em></td>
<td>This gene is responsible for the synthesis of trichotheccene which inhibits the protein and DNA synthesis in the cells of the pathogens and inhibits their growth.</td>
<td></td>
</tr>
<tr>
<td><em>TgaA</em> and <em>TgaB</em></td>
<td><em>Trichoderma virens</em></td>
<td>This gene exhibits antagonist activity against <em>R. solani</em> and <em>Sclerotium rolfsii</em></td>
<td></td>
</tr>
<tr>
<td><em>ThPG1</em></td>
<td><em>Trichoderma harzianum</em></td>
<td>This gene encodes for endopoly-galacturonase. This enzyme is involved in the cell wall degradation of the pathogens like <em>R. solani</em> and <em>P. ultimum</em></td>
<td></td>
</tr>
<tr>
<td><em>Th-Chit</em></td>
<td><em>Trichoderma harzianum</em></td>
<td>This gene is responsible for the antifungal activity in transgenic tobacco plant.</td>
<td></td>
</tr>
<tr>
<td><em>tri5</em></td>
<td><em>Trichoderma brevicompactum</em></td>
<td>This gene helps in the production of <em>Trichoderma</em> in which shows antifungal activity against <em>S. cerevisiae, Kluyveromyces marxianus, Candida albicans, C. glabrata, C. tropicalis and Aspergillus fumigates</em>.</td>
<td></td>
</tr>
<tr>
<td><em>erg1</em></td>
<td><em>Trichoderma harzianum</em></td>
<td>This gene encodes an enzyme named squalene peroxidase, which helps in the synthesis of ergosterol and silencing of this gene provides resistance to terbinafine, an antifungal compound.</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Contd.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Origin</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TvgST</td>
<td>Trichoderma virens</td>
<td>This gene is responsible for cadmium tolerance</td>
</tr>
<tr>
<td>Thkel1</td>
<td>Trichoderma harzianum</td>
<td>This gene codes for putative kelch-repeat protein which helps in regulating the glucosidase activity and enhances tolerance to salt and osmotic stresses in Arabidopsis thaliana plants</td>
</tr>
<tr>
<td>egl1.</td>
<td>Trichoderma longibrachiatum</td>
<td>This gene showed biocontrol activity against P. ultimum in damping-off of cucumber</td>
</tr>
<tr>
<td>qid74</td>
<td>Trichoderma harzianum CECT 2413</td>
<td>This gene plays a significant role in cell protection and provide adherence to hydrophobic surfaces that helps the fungus in mycoparasitic activity against R. solani pathogen</td>
</tr>
<tr>
<td>Taabc2</td>
<td>Trichoderma atroviride</td>
<td>This gene has a significant role in ATP binding cassette (ABC) transporter in cell membrane pump that helps in the mycoparasitic activity</td>
</tr>
<tr>
<td>tac1</td>
<td>Trichoderma virens</td>
<td>This gene has its role in mycoparasitic activity against R. solani and P. ultimum</td>
</tr>
<tr>
<td>TrCCD1</td>
<td>Trichoderma reesei</td>
<td>This gene is involved in carotenoid metabolism that helps in the development of conidiospores and hyphal growth in T. reesei</td>
</tr>
</tbody>
</table>

Table 3. Summary of some features of the sequenced genomes.

<table>
<thead>
<tr>
<th>Feature</th>
<th>T. reesei</th>
<th>T. virens</th>
<th>T. atroviride</th>
<th>T. harzianum</th>
<th>T. asperellum</th>
<th>T. longibrachiatum</th>
<th>T. ctrinoviride</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genome size (Mb)</td>
<td>34.1</td>
<td>39</td>
<td>36.1</td>
<td>40.98</td>
<td>37.46</td>
<td>32.24</td>
<td>33.48</td>
</tr>
<tr>
<td>No of predicted genes</td>
<td>9129</td>
<td>12427</td>
<td>11863</td>
<td>14095</td>
<td>12566</td>
<td>10792</td>
<td>9397</td>
</tr>
<tr>
<td>Glycosyl hydrolases</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chitinases</td>
<td>23</td>
<td>41</td>
<td>34</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Glucanase</td>
<td>15</td>
<td>18</td>
<td>18</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Secondary metabolites biosynthesis, transport and catabolisms (KOG)</td>
<td>262</td>
<td>440</td>
<td>349</td>
<td>438</td>
<td>358</td>
<td>253</td>
<td>285</td>
</tr>
<tr>
<td>PKS</td>
<td>11</td>
<td>18</td>
<td>18</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>NRPS</td>
<td>10</td>
<td>28</td>
<td>16</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>PKS-NRPS</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>NA</td>
<td>NA</td>
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<td>NA</td>
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<td>SSCP3</td>
<td>260</td>
<td>319</td>
<td>301</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Xenobiotics sidegradation and metabolisms (KEGG)</td>
<td>327</td>
<td>519</td>
<td>453</td>
<td>610</td>
<td>432</td>
<td>232</td>
<td>359</td>
</tr>
<tr>
<td>Mating types</td>
<td>MAT1-2</td>
<td>MAT1-2</td>
<td>MAT1-2</td>
<td>MAT1-2</td>
<td>MAT1</td>
<td>MAT1-1</td>
<td>MAT1-2</td>
</tr>
</tbody>
</table>
cDNA collection and bacterial artificial chromosome (BAC) libraries for academic researchers. DNA mediated transformations and gene protocols have been developed for the genomic study of *Trichoderma*.

**MASS MULTIPLICATION OF TRICHODERMA**

Grains are cheap, easily available and act as best nutritive media for the mass multiplication of many microorganisms. Bajra (*Pennisetum typhoides*) grains should be completely soaked in 2% sucrose solution in water for 6 h. After draining out the excess water, the soaked 250 g seeds of bajra should be filled in autoclavable polypropylene (PP) bags of 30 × 20 cm². The PP bags should be plugged with nonabsorbent cotton followed by autoclaving at 15 lbs pressure for 30 min. After autoclaving, the bags should be left for cooling overnight. The next day, the bags should be individually inoculated by using 5 ml stock solution (10⁶ to 10⁸ CFU/ml) of starter culture grown for 100 days, with syringe. Before inoculation, the place from where the inoculation is to be made should be marked out with a small circle with the help of marker pen. Punctured place of injection of the PP bag must be sealed with cellophane tape. The bags should be incubated at 25 ± 2°C for 15 days in a temperature controlled room. After 15 days of incubation, the contents of the bags should be taken out and kept in hot air oven for drying overnight at 35°C. During the 15 days of incubation visual check every day is essential to ensure detection and elimination of contaminated PP bag(s). Formulation thus prepared should be ground to fine powder, while ensuring that during the process temperature does not go beyond at 35°C. The powdered formulation thus obtained should be mixed with pre-sterilized talc in 1:9 (Trichoderma spore:talc) ratio. Three samples should be taken from each, but lot during production and tested using a standardized method to determine the viability of the active ingredient expressed as colony forming units (CFU). The product thus prepared is ready for packaging at this stage. For storage, the finished product should be stored in vacuum filled plastic bags, covered by paper cartons of different sizes (250, 500 and 1000 g). These packets should then be kept in sealed cartons for transportation purpose.

**ADVANTAGES OF TRICHODERMA**

(1) *Trichoderma* spp. are very useful for fabric detergent, animal feed production, fuel production, alternative to conventional bleaching, effluent treatment, degradation of organochlorine pesticides and biocontrol of crop diseases.

(2) It is a potential bioagent for the management of fungal seed and soil borne pathogens. It is well known for its antagonistic activity against soil borne pathogens, such as *Fusarium*, *Pythium*, and *Rhizoctonia*.

(3) It is also known to suppress plant parasitic nematodes.

(4) It does not lead to development of resistance in plant pathogens, no phytotoxic effects, do not create any pollution problems as it is eco-friendly, promote plant growth, induces resistance in host, solubilize phosphorus.

**Figure 1.** Scanning electron micrograph on mycoparasitism of the *F. oxysporum ciceri* hyphae by the hyphae of *T. harzianum* with pincer shaped structure.
and micronutrients and hence increase soil fertility. (5) It significantly minimizes losses due to crop diseases and reduces cost of production, increases yield, quality and profit. (6) Many Trichoderma spp. are of great economic importance producing hydrolytic enzymes, namely, cellulases, chitinases and xylanases, biochemicals and antibiotic products which have been applied to fields, such as food processing and pulp bleaching. In addition, some species produce heterologous proteins and others have been successfully used as biological control agents against a range of phytopathogens.

DISADVANTAGES OF TRICHODERMA

There are many advantages associated with the use of Trichoderma. However, in addition to their useful properties, there are some disadvantages associated with the use of Trichoderma.

(1) Some species of Trichoderma pose a threat to the horticultural industry. For example, reduction in mushroom yield by as much as 50% have been attributed to Trichoderma infection and hence it is considered as a harmful parasite of mushroom.

(2) It also affects the organ (liver) transplanted in human.

(3) The disease is the major constraint in economical production as it inflicts heavy crop losses.

CONCLUSION AND FUTURE RESEARCH

Chemical based control is very effective, but there are some disadvantages associated with the use of these chemicals. The most dangerous thing with the use of these chemicals is the toxicity which they impart to the soil. That is why today people avoid the use of chemical based fungicide. Biosynthetic design of fungicide has present a new era in the development of fungicide. The genes present in the fungi Trichoderma has the ability to enhance host plant’s resistance against phytopathogenic fungi.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

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Antimicrobial activity of *Taverniera Abyssinica* A. Rich against human pathogenic bacteria and fungi

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Medicinal plants represent a rich source of antimicrobial agents. Even though hundreds of plant species have been tested for antimicrobial activities, the enormous mass of them have not been adequately evaluated. *Taverniera abyssinica* A. Rich is a widely used Ethiopian endemic medicinal plant commonly known under the local name of “Dingetegna”. Medicinal plant preparations are generally very popular in developing countries with a long tradition in the use of them. Root of the medicinal plant was extracted by maceration method using three different extraction solvents. Disc diffusion assay and agar dilution method were used to determine antimicrobial activity against *Staphylococcus aureus*, *Enterococcus faecalis* and *Escherichia coli* and clinical isolate of *Candida albicans* and *Aspergillus flavus*. To compare extraction solvents and the difference in sensitivity of test microorganisms, one-way analysis of variance was used. *T. abyssinica* A. Rich extracts exhibited remarkable difference in antimicrobial activity between water and alcohol extract. On the other hand there were little differences in antimicrobial activities of extracts obtained using ethanol and methanol as solvents. As a whole, extracts showed better antimicrobial activity against *S. aureus*, *E. faecalis* and *C. albicans* while *E. coli* and *A. flavus* were the most resistant microorganisms to this medicinal plant. Antimicrobial activity of the medicinal plant varies with extraction solvent and tested microorganisms. Even though the local people are using this medicinal plant in treatment of various types of infectious disease, the medicinal plant has little antimicrobial activity. *S. aureus* was the most sensitive microorganism as compared to other tested microorganisms.

**Key words:** Antibacterial, Antifungal, Crude extract, Ethiopia, *Taverniera abyssinica* A. Rich.

**INTRODUCTION**

Medicinal plants represent a rich source of antimicrobial agents. In different countries, plants are used medicinally and are the source of several effective and powerful drugs (Rahmoun et al., 2012; Gemechu et al., 2015). Even though hundreds of plant species have been tested for antimicrobial activities, the enormous mass of them have not been adequately evaluated (Das et al., 2010; Rahmoun et al., 2013). The antimicrobial agent contained in plants usually extracted using different solvents and the antimicrobial properties of the extracts may vary...
were tested for their antipyretic and analgesic properties extracts showed strong nematicidal activities towards been identified by the findings of fever, discomfort, stomachache and for many other pains been traditionally used for the treatment of various diseases in Ethiopia. A small bundle of the roots are compounds isolated (Kelbessa et al., 1992; Thulin, 1989). 

The medicinal importance of *T. abyssinica* A. Rich has been identified by the findings of different chemical compounds isolated from the rootstocks (Noamési et al., 1990; Abera, 2010). The study done on crude extracts and purified substances of *T. abyssinica* A. Rich plant were tested for their antipyretic and analgesic properties (Dange et al., 1990). In other study carried out, the extracts showed strong nematicidal activities towards *C. elegans*. Medicarpin and 4-hydroxymedicarpin were isolated as nematicidal constituents from the extracts (Stadler et al., 1995). The local people are also using this medicinal plant in treatment of various types of infectious disease. Medicinal plant preparations are generally very popular in developing countries with a long tradition in the use of them (Sharma et al., 2013). On the other hand, scientific evidence carried out to assess the antimicrobial activity of medicinal plant is limited. The aim of this study was to evaluate antibacterial and antifungal activity of root of *T. abyssinica* A. Rich (Dingetegna) which is well known endemic medicinal plant in Ethiopian.

**MATERIALS AND METHODS**

**Study design**

*In-vitro* experimental study of antibacterial and antifungal activity of *T. abyssinica* A. Rich was carried out by disc diffusion method and agar dilution method to determine minimum inhibition concentration, minimum bactericidal and fungicidal concentration, respectively. Positive and negative controls were used to monitor antimicrobial activity in all assays. All measurements were repeated three times and mean ± SD was used to describe the measurements.

**Collection and extraction of Plant Materials**

Root of *T. abyssinica* A. Rich was purchased from local market and authenticated by taxonomist and specimen was deposited at the National Herbarium, Department of Biology, Addis Ababa University Herbarium. The plant materials were washed three times under running tap water followed by rinsing twice with sterile distilled water and then air-dried in an oven at 40°C. Then ground into fine powder with electric grinder (Figure 1). About 25 g of fine powder of the medicinal plant was dissolves in 250 mL of solvents (ethanol, methanol and distilled water) separately in sterilized screw capped 500 mL glass bottles. Then the mixtures were kept in orbital shaker for 12 h at room temperature. Then the extracts were filtered by Whatman No. 1 filter paper. After having filtered extracts, they were evaporated to remove the solvent under vacuum in Rotary Evaporator kept at 40°C. Then the residues from rotary evaporator were allowed to dry in room temperature. The powdered extracts were weighed and dissolved in distilled water to gate stock solution of 200 mg/mL by labeling for each extraction solvents and stored in deep freezer at -20°C for further use (Parekh et al., 2005; Handa et al., 2008).

**Determination of disc diffusion assay**

To determine disc diffusion assay, *S. aureus* (ATCC-25923), *E. faecalis* (ATCC-29212) and *E. coli* (ATCC-25922), and clinical isolate of *C. albicans* and *A. flavus* were used to screen antimicrobial activity of the medicinal plant. Mueller Hinton agar medium and Sabouraud’s dextrose agar (SDA) were used to carry out disc diffusion assay antibacterial and antifungal activity respectively.

Diffusion discs of approximately 6 mm diameter were prepared from Whatman No. 1 filter paper by puncher and sterilized by autoclave then oven dried in sterile way and each solvent extracts were prepared into a series of concentrations: 10, 20, 40 and 80 mg/mL to determine disc diffusion assay. A 10 µl of each concentration of crude medicinal plant extracts was impregnated in separate sterile disc using sterile micropipette tips and stored at 4°C in separate sterile containers according to their extraction solvents and concentrations. Then disc diffusion assay was carried out using Kirby- Baur disk diffusion method (CLSI, 2009). Gentamycin (10 µg/mL) and Ketoconazole (10 µg/mL) disc were used as positive control for bacteria and fungi respectively. A blank disc impregnated with each solvent was used as negative control. All the tests were conducted in triplicate and the average of the three measurements was used.

**Determination of minimum inhibitory concentration**

Agar dilutions method was used to determine minimum inhibitory concentration (MIC) of the medicinal plant extracts. For each plant extract, a stock solution of 200 mg/mL was added into a sterilized molten Mueller Hinton agar and SDA after cooled to 45°C in water bath. Then two fold serial dilutions was used to obtain 100, 50, 25, 12.50, 6.25, 3.125 and 1.56 mg/mL concentration of medicinal plant extracts in agar medium. Then the mixture of plant extract and molten agar medium were poured to 90 mm Petri dish and solidified.

Then the plates were inoculated with a loopful of 0.5 McFarland standards diluted suspension of each test microorganisms in small spot. The plates were incubated at 37°C for 24 h and at 27°C for 48
h for bacteria and fungi, respectively. Then the minimum dilution of the plant extracts completely inhibiting the growth of each organism was taken as the MIC. A control comprising the test organism grown on fresh agar medium and agar medium with each solvent were used as control. All the tests were done in triplicates (CLSI, 2009).

**Determination of minimum bactericidal and fungicidal concentration**

Concentrations of the plant extract determined as MIC, the preceding one and one more concentration between the two concentrations were used to determine of MBC and MFC. Then these concentrations of the plant extracts were adjusted in nutrient broth and a Sabouraud’s dextrose broth and inoculated with test microorganisms then incubated at 37°C for 24 and 48 h for bacteria and fungi, respectively. Then after, a loopful of all broth media was sub-cultured on Mueller-Hinton agar and SDA plates. The inoculated plates were incubated at 37°C for 48 h and at 27°C for 72 h for bacteria and fungi, respectively. A control comprising the test organism grown on fresh agar medium and agar medium containing each solvent were used as control. All the tests were done in triplicate.

**Statistical analysis**

For each assay, all the measurements were replicated three times and the results were presented as mean ± SD. One way ANOVA followed by Tukey’s test was used to compare extraction solvents and the difference in the sensitivity of the test microorganisms using the statistical package for social sciences (SPSS) version 16 and P-value ≤ 0.05 were considered as statistically significant.

**RESULTS**

*T. abyssinica* A. Rich extracts exhibited remarkable difference in antimicrobial activity between water extract and alcohol extract. On the other hand there were little differences in antimicrobial activities of extracts obtained using ethanol and methanol as solvents. In disc diffusion assay, extracts of *T. abyssinica* A. Rich in water exhibited nearly the same amount of antimicrobial activities against *S. aureus* and *E. faecalis*. From tested fungal species, *T. abyssinica* A. Rich showed antifungal activity against *C. albicans* only at 80 mg/mL in water extract. Furthermore, the extracts in water did not exhibit any activity against *E. coli* and *A. flavus* up to 80 mg/mL (Table 1).

*T. abyssinica* A. Rich root extract in ethanol showed weak antimicrobial activity against *E. coli* and *A. flavus* at 80 mg/mL whereas the plant extract against *S. aureus* and *E. faecalis* were showed antimicrobial activity starting from 20 mg/mL. Likewise, extracts in methanol also exhibited antimicrobial activities at 80mg/mL against all tested microorganism. In methanol extract, the maximum antimicrobial activity was observed against *S. aureus*. There was significant difference (P < 0.5) between antimicrobial activity of water based and alcohol based extracts of the medicinal plant (Table 1).

The minimum inhibition concentration assay also showed water based extracts had weak antimicrobial activity than alcohol based extracts. In this assay, *T. abyssinica* A. Rich showed better antimicrobial activity against *S. aureus* and *C. albicans* when compared with *E. coli*. On the other hand, *E. faecalis* and *A. flavus* were inhibited at 100 mg/mL of water based extract of the medicinal plant. In methanol based extract, the least MIC (6.25mg/mL) was observed against *C. albicans* whereas the highest MIC of 25mg/mL was observed against *E. coli*. Generally, there was no significant difference (P > 0.05) in antimicrobial activity of ethanol based and methanol based extracts of *T. abyssinica* A. Rich against selected pathogenic bacterial and fungal species. The plant extracts also showed less antimicrobial activity against *E. coli* and *A. flavus* than other tested microorganisms (Table 2).

In bactericidal and fungicidal determination assay, the extracts of *T. abyssinica* A. Rich, exhibited varying degrees of antimicrobial activities against the tested organisms. The water based extracts exhibited bactericidal and
Table 1. Antimicrobial activity of *T. abyssinica* root extract obtained using three different extraction solvents with four different concentrations.

<table>
<thead>
<tr>
<th>Extraction solvents</th>
<th>Extract concentration (mg/ml)</th>
<th>Inhibition zone (mm)</th>
<th>Bacterial species</th>
<th>Fungal Species</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>S. aureus</td>
<td>E. coli</td>
</tr>
<tr>
<td>Water</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>8.66 ± 0.57</td>
<td>-</td>
<td>8.33 ± 0.57</td>
</tr>
<tr>
<td>Ethanol</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>8.33 ± 0.57</td>
<td>-</td>
<td>8.00 ± 1.00</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>9.33 ± 0.57</td>
<td>9.33 ± 0.57</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>11.00 ± 1.00</td>
<td>8.33 ± 0.57</td>
<td>12.66 ± 0.57</td>
</tr>
<tr>
<td>Methanol</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>8.00 ± 0.00</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>9.66 ± 1.15</td>
<td>-</td>
<td>9.00 ± 1.00</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>13.00 ± 1.00</td>
<td>8.66 ± 1.155</td>
<td>12.33 ± 0.57</td>
</tr>
<tr>
<td>Positive control</td>
<td></td>
<td></td>
<td>22.33 ± 0.57</td>
<td>20 ±0.00</td>
</tr>
<tr>
<td>Negative control</td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(-) = No activity; Values are mean of inhibition zone (mm) ± S.D of three replicates.

Table 2. Average minimum inhibitory concentrations, minimum bactericidal and fungicidal concentrations of *T. abyssinica* in three different extraction solvents

<table>
<thead>
<tr>
<th>Assay methods</th>
<th>Extract solvent</th>
<th>MIC, MBC and MFC (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bacterial species</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S. aureus</td>
</tr>
<tr>
<td>MIC</td>
<td>Water</td>
<td>50.00</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>12.50</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>12.50</td>
</tr>
<tr>
<td>MBC/MFC</td>
<td>Water</td>
<td>100.00</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>25.00</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>12.50</td>
</tr>
</tbody>
</table>

(*) = No inhibitory or bactericidal/fungicidal activity at 100 mg/ml; MIC=minimum inhibition concentration; MBC=minimum bactericidal concentration; MFC=Minimum fungicidal concentration.

fungicidal effects at 100mg/mL against *S. aureus* and *C. albicans*, respectively. The strongest fungicidal activities were observed in alcohols extracts against *C. albicans*, *S. aureus* and *E. faecalis* while the weakest bactericidal effects were recorded against *E. coli* (Table 2).

**DISCUSSION**

Antimicrobial activity of *T. abyssinica* A. Rich varied with extraction solvent used. The successful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction procedure (Muhsin and Hussein, 2014). Antimicrobial activity of water extract of *T. abyssinica* A. Rich obtained in the current study was relatively low as compared to positive controls and alcohol extracts. However, this extract showed antimicrobial activity on *S. aureus*, *E. faecalis* and *C. albicans* at high concentration. This indicates that water extract of this medicinal plant at higher concentration can be effective antimicrobial agent against these microorganisms.

Though traditional healers use primarily water but plant extracts from alcoholic solvents have been found to give
more consistent antimicrobial activity compared to water extract. Water soluble flavonoids have no antimicrobial significance and water soluble phenolics are only important as antimicrobial compound in water extract (Lapornik et al., 2005; Das et al., 2010). In contrast, ethanol and methanol extract of T. abyssinica A. Rich showed better antimicrobial activity against tested microorganisms that generally increased with the increase in the concentration of the extract. It means that they are more efficient in cell walls degradation which has non-polar character and cause polyphenols to be released from cells (Wang, 2010). In addition to this enzyme polyphenol oxidase are inactivated in methanol and ethanol extract (Karmegam et al., 2012). This may be the reason why the antimicrobial activities of selected medicinal plant showed lower in water extract in our study.

In general, this medicinal plant showed a low antimicrobial activity against E. coli and A. flavus through all the three extraction solvent used in the current study as compared to other microorganisms. Antimicrobial studies also showed as Gram-negative bacteria show a higher resistance to plant extracts than Gram-positive bacteria. This may be due to the variation in the cell wall structures of Gram-positive and Gram-negative bacteria. Gram-negative bacteria have an outer membrane that is composed of high density lipopolysaccharides that serves as a barrier to many environmental substances including antibiotics (Palombo and Semple, 2001; Robinson et al., 2009).

In Ethiopia, most of people have been using T. abyssinica A. Rich for toothbrush and on the other hand E. faecalis has been frequently found in root canal-treated teeth in prevalence values ranging from 30% to 90% of the cases (Kunin, 1993). Therefore, without having this information, people have been benefiting from this medicinal plant. Plant materials remain an important resource to combat serious diseases in the world (Vlietinck et al., 1995; Regassa, 2013). People living in rural areas from their personal experience also know that this medicinal plant is valuable source of natural products of health care. However, they may not understand the scientific facts behind this medicinal plants and their effective way of extraction (Mohammed and Berhanu, 2011; Regassa, 2013). There is no study done on antibacterial and antifungal cility of this medicinal plant so far but study showed that it has strong nematicidal activity (Stadler et al., 1995).

Conclusion

Even though the local people are using this medicinal plant in treatment of various types of infectious disease, the medicinal plant has little antimicrobial activity. Antibacterial and antifungal activity of T. abyssinica A. Rich varies in extraction solvent used. Water was the weakest extraction solvent whereas ethanol and methanol were good solvents to obtain antimicrobial phytochemical from this medicinal plant. Ethanol and methanol extracts showed better antimicrobial activity against S. aureus, E. faecalis and C. albicans while E. coli and A. flavus were the most resistant microorganisms to this medicinal plant.

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Conflict of interests

The authors did not declare any conflict of interest.

Authors’ contributions

GA participated in the design of the study, coordinated and was involved in data collection, experimental work, and also analyzed the data, and drafted the paper. AG and ED participated in the analysis and revised subsequent drafts of the paper. All authors read and approved the final manuscript.

REFERENCES

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Full Length Research Paper

Aflatoxin B1 in commercial granolas

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The aim of this study was to measure aflatoxin B1 in granola sold in stores in the city of Teresina, Piauí. Sixty samples of granola sold in four supermarkets were used. The mycological evaluation was carried out immediately, and then the samples were stored at -4°C, for later analysis of aflatoxin B1. Thirty-one strains of Aspergillus flavus were isolated. Two strains demonstrated capacity for aflatoxin production with aflatoxin B1 concentration of 2.30 and 3.90 µg/g. Sixty percent of samples tested positive for aflatoxin. Granolas may have different levels of aflatoxin B1, a risk for the consumer. Therefore, there must be better control of the quality of raw materials used to make granola, because they may be the source of contamination by aflatoxins.

Key words: Aspergillus flavus, cereals, fungi, grains, mycotoxins.

INTRODUCTION

Granola is an increasingly popular tasty food with high energetic value, and nutritional and functional properties. It is rich in fiber and consists of a mixture of cereal grains, dried fruits, linseed, wheat, corn and rice flakes, and oily seeds such as peanuts and Brazil nuts. Diets high in fiber protect against obesity, cardiovascular disease, diabetes, dyslipidemia and some cancers (Neutzling et al., 2007). Granola is among the foods shown to help treat these diseases (Granada et al., 2003; Vecchia and Castilhos, 2007).

Despite the many benefits granola provides, its composition requires special attention because according to Gimeno (2000) and Dantigny et al. (2005), cereal products are an ideal substrate for fungi growth and mycotoxin production if humidity and storage temperature during processing are not controlled (Pereira et al., 2002;

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The effects of fungal growth in foodstuffs include decreased germination power, visible molding, discoloration, unpleasant odor, loss of dry matter, heating, chemical and nutritional changes, loss of quality and mycotoxin production (Lazzari, 1998; Junior and Zappa, 2009).

The genera Aspergillus, Penicillium and Fusarium are some of the mycotoxigenic fungi involved in the human and animal food chain, and produce the vast majority of mycotoxins (Sweeney and Dobson, 1998; Hermanns et al., 2006; Pitt and Hocking, 2009). Mycotoxins in food are a concern for public health because they can damage health and bring about economic losses when present in the animal food production chain (Pereyra et al., 2010).

Aflatoxins are produced mainly by strains of Aspergillus flavus, A. parasiticus and A. nomius. These species are found in soil, which is the main source of contamination. They are capable of growing on various types of cereals, especially those containing corn and peanuts (Rodriguez-Amaya and Sabino, 2002; Pitt and Hocking, 2009). Aflatoxin B1 is the most important and has carcinogenic potential in humans (IARC, 2006).

Vecchia and Castilhos-Fortes (2007) established the presence of mycotoxin producing fungi, especially Aspergillus, Fusarium and Penicillium, in granola marketed in Porto Alegre. The predominant genera was Aspergillus in three seasons of the year and their results show the need for greater control and supervision in order to eliminate toxin-producing microorganisms in granola.

Granada et al. (2003) evaluated the microbiology and composition of commercial granola and detected mold and yeast counts ranging from 12 x 104 and 33 x 106 CFU/g. The authors stated that the high score was due to the low pH found in the samples, a factor that must be controlled to avoid the proliferation of fungi and production of mycotoxins.

Given the above and considering that there are few studies on fungi and mycotoxins in granola, the objective of this study was to measure aflatoxin B1 in granola sold in stores in the city of Teresina, Piauí.

MATTERIALS AND METHODS

Sample collection

Samples were purchased in a supermarket chain where granola is regularly found in the city of Teresina, Piauí. Just one supermarket was randomly selected to collect the samples, because the same commercial brands were sold in all supermarkets. During data collection the integrity of packaging, labeling instructions, hygiene conditions and room temperature were verified.

Fifteen samples of four different brands (N=60) were collected. The packets were 250 or 300 g. The samples were characterized as "A", "B", "C" and "D". The quantification of fungi, analysis of the toxigenic capacity of the strains of A. flavus and detection of aflatoxins B1 were carried out at the Laboratory of Microbiological Control of Food of the Nucleus of Studies, Research and Processing (NUEPPA) at the Center for Agricultural Sciences, Federal University of Piauí.

Determination of mycobiota and identification of Aspergillus species

The fungi colonies were observed under microscope to identify the fungal genera. Colonies belonging to genera Aspergillus and Penicillium were isolated and identified using identification keys described by Klich (2002) and Pitt (2004), in four basic media: Czapek yeast extract agar (CYA); malt extract agar (MEA); Czapek yeast extract agar 20% sucrose (CY20S) and Glycerol Nitrate Agar (G25N).

A conidial suspension from each strain was prepared in 0.5 ml of medium consisting of 0.2% agar-agar and 0.05% Tween 80TM, distributed in Eppendorf microtubes previously sterilized at 121°C for 15 min. Afterwards, the isolated A. flavus strains were tested for production of aflatoxin B1, following the method recommended by Geiser et al. (1998) as follows: strains were cultivated on MEA slides at 28 °C for seven days.

After the incubation period, the mycelium was transferred to a microtube and 1000 μL of chloroform added. The mixture was stirred for 10 min in a microcentrifuge at 1400 rpm; the mycelium was removed and the chloroform extract was evaporated under N2 flow. The residue was re-dissolved in 200 μL of chloroform. The detection and quantitation of aflatoxin B1 of the extracts were carried out using high-performance liquid chromatography with a Prominenpose model Shimadzu gas chromatograph with fluorescence detector (RF-10AXL SUPER model) according to the methodology proposed by Truckess et al. (1994). An aliquot of 200 μL of sample extract was derivatized with 700 μL trifluoroacetic acid/acetic acid/water (20:10:70, v/v/v). Chromatographic separations were carried out on a reversed phase column (silica gel, 150 x 4.6 mm id., 5.0μm particle size, Varian, Inc., Palo Alto, USA). The mobile phase used was acetonitrile, methanol and water (17:17:66 v/v/v) at a flow rate of 1.5 mL min⁻¹. The fluorescence of aflatoxin derivatives was recorded at excitation and emission wave lengths of 360 and 460 nm, respectively. The standard curve was constructed at different standard levels of AFB1: 1.01, 2.02 and 4.04 ng/ml (Sigma Aldrich® Co., St. Louis, MO USA, purity> 99%). The toxin was quantified using the correlation between the heights of peaks of aflatoxin B1 in the sample extract and the standard curve (y = 0.0003x – 0.0077; R2 = 0.99). The detection limit of the analytical method was 0.4 ng/g, based on the signal-to-noise ratio (3:1) and the limit of quantification was set at 3 times the detection limit (1.4 ng/g).

Detecting Aflatoxin B1

Extraction of aflatoxins from samples was carried out using MycoSep 228 Multifunctional columns (MFC, Romer Labs® Inc., MO, USA), following the manufacturer’s instructions as follows: for granola aflatoxin extraction, 25 g each sample was added to 100 mL of acetonitrile: water (84:16, v/v) and homogenised in a domestic blender for 3 min. Afterwards, the mixture was filtered using Whatman #4 paper (Whatman, Inc., Clifton, New Jersey,
Table 1. Identification of fungal species in granola commercialized in Teresina, Piauí.

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>A. niger agregados</td>
<td>5</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A. carbonarius</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A. japonicus</td>
<td>10</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A. flavus</td>
<td>27</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>A. tamari</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A. terreus</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>A. penicillioides</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>44</td>
<td>5</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Penicillium</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>P. citrinum</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>P. simplicissimum</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P. funiculosum</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P. islandicum</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P. variabile</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Total geral por marcas</td>
<td>48</td>
<td>7</td>
<td>2</td>
<td>6</td>
</tr>
</tbody>
</table>

n= number of individuals.

USA) and 8.0 mL from the filtrate were transferred to a 10 ml tube. The filtrate was purified with the use of Multifunction MycoSep 224 columns (MFC, Romer Labs®, Inc., MO, USA). After the purification, 4.0 ml of the purified extract were evaporated in a water bath at 70°C.

An aliquot (200 μL) was derivatized with 700 μl trifluoroacetic-water-acetic acid (20:10:70, v / v). Chromatographic separations were performed on a reverse phase column (Silica Gel, 150 x 4.6 mm id, 5 um particle size, Varian, Inc., Palo Alto, USA). Mobile phase used was acetonitrile: methanol: water (1: 1: 4 v / v / v); the injected volume was 20 μL, and the speed flow was 1.5 mL per minute. Detection of aflatoxins was by fluorescence using a wavelength of 360 nm excitation and 440 nm emission. Standard curves were constructed with different levels of aflatoxins. The limit detection of the method was 0.4 ng / mL.

The detection and quantification of aflatoxin B1 from the extracts were carried out using high-performance liquid chromatography with a Prominence model Shimadzu® gas chromatograph with fluorescence detector (RF-10AXL SUPER model) according to the methodology proposed by Truckssess et al. (1994).

Statistical analysis

The results of the counts were transformed to log10(x+1). Correlation analysis and ANOVAs were carried out using Sigma Stat statistical software (1994) with 5.0% significance.

RESULTS AND DISCUSSION

At the time of acquisition of the samples the temperature in the stores ranged from 26.2 to 29.5°C. The granola products were on clean shelves in an aired, well-lit and sanitized location according to ANVISA’s RDC 216/04 for dry goods (Brazil, 2004). The packages were clean and undamaged, and listed the lot number, expiration date, and weight between 250 and 300 g. The nutritional information met the labeling standards for food in the RDC Number 360/2003 of ANVISA (Brazil, 2003).

The granola brands sold in Teresina, had different formulations depending on the manufacturer, using between 4 to 13 different ingredients. However, all granolas used oats, raisins, wheat and its derivatives. According to Dantigny et al. (2005) these ingredients can encourage the growth of pre-existing fungi in various stages of processing, due to intrinsic nutritional factors and storage conditions that favor the increase in relative humidity.

Table 1 shows the isolated species of Aspergillus and Penicillium of the different granola brands marketed in Teresina, Piauí. Thirty-one A. flavus strains were isolated and 27 of them were isolated from brand "A". This species is considered the leading producer of aflatoxin (Pitt and Hocking, 2009). Two strains were shown to produce aflatoxin B1, as shown in Table 2. Brand "A" presented two strains of A. flavus, which have the potential to produce aflatoxin B1; they were classified as "Strain 21" and "Strain 26 " (Table 2). The concentration of aflatoxin B1 was 2.30 and 3.90 μg /g, respectively. Despite the low incidence of aflatoxin B1 in this sample (Table 4), there is a need for greater humidity control and better storage conditions, otherwise the environment is conducive for the development of potentially aflatoxin strains and subsequent production of their metabolites.
### Table 2. Strains of *Aspergillus flavus* aflatoxin B1 producers isolated from granola commercialized in Teresina, PI.

<table>
<thead>
<tr>
<th>Brands</th>
<th>N</th>
<th>+</th>
<th>Aflatoxin B1 amount by strain in µg / g (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>27</td>
<td>2</td>
<td>2.30</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>0</td>
<td>3.90</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>0</td>
<td>2.30</td>
</tr>
<tr>
<td>D</td>
<td>2</td>
<td>0</td>
<td>2.30</td>
</tr>
<tr>
<td>Total</td>
<td>31</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

N = number of strains isolated from the granola samples; + = Producer strains of aflatoxin B1; µg/g = micrograms per gram.

### Table 3. Positive samples for aflatoxin B1 in granola commercialized in Teresina, PI.

<table>
<thead>
<tr>
<th>Granola brands</th>
<th>Number of positive samples for aflatoxin B1</th>
<th>% Positive sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;A&quot; (N=15)</td>
<td>12</td>
<td>80.0</td>
</tr>
<tr>
<td>&quot;B&quot; (N=15)</td>
<td>1</td>
<td>6.6</td>
</tr>
<tr>
<td>&quot;C&quot; (N=15)</td>
<td>8</td>
<td>53.3</td>
</tr>
<tr>
<td>&quot;D&quot; (N=15)</td>
<td>15</td>
<td>100.0</td>
</tr>
<tr>
<td>Sample analyzed</td>
<td>Total Positive</td>
<td>% positive</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>60.0</td>
</tr>
</tbody>
</table>

N = number of samples; % = percent.

Brand "D" presented contamination in 100% of the analyzed samples with concentrations ranging from 3.42 to 4.52 ng/g. Brand "B" only had one contaminated sample (Table 3).

According to the National Health Surveillance Agency (Brazil, 2010), which manages and monitors the National Program for Monitoring Food Sanitary Quality (PNMQSA), the presence of aflatoxin is one of the parameters used to assess product quality. Current legislation does not have a specific parameter for granolas. However, the maximum permitted level of aflatoxin B1, B2, G1 and G2 for food containing cereals and cereal products (except corn and derivatives including malted barley) is 5.0 µg/kg (Brazil, 2011). Thus, the results of this study are within such limits. The contamination is likely to have occurred due to the use of raw materials previously contaminated with aflatoxin B1.

There were differences between the brands with respect to the amount of aflatoxin B1 detected. Brand "D" had the highest contamination level with a mean concentration of 4.00 µg/kg (Table 4). Therefore, a good quality control of the ingredients is necessary, to prevent contamination risks and consumer health problems.

It is important to identify the fungal mycobiota and its mycotoxins in foods so that these studies can provide information inherent to product quality, with respect to the presence of fungi and their mycotoxins. Furthermore, preventive actions must take place during all food production stages, from harvesting, storage, processing, initial steps for controlling fungi and their metabolites, as mycotoxins can potentially be found in the samples. Additional practices are required to reduce the contamination of food, as well as procedures for the treatment of food which are needed to prevent health problems.

Granola products may have different levels of aflatoxin B1, and are a risk for the consumer. Better control of the quality of raw materials used is necessary because they may be sources of contamination by aflatoxins in granola.
Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES


