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Fungal and Aflatoxin Contamination in Soybean Paste Collected from Ban Village, Hung Yen Province, Vietnam

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Abstract: Soybean paste is a fermented food produced in many countries around the world by various ways. In Vietnam, *Aspergillus oryzae* was used as a fermentation agent in soybean paste process. This study was carried out to investigate fungi and aflatoxin contaminations in soybean paste samples. Several fungal strains were isolated. Among them, eleven selected fungal isolates were identified by phenotypic characteristics and molecular analysis using the ITS (internal transcribed spacer) sequence and revealed eight species from two genera *Aspergillus* and *Penicillium.* The results also showed that aflatoxin was not detected in all soybean paste samples, however, one isolated strain of the *A. oryzae*/*flavus* group was found to be an aflatoxigenic fungal strain.

Keywords: Aflatoxin, *Aspergillus*, Fungi, Soybean Paste

1. Introduction

Aflatoxins are secondary metabolites produced mainly by members of the genus *Aspergillus* such as *A. flavus*, *A. parasiticus*, and *A. nomius*. These fungi normally grow in the tropical countries, especially in hot and humid region such as Vietnam. They usually infect cereal crops such as rice, corn, peanuts, tree nuts, and soybean at different stages including pre-harvest, harvest, processing and handing [1, 2]. There are more than 20 known aflatoxins, but the four common ones are B1, B2, G1, and G2 [3]. When aflatoxins are contaminated in foods, they cannot be destroyed by normal cooking process because of their extreme heat resistance. For example, aflatoxin B1 can only be degraded at temperature higher than 160 °C [2, 4]. Contamination of foods by aflatoxins poses a risk to human health because these compounds are highly carcinogenic and can directly influence the structure of DNA. The defects of genetic materials can lead to fetal misdevelopment and miscarriages. Moreover, aflatoxins are also known as toxic compounds and can lead to serious threats to human and animal health such as reduced weight gain and suppression of the immune system [1, 2].

Soybean paste (*Tương* in Vietnamese) is a traditional fermented food used in Vietnamese cuisine that made by ground soybean and sticky rice. *Tương* was prepared by mixing of mashed soybeans with salt solution and steamed sticky rice containing fungal species *Aspergillus oryzae*. The mixture was then incubated for at least 15 to 20 days to create fermented soybean paste. During the incubation time, *A. oryzae* performs an important role in decomposition of organic compounds such as protein and starch in soybean and sticky rice into small nutrient function molecules as amino acids, sugars, organic acids, and esters, which form the typical flavor of soybean paste [5]. The fungal species *A. oryzae* use for soybean paste production is commonly evaluated as being safe. However, it is possible to be

contaminated with aflatoxin producing fungi from surrounding environment during production process. This study was conducted to identify fungi species isolated from soybean paste samples collected from Ban village, HungYen province, Vietnam and to determine which isolates will be potential aflatoxigenic strains.

2. Materials and Methods

2.1. Sampling

The samples were taken at public market in Ban village, Hung Yen province, South Vietnam. The Bần village is one of most famous regions in Vietnam for soybean paste product since the late nineteenth century.

2.2. Isolation and Morphological Characterization of Fungal Strains

Soybean paste samples were serially dilluted with sterile 1% sodium chloride solution, and then 100 µl of the dilution were spread on Czapek Dox Agar (CZ) medium. The plates were incubated at 30 °C for 5 to 7 days. Typical fungal colonies were counted and subcultured onto a fresh CZ medium to obtain a pure culture.

Morphological characteristics such as colony growth, colony colour, and colony texture of each pure culture was identified according to a method described by Diba et al [6]. For microscopic analysis such as conidiophores, vesicles, matulae, phialides, and conidia, Riddell's classic slide culture method was used [7].

2.3. Phylogenetic Characterization of Selected Fungi

For phylogenetic studies, the ITS (internal transcribed spacer) primers: ITS 5 (5′-GGAAGTAAAAGTCGTAACAAGG) and ITS 4 (5′-TCCTCCGCTTATTGATATGC) were used to amplify the ITS region I between the 18S and 5.8S rDNAs, the 5.8S rDNA, the ITS region II, and a portion of the 28S rDNA. Sequencing of the amplified DNA fragment was performed at 1st Base (Singapore). GenBank were used to seek for ITS gene similarities. Phylogenetic analysis based on the ITS gene was performed with the aid of the Mega 6 software package using the neighbor-joining distance correction methods.

2.4. Detection of Aflatoxin

Aflatoxin in soybean paste product was tested by using Total Aflatoxin Test kit (Green Age, Vietnam). The lowest level can be detected by this kit is 5 ppm. The Aflatoxin Mix 4 solution (Sigma) was used as positive control.

The ability for aflatoxin production by selected fungal strains was also tested. Fungal strains were first grown on steamed sticky rice at 30 °C to make the rice mold. After 7 days, samples were then taken for aflatoxin detection by using Total Aflatoxin Test kit.

3. Results and Discussion

3.1. Fungi Isolation and Morphological Characterization

The soybean paste samples were diluted and inoculated on solid Czapek Dox medium. After 5 days of incubation at 30 °C, the isolated fungal colonies were counted and transferred to new CZ plate and incubated for 3 days to obtain a pure culture. The number of fungal cells in soybean paste was ranged from 10^4 to 10^6 cells/g. Based on characteristics of colony morphology, growth rate, pigmentation, and microscopic analysis, most of the isolates were assigned into two genera: *Aspergillus* (Figure 1A) and *Penicillium* (Figure 1B). *Aspergillus* species were doninant and can be found in all collected sample. Previous studies were also reported that *Aspergillus* and *Penicillium* are two main genera were found in *meju* samples – a starter material for Korean traditional fermented soybean product [8, 9]. *Aspergillus*, *Fusarium* and *Penicillin* are there main genera were found in maize samples collected from Vietnam. Among them, *Aspergillus* was the most frequent genus, it can be found in all maize samples [10]. Vietnam located in both tropical and subtropical zones. It is characterized by high temperature, high rainfall, and high mumidity. This kind of climate is well known to be a favourable conditions for mould growing, especially *Aspergillus* genus. These species are common contaminants of starchy foods and are responsible for both crop spoilage and mycotoxin contamination of food stuffs [1, 10].

In this study, eleven fungal isolates were randomly chosen for further identification using molecular technique.

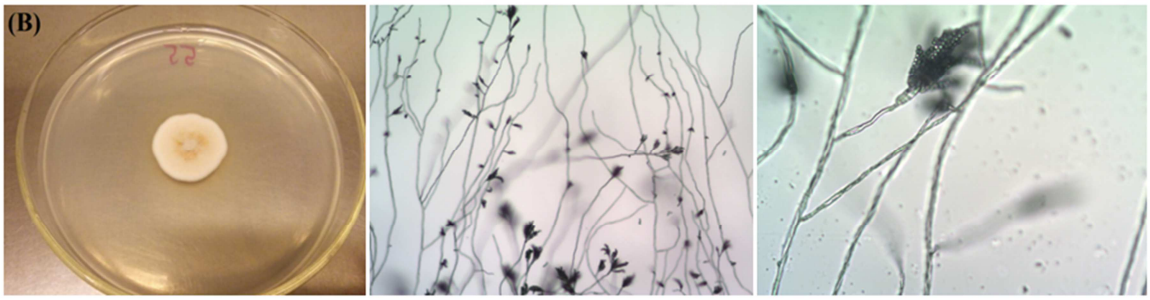

Figure 1. *Colony surface and conidial head of isolated strains: (A) a Aspergillus species - strain Tb11, and (B) a Penicillium species – strain Tb55.*

3.2. Phenogenetic Studies Based on ITS Sequence of the Selected Fungal Strains

In order to identify the species of the 11 isolates, molecular analysis was conducted using the rDNA-ITS gene. The phynogenetic tree constructed rDNA-ITS sequence showed that 11 fungal isolates were identified as belonging to 2 genera and 8 species (Figure 2). The sequences of three strains Tb5, Tb55, and Tb61 share a close relationship with sequences of *Penicillium* species. Strain Tb5 and *P. oxalicum* clustered together and had a ITS gene similarity of 99.7%, whereas, the ITS sequences of the strains Tb55 and Tb61 were 99.6% similar to the sequences of *P. chrysogenum* and *P. citrinum*, respectively.

Eight strains belonged to the *Aspergillus* genus. The sequence of strain Tb63 were 99% similar to the sequence of *A. unguis*. Strains Tb64 and Tb76 clustered together and had ITS sequence similarity of 99%. The sequence of these two strains were also 99% similar to the sequences of *A. niger* and *A. tubingensis*. The remaining 5 strains (Tb3, Tb11, Tb32, Tb36, and Tb37) clustered together and gathered with both *A. oryzae* and *A. flavus*. It is difficult to distinguish them because they had ITS similarity of 99.5 to 99.8%. Moreover, the morphological characteristics of five strains showed similar colony growth, colony colour, and conidial heads.

Both *A. oryzae* and *A. flavus* belong to the *Aspergillus* section Flavi, and have many morphological similarities [11]. Previous studies suggested that *A. oryzae* may be a variant morphotype of typical *A. flavus* and the gene homology of the two species was 99.5% [12]. They can only be divided into *A. oryzae* or *A. flavus* according to the difference in aflatoxigenicity. *A. flavus* can produce aflatoxins while *A. oryzae* does not [13]. The aflatoxigenicity of five fungal strains belonging to *A. oryzae/flavus* group was then determined.

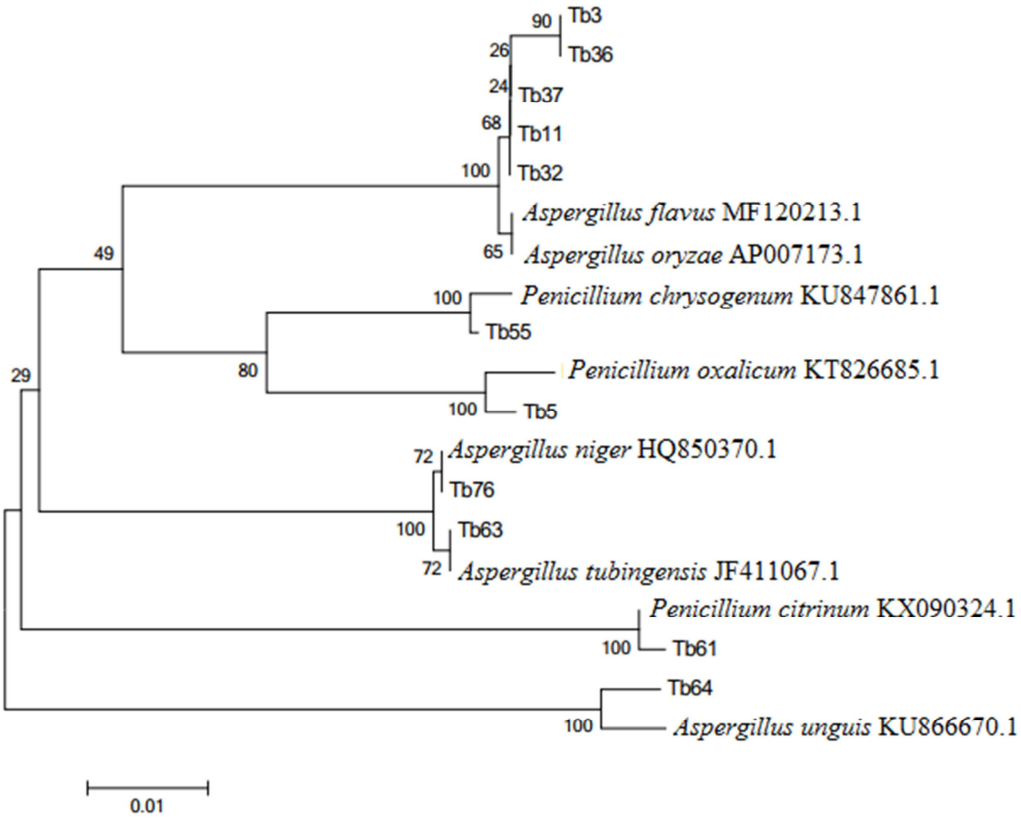

Figure 2. *Phylogenetic tree constructed using ITS gene sequences of 11 fungal strains isolated from soybean paste samples. The number above the nodes represent bootstrap value (out of 1000 bootstrap replications).*

3.3. Detection of Aflatoxin

The five strains belonging to *A. oryzae*/*flavus* group were grown on the steamed sticky rice and samples were collected after 7 days of cultivation. The aflatoxigenicity of soybean paste samples and 5 rice mold samples were then examined using Total Aflatoxin Test kit (Green Age, Vietnam). The results showed that aflatoxins were not detected in all collected soybean paste samples. However, among 5 tested strain, one strain was found to be aflatoxinogenic. As shown in Figure 3, the negative control had two bands, while positive control and strain Tb37 had one band. The presence of aflatoxin producing fungal strain in collected soybean paste sample suggested that aflatoxin may contaminate in the soybean paste products but its concentration may not be high enough to be detected by the kit.

It should be noted that the kit used in this study could detect the aflatoxin at a certain concentration, it not sensitive enough for the detail quantification of the toxin, therefore it can only be used for initial screening. For accurate identification and quantification of aflatoxins, a more sensitive method such as high-performance liquid chromatography with fluorescence detection or enzyme-linked immune-sorbent assay (ELISA) need to be used [14].

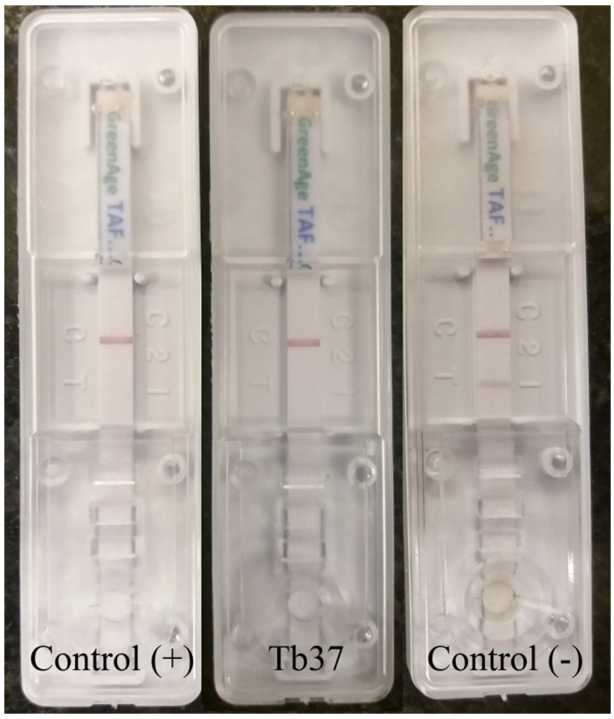

Figure 3. *Aflatoxin detection by using Total Aflatoxin Test kit. Strain Tb37 showed positive result.*

4. Conclusion

The current study examined fungi and aflatoxin contaminations in soybean paste samples collected from a region of Vietnam. The results shown that *Aspergillus* and *Penicillium* were two dominant genera found in soybean paste. Among 11 selected fungal isolates, 3 isolates belonged to the *Penicillium* genus and 8 isolates belonged to the *Aspergillus* genus. Five of eight *Aspergillus* species clustered together and belonged to the *A. oryzae/flavus* group. Aflatoxins at level of 5 ppm were not detected in all collected soybean paste samples, but one strain of the *A. oryzae/flavus* group was found to be an aflatoxigenic fungal species.

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