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Article

Prevalence, Characterization and Mycotoxin Production Ability of *Fusarium* Species on Korean Adlay (*Coix lacrymal-jobi* L.) Seeds

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Abstract: Adlay seed samples were collected from 3 adlay growing regions (Yeoncheon, Jeonnam and Eumseong regions) in Korea during 2012. Among all the samples collected, 400 seeds were tested for fungal occurrence by standard blotter and test tube agar methods and different taxonomic groups of fungal genera were detected. The most predominant fungal genera encountered were *Fusarium*, *Phoma*, *Alternaria*, *Cladosporium*, *Curvularia*, *Cochliobolus* and *Leptosphaerulina*. The occurrence of *Fusarium* species were 45.6% and based on the combined sequences of two protein coding genes, EF-1a, Beta-tubulin and phylogenetic analysis, 10 species were characterized as *F. incarnatum* (11.67%), *F. kyushense* (10.33%), *F. fujikuroi* (8.67%), *F. concentricum* (6.00%), *F. asiaticum* (5.67%), *F. graminearum* (1.67%), *F. miscanthi* (0.67%), *F. polyphialidiom* (0.33%), *F. armeniacum* (0.33%) and *F. thapsinum* (0.33%). The ability of these isolates to produce mycotoxins fumonisin (FUM) and zeralenone (ZEN) were tested by ELISA quantitative analysis method. The result revealed that fumonisin (FUM) was produced only by *F. fujikuroi* and zeralenone (ZEN) by *F. asiaticum* & *F. graminearum*. Mycotoxigenic species were then examined for their morphological characteristics to confirm their identity. Morphological observations of the species correlated well with their molecular identification and confirmed as *F. asiaticum*, *F. fujikuroi* and *F. graminearum*.

Keywords: Adlay seeds; ELISA; *Fusarium*; morphological data analysis; mycotoxins; phylogenetic analysis S

1. Introduction

Herbal medicine is kind of herbal products (plants products such as flowers, seeds, plants, shrubs, tree branches, moss, lichens, algae, mushroom, sea weed and fungus) used for the purpose of medicine. According to the World Health Organization, people living in developing countries mostly rely on herbal and/or traditional medicines (80% of people) [1]. But astonishingly, herbal or alternative medicines are popular in developed countries too. China and India are the largest producers and uses of herbal and traditional medicines. Barnes et al. [2] mentioned that about half of the Australian and one third of Americans use alternative medicines. Korea is the country, which produces medicinal plants and uses them in different form. Korean ginseng (*Panax ginseng*) is very popular all over the world which is known to have the ability to increase work efficiency and physical stamina. Korean adlay (*Coix lacrymal-jobi* L.) plant is another herbal plant and seeds are

used as a traditional medicinal plant in Korean peninsula only. The plant is grown tropical, subtropical, temperate regions and popular in Korea, China and Japan [3]. The seed production in Korea ranks fifth among other medicinal plants with an annual income of 26.5 billion won (MAFRA 2015). Compare to other cereals in contains higher protein, high lipid and fiber content [4] large amounts of calcium, iron, vitamin B1 which makes this plant as a alternative food source [5-6]. The powdery form of seeds is known to have the disease reducing ability. Some believes that adlay plant seeds could reduce the risk of the occurrence of cancer, lower the level of blood cholesterol and produces coixol a functional material of some medicines [7-9].

Adlay seeds are infected with different pathogens in the field and after harvest. The main production barriers are *Bipolaris coicis* and *Septoria* sp. produces leaf blight disease. *Fusarium* species (*F. graminearum*) also reported from the plant [10-11]. *Fusarium* is one of the most important fungal genera produces diseases on cereal and in the mycological taxonomy.

Fusarium species are capable of producing a wide variety of mycotoxins in pre-harvest infected plants in fields and in store house [12]. Mycotoxins which pose a significant risk to human and animal health are generally produced by five fungal genera: *Alternaria*, *Aspergillus*, *Claviceps*, *Fusarium* and *Penicillium* [13]. *Fusarium* mycotoxins are important as they are the infective agents of plants, animals and human [14]. *Fusarium* species which are the producers of mycotoxins are *Fusarium oxysporum* species complex, *F. graminearum* species complex, *F. solani* species complex, *F. poae*, *F. verticilliodes* etc. [15].

So, the objective of the present study was to study the prevalence and molecular characterization (by combined analysis of two protein coding genes, EF-1a and Beta-tubulin) of *Fusarium* species in adlay (*Coix lacrymal-jobi* L.) seeds in Korea and to study the main mycotoxin production ability of *Fusarium* species (FUM and ZEN) by ELISA quantitive analysis.

2. Results

2.1. Mycofloral incidence

A total of 400 fungal isolate belonging to 7 genera and 20 species were detected from adlay seeds by the standard blotter and test tube agar methods. The differences of fungal populations isolated from the medicinal plant are shown in Figure 1. Seven fungal genera detected were *Alternaria*, *Cladosporium*, *Cochlibolus*, *Curvularia*, *Fusarium*, *Leptosphaerulina* and *Phoma* (Figure 1). Among the isolated fungi, 45.6% were *Fusarium* species followed by *Phoma* (17.33%) and *Alternaria* (8.33%), *Cladosporium* (7.00%), *Curvularia* (1.00%), *Cochlobolus* (0.67%) and *Leptosphaerulina* (0.33%).

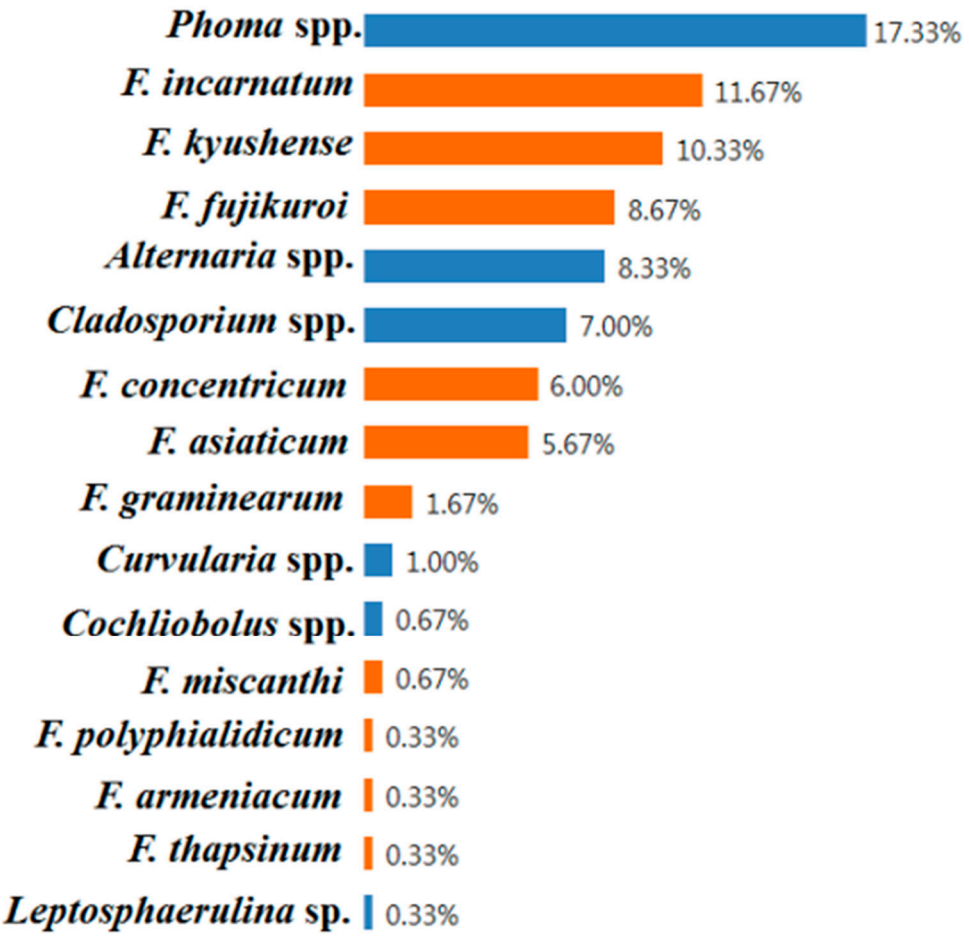

Figure 1. Percentage incidence of seed-borne fungi on adlay based on morphology and ITS gene sequence analysis from 400 seeds

2.2. Incidence of Fusarium

Fusarium was superficial fungi isolated from adlay seeds. Fusarium fungi were grouped into different groups. Among different groups representative isolates were selected and sequenced with different primers. Based on internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA), Elongation factor 1-alpha (EF 1a) gene and beta-tubulin (BT2) and phylogenetic analysis by maximum likelihood (ML) method, 10 species of *Fusarium* were characterized. Identified fungal species and incidence percentage were *F. incarnatum* (11.67%), *F. kyushense* (10.33%), *F. fujikuroi* (8.67%), *F. concentricum* (6.00%), *F. asiaticum* (5.67%), *F. graminearum* (1.67%), *F. miscanthi* (0.67%), *F. polyphialidiom* (0.33%), *F. armeniacum* (0.33%) and *F. thapsinum* (0.33%). Representative isolates were then grown on synthetic nutrient-poor agar (SNA), carnation leaf agar (CLA) and potato-dextrose agar (PDA) to examine morphological characteristics. Morphology studies of these representatives' fungi correlated well with the molecular analysis (data not shown).

2.3. Occurance of mycotoxin in different Fusarium species

Collected Fusarium samples were investigated for toxin producing ability by ELISA quantitative analysis. The result confirmed that among the ten species of Fusarium, FUM production was highly associated with *F. fujikuroi.* Among the 10 species, other did not produce FUM, even the experiment

was conducted three times with 3 replications. The average production of FUM was 9.92 ppm, 7.02 ppm and 4.52 ppm, respectively in three different experiments (Figure 2). Zearalenone (ZEN) production was restricted to *F. asiaticum* & *F. graminearum* species among all other *Fusarium* species isolated from adlay seeds in Korea. However, only three species produced FUM or ZEN, which are *F. fujikuroi*, *F. asiaticum* and *F. graminearum*. There were no detectable amounts of FUM and ZEN produced by isolates of other eight *Fusarium* species. A high amount of ZEN was produced by the species of *F. graminearum* (398.94 ppb, 212.53 ppb and 161.85 ppb in three replications) and the lower amount was by *F. asiaticum* (64.94 ppb) (Figure 3).

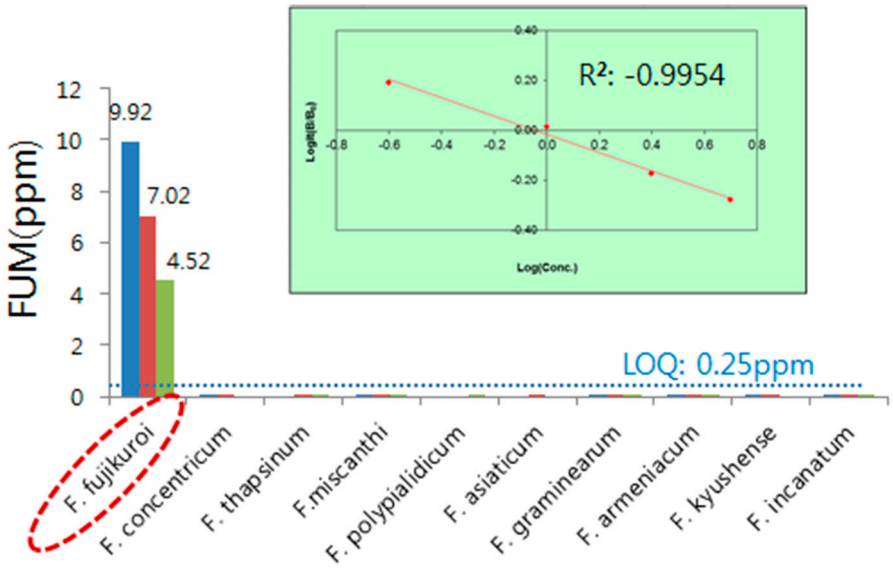

Figure 2. Fumonisin producing ability of ten *Fusarium* species isolated from adlay seeds. LOQ; limit of quantitation. Fum; Fumonisin.

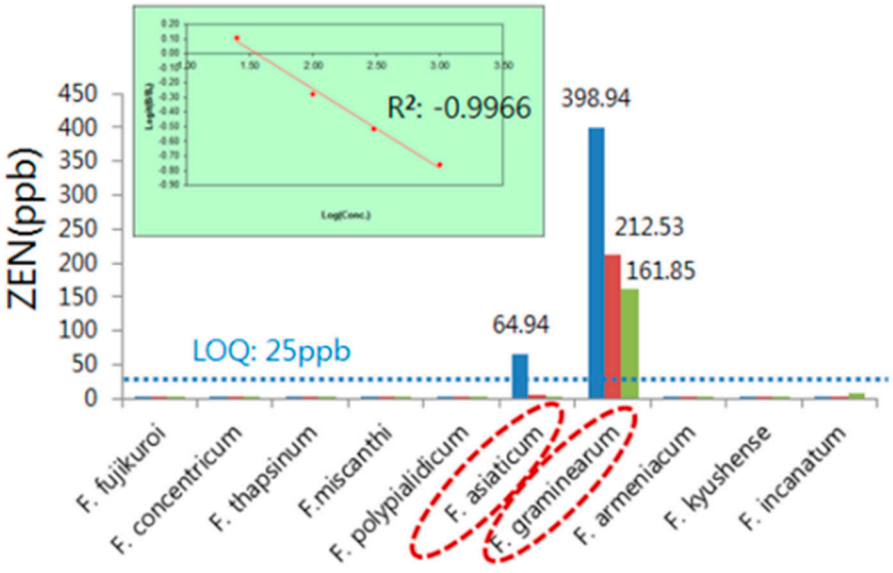

Figure 3. Zeralenone producing ability of ten *Fusarium* species isolated from adlay seeds. LOQ; limit of quantitation, ZEN; Zeralenone.

3. Discussion

Adlay (*Coix lacrymal-jobi* L.) is one of the most common medicinal resources which give food materials as well as drinks as tea in Korea. This plant is grown well in oriental countries, where traditional medicines are well known, popular and consumed as medicinal purposes. This plant seeds showed the prevalence of diversified fungal flora in the present study. Among them *Fusarium* (45.6%) and *Phoma* (17.33%) were predominant fungal genera. Study revealed that *Fusarium*, *Phoma*, *Alternaria*, *Penicillium*, *Aspergillus*, *Cladosporium* spp. [12, 16-19] are the commonly isolated mycoflora from cereal seeds and other plant seeds. Almost all the common species were detected except *Penicillium* and *Aspergillus*. These two fungi are most common in many seeds. There might have reason, that is competitive interactions exist among *Fusarium*, *Aspergillus* and *Penicillium*. Studies revealed the same, where negative correlation between *Fusarium* spp. and *Penicillium* spp. were reported in grains by Martin et al. [20] and Barros et al. [21]. Prevalence of *Fusarium*, occurred in similar trend explained by Chen et al. [16] in, where *Aspergillus* and *Penicillium* were not recorded.

The species predominantly found associated with seed diseases of Adlay were *Fusarium*-45.6% of total fungi isolated. The biggest number of species was held to the genus. Ten species of *Fusarium* were characterized. *Fusarium* species are difficult to identify and have to apply multilocus molecular data analysis along with morphological characterization. Internal transcribed spacer (ITS) and two protein coding genes Elongation factor 1-alpha (EF 1a) gene and beta-tubulin (BT2) and their phylogenetic analysis grouped the isolated Fusarium into 10 different species group. Refai et al. [22] and Herron et al. [15] recommended the similar molecular characterization methods to identify Fusarium. In the present study, morphological characterization perfectly correlated with their molecular data analysis. By the two analytical methods, 10 species recovered were *F. incarnatum*, *F. kyushense*, *F. fujikuroi*, *F. concentricum*, *F. asiaticum*, *F. graminearum*, *F. miscanthi*, *F. polyphialidicm*, *F. armeniacum* and *F. thapsinum*. Among all Fusarium species, *F. incarnatum* and *F. kyushense* encountered in 11.67% and 10.33%, respectively. The prevalence of *F. fujikuroi*, *F. concentricum* and *F. asiaticum* were 8.67%, 6.00% and 5.67%, respectively. *F. graminearum*, *F. miscanthi*, *F. polyphialidiom*, *F. armeniacum* and *F. thapsinum* were recorded lower in percentage as 1.67%, 0.67%, 0.33%, 0.33% and 0.33%. Other research focuses a number of *Fusarium* species too. Bottalico et al. [12] encountered 15 species of *Fusarium* associated head blight in small grain cereals in Europe. Chen et al. [16] claimed that 51% of fungi were *F. incarnatum* associated with spider flower seed. Eight most common *Fusarium* species were isolated from Norwegian cereals by Langseth et al. [17].

In the present study production of the toxins of FUM and ZEN was investigated. Among all the species it was found that only one species- *Fusarium fujikuroi* produced FUM, whereas only two species of isolates produced ZEN. Species of the ZEN producing *Fusarium* were *F. asiaticum* & *F. graminearum*. Toxin production of *Fusarium* in Korea were monitored in staple foods and also checked in barley and maize for *Fusarium* occurrence and found out that in barley and rice bore 29.8, 6.4, 36.2% and 2.3, 55.8, 34.9% *Fusarium* frequently. Among the all, only one sample from corn detected FUM in a permissible threshold level of 100.90 μg/kg [23]. Prevalence of Fusarium species and their ability to produce the mycotoxins (Zzearalenone, moniliformin and fumonisin B1) were examined in Zimbabwean corn and observed that only one produced all the three mycotoxins simultaneously whilst most produced fumonisin B1 ans/or moniliformin. Only nine isolates produced ZEN [24]. In Europe, mycotoxin production by *Fusarium* were examined in head blight of small-grain cereals and revealed that *F. graminearum* & *F. culmorum* produced highest amounts of ZEN in north European areas [12]. High occurrence of ZEN was described by Srobarova and Pavlova [25] is ears of winter wheat highly contaminated with *F. graminearum.* In Austria, kernel samples or durum wheat predominantly infected by *F. graminearum* with a lower presence of *F. culmorum*, contained low levels of ZEN [26]. Different Fusarium species have been claimed to produce zearalenone, which might be the result of estrogenic effect.

4. Conclusions

A small scale research has been conducted worldwide to characterize the mycobiota and toxigenic effect of fungi, especially *Fusarium* from medicinal plant seeds. In oriental region (Korea,

Japan and China) medicinal plants and their tissues values a lot for traditional oriental medicine. So, the prevalence of fungi (special focus was on *Fusarium*) was investigated for the first time from adlay seeds in Korea. Adlay is the host species of different fungal taxa- *Fusarium*, *Phoma*, *Alternaria*, *Cladosporium*, *Curvularia*, *Cochliobolus*, *Leptosphaerulina* etc. among the fungi detected a maximum number of fungi were detected as Fusarium, which constitutes 45.6%. Collected *Fusarium* samples were investigated for toxin producing ability by ELISA quantitative analysis. The result confirmed that FUM was produced by *F. fujikuroi* and ZEN by *F. asiaticum* & *F. graminearum*.

5. Materials and Methods

5.1. Plant species and sample collection

Adlay (*Coix lachrymal-jobi* L.) is commonly known as job's tears but commonly sold as Chinese pearl barley in Asian supermarkets. Besides the use of ornamental purposes, adlay grains are useful as a source of cereal foods and traditional folk medicine. In Korea, the adlay tea (yulmu cha) is popular drink. The plant is grown in some part of Korea. Adlay seeds were collected from 3 adlay growing regions (Yeoncheon, Jeonnam and Eumseong regions) during 2012.

5.2. Mycofloral isolation and analysis

Among all the samples collected, 400 seeds were tested for fungal occurrence by standard blotter and test tube agar methods. Seeds were surface sterilized by the methods described by Paul et al. [27]. By the blotter method, adlay seeds were incubated on wet filter paper for 7 days at 20 C temp. under 12/12 NUV/dark cycle and by test tube agar method, seeds were incubated for 3 weeks on test tube poured with PDA media at 20C temp. at 12-12 hr light/dark condition cycle. After 2 days, seeds were checked under stereomicroscope for fungal hyphae and conidial growth. Hyphae and conidia were then transferred to PDA media and finally prepared pure culture.

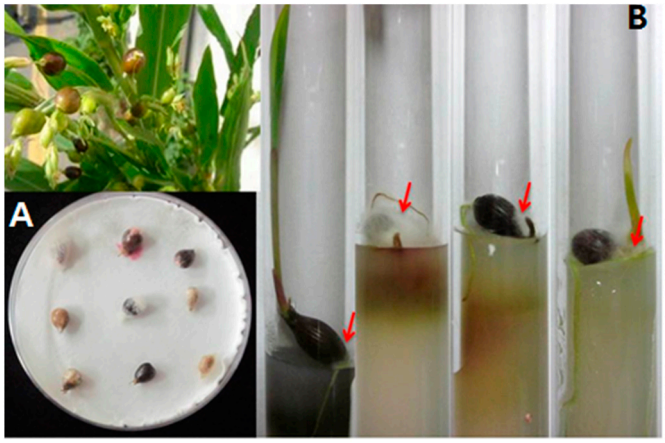

Figure 1. Incubation of adlay seeds. A, The Blotter method. Adlay seed incubated for 7 days at 20℃ under 12/12 h NUV/dark cycle; B, Test tube agar mtehod. Adlay seed incubated for 3 weeks at 20℃ under 12/12 h daylight/dark cycle.

5.3. Molecular identification of the isolates

5.3.1. DNA extraction, PCR and Purification

All the fungal isolates were grown on PDA for 7 days. Genomic DNA was extracted by the method described by Paul et al. [28]. The internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) was used in this study for PCR amplification of all isolates [29]. The ribosomal DNA of the *Fusarium* isolates was then separated and done PCR amplification by using two other-Elongation factor 1-alpha (EF 1a) gene and beta-tubulin (BT2) . The amplification reaction for each gene was performed in 50μL reaction volume and carried out in a GeneAmp PCR System 2700 thermo cycler (Applied Biosystems, Foster City, CA, USA), conditions described by Deng *et al.* [30].

The Wizard PCR prep. kit (Promega, Madison, WI, USA) was used for purification of successfully amplified PCR products. Sequencing of strands was performed with an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) with the same primer used for PCR amplification.

Table 1. Nucleotide sequences of primer sets used for amplifying target genes

Primers	Primer sequences	References
ITS5	GGAAGTAAAAGTCGTAACAAGG	White *et al.* 1990
ITS4	TCCTCCGCTTATTGATATGC	
EF1	ATGGGTAAGGAAGACAAGAC	O,Donnell *et al.*, 2000
EF2	GGAAGTACCAGTGATCATGTT	
EF3	GTAAGGAGGASAAGACTCACC	
EF2T	GGAAGTACCAGTGATCATGTT	
Btu-F-F01	CAGACCGGTCAGTGCGTAA	Watanabe *et al.*, 2011
Btu-F-R01	TTGGGGTCGAACATCTGCT	

5.3.2. Sequencing and phylogenetic analysis

The obtained sequences and the other sequences retrieved from the previous study or in GenBank were initially aligned with the CLUSTAL X program [31], were edited in BioEdit v 7.0.1 and were completed with manual adjustment. For the combined analysis, the genes were concatenated in a single nucleotide alignment. Maximum likelihood analysis was conducted using RAxML 7.2.8 HPC by employing the GTRGAMMA model of nucleotide substitution. The robustness of the phylogram in the maximum likelihood analyses was evaluated by 1000 bootstrap replications. The best tree obtained from this search was edited in Mega v 5.05 [32].

5.4. Analysis of mycotoxins

5.4.1. Fusarium inoculation for ELISA

Adlay seeds were dehulled and 10 g seeds were mixed with 5 ml sterilized distilled water in a 50 ml tube. Tubes were then autoclaved for 30 minutes at 121°C. After cooling, 5-6 mycelial plugs (6 mm) of 5 day cultured *Fusarium* species on PDA media were transferred to the tube. Tubes were incubated for 30 days at 15°C. Before toxin analysis, all the samples were dried in a dryer at 60°C until the weight of each sample to be reached 10 g again and then stored at -20°C prior to the toxin analysis.

Table 2. PCR condition for amplifying target genes used in this study

Gene	Initial denaturing	Denaturing	Annealing	Extension	Final extension	Cycle
ITS (ITS5, ITS4)	94℃, 10 m	94℃, 30 s	55℃, 30 s	72℃, 1 m	72℃, 10 m	30
EF (EF1, EF2)	94℃, 5 m	94℃, 30 s	52℃, 40 s	72℃, 1 m	72℃, 3 m	35
EF (EF3, EF2T)	94℃, 5 m	94℃, 30 s	53℃, 30 s	72℃, 1 m	72℃, 5 m	40
β-tubulin (BT2a, BT2b)	94℃, 5 m	94℃, 30 s	60℃, 30 s	72℃, 1 m	72℃, 3 m	35

5.4.2. Sample Preparation and Toxin analysis

To analyze and quantify levels of FUM and DON, Enzyme-linked immunosorbent assay (ELISA) was used in adlay. ELISA was performed using commercial kits- AgraQuant(R) Fumonisin Test Kit & AgraQuant(R) Deoxynivalenol Test Kit (Romer Labs GmbH, Tulln, Austria). Sample preparation and analysis were done according to manufacturer instructions written in the handbook. All samples were ground to a fine powder, so that over 70% of the powder passed through a 0.5mm mesh sieve. Prepared samples of 10 g were mixed with 50 ml of distilled water for FUM & DON and then homogenized in a Waring blender at high speed for 3 min. Extracts to be allowed to be filtered through Whatman filter paper No. 1 (Whatman, Maidstone, UK).

5.5. Morphological characterization of toxigenic Fusarium

Fusarium species, isolated from adlay seeds were grown on potato dextrose agar (PDA; Difco, Montreal, Canada) at 20°C in the dark to describe aerial mycelium and pigmentation. Colony diameters were measured after 7 days of incubation and other characteristics such as texture, color and pigmentation were also recorded. For describing conidial morphology, isolates were grown on synthetic nutrient-poor agar (SNA) beneath fluorescent lights (12/12 light/dark) at 20°C to induce sporulation. Randomly selected conidia (50) from 7-day-old cultures were used to obtain conidial measurements and photographed using an OLYMPUS BX50 light microscope (OLYMPUS, Tokyo, Japan) with an Artcam 300MI digital camera (ARTRAY, Tokyo, Japan). Morphological characteristics of the isolate were then compared with previous descriptions.

5.6. Statistical analysis

Data were analyzed using SAS Enterprise 4.3 (SAS Institute Inc., Cary, NC, USA) program and ANOVA (analysis of variance) was by DMRT (Duncan's Multiple Range Test, P=0.05, 0.01).

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Conflicts of Interest: "The authors declare no conflict of interest."

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