Fungal occurrence in physic nut *((Jatropha curcas)* seeds during storage and possibility aflatoxin production by *Aspergillus flavs* and *Aspergillus paraziticus* isolates.

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Abstract: The aims of the current study were to determine the seed quality of *J. curcas* of peeled and non peeled nut during storage and the capability of *Aspergillus flavus* and *Aspergillus paraziticus* isolates to produce aflatoxin. Seeds of peeled or non peeled nuts stored for one year and the recent collected seeds of non peeled nut were contaminated with fungi with an average of 37.8% on PDA medium and with an average of 48.9% on Czapek's agar medium. The percentage of fungal infection was higher in seeds stored for one year than in the seeds of the other two sources of non peeled nuts. The obtained results also showed that the number of fungi as cfu per seed was ranged from 0.8 to 6.7cfu/seed with an average of 2.93 and 3.3 cfu/seed on PDA and salt Czapek's agar media respectively. Twelve species which belong to seven genera were detected and they were classified as, *Alternaria tenuis* (11.03%), *A. flavus* (11.77%), *A. niger* (9.77%), *A. paraziticus* (2.05%), *A. terreus* (3.05%), *Fusarium* spp. (22.13%), *Mucor* spp. (6.20%), *Penicillium* spp. (11.03%), *Rhizopus* spp. (8.73%) and *Rhizoctonia solani* (11.87 %). Among the so called storage fungi, *Aspergillus* spp. was the most dominant fungi occurred in highest frequent (30.16%), beside the field fungi (damping –off, root rot and wilt pathogens), i.e., *Fusarium* spp and *Rhizoctonia solani* were also found in high frequencies. The current data showed that the highest percentage of contamination with *A. flavus* was detected in seeds of non peeled nut stored for one year (12.5%) followed by seeds of recent collected non peeled nut (12.1%), while *A. Paraziticus* were detected only in seeds of recently collected of non peeled nut by 6.1%. 62. Percentae of *A. flavus* isolates had a higher ability to produce aflatoxin B_1 with maximal level reached to 20.6µg /100ml, while 60.0% isolates of *A. paraziticus* were able to produce aflatoxin in lower level than *A. flavus*, where the maximum level reached to 17.2µg /100ml.
[Ahmed F. Sahab, Soher E. Aly، Lobna S. Nawar, and Sawsan Y. El-Faham. Fungal occurrence in physic nut (*Jatropha curcas*) seeds during storage and possibility aflatoxin production by *Aspergillus flavs* and *Aspergillus paraziticus* isolates. Journal of American Science 2011;7(5):511-516]. (ISSN: 1545-1003).
http://www.americanscience.org.

Keywords: physic nut, seedborne fungi, aflatoxin, biodiesel, frequency occurrence

1- Introduction

Physic nut (*Jatropha curcas* L.) globally known as jatropha belongs to the family Euphobiaceae. It is a large shrub or small tropical tree widely distributed in arid and semiarid areas. Jatropha is a main commodity source for biodiesel in several countries. The tree has only a few pest and disease problems. However, recently due to the expansion of this crop throughout the plant pathogens may be spreads through infected seeds. The mycological profiles of jatropha seeds have been studied. Neves *et al*. (2009) reported that *Fusarium* , *Rhizoctonia and Alternaria* spp. were the major fungi detected in the blotter test.While, Worang (2008) isolated sixteen fungal species from physic nut seeds (jatropha) during six months of storage. They also reported that at the beginning of storage , most of the fungi that infected the seeds were classified as field fungi such as *Colletotricum* spp. *Cladosporium* spp. and *Fusarium* spp. after three months of storage , the existence of field fungi was generally replaced by storage fungi, such as *Aspergillus* and *Penicillium* spp. dominate the population.

However, recently root rot caused by *Rhizoctonia bataticola* has been recorded as one of the most divesting disease of jatropha (Sharma and Kumar, 2009 and Kumar *et al*., 2011). In the same direction, Hedge *et al*. (2009) isolated *Sclerotium rolfsii* from seed and seedling of *J. curcas*.

It has been well established that fungal that fungal invasion of different agricultural commodities is occasionally associated with the development of mycotoxins. Along with this view, Lillard *et* al. (1970) isolated 71 cultures of *Aspergillus flavus* and *A. paraziticus* most of which produced aflatoxins *in-vitro*. Aflatoxins producing fungi have been isolated from cereal grains, wheat, oil seeds and nut products (Yu *et al*., 2004). *A. flavus* are natural contaminants of many feeds and raw materials for human and animal consumption. On a survey conducted during

four years, Martins (1987) found *A. flavus* in about 70.0% of feeds (1,103 samples), and in 42.6% of raw materials. Martins (1989) in a study about *in vitro* aflatoxin production by *Aspergillus flavus* (114 strains) isolated from raw materials and mixed feed found 60.5% toxigenic strains and the range of the aflatoxin B_1 was 0.004 to 391.9 mg/kg. Those aflatoxins producing fungi are widely distributed in nature and can grow over wide range environmental conditions (Holmquist *et al*. 1983). They are known to be hepatotoxic , carcinogenic and teratogenicto different species of animals (Yu *et al.,* 2002)

Therefore, it seems of importance to study the fungal flora of jatropha seeds and their percentage occurrence. The study also include the capability of *A. flavus* and *A. paraziticus* isolates isolated from jatropha seeds to produce aflatoxins.

2- Materials and Methods

1- Seeds of Jatropha nut samples

Jatropha nut samples were collected from environmental farm at Aloksour Governorat (five samples). The nut samples were tested for current studied for isolation of fungi from jatropha seeds and aflatoxin determination.

2- Recovery, identification and enumeration of the mycoflora

Fungi associated with seeds were isolated according to the Foor *et al*. (1976). Each five seeds were placed in 20 ml sterile container, then sterilized distilled water was added and left for 2 minutes, to allow seeds to sink. Water was drained off and 2.5% equal solution of sodium hypochlorite was added and left for 3 minutes. Drain of excess solution and immediately rinse in 3 changes sterilized distilled water. Drain off water, dried between two layer of sterilized filter papers. The seeds of 50 pods were plated on potato dextrose streptomycin agar medium (A.T.C.C., 1978) at rate of five seeds / dish. Plates were incubated at 28ºC± 2 for 5-8 days.

The fungal colonies were microscopic examined by observing the colonial morphology color of colony, texture, shape and surface appearance and cultural characteristic - a sexual and sexual reproductive structures like sporangia, conidial head, arthrospores, the vegetative mycelia, septate or non-septate. All fungal isolates were identified to the generic or species level according to Gilman (1957); Nelson *et al*. (1983) and Barnett and Hunter (1986).

The percentage of natural seeds infection, the total fugal counts and the frequency occurrence of different fungi associated seeds were determined.

3- Efficacy of isolates to produce Aflatoxin

3-1: *Chemicals:* aflatoxin B_1 (AFb_1) standers were purchased from sigma chemical Comp. (St. Louis, Mo). The purity was confirmed by capillary GC-Mass spectroscopy and UV spectrophotometry.

3-2: *Media used*: the cultures were cultivated on yeast extract sucrose broth. All chemicals used were in high purity.

3-3: *Preparation of spore suspension*: the pure isolated and identified of *A. flavus* and *A. paraziticus* were grown on slants of potato dextrose agar medium and incubated for 7 days at 28ºC± 2 .Spores were harvested by adding sterilizes water with few drops of Tween 80 solution (0.42% v/v) filtered through several layers of cheese cloth. The number of conidia was estimated by Hemacytometer and the suspension was adjusted to contain approximately 10^6/ ml.

3-4: *Culture preparation*: the ability of *A. flavus* and *A. paraziticus* to produce aflatoxin were tested on yeast extract sucrose broth (YES medium) A set of three flasks as replicates containing 100ml of YES broth were inoculated with 1ml of spore suspension (10^6 spores/ ml) and then incubated at 28ºC± 2 for 7 days in dark. The aflatoxin was determined in the culture broth as described in AOAC (1995).

3-5: *Aflatoxin determination*: aflatoxin was determined in the culture broth as described in AOAC (1995) using HPLC analyses according to the Hutchins and Hagier (1983). Water HPLC (EX. 365, EM, 450nm). Millennium soft ware programe was applied for calculation NOVA Pak C18 column (3.4x 150 mm 4µ). Mobile phase A: Acetonitrile : H_2O, 15: 85(v/v). Mobile phase B: (Methanol 100%)

3- Results and Discussions

1- Percentage of the natural fungal infection

It is clear from the data presented in Table(1) that seeds of peeled or non peeled nuts stored for one year and the recent collected seeds of non peeled nut were contaminated with fungi with an average of 37.80% on PDA medium and with an average of 48.9% on Czapek's agar medium.

The percentage of fungal infection was higher in seeds stored for one year than in the seeds of the other two sources of non peeled nuts, as the mean percentage of infection were 56.70, 26.70 and 46.65 in seeds stored for one year and seeds of non peeled nuts stored for one year or recently collected respectively.

Table 1. Percentage of fungal infection and fungal count (cfu/seed) of jatropha seeds recently collected or stored for one year on PDA and salt Czapek's agar media

Source	PDA medium		Czapek's agar medium		Mean of % infection
	% infection	Count of CFU/seed	% infection	Count of CFU/seed	
Seed stored for one year	46.7	4.7*	66.7	6.7	56.70
Seeds of non peeld nut stored for one year	26.7	0.8	26.7	0.6	26.70
recent seeds of non peeled nut	40.0	3.3	53.3	2.6	46.65
Average	37.80	2.93	48.90	3.3	

- Tests were rune in quadruplicate
- Counts represent the number of fungi / seed incubated at 28±2ºC for 7 days

On the other hand, seeds of non peeled nut stored for one year represented the lowest densities of contamination on the two tested media. Almost similar results were obtained by earlier finding by Worang (2008) and Neves (2009).

It is clear also from Table 1 that the number of fungi as cfu per seed was ranged from 0.8 to 6.7cfu/seed with an average of 2.93 and 3.3 cfu/seed on PDA and salt Czapek's agar media respectively. Moreover, seeds of non peeled nut showed low fungal densities on the two tested media than the corresponding figures of seed from peeled nut and stored for one year. As, the count were decreased on PDA medium from 4.7 to 0.8 cfu/seed (82.9% reduce) in seeds of peeled and non peeled stored for one year respectively. These results can explained by the finding of Worang (2008) who reported that at the beginning of storage, most of the fungi that infected the seeds were classified as field fungi, while after three months of storage , the existence of field fungi was generally replaced by storage fungi ,or may be due to the antifungal activity of jatropha seed oil on fungal growth.

2- Frequency occurrence of fungi infected jatropha seeds

The data of all samples per principle fungal genera or species given in Table (2) indicate that the surface contamination occurred within intact nuts of each sample. Great variation in types and in numbers of propagation s among samples was noted and many fungal isolates which found in seeds of recently collected nuts were absent in seeds of non peeled nuts stored for one year. Data also showed that fungi were much more abundant in seeds stored for one year due to apparently to the absence of cortex of the nut consequently infection with soil fungi were occurred. Twelve species which belong to seven genera were detected and they were classified as, *Alternaria tenuis* (11.03%), *Aspergillus flavus* (11.77%), *Aspergillus niger* (9.77%), *Aspergillus paraziticus* (2.05%), *Aspergillus terreus* (3.05%), *Fusarium* spp. (22.13%), *Mucor* spp. (6.20%), *Penicillium* spp. (11.03%), *Rhizopus* spp. (8.73%) and *Rhizoctonia solani* (11.87 %). The obtained results agree with Sharma and Komar (2011) and differed than results obtained by Hedge *et al*. (2009), they isolated *Sclerotium rolfsii* from seed and seedling of *J. curas*.

Depending upon their frequent of occurrence the genera and species were grouped as major and minor component include: *Fusarium* spp. (22.13%) and *Aspergillus* (30.16%), were the most frequent isolated fungi. Species of *Fusarium* and *Aspergillus* also found in high incidence in seeds of jatropha study by Melo *et al.* (2007) and Neves *et al.* (2009) which agrees with the obtained result here.

Among the so called storage fungi, *Aspergillus* spp. was the most dominant fungi occurred in highest frequent (30.16%). On the other hand, the field fungi (damping –off, root rot and wilt pathogens), i.e., *Fusarium* spp and *Rhizoctonia solani* were also found in high frequencies. This important plant pathogenic fungus is consistently found associated with seeds of different plant species and have the potential cause sever damage (Tanaka, 2001 and Neves *et a*l., 2009). Species of *Fusarium* have also found in association with seeds of caster bean (*Ricinus communities* L.) another important plant for production of biodesel and belonging to the family Euphrbiaceae, like jatrpha (Mariotti *et al.,* 1987).

Besides the potential of pathogenic fungi induce disease and cause damage, seed analyzed of different sources detected fungi that produce mycotoxins and the presence of such harmful substances is another major problem associated with seeds. As, the current data showed that the highest percentage of contamination with *Aspergillus flavus* was detected in seeds of non peeled nut stored for one year (12.5%) followed by seeds of recent collected non peeled nut (12.1%), while *A. Paraziticus* were

detected only in seeds of recently collected of non peeled nut by 6.1%. Mycotoxigenic species can be found in all major groups fungi, specially the genera *Aspergillus, Penicillium* and *Fusarium* (Scussel, 1998). Such fungi were found in the analysis made of the seeds which, although not edible may cause other types of toxicity to be handled.

Table 2. The frequency occurrence percentage of fungi isolated from jatropha seeds recently collected or stored for one year of peeled and non peeled nut on PDA medium

Fungi	Seeds stored for one year	Seeds of non peeled nut stored for one year	Recently collected seeds of non peeled nut	Mean %
Alternaria tenuis	14.9	-	18.2	11.03
Aspergillus flavus	10.7	12.5	12.1	11.77
Aspergillus niger	10.7	12.5	6.1	9.77
Aspergillus paraziticus	-	-	6.1	2.05
Aspergillus terreus	-	-	6.1	3.05
Aspergillus spp.	10.7	-	-	3.57
Fusarium spp.	10.7	37.5	18.2	22.13
Mucor spp.	-	12.5	6.1	6.20
Penicillium spp.	14.9	-	18.2	11.03
Rhizopus spp.	10.7	12.5	3.0	8.73
Rhizoctonia solani	17.0	12.5	6.1	11.87

* Tests were run in quadruplicate.

* Counts represent the number of fungi / seed incubated at 28±2ºC for 7 days

3- Efficacy of *A. flavus* and *A. paraziticus* to produce aflatoxin

The results in Tables 3 and 4 revealed that 5 out of 10 isolates of *A. flavus* had a higher ability to produce aflatoxin B_1 with maximal level reached to 20.6µg /100ml, while 6 out of 10 isolates of *A. paraziticus* were able to produce aflatoxin in lower level than *A. flavus*, where the maximum level reached to 17.2µg /100ml. Data also indicated that the ability of all positive isolates of *A. flavus* were in a wide range that the aflatoxin was produced in amount ranged between 3.3 to 20.6 µg /100ml. Results agree with Martins (1989) reported in a study about *in-vitro* aflatoxin production by *Aspergillus flavus* (114 strains) isolated from raw materials and mixed feed found 60.5% toxigenic strains and the range of the aflatoxin B_1 was 0.004 to 391.9 mg/kg. On the other hand, the aflatoxin B_1 was not detected in 5 of 10 (50%) and in 4 of 10 (60.%) of the total isolates of *A. flavus* and *A. paraziticus* respectively.

Moreover, data presented in Table (4) revealed that *A. flavus* isolate No. 7 was the higher producer compared to the other *A. flavus* isolates while, the lowest producer being isolate No. (4). On the other hand, *A. paraziticus* isolate No. 5 showed the higher aflatoxin production, whereas isolate No. 3 was the lowest producer.

Table 3 Efficacy of isolated *A. flavus* and A. paraziticus to produce aflatoxin B_1

Fungal species	No. of tested isolates	No. of positive isolates	Range (µg /100ml)
Aspergillus flavus	10	5 (50.0%)	3.3 – 20.6
Aspergillus paraziticus	10	6 (60.0%)	5 – 17.2

The variation in the amount of aflatoxin B_1 may be related to genetic criteria in the fungal species. These reasons were confirmed by the results obtained previously by Aly and Nader (2003) who found that 25% of *A. flavus* isolates have the ability to produce aflatoxin B_1 in amount ranged between 10 to 100 µg/Kg on corn culture. This discrepancy might be due to the variation in the origin of raw material, which play an important role in distribution of mycotoxin producing fungi (Llewellyn *et al*., 1981). Also, our results in good agreements with those reported by Lillard et al. (1970), Abbas *et al*. (2004) and Yu *et al.* (2004). In this concern, Martins *et al*. (2000) reported that both *A. flavus* and *A. paraziticus* showed a variation in aflatoxin production which mainly depend on abiotic and genetic parameters.

Table 4 Aflatoxin B_1 production in different *Asp. flavus* and *Asp. paraziticus* isolates (mean ± standard error).

Isolates No	*Asp. flavus*	*Asp. parasitcus*
1	-	-
2	8.17 ± 3.5	6.37 ± 0.55
3	10.7 ± 0.9	5.13 ± 0.46
4	6.87 ± 0.72	-
5	6.7 ± 3.26	17.17 ± 0.47
6	-	15.4 ± 0.42
7	20.67 ± 0.86	6.1 ± 0.38
8	-	-
9	-	-
10	-	6.73 ± 0.33

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5/1/2011