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Research Paper

FUNGI ASSOCIATED WITH SEEDS OF ASHFORD VARIETY OF GROUNDNUT GROWN IN YEMEN AND ITS DISINFECTION *IN VITRO* USING SODIUM HYPOCHLORITE

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Abstract

This present study was undertaken to study the seed-borne fungi of groundnut that attack the plants and reduce their yield in Yemen. The aim of the present study was to isolate and identify seed borne fungi associated with groundnut seeds and to assess the effect of surface disinfection time on percent of seed infection and germination, a single variety of groundnut "Ashford" obtained from agricultural research stations in Seiyun and Al-kod was used for study. Seed borne fungi associated with groundnut were detected using agar plate and blotter methods. Three disinfection times by using sodium hypochlorite (0 minute, 3 minute and 6 minute), were used in the study. Two hundred seed from each location of Ashford variety maintaining 20 replicates for each treatment in blotter and agar plate technique. The results of study showed four fungi species comprising, three genera namely *Aspergillus niger*, *Aspergillus flavus*, *Macrophomina phaseolina*, and *Rhizopus* sp were isolated from groundnut seed, the percentage frequency were (37%) for *A. niger*, followed by *A. flavus*, representing (34.5%), *M. phaseolina* (15%) and *Rhizopus* sp (11%) of total isolates by using agar plate method, and *A. niger* 36.5%, *A. flavus* 32.5% and *M. phaseolina* 21% by using blotter method. The study also indicate there was significant difference ($p \leq 0.05$) between disinfection time period for 6 minute, 0 minutes and 3 minutes where the disinfection time period for 6 minutes are more effective comparable with 0 minutes and 3 minutes, the percent of seed infection was reached 46.2% and 71.9% in blotter and agar plate method, and increase the seed germination percentage to 76% and 64% in blotter and agar plate method respectively.

Key words: Groundnut, Seed infection, Disinfection, Germination, Seed borne fungi.

INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is an important legume crop cultivated world over for food & its oil use. Being a legume, it is also valued for its nitrogen fixing capacity through the root nodule bacteria (Rhizobium). Groundnut is the 6th most important oilseed crop in the world. It contains 48-50% oil and 26-28% protein. It provides 12% recommended nutrients and has dietary fibres that reduce the risk of some kinds of cancer and helps control blood sugar. Among 13 essential vitamins, 7 are found in groundnut and among 20 minerals necessary for growth, 7 are present in groundnut (Rani, 2014).

Groundnut is grown on nearly 21.70 million ha with the production of 41.18 million tons and an average yield of 1667 kg/ha in 2012 (FAOSTAT, 2012). In Yemen groundnut is an important leguminous agricultural plant, it is cultivated for seeds. Groundnut available legume crop is cultivated over an area of 2357 hectares with a production of a bout 1678 kg per hectare. The crop is popularly grown in Southern and Eastern part of Yemen (Abyan and Wadi Hadhramout Governorates). (Jehlan, 2013). This important crop is attacked by a number of pathogenic fungi of economic importance. Seed- borne disease have been found to affect the growth and productively of crop plants. A seed-borne pathogen present externally or internally or associated with the seed as contaminate may cause seed abortion, seed rot, seed necrosis, reduction or elimination of germination capacity as well as seedling damage resulting in development of disease at later stages of growth by systemic or local infection (Sayed *et al.,* 2013). The seeds are found to be responsible for disease transmission because they carry a number of pathogens, which get associated either in the field or in the post harvest storage condition (Manimurugan, 2003)

Fungi like *Aspergillus niger, Aspergillus flavus, Alternaria dianthicola, Curvularia lunata, Curvularia pellescens, Fusarium oxysporum, Fusarium equiseti, Macrophomina phaseolina, Rhizopus stolonifer, Penicillium digitatum and Penicillium chrysogenum* causes discoloration, rotting, shrinking, seed necrosis, loss in germination capacity and toxification to oilseeds (Chavan and Kakde, 2008).

Ihejirka *et al*.(2005); Aliyu and Kutama, (2007); Hedayati *et al.,* (2010); Syed *et al.,* (2013) and Akinnibosun and Osawaru (2015), reported that *Fusarium spp*., *Aspergillus spp. Rhizopus spp, Mucor spp* and *Penicillium spp*., were the most abundant fungi encountered in groundnut seeds tested before harvest. Umechuruba (1986), Isolated *Macrophomina phaseolina*, *Penicillium spp.*, *Fusarium equiseti*, *F. solani*, *F. moniliforme var. subglutinans*, *F. sambicum*, *F. semitectum*, *F. moniliforme*, *Colletorichum dematium*, *Aspergillus niger* and *A flavus* from thirteen unshelled groundnut samples from Nigeria while Rasheed *et al* ., (2004), isolated *Aspergillus niger*, *Aspergillus flavus*, *Fusarium oxysporum*, *Fusarium semitectum*, *Macrophomina phaseolina*, *Rhizoctonia solani* from seven samples of groundnut were collected from different localities of Pakistan.

Surface disinfection for the purpose of determining the presence of internal fungi in grains or seeds gave variable and sometimes erroneous results. A wide range of surface disinfectants, such as ethanol, hydrogen peroxide, bromine water, mercuric chloride, silver nitrate, and antibiotics are used for surface sterilization; however sodium hypochlorite (commercial bleach) has been most widely used. Nakagawara, (1998) reported that the hypochlorite is known to be a very effective to killer of bacteria; even micro molar concentrations are enough to reduce bacterial populations significantly. Bloomfield and Arthur (1991) described the sodium hypochlorite is highly effective against all kinds of bacteria, fungi, and viruses. It kills microbes by oxidizing biological molecules such as proteins and nucleic acids.

The present study was conducted to detect and identify the seed borne fungi associated with Ashford cultivar seeds and to asses the effect of surface disinfection time on seed infection under in vitro condition

2. MATERIAL AND METHODS

2.1. Materials and experimental location area

Groundnut seeds with their pericarp removed of Ashford variety were screened to determine the seed-borne fungi associated with seed variety. These seeds were collected in July, 2014 from Seiyun Agricultural Research Station and Al-kod Agricultural research Station, the study was conducted in Faculty of Applied sciences laboratory, Hadhramout University.

2.2 Detection and identification of fungi

Seed samples were analyzed for the detection of seed-borne fungi by blotter method and agar plate method following International Rules for Seed Health Testing, the following two methods were used in this experiment. For proper identification of fungi temporary slides were prepared from the fungal colony and observed under compound microscope at 100 X and 400 X and

identified with the help of colony characteristics such as color and texture of mycelia and type of pigmentation. Microscopic characteristics of spores such as shape and color also used to identify the pathogens associated with the seed of Keys suggested by Malone and Muskette, (1964), Barnett and Hunter (1972), Singh *et al,* 1991, Larone, (2002) and Klich, (2002) .

2.2.1 Blotter method

The collected seed samples of groundnut variety of Ashford were analyzed for the presence of seed-fungal pathogens by blotter method following International Rules for Seed Testing (ISTA, 2001). Two hundred seeds were tested for each location of Ashford variety maintaining twenty replications, ten seeds were placed on three layers of moist filter paper were soaked in sterilized distilled water and place at the bottom of 9cm diameter Petri dish groundnut seed sample from each location were taken randomly and then placed on the moist filter paper at the rate of 10 seeds per dish. The Petri dish were then incubated at 25^0 C for 7 days under 12 hours alternating cycle of light at darkness, the seeds were examined under stereomicroscope for recording the seed borne fungal infection grown on the incubated seeds.

2.2.2 Agar plate method

In the agar plate method, two hundred seeds were tested for each maintaining 20 replications, surface disinfected seeds 1.5% hypochlorite sodium were plated on Potato Dextrose Agar (PDA) medium and the plated seeds were usually incubated for 5 – 7 days at 25^0 C±2 under cycles of light and darkness. At the end of the incubation period fungi growing out from the seeds on the agar medium were examined and identified.

Each seed in the blotter method and agar method was observed under stereo microscope in order to record the presence of fungal colony 7 days after incubation based on growth habit. In doubtful cases temporary slides were prepared from fungal colony and observed under compound microscope. The results were presented as percent incidence for individual pathogen germination of the seed was also recorded. Each individual incubated seed was observed under stereomicroscope at 16X and 25 X magnifications in order to record the incidence of seed-borne fungi. Pure cultures of individual fungal isolates were critically examined and identified fungi were identified based on growth characters on the incubated seeds. Fungi were identified based on gross colony morphology and microscopic characters. Colony identification was based on colony characters such as color and texture of mycelia and type of pigmentation. Microscopic characters of spores such as shape and color.

2.2.3 Data Collected

Fungal species found growing on the surface of seeds, Type and frequency of occurrence of identified fungal species was recorded. Percentage frequency (PF) of occurrence of fungal was calculated by using the following formula:

PF= (No. of seeds on which fungus appear / Total number of seeds) X 100

Percent of germination (PG) of seed varieties are determined as proportion of germinated seed over the total number of seed and computed by using the following formula:

PG= No. of seeds germination/ Total number of seeds) X 100.

2.2.4 Evaluation of surface disinfection time on seed infection.

A total of 200 groundnut seed were surface sterilized in 1.5% aqueous solution of sodium hypochlorite (NaOCl) at different time interval (O min, 3min and 6 min). Following this the seeds were rinsed in three change of sterile distilled water and then dried between two layers of soft paper. The treated seeds were placed in a Petri dish containing potato dextrose agar (PDA) media for agar plate method and also placed on the moist filter paper for blotter method, at the rate of 10 seed per Petri dish and then incubated for 7 days at25^0 C under usual day/night regime.

2.2.5 Statistical analysis

Data on percent of seed infection and seed germination were subject to analysis at variance (ANOVA) using Genstat statistical package, (1995). Mean were compared using Lest Significance Difference (LSD) at 5% probability level.

3. RESULTS AND DISCUSSION

3.1. Prevalence of Seed Borne Fungi on peanut

A total of four fungi species comprising three genera namely *Aspergillus flavus, Aspergillus niger, Macrophomina phaseolina,* and *Rhizopus sp.* were isolated from groundnut seed samples of Ashford variety collected from Seiyun agricultural research station and Al-kod agricultural research station (Table 1and Table 2). Four species were isolated by the agar plate method and three were isolated by the blotter method. Fungal specie which was not isolated by the blotter method was *Rhizopus sp.* the percentage frequency were (37%) for *A. niger*, followed by *A. flavus*, representing (34.5%), *M. phaseolina* (15%) and *Rhizopus* sp (11%) of total isolates by using agar plate method, and *A. niger* 36.5%, *A. flavus* 32.5% and *M. phaseolina* 21% by using blotter method.

Table 1Type and percentage frequency of seed-borne fungi associated with cultivar Ashford of groundnut seed on blotter method from Al-kod and Seiyun Agricultural Research Stations

Fungal species				
Location	*Aspergillus niger*	*Aspergillus flavus*	*Macrophomina phaseolina*	Mean
Al-kod	36.00	32.00	26.00	31.3
Seiyun	37.00	33.00	16.00	28.7
LSD 0.5	**12.08**			**6.98**
Mean	36.50	32.50	21.00	
LSD 0.5	**8.55**			

There are no significant different($p \leq 0.05$) between the frequency of fungi in agricultural research stations in Al-kod and Seiyun in blotter method, it reached 31.3% and 28.7% respectively while in agar plate method reached 24% and 24.75% respectively. Also there are significant difference at ($p \leq 0.05$) between type and frequency of fungi, were found that *A. niger* and *A. flavus* are more frequent compared to the rest of fungi, it reached 36.5% and 32.5% in blotter method and 37% and 34.5% in agar plate method.

Table 2 Type and percentage frequency of seed-borne fungi associated with cultivar Ashford of groundnut seed on agar plate method (PDA) from Al-kod and Seiyun Agricultural Research Stations

Fungal species					
Location	*Aspergillus niger*	*Aspergillus flavus*	*Macrophomina phaseolina*	*Rhizopus* sp	Mean
Al-kod	41.00	30.00	18.00	7.00	24.00
Seiyun	33.00	39.00	12.00	15.00	24.75
LSD 0.5	**11.87**				**5.93**
Mean	37.00	34.50	15.00	11.00	
LSD 0.5	**8.39**				

Results of the present investigation revealed that at least four important seed-borne fungal pathogens on groundnut seeds namely *A. niger*, *A. flavus* and *M. phaseolina* and *Rhizopus sp* in Yemen, Among the fungi *A. niger* and *A. flavus* was the most frequently isolated species in groundnut seeds on both agar plate and blotter method. Mukherjee *et al.,* (1992); Hedayati *et al*. (2010); Oladipupo, (2011); Ibiam and Egwu(2011); Abdulla, (2013); Syed *et al*., (2013); Akinnibosun and Osawaru, (2015) also found that *A.niger* and *A. flavus* were the predominant storage fungi of ground seed. Such similar reports have been made by Alemu, (2014) on soybean seed. *A. niger, M. phaseolina and Penicillium* have also been reported on groundnut seed (Jehlan, 2013). While Rasheed *et al.* (2004) found *M. phaseolina*, *Rhizoctonia solani*, *F. solani*, *F. oxysporum, A. niger* and *A. flavus* were predominant in groundnut and seed coat was greatly

infected by fungi followed by cotyledon and axis. Aliyu and Kutama, (2007) identified six fungal taxa, namely *Aspergillus*, *Rhizopus*, *Penicillium*, *Curvularia*, *Fusarium* and *Mucor* in groundnut under different storage conditions. Rosetto *et al*. (2005); Ihejirika *et al*., (2005) indicated that the fungi responsible for storage rot of groundnut were *A. flavus* and *A. niger*.

3.2. Influence of Disinfection Time on groundnut seed infection

The effect of different disinfection times with sodium hypochlorite1.5% (0 minute, 3 minute and 6 minute) on fungal seed contamination on both blotter and agar plate method are depicted in Table 3 and Table 4. The result showed that there are no significant differences in disinfection time period to reduce the seed-borne disease in both the agricultural research stations in Seiyun and Al-kod, where the percent of seed infection reducing(dropped) to 70.33% and 73.9% in blotter method and 79.47% and 81.4% in agar plate method respectively.

The result also revealed that time period of seed disinfection with sodium hypochlorite1.5% has significant effect ($p \leq 0.05$) on percent of seed infection, the highest seed infection was observed in disinfection time 0 minute (78.5% and 87.9%) on blotter and agar plate method respectively while the lowest seed infection was recorded in blotter and agar plate method containing seed disinfection with longer time for 6 minute (64.2% and 71.9%) respectively. Disinfection time period for 6 minutes more effective in reducing the seed-borne fungi compare to 0 minutes (78.5% and 87.9%) and 3 minutes (73.6% and 81.5%), in blotter and agar plate method respectively, the seed infection was reached 64.2% in blotter method and 71.9% in agar plate method, and can be recommended by using a disinfection time period for 6 minutes of sodium hypochlorite 1.5%.

Table 3 Influence of disinfection time on percent of seed infection using blotter method under laboratory conditions.

Agricultural Research Stations			
Disinfection time	Al-kod	Seiyun	Mean
0 minute	80.00	77.00	78.50
3 minute	75.00	72.20	73.63
6 minute	66.60	61.80	64.20
LSD 0.5	**4.12**		**2.91**
Mean	73.9	70.33	
LSD 0.5	**2.38**		

Table 4. Influence of disinfection time on percent of seed infection using agar plate method under laboratory conditions.

Agricultural Research Stations			
Disinfection time	Al-kod	Seiyun	Mean
0 minute	89.00	86.80	87.90
3 minute	83.00	80.00	81.50
6 minute	72.20	71.60	71.90
LSD 0.5	**3.77**		**2.67**
Mean	81.40	79.47	
LSD 0.5	**2.18**		

Hypochlorite is also routinely used as a sanitizer for domestic uses, as well as in food-processing plants to remove surface contaminants which can alter food quality or lead to food-borne diseases (Andrews, 1995).

In general, the observed increase in percent seed infection in agar and blotter method especially when short disinfection period time, this is due to the fact that short surface disinfection time, give rise (produce) saprophytic fungi to grow. On otherwise, the results showed that the disinfection time for a long time leads to a decline in the percent seed infection. This is due the fact that the ability of sodium hypochlorite to remove the saprophytic fungi of the surface of

seeds, it was reported that (Cram and fraedrich, 2010) disinfection by sodium hypochlorite can actually eliminate seed coat contamination of seed-borne fungi and can effective to reduce the percent of seed infection. Like wise, Dawar *et al.,* 2007 reported that surface disinfection reduces fungal contamination are associated externally. In this study the surface disinfection time didn't give favorable results, similar to the present study the work of Alemu, (2014) on soybean seed. This is due to the fact that report of (Souer and Burroughs, 1986), that the effectiveness of disinfection not only depends on duration but also depends on the PH, formulation and concentration of sodium hypochlorite solution.

3.3. Influence of Disinfection Time on Seed Germination

The influence of different disinfection times on seed germination on both blotter and agar plate method are depicted in (Table 5 and Table 6) The result showed that there are no significant difference ($p \leq 0.05$) for disinfection times period in the percentage of germination in both agricultural research stations in Seiyun and Al-kod, in blotter method the percentage of germination was reached 49.3% and 50.7% and in agar plate method reached 46.7% and 49.3%.

The result also revealed that time period of seed disinfection with sodium hypochlorite1.5% has significant effect ($p \leq 0.05$) on percent of seed germination percentage, the highest seed germination percentage been observed in disinfection time period for 6 minute 70.0% and 64.0% on blotter and agar plate method respectively. While the lowest seed germination percentage was recorded in blotter and agar plate method containing seed disinfection times with 0 minute 22.0% and 38.0% respectively. Disinfection time period for 6 minutes more effective in increasing the germination percentage compare to 0 minutes (22.0% and 38.0%) and 3 minutes (58.0% and 42.0%) in blotter method and agar plate method respectively, the percentage of germination was reached 70.0% in blotter method and 64.0% in agar plate method, and can be recommended by using a disinfection time period for 6 minutes of sodium hypochlorite 1.5%.

Table 5 Influence of disinfection time on percent of seed germination using blotter method under laboratory conditions

Agricultural Research Stations			
Disinfection time	Al-kod	Seiyun	Mean
0 minute	16.00	28.00	22.00
3 minute	60.00	56.00	58.00
6 minute	76.00	64.00	70.00
LSD 0.5	**30.31**		**21.44**
Mean	50.70	49.30	
LSD 0.5	**17.50**		

Table 6 Influence of disinfection time on percent of seed germination using agar plate method under laboratory conditions

Agricultural Research Stations			
Disinfection time	Al-kod	Seiyun	Mean
0 minute	44.00	32.00	38.00
3 minute	40.00	44.00	42.00
6 minute	64.00	64.00	64.00
LSD 0.5	**17.90**		**12.65**
Mean	49.30	46.70	
LSD 0.5	**10.33**		

Aspergillus species were predominant fungi on groundnut and those species were serious in reducing germination of seed and those species were reported to be responsible for a number of mycotoxins in groundnut. Mathur and Jorgensen, (1992); Bahattcharya and Raha, (2002);

Oladipupo, (2011), these studies agreement with present study that showed those fungi were associated with the pathological effect on groundnut seed, such seed decay, low percentage germination was as a result of infection of seed-borne fungi on those seeds and the presence of *Aspergillus* spp which are the predominate fungi observed suggest that it inhibited the growth of other fungi due to competition for infection. The Aspergillus species (*A. niger* and *A. flavus*) reduced the germination of seed and damage the seed in the storage (Christensen, 1973). In present study the germination rate of groundnut ranged from 22 to 70%% and from 38 to 64% in blotter and agar plate methods respectively. This result almost similar to Abdul *et al.,* (2009) reported that , pretreatment of seed with sodium hypochlorite significantly increase the rate of seed germination percentage the increase germination probably due to seed coat scratch seed hydration and aeration seeds.

CONCLUSION

The present study revealed at least three fungal genera were encountered in high percent frequencies of seed-borne fungal pathogen and infection percentage in samples of groundnut collected from Seiyun and Al-kod Agricultural Research stations. *Aspergillus*, *Macrophomina*, and *Rhizopus*. were the main fungi occurring frequently in groundnut seeds. Out of the fungus *Rhizopus* sp was recorded for the first time in agar plate method. Of the fungi isolated Aspergillus sp. is an important mycotoxins producer and produces aflatoxin B1 is the most toxic aflatoxin, being a potent genotoxic carcinogen in laboratory animals and there is strong evidence for its liver carcinogenity in humans (Varga *et al.,* 2009). Because the isolation of high percentage of *A. niger* and *A. flavus* from the samples in the present study, we recommended good storage conditions in order to reducing the mold growth and aflatoxin production in groundnut seeds. The presence of these fungi indicates a clear need for field surveys for these and other pathogens. Testing seeds health of major crops should introduce in the national seed quality control system.

The present study also revealed that different fungal pathogens are associated with groundnut seed and disinfection of seed with sodium hypochlorite had the potential for reduction of fungal inoculum which are mainly associated externally on the seed coat. It was also observed that, the duration of surface disinfection time can considerably affect quality the seed.

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