Journal of Biology, Agriculture and Healthcare
ISSN 2224-3208 (Paper) ISSN 2225-093X (Online)
Vol.4, No.25, 2014

Seed Borne Fungal Pathogen Associated with Soybean (*Glycine max* L.) and their Management in Jimma, Southwestern Ethiopia

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Abstract
The present study was amid to detect and identify seed borne fungi associated with soybean seeds and to assess the effect of surface disinfection time on percent of seed infection. A single variety of soybean "Clark" of two batches obtained from Jimma agriculture research center was used for the study. Seed borne fungi associated with soybean were detected using agar plate and blotter technique. Four disinfection times (0 minute, 2 minute, 4 minute and 6 minute) were tested in the study. A total of five fungi species comprising four genera namely Aspergillus flavus, Aspergillus niger, Fusarium spp., Penicillium sp. and Rhizopus spp. were isolated from soybean seed. Among which Aspergillus flavus and Aspergillus niger were found to be the most prevalent. The study also indicate that time of seed disinfection has significant effect ($p>0.05$) on percent of seed infection. The highest seed infection was observed in the un-disinfected seed lots while the lowest seed infection was recorded in the plate containing seed disinfected with longer time 6 minute. However the difference in disinfection time has no relation with the germination potential of the grain. Generally, the present study reviled that a wide range of pathogens are associated with soybean seed, and the time of surface disinfection can considerably affect the percent of seed infection. Moreover, extensive similar researches needs to be conducted to develop safe and effective management tools to tackle problems related with seed borne fungi.
Keywords: Soybean, Seed infection, Disinfection, Germination, Seed borne fungi

1. Introduction
Soybeans (*Glycine max* L.) serve as one of the most valuable crops in the world, not only as an oil seed crop and feed for livestock and aquaculture, but also as a good source of protein for the human diet and as a feedstock (Masuda and Goldsmith, 2009). The crop can be grown in tropical, sub- tropical as well as the temperate regions. It is a primary source of vegetable oil and protein concentrates. Soybean is an excellent source of major nutrients, about 40% of dry matter is protein and 20% fat (Lakshmeesha *et al*., 2013). Worldwide production of soybean was projected to be 250.39 million metric ton in 2009-10 (USDA 2009).

Even if soybean is recently domesticated crop, it has a great potential for Ethiopia as it has been dully recognized by many researchers and organization for its economic importance and its domestic demands for various uses. Production of this crop is indispensable in the country to enrich the stable cereal crop with sufficient and high quality protei9n in order to overcome the problem of malnutrition. However, as other grain legumes, soybean, are attacked by a wide range of diseases many of which are seed borne (Lakshmeesha *et al*., 2013).

Seed borne mycoflora are significant destroyers of foodstuffs and grains during storage rendering them unfit for human consumption by retarding their nutritive value and often by producing mycotoxins. Seed borne pathogens have been involved in seed rots during germination and seedling mortality leading to poor crop stand reduction in plant growth and productivity of crops (Kubiak and Korbas, 1999; Dawson and Bateman, 2001; Akranuchat *et al.,* 2007). Infected seeds play considerable role in the establishment of economically important plant diseases in the field resulting in heavy reduction of crop yields. Apart from this, infected seeds act as a vehicle in carrying pathogens to uninfected areas within a country and from one country to the other (Waller, 2002).

The detection and identification of microorganisms associated with seeds of the major crops in a country is the first and major step towards efficient seed health testing system. In Ethiopia, Some investigations have been made intermingle on soybean in various aspects for the past two decayed. But, the information on the seed borne fungi is meager. Thus, the present study was conducted to detect and identify the seed borne fungi associated with soybean seeds and to assess the effect of surface disinfection time on seed infection.

2. Material and Methods
2.1. Description of study Area
The study was conducted in Plant Pathology laboratory of Jimma University College of Agriculture and Veterinary Medicine (JUCAVM), during 2013 main cropping season.
2.2. Sample collection
Soybean seed sample of single Varity "Clark" was obtained from Jimma Agriculture Research Center and used

for the entire experiment. The seeds were of two batches *i.e*. of the year 2012 and 2013. The seed samples were brought to Plant Pathology Laboratory of Jimma University and stored at room temperature for subsequent studies.

2.3. 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 with some modifications.

2.3.1. Blotter method

The fungi associated with soybean seeds were detected by Blotter method following the International Rules for Seed Testing Association. In this method, three pieces of filter paper were soaked in sterilized distilled water and placed at the bottom of 9 cm diameter Petri dish. Soybean seeds sample from each batch were taken randomly and then placed on the moist filter paper at the rate of 10 seeds per Petri dish. The Petri dishes were then incubated at 25 $^{\circ}$C for seven days under 12 hour alternating cycle of light and darkness. After incubation, the seeds were examined under microscope for recording the seed borne fungal infections grown on the incubated seeds.

2.3.2. Agar plating method

Soybean seeds were surface sterilized in 1% aqueous solution of sodium hypochlorite (NaOCl) for four minute followed by rinsing 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, 10 seed per Petri dish, and then incubated for seven days at 25°C. Fungal pathogen associated with soybean seed were identified following sporulation.

2.3.3. Identification of fungi

Pure cultures of individual fungal isolates were critically examined and identified. Fungi were identified based on gross colony morphology and microscopic characters. Colony identification was based on 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 grain.

2.4. Evaluation of Surface Disinfection Time on Seed Infection

A total of 100 soybean seeds were surface sterilized in 1% aqueous solution of sodium hypochlorite (NaOCl) at different time interval (0min, 2 min, 4 min 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 seven days at 25°C under usual day/night regime. The experiment was arranged in Completely Randomize Design (CRD) with three replications.

2.5. Data Collected

Type and frequency of occurrence of identified fungal species was recorded. Frequency of occurrence of a particular pathogen was computed by dividing occurrence of the individual pathogen to the total population. Percent of seed infection is determined as the proportion of soybean seed showing any symptom of infection and calculated as:

$$\%\text{of seed infection} = \frac{\text{Infected seed}}{\text{total seed}} * 100$$

Percent of germination is determined as proportion of germinated seed over the total number of seed and computed using the following formula:-

$$\%\text{of seed germination} = \frac{\text{Germinated seed}}{\text{Total seed}} * 100$$

2.6. Statistical Analysis

Data on percent of seed infection and seed germination were subjected to analysis of variance (ANOVA) using SAS software. Mean were compared using List Significance Difference (LSD) at 5% probability level.

3. Results and Discussion

3.1. Prevalence of Seed Borne Fungi on Soybean

A total of five fungi species comprising four genera namely *Aspergillus flavus, Aspergillus niger, Fusarium spp.*, *Penicillium* sp. and *Rhizopus spp.* were isolated from soybean seed samples of Clark variety collected from Jimma agricultural research center (Table 1and Table 2). Five species were isolated by the agar plate method and four were isolated by the blotter method. Fungal specie which was not isolated by the blotter method was *Fusarium species.*

Aspergillus flavus occurred on 29.18% and 28.04% of the samples tested on blotter and agar plate method, respectively; while *Aspergillus niger* occurred in 23.81% of the samples tested on blotter method and 36.06% of the samples tested on agar plate method. Another important storage pathogen *Penicillium* sp occurred in 14.59% and 15.87% of the samples tested on blotter method and agar plate method, respectively.

Present study showed that saprophytic fungi viz., *A. flavus* and *A. niger* were predominant among the fungi

isolated on both agar plate and blotter method. Such similar reports have been made by Dawar & Ghaffar (1991) on sunflower seed, Rasheed *et al*., (2004) on groundnut seed. *A. flavus* and *A. niger* were the predominant storage fungi of soybean seed (Tariq *et al*., 2005). These species have been reported to reduce the germination of seed and damage the seeds in storage. It was also reported that *A. flavus* was the important mycotoxin producer and produce aflatoxin B1, B2, G1 and G2 which are hepatocarcinogenic. Mycotoxins can cause severe damage to the liver, kidneys and nervous system of man even in low dosages (Rodricks, 1976). There is therefore need for reducing the pathogenic fungi by treatment of seed for obtaining the good quality of seed and also reduce the mould fungi and mycotoxins production by improving the storage conditions.

Pensillium spp. and *Fusarim* spp. were isolated from the sample seed lots. Similar, to the present study, these fungi were reported to be seed borne in soybean by a number of other workers (Shovan *et al*., 2008; Lević *et al*., 2012). Fungi such as species of *Rhizopus spp.* occur in higher percentages on blotter method compared (13.94%) to that of the agar plat method (7.30%) this is due to the fact that seed which were placed on PDA had been subjected to surface sterilization with sodium hypochlorite, which reduces initial inoculum of the pathogen.

Other fungal species such as *Acremoniurn, A. alternata, Arthobotrys, Ascochyta pinodella, Cercospora kikuchi, Cheatomium, Cheatophoma, Cladosporium, Colletorichum truncatuin C. dematium, Diaporthe, Diplodia, Egicoccum, Monilia, Myrothecium, Nigrospora, Phomopsis, Pythium, Botrytis, Sclerotinia, Septocylindricum, Sphaceloma, Tricothecium*, which were found as frequent contaminants of soybean seeds (Begum *et al*., 2008; Ahammed *et al*., 2006) were not reported in the present studies. Generally, in comparing the two isolation procedures, it was found that more isolates were obtained from the agar plate method; this is expected, since PDA is more sensitive in detecting even traces of infection. Khattak *et al*. (1993) reported that, the percentage of mycoflora isolated by agar plate was higher than blotter method in soybean. Solanke *et al*. (1997) also stated that agar plate method yield more mycoflora than blotter method.

3.2. Effect of Disinfection Time on Grain Quality

The effect of different disinfection times (0 minute, 2 minute, 4 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 reveled that time of seed disinfection has significant effect ($p < 0.05$) on percent of seed infection. The highest seed infection was observed in seed lots without disinfection while the lowest seed infection was recorded in the plate containing seed disinfected with longer time 6 minutes; indicating that disinfection time has a significant effect on fungal pathogen associated with seed. However the difference in disinfection time has no relation with the germination potential of the grain

In the 2004 seed sample percent of seed infection was reached 100% and 53.33% on plate which contained the untreated seed lots, on agar plate and blotter method respectively, which are statistically not significant with seeds which are disinfected for 2 minutes (Table 3 and 4). This level was reduced significantly to 76.67% and 40.00% on agar plate and blotter method respectively, by increasing the disinfection time to 6 minute. Similarly in 2005 seed sample present of seed infection reached 93.33 % and 50% on agar plate and blotter method respectively on plate which contained the untreated seed lots. This level was reduced significantly to 73.33% and 36.67% by increasing the disinfection time. Generally, high seed infection were observed in plate containing seed which has short disinfection time, this might be due to the fact that the short time of surface disinfection give rise saprophytic organism to grow. On the other hand, the petri plate containing seed sample disinfected for long time showed relatively low percent of seed infection. This is due to the fact that, the NaOCl will wash those saprophytic organisms which are attached on the surface of the seed. It was also reported that disinfection of the seed lot with sodium hypochlorite can virtually eliminate seed coat contamination of seeds by seed borne fungi and can be effective for reduce percent of seed infection (Cram and Fraedrich, 2010). Similarly, Dawar *et al*. (2007) also reported that surface disinfection reduces fungal contamination mainly which are associated externally. In the present study the surface disinfection time didn't give satisfactory results. This is due to the fact that the effectiveness of surface disinfection depends not only on the duration but also depends on the pH, formulation and concentration of the hypochlorite solution (Sauer and Burroughs, 1986).

3.3. Effect of Disinification Time on Seed Germination

The effect of different disinfection times on seed germination on both blotter and agar plate method are depicted in figure 1 and figure 2, respectively. The result reviled that there is no significant difference among treatments receiving different disinfection time on seed germination. Indicating, disinfection time has no impact on seed germination of soybean seed. In seed sample taken form 2004 batch the germination rate of soybean ranged from 43.33 to 53.33% and from 43.33 to 50% in blotter and agar plate methods respectively. Similarly in the seed sample taken for 2005 the germination rate of soybean seed ranged from 66.67 to 76.66% on blotter method and it ranges from 53.33 to 63.33% in agar plate method.

Unlike the present study, Abdul *et al*. (2009) reported that, pretreatment of seeds with NaOCl significantly increase the rate of seed germination. The enhanced germination probably due to seed coat scarification leading to increased seed hydration and aeration (Yildiz & Er, 2002). However, the concentrations of the disinfectant the environmental condition prevail up on treatment have great influence on their efficacy. Despite the above facts

that various techniques such as application of various chemicals, seed priming and brief seed pretreatment with various chemicals can used to improve seed germination (Ashraf & Foolad, 2005). In the present study increase disinfection time doesn't increase the rate of seed germination these may be due to the fact that the concentration of the disinfectant agent (NaOCl) is low to create significant difference in germination potential.

4. Conclusion

The present study reviled that different fungal pathogens are associated with soybean 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. However, further extensive similar research needs to be conducted to identify other major pathogenic fungi associated with soybean seed across different location to come with tangible recommendation to develop appropriate management tool to reduce the losses due to seed borne fungi.

Acknowledgment

The author is grateful to Jimma University College of Agriculture and Veterinary Medicine for providing laboratory facility. The author would like to extend his heart-felt thanks to Dr. Girma Adugna for his technical support throughout the study.

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Table 1. Type and frequency of fungi associated with soybean seed on blotter method

Fungal species	Seed batch		Average
	2012	2013	
Aspergillus flavous	26.92*	29.15	28.04
Aspergillus niger	34.62	37.50	36.06
Fusarium spp	0.00	0.00	0.00
Pencillium spp	19.23	12.50	15.87
Rhizopus spp	15.38	12.5	13.94
Unidentified	3.85	8.33	6.09
Total			**100**

*Percentage frequency of fungi computed as individual isolates divided by total isolates

Table 2. Type and frequency of fungi associated with soybean seed on agar plate method

Fungal species	Seed batch		Average
	2012	2013	
Aspergillus flavous	27.59*	30.77	29.18
Aspergillus niger	24.14	26.92	23.81
Fusarium spp	13.79	11.54	12.67
Pencillium spp	17.24	15.38	14.59
Rhizopus spp	6.90	7.69	7.30
Unidentified	10.34	7.69	9.02
Total			**100.00**

*Percentage frequency of fungi computed as individual isolates divided by total isolates

Table 2: Effect of disinfection time on percent of seed infection using blotter method

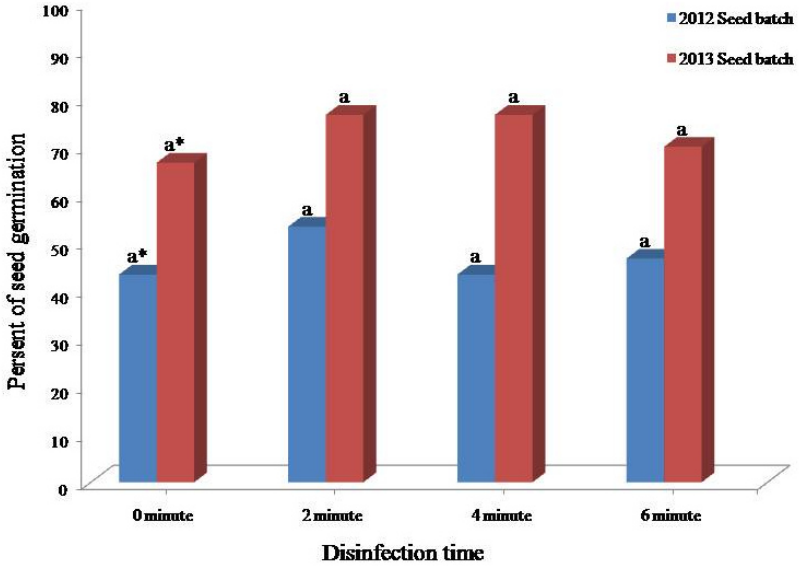
Disinfection time	Seed batch	
	2012	2013
0 minute	53.33^{a*}	50.00^{a}
2 minute	50.00^{ab}	46.67^{ab}
4 minute	43.33^{bc}	36.67^{b}
6 minute	40.00^{c}	36.67^{b}
LSD	7.69	13.31
CV	8.75	16.64

*Values are mean of three replications
Means followed by the same letter are not significantly different ($P < 0.05$) with in lots

Table 3: Effect of disinfection time on percent of seed infection using agar plate method

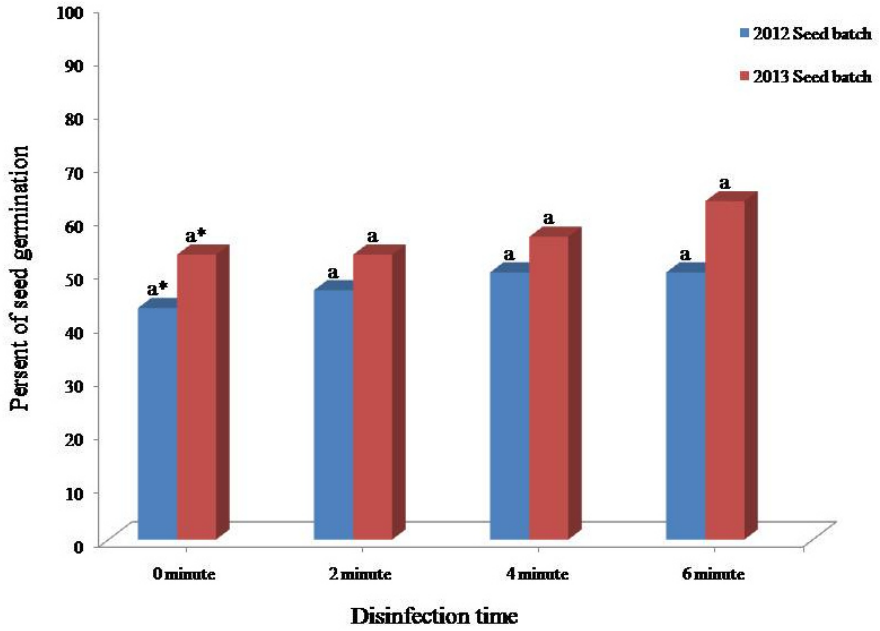
Disinfection time	Seed batch	
	2012	2013
0 minute	100.0^{a*}	93.33^{a}
2 minute	90.00^{ab}	86.67^{a}
4 minute	86.67^{bc}	73.33^{b}
6 minute	76.67^{c}	73.33^{b}
LSD	12.15	10.87
CV	7.31	7.07

*Values are mean of three replications
Means followed by the same letter are not significantly different ($P < 0.05$) with in lots



Values are mean of three replications
* Means followed by the same letter are not significantly different (P < 0.05)

Figure 1. Effect of disinfection time on percent of seed germination using blotter method



Values are mean of three replications
* Means followed by the same letter are not significantly different (P < 0.05)

Figure 2. Effect of disinfection time on percent of seed germination using agar plate method

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