ASSESSMENT OF SEEDBORNE MYCOFLORA OF SUNFLOWER (*Helianthus annuus* L.)

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DHAKA-1207

DECEMBER, 2021

ASSESSMENT OF SEEDBORNE MYCOFLORA OF SUNFLOWER (*Helianthus annuus* L.)

BY

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Registration No. 19-10291

A Thesis
Submitted to the faculty of Agriculture
Sher-e-Bangla Agricultural University, Dhaka,
in partial fulfillment of the requirements
for the Degree of

MASTER OF SCIENCE
IN
PLANT PATHOLOGY

SEMESTER: July-December, 2021

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CERTIFICATE

This is to certify that the thesis entitled, **"ASSESSMENT OF SEEDBORNE MYCOFLORA OF SUNFLOWER (*Helianthus annuus* L.)"** *submitted to the Department of Plant Pathology, Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirement for the degree of* **MASTER OF SCIENCE IN PLANT PATHOLOGY** *embodies the results of a piece of bona fide researchwork carried out by bearing Registration No.* **19-10291** *under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma, elsewhere in the country or abroad.*

I further certify that such help or sources of information, as have been availed of during the course of this investigation has duly been acknowledged.

Dated:

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DEDICATED TO MY BELOVED PARENTS

ACKNOWLEDGEMENTS

All praises to Almighty ALLAH who kindly enabled the author to complete the research work and prepare this thesis successfully.

The author humbly takes this opportunity to place his deep sense of gratitude to her supervisor ***Prof. Dr. Fatema Begum****, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, for her scholastic guidance, valuable suggestions, constant encouragement, affection, immeasurable help and constructive criticism during the entire period of research work and preparation of the thesis.*

The author equally and deeply indebted to his co-supervisor, ***Prof. Dr. Khadija Akhter****, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, for her kind co-operation, cordial suggestions, constructive criticisms and valuable advice to complete the thesis.*

The author would like to express his greatfulness to the Chairman, ***Prof. Abu Noman Faruq Ahmmed*** *and other respected teachers,* ***Prof. Dr. Md. Rafiqul Islam****, Prof. Dr. M. Salahuddin M. Chowhury, Prof. Dr. Nazneen Sultana, Prof. Dr. F.M. Aminuzzaman and Prof. Dr. Md. Belal Hossain, Department of Plant Pathology, Faculty of Agriculture, Sher-e–Bangla Agricultural University, for their valuable suggestion, direct and indirect advices, encouragement and co-operation during the whole period of the study.*

The author would like to thank the office staff of the Department of Plant Pathology and the Farm Division of SAU for their cooperation and help to complete this research work.

The author recalls his beloved friends ***Rifat ara Sultana Bindu*** *,* ***Md. Moudud Ahmod****,* ***Atkiya Rahman Mithi, Tohidur Rahman*** *and* ***Golam Sharowar*** *for their great support, help and encouragement to complete this study with pleasure.*

The author deeply expresses his heartfelt respect and gratitude to his beloved father Md. Shahidul Islam, mother Mst. Salina Begum and elder brother Md. Salim Reza whose everlasting love, unfading faith, continuous inspiration, well wishes and blessings kept him enthusiastic throughout his life and molded him to the current position without which this work could not be completed.

The author

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ABSTRACT

An experiment was conducted in Dr. M. A Wazed Miah Central laboratory, Department of Plant Pathology, Faculty of Agriculture, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207 during the period from June' 2020 to December' 2020 to detect, identify and isolating associated mycoflora in three varieties of sunflower for fullfilling the objectives with five methods viz., Blotter paper pethod, Deep freezing method, Washing test, PDA media method and Water agar plate method were used. The experiment was laid out in Completely Randomized Design (CRD) with three replications for all methods. The species composition, percentage of seed infection and seeds germination percentage differed among cultivars as well as method. A total of 9 different fungal species belonging to six genera were detected from sunflower seed through five different methods. Among them BARI Sunflower 3 showed the highest infection in washing test compared to other methods. A number of fungi isolated in the present study specially those in the genera viz. *Alternaria sp, Curvularia sp, Aspergillus sp, Fusarium sp, Rhizopus sp, and Stemphyllum sp* are known to be potent mycoflora producers. Among the fungi detected, frequency of *Alternaria alternata* was found significantly the highest in the all methods. The highest percentage of germination was found in BARI Sunflower 3 (86.67%) by Blotter paper method. The highest percentage of infection was found in BARI Sunflower 3 (73.33%) in both Blotter and Washing test and the percentage of dead seed after incubation was least in BARI Sunflower 3 (13.33%) by Blotter paper method. Of the five methods tested for detection of seedborne mycoflora, Blotter paper, PDA media, and Washing test were highly effective for mycoflora study.

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ABBREVIATION AND ACRONYMS

SAU	Sher-e-Bangla Agricultural University
BBS	Bangladesh Bureau of Statistics
USDA	United States Department of Agriculture
WHO	World Health Organization
FAO	Food and Agriculture Organization
MS	Master of Science
CRD	Completely Randomized Design
et al.	And others
Viz.	Namely
e.g.	exempli gratia (L), for example
etc.,	Etcetera
i.e.	id est (L), that is
%	Percentage
°C	Degree Celsius
mg	Milligram
l	Liter
cm	Centimeter
ml	Milliliter
g	Gram (s)
Kg	Kilogram (s)
LSD	Least Significance Difference
No.	Number
PDA	Potato Dextrose Agar
SB	Standard Blotter
DFB	Deep Freezing Blotter

CHAPTER I

INTRODUCTION

Sunflower (*Helianthus annuus* L.), is an herbaceous annual plant, an important member of the family Asteraceae and is one of the major oilseed crops ranked fourth among vegetable oil seed production mainly grown for edible oil in worldwide. The genus Helianthus is named from the Greek word “helios” meaning sun and “anthos” meaning flower (Kindscher, 1987). Although the center of origin of the Sunflower is thought to be in the southern USA and Mexico (Heiser, 1951), it is extensively grown in Ukraine, Russia, Argentina, Romania, China, Turkey, Bulgaria, Hungary, France, USA, Spain, and India. The largest suppliers of sunflower seeds in the world market are the European Union, Russia, Ukraine, Argentina, the United States, China, India and Turkey. Sunflower oil contributes to about 13% of the world's edible oil production with high value (Gabagambi *et al*., 2010). In 1969, the sunflower (*Helianthus annuus* L.) was first planted as an oilseed plant in India.

In Bangladesh, Sunflower is being cultivated as an oil seed crop since 1975 (Islam *et al.,* 2004). It can be a profitable first crop in double or multiple cropping systems (Sheaffer *et al*., 1977) as it can be cultivated at a wide range of geographical conditions. The plant can be cultivated both as an irrigated or rain-fed crop in different areas. Now-a-days special emphasis has been given to extending sunflower production in saline and char regions in the country. The plant can be harvested in 90-110 days and in 2018-19, Bangladesh produced about 1975 metric tons of sunflower from 1290 ha of land (BBS, 2020).

These sunflower crops are of two types based on the objectives of cultivation and use. One type is grown for the edible seeds, while the other- which is the majority farmed is grown for the oil. Its seed is highly nutritious containing about 20% protein and 40 to 50% vegetable oil associated with a very high calorific value. The Sunflower edible seeds have a mild, nutty flavor and a firm but tender texture. Those are often roasted to enhance the flavor, though these can also buy them raw.

They’re rich in healthy fats, beneficial plant compounds, several vitamins and minerals which play a role in reducing the risk of common health problems, including heart disease and type 2 diabetes.

Additionally, sunflower edible seeds are a good source of phenolic acids and flavonoids which also function as antioxidants. The oil obtained from sunflower seeds contain about 45–50%. The oil is healthy because of light and odorless characteristics with an ample amount of vitamin E especially recommended for heart patients due to its high quality and non-cholesterol properties. It is an excellent source of unsaturated fats (60 to 73% linoleic acid), with a sufficient amount of calcium, iron, and vitamins like A, B, E, and K (Gosal *et al*., 1988). Besides, it also contains crude protein, fiber and important nutrients like selenium, copper, and zinc (Weiss, 2000; Gonzalez-Matute *et al*., 2002).

As a source of high-quality edible oil, sunflower oil is used in cooking in different food preparations (Joksimovic *et al*., 2006). Besides this the oil can be used in the production of vanaspati ghee, soap and cosmetics. The plant of sunflower is not only a source of food and energy but also has phytoremediation potential (Mukhtar *et al.,* 2010) feed. It can also be used as manure (Agy *et al*., 2013) and around 25% protein-containing meal can be used for animal preparation. This sunflower species is also used as wild bird food, in some industrial applications, and as ornamental in domestic gardens.

However, these high-value crops are infected by many diseases caused by various types of pathogen like fungi, bacteria, and viruses. It is estimated that mold or fungal development known as mycoflora affects 25% of the world’s crop. The sunflower plant can harbour more than 30 types of the pathogen (the majority are fungi) which can reduce the yield and quality of oil by adversely affecting normal physiological processes (Gulya *et al*., 1994).

Some of the important reported fungal diseases of sunflower are Alternaria leaf blight (*Alternaria sp*.), Powdery mildew (*Erysiphe cichoracearum*), Downy mildew (*Plasmopara halstedii*), Rust (*Puccinia helianthi*), Root rot (*Macrophomina phaseolina*), Collar rot (*Sclerotium rolfsii*), Head rot (*Rhizopus*

sp.), Verticillium wilt (*Verticillium dahliae*) and Leaf spot (*Helminthosporium helianthi*) etc.

Notable many of these diseases are seed-borne whereas the healthy and pathogen-free seeds are the prerequisite for better crop production as seed is the most important input. Pathogen-free healthy seeds are essential for desired plant populations and a good harvest. Seeds are the carrier of disease dissemination that gets associated in the fields or at storage conditions (Begum *et al*., 2010).

Moreover, saprophytic fungi increase free fatty acid in seeds lowering the oil quality and thus reducing the economic value of sunflower seed (Singh and Prasad, 1977; Vijayalakshmi and Rao, 1986) or produce mycotoxins (Abdel-Malek *et al*., 1994, Abdullah and Al-Mosawi., 2010).

The different extensive study of sunflower seed confirms presence of *Alternaria alternata, A. helianthi, Aspergillus flavus, A. niger, A. fumigatus, Curvularia lunata, Fusarium moniliforme, F. solani, Drechslera tetramera, Cladosporium spp., Penicillium citrinum, Macrophomina phaseolina, Mucor mucedo* and *Rhizopus nigricans* etc., in the seeds (Vijayalakshmi and Rao, 1985; Kaur *et al*., 1990; Reddy 1993; Nahar *et al*., 2005; Afzal *et al*., 2010).

Generally the seed borne fungi manipulate biochemical changes in grain, reduce oil, carbohydrate and protein content, most importantly reduce the germination (Ijaz *et al*., 2001). Fat and protein contents were reported to reduced by *A. flavus, A. terreus, A. nigar, A. fumigates, A. versicolor* (Chavan, 2011). *F. equiseti, F. oxysporum* and *Curvularia lunata* were reported to reduce sugar content of sunflower seed (Chavan, 2011). The fungus under *Fusarium* sp. produces aflatoxin has devastative impact on seed germination and health (Ozcelik *et al*., 1990; Frisvad and Thrane, 2004).

The detection and study of mycoflora in seed lot is very important for maintaining proper seed health, germination, seed properties, previous inference about which seed borne disease may prevails in the cultivating crops. Analysis of seed infection level is a valid investigation tool to foresee the disease development transmitted by seeds (Taylor *et al*., 2001).

Several methods have been developed to detect the seed borne mycoflora in seed lots (Begum and Momin, 2000; Boughalleb and El Mahjoub, 2006; Kunwar *et al*., 1986; Krishnappa and Shetty, 1990; Moore, 1984; Neergaard, 1977; Raut, 1987; Sadashivaiah *et al*., 1986; Shahda *et al*., 1995;). The emphasis has been on methods, which are simple, easy, economic, sensitive, reproducible and efficient, but some methods such blotter and deep freezing suppress seed germination (Limonard, 1968).

However, studies are limited on seed-borne diseases of Sunflower in Bangladesh. The seed borne fungal diseases are transmitted by seeds, where the fungi can survive as conidia or mycelia on the seed coat or surface (Champion, 1997; Gargouri *et al*., 2000). The study of mycoflora of popular Sunflower variety in Bangladesh using different isolation methods can be fruitfull for revealing insight about the mycoflora associated with seed borne diseases those can be threated to production. If this research method become succeed the poor farmers of the country will get advantages and the production will be increased, consequently will increase the national economy of our country.

Considering the above facts and points, this research work was designed to achieve the following specific objectives:

- To evaluate different methods for seed health test of sunflower seed and
- To determine the frequency of their occurrence.

CHAPTER II

REVIEW OF LITERATURE

Sunflower (*Helianthus annus* L.) is well known as a flower as well as a promising oil seed crop in Bangladesh. It plays an important role in oil production through out the world. The oil, produced, are more beneficial to health than other type of oil.

2.1 Nutritional value of sunflower seed

There is high demand for sunflower because its oil is good for health as it contains low cholesterol. It contains 60- 73% linoleic acid with a sufficient amount of calcium, iron and vitamins like A, B, E and K. Sunflower seed is the source of high-quality oil (45-52%) have a higher content of polyunsaturated fatty acid. It is one of the most widely studied plants for heavy metal phytoremediation (Kara *et al*., 2013).

However, it is well known that sunflower can contain, degrade or eliminate metals (Chen *et al*., 2012; Ker and Charest, 2010; Lee and Yang, 2010), polycyclic aromatic hydrocarbons (Tejeda-Agredano *et al*., 2013) and polychlorinated biphenyls (Fiebig *et al*., 1997) from soil or water. Sunflower species are allelopathic in nature; as well as cultivated sunflower has great allelopathic potential and inhibits weed-seedling growth of velvet leaf, thorn apple, morning glory, wild mustard and other weeds (Macías *et al*., 1998).

Farmers are cultivating sunflowers as an adaptation practice of climate change in the coastal region of Bangladesh. Due to its larger adaptation capability and higher oil quality, sunflower can be grown almost in all regions of the world with high seed yield and oil content (Sencar *et al*., 1991). One kg of sunflower seeds yields 500 to 600 grams of oil, which is more than that of any other oilseeds. It also contains good quality protein (19 to 25%) in seeds (Gosal *et al.,* 1988).

2.2. Fungal mycoflora associated with sunflower Seed

The most important input material for crop production is seed, which is deliberately infected by various pathogens. Among the pathogen, fungi or mycoflora infect the seed most frequently.

Ingle, (2020) isolated total thirteen species belongs to six genera of fungi from groundnut seed name *Aspergillus niger, Aspergillus flavus, Aspergillus terreus, Aspergillus Fumigatus, Aspergillus nidulans, Aspergillus versicolor, Aspergillus wentii, Aspergillus humicola, Rhizopus stolonifera, Curvalaria lunata, Penicillium* sp, *Fusarium moniliforme, Mucor* sp.

Erdenetsogt *et al*., (2019) isolated a total of 7 fungal species from two wheat cultivar including *Fusarium sp., A.alternata, A.niger, A.candidus, T.laevis, T.tritici, Penicillium sp.*

Khani *et al*., (2019) isolated a total of 10 different fungal species belonging to different genera from peas seeds name *Alternaria alternata, A. flavus, A. fumigates, A. niger, Curvularia lunata, F. oxysporum, Penicillium, Rhizopus stolonifera, Stemphylium and Trichoderma viride*.

The previous studies at different parts of the world on mycoflora of sunflower showed varied result, especially differ based on technique used for the isolation. Irshad *et al*. (2017) detected a total of 12 genera of fungi including; *Alternaria alternata, Aspergillus flavus, A. niger, Cladosporium* sp., *Stemphylium helianthi, Penicillium* sp., *Fusarium oxysporum, Mucor* sp., *F. moniliforme, F. solani, Rhizocotonia solani and Rhizopus* sp.

Ghoneem *et al*., (2014) isolated 20 genera and 31 species of fungi from Sunflower seed, of which most abundant mycflora were *Alternaria alternata, Aspergillus flavus, Rhizopus stolonifer, Penicillium* sp. *and Cladosporium* sp.

Ramesh *et al.*, (2013) isolated 11 fungi name *Macrophomina phaseolina, Fusarium oxysporum, Aspergillus flavus, A. niger, Phoma* spp., *Sclerotinia sclerotiorum, F. solani, F. moniliformae, Rhizophus* spp., *Botrytis cinerea and Cercospora Kikuchi* from soybean in kerala.

Islam *et al*., (2013) studied mesta seeds and detected nine different fungi using standard blotter method. The fungi were *Macrophomina phaseolina*, *Botryodiplodia theobromae*, *Colletotrichum corchori, Curvularia lunata*, *Fusarium* spp., *Aspergillus favus, A. niger, Penicillium* spp. *and Alternaria* spp.

In 2012 Pushpavathi *et al*. analyzed the seed mycoflora of 12 safflower cultivars and showed the association of 10 fungal species among them *Alternaria carthami* (2-54%) was detected by all the three methods.

The mycoflora of sundried okra (*Abelmoschus esculentus*) was studied by Fagbohun & Faleye, (2012) and they reported the association of six fungi with the seeds, namely: *Rhizopus* sp., *Mucor* sp., *Aspergillus niger*, *Aspergillus flavus* and *Neurospora crassa*.

Afzal *et al*., (2010) isolated fungi associated with seeds of seven cultivars of sunflower by using agar and blotter paper methods. A total of 13 phytopathogenic fungal species including *Alternaria alternata, A. helianthi, Aspergillus flavus, A. fumigatus, A. niger, Curvularia lunata, Drechslera tetramera, Fusarium solani, F. moniliforme, Macrophomina phaseolina, Mucor mucedo, Penicillium and Rhizopus* sp. were identified.

Abdullah and Al-Mosawi, (2010) identified sunflower seeds associated 48 species of fungi belonging to 19 genera by studying 9 variety of Sunflower. Among these species of mycoflora *Aspergillus niger, A. flavus, Chaetomium globosum, Alternaria alternata, A. fumigatus, C. atrobrunneum, A. terreus, Penicillium*

expansum, P. brevicompactum, Fusarium oxysporum, F. solani, Rhizopus stolonifer, Mucor hiemalis and *A. ochraceus* were the most frequent

A study was conducted on seed borne mycoflora in Maize. A total number of 56 species belonging to 23 genera of fungi from maize seed was isolated by using blotter, agar plate and deep-freezing methods as recommended by ISTA. Among these species of mycoflora about 70% of the samples were infested with *Aspergillus flavus, A. niger, A. wentii* and *Penicillium* spp. (Niaz & Dawar, 2009).

Prasad *et al*., (2008) studied seed borne nature of *Alternaria carthami* in safflower and found maximum infection at the seed coat (76.6%) followed by endosperm (38.3%) and embryo (20.4%).

Sultana and Ghaffar, (2007) isolated 15 genera and 29 fungal species from bitter gourd seeds in Pakistan using ISTA techniques. Several seed borne fungi prevail on cucurbits including: *Botryodiplodia theobromae, A. alternata, C. lunata, Chaetomium spp., D. tetramera, F. equiseti, F. solani* and *F. moniliforme* on gourd seeds and on squash, watermelon, bitter gourd, muskmelon and cucumber (Mathur, 1990).

Sharfun Nahar *et al.*, (2005) reported the association of large number of fungi with sunflower and zinnia seeds includes: *Aspergillus flavus, A. niger, A. ocheraceus, Alternaria alternata, Fusarium solani, Penicillium digitatum, Rhizopus arrihizus, Acremonium fusidioides, Arthrobotrys oligospora, Bipolaris bisepta, Cephaliophora tropica, Chaetomium spinosum, Cladobotryum varium, Cladosporium cladosporioides, Emericella nidulans, Gonatobotrys simplex, Humicola grisea, Memnoniella echinata, Mucor mucedo, Myrothecium verrucaria, Phialophora verrucosa* and *Syncephalastrum racemosum.*

In Safflower varities the seed germination and vigour was adversely affected by *Alternaria, Fusarium, Aspergillus* sp. (Raghuwanshi et al., 2002).

Awadhiya (1992) studied seed mycoflora associated with 50 varieties of safflower and recorded the occurrence of *Alternaria, Fusarium,* and *Macrophomina* sp. and found occurrence of *Alternaria carthami* was found to be predominant (100%).

In 1989, Borkar and Shinde reported that externally seed borne *Alternaria carthami* in safflower not only reduced the seed quality by causing seed rot but also seedling decay, pre and post-emergence mortality of seedlings.

A study on seed mycoflora associated with 13 varieties of safflower was conducted by Singh *et al*. (1987) and they recorded 11 fungal species associated with seeds. Among the species detected, the occurrence of *Alternaria* spp and *Rhizoctonia* spp were found to be high with 40% and 30% respectively.

Rajagopalan and Shanmugam (1983) isolated *Alternaria carthami* from surface sterilized safflower seeds and observed that the pathogen is externally seed borne and seldom carried internally. Prasad (1985) tested 35 varieties of safflower by standard blotter method for the detection of *Alternaria carthami* pre and post emergence seedling mortality.

Padaganur and Anil kumar observed *Curvularia* sp, *Alternaria* sp, *Aspergillus flavus, Aspergillus niger, Fusarium* sp. on different seed lots of two varieties of safflower in 1976.

The economic value of sunflower seeds is greatly influenced by the associated saprotrophic fungi, which may reduce oil quality due to increase of free fatty acids amount in seeds during storage (McGee and Christensen 1970, Singh and Prasad 1977; Vijayalakshmi and Rao, 1986; Bhutta *et al*. 1997) or produce mycotoxins (Shahnaz and Ghaffar 1991; Abdullah and Al-Mosawi, 2010).

2.3 Methods of Mycoflora study in sunflower seed

To study the association of mycoflora with seeds of different crops, various techniques have been employed around the world. The standard techniques those are recommended by the ISTA are used most frequently.

2.3.1 Standard Blotter Method

Standard blotter method is a widely used standard seed health testing method due to its simplicity and low-cost operation. Ingle (2020) isolated a total of 13 species fungi belonging to 6 genera from groundnut seed sample using standard blotter test. According to study the percent of incidence is lower in blotter method than seed washing test.

Khani *et al*., (2019) studied mycoflora of pea and extracted 10 different fungal species belonging to different genera through blotter paper. The extent of fungus extracted from blotter paper was lower compared to the agar plate method.

Irshad *et al*., (2017) recovered 12 genera of fungi from sunflower and Zinnia using blotter method and suggested agar plate method was more effective than blotter paper method. Patil *et al*., (2018) tested five STH methods to study mycoflora of sunflower and found blotter paper method as most efficient followed by the methods viz. 2-4 D blotter paper, Modified PDA, Agar plate and Paper towel method.

Ghoneem *et al*., (2014) isolated a large number of (19 genera 30 species) fungus from sunflower seed by blotter method than deep freezing. The untreated seed was found to produce more fungal species than the treated seeds, and blotter method was recommended for isolating saprophytes like *A. flavus, A. Niger, Rhizopus*. In 2014, Singh, studied seed borne fungi associated with chickpea seeds following standard blotter methods.

Islam *et al*., (2013) detected 9 different fungi in samples of mesta seeds using standard blotter method. Ramesh *et al*., (2013) isolated 11 fungi from the seed of soybean by using blotter and recommended the technique for routine enumeration.

Abdullah and Al-Mosawi, (2010), used blotter method and isolated 48 fungal species from seeds of 9 sunflower cultivars in Iraq. Afzal *et al*., (2010) isolated 11 fungi from sunflower seed by blotting paper method under unsterilized conditions and confront the method effectively, routinely and consistently applicable to obtain reliable results.

Niaz and Dawar, (2009) extracted mycoflora of sunflower using blotter, deep freezing and agar method and found agar plate method yielded the highest number of fungi as compared to blotter and deep-freezing methods.

Sharfun-Nahar *et al.*, (2005) extracted 45 fungal species belonging to 27 genera by using blotter method. In cucurbits, blotter method was useful for the detection of most infectious fungi (Begum and Momin, 2000; Elwakil and El-Metwally, 2001; Avinash and Ravishankar, 2013). Squash seed results confirm the previous recorded results in Pakistan (Rahim and Hasan, 2013).

Solanke *et al*. (1997) stated that agar plate technique yields high mycoflora than the blotter method. Irrespective of the method of isolation and variety used, unsterilized seed gave a greater number of mycoflora than the sterilized seed (Ahammed *et al*. 2006). The blotter method has shown better results than the agar plate technique (Rasheed *et al*. 2004; Dawar *et al*., 1997).

2.3.2 Deep freezing blotter method

Deep freezing blotter (DFB) is a modification of standard blotter (SB) technique where seeds are incubated at low temperature for a short period and then incubated in blotter as usual. The method differs in efficiency to extract mycofolora from seed than SB method.

Ghoneem *et al*., (2014) extracted 28 species of fungi from 18 genera from sunflower seed following deep feezing blotter (DFB) method. DFB was found to be enhanced recovery of *A. flavipens, A. glaucans, Macrophomina phaseolina, Trichothecium rosum*.

Niaz and Dawar, (2009) isolated 56 species of fungi belonging to 23 genera from sunflower seed using three different techniques and suggested deep freezing

method for the detection of deep seated as well as slow growing seed borne fungi like *Drechslera spp., Fusarium spp., Penicillium spp., Nigrospora oryzae, Monilia* sp., *Macrophomina phaseolina, Alternaria alternata, Syncephalastrum racemosum.*

Sharfun-Nahar *et al*., (2005) used deep-freezing technique and isolated 38 fungal species belonging to 23 genera from 35 samples of *Helianthus annuus*. Deep freezing technique appeared more suitable as compared to standard blotter technique for the detection of *Fusarium* spp.

2.3.3 Washing method

Ingle, (2020) isolated thirteen species belonging to six genera of fungi from groundnut seed samples and found the percent of incidence is highest in seed washing test as compared to standard blotter test and agar plate method.

Billingsley *et al*., (2017) sterilized the seeds using 0.5% Clorox before incubating in malt extract agar to understand the diversity and function of seed-borne fungi in alpine tundra.

Irshad *et al*., (2015) used blotter methods following washing of seed with 2% Clorox to extract Mycoflora of citrus and found 11 fungal species.

Saleem *et al*.,(2014) investigated seed borne mycoflora associated with through standard blotter paper and recovered thirteen fungal genera from seeds. The seed treated with Clorox before placing in blotter resulted in less frequency of the fungi.

El-Wakil *et al.,* (2011) isolated 15 fungal species from 11 genera in flax cultivars using standard blotter method. Before placing the seeds in the blotter, the seeds were washed using 2.5% Clorox for surface sterilization.

Ahmad and Iram, (2008) washed the plant samples with 1% chlorox before incubation in PDA media to isolate soil borne mycoflora present in rice-wheat cropping system.

2.3.4 Agar plate method

Agar plate method is a recommended method by the International Seed Testing Association (ISTA) for fungal isolation. An agar plate is a Petri plate containing a medium of growth (typically agar plus nutrients) used to cultivate microorganisms. Many study have successfully used the method to isolate fungi from different crop seeds.

Khani *et al*., (2019) isolated 10 different fungal species from seeds of peas seeds through blotter paper and agar plate methods. Although the trend of frequency was similar in two method, the extent was higher in agar plate method as the majority of the fungal isolates preferred to grow on agar plates instead of blotter paper.

Patil *et al*., (2018) attempted five STH method in sunflower seed and Blotter paper was observed the most efficient method followed by 2-4,D blotter paper, Modified PDA, Agar plate and Paper towel.

Irshad *et al*., (2017) isolated 12 genera of fungi from sunflower & zinnia and maximum number of fungi using agar plate method than blotter paper method.

Singh, (2014) used agar plate method and isolated seven fungal species from Cicer arietinum seed; such as *Alternaria alternata, Aspergillus flavus, A niger, A fumigatus, Curvularia lunata, Fusarium moniliforme* and *Rhizoctonia solani*.

Ramesh *et al*., (2013) isolated 11 fungi from soybean seeds by using agar and blotter technique.

Afzal *et al.*, (2010) isolated a total of 12 fungi by agar plate method from sunflower seed and found this method of fungal isolation are effective, routinely and consistently applied.

Niaz and Dawar, (2009) isolated 56 species of fungi belong to 23 genera from sunflower seeds using agar plate method. Of the three methods used, agar plate method yielded the highest number of fungi as compared to blotter and deep-freezing methods.

The agar plate method was found to be suitable for the detection of *Aspergillus spp., Cladosporium spp., Curvularia spp., and Rhizopus spp.*

2.3.5 Water agar method

Seed borne mycoflora of cowpea were isolated by Zanjare *et al*., (2020) by using blotter method, agar plate method, deep freezing, 2-4, D method and Test tube water agar seedling symptom test as recommended by ISTA. They found water agar method as least effective compared to other techniques.

Gohari *et al*., (2007) investigated the mycoflora of stored grain of wheat using the moist chamber method and agar test. The single-spore culture was established on 2% distilled water agar that results in 9 species of fungi.

Freire *et al.,* (2000) surface sterilized the seeds in 2% (v/v) sodium hypochlorite (0.5% active chlorine) for 2 min and rinsed twice in sterile distilled water before being plated onto tap water agar (TWA) to study the mycoflora of black pepper, white pepper and Brazil nuts.

2.4 Extent of germinated, infected and dead seed

Mycobiota associated with seeds of 9 sunflower cultivars/inbreed (*Helianthus annuus*) viz., ‘Akmar’, ‘Eurofflore’, ‘AS 508’, ‘Mannon’, ‘AS 615’, ‘Florasol’ and three unidentified local cultivars were studied. The seeds were associated with 48

species of fungi belonging to 19 genera. The species composition, percentage of seed infection and seed germination percentage differed among cultivars. ‘Akmar’ cultivar showed the lowest number of detected species (17 species), whereas the highest number (48 species) was isolated from the unidentified local cultivar 3. Maximum seed germination was observed in ‘Akmar’ (100%) and minimum in unidentified local cultivar 3 (38%) (Abdullah and Al-Mosawi, 2010).

CHAPTER III

MATERIALS AND METHOD

The experiment was conducted to detect mycoflora asscociated with sunflower seed following different techniques. The materials used and methodology followed in the studyare described in this chapter under heading and Sub-heading.

3.1 Experimental site

The laboratory experiment was conducted Dr. M. A Wazed Miah Central laboratory, Department of Plant Pathology, Faculty of Agriculture Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207.

3.2 Experimental period

The experiment was conducted during June' 2020 to December' 2020.

3.3 Design of the experiment

The experiment was carried out following Completely Randomized Design (CRD) with three replications for all methods.

3.4 Materials

The following materials were used in the experiment

1) **Tools** viz., scissor, needles, knife, blade, scale, forceps, sprit lamp, electronic balance, wrapping tape, blotter paper, Aluminum foil.

2) **Large equipment's** viz., Incubator, Autoclave, Laminar Air Flow, Oven, Refrigerator.

3) **Chemicals** viz., 70% Ethanol, Hexisol, Agar powder, Dextrose powder, Lactic acid, Glycerin, Cotton blue, Clorox (4-6%), paraffin wax.

4) **Glassware** viz., Petri dish, Funnel, Test tube, Beaker, Conical flask, Slide, Cover slip.

5) **Data recording materials** viz., microscope (Sterio & Compound), computer, camera.

3.5 Collection of Sunflower seeds

About total of three (03) varieties were used to perform this experiment to observe the flourish of mycoflora on seeds. A local variety of Sunflower was collected from Siddik bazar of Gulistan and other two variety i.e., BARI Sunflower 2 and BARI Sunflower 3 were collected from Oilseed Research Center (ORC) of Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur, Bangladesh.

3.6 Preservation of collected seed

Collected seeds were stored in a refrigerator for few days to manage all other equipments to start the experiment.

3.7 Seed characteristics

From the collected seeds, 100 seed of each variety was taken separately to grade them based on study of their morphological characteristics. The grading result was finding of different size and shape of seed sample viz. large robust seeds, medium sized seeds, deformed, small robust, discolored and slandered type.

3.8 Detection Techniques for identification of mycoflora associated with Sunflower seeds

Five inoculation techniques were used as treatments viz. Blotter paper method, Potato Dextrose Agar (PDA), Water agar plate method, Deep freezing method and Washing test for growing and analyzing growth of the mycoflora on three (03) selected sunflower varieties.

3.9 Seed Placement technique

For growing the seeds and mycoflora, petri plates with 9cm diameter were taken. In each of the petri plates providing with the method, 10 seeds were incubated at the media following the International Rules for Seed Testing (ISTA, 2001) and each method was replicated 03 times. Thus, a total of 45 petri plates were placed for the

study with five incubation techniques on three sunflower varieties. The mycoflora was observed on the germinating seeds and isolation and identification was done using stereo microscope and compound microscope.

3.10 Layout for the laboratory experiment

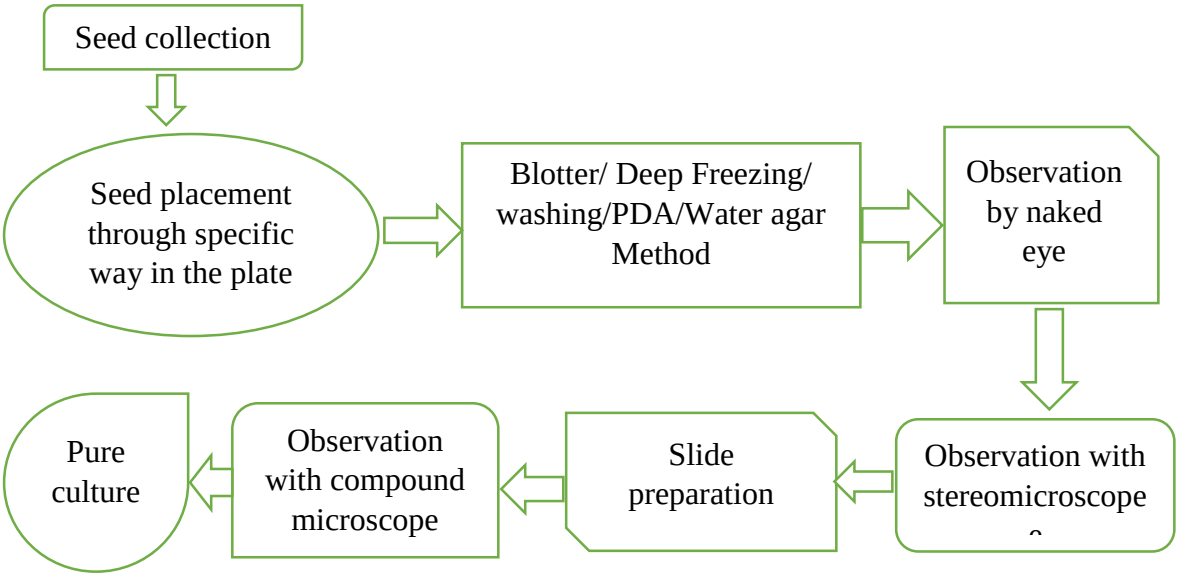


Fig 1: Flowchart process of isolation of fungi by different method

3.10.1 Different Media preparation

Five kinds of techniques were used for this experiment. The standard protocols for the different techniques were followed to fulfill the procedures.

3.10.1.1 Blotter Paper method

In Blotter paper method, petri plates with 9 cm diameter were taken and sterilized by washing with 70% ethanol first. Then, sterilized petri plates were dried and Blotter paper (9cm) were placed inside the plates in single layer giving appropriate size according to the plates through cutting. The blotter was sprayed with distilled water and 10 untreated seeds from one variety were placed using sterilized forceps. A total of three petri plates were prepared for the three-varieties following the procedure. The plates were replicated three times. All the work was performed inside the laminar airflow cabinet to avoid the contamination. The plates were placed under ideal condition for germination. All the plates were incubated for three

to five days. Seeds were germinated after three days of placement and data on mycoflora were recorded after five days of incubation.

3.10.1.2. Deep freezing method

In this method of incubation, nine petri plates were prepared as usual like blotter paper method. There after the prepared then all the petri plates were incubated for 24 hours at 20±2°C. After completion of incubation, the petri plates were incubated at -20°C in dark at a freezer and then kept back under original conditions for five days. The plates were checked after three days for germination and study of mycoflora after five days of incubation.

3.10.1.3. Washing test

The seeds of three varieties of Sunflower were emerged in a 4-6% Clorox solution for 30 second. After that, the seeds were taken off and washed with distilled water for 2-3 minutes. Nine petri plates were sterilized by washing with 70% ethanol and prepared for incubation placing blotter paper inside it. The blotter was sprayed with distilled water and the treated seeds were placed using a sterile forceps in the petri plates from distilled water bath. The ready petri plates were incubated at 25±2°C temperature and under favorable condition of germination. Plates were incubated for five days. The germination was examined after three days and mycoflora growth was examined after five days of incubation.

3.10.1.4. PDA Method

PDA media was prepared according to standard protocol described by Ricker and Ricker (1936). The composition of PDA media is given below:

Ingredient	Quantities
Potato	200 g
Dextrose	20 g
Agar	17 g
Distilled Water	1 L

To prepare the PDA media firstly, the potatoes were washed under running tap water and then peeled off. For preparing 250 ml of PDA media an amount of 50 gm peeled potato was measured by electric balance after cutting into small pieces using sharp knife. The potato blocks were taken into a pan containing 250 ml of water and boiled for 15 minutes. The starch extract was filtered into a conical flask using a sieve. In the conical flask 5 gm of agar powder and 5 gm of dextrose was taken. The mixture was shaken to make the solution and the opening of the conical flask was closed using cotton and aluminium foil. The media was autoclaved for 45 minutes in 121˚C temperature under 15 psi pressure for sterilization. In the same time, nine petri plates were wrapped with foil paper and placed inside the autoclave machine for sterilizing purpose. After autoclaving, when the temperature was suitable 14 drops of lactic acid were added into the media and mixed well. The sterilized PDA media was poured into nine petri plates. After settling down of the media, 10 seeds from each of the sunflower variety were placed in three different plates. The plates were replicated three times and then incubated for five days under favourable condition.

3.10.1.5. Water Agar plate method

For preparing 250 ml of water agar media an amount 5 gm of agar powder was taken in a conical flask containing 250 ml of distilled water. The mixture was shaken to make the solution and the opening of the conical flask was closed using cotton and aluminium foil. The media was autoclaved for 45 minutes in 121˚C temperature under 15 psi pressure for sterilization. In the same time, nine petri plates were wrapped with foil paper and placed inside the autoclave machine for sterilizing purpose. The sterilized water agar media was poured into nine petri plates. After settling down of the media, 10 seeds from each of the sunflower variety were placed in three different plates. The plates were replicated three times and then incubated for five days under favorable condition.

3.10.2 Data collection

In the period of 5-7 days long incubation period all 45 petri plates were examined at three days for taking data on germination. During the data collection dead seed and germinated seed were counted. The percentage of dead and germinated seeds were calculated following the equation. To calculate percent seed infection (Aslam *et al.*, 2015) the following formulae were used-

$$\% \text{ Germinated seed} = \frac{\text{Number of germinatd seed}}{\text{Total number of seed}} \times 100 \ldots\ldots (1)$$

$$\% \text{ Infected seed} = \frac{\text{Number of infected seed}}{\text{Total number of seed}} \times 100 \ldots\ldots\ldots\ldots (2)$$

$$\% \text{ Dead seed} = \frac{\text{Number of dead seed}}{\text{Total number of seed}} \times 100 \qquad \ldots\ldots\ldots\ldots (3)$$

3.10.3 Comparison of infection percentage by different method

Data on number of seeds infected by different fungi and specific fungi were recorded separately to calculate percent seed infection and frequency respectively.

3.10.4 Isolation of the mycoflora

Isolation of mycoflora was done from all tested seed by some subsequent methods at the five days of incubation. Firstly, the growth of mycoflora was tried to identify by visual assessment. Then stereomicroscope and compound microscopic study was performed to identify different fungal growth and taking pictures. First the plates were observed at 10X magnification to locate the growths. After that 40X magnification was used to study the growth intensively and taking the picture of mycofloral growth.

3.10.5 Identification of pathogens

Temporary slides of fungal isolates from seeds were made and observed under a light microscope. Morphological characters of fungi were recorded and compared with standard keys for establishing their identity (Barnett and Hunter 1972; Nelson *et al*. 1983). In addition, internet databases were also used to compare the morphological characteristics of isolates.

3.10.6 Determination of extent of seed mycoflora

Determination of extent of seed associated mycoflora with sunflower crops were evaluated from all methods. The extent of seed associated mycoflora from sunflower seed was calculated by using the following formula from Sidra *et al*., (2019).

$$\% \text{ frequency} = \frac{\text{Number of pieces colonized}}{\text{Total number of pieces studied}} \times 100 \quad \ldots\ldots\ldots\ldots \quad (4)$$

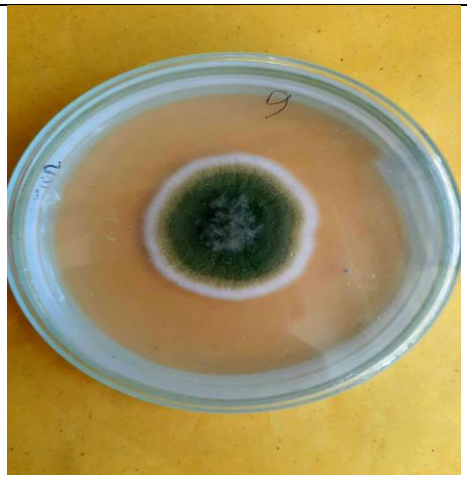
3.10.7 Pure culture of identified fungi on PDA media

Two slice were cut from each of the sample with the help of sterilized forceps and it was cultured in separate petri plates which was containing freshly prepared PDA. The petri plates were kept in an incubator which was preset at 25°C for 48 hours. For obtaining pure cultures of the fungal isolates the developing fungal cultures were antiseptically sub cultured repeatedly into freshly prepared PDA plates until cultures consisting of only one type of fungus was reached. A small portion of fungal growth from each pure petri plates was teased with a sterilized needle into 1-2 drops of glicerin on a clean slide for identifying each of the fungus. It was covered with a coverslip and it was examined under a compound microscope. It was finished by comparing the morphological features of each of the prepared fungal slides as examined under microscope as well as their corresponding pure plate with the descriptions by Talbot (1971) and Deacon (1980).

A

B

C



D

E

Plate 1. Fungal growth (pure culture) on PDA media. A. *Alternaria alternata*; B. *Aspergillus niger*; C. *Fusarium sp.*; D. *Aspergillus flavus*, and E. *Rhizopus sp.*

3.11 Statistical analysis

The collected data were preprocessed using microsoft office excel. The organized data were then analyzed by Statistix-10 computer package program. The Analysis of variance (one way ANOVA) was performed to find out the variation of result from experimental treatments. Mean separation was done by LSD test at 5% level of significance.

CHAPTER IV

RESULTS

The experiment extracted various information about mycoflora associated with the Sunflower seed, the ability of three Sunflower varieties on germination (%), Infected seed(%) and Dead seed(%), and also the extent of seed associated mycoflora with those varieties of Sunflower when following five different techniques of seed heath testing. The findings are presented in this chapter.

4.1 Visual observation method

From the collected Sunflower seeds 100 seeds from each variety were graded on the basis of some criteria like size, shape and colour. The results found are shown in table 1.

The highest number of large robust seeds were found in BARI Sunflower 2 and the lowest was found in Local variety (Table 1). The number of highest medium sized seeds were found in BARI Sunflower 3 and the lowest was found in local variety. The number of highest deformed sized seeds were found in local variety and the lowest was in BARI Sunflower 2.The highest number of small robust seeds were found in BARI Sunflower 3 and lowest was found in BARI Sunflower 2. The highest number of discolored seeds were found in local variety and the lowest was found in BARI Sunflower 2. And Slandered type seeds were also observed in the inspection where the highest number of seeds of this type was found in BARI Sunflower 2 and the lowest in local variety.

Table 1. Visual observation of sunflower seed by inspection method

Seed type	Number of Seed		
	BARI Sunflower 2	BARI Sunflower 3	Local Variety
Large Robust seeds	18	14	9
Medium sized seeds	20	24	13
Deform	15	18	27
Small Robust	17	32	22
Discolored	5	4	25
Slandered	25	8	4
Total	**100**	**100**	**100**

4.2 Detection of fungal mycoflora associated with sunflower seeds by different methods

The mycoflora isolated from seeds of three varieties of Sunflower viz., BARI Sunflower 2, BARI Sunflower 3 and local variety showed great variability in associated mycoflora extracting through five seed heath testing incubation techniques viz., Blotter paper, Deep freezing, Washing method, PDA and Water agar method. In tota 09 genera of fungi was isolated from the seed of selected varieties (Table 3).

4.2.1 Germinated, Infected and Dead seed Percentage of Sunflower seed in Blotter paper method

The influence of different seed health testing incubarion technique was separately evaluated on seed of three Sunflower varieties in regards to % germination, % infected seed and % dead seed. In Blotter paper method significant variation ($p< 0.05$) was observed in % germinated seed, % infected seed and % dead seed among the varieties. The results are presented in Figure 2.

The highest percentage of germination was found in BARI Sunflower 3 (86.67%) followed BARI Sunflower 2 (76.67%) and Local varieties (63.33%). Significant lowest percentage of dead seed was also in BARI Sunflower 3 (13.33%) followed

by statistically similar BARI Sunflower 2(23.33%) and Local (36.67%). Interestingly, though % infected seed did not varied among the varieties but the highest percentage of infection was in BARI Sunflower 3 (73.33%) followed by BARI Sunflower 2 (63.33%) and Local (63.33%).

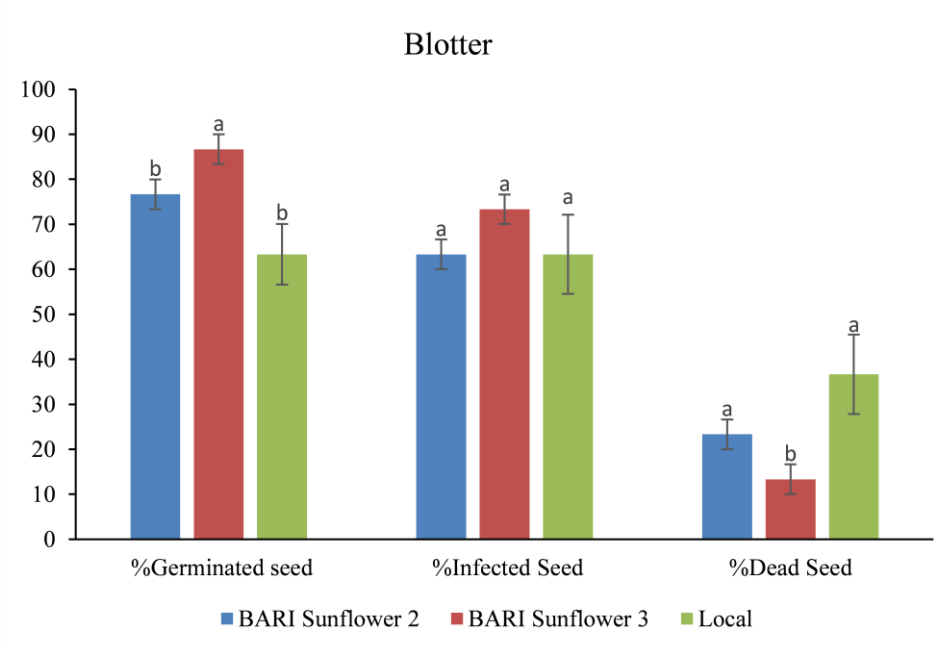


Figure 2. Germinated seed, Infected seed and Dead seed percentage of seed borne mycoflora of Sunflower by Blotter paper method

4.2.2 Germinated, Infected seed and Dead seed Percentage of Sunflower seeds in Deep Freezing method

The influence of different seed health testing techniques of incubation was separately evaluated on seed of three Sunflower varieties in regards to %germination, %infected seed and % dead seed. In deep freezing method significant variation ($p< 0.05$) was observed in %germinated seed, %infected seed and % dead seed among the varieties. The results are presented in Figure 3.

In deep freezing method, significant variation among the varieties was only observed in % infected seed, not in % germinated and % dead seed.

Although the germinated seed (%) did not vary significantly among the varieties in deep freezing method, the maximum germinated seed (%) was in BARI Sunflower 3 (83.33%) followed by BARI Sunflower 2 (73.33%) and Local (66.67%). The

infected seed (%) was maximum in BARI Sunflower 3 (70%) followed by BARI Sunflower 2 (60%) and Local (53.33%). The dead seed (%) was similar among the varieties but lowest was observed in BARI Sunflower 3 (16.67%).

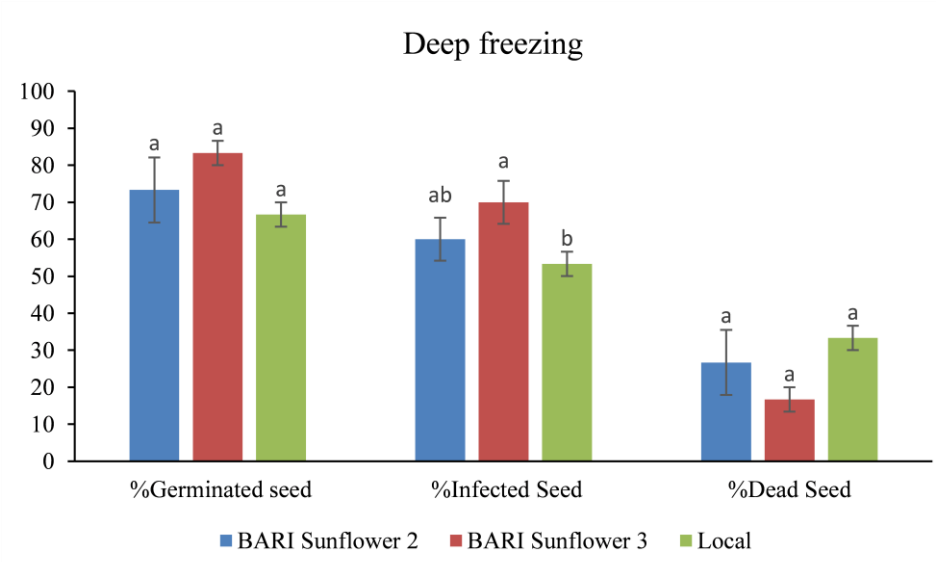


Figure 3. Germinated seed, Infected seed and Dead seed percentage of seed borne mycoflora of Sunflower by Deep freezing method

4.2.3 Germinated, Infected seed and Dead seed Percentage of Sunflower seeds in washing test

The influence of different seed health testing technique of incubation was separately evaluated on seed of three Sunflower varieties in regards to germination (%),infected seed (%) and dead seed (%). In washing method significant variation ($p< 0.05$) was observed in germinated seed (%), infected seed (%) and dead seed (%) among the varieties. The results are presented in Figure 4.

No significant variation was resulted among the varieties in regards to germinated seed (%), Infected seed (%) and dead seed (%) following the washing method for germination.

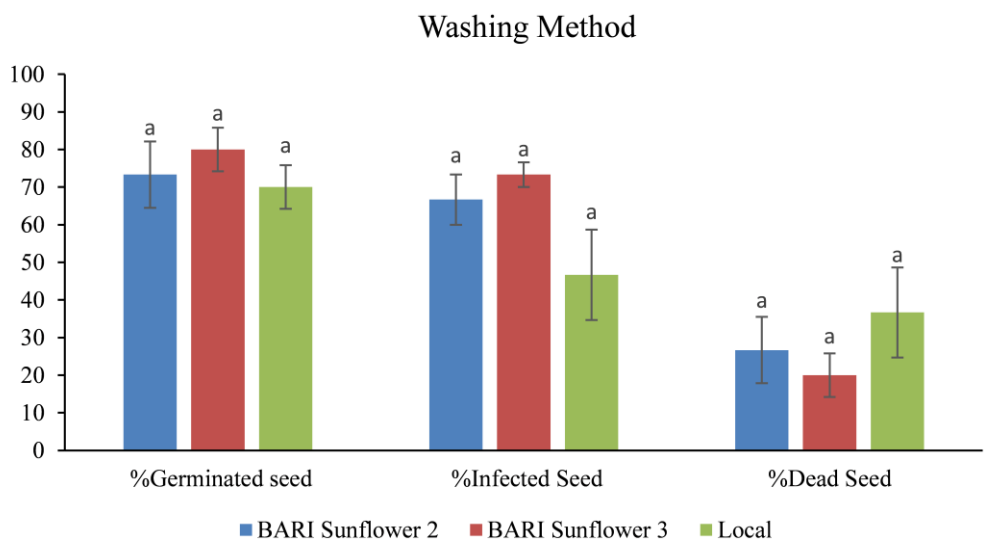


Figure 4. Germinated seed, Infected seed and Dead seed percentage of seed borne mycoflora of Sunflower by Washing method

The germinated seed (%) as well as infected seed (%) was maximum in BARI Sunflower 3 (80% & 73.33%) followed by BARI Sunflower 2 (73.33% & 66.67%) and Local variety (70% & 46.67%) respectively. The dead seed (%) was lowest in BARI Sunflower 3 (20%) followed by BARI Sunflower 2 (26.67%) and Local variety (36.67%).

4.2.4 Germinated, Infected seed and Dead seed Percentage of Sunflower seeds in PDA method

The influence of different seed health testing technique of incubation was separately evaluated on seed of three Sunflower varieties in regards to germination (%), infected seed(%) and dead seed (%). In washing method significant variation ($p< 0.05$) was observed in germinated seed (%), infected seed (%) and dead seed (%) among the varieties. The results are presented in Figure 5.

The varieties of sunflower regarding % germinated seed, % infected seed and % dead seed were statistically similar when germinating following PDA technique.

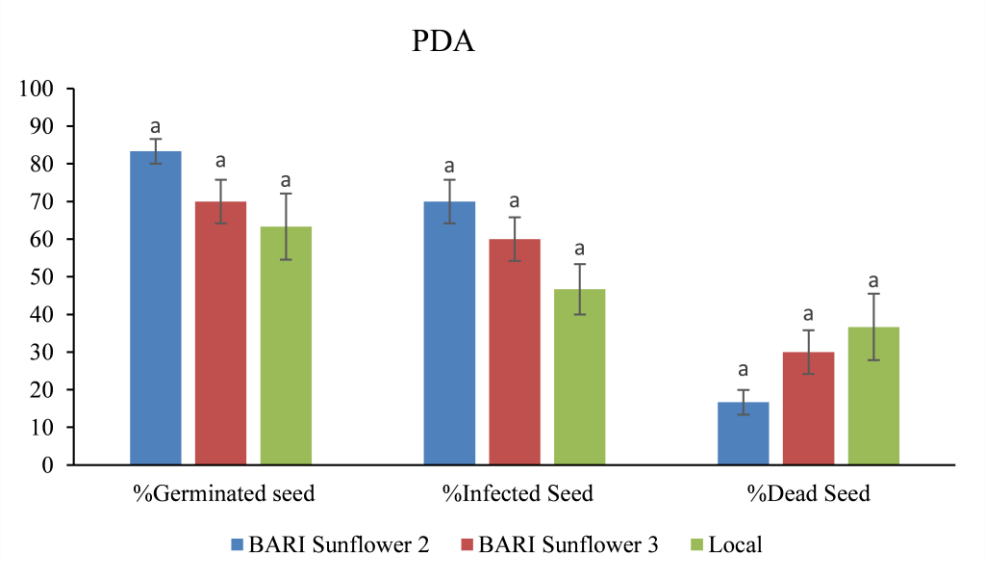


Figure 5. Germinated seed, Infected seed and Dead seed percentage of seed borne mycoflora of Sunflower by PDA method

In the PDA method, the highest percentage of germination in seed was obtained from BARI Sunflower 2 (83.33%) followed by BARI Sunflower 3 (70%) and Local variety (63.33%). Similar trend was observed in % infected seed where it was maximum in BARI Sunflower 2 (70%) followed by BARI Sunflower 3 (60%) and Local variety (46.67%). The lowest percentage of dead seed was in BARI Sunflower 2 (16.67%) followed by BARI Sunflower 3 and Local variety.

4.2.5 Germinated, Infected seed and Dead seed Percentage of Sunflower seeds in water agar plate method

The influence of different seed health testing technique of incubation was separately evaluated on seed of three Sunflower varieties in regards to germination (%), infected seed (%) and dead seed (%). In washing test significant variation ($p< 0.05$) was observed in germinated seed (%), infected seed (%) and dead seed (%) among the varieties. The results are presented in Figure 6.

In Water agar method of seed germination, the variation among the varieties was not significant in regards to germinated seed (%), infected seed (%) and dead seed (%) (Figure 5). But similar to PDA, the highest germinated seed (%) was seen in BARI Sunflower 2 (70%) followed by BARI Sunflower 3 (66.67%) and Local variety (60%). Incase of infected seed (%) and dead seed (%) the maximum

percentage was in BARI Sunflower 2 (66.67%) followed by BARI Sunflower 3 (60%) and Local variety (43.33%). The lowest % dead seed found was in BARI Sunflower 2 (30%) followed by BARI Sunflower 3 (33.33%) and local (40%).

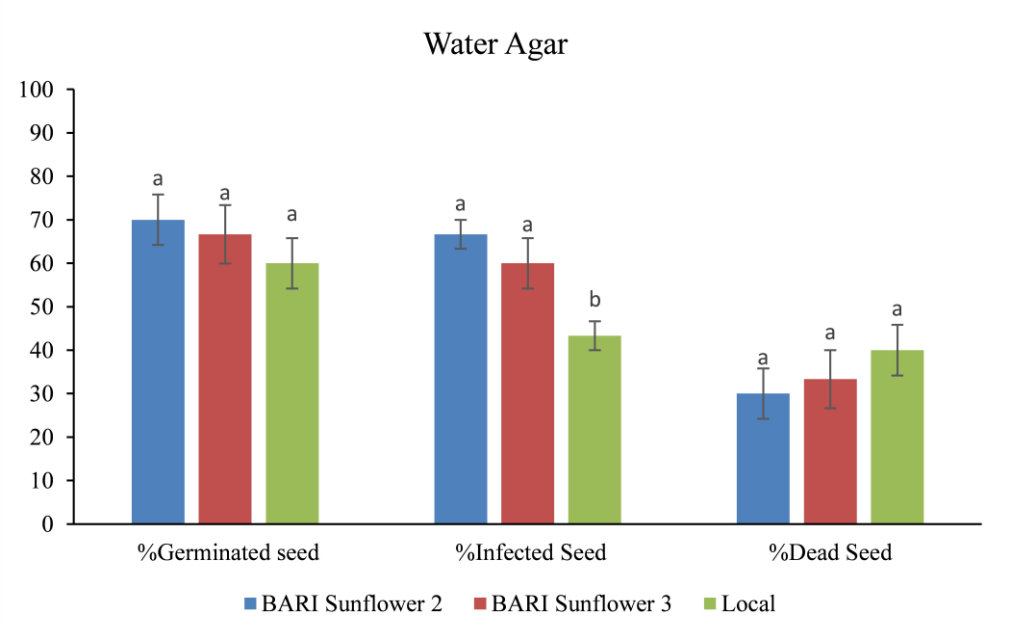


Figure 6. Germinated seed, Infected seed and Dead seed percentage of seed borne mycoflora of Sunflower by Water Agar method

4.3 Comparison of infection percentage of sunflower seeds by different method

The infection percentage was calculated to campare between the variety with different method to assess the highest and lowest infection containing variety and method and the obtained result shown in the below (table 2).

Table 2. Mean percent infection of sunflower seed by mycoflora

Sl. No.	Detection method	Variety	% Seed infection
1	Blotter paper	BARI Sunflower 2	63.33
		BARI Sunflower 3	73.33
		Local Variety	63.33
2	Deep freezing	BARI Sunflower 2	60.00
		BARI Sunflower 3	70.00
		Local Variety	53.33
3	Washing test	BARI Sunflower 2	66.67
		BARI Sunflower 3	73.33
		Local Variety	46.67
4	PDA	BARI Sunflower 2	70.00
		BARI Sunflower 3	60.00
		Local Variety	46.70
5	Water Agar plate	BARI Sunflower 2	66.67
		BARI Sunflower 3	60.00
		Local Variety	43.33

4.4 The extent of fungal mycoflora associated with sunflower seeds

A total nine species of six genera of fungus identified from collected the seed samples. Then they were irrespective of Sunflower varieties and techniques of incubation. No methods were fully sufficient to reveal all nine fungi where washing method extracted maximum of eight. The separately analyzed result (one way ANOVA) of each sunflower variety under different techniques showed diverse result where some of the frequency of associated mycoflora varied significantly ($P< 0.05$). The results are presented in Table 3,4,5,6 and 7.

4.4.1 In Blotter paper method

Using the blotter paper method, seeds of BARI Sunflower 2 variety showed significantly the highest frequency of *Rhizopus* sp. (76.7%) was observed followed by *A. alternata* (63.3%), *Fusarium* sp. (60%), *A. flavus* (36.7%) and *Aspergillus* sp. (36.7%). In case of BARI Sunflower 3, only *Rhizopus* sp. (53.3%), *A. alternata* (50%) and *Fusarium* sp. (43.3%) were found in blotter paper frequency of which was statistically similar. Same three fungus was found in Local variety but frequency of Rhizopus was significantly higher (56.7%) than *Fusarium* sp. (30%) and *A. alternata* (16.7%) (table 3).

Table 3. The extent of mycoflora associated with Sunflower isolated through blotter paper method

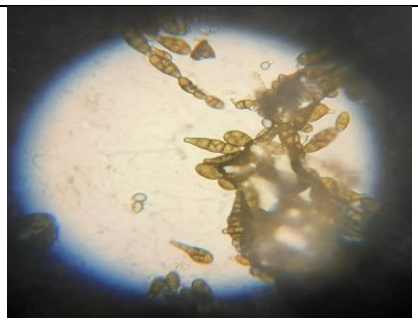
Mycoflora	Overall Frequency Percent		
	BARI Sunflower 2	BARI Sunflower 3	Local variety
Alternaria alternata	63.3±3.3^{b}	50±5.8^{a}	16.7±3.3^{b}
Curvularia sp.	-	-	-
Aspergillus flavus	36.7±3.3^{c}	-	-
A. helianthi	-	-	-
A. niger	-	-	-
Aspergillus sp.	36.7±3.3^{c}	-	-
Fusarium sp	60±5.8^{b}	43.3±6.7^{a}	30±5.8^{b}
Rhizopus sp.	76.7±3.3^{a}	53.3±3.3^{a}	56.7±3.3^{a}
Stemphyllum sp.	-	-	-
Significance	***	NS	*
Pr(>F)	0.00014	0.373	0.0123
LSD	11.40	17.72	19.99

Note: *Figures following the similar letter within a column are not significantly varied according to the LSD (least significant difference) test at P<0.05.*

A

B

C



D

E

F

G

H

Plate 2. Extent of fungi in blotter paper method. A. Placement of seeds; B. incubation of seeds; C, E, G: Stereo and D,F,H: Compound microscopic view of *Alternaria alternata*, *Fusarium* sp. and *Rhizopus* sp. respectively

4.4.2 In Deep freezing method

Incubating by deep freezing techniques for BARI Sunflower 2 only 03 fungi wre observed. Where significantly higher frequency was observed for *A. niger* (63.3%) than *A. alternata* (40%) and *Rhizopus* sp. (26.7%). Same three fungus was found in BARI Sunflower 3 with a frequency of 40%, 30% and 23.3% respectively which were statistically similar. However, following the same techniques of incubation five types of fungi were isolated from the plates of Local variety. Among these, *Rhizopus* sp. (46.7%) and *Aspergillus* sp. (43.3%) were statistically similar but highest frequency and the lowest frequency was observed for *A. alternata, A. niger and A. flavus* (Table 4).

Table 4. The extent of mycoflora associated with Sunflower isolated through Deep freezing method

Mycoflora	Overall Frequency Percent		
	BARI Sunflower 2	BARI Sunflower 3	Local variety
Alternaria alternata	40 ± 5.8^{b}	30 ± 5.8^{a}	30 ± 5.8^{b}
Curvularia sp.	-	-	-
A. flavus	-	-	20 ± 0^{b}
A. helianthi	-	-	-
A. niger	63.3 ± 3.3^{a}	40 ± 10^{a}	26.7 ± 3.3^{b}
Aspergillus sp.	-	-	43.3 ± 3.3^{a}
Fusarium sp.	-	-	-
Rhizopus sp.	26.7 ± 3.3^{b}	23.3 ± 3.3^{a}	46.7 ± 3.3^{a}
Stemphyllum sp.	-	-	-
Significance	*	NS	**
Pr(>F)	0.0131	0.0878	0.00334
LSD	18.51	15.11	11.66

Note: *Figures following the similar letter within a column are not significantly varied according to the LSD (least significant difference) test at P<0.05.*

A

B

C

D

E

F

G

Plate 3. Extent of fungi in Deep freezing method. A. naked eye view of the plate after incubation of seeds; B, D and F the stereo and C,E and G the compound microscopic view of *Alternaria alternata*, *Aspergillus niger* and *Rhizopus* sp. respectively

4.4.3 In washing test

In washing test, all the plates yielded most of the 09 fungi (Table 5). Seven fungi except *Fusarium* sp. and *Stemphylium* sp. were found in BARI Sunflower 2 seeds, where significantly higher frequency was observed for *A. alternata* (56.7%), followed by *A. helianthi* (40%), *Rhizopus* sp. (23.3%), *Aspergillus* sp. (23.3%), *A. niger* (23.3%), *A. flavus* (23.3%) and *Curvularia* sp. (20%). BARI Sunflower 3 seeds results in *A. alternata, A. helianthi, A. niger, Fusarium*, and *Rhizopus* sp. With the 26%-36% frequency, where all are statistically similar. The seeds of Local variety were infected with 6 fungal species where, *A. alternata* (36.7%), *Rhizopus* sp. (33.3%), *Aspergillus* sp. (30%), *A. flavus* (30%), *A. helianthi* (30%) and *A. niger* (26.7%) were statistically similar.

Table 5. The extent of mycoflora associated with Sunflower isolated through washing method

Mycoflora	Overall Frequency Percent		
	BARI Sunflower 2	BARI Sunflower 3	Local variety
Alternaria alternata	56.7±8.8^{a}	36.7±6.7^{a}	36.7±3.3^{a}
Curvularia sp.	20±0^{c}	-	-
A. flavus	23.3±3.3^{c}	-	30±0^{a}
A. helianthi	40±5.8^{b}	26.7±3.3^{a}	30±5.8^{a}
A. niger	23.3±3.3^{c}	26.7±3.3^{a}	23.3±3.3^{a}
Aspergillus sp.	23.3±3.3^{c}	-	30±0^{a}
Fusarium sp.	-	26.7±3.3^{a}	-
Rhizopus sp.	23.3±3.3^{c}	33.3±3.3^{a}	33.3±6.7^{a}
Stemphyllum	-	-	-
Significance	***	NS	NS
Pr(>F)	0.00052	0.0825	0.406
LSD	13.35	8.76	13.15

Note: *Figures following the similar letter within a column are not significantly varied according to the LSD (least significant difference) test at P<0.05. Here V1= BARI Sunflower 2, V2= BARI Sunflower 3, V3= Local variety.*

A

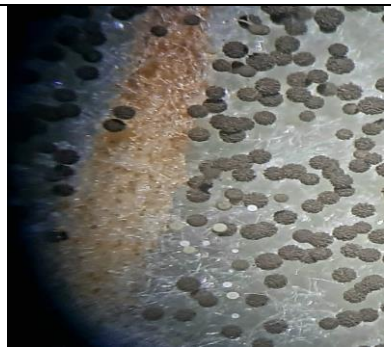
B

C

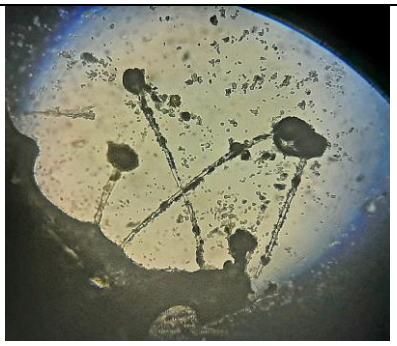
D

E

F



G



H

Plate 4. Extent of fungi in seed washing method. A. Washing of the seeds by NaOCl; B. placement of seeds; C, E, G: stereo and D, F, H: compound microscopic view of *Alternaria alternata, Alternaria helianthi* and *Rhizopus* sp. respectively

4.4.4 In PDA Method

Incubating seeds of sunflower on PDA media yielded 4 different kinds of fungi, where *A. alternate* and *Fusarium* sp. Were common for all the sunflower varieties (Table 6). In BARI Sunflower 2 (Table 6). Among these, *A. alternata* occurs at highest frequency (56.7%) than *Stemphylium* sp. (33.3%), *Rhizopus* sp. (30%), and *Fusarium* sp. (26.7%). However, in BARI Sunflower 3, the fungi having higest frequency was *Rhizopus* sp. (66.7%) that is followed by *Fusarium* sp. (43.3%) with lowest frequency of *A. helianthi* (26.7%) and *A. alternata* (23.3%). The seeds of Local variety yielded 5 types of fungi where *Aspergillus* sp. (36.7%), *A. flavus* (36.7%), *A. helianthi* (33.3%), *A. alternata* (30%) and *Fusarium* sp. (30%) which weren't varied statistically.

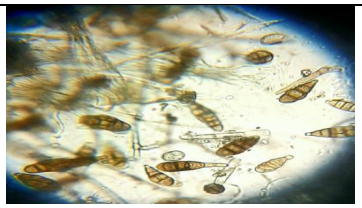
Table 6. The extent of mycoflora associated with Sunflower isolated through PDA method

Mycoflora	Overall Frequency Percent		
	BARI Sunflower 2	BARI Sunflower 3	Local variety
Alternaria alternata	56.7 ± 3.3^{a}	23.3 ± 3.3^{c}	30 ± 5.8^{a}
Curvularia sp.	-	-	-
A. flavus	-	-	36.7 ± 6.7^{a}
A. helianthi	-	26.7 ± 3.3^{c}	33.3 ± 13.3^{a}
A. niger	-	-	-
Aspergillus sp.	-	-	36.7 ± 6.7^{a}
Fusarium sp.	26.7 ± 3.3^{b}	43.3 ± 3.3^{b}	30 ± 5.8^{a}
Rhizopus sp.	30 ± 0^{b}	66.7 ± 3.3^{a}	-
Stemphyllum sp.	33.3 ± 3.3^{b}	-	-
Significance	**	***	NS
Pr(>F)	0.00205	0.000413	0.958
LSD	11.04	12.01	28.14

Note: *Figures following the similar letter within a column are not significantly varied according to the LSD (least significant difference) test at P<0.05.*

A

B



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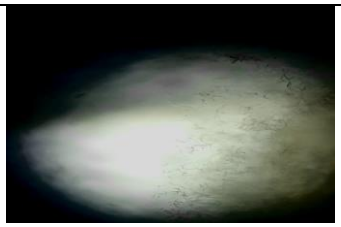

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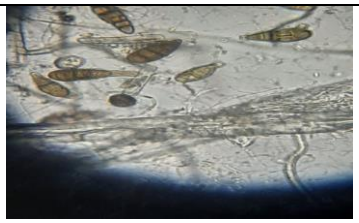

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F

G

H

I

Plate 5. Extent of fungi in PDA media method. A. Naked eye view of seeds after incubation; B, D, F, H: stereo and C,E,G, I: compound microscopic view of *Alternaria alternata*, *Alternaria helianthi*, *Fusarium* sp. and *Stemphylium* sp. respectively

4.4.5 In Water Agar plate Method

The plates of sunflower seed having water agar media showed varied results where 5 types of fungi were cmmon for all the varieties (Table 7). Out of 9 fungi only 7 types of fungi were obsereved in the plates containing BARI Sunflower 2. The plates containing BARI Sunflower 2 results in seven types of fungi out of nine. The significantly higher frequency was observed in *A. alternata* (46.7%), *A. flavus* (33.3%) and *Aspergillus* sp. (33.3%), followed by *Fusarium* sp. (23.3%), *Rhizopus* sp. (20%) and *A. helianthi* (20%). The lowest frequency was observed in *A. niger* (16.7%). In seeds of BARI Sunflower 3, five types was found viz. *A. alternata* (36.7%), *A. flavus* (30%), *Aspergillus* sp. (30%), *A. helianthi* (23.3%) and *Rhizopus* sp. (20%). The seeds of local sunflower variety resulted six types of fungi. Among these, *Aspergillus* sp. (53.3%) was most prevalent with *A. flavus* (43.3%) and *A. alternata* (40%) followed by *A. helianthi* (30%) and *A. niger* (26.7%). However, lowest frequency was observed for *Rhizopus* sp. (20%).

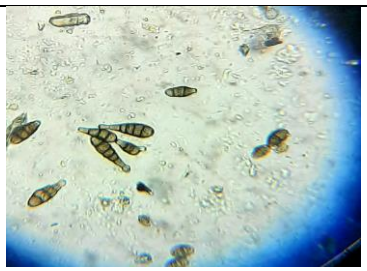
Table 7. The extent of mycoflora associated with Sunflower isolated through water agar

Mycoflora	Overall Frequency Percent		
	BARI Sunflower 2	BARI Sunflower 3	Local variety
Alternaria alternate	46.7 ± 3.3^{a}	36.7 ± 3.3^{a}	40 ± 5.8^{abc}
Curvularia sp.	-	-	-
A. flavus	33.3 ± 8.8^{ab}	30 ± 5.8^{a}	43.3 ± 8.8^{ab}
A. helianthi	20 ± 5.8^{bc}	23.3 ± 3.3^{a}	30 ± 5.8^{bcd}
A. niger	16.7 ± 3.3^{c}	-	26.7 ± 3.3^{cd}
Aspergillus sp.	33.3 ± 8.8^{ab}	30 ± 5.8^{a}	53.3 ± 3.3^{a}
Fusarium sp.	23.3 ± 3.3^{bc}	-	-
Rhizopus sp.	20 ± 0^{bc}	20 ± 5.8^{a}	20 ± 0^{d}
Stemphyllum sp.	-	-	-
Significance	**	NS	**
Pr(>F)	0.0095	0.228	0.00507
LSD	14.87	15.94	14.73

Note: *Figures following the similar letter within a column are not significantly varied according to the LSD (least significant difference) test at P<0.05.*

A

B



C

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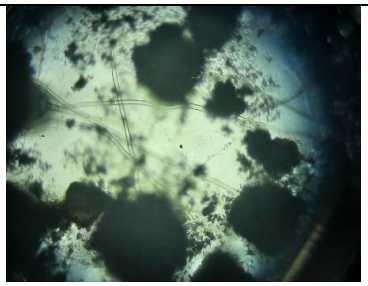

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Plate 6. Findings from water agar media method. A. Naked eye view of seeds after incubation; B, D & F: are the stereo and C,E& G: compound microscopic view of *Alternaria helianthi*, *Aspergillus flavus* & *Rhizopus* sp. respectively.

CHAPTER V

DISCUSSION

Sunflower (*Helianthus annuus* L.) is an important oil seed crop which is originated from Central and North America. A wide range of uses of sunflower have been reported throughout the world such as ornamental plant, medicinal, alimentary, feedstock, fodder, dyes for textile industry, body painting, decorations and so on.

The experiment was carried to find out associated mycoflora of sunflower seeds in three sunflower viz., BARI Sunflower 2, BARI Sunflower 3 and one Local varieties by through five seedborne mycoflora health testing techniques of incubation such as Blotter Paper Method, Deep Freezing Method, Washing test, PDA media Method and Water Agar plate Method.

In visual observation method, from 100 seed counting, it was observed that BARI Sunflower 2 variety showed the maximum type slandered and minimum types of discolored seed, BARI Sunflower 3 variety showed the maximum type of small robust seed and lowest discolored seed and in Local variety maximum number of deformed and lowest number of discolored seed (Khare, 1996).

The results of this experiment clearly depict influence of different methods on germination, infection and dead seed percentage of Sunflower seeds. The highest germination was observed in BARI Sunflower 3 (86.67%) while incubating in blotter paper methods followed by deep freezing. BARI Sunflower 2 germinated most in PDA media (83.33%) followed by blotter paper method (76.67%) while Local variety showed most germination by seed washing method. Water agar method showed the lowest germination in case of all varieties. It was cleared that for a better germination of seed the blotter paper method was fairly efficient followed by variety of BARI Sunflower 3 (86.67%), PDA method and variety is BARI Sunflower 2 (83.33%), Deep freezing method and variety is BARI Sunflower

3 (83.33%) , Washing method and variety is BARI Sunflower 3 (80%), Water agar method and variety is BARI Sunflower 2 (70%) . Similar, works also done by different scientist. Islam *et al*., (2013) reported maximum of 84% germination in mesta seed incubation as untreated seed in blotter paper which was closely confront with the germination of BARI Sunflower 3 at blotter paper method under untreated condition. Afzal *et al.*, (2010) reported germination of untreated sunflower seed under blotter and PDA method of about 75% that is closely resemble with the germination in BARI Sunflower 2 in blotter paper method. Irshad *et al.*, (2017) reported a 10-30% reduction in germination of Sunflower seed in PDA and blotter paper method. In 2010, Abdullah and Al-Mosawi reported the degree of seed germinated seeds ranged from 38 to 100% investigating nine sunflower cultivars. The percentage of infected seed by fungi indicates how efficient a techniques is to isolate the seed borne mycoflora.

The maximum infected seed was found in Blotter paper method as well as seed Washing method for BARI Sunflower 3 (73.3%). Seventy precent infected seeds were obtained on PDA media with BARI Sunflower 2. For BARI Sunflower 2 about 66.7% infected seeds were observed on Water agar method. BARI Sunflower 2 yielded 60% infected seed on Deep freezing method.

BARI Sunflower 2 seed infected most in PDA method (70%) while local seed infected most in blotter paper method (63.33%). BARI Sunflower 2 infected least in deep freezing method while it was water agar techniques that yield lowest infection in seeds of both BARI Sunflower 3 and Local variety. Similar results were also found by Abdullah and Al-Mosawi (2010), where the degree of infected seeds ranged from 10 to 45% in nine sunflower cultivars. Srinivas *et al*., (2017) reported a mean percentage of infection by standard blotter method was 66.75% and in agar plate method was 60.25%.

The percentage of dead seed after incubation was maximum in water agar method for all three varieties ranging from 30-40%. It was least in blotter paper method and for BARI Sunflower 3 (13.33%), followed by PDA method in BARI Sunflower 2 (16.67%) Deep freezing method in BARI Sunflower 3 (16.67%), Washing

method with BARI Sunflower 2 (20%) and the water agar method for BARI Sunflower 2 (20%). Irshad *et a*l., (2017) reported 10-20% seedling mortality in sunflower seed and examined the sample having *A. alternata* usually had higher amount of dead seed. Regarding the percentage of germination, infected and dead seed, Blotter paper and PDA were recommended as best during mycoflora isolation. While seed washing and deep freezing can be used fairly. The blotter paper and PDA method were also recommended by many research works.

A total of 9 fungal species were isolated from seeds of sunflower. It was observed that the higher number of fungal isolates was preferably grown on water agar and washing method. The association of associated fungi isolated through water agar and washing method. The present study also showed variability. Our study is consistent with Sheela, (2017), she reported both Blotter paper method and Agar plate method as the most suitable technique for detection of fungi. A total of 9 fungi were found viz. *Alternaria alternata, A. helianthi, Curvularia* sp., *Aspergillus flavus, A. Niger, Aspergillus* sp, *Fusarium* sp., *Rhizopus* sp., *and Stemphyllum* sp. were isolated through five methods on three selected sunflower varieties. The Mean frequency of fungi recorded was found to be Significant variation in all method. The fungi *A. Alternata* was recorded with significantly high frequency by all method. The blotter method was found to be superior in recovery *A. alternata* fungi associated with Sunflower seed. Similar result was found by Dawar and Ghaffar (1991) and Salustiano *et al*., (2006) in sunflower.

Associated fungi found considering all five techniques of isolation from three varities of Sunflower were nine (09) viz. *Alternaria alternata, A. helianthi, A. Curvularia* sp., *Aspergillus flavus, A. Niger, Aspergillus* sp, *Fusarium* sp., *Rhizopus*sp., *and Stemphyllum* sp. All of these fungi were present in BARI Sunflower 2, but BARI Sunflower 3 and Local variety lacks *Curvularia* and *Stemphyllum* in their seeds. The association of these mycoflora was previously reported in seeds of Sunflower (Abdullah and Al-Mosawi, 2010; Afzal *et al*., 2010; Dawar and Ghaffar, 1991; Ghoneem *et al*., 2014; Irshad *et al*., 2017; Patil *et al*., 2018; Sharfun-Nahar *et al*., 2005; Srinivas *et al*., 2017).

However, the washing method was found to be useful in isolation of maximum eight types of fungus except *Stemphyllum*. The occurance of *Curvularia* was only observed in washing method. Similar to the current work, Abdullah and Al-Mosawi, (2010) reported occurance of *Curvularia* in seed washing techniques but Srinivas *et al*., (2017) reported the fungus in standard blotter method too. In Water agar isolated seven types of fungi were isolated except *Stemphyllum* and *Curvularia*. PDA was suitable for isolating seven types of fungi including *Stemphyllum*, excluding *Curvularia* and *A. niger*. Srinivas *et al*., (2017) reported *Curvularia* and *A. niger* in sunflower seeds incubating in agar plate method.

In blotter paper method, five types of fungi were isolated viz., name *Alternaria alternata, Aspergillus flavus, Aspergillus* sp., *Fusarium* sp*, and Rhizopus* sp. which was also found by Srinivas *et al*., (2017) with many others. In this study *Stemphyllum* was only observed in PDA but this fungus was previously reported in SB and DFB method (Ghoneem *et al*., 2014). The SB techniques was reported to be efficient in seed borne saprophyte isolation (Ghoneem *et al.*, 2014).

The deep freezing method was suitable for isolating *Alternaria alternata, Aspergillus flavus, A. Niger, Aspergillus, and Rhizopus.* The fungus found in SB and DBF in this study were also reported by Sharfun-Nahar *et al.*, (2005) previously using same methods. Considering the fact of isolating maximum types of fungal species from the seeds of Sunflower the seed washing, PDA and water agar techniques were found promising. The blotter and deep freezing method were resulting in common types of fungi like *Alternaria alternata*, *Rhizopus*, and *Fusarium*.

The evaluation of extent of mycoflora association with Sunflower seeds in different techniques depict fungus specific suitable techniques for isolation. The *Alternaria alternata, Rhizopus, Aspergilllus flavus* and *Aspergillus* were found to be most common in the seeds of Sunflower which were isolated by all five methods. The high abundance of these fungus was previously reported (Ghoneem *et al*., 2014).

Patil *et al*., (2018) reported *A. alternata* as most frequent fungi that resemble the current work. Abdullah and Al-Mosawi, (2010) reported *Aspergillus* sp. *, Alterneria*

and *Fusarium* as most common genera in the seeds of Sunflower that explains higher frequency of these fungus in the findings of current work. The highest frequency of *Alternaria alternata* (66.3%) and *Rhizopus* (76.7%) was observed in blotter paper method, while highest frequency of *Aspergillus flavus* (43.3%) and *Aspergillus* sp. (53.3%) was observed in water agar method. Sharfun-Nahar *et al*., (2005) reported the isolation of these fungus by both SB and DFB method.

The *Fusarium* sp. observed in four methods except deep freezing, *A. helianthi* did not occurred in blotter paper and deep freezing method, *A. niger* did not occurred in blotter paper and PDA method. *A. helianthi* was previously reported in PDA method like the current work (Afzal *et al*., 2010). The maximum fequency of Fusarium were seen in blloter paper method, *A. helienthi* at seed washing technique and *A. niger* at deep freezing method. Though Sharfun-Nahar *et al*., (2005) found *Fusarium* in both SB and DFB method but DFB was referred to be more suitable for *Fusarium* isolation. Ghoneem *et al.*, (2014) mentioned occurance of fusarium in both SB and DFB method.

The frequency of occurance of different fungi varied with the differenet techniques indicating efficiency of a technique to isolate a fungi. Though some fungi were behaved like selective to some techniques, the most common and frequently occuring fungus was isolated by all five techniques. But considering all the facts like percentage of germination, infection and dead seed, ability of a techniques to isolate maximum species under diffreent genera and frequency of occuring by a specific fungus to the techniques it was evident that blotter paper, PDA, and washing method was highly effective for mycoflora study.

The deep freezing method can be employed for deep hibernating fungi while water agar can isolate various types of fungi but at lower frequency and with higher seed mortality. In 2017, Irshad *et al.*, reported Agar plate method as a very effective as compared to the blotter paper method for isolating seed borne mycoflora. Srinivas *et al.*, (2017) reported standard blotter as superior techniques to agar plate in recovering most of the other fungi associated with sunflower seeds. Patil *et al*. in 2018 recommened blotter paper, 2,4-D blotter and Modified PDA methods as

efficient techniques to study the mycoflora. Dawar and Ghaffar (1991) isolated more fungi by blotter paper method followed by agar plate and deep freezing method.

CHAPTER VI

SUMMARY AND COCLUSION

The sunflower is an annual herbaceous flowering plant grown for its edible oily seeds. It is cultivated at a wide range of geographical conditions. Bangladesh produced about 1975 metric tons of sunflower from 1290 ha of land. Sunflower edible seeds are a good source of phenolic acids and flavonoids which also function as antioxidants. Sunflower plant can also be used as manure and around 25% protein-containing meal can be used for animal preparation.

The experiment was carried out with 3 different sources of sunflower seeds of BARI Sunflower 2, BARI Sunflower 3 and a Local variety with five methods viz., Blotter Paper Method, Deep Freezing Method, Washing Method, PDA media Method and the Water Agar Method to find out, to isolate and identify fungal mycoflora associated with sunflower seeds and determination the frequency of their occurances.

The percentage of germination of seed, how much seed get infected during mycoflora isolation and the percentage of dead seed in the process are important to know for understanding the suitability of a techniques for seed testing.

The blotter paper method was fairly efficient to obtain a better germination BARI Sunflower 3 (86.67%). For all three varieties of sunflower seeds germination percentage ranging from 64-87%.

The maximum percentage of infected seed was found in blotter paper method and seed washing method for BARI Sunflower 3 (73.33%). For all three varieties seed infection ranging from 10-45%.

The percentage of dead seed after incubation was least in blotter paper method for the variety of BARI Sunflower 3 (13.33%) and maximum in water agar method occurance of percent dead sees in all three varieties ranging from 30-40%.

The washing method was found to be useful in isolation of maximum eight types of fungi except *Stemphyllum* sp. The blotter and deep freezing methods were resulting in common types of fungi like *Alternaria alternata*, *Rhizopus* sp., and *Fusarium* sp. The highest frequency of *Alternaria alternata* (66.3%) and *Rhizopus* (76.7%) was observed in blotter paper method while the highest frequency method.of *Aspergillus flavus* (43.3%) and *Aspergillus* sp. (53.3%) was observed in water agar method.

Considering different facts, blotter paper, PDA and seed washing test were found to be most suitable for the mycoflora isolation while deep freezing method was suitable for deep hibernating fungi. It is obvious that seedborne pathogens can cause severe losses to the crop by reducing seed germination and developing seedborne diseases. From the above findings of research the following conclusions can be done-

- A total of 9 different fungal species belonging to different genera were identified from sunflower seeds through five different seed health testing incubation methods. Among them BARI Sunflower 3 showed the highest infection in washing method compared to other method.
- A number of fungi isolated in the present study specially those in the genera viz., *Alternaria, Curvularia, Aspergillus,, Fusarium, Rhizopus, and Stemphyllum* were known to be potent mycoflora producers.
- Among the fungi identified, frequency of *Alternaria alternata* was found significantly highest in all the five methods.

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CHAPTER VIII

APPENDICES

Appendix 1: ANOVA of % germinated seed in blotter method

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	355.6	177.78	16	0.0123 *
Variety	2	288.9	144.44	13	0.0178 *
Residuals	4	44.4	11.11		

Appendix 2: ANOVA of % Infected seed in blotter method

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	466.7	233.33	7	0.0494 *
Variety	2	200.0	100.00	3	0.1600 *
Residuals	4	133.3	33.33		

Appendix 3: ANOVA of % Dead seed in blotter method

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	355.6	177.78	16	0.0123 *
Variety	2	288.9	144.44	13	0.0178 *
Residuals	4	44.4	11.11		

Appendix 4: ANOVA of % Germinated seed in Water Agar method

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	88.9	44.44	0.308	0.751
Variety	2	155.6	77.78	0.538	0.621
Residuals	4	577.8	144.44		

Appendix 5: ANOVA of % Infected seed in Water Agar method

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	266.7	133.3	8	0.0400 *
Variety	2	866.7	433.3	26	0.0051 **
Residuals	4	66.7	16.7		

Appendix 6: ANOVA of % Dead seed in Water Agar method

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	88.9	44.44	0.308	0.751
Variety	2	155.6	77.78	0.538	0.621
Residuals	4	577.8	144.44		

Appendix 7: ANOVA of %Germinated seed in PDA method

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	155.6	77.78	0.538	0.621
Variety	2	622.2	311.11	2.154	0.232
Residuals	4	577.8	144.44		

Appendix 8: ANOVA of %Infected seed in PDA method

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	22.2	11.1	0.069	0.934
Variety	2	822.2	411.1	2.552	0.193
Residuals	4	644.4	161.1		

Appendix 9: ANOVA of %Dead seed in PDA method

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	155.6	77.78	0.538	0.621
Variety	2	622.2	311.11	2.154	0.232
Residuals	4	577.8	144.44		

Appendix 10: ANOVA of %Germinated seed in Deep freezing method

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	422.2	211.11	4.75	0.0878
Variety	2	422.2	211.11	4.75	0.0878
Residuals	4	177.8	44.44		

Appendix 11: ANOVA of %Infected seed in Deep freezing method

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	355.6	177.78	6.4	0.0567
Variety	2	422.2	211.11	7.6	0.0434*
Residuals	4	111.1	27.78		

Appendix 12: ANOVA of %Dead seed in Deep freezing method

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	422.2	211.11	4.75	0.0878
Variety	2	422.2	211.11	4.75	0.0878
Residuals	4	177.8	44.44		

Appendix 13: ANOVA of %Germinated seed in washing method

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	22.2	11.11	0.053	0.949
Variety	2	155.6	77.78	0.368	0.713
Residuals	4	844.4	211.11		

Appendix 14: ANOVA of %Infected seed in washing method

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	155.6	77.8	0.298	0.758
Variety	2	1155.6	577.8	2.213	0.225
Residuals	4	1044.4	261.1		

Appendix 15: ANOVA of %Dead seed in washing method

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	155.6	77.8	0.298	0.758
Variety	2	422.2	211.1	0.613	0.586
Residuals	4	1377.8	344.4		

Appendix 16: ANOVA of Mycoflora frequency in BARI Sunflower 2 variety growing in PDA media.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	16.7	8.3	0.273	0.77025
Mycoflora	3	1666.7	555.6	18.182	0.00205 **
Residuals	6	183.3	30.6		

Appendix 17: ANOVA of Mycoflora frequency in BARI Sunflower 3 variety growing in PDA media.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	50	25	0.692	0.536377
Mycoflora	3	3533	1177.8	32.615	0.000413***
Residuals	6	217	36.1		

Appendix 18: ANOVA of Mycoflora frequency in Local variety growing in PDA media.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	213.3	106.67	0.478	0.637
Mycoflora	4	133.3	33.33	0.149	0.958
Residuals	8	1786.7	223.33		

Appendix 19: ANOVA of Mycoflora frequency in BARI Sunflower 2 variety growing in Blotter.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	173	86.7	2.364	0.156106
Mycoflora	4	3707	926.7	25.273	0.000136 ***
Residuals	8	293	36.7		

Appendix 20: ANOVA of Mycoflora frequency in BARI Sunflower 3 variety growing in Blotter.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	288.9	144.44	2.364	0.210
Mycoflora	2	155.6	77.78	1.273	0.373
Residuals	4	244.4	61.11		

Appendix 21: ANOVA of Mycoflora frequency in Local variety growing in Blotter.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	22.2	11.1	0.143	0.8711
Mycoflora	2	2488.9	1244.4	16.000	0.0123 *
Residuals	4	311.1	77.8		

Appendix 22: ANOVA of Mycoflora frequency in BARI Sunflower 2 variety growing in water agar media.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	495.2	247.6	3.545	0.0617
Mycoflora	6	2047.6	341.3	4.886	0.0095 **
Residuals	12	838.1	69.8		

Appendix 23: ANOVA of Mycoflora frequency in BARI Sunflower 3 variety growing in water agar media.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	160.0	80.00	1.116	0.374
Mycoflora	4	506.7	126.67	1.767	0.228
Residuals	8	573.3	71.67		

Appendix 24: ANOVA of Mycoflora frequency in Local variety growing in water agar media.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	344.4	172.2	2.627	0.12107
Mycoflora	5	2244.4	448.9	6.847	0.00507 **
Residuals	10	655.6	65.6		

Appendix 25: ANOVA of Mycoflora frequency in BARI Sunflower 2 variety growing following deep freezing method.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	66.7	33.3	0.5	0.6400
Mycoflora	2	2066.7	1033.3	15.5	0.0131 *
Residuals	4	266.7	66.7		

Appendix 26: ANOVA of Mycoflora frequency in BARI Sunflower 3 variety growing following deep freezing method.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	688.9	344.4	7.75	0.0421 *
Mycoflora	2	422.2	211.1	4.75	0.0878 .
Residuals	4	177.8	44.4		

Appendix 27: ANOVA of Mycoflora frequency in Local variety growing following deep freezing method.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	93.3	46.7	1.217	0.34548
Mycoflora	4	1533.3	383.3	10.000	0.00334 **
Residuals	8	306.7	38.3		

Appendix 28: ANOVA of Mycoflora frequency in BARI Sunflower 2 variety growing following washing method.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	257	128.6	2.282	0.144608
Mycoflora	6	3267	544.4	9.662	0.000519 ***
Residuals	12	676	56.3		

Appendix 29: ANOVA of Mycoflora frequency in BARI Sunflower 3 variety growing following washing method.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	360.0	180.00	8.308	0.0112 *
Mycoflora	4	266.7	66.67	3.077	0.0825 .
Residuals	8	173.3	21.67		

Appendix 30: ANOVA of Mycoflora frequency in Local variety growing following washing method.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Replication	2	77.8	38.89	0.745	0.499
Mycoflora	5	294.4	58.89	1.128	0.406
Residuals	10	522.2	52.22		