MICROFLORA ON BLACK GRAM [*Vigna mungo* (L.) Hepper] SEEDS FROM DIFFERENT STORAGE CONDITION

M.Sc. Dissertation Submitted for partial fulfillment of Master's Degree in Botany (Plant Pathology and Applied Mycology)

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May 22, 2018

LETTER OF RECOMMENDATION

This is to certify that the dissertation work entitled **"Microflora on black gram [*Vigna mungo* (L.) Hepper] Seeds from different storage condition"** was conducted by **Ms. Laxmi Acharya** under my supervision. To the best of my knowledge, the results of this work have not been submitted to any other academic degree in any other institutions. Therefore, I recommend this dissertation for the final evaluation and acceptance for the partial fulfillment of Master's degree in Science.

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LETTER OF APPROVAL

The Central Department of Botany of Tribhuvan University certifies that **Laxmi Acharya** has passed the Final Examination of Master's degree in Science as of May 19, 2018. This is the final and approved form of the dissertation.

"Microflora on black gram [*Vigna mungo* (L.) Hepper] Seeds from different storage condition"

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ACKNOWLEDGEMENT

I would like to express my sincere gratitude and cordial thanks to my supervisor Dr. Sanjay Kumar Jha, Central Department of Botany, T.U. for his invaluable guidance and encouragement for the completion of this dissertation work. I would also like to express my sincere gratitude and special thanks to my respected teacher Dr. Lal Bhahadur Thapa for his invaluable support and advice.

I would like to acknowledge Prof. Dr. Mohan Siwakoti, Head of Department of Botany and Prof. Dr. Pramod Kumar Jha, former Head of Department of Botany for their support. My sincere thanks to all non-teaching staffs for their assistance in experimental work to finish this dissertation work.

I am equally thankful to my friends Mr. Roshan Gaudel Chhetri, Mr. Santosh Thapa, Miss Manisha Awasthi and Mr. Sujan Balami for their invaluable support to complete this dissertation work.

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May 22, 2018

ABSTRACT

Black gram is a most important summer pulse, which has a great market demand with a good potentiality for trade and export in Nepal. The production of black gram has ceased at higher rate by many fungi, as fungi infest in seeds. In this research, contact and systemic fungicides were applied in different concentrations to prevent its post-harvest lose.

Seeds of black gram from two different storage sources were tested for seed-borne fungi. PDA, Blotter and Sand methods were used to isolate seed-borne fungi, in which PDA method was found to be the best. Seeds from traditional storage were found to be more infested with seed-borne fungi in comparison to seeds from the market. Among 25 isolated fungi species, four dominant species such as *Aspergillus flavus*, *Aspergillus niger*, *Alternaria alternata* and *Fusarium sp* were taken for treatment with chemical fungicides. *Aspergillus flavus* and *Aspergillus niger* were best controlled by Carbendazim, and *Fusarium* sp. and *Alternaria alternate* were best controlled by Mancozeb. Carbendazim was found to be most effective at the concentration of 1600 ppm. These chemicals have shown good inhibitory action against fungi. In addition to this, seeds were treated with plant extracts of *Azadirachta indica* and *Ageratum haustonianum* to reduce the presence and frequency of pathogens. Such seed treatment showed effective control over different selected seed-borne fungi. Plant extracts, which are residue free products and pose lower risk to pollution, were found to be good for reducing the pathogens.

Keywords: Synthetic, Fungicide, Frequency, Pathogen

ABBREVIATIONS

µm	micrometer
$CaOCl_2$	Calcium hypochlorite
CDB	Central Department of Botany
FAO	Food and Agricultural Organization
Gm	gram
ISTA	International rules for Seed Testing
lb	Pound
Ml	Milliliter
Mm	Millimeter
NaOCl	Sodium Hypochlorite
PDA	Potato Dextrose Agar
ppm	Parts per million
psi	Pressure
SS	Sterilized seeds
TU	Tribhuvan University
USDA	United States Department of Agriculture
USS	Unsterilized seeds

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CHAPTER ONE: INTRODUCTION

1.1 Background

Black gram [*Vigna mungo* (L.) Hepper] is excellent source of easily digestible protein. Several factors are responsible for low production of black gram. Among them, diseases play an important role (Nine, 1980; Pal, 1996). Black gram is mainly grown in South Asia and to a limited extent in Southeast Asia. Black gram in India, where it is known as Urd bean, is a common pulse for soups and curry dishes. In Thailand, black gram is cultivated for export. In Nepal black gram is called Mass or Kalo Dal, which is the most loved Dal by Nepalese. It is believed that black gram was domesticated in North South Asia from *Vigna mungo* var. *silvestris* that commonly grows there (Luloki *et al.*, 1980; Fuller 2002)

Black gram is an erect, fast growing annual, herbaceous legume reaching up to 30-100 cm in height. It has a well-developed taproot and its stems are diffusely branched from the base. Occasionally it has a twining habit and it is generally pubescent. The leaves are trifoliate with ovate leaflets. Flowers are small, auxiliary and bright yellow in color .The fruit is cylindrical, erect pod (Ecocrop, 2011).The pod is hairy which contains 4-10 black seeds.

Economic importance of Black gram

Seeds of leguminous plants are used as protein rich food. Pods containing grains are the economic portion. The wastes or stalks are called the 'haulm' or 'stover'. Haulm is used as green manure and has high value cattle feed. The genus *Vigna Fabaceae*, formerly *Leguminosae*, compose more than 200 species that are native to the warm regions of both the old world and new world. The genus *Vigna* contains several species that are of considerable economic importance in many developing countries (Shahjahan, 1995).

Seed sprout and green pods are edible and much appreciated for their high digestibility and lack of flatulence induction (Jansen, 2006; Fery, 2002). *Vigna mungo* is usually used as cattle food as a fodder, but the plant, seeds and by-products are also consumed by other species (Fuller, 2004). It can be used as cover crop and green manure, and is often used as dry season intercrop in rice and wheat as it has a beneficial effect on soil nutrient status (Jansen, 2006; Parashar, 2006). Harvesting of *Vigna mungo* seeds can be done by picking the pods or by uprooting or cutting the whole plants. The crop residues (stems, leaves and empty pods) are then available for fodder (Fuller, 2004).

Nutritional importance of Black gram

Vigna mungo has a taproot. It is an N-fixing legume that improves soil fertility and soil physical properties (Parashar, 2006). Its cultivation does not require N fertilization but N fixation is improved by inoculation with local rhizobium strains (Sharma *et al.*, 2013). It provides supplementary food to the farmers and nitrogen to the other crop. Associations with maize, groundnut or cajan pea can improve productivity of those crops by 42-53% (Krishna, 2010). Black gram is very nutritious as it contains high levels of protein (25g/100g), potassium (983 mg/100g), calcium (138 mg/100g), iron (7.57 mg/100g), niacin (1.447 mg/100g), thiamine (0.273 mg/100g), and riboflavin (0.254 mg/100g) (Bhagwat *et al.*, 2008). It complements the essential amino acids provided in most cereals and plays an important role in the diets of the people of Nepal and India (Ahmedani *et al.*, 2007). It has been shown to be useful in mitigating elevated cholesterol levels (Menon and Kurup, 1976; Indira and Kurup, 2013). The seeds are rich in protein (24-26%) and starch (35%). It also contains trypsin inhibitors and condensed tannins, sometimes in larger amounts than chickpeas, faba beans and peas (Wiryawan, 1997).

Global and national status of Black gram

About 66% of population depends on agriculture for their livelihood and share 38.81% GDP (MOAC, 2004). Grain legumes covers (316,010 ha) about 10% total cultivated land (309,100 ha) and ranked 4th in terms of area after rice, maize and wheat and the average productivity is 840 kg/ha (MOAC, 2004). Among them black gram has occupied the 2nd position after lentil in area (32,152 ha) and with the productivity 793 kg/ha (MOAC, 2004). The production of black gram globally is around 8.5 million tones. India contributes nearly 70% of world production followed by Myanmar and Thailand. Grain legumes play an important role in Nepalese agriculture contributing towards foods and nutritional security, nitrogen economy, crop intensification, diversification and sustainable farming systems. Grain legumes rank fourth in terms of acreage (about 10.8% of total cultivated land) and 5th in production. The current estimates for area, production and productivity of grain legumes in Nepal are 334,323 ha 319,770 metric tons and 956 kg/ha respectively. Area, production and productivity of lentil have been increased by 111%, 257% and 69% respectively in between 1985/86 and 2012/13 major grain legume and accounts for 62% of area and 65% production of total grain legumes and have emerged as an important agricultural export commodity (MoAD, 2013).

Storage condition and seed-borne fungi

Agricultural production mainly depends on the quality of seeds (Rajbhandary, 1991). There is a need to search for alternative approaches to store grains/cereals for human consumption without toxicity problems that are eco-friendly and not capital intensive. Plant extracts of many higher plants have been reported to exhibit antibacterial, antifungal and insecticidal properties under laboratory trails (Satish *et al.,* 1999; Okigbo and Ogbonnaya, 2006; Ergene *et al.,* 2006; Kiran and Raveesha, 2006; Mohana and Raveesha, 2006).

Seed borne fungi are defined as those "that are dispersed with seeds of the host" (Ingold, 1971). Seeds sheltering fungi can be defined as being either contaminated or infected (Ingold, 1953). Quality of seeds can be determined by presence or absence of seed borne fungi on seed surface. Mycotoxins are toxic substances produced mostly as secondary metabolites by filamentous fungi that grown on seeds, grains, feed and contaminate them during storage and pose the most serious threads to human and animals health while consumption. The world Health Organization estimated that approximately 25% of the world's grains and crops were contaminated by mycotoxins, and more than 300 fungal metabolites are reported to be toxic to man and animals (Galvano *et al.,* 2001).

Toxins produced by some fungi like *Aspergillus flavus* render the food grains inedible and some may induce internal hemorrhage or even carcinogenesis (Salesh *et al.,* 1988; EL *et al.,* 2007). The deterioration of stored seed becomes faster if the seeds are not properly dried and the moisture is not controlled (Bass, 1973 and Delouche *et al.,* 1973). Many factors are responsible for seeds longevity in storage these includes moisture content, temperatures, relative humidity, initial viability, stage of maturity at harvest, storage gas and initial moisture content of seed entering into the storage (Harrington 1972 and Barton 1961).

Many plant pathogens are transported by botanical seed and other propagating materials, while the oral diseases are mainly transmitted through vegetative propagating material. Seed borne diseases are commonly observed in cereals and pulses. There are specially two types of seed born pathogen.

1) Adherent to the outer covering of the seed and
2) Borne inside the seed.

About 90% of the food crops propagated from the seeds and rest 10% are propagated by the vestigial body (Neergard, 1979).

Major seed borne diseases of black gram:

Black gram is an important summer grain in mid hills. The disease may be fungal, bacterial, viral and nematodes. The major disease of black gram are given in table one.

Table 1. List of common black gram diseases and their causing agents

S.N	Common name	Causal Organism
1	Powdery mildew	*Erysiphe polygoni*
2	Cercospora leaf spot	*Cercospora canescens*
3	Stem canker	*Macrophomina phaseolina*
4	Leaf crinkle disease	Leaf crinkle virus
5	Rust	*Uromyces phaseoli*
6	Anthracnose	*Colletotrichum lindemuthianum*
7	Bacterial leaf blight	*Xanthomona sphaseoli*
8	Root rot and leaf blight	*Rhizoctonia solani*

Reduced germinating capacity of seeds is one of the most important damage caused by seed borne fungi. In case of deep seated fungi, the embryo gets infected by the toxins. The metabolites produced by degrading the viable cells. This potentially alters or lowers the germination seeds by killing the embryo. Mycotoxins cause adverse effects on seed germination (Deshpande and Gajewar, 1976). If seeds are infected, with fungi then certain seed borne fungi produce disease symptoms in mature plant (Rajbhandari, 1991). Seeds are most efficient and dominant means of plant propagation and are used by people to cultivate new plant in the same area from its harvest or completely at new area therefore the infested seeds spreads to new area becomes more virulent under new hosts availability. Seed borne fungi may discolor or damage the embryo or the whole seed and thus reduces the quality for consumption and for price as seeds become unattractive and unfit (Agrios, 2005). Toxin produced by fungi like *Aspergillus flavus*, *A. nidulans*, *A. niger*, *Trichoderma viridi* and *Alternaria alternata* also affect the seeds germination (Kamal and Verma, 1987).

Healthy seed is the foundation of healthy plant, a necessary condition for good yield (Diaz *et al.,* 1998). Among various factors which affect seed health, the most important are seed borne fungi that caused reduction in seed germination and seed vigor. Seed borne pathogens reduce

yield up to 15-90% if untreated seeds are grown in the field. The main objective of the present study is to see the effect of seed borne mycoflora in Black gram seeds during different storage condition.

1.2 Rational of the study

Plant disease is simply defined as the impairment of the normal state of plant that interrupts or modifies its vital function. All species of plants, either wild or cultivated alike are subject to diseases. Plant diseases markedly affect social development and man's economy by decreasing in quality and quantity of plant product. The bible and other easily writings mention diseases, such as rusts, mildews blights and blast that have caused famine and other drastic changes in the economy of nation since the dawn of recorded history. Loss of crops from plant diseases may result in hunger and starvation, especially in less developed country where access to disease control method is limited.

Nepal is an agriculture country. Agriculture system of country is still traditional have a few knowledge about the plant disease and their cause and control. But there has various investigations of different species of pathogens from different cultivated as well as wild host. There are lots of factor like climatic condition, local environment etc. which affect the severity and occurrence. Therefore, the present work entitled as microflora on black gram seeds from different storage condition of Chitwan district.

1.3 Objective of the Study

Major objective

1. Identification of fungi from seed of two different storage conditions and control of some dominant fungi from chemical fungicide.

Specific objective

1. To evaluate the frequency of fungi from the seeds.
2. To compare the efficacy of chemical fungicides.
3. To study the effectiveness of fungicides on the pathogen.

1.4 Limitation of the study

1. Isolated fungi were identified to the species level in the basis of morphological characteristics. Some fungi showed peculiar morphological characteristics, which made us difficult to identify them to the species level.
2. We used Agar plate, Blotter, and Sand methods for isolation of fungi. PDA media is used for the Agar plate method, which may limit the isolation of slow growing pathogens.

CHAPTER TWO: LITERATURE REVIEW

All available literature, which seems to meet the requirement of present work had been reviewed as much as possible through various related papers and cited.

Nair and Arora (1994) isolated nine seed borne fungal pathogens from legume crops. *Aspergillus niger, Verticillium* sp., *Alternaria alternata, A. flavus, Phome* sp., *Rhyzoctinia bactaticola, Gliocladium virens* and *Botrydiplodia theobromae* were obtained from diseased and wrinkled seeds of legume crops. The germination percentage was highest in all the healthy seeds followed by wrinkled diseased seed.

Nayak B.K. *et al.,* (1950) observed the seed borne fungi and their effects on germination and seedling mortality in black gram. They had used agar plate and blotter method for the isolation of fungi total of 37 fungi taxa including with *Aspergillus* spp were isolated which were responsible for seedling rot during germination.

Hussain *et al.,* (2007) studied seed borne fungi of Gram after surface disinfecting with either 10% sodium hypo chloride or mercuric chloride for five minutes using Agar plating, Blotter, Deep freezing and component plating methods. The fungal species isolated were *Alternaria alternate, A. flavus, A. niger, Chaetomium* sp., *Cladosporium herbarium, Curvularia lunta, F. oxysporium, Gliocladium* sp., *Helminthosporium* sp., *Nigrospora* sp., *Penecillium* sp., and *Rhizoctina bataticola.*

Raju (2013) tested lentil seeds from four types of storage containers and two storage conditions and isolated *Fusarium oxysporum* (3.50%), *Aspergillus niger* (19.75%), *Aspergillus flavus* (5.75%), *Penicillium* sp. (2.50%) and *Rhizopus* sp. (2.89%). From two types of storage conditions (three months after storage, six months after storage) and four types of storage containers (tin pot, polythene bag, paper bag and gunny bag) it was observed that the percentage germination and normal seedlings were found decreased as the storage time increased. Ghangaokar and Kshirsagar (2013) used blotter technique and isolated *Aspergillus flavus, Aspergillus niger, Aspergillus terreus, Alternaria alternate, Fusarium moniliforme, F. oxysporum, Rhizopus nigricans, Trichoderma viridae, Humicola* sp., *Penicillium chrysogenum, Mucor hiemalis, Moniliasito phila, Nigrospora oryze* from untreated seeds and only seven species from treated seeds with 0.1% NaOCl for ten minutes.

Hussain *et al.,* (2007) isolated *Alternaria alternata, Aspergillus* spp.*, Fusarium moniliforme, Mucorhiemalis, Chaetomium* spp.*, Penicillum citrinum, Aspergillus niger, A. flavus, A. terreus* and *Nigrospora* spp.*,* from treated seed samples collected from various locations of the Punjab, Pakistan and were analyzed for externally and internally seed borne fungi. Whereas, *F. moniliforme, A. alternata, M. hiemalis, Chaetomium* spp.*,* and *Aspergillus niger* were common in all samples while *P. citrinum, A. flavus, A. terreus*and *Nigrospora* spp., were only isolated from untreated seeds.

Three pea varieties were treated with Chlorox (1%), hot water (52^0C for 12-13 min) and fungicide Vitvax, 12 fungi isolated. Among them *Alternaria* sp.*, Fusarium* sp.*, Aspergillus* sp.*, Penicillium* sp.*,* and *Rhizopus* sp, were found in high frequencies (Begum *et al.,* 2004). Mycoflora consisting of *Cephalosporium, Alternaria, Aspergillus, Penicillium, Rhizopus, Acrothecium, Fusarium, Rhizoctonia, Curvularia, Pythium* and *Trichoderma* are isolated and identified from root nodules of leguminous plants (Chhonkar and Subba, 1966).

The inherent longevity of seeds, the conditions to which they are exposed to prior to harvest and their treatment during harvest and threshing (drying), are the main determinants of the responses of seeds to storage (Agrawal, 1980; Delouche *et al.,* 1973). The germination percentage of Mung bean, cowpea, snap bean etc. seeds were treated with four fungicide increases, and amount of pathogen decreases. Benlate and Captan were the most effective against *Colletotrichum lindemuthianum, Macrophomina phaseolina, Cercospora cruenta, Fusarium moniliforme, Fusarium oxysporum, Aspergillus* spp. and *Penicillium* spp. (Rodriguez, 1984).

Several methods for controlling such fungal borne diseases have been evaluated by studying the use of resistant varieties of crop (Brisa *et al.,* 2007), chemical control of pathogens, novel agricultural practices (Punja *et al.,* 1986) by applying various plant volatile compounds (El-Mougy *et al.,* 2007), and plant extracts (Kumar and Tripathi, 1991).

Agar plate method and blotter method were used to isolate the fungi from seeds of Mung bean. Total eight fungi was isolated and identified as *Alternaria alternata, Aspergillus flavus, Aspergillus niger, Aspergillus candidus, Fusarium semitectum, Penicillium citrium, Macrophomia phaseolina* and *Rhizopus stolonifer.* Between these two methods blotter method yielded higher number of fungi (Sarita *et al.,* 2014).

Rathod *et al.,* (2012) used three methods as agar paper method, standard blotter paper and seed washes methods to identify the seed mycoflora of different varieties of legumes.

Among the three methods, the agar paper method was found to be suitable as in less incubation; there was higher percent incidence of seed mycoflora.

Nik and Parbery (1977) tested 22 tropical and 3 temperate pasture legume species 42 species of fungi were isolated. Most of species were isolated from blotter method, which required supplementing with procedures such as isolation onto PDA and Nash-Snyder ager to ensure the isolation of as many species as possible from each seed sample. Eight species were *Fusarium acuminatum, F. avenaceum, F. equiseti, F. fusarioides, F. oxysporum, F. poae, Diaporthepha seolorum* and *Phoma sorghina.*

Embaby and Abdel-Galil (2006) isolated 200 fungal species belonged to five genera namly as *Alternaria, Aspergillus, Epicoccum, Fusarium* and *Trichoderma.* They concluded that agar plate (PDA medium) was enhanced for seed testing than blotter method and gave higher numbers of fungal colony. Seed borne fungi in bean were *Aspergillus niger, A. ochraceus, A. parasiticus, A. flavus, Aspergillus* spp. The fungi most frequently isolated from the cowpea seeds were *Aspergillus niger, A. parasiticus, Aspergillus* spp. and *Fusarium* spp. The frequency of isolated fungi from lupine seeds were *Aspergillus niger, A. flavus, Aspergillus* spp., *F. oxysporum, Fusarium* spp., *Alternaria* spp. and *Trichoderma* spp., respectively.

The predominant species of the genera isolated from black bean were *Alternaria alternata, Sclerotinia sclerotiorum, Rhizoctonia solani, Fusarium semitectum and Acremonium strictum. Alternaria alternata, Lasiodiplodia theobrome, Drechslera tetramera* were isolated from cowpea seeds in standard blotter and agar plate methods (Castillo *et al.*, 2004). The most common seed-borne fungi on dry beans (*P. vulgaries)* in Croatia were *Alternaria* spp., *Aspergillus* spp., *Fusarium* spp., *Botrytis* spp., *Chaetomiumm* spp., *Penicillum* spp., *Rhizopus* spp., *Cladosporium* spp. *and Trichothecium* spp. (Domijan *et al.*,2005). Nasir (2003) tested the four incubation method for isolation of seed borne fungi. Among four incubation method, incubation by PDA method yielded the highest number of fungal species either with or without the treatment.

Ghangaokar and Kshirsagar (2013) studied seed borne fungi on different legumes. The legume seed born fungi was screened by using blotter plate method from selected untreated and treated seeds. The untreated seeds were found to be associated with highest number of seed borne fungi. The isolated fungi are *Alternaria alternata, Chaetomium* spp., *Penicillium citrinum, Aspergillusniger, A. fumigatum, A. flavus, Rhizopus nigricans,*

Fusarium oxysporum, F. moniliform, F. solani, Chaetomium sp., *Curvularia lunata, Macrophominsp, Moniliasp, Penicillium* sp., *Rhizoctonia* sp., *Trichoderma.*

Rodrigues (1984) tested the germination percentage of Mung bean, snap bean, cowpea etc. treated with four fungicide, increases the germination percentage and decreases the amount of pathogen. Fungicide Benlate and Captan were the most effective against *Colletotrichum lindemuthianum, Macrophomina phaseolina, Cercospora cruenta, Fusarium moniliforme, Fusarium oxysporum, Aspergillus* spp., and *Penicillium* spp.

Ellis *et al.,* (1975) treated soybean seed with three fungicides. Seeds treated with fungicide had a higher germination in vitro and emergence in vermiculite and field soil than non-treated controls. Nasir (2003) found that the growth *Alternaria alternata, Cladosporium cladosporoides, Macrophomina phaseolina, Drechlera specifera, Fusarium oxysporium* and *Rhizoctonia solani* were significantly reduced on PDA media amended with 0.1% of either Captan, Vitavax, Dithane M45, Thiramand Benomyl.

Rasheed *et al.,* (2004) studied fungi of groundnut seed using ISTA techniques. Component plating of groundnut seed showed that seed coat was greatly infected by fungi viz. *Alternaria citri, Aspergillus flavus, A. niger, Fusarium oxysporium, F. semitectum, F. solani, Macrophomina phaseolina* and *Rhizoctina solani.* Reduced number of fungal species in surface sterilized seed indicated that most of the fungi were located on seed coat. In seeding symptoms, it showed *Macrophomia phaseolina, Fusarium* sp., *Rhizoctonia solani, Aspergillus flavus* and *A. niger* resulting pre and post emergence rot.

Shah *et al.,* (2006) reported that *Fusarium oxysporum* against Carbendazim, Mancozob conjoint Carbendazim and Sulphur. All the treatments significantly reduced the growth of the *F. oxysporum* as compared to control but it was observed that the growth of the fungus were significantly less in 10000 ppm concentration as compared to 10, 100 and 1000 ppm concentration of fungicides. On comparative analysis of different fungicides tested mancozeb showed maximum inhibition of *F. oxysporium* as compared to other fungicides. Sudha *et al.,* (2013) reported Carbendazim (73%) was effective and superior to other fungicides at 0.15% concentration, whereas benomyl, triademefon and thiophanate methyl were not effective in inhibiting the mycelia growth of *Aspergillus flavus.*

According to Pathak and Zaidi (2013) study the effect of various fungicides viz. Carbendazim and Cancozeb on the incidence of seed borne fungi and their effect on seed

germination. The seed treatment by the fungicides showed that Mancozeb increased their germination percentage and reduced seed mycoflora.

Seeds borne fungi like *Alternaria alternata, Aspergillus niger, Aspergillus flavus, Rhizopus* spp, *Fusarium moniliforme* were detected from seeds of wheat treated with Carbendazim and Mancozeb showed the very lowered frequency values (Siddiqui and Arif, 2004). KB and Moeng (2015) studied the effect of fungicides were Dithane M-45 and copper oxychloride, both the fungicides were not so effective in reducing fungal incidence and increasing seed germination. Rathod *et al.,* (2010) and Tu (1982), Carbendazim and Mancozeb were found more effective against seed borne fungi *Aspergillus flavus* in groundnut and soybean.

Singh et al., (1980) owed the fungicidal and bactericidal properties of extract from neem leaves either in vitro or in vivo trials to the presence of several antimicrobial active ingredients in leaves of neem tree such as desactylimbin, quercetin and sitosterol.

Javed and Bashir (2012) tested methanolic, aqueous and n-hexane extract of *Ageratum conyzoide*s form leaf, infloraroscence and root and found methanolic extract of all parts to be effective on control of *Fusarium solani*.

Leaves extract of *Ageratum conyzoides* essential oil possesses antifungal activities against *Aspergillus flavus* and inhibitor effect on aflatoxin production (Nogueira et.al., 2010).

CHAPTER THREE: MATERIAL AND METHODS

3.1 Mycoflora isolation and identification

3.1.1 Seeds sample collection

In this study the seed samples of black gram were collected from local market (Geetanagar) and traditional storage from village (Shivanagar) of Chitwan district. Seeds were tagged as local market and as traditional storage.

3.1.2 Fungi isolation

a) Standard Blotter Technique

In this method, the seeds were plated in the sterilized Petri plates on three layers of moistened and sterilized blotters. For this at first the blotters were cut into appropriate size and autoclaved at 121^0C for 30 minutes at 15 psi. The Petri plates were also sterilized in a hot air oven at 160^0C for two hours then the sterile blotters were placed within Petri plates aseptically (i.e inside laminar Airflow chamber) followed by moistening them with sterile water removing the excess water later on. Excess water was removed to avoid extra moisture that could check or minimize the fungal growth on blotters. The plates were then plated or inoculated with sterilized and non-sterilized seeds separately with the help of needle and Bunsen burner, five replicas for each treatment. The seeds were placed at equal distance from each other. For sterilization process, seeds were treated with 2% NaOCl for two minutes (sterilization of seeds are done for the isolation of entophytic fungi.) and were washed with sterile water for three times for rinsing. Finally the Petri plates were incubated at 22 ± 2^0 C in incubator .Fungi grow more successfully at lower temperature.

b) Agar Plate Method

In this method, seeds were placed in sterilized Petri dishes containing synthetic Potato Dextrose Agar media at the recommended ratio of 39 gm/1000 ml. The media was sterilized in autoclave at 121^0C and 15 psi for 30 minutes. Prepared media was added with antibiotic to protect the media from bacterial contamination. About 10 ml of media was poured in the dishes aseptically near the Bunsen burner to avoid fungal contaminations. Then sterilized and non-sterilized seeds were inoculated aseptically on solidified media at equal distance from each other with five replicas of each treatment. Finally, the plates were sealed with cello tape and incubated at $22\pm2\ ^0$C.

c) Sand method

In this method, fine Sand was sterilized in autoclave at 121°C and 15 lbs/sq inch pressure for 20 minutes. Then the sterilized plastic pots were filled with this sterilized sand. Then sterilized seeds and non-sterilized seed from village and market were inoculated in the pots according to the size of the seeds at equal distance. Finally each pots are placed in incubator for incubation at maintain temperature of 22±2°C. All the pots should be moistened with sterilized water throughout the experiment.

Observation of seeds microflora were carried out after one week to 4 complete weeks. During the observations period, the sign of fungal infection, Frequency, Color were recorded. The fungal colonies observed on the seeds were culture and sub culture on PDA media slant by using single hyphal tip method. These slants were stored in refrigerator for further study.

3.1.3 Fungi identification

The isolated fungi were carefully and aseptically maintained in PDA culture slants. The fungi were identified by photographs, preparing slides with the help of monographs, literatures and following books:

- Thom and Raper 1945.
- Barnett and Hunter 1960.
- Gilman 1975.
- Watanabe 2010.

3.1.4 Chemical control

Chemical control is an most important method used to control plant diseases (Agrios,1997; Cline et al.,1988). Fungicides are easily available in store and inexpensive. Fungicides in seed borne pathogen are used to kill or inhibit growth of seed borne fungi. But the effect of fungicides depends upon a type and nature of fungi being associated and broadly on the spectrum of fungicide used. Fungicides can either be contact, translaminar or systemic. Contact fungicides are not taken up into the plant tissue and protect only the plant where the spray is deposited for example Mancozeb, Copper etc. Translaminar fungicides redistribute the fungicides from the upper, sprayed leaf surface to the lower, unsprayed surface. Systemic fungicides are taken up and redistribute through the xylem vessel for example Carbendazim. Systemic fungicides are incubating fungi that are out of reach of contact and translaminar fungicides.

Fungicides Mancozeb, Carbendazim and Copperoxy chloride were used in this study in concentrations of 800 ppm, 1200 ppm, 1600 ppm for the most dominant pathogens *Aspergillus flavus* , *Aspergillus niger, Alternaria alternata* and *Fusarium* sp. Fungicide were mixed with synthetic PDA and inoculation of Pathogen were done ascetically (i.e. in laminar air flow chamber).

3.1.5 Seed treatment

The quality of seed used by the farmer determines the structure of their agricultural practice. Seed treatment has been considered as the cheapest and the best method for the direct control of plant disease (Neergaard 1971). In this experiment, seeds are treated with Plant extracts *Azadirachta indica* and *Azeratum houstonianum*. The leaves of both *Azadirachta indica* and *Ageratum houstonianum* collected from local area of Chitwan district were washed and dried for seven days. These dried leaves were then crushed and converted into powder form by using Blender. Such power was mixed with seeds, and the mixture was stored for up to five to seven days. Such treated seeds were then assayed for seed mycoflora by employing Agar plate method and following the International Rules for Seed Testing. According to International Seed Testing Association (ISTA,1950), Seeds of each sample should be placed equidistantly and aseptically on Potato dextrose Petri plates at the rate of 10-12 seeds per plate and the plates should be incubated at room temperature (22±2 °C) for seven days under diurnal cycles of 12 h. light and 12 h darkness. Seeds without treatment served as control. Our observations on seed mycoflora were recorded after eighth day of incubation. We observed fungal growth on seeds by using stereo binocular microscope.

CHAPTER FOUR: RESULTS

4.1 Comparison of frequency on isolated fungi

Altogether 25 species of fungi were isolated from seed of black gram. Among isolated fungi *Aspergillus, Alternaria, Fusarium, Cladosporium, Rhizoctinia, Rhizopus, Penicillium, Curvularia,* and *Trichoderma* were more common. Most frequently appeared genus was *Aspergillus* and followed by *Fusarium* sp. *Aspergillus flavus* shows the highest presence among all isolated fungi on seeds followed by *Aspergillus niger*, *Alternaria alternata, Fusarium* sp. 6 species of *Aspergillus*, 4 species of *Fusarium*, 2 species of *Cladosporium* ,2 species of *Penicillium*, 2 species of *Alternaria* , 1 *Curvularia* sp, 1 *Rhizoctonia* sp, 1 *Rhizopus*, 1 *Mucor* sp, 1 *Trichoderma* sp and 4 Unidentified species were isolated from seeds sample. Mycoflora were mostly isolated from traditional storage than local market (See Annx1).

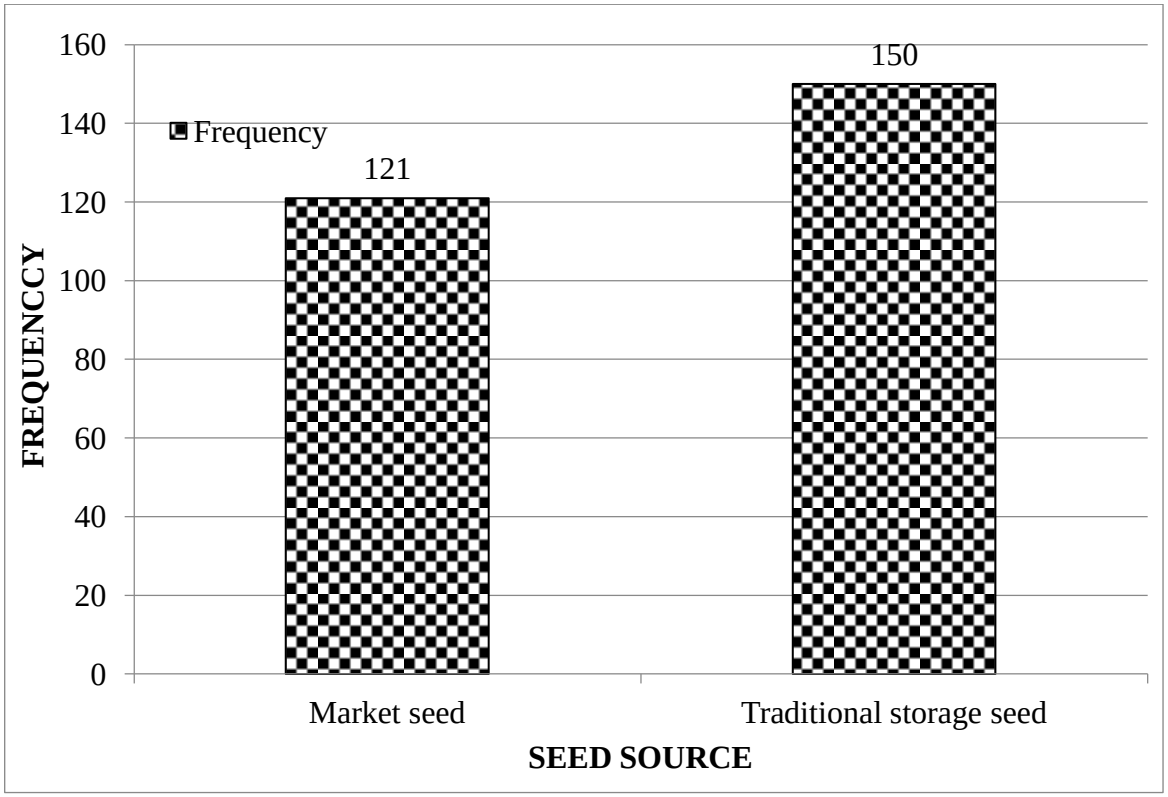

Figure 1: Total frequency of seed fungi

It was found that frequency of fungi more prominent or intense in seeds from source two followed by market seed (Fig.1). Seeds from traditional storage seed were infested with highest frequency.

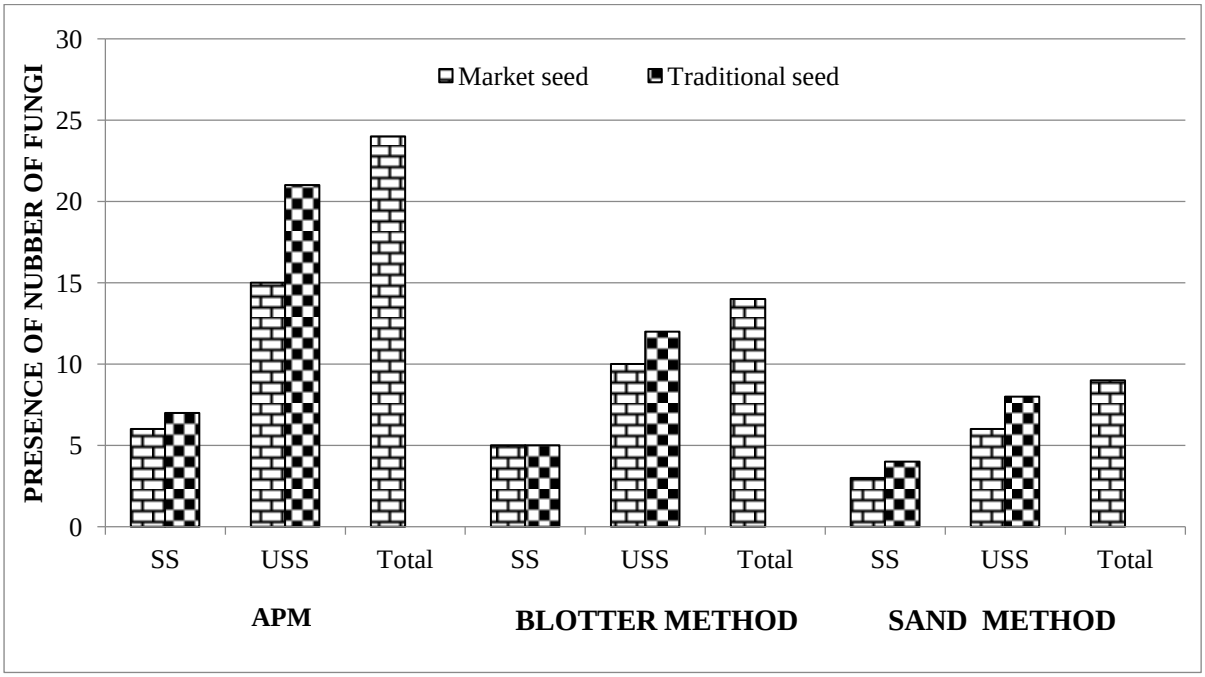

Figure 2: Presence of fungi from different seed source

In both sterilized and unsterilized seed, fungal presence was higher in traditional storage seeds, with compare to market seeds in all three methods. Fungal presence was higher in Agar plate method followed by Blotter and Sand method (Fig.2).

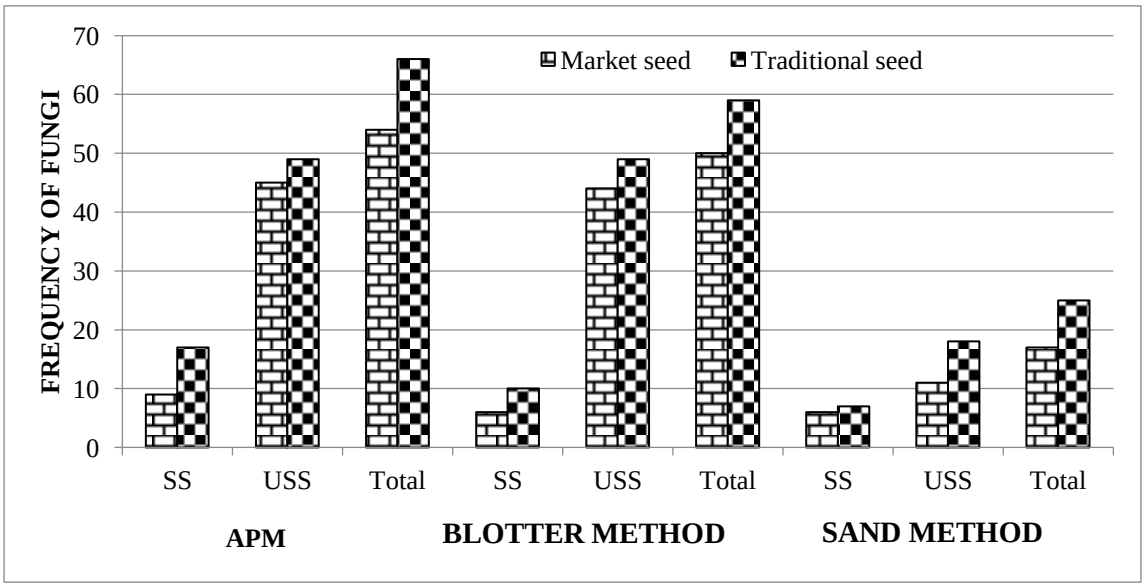

Figure 3: Frequency of fungi from different seed source

In both sterilized and unsterilized seed, fungal frequency was higher in traditional storage seeds, with compare to market seeds in all three methods. Fungal frequency was higher in Agar plate method followed by Blotter and Sand method (Fig.3).

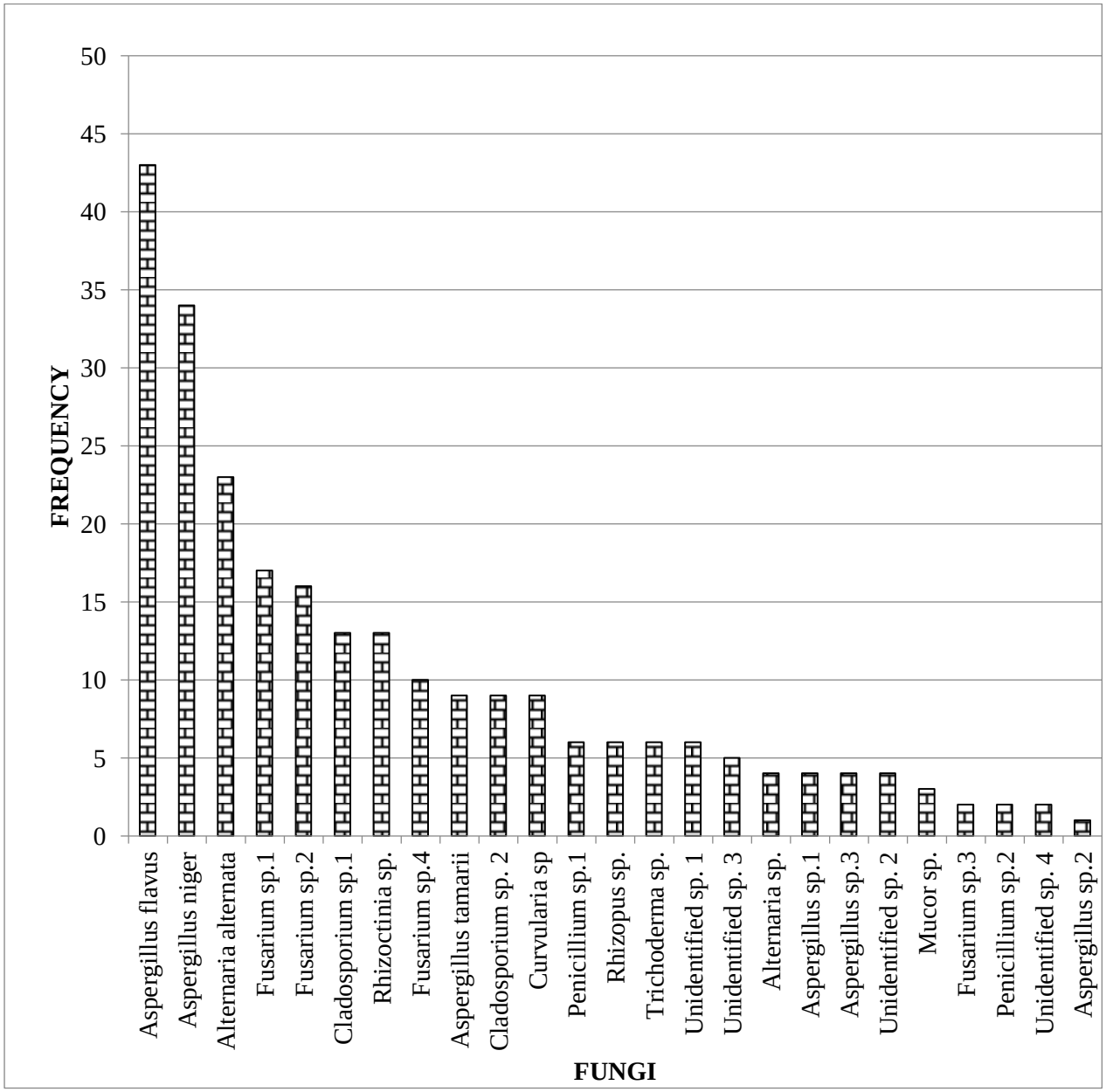

Figure 4: Frequency status of fungi

The frequency graph reveals that the most repetitive seed-borne fungi were *Aspergillus flavus* which occurred for 43 times. *Aspergillus flavus* was followed by *Aspergillus niger, Alternaria alternata* and *Fusarium* sp.1. Genus *Aspergillus* followed by *Fusarium* were most present.

4.2 Efficacy of fungicides

4.2.1 Percentage inhibition of fungicides on test fungi

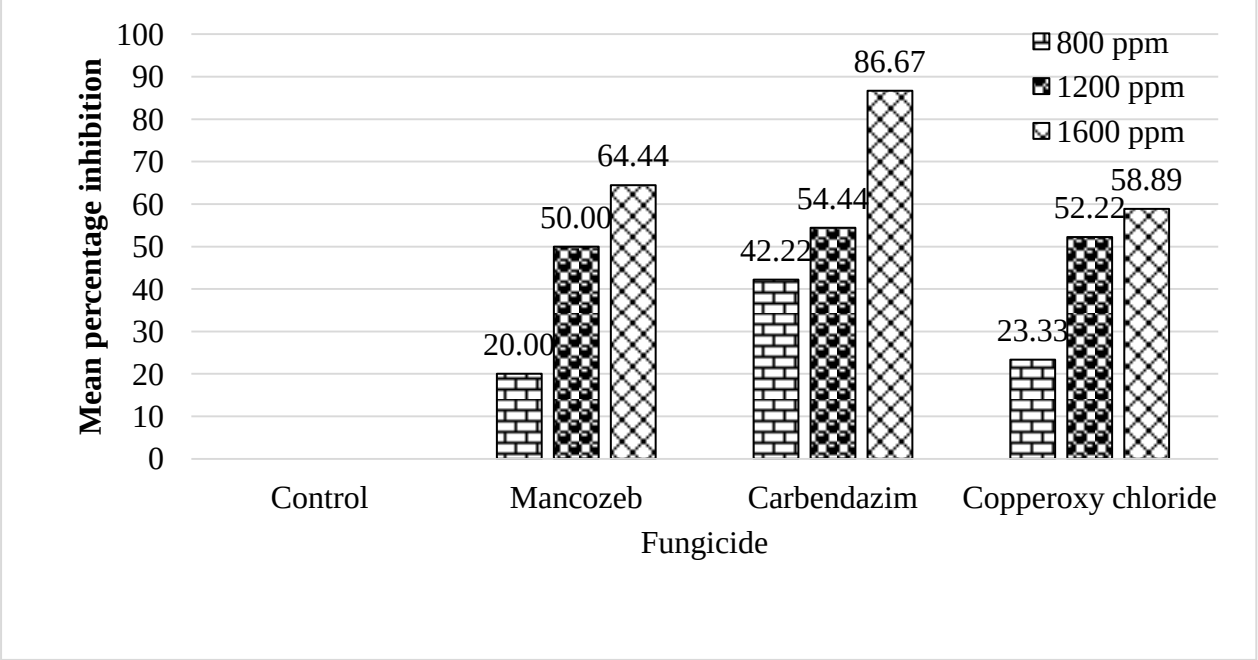

Figure 5: Percentage inhibition of *Aspergillus flavus*.

Among three chemical fungicides best result was found by Carbendazim with 86.67% at 1600 ppm in *Aspergillus flavus* which was higher than the inhibition done by remaining other chemicals (Fig.5).

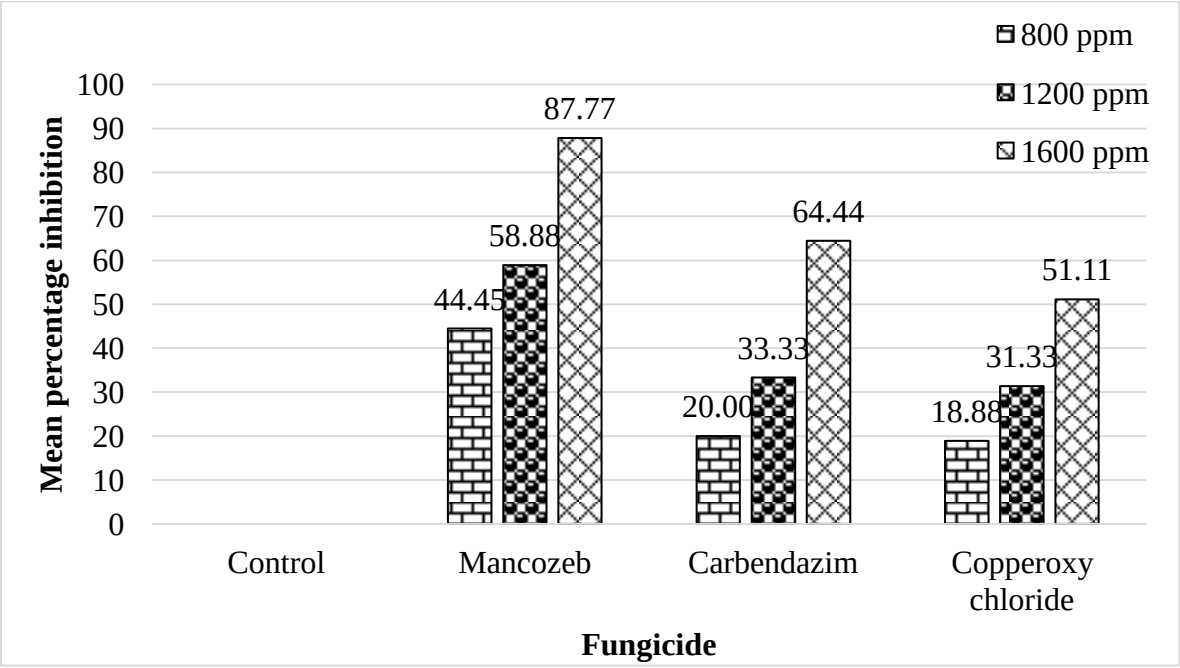

Figure 6: Percentage inhibition of *Fusarium* sp.

Fusarium sp. was best inhibited at 1600 ppm concentration by Mancozob (87.77 %) and by Carbendazim (64.44 %) which was higher than inhibition shown by Copperoxy chloride (52.44 %). The least inhibition was shown by Copperoxy chloride at 800 ppm concentration with 19.11 % (Fig.6).

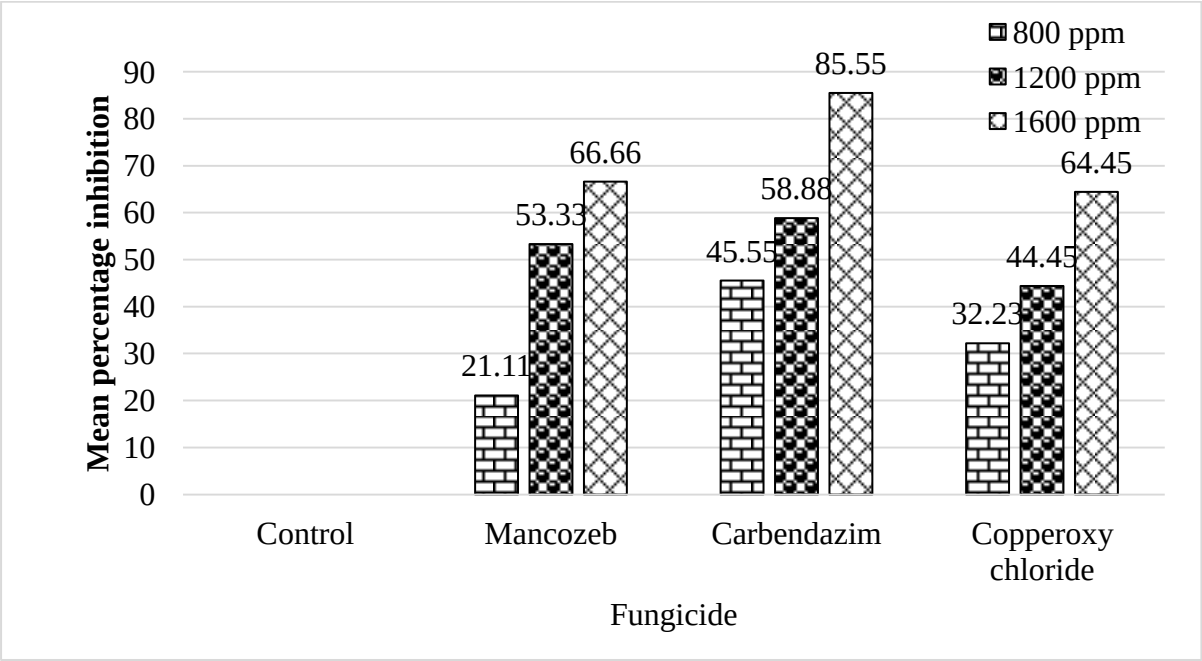

Figure 7: Percentage inhibition of *Apergillus niger*

Aspergillus niger was best controlled by Carbendazim at the concentration of (85.56%) at 1600 ppm and least inhibited by Copperoxy chloride (64.44%) (Fig.7).

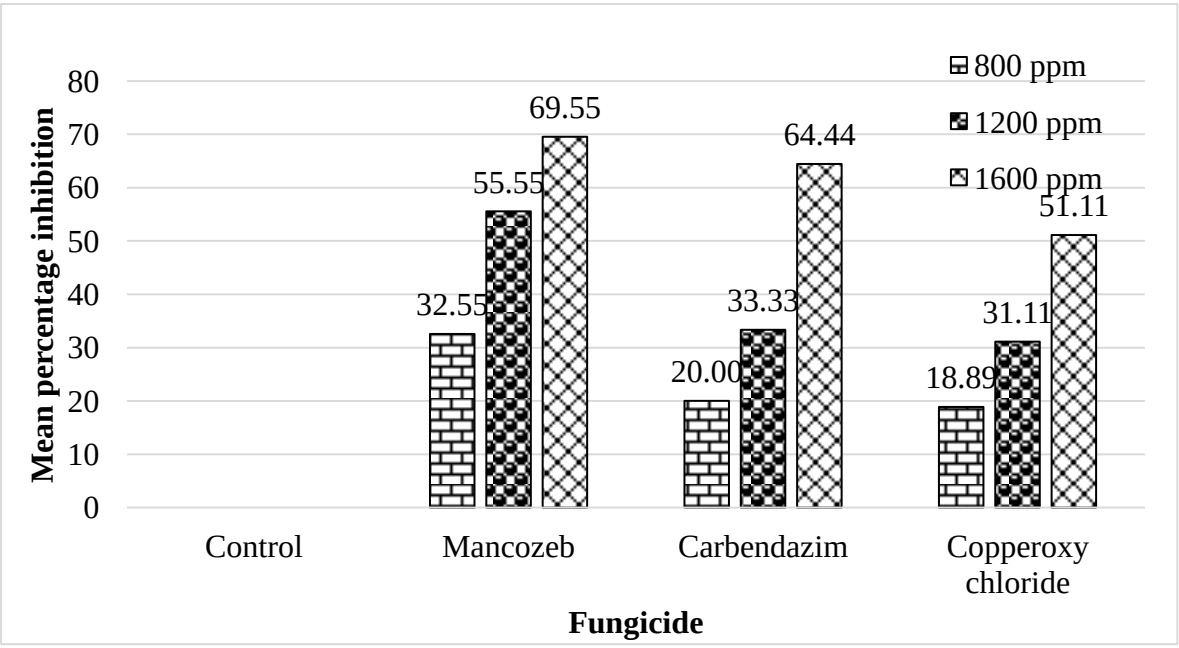

Figure 8: Percentage inhibition of *Alternaria alternata*.

Alternaria alternata was best controlled by Mancozeb at1600 ppm, inhibited to 75.45% and least by Copperoxy chloride at 800 ppm (Fig.8).

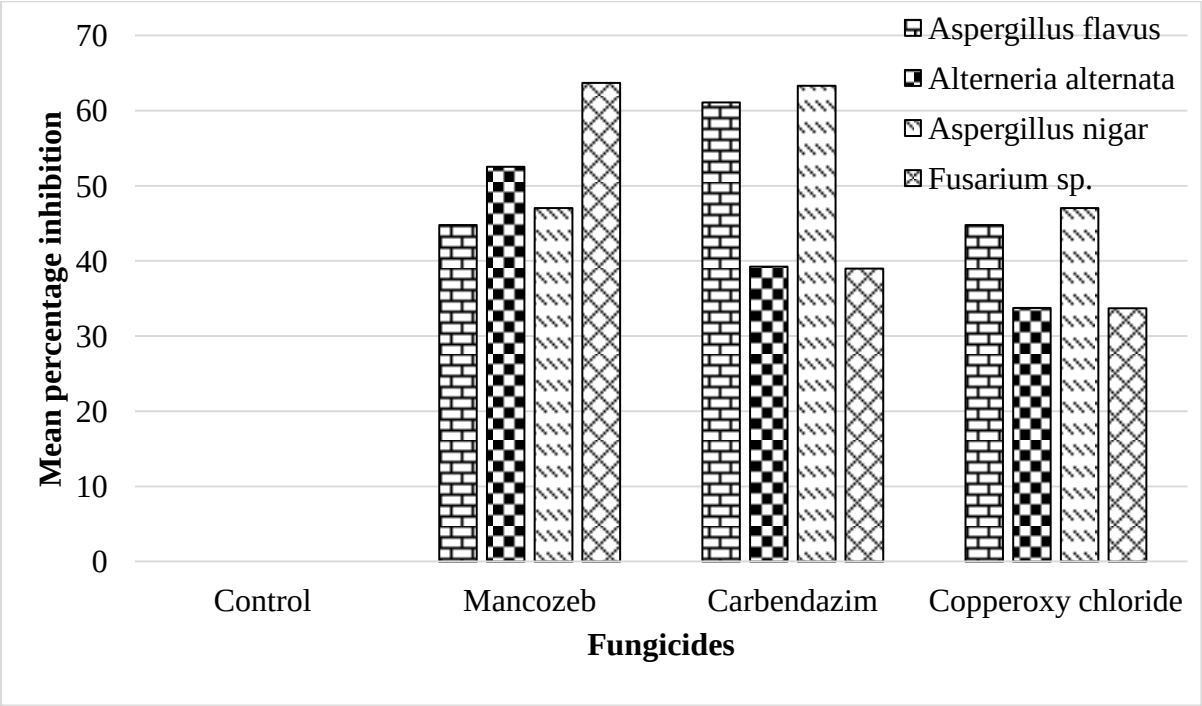

Figure 9: Comparisons of all test fungi against chemical fungicides

Among three fungicides carbendazim was most effective against *Aspergillus flavus* followed by, *Aspergillus nigar, Alternaria alternate, Fusarium* sp. and Mancozeb was most effective for *Fusarium* sp. and for *Alternaria alternata* (Fig.9).

4.3 Efficacy of Plant extract on pathogen

4.3.1 *Azadirachta indica* treated seeds

Table 2: Frequency and incidence of fungi plant extract of *Azadiracta indica* treated seeds; (the value was from 10 plates).

Pathogen	APM			
	Market seed		Traditional seed	
Aspergillus flavus	+	2	+	4
Aspergillus niger	+	2	+	3
Rhizoctinia sp.	+	2	+	2
Penicillium sp.1	-	0	+	1

(+) = Presence, (-)= Absence, Number = Frequency

Table 2 shows the four fungi, which were isolated from seeds treated with *Azadirachta indica* using Agar plate method. Three fungi were isolated from market seeds, and four fungi were isolated from traditional storage seeds. *Aspergillus flavus, Aspergillus niger* and *Rhizoctinia*

sp. are common fungi in both market seeds and traditional storage seeds. *Penicillium* sp.1 was isolated from traditional storage seeds only.

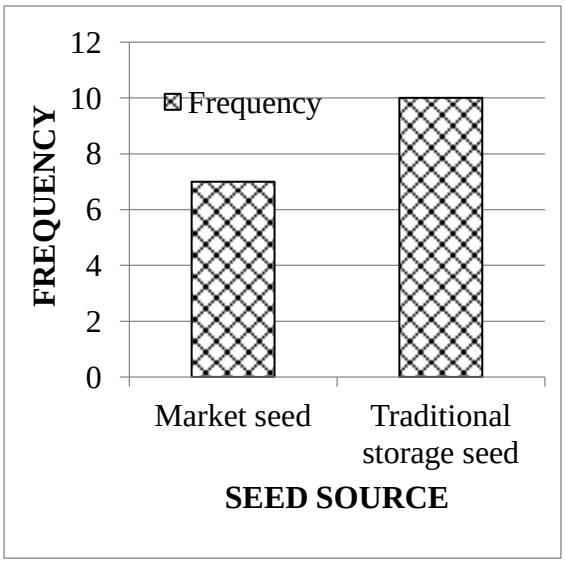

Figure 11: Total frequency of fungi *on Azadirachta indica* treated seeds

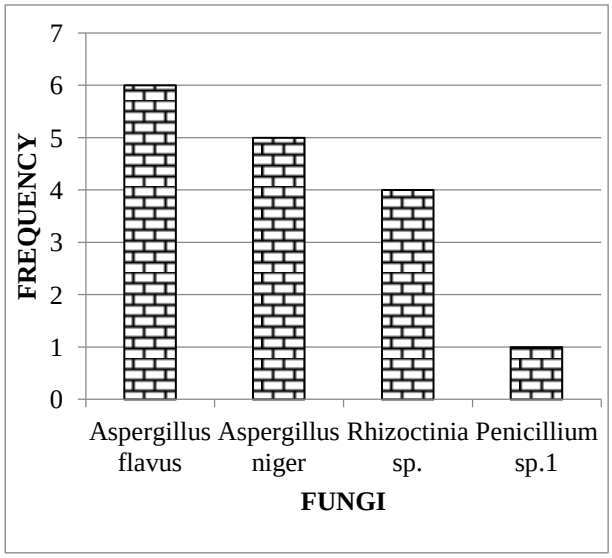

Figure 10: Frequency of individual fungi on *Azadirachta indica* treated seeds

While treating seeds with plant extract *Azadirachta indica*, the presence and frequency of fungi were more in traditional storage seeds than in market seeds (Fig.10). Among the isolated fungi, *Aspergillus flavus* and *Aspergillus niger* were the most frequently encountered (Fig.11).

4.3.2 *Ageratum houstonianum* treated seeds

Table 3: Frequency and incidence of fungi on *Ageratum houstoniaum* treated seeds, (the value was from 10 plates).

Pathogen	APM			
	Market seed		Traditional seed	
Aspergillus niger	-	0	+	2
Cladosporium sp.1	+	2	+	2
Fusarium sp.1	+	1	+	3
Fusarium sp.2	-	0	+	1

(+) = Presence, (-) = Absence, Number = Frequency

Total four fungi were isolated from seeds treated with *Ageratum houstoniumon* using Agar plate method (Table 3). Two fungi were isolated from source one and four fungi were isolated from source two. *Cladosporium* sp.1 and *Fusarium* sp.1 were common for both sources. *Aspergillus niger* and *Fusarium* sp.2 were isolated in source two only.

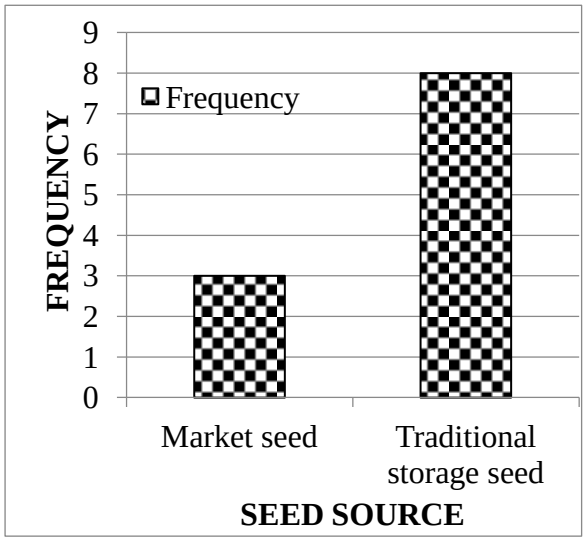

Figure 13: Total frequency of fungi on *Ageratum houstonianum* treated seeds

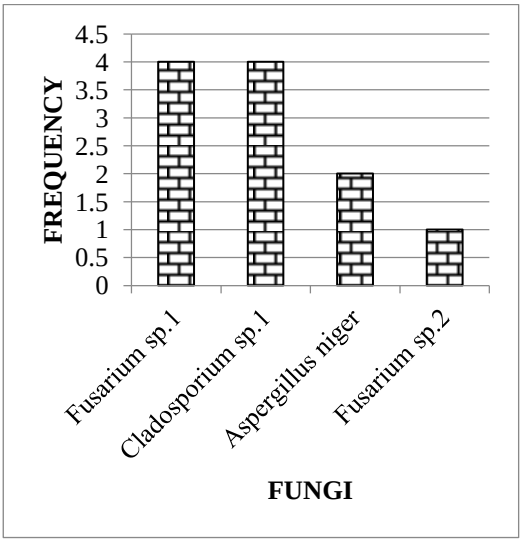

Figure 12: Frequency of individual fungi on *Ageratum houstonianum* treated seeds

Even after seed treatment, the frequency of fungi in traditional storage seeds was higher than in market seeds (Fig.12). The most frequent fungi were *Fusarium* sp.1 and *Cladosporium* sp. 1, and least frequent fungi was *Fusarium* sp. 2 (Fig. 13).

CHAPTER FIVE: DISCUSSION

The present investigation deal with the isolation, identification, comparison and control of seed mycoflora of black gram (Mass) from two different storage sources, namely local market (source one) and traditional village storage (source two). In this study, Carbendazim (800, 1200, and 1600 ppm), Mancozeb (800, 1200, and 1600 ppm) and Copperoxy chloride (800, 1200, and 1600 ppm) fungicides were used for the chemical control of fungi. Fungi such as *Aspergillus niger, Alspergillus flavius, Alternaria alternata and Fusarium* sp. were treated with chemicals. Seed were treated with extracts of *Azadirachta indica* and *Ageratum houstonianum* plants.

Agar plate method, Standard Blotter Technique and Sand method were used for isolation of fungi. 25 different fungi were isolated which include *Aspergillus flavus, Aspergillus niger, Alternaria alternata, Fusarium* sp, *Fusarium* sp., *Cladosporium* sp. 1, *Rhizoctinia* sp, *Fusarium* sp 4. , *Aspergillus tamari, Cladosporium* sp 2, *Curvularia* sp, *penicillium* sp 1 , *Rhizopus* sp, *Trichoderma* sp., unidentified sp. 1, unidentified sp. 3, *Alternaria* sp, *Aspergillus* sp. 1, *Aspergillus* sp. 3, unidentified sp. 2, *Mucor* sp, *Fusarium* sp. 3, *Penicillium* sp. 2, unidentified sp. 4, and *Aspergillus* sp. 2. Among the isolated fungi *Aspergillus flavus* was, the most frequent fungi (43) is followed by *Aspergillus niger* (34), *Alternaria alternata* (23) *Fusarium* sp.1 (17). Bilgrami *et.al*., (1976) reported the highest frequency of *Aspergillus flavus* in pulse seeds.

From all these three methods of isolation, only three *Aspergillus flavus*, *Aspergillus niger* and *Fusarium* sp. 1 are the common species which present in all methods in all sterilized and unsterilized condition. Usually unidentified species of fungi were mostly isolated from seeds source two and were present only on seeds treated with Sodium hypochlorite (NaOCl). Due to their only occurrence in sterilized seeds, it can be estimated that they might be pathogenic. The genus like *Aspergillus flavus, Aspergillus niger, Penicillium* and *Fusarium* were isolated from unsterilized seeds at a higher ratio as compared to sterilized seeds. Surface sterilization of seed with 1% $CaOCl_2$ reduced the incidence of *Aspergillus* and *Cladosporium* species (Tempe, 1970). Similar type of result was found on lentil by Tariq *et al.,* (2005), on sunflower seed by Dawar and Ghaffar (1991) and on maize by Naiz and Dawar (2009). Fakhrunnisa *et al.,* (2006) found that deep freezing method

showed better results for isolation of *Alternaria alternate, Cladosporium herbarum, Drechslera* spp., and *Fusarium* spp.

Since the seed sample first was collected from the market and seed sample second was collected from the traditional storage in Chitwan district, the difference in their storage condition might be a reason for variation in the presence and frequency of fungi in these seed samples. Majority of fungi were observed in seeds collected from source two followed by source one. The fungal dominancy in seeds from source two might be due to the improper traditional storage condition during post-harvest management and non-commercial agricultural practice. Farmers, who do non-commercial agriculture, only cultivate the crop for household consumption and do not use fungicides at proper time, which results the presence of pathogens in the seeds. Another possible reason could be the adherence of soil particles to the seeds which might not have been removed completely during seed rising and washing at the time of incubation. On the other hand, the reason behind least fungal incidence and prevalence in seeds from source one might be due to the treatment with fungicides, healthy storage condition, fungicides used in field and sowing of healthy seeds. Another reason for least fungal visibility could be due to seeds produced from the resistant varieties.

In this experiment, Agar plate method was found to be best than Standard Blotter method and Sterilized Sand method for the isolation of fungi. Both the figures and frequency of presence were slightly more in Agar plate method than in Blotter method and Sand method. Least numbers of fungi were isolated from the Sterilized sand method. The total frequency of fungi on Agar plate Method, Blotter method and Sterilized Sand method were 120, 109 and 42 respectively.

Altogether 25 species of fungi were isolated. Among them, *Aspergillus*, *Fusarium* and *Alternaria* were dominant. The number of fungi species found from Agar plate method, Blotter method, and Sterilized Sand method were 24, 14 and 9 respectively. Kassim *et al.*, (1987) found that Agar Plate method was more suitable for the isolation of mycoflora of barley than Blotter method in Saudi Arabia. According to Agrwal, Mathur and Neergaard, (1971), Standard Blotter Technique was found to be suitable method for the isolation of mycoflora. It was also proved to be most suitable method for isolation of seed borne fungi in rice, wheat, black gram, green gram Suhag (1973) and Naize and Dawar (2009) reported that Agar plate method is as good as Blotter method for fungus isolation on maize seed. Additionally, Nasir (2003) reported that PDA method yield highest number of fungus from

soybean among four methods. Such results might imply that the nutrients in the media might have an important role for the initiation of growth of fungi on some pulses. This implication can further imply that media composition in Agar plate is much favorable than in Blotter method. It was also found that growth of some notable fungi was suppressed due to the increase in the surface mycoflora, it indicated that pathogenic fungi might be weak competitors in comparison of the surface mycoflora.

Fungicides are heterogeneous group of organic compounds, which are usually unrelated, both chemically and in their mode of action against pathogens. The fungicides may have a significant influence on the production and activity of cell wall degrading enzymes produced by plant pathogenic fungi. Mehta *et al.,* (1990) reported the effect of fungicides on the production of pectolytic and cellulolytic enzymes by the fungi, thereby reducing the incidence of fungal pathogen. These observations support the present findings, in which fungicides reduced the incidence of mycoflora in the seeds. Ghosh and Das (1999) and Misra found that reduction in *Fusarium* wilt and collar rot were reported due to the treatment of seeds with fungicides.

According to Shah and Jain (1993) and Mcgee (1995) fungicidal treatment reduced seed mycoflora and improved seed germination. The control done by fungicides depends upon the type and nature of associated fungi. On the same way, the control also depends on the types of fungicides i.e. Contact, Translaminar and Systemic. Contact fungicides like Mancozeb and copper act on the outside of the plant mostly by interfering spore germination whreas systemic fungicides act within the plant after being taken up in tissue and translocated through the plant. In vitro evaluation of fungicides by poison food technique showed that combination of Carbendazim and Mancozeb were effective in inhibiting the mycelial growth which is followed by difenconazole (Madhavi and Bhattiprolu, 2011), and similar result was found in this study.

Carbendazim inhibited the growth of *Aspergillus flavus* to 88.89%, *Aspergillus niger* to 86.67% and *Fusarium* sp. to 85.56%. The difference in fungi inhibition might be due to the specificity of fungicides used in this study. Bavistin checked the 20, 30 and 40mg/10 ml fungicides effect on three different fungi *Aspergillus niger*, *Alternaria alternate* and *Fusarium solani* during seed treatment. The fungicide totally restricted the incidence of all the fungi at 40 mg/l (Sakoor *et al.,* 2011).

According to Ibiam *et al.,* (2000 and 2006) bavistin was effective against *Alternaria alternata*, *Fusarium solani*, *Aspergillus niger* and *Aspergillus flavus* at 40 mg/ml. Ibiam *et al.,* (2006) stated that systemic fungicides either inactivate or killed the pathogen in the seeds or seedlings as the germination of seeds start. The metabolic activities of fungi could not be arrested at lower concentration of fungicides which may be due to the fact that fungicides are unable to destroy few fungi at lower concentrations. As the concentration of fungicides increases, the metabolic activities of the fungi were completely destroyed.

Morshed (1995) also conducted experiments on *Phaseolus vulgaris* and reported that bavistin was efficient for the control of seed borne fungi like *Fusarium* sp. and *Alternaria* sp. Dodan *et al.,* (1991) used Bavistin (Carbendazim) to radicate *Fusarium monilforme* which is causal agent of root rot and bakanae disease of rice.

Another chemical fungicide used was Mancozeb, which inhibited the growth of *Alternaria alternata* to 87.78 % and *Fusarium* sp. to 66.67 %, *Aspergillus flavus* to 66.68% and *Aspergillus niger* to 64.44 %. Similar study was done by Shirurkar *et al.* (2012) against some seed-borne fungi of maize. That group also used Mancozeb and Carbendazim, in which Mancozeb was found to be better for the control of fungi like *Fusarrium solani*, and Carbendazim was found to be better for the control of *Aspergillus flavus, A. niger, A. funigatus* and *A. terreus*.

Vikas *et al.,* (2013) found that Mancozeb inhibited *Alternaria alternata* to 74.80% in concentration of 0.25% and Carbendazim inhibited to 73.80 % in concentration of 0.2 %. Also, according to Sudha *et al.,* (2013), Mancozeb inhibited radial mycelial growth *Aspergillus flavus* to 91.1 % in concentration of 0.3 %. The differences found in inhibition percentage may be due to the different concentration used in the experiments.

Nandeesha *et al.,* (2013) reported that four systemic fungicides completely inhibited the mycelial growth in all the concentrations including 250 ppm. When non-systemic fungicides were tested, complete inhibition of mycelial growth was observed at 1000 ppm with Mancozeb. However, Mancozeb was found highly compatible with *Trichoderma* sp. (TAG-2) compared to *Aspergillus niger*. The use of systemic fungicides, for example Mancozeb and Carbendazim, were considered very effective. The inhibition of fungi by fungicides was less in low concentration i.e. at 800 ppm and as concentration increases rate of inhibition also increases i.e. at 1600 ppm.

Mancozeb, Carbendazim and Copperoxy chloride are the three fungicide used to control the test fungi in this work. From the fungicide Carbendazim (1600 ppm) *Aspergillus flavus* was inhibited to 86.66%, *Fusarium* sp. to 64.56% *Aspergillus niger* to 85.56% and *Alternaria alternata* to 64.44%. Ouf (1993) controlled seed borne fungi like *Aspergillus niger, A. flavus, Penicillium* sp and *Fusarium* sp. when treated with chemicals. Singh and Kang (1992) reported that laboratory evolution of seed treatment of rice with Carbendazim (bavistin) controlled seed rot and significantly decreased seedling mortality.

Copperoxy chloride is the last used fungicide against the test fungi and *Aspergillus flavus* inhibited to 58.89%, *Fusarium* sp. to 64.44%, *Aspergillus niger* to 55.55% and *Alternaria alternata* to 51.1%. In vitro evaluation of fungicides by poison food technique showed that combination of Carbendazim and Mancozeb were effective in inhibiting the mycelial growth which is followed by difenconazole (Madhavi and Bhattiprolu, 2011) and similar result was found in this study.

Nandeesha *et al.,* (2013) reported that four systemic fungicides completely inhibited the mycelial growth in all the concentrations including 250 ppm. When non-systemic fungicides were tested, complete inhibition of mycelial growth was observed at 1600 ppm with Mancozeb. However, Mancozeb was found highly compatible with *Trichoderma* sp. (TAG-2) compared to *Aspergillus niger*. The use of systemic fungicides, i.e. Carbendazim was considered very effective.

Plant extract of *Azadirachta indica* and *Ageratum houstonianum* were used for seeds treatment. From this study it was conform that fungi associated with seeds were reduced by treatment to plant extract. According to Anon (2009), neem oil is an effective fungicide for the prevention and control of various fungal diseases including powdery mildew, black spot, downy mildew, anthracnose, rust, leaf spot, botrytis and needle rust. Kumar (2014) worked on essential oil of *Ageratum houstonianum* and found the oil to be fungicidal at 500 ppm against *Candida albicans* and *Trichophyton rubrum*. . Bio-control technology has been considered as viable and promising approach for disease reduction (Cook and Baker, 1983; Papavizas and Lewis, 1988).

CHAPTER SIX: CONCLUSION AND RECOMMENDATION

6.1 Conclusions

Twenty-five species of seed borne fungi were isolated from black gram collected from local market and traditional storage. Seeds from traditional storage were more infested with fungi in comparison to seeds from local storage. The difference in fungi infestation might be due to the difference in storage conditions of the seeds. For traditional storage seeds, more infestation with fungi might be due to poor storage condition and exposure with field (soil) fungi. For the isolation of fungi, Agar plate method was found to be best compared to the Standard Blotter and Sand methods. Notable results found while controlling the fungi were Carbendazim (systematic) best controlled *Aspergillus flavus* and *Aspergillus niger*, and Mancozeb (contact) best controlled *Fusarium* sp. and *Alternaria alternate*. Among three chemical fungicides, Carbendazim (systemic) was found to be most effective. The reduction of pathogens by seed treatment using plant extracts was also found to be very effective.

6.2 Recommendations

In this study, the highest numbers of microflora were isolated from the Agar plate method. Carbendazim was found to be most effective against the common seed-borne fungi of Black gram. To improve productivity or yield of the Black gram, surface sterilization of seeds can be done by synthetic chemical fungicides. Also, Plant extracts, which act as seed protectants, can be used during seed storage. Plant extracts are residue free and eco-friendly to the environment.

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APPENDIX

Organisms	APM (Agar plate method)								Blotter method								Sand method							
	Market seed				Traditional storage seed				Market seed				Traditional storage seed				Market seed				Traditional storage seed			
	SS		USS		SS		USS		SS		USS		SS		USS		SS		USS		SS		USS	
Alternaria alternate	-	0	+	5	-	0	+	4	-	0	+	1	+	2	+	4	-	0	+	3	+	2	+	2
Alternaria sp.	-	0	-	0	-	0	+	2	-	0	-	0	-	0	+	2	-	0	-	0	-	0	-	0
Aspergillus flavus	+	2	+	5	+	2	+	6	+	2	+	8	+	2	+	7	+	2	+	1	+	2	+	4
Aspergillus niger	+	2	+	7	+	2	+	3	+	1	+	5	-	0	+	6	+	2	+	1	+	2	+	3
Aspergillus sp.1	-	0	+	3	-	0	+	1	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0
Aspergillus sp.2	-	0	+	1	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0
Aspergillus sp.3	-	0	+	1	-	0	+	1	-	0	-	0	-	0	+	2	-	0	-	0	-	0	-	0
Aspergillus tamari	-	0	+	2	-	0	+	4	-	0	-	0	-	0	+	2	-	0	-	0	-	0	-	1
Cladosporium sp.1	+	1	+	4	-	0	+	3	-	0	+	5	-	0	-	0	-	0	-	0	-	0	-	0
Cladosporium sp. 2	-	0	-	0	-	0	+	4	-	0	-	0	-	0	+	3	-	0	-	0	-	0	+	2
Fusarium sp.1	+	1	+	2	+	1	+	2	+	1	+	2	+	1	+	2	+	2	+	1	+	1	+	1
Curvularia sp	-	0	+	2	-	0	+	1	-	0	+	2	-	0	+	1	-	0	+	3	-	0	-	0
Fusarium sp.2	-	0	-	5	-	0	-	0	-	0	+	5	+	2	-	0	-	0	+	2	-	0	+	2
Fusarium sp.3	-	0	-	0	-	0	+	2	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0
Fusarium sp.4	-	0	+	6	+	2	+	2	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0
Penicillium sp.1	-	0	-	0	-	0	+	3	-	0	-	0	-	0	+	3	-	0	-	0	-	0	-	0
Penicillium sp.2	-	0	+	2	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0
Rhizoctinia sp.	+	2	-	0	+	5	+	1	+	2	+	1	+	2	-	0	-	0	-	0	-	0	-	0
Rhizopus sp.	-	0	+	0	-	0	+	2	-	0	+	2	-	0	+	2	-	0	-	0	-	0	-	0
Trichoderma sp.	-	0	+	2	-	0	+	2	-	0	-	0	-	0	-	0	-	0	-	0	-	0	+	2
Mucor sp.	-	0	+	2	-	1	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0
Unidentified sp. 1	+	2	+	2	-	0	+	1	+	1	-	0	-	0	-	0	-	0	-	0	-	0	-	0
Unidentified sp. 2	-	0	-	0	+	2	+	2	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0
Unidentified sp. 3	-	0	-	0	+	2	+	1	-	0	-	0	-	0	+	1	-	0	-	0	-	0	+	1
Unidentified sp. 4	-	0	-	0	-	0	+	1	-	0	+	1	-	0	-	0	-	0	-	0	-	0	-	0

SS = Sterilized seed, USS= Unsterilized seed,(+)= Presence, (-) = Absence, Number = Frequency

PHOTO PLATES

1. Black gram seed

Fig.1: Market seed sample

Fig.2: Traditional storage seed sample

2. Fungi isolation from PDA, Standard Blotter and Sand Methods

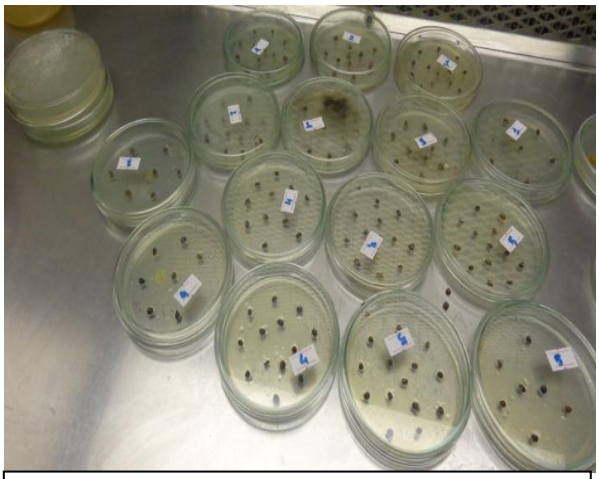


Fig.3: PDA method

Fig.4: Blotter method

Fig.5: Sand method

3. Fungi under microscopic view:

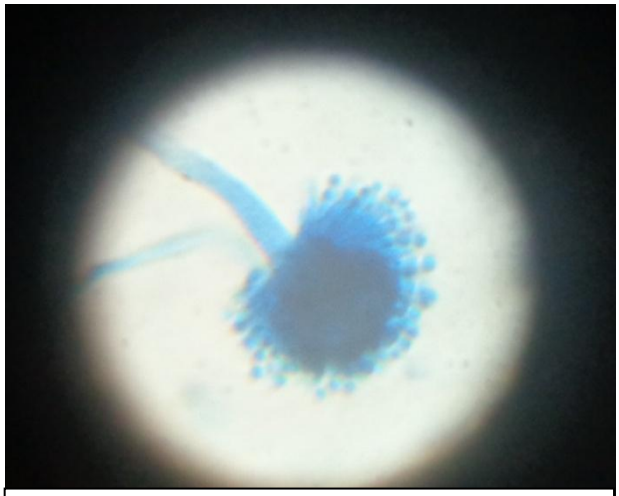


Fig.6: *Aspergillus flavus*

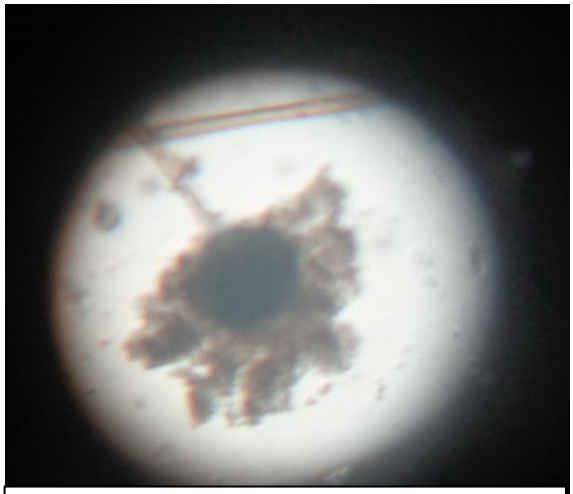


Fig.7: *Aspergillus niger*

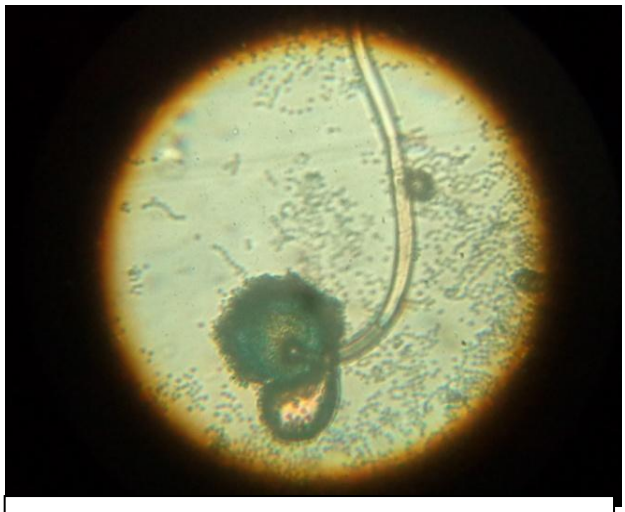


Fig.8: *Aspergillus tamarii*

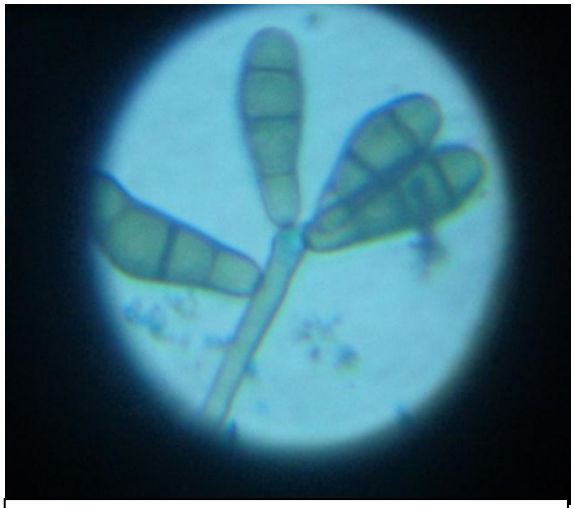


Fig.9: *Curvularia* sp.

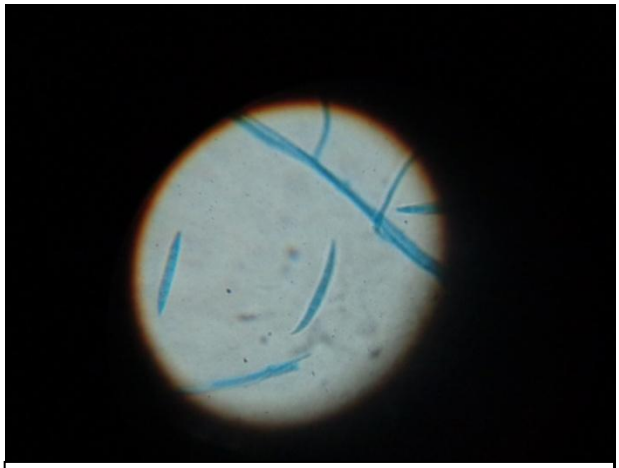


Fig.10: *Fusarium* sp.

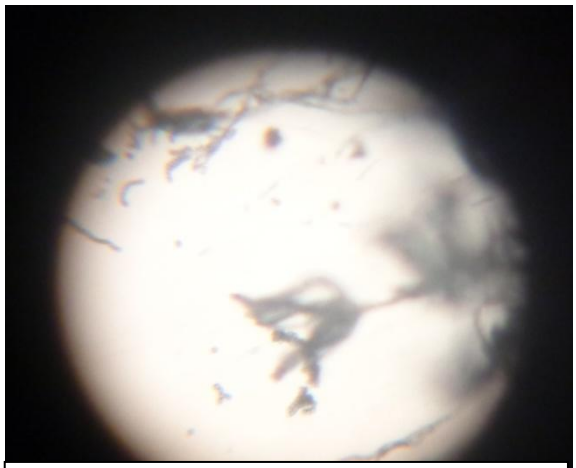


Fig.11: *Penicillium* sp.1

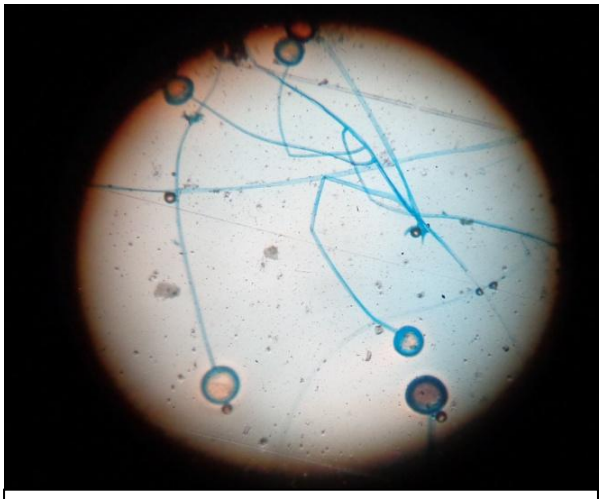


Fig.12: *Mucor* sp.

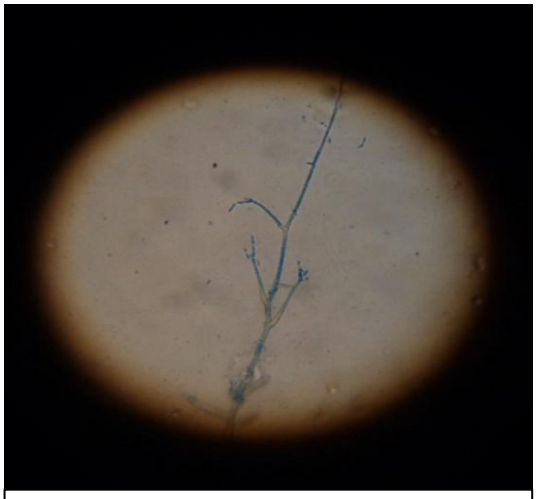


Fig.13: Unidentified sp. (dirty green)

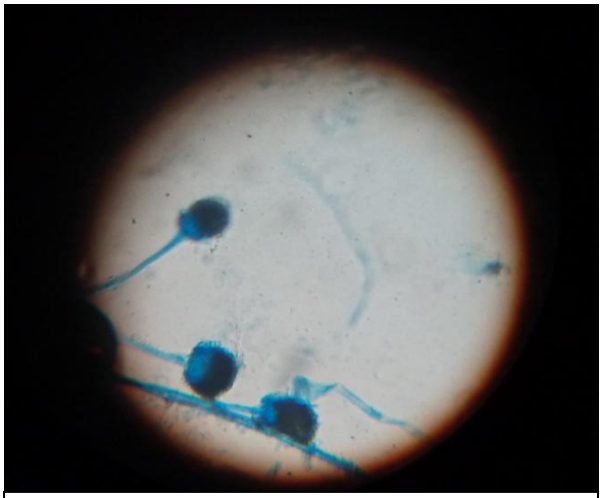


Fig.14: Unidentified 1(blueish white)

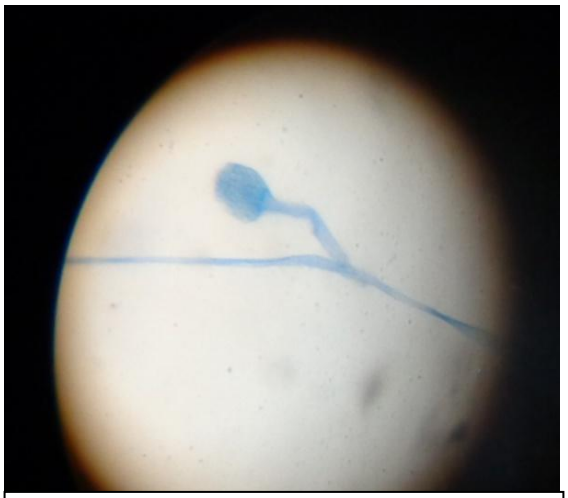


Fig.15: Unidentified 2

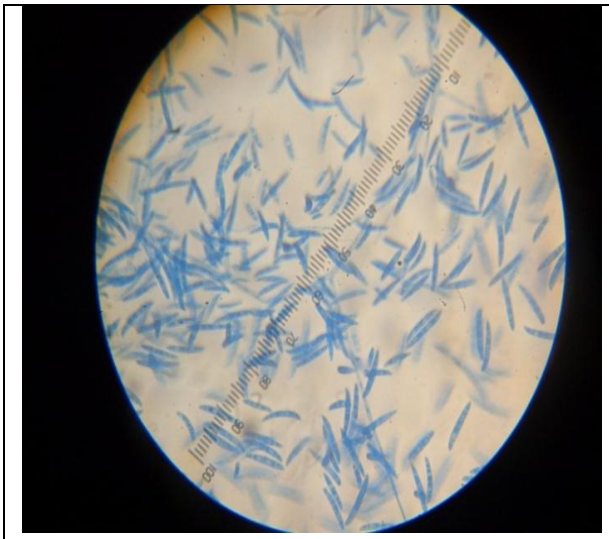


Fig 16: *Fusarium* sp

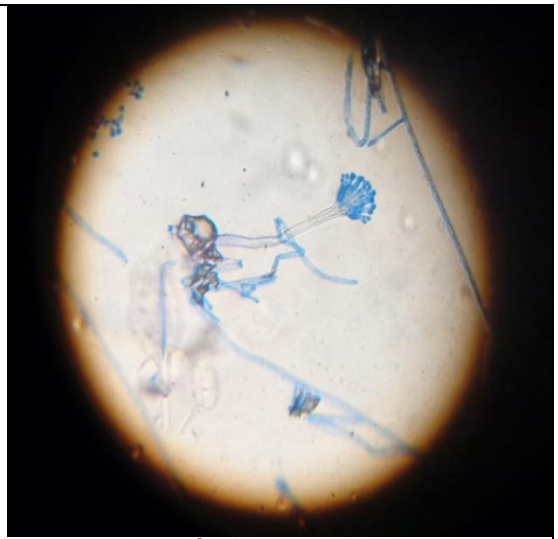


Fig7: Unidentified species

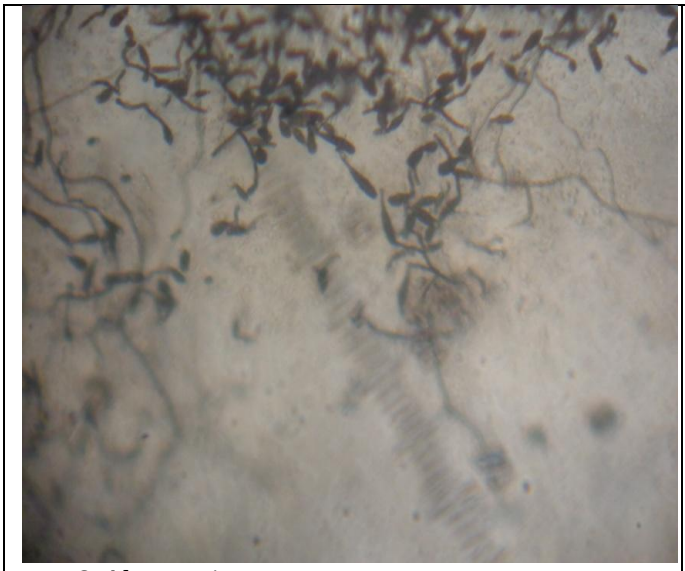


Fig18 *Alternaria* sp

Fig 19 observation on microscope

Fig. 20: Storage of fungi slant in tube

Fig 21: pure culture of *Fusarium* sp

Fig 22:pure culture of *Aspergillus* sp

3. Control of test fungi through chemical fungicide:

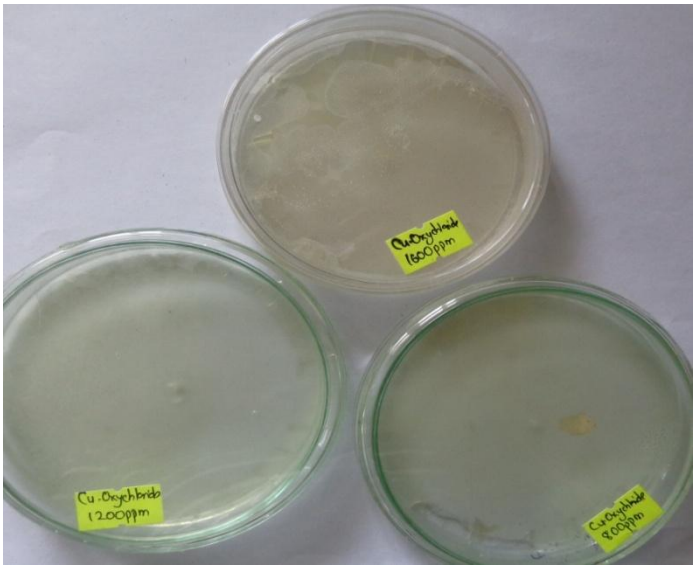


Fig. 23: Inoculation of pathogen in PDA plates with fungicide

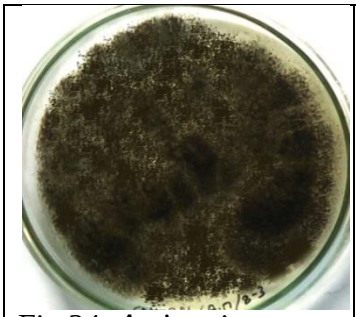
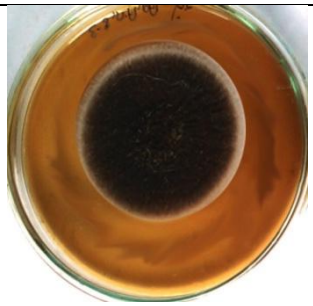
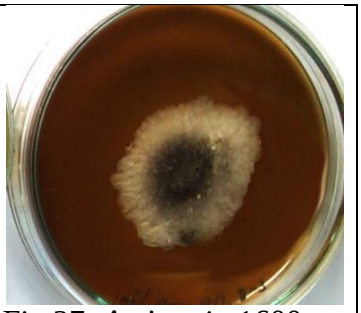
 Fig 24: *A.niger* in control	 Fig 25 :*A.niger* in 800ppm	 Fig 26: *A.niger* in 1200 ppm	 Fig 27: *A niger* in 1600 ppm

Fig.24: *Aspergillus niger* treated by carbendazim