Studies on utilization of rhizospheric pseudomonads in preventing seed biodeterioration of *Arachis hypogaea* L, enhancement in seed germination and crop production

Thesis

SUBMITTED TO

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

LUCKNOW

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UTTAR PRADESH, INDIA

2019

Dedicated to

My Beloved Parents

CERTIFICATE

This is to certify that the thesis titled "**Studies on utilization of rhizospheric pseudomonads in preventing seed bio-deterioration of *Arachis hypogaea L*, enhancement in seed germination and crop production**" submitted by **Mr. Rishabh Chitranshi** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

The thesis submitted to Babasaheb Bhimrao Ambedkar University, Lucknow satisfies all the requirements as stipulated in the Doctor of Philosophy (Ph.D.) University regulations 1999 as amended in 2010 and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

Head of the Department

Supervisor

DECLARATION

This is to certify that I have worked on the research thesis entitled "**Studies on utilization of rhizospheric Pseudomonads in preventing seed bio-deterioration of *Arachis hypogaea L*, enhancement in seed germination and crop production**". The data mentioned in this thesis were collected and obtained during genuine work done by me. Data obtained from other agencies have been duly acknowledged. None of the findings pertaining to the work has been concealed. The result embodied in this report has not been submitted to any other University, Institution or Research Centre for the award of any degree. **"The thesis is essential free from all kinds of plagiarism"**

Date:

Place: **RISHABH CHITRANSHI**

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Rishabh Chitranshi

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LIST OF ABBREVIATIONS

%	Percent
° C	Degree Celsius
° E	Degree East
° N	Degree North
µg	Micro Gram
µL	Micro Liters
µS/cm	Micro-Siemens per Centimeter
ACC	1-Aminocyclopropane-1-Carboxylate
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Sequence Tool
BNF	Biological Nitrogen Fixation
bp	Base Pairs
BTB	Bromothymol Blue
CAS	Chrome Azurol S
CFU	Colony Forming Unit
cm	Centimeters
CMC	Carboxymethylcellulose
CRYEMA	Congo Red Yeast Extract Mannitol Agar Media
DAS	Days After Sowing
DMRT	Duncan's Multiplicity Range Test
DNA	Deoxy Ribonucleic Acid
EC	Electrical Conductivity
EDS	Energy Dispersive Spectroscopy
EPS	Exopolysaccharides
FT-IR	Fourier Transmission Infra-Red
g	Gram
GAs	Gibberellins
GPA	Glucose Peptone Agar
ha	Hectare

HAM	Hofer's Alkaline Media
HCN	Hydrogen Cyanide
HDTMA	Hexadecyltrimethylammonium
hrs	Hours
IAA	Indole-3-Acetic Acid
IAR	Intrinsic Antibiotic Resistance
K	Potassium
Kg	Kilogram
KNO_3	Potassium Nitrate
LPWG	Legume Phylogeny Working Group
m	Meter
mg	Milligram
Mha	Million Hectares
ml	Mili Liters
MR-VP	Methyl-Red and Voges-Proskauer
MTCC	Microbial Type Culture Collection
N	Nitrogen
N_2	Dinitrogen
$NaNO_3$	Sodium Nitrate
NBAIM	National Bureau of Agriculturally Important Microorganisms
NH_3	Ammonia
NH_4Cl	Ammonium Chloride
NH_4SO_4	Ammonium Sulphate
nm	Nano Meter
PDA	Potato Dextrose Agar
PGP	Plant Growth Promoting
PGPR	Plant Growth Promoting Rhizobacteria
PHB	Poly-P-Hydroxybutyrate
PIPES	Piperazine-N, N'-Bis (Ethane Sulfonic acid)
PSB	Phosphate Solubilizing Bacteria
PSI	Phosphate Solubilization Index
PSU	Percent Siderophore Unit
RAPD	Random Amplified Polymorphic DNA

rpm	Rotation per Minutes
rRNA	Ribosomal Ribonucleic Acid
SD	Standard Deviation
SEM	Scanning Electron Microscopy
TLC	Thin Layer Chromatography
UV	Ultra Violet
WHO	World Health Organization
YEMA	Yeast Extract Mannitol Agar
Zn	Zinc
ZSI	Zinc Solubilization Index
α	Alpha
β	Beta
ϒ	Gama

Chapter 1

Introduction

INTRODUCTION

India is one of the largest oilseeds producers in the world and that's why oilseeds are known as a backbone of Indian agriculture and economy. In the of oilseeds production, India has second place after China (Rai et. al. 2016). Oilseed crops play a significant role in Indian agronomical trends apart from grains in terms of area and production (Jha et.al. 2012). India produces total 9 oilseeds crops, in which soybean, groundnut, rapeseed-mustard, sunflower, sesame, safflower and niger are edible while castor and linseed are non-edible (Meena et. al 2017). A report of the Department of Agriculture Statistics (GOI-2018) stated that the country imparts approximately 12-15% for total area of oilseed production, while it produces only 7% of vegetable oils globally. As per the 4th advance estimates (GOI- 2018) for 2017-18, the total production of oilseed crops is about 32 metric ton (MT), and around 14% (more than 27 million hectares Mh) of its total agricultural land is used for oilseed crops. The productions of oilseeds slightly increase over the previous year (Kumar 2016). Moreover, the area for oilseeds production has been increasing over the time, but the production of oilseeds is still very low; it is not suitable scenario to sustain and maintain the financial stability for oil seed growers as compared to other oilseed producing countries in the world (FAO-2016). In recent agronomical trends, a number of researches focus on some particular ways and methods to achieve the goal of increasing productivity in India (Manjunatha et.al 2018). The gap between productivity and demand of oilseeds is primarily because the cultivation of oilseed crops is mostly practiced on marginal lands;

which are lacking in sufficient irrigation and have low levels of inputs such as fertilizers, pesticides and new techniques. However, India still imports a significant proportion of its requirement of edible oil (Madhusudhana et. al. 2013).

As for as Indian agrological concern, groundnut, soybean and rapeseed-mustard are responsible for 80% of the total area, that is used for the cultivation of oilseeds in the country. In India, groundnut is considered one of the major cash crops for the farmers. Apart from India, other countries such as Nigeria, Senegal, Sudan, Burma and USA also produce groundnut in significant amount (Fletcher and Shi 2016). Groundnut is an annual crop, cultivated in 5 Million hectares area approximately and total groundnut production stands for about 6 million tonnes. India is also export the groundnut, a total of 7, 26,535.91 MT of groundnuts exported for the year 2016-17 with the cost of Rs. 5,456.72 carore / 813.45 USD Millions (APDA 2018; Avali 2017).

Groundnut (*Arachis hypogaea L.*) basically belongs to the family *Fabaceae*is also known as *Leguminosae.* Groundnut is native of Brazil to Peru, Argentina and Ghana, from where it was introduced to other West Indies islands. Further Portuguese introduced groundnut into Africa from where it was introduced into North America. In the first half of the sixteenth century, groundnut introduced into India from one of the Pacific islands of China, Central America or South America (Zargar et al. 2016).

Groundnuts in India are grown mostly under rain-fed conditions and are available throughout the year due to a two-crop cycle harvested in March and

October. The main groundnut varieties produced in India are Kadiri-2, Kadiri-3, BG-1, BG-2, Kuber, GAUG-1, GAUG-10, PG-1, T-28, T-64, Chandra, Chitra, Kaushal, Parkash, and Amber (APDA-2018) Gujarat, Andhra Pradesh, Tamil Nadu, Karnataka, Maharashtra and Uttar Pradesh are major groundnut producing states of India (APDA 2018).

Groundnut seeds have a good source of dietary fiber, protein, vitamins B, E and K, minerals (phosphorus, calcium, magnesium and potassium) and phytosterols along with 48-50% oil in it (Savage and Keenan 1994; Arya et. al. 2016; Usman et. al. 2013). Groundnut seeds are used as dry fruits, peanut butter and cooking oil are some of the by-products of these seeds (Zhao et. al. 2012; Chang et.al. 2013). Groundnut seeds also have some medicinal values, Beta-sitoserol (SIT) a substitute of an antioxidant (phytosterol) found in groundnut seeds which make them capable enough to control the cholesterol problems and help in preventing cardiac diseases in humans (Özcan 2003; Lopes et. al. 2011). Moreover, groundnut seeds are used as a defender against cancer because it may help to inhibit the growth and formation of tumor cells (Bishayeeet al.2010; Arya et. al. 2016). These seeds are also very useful for skin related problems, due to the presence of omega 3 fatty acids which helps in reducing inflammation and prevent skin eruptions (Lin et. al. 2017). Beta carotene, found in groundnut is very critical for skin's health. It is converted into Vitamin A in the body which helps in the growth and repair of body tissues. Thus peanuts help to heal wounds and bruises at a faster pace (Xavier et. al.2017).

Despite being commercially viable, highly productive and nutritious crop, groundnut seeds have some drawbacks such as due to the presence of excessive oil, vitamins, protein and mineral in it; these seeds have a high risk of infection of harmful micro-organisms during storage (Sudini et. al. 2015).

Groundnut is kept in the storage immediately after harvesting and it is mostly used in sowing of the next crop. Moisture, temperature and fresh air are some parameters that maintain the quality of oilseeds and their sprouting sprout (Singh et.al.2017). If there is no proper arrangement of these things in the warehouse, then the seeds became deteriorated. It has a great impact on the oil holding capacity, seedling and germination; sometimes even this causes the death of seeds (Jyoti and Malik, 2013; Sepehri and Rouhi2016).

Deterioration is a natural and undesirable phenomenon of agriculture which can be defined as "deteriorative alterations occurring with the time that increase the seed's exposure to external challenges and decrease the ability of the seed to survive". According to Hussin (2011); Jyoti and Malik (2013) deterioration is known as damage and loss to the variety, quality and capability to the seed. It is also linked with various metabolic and chemical alterations including lipid-per-oxidation, membrane disruption and protein synthesis. Annual Losses due to seed deterioration is about 25% (Shelar et al. 2008; Khaliliaqdam et. al 2012). Deterioration of oilseeds is a process in which various physiological, biochemical and physical changes viz; membrane digitation, DNA damage irregular RNA and Protein synthesis, reduced metabolic activities, the formation of free radicals are included (Kumar 2017).

Further, the seeds having excessive moisture content, high nutritive value are more likely to deteriorate because they provide favorable conditions to the microorganism during storage (Kolte 2018)

A wide range of micro-organisms like bacteria, fungi, mites, insects and rodents attacks the groundnut crop (Jyoti and Malik 2013). Some species of *Aspergillus* (*A. candidus, A. flavus, A. niger, A. terreus, and A. ruber*) are dominant as bio-deteriorating agents of groundnut seeds followed by *Rhizopus, Penicillium, Curvularia, Fusarium, Alternaria* (Chavan, 2011; Sugui et al. 2015). *A. flavus* is responsible for aflatoxin contamination of crops prior to harvest or during storage (Gachara et al. 2018). In storage, *A. flavus* is more susceptible for colonization of groundnut (Adithya 2016). *A. flavus* in association with *A. niger* cause storage rot of groundnut (Ibiam and Egwu 2011;Sharma 2012). It has also been reported that moisture content in seeds increased gradually and deteriorating agents are more active with long term period in groundnut while the viability of seed decreases (Bewley and Black 2012).

In recent agro-economic trends, the main aim of agriculture is to develop new and innovative techniques to fulfil the requirement of demand and supply of oilseeds in India. Currently, Indian agriculture and allied sectors grow itself very rapidly in various fields as like crop production, crop management and disease control. Moreover, there are numerous challenges yet to be faced.

A number of products are available in the market which are very effective in crop production and widely used by the formers to improve the

quality of their respective crops (Oliveira et al. 2014). Most of the products are chemically synthesized and may cause serious health issues. Chemical-based pesticides are very harmful products and can enhance plant growth or protect plants from external factors, but also cause many dangerous diseases in animals and humans (Nicolopoulou et al. 2016). There are numerous reports that indicated the negative or harmful effects of pesticides; even some portions of pesticides are reported to be retained on (Kim et al. 2017).

To overcome these problems, an economical, eco-friendly and viable tool is available in the form of plant-associated microorganisms which can enhance growth, productivity and improve soil health (Ju et al. 2018). This method can also help in the reduction of chemical-based fertilizer and pesticides; and also helps in promoting sustainable agriculture.

Microorganisms are cosmopolitan; found to be every possible habitat in the world, but some are pathogenic or some are beneficial. Beneficial microbes are very useful in agriculture; and metabolites obtained from these micro-organisms are proved to be best, cost effective and environment-friendly; which can be a good alternative of chemical pesticides and fungicides (Glare et al.2012; Kumar et al. 2018). A group of microbes which are present in the rhizospheric zone of plants is known as rhizospheric bacteria or rhizobacteria (Arora et al. 2016). Rhizobacteria are mainly found in the root section of plants and they have the capability to uptake nutrients from soil (Piietal. 2015). So that they are very useful for plants, because of them the plants easily and naturally increase the size and number of root for proper nutrient uptake. The group of

these beneficial bacteria is also known as plant growth promoting rhizobacteria (PGPR), a term coined by Kloepper and Schroth (1978). PGPR induces the plant growth in two different ways, indirectly or directly; in direct promotion of plant growth includes synthesizing and releasing some compounds such as phytohormones (IAA) which are directly available to plant roots, or facilitating the uptake of certain nutrients from the environment (Glick, 1995; Ahemad M, Kibret 2014). While in indirect promotion PGPR lessen or prevent the deleterious effects of one or more phytopathogenic organisms (Ahemad M, Kibret 2014). This can happen by producing antagonistic substances or by inducing resistance to pathogens (Glick, 1995; Ahmed and Holmström 2014). Indirect mechanism of PGPR also include a variety of plant growth enhancing functions such as increase nutrient uptake availability of plants by fixing nitrogen (Kumar et al. 2015), by producing siderophore (Gull and Hafeez 2016), by solubilizing phosphate in soil (Deshwal and Kumar 2013) and by protecting from various deteriorating agents.

PGPR include bacteria of diverse genera, of which *Pseudomonas* sp. are predominant (Fouzia et al. 2015). Fluorescent pseudomonads are highly efficient to inhibit the growth of harmful microbes and fungi (Lukkani et al. 2014). *Pseudomonas fluorescens* is known as highly specialized in aggressive root colonization, induction of systemic resistance in the plant, and production of antifungal and antibiotic substances (Berendsen et al. 2012). The ability of *P. fluorescens*, like the production of siderophore, antibiotics to suppress phytopathogens could be of significant agronomic importance (Sahuet al.2018).

These mechanisms of *P. fluorescens* have essential functions in microbial antagonism but also are able to elicit induced resistance. Resistance-inducing and antagonistic rhizobacteria might be useful in formulating new inoculants, offering an attractive alternative of eco-friendly biological control of plant disease and improving the cropping systems into which it can be most profitably applied (Molloy et al. 2013).

Hence, the present study is formulated for the application of bacteria or their metabolites with a systematic strategy designed to fully utilize all these beneficial factors to prevent oilseeds from various deteriorative effects in groundnut seeds under storage condition. This study also includes enhancement in seed germination and crop production by using PGPR, Pseudomonads.

OBJECTIVES:

- Isolation and characterization of pseudomonad from rhizospheric soil of *Arachis hypogaea L.*
- Check the bio-control potential against bio-deteriorating agents of *Arachis hypogaea L.*
- To determine the mechanism of action of selected*Pseudomonas* strains against bio- deteriorating agents.
- To investigate the effect (in vitro) of selected *Pseudomonas*strain on seed germination and plant growth.
- Check the quality of seeds (oil content) before and after treatment.
- Field trial of treated seeds with selected *Pseudomonas* strain.

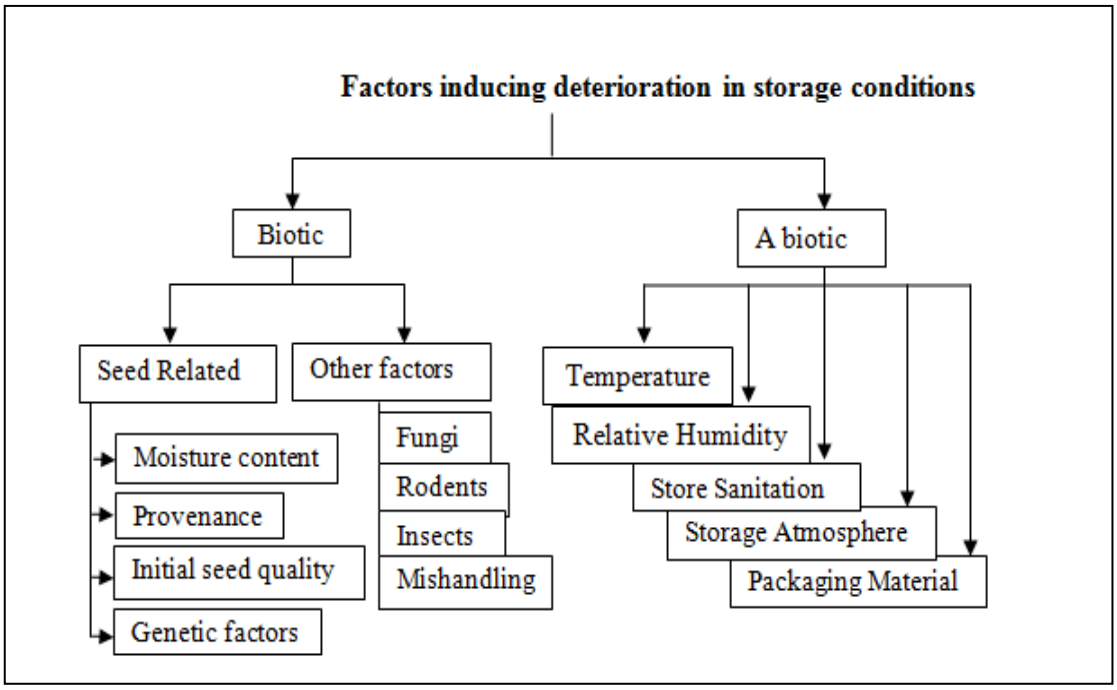

Figure-1 Factors effecting seed deterioration under storage conditions

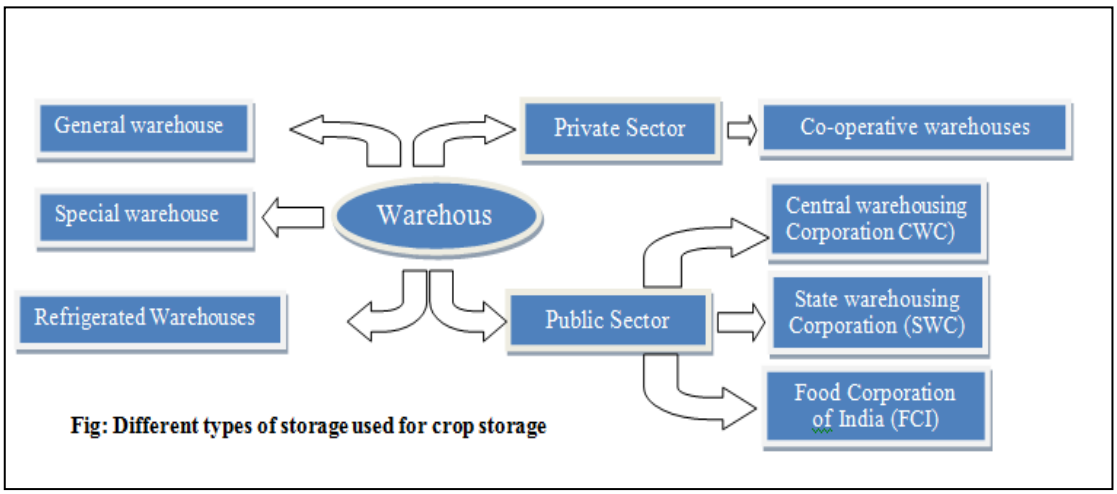

Figure-2 Type of stores for crop storage

Chapter 2

Review of Literature

Oilseedsare mainly cultivated for the production of edible oils. They have ample amount of high quality natural oil in it. Along with this, they are good source of renewable energy with high nutritive values. Due to the high and natural nutritive impact, oilseeds are very demanding in food, pharmaceuticals, biofuels, and livestock industries (Anilakumaret al. 2012).That's why oilseeds are known as agro- economical crops because they are just like a backbone of several agricultural economies worldwide(Krishiworld 2014).

The majorly grown oilseeds are soybean, groundnut, sunflower, safflower, sesame and rapeseed mustard worldwide. Europe and America jointly contributes above 60% in total oil seed production throughout the world. Africa, Malaysia, and Indonesia come from tropical regions contribute only about 5% for oilseed production (Sharma et al. 2012). Since 2010, the total production of vegetable oil has increased at the average annual rate of about 3.8 % with net increase of 31% in total production area around the world (Figure 3).

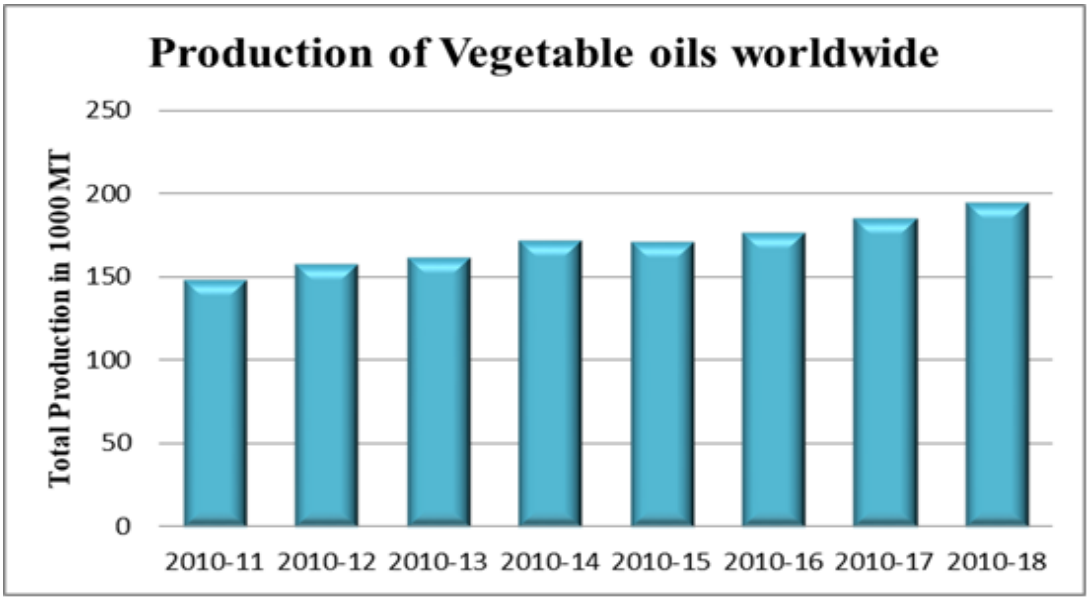

Figure-3 Total vegetable oil production world wide

It is observed that soybean (*Glycine max* L.*Merr*.) and Brassica sp., surpassing peanut (*Arachis hypogaea L.*), sunflower (*Helianthus annuus L.*), and cottonseed (*Gossypiumhirsutum L.*) which were leading oil seed crops since couple of decades in international market (Seiler et al. 2017).

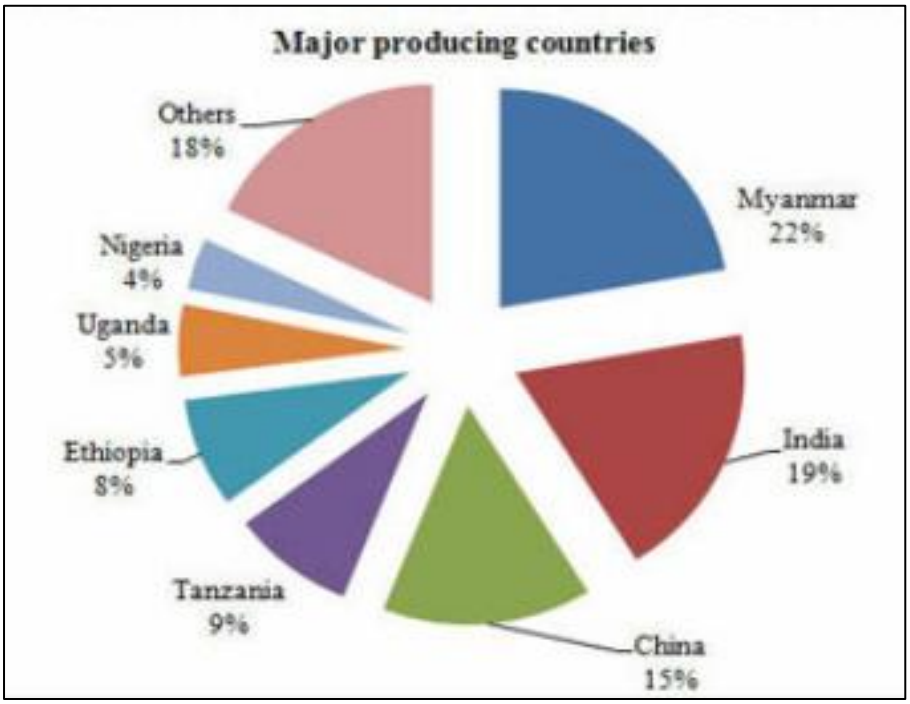

Figure 4 Total oil seed share worldwide 2016-17 (Statica -2018)

According to Ferguson et al. (2005) Groundnut (*Arachis hypogaea L*) is the native of South America and its distribution is confined to Argentina, Bolivia, Brazil, Paraguay, and Uruguay. The overall production area of groundnut was 26.15 about million hectors with the productivity of 45.46 million metric tons till July 2018(USDA-2018). Groundnut seeds are excellent sources of nutrition they contain vitamins B1 (thiamine), B2 (riboflavin), B3 (niacin) and vitamin E. they also have minerals such as- Mg, Fe, Zn and Ca in it (Gupta 2015).These oilseeds have both monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), presence of various desirable fatty acids determines the quality of oil(Orsavova et al. 2015). The most important unsaturated fatty acids are oleic (C18:1), linoleic (C18:2), and palmitic (C16:0) acids, which contribute nearly 80% of total fatty acids (Dwivedi et al. 1993; Ramos et al. 2009). Further, the sunflower seed used as vegetable oil, followed by edible snack nut and used in bird feed mixtures. The seed contains oil in an average of about 4%. ω-6, PUFA linoleic acid, MUFA and oleic acid are some of the major components of sunflower oil (Khan et al. 2015). Soybean oil is the major source for vegetable oil. It content about 21% of oil, the fatty acid composition of soyabean oil is oleic (23%), linoleic acid (55%), palmitic (11%) and (7%) of linolenicacid (Huth et al. 2015). Safflower seed, an annual crop, is grown principally in the United States, Mexico, India, and the Middle East. It contains about 32–40% oil. According to Sabikhi and Kumar (2012) safflower oil consisted of 5.53% palmitic, 1.62% stearic, 11.00% oleic, 81.5% linoleic, and 0.40% linolenic acids.Sesame seeds are known for their high quality oils

these seeds are basically grown in tropical countries with yields ratio of about 47–50% (w/w)(Mohammed et al. 2018). The sesame seeds oil contain less than 20% SFA with palmitic (7.9–12%) and stearic (4.8–6.1%) acids(Gharby et al. 2017) Oleic and linoleic acids are the key components for 80% of total fatty acids composition (Ariffin 2009). According to Singha et al. (2014), in term of production area Indian share in global oilseed market is about 15-20%. India ranks first in the production of most of the minor oilseeds (castor, niger, safflower and sesame). In the case of major oilseeds, India ranks first in the production of groundnut, third in rapeseed-mustard and fifth in soyabean(Rai et al. 2016). (Fig-4,5&6)

Apart from all it is observed that the consumption and production of oilseeds increased globally but there is no proper arrangements for the protection and prevention of oilseeds from various deteriorative agents under storage conditions. A global loss of total oilseeds during storage is about 26% (Shelar et al., 2008).Deterioration of seed leads to decreased germination percentage, which in turn produces pathetic seedlings making them less viable and ultimately seed death (Tilebeni HG, Golpayegani 2011;Kumar 2017). Field weathering, harvesting and storage also contributes in loss of seed quality (Farhadi et al. 2012; Delouche2016). Numerous species of *Aspergillus* (*A. candidus, A. flavus, A. ruber, A. niger, A. terreus,Rhizopus, Penicillium, Curvularia, Fusarium and Alternaria* are responsible for bio-deterioration of seeds in storage condition (Kaur2017).

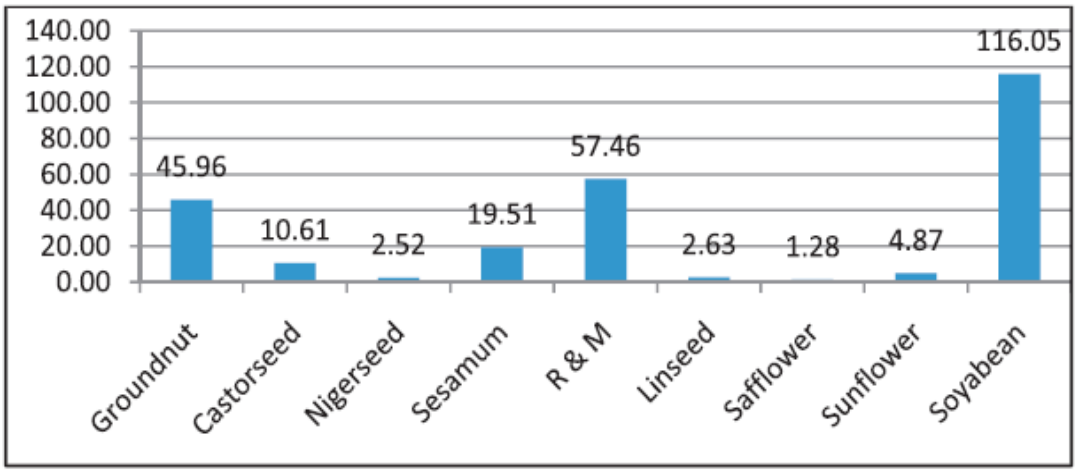

Figure-5 Total area of oilseed production in India 2015-16, DAS-GOI (2016)

(In lakh ht.)

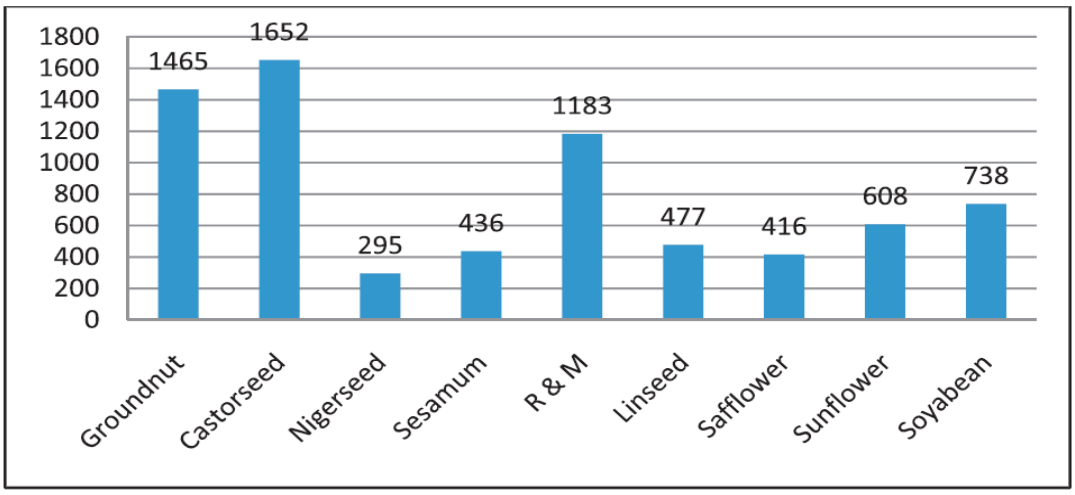

Figure-6 Total productivity of oilseeds in India 2015-16, DAS-GOI (2016)

(In lakh ht.)

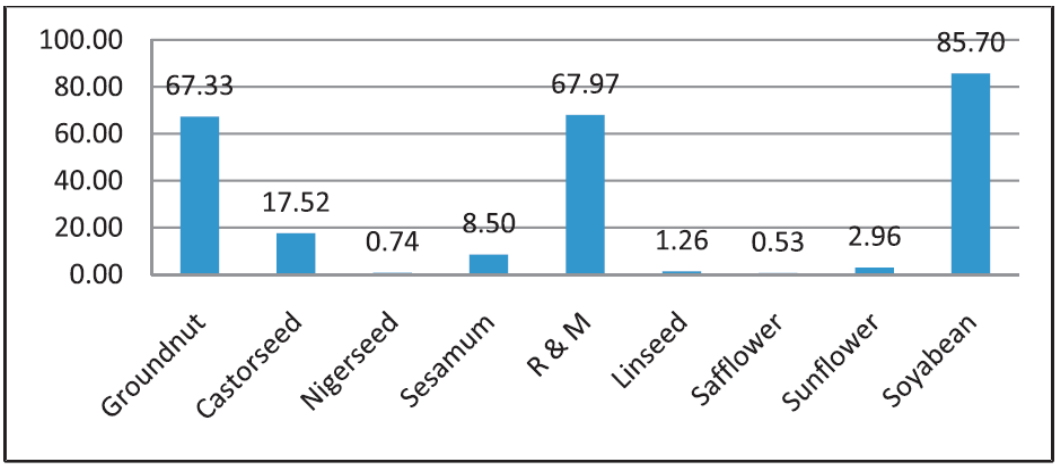

Figure-7 Total production of oilseeds in India 2015-16, DAS-GOI (2016)

(In lakh ht.)

2.1 DETERIORATION OF OIL SEEDS DURING STORAGE:

Deterioration and loss of seed quality is explained by Jyoti and Malik (2013) as "deteriorative alterations occurring with time that increase the seed's exposure to external challenges and decrease the ability of the seed to survive". However, deterioration is more likely described as modifications that occur in physiological, biochemical properties of seeds followed by physical cytological conditions (Sisman, 2005; Wang et al., 2016).

There are several environmental factors that cause seed deterioration such as changes in seed development, seed variety-quality, viability, temperature, moisture, environmental and geographical situations(Shaban 2013). Moreover, deterioration of seed can be linked with changes in various metabolic and chemical processes such as lipid peroxidation, membrane disruption and protein synthesis inhibition (Eskin and Shahidi 2012). These types of alterations during the whole process of deterioration may cause annual losses up to 25% of total crop production (Shelaret al. 2008). Once deterioration begins in seed, the process can't be reversible (Kapooret al. 2010). Farhadi et al. (2012) observed that seed deterioration stimulated the changes in physical, physiological, biochemical and cytological processes, which affects the viability of seeds and further cause the death of seed.

Moreover weather during harvesting, might be responsible for deterioration. During preceding harvesting, seed can be expose to adverse environmental conditions also affects the physiological quality of groundnut seeds(Bewley and Black 2012). The rate of deterioration varies from one

cultivar to another whereas the degree of deterioration in deteriorated seeds is more than healthy seeds. Thus, deteriorated seeds produce spotty fields and fewer plants in comparison to healthy seeds, which are caused by reduced growth rate (Biabaniet al. 2011;Kapooret al. 2010).

Apart from that it has also observed that when seed deterioration progress, seed germination decreases respectively. The process of seed deterioration can be observed from seed development and germination. Further, environmental elements contribute widely to seed-deterioration therefore; maintain favorable conditions in which seeds remain viable during storage are very critical (Mbofung et al. 2013). However, seed longevity, which is adopted from nature, is determined by environmental and geographical conditions. On the other hand seeds that develops in drought lack nutrient and become more susceptible to rapid deterioration. (Kibinza et. al.,2006).Thus, if the seeds are stored at higher moisture content, the deterioration takes place more readily and may affect the longevity of seed survival.

2.2 FACTORS AFFECTING OIL SEED DURING STORAGE

2.2.1 PHYSICAL

2.2.1.1 TEMPERATURE

To prevent from seed deterioration, temperature is crucial factor for during storage. Before storage, oil seeds undergo to drying to maintain physiological quality and then stored under the recommended temperature such as temperatures below 20 ºC and relative humidity below 60% (Balešević-Tubić 2010). Although drying necessary before storage but without proper care,

it could have negative impact on seed quality in terms of their germination and vigor which further leads to seed deterioration(Rathinavel, 2014;Mahjabin and Abidi, 2015).

2.2.1.2 MOISTURE CONTENT AND RELATIVE HUMIDITY

For maintain seed quality, a minimum moisture content required during storage. It is observed that seed deterioration increases as moisture content is increased. During storage, moisture content of oil seeds affected by the relative humidity; if relative humidity alters, it also could increase or decrease moisture content of the seeds(Delouche 2016). To deliberate the role of moisture content in seed deterioration, there is also need to understand the factors which affect the water absorption and retention as well as their effects. However thickness, structure and chemical composition of the seed coat of oil seeds could affect the rate of water absorption and retention by oil seeds; generally seeds having hard seed coat such as sunflower and soybean restrict the moisture uptake from the storage atmosphere(Copeland and McDonald 2012). (Table -1) Further,it was found that relative humidity should be maintained below 60% to prevent seed deterioration. Relative humidity reflects the amount of moisture actually in the air as a percentage of the amount of moisture that the air is capable of holding at the same temperature. It is well observed that warm air can contain more water in compared to cool air. During storage, when a proper time is given, moisture content of the seed would be in equilibrium with the surrounding environment through air filled in the interstitial spaces among the seeds. Finally it could be fully saturated when the net movement of moisture from air to seed, or from seed to air is zero.

Major storage fungi	Oil seeds	Moisture %
Aspergillus flavus	Soybean, Starchy carels	17.0-18.0 17.0-17.5
Penicillium	Starch grain Soybean Sunflower	16.5-20.0 17.0-20.0 10.0-15.0
Aspergillus restrictus	Starchy carels Soybean Sunflower,safflower,groundnuts	14.0-14.5 12.0-12.5 8.0-9.0
Eurotium glaucus	Starchy carels Soybean Sunflower,safflower,groundnuts	14.5-15.0 12.5-13.0 9.0-9.5
A candidus	Starchy carels Soybean Sunflower	15.0-15.5 14.0-14.5 9.0-9.5
A ochraceus	Starchy carels Soybean Sunflower	15.0-15.5 14.0-14.5 9.0-9.5

Table1- Major storage fungi and moisture percent under storage conditions

2.2.2. CHEMICAL

2.2.2.1 LIPID PEROXIDATION

Fatty acids are the most important contain of oil seeds, during storage when Lipid-auto-oxidation increases in seeds, the level of free fatty acid contents hike gradually and cause damage to oily plant species (Hosseinzadeh et al. 2007) Therefore, vigor testing becomes essential in seed stored under known and unknown or adverse conditions. Fatty acid imbalance can be viewed during seed ageing to test the damage caused. The parameters such as oil content, fatty acid composition and protein content of the seed are eventually influenced by storage condition and time ((Wood et al. 2008). The rate of water absorption by seed having a low water potential is also known to imbalance fatty acid content thus contributing to less viable seeds (Smýkalet al.2014). Fatty acid composition of seeds could be essential parameter, which

can be helpful in determining the susceptibility of oil content to oxidation. Shaban (2013) observed that sunflower seed needs special care during storage due to its high oil content. It is also observed that seeds having low water potential retain less water during imbibition, that can be determined by its osmotic and matrix potential (Trivadi, 2015). Different kinds of fatty acid found in seed oil like saturated and unsaturated fatty acid, which determines the types and severity of chemical reaction that takes place during storage period (Singh, 2017). Some of the seeds which are oil rich, there are more risk of auto-oxidation, mainly contain oleic (18:1), linoleic (18:2) and linolenic (18:1) fatty acid chain (Matthäus and Musazcan 2015)Degree of unsaturation significantly influences the degree of degradation(Morelloet.al.2004).Pouring evidence indicates that the relationship between length and environment of storage, and changes of fatty acid and seed-oil-contents depend upon natural soybean seed ageing (Singh, 2017). In longer period of storage of sunflower seeds, linoleic acid content of seeds has been reduced. In some ecotypes of soybean decrease under conventional storage situation has been found to have high co-relation in changes of total oil, oleic and linoleic acid contents and intensity (Orsavova et al. 2015). Oxidative reaction taking place in plant cell are linked with free toxic radicals, reduction of molecular oxygen and formation of toxic products. The toxic product formed due to degradation of lipids is a result of series of reactions. Oxidative damage occurs due to fatty acid changes and loss in germination capability of seeds. Free radicals available in seeds are closely related with water content and seed component for their activity (Ghassemi et

al. 2010).Thus slow-growth, abnormal seed-lings and un-germinated seeds are the result of changes taking place in membrane cells (Tian et al. 2014).

2.2.2.2 CHANGES IN THE ACTIVITY OF ENZYMES

Due to improper storage conditions, various enzyme activities present in the seeds affected; leads to alterations in the activity of enzymes. This could be due to configurational changes in their structure of oil seeds. Some of the configurational changes are partial folding or unfolding, degradation to subunits and condensation to form polymers. Hydrolytic enzymes such aslipase, phospholipase, protease, DNase, phosphatase and amylase; are primary enzymes which activated by high moisture levels. In longer period of storage, other enzymes such as superoxide dismutase, catalase, peroxidase, glutathione reductase and ascorbate peroxidase; responsible for scavenging of free radical and peroxide also imparts in seed deterioration.

2.2.3 BIOLOGICAL

2.2.3.1 INSECTS AND MITES

Some insects and mites such as Rust-red flour beetle (*TriboliumCastaneum*), Indian meal moth (*Plodia*interpu*n*ctella), Warehouse moths (*Ephestia* sp.) and Psocids (*Liposcelis* sp.) which also cause or enhanced the seed deterioration.

2.2.3.2 MICROBIAL AGENTS

Microbes like Bacteria, fungi, mites, insects and rodents are closely linked with seeds. Once seeds are harvested from fields, a wide range of micro-

organisms attacks on them (Table 1) (Jyoti and Malik, 2013). Species of *Aspergillus* (*A. flavus, A. niger, A. ruber A. terr*eusand *A. candidus*) are closely related and dominant as bio-deteriorating agents followed by *Alternaria, Curvularia, Fusarium, Rhizopus*, *Penicillium* etc. (Rasheed et al., 2004; Chavan, 2011). Further, most probable aflatoxin contaminations caused in oil seed crops is due to the presence of *A. flavus* prior to harvest or during storage (Torres et al. 2014). Moreover, in storage *Aspergillus flavus* is more susceptible for colonization of groundnuts (Nayaket al.2017)However, an association of *Aspergillus flavus* with *Aspergillus niger* cause storage rot of groundnut (Jadon et al. 2015). The oil holding capability of groundnut seeds decrease within a short period due to the irreversible phenomena of ageing. Under such conditions seeds were also susceptible to fungi, insects and other microorganisms (Santos et al. 2016).It has also been reported that moisture and sugar content in seeds increased gradually and deteriorating agents are more active with long term period in groundnut while the viability of seed decreases (Bewley and Black, 2012).According to Mosavi et al. (2011) and Rasheed et al. (2004),it was observed that moisture level increased during rainy season and decrease during long term storage. However, post harvested treatment may have impact on the quality of seed.

(Ibiam and Egwu, 2011).

2.3 PREVENTION OF OIL SEEDS FROM DETERIORATION DURING STORAGE:

2.3.1 CHEMICAL METHODS AND DISADVANTAGES

There are some chemicals which are used for prevention of seed deterioration in oil seeds. Phosphine, Pyrethrins, Diatomaceous earth (DE) and Ethyl Formate (Vapormate) are the some chemicals which recommended. Pyrethrins and DE use should be limited to storage area treatments and Vapormate is restricted for use by licensed fumigators only. This leaves phosphine as the key farm storage treatment for oilseed storage pests. Excess and irresponsible use of these chemical might be retained by the stored seeds, which could further a concern for the public health.

Long term use of the chemical may cause severe health issues such as:- cancer, liver damage, dominant health problems in females and feminizing effects in males, including lowered sperm counts and abnormal breast development. Long term use of DE may cause various skin related problems including lungs infection such as asthma, chronic obstructive pulmonary disease. While Ethyl formate is readily absorbed into the blood via the alveoli of the respiratory system. It is also reported to be absorbed from the gastrointestinal tract and slightly through the skin.

2.4 BIOLOGICAL APPROCHES

Today's agriculture is mostly based on technologies so that up gradation of strategies for prevention and control of bio-deterioration in oil seeds is

seems to be a serious issue. In recent agro-economic trends where nature friendly subway is needed for cure and control of oil seeds from various deteriorating agents, use of microbes is a better approach. In support of this phenomenon some of the beneficial soil microbes known as plant growth promoting rhizobacteria (PGPR) are *Pseudomonas, Azotobacter, Azospirillium, Burkholderia, Bacillus, Enterobacter, Erwinia, Flavobacterium and Rhizobium* (Deshwal et al.2013). Among all, genus *Pseudomonas* is one of the leading bacteria which inhibit growth of pathogenic fungus in agriculture fields (Lanteigne et al. 2012; Novik et al. 2015) and there are number of reports available regarding the potential use of Pseudomonas in maintenance of soil health and protection from pathogens. Choudhury et al. (2009) and Arora et al. (2013) suggested that these microbes are metabolically and functionally more diverse which makes them very useful in sustainable agriculture. In the recent era of sustainable agricultural production, considerable attention has been given to the immense potential of using fluorescent pseudomonas as biocontrol agents against bio-deteriorating agents. Moreover, a considerable work carried out in relation to use the fluorescent pseudomonads for enhancing crop growth and yield in a sustainable manner which has been shown to improve plant health or increase yield, which are usually referred the interactions in the rhizosphere (Montaño et al. 2014). However, florescent pseudomonads are very significant in enhancing availability of soil nutrients through transformation, mobilization and solubilization(Ahemad and Kibret, 2014). Bhakthavatchalu et al. (2013)conclude that plant growth promoting Pseudomonas strains produced

IAA, HCN and siderophore activity. Hence, for present days we can say that the treatment of seed deterioration by biological way is seems to be very beneficial for humanity and nature as well.

2.5 FUTURE PROSPACTS:

Due to the explosion demand of food is increasing day by day and for that there is a need of healthy plant resources. There are several kinds of pathogens that attack food plants in field and also during storage. So that it is necessary to control the pathogenic attack by using some measures. Chemical based fungicides and pesticides are widely used in crop protection but they also have some limitations such as toxicity resentence, polluted environment etc. So that in recent agro-economic trends it is needed to find out some environment friendly alternatives for disease control or pest management for field and storage crops. Biological control is a batter alternative for recent agriculture systems in this system chemical based pesticides are replaced by microbial pesticides commonly known as bio-pesticides which are natural products plant incorporated protectants (transgenic crops).

Plant growth promoting rhizobactria (PGPR) the most valuable biocontrol agent in today's agriculture seems quite relevant about its action mechanism for strategical crop protection and improving the efficacy of particular bio-control agent. The microbes used in bio-control treatment are basically nontoxic and environment friendly, have no pollutant and no other relevant side effects in comparison to chemical based pest or disease control

methods. Bio-control agents are also seemed nontoxic of mankind as well. Hence, after this study it was observed that biological treatments of disease management and crop protection have a healthy future for sustainable agriculture with lower investment and less harmful effects.

Table -2 Crop-wise Area covered under Oilseeds for last 05 years (2013-14 to 2017-18) in India

Crops	Season	Production ('000 tonne)					
		2013-14	2014-15	2015-16	2016-17	2017-18*	Normal
Groundnut	Kharif	8058	5930.5	5367.5	6047.64	7365.3	6553.8
	Rabi	1655.9	1471.2	1365.8	1413.69	1577.2	1496.8
	Total	9713.9	7401.7	6733.3	7461.53	8942.5	8050.6
Castorseed	Kharif	1726.6	1870	1751.8	1376.42	1489.7	1642.9
Nigerseed	Kharif	97.8	76.2	74.3	85.14	75.3	81.7
Sesame	Kharif	714.6	827.8	850.1	747.03	743.9	776.7
Rapeseed & Mustard	Rabi	7876.7	6282.4	6796.7	7917.23	8041	7382.8
Linseed	Rabi	141.7	154.6	125.5	184.25	172.8	155.8
Safflower	Rabi	113.4	90.1	53	93.9	45.1	79.1
Sunflower	Kharif	154.1	110.9	66.2	98.3	73.6	100.6
	Rabi	349.9	323.3	230.1	153.08	120.4	235.4
	Total	503.9	434.2	296.3	251.38	194	336.0
Soybean	Kharif	11860.8	10373.8	8569.8	13158.73	10933.7	10979.4
Edible Oilseeds	Kharif	20885.3	17319.2	14927.9	20136.84	19191.8	18492.2
	Rabi	9995.8	8167.1	8445.6	9577.9	9783.7	9194.0
	Total	30881.1	25486.3	23373.5	29714.74	28975.5	27686.2
Non Edible Oilseeds	Kharif	1726.6	1870	1751.8	1376.42	1489.7	1642.9
	Rabi	141.7	154.6	125.5	184.25	172.8	155.8
	Total	1868.3	2024.6	1877.3	1560.67	1662.5	1798.7
Total Nine Oilseeds	Kharif	22611.8	19189.2	16679.7	21513.26	20681.5	20135.1
	Rabi	10137.6	8321.7	8571.1	9762.15	9956.5	9349.8
	Total	**32749.4**	**27510.8**	**25250.8**	**31275.41**	**30638**	**29484.9**

*4thAdvance Estimates of DES GOI

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*4th Advance Estimates of DES

Chapter 3

Material and Methods

3.1 Sample collection

In this study all the groundnut samples were collected as described by Arora et al. (2008) from three different districts of Uttar Pradesh, India including Unnao (26º.54'22'73" N latitude 80º.48'36'64" E longitude), Manpuri (27.31'34'18" N latitude, 79.16'56'69" E longitude) and Farrukhabad (27º.32'81'55" N latitude and 79º.61'56'47" E longitude). Sample collection was completed during July-October growing sessions with the optimum climatic conditions. At the time of sampling the temperature is about 30ºC with an average rainfall of about 55-60 m. Three different fields are targeted for the collection of plants from each district. Further, the rhizospheric soil samples were also collected from the same fields as described by Arora et al. (2008). All the samples were collected separately in sterilized biohazard bags. All the samples were stored in our laboratory within 48 hrs.

3.2 Isolation and purification

3.2.1. Isolation of seed deteriorating pathogen:

Fungal isolation was complete according to Arora et al. (2008). The seeds of groundnut were obtained from collected samples. Seeds were washed in running tap water, followed by distilled water. To remove epiphytic microbes, samples were treated with Hydrogen peroxide solution for surface cleaning. all the seeds which shells were dipped in 3% H_2O_2

solution for 2 minutes then washed with double distilled water for three times and let them air dry under striate conditions. After successful sterilization, ground nut seed were placed on pre- prepared Potato dextrose agar (PDA) plates. All the plates were incubating at 25 degree centigrade for 07 days. Pure fungus colonies were separated on PDA plates for further examining.

3.2.2. Isolation of bacteria:

Isolation of bacteria was completed according to Kumar et al. (2012), in which rhizospheric soils were detached from roots of collected groundnut plants. The soil were serially diluted, two different serial dilution methods were used for this. In first process 10 germs of soil sample were suspended into 90 ml double distilled autoclaved water in 250 ml flask. Secondly, 01gm of soil sample was mixed with 09 ml of autoclaved DDW in test tube. Dilutions in test tubes from 10^{-9} to 10^{-4} of both soil suspensions were made. Mix them gently with the help of micropipette and spread 10µl for 10^{-4}, 10^{-5} dilution on pre prepared nutrient agar plate respectively, followed by King's B medium (Hi Media) King et al. 1954, with added glycerol. Incubate plates at 28ºC for 48 hrs. Bacterial colonies appeared on petri plate was further purified on Fluorescent Pseudomonas agar (Hi-Media) specific media for the isolation of *Pseudomonas fluorescence*. Fresh colonies were separated and examined under UV light for detection of fluorescent property of bacterial isolate.

3.3. Characterization of bacteria

3.3.1. Biochemical characterization

i) Catalase test

To determine catalase activity, fresh broth cultures (24 h old) of bacterial isolates was used and a loopfull of bacterial culture was placed on a glass slide and one drop of H_2O_2 (5-10%) was added. The appearance of gas bubbles is the indicator of catalase enzyme (McFadden, 1980).

ii) Oxidase test

This activity is determined by using oxidase discs. Fresh bacterial culture was spotted on the disc placed on a glass slide. Appearance of dark purple colour was observed within 10 second for positive oxidase test as described by Babu and Paramageetham 2013.

iii) Ammonia production

Qualitative detection of ammonia production was done by the method given by Cappuccino and Sherman (1992). Isolates were grown in peptone water at 28 ºC for 48-72 h. After incubation, 1ml of Nessler's reagent was added in each tube. Change of broth color from slight yellow to orange and brown indicate ammonia production.

3.3.2 Plant growth promoting activity

i) IAA activity

IAA production by rhizospheric isolates was determined according to the method of Khamna et al. (2009). Isolates were grown in 5 ml of

Glucose Yeast Extract (GYE) broth, supplemented with 0.2% (w/v) L-tryptophan at 28°C and 170 rpm for 7 days in the dark. After incubation cultures were centrifuged at 5000 rpm for 10 minutes at 4°C to harvest the cells. Then 1 ml of culture supernatant was taken into a fresh test tube and 1 ml of Salkowski's reagent (prepared according to Gordon and Weber, 1951) was added. Reaction mixture was incubated for 30 minutes at 28°C. Development of pink color in reaction mixture indicated the presence of IAA which was quantitatively estimated by measuring the absorbance at 530 nm in a spectrophotometer. Amount of IAA produced (µg/ml) was calculated with the help of a standard curve of 0.5-100 µg/ml concentration.

ii) Siderophore production

Siderophore production by isolates was assayed on the Chrome azurol Sulphonate (CAS) agar medium as described by Schwyn and Neilands (1987) with some modification as proposed by Perez-Miranda et al. (2007). Modified overlaid-CAS agar (O-CAS) plates were inoculated with test organism and uninoculated plates were kept as control. All plates were incubated at 28°C for 48–72 h. Development of halo zone or change in color from blue to purple or orange around the colony was considered as positive for siderophore production.

iii) HCN test

Hydrogen cyanide (HCN) production was evaluated by streaking the bacterial isolates on NA medium amended with glycine. Whatman No.1 filter paper soaked in picric acid (0.05% solution in 2% sodium carbonate)

was placed in the lid of each Petri plates. The plates were then sealed air-tight with parafilm and incubated at 30o C for 48 h. A color change of the filter paper from deep yellow to reddish-brown color was considered as an indication of HCN production (Bakker and Schippers, 1987).

3.4. Antagonistic activity of bacterial isolates against deteriorating agents:

3.4.1. Dual culture assay

The dual culture plate assay was carried out in triplicates for antifungal activity of potent bacterial strain culture against selected pathogenic fungi by dual culture method (Dennis and Webster 1971; Pal and Gardener 2006). The experiment was performed by inoculate 5mm disc of 7 days old culture of fungal pathogen on 1.5 cm from the left edge of the PDA containing petri dish. The bacterial culture was inoculated at 1.5 cm from the right edge of same petri dish. One of the petri dish inoculated fungus is used as control plate. The plates were incubated then at 29ºC for a week. The antagonistic activity of potent bacterial strain was observed by inhibition of the growth of pathogen.

3.4.2. Broth spray assay

Bacterial culture was grown in conical flask with king's B broth medium and incubated at 28^{o}C in incubator shaker for 2 days. The optical density was measured with the help of spectrophotometer for bacterial growth (Day et al. 2004). Further, supernatant of the bacterial culture was

extracted after centrifugation. Bacterial culture and supernatant were spray on 100g each of surface sterilized groundnut seeds and incubated at 28^0C for 30 days with following treatments:

Untreated seeds, seeds + RF-07 (fungal pathogen) seeds + RC-07 (bacterium), seeds + RF-07+ RC-07, seeds + RF-07+RC-07 + supernatant, seeds +RF-07+ supernatant Growth and inhibition of fungal contaminants was observed with regular intervals of 10, 20 and 30 days respectively.

3.5. Molecular characterization

Molecular identification of selected bacterial strains was performed on the basis of promising plant growth promoting and biocontrol potential against fungal pathogens, one of the best performing isolates RC-07 was selected for further study and identified on the basis of rRNA gene sequencing. Whereas, three dominant seed deteriorating fungi were go through the 18S rRNA sequencing for further identification.

3.5.1. 18S rRNA of gene sequencing

Pure fungal isolates RF-02, RF-03 and RF-07 were further analyzed for microscopic identification by using lactophenol cotton blue staining followed by 18S rRNA sequencing for confirmation on genus and species level. In that process standard protocols were followed at each level of isolation of fungal genomic DNA and RNA. Moreover, PCR amplification and purification of PCR product was completed as per the instruction of primer manufactures on the kit (Sigma Aldrich). After sequencing of

purified PCR product result sequences were go through the BLAST search tool providing by National Center for Biotechnology Information (NCBI).

3.5.2. 16S rRNA gene sequencing

Genomic DNA of WRE-1 was isolated by using the InstaGene TM Matrix Genomic DNA isolation kit. Using universal primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTG TTACGACTT), 16S rRNA gene fragment was amplified using MJ Research Peltier Thermal Cycler. Single-pass sequencing was performed on each template using 16s rRNA universal primers 518F (CCAGCAGCCGCGGTAATACG) and 800R (TACCAGGGTATCTA ATCC).

3.7 Analysis of Mechanism of action for bacterial antagonism:

Isolate RC-07 was analyzed further by Gas Chromatography and mass spectrometry (GC-MS) technique for the detection of metabolites, produced during the dual culture assay and spray assay. Fresh culture of isolate RC-07 was inoculated in an Erlenmeyer flask containing Nutrient broth medium and incubated for 3 to 4 days. Further, for the fermentation the flask was incubated at room temperature for 7 days on a rotary shaker with 110 rpm. After fermentation the culture broth was filtered and the filtrates were added with solvents chloroform and ethanol separately in a ratio of 1:1. Chloroform added culture broth formed two layers; the solvent layer was separated using separating funnel and stored in sterile vials. Ethanol added culture broth was retained as aqueous extract.

3.8 *In Vitro* study

Based on antagonistic activity in dual plate culture and bio-spray assay, bacterial isolate RC-07 was selected for pot study of groundnut seeds to observe the growth inhibition of *A. niger.* 100 g of groundnut seeds were soaked overnight in 500 mL distilled water (autoclaved at 121°C for 15 min) in 1 L conical flask, each for the following treatments such as: (a) Untreated seed, (b) seed+ *A. niger* RF-07, (c) seed + RC-07,(d) seed+ *A. niger* RF-07 + RC-07, (e) seed + *A. niger* RF-07+ RC-07+ supernatant and (f) seeds+ *A. niger* RF-07+ supernatant. Four-five seeds from each treatment were sown in pot (triplicates) designated as specific seed treatment in sowing sessions for groundnut seeds and allowed to grow in at 30-32°C for 90 days.

3.9 *In Vivo* study

After laboratory studies the treatments were applied on field located in Yaquitgang 27º.32’81’55” N latitude and 79°.61’56’47” E longitude) district Farrukhabad, Uttar Pradesh. Isolate RC-07 was applied on groundnut seeds to observe the growth inhibition of *A. niger.* 100 g of groundnut seeds were soaked overnight in 500 mL distilled water (autoclaved at 121°C for 15 min) in 1 L conical flask, each for the following treatments such as: (a) Untreated seed, (b) seed+ *A. niger* RF-07, (c) seed + RC-07,(d) seed+ *A. niger* RF-07 + RC-07, (e) seed + *A. niger* RF-07+ RC-07+ supernatant and (f) seeds+ *A. niger* RF-07+ supernatant. Four-five seeds from the each treatment were taken and they were sown in

pot (triplicates) designated as specific seed treatment in sowing sessions for groundnut seeds and allowed to grow in at 30-32°C for 90 days.

3.10 Soil analysis of experimental field

Soil analysis was executed after the completion of field trail on the targeted experimental field. The rhizospheric soil sample of control block and treatment block (Supernatant+ seed) was collected from each three blocks and stored at laboratory conditions as required. The soil samples were homogenized thoroughly and make to samples of control block and treated block respectively for the following types physico-chemical parameters:-

I) Estimation of Total Nitrogen

Nitrogen estimation was done according to Black (1965). 5gm of processed soil sample was taken in 500 mL flask and add 100 mg CuSO4, 1 g K_2SO4 and 25 mL Con. H_2SO4 in the flask. Thoroughly mix all content and then digest. Maintain the cooling temperature after digestion of the content. Fit the flask with two neck joints to one neck dropping funnel is connected for adding 40 % NaOH while to the other neck Kjeldahl trap, which is used to trap the NaOH coming with the distillate. The trap is connected to the condenser with a delivery tube which dips into 25 mL of 0.1 N HCl contained in a conical flask, with one or two drops of methyl red indicator. Add about 25 mL of 40 % NaOH solution till the content are alkaline in reaction. Heat the conical flask. Allow the ammonia formed to be absorbed in standard HCl. When no more ammonia is received stop the

distillation. Titrate the excess of the acid with 0.1 N NaOH solutions till the pink color changes to greenish. From the titrate value calculate the multi equivalence of the acid participating in the process of ammonia absorbing during digestion.

II) Estimation of phosphate

The estimation of phosphate content of soil sample was determined as Nagul, (2015). The principle of this method involves the formation of molybdophosphoric acid, which is reduced to the intensely colored complex, molybdenum blue. This analytical method is extremely sensitive and is reliable down to concentrations below 0.1 mg phosphorus per litter. Standard Phosphate solution was prepared from phosphate solution. Five standard solutions and the blank should now be treated according to the following "color development" procedure. After measuring the absorbance at wavelength of 650 nanometers make a standard graph of absorbance versus concentration. The sample was placed in flask 25 mL and put 1.00 mL of ammonium molybdate solution into the flask and swirls to mix. To the flask add 2 drops of stannous chloride solution and mix by swirling. If phosphate is present, a blue color was developed to a maximum in 5 minutes. Measurements should be taken anywhere from 5 to 15 minutes after addition of stannous chloride. After the blue color formed, Optical density was taken at wavelength to 650 nm on the spectrophotometer. Use the blank solution to set it to read zero absorbance**.**

III) Estimation of potassium

The total potassium of soil sample was estimated by using flame photometry assay as describe in IS9497: 1980 by Biotech park Lucknow.

IV) Estimation of total carbon

Estimation of total organic carbon was completed according to Schumacher (2002) by UV/visible spectrophotometric from soil required 1N potassium dichromate, 98% conc. Sulphuric acid and standard glucose solution. Five mL of freshly prepared potassium dichromate was added in 500 mg of soil sample. Concentrate sulphuric acid was added in sample and mixed slowly. The solution was kept overnight and Optical density was taken at 660 nm. All experiment was performed in triplicate and total carbon was estimated by comparing with standard graph of glucose.

V) pH Value

The soil pH reflects the nature wither it may be acidic, neutral, basic or alkaline. the pH meter (Labman- LMPH 10) was calibrated first of all by using two buffer solutions of different pH values, 4.0 and 7.0. First of all the temperature of the solution was measured and the temperature knob was adjusted. Then the combined electrode rod was dipped in pH 7.0 buffer solution, actual pH was checked at measured temperature and adjusted/calibrated with buffer knob. Then the combined electrode was dipped in pH 4.0 buffer solution and adjusted with sensitivity Knob. These were repeated until pH meter gave correct reading of both the test buffer

solutions. pH value of treated and untreated soil was measured as per Bruckner, (2012).

VI) Conductivity

Electrical conductivity can be measured as described by Bruckner (2012) with the help of EC meter. The probe or sensor consists of two metal electrodes and a constant voltage is applied across the electrodes resulting in an electrical current flowing through the sample. Since the current flowing through the soil is proportional to the concentration of dissolved ions in the soil, the electrical conductivity can be measured. The higher the dissolved salt/ion concentration, the more conductive the sample and hence the higher the conductivity reading.

3.11Analysis of Oil Content

3.11.1 High pressure liquid chromatography

Oil content analysis of groundnut oil was carried out by using HPLC technique. For Vitamins analysis by HPLC, 500 µl of oil extracts were prepared in acetone: chloroform (30:70), dried and finally dissolved in methanol. For Standard 100µg/ml of each Fat soluble vitamin (1000 µl) is used. A linear gradient methanol, acetonitrile and water mixed in the ratio (84:14:2) at a constant flow rate of 1 ml/min with 2300 pressure and a UV (2487) detector was employed for the detection of peaks, using two channels simultaneously at a wavelength of 292 nm, a bandwidth of 5 nm and another wavelength of 280 nm.

Analysis of fatty acid methyl ester (FAME)

For fatty acid analysis of groundnut oil through GC, weigh approximately 160 g of NaCl into a beaker. Use a scuttle to transfer NaCl into a 1000 mL Erlenmayer flask. Add 400 mL of distilled water and heat the flask on an electric hot plate, shaking the content occasionally. Further, Prepare fatty acid methyl esters by taking 250 mg of sample into a 100 mL round bottomed flask using a Pasteur pipette. Add a boiling stone, and 6 mL of methanolic solution of NaOH (c = 0.5 mol/L). Attach the flask to a side-arm Y-piece and reflux condenser, and heat the content. Add 10 mL of heptane using the side-arm of the Y-piece, and heat for one more minute. Switch the heater off, add saturated solution of NaCl and shake in a circular fashion. Fill the flask with saturated solution of NaCl such that the heptane layer reaches the narrow part of the flask. Let the flask cool down and the phases separate. Using a Pasteur pipette transfer 1 mL of the upper (heptane) layer into a GC vial.

3.12 statistical Analyses

Statistical analysis of the data was carried out by analysis of variance (ANOVA) method using PASW Statistics 18 (IBM SPSS, Chicago, IL, USA). Means and standard errors were calculated for data of three replicates for pot and field experiments. Comparisons between means were carried out using Duncan's multiple-range tests (DMRT) at a significance level of $p < 0.05$.

Chapter 4

Results

4.1 Fungal pathogens of storage groundnut

Total 7 fungi were isolated from stored groundnut seeds in which 3 fungal colonies were dominant on groundnut seeds grew rapidly and produced colors of white, yellow, yellow-brown, brown to black or shades of green, mostly consisting of a dense felt of erect conidiophores were broadly classified as *Aspergillus species* after morphological identification as described by Klich 2002. During the morphology study for RF-02 and RF-03 almost similar results were found like the conidiophores were, thick walled, and coarsely roughened or pitted and was vesicle bearing while conidia were globose with thin walls, which were slightly roughened. All the 3 fungal strains show highly dominant nature throughout the study. (Fig-9,10)

4.1 Bacterial isolates and their morphological properties

In this study total 7 bacteria were isolated from rhizospheric soil of groundnut. All the isolates showed mucilaginous colonies with smooth margins, and produce florescence on Hi media fluorescent pseudomonads specific agar medium plates (Fig -11). Among all isolates RC04,RC 05, RC06 and 07 were showing fluorescens in UV light (Fig-12) all of them are Gram negative and rod shaped (Fig -13).

4.3 Biochemical identification of bacterial isolates

All seven bacterial isolates were further analyzed for various bio-chemical activities for most of the analyses KB-002 Hi media biochemical test kit specific for Gram negative rods were used. All seven isolates were positive

for catalase activity analyzed by adding drop of hydrogen peroxide (Fig-14). All the isolates showed positive oxidase, nitrate reductase, urease production and citrate utilization while none of the isolates showed positive H_2S Production activity. Along with this enzymatic activity of lipase showed negative by all the isolates and TDA shows more likely to be positive. Presence of glucose was positive for all the isolates but in case of lactose it was about 89% positive and in arabinose in was not actually determined by the biochemical test kit (Hi Media-KB-002) On the basis of these biochemical tests all the isolates were observed to be very close with *P. fluorescens*. (Fig-15)

4.4 Plant growth promoting properties of isolates

4.4.1 IAA production:

All of the 07 bacterial isolates show positive IAA production ability when observed by UV visible spectrophotometer (Evolution 201 UV visible) analysis and colour change. Among all RC-07 was the best IAA producer at optimum cultural conditions. (table-5; Fig-16)

4.4.2 Siderophore production

Only one isolate RC-07 shows positive result on CAS agar plates during qualitative analysis with yellowish zone on CAS plate. (Fig-16)

4.4.3 HCN production

Isolate RC-01, RC-04 and RC-07 was showing positive results as observed by colour change of filter paper soaked in picric acid from yellow to orange.(Fig-16)

4.5 Anti-fungal assay

The antagonistic potential of the isolate RC-07 was checked on fungal deteriorating agent RF-07 *Aspergillus niger* through dual culture and spray dilution antifungal assays. In dual culture assay the zone of inhibition was measured in mm and compared with growth of deteriorating pathogen in control plate (Fig-17). Further, in spray dilution assay fresh culture of isolate RC-07 was spray on groundnut seeds and incubates for 30 days (Fig-22). Results recorded after regular intervals of 10, 20, and 30 day shows that the supernatant of isolated *P. fluorescens* strains was more potent as compared with bacterial suspension with no growth of pathogens after 30 days (table-7). Isolate RC-07 shows positive and maximum inhibition of fungal deteriorating agent *A. niger*. So that the isolate RC-07 found as best biocontrol agent against storage seed deteriorating fungi.

4.6 Molecular characterization

4.6.1 16S rRNA identification of bacteria

Antagonistically active bacterial isolate RC-07 was screened further on various basic tests among all other seven strains. The isolate was gram negative and rod shaped shows positive oxidase and catalase tests with fluorescent property under UV light. Further, in 16s rRNA sequencing strain was showing 99% similarities with genus *Pseudomonas*. Moreover, a comparative study with available database on NCBI and The phylogenetic analysis (figure) of that strain RC-07 shows the direct relation with *Pseudomonas fluorescens*. Sequence was submitted with the accession number LC375795.(Fig-18)

4.6.2 18S rRNA identification of deteriorating fungi

Among all the isolates three fungal strains were picked up for further study (RF-02, RF-03 and RF- 07) (Figure). These three isolates were very dominant on seeds during the period of isolation and incubation as well. The identity of these isolated fungi were determined by amplification and sequencing of the 18S rRNA. The edited sequences were deposited in Gene Bank, under accession KY933394, MF120213.1 and KY357318 respectively (Fig-19, 20a and 20b). RF-02 with RF-03 was identified as *Aspergillus flavus* and RF-07 as *Aspergillus niger* by 18S rRNA sequencing.

4.7 Metabolite production analysis

It was observed that *Pseudomonas fluorescens* strain RC-07 protect groundnut seeds very effectively against *A. niger* during spray assay. However, supernatant of *P. fluorescens* strain RC-07 shows best inhibition in comparison to broth spray. Further, the supernatant was analyzed through gas chromatography and mass spectrometry technique. A peak shown at a retention time of 6.780 min confirm the presence of 2,4-diacetylphloroglucinol (2-4 DAPG) while another peak at RT 18.679 confirm the presence of Phenazine-1-Carboxylic Acid in the supernatant. (Fig-24).

4.8 *In Vitro* pot studies

During the pot study it was observed after regular intervals of 45 and 90 days that the groundnut seed treated with supernatant spray of *P. fluorescens* RC-07 shows best results against *A. niger* RF-07 pathogen in comparison to direct broth spray on seeds. After maturation, the difference between control

and supernatant treated seed are clearly shown the enhancement of crop. While seeds infected with pathogen was not even grow properly. Direct bacterial contact with seed was not as effected as needed in bacteria+ seeds and bacteria+ fungus+ seeds. The control grows in normal speed during pot study. So that this study found that the spray method is more effective for prevention of seed to be sown in pot from *A.niger*. .(table-8; Fig-23)

4.9 *In Vivo* field Studies

The field study was also observed with same intervals of 45 and 90 days that groundnut seed treated with supernatant spray of *Pseudomonas fluorescens* RC-07 shows best results against *Aspergillus niger* RF-07 pathogen in comparison to direct broth spray on seeds. After maturation, the difference between control and supernatant treated seed are clearly shown the enhancement of crop. While seeds infected with pathogen was not even grow properly. Direct bacterial contact with seed was not as affected as needed. While, the control plant was grow in normal speed. So that this study found that the spray method is more effective for prevention of seed to be sown in fields or pot from *A.niger*.(table-9; Fig-26)

4.10 Oil content analysis

4.10.1 High performance liquid chromatography

High performance liquid chromatography profile of ethyl acetate extract of groundnut seeds oil clearly showed the presence of vitamins E, D2 & K which was confirmed through the compression with standard references of fat soluble vitamins. (Fig-27)

4.10.2 Fatty acid methyl easter analysis (FAME)

Fatty acid methyl Easter analysis (FAME) by GC of groundnut oil clearly showed the presence of oleic, linoleic and some other oils which was confirmed through the slandered libarary with an oil content of about 43%. Different characteristic peaks were obtained in chromatogram between retention times (RT) as depicted in the chromatogram.(Fig-28)

Figures and tables

Figure-8 Sampling from Unnao, Farrukhabad and Mainpuri district

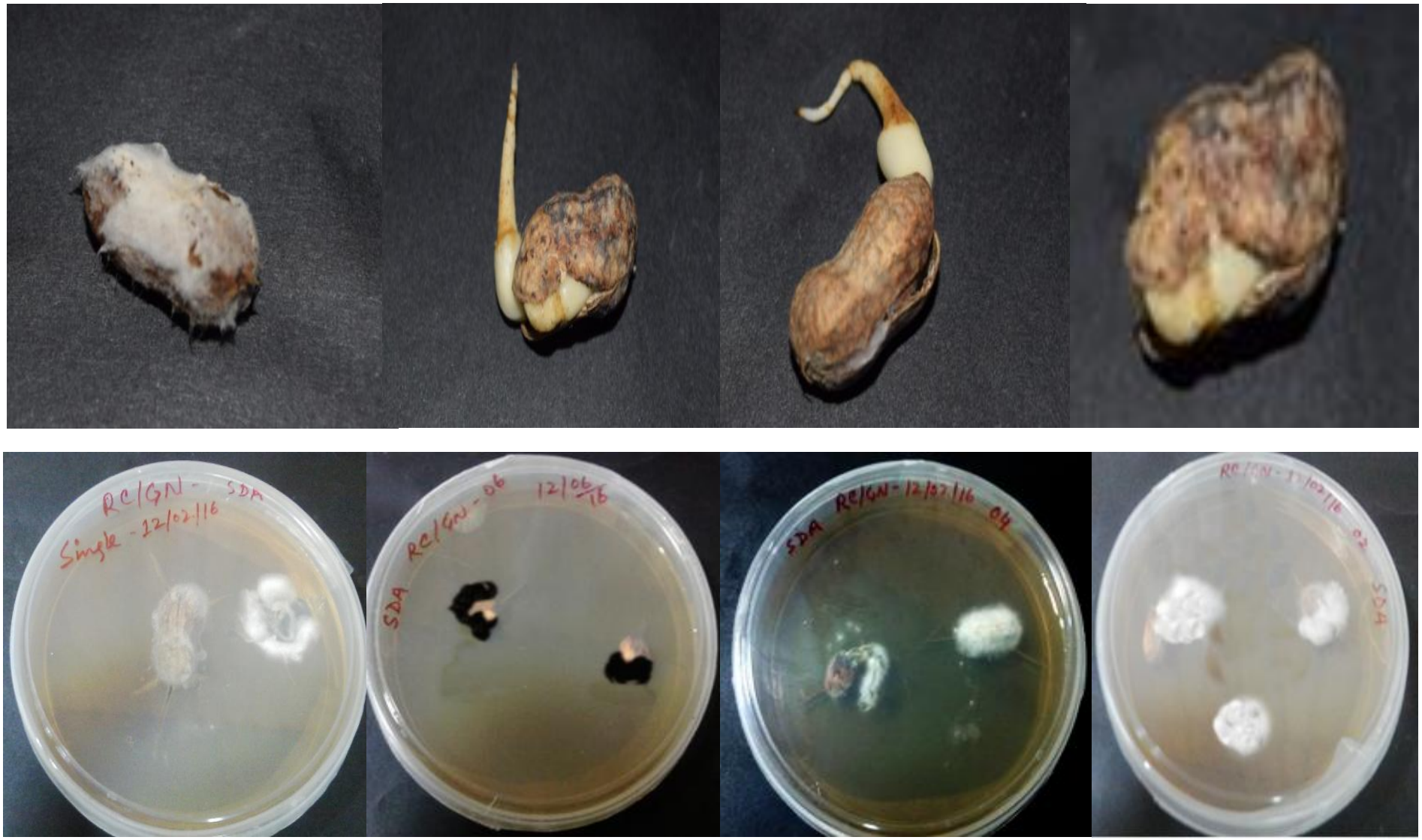


Figure-9 Isolation of deteriorating fungi on PDA plates

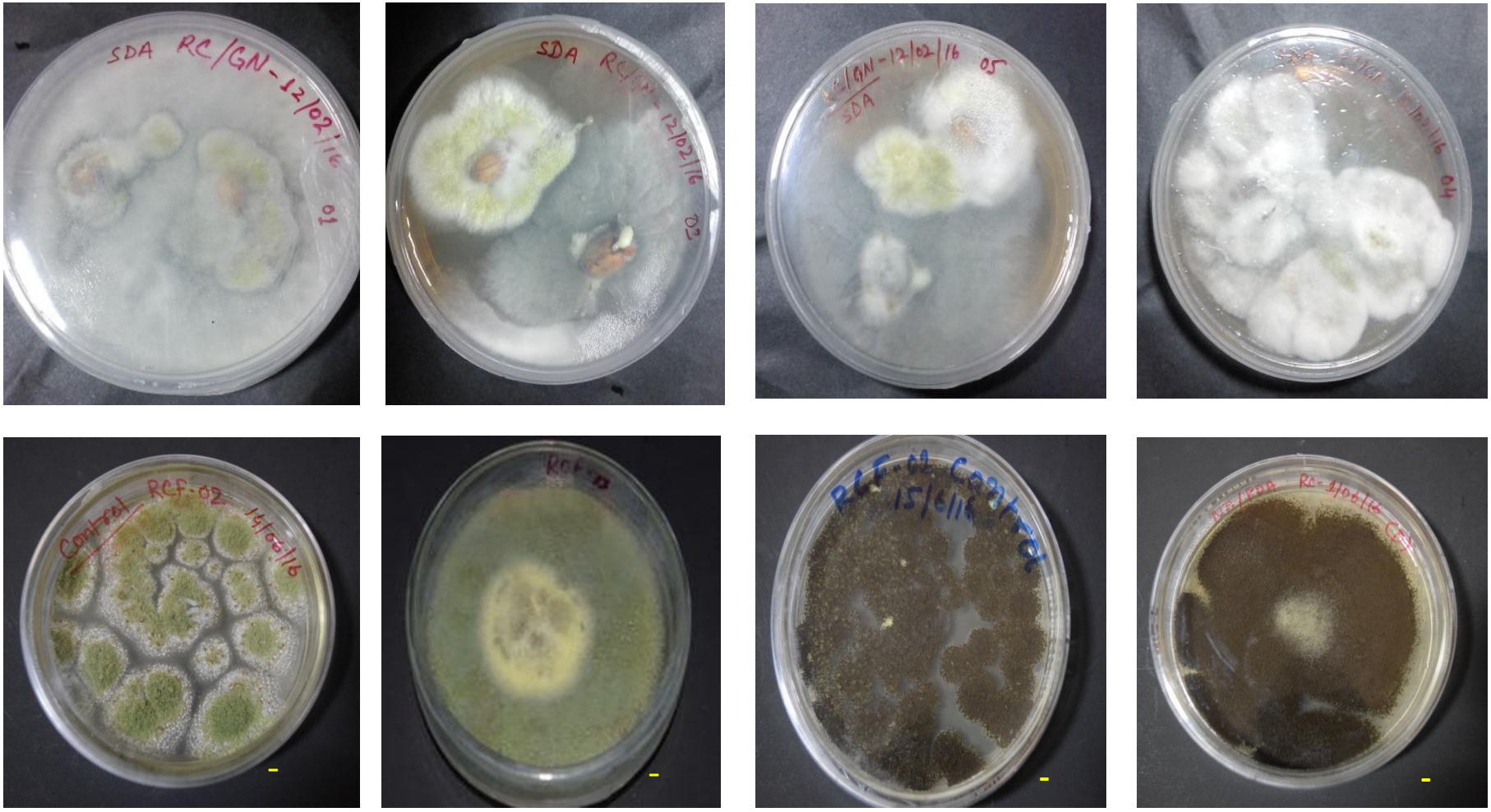


Fig-10 Isolated and purified fungal colonies on PDA plates

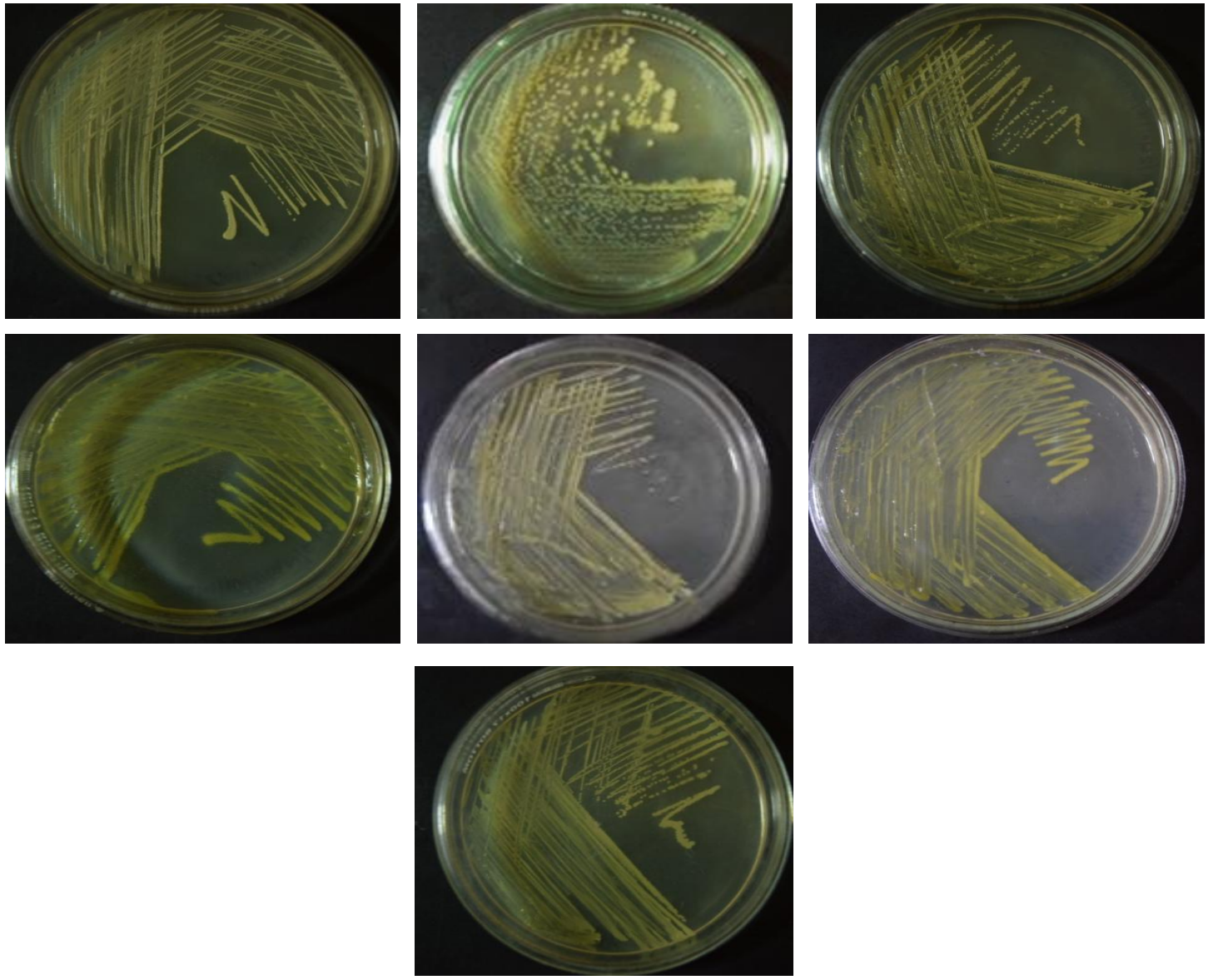


Figure -11 Colony morphology and pigmentation onto the Fluorescent Pseudomonas Medium

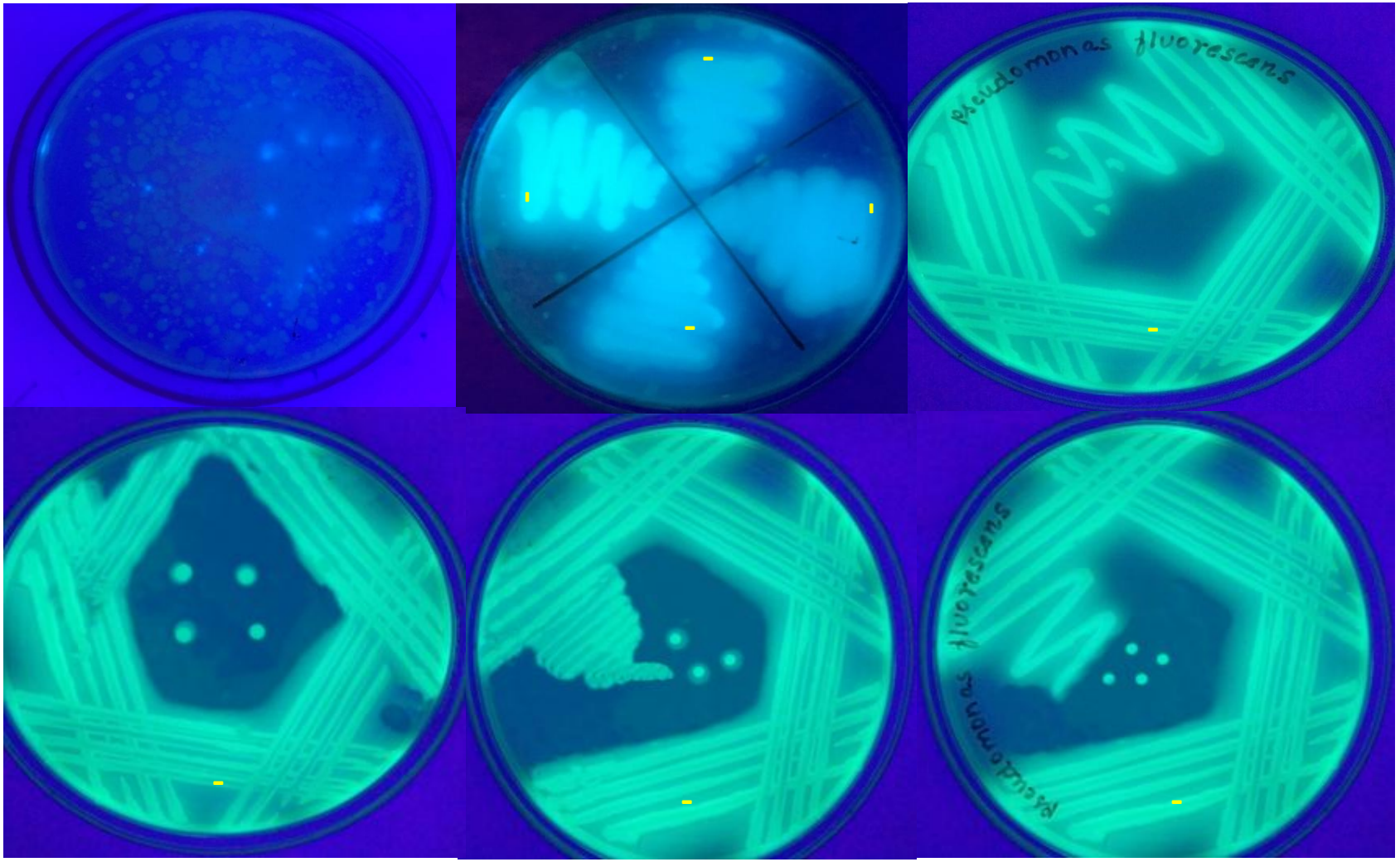


Figure-12 Plates showing positive UV visibility test of bacterial isolates

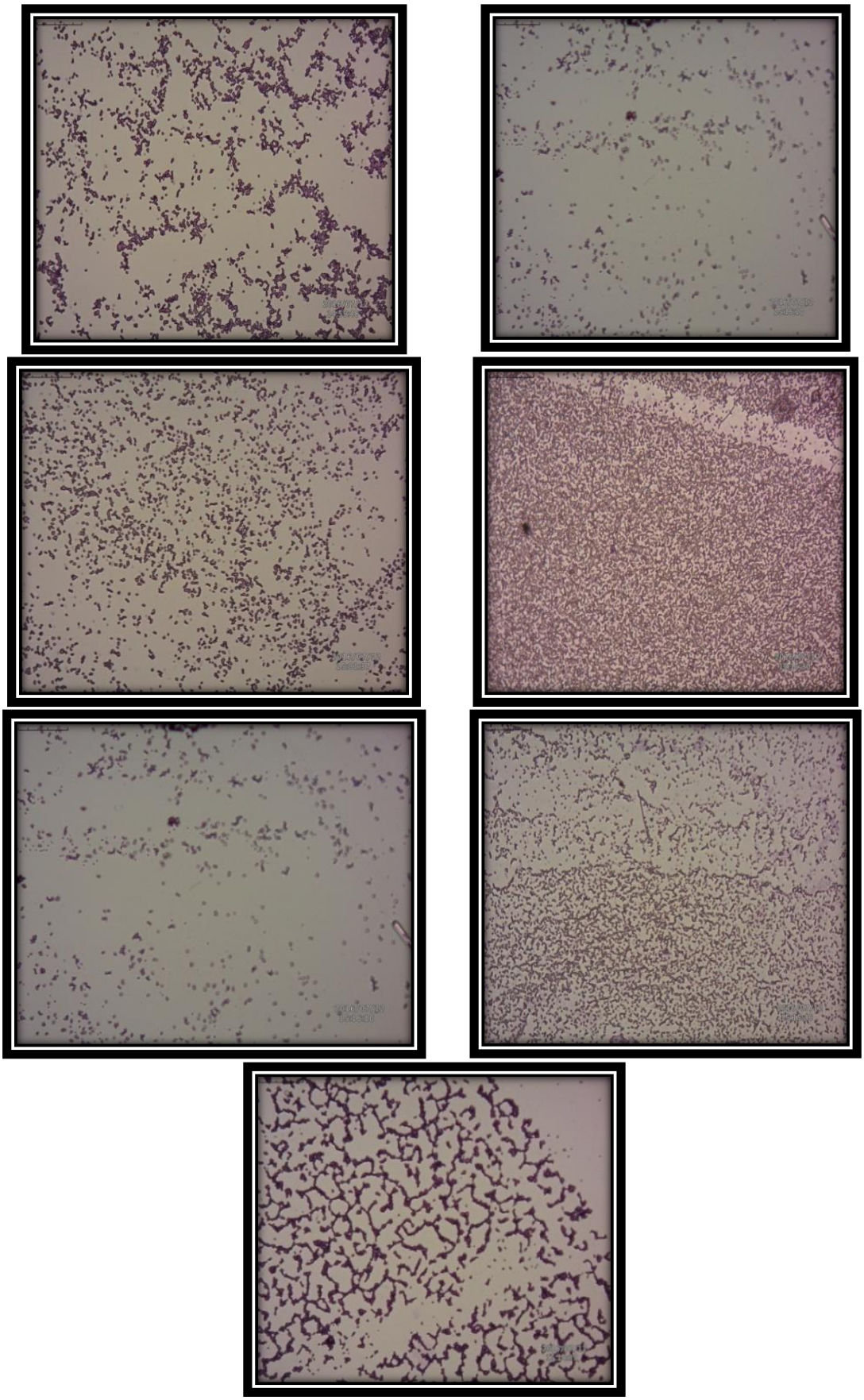


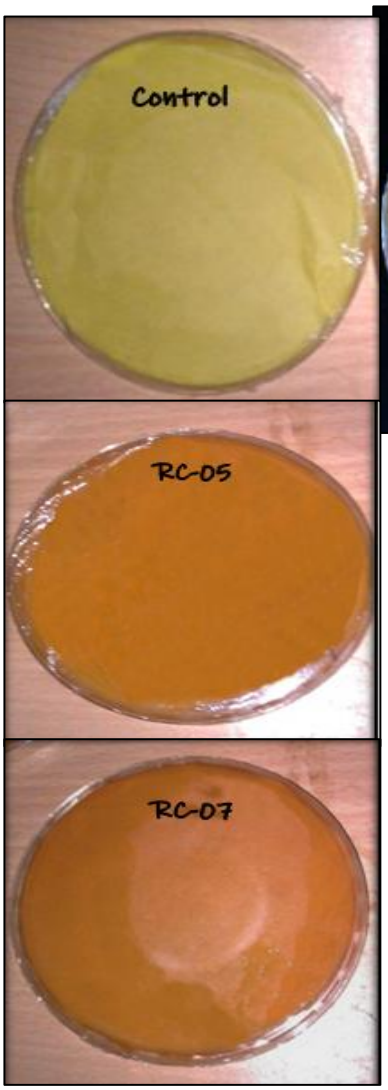
Figure-13 Gram's staining of bacterial isolates

Sample	Gram's Nature	Morphology
RC-01	Gram negative	Rod shape
RC-02	Gram negative	Rod shape
RC-03	Gram negative	Rod shape
RC-04	Gram negative	Rod shape
RC-05	Gram negative	Rod shape
RC-06	Gram negative	Rod shape
RC-07	Gram negative	Rod shape

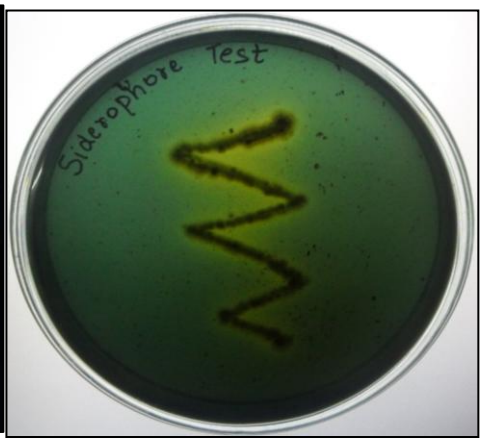
Table -4 Morphological characteristic of bacterial isolates

Qualitative Estimation of Plant Growth Promoting Activity					Quantitative Estimation
	IAA	**Siderophore**	**Catalase production**	**HCN**	**IAA**
RC-01	++	-	+	+	0.746±0.29
RC-02	+	-	+	-	01.164±0.42
RC-03	+	-	+	_	0.918±0.38
RC-04	+	-	++	_	4.668±1.90
RC-05	+	-	++	++	2.465±0.90
RC-06	++	-	++		1.14±0.43
RC-07	+++	+++	++	++	10.925±3.69

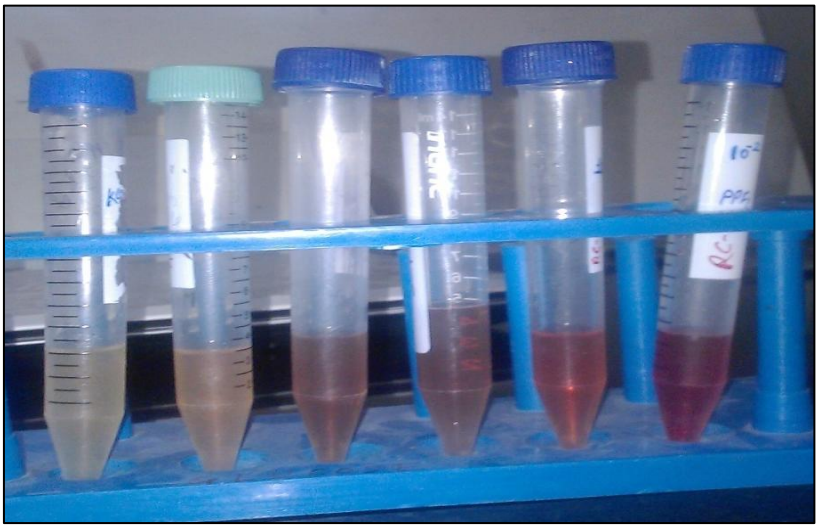
Table -5 Plant growth promoting traits of isolates



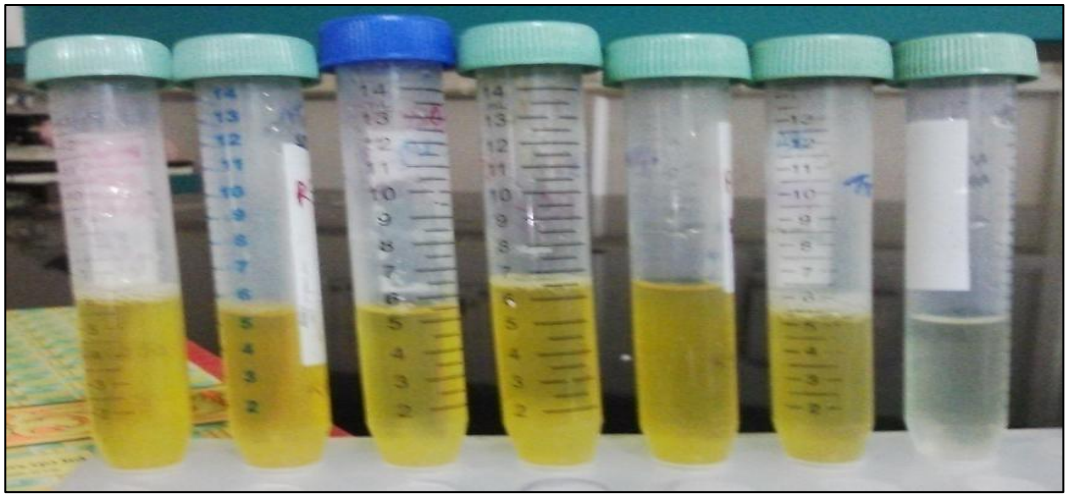
HCN production



Siderophore production



IAA production



Ammonia production

Figure-14 Plant growth promoting traits of isolates

Sample-	RC-01	RC-02	RC-03	RC-04	RC-05	RC-06	RC-07
Citrate Utilization	+	+	+	+	+	+	+
Lysine	-	-	-	-	-	-	-
Urease	+	+	+	+	+	+	+
TDA	v	v	v	v	v	v	v
Nitrate reduction	v	v	v	v	v	v	v
H2S Production	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	+
Adonitol	nd	nd	nd	nd	nd	nd	nd
Lactose	v	v	v	v	v	v	v
Arabinose	v	v	v	v	v	v	v
Sorbitol	-	-	-	-	-	-	-

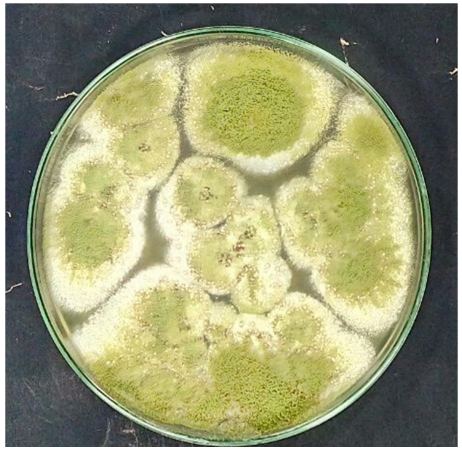
Table-6 Biochemical characterization of bacterial isolates isolate

No.	Test	Original colour of the medium	Positive reaction	Negative reaction
1	Citrate utilization	Green	Blue	Green
2	Lysine utilization	Olive green to Light Purple	Purple / Dark Purple	Yellow
3	Ornithine utilization	Olive green to Light Purple	Purple / Dark Purple	Yellow
4	Urease	Orangish yellow	Pink	Orangish yellow
5	Phenylalanine Deamination	Colourless	Green	Colourless
6	Nitrate reduction	Colourless	Pinkish Red	Colourless
7	H_2S production	Orangish yellow	Black	Orangish yellow
8	Glucose	Pinkish Red / Red	Yellow	Red / Pink
9	Adonitol	Pinkish Red / Red	Yellow	Red / Pink
10	Lactose	Pinkish Red / Red	Yellow	Red / Pink
11	Arabinose	Pinkish Red / Red	Yellow	Red / Pink
12	Sorbitol	Pinkish Red / Red	Yellow	Red / Pink

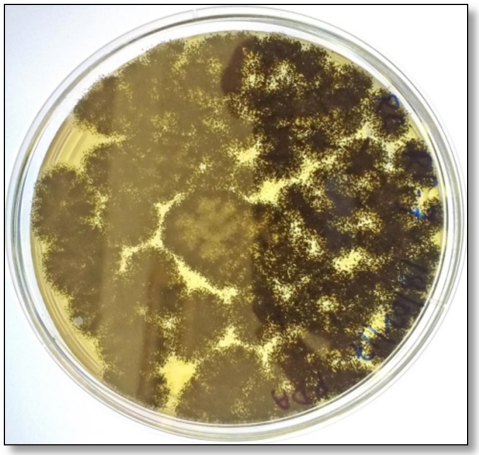
Identification Index for Gram-negative rods

Tests	Citrate utilization	Lysine	Ornithine	Urease	TDA	Nitrate reduction	H_2S production	Glucose	Adonitol	Lactose	Arabinose	Sorbitol
Pseudomonas aeruginosa	+	-	-	V	-	+	-	+	nd	-	-	-
Pseudomonas fluorescens	+	-	-	V	-	V	-	+	nd	V	V	-
Pseudomonas putida	+	-	-	V	-	-	-	+	nd	V	V	nd

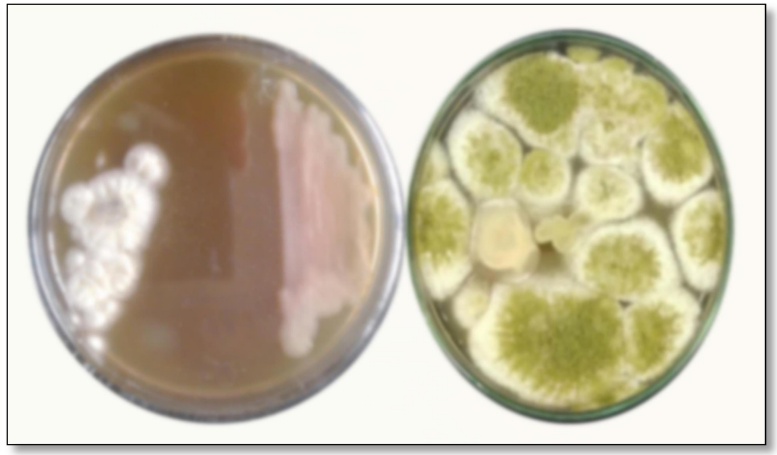
Figure-15 Biochemical characterization of isolates through hi media test kit KB002



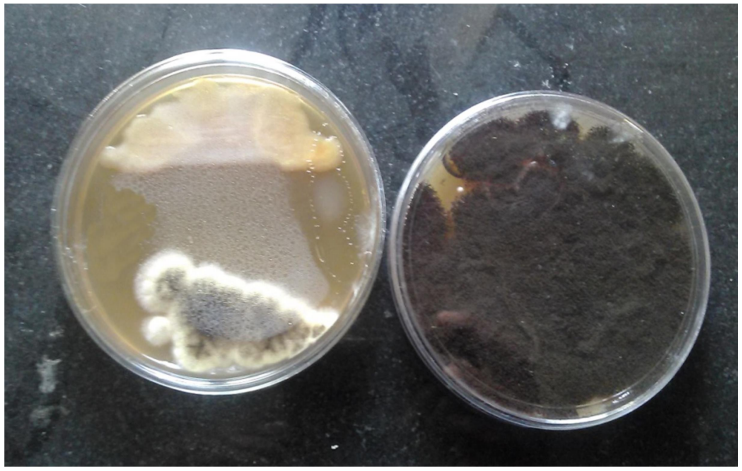
Aspergillus flavus
RF-02 Control



Aspergillus niger
RF-07 Control



Biocontrol of RF-02 by RC-07
with Control RF-02



Biocontrol of RF-07 by RC-07
with Control RF-07 (A)

Biocontrol of RF-07 by RC-07
with Control RF-07 (B)

Figure-16 Bio-control activity analysis by dual culture method

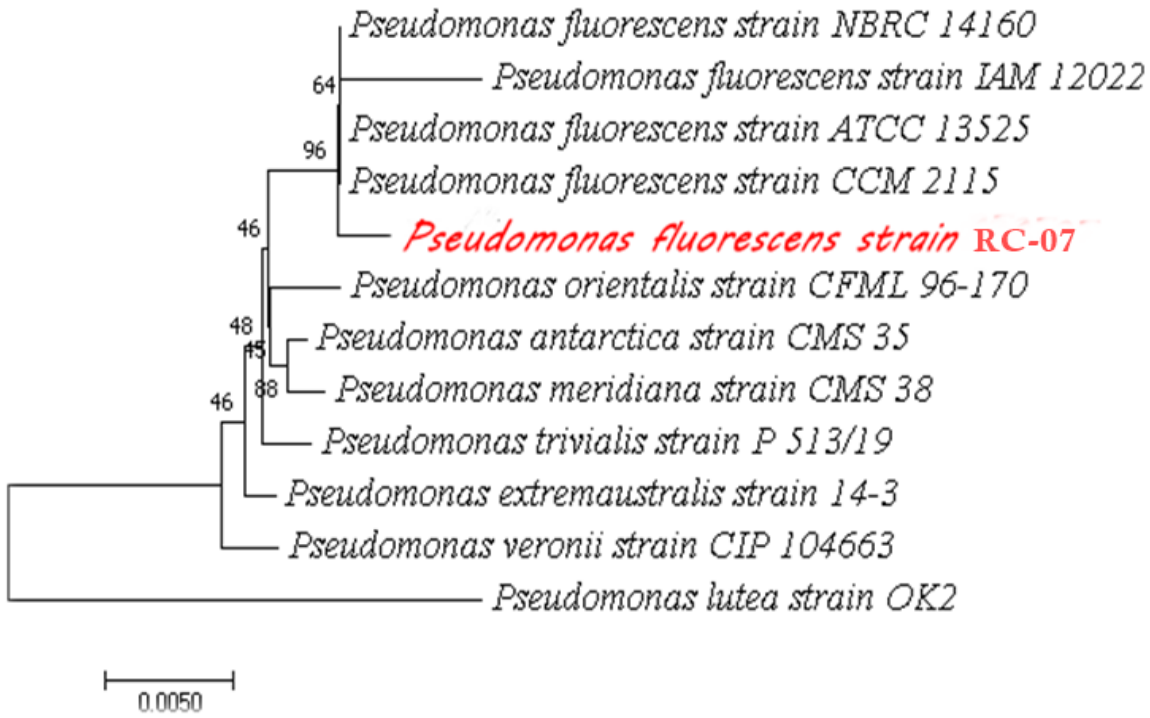


Figure-17 Phylogenetic analyses of RC-07 (*Pseudomonas fluorescens*) Accession no. LC375795

Treatments	10 Days	20 days	30 days
T1	++	++	++
T2	++	+++	+++
T3	-	-	+
T4	-	-	+
T5	-	-	-
T6	-	-	-

Table-7 Broth spray assay (+) = slow growth of pathogen,(++)= medium growth (+++) high growth while (-) denotes no growth of pathogen

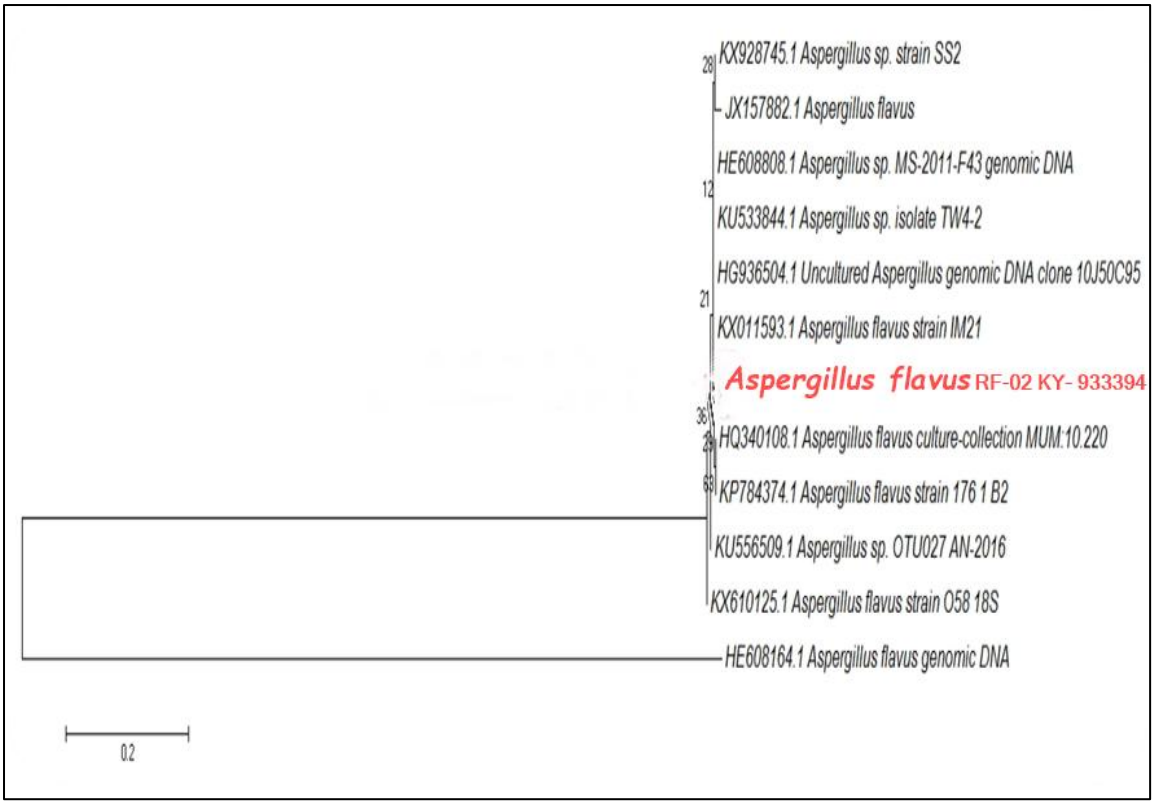


Figure-19a Phylogenetic analyses RF-03 (*Aspergillus flavus*) NCBI Accession no. KY933394

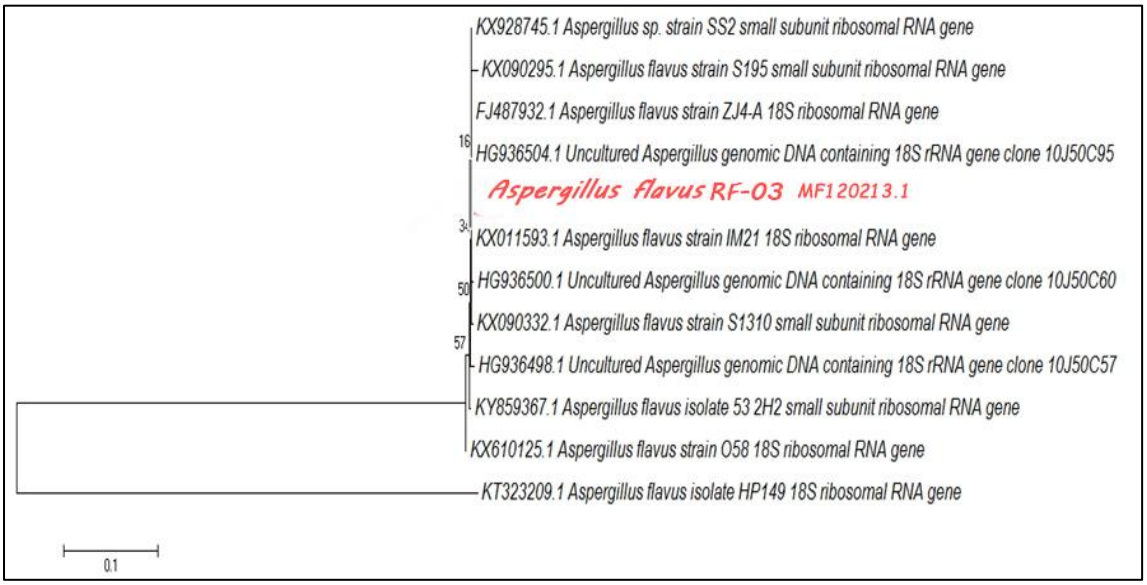


Figure -19b Phylogenetic analyses RF-02 (*Aspergillus flavus*) NCBI Accession no. MF120213.1

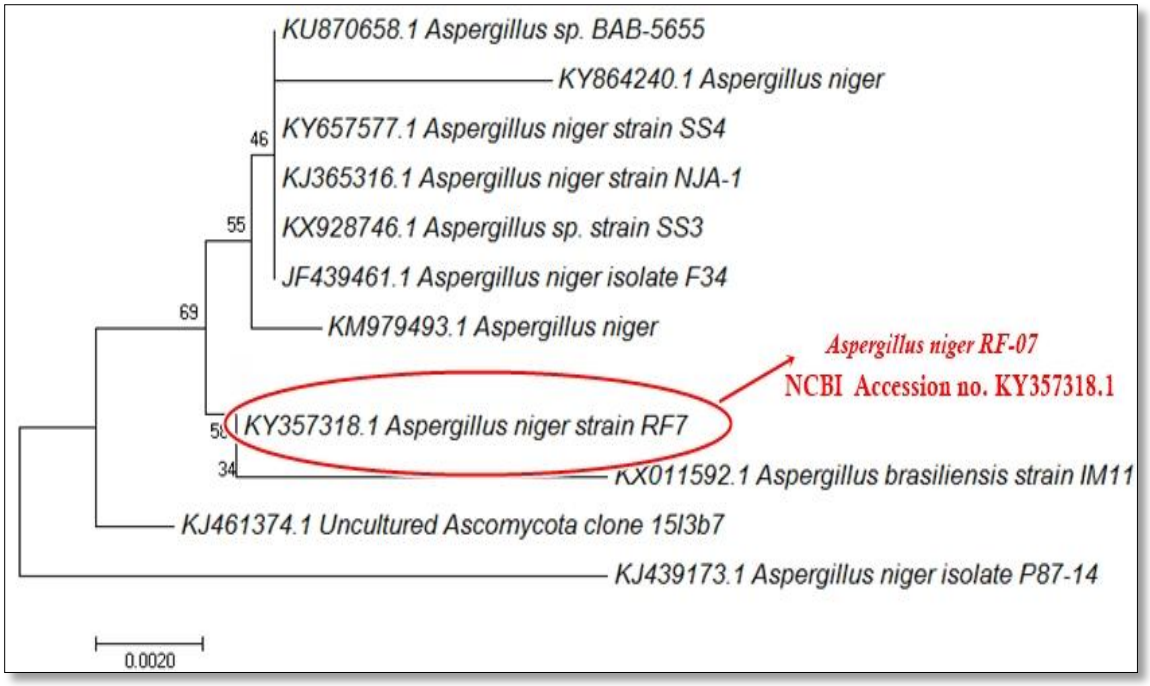


Figure- 20 Phylogenetic analyses RF-07 (*Aspergillus niger*) NCBI Accession no. KY357318

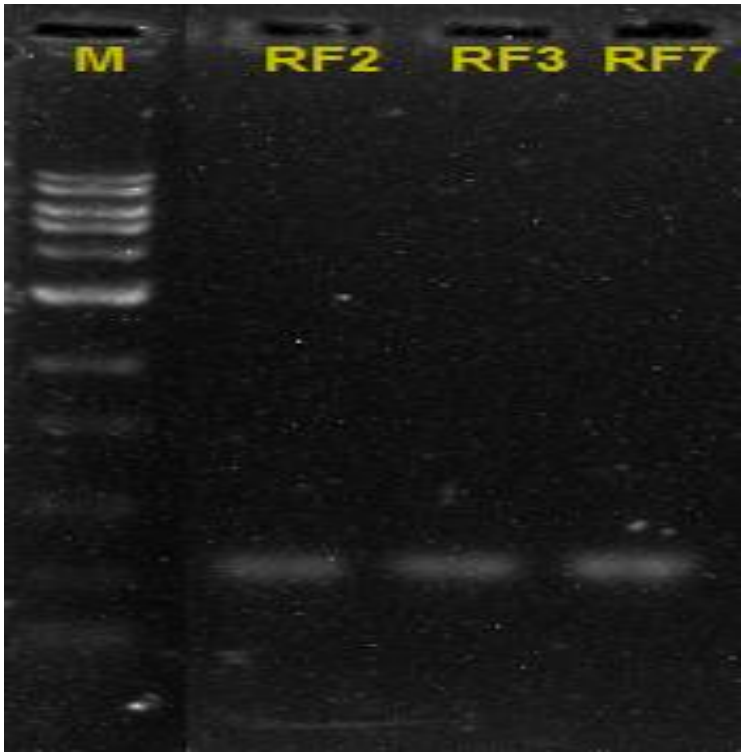


Figure -21 PCR amplification of 18S rRNA gene of isolated fungal strains

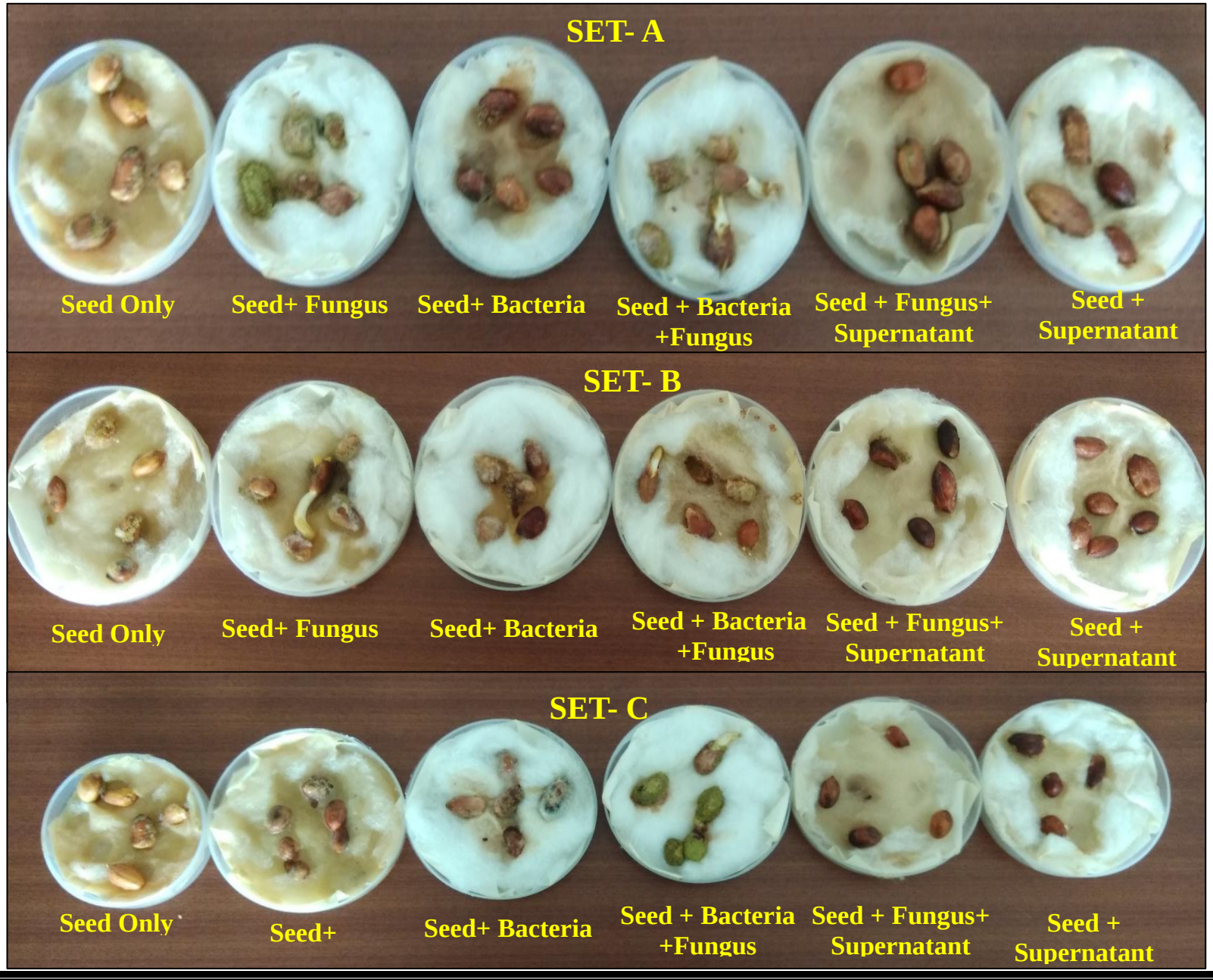
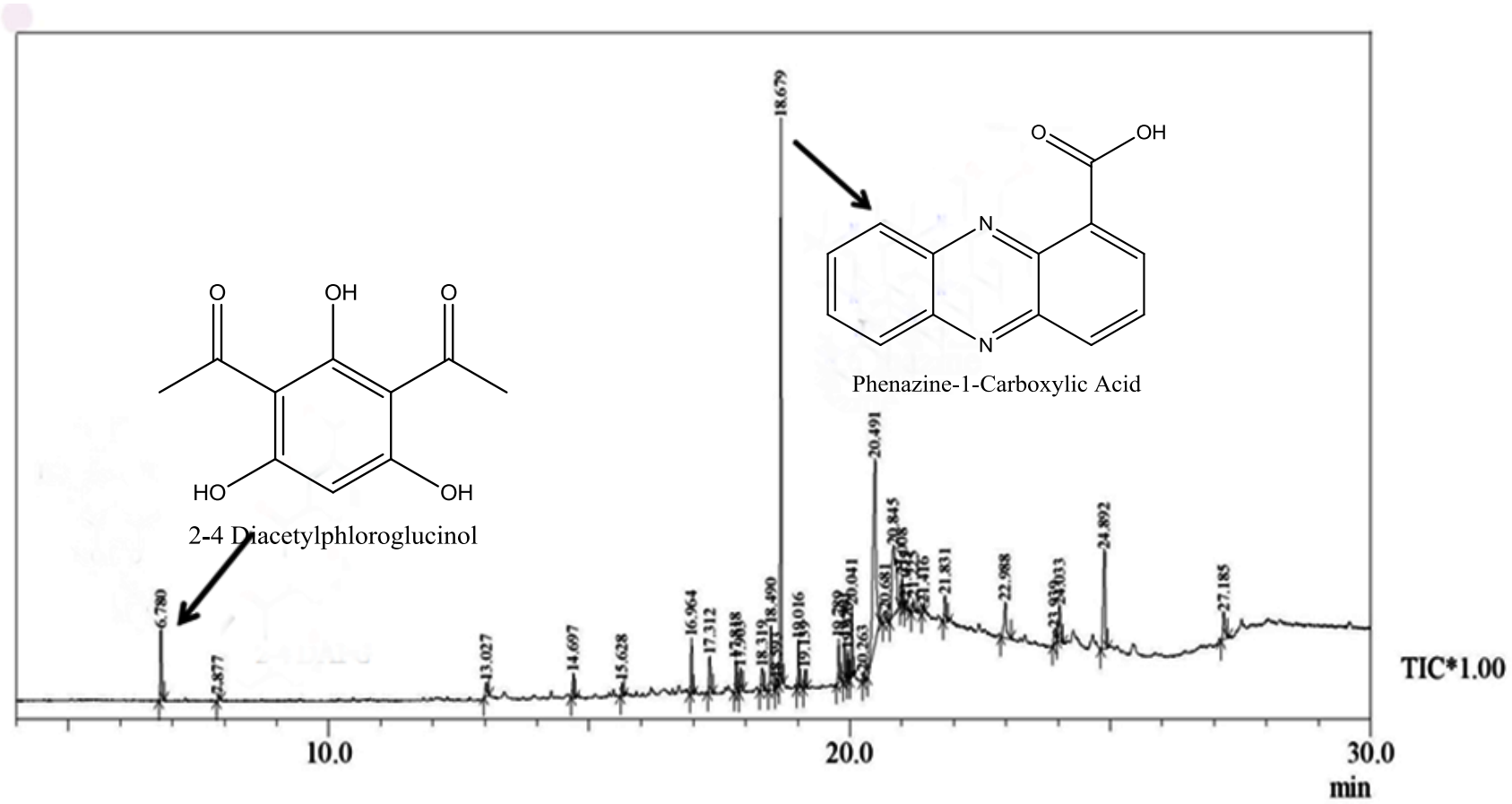

SET- A
Seed Only
Seed+ Fungus
Seed+ Bacteria
Seed + Bacteria +Fungus
Seed + Fungus+ Supernatant
Seed + Supernatant
SET- B
Seed Only
Seed+ Fungus
Seed+ Bacteria
Seed + Bacteria +Fungus
Seed + Fungus+ Supernatant
Seed + Supernatant
SET- C
Seed Only
Seed+
Seed+ Bacteria
Seed + Bacteria +Fungus
Seed + Fungus+ Supernatant
Seed + Supernatant

Figure-23 *In vitro* analyses of treatments on groundnut seeds (Pot studies)

-	**45 days**		**90 days**	
Treatments	**Shoot length (cm)**	**Root length (cm)**	**Shoot length (cm)**	**Root length (cm)**
Seed (Control)	9.03 ± 0.25 e	4.83 ± 0.47 d	16.03 ± 0.25 d	11.80 ± 0.36 de
Seed+ AN	4.80 ± 0.45 f	2.83 ± 0.25 e	13.66 ± 0.49 e	10.03 ± 0.47 e
Seed+ AN+ Bact	11.20 ± 0.36 d	6.10 ± 0.26 c	21.13 ± 0.60 c	16.73 ± 0.32 d
Seed+ Bact	13.46 ± 0.37 c	9.26 ± 0.35 c	25.93 ± 0.47 b	20.36 ± 0.40 c
Seed+ AN+ Supr	18.20 ± 0.88 b	10.36 ± 0.55 b	26.83 ± 0.30 bc	21.60 ± 0.26 bc
Seed + Supr	21.00 ± 0.65 a	12.40 ± 0.30 a	29.26 ± 0.40 a	22.26 ± 0.47 a

Data are mean of three replicates ± standard error of means. Means, followed by the same letter in a column are not significantly different (P = 0.05) by Duncan's multivariate test.

Table-8 Effects of treatments on groundnut during pot study



RT	Compound Name
6.780	2-4 Diacetylphloroglucinol (DAPG)
18.679	Phenazine-1-Carboxylic Acid

Figure-24 GC-MS of metabolite analyses

SET- A BLOCK-1	BLOCK-2	BLOCK-3	BLOCK-4	BLOCK-5	BLOCK-6
SET-B BLOCK-1	BLOCK-2	BLOCK-3	BLOCK-4	BLOCK-5	BLOCK-6
SET- C BLOCK-1	BLOCK-2	BLOCK-3	BLOCK-4	BLOCK-5	BLOCK6

SET-A (Control, seed+ Fungus, Seed+ Bacteria, Seed+ fungus + Bacteria, Seed+ Fungus+ supernatant, Seed+ Supernatant)
SET-B (Seed+ Fungus, Seed+ Bacteria, Seed+ fungus + Bacteria, Control, Seed+ Supernatant, Seed+ Fungus + Supernatant)
SET-C (Seed+ Supernatant, Seed+ Fungus+ Supernatant, Seed+ Fungus, Seed+ Bacteria, Seed+ Fungus+ Bacteria, Control)
* Field arrangements are from Left to Right for all sets.

Figure-25 Field study design map

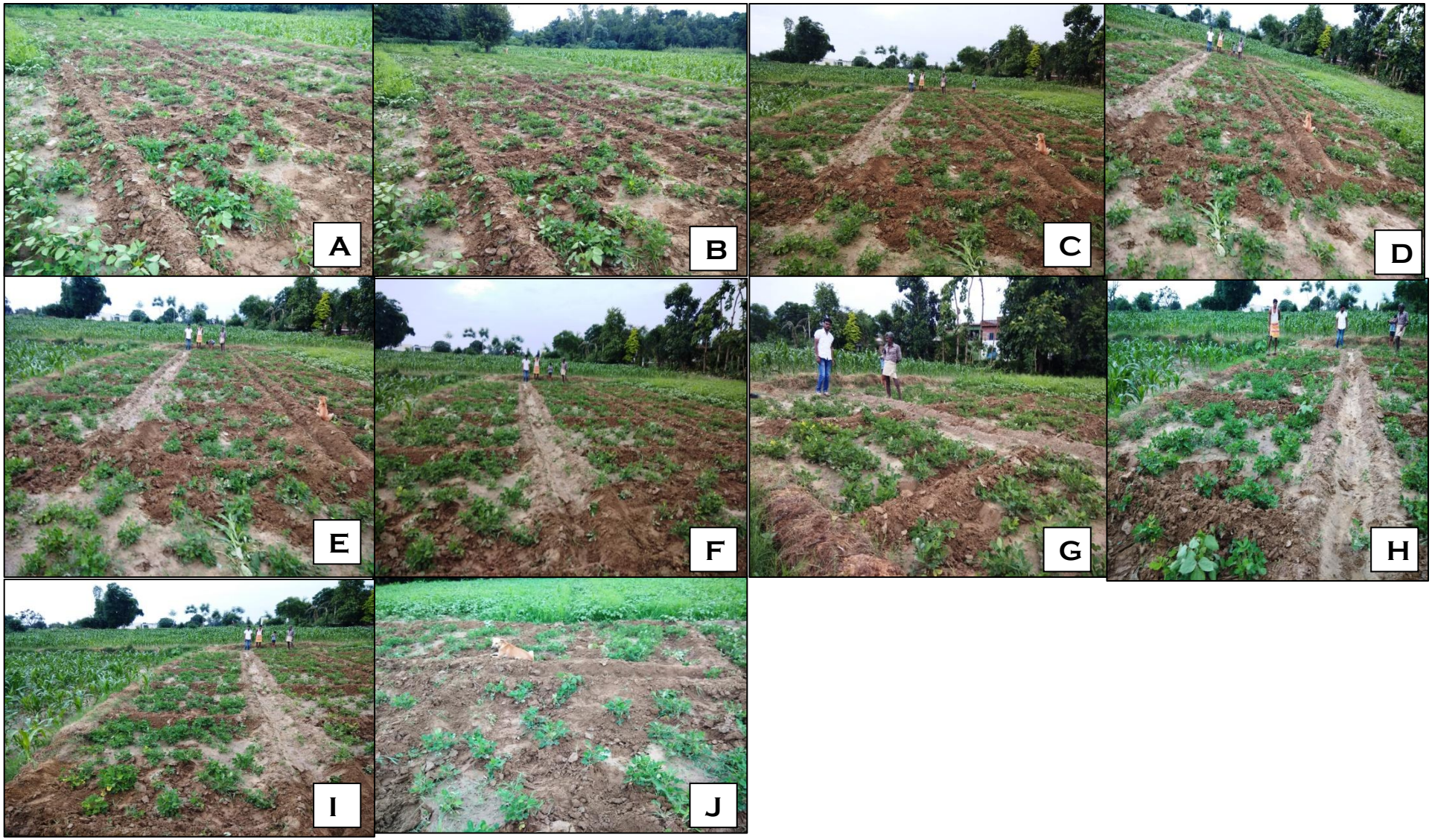


Figure-26 *In vivo* analyses of treatments on groundnut seeds (Field study) at Yaqutganj, Farrukhabad

Treatment	45 Days		90 Days	
	Root Length (Cm)	**Shoot Length (Cm)**	**Root Length (Cm)**	**Shoot Length (Cm)**
Seed Only	4.8±0.4	9.0±0.3	11.8±0.3	16.5±0.2
Seed+ AN	2.8±0.2	4.8±0.4	10.0±0.4	13.6±0.4
Seed + Bacteria+ AN	6.1±0.2	11.2±0.3	16.7±0.3	21.1±0.6
Seed + Bacteria	9.2±0.3	13.4±0.3	20.3±0.4	25.9±0.4
Seed+ AN+ supernatant	10.3±0.5	18.4±0.3	21.6±0.2	26.8±0.3
Seed+ supernatant	12.4±0.3	21.0±0.6	22.2±0.4	29.2±0.4

Table-9 Effects of treatments on groundnut during field study

S. No.	Parameters	Control	Treated	References ranges
1	Total Nitrogen	0.378%	0.0952%	0.5-1.5%
2	Phosphate content	2.194 µg P/mL	2.638 µg P/mL	0.6-5.0%
3	Total Potassium	0.042%	0.049%	0.01-1.5%
4	Organic Carbon	10%	12.5%	5-15%
5	pH	6.8	6.9	4.0-10
6	Conductivity	0.372 ms/cm	0.468 ms/cm	0.3-1.2 ms/cm

Table- 10 Physicochemical analysis of control and treated soil

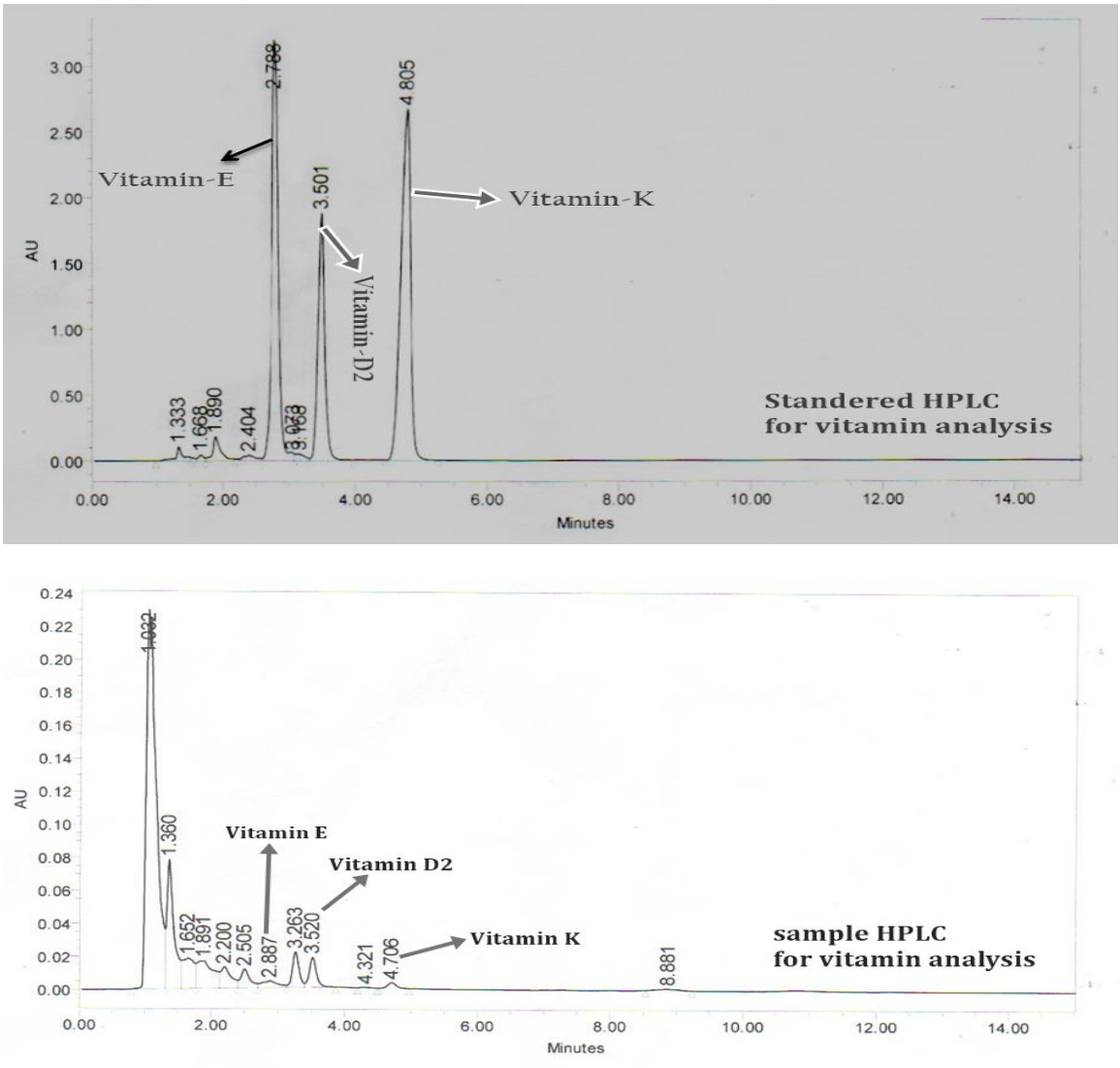


Figure-27 HPLC analyses of groundnut oil

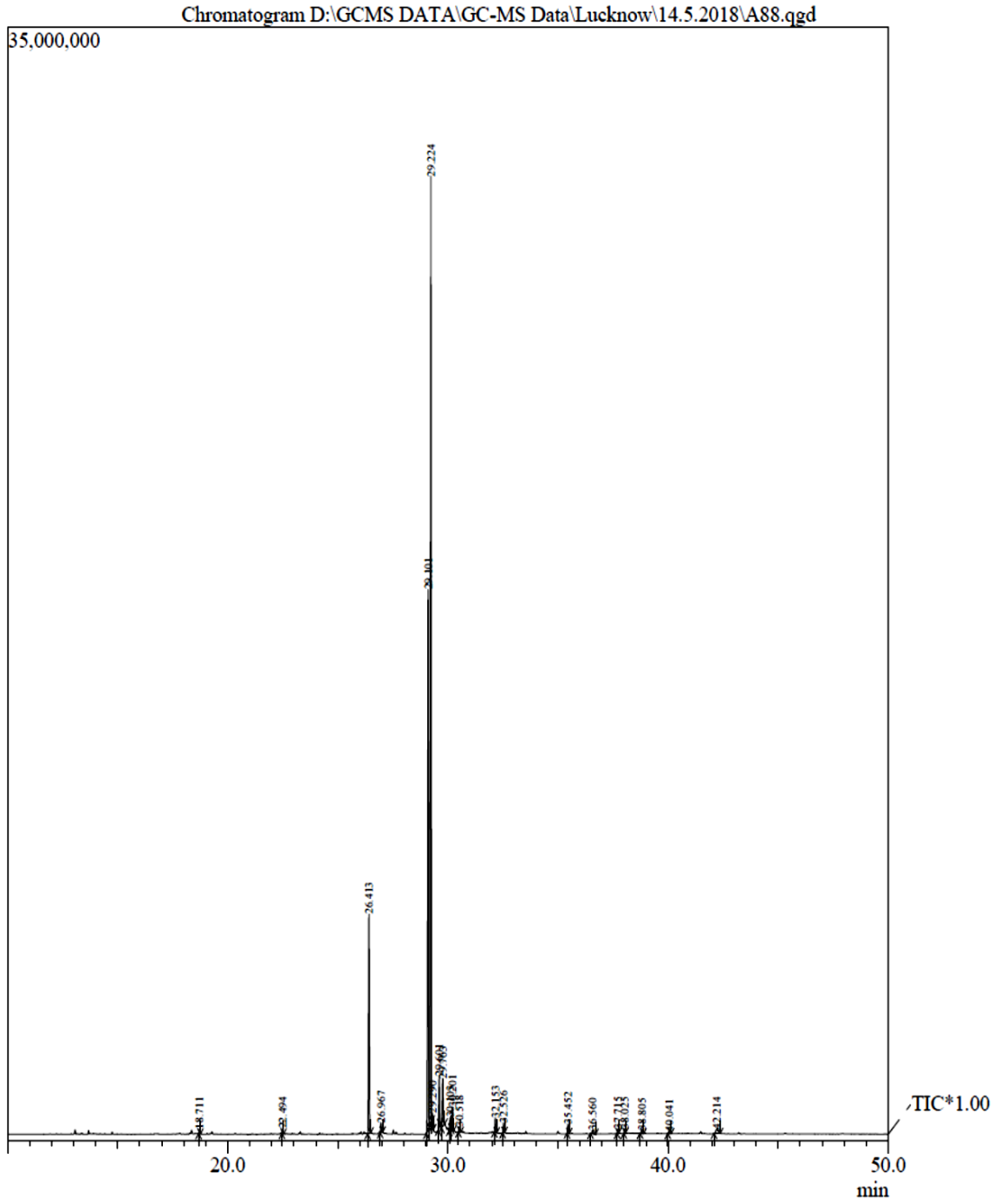


Figure-28 FAME analyses of groundnut oil

Peak Report TIC

Peak#	R.Time	Area	Area%	Name
1	18.711	299936	0.20	PHENOL, 2,4-BIS(1,1-DIMETHYLETHYL)-
2	22.494	340484	0.22	Dodecane, 4,6-dimethyl-
3	26.413	16306226	10.74	Hexadecanoic acid, methyl ester
4	26.967	807903	0.53	n-Hexadecanoic acid
5	29.101	42260396	27.83	9,12-Octadecadienoic acid, methyl ester, (E,E)-
6	29.224	75625492	49.81	9-Octadecenoic acid (Z)-, methyl ester
7	29.290	561908	0.37	10-Octadecenoic acid, methyl ester
8	29.601	3463321	2.28	Methyl stearate
9	29.763	4309387	2.84	OCTADEC-9-ENOIC ACID
10	30.103	904382	0.60	Linoelaidic acid
11	30.201	1645755	1.08	(E)-9-Octadecenoic acid ethyl ester
12	30.518	432729	0.28	9,11-Octadecadienoic acid, methyl ester, (E,E)-
13	32.153	1149446	0.76	cis-Methyl 11-eicosenoate
14	32.526	681784	0.45	EICOSANOIC ACID, METHYL ESTER
15	35.452	785096	0.52	DOCOSANOIC ACID, METHYL ESTER
16	36.560	213627	0.14	Heneicosane
17	37.715	247635	0.16	Heptadecane, 2,6,10,15-tetramethyl-
18	38.025	276267	0.18	Tetracosanoic acid, methyl ester
19	38.805	304381	0.20	Heptadecane, 2,6,10,15-tetramethyl-
20	40.041	207265	0.14	Heptadecane, 2,6,10,15-tetramethyl-
21	42.214	1011699	0.67	Tris(2,4-di-tert-butylphenyl) phosphate
		151835119	100.00	

Chapter 5

Discussion

Oilseeds are one of the most important crops cultivated worldwide. The major oilseed crops are groundnut, soyabean, sesame, rapeseed, sunflower, and safflower. The products of oilseed crops directly or indirectly involves in human and animal diets due to its nutrient compositions. Apart from the food value of oilseeds, several industrial products such as biodiesel, fertilizer, medicine, cosmetics, animal feeds, fibers, paint, button have also been reported (Abiodun, 2017). The quality and viability of an oilseed is directly influenced by fungi, insects and other microorganisms actively present in warehouse (Rai et al. 2008).

Groundnut (*Arachis hypogaea L.*) is an important oilseed crop in India, also known as a major cash crop for Indian agriculture sector (Singh et al., 2017). But being an oilseed groundnut has many drawbacks. Usually, groundnut are stored dry in a warehouse, but due to lack of proper infrastructure of storage in village areas, groundnut seed loses its viability within a short period during the irreversible phenomena of ageing. Under such conditions, seeds are also susceptible to fungi, insects and other microorganisms. Therefore, it is important to protect oil seeds from these pathogens for global concern of food security.

The major problem related with groundnut seeds under storage condition is aflatoxin contamination caused by fungal pathogens (Waliyar et al.

2008). It is mainly caused by *Aspergillus* spp *A. flavus* and *A. niger* are the most common species allied with aflatoxin contamination of agricultural crops (Wu, 2014). Aflatoxin infections have been found in crop such as groundnut, maize, and cotton used for human and animal food (Goldblatt, 2012).

According to WHO 2018, about 25-30% of total produced oil seed crop are destroyed due to aflatoxin contamination. However, pre- and postharvest aflatoxin contamination in groundnuts is a major issue for food safety and human health. The aflatoxin contamination is harmful mainly due to crop quality value and food stuffs may charge in aflatoxin. Aflatoxin is basically a group of toxin such as G1, G2, B1, B2, M1, and M2 that are produced by the plant pathogen (Amaike and Keller, 2011). These toxins occur naturally and have been found in a wide range of commodities, including peanuts used for animal and human consumption (Williams et al. 2004). Aflatoxins are toxic, mutagenic, and carcinogenic compounds (Chen et al. 2013). Depending on their levels, toxins can severely affect human beings.

To handle this issue, in present study three predominant fungal pathogens were isolated from infected storage groundnut seeds, collected from local warehouse located at Yaqutganj district Farrukhabad and were identified by 18S rRNA sequencing. Two of them were identified as *A. flavus* (RF-02,03) and the other one was identified as *A. niger* (RF-07). Similarly, Rathod et al. (2012) reported that agar plate method for the isolation of seed borne fungi in

groundnut with the highest occurrence of *A. flavus and A. niger*. Aseefa et al. (2012) and Naqui et al. (2013) found that *A. flavus and A. niger* are dominating pathogen of groundnut seeds. From the several fungal species isolated from the groundnuts Guchi (2015) found *A. niger* with 85.65% occurrence then *A. flavus* with 81.80% and *A. tamari* with 63.57%.

Secondly, total 7 bacteria were isolated from rhizospheric soil of groundnut and characterized morphologically and biochemically. All isolates were found to be Gram-negative rod shaped, showing fluorescens in specific agar medium with yellowish green pigmentation. All the isolates were positive for oxidase test, citrate utilization, and catalase fermentation. Further, in present study these bacteria were analyzed through biochemical test kit for gram-negative bacteria powered by Hi Media having various biochemical test like Citrate utilization, Lysine utilization, Ornithine utilization, Urease, Phenylalanine Deamination, Nitrate reduction, H_2S production, Glucose, Adonitol, Lactose, Arabinose and Sorbitol. Identification of various *Pseudomonas* species was performed by using Result interpretation chart.

The kit KB002 was standardized, colorimetric system based on carbohydrate utilization and other biochemical tests specific for the identification of *Pseudomonas* species. The test were based on the principle of pH change and substrate utilization. *Pseudomonas* species on incubation exhibit metabolic changes which are indicated by a colour change in the media that can either interpreted as negative or positive result.

Similarly, Deshwal et al., (2013) isolated 140 fluorescent and non-fluorescent strains of *Pseudomonas* on King's medium and found that all strains were gram negative and rod shaped. Isolated strains showed growth at 42 ^{o}C. Manasa et al. 2017 isolate 15 *P. fluorescens* form different rhizospheric soil on behalf of their colony morphology and Gram reaction.

After the isolation and biochemical identification of all isolates, the isolates were further analyzed for various PGP characteristics such as IAA, HCN and Siderophore. IAA production has been reported by other researchers (Malleswari and Bagyanarayan, 2013). Among all in our investigations RC-07 isolate was found the as highest IAA production. Previously the production of IAA has been reported in *Bacillus, Pseudomonas* and other rhizobacterial isolates. Malleswari and Bagyanarayana (2013) reported a production of 15 µg/ml of IAA by Bacillus sp. (Cf 60) which is in correlation to our results. Our isolate Bacillus strain CHIII(I)Y6 reported a production of 14 µg/ml of IAA. Saranraj et al. (2013) tested the *Pseudomonas* and *Bacillus* strains isolated from paddy rhizosphere for the production of IAA. Kochar et al. (2011) analyzed the biocontrol strain *Pseudomonas fluorescens* for IAA biosynthesis and studied the effect of its consequent manipulation on its plant-growth-promoting (PGP) potential. The IAA production by *Bacillus subtilis* was relatively low when compared to *Pseudomonas fluorescens* (Sivasakthi, 2013). In our investigation, the maximum production of IAA was recorded by the isolate RC-07 ranged from 10.9 µg/ml. similarly, Kannahi and Kowsalya,

(2013) reported 3.8 µg/ml of IAA production by *Bacillus subtilis* and 5.3 µg/ml IAA by *Pseudomonas fluorescens*. The isolate *Pseudomonas aeruginosa* was found to produce 80 µg/ml of IAA on tryptophan supplementation (0.1g/L) (Bakthavatchalu et al. 2012).

Pseudomonas fluorescens produces various chemical compounds with different benefits for the plant. Among them, HCN is recognized as a biocontrol agent, based on its ascribed toxicity against plant pathogens. It was found that HCN does not act as a biocontrol agent, but involves in geochemical processes in the substrate and indirectly increasing the availability of phosphate. So that the main contribution of HCN is to increase the nutrient availability, which is beneficial for the rhizobacteria and their plant hosts. Similarly in previous studies it was observed that certain strains of rhizosphere *Pseudomonas* spp. produce metabolites such as HCN to inhibit the growth of pathogens and able to enhance plant establishment (Schippers et al.1990). Further, Ramette et al. (2003) reviewed HCN as a broad spectrum antimicrobial compound released naturally from fluorescent *Pseudomonads* and biologically control several root diseases by many plant associated. Strong HCN production was recorded by isolate *P. aerugonisa* FP6, evidenced by change in colour of filter paper to reddish brown after 2-3 days of incubation (Bhakthvatchalu et al. 2013). That's why in this study HCN producing isolates were important for prevention and control of various pathetic fungi of storage

area of groundnut. In our study, *Pseudomonas fluorescens* strain RC-07 isolate exhibited HCN production by changing the colour of the filter paper from orange to brown colour.

However, siderophore producing strain become more efficient because siderophore is an organic compound with low molecular weight produced by microorganisms during iron deficient conditions to chelate the ferric iron Fe (III) from different habitats and make it available for plant cells. In recent trends of using bio-active compounds siderophore become popular due their potential ability to bind a variety of metals in addition to iron with a wide range of chemical structures and specific properties (Johnstone and Nolan, 2015). Similarly siderophore production on CAS assays (Tank & Saraf, 2010) was observed by Beneduzi et al. 2012. Siderophore mediated antagonism was observed against common phytopathogens viz., *A. flavus, A. niger, C. capsicum and F. oxysporum* (Prashant et al. 2009). Bakthavatchalu et al. (2012) reported the production of siderophore (2×105 moles/l) by *P. aeruginosa* FP6 and the nature of siderophore was hydroxamate type. Similarly, Sen et al. (2006) reported hydroxamate type of siderophore produced by *fluorescent Pseudomonas* BRL-1 evidenced by the formation of orange zone around the colony produced by the strain BRL-1 in CAS agar 140 plate (Sen et al., 2006). So that siderophore production is an important PGP trait exhibited by *Pseudomonas* sp. and that is why we choose strain producing siderophore for further studies.

In the next step after biochemical and PGP characterization of isolated bacterial strains the best strain RC-07 was selected for antagonistic activity analysis against deteriorating fungal pathogens. Two types of assays were used (dual culture assay and spray dilution assay) to check the antagonistic efficacy of RC-07 against dominant fungal deteriorating agents. Dual culture assay on petri dish shows the growth inhibition of fungal pathogen during and after incubation of 7 days while in control plates almost 99% of fungal growth was observed after same incubation period. A number of researchers observed the similar results during their study of controlling plant pathogens using antagonistically active microorganism. Fungal pathogen, *A. niger* can cause several chronic diseases in fruits and vegetables such onions and peanuts. The results showed significant antagonistic potential *of P. fluorescens* against *A. niger* with the reduction of 65.1%-81.3% in the fungal colony diameter (Yunus et al. 2016). In a separate study Gajera et al. 2011 isolated *P. fluorescens* from soil samples and inhibited growth of *A. flavus* and *A. niger* at the levels of 37.85%, and 32.13 % respectively.

However, in spray dilution assay significant results was observed with no fungal contaminations in seeds treated with supernatant of *P. fluorescens*. In comparison of control and also with broth spray dilution. Neidig et al. 2011 reviewed those bacterial secondary metabolites responsible for the suppression of plant pathogens strongly inhibit predators of soil and enhance the resistance of beneficial microbes. The production of 2, 4- diacetylphloroglucinol (2-4

DAPG) has been reported as an antagonistic metabolite secreted by *P. fluorescens* (Sajeli et al. 2014). Phenazine production has previously been studied in *Pseudomonas spp*. as competent biocontrol agents (Morrison et al.2016).

Microbial metabolites are the compounds those are responsible for antagonistic activity against pathogens. In our study, after considerable results given by RC-07 during *in vitro* studies, the bacterial strain RC-07 was go through the GC-MS analysis and potential presence of 2-4 DAPG with Phenazine was clearly observed. Schnider et al. 2000 in a study identified 2,4 DAPG as the active compound in extracts from *P. fluorescens* culture supernatants. In continuation, Wu et al. (2012) studied that *P. fluorescens* produces the antibiotic phenolic compound 2,4-DAPG for prevention and control against fungal pathogens. *P. fluorescens* produced 2,4-DAPG when the hydrophobic portion enters into the targeted cell membrane and stimulate the antifungal activity of phloroglucinol by increasing the volume of hydrophobicity portion.

On the other hand Phenazine is also an organic compound with the formula $(C_6H_4)_2N_2$, it has a wide range of nitrogen-containing heterocyclic compounds produced by *P. fluorescens* and act as a bioactive compound against fungal pathogens (Mavrodi et al. 1998). Phenazines and their derivatives are widely used by pharma industries and clinical research. It may

be very useful for plants because of its ability to induce plant growth and systemic resistance.

In the present investigation, based on the maximum number of plant growth promoting activities exhibited by the isolates, only one isolate RC-07 was selected for molecular characterization. After 16S r RNA sequencing the phylogenetic analysis was prepared by MEGA7. The isolate RC-07 showed 99% homology with *Pseudomonas* sp.

Bases on above RC-07 were further taken for *in vivo* (Pot) and *in vitro* (field) study. It was observed that seeds treated with supernatant of RC-07 showing best results in compression control and seeds treated with broth culture. Further, significant enhancement was found in plant growth promoting activity of plants treated with RC-07 in both pot and field studies. Khare and Arora in 2010 reported that *P. fluorescens* shows plant growth promotion and root elongation in plants with disease management as well. Further, these microbes potentially inhibit the infection of *A. flavus* with less aflatoxin level. The salient feature of *P. fluorescens* is the enhancement in seed quality variables can be improved by reducing the seed borne fungal infection and by lowering the aflatoxin level. This type of biocontrol method can be applied to manage *A. flavus* infection and aflatoxin production in storage laves by increasing the defensive nature of seeds of groundnuts.

Groundnut seeds were further analyzed for their oil containing capacity and various nutritional parameters such as estimation of fat soluble vitamins

and fatty acid profiling through HPLC and FAMEs techniques. In generally palmitic acid (16:0) constitutes nearly 10% and the oleic (18:1) and linoleic acid (18:2) proportions together make up 80% of the fatty acid composition in groundnut (Shasidhar et al. 2017).

Several marketable benefits of oleic acid drive the breeding effort toward producing high oleic peanuts. First, oleic acid, a monounsaturated fatty acid, has 10-fold higher auto-oxidative stability than linoleic acid. Saturated fatty acids (SFA) have been generally la-beled as the cause of cancers and coronary heart disease.

The mean ratio of polyunsaturated fatty acids (PUFA) to SFA recommended by the British Department of Health is more than 0.45, and WHO/FAO experts have reported guidelines for a "balanced diet" in which suggested ratio of PUFA/SFA is above 0.4 (Johnson et al. 2009). It has been estimated that the present Western diet is deficient in ω3 fatty acids, with a ratio of ω6 to ω3 of 15 − 20/1, instead of 1/1 as is the case with wild animals and presumably human beings (Si-mopoulos, 2008). Nutritional advice for today's ω6/ω3 ratio is less than 4 (Simopoulos, 2001). Considerable importance has been ascribed to the role of the O/L and iodine value (IV) in governing product shelf life. High O/L ratio and low IV have been associated with greatly enhanced shelf life and decreased rancidity of the product (Makeri et al. 2011). The fatty acid composition of groundnut oil varies depending on the genotype, seed maturity, climatic condi-tions, growth location, and

interactions between these factors (Andersen and Gorbet, 2002). it has been reported that oleic acid increases and linoleic acid decreases with seed maturity. In groundnut as seeds progressed from intermediate through nearly-mature to mature stages, palmitic and linoleic acid de-creased while oleic acid increased (Casini et al. 2003). The high smoke point of groundnut oil (230 C) makes it suitable for deep frying of foods. Southern and western Indian population prefers groundnut oil for consumption (Vyas et al. 2013). In India, it is also used for processing of vanaspati. Groundnut oil fatty acid profile exhibited high MUFA content with oleic acid the predominant fatty acid. Oleic acid was found in good proportion in all edible oil sample evaluated ranging from 7.24% in coconut oil to as high as 53.77% in groundnut oil. This fatty acid is associated with long shelf life due to high oxidative stability (O'keefe et al.1993). Groundnut oil also contained moderate amount of linoleic acid and varied from 22.39 to 30.51% with average value of 26.96% (Dorni et al. 2018). It was also contained saturated fatty acid palmitic (10.46%) and stearic (3.37%) in notable amount, while arachidic, behenic and lignoceric were noticed in trace amount. NVIF (1989) reported palmitoleic acid (1.4%) which was not found in the present study. This may be due to the fact that the present study employs capillary column which provides better separation of all fatty acids. Kostik et al. (2013) reported a slightly higher value of MUFA (58.5%) but almost the same TPUFA/TSFA ratio (1.04) as obtained in present investigation. On the other hand, Li et al. (2016) reported a higher value of

MUFA (68%) in groundnut oil collected from Zhengzhou (China). This may be again attributed to difference in genotypes used for oil extraction, growing conditions and refining process used.

It was clearly observed from the above study that *A. flavus* and *A.niger* are dominant deteriorating contaminants of groundnut seeds during storage. That's why cure and control of oil seeds from deteriorative fungal pathogens is also very important part of agriculture. For control of these types of storage pathogens, bacterial metabolites seem to be very helpful as they are non-toxic and environmental friendly. Hence, In this study basically the role of PGPR-(*P. fluorescens)* on storage seed deteriorating fungi (*A. niger*) was observed with different ways of antifungal assay and found that *P. fluorescens* has a potential to control and overcome the harmful effects of deteriorating fungi on groundnut seeds during storage. It was also found that application of metabolites on seeds may improve the seed quality of oilseed. Microbial metabolites are very useful in agriculture because they have no adverse effect on environment or human beings and a good alternative of chemical based pesticides or fungicides.

Chapter 6
Conclusion

On the bases of this study, it is concluded that *Pseudomonas fluorescens* suppress the growth of fungal pathogens on storage seeds of groundnut. Moreover, there is no damage found in its morphological and chemical composition. However, supernatant of *P. fluorescens* seems to be more effective in comparison to bacterial suspension. As bio- control agent, *P. fluorescens* have been shown beneficial prospects on seed health during storage. Various scientific reports are published worldwide and described the ability of different pseudomonas strains to significantly control a number of fungal, bacterial and nematode diseases in cereals, horticultural crops, oil seeds and others. However, the bacterial antagonism in storage area for oil seed crops need more attention in that case this work seems to be very beneficial in controlling deterioration and their after effects on crop production. Besides preventing seed deterioration, it may also improve seed health which helps in healthy seedling and fulfill the yield gap for sustainable agro-economic development worldwide.

Chapter 7

Summary

Groundnut (*Arachis hypogaea L.*) is an economical crop worldwide, having a rich source of all the nutrients. Being an oilseed, groundnut seeds are very much affected by predominant storage deteriorating fungi *A. niger* and *A. flavus*, which causing aflatoxin contaminations in storage seeds. These fungal agents cause various physiological and biochemical changes in the nutritive composition of seeds. Moreover, it is very difficult to pause or stop deteriorative alterations permanently. The after effects are clearly seen immediately or after sowing such as weak seedling, unhealthy plant, immature seeds and various plant diseases at the time of maturation.

Aflatoxin is basically a group of toxin such as G1, G2, B1, B2, M1, and M2 that are produced by the plant pathogen. These toxins occur naturally and have been found in a wide range of commodities, including groundnuts used for animal and human consumption. Aflatoxins are toxic, mutagenic, and carcinogenic compounds. Depending on their levels, toxins can severely affect human beings.

A group of bacteria found in the vicinity of plant roots network called rhizobacteria which helps in enhancing plant growth. So this group is also known as PGPR (Plant Growth Promoting Rhizobacteria). PGPRs include a large number of bacterial populations, in which *Pseudomonas* is one of a very efficient member of this group. They produce antibiotics and other metabolites like production of siderophore, hydrolytic enzymes such as phenazine (PHE), 2,4-diacetylphloroglucinol (PHL), pyoverdin (PYO) (pseudobactin),

indoleacetate and pyoluteorin (Plt) plays an important role in generating immune system of plants against various pathogens. *P. fluorescent* shows antagonistic property against plant pathogens. It is also reported that application of these beneficial microbes in plants under stress conditions may resist them against various pathogenic fungi. The bio-priming of seeds with microbial metabolites are very useful to protect seeds against pathogens during storage; they are more effective target based environmental friendly and cost effective alternatives of harmful chemicals based pesticides. *P. fluorescence* shows biocontrol activity against several phytopathogens such as *Alternaria, Aspergillus, Fusarium, Rhizoctonia, Phytophthora*.

In the present work thus, the utilization of *P. fluorescens* in prevention and control of seed deteriorating fungi (*A. niger*) during storage of groundnut seeds were analyzed. The preferred area for this study is natural environmental and agriculture conditions and the targeted sampling sites for collection of rhizospheric soil were located in three different districts (Mainpuri, Farrukhabad and Unnao) of Uttar Pradesh, India. Further, for the isolation of fungal pathogens, contaminated groundnut seeds were singled out form the warehouse of a local farmer situated near by the sampling site in district Farrukhabad.

For the isolation of fungal pathogen, surface sterilized groundnut seeds were placed on PDA plats and incubated at 29ºC for one week. After incubation different fungal colonies were observed on groundnut seeds which were further purified on PDA plates. Among all, three dominant fungal isolates

were identified as *A. flavus* (RF-02, RF-03) and *A. niger* (RF-07) after 18S rRNA sequencing. These identified fungal strains were separated for further study. Secondly, total 7 bacteria were isolated from rhizospheric soil of groundnut on fluorescent pseudomonas specific agar medium (Hi-media). These bacterial isolates were further analyzed for morphological, biochemical and PGP activity tests.

All the bacterial isolates have mucilaginous colonies with smooth margins. Moreover, the isolates were identified as Gram negative and rod shaped bacteria. All the isolates showing yellowish fluorescent and were fast growers. These isolates were further analyzed for various bio-chemical activities, for most of the analyses KB-002 Hi media biochemical test kit specific for gram negative rods were used. All seven isolates were positive for catalase activity, positive oxidase, nitrate reductase, urease production and citrate utilization while none of the isolates showed positive H_2S Production activity. Along with this enzymatic activity of lipase showed negative by all the isolates and TDA shows more likely to be positive. Presence of glucose was positive for all the isolates but in case of lactose it was about 89% positive and in arabinose it was not actually determined by the biochemical test kit. On the basis of these biochemical tests all the isolates were observed to be very close with *P. fluorescens*.

All the bacterial isolates were further go through with PGP activity analyses and observed that all bacterial isolates show positive IAA production ability checked by UV visible spectrophotometer (Evolution 201 UV visible)

analysis and colour change. Among all, RC-07 was the best IAA producer at optimum cultural conditions. Only one isolate RC-07 shows positive siderophore production on CAS agar plates during qualitative analysis with yellowish zone. Strain RC-01, RC-04 and RC-07 were showing positive HCN production by colour change of filter paper soaked in picric acid from yellow to orange.

On the bases of biochemical and PGP activity RC-07 with best results was selected for further studies. The antagonistic potential of RC-07 was checked on fungal deteriorating agent RF-07 (*Aspergillus niger*) through dual culture and spray dilution antifungal assays. In dual culture assay inhibition was clearly observed when compared with growth of deteriorating pathogen in control plate. Further, in present study RC-07 shows positive and maximum inhibition of fungal deteriorating agent *A. niger*. It was observed that *Pseudomonas* sp. secreted various enzymes and metabolites such as phenazine and 2,4-diacetylphloroglucinol, which not only inhibit the pathogens but also promote plant growth. The presence of phenazine and 2,4-diacetylphloroglucinol (2,4 DAPG) in the supernatant of RC-07 ware clearly observed in gas chromatography and mass spectrophotometry (GCMS).

Antagonistically active bacterial strain RC-07 was screened by 16s rRNA sequencing. The strain was showing 99% similarities with genus *Pseudomonas*. Moreover, a comparative study with available database on NCBI and The phylogenetic analysis of that strain RC-07 shows the direct

relation with *Pseudomonas fluorescens*. Sequence was submitted in DDBJ data base with the accession number LC375795.

During the pot study it was observed that groundnut seed treated with supernatant of *P. fluorescens* RC-07 shows best results against *A.niger* RF-07 pathogen in comparison to direct application of broth on seeds. After maturation, the difference between control and supernatant treated seed are clearly shown the enhancement of crop. While seeds infected with pathogen was not even grow properly. Direct bacterial contact with seed was not as effective as needed. The control grows in normal speed during pot study. So that this study found that the supernatant spray method is more effective for prevention of seed to be sown in pot from *A.niger*.

Similar results were obtained from field study of groundnut seeds. The supernatant spray of *P. fluorescens* RC-07 shows best results against *A. niger* RF-07 pathogen in comparison to application of broth spray on seeds. After maturation, the difference between control and supernatant treated seed are clearly visible.

High performance liquid chromatography profile of ethyl acetate extract of groundnut seeds oil clearly showed the high level presence of vitamins E, D2 & K which was confirmed through the compression with standard references of fat soluble vitamins. Fatty acid methyl Easter analysis (FAME) by GC of groundnut oil clearly showed the presence of oleic, linoleic and some other oils which was confirmed through the slandered library with an oil

content of about 43%. Different characteristic peaks were obtained in chromatogram between retention times (RT) as depicted in the chromatogram.

In concluding remark, it was observed during this study that *Pseudomonas fluorescens* was potentially able to suppress the growth of fungal pathogens on storage groundnut seeds. However, supernatant of *P. fluorescens* seems to be more effective in comparison to bacterial suspension. As bio-control agent, *P. fluorescens* have been shown beneficial prospects on seed health during storage. Various scientific reports are published worldwide and described the ability of different *pseudomonas* strains to significantly control a number of fungal, bacterial and nematode diseases in cereals, horticultural crops, oil seeds and others. However, the bacterial antagonisms in storage area for oil seed crops need more attention and in that case this work seems to be very beneficial in controlling deterioration and their after effects on crop production. Besides preventing seed deterioration, it may also improve seed health which helps in healthy seedling and healthy crop production to fulfill the yield gap for sustainable agro-economic development worldwide.

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Appendix

NUTRIENT AGAR -

Ingredients	gms/liter
Peptone	5.0
Yeast extract	1.5
Beef extract	1.5
NaCl	5.0
AGAR	16.0
pH	7.0
Distilled water	1000ml

NUTRIENT BROTH (NB) –

Ingredients	gms/liter
Peptone	5.0
Yeast extract	1.5
Beef extract	1.5
NaCl	5.0
pH	7.0
Distilled water	1000 ml

POTATO DEXTROSE AGAR

Ingredients	gms/liter
Potato	20.0
dextrose	20.0
Agar	20.0
Distilled water	1000 ml

PSEUDOMONAS AGAR (FOR FLUORESCEIN)

Ingredients	gms/liter
Casein enzymic hydrolysate	10.0
Proteose peptone	10.0
Dipotassium phosphate	1.5
Magnesium sulphate	1.5
Agar	15
pH	7.0

CHEMICALS AND GLASSWARE

Chemical and glassware used were procured from the following sources:

- Bangalore Genei Pvt. Ltd., India
- Eppendorf India Ltd., India
- Genexy Scientific Pvt. Ltd., India
- Himedia Biosciences, India
- Sigma Chemicals Co., USA
- Tarsons Products Pvt. Ltd., India
- Thermo Scientific Pvt. Ltd., India

STAINS, INDICATORS, AND REAGENTS

a) Gram staining

Crystal violet solution: Dissolve 2.0 gm crystal violet in 20.0 m of 95% ethyl alcohol

Gram's Iodine solution: Mixed 1.0 gm Iodine, 2.0 gm potassium iodide in 300.0 ml of distilled water

Safranin: Dissolved 10.0 ml of safranin into 100.0 ml of distilled water

b) Indole production test

Kovac'sreagent: Dissolve the 5.0 gm of diaminobenzaldehyde in the 75.0 ml of amyl alcohol. Then add 25.0 ml of hydrochloric acid to the above preparation. Store the reagent in the refrigerator.

c) IAA production

Salkowski reagent : 2 ml 0.5M $FeCl_3$ and 49 ml water and 49 ml 70% perchloric acid.

d) Ammonia production

Nessler'sreagent : Dissolve 50.0 gm of potassium iodide in 35 ml of distilled water and added saturated solution of mercuric chloride. Added 400 ml of potassium hydroxide. Dilute to 1000 ml by addition of distilled water. Allow to settle for one week. Stored in tightly stopper brown bottles.

e) HCN production

2% Sodium carbonate solution: Dissolve 2 gm of $NaCO_3$ in 100 ml of distilled water.0.5% Picric acid solution Mix 0.5 gm picric acid in 100 ml of water.

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Section A: Green Chemistry

Research Article

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Isolation and characterization of storage seed deteriorating fungi and their effect on groundnut seeds

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Abstract: Groundnut (*Arachis hypogaea L.*) is an important oilseed crop in India. It has a good contribution to Indian economy. Oil seeds are highly susceptible for fungal infections under storage condition as they have ample amount of nutrients in them. Different fungal species of *Aspergillus* were dominant in coursing infections in groundnut. *Aspergillus* species cause various deteriorative alterations by physical and chemical means in groundnut seeds. Storage fungi cause chronic infection in seeds due to favorable environmental conditions such as moisture, relative humidity and temperature. In current study, fungal pathogens were isolated from Groundnut seeds. Groundnut seeds were singled out from warehouse located in Farrukhabad, Uttar Pradesh, India. Two dominant fungal pathogens RF-02 and RF-07 which cause major destruction in storage groundnut seeds were selected for further analysis at genus and species level by using 18S rRNA sequencing technique. Fungal strains dominantly covered up about 90 percent of groundnut seeds. After 18s rRNA, sequencing RF-02 and RF-07 strains were identified as *Aspergillus flavus* and *Aspergillus niger*. These identified sequences were submitted in NCBI and got the accession numbers KY933394 and KY357318 respectively.

Keywords: Seed deterioration, oil seeds, groundnut seeds, storage oil seeds.

INTRODUCTION

The quality and viability of an oilseed is directly influenced by fungi, insects and other microorganisms actively present in warehouse[1]. Groundnut (*Arachis hypogaea L.*) is an important oilseed crop in India. It has a good contribution to Indian economy. Moreover, groundnut has also known as a major cash crop for Indian agriculture sector[2]. But being an oilseed groundnut has many drawbacks. In Indian agriculture systems crops are stored in warehouse immediately after harvesting. The major problem related with groundnut seeds under storage condition is aflatoxin contamination caused by fungal pathogens[3]. It is mainly caused by *Aspergillus spp.*[4]

A. flavus is the most common species allied with aflatoxin contamination of agricultural crops[5, 6, 7]. Aflatoxins infections have been found in crop such as groundnut, maize, and cotton used for human and animal food [8-9]. According to WHO 2018 about 25-30% of total produced oil seed crop are destroyed due to aflatoxin contamination[10]. Groundnut is an excellent source of multiple nutrients including vitamin E and magnesium followed by lesser amount of carbohydrates [11]. Apart from that, theses seed has oil and protein in ample amount which further break down into simple sugars and amino acids[11]. Moreover, they are essential for germinating seed as an energy source [12].

Usually, groundnut are stored dry in a warehouse, but due to lack of proper infrastructure of storage in village areas, groundnut seed loses its viability within a short period during the irreversible phenomena of ageing. Under such conditions, seeds are also susceptible to fungi, insects and other microorganisms [13]. Therefore, it is important to protect oil seeds from these pathogens for global concern of food security. Many studies suggested that the changes in lipid components of seeds were associated with seed deterioration. According to Bhattacharya and Raha[14] in 2002 during the process of ageing carbohydrate, oil content decreases gradually and an increment may found in free fatty acid content with subsequent gradual loss.

Moreover, a gradual decrease is found in protein content of oil seeds like maize, groundnut and soybean seeds due to storage fungi. The moisture content of the seed is one of the major character stick features which may be influenced by genetic and environmental fluctuations[15]. The initial moisture content is directly responsible for the quality of seed in groundnut[16-17] Deteriorating fungi that occur in warehouses cause changes in biochemical constituents of seeds which may result in low seedling strength and quality. Thus, the main aim of this study is to isolate, purify and characterize dominant fungal seed deteriorating agents and their impact on storage seeds of groundnut under laboratory conditions.

2. EXPERIMENTAL

2.1 Study area and cultivar: The preferred area for this study is natural environmental and agriculture conditions and the targeted experimental fields are located in a village Yaquitganj (27º.32’81’55” N latitude and 79º.61’56’47” E longitude) in district Farrukhabad, Uttar Pradesh, India. The cultivar used for whole experiment was Chitra, a variety of groundnut which is commonly cultivated in Uttar Pradesh.

2.2 Isolation and Purification of groundnut deteriorating fungi:

2.2.1. Isolation of fungi: Raw groundnut (*Arachis hypogaea L.*) was singled out in an indiscriminate manner at the time of second harvesting session (July- October) in year 2016. All collected ground nut seeds were stored in the laboratory conditions within 48 h at 4^0C. Further, the groundnut seed was washed with tap water for removing unwanted soil and adhesive particles. After air dry seeds were separated from shells and placed on potato dextrose agar (Hi-media) plates (three seeds per plate) and incubated at 29°C for one week. After incubation the plates were observed; different fungal colonies appeared on groundnut seeds were further purified on PDA plates. Among all, two dominant fungal isolates RF-02 and RF-07 were separated for further study. **(Figure-1)**

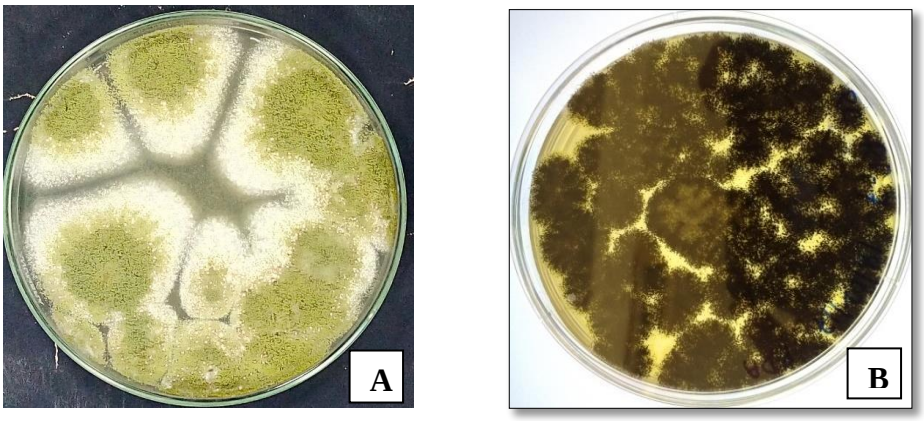

Fig-1 :(A) RF-02 *Aspergillus flavus* (**B)** RF-07 *Aspergillus niger*
Plates of isolated fungal deteriorating agents of Groundnut.

2.2.2. Characterization of groundnut deteriorating fungi: Pure fungal isolates RF-02 and RF-07 were further analyzed for microscopic identification by using lactophenol cotton blue staining **(Figure-2),** followed by 18S rRNA sequencing for confirmation on genus and species level. In that process standard protocols were followed at each level of isolation of fungal genomic DNA and RNA[18]. Moreover, PCR amplification and purification of PCR product was completed as per the instruction of primer manufactures on the kit (Sigma Aldrich). After sequencing of purified PCR product result sequences were go through the BLAST search tool providing by National Center for Biotechnology Information (NCBI).

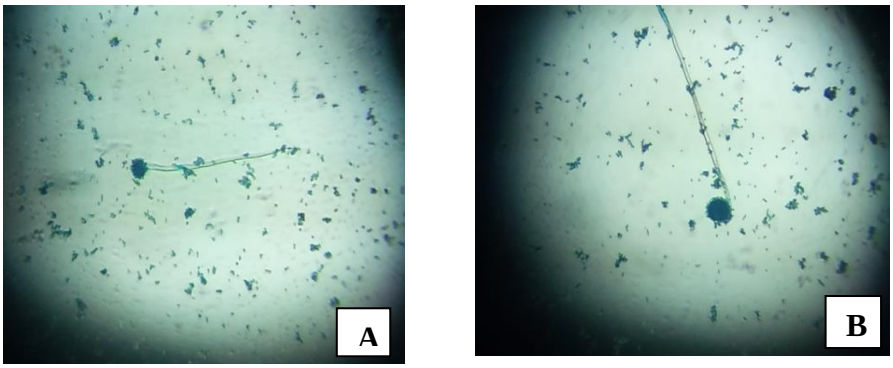

Fig-2 (A) RF-02 *Aspergillus flavus* (**B)** RF-07 *Aspergillus niger* microscopic images

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2.3 *In vitro* pathogenicity on aged seeds: One year aged groundnut seeds were collected from storage area of the owner of targeted experimental field with same variety. The collected groundnut was stored in the laboratory conditions for 48 h at room temperature. The groundnut was washed with tap water and sterilized by using 2% of mercuric chloride solution followed by three washes with double distilled water and air dry for surface cleaning[19]. In continuation of the experiment, five seeds were placed in one Petri dish (in triplicate). The disc of fungal isolate was placed in center of the Petri plate with the help of well borer (one disc in each plate). Same process was repeated with second fungal isolate with one control plate without fungal discs. All plates were carefully placed into the incubator for incubation at 28-29°C for one week.

3. RESULTS AND DISCUSSION

3.1 Characterization of deteriorating fungi: Among all the isolates two fungal strains were picked up for further study (RF-02 and RF- 07) (**Figure 3)**. These two isolates were very dominant on seeds during the period of isolation and incubation as well. The identity of these isolated fungi were determined by amplification and sequencing of the 18S rRNA. The edited sequences were deposited in GeneBank, under accession KY933394 and KY357318 respectively **(Figure-4 and 5).** RF-02 was identified as *Aspergillus flavus* and RF-07 as *Aspergillus niger* by 18S rRNA sequencing.

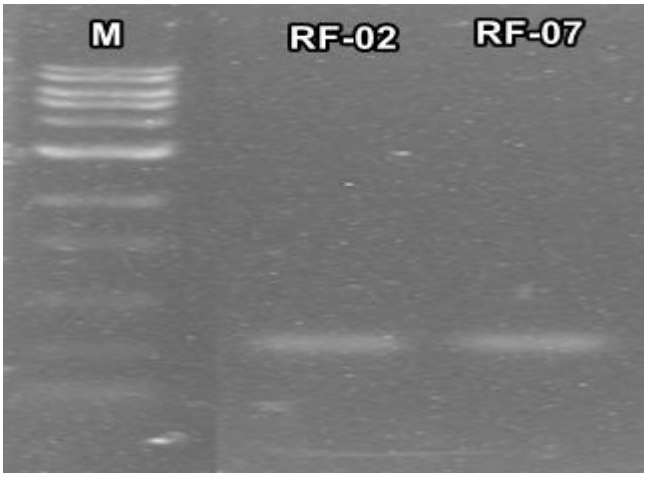

Fig.-3: Agrose gel bands of RF-02 and 07 with comparative markers

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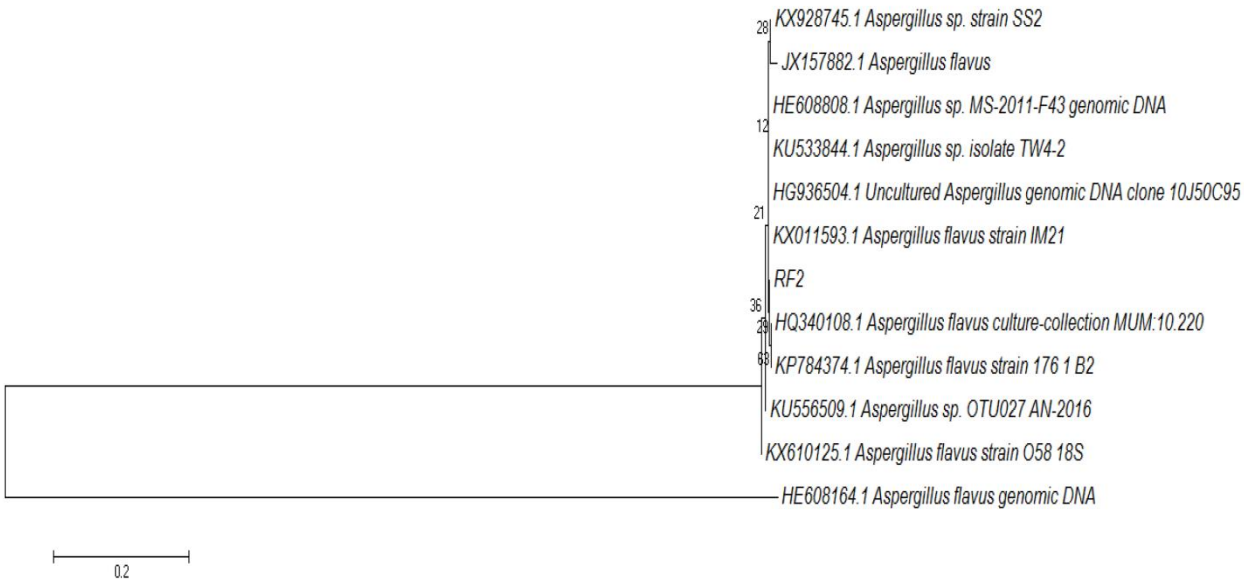

Fig. 4: Phylogenetic tree of RF-02(*Aspergillus flavus*)

Fig. 5: Phylogenetic tree of RF-07(*Aspergillus niger*)

3.2 Effect of deteriorating fungi on groundnut seeds: In this study, it was observed that the fungal discs which were placed in the center of Petri plate; *A. flavus* and *A. niger* fully spread on to the plate after one week of incubation and almost 90% of groundnut seeds were infected equally with their respective fungal inoculants. *A. flavus* and *A. niger* predominantly affect the seeds of ground nut in both field level and under storage conditions with significant damage. Raju and Krishnamurthy[20] found that the infection of *A. niger* in groundnut increased with the time of storage period. Further, Krishnappa et al.[21] reported that groundnut

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pods stored in gunny bag; had recorded maximum infection ranged between 16 and 18% of *A. flavus*. According to [22-23] *A. flavus* is frequently associated with aflatoxin contamination of agricultural crops [22-23]. Jolly et al.[24] concluded that presence of aflatoxins in food material is very dangerous for humans because they may cause cancer and other health problems. Bhatnagar-Mathur, et al.[25] found that the infection probability of *A. flavus* in groundnut increases during stress conditions and at the time of crop harvesting. Further, Lai, et al.[26] found Aflatoxin B1 as one of the toxic and potent carcinogen produced by fungal contaminations. Chiewchan, et al.[27] recommended that aflatoxin contamination should be avoided during cultivation, storage, transport and processing.

The present study thus reports two fungal strains of *A. flavus* and *A. niger* which cause deterioration in groundnut seeds during storage and in fields. It is important to find out biological methods to combat such fungal pathogesn so as to prevent the use of pesticides/fungicides[28-29]. Many biocontrol agents are known which should be exploited and used for control of such seed deteriorating and harmful fungi.[30,31,32]

CONCLUSION

In the concluding remarks on the above study, authors' clearly observed *A. flavus* and *A.niger* as dominant deteriorating contaminants of groundnut seeds during storage. Moreover, it was also found that the seeds on which the fungal growth was observed are totally deteriorated with the maturation of fungal contaminant. That's why cure and control of oil seeds from deteriorative fungal pathogens is also very important part of agriculture. For control of these type of storage pathogens, bacterial metabolites seem to be very helpful as they are non-toxic and environmental friendly. A quality research is needed for storage seeds and their environmental conditions to reduce the use of harmful chemical based preservatives and increase the use of biological subways for healthy and sustainable agriculture in India and all over the world for better food security.

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ISOLATION AND CHARACTERIZATION OF DOMINANT PATHOGENIC FUNGUS *ASPERGILLUS FLAVUS* FROM STORAGE SEEDS OF GROUNDNUT

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Abstract: *Aspergillus flavus* is one of the well-known storage fungal pathogen. It causes very harmful effect for agricultural crops. Infection spreads dangerously, especially in the oilseeds having excessive moisture and nutrients. Keeping in view of the availability of fungus and its pathogenicity, traces of dominant fungal strain RF-03 have been taken from the infected groundnut seeds. These seeds were collected form warehouse of a local farmer of district Manpuri. The isolated strain dominantly covers the petri plates and also affects the development and quality of seed. Pure culture of RF-03 was analyzed firstly through electron microscope for morphological characterization. Further, the fungal strain was identified for genus and species level and resulted as *A. flavus* by 18S rRNA sequencing, the obtained sequence was then submitted in NCBI under the accession number MF120213.1.

IndexTerms - Aspergillus flavus, storage oil seeds, fungal pathogens, SEM analysis.

I. INTRODUCTION

In concern of oil seeds, Groundnut (*Arachis hypogaea L*) is one of the most important oil seed crop worldwide and Indian is second largest producer of groundnut after china Pandey et al, (2012).It covers more than 82% of total oil seed production in India. Groundnut typically contains ample amount of protein and oil in it. Moreover, it is a rich source of multiple nutrients including Vitamin E and magnesium. Shalini et al. (2016). According to Adeyeye and Ajewole (1992); Sadasivam and Manickam (2008) the quality parameters for oil and products of groundnut are directly influenced by fatty acid profile along with growing conditions. That's why the crop is more susceptible to a variety of plant pathogens. Li et al. (2013).

Aspergillus flavus is one of the predominant pathetic fungi for oilseed crops also known as opportunistic pathogenesis in plants. A. flavus is one of the major factor for inhibit good quality seeds. Considerable economic loss takes place during international trade is only because of lower quality seed production. The seeds can be contaminated by Aspergillus spp. during harvest, pre and post-harvest, in storage or during transport. Shekhar et.al (2018); Vankayalapati (2018). The aflatoxin levels increases due to improper harvesting. The attacks of pathogens resulting yield losses and lower quality of the produce. Romani (2004). A yield loss due to occurrence of the diseases in groundnut is higher than 50%. Oilseeds may moderated there chemical composition due to the production of the potent carcinogen aflatoxin by fungal pathogens. Aflatoxins are toxic, mutagenic, and carcinogenic compounds Chen et al. (2013). These toxins occur naturally and have been found in a wide range of commodities, including groundnuts used for animal and human consumption. Williams et al. (2004)

The presence of pathetic fungi in storage area is a major threat of quality seeds production and fulfills the yield gap between supplies and demand. For sustainable agriculture and healthy food production it is very important to identify the dominant pathogens of storage area. Hence, the present study is formulated to identify and characterize dominant fungal pathogen from actual environmental conditions found in warehouse located within the groundnut producing area.

II. RESEARCH METHODOLOGY

II.1 Sample Collection

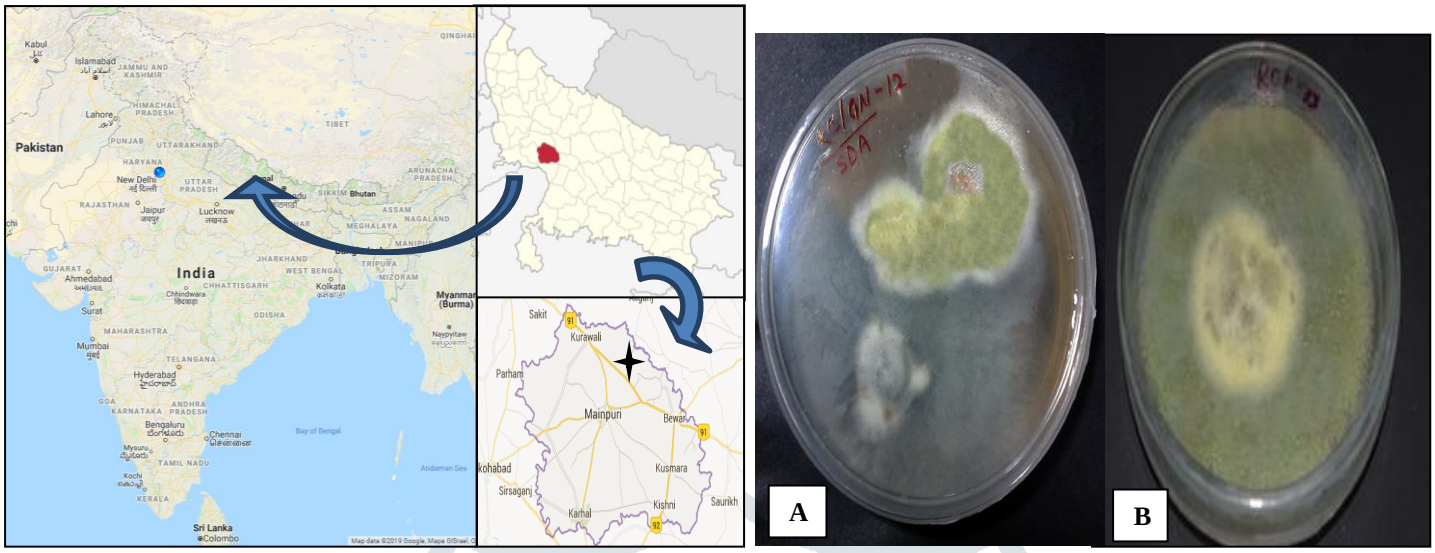
For the isolation of fungal pathogens, one year aged groundnut seeds were collected from the natural agriculture environment at village Alipurkheda district Manipuri, Uttar Pradesh (**Figure-1**). Infected seeds were collected in polybags and stored at room temperature in laboratory conditions.

II.2 Isolation of pathogen

Fungal pathogens were isolated from infected seeds and shells of groundnut. All the infected groundnuts were going through the surface sterilization with 1% of sodium hypochlorite for 30 seconds, followed by three thorough rinses with sterilized distilled water. Air dried infected seeds were separated from shells, and then placed on potato dextrose agar (Hi media) plates. After incubation on 28°C for 5-7 days fungal colonies appeared on seeds and shells were picked up and purified further on PDA plates. Among all RF-03 was found in both shells and seeds were separated for further study (**Figure-2**)

Fig-1 Site map of sampling In District Manpuri UP **Fig-2 (A) isolate *Aspergillus flavus* (B) RF-03 *Aspergillus flavus***

plates of isolated fungal deteriorating agents of Groundnut.



II.3 SEM Analysis

Purified fungal strain RF-03 was analyzed through surface electron microscope , fresh fungal samples were prepared and fixed by using 2% glutaraldehyde in 0.2 M phosphate buffer (pH 6.8) at room temperature for 4 to 6 h. further, the fixed samples were carefully rinsed with 0.2 M phosphate buffer (pH 6.8) for 1–2 h, and then dehydrated in a graded acetone series (30, 50, 70, 80, 90, and 100%), each grade for 30 min and three times for 100% acetone. Fully dehydrated samples were completely dried and mounted on stubs for examination under SEM (**Figure-3**).

Fig-3 SEM micrographs of isolated fungal deteriorating agents of Groundnut.

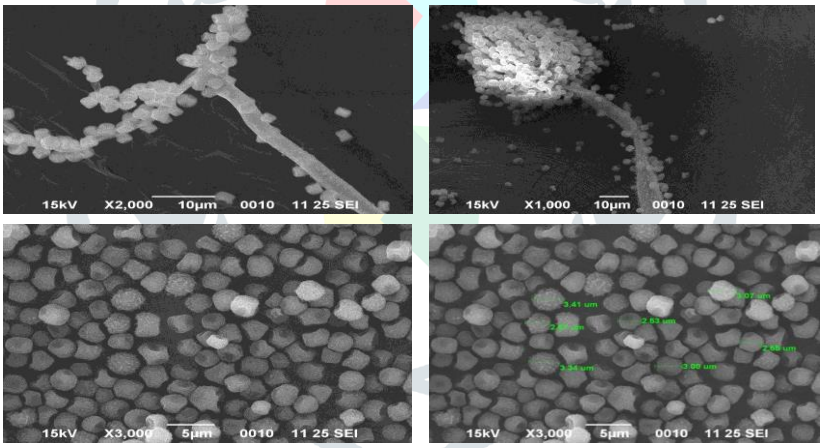
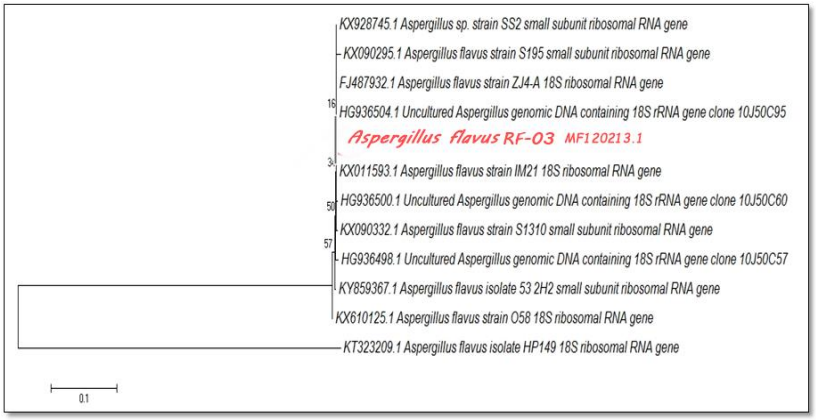


Fig-4 Phylogenetic analysis of isolate RF-03



II.4 18S rRNA gene sequencing

Fungal pathogen was further identified at genus and species level by using the 18S rRNA sequencing technique. Genomic DNA was isolated as described by Smit et al. in (1999). Moreover, amplification and purification of PCR product was completed as per the instruction of primer manufactures on the kit (Sigma Aldrich). 18S rRNA ITS Region universal primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC), PCR product was sequenced using the ITS1/ITS4 primers. Result sequence was further submitted to NCBI.

III. RESULTS AND DISCUSSION

III.1 Identification of pathogen

Among all isolate the dominant fungal pathogen RF-03 was resulted as Aspergillus flavus after 18S rRNA sequencing. The FASTA format of sequence was submitted to NCBI under the accession number MF120213.1.(**Figure-4)** *A. flavus* highly contagious and covered full petri dish during isolation process further, the fungi is responsible for foodborne disease along with aflatoxin production in seeds under storage conditions. Chen et al, (2002) ; Gupta and Chauhan (1970) isolated A. flavus from groundnut seeds. Similarly, Lalithakumari et al. (1971) observed presence of A. flavus as dominant fungi on groundnut seeds. Bhattcharya and Raha (2002) reported that in groundnut seeds Aspergillus niger, A. rubber and A. flavus were initially very abundantly found in storage condition.

III.2 Surface morphology of pathogen

Scanning Electron Microscopy is done for revealing the surface morphology of isolated fungi. SEM creates various images by focusing a high energy beam of electrons onto the surface of a sample and detecting signals from the interaction of the incident electron with the sample's surface. Conidia of *A. flavus* have relatively thin walls which are finely to moderately rough ened. Their shape can vary from spherical to elliptical. Scanning Electron Micoroscopy (SEM) micrographs clearly show these ornamentation differences Furthermore, once SEM micrographs have been studied and compared, then with practice these differences become apparent using light microscopy. Fungal species have been detected and also identified through SEM.

The present study thus reports fungal strain RF-03 as *A. flavus* which cause savior infections in groundnut seeds during storage. Biological methods are seems to be very useful and environmental friendly alternative to combat such fungal pathogens so as to prevent the use of pesticides/fungicides (Arora et al. 2018; Mishra et al. 2016). Many biocontrol agents are known which should be exploited and used for control of such seed deteriorating and harmful fungi (Verma et al. 2019; Mishra and Arora 2017;Tiwari and Arora 2018)

IV. CONCLUSION

At the end of this study, the authors concluded that *A. flavus* is a very infectious storage fungi, which effectively causes infections in nutrient rich seeds during long term storage. Because of these lethal infections, groundnut seeds can't be properly developed. Moreover, their ability to wear oil becomes very less. However, prevention of groundnut seeds from mycobial contamination environmental friendly subways i.e. microbial metabolites should be used for better storage of groundnut seeds. Microbial metabolites are naturally according substances with no harmful effect to humans and animals as well. The use of metabolites for cure and control of fungal pathogens during storage seems to be very cheap and best alternatives of chemical based fungicides. Quality research is needed for proper storage treatments of oil seeds worldwide.

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