Isolation, tolerance, bioaccumulation and plant growth promoting activities of selenium tolerant fungal isolates in vitro and in vivo.

A thesis
submitted in fulfilment of the requirement for
the award of degree of

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IN
BIOTECHNOLOGY

Under the guidance of

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Certificate

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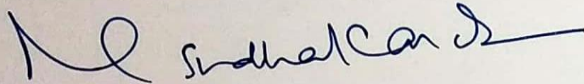

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DECLARATION

I hereby declare that the work which is being presented in the thesis "**Isolation, tolerance, bioaccumulation and plant growth promoting activities of selenium tolerant fungal isolates in vitro and in vivo"** submitted by me for the award of the degree of Master of Science in the department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab is true and original record of my own independent and original research work carried out under the supervision of **Prof. Dr. M. Sudhakara Reddy**, Professor, Department of Biotechnology. The matter embodied in this thesis has not been submitted in part or full to any other institute or university for the award of any degree in India or Abroad.

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Place: PATIALA

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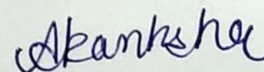


AKANKSHA

TABLE OF CONTENTS

S.No.	Chapters	Page No.
1.	**INTRODUCTION**	1-3
	Objectives	
2.	**REVIEW OF LITERATURE**	4
		5
	2.1 Selenium	6-7
	2.2 Selenium in environment	7-8
	2.3 Seleniferous regions	9-14
	2.4 Selenium in soil	
	2.4.1 Selenium speciation in soil	
	2.4.2 pH and redox potential of soil	
	2.4.3 Selenium metabolism in plants	
	2.4.4 Total selenium and bioavailability in soil	
	2.5 Selenium in plants	14-15
	2.5.1 Selenium uptake in plants	
	2.5.2 Selenium transport and accumulation within in plants	
	2.5.3 Biotransformation of Se in soil-plant environment	
	2.5.4 Selenium metabolism in plants	
	2.6 Microbial transformation in soil	15-16
	2.7 Filamentous fungi in selenium bioaccumulation	16-20
	2.7.1 Selenium reduction and tolerance by fungi	
	2.7.2 Selenium bioaccumulation and biovolatilization by fungi	
	2.8 Plant growth promoting activities by fungi	20-22
	2.8.1 Indole acetic acid (IAA) production by fungi	
	2.8.2 Phosphate solublization by fungi	
	2.8.3 Siderophore production by fungi	
3.	**MATERIALS AND METHODS**	23
	3.1 Collection of soil sample	24
	3.2 Isolation of selenium tolerant fungal strain	24-25
	3.2.1 Isolation of selenium tolerant fungal strains from seleniferous soil by serial dilution method	
	3.3 Subculturing of isolation of selenium tolerant fungal strains	25
	3.4 Determination of selenium tolerance level of pure fungal isolates	25-27

3.4.1 To check selenium tolerance level of fungal isolates
3.5 Plant growth promoting activities of highly tolerant fungal isolates 27-31
3.5.1 Quantification of Indole acetic acid (IAA) by fungi
3.5.2 Qualitative estimation of phosphate solublization by fungi
3.5.3 HCN production by fungi
3.5.4 Siderophore production by fungi
3.5.5 Ammonia production by fungi
3.6 Effect of fungi on plant growth promotion 31-32
3.6.1 Field experiment
3.6.2 Inoculation of seeds
3.6.2.1 Methodology for inoculum and seed preparation
3.6.2.2 Experimental field and plant cultivation
3.6.2.3 Analysis of plants after harvesting
3.7 Estimation of selenium bioaccumulation 33-36
3.7.1 Estimation of selenium in fungal tissue/roots/plant tissue
3.8 Morphological characterization of highly tolerant fungal isolates 36
3.9 Molecular methods for identification of highly Se tolerant fungal isolates 37-44
3.9.1 Isolation of genomic DNA from Se tolerant fungal isolates
3.9.2 Qualitative analysis of isolated DNA
3.9.3 Quantitative analysis of isolated DNA
3.9.4 Amplification of ITS region by PCR
3.9.5 Purification of PCR products
3.9.6 Ligation of ITS in TA cloning vector pMD20T
3.9.7 Preparation of competent cells
3.9.8 Blue-white screening of recombinant plasmids
3.9.9 Colony PCR
3.9.10 Plasmid isolation (alkali lysis method)
3.9.11 Sequencing of ITS region
3.9.12 Phylogenetic relatedness
3.10 Estimation of selenium in soil sample 44-47
3.11 Physiochemical characteristics of soil 47-53
3.11.1 Available phosphorus (P)
3.11.2 Total phosphorus
3.11.3 Organic carbon
3.11.4 Total nitrogen
4. **RESULTS & DISCUSSION**
4.1 Collection of soil sample 55
4.2 Isolation of selenium tolerant fungal isolates 55-56

4.3 Subculturing of selenium tolerant fugal isolates 56-57
4.4 To check selenium tolerance level of fungal isolates 57-61
4.5 Plant growth promoting of highly tolerant fungal isolates 61-69
4.5.1 Quantification of Indole acetic acid
4.5.2 Phosphate solubilisation by fungi
4.5.3 HCN production by fungi
4.5.4 Siderophore production by fungi
4.5.5 Ammonia production by fungi
4.6 Estimation of selenium bioaccumulation 69-72
4.7 Morphological characterization of highly selenium tolerant fungal isolates 72-73
4.8 Molecular methods for identification of highly selenium tolerant fungal isolates 74
4.8.1 Analysis of isolated genomic DNA
4.8.2 Quantitative analysis on Nanodrop
4.8.3 Amplification of ITS region
4.8.4 Genetic transformation and blue-white screening
4.8.5 Plasmid isolation (alkali lysis method)
4.8.6 Sequencing of ITS region
4.9 Physiochemical characteristics of soil 81
4.10 Effect of fungi on plant growth promotion 81-84
4.10.1 Field experiment
4.10.2 Seed inoculation
4.10.3 Experimental field preparation and cultivation of plants
4.10.4 Analysis of maize plant after harvesting
5. **DISCUSSION** 85-87
6. **CONCLUSIONS** 88-89
7. **REFERENCES** 90-97

ABBREVIATIONS

Se	Selenium
SeCys	Selenocysteine
SeMet	Selenomethionine
DMSe	Dimethylselenide
DMDSe	Dimethyldiselenide
CH_3SeH	Methaneselenol
Se2-	Selenide
SeO_4^{2-}	Selenite
Se (0)	Elemental selenium
S	Sulphur
APS	ATP Sulphurlase
APR	ATP reductase
CS	Cysteine synthase
SL	Selenocysteine lyase
SMT	Selenocysteinemethyltransferases
CTAB	Cetyl trimethyl ammonium bromide
CAS	Chrome Azurol 'S'
MMN	Modified Melin-Narkarne
NCBI	National Centre of biotechnology and Information
rRNA	Ribosomal RNA
DNA	Deoxyribonucleic acid
µl	microliter
µg	microgram
rpm	revolution per minute
PCR	Polymerase chain revolution

LIST OF FIGURES	Page No
Fig 2.1 Flow chart showing natural selenium cycle	6
Fig 2.2 Presence of seleniferous areas in Punjab region of northwestern India	8
Fig 2.3 Selenium accumulation in soil depending upon its accumulation in plants	11
Fig 2.4 Transformation of selenium in soil-plant system (selenomethionine; selenocysteine; dimethylselenide; dimethyldiselenide)	13
Fig 2.5 Schematic representation of Se metabolism inside the plant cells ATP sulfurylase; APS reductase; Cysteine synthase; Selenocysteine lyase; Selenocysteine methyl transferase; A-Accumulation; T,-tolerance	15
Fig 2.6 Fungi-metal interactions	17
Fig 4.1 Field soil of Jainpur, Nawanshahr region	55
Fig 4.2 Linear graph showing emission spectra of Se-ICP standard solution at different concentrations and that of seleniferous soil sample	56
Fig 4.3 Different fungal isolates obtained from seleniferous soil sample a) SeF1 b) SeF2 c) SeF4 d) SeF5 e) SeF6 f) SeF7 g) SeF9 h) SeF10 i) SeF12	57
Fig 4.4 Effect of sodium selenite at different concentrations (20-100 mM) in MMN broth on fungal isolates. (a) SeF5 fungal isolate in MMN broth, (b) SeF12 fungal isolate in MMN broth	59
Fig 4.5 Effect of different concentrations of Se on fungal biomass (mg/50ml) SeF5 and SeF12	60
Fig 4.6 Standard curve obtained from the known concentrations of IAA	62
Fig 4.7 Standards of known concentrations of IAA a) 20 µg/ml b) 10 µg/ml c) 30 µg/ml d) 40 µg/ml e) control 0 µg/ml	63
Fig 4.8 IAA production by selenium tolerant fungal isolates, a) negative control b) SeF5 c) SeF12	63
Fig 4.9 Phosphate solubilization a) Control plate b) SeF12 c) SeF5	64
Fig 4.10 SeF5 and SeF12 showed negative results for HCN production as there is no change in filter paper colour from yellow to brown. Plate a) negative control, plate b) SeF5, plate c) SeF12.	65
Fig 4.11 Siderophore production in broth a) SeF12 b) SeF5 c) SeF4 d) SeF3	67

Fig 4.12 Siderophore production a) SeF5 (bluish coloured zone) b) CAS plate without inoculation c) SeF12 (purple coloured zone).	67
Fig 4.13 Ammonia production shown by SeF5 and SeF12 by giving brown colour. a) Control; b) SeF5; c) SeF12.	68
Fig 4.14 Selenium accumulation in fungal biomass (mg) of SeF5 and SeF12	70
Fig 4.15 Se bioaccumulation in root biomass (mg) inoculated with SeF5 and SeF12	71
Fig 4.16 Selenium bioaccumulation in shoot biomass (mg) inoculated with SeF5 and SeF12.	72
Fig 4.17 Microscopic view of *Pseudopestalotiopsis thecae* (SeF12) 100X magnification.	73
Fig 4.18 Microscopic view of *Fusarium equiseti* (SeF5) 100X magnification	73
Fig 4.19 Agarose gel showing DNA bands of SeF12 and SeF5, Ladder: 1kb Lane 1; SeF5: Lane 2; SeF12: Lane 3.	74
Fig 4.20 Amplification of ITS-PCR products of two isolates. Lane 1: 1 kb; Lane 2: SeF5; Lane 3: SeF5; Lane 4: SeF12: Lane 4: SeF12.	75
Fig 4.21 LA and Ampicillin plates showing blue white colonies.	76
Fig 4.22. Plasmid DNA of ITS region of SeF5 and SeF12. Lane 1: 1 kB ladder; Lane 2: SeF_5 plasmid; SeF_{12} plasmid.	77
Fig 4.23 Blast results of SeF5 fungal isolate	78
Fig 4.24 Multiple sequence alignment pf SeF5 fungal isolate.	78
Fig 4.25 Phylogenetic tree of *Fusarium equiseti* (SeF5)	79
Fig 4.26 Blast results of ITS 18S RNA region of SeF12 fungal isolate	80
Fig 4.27 Multiple sequence alignment of SeF12 fungal isolate *Pseudopestalotiopsis theae* (SeF12)	80
Fig 4.28 Phylogenetic tree of *Pseudopestalotiopsis theae*	81
Fig 4.29 Comparing SeF12 and SeF5 with control plant after 2 months. (a) SeF12 with control; (b) SeF5 with control	83
Fig 4.30 Roots and whole maize plant of SeF12 and SeF5 with control plant. (a) SeF5 whole plant; (b) SeF12 whole plant; (c) SeF5 roots(d) SeF12 roots.	84

LIST OF TABLES	Page.No.
Table 2.1 Selenium speciation in soil	9
Table 2.2 Presence of selenium in soil	11
Table 3.1 Standards of known selenium concentrations	35
Table 3.2 Components of extraction buffer	37
Table 3.3 Reaction mixture for PCR amplification	40
Table 3.4 Conditions of PCR reactions for 35 cycles	40
Table 3.5 Reaction mixture for ligation of ITS region	41
Table 3.6 Reaction mixture of colony PCR for 35 cycles	42
Table 3.7 PCR conditions for colony PCR	43
Table 3.8 Standards of known selenium concentrations	47
Table 4.1 Tolerance studies of dry weight of fungal biomass (mg/ml) at different concentrations of sodium selenite (mM)	58
Table 4.2 Phosphate solubilization index of SeF5 and SeF12.	64
Table 4.3 Showing % of siderophore production by SeF5 and SeF12	66
Table 4.4 Selenium bioaccumulation in fungal biomass (mg) of SeF5 and SeF12 at different concentrations	69
Table 4.5 Two factor ANOVA analysis of fungal biomass for Se uptake in SeF5 and SeF12	70
Table 4.6 Root biomass (mg) of SeF5 and SeF12 plant in selenium contaminated soil	71
Table 4.7 Shoot biomass (mg) of SeF5 and SeF12 plant in selenium contaminated soil	72
Table 4.8 Quantitative analysis of isolated DNA by Nanodrop	75
Table 4.9 Physiochemical characteristics of wheat soil sample	81
Table 4.10 Effect on various plant tissues inoculated with fungal isolates (SeF5 and SeF12)	82

ABSTRACT

Due to increased human activities like crude oil refining, combustion and various human activities, industrial waste release a large amount of gases and wastes. Wastes include different types of metalloids like Cd, Pb, Se, Te and Sb. Among these metalloids, selenium is required by the body in trace amount. The larger concentration of selenium is harmful to humans and leads to various diseases. Nowadays most of the areas are affected by selenium contamination and produces harmful effects on animals and humans. The objective of the present work was to isolate fungi from seleniferous soil means rich in selenium concentration. Microbes undergo various mechanisms to detoxify the concentration of metal and metalloid by various processes like complexation, biosorption, reduction and metal-fungal interaction. Fungal isolates can be used for bioremediation process means removal of heavy metals by living organisms. The objective of present work was to exploit the fungal strains *Fusarium equiseti* and *Pseudopestalotiopsis theae* and checked their tolerance level at different concentrations of sodium selenite tolerance level at higher concentration of selenium and showed plant growth promoting activities like phosphate solubilization, IAA production, HCN production and siderophore production. Fungal isolates act as a plant growth promoting fungi and good biofertilizers for plant growth. Use of microorganisms as a PGPR (Plant Growth Promoting Activities) and removal of Se from contaminated areas was cheapest method.

INTRODUCTION

Introduction

In 1817, Selenium was discovered by Swedish chemist Jons Jacob Berzelius. The first publication on this element was discovered in 1818 named Selenium (Watts 1994). In the periodic table, it belongs to the oxygen family. Selenium (Se) is known as a metalloid due to the properties of both metal and non-metal. Se takes up 70th position in a naturally occurring element. It is known as a trace element due to present in low amount in earth crust (0.05 ppm). Naturally, Se is present in volcanic matter, rocks, minerals, soil, coal, coal ash and dust. Various natural and anthropogenic activity in an atmosphere like crude oil refining, combustion of coal, synthesis of lead and copper ores gives selenium as a byproduct (Winkel et al. 2012). Naturally, this element exists in inorganic forms (selenate, selenite and selenide) and Selenocysteine and Selenomethionine are various organic forms. Among above two forms, selenate is more water soluble then selenite (Bodnar et al. 2012). Various volatile organic (dimethylselenide, dimethyldiselenide, methaneselenol) and inorganic (Selenium dioxide) derivatives of selenium which is present in the atmosphere (Mehdi et al. 2013).

Selenium is an important compound for both animals and humans. Se shows antioxidant and anticancer properties when taken in a small amount (Reid et al. 2008). It is one of the essential micronutrients for humans because it is the main component in selenoproteins (glutathione peroxidases (GSHPx), iodothyronine-deiodinase and thioredoxin reductase). It plays a significant role in strengthening and defensive mechanism of immune system for oxidative stress (Kaur et al. 2014).

Dose and chemical form shows a necessary part in the biological activity of Se. Recommended level of Se in human diet by World Health Organization (WHO) is from 50-55 µg/day. Se uptake (<40 µg/day) causes its deficiency and overdose (>400 µg/day) causes chronic Se toxicity known as selenosis (Winkel et al. 2012). Various symptoms observed due to Se deficiency are: abnormalities in thyroid function, growth retardation and impaired bone metabolism. It produces a toxic effect at higher concentration causing DNA damage by producing oxidative stress (Izquierdo and Herrero 2010). Other symptoms are defective nails, skin lesions and alopecia (Gupta and Gupta 2017).

Northeastern parts of Punjab and Haryana is rich in seleniferous soil. Se concentration in soil of Nawanshahr- Hoshiarpur region of Punjab varies from 0.25-4.5 mg Se/kg. (Sharma et al. 2009). Agricultural land of 1000 hectares is affected by Se higher level. Consumption of crops in this region produces Se toxicity. Amount of Se in soil and crop products is ranging from 2.7- 6.5 mg Se/kg and 13-670 mg Se/kg. Various crops are grown in this region like wheat grains, wheat husk, rice, maize and mustard. Fodder and grains produce in these regions contain Se from 5-160 and 4-66 mg Se/kg. Various selenosis symptoms are shown by wheat crop like chlorosis, pink colouration on a lower side of leaves. The concentration of Se in soil ranging from 0.5 mg Se/kg produces toxic effects in fodder too leading to selenium toxicity in animals. Crop productivity reduces by 10% due to Se toxicity (Dhillion and Dhillion 2000).

Soil is rich in various microorganisms. These microorganisms help in selenium removal from soil, water and sediments by special mechanisms. They show various microbial or biological activities like methylation, reduction, oxidation and demethylation (Dungan and Frankenberger 1999). These microbes used in remediation process are low cost and ecofriendly (Joshi et al. 2011). Tolerant fungi have been found which can survive in metal and the metalloid toxic environment by using special mechanism. Use of fungi in bioremediation process is due to following reasons: the ability to grow and survive under extreme conditions like high temperature, pH and metal concentrations (Siddiquee et al. 2015). Removal of metalloid by microorganisms is known as a bioremediation. Most of the Se bioremediation studies have been done on bacteria but researchers have found the contribution of fungi too in selenium bioremediation process. Recently researchers have exploited various filamentous fungi which plays a key role in Se remediation process (Urik et al. 2014).

Filamentous fungi show unique metabolic pathways by which they act as a bio accumulators for metalloid and convert the toxic state of metalloid to innocuous state or volatile derivatives (Urik et al. 2016). Microbes-metalloid interaction involves various processes like adsorption, complexation, oxidation precipitation and reduction. Free-living fungal system undergoes six distinct mechanisms like metal mobilizing, biosorption to cell wall, metal uptake, metal transformations and intracellular metal immobilization to detoxify the metal and metalloid toxicity (Gadd 1933; Harms et al 2011). Fungal structure has an

ability to mobilize the selenium which further helps in removal of Se contamination from wastewater, sediments and soil. Fungi undergo various metabolic processes like bioalkylation, leaching process, accumulation, reduction, biosorption of metal (loid) and metal uptake to reduce the toxic effect of metals. These detoxification mechanisms which causes the efficient removal of Se (Littera et al. 2011). Fungi contribute to biovolatization and biotransformation of metal. Fungal species *Aspergillus clavatus* is involved in the active and passive movement of Se (VI) oxyanions to environment-friendly selenium species (Urik et al. 2016).

Pandya and Saraf (2010) Plant system involves two mechanisms for their growth: direct mechanism involves the production of various growth regulators (auxins, cytokinin, ethylene and gibberelins), phosphate for mineral solubilization and nitrogen fixation. Various actions like cell wall synthesis, production of siderophores, HCN production, antibiotics and competition with harmful microorganisms come under indirect plant growth mechanism. Rhizospheric fungi can act as a PGPF (Plant Growth Promoting Fungi) which promotes both indirect and direct mechanisms for plant growth. Fungal species belonging to genera *Fusarium, Trichoderma*, *Aspergillius* and *Phoma* act as a PGPF which are non-pathogenic in nature and prove to be beneficial for the growth of various crop plants and shield the plants from various diseases.

OBJECTIVES

- Isolation of selenium tolerant fungi from seleniferous soil.
- Selenium tolerance and bioaccumulation of selenium and plant growth promoting activities of selected fungi.
- Plant growth promotion by fungal isolates in selenium contaminated soil.

REVIEW OF LITERATURE

Review of Literature

2.1 Selenium

Selenium (Se) well known as a metalloid was spotted in 1817 by one of famous Swedish chemist Jon Jacob Berzelius. Se originated from Greek word Selene means goddess of moon due its resemblance with tellurium (Watts 1994). In, earth crust its concentration is 0.05 ppm due which it is known as a trace element. Naturally, Se is present in volcanic matter, rocks, minerals, soil, coal, coal ash and dust (Fig 2.1). It belongs to oxygen family in the periodic table. Se is present in five stable oxidation state: -1, -2, 0, +4, +6 (Winkel et al. 2012). At room temperature, Se is present in solid form and precipitation of Se in liquid medium gives brick red powder. Its various physiochemical properties like atomic size, ionization state, oxidation states and bond energies are analogous to sulphur (S) in periodic table. It shows both the properties of metal and non-metal due to which it is semiconductive in nature. It remains stable at room temperature (Mehdi et al. 2013).

Se is present all over the biosphere and non-living environment like hydrosphere, lithosphere and atmosphere (White et al. 2004). Se is present in form of volatile derivatives in the atmosphere. There are two types of Se volatile derivatives: organic forms includes dimethylselenide, dimethyldiselenide, methaneselenol, dimethyl selenone and inorganic hydrogen selenide volatile derivatives (Mehdi et al. 2013). Naturally, it is categorized into two forms which are organic and inorganic forms. Selenomethionine (Semeth) and selenocysteine (Secys) comes under the category of organic forms present in humans. Inorganic forms contain selenite (+4), selenate (+6) and elemental selenium Se (0) which is mostly present in the soil, water and wastes (Sors et al. 2005). There is a structural difference between the two forms, inorganic forms, Se is directly bonded to a carbon atom and covalently bonded in inorganic forms. Between these two forms, organic one is less toxic than inorganic form (Bodnar et al. 2012).

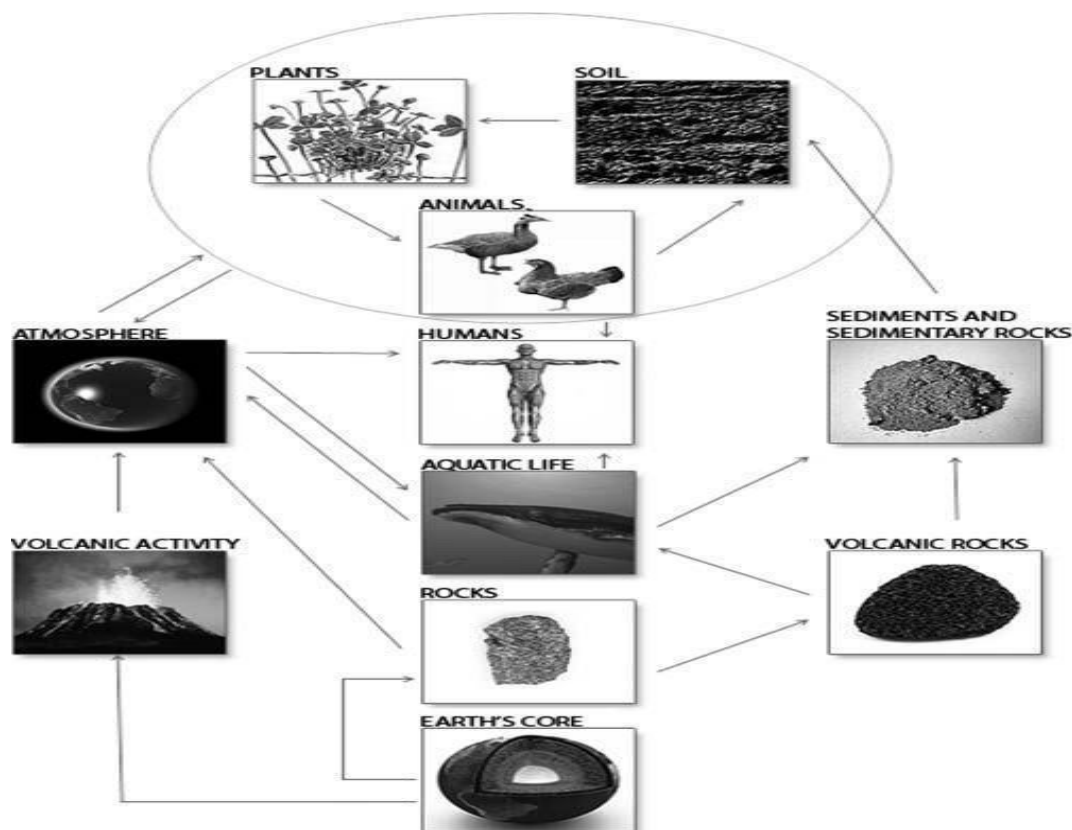


Fig 2.1 Flow chart showing natural selenium cycle (Bodnar et al. 2012)

2.2 Selenium in environment

In biosphere activities like soil erosion, burning of fossil fuels is important for biogeochemical cycling of Se in an environment. Distribution of Se all over the globe is not uniform. It depends upon two factors: natural and Se transport processes. In plants and animals distribution depend upon the amount of Se in the soil. The concentration of Se in non-seleniferous soil is less than 0.05-0.1 mg Se/kg and in seleniferous soil is between 2-5 mg Se/kg (Dhillion et al. 1997). Presence of Se also depends upon various factors like pH, temperature conditions and soil properties like organic carbon, iron and clay contents in an environment.

Se occurs in two oxyanions form i.e. selenate and selenite in the environment. Selenate remains stable at higher oxidation state does not form complex with clay particles and absorption on a surface. But, selenite remain stable under moderate oxidizing and reducing conditions, show irreversible reaction with soil particles and organic matter (Fukushi and Sverjensky 2007).

The concentration of Se in food sources depends on the geographical conditions, uptake and accumulation of Se by soil present in the soil. A concentration of Se is more in vegetables than fruits. Se content in cereals is between 0.01-0.55 µg/g and dairy products like milk contains Se between 0.001-0.17 µg/g. Food products which are rich sources of Se are Brazil nuts, brassica and garlic (Dummon et al. 2006). Se concentration in fresh and sea water varies from 4000-12000 µg/ml. Recommended level of Se in drinking water should not be more than 10 µg/ml. The concentration of Se in air varies from 1-10 ng/m^3 (Mehdi et al. 2013).

2.3 Seleniferous regions

"Seleniferous" means elevated in Se. There are two types of soil, seleniferous and non-seleniferous depending on the Se concentration present in agricultural products grown in the soil. Seleniferous soils are those where Se concentration is found to be more than 5 mg/kg. Soil containing Se concentration from 0.1-0.5 mg/kg is known as a seleniferous soil because crops grown on that soil contains Se concentration greater than 4 mg Se/kg (Dhillon and Dhillon 1997). Seleniferous soil shows characteristics heavy or clayey texture. Various anthropogenic and natural activities like weathering of rocks, soil erosion, leaching processes play a very important role in the formation of the seleniferous region all over the globe.

Countries like Australia, India, China, Ireland and the USA shows a higher concentration of Se in soil (Dhillon and Dhillon 2003). Northeastern parts of Punjab and Haryana in India have been identified the pockets of seleniferous soil (Singh and Kumar 1976; Ruby and Punj 2001). Agricultural land of 1000 hectares is affected by the high level of selenium. The soil of Jainpur and Barwa village of Punjab is affected by the elevated level of Se (Fig 2.2). The range of Se in soil and crops varies from 2.7-6.5 mg Se/kg and 13-670 mg Se/kg respectively (Sharma et al. 2009). The main reason for higher Se concentration in this region is that it is situated in foothills of Shivalik range which contains rocks rich in Se. Leaching of Se from rocks is occurring in this area. The main water irrigation source in this region is groundwater, in which Se varies from 479 µg/L with a mean value of 170 µg/L. Se concentration is found to be higher in Shivalik foothills zone. So, due to higher Se concentration in groundwater it is not reported good for irrigation purpose (Srivastava et al. 2006). This groundwater contaminated with Se is

also making the soil in this region seleniferous. Consumption of crops of selenium affected region is responsible for the selenium toxicity in humans.

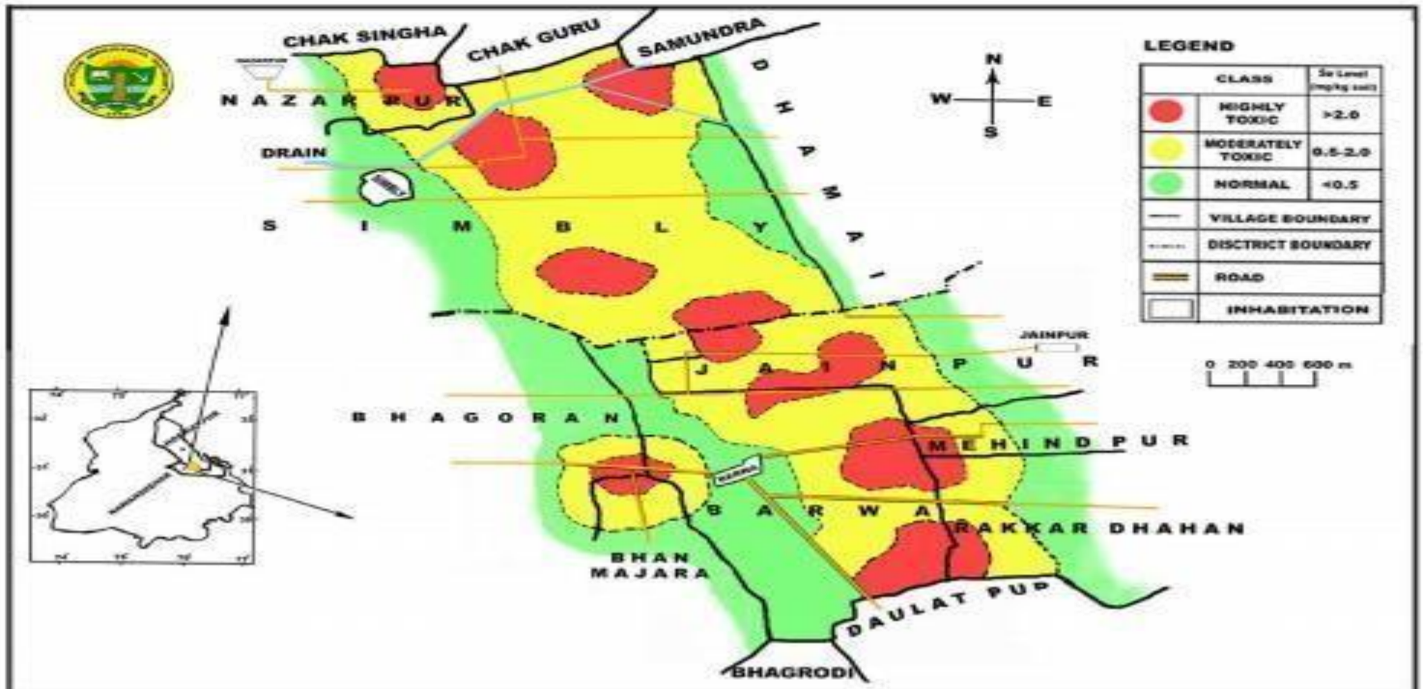


Fig 2.2 Presence of seleniferous areas in Punjab region of northwestern India (Dhillon KS and Dhillon SK 2003)

They consume Se up to 400 µg/day but, daily requirement is just 40 µg/day (Hira et al. 2004). Intake of these crops causes diseases known as a selenosis. Selenosis is divided into two categories: chronic and acute selenosis. Symptoms shown by acute selenosis are: oedema and tissue hamorrhage and chronic selenosis involve loss of hairs, brittle nails, lesions on nails, horny skin (Goldhaber 2003). Various selenosis symptoms observed in humans of northeastern parts of Punjab due Se toxicity are a deformation of fingertips, blood coming out from fingertips visible, headache and loss of body hairs. Selenium toxicity is also seen in plants growing in this region. The first symptom of Se toxicity was reported in wheat plants in seleniferous region of Punjab. There is the formation of pink colour or snow white chlorosis on the sheath and lower side of leaves (Dhillon and Dhillon 1991).

2.4. Selenium in soil

2.4.1 Selenium speciation in soil

Naturally, Se exits in two different forms: organic and inorganic. These forms are present in different oxidation states: -2, 0, +4 and +6. Inorganic forms are present in the soil in form of $SeO_3^{2-}$and SeO_4^{2-}. These forms are present in aqueous phase. Reduction of selenite and selenate gives Se (0) which is known as an elemental selenium (Table 2.1). It is one of the most stable form of Se (Barcelóux 1999).

Table 2.1 Selenium speciation in soil (Barcelóux 1999).

Se species	Chemical formulae	Oxidation number	Present
Elemental selenium	Se	0	Acidic and low redox potential soil.
Selenide	Se^{2-}	-2	Acidic and low redox potential soil.
Selenite	SeO_4^{2-}	+4	Rich in acidic, moderate redox soils.
Selenate	SeO_3^{2-}	+6	Rich in alkaline, elevated redox soils.

Presence and movement of Se in soil depend on the chemical form of Se present in the soil and presence and absence of oxygen conditions. Selenate form of Se is more mobile in oxic (presence of oxygen) conditions than anoxic (absence of oxygen) conditions (Lakin 1972). Among the two forms, selenite is strongly adsorbed on the surface of soil and selenate is highly soluble more available to plants, but it is more toxic than selenate form (Ahlrichs and Hossner 1987).

2.4.2 Soil pH and redox potential

Different form of Se in soil depend on the two factors: pH and redox (oxidation and reduction) potential (Mayland et al. 1989). Adsorption on the surface of soil depend upon the acidity and alkalinity of the soil. Selenate form is more prominent in alkaline soil and in acidic soil selenite form dominate over selenate form (Barrow and Whelan 1989). Soil pH plays a very important role in the adsorption of selenite and selenate. Adsorption of selenate and selenite decreases

with increase in soil pH due to present positive charges on the surface of the acidic soil and creates attractive forces between Se oxyanions and in alkaline soil more negative charges are present on the soil surface. Se forms are more prominent in soil conditions like organic, acidic and reducing (Fordyce 2005). Movement and conversion of selenium in the atmosphere are controlled by redox potential of the soil. Presence of Se different forms in soil depends on the different redox potentials shown by selenate and selenite. At, higher redox potential selenate (SeO_4^{2-}) form more common, medium redox range selenite (SeO_3^{2-}) form is present and at low redox potential selenide is present in the soil (Elrashidi et al. 1987). Adsorption process helps in the mobility and solubility of Se under oxidizing and reducing conditions. There is the formation of insoluble and immobile selenium species in reducing conditions due to this reason Se shows more mobility in oxidizing conditions than reducing conditions (Masscheleyn et al. 1991).

2.4.3 Total selenium and bioavailability in soil:

Selenium is one of the essential trace element for humans and animals. The source of Se to the humans is in the form of forage crops. The concentration of Se present in the soil is 0.01-2 mg Se/kg (Table 2.2), but selenium is present at different concentration depending on the total Se present in the soil (Kabata and Pendias 1992). But, at high concentration of Se in soil this relationship is not linear (Sun et al. 2007). Total Se concentration in soil do not give proper information about natural effect of Se due to separation of solid phases in the soil. The factors like pH, oxide, clay and calcium carbonate ($CaCO_3$) content plays an important role in spreading of Se in the soil (Wang et al. 2010).

Table 2.2 Presence of selenium in soil (Tan et al.1989).

Se concentration in soil (mg/kg)	Classification
<0.125	Insufficient
0.125-0.175	Negligible
0.175-3	Tolerable – High
>3	Unmeasurable
>5	Seleniferous

There is more adsorption of Se in uncultivated soil and in cultivated soil, mobility and transformation of Se is more because of competing anions. Addition of phosphate as a fertilizer helps to desorb the Se in the soil (Lessa et al. 2016).

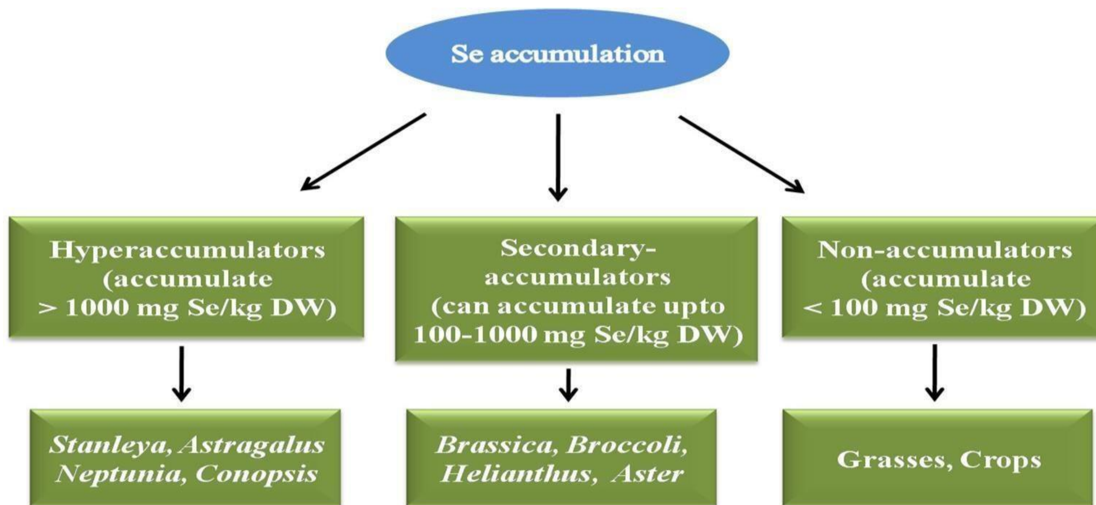


Fig 2.3 Selenium accumulation in soil depending upon its accumulation in plants (Mayland et al. 1989).

Accumulators are subdivided into primary and secondary accumulators. Difference between primary and secondary accumulator is Se concentration. Primary accumulators contain Se

concentration up to 1000 mg/kg, grow well on the soil. But in secondary accumulators, Se concentration ranges from 50-100 mg/kg (Mayland et al. 1989).

Example of secondary accumulators are *Grindelia Castilleja, Atriplex and Gutierrezia. Astragalus bisulcatus* and *Stanleya pinnata* (Quinn et al. 2010). These plants known as hyperaccumulator plants, containing Se concentration between 1000-15000 mg Se/kg. Nowadays, hyperaccumulating plants are used for bioremediation process. These plants are used for elimination of Se contamination from water, soil and sediments known as a phytoremediation (Bañuelos et al. 1995). The concentration of Se is more in grasses than leguminous plants. Vegetables like turnips, peas, beans, carrot, beets, potatoes and cucumber are high in Se concentration of 6000 mg Se/kg than fruits (Mehdi et al. 2013). Brazil nuts are higher in Se concentration (Navarro-Alarcon 2008). Plants like wheat, maize and mustard grow well on seleniferous soil and contains high availability of Se in the soil (Eiche et al. 2015).

2.4.4 Selenium transport and accumulation within the plants

Different transporters like sulfate and phosphate transporters of plants help in transport of selenate and selenite inside the plant. Selenate is transported through sulphate transporters by passive absorption in the plasma membrane of the plant roots and selenite is transported via phosphate transporters by active absorption (Sors et al. 2005). There is the difference in the Se transport in accumulators and non-accumulators. In accumulator plants, Se is transported to the leaves before accumulating in seeds and fruits and in non-accumulator plants accumulation is similar in roots, fruits and seeds (Terry et al. 2000). In, *Astralagus bisculcatus* concentration of Se is more in seeds than roots and leaves while in *Stanley pinnata*, seeds have higher Se concentration than roots (Freeman et al. 2012).

Pyrzynska et al (2009) suggested mechanisms like symplastic and apoplastic transport for transport of the Se within the cytoplasm of the plants. Selenate is transported via apoplastic mechanisms through inert transport by microcapillaries in the peripheral tissue of roots without infiltration of selenium compounds and is not transformed to other chemical forms. Due to this reason, selenate is more available to plants while selenite gets accumulated in the plant roots because it is not easily transported and changes to other chemical forms of Se easily. Absorption of selenite take place through apoplastic mechanism inside the cytoplasm and

transformed to selenide (Zayed et al. 1998). Se concentration increases in rice grain on an addition of selenate to the soil than selenite (Boldrin et al. 2013).

2.4.5 Biotransformation of Se in soil-plant environment

Researchers have conducted various experiment to show the evidence of Se transformation in plants. Results have shown that selenate is the main form of Se present in the plants after the harvesting. They contain a lesser amount of organic forms (Selenomethionine and selenocysteine) in plants parts (roots and shoots). This shows that inorganic forms are converted into organic forms in plant system (Funes-Collado et al. 2013). Various organic forms to which selenocysteine is transformed are selenomethionine (SeMet), methylselenocysteine (SeMeCys), and methylselenomethionine (MeSeMet) (Mehdi et al. 2013).

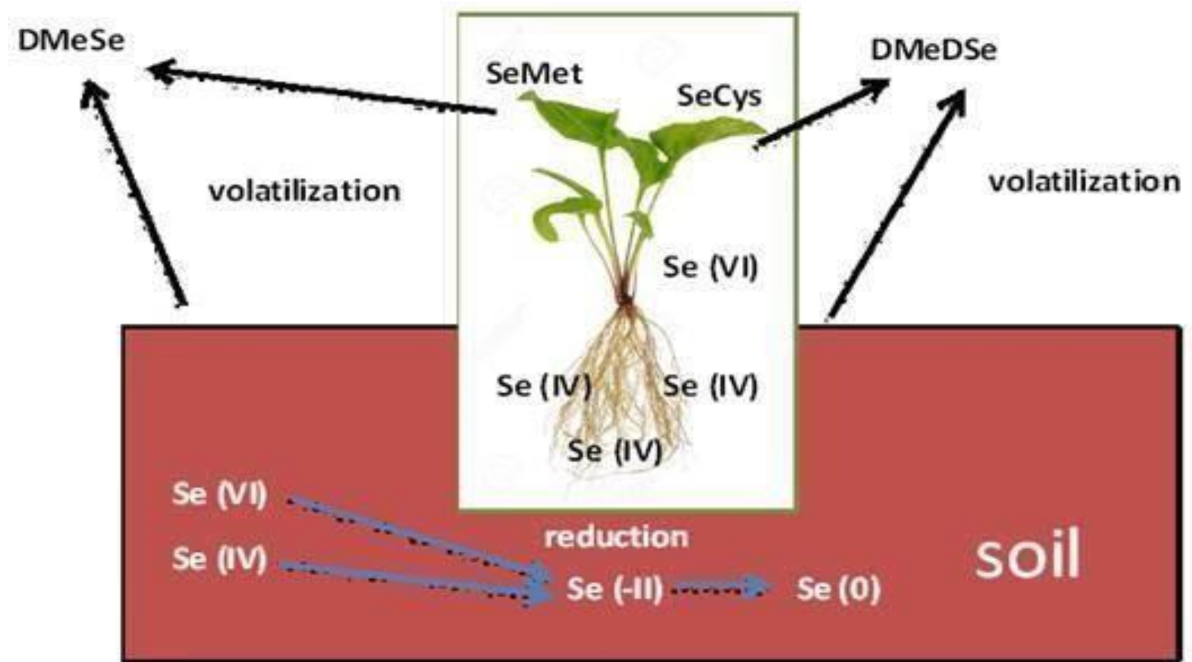


Fig 2.4 Selenium transformation in soil-plant system (selenomethionine; selenocysteine; dimethylselenide; dimethyldiselenide) (Mehdi et al. 2013).

Conversion of Selenocysteine to various organic Se species is known as a detoxification mechanism (Fig 2.4). Detoxification mechanism did not allow the adding of Se in the proteins. Hyperaccumulator plant *Astralagus bisculatus*, hyperaccumulator shows detoxification

mechanism by biotransformation of SeCys to SeMetCys, methylated form of Se organic species present in the young leaves of the plant. In, detoxification mechanism inorganic form is converted into organic form which is adapted by plants grown on the seleniferous soil and Se hyperaccumulators for survival (Pickering et al. 2003). Volatilization is a process which remove the Se from soil-plant system. The conversion of these methylated Se organic species (SeMeth and SeMeCys) into volatile selenide species (DMeSe or DMeDSe) is a detoxification process in plants (Dhillon et al. 2010).

2.5 Selenium in plants

2.5.1 Selenium uptake in plants

Classification of plant species in three main categories on the basis of Se present in their tissue are: non-accumulators, accumulators and hyperaccumulators. Non-accumulator plants contain Se ranging from of 5-50 mg/kg in plant tissue. Grains and grasses come under the category of non-accumulator plants.

2.5.2 Selenium metabolism in plants

Selenate is more available for uptake by the plant, than selenite form of Se because due to weak electrostatic attraction it is weakly attached to the soil surface. Selenate metabolism in plant system take place via sulphate assimilation pathway inside the chloroplast due to the similarity with the sulphur. Five steps are involved in the Sulphur (S) metabolism pathway for Se metabolism in plants. In first step inorganic Se is transformed into selenite form. This step is controlled by two enzymes: ATP sulphurlase (APS) and ATP reductase (APR). Hydrolysis reaction takes place in which ATP forms adenosine phosphoselenate controlled by APS which reduces to selenate inorganic form of Se (Sors et al. 2005). In next conversion selenite is converted into selenide form by enzyme sulphide reductase (Wallenberg et al. 2010). The third conversion involves coupling reaction with O-Acetyl serine enzyme in presence of cysteine synthase in which selenide is converted to SeCys. Cysteine synthase enzyme shows powerful attraction with S^{2-} than sulfide. In this step, SeCys form is converted to elemental Se (0) by SeCys lyase enzyme to methyl-SeCys by selenocysteine methyltransferases or SeMeth by various enzymes. In the last step, SeCys or SeMeth is converted DMSe or DMDSe (volatile Se

species) by methylation (Pilon and Quim 2010). End products of metabolic processes (Fig 2.5) depend on the accumulating plants species (Mehdi Y et al. 2013). In non Se accumulating plants selenomethionine (SeMeth) is converting to dimethylselenide (DMSe) by five steps

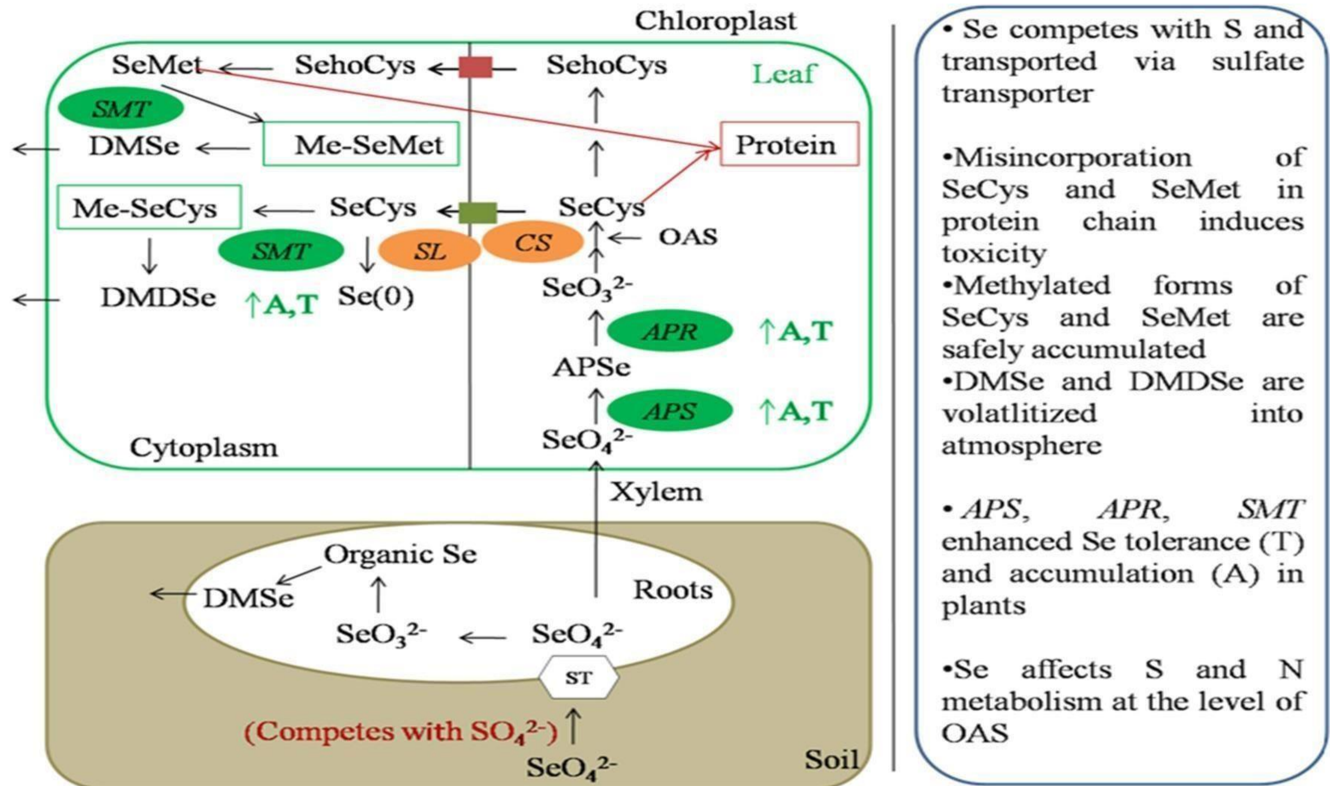


Fig 2.5 Schematic representation of Se metabolism in the plant cells ATP sulfurylase, APS reductase, Cysteine synthase, Selenocysteine lyase, Selenocysteine methyl transferase, A-Accumulation, T-tolerance (Pilon and Quim 2010).

Metabolism and in Se accumulating plants it is converted into dimethydiselenide (DMDSe) involving four steps (Pilon and Quim 2010). This metabolism process in plants acts as a detoxification mechanisms for Se toxic species. Incorporation of selenocysteine (SeCys) and selenomethionine (SeMeth) in proteins proves fatal for plants as it alters its protein structure and causes toxicity (Dhillon et al. 2010).

2.6 Microbial transformations in soil

A number of microorganisms are present in the soil. Soil microorganisms show various microbial or biological activities like methylation, reduction, oxidation and demethylation reactions which are controlled or mediated by microorganisms like bacteria and fungi (Dungan and Frankenberger 1999). Conversion selenite and selenate to nontoxic forms (elemental Se

and selenide) is done by a change in pH and redox potential. Retention of Se in soil is increased by microbial activities. Transformation of Se by microorganisms take place in both oxic and anoxic conditions (Darcheville et al. 2008). The transformation of chemical forms (selenate, selenite, elemental Se (0), selenide) into the volatile derivatives (dimethyldiselenide, dimethyl selenide, dimethylselenone and methaneselenol) in the atmosphere is carried out by microorganisms and roots. Between selenate and selenite, selenite is more available to plants and is water soluble (Zieve and Peterson 1984).

The soil is rich in various microorganisms like bacteria and fungi. These microorganisms help in selenium removal from soil, water and sediments by special mechanisms. Most of the Se bioremediation studies have been done on bacteria but researchers have found the contribution of fungi too in selenium bioremediation process. Recently researchers have exploited various filamentous fungi which play a key role in Se bioremediation process (Urik et al. 2014).

2.7 Filamentous fungi in selenium bioaccumulation

Filamentous fungi help in selenium immobilization. In, metal contaminated environments, free-living fungi undergo various metabolism processes like reduction, accumulation, bioalkylation, leaching process, demethylation and physiochemical interaction of metals with fungal surfaces and metabolites (Urik et al. 2014). Filamentous fungi are selected over other fungal species because they act as a biosorption agent and can accumulate the Se from liquid media. Conversion of organic and inorganic forms of Se to non-toxic forms and volatilized in the atmosphere. Fungal biomass uses biosorption mechanism for accumulation and degradation of toxic. Biosorption processes undergo various mechanisms like ion exchange, chelation, complexation, adsorption, ion entrapment and diffusion by cell walls and membranes (Volesky and Holan 1995). These fungi can survive at high pH, extreme conditions and a high concentration of metal. Various mechanisms shown by filamentous fungi for tolerance and detoxify the metal are explained as follows (Fig 2.6):

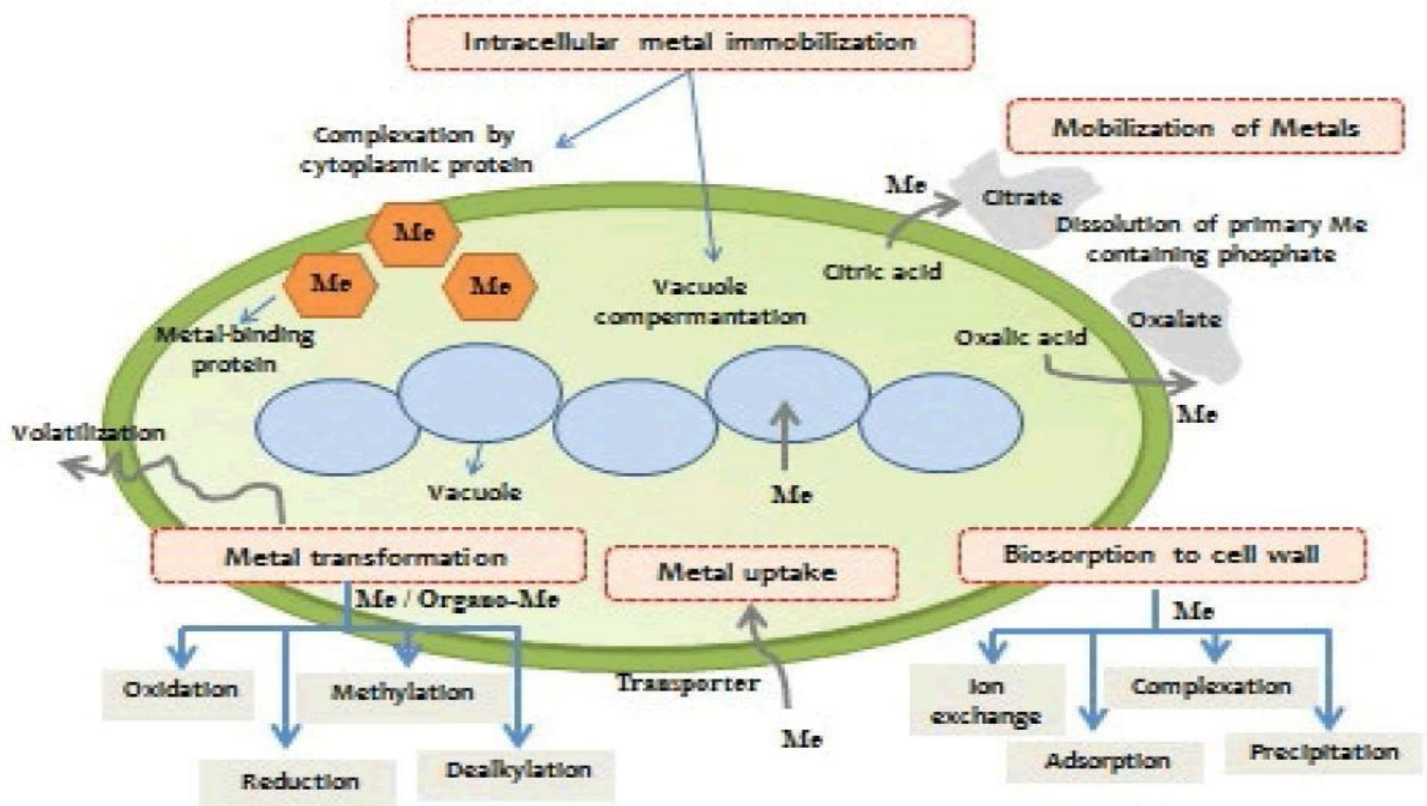


Fig 2.6 Fungi-metal interactions (Gadd 1993; Harms et al 2011)

- **Mobilization of metal:** Various organic acids like citric acid and oxalic acid are secreted out by the fungus. Citric acid chelates the metal ions and oxalic acid produce insoluble oxalate which helps in the dissolution of metal containing phosphate. Organic acids production help in metal dissolution by mycosphere acidification and metal complex structure formation (Gadd 1993).
- **Biosorption to cell wall:** The word biosorption means the accumulation of metalloid by biological material. This process involves various mechanisms like ion exchange, complexation, adsorption, crystallization and precipitation. Biomass concentration and type of metalloid shows a necessary role in this mechanism. The fungal cell wall first layer which comes in contact with metal it forms the protective layer and barrier which help in uptake an of metal inside the cell (Gadd 1993).

- **Metal uptake:** This mechanism involves the transporters like metabolically-coupled and H^+ transport system. These transporters react with metal for metal uptake (Gadd 1993).
- **Intracellular metal immobilization:** The phenomena is divided into two processes: vacuole compartmentation and complexation by cytoplasmic proteins known as a metallothionenis. These proteins maintain intracellular modulation by binding with the metal. Fungal vacuole shows various processes like storage of metal, detoxification of the metalloid and molecular degradation (Harms et al. 2011)
- **Metal transformations:** Biotransformation is a process in which toxic form of metalloid is converted to a less toxic form in form of volatile derivatives which is not harmful to the environment in form of volatile derivatives. It helps in reduction of metalloid toxicity by various reactions like oxidation, reduction, methylation and dealkylation. Also, distribution of the metalloid in fungal mycelium, vacuoles and vesicles (Harms et al. 2011).

2.7.1 Selenium reduction and tolerance by fungi

Microbial processes helps in Se distribution and its forms. Microorganisms help in removal of selenium toxicity from the polluted area by processes like volatilization and changing of Se to a non-toxic form of elemental Se (0). Microbes show detoxification mechanisms in which (SeO_6^{2-}) a form of Se changes to Se (0) by painter type reaction in which reaction takes place with a reactive thiol group (Kausch et al. 2012). Various microorganisms present in environment use selenite and selenate form of Se for growth and dissimilatory reduction in this non-toxic form of Se is produced by internal uptake or releases extracellularly in the medium. There is no formation of bigger crystals, spherical shape nanoparticles are formed known as an elemental Se (0) (Lenz et al. 2011).

Most of the research for Se is focused on bacteria, rather than fungi. But, many researchers have started shifting their focus on fungi. Many fungi show metalloid tolerance. Continuous exposure of environment (soil, water and sediments) to metalloids, become a source for isolation of tolerant microbial species.

(Rosenfeld et al. 2017) had screened six fungal species for Se tolerance at different Se concentration (0-10 mM). All six fungal species reduced selenite and selenate form of Se to elemental Se (0) by volatilization. All fungal species shows one detoxification mechanism. Removal of Se occurred in oxic environment. In another research, three fungal strains, *Aspergillus sp, Fusarium sp., Penicillium sp*. were isolated from seawater present near the industrial area contaminated with Se. Among isolated fungal isolates *Penicillium* could survive up to a higher concentration of 50 ppm of Se concentration (Jaya et al. 2013).

2.7.2 Selenium bioaccumulation and bio-volatilization by fungi

Bioaccumulation is a phenomenon in which there is active transformation of metal and metalloid in microbial cells. This causes a change in speciation, bioavailability, toxicity, mobility and volatility of metals. Bioaccumulation takes place only in living cells of microbes (Chojnacka 2010). Most of the bioaccumulation and biovolatization processes are observed in the metals like Cr, Zn, Cu and Cd, less information is available for metalloids like selenium (Se), antimony (An), mercury (Hg), tellurium (Te), aluminium (Al) for bioaccumulation by microbial biomass specially by filamentous fungi (Haegman et al. 2013) Filamentous fungi acts as an efficient metalloid bioaccumulators because it shows unique metabolic pathways which help in conversion of the metalloid to volatile derivatives (Rabb and Feldmam 2013).

Fatoki OS (1997) suggested that accumulation of metalloid by microbial cells take place by biomethylation process in which metal changes to volatile derivatives (alkylated or methylated). Biomethylation in living organisms takes place by direct linkage of metalloid by enzymatic transfer of methyl group. This involves series of oxidative and reductive reaction resulting in the formation of methylated products. The release of these methylated products in atmosphere is known as a bio-volatilization (Boriova et al. 2014) The authors had quantified the metalloids like arsenic (III), arsenic (V), selenium (IV), selenium (VI), antimony (III), antimony (V), tellurium(IV), mercury (II), thallium (I), bismuth (III) biotransformation and biovolatilization by using filamentous fungi named *Scopulariopsis brevicaulis*. The amount of metalloid biotransformed was analysed using the technique ICP AAS.

The decreasing order of bioaccumulation of metalloids at different concentrations was as follows:

Bi (III)> Te (IV)> Hg (II)> Se (IV)> Te (VI)> Sb (III)

The highest percentage of biovolatilization was found in mercury (II) 50% and selenium (IV) 46.5%. They suggested the removal of metalloids by *Scopulariopsis brevicaulis* can be used in bioremediation of contaminated sites.

Fungus named *Aspergillus clavatus* was investigated for bioaccumulation and biotransformation of sodium selenate form of Se from an aqueous medium. Sodium selenate was removed efficiently at lower concentration and at higher concentration it was biotransformed to volatile derivatives. They used inductively coupled plasma optical emission spectrometry for estimation of Se in fungal biomass after growing fungus for 14 days in medium supplemented with sodium selenate. *Aspergillus clavatus* bioaccumulate of sodium selenate up to 2.3 mg/g dry weight of fungal biomass and biovolatilized up to 2.8 mg/g. The fungus removed the Se even at a higher concentration of 89 mg/L from aqueous media by bioaccumulation. These filamentous fungi proved to be of great benefit for removal of sodium selenate from Se contaminated water (Urik et al. 2016).

2.8. Plant growth promoting activities by fungi

2.8.1. Indole acetic acid (IAA) production by fungi

Indole acetic acid is one of the most important auxins and produced from L-tryptophan metabolism by various plant growth promoting rhizobacteria. IAA helps the plants adjust the plants in changing environmental conditions. Various activities which are regulated by IAA in plants are: longer roots production, increased in lateral roots and root hairs, nutrient uptake. Various micoorganisms produces IAA habitat in the rhizosphere of plants (Datta and Basu 2000). Mwashasha (2013) isolated 120 pure fungal isolates and screened them for IAA production. Six isolates show 5% positivity for IAA production and provided great benefit in the growth of rice plants.

2.8.2 Phosphate solublization by fungi

Phosphorus is necessary macronutrient need for the plant growth. It is an important nutrient for agricultural aspects. It helps in N_2 fixation in the atmosphere. It is made available to the plants in form of phosphate fertilizers. Only 1% is used by the plant and rest changes to insoluble complexes like aluminium phosphate, calcium phosphate and iron phosphate. But, this method of fertilisation is costly and not environment friendly. There is a need for the development of cost-effective and environmentally friendly method. Nowadays rock phosphate is used as a fertilizer for phosphorus (Chuang et al. 2007). Various microorganisms like bacteria and fungi are reported which are capable of solubilizing the rock phosphate and provide soluble phosphorus to plants by releasing various organic acids by fungi like the production of enzymes, chelation of oxo acids from sugar and reduction of pH. These microorganisms are used as biofertilizers and named as phosphate solubilizing microorganisms (Xiao et al. 2008).

Reddy et al (2002) reported two fungal isolates named *Aspergillus tubingenesis* and *Aspergillus niger* with the ability to solubilize the rock phosphate. Among all the isolates *Aspergillus tubingenesis* showed maximum phosphate solubilization than all other isolates. Maximum solubilization was shown in the presence of 2% rock phosphate.

Among all the fungus species, filamentous fungi are the greatest producers of organic acids. Fungal species named *Aspergillus niger* and *Penicillium* have been tested for rock phosphate solubilization. These species play an important role in biocontrol, biodegradation and P fertilizers (Richa et al. 2007; Pandya et al. 2008). Microorganisms help in the solubilization of bound phosphate present in roots and make it available to plants (Gaur 1990). In one of study has found that *Aspergillus niger* and *Trichoderma harzianum* act as effective phosphate fertilizers for the growth of chickpea plant. These species act as a great biofertilizer for the growth of chickpea (*Cicer arietinum*) plant (Yadav et al. 2011).

2.8.3 Siderophore production by fungi

Siderophores are ferric specific ligands produced by microbes in low iron conditions. They have a low molecular weight (<10,000D). Siderophores absorb ferric ions from insoluble hydroxide the Fe bound to the soil surface in iron stress conditions. Complex formation takes place with both soluble and insoluble Fe compounds like ferric phosphate, ferritin, chelators (EDTA), flavone pigments (Winkelmann 2002). Chrome azurol S (CAS) dye is used for the detection of siderophore production in which ternary complex chrome azurol S/ iron (lII)/ hexadecyltrimethylammonium bromide used as an indicator for the siderophore production in microbes. There is a colour change from orange to blue when Fe is removed from the CAS dye by fungi (Schwyn and Neilands 1987). Fungi show siderophore production under oxic conditions during iron stress. There are three types of siderophores depending upon the colour produced. Hydroxamate type, carboxylate type and phenolate type. Mostly fungi produce hydroxamate-type of siderophore (Thieken et al. 1992; Fekete et al. 1989). Use of siderophore producing fungi helps in improving the iron deficiency, promotes plant growth and improves the economic value of crops. Some fungi prove beneficial for iron nutrition to non-leguminous plants. Fungal species named *P. chrysogenium* and *Rhizopus arrhizus* improves Fe nutrition in crops like cucumber, maize and tomatoes and protects the crops from plant pathogens (Berg et al. 2002; Wolfgang et al. 2000).

In another study, the authors have studied siderophore production by two fungal species named *Aspergillus niger* and *Aspergillus parasiticus* used as a Plant Growth Promoting Fungi (PGPF). *A. niger* shows 80% and *A. parasiticus* showed 73% siderophore units. Both the fungal isolates show maximum growth at pH-7.0. These fungal species improved the growth of mungbeans plant (Patel et al. 2017).

MATERIALS AND METHODS

Materials and Methods

3.1. Collection of soil sample

To isolate selenium (Se) tolerant fungal strains, the soil sample was collected from agricultural land affected by the elevated level of Se concentration. The rhizospheric soil of wheat (cereal crop) (Triticum aestivum) plant, grown in the agricultural land of Jainpur (31.13539N, 76.18536E) village situated in Nawanshahr, Punjab. The soil sample was drawn carefully up to 10 cm in depth and was stored in sterile plastic bags at 4℃ till not used for isolation of selenium tolerant fungal strains and rest of the soil was used for determining various physiochemical characteristics and selenium estimation.

3.2. Isolation of selenium tolerant fungi

Selenium tolerant fungi were isolated by serial dilution method on Czapek Dox agar plates.

Composition of Czapek's Dox agar medium:

Components	**g/L**
Glucose	30
Sodium nitrate	2
Dipotassium phosphate	1
Magnesium sulphate	0.500
Potassium phosphate	0.500
Ferrous sulphate	0.010
Final pH (at 25℃)	7.2
Agar	15.000

Czapek Dox media was prepared by replacing sucrose with glucose as a sole carbon source.

The components of czapek agar medium were added in 1000 ml double distilled water, pH adjusted to 7.2 and autoclaved for 15 minutes at 15 Psi. After, autoclaving the medium filter

sterilized sodium selenite (5 mM) and chloramphenicol (100 μl/ml) antibiotic was added. Poured in sterile petriplates in aseptic conditions.

3.2.1. Isolation of Selenium tolerant fungi from the seleniferous soil by serial dilution method

- For isolation of Se tolerant fungi, 10 g soil sample was added in 100 ml normal saline solution (0.85% NaCl in distilled water) in each test tube and mixed thoroughly for 5-10 minutes.
- Dilutions of starter culture were made up to 1×10^{-3} in test tubes containing 9 ml of sterile physiological normal saline.
- From each dilution, after proper vortexing 100 μl culture was taken and spread on czapek dox agar plates amended with sodium selenite (5 mM) and chloramphenicol (100 μl/ml).
- Plates were incubated for 21 days and were examined every 2-3 days for fungal growth.

3.3. Subculturing of isolated Se tolerant fungi

Subculturing was done for isolation of pure strains from the mixed culture obtained.

Procedure:

- Fungal disc of 5 mm in diameter was cut from the periphery of actively growing fungi which were visibly different from each other and subcultured on fresh plates.
- Colonies grown on czapek dox agar plate amended with sodium selenite (5 mM) was maintained by repeating subculturing on czapek dox agar plates and stored at 4˚C.

3.4. Determination of selenium tolerance level of pure fungal strains

The tolerance level of pure fungal isolates was checked at different concentrations of sodium selenite (10-100 mM) in Modified Melin-Norkrane medium.

Composition of Modified Melin- Norkrane medium

Components	**Stock (100 ml)**	**g/L**
Potassium hydrogen phosphate	10g	5 ml
Ammonium hydrogen phosphate	5g	5 ml
Magnesium sulphate	3g	5 ml
Calcium chloride	1g	5 ml
Sodium chloride	0.5g	5 ml
Ferric chloride	1g	1.2 ml
Thiamine	100mg	1.0 ml
Micronutrients	10ml	10 ml
Glucose	2.5 g	2.5 g

3.4.1. Selenium tolerance level of fungal isolates:

- Purified prominent fungal isolates were screened for their selenium tolerance.
- 2-3 fungal discs of diameter 5mm was inoculated in a 250 ml flask containing 50ml czapek dox broth supplemented with different concentration of sodium selenite (10-100 mM) with the interval of 10 mM.
- Flasks were kept on a rotary shaker (130 rpm) at 25℃ for 14 days.
- After 14 days fungal mycelium was filtered through Whatmann filter paper.
- Fungal mycelium was dried in a hot air oven.
- Mycelium was scraped with help of forceps and placed on aluminium foil.
- Fungal biomass was weighed at different concentrations of sodium selenite.
- The highly tolerant fungal isolate was determined by the weight of fungal biomass against sodium selenite concentration.
- Tolerance was checked up to concentration at which there was no mycelium growth.
- As the concentration of sodium selenite increases the mycelium growth decreases.
- Fungal strains tolerant at higher concentration of sodium selenite and showing good mycelium growth was labelled as a highly Se tolerant fungal strain.
- From all the isolates two Se highly tolerant fungal strains were selected for further screening.
- The experiment should be repeated in triplicates.

- The dry weight of fungus biomass was calculated using following formulae:

Dry weight of fungus biomass (g) = (weight of aluminium foil + mycelium) – (weight of aluminium foil)

- The standard curve was prepared using data obtained.

3.5. Plant growth promoting activities of highly tolerant fungal isolates

3.5.1. Quantification of Indole Acetic Acid

Quantitative analysis of Indole acetic acid was done according to by Brick et al. (1991).

Composition of Czapek's dox broth

Components	g/L
Glucose	30
Dipotassium phosphate	2
Magnesium sulphate	1
Potassium phosphate	0.500
Ferrous sulphate	0.500
Final pH (at 25℃)	7.2

Czapek dox broth for IAA quantification was prepared by replacing sodium nitrate and Ltryptophan (1000 µg/ml) was added in its placed.

Reagents

1. L-Tryptophan (1000 µg/ml)
2. Salkowski's reagent (2% 0.5 M $FeCl_3$ in 35% perchloric acid).

Procedure

- The fungi was grown in a czapek-dox broth amended with 1000 µg/ml L-tryptophan.
- For each fungi, 3 czapek dox agar disks about 5 mm of actively growing mycelium were inoculated in czapek-dox broth and incubated at 30℃ for 3 days.
- After 3 days, the culture was centrifuged at 8000 rpm for 10 minutes.

- 1 ml supernatant was taken in a test tube to which 2 ml salkowski's reagent was added, kept in dark room for 30 minutes.
- Absorbance was noted at 540 nm using spectrophotometer after pink colour was obtained.
- To prepare a standard curve of 5, 10, 50 and 100 μg/ml from IAA stock solution (Gordon & Weber 1951), added 2ml of salkowski's reagent and stored in dark for 30 minutes. Absorbance was measured at 540 nm after the formation of pink colour took place.
- The concentration of unknown sample was drawn from the standard curve by making regression equation.
- The experiment was carried out in three replicates.

3.5.2. Qualitative estimation of phosphate solubilization by fungi

Method for qualitative estimation of phosphate solubilization was done on Pikovskaya medium containing insoluble tricalcium phosphate suggested by (Pikovskaya 1948).

Composition of Pikovskaya agar medium:

Composition	**g/L**
Glucose	2.5
Ammonium sulphate	13
Sodium chloride	0.2
Magnesium sulphate	0.1
Potassium chloride	0.2
Yeast extract	0.5
Manganese sulphate (trace)	0.0001
Ferrous sulphate	0.0001
Agar	15
pH	7.2

Procedure:

- Czapek dox agar disks covered with actively grown fungal mycelium was placed in the centre of Pikovskaya agar plates.
- Plates were incubated at 25˚C for 3-6 days.
- Colonies forming halo zone around them indicated the phosphate solubilization.
- Then phosphate solubilization index (SI) was calculated using following formulae (Premono et al. 1996):

$$\textbf{Solubilization index (SI)} = \frac{\text{Colony diameter} + \text{Halozone diameter}}{\text{Colony diameter}}$$

3.5.3. HCN production by fungi

Determination of HCN production by fungi was one according to (Bakker and Schippers 1987).

Reagents

- 2% (w/v) of sodium carbonate
- 0.5 % (w/v) of picric acid
- 4.4 g/l glycine

Procedure

- Inoculation of fungal culture was done on czapek dox agar plates amended with 4.4 g/l glycine.
- Whatmann filter paper was dipped in 2 % w/v sodium carbonate in 0.5 % (w/v) picric acid solution and was placed inside the lid of the petriplates.
- Plates were sealed with parafilm and incubated at 25℃ for 3 days. Colour changes of the filter paper from yellow to brownish indicates the HCN production.

3.5.4. Siderophore production by fungi

Detection of siderophore production in fungal isolates was checked by using Chrome-Azurol 'S' (CAS) Agar medium (Schwnyn and Neilands. 1987).

Reagents

- **Preparation CAS dye**
  - 60.5 mg of Chrome-Azurol 'S' dye was added to 50 ml of distilled water and constantly mixed 1mM $FeCL_3$. $6H_20$ solution prepared in 10 ml of 10mM HCL. It results in dark reddish colour.
  - 72.9 mg Cetyl trimethyl ammonium bromide (CTAB) was dissolved in 40 ml of distilled water and slowly mixed in CAS dye solution with constant stirring before autoclave. This results in dark blue colour.
- **Preparation of CAS agar medium**
  - Mix 100 ml CAS dye was mixed in 300 ml czapek dox broth, before pouring pH was checked if pH was below 7.0 it was maintained with NaOH (autoclaved).

Procedure

- 5 mm diameter discs of fungal isolates were spot inoculated in the centre of CAS dye agar plates.
- Plates were incubated at 28℃ for 14 days.
- Isolates showing blue/wine halo zones were considered as siderophore producers.
- In a similar way siderophore was checked in czapek dox broth.
- After, 14 days of culture broth was centrifuged at 10,000 rpm for 15 minutes.
- 1.5 ml culture supernatant was taken and 1.5 ml CAS dye was added.
- Blank for the sample was taken uninoculated broth.
- Reference was taken which contains 1.5 ml uninoculated broth and 1.5 ml CAS dye used against the supernatant culture.
- Absorbance was noted at 630 nm on the spectrophotometer.
- Siderophore content in broth was calculated by using following formulae (Payne 1994):

$$\textbf{\% Siderophore units} = \frac{Ar - As}{Ar} \times 100$$

Where

Ar = Absorbance of reference at 630 nm (CAS uninoculated broth & CAS dye)
As = Absorbance of the sample at 630 nm.

3.5.5. Production of ammonia by fungi

Ammonia production in fungal isolates was according to (Cappuccino and Sherman 2005).

Composition of peptone water:

Components	g/L
Peptone	10
NaCl	5
pH	7.2

Procedure:

- 15 ml peptone water was added in a 50 ml test tube.
- Test tubes were autoclaved at 121℃ for 15 minutes.
- Freshly grown fungal discs were inoculated in peptone water.
- Then, test tubes were incubated at 28℃ for 48-72 hours.
- After incubation 0.5 ml nesseler's reagent was added, brown to yellow colour showed positive for ammonia production.

3.6. Effect of fungi on plant growth promotion

3.6.1. Field experiment

To check the effect of isolated selenium tolerant fungal strains on crop production, field experiment of *Zea mays* (maize crop) was carried at TIFAC Core, Thapar Institute of Engineering and Technology, Patiala, Punjab. This site is situated at 30.30°N latitude and 76.38°E longitude. This site present under the agroclimatic region of Punjab. The soil used in the experiment was brought from seleniferous region of Jainpur village of Nawanshahr region of Punjab having sandy loamy texture.

3.6.2. Inoculation of seeds

Seeds of selenium tolerant fungal isolates were inoculated in slurry containing 10% sugar and 40% guar gum and seeds were dipped to form uniform coating of fungal inoculum around the seeds. The seeds coated with slurry were dried overnight before they were ready to sow.

3.6.2.1. Methodology for fungal inoculum and seed preparation

- Selenium tolerant fungal isolates were grown on czapek dox agar plates, incubated at 25℃ for 21 days.
- Fungal growth was scraped from plates by using scalpel and suspended in sterile distilled water.
- Then, 50 g sugar was added in 500 ml beaker and solution was heated for 15 minutes.
- 200g of guar gum was added in hot sugar solution, mixture was allowed to cool to room temperature.
- Surface sterilization of coated seeds was done by treating seeds with 95% ethanol for 3 minutes followed by 3 % sodium hypochlorite for 5 minutes, then 5 times washing was done with sterile distilled water.
- Surface sterilized seeds were added in slurry containing fungal inoculum for 5 minutes to make a uniform coating around the seeds.
- Seeds coated with 40% gum arabic and 10% sugar was served as a control.
- Seeds coated with slurry was dried overnight before using them for sowing.

3.6.2.2. Experimental field preparation and cultivation of plants

Field trials were conducted at Core, Thapar Institute of Engineering and Technology. In field trails, plastic cups were used and 3 treatment with 10 replicates.

Treatment consisted of:

- Soil;
- Soil + SeF_5 (Se tolerant fungal isolate no.1).
- Soil + SeF_{12} (Se tolerant fungal isolate no. 2).

Maize variety was cultivated in seleniferous soil in the summer season. All cups were irrigated regularly.

3.6.2.3. Analysis of plants after harvesting

After 2 months of harvesting, various parameters were measured: root and shoot length, dry weight of shoot and root was measured and recorded.

3.7. Estimation of selenium bioaccumulation

Selenium bioccumulation by fungal isolates was checked by using fluorescence spectrometry (Perkin Elmer).

Procedure

- Fungal isolates were inoculated in czapek dox broth containing sodium selenite concentration from 20-100 mM.
- Flasks were incubated at 25℃ for 14 days on incubator shaker.
- After 14 days, the broth containing fungal mycelium filtered on sterile filter paper and shifted on preweighed aluminium foil.
- The fungal mycelium in aluminium foil was dried overnight at 60℃ in hot air oven.
- Then, selenium accumulation was estimated by fluorescence spectrometry by taking 100 mg fungal mycelium grow at 20-100 mM sodium selenite concentration and crushed maize plant tissue (roots, shoots).
- Free selenium which was not accumulated by fungal isolates was also estimated by taking 100 µl supernatant.
- Gives the exact concentration of selenium accumulated by fungal isolates.

3.7.1. Estimation of selenium (Se) in fungal mycelium/roots/shoots

Selenium in fungal mycelium, roots and shoots was estimated by using fluorescence spectrometry (Perkin Elmer).

Reagents

- **Fungal mycelium (10 mg); roots and shoots (200 mg)**
- **HNO_3 + $HCLO_4$ (3:1)**
- **6N HCL**
- **Double distilled water**
- **MQ water (pH- 1.8 made with HCL)**

- **MQ water**
- **DAN (2,3-diamino naphthalene) solution- 0.1% in 0.1 N HCL**
  - 100 ml of 0.1 N HCl was taken in brown color bottle covered with aluminium foil.
  - Added 100 mg of DAN (powder form) and 500 mg hydroxylaminehydrochloride to it and mixed thoroughly by shaking.
  - Then bottle was placed in water bath at 50℃ for 30 minutes.
  - Poured crude mixture in a properly washed separating funnel.
  - Cyclohexane was added at the top of the crude DAN solution in the separating funnel.
  - Shaked properly, allowed aqueous and cyclohexane layers to separate properly and settled.
  - All the Dan dye was collected in aqueous layer (downward layer).
  - DAN dye was collected in the bottle, upper layer of cyclohexane was discarded.
  - Again fresh cyclohexane was added to the collected DAN (added to the separating funnel).
  - Shaking step was repeated again and collecting 3-4 times till cyclohexane layer came out to be clear from brown.
  - Extracted DAN solution was collected in white plastic bottle or glass bottle covered with alunimium foil, stored at 4°C.
- **Stabilising solution**
  - Take 40 ml double distilled water, 10 g hydroxylamine hydrochloride powder was added to it mixed properly to dissolve.

- Added 4 ml of 1M EDTA solution and volume was make up to 100 ml by double distilled water.

- **Teflon tubes**
- **Microprocessor digestor (Mars)**
- **Fluorescence spectrometer (Perkin Elmer)**

Procedure

- 100 mg/0.1 g liquid N_2 crushed fungal tissue/roots/plant tissues (fine powder form). Tubes were kept in microprocessor digestor by tightening the tubes using tightner and placed in the rods.
- The mixture was digested for 30 minutes at 200℃.
- After digestion teflon tubes were cooled and 5 ml 6N HCl was added carefully.
- Teflon tubes was again tightened and replaced in the digestor. Digestion was performed at 150℃ for 15 minutes.
- After completion of digestion, volume was made up to 50 ml with distilled water.
- The solution was mixed gently by inversion and allowed the debris to settle down.
- Four glass tubes were taken per sample.
- In each test tubes 10 ml of HCl (pH-1.8), 500µl-1 ml of digested sample and 100 µl of stabilizing solution was added.
- Tubes were vortexed for 1 minute and placed at 50̊ C in a water for 10 minutes.
- Standard stock solution (Table 3.1) of sodium selenate (100 ppb=100ng/ml) was made. It was used to make standard sodium selenite solutions ranging from 10ng-50ng as follows:

Table 3.1 Standards of known selenium concentrations

Standard(ng/ml)	Stock solution(µl)	MQ. Water (pH-1.8)µl
10	300	2700
20	600	2400
30	900	2100
40	1200	1800
50	1500	1500

- 100 µl of stabilizing solution was added to tubes containing standards and again step 11 was repeated.
- All the test and standard's tubes were cooled down and 500µl of DAN was added to each tube. Tubes were votexed for 30 seconds.
- Tubes were again placed at 30˚C for half an hour.
- Tubes were cooled down and 3 ml of cyclohexane was added in each tube carefully keeping the cyclohexane volume similar in each tube.
- Each tube was vortexed for 1-2 minutes properly.
- The upper cyclohexane layer in which all the selenium (in the form of piazselenol) gets extracted, was taken in cuvette to check the emission spectra at 520 nm using fluorescence spectrometer.
- Regression coefficient was calculated and a calibration curve was drawn using the emission spectra of standards (10-50 ng sodium selenite)
- The concentration of selenium present in the samples was calculated against the absorbane using the regression equation.
- For each sample, the test was performed in 4 replicates.

3.8. Morphological characterization of highly Se tolerant fungal isolates

To study the morphology of fungal isolates, lactophenol cotton blue was used for staining.

Procedure:

- Fungal mycelium was picked from periphery of actively growing mycelium with help of sterile needle.
- Placed over the clean slide and 1-2 drop of lactophenol cotton blue was added.
- Identification of fungal isolates was done by visualizing inception by bright field microscope under 40X magnification (Aneja 2006).

3.9. Molecular methods for identification of highly Se tolerant fungal isolates

3.9.1. Isolation of genomic DNA from Se tolerant fungal isolates (Allen et al. 2006)

Table 3.2 Components of extraction buffer

Components	Stock concentration	Working concentration
Tris- HCL (pH-8.0)	1M	100 ml
NaCl	5M	280 ml
EDTA	0.5 M	40 ml
CTAB	5g	20 g
Final volume with double distilled water		1000 ml

Procedure:

- Fungal isolates were grown in the czapek dox broth and incubated at 25℃ for 14 days.
- After 14 days mycelium was filtered through muslin cloth, medium was removed from the mycelium.
- Mycelia was air dried in laminar air flow and stored at -80℃ for further use.
- Mycelium was crushed with help of pestle mortar using liquid nitrogen.
- Rapidly transferred the powdered mycelia in sterile eppendorfs and stored at -80℃.
- About 600µl preheated (65℃) extraction buffer was added to 100 mg liquid N_2 crushed mycelia and vortexed for 1 min to mix thoroughly.
- The eppendrofs were incubated at 65℃ for 1 hour in a water bath and were inverted after every 10 min.
- Centrifuged at 13500g for 15 min at 4℃ to remove the non-soluble debris.
- The, supernatant obtained after centrifugation was transferred in the 2 ml eppendorfs.
- RNase treatment was given by adding 3µl RNAases in supernatant and incubation at 37℃ for ½ hour.
- 800 µl of phenol:chloroform:isoamyl alcohol was added to the supernatant. Mixed gently by inverting the tube a few times while keeping at room temperature.
- The mixture was centrifuged the mixture at 13500 g for 10 minutes. Carefully transferred the upper aqueous layer in fresh tube without disturbing the interface layer.

- Polyvinyl propylene was added after this step and kept for 15 minutes in case of dark tissue followed by chloroform isoamyl alcohol extraction step.
- If aqueous layer obtained was fuzzy, chloroform: isoamyl alcohol step was repeated till clear aqueous layer was obtained.
- 800µl of chilled isopropanol was taken in 1.5 ml eppendorf tube and stored previously at -20℃ for 20 minutes.
- Clear aqueous layer was added to chilled isopropanol to precipitate the DNA**.**
- Tubes were centrifuge at 13500 g for 15 minutes.
- In this step supernatant was decant while keeping look at the pellet (Pellet can white or invisible at this step).
- The pellet was resuspended 3M sodium acetate by gently flicking with fingers. It would take time, but ultimately the pellet would disappear.
- Tubes were incubated at 4℃, overnight.
- 600 µl of prechilled (-20℃) ethanol was added and incubated at -20℃ for 1 hour to precipitate the DNA (white tiny ppts are sometimes visible).
- Then, mixture was centrifuged at 13500 g for 20 minutes at 4℃.
- Carefully removed the supernatant by decanting and 500 µl of chilled (-20℃) 70% ethanol was added.
- Pellet was dislodged by quick 2 seconds vortexing or by flicking with fingers.
- Centrifuged at 13500 g for 10 minutes in a cooling centrifuge at 4℃ and removed the 70% ethanol supernatant without disturbing the pellet.
- Air dry the pellet at room temperature.
- Resuspended the pellet in 25 µl-30 µl of sterile MQ water.
- Isolated genomic DNA sample was stored at -20℃ for further use.

3.9.2. Qualitative analysis of isolated DNA

Qualitative analysis of isolated DNA was performed by agarose gel electrophoresis.

Reagents: 0.5X TBE buffer, agarose gel (1%), DNA (100 ng) sample, loading dye, DNA ladder, ethidium bromide staining solution, gel doc, gel tray, cast tray, power pac,

electrophoresis unit (0.5 V/cm) glass flasks, measuring cylinder, gel doc, micropipettes, sterilized tips.

Procedure:

- 100 ng DNA sample was loaded on 1% agarose gel prepared in 40 ml TBE (0.5X) buffer, ethidium bromide was added in the gel.
- After the gel solidified, DNA sample was loaded with 6X loading dye.
- Nucleic acids were electrophoresed at 50 volts (3volts/cm) for 60 minutes.
- Gel was visualized on U.V transilluminator.

3.9.3. Quantitative analysis of isolated DNA

Quantitative analysis was done by using a Nanodrop 1000 spectrophotometer (Thermo scientific, Wilmington, DE). Quantity of DNA sample was evaluated by measurement of A260/280 ratio. Ideally, A260/A280 ratio should be in range of 1.8-2.0. Ratios less than 1.8 indicates protein or phenol contamination, while ratios greater than 2.0 indicate the presence of RNA.

3.9.4. Amplification of ITS region by PCR (polymerase chain reaction):

3.9.4.1 Steps involved in Ampilification of ITS region

Materials used: Eppendorf, DNA samples (template), Deoxynucleotide triphosphates (dNTPS), 10X Buffer, DNA polymerase (Taq polymerase), Magnesium chloride (Fermentas), Forward and Reverse primer, MQ sterile water, PCR tubes, Micropipettes, Sterilized tips.

Procedure:

- Fragment spanning ITS-1 and ITS -2 region of the rRNA gene from the genomic DNA were amplified by using the PCR primers ITS1 (5'- TCC GTA GGT GAA CCT) GCG G- 3' and ITS4 (5'-TCC GCT TAT TGA TAT GC-3') as describe by (White et al.1990).

Table 3.3 Reaction mixture for PCR amplification

Components	**Concentrations**
Buffer	10X
DNA	40 ng
$MgCl_2$	1.5 mM
Forward primer	0.5 mM
Reverse primer	0.5 mM
Taq polymerase (Fermentas, USA)	2.5 units

- Amplification were performed in GenAmp 2700 thermocycler (Appiled biosystem USA).
- Controls containing no DNA template was included in every amplification to test for the presence of contamination of reagents and buffer.
- Aliquots (6µl) of amplification products were electrophoresed in 1% agarose gel and visualized on a UV transilluminator.

Table 3.4 Conditions of PCR reactions for 35 cycles

PCR Conditions	**Temperature(℃)**
Initial denaturation	94 ℃ (1 min)
Denaturation	94 ℃ (1 min)
Annealing	50 ℃ (1 min)
Elongation	72 ℃ (1: 30 min)
Final elongation	72 ℃ (8 min)
Dwelling temperature	4 ℃

35 cycles

3.9.4.2. Purification of PCR products

PCR products were purified by agarose gel (0.8%) electrophoresis prior to cloning. Purification was a done to obtain the PCR product on basis of band size. Besides removing surplus primers, nucleotides and salts. Gel extraction kit method possessed the advantage that incomplete (shorter) amplification fragment was removed prior to the cloning. QIAquick gel

extraction kit (Qiagen Inc., USA) used as per manufacturer's instructions. Purified PCR products were suspended in 30 µl sterile MQ water and used for TA cloning.

3.9.4.3. Ligation of ITS in TA cloning vector pMD20T

The ITS region containing PCR purified products were cloned using the restriction independent cloning kit, followed by manufacturer's protocol. The ITS amplicon was ligated into pMD20T vector. The reaction mixture was kept in chiller at -16℃ for 2 hours and incubated at 4℃ overnight.

Table 3.5 Reaction mixture for ligation of ITS region

Components	**Concentration**
Plasmid (pMD20T)	1 µl
Insert(SeF_5 (56.8ng/ml), SeF_{12} (49.1 ng/ml)	2 µl
Mighty mix	5 µl
MQ water	2 µl

3.9.4.4. Preparation of competent cells

- Inoculated 100 µl of DH10β cells in 20 ml Luria broth and incubated it for 20 hours at 37℃ with vigorous shaking.
- Aseptically transferred 1% of the above saturated culture into a fresh 20 ml LB and incubated at 37ºC for 2-3 hours with shaking.
- O.D. was taken at 600 nm which should be 0.5.
- Transferred the culture to a sterile ice cold 50 ml falcon.
- Placed the falcons on ice for 10 minutes. Recovered the cells by centrifugation at 5000 rpm for 10 minutes at 4ºC.
- Decanted the media and allowed it to stand for 1 minute.
- Resuspended the cells in 1 ml ice cold 0.1 M $CaCl_2$.
- 2-3 times washing was done with 0.1 M $CaCl_2$.
- The cells were be stored on ice for 24 hours.
- Transferred 100 µl of suspension of competent cells was added in 80% glycerol to a sterile and pre-chilled eppendorfs.

3.9.4.5. Blue- white screening of recombinant plasmid

- Added the cloned product in 100µl of E.coli competent cells including negative control with no plasmid and positive control with insert.
- Mixed the contents of the eppendorfs gently.
- Stored the eppendorfs on ice for 30 minutes for binding of the plasmids.
- Incubated the eppendorfs in water bath set at 42℃ for 2 minutes for heat shock.
- Rapidly transferred the eppendorfs on ice for 2 minutes.
- Added 1 ml of LB and incubated at 37˚C for 45 -60 minutes to allow bacteria to recover and express antibiotic resistance marker encoded by plasmid.
- Spread 100 µl of transformed cells on L.A. and ampicillin (100 µg/ml) plates which were spread with 40 µl of isopropyl beta-D-thiogalactopyranoside (IPTG) and 40 µl 5-bromo-4-chloro-3-indoyl β-D galactosidase (X-gal) and incubated it for overnight at 37℃.
- Colonies appeared after 20 hours.
- Two types of colonies appeared: blue and white.
- White colonies indicated transformed and recombinant colonies with gene of interest while blue colonies indicated the untransformed ones.

3.9.4.6. Colony PCR

- White colonies were picked and the colony PCR was performed using M13 primers M13 F 5'-GGTTTCCCAGTCACGAC-3' and M13 R 5'-GGAAACAGCTATGACCATG-3'.
- The reaction mixture was made and heated at 98℃ for allowing the lysis of cells.

Table 3.6 Reaction mixture of colony PCR for 35 cycles

Components	Concentration
M.Q water	6 µl
Buffer	2 µl
DNTPs	1.5 µl
MgCl2	1 µl
Forward primer	1 µl
Reverse primer	1 µl
Template	3 µl

Table 3.7 PCR conditions for colony PCR

Components	Temperature(℃)
Initial denaturation	92℃ (2 minutes)
Denaturation	92℃ (1 minutes)
Annealing	50℃ (30 seconds)
Elongation	72℃ (2 minutes)
Final elongation	72℃ (7 minutes)
Dwelling temperature	4℃

35 cycles

3.9.4.7. Plasmid isolation (alkali lysis method)

Reagents

- **Solution I** = 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl**.**
- **Solution II** = 0.2 M NaOH, 1% sodium dodecyl sulphate (SDS) should be freshly prepared.
- **Solution III** = 60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid, 28.5 ml of distilled water for 100 ml solution III (pH- 5.8).
- **Phenol: Chloroform: Isoamyl alcohol (25:24:1)**

Procedure

- Prepared 10 ml L.A. in a test tube and autoclaved it.
- Allowed the media to cool and added ampicillin (100 µg/ml) to the media.
- Inoculated a loopful bacterial colony and incubated at 37°C for 24 hours.
- Took 5 ml of culture and centrifuged at 8000 rpm for 5 minutes to obtain pellet.
- Added 200µl of ice-cold solution I followed by vortexing it (to ensure that bacterial pellet was completely dispersed in the solution). Placed it at room temperature for 3 minutes.
- Added 400 µl of freshly prepared solution II and mix by inversion.
- Kept the tubes on ice for 10 minutes.
- Added 300 µl of ice-cold solution III and mix by inversion.
- Placed the tubes on ice for 10 minutes.

- Centrifuged the contents at 12000 rpm for 10 minutes at 4℃.
- Transferred the supernatant to a fresh tube and added equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). Centrifuged at 10,000 rpm for 20 minutes.
- Aqueous layer was transferred to a fresh tube and equal volume of isopropanol was added to it.
- Gently inverted the tubes a few times and allowed them to stand for 10 minutes at room temperature.
- Centrifuged the contents 8000 rpm for 10 minutes and discarded the supernatant.
- Added 300 µl of 70% ethanol to the pellet and centrifuged it at 8000 rpm for 5 minutes.
- Decant the supernatant and allowed the pellet to air-dry.
- Suspended the pellet in 30 µl of MQ water.

3.9.4.8 Sequencing of ITS region

The fragments of ITS-5.8S-ITS2 amplified by PCR were sequenced according to Sanger et al. (1977) using an automated DNA sequencer (DNA sequencing Facility, Department of Biochemistry, South Campus, Delhi University, New Delhi, India).

3.9.4.9. Phylogenetic relatedness

The ITS region gene sequences of isolates were compared with those available in NCBI/GenBank/EMBL databases using "BLAST N" program. The strains with closely related sequences were aligned by using CLUSTALW program. Phylogenetic tree was constructed by maximum parsimony by using MEGA-5 program.

3.10. Estimation of selenium (Se) in soil sample

Selenium in soil sample was estimated by using fluorescence spectrometry (Perkin Elmer).

Reagents

- **Soil sample – 1g**
- **HNO_3 + $HCLO_4$ (3:1)**
- **6N HCL**
- **Double distilled water**
- **MQ water (pH- 1.8 made with HCL)**

- **MQ water**
- **DAN (dye solution)- 0.1% in 0.1 N HCL**
  - 100 ml of 0.1 N HCl was taken in brown color bottle covered with aluminium foil.
  - Added 100 mg of DAN (powder form) and 500 mg hydroxylaminehydrochloride to it and mixed thoroughly by shaking.
  - Then bottle was placed in water bath at 50°C for 30 minutes.
  - Poured crude mixture in a properly washed and rinsed with double distilled water by separating funnel.
  - Cyclohexane was added at the top of the bottle.
  - Shaked properly, allowed two layers to separate properly and settled.
  - All the Dan dye was collected in aqueous layer (downward layer).
  - DAN dye was collected in the bottle, upper layer of cyclohexane was discarded.
  - Again fresh cyclohexane was added to the collected DAN (added to the separating funnel).
  - Shaking step was repeated again and collecting 3-4 times till cyclohexane layer came out to be clear from brown.
  - Extracted DAN solution was collected in white plastic bottle or glass bottle covered with alunimium foil, stored at 4°C.
- **Stabilising solution**
  - Take 40 ml double distilled water, 10 g hydroxylamine hydrochloride powder was added to it mixed properly to dissolve.

- Added 4 ml of 1M EDTA solution and volume was make up to 100 ml by double distilled water.

- **Teflon tubes**
- **Microprocessor digestor**
- **Fluorescence spectrometer**

Procedure

- 1g soil sample was taken in teflon tubes, 3ml of HNO_3+ $HClO_4$ (3:1) was added to the tubes.
- Tubes were kept in microprocessor digestor by tightening the tubes using tightner and placed in the rods.
- The mixture was digested for 30 minutes at 200℃.
- After digestion teflon tubes were cooled and 5 ml 6N HCl was added carefully.
- Teflon tubes was again tightened and replaced in the digestor. Digestion was performed at 150℃ for 15 minutes.
- After completion of digestion, volume was made up to 50 ml with distilled water.
- The solution was mixed gently by inversion and allowed the debris to settle down.
- Four glass tubes were taken per sample.
- In each test tubes 5-10 ml of HCl (pH-1.8), 500 µl of digested sample and 100 µl of stabilizing solution was added.
- Tubes were vortexed for 1 minute and placed at 50℃ in a water for 10 minutes.
- Standard stock solution (Table 3.8) of sodium selenate (100 ppb=100 ng/ml) was made. It was used to make standard sodium selenite solutions ranging from 10ng-50ng as follow:

Table 3.8 Standards of known selenium concentrations

Standard (ng/ml)	Stock solution (µl)	MQ. Water (pH-1.8)
10	300	2700
20	600	2400
30	900	2100
40	1200	1800
50	1500	1500

- 100 µl of stabilizing solution was added to tubes containing standards and again step 11 was repeated.
- All the test and standard's tubes were cooled down and 500 µl of DAN was added to each tube. Tubes were votexed for 30 seconds.
- Tubes were again placed at 30℃ for half an hour.
- Tubes were cooled down and 3 ml of cyclohexane was added in each tube carefully keeping the cyclohexane volume similar in each tube.
- Each tube was vortexed for 1-2 minutes properly.
- The upper cyclohexane layer in which all the selenium (in the form of piazselenol) is extracted, is taken in cuvette to check the emission spectra at 520 nm using fluorescence spectrometer.
- Regression coefficient was calculated and a calibration curve was drawn using the emission spectra of standards (10-50 ng sodium selenite).
- The concentration of selenium present in the samples was calculated against the absorbance using the regression equation.
- For each sample, the test was performed in 4 replicates.

3.11. Physiochemical properities of soil:

3.11.1. Determination of soil pH:

- 2 g soil sample was weighed and taken in 100 ml beaker.
- 50 ml of distilled water was added and mixed thoroughly using glass rod.
- pH of soil sample was measured by dipping standardized knob in soil sample.

- Knob should be cleaned with distilled water and wiped with filter paper after every sample.

3.11.2. Available phosphorus (P)

Available phosphorus in the seleniferous soil was determined according to given by (Olsen et al. 1954).

Reagents:

- **0.5 M $NaHCO_3$ extracting solution**: 42 g of sodium bicarbonate was added in distilled water and final volume was made up to 1 litre. The pH was adjusted to 8.5 with the help of 1N NaOH.
- **Sulphuric acid (2.5 M):** 140 ml of conc. H_2SO_4 was diluted with 1 litre of distilled water.
- **Reagent A**: 12 g of ammonium molybdate was added to 250 ml of distilled water and 0.2908 g of antimony potassium was added to 100 ml of distilled water. Both the solutions were added to 1000 ml of 2.5 M of sulphuric acid, mixed thoroughly and the volume was made up to 2 litre with distilled water.
- **Reagent B (freshly prepared):** 1.058 g of ascorbic acid was added to 200 ml of reagent A and mixed thoroughly.
- **Stock standard PO_4-P solution (1000 mg/l):** 0.4394 g of KH_2PO_4 was dissolved in 100 ml of distilled water.
- **Working standard PO_4-P solution (10 mg/l):** 0.7 ml of stock 1000 mg/l was diluted with 70 ml distilled water.

Procedure:

- 2.5 g of soil sample was weighed and 50 ml of extracting solution was added to it.
- The suspension was kept on shaker for 30 minutes followed by filtration with the help of whatman filter paper no. 42.
- 10 ml of filtrate was taken in a beaker and 1 ml of 2.5 M H_2SO_4, 15.5 ml distilled water, 8 ml of reagent B and again 15.5 ml of distilled water were added.
- After 10 minutes blue color was developed, O.D. of the sample was measured at 882 nm against blank.

- Blank was prepared as above but does not contained the soil.
- To prepare standard curve of 0.1-1.0 mg/L of 10 mg/L working standard solution of phosphorus was added. Added 10 ml of extracting solution, 1 ml of 2.5 M H_2SO_4, 8 ml of reagent B and the final volume was made upto 50 ml with the help of distilled water. After 10 minutes, O.D. of these solutions were taken at 882 nm.
- Experiment was carried out in triplicates.

3.11.3. Total phosphorus (Kitson & Melon. 1944):

(Vandomolybdophosphoric Yellow color method)

Ammonium molybate reacts with acidic soil and form a heteropoly acid and molybdophosphoric acid. Yellow colour vanadomolybdophosphoric acid is formed. Intensity of yellow colour is directly proportional to phosphate concentration.

Reagents

- **Vanadomolybdate solution:**
  Solution A- 25 g ammonium molybdate [($(NH_4)_6Mo_7O_{24}.4H_2O$] was dissolved in 300 ml distilled water in 500 ml beaker.
  Solution B- 1.25 g ammonium (meta) vandate (NH_4VO_3) was dissolved in 300 ml boiling water. Cooled, added 250 ml concentrated HNO_3 and cooled again. Then solution A was added to solution B and final volume was made up to 1000 ml in volumetric flask.
- **Phosphorus stock standard solution (50 mg/I P):** Dried KH_2PO_4 was dissolved in distilled water and mixed thoroughly. It was acidifed with 7N H_2SO_4 and volume was made 1 litre to get 50 mg/I P solution. Toulene was added to prevent the microbial activity.

Procedure

- Digestion of soil sample was done by Kjeldahl method in which 2 g soil sample was taken in kjeldahl flask and 10 g sodium sulphate, 0.5 g copper sulphate and 30 ml concentrated H_2SO_4 was added to it.

- Then, flask was kept on digestion apparatus till volume get reduces and blue residues was formed.
- After digestion 50 ml volume make up was done with distilled water.
- 10 g acid digested soil sample was taken in 50 ml volumetric flask. Added 10 ml of vanadomolybdate solution and diluted to 50 ml.
- Mixed properly and phosphorus concentration was noted on spectrophotometer after 10 minutes at 420 nm.
- Working concentration of 0, 1, 2, 3, 4 and 5 mg from per litre stock solution in 50 ml volumetric flasks and developed the yellow color mentioned above.
- Spectrophotometer was calibrated with known standard phosphorus concentration and calculated for sample.
- Experiment was carried out in replicates.

Calculation

$$\textbf{P (mg/kg)} = \frac{\text{Vol makeup after digestion}}{\text{weight of sample (g)}} \times \frac{50}{\text{vol of digest used to develop color (ml)}} \times \text{P (mg/l)}$$

3.11.4. Organic carbon in soil

The total organic carbon in soil was estimated by the method given by (Walkley and Black 1934).

Reagents

- **1 N potassium dichromate:** 49.04 g of potassium dichromate was dissolved in distilled water and volume was made up to 1 litre.
- **0.5 N ferrous ammonium sulphate:** Dissolved 198 g of ferrous ammonium sulphate in distilled water and volume was made up to 1 litre.
- **Diphenyl amine indicator:** 0.5 g of diphenyl amine indicator was dissolved in 20 ml water and 100 ml concentrated H_2SO_4.
- **Concentrated sulphuric acid**

Procedure:

- 10 ml of 1N $K_2Cr_2O_7$ was added in 500 ml conical flask and to it 1 g soil sample was added.
- Mixing of soil sample and reagent was done by swirling.
- 20 ml of concentrated H_2SO_4 was added, flask was allowed to stand undisturbed for 30 minutes.
- Then, 1 ml of diphenyl amine indicator was added.
- Contents were titrated against 0.5 N ferrous ammonium sulphate, till end point is observed from blue to violet green.
- Blank was run without the soil sample.
- Experiment was carried out in replicates.

Calculation

$$\textbf{Organic carbon (\%)} = \frac{10\,(B-T) \times 0.003 \times 100}{B \times \text{weight of soil (g)}}$$

Where
B is volume of ferrous ammonium sulphate required for blank titration.

T is volume of ferrous ammonium sulphate required for soil sample.

Because organic matter contains 58% carbon, so

Calculation

Organic matter (%) = Organic carbon (%) × 1.724 (van Bemnelen factor)

3.11.5. Total nitrogen in soil sample

Total nitrogen in soil sample was estimated by Kjeldahl method by (Piper 1960).

Reagents

- **Anhydrous sodium sulphate**
- **0.5 gm Copper sulphate (catalyst)**
- **Concentrated sulphuric acid**

- **0.02 N Sulphuric acid:** 0.272 ml of concentrated sulphuric acid was added in 500 ml distilled water.
- **Boric acid solution:** 20g of boric acid dissolved in 1000 ml distilled water.
- **Mixed indicator solution:** 0.066 g of methyl red and 0.099 g of bromo cresol green was dissolved in 100 ml distilled water.
- **0.25 N standard sodium hydroxide solution:** 13.201 ml of NaOH solution in 200 ml distilled water and final volume made upto 1 litre.
- **Digestion mixture:** 10 g sodium sulphate, 0.5 gm copper sulphate, 30 ml concentrated H_2SO_4.

Procedure

- 2 g of soil sample was taken in kjeldahl flask, 0.5 g copper sulphate was added in 30 ml H_2SO_4 and after digestion 50 ml water was added.
- Flask was kept in digestion chamber at 100℃ for 3 hours.
- Color changed was monitored from dark brown to greenish white, contents were cooled and 50 ml distilled water was added.
- Boric acid was taken in a beaker into which tubes were dipped.
- Glass beads were added in distillation flask containing digested sample.
- The volume of liquid in the distillation flask should not exceed half the capacity of flask to prevent the frothing.
- Sodium hydroxide was added to the distillation flask through the open end of the condenser attachment and stoppered. Water flow was maintained through the condenser.
- Condenser should be arranged to dip the tube in the beaker containing 50 ml boric acid.
- Distillate one third of the total volume of the solution in the flask.
- Assembly was cooled and dismantle.
- Distillate was collected through receiver tube containing 15 ml boric acid and 3 drops of mixed indicator was added.

- The distillate containing ammonia was titrated against sulphuric acid till grass green colour changes to steel grey.

Calculation

$$\textbf{Nitrogen (\% by mass)} = \frac{1.4 \times V \times N}{W}$$

Where, V = volume in ml of standard sulphuric acid used in titration, **N** = normality of standard sulphuric acid, **W** = mass in g of sample taken for the test.

RESULTS

RESULTS

4.1. Collection of soil sample

Fig 4.1 Field soil of Jainpur, Nawanshahr region.

Wheat rhizospheric and bulk soil samples were collected from Jainpur village of Nawanshahr-Hoshiarpur region which is reported as a seleniferous region in Punjab. This agricultural land is affected by the elevated level of Se concentration. The soil samples were collected in plastic bags and brought to the laboratory. The rhizospheric soil samples were stored at 4℃ till further processing. The rhizospheric soil samples were used for isolation of selenium tolerant fungi and analysis of physiochemical characteristics of soil. The bulk soil was used for nursery experiments (Fig 4.1).

4.2. Estimation of selenium content in soil sample

Estimation of selenium content in rhizospheric soil sample was done by using fluorescence spectrometery. The emission spectrum of the complex called piazselenol formed by complexing of DAN dye with Se^{4+} was measured at an excitation wavelength of 360 nm and an emission wavelength of 520 nm. Se concentration in soil samples was determined by drawing a standard

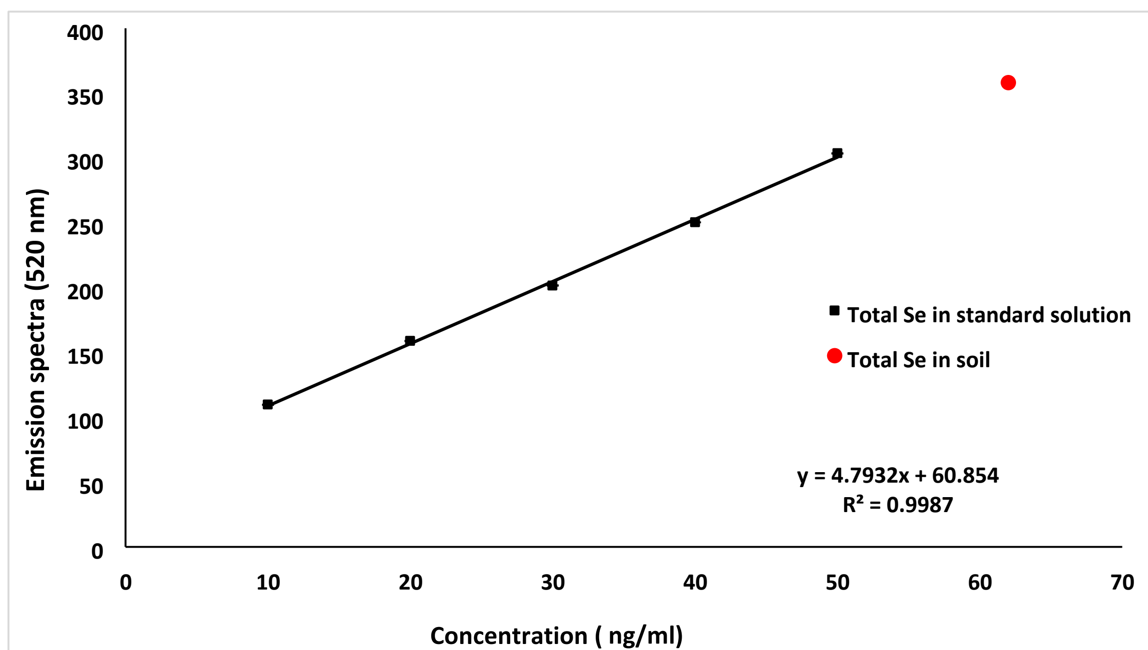


Fig 4.2 Linear graph showing emission spectra of Se-ICP standard solution at different concentrations and that of seleniferous soil sample.

curve of emission spectra obtained of National Institute of Standards and Technology (NIST) certified Se-ICP standard solution at 5 different concentrations (10ng/ml-50ng/ml). The value of Se in collected soil sample was 5 mg/kg (Fig 4.2).

4.3. Isolation of selenium tolerant fungal isolates

Selenium tolerant fungal strains were isolated from rhizospheric soil of wheat (Triticum aestivum) by serial dilution method. Briefly, 10 g of soil sample was added to 100 ml of sterile normal saline (0.8% NaCl) and kept on a shaker at 120 rpm for about 30 minutes. After that, 1 ml of primary inoculum was added to 9 ml of sterile normal saline and so on upto 1×10^{-4} dilution. From each dilution, a 100 µl inoculum was spread on czapek dox agar plates amended with sodium selenite (5 mM) to select for selenium tolerant fungal isolates and chloramphenicol (100 µl/ml) to prevent undesired bacterial growth. Plates were incubated at 25℃ for 21 days. After about 8 days, mixed Se tolerant fungal isolates were obtained on czapek dox agar plates (Fig 4.3).

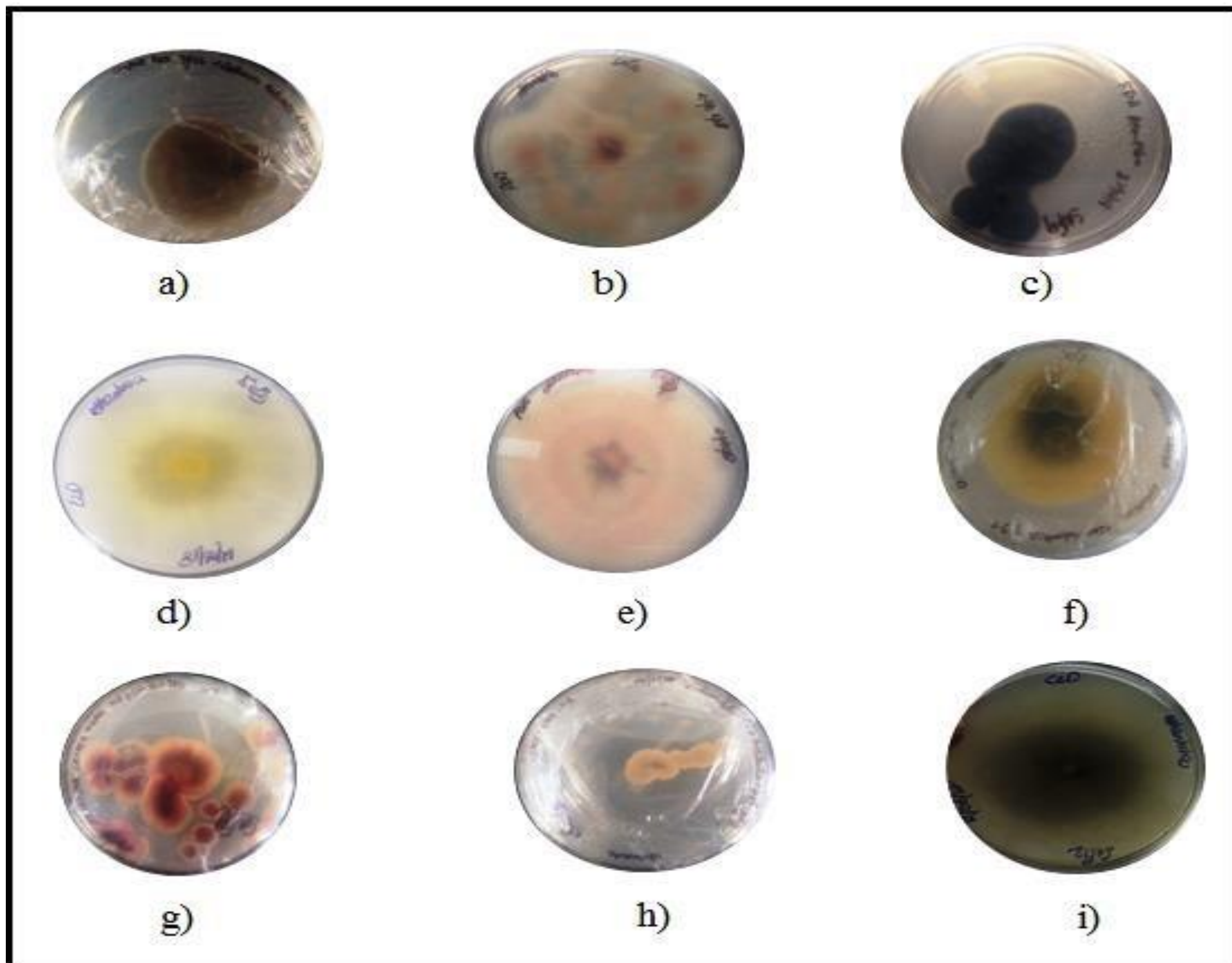


Fig 4.3 Different fungal isolates obtained from seleniferous soil sample a) SeF1 b) SeF2 c) SeF4 d) SeF5 e) SeF6 f) SeF7 g) SeF9 h) SeF10 i) SeF12.

4.4 Selenium tolerance levels of fungal isolates

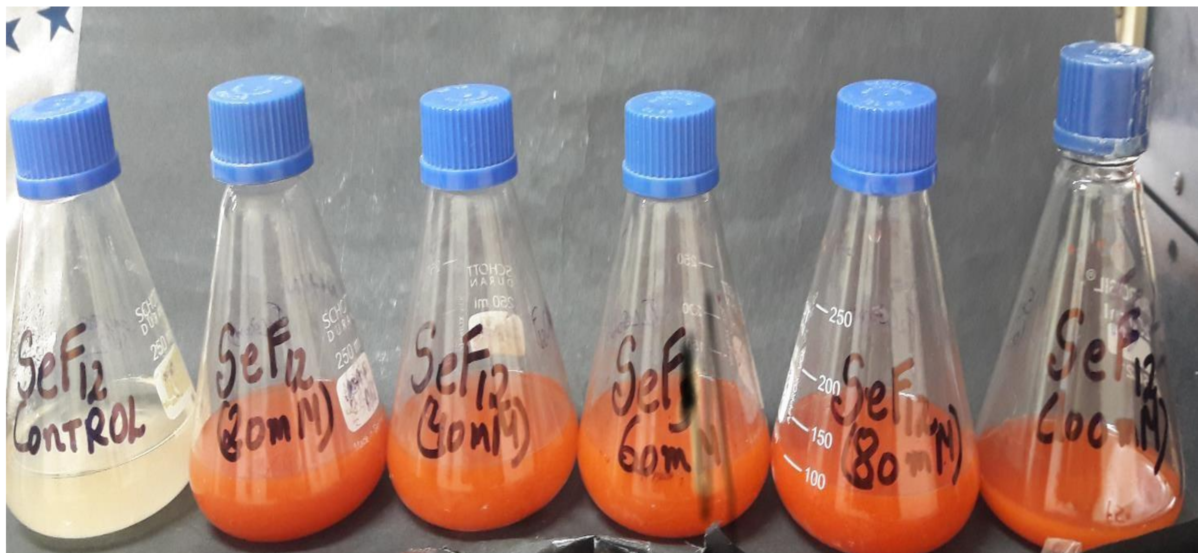
Selenium tolerance levels of all the 9 fungal isolates (SeF1, SeF2, SeF4, SeF5, SeF6, SeF7, SeF9, SeF10 and SeF12) was checked in modified melin-norkrans (MMN) broth supplemented with different concentrations (10 mM-100 mM) of sodium selenite. The flasks with 5 fungal discs of 5 mm were incubated at 25˚C on an incubator shaker for 14 days. The broth was filtered through filter paper using funnel and fungal biomass was transferred to pre-weighed aluminium foil. The supernatant was collected in 50 ml falcons. Fungal mycelium was air dried in an oven at 60℃. The dry weight of fungal biomass at different concentration of sodium selenite was noted down (Table: 4.1).

Table 4.1 Tolerance studies of dry weight of fungal biomass (mg/50ml) at different concentrations of sodium selenite (mM)

Fungal isolates	**Concentrations (in mM)**					
	CONTROL	20	40	60	80	90
SeF1	15.0±0.5	13.3±1.1	11.3±1.0	9.4±0.7	8.5±0.7	5.3±1.0
SeF2	11.3±0.8	9.2±1.0	7.7±0.4	6.4±0.8	5.1±0.7	3.3±1.1
SeF4	16.4±1.1	11.0±0.5	9.7±0.640	8.0±0.7	7.0±0.3	5.7±0.6
SeF5	**35.4±5.0**	**31.1±4.0**	**27.0±0.9**	**23.8±1.6**	**22.6±3.0**	**20.9±1.5**
SeF6	21.6±1.1	17.4±1.0	15.5±0.8	13.5±1.0	11.6±0.5	8.9±1.4
SeF7	22.7±3.1	17.4±1.1	15.4±1.0	14.1±0.5	10.4±0.9	8.4±0.8
SeF9	23.0±1.8	18.5±1.0	15.3±1.0	14.3±1.5	14.0±1.5	14.0±1.3
SeF10	17.4±1.0	15.4±1.0	14.6±0.9	11.4±0.8	9.2±1.4	5.4±1.0
SeF12	**31.1±0.9**	**29.4±1.0**	**26.6±2.2**	**22.3±1.7**	**20.4±1.0**	**19.3±0.9**

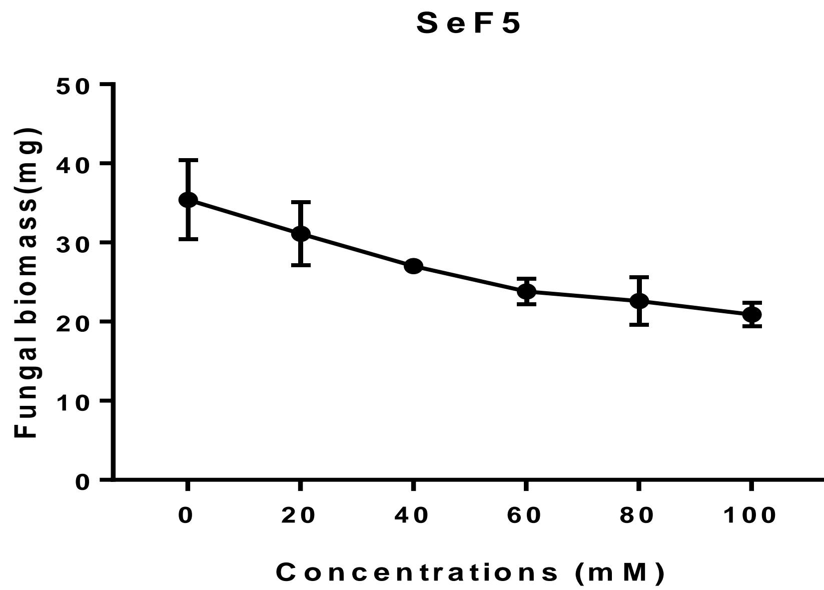
It was observed that except two strains, none of the strains were capable of growing in medium spiked with more than 20 mM sodium selenite. Dry weight of fungal mycelium was weighed. On basis of dry weight of fungal mycelium, two fungal isolates named SeF5 and SeF12 was selected as a high selenium tolerant fungal isolates because from all the 9 isolates these two isolates showed mycelium growth up to the 100 mM concentration of sodium selenite. There was no mycelium growth in medium containing 120 mM sodium selenite. The colour of broth turns red due to the reduction of sodium selenite to Se (0) by fungal isolates known as red elemental selenium (Fig 4.4). With increase in the concentration of sodium selenite fungal biomass of SeF5 and SeF12 decreases (Fig 4.5).

(a)



(b)

Fig 4.4 Effect of sodium selenite at different concentrations (20-100 mM) in MMN broth on fungal isolates. (a) SeF5 fungal isolate in MMN broth, (b) SeF12 fungal isolate in MMN broth.



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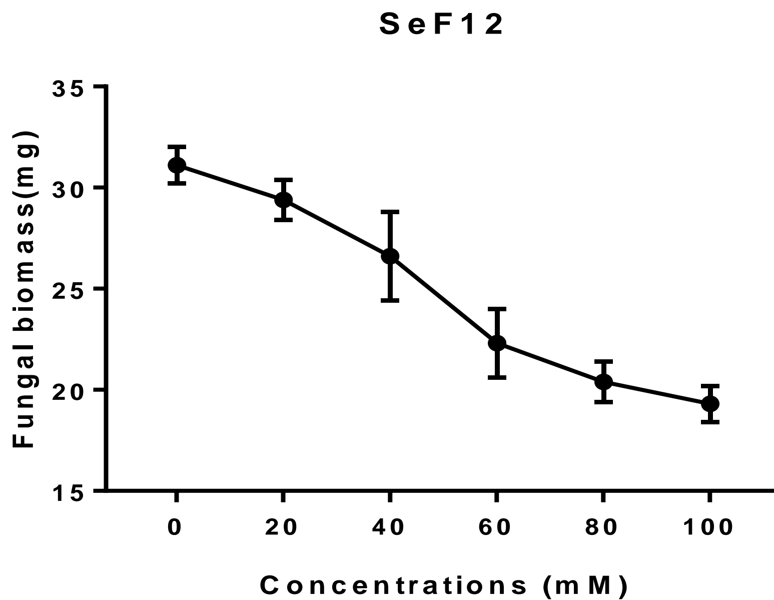

b)

Fig 4.5 Effect of different concentrations of Se on fungal biomass SeF5 and SeF12.

The colour of the broth turns red due to the reduction of sodium selenite to Se (0) by fungal isolates known as red elemental selenium (Fig 4.4). With increase in the concentration of

sodium selenite fungal biomass of SeF5 and SeF12 decreases. The growth of mycelium was maximum at 20 mM and very less at 100 mM (Fig 4.5).

4.5. Effect of fungi on plant growth promotion

Nine isolates were examined for showing various plant growth promoting activities in vitro. The reason was to find if these selenium tolerant fungal isolates could be used as biofertilizers for the plants growing in selenium impacted soils so that they could be saved plants from toxic effects of selenium present in soil at such high concentrations.

4.5.1. Quantification of Indole Acetic Acid (IAA)

Fungal discs were inoculated in czapek dox broth containing 1000 µg/ml tryptophan. The broth was incubated at 25℃ for 5 days. After 5 days, the culture was taken in sterile falcons and centrifuged at 10,000 rpm for 5 minutes. 1 ml culture supernatant was taken in a test tube and 2 ml salkowski reagent was added. Test tubes were incubated in dark for 30 minutes. The supernatant obtained from fungal isolates showing IAA production produced dark to light pink colour. The absorbance of the resulting solution was measured at 540 nm. The quantification of IAA produced by fungal isolates was determined by regression analysis of standard linear plot drawn by using standard IAA solution at different concentrations and drawing a standard regression equation.

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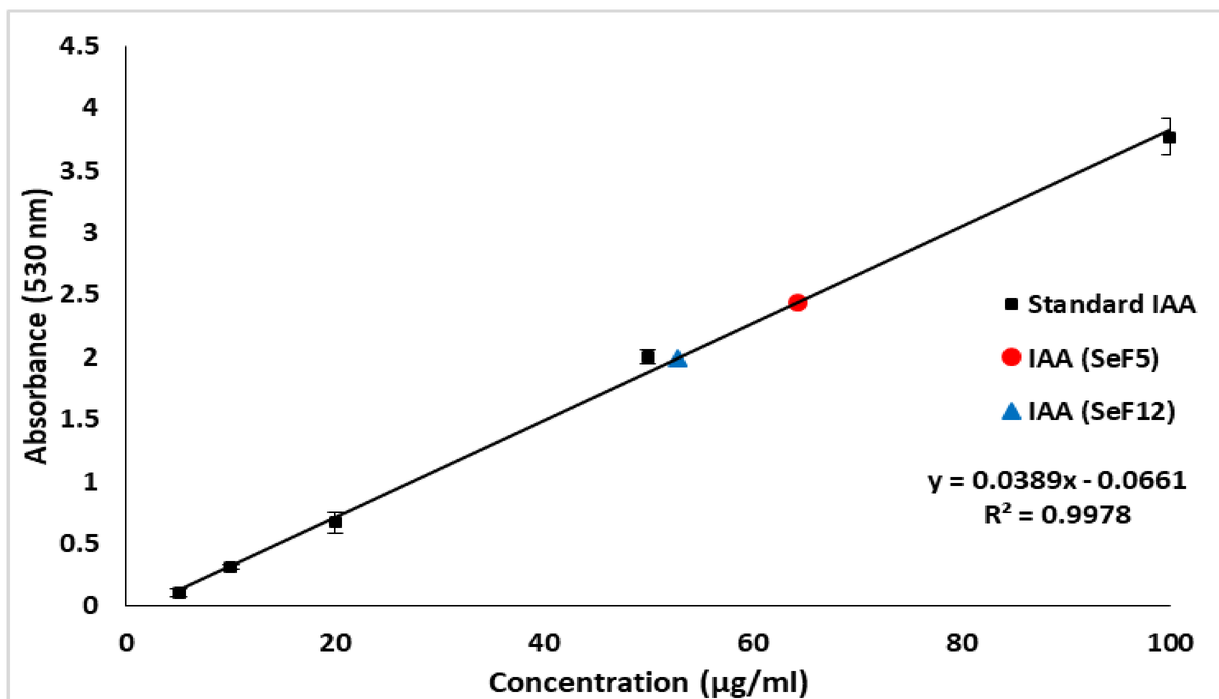
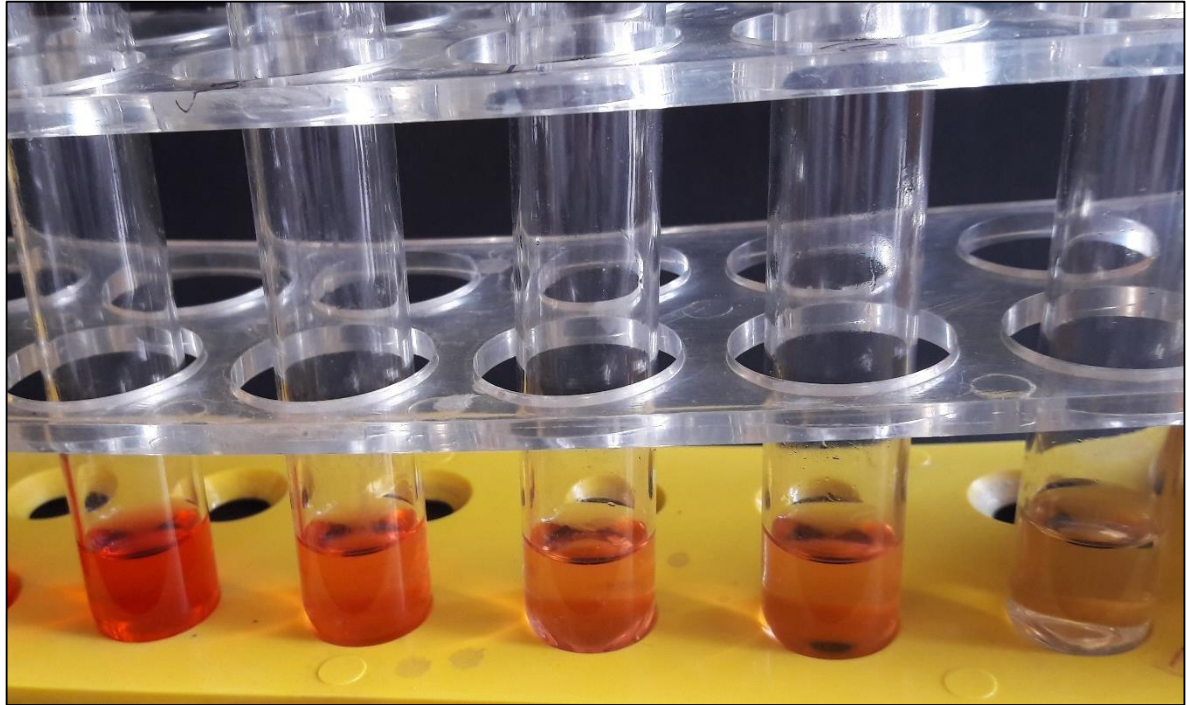


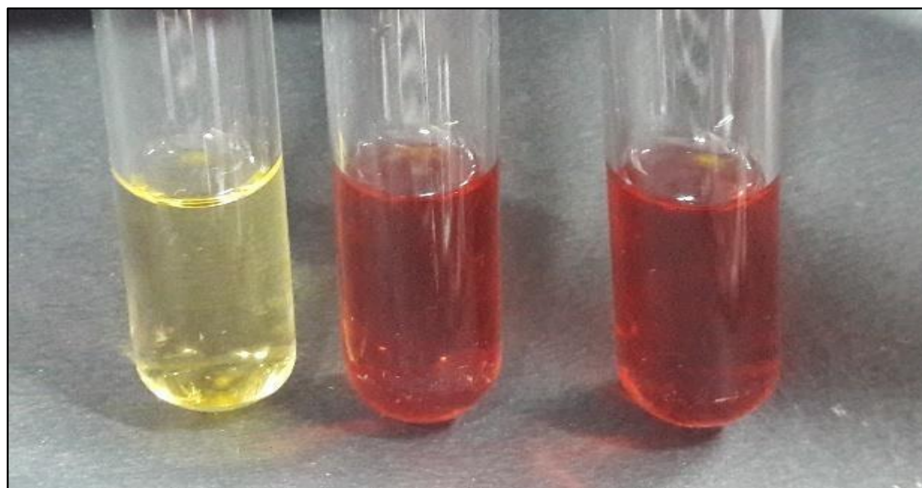
Fig 4.6 Standard curve obtained from the known concentrations of IAA

From all nine isolates (SeF1, SeF2, SeF4, SeF5, SeF6, SeF7, SeF9, SeF10, SeF12). SeF5 and SeF12 showed a positive test for IAA production indicated by pink colour. No other isolates showed IAA production. The concentration of IAA depended upon the intensity of pink colour produced which was determined by using spectroscopy. The IAA concentration of SeF5 and SeF12 was calculated from linear regression equation obtained from the known concentration of IAA standards (Fig 4.6). The concentration of IAA produced by SeF5 was 64.278 µg/ml and SeF12 was 52.89 µg/ml. Pink colour is produced in this case due to the formation of tris-(indole-3-aceto) iron (III) complex which gives dark pink colour (Fig: 4.7; Fig: 4.8). Indole 3-acetic acid production by fungi helps in plant growth promoting by cell elongation and division.



a) b) c) d) e)

Fig 4.7 Standards of known concentrations of IAA a) 20 µg/ml b) 10 µg/ml c) 30 µg/ml d) 40 µg/ml e) control 0 µg/ml.



a) b) c)

Fig 4.8 IAA production by selenium tolerant fungal isolates, a) negative control b) SeF5 c) SeF12

4.5.2. Phosphate solubilization by fungi

Nine fungal isolates were screened for the phosphate solubilization. Qualitative and quantitative estimation of phosphate solubilization was estimated on pikovskaya agar medium. Fungal isolates were inoculated in the centre of PVK agar plates. Plates were incubated at 25℃ for 5 days. Phosphate solubilization index was calculated for all the nine fungal isolates (Table 4.2).

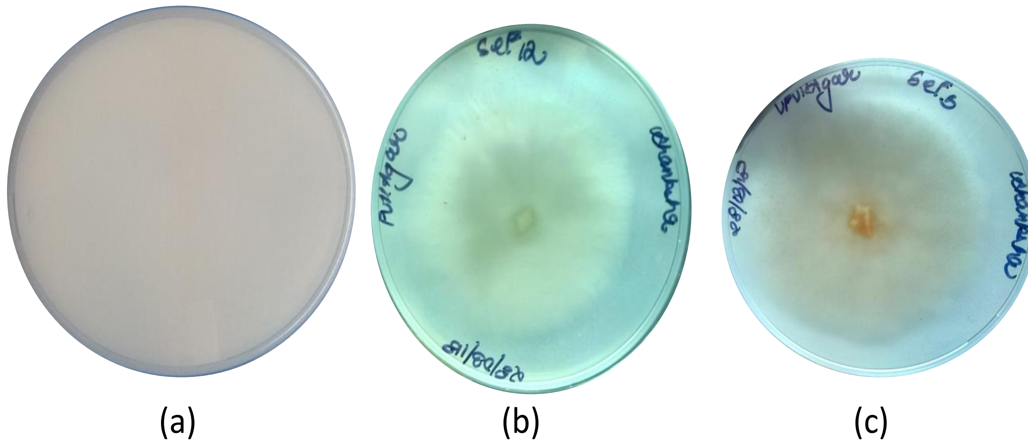


Fig 4.9 Phosphate solubilization a) Control plate b) SeF12 c) SeF5

Table 4.2 Phosphate solubilization index of SeF5 and SeF12.

S.No.	Fungal isolates	Soluibilzation index (mm)
1	SeF1	No halozone formation
2	SeF2	No halozone formation
3	SeF4	5.0
4	SeF5	4.5
5	SeF6	No halozones formed
6	SeF8	No halozone formation
7	SeF9	No halozones formed
8	SeF10	No halozones formed
9	SeF12	10

From all the nine isolates (SeF1, SeF2, SeF4, SeF5, SeF6, SeF7, SeF9, SeF10, SeF12) only three isolates named SeF5 (10 mm), SeF12 (10 mm) and SeF4 (5 mm) showed phosphate solubilization by formation of halo zone around them (Fig 4.9). The halo zone formation takes place by conversion of insoluble phosphate to soluble phosphate by phosphate solubilizing fungi. This property of fungal inoculum as biofertilizer helps the plants in using otherwise unavailable phosphorus for various processes like cell division, respiration and photosynthesis by providing soluble phosphate. Phosphate solubilizing organisms also solubilize the bound phosphate present in roots and makes it available to plants.

4.5.3. HCN production by fungi

Czapek dox agar plates supplemented with 4.4 g/l glycine and whatmann filter paper no-1 was soaked in 2% sodium carbonate in 0.5% picric acid solution, the filter paper was placed inside the lid of petriplates and incubated at 25℃ for 3 days for visualizing HCN production.

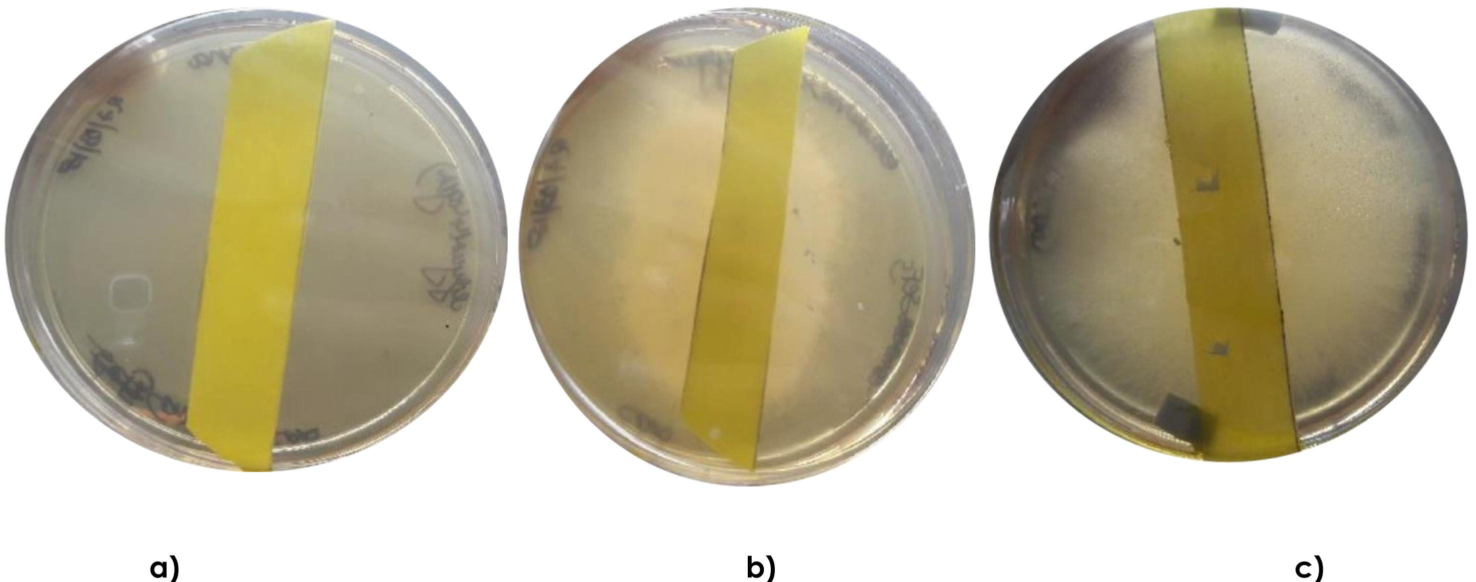


Fig 4.10 SeF5 and SeF12 showed negative results for HCN production as there is no change in filter paper colour from yellow to brown. Plate a) negative control, plate b) SeF5, plate c) SeF12.

All the nine isolates (SeF1, SeF2, SeF4, SeF5, SeF6, SeF7, SeF9, SeF10 and SeF12) were screened for HCN production and none of them had showed positive test for HCN production.

There was no change in colour of filter paper from yellow to reddish brown. Hence, both the isolates were negative for HCN production (Fig. 4.10). Hydrogen cyanide helps in plant growth promoting activities by a production of antimicrobial products which protects the plants from pathogens by inhibiting their growth in plants.

4.5.4 Siderophore production by fungi

Screening of nine isolates for siderophore production. SeF5 and SeF12 were found to be positive for the siderophore production from all other seven isolates (SeF1, SeF2, SeF4, SeF5, SeF6, SeF7, SeF9, SeF10 and SeF12). Siderophore production for both the isolates was checked on CAS (chrome auzrol S) agar plates for the development of coloured halo zone around the isolates. Isolates were inoculated in the centre of the CAS pertiplates and incubated for 14 days. After 14 days there was formation of different coloured halo zones around the isolates. SeF5 showed purple or wine coloured halo zone, on the other hand, SeF12 showed blue coloured halo zone (Fig 4.12, Table 4.3).In, similar way siderophore production was checked in CAS broth (Fig 4.11).

Table 4.3 % of siderophore production by SeF5 and SeF12

S.No	**Fungal isolates**	**Siderophore production (%)**
1	SeF5	15.50
2	SeF12	31.68
3	SeF3	9.50
4	SeF4	8.5

a) b) c) d)

Fig 4.11 Siderophore production in broth a) SeF12 b) SeF5 c) SeF4 d) SeF3

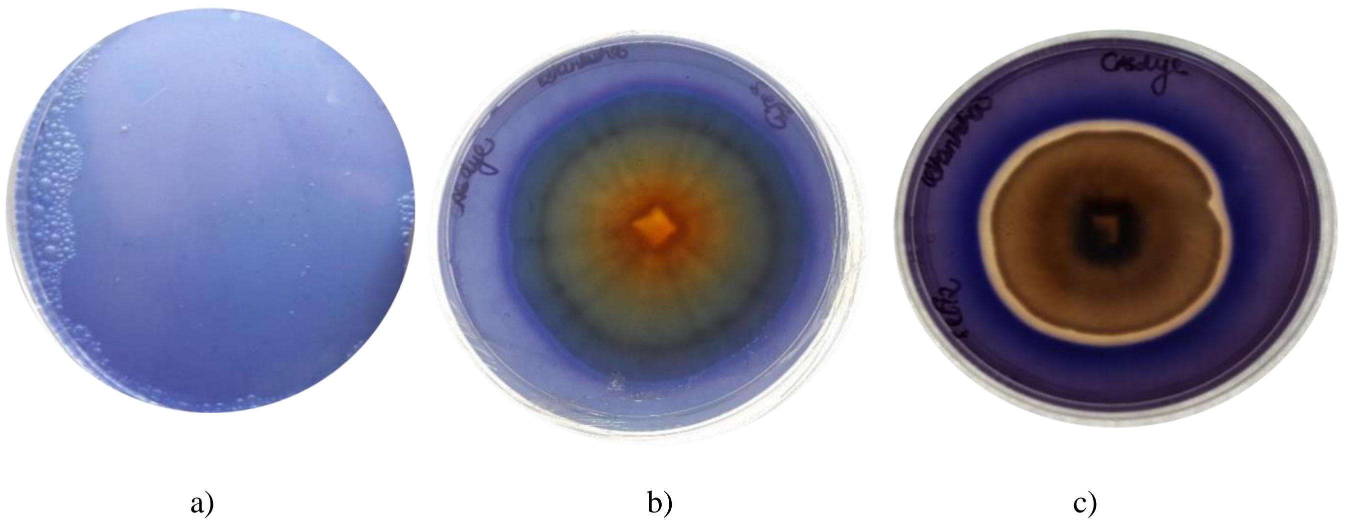


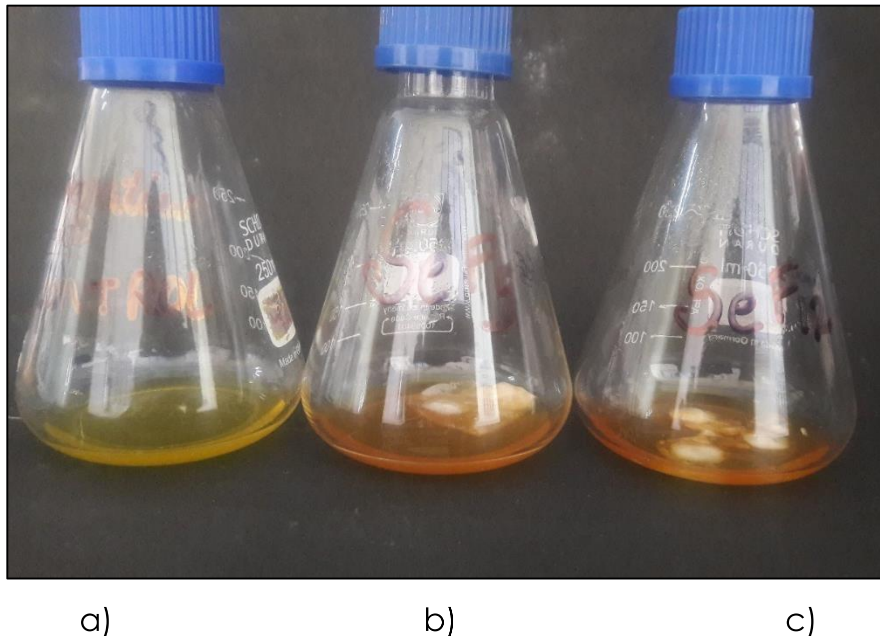
Fig 4.12 Siderophore production a) SeF$_5$ (bluish coloured zone) b) CAS plate without inoculation c) SeF$_{12}$ (purple coloured zone).

From nine fungal isolates (SeF1, SeF2, SeF4, SeF5, SeF6, SeF7, SeF9, SeF10, SeF12) only 4 isolates showed siderophore production by formation of halo zones. But, from four isolates (SeF5, SeF12, SeF4 and SeF3) only 2 showed maximum siderophore production. SeF4 and SeF12 gives halozone but its siderophore production % was less than 10. So they were not

included for further analysis. The different coloured zone during siderophore production by fungal isolates is due to the formation of ternary complex (Hexadecyltrimethylammoniumbromide) with chrome azurol S dye used as an indicator to test the siderophore production by fungi. Siderophores produced by fungi helps in plant growth promotion by providing ferric ions and absorption of bound iron from soil surface during the iron stress conditions by the plants. Siderophores also protect crop plants from plant pathogens.

4.5.5. Ammonia production by fungi

Ammonia production was checked in 15 ml of peptone water. Fungal discs were inoculated in a flask containing peptone water. The flasks were incubated at 25℃ for 48-72 hours. After incubation 1 ml nesselar's reagent was added.



a) b) c)

Fig 4.13 Ammonia production shown by SeF5 and SeF12 by giving brown colour. a) Control; b) SeF5; c) SeF12.

From all the nine isolates only two isolates showed ammonia production by producing brown colour obtained after adding nessler's reagent in media containing SeF5 and SeF12 shows a positive test for ammonia production (Figure: 4.13). pH of nesselar's reagent is alkaline in nature due to this reason when fungi produces free ammonia it reacts with nesselar 's reagent and gives brown colour. Ammonia production by fungal isolates has plant growth promoting activities.

Selection of fungal isolates was done on basis of tolerance studies and plant growth promoting activities (PGPR). Total nine fungal isolates were screened for plant growth promoting activities. A few isolates showed plant growth promoting activities like IAA production, phosphate solubilization and siderophore production, but not all were tolerant to high concentrations of selenium in the form of sodium selenite. All the nine fungal isolates were screened for tolerance level and PGPR activities. We had selected two isolates (SeF5 and SeF12) from all the nine fungal isolates (SeF1, SeF2, SeF4, SeF5, SeF6, SeF7, SeF9, SeF10, SeF12) because it was tolerant upto 80 mM of sodium selenite and showing good mycelium growth. PGPR activities like IAA production, phosphate solubilization, HCN, ammonia and siderophore production were showed by both the isolates and in vitro plant growth promoting activities for further analysis. SeF5 and SeF12 was selected for estimation of selenium bioaccumulation.

4.6 Estimation of selenium bioaccumulation

Fungal biomass and supernatant obtained when tolerance level of SeF5and SeF12 was checked. Biomass was dried in oven at 50 ℃ for overnight. Two high concentrations and one lower concentration of sodium selenite were selected for studying selenium bioaccumulation in fungal isolates. Bioaccumulation was checked by the fluorescence spectrometer. Digestion was done for all the samples. In a similar way, bioaccumulation was checked by biosorption mechanism in different plant parts by taking dried, crushed 10 mg fungal biomass, 200 mg roots, shoots used too to determine the effect of inoculated fungal isolates on selenium bioaccumulation by different plant parts. Two way ANOVA analysis was done to check the Se uptake in fungal biomass, roots and shoots sample (Table 4.4; Table 4.5).

Table 4.4 Selenium bioaccumulation in fungal biomass (mg) of SeF5 and SeF12 at different concentrations

Concentrations (mM)	**SeF5**	**SeF12**
20	561.7±7.6	662.9±5.9
60	447.6±9.2	466.3±4.6
100	185.6±3.3	371.3±8.0

Table 4.5 Two factor ANOVA analysis of fungal biomass for Se uptake in SeF5 and SeF12

Source of variation	**Sum of squares**	**Degree of Freedom**	**Mean sum of squares**	**F (DFn, DFd)**	**P value**
Interaction	19420	2	9710	F(2.12)=302.4	P<0.0001
Concentration	33847	2	166924	F(2,12)=5199	P<0.0001
Fungal	48928	1	48928	F(1,12)=1524	P<0.0001
Residual	385.3	12	32.11	-	P<0.0001

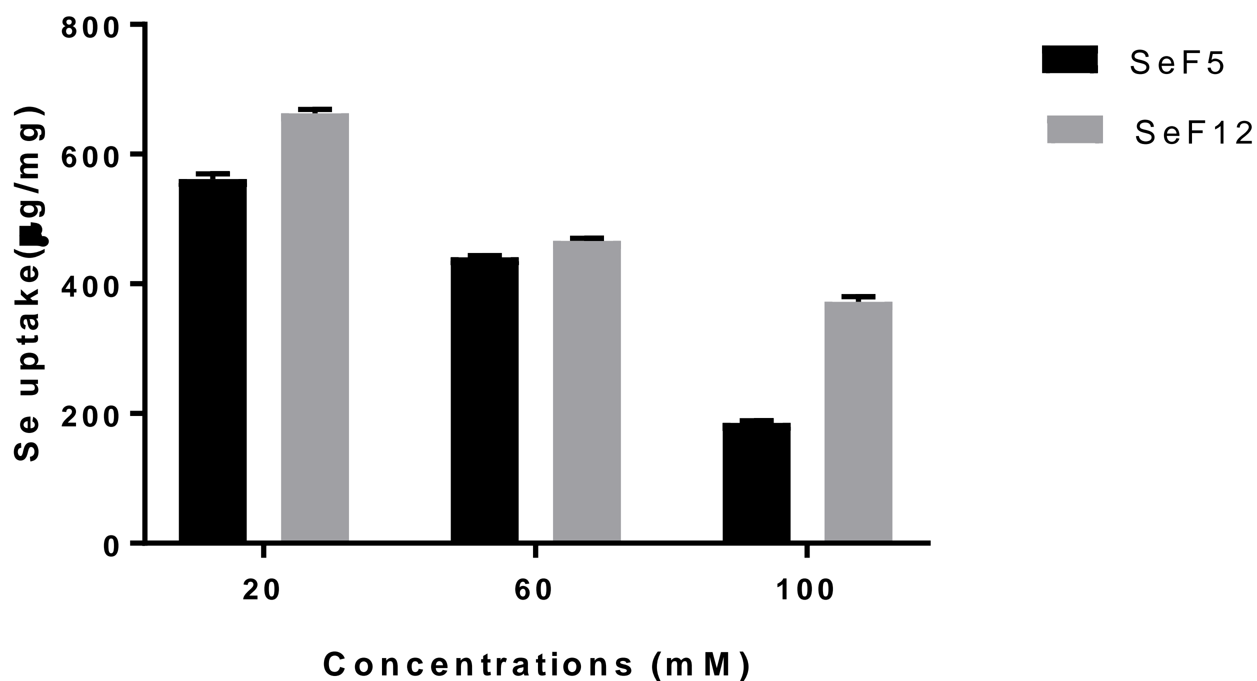


Fig 4.14 Selenium bioaccumulation in fungal biomass (mg) of SeF5 and SeF12

Table 4.6 Root biomass (mg) of SeF5 and SeF12 plant in selenium contaminated soil

Treatments	**Root biomass (mg)**
Control	6.1±0.1
SeF5	6.1±0.1
SeF12	52.8±0.6

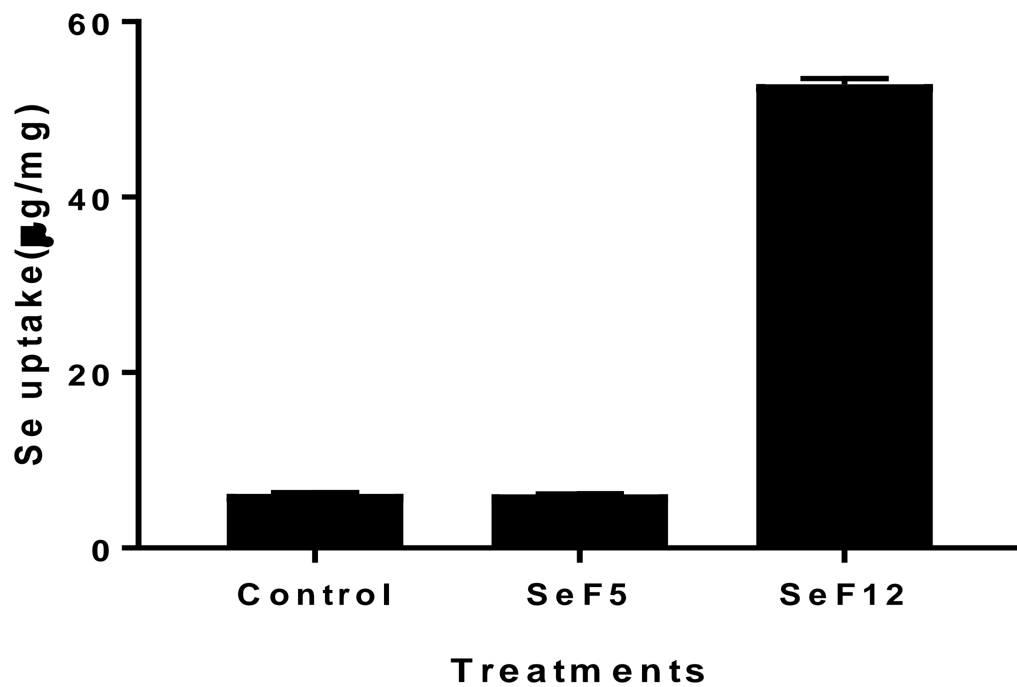


Fig 4.15 Se bioaccumulation in root biomass (mg) inoculated with SeF5 and SeF12

Table 4.7 Shoot biomass (mg) of SeF5 and SeF12 plant in selenium contaminated soil

Treatment	**Shoot biomass(mg)**
Control	4.2±0.1
SeF5	9.4±0.2
SeF12	8.1±0.6

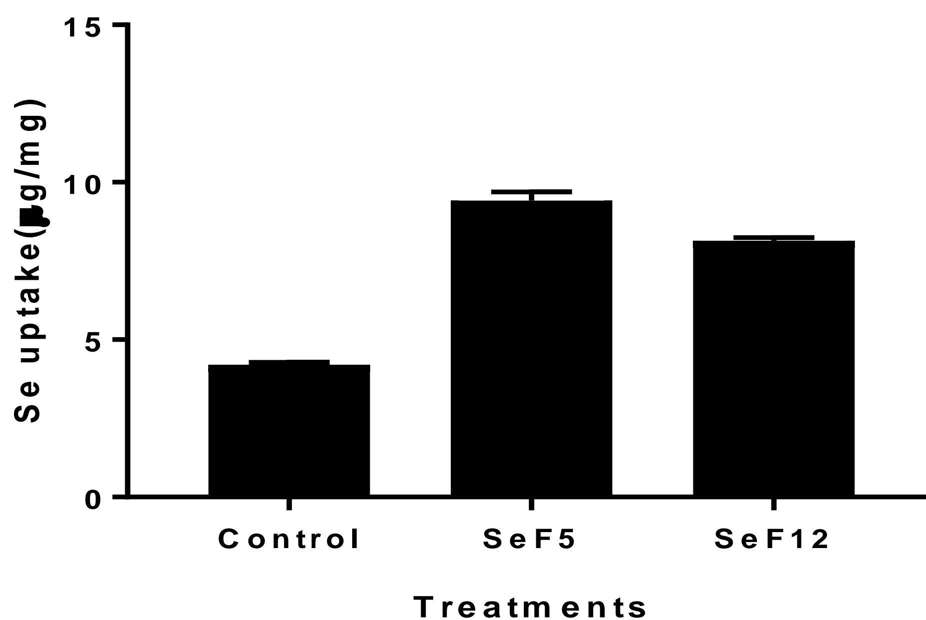


Fig 4.16 Se bioaccumulation in shoot biomass (mg) inoculated with SeF5 and SeF12

4.7. Morphological characterization of highly Se tolerant fungal isolates:

SeF12 *Pseudopestalotiopsis thecae*

SeF12 belongs Amphisphaeriaceae family known as *Pseudopestalotiopsis thecae*. The fungal mycelium had radial growth and was white in colour on czapek dox agar plate. It is a fast growing fungi, fully covering the petriplate within 6 days.

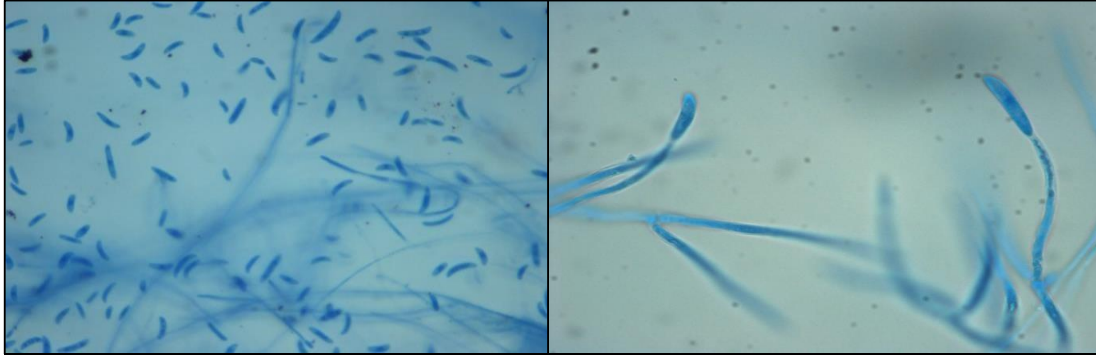


Fig 4.17 Microscopic view of *Pseudopestalotiopsis* thecae (SeF12) 100X magnification.

As evident by Lactophenol cotton blue staining, it consists single conidia and not well differrentiated conidia. Cells were hyaline shaped at the terminal end. It consists of banana shaped conidiophores with the presence of 4 septa (Figure: 4.17).

SeF5 *Fusarium equiseti*

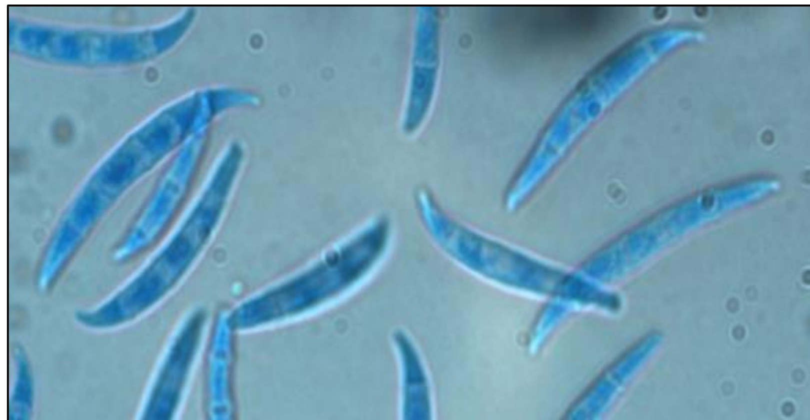


Fig 4.18 Microscopic view of *Fusarium equiseti* (SeF5) 100X magnification

SeF5 belongs to the Nectriaceae family and known as a *Fusarium equiseti*. *Fusarium* is known as a filamentous fungi and is widely spread in the soil. Spores of *Fusarium* are spindle or sickle

shaped with 5 septa. It is also a fast growing fungi and light violet colour on the czapek dox agar plate and yellow pigmented at the back of petriplate. Curved macroconidia cells were visualized under a microscope (Fig 4.18).

4.8. Molecular identification of highly Se tolerant fungal isolates:

4.8.1. Analysis of isolated genomic DNA of ITS region:

Mycelia of SeF_5 and SeF12 fungal isolates DNA was visualized on 0.8% agarose gel electrophoresis. Results showed both the isolates single band of approximately 550bp (Figure: 4.19).

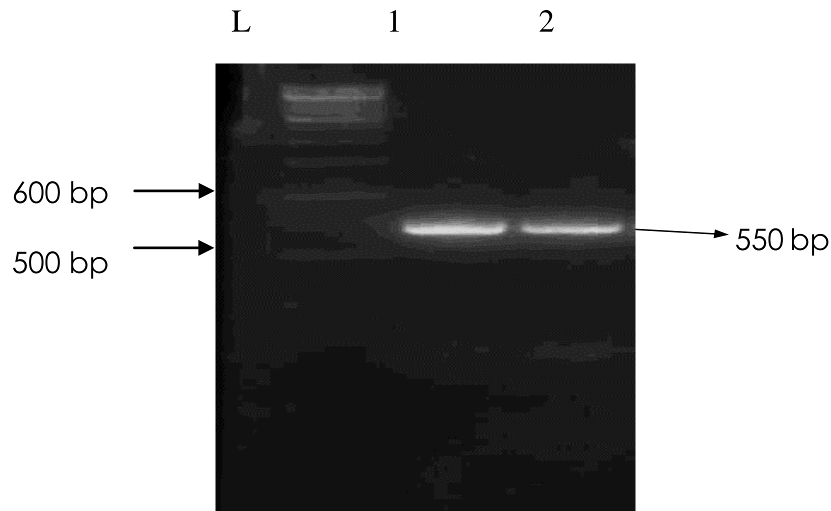


Fig 4.19 Agarose gel showing DNA bands of SeF12 and SeF5, Ladder: 1 kb: Lane 1; SeF5: Lane 2; SeF12: Lane 3.

4.8.2. Quantitative analysis on Nanodrop:

Analysis of isolated DNA was done by using a Nanodrop 1000 spectrophotometer (Thermo scientific. Wilmington, DE). Quantity was measured by A260/280 ratio. The ratio should be between 1.8-2.0. The ratio less than 1.8 shows protein contamination, while ratio of DNA more than 2.0 shows RNA contamination.

Table 4.8 Quantitative analysis of isolated DNA by Nanodrop

S.No.	Fungal isolates	A(260/280) ratio	Concentration of DNA(ng/µl)
1	SeF5	1.81	221.0
2	SeF12	1.87	260.1

Results showed that SeF5 and SeF12 A (260/280) ratio was betweem 1.8-2.0. DNA sample was pure. There was no protein and RNA contamination (Table: 4.10).

4.8.3. Amplification of ITS region

SeF5and SeF12 genomic DNA was amplified by using PCR primers (5'- TCC GTA GGT GAA CCT) GCG G- 3' and ITS4 (5'- TCC GCT TAT TGA TAT GC- 3'). Results were viewed on 0.8% agarose gel electrophoresis. Amplification products of both the isolates showed band size of approximately 550 bp (Figure: 4.20). Pooling of amplified product was done to increase the concentration of DNA for purification of PCR products. After amplification purification of PCR products was done by using the gel extraction kit method to remove the extra nucleotides before cloning.

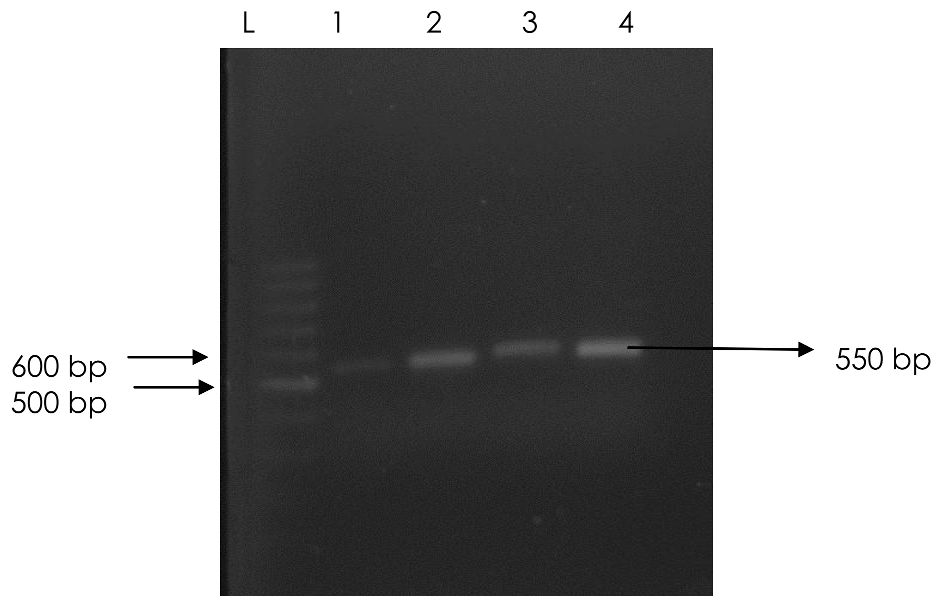


Fig 4.20 Amplification of ITS-PCR products of two isolates. Ladder: 1 kb; Lane 2: SeF5; Lane 3: SeF5; Lane 4: SeF12: Lane 4: SeF12.

4.8.4. Genetic transformation and blue-white screening:

PCR products obtained after amplification contained dA overhang added to 3' end. In, TA cloning method utilizes T- vector containing dT overhang which complements with dA overhang of PCR product to form a cloned product. Cloned product was inserted into competent cells to get the transformed product. The genetic transformation was done using DH10β cells. Colony PCR was done to evaluate the transformation of ligated product.

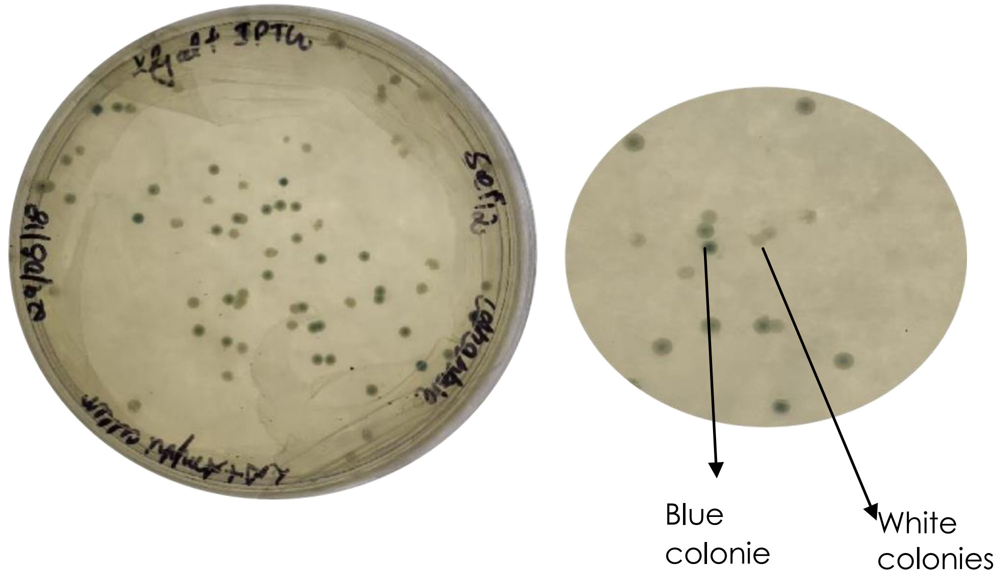


Fig 4.21 LA and Ampicillin plates showing blue white colonies.

White and blue colonies were obtained on LA and Ampicillin plates (Figure: 4.21). Colonies with the insert containing plasmid have a non-functional β- galactosidase and give a whitish creamy colour of E.coli cells known as transformed colonies. X-gal produces pigment due to intact β-galactosidases, turning bacterial colony blue known as untransformed colonies. IPTG helps in the expression of the gene of interest.

4.8.5. Plasmid isolation (alkali lysis method)

After confirming the transformed colonies by colony PCR. Plasmid isolation was done of transformed colonies. Results were viewed on 0.8% agarose gel electrophoresis.

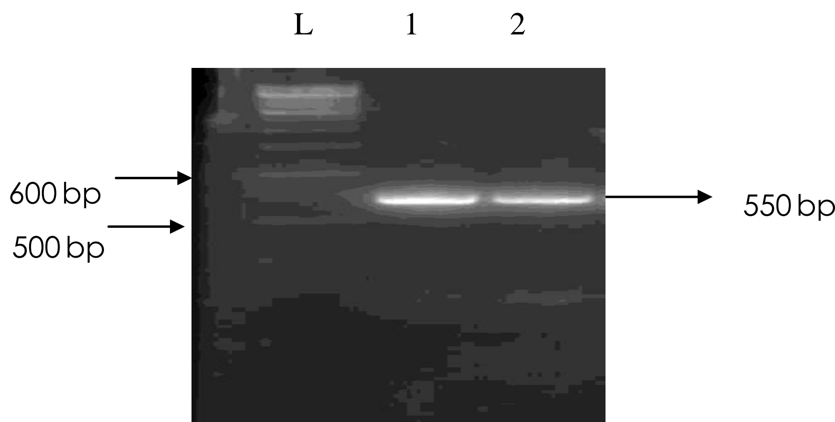


Fig 4.22 Plasmid DNA of ITS region of SeF5 and SeF12. Lane 1: 1 kB ladder; Lane 2: SeF_5 plasmid; SeF_{12} plasmid.

Sharp bands were obtained around the 550 bp. Plasmid DNA is present in circular forms and smaller in size than a linear form of DNA. Plasmid DNA contains insert. Plasmid isolation was done to check the presence of an insert in a plasmid (Fig 4.22).

4.8.6. Sequencing of ITS region

The fragment of ITS-5.8S-ITS2 amplified by PCR were sequenced. Sequenced obtained is as follows:

SeF5

TCCGTAGGTGAACCTGCGGAGGGATCATTACCGAGTTTACAACTCCCAAACCCCT
GTGAACATACCTATACGTTGCCTCGGCGGATCAGCCCGCGCCCCGTAAAACGGG
ACGGCCCGCCCGAGGACCCCTAAACTCTGTTTTTAGTGGAACTTCTGAGTAAAAC
AAACAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGA
AGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATC
GAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGA
GCGTCATTTCAACCCTCAAGCTCAGCTTGGTGTTGGGACTCGCGGTAACCCGCGT
TCCCCAAATCGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAATCATACACC
TCGTTACTGGTAATCGTCCGGCCACGCCGTTAAACCCCAACTTCTGAATGTTGAC
CTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

	Description	Max score	Total score	Query cover	E value	Ident	Accession
	Fusarium equiseti 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sec	1011	1011	75%	0.0	100%	KT277307.1
	Fusarium equiseti isolate TMC small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed s	1009	1009	74%	0.0	100%	MG515225.1
	Fusarium sp. 141GP/S internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribos	1009	1009	74%	0.0	100%	GQ352488.1
	Fusarium sp. BAB-3343 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, comple	1007	1007	75%	0.0	99%	KU504317.1
	Fusarium sp. strain L-TA-BP22-F 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer	1003	1003	74%	0.0	99%	KX856913.1
	Fusarium equiseti genomic DNA sequence contains 18S rRNA gene, ITS1, 5.8S rRNA gene, isolate RA1_3, clone R_A_1	1003	1003	74%	0.0	99%	LT617634.1
	Fusarium equiseti isolate T34 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, c	1003	1003	74%	0.0	99%	FJ459976.1
	Fusarium equiseti isolate MO157 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer	1002	1002	74%	0.0	99%	KX197955.1
	Fusarium sp. BAB-3607 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, comple	1002	1002	75%	0.0	99%	KM066588.2
	Fusarium sp. BAB-3867 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, comple	1002	1002	74%	0.0	99%	KM066540.2
	Fusarium sp. BAB-4036 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, comple	1002	1002	74%	0.0	99%	KM051401.1
	Fusarium equiseti 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sec	1002	1002	75%	0.0	99%	HQ332532.1
	Fusarium sp. FIESC_22a strain NRRL 34002 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcri	1002	1002	75%	0.0	99%	GQ505715.1
	Fusarium sp. NRRL 31160 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, com	1002	1002	75%	0.0	99%	GQ505696.1
	Fusarium incarnatum isolate Cjmgnf2 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transc	1000	1000	75%	0.0	99%	KY436233.1

Fig 4.23 Blast results of SeF5 fungal isolate

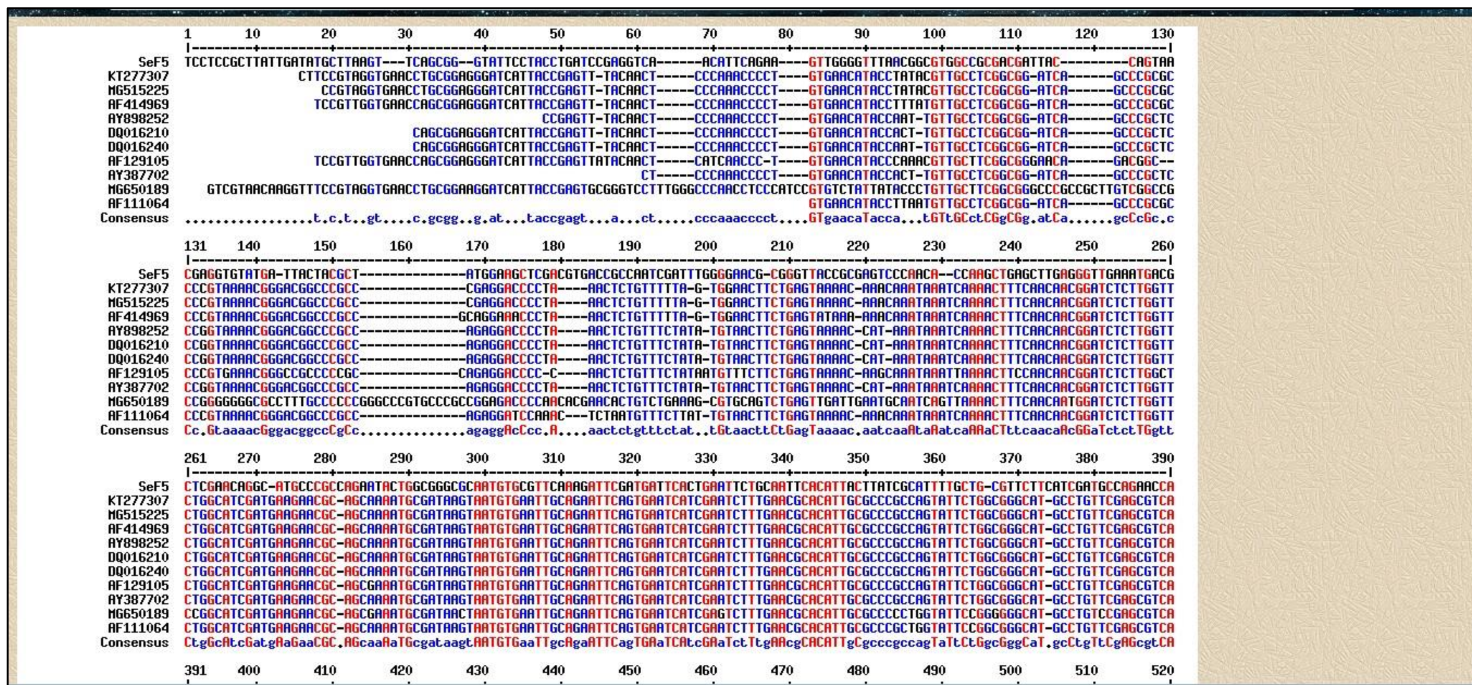


Fig 4.24 Multiple sequence alignment pf SeF5 fungal isolate

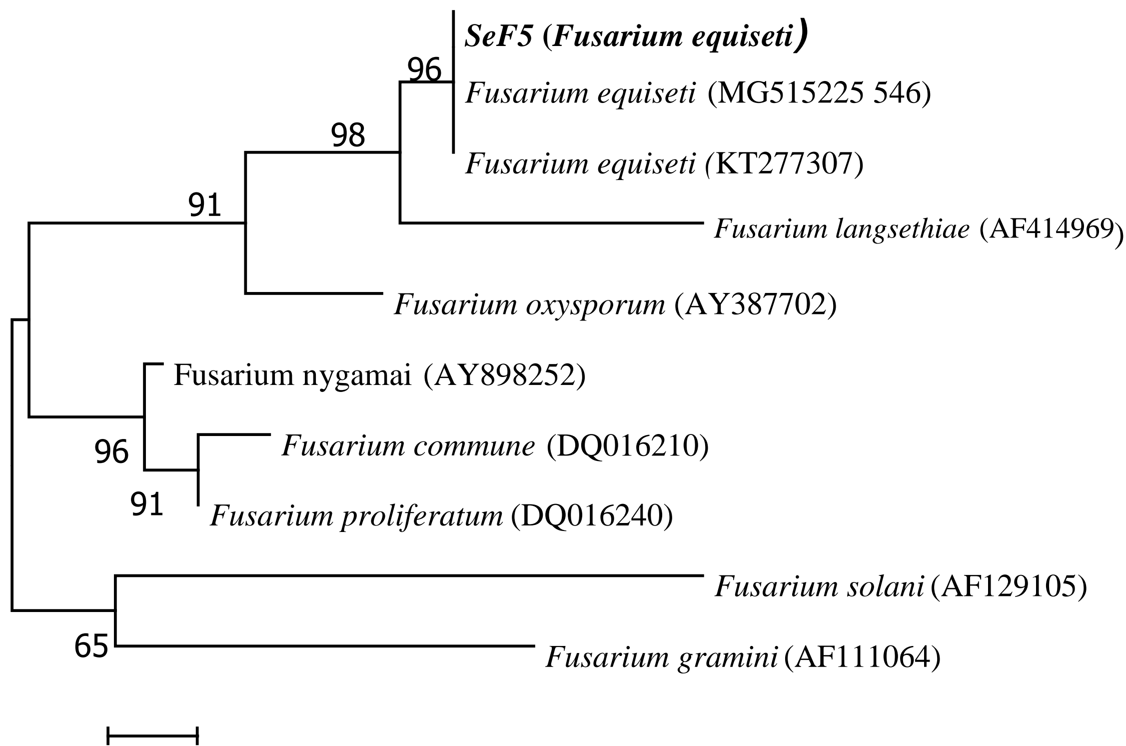


Fig 4.25 Phylogenetic tree of *Fusarium equiseti* (SeF5)

SeF12 R

TCCGTAGGTGAACCTGCGGAGGGATCATTATAGAGTTTTCTAAACTCCCAACCCA
TGTGAACTTACCTTTTGTTGCCTCGGCAGAGGTTACCTGGTACCTGGAGACAGGT
TACCCTGTAGCAGCTGCCGGTGGACTACTAAACTCTTGTTATTTTATGTAATCTG
AGCGTCTTATTTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA
TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTG
AATCATCGAATCTTTGAACGCACATTGTGCCCATTAGTATTCTAGTGGGCATGCC
TGTTCGAGCGTCATTTCAACCCTTAAGCCTAGCTTAGTGTTGGGAATTTACAGTT
ATGTAATTCCTGAAATACAACGGCGGATCTGTGGTATCCTCTGAGCGTAGTAAAT
TATTTCTCGCTTTTGTCAGGTGCTGCAGCTCCCAGCCGCTAAACCCCCAATTTTTT
GTGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAA
GCGGAGGAA.

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

	Description	Max score	Total score	Query cover	E value	Ident	Accession
	Pseudopestalotiopsis theae strain BPC50 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed space	1020	1020	100%	0.0	99%	KM510412.1
	Pestalotiopsis theae 18S small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, con	1014	1014	100%	0.0	99%	EU833970.1
	Pseudopestalotiopsis theae isolate LH13 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer	1009	1009	100%	0.0	99%	HQ832793.1
	Pseudopestalotiopsis camelliae-sinensis isolate LB06 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transc	1007	1007	99%	0.0	99%	KX757712.1
	Pseudopestalotiopsis camelliae-sinensis isolate LB05 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transc	1007	1007	99%	0.0	99%	KX757711.1
	Pseudopestalotiopsis camelliae-sinensis isolate LB01 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transc	1007	1007	99%	0.0	99%	KX757707.1
	Pseudopestalotiopsis camelliae-sinensis isolate LB16 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transc	1007	1007	99%	0.0	99%	KY319133.1
	Pseudopestalotiopsis camelliae-sinensis isolate LB15 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transc	1007	1007	99%	0.0	99%	KY319132.1
	Pestalotiopsis theae HA-33 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete s	1007	1007	99%	0.0	99%	AY924293.1
	Pestalotiopsis sp. 29V 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete seque	1005	1005	98%	0.0	99%	JF304634.1
	Pseudopestalotiopsis theae strain BPC11 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed space	1003	1003	100%	0.0	99%	KM510413.1
	Pseudopestalotiopsis camelliae-sinensis isolate LB10 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transc	1002	1002	99%	0.0	99%	KX757716.1
	Pseudopestalotiopsis camelliae-sinensis isolate LB09 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transc	1002	1002	99%	0.0	99%	KX757715.1
	Pseudopestalotiopsis camelliae-sinensis isolate LB04 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transc	1002	1002	99%	0.0	99%	KX757710.1
	Pseudopestalotiopsis camelliae-sinensis isolate LB03 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transc	1002	1002	99%	0.0	99%	KX757709.1
	Fungal endophyte isolate 308 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complet	1002	1002	99%	0.0	99%	KP015371.1

Fig 4.26 Blast results of ITS 18S RNA region of SeF12 fungal isolate

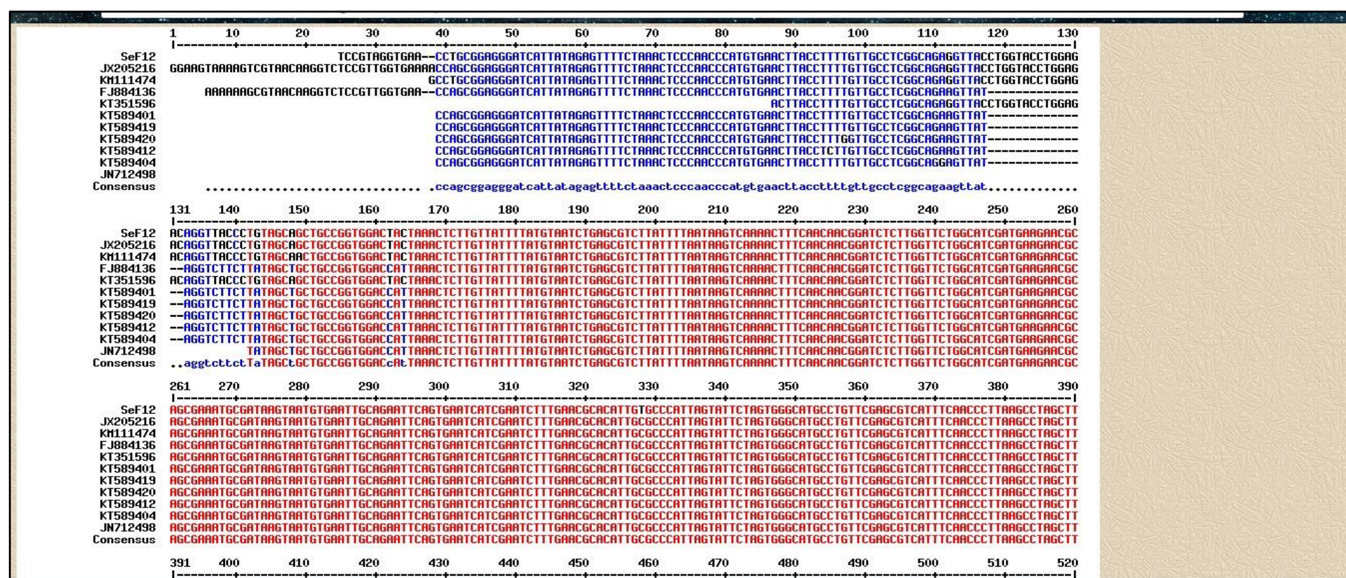


Fig 4.27 Multiple sequence alignment of SeF12 fungal isolate *Pseudopestalotiopsis theae* (SeF12)

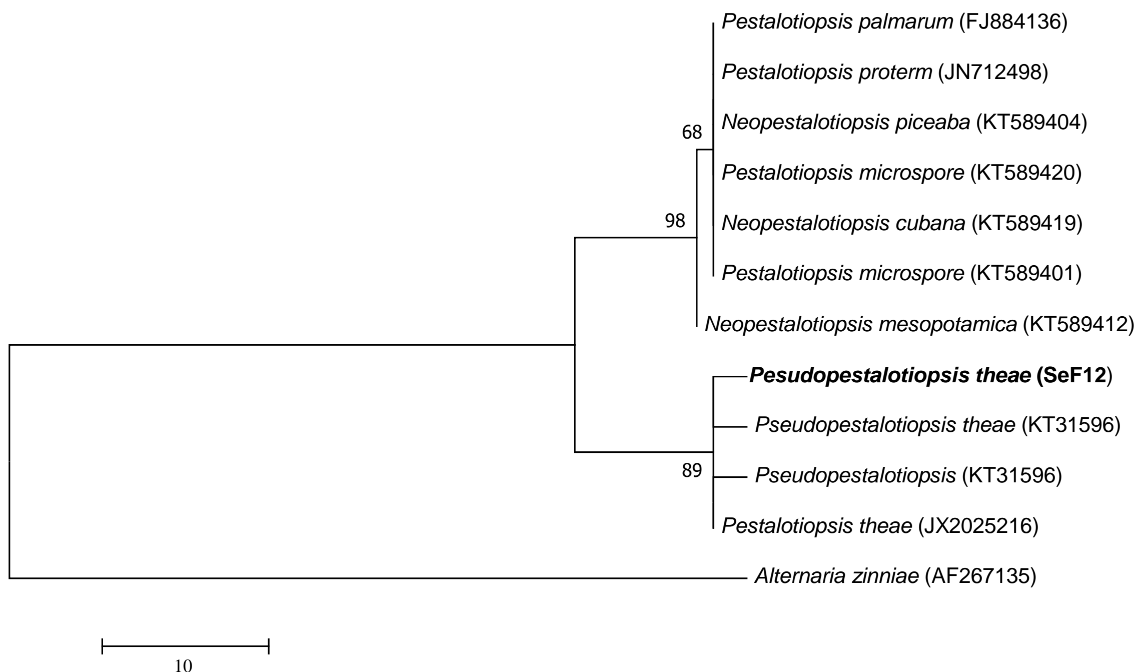


Fig 4.28 Phylogenetic tree of *Pseudopestalotiopsis theae*

4.9. Physiochemical charactersitics of soil:

Table 4.9 Physiochemical characteristics of wheat soil sample

S.No.	Physiochemical characterisitics	Wheat soil sample	
1	Soil pH	8.38±0.21	-
2	Available phosphorus	0.3021±0.491	
3	Total phosphorus	2.148 ± 0.0044.	
4	Organic carbon	15.50% 4.35	
5	Nitrogen	0.48± 0.03	

4.10. Effect of fungi on plant growth promotion

4.10.1. Field experiment

To check the effect of SeF5 and SeF12 on crop production**,** field trails for *Zea mays* plant was conducted at TIFAC Core, TIET. The seleniferous bulk soil used in the experiment was brought from the seleniferous region of Jainpur village situated in Nawanshahr region of Punjab. Surface sterilization of maize seeds was done by washing with 95% ethanol and 3% sodium hypochlorite for 10 minutes. After surface sterilization, washing was done with sterile distilled water for 4-5 times. Seed inoculation for SeF5 and SeF12 was done by mixing the fungal spores

in 10% sugar and 40% guar gum to form a slurry in which maize seeds were dipped to form the uniform coating of fungal inoculum around the seeds. These seeds were dried overnight in shade and used for sowing next day. Maize seeds coated with 40% gum arabic and 10% sugar was served as a control for both the isolates.

4.10.2. Analysis *Zea mays* plants after harvesting

After 2 months of planting of maize plants, they were harvested and various parameters were measured for control plants, plants inoculated with SeF5 and SeF12. Measurements like root length, shoot length and whole plant length of all the test plants was noted down (Table 4.12). The height of inoculated maize plants was compared with control maize plant (Figure 4.19). Roots and shoots of test plants were compared with those of control plants (Figure 4.20). The roots and shoots were separated and kept in oven at 50˚C for removal of all the water content inside the plant tissue. The dry weight of roots and shoots was noted (Figure 4.21).SS

Table 4.10 Effect on various plant tissues inoculated with fungal isolates (SeF5 and SeF12)

Treatment	**Parameters**			
	Shoot height (cm)	**Root length (cm)**	**Dry shoot weight (mg)**	**Dry root weight (mg)**
Control	17.1±0.03c	13.0±0.05b	505±0.03c	108±0.07c
SeF5	19.2±0.12a	29.9±0.09a	690±0.06a	197±0.03a
SeF12	19.0±0.15a	22.8±0.07b	672±0.05b	132±0.03b

Values are Mean±SD (n= 3). Means sharing common letter within the column are not significantly different at P<0.05.

Shoot length, root length and whole plant length of both the isolates were more as compared to control plant. Isolates showed the property of good biofertilizers and use of micoorganisms in plant growth promoting is not harmful for plants and environment. Both the isolates helped the plant in selenium stress conditions. Out of both the isolated SeF5 showed more shoot length as compared to SeF12. The comparision of SeF5 and SeF12 maize plant was done with control maize plant without fungal inoculum. Seleniferous soil brought from the Jainpur village

situated in Nawanshahr region of Punjab was used for the experiment. Both the fungal isolates act as a biofertilizers and showed all the plant growth promoting activities like IAA production, HCN production, siderophore production, ammonia production which help the plant in its growth and development. Both the isolates provided nutrients to the maize plant, no chemical fertilizers was added externally. SeF5 and SeF12 showed maximum growth as compared to control. Selenium estimation was done in dry, shoots and roots of maize plant.

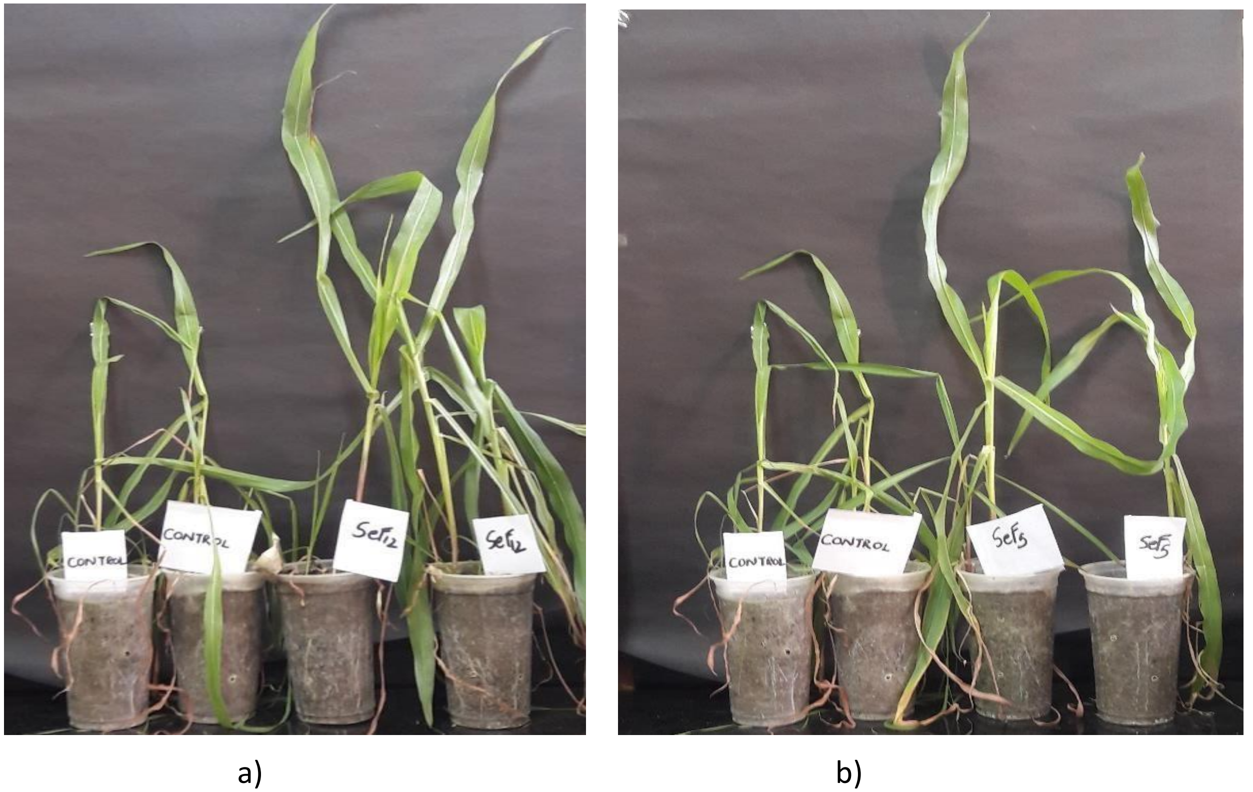


Fig 4.29 Comparing SeF12 and SeF5 with control plant after 2 months. (a) SeF12 with control; (b) SeF5 with control.

Control maize plant without fungal inoculum

Maize plant inoculated with SeF5 fungal inoculum

c)

Control maize plant without fungal inoculum

b)

Maize plant inoculated with SeF12 fungal

Control maize plant roots without SeF12 fungal inoculum.

c

Maize plant roots with SeF12 fungal inoculum

Control maize plant without SeF12 fungal inoculum

d

Maize plant roots SeF12 fungal inoculum

Fig 4.30 Roots and whole maize plant of SeF12 and SeF5 with control plant. (a) SeF5 whole plant; (b) SeF12 whole plant; (c) SeF5 roots (d) SeF12 roots.

DISCUSSION

Seleniferous” means elevated in Se. Seleniferous soils are those where Se concentration is found to be more than 5mg/kg. Soil containing Se concentration from 0.1-5 mg/kg is known as a seleniferous soil because crops grown on that soil contains Se concentration greater than 4 mg Se/kg (Dhillon and Dhillon 1997). Agricultural land of 1000 hectares is affected by the high level of selenium. The soil of Jainpur and Barwa village of Punjab is affected by the elevated level of Se. The range of Se in soil and crops varies from 2.7-6.5 mg Se/kg and 13-670 mg Se/kg respectively (Sharma et al. 2009). In our study we had estimated 5 mg Se/kg in soil sample.

Most of the Se bioremediation studies have been done on bacteria but researchers have found the contribution of fungi too in selenium bioremediation process. Recently researchers have exploited various filamentous fungi which play a key role in Se bioremediation process (Urik et al. 2014). We had isolated two fungal strains *Fusarium equiseti* and *Pseudopestaliotiopsis theae* from soil sample rich in selenium concentration. Se tolerance of fungal isolates was checked at different Se concentration (20-100 mM) of sodium selenite. Both the isolates were tolerant upto 80 mM concentration of sodium selenite. Transformation the sodium selenite to elemental Se (0) by producing red colour broth (Rosenfeld et al. 2017) had screened six fungal species for Se tolerance at different Se concentration (0-10 mM). In another research, three fungal strains, *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp. were isolated from seawater present near the industrial area contaminated with Se. Among isolated fungal isolates *Penicillium* could survive up to a higher concentration of 50 ppm of Se concentration (Jaya et al. 2013).

In our work Se in fungal biomass was estimated by Fluoresence spectrometry after growing fungus for 14 days in MMN medium supplemented with sodium selenite. Accumulation level of *Fusarium equiseti* and *Pseudopestaliotiopsis theae* was checked at 20mm, 60mm and 100mm. As the concentration of sodium selenite increases bioaccumulation of selenium by fungal isolates decreases. *Fusarium equiseti* bioaccumulate sodium selenite by biosorption mechanism up to 569.35 µg Se/mg dry weight of fungal biomass at 20 mM concentration of sodium selenite in liquid broth. But at higher concentration of 100 mM of sodium selenite it accumulates only 185.15 µg Se/mg dry weight of fungal biomass.

Pseudopestaliotopsis theae bioaccumulate concentration of sodium selenite up to 669.5 µg Se/mg) at 20mM. Another study reported fungi named *Aspergillus clavatus* was investigated for bioaccumulation and biotransformation of sodium selenate form of Se from an aqueous medium. Sodium selenate was removed efficiently at lower concentration and at higher concentration it was biotransformed to volatile derivatives. They used inductively coupled plasma optical emission spectrometry for estimation of Se in fungal biomass after growing fungus for 14 days in medium supplemented with sodium selenate. *Aspergillus clavatus* bioaccumulate of sodium selenate up to 2.3 mg/g dry weight of fungal biomass and biovolatilized up to 2.8 mg/g. The fungus removed the Se even at a higher concentration of 89 mg/L from aqueous media by bioaccumulation. These filamentous fungi proved to be of great benefit for removal of sodium selenate from Se contaminated water (Urik et al. 2016).

Fusarium equiseti and *Pseudopsetaliotiopsis theae* had showed plant growth promoting activities like IAA production, siderophore production, HCN production and phosphate solubilization. Pandya and Saraf (2010) Fungal species belonging to genera *Fusarium, Trichoderma, Aspergillius* and *Phoma* act as a plant growth promotinf fungi (PGPF) which are non-pathogenic in nature and prove to be beneficial for the growth of various crop plants and shield them from diseases. In our study *Fusarium* (64.278 µg/ml) and *Pseudopestatiloposis theae* (52.89 µg/ml) produces this much concentration of IAA helps in growth of *Zea mays* plant. Mwashasha (2013) isolated 120 pure fungal isolates and screened them for IAA production. Six isolates show 5% positivity for IAA production and provided great benefit in the growth of rice plants. Phosphate solubilization of 10 mm was showed by both the fungal isolates.

Reddy et al (2002) reported two fungal isolates named *Aspergillus tubingenesis* and *Aspergillus niger* with the ability to solubilize the rock phosphate. Among all the isolates *Aspergillus tubingenesis* showed maximum phosphate solubilization than all other isolates. Maximum solubilization was shown in the presence of 2% rock phosphate. Filamentous fungi are greatest producers of organic acids. Siderophores was produced in low iron stress conditions. *Fusarium equiseti* and *Pseudopestaliophosis theae* produces siderophore by showing blue/purple halo zone. The percentage of siderophore produced by both the isolates were 15.50% and 31.68%

respectively. In another study, the authors have studied siderophore production by two fungal species *Aspergillus niger* and *Aspergillus parasiticus* used as a Plant Growth Promoting Fungi (PGPF). *A. niger* shows 80% and *A. parasiticus* showed 73% siderophore units.

CONCLUSIONS

“Seleniferous” means elevated in Se. There are two types of soil, seleniferous and non-seleniferous depending on the Se concentration present in agricultural products grown in the soil. Seleniferous soils are those where Se concentration is found to be more than 5mg/kg. Soil containing Se concentration from 0.1-0.5 mg/kg is known as a seleniferous soil because crops grown on that soil contains Se concentration greater than 4 mg Se/kg (Dhillon and Dhillon 1997). Four study we had collected seleniferous soil from Jainpur village situated in Nawanshahr region, Punjab. This region soil is rich in Se concentration and by using fluoresence spectromertry we had found that Se concentration in soil was 5 mg/kg. Various phsysiochemical characteristics of soil was measured like pH, available phosphorus (0.3021±0.4), total phosphorus (2.148±0.004), carbon (15.50%) and nitrogen (0.48±0.03). The soil pH was 8.38±0.21, soil is alkaline in nature due to pH more than 7.0.

Nine fungal strains were isolated from the seleniferous soil named SeF1, SeF2, SeF4, SeF5, SeF6, SeF7, SeF9, SeF10 and SeF12. Tolerance level of all the isolates were checked at different concentrations of sodium selenite (10-100 mM) in MMN broth. Then, plant growth promoting activities like IAA production, phosphate solubilisation, ammonia production, HCN production and siderophore production was checked for all the isolates. From all the isolates, two strains SeF5 (64.278 µg/ml) and SeF12 (52.89µg/ml) showed IAA production. Three isolates SeF5 SeF12 and SeF4 showed phosphate solublization but SeF5 and SeF12 showed more amount of phosphate solubilization index. Siderophore production was showed by SeF5, SeF3, SeF12, SeF4. Maximum siderophore units were given by SeF5 (15.50%) and SeF12 (31.68%). HCN production was negative for all the isolates. Ammonia production was showed by SeF5 and SeF12 by giving dark brown colour. Both the isolates showed plant growth promoting activities.

Isolates named SeF5 and SeF12 was selected for nursery experiments because these both strains was highly tolerant and showed fungal mycelium up to 80 mM. Filamentous fungi are great bioacccumulators and help in plant growth promotion in Se stress conditions by showing detoxification mechanism which changes Se into less toxic form. In our work Se in fungal biomass was estimated by Fluoresence spectrometry after growing fungus for 14 days in MMN medium supplemented with sodium selenite. Accumulation level of *Fusarium equiseti* and

Pseudopestaliotiopsis theae was checked at 20 mM, 60 mM and 100 mM. As the concentration of sodium selenite increases bioaccumulation of selenium by fungal isolates decreases. *Fusarium equiseti* showed (569.35 µg Se/mg) dry biomass of bioaccumulation at 20 mM sodium selenite and *Pseudopestaliotiopsis theae* showed (669.5 µg Se/mg) of dry biomass of bioaccumulation at 20 mM. *Fusarium equiseti* root biomass (6.15 µg Se/mg) and shoot biomass (9.315 µg Se/mg) and *Pseudopestaliotiopsis theae* showed root biomass (53.36 µg Se/mg) and shoot biomass (8.195 µg Se/mg). With increase in the concentration of sodium selenite bioaccumulation decreases with decreases in fungal mycelium.

These fungal strains showed all the plant growth promoting activities. Nursery experiment was conducted for both the isolates on maize plants with 10 replicates and comparision was done with control maize plant. Various parameters like shoot length, root length, dry weight of shoots and roots were measured and compared with control maize plant. Roots and shoots of both the isolates was more in weight and longer than control plant root and shoots. Fungal strains isolated from seleniferous soil helped in plant growth promotion by providing all the nutrients to *Zea mays* plant inoculated with seeds coated with *Fusarium* and *Pseudopestaliotiopsis theae* showed more shoot length, root length, dry weight of shoots and roots as compared to control plant. Both the isolates act as a great biofertilizers by providing all the nutrients to the plants. Fertilizers were not added from outside in the plant.

The future prospects of study, these fungal isolates can be used in bioremediation of Se from contaminated areas. Fungi help in removal of Se by detoxification mechanisms. Plant growth promotion of crops and helps the plants to grow in stress conditions. Both the fungal isolates can survive upto 80 mM concentration of sodium selenite. Isolates helps in selenium removal by accumulation and changing it into less toxic form which is not harmful for environment.

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