DIVERSITY OF FUNGAL ENDOPHYTES IN DIFFERENT VARIETIES OF WHEAT GROWN IN PUNJAB

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THESIS SUBMITTED
IN PARTIAL FULFILMENT OF THE REQUIREMENT OF THE DEGREE OF

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IN

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By

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CERTIFICATE

This is to certify that the thesis entitled ***"Diversity of fungal endophytes in different varieties of wheat grown in Punjab"*** being submitted by **Ms. Harleen Kaur Walia (Roll No-601504004)** in partial fulfillment of the requirements for the award of degree of Master of Technology in Biotechnology, Thapar University, Patiala, Punjab is a bonafide work carried out under the supervision and conception of Dr. Sanjai Saxena and that no part-of this thesis has been submitted for the award of any other degree.

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CANDIDATE'S DECLARATION

I hereby declare that the work being presented in the thesis entitled ***"Diversity of fungal endophytes in different varieties of wheat grown in Punjab"*** in partial fulfilment of the requirements for the award of degree of Master in Biotechnology, Department of Biotechnology, Thapar University, Patiala is my own laboratory work during the period of July 2016 to July 2017, under the conception and supervision of **Dr. Sanjai Saxena,** Professor, Department of Biotechnology (DBT), Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.

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This is to certify that the above statement made by the candidate is correct and true to the best of my knowledge

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Date: Harleen Kaur Walia

Place:

This thesis is truly dedicated to my parents and my senior...

For their love, support and encouragement

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ABBREVIATIONS

S.No.	Abbreviation	Full form
1.	µg	Micro gram
2.	µl	Micro litre
3.	µM	Micro molar
4.	DNA	Deoxyribonucleic acid
5.	dNTP	Deoxynucleotide triphosphate
6.	DPPH	1,1-diphenyl-2-picrylhydrazyl
7.	EDTA	Ethylene diamine tetra acetic acid
8.	Et Br	Ethidium bromide
9.	HPLC	High performance liquid chromatography
10.	ITS	Internal transcribed spacer
11.	L	Litre
12.	LSU	Large subunit
13.	MEA	Malt extract agar
14.	mg	Milli gram
15.	MHA	Muller hinton agar
16.	MHB	Muller hinton broth
17.	ml	Milli litre
18.	ng	Nano gram
19.	PCR	Polymerase chain reaction
20.	PDA	Potato dextrose agar
21.	PDB	Potato dextrose broth
22.	pH	Potential of hydrogen
23.	PLA	Pine leaf agar
24.	rpm	Revolutions per minute
25.	RT	Room temperature
26.	SNA	Synthetic nutrient deficient agar
27.	TAE	Tris acetate EDTA

28.	UV	Ultra violet
29.	WA	Water agar

EXECUTIVE SUMMARY

Abiotic and biotic stresses are reducing the crop yield and productivity. There is an urge to increase abiotic and biotic stress tolerance and nutrient uptake efficiency of plants due to climatic changes in order to meet the global food demand. The earlier methods which aimed at improving crop cultivars viz. the introduction of the hybrids, increasing the agricultural inputs in terms of fertilizer, pesticides, water, herbicides and the practices of crop management. These methods helped in increasing the photosynthetic efficiency, growth and yield of the plant. Due to increase in population there is need to increase the production to meet the ever increasing demand. The method which is not detrimental to health of the human beings and for the soil. So, there is an urge to explore the "endophytic fungal" diversity associated with plant.

The endophytes are the micro- symbionts which reside within plant without causing any harm to plant. Endophytic fungi are considered to be ware houses of plethora of bioactive compounds which exhibits antimicrobial, antifungal and immunosuppressive activities. The present study reports the exploration of diversity of fungal endophytes from the two high yielding varieties viz. HD2967, WH1105 of wheat (*Triticum aestivum*) grown in Punjab. In this study the isolation was done after every 15 days from the day of sowing till the flowering stage. The isolates obtained in the study were then subjected to production in potato dextrose broth for screening its biological activity in terms of the antimicrobial and antioxidant activity of culture filtrates. The polyphenols present in the culture filtrate were further ascertained using HPLC, wherein 8 standard polyphenols as well as the crude wheat grass extract was also analyzed.

The potential endophytic fungus isolated were identified through classical tools. ITS region of approximately 500 bp-600 bp was amplified and further speciation of one fungal isolate was deduced after analyzing sequencing data of ITS region and rest are under process.

Chapter – 1

INTRODUCTION

1. INTRODUCTION

Abiotic stresses such as extreme temperature, heavy metal toxicity, drought and salinity are the leading cause of crop loss globally which are causing a reduction by more than 50% (Latef et al., 2016; Rodriguez et al., 2004). As the human population globally is increasing there is an enhanced demand of increasing food supplies. Wheat is a staple crop of the world, the cultivation of which dates back more than 5000 years ago during the Indus civilization, wherein, the original species *Triticum sphaerococcum* popularly known as Indian wheat was grown. However, with advances in agriculture and breeding sciences the properties of wheat were improved through generations (Reynolds et al., 2009; Dodd et al., 2011) and the present day species are *Triticum aestivum* (common bread wheat), *Triticum durum* (macroni wheat) and *Triticum dicoccum* (emmer wheat) which are presently grown (Singh et al., 2015).

India is the second largest producer of wheat in world and Punjab, Haryana and Uttar Pradesh are the three states which are referred to as the predominant wheat producers apart from Madhya Pradesh (Directorate of wheat annual report 2011-2012, 2012-13). However, there is an increasing concern with respect to the productivity and yield of wheat with increase in environmental temperature and weathering water supply for irrigation in these states. There are reports wherein a 5.5% drop in worldwide production and yield has been reported due to climatic variability (www.world-grain.com). Thus, it becomes imperative to understand the impact of climate change on these crops and further elevate these stresses to maintain and meet the productivity of the crops (Kumar et al., 2013; Saxena et al., 2013). It is widely recognized that fungal symbionts are associated with every plant in the natural ecosystem wherein, they colonize completely or partially in the internal tissues of host plant (Strobel et al., 2003; Lee et al., 2012; Arnold et al., 2007). This suggests that the unique niche of the symbiotic micro-organisms present inside the plant plays significant role in adaptation to overcome different biotic and abiotic stresses. It has also been reported by recent literature survey that mutualistic or symbiotic endophytic fungi contribute to fitness of plant by providing tolerance to adopt abiotic stress and promote nutrient acquisition (White et al., 1992; White et al., 2010; LópezRáez et al., 2010; Latef et al., 2016). This is very important aspect in natural and agricultural systems for plants to cope with increasingly adverse growing conditions.

Thus, there is an increase interest in understanding the diversity of the fungal endophytes associated with the monocot and dicot plants for developing biotechnological application to improve plant stress tolerance and sustainable food production by the staple crops (Xie et al., 2014; Bonfante et al., 2010). This concept is new in India as staple crops have not been bio-prospected for the presence of fungal symbionts during different stages of growth and screen for their prospective use for combating abiotic stress and for improvement of crop yield (LópezRáez et al., 2010; Latef et al., 2016; Bonfante et al., 2010; Xie et al., 2014).

Thus, it is imperative to explore the myco-symbiont of the Indian wheat variety (viz. HD 2967, WH 1105) which are grown as major crop in Punjab.

Chapter – 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. WHEAT

Wheat is one of the most important cereal food in India after rice and is most widely cultivated staple food crop in the world. It is a good source of nutrition, protein, vitamin of group-B, mineral and dietary fiber; but the nutritional composition of wheat grain may be affected due to the environmental conditions (Simmonds, 1989). Wheat is rich source of minerals like iron and zinc, the minerals which led to the most widespread nutrient deficiency in the world. The cereals including wheat is rich source of these two minerals. The daily uptake of wheat contains approximately 25% zinc and 44% of the iron (Henderson et al., 2007). Wheat has application in animal feed, brewing of wheat beer, ethanol production and for manufacturing various bakery products. The dough which is made from wheat flour gives it an advantage over other crops, which thus can be processed into a range of breads, bakery products, noodles, pasta and other processed foods. These properties is due to the gluten protein fraction. The predominant carotenoid present in wheat is lutein and in comparison to the endosperm fraction, germ/bran fractions of wheat contains carotenoids and the antioxidant activity in greater amounts (Alan et al., 2000).

The America's USDA's Dietary Guidelines states that it is essential for all adults to consume whole grains three times at least every day. It helps in reduction of death rates as it prevents from both cancer and heart diseases. The whole grains, antioxidant, vitamins, fiber or trace minerals, phytochemicals may provide protection against heart disease (Kumar et al., 2011).

2.1.1. Classification of Indian Wheat

Wheat belongs to the poaceae family and is an annual plant. It belongs to genus *Triticum*. Percival (1921) was the person who described and recognized 18 species of wheat. The 3 common classification of Indian wheat is given below:

a) Common bread wheat (*Triticum aestivum*) (2n = 6x = 42): it is grown in Punjab, Uttar Pradesh, Bihar and Parts of Rajasthan; the Indo-Gangetic plains which contains alluvial soil.
b) Emmer or Khapli wheat (*Triticum dicoccum*) (2n = 2x = 14): It is said that it is developed from *T. diccoides* Koru (a wild form). It's grown mostly in Maharashtra, Tamil Nadu, Karnataka;

the southern part of India. It is also grown in Spain, Italy, Germany and Russia. The contribution by this type of wheat grown in India is about 2%.

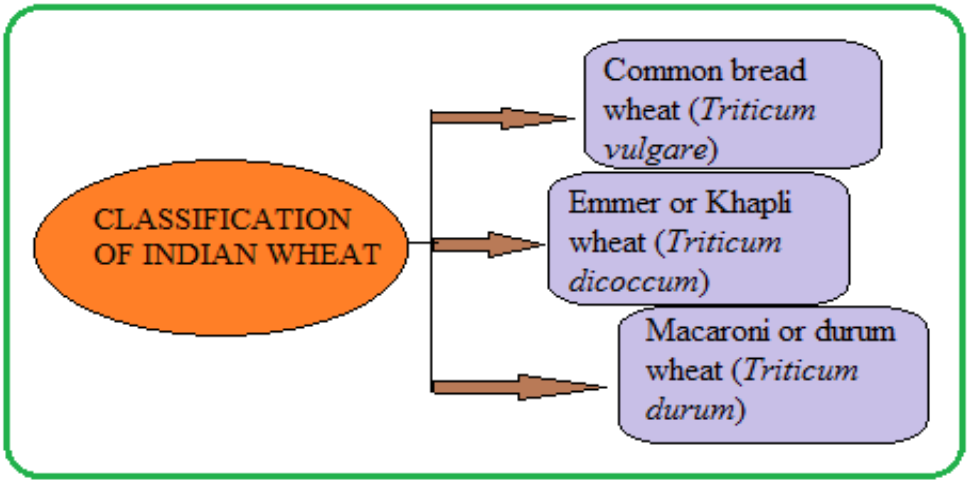


Fig. 2.1 : Classification of Indian wheat

c) Pasta or macroni wheat (*Triticum durum*) (2n=4x=28): This kind of wheat is suitable for drought conditions or in states where there is condition of restricted irrigation like West Bengal, Karnataka, Punjab, Tamil Nadu, Madhya Pradesh, Gujarat and Himachal Pradesh. It is also grown in other countries viz. Italy, USA, Canada, and Russia. The contribution by this type of wheat grown in India accounts for about 12% (Singh et al., 2015).

2.1.1. Wheat growing zones in India

Wheat being one of the major crop of India but, there is variation in climatic conditions. Wheat being second most important staple food, it is grown almost in all parts of India. Geographically, India has been divided into six zones where it is grown, namely :

1. Northern hills zone (NHZ)
2. North Eastern Plain Zone (NEPZ)
3. North Western Plain Zone (NWPZ)
4. Peninsular Zone (PZ)
5. Central Zone (CZ)
6. Southern Hills Zone (SHZ)

Table 2.1 : Different zones including, the states, area covered , average productivity and major constraints in the growth of wheat

ZONE	STATE	AREA COVERED	AVERAGE PRODUCTIVITY	MAJOR CONSTRAINTS
Northern Hills Zone (NHZ)	Himachal Pradesh, Jammu and Kashmir, Uttaranchal, north eastern states, Sikkim and hills of West Bengal (WB)	0.8 million hectares	16.64 q/ha	water stress, late sowing, rusts like yellow and brown and plant population is low
North Eastern Plain Zone (NEPZ)	East part of Uttar Pradesh, Jharkhand, Bihar, Assam, Orissa, WB, Sikkim and plains of far eastern states which are under condition of irrigation	9.5 million hectares	25.1 q/ha	Delayed sowings, weeds, leaf blight and brown rust, lack of seeds of improved varieties, plant population low and deficiency of zinc
North Western Plain Zone (NWPZ)	Punjab, Haryana, Delhi, Rajasthan and Western U.P, Jammu and Kathua district J&K, Paonta and una district valley of HP and Uttaranchal Pradesh's tarai region	9.5 million hectares	39.4 q/ha	Yellow and brown rusts, weeds, termites, powdery mildew, Karnal bunt and foliar blight
Peninsular Zone (PZ)	Southern states of Maharashtra, Karnatka, Goa, Andhra Pradesh and plains of Tamil Nadu	1.5 million hectares	29.8 q/ha	Grain discoloration, attack of aphid, leaf and brown rust and water stress
Central Zone (CZ)	Chattisgarh, Jhansi division of UP, MP, Gujarat and Kota and Udaipur division of Rajasthan	4.5 million hectares	24.1 q/ha	Termites, pervalant drought conditions, leaf and stem rust and rodents
Southern Hills Zone (SHZ)	Hills of Kerala and Tamil Nadu comprising the Palni and Nilgiri hills of southern plateau	0.2 million hectares	10 q/ha	attack of birds, black rust, attack of termites followed by lodging and delayed sowing

(Source : http://www.krishisewa.com/agroclimatic-zones/wheat-growing-zones.html)

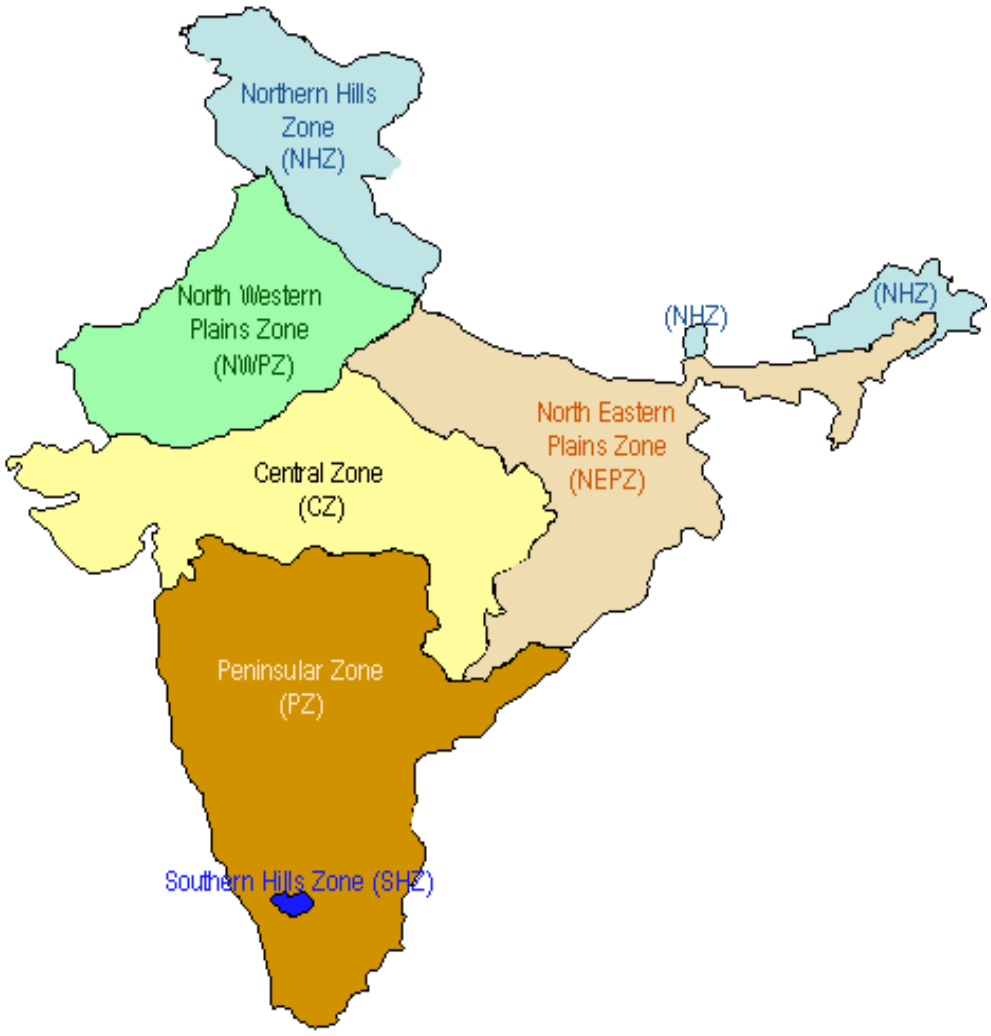


Fig. 2.2 : Map depicting different zones in India where wheat is grown

(Source : http://www.krishisewa.com/agroclimatic-zones/wheat-growing-zones.html)

2.1.2. Nutritional Value Of Wheat

Wheat is rich in mineral salts, magnesium, calcium, potassium, silicon, zinc, chlorine, manganese, arsenic, iodide, copper, vitamin B, and vitamin E. Being a rich source of nutrients it is often used as a major source of nourishment. Thus, various problems can be resolved by consumption of whole wheat, problems viz., gallstones, anemia, chronic inflammation, mineral deficiencies, breast cancer, obesity, tuberculosis, asthenia, problems related to pregnancy and breastfeeding (Hadjivassiliou et al., 2003). It is also recommended to treat sterility. The seeds are useful for treating skin diseases, cardiovascular ailments, gastrointestinal conditions and respiratory illnesses. It is also known to

reduce the risk of heart attack as it helps in balancing the cholesterol levels (Garvin et al., 2006; Grant, 1979; Reddy et al., 2000)

2.1.3. Loss in wheat productivity

The nature's wrath in terms of biotic and abiotic stress adversely affect the productivity of plant. Plants are generally exposed to stress conditions such as salt, flood, drought, high and low temperature, heavy metal toxicity and oxidative stress. There is huge loss worth hundreds of million dollars every year caused by abiotic stresses due to crop failure and loss in productivity. Thus, the sustainability of agricultural industry is threatened due to these stresses (Mahajan et al., 2005). Abiotic stress of one or other kind affects nearly about 90% of the total arable land.

Stress in physical terms is defined as the average amount of force exerted per unit area. On the other hand, the force applied by stress is hard to measure in plants and also another condition could be that it might be optimum condition for one plant while a stress factor for another plant. The stress to be defined in biological terms is difficult. Thus, an overpowering pressure of some adverse force or condition defines the biological stress that inhibits growth, normal functions and well-being of biological systems (Khan et al., 2001).

Throughout the entire life span of a plant, plant is being subjected to various environmental stresses and are thus, exposed frequently to a different stresses:

a) abiotic stresses such as salinity, heat, heavy metals, flooding, radiation and soil structure;
b) biotic stresses including weeds, pathogens and herbivores.

As plants are immobile in nature, they are affected by these environmental stress factor, thus, affecting metabolism and normal growth of plants and cause reduction in crop productivity worldwide (Mahajan et al., 2005).

2.2. Need of exploring novel source for yield improvement

Due to global food security challenge there is requirement of continuous improvement in crop yields and agricultural production practices. The climatic changes and the increase in agricultural production requires innovative ways to increase abiotic and biotic stress tolerance and improve nutrient uptake efficiency in crop plants to meet the future global demand of food. The breeding efforts led to the "Green Revolution" in the mid-late 20^{th} century, which aimed at improving crop cultivars, the introduction of the hybrids, increasing the agricultural inputs in terms of fertilizer, pesticides, water, herbicides and the practices of crop management. The various management practices and genetic approaches are only able to increase the growth, yield and the photosynthetic efficiency of wheat (Reynolds et al., 2009; Dodd et al., 2011). As the human population is increasing day by day and it is estimated that it will reach nine billion by 2050. There will be an increase in food production due to increase in population along with change in climatic changes. Thus, the need to work upon the novel source of "endophytic fungi" which is associated with the crop plant, which can be used as the microbial inoculant and might have the potential to improve quality of seed and thus, further improve abiotic tolerance capacity of wheat (Hubbard et al., 2013).

2.3. Endophytic fungi

Most of the plants present on this planet have a kind of symbiotic relationship with the microbes. Thus, the microbes can be epiphytic - those which reside on the surface of the plant especially leaves and thus can be detrimental to the plants health; secondly those associated with roots are known as mycorrhizas and lastly the endophytes – those present inside the plant part (Strobel et al., 2003; Arnold et al., 2007). Thus, the endophytes can be defined as the micro-organisms which reside within the plant tissue without showing the symptoms of their existence and are present virtually in all parts of the plant. These obtain nutrients from the host plant and contribute positively to the host fitness and are thus known as mutual symbionts (Larran et al., 2007; Sadrati et al., 2013). The endophytes or symbiotic microbes can be prokaryotic (i.e. bacteria) or eukaryotic (i.e. fungi) in nature.

These symbiotic microbes have been of interest due to the potential of fulfilling the discovered or undiscovered microbial diversity associated with the host plant (Tan et al., 2001). Many microbes

have been isolated and evaluated for the presence of the potientially bioactive natural products from the epiphytes. But, from past few years the interest has been shifted towards the less explored group of "endophytic fungi". The association of endophytic microbes with plant is known to exist from million of years ago and this may have begun since the plants have first appeared on the earth and thus, this association was important for movement of plant from aquatic system to the terrestrial ecosystem (Kharwar et al., 2015).

As already defined the endophytes are the endosymbionts i.e, the micro-organisms that form symptomless infections within healthy plants tissues (Carroll et al., 1977). It is said to be endophyte if it lives within a plant for at least part of its life cycle without causing any apparent disease. The transmission of endophytes is thought to be by two methods (Bright et al., 2010):

(a) horizontally (among individuals) or

(b) vertically (directly from parent to offspring).

Thus, the transmission of endophytes whether horizontally (Fig. 2.4) or vertically should be well understood during the study of diversity of endophytes associated with the plant.

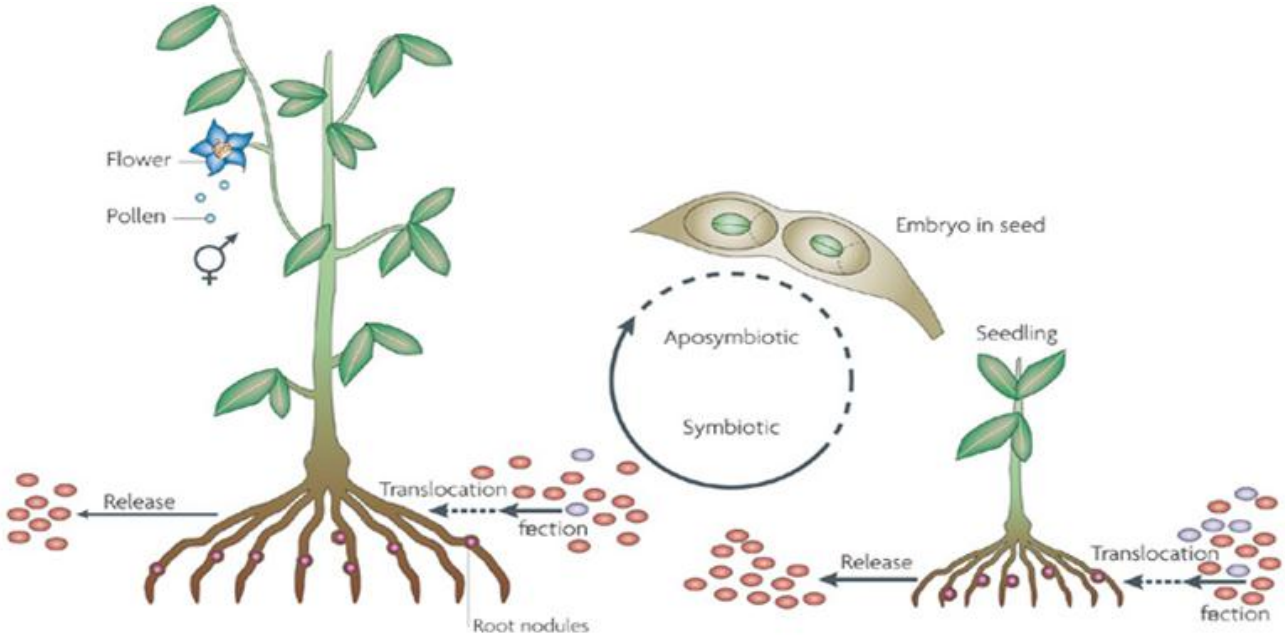


Fig. 2.3 : The horizontal transmission of endophytes in plant

The endophytic fungi has gained the attention of scientists to explore them as the potential producers of novel and bioactive compounds (Strobel et al., 2003). Over past two decades, different valuable bioactive compounds possessing insecticidal, antimicrobial, cytotoxic and anti-cancer activities have been discovered from endophytic fungi (Table 2.2). These bioactive compounds could be classified mainly as steroids, alkaloids, terpenoids, isocoumarins, quinones, phenylpropanoids, lignans, lactones and phenols (Zhang et al., 2006; Xu et al., 2008).

Table 2.2 : Some examples of bioactive compounds isolated from endophytic fungi

COMPOUND	SOURCE ORGANISM	ACTIVITY	REFERNCES
Taxol	*Taxomyces andreanae*	Prostate, ovarian, breast and lung cancers	Stierle et al., (1993)
Helvolic acid	*Pichia guilliermondii* Ppf9	Antimicrobial agent	Zhao et al., (2010)
Camptothecin	*Entrophospora infrequens*	Ovarian, small lung and refractory ovarian cancers	Puri et al., (2005)

2.3.1 The endophyte – host interaction

The endophytes are considered to be the synthesizer of various chemical substances inside the host plant predominantly the bioactive compounds which help in prevention of plants against pathogen (Sadrati et al; 2013). Thus, endophytes have various benefits which it confers to the host plant. This symbiotic relationship exist because both of them share some mutualistic benefits.

The endophytic association is found with every host studied till date, including the ones belonging to *Poaceae* family where these colonize the single cells or the tissues. The plant provides the environmental and nutritional requirements to the fungus for their survival and thus in return the endophytes protect the host plant. Hence, due to this the interest has grown towards exploring interactions between the host and endophyte (Dingle *et al.*, 2003). Thus, one interaction has been studied in depth, the fungi of genus *Neotyphodium* (*Balansiae, Clavicipitaceae*), forms the systemic association with their grass host. The fungus of this genus (i.e. *Neotyphodium*) provides host protection against herbivores, pathogens, drought, improves survival , stimulates seed germination and plant growth of grasses (Crous et al., 1995; Marshall et al., 1999).

2.3.1.1. Reduction in leaf rust disease by endophytic fungi

The endophytic and pathogenic fungi coexist in host tissue, yet the interaction between them is unknown in most cases. The interaction between the endophytic fungi and *Puccinia recondita* f. sp. *tritici* in wheat was examined in laboratory experiments (Dingle et al., 2003). This was carried out to examine whether the presence of endophytic fungi reduces the leaf rust disease caused by *Puccinia recondita* f. sp. *tritici*. It was observed that the endophytes and the cell –free washings from culture plates on endophytes reduced the density and size of pustules when inoculated 3,7 and 14d prior to the pathogen. The endophytes which colonise wheat tissue before *Puccinia tritinica* might release some of the phenolic metabolites and other defense-related compounds that may reduce the disease. Thus, to some extent the endophytes may benefit host plants by preventing pathogenic or parasitic organisms from colonizing them. Can also say that, endophytes may also produce chemicals which inhibit the growth of competitors (Jeffries et al., 1994; Larsen et al., 2001).

2.3.1.2. Endophytic fungi combat abiotic stress

Another study was carried out by Hubbard et al., (2013) where they evaluated the impact of fungal endophyte symbiosis on the growth, eco-physiological and reproductive success of wheat exposed to heat and drought. Thus, the endophytic fungi tested in their study helped in increasing the tolerance of wheat for drought and heat. The most beneficial endophyte found was SMCD 2206, followed by SMCD 2210 and 2215. It was observed that the seeds of second-generation which were produced by wheat which was dought stressed colonized by these endophytes had decreased efficiency of water use as compared to those produced by endophyte free wheat. The germination of these seeds as compared to endophyte –free seeds was also fast. Thus, the endophytes have also been found in combating abiotic stresses, including drought, salt or heat stresses (Sherameti et al. 2008; Sun et al. 2010) (Fig. 2.5).

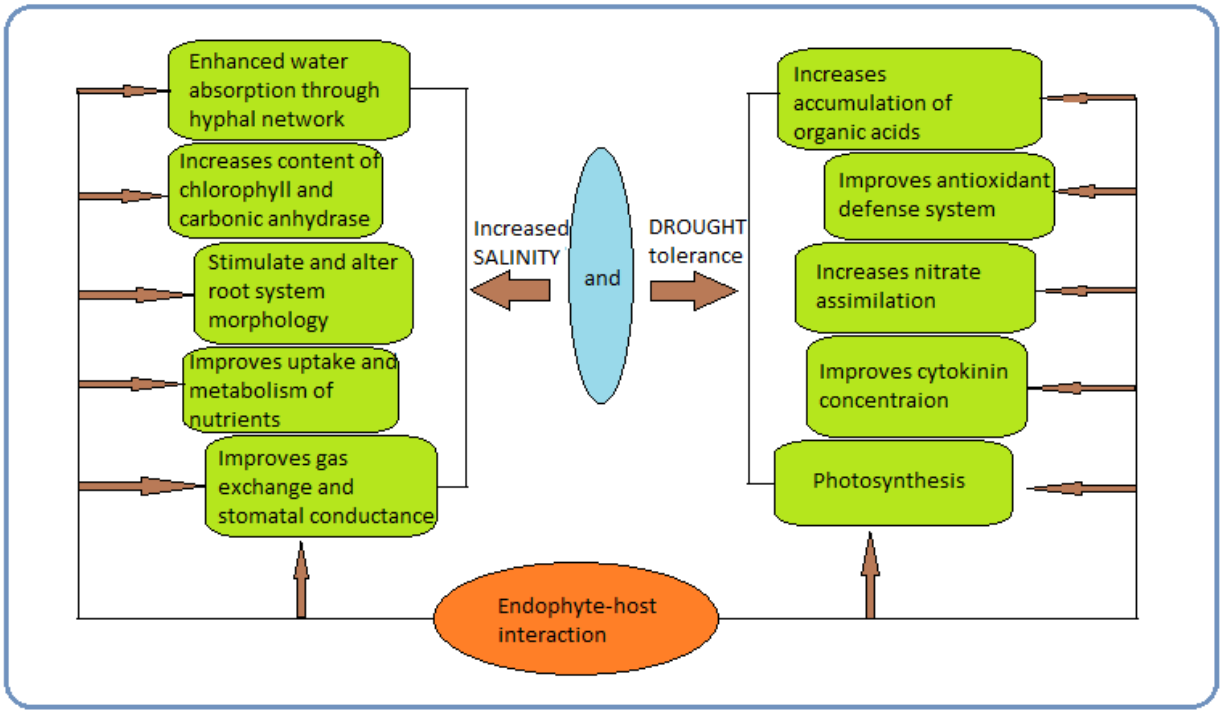


Fig. 2.4 : Schematic representation of major mechanisms underlying endophytic fungi-mediated improved tolerance in plants under salinity and drought stresses

2.3.1.3. Endophytic fungi increases plant growth

Some fungal and bacterial endophytes have proven to increase plant growth, seedling establishment, yield and grain quality (protein content and mineral composition). In a study the seed was coated with microbial consortium based on the arbuscular mycorrhizal (AM) fungus *Glomus intraradices* BEG72, *Glomus mossae* and *Trichoderma atroviride* MUCL 45632. The observation was that the coated seeds expressed higher number of shoot, leaves and root dry biomass of seedlings by 28.6%, 10.0%, 23.1% and 64.2% as compared to uncoated seeds of wheat. A decrease by 24.3% and 7.7% was observed in the grain yield in open field trials during the first and second season of growing respectively, of the uncoated seeds than the coated seeds. The inoculation of seeds by *Trichoderma* and AM fungi also increased the quality of wheat, in terms of protein content viz., P, Zn, K and Fe concentrations were also improved. It was also observed that the grain yield and stability of the coated seeds increased which was associated with increased level

of nutrient uptake both macro and micro nutrient. The application of coated seeds containing *Glomus* and *Trichoderma* can improve the crop performance of wheat in a sustainable way (Collaa et al., 2015).

Thus, due to all these benefits study on the endophytic diversity from wheat has gained a lot of attention. There is a need to increase the abiotic tolerance of wheat grown in Punjab in order to increase the productivity to meet the demand of ever increasing population. Due to change in environmental conditions it is very necessary to understand the biological diversity associated with the wheat, so that the culture which might be potent and used as inoculant for increasing the abiotic tolerance of the wheat can be identified. Table 2.3 shows some of the examples of endophytes which have been identified and used as inoculants to increase the tolerance against drought, water stress, heat and salinity in different host plants.

Table 2.3 : Some examples of fungal endophytes that conferred abiotic stress tolerance

FUNGAL ENDOPHYTE	ABIOTIC STRESS	HOST PLANT	REFERENCE
N. coenophialum	Drought/Water Stress	Tall fescue	Belesky et al., (1989) and de Battista *et al*., (1990)
N. uncinatum	Water Stress	Meadow fescue	Malinowski, (1995)
Neotyphodium sp.	Drought	*Festuca pratensis* Perennial Ryegrass *F. arizonica*	Malinowski et al., (19970 Barker et al., (1997) Morse et al., (2002)
Colletotrichum magna	Drought	L. esculentum Capsicum annuum	Redman et al., (2001)
Curvularia protuberate	Heat	Dichanthelium lanuginosum	Redman et al., (2002)
F. culmorum	Salinity	*L. mollis* *O. sativa*	Rodriguez et al., (2008)

		L. esculentum *D. lanuginosum*	
C. magna	Drought	*Triticum aestivum* Watermelon	Rodriguez and Redman, (2008)

Chapter – 3

AIM OF THE STUDY

3. AIM OF THE STUDY

1. Isolation of fungal endophytes from different stages from wheat (*Triticum aestivum*) grown in Punjab
2. Diversity analysis of fungal endophytes using classical and molecular taxonomic tools
3. Screening of the fungal isolates for their in-vitro antimicrobial and the antioxidant activity

Chapter – 4

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1. Plant material and growth

The two most widely grown and high yielding varieties of wheat in Punjab were choosen for analyzing the diversity of fungal endophytes from wheat grown in Punjab. The two varieties were: 1) HD 2967 and 2) WH 1105.

The hard wheat berries were sown in two separate experimental fields of dimension 5m X 5m. The sown wheat was watered after every 2-3 days.

4.2. Isolation of fungal endophytes

The two wheat varieties HD 2967 and WH 1105 were grown and after every 15 days the sample was collected for isolation. Samples of each organ: leaves, internode, roots and spikes were collected and immediately transported for processing. Four healthy plant part samples (i.e. leaf, internode, root, spike) were collected each time of each variety and washed under running tap water and then the surface sterilization was carried out by immersion firstly in 1% sodium hypochlorite for 1-2 min, then in 70% ethanol for 1 min followed by immersion in 30% ethanol for 45 sec and finally washing was done in sterile distilled water (Schulz et al., 1993). The surface sterilized samples were cut into small pieces of 2-3 mm with the help of sterile forceps and surgical blade. 6-8 pieces per organ were placed in each petri plate containing Potato Dextrose Agar (PDA; Hi-Media, India) supplemented with Kanamycin (1mg/ml; Hi-Media, India) with the ventral side of sample facing the medium. Two plates of each plant organ was sampled each time. The petri plates were incubated in BOD incubator (Metrex Scientific Instruments, India) at 26 ± 2˚C for 10 – 15 days with 12 h light / dark cycles and checked every two to three days for emergence of fungal endophytes. The efficiency of surface sterilization was confirmed by imprinting the surface sterilized plant tissue on the PDA plate. The absence of fungal growth after incubation confirms the efficacy of surface sterilization. The individual colonies were picked after 10 days incubation from the edge of the colony and transferred to the PDA with the help of sterile loop to get the pure culture.

4.2.1. Preservation of the endophytic fungi

The purified endophytic isolates were transferred aseptically to PDA slants and vials containing 10% glycerol for long term preservation.

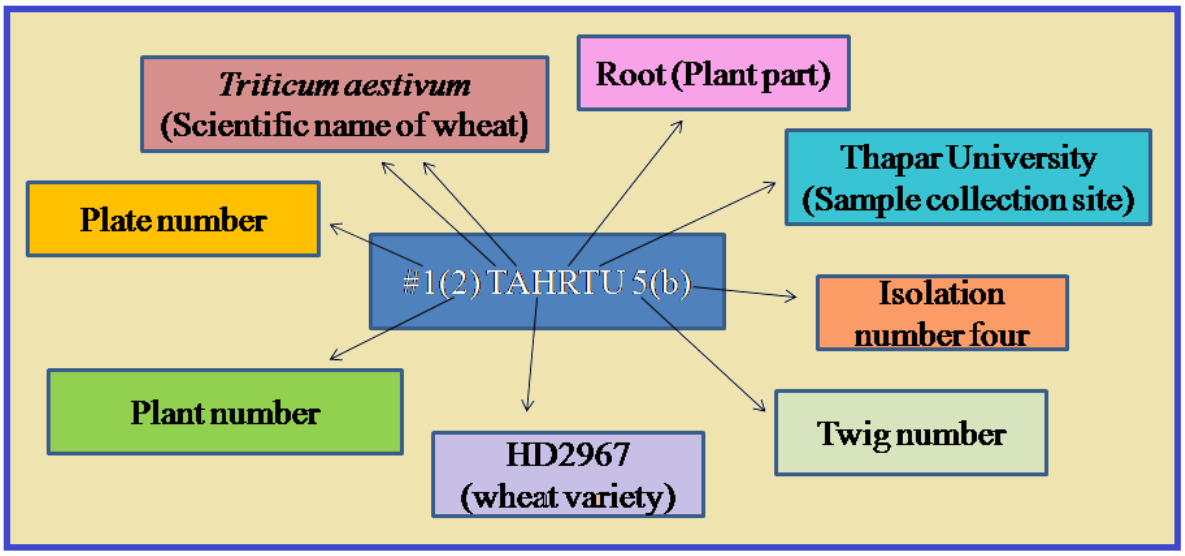


Fig. 4.1 : Method of coding the pure isolates of endophytic fungi

4.2.2. Statistical analysis

4.2.2.1. Calculation of colonization frequency

The percentage colonization frequency (%CF) of the fungal endophytes isolated during the study at different time intervals was also calculated (Hata et al., 1995). The %CF was calculated using the formula :

$$\% \text{ Colonization frequency} = \frac{\text{No.of endophytic fungi isolated}}{\text{No.of twigs inoculated}} \text{ X } 100$$

4.3. Production of culture filtrates

The endophytic cultures were subjected to culture filtrate production by inoculating 5 mm mycelial disc of 7-10 day old fungal culture to 25 ml of the pre-sterilized Potato Dextrose Broth (PDB; Hi-Media, India) medium for the induction of secondary metabolites. The flasks were incubated in orbital incubator shaker (Medilab Enterprises, India) at 26 ± 2˚C, 120 revolutions per minute (rpm) for 8 days. After the incubation was over, the spent broth was separated from mycelia by filtration through Whatman filter paper 4 (GE Healthcare Life Sciences, India) followed by centrifugation (Hitachi, Japan) at 12,000 rpm for 10 min to get the cell free filtrate (Rodrigues et al., 2000, Vicente et al., 2001). The supernatant obtained was then stored in sterile vials at -20˚C till further use.

4.4. Identification of Endophytic Fungi

The endophytic fungi isolated was identified by classical and molecular taxonomic tools.

4.4.1. Morphotaxonomy

The tentative identification of the obtained endophytic fungi was done using Nikon microscope (Japan) on the basis of their morphological and microscopic characters. If identification was not possible on the PDA plate, the endophytic fungi were incubated for further study on different media viz. 2% Malt extract agar (MEA; 30 g malt extract, 5 g mycological peptone, 20 g agar and 1000 ml distilled water with pH 5.4 ± 0.2), Synthetic nutrient deficient agar (SNA; 0.2 g glucose, 0.2 g sucrose, 1 g potassium dihydrogen phosphate, 1 g potassium nitrate, 0.25 g magnesium sulphate anhydrous, 0.5 g potassium chloride, 15 g agar and 1000 ml distilled water with pH 5.4 ± 0.2), Water agar (WA; 20 g agar in 1000 ml distilled water), Pine leaf agar (PLA; pine leaves, 20 g agar in 1000 ml distilled water). After inoculating the fungi the plates were incubated at 26 ± 2˚C for 20 – 25 days with 12 hour light / dark photo period until sporulation was obtained. Morphological characteristics such as colony color, appearance, growth rate was observed carefully and noted. For microscopic features, a drop of water was put on clean glass slide, upon which the mycelial mass was placed and teased properly with the help of fine tip needle. It was then stained with Lactophenol cotton blue (Hi Media, India). The slide was covered with 18 x 10 mm cover slip avoiding the formation of air

bubble. The slide was observed at 10X, 40X and 100X using Nikon binocular microscope (Barnett et al., 1998).

4.4.2 Molecular Identification of endophytic fungi

The identity of fungi was confirmed by using molecular tools, fungi was subjected to DNA isolation followed by amplification of ITS and LSU region and further construction of phylogenetic tree to ensure accurate taxonomic placement.

4.4.2.1 DNA Isolation

The fungal genomic DNA was isolated from 5-7 day old culture grown on PDA plate using Wizard® Genomic DNA purification kit (Promega, USA). 6-7 mycelial plugs of 5 mm diameter were scooped out. The mycelia plugs were crushed in mortar-pestle using liquid nitrogen (N_2). 1ml cell lysis solution and 600µl of nuclei lysis solution was added and crushed again in mortar-pestle. The solution was transferred to the fresh eppendorf and incubated at 65°C for 15 min with intermittent mixing. After incubation the eppendorfs were kept at room temperature (RT) for 5 min. The eppendorfs were then centrifuged at 12,000 rpm for 3 min to remove the cell debris. Then 5µl of RNAse was added and incubated at 37°C for 5 min followed by addition of 200µl of protein precipitation solution. The eppendorfs were centrifuged at 12,000 rpm for 3 min to remove contaminating proteins. The aqueous phase containing DNA was transferred to 600-700µl of chilled iso-propanol in the eppendorf and centrifuged at 12,000 rpm for 3 min. The DNA pellet was rinsed with 70% ethanol followed by centrifugation at 13,000 rpm for 1 min. Then pellet was air dried and dissolved in 50µl of DNA dehydration buffer (Tris EDTA buffer (pH 8)). The qualitative estimation of the DNA isolated was done by agarose gel electrophoresis.

4.4.2.2 Agarose gel electrophoresis

0.8% agarose gel containing 0.5µg/ml of ethidium bromide (EtBr, visualizing dye) was prepared in 1X TAE (Tris Acetate EDTA) buffer and then the gel was casted in the electrophoretic apparatus. The gel was allowed to solidify and the comb was removed off carefully. The running buffer (1X TAE) was poured into the electrophoretic tank. The DNA sample (4µl) was mixed with the 6X loading dye (3µl) and subsequently loaded into wells and allowed to run at 60V for 1h. The DNA fragments were Documentation System using Quantity-1-D analysis software.

Quantitative estimation of the genomic DNA sample was done by using nanodrop and spectrophotometer. The absorbance of the sample was taken at 260nm and 280nm. 1 O.D is equivalent to 50 µg/ml of DNA sample. The concenteration of DNA was calculated by following formula–

$$\text{Concentration } (\mu g/ml) = O.D_{260\ nm} \times 50\mu g/ml \times \text{Dilution factor}$$

If the ratio (260/280) is less than 1.6, then there is RNA contamination, if the ratio lies between 1.6-1.8, then DNA sample is pure. If the ratio is more than 1.8, the DNA might be contaminated with protein.

4.4.2.3 PCR amplification

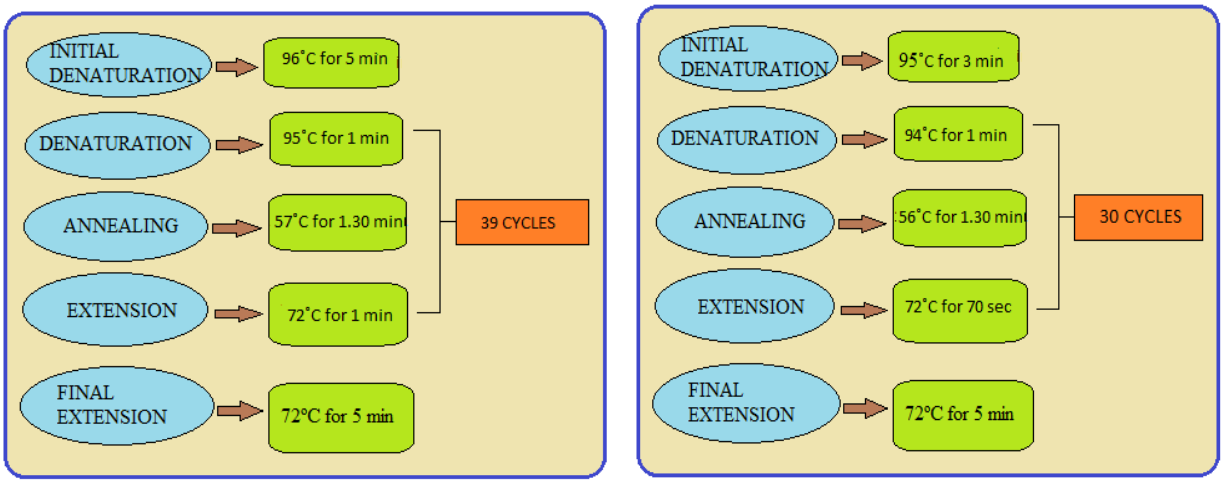
ITS1-5.8S-ITS2 rDNA sequence was amplified using the pair of universal primer i.e. ITS 1 and ITS 4 , synthesized by Integrated DNA Technologies (IDT), USA, in a Thermocycler (My Cycler, Bio- Rad Laboratories, Inc.). Amplification reaction was carried out by using the primers ITS1 (5' TCC GTA GGT GAA CCT GCG G 3') and ITS 4 (5' TCC TCC GCT TAT TGA TAT GC 3') (White *et al.*, 1990). rDNA sequence was also amplified using the LSU primers i.e. LROR and LR 5 (LSU primers), synthesized by Integrated DNA Technologies (IDT), USA, in a Thermocycler (My Cycler, Bio- Rad Laboratories, Inc.). Amplification reaction was carried out by using the primers LROR (5' ACCCGCTGAACTTAAGC 3') and LR 5 (5' TCCTGAGGGAAACTTCG 3') (Vilgalys and Hester, 1990). Amplification was performed in 25µl reaction mixture containing DNA sample in concentration range of 25-50ng, 0.8 µM of each primer (LROR and LR 5), 2.5mM of dNTP (Bangalore GeNei), 1.5 mM $MgCl_2$ (Bangalore GeNei), 1.5 U of Taq DNA Polymerase (Bangalore GeNei) in 10 X Taq buffer (Bangalore GeNei).

The Thermal cycling conditions for ITS consisted of initial denaturation at 96˚C for 5 min followed by 39 cycles of 95˚C for 1 min, 57˚C for 1.30 min, 72˚C for 1 min followed by final extension at 72˚C for 5 min (Fig. 4.4). The PCR amplicons were examined using gel electrophoresis in a 1.5 % agarose gel at 60V for 1h. Gel imaging was performed under UV light in Bio- Rad Gel documentation System.

The Thermal cycling conditions for LSU consisted of initial denaturation at 95˚C for 3 min followed by 30 cycles of 94˚C for 1 min, 56˚C for 1.30 min, 72˚C for 70 sec followed by final extension at 72˚C

for 5 min (Fig. 4.4). The PCR amplicons were examined using gel electrophoresis in a 1.5 % agarose gel at 60V for 1h. Gel imaging was performed under UV light in Bio- Rad Gel Documentation System.

An approximate 900-1100 bp PCR amplicon using LSU primers was purified by using the Wizard® SV Gel and PCR clean up system kit (Promega, USA). The purified amplicon was sent for direct sequencing to Eurofins, Bangalore.



a) b)

Fig. 4.3 : Thermal cycling conditions of PCR for amplifying rDNA of endophytic fungi using a) ITS primer, b) LSU primer

4.4.2.4. Sequence assembly and phylogenetic analysis

The obtained chromatogram was manually edited and the sequence obtained was submitted in the GenBank. The homology of the sequence was obtained by subjecting the final sequence to similarity search using BLAST. The final sequence and the reference taxa obtained was aligned using CLUSTAL W in MEGA-5 (Tamura et al., 2011). The evolutionary relationship was inferred using Maximum Parsimony method. The Maximum Parsimony tree was obtained by using the Close-Neighbor-Interchange algorithm (Nei et al., 2000) with search level 3 in which the initial trees were obtained with the random addition of sequences (20 replicates). The tree was drawn to scale, with branch lengths calculated using the average pathway method (Nei et al., 2000) and are in the units of the number of changes over the whole sequence. Gaps and missing data were eliminated. 1000 bootstrap replicates were taken to infer evolutionary relationship (Felsenstein , 1985).

4.5. Biological activity

4.5.1. In-vitro Antimicrobial Assay

The in-vitro antimicrobial assay was performed using agar well diffusion assay. The endophytic fungi were screened for its antimicrobial activity against three microbial cultures, 2 gram positive bacteria MTCC 737 (*Staphylococcus aureus*), MTCC 96 (*S.aureus*) and 1 gram negative bacteria (*Escherichia coli*). Test organisms were procured from pre-existing repository maintained by Dr. Sanjai Saxena, Professor, Thapar University, Patiala. The cultures were revived by inoculating them in Muller Hinton Broth (MHB; Hi-Media, India) one day before performing the assay. The test micro-organisms were diluted in 0.9% w/v saline and visually adjusted with 0.5 McFarland solution to achieve 10^6 CFU/ml. The test organism was then spread on the Muller Hinton Agar (MHA, Hi-Media, India) with the help of cotton swab. Subsequently 5mm wells were scooped out with sterile cork-borer on pre-made MHA plates and 30 µl of the sample was poured in each well. The inoculated plates were incubated at 37˚C for 16-24 hr. Streptomycin (1mg/ml; Hi-Media, India) was used as the positive control and the uninoculated PDB as the negative control. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms. All the tests were performed in triplicate and the results expressed as mean ± SD (Baris *et al*., 2006).

4.5.2. In-vitro Antioxidant Assay

The antioxidant activity of the culture filtrate/solvent fractions i.e. the ability to scavenge free radicals was performed using DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals, according to Kitts et al.,(2000) with slight modification. Briefly, 20 µl of the test sample (1 mg/ml) was added to 230 µl of DPPH (100 µM in methanol) and mixed thoroughly. The mixture was incubated for 30 min at room temperature in dark. After incubation is complete the absorbance was measured at 517 nm. Quercetin (10-50 µg/ml) was used as standard and working DPPH used as the control. The DPPH radical scavenging capacity was expressed as microgram of quercetin equivalents per milligram of extract.

The percentage free radical scavenging activity of the fungal extract was calculated as :

$$\% \text{ free radical scavenging activity} = \frac{(\text{O.D.control} - \text{O.D.test sample})}{\text{O.D.control}} \text{ X } 100$$

4.6. Liquid - Liquid extraction

The cultures exhibiting potent antioxidant activity were then subjected to liquid-liquid extraction for the isolation of the bioactive compound. The culture filtrate was extracted with ethyl acetate in 1:2 (culture extract : ethyl acetate) ratio. The organic layer was pooled out and anhydrous sodium sulphate added to absorb water molecules present in it. The aqueous layer or filtrate was extracted again, the extraction was done thrice. The organic layer obtained was evaporated using Rotatory Evaporator (D-Lab, India) at 30˚C to dryness to obtain the crude bioactive compound. The compound obtained was weighed and reconstituted in methanol and screened using HPLC to determine the polyphenols present in it (Kjer et al., 2010).

4.6. High Performance Liquid Chromatography

The samples showing potent antioxidant activity were evaluated for the presence of polyphenols by using HPLC (Perkin Elmer- 200 series pump). About 30µg of crude bioactive fraction was dissolved in methanol and injected into the HPLC column. The extract of wheat grass (1 mg/ml) in methanol was also prepared and injected into column to determine the different types of polyphenols present in wheat. Gradient elution of methanol and acetic acid (2%) ranging from 10% to 90% was used as mobile phase with a flow rate of 1 ml/min. To determine the type of polyphenol present 8 polyphenols were prepared (Stock- 1mg/ml) in HPLC grade methanol. 20µl of each polyphenol was injected into C18 (5 µm) reverse phase Discovery column (Sigma Aldrich) with 4.6 mm Internal Diameter x 150 mm Length. The data of the peak area vs. concentration of the standard polyphenols obtained was used to estimate the type of polyphenol present in the wheat grass and thus, the endophytic fungal extract in order to correlate that the fungal endophytes isolated from wheat possess the same polyphenols as present in wheat grass.

Chapter – 5

RESULTS

5. RESULTS

5.1. Isolation of endophytic fungi

In the present study, the endophytic fungi was isolated from wheat (Fig. 5.1, a)) and a total of 214 endophytic fungi were isolated from two wheat varieties. 112 isolates from HD2967 (Fig. 5.1, b)) and 102 endophytic fungi from WH1105 (Fig. 5.1, c)) variety of wheat were isolated. The isolation was done after every 15 days to know about the diversity present in wheat at different intervals during the growth of plant. Since, wheat is the major staple crop, its productivity and yield is decreasing due to various biotic and abiotic stresses. The earlier strategies adopted have helped in increasing the photosynthetic efficiency, growth, yield. But the use of these myco symbionts might help in increasing the tolerance to abiotic stresses and the seed quality which are the major barrier in reducing the yield. Hence, the present investigation undertakes the isolation of endophytic fungi from wheat and evaluating its antimicrobial and antioxidant property.

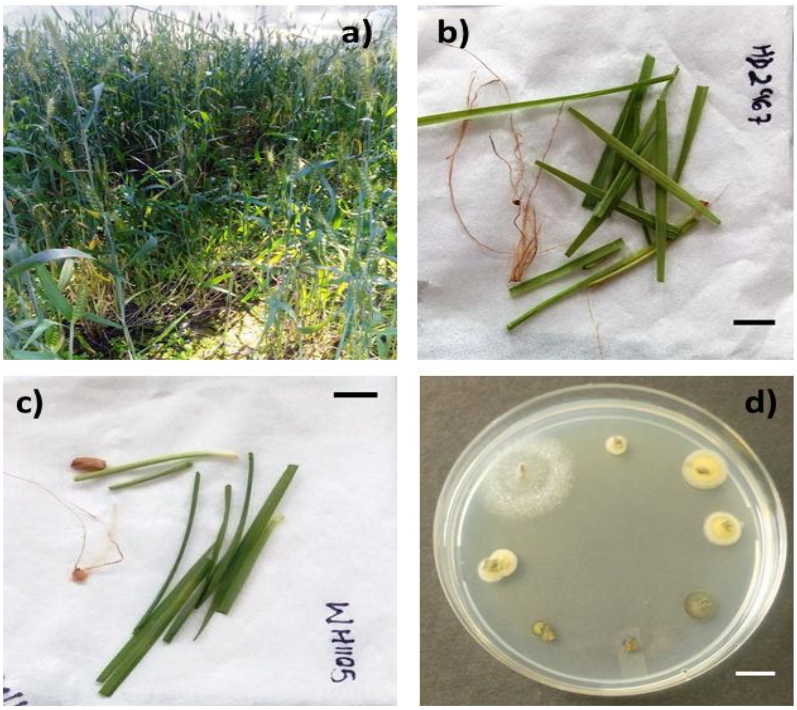


Fig. 5.1: a) Wheat grown to study the diversity of endophytic fungi; b) and c) the two wheat varieties; d) endophytic fungi emerging out from the twigs inoculated on the PDA plate (Bar : 10mm)

Out of 112 isolates from the variety HD 2967, 67 were isolated from roots, 21 from leaf, 14 from internode and 10 were isolated from spike and out of 102 isolates from WH 1105 variety of wheat 50 were isolated from root, 17 from leaf, 21 from internode and 14 were isolated from spike. These endophytic fungal isolates were tentatively identified on the basis of morphological and microscopic characteristics (Barnett and Hunter, 1998), wherein (Fig. 5.2) represents some of the pure cultures isolated during investigation, whereas, (fig. 5.3) represents the microscopic features of different types of fungal isolates.

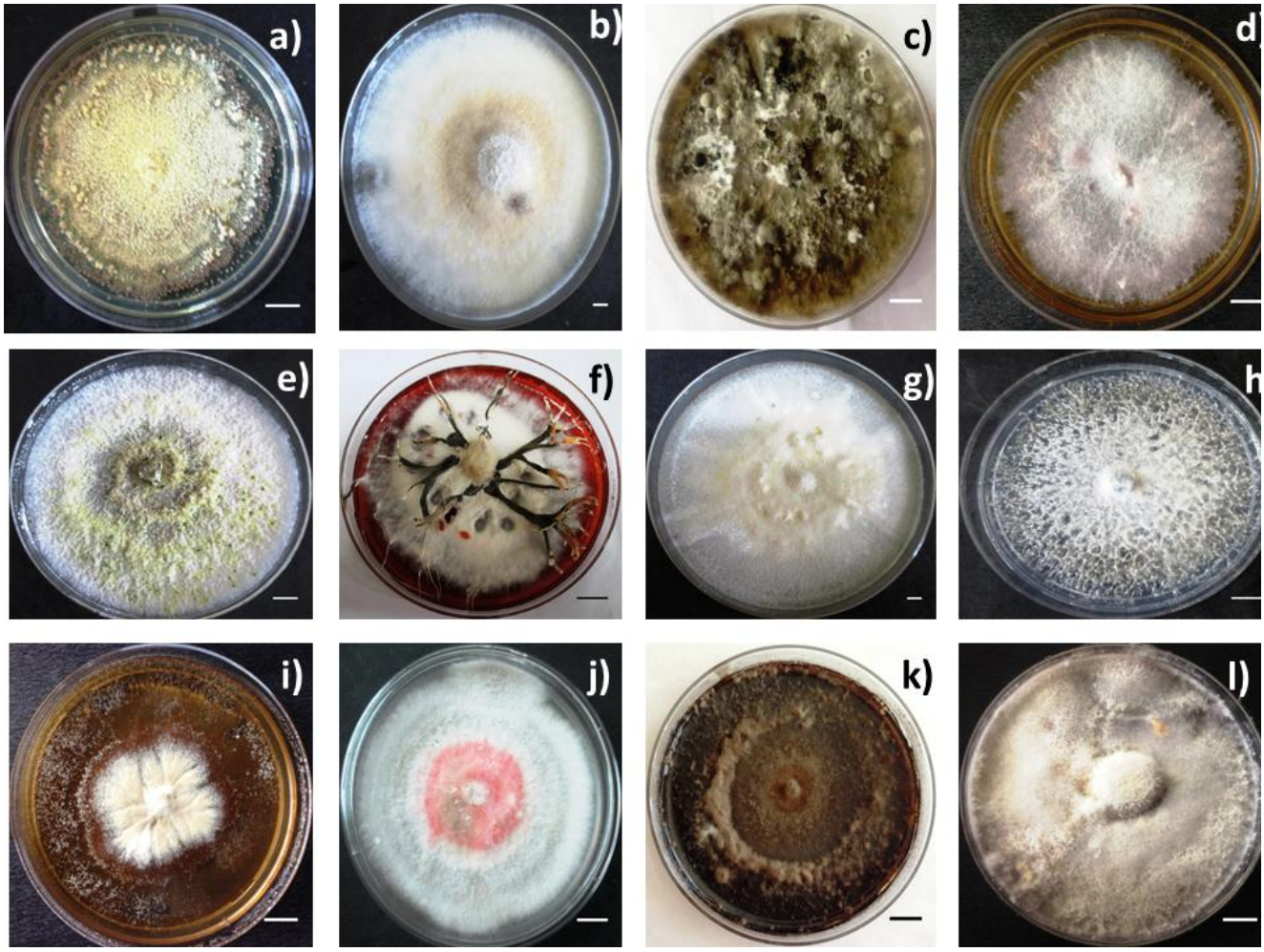


Fig.5.2 : Pure cultures of different endophytic fungi isolated from wheat a) *Aspergillus* sp.; b) *Fusarium* sp.; c) *Botryosphaeria* sp.; d) *Arthrinium* sp.; e) *Alternaria* sp.; f) *Chaetomium* sp.; g)-i) Unidentified; j) *Fusarium* sp.; k) *Nigrospora* sp.; l) Unidentified (Bar : 10mm)

CULTURE CODE	HOST PLANT	PLANT PART	SITE OF SAMPLE COLLECTION	TENTATIVE IDENTIFICATION
#6TAHRTU	*Triticum aestivum*(HD2967)	Root	Thapar University	*Botryosphaeria* sp.
#8TAHRTU	*Triticum aestivum*(HD2967)	Root	Thapar University	*Botryosphaeria* sp.
#3TAHLTU	*Triticum aestivum*(HD2967)	Leaf	Thapar University	Unidentified
#10TAHLTU	*Triticum aestivum*(HD2967)	Leaf	Thapar University	*Nigrospora* sp.
#13TAHLTU	*Triticum aestivum*(HD2967)	Leaf	Thapar University	*Nigrospora* sp.
#28TAHLTU	*Triticum aestivum*(HD2967)	Leaf	Thapar University	*Nigrospora* sp.
#1(1)TAHSTU 1	*Triticum aestivum*(HD2967)	Internode	Thapar University	*Penicillium* sp.
#6TAHLTU 1	*Triticum aestivum*(HD2967)	Leaf	Thapar University	Unidentified
#1(1)TAHRTU 2	*Triticum aestivum*(HD2967)	Root	Thapar University	*Fusarium* sp.
#2(1)TAHRTU 12	*Triticum aestivum*(HD2967)	Root	Thapar University	*Penicillium* sp.
#1(2)TAHRTU 3	*Triticum aestivum*(HD2967)	Root	Thapar University	*Arthrinium* sp.
#1(3)TAHRTU 7	*Triticum aestivum*(HD2967)	Root	Thapar University	*Collectotrichum* sp.
#1(4)TAHRTU 6	*Triticum aestivum*(HD2967)	Root	Thapar University	Unidentified
#1(1)TAHSTU 2(a)	*Triticum aestivum*(HD2967)	Internode	Thapar University	*Fusarium* sp.
#2(3)TAHSTU 16(a)	*Triticum aestivum*(HD2967)	Internode	Thapar University	Unidentified
#1(4)TAHLTU 2(a)	*Triticum aestivum*(HD2967)	Leaf	Thapar University	*Nigrospora* sp.
#3(2)TAHLTU 24(a)	*Triticum aestivum*(HD2967)	Leaf	Thapar University	Unidentified
#1(1)TAHRTU 1(a)	*Triticum aestivum*(HD2967)	Root	Thapar University	*Botryosphaeria* sp.
#2(2)TAHRTU 10(a)	*Triticum aestivum*(HD2967)	Root	Thapar University	*Phaeoacremonium* sp.
#1(3)TAHRTU 5(a)	*Triticum aestivum*(HD2967)	Root	Thapar University	Unidentified
#1(1)TAHRTU 6(b)	*Triticum aestivum*(HD2967)	Root	Thapar University	*Phaeoacremonium* sp.
#2(1)TAHRTU 10(b)	*Triticum aestivum*(HD2967)	Root	Thapar University	Unidentified
#2(1)TAHRTU 15(b)	*Triticum aestivum*(HD2967)	Root	Thapar University	*Fusarium* sp.
#1(2)TAHRTU 5(b)	*Triticum aestivum*(HD2967)	Root	Thapar University	*Fusarium* sp.
#2(2)TAHRTU 13(b)	*Triticum aestivum*(HD2967)	Root	Thapar University	*Acremonium* sp.
#2(1) TAHLTU 12(c)	*Triticum aestivum*(HD2967)	Leaf	Thapar University	*Alternaria* sp.
#2(1)TAHSTU 14(c)	*Triticum aestivum*(HD2967)	Internode	Thapar University	Unidentified
#1(1)TAHRTU 6(c)	*Triticum aestivum*(HD2967)	Root	Thapar University	*Botryosphaeria* sp.
#2(1)TAHRTU 14(c)	*Triticum aestivum*(HD2967)	Root	Thapar University	*Fusarium* sp.
#2(1)TAHRTU 15(c)	*Triticum aestivum*(HD2967)	Root	Thapar University	*Alternaria* sp.
#1(2)TAHRTU 1(c)	*Triticum aestivum*(HD2967)	Root	Thapar University	Unidentified
#1(2)TAHRTU 8(c)	*Triticum aestivum*(HD2967)	Root	Thapar University	Unidentified
#2(2)TAHRTU 14(c)	*Triticum aestivum*(HD2967)	Root	Thapar University	*Alternaria* sp.
#1(3)TAHRTU 7(c)	*Triticum aestivum*(HD2967)	Root	Thapar University	*Botryosphaeria* sp.
#2(3)TAHRTU 14(c)	*Triticum aestivum*(HD2967)	Root	Thapar University	Unidentified

#2(4)TAHRTU 11(c)	*Triticum aestivum*(HD2967)	Root	Thapar University	*Alternaria* sp.
#3TAHSPTU 17(d)	*Triticum aestivum*(HD2967)	Spike	Thapar University	Unidentified
#4TAHSPTU 29(d)	*Triticum aestivum*(HD2967)	Spike	Thapar University	Unidentified
#2(1)TAHLTU 9(d)	*Triticum aestivum*(HD2967)	Leaf	Thapar University	Unidentified
#2(4)TAHLTU 13(d)	*Triticum aestivum*(HD2967)	Leaf	Thapar University	Unidentified
#2(1)TAHSTU 9(d)	*Triticum aestivum*(HD2967)	Internode	Thapar University	Unidentified
#1(2)TAHSTU 3(d)	*Triticum aestivum*(HD2967)	Internode	Thapar University	Unidentified
#1(1)TAHRTU 1(d)	*Triticum aestivum*(HD2967)	Root	Thapar University	Unidentified
#2(3)TAHRTU 11(d)	*Triticum aestivum*(HD2967)	Root	Thapar University	Unidentified
#1TAHSPTU 5(e)	*Triticum aestivum*(HD2967)	Spike	Thapar University	Unidentified
#1(4)TAHLTU 1(e)	*Triticum aestivum*(HD2967)	Leaf	Thapar University	Unidentified
#2(4)TAHLTU 15(e)	*Triticum aestivum*(HD2967)	Leaf	Thapar University	Unidentified
#2(1)TAHSTU 16(e)	*Triticum aestivum*(HD2967)	Internode	Thapar University	Unidentified
#1(1)TAHRTU 7(e)	*Triticum aestivum*(HD2967)	Root	Thapar University	Unidentified
#1(4)TAHRTU 2(e)	*Triticum aestivum*(HD2967)	Root	Thapar University	Unidentified
#1(4)TAHRTU 6(e)	*Triticum aestivum*(HD2967)	Root	Thapar University	*Fusarium* sp.
#1 TAHSPTU 2(f)	*Triticum aestivum*(HD2967)	Spike	Thapar University	Unidentified
#6 TAHSPTU 45(f)	*Triticum aestivum*(HD2967)	Spike	Thapar University	Unidentified
#2(1) TAHSTU 10(f)	*Triticum aestivum*(HD2967)	Internode	Thapar University	Unidentified
#1(4) TAHSTU 5(f)	*Triticum aestivum*(HD2967)	Internode	Thapar University	Unidentified
#1(1)TAHLTU 4(f)	*Triticum aestivum*(HD2967)	Leaf	Thapar University	Unidentified
#1(4) TAHLTU 7(f)	*Triticum aestivum*(HD2967)	Leaf	Thapar University	Unidentified
#2(1) TAHRTU 12(f)	*Triticum aestivum*(HD2967)	Root	Thapar University	Unidentified
#2(4) TAHRTU 10(f)	*Triticum aestivum*(HD2967)	Root	Thapar University	Unidentified
#1(3) TAHSPTU 3(g)	*Triticum aestivum*(HD2967)	Spike	Thapar University	Unidentified
#2(3) TAHSPTU 10(g)	*Triticum aestivum*(HD2967)	Spike	Thapar University	Unidentified
#2(2) TAHSTU 16(g)	*Triticum aestivum*(HD2967)	Internode	Thapar University	Unidentified
#1(2) TAHLTU 7(g)	*Triticum aestivum*(HD2967)	Leaf	Thapar University	Unidentified
#2(2) TAHRTU 10(g)	*Triticum aestivum*(HD2967)	Root	Thapar University	Unidentified
#1(3) TAHRTU 8(g)	*Triticum aestivum*(HD2967)	Root	Thapar University	Unidentified
#14TAWRTU	*Triticum aestivum (WH1105)*	Root	Thapar University	*Nigrospora* sp.
#19TAWRTU	*Triticum aestivum (WH1105)*	Root	Thapar University	*Botryosphaeria* sp.
#26TAWRTU	*Triticum aestivum (WH1105)*	Root	Thapar University	Unidentified
#31TAWRTU	*Triticum aestivum (WH1105)*	Root	Thapar University	*Fusarium* sp.
#22TAWLTU	*Triticum aestivum (WH1105)*	Leaf	Thapar University	*Nigrospora* sp.
#2TAWLTU 7	*Triticum aestivum (WH1105)*	Leaf	Thapar University	Unidentified

#1(2)TAWSTU 2	*Triticum aestivum (WH1105)*	Internode	Thapar University	Unidentified
#1(2)TAWRTU 5	*Triticum aestivum (WH1105)*	Root	Thapar University	Unidentified
#1(3)TAWRTU 2	*Triticum aestivum (WH1105)*	Root	Thapar University	*Alternaria* sp.
#1(3)TAWRTU 4	*Triticum aestivum (WH1105)*	Root	Thapar University	Unidentified
#2(1) TAWLTU 12(a)	*Triticum aestivum (WH1105)*	Leaf	Thapar University	Unidentified
#2(1)TAWRTU 10(a)	*Triticum aestivum (WH1105)*	Root	Thapar University	Unidentified
#2(1)TAWRTU 15(a)	*Triticum aestivum (WH1105)*	Root	Thapar University	Unidentified
#2(1)TAWRTU 16(a)	*Triticum aestivum (WH1105)*	Root	Thapar University	Unidentified
#1(1)TAWSTU 7(b)	*Triticum aestivum (WH1105)*	Internode	Thapar University	Unidentified
#2(3)TAWSTU 12(b)	*Triticum aestivum (WH1105)*	Internode	Thapar University	*Aspergillus* sp.
#2(1)TAWLTU 11(b)	*Triticum aestivum (WH1105)*	Leaf	Thapar University	Unidentified
#1(3) TAWLTU 1(b)	*Triticum aestivum (WH1105)*	Leaf	Thapar University	*Nigrospora* sp.
#1(3)TAWRTU 2(b)	*Triticum aestivum (WH1105)*	Root	Thapar University	*Alternaria* sp.
#1(3)TAWRTU 5(b)	*Triticum aestivum (WH1105)*	Root	Thapar University	*Fusarium* sp.
#2(3)TAWRTU 16(b)	*Triticum aestivum (WH1105)*	Root	Thapar University	Unidentified
#2(4)TAWRTU 8(b)	*Triticum aestivum (WH1105)*	Root	Thapar University	Unidentified
#1(2)TAWLTU 7(c)	*Triticum aestivum (WH1105)*	Leaf	Thapar University	Unidentified
#1(2)TAWLTU 7(c)	*Triticum aestivum (WH1105)*	Leaf	Thapar University	Unidentified
#2(2)TAWSTU 9(c)	*Triticum aestivum (WH1105)*	Internode	Thapar University	Unidentified
#2(3)TAWSTU 10(c)	*Triticum aestivum (WH1105)*	Internode	Thapar University	Unidentified
#1(1)TAWRTU 3(c)	*Triticum aestivum (WH1105)*	Root	Thapar University	Unidentified
#2(1)TAWRTU 13(c)	*Triticum aestivum (WH1105)*	Root	Thapar University	Unidentified
#1(4)TAWRTU 7(c)	*Triticum aestivum (WH1105)*	Root	Thapar University	*Aspergillus* sp.
#2(4)TAWRTU 9(c)	*Triticum aestivum (WH1105)*	Root	Thapar University	Unidentified
#(5)TAWRTU 4(c)	*Triticum aestivum (WH1105)*	Root	Thapar University	*Alternaria* sp.
#1TAWSPTU 6(d)	*Triticum aestivum (WH1105)*	Spike	Thapar University	Unidentified
#2(2)TAWLTU 11(d)	*Triticum aestivum (WH1105)*	Leaf	Thapar University	*Botryosphaeria* sp.
#2(3)TAWLTU 10(d)	*Triticum aestivum (WH1105)*	Leaf	Thapar University	Unidentified
#2(1)TAWSTU 9(d)	*Triticum aestivum (WH1105)*	Internode	Thapar University	*Botryosphaeria* sp.
#2(1)TAWSTU 16(d)	*Triticum aestivum (WH1105)*	Internode	Thapar University	*Botryosphaeria* sp.
#2(2)TAWRTU 15(d)	*Triticum aestivum (WH1105)*	Root	Thapar University	Unidentified
#2(3)TAWRTU 16(d)	*Triticum aestivum (WH1105)*	Root	Thapar University	Unidentified
#1(4)TAWRTU 1(d)	*Triticum aestivum (WH1105)*	Root	Thapar University	Unidentified
#1TAWSPTU 2(e)	*Triticum aestivum (WH1105)*	Spike	Thapar University	Unidentified
#3TAWSPTU 19(e)	*Triticum aestivum (WH1105)*	Spike	Thapar University	Unidentified
#1(1)TAWLTU 5(e)	*Triticum aestivum (WH1105)*	Leaf	Thapar University	Unidentified

#1(2)TAWSTU 6(e)	*Triticum aestivum (WH1105)*	Internode	Thapar University	Unidentified
#2(3)TAWSTU 12(e)	*Triticum aestivum (WH1105)*	Internode	Thapar University	Unidentified
#2(2)TAWRTU 16(e)	*Triticum aestivum (WH1105)*	Root	Thapar University	Unidentified
#1(4)TAWRTU 2(e)	*Triticum aestivum (WH1105)*	Root	Thapar University	Unidentified
#1 TAWSPTU 3(f)	*Triticum aestivum (WH1105)*	Spike	Thapar University	Unidentified
#6 TAWSPTU 42(f)	*Triticum aestivum (WH1105)*	Spike	Thapar University	Unidentified
#1(3) TAWRTU 1(f)	*Triticum aestivum (WH1105)*	Root	Thapar University	Unidentified
#2(4) TAWRTU 16(f)	*Triticum aestivum (WH1105)*	Root	Thapar University	Unidentified
#1(2) TAWSPTU 1(g)	*Triticum aestivum (WH1105)*	Spike	Thapar University	Unidentified
#2(3) TAWSPTU 15(g)	*Triticum aestivum (WH1105)*	Spike	Thapar University	Unidentified
#1 TAWSTU 2(g)	*Triticum aestivum (WH1105)*	Internode	Thapar University	Unidentified
#1 TAWLTU 4(g)	*Triticum aestivum (WH1105)*	Leaf	Thapar University	Unidentified
#1 TAWLTU 8(g)	*Triticum aestivum (WH1105)*	Leaf	Thapar University	Unidentified
#1(1) TAWRTU 2(g)	*Triticum aestivum (WH1105)*	Root	Thapar University	Unidentified

Table 5.1: List of some endophytic fungi isolated during the study

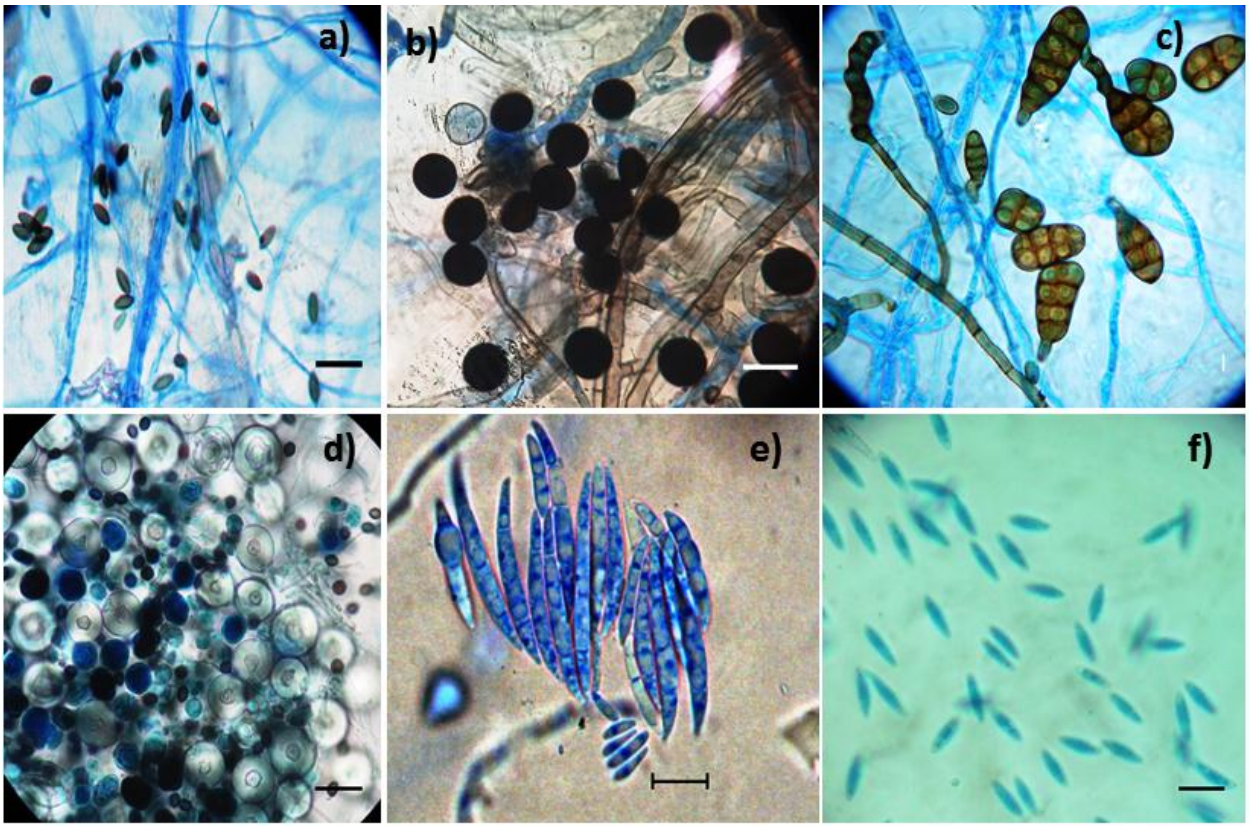


Fig. 5.3 : Microscopic feature of endophytic fungi isolated during study a) conidia of *Arthrinium* sp.; b) conidia of *Nigrospora* sp.; c) micro and macro conidia of *Alternaria* sp.; d) *Aspergillus* sp.; e) micro and macro conidia of *Fusarium* sp.; f) conidia of *Collectotrichum* sp. (Bar : 10µm)

5.1.1. Calculation of colonization frequency

The percentage colonization frequency (%CF) of endophytic fungi depicted that the endophytic load was maximum at 75 days in both the varieties of wheat, which is mainly contributed by the endophytes isolated from root. But, as the growth of wheat advances towards flowering stage the endophytic load increased in the spikes as compared to other parts of the plant (Fig. 5.4,5.5). The %CF of root, leaf, internode, spike as well as the cumulative %CF at different time intervals is illustrated in (table 5.2, 5.3).

Table 5.2 : %CF of root, leaf, internode, spike and cumulative frequency of endophytic fungi isolated from HD2967 variety of wheat

TIME INTERVAL	ROOT %CF	LEAF %CF	INTERNODE %CF	SPIKE %CF	CUMMULATIVE %CF
15 days	3.13	7.81	0	0	10.94
30 days	17.19	1.56	1.56	0	20.31
45 days	9.38	3.13	3.13	0	15.64
60 days	10.94	0	0	0	10.94
75 days	29.69	3.13	3.13	0	35.95
90 days	9.38	3.13	4.69	3.13	20.33
105 days	12.5	4.69	1.56	1.56	20.31
120 days	7.81	7.81	6.25	6.25	28.12
135 days	4.69	1.56	1.56	4.69	12.5

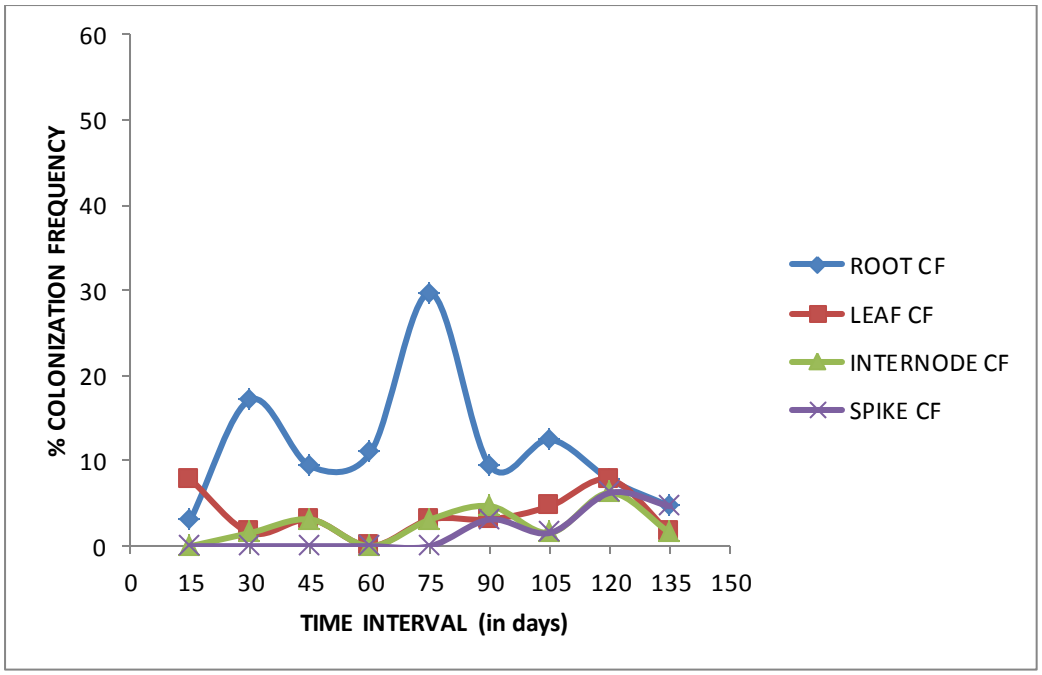


Fig. 5.4 : The %CF of HD2967 at different time intervals and from different parts of the wheat viz. root, leaf, internode and spike

Table 5.3 : %CF of root, leaf, internode, spike and cumulative frequency of endophytic fungi isolated from WH1105 variety of wheat

TIME INTERVAL	ROOT %CF	LEAF %CF	INTERNODE %CF	SPIKE %CF	CUMMULATIVE %CF
15 days	6.25	1.56	0	0	7.81
30 days	10.94	1.56	3.13	0	15.63
45 days	7.81	1.56	0	0	9.37
60 days	18.75	7.81	3.13	0	29.69
75 days	18.75	4.69	6.25	0	29.69
90 days	4.69	4.69	10.94	1.56	21.88
105 days	4.69	1.56	7.81	4.69	18.75
120 days	4.69	0	0	9.38	14.07
135 days	1.56	3.13	1.56	6.25	12.5

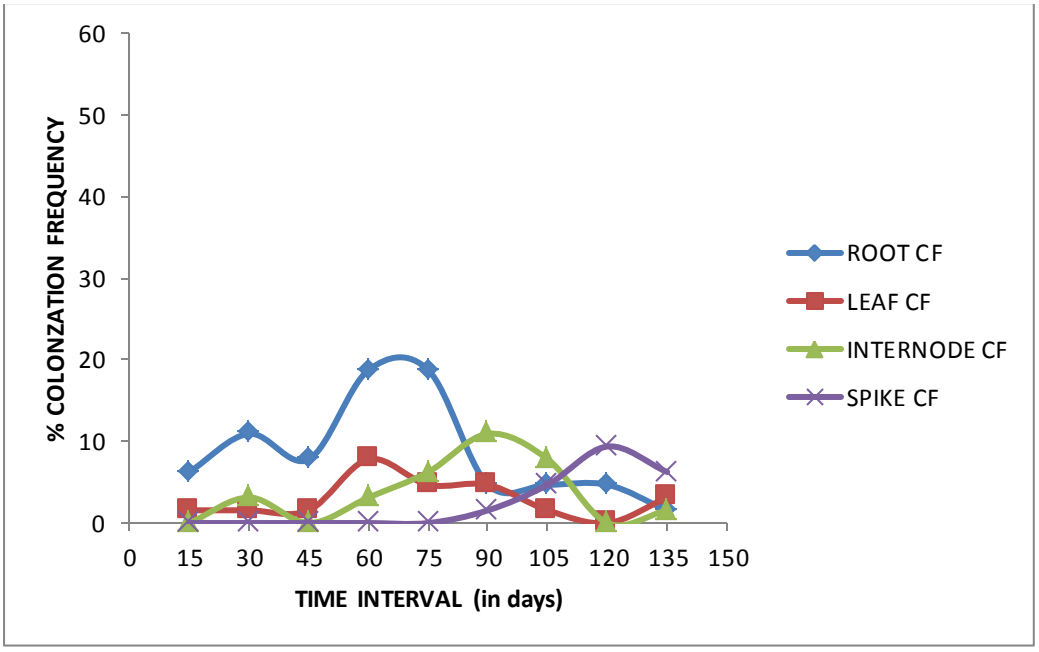
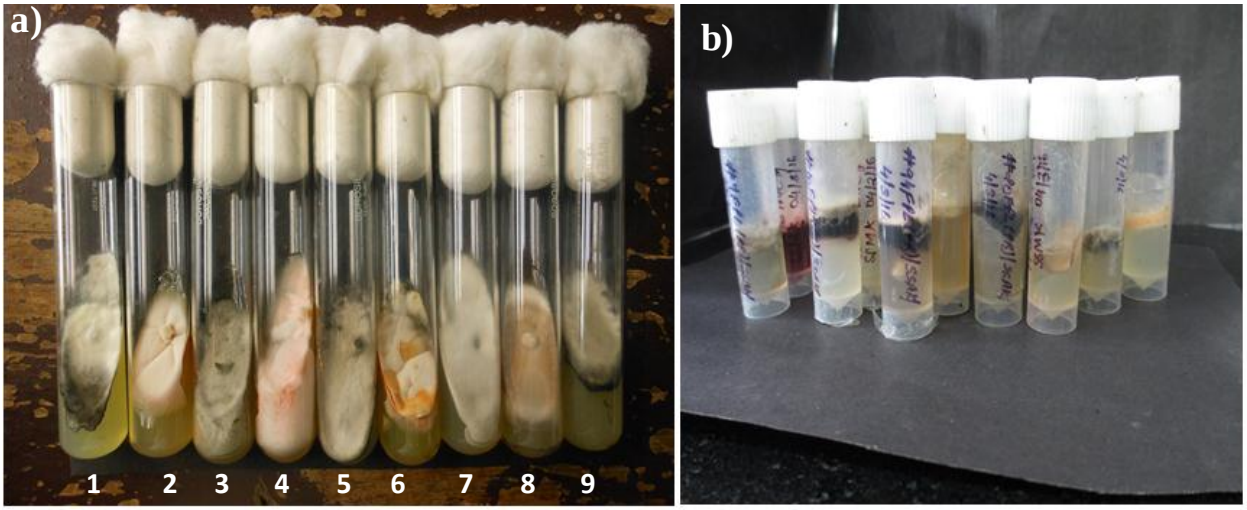


Fig. 5.5 : The %CF of WH1105 at different time intervals and from different parts of the wheat viz. root, leaf, internode and spike

5.1.2. Preservation of endophytic fungi

The isolated endophytes were aseptically maintained in the PDA vials and slants (Fig. 5.6) containing 10% glycerol for preserving them for longer period as it is not possible to preserve cultures in plates for longer duration.



1. #8TAHRTU
2. #3TAHLTU
3. #2(2)TAWLTU11(d)
4. #2(1)TAWSTU9(d)
5. #1(1)TAHRTU6(c)
6. #1(2)TAHRTU5(b)
7. #2(1)TAWRTU13(c)
8. #1(4)TAHLTU2(a)
9. #1(1)TAHSTU2(a)

Fig. 5.6 : Endophytic isolates preserved for long term storage by different methods, a) PDA slants; b) PDA vials

5.2. Production of culture filtrates

The isolates obtained from wheat in this study were subjected for the production of secondary metabolites in PDB medium. The cultures vary in their growth rate as some were slow growing and some fast growing which produced more biomass as compared to other cultures. The filtrates obtained also varied in their pH, the pH of the cultures ranged mostly between pH 4-8, except for the isolate #1(1) TAHSTU 2(a). The biomass produced of some filtrates from higher to lower along with their pH is given in (table 5.4).

Table 5.4 : Biomass production along with pH of some fungal isolate

S.No.	CULTURE CODE	BIOMASS (gm)	pH
1	#8 TAHRTU	1.51	5.10
2	#3 TAHLTU	1.31	7.32
3	#2(2) TAWLTU 11(d)	1.04	6.94
4	#2(1) TAWSTU 9(d)	0.54	7.87
5	#1(1) TAHRTU 6(c)	0.37	7.25
6	#1(2) TAHRTU 5(b)	0.20	6.14
7	#2(1) TAWRTU 13(c)	0.14	5.81
8	#1(4) TAHLTU 2(a)	0.015	5.49
9	#19 TAWRTU	0.05	4.41
10	#1(1) TAHSTU 2(a)	0.003	8.61

Fig. 5.7 : Production of culture filtrates by various fungal cultures

5.3. Biological activity

5.3.1. In-vitro Antimicrobial Assay

The antimicrobial activity of the cultures was carried out. Of the 125 cultures screened only 38 cultures showed the antimicrobial activity. The zone of inhibition of the cultures varied between 6mm to 16mm, the maximum zone obtained was of #1(1)TAHRTU 2 against MTCC 96 which was 18.33 ± 0.58 and the lowest zone of inhibition obtained was 6.33 ± 0.58 of #1(4)TAHRTU 1 against

MTCC 737. The cultures which showed positive result or the antimicrobial activity are illustrated in (Table 5.5).

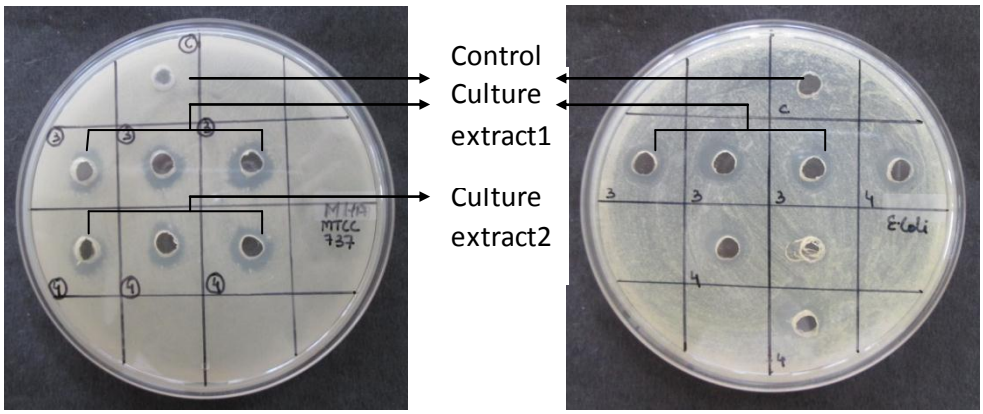


Fig. 5.8 : Plates depicting antimicrobial assay of the culture against MTCC 737 and *E.coli*

Table 5.5 : List of cultures which possess antimicrobial activity

S.No.	CULTURE CODE	MTCC 96 *	MTCC 737 *	*E.coli**
1	#6TAHRTU	9 ± 1	7.67 ± 0.58	8 ± 0
2	#1(1)TAHSTU 1	X	X	6.67 ± 0.58
3	#6TAHLTU 1	X	8.33 ± 0.58	9.33 ± 0.58
4	#1(1)TAHRTU 2	18.33 ± 0.58	15.33 ± 1.53	10.67 ± 0.58
5	#1(2)TAHRTU 3	X	6.67 ± 0.58	9.33 ± 0.58
6	#1(2)TAHRTU 8	X	6.67 ± 0.58	9.33 ± 0.58
7	#1(3)TAHRTU 1	X	10.67 ± 0.58	8 ± 0
8	#1(3)TAHRTU 3	10.33 ± 1.15	8.67 ± 0.58	9± 1
9	#1(3)TAHRTU 6	8.33 ± 0.58	10.67 ± 0.58	13.67 ± 0.58
10	#1(3)TAHRTU 7	10.67 ± 0.58	10 ± 1	8.33 ± 0.58
11	#2(3)TAHRTU 11	7.33 ± 0.58	X	X
12	#1(4)TAHRTU 1	X	6.33 ± 0.58	9.67 ± 0.58
13	#1(4)TAHRTU 6	X	7.67 ± 0.58	9 ± 0
14	#1(2)TAHRTU 5(b)	10 ± 0	X	X
15	#2(1) TAHLTU 12(c)	7.67 ± 0.58	X	10 ± 1
16	#1(1)TAHRTU 6(c)	X	10.33 ± 0.58	8.67 ± 0.58
17	#2(1)TAHRTU 11(c)	10.33 ± 0.58	8 ± 0	12.33 ± 0.58
18	#2(1)TAHRTU 15(c)	8.33 ± 0.58	X	8.67 ± 0.58

19	#2(2)TAHRTU 14(c)	11 ± 1	10 ± 1	9.33 ± 0.58
20	#1(3)TAHRTU 7(c)	8.33 ± 0.58	8.67 ± 0.58	8.33 ± 0.58
21	#4TAHSPTU 29(d)	8.33 ± 1.15	10 ± 0	10 ± 1
22	#26TAWRTU	X	6 ± 0	7.33 ± 0.58
23	#2TAWLTU 7	X	7 ± 0	8.33 ± 0.58
24	#2(4)TAWSTU 16	X	X	9.33 ± 0.58
25	#1(1)TAWRTU 5	X	10.33 ± 1.15	9.67 ± 0.58
26	#1(3)TAWRTU 4	8.67 ± 0.58	9.67 ± 0.58	12.67 ± 0.58
27	#1(3)TAWRTU 8	9.67 ± 0.58	9.67 ± 1.15	8.33 ± 0.58
28	#2(3)TAWRTU 9	10.67 ± 0.58	10 ± 1	10.67 ± 0.58
29	#2(3)TAWRTU 16	9 ± 0	9.33 ± 0.58	10.67 ± 0.58
30	#2(1)TAWRTU 15(a)	11.67 ± 0.58	12.33 ± 0.58	X
31	#2(1)TAWRTU 16(a)	10 ± 0	X	X
32	#2(2)TAWRTU 12(b)	11.67 ± 0.58	10.33 ± 0.58	X
33	#1(1)TAWRTU 3(c)	X	8.67 ± 0.58	10.33 ± 0.58
34	#2(1)TAWRTU 13(c)	7.67 ± 0.58	7.67 ± 0.58	11 ± 1
35	#2(1)TAWRTU 14(c)	X	9.67 ± 0.58	9.33 ± 0.58
36	#(5)TAWRTU 4(c)	12 ± 0	9.67 ± 0.58	11.67 ± 0.58
37	#2(1)TAWSTU 9(d)	8.33 ± 0.58	11.67 ± 0.58	9.33 ± 0.58
38	#2(1)TAWSTU 16(d)	X	X	8 ± 0

(*Data represented as mean ± SD, x- represents no antimicrobial activity)

5.3.2. In-vitro Antioxidant Assay

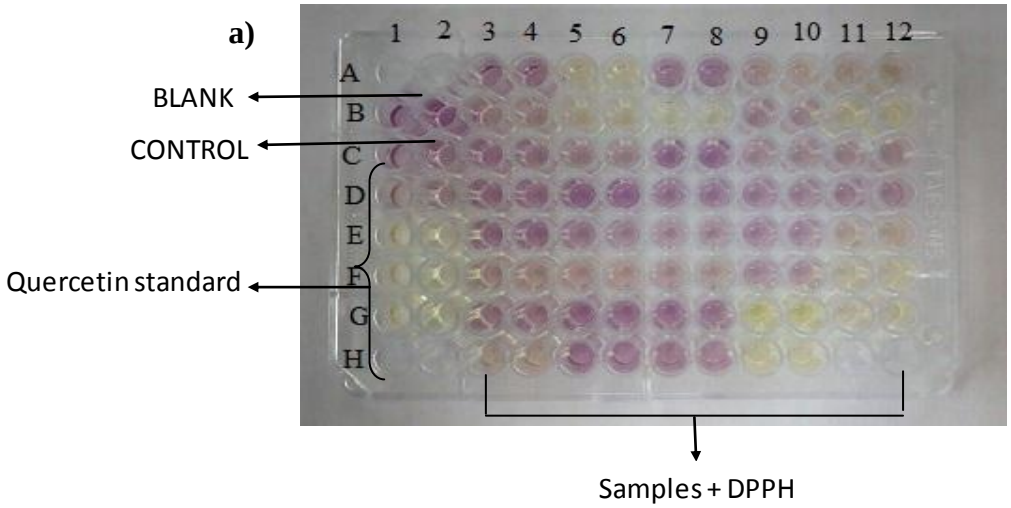
The culture filtrates of the isolated endophytic fungi were evaluated for their capacity of antioxidant activity using the DPPH free radical scavenging system. After incubation, the results depicted that some extracts do have good scavenging activity while others showed less activity to scavenge the free radicals. Activity as high as 90.9 ± 0.63%, 90.41 ± 0.001% and 89.75 ± 0.004% of the isolates #1(3)TAHRTU 1, #2(1)TAHRTU 12 and #(5)TAWRTU 4(c) respectively, was obtained which is higher than the Quercetin (Fig. 5.9, a)-b)) used as standard, which showed scavenging activity of 84.71 ± 0.006% (50 µg/ml). The filtrates which possess activity above 60% are illustrated in table 5.6.

Table 5.6 : The antioxidant activity of cultures exhibiting activity above 60%

S.No.	CULTURE CODE	% FREE RADICAL SCAVENGING *(%)	CONCENTRATION (µg Quercetin equivalent / mg extract)
1	#6TAHRTU	65.4 ± 1.23	36.9
2	#1(1)TAHSTU 1	76.46 ± 1.46	43.64
3	#1(1)TAHRTU 2	87.33 ± 1.41	50.26
4	#2(1)TAHRTU 12	90.41 ± 0.001	52.13
5	#1(3)TAHRTU 1	90.9 ± 0.63	52.43
6	#1(1)TAHRTU 4(c)	60.36 ± 1.22	33.84
7	#2(1)TAHRTU 14(c)	69.72 ± 0.026	39.53
8	#1(2)TAHRTU 1(c)	72.2 ± 0.02	41.35
9	#4TAHSPTU 29(d)	80.52 ± 0.085	46.11
10	#1(1)TAHRTU 4(c)	60.36 ± 1.22	33.84
11	#2(1)TAHRTU 14(c)	69.72 ± 0.026	39.53
12	#1(2)TAHRTU 1(c)	72.2 ± 0.02	41.35
13	#4TAHSPTU 29(d)	80.52 ± 0.085	46.11
14	#2(1)TAHSTU 9(d)	77.79 ± 1.57	44.45
15	#1(2)TAHSTU 3(d)	85.2 ± 0.47	48.96
16	#1(2)TAHRTU 7(d)	78.29 ± 1.84	44.75
17	#1(2)TAHRTU 8(d)	78.25 ± 0.007	44.73
18	#1(2)TAHRTU 8(e)	86.17 ± 1.79	49.55
19	#1(2)TAWRTU 5	62.95 ± 1.57	35.41
20	#1(3)TAWRTU 8	61.65 ± 0.99	34.62
21	#2(3)TAWRTU 9	60.32 ± 1.28	33.81
22	#2(1) TAWLTU 12(a)	87.38 ± 0.004	50.29
23	#2(1)TAWRTU 15(a)	89.55 ± 0.01	45.52
24	#2(3)TAWSTU 12(b)	78.22 ± 0.01	44.71
25	#2(2)TAWRTU 12(b)	72.5 ± 0.021	41.23
26	#1(2)TAWRTU 3(c)	60.99 ± 0.018	34.22
27	#(5)TAWRTU 4(c)	89.75 ± 0.004	51.73
28	#2(1)TAWSTU 12(d)	63.79 ± 0.022	35.92

(*Data represented as mean ± SD)

a)



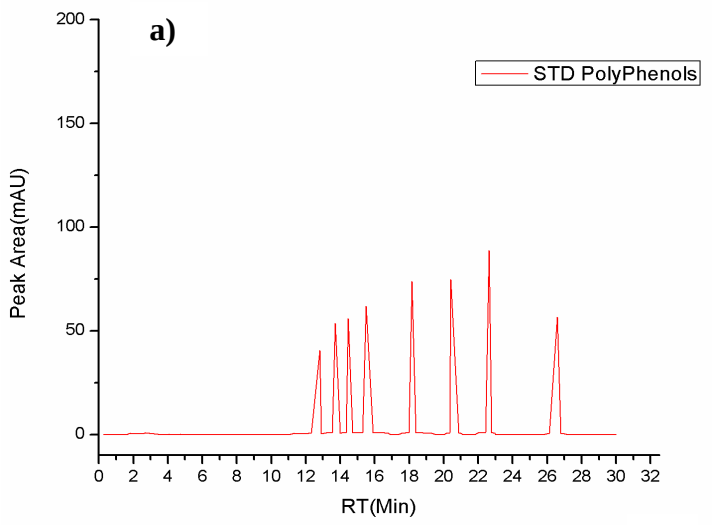
b)

Blank	Blank	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33
Control	Control	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
Q 1	Q1	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
Q2	Q2	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
Q 3	Q 3	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
Q 4	Q 4	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
Q 5	Q 5	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
		S8	S8	S16	S16	S24	S24	S32	S32	S40	S40

Fig. 5.9 : a) ELISA plate of DPPH antioxidant activity; b) description of each well of the plate (Control contains DPPH; Q1,Q2,Q3,Q4,Q5- Quercetin used as standard at different concentrations of 10,20,30,40,50 µg/ml respectively; S1-S40 are the different culture extracts + DPPH)

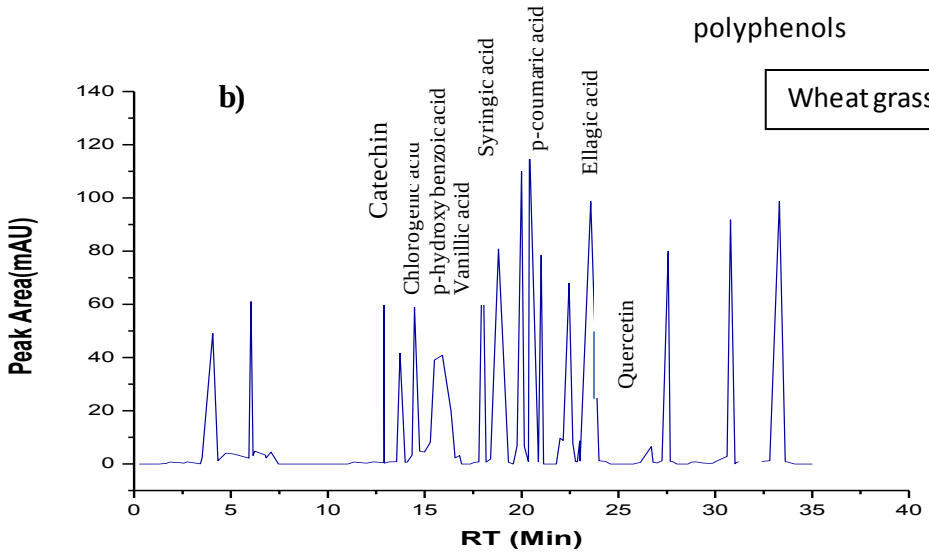
5.4. HPLC of different polyphenols, wheat grass extract and culture extracts

The cultures which showed DPPH activity above 80% were undertaken for HPLC analysis to determine different polyphenols present in it. Eight standard polyphenols (1mg/ml) were injected into the column and analyzed. The wheat grass extract was also analyzed to determine the types of polyphenols present in it along with the culture extracts to correlate that the endophytes isolated from the wheat might possess same polyphenols as present in wheat grass.



Type of polyphenol	Retention time (min)
Catechin	12.816
Chlorogenic acid	13.719
p-hydroxy benzoic acid	14.409
Vanillic acid	15.976
Syringic acid	18.169
p-coumaric acid	20.402
Ellagic acid	22.606
Quercetin	26.550

Table 5.7 : Retention time of standard polyphenols



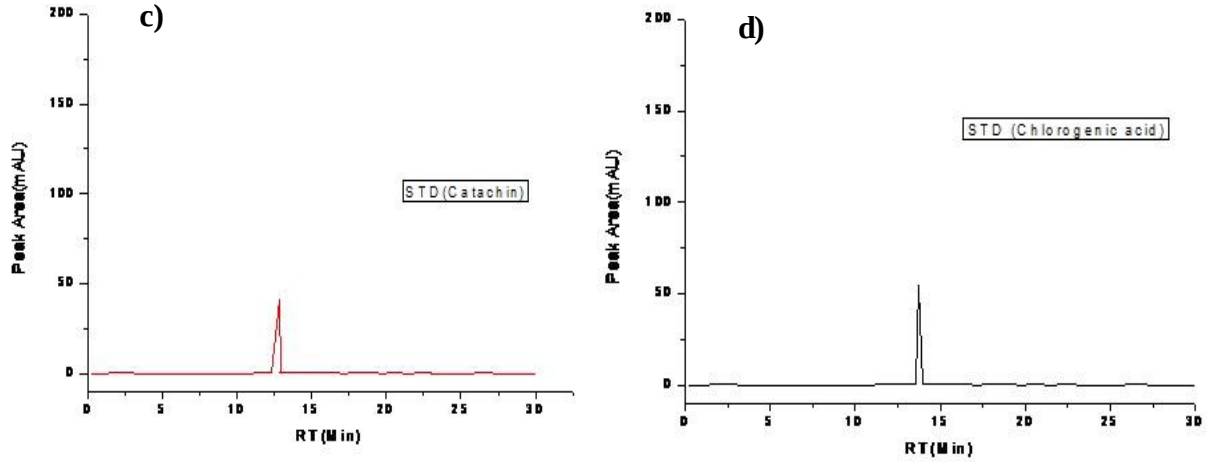


Fig. 5.10 : HPLC spectra of a) eight different polyphenols; b) wheat grass extract; c) catechin; d) chlorogenic acid

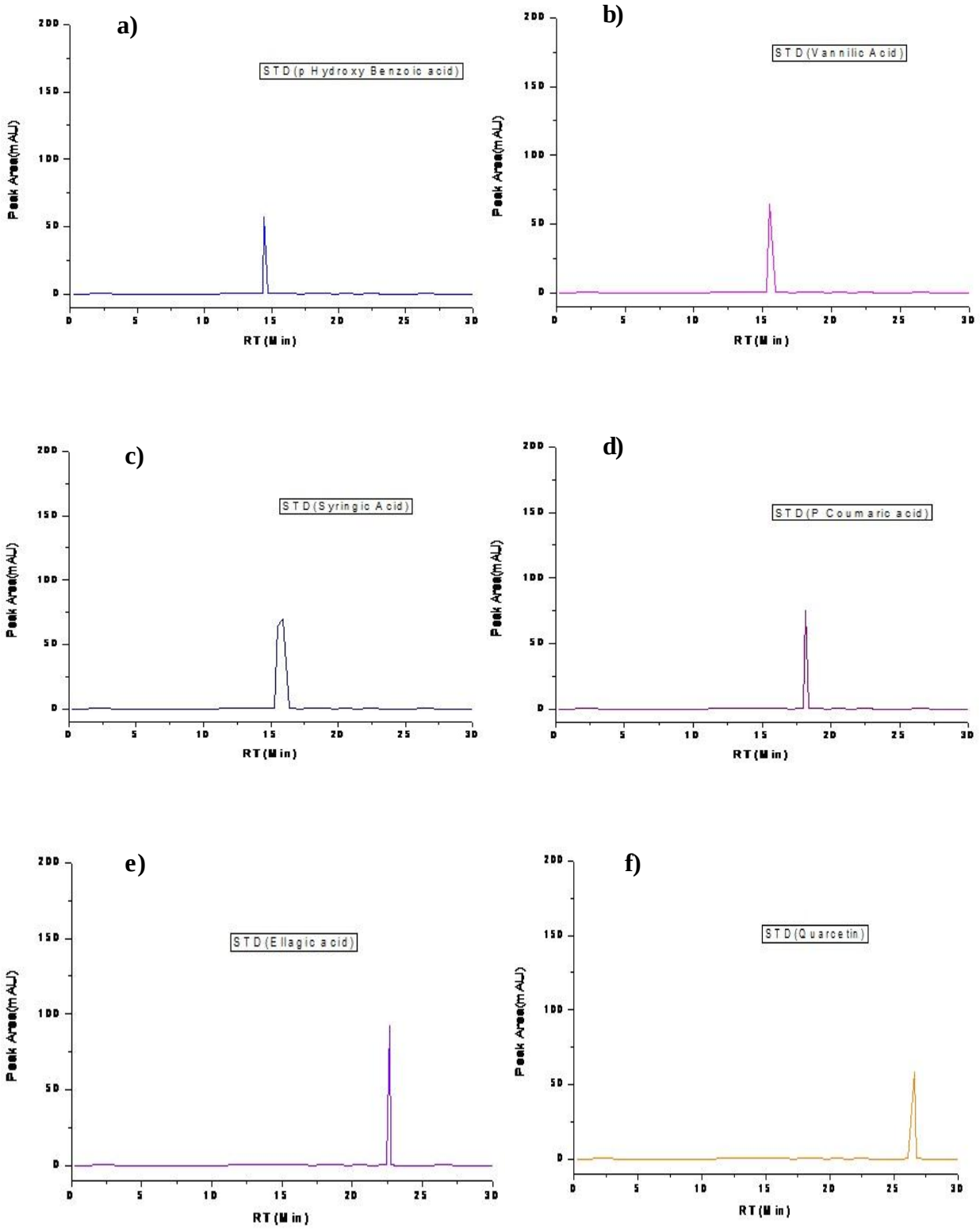


Fig. 5.11 : HPLC spectra of standards of a) p-hydroxy benzoic acid; b) vanillic acid; c) syringic acid; d) p-coumaric acid; e) ellagic acid; f) quercetin

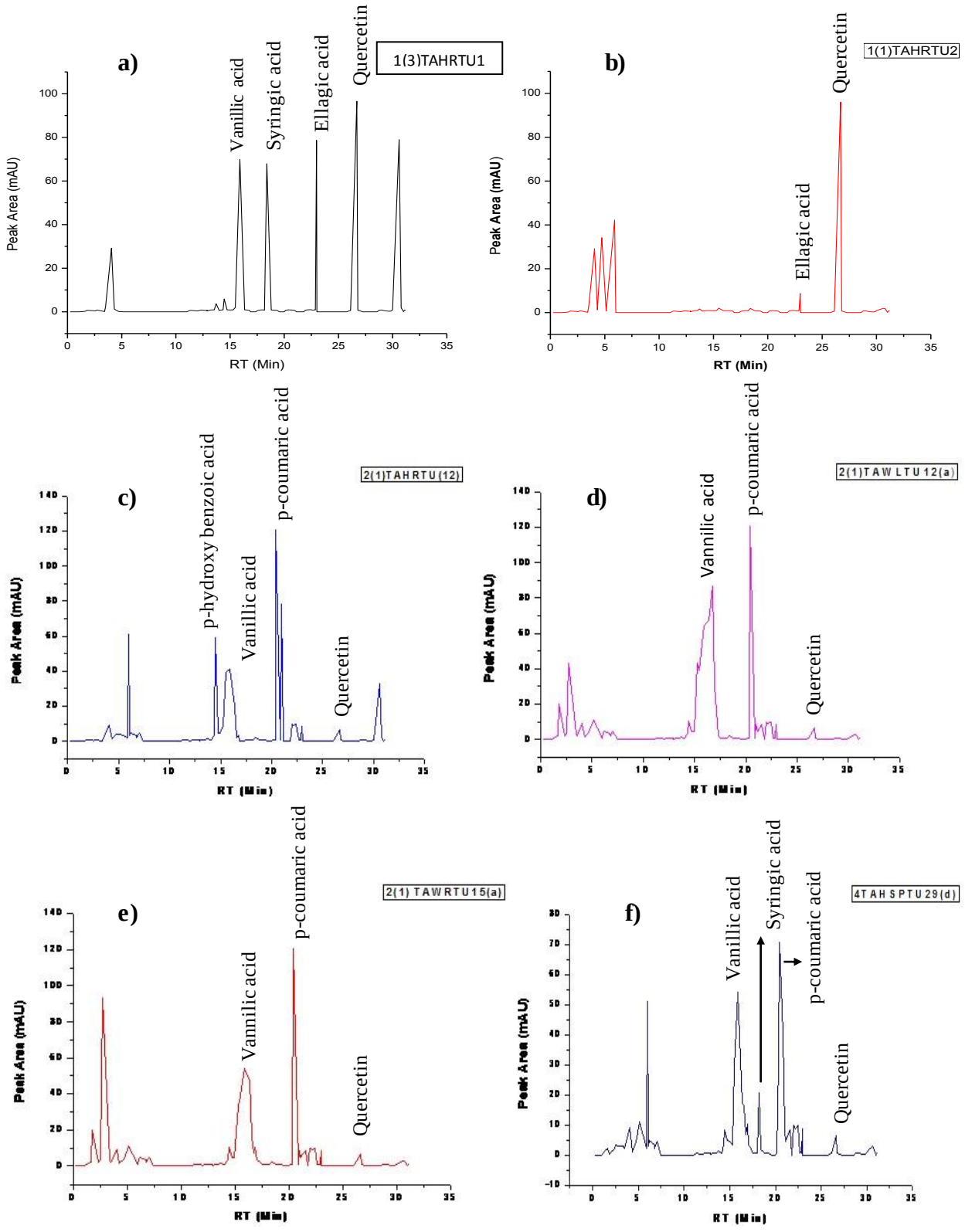


Fig. 5.12 : HPLC spectra of extracts of a) #1(3) TAHRTU 1; b) #1(1) TAHRTU 2; c) #2(1) TAHRTU 12; d) #2(1) TAWLTU 12(a); e) #2(1) TAWRTU 15(a); f) #4 TAHSPTU 29(d)

The crude wheat grass extract has retention time viz. 12.807, 13.721, 14.412, 15.981, 18.163, 20.408, 22.601, 26.545 (Fig. 5.10, b)) resembling to all the eight polyphenols (Fig. 5.10, a), c), d) Fig. 5.11, a)-f)) examined during the study. In the experiment the crude culture extract of #1(3) TAHRTU 1 exhibited retention time of 15.991, 18.172, 22.613, 26.557 (Fig. 5.12, a)) which resembles to vanillic acid, syringic acid, ellagic acid and quercetin; extract of #1(1) TAHRTU 2 exhibited retention time of 22.614, 26.549 (Fig. 5.12, b)) which resembles to ellagic acid and quercetin; extract of #2(1) TAHRTU 12 exhibited retention time of 14.415, 15.983, 20.424, 26.563 (Fig. 5.12, c)) which resembles to p-hydroxy benzoic acid, vanillic acid, p-coumaric acid and quercetin; extract of #2(1) TAWLTU 12(a) exhibited retention time of 15.992, 20.413, 26.548 (Fig. 5.12, d)) which resembles to vanillic acid, p-coumaric acid and quercetin; extract of #2(1) TAWRTU 15(a) exhibited retention time of 15.987, 20.412, 26.556 (Fig. 5.12, e)) which resembles to vanillic acid, p-coumaric acid; extract of #4 TAHSPTU 29(d) exhibited retention time of 15.989, 18.162, 20.409, 26.562 (Fig. 5.12, f)) which resembles to vanillic acid, syringic acid, p-coumaric acid and quercetin. It was observed that Quercetin and Vanillic acid were the two polyphenols mostly present in all the fungal endophytes analyzed using HPLC.

5.5. Identification of endophytic fungi

The isolate #1(2) TAHRTU 5(b) was identified on the basis of both morphotaxonomy and molecular taxonomy tools.

5.5.1. Morphotaxonomy

The endophytic fungus #1(2) TAHRTU 5(b) produced white, fast growing (90 ± 0) floccose aerial mycelium over PDA and MEA. Initially white in color and later becomes pale brown with smooth margin over PDA (Fig. 5.13, a)) and MEA (Fig. 5.13, b)). Over Pine Leaf Agar (PLA) and Water Agar (WA) the margins were flat. On SNA medium it was white in color with wooly appearance (Fig. 5.13, c)). On PLA medium it was brown in color (Fig. 5.13, d)) (Table 5.8).

On PDA and MEA hypha were thick, septate and branched. Conidiophores were present in the aerial mycelium. Conidia developed over aerial conidiophores are generally fusi form to falcate

Table 5.8 : Colony morphology of #1(2) TAHRTU 5(b) on different medium after 20 days, 28˚C, 12h dark

Medium	Colony color		Colony diameter*(mm)	Margin	Pigment	Odour
	Front	Back				
PDA	White	Pale brown	90 ± 0	Circular, smooth	Pale brown	No odour
MEA	Whitish brown	Pale brown	90 ± 0	Circular, smooth	Pale brown	No odour
PLA	Whitish brown	Brown	49 ± 2	Flat	No pigment	No odour
SNA	White	White	85 ± 3	Smooth	No pigment	No odour
WA	White	White	42 ± 2	Flat	No pigment	No odour

(*Data represented as mean ± SD)

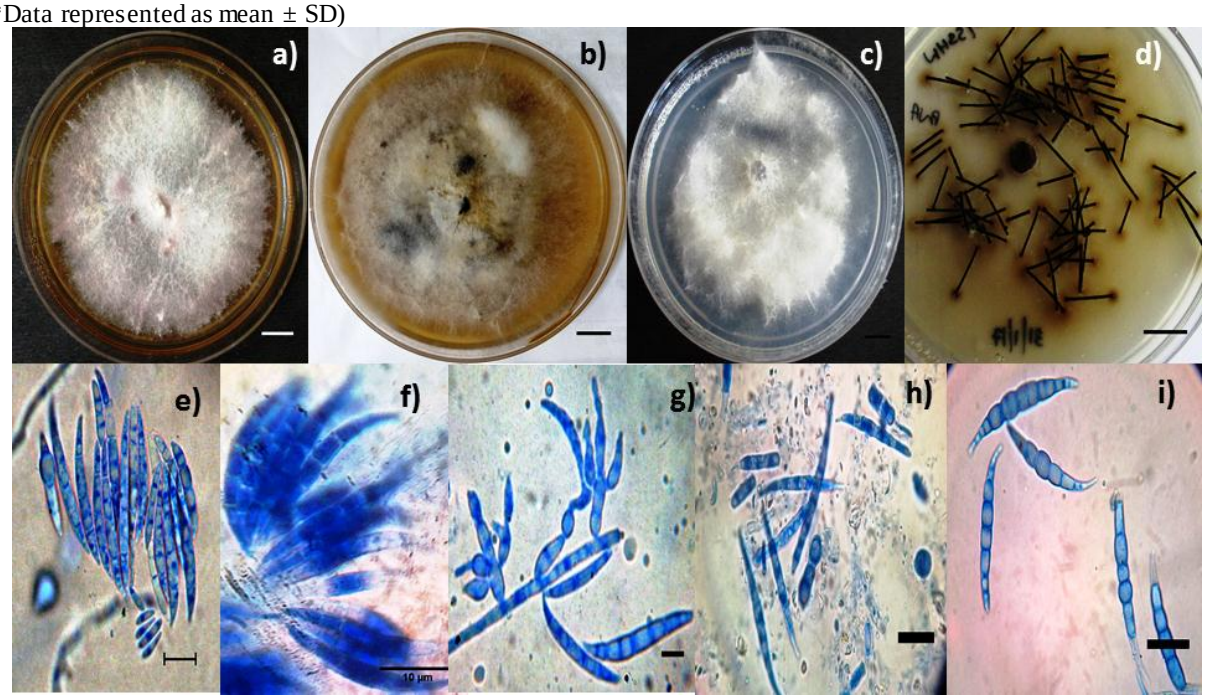


Fig. 5.13 : Morphological and microscopic feature of #1(2) TAHRTU 5(b); colony morphology on a) PDA medium; b) MEA medium; c) SNA medium; d) PLA medium (Bar : 10mm); f) and i) macro conidia over PDA medium; e) micro and macro conidia on SNA medium; g) and h) macro conidia with chlamydospores (Bar : 10µm)

in shape, usually 3-7 septate and both macro and micro conidia were present (Fig. 5.13, f) and i)). On WA and SNA medium, colonies were moderate to fast growing, white in color, hypha were septate and thick, conidia were fusi form to slightly curved with foot shell, mostly 5-6 septate and both macro and micro conidia were present (Fig. 5.13, e)). Based on these morphological features the fungus # 1(2) TAHRTU 5(b) was identified as *Fusarium* sp.

5.5.2. Molecular identification

5.5.2.1. Genomic DNA isolation and PCR amplification

The genomic DNA isolation of 82 fungal endophytes isolated during study was carried out and the size of the genomic DNA was more than approximately 10kb. The concentration of the genomic DNA isolated viz. # 1(2) TAHRTU 5(b) was 32ng/µl and absorbance at 260nm/280nm was taken which was 1.67 to determine the quantity and purity of the DNA isolated.

The PCR of approximately 40 cultures was done and the PCR amplicon was resolved on 1.5% agarose gel to check the size of the amplicon on the basis of its mobility and compared with ladder of 500 bp. The size of the PCR amplicon was found to be in the range of 500-600 bp for DNA amplified using ITS primer (Fig. 5.14) and 900-1100 bp for DNA amplified using LSU primer.

The bulk PCR amplification of culture #1(2) TAHRTU 5(b) using LSU primer was carried out, pured and was sent for sequencing. The rest of the samples are under process.

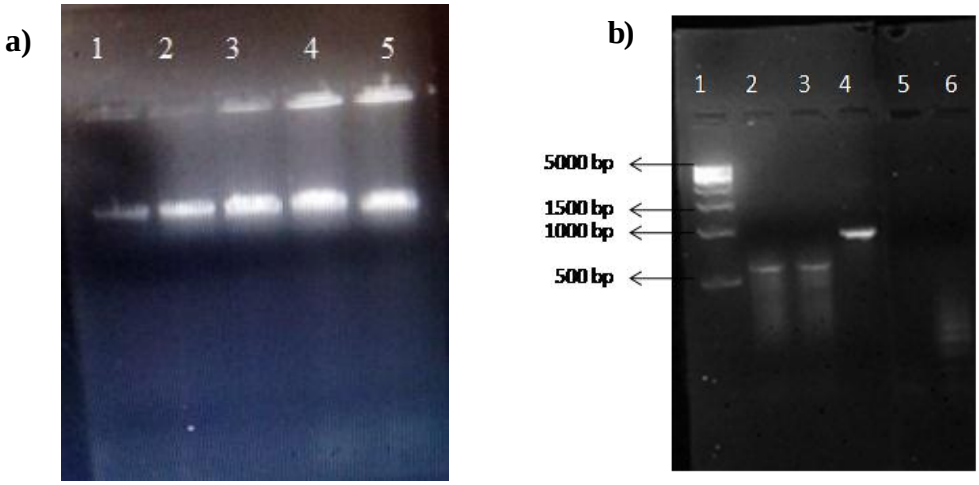


Fig. 5.14 : a) Genomic DNA of #1(2) TAHRTU 5(b); b) PCR amplicon of ITS1-5.8S-ITS2 and LSU rDNA region of #1(2) TAHRTU 5(b), Lane 1: ladder of 500 bp, Lane2-3 PCR amplicon of ITS region, Lane 4 PCR amplicon of LSU region

5.5.2.2. Sequence assembly and alignment

The PCR amplicon of #1(2) TAHRTU 5(b) was sent for sequencing and final sequence was submitted to GenBank under the accession number H8MM3U3Y014. The positional homology of final sequence of #1(2) TAHRTU 5(b) was established by subjecting it to sequence similarity search by using BLAST. BLAST analysis exhibited 100% sequence similarity with *Fusarium oxysporum* species, followed by 99% sequence similarity with *Fusarium proliferatum, Fusarium fujikuroi* and *Fusarium verticillioides* (Table 5.9). The final sequence of #1(2) TAHRTU 5(b) is as follows:

Fusarium sp. #1(2) TAHRTU 5(b)_LSU

TGCCCTAGTAACGGCGAGTGAAGCGGCAACAGCTCAAATTTGAAATCTGGCTCTCGGGCCCGAGTTGTAATT
TGTAGAGGATACTTTTGATGCGGTGCCTTCCGAGTTCCCTGGAACGGGACGCCATAGAGGGTGAGAGCCCC
GTCTGGTTGGATGCCAAATCTCTGTAAAGTTCCTTCAACGAGTCGAGTAGTTTGGGAATGCTGCTCTAAATGG
GAGGTATATGTCTTCTAAAGCTAAATACCGGCCAGAGACCGATAGCGCACAAGTAGAGTGATCGAAAGATG
AAAAGCACTTTGAAAAGAGAGTTAAAAAGTACGTGAAATTGTTGAAAGGGAAGCGTTTATGACCAGACTTG
GGCTTGGTTAATCATCTGGGGTTCTCCCCAGTGCACTTTTCCAGTCCAGGCCAGCATCAGTTTTCCCCGGGGG
ATAAAGGCGGCGGGAATGTGGCTCTCTTCGGGGAGTGTTATAGCCCACCGTGTAATACCCTGGGGGGGACT
GAGGTTCGCGCATCTGCAAGGATGCTGGCGTAATGGTCATCAACGACCCGTCTTGAAACACGGACCAAGGA
GTCGTCTTCGTATGCGAGTGTTCGGGTGTCAAACCCCTACGCGTAATGAAAGTGAACGCAGGTGAGAGCTTC
GGCGCATCATCGACCGATCCTGATGTTCTCGGATGGATTTGAGTAAGAGCATACGGGGCCGGACCCGAAAG
AAGGTGAACTATGCCTGTATAGGGTGAAGCCAGAGGAAACTCTGGTGGAGGCTCGCAGCGGTTCTGACGTG
CAAATCGATCGTCAAATATGGGCATGGGGGCGAAAGACTAATCG

Table 5.9: BLAST analysis of final sequence of #1(2) TAHRTU 5(b)

S.No.	Species name	Accession no.	Query coverage (%)	Sequence similarity (%)
1.	*Fusarium* sp. strain NK-NH2 large subunit ribosomal RNA gene, partial sequence	KY421623.1	100%	100%
2.	*Fusarium oxysporum* f. sp. lycopersici 4287 28S ribosomal RNA rRNA	XR_001936475.1	100%	100%
3.	*Fusarium oxysporum* f. sp. lycopersici 4287 28S ribosomal RNA rRNA	XR_001936472.1	100%	100%

4.	*Fusarium oxysporum* f. sp. lycopersici 4287 28S ribosomal RNA rRNA	XR_001936471.1	100%	100%
5.	*Fusarium oxysporum* f. sp. lycopersici 4287 28S ribosomal RNA rRNA	XR_001936467.1	100%	100%
6.	*Fusarium oxysporum* f. sp. lycopersici 4287 28S ribosomal RNA rRNA	XR_001936466.1	100%	100%
7.	*Fusarium oxysporum* f. sp. lycopersici 4287 28S ribosomal RNA rRNA	XR_001936465.1	100%	100%
8.	*Fusarium oxysporum* f. sp. lycopersici 4287 28S ribosomal RNA rRNA	XR_001936463.1	100%	100%
9.	*Fusarium oxysporum* f. sp. lycopersici 4287 28S ribosomal RNA rRNA	XR_001936461.1	100%	100%
10.	*Fusarium oxysporum* f. sp. lycopersici 4287 28S ribosomal RNA rRNA	XR_001936459.1	100%	100%
11.	*Fusarium oxysporum* f. sp. lycopersici 4287 28S ribosomal RNA rRNA	XR_001936455.1	100%	100%
12.	*Fusarium oxysporum* f. sp. lycopersici 4287 28S ribosomal RNA rRNA	XR_001936453.1	100%	100%
13.	*Fusarium proliferatum* strain PP74 28S large subunit ribosomal RNA gene, partial sequence	FJ890385.1	100%	99%
14.	*Fusarium fujikuroi* isolate H2 28S ribosomal RNA gene, partial sequence	KX375765.1	100%	99%
15.	*Fusarium verticillioides* 7600 28S ribosomal RNA rRNA	XR_001936440.1	100%	99%

Thus, the isolate #1(2) TAHRTU 5(b) was identified as *Fusarium oxysporum*.

Chapter – 6

DISCUSSION

6. DISCUSSION

The environmental stresses viz. abiotic and biotic stresses leads to reduction in the productivity and yield of the plants. Various methods have been adopted to increase the productivity of the plant viz. genetic sciences and the management practices (i.e. use of chemical fertilizers, pesticides, insecticides etc.), these have helped in increasing the plant growth, yield and photosynthetic efficiency (Dodd et al., 2011; Reynolds et al., 2009). But, these methods have some side effects on human beings as well as the environment. This has stimulated research for identifying new source for increasing the yield and the productivity by increasing the tolerance to abiotic stresses.

Endophytic fungi has gained attention from last two decades for exploring the diversity associated with the plant (Strobel et al., 2003). The endophytes produce metabolites, some of these have an inhibitory action on pathogens and pests (Arnold et al., 2003; Schulz et al., 2002). Endophytes have also been associated in increasing growth, yield and increases tolerance for stress contributed to plant (Rodriguez et al., 2008; Yuan et al., 2010; Waller et al., 2005).

Wheat being staple food there is an urge to increase its yield in order to meet the global food demand. For this reason, the diversity of fungal endophytes in wheat grown needs to be understood in order to find the potent fungal inoculate which might help in increasing tolerance to abiotic stresses. Hence, in the present study the diversity of fungal endophytes was explored from wheat grown in Punjab in order to combat the abiotic stresses and screening the in-vitro antimicrobial and antioxidant activity.

In the present study two varieties of wheat viz. HD 2967 and WH 1105 were selected for isolation of fungal endophytes and in total 214 isolates have been isolated, 112 isolates from HD2967 and 102 endophytic fungi from WH1105 variety of wheat. The fungal endophytes are known to occur in numerous hosts in different plant parts, including the members of *Poaceae* (Carroll et al., 1977; Petrini et al., 1982). Previous studies of endophytes on wheat have been conducted where Crous et al., (1995) isolated fungal endophytes belonging to 55 different taxa, from four cultivars of wheat viz. SST66, Dias, Nantes and Palmiet. Another study in Argentina was carried out where, 130 fungal isolates were isolated from healthy leaf of three wheat cultivars of which 19 fungal species were identified and 3 bacterial isolates which were *Bacillus* sp.(Larran et al., 2002); another study where 722 isolates from leaves, glumes, stems and grains were recovered (Larran et al., 2006). Whereas,

Lalzar et al., (2016) examined the endophytic diversity in wild species in comparison to the diversity of endophytes in wheat and analyzed 514 intergenic spacer region sequences .

The percentage colonization frequency of the isolates from two varieties was calculated and the number of isolates were greater in the roots than those from other plant organs and the maximum load of endophytic fungi was obtained after 75 days which is contributed by the endophytes isolated from root after which the endophytic load increased in spikes. Whereas, in earlier studies on wheat the number of isolated taxa was found to be greater in leaves than other parts (Crous et al., 1995; Larran et al., 2006).

The tentative identification of some of the fungal endophytes was carried out. *Aspergillu*s sp., *Alternaria* sp., *Arthrinium* sp., *Acremonium* sp., *Botryosphaeria* sp., *Collectotrichum* sp., *Fusarium* sp., *Nigrospora* sp., *Penicillium* sp., *Phaeoacremonium* sp. were the different endophytes colonizing different parts of wheat plant. Colonization of wheat by *Alternaria* sp., *Arthrinium* sp., *Acremonium* sp., *Fusarium* sp., *Nigrospora* sp., *Penicillium* sp., *Phoma* sp., *Epicoccum* sp., *Chaetomium* sp. has already been reported (Crous et al., 1995; Larran et al., 2002; Larran et al., 2006).

Most of the endophytic fungi produce secondary metabolite and some of these compounds are antibiotics having antibacterial, antifungal and anti-insecticidal property which inhibit growth of other micro organisms, including plant pathogens (Chareprasert et al., 2006; Larran et al., 2007). The culture filtrates in the study were subjected to in-vitro antimicrobial assay (Baris et al., 2006) to screen the cultures which are having inhibitory potential against *S. aureus* and *E. coli.* The isolate #1(1)TAHRTU 2 which is tentatively identified as *Fusarium* sp., possess best antimicrobial activity. Thus, this assay can help in choosing the inoculum which can in-turn help in protecting wheat from pathogens and pest. In previous studies 67 endophytic fungi isolated from *Q. variabilis* showed significant antimicrobial activity (Wang et al., 2007). The endophytic fungi isolated from rain trees and teak also exhibited the antimicrobial activity (Chareprasert et al., 2006). The endophytic fungi and actinomycetes isolated from wheat (*Triticum durum*) showed antimicrobial activity and the maximum inhibition was by *Penicillium* sp. against *C. albicans* (Sadrati et al., 2013).

An enormous variety of plants have been screened for new source of natural antioxidants, phenolics and flavanoid derivative compounds which were proved to be potent antioxidant and free radical scavengers. Significant correlation between phenolic compounds and antioxidant properties

of wheat plant was noted (Khennouf et al., 2010; Baghiani et al., 2010). The same was seen in the studies on endophytes, in present study the in-vitro antioxidant assay (Kitts et al., 2000) of the fungal endophytes was carried out and activity as high as 90.9 ± 0.63%, 90.41 ± 0.001% and 89.75 ± 0.004% of the isolates #1(3)TAHRTU 1, #2(1)TAHRTU 12 and #(5)TAWRTU 4(c) respectively, was obtained which is higher than the Quercetin used as standard, which showed scavenging activity of 84.71 ± 0.006% (50 µg/ml). Sadrati et al., (2013) isolated 23 endophytic actinomycetes and 20 endophytic fungi from wheat (*Triticum durum*). The antioxidant activity was seen in *Aspergillus* and *Penicillium* crude extract with an inhibition percentage of 73.97 and 78.96, respectively.

Liquid Chromatography is the most widely used analytical technique for the qualitative and quantitative screening of different bioactive compounds under the condition of the determinate chromatographic system and the operation, each material has ascertained retention time. Thus, the unknown material and the standard substance can be analyzed preliminary. The same material owe to the similar retention time under the same chromatographic condition (Petrovic et al., 2005; Cia et al., 2004). The crude methanolic wheat grass extract and 6 isolates having activity above 80% were evaluated using HPLC to evaluate the types of polyphenols present and correlate that the endophyte possesses same polyphenols as present in wheat grass. Quercetin and Vanillic acid were the two polyphenols commonly found in fungal endophytes evaluated. The HPLC analyses revealed the presence of various phytochemicals such as Quercetin, Vanillic acid, p-coumaric acid, Ellagic acid. These phytochemicals are known to be antioxidant molecules and are used in therapeutic studies (Cia et al., 2004). Our study also reports similar result highlighting the association of micro-organisms with wheat plant and its properties.

Thus, the endophytes are considered to be the potential source for the novel bioactive compounds (Strobel et al., 2003).

Chapter – 7

CONCLUSION

6. CONCLUSION

The fungal endophytic diversity was explored in wheat as it being important cereal crop especially in the Northern India.

1. In the current study the fungal endophytes from two high yielding varieties grown in Punjab viz. HD 2967, WH 1105 were isolated in order to determine the diversity present in the wheat. In total 214 isolates have been recovered from different stages of wheat.
2. The antioxidant and antimicrobial potential of the isolates was determined. The culture isolate #1(1)TAHRTU 2 possess good antimicrobial activity. The isolates #1(3)TAHRTU 1, #2(1)TAHRTU 12 and #(5)TAWRTU 4(c) showed scavenging activity of above 89%. Quercetin and Vanillic acid were the two polyphenols commonly found in fungal endophytes analyzed using HPLC.
3. The further study focuses on identifying them and choosing the isolate which could be used as the inoculum in coating seeds and can thus, help in increasing the abiotic stress tolerance in wheat crop.

Chapter – 8

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BIBLIOGRAPHY

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APPENDIX

Media

1. **Potato dextrose agar**

   Potato dextrose agar – 39gm

   Distilled water – 1L

   Final pH (at 26°C)- 5.6±0.2

   Autoclave at 121ºC for 15 min

2. **Malt extract agar**

   Malt extract – 30gm

   Mycological peptone – 5gm

   Agar – 15gm

   Distilled water – 1L

   Final pH - 7.6 ± 0.2 at 37°C

   Autoclave at 121ºC for 15 min

3. **Synthetic nutrient deficient agar**

   Glucose – 0.2gm

   Sucrose – 0.2gm

   Pot. dihydrogen phosphate – 1gm

   Potassium nitrate – 1gm

   Magnesium sulphate – 0.25gm

   Potassium chloride – 0.5gm

   Agar – 15gm

   Distilled water – 1L

   Final pH – 5.4 ± 0.2 at 26°C

   Autoclave at 121ºC for 15 min

4. **Water agar**

   Agar – 15gm

   Distilled water – 1L

   Autoclave at 121ºC for 15 min

5. **Pine leaf agar**

   Pine leaves

   Agar – 15gm

   Distilled water – 1L

   Autoclave at 121ºC for 15 min

Buffers

1. **50XTAE**

   Tris base – 242g

   Glacial acetic acid – 57.1ml

   0.5M EDTA – 10ml

   Distilled water – 1L

2. **1X TE Tris-HCl (pH 8.0)**

   10 mM EDTA - 0.1 mM

   Distilled water - 100ml

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