Evaluation of Fungal Endophytes to induce Abiotic Stress tolerance in Wheat *(Triticum aestivum)*

A Thesis

submitted in partial fulfillment of the requirements for the award of the degree of

Master of Science
In
Biotechnology

By

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Under the Supervision of

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June 2018

Certificate

This is to certify that the thesis entitled **Evaluation of Fungal Endophytes to induce Abiotic Stress tolerance in Wheat *(Triticum aestivum)*** being submitted by **Gurleen Kaur Sodhi (Reg. No. 301601008)**, in partial fulfillment of the requirements for the award of the degree of **Master of Science** in Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab is a bonafide work carried out under the guidance and conception of Dr. Sanjai Saxena and that no part of this thesis has been submitted for the award of any other degree.

Date: 23 August 2018

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Candidate's Declaration

I hereby certify that the work, which is being presented in the thesis, entitled **Evaluation of Fungal Endophytes to induce Abiotic Stress tolerance in Wheat *(Triticum aestivum)*** , in partial fulfillment of the requirements for the award of the degree of **Master of Science** and submitted to the institution is an authentic record of my own work carried out during the period **January, 2018** to **June, 2018** under the supervision of **Dr. Sanjai Saxena, Professor, Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab India**. I have also cited the references of the text(s)/figure(s)/table(s) from where they have been taken.

The matter presented in this thesis has not been submitted elsewhere for the award of any other degree or diploma from any institution.

Date: 23 August 2018

Gurleen Kaur Sodhi
Candidate

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

Date: 23 August 2018

Dr. Sanjai Saxena
Supervisor

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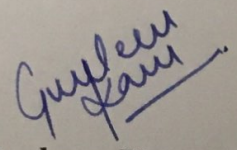

Gurleen Kaur Sodhi

This thesis is dedicated to Scooby Sodhi

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List of Abbreviations

μg	Micro gram
μl	Micro litre
μM	Micro molar
ABTS	2, 2-azino-bis-3-ethylbenzothiazoline 6-sulphonic acid
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DPPH	1,1-diphenyl-2-picrylhydrazyl
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
Et Br	Ethidium bromide
FAO	Food and Agriculture Organization
FRAP	Ferric ion reducing antioxidant power
FRS	Free radical scavenging
HYV	Hight yielding variety
ITS	Internal transcribed spacer
LAF	Laminar air flow
MEA	Malt extract agar
mg	Milli gram
ml	Milli litre
mM	Milli molar
ng	Nano gram
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
pH	Potential of hydrogen
rpm	Revolutions per minute
RT	Room temperature
SD	Standard deviation
TAE	Tris acetate EDTA
TEAC	Trolox equivalent antioxidant capacity
TFC	Total lavonoid content
TPC	Total phenolic content

Executive Summary

The stress caused due inanimate things is termed as abiotic stress which causes negative impact on the living organisms in a specific environment. At present abiotic stress is one of the leading causes of crop yield loss. The abiotic stress factors when occur in combination have the most detrimental impact on the plants. The rapid urbanisation and global hunger has lead to increase in the demand of food supply. Wheat being the most widely consumed staple food has always been the subject for yield improvement in order to meet the growing demand. The prevailing methods such as use of hybrid seeds, improving crop fertilizers etc., helped in yield improvement but there is a need of exploring a sustainable source for yield improvement. The diverse ecological niche of endophytic fungi has been of keen interest due to its symbiotic relationship with the host plant. These fungi have co-existed with the plants since ancient times and have shown potential in conferring stress tolerance to the host plants. In this study, endophytic fungi pre-isolated from different parts of two high yielding wheat varieties HD2967, WH1105 (grown in Punjab) were screened for abiotic stress tolerance. Two main abiotic stresses chosen were salinity and extreme temperature. The endophytic cultures were observed for growth till 10 days at different concentrations of NaCl (ranging from 5-20%) and temperature range (16°C-40°C). These were then compared with a control to screen out the best isolate that tolerates stress. The potent isolates #2(1)TAHRTU 12, #2(4)TAHLTU 15(e) and #2(1)TAWLTU 12(a) were subjected to production in potato dextrose broth for screening of its antioxidant activity. The isolate #2(1)TAWLTU 12(a) exhibited best antioxidant activity among the three. The total phenol content of the three isolates was also estimated in which isolate #2(1)TAWLTU 12(a) exhibited highest content of polyphenols and flavonoids. Further classical tools were used to identify the potent cultures; their genomic DNA was isolated and ITS region of approximately 500-600 bp was amplified before sending it for sequencing.

Keywords: Abiotic Stress, wheat, endophytic fungi, salinity, temperature.

Chapter 1

Introduction

Abiotic stresses such as extreme temperatures, heavy metal toxicity, and oxidative stress etc., have been reported to cause a reduction in crop yield by more than 50% [Abdel Latef and Tran, 2016; Rodriguez et al., 2004]. These stresses not only affect the growth and productivity but also bring in morphological, physiological, biochemical and molecular changes in plants [Mahajan and Tuteja, 2005]. According to a report by world grain news, climatic variability alone contributes 5.5% to the loss in yield worldwide [World-Grain-Newsletter, 2017]. As the global population is going to be $\sim$ 7.8 billion by the year 2020 the demand of food production will also increase simultaneously. An estimated rise of 70% in food production is required to meet the demand as reported by the Food and Agriculture Organization (FAO). In 2016 the FAO reported that, out of the 50,000+ edible plants only three of them, namely rice, maize, and wheat provide 60% of the worlds food energy intake. Wheat is a widely consumed staple food whose cultivation dates back to 10,000 years ago [Shewry, 2009]. It is a good source of nutrition, proteins, vitamins of group-B, minerals and dietary fibers; but environmental conditions may affect the nutritional composition of wheat grain [Simmonds, 1989]. With advancements in agricultural techniques and Green revolution has lead to production of high yielding variety (HYV) seeds.

Prolific wheat producing states like Punjab, Haryana, Uttar Pradesh etc., make India the second largest wheat producer worldwide [ICAR, 2013]. However, there is this growing concern about the productivity and yield of wheat due to environmental factors which not only cause stress on plants but have affected irrigation by disturbing the water table level in these states [Jain and Kumar, 2007]. In India, particularly in Punjab, the wheat varieties which are generally grown are irrigation fed, which not only uses ample amount of water but also decreases the water table due to which the soil is no longer leached and salinity problems develop [Pandey, 2016; Bauder and Brock, 2001]. Similarly, the temperature has also increased since a decade which has affected the crop productivity [Kalra et al., 2008]. Hence today the demand is for drought, salinity and temperature resistant varieties which are high yielding as well.

Over the years many methods such as production of high yielding variety seeds, up-

regulation of specific genes etc., have been employed to increase the plant yield in a sustainable manner and to meet the rising demand of food production [Dodd et al., 2011]. Wheat has been a target for yield improvement throughout history, first by domestication and later by selection and breeding [Abbo et al., 2012]. The decline in arable land area due to degradation of soil and the increase in demand of food production, demands exploration of alternative approaches for improving productivity of wheat [Lal, 1998]. One such emerging strategy is the use of symbiotic relationship between the endophytic fungi and plants. Researchers have indicated that plants cannot function as souvenir individuals and have one or more type of endophytes present in them [Strobel and Daisy, 2003]. The endophytic fungus resides within the host plant in various ecological relationships. They obtain nutrients from the host plant and contribute positively to the host fitness and are thus termed as mutual symbionts [Larran et al., 2007; Sadrati et al., 2013].

The association between endophytic fungi and plants is ecologically important and is being exploited to confer resistance in plants to adapt or overcome these stresses. The endophytes have been of interest due to the potential of fulfilling the microbial diversity associated with the host plant [Tan and Zou, 2001] and to produce novel and bioactive compounds [Strobel and Daisy, 2003]. Recent studies have shown that endophytic fungus helps in overall fitness of plants by providing tolerance against abiotic stresses [Pozo et al., 2010; Abdel Latef and Tran, 2016; Bonfante and Genre, 2010; Xie et al., 2014] and enhancing nitrogen uptake [White et al., 1992; White and Torres, 2010; López-Ráez et al., 2010; Abdel Latef and Tran, 2016]. The habitat adapted symbiosis is known to be the major contributor in inducing stress tolerance in plants [Rodriguez et al., 2008]. Timely seedling establishment in stressful environment is critical life stage of plants. Improve in wheat seed germination on colonizing with fungal endophytes has been seen by hydrothermal time model [Hubbard et al., 2012]. Thus, endophytic fungi have been of keen interest to improve plant stress tolerance and for production of sustainable food crops.

Therefore, in this study we propose to screen an endophytic fungus which can be used as seed inoculant in order to confer abiotic stress tolerance in wheat plant and eventually help in improving crop yield in a sustainable manner.

Chapter 2

Review of literature

2.1 Wheat

Wheat is a worldwide consumed staple food belonging to family Poaceae of plant Kingdom [Kheyrodin, 2016]. Neolithic revolution which occurred about 10,000 years ago first saw the cultivation of wheat [Parry et al., 2017]. It is counted among the big three cereals with over 93 million tonne produced annually in India itself [Shewry, 2009; FAO-report, 2017]. Percival Baker (1921) identified 18 species of wheat out of which three commonly grown in India are *Triticum aestivum* (2n =6x=42), *Triticum dicoccum* (2n=2x=14) and *Triticum durum* (2n=4x=28). According to USDA's (United States Department of Agriculture) guidelines all adults must consume whole grains at least thrice every day. *Triticum aestivum* is the major wheat species grown worldwide and is thus called common bread or wheat bread [Parry et al., 2017]. Since cereals and breads are main source of energy for all age groups, wheat is the most prominent choice of consumers. The increase in demand is also due to the ability to make unique food products using wheat. Also the vast variety of food products processed from wheat are convenient to produce as well as consume as compared to the traditional products [Parry et al., 2017]. In Figure 2.1 we can see the increase in amount of wheat produced each year globally [FAO-report, 2017].

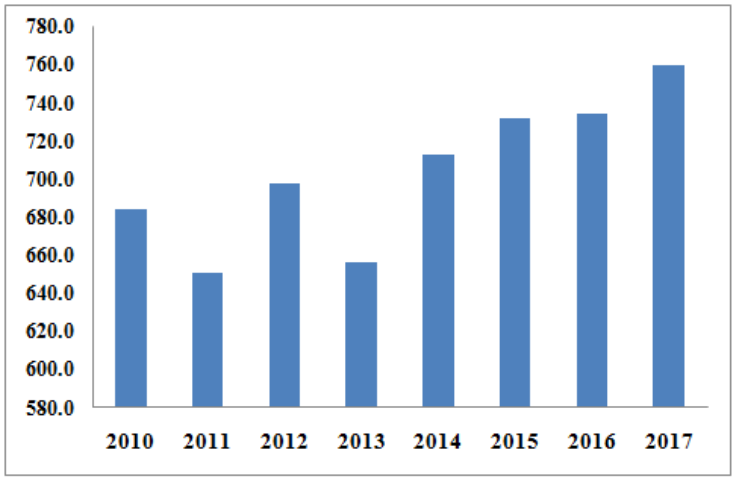

Figure 2.1: Global wheat production (in million tonne)

Wheat consumption has increased rapidly mainly due to urbanization and the shift in taste and preference over rice and other coarse grains [Curtis, 2002]. It has become a global commodity and adoption of western lifestyle due to industrialization and urbanization [Parry et al., 2017]. According to the latest report by Punjab data [ICAR, 2013], Punjab contributes 12% to India's total grain production. In the year 2017, a total of 17636 thousand metric ton wheat was produced in Punjab [PunjabData, 2018]. Over the years it has proved to be one of the best examples of Green revolution in India. The main achievements involved production of High yielding wheat variety and rust resistant wheat variety [ICAR, 2013].

2.2 Nutritional value and health benefits of wheat

Wheat is a good source of nutrition, protein, vitamin of group-B, mineral and dietary fiber [Simmonds, 1989]. Research has shown that these fibres may reduce the risk of cardiovascular diseases, type-2 diabetes and certain cancer [Fung et al., 2002; Koh-Banerjee et al., 2004; Sahyoun et al., 2006; Seal, 2006; de Munter et al., 2007; Schatzkin et al., 2007; Mellen et al., 2008]. The fibres also play an important role in good health of our gut [Slavin, 2013]. Wheat is also rich in minerals like iron and zinc, the minerals which led to the most widespread nutrient deficiency in the world. The properties possessed by gluten protein fraction of protein has enabled to process a wide variety of goods such as bread, noodles, pasta, baked good etc. [Parry et al., 2017]. 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]. Consuming wheat as a breakfast cereal has shown prebiotic effect on the gut microbiota. As a result the growth of beneficial intestinal micro-organisms increases [Costabile et al., 2008]. It also reduced the duration of inflammatory diarrhea, inflammation due to bowel disease whereas an increase in bio-availability and uptake of nutrients was observed on research [Slavin, 2013]. Besides this wheat has presence of polyphenolic compounds [Baghiani et al., 2010]. These polyphenolic compounds are known to have antioxidant properties which help in removing potentially damaging oxidizing agents from human body [Dykes and Rooney, 2007]. More than 5000 flavonoids are found in nature [Dykes and Rooney, 2007] many of which are found in pericarp of pigmented cereals such as wheat [Dykes and Rooney, 2007]. Other than antioxidant properties they have been reported to show anti-carcinogenic and anti-allergic properties as well [Dykes and Rooney, 2007].

2.3 Loss in wheat productivity

According to the latest report released by The Cereal Supply and Demand Brief of FAO in June, 2018 the world cereal production is falling whereas the demand remains ample. There are many factors that contribute to the loss in wheat productivity. Since plants are immobile in nature they are exposed to nature's wrath in terms of environmental stresses [Mahajan and Tuteja, 2005]. These stresses can be of two types:

1. Biotic stress: It is the stress caused by the damage done by other organism such as parasites, weeds etc.
2. Abiotic stress: It is the stress caused due to inanimate things such as extreme temperature conditions, salinity of soil, oxidative stress etc.

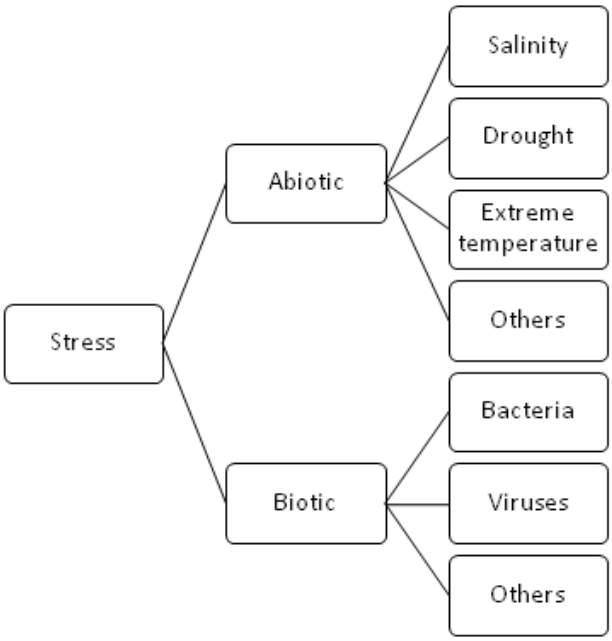

Figure 2.2: Abiotic and Biotic stress factors

Abiotic stress of one or other kind affects nearly about 90% of the total arable land. Thus, the sustainability of agricultural industry is threatened due to these stresses [Mahajan and Tuteja, 2005]. In India, Punjab is one of the major producers of wheat along with Uttar Pradesh and Haryana. It is also called the bread basket of India and is currently facing major depletion in cereal productivity specially wheat due to various abiotic factors [PunjabData, 2018]. Since most of the crops grown in Punjab are irrigation fed, reports indicate the increase in water logged area from 0.55 m ha in 2005-06 to 0.87 m ha in 2008-09. As a result of increased water logging area the amount of salinity in soil is increasing. While some regions of Punjab are facing water logging problems others are

facing a decrease in groundwater level that too at alarming rates [Mihir Shah, 2013]. As the temperature is rising the yield of wheat is also affected by the stress caused due to high temperatures [Kalra et al., 2008]. In Figure 2.3, we can see the major factors responsible for crop yield loss [Mahajan and Tuteja, 2005].

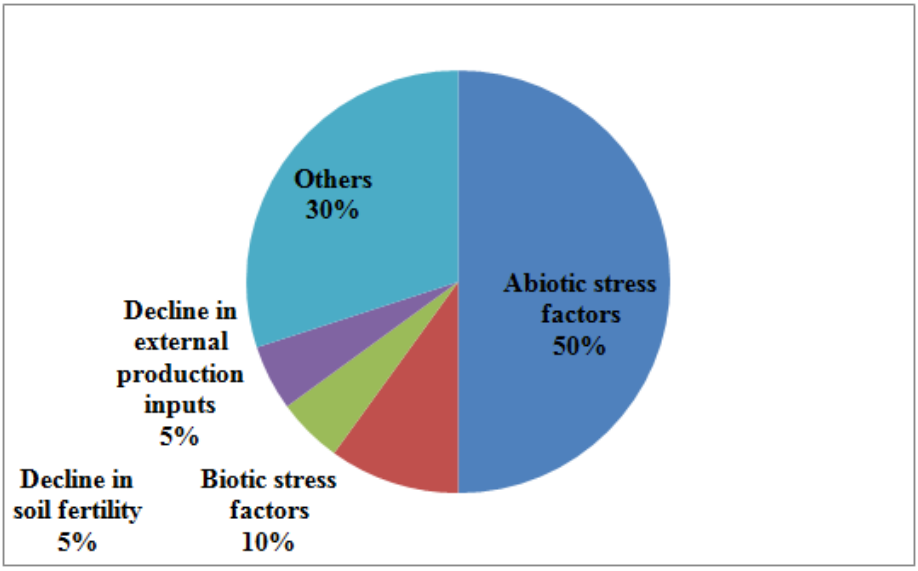

Figure 2.3: Causes of crop yield loss

2.4 Need to explore novel source for yield improvement

There are various reasons that demand the need to explore a novel source for yield improvement of wheat plant. The rise in global hunger due to the increase in global population is one of the reasons [FAO-report, 2017]. With the increase in global population and urbanization the agricultural land is also decreasing. To meet the rapid increase in demand a variety of wheat that produces high yield is require. A unique milestone was achieved with the onset of Green revolution which saw the discovery of HVY seeds [Yadvendra Singh, 2018]. But only high yielding variety is not sufficient to overcome the global food security challenge. Environmental factors such as biotic and abiotic stress are major deal breakers in the race between demand and supply [Mittler, 2006]. These stresses not only affect the yield of the plant but also bring in morphological changes in plants [Mahajan and Tuteja, 2005]. In order to overcome these issues we need to explore a novel source that not only helps in tolerating the environmental stresses but also help in producing a sustainable yield. Thus, we chose to explore the symbiotic relationship between endophytic fungi and plants.

2.5 Endophyte

Micro-organisms occur in various ecological relationships with the plant. In 1809 German botanist Heinrich Friedrich link first described endophytes. An endophyte is an organism that resides with in the body of the host plant and causes no apparent harm to the host plant and is thus known as endosymbiont. They are ubiquitous in nature. They obtain nutrients from the host plant and contribute positively to the host fitness [Kharwar et al., 2009] and are thus known as mutual symbionts [Sadrati et al., 2013]. The transmission of endophytes is occurs by two methods [Bright and Bulgheresi, 2010].

(A) Horizontally (among individuals) or

(B) Vertically (directly from parent to offspring).

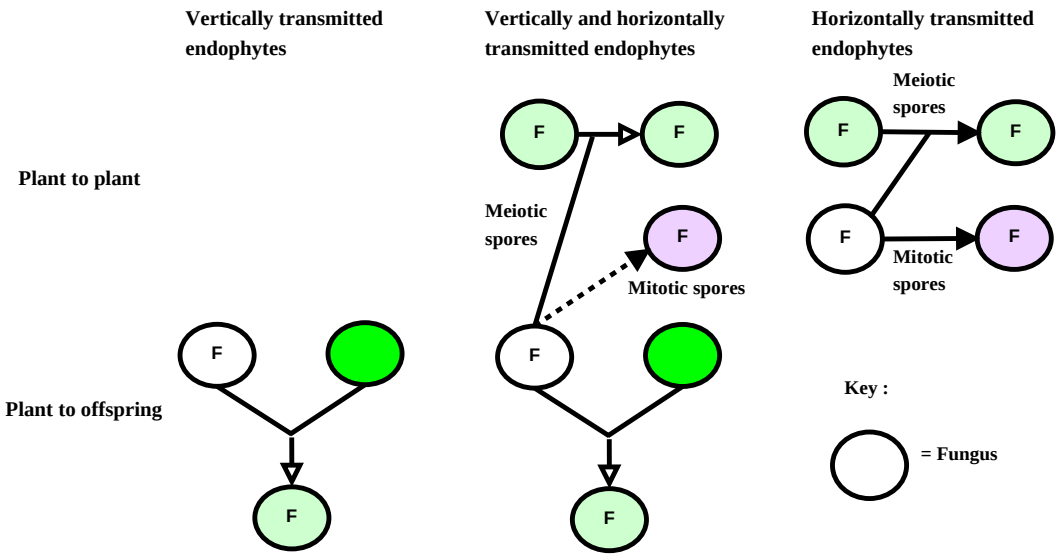

Figure 2.4: Transmission of endophytes

The endophytes are being exploited because they are expected to nurse many of the undiscovered fungal species [Saikkonen et al., 2004]. They are a source of novel bioactive compounds [Strobel, 2003] and secondary metabolites [Saikkonen et al., 2004]. And they are known to confer biotic and abiotic resistance in plants [Latch, 1993; Hutton et al., 1996].

2.6 Endophyte-host interaction

The endophytic fungi and host exist in various ecological relationships. The host plant helps in fulfilling the environmental and nutritional requirements of the fungus for their survival and in return the endophytes protect the host plant [Lee et al., 2012]. On studying the petrified remains it was found that the fungi and plant association dates back to 400 million years ago [Rodriguez and Redman, 2008]. And their symbiotic relationship is thought to be the reason of movement of plants onto the land [Remy et al., 1994].

Previously many studies have been carried out on endophytic fungi. Stress tolerance mechanism study was carried out in which it was observed that endophytes follow two mechanisms i.e. activation of host stress response system [Schulz et al., 1999; Redman et al., 1999], biosynthesis of antistress chemicals [Miller et al., 2002; Raheleh et al., 2012]. It was observed that some non cultivable microbial components which represent a large reservoir of heritable DNA are transmitted vertically from generation to generation that are responsible for enhancing the performance, longevity and adaptation in plants [Singh et al., 2011]. In another study the endophytic fungi helped in increasing the plant growth. A study was conducted in which the seeds of plants were coated with endophhytic fungi *Glomus intraradices* BEG72, *Glomus mossae* and *Trichoderma atroviride* MUCL 45632. It was observed 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 the uncoated seeds. The uncoated seeds showed a decrease in yield by 24.3% and 7.7% when tested in open fields. Along with increased yield the stability of the coated seeds also increased as a result of which increased level of nutrient uptake was observed [Colla et al., 2015].

Evidence has shown that the symbiotic relationship between endophytic fungi and plant can combat plant pathogen attacks. Various mechanisms such as induction of plant defence mechanism, production of antibiotics that inhibit growth of pathogen [Schulz and Boyle, 2005; Strobel et al., 2002; Zhang et al., 2007]. Various experiments were conducted against plant pathogenic fungi, nematods, bacteria, viruses etc., to check if endophyte induced plant show reduced effect caused by these plant pathogens. As a result it was seen that the ecological niche of endophytes hold a good potential in plant improvement and disease control [Zabalgogeazcoa, 2008]. In another experiment the interaction between endophytic fungi and wheat plant were studied in laboratory experiments to check if the endophyte reduces the leaf rust disease caused by *Puccinia recondite*. It was observed that the endophyte reduced the density and size of pustules when inoculated 3, 7 and 14 days prior to the pathogen. The endophyte has been speculated to have released some defence related chemical that resulted in reduction the disease [Dingle and Mcgee,

2003].

The endophytic fungi are also a source of natural products. A study was carried out in which various compounds were isolated from the endophytic fungi such as antibiotics, antiviral compounds, anticancer agents, compounds having antioxidant properties, insecticidal activities, antidiabetic activities and immunosuppressive compounds. As a result the poorly studied endophytic diversity should be explored further to understand the mechanism of action and their actual potential [Strobel et al., 2004]. A study conducted in Yuanmou county of China on medicinal plants showed that endophytic fungi exhibit antitumour and antifungal properties. Healthy plant samples were procured and 130 endophytic isolates were tested for antitumour activities on human gastric tumour cell line BGC-823 and for antifungal properties against 7 phytopathogenic fungi. As a result 12 isolates showed inhibition in growth against tumour ranging from 39-100% whereas 7.7% isolates showed antifungal activity against 6 phytopathogenic fungi [Li et al., 2005].

In a study conducted on geothermal soils, it was seen that the soils faced annual temperature fluctuations ranging from 20°C-50°C. A specie of grass *Dichanthelium lanuginosum* also known as panic grass was tested for habitat-adapted fungal symbiosis. The grass was procured and fungal endophytes were isolated. It was seen that a dominant fungal endophyte *C. protuberate (Curvularia protuberata)* was conferring heat tolerance in the panic grass. When grown individually neither the plant nor the endophyte was able to tolerate the stress conditions. Thus, it indicated that habitat adapted symbiosis is the contributor of stress tolerance. In another study conducted on coastal and geothermal soils, endophytic fungi were isolated from *Leymus mollis*, commonly known as the American dune grass. The fungal endophytes were used to inoculate test plants and subjected to abiotic stress in temperature controlled magenta boxes. The plants were studied for their change in adaptations to the stress induced on them. As a result it was seen that the endophytes conferred same tolerance to the test plants that they originally did in the grass specie that they were isolated from. It implied that habitat adapted symbiosis can result in stress tolerance [Rodriguez et al., 2008].

In another study two endophytic fungi were examined for their ability to secrete phytohormones i.e. gibberellins (GAs) and indole acetic acid (IAA). These phytohormones are known to mitigate the abiotic stress. 18 endophytes were isolated from roots of cucumber plant and quantified for production of phytohormones. Both the strains were found to be growth promoting. As a result increased shoot length,chlorophyll content, dry weights were observed on comparison with a control in which they endophytes were not induced. On phylogenetic analysis the two species were found to be of *Phoma glomerata and Penecillium sp.* [Waqas et al., 2012]. A similar study was conducted in which 9

endophytic fungi were isolated from roots of cucumber plant. Their culture filtrates were used for screening. On the basis of phylogenetic analysis the potent culture was identified as a strain of *Paecilomyces formosus* LHL10. On inoculation of *P. formosus* in host plant, it enhanced shoot length, enhanced the accumulation of proline and antioxidants and maintained plant water potential [Khan et al., 2012].

Salt tolerance and higher yield was seen in barley on induction of endophytic fungi *Piriformospora indica*. Non-infested and infested barley seedlings were exposed to moderate (100 mM NaCl) and high (300 mM NaCl) salt concentrations for 2 weeks, the non-infested seedlings showed increasing leaf chlorosis and reduced growth. The detrimental effect of moderate salt stress was completely abolished when induced by *P. indica*. The plant also showed enhanced yield, higher antioxidant capacity, and induced systemic disease resistance [Waller et al., 2005]. In another experiment 18 day old *Arabidopsis* seedlings were co-cultivated with *Piriformospora indica* and exposed to drought stress for 84 hours. After 72 hours half of the seedlings were transferred to soil while the other half was transferred after 84 hours. As a result none of the 72 hour and 84 hour uncolonized seedlings produced any flower or seeds whereas 59% of 72 hour and 47% of 84 hour colonized seeds produced flowers and seeds. Thus, *P. indica* is associated with a diverse set of stress related genes which confer resistance to the plant [Sherameti et al., 2008].

The fungal endophytes have potential of providing a novel strategy for conferring abiotic stress tolerance in plants in order to adapt to the negative impact of global climate change on agriculture [Rodriguez et al., 2008].

Chapter 3

Aim of the Study

The present study focuses on:

1. Screening of pre-isolated endophytic fungi on abiotic stress factors i.e. salinity and temperature stress.
2. Identification of the potent endophytes using classical morphotaxonomy and molecular tools.
3. Testing the potent cultures for antioxidant activity using different assays.

Chapter 4

Materials and Methods

Procurement of endophytic cultures was done from pre-existing repository maintained by Dr. Sanjai Saxena, Professor, Thapar Institute of Engineering and Technology, Patiala, Punjab.

4.1 Preparation of Potato dextrose agar (PDA) plates:

39 g of PDA (Hi-media, India) was dissolved in 1000ml of distilled water and its pH was maintained at 5.2. It was then dispensed in 250 ml flasks and covered using cotton plugs. The media was autoclaved at 121°C, 15 psi for 15 minutes. The autoclaved media was poured in sterile petri plates under aseptic conditions in lamina air flow hood (LAF)(Thermodyne Pvt. Ltd., India) and allowed to solidify before use.

4.2 Sub-culturing and Maintenance of Endophytic Isolates:

A total of 100 endophytic fungi previously isolated from two high yielding varieties (HD2967 and WH1105) of wheat grown in Punjab were procured and sub-cultured on sterile PDA media plates under aseptic conditions in laminar air flow hood (LAF) and incubated at 28 ± 2 °C for 7-8 days. After incubation a mycelial plug of actively growing endophytic culture was point inoculated on fresh media and this process was repeated to maintain the pure culture.

4.3 Preservation of Fungal Isolates:

The fungal isolates were preserved in 10%(v/v) glycerol vials. 7-8 day old actively growing fungal culture was point inoculated in sterile PDA vials containing 10% glycerol under aseptic conditions in LAF. The vials were kept at 28°C for 10 days before storing them at 4°C. The glycerol acts as cryoprotectant and protects the sample from freezing damage.

4.4 Evaluation of Endophytic Fungi for Abiotic Stress Tolerance:

The endophytic isolates were subjected to two abiotic stresses namely, salinity and temperature stress.

1. To induce salinity stress 5%, 10%, 15%, 20% (w/v) NaCl (Hi-media, India) was added to PDA media before autoclaving it. The media was then dispensed in sterile petri plates under aseptic conditions in LAF. The media was allowed to solidify. Then a mycelial disc of 7-8 day old actively growing endophytic culture was then point inoculated facing the media surface and the plate was kept at 28 ± 2°C for 10 days. The growth rate of fungi was measured by noting the mean diameter every day till 10 days. It was compared against a control which had no NaCl [Tresner and Hayes, 1971].
2. To induce temperature stress the media was prepared as described in section 4.1. Then a mycelial disc of 7-8 day old actively growing endophytic cultures was point inoculated on PDA plates and kept at different temperatures ranging from 16°C, 22°C, 34°C, 40°C for 10 days. The growth was measured by noting the mean diameter every day till 10 days and compared against a control plate kept at 28°C. [Devi et al., 2005].

4.5 Production of Filtrate of Potent Culture:

The cultures were subjected for production of filtrates. 5mm mycelial plugs of 7-8 days old actively growing culture were inoculated in a flask containing 60 ml presterilised Potato dextrose broth (PDB) (Hi-media, India) and incubated at 120 rpm for 10 days at 28°C. It was then filtered using Whatman filter paper number 4 (G.E Healthcare) and centrifuged at 10,000 rpm for 10 min to obtain cell free extract and kept at -20°C till further use.

4.6 Liquid-liquid Extraction:

The culture filtrates were then subjected to liquid-liquid extraction using ethyl acetate as a solvent. Ethyl acetate (RANKEM, India) was added to the culture filtrate in the ratio 2:1 and shaken vigorously. The organic layer was collected in a crucible, dehydrated using anhydrous sodium sulphate and evaporated using rotator evaporator. The dried crude fraction was reconstituted in methanol (RANKEM, India).

4.7 Identification of Potent Endophytic Fungi

4.7.1 Classical Morphotaxonomy:

The potent cultures were studied for microscopic properties. Different characteristics such as colony shape, color, pigment production, growth rate etc., were studied. For microscopic examination, a clean glass slide was taken and a drop of water was placed over it. Using a fine needle, mycelial mass was taken and placed over the drop of water. It was teased properly using a fine tip needle and stained using Lacto-phenol cotton blue (Hi-media, India). A cover slip of dimensions 18 × 10 mm was used to cover it while avoiding the formation of air bubbles. The slide was further mounted with DPX and observed under 10X, 40X and 100X using Nikon binocular microscope [Barnett and Hunter, 1998].

4.7.2 Molecular Identification:

4.7.2.1 DNA Isolation of Endophytic Fungi:

Fungal cultures were grown on PDA plates for 4-5 days at 28°C using Wizard Genomic DNA puification kit (Promega, USA). 0.5-1g of mycelium was grounded to fine powder using liquid nitrogen in pestle and mortar. 1 ml of cell lysis buffer was added to 660-750 μl of extraction buffer was added and crushed again. It was the transferred to 2 ml micro-centrifuge tube. Vortexed and incubated at 65°C in water bath for one hour (mixing after every 15 mins). Centrifuged at 10,000 rpm for 15 min to remove all cell debris. Further, 6l of RNase was added and incubated at 37°C for 30 min. Then 200 μl of protein precipitation solution was added. Further it was centrifuged at 12,000 rpm for 15 min to obtain DNA pellet. The pellet was washed with 70% chilled ethanol, centrifuged at 12000 rpm for 2 min. The supernatant was decanted and allowed to air dry. Finally, the pellet was dissolved in 20 μl of TE buffer. Agarose gel electrophoresis was done to perform the qualitative estimation of DNA.

4.7.2.2 Quantitative and Qualitative Estimation of Genomic DNA:

Agarose gel (0.8%) was prepared in 1X TAE buffer and 0.5 μg/ml of EtBr (visualization dye) was added to it. This gel was poured in gel castng tray and comb was inserted to make wells. The gel was allowed to polymerise. After polymerisation of gel, samples were mixed with 6X loading dye and were loaded into the wells. Gel was allowed to run at 70 V for 1 hour. Further, the gel was observed under UV trans-illuminator. For imaging of DNA, Bio Rad Gel Documentation System which uses 1 D Quantity analysis software

was used. For the quantitative analysis of DNA, the absorbance of DNA was taken at 260 and 280 nm. 50 μg/ml of DNA sample is equal to 1O.D. The concentration of DNA was calculated by following formula-

$$Concentration(\frac{\mu g}{ml}) = O.D_{260nm} * 50(\frac{\mu g}{ml}) * Dilution factor$$

The purity of the DNA sample was analyzed by taking ratio of O.D. at 260 and 280 nm. If the value comes below 1.6, the DNA is contaminated with protein. If it is between 1.6 1.8, the DNA sample is pure. If the value comes above 1.8, the DNA sample is contaminated by RNA.

4.7.2.3 PCR Amplification of Genomic DNA:

Amplification of ITS1-5.8S-ITS2 rDNA sequence was carried out using universal primer pair i.e. ITS 1 and ITS 4 [White et al., 1990] synthesized by Integrated DNA Technologies (IDT), USA, in a Thermocycler (My Cycler, Bio-Rad Laboratories, Inc.). Amplification was performed in 25 μl of reaction mixture containing 25ng of extracted fungal DNA, 0.8 μM of each primer (ITS1 and ITS4), 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 conditions for Thermal cycler consisted of initial denaturation at 96°C for 5 min followed by 39 cycles of 95°C for 1 min, 58°C for 1.30 min, 72°C for 1 min followed by final extension at 72°C for 5 min. The PCR amplicons were examined using gel electrophoresis in a 1.5 % agarose gel at 40V for 1.30 hr. Gel imaging was performed under UV light in Bio- Rad Gel documentation System. The amplicons were purified using Wizard SV Gel and PCR clean up system kit (Promega, USA) and sent for sequencing to GeNei, Bangalore.

4.8 Biological Activity of Potential Isolates:

4.8.1 *In-vitro* Antioxidant Assay:

4.8.1.1 DPPH 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 with slight modification [Kitts et al., 2000]. Briefly, 20 μl of the 1mg/ml test sample (200-1000mg/ml dilution) 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 using ELISA reader (Biotek, USA). 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:

$$\%FRS = \frac{Absorbance(Control) - Absorbance(Sample)}{Absorbance(Control)} * 100$$

It was further used to calculate IC_{50} The antioxidant activity was expressed as μg of Quercetin per mg of extract. The test was performed in triplicate and the data was reported as mean ± SD (Standard deviation).

4.8.1.2 Trolox Equivalent Antioxidant Capacity (TEAC Assay):

It uses the ability of an antioxidant to scavenge ABTS to determine the level of its activity [Re et al., 1999]. ABTS radical was generated by mixing 7 mM ABTS (2, 2-azino-bis-3-ethylbenzothiazoline 6-sulphonic acid) made in 0.1 M PBS of pH-7.4 with 2.45 mM potassium persulphate in equal volume followed by 16 hour incubation at room temperature in dark. This ABTS radical was diluted in PBS to an absorbance of 0.9-1.0. Then 1ml of working ABTS solution was added to 10 μl of the fungal extract (200-1000mg/ml dilution) and the reaction mixture was incubated for 6 minutes. The decrease in absorbance was recorded at 734 nm using ELISA reader. Phosphate buffer saline was used as blank and ABTS was used as control. Percentage free radical scavenging (%FRS) at different concentration was calculated using the formula.

$$\%FRS = \frac{Absorbance(Control) - Absorbance(Sample)}{Absorbance(Control)} * 100$$

A linear regression was plotted between the different concentrations and their respective %FRS which was then used to calculate the IC_{50}. The test was performed in triplicate and the data was reported as mean ± SD.

4.8.1.3 Ferric ion Reducing Antioxidant Power (FRAP Assay):

It uses a mixture of 0.3M sodium acetate buffer, 10 mM 2, 4, 6-tripyridyl-s-triazine and 20 mM Iron (III) chloride hexahydrate in the ratio 10:1:1 as FRAP reagent [Benzie and Strain, 1996]. 1 ml of FRAP reagent was added to 10 μl of 1mg/ml extract and mixed thoroughly. The reaction mixture was incubated for 30 minutes at room temperature

before checking the absorbance at 595 nm using ELISA reader. Ascorbic acid at concentration ranging from 10-50 μg/ml was used as a standard whereas working solution of FRAP with deionised water was used as blank. A linear regression was plotted between the concentrations of standard and their absorbance using which the concentration of sample was calculated. The ferric reducing antioxidant power was measured as μg ascorbic acid equivalent per mg of extract. The test was performed in triplicate and the data was reported as mean ± SD.

4.8.1.4 Total Phenolic Content (TPC) Estimation:

The total phenolic content was determined using Folin-Ciocalteu (FC) reagent [Ainsworth and Gillespie, 2007]. To 100 μl of 1mg/ml fungal extract 1.5ml of deionised water and 100 μl FC reagent was added. The mixture was incubated at room temperature for 10 minutes. Then, 200 μl of Na_2CO_3 (6% w/v) was added to the reaction mixture and allowed to stand at room temperature for 1 hour. Gallic acid (10-100 μg/ml) was used as a standard. The absorbance was noted at 760 nm after incubation using ELISA reader. A linear regression was plotted between the concentrations of standard and their absorbance using which the concentration of sample was calculated. Total phenolic content was expressed as μg of Gallic acid equivalent per mg of sample. The test was performed in triplicate and the data was reported as mean ± SD.

4.8.1.5 Total Flavonoid Content (TFC) Estimation:

The total flavonoid content of the fungal extract was determined [Bahorun et al., 2004]. To 200 μl of 1mg/ml fungal extract 800 μl deionised water and 60 μl $NaNO_2$ (5%w/v) was added. The reaction mixture was incubated for 5 minutes at room temperature. Then 60 μl $AlCl_3$ (10%w/v) was added and the reaction mixture was again incubated for 1 minute. 400 μl of 1N NaOH was added to the reaction mixture and the volume was made up to 2 ml using distilled water. Quercetin (50-250 μg/ml) was used as standard. The absorbance was noted at 510 nm. A linear regression was plotted between the concentrations of standard and their absorbance using which the concentration of sample was calculated. Total flavonoid content was expressed as μg of Quercetin equivalent per mg of sample. The test was performed in triplicate and the data was reported as mean ± SD.

Chapter 5

Results

5.1 Sub-culturing and maintenance of pure cultures:

A total of 100 endophytic fungi isolates were procured from the pre-existing repository maintained by Dr. Sanjai Saxena, Professor, T.I.E.T, Patiala, Punjab. The isolates were sub-cultured on PDA plates and kept for incubation at 28 ± 2°C for 7-8 days before further use. To maintain the cultures were sub-cultured on fresh PDA media repeatedly. The figure 5.1 shows some of the endophytic fungi grown during this study and figure 5.2 shows microscopic features of some of the endophytes screened during this study.

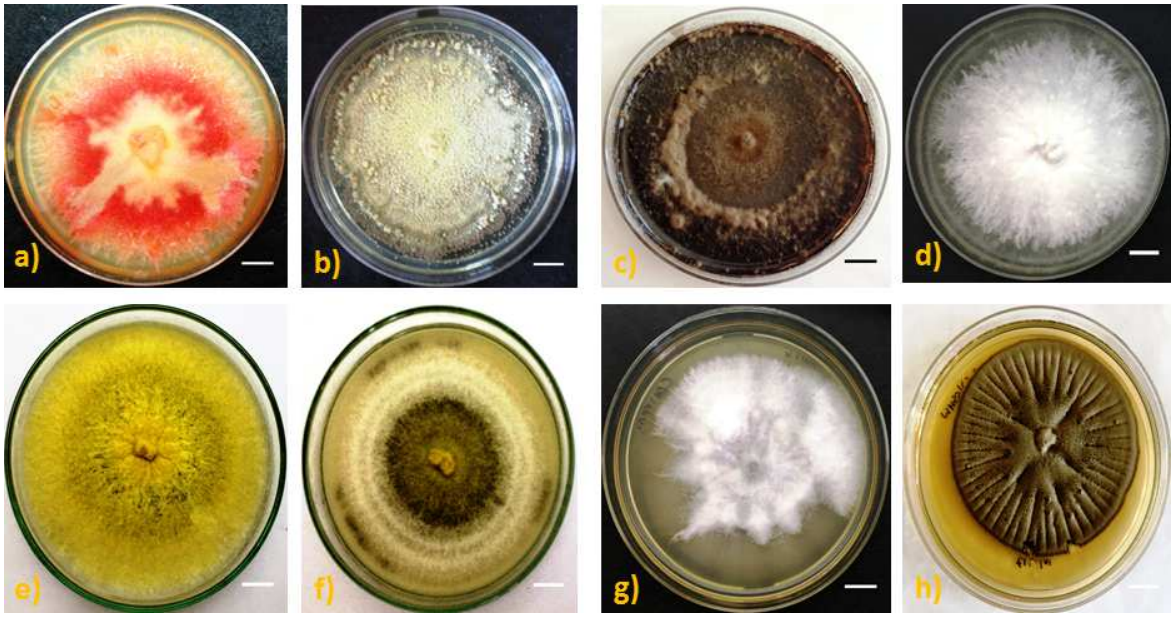

Figure 5.1: Pure culture of different endophytic fungi procured from lab. a) *Fusarium sp.* b) *Asperigillus nidulans* c) *Lasidoplodia* d) *Alternaria* e) Unidentified f) *Nigrospora* g) *Fussarium sp.* h) *Aspergillus sp.* (Bar: 10 mm)

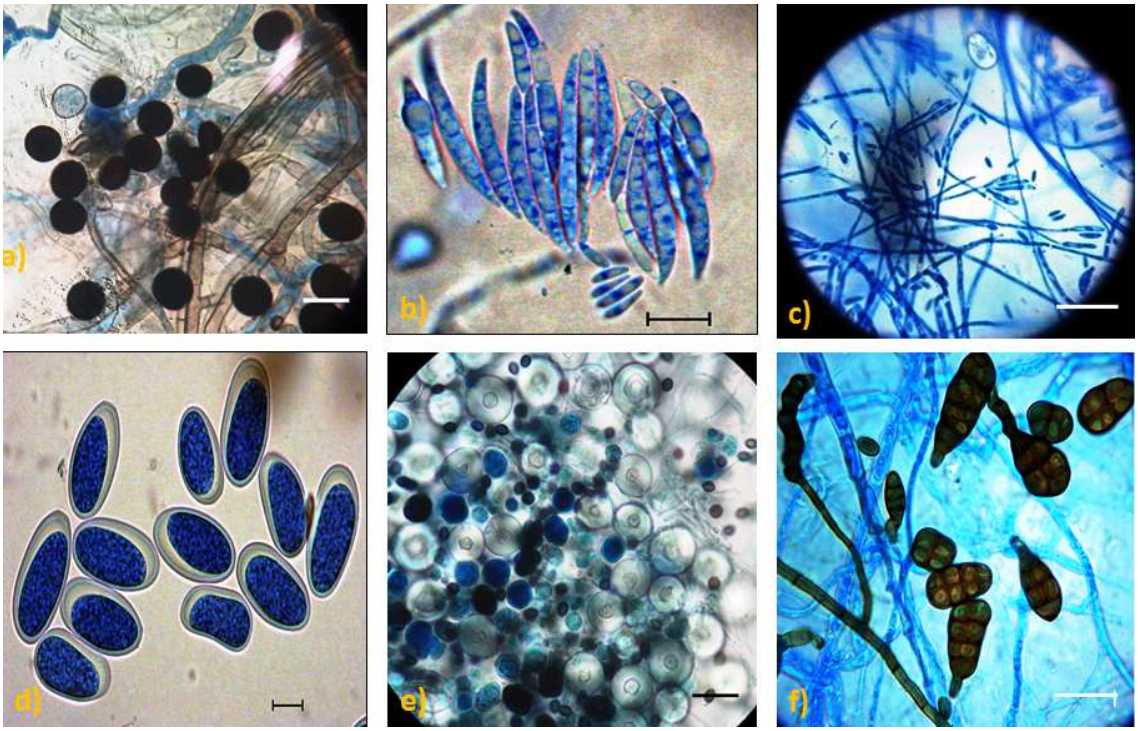

Figure 5.2: Microscopic features of some of the endophytic fungi screened during this study. a) *Nigrospora sp.* b) *Fusarium sp.* c) *Fusarium sp.* d) *Lasiodiplodia sp.* e) *Aspergillus nidulans* f) *Alternaria sp.* (Bar: 10 μm)

5.2 Preservation of endophytic fungi:

To preserve the isolates PDA vials or slants contacting 10% (v/v) glycerol were used. The 7-8 day old culture was point inoculated in autoclaved vials and kept for incubation at 28±2°C for 10-12 days. The vials were finally stored at 4°C.

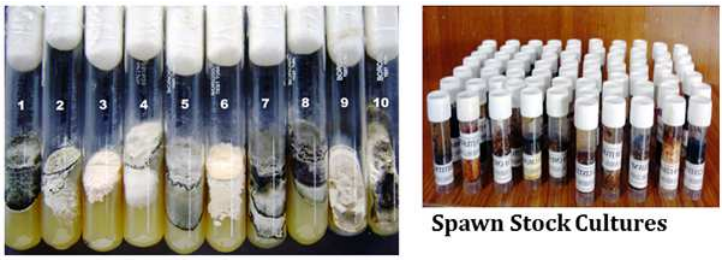

Figure 5.3: PDA slants and vials for preservation of fungal endophytes

5.3 Evaluation of Endophytic Fungi for Abiotic Stress Tolerance:

Two parameters of abiotic stress i.e. salinity and extreme temperature stress were studied on the fungal endophyte isolates. The results obtained on screening the endophytes are given in Table 5.1. The isolates #2(1)TAHRTU 12, #2(4)TAHLTU 15(e) and #2(1)TAWLTU 12(a) were selected as they showed tolerance against the highest parameters of each abiotic stress factor.

Table 5.1: Abiotic stress

S.No.	**Culture Code**	**Plant part**	**Wheat variety**	**Maximum salinity(w/v)**	**Maximum Temperature °C**
1	#6TAHRTU	Root	HD 2967	15%	34°C
2	#8TAHRTU	Root	HD 2967	10%	34°C
3	#3TAHLTU	Leaf	HD 2967	15%	34°C
4	#10TAHLTU	Leaf	HD 2967	15%	28°C
5	#13TAHLTU	Leaf	HD 2967	15%	28°C
6	#28TAHLTU	Leaf	HD 2967	-	28°C
7	#1(1)TAHSTU1	Internode	HD 2967	10%	34°C
8	#6TAHLTU 1	Leaf	HD 2967	15%	34°C
9	#1(1)TAHRTU 2	Root	HD 2967	10%	40°C
10	#2(1)TAHRTU12	Root	HD 2967	20%	40°C
11	#1(2)TAHRTU3	Root	HD 2967	10%	28°C
12	#1(3)TAHRTU7	Root	HD 2967	15%	34°C
13	#1(4)TAHRTU6	Root	HD 2967	10%	34°C
14	#1(1)TAHSTU2(a)	Internode	HD 2967	10%	34°C
15	#2(3)TAHSTU16(a)	Internode	HD 2967	15%	28°C

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Table 5.1: Abiotic stress (Cont.)

S.No.	Culture Code	Plant part	Wheat variety	Maximum salinity(w/v)	Maximum Temperature °C
16	#1(4)TAHLTU2(a)	Leaf	HD 2967	10%	28°C
17	#1(1)TAHRTU8(a)	Root	HD 2967	10%	28°C
18	#1(3)TAHRTU5(a)	Root	HD 2967	-	40°C
19	#1(1)TAHRTU6(b)	Root	HD 2967	15%	28°C
20	#2(3)TAHRTU10(b)	Root	HD 2967	10%	28°C
21	#2(1)TAHRTU15(b)	Root	HD 2967	10%	28°C
22	#1(2)TAHRTU5(b)	Root	HD 2967	10%	28°C
23	#2(2)TAHRTU13(b)	Root	HD 2967	10%	28°C
24	#2(1)TAHSPTU14(c)	Internode	HD 2967	10%	28°C
25	#2(1)TARTU14(c)	Root	HD 2967	10%	28°C
26	#2(1)TAHRTU15(c)	Root	HD 2967	15%	28°C
27	#1(2)TAHRTU1(c)	Root	HD 2967	5%	28°C
28	#1(2)TAHRTU8(c)	Root	HD 2967	5%	28°C
29	#2(2)TAHRTU14(c)	Root	HD 2967	10%	28°C
30	#2(3)TAHRTU14(c)	Root	HD 2967	10%	28°C
31	#2(4)TAHRTU11(c)	Root	HD 2967	10%	28°C
32	#3TAHSPTU17(d)	Spike	HD 2967	10%	28°C
33	#2(1)TAHLTU9(d)	Leaf	HD 2967	20%	28°C
34	#1(1)TAHLTU1(d)	Leaf	HD 2967	5%	28°C
35	#2(1)TAHSTU9(d)	Internode	HD 2967	15%	28°C
36	#2(1)TAHSTU15(d)	Internode	HD 2967	15%	28°C
37	#1(1)TAHRTU1(d)	Root	HD 2967	5%	40°C
38	#2(3)TAHRTU11(d)	Root	HD 2967	10%	34°C
39	#2(1)TAHRTU14(d)	Root	HD 2967	10%	28°C
40	#2(3)TAHRTU16(d)	Root	HD 2967	10%	28°C
41	#1(4)TAHLTU1(e)	Leaf	HD 2967	10%	28°C
42	#2(4)TAHLTU15(e)	Leaf	HD 2967	20%	40°C
43	#1(1)TAHRTU7(e)	Root	HD 2967	10%	34°C
44	#1(4)TAHRTU6(e)	Root	HD 2967	10%	28°C
45	#1(4)TAHRTU8(e)	Root	HD 2967	10%	28°C
46	#1(2)TAHRTU8(e)	Root	HD 2967	15%	28°C
47	#2(1)TAHRTU13(e)	Root	HD 2967	15%	28°C
48	#2(2)TAHRTU9(e)	Root	HD 2967	15%	28°C
49	#1(1)TAHRTU5(e)	Root	HD 2967	20%	28°C
50	#1(4)TAHRTU4(e)	Root	HD 2967	10%	28°C
51	#6TAHSPTU42(f)	Spike	HD 2967	-	28°C
52	#2(1)TAHSTU10(f)	Internode	HD 2967	5%	34°C
53	#1(1)TAHSTU4(f)	Internode	HD 2967	5%	28°C

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Table 5.1: Abiotic stress (Cont.)

S.No.	Culture Code	Plant part	Wheat variety	Maximum salinity(w/v)	Maximum Temperature °C
54	#1(2)TAHSTU7(f)	Internode	HD 2967	5%	28°C
55	#2(1)TAHRTU12(f)	Root	HD 2967	5%	28°C
56	#1(3)TAHRTU4(f)	Root	HD 2967	15%	28°C
57	#1(3)TAHRTU8(f)	Root	HD 2967	5%	28°C
58	#2(4)TAHRTU12(f)	Root	HD 2967	5%	28°C
59	#1(2)TAHLTU7(g)	Leaf	HD 2967	10%	34°C
60	#2(2)TAHRTU10(g)	Root	HD 2967	5%	28°C
61	1(3)TAHRTU8(g)	Root	HD 2967	15%	28°C
62	#14TAWRTU	Root	WH 1105	5%	34°C
63	#19TAWRTU	Root	WH 1105	-	40°C
64	#26TAWRTU	Root	WH 1105	15%	34°C
65	#31TAWRTU	Root	WH 1105	15%	34°C
66	#22TAWRTU	Root	WH 1105	15%	28°C
67	#2TAWLTU7	Leaf	WH 1105	15%	28°C
68	#1(2)TAWRTU5	Root	WH 1105	20%	28°C
69	#1(3)TAWRTU4	Root	WH 1105	15%	34°C
70	#2(1)TAWLTU12(a)	Leaf	WH 1105	15%	40°C
71	#2(1)TAWRTU10(a)	Root	WH 1105	20%	34°C
72	#2(1)TAWRTU15(a)	Root	WH 1105	15%	34°C
73	#2(1)TAWRTU16(a)	Root	WH 1105	15%	34°C
74	#1(1)TAWSTU7(b)	Internode	WH 1105	5%	28°C
75	#2(3)TAWSTU12(b)	Internode	WH 1105	15%	28°C
76	#2(1)TAWLTU11(b)	Leaf	WH 1105	-	28°C
77	#1(3)TAWLTU1(b)	Leaf	WH 1105	5%	28°C
78	#1(3)TAWRTU2(b)	Root	WH 1105	-	28°C
79	#1(3)TAWRTU5(b)	Root	WH 1105	15%	28°C
80	#2(3)TAWRTU16(b)	Root	WH 1105	10%	28°C
81	#2(4)TAWRTU8(b)	Root	WH 1105	15%	28°C
82	#2(3)TAWSTU10(c)	Internode	WH 1105	15%	28°C
83	#1(1)TAWRTU3(c)	Root	WH 1105	10%	40°C
84	#2(1)TAWRTU13(c)	Root	WH 1105	10%	28°C
85	#1(4)TAWRTU7(c)	Root	WH 1105	10%	28°C
86	#2(4)TAWRTU9(c)	Root	WH 1105	10%	28°C
87	#5TAWRTU4(c)	Root	WH 1105	15%	28°C
88	#2(3)TAWLTU10(d)	Leaf	WH 1105	5%	28°C
89	#2(2)TAWRTU15(d)	Root	WH 1105	15%	28°C
90	#2(3)TAWRTU16(d)	Root	WH 1105	5%	28°C
91	#1(4)TAWRTU1(d)	Root	WH 1105	10%	28°C

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Table 5.1: Abiotic stress (Cont.)

S.No.	Culture Code	Plant part	Wheat variety	Maximum salinity(w/v)	Maximum Temperature °C
92	#3TAWSPTU19(e)	Spike	WH 1105	15%	34°C
93	#1(2)TAWRTU2(e)	Root	WH 1105	10%	28°C
94	#1(4)TAWRTU2(e)	Root	WH 1105	10%	28°C
95	#1TAWSPTU3(f)	Spike	WH 1105	10%	34°C
96	#2(4)TAWRTU16(f)	Root	WH 1105	10%	28°C
97	#2(3)TAWSPTU15(g)	Spike	WH 1105	15%	28°C
98	#1TAWSTU2(g)	Internode	WH 1105	-	34°C
99	#1TAWLTU4(g)	Leaf	WH 1105	15%	28°C
100	#1TAWLTU8(g)	Leaf	WH 1105	20%	28°C

The average growth on salinity and temperature stress of potent cultures is shown from Figure 5.4 to Figure 5.9.

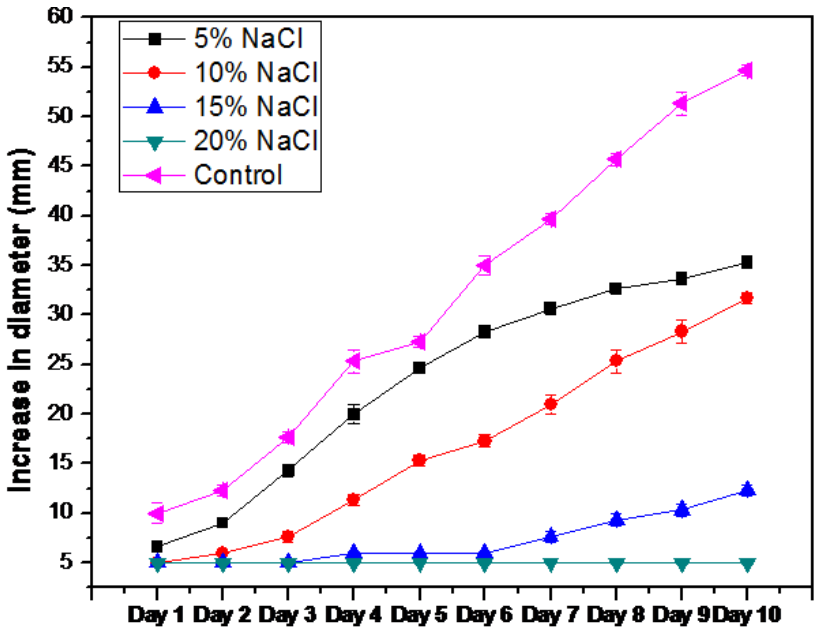

Figure 5.4: The average change in diameter of #2(1)TAWLTU 12(a) at different concentrations of NaCl.

The isolate #2(1)TAWLTU 12(a) did not show any growth at 20% NaCl concentration but approximately 20% growth at 15% NaCl was observed as compared to control. Whereas 50-55% growth at 10% and 5% NaCl concentration was observed as compared to control (As shown in Figure 5.4).

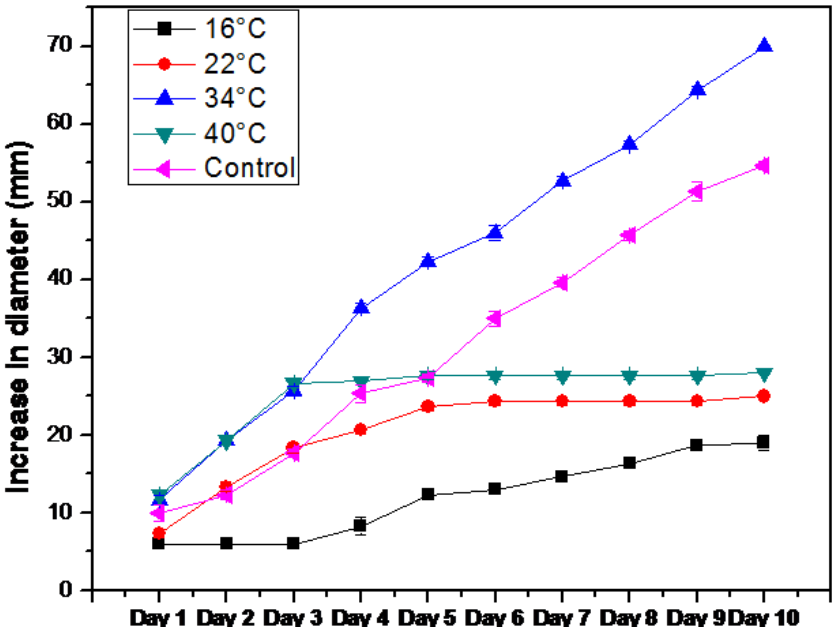

Figure 5.5: The average change in diameter of #2(1)TAWLTU 12(a) at different temperatures.

The same isolate exhibited no growth at 40°C initially but after day 3, 20% growth at 40°C as compared to control was seen. Whereas 35-70% growth as compared to control was seen at lower temperatures. It also exhibited better growth at 34°C as compared to control (As shown in Figure 5.5).

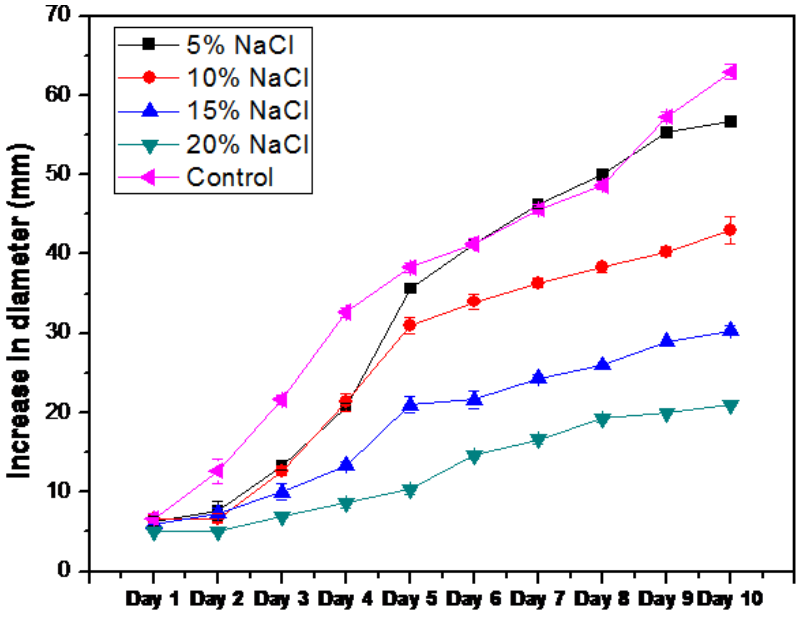

Figure 5.6: The average change in diameter of #2(1)TAHRTU 12 at different concentrations of NaCl.

The isolate #2(1)TAHRTU 12 exhibited approximately 30% growth at 20% NaCl as compared to control. At lower concentrations of NaCl approximately 35-80% growth was observed as compared to control (As shown in Figure 5.6).

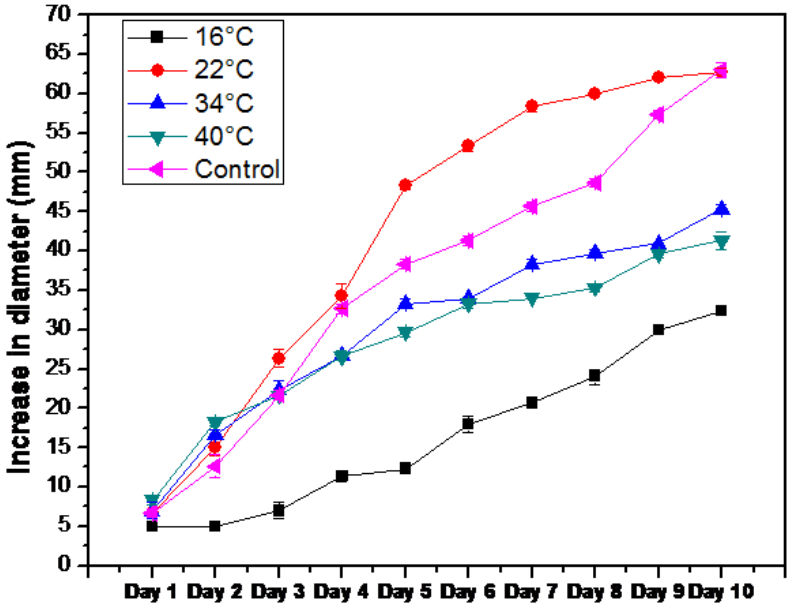

Figure 5.7: The average change in diameter of #2(1)TAHRTU 12 at different temperatures.

The isolate #2(1)TAHRTU 12 exhibited 50-55% growth at 34°C and 40°C as compared to control (As shown in Figure 5.7).

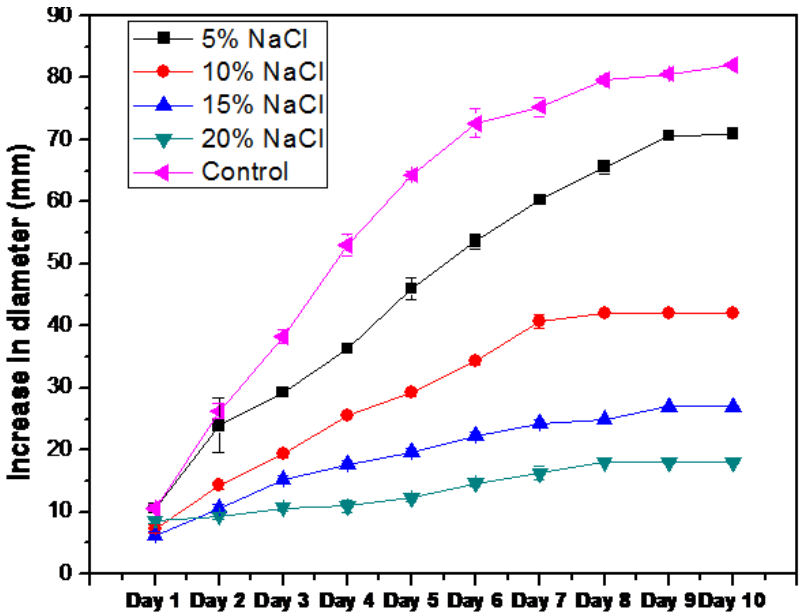

Figure 5.8: The average change in diameter of #2(4)TAHLTU 15(e) at different concentrations of NaCl.

A growth of approximately 20-30% was seen of isolate #2(4)TAHLTU 15(e) at 15-20% NaCl as compared to control (As shown in Figure 5.8).

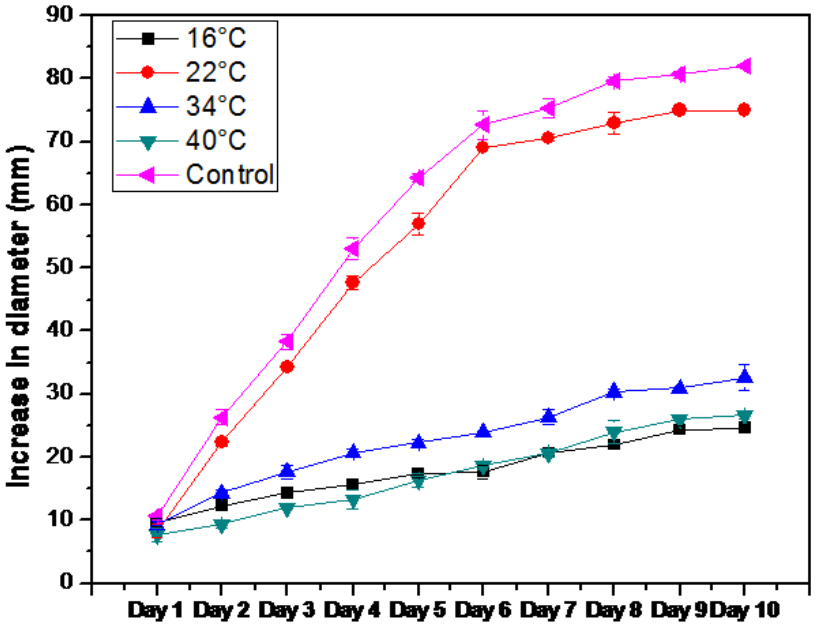

Figure 5.9: The average change in diameter of #2(4)TAHLTU 15(e) at different temperatures.

The same isolate exhibited a growth of approximately 20% at 34-40°C as compared to control (As shown in Figure 5.9).

5.4 Production of Filtrate of Potent Cultures:

The cultures #2(1)TAHRTU 12, #2(4)TAHLTU 15(e) and #2(1)TAWLTU 12(a) were subjected to production of secondary metabolites in PDB medium.

Figure 5.10: Production of culture filterate

5.5 Identification of Potent Endophytic Fungi:

The isolates #(1)TAHRTU 12, #2(4)TAHLTU 15(e) and #2(1)TAWLTU 12(a) which are the potent cultures screened in the study, were identified using both morphotaxonomy and molecular biology tools.

5.5.1 Morphotaxonomy:

1. **Morphotaxonomy of #2(1) TAHRTU 12:** The endophytic fungus #2(1) TAHRTU 12 produced white, white-yellow (90 ± 0) mostly consisting of a dense felt of erect conidiophores over PDA and MEA. Colonies are granular to cottony or powdery white in color initially and later becomes brown to black with shades of green or with a white apron at margin over PDA and MEA. The reverse side is white to tan unlike most species its grows at 40°C. Conidiophores are long approx. 200-450 μ in diameter. Vesicles are uniseriate and are covered by phialides/candida on only disital half. Conidia are single celled, smooth or rough walled, hyaline, and produced in long dry chains which may radiate in compact columns. Morphological and microscopic features of the isolate can be seen in Figure 5.11. Based on these morphological features the fungus was identified as *Aspergillus sp.*

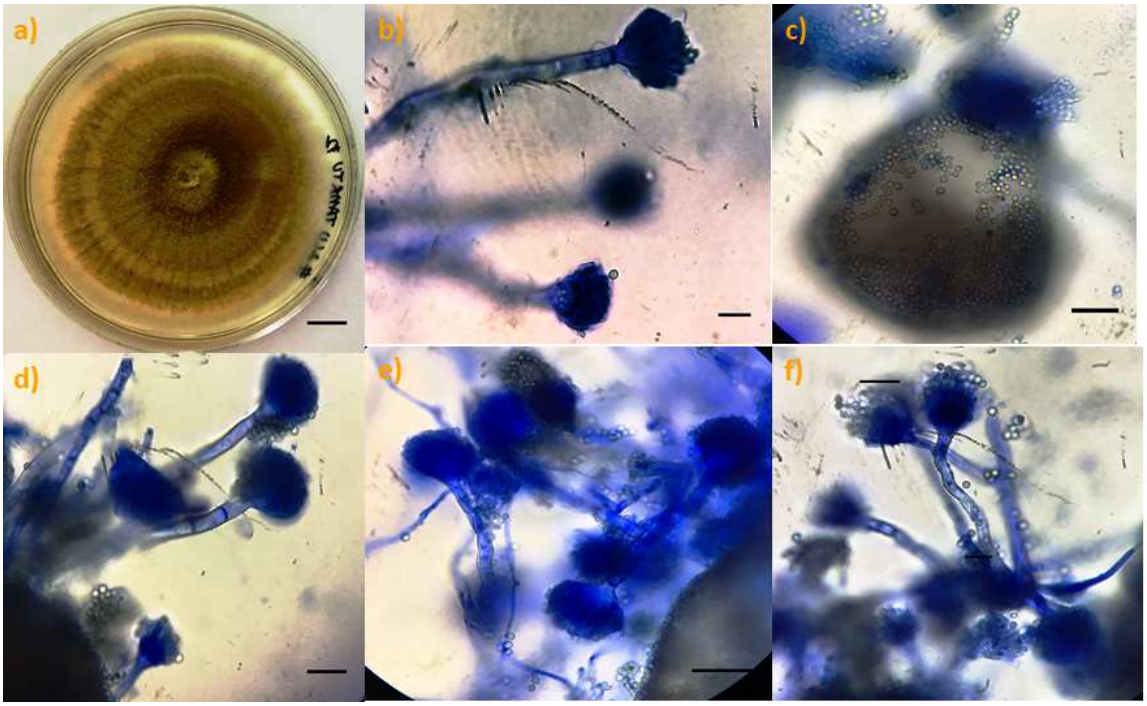

Figure 5.11: Morphological and microscopic feature of #2(1) TAHRTU 12. Colony morphology on PDA medium (Bar: 10mm), c) Conidia in the conidiophore over PDA medium, b), d-e) Conidial heads with conidia on MEA medium, f) Vesicle formation (Bar: 10 μm)

2. **Morphotaxonomy of #2(1) TAWLTU 12 (a):** The endophytic fungus #2(1) TAWLTU 12 (a)produced white, velvety-white (90 ± 0). Initially it was white gradually turned to yellow and after that dark brown in color. Conidiophores are short in length and brown in color. Conidia are short, rough walled, globose, light brown in color, 2-5 μm in diameter. The ascospores are also rough walled, dark brown in color with 4-7μm in diameter. Morphological and microscopic features of the isolate can be seen in Figure 5.12. Based on these morphological features the fungus was identified as *Aspergillus nidulans.*

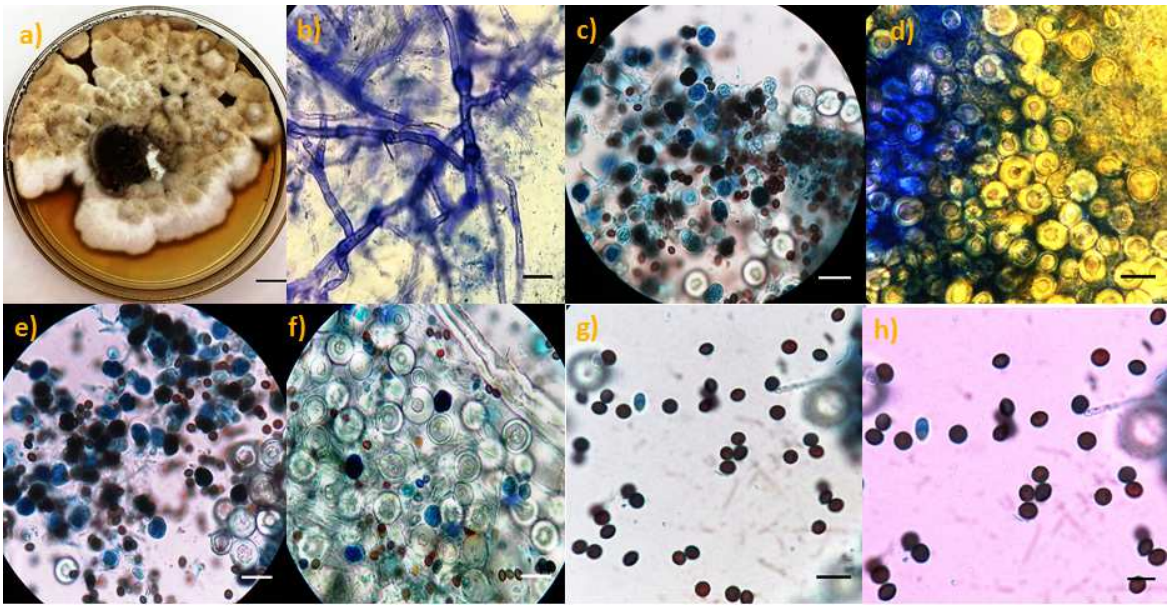

Figure 5.12: Morphological and microscopic feature of #2(1) TAWLTU 12(a). a) Colony morphology on PDA medium (Bar: 10 mm), b) Formation of conidial head, c) - f) Smooth walled ascospores with conidia over PDA medium, g)-h) Conidia (Bar: 10 μm)

3. **Morphotaxonomy of #2(4) TAHLTU 15 (e):**The endophytic fungus #2(1) TAHLTU 15(e)produced white, white-yellow (90 ± 0) mostly consisting of a dense felt of erect conidiophores over PDA and MEA. Colonies are granular to cottony or powdery white in color initially and later becomes brown to black with shades of green or with a white apron at margin over PDA and MEA. The reverse side is white to tan unlike most species its grows at 40°C. Conidiophores are long approx. 200-450μ in diameter. Vesicles are uniseriate and are covered by phialides/candida on only disital half. Conidia are one celled, smooth or rough walled, hyaline, and produced in long dry chains which may be radiate in compact columns. Morphological and microscopic features of the isolate can be seen in Figure 5.13. Based on these morphological features the fungus was identified as *Aspergillus sp.*

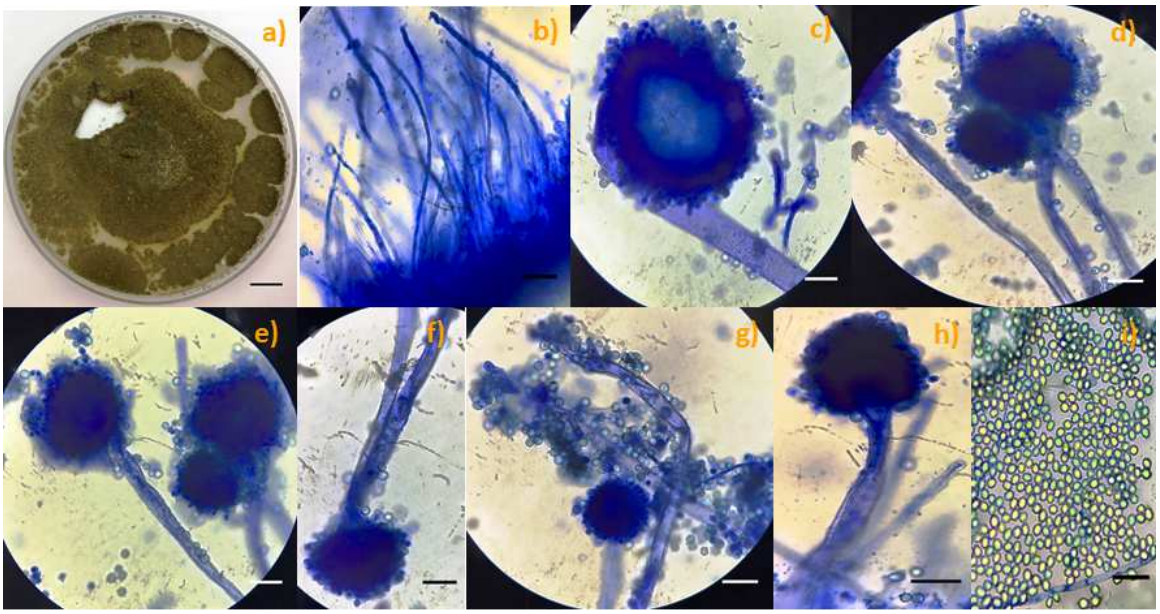

Figure 5.13: Morphological and microscopic feature of #2(4) TAHLTU 15 (e). a) Colony morphology on PDA medium (Bar: 10 mm), b-c) Vesicle formation over PDA medium, d-h) Conidial heads with conidia on MEA medium, i) Irregular conidia formation (Bar: 10 μm)

5.5.2 Molecular Identification:

5.5.2.1 Genomic DNA Isolation:

The genomic DNA of #2(1)TAHRTU 12, #2(4)TAHLTU 15(e) and #2(1)TAWLTU 12(a) were isolated and the size of genomic DNA was found to be 10 kb approximately.

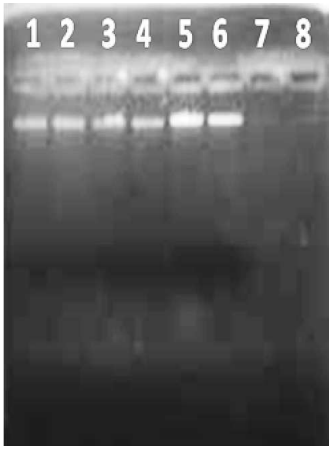

Figure 5.14: Lane 1-2) Genomic DNA of #2(1)TAHRTU 12, Lane 3-4) Genomic DNA of #2(4)TAHLTU 15(e), Lane 5-6) Genomic DNA of #2(1)TAWLTU 12(a)

5.5.2.2 Quantitative and Qualitative Estimation of Genomic DNA:

The concentration of Genomic DNA of #2(1)TAHRTU 12, #2(4)TAHLTU 15(e) and #2(1)TAWLTU 12(a) were found to be 386.2 ng/μl, 101.3 ng/μl and 211 ng/μl respectively. The absorbance at 260/280nm was 1.69, 1.64 and 1.67 respectively.

5.5.2.3 PCR Amplification of Genomic DNA:

PCR amplification of the cultures has been performed and the amplicons were resolved in 1.5% agarose gel to check the size of the amplicon on the basis of its mobility and compared with a ladder of 100 bp. The size of the PCR amplicon was found to be in the range of 500-600 bp for DNA amplified using ITS primer. The bulk PCR amplification of culture #2(1)TAHRTU 12, #2(4)TAHLTU 15(e) and #2(1)TAWLTU 12(a) using ITS primer was carried out, purified and was sent for sequencing. The sequencing results are still awaited.

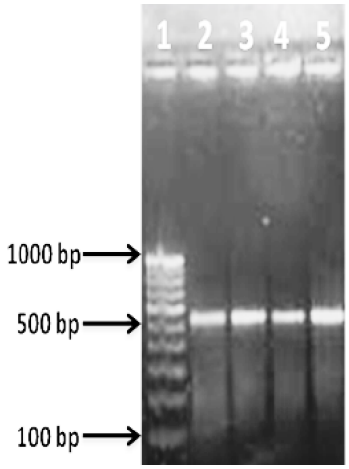

Figure 5.15: Lane 1) Ladder of 100 bp, Lane 2) Amplified DNA of #2(1)TAHRTU 12, Lane 3) Amplified DNA of #2(4)TAHLTU15(e), Lane 4-5) Amplified DNA of #2(1)TAWLTU12(a)

5.6 Biological Activity:

5.6.1 *Invitro* Antioxidant Assay:

The potent cultures screened during this study were tested for their antioxidant capacity using the following assays.

5.6.1.1 DPPH Antioxidant Assay:

The culture #2(1)TAWLTU12(a) showed highest antioxidant capacity as compared to the other two samples and the standard quercetin The IC_{50} value of #2(1)TAWLTU12(a) came out to be 83.01 ± 7.8 mg/ml followed by #2(1)TAHRTU 12, exhibiting IC_{50} value of 274.5 ± 45.8 mg/ml which was even higher than the Quercetin (IC_{50} = 374.65 ± 10.5) used as the standard and #2(4)TAHLTU15(e) exhibited least IC50 value of 657.2 ± 25.04 mg/ml (As shown in Table 5.2).

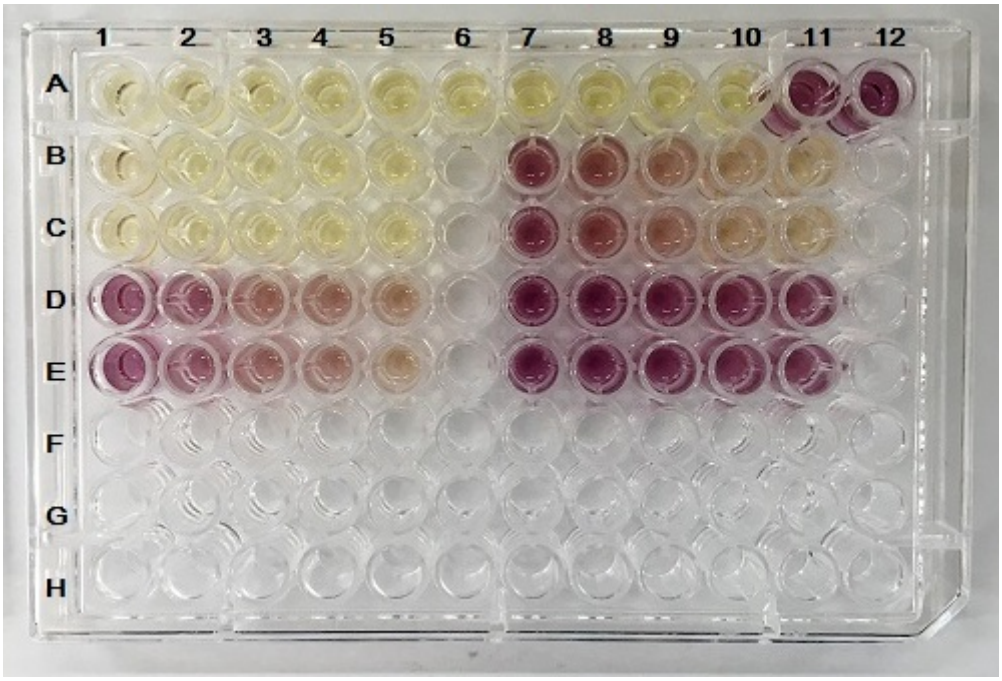

Figure 5.16: ELISA plate showing DPPH assay. A1-A10) Concentration of Quercetin (200-1000 μg/ml). A11-12) Working solution og DPPH. B1-B5, C1-C5) #2(1)TAWLTU 12(a). B7-B11, C7-C11, D1-D5, E1-E5) #2(1)TAHRTU 12. D7-D11, E7-E11) #2(4)TAHLTU 15(e)

In the Figure 5.16 we can see the change in colour from purple to yellow caused over time. This change in colour is due to the reduction of DPPH radical caused by the antioxidants present in the fungal extract. The pale yellow colour indicates higher antioxidant activity present in that particular extract.

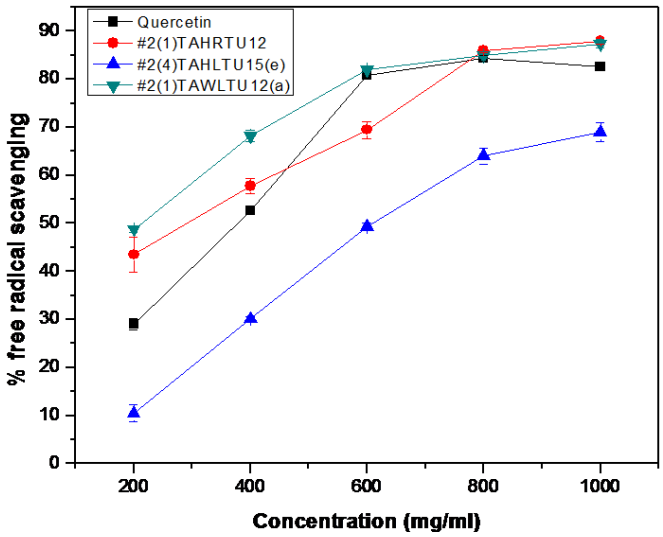

Figure 5.17: % FRS of potent cultures at different concentration (mg/ml) examined using DPPH assay

5.6.1.2 Trolox Equivalent Antioxidant Capacity (TEAC Assay):

In this assay the sample #2(1)TAHRTU12(a) exhibited highest scavenging at IC_{50} of 95.7 ± 9.9 mg/ml followed by #2(1)TAHRTU12 which exhibited IC_{50} of 195.6 ± 4.9 mg/ml and #2(4)TAHLTU15(e) which exhibited IC_{50} of 368.9 ± 6.2 mg/ml. All the isolates exhibited better scavenging as compared to Trolox (IC_{50}=698.5±12.8) which was used as a standard.

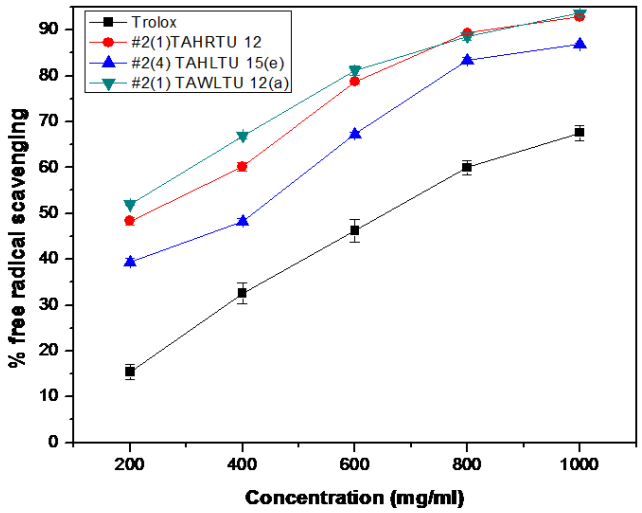

Figure 5.18: % FRS of potent cultures at different concentration (mg/ml) examined using TEAC assay

Table 5.2: IC_{50} value of top endophytic fungi screened

S.No.	Sample	IC_{50} (mg/ml)	
		DPPH	TEAC
1.	Standard	374.65 ± 10.50	698.50 ± 12.84
2.	#2(1) TAHRTU 12	274.46 ± 45.80	195.62± 4.88
3.	#2(4) TAHLTU 15(e)	657.19 ± 25.04	368.88 ± 6.16
4.	#2(1) TAWLTU 12(a)	83.01 ±7.79	95.73 ±9.94

5.6.1.3 Ferric ion Reducing Antioxidant Power (FRAP Assay):

The maximum Fe(II) ion reducing capacity was that of #2(1)TAWLTU 12(a) at 110.5 ± 1.9 μg Fe(II) equivalent per mg of sample, followed by #2(1) TAHRTU 12 at 98.6 ± 0.03 μg Fe(II) equivalent per mg of sample which was evaluated from the equation (y=0.0374x-0.1607). The least reducing capacity was that of #2(4)TAHLTU15(e) at 44.9 ± 0.8 μg Fe(II) equivalent per mg of sample (As shown in table 5.3).

5.6.1.4 Total Phenolic Content (TPC) Estimation:

The total phenolic content was highest in #2(1)TAHRTU12(a) at 129.9 ± 3.5 μg Gallic acid equivalent per mg of sample followed by #2(1)TAHRTU12 at 107.6 ± 2.5 μg Gallic acid equivalent per mg of sample. The least phenolic content was seen in #2(4)TAHLTU15(e) at 80.7 ± 0.6 μg Gallic acid equivalent per mg of sample (As shown in Table 5.3). The concentration was evaluated from the equation (y=0.0104x-0.1915)

5.6.1.5 Total Flavonoid Content (TFC) Estimation:

Total flavonoid content was seen maximum in the isolate #2(1)TAHRTU12(a) at 346.08 ± 27.4 μg Quercetin equivalent per mg of sample followed by #2(1)TAHRTU12 at 310.1 ± 12.7 μg Quercetin equivalent per mg of sample. The least flavonoid content was seen in #2(4)TAHLTU15(e) at 192.5 ± 15.5 μg Quercetin equivalent per mg of sample (As shown in Table 5.3). The concentration was evaluated from the equation (y=0.0007x+0.0569)

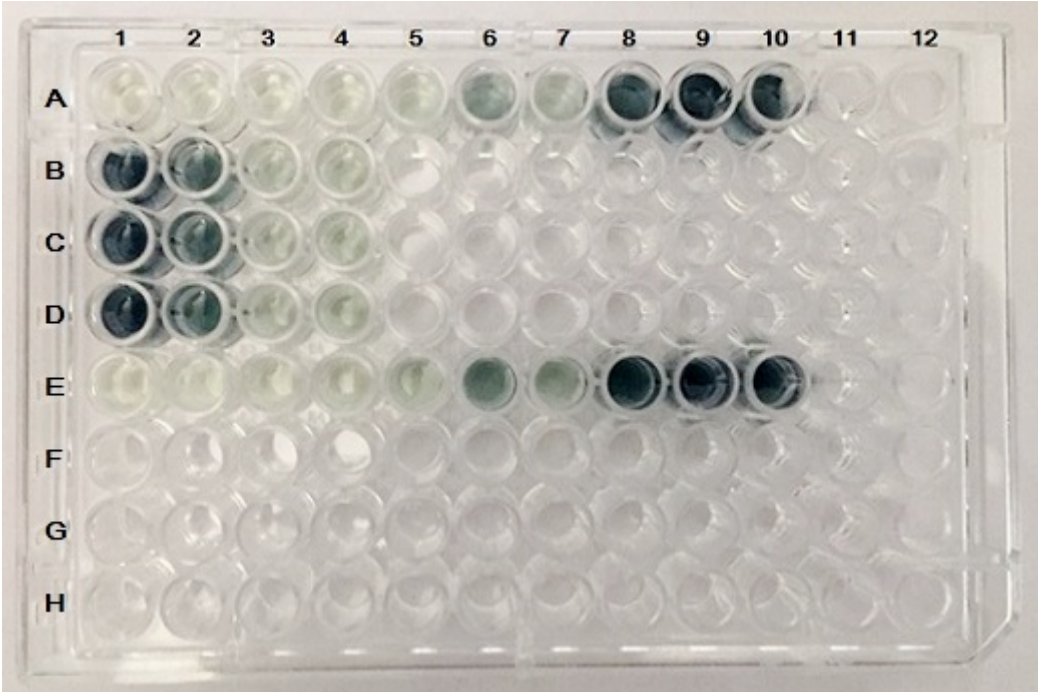

Figure 5.19: ELISA plate showing TFC estimation. A1-A10, E1-E10) Concentration of Gallic acid (10-100 μg/ml). B1-2, C1-2, D1-2) #2(4)TAHLTU 15(e). B3, C3, D3) #2(1)TAHRTU 12. B4, C4, D4) #2(1)TAWALTU 12(a)

In Figure 5.19 we can see the change in colour from dark green to pale yellow. The dark green colour signifies high amount of flavonoid content present in that particular extract.

Table 5.3: TPC, TFC and FRAP of the top endophytic fungi screened

S.No.	**Sample**	**TPC** Conc.(μg Gallic acid equivalent / mg of sample)	**TFC** Conc.(μg Quercetin equivalent / mg of sample)	**FRAP** Conc.(μg Fe(II) equivalent / mg of sample)
1.	#2(1) TAHRTU 12	106.26 ± 4.30	324.43 ± 11.69	98.65 ± 0.55
2.	#2(4) TAHLTU 15(e)	79.72 ± 2.23	201.10 ± 18.59	44.71 ± 0.22
3.	#2(1) TAWLTU 12(a)	128.34 ± 6.43	361.10 ± 16.74	110.08 ±0.60

Chapter 6

Discussion

Over the time various environmental factors such as biotic and abiotic stress have lead to loss in the yield of various food crops. In physical terms stress can be defined as the average amount of force per unit area but when it comes to living organisms the scenario is different as stress for one being might be optimum condition for the other. Abiotic stress of one or other kind affects nearly about 90% of the total arable land. These stresses range from salinity, drought, and oxidative stress to extreme temperatures. Many strategies have been exploited to overcome these environmental stresses such as the use of hybrids, HYV seeds, improvement of fertilisers, genetic engineering etc. But the rising problem of abiotic stress has threatened the sustainability of agriculture industry [Mahajan and Tuteja, 2005].

Cultivation of wheat dates back to ancient time and now has become one of most widely cultivated, harvested and consumed food crop. It is consumed by all age groups because of its unique bread making property [Veraverbeke and Delcour, 2002]. Visco-elastic properties of gluten proteins present in wheat enables to make great varieties of food [Khan, 2016]. Since the demand of wheat is increasing with time there is a need to explore a sustainable method to meet this increasing demand.

The diversity of fungal endophytes has gained popularity due to the positive health benefits they confer to the plant via symbiosis [Latef et al., 2016]. The endophytes are known to produce some secondary metabolites that act as inhibitors of pathogen [Rodriguez et al., 2009; Schulz et al., 2002]. Besides metabolites they also produce some natural products having anti-cancerous, anti-diabetic, anti-oxidant properties etc. [Strobel et al., 2004]. The major prominent benefit that they confer to the plant is the ability to tolerate stress [Sherameti et al., 2008]. The habitat adapted symbiosis has proved to play a major role in helping the plants combat abiotic stresses.

An example of host benefits because of endophytic association was seen when two endophytic fungi *Phoma glomerata* LWL2 and *Penecillium sp.* LWL3 on induction in host cucumber plant exhibited positive health benefits to the host plant. The symbiosis resulted in increased nutrient uptake and reduced sodium toxicity by reducing the production of polyphenol oxidases, peroxidases etc. As a result significant growth of host pant during

stress conditions was observed [Waqas et al., 2012]. In another study, the wheat plant was inoculated with endophytic fungi SMCD 2206, 2210 or 2215 which helped the wheat plant tolerate heat stress. Significant increase in plant height and germination of second generation seeds was observed [Hubbard et al., 2014]. To check the hypothesis that the endophytic colonization causes epigenetic changes which maybe the reason of elevated plant tolerance, Methyl-sensitive amplified polymorphism (MSAP) was used. The wheat plant was co-cultured with endophyte SMCD 2206 and the DNA methylation patterns were observed. A close link between the stressed and unstressed host plant was seen as compared to the endophyte free, stressed plant [Hubbard et al., 2014].

In this study, the fungal endophytes were pre isolated from two high yielding wheat varieties (HD2967 and WH1105) grown in Punjab. These endophytes were screened for salinity and extreme temperature tolerance. On screening the fungal isolates for salinity and temperature tolerance it was found that three of the isolates #2(1)TAHRTU 12, #2(4)TAHLTU 15(e) and #2(1)TAHRTU 12(a) showed good results at both salinity stress as well as temperature stress. Tolerance for 15-20% salinity and 40±2°C temperature was observed.

Previous studies on endophytic fungi have been done where it was seen that *Piriformospora indica* isolated from host plant present in Thar Desert, India was able to confer moderate (100 mM) to high (300 mM) salt tolerance in Barley [Baltruschat et al., 2008]. Studies have also shown that antioxidant enzymes play an important role in inducing tolerance to host plant [Sharma et al., 2012; Hediye Sekmen et al., 2007; Bor et al., 2003]. Studies have also been done to check the tolerance of high temperature conditions. It was seen that *B. bassiana* whose optimum temperature was found to be 25 °C, showed 25% relative growth at 42±1°C on 8 hour photoperiod [Devi et al., 2005].

These potent isolates were further pursued for identification using classical morphotaxonomy and molecular tools. Different characteristics such as structure, shape, colony size, pigment produced etc. is studied and used as a basis for classification of organisms. Whereas in molecular tools essential elements such as nucleic acids, proteins are used for sequencing and identifying a certain organism. Morphologically all the three isolates i.e. #2(1)TAHRTU 12, #2(4)TAHLTU 15(e) and #2(1)TAWLTU 12(a) were identified as *Aspegillus sp.*. The molecular identification is pending. The isolates were also tested for their antioxidant properties. On testing the top three isolates for antioxidant capacity, phenolic content and flavonoid content it was found that the isolate #2(1)TAHRTU 12(a) exhibited highest antioxidant capacity (DPPH IC_{50}=274.46±45.80 TEAC IC_{50}=195.62±4.88), phenolic content (106.26±4.30) and flavonoid content (324.43± 11.69) followed by #2(1)TAHRTU 12 and #2(4)TAHLTU 15(e).

Previous studies have shown that the endophytes have presence of antioxidants which help in tolerating abiotic stress. Formation of reactive oxygen species (ROS) is seen when the plant undergoes abiotic stress. These ROS are detrimental to the plant. The presence of antioxidants helps in combating these ROS. A *Xylaria sp.* (Strain number YX-28) isolated from *G. biloba* in China was subjected to production of culture filtrate and tested for presence of phenols, flavonoids and antioxidants. As a result 54.51 ± 1.05 mg GAE/g dw phenolics and 86.76 ± 0.58 mg RE/g dw flavonoids were reported in methanol extract of *Xylaria sp.*[Liu et al., 2007].

In another study the phenolic content and antioxidant capacity of 292 morphologically distinct endophytic fungi which were isolated from 9 different Chinese medicinal plants was tested. It was seen that the endohytic fungi exhibiting high antioxidant capacity also exhibited phenolic content thereby suggesting that the two are correlated [Huang et al., 2007].

Therefore, we can further test these isolates as seed inoculants for their potential in conferring abiotic stress tolerance to the host wheat plant and also test their potential in conferring biotic stress tolerance.

Chapter 7

Conclusion

The endophytic fungi screened during the study hold potential in conferring abiotic stress tolerance to the wheat plant.

1. A total of 100 endophytic fungi pre-isolated from two high yielding wheat varieties (HD2967, WH1105) grown in Punjab were tested on two abiotic stress factors. The isolate #2(1)TAHRTU 12, #2(4)TAHLTU 15(e) and #2(1)TAHRTU 12(a) exhibited best result on the two abiotic factors i.e. salinity and extreme temperature stress. Tolerance of 15-20% salinity and 40±2°C temperature was observed.
2. The isolates were further tested for their antioxidant activity, phenolic and flavonoid content. The isolate #2(1)TAHRTU 12(a) exhibited the highest antioxidant activity as well as phenolic and flavonoid content.
3. The further study involves using these isolates as seed inoculums to test the abiotic stress tolerance of host plant i.e. wheat (*Triticum aestivum*).

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