“Investigation of efficacy of endophytes for the disease management and growth enhancement of endangered medicinal plant *Withania somnifera*”

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Submitted By
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CERTIFICATE

This is to certify that the thesis titled **"Investigation of efficacy of endophytes for the disease management and growth enhancement of endangered medicinal plant *Withania somnifera*"** submitted by **Miss Rachna Singh** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

The thesis submitted to Babasaheb Bhimrao Ambedkar University, Lucknow satisfies all the requirements as stipulated in the Doctor of Philosophy (Ph.D.) regulations - 1999 as amended in 2008/2010/2013 and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

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DECLARATION

This is to certify that I have worked on the research thesis entitled "**Investigation of efficacy of endophytes for the disease management and growth enhancement of endangered medicinal plant *Withania somnifera***". The data mentioned in this thesis were collected and obtained during genuine work done by me. Data obtained from other agencies have been duly acknowledged. None of the findings pertaining to the work has been concealed. The result embodied in this report has not been submitted to any other University, Institution or Research Centre for the award of any degree.

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Rachna Singh

ABBREVIATIONS

ACC	1-Aminocyclopropane-1-carboxylic acid
AMF	Arbuscular Mycorrhizal Fungi
BCAs	Biocontrol agents
CFU	Colony forming units
CTAB	N-Cetyl N, N, N-trimethylammonium bromide
DMRT	Duncan’s multiple-range test
DNSA	Di-nitrosalicylic acid
FAO	Food and Agricultural Organisation
GAE	Gallic acid equivalent
IAA	Indole acetic acid
MAPs	Medicinal and aromatic plants
PCR	Polymerase Chain Reaction
RT-PCR	Real Time Polymerase Chain Reaction
SEM	Scanning electron microscope
WHO	World Health Organization

LIST OF TABLES

LIST OF FIGURES

CONTENTS

As human population is growing rapidly, demand for easily available and cheaper medicines to fulfill their health care needs is also increasing. Western medical system, which is practiced by most health care providers at present, is not only costly but its side effects also begin to outweigh the benefits. With frequently reported cases of multiple drug resistance among human pathogens and life-threatening damage to human organs by allopathic medicines in long run, traditional herbal health care systems regained a wide recognition over past few years (Gesler, 1992; Pal and Shukla, 2003; Nasri and Shirzad, 2013). Meanwhile drug discovery process is becoming extremely expensive, riskier and critically inefficient and pharmaceutical industry is facing serious challenges. Where scientists and pharmaceutical companies are urgently looking for new drug sources, herbal medicines are making a huge comeback as an alternative to allopathic medicines. Herbal medicines are not only comparatively cost-effective, possess lesser side effects but bioactive compounds obtained from medicinal and aromatic plants also reduce the burden for developing more effective drugs at economic investments with better safety (Patwardhana and Vaidya, 2010).

1.1 Global herbal market and Indian prospects

Medicinal plants constitute up to 80% of raw material of herbal drugs, herbal food supplements and herbal cosmetics in both domestic and international market. According to an estimate of World Health Organization (WHO, 2005), 80% of the human population in developing countries rely upon plant-based products for their primary health care needs. At present, the global market for all herbal supplements and remedies is estimated to be US$ 62 billion which could reach US$ 115 billion by 2020 and US$ 5 trillion by 2050, with Europe the largest and the Asia-Pacific the fastest growing markets (Nagpal and Karki, 2004). According to COMTRADE (Commodity Trade Statistics) database by the United Nations Statistics Division, New York, average trade of

Medicinal and Aromatic Plants (MAPs) during 1991-2003 was dominated by twelve countries. The reported annual global export of pharmaceutical plants amounted on average to 4, 67,000 tonnes valued at US$ 1.2 billion. About 80% of the worldwide import and export of MAPs is dominated by temperate Asian and European countries where temperate Asia are responsible for 41% of the annual global imports and even 48% of the annual global exports (Lange, 2006**)** ***(Figure 1.1).***

India with 16 agro-climatic zones, 10 vegetative zones, 15 biotic provinces and 426 biomes, is one of the 17 mega-diversity countries in the world and has 17000-18000 species of flowering plants of which 6000-7000 are estimated to have medicinal usage in folk and documented systems of medicine, like Ayurveda, Siddha, Unani and Homoeopathy. The domestic trade of the AYUSH industry is of the order of Rs. 80 to 90 billion and export of Indian medicinal plants and their products is of worth Rs. 10 billion (National Medicinal Plant Board, Govt. of India). Analysis of the foreign-trade statistics of commodity pharmaceutical plants for the period 1991-2003 by United Nations Statistics Division, New York revealed that in 2003, India was the USA’s most important source country for MAPs, with an import share of 28%. India, on the second place of the world’s most important source countries, exported annually on average 40,400 tonnes of pharmaceutical plants valued at 61.7 million US$. The exports fluctuated between 31,000 t and 49,000 t during the period 1991-2003. The country exported to at least 95 countries, main destinations were the USA and Europe (Lange, 2006**)**.

Indian climatic conditions are suitable for the cultivation of a wide variety of valuable medicinal plants, holding a great potential to establish in global herbal market by promoting the production of standardized raw material of guaranteed content and quality.

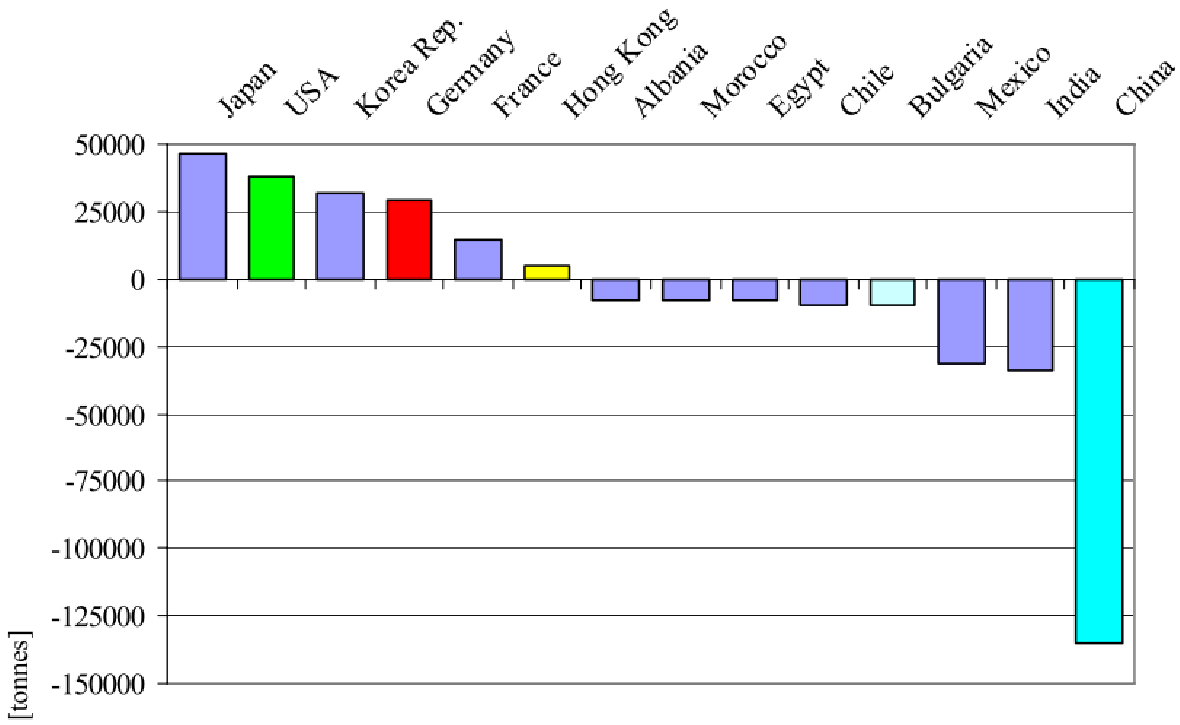


***Figure 1.1*) Graph showing average international trade of medicinal and aromatic plants of selected countries for the period 1991-2003 (Consumer countries are on the left-hand side and source countries on the right)**

Source: COMTRADE database, United Nation Statistics Division, New York (Lange, 2006)

1.2) *Withania somnifera*: Rejuvenating herb with high medicinal value

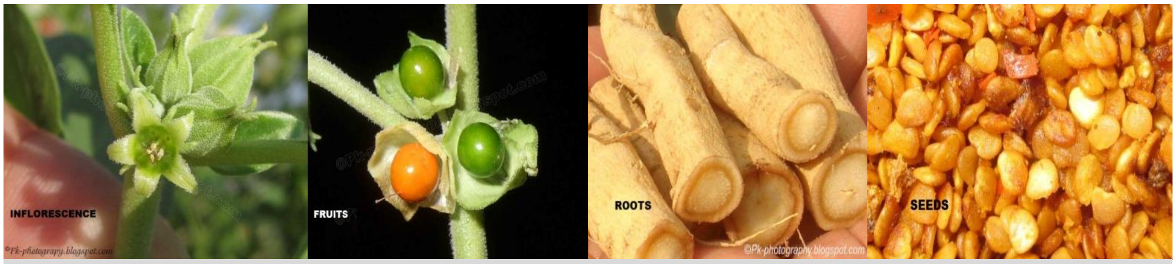
Withania somnifera (L.) Dunal is a popular and traditional Indian medicinal herb belonging to family Solanaceae and commonly known as Ashwagandha (Hindi), winter cherry or Indian ginseng (English). It is an important medicinal crop, ecologically as well as economically, as besides having enormous commercial benefits due to its high medicinal value, the plant is able to survive harsh environmental conditions. *W. somnifera* is remarkably drought-resistant and successfully grows in almost all soil types including those which are difficult to irrigate (Kiran et al., 2009). *W. somnifera* also helps in soil reclamation of arid and nutrient-poor alkaline soils (Jaleel, 2009).

W. somnifera is widely distributed in the drier parts of tropical and subtropical zones, ranging from the Canary Islands, the Mediterranean region and Northern Africa to Southwest Asia (Warrier et al., 1996; Mirjalili et al., 2009). It is a perennial herb with angular and branched stem, alternate and ovate leaves, axillary flowers in cluster of 2-5, berry fruits (green colored when young and turned to orange at maturity) and light to yellowish brown seeds **(*Figure 1.2*).**

1.2.1) Therapeutic significance of *W. somnifera*

W. somnifera has been used in indigenous medicines by Indian tribal and rural people for over 3,000 years (The wealth of India, 1976) and holds an enormous therapeutic potential due to its potent aphrodisiac, sedative, rejuvenative and life prolonging properties. Ashwagandha root is an important constituent of over 200 formulations in Ayruvedha, Siddha and Unani medicine to cure various physiological disorders (Asthana and Raina, 1989) ***(Figure 1.3***). Medicinal properties of ashwagandha (e.g. antitumor, anti-inflammatory, immunomodulatory, anti-ageing and rejuvenating properties) are primarily attributed to a group of naturally occurring C_{28}-steroidal lactone triterpenoids known as withanolides (Mirjalili et al., 2009). Pharmacological properties of ashwagandha are quite similar to *Panex ginseng* (famous as Korean ginseng or Chinese ginseng) which has led to ashwagandha roots being called Indian ginseng. In addition, pharmacological investigations suggest better and safe utility of ashwagandha than *P. ginseng* (Grandhi et al., 1994). Moreover, ashwagandha has shorter life cycle of about 6-8 months to reach maturity while Korean ginseng take 8 years to grow completely. Despite aforementioned facts, international market of Korean ginseng is worth over US $ 800 M while that of Indian ginseng is less than US $ I M due to poor marketing strategies (Department of Scientific and Industrial research, Govt. of India). According to an estimate, the annual requirement of *W. somnifera* for alkaloid production is about 9127.5 tons which far exceeds the annual production of about 5905.1 tons under cultivation (National Medicinal Plant Board (NMBP), Ministry of Ayush, Govt. of India). Ashwagandha is selected as one of the thirty-two prioritized medicinal plants of India by NMBP which are in great demand in domestic and international market (Ved and Goraya, 2007; Sharda et al., 2007). Because of its enormous medicinal significance, *W. somnifera* also appears in WHO monographs on Selected Medicinal Plants and an American Herbal Pharmacopoeia monograph (WHO, 2009).

A plant of *Withania somnifera*



Inflorescence* **Fruits*** **Roots*** **Seeds***

***Pictures source: pk-photography.blogspot**

***Figure 1.2*) *W. somnifera* plant and various plant parts**

***Figure 1.3*) Pictures of some commercially available ayurvedic formulation of *W. somnifera* in Indian market**

1.2.2) Need for cultivation and conservation

For its alkaloids and other phytochemicals, ashwagandha has been overexploited from natural habitats by local people and pharmaceuticals, due to which this valuable herb has reached near extinction and facing an uncertain future (Sivanesan and Jeong, 2007). Balaguru et al. (2006) reported *W. somnifera* under vulnerable category of red listed plants. *W. somnifera* has been reported as endangered species by Antonisamy and Manickam (1999) and Haq (2011).

Medicinal plants, collected from wild resources not only destruct their natural habitats but also degrade the quality of raw material due to more chances of adulteration and mis-identification of plant material. Cultivated medicinal plants are always more suitable and reliable for production of drugs as it ensures the content and quality of raw material according to international standards and helps in maintaining a continuous and consistent supply.

1.2.3) Major constraints in cultivation of *W. somnifera*

Despite ample scope and high demand of *W. somnifera* in domestic and international market, cultivation of the medicinal crop is comparatively low. Several factors are responsible for the lack of awareness and interest of farmers in the cultivation and production of this valuable medicinal crop as discussed below:

1.2.3.1) Low seed germination and high seedling mortality

W. somnifera self-propagate by seed however germination potential of stored seeds has been reported to be very low (Karnick, 1978; Farooqi and Sreeramu, 2001). Seed viability of ashwagandha is limited to one year, making the long duration seed storage futile (Farooqi and Sreeramu, 2001). Presence of some inhibitory substances in fruit is responsible for poor seed germination despite high viability of seeds (De Silva and Senarath, 2009). Generally, spoilage of ashwagandha seed also occurs due to fungal infection which is responsible for poor seed germination and high seedlings mortality rate (Kumar et al., 2001).

1.2.3.2) Crop destruction by fungal pathogens

W. somnifera is attacked by a number of phytopathogens which cause considerable damage to the crop and decrease its quality for medicinal usage. Naturally growing plants are systematically infected by fungal pathogens responsible for leaf spot, leaf rust, root rot, stem rot, damping off of seedlings caused by *Alternaria alternata, Fusarium solani, Aecidium withaniae, Mucor mucedo, Rhizoctonia solani* etc. (Gupta et al., 2004; Pati et al., 2008; Chavan and Korekar, 2011; Dadwal and Savitri, 2011). Leaf spot of ashwagandha is the most prevalent disease, which is severe in the plains of Punjab, Haryana and Himachal Pradesh (Pati et al., 2008).

1.2.3.3) Restricted use of synthetic agrochemicals

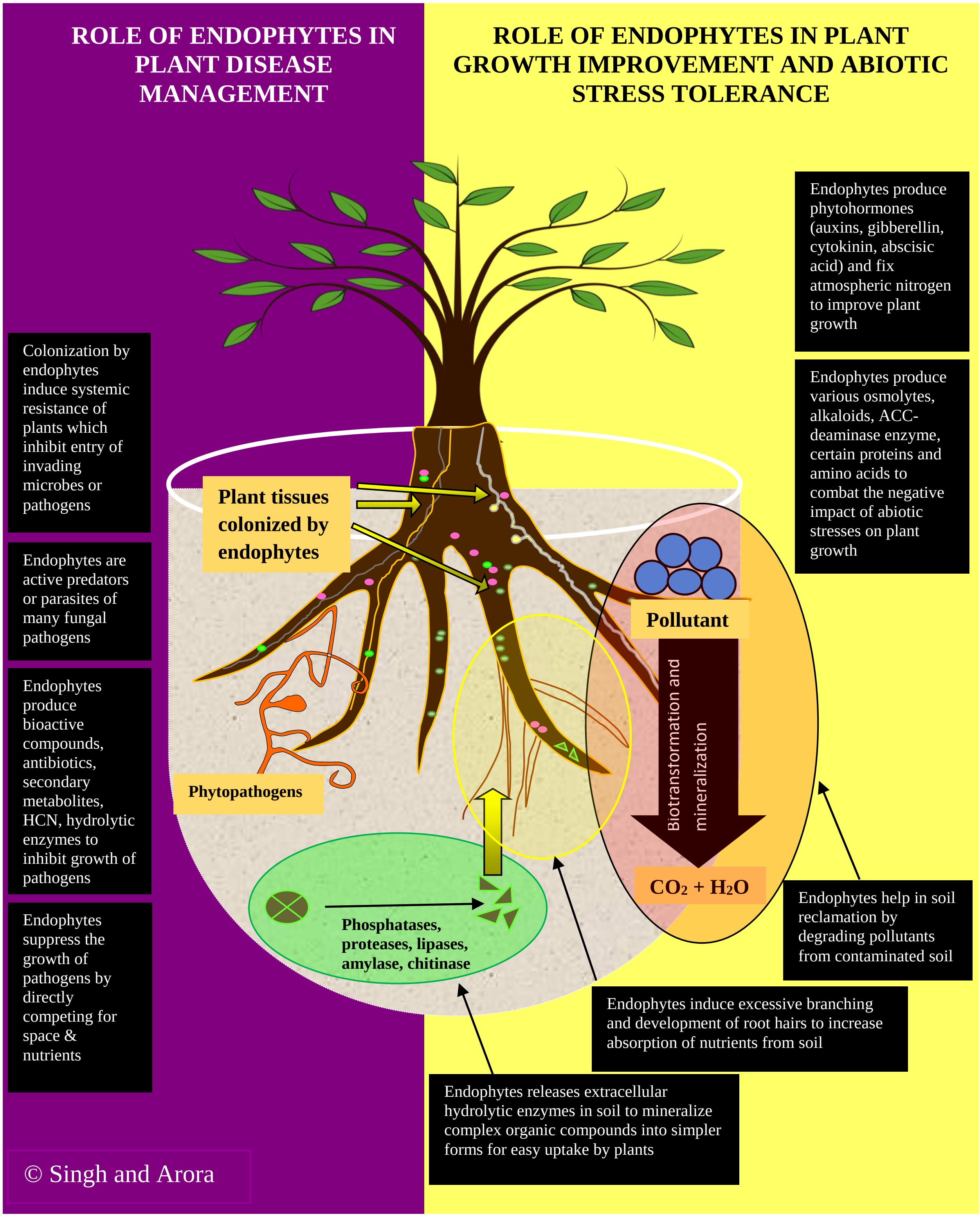
Pesticides when applied to medicinal crops to protect them from a range of pests, often render the plant inappropriate for medicinal use. It has been found that crude medicinal plants as well as infusions, decoctions, tinctures and essential oils contain pesticide residues thus many countries have their own restriction for the use of pesticides. In some studies, the concentration of organophosphorous pesticides including parathion, malathion and diazinon are detected in some medicinal plant (Abou-Arab and Abou-Donia, 2001). Pesticides are restricted in cultivation of medicinal crops mainly because the root, stem, leaves or berries are used for the purpose of making medicines which may have chances of being contaminated by pesticides leading to various diseases and disorders (Fatma et al., 2010).

Organic farming is preferred for medicinal plant cultivation, however lack of effective alternatives of synthetic agrochemicals (to protect plants from destructive phytopathogens and to enhance crop yield) discourage farmers and key reason behind less cultivation of medicinal plants.

1.3) Endophytes: Potent bioinoculants for cultivation of medicinal plant

Endophytic microorganisms are plant's mutualistic partners that inhabit the plants inter or intracellularly without any apparent harm to host (Petrini, 1991). These are important plant growth promoting microbes which possess the capability of colonizing internal host tissues as well as outstanding potential to promote plant growth and to protect host plant from phytopathogens. The potential of vigorous colonization of host tissues by endophytes makes them a valuable tool for agricultural practices which are gaining considerable importance as bioinoculants these days because they are less affected by fluctuating environmental conditions.

Endophytic microorganisms can be potentially used in controlling phytopathogens as they are known to produce various antibiotics, lytic enzymes, bioactive secondary metabolites. Some endophytes are also reported as parasites of insect pests and fungal pathogens of plants. Endophytic microbes induce systemic resistance in host plant (Picard et al., 2000; Schulz et al., 2002) and also increase their fitness by enhanced nutrient acquisition (Kimura et al., 1992). Various roles, performed by endophytes in conferring plant growth promotion, protection from phytopathogens and in enhanced survival to their host plant under harsh environmental conditions are discussed and diagrammatically represented in ***figure 1.4***.



ROLE OF ENDOPHYTES IN PLANT DISEASE MANAGEMENT

ROLE OF ENDOPHYTES IN PLANT GROWTH IMPROVEMENT AND ABIOTIC STRESS TOLERANCE

Colonization by endophytes induce systemic resistance of plants which inhibit entry of invading microbes or pathogens

Endophytes are active predators or parasites of many fungal pathogens

Endophytes produce bioactive compounds, antibiotics, secondary metabolites, HCN, hydrolytic enzymes to inhibit growth of pathogens

Endophytes suppress the growth of pathogens by directly competing for space & nutrients

Endophytes produce phytohormones (auxins, gibberellin, cytokinin, abscisic acid) and fix atmospheric nitrogen to improve plant growth

Endophytes produce various osmolytes, alkaloids, ACC-deaminase enzyme, certain proteins and amino acids to combat the negative impact of abiotic stresses on plant growth

Endophytes help in soil reclamation by degrading pollutants from contaminated soil

Endophytes induce excessive branching and development of root hairs to increase absorption of nutrients from soil

Endophytes releases extracellular hydrolytic enzymes in soil to mineralize complex organic compounds into simpler forms for easy uptake by plants

Figure 1.4) **Diagrammatic representation of various roles of endophytes in conferring fitness and survival of their host plant** **(*Image is copyright of Singh and Arora)**

Several studies have shown the positive effects of endophytes as bioinoculants in the cultivation of medicinal plants (Tiwari et al., 2010; Chandra et al., 2010; Verma et al., 2014). Therefore, the study of endophytic microorganisms, associated with different plant parts of *W. somnifera* to explore their possible role in promoting plant growth and in suppression of fungal infections is prime focus of present study. Objectives of the study are:

- Isolation of endophytes from selected medicinal plants.
- Isolation of fungal pathogens of *W. somnifera* (*A. alternata*, *F. solani*)
- Screening of isolated endophytes for their plant growth promoting and antimicrobial attributes
- Study of mechanisms involved in biocontrol and plant growth enhancement activities
- *In vitro* and *in vivo* study of biocontrol activities of selected endophytic strains against isolated fungal pathogens of *W. somnifera*
- Extraction and characterization of bioactive compounds produced by selected endophytes.
- Effect of isolated endophytes on plant growth, yield and alkaloid content of test crop as well as on rhizospheric microbial population.

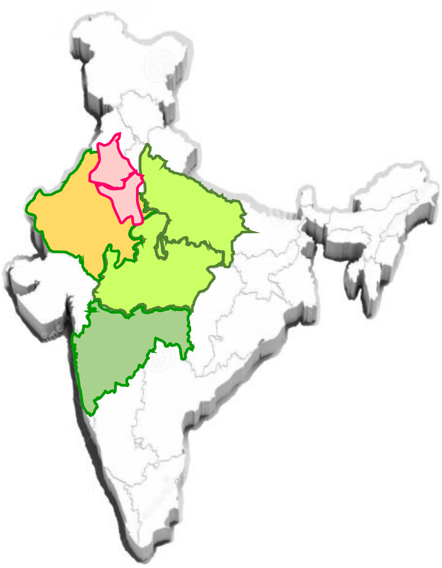
W. somnifera, an important medicinal crop with high demand in domestic and international market is still under-cultivated and less popular among farmers despite its huge economic benefits. *W. somnifera* crop suffer a colossal loss under wild and cultivated conditions due to attack of various phytopathogens and disease management in medicinal plants using synthetic pesticides is strictly prohibited. This has generated a need for economic, eco-friendly and safe alternative of synthetic pesticides and microbial inoculants, particularly endophytes, are emerging as ideal choice.

2.1) Cultivation of *W. somnifera* in India

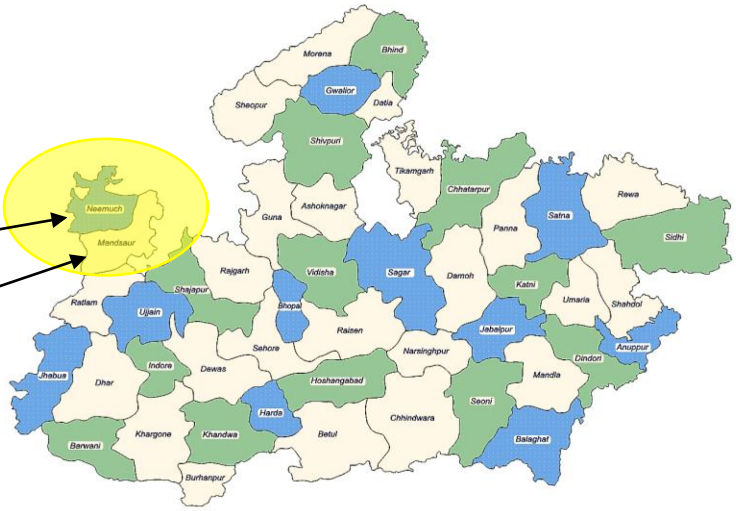
W. somnifera naturally found in forests, disturbed soil, along roadsides or in cultivated lands with 600-750 mm annual rainfall and well drained soils (Patra et al., 2004). Sandy loams and stony red clay soils with pH 7.5 to 8.0 are suitable for their growth (Thomas et al, 2000). Drier regions of India including foot hills of Punjab and Himachal Pradesh, north-western region of Madhya Pradesh are natural source of *W. somnifera* plant (Shrivastava and Sahu, 2013). According to an estimate, about 229 tonnes of ashwagandha roots has been consumed by 63% pharmacies in Gujarat state alone, which is mainly procured from naturally growing plants (Singh and Parabia, 2003).

Due to this widening gap between demand and supply worldwide and rapid destruction of natural habitats of *W. somnifera*, efforts are being made by Indian government to promote the cultivation of this plant. In India, *W. somnifera* is cultivated in around 10,780 hectares with a production of 8429 tonnes where Rajasthan, Punjab, Haryana, Uttar Pradesh, Maharashtra and Madhya Pradesh are major states for *Withania* cultivation. In Madhya Pradesh, ashwagandha is cultivated over 5000 hectares in the drier parts including Manasa, Neemuch and Jawad tehsils of Mandsaur district (Shrivastava and Sahu, 2013). The Neemuch and Mandsaur markets of Madhya Pradesh are popular world over for ashwagandha supply. Importers and buyers within the country as well as traditional practitioners, Ayurvedic and Siddha Drug manufacturers visit these markets for procurement of ashwagandha roots every year (National Bank for Agriculture and Rural Development (NABARD), Govt. of India) ***(Figure 2.1)***.

Pandey and Patra (2001) reported that cultivated ashwagandha crops yielded better quality roots with improved alkaloid content. ‘WS-20’ or ‘Jawahar Ashgandh 20’ and ‘WS 22’ or ‘Jawahar 22’ are high-yielding selection varieties of *W. somnifera*, released by Jawaharlal Nehru Krishi Vishwavidyalay, Madhya Pradesh (Das et al., 2010). WS-20 variety is short in stature thus most amenable for high density planting and possess average stability over a wide range of environmental conditions. The variety yields total withanolides content of 0.30% in dry roots on cultivation for 180 days (Nigam et al., 1991). ‘Poshita’ a selection variety, developed in Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow which take about 180 days to mature. Estimated average dry root yield of ‘Poshita’ variety is about 1400 kg/ha, total alkaloid recovery is 1.292 kg/ha and total withanolides yield is 3.5 kg/ha (Mishra et al., 2001). ‘Rakshita’ is another high-yield variety of *W. somnifera*, released by CIMAP with 8000-10000 kg/ha dry root production and 0.5% of root alkaloid content (Mathew et al., 2005; Datta et al., 2011).



Nagaur district of Rajasthan



Neemuch and Mandsaur district of Madhya Pradesh

🟧 **Tropical desert**

🟩 **Humid sub-tropical**

🟩 **Tropical wet & dry**

🟥 **Semi-arid of steppe**

Prevailing climatic conditions in ashwagandha producing states

***Figure 2.1*) Ashwagandha producing states of India and popular markets for worldwide import and export of ashwagandha**

2.2) Crop destruction by fungal pathogens

W. somnifera crop is susceptible to attack by fungal pathogens which significantly reduce the yield and alkaloid content of the plant. Destruction of ashwagandha plants due to fungal infections e.g. leaf spot by *Myrothecium roridium, Pseudocercospora withanii* (Mahrshi, 1986; Aneja and Kaur, 1995), *Alternaria alternata* (Pati et al., 2008), leaf blight by *A. dianthicola* (Maiti et al., 2007), leaf rot by *Choanephora cucurbitarum* (Saroj et al., 2012), die-back disease by *A. alternata* (Gupta et al., 1993), root rot and wilt by *Fusarium solani* (Gupta et al., 2004), seedling damping off by *Sclerotium rolfsii* (Dadwal and Savitri, 2011) and *F. solani* (Gupta et al., 2004) have been reported from various regions in India. High seedling mortality of *W. somnifera* in Mandsaur district of Madhya Pradesh was reported by Pandey et al. (1988). Similarly, seedling mortality due to damping off has also been a major barrier in successful cultivation of *W. somnifera* in Jammu region (Gupta et al., 1993). Seedling mortality is severe under high temperature and humid conditions. To control fungal infections in *W. somnifera* crop, chemical fungicides are commonly used by farmers. Seed treatment of *W. somnifera* with Thiram^{TM} or Deltan^{TM} and foliar spray of Fytolon$^{®}$, Dithane Z-78 or M-45, Malathion, Kelthan, Carbofuran generally applied to minimize fungal infections in India (Patra et al., 2004; Datta et al., 2011).

Pest control, now days, is a matter of critical concern and cultivation of medicinal plants without using synthetic agrochemicals is preferred. Pesticide use in medicinal plant cultivation is strictly regulated and plant material with pesticide residues beyond maximum permissible limits are not considered safe for use in medicines (United State Pharmacopeia (USP), 2010). The occurrence of pesticides residues in herbal medicines has been widely reviewed and revealed that organochlorines and organophosphate pesticides are largely detected in herbal drugs worldwide

(Zuin and Vilegas, 2000; Zuin et al., 2003; Leung et al., 2005; Jeon et al., 2007; Mishra et al., 2007, Sun et al., 2007; Abhilash and Singh, 2008). Nguyen et al. (2010) investigated Korean herbs for the presence of pesticide residues and detected the presence of chlorfenapyr, chlorfluazuron, metalaxyl, pyridalyl, fenvalerate, tebuconazole and tebufenozide residues in several plants. Residues of *p, p′*-DDE were also detected as main contaminants.

Lack of effective alternative cultivation techniques in order to control fungal diseases is major constraint to achieve the target production of *W. somnifera*. With increasing demand for organic farming practices, microbial bioinoculants are gaining popularity as biofertilizers and biopesticides. Endophytes which are viewed as promising bioinoculants for sustainable agricultural practices, role in increasing fitness, yield and quality of their host plants with enhanced resistance towards fungal pathogens and abiotic stresses has been discussed in present chapter.

2.3) Endophytes as plant growth promoting mutualistic partners of plants

Plants attract diverse heterotrophic microorganisms in soil by releasing readily-available nutrient-rich compounds through a process of root exudation into the rhizosphere region (Lugtenberg and Dekkers, 1999). This nutrient-rich zone promotes the establishment of a mutualistic relationship with beneficial microorganisms which possess a wide range of metabolic properties that may modulate plant growth (Heeb and Haas, 2001; Wakelin et al., 2004; Compant et al., 2005).

Endophytic microorganisms possess the ability to colonize healthy plant tissue, residing within a plant cell or between plant tissues with no apparent symptoms of disease (Nair and Padmavathy, 2014). Synergistic interaction between endophytes and their host plants has been recently demonstrated as a double-fitness trait and sequence of events, leading to the colonization of a plant by an endophytic microorganism, is presumably similar, at least in the early stages, to that observed for rhizospheric microorganisms. Indeed, microorganisms belonging to the so-called ‘root-colonizing rhizosphere-competent microorganisms’ are often found as endophytes (Gaiero et al., 2013). A plant-endophyte mutualism established after coordinated invasion by microbes on the root surface from rhizospheric region which involves multiple signalling pathways and reciprocal signalling between plants and endophytes and between endophytes (Morris and Monier, 2003; Rosenblueth and Martínez-Romero, 2006; Rudrappa et al., 2008). Microbe–microbe signalling takes place through quorum sensing which is a cell density-dependent regulator mechanism of microbial behaviour (Teplitski, 2000). Quorum sensing involves the production and perception of low molecular weight molecules, called autoinducers, which diffuse in and out of individual bacteria cells and transmit signals (Chernin, 2011). In addition to communication between microbes via extracellular molecules, plants releases host-specific compounds such as flavonoids that influence endophyte colonization (Balachandar et al., 2006). The release of specific flavonoids enables certain bacteria to preferentially colonize a host plant through the activation of gene expression required for colonization (Bais et al., 2004). Sometimes certain growth hormones are also involved in colonization process (Balachandar et al., 2006).

“Endophyte” is a generic term which represents a group of organisms, lives inside the plant. The term ‘endophyte’ was first coined by German scientist De Bary in 1866. Taken literally, the word endophyte means “in the plant” (endon Gr. = within, phyton = plant). Azevedo (1998) defined endophytes as “those microorganisms that inhabit the interior of plants, especially leaves, branches and stems, showing no apparent harm to the host”. Petrini (1991) considers the term endophyte to be purely topographical: ‘‘Endophytes colonize symptomlessly the living, internal tissues of their host, even though the endophyte may, after an incubation or latency period, cause disease.” This latter definition is broad enough to include virtually any microbe that colonizes the internal tissues of plants.

Hirsch and Braun (1992) defined endophytes as “fungi colonizing the living plant tissues without causing any immediate, overt negative effects”. This definition includes virtually the entire spectrum of symbiotic interactions in which fungi and plants participate: parasitism, commensalism, and mutualism (Bills, 1996) however this fails to include prokaryotic microbes, such as bacteria and blue-green algae, or endophytic vascular plants (Fisher et al., 1992; Mauseth et al., 1985). Wilson (1995) describe endophyte as “Fungi and bacteria which, for all or part of their life cycle invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues, but cause no symptoms of disease”.

2.3.1) Co-evolution of endophytes with plants

Plants have a long history of co-evolution with endophytes. Mutualistic association of plants with endophytes and their co-evolution on earth was first evidenced from fossilized tissues of tree *Amyelon radicians* from the Palaeozoic era (Bacon and Hill 1996). Plant-endophyte relationships may have begun to evolve from the time when higher plants first appeared on the earth, hundreds of million years ago (Taylor and Taylor, 2000). During the long period of co-evolution, a friendly relationship was formed between each endophyte and its host plant. In addition, some endophytes have the ability to produce the similar bioactive compounds as those originated from their host plant. It has been suggested that the reason some endophytes produce certain phytochemicals, originally characteristic of the host, might be related to a genetic combination of the endophyte with the host that occurred in evolutionary time (Tan and Zou, 2001). This is a concept that was originally proposed as a mechanism to explain why *Taxomyces andreanae* may be producing taxol, the most promising natural bioactive molecule discovered against cancer (Stierle et al., 1993). Thereafter, endophytes have been widely studied and reported to produce secondary metabolites similar to their host plants including podophyllotoxin (Eyberger et al., 2006; Puri et al., 2006), deoxypodophyllotoxin (Kusari et al., 2009), hypericin and emodin (Kusari et al., 2008), camptothecin and structural analogs (Puri et al., 2005; Shweta et al., 2010), azadirachtin (Kusari et al., 2012), vinblastine and vincristine (Kumar et al., 2013).

2.4) Endophytes as potential bioinoculants for sustainable agriculture

As environmental contaminations by toxic agrochemicals have increased, alternative approaches for controlling pest population and increasing crop productivity have become research priorities. Numerous studies have suggested the beneficial effects of endophytes on plants which may include enhanced insect and nematode resistance, fungal disease resistance, enhanced vegetative growth, and increased tolerance to abiotic stresses.

2.4.1) Biocontrol potential of endophytes

It was not earlier than 1977, an important year in the history of endophyte research, when endophytic microorganisms received considerable attention from scientists. In 1977, Charles Bacon and colleagues found that the cattle fed in pastures of the grass *Festuca arundinacea* suffered from a syndrome - fescue toxicosis (Bacon et al., 1977). These researchers concluded that, although there were no apparent disease symptoms, most plants of *Festuca arundinacea* from pastures where cattle suffered intoxications, had their leaves and stems systemically colonized by a fungus which was later identified as *Neotyphodium coenophialum.* It was found that infected *Festuca* plants contained several toxic alkaloids, and that *Neotyphodium* species can be beneficial to their plant hosts, increasing their tolerance of biotic and abiotic stress factors (Schardl et al., 2004). Webber (1981) was among the earlier researchers who reported plant protection ability of endophytic fungi against insect pests. He observed that endophytic fungi *Phomopsis oblonga* was able to reduce the spread of Dutch elm disease (causal agent *Ceratocystis ulmi)* by controlling its vector beetle *Physocnemum brevilineum*. More recent studies have indicated that endophytic microorganisms seem to be excellent candidates for use as biological control agents (BCAs).

According to Backman et al. (1997), the effectiveness of endophytes as biological control agents (BCAs) is dependent on many factors. These factors include: host specificity, the population dynamics and pattern of host colonization, the ability to move within host tissues, and the ability to induce systemic resistance. Endophytic bacteria strains isolated from potato stem tissues were found effective biocontrol agents against *Clavibacter michiganensis* subsp. Sepedonicus (Van Buren et al., 1993). Endophytic bacteria were also studied to suppress bacterial blight of rice (Poon et al., 1977) and oak wilt pathogen *Ceratocystis fagacearum* (Brooks et al., 1994). Colonization of multiple hosts has been observed with other species of endophytes and plants. For example: *Pseudomonas putida* 89B-27 and *Serratia marcescens* 90-166 reduced Cucumber Mosaic Virus in tomatoes and cucumbers (Raupach et al., 1996) as well as anthracnose and *Fusarium* wilt in cucumber (Liu et al., 1995). Narisawa et al. (2000) found that the root endophytic fungi *Heteroconium chaetospira* suppressed *Verticillium* yellows in Chinese cabbage in the field. *Verticillium* wilt is one of the most destructive diseases of aubergine. Endophytic fungi, especially *Heteroconium chaetospira, Phialocephala fortinii, Fusarium, Penicillium* and *Trichoderma* after being inoculated onto axenically raised aubergine seedlings, almost completely suppressed the pathogenic effects of a post-inoculated, virulent strain of *Verticillium dahliae* (Narisawa et al., 2000). *Pseudomonas* sp. strain PsJN, an onion endophyte, inhibited *Botrytis cinerea* Pers. and promoted vine growth in colonized grapevines, demonstrating that divergent hosts could be colonized (Barka et al., 2002). Eggplant wilt caused by *Ralstonia solanacearum* was reduced by 70% after seeds were inoculated with an antimicrobial compound 2, 4-diacetylphloroglucinol (DAPG)-producing endophytic isolates (Ramesh et al., 2008). Ownley et al. (2008) demonstrated the suppression of tomato and cotton seedling wilt by seed application of endophytic fungi *Beauveria bassiana* which exhibited strong antagonistic activity against pathogenic fungi *Rhizoctonia solani* and *Pythium myriotylum*. Kefi et al. (2015) isolated endophytic *Bacillus* strains from tomato plants which showed strong inhibitory effect against phytopathogenic fungi *Botrytis cinerea.* Antifungal activity of *Bacillus* strains was due to their ability to produce and secrete antifungal lipopeptides. Ray et al. (2015) demonstrated successful biocontrol potential of biofilm-forming endophytic *Alcaligenes*, isolated from *Abelmoschus esculentus* (okra plant) against collar rot pathogen *Sclerotium rolfsii*.

2.4.1.1) Biocontrol mechanisms of endophytes

The mechanism by which endophytes can act as biocontrol agents include the production of antifungal or antibacterial agents (Lambert et al., 1987; Maurhofer et al., 1992), siderophore production (Kloepper et al., 1980; Schroth and Hancock, 1981; Duijff et al., 1993), competition for nutrient (Lockwood, 1990), niche exclusion (Cook & Baker, 1983) and indirectly through the induction of systemic acquired host resistance or immunity (Tuzun and Kloepper, 1994; Chen et al., 1995; Liu et al., 1995). The most commonly reported mechanism of biological control by endophytes is antagonism. Antagonism includes the more specific mechanisms of biological control by antibiosis, competition and predation or mycoparasitism.

Antibiosis describes the ability of an endophyte to inhibit the growth of phytopathogens by producing antimicrobial compounds or toxins. A broad spectrum of endophytic bacteria and fungi has been described to control fungal plant pathogens on different plant species. The majority of antagonistic endophytic bacteria are Gram-negative and belong to the group of fluorescent pseudomonads. Control potential in terms of reduced disease severity can approach 80% under greenhouse conditions (Pleban et al., 1995). Besides pseudomonads, other Gram-negative species with biocontrol activity against fungal pathogens are *Phyllobacterium rubiacearum*, *Burkholderia solanacearum* (Chen et al., 1995), *Sphingomonas trueperi* (Adhikari et al., 2001) and *Serratia plymuthica* (Faltin et al., 2004). Cho et al. (2007) analyzed the antifungal activity of endophytes, isolated from ginseng against *Rhizoctonia solani, Fusarium oxysporum, Pythium ultimum* and *P. capsici* and observed the broad-spectrum antifungal activity of three isolates *Bacillus* sp., *Paenibacillus polymyxa* and *Pseudomonas poae* against all tested pathogens.

One of the biocontrol mechanisms by endophyte is competition for nutrients. Competition is considered an important factor in the control of pathogens by endophytes since both the microorganisms colonize similar niches and utilize same nutrients. Under iron-limiting conditions, endophytic microorganisms produce siderophores with high affinity for ferric ions (Nudel et al., 2001). Inhibition of growth of phytopathogens by siderophore-producing endophytes is well documented (Lacava et al., 2008; Verma et al., 2011; Rybakova et al., 2015).

Predation or lysis is another method through which endophytes inhibit fungal pathogens in the rhizosphere include physical destruction of fungal cell walls by the action of hydrolytic enzymes produced by the endophytes (Taechowisan et al., 2009). *Trichoderma koningii* has been found to produces cell wall degrading enzymes; chitinases, ß - 1, 3- glucanases and cellulases which aid in the colonization of their host cells (Salehpour, 2005). Mycoparasitism is an important biocontrol mechanism of endophytic fungi against fungal plant pathogens (Benyagoub et al., 1994; Cao et al., 2009)

A near-future application may consider the use of genetically engineered endophytes with biological control potential in agricultural crops. The endophytes *Herbaspirillum seropedicae* and *Clavibacter xylii* have been genetically modified to produce and excrete the δ-endotoxin of *Bacillus thuringensis* to control insect pests (Turner et al., 1991; Downing et al., 2000).

Qi and Lan (2011) developed a recombinant endophyte against sap-sucking pests. The fungal endophyte *Chaetomium globosum* YY-11 with antifungal activities was isolated from rape seedlings. Pinellia ternate agglutinin (*pta*) gene was cloned into YY-11 mediated by *Agrobacterium tumefaciens*. Products of pta gene are Pinellia ernata agglutinin (PTA) which are plant lectins having insecticidal properties.

2.4.2) Role of endophytes in plant growth enhancement

Plant growth promotion mediated by endophytic bacteria may be exerted by several mechanisms, e.g. production of plant growth hormones, synthesis of siderophores, nitrogen fixation, solubilisation of minerals such as phosphorous and zinc, or via enzymatic activities, for example suppression of ethylene by 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Chernin and Chet, 2002; Gaiero et al., 2013; Mishra et al., 2015). Endophytic organisms can also supply essential vitamins to plants (Pirttila et al., 2004),

New research efforts have focussed on the use of endophytic diazotrophs in agriculture that are able to supply biologically fixed nitrogen (BNF) directly to their host. Diazotrophic endophytic bacteria such as *Burkholderia* spp. and *Azospirillum brasilense* have been reported to significantly increase the host biomass production under controlled conditions by N_2 fixation (Bhattacharjee et al., 2008). A variety of endophytic nitrogen-fixing bacteria have already been found that colonize the interior roots of rice, maize and grass (Cocking et al., 1990; Hurek et al., 1991; Barraquio et al.,1997) and are believed to be capable of contributing directly to the nitrogen requirement in sugarcane (Boddey et al., 1995), rice (Ladha and Reddy, 1995; Yanni et al., 1997) and wheat (Webster et al., 1997). Promising endophyte candidates identified for associative (nonsymbiotic) N_2 fixation are *Acetobacter diazotrophicus, Herbaspirillum* sp. and *Azoarcus* sp. (Ladha and Reddy, 1995). Endophytic fungus *Piriformospora indica* is involved in nitrogen accumulation in the shoots of *Nicotiana tobaccum* and *A. thaliana* (Sherameti et al., 2005). N content in *N. tobaccum* was increased by 22%, indicating a transfer of about 60% substrate nitrogen into the plants. This nitrogen content increase was correlated with a 50% increase in nitrate reductase activity, a key enzyme in nitrate assimilation, in *N. tobaccum* and a similar 30% increase in *A. thaliana* (Sherameti et al., 2005).

Endophytes possess the potential to mineralize complex and insoluble forms of macro- and micronutrients by secreting extracellular hydrolytic enzymes and enhance the availability of nutrients to plants. Several reports are available on phosphate solubilizing potential of endophytic bacteria and their positive impact on plant growth (Wakelin et al., 2004; Puente et al., 2009; Ray et al., 2015; Oteino et al., 2015).

Endophytes may enhance growth by producing phytohormones without any apparent facilitation of host nutrient uptake or stimulation of host nutrient metabolism. The endophytic fungi may enhance biomass by producing growth hormones or by inducing the host-hormone production (Petrini, 1991; Schulz and Boyle, 2005). Endophytic fungi *Piriformospora fortinii* is reported to increase the root and shoot biomass (Römmert et al., 2002). The mycelial culture extract of *P.*

fortinii induced a similar increase in *Larix decidua* shoot and root biomass as did the fungus itself. Most likely the growth promotion was attributable to indole acetic acid (IAA) as the fungus synthesized the hormone *in vitro*. A similar effect has also been observed with *P. indica*, when a fungal filtrate (1% w/v) was added to maize seedlings three times a week for 4 weeks, shoot biomass increase was similar to that observed in inoculation experiments with living cultures of the fungus (Varma et al., 1999). Strains of *Pseudomonas*, *Enterobacter*, *Staphylococcus, Azotobacter* and *Azospirillum* produce plant growth regulators such as ethylene, auxins or cytokinins, which are assumed to promote plant growth (Arshad and Frankenberger, 1991). Harish et al. (2007) isolated IAA producing endophytic bacteria from the corm and banana plants to investigate their efficacy on plant growth promotion and observed significant increase in plant height, fresh and dry weights and number of leaves per plant. Sziderics et al. (2007*)* studied the effect of endophytes of genera *Arthrobacter*, *Bacillus* and *Microbacterium* in sweet pepper plant under *in vitro* conditions and observed 75% increase in total biomass. Rogers (2008) investigated the plant growth promoting effect of endophyte *Enterobacter* sp. and found 55 % greater total biomass of endophyte-treated poplar plants than un-inoculated control plants. There was also marked increase in root growth and significant increase in root to shoot ratio. Jaber and Enkerli (2016) studied the effect of increased duration of seed treatment with two endophytic fungal entomopathogens *Beauveria bassiana* and *Metarhizium brunneum* on the growth and colonization of broad bean (*Vicia faba*). Significant enhancement of plant height, number of leaf pair, fresh root and shoot weight was observed at 14 and 28 days' post inoculation

2.4.3) Role of endophytes in abiotic stress tolerance

Studies also revealed great potential of endophytes in abiotic stress tolerance. Abiotic stresses such as, drought, high soil salinity, heat, cold, oxidative stress and heavy metal toxicity are the common adverse environmental conditions that affect and limit crop productivity worldwide. It may be a promising alternative strategy to exploit endophytes to overcome the limitations to crop production brought by abiotic stress. Studies on endophytes-host interactions suggested that endophytes induce various morphological and physiological changes in host plant which leads to better survival of plants under abiotic stresses.

Numerous studies on endophytes have documented that fungal endophytes confer some level of drought tolerance to plants (Clay and Schardl, 2002). Endophytic fungi have been demonstrated to ameliorate drought stress for a few species of agronomically important forage and turf grasses, namely *Lolium* and Festuca spp. (Malinowski and Belesky, 2000; Clay and Schardl, 2002; Malinowski et al., 2005; Muller and Krauss, 2005; Saikkonen et al., 2006). Under drought conditions, the systemic endophyte *Neotyphodium coenophialum* in tall fescue grass (*Lolium arundinaceum*) can enhance host growth and survival relative to uninfected plants (Arachevaleta et al., 1989; Bouton et al., 1993; West, 1994; Malinowski & Belesky, 2000). Drought stress leads to various morphological and physiological adaptations in endophyte-infected plants. Malinowski et al. (1999) showed that endophyte infection increased root hair length and decreased root diameter in tall fescue under drought stress. Endophyte-mediated reductions in root diameter, increases in root hair length, and reductions in root production near the soil surface may increase plant water absorption and decrease susceptibility to drought (Richardson et al., 1990; Malinowski et al., 1999; Crush et al., 2004). Similarly, endophytes also affect stomatal behaviour in tall fescue (Elmi and West, 1995; Buck et al., 1997) and meadow fescue (Malinowski et al., 1997). However, the short-term benefits of rapid stomatal closure could be off-set by eventual reductions in photosynthetic rates (Belesky et al., 1987a; Elmi et al., 2000). Endophyte-infected plants exhibited leaf rolling sooner than endophyte-free plants of the same genotype during the onset of drought stress (Arachevaleta et al., 1989). In addition, endophyte-infected tall fescue adjusts to water deficit by reducing leaf area and thus reducing the area contributing to transpirational loss (Belesky et al., 1989; West et al., 1990). Endophyte-grass mutualistic associations may also involve in the maintenance of higher water content than those in noninfected plants (Buck et al., 1997). This high-water content may be due to accumulation of solutes in the tissues of endophyte-infected plants or reduced leaf conductance and slowdown of transpiration or thicker cuticle.

Hill et al. (1990) reported that endophyte-infected plants had greater concentrations of total non-structural carbohydrates than noninfected plants when grown under non-limiting water supply. Richardson et al. (1992) reported that water- stressed endophyte-infected plants of tall fescue genotype CB1 accumulated more glucose and fructose in leaf blades and leaf sheaths than noninfected plants. These carbohydrates are osmotically active and accumulated in plants during growth under non-drought condition and translocated during imposed drought stress and contribute to drought tolerance. Osmotic adjustment appears to occur, in part, through increased accumulation of solutes in the presence of the endophyte (Richardson et al., 1992). Solutes that contribute to osmotic adjustment in endophyte-infected grasses are water soluble sugars (Richardson et al., 1992) and fungal metabolites like mannitol and arabitol (Richardson et al., 1992). Hsiao (1973) reported that accumulation of proline amino acid in plants during drought is correlated with drought resistance. However more recently, specific fungal secondary metabolites have been implicated in growth response and drought-tolerance mechanisms, such as the production of loline alkaloids that affect osmotic potential that reduces the effects of drought stress (Bush et al., 1997). Loline alkaloids, produced by some endophyte-infected grass species (Bush et al., 1993), are water soluble and may act as osmoregulators (Bacon, 1993). This suggests that the loline alkaloids have a dual role in grass protection, from insect pests and drought stress. There is an association of endophyte status with dehydrins, a group of intrinsically unstructured proteins abundant during late embryogenesis (Carson et al., 2004), that is associated with protection from drought and temperature stresses in several grasses, including tall fescue.

Endophytes also alter the concentration of certain alkaloids and secondary metabolites in response to biotic or abiotic stress. Gentry et al. (1969) reported the first documented evidence that mineral nutrition affect the concentration of certain alkaloids in tall fescue. He found that the concentration of perloline alkaloid was increased by nitrogen fertilization but decreased by phosphorous and potassium when compared to nonfertilized controls. However, occurrence of endophytes was not known at the time. After the discovery of endophytes and their role in alkaloid synthesis in the host grass, management practices were developed to reduce alkaloid production and toxicity of tall fescues. Alkaloids, generally produced by endophyte infected grasses in response to biotic stresses are nitrogen-rich compounds (Porter, 1994). Lyons and Bacon (1984) reported an increase in the concentration of ergopeptine alkaloid in response to high rates of nitrogen fertilization under controlled environment. Similarly, Belesky et al. (1987b) also reported an increase in pyrrolizidine alkaloid concentration in response to nitrogen fertilization. Lyons et al. (1990) found that endophyte-infected tall fescue plants had greater ammonium and amino acid levels and fewer nitrates than noninfected plants. In addition, infected plants were more efficient to use available nitrogen than noninfected plants under N limiting conditions.

2.4.4) Role of endophytes in soil reclamation

A promising field to exploit plant-endophyte partnerships is reclamation of degraded or contaminated soils. Endophytes also play an integral role in nutrient mineralization in soil by various producing various extracellular hydrolytic enzymes, secretion of organic acids, production of siderophores to enhance phosphorous availability by chelating cations such as Fe, Al or Ca that are involved in the formation of insoluble phytates (Singh et al., 2011). Extracellular enzymes such as phosphatases, proteases, lipases, amylases, cellulases, chitinase and urease regulate mineralization of organic compounds in soil (Das and Varma, 2011). Endophytes possess the potential to mineralize complex and insoluble forms of macro- and micronutrients by secreting extracellular hydrolytic enzymes and enhance the availability of nutrients to plants (Tabatabai, 1994; Wakelin et al., 2004; Sharma et al., 2013; Oteino et al., 2015). Soil enzyme activity measurements have been used as indicator of soil quality and health (Bandick and Dick, 1999; Badiane et al., 2001).

Many plant growth promoting endophytes can assist their host plant to overcome contaminant-induced stress responses, thus providing improved plant growth. During phytoremediation of organic contaminants, plants can further benefit from endophytes possessing appropriate degradation pathways and metabolic capabilities, leading to more efficient contaminant degradation and reduction of both phytotoxicity and evapotranspiration of volatile contaminants. For phytoremediation of toxic metals, endophytes possessing a metal-resistance/sequestration system can lower metal phytotoxicity and affect metal translocation to the above-ground plant parts. Endophytes may assist in the phytoremediation of recalcitrant PAHs. Bacteria with high tolerance to PAHs were discovered in several willows (*Salix* spp.) and poplar (*Populus* spp.) clones (Moore et al., 2006).

2.5) Role of endophytes in enhancing seed germination

Seed germination Plant propagation is an important stage in production technology of herbal medicines as quality of herbs largely depends upon the germplasm with desired marker compounds. Thus outsourcing of right propagules of medicinal plants is a key requirement for developing successful cultivation and conservation techniques (Hamilton et al., 2003). Propagation through seeds is considered as a best multiplication method for most of the medicinal plants (Miller, 1914). Traditionally *W. somnifera* is propagated through seeds however germination rate is very low and seedling mortality is high due to seed borne-infections (Vakeswaran and Krishnaswamy, 2003).

It is believed that endophytes may change the capacity of host seeds to sense the external environment, thus altering physio-ecological processes such as dormancy, germination and/or seed survivorship (Gundel et al., 2006). Seed germination enhancement by endophytes has been observed in *Lolium perenne* (Clay, 1987) and *Bromus setifolius* (Novas et al., 2003). Endophytic colonization at the seed state and their effect on seed germination and seedling vigor has been investigated by many researchers (Muhlmann and Peintner, 2000; Baskin and Baskin, 2004; Adriaensen et al., 2006; White and Torres, 2010). Thus use of endophytes in developing methods by which seedling emergence can be enhanced and protected under the limitations of biotic and abiotic stresses could be helpful in the cultivation of medicinal plants (Vujanovic et al., 2000; Vujanovic and Vujanovic, 2006).

Ren et al. (2002) observed 98% germination of endophyte-infected seeds of *Lolium perenne* in comparison to 78% germination recorded in non-infected seeds. Vetrivelkalai et al. (2010) reported that on seed bacterization with endophytic bacterial isolates significantly enhanced the germination percentage and vigour index of bhendi seedlings by roll towel technique and pot culture studies. Dochhil et al. (2013) studies on seed germination enhancing activity of endophytic *Streptomyces* from medicinal plant *Centella asiatica* revealed high germination rate induced by endophytes. Similarly Chandrashekhara et al. (2007) demonstrated that seed bacterization with endophytic bacteria *Pseudomonas fluorescens* ISR34 and *Bacillus* sp. ISR37 not only significantly improve seed germination and plant growth but also showed enhanced resistance of seedlings against downy mildew disease caused by *Sclerospora graminicola*. Dalal and Kulkarni (2015) studied the effects of indigenous endophytic microorganisms *Pseudomonas* sp., *Bacillus* sp., *Burkholderia* sp. *Streptomyces* sp., *Actinoplanes* sp., *Alternaria* sp. and *Fusarium* sp. in soybean and found significant improvement in seed germination, seedling vigour index, plant growth and in disease suppression against *Rhizoctonia solani*.

2.6) Endophytes-mediated growth promotion and yield improvement in medicinal plants

Liu et al. (2001) demonstrated successful antifungal activity of endophytes, isolated from medicinal plant *Artemisia annua* against crop-threatening fungi *Gaeumannomyces graminis var. tritici*, *Rhizoctonia cerealis, Helminthosporium sativum, Fusarium graminearum, Gerlachia nivalis* and *Phytophthora capsici.* Gangwar et al. (2011) studied the biocontrol potential of endophytic actinomycetes, isolated from medicinal plants *Aloe vera, Mentha arvensis* and *Ocimum* and found that isolates displayed a wide spectrum activity against fungal phytopathogens *Aspergillus niger, Aspergillus flavus, Alternaria brassicicola, Botrytis cinerea, Penicillium digitatum, Fusarium oxysporum, Penicillium pinophilum, Phytophthora dresclea* and *Colletotrichum fulcatum.* Chowdhary and Kaushik (2015) explored and investigated endophytic fungal population, isolated from traditional medicinal herb Tulsi (*Ocimum sanctum*) for their antiphytopathogenic activity against widespread plant pathogens *Botrytis cinerea, Sclerotinia sclerotiorum, Rhizoctonia solani* and *Fusarium oxysporum.*

Recently an ACC-Deaminase-producing salt tolerant endophytic bacteria *Brachybacterium paraconglomeratum* strain SMR20, isolated from the roots of a medicinal plant *Chlorophytum borivilianum* was investigated for its ability to alleviate negative impact of salt stress-induced damage. Endophyte-treated plants showed decreased production of ethylene, delayed chlorosis and senescence that resulted in improved yield of plants. Apart from reducing ethylene, the presence of the endophyte also enhanced the production of indole-3-acetic acid and abscisic acid in plants. Amount of total leaf pigments, proline, malondialdehyde and foliar nutrient uptake was also improved (Barnawal et al., 2016).

Pandey et al. (2016) investigated the role of endophytes in the modulation of plant productivity and benzylisoquinoline alkaloid (BIA) biosynthesis in opium poppy (*Papaver somniferum)* and revealed that endophytes from leaf were involved in improving photosynthetic efficiency thus crop growth and yield whereas the endophytes from capsule were involved in enhancing BIA biosynthesis. Capsule endophytes, isolated from an alkaloid-rich *P. somniferum* variety ‘Sampada’ enhanced BIA production even in alkaloid-less variety ‘Sujata’. Expression study of the genes involved in BIA biosynthesis conferred the differential regulation of their expression in the presence of capsule endophytes. The capsule endophyte SM1B (*Acinetobacter*) was found to play a key role in the upregulated expression of the key genes for the BIA biosynthesis.

Most plants are colonised by a broad spectrum of endophytic bacteria that are potentially antagonistic towards fungal plant pathogens. This enormous potential needs to be further explored for its use in modern plant disease control strategies. Endophytes hold an enormous potential to develop effective bioinoculants for more successful biological control of plant diseases and improved crop production especially in the cultivation of medicinal plant.

3.1) Sample Collection

Endophytic microorganisms were isolated from healthy and asymptomatic plant parts of *W. somnifera* including leaves, stem and root tissues (***Figure 3.1***) which were collected from the plants growing in and around campus premises of Babasaheb Bhimrao Ambedkar University, Lucknow (26.7679° N, 80.9263° E) during March 2012-August 2013 in all growing seasons. Samples were also collected from CSIR-CIMAP, Lucknow (26.8° N, 80.9° E) and Forest research institute, Dehradun (30.318° N, 78.029° E) during 2013-14.

3.2) Isolation of endophytes

Endophytes were isolated according to the procedure of Hallmann et al. (2006). Samples were washed in running tap water, followed by distilled water. To remove epiphytic microbes, samples were treated with 70% ethanol for 30 seconds and then excised to $1cm^2$ pieces with the help of a sterile blade. These sections were surface sterilized in 3% sodium hypochlorite for 10 minutes followed by 5 times thorough rinses with sterilized distilled water. Sterility after surface sterilization was checked by spreading 100µl of aliquot from the final wash on nutrient agar (NA) and Potato dextrose agar (PDA) plates and incubated at 28°C for 24–48 h to examine microbial growth. After successful sterilization, plant tissues were macerated using a sterile mortar and pestle in a small volume of sterile phosphate buffered saline (pH 7.4). 100 µl tissue extract of different dilutions (up to 10^{-6}) were plated onto NA, King’s B, yeast extract mannitol agar (YEMA) and PDA plates with three replications each. After growth of bacterial and fungal colonies, morphologically different colonies were picked and maintained as pure cultures. For long term storage, glycerol stock (20%) of pure cultures of isolates were prepared and maintained at -20°C.

3.3) Isolation of phytopathogens

During study of disease profile of *W. somnifera*, leaf spot disease was found most prevalent in all growing seasons. For the isolation of associated fungal pathogens from infected tissues, samples were repeatedly collected from the diseased plants growing in and around BBAU campus and CSIR-CIMAP, Lucknow (***Figure 3.2***). Fungal pathogens were isolated from infected leaf portions with brown necrotic spots. Diseased portions of leaves were cut and surface sterilized with 1% sodium hypochlorite for 30 seconds, followed by three thorough rinses with sterilized distilled water. Surface sterilized infected leaf portions were then placed on PDA media and plates were incubated at 28°C for 5-7 days. After growth on PDA, each fungal colony was picked and transferred to fresh PDA plate to maintain pure culture.

Fungal pathogen *Fusarium solani* which causes leaf spot and root rot disease in *W. somnifera*, was procured from the laboratory of Microbial Technology Division, CSIR-CIMAP, Lucknow.

3.3.1) Pathogenicity test

Pathogenicity of fungal pathogens was checked *in vitro* by detached leaflet assay and *in vivo* by pot experiments.

3.3.1.1) *In vitro* pathogenicity test

Conidial suspension of ten-day old culture of each isolated fungal pathogen was prepared by flooding the culture plate with sterile distilled water containing 0.01% tween 80. After gentle rubbing of mycelium, suspension was filtered through cheesecloth and conidial count was determined by haemocytometer and adjusted to 10^6 conidia ml^{-1} using sterile distilled water. *In vitro* pathogenicity of each isolated fungal pathogens was determined according to Dhingra and Sinclair (1995) for which medium sized healthy leaves of *W. somnifera* were selected and surface sterilized for 5 minutes in 3.5 % sodium hypochlorite solution and washed several times with sterile water. After sterilization, leaves were carefully placed into sterile petri plates with moistened filter paper at the base. 1 ml of aqueous conidial suspension (10^6 conidia ml^{-1}) of each fungal pathogen was sprayed over surface sterilized leaves and plates were kept in BOD incubator for 5-7 days at 25°C. Leaves sprayed with sterilized distilled water were kept as control. After incubation disease severity was recorded according to the method of Xue et al. (2013). Disease severity as a percentage of the lesion area on the leaf surface was determined from a score of 0–4 where score 0= no symptoms; score 1= 1–25% of leaf area was infected; score 2= 26–50% of leaf area was infected; score 3= 51–75% of leaf area was infected and score 4= more than 75% of leaf area was infected. Disease severity and biocontrol efficacy were calculated according to the following formula as described by Xue et al. (2013):

Disease severity = [∑ (disease score x number of leaves with that score) / (total number of leaves investigated x 4)] x 100

where the number 4 represents the highest disease score.

Re-isolation of pathogens was carried out and the experiment was repeated twice.

3.3.1.2) *In vivo* pathogenicity test

Fungal pathogens which successfully caused disease symptoms on healthy leaves during *in vitro* pathogenicity test were further examined for their pathogenic nature *in vivo*. Conidial suspension of pathogen was inoculated on intact, healthy plants of *W. somnifera* by pot experiment in green house. Foliar spray of conidial suspension was applied three times at an interval of 48 h and plants were kept under moist condition at 28°C for 15- 20 days. After incubation, fungal pathogens which successfully caused disease symptoms during *in vitro* and *in vivo* pathogenicity test, were selected for further study.

***Figure 3.1*) Samples of *W. somnifera* plant collected for the isolation of endophytes**

***Figure 3.2*) Picture of field-grown *Withania* crop (from CIMAP, Lucknow) suffered from leaf spot disease**

3.4) Screening and characterization of endophytic microbes for plant growth promoting (PGP) traits *in vitro*

Each endophytic isolate was screened and characterized individually for their PGP potential *in vitro* by using standardized protocols as described below.

3.4.1) Phosphate solubilization

Phosphate solubilization test was conducted qualitatively (according to Gaur, 1990) by inoculating the isolated endophytes on Pikovskaya's (PVK) agar media. Plates were incubated at 28°C for 5 days. After incubation, inoculated plates were observed for clear zone around bacterial colonies. Phosphate solubilization index (PSI) was evaluated according to the ratio of the total diameter (colony diameter + halo zone) and the colony diameter (Edi-Premono et al., 1996).

3.4.2) Indole acetic acid (IAA) production

IAA production by endophytic isolates was determined according to the method of Khamna et al. (2009). Isolates were grown in 5 ml of Glucose Yeast Extract (GYE) broth, supplemented with 0.2% (w/v) L-tryptophan at 28°C and 170 rpm for 7 days in the dark. After incubation cultures were centrifuged at 5000 rpm for 10 minutes at 4°C to harvest the cells. Then 1 ml of culture supernatant was taken into a fresh test tube and 1 ml of Salkowski's reagent (prepared according to Gordon and Weber, 1951) was added. Reaction mixture was incubated for 30 minutes at 28°C. Development of pink color in reaction mixture indicated the presence of IAA which was quantitatively estimated by measuring the absorbance at 530 nm in a spectrophotometer. Amount of IAA produced (µg/ml) was calculated with the help of a standard curve of 0.5-100 µg/ml concentration.

3.4.3) Siderophore production

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

For quantitative estimation of siderophore production, bacterial isolates were grown in nutrient broth (NB) and fungal isolates in potato dextrose both (PDB) at 28°C for 3-5 days. After incubation, culture broth was centrifuged at 8000 rpm for 10 minutes and 0.5 ml of culture supernatant was transferred to fresh test tube. To the supernatant, 0.5 ml of CAS reagent was added and absorbance was measured at 630 nm in a spectrophotometer. Uninoculated broth with CAS reagent was used as blank. Siderophore content was calculated according to the formula:

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

Where Ar = Absorbance of blank at 630 nm; As = Absorbance of sample at 630 nm

3.4.4) Zinc solubilization

Zinc solubilization potential of isolates was determined according to method of Saravanan et al. (2007) using mineral salts medium supplemented with 0.1% insoluble Zn compound (ZnO and $ZnCO_3$). Isolates were spot-inoculated on agar plates and incubated at 28°C for 48 h. After incubation, plates were observed for clearing zone around colony and diameter was calculated.

3.4.5) Alkaline phosphatase activity of phosphate-solubilizing endophytes

Alkaline phosphatases play key role in solubilizing organic P in neutral and alkaline soils. Since pot and field trials were conducted in alkaline soil (pH 8.2), alkaline phosphatase activity of isolated endophytes was also investigated.

Phosphate solubilizing endophytes were inoculated in Pikovskaya's broth containing 5g/L of tri-calcium phosphate as a phosphorus source and incubated for 5-6 days. Phosphatase activity of endophytes were assayed according to the method of Tabatabai and Brammer (1969) in which *p*NP linked substrate and enzymatic activity is determined from colorimetric measurement of *p*NP released in buffered substrate solution during incubation and results were reported in units of µmol *p*NP released in reaction mixture. The cultures were filtered after centrifugation at 8000 rpm for 10 minutes and supernatant was separated for phosphatase assay. To 3 ml of the supernatant, 1 ml of Tris–HCl buffer (pH 10.0) was added, followed by addition of 100 µl of *p*NPP solution. Reaction mixtures were incubated for 20 min at 37^0 C. Reaction was then terminated by addition of 2 ml of 1M NaOH solution. Uninoculated broth or distilled water was used as control. Release of *p*NP was measured spectrophotometrically at wavelength 410 nm (Verchot and Borelli, 2005).

3.5) Antagonistic activity of endophytic strains against *A. alternata* and *F. solani*

In vitro antagonistic activity of isolated endophytic strains against *A. alternata* and *F. solani* was evaluated using dual culture plate assay on petri plates containing modified PDA medium. From 10-days old culture of pathogenic fungi, mycelial disc was cut with the help of a cork borer and placed in the center of agar plate. Endophytic isolates were inoculated 2 cm. away from fungal disc and plates were incubated at 28^{O}C for 5-7 days. Plates only with disc of pathogenic fungi served as control. Percent mycelial inhibition of fungal pathogens was measured using the formula (Taechowisan et al., 2005):

$$\textbf{\% mycelial inhibition} = \mathbf{C – T/C \times 100}$$

Where C= diameter of mycelial growth of pathogenic fungi in control plate, T= mycelial growth of pathogenic fungi in treatment.

3.5.1) Detached leaflet assay to determine biocontrol potential of selected antagonistic isolates

Healthy and asymptomatic leaves of *W. somnifera* were detached from healthy plants, washed in running tap water and surface-sterilized according to the procedure of Palaniyandi et al. (2011). The detached leaf assay to determine the biocontrol potential of two selected isolates WRE-1 and WSEF-4 was carried out using following treatments:

(1) Leaves soaked with conidial or cell suspension of endophyte isolates WRE-1 and WSEF-4 (10^8 spores/ml) for 60 min

(2) Leaves soaked with conidial/cell suspension of endophytic isolate and then sprayed with spore suspension of pathogenic fungi *A. alternata* and *F. solani* in separate treatments (10^5 spores/ml)

(3) Leaves sprayed with spore suspension of pathogenic fungi (10^5 spores/ml)

(4) Leaves sprayed with sterile distilled water (as control)

All treatments were kept in sterile Petri dishes with wet filter paper and moisture level in the leaves was maintained at regular intervals. Plates were incubated at 28°C for 5-7 days. Ten leaves were used per treatment and effect of endophytes on disease suppression was calculated by recording the percentage of infected leaf area (according to Xue et al., 2013). Disease severity was calculated according to the procedure as mentioned in section 3.3.1.1.

3.5.2) Exploration of biocontrol mechanisms by isolates

Isolates were investigated for various biocontrol mechanisms *in vitro* including production of cell-wall degrading enzyme chitinase, bioactive secondary metabolites, HCN, siderophores and parasitic interaction of endophytes with fungal pathogens.

3.5.2.1) Chitinase activity

All endophytes were screened for chitinase activity on chitin agar plate in which 1% of colloidal chitin was used. Plates were incubated at 37 ˚C for 5-7 days and observed for clear zone around colonies.

3.5.2.2) Production of β-1, 3-glucanase enzyme

Production of cell-wall degrading enzyme β-1, 3-glucanase was assayed according to Morohashi and Matsushima (2000). To estimate β-1, 3-glucanase enzyme production quantitatively, laminarin was used as substrate and glucose released after hydrolysis of laminarin was calculated by DNS method for the quantification of reducing sugars.

The reaction was performed using 200 μL of phosphate buffer (pH 6.0), 100 μL of the culture sample and 100 μL of laminarin (4 mg/mL). The reaction mixture was incubated at 50°C for 1 h then 200 μL of DNS reagent was added. Absorbance of reaction mixture at 540 nm was measured using spectrophotometer and buffer solution in culture medium was used as blank. Released amount of glucose was quantified using standard curve of glucose and the enzymatic activity was expressed as U/L, where a unit of activity (U) was defined as 1.0 g of reducing sugar released from laminarin under the assay conditions used.

3.5.2.3) HCN production

Hydrogen cyanide (HCN) production was evaluated by streaking the bacterial isolates on NA medium amended with glycine. Whatman No.1 filter paper soaked in picric acid (0.05% solution in 2% sodium carbonate) was placed in the lid of each Petri plates. The plates were then sealed air- tight with parafilm and incubated at 30^o C for 48 h. A color change of the filter paper from deep yellow to reddish-brown color was considered as an indication of HCN production (Bakker and Schippers, 1987).

3.5.2.4) Parasitic interaction of endophytes with fungal pathogens

From 3-7 days, old dual-culture plates (where antagonistic endophyte and fungal pathogen had grown together), hyphae in the contact zone between colonies of inoculated endophyte and fungal pathogen were examined under light microscope (magnifications of up to 400x) for any morphological change in hyphal structure, development of coils, penetrating structures around hyphae of fungal pathogens or hyphal disintegration in pathogens. For further investigation of parasitic behavior of endophytes, hyphae of fungal pathogens from the contact zone were picked and examined under scanning electron microscope (SEM). For SEM analysis, samples were fixed for 4–6 h in 2% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 6.8) at room temperature. Samples were rinsed thoroughly for 1–2 h with 0.2 M phosphate buffer (pH 6.8), and then dehydrated in a graded acetone series (30, 50, 70, 80, 90, and 100%), each grade for 30 min and three times for 100% acetone. Fully dehydrated samples were completely dried and mounted on stubs for examination under SEM.

3.5.2.5) Production of bioactive secondary metabolites

Antagonistic activity using cell-free culture filtrate of endophytic bacteria was also checked for which log phase broth culture of isolates was harvested, centrifuged at 8000 rpm at 4^{O}C for 10 minutes. Supernatant was filtered using syringe filter and 100 µl was aliquoted into wells made on 2 cm away from the center and disc of pathogenic fungi was placed at center of an agar plate. All the plates were incubated at 28^{O}C for 5-7 days and zone of inhibition of colony growth was measured after incubation.

3.6) Phenotypic and biochemical characterization of selected endophytes

After screening of isolates for PGP and biocontrol potential, selected endophytes were phenotypically and biochemically characterized according to Bergey's manual of systemic biology, on the basis of Gram staining, endospore staining, lactophenol cotton blue staining of fungi, catalase activity, amylase, urease, cellulase, lipase and protease production, ammonia production and citrate utilization. Gram staining and endospore staining was performed according to the method proposed by Aneja (2005). Morphology of fungal isolates was examined after staining of hyphae with lactophenol cotton blue stain. To determine catalase activity, fresh broth cultures (24 h old) of bacterial isolates was used and a loopful of bacterial culture was placed on a glass slide and one drop of H_2O_2 (5-10%) was added. The appearance of gas bubbles is the indicator of catalase enzyme (McFadden, 1980). Standardized protocols for other biochemical tests are described below:

3.6.1) Amylase test

Amylase activity was assayed on starch agar media. Endophytes were inoculated on starch agar plates and incubated at 28°C for 2-5 days. After incubation, plates were flooded with iodine solution and observed for clear zone around the colonies (Aneja, 2005).

3.6.2) Cellulase activity

To determine the extracellular cellulase activity, screening was done on Czapek-mineral salt agar (CMSA) media. CMSA plates were inoculated with endophytes and incubated at 28°C for 5-7 days. After incubation plates were flooded with an aqueous solution of 1% congo red solution for 4-5 minutes (Kasana *et al.,* 2008) and then washed with 1M sodium chloride solution for 15 minutes. The diameter of halo zone around each colony was measured.

3.6.3) Protease activity

For proteolytic activity, endophytes were inoculated on skimmed milk agar (SMA) media and inoculated SMA plates were incubated at 28°C for 4 days. After incubation period, SMA plates were observed for the clear zone around bacterial colonies (Aneja, 2005).

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3.6.4) Lipase activity

Lipase activity of isolated endophytes was checked on Tween-20 agar medium. Endophytes were inoculated on tween 20 agar media and incubated for 3 days at 28°C. After incubation, plates were observed for the zone of precipitation around colonies (Devender et al., 2012)

3.6.5) Urease Test

Urease activity was determined on urea agar by preparing urea agar slant tubes. Isolates were streaked on agar surface and tubes were incubated for 24-48 h at 28 ºC. After incubation, tubes were observed for change of media color from yellow to pink as compared with control which indicates the positive result for urease test (Aneja, 2005).

3.6.6) Citrate utilization test

Citrate utilization was assayed on Simmon’s citrate agar slants. Slant tubes were inoculated with endophyte isolates and incubated at 28ºC for 48 h. Appearance of blue color in tubes indicated positive citrate utilization activity (Aneja, 2005).

3.6.7) Ammonia production

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

3.7) Molecular identification and phylogenetic analysis

Molecular identification of selected endophytic strains and fungal pathogen was performed by Yaazh Xenomic services, Madurai, Tamil Nadu. On the basis of promising plant growth promoting and biocontrol potential against fungal pathogens, two best performing isolates WRE-1 and WSEF-4 were selected for further study and identified on the basis of rRNA gene sequencing. Identity of most pathogenic fungal strain, isolated from infected *Withania* leaves was also confirmed after sequencing of 18S rRNA gene sequencing.

3.7.1) 16S rRNA gene sequencing

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

3.7.2) 18S rRNA gene sequencing

Genomic DNA of endophyte WSEF-4 and fungal pathogen was isolated by using the InstaGene^{TM} Matrix Genomic DNA isolation kit (Catalog # 732-6030). Using 18S rRNA ITS Region universal primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC), gene fragment was amplified using MJ Research PTC-225 Peltier Thermal Cycler. PCR product was sequenced using the ITS1/ITS4 primers.

3.7.3) Sequence BLAST and phylogenetic analysis

16S and 18S rRNA gene sequences were blast with their closely related sequence using NCBI blast tool. The phylogeny analysis of sequence with the closely related sequence of blast results was performed using PhyML program, followed by multiple sequence alignment using the program MUSCLE 3.7.

3.8) Determination of non-pathogenic nature of strain WRE-1 and antibiotic susceptibility

Clinical isolates of *P. aeruginosa* are generally reported as opportunistic human pathogen and simply characterized on the basis their hemolytic activity on blood agar media, mesophilic growth (above 37°C), hydrolytic activity for elastin protein and development of antibiotic resistance (Liu, 1974; Stover et al., 1983; Girlich et al., 2004; OECD, 2015). Isolates of *P. aeruginosa* from environment have an optimal temperature growth range of 25 to 30°C and are not virulent to human cells, but strains isolated from clinical samples, have a higher permissive growth range, up to 37°C, and show increased virulence against human cells. Thus, non-pathogenic nature of *P. aeruginosa* WRE-1 (identified after molecular sequencing) was confirmed from non-haemolytic activity on blood agar media and negative activity for elastase. Pyocyanin production was determined according to Price-Whelan et al. (2006). Protease production was also examined (see section 3.6.3). Antibiotic susceptibility for twenty different antibiotics, belonging to different groups was also investigated by agar-disc method.

3.9) Extraction and characterization of secondary metabolites

Extraction of metabolites from the broth culture of selected endophytes was performed as described by Choudhary et al. (2004). A 5-day-old broth culture of WRE-1 and 20-day-old broth culture of WSEF-4 was centrifuged at 10,000 rpm for 15 minutes to remove cells. Obtained supernatant was extracted with organic solvents methanol, chloroform, butanol and ethyl acetate sequentially. After extraction, solvent was evaporated and obtained compounds were checked for their antifungal activity on PDA plate by agar-well diffusion method. Compounds extracted with ethyl acetate exhibited maximum antifungal activity which were characterized using FTIR.

3.10) Determination of endophytic nature of isolates

Endophytic nature of selected isolates was determined according to the method proposed by Etesami et al. (2014). Seeds of *W. somnifera* (‘Poshita’ variety) were made endophyte-free by soaking overnight in aqueous suspension of antibacterial and antifungal compounds. After soaking, seeds were crushed with the help of sterile mortar and pestle, serially diluted and 100 µl of suspension was spread over NA plates to ensure the elimination of endophytic microflora of seeds. Thereafter endophyte-free seeds were raised under gnotobiotic conditions in culture tubes using sterilized soil. Endophyte-free seeds were coated with selected endophytic isolates and incubated at 28°C for 60 days. After incubation, endophytic strains were re-isolated from plant tissues.

3.11) Effect of endophytes on seed germination

Effect of endophytes on seed germination of *W. somnifera* and their role in controlling seed borne infections was examined *in vitro* as well as *in vivo* for which seeds of 'Poshita' variety were obtained from CSIR-CIMAP, Lucknow, India. Prior to germination test, seeds were surface sterilized for which mature and healthy seeds were washed thoroughly under running tap water for 30 min. The seeds were then surface disinfected with an aqueous solution of 0.1% mercuric chloride for 5 min followed by 5 times rinsing with double distilled water.

A dressing of the endophytes WRE-1 and WSEF-4 was applied to the seeds in the form of a thin layer of culture suspension. To prepare culture suspension of endophyte WRE-1, 24 h old broth culture was centrifuged at 8000 rpm for 15 min, supernatant was discarded and pellet was suspended in sterilized distilled water. Cell population of 2.8×10^8 per ml was estimated by counting of bacterial cell number using haemocytometer. Spore suspension of fungal endophyte WSEF-4 was prepared by harvesting spores from a 5-days old culture plate by adding 5 ml of sterilized distilled water to the culture plate and gentle rubbing of colony surface using glass rod or spreader. Spore population was maintained up to $1.5 \times 10_6$ per ml by counting spore number using haemocytometer. Surface sterilized seeds were kept overnight in spore/cell suspension of endophyte isolate. Thereafter according to the treatment seeds were also treated with spore suspension of fungal pathogen and then placed on Petri dishes. Experiment set and treatments are mentioned below.

1) Control
2) *A. alternata* (AA)
3) *F. solani* (FS)
4) WRE-1
5) WSEF-4
6) WRE-1+ *A. alternata*
7) WRE-1+ *F. solani*
8) WSEF-4+ *A. alternata*
9) WSEF-4+ *F. solani*

In vitro seed germination was carried out according to Khanna et al. (2013). Twenty-five seeds were used in each case with four replications in Petri dishes (90 mm diameter) lined with Whatman No. 1 filter paper moistened with sterile distilled water. Germination was checked every 24 h under controlled conditions. Effect of endophytes on seedling vigor was examined by pot experiment using soil: compost (3:1) as potting mixture. Seedling and radicle length was recorded on the basis of randomly selected ten seedlings per treatment after 30 days of incubation.

3.12) Pot and field experiments

Pot experiments were carried out during year 2014-15, each for 60 days (from October to December, 2014 and February to April, 2015) in green house at BBAU, Lucknow. Field experiments were also conducted twice during year 2015-16, each for 120 days (from February to May, 2015 and October, 2015 to January, 2016) in a local farm of Lucknow district. The experimental design was complete randomized block design with five replications.

3.12.1) Pot preparation

The potting mixture in all experiments consisted of soil: compost (3:1) sieved through 2.5 mm mesh and then sterilized by autoclaving at 121ºC for 3h in autoclavable plastic bags. The soil used was a sandy loam with pH 8.5, EC 343 µs, 3.2 g/kg organic carbon, 2.3 g/kg available N, 69.5 mg/kg available P and 187.2 mg/kg available K. For field experiments, 50 days old seedlings were transplanted into the field at a spacing of 60x45 cm using randomized block design with five replications for each treatment. Planting holes were prepared with depth and diameter of about 10-15 cm. Before transplanting seedlings, soil samples were collected and analyzed for initial microbial population and physico-chemical parameters (pH, EC, available organic C, N, P, K).

3.12.2) Seedlings of *W. somnifera*

Seedlings of *W. somnifera* ‘Poshita’ variety were raised in pots (containing sterilized soil) using endophyte-free seeds and healthy seedlings with uniform length were selected for pot and field experiments.

3.12.3) Bioinoculum preparation

To prepare inoculums of fungal endophyte WSEF-4 and fungal pathogens *A. alternata* and *F. solani*, fungi were mass multiplied on potato dextrose broth at 28°C for 7 days. After incubation, mycelia of each fungus was separated from broth and homogenized in 500 ml of 100 mM phosphate buffer to obtain a cfu of 1.5×10^6 per ml buffer suspension.

Inoculum of bacterial endophyte WRE-1 was prepared by inoculating the bacteria in nutrient broth for 24 h at 210 rpm at 28°C. After incubation, bacterial suspension was centrifuged at

8,000 rpm for 15 min. the supernatant was discarded and pellets containing bacterial cells were suspended in 500 ml of 100 mM of phosphate buffer. Bacterial count in the suspension was 2x10^8 per ml buffer suspension.

3.12.4) Compatibility of endophytic bioinoculants with arbuscular mycorrhizal fungi (AMF) *Glomus intraradices* and co-inoculation effect on plant growth

Compatibility of endophytes with mycorrhizal fungi was also investigated under field condition for which AM fungi *Glomus intraradices* was purchased from Ambika Biotech (ISO 9001:2008). Product (under the trade name Root care) contained 1,00,000 infectious propagules per Kg. Endophytes WRE-1 and WSEF-4 were co-inoculated with *G. intraradicus* and their effect on plant growth was examined.

3.12.5) Treatments

Seedlings for each treatment were raised separately in small pots in sterilized soil by inoculating the soil accordingly. 50-days old seedlings from each treatment were transplanted to field for field trials. No pathogenic strains were inoculated directly to the field soil. Seedlings treated with selected endophytes, test pathogens and mycorrhizal fungi, inoculated individually or co-inoculated in various combinations. For pot experiments treatment number 1 to 9 were followed while field experiments were performed using treatments from 1 to 16.

1) Control
2) AA (*A. alternata)*
3) FS (*F. solani*)
4) WRE-1
5) WRE-1+AA
6) WRE-1+FS

7) WSEF-4
8) WSEF-4+AA
9) WSEF-4+FS
10) GI (*G. intraradices*)
11) WRE-1+GI
12) WRE-1+GI+AA
13) WRE-1+GI+FS
14) WSEF-4+GI
15) WSEF-4+GI+AA
16) WSEF-4+GI+FS

3.13) Growth observations and harvesting

After harvesting, plant height, root length, fresh weight and dry weight for each treatment were recorded. Dry weight was determined after drying plants in a hot air oven at 60°C. Rhizospheric soil was also collected for the estimation of nutrient level in soil.

3.14) Phytochemical analysis after harvesting

Phytochemical analysis of plants (including total chlorophyll, proline, reducing sugars, total phenolics and total withanolides) was done after 45 days of transplantation and after harvesting.

3.14.1) Reducing Sugar

0.5 g fresh leaf was ground in a cold mortar and pestle and homogenized in 10 ml 80 % ethanol. The suspension was centrifuged at 14000 rpm for 30 min at 4 ºC. The supernatant was taken for the reducing sugar estimation. To 3 ml of alcoholic plant extract, 3 ml of DNSA (Di-nitrosalicylic acid) was added and heated for 5 mins in boiling water bath. After boiling it developed colour, to this 1 ml of 40% Rochelle salt (Sodium potassium tartarate) was added and OD was taken at 515 nm (Miller, 1972).

3.14.2) Estimation of chlorophyll content

Chlorophyll content was measured according to the method of Chen et al. (2009) in which 300 mg of fresh leaves were collected and partially dehydrated by soaking them in 95% ethanol for 2-5 minutes. Thereafter leaves were sliced into small pieces, grinded with the help of mortar and pestle. 5 ml of 80% acetone was added to a 15 ml Falcon tube, leaf extract was transferred to it and kept in dark for 15-30 min. then tubes were centrifuged at 4℃ for 15 min (3,000 rpm), the supernatant was transferred to a new centrifuge tube and kept in dark. Then OD was measured at 663 nm and 645 nm using spectrophotometer. The chlorophyll concentrations were calculated as follows (80% acetone was used as a blank control):

Chlorophyll a (mg/g) = [(12.25 x A_{663}) – (2.55 x A_{645})] x mg/ml leaf tissue

Chlorophyll b (mg/g) = [(20.31 x A_{645}) – (4.91 x A_{663})] x mg/ml leaf tissue

Total chlorophyll = chlorophyll a + chlorophyll b = (17.76 x A_{645}) + (7.34 x A_{663}) x mg/ml leaf tissue

3.14.3) Total Proline Content

The total proline in the leaves was measured using the modified procedure of Bates et al. (1973). 100 mg of plant leaves were homogenized in 1.5 ml of 3% sulphosalicylic acid and residue was removed by centrifugation. 100 µl of extract was reacted with 2 ml glacial acetic acid and 2 ml acid ninhydrin for one hour at 100°C. The reaction was terminated in an ice-bath. The reaction mixture was extracted with 1 ml toluene. The chromophore containing toluene was warmed to room temperature and its absorbance was measured at 520 nm. The amount of proline was determined from a standard curve.

3.14.4) Preparation of leaf extract for estimation of total phenolics and total withanolides

Preparation of plant extracts to determine total phenolics and withanolide-A content was done according to Dhanani et al. (2013). Leaves of *W. somnifera* were properly washed and air dried. The air-dried plant material was finely powdered with the help of an electrical grinder. Then 5 g of powdered plant material was extracted with 50 ml of ethanol and filtered. Liquid extract was concentrated by evaporation and dried extract was used for the determination of total phenolics and alkaloid content.

3.14.4.1) Total phenolics content

Total phenolics content was determined according to Kaur and Kapoor (2002). Dried extract of leaves was dissolved in distilled water (1mg/ml) and 0.5 ml of Folin-Ciocalteau reagent was added to it. Total volume was adjusted to 8.5 ml using distilled water and tubes were incubated at 25°C for 10 min. Then 1.5 ml of 20% sodium carbonate solution was added to each tube and tubes were incubated in a water bath at 40°C for 20 min. After incubation, blue color was developed in reaction mixture, absorbance of which was measured using UV-Visible spectrophotometer at 755 nm. Phenolic content was quantified using standard curve of gallic acid and expressed as gallic acid equivalent (GAE) mg/g of the extract.

3.14.4.2) Total withanolides content

Withanolides content from dried extract of leaves was determined using LC-10 system on using a reverse-phase C_{18} column (4 mm, 3.9x150 mm; Nova-Pack, Waters, Milford, MA) and a solvent system (0.6 ml min $^{-1}$) comprised of methanol and water (each containing 0.1% acetic acid) in the gradient mode - 45:55 to 65:35 (45 min). Detection was done at 227 nm using an online UV detector (SPD-10A) and the chromatogram reports were generated through integrated software (Class-LC10, version 1.63). 10 µl of withanolide sample (methanolic solution) or 5 µl of marker withanolide was injected for each run (Sangwan et al., 2008).

3.15) Root colonization by AM fungi *G. intraradices*

The root samples were cleared using 10% KOH for 1 h at 90°C and stained with lactophenol cotton blue by standard method (Phillips and Hayman, 1970). Positive counts for AM colonization included the presence of vesicles or arbuscules or typical mycelium within the roots. Colonization percentage was calculated according to Mcgonigle et al. (1990). Formula for calculating root colonization percent is as mentioned below:

Percent root colonization= (Number of root bits shows colonization/total number of root bits observed) x 100

3.16) Soil analysis

At the time of harvesting, rhizospheric soil from each treatment was collected from 0-15 cm depth. Soil samples in triplicate were collected plot-wise and stored at 4°C. Prior to its examination for physico-chemical parameters, each soil sample was air dried and sieved (2 mm). To measure pH and electrical conductivity (EC), soil was dissolved in double distilled water at the ratio of 1:2.5 (weight/volume) and kept for 30 min at room temperature. Thereafter suspension was filtered through filter paper and filtered extract was used to measure the pH and EC by respective electrodes. Total organic carbon in soil samples was measured according to the dichromate-oxidation method and available nitrogen by Kjeldahl method (Black, 1965). The available phosphorous content was measured according to ascorbic-acid method following the method of Bray and Kurtz (1945) and available potassium was measured by flame photometer after extraction of soil with 2% (weight/volume) ammonium acetate solution (Black, 1965).

3.17) Statistical analysis

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

4.1) Endophyte isolates of *W. somnifera*

In total 72 isolates (including bacteria and fungi) were obtained from leaves, shoot and root tissues of *W. somnifera*. Maximum numbers of endophytes were isolated from stem tissues, followed by leaves and roots. Population of bacterial endophytes was found comparatively higher than fungal endophytes. Endophytic bacteria and fungi, belonging to the genus *Pseudomonas, Bacillus, Serratia, Micrococcus, Aspergillus, Alternaria, Chaetomium, Fusarium, Cladosporium, Bipolaris* and *Verticillium* (as identified later on the basis of phenotypic, biochemical characterization and microscopic observation) were obtained from different plant parts of collected samples ***(Figure 4.1)***. Detail of endophytic microorganisms isolated from different plant parts is given in ***Table 4.1.***

4.2) Fungal pathogens of *W. somnifera*

Most of the fungi isolated from diseased leaf tissues were able to produce necrotic brown lesions during *in vitro* pathogenicity assay. Pathogenic fungi P1 showed highest disease severity index during *in vitro* assay. Appearance of similar disease symptoms on healthy plants after foliar spray of pathogenic fungi P1 and re-isolation of same pathogen from infected leaf tissues confirmed the pathogenic nature of fungi P1. On the basis of *in vitro* and *in vivo* pathogenicity assay, P1 was found the most virulent fungi of *W. somnifera,* which was identified as *Alternaria alternata* and selected for further study. Pathogenic fungi *F. solani* was procured from the laboratory of Microbial Technology Division, CSIR-CIMAP, Lucknow. *F. solani* was also investigated for its pathogenic nature *in vitro* as well as *in vivo* (***Figure 4.2).*** Disease severity index of *F. solani* was recorded up to 75% and for *A. alternata* was up to 83%.

4.3) Plant growth promoting traits of endophytic isolates

Screening of endophytic isolates for their capability to fix nitrogen, phosphate and zinc solubilization, phytohormone IAA and siderophore production, total thirty endophytes including bacteria and fungi showed positive activity for one or more PGP traits ***(Figure 4.3, Table 4.2)***. Phosphate solubilization was found most common PGP trait among endophytes as 56.6% isolates were phosphate solubilizers. Fungal endophytes were highly efficient and most of the isolated fungi solubilized phosphate on PVK agar media. Highest level of PSI was observed for bacterial isolate WRE-1 (2.72±0.05) and fungal isolate WLEF-2 (3.03±0.15).

Phytohormone IAA production was the second most common PGP trait among isolates and was exhibited by 50% of the endophytes. Most of the IAA producing bacterial strains showed slight or average activity while three fungal isolates WSEF-4, WSEF-9 and WLEF-7 were found adequately efficient IAA producers as 32.5±0.2 ppm, 34.3±0.5 ppm and 35.2±0.2 ppm concentrations were recorded respectively, after quantitative estimation of IAA production.

Siderophore production was exhibited by 33.3% of endophyte isolates. Fungal isolates were more efficient siderophore producers in comparison to bacteria. Bacterial isolate WRE-1 and Fungal isolate WSEF-3 and WSEF-10 showed strong iron-chelating potential. Siderophore units recorded for WRE-1, WSEF-3 and WSEF-10 were 34.8%, 46.2% and 43.8% respectively.

Zinc solubilization activity was showed by 50% of the isolates. Bacterial isolate WRE-1 and WSE-6 were best zinc solubilizers. Both these strains also showed strong alkaline phosphatase activity (at pH 10.0). Released *p*NP concentration for WRE-1 was >500 mg L^{-1} and for WSE-6 was 530 mg L^{-1} as recorded spectrophotometrically. In case of fungal isolate WSEF-5, maximum phosphatase activity was recorded with 630 mg L^{-1} of released *p*NP.

4.4) Endophytic isolates antagonistic against A. *alternata* and *F. solani*

In total 30% of isolates exhibited antagonistic activity against both the test fungi by inhibiting mycelial growth by 50% or higher. Bacterial isolate WRE-1 was most potent biocontrol agent which suppressed mycelial growth of *F. solani* and *A. alternata* by 66.6% and 72.2% respectively in comparison to control as recorded by dual culture assay. Fungal isolate WSEF-4 suppressed the growth of *F. solani* most effectively by inhibiting 77.7% of mycelial growth while inhibition of *A. alternata* was 61.1% ***(Figure 4.4)***. Biocontrol potential of two most potent antagonistic endophyte isolates WRE-1 and WSEF-4 (in suppression of leaf spot disease) was examined *in vitro* by detached leaflet assay ***(Figure 4.5)***. Results obtained showed significant suppression of fungal pathogens and disease severity by both the endophytes. Disease severity recorded for pathogenic fungi *F. solani* and *A. alternata* was 62.5% and 75% respectively. Endophyte WRE-1 reduced the disease severity while co-inoculated with *F. solani* and *A. alternata* by 93.3% and 83.3% respectively. WSEF-4 on co-inoculation suppressed disease severity by *F. solani* and *A. alternata* by 86.72% and 77.86% respectively.

Exploration of biocontrol mechanism revealed that mycoparasitic interaction of fungal endophytes with pathogenic fungi might contribute to their antagonistic activities. Regular observation of hyphae from dual culture plates under light microscopy revealed that fungal endophytes tended to grow along the hyphae of the pathogen as soon as two colonies came into contact. Coiling of hyphae of WSEF-4 around hyphae of *A. alternata* and *F. solani* was observed ***(Figure 4.6)***. Fungal isolate WREF-4 showed rigorously coiled hyphal structures around hyphae of *A. alternata*. Observation of hyphae from the contact zone between two fungi under scanning electron microscope revealed extensive branching and coiling of hyphae of WSEF-4 around growing hyphae of *A. alternata*. SEM analysis of hyphae from contact zone between WRE-1 and *A.*

alternata revealed the presence of holes on cell wall of pathogenic fungi while that from WRE-1 and *F. solani* displayed disintegration of hyphae ***(Figure 4.7)***.

Another biocontrol mechanism by antagonistic endophytes, observed in case of isolates WRE-1 and WSEF-4 was production of bioactive secondary metabolites as determined by using cell-free culture supernatant (CFCS) (figure 4.4). CFCS of both the isolates showed similar inhibitory effect on both the pathogenic fungi. HCN production was reported negative among endophyte isolates. Production of extracellular hydrolytic enzymes amylase, protease, lipase, β-1,3-glucanase and chitinase production by both the isolates showed that these enzymes might contribute towards their inhibitory effect on fungal pathogens ***(Figure 4.8).*** Fungal cell-wall degrading enzyme chitinase was extracellularly produced by isolate WRE-1 and WSEF-4 on colloidal chitin agar media. From a dual culture plate, hyphae of both the leaf pathogen from contact zone (between leaf pathogens and antagonistic endophyte) were analyzed under SEM. Mycelial observation under SEM revealed that cell wall of fungal pathogens was degraded by isolate WRE-1 and WSEF-4 as pore formation and highly fragile and broken hyphae were observed in most of the analyzed samples ***(Figure 4.7)***.

4.5) Phenotypic and biochemical characterization of selected endophytes

Phenotypic and biochemical characterization of endophytes, screened on the basis of their PGP potential and antagonistic activity against fungal pathogens was done. Bacterial endophytes were characterized according to Bergey's Manual Of Systemic Bacteriology on the basis of colony morphology, cell shape, cell wall type, presence or absence of endospore, enzymatic and physiological activities, as described in ***table 4.3***. Fungal endophytes were characterized on the basis of their colony morphology, hyphal structure (branched/unbranched, septate/aseptate, coenocytic/multicellular), structure of spores and their enzymatic or physiological activities ***(Table***

4.4 and 4.5, Figure 4.9). Morphological identification of endophytic fungi was done according to Watanabe (2002).

Although the levels of probability found from these results do not permit to draw any reliable conclusion towards accurate identifications of the isolates but obtained results can provide us a rough idea of diversity of endophytes at least at genus level. Obtained results suggest that *W. somnifera* is found to be associated with various endophytic bacteria and fungi, represented by the genus *Pseudomonas, Bacillus, Serratia, Micrococcus, Aspergillus, Alternaria, Colletotrichum, Chaetomium, Fusarium, Cladosporium, Bipolaris* and *Verticillium*.

4.6) Molecular identification and phylogenetic analysis

A comparison of 16S rRNA gene sequence of isolate WRE-1 with the sequences present in NCBI database revealed its maximum homology with *Pseudomonas aeruginosa*. Comparison of 18S rRNA gene sequences of endophyte isolate WSEF-4 showed maximum sequence similarity with *Chaetomium globosum.* Fungal pathogen P1 was identified as *Alternaria alternata* on the basis of 18S rRNA gene sequencing. All three sequences were submitted to Genbank (NCBI) (details given in the Table 4.6) and phylogenetic tree were constructed according to the 16S and 18S rRNA gene sequences (***Figure 4.10)***.

4.7) Confirmatory tests for biosafety of strain *P. aeruginosa* WRE-1

Since *P. aeruginosa* is reported for its opportunistic pathogenic nature for humans, non-virulent nature of endophyte isolate *P. aeruginosa* WRE-1 was confirmed before further study and their field applicability. *P. aeruginosa* WRE-1 showed no hemolytic activity on blood agar, negative activity for pyocyanine production, protease production and elastin protein hydrolysis. Optimum growth temperature was 20-28°C and growth reduced at further temperature increase. Antibiotic sensitivity was also investigated for twenty antibiotics of different groups ***(Table 4.7, Figure 4.11)***.

4.8) Extraction and characterization of secondary metabolites

Characterization of secondary metabolites, extracted (with ethyl acetate) from strain WRE-1 and WSEF-4 for their bioactive compounds was done using FTIR. Functional groups in the metabolites were identified using absorption spectra ***(Figure 4.12).*** The FTIR spectrum of metabolite from WRE-1 showed a peak at 3355.9 cm-1 indicating presence of –NH (amine), peaks at 2,924.5 and 2854.5 cm-1 indicating C-H stretch in methylene group, peak at 1720 cm-1 showed the presence of C=O (saturated aliphatic/ aromatic compound) group, peaks at 1632.3, 1463.5, 1371.6 showed C=C and C=N stretch, vibration at 1244.3, 1153.6, 1073.8, 1034.0, 911.3 indicate C=C (monosubstituted alkene), 833.5 indicate para and 771.9 indicate meta substituted benzene ring.

On the basis of absorption frequencies, secondary metabolite of *Pseudomonas* strain WRE-1 was partially characterized as a derivative of phenazine compound. The IR spectra of phenazine and twenty-eight of its derivatives have been recorded in the region 3800-650 cm^{-1}. Interpretation of absorption spectra of bioactive secondary metabolite of strain WRE-1 was prepared on the basis of comparison with IR spectra of phenazines compound for which review article by Stammer and Taurins (1963) and Kadam et al. (2013) were used as reference.

FTIR spectra for metabolite WSEF-4 and observed peaks showed the presence of NH stretch, C=O group (from aromatic compound), C=C stretch, O-H group (in benzene ring) and –C-H bending. These functional groups indicate that the compound was aromatic in nature however due to unavailability of reference spectra in available literature, the compound was not identified and requires further studies for complete identification.

4.9) Determination of endophytic nature of isolates

Colonization efficiency of two selected isolates was investigated prior to field trials. Both the endophytes were capable to colonize their host plant and proliferate as endophyte as confirmed after their re-isolation from treated seedlings and microscopic examination of plant tissues ***(Figure 4.13).*** Colonization efficiency of WRE-1 and WSEF-4 was recorded up to 60% and 80% respectively, calculated on the basis re-isolation of endophyte from the tissues of seedlings inoculated with the same strain. Colonization of roots by fungal endophyte WSEF-4 was also confirmed after microscopic investigation of root tissues of WSEF-4 inoculated seedlings and identified on the basis of structure of hyphae and spores.

4.10) Effect of endophytes on seed germination

Seed germination results of *W. somnifera* and effect of various treatments on germination percentage and seedling vigor index are given in Table 4.8. Results obtained suggest positive influence of endophytes on seed germination and development of seedlings. *W. somnifera* seeds showed poor germination rate. Both the test pathogens reduced the germination as well as seedling development. *F. solani* treated seedlings displayed seedlings damping off which suppressed the germinated seeds to further develop, *A. alternata* treated seeds displayed suppressed germination and most seeds failed to germinate. In both the cases germination percentage and seedling vigor was highly reduced ***(Figure 4.14).***

Endophyte WRE-1 and WSEF-4 improved the germination by about 15% and 38% respectively in comparison to control. Both the isolates also suppressed the negative impact of pathogens on seed germination when co-inoculated. WRE-1 when coinoculated with *A. Alternaria* and *F. solani*, improved the germination by 33% and 150% as compared with *A. alternata* and *F. solani* (alone) treated seeds. Similarly, co-inoculation of WSEF-4 with *A. alternata* and *F. solani* showed 66% and 133% higher germination in comparison to *A. alternata* and *F. solani* treated seeds. Effect of endophytes on seedling development was examined after 30 days of seed sowing in pots. Seedling and radicle length was found improved in WRE-1 and WSEF-4 treated seeds. WSEF-4 treated seeds showed completely healthy seedlings with improved seedling and radicle length by 31% and 17% in comparison to control. Pre- or post-emergent seedling mortality was not observed in WRE-1 and WSEF-4 treated seeds when co-inoculated with *A. alternata* and *F. solani*. Seedlings developed from pathogen-treated seeds showed reduced seedling and radicle length, reduced surface area of leaves and shrinking and distortion in leaves ***(Figure 4.15).***

4.11) Effect of endophytes on growth and yield of *W. somnifera* and role in disease suppression

As observed from pot experiments and field trials, plant height, root length, fresh and dry weight of plants improved significantly ($P \leq 0.05$) as a result of application of endophyte *Pseudomonas aeruginosa* WRE-1 and *Chaetomium globosum* WREF-4 in *W. somnifera*. Both the strains effectively suppressed the fungal pathogens *A. alternata* and *F. solani*.

4.11.1) Results obtained from pot experiment

Results obtained from pot experiment clearly showed that fungal pathogens *A. alternata* and *F. solani* severely affect plant growth and yield by reducing root development and by enhancing leaf fall. The disease symptoms were observed mainly on leaves in the form of brown necrotic leaf spots. Spots were gradually enlarged with ageing and severely infected leaves dried and shed. Both the fungal pathogens affect plant height, root length, fresh weight and dry weight of *Withania* plants. Number of branches were also reduced *A. alternata* and *F. solani* reduced the height of treated plants by 23% and 15% respectively in comparison to control plant. *A. alternata* reduced plant height, root length, fresh weight and dry weight of treated plants by 23%, 30%, 51% and

54% respectively. Similarly, *F. solani* showed 15%, 21%, 31% and 35% reduction in plant height, root length, fresh and dry weight of plants respectively. Endophytes WRE-1 and WSEF-4 significantly improved plant growth parameters. Both the isolates also effectively suppressed the negative impact of pathogens *A. alternata* and *F. solani* and no disease symptoms were observed in plants co-inoculated with endophytes and pathogens. WRE-1 enhance crop productivity by increasing plant's height, root length, fresh and dry weight by18%, 29%, 22%, 21% respectively in comparison to control. WSEF-4 showed maximum yield enhancement by increasing plant's height, root length, fresh and dry weight by 31%, 44%, 30%, 29% respectively in comparison to control ***(Table 4.9, Figure 4.16, 4.17).***

4.11.2) Results obtained from field experiment

Analysis of plant growth parameters and phytochemical analysis revealed that both the fungal pathogens reduced the root and shoot development in their host plant also under field conditions. Roots of *W. somnifera* are major plant part that are used in medicines. However, colonization of fungal pathogens highly reduced the root development and further suppressed the plant growth ***(Figure 4.18).*** *A. alternata* displayed 49% reduction in dry weight of root in comparison to control and *F. solani* showed 26% reduction in root dry weight. Strain WRE-1showed an increase of 41% in root dry weight in comparison to control. Maximum increase was observed in WSEF-4 and WSEF-4+GI inoculated plants with 83% and 93% increase in root dry weight respectively in comparison to control. WSEF-4 also significantly improved the growth of pathogens-treated plants when co-inoculated. An increase of 275% and 103% in root dry weight was observed in plants when treated with WSEF-4+AA and WSEF-4+FS in comparison to *A. alternata* and *F. solani* treated plants, respectively. WSEF-4+GI combination was most effective against both the fungal pathogens as and an increase of 242% and 149% plant dry weight was obtained in comparison to *A. alternata* and *F. solani* treated plants respectively.

Phytochemical analysis revealed that both the leaf pathogens are highly destructive and significantly reduced phytochemicals in affected plants. Total withanolides content was reduced up to 45% and 32% in *A. alternata* and *F. solani* treated plants in comparison to control. WRE-1 and WSEF-4 inoculated plants showed an increase of 40% and 53% in withanolides content in comparison to control. Significant improvement in reducing sugars, total phenolics and withanolides content in endophyte-treated plants in comparison to control was observed. Highest yield of phytochemicals was obtained from ashwagandha plants, co-inoculated with endophyte WSEF-4 and mycorrhizal fungi *G. intraradices*. No significant difference of proline content was observed in plants of various treatments ***(Table 4.10, 4.11; Figure 4.20)***.

4.12) Compatibility of mycorrhizal fungi with endophyte bioinoculants

Percent root colonization and spore count of *G. intraradices* was calculated and obtained results suggests that endophyte WRE-1 which has been identified as *Pseudomonas aeruginosa* suppressed the colonization by mycorrhizal fungi as only 24% root colonization was observed. Highly reduced root colonization percent and spore count of *G. intraradices* in WRE-1 co-inoculated plants suggests unsuitability of the strain WRE-1 as a bioinoculant along with *G. intraradices*. On the other hand, endophyte strain WSEF-4 showed no significant negative effect on mycorrhizal fungi. Comparative analysis of co-inoculation of WSEF-4 and *G. intraradices* with *G. intraradices* inoculated plants and rhizospheric soil displayed a slight difference in root colonization percent and spore count which confirmed the compatibility of WSEF-4 with *G. intraradices* ***(Table 4.11; Figure 4.19)***.

4.13) Soil analysis

Endophytic bioinoculants significantly improved the soil properties by balancing the pH, EC and by increasing nutrients level in soil. The uncultivated soil was alkaline with a pH of 8.5 and EC value 343 µs. Total organic C, available N, P, K estimated in uncultivated soil was recorded up to 3.2 g/kg, 2.3 g/kg, 69.5 mg/kg and 187.2 mg/kg respectively. *Withania* plantation improved the soil properties by lowering the pH up to 7.8 and EC up to 294 µs. Total organic C, N, P, K in rhizospheric soil of control treatment was 4.35 g/kg, 3.4 g/kg, 85.5 mg/kg and 278.2 mg/kg respectively. Maximum improvement in soil properties was found in WSEF-4+GI treated soil where pH and EC was reduced up to 7.32 and 217 µs and an increase of 26% of total organic C, 21% of available N, 52% of available P and 12% of available K was recorded in comparison to control. In WREF-4+GI treated soil, pH 7.47, EC 224 µs, percent increase of 25.28% of total organic C, 11.76% of available N, 46% of available P and 9.41% available K in comparison to control was recorded.

Table 4.1) **Isolated endophytes from different plant parts of *W. somnifera***

Plant parts	Isolated bacteria	Isolated fungi	Total isolates
Stem	28	9	37
Leaves	14	8	22
Roots	8	5	13

Table 4.2) **Plant growth promoting traits of isolates**

Isolate	**P solubilization**	**Alkaline phosphatase activity**	**N_2 fixation**	**Siderophore production**	**IAA activity**	**Zn solubilization**
WRE-1	+	+	-	+	-	+
WRE-2	+	-	+	-	+	+
WRE-3	+	-	+	-	-	-
WRE-b1	-	-	+	-	-	+
WRE-b2	-	-	-	-	+	+
WSE-4	+	-	-	+	-	-
WSE-5	-	-	-	-	+	+
WSE-b5	+	+	-	-	-	+
WSE-8	-	-	-	-	+	+
WSE-2	-	-	+	-	-	+
WSE-3	-	-	-	-	+	+
WSE-6	+	+	-	+	-	+
WLE-1	-	-	+	-	+	-
WLE-2	-	-	-	-	+	+
WLE-5	-	-	+	-	-	+
WLE-b6	+	-	-	-	+	-
WLE-8	-	-	+	-	+	-
WSEF-1	+	-	-	+	-	-
WSEF-3	+	-	-	-	+	-
WSEF-4	+	-	-	+	+	-
WSEF-5	+	+	-	+	-	-
WSEF-8	-	-	-	-	+	+
WSEF-9	+	-	-	+	+	-
WSEF-10	+	-	-	+	-	+
WSEF-13	+	-	-	-	-	-
WREF-4	+	-	-	+	-	+
WREF-6	+	+	-	+	-	-
WLEF-1	-	-	-	-	-	-
WLEF-2	+	-	-	-	+	-
WLEF-7	-	-	-	-	+	-

+ and - indicate positive and negative activity respectively

Table 4.3) **Phenotypic and biochemical characterization of bacterial isolates**

Isolate	**Gram stain. & morphology**	**Endospore**	**Catalase**	**Amylase**	**Protease**	**Lipase**	**Cellulase**	**Urease**	**Ammonia production**	**Citrate utilization**
WRE-1	- rods	-	+	+	-	-	-	+	+	+
WRE-2	+ rods	+	-	+	-	+	+	+	+	-
WRE-3	- rods	-	+	-	+	+	-	-	+	+
WRE-b1	+ cocci	-	-	-	-	-	-	+	-	+
WRE-b2	- cocci	-	+	-	+	-	-	+	+	+
WSE-4	- cocci	-	+	-	-	-	+	+	-	+
WSE-5	+ cocci	-	+	-	-	+	-	+	+	-
WSE-b5	+ cocci	-	+	-	-	+	-	+	+	+
WSE-8	- cocci	-	+	-	-	-	-	+	+	-
WSE-2	+ rods	+	+	+	+	+	-	+	+	-
WSE-3	- cocci	+	+	-	-	+	-	+	+	-
WSE-6	- rods	-	+	-	+	+	-	+	+	+
WLE-1	- rods	-	+	-	-	-	-	+	-	+
WLE-2	- cocci	-	-	+	-	-	-	+	+	+
WLE-5	+ rods	+	+	+	+	+	-	-	+	-
WLE-b6	+ cocci	+	+	-	-	-	-	+	+	+
WLE-8	- rods	-	-	+	-	-	-	+	-	+

+ indicate positive activity and – indicate negative active

Table 4.4) **Morphological and physiological characterization of fungal endophytes**

Isolate	**Hyphal morphology**			**Amylase**	**Protease**	**Lipase**	**Cellulase**	**Ammonia**
	Branching	**Septa**	**Spores**					
WSEF-1	Branched	Septate	+	+	+	+	-	-
WSEF-3	Branched	Septate	+	+	+	+	+	-
WSEF-4	Branched	Septate	+	-	-	+	+	+
WSEF-5	Branched	Septate	+	+	+	+	+	-
WSEF-8	Branched	Septate	+	-	+	+	+	+
WSEF-9	Branched	Septate	+	+	-	+	+	+
WSEF-10	Branched	Septate	+	+	-	+	+	-
WSEF-13	Branched	Septate	+	+	+	+	+	+
WREF-4	Branched	Septate	+	+	+	+	+	+
WREF-6	Branched	Septate	+	+	+	-	+	-
WLEF-1	Branched	Septate	+	+	-	-	+	+
WLEF-2	Branched	Septate	Not clear	+	-	+	+	-
WLEF-7	Branched	Septate	+	-	+	-	+	+
+ indicate positive activity and – indicate negative active								

Table 4.5) **Identification of endophytic fungi on the basis of colony morphology and mycelial structure (Watanabe, 2002)**

Fungal strain	**Mycelial characteristics observed**	**Colony morphology on PDA**	**Identified as**
WLEF-1	Hyphae septate and branched. Conidiophores brown, erect, simple or branched, bearing conidia apically and laterally on apical fertile parts. Conidia brown, solitary, ellipsoidal, usually 3-, rarely 4-5 distoseptate without hilum basally	Fast growing grey colonies	***Bipolaris* sp.**
WREF-4	Hyphae septate and branched. Conidiophores colorless, hyaline, inflated globosely forming vesicle. Conidiophores bear numerous conidia-bearing cells, called uniseriate phialides with conidia in long chains	Highly sporulating fungi with white colonies during hyphal growth and turned to yellow or golden at the stage of sporulation	***Aspergillus* sp.**
WSEF-3	Hyphae septate and branched. Acervuli are rounded or elongated, separate or confluent, superficial, erumpent, conspicuously multicellular with darkly pigmented setae. Conidia are hyaline, single-celled, falcate, fusiform, spindle shaped with acute apices.	Grey colonies with black spots due to the formation of sclerotia	***Colletotrichum* sp.**

WSEF-1, WSEF-8 and WSEF-13	Hyphae septate and branched. Conidiophores are dark colored, simple or branched, bearing catenulate conidia at the apex and apical fertile parts. Conidia dark brown, cylindrical, muriform, composed of 3-4 transverse walls and 1-2 longitudinal walls.	Highly-sporulating green colonies with very less visible hyphal growth	***Alternaria* sp.**
WLEF-7 and WSEF-4	Hyphae septate and branched, perithecia globose or barrel-shaped, covered with many stiff dark, coiled, terminal hairs. Asci hyaline, bearing 8 ascospores arranged in one row, ascospores are ellipsoidal, apiculate at both ends	Colonies of WLEF-7 were grey colored, hyphal growth in concentric rings. WSEF-4 colonies are pale yellow turned to grey-green colored during sporulation	***Chaetomium* sp.**
WSEF-5	Hyphae branched and septate. Mycelia are characterized by erect conidiophores without nodes and aseptate or inconspicuously septate non-spherical conidia. Conidiophores pale brown, erect, branched 2–3 times at the apical parts, bearing catenulate conidia in each branch. Conidia blastosporous, often not well differentiated from branches, hyaline or brown, ovate, ellipsoidal,	Colonies are slow growing, mostly olivaceous-brown to green, often becoming powdery due to the production of abundant conidia. Colonies appear compact, convex, and effused and furrowed	***Cladosporium* sp.**

	cylindrical, subglobose, irregular in shape, apiculate at one end, often truncate at another end		
WREF-6	Hyphae branched and septate, conidiophores (phialides) solitary on aerial hyphae, bearing spore masses apically. Conidia phialosporous, hyaline, cylindrical with rounded apex, unicellular	Colonies are slow growing, on PDA pure white with talc-like appearance	***Verticillium* sp.**
WSEF-10	Hyphae were branched, hyaline and septate, macroconidia were sickle-shaped to almost straight and 3-4 sepatate. Microconidia were club-shaped and single-celled	Abundant mycelium with white colonies on PDA, which became purple-orange with age	***Fusarium* sp.**
WSEF-9	Hyphae were branched and septate, Conidia small, unicellular or with 2-3 septa. Septate conidia acropetally developed, unicellular conidia are globose and apiculate basally	Colonies white with thick threads emerging upward	Unidentified
WLEF-2	Hyphae highly branched and septate however clear spores were not observed. Knob-like structures were present on hyphae	Colonies were pinkish-white, appear compact, convex, and effused and furrowed	Unidentified

Table 4.6) **Details of molecular sequences submitted to Genbank (NCBI)**

Strain name	**GenBank Acc. no.**	**Query coverage**	**% sequence similarity**	**Organism with the highest sequence identity**
WRE-1	KT761191	77%	99%	*Pseudomonas aeruginosa*
WREF-4	KX494863	98%	97%	*Chaetomium globosum*
P1	KX494864	73%	97%	*Alternaria alternata*

Table 4.7) **Antibiotic-susceptibility of strain *P. aeruginosa* WRE-1**

Antibiotic	**Sensitivity**	**Zone of inhibition (diameter in cm.)**
Neomycin (N^{30})	+	1.3
Ofloxacin (OF^{5})	+	2.6
Ciprofloxacin (CIP^{5})	+++	3.0
Cefotaxime (CTX^{30})	+	1.5
Norfloxacin (NX^{10})	+++	3.0
Gentamycin (GEN^{10})	+	1.8
Metronidazole (MT^{5})	**-**	**No inhibition**
Erythromycin (E^{15})	**-**	**No inhibition**
Amikacin (AK^{30})	+	2.0
Streptomycin (S^{10})	+	1.3
Tetracycline (TE^{30})	+	2.2
Polymyxin B (PB^{300})	+	1.5
Chloramphenicol (C^{30})	+	1.5
Colistin (CL^{10})	+	1.5
Penicillin-G (P^{10})	**-**	**No inhibition**
Oxytetracycline (O^{30})	+	1.0
Piperacillin (PI^{100})	+	1.3
Imipenem (IPM^{10})	+	2.5
Nalidixic acid (NA^{30})	+	1.2
+ indicate susceptibility and – indicate resistance for antibiotic		

Table 4.8) **Effect of selected endophytes on seed germination and seedling development (15 days incubation for germination percent and 30 days of incubation for seedling development)**

Treatment	Seedling length (cm)	Radical length (cm)	Seedling vigor Index	Germination percent
Control	4.18± 0.13^{d}	2.40±0.07^{d}	227.76	52%
A. alternata	2.92±0.58^{f}	1.94±0.11^{e}	123.12	36%
F. solani	3.36±0.2^{e}	2.14±0.20^{e}	96.48	24%
WRE-1	4.60±0.18^{c}	2.50±0.07^{c}	276.67	65%
WSEF-2	5.10±0.13^{a}	2.82±0.08^{a}	360.00	78%
WRE-1+AA	4.42±0.16^{c}	2.46±0.11^{c}	262.00	52%
WRE-1+FS	4.53±0.10^{c}	2.60±0.18^{bc}	269.21	60%
WSEF-2+AA	4.96±0.15^{b}	2.68±0.08^{b}	288.00	72%
WSEF-2+FS	4.72±0.16^{b}	2.66±0.05^{b}	278.32	65%

Values are expressed in mean of 5 replicates± Standard deviation (SD)

Mean in each column followed by same alphabets do not differ significantly ($P \leq 0.05$) according to Duncan's multiple range test (DMRT)

***Table 4.9)* Effect of endophytes on plant growth and disease control (60 days, pot experiment)**

Treatment	**Plant height (cm.)**	**Root length (cm.)**	**Weight**		**Number of branches**
			Fresh wt. (gm)	**Dry wt. (gm)**	
Control	32.50±.10^{f}	12.43±.11^{e}	382.7±7.8^{e}	95.58±1.94^{d}	2.67±.57^{f}
A. alternata	25.80±.30^{h}	8.70±.20^{g}	187.46±6.48^{g}	43.63±1.97^{f}	1.33±.57^{g}
F. solani	27.76±.40^{g}	9.73±.45^{f}	260.44±4.35^{f}	62.40±1.09^{e}	2.00±.00^{gf}
WRE-1	38.46±.37^{c}	16.03±.15^{c}	466.91±11.14^{c}	115.30±4.98^{b}	3.33±.57^{cde}
WSEF-4	42.56±.20^{a}	17.90±.20^{a}	498.61±10.80^{a}	122.92±4.18^{a}	5.33±.57^{a}
WRE-1+ *A. alternata*	35.86±.35^{e}	15.26±.40^{d}	421.70±2.04^{d}	105.25±0.50^{c}	3.00±.00de
WRE-1 + *F. solani*	37.13±.40^{d}	15.06±.37^{d}	432.97±3.74^{d}	108.16±.87^{c}	3.67±.57^{bcd}
WSEF-4 + *A. alternata*	40.00±.40^{b}	17.00±.26^{b}	482.06±8.00^{b}	120.41±2.02^{a}	4.00±.00^{bc}
WSEF-4 + *F. solani*	40.36±.50^{b}	17.30±.34^{b}	491.36±5.64^{ab}	122.75±1.39^{a}	4.33±.57^{b}

Values expressed in means ± SD (n=3)

Mean in each column followed by same alphabets do not differ significantly ($P \leq 0.05$) according to Duncan's multiple range test (DMRT)

Table 4.10) **Effect of selected endophytes on plant growth parameters (field trial, 120 days)**

Treatments	**Plant height**		**Plant weight**	
	Shoot (cm)	**Root (cm)**	**Fresh (g)**	**Dry (g)**
Control	112.26±1.17^{ef}	24.16±0.15^{g}	530.07±8.32^{f}	132.23±3.83^{h}
AA	105.76±1.34^{h}	23.03±0.15^{f}	275.34±9.02^{h}	68.01±3.67^{j}
FS	109.53±1.36^{g}	22.73±0.15^{h}	389.41±8.88^{g}	97.66±3.11^{i}
GI	115.30±0.89^{d}	27.26±0.05^{c}	692.22±7.53^{e}	173.60±1.17^{e}
GI+AA	113.9±0.82^{e}	26.8±0.07^{d}	642.31±4.12^{e}	160.57±0.89^{f}
GI+FS	116.24±0.51^{d}	25.9±0.8^{e}	591.16±11.4^{f}	147.79±2.01^{g}
WRE-1	123.60±1.06^{b}	29.06±0.23^{b}	749.13±18.12^{c}	186.22±5.81^{d}
WRE-1+GI	121.91±1.09^{b}	27.40±0.17^{c}	742.14±10.22^{c}	184.76±1.74^{cd}
WRE-1+AA	113.16±1.02^{e}	25.60±0.34^{e}	721.95±11.29^{d}	179.28±3.03^{e}
WRE-1+FS	115.70±0.60^{d}	25.36±0.15^{e}	736.82±8.41^{cd}	183.93±1.94^{cd}
WRE-1+GI+AA	119.63±1.05^{c}	26.50±0.20^{d}	724.44±8.45^{d}	180.53±1.65^{c}
WRE-1+GI+FS	120.83±0.65^{c}	26.83±0.15^{d}	738.24±6.83^{cd}	184.44±2.40^{cd}
WSEF-4	126.33±0.47^{a}	31.36±0.11^{a}	969.27±16.5^{a}	242.26±4.92^{a}
WSEF-4+GI	128.60±0.62^{a}	33.96±0.60^{a}	1020.31±21.77^{a}	255.22±6.55^{a}
WSEF-4+AA	120.83±1.12^{c}	28.56±0.25^{b}	792.69±14.33^{b}	198.70±3.33^{b}
WSEF-4+FS	122.16±0.49^{b}	27.13±0.20^{c}	834.03±3.92^{b}	208.89±4.28^{b}
WSEF-4+GI+AA	121.83±.25	29.90±0.20	928.29±3.11	232.50±2.26
WSEF-4+GI+FS	120.93±.23	29.50±0.20	972.79±3.21	243.99±2.68

Values expressed in means ± SD (n=3)

Mean in each column followed by same alphabets do not differ significantly ($P \leq 0.05$) according to Duncan's multiple range test (DMRT)

Table 4.11) **Phytochemical analysis of plants (field trial, 120 days)**

Treatments	**Total chlorophyll (mg/g)**	**Reducing sugars (mg/gm)**	**Total phenolics (mg/g GAE)**	**Total withanolides (µg/mg)**
Control	1.61±0.05^{f}	3.25±0.03^{e}	17.53±0.07^{d}	2.13±0.01^{f}
AA	0.56±0.10^{h}	1.83±0.06^{g}	15.26±0.04^{f}	1.19±0.11^{g}
FS	0.72±0.025^{h}	2.03±0.07^{f}	14.41±0.06^{g}	0.86±0.09^{h}
GI	1.90±0.01^{g}	3.80±0.02^{c}	17.99±0.45^{c}	2.68±0.02^{e}
WRE-1	2.86±0.07^{b}	3.91±0.02^{c}	18.97±0.21^{b}	3.83±0.05^{b}
WRE-1+GI	2.59±0.14^{c}	3.32±0.07^{e}	17.49±0.06^{d}	3.13±0.04^{d}
WRE-1+AA	2.23±0.09^{e}	3.46±0.03^{d}	17.38±0.06^{d}	3.16±0.02^{d}
WRE-1+FS	2.12±0.13^{e}	3.37±0.03^{d}	16.56±0.48^{e}	2.98±0.01^{de}
WRE-1+AA+GI	2.48±0.21^{d}	3.44±0.02^{d}	17.42±0.10^{d}	3.14±0.04^{d}
WRE-1+FS+GI	2.32±0.13^{d}	3.45±0.06^{d}	17.96±0.20^{c}	3.23±0.04^{cd}
WSEF-4	3.08±0.10^{a}	4.73±0.04^{a}	20.73±0.32^{a}	4.19±0.05^{a}
WSEF-4+GI	3.23±0.07^{a}	5.34±0.13^{a}	21.10±0.10^{a}	4.60±0.03^{a}
WSEF-4+AA	2.81±0.09^{b}	3.81±0.14^{c}	17.90±0.48^{c}	3.33±0.12^{c}
WSEF-4+FS	2.74±0.14^{c}	3.92±0.04^{c}	17.67±0.19^{c}	3.21±0.02^{cd}
WSEF-4+AA+GI	3.02±0.12^{a}	4.21±0.06^{b}	18.83±0.12^{b}	3.80±0.02^{b}
WSEF-4+FS+GI	2.89±0.08^{b}	4.48±0.03^{b}	18.63±0.08^{b}	3.47±0.04^{c}

Values expressed in means ± SD (n=3)

Mean in each column followed by same alphabets do not differ significantly (P ≤ 0.05) according to Duncan's multiple range test (DMRT)

Table 4.12) **Percent root colonization of *G. intraradices***

Treatments	**Percent root colonization**	**Spore count (per 50 gm of soil)**
GI	80.00±2.88^{a}	283.33±3.78^{a}
GI+AA	72.00±3.78^{b}	271.34±6.41^{b}
GI+FS	65.00±2.87^{c}	267.51±5.22^{c}
WRE-1+GI	24.00±3.60^{d}	62.67±4.72^{d}
WRE-1+AA+GI	25.33±3.78^{d}	60.00±3.60^{ef}
WRE-1+FS+GI	5.67±3.13^{e}	55.00±5.00^{f}
WSEFE-4+GI	76.33±2.88^{a}	273.67±5.13^{b}
WSEF-4+AA+GI	67.67±2.51^{c}	261.33±3.51^{c}
WSEF-4+FS+GI	69.00±3.46^{c}	257.33±3.51^{c}

Values expressed in means ± SD (n=3)

Mean in each column followed by same alphabets do not differ significantly ($P \leq 0.05$) according to Duncan's multiple range test (DMRT)

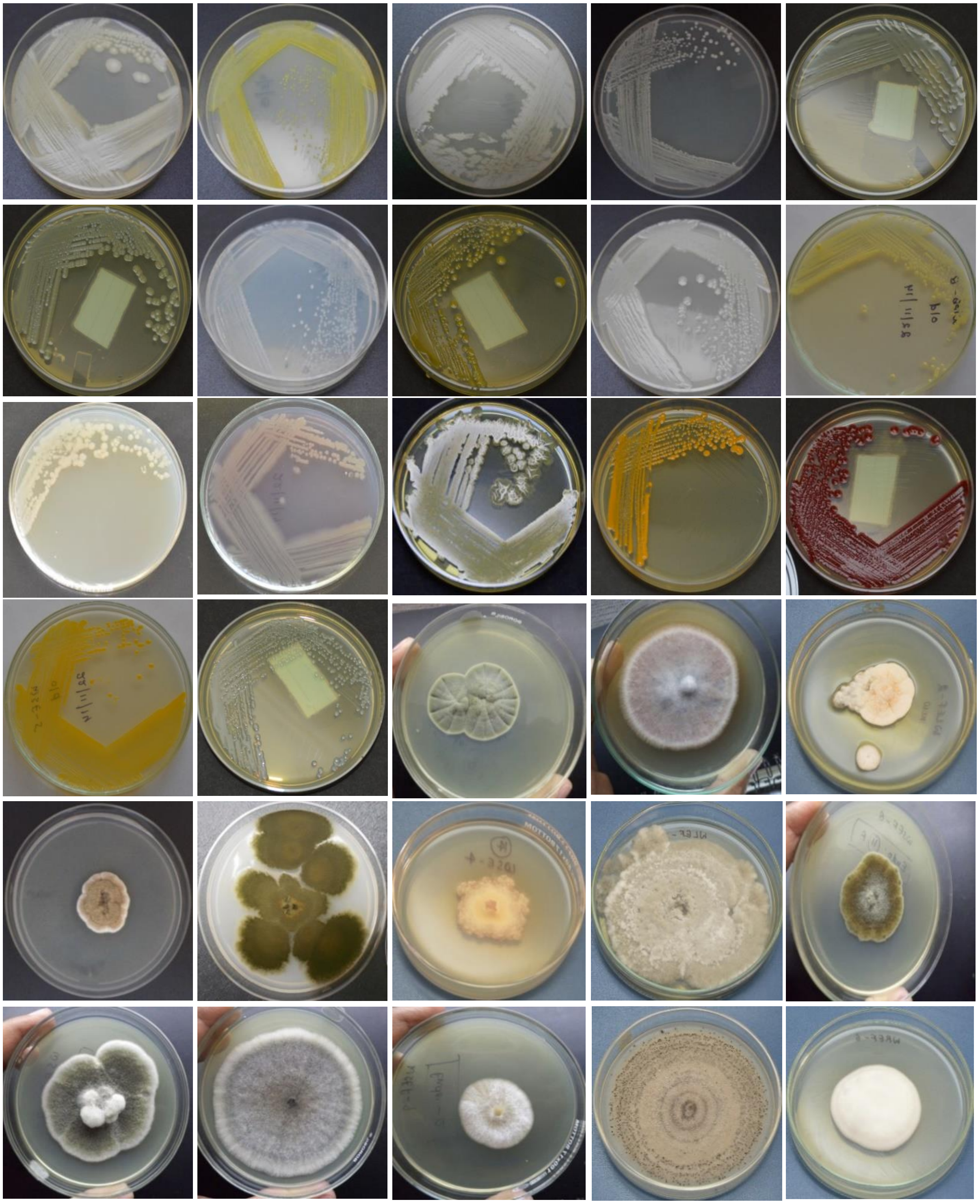


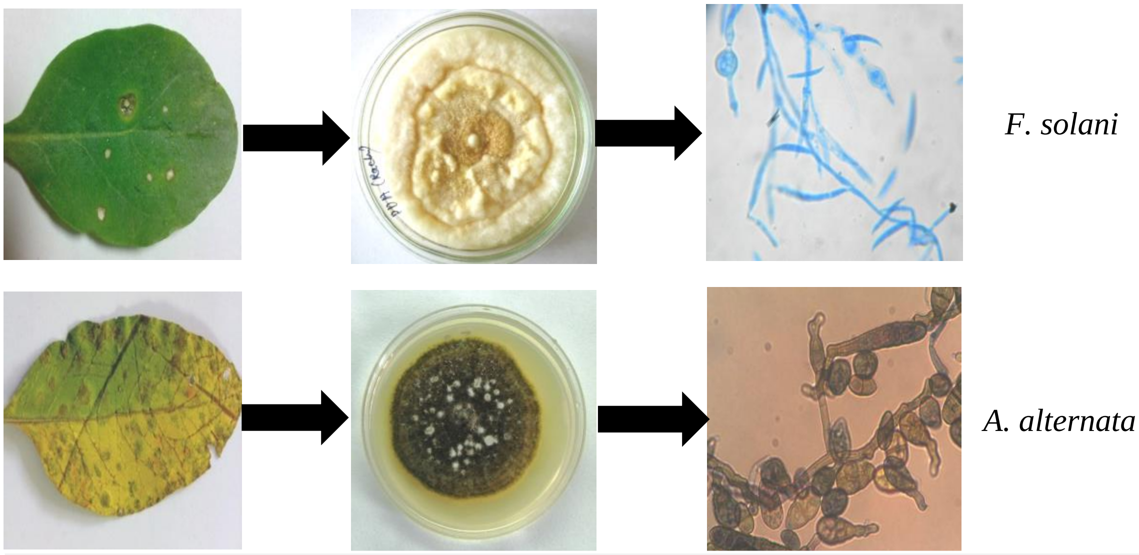
Figure 4.1*) Endophytes, isolated from various plant parts of *W. somnifera

In vitro pathogenicity assay for *A. alternata*

In vivo pathogenicity assay for *A. alternata*

In vitro pathogenicity assay for *F. solani*

In vitro pathogenicity assay for *F. solani*



Re-isolation of fungal pathogens from diseased leaves of *W. somnifera*

***Figure 4.2)* Pathogenicity test of isolated fungal pathogens**

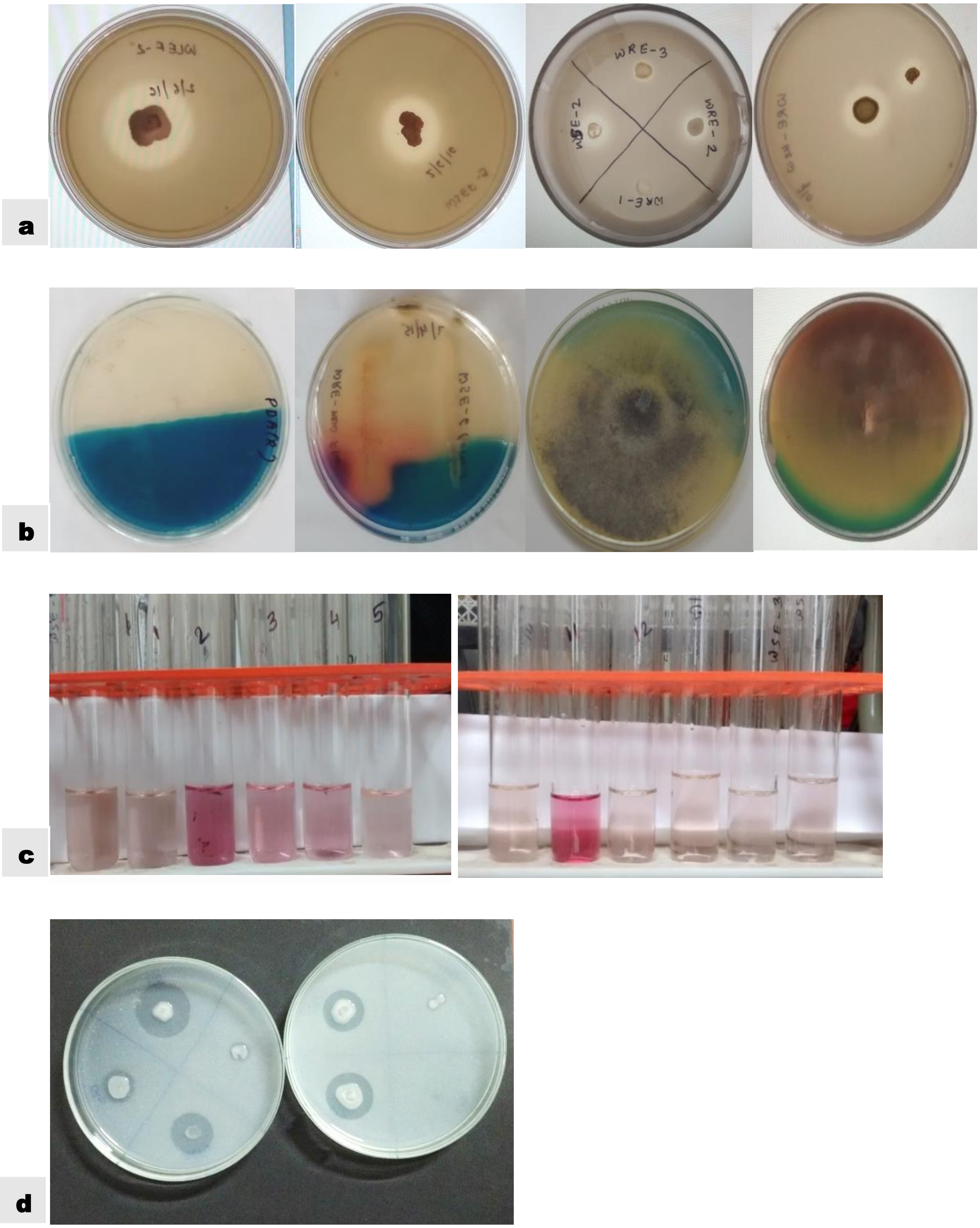
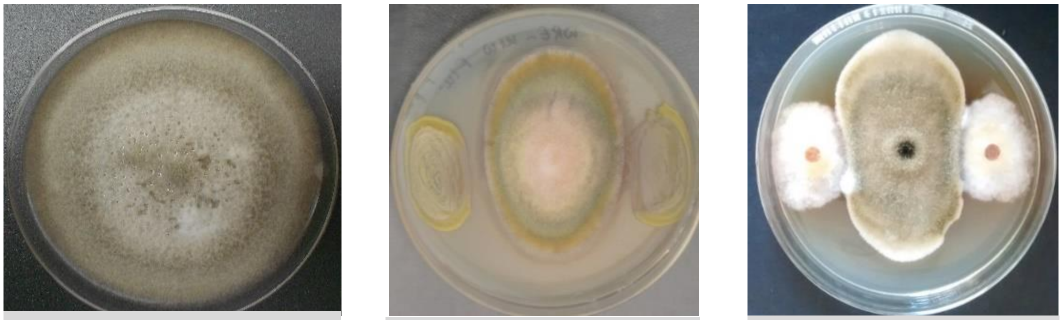


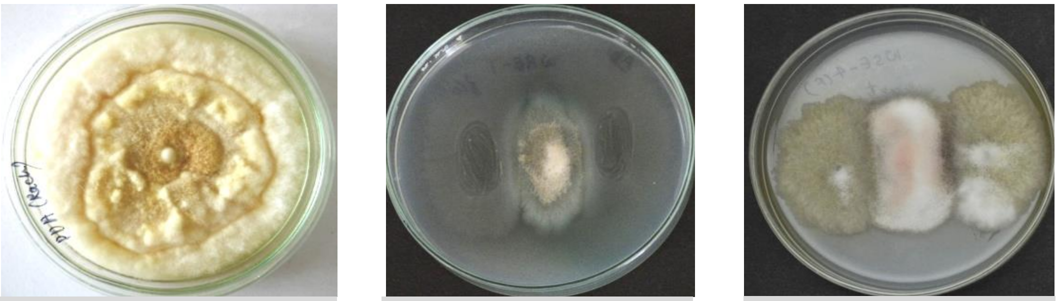
Figure 4.3) **PGP characteristics of isolates a) phosphate solubilization, b) siderophore production, c) Indole acetic acid production, d) zinc solubilization**



Control (A. alternata)

WRE-1+ *A. alternata*

WSEF-4+ *A. alternata*



Control (F. solani)

WRE-1+ *F. solani*

WSEF-4+ *F. solani*

Control (*A. alternata*)

CFCS of WRE-1+ *A. alternata*

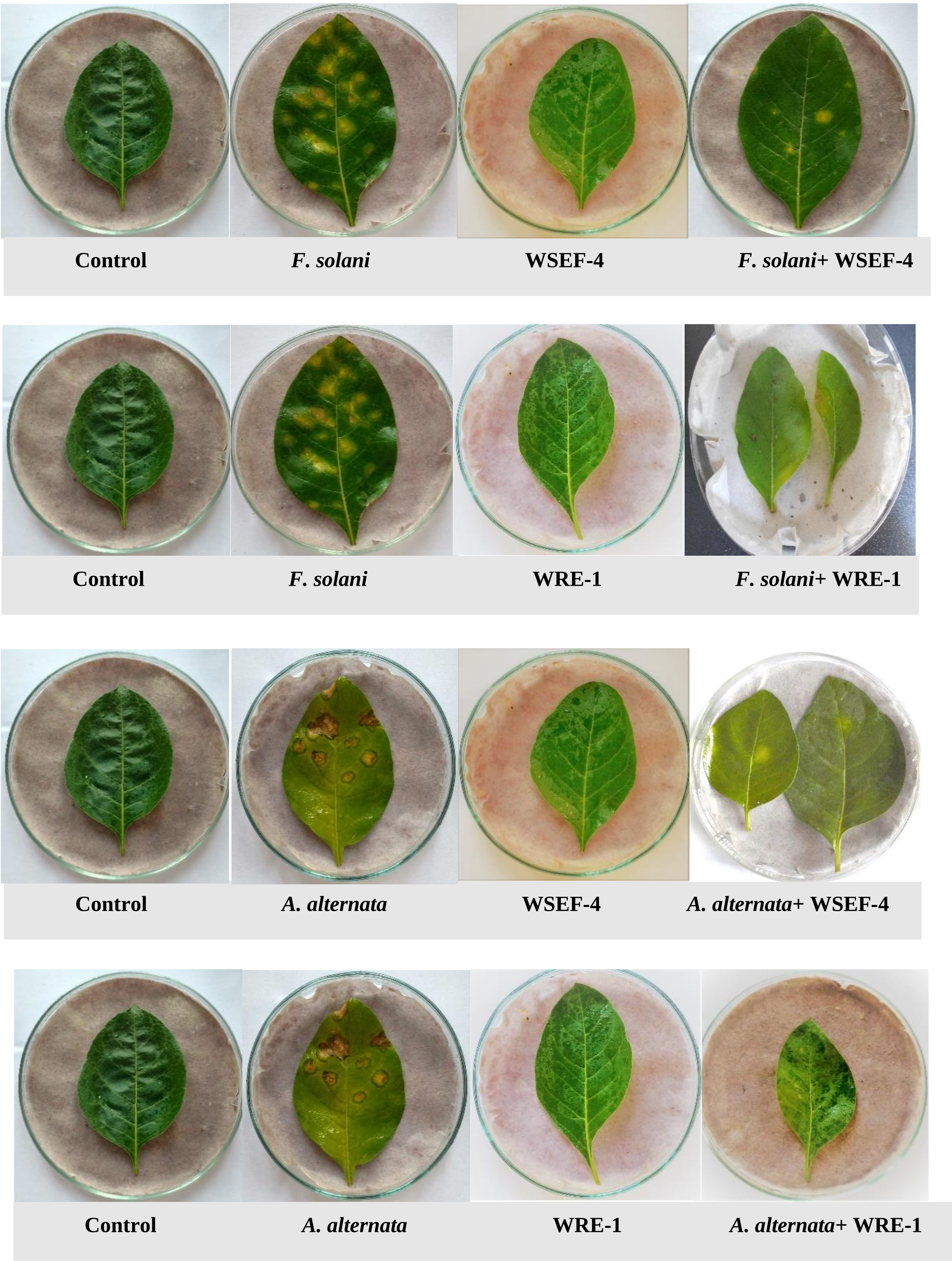
CFCS of WSEF-4 against *A. alternata*

Control (*F. solani*)

CFCS of WRE-1+*F. solani*

CFCS of WSEF-4 + *F. solani*

Figure 4.4) **Antagonistic activity of endophytes against fungal pathogens**



Control ***F. solani*** **WSEF-4** ***F. solani*+ WSEF-4**

Control ***F. solani*** **WRE-1** ***F. solani*+ WRE-1**

Control ***A. alternata*** **WSEF-4** ***A. alternata*+ WSEF-4**

Control ***A. alternata*** **WRE-1** ***A. alternata*+ WRE-1**

Figure 4.5) **Detached leaflet assay to determine biocontrol potential of isolate WRE-1 and WSEF-4 against pathogenic fungi *A. alternata* and *F. solani***

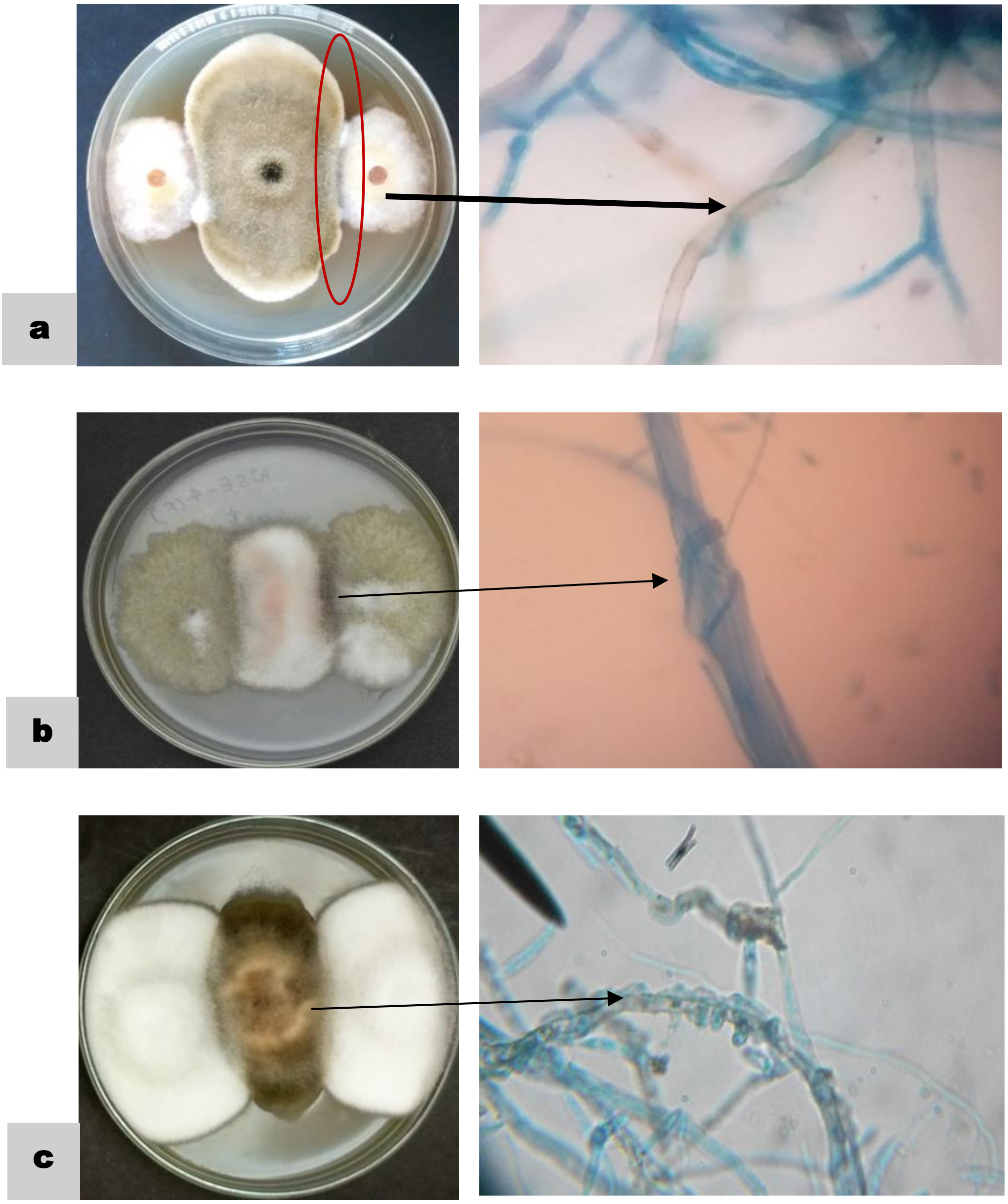


Figure 4.6) **Mycoparasitic interaction of endophyte with fungal pathogens (under light microscope)**

a) coiling of WSEF-4 hyphae around hyphae of *A. alternata*
b) coiling of hyphae of WSEF-4 around hyphae of *F. solani*
c) coiling of endophyte WREF-4 around hyphae of *A. alternata*

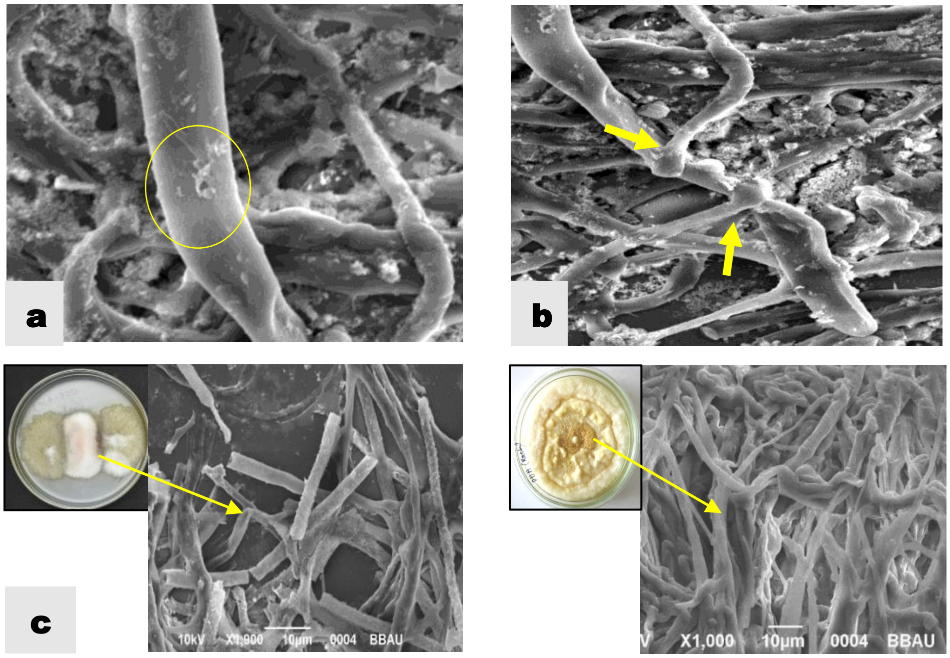
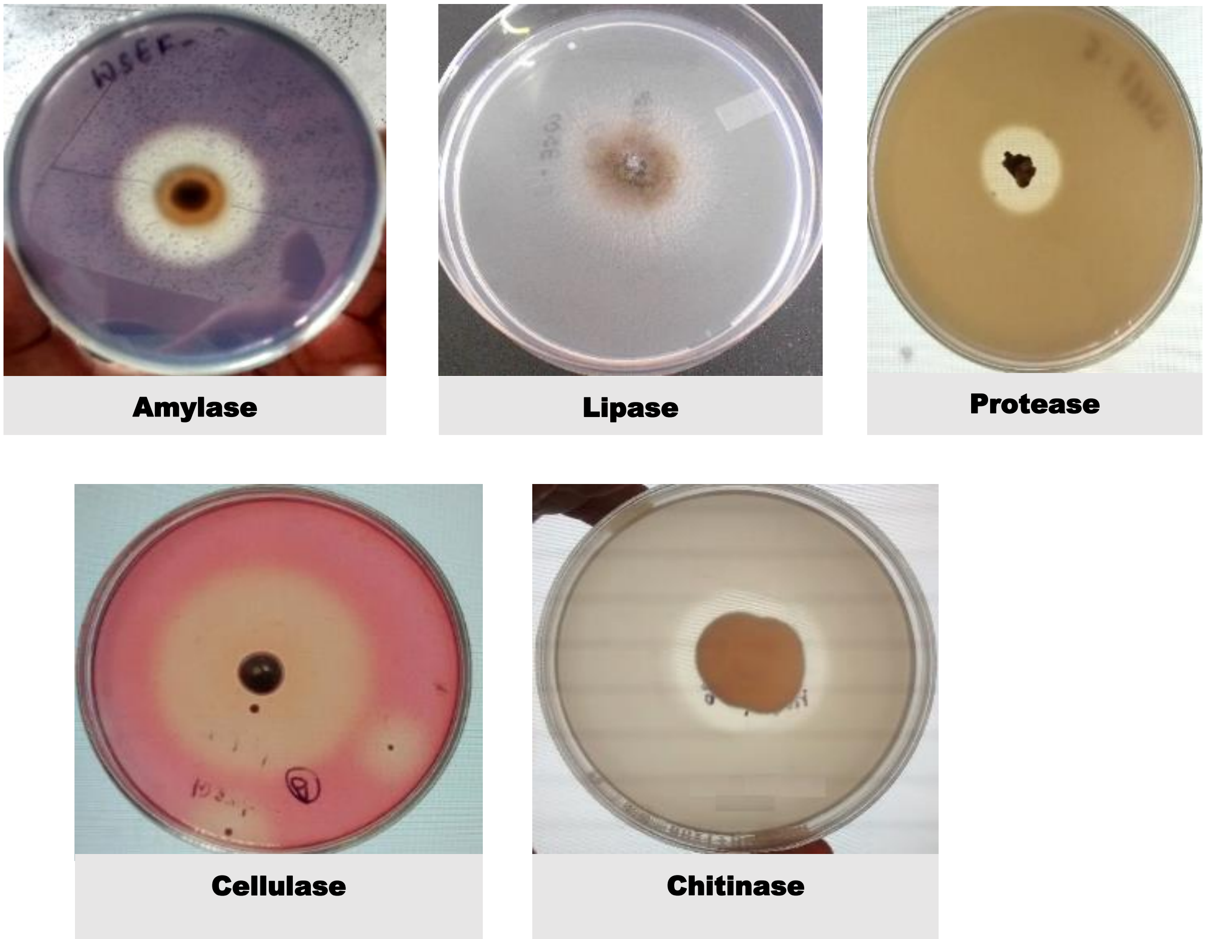


Figure 4.7) **SEM analysis of degenerated hyphae of pathogenic fungi at contact zone with antagonistic endophytes**

a) presence of pores on cell wall of *A. Alternaria* at contact zone with WSEF-4

b) coiling of WSEF-4 hyphae around *A. alternata* hyphae

c) disintegrated hyphae of *F. solani* at contact zone with WRE-1 in comparison to healthy mycelia of control plate



Amylase

Lipase

Protease

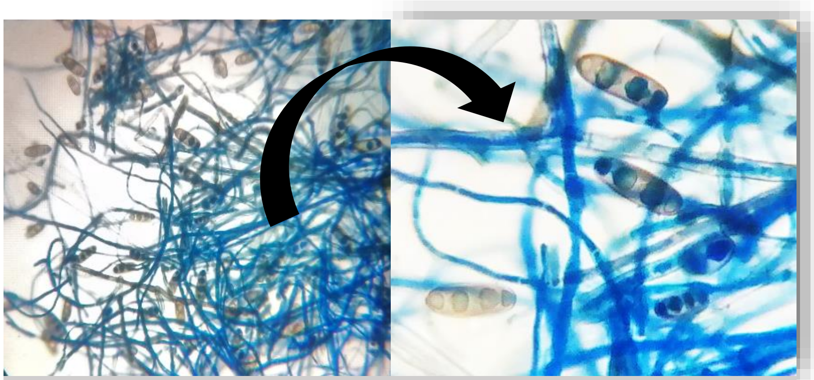
Cellulase

Chitinase

Figure 4.8) **Production of extracellular hydrolytic enzymes by isolate WSEF-4**

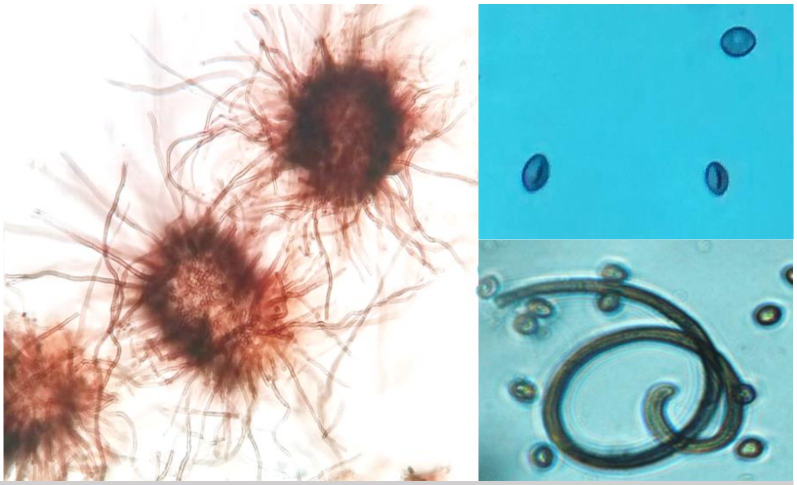
a) Amylase, b) Lipase, c) Protease, d) Cellulase, e) Chitinase

FIGURES

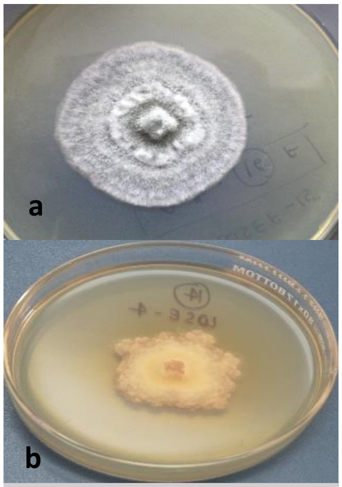


Bipolaris **mycelia with enlarged view of 4-celled spores**

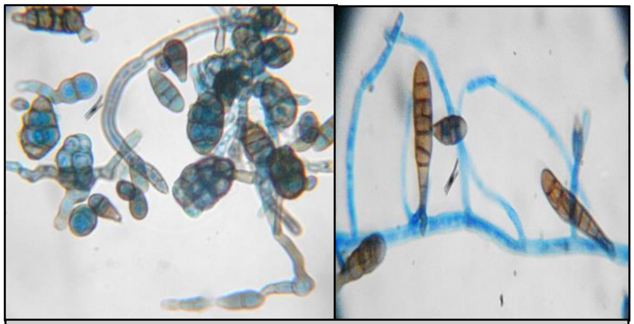
WLEF-1 (identified as *Bipolaris* sp.)



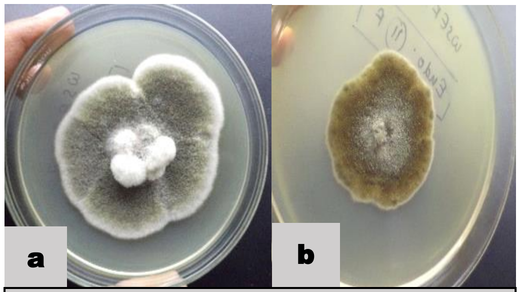
Perithecia of *Chaetomium* with enlarged view of ascospores and coiled hairs



WLEF-7 (a)& WSEF-4 (b) (identified as *Chaetomium* sp.)



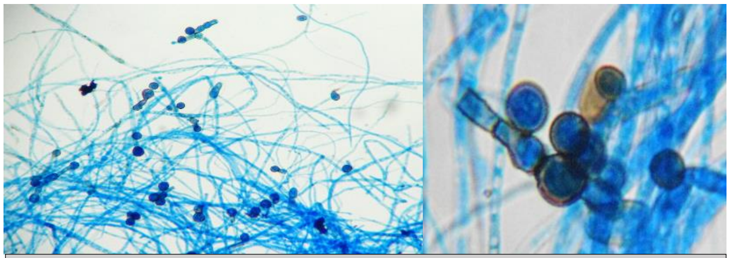
Alternaria **hyphae bearing septate conidia**



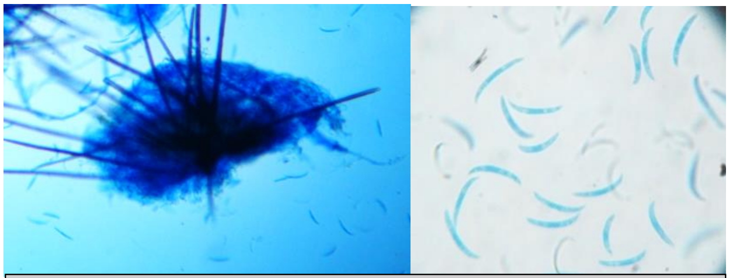
WSEF-1 (a) & WSEF-13 (b) identified as *Alternaria*

Continued……….

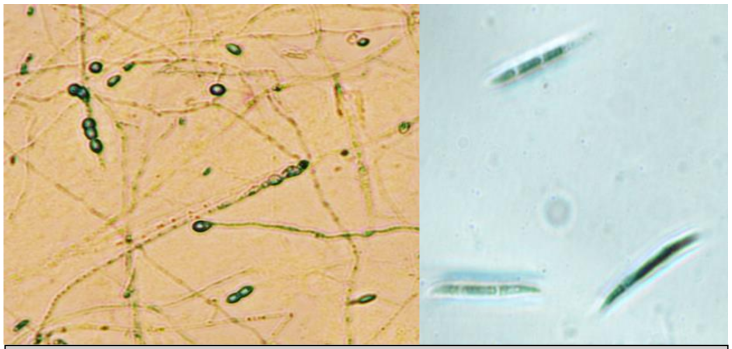
FIGURES


***Cladosporium* hyphae with enlarged view of spores**

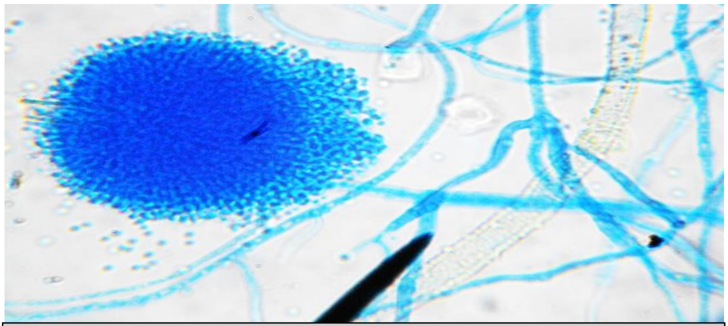

WSEF-5 (identified as *Cladosprium sp.)*

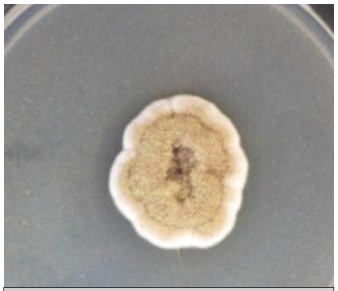

***Colletotrichum* acervuli with sickle shaped aseptate spores**


WSEF-3 identified as (*Colletotrichum* sp.)


***Fusarium* mycelia with chlamydospores and sickle shaped septate conidia**


WSEF-10 (identified as *Fusarium* sp.)


***Aspergillus* sporangiophore**


WREF-4 (identified as *Aspergillus* sp.)

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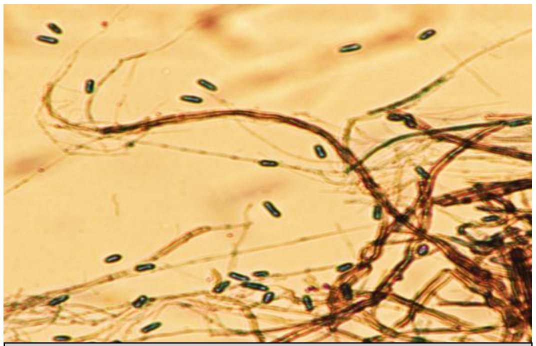
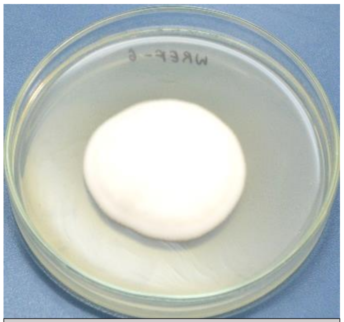
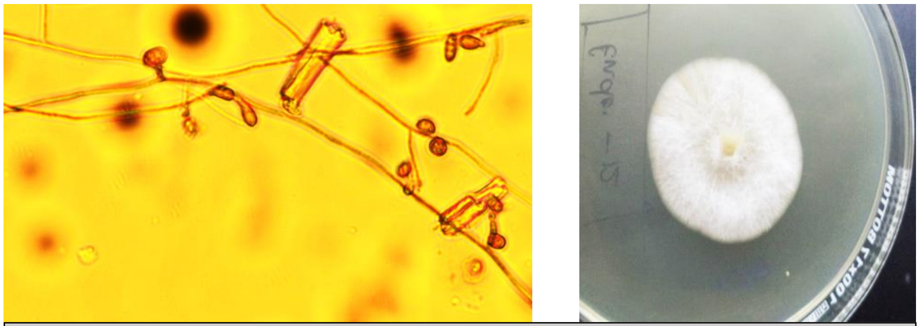


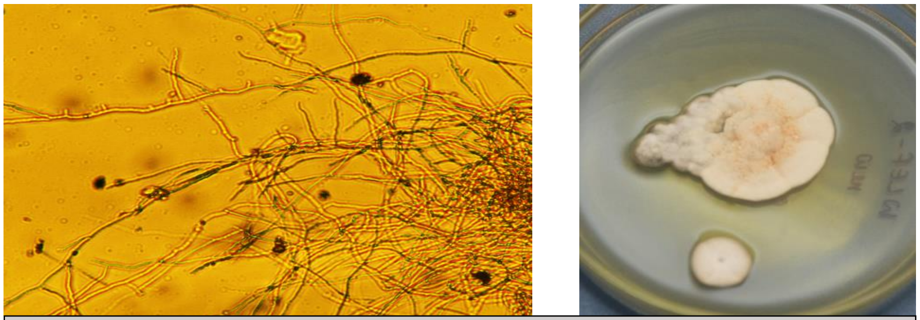
***Verticillium* mycelia with spores**



WSEF-6 (identified as *Verticillium* sp.)

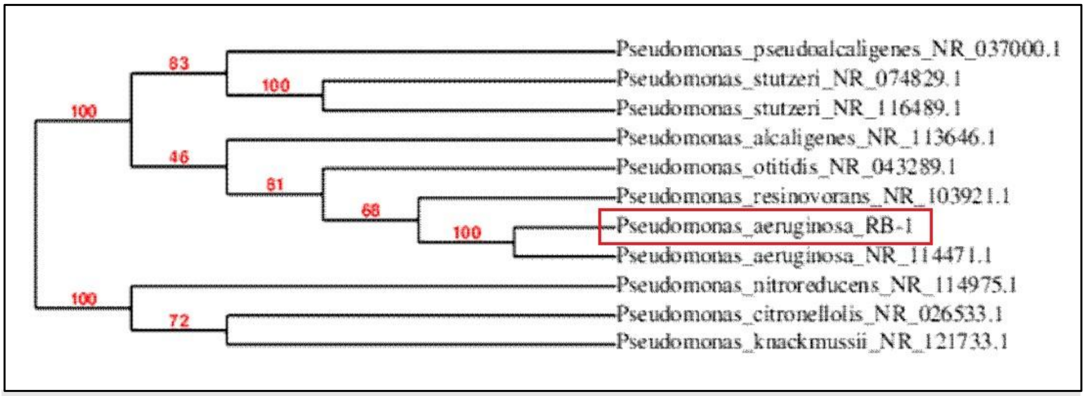


Mycelial structure and colony morphology of WSEF-9 (unidentified)

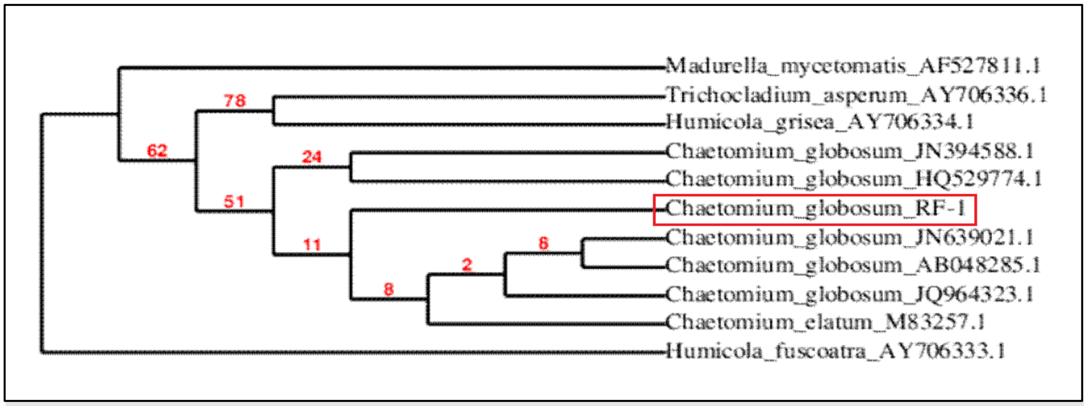


Mycelial structure and colony morphology of WLEF-2 (unidentified)

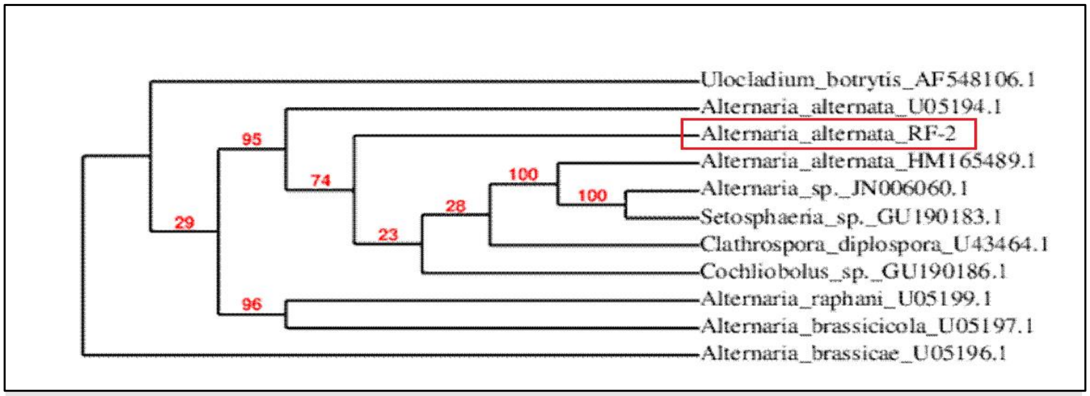
Figure 4.9) **Colony morphology (on PDA) and microscopic observation of endophytic fungi**



Phylogenetic analysis of endophyte isolate WRE-1 on basis of 16S rRNA gene sequence

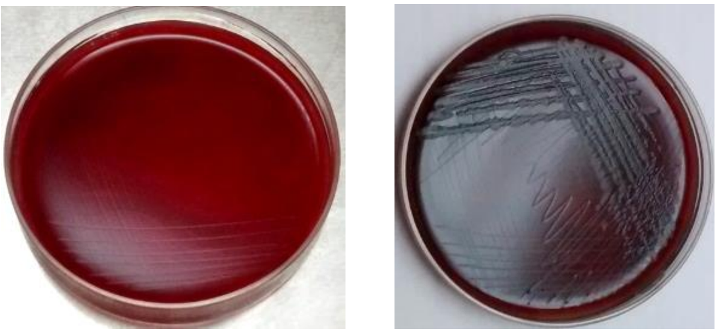


Phylogenetic analysis of endophyte isolate WSEF-4 on basis of 18S rRNA gene sequence



Phylogenetic analysis of isolated leaf pathogen of *W. somnifera* on basis of 18S rRNA gene sequence

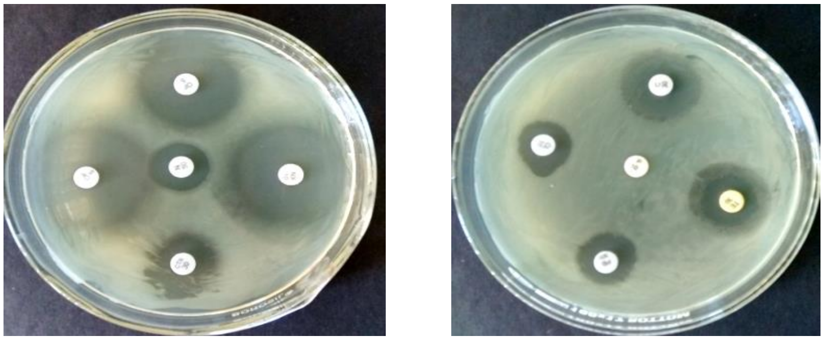
Figure 4.10) **Phylogenetic analysis of endophytic isolates and isolated leaf pathogen (selected for pot and field study)**



Blood agar (Control)

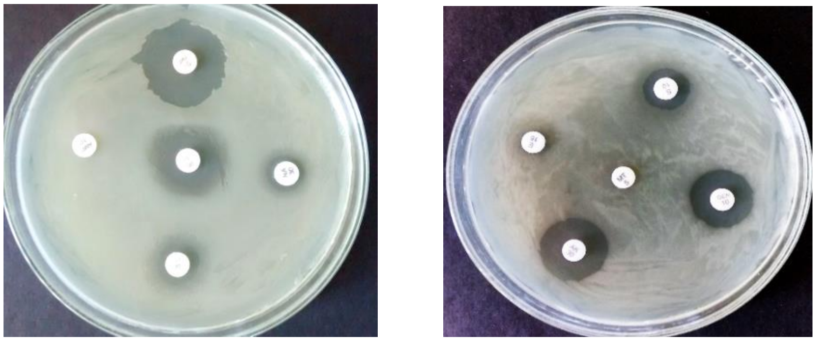
Blood agar inoculated with *P.*

Figure 4.11-a) **Non-hemolytic activity of isolate *P. aeruginosa* WRE-1 on blood agar**



Sensitivity for N^{30}, OF^{5},
CIP^{5}, CTX^{30}, NX^{10}

Sensitivity for TE^{30}, PB^{300},
C^{30}, CL^{10}, P$^{10\ (resistant)}$

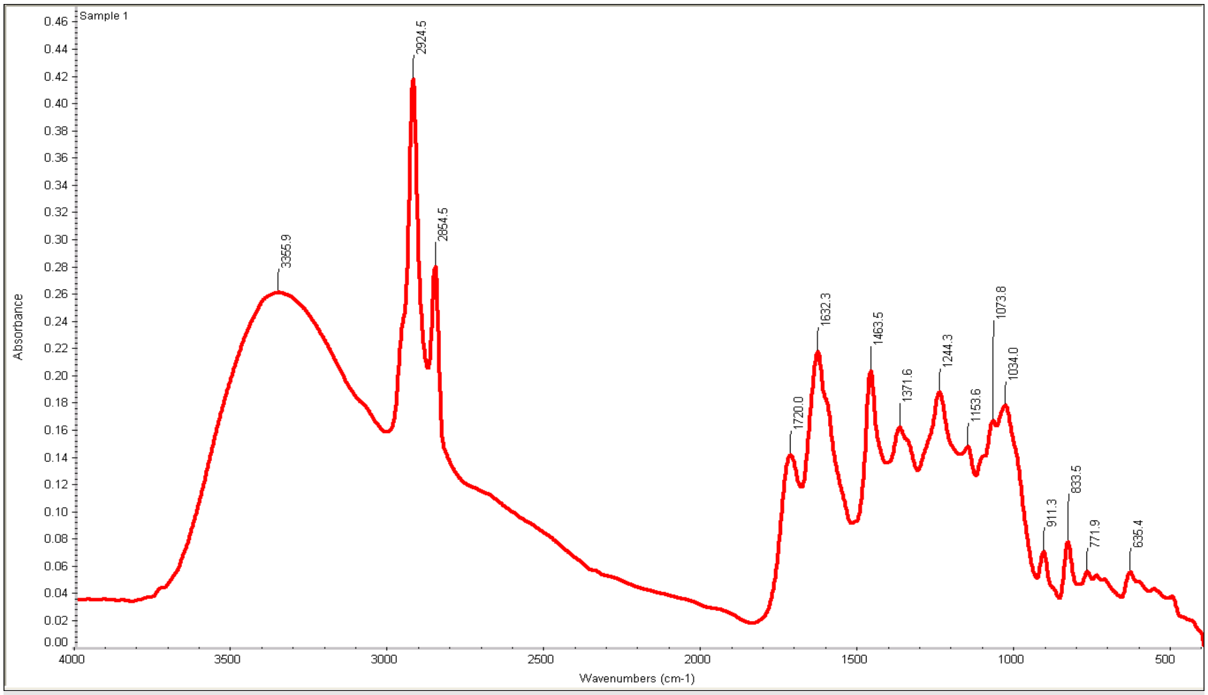


Sensitivity for O^{30}, PI^{100},
NA^{30}, AMP$^{10\ (resistant)}$, IPM^{10}

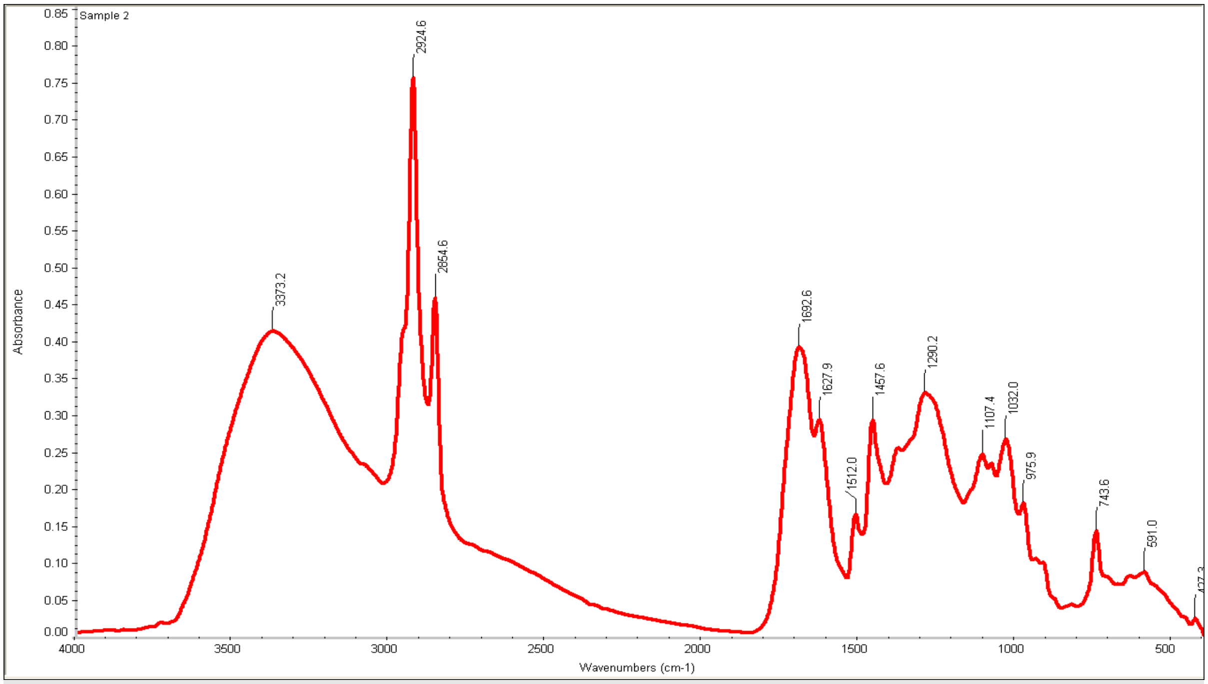
Sensitivity for GEN^{10}, MT$^{5\ (resistant)}$,
E$^{15\ (resistant),}$ AK^{30}, S$^{10\ (resistant)}$

Figure 4.11-b) **Susceptibility of strain *P. aeruginosa* WRE-1 towards different antibiotics**

Figure 4.11) **Pathogenicity test for isolate *P. aeruginosa* WRE-1**



Absorption spectra of secondary metabolite of strain WRE-1 (extracted with ethyl acetate)



Absorption spectra of secondary metabolite of strain WSEF-4 (extracted with ethyl acetate)

Figure 4.12) **FT-IR analysis of bioactive secondary metabolites of endophytes**

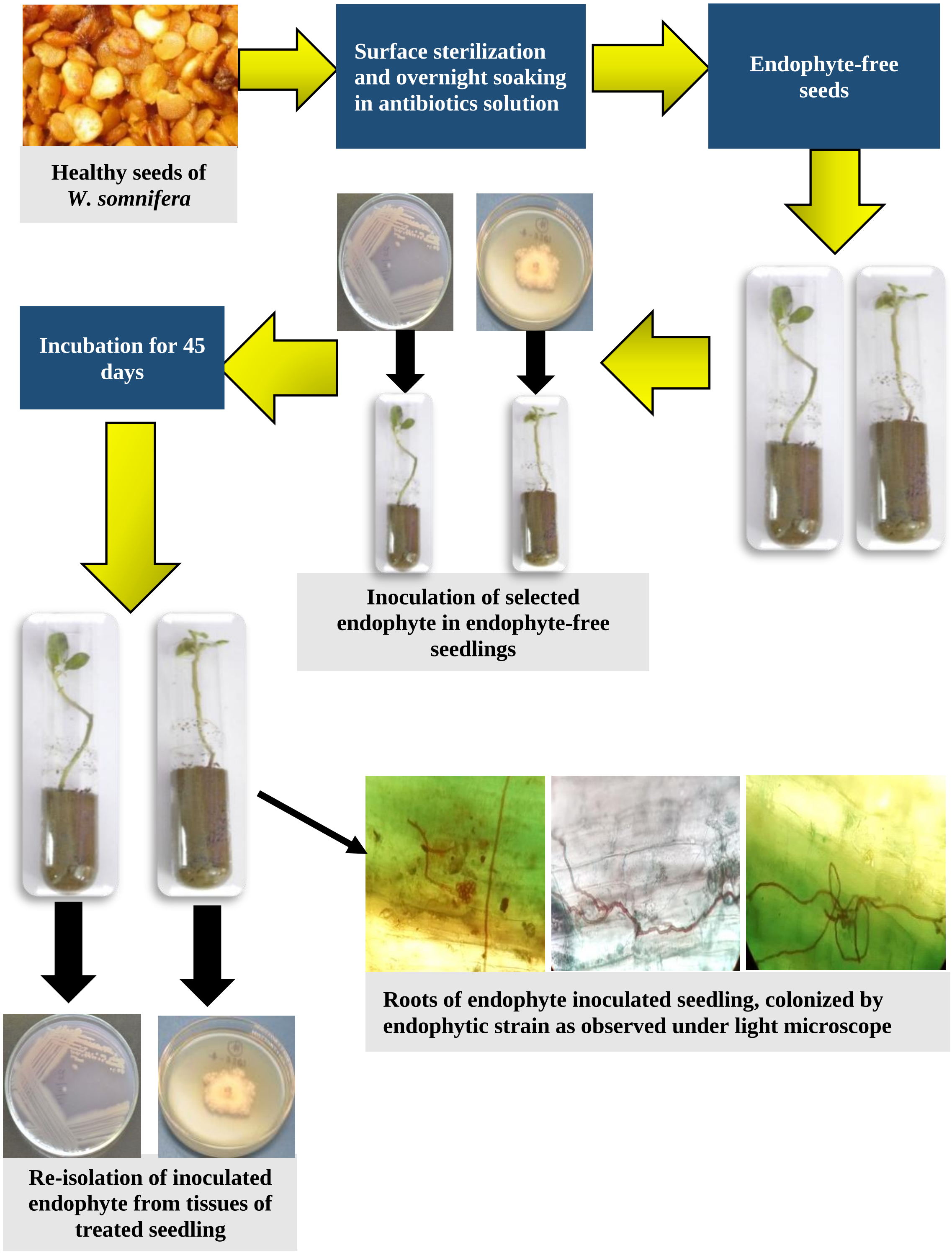


Figure 4.13) **Scheme of experiment done to determination of endophytic nature of isolates WRE-1 and WSEF-4**

Control | ***A. alternata*** | ***F. solani***

WRE-1 | **WRE-1+ *A. alternata*** | **WRE-1+ *F. solani***

WSEF-4 | **WSEF-4+ *A. alternata*** | **WSEF-4+ *F. solani***

Figure 4.14) **Effect of endophytes on seed germination (*in vitro* assay by paper towel method)**

Control **WSEF-4** ***F. solani*** **WSEF-4+ *F. solani***

Control ***A. alternata*** **WSEF-4** **WSEF-4+ *A. alternata***

Control **WRE-1** ***A. alternata*** **WRE-1+ *A. alternata***

Control **WRE-1** ***F. solani*** **WRE-1+*F. solani***

Figure 4.15) **Effect of endophytes on seedling development (pot experiment)**

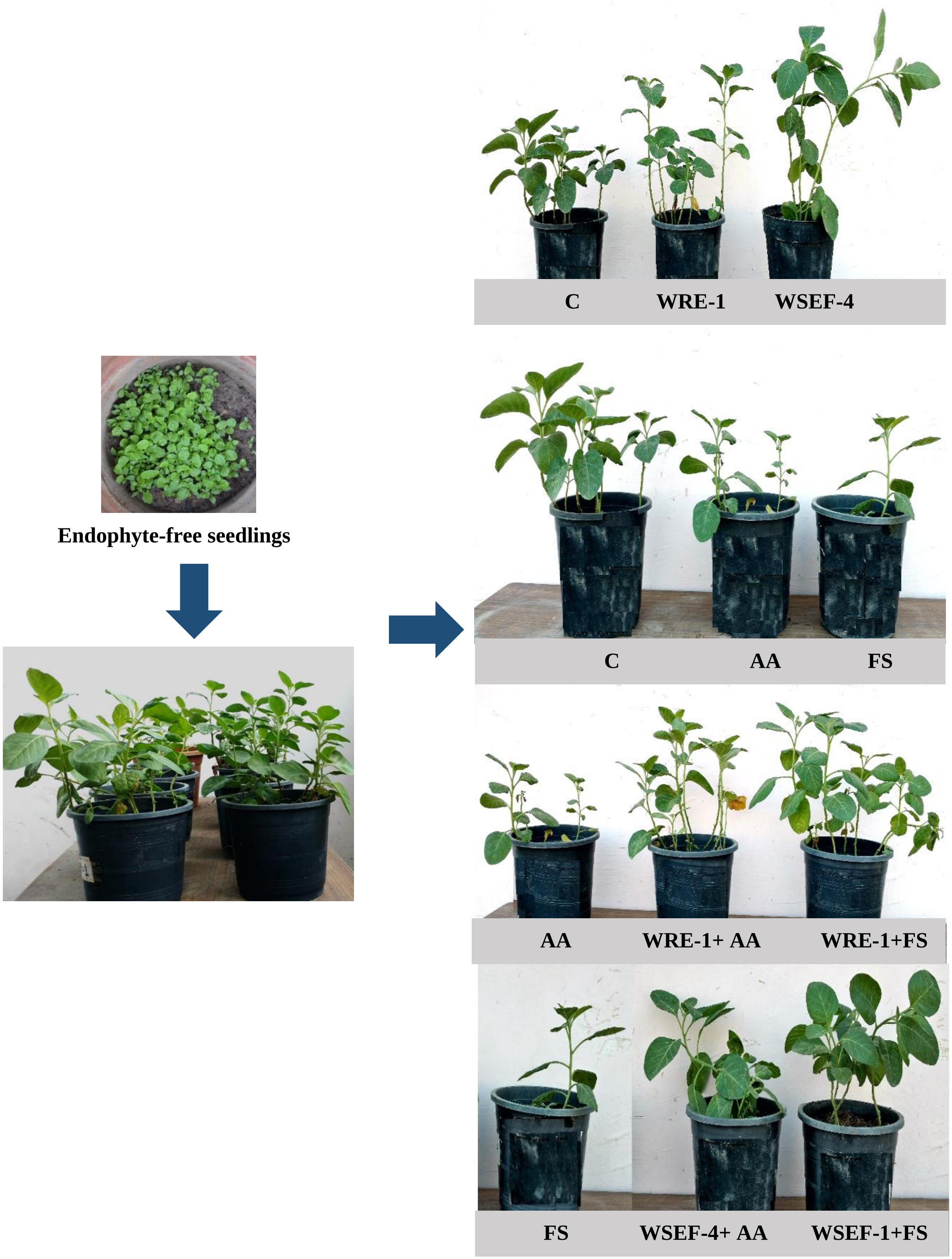


Figure 4.16) **Effect of isolates WRE-1 and WSEF-4 on growth of *W. somnifera* (Pot experiment)**

Field trial (60 days old)

Field trial (120 days old)

Figure 4.17) **Pictures of field experiment**

Leaf spot disease in *A. alternata* and *F. solani* treated plants during field experiment

Distorted roots in *F. solani* treated plants during field experiment

Figure 4.18) **Leaf spot symptoms observed in pathogen-inoculated plants during field experiment**

Control ***A. alternata*** ***F. solani***

G. intraradices **WRE-1** **WSEF-4**

WSEF-4 + *G. intraradices*

Figure 4.19) **Root development in *W. somnifera* plants under the influence of various treatments during field trials**

Control ***A. alternata*** ***F. solani***

G. intraradices **WSE-1** **WSEF-4**

WSE-1+ *G. intraradices* **WSEF-4+ *G. intraradices***

Figure 4.20) **Field trials to evaluate PGP and biocontrol potential of endophytes of *W. somnifera***

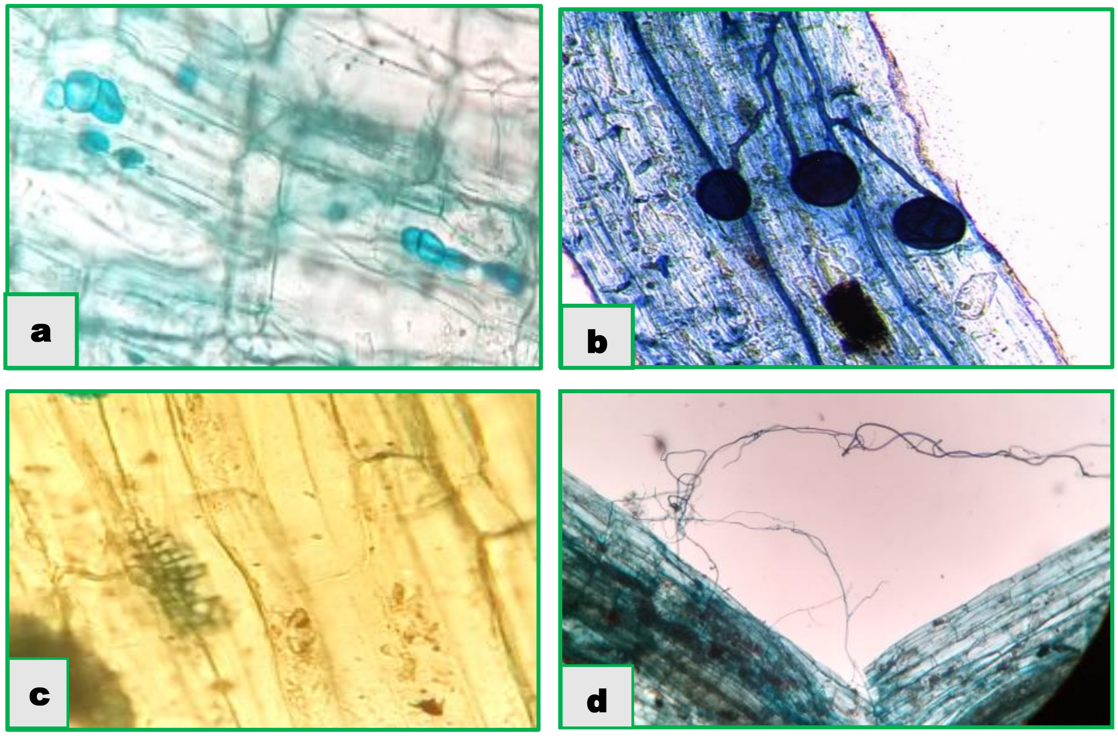


Figure 4.21) **Colonization of *W. somnifera* roots by mycorrhizal fungi *G. intraradices* showing a, b) vesicles, c) arbuscules, d) hyphal growth inside roots**

W. somnifera is an important cash crop due to its wide use in pharmaceutical industry and traditional health care practices. It is in high demand in domestic and international market (Datta et al., 2011). Cultivation of *W. somnifera* holds ample scope for farmers because of its demand in market at attractive price. Furthermore, the plant is well-adapted to grow in disturbed soils and help in soil reclamation hence unused waste or degraded lands can be used for its cultivation. The crop can also be cultivated in integration with traditional crops. Despite this *W. somnifera* is under-cultivated crop and most of the commercial demand is accomplished from wild sources through destructive harvesting and such collection practices cause definite threat to germplasm and diversity of the plant. Lack of simple and appropriate agro-techniques is the major reason behind the negligence and lack of interest of the farmers towards cultivation of ashwagandha. *W. somnifera* is highly susceptible to various fungal pathogens however the disease protection measures are strictly restricted towards the use of synthetic fungicides which can contaminate the plant and reduce its suitability for medicinal use. The plant is reported to be affected by various fungal pathogens causing leaf spot, leaf rust and blight, root rot and wilt diseases. These infections are transmitted through infected seeds or soil. Seed-borne and soil-borne infections often results into seed rotting and hamper seed germination (Khalid et al., 2001). Seedlings of the plant are destroyed by fungal pathogens which causes seedling blight and damping off disease (Datta et al., 2011). Such fungal infections in *W. somnifera* adversely affect the plant growth at early stages by reducing seed germination and seedlings vigor, by suppressing root development and destroying aerial plant parts at maturity. This often resulted in great loss of yield and pharmaceutically-important alkaloids (Shivanna et al., 2014). *W. somnifera* which propagated mainly through seeds, also shows poor germination capacity due to the presence of inhibitory substances in its fruits and germination is reported to be 40-60% (Shanmugaratnam et al., 2013;

Shravankumar and Datta, 2014). Foliar sprays and seed coating with chemical fungicides malathion, thiram/mancozeb, copperoxychloride, kelthane etc. are common disease management practices though commercially-unsuitable (Ashashri et al., 2015). Reducing the impact of fungal pathogens on yields and quality of *W. somnifera* remains an intractable problem because of lack of effective fungicides and their restrictive use.

During sampling for the isolation of pathogenic fungi from infected plants, it was found that *W. somnifera* is prone to attack by several fungal pathogens under field conditions which are responsible for serious crop damage and affect all plant parts. After repeated isolation of fungal pathogens from infected leaves, *in vitro* and *in vivo* pathogenicity assay and microscopic observation of pathogenic strains, it was found that *Alternaria* infection is quite severe and obtained from most of the infected tissues. Prior studies on fungal diseases of *W. somnifera* revealed that the crop is prone to destruction by leaf rust, leaf spot, stem and root rot, seed rot and seedling damping off. Leaf spot disease is most prevalent and caused by many fungal pathogens such as *Alternaria alternata* (Pati et al., 2008), *Pithomyces chartarum* (Verma et al., 2007), *Fusarium solani* (Chavan and Korekar, 2011) and *Alternaria dianthicola* (Maiti et al., 2012).

Fungal pathogens *A. alternata* and *F. solani* were selected for the study because of their prevalence in Indian climate and deleterious effect on ashwagandha crop. Both the pathogens are also responsible for seed-borne infections (Shivanna et al., 2013). *F. solani* also causes root rot and wilt disease (Gupta et al., 2004). Wilting leads to death and decay of underground parts of mature plant as well as infected seeds also showed symptoms of yellowing, drooping and decay at seedling stage, leading to 30-50% mortality (Gupta et al., 2004). *A. alternata* infection adversely affects plant growth by destroying leaf tissues and chloroplast which hampers photosynthesis in infected plants (Hussien et al., 2001). Studies also reported reduced content of reducing sugar, phelnolic content, chlorophyll, carotenoids and withanolides in infected plants (Pati et al., 2008; Shivanna et al., 2013).

The diversity of endophytic microorganisms, their intimate mutualistic association with their host plant and enormous potential in plant growth promotion and protection from invading phytopathogens have been examined extensively (Thakore, 2006; Gaiero et al., 2013; Khalifa et al., 2015). Studies on PGP and biocontrol ability of endophytic microorganisms provide evidences for their excellent potential as bioinoculants in crop cultivation, especially for medicinal plants where use of synthetic agro-chemicals is strictly restricted (Horrigan et al., 2002). However, use of endophytes as biofertlizer and biopesticide in agriculture, their field applicability and commercial production is in initial stages. In present study, bacterial and fungal endophytes were isolated from different plant parts of *W. somnifera* and screened for their plant growth promoting and biocontrol potential against two destructive fungal pathogens of *W. somnifera* i.e. *A. alternata* and *F. solani* under laboratory as well as field conditions.

In total, seventy-two morphologically distinct isolates were found as endophytes, associated with leaf, stem and root tissues of *W. somnifera*. Since most of the endophytic microorganisms are non-culturable and need culture-independent molecular techniques to detect and identify (Arnold, 2007), examined plant tissues may contain greater diversity of associated endophytes but isolation in this work, is culture-dependent and only those isolates which have shown clearly visible growth under *in vitro* conditions on semi-synthetic culture media were further studied. All morphologically distinct isolates were selected for the screening of their PGP and biocontrol potential. Isolates with effective antagonistic activity against test pathogens, were further explored for their biocontrol mechanisms. After screening, thirty isolates with positive PGP traits or antagonistic activity were further characterized.

Endophytes induce growth promotion in their host plant by three interrelated mechanisms which are phytostimulation, biofertilization and biocontrol of invading pathogens (Bloemberg and Lugtenberg, 2001). Phytostimulation include mechanisms for direct promotion of plant growth by production of phytohormones. Indole acetic acid (IAA) is a naturally occurring phytohormone, belonging to auxin group. IAA helps in promoting plant growth by positively affecting cell elongation and division, root initiation and apical dominance (Forchetti et al., 2007; Spaepen, 2015). IAA production by plant-associated mutualistic microorganisms is closely linked to plant growth promotion and resulted in increased root and shoot biomass as evidenced from several studies (Xie et al., 1996; Mayak et al., 1999; Patten and Glick, 2002; Ji et al., 2014; Ray et al., 2015). Studies suggests that IAA biosynthesis by endophytes induce morphological changes in plant roots by shortening of primary root and increasing number of lateral roots and root hairs (Spaepen, 2015). These alterations in root structure leading to an increased root surface allowing better uptake of nutrients. The changes in root architecture are also translated into higher biomass of the shoot (Compant et al., 2005; Rajkumar, 2009). Among endophytes of *W. somnifera*, IAA production was exhibited by 50% of the isolates. Two endophytic fungi WSEF-4 and WLEF-7 showed maximum IAA production and both were belonging to the genus *Chaetomium* as identified on the basis of microscopic observation. Strain WSEF-4 was further identified as *Chaetomium globosum* on the basis of 16S rRNA gene sequencing. Under pot and field trials, WSEF-4 showed positive impact on root development as WSEF-4 treated plant roots were highly branched with well-developed lateral root system.

Biofertilization includes all the mechanisms of plant growth promotion by which accessibility or supply of major nutrients increases (Bashan, 1998). A well-studied form of biofertilization is nitrogen fixation through which plant-associated microbes convert atmospheric nitrogen to ammonia (Bloemberg and Lugtenberg, 2001; Gupta et al., 2012). Phosphorus solubilization is another important mechanism of biofertilization in which microorganisms release low molecular weight acids (to chelate metal cations attached to phosphorus) or secrete extracellular phosphatase enzymes to solubilize complex form of phosphate and enhance its accessibility to plants (Kpomblekou and Tabatabai, 2003). In present study, endophytic isolates were found potent phosphate solubilizers and most fungal isolates solubilize insoluble phosphate source under *in vitro* assay. We focused our study on alkaline phosphatase production as a phosphate solubilization mechanism. During our examination of extracellular alkaline phosphatase, fungal isolate WSEF-5 was found most promising which releases substantial amount of released *p*NP. Bacterial isolates WRE-1 and WSE-6, identified as *Pseudomonas* sp., were also effective. Typical phosphate solubilization values among phosphate solubilizing microbes ranges from 10 to 800 mg L-1 (Rodriguez and Fraga, 1999; Stephen and Jisha, 2011, Hussain et al., 2013; Oteino et al., 2015). Released *p*NP in case of three most effective alkaline phosphatase producing isolates WRE-1, WSE-6 and WSEF-5 was >500 mg L^{-1}, 530 mg L^{-1} and 630 mg L^{-1} respectively.

Phosphate solubilization among endophytes and their role in enhanced plant growth under field condition has been widely studied. Forchetti et al. (2007) isolated, characterized, and quantified the phosphate solubilization abilities of endophytes *Achromobacter xiloxidans* and *Bacillus pumilus* in sunflower. Yazdani and Bahmanyar (2009) found that the use of endophytic bacteria as biofertilizer in corn production reduced the need for phosphorus application by 50% without significant loss in grain yield. Oteino et al. (2015) studies on phosphate solubilizing endophyte *Pseudomonas fluorescens* revealed its significant role in plant growth promotion of *Pisum sativum*. In present study, efficient phosphate solubilizer bacteria WRE-1 (identified as *Pseudomonas aeruginosa*) was studied under pot and field conditions and significantly increase plant biomass. P solubilization by *P. aeruginosa* and other fluorescent pseudomonads and their role in plant growth promotion has been widely studied (Cattelan et al., 1999; Pandey et al., 2006; Tewari and Arora, 2014).

Production of siderophores (iron-chelating compounds) is another mechanism which play an important role in plant growth promotion as well as in suppressing the growth of phytopathogens (Verma et al., 2011). Iron is a crucial element for plant development as it is a cofactor of many metabolic pathways. Iron deficiency in soil may lead to disruption of respiration and photosynthesis (Guerinot 2010; Zuo and Zhang 2011). Plants utilizes iron from soil by acidification of the rhizosphere or by secreting phytosiderophores to chelate iron which is then transported into root (Altomare and Tringovska, 2011). However, these strategies are often not efficient for sufficient supply of iron especially in calcareous and alkaline soils. Microbial siderophores reported to be used by plants during iron-limiting conditions (Crowley et al., 1992; Johnson et al., 2002; Fernandez et al., 2005). These compounds also exert beneficial effects by solubilizing phosphates and other micronutrients in soil (Ramos Solano et al., 2008). Present study revealed that fungal endophyte WSEF-3 (*Colletotrichum* sp.), WSEF-10 (*Fusarium* sp.) and bacterial endophyte WRE-1 (*Pseudomonas aeruginosa*) were potent siderophore producing strains. WRE-1 showed promising growth promoting and biocontrol capability under field conditions which might be attributed due to its siderophore production potential. Siderophore production may also be highly important for endophytes as they have to outcompete the invading pathogens as well as must compete with plant cells for iron supply (Idris et al., 2004; Lacava et al., 2008).

Endophytes also contributed towards plant growth by solubilizing zinc in soil and facilitating its uptake by their host plants. Zn is an essential micronutrient and functions as enzyme cofactor which play key role in the growth and productivity of the plants and its deficiency can hinder the metabolic and physiological activity in plants. Since many Indian soils are Zn deficient with the content much below the critical level of 1.5 ppm and soil conditions such as high pH, low organic matter content, high usage of P fertilizer often resulted in unavailability of zinc, even after applying synthetic fertilizer to reduce Zn deficiency. Therefore, Zn-solubilizing bioinoculants provide an alternative approach to overcome Zn deficiency (Goteti et al., 2013; Kumari et al., 2014). Zn solubilizing endophytes help to solubilize unavailable form of Zn and increases nutrient availability to their host plants. Zn solubilization was exhibited by 50% of the endophytes isolated from *W. somnifera* which suggest their possible roles in biofertilization.

An important mechanism by which endophytes enhance the survivability of their host plant is through suppressing the growth of phytopathogens. Selection of effective antagonistic microorganism is foremost step in development of biopesticides. Isolates of *W. somnifera* were screened for their antagonistic potential against fungal pathogens *A. alternata* and *F. solani*. Dual culture plate bioassay revealed the association of *W. somnifera* with many antagonistic bacteria and fungi as endophytes. Strain WRE-1 (*Pseudomonas aeruginosa*) and WSEF-4 (*Chaetomium globosum*) were most potent antagonists against both the pathogens. Biocontrol potential of endophytic *C. globosum* has already been widely investigated and well documented (Fatima et al., 2016). Commercial formulation of this fungi has also been developed (Soytong et al., 2001). Biocontrol potential of *C. globosum* has been reported against *Pythium ultimum*, *Phytophthora* *infestans* (Zhang et al., 2012), *Candida albicans, Cryptococcus neoformans* (Guo et al., 2008), *Rhizoctonia solani* and *Alternaria alternata* (Naik et al., 2009), *Fusarium oxysporum* (Kumar et al., 2011) and *F. sulphureum* (Li et al., 2011). Biocontrol mechanisms explored for the fungi *C. globosum* involve cell wall degradation of *P. ultimum* caused by β-glucanases and cellulases (Inglis and Kawchuk, 2002). Several antifungal compounds have also been reported including chaetomin, gliotoxin, cheatoglobosin, chaetocyclinones, chaetoviridins etc. (Fatima et al., 2016).

Isolate WSEF-4 exhibited mycoparasitism as an important biocontrol mechanism as coiling of *C. globosum* hyphae around hyphae of both the fungal pathogens was confirmed from mycelial observation under light microscope and SEM analysis. Possible mechanisms behind this mode of parasitism might be the secretion of lytic enzymes (protease, lipase, β-1,3-glucanase, chitinase) and antifungal compounds. Chitinase production is widely demonstrated as a biocontrol mechanism to inhibit fungal pathogens (Inbar and Chet 1991; Gupta et al., 1995; Quecine et al., 2008). Chitinase production by *C. globosum* and its inhibitory effect on phytopathogenic fungi *Rhizoctonia solani*, *Fusarium oxysporum*, *Sclerotinia sclerotiorum*, *Valsa sordida*, and *Phytophthora sojae*, has been reported by Liu et al. (2008). Endophytes possess greater ability to penetrate and colonize their host plant and it has been demonstrated that extracellular cellulases, produced by endophytes play a key role in their entry by hydrolyzing plant cell-wall (Hallmann et al., 1997). Cellulase production by endophytes not only provides them competitive advantage to colonize their host but colonization by endophytes also induces systemic resistance in their hosts. Induced systemic resistance is endophyte-mediated activation of the host plant’s physical or chemical defenses which resist further attack by invading phytopathogens (Quadt-Hallmann et al., 1997). Among plant-associated growth promoting microorganisms, endophytes are considered as most suitable candidates for eliciting ISR as endophytes which are originally isolated from plant tissues exhibit a stronger interaction with plants than epiphytes (Yi et al., 2013). No HCN activity was shown by endophyte isolates. Although HCN production is an important biocontrol mechanism for the suppression of root pathogens (Drfago et al., 1990), negative HCN activity among endophytes might be beneficial for plants since endophytes colonize the internal plant tissues and HCN production could have deleterious effect on plants as well. Bakker et al. (1989) observed the inhibitory effect of cyanide producing *Pseudomonas* spp. on plant growth in potato. Similarly, growth inhibition in lettuce and bean (Alstrrm and Burns, 1989) and in several other crops by cyanide producing rhizospheric pseudomonads has been claimed by many researchers (Schippers et al., 1987).

After screening for their PGP and biocontrol potential, endophyte isolates were partially characterized on the basis of biochemical, morphological and physiological characteristics. In our study, we found that *W. somnifera* tissues are colonized by variety of microorganisms belonging to the genus *Bacillus, Pseudomonas, Serratia, Micrococcus, Aspergillus, Fusarium, Alternaria, Colletotrichum, Bipolaris and Chaetomium.* Diversity of endophytes of *W. somnifera* have already been investigated by many researchers. Qadri et al. (2013) explored the diversity of fungal endophytes and reported the presence of *Gibberella moniliformis, Cochliobolus lunatus, Fusarium sp., Fusarium equiseti, Gibberella moniliformis, Hypoxylon fragiforme, Nigrospora sphaerica, Cercophora caudate* and *Cladosporium cladosporioides* as endophytes. Similarly Khan et al. (2010) isolated *Chaetomium bostrycodes, Eurotium rubrum, Melanospora fusispora, Aspergillus awamori, Aspergillus auricomus, Aspergillus flavus, Aspergillus niger, Aspergillus pulvinus, Aspergillus terreus, Aspergillus terreus var. aureus, Aspergillus terricola, Aspergillus thomii, Alternaria alternata, Cladosporium cladosporioides, Curvularia oryzae, Drechslera australiensis, Fusarium moniliforme, Fusarium semitectum, Myrothecium roridum, Penicillium* *corylophilum, Penicillium, Phoma sp.* from different plant tissues of *W. somnifera.* Bacterial endophytes *Alcaligenes faecalis* (Abdallah et al., 2016), *Serratia marcescence* and *Bacillus subtilis* (Sanjaykumar Ingle, 2011) were also reported as endophytes, associated with *W. somnifera*.

On the basis of PGP and biocontrol potential of isolates, two best performing strains were further identified on the basis of 16S rRNA gene sequencing. Strain WRE-1 which was identified as *Pseudomonas aeruginosa* is generally considered as opportunistic human pathogen thus its non-pathogenic nature was confirmed by negative hemolytic activity on blood agar media, negative protease, elastase activity, negative activity for pyocyanin production. Optimum growth temperature ranged between 20-28°C and growth reduced above 30°C. Susceptibility of isolate WRE-1 for twenty different antibiotics belonging to different groups, was also investigated by antibiotic-sensitivity assay and was found susceptible to most of the antibiotics. Colonization of rhizosphere and internal plant tissues by *P. aeruginosa* and its plant growth enhancing potential is well documented. *P. aeruginosa* support plant growth by inducing systemic resistance of host plant (Meyer and Hofte, 1997; 1999), by IAA, siderophore, HCN, ammonia, exo-polysaccharide production, phosphate solubilization (Ahmad and Khan, 2012, 2011; Naik and Dubey, 2011; Braud et al., 2009; Rajkumar and Freitas, 2008), by inhibiting the growth of phytopathogens (Bano and Musarrat, 2003), by improving seed germination percentage (Adesemoye et al., 2008) and overall increase in yield and growth of their host (Ahemad and Kibret, 2014; Adesemoye and Ugoji, 2009). Although their opportunistic pathogenic nature to humans is major barrier in their use as biofertilizers and biopesticides but common occurrence of *P. aeruginosa* in agricultural soils, their existence in the form of rhizobacteria and endophyte and successful study of non-pathogenic strains for growth enhancing and biocontrol efficiency under field trials support their use in sustainable agriculture. *P. aeruginosa* is highly successful in adapting to diverse environments. Functional analysis of *P. aeruginosa* genome shows that the bacterium possesses highest proportion of regulatory genes observed for a bacterial species to date (Stover et al., 2000). As also confirmed from our study, isolate *P. aeruginosa* WRE-1 successfully improved plant growth parameters and effectively suppress the growth of fungal pathogens. Strain WSEF-4 was the best performing strain which showed maximum growth promoting potential. Fresh weight of WRE-1 and WSEF-4 inoculated plants showed 74.1% and 87.6% increase in comparison to control. Both the strains when co-inoculated with fungal pathogens *A. alternata* and *F. solani* completely suppressed the disease as no disease symptoms were observed during pot experiment and in field trials.

Both the isolates also significantly inhibited seed-borne infections as confirmed from seed-germination assay. The ability of seeds to germinate in favorable conditions is critical for plant adaptability, survival, growth, and reproduction (Baskin and Baskin 2004). Seed germination enhancing capacity of isolates WRE-1 and WSEF-4 can be very useful trait as *W. somnifera* seeds naturally possess poor germination rate due to the presence of certain inhibitory substances in fruits. Moreover, effective control of both the destructive seed-borne fungal pathogens by selected endophytes might be helpful in disease management of *W. somnifera* by controlling infections at initial stages.

Mycorrhizal fungi are common inhabitants of medicinal plants and their association has been widely investigated in different soil ecosystems (Taber and Trappe 1982; Waheed 1982; Iqbal and Nasim, 1986; Wei and Wang, 1989; Udea et al., 1992; Haq and Hussain, 1995; Gorsi 2002). This close association plays many significant roles in increasing plant growth and productivity of medicinal plants (Smith and Read 2008). Diversity of arbuscular mycorrhizal fungi in the rhizosphere also have profound effect on medicinal value of the plant (Wubet et al., 2003; Karthikeyan et al., 2009; Kumar et al., 2010; Zeng et al., 2013).

Compatibility analysis of selected antagonistic isolates WRE-1 and WSEF-4 with mycorrhizal fungi *G. intraradices W. somnifera* revealed that *Pseudomonas aeruginosa* WRE-1 might have negative impact on root colonization by mycorrhizal fungi *G. intraradices*. Co-inoculation of WRE-1 with *G. intraradices* highly suppressed its colonization. On the other hand, WSEF-4 was found sufficiently compatible with *G. intraradices* and their co-inoculation exhibited most promising results. WSEF-4 in combination with *G. intraradices* showed profound effect on plant growth as shoot and root length, fresh and dry weight of plants was significantly higher in comparison to control. Phytochemicals e.g. total phenolics, total withanolides, chlorophyll content was also improved.

Plants, colonized by mycorrhizal fungi have higher nutrient uptake capacity than non-mycorrhizal plants (Nisha and Rajeshkumar, 2010). Studies on mycorrhizal inoculation with medicinal plants *Abelmoschus moschatus, Clitoria ternatea, Plumbago zeylanica, Psoralea corylifolia* and *Withania somnifera* suggests that mycorrhizal inoculation increased the dry matter of medicinal plants in different types of soil (Chandra et al., 2010). Similar studies for analysis of impact of mycorrhizal on shoot height and root biomass of *Poncirus trifoliata, Piper longum, Salvia officinalis and Plectranthus amboinicus* medicinal plants also suggests that plant growth is promoted by mycorrhizal colonization (Wang et al., 2006; Rajeshkumar et al., 2008; Geneva et al., 2010; Gogoi and Singh 2011).

AM fungi also play important role in protection from phytopathogens as revealed from several reports (Wehner et al., 2009). In our study endophytes WREF-4 and *G. intraradices* when co-inoculated with *A. alternata* and *F. solani* showed significantly improved shoot and root length, fresh and dry weight of plant and content of phytochemicals. However, a slight reduction in percent root colonization by *G. intraradices* was also observed in pathogen inoculated plants.

Singh et al. (2013) investigated biocontrol potential of AM fungi *Glomus fasciculatum* in combination with rhizospheric antagonistic bacteria *Pseudomonas monteilii* against root pathogens *Fusarium chlamydosporum* and *Ralstonia solanacearum* (causes rot and wilt) of medicinal plant *Coleus forskohlii.* Obtained results revealed that co-inoculation of *P. monteilii* with *G. fasciculatum* significantly improved the AM root colonization percent and spore numbers. The alkaloid forskolin content of *C. forskohlii* was also significantly increased by co-inoculation of *P. monteilii* with *G. fasciculatum.* Improvement of secondary metabolite content in response to mycorrhizal colonization in medicinal plants has been observed earlier which might be due to due to enhanced nutrient availability or altered phytohormone level in plant tissues (Toussaint, 2007; Koeberl et al., 2013). Several studies have reported positive impact of AM fungi on alkaloid content and other phytochemicals of medicinal plants and reported increased content of essential oil and its composition in medicinal plant *Ocimum basilicum* (Copetta et al., 2006), high content of alkaloid andrographolide in the leaf extracts of *Andrographis paniculata* (Radhika and Rodrigues, 2011), higher phenolics content in leaves and flower heads of *Cynara cardunculus* in *G. intraradices*-inoculated plants (Ceccarelli et al., 2010), flavonoids in *Trifolium repens* (Ponce et al., 2004) and total coumarin and imperatorin in *Angelica dahurica* (Zhao and He, 2011).

The isolate WSEF-4 individually and in combination with *G. intraradices* significantly improved soil properties by lowering the pH from alkaline (pH 8.3 of and EC 343µs before plantation), indicating their role in sodic soil amelioration. *W. somnifera* itself is well-known for its soil-reclamation which might be attributed to its root-exudates. Soil pH, EC, total organic C, available N, P and K in rhizospheric soil of control treatment was found improved in comparison to uncultivated soil. Soil quality was further improved in rhizosphere of endophyte-inoculated plants. Increased microbial activity and their extracellular enzymes play a key role in improvement of soil texture and nutrient level which support prolific root growth in plants (Gill et al., 2009). Increased microbial activity also improve nutrients in soil by secretion of mucilage/polysaccharides and other exudates in the rhizosphere which may bind soil particles into aggregates (Shukla et al., 2008). Improved physico-chemical parameters of soil from the rhizosphere of endophyte-inoculated plants of strain WSEF-4 and *G. intraradices* may also contribute to plant growth enhancement through the possible production and release of secondary metabolites (plant growth regulator, phytohormones and beneficial mycelial exudates). Microbial enzymes also facilitate the availability of soil nutrients in the plant root-zone soil (Srinivasan et al., 2012). Root elongation induced by phytohormone IAA production by microorganisms also help in improving soil texture. Mineralization of organic P by alkaline phosphatases activity by microbes might have solubilizing effects on P substrates in soil (Yadav et al., 2009). Endophytic bacteria in root zone are capable of increasing the availability of soil P to plant growth (Duangpaenga et al., 2013). Therefore, the endophyte bioinoculants may be recommended as an efficient soil amendment for reclaiming sodic soil and supporting cultivation with better growth and yield of crops.

In present study, role of endophytes in plant growth, nutrient availability, disease resistance, yield and quality of medicinal plant *W. somnifera* has been demonstrated and concluded that isolate *Chaetomium globosum* WSEF-4 could be a useful biological tool for growth promotion and disease management of the plant. The results obtained from this study support the notion that endophytes hold a great potential as biofertilizer and biopesticide in the cultivation of medicinal plants. Use of *C. globosum* WSEF-4 (which effectively enhance seed germination and also control seed-borne infections in *W. somnifera*) in seed coating can not only contribute to enhance vegetative propagation of *W. somnifera* but also can be helpful in the management of pre- and post-emergent seedling mortality. Use of endophyte *C. globosum* WSEF-4 as bioinoculants provides a promising alternative to synthetic fertilizers and pesticides for the cultivation of medicinal plants in an eco-friendly manner.

With increasing human population, demand for medicinal plants to fulfill their health care needs is also increasing. Substantial growth in herb and herbal product market across the world has opened-up the doors for new income-generating opportunities for rural populations through the cultivation of medicinal and aromatic plants. However, cultivation of medicinal plants is generally neglected by farmers in India due to restricted use of synthetic agrochemicals, lack of alternative cultivation techniques and less popular organic farming practices. Thus, it is the need of the hour to develop some effective cultivation strategies, not only to conserve our forests from destructive harvesting of medicinal plants but also to attract farmers towards medicinal plant cultivation. Biotic and abiotic stress factors largely affect the quality and quantity of pharmaceutically important compounds of medicinal plants. The prime focus of agronomists is to develop sustainable cultivation techniques and in search of safe alternative of synthetic agrochemicals, endophytes are viewed as promising plant growth promoting bioinoculants. Endophyte *Chaetomium globosum* WSEF-4 made prominent impact on reduction of crop destruction by fungal pathogens and also enhanced plant growth and alkaloids in *W. somnifera*. Consortium of *C. globosum* WSEF-4 with AM fungi escalated the positive impact on plant growth. Endophytes play an important role in management of seed-borne and soil-borne fungal infections as also evidenced from present study. Bioformulations of endophytes can effectively transfer these PGP microbes to the field for enhancing productivity and such techniques can be adopted for safe and eco-friendly cultivation of medicinal plants. However, selection and inoculation of efficient endophytic bioinoculant is a crucial step for successful cultivation and to obtain high-quality secondary metabolites. Therefore, extensive research is recommended to better understand the role of endophytes of medicinal plants in agroecosystems and how their genetic and functional diversity affect the internal chemical nature of plant tissues and how it can be helpful in the increasing productivity of medicinal plants.

Development of bioformulations of effective PGP microbes such as *C. globosum* WSEF-4, *Pseudomonas* WRE-1, AM fungi *G. intraradices* for the cultivation of important medicinal plants such as *W. somnifera* can go a long way in ensuring sustainable supply of the plant in an eco-friendly manner. This will lead to the production of quality drugs from the medicinal plants, devoid of chemical residues which is an essential requirement. Farmers will also be encouraged to cultivate medicinal plants if high yield and appropriate market values are obtained.

The present study thus can be an important milestone in development of bioformulations for medicinal plants to make uninterrupted high quality supply of drugs for pharmaceutical industries and traditional Indian medicine system.

SUMMARY

The present study "Investigation of efficacy of endophytes for the disease management and growth enhancement of endangered medicinal plant *Withania somnifera*" was aimed to explore the roles of endophytic microflora, associated with the medicinal plant *W. somnifera,* in enhancing growth and yield of their host. Possible mechanisms by which endophytes increase fitness and survivability of their hosts and provide protection from destructive fungal pathogens were duly investigated. In present study, focus was on the screening and selection of potential growth promoting and antagonistic endophytes which not only possess better field applicability but also could replace the use of synthetic agro-chemicals in medicinal plant cultivation as we can not afford harmful chemicals to cultivate medicinal plants because they are mainly used for medicinal purpose and as dietary supplements.

Objectives

- Isolation of endophytes from selected medicinal plants.
- Isolation of fungal pathogens of *W. somnifera* (*A. alternata*, *F. solani*)
- Screening of isolated endophytes for their plant growth promoting and antimicrobial attributes
- Study of mechanisms involved in biocontrol and plant growth enhancement activities
- *In vitro* and *in vivo* study of biocontrol activities of selected endophytic strains against isolated fungal pathogens of *W. somnifera*
- Extraction and characterization of bioactive compounds produced by selected endophytes.

- Effect of isolated endophytes on plant growth, yield and alkaloid content of test crop as well as on rhizospheric microbial population.

Important findings from the study

Endophytes were isolated from different plant parts of *W. somnifera.* In total 72 isolates (including bacteria and fungi) were obtained from leaves, shoot and root tissues. Maximum endophytes were obtained from stem tissues, followed by leaves and roots. Population of bacterial endophytes was found comparatively higher than fungal endophytes. Fungal pathogens, responsible for destruction of *W. somnifera* crop were also isolated for which locally distributed plants and field crops of *W. somnifera* were surveyed during all the seasons. It was observed that leaf spot disease in *W. somnifera* most prevalent in Lucknow district. Fungal pathogen P1 isolated from diseased leaves was found highly destructive when investigated for its pathogenicity *in vitro* and *in vivo*. Fungal pathogen *F. solani* which is responsible for leaf spot, root rot and post-emergent seedling damping off disease in *W. somnifera*, was procured from the laboratory of Microbial Technology Division, CSIR-CIMAP, Lucknow and both the pathogens were selected for further studies. Isolated fungal pathogen P1 was identified as *Alternaria alternata* on the basis of 18S rRNA gene sequence. Sequence has been submitted to NCBI (accession number: KX494864). Endophytes isolated from *W. somnifera* were screened for their PGP traits and antagonistic activity including nitrogen fixation, phosphate solubilization and alkaline phosphatase activity, indole acetic acid production, siderophore production, zinc solubilization and antagonism against fungal pathogens *A. alternata* and *F. solani*. In total, thirty endophytes showed positive activity for one or more PGP traits and antagonism against fungal pathogens. Fungal endophytes were found effective phosphate solubilizers, IAA and siderophore producers as well as biocontrol agents. During investigation of biocontrol mechanisms of antagonistic endophytes, it was found that mycoparasitism was an important mechanism by which endophytic fungi inhibited the growth of pathogenic fungi. Isolate WSEF-4 showed parasitic interaction with both the pathogenic fungi and was found active producer of cell wall degrading enzymes such as β-1, 3-glucanase, cellulase, chitinase, protease, and lipase. Bacterial endophyte WRE-1 (isolated from root) and fungal endophyte WSEF-4 (isolated from stem tissues) were selected for further studies on the basis of their multiple PGP traits and effective biocontrol potential against fungal pathogens. Endophytes were partially characterized and identified on the basis of biochemical, morphological and physiological characteristics. Identified bacterial endophytes included the members from genus *Bacillus, Serratia, Pseudomonas, Micrococcus and* fungal endophytes included genus *Aspergillus, Alternaria, Chaetomium, Bipolaris, Verticillium, Cladosporium, Fusarium* and *Colletotrichum.* Selected isolates WRE-1 and WSEF-4 were also identified through 16S rRNA and 18S rRNA gene sequencing as *Pseudomonas aeruginosa* and *Chaetomium globosum* respectively. *P. aeruginosa* species is widely distributed in nature as an endophyte or as rhizospheric bacteria which are non-pathogenic to human but clinical isolates of *P. aeruginosa* have also been reported as opportunistic human pathogens. Thus isolate *P. aeruginosa* WRE-1 was checked for pathogenicity to carry out further study. It was found to be non-haemolytic, negative for pyocyanin production, unable to hydrolyze elastin protein and exhibited negative protease activity. WRE-1 showed reduced growth above 35°C and growth observations showed that optimum temperature for the growth of *P. aeruginosa* WRE-1 was 20-28°C. Antibiotic susceptibility assay showed that *P. aeruginosa* WRE-1 was susceptible to most of the antibiotics and multiple drug resistance was not observed.

Culture filtrates of isolates WRE-1 and WSEF-4 showed inhibitory effect on fungal pathogens thus their bioactive secondary metabolites were extracted and partially characterized using FTIR analysis. Ethyl acetate extracts of both the isolates showed maximum inhibitory effect on fungal pathogens. Metabolite produced by isolates WRE-1 was partially characterized as a derivative of phenazine and bioactive secondary metabolite of WSEF-4 was characterized as an aromatic compound. Effect of selected strains on seed germination and seedling vigor was also examined. Isolates WRE-1 and WSEF-4 significantly improved percent seed germination in comparison to control. Fungal pathogen *A. alternata* inoculated seeds showed pre-emergent seedling mortality and most seeds failed to germinate. *F. solani* treated seeds showed post-emergent seedling mortality and damping off of seedlings. Both the pathogens highly reduced germination percentage. Seeds treated with *A. alternata* and *F. solani* when co-inoculated with isolates WRE-1 and WSEF-4, showed great improvement in germination and seedling vigor index with no apparent seedling mortality or damping off. Endophytic nature and colonization ability of both the selected endophytes was determined and their plant growth promoting and biocontrol potential was investigated by pot experiments and field trials. Pot experiments and field trials (during October 2014 to January 2015; February to May 2015; October 2015 to February 2016) were conducted using endophyte-free seedlings of 'Poshita' variety of *W. somnifera*. Pot and field experiments showed promising results for growth enhancing and biocontrol potential of endophytes WRE-1 and WSEF-4. Plants treated with endophytes showed significantly improved shoot and root length, fresh and dry weight and improved content of chlorophyll, total phenolics, reducing sugar and total withanolides. Endophytes when co-inoculated with fungal pathogens were found able to control the disease development in treated plants and increased plant growth. Compatibility of both the endophyte isolates was also examined with an important rhizospheric

AM fungi *Glomus intraradices*. Isolate WRE-1 showed inhibitory effect on *G. intraradices* and reduced the root colonization. On the other hand, WSEF-4 showed great compatibility with *G. intraradices* and no inhibition was observed for root colonization. Co-inoculation of endophyte WSEF-4 with AM fungi *G. intraradices* also showed maximum improvement in plant growth. Disease development in WSEF-4+GI treated plants was completely suppressed and plants showed significantly higher yield and quality in comparison to control. Soil analysis also revealed significant improvement in soil properties after inoculation of endophytes WRE-1 and WSEF-4. *W. somnifera* itself is capable to improve the soil properties as observed during comparative analysis of soil from uncultivated land and rhizospheric soil after plantation of *W. somnifera*. All the microbial inoculants including WRE-1, WSEF-4 and G. *intraradices* improved nutrient level and significantly reduced the alkaline nature of soil. Maximum improvement of total organic C, available N, P and K was observed in WSEF-4+GI amended soil.

From above mentioned findings, it can be concluded that endophytes hold a great potential for their application as biofertilizers and biopesticides in the commercial cultivation of medicinal plants. Less cultivation of *W. somnifera* despite its high demand in market is due to low seed germination, high seedling mortality and high yield loss due to fungal pathogens. Moreover, use of synthetic agrochemicals is restricted and alternative techniques to promote plant yield and to control pests and fungal pathogens are costly and not easily accessible to farmers. Present study provided an initial assessment of the potential of endophytes associated with *W. somnifera*, in plant growth promotion and biocontrol of destructive fungal pathogens. Isolates WRE-1 and WSEF-4 were not only found capable of enhancing seed germination in *W. somnifera* but also effectively controlled seed-borne fungal infections and significantly reduced pre- and post-

emergent seedling mortality. Both the isolates were efficient colonizers of *W. somnifera* and showed plant growth promoting and biocontrol potential under controlled conditions in green house as well as under field conditions. Results obtained from our study suggest that endophytes can be used as alternative of synthetic agrochemicals for the cultivation of medicinal plants. Studies also suggest that endophytes possess high colonization efficiency and survivability under field conditions thus less prone to fluctuations in environmental conditions. Endophytes-based products can support the organic farming practices for medicinal plants and can be adopted for safe and eco-friendly sustainable agriculture.

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2. Arora NK, Tewari S, Singh R (2013) Multifaceted plant associated microbes and their mechanisms diminish the concept of direct and indirect PGPRs. In: Arora NK (ed) Plant Microbe Symbiosis- Fundamentals and Advances, Springer publication, Netherland. Pp 411-449
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Bacterial Formulations and Delivery Systems against Pests in Sustainable Agro-Food Production

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Introduction

The world's human population has now passed 7 billion, and it is expected to reach up to 11 billion by 2100 with a prediction of a 70% chance of a continuous increase in population (EEA, 2015). Such population increases lead to a greater demand for food. Since food is the basic necessity for life, the human population cannot compromise on food security, not even at the cost of earth's sustainability. It has been estimated that the current global population, is two to three times higher than can be sustained by current food production levels and is already utilizing 50% more resources than the earth is producing (http://www.worldpopulationbalance.org). Consequently, our overburdened resources are declining very rapidly. Current agricultural practices are contributing toward ecological degradation but as the issue of food security is of prime importance researchers are concerned to find better and safe alternatives to synthetic agrochemicals.

Pests: Major Constraint toward Food Security

A substantial amount of agricultural production is destroyed annually by a variety of insect pests, bacterial, fungal, and viral pathogens which result in the loss of 30–40% of food and fiber crops and account for huge economic losses. Although there is limited quantifiable data about global pests and their effect on crop production, several reports, research and surveys on pest outbreaks help to illustrate the global impact of pests on food production. Worldwide there are ~9000 species of insects and mites; 50 000 species of bacterial and fungal phytopathogens; and 8000 species of weeds which largely reduce crop yield and quality (Ortiz-Hernandez et al., 2013). Examples of pest outbreaks with historically devastating effects on food crops are given in **Table 1**.

Several studies have suggested that specific crop losses due to pests may vary between 10% and 90% (Youdeowei, 1989). Singh and Shekhawat (1999) stated that in India, crop losses due to pests may be as high as 80% if the crop is not well protected. In India, using with current disease management practices including the heavy use of synthetic pesticides, crop loss of 30–40% due to insect pests, weeds, and diseases was estimated to be approximately US$ 2 billion in 1995 (Gautam and Mishra, 1995). Worldwide crop loss due to pests in 1996 was estimated to be approximately $ 500 billion per year even after the annual application of 2.5 million metric tons of pesticides and synthetic chemicals which approximately was valued at $ 31.25 billion (Pimentel, 1997).

Over the history of agriculture, farmers have always been challenged by pests particularly in tropical and subtropical regions where climatic conditions are highly favorable for the reproduction of a wide range of pests and phytopathogens. A high population density of pests requires considerable efforts from farmers to achieve adequate production of food. Moreover the high pest population in developing countries is a complex problem as these countries are also facing a rapid increase in the human population at the rate of 1.2% annually (Reece et al., 2011). Pest populations in the near future will become more challenging. A recent study by researchers at the University of Exeter and the University of Oxford has revealed that crop pests are moving

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Chapter 16
Multifaceted Plant-Associated Microbes and Their Mechanisms Diminish the Concept of Direct and Indirect PGPRs

Naveen Kumar Arora, Sakshi Tewari, and Rachna Singh

Contents

Abstract It is an old saying that when we take from nature, we have to give back also; this give-and-take phenomenon leads to sustainability and is important for growth of a relationship. This is also applicable in plant–microbial world. The association of microbes with plants can be exploited and used to gain the benefits not only for the associated organisms but also for the ecosystem as a whole. When we view it in a holistic way, it is clear that multifaceted and diverse mechanisms of

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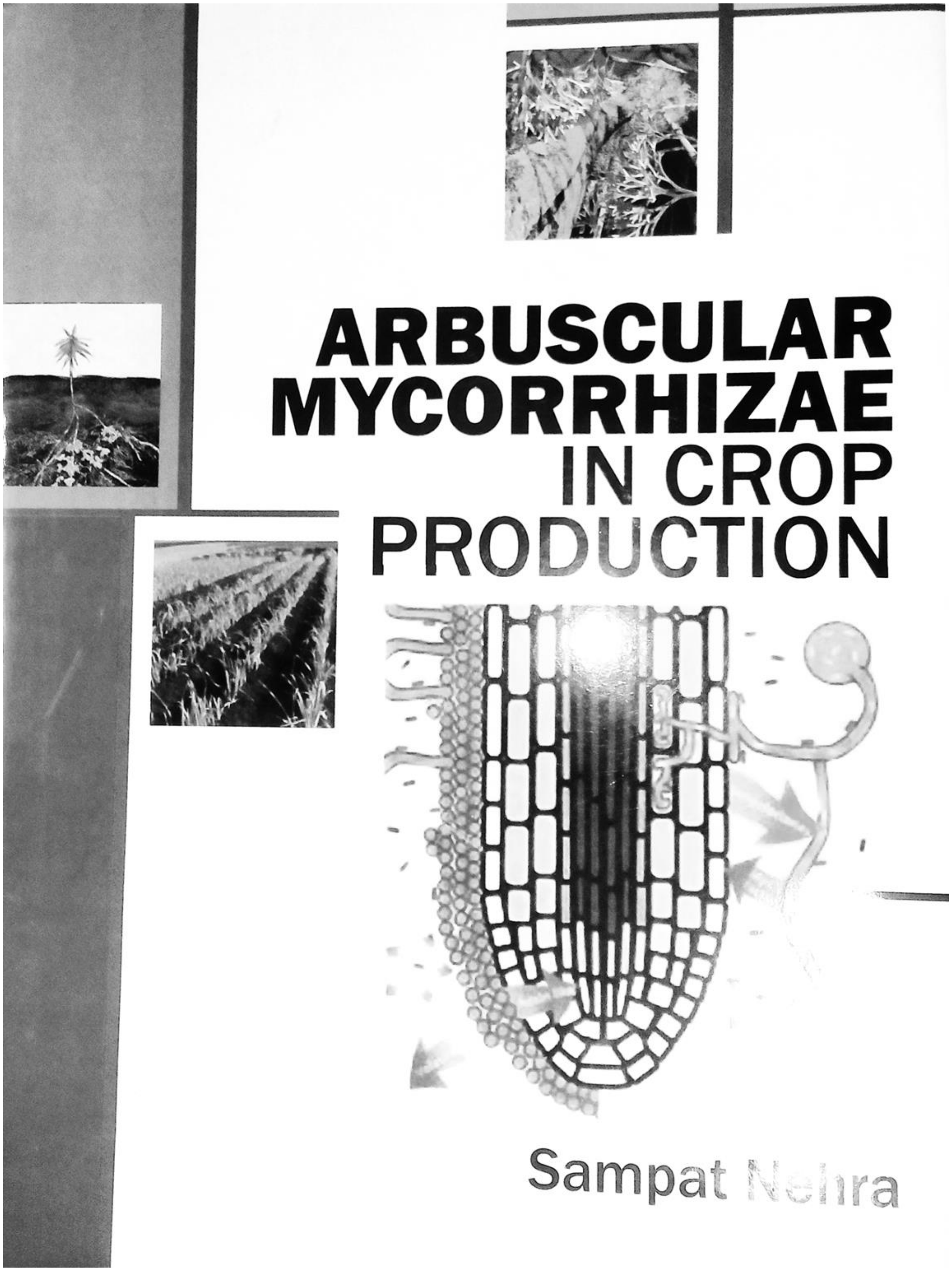
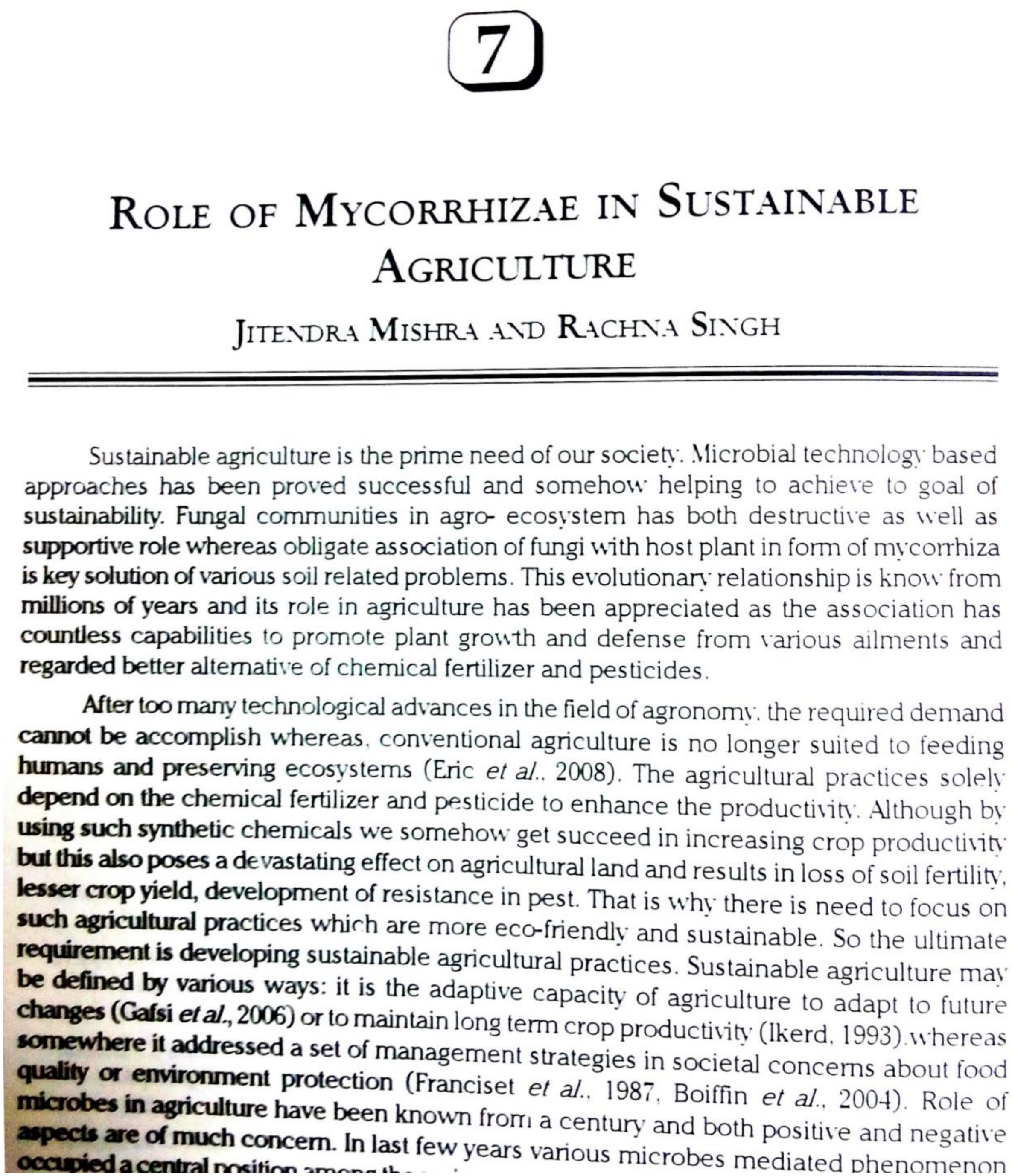


ARBUSCULAR
MYCORRHIZAE
IN CROP
PRODUCTION

Sampat Nehra



7

ROLE OF MYCORRHIZAE IN SUSTAINABLE AGRICULTURE

JITENDRA MISHRA AND RACHNA SINGH

Sustainable agriculture is the prime need of our society. Microbial technology based approaches has been proved successful and somehow helping to achieve to goal of sustainability. Fungal communities in agro- ecosystem has both destructive as well as supportive role whereas obligate association of fungi with host plant in form of mycorrhiza is key solution of various soil related problems. This evolutionary relationship is know from millions of years and its role in agriculture has been appreciated as the association has countless capabilities to promote plant growth and defense from various ailments and regarded better alternative of chemical fertilizer and pesticides.

After too many technological advances in the field of agronomy, the required demand cannot be accomplish whereas, conventional agriculture is no longer suited to feeding humans and preserving ecosystems (Eric *et al.*, 2008). The agricultural practices solely depend on the chemical fertilizer and pesticide to enhance the productivity. Although by using such synthetic chemicals we somehow get succeed in increasing crop productivity but this also poses a devastating effect on agricultural land and results in loss of soil fertility, lesser crop yield, development of resistance in pest. That is why there is need to focus on such agricultural practices which are more eco-friendly and sustainable. So the ultimate requirement is developing sustainable agricultural practices. Sustainable agriculture may be defined by various ways: it is the adaptive capacity of agriculture to adapt to future changes (Gafsi *et al.*, 2006) or to maintain long term crop productivity (Ikerd, 1993).whereas somewhere it addressed a set of management strategies in societal concerns about food quality or environment protection (Franciset *et al.*, 1987, Boiffin *et al.*, 2004). Role of microbes in agriculture have been known from a century and both positive and negative aspects are of much concern. In last few years various microbes mediated phenomenon occupied a central position among

Chemicals and glassware sources

Chemical and glassware used were procured from the following sources:

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

Media and nutrients

1. Nutrient agar media

Constituents	Amount (l^{-1})
Peptone	5.0 g
Yeast extract	1.5 g
Beef extract	1.5 g
NaCl	5.0 g
Agar	16 g
H_2O	1000 ml
pH	7.0

2. Nutrient Broth (NB)

Constituents	**Amount (l^{-1})**
Peptone	5.0 g
Yeast extract	1.5 g
Beef extract	1.5 g
NaCl	5.0 g
H_2O	To 1000 ml
pH	7.0

3. Luria Agar (LA) medium

Constituents	Amount (l^{-1})
Proteose peptone	20 g
Glycerol	10 ml
K_2HPO_4	1.5 g
$MgSO_4$	1.5 g
Agar	16 g
H_2O	To 1000 ml
pH	7.0

4. Luria Broth (LB)

Constituents	Amount (l^{-1})
Proteose peptone	20 g
Glycerol	10 ml
K_2HPO_4	1.5 g
$MgSO_4$	1.5 g
H_2O	To 1000 ml
pH	7.0

5. Potato Dextrose Agar

Constituents	Amount (l^{-1})
Potato	200 g
Dextrose	20.0 g
Agar	20.0 g

6. Pikovskaya’s Agar medium

Constituents	Amount (l^{-1})
Yeast extract	0.5 g

Dextrose	10.0 g
$Ca_3(PO_4)_2$	5.0 g
$(NH_4)_2SO_4$	0.5 g
KCl	0.2 g
$MgSO_4$	0.1 g
$MnSO_4$	0.0001 g
$FeSO_4$	0.0001 g
Agar	15.0 g
H_2O	1000 ml
pH	7.0

7. Acetate buffer

Constituents	Amount (l^{-1})
Acetic Acid	11.55 g
Sodium Acetate	16.40 g
H_2O	To 1000 ml

8. Tryptan blue stain

Constituents	Amount (l^{-1})
Glycerol	500 ml
HCl (1%)	50 ml
Trypon blue	0.5 g
H_2O	To 1000 ml

9. Reagents and stains

Saline Water

8.5 g NaCl in 1000 ml water

Salkowaski reagent

12 g $FeCl_3$ in 1 liter H2SO4 (7.9M)