Enhancing efficacy of biocontrol of Fusarium wilt and Early blight of Tomato (*Lycopersicon esculentum* Mill.) by *Trichoderma* and *Pseudomonas*

THESIS

SUBMITTED TO

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

LUCKNOW

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(A Central University, NAAC Accredited ‘A’ Grade)

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Dedicated to
My Beloved Parents

DECLARATION

This is to certify that the material embodied in the present work entitled "**Enhancing efficacy of biocontrol of Fusarium wilt and Early blight of Tomato (*Lycopersicon esculentum* Mill.) by *Trichoderma* and *Pseudomonas***" is based on candidate's original research work. It has not been submitted in part or full for any other diploma or degree of any University. The indebtedness of the candidate to others has been duly acknowledged at relevant places. I hereby also undertake that the thesis is essentially free from all kinds of plagiarism.

Swati Sachdev
5/10/18

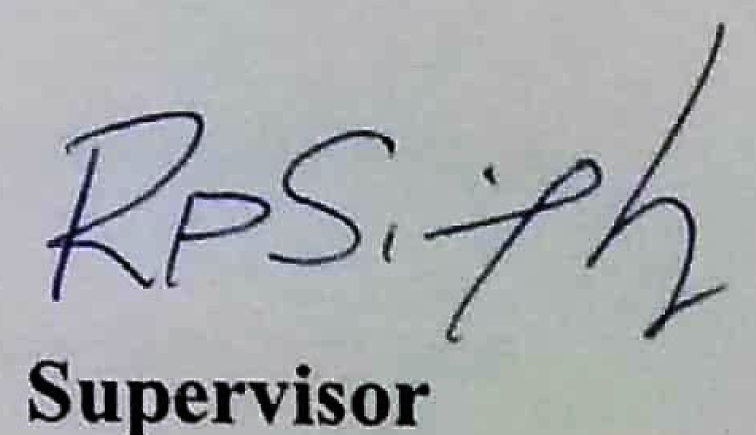
Name and Signature

(Candidate)

CERTIFICATE

This is to certify that the thesis titled "**Enhancing efficacy of biocontrol of Fusarium wilt and Early blight of Tomato (*Lycopersicon esculentum* Mill.) by *Trichoderma* and *Pseudomonas***" submitted by **Ms. Swati Sachdev** 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.) regulation-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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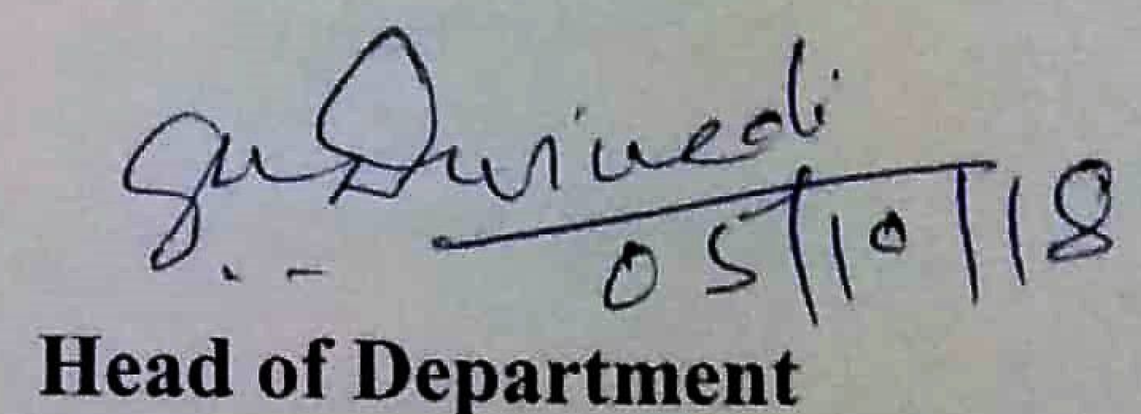

Head of Department

PREFACE

Production of sufficient agricultural produce to achieve food security for gradually increasing population in a sustainable manner is an arduous task. The unsustainable agricultural practices such as the excessive and injudicious use of chemical pesticides and fertilizers coupled with continuous climate change had resulted in depletion of soil nutrients, increased salinity, and altered geographical distribution of pests and pathogens which in turn has enhanced plant's susceptibility to pests and pathogens infestation; and reduced growth. Tomato is the most common crop cultivated all around the world and has earned the status of "functional food" due to its nutritive contents which posses ability to reduce the risk of cardiovascular diseases, cancer, and several disorders. The growth and nutritional content of tomato are severely influenced by the occurrence of pathogenic diseases. To control pathogens, farmers use chemical pesticides indiscriminately, that often become a part of other ecosystems and incorporate in food chains and ultimately leads to hazardous effects on living beings and environment. Thus, there is a need to shift agricultural practices of using chemical pesticides for disease management to an alternative approach that are eco-friendly and sustainable. The microorganisms residing in the rhizosphere of plants posses an ability that confers beneficial effects on plant growth either directly by enhancing acquisition of water and nutrients, production of phytostimulating hormones, solubilization of inorganic minerals and indirectly by curbing the growth of deleterious microorganisms. Harnessing the potential of these microorganisms for controlling pathogens as well as promoting the growth of plants effectively and enhancing their efficacy by use of consortium or their metabolites can pave a way for sustainable agriculture that not only provide food security to present generation but also maintain the ability of agro-ecosystem to produce food for the future generation.

In present work, an attempt was made to identify the potential of native rhizospheric microbial isolates to reduce the incidence of fusarium wilt and early blight of tomato and to enhance their efficacy by developing the consortium of microbial isolates and/or their metabolite.

Swati Sachdev
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Swati Sachdev

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LIST OF ABBREVIATIONS

AIMs	Agriculturally important microorganism
AMF	Arbuscular mycorrhizal fungi
ANOVA	Analysis of Variance
ASC-GSH	Ascorbate –glutathione
BCA	Biocontrol agents
CAS	Chrome azurol sulphonate
CAT	Catalase
CMC	Carboxy methyl cellulose
CTAB	Cetyltrimethyl ammonium bromide
CWDE	Cell wall degrading enzymes
DAP	Di-ammonium phosphate
DAPG	2, 4- Diacetylphoroglucinol
DAS	Days after sowing
DAT	Days after transplantation
DNA	Deoxyribonucleic acid
DW	Distilled water
dw	Dry weight
E_{24}	Emulsification activity
EC	Electrical conductivity
EPS	Exopolysaccharides
ET	Ethylene
FAO	Food and Agriculture Organization
Fol	*Fusarium oxysporum* f. sp. *lycopersici*
FTIR	Fourier transform infrared spectroscopy
fw	Fresh weight
GDP	Gross Domestic Product
GHI	Global hunger index
HCN	Hydrogen cyanide
HDTMA	Hexadecyltrimethylammonium bromide
HVA	High-value agriculture
H_2S	Hydrogen sulfide

IAA	Indole-3-acetic acid
IPCC	Intergovernmental panel on climate change
IPM	Integrated Pest Management
ISR	Induced systemic resistance
IST	Induced systemic tolerance
ITS	Internal transcribed spacer
JA	Jasmonic acid
KBr	Potassium bromide
KOH	Potassium hydroxide
MR-VP	Methyl Red and Voges Prokauer
MSM	Minimal salt medium
NA	Nutrient agar
NB	Nutrient broth
NCBI	National Center for Biotechnology Information
ND	Not detected
NO	Nitric oxide
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PDI	Percentage disease index
PGP	Plant growth promotion
PGPF	Plant growth promoting fungi
PGPM	Plant growth promoting microorganisms
PGPR	Plant growth promoting rhizobacteria
PIPES	Piperazine-N,N′-bis(2-ethanesulfonic acid)
PIRG	Percentage inhibition of radial growth
PO	Peroxidase
PPE	Personal protection equipment
PPO	Polyphenol oxidase
PSI	Phosphate solubilization index
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
RWC	Relative water content

SA	Salicyclic acid
SAR	Systemic acquired resistance
SDW	Sterilized distilled water
SE	Standard error
SEM	Scanning electron microscope
SMA	Skimmed Milk Agar
TSI	Triple sugar iron test
VI	Vigor index
VOCs	Volatile organic compounds
ZSI	Zinc solubilization index
6PP	6n-Pentyl-α-pyrone

Introduction

Agricultural is the major sector that determines the economic growth of a country. It is the main source of livelihood for many people and incurs national income for most of the developing countries. Agriculture fulfills the demand for food, feed, and fodder of ever-increasing population and its stability ensure food security of the country. Food security is attained when a country is able to produce food sufficient to feed its population and fulfills their dietary needs. Attaining food security is a challenging task as agriculture sector is highly vulnerable to fluctuation in climatic conditions (Khanal and Mishra, 2017), biotic stress (Gimenez et al., 2018), shrinking arable land due to degradation and desertification, and conversion of agricultural lands into commercial landscape for developing infrastructure to accommodate and avail modern facilities to growing population (Meena and Meena, 2017). It has been estimated that the world population will reach 9 billion by 2050. According to FAO (2009), the major challenge for the agriculture sector will be to produce 70% more food crop for an extra 2.3 million population by 2050.

India is an agriculture-based country where around 68% of the population is dependent upon agriculture and contributes nearly 18% to India's GDP (Economic survey, 2015). India has a second largest agrarian economy at the world level (Kumar et al., 2017). Being a center for the growth of diverse crop plants, accessibility of sufficient food and nutrition is far away from the reach of millions of Indians. In the world, around 800 million people suffer from hunger and the majority of this population lives in developing countries (FAO, 2015). India holds the 100th position in Global hunger index (GHI) flaunting serious hunger problem in the country (Grebmer et al., 2017). In the context of the Indian scenario of population growth, it has been anticipated that 450 million tonnes of food product will be required to feed 1.65 billion of Indian population by 2050 (Thind, 2015). Thus there is an urgent need

for finding sustainable solutions for the production of more food to feed present and future generation (Meena and Meena, 2017).

1.1. Vegetable crop cultivation in India

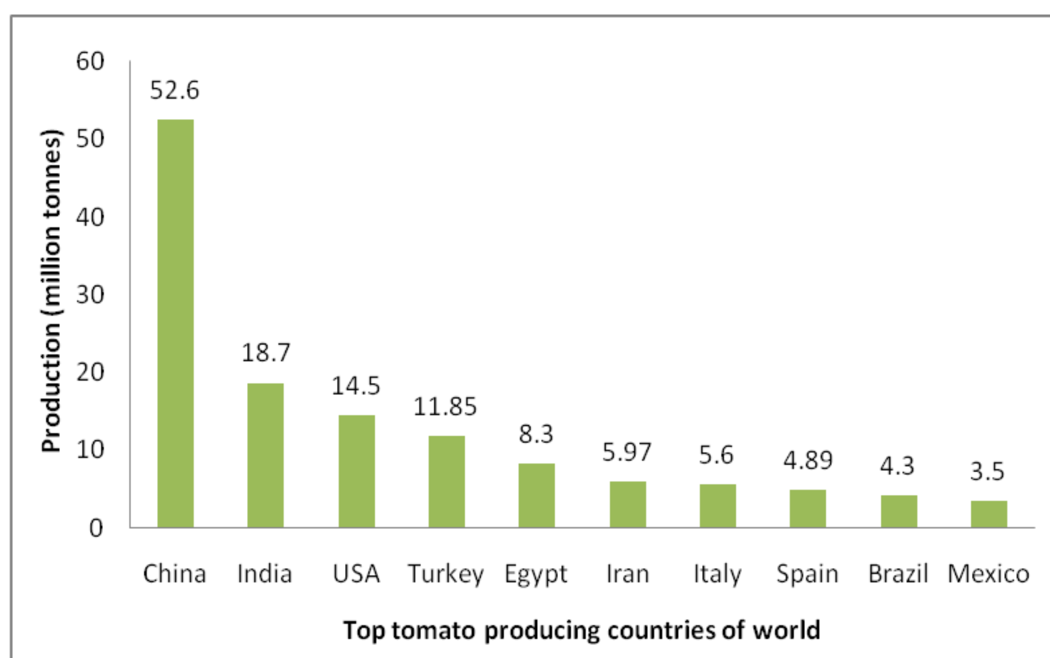
India is bestowed with different bio-geographical realms that favor diverse ecological conditions representing growth of several exotic and indigenous crop plants (Singh, 2017). Out of 329 million hectares of total land in India, 55% is indulged in agriculture for growing different crops (Government of India, 2013). Today, horticulture is a major agricultural sector in our country and 90% share of horticulture crops comprise of fruits and vegetables. Fruits and vegetables are considered as high-value agriculture (HVA) crops that possess the potential to accelerate the growth of the agriculture sector (Pandey and Suganthi, 2015). The total area and productivity of horticulture crops in India in the year 2016-17 was 24.9 million hectares and 295.2 million tonnes, respectively (Government of India, 2017a).

Vegetables are an important component of horticulture as well as Indian agriculture. It constitutes about 59-61% of horticulture produce (Government of India, 2017a). India is the second largest producer of vegetable crops in the world, next to China. As per the Government of India (2017b), per capita availability of vegetables during 2016-17 was 355 gm/person/day. The area under vegetable cultivation has been increased in India in past few years probably due to increase in per capita income, increase in consciousness for consumption of healthy foods and shifting of farmers focus to grow high-value crops that give higher returns (Choudhary and Kundal, 2015). Cultivation of vegetables not only helps to improve the nutritional status of the population but also a good source of employment and income. The area under vegetable cultivation in 2016-17 was estimated to be 10.3 million hectares with

175 million tonnes of production (Government of India, 2017a). Among several states, Uttar Pradesh has been reported as highest vegetable producing state in India during 2016-17, representing 15% of total production of the country as per the report, Horticulture statistics at a glance 2017 of Government of India (2017a). 1.4 million hectares of the area was documented under vegetable cultivation with 26.4 million tonnes of production in the state (Government of India, 2017a).

1.2. Tomato cultivation

Tomato (*Lycopersicon esculentum* Mill.), the second most cultivated vegetable crop in the world, is one of the most versatile and popular vegetables with wide usage (Bawa, 2016). Tomato belongs to genus *Lycopersicon* of family Solanaceae, also known as nightshade family that covers several commercially important crops *i.e.,* potato, brinjal, and pepper (Piquerez et al., 2014). Tomato is native to Mexico, but now it is cultivated and consumed all over the world (Arah et al., 2015).



Source: FAOSTAT, 2016

Figure 1.1: Top 10 tomato producing countries in 2014

The world production of tomato was recorded as 177 million tonnes in 2016 (Anonymous, 2018). It is a short duration crop that gives high yield hence it is economically attractive. In tropical Asia, it is an important cash crop for small farmers (Nagaraju et al., 2002) providing the main source of employment in rural activities (Fontenella et al., 2011).

1.2.1. Environmental conditions affecting tomato production

Tomato is a day-neutral plant and can be grown all year around. Climatic conditions greatly influence tomato productivity and quality of produce. Temperature, light intensity, water availability and soil properties impinge on tomato productivity. High as well as low climatic condition limits tomato productivity. Tomato is a temperature sensitive crop that requires optimum temperature ranging 23-27ºC for high yield and good quality (Nicole et al., 2009). The temperature below 15ºC or above 35ºC detrimentally affects the growth and yield of the plant. Very low-temperature results in a delay in fruit color development and ripening whereas high temperature hampers fruit set; development of flavor and lycopene (Bawa, 2016). The low light intensity also affects fruit set, ripening, pigmentation, and nutritive value.

The dry spells and heavy rainfall both affect the growth of the tomato plant. They grow well during low and medium rainfall condition with proper irrigation provided during the off-season. Excess moisture conditions increase diseases incidence and distress fruit ripening (Bawa, 2016). Soil is another criterion that influences the tomato growth. Tomatoes can grow in a wide range of soil having high organic matter, however, prefer well-drained sandy loam soil with pH 5-7.5 (Waiganjo et al., 2006).

1.2.2. Nutritional requirements of the tomato plant

Availability and accessibility of macro- and micro-nutrients to tomato plant reported to play a significant role in plant growth and high yield of tomato (Ordookhanil et al., 2010). Tomato plants have high requirement of some nutrients including Potassium (K), Calcium (Ca), Zinc (Zn), Iron (Fe) and Manganese (Mn) for good qualitative and quantitative production (Kisetu and Heri, 2014). Insufficient availability of potassium and calcium, affect soluble sugar content of tomato fruits and make them susceptible to various physiological disorders like blossom end rot (Hinamn et al., 2012). Potassium also directly correlates with the lycopene content of fruits, thus access to sufficient potassium result in tomato fruit with a good amount of lycopene content (Taber et al., 2008). Tomato plant also requires an adequate amount of Nitrogen (N) and Phosphorus (P) which are essential for root development, early flowering and fruit setting (Republic of South Africa, 2010). Other micro nutrients *i.e.,* Copper (Cu), Magnesium (Mg) and Boron (B) are although required in low quantities, but essentially required for fruit setting and dry matter partitioning (Kisetu and Heri, 2014).

1.2.3. Nutritive values of tomato

Tomato is one of the most versatile and economically important vegetable crops that not only has culinary importance but also tops the list of the industrial crop because of processing qualities (Christopher et al., 2010). Tomato is consumed in different forms such as salad, soup, puree, ketchup, sauce, juice, *etc*. Due to high nutritive values tomato is considered an important part of the human diet and it is believed that consuming one tomato in a day improves the health of an individual (Yadav, 2017).

A healthy diet is identified as a significant factor in preventing chronic diseases, and in improving energy balance and weight management (Dorais et al., 2008). Tomato is a good appetizer and its soup helps to prevent constipation. The low calories content, relatively high fiber content and various vitamins, minerals, phenols and antioxidants such as lycopene, β-carotene, flavonoids make tomato an excellent functional food providing additional physiological benefits along with basic nutritional requirements (Doris et al., 2008). Thus tomato occupies a prime position in the list of protective food (Arah et al., 2015; Babu et al., 2015). Several scientific workers have documented the effective role of a tomato in the reduction of risk of certain types of cancer, cardiovascular diseases, hypertension, respiratory disorder, skin diseases, neurodegenerative disorders, infertility in males and age-related macular degeneration due to the presence of essential nutrients and antioxidants (Rao et al., 2018). These nutrients and antioxidants eliminate active oxygen species that cause oxidative stress and are responsible for the critical damages to biomolecules (Dorais et al., 2008; Babu et al., 2015).

1.3. Abiotic and biotic constraints affecting tomato cultivation

Plants are often get exposed to several abiotic and biotic stresses simultaneously under natural environmental conditions that have a devastating effect on plant growth and productivity. The biotic and abiotic factors driven by climate change have resulted in a 1-5% reduction in agricultural production in the last 30 years (Newbery et al., 2016). The occurrence of environmental stress alone can reduce potential plant yield upto 70% that poses a challenge to the sustainable production of plant products (Agrawal et al., 2006). The broad range of abiotic stress that influences plant growth and productivity includes drought, salinity, flood, chilling, heat shock, heavy metal contamination, UV radiation, etc. (Suzuki et al., 2014). Exposure of plants to abiotic

stresses as a consequence leads to oxidative stress that strikingly alters or reduces the nutritional status and the antioxidant activity of plants (Apel and Hirt, 2004).

Plants are also constantly encountered with various biotic stresses that affect crop productivity worldwide. On an average 26% of the plan yield is annually lost due to pest infestation and disease incidences (Piquerez et al., 2014). The plant diseases caused by phytopathogens are considered as one of the major constraints in the production of food material (Agrios, 2005). It has been predicted that due to climate change, a poleward moment of pathogens could take place (Bebber et al., 2013). This poleward moment may result in the development of new diseases that could become endemic if counteract measures are not deployed on time and as a consequence lead to worst scenarios of crop productivity loss (Piquerez et al., 2014).

1.3.1. Abiotic stress

Abiotic stress is a major challenge that limits plant growth and productivity and has negative ecological footprints on the environment. An array of abiotic factors impinges on crop cultivation. Among them continuous change in climatic conditions, exacerbating the situation of water scarcity, soil pollution and degradation has been identified as the potential limiting factor that obstructs crop productivity and has become a matter of concern worldwide. The anthropogenic activities such as modern agricultural practices adopted as a part of the green revolution, industrialization and urban sprawl are the major sources of soil contamination and degradation (Ullah et al., 2015). The irrigation techniques, use of agrochemicals and dumping of industrial waste has escorted agriculture to become more vulnerable to various biotic and abiotic stresses resulting in lower productivity and quality.

1.3.2. Biotic stress

Tomatoes are susceptible to a broad range of phytopathogens and pests comprising several fungi, oomycetes, bacteria, viruses, nematodes, and insects. Owing to the vulnerability to a range of pathogenic genera, studying plant-pathogen interaction in tomato helps to establish an effective control against these diseases and to enhance tomato productivity worldwide (Piquerez et al., 2014). Therefore, the tomato is considered as a research model which provides an excellent example of how to craft microbial biocontrol agents as an integral part of Integrated Pest Management (IPM) strategy (Minuto et al., 2006).

The pests that commonly feed on tomato plants and check their growth include fruit borer (*Helicoverpa armigera*), common army worm (*Spodoptera* spp.), whitefly (*Bemisia tabaci*), Leaf miner (*Liriomyza trifolii*) and spider mites (*Tetranychus urticae*). These pests affect tender foliage and mesophyll tissues of leaves, flower buds, and fruit during tomato cultivation, resulting in severe productivity loss. The tomato diseases caused by soil-borne, seed-borne and foliar pathogens are distributed worldwide that include vascular wilts, damping off, *Fusarium* crown rot, corky root rot, black dot root rot, bacterial canker, root knot, gray mold early blight, late blight, bacterial speck, bacterial spot, spotted wilt, mosaic, yellow leaf curl, *etc*. These pathogens pose a challenge to sustainable cultivation of tomato and bar its yield. The common diseases and disorders of tomato and sources responsible are presented in **table 1.1**.

Table 1.1: Common diseases and disorders of tomato

Diseases/disorders	Causal agent of infection(s)/disorder(s)	Type of disease/source of infection
Fungal diseases		
Fusarium wilt	*Fusarium oxysporum* f. sp. *lycopersici*	Soil-borne
Crown and root rot	*Fusarium oxysporum f. sp. radicis-lycopersici*	Soil-borne
Verticillium wilt	*Verticillium dahliae* *Verticillium albo-atrum*	Soil-borne
Damping off	*Pythium aphanidermatum* *Rhizoctonia solani*	Soil-borne
Southern blight	*Sclerotium rolfsii*	Soil-borne
Corky root rot	*Pyrenochaeta lycopersici*	Soil-borne
Buckeye-rot	*Phytophthora nicotinae*	Soil-borne
Early blight	*Alternaria solani*	Soil-borne and Foliar
Late blight	*Phytophthora infestans*	Soil-borne and Foliar
Septoria leaf spot	*Septoria lycopersici*	Foliar
Gray mold	*Botrytis cinerea*	Foliar
Leaf mold	*Fulvia fulva*	Foliar
Gray leaf spot	*Stemphyllium solani*	Foliar
Anthracnose	*Colletotrichum coccodes*	Fruit attacking disease
Bacterial diseases		
Stem rot	*Erwinia carotovora* subsp. *carotovora*	Soil-borne
Bacterial wilt	*Ralstonia solanacearum*	Soil-borne
Bacterial canker	*Clavibacter michiganensis* subsp. *Michiganensis*	Foliar
Bacterial speck	*Pseudomonas syringae pv. Tomato*	Foliar
Bacterial spot	*Xanthomonas campestris pv. vesicatoria*	Foliar

Viral diseases		
Spotted wilt	*Tomato spotted wilt virus*	Transmitted by Thrips
Yellow leaf curl	*Tomato Yellow leaf curl virus*	Transmitted by whitefly
Mosaic	*Tomato mosaic virus*	Seed-borne
Nematode disease		
Root knot	*Meloidogyne* spp.	Soil-borne
Physiological disorders		
Blossom end rot	Calcium deficiency	Fruit affecting disorder
Fruit cracking	Heavy rain preceded by dry weather	Fruit affecting disorder
Catfaced fruit	Exposure to low temperature	Fruit affecting disorder
Sunscald	Exposure to high temperature	Fruit affecting disorder
Blotchy ripening	Potassium and boron deficiency, high level of nitrogen and high temperature	Fruit affecting disorder

1.4. Conventional practices for management of biotic stress

To enhance the food productivity and to control plant pests and diseases, agrochemicals such as fertilizers and pesticides are being used since the green revolution. The adoption of green revolution enhanced calorie supply from 2396 Kcal/day/capita to 2589 Kcal/day/capita in a decade from 1975 to 1985 (FAOSTAT, 2013). Since green revolution, modern agricultural practices raised agricultural production manifolds, however as a consequences deteriorated environmental quality/multi-functionality, depleted natural resources, caused genetic erosion, loss of biodiversity and socio-economic problems (Chowdappa et al., 2013; Babu et al., 2015). The imbalances in the microbial community have created unfavorable

conditions for the activity of beneficial organisms (El Hassan et al., 2013) that play a crucial role in the growth and productivity of both plants and soil. Overuse of agrochemicals coupled with uncertainty in climatic behavior, resource depletion, land fragmentation and degradation, lack of awareness among farmers, increased incidence of abiotic stress and accelerated rate of pests and disease occurrence has challenged agricultural sustainability (Sachdev and Singh, 2016a, b; Kashyap et al., 2017). To curb the negative consequences of the use of chemicals pesticides and produce residue free food, the focus of researchers has shifted toward alternative and sustainable approaches to control plant diseases and enhance food production. The alternative methods that makes use of organic amendments to soil involving compost, manures, agricultural waste, *etc*.; cultural practices including tillage, crop rotation and burning; soil solarization; steam sterilization; use of resistant cultivars and biological methods are considered as powerful tool for management of plant diseases (Mokhtar and El-Mougy, 2014).

The alternative methods like organic amendments to soil, cultural practices, soil solarization, and resistant cultivars are the most practical and cost-efficient strategies, however, these practices do not control plant diseases effectively; their efficiency is limited by the diversity of pathogen-host plants, the ability to colonize the rhizosphere of non-host plants and persistence in soil for long *via* resting spore (Jabnoun-Khiareddine et al., 2009). Even the efficiency of resistant cultivars is limited by pathogenic variability due to the emergence of new pathogenic races and pathotypes (Jimenez-Gasco et al., 2004). In search of sustainable, long lasting, cost-effective and eco-friendly approach for managing plant diseases, research has been geared toward biocontrol agents that include the use of naturally occurring

microorganisms which are considered safe and compatible with other organic methods of pest and disease management (Motlagh and Samimi, 2013).

1.5. Rhizospheric microorganisms: an alternative approach for the management of biotic stress

The microorganisms residing in the vicinity of plant roots are termed as Rhizospheric microorganisms. The microbes present in plant rhizosphere show a different level of interaction with each other. The interaction between rhizospheric microorganisms may be associative, mutualistic, competitive or antagonistic (Jain et al., 2016), resulting in neutral, beneficial as well as the detrimental effect on plants. The gigantic body of literature evidently supports role of beneficial rhizospheric microorganisms in biocontrol of plant diseases, plant growth promotion and improve resistance in plants to withstand under abiotic stress conditions (Egamberdieva et al., 2013; Jain et al., 2016). These microorganisms display a plethora of mechanisms as well as possess the ability to colonize plant roots that aid their capacity to provide protection to plants against stressful circumstances (Jain et al., 2016). The beneficial rhizospheric microorganisms combat with phytopathogens either by inhibiting or suppressing their growth by the deployment of an array of mechanisms including competition for nutrition and space, mycoparasitism, antibiosis and Inducing systemic resistance (ISR) either individually or simultaneously (Babu et al., 2015; Patel and Saraf, 2017). Several rhizospheric bacteria and fungi such as species belonging to genera *Bacillus*, *Pseudomonas*, *Trichoderma*, Arbuscular mycorrhizal fungi (AMF) utilizes one or more of these biocontrol mechanisms for management of phytopathogens and tailoring abiotic stress that ultimately leads to enhanced plant productivity (Babu et al., 2015; Karimi et al., 2017).

Adoption of biological methods *i.e.,* use of microorganisms as biofertilizers and biopesticides have emerged as a safer and effective alternative to replace or supplement the existing agricultural practices to enhance productivity, reduce or eliminate plant diseases, bio-remediate contaminated soil and provide tolerance to plant withstand under influence of abiotic stress. These microorganisms positively stimulate plant growth, either directly or indirectly *via* different modes of action, however, they still lack their position in actual field conditions and in agrochemical markets. The major reasons behind their unsuccessful stories are their poor and inconsistence field performances and inability to survive under prevailing sets of environmental conditions. These hurdles could be resolved by adopting improved methods such as isolating native microbial strains and the use of their consortia in place of individual strain that show better field performance due to the synergistic effect of various mechanisms.

The biocontrol agents are greatly influenced by biotic and abiotic factors. The mechanisms used by biocontrol agents may be dissimilarly affected by the environmental conditions, and if multiple mechanisms are involved under a particular set of conditions then it may be possible that one of the mechanisms may compensate for the other (Guetsky et al., 2002). Therefore the maximum percentage of success in disease control by biocontrol agents can be achieved if a consortium of microorganisms is used for disease control or suppression having several different mechanisms that could have either additive or synergistic effects (Siddiqui and Akhtar, 2009). Thus, the present study was conducted to isolate native rhizospheric microbial isolates possessing the ability to manage fungal diseases of tomato and development of consortium for enhancing their efficacy.

The objectives of the study:

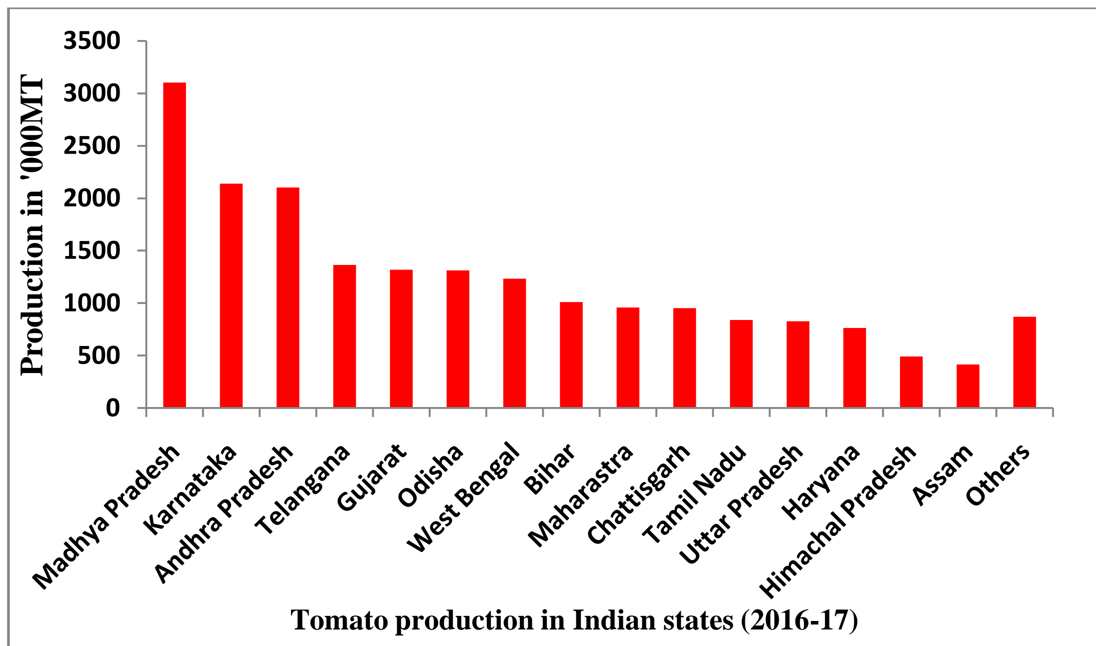
1. Survey on trends in use of pesticides and fertilizers by tomato growers in the vicinity of Lucknow city.
2. Isolation and identification of fungal pathogens obtained from tomato crop.
3. Isolation, identification, and characterization of rhizospheric microbial isolates possessing antagonistic activity.
4. Screening of isolated rhizospheric microbial isolates for antagonistic activity against fungal phytopathogens of tomato under pot conditions.
5. Assessment of antagonistic and plant growth promoting activities of best performing rhizospheric microbial isolates individually and in consortia in pot condition.
6. Extraction and characterization of biosurfactant from microbial biocontrol agents and *in vitro* assessment of its efficacy on biocontrol of fungal phytopathogens.
7. Assessment of biosurfactant and microbial biocontrol agents on disease control, plant growth promotion, and yield of tomato.

Review of Literature

Food security is a prior concern for policy makers of the world. Climate change, disease occurrence, contamination of agricultural land with non-degradable xenobiotic materials, increasing salinity and gradually depleting non-renewable resources have ramified the problem of adequate food production. The continuous change in temperature and rainfall pattern has been enhanced and future projections for climate change by IPCC has alarmed for increased susceptibility of crop plants toward pest and pathogenic infections posing threat to food security for growing population (Altieri et al., 2015). India, a developing country is also not unaffected with a crisis of food insecurity. Development of resistance in pests and pathogens, uncertain climatic phenomenon, resource degradation, land fragmentation, poor market connections, poor monetary gains to farmers, inaccessibility to technology (Manneh et al., 2016) and paucity of knowledge has detrimentally impinged on agricultural output in many Indian states (Sachdev and Singh, 2016a).

Tomato (*Lycopersicon esculentum* Mill.) is an important tropical and sub-tropical vegetable crop for both tomato growers and consumers due to high economic and nutritional values (Solanki et al., 2014). Around 25,000 varieties of tomato are available that differ in shape, size, fruit color, type of leaves and resistance to diseases (Jankowska et al., 2016). Tomato is consumed fresh as well as in processed form all over the world as soup, salad, pickles, ketchup, puree, sauces, *etc*. The high minerals, vitamins, and carotenoids content with low fat, calories and cholesterol make their consumption suitable for a healthy lifestyle (Krumbein et al., 2006; Linero et al., 2015). In India due to nutritional properties tomato is known as “Poor Man’s Orange” (Chopada et al., 2014). The productivity of tomato is greatly hampered by environmental and biotic factors. The environmental factors such as radiation intensity, temperature, Carbon dioxide (CO_2) level, availability of nutrients (Krumbein et al., 2006) and biotic factors *i.e.,* pathogen infestation (Fakhro et al., 2010) limits growth, productivity, and quality of tomato fruits.

2.1. Tomato cultivation in Indian Context



Source: Government of India, 2017a

Figure 2.1: State-wise tomato production in the year 2016-17

India holds the second position in tomato cultivation in the world. The production share of tomato among major vegetable crops in India in 2012-2013 was 11.2%. Tomato is grown all over the country. The area and production of tomato in India and in Uttar Pradesh during 2016-17 was recorded as 0.8 million hectares and 19.7 million tonnes; and 0.02 million hectare and 0.8 million tones, respectively (Government of India, 2017a). Madhya Pradesh, Karnataka, Andhra Pradesh, Telangana, and Gujarat were documented as five top highest tomato producing states of India during 2016-17 (Government of India, 2017a).

2.2.Fungal phytopathogens affecting tomato production

Plants being sessile are often subjected to numerous biotic challenges (Jain et al., 2016). Tomato is a host to several bacterial, fungal, nematodes and viral phytopathogens (Chan et al., 2005), however, few to them results in considerable

productivity losses that include major fungal diseases like Fusarium wilt, early blight, root rot and damping off, and late blight caused by fungal pathogens *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*), *Alternaria solani*, *Rhizoctonia solani*, *Phytophthora infestans*, respectively (Solanki et al., 2014).

2.2.1. Fusarium wilt

Fusarium wilt is a host-specific fungal disease affecting several economically important crops in the world. Fusarium wilt of tomato is the most destructive diseases occurring all over the world and accounts 30-40% yield loss (Yeole et al., 2016). In India also *Fusarium* wilt is a major threat to tomato production that has been reported to cause 25-55% productivity loss in different parts of the country (Nirmaladevi et al., 2016). *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) is the causal agent of *Fusarium* wilt of tomato. It's a cosmopolitan soil borne pathogen that persists in soil for an indefinite time by producing resilient structures which remain viable for several years in soil and plant debris, even in the absence of host plant. Hence, it is difficult to control wilt caused by *Fol* in tomato. It has white to pink or purple color mycelia colony. It produces three types of asexual spores *i.e.,* macroconidia, microconidia, and chlamydospores. Macroconidia are hyaline, fusiform, slightly curved from edges and pointed, 3-7 septate with size ranging from 30-60 x 3-5µm (Ignjatov et al., 2012). Microconidia are unicellular, non-septate, elliptical or cylindrical in shape clustered into the false head, with size ranging between 5-12 x 2.3-3.5µm (Ignjatov et al., 2012). Chlamydospores are an intercalary or terminal structure that has a smooth or rough wall.

High temperature, acidic soil, low nitrogen, and phosphorus content and high potassium level in soil favor infection of the pathogen in tomato. The pathogen

invades in the plant through roots from where it moves toward and proliferates in vascular tissues. It clogs xylem vessels and inhibits water conductance leading to wilting (Chopada et al., 2014; Nirmaladevi et al., 2016). *Fusarium* wilt is characterized by drooping leaves followed by chlorosis, necrosis, browning or discoloration of vascular tissues finally leading to the death of the plant. The three pathogenic races of *Fol* have been identified depending upon pathogenicity on tomato cultivars (Kawabe et al., 2005; Nirmaladevi et al., 2016).

2.2.2. Early blight

Early blight disease incited by fungal pathogen *Alternaria solani* (Ellis and Martin) Sorauer, is another destructive and frequently encountering disease of tomato occurring all over the world that cause qualitative and quantitative productivity loss (Jambhulkar et al., 2012; Joseph et al., 2017). Early blight has been recorded to claim upto 80% yield losses (Joseph et al., 2017). *A. solani* is a seed, soil and air borne pathogen that affects foliar parts of the plant (Jambhulkar et al., 2012). The catenate or muriform conidia are 12-20 x 35-75µm in size with 2-7 transverse septa, 1-4 longitudinal septa and a filiform long beak (Alhussaen, 2012; Chohan et al., 2015). Conidia are generally found either solitary or in the acropetal chain (Chohan et al., 2015). The pathogen can infect tomato plant during all growing stages, cultivated in any season resulting in qualitative as well as a quantitative loss (Sahu et al., 2013). The immense variability among various pathogenic isolates of *A. solani*, the prolonged phase of disease cycle and ability to infect a broad range of plant species makes their management difficult (Joseph et al., 2017).

The disease is characterized by bull's eye leaf spots which first appears on older leaves as small, isolated, scattered pale brown lesions and later on develop on

stem and fruits. Fully developed spots are irregular, brownish to black in color with concentric rings inside the spot. The production of melanin-like pigment on the infected host by the pathogen is one of the distinguishing characters (Chohan et al., 2015). The disease results in reduced photosynthesis rate and under severe condition cause defoliation of the entire plant (Chaerani et al., 2007). The disease severity enhances with an increase in wetness on the leaf surface and plants are more susceptible when the temperature ranges around 24-29˚C (Jambhulkar et al., 2012; Sahu et al., 2013).

2.3. Conventional methods for management of fungal pathogens of tomato

To increase agricultural productivity, since the green revolution the agricultural practices are heavily dependent upon synthetic agrochemicals. However, their excessive and injudicious use has damagingly affected environment and life on earth. Eutrophication of water bodies; contamination of soil and air with toxic residues and their incorporation in food chains are fate of application of agrochemicals that has threatened life of biodiversity; resulted in decline in soil micro flora and fauna and has increased resistance in pests and pathogens (Jain et al., 2016). This destructive effect is augmented by climate change that has been witnessed from last decade and is anticipated to continue in the future (IPCC, 2007). The continuous change in climatic conditions has enhanced the occurrence of extreme events of temperature, abrupt changes in rainfall pattern, soil salinity, loss of soil nutrients and incidence of pests and pathogens manifestation; heading toward severe biotic and abiotic stresses in plants.

Tomato is grown in both open fields and in greenhouse. In both conditions, they are attacked by various fungal pathogens that affect yield and quality of produce.

To check fungal pathogens fungicides are like Carbendazim, Mancozeb, Pyrimethanil, Iprodione, *etc*. are generally used at different growth stages by tomato growers (Zhu et al., 2016). Tomato is regarded as one of the major vegetable crops that registered for a maximum input of pesticides for management of pathogens (Ali et al., 2016). The tomato plants grown in greenhouse accumulate the higher level of toxic residues of pesticides as compared to tomatoes grown in open field due to prevailing conditions of greenhouse (Zhu et al., 2016). Extensive use of fungicides on tomatoes has also resulted in the development of resistance in phytopathogenic fungus (Zhu et al., 2016). The negative footprints of conventional agrochemicals and increasing awareness among consumers for residue-free food commodities has oriented toward sustainable food production approaches such as organic farming, Integrated Nutrition Management, and Integrated Pest Management practices. Use of natural occurring microbiota having beneficial effects on plant productivity is considered as one of the significant alternative approaches. In natural conditions, plants harbor enormous biota in or around roots called rhizosphere by offering nutrition to them in form of root exudates. Harnessing potential of such beneficial Rhizospheric microorganisms is considered eco-friendly and cost-effective breakthrough from conventional pesticides and fertilizers.

2.4. Rhizospheric microorganisms: a sustainable alternative for biotic stress management

Soil, the most influential component of plant ecosystem stimulates the plant growth and productivity. The narrow zone of soil surrounding plant roots termed as “Rhizosphere” is a tempting denizen for several soil-borne microorganisms influenced by the activity of plant roots (Podile et al., 2014). The microbial population in rhizosphere display competition as well as co-operation with other

existing living entities to sustain themselves. The beneficial rhizospheric microorganisms also are known as Agriculturally important microorganisms (AIMs) displaying plant-microbe relationship facilitate plant growth by acquisition of inaccessible nutrients, stimulating growth by synthesis of phytohormones and alleviating ecological stress (Singh et al., 2016) without any "trade-off" that otherwise can have a negative effect on the plant. The plant growth promoting rhizobacteria (PGPR), Plant growth promoting fungi (PGPF), biotic stress alleviating fungi and arbuscular mycorrhizal fungi (AMF) are some beneficial microorganisms that survive in rhizosphere as free-living entities (in the vicinity of roots either in soil or on root surface) or as endophytes. Exploitation of AIMs for sustainable agriculture is an efficient and cost-effective strategy. Interaction of agriculturally important fungi with plant demonstrates paramount importance in the sustainability of agriculture and ecosystem (Ansari et al., 2013).

2.4.1. Plant growth promoting rhizobacteria

Plant Growth Promoting Rhizobacteria (PGPR), the term was coined by Kloepper and Scroth (1978) for free-living and root-colonizing bacteria that includes species belonging to genus *Achromobacter, Acinetobacter, Aeromonas, Agrobacterium, Alcaligens, Azoarcus, Azospirillum, Azotobacter, Bacillus, Bradyrhizobium, Cellulomonas, Clostridium, Enterobacter, Erwinia, Flavobacterium, Frankia, Gluconacetobacter, Klebsiella, Methylobacterium, Microbacterium, Micromonospora, Paenibacillus, Pseudomonas, Rhizobium, Serratia, Streptomyces, Variovorax, Xanthomonas* (Niranjan and Hariprasad, 2014; Kumar et al., 2014) that possess ability to promote plant growth. PGPR show panoply of traits that embrace plant growth promotion, management of plant diseases, alleviation of abiotic stresses and degradation of xenobiotic compounds (Kloepper et al., 2004) which has received

global attention. These attributes of PGPR involve exhibition of array of mechanisms including well colonization of plant roots, antibiosis, competition for nutrition and niche, production of phytohormones, enzymes and metabolites; and/or induced systemic resistance (ISR) and Induced Systemic Tolerance (IST) (Yang et al., 2009; Almaghrabi et al., 2013).

Growth promotion, biocontrol and abiotic stress management traits of PGPR are studied well and cited in the literature. Strains of PGPR have shown positive effects on seed germination rate, tolerance to drought, weight of shoots and roots, yield, and plant growth under salt stress (Van Loon et al., 1998; Almaghrabi et al., 2013), fruit quality attributes such as size and texture of tomato (Mena-Violante and Olalde-Portugal, 2007), reduction in population size of root-knot nematodes, incidence of root galling, egg masses on root and damages caused (Almaghrabi et al., 2013). The PGPR strains, *Serratia* sp., fluorescent Pseudomonad, and *Bacillus* sp. were reported by Guo et al., (2004) to provide effective control against bacterial wilt of tomato as well as promoted plant growth under the different environmental condition and their formulated product retained biocontrol and plant growth promoting activities even after two years of storage.

2.4.2. Biocontrol and plant growth promoting fungi: *Trichoderma*

Trichoderma (teleomorph *Hypocrea*) is a ubiquitous genus, belongs to ascomycota division of fungi (Bae et al., 2016). Till present 1100 strains of *Trichoderma* including teleomorphic (*Hypocrea*) and anamorphic stages are identified from 75 molecularly characterized species (Contreras-Cornejo et al., 2016). The new strains are continuously being studied and recognized (Contreras-Cornejo et al., 2016). *Trichoderma* sustains its life under diverse climatic conditions and has wide

geographical distribution ranging from tropical climate to polar (Hermosa et al., 2004). *Trichoderma* strains are filamentous, saprophytic fungi that proliferate freely in soil and/ or show a symbiotic relation with plant roots and foliar parts (Reino et al., 2008). *Trichoderma* spp. is an important player in agriculture that poses potential to act as biocontrol agents (Saber et al., 2017), plant growth promoter (Hermosa et al., 2012) and has been recognized as Plant Growth Promoting Fungi (PGPF) (Doni et al., 2013). The role of *Trichoderma* spp. in physiological stress alleviation (Shoresh et al., 2010) and soil bioremediation (Tripathi et al., 2017) is also well documented in the literature. Harnessing the potential of *Trichoderma* spp. efficiently can reduce dependency on agrochemicals. The principle biocontrol attribute of *Trichoderma* is believed to involve mechanism of mycoparasitism, antibiosis and niche exclusion, however, other indirect mechanisms of induced systemic resistance and growth promotion are also acknowledged to play a crucial role in biocontrol as well as in amelioration of abiotic stresses; enhancing plant growth and yield (Bae et al., 2016; Youssef et al., 2016). Another mechanism that enhances resistance against biotic and abiotic stress is production of enzymes such as superoxide dismutase, catalase and peroxidase involved in scavenging of reactive oxygen species (ROS) (Shoresh et al., 2010; Chowdappa et al., 2013; Youseef et al., 2016), that are produced in high concentration during plant growth under stressful conditions, having damaging effects on plants (Mastouri et al., 2012).

Trichoderma spp. are versatile fungi with diverse genetic variability, ubiquitousness, synthesize variety of secondary metabolites, colonize substrate effectively and grow under extremes of climatic regimes, show rapid growth, establish chemical communication with other soil microbiota and plants and posses

ability to serve as plant's safeguard and growth promoter (Contreras-Cornejo et al., 2016; Kashyap et al., 2017).

2.5. Mechanisms employed by rhizospheric microorganisms

The biocontrol potential of PGPR and *Trichoderma* spp. is an attribute that involves mechanisms of antibiosis, parasitism, competition for nutrition and space, modification in environmental conditions and induced systemic resistance (Reino et al., 2008; Gupta et al., 2015; Saravanakumar et al., 2016). These mechanisms either act singularly or synergistically against pathogens after mutualistic association between *Trichoderma*/PGPR and plant. The biocontrol activity of PGPR and *Trichoderma* depends on their strain, host plant, pathogen and prevailing environmental conditions including temperature, availability of nutrients, the concentration of iron and pH (Benitez et al., 2004; Gouda et al., 2018). Activation of these mechanisms involves production of several metabolites and enzymes such as cell wall degrading enzymes (CWDE), siderophores, carbon and nitrogen permeases, cyanide etc. (Sharma et al., 2012; Gupta et al., 2015) and efficient root colonization (Harman, 2011; Gupta et al., 2015). Production of metabolites (Marra et al., 2006) and root colonization (Lakshmanan et al., 2013) by *Trichoderma* spp. and PGPR brings changes in gene expression of the host plant, responsible for providing protection or responding toward stress. In-depth understanding of mechanisms and expression of genes involved in regulation of mechanisms is pre-requisite for harnessing the potential of PGPR's and *Trichoderma* species effectively for sustainable agro-ecosystem.

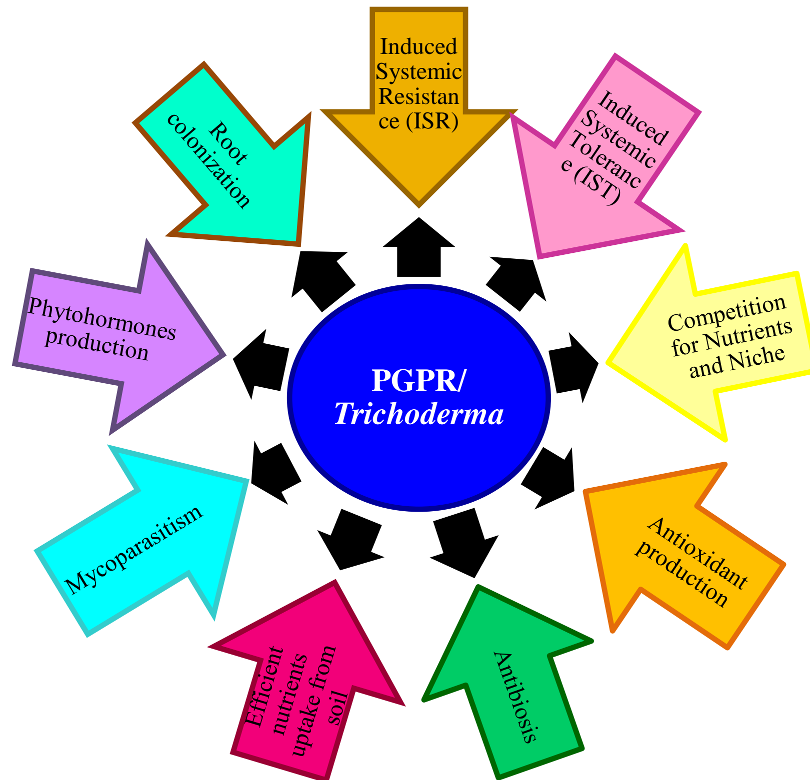


Figure 2.2: The Multifacet traits of PGPRs and *Trichoderma* spp. for plant growth promotion and alleviation of ecological stress

2.5.1. Root colonization

Root colonization by PGPR and *Trichoderma* is prior among all mechanisms that determine their efficiency to increase plant growth, biocontrol potential and ability to induce tolerance against abiotic stress (Benitez et al., 2004; Gupta et al., 2015). The root exudates produced by plants are a major attractant for PGPR and *Trichoderma* spp. as a source of nutrition to establish themselves in the rhizosphere (Hermosa et al., 2012; Yuan et al., 2015). The pathogenic soil-borne microorganisms primarily colonize plant roots to initiate infection, however, colonization of plant roots by beneficial microorganisms prior to pathogens results in competition for ecological niche leading to the elimination of later. Root colonization by beneficial rhizospheric microorganisms is attained by recognizing and adhering to the root surface,

penetrating in root, establishing communication with the plant via chemical signals *i.e.,* chemotaxis (Harman, 2011) and enduring presence of toxicants produced by plants due to their invasion (Benitez et al., 2004; Hermosa et al., 2012). The adherence to root surface is mediated by extracellular polymeric substances (Yuan et al., 2015), hydrophobins and expansin-like proteins, peptides and cell wall degrading enzymes (Hermosa et al., 2012). For successful colonization, microbial species proliferate along with growth and expansion of plant roots and form biofilm (Yuan et al., 2015).

Several studies provide evidence for the role of root colonization by beneficial microorganisms in the alleviation of biotic and abiotic stress in plants. Mastouri et al., (2012) in their work demonstrated the effect of colonization of tomato roots by *T. harzianum* T-22 on antioxidant production activity of the plant. The gene expressions encoding for antioxidant enzymes (Superoxide dismutase, Ascorbate –glutathione (ASC-GSH) cycle enzymes) were found to be significantly increased that enhanced redox buffering capacity and plant's ability to tolerate a wide range of environmental stresses such as water deficit, sub-optimal temperature, and salinity. Similarly, inoculation of *T. harzianum* NBRI-1055 to sunflower confers protection against *Rhizoctonia solani* by reprogramming oxidant and antioxidant compounds (Singh et al., 2011).

Inefficient plant roots colonization by biocontrol strains of PGPR have been reported to show inconsistent performance in the management of soilborne diseases (Schippers et al., 1987). An inverse correlation between a number of beneficial bacteria and number of diseased plants has been documented by Bull et al., (1991), depicting correlation of inefficient root colonization on reduced biocontrol potential of bacteria (Schippers et al., 1987). In support of this, the study of Guo et al., (2004)

suggested that PGPR strains that possess the ability to produce inhibition zone and colonize roots efficiently can be promising biocontrol agents.

2.5.2. Mycoparasitism

It is one of the major mechanisms displayed by *Trichoderma* spp. against soil-borne pathogens and extensively exploited in agriculture. The mechanism of mycoparasitism is a complex process that involves the direct physical interaction of *Trichoderma* with pathogens followed by secretion of antibiotics and cell wall degrading enzymes (CWDE) such as chitinase, β-glucanase, and protease (Benitez et al, 2004; Sarvanakumar et al., 2016). Several events occur during this process that includes recognization of pathogens, attack, penetration, and finally lysis of host-pathogen. During mycoparasitism *Trichoderma* recognizes the host pathogens through signal received from host-pathogen, coil around it and produces appressoria like structure, which with the help of cell wall degrading enzymes disintegrate cell wall polysaccharides and penetrate inside the host cytoplasm, utilizes its cell content as a source of nutrition and finally cause death of the pathogen (Troian et al., 2014; Saravanakumar et al., 2016). Mycoparasitic activity of *Trichoderma* has been reported against several pathogens like *Alternaria alternate*, *Sclerotinia sclerotiorum, Rhizoctonia solani, Pythium* spp.*, Fusarium* spp.*, Sclerotium rolfsii*, root-knot nematodes (Troian et al., 2014; Contreras-Cornejo et al., 2016).

2.5.3. Antibiosis

Antibiosis involves low molecular weight diffusible volatile and non-volatile secondary metabolites with antibiotic properties that are toxic to other microorganisms and impedes their growth (Vinale et al., 2014). It is an important mechanism employed by PGPR and *Trichoderma* spp. to curtail the proliferation of

phytopathogens. PGPRs and *Trichoderma* spp. secrete several secondary metabolites such as siderophores, Hydrogen cyanide (HCN), fungal cell wall degrading enzymes (CWDEs) and antibiotics preferably, volatile organic compounds (VOCs) and diffusible compounds, that play significant role in plant disease suppression by phenomenon of antibiosis and competition for niche and essential nutrients such as iron, which are considered as plausibly effective mechanisms against pathogens (Vinale et al., 2014; Shrestha et al., 2015). CWDEs secreted by PGPRs such as proteases, lipases, chitinase, and β-1,3-glucanase, are found to be involved in lysis of fungal cell by disintegration of cell wall (Shrestha et al., 2015) and degradation of gut lining of insects by chitinase (Sharp, 2013). Chitinase produce by *Cellulosimicrobium cellulans* 191 and *Bacillus licheniformis* participate in biocontrol of pathogenic fungi by degrading their cell wall (Fleuri et al., 2009; Xiao et al., 2009). *Pseudomonas cepacia* strain producing β-1,3-glucanase was found to restrict the growth of many pathogenic fungi in rhizosphere counting *Rhizoctonia solani*, *Pythium ultimum* and *Sclerotium rolfsii* (Fridlender et al., 1993). Chitinase, protease and β-1,3-glucanase activity of *Bacillus thuringiensis* C25 disintegrated and lysed hyphae of *Sclerotinia minor* and *S. sclerotiorum* (Shrestha et al., 2015).

Trichoderma species are able to produce variety of volatile and non-volatile compounds such as Nitrogen heterocyclic compounds (harzianic acid), peptaibols (alamethicin), tricholin, viridins (viridin, viridiol), diketopiperazines (gliovirin, gliotoxin), pyrones (6-pentyl-α-pyrone), terpenoids, trichothecene and others which are capable to inhibit growth of various microorganisms (Vinale et al., 2014; Contreras-Cornejo et al., 2016). The metabolites produced by *Trichoderma* help plants to withstand in presence of pathogens and stimulate plant defense mechanism and promote plant growth (Vinale et al., 2012). The metabolites produced by

Trichoderma vary from isolates to isolates and even within species (Vinale et al., 2009a). Similar to *Trichoderma* spp., the PGPRs species preferably, *Bacillus* and *Pseudomonas* which are cosmopolitan in their occurrence, synthesize the diversity of secondary metabolites with antibiotic activity. Several species of *Bacillus* such as *B. subtilis, B. amyloliquifaciens, B. cereus, B. licheniformis, B. mycoides, B. pumilus* and *B. megaterium* inhibit growth of phytopathogens by secreting many toxic compounds like catabolic enzymes, peptide and antibiotics (Yu et al., 2002; Choudhary and Johri 2008). Iturin A and Surfactin secreted by *Bacillus subtilis* and Zwittermicin A synthesized by *B. cereus* are documented to suppress damping off of Tomato and alfalfa, respectively (Choudhary and Johri, 2009).

The secondary metabolites isolated from *T. harzianum* such as Harzianic acid, Harzianolide, T39butenolide, T22azaphilone, Harzianopyridone have shown marked inhibitory effect on mycelia growth of various fungal phytopathogens (*Rhizoctonia solani*, *Pythium ultimum*, *Gaeumannomyces graminis* var. *tritici*, *Pythium irregular*, *Sclerotinia sclerotiorum*, *Botrytis cinerea*) (Vinale et al., 2009b; Vinale et al., 2014). Fluorescent *Pseudomonas* producing a variety of metabolites such as HCN, Phloroglucinol, pyoluteorin, and pyrrolnitrin are instrumental in disease suppression (Maurhofer et al., 1992). Secretion of 2,4-diacetylphloroglucinol (DAPG), a polyketide antibiotic by strains of fluorescent *pseudomonas* are reported to play significant role in reduction of plant diseases (Ahmadzadeh and Tehrani, 2009), demonstrated by suppression of *Rhizoctonia solani* causing damping off disease in common bean under *in vitro* as well as green house condition by 2,4-DAPG, siderophore and proteolytic enzyme producing strains of fluorescent *pseudomonas*, UTPF 16 and UTPF 26 (Ahmadzadeh and Tehrani, 2009). This result was supported by an experiment where a genetic mutant of biocontrol strain of *Pseudomonas*

fluorescens 2P24 defective in 2,4-DAPG was incapable of controlling soil-borne diseases of plants (Zhang, 2011).

Volatile compounds secreted by PGPR reported to inhibit the growth of pathogens and provide tolerance against environmental stress. *Pseudomonas chlororaphis* O6 strain producing volatile compounds, 2R, 3R-butanediol showed tolerance against biotic and abiotic stress in *Arabidopsis thaliana* (Cho et al., 2008). The effect of volatile compounds and culture filtrate of two strains of *Pseudomonas* PS1 and PS2 evaluated under *in-vitro* conditions that showed restriction of colony growth of pathogen *Macrophomina phaseolina* by volatile compounds to 25 and 32%, respectively and 57 and 61%, respectively, by culture filtrate at 20% concentration (Bhatia et al., 2003).

The secondary metabolites are often reported to act synergistically with cell wall degrading enzymes (Blaszczyk et al., 2014). The dual effect of lytic enzymes and antibiotics enhance the antagonistic activity of biocontrol agents suggesting synchronization between the mechanism of mycoparasitism and antibiosis in pathogen control (Li et al., 2016). *Trichoderma* is known to exploit hydrolytic enzymes and secondary metabolites simultaneously to antagonize target microorganisms. Synergism of lytic enzymes and secondary metabolites of *T. harzianum* have been reported against fungal pathogens (Howell, 1998).

2.5.4. Competition

Competition for space and nutrition is an important mechanism for biocontrol of phytopathogens. *Trichoderma*, as well as PGPRs, are efficient competitors for limited nutrients that deprive other organisms from essential life-sustaining nutrients, leading to starvation, resulting in death and elimination (Dwivedi and Johri, 2003; Benitez et

al., 2004; Loaces et al., 2011). Competition for a nutrient is the best mechanism for controlling the growth of phytopathogen *Botrytis cinerea*, which is sensitive to low nutrient level (Benitez et al., 2004). Iron is an essential micronutrient required for the growth and viability of organisms. During limited availability of iron in the soil, microorganism secret low molecular weight iron chelating compound-siderophores that binds with unavailable forms of iron (III) and regulate its availability in the rhizosphere (Vinale et al., 2013). The competition for iron binding in the rhizosphere is determined by the affinity of siderophores for iron (Vinale et al., 2013). The iron complexes formed by chelation are recognized and taken up by plant species and this plays an important role in satisfying the requirement for iron by plants, mainly in calcareous soil (Vinale et al., 2013). Some PGPRs and *Trichoderma* strains are efficient siderophore producers that chelate iron and check the growth of other organisms (Dwivedi and Johri, 2003; Benitez et al., 2004). *Trichoderma harzianum* produces harzianic acid that binds with Fe^{3+}, making it soluble and available for plants and influenced the growth of plant colonized by *T. harzianum* even under iron-deficient condition (Vinale et al., 2013).

Burkholderia colonizing inside young rice plant prevented infection of *Sclerotium oryzae* and *Rhizoctonia oryzae* (Loaces et al., 2011). Catechol type siderophore secreted by *Azospirillum brasilens* REC2 and REC3 inhibited the growth of *Colletotrichum acutatum* M11 and reduced anthracnose disease severity on the strawberry plant under *in vitro* and *in planta* condition, respectively (Tortora et al., 2011). Many PGPRs secrete siderophores, however, Pseudomonads are leading producers (Sharma and Johri, 2003). Siderophore pyoverdin produces by *Pseudomonas* species poses ability to suppress the growth of *Pythium* induced post-emergence damping-off of seedlings in many crops (Buysens et al., 1996). The

mutant strain *P. aeruginosa* 7NSK2 deficient either in siderophore pyoverdin or pyochelin, or both, when compared to wild strains showed that one of these siderophores are essential for antagonism of *Pythium* causing damping-off in tomato (Buysens et al., 1996).

2.5.5. Induced Systemic Resistance

In natural ecological conditions, plants suffer from multiple biotic stresses and respond to such complex situation in an integrated manner by adopting a mechanism of Induced resistance or enhanced state of defense. Induced resistance is an additional mechanism displayed against pathogenic or pest attack that involves activation of signal transduction pathway. This signaling pathway involve four hormones: Salicylic acid (SA), Nitric oxide (NO), Jasmonic acid (JA) and Ethylene (ET) (Choudhary and Johri, 2009; Shavit et al., 2013). ISR activated by non-pathogenic strains of microorganism enhance plant's "immunity" against phytopathogens and pests *via* Jasmonic acid (JA) and Ethylene (ET) signal pathways.

Table 2.1: Summary of the mode of action displayed by PGPRs and *Trichoderma* for management of biotic stress

PGPR/*Trichoderma* spp.	Target plant	Diseases/Pathogen	Mode of action	References
Trichoderma asperellum CCTCC-RW0014	Cucumber	*Fusarium oxysporum* f. sp. *cucumerinum* (FOC)	Production of CWDE and secondary metabolites	Saravanakumar et al., 2016
B. thuringiensis	-	*Sclerotinia minor*, *S. sclerotiorum*	Chitinase, Protease, β- 1,3-glucanase	Shrestha et al., 2015
T. viride	Wheat	*Fusarium oxysporum*	Production of antioxidants	Mohapatra and Mittra, 2017
Trichoderma atroviride/ *petersenii* KACC 40557	Pepper and tomato	*Phytophthora* spp.	Antibiosis, Induced systemic resistance genes and plant hormonal changes	Bae et al., 2016
Trichoderma harzianum	Tomato	*Rhizoctonia solani*	Production of ROS scavenging antioxidant enzymes	Youssef et al., 2016
Trichoderma harzianum ALL42	-	*Rhizoctonia solani* and *Macrophomina phaseolina*	Mycoparasitism by the production of CWDE	Monteiro et al., 2010
Pseudomonas aeruginosa 2apa	Tomato	Root and foliar pathogens	ISR, production of antimicrobial compounds	Hariprasad et al., 2014
P putida S1; *P. aeruginosa* cgr	Chickpea	*Sclerotinia sclerotiorum*; Salinity stress	HCN, IAA, siderophore, defense enzymes, and phenolic compounds	Sarkar et al., 2014
P. fluorescens WCS417r	Arabidopsis thaliana	Bemisia tabacci, necrotrophic pathogen	ISR	Shavit et al., 2013; Van Oosten et al., 2008
Fluorescent *Pseudomonas* CMR12a	Bean	Root rot-*Rhizoctonia solani*	Lipopeptide and antibiotic	D'aes et al., 2011
Pseudomonas fluorescens	Barley	Head blight- *Fusarium*	IAA	Petti et al., 2012
Pseudomonas fluorescens	Wheat, Tomato	Take all disease- *Gaeumanomyces graminis* var. *tritici*; Tomato bacterial wilt	Quorum sensing via *pcoR* and *pcoI* system	Zhang 2011; Wei and Zhang, 2006

PGPR/*Trichoderma* spp.	Target plant	Diseases/Pathogen	Mode of action	References
Azospirillium brasilens REC2 and REC3	Strawberry	Anthracnose-*Colletotrichum aculatum* M11	Catechol type siderophores, salicylic acid	Tortora et al., 2011
Cellulosimicrobium cellulans 191*; B. licheniformis*	-	Pathogenic fungi	Chitinase	Fleuri et al., 2009; Xiao et al., 2009
T. longibrachiatum	Onion	*Fusarium oxysporum* f. sp. *cepa* and oxidative stress	Antioxidant production and induce systemic resistance	Abdelrahman et al., 2016
T. harzianum/ T. atroviride	Grapes (*Vitis vinifera*)	*Uncinula necator*	Secondary metabolites, antioxidants and polyphenols production	Pascale et al., 2017
Fluorescent *Pseudomonas* UTPF16; UTPF26	Common bean	Damping off-*Rhizoctonia solani*	2,4-DAPG, siderophores, proteolytic enzymes	Ahmadzadeh and Tehrani, 2009
Bacillus subtilis, B. cereus	Tomato Alfalfa	Damping off	Iturin A, Surfactin, Zwittermicin A	Choudhary and Johri, 2009
P. fluorescens CHAO	Tomato	Root-knot nematode- *Meloidogyne javanica*	HCN	Siddiqui et al., 2006
Pseudomonas PS1 and PS2	-	*Macrophomina phaseolina*	Volatile and diffusible metabolites	Bhatia et al., 2003
Pseudomonas aeruginosa 78	-	Root-knot nematode- *Meloidogyne javanica*	Heat liable polar substance; Antibiotic 2,4-DAPG mediated ISR	Siddiqui and Shaukat, 2003
Fluorescent *Pseudomonas* RBL101, RSI125	-	Bacterial wilt	Fluorescent siderophores	Jagdeesh et al., 2001
Bacillus subtilis	Chickpea	Fusarium wilt-*Fusarium oxysporum* f. sp. *ciceris*	Antifungal antibiotic	Kumar, 1999
Burkholderia cepacia	Cotton	Damping off- *Rhizoctonia solani*	Novel lipopeptide antibiotic (AFCBC11)	Kang et al., 1998
Pseudomonas aeruginosa 7NSK2	Tomato	Damping off and Root rot-*Pythium* sp.	Siderophores-pyochelin, pyoverdin, salicylic acid	Buyens et al., 1996

Many strains of PGPR including *Bacillus* spp., *Pseudomonas* spp., *Serratia marcescens* and *Trichoderma* spp. are reported to induce ISR against many foliar pathogens (Choudhary and Johri, 2009; Segarra et al., 2009). The *Pseudomonas* sp. and *Bacillus* sp. are reported to induce systemic resistance in plants against root-knot nematodes (Kloepper and Ryu, 2006).

2.5.6. Plant growth promotion

Negatively stimulated plant growth results in a deteriorated yield of agricultural produce qualitatively and quantitatively. Many soil-dwelling microorganisms possess the ability to promote plant growth in numerous ways such as by facilitating nutrients uptake, enhancing root growth and development, phytohormones production and alleviating ecological stress. PGPRs and *Trichoderma* are some soil-dwelling microbes that acquire capacity to colonize plant roots (Mendis et al., 2018) and subsequently stimulate plant growth and yield production by facilitating nutrient uptake/enhanced fertilizers use efficiency and increased seed germination rate (Pavlova et al., 2017; Kashyap et al., 2017). PGPRs and *Trichoderma* on colonizing plant roots grow along the root length with continuous growth, mediate positive alteration in root architecture and provide anticipated beneficial effects including better anchorage, enhanced water and nutrients uptake/use efficiency (Contreras-Cornejo et al., 2009; Pavlova et al., 2017).

Nutrient like Phosphorus, Nitrogen, Potassium and other micronutrients (Fe, Mn, Cu, and Zn) are essential for balanced plant growth. Unavailability or inaccessibility to these nutrients leads to productivity loss. Several soil inhabiting microbes including *Trichoderma* and plant growth promoting Rhizobacteria (PGPR) have the capacity to solubilize insoluble phosphate and zinc, mineralize organic

phosphate, forming iron chelates, *etc.* and making them accessible for plant's uptake (Li et al., 2015). These microorganisms catalyze many chemical and biochemical reactions by altering soil pH (Li et al., 2015). Li et al., (2015) investigated the role of a commercially available strain of *Trichoderma*, *T. harzianum* SQR-T037 in improving nutrient uptake (P, Mn, Zn, Fe, and Cu) by tomato plant and the mechanisms govern their PGP activity. The results revealed that the strain studied was able to solubilize insoluble compounds of iron, zinc, and phytate, however, unable to solubilize phosphate and manganese insoluble compound in *vitro* condition thus when inoculated with tomato seedlings in hydroponic condition, improved biomass in copper-deficient condition, did not showed significant effect in iron and zinc deficient condition whereas competed and suppressed seedling growth in phosphorus deficient condition. Concomitantly under glasshouse, treatment of bacterial isolate *Brevibacterium frigrotolerans* was observed to increase growth and nutrient uptake by wheat (Tara and Saharan, 2017).

The volatile metabolites of many isolates of *Trichoderma* also possess the potential to enhance plant growth. The volatile metabolites of *Trichoderma harzianum* and *T. asperellum* were found to participate in activation of Fe uptake in Arabidopsis and Tomato by modulating expression of transcription factor *MYB72* (Martinez-Medina et al., 2017). A parallel study undertaken by Hung et al., (2013) demonstrated increased biomass and chlorophyll content of *Arabidopsis thaliana* by the volatile metabolites produced by *Trichoderma viride*. Concomitantly, the growth of Arabidopsis was positively influenced by the volatiles produced by *T. virens* Gv29., *T. asperellum* LU1370, *T. atroviride* IMI206040 and *T*. sp. "*atroviride B*" LU132 (Nieto-Jacobo et al., 2017).

Plant hormones play a crucial role in plant growth promotion and development under normal and stressed environmental circumstances (Peleg and Blumwald, 2011). Auxin, cytokinins, ethylene, and abscisic acid are major plant hormones that regulate the growth and development of plants (Matinez-Medina et al., 2014). There are several pieces of evidence lined in the literature that indicates production of phytohormones by several microorganisms lead to plant morphogenesis (Matinez-Medina et al., 2014). Some *Trichoderma* spp. are known to synthesize growth regulating metabolites that alter phytohormonal network of host plant leading to growth promotion as well as resistance to diseases (Hermosa et al., 2013; Matinez-Medina et al., 2014).

IAA, most abundantly occurring auxin compound is an important player in plant growth and known to be responsible for lateral root growth and root hairs development in plants. An increase in auxin (IAA) content and a decrease in cytokinin and abscisic acid contents in shoots of melon were observed to be responsible for increased plant fitness (Martinez-Medina et al., 2014). Contreras-Cornejo et al., (2009) also detailed out the role of IAA synthesized by *Trichoderma viride* in enhanced growth of *Arabidopsis thaliana*. The crude extracts of *Trichoderma* spp. showing the presence of IAA, the improved growth potential of Quinoa with grain yield, lettuce and radish (Ortuno et al., 2017). Analogously, Indole Acetic Acid (IAA) produced by PGPR have the ability to alter root architecture (Kloeper et al., 2007) that result in increased number of root tips and surface area for deep anchorage in soil and better uptake of nutrients and water that provides resistance against nutrient deficiency, drought and salt stress. Egamberdieva et al., (2015) in their study reported, PGPR strain *Pseudomonas putida* R4 and *Pseudomonas chlororaphis* R5 producing IAA mediated alleviation of salt stress and biocontrol of *Fusarium solani* causing root

rot of cotton under the gnotobiotic condition and recorded improved chlorophyll, malondialdehyde, proline, and soluble sugar content. The PGPR *Pseudomonas fluorescens* having ability to produce IAA has reported controlling head blight disease of barley caused by *Fusarium* (Petti et al., 2012). Cytokinin also reported increasing shoot biomass in lettuce grown in drying soil when inoculated with *Bacillus subtilis* producing cytokinin (Arkhipova et al., 2007).

2.5.7. Alleviation of abiotic stress

In natural conditions plants are frequently encountered with several abiotic stresses such as salinity, acidic condition, drought (water deficiency), flooding (anoxia), temperature fluctuation (heat/chilling stress), poor nutrition level in soil, light intensity, toxic levels of heavy metals which causes nutrient deficiency, oxidative stress, early senescence, impaired seed germination, plant dehydration, membrane dysfunction, photosynthesis inhibition, disturbed ion and osmotic homeostasis in plants (Contreras-Cornejo et al., 2014; Meena et al., 2017). Under abiotic stress conditions, plants become more susceptible to phytopathogens. Abiotic stress bring about changes in the physiological process of plants causing imbalance in plant hormones level, reduced photosynthesis and protein synthesis that ultimately leads to reduced plant growth (Egamberdieva et al., 2015) and this was also ascertained by Triky-Dotan et al., (2005) by demonstrating enhanced root rot severity in tomato caused by *Fusarium oxysporum* f. sp. *Radicis lycopersici* when irrigated with saline water having EC= 4.6dSm.

Plants are gifted with intrinsic capabilities to cope up with environmental stresses which work on the cost of their growth and productivity resulting in “trade-off” (Huot et al., 2014). Plant under stress (biotic and abiotic) conditions switch

production of reactive oxygen species (ROS), ethylene production, a shift in hormones production and deficiency of nutrients required for normal growth. These ROS provide tolerance to unfavorable conditions and play a significant role in signal transduction (Jaspers and Kangasjarvi, 2010). ROS limit growth of pathogens and provide protection by the production of compounds such as phytoalexin, pathogenesis-related proteins, strengthening plant cell wall, *etc*. (Vicente et al., 2014). However, the ROS are considered toxic molecules that cause oxidative damage in plants. To prevent oxidative damage the plant constitutes antioxidant machinery (comprising several enzymatic compounds such as peroxidase, catalase, ascorbate-glutathione (ASC-GSH) cycle enzymes, *etc*. and non-enzymatic compounds) which controls production and utilization of ROS (Vicente et al., 2014). The soil natural microbial biome support plants to render effect of abiotic stresses by evoking resistance in plants termed as Induced Systemic Tolerance (IST) that improve metabolic activity (Meena et al., 2017), thereby clinch plants from reaching to deteriorating level of trade-off.

Colonization of plant roots by *Trichoderma* are reported to fortify antioxidant production system of plants, thereby alleviating oxidative damages occurred due to biotic and biotic stress conditions (Vicente et al., 2014). The increase in antioxidant activity and ascorbic acid content in *Brassica rapa* plant treated with *T. harzianum* TM10 were reported by Gallo et al., (2013). Jain et al., (2015) reported biocontrol agents (BCA) *Trichoderma harzianum* TNHU27, *Pseudomonas aeruginosa* PJHU15 and *Bacillus subtilis* BHHU100 both alone or in consortium provided protection against *Sclerotinia sclerotiorum* and reduced pea plant cell death. This protection was mediated *via* modulation of oxidative burst by suppressing production of oxalic acid and partially by enhancing production of hydrogen peroxide (H_2O_2). The PGPR

Pseudomonas mendocina when inoculated with lettuce found to augment production of antioxidant catalase (CAT) under severe drought condition reducing oxidative damage (Kohler et al., 2008). The *B. subtilis, Bacillus amyloliquefaciens*, *B. thuringiensis*, *B. licheniforms,* and *Paenibacillus favisporus* inoculated maize seedlings under drought conditions were found to have high proline content indicating upregulation of proline biosynthesis to maintain cell water content, protein and protection of cellular membrane, thereby reducing oxidative damage and high soluble starch content by degradation of starch for osmotic adjustment to alleviate drought stress whereas the antioxidant enzymes activity was found to be reduced (Vardharajula et al., 2011). Co-inoculation of *Rhizobium* and *Pseudomonas* with *Zea mays* was instrumental in salt tolerance by increasing proline content, reducing electrolyte leakage, maintained Relative water content (RWC) of leaves and selective K^+ uptake by the plant (Bano and Fatima, 2009).

2.6. Biosurfactant: Microbial bioactive compounds

The amphiphilic surface active substances synthesized by bacteria, actinomycetes, fungi, yeast, and plants are known as Biosurfactants (Thavasi et al., 2014; Sarubbo et al., 2015; Das et al., 2017). Biosurfactants show diversity in chemical structures and properties (Sarubbo et al., 2015). They are ionic, eco-friendly, ecologically compatible, less toxic, highly specific and biodegradable compounds that show better tolerance and stability than synthetic surfactants at extreme conditions of temperature, pH (ranging 2-12) and salt concentration (up to 10%) (Sachdev and Cameotra, 2013; Thavasi et al., 2014; De et al., 2015; Sarubbo et al., 2015; Das et al., 2017; Liu et al., 2018). Biosurfactant found applicability in management of phytopathogens as antimicrobial agent, microbial growth enhancement, positively improve plant-microbe interaction resulting in indirect plant growth promotion, remediation of heavy metal

and petroleum contaminated soil, in pharmaceuticals, cosmetic and food industries (Sachdev and Cameotra, 2013; Thavasi et al., 2014; De et al., 2015; Das et al., 2017). The multiple ecological services provided by biosurfactant is demonstrated in **figure 2.3.**

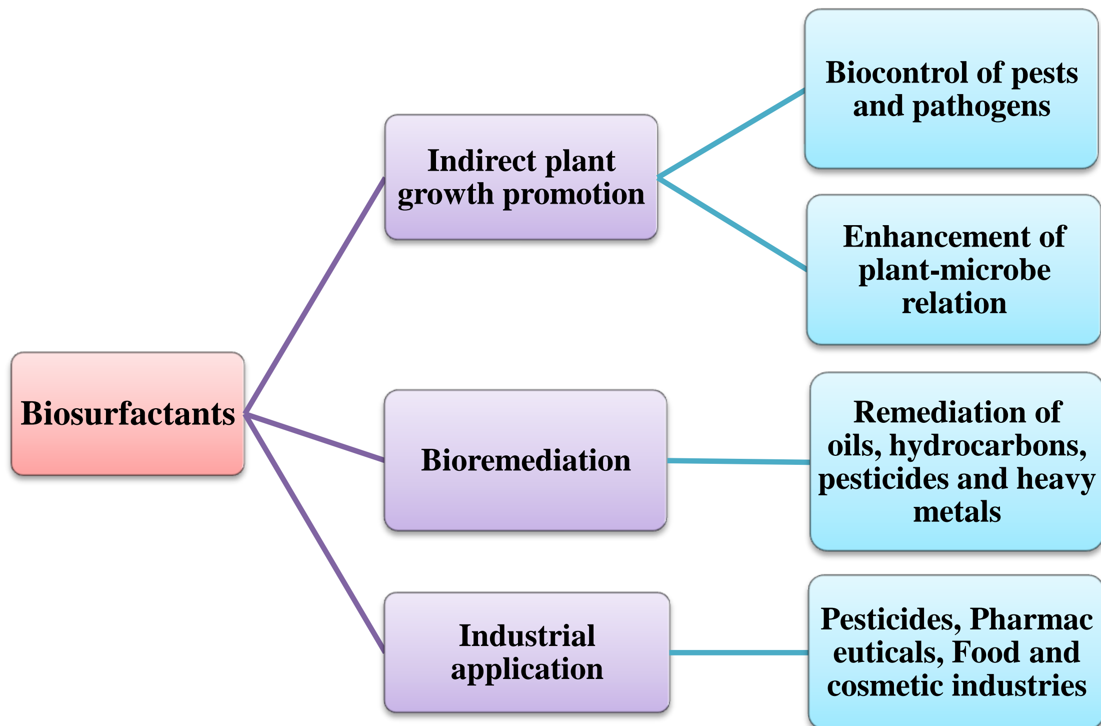


Figure 2.3: Multiple beneficial services provided by Biosurfactants

Biosurfactants are classified into two categories on the basis of molecular mass as low molecular mass biosurfactant including glycolipid, phospholipid, and lipopeptides; and high molecular mass biosurfactant *i.e.,* polymeric and particulate biosurfactants (Das et al., 2017). The example of some biosurfactants and their source microorganisms are: glycolipid biosurfactants such as rhamnolipid and sophorolipids are produced by bacterial species *Pseudomonas aeruginosa* and yeast *Candida* spp., respectively; lipopeptides *i.e.,* viscosin, surfactin, and polymyxin are produced by bacteria *Pseudomonas fluorescens*, *Bacillus subtilis* and *B. polymyxia*, respectively;

Phospholipid biosurfactant produced by *Thiobacillus thiooxidans*; Polymeric biosurfactant such as Emulsan and Alasan are produced by *Acinetobacter calcoaceticus* and *A. radioresistens*; and Particulate biosurfactants like Vesicles by *Acinetobacter calcoaceticus* (Sarubbo et al., 2015).

2.6.1. Biosurfactant as a biocontrol agent

The biosurfactant synthesized by rhizobacteria have been documented as biocontrol agents (Nihorimbere et al., 2011; Sachdev and Cameotra, 2013). The biosurfactant can curb the growth of both pathogens and pests affecting crop productivity (Krzyzanowska et al., 2012). Rodriguez and Mahoney (1994) demonstrated the inhibitory effect of surfactant on the production of aflatoxin by *Aspergillus* sp. responsible for causing post- and pre-harvest infection in many plant crops. The lipopeptide biosurfactant produced by *Bacillus* was reported by Velho et al., (2011) to inhibit the growth of many pathogenic fungi like *Fusarium*, *Aspergillus*, *etc*. The green surfactants (biosurfactants) are also known to accelerate biocontrol mechanisms of beneficial microorganisms such as antibiosis, induced systemic resistance, parasitism, etc. and provide conditions that support their proliferation resulting in improved biocontrol potential (Zhang et al., 2011; Sachdev and Cameotra, 2013). The combined application of surfactant with fungus *Myrothecium verrucaria* has been documented to cause eradication of weed *Pueraria lobata* (Kudzu) from the agricultural field (Boyette et al., 2002). Biosurfactants can regulate motility, signaling, differentiation, and biofilm formation process of microorganisms (Ron and Rosenberg et al., 2001) and increase the availability of nutrients thereby promote plant growth (Sachdev and Cameotra, 2013).

The *Pseudomonas* spp. are known to produce rhamnolipid, a glycolipid biosurfactant (Hajfarajollah et al., 2015). Rhamnolipid possess the ability to emulsify oil and reduce water surface tension (El-Sheshtawy and Doheim, 2014); control process of Quorum sensing in *Pseudomonas* spp. (Dusane et al., 2010); improve plant growth by reducing the deleterious effects of pathogens, pests and increasing the availability of nutrients by solubilizing hydrophobic compounds (Paulino et al., 2016; Tiso et al., 2017). Rhamnolipids are considered as potential new generation biopesticides. *In vitro* study revealed the antimicrobial activity of rhamnolipid against *Fusarium verticillioides* responsible for causing stalk and ear rot in maize (Borah et al., 2016). Rhamnolipid inhibited the growth of *F. verticillioides* by damaging mycelia of the pathogen. Analogous to this observation, application of rhamnolipid was recorded to curb the growth of fungal pathogen *Fusarium oxysporum* responsible for causing *Fusarium* wilt in tomato by Deepika et al., (2015). Several workers have also identified effectiveness of rhamnolipid in inhibition of zoospore formation by phytopathogens that have developed resistance to conventional pesticides in due course of time and boost plant's immunity against plant pathogens (Tiso et al., 2017). Debode et al., (2007) found viability inhibition of *Verticillium* spp. by use of biosurfactants produced by *P. aeruginosa* under *in vitro* condition. Rhamnolipid have shown priming effect on seeds hence they could be used as priming agents and stimulate plant immune system resulting in the development of resistance against pathogens (Sanchez et al., 2012). The combination effect of rhamnolipid with microbial inoculants (*Phanerochete chrysosporium* and *Azotobacter chrococcum*) was found to be stimulatory on growth rate and number of earthworms (*Eisenia foetida*) that resulted in the production of improved quality of vermicompost over individual treatment (Gong et al., 2017).

2.7. Consortia: way to enhance the potential of biopesticides

In natural conditions, plants often exist in communities (Vacheron et al., 2013). This community structure many times on interaction with plants result in beneficial effects. Thus inoculation of beneficial microbial inoculants in soil mimics as natural ecosystems and hence as consequence leads to better outcomes (Sarma et al., 2015). Several studies have demonstrated that consortia improve the efficacy of a system in comparison to monoculture as each member of consortium cooperate with other and as resultant improve growth and survivability of all individual (Lawniczak et al., 2013). Thus the application of consortia for management of phytopathogens, plant growth promotion and biodegradation of organic matters is found most suitable and efficient strategy (Lawniczak et al., 2013; Rajasekhar et al., 2016). Suleiman et al., (2017) reported consortium of *Bacillus subtilis* Bf12 and *Pseudomonas fluorescens* Pf2 had better antagonistic and plant growth promoting effect on *Alternaria solani* (63% disease reduction) and tomato seedlings, respectively, in comparison to individual treatment under *in vivo* condition. This result was may be due to the synergistic effect of two microbial agents on each other (Sarma et al., 2015). The soil application of three different microbial consortia *i.e.,* consortia of *Trichoderma harzianum* + *Pseudomonas fluorescens* + *Bacillus subtilis* + *Rhizobium*; *T. harzianum* + *B. subtilis* and *P. fluorescens* + *Rhizobium* reduced diseases severity on pigeonpea caused by soil-borne pathogens by 86, 82 and 77%, respectively (Rajasekhar et al., 2016). In parallel to the above findings, the study of Singh et al., (2013) showed 53.23% of disease reduction in tomato caused *Sclerotium rolfsii* by application of consortium of *Trichoderma harzianum* and *Pseudomonas* sp. The combined application of *Fusarium equiseti* with *Glomus mosseae* was observed to reduce anthracnose and damping off disease incidences but also found to reduce the rate of root colonization significantly by *G. mosseae* (Saldajeno and Hyakumachi, 2011). Use of consortia not only reduce the severity and enhance plant growth promotion but

their role in the biodegradation process has also been documented in the literature. Kadali et al., (2012) reported an increased rate of mineralization (18.5%) by consortia over an individual culture that showed 4.7-10% mineralization.

Development of consortium for use should focus on factors that affect their potential activity as sometimes use of incompatible inoculations leads to negative or null effects instead of positive effects (Sarma et al., 2015). Several findings in the literature advocate the negative or null effect of consortia on plant growth and disease control (Flor-Peregrin et al., 2014). The three microbial component consortium consisting of *Azospirillum lipoferum* CRT1, *Glomus intraradices* JJ291 and *Pseudomonas fluorescens* F113 in comparison to individual treatment of *G. intraradices* does not raise production of secondary metabolites in maize roots (Walker et al., 2012). Similarly co-inoculation of two microbial inoculants *i.e., Bacillus subtillis* 101 and *Azospirillum brasilense* Sp245 showed lower plant biomass accumulation as compared to individual treatment and were at par with control (Felici et al., 2008). Analogously, no significant difference was observed in biocontrol of *Rhizoctonia solani* by application of *Trichoderma harzianum* and *Serratia proteamaculans* individually or in combination (Youssef et al., 2016). The negative effect of co-inoculation of Arbuscular mycorrhiza and *Rhizobium* was observed under moderate drought conditions on growth of *Phaseolus vulgaris* which was may be due to a reduction in nodule formation and atmospheric Nitrogen fixation efficiency (Franzini et al., 2010). Therefore, some selection criteria are supposed to be framed for consortia development including search for suitable partners that show compatibility with each other (Jain et al., 2012; Sarma et al., 2015), diversity in mechanisms (Sarma et al., 2015), *etc.* that as a resultant enhances the activity spectrum, reliability and consistency under diverse environmental and soil conditions (Sarma et al., 2015).

Materials & Methods

3.1. Survey methodology

The primary data was collected through a random field survey based on pre-structured questionnaire to reveal out the present scenario of tomato cultivation *i.e.,* trends followed regarding use of pesticides and fertilizers in study areas and problems encountered by farmers. Personal interviews with farmers were conducted randomly in and around Lucknow city (26.85° N, 80.95° E), including districts Unnao (26.54° N, 80.49° E), Raebareli (26.23° N, 81.24° E) and Barabanki (26.99° N, 81.25° E) of Uttar Pradesh, (India), during different tomato growing seasons, 2014-2016.

3.2. Sample collection

The plant and rhizopheric soil samples of tomato, chilli, brinjal, beans, ladyfinger, ridge gourd and vetiver were collected from different agricultural fields of Lucknow during 2014-2015 for isolation of pathogenic and antagonistic microbial isolates (**Figure 3.1**). The samples were collected in sterilized polyethylene bags (**Figure 3.2)** and stored in refrigerator at 4°C for further use.

Figure 3.1: Sample collection from agricultural field

Figure 3.2: Collected samples of soil and plant

3.3. Isolation of pathogenic fungi

The pathogenic fungi *Alternaria solani* and *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) were isolated from infected tomato leaves, roots and soil samples collected from various tomato growing fields of Lucknow, U.P. (India) during different seasons. From soil, pathogens were isolated by serial dilution method. 10 gm of each soil sample was mixed with 100 ml of sterilized distilled water (SDW) and then serially diluted upto 10^{-5}. 0.1 ml of each dilution *i.e.,* 10^{-3}-10^{-5} was spread on Petri plates containing media Potato dextrose agar (PDA). Media before pouring into sterilized Petri plates was autoclaved at 121℃ for 15 min. For isolation from roots and leaves collected, they were washed thoroughly under tap water to remove adhering soil and rinsed thrice with SDW. After washing roots and leaves were cut into small pieces, dried on blotter sheet and aseptically placed on Petri plates containing solidified sterilized PDA media. Inoculated plates were incubated at 28±2°C. The colonies were identified on morphological basis and stored on PDA slants at 4°C for future use.

3.3.1. Identification of pathogens

The pathogenic fungi were identified on the basis of colony appearance and the spores produced by them by visualizing under light microscope. The isolates showing characters similar to *Fusarium oxysporum* and *Alternaria solani* were further assayed for their ability to cause pathogenicity in tomato seedlings under *in vitro* condition.

3.3.2. *In vitro* pathogenicity assay

3.3.2.1. Conidial suspension of pathogens: The fungi were cultured on Potato dextrose agar (PDA) plates for seven days and then the spores were scraped off with the help of glass rod by adding 10 ml sterilized distilled water (SDW) to each plate. The spore count was determined by using Neubauer's hemocytometer and adjusted according to the spore concentration required.

3.3.2.2. Detached leaf assay for *Alternaria solani*: The detached leaf assay for assessing pathogenicity of *Alternaria solani* was performed by following method described by Singh et al., (2016) with some modifications. The medium sized health leaflet of tomato was surface sterilized with sodium hypochlorite for 5 min and then rinsed thrice with sterilized distilled water. The leaflet was placed on sterilized Petri plates lined with moist filter paper. 1 ml of conidial suspension of pathogen *Alternaria solani* containing 1 x 10^6 spores per ml was sprayed over the leaflet and the Petri plates was sealed with parafilm. The plates were maintained at 27˚C for 7 days. In case of control sterilized distill water was sprayed over the leaflet. Development of disease symptoms confirmed pathogenicity of fungal isolates.

3.3.2.3. Cup assay for *Fusarium oxysporum* f. sp. *lycopersici*: The 10 ml spore suspension of *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) containing 1 x 10^6 spores

was added in 50 gm sterilized soil in plastic cups. The soil was covered with muslin cloth and incubated for one week at room temperature for growth and establishment of pathogens in soil (Pandey and Dubey, 1994), and then sterilized seeds of Tomato cv. Pusa ruby were sown in soil to assess the pathogenicity of fungal isolate. Soil without spore suspension of fungal isolates was used as control. The cups were kept in Net house and watered daily till 30 days after sowing (DAS). The isolates that showed positive disease symptoms *i.e.,* wilting in seedlings after 30 DAS were confirmed as pathogenic strains.

3.4. Isolation of rhizospheric antagonistic microorganisms

Rhizospheric soil and roots of healthy plants were collected randomly from different crops (tomato vetiver, chilli, beans, brinjal, ladyfinger and ridge gourd) fields of Lucknow, Uttar Pradesh (India) for isolation of strains possessing antagonistic properties (**Figure 3.3**). The soil sample was serially diluted upto 10^{-9} similarly as mentioned in **section 3.3**. The 0.1 ml of each dilution *i.e.,* 10^{-7}-10^{-9} for bacteria and 10^{-3}-10^{-5} for fungi, were spread on Petri plates containing media Nutrient agar (NA) and Potato dextrose agar (PDA) for isolation of rhizospheric bacteria and fungi, respectively. Biocontrol strains from healthy plant roots were isolated by following the protocol mentioned above in section isolation of pathogens. The colonies of which were visually distinguished were identified and transferred on respective media plates and incubated at 28±2°C for identification and future use.

Figure 3.3: Healthy plant root collected for isolation of rhizospheric microbial isolates

3.4.1. Phenotypic characters of antagonistic fungi

On the basis of colony growth different fungal isolates were purified. These isolates were identified on the basis of their microscopic structure.

3.4.1.1. Identification of *Trichoderma*- Further the isolates belonging to genus of *Trichoderma w*ere confirmed on the basis of morphological aspect like colony appearance and sporulation pattern, by growing cultures on Potato dextrose agar (PDA). The colony characteristics and growth rate was observed by placing 7 mm disc of *Trichoderma* spp. at centre of all Petri plates taken from the actively growing margin of seven days old culture. The Petri plates were kept for incubation at 28°C±1 in darkness. The radial growth was measured at every 24 h interval till 4 days.

The microscopic examination was done under light and Scanning electron microscope (SEM) after culture grown for 5-7 days on PDA. The preliminary microscopic structure of *Trichoderma* spp. was observed under light microscope by preparing slide in drop of 3% KOH, then replaced with water and stained with lactophenol cotton blue. The structure of phialides and conidia was studied through

Scanning Electron Microscope (SEM). The samples were prepared according to the method described by Mycock and Berjak, (1991) with some modifications. The mycelia of *Trichoderma* was fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.0) and kept at 4ºC for 6 h. After incubation the sample was washed 3 times in 0.1 M buffer for 15 min each at 4ºC to remove un-reactive fixative. After washing the sample was dehydrated for 20 min in each graded series *i.e.,* 30, 50, 70, 90, 95 and 100% of ethanol. Then samples were dried at Critical point drying and mounted on aluminum stub with carbon tape. Further sample was coated with platinum particles using Sputter coater and analyzed under SEM (Model: Joel Quanta 250) at various resolution (2000-6000X).

3.4.2. Phenotypic and biochemical characteristics of isolated rhizobacterial isolates

The bacteria different in colony morphology were purified and subjected to biochemical test for identification according to Aneja, (2003). The biochemical tests performed as given below.

3.4.2.1. ***Gram Staining***- Gram staining was performed by the following the Gram's method. The log phase culture of bacterial strains isolated were smeared on glass slide, air dried and heat fixed. The smear was stained with crystal violet and kept for 1 min, then rinsed with distilled water (DW) and then few drops of iodine was added and kept for next 45 sec. The iodine was again washed with DW, followed by washing with 95% ethyl alcohol and then rinsed with DW. After washing smear was flooded with safranin for 1 min and washed with DW. The smear was air dried and observed under light microscope at 100x (oil immersion lens) by adding drop of immersion oil to determine shape and gram reaction.

3.4.2.2. Amylase- Amylase activity was examined by inoculating bacterial isolates on starch agar plates. The plates were incubated at 30°C±1 for 48 hrs. After incubation, plates were flooded with iodine solution. Formation of clear zone around the colonies indicated positive amylase activity.

3.4.2.3. Urease- Urease activity was assayed on urea agar slant tubes. Isolates were streaked on agar surface and tubes were incubated for 24-48 hrs at 30ºC±1. Change in colour of media from yellow to pink after incubation indicated positive result for Urease activity.

3.4.2.4. Citrate utilization- Citrate utilization was determined on Simon's citrate agar slants. Isolates were streaked on media and incubated at 30ºC±1 for 48 hrs. Change in colour from green to blue indicated positive citrate utilization by isolates.

3.4.2.5. Oxidase- The Oxidase test was performed by adding oxidase discs (saturated with 1% aqueous tetra-methyl-para-phenylene-diamine solution) in nutrient broth containing 24 hrs old bacterial cultures. Development of dark purple colour on disc within 10 sec, indicated positive result for oxidase.

3.4.2.6. Catalase- Catalase test was performed by adding a drop of 3% hydrogen peroxide on 48 hrs old bacterial colony on a clean glass slide and mixed using a sterile tooth-pick. The effervescence indicated positive catalase activity.

3.4.2.7. Methyl Red and Voges Proskauer (MR-VP)- The bacterial isolates were inoculated in 5 ml of MR-VP broth and incubated for 48-72 hrs at 35-37°C. After incubation to 1 ml of the broth, 2-3 drops of methyl red was added. Development of red colour indicated positive while yellow colour indicated negative result for methyl red. Take 1 ml of remaining part of broth and add 0.6 ml of 5% α-naphthol in ethanol

and 0.2 ml of 40% potassium hydroxide. The test tube was then shaken gently and kept undisturbed for 15 min. The positive VP test was indicated by development of a red colour starting from the liquid-air interface while no colour change indicated negative VP test.

3.4.2.8. Triple Sugar Iron test (TSI)- The TSI slant was inoculated with bacterial isolates and incubated at 37°C for 24 hrs. After incubation observations were made. The production of gas was determined by cracking of the medium; formation of H_2S gas was determine by the blackening of the whole media or a streak of ring of blackening at the slant butt junction, glucose fermentation was determine by the yellowing of the butt. The fermentation of lactose or sucrose both was determined by the yellowing of the slant and the butt.

3.4.2.9. Motility- The motility test was performed by stabbing inoculating needle containing bacterial isolate in Motility medium in a test tube. The tube was incubated for 24 hrs at 27˚C. After incubation motility was determined by observing the line inoculation. The hazy and diffuse growth throughout the media indicated positive motility.

3.4.2.10. Nitrate reductase- Nitrate broth was inoculated with bacterial isolate and incubated at 30±2ºC. After incubation a dropper full of sulfanilic acid and α-naphthylamine was added. Conversion of medium red after the addition of the nitrate reagents indicated positive result for nitrate reduction.

3.4.2.11. Gelatin hydrolysis- The gelatin agar was inoculated with bacterial isolates and incubated at 30˚C for 4 days. The media liquefied was considered positive for production of glucanase enzyme.

3.4.2.12. Indole test- The Kovac strips were added at the mouth of test tube containing Tryptophan media inoculated with bacterial isolates was incubated at 30˚C for 24 hrs. Change in color of Kovac strips indicated positive indole test.

3.4.3. Screening biocontrol potential of isolates by dual culture

The dual culture assay was performed by the method described by Dennis and Webster (1971). 90 mm Petri plates containing PDA were set up by placing 7 mm plugs of pathogens *F. oxysporum* f. sp. *lycopersici* and *A. solani,* separately on plates. *Trichoderma* isolates were placed opposite to each pathogen at equal distances from edges and for bacterial isolates, pathogen plug was placed in centre and a loopful of each bacterial isolate was streaked on PDA plates separately, 1 cm from opposite edges. As controls, pathogens were placed on PDA plates without *Trichoderma* and rhizobacterial isolates. The inoculated plates were monitored daily in order to estimate the time taken by each antagonist to confront with the pathogens and restrict their growth. From 3-7 days old dual-culture plates, hyphae in the contact zone between colonies of *Trichoderma* and pathogen were examined under light microscope (magnifications of up to 40X) for development of coils around pathogen mycelia by antagonistic fungi.

Petri plates were incubated at 28±2°C for 7 days and the experiment was conducted in triplicates and repeated twice. The percent inhibition of mycelia growth of the pathogens was calculated by using formula given by Behzad et al., (2008):

$$I\% = \frac{C2 - C1}{C2} X\, 100$$

Where:

I% = growth inhibition of pathogen

C1= radial growth of the pathogens (mm) in dual culture plate

C2 = radial growth of pathogen (mm) in control plate

3.4.4. Molecular identification of antagonistic microorganisms

The strains that showed positive biocontrol activity under *in vitro* dual culture test were identified at genomic level. Molecular characterization of bacterial isolates was done by 16S rRNA sequencing method as described by Maji et al., (2013) and 18S rRNA ITS sequencing was performed for *Trichoderma* isolates as described by Maji et al., (2015).

3.4.4.1. DNA extraction and amplification- The genomic DNA of bacteria was isolated from overnight grown culture and fungal genomic DNA was isolated from 4 days old grown culture. The isolated genomic DNA was quantified spectrophotometrically by using NanoDrop ND1000 and its quality was checked through agarose gel electrophoresis. The amplification was done by using universal primers (forward and reverse).

3.4.4.2. Amplification primers used

Amplification of	Forward Universal Primer	Reverse Universal Primer
16S rRNA	5´-AGAGTTTGATCCTGGCTCAG-3´	5´-ACGGCTACCTTGTTACGACTT-3´
ITS gene	ITS 1 (5´-TCCGTAGGTGAACCTGCGG-3´)	ITS 4 (5´-TCCTCCGCTTATTGATATGC-3´)

For both bacterial and fungal isolates approximately 25 ng of genomic DNA and 5 pmol l^{-1} of each primer was used for amplification in a thermocycler. The thermocycler for bacterial isolate was programmed as 94˚C for 5 min; 34 cycles of 94˚C for 1 min, 57.4˚C for 1 min, 72˚C for 2 min; 72˚C for 10 min; 4˚C for an infinite period. For fungal isolates thermocycler was programmed as 95˚C for 5 min; 32

cycles of 95˚C for 30 sec, 55˚C for 30 sec, 72˚C for 1 min; 72˚C for 10 min; 4˚C for an infinite period.

3.4.4.3. Purification of PCR product- After amplification PCR products were purified with PCR cleanup Kit (Axygen Union city, CA) and sequenced directly by using forward universal primer and Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystem, USA) on a 3130 x 1 Genetic Analyzer (Applied Biosystem, USA) using manufacturer's protocol.

3.4.4.4. Sequence analysis and accession number- The sequences obtained were analyzed by using nucleotide BLAST (NCBI- BLASTN). The accession number of isolated bacterial and fungal strains was obtained from NCBI. The phylogenetic tree of bacterial and fungal antagonistic isolates were constructed using neighbor-joining tree based on analysis of partial 16s rRNA and 18s rRNA nucleotide sequence, respectively showing relationship between isolated and other strains documented in literature possessing biocontrol, PGP and stress alleviation activity.

3.4.5. Characterization of plant growth promoting and biocontrol activities

3.4.5.1. Indole-3-acetic acid (IAA)- Indole acetic acid production assay was performed by modified method described by Brick et al., (1991). The bacterial cultures were grown for 48 h and fungal cultures for 7 days on minimal medium amended with 0.2 g/l L-tryptophan at 28±2˚C. Fully grown cultures were centrifuged at 10,000 rpm for 10 min at 4˚C. 2 ml of supernatant was mixed with two drops of orthophosphoric acid and 2 ml of Salkowski reagent. 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.

3.4.5.2. Phosphate solubilization- Phosphate solubilization by microbial isolates was screened on Pikovskaya agar media as described by Gaur, (1990). Microbial isolates were inoculated on plates and incubated at 28±2ºC for 4 days. Development of halo zone around bacterial/fungal colony indicated ability of isolates to solubilize phosphate. 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).

3.4.5.3. Ammonia production- It was detected by following method of Cappuccino and Sherman, (1992). Microbial isolates were cultured in 10 ml peptone water in each test tube and incubated for 48-72 hrs at 28±2˚C. After incubation 0.5 ml of Nessler's reagent was added in each test tube. Development of brown to yellow color indicated positive test for ammonia production.

3.4.5.4. Siderophores- Siderophore production was estimated on Chrome azurol sulphonate (CAS) agar media, prepared as described by Kumari and Kaviyarasan, (2014) by mixing 60.5 mg CAS dye dissolved in 50ml distilled water with 10 ml of 1mM $FeCl_3.H_2O$ in 10mM HCl and 72.9 mg of Hexadecyltrimethylammonium bromide (HDTMA) in 40 ml distilled water. CAS media and PDA (*Trichoderma* isolates) or NA (bacterial isolates) after autoclaving was mixed and pH was maintained to 6.8 by adding PIPES buffer (4.032 g/100ml). The inoculated plates were incubated at 30˚C for 2-3 days and formation of orange halos indicated positive result.

3.4.5.5. 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). Isolates were spot-inoculated on agar plates and incubated at 28°C for 48 hrs. After incubation, plates were observed for clearing zone around colony and diameter was calculated.

3.4.5.6. Cellulase- Microbial isolates were screened for cellulase production by streaking isolates on cellulose congo-red agar media as described by Gupta et al., (2012). Discoloration of congo-red around bacterial colonies was taken as positive for cellulose degradation.

3.4.5.7. Protease- For proteolytic activity, microbial isolates 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.

3.4.5.8. Chitinase- Chitinase production was estimated on colloidal chitin agar which was prepared by the method described by Agrawal and Kotasthane, (2012) by mixing 4.5 g of colloidal chitin with 3 g $(NH_4)_2SO_4$, 2.0 g KH_2PO_4, 1.0 g citric acid monohydrate, 0.3 g $MgSO_4.5H_2O$, 0.15 g Bromocresol purple, 200 µl Tween-80 and 20 g agar in 1000 ml distilled water. The pH of media was adjusted to 4.7 with 50% KOH and autoclaved. The plated were inoculated with antagonistic isolates and incubated at 30˚C for 3-4 days. Chitinase production was observed by formation of purple colored zone around the colony.

3.4.5.9. HCN- Hydrogen cyanide (HCN) production by bacterial isolates was screened by following method of Lorck, (1948). Bacterial isolates streaked on nutrient agar (NA) amended with 4.4 g/l glycine. A Whatman filter no. 1 soaked in 2% sodium

carbonate in 0.5% picric acid solution was placed inside the upper lid of inoculated plates and sealed with parafilms. Petri plates were incubated at 28˚C for 4 days. Change in color of filter paper yellow to brown was positive for HCN production.

3.4.6. Root colonization potential of antagonists

3.4.6.1. Biofilm production- 10 ml of trypticase soya agar was inoculated with loopful of overnight grown bacterial culture or with a plug of fresh cultured fungal isolate and incubated at 30˚C for 48 hrs. Post inoculation contents were decanted and washed with phosphate buffer saline (pH 7.3) and dried. Dried tubes were stained with crystal violet (0.1%). Excess of stain was washed with deionized water. Tubes were dried again in inverted position and observed for biofilm formation (Yadav and Sundari, 2015).

3.4.6.2. Exopolysaccharide (EPS) production- In a nutrient broth amended with sucrose or tryptone soy broth bacterial and fungal isolates was inoculated for 48-72 hrs. After incubation broth was centrifuged at 10,000 rpm for 10 min and supernatant collected was mixed with equal amount of chilled acetone/absolute ethanol and kept in refrigerator overnight. Formation of precipitate indicates presence of EPS.

3.4.6.3. Root section analysis under SEM- The plants inoculated with best performing antagonistic isolates were analyzed for their root colonization activity under *in vivo* conditions. The root of plants were harvested 90 DAT. Washed thrice thoroughly with distilled water and then cut transversely into thin sections. The sections were prepared for SEM analysis as described in **section 3.4.1.1.** After sample preparation, the root sections were observed under SEM.

3.4.7. Effect of volatile and non-volatile metabolites on pathogens

The volatile and non-volatile metabolite assay was performed by following the method given by Dennis and Webster, (1971). The volatile assay was performed by inoculating two bottoms of Petri plates containing PDA/PDA individually with 7 mm disc of pathogen and *Trichoderma* isolates or PDA/NA individually with 7 mm disc of pathogen and bacterial isolate, respectively. The inoculated bottoms were adjusted one over another in such a manner that pathogen inoculated plate lie above the antagonistic inoculated plate and sealed together by parafilm. In case of control only pathogen was inoculated and was devoid of antagonist.

For non-volatile assay the three discs (7 mm diameter) of *Trichoderma* spp. taken from the active margin of 7 days old colonies was inoculated in 100 ml sterilized potato dextrose broth (PDB) in 250 ml Erlenmeyer flask and incubated at 28±2°C without shaking for 12 days. For bacterial isolates the culture filtrate was prepared by inoculating Nutrient Broth (NB) with loopful cultures of bacteria and incubated for 48 hours at 37±2°C. After incubation, culture broths were filtered through Whatman filter paper no. 1 and re-filtered through Millipore membrane filter (0.22 μ) to obtain cell-free culture filtrates. Culture filtrates (1, 2 and 4 ml) of each antagonist was poured in sterilized Petri plate followed by pouring 19, 18 and 16 ml of PDA, respectively, to make concentrations 5%, 10% and 20% and inoculated with pathogen plug. In control, sterilized water was added to PDA instead of culture filtrate.

The inoculated plates were incubated at 28±2°C for 7 days and monitored daily to keep record of growth of the pathogens. The experiment was conducted in

triplicates and repeated twice. The percent inhibition of mycelia growth of the pathogens was calculated in a similar manner as mentioned above in **section 3.4.3.**

3.4.8. Effect of antagonistic microbial isolates on seed germination and vigor index

Effect of microbial antagonists on seed germination was assessed. Tomato seeds (*Lycopersicon esculentum* var. Pusa ruby) purchased from local market was surface sterilized by 1% mercuric chloride ($HgCl_2$) and washed thrice with sterile distilled water. Seeds were coated individually with *Trichoderma* TvR1, *Trichoderma* TbS2, *Pseudomonas* Tr20, *Bacillus* BS6 and *Bacillus* CS13 with 1% carboxymethyl cellulose (CMC) as adhesive for one hour. For control seed were soaked only in sterilized distilled water containing 1% CMC as adhesive for one hour. Seeds were then dried overnight at room temperature in Laminar flow. Thirty seeds were used in each case to assess *in vitro* seed germination effect of antagonistic microbial isolates. Experiment was performed in replicated of three. The Petri plates lined with Whatman filter paper no. 1 moistened with sterilized distilled water and kept for germination under controlled conditions. The germination was checked after every 24 hours for twenty days and thereafter germinated seedlings were taken and their shoot and root length was measured.

The germination index and vigor index was calculated was using formula described by Thakkar and Saraf, (2014) as given below:

$$\text{Germination percentage} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds}} \text{ X } 100$$

$$\text{Vigor index (VI)} = \text{Germination percentage X mean of seedling length (cm)}$$

3.5. Screening test for biosurfactant production

3.5.1. CTAB agar plate method

This method was developed by Siegmund and Wagner, (1991) which is used for semi-quantitative screening of extra cellular anionic surfactants. Mineral salt agar media supplemented with 2% glucose was used. 0.5 g/l Cetyltrimethylammonium bromide (CTAB) and 0.005g/l methylene blue was added for detection of anionic biosurfactant. A loopful of bacteria was inoculated on agar plates and incubated at 37±2ºC for 48-72 hrs. Development of dark blue halo zone around bacterial colony was considered positive.

3.5.2. Haemolysis of blood agar

Biosurfactant cause lysis to erythrocytes, thus blood haemolysis is used as preliminary screening method to detect ability of microorganisms to produce biosurfactant. Haemolysis of blood agar was done on the basis of method given by Mulligan et al., (1984). The bacterial isolate was inoculated on sheep blood agar plates and incubated at 30±2ºC for 48 hrs. Development of clear zone around colonies represented positive blood haemolysis.

3.6. Extraction of Biosurfactant

The bacteria *P. aeruginosa* Tr20 was grown in NB for 24 h at 35ºC. 5 ml of bacterial culture with cell density adjusted to OD_{600}= 1.0 was added to 100 ml of sterilized Minimal salt medium (MSM) containing glucose as sole carbon source (2% w/v) in 500 ml conical flask and kept for 96 h in rotary shaker at 35ºC and 150 rpm.

After incubation the crude biosurfactant was extracted from media by adopting protocol described by El-Sheshtawy and Doheim, (2014) with slight modification.

The media was centrifuged at 10,000 rpm for 30 min. The supernatant was collected and the pellet containing cells was discarded. The supernatant collected was acidified to pH 2.0 by adding 6N HCl. Then it was kept at 4°C overnight for precipitation. After precipitation, the precipitate was centrifuged at 10,000 rpm for 15 min and the pellet was collected and dissolved in 0.05M sodium bicarbonate (pH 8.6). Again the sample was acidified and centrifuged at 12,000 rpm for 20 min. The precipitate was mixed with 2:1 ratio of Chloroform: methanol and vigorously shaken and allowed to form layers. Then the organic layer containing biosurfactant was separated with the help of separating funnel. The solvent was evaporated in rotary evaporator at 40ºC and viscous honey colour residue was obtained.

3.6.1 Characterization of Biosurfactant

3.6.1.1. Foam height- The foam height of biosurfactant was measured by vigorously shaking 10 ml of supernatant containing biosurfactant (after 96 hrs of incubation) in a glass vial for 2 min. The foaming height was calculated by using formula:

$$\text{Foaming } \frac{\text{Height of foam}}{\text{Total height}} \text{ X } 100$$

3.6.1.2. Emulsification assay- The Emulsification activity of biosurfactant was performed by adopting method of Cooper and Goldenberg, (1987) with some modifications. 5 ml supernatant was mixed with 5ml kerosene in a screw cap vial. The mixture was vortexed at high speed for 2 min and kept for 24 h. After 24 h, the emulsification activity was calculated by using formula:

$$\text{Emulsification activity } (E_{24}) = \frac{\text{Height of emulsion layer}}{\text{Total height}} \text{ X } 100$$

3.6.1.3. Oil displacement test- Oil displacement test was performed by following method described by El-Sheshtawy and Doheim, (2014) to assess the surface activity of biosurfactant by measuring the diameter of clear zone produced after adding drop of biosurfactant on thin film of oil on water. The experiment was performed at room temperature by adding 20 ml of water in 10 cm diameter Petri plate. 20 µl of oil was added above water to form thin film, and then a 10 µl of test solution was added on the surface of oil. The diameter of oil cleared by test solution was measured.

3.6.1.4. Fourier Transform Infrared Spectroscopy (FTIR)- FTIR analysis of crude surfactant was performed to identify functional groups. 1 mg crude biosurfactant was ground with 100 mg of KBr and pressed by subjecting to physical pressure for 30 sec to produce translucent pellets. Infrared absorption spectra were recorded on FTIR model Nicolet 6700 (Thermo Scientific, USA) with spectral resolution and wave number accuracy of 4 and 0.01 cm^{-1}, respectively.

3.6.2. *In vitro* assessment of effect of biosurfactant on growth of pathogens

Effect of different concentration of crude biosurfactant *i.e.,* 100 mg/l, 200 mg/l, 400 mg/l and 800 mg/l on growth of *Fol* and *A. solani* was assessed by adopting method mentioned in **section 3.4.7.** for assessing effect of non-volatile metabolites on pathogens. The 7 mm plug of pathogen was placed in centre of PDA plates containing different concentration of biosurfactant. The plates were incubated at 28±2°C for 7 days. The mycelia growth inhibition was assessed after 7 days of inoculation by using formula given by Behzad et al., (2008) mentioned above in **section 3.4.3.**

3.6.3. Compatibility assay

The compatibility between antagonistic microbial isolates and biosurfactant was tested by dual culture technique.

3.6.3.1. Compatibility among bacterial isolates- For assessing compatibility among bacterial isolates, the two isolates were streaked on NA plates, in a perpendicular manner making sign of "+".

3.6.3.2. Compatibility among isolates of *Trichoderma*- Compatibility between two isolates of *Trichoderma* was assessed by placing 7 mm mycelia plug of both of the isolates of *Trichoderma* on two opposite edges of Petri plates containing PDA.

3.6.3.3. Compatibility among bacterial and *Trichoderma* isolates- Compatibility between bacterial and *Trichoderma* isolates was assessed by placing 7 mm plug of *Trichoderma* at the centre of Petri plates containing PDA and streaking bacteria on two opposite edges of the plates.

The plates were incubated at 28±2ºC and observations were done on daily basis for 7 days. Presence of no inhibition zone between two isolates was marked as compatible strains.

3.6.4. Compatibility between biosurfactant and *Trichoderma* isolate

Compatibility of *Trichoderma lixii* TvR1 with different concentration of crude biosurfactant was assessed by following protocol adopted in section *in vitro* assessment of effect of Biosurfactant on growth of pathogens. The growth rate and colony appearance of *T. lixii* TvR1was compared with that of control to assess compatibility of biosurfactant with *T. lixii* TvR1.

3.7. Pot experiment

Three pot experiments were conducted during December, 2015 - March, 2018 with different treatments mentioned in **section 3.7.3.1. – 3.7.3.3.**

3.7.1. Nursery preparation

The nursery of tomato seedlings was prepared one month before transplantation. The tomato seeds were coated with different antagonists individually and/or with consortium (in second and third pot experiment) and were sown in germination tray with sterilized soil and watered regularly for 28 days.

Figure 3.4: Seedling grown in seedling tray

3.7.2. Soil preparation and seedling transplantation

The soil used in pots and trays was sterilized in autoclave at 15 Psi at 121˚C for one hour for three consecutive days. The physicochemical parameters of soil *i.e.,* pH, electrical conductivity, organic carbon, bulk density, available N, P, K and water holding capacity were assessed and their values were found to be 7.24, 0.729 dS/m, 1.14 %, 154.2 mg/kg, 11.33 mg/kg, 132.81 mg/kg and 39.9 %, respectively. After 28 days of germination, seedlings were transplanted in the earthen pots containing sterilized soil.

3.7.3. Pathogen inoculums preparation and inoculation

Fol was cultured on sterilized wheat porridge for mass multiplication for 2 weeks and then inoculated in soil @ 5 g/kg. The soil was challenged with *Fol* one week prior to transplantation for the establishment of pathogen in soil. In case of *A. solani*, spore suspension was prepared in a similar manner as described in **section 3.5.1.** After 7

days of transplantation, *A. solani* was inoculated by spraying spore suspension (10^5spores/ml) on adaxial or abaxial surface of leaves. The plants were covered with polybags for 48 hrs to maintain moisture and development of disease incidence. For each treatment, three such pots were maintained and experiment was performed in complete randomized design. After 60 days of pathogen challenge (in first pot trial) and 90 days post pathogen challenge (in second and third pot experiment), observations were recorded for root length (cm), shoot length (cm), shoot fresh and dry weight (g) and root fresh and dry weight (g), number of leaves and fruits/flower for each treatment. The experimental setup was laid in completely randomized design in replicates of four. The experimental setup with different treatments is given below:

3.7.3.1. First pot trial to screen biocontrol activity of isolated antagonistic microbes

1.	Control (Non-challenged)
2.	*Bacillus subtilis* BS6
3.	*B. subtilis* CS13
4.	*Pseudomonas aeruginosa* Tr20
5.	*Trichoderma lixii* TvR1
6.	*T. brevicompactum* TbS2
7.	*Fusarium oxysporum* f. sp. *lycopersici* (*Fol*)
8.	*Fol* + *B. subtilis* BS6
9.	*Fol* + *B. subtilis* CS13
10.	*Fol* + *P. aeruginosa* Tr20
11.	*Fol* + *T. lixii* TvR1
12.	*Fol* + *T. brevicompactum* TbS2
13.	*Alternaria solani*
14.	*A. solani* + *B. subtilis* BS6
15.	*A. solani* + *B. subtilis* CS13
16.	*A. solani* + *P. aeruginosa* Tr20
17.	*A. solani* + *T. lixii* TvR1
18.	*A. solani* + *T. brevicompactum* TbS2

3.7.3.2. Second pot experiment to assess biocontrol and PGP activity of best performing antagonistic isolates

1.	Control
2.	*Trichoderma lixii* TvR1
3.	*Pseudomonas aeruginosa* Tr20
4.	*T. lixii* TvR1 + *P. aeruginosa* Tr20
5.	*Fusarium oxysporum* f. sp. *lycopersici* (*Fol*)
6.	*Fol* + *T. lixii* TvR1
7.	*Fol* + *P. aeruginosa* Tr20
8.	*Fol* + *T. lixii* TvR1 + *P. aeruginosa* Tr20
9.	*Alternaria solani*
10.	*A. solani* + *T. lixii* TvR1
11.	*A. solani* + *P. aeruginosa* Tr20
12.	*A. solani* + *T. lixii* TvR1 + *P. aeruginosa* Tr20

3.7.3.3. Third pot experiment to assess biocontrol and PGP activity of microbial biocontrol isolates and biosurfactant

1.	Control
2.	*Trichoderma lixii* TvR1
3.	*Pseudomonas aeruginosa* Tr20
4.	Biosurfactant @ 200mg/ml
5.	*T. lixii* TvR1 + *P. aeruginosa* Tr20
6.	*T. lixii* TvR1 + Biosurfactant @ 200mg/ml
7.	*Fusarium oxysporum* f. sp. *lycopersici* (*Fol*)
8.	*Fol* + *T. lixii* TvR1
9.	*Fol* + *P. aeruginosa* Tr20
10.	*Fol* + Biosurfactant @ 200mg/ml
11.	*Fol* + *T. lixii* TvR1 + *P. aeruginosa* Tr20
12.	*Fol* + *T. lixii* TvR1+ Biosurfactant @ 200mg/ml
13.	*Alternaria solani*
14.	*A. solani* + *T. lixii* TvR1
15.	*A. solani* + *P. aeruginosa* Tr20
16.	*A. solani* + Biosurfactant @ 200mg/ml
17.	*A. solani* + *T. lixii* TvR1 + *P. aeruginosa* Tr20
18.	*A. solani* + *T. lixii* TvR1+ Biosurfactant @ 200mg/ml

3.7.4. Percentage Disease Index (PDI)

The percentage of disease index (PDI) for *Fusarium* wilt and early blight was calculated after 90 days of treatment (60 days after pathogen challenge in case of first pot experiment performed to screen effective isolates) as per the formula described by Akkopru and Demir, (2005) and Wheeler, (1969), respectively using disease severity score given by Bora et al., (2004) for *Fusarium* wilt and Horsfall and Barratt, (1945) with some modification as described by Sahu et al., (2013) for early blight.

$$\text{PDI} = \frac{\Sigma(\text{Disease score X Number of plants rated})}{\text{Total number of plants X 4}} \text{ X } 100$$

Where 4= highest disease score for incidence of fusarium wilt given by Bora et al. (2004)

$$\text{PDI} = \frac{\Sigma(\text{Disease score X Number of leaves rated})}{\text{Total number of leaves X 5}} \text{ X } 100$$

Where 5= highest disease score for incidence of early blight given by Horsfall and Barratt (1945).

3.7.5. Phytochemical parameters

3.7.5.1. Estimation of Total Chlorophyll and Carotenoid content- Total Chlorophyll and carotenoid content were estimated 90 days post pathogen challenge. The Chlorophyll was estimated by using method described by Arnon, (1949) and Carotenoid content by following method described by Duxbury and Yentsch, (1956). About 0.5 gm of fresh leaf was crushed in 10 ml of 80% (v/v acetone/water) chilled acetone with the help of pestle mortar in dark. Centrifuged at 5000 rpm at 10˚C for 15 min. The supernatant was taken and optical density was measured at wavelength 663 nm, 645 nm and 480 nm by spectrophotometer. 80% acetone was used as blank. The

chlorophyll and carotenoids (in mg/g fresh weight) were determined by following formula:

$$\text{Chlorophyll a} = \frac{[12.7\,(A_{663}) - 2.69\,(A_{645})] \text{ x V}}{1000 \text{ x W}}$$

$$\text{Chlorophyll b} = \frac{[22.9\,(A_{645}) - 4.68\,(A_{663})] \text{ x V}}{1000 \text{ x W}}$$

$$\text{Total chlorophyll} = \frac{[20.2\,(A_{645}) + 8.02\,(A_{663})] \text{ x V}}{1000 \text{ x W}}$$

$$\text{Carotenoid} = \frac{[7.6\,(A_{480}) - 2.63\,(A_{645})] \text{ x V}}{1000 \text{ x W}}$$

Where:

V = Volume of acetone = 10 ml

W = weight of sample = 0.5 gm

A = Absorbance

3.7.5.2. Estimation of Carbohydrate content- Carbohydrate content was estimated by anthrone method. 100 mg of leaves were homogenized in 5 ml 2.5 N HCl and centrifuged. To 0.1 ml extract made up to 1 ml by adding distilled water and then 4 ml 0.2% anthrone prepared in 95% ice cold H_2SO_4 was added and boiled for 10 min in water bath. Reading was taken at 630 nm. The standard curve of graded glucose was prepared. The carbohydrate content was expressed in mg/g fw.

3.7.5.3. Estimation of Protein content- Protein was estimated by Bradford method. 0.5 g of leaves was homogenized in 1.5 ml of 0.1M phosphate buffer and centrifuged at 5000 rpm for 20 min at 4°C. 0.5 ml of supernatant taken and equal amount of 10% chilled TCA added and again centrifuged at 3300 rpm for 30 min. The supernatant

was discarded and pellet was washed with acetone and then dissolved in 1ml 0.1N NaOH. 100 µl of sample was taken in tube and 5 ml Bradford reagent (1:4) was added in tubes. The tubes were kept for 10 min for color development. The absorbance was taken at 595 nm. In control in place of extract SDW was used. The standard curve of graded Bovine serum albumin was prepared. The protein content was expressed in mg/g fw.

3.7.6. Estimation of production of defense-related enzymes

Enzyme activity in leaves after 7 days of pathogen challenge was performed by homogenizing 1 gm of sample with 2 ml 50mM sodium phosphate buffer (pH 6) at 4˚C or 1.5 ml of 50mM Tris HCl buffer (pH 7.5) at 4˚C. The sample was centrifuged at 4000 rpm for 15 min and supernatant was collected which was stored in 2 ml sterilized eppendorf in deep freezer.

3.7.6.1. Peroxidase (PO)- Peroxidase activity was performed by mixing 1.5 ml of 0.05M freshly prepared pyrogallol with 0.5 ml of 1% H_2O_2 and 0.5 ml of supernatant at the last. Change in absorbance was recorded in every 30 seconds for 3 min at 420nm. For control boiled enzyme/distill water was used. Values were expressed as change in absorbance as Unit/min/g fresh weight.

3.7.6.2. Polyphenol oxidase (PPO)- Polyphenol oxidase acitivity was performed by following method given by Murthy et al., (2013). 1.5 ml of 0.1 M sodium phosphate buffer (pH 7) was mixed with 200 µl of enzyme extract. 200 µl of 0.01M catechol (freshly prepared) was added in the end and absorbance was taken at 546 nm at 30 sec time interval for 3 min. Values were expressed as change in absorbance as Unit/min/g fresh weight.

3.7.7. Fruit yield per plant – Fruits were harvested twice a week from 90-120 days after transplantation (DAT). The total yield included all fruits harvested. Fruit yield was estimated by multiplying average weight of fruit (average of three fruits) with mean number of fruits per plant.

3.7.8. Estimation of fruit quality

3.7.8.1. pH- The pH of 50 g of homogenized sample of tomato pulp was measured by using pH meter.

3.7.8.2. Titratable acidity- The titratable acidity of tomato pulp was estimated by titrating 10 g sample mixed with 50 ml of distilled water with 0.1N NaOH till the sample reached to pH 8.1. The titratable acidity content as % of citric acid was determined by using formula:

$$\text{Titratable acidity} = \frac{\text{Amount of NaOH used (ml) X 0.1N NaOH X 0.064 X 100}}{\text{grams of sample used}}$$

3.7.8.3. Carotenoids content- β-Carotene and lycopene content in ripe tomato fruits were estimated as described by Nagata and Yamashita, (1992). The 1 g of tomato extract was mixed with 20 ml of hexane: acetone mixture in ratio 6:4 and shaken vigorously for 1 min and filtered through Whatman filter paper No. 4. The absorbance was measured at 453, 505, 645 and 663 nm. The carotenoids content was calculated by using formula:

$$\beta - \text{Carotene} = 0.216 \text{ X } A_{663} - 1.220 \text{ X } A_{645} - 0.304 \text{ X } A_{505} + 0.452 \text{ X } A_{453}$$

$$\text{Lycopene} = -0.0458 \text{ X } A_{663} + 0.204 \text{ X } A_{645} + 0.372 \text{ X } A_{505} - 0.0806 \text{ X } A_{453}$$

The final result was expressed in mg 100^{-1} gm.

3.7.8.4. Moisture content- 100 g of tomato was dried for 2 hrs at 105±2°C in hot air oven and then its weight was recorded till constant weight obtained.

$$\text{Moisture content (\%)} = \frac{\text{Total weight} - \text{weight after drying}}{\text{Total weight}} \text{ X } 100$$

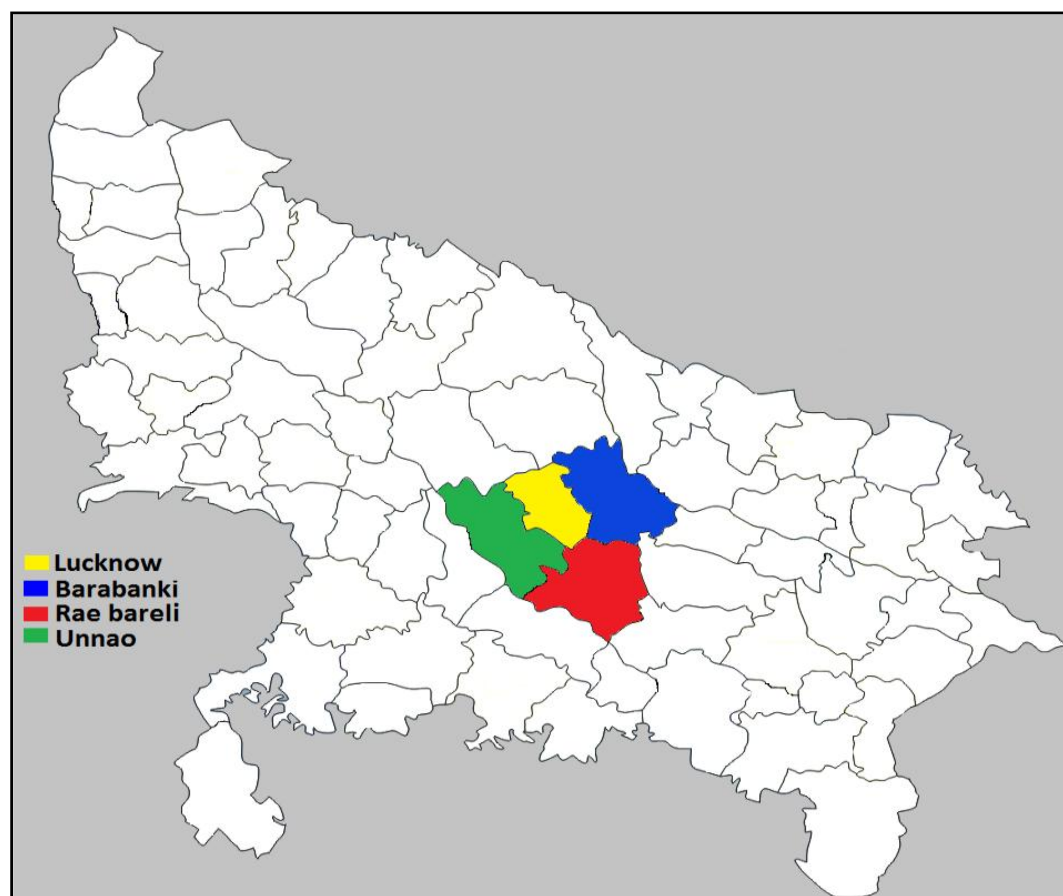
3.8. Statistical analysis

Data were statistically tested to identify significant treatment by using Analysis of Variance (ANOVA) followed by DUNCAN test at significance level of 5% using SPSS 16. The mean (n=3) and standard error were calculated on Microsoft office 2007. The data were computed in percentage and graphs were also plotted with the help of Microsoft office 2007.

Results

4.1. Survey on trends in use of agrochemicals

The data was collected randomly from tomato growers of some districts of Uttar Pradesh including Lucknow (Mohanlalganj, Chinhat, Bakshi ka Talab, Sarojini nagar, Bijnor, Bachhrawan), Unnao, Barabanki and Raebareli (**Figure 4.1**). Total 97 tomato growers (**n=97**) were interviewed (**Figure 4.2**) using pre-structured questionnaire (**Appendix**). The data regarding age, educational status, awareness among farmers, safety measures taken by farmers while handling chemical fertilizers and pesticides were also documented along with ecological stresses affecting productivity and trend in use of pesticides and fertilizers by them in order to gather information regarding farmer's dependency on agrochemicals for enhancing growth and productivity of tomato in this region.



Map Source: Google

Figure 4.1: Map of Uttar Pradesh showing study areas

Figure 4.2: Tomato fields visited in and around Lucknow city and survey conducted

4.1.1. Age and education status

The survey revealed that the majority of adults between the age group of 21-50 years were involved in agricultural activities and approximately only 14% of farmers above 50 years of age were cultivating tomato (**Table 4.1**). Nearly 40 % of the farmers engaged in tomato cultivation were illiterate and 31 % were having education up to primary level (**Table 4.2**).

Table 4.1: Age group of tomato cultivars in study area

Age group (in years)	≤ 20	21-30	31-40	41-50	51-60	>60	Data unavailable
Percentage of farmers (%)	3.09	22.68	27.84	24.74	13.40	1.03	7.22

Table 4.2: Education level of tomato cultivars in the study area

Education (grades)	Illiterate	Primary level (up to 8th)	Secondary level (9th to 12th)	Graduate	Post-graduate
Percentage of farmers (%)	40.21	30.96	18.56	9.28	1.03

The relationship between age and educational status of tomato growers (**Figure 4.3**) revealed that the most of the elder farmers (above the age of 40 years) were illiterate and few were educated up to primary level (up to 8th grade). However, nearly 19 % farmers of age group 31-40 years were having primary education and 16% of farmers below the age of 30 years were found to be well educated (education above 8th grade).

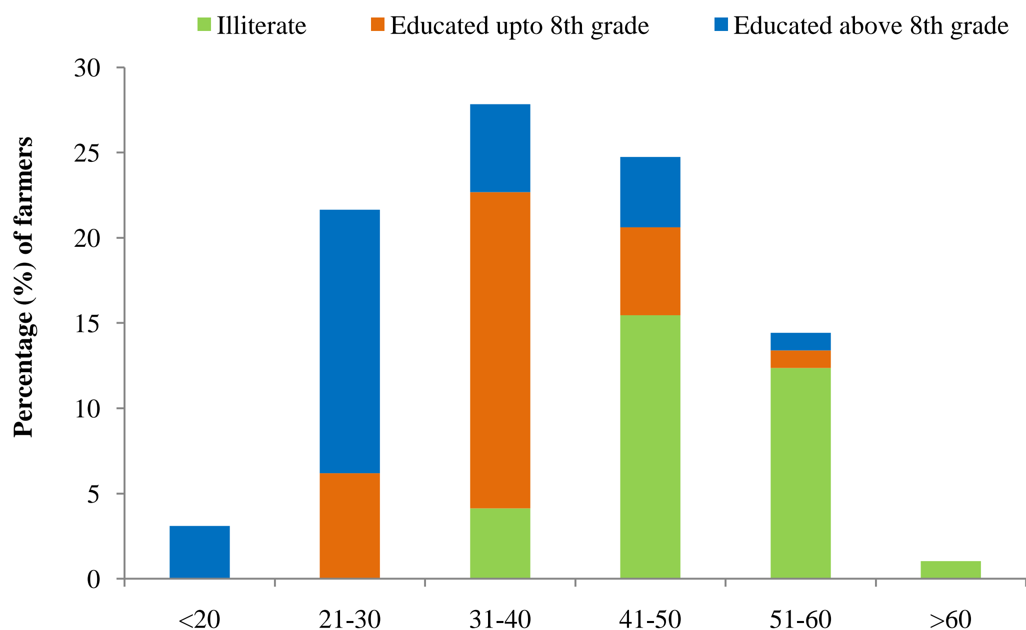


Figure 4.3: Showing relation between the age group of farmers and education level

4.1.2. Tomato farming land size and socio-economic status of farmers

Through data analyses of a survey it was observed that in the study area the tomato cultivation was done in small parts only. Farmers were growing tomato randomly in small patches. 55.67% of the total tomato cultivation was done in the area equal to or less than 0.13 hectare (0.3 acres) (**Table 4.3**). However, only 2.06% of farmers were cultivating tomato in an area above 1.3 hectares (3.1 acres).

Table 4.3: Land size under tomato farming

Land size in hectares (acres)	**≤ 0.13 (≤0.3)**	**0.13-0.26 (0.3-0.6)**	**0.26-1.3 (0.6–3.1)**	**>1.3 (>3.1)**	**Data unavailable**
Percentage of farmers (%)	55.67	4.12	15.46	2.06	22.68

4.1.3. Types of tomato seeds used and preferable time of tomato cultivation

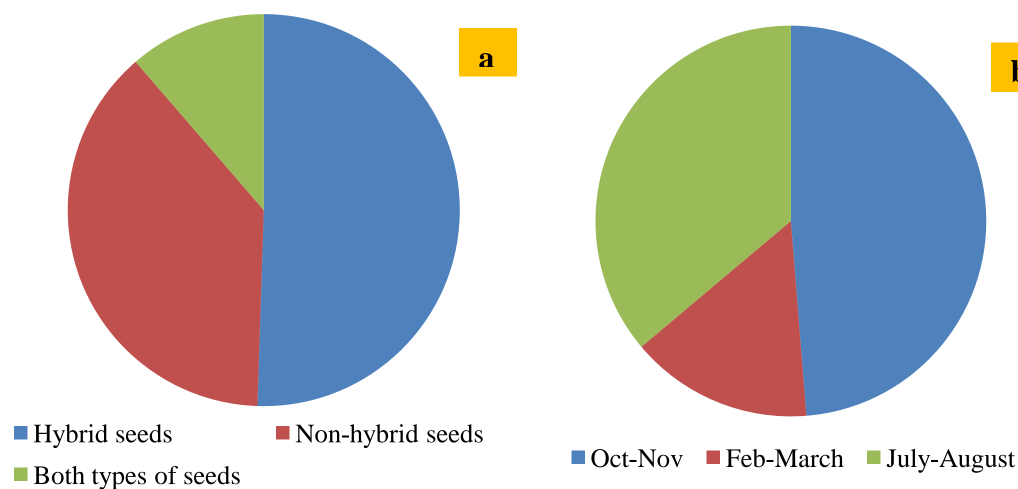


Figure 4.4: Farmer's preference for types of seeds and time of tomato cultivation in and around Lucknow city. a) Types of tomato seeds preferred; b) percentage of farmers cultivating tomato during different growing seasons

Both hybrid and non-hybrid tomato seeds were used by the farmers for cultivation; however, the use of hybrid seeds was higher than non-hybrid seeds (**Figure 4.4a**). The 51% of farmers preferred hybrid seeds for high yield, however, 38% farmers were using non-hybrid seeds in their fields and rest 11% farmers were using both hybrids as well as non-hybrid seeds for tomato cultivation. Data revealed that tomato was cultivated in all three crop growing seasons *i.e.,* Rabi, Kharif, and Zaid. However, Rabi season was the most preferred season for tomato cultivation (**Figure 4.4b**). The sowing was preferably done in during the months of October-November (59.79 %), followed by July-August (44.33 %) and February-March (18.56 %).

4.1.4. Abiotic stresses and their management

Abiotic stresses such as temperature, rainfall, and water availability were found to be major factors affecting tomato productivity. Extreme conditions of temperature were

reported to reduce the total yield of tomato. Heavy rainfall was also complained to be a reason for the damage of the crop resulting in lower yield. Similarly, scarcity of water was another major problem for tomato cultivation. The fields were irrigated by flood irrigation and pumping by using tubewell engines that run at the cost of Rs. 110-150 per hour. This extra cost increases the burden on the shoulders of farmers already suffering from other problems in the crop cultivation.

4.1.5. Biotic stress encountered and types of pesticides used by farmers

Diseases and pests infestation were the most common biotic factors affecting tomato productivity in the study areas. Many farmers also reported wild animals *e.g.,* Nilgai (*Boselaphus tragocamelus*) destroying crops. The common tomato pests and pathogenic diseases occurred and pesticides used for their management by farmers in the study area are outlined in **table 4.4**.

Table 4.4: The biotic stress affecting tomato cultivation and types of pesticides used by farmer's in the study area

Types of diseases and pests reported in study areas	Major Pesticides used by farmers
Fungal and bacterial diseases (bacterial and fungal wilt; damping off, early blight) resulting in wilting, necrosis, damping off, dark spots on leaves and fruits	Mancozeb (Dithane M-45), Metalaxyl, Carbendazim, Thiram, Copper oxychloride (Blitox), Chlorothalonil (Docket), *etc*.
Viral diseases like leaf curl and dwarfness and Pests infestation *e.g.,* caterpillar, aphids, termites, and bugs	Imidacloprid, Carbosulfan (Marshal), Chloropyrifos, Quinolphos, Phorate, Acephate (starthene), Lambda-Cyhalothrin (Corolambda), *etc*.

4.1.6. Trends in the use of conventional agrochemicals and alternative methods by farmers

To protect the tomato crops from pests and pathogens infestation farmers were heavily reliant on chemical pesticides. The most common pesticides being used by the tomato growers in the study area were the new generation pesticides belonging to class of synthetic pyrethroids (Lambda-Cyhalothrin), neonicotinoids (Imidacloprid), organophosphates (Chloropyrifos, Quinolphos, Phorate, Acephate) and carbamates (Mancozeb, Carbosulfan, Carbendazim, Thiram).

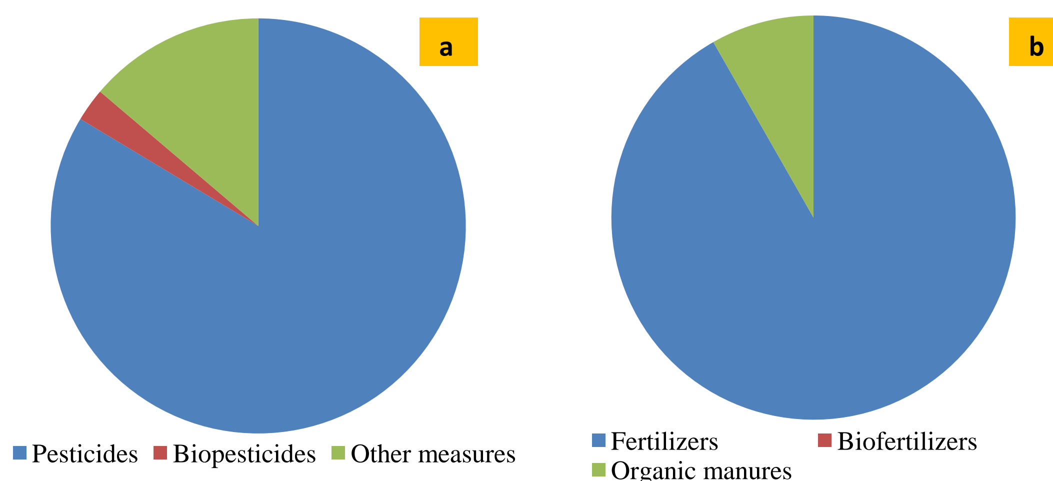


Figure 4.5: Use of various methods for disease control and plant growth promotion. a) Percentage of different control measures used for controlling pathogens; b) Percentage of different fertilizers and methods used for plant growth promotion

Indiscriminate use of pesticides was very high. Application of pesticides was done as recommended by the sellers. Through the survey, it was also observed that in some areas farmers were being gullied by the sellers to make their own profits. Use of biopesticides by farmers was negligible however some farmers were found to use other alternatives such as the use of flyash, removal of diseased plants, rotation of crops, *etc*. to protect from damages caused by pests and pathogens (**Figure 4.5a**).

Application of chemical fertilizers in the study areas for tomato cultivation was high owing to the general belief that high inputs could result in higher yield. Di-ammonium phosphate (DAP) and Urea were the most common chemical fertilizers used while other chemical fertilizers were also used in a smaller portion including Potash, Zinc, Sulphur, and NPK. None of the farmers revealed the use of biofertilizers, but cow dung and compost were reported to be used by around 9% of the farmers in the study areas (**Figure 4.5b**).

4.1.7. Total benefits received by farmers

No exact estimate regarding the total input cost and returns could be documented. In general for tomato production in 0.13 hectare (0.3 acres) acreage, the total input cost of Rs. 15,000 to 20,000 was roughly estimated. In case of good productivity, without major loses, profit in monetary terms was documented to be around 5,000 to 10,000 for this acreage whereas in case of loses it becomes hard for the farmers to cover the input cost even.

4.1.8. Safety measures taken by the farmers

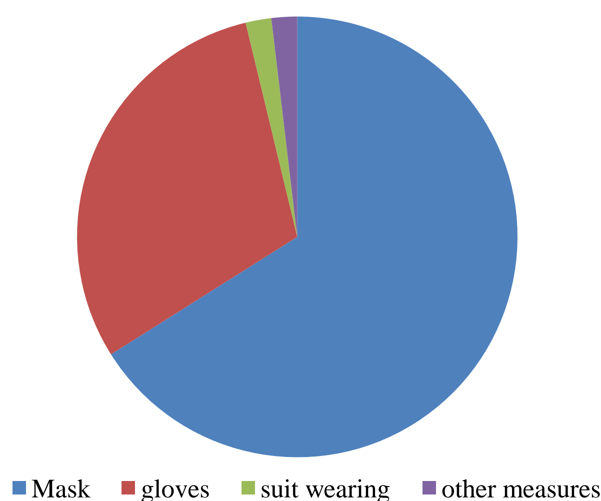


Figure 4.6: Percentage of tomato cultivating farmers using safety measures during pesticide application

As knowledge pertaining to the ill effects of pesticides use among farmers was low, very few farmers (~36%) were found to take safety measures while spraying pesticides in the field (**Figure 4.6**). Among those farmers, most of them were recorded to cover their faces with a cloth as a mask. Only a few of them (16.5 %) were stated use of gloves while spraying pesticides in fields. However, the majority of farmers were reluctant to use any safety measures before applying pesticides in their fields. Out of 97 farmers interviewed only one farmer was documented to use safety suit and study the direction of air flow before pesticide spraying as a step toward safety measure.

4.2. Isolation and characterization of pathogenic fungi of tomato

Table 4.5: *Fusarium* and *Alternaria* species isolated from infected tomato plant and soil

Samples collected	*Fusarium* spp.	*Alternaria* spp.
Soil	5	3
Root	3	0
Leaves	0	8
Total	**8**	**11**

The infected tomato plants (**Figure 4.7**) and soil samples were collected from different tomato growing areas to isolate fungal pathogens belonging to genera *Fusarium* and *Alternaria*. Total eight isolates of *Fusarium* spp. and eleven isolates of *Alternaria* spp. were obtained from various samples collected (**Table 4.5**). The fungal strains isolated were identified on the basis of morphological characters *i.e.,* colony appearance color of mycelium on culture media (**Figure 4.8 and 4.9**) and spores produced by them (**Figure 4.10**). After identification, the strains were assessed for their ability to cause infection *i.e.,* pathogenicity in tomato plants under *in vitro* condition.

Figure 4.7: Infected tomato plants

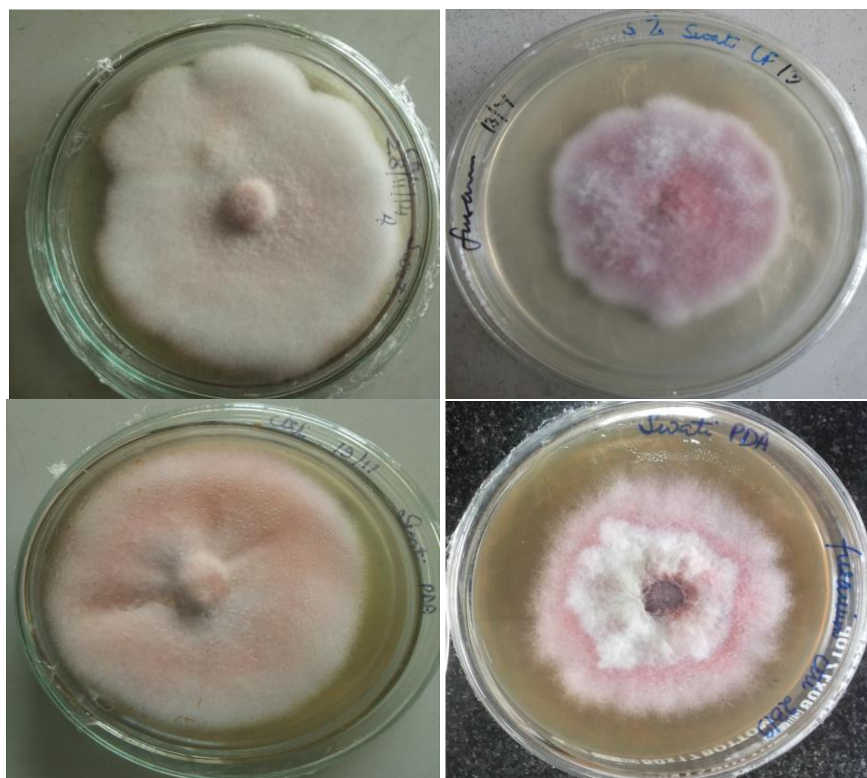


Figure 4.8: Different isolates of *Fusarium oxysporum* isolated from soil and parts of infected tomato plants

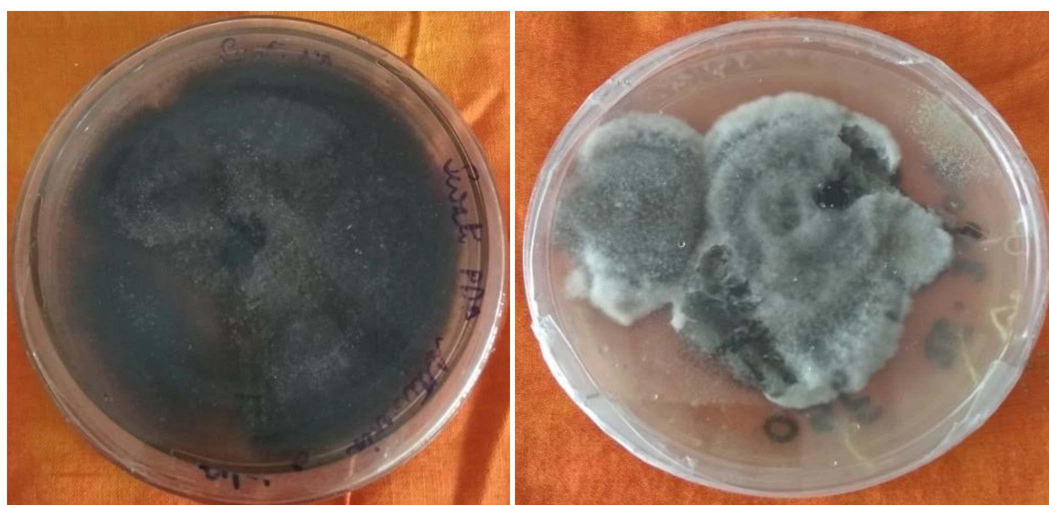


Figure 4.9: Different isolates of *Alternaria* spp. isolated from infected tomato plants

Figure 4.10: Microscopic structure of fungal pathogens isolated. a) Macroconidia of *Fusarium oxysporum* strains; b) spores of *Alternaria* sp.

Figure 4.11: Pathogenicity assay for *Fusarium oxysporum* f. sp. *lycopersici*. Left: comparison between control and infected seedling; right: the enlarged image of infected seedlings

Figure 4.12: Pathogenicity assay for *Alternaria solani*. Left: *in vitro* pathogenicity assay; right: the enlarged image of an infected leaf

The pathogenicity assay revealed that out of eight strains of *Fusarium oxysporum* isolated, two were positive for pathogenic activity and one strain showed the highest disease severity index (58%) (**Figure 4.11**). Similarly, five strains of *Alternaria* isolated were found to be pathogenic with the most virulent strain showing disease severity index of 49.3% (**Figure 4.12**). On the basis of morphological characters and the characteristic symptoms produced by them on tomato plants under *in vitro* condition, the highly pathogenic strains were identified as *Fusarium oxysporum* f. sp. *lycopersici* and *Alternaria solani* which were used further in the study.

4.3. Isolation, identification, and characterization of rhizospheric microbial isolates

Healthy plant roots and soil samples were collected from various fields to isolate rhizospheric microbial strains (Plant growth promoting rhizobacteria (PGPR) and *Trichoderma*) possessing antagonistic activities. In total seven isolates of fungi (two *Trichoderma* spp.) and fifteen isolates of bacteria were isolated (**Table 4.6**).

Table 4.6: Rhizospheric microbial isolates

	Samples collected	Number of isolates	Identified as
Fungal Isolates	Soil	6	*Aspergillus* spp.
			Penicillium spp.
			Trichoderma sp.
			Rhizopus sp.
	Root	1	*Trichoderma* sp.
	Total	**7**	
Bacterial Isolates	Soil	12	*Bacillus* spp.
			Escherichia coli
			Streptococcus sp.
			Citrobacter sp.
			Proteus sp.
			Enterobacter sp.
	Root	3	*Pseudomonas* sp.
			Bacillus spp.
	Total	**15**	

4.3.1. Morphological identification of fungal Isolates

The six fungal strains were isolated from rhizospheric soil and one from the root surface. All the fungal strains isolated *i.e.,* TvR1, TbS2 were identified as species belonging to genus *Trichoderma*, AcS4, AbS5 as *Aspergillus*, PbS3, PrS7 as *Penicillium* and RcS6 as *Rhizopus* on the basis of colony appearance and microscopic structures (**Figure 4.13 and 4.14**). The two isolates TvR1 and TbS2 identified as *Trichoderma* species were further studies and identified in detail for confirmation.

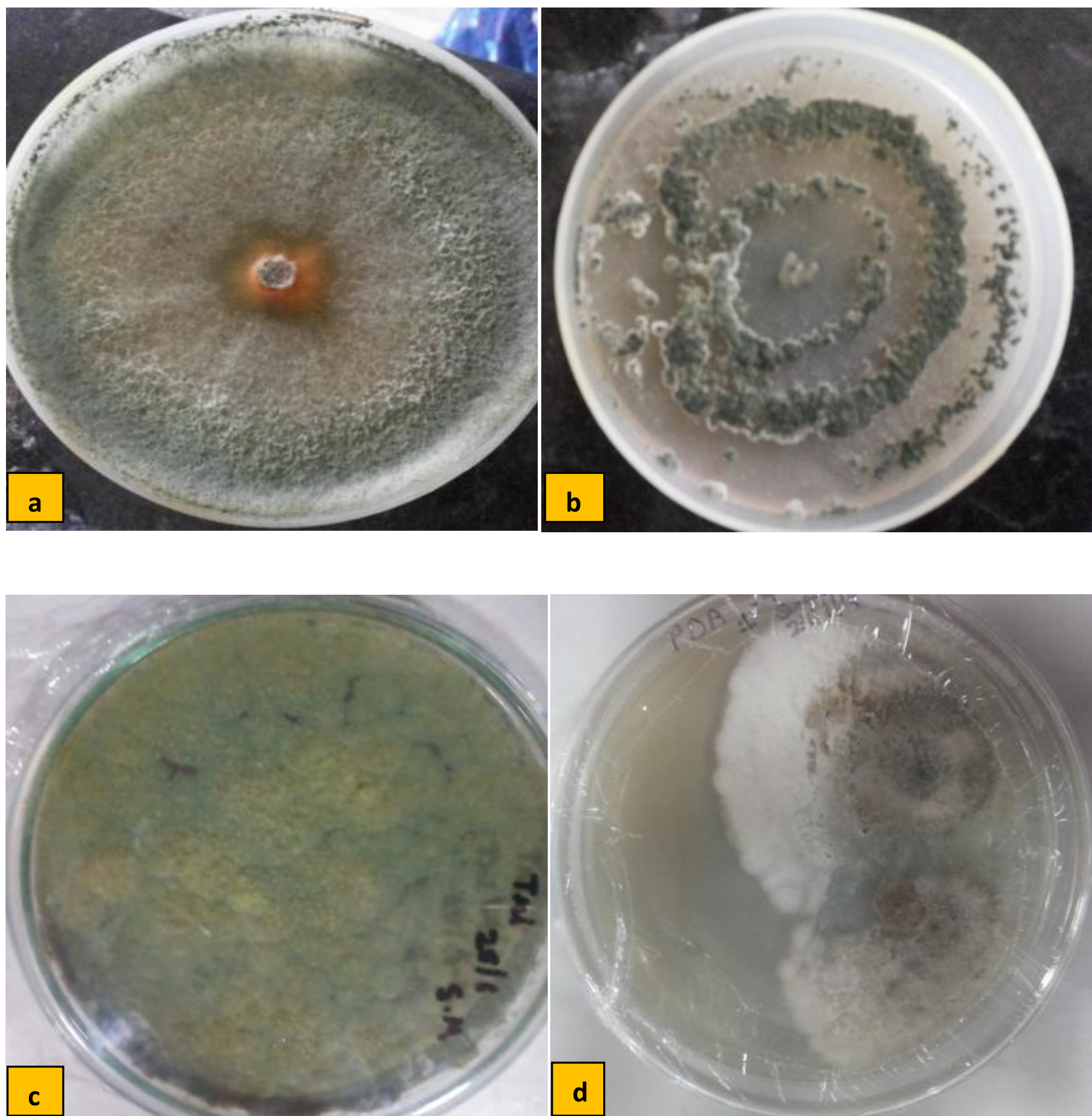


Figure 4.13: Rhizospheric fungi isolated from healthy plant soil and roots. a) TvR1; b) TbS2; c) AcS4; d) PbS3

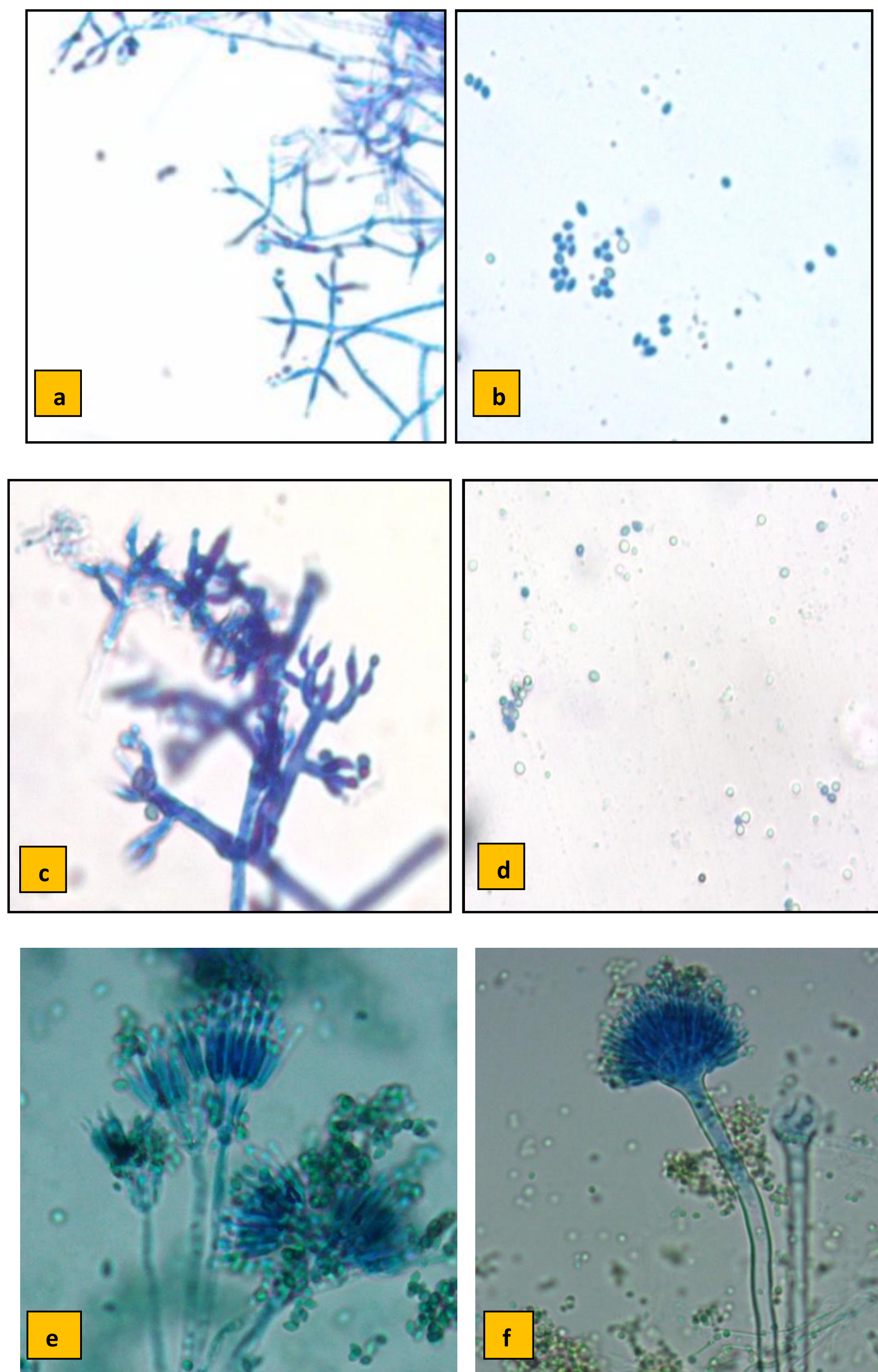


Figure 4.14: Microscopic structure of isolated rhizospheric fungi at 40X (Light microscope). a) Phialides and conidiophores of TvR1; b) conidia of TvR1; c) Phialides and conidiophores of TbS2; d) Conidia of TbS2; e) PbS3; f) AcS4

4.3.2. Phenotypic and microscopic identification of *Trichoderma* isolates

The isolates of *Trichoderma* were further identified in detail on the basis of phenotypic characters. The morphological identification of *Trichoderma* isolate TvR1 and TbS2 was done on basis of the colony appearance, growth rate, and microscopic structures. After 96 hrs of incubation, the radial growth of TvR1 and TbS2 were reported to be 45 mm and 31.1 mm respectively (**Table 4.7**).

Table 4.7: A morphological feature of *Trichoderma* isolates TvR1 and TbS2 after 92 hours of incubation

Parameters	TvR1	TbS2
Conidia	Whitish green spores grown over full plate	White spores arranged in concentric rings
Mycelium	White mycelia	White mycelia
The reverse side of the colony	Yellowish	White
Radial growth (mm)	45.0	31.1

The isolate TvR1 found to grow faster as compared to isolate TbS2. The green color subglobose to ovoidal conidia were produced by a mature colony of both of the isolates. The conidia of TvR1 were found effused in mycelia and spread on Petri plate, densely in the center and at periphery without pustules. In case of TbS2 conidia were arranged in two concentric undulating rings forming pustules (**Figure 4.13 a, b**). The microscopic features (**Figure 4.14 a-d; 4.15**) revealed the shape, size, and arrangement of phialides that were flask-shaped, constricted toward tip and base and swollen in middle. On the basis of morphological and microscopic characteristics described by Samuels et al. (2002, 2014) the fungal isolates were confirmed as *Trichoderma* spp.

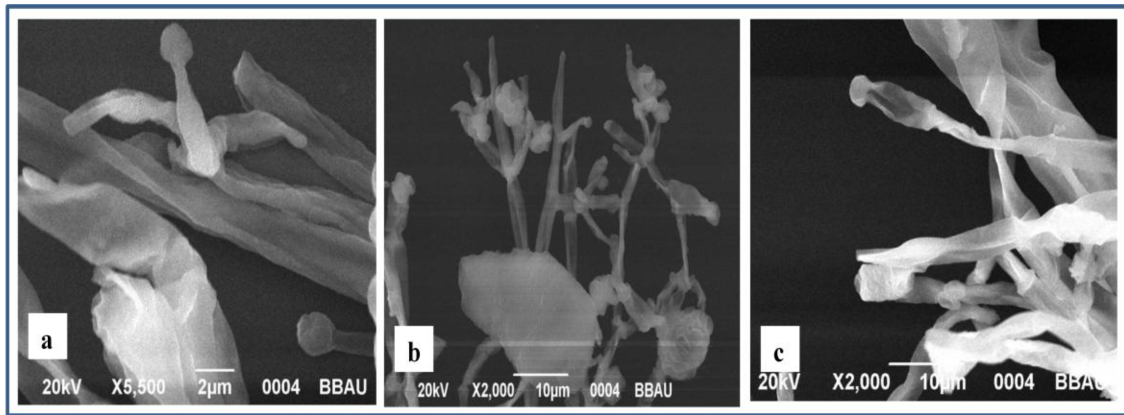


Figure 4.15: Scanning Electron Microscopic (SEM) images of the microscopic structure of *Trichoderma* isolates. a and b) TvR1; c) TbS2

4.3.3. Biochemical identification of rhizobacterial isolates

The twelve rhizobacteria were isolated from soil and three from root surfaces of a different plant. The isolated strains were distinguished on the basis of colony morphology and were purified (**Figure 4.16**). The purified isolates were initially identified on the basis of biochemical characteristics (Aneja, 2003). On the basis of biochemical tests, 47% of total isolated rhizobacterial strains were identified as species belonging to genus *Bacillus*. The single strain isolated from healthy tomato roots was characterized as *Pseudomonas* sp. The remaining isolates were identified as *Proteus*, *Streptococcus*, *Enterobacter,* and *Escherichia*. The biochemical reaction displayed by all the isolated strains is presented in **table 4.8**.

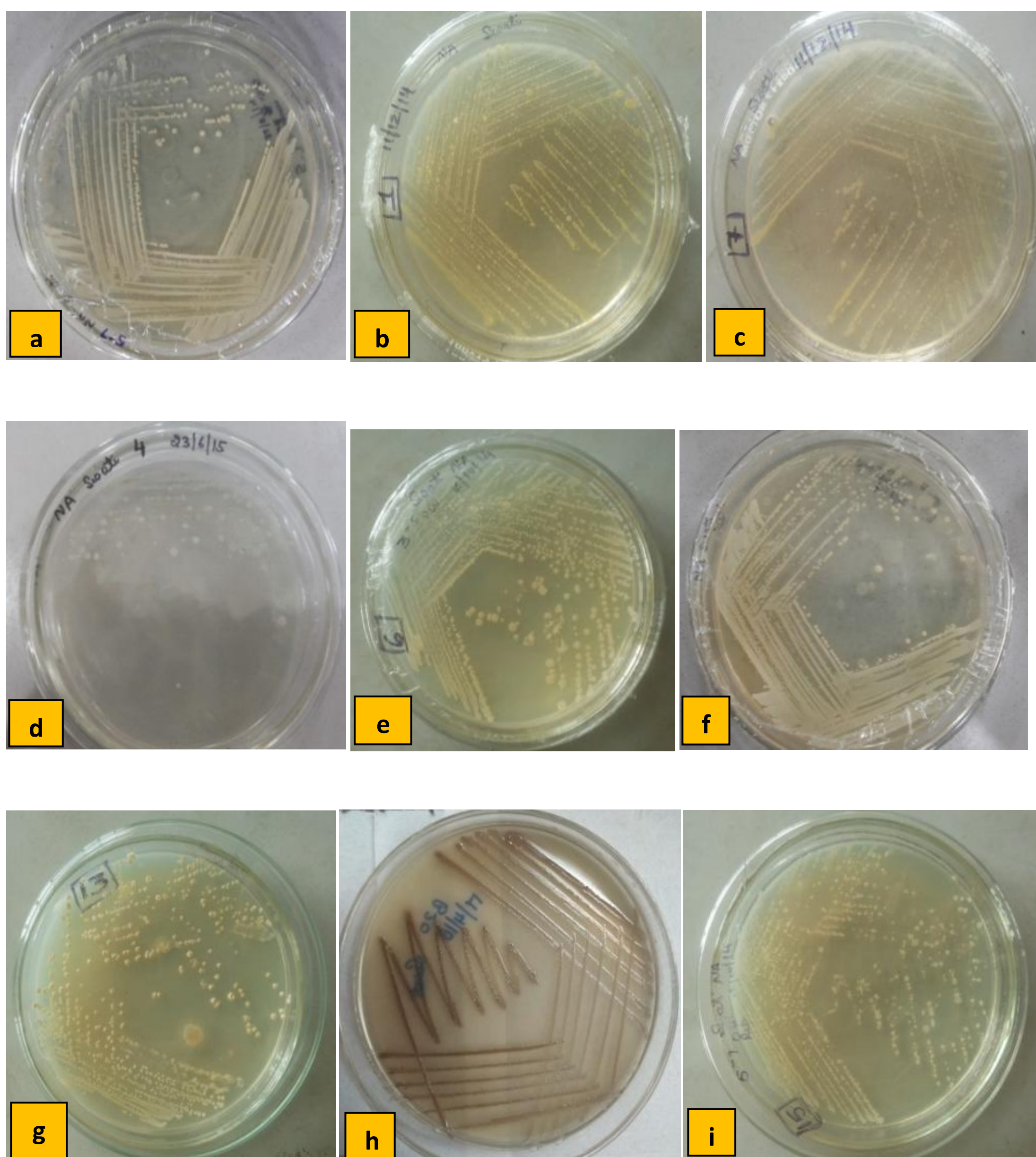


Figure 4.16: The purified colony of rhizobacterial strains isolated from healthy plants soil and root. a) TS1; b) LS2; c) LS3; d) LS4; e) BS6; f) ES4; g) CS13; h) Tr20; i) Ts23

Table 4.8: Biochemical characteristics of isolated rhizobacteria

Biochemical characters	TS1	LS2	LS3	LS4	BS5	BS6	ES1	ES2	ES3	ES4	CS1	CS2	CS13	Tr20	Ts23
Gram reaction	+	+	+	+	+	+	-	-	-	+	-	-	+	-	+
Cell shape	rod	cocci	rod	cocci	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod
Motility	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Nitrate reductase	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
Catalase	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-
MR test	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-
VP test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrate utilization	-	+	-	-	-	+	-	+	-	-	+	-	+	+	+
Urease	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Amylase hydrolysis	+	-	+	+	-	+	-	+	+	+	-	-	+	-	-
Gelatin hydrolysis	-	-	-	-	-	-	-	+	-	+	+	-	+	+	+
Glucose fermentation	ND	+	-	+	-	+	+	ND	-	-	+	+	+	-	-
Lactose/ Sucrose fermentation	+	-	-	+	-	-	+	ND	-	-	+	-	-	-	-
H_2S gas production	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-
	Bacillus	***Streptococcus***	***Bacillus***	***Streptococcus***	***Bacillus***	***Bacillus***	***Escherichia***	***Citrobacter***	***Proteus***	***Bacillus***	***Enterobacter***	***Escherichia***	***Bacillus***	***Pseudomonas***	***Bacillus***

ND= Not Detected as butt and slant was masked black due to H_2S production; - = negative; + = positive; MR=Methyl Red; P=Vogues Proskauer

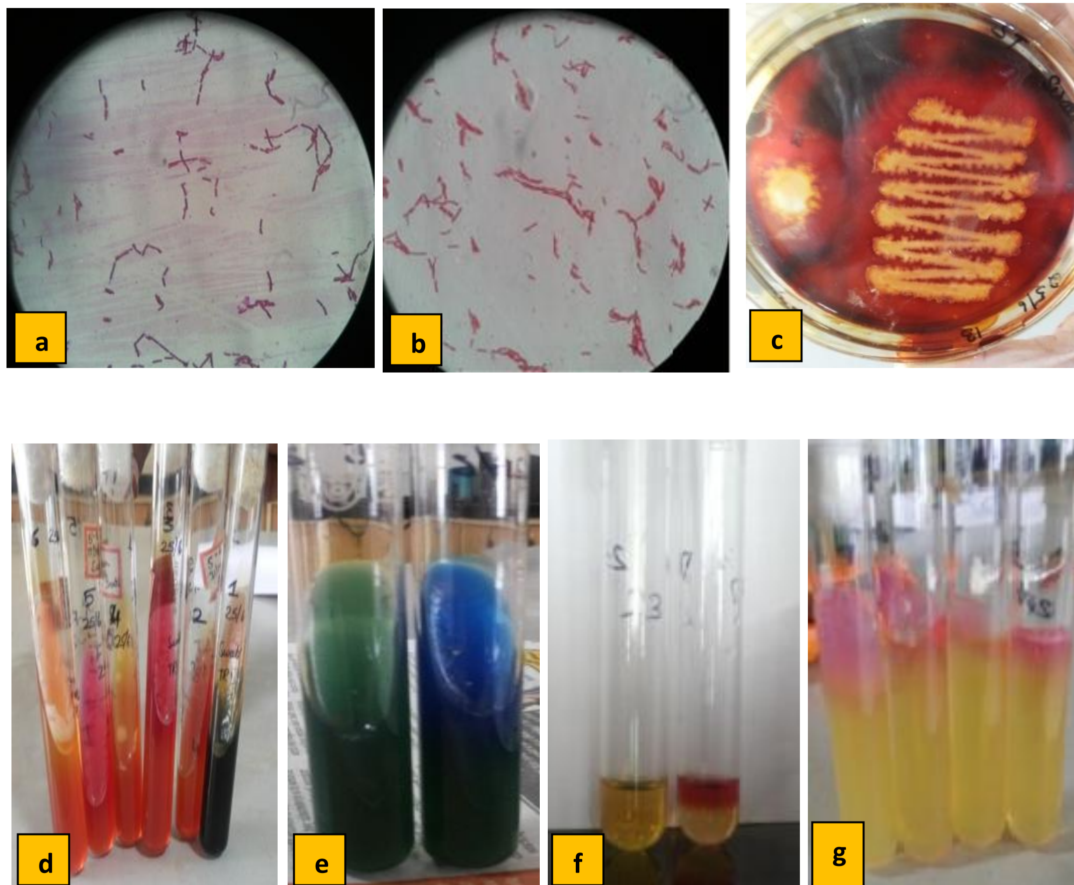


Figure 4.17: Biochemical characteristics of isolated rhizobacteria. a) Gram-positive rhizobacteria; b) Gram-negative rhizobacteria c) Amylase activity; d) Triple sugar Iron test; e) Citrate utilization test; f) Methyl red test; g) Urease test

4.3.4. Dual culture assay

The antagonistic assays of *Trichoderma* and rhizobacterial isolates on *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) and *A. solani* were assessed by exploiting the dual culture technique. Both the *Trichoderma* isolates (TvR1 and TbS2) and three rhizobacterial isolates BS6, CS13 and Tr20 were found possessing antagonistic potential against pathogens. The data obtained are presented in **table 4.9**.

Table 4.9: Percentage inhibition of radial growth of pathogens (PIRG) by isolated *Trichoderma* and rhizobacterial isolates

	Isolates	*Fusarium oxysporum* f. sp. *lycopersici*	*Alternaria solani*
Trichod-erma	TvR1	55.31^{d} ± 0.75	66.54^{d} ± 1.15
	TbS2	43.22^{c} ± 1.63	49.81^{a} ± 0.71
Rhizobacteria	TS1	ND	ND
	LS2	ND	ND
	LS3	ND	ND
	LS4	ND	ND
	BS5	ND	ND
	BS6	28.64^{a} ± 0.37	51.67^{a} ± 0.99
	ES1	ND	ND
	ES2	ND	ND
	ES3	ND	ND
	ES4	ND	ND
	CS1	ND	ND
	CS2	ND	ND
	CS13	31.64^{b} ± 0.41	56.12^{b} ± 1.01
	Tr20	54.09^{d} ± 0.76	61.54^{c} ± 0.96
	Ts23	ND	ND

Values are mean of triplicate; ND= Not Detected; ± = Standard error; values followed by different letters are significantly different according to DUNCAN test at 5% significance level

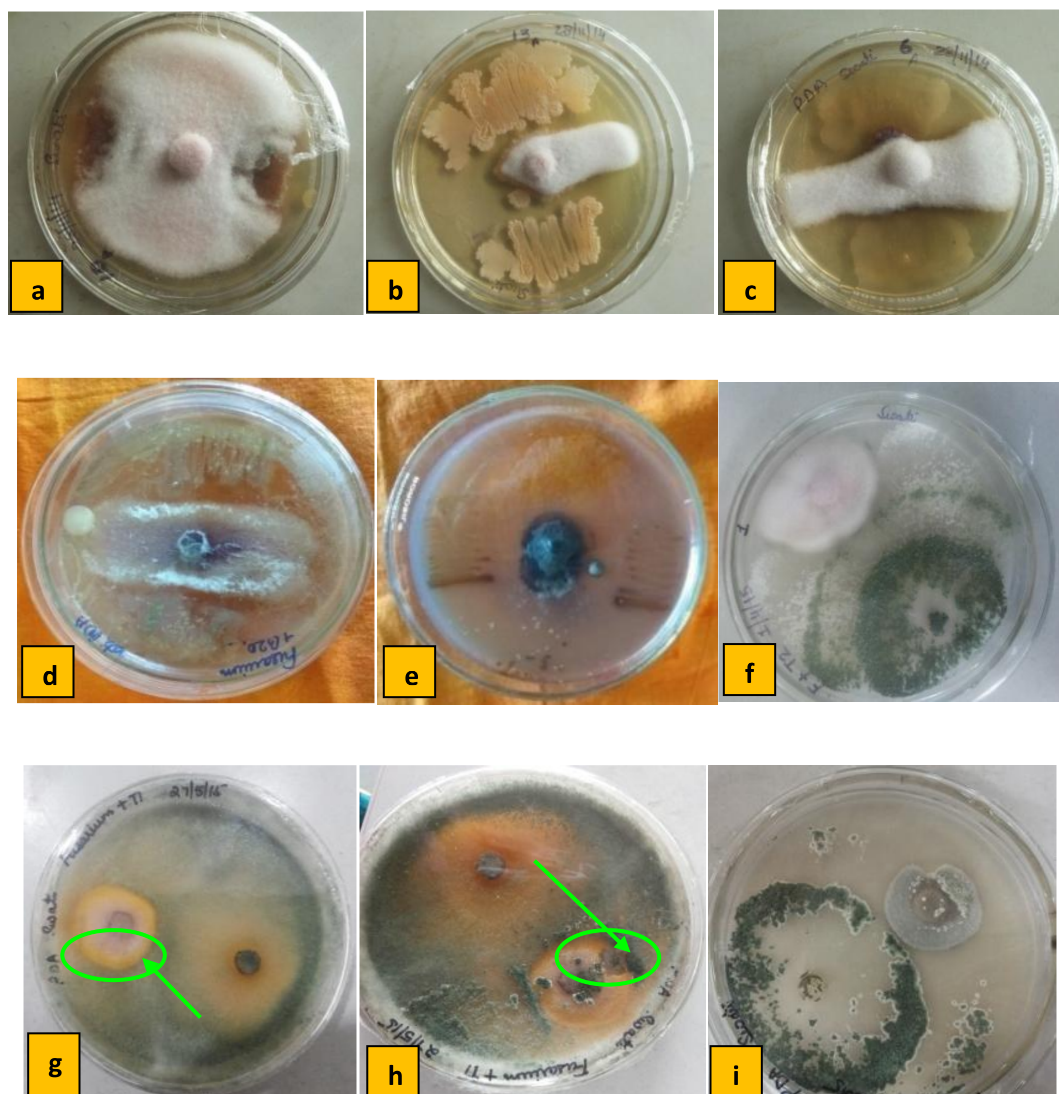


Figure 4.18: Dual culture of rhizospheric isolates and pathogenic fungi. a) Growth of *Fol* over rhizobacteria CS1; b) *Fol* mycelia growth inhibition by CS13; c) *Fol* mycelia growth inhibition by BS6; d) *Fol* mycelia growth inhibition by Tr20; e) *Alternaria solani* mycelia growth inhibition by Tr20; f) *Fol* mycelia growth inhibition by TbS2; g) The arrow shows production of antibiotic by TvR1 around colony of *Fol* resulting growth inhibition; h) *A. solani* mycelia growth inhibition by *Trichoderma* TvR1; i) Overgrowth of TvR1 on *A. solani*

Among all, the two isolates *Trichoderma* TvR1 and rhizobacteria Tr20 showed maximum radial growth inhibition of *Fol* and *A. solani*. Percentage mycelial growth inhibition of *Fol* and *A. solani* by TvR1 was recorded 55.31 and 66.54%, respectively and Tr20 showed 54.09 and 61.54% inhibition, respectively (**Figure 4.18**). Dual culture assay revealed the better antagonistic potential of *Trichoderma* spp. and rhizobacterial isolates against *A. solani* as compared to *Fol* under *in vitro* condition.

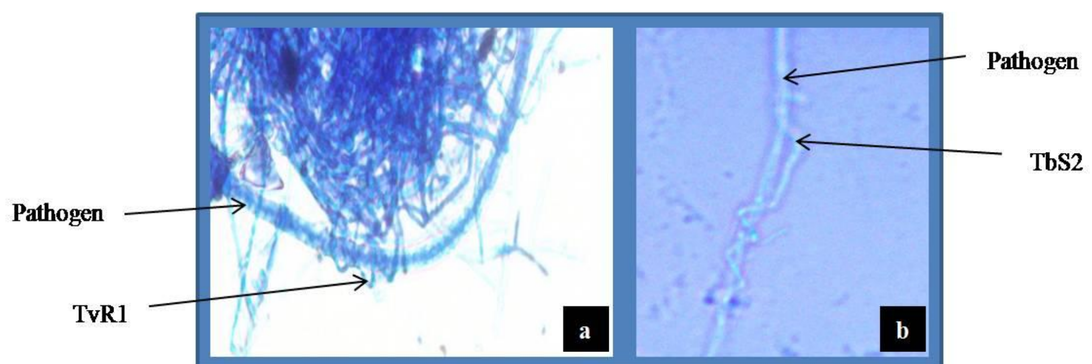


Figure 4.19: Showing coiling around pathogen mycelia by a) *Trichoderma* TvR1; b) *Trichoderma* TbS2

The isolates of *Trichoderma* TvR1 and TbS2 were fast growing compared to pathogens, and their first contact with pathogens was noticed on 3rd and 5th days of inoculation, respectively. TvR1 and TbS2 restricted the growth of pathogens, depicting the possible involvement of a mechanism of competition for space. Isolate TbS2 was found only to arrest the growth of pathogens (**Figure 4.18 f**) whereas isolate TvR1 restricted the growth of pathogens and formed a clear inhibition zone suggesting a role of antibiotic compounds in growth inhibition (**Figure 4.18 g**). Formation of an inhibition zone around pathogens by TvR1 indicates secretion of non- volatile diffusible metabolites having an inhibitory effect on pathogen growth. With the increase in incubation time, *Trichoderma* isolates overgrew on a colony of

pathogens (**Figure 4.18 i**), revealing the involvement of mycoparasitic mode of action which was confirmed by a microscopic study showing coiling of *Trichoderma* hyphae around pathogens hyphae (**Figure 4.19**).

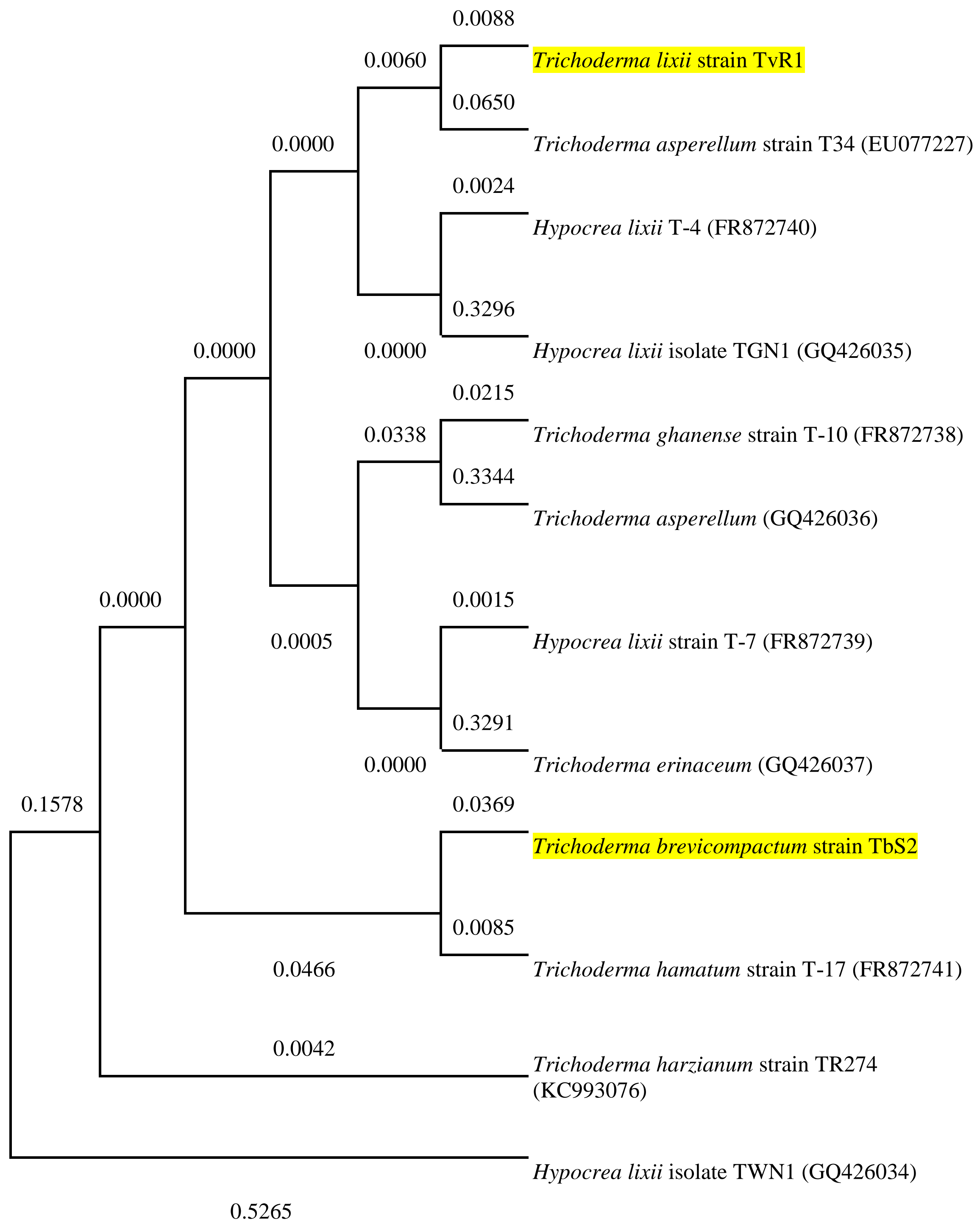
4.3.5. Identification of microbial isolates at the genomic level

The strains showing positive antagonistic ability in dual culture assay were identified at a genetic level on the basis of 16s RNA and ITS region sequencing. The isolated rhizobacterial strains BS6 and CS13 were identified as *Bacillus subtilis*, Tr20 as *Pseudomonas aeruginosa*, *Trichoderma* TvR1 and TbS2 as *Trichoderma lixii* and *Trichoderma brevicompactum*, respectively.

Table 4.10: Accession number and identified species of isolated antagonistic rhizospheric strains at the genomic level

Bacterial/ Fungal Isolates	Identified as	Accession no.
BS6	*Bacillus subtilis*	MF780728
CS13	*Bacillus subtilis*	MF678835
Tr20	*Pseudomonas aeruginosa*	MF797805
TvR1	*Trichoderma lixii*	MF780730
TbS2	*Trichoderma brevicompactum*	MF780729

The accession number of identified isolates was obtained from NCBI and their phylogenetic tree was constructed which are presented in **Table 4.10** and **Figure 4.20**, respectively.



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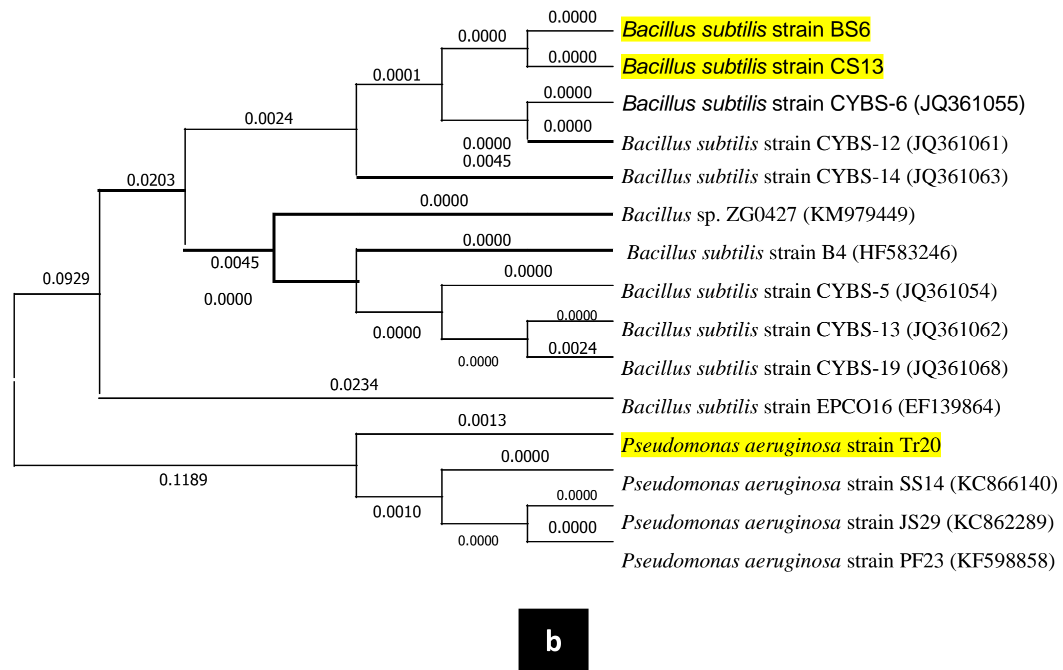

Figure 4.20: Phylogenetic trees were constructed using neighbor-joining tree based on analysis of partial 18S rRNA nucleotide and 16S rRNA nucleotide sequence of fungi and rhizobacterial isolates respectively, showing the relationship between isolated and other strains documented in literature possessing biocontrol, PGP and stress alleviation activity. a) Phylogenetic tree of *Trichoderma* isolates; b) Phylogenetic tree of bacterial isolates

4.3.6. Characterization of microbial isolates for production of secondary metabolites

Trichoderma and rhizobacterial isolates were screened for production of Indole acetic acid, ammonia, siderophore, hydrogen cyanide, ability to solubilize inorganic phosphate and zinc, and hydrolytic enzymes *i.e.,* chitinase, protease, and cellulase (**Table 4.11**). Both the species of *Trichoderma* and three bacterial strains *Pseudomonas* Tr20, *Bacillus* Tr23 and *Bacillus* CS13 showing antagonistic activity under *in vitro* condition displayed plant growth promoting (PGP) ability as well as the

production of enzymes involved in biocontrol of phytopathogens (**Figure 4.21 and 4.22**).

The isolates that showed only PGP activities include rhizobacterial isolates TS1, LS2, ES1, ES2, ES3, ES4, and CS1. The *Trichoderma* isolates TvR1 and TbS2 and rhizobacterial isolates Tr20, ES2, Ts23 and ES1 displayed ability to produce plant growth promoting hormone Indole Acetic Acid (IAA). The production of ammonia was reported by *Trichoderma* isolates TvR1 and TbS2 and four rhizobacterial isolates *i.e.,* TS1, ES4, CS13, and Tr20. None of the two *Trichoderma* isolates showed a positive result for inorganic phosphate solubilization, whereas rhizobacterial isolates TS1, ES1, ES4, CS1, and Tr20 showed the capability to solubilize inorganic phosphate. Another PGP trait, Zn solubilization was reported to be displayed by the *Trichoderma* TvR1 and TbS2 and rhizobacteria Tr20.

The mechanisms of mycoparasitism and competition for nutrients involve the production of cell wall degrading enzymes (CWDEs) like chitinase, β-1,3 glucanase, protease, cellulase, and low molecular compound siderophores and hydrogen cyanide (HCN) that are instrumental in limiting the growth of pathogens (Shrestha et al. 2015). The *Trichoderma* spp. TvR1 and TbS2 and rhizobacterial isolate Tr20 studied in present work displayed the positive result for chitinase and siderophore production; protease production was reported in BS6, CS13 and Tr20 and cellulase production were observed for TvR1, TbS2, Tr20, and Ts23. The rhizobacteria Tr20 was the only isolate that was reported to produce Hydrogen cyanide (HCN).

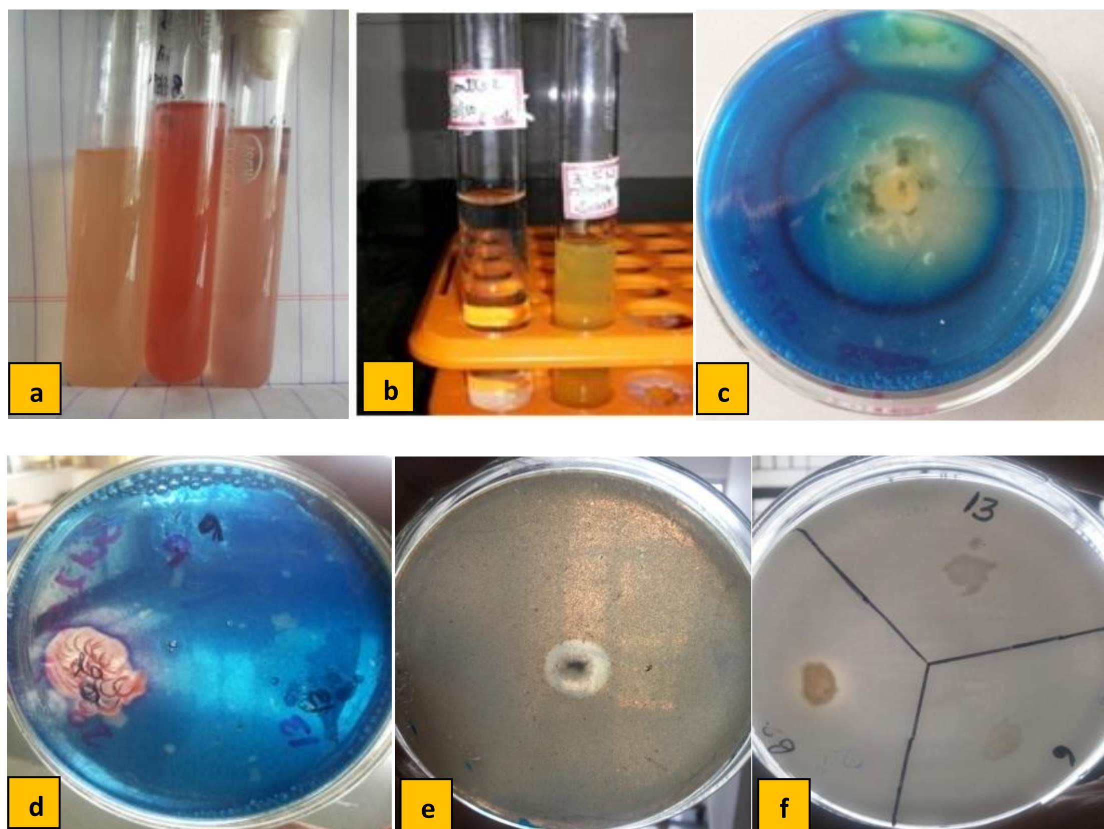

Figure 4.21: Plant growth promoting the activity of rhizospheric isolates. a) Indole acetic acid production; b) Ammonia production (left: control; right: positive); c) Siderophore production by *Trichoderma* TvR1; d) Siderophore production by *Pseudomonas* Tr20; e) Zn solubilization activity of *Trichoderma* TbS2; f) Inorganic phosphate solubilization by *Pseudomonas* Tr20

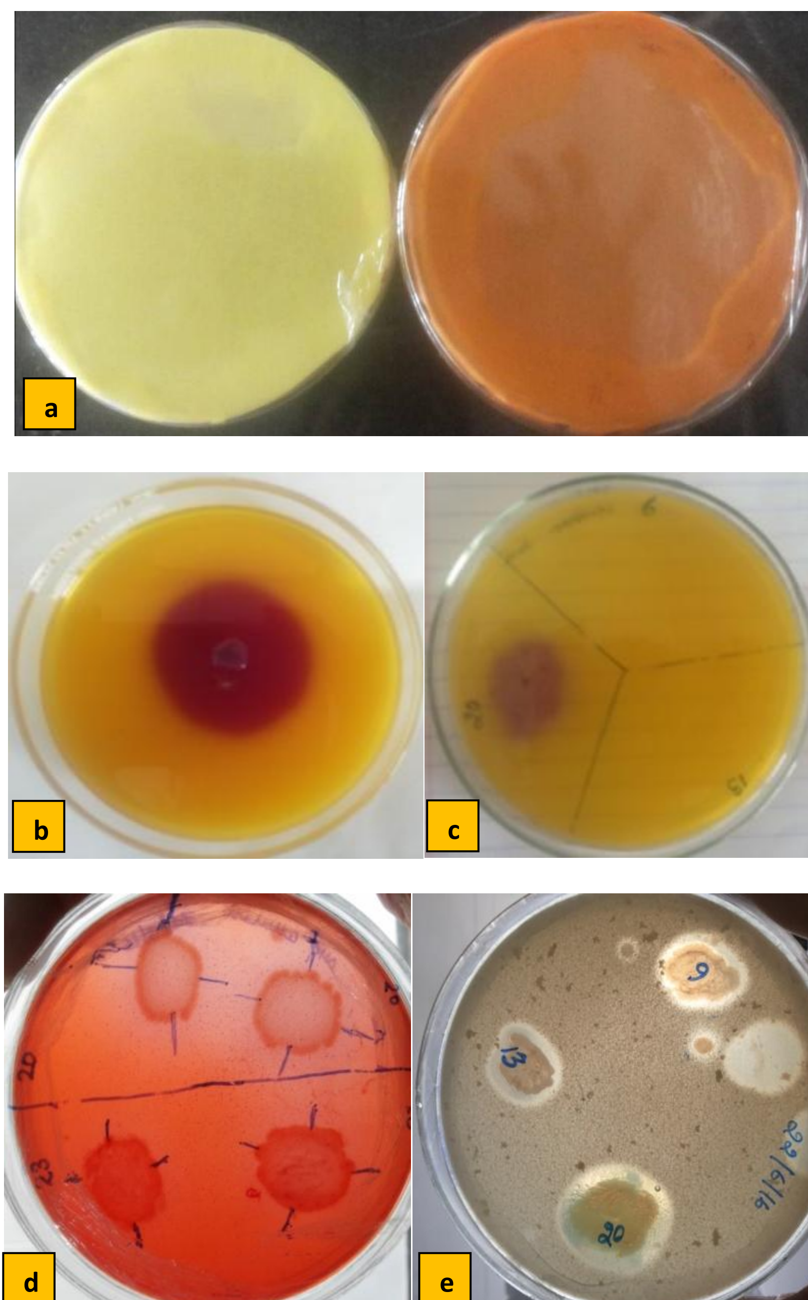

Figure 4.22: Production of lytic enzymes by rhizospheric microbial isolates. a) HCN production by *Pseudomonas* Tr20 (left: control; right: positive); b) Chitinase production by *Trichoderma* TvR1; c) Chitinase production by *Pseudomonas* Tr20; d) Cellulase production *Pseudomonas* Tr20 and *Bacillus* Ts23; e) protease production by *Bacillus* BS6 and CS13 and *Pseudomonas* Tr20

Table 4.11: Plant growth promoting (PGP) and biocontrol attributes of *Trichoderma* and rhizobacterial isolates

PGP/Biocontrol activity	TvR1	TbS2	TS1	LS2	LS3	LS4	BS5	BS6	ES1	ES2	ES3	ES4	CS1	CS2	CS13	Tr20	Ts23
IAA	+	+	+	-	-	-	-	-	+	+	+	-	-	-	-	+	+
Ammonia	+	+	+	-	-	-	-	-	-	-	-	+	-	-	+	+	-
Phosphate solubilization	-	-	+	+	-	-	-	-	+	-	-	+	+	-	-	+	-
Zinc solubilization	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Siderophore	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
HCN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Cellulase	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Chitinase	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Protease	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	-

- = negative; + positive

4.3.7. Quantitative estimation of Indole acetic acid and solubilization index of inorganic mineral

The isolates that displayed positive antagonistic abilities were assayed for quantitative estimation of Indole acetic acid produced and the inorganic mineral (phosphate and Zinc) solubilization index was calculated. **Table 4.12** outlines the data obtained through the study.

Table 4.12: Quantitative estimation of IAA and Solubilization index value for inorganic minerals (Phosphate and Zinc)

Isolates	Phosphate solubilization index (PSI value)	IAA (µg/ml)	Zinc solubilization index (ZSI value)
Bacillus BS6	-	-	-
Bacillus CS13	-	-	-
Pseudomonas Tr20	1.65±0.02	1.37±0.02[b]	1.53±0.01[b]
Trichoderma TvR1	-	1.82±0.01[c]	1.62±0.02[c]
Trichoderma TbS2	-	1.28±0.03[a]	1.44±0.01[a]

Values are mean of triplicate; ± Standard error; Values followed by different letters are significantly different according to DUNCAN test at 5% significance level

Pseudomonas Tr20 was found to be only rhizospheric isolate that possesses the ability to solubilize inorganic phosphate. The phosphate solubilization index value was observed to be 1.65 (±0.02) after 10 days of incubation. The isolate *Trichoderma* TvR1 (1.82±0.01 µg/ml) was observed to produce the maximum amount of IAA followed by *Pseudomonas* Tr20 (1.37±0.02 µg/ml) and *Trichoderma* TbS2 (1.28±0.03

µg/ml). *Trichoderma* TvR1 was found to produce significantly ($p \leq 0.05$) higher amount of IAA than other two IAA producing isolates. Likewise, the trend observed for production of IAA, Zinc solubilization index was also recorded. The maximum and significant Zn solubilization index value was reported for isolate *Trichoderma* TvR1 (1.62±0.02) followed by *Pseudomonas* Tr20 (1.53±0.01) and *Trichoderma* TbS2 (1.44±0.01).

4.3.8. Exopolysaccharides production and biofilm formation

The ability of antagonistic isolates *Trichoderma* TvR1, *Trichoderma* TbS2, *Bacillus* BS6, *Bacillus* CS13, and *Pseudomonas* Tr20 to produce exopolysaccharides (EPSs) and forming biofilms were assessed under *in vitro* conditions. The isolates *Trichoderma* TvR1, *Trichoderma* TbS2, and *Pseudomonas* Tr20 were found positive for EPS production and formation of biofilm (**Table 4.13; Figure 4.23**), however, none of the isolates of *Bacillus* displayed the ability to produce EPS and forming a biofilm. The isolates with positive EPS production and biofilm formation ability determined their capability to colonize plant roots efficiently.

Table 4.13: Production of exopolysaccharides and biofilm forming ability of antagonistic isolates

Isolates	EPS production	Biofilm formation
Bacillus BS6	-	-
Bacillus CS13	-	-
Pseudomonas Tr20	+	+
Trichoderma TvR1	+	+
Trichoderma TbS2	+	+

- = negative; + positive

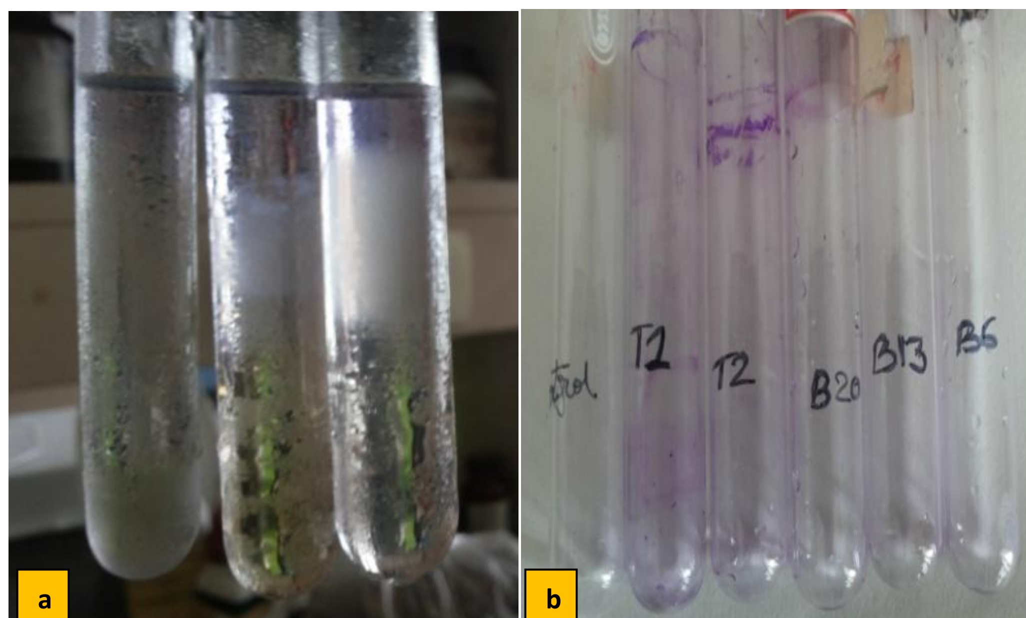

Figure 4.23: Production of exopolysaccharides and biofilm formation by antagonistic isolates. a) EPS production (left to right: control; *Trichoderma* TvR1; *Pseudomonas* Tr20); b) Biofilm formation (left to right: control; *Trichoderma* TvR1; *Trichoderma* TbS2; *Pseudomonas* Tr20; *Bacillus* CS13; *Bacillus* BS6)

4.3.9. Volatile and non-volatile metabolites assay

On the basis of the result obtained through dual culture test, the isolates with positive biocontrol potential were assayed to determine the activity of their metabolites on the growth of fungal pathogens. The volatile metabolite assay was undertaken to establish the fact that volatile secondary compounds secreted by isolated antagonists play role in inhibition of fungal pathogens growth. The percentage inhibition of radial growth (PIRG) of pathogens by metabolites is presented in **table 4.14**. Results revealed the volatile metabolites produced by *Trichoderma* TvR1 had a most significant inhibitory effect on the growth of pathogen *Fol* (22.24%) over other isolates, however, volatile metabolites of *Bacillus* BS6 and *Pseudomonas* Tr20 displayed more significant

antagonistic effect on *A. solani*. The PIRG of *A. solani* by *Bacillus* BS6 and *Pseudomonas* Tr20 were observed to be 30.93 and 34.20%, respectively higher than PIRG displayed by *Trichoderma* TvR1, which in case of *Fol* resulted in highest growth inhibition.

Table 4.14: Percentage inhibition of radial growth (PIRG) of pathogens by Volatile metabolites

Pathogens	*Bacillus* BS6	*Bacillus* CS13	*Pseudomonas* Tr20	*Trichoderma* TvR1	*Trichoderma* TbS2
Fol	9.56±0.72[b]	3.21±0.52[a]	10.06±0.13[b]	22.24±0.72[d]	18.65±1.24[c]
A. solani	27.18±0.42[c]	23.04±0.91[b]	27.86±1.05[c]	20.76±0.78[b]	15.19±1.02[a]

Values are mean of triplicate and presented as a percentage; ± = Standard error; values followed by different letters are significantly different according to DUNCAN test at 5% significance level

Table 4.15: Percentage inhibition of radial growth (PIRG) of pathogens by culture filtrate

Antagonists/pathogens	***Fusarium oxysporum* f. sp. *lycopersici* (%)**			***Alternaria solani* (%)**		
	5%	10%	20%	5%	10%	20%
***Bacillus* BS6**	3.76 ± 0.25[a]	9.65 ± 0.21[b]	14.41 ± 0.25[d]	2.6 ± 0.21[a]	7.43 ± 0.24[b]	10.96 ± 0.30[c]
***Bacillus* CS13**	12.78 ± 0.33[c]	23.43 ± 0.23[g]	46.12 ± 0.19[k]	11.49 ± 0.26[c]	18.73 ± 0.19[f]	31.27 ± 0.44[i]
***Pseudomonas* Tr20**	19.76 ± 0.23[f]	26.79 ± 0.23[h]	51.38 ± 0.19[l]	14.19 ± 0.23[d]	20.41 ± 0.16[g]	38.52 ± 0.29[k]
***Trichoderma* TvR1**	57.65 ± 0.24[m]	64.97 ± 0.38[n]	67.85 ± 0.31[o]	23.44 ± 0.29[h]	37.71± 0.28[j]	41.74 ± 0.39[m]
***Trichoderma* TbS2**	18.44 ± 0.06[e]	34.91 ± 0.26[i]	41.97 ± 0.31[j]	17.55 ± 0.20[e]	31.12 ± 0.17[i]	39.42 ± 0.26[l]

Values are mean of triplicate and presented as a percentage; ± = Standard error; values followed by different letters are significantly different according to DUNCAN test at 5% significance level

Similar to a volatile metabolite assay, the culture filtrate assay of *Trichoderma* spp. and rhizobacterial isolates was done to assess the effect of non-volatile diffusible compounds produced by these antagonistic isolates on pathogens. The result obtained (**Table 4.15**) shows that the PIRG was directly proportional to the concentration of filtrate. The maximum inhibition of mycelia growth of *Fol* and *A. solani* was reported to be 67.85 and 41.74% respectively at 20% concentration of culture filtrate of *Trichoderma* TvR1 whereas minimum inhibition of pathogens was reported to be 14.41 and 10.96% respectively by culture filtrate of *Bacillus* BS6 at the same concentration. At 20% concentration of culture filtrate of *Pseudomonas* Tr20 and *Trichoderma* TvR1 more than 50% of the radial growth inhibition of *Fol* was recorded.

4.3.10. *In vitro* seed germination assay

Table 4.16: Effect of rhizospheric microbial antagonists on seed germination and vigor index

Treatments	Mean Seedling Length (cm)	Germination Percentage (%)	Seedling Vigor Index
Bacillus BS6	7.60±0.27[b]	70	532.33±18.85[b]
Bacillus CS13	9.42±0.31[c]	76.67	722.36±23.80[c]
Pseudomonas Tr20	10.39±0.31[d]	86.87	902.78±26.76[d]
Trichoderma TvR1	11.10±0.26[d]	90	999±22.96[e]
Trichoderma TbS2	6.49±0.25[a]	66.67	432.69±16.37[a]
Control[#]	6.66±0.30[a]	66.67	444.02±16.08[a]

[#] = treated only with distilled water; Values are mean of the number of seeds germinated; ± = Standard error; values followed by different letters are significantly different according to DUNCAN test at 5% significance level

The plant growth promoting the effect of isolated antagonistic strains was assessed under *in vitro* condition (**Figure 4.24**). All the antagonistic isolates were observed to promote seedling length, germination percentage and vigor index over no amendments except one isolate *Trichoderma* TbS2 (**Table 4.16**).

Figure 4.24: *In vitro* seed germination assay

Inoculation of seeds with *Trichoderma* TvR1 and *Pseudomonas* Tr20 was reported to promote seedling length, the percentage of germination and vigor index significantly than other isolates and control. Inoculation of tomato seeds with *Trichoderma* TvR1 and *Pseudomonas* Tr20 increased seedling length by 66.67 and 56%, respectively over control. Similarly, the vigor index was increased by 125.22

and 103.32% by *Trichoderma* TvR1 and *Pseudomonas* Tr20, respectively over seeds inoculated with distilled water only.

4.4. Pot experiment for screening antagonistic potential of isolated strains

Under *in vivo* conditions, biocontrol activity and plant growth promoting attributes of isolated rhizospheric strains were assessed (**Figure 4.25**). The percentage of disease incidence, percentage of disease reduction and increase in biometric parameters of plants were analyzed after 60 days of transplantation (DAT). The data is presented in **table 4.17 and 4.18**. The pot experiment conducted to screen potential of isolates revealed that all strains showing positive biocontrol activity under *in vitro* conditions were effective in controlling pathogens under pot condition.

All the isolates were observed to improve the growth parameters of tomato plants over control except *Trichoderma brevicompactum* TbS2. The tomato seeds coated with isolate TbS2 showed growth at par with control. The maximum plant height was reported in case of seeds coated with isolate *Trichoderma lixii* TvR1 followed by *Pseudomonas aeruginosa* Tr20. The plant's height was recorded to be increased by 47.3 and 41.5% over control treated with *Trichoderma* TvR1 and *Pseudomonas* Tr20, respectively. Even in the case of pathogens challenged plants *Trichoderma* TvR1 was found to improve growth significantly than other isolates and control which was followed by *Pseudomonas* Tr20. The trend for plant biomass was analogous to the trend observed for plant height.

The inoculation of *Trichoderma lixii* TvR1 was reported to increase the biomass of plants challenged with *Fol*, *A. solani,* and non-challenged plants by 48.5, 40.6 and 35% over their respective control. After *Trichoderma*, *Pseudomonas aeruginosa* Tr20 was found to be the most effective and significant microbial

biocontrol agent that displayed plant growth. It increased the biomass of *Fol*, *A. solani,* and non-challenged plants by 39.4, 34.8 and 31% over their respective control. The result recorded showed that all biocontrol agents were more effective in promoting plant biomass when challenged with pathogens rather than non-challenged plants. The results obtained through the screening experiment demonstrated *T. lixii* TvR1 and *P. aeruginosa* Tr20 as most effective strains among all isolated microbial biocontrol agents. Thus these two individual strains were selected for further study.

Figure 4.25: Pot experiment to screen biocontrol and plant growth promoting attribute of isolated rhizospheric microbes

Table 4.17: Plant growth after 60 days after transplantation (DAT)

Treatments	Plant height (cm)	Shoot fw (g)	Root fw (g)	Shoot dw (g)	Root dw (g)
Control	52.6±2.6^{cde}	29.8±0.8^{b}	2.2±0.1^{abc}	6.8±0.3^{ab}	0.6±0.04^{bc}
Bacillus BS6	57.2±2.3^{efg}	32.4±1.1^{bc}	2.7±0.3^{cde}	7.9±0.2^{de}	0.8±0.01^{de}
Bacillus CS13	63.7±2.2^{hij}	37.7±1.6^{d}	3.3±0.3^{fghi}	8.0±0.2^{def}	0.9±0.03^{ef}
Pseudomonas Tr20	74.4±1.8^{lm}	42.2±1.3^{e}	3.8±0.2^{ij}	8.6±0.3^{fg}	1.1±0.07^{g}
Trichoderma TvR1	77.5±1.8^{m}	43.9±1.6^{e}	4.0±0.2^{j}	8.9±0.2^{g}	1.1±0.12^{g}
Trichoderma TbS2	51.3±1.2^{cd}	29.2±1.1^{b}	2.0±0.2^{ab}	6.6±0.1^{ab}	0.7±0.03^{cd}
Fol	40.4±0.9^{a}	22.2±1.0^{a}	1.8±0.1^{a}	6.2±0.2^{a}	0.4±0.02^{a}
Fol + *Bacillus* BS6	54.4±1.4^{de}	31.6±0.7^{b}	2.9±0.2^{defg}	7.1±0.2^{bc}	0.7±0.05^{cd}
Fol + *Bacillus* CS13	60.4±1.3^{fgh}	35.8±1.9^{cd}	3.1±0.2^{efgh}	7.6±0.2^{cd}	0.7±0.03^{cd}
Fol + *Pseudomonas* Tr20	68.3±1.2^{jk}	43.1±0.7^{e}	3.5±0.2^{hij}	8.3±0.2^{defg}	0.9±0.04^{ef}
Fol + *Trichoderma* TvR1	70.3±1.7^{kl}	45.7±1.1^{e}	3.7±0.1^{ij}	8.8±0.1^{g}	1.0±0.06^{fg}
Fol + *Trichoderma* TbS2	45.6±1.2^{ab}	21.4±1.2^{a}	2.0±0.2^{ab}	6.5±0.1^{ab}	0.6±0.04^{bc}
A. solani	45±1.5^{ab}	22.8±1.2^{a}	2.2±0.1^{abc}	6.4±0.2^{a}	0.5±0.08^{ab}
A. solani + *Bacillus* BS6	55.7±2.6^{def}	30.3±1.0^{b}	2.8±0.1^{def}	7.1±0.3^{bc}	0.6±0.04^{bc}
A. solani + *Bacillus* CS13	61.6±1.7^{ghi}	35.8±1.2^{cd}	3.1±0.2^{efgh}	7.8±0.2^{de}	0.7±0.03^{cd}
A. solani + *Pseudomonas* Tr20	67.1±1.6^{ijk}	42.5±1.1^{e}	3.4±0.2^{ghi}	8.4±0.2^{efg}	0.9±0.06^{ef}
A. solani + *Trichoderma* TvR1	67.9±2.3^{jk}	44.3±1.6^{e}	3.8±0.2^{ij}	8.7±0.3^{g}	1.0±0.08^{fg}
A. solani + *Trichoderma* TbS2	47.3±2.7^{bc}	20.6±0.6^{a}	2.4±0.1^{bcd}	6.4±0.3^{a}	0.6±0.04^{bc}

Values are mean of triplicate; ± = Standard error; values followed by different letters are significantly different according to DUNCAN test at 5% significance level; fw= Fresh weight; dw= Dry weight

Figure 4.26: Infected plant parts showing symptoms of diseases. Left: Root browning resulted by infection of *Fusarium oxysporum* f. sp. *lycopersici*; Right: Characteristic symptoms on leaf caused by *Alternaria solani*

4.4.1. Percentage disease reduction

The inoculations of pathogens challenged plants with rhizospheric biocontrol isolates were found to reduce diseases symptoms (**Figure 4.26**). The highest percentage of disease reduction in case of *Fol* challenge plants was reported by *Trichoderma lixii* TvR1 (31.58%) followed by *Pseudomonas aeruginosa* Tr20 (26.32%). The lowest reduction of fusarium wilt (10.53%) was displayed by the two rhizospheric biocontrol isolates *Bacillus subtilis* BS6 and *B. subtilis* CS13. A similar trend of reduction of early blight incidence by these microbial biocontrol agents was observed. The *Trichoderma lixii* TvR1 was documented to show the highest disease reduction of 30.50% whereas *Bacillus subtilis* BS6 was found to be less effective in controlling phytopathogens in pot conditions. It reported to reduced incidence of early blight only by 13.55%.

Table 4.18: Percentage of disease incidence and disease reduction

Treatments	Percent disease index	Disease reduction (%)
Control	0	0
Fusarium oxysporum f. sp. *lycopersici (Fol)*	52.78	-
Fol + *Bacillus* BS6	47.22	10.53
Fol + *Bacillus* CS13	44.44	15.80
Fol + *Pseudomonas* Tr20	38.89	26.32
Fol + *Trichoderma* TvR1	36.11	31.58
Fol + *Trichoderma* TbS2	42.00	20.42
Alternaria solani	43.70	-
A. solani + *Bacillus* BS6	37.78	13.55
A. solani + *Bacillus* CS13	36.88	15.61
A. solani + *Pseudomonas* Tr20	31.52	27.87
A. solani + *Trichoderma* TvR1	30.37	30.50
A. solani + *Trichoderma* TbS2	31.80	13.90

4.4.2. Compatibility of isolated microbes

The compatibility between the strains is a paramount factor which decides their ability to form successful consortia with each other. The compatibility assay between all isolated strains displaying positive antagonistic effect was assessed under *in vitro* (**Figure 4.27**) for the formulation of effective consortia. The result obtained is presented in **table 4.19**.

Table 4.19: Compatibility between isolated rhizospheric microbial strains

Strains combination	Compatibility
Bacillus subtilis BS6 + *B. subtilis* CS13	+
B. subtilis BS6 + *Pseudomonas aeruginosa* Tr20	-
B. subtilis CS13+ *P. aeruginosa* Tr20	-
Trichoderma lixii TvR1 + *T. brevicompactum* TbS2	-
T. lixii TvR1 + *B. subtilis* BS6	-
T. lixii TvR1 + *B. subtilis* CS13	-
T. lixii TvR1 + *P. aeruginosa* Tr20	+
T. brevicompactum TbS2 + *B. subtilis* BS6	-
T. brevicompactum TbS2 + *B. subtilis* CS13	-
T. brevicompactum TbS2 + *P. aeruginosa* Tr20	-

+ = compatible strains; - = Not compatible strains

Among all possible combinations, only two combinations of isolated strains were found compatible. The strain *Bacillus subtilis* BS6 was found compatible with isolated strain *Bacillus subtilis* CS13 and *Trichoderma lixii* TvR1 was found compatible with *Pseudomonas aeruginosa* Tr20. Rest other combinations were incompatible with each other, hence rules out the possibility of forming consortia.

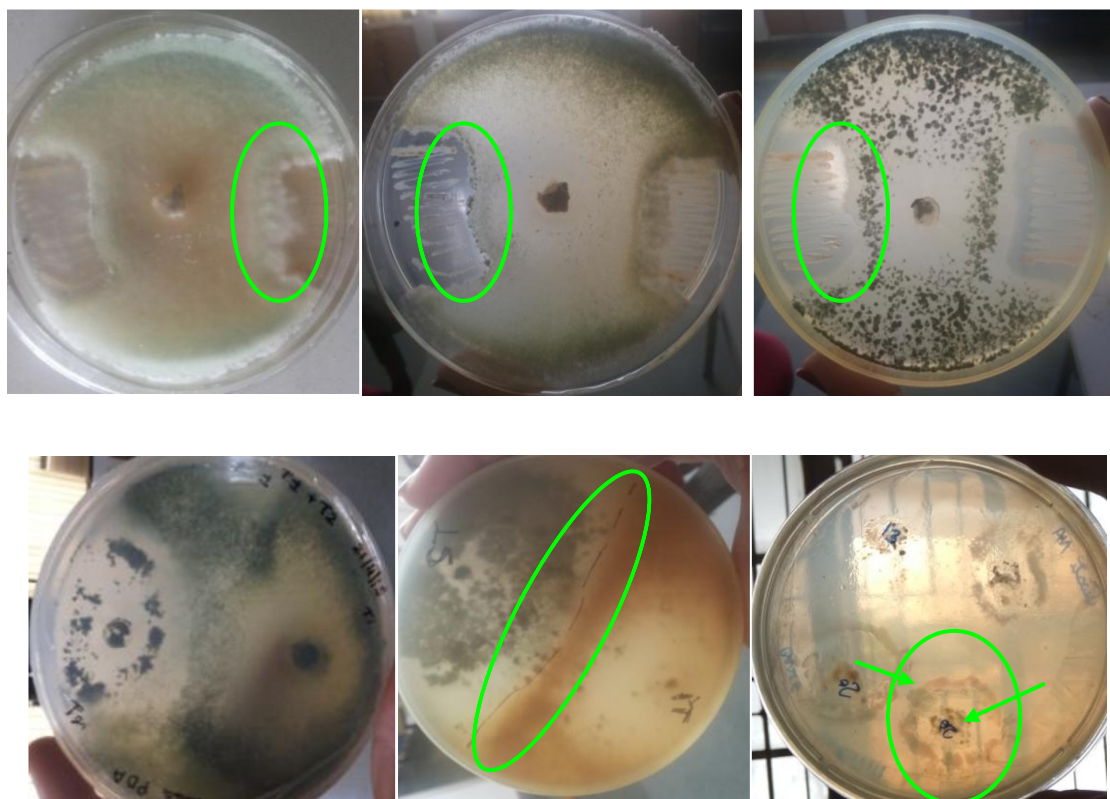

Figure 4.27: Compatibility test between isolated rhizospheric antagonistic isolates. Left to right (upper side): Compatible strains (*Trichoderma lixii* TvR1 and *Pseudomonas aeruginosa* Tr20); Non-compatible strains (*Trichoderma lixii* TvR1 and *Bacillus subtilis* BS6); Non-compatible strains (*Trichoderma lixii* TbS2 and *Pseudomonas aeruginosa* Tr20); Left to right (lower side): Non-compatible strains (*Trichoderma lixii* TvR1 and *Trichoderma brevicompactum* TbS2) front view; Non-compatible strains (*Trichoderma lixii* TvR1 and *Trichoderma brevicompactum* TbS2) reverse side view; Non-compatible strains (*Pseudomonas aeruginosa* Tr20 and *Bacillus subtilis* CS13)

4.5. Pot experiment with best performing isolates and their consortium

On the basis of the *in vivo* screening experiment and compatibility assay the two isolates *i.e., Trichoderma lixii* TvR1 and *Pseudomonas aeruginosa* Tr20 were selected for the further experiment. 90 days pot experiment was performed to assess the biocontrol and plant growth promoting effect of two isolates individually as well as in consortium (**Figure 4.28**). After 90 days of transplantation, the result revealed that all treatments were significantly effective in controlling diseases and promoting the growth of tomato plant (**Table 4.20**). However, the consortium showed the more pronounced effect on plant growth and disease management. The consortium was observed to promote plant height of non-challenged, *Fol* challenged and *A. solani* challenged plants by 33.5, 50.9 and 36.3%, respectively over respective control.

The plant biomass was also found to be increased significantly on inoculation of rhizospheric biocontrol agents. The result revealed that biocontrol agents increased the biomass of plants challenged with *Fol* more than the plants challenged with *A. solani* and non-challenged plants. The consortium was reported to show a maximum increase in biomass followed by plants individually treated with *T. lixii* TvR1 which was again followed by plants treated with *P. aeruginosa* Tr20. The biomass increase over their respective control *i.e., Fol*, *A. solani,* and the positive control was recorded as 60.5, 25.2 and 29.8% by the consortium.

The numbers of fruits and leaves recorded per plant were also increased significantly in challenged and non-challenged plants treated with the consortium as compared to their respective control and individual treatments. The maximum numbers of fruits (8.3±0.3) and leaves (25.7±0.9) were documented in non-challenged plants treated with a consortium of *Trichoderma lixii* TvR1 + *Pseudomonas*

aeruginosa Tr20. However, the number of fruits and leaves per plant treated with individual biocontrol agents *i.e., T. lixii* TvR1 and *P. aeruginosa* Tr20 were significantly the same.

Figure 4.28: Pot experiment to assess biocontrol and plant growth promoting attribute of selected rhizospheric isolates. Left to right: Control; Pathogen challenged plant; Inoculated with *Trichoderma lixii* TvR1; Inoculated with *Pseudomonas aeruginosa* Tr20; Inoculated with consortia of *T. lixii* TvR1 + *P. aeruginosa* Tr20

Table 4.20: Pot experiment: plant growth after 90 days of transplantation

Treatments	Shoot length (cm)	Root length (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)	Root dry weight (g)	No. of leaves	No. of fruits
Control	69.8 ±2.4^{c}	27.2±0.7^{c}	50.8±0.5^{c}	11.0±0.6^{bc}	5.8±0.2^{b}	1.2 ±0.06^{b}	17±1.5^{bc}	5.33±0.7^{bcd}
Trichoderma lixii TvR1	82.4 ±1.7^{g}	36.3±0.9^{ef}	57.3±0.6^{fg}	12.7±0.6^{cd}	7.7±0.2^{def}	2.0±0.12d^{e}	23.3±0.9^{ef}	6±0^{bcd}
Pseudomonas aeruginosa Tr20	82.2 ±1.4^{fg}	35.9±0.7^{ef}	54.5±0.9^{def}	12.4±1.0^{cd}	7.6±0.1^{de}	1.8±0.08^{cd}	24±1.2^{ef}	6.3±0.7^{bcd}
T. lixii TvR1 + *P. aeruginosa* Tr20	91.3 ±2.2^{h}	38.2±1.1^{f}	61.4±1.2^{h}	13.6±0.7^{d}	8.2±0.1^{f}	2.2±0.07^{e}	25.7±0.9^{f}	8.3±0.3^{e}
Fusarium oxysporum f. sp. *lycopersici (Fol)*	50.7 ±1.0^{a}	15.7±0.8^{a}	39.9± 1.0^{a}	8.3±0.5^{a}	4.6±0.2^{a}	0.9±0.06^{a}	14.3±0.9^{ab}	2.7±0.3^{a}
Fol + *T. lixii* TvR1	77.6 ±1.2^{ef}	31.4±0.7^{d}	52.8± 1.4^{cde}	11.7±1.0^{bcd}	7.2±0.2^{c}	1.7±0.06^{c}	21.3±1.2^{de}	5.7±0.3^{bcd}
Fol+ *P. aeruginosa* Tr20	74.6 ±0.87^{de}	30.0±1.7^{d}	51.5± 0.9^{cd}	11.3±0.6^{bcd}	7.0±0.1^{c}	1.6±0.05^{c}	21±1.5^{de}	6±0.6^{bcd}
Fol+ *T. lixii* TvR1 + *P. aeruginosa* Tr20	81.0 ±1.0^{fg}	34.9±1.0^{e}	55.6± 1.2^{efg}	12.8±0.8^{cd}	7.9±0.2^{de}	2.0±0.09^{de}	23.3±1.2^{ef}	7±0.6^{de}
Alternaria solani	62.5 ±1.2^{b}	20.7±0.9^{b}	45.4±1.2^{b}	9.6±0.8^{ab}	5.6±0.2^{ab}	1.1±0.07^{ab}	13±1.0^{a}	2.3±0.7^{a}
A. solani+ *T. lixii* TvR1	72.0 ±0.8^{cd}	31.2±0.9^{d}	54.4± 0.9^{def}	11.4±0.7^{bcd}	7.6±0.2^{c}	1.7±0.04^{c}	19.7±0.7^{cd}	5±0.6^{bc}
A. solani+ *P. aeruginosa* Tr20	71.8 ±1.1^{cd}	30.3±0.6^{d}	53.1±1.2^{cde}	11.3±0.6^{bcd}	7.5±0.2^{c}	1.7±0.05^{c}	19±0.6^{cd}	4.7±0.7^{b}
A. solani+ *T. lixii* TvR1 + *P. aeruginosa* Tr20	78.1 ±1.8^{efg}	35.3±0.5^{e}	58.2±1.2^{g}	12.6±1.0^{cd}	8.0±0.2^{e}	2.1±0.10^{e}	21.7±0.3^{de}	6.7±0.9^{cde}

Values are mean of triplicate; ± = Standard error; values followed by different letters are significantly different according to DUNCAN test at 5% significance level

4.5.1. Effect on chlorophyll and carotenoid content

The effect of pathogens and selected strains on physiological activity of plants *i.e.,* chlorophyll and carotenoid content in plant leaves was determined after 90 days of transplantation (**Table 4.21**).

Table 4.21: Chlorophyll and Carotenoid content

Treatments	Chlorophyll a	Chlorophyll b	Total Chlorophyll	Carotenoid
Control	0.13±0.002[b]	0.09±0.006[a]	0.22±0.008[b]	0.24±0.014[cd]
Trichoderma lixii TvR1	0.33±0.012[f]	0.33±0.010[e]	0.65±0.006[g]	0.26±0.004[de]
Pseudomonas aeruginosa Tr20	0.33±0.008[f]	0.32±0.002[e]	0.64±0.008[g]	0.23±0.005[bc]
T. lixii TvR1+ *P. aeruginosa* Tr20	0.65±0.015[i]	0.46±0.013[f]	1.10±0.028[j]	0.34±0.011[g]
Fusarium oxysporum f. sp. *lycopersici* (*Fol*)	0.07±0.001[a]	0.09±0.003[a]	0.17±0.003[a]	0.18±0.005[a]
Fol + *T. lixii* TvR1	0.28±0.008[e]	0.29±0.005[d]	0.57±0.013[e]	0.32±0.004[g]
Fol + *P. aeruginosa* Tr20	0.43±0.002[g]	0.18±0.004[b]	0.61±0.005[f]	0.32±0.004[g]
Fol + *T. lixii* TvR1+ *P. aeruginosa* Tr20	0.67±0.001[j]	0.28±0.003[d]	0.95±0.004[h]	0.27±0.004[e]
A. solani	0.1±0.001[a]	0.11±0.004[a]	0.2±0.005[b]	0.21±0.005[b]
A. solani + *T. lixii* TvR1	0.25±0.011[d]	0.21±0.004[c]	0.46±0.011[d]	0.21±0.003[b]
A. solani + *P. aeruginosa* Tr20	0.17±0.004[c]	0.18±0.009[b]	0.35±0.005[c]	0.24±0.002[c]
A. solani + *T. lixii* TvR1 + *P. aeruginosa* Tr20	0.53±0.010[h]	0.5±0.003[g]	1.03±0.011[i]	0.30±0.006[f]

Values are mean of triplicate; ± = Standard error; values followed by different letters are significantly different according to DUNCAN test at 5% significance level

The maximum total chlorophyll and carotenoid content were recorded in plants treated with consortium either challenged with pathogens or non-challenged. Among individual treatments, the highest chlorophyll content in *A. solani* challenged plants was reported by *Trichoderma lixii* TvR1 (0.46±0.011) inoculated plants followed by *Pseudomonas aeruginosa* Tr20 (0.35±0.005) treated plants whereas carotenoid content was found significantly higher on inoculation of *P. aeruginosa* Tr20 (0.24±0.002) than *T. lixii* TvR1 (0.21±0.003). In case of *Fol* challenged plants maximum total chlorophyll content among individual treatments, was recorded in plants treated with *P. aeruginosa* Tr20 (0.61±0.005) followed by *T. lixii* TvR1 (0.57±0.013) and the carotenoid content was observed to be same in plants treated either with *T. lixii* TvR1 or *P. aeruginosa* Tr20 (0.32±0.004). The total chlorophyll content in non-challenged plant treated with individual biocontrol agents does not bear any significant difference, however, the higher carotenoid content in leaves of *T. lixii* TvR1 (0.26±0.004) inoculated plants than *P. aeruginosa* Tr20 (0.23±0.005) inoculated plants were reported.

4.5.2. Percentage disease reduction

The percentage of disease incidence occurred and disease severity reduced by different treatments in presented in **table 4.22**. Similar to plant growth promotion, the consortium was observed to show the highest percentage of disease reduction. The percentage of reduction of fusarium wilt and early blight in challenged plants was reported to be 40 and 30.71%, respectively by the use of consortium. The treatment of challenged plants with *T. lixii* TvR1 and *P. aeruginosa* Tr20 isolates individually reduced the percentage of fusarium wilt by 30 and 26.66%; and early blight by 25.14 and 22.06%, respectively.

Table 4.22: Percentage of disease incidence and disease reduction

Treatments	Percentage Disease Index	Disease reduction (%)
Control#	0	0
Fusarium oxysporum f. sp. *lycopersici* (*Fol*)	50	-
Trichoderma lixii TvR1	35	30
Pseudomonas aeruginosa Tr20	36.67	26.66
T. lixii TvR1 + *P. aeruginosa* Tr20	30	40
Alternaria solani	35	-
T. lixii TvR1	26.20	25.14
P. aeruginosa Tr20	27.28	22.06
T. lixii TvR1 + *P. aeruginosa* Tr20	24.25	30.71

4.5.3. Scanning electron microscopic study for root colonization

The ability of the best performing rhizospheric isolates to colonize plant roots successfully was confirmed by analyzing treated plant roots sections under a scanning electron microscope (SEM). The study of root sections under SEM showing spores of *T. lixii* TvR1 and cells of *P. aeruginosa* Tr20 confirmed their ability to colonize tomato roots successfully. The results obtained are presented in **figure 4.29**.

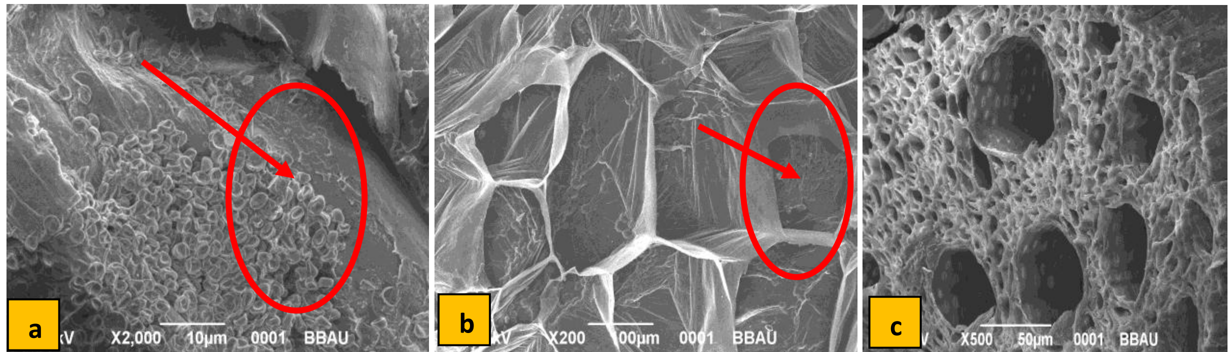

Figure 4.29: Colonization of tomato roots by rhizospheric isolates. a) Spores of *Trichoderma lixii* TvR1; b) *Pseudomonas aeruginosa* Tr20; c) Control root without microbial treatment

4.6. Screening, extraction, and characterization of biosurfactant

The isolate *Pseudomonas aeruginosa* Tr20 was assessed to determine its ability to produce biosurfactant and use of biosurfactant as a biological means of management of pathogens. The qualitative studies were performed to screen the ability of *P. aeruginosa* Tr20 to produce biosurfactant. The development of dark blue zone around a colony of *P. aeruginosa* Tr20 inoculated on mineral salt agar media amended with methylene blue and CTAB showed a positive result for production of anionic biosurfactant. The clear zone production around a colony of *P. aeruginosa* Tr20 on blood agar that signifies hemolysis of blood by biosurfactant confirmed its ability to produce biosurfactant (**Figure 4.30**). After confirmation, biosurfactant was extracted and its activity was characterized (**Figure 4.31**). The data obtained including emulsification index, foam forming ability, and oil displacement activity demonstrated that the compound extracted possess attributes of surfactant. The result is detailed in **table 4.23.**

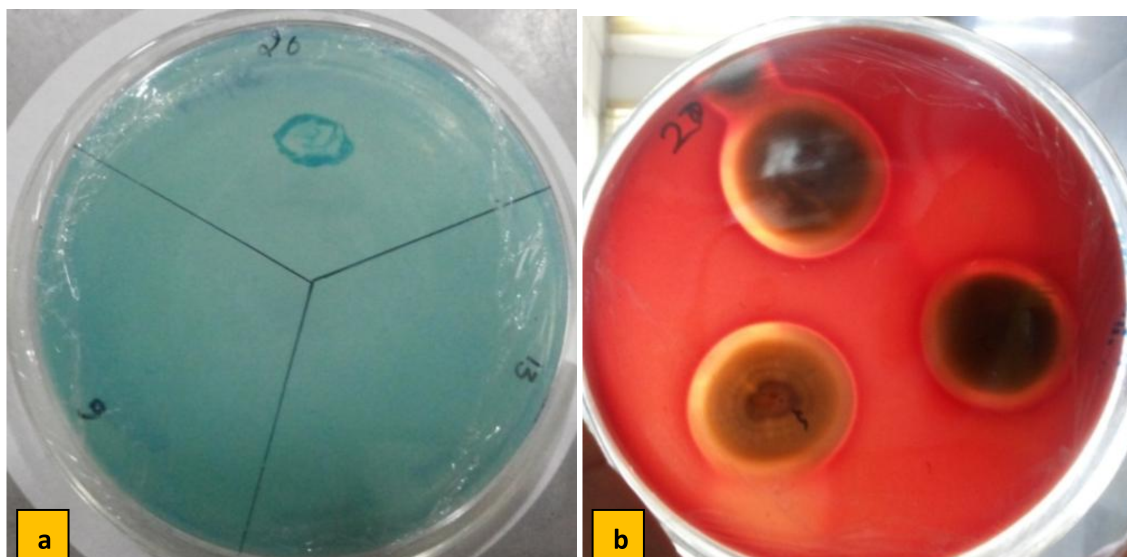

Figure 4.30: Screening test for biosurfactant producing ability of *Pseudomonas aeruginosa* Tr20. a) Dark blue zone development on CTAB agar plates; b) Haemolysis of blood agar

Table 4.23: Characterization of biosurfactant extracted from *Pseudomonas aeruginosa* Tr20

Parameters	Values
Foam height (%)	52±1.01
Emulsification index (%)	46.67±0.93
Oil displacement (cm)	6.8±0.15
Per litre production (g/L)	0.815±0.007

Values are mean of triplicate; ± = Standard error

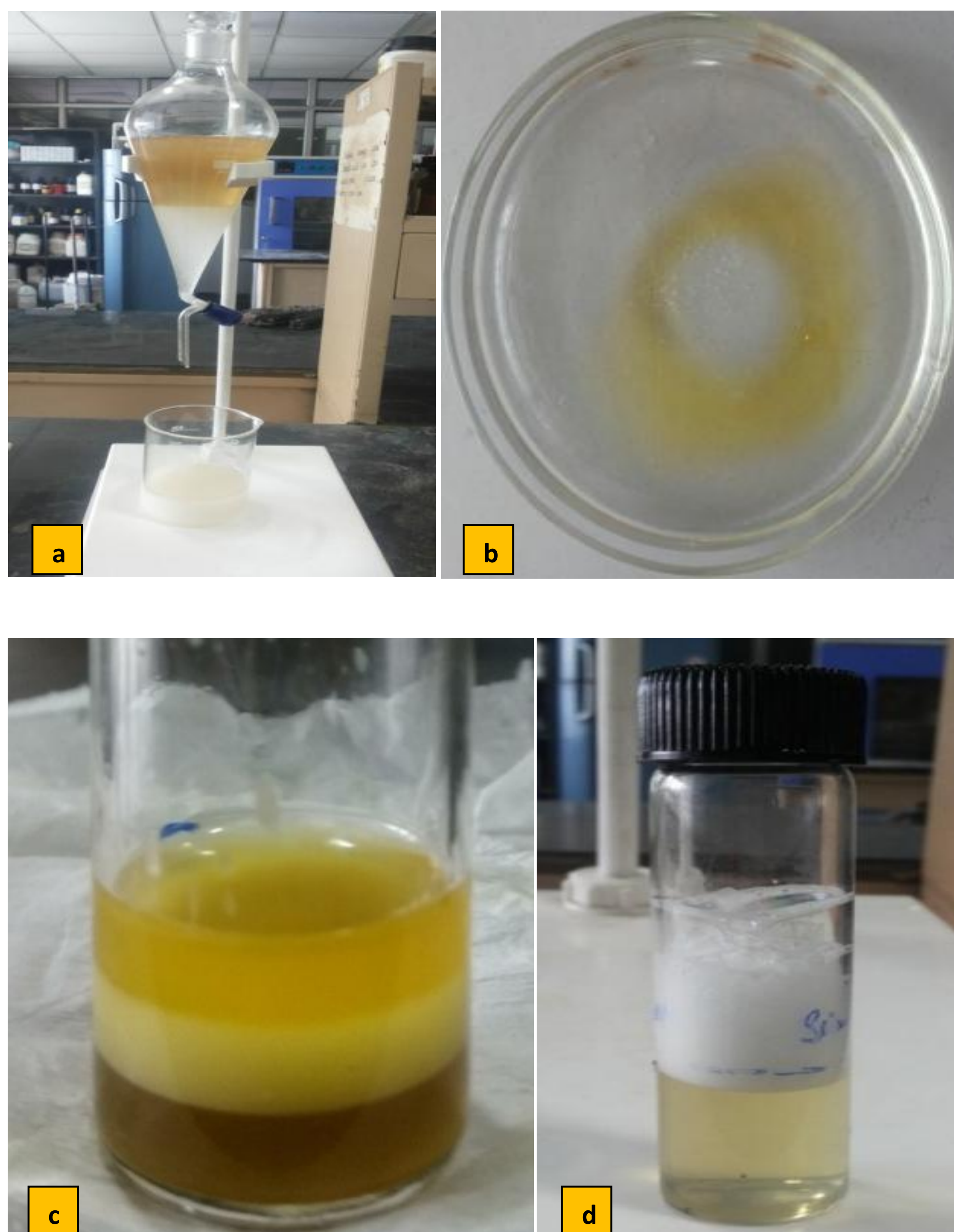

Figure 4.31: Extraction and characterization of biosurfactant. a) separation of the organic layer containing biosurfactant from media; b) oil displacement; c) emulsification activity; d) foam height produced by biosurfactant

The extracted biosurfactant was further characterized through FTIR analysis. The functional group bands positioned at located at 3400, 2928, 2860, 1727, and 1310-1061 cm^{-1} in FTIR image shows chemical structural resemblance to rhamnolipid that confirmed its as anionic biosurfactant Rhamnolipid (**Figure 4.32**).

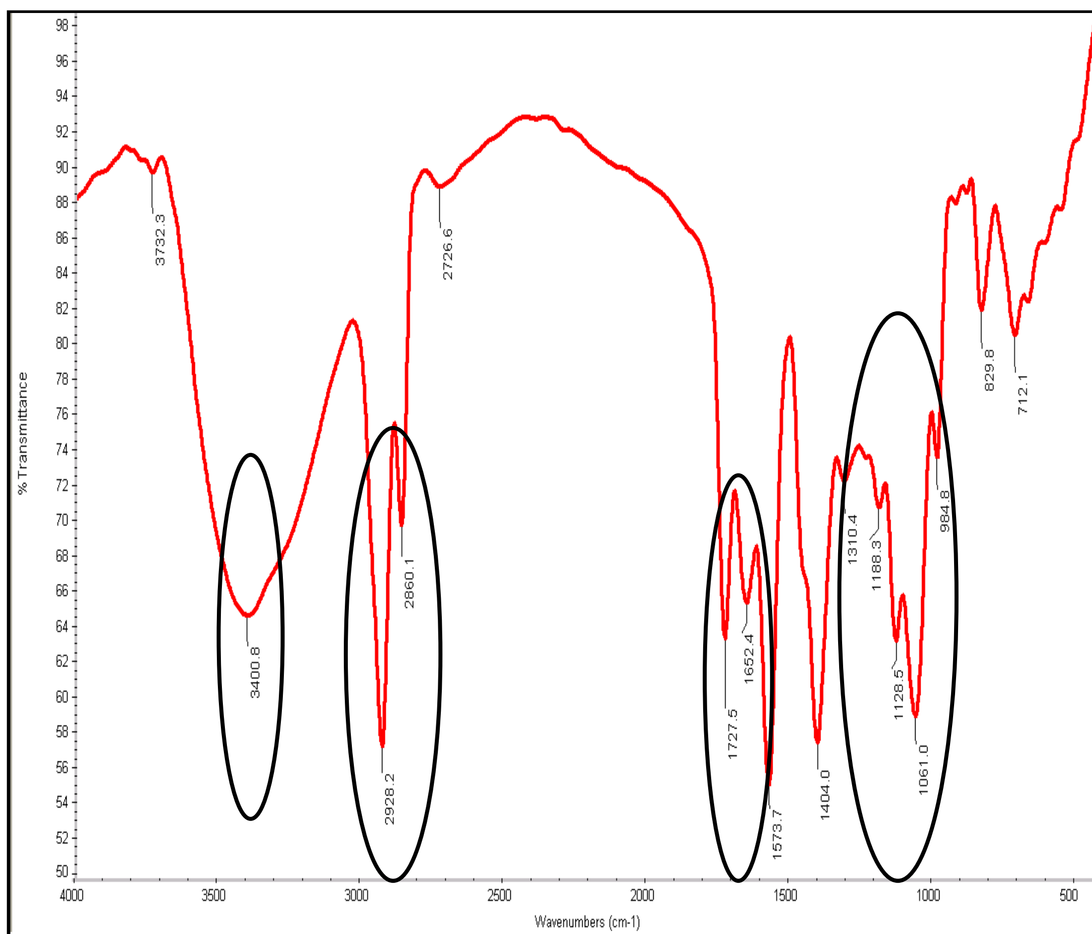

Figure 4.32: FTIR image of Biosurfactant

4.6.1. *In vitro* biocontrol activity of biosurfactant

The ability of biosurfactant extracted in controlling the growth of pathogens was assessed under *in vitro* condition at different concentrations (**Figure 4.33**). The data obtained (**Table 4.24**) from assay revealed biosurfactant that with an increase in concentration, the biocontrol activity was increased considerably. The maximum growth inhibition of *Fol* (36.82%) and *A. solani* (39.90%) was observed at concentration 800 mg/l.

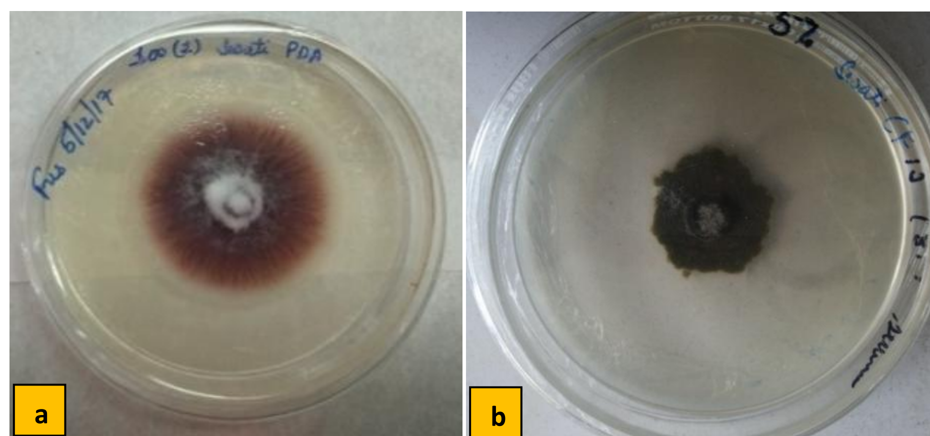

Figure 4.33: Biocontrol activity of Biosurfactant. a) Growth inhibition of *Fol*; b) growth inhibition of *A. solani*

Table 4.24: Percentage inhibition of radial growth (PIRG) of pathogens by biosurfactant

Concentration (mg/l)	*Fol*	*A. solani*
100	27.30±0.24^{a}	29.27±0.27^{a}
200	32.68±0.30^{b}	35.42±0.43^{b}
400	35.76±0.18^{c}	38.89±0.25^{c}
800	36.82±0.34^{d}	39.90±0.21^{c}

Values are mean of triplicate; ± = Standard error; values followed by different letters are significantly different according to DUNCAN test at 5% significance level

4.6.2. Compatibility of biosurfactant with *Trichoderma lixii* TvR1

The compatibility of *T. lixii* TvR1 (which was found compatible with *P. aeruginosa* Tr20) with biosurfactant was assessed in order to formulate their consortium and replace living microbial cells of *P. aeruginosa* Tr20 with its biosurfactant. The compatibility was assessed at concentration 100, 200, 400 and 800 mg/l of biosurfactant. The *Trichoderma* displayed compatibility with biosurfactant at concentration 200 mg/l, however, a further increase in concentration was observed to

inhibit the growth of later. The higher concentration of biosurfactant (above 200 mg/l) also affected the colony appearance of *T. lixii* (**Figure 4.34**).

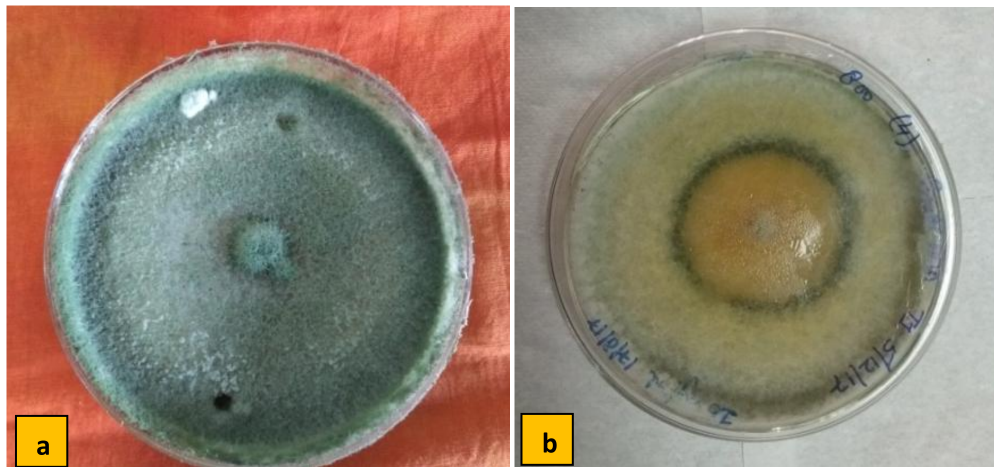

Figure 4.34: Compatibility test between biosurfactant and *T. lixii* at different concentration. a) The growth of *T. lixii* at concentration 200 mg/l; b) The growth of *T. lixii* at concentration 800 mg/l

4.7. Pot experiment with microbial biocontrol agents, biosurfactant and their consortia

The pot experiment was performed to assess and compare the effect of different treatments on biocontrol activity, growth promotion ability and yield obtained (**Figure 4.35**). The 90 days pot experiment after transplantation was performed and data was recorded is presented in **table 4.25**. The data revealed that the plant height of plants either challenged or non-challenged with pathogens was significantly higher on treatment with a consortium of *T. lixii* TvR1 and *P. aeruginosa* Tr20 (**Figure 4.36**).

The increase in plant biomass of non-challenged; challenged with *Fol* and *A. solani* plants by the application of consortium of *T. lixii* TvR1 + *P. aeruginosa* Tr20 and consortium of *T. lixii* TvR1 + biosurfactant @ concentration 200 mg/l was documented as 49.3 and 27.6; 59.3 and 47.5; and 43.9 and 26.5%, respectively. All

the treatments were found to promote plant fresh weight and dry weight compared to both negative and positive control. The consortium of *T. lixii* TvR1 + *P. aeruginosa* Tr20 was the most effective treatment, which improved plant fresh weight and dry weight significantly. It increased the fresh and dry weight of *Fol* challenged; *A. solani* challenged; and non-challenged plants by 52.8 and 58.8; 30.8 and 42.7; and 38.8 and 52.6% over their respective control. This growth promoting trend was followed by a consortium of *T. lixii* TvR1 and biosurfactant @ concentration 200 mg/l, except the dry weight of non-challenged *T. lixii* TvR1 treated plants that displayed second highest value (31.6 %).

The maximum number of fruits were found in non-challenged plants treated with consortium of *T. lixii* TvR1 + *P. aeruginosa* Tr20 (7.7±0.3), followed by non-challenged plants inoculated with consortium of *T. lixii* TvR1 (6.7±0.9) and *Fol* challenged plant inoculated with consortium of *T. lixii* TvR1 + *P. aeruginosa* Tr20 (6.7±0.7). In case of a number of leaves, the highest number of leaves were documented in non-challenged plants treated with a consortium of *T. lixii* TvR1 + *P. aeruginosa* Tr20 (29.3±0.3), followed by *Fol* challenged plants treated with *T. lixii* TvR1 + *P. aeruginosa* Tr20 (27.03±0.3).

Table 4.25: Plant growth data after 90 days of transplantation

Treatments	Shoot length (cm)	Root length (cm)	Shoot fw (g)	Shoot dw (g)	Root fw (g)	Root dw (g)	No. of leaves	No. of fruits
Control	65.5±2.1^{b}	30.7±0.9^{cd}	42.5±1.1^{b}	8.5±0.2^{b}	4.9±0.11^{bc}	1.0±0.04^{ab}	17.7±0.3^{b}	4.3±0.3^{b}
Trichoderma lixii TvR1	88.2±1.8^{g}	37.9±1.2^{fg}	51.2±1.0^{efg}	10.7±0.3^{ef}	6.6±0.09^{ghi}	1.8±0.02^{fg}	26.7±1.5^{de}	6.3±0.7^{cde}
Pseudomonas aeruginosa Tr20	87.3±1.0^{g}	35.2±0.6^{ef}	50.5±2.1^{defg}	10.4±0.2^{de}	6.6±0.09^{ghi}	1.4±0.06^{cd}	26±1.2^{de}	6.7±0.9^{de}
Biosurfactant	67.8±0.8^{bc}	32.1±0.8^{cde}	42.8±0.8^{b}	8.6±0.8^{b}	5.0±0.13^{c}	1.0±0.05^{ab}	19.3±0.9^{bc}	4.7±0.3^{bc}
T. lixii TvR1 + *P. aeruginosa* Tr20	94.5±1.3^{h}	40.1±1.6^{g}	58.4±1.3^{h}	12.6±0.2^{h}	7.4±0.15^{k}	1.9±0.04^{g}	29.3±0.3^{f}	7.7±0.3^{e}
Biosurfactant + *T. lixii* TvR1	88.9±0.9^{g}	38.2±0.9^{fg}	51.1±1.8^{defg}	10.3±0.1^{de}	6.8±0.05^{hij}	1.7±0.07^{efg}	26.3±0.3^{de}	6.3±0.3^{cde}
Fusarium oxysporum f. sp. *lycopersici (Fol)*	59.2±1.3^{a}	20.4±1.3^{a}	36.4±1.5^{a}	7.7±0.1^{a}	4.1±0.08^{a}	0.8±0.07^{a}	14.3±1.2^{a}	1.7±0.3^{a}
Fol + *T. lixii* TvR1	82.1±1.2^{ef}	33.2±1.0^{cde}	49.9±1.3^{def}	10.7±0.7^{ef}	6.3±0.09^{efg}	1.5±0.08^{de}	24.7±1.2^{de}	5.7±0.3^{bcde}
Fol + *P. aeruginosa* Tr20	80.9±1.2^{ef}	32.1±1.0^{cde}	48.3±1.4^{cde}	10.3±0.1d^{e}	6.1.0±0.10^{e}	1.4±0.04^{cd}	24±1.2^{d}	5.7±0.9^{bcde}

Treatments	Shoot length (cm)	Root length (cm)	Shoot fw (g)	Shoot dw (g)	Root fw (g)	Root dw (g)	No. of leaves	No. of fruits
Fol + Biosurfactant	69.3±1.6^{bc}	30.7±1.0^{cd}	44.2 ±2.1^{bc}	9.1±0.1^{c}	5.2±0.19^{cd}	1.0 ±0.04^{ab}	20.3±0.3^{bc}	4.3±0.3^{b}
Fol + *T. lixii* TvR1 + *P. aeruginosa* Tr20	93.5±0.9^{h}	39.8±1.5^{g}	55.0±2.0^{fgh}	11.9±0.1^{g}	6.9±0.14^{ij}	1.6±0.12^{def}	27.3±0.3^{ef}	6.7±0.7^{de}
Fol + *T. lixii* TvR1 + Biosurfactant	82.7±1.2^{f}	34.1±1.0^{de}	50.2±1.7^{defg}	10.9±0.1^{f}	6.5±0.05^{fgh}	1.8±0.09^{fg}	25.7±1.2^{de}	6.3±0.7^{cde}
Alternaria solani	66.7±1.0^{bc}	25.9±1.2^{b}	43.2±1.5^{bc}	8.6±0.2^{b}	4.6±0.13^{b}	1.0±0.06^{ab}	12.3±0.7^{a}	2.3±0.3^{a}
A. solani+ *T. lixii* TvR1	78.2±2.0^{de}	33.9±1.4^{de}	50.9±2.1^{defg}	10.2±0.1^{de}	6.4±0.11^{efg}	1.5±0.04^{de}	21±1^{bc}	5.7±0.9^{bcd}
A. solani+ *P. aeruginosa* Tr20	75.4±1.7^{d}	34.1±1.2^{de}	48.3±2.2^{cde}	10.1±0.1^{d}	6.2±0.09^{ef}	1.5±0.07^{de}	20.3±0.3^{bc}	5±0.6^{bcd}
A. solani + Biosurfactant	70.2±1.7^{c}	29.8±1.5^{c}	45.7±1.5^{bcd}	9.2±0.2^{c}	5.4±0.15^{d}	1.2±0.07^{bc}	19±0.6^{bc}	4.7±0.3^{bc}
A. solani+ *T. lixii* TvR1 + *P. aeruginosa* Tr20	88.6±1.0^{g}	38.4±1.0^{fg}	55.4±1.3^{gh}	11.9±0.3^{g}	7.1±0.13^{jk}	1.8±0.10^{fg}	24.7±0.7^{de}	6.3±0.3^{cde}
A. solani + *T. lixii* TvR1 + Biosurfactant	79.4±1.0^{def}	35.2±1.4^{ef}	51.8±2.0^{efg}	10.3±0.1^{de}	6.4±0.03^{efg}	1.7±0.09^{efg}	21.3±0.3^{c}	5.3±0.7^{bcd}

Values are mean of triplicate; ± = Standard error; values followed by different letters are significantly different according to DUNCAN test at 5% significance level; fw = Fresh weight; dw = Dry weight

4.7.1. Percentage disease reduction

The disease severity index and disease reduction were assessed after 90 DAT. The data presented in **Table 4.26** reveal that maximum reduction of fusarium wilt and early blight was displayed by a consortium of *T. lixii* TvR1 + *P. aeruginosa* Tr20 by 33.75 and 34.57%, respectively, followed by a consortium of *T. lixii* TvR1 + biosurfactant *i.e.,* 27.71 and 29.43%, respectively. Formulation of a consortium of *T. lixii* TvR1 + biosurfactant was observed to increase the biocontrol activity of individual strain *T. lixii* TvR1.

Table 4.26: Disease incidence and disease percentage reduction

Treatments	Percentage Disease Index	Disease reduction (%)
Control	0	0
Fusarium oxysporum f. sp. *lycopersici (Fol)*	48	-
Fol + *Trichoderma lixii* TvR1	35.4	26.25
Fol + *Pseudomonas aeruginosa* Tr20	36	25
Fol + Biosurfactant	38	20.83
Fol + *T. lixii* TvR1 + *P. aeruginosa* Tr20	31.8	33.75
Fol + *T. lixii* TvR1 + Biosurfactant	34.7	27.71
Alternaria solani	35	-
A. solani + *T. lixii* TvR1	25	28.57
A. solani + *P. aeruginosa* Tr20	25.7	26.57
A. solani + Biosurfactant	27	22.86
A. solani + *T. lixii* TvR1 + *P. aeruginosa* Tr20	22.9	34.57
A. solani + *T. lixii* TvR1 + Biosurfactant	24.7	29.43

Figure 4.35: Pot experiment set up. a) Left to right: Control; biosurfactant; *T. lixii* TvR1; *P. aeruginosa* Tr20; *T. lixii* TvR1 + biosurfactant; *T. lixii* TvR1 + *P. aeruginosa* Tr20; b) Left to right: Control; pathogen challenged; pathogen challenged + biosurfactant; pathogen challenged + *P. aeruginosa* Tr20; pathogen challenged + *T. lixii* TvR1; pathogen challenged + *T. lixii* TvR1 + biosurfactant; pathogen challenged + *T. lixii* TvR1 + *P. aeruginosa* Tr20

Figure 4.36: Plant height and root length. a) Left to right: Control; pathogen challenged; pathogen + *T. lixii* TvR1; pathogen + biosurfactant; pathogen + *T. lixii* TvR1 + biosurfactant; pathogen + *P. aeruginosa* Tr20; pathogen + *T. lixii* TvR1 + *P. aeruginosa* Tr20; b) Left to right: Root length of control; pathogen challenged; pathogen + *T. lixii* TvR1; pathogen + *T. lixii* TvR1 + biosurfactant; pathogen + *P. aeruginosa* Tr20; pathogen + biosurfactant; pathogen + *T. lixii* TvR1 + *P. aeruginosa* Tr20

4.7.2. Physiological parameters

The physiological parameters *i.e.,* chlorophyll, carotenoid, carbohydrate and protein content in plants leaves was assessed after 90 DAT. The chlorophyll and carotenoid content is outlined in **table 4.27**. The maximum values for total chlorophyll was obtained for non-challenged plants treated with consortium of *T. lixii* TvR1 + *P. aeruginosa* Tr20 (1.18±0.04 mg/g fw) followed by consortium of *T. lixii* TvR1 + Biosurfactant (1.02±0.01 mg/g fw), however, no such trend was notice in case of carotenoid content. The highest carotenoid content was recorded in non-challenged (0.37±0.003 mg/g fw) and *A. solani* challenged (0.37±0.001 mg/g fw) plants treated with consortium of *T. lixii* TvR1 + *P. aeruginosa* Tr20, proceeded by *A. solani* challenged (0.33±0.01 mg/g fw) plants treated with consortium of *T. lixii* TvR1 + biosurfactant.

The data for carbohydrate and protein content obtained (**Table 4.28**) showed a similar trend as above. Maximum values of carbohydrate (11.37±0.28 mg/g fw) and protein content (61.84±0.23 mg/g fw) in leaves of non-challenged plants were observed when treated with consortium of two microbial isolates *i.e., T. lixii* TvR1 + *P. aeruginosa* Tr20, which was followed by consortium of *T. lixii* TvR1 + biosurfactant for carbohydrate content (10.19±0.22 mg/g fw) and by *T. lixii* TvR1 alone for protein content (52.46±1.08 mg/g fw). In *Fol* and *A. solani* challenged plants the maximum protein content (58.29±0.70 and 59.09±0.37 mg/g fw) and carbohydrate content (9.9±0.24 and 9.9±0.22 mg/g fw) was recorded by inoculation of *T. lixii* TvR1 + *P. aeruginosa* Tr20. Though the consortium of *T. lixii* TvR1 + biosurfactant was found effective; the application of biosurfactant alone produced the least significant effect.

Table 4.27: Chlorophyll and Carotenoid content

Treatments	Chlorophyll a (mg/g fw)	Chlorophyll b (mg/g fw)	Total chlorophyll (mg/g fw)	Carotenoid (mg/g fw)
Control	0.37 ±0.01^{de}	0.25±0.01^{bc}	0.61±0.01^{d}	0.24±0.01^{bcd}
Trichoderma lixii TvR1	0.64±0.02^{hi}	0.24±0.02^{b}	0.87±0.01^{hi}	0.25±0.02^{bcdef}
Pseudomonas aeruginosa Tr20	0.60±0.01^{h}	0.24±0.01^{b}	0.85±0.04^{gh}	0.26±0.01^{bcdef}
Biosurfactant	0.23±0.01^{ef}	0.23±0.01^{fg}	0.23±0.0^{1f}	0.23±0.003^{bcd}
T. lixii TvR1 + *P. aeruginosa* Tr20	0.70±0.03^{j}	0.53±0.01^{h}	1.18±0.04^{l}	0.37±0.03^{h}
Biosurfactant + *T.lixii* TvR1	0.66±0.01^{i}	0.39±0.01^{g}	1.02±0.01^{k}	0.27±0.04^{cdef}
Fusarium oxysporum f. sp. *lycopersici (Fol)*	0.13±0.01^{a}	0.09±0.01^{a}	0.20±0.003^{a}	0.16±0.01^{a}
Fol + *T.lixii* TvR1	0.43±0.01^{fg}	0.07±0.01^{a}	0.50±0.01^{bc}	0.23±0.01^{bcd}
Fol+ *P. aeruginosa* Tr20	0.42±0.01^{fg}	0.30±0.01^{de}	0.71±0.02^{e}	0.29±0.01^{defg}
Fol + Biosurfactant	0.24±0.01^{c}	0.29±0.01^{cde}	0.53±0.01^{c}	0.30±0.01^{efg}
Fol+ *T.lixii* TvR1 + *P. aeruginosa* Tr20	0.63±0.01^{hi}	0.32±0.01^{ef}	0.94±0.01^{j}	0.30±0.02^{efg}
Fol+ *T.lixii* TvR1 +Biosurfactant	0.44±0.01^{fg}	0.34±0.01^{f}	0.78±0.01^{f}	0.25±0.01^{bcde}
Alternaria solani	0.17±0.01^{b}	0.28±0.02^{cd}	0.45±0.01^{b}	0.21±0.003^{ab}
A. solani+ *T.lixii* TvR1	0.46±0.01^{g}	0.35±0.02^{fg}	0.81±0.04^{fg}	0.27±0.01^{cdef}
A. solani+ *P. aeruginosa* Tr20	0.45±0.01^{g}	0.24±0.02^{b}	0.69±0.01^{e}	0.31±0.01^{fg}
A. solani +Biosurfactant	0.36±0.02^{d}	0.29±0.01^{cde}	0.61±0.01^{d}	0.23±0.02^{bc}
A. solani+ *T.lixii* TvR1 + *P. aeruginosa* Tr20	0.64±0.01^{hi}	0.26±0.01^{bc}	0.91±0.01^{ij}	0.37±0.01^{h}
A. solani + *T.lixii* TvR1 +Biosurfactant	0.42±0.01^{fg}	0.35±0.01^{fg}	0.78±0.01^{f}	0.33±0.01^{gh}

Values are mean of triplicate; ± = Standard error; values followed by different letters are significantly different according to DUNCAN test at 5% significance level

Table 4.28: Protein and Carbohydrate content in tomato leaves after 90 DAT

Treatments	Protein (mg/g fw)	Carbohydrate (mg/g fw)
Control	44.27±0.36^{e}	8.35±0.25^{b}
Trichoderma lixii TvR1	52.35±1.08^{i}	10.19±0.26^{fg}
Pseudomonas aeruginosa Tr20	48.70±0.34^{g}	9.44±0.48^{def}
Biosurfactant	38.63±0.35^{c}	8.55±0.24^{bc}
T. lixii TvR1 + *P. aeruginosa* Tr20	61.85±0.23^{k}	11.38±0.28^{h}
Biosurfactant + *T. lixii* TvR1	50.54±0.42^{h}	10.22±0.22^{g}
Fusarium oxysporum f. sp. *lycopersici (Fol)*	29.05±0.16^{a}	6.18±0.18^{a}
Fol + *T. lixii* TvR1	46.98±0.29^{f}	9.14±0.22^{bcde}
Fol + *P. aeruginosa* Tr20	48.30±0.20^{g}	8.54±0.23^{bc}
Fol + Biosurfactant	41.07±0.32^{d}	8.81±0.19^{bcd}
Fol + *T. lixii* TvR1 + *P. aeruginosa* Tr20	58.30±0.35^{j}	9.90±0.24^{efg}
Fol + *T. lixii* TvR1 + Biosurfactant	47.83±0.20^{fg}	9.21±0.24^{cde}
Alternaria solani	34.92±0.17^{b}	6.52±0.30^{a}
A. solani + *T.lixii* TvR1	47.87±0.31^{fg}	8.76±0.12^{bcd}
A. solani + *P. aeruginosa* Tr20	48.65±0.23^{g}	8.92±0.14^{bcd}
A. solani + Biosurfactant	41.12±0.17^{d}	8.56±0.17^{bc}
A. solani + *T. lixii* TvR1 + *P. aeruginosa* Tr20	59.08±0.15^{j}	9.90±0.22^{efg}
A. solani + *T. lixii* TvR1 + Biosurfactant	48.83±0.24^{g}	9.11±0.15^{bcd}

Values are mean of triplicate; ± = Standard error; values followed by different letters are significantly different according to DUNCAN test at 5% significance level

4.7.3. Defense-related enzymes production

The production of defense-related enzymes Polyphenol oxidase (PPO) and Peroxidase (PO) by the microbial isolates individually and in the consortium were determined 7 DAT. The result obtained is presented in **figure 4.37 a, b**. In *Fol* challenged plants maximum PPO activity was displayed by *Pseudomonas aeruginosa* Tr20 whereas highest PO activity was demonstrated by *Trichoderma lixii* TvR1 which were found 42.9 and 53.5% higher than their respective controls. In case *A. solani* challenged plants highest PPO and PO activity was observed in seedlings inoculated with a consortium of *T. lixii* TvR1 + *P. aeruginosa* Tr20 that were 54.6 and 48.2% more than their respective controls.

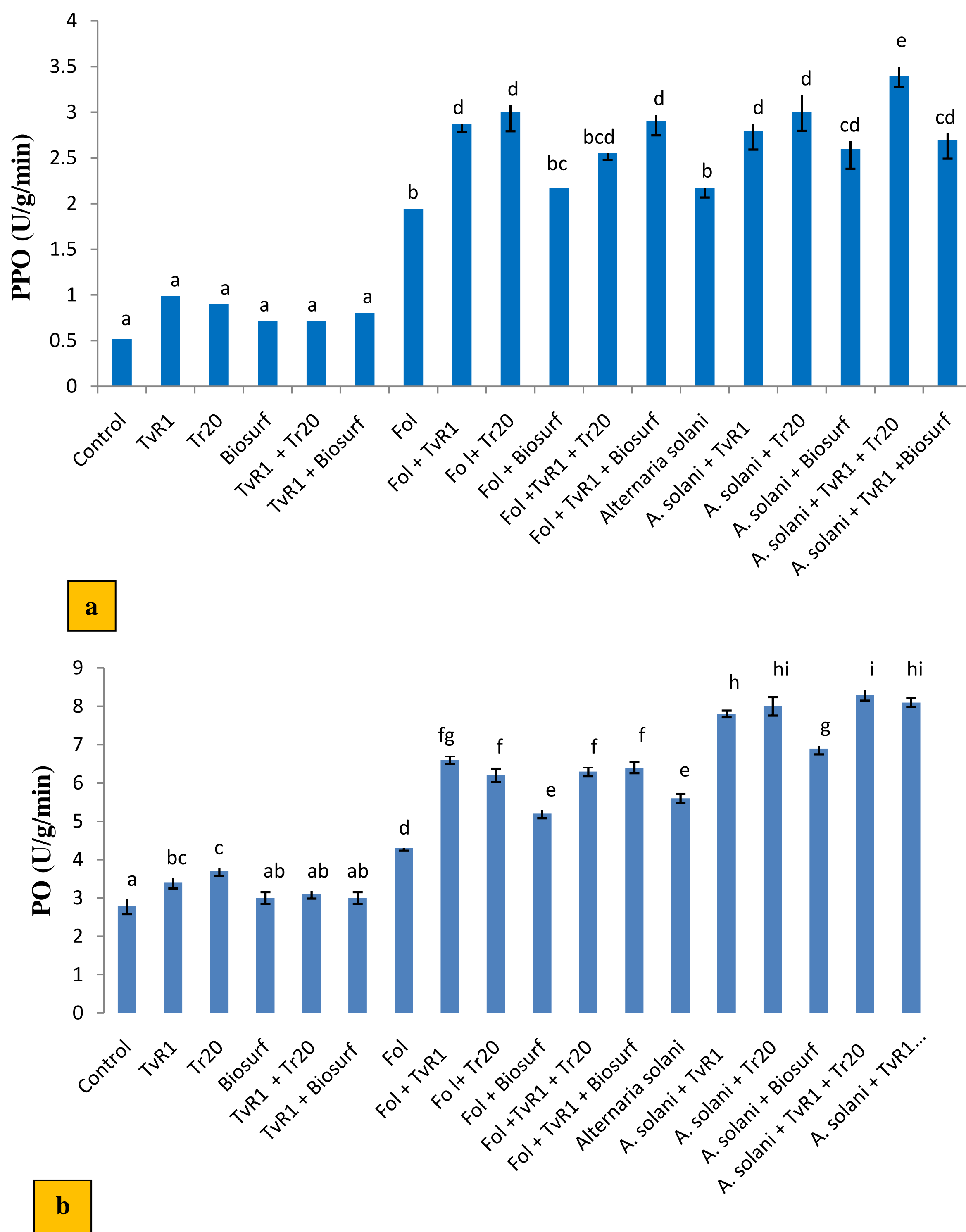

Figure 4.37: Production of defense-related enzymes in tomato seedlings. a) Polyphenol oxidase activity; b) Peroxidase activity. Bars are means of three replicates ± standard error of means. Columns followed by different letters are significantly different according to DUNCAN test at 5% significance level. Biosurf = biosurfactant used @ 200 mg/L.

4.7.4. Fruit yield and nutrient content

The average fruit yield and nutrient content were analyzed at the end of the experiment. The data is presented in **table 4.29**. The maximum average fruit yield per plant (287.8±11.0 g) was recorded in non-challenged plants treated with *Pseudomonas aeruginosa* Tr20. Among pathogens infected plants maximum fruit yield was recorded in plants treated with a consortium of *T. lixii* TvR1 + *P. aeruginosa* Tr20. The highest fruit yield of 256.0±13.0 and 236.6±19.2 g per plant was documented in *Fol* and *A. solani* challenged plants, respectively, treated with a consortium of *T. lixii* TvR1 + *P. aeruginosa* Tr20.

The pH value, titratable acidity and moisture content of tomato fruits were found to be ranging 4.25-4.47; 0.41-0.5% citric acid and 91.8-95.67%. The result revealed that the inoculation of the plant with biocontrol agents reduces the pH value of tomato fruits. The lowest pH value was documented in fruits of non-challenged plants treated with a consortium of *T. lixii* TvR1 + *P. aeruginosa* Tr20 whereas in case of *Fol* and *A. solani* challenged plants lowest pH value of fruits was observed by inoculation of *T. lixii* TvR1 alone and a consortium of *T. lixii* TvR1 + *P. aeruginosa* Tr20, respectively. However, no significant effect of inoculation of biocontrol agents was observed on the titratable acidity of fruits. In case of the moisture content of fruits, the values of different treatments were found significantly different however this difference was not due to the inoculation of biocontrol agents.

Table 4.29: Yield and quality of tomato fruits

Treatments	Average fruit yield per plant (g)	pH	Titratable acidity (% citric acid)	Moisture content (%)	Lycopene (mg 100^{-1} g)	β-Carotene (mg 100^{-1} g)
Control	106.6±5.0^{b}	4.44±0.01^{f}	0.42±0.01^{a}	92.53±0.27^{abcde}	5.45±0.37^{b}	0.30 ±0.02^{ab}
Trichoderma lixii TvR1	196.7±7.4^{fgh}	4.34±0.01^{bcde}	0.50±0.03^{a}	93.7±0.27^{fg}	5.95±0.26^{hi}	0.43±0.03^{e}
Pseudomonas aeruginosa Tr20	213.2±13.0^{hi}	4.40±0.08^{def}	0.47±0.02^{a}	91.8±0.16^{ab}	5.67±0.29^{i}	0.37±0.3^{bcde}
Biosurfactant	115.2±6.6^{b}	4.41±0.03^{ef}	0.42±0.02^{a}	93.92±0.72^{g}	5.54±0.32^{c}	0.32±0.03^{bcde}
T. lixii TvR1 + *P. aeruginosa* Tr20	287.8±11.0^{k}	4.25±0.02^{a}	0.51±0.01^{a}	95.13±0.34^{h}	5.83±0.03^{i}	0.41±0.02^{e}
T. lixii TvR1 + Biosurfactant	205.8±11.3^{gh}	4.40±0.02^{ef}	0.43±0.01^{a}	92.75±0.40^{bcdef}	5.61±0.4^{de}	0.38±0.02^{de}
Fusarium oxysporum f. sp. *lycopersici* (*Fol*)	29.1±5.4^{a}	4.38±0.01^{bcdef}	0.38±0.04^{a}	92.05±0.33^{abc}	4.96±0.38^{a}	0.26±0.03^{a}
Fol + *T. lixii* TvR1	176.9±8.2^{def}	4.30±0.01^{ab}	0.49±0.05^{a}	92.39±0.37^{abcd}	5.86±0.45^{fg}	0.34±0.04^{bcde}
Fol + *P. aeruginosa* Tr20	170.0±10.8^{de}	4.32±0.01^{abc}	0.46±0.01^{a}	93.18±0.45^{cdefg}	5.73±0.24^{efg}	0.39±0.02^{de}

Treatments	Average fruit yield per plant (g)	pH	Titratable acidity (% citric acid)	Moisture content (%)	Lycopene (mg 100^{-1} g)	β-Carotene (mg 100^{-1} g)
Fol + Biosurfactant	109.0±8.0^{b}	4.42±0.02^{f}	0.43±0.03^{a}	93.31±0.27^{defg}	5.49±0.36^{cd}	0.40±0.04^{de}
Fol + *T. lixii* TvR1 + *P. aeruginosa* Tr20	256.0±13.0^{j}	4.33±0.01^{bcd}	0.49±0.04^{a}	92.78±0.34^{bcdef}	5.99±0.31^{i}	0.36±0.02^{de}
Fol + *T. lixii* TvR1 + Biosurfactant	180.8±18.8^{efg}	4.36±0.01^{bcdef}	0.44±0.06^{a}	91.53±0.30^{a}	5.64±0.43^{ef}	0.35±0.02^{bcd}
Alternaria solani	44±3.7^{a}	4.42±0.01^{f}	0.42±0.07^{a}	91.84±0.24^{ab}	5.31±0.33^{ef}	0.29±0.03^{ab}
A. solani + *T. lixii* TvR1	155.1±17.1^{cd}	4.31±0.01^{ab}	0.49±0.02^{a}	92.5±0.20^{abcde}	6.03±0.34^{gh}	0.38±0.03^{cde}
A. solani + *P. aeruginosa* Tr20	141.2±3.2^{c}	4.37±0.01^{bcdef}	0.46±0.04^{a}	92.12±0.21^{abc}	5.77±0.47^{fg}	0.41±0.03^{e}
A. solani + Biosurfactant	112.7±8.2^{b}	4.39±0.01^{cdef}	0.44±0.03^{a}	93.57±0.25^{efg}	5.68±0.38^{c}	0.33±0.02^{de}
A. solani+ *T. lixii* TvR1 + *P. aeruginosa* Tr20	236.6±19.2^{ij}	4.25±0.02^{b}	0.48±0.01^{a}	95.67±0.33^{h}	5.94±0.26^{hi}	0.39±0.04^{de}
A. solani + *T. lixii* TvR1 + Biosurfactant	145.2±16.4^{c}	4.32±0.01^{abc}	0.45±0.01^{a}	92.3±0.48^{abcd}	5.90±0.29^{efg}	0.36±0.03^{bcde}

Values are mean of triplicate; ± = Standard error; values followed by different letters are significantly different according to DUNCAN test at 5% significance level

The lycopene and β-Carotene content were documented maximum in plants inoculated with biocontrol agents as compared to their respective control. The maximum lycopene content was recorded in fruits inoculated with *T. lixii* TvR1 individually except in *Fol* challenged plants where highest lycopene content in fruits was documented in the consortium (*T. lixii* TvR1 + *P. aeruginosa* Tr20) treated plants. The maximum β-Carotene content (0.43±0.03 mg/100 g) in fruits of non-challenged plants was recorded on inoculation with *T. lixii* TvR1 alone. In the case of *Fol* and *A. solani* challenged plants, highest β-Carotene was produced by application of biosurfactant individually (0.40 mg/100 g) and *P. aeruginosa* (0.41 mg/100 g), respectively.

Discussion

Tomato is one of the most cultivated vegetable crops in the world. Like any other crop, tomato growth and yield is affected by several pests and pathogens and nutritional status of the soil. To manage diseases and increase yield, farmers take several counter measures that include the use of various pesticides and fertilizers. The extent of use of agrochemicals and other sustainable approaches for tomato production and challenges faced by farmers while tomato cultivation in and around Lucknow city were addressed in present work through a survey. The study also focused on finding sustainable approaches to protect tomato crop from fungal pathogens and improve its production while reducing inputs of agrochemicals.

5.1. Survey on trends in use of agrochemicals

A pre-structured questionnaire-based survey was conducted during different tomato farming seasons (2014-2016) and farmers were interviewed randomly. The data collected regarding age groups of farmers indulged in agricultural practices in our study was analogous to data reported by Brar et al., (2018). The maximum numbers of farmers belonging to 21-50 years age group were involved in farming practices and only 11.48% of farmers were above the age of 50 years. Our study revealed that approximately 40% of the farmers were illiterate, lacking knowledge about the use of pesticides, quantity required; safety measures to be taken; the health impacts associated with their indiscriminate use and alternative approaches can be used for plant cultivation. Conversely, 16% of farmers below the age of 30 years were found well educated, indicating that the younger age group farmers were cognizant about education. In contrast to our study, Brar et al., (2018) reported comparatively high literacy rate with more number of farmers receiving education up to the secondary level or above. The survey highlighted the role of education in agricultural practices. Understanding among young farmers about the role of education in their life could

have a noteworthy impact on agricultural practices as well as on their socio-economic status. The good educational status assists farmers to understand and implement best agricultural practice and technology in their farms (Mittal and Meher, 2015) and encourage other farmers to opt sustainable and safe farming methods.

The study also revealed that most of the tomato growers were marginal farmers having less than one-hectare acreage under tomato cultivation predicting the low socio-economic status of farmers. The results obtained corroborates with the study of Kar and Dhara, (2007) who mentioned education and economic conditions being instrumental in determining the socio-economic status of farmers and found that low education and status of farmers were closely related with lack of awareness regarding occupational diseases, their treatments and counteractive actions (Kar and Dhara, 2007).

The choice for the type of tomato seeds preferred by the farmers was dependent on total yield potential, the time required for fruit maturation, the timing of cultivation, better shelf life and availability with ease in the market. The hybrid seeds were also preferred due to their intrinsic characteristics to tolerate biotic and abiotic stress (Selvaraj, 2008) and better quality (da Silva Dias, 2010). The October-November was reported to be the highest tomato sowing season in the study area. The rabi season (October-march) is the main season for tomato cultivation in northern India due to the prevailing conditions of temperature and humidity that are suitable for its growth and higher productivity. The tomato crop sown in late season reported to have better quality but low productivity due to improper growth and fruit set under unfavorable climatic conditions *i.e.,* high temperature (Garg et al., 2008). High and/or too low temperature and high soil moisture affect the plants physiological and biochemical processes, thereby reducing productivity (Gunawardena and De Silva,

2015) and enhance plant's susceptibility to various pathogenic diseases. Therefore, farmers cultivate tomato mostly in winters when conditions are favorable. However, some farmers also reported cultivating tomato during Kharif and Zaid season, to earn more during the off-season, when prices are high due to the scarcity of tomatoes in the market (Garg et al., 2008).

Infection caused by pests and pathogens is common in the natural and domesticated ecosystem. To check their growth farmers use excessive agrochemicals. In study area occurrence of several pests (caterpillar, aphids, bugs), fungal and bacterial pathogens (causing wilt, damping off, leaf spots) and viral pathogens (causing leaf curl and dwarfness) were reported. For control of these pests and pathogens, heavy use of pesticides was documented. The chemical pesticides have often reported causing negative impacts on humans and the ecosystem. Some commonly used pesticides such as Mancozeb and chlorpyrifos are carcinogenic in nature (Dabrowski et al., 2014; Mostafalou and Abdollahi, 2013), Lambda-cyhalothrin is neurotoxic (Tomar et al., 2015) and Acephate is endocrine disrupter (Dabrowski et al., 2014) that severely impair mammalian body functioning. Therefore, alternative approaches to diseases and pests management are necessary. Use of biopesticides, flyash, rotation of crops, *etc*. was reported as alternative biocontrol measures however their percentage was negligible. Similar to chemical pesticides, the input of chemical fertilizers was high in areas surveyed. The application of cow dung and compost was also documented however none of the farmers were using biofertilizers. Farmers were reluctant to use biopesticides and biofertilizers due to their narrow activity spectrum, inconsistence field performance and poor quality (Singh et al., 2016; Timmusk et al., 2017).

The abiotic and biotic factors, both were found to impede the growth and productivity of tomato in the study area. The farmers reported that during times of losses it becomes difficult to cover input cost. Low educational status, lack of knowledge and guidance were the main factors for losses occurred due to improper management. These factors were also responsible for not using personal protection equipment (PPE) such as using masks, gloves, suits, *etc*. by the farmers while handling and using agrochemicals. The only single respondent who was well educated was reported to take all necessary precautionary measures including analyzing the direction of flow of air before spraying pesticides in his field. Singh and Gupta, (2009) also reported the similar issue of not using safety measures by farmers and suggested the need for organizing training and programs for increasing farmer's awareness.

5.2. Fungal pathogens of tomato affecting growth

Tomato plant cultivated in India are prone to array of fungal pathogens including *Fusarium oxysporum* f. sp. *lycopersici*, *Alternaria solani*, *Macrophomina phaseolina*, *Phytophthora infestans*, *Pythium aphanidermatum*, *Sclerotium rolfsii* and *Septoria lycopersici* responsible for causing *Fusarium* wilt (Sharma et al., 2018), Early blight (Deshpande et al., 2017), Charcoal rot (Deshpande et al., 2017), Late blight (Chowdappa et al., 2015), damping off (Ramamoorthy et al., 2002), collar rot (Mahato et al., 2017) and Septoria leaf rot (Deshpande et al., 2017), respectively. During the survey, the incidence of fungal diseases in tomato plants in and around Lucknow city was reported. Several pathogenic fungi were isolated from tomato plants collected from different survey sites showing varying degrees of infections and were purified. The morphology of fungal colony, microscopic structure, and their pathogenicity was established under *in vitro* conditions. The soil-borne and foliar

disease pathogens fungal pathogens *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) and *Alternaria solani,* respectively were found most common and predominant in samples collected. These two pathogenic fungi were selected for the study as these were the most prevalent pathogens of tomato in existing environmental conditions and having two different sites of infection *i.e.,* roots and leaves.

5.3. Rhizospheric microorganisms as biocontrol agents

The microbial isolates inhabiting in environs of plant roots are called Rhizospheric microorganisms. These microbes show the positive, neutral or detrimental effect on plant growth. The rhizospheric microbes possessing positive attributes promote plant growth directly by enhancing acquisition of water and minerals, production of phytohormones, solubilizing and increasing accessibility to inorganic compounds and indirectly by curtailing the growth of phytopathogens. Several rhizobacteria and fungi including species of genera *Pseudomonas*, *Trichoderma*, *Bacillus*, *etc*. are well known rhizospheric microorganisms that exert a positive effect on plants growth (Sachdev and Singh, 2017).

In the study, an attempt was made to isolate beneficial rhizospheric bacteria and fungi possessing the ability to control the growth of fungal pathogens of tomato. The fifteen strains of rhizobacteria and seven fungal strains were isolated that initially identified on biochemical, phenotypic and microscopic characters. Among fungal strains, species of *Aspergillus*, *Penicillium*, *Rhizopus,* and *Trichoderma* were isolated. Only strains of *Trichoderma* were included in the study as other isolates such as *Aspergillus*, *Penicillium* and *Rhizopus* are reported to have deleterious effects on humans (Prabhu and Patel, 2004; Egbuta et al., 2017). Species of *Aspergillus*, *Penicillium,* and *Rhizopus* produce numerous mycotoxins which cause aspergillosis,

penicilliosis and mucormycosis, respectively (Prabhu and Patel, 2004; Egbuta et al., 2017). The microscopic characters of the two isolates of *Trichoderma* were studied under scanning electron microscope (SEM). The phialides of both *Trichoderma* strains were flask-shaped, constricted toward tip and base and swollen in middle and arranged in a whorl. The conidia were greenish to grey in color with subglobose to the ovoidal structure. The orientation and shapes of phialides and shape and color of conidia were found resembling with that of *Trichoderma* species as described by Samuels et al., (2002, 2014), that formed the basis for confirmation. Similarly, the bacterial strains were identified primarily on the basis of the biochemical test described by Aneja, (2003). It was found that strains of *Bacillus* were dominant in agricultural soil. The strains with positive biocontrol attributes were later confirmed at the genomic level.

The rhizospheric bacteria and isolates of *Trichoderma* were assessed for their biocontrol attribute under *in vitro* condition and found that three rhizobacterial and both of the *Trichoderma* isolates were positive biocontrol microbes showing inhibition of pathogens (*Fol* and *A. solani*) mycelia. The maximum percentage inhibition of radial growth (PIRG) of pathogen mycelia was recorded for *Trichoderma* TvR1 and rhizobacteria Tr20. *Trichoderma* species are well known to produce several cell wall degrading enzymes (CWDEs) (Brunner et al., 2003) and antibiotics that are instrumental in inhibition of pathogens growth *via* mycoparasitism, competition, and antibiosis (El-Hassan et al., 2013).

In the present study both the isolates of *Trichoderma* were observed growing around and over a colony of pathogens, whereas isolate TvR1 also formed a clear zone of inhibition around the pathogen colony. Inhibition of pathogen colony by the growth of antagonists reveal competition, whereas formation of an inhibition zone

around pathogen indicates secretion of non- volatile diffusible metabolites having an inhibitory effect on the pathogen growth and suggests involvement of mechanism antibiosis for biocontrol. Overgrowth of *Trichoderma* on a colony of pathogens and the microscopic study of the hyphal interaction between pathogens and *Trichoderma* isolates revealed coiling of hyphae of *Trichoderma* around pathogens hyphae depicting possibly employment of mechanism of mycoparasitism in antagonism of pathogens. The observation recorded in our study corroborates with those presented by Jabnoun-Khiareddine et al., (2009) who observed 25.97 % to 62.15 % reduction of mycelia growth of *Verticillium dahliae* vd82 challenged with *Trichoderma harzianum* and *T. virens* and observed invasion of *Trichoderma* in the colony of pathogen demonstrating the role of competition for space and nutrients. Similarly, the reduced mycelial growth and sporulation of *F. oxysporum* f. sp. *lentis* due to competition for available space and nutrients by *T. hamatum* was reported by El-Hassan et al., (2013). The microscopic findings revealed direct and aggressive contact of *T. hamatum* with *Fusarium*, coiling around the hyphae of latter and ultimately resulting in the collapse of hyphae of pathogens by the production of hydrolytic enzymes.

The rhizobacteria were also effective in reducing the growth of pathogens under *in vitro* condition. The maximum inhibition of pathogens was displayed by Tr20 followed by CS13 and BS6. Rhizospheric bacterial isolates such as *Pseudomonas*, *Bacillus* are well known for their ability to maintain plant and soil health and offering protection against diseases (Hol et al., 2013; Goswami et al., 2013). Mishra et al., (2016) reported biocontrol of *Fusarium moniliforme* causing ear rot in maize by fluorescent Pseudomonad. Similarly, Sarkar et al., (2014) reported biocontrol of phytopathogen *Sclerotinia sclerotiorum* by *Pseudomonas putida* and *P. aeruginosa* possessing ability to produce Indole acetic acid (IAA), Hydrogen cyanide

(HCN) and ammonia. These studies support the findings of the present study and state that the biocontrol attribute of isolated rhizospheric bacteria may be anticipated by the production of secondary metabolites.

The five isolates that displayed positive biocontrol potential under *in vitro* condition were identified at genomic level for confirmation. The five isolates were identified as *Bacillus subtilis* BS6, *B. subtilis* CS13, *Pseudomonas aeruginosa* Tr20, *Trichoderma lixii* TvR1, and *T. brevicompactum* TbS2. The phylogenetic trees were constructed using neighbor-joining tree based on analysis of partial 18S rRNA nucleotide and 16S rRNA nucleotide sequence of fungi and rhizobacterial isolates respectively, showing the relationship between isolated and other strains documented in literature possessing biocontrol, plant growth promoting and stress alleviation activities.

5.4. Characterization for production of secondary metabolites

Production of secondary metabolites, phytohormones and solubilization of inorganic minerals by microorganisms are significant for plant growth promotion and disease management. Nutrient solubilization (inorganic phosphate, zinc and potassium) and production of ammonia increases the availability of nutrients for plants in soil whereas production of phytohormones such as Indole Acetic Acid (IAA) modifies root architecture that increases number of root tips and surface area for deep anchorage in soil and better uptake of nutrients and water (Vacheron et al., 2013; Li et al., 2015) that in turn provides resistance against nutrient deficiency and tolerance to abiotic stresses.

Siderophores, iron chelating low molecular weight compounds show two ways benefits for plants. Siderophores reduces the iron deficiency in plants and also knock out the pathogenic microbes from the same niche by depriving them from availing

iron, ensuing competition for nutrients and space (Dwivedi and Johri, 2003). The elimination of competing microbes due to competition for nutrients and niche by siderophore producing plant growth promoting rhizobacteria (PGPR) that colonize plant roots was reported by Loaces et al., (2011) in their work. Similarly, *Azospirillum brasilens* REC2 and REC3 were reported to inhibit the growth of *Colletotrichum acutatum* M11 and reduced anthracnose disease severity on the strawberry plant under *in vitro* and *in planta* by synthesizing catechol type siderophore and salicylic acid (Tortora et al., 2011). The harzianic acid produced by *Trichoderma harzianum* act as siderophore was reported to stimulate the growth of competing microbes in soil either positively or negatively (Vinale et al., 2013).

The isolated microbes were found to produce secondary metabolites (Hydrogen cyanide, Chitinase, cellulase, protease) that participate in inhibition of pathogens *via* mechanisms of mycoparasitism, competition and antibiosis (Shrestha et al., 2015). The hydrolytic enzymes assist antagonistic isolates to degrade polysaccharides of pathogen cell wall (Saravanakumar et al., 2016); and cellulose present in plant cells that assist in successful root colonization (Strakowska et al., 2014). Disintegrated and hyphae lysis of *Sclerotinia minor* and *S. sclerotiorum* by chitinase, protease and β-1,3-glucanase activity of *Bacillus thuringiensis* C25 was documented by Shrestha et al., (2015). Ahmad et al., (2008) reported siderophores and HCN produced by isolates of *Azotobacter*, *Pseudomonas* and *Bacillus*, either alone or synergistically with other metabolites displayed biocontrol effect against plant pathogens.

5.5. Root colonization by antagonistic isolates

The ability to produce Exopolysaccharides (EPS) and form biofilm was displayed by antagonistic isolated strains TvR1, TbS2, and Tr20. The EPS are a significant

structural constituent of biofilm which shows several ecological benefits. EPS helps in adherence to different surfaces including plant roots, aggregation, formation of biofilms and root colonization; provides protection against biotic/abiotic stresses; enhance nutrient availability and mediate communication with other microbial communities (Sathiyanarayanan et al., 2017). The multispecies interaction results in the production of new varieties of polysaccharides with variable composition (Andersson et al., 2011) that play important role in better colonization of soil and plants and maintaining soil ecosystem equilibrium (Burmolle et al., 2014; Velmourougane et al., 2017). The higher values for EPS production during biofilm formation was observed when *Azotobacter chroococcum* co-cultured with *Trichoderma viride* than individual cultures. The study suggests a synergistic effect of bacterial and fungal association on growth, biofilm formation and EPS production that may result in better colonization (Velmourougane et al., 2017). EPS also helps in enhancing water uptake and acquisition of nutrient from the soil by forming soil aggregates and increasing their stability, thereby resulting in better plant growth. This plant growth promoting effect of EPS was reported by Kumar et al., (2012) on inoculation of sorghum seedlings with EPS producing strain P13 in combination with arbuscular mycorrhizal fungi.

The root colonization ability of *T. lixii* TvR1 and *P. aeruginosa* Tr20 was studied under scanning electron microscope. Colonization of inoculated roots with their spores/cells confirmed the role of root colonization in effective biocontrol and PGP activity. The findings on effective root colonization by microbial isolates were supported by the studies of Chacon et al., (2007); Alonsa-Ramirez et al., (2014) and Ruano-Rosa et al., (2016) who reported the ability of *Trichoderma* CECT 2413 to colonize roots of tomato, *Arabidopsis thaliana* and olive, respectively.

5.6. Biocontrol ability of volatile metabolites and non-volatile metabolites of antagonistic isolates

The result obtained revealed that the volatile metabolite produced by isolates TvR1, TbS2 and Tr20 had a significant inhibitory effect on growth of pathogen *Fol*, however volatile metabolites of all the five isolates *i.e.,* TvR1, TbS2, BS6, CS13, and Tr20 displayed good antagonistic effect against *A. solani*. The volatile metabolites produced by rhizobacteria and *Trichoderma* are well known for their activity to suppress the growth of pathogenic fungi (Kanchiswamy et al., 2015). Asad et al., (2014) reported less than 20% inhibition of *Rhizoctonia solani* by volatile metabolites of *T. asperellum*, *T. harzianum,* and other *Trichoderma* spp. Volatile compounds emitted by *Pseudomonas trivialis* 3Re2-7, *Bacillus subtilis* B2g, *Serratia plymuthica* HRO-C48, *etc*. were found to negatively affected the growth of soil-borne fungal pathogen *Ralstonia solani* (99-80%) (Kanchiswamy et al., 2015). Identification and application of volatile compounds emitted by rhizobacteria can provide better opportunity to formulate an eco-friendly and effective product for crop protection.

The non-volatile metabolites (culture filtrate) assay displayed that the percentage inhibition of pathogen growth was directly proportional to the concentration of filtrate. The non-volatile diffusible compounds by *Trichoderma* spp. in the present study found to have an inhibitory effect on the radial growth of pathogens. The significant reduction in the sporulation, germination of conidia and length of the germ tube of *Fol*, *Verticillium dahliae* and *A. solani* by culture filtrate of *T. harzianum and T. hamatum* was reported by El- Rifai et al., (2003). Choudary et al., (2007) extracted the crude antifungal metabolites from the culture filtrate of *T. harzianum* and observed complete growth inhibition of the pathogens (*Sclerotium rolfsii, Ralstonia solani,* and *Fusarium oxysporum*) at 2.5% concentration.

5.7. Seed germination assay

The inoculation of seeds with all different treatments was found to have a significant effect on the seedling growth and germination rate over control. Several workers had reported a significant increase in the rate of seed germination and seedling vigor index of various plant seeds by use of microbial inoculants either individually or in the consortium (Rudolph et al., 2015). In the present study, all isolated antagonists significantly improved the rate of seed germination and vigor index over non-inoculated seeds with an exception where treatment of seeds with isolate TbS2 does not exhibit any significant effect on the seed germination and the vigor index. Some *Trichoderma* isolates are found to display stimulating effect on seed germination and vigor index, however, in some case neutral or even negative effects have been observed (Hajieghrari and Mohammadi, 2016). The significant increase in germination rate and vigor index of maize was reported by Gholami et al., (2009) on inoculation with six strains of *Pseudomonas* and *Azospirillium*. Similarly, inoculation of cucumber seeds with ten isolates of rhizobacteria resulted in increased germination rate by 8.07-15.32% and vigor index by 98.62-148.05% (Islam et al., 2016). The seed germination rate and vigor index depend on the production of seed germinating compounds including phytohormones and enzymes that participate in the acquisition of nourishment (Hajieghrari and Mohammadi, 2016).

5.8. Compatibility between antagonistic strains and with biosurfactant for development of consortia

Recently several studies have demonstrated that use of consortia has broader activity spectra and possess holistic approach by providing protection against phytopathogens, mobilizing plant nutrients, increasing the availability of N and P, releasing phytohormones, *etc*. (Mishra and Sundari, 2017). The consortia applied, results in

synergistic effects only when different microbial isolates constituting consortium are compatible with each other (Mishra and Sundari, 2017). In the present study among microbial antagonistic isolates, only *Trichoderma lixii* TvR1 was found compatible with *Pseudomonas aeruginosa* Tr20 and *Bacillus subtilis* BS6 with *B. subtilis* CS13. Rest other combinations were observed to inhibit the growth of one another.

The biosurfactants are known to have both the positive and the negative effects on the growth of the microbes (D'aes et al., 2010). The biosurfactant extracted from *P. aeruginosa* Tr20 was also reported to be compatible with the *T. lixii* TvR1 @ concentration 200 mg/L, however, beyond this concentration, biosurfactant was found to affect the growth and colony morphology of *T. lixii* TvR1. The result depicted the concentration-dependent effect of biosurfactant on *T. lixii* TvR1. The biosurfactant has been reported to facilitate and/or enhance the biocontrol activity of biocontrol agents and plant growth promoting microorganisms (PGPMs) (Singh et al., 2007; Sachdev and Cameotra, 2013).

5.9. Characterization and biocontrol activity of Biosurfactant

Owing to the pathogenicity of *Pseudomonas aeruginosa* Tr20 to human beings, as an alternative, their ability to produce biosurfactant was assessed. The biosurfactant production ability was assessed with the idea to replace efficient biocontrol agent with its secondary metabolites and assessing their capability to perform as a biocontrol agent. The isolate Tr20 were screened for the production of biosurfactant on CTAB agar plates and blood agar plates. The anionic biosurfactant produced by the microbe results in the formation of an insoluble ion pair with methylene blue and CTAB that give blue color around the colony (Walter et al., 2010). The production of a clear zone around blood agar confirms the production of biosurfactant that possesses the ability to cause lysis of the blood cells (Walter et al., 2010).

The ability to form foam, surface activity against oil, reduction of surface tension and emulsify hydrocarbons are some physiochemical characteristics of biosurfactant (Pornsunthorntawee et al., 2007). The oil displacement by extracted compound demonstrated its surface activity against oil. Similarly, the formation of foam on vigorous shaking and emulsification of oil by the compound isolated confirmed it as biosurfactant. The yield of biosurfactant obtained was 0.815 g/L in the present study. El-Sheshtawy and Doheim (2014) also reported a lower yield of biosurfactant about 1 g/L, however, Singh and Cameotra, (2013) reported high yield approximately 4.5 g/L.

Further to elucidate the chemical structure of biosurfactant FTIR spectroscopy was performed that assisted identification of the chemicals bonds and functional groups present (Pornsunthorntawee et al., 2007). The absorption band located at 3400, 2928, 2860, 1727, and 1310-1061 cm^{-1} on FTIR graph shows chemical structural resemblance to rhamnolipid. The band at position 3400 cm^{-1} depicted O-H stretching vibration of the hydroxyl group of biosurfactant. The peak at 2928 and 2860 cm^{-1} corresponds to C-H stretching vibrations of the hydrocarbon chain. The peak at 1727 cm^{-1} resembles C=O stretching vibrations of the carbonyl group and stretching band in region 1300-1060 cm^{-1} show bond between carbon and hydroxyl group of rhamnose rings of rhamnolipid. This result was analogous to the FTIR results for Rhamnolipid documented by Pornsunthorntawee et al., (2007) and Singh and Cameotra, (2013).

The biosurfactant displayed positive antagonistic activity against pathogens *Fol* and *A. solani*. The result revealed that the antagonistic activity was concentration dependent. With the increase in concentration, the PIRG of pathogens was increased. The antimicrobial activity of biosurfactant is evaluated by many workers. Inhibition

of several tested microbial strains by biosurfactant was reported by El-Sheshtawy and Doheim (2014) under *in vitro* condition. The inhibition of pathogens *Fusarium*, *Aspergillus* and *Bipolaris sorokiniana* by the biosurfactant produced by *Bacillus* was reported by Vehlo et al., (2011). The addition of biosurfactant provides favorable conditions for the growth of other microbes that enhance their activity (Zhange et al., 2011).

5.10. Biocontrol and plant growth promoting activity of isolated microbial antagonists, biosurfactant and their consortium under pot conditions

The strains with positive biocontrol activity under *in vitro* conditions were screened for biocontrol and plant growth promoting (PGP) activity in pot conditions. The isolated strains displayed significant biocontrol and PGP activity with respect to control except the isolate *Trichoderma brevicompactum* TbS2. The isolate *T. brevicompactum* TbS2 with effective biocontrol potential was unable to promote plant growth may be due to the production of phytotoxic compounds. *T. brevicompactum* have been reported to secrete metabolite Trichodermin that play an influential role in biocontrol (Hermosa et al., 2013), however, are phytotoxic in nature that inhibits seed germination and plant growth promotion. This was evidently reported by Tijerino et al., (2011) who observed reduced plant size, root length and a number of lateral roots due to inoculation of *T. brevicompactum* as compared to control tomato seedlings.

The other four isolates were found to reduce the incidence of fusarium wilt and early blight caused by soil-borne pathogen *Fol* and foliar pathogen *A. solani*, respectively. The rhizospheric bacteria (Wu et al., 2005) and *Trichoderma* species are known to colonize plant roots effectively and proliferate which result in suppression of pathogens directly by the mechanism of mycoparasitism, antibiosis, competition

for space and nutrition and/or indirectly by induction of systemic resistance (ISR) (Chowdappa et al., 2013). Babu et al., (2015) in their study reported a reduction of incidence of early blight in tomato plants inoculated with PGPRs.

The isolate *Trichoderma lixii* TvR1 and *Pseudomonas aeruginosa* Tr20 were found the most effective strains that displayed the highest percentage of disease reduction as well as promoted plant growth significantly. The strain *T. lixii* TvR1 and *P. aeruginosa* Tr20 were also found compatible with each other and thus were formulated in the consortium and their biocontrol, as well as plant growth promoting effect, was assessed. The isolates TvR1 and Tr20 were found to produce Exopolysaccharide (EPS) and possess the ability to form a biofilm which confirmed their efficient root colonization potential. Ahn et al., (2007) in their study reported that the biocontrol bacterium *Pseudomonas putida* 06909 possessing ability to form a biofilm on citrus root resulted in inhibition of fungal phytopathogen *Phytophthora parasitica*. After successful root colonization plant growth promoting microorganisms (PGPMs) also ameliorates the deleterious effect of pathogens on plants by secreting low molecular weight siderophores that deprive pathogens from assessing iron essential for their growth and proliferation; hydrogen cyanide, antibiotic and secondary metabolites that also indirectly increases plant growth.

In addition to root colonization, these two isolates were also observed to produce Indole acetic acid, siderophores, hydrolytic enzymes and mineralize inorganic compounds. Several studies provide strong evidence on the role of auxin (IAA) in development of plant root system *i.e.*, lateral root and root hairs. Many PGPR and *Trichoderma* species synthesize IAA (Patten and Glick, 2002; Contreras-Cornejo et al., 2009; Nieto-Jacobo et al., 2017). Microbial IAA promotes root growth

directly by stimulating cell elongation or cell division and indirectly by influencing ACC deaminase activity (Patten and Glick 2002; Gravel et al., 2007). In our study, *T. lixii* TvR1 and *P. aeruginosa* Tr20 both displayed IAA production which may be responsible for root growth and development that induce significant growth by better nutrient and absorption from the soil. Patten and Glick, (2002) also demonstrated the role of IAA in development of plant root system. The mung bean cuttings soaked in a suspension of a wild-type strain of *Pseudomonas putida* GR12-2 containing a high level of IAA, stimulated the formation of several, very small, adventitious roots, whereas the IAA deficient mutant resulted in the formation of fewer roots. Contreras-Cornejo et al., (2009) showed the indolic compounds synthesized by *Trichoderma virens* increased the biomass of wild-type *Arabidopsis thaliana* and lateral root development.

The antagonistic isolate *P. aeruginosa* Tr20 was found to produce HCN, siderophores, ammonia and hydrolytic enzymes and solubilize phosphate and zinc whereas *T. lixii* TvR1 possess the ability to synthesize siderophore, hydrolytic enzymes, ammonia production and solubilization of zinc. Nitrogen is a major nutrient required by plants for growth however, plants are unable to utilize atmospheric nitrogen (Rodrigues et al., 2016). Soil-dwelling microbes can fix atmospheric nitrogen by converting them into ammonia. Thus the production of ammonia by microbes improves soil fertility, fulfills plant's demand for nitrogen that stimulates their growth and also enhances plant's defense towards pathogen colonization (El-Din Hassan, 2017). These attributes may be possibly the reason for the biocontrol activity of the two isolates used in the present study.

The biosurfactant was used in the present study as safer and eco-friendly approach for disease control or plant growth promotion. Goswami et al., (2015) in

their study reported the effective role of rhamnolipid biosurfactant extracted from *P. aeruginosa* in the reduction of red rot disease of sugarcane caused by *Colletotrichum falcatum*. In the present study, the individual application of biosurfactant does not display significant biocontrol effect. However, many workers have reported plant growth promoting effects of biosurfactant. The biosurfactant increases the availability of nutrients in the soil and improves the soil quality by reducing hazardous pesticides, heavy metals and hydrocarbons from soil that results in plant growth promotion (Sachdev and Cameotra, 2013). In our study, the combined application of biosurfactant with *T. lixii* TvR1 was found to reduce the percentage of disease incidence more as compared to the individual treatment of *T. lixii* TvR1, and biosurfactant. This suggests a role of biosurfactant in promoting biocontrol activity of microbial isolates. The biosurfactant has been reported to regulate the process of quorum sensing that affects the root colonization activity of microorganisms (Sachdev and Cameotra, 2013). This attribute may be the probable reason for improving biocontrol activity of *T. lixii* TvR1 in presence of biosurfactant.

The application of microbial biocontrol agents and biosurfactant either individually or in the consortium also resulted in the production of pathogenesis-related enzymes that are known to induce systemic resistance in plants against pathogens (Segarra et al., 2009; D'aes et al., 2010). Inoculation of cucumber plants by *Trichoderma asperellum* T203 conferred protection against *Pseudomonas syringae* pv. *lachrymans* by eliciting induced resistance *via* jasmonic acid and ethylene signaling pathway (Shoresh et al., 2005). In the present study, all the biocontrol agents increased the activity of polyphenol oxidase (PPO) and peroxidase (PO). However, the maximum upregulation of PPO and peroxidase PO *Fol* infected plants were induced by *P. aeruginosa* Tr20 and *T. lixii* TvR1, respectively, whereas in case

of foliar pathogen *A. solani* infected plants the maximum PPO and PO were induced by a consortium of *T. lixii* TvR1 + *P. aeruginosa* Tr20. The result demonstrated that the production of defense-related enzymes was dependent upon the type of pathogen infection. In *A. solani* challenged plants, a correlation between the activity of pathogenesis-related compounds and disease reduction was observed. A similar result was observed by Babu and co-workers, (2015), who found significant upregulation of peroxidase and polyphenol oxidase genes after treatment of *A. solani* infected plants with biocontrol agent TN-Vel-35. The enhanced ISR in cucumber by a combination of *Trichoderma harzianum* Tr6 with *Pseudomonas* sp. Ps14 against *Fusarium oxysporum* f. sp. *radicis cucumerinum* and *Botrytis cinerea* in cucumber and *Arabidopsis thaliana*, respectively was reported by Alizedah et al., (2013).

Chlorophyll estimation is an important parameter that indicates the photosynthetic and metabolic activity of plants (Hartmann et al., 2009; Mathivanan et al., 2017). In the present study, it was found that infestation of *Fol* and *A. solani* significantly reduced the total chlorophyll content in leaves. The highest chlorophyll content was reported in plants treated with consortium than individual treatment. Increase in chlorophyll content of plants may be due to enhanced uptake of nutrients (microbial fixed nitrogen) from soil and increased rate of photosynthesis (Bashan et al., 1990; Premachandra et al., 2016). The effect of PGPRs on chlorophyll content of groundnut was studied by Mathivanan et al., (2017) and observed maximum chlorophyll in plants treated with a consortium of *Pseudomonas*, *Rhizobium,* and *Bacillus*. Another evidence of an increase in chlorophyll content by application of consortium of *Trichoderma harzianum* BHU-51 and BHU-105 was reported by Singh and Singh (2012) which was significantly higher than non-pathogen inoculated and pathogen inoculated control. The increased rate of photosynthesis and chlorophyll content in broccoli was reported on inoculation with *T. viride* alone and a consortium

of *Glomus intraradices*, *Acaulospora laevis*, *T. viride,* and *Pseudomonas fluorescens*. This increase may be attributed by the increase in stomatal conductance (Tanwar et al., 2013). *Trichoderma* species are reported to set chemical communication on efficient root colonization, which alters gene expression and provides several benefits including increased nitrogen use efficiency, resistance to biotic and abiotic stress and enhanced photosynthetic activity (Hermosa et al., 2010; Harman et al., 2012).

Carotenoid is an accessory photosynthetic pigment that acts as light harvesting pigment and protects photosynthetic pigments from harmful radiations (Hashimoto et al., 2016). The carotenoid content in plant leaves after 90 days of inoculation was assessed and results obtained showed maximum content in leaves treated with the consortium. Our findings were supported by the work reported by Hernandez et al., (2014), who found a positive effect of combined use of *Azospirillium brasiliense* and *Pantoea dispersa* on carotenoid content of lettuce. Similarly, treatment of pea plants with a consortium of *P. aeruginosa* PJHU15, *T. harzianum* TNHU27, and *Bacillus subtilis* BHHU100 showed maximum carotenoid and chlorophyll content in presence as well as the absence of pathogen *Sclerotinia sclerotiorum* (Jain et al., 2015).

The treatment of plants with different biocontrol agents either challenged or non-challenged were found to improve carbohydrate and protein content as compared to respective control. The maximum carbohydrate and protein content were found in plants treated with a consortium of *T. lixii* TvR1 + *P. aeruginosa* Tr20 followed by a consortium of *T. lixii* TvR1+ biosurfactant. The results corroborate with that reported by Kalita and co-worker, (2015) who found increased protein, carbohydrate, lipid and amino acid content in cauliflower treated with a consortium of *Bacillus cereus* (MTCC 8297) and two strains of *Pseudomonas rhodesiae* (MTCC 8299 and 8300). The improved carbohydrate and protein content were may be due to the increased

growth, enhanced photosynthetic activity and uptake of nitrogen from the soil in plants (Alamri et al., 2018; Sachdev and Singh, 2018).

Inoculation of biological agents was found to increase the yield of tomato plants either pathogen challenged or non-challenged. The study revealed the growth promoting effect of the microbial strains and their metabolites on tomato yield. Further, the maximum fruit yield per plant was recorded for a set of plants treated with a consortium of *T. lixii* TvR1 + *P. aeruginosa* Tr20 followed by a consortium of *T. lixii* TvR1 + biosurfactant. The use of consortia of biological agents or their metabolites were found to be more effective in increasing yield than the individual. Our result corroborates with the study of several workers who reported the pronounced effect of the consortium on fruit yield over individual treatment. The titratable acidity and moisture content of tomato fruits were not influenced by the treatment of different biocontrol agents. The low pH is considered as one of the qualities of tomato fruits that makes them more valuable for industrial purposes and reduces the chances of contamination of the processed products with deleterious microorganisms thereby increasing their shelf life (Caliman et al., 2010). Reduction in pH value on treatment with biocontrol agents was observed. This result was supported by Bona et al., (2017), who found lower pH value for the tomato fruits treated with arbuscular mycorrhizal fungi (AMF) as compared to control.

β-carotene and lycopene, responsible for imparting red color, are two carotenoids found in tomato fruits. The content of lycopene and β-carotene was found to be affected by the inoculation of biocontrol agents. The results were in agreement with that presented by Molla et al., (2012), who found higher values for lycopene and β-carotene in fruits on treated with biofertilizers as compared to untreated control. However, In contrast to the result obtained, no effect of the inoculation of microbial agents on the carotenoid content was reported by Bona et al., (2017).

Summary & Conclusion

Agriculture is an oldest and major occupation of the human beings that fulfills the basic needs of the human. In natural or man-made agro-ecosystems, the occurrence of diseases is a common phenomenon. However, the rapid increase in world's population, deterioration of the soil quality and continuous climate change has coupled with the phenomenon of disease and pest incidences that has aggravated the negative impact on plant growth, their yield, and nutritional values. Tomato is one of the most cultivated vegetable crops in the world. Due to its nutritional content and processing values, it is considered as a functional food crop and ranked top in industrial food products.

Tomato crop is sensitive to stresses and its productivity, quality of fruit content is greatly influenced by the biotic and abiotic factors. The occurrence of pathogenic diseases in tomato is common that hampers its yield and reduces market value. Thus the high demand of good quality of tomatoes in industrial sectors as well as for domestic purposes, compel farmers to input excessive amounts of agro-chemicals to obtain high yield. The excessive use of agro-chemicals is no more a sustainable approach due to their intrinsic negative externalities on the environment and living beings. The present study focused on assessing the problems faced by the farmers growing tomato in and around Lucknow city, the trend in use of fertilizers and pesticides followed by them to enhance productivity and to enhance the efficacy to biological approaches in reducing the incidence of fungal diseases on tomato. The major findings of the study were:

A personal interview was conducted with 97 tomato growers based on the pre-structured questionnaire, during different tomato growing seasons (2014-2016). The data regarding farmers age and education was collected that revealed that maximum farmers who indulged in tomato cultivation were between the age of 21-50 years and

about 40% of the farmers surveyed were illiterate. However, most of the farmers below the age of 30 years were found to be educated upto 8th standard. The data show that the low level of literacy was the main reason for the lack of knowledge about the use of modern technologies for sustainable crop cultivation and safety measures while handling agro-chemicals. This was proved by the data of single respondent collected, who was well educated and reported to use sustainable methods for disease management and plant growth and using proper safety measures *i.e.,* use of mask, gloves, studying the direction of wind before spraying agro-chemicals.

The majority of the farmers were marginal and cultivating tomato in small areas, less than a hectare, determining their low socio-economic conditions. The choice of seeds and best timing preferred for tomato cultivation by the farmers was also surveyed and found that maximum numbers of farmers willing to use hybrid seeds over non-hybrid seeds and October-November was the most preferable season for nursery preparation, although tomato can be cultivated all year round as Kharif and Zaid crop. The hybrid seeds were preferred due to their high yield potential, less time required for yield harvesting, better shelf life and quality of fruit content whereas winter or rabi season (October-March) was preferred due to favorable prevailing environmental conditions.

The abiotic, as well as biotic factors, were reported responsible for affecting tomato cultivation during the survey. The fluctuation in temperature, scarcity of water, land fragmentation, low monetary returns were the main abiotic factors whereas infestation of pests like caterpillars, termites, bugs, aphids, and fungal, bacterial, viral pathogens were the biotic agents found responsible for challenging tomato cultivation in the study area. The farmers were heavily reliant on agrochemicals such as organophosphates, synthetic pyrethroids carbamates, and

neonicotinoids to increase reduce plant pests and pathogens and Di-ammonium phosphate (DAP), urea, potash to increase the tomato productivity. The use of biofertilizers and biopesticides were negligible. The reason behind high inputs of the agro-chemicals and the negligible use of sustainable biological approaches was the low educational status of the farmers. This highlights the fact that educational status plays a most influential role in the trend in use of approaches for disease control and plant growth promotion.

Further, the pathogenic fungi responsible for causing biotic stress on tomato cultivation in and around Lucknow city were studied. The infected tomato plant and soil samples were collected from various tomato fields and cultured in laboratory conditions to assess the major fungal pathogens affecting the growth in the study area. The two pathogens *i.e*., *Fusarium oxysporum* f. sp. *lycopersici* and *A. solani*, responsible for fusarium wilt and early blight, respectively in tomato were found prevalent in and around Lucknow city. The pathogenic nature of isolated strains was established under *in vitro* conditions.

In search of sustainable approach for controlling the growth of these fungal pathogens, a further attempt was made to isolate rhizospheric microbial strains possessing biocontrol ability. The microorganisms such as rhizobacteria, fungi, arbuscular mycorrhiza that colonize rhizosphere and plant roots are known as rhizospheric microbes. The association of several rhizospheric microorganisms with plants is reported to be a beneficial association. These microbes have demonstrated a significant role in the management of plant diseases and tailoring abiotic stress, ultimately leading to enhanced plant productivity. The seven fungal and fifteen rhizobacterial strains were isolated from roots and soil from the vicinity of healthy plants. The isolated fungal strains were identified on the basis of phenotypic

characters whereas rhizobacterial strains identified on biochemical tests. Among seven fungal isolates, two strains TvR1 and TbS2 were identified as *Trichoderma*, two strains AcS4 and AbS5 as *Aspergillus*, other two strains PbS3 and PrS7 as *Penicillium* and single isolate RcS6 as *Rhizopus*. The *Trichoderma* strains were selected for further study and rest were discarded owing to their ability to produce mycotoxins. The phenotypic characters of the isolates of *Trichoderma* were further studied in details for confirmation. Among isolated Rhizobacteria, 47% of the isolates were found to be *Bacillus* species (TS1, LS3, BS5, BS6, ES4, CS13, and Ts23) and only single strain of *Pseudomonas* (Tr20) was isolated.

The isolated strains observed for their antagonistic/biocontrol potential under *in vitro* condition. Total five isolates including two isolates of *Trichoderma* (TvR1 and TbS2) and three rhizobacteria (BS6, CS13, and Tr20) displayed positive biocontrol activity. The isolates of *Trichoderma* displayed mechanism of competition, antibiosis and mycoparasitism against pathogens. Similarly, the rhizobacterial isolates involved mechanism of antibiosis and competition. The strains BS6, CS13, Tr20, TvR1 and TbS2 with positive biocontrol attributes were further identified at a genomic level on the basis of 16s rRNA and ITS region sequencing as *Bacillus subtilis* BS6, *B. subtilis* CS13, *Pseudomonas aeruginosa* Tr20, *Trichoderma lixii* TvR1, and *T. brevicompactum* TbS2, respectively. The sequences were submitted to NCBI for obtaining their accession numbers. The accession numbers obtained were *Bacillus subtilis* BS6 (MF780728), *B. subtilis* CS13 (MF678835), *Pseudomonas aeruginosa* Tr20 (MF797805), *Trichoderma lixii* TvR1 (MF780730) and *T. brevicompactum* TbS2 (MF780729).

The isolated strains were characterized for their ability to produce secondary metabolites, phytohormones, and solubilization of inorganic minerals involved in

biocontrol and plant growth promoting activity. Among antagonistic isolates, production of hydrolytic enzymes chitinase and cellulase; siderophores; phytohormones Indole acetic acid and solubilization of Zn was reported by *Trichoderma lixii* TvR1, *T. brevicompactum* TbS2, and *Pseudomonas aeruginosa* Tr20. The production of Hydrogen cyanide and solubilization of inorganic phosphate was displayed only by *P. aeruginosa* Tr20. All the three rhizobacterial antagonistic isolates found producing protease and the ammonia production was documented for *T. lixii* TvR1, *T. brevicompactum* TbS2, *B. subtilis* CS13, and *Pseudomonas aeruginosa* Tr20. The production of exopolysaccharides (EPS) and biofilm forming ability that participates in root colonization was assessed for antagonistic isolates. The *Trichoderma* isolate TvR1 and TbS2 and rhizobacterial isolates Tr20 were found positive for the production of EPS and biofilm formation. The volatile and non-volatile metabolites of antagonistic isolates were also found instrumental in their biocontrol activities.

The seed germination and seedling vigor index was promoted by all the antagonistic isolates except *T. brevicompactum* TbS2, over control. However, *T. brevicompactum* TbS2 displayed plant growth promoting (PGP) activities *i.e.,* ability to produce IAA, ammonia, siderophores, EPS, solubilization of Zn and formation of biofilm under *in vitro* condition. Similar results were reported in pot experiment where *T. brevicompactum* TbS2 reduced the incidence of fusarium wilt and early blight but does not produce a significant effect on plant growth promotion. The strain *T. brevicompactum* is documented to produce a phytotoxic metabolite that assists in biocontrol activity but hinders plant growth. The overall results of the *in vitro* study displayed that the two isolates *Trichoderma* TvR1 and *Pseudomonas* Tr20 were the most effective strains that possessed biocontrol as well as PGP activities.

Further, the pot experiment was undertaken to screen the biocontrol as well as PGP activity of isolated antagonistic isolates under *in vivo* conditions. The results obtained were in line with the result obtained under *in vitro* conditions. The maximum percentage of disease reduction and plant growth promotion was displayed by *T. lixii* TvR1 and *P. aeruginosa* Tr20. *T. lixii* TvR1 and *P. aeruginosa* Tr20 reduced the incidence of fusarium wilt and early blight by 31.58 and 26.32; and 30.50 and 27.87%, respectively, and increased plant height by 47.3 and 41.4; 74.0 and 69.1; and 50.9 and 49% over positive control; *Fol* infected control and *A. solani* infected control, respectively. The use of consortia is known to have broader activity spectra due to the synergistic effects of the compatible members. The isolates *Trichoderma lixii* TvR1 and *Pseudomonas aeruginosa* Tr20 were found compatible with each other and formulated in a consortium for effective management of fusarium wilt and early blight.

On the basis of the result obtained through screening experiment and compatibility between antagonistic isolates, *T. lixii* TvR1, *P. aeruginosa* Tr20 and their consortium were investigated for management of fusarium wilt and early blight and promotion of growth of tomato plants. During the investigation, all the three treatments were found effective in reducing disease incidences and promoting growth. However, the consortium of two increased the biocontrol and PGP activity of the individual microbial isolates. These results confirmed that the application of consortium of compatible isolates has synergistic effects that promote the efficacy of individual isolate. The consortium also improved the photosynthetic pigments of tomato plants over positive and negative control. The results confirmed the biocontrol attributes of isolated antagonistic strains and further examination of roots sections under SEM revealed the effective root colonization ability of *T. lixii* TvR1 and *P.*

aeruginosa Tr20 suggesting root colonization as one of the mechanisms for biocontrol and PGP.

The antagonistic isolate *Pseudomonas aeruginosa* Tr20 was reported as effective biocontrol and PGP strain, however, the *P. aeruginosa* is an opportunistic human pathogen. Therefore to minimize the risk associated with the use of living cells of *P. aeruginosa*, an attempt was made to replace it with its biosurfactant. The biosurfactant was extracted from *P. aeruginosa* Tr20 and characterized on the basis of some physiochemical parameters and FTIR analysis. Through FTIR studies, the biosurfactant was identified as Rhamnolipid. The biosurfactant obtained was found to be effective in reducing the growth of *Fol* and *A. solani* under *in vitro* condition and its effect was concentration dependent. The compatibility of biosurfactant with *T. lixii* TvR1 was evaluated to develop consortium and their activity was investigated in pot conditions. The biosurfactant was found to be compatible with *T. lixii* TvR1 @ concentration 200 mg/L, a further increase in the concentration of biosurfactant observed to limit the growth of the latter.

The pot experiment was undertaken to assess the effect of biosurfactant and its consortium with *T. lixii* TvR1 @ concentration 200 mg/L on biocontrol of pathogens and PGP. The analysis of result demonstrated that consortium of *T. lixii* TvR1 and biosurfactant @ concentration 200 mg/L enhanced the efficacy of *T. lixii* TvR1, however, the maximum disease control was reported by the inoculation of a consortium of *T. lixii* TvR1 + *P. aeruginosa* Tr20. The consortium *T. lixii* TvR1 + biosurfactant does not displaed significant effect on PGP and fruit yield whereas photosynthetic pigment, carbohydrate and protein content was increased significantly in leaves as compared to the individual treatment of *T. lixii* TvR1.

The inoculation of microbial agents and biosurfactant individually or in the consortium was reported to induce production of pathogenesis-related enzymes. The consortium of *T. lixii* TvR1 + *P. aeruginosa* Tr20 displayed the highest production of polyphenol oxidase (PPO) and peroxidase (PO) enzymes. The PPO production by consortium *T. lixii* TvR1 + biosurfactant was low in comparison to individual inoculation of *T. lixii* TvR1 and *P. aeruginosa* Tr20 in pathogen-challenged plants. The production of PO in plants treated with a consortium of *T. lixii* TvR1 + biosurfactant was significantly same as reported to a consortium of *T. lixii* TvR1 + *P. aeruginosa* Tr20. Investigation of the effect of biocontrol agents and biosurfactant on quality of tomato fruits revealed that microbial agents were effective in improving values for pH, lycopene, and β-carotene than biosurfactant with an exception, where biosurfactant alone found to increase β-carotene content in plants challenged with *Fol*. The treatment of biocontrol agents and biosurfactant did not displayed any effect on the titratable acidity and moisture content of the fruit.

From the findings of the study, it was concluded that the lack of knowledge among farmers in the study area was the major reason for the excessive use of agro-chemicals. Therefore there is a need for government and non-governmental organization to organize awareness camps for farmers to educate them about the toxicity and negative impacts of agrochemicals on their health as well as on the health of the consumers and environment and promote the use of sustainable approaches for agricultural activities. Further, the use of native rhizospheric microbial isolates was found as an efficient approach for biocontrol of fusarium wilt and early blight. With this, the study also confirmed that use of consortium in place of individual treatment is more effective in biocontrol of pathogens and plant growth promotion. The use of the microbial metabolites in place of living microbial cells can be another eco-friendly and safer option for sustainable agricultural practices.

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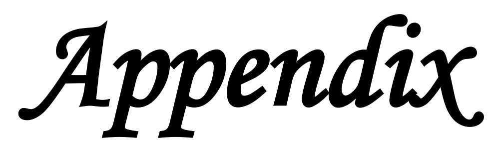

Questionnaire

Topic: Survey on use of Conventional Chemical Pesticides, Biopesticides and other disease management practices adopted by tomato growers

Personal Information:

Name: ………………………………

Gender: ……………………...........

Age: ………………………………..

Location: ……………………………

Education: …………………………..

Address: ……………………………

Contact no: ………………………….…

Signature & Date: ………………….

Specific Information:

1) Area of field ……………………………………………………….
2) Seeds
   (i) Name of variety (s)……………………………………
   (ii) Cost…………………………………………………..
   (iii) Amount of seeds used………………………………...
3) Season & Time of tomato cultivation………………………………
4) Disease Occurrence
   (i) YES/NO………………………………………………...
   (ii) Types of disease occur………………………………….
   (iii) Name of Diseases……………………………………
   (iv) Time of Occurrence…………………………………..
   (v) Other relevant information……………………………...
5) Use of Chemical Pesticides
   (i) YES/NO………………………………………………...
   (ii) Name of pesticides……………………………………
   (iii) Amount of pesticides used………………………………
   (iv) Total Cost……………………………………………….
6) Use of Biopesticides
   (i) YES/NO………………………………………………...
   (ii) Name of biopesticides…………………………………
   (iii) Amount of biopesticides used…………………………

(iv) Total Cost……………………………………………….

7) Use of other alternative methods for disease management

(i) YES/NO……………………………………………...

(ii) Please, specify………………………………………..

(v) Detail of Alternative methods…………………………..

(vi) Total Cost……………………………………………….

8) Use of Chemical Fertilizers

(i) YES/NO……………………………………………...

(ii) Name of fertilizers………………………………………

(iii) Amount of Fertilizers used…………………………...

(iv) Other Alternative methods…………………………...

(v) Total cost………………………………………………..

9) Total Productivity…………………………………………………

10) Problems Occur during tomato cultivation…………………………

……………………………………………………………………..

11) Total Cost

(i) Input…………………………………………………......

(ii) Output……………………………………………….......

12) Use of Safety Measures

(i) YES/NO……………………………………………...

(ii) Type of measure taken

(a) Gloves …………………………….

(b) Mask ………..................................

(c) Suit ………………………………..

(d) Other measures …………………...

THANK YOU

Publications

Research articles

Swati Sachdev and Rana Pratap Singh (2016). Studies on trends in use of pesticides and fertilizers for tomato cultivation in the vicinity of Lucknow, India. **International Journal of Science, Technology & Society (IJSTS).** 2(1&2): 49-54.

Swati Sachdev and Rana Pratap Singh (2018). Isolation, characterization and screening of native microbial isolates for biocontrol of fungal pathogens of tomato. **Climate Change and Environmental Sustainability (CCES).** 6(1):46-58.

Swati Sachdev and Rana Pratap Singh (2018). Optimization of culture conditions for mass production of *Trichoderma* using Response Surface Methodology and assessment of its effect on growth of Spinach (*Spinacia oleracea*). **3 Biotech**. 8:360. (Impact factor: 1.497).

Review articles

Swati Sachdev and Rana Pratap Singh (2016). Current Challenges, Constraints and Future Strategies for Development of Successful Market for Biopesticides. **Climate Change and Environmental Sustainability (CCES).** 4(2): 129-136.

Swati Sachdev and Rana Pratap Singh (2017). Sustainable management of soil borne pathogens of Tomato. **International Journal of Science, Technology & Society (IJSTS).** 3(2):36-40.

Swati Sachdev and Rana Pratap Singh (2018). Root colonization: Imperative mechanism for efficient plant protection and growth. **MOJ Ecology and Environmental Sciences.** 3(4): 240-242.

RESEARCH ARTICLE

International Journal of Science, Technology and Society
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Studies on trends in use of pesticides and fertilizers for tomato cultivation in the vicinity of Lucknow, India

Swati Sachdev and Rana Pratap Singh*

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Agrochemicals, biofertilizers, biopesticides, biotic stress, illiteracy, survey.

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ABSTRACT

The present work was undertaken to study the trends in use of chemical pesticides and fertilizers for tomato cultivation in vicinity of Lucknow city, India; to gather information regarding knowledge among farmers related to use of biological fertilizers and pesticides; and the constraints faced by them during tomato cultivation through questionnaire based survey. The study revealed the high consumption rate of agrochemicals suggesting farmer's sole dependency on them to protect tomato crop from pests and diseases, and to enhance productivity. The knowledge pertaining to biofertilizers and biopesticides among farmers was negligible. Most of the farmers were either illiterate or having low education level which was the major cause for indiscriminate use of agrochemicals. The area under tomato cultivation was observed to be reduced due to land fragmentation, urbanization, biotic and abiotic stresses. Biotic factors like occurrence of fungal and viral diseases, pest infestation and abiotic factors like scarcity of water, fluctuation in temperature conditions were major reasons for productivity loses.

1. INTRODUCTION

Food security is a prior concern for policy makers of the world. Climate change and gradually depleting non-renewable resources has ramified the problem of adequate food production. India, a developing country is also not unaffected with crisis of food insecurity. Uncertainty in climatic phenomenon, land fragmentation, resource degradation, poor market linkages, low monetary returns to farmers, poor access to technology and lack of knowledge has detrimentally impinged on agricultural production in many states of India. In Western India, yield of many rainfed crops have been reported to be reduced once in three years due to water stress (Raju and Chand, 2010; Kurothe et al., 2014).

India holds second position in tomato cultivation, next to potato among cultivated vegetable crops. Uttar Pradesh is one of the major Indian states that produce tomato. The area for tomato production and its productivity in 2010-2011 was recorded 6690 hectare and 248633 tonnes respectively in U.P. and in Lucknow, 807 hectares and 31159 tonnes respectively, by State Horticulture Mission, Government of Uttar Pradesh (Vanitha et al., 2013). The productivity of tomato is highly influenced by pests and diseases infestation and is also sensitive to environmental stress including very high or low temperature, dry or water logging conditions.

Heavy inputs of agrochemical by farmers in India is a common practice, however, excessive use of agrochemicals has raised questions in terms of sustainability of soil to support plant growth in future. Increasing demand of water for irrigation has raised another question to sustainability of agriculture when water has been a resource for conservation. Negative effects of agrochemicals and scarcity of water with continuously changing environmental conditions has urged to shift toward organic farming practices. But before shifting toward sustainable approach there is a need to understand the problems being faced by farmers, approaches being used by them and their level of knowledge. Keeping in view these aforesaid facts, present study was undertaken on a small scale to analyze the scenario of tomato cultivation with specific objectives: 1) Assessment of agrochemicals loads on tomato fields, 2) Evaluation of factors affecting productivity of tomato and 3) knowledge pertaining among farmers regarding benefits

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DOI: 10.5958/2320-642X.2018.00006.6

RESEARCH ARTICLE

Isolation, Characterisation and Screening of Native Microbial Isolates for Biocontrol of Fungal Pathogens of Tomato

Swati Sachdev[1] • **Rana Pratap Singh**[2]*

Abstract The native strains of *Trichoderma* and Rhizobacteria were isolated from rhizosphere of seven different crops from agroecosystem of peri-urban area of Lucknow, Uttar Pradesh, (India). The five isolates *Trichoderma lixii* TvR1, *T. brevicompactum* TbS2, *Bacillus subtilis* BS6, *B. subtilis* CS13 and *Pseudomonas aeruginosa* Tr20 displayed positive antagonistic potential against *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) and *Alternaria solani* under *in vitro* conditions. The biocontrol agents (BCAs) were found to produce cell wall degrading enzymes (CWDEs) including, chitinase, protease and cellulase. *T. lixii* TvR1 and *P. aeruginosa* Tr20 were found to be the most effective strains that showed significant inhibitory effect on pathogens. Dual culture, volatile metabolite and culture filtrate assay revealed percentage radial inhibition of *Fol* (55.31%, 54.09%; 22.24%, 10.06% and 67.85%, 51.38%) and *A. solani* (66.54%, 61.54%; 20.76%, 27.6% and 41.74%, 38.52%) by *T. lixii* TvR1 and *P. aeruginosa* Tr20, respectively. Antagonistic assays revealed probable involvement of mechanism of mycoparasitism and production of secondary metabolites such as antibiotics by both biocontrol agents. In addition to biocontrol activity, the BCAs displayed plant growth promoting (PGP) activities such as production of indole acetic acid (IAA), siderophores and ammonia and phosphate solubilisation.

Keywords: *Trichoderma*, Rhizobacteria, Cell wall degrading enzymes, Antibiotics, Mycoparasitism, Plant growth promoting activity

1. Introduction

Tomato (*Lycopersicon esculentum* Mill.) is a commercially important and second most cultivated vegetable crop in the world. Global production of Tomato in 2014 was recorded as 170.8 million tonnes and Indian contribution to the world's production was approximately 19 million tonnes. India holds second position in tomato cultivation and Uttar Pradesh (U.P.) is one of the important tomato producing state of India (Sachdev and Singh, 2016a). The area under tomato production and its productivity in 2010–2011 were recorded 6690 hectares and 248,633 tonnes, respectively in U.P. and in Lucknow was recorded 807 hectares and 31,159 tonnes, respectively, by State Horticulture Mission, Government of Uttar Pradesh (Sachdev and Singh, 2016a).

Fusarium oxysporum f. sp. *lycopersici* (*Fol*) and *Alternaria solani* are major destructive fungal pathogens of tomato causing *Fusarium* wilt and early blight, respectively (Gowtham *et al.,* 2016; Babu *et al.,* 2015). The diseases caused by these pathogens show significant impact on tomato production and present challenges for tomato growers worldwide (Patel and Saraf, 2017). *Fusarium* wilt causes extensive productivity loss in both field as well as in greenhouse condition (McGovern, 2015). *A. solani* affects foliar parts of plant ensuing severe defoliation and reduced productivity with poor fruit quality (Babu *et al.,* 2015). In India upto 45% and 80% of yield loss has been reported due to incidence of *Fusarium* wilt and early blight, respectively (Latha *et al.,* 2009; Ramyabharathi *et al.,* 2012). Global warming is another threat that could negatively influence the agricultural productivity. Change in temperature could favour the development of dormant pathogenic species that can become endemic and can shift the geographical

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3 Biotech (2018) 8:360
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ORIGINAL ARTICLE

Optimization of culture conditions for mass production and bio-formulation of *Trichoderma* using response surface methodology

Swati Sachdev[1] · **Anupriya Singh**[1] · **Rana Pratap Singh**[1]

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Abstract

Use of agro-waste for production of value added products is a good alternative for developing low-cost carriers for formulation of *Trichoderma*-based bio-products. It provides avenues for safe utilization of wastes, while reducing cost and environment pollution load of waste disposal. The present study was undertaken to find suitable agro-waste for economical and higher mass production of *Trichoderma lixii* TvR1 under solid-state fermentation, optimizing culture conditions using mathematical model and assessing effect of formulated bio-product on growth of Spinach (*Spinacia oleracea*). Among various agro-wastes screened, sugarcane bagasse was observed to support maximum growth (20.08×10^7 spores/g) of *T. lixii* TvR1 which was significantly ($p \leq 0.05$) higher than the others. The Response Surface Methodology (RSM) was used to optimize culture conditions using optimal point prediction analysis which predicted that maximum spore production of *T. lixii* TvR1 (19.1245×10^7 spores/g) will be obtained at 30 °C and 68.87% of moisture content after 31 days of incubation. Amendment of formulated bio-product of *T. lixii* TvR1 in soil at concentration 15% w/w promoted biomass, photosynthetic pigments, and protein content of spinach (significant at $p \leq 0.05$). After 6 weeks of sowing the shoot length, root length, and photosynthetic pigments of plants irrigated daily and on alternate days were reported to be increased by 66.97, 185.03, and 82.80%; and 56.56, 71.36, and 74.64%, respectively; over the no amendment.

Keywords Agro-waste · Sub-optimal irrigation · *Trichoderma* · Optimal point prediction analysis · Photosynthetic pigments

Introduction

Trichoderma is outstanding and versatile genus of fungi that has agricultural as well as industrial importance. It possess ability to promote plant growth and productivity, manage pests and pathogens, alleviate abiotic stresses, biodegrade xenobiotic compounds and produce industrially important metabolites (Mastouri et al. 2010; Blaszczyk et al. 2014; Keswani et al. 2014; Hyder et al. 2017). Earlier species of *Trichoderma* were generally considered as biocontrol agents that promote plant growth indirectly by inhibiting growth of pathogens, however recently many studies supported their direct role in plant growth. Certain beneficial strains of *Trichoderma* release several proteinaceous and auxin-like compounds that modify phytohormones leading to plant growth and development on interaction with plants (Garnica-Vergara et al. 2015; Contreras-Cornejo et al. 2016). The vibrant traits of *Trichoderma*, craft them as a potential candidate for commercial use in agriculture and industries worldwide. For extensive application of *Trichoderma* based products, large scale production of micropropagules is required. The developmental cost and technological challenges are major hindrance for development of successful product (Singh and Nautiyal 2012; Sachdev and Singh 2016). The cost effective large scale production can be achieved through solid state fermentation. Solid state fermentation (SSF) is a cost effective process, widely used for the mass production of filamentous fungi, their enzymes and/or other metabolites on solid substrates with sufficient moisture but not in free-state (Cavalcante et al. 2008). The raw material used as substrate for biomass production accounts 35–40% of production cost (Eltem et al. 2017). Therefore, utilization of

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REVIEW ARTICLE

Current Challenges, Constraints and Future Strategies for Development of Successful Market for Biopesticides

Swati Sachdev[1] • Rana Pratap Singh[2]*

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Abstract Biopesticides are revolutionary crop protectants that protect crops from vast array of pests and pathogens in eco-friendly manner. They show plethora of benefits over synthetic pesticides, such as target specificity, less toxicity and biodegradability; however, they lack position in crop protection market and represent only 3.5% of global pesticides market (Olson *et al.*, 2013). Though biopesticides lag behind in commercial pesticide market, they have great deals to offer for development of sustainable agriculture. The successful market for biopesticides can be achieved through understanding the major challenges and constraints faced by them and developing new strategies such as optimising delivery system; screening new and better strains; educating farmers and others to cope up with encountering hurdles.

Keywords Crop protectant, Target specificity, Optimising delivery system, Educating farmers

1. Introduction

Food and fibre are two most crucial products of agriculture. Their demand is increasing gradually with gradual increase in world population. It has been estimated that world population will rise to 10.12 billion by the year 2100 (Nawaz *et al.*, 2016). The agricultural sector has been engulfed with various constraints such as continuous climate change, low soil fertility, increased incidence and intensity of pest and disease occurrence. These constraints have reduced productivity and resulted in shortage of food and fibre in many parts of the world, posing question mark on availability of basic needs of living for future generation. To address these problems, modern and advanced strategies must be adopted that not only meet the demand of present and future generation but restore the productivity potential of agricultural sector.

Incidence of pest and pathogen occurrence is a natural phenomenon and often passed unnoticed. However, they become a matter of consideration when their spectra increase resulting in severe losses. Use of chemical pesticides to reduce impact of such severe damages has become a part of routine agricultural practices. Undoubtedly, application of chemical pesticides has posed risk to sustainability and health of ecosystem as well as humans. Therefore, to rescue agriculture from debt of disease occurrence and pest infestation, incorporation of biopesticides in pest management (PM) programme has been cited as sustainable strategy.

Biopesticides are living origin products that are derived from plants, animals and microorganisms such as bacteria, fungi, protozoa, algae and viruses. Depending on their origin, Environmental Protection Agency (EPA) has classified biopesticides into three categories: (1) microbial biopesticides that include living cells of fungi, bacteria, viruses and others; (2) biochemical biopesticides based on byproducts or extracts of plants and other living organisms and (3) plant incorporated protectants include transgenic plants incorporated with genetic material for compound that helps to express protection against pests (EPA, 2011). Further, biopesticides can also be identified as biofungicides, bionematicides, bioherbicides and bioinsecticides on the basis of target species. Most commonly registered biopesticides are based on *Trichoderma* spp., *Bacillus thuringiensis*, neem-based products, nucleopolyhedrovirus, *Trichogramma*, *Pseudomonas fluorescens*, *Bacillus subtilis* and *Beauveria bassiana*.

2. Global Scenario of Biopesticides Market

The biopesticides market holds a very small share in crop protection market of world. In 2009, global biopesticides market was approximately 3.5% ($1.6 billion) of total pesticide market of world (BCC Research, 2010)

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REVIEW ARTICLE

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Sustainable management of soil borne pathogens of tomato

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ABSTRACT

Tomato is the second most cultivated vegetable crop in world, which not only used in culinary but also has industrial and medicinal value. Tomato plants are susceptible to various soil borne diseases that severely affect its growth and fruit quality either grown in fields or cultivated in greenhouse under controlled conditions. The use of rhizospheric microorganisms as a biocontrol agent (BCA) against phytopathogens is considered as one of the most promising eco-friendly approach for sustainable management for tomato cultivation. The rhizopsheric microorganisms caters several mechanisms to control or suppress soil borne phytopathogenic fungi, bacteria and nematodes, via direct mechanism of mycoparasitism and/or indirect mechanisms such as antibiosis through production of secondary metabolites or antibiotics; and competition for nutrients and ecological niche. The present work outlines role of rhizospheric microbial isolates in management of phytopathogens affecting quantitative and qualitative production of tomato.

INTRODUCTION

Tomato (*Lycopersicum esculentum* L.) is commercially important and the second most cultivated vegetable crop in the world belonging to the Solanaceae family that encompasses several other important crops *i.e.,* potato, eggplant and pepper (Piquerez *et al.* 2014). Tomato is native species to Mexico, but cultivated and consumed all around the world (Arah *et al.* 2015). It's a warm season crop that requires optimum range of temperature *i.e.,* 21-24°C, however temperature ranging between 10°C to 30°C is suitable for the growth. Tomato plants require well drained soil with preferred pH range 5.5-6.8. Wet conditions increase incidence of diseases and affect fruit ripening.

Commercial and medicinal value of Tomato

It is one of the most versatile, economically important vegetable that not only found space in kitchens but also tops the list of industrial crop because of its outstanding processing qualities (Christopher *et al.* 2010). Tomato has beneficial effects on human health due to presence of antioxidants and essential nutrients such as lycopene, β-carotene, flavonoids, vitamins and essential minerals, and therefore considered as one of the most important "protective foods" (Babu *et al.* 2015; Arah *et al.* 2015). Several studies have demonstrated that consumption of tomato has strong inverse correlation with the risk of certain types of cancer, cardiovascular diseases and age-related macular degeneration (Babu *et al.* 2015; Arah *et al.* 2015).

Factors affecting tomato productivity

Plants are constantly encountered with various biotic stresses that affect crop productivity worldwide. Biotic stresses are the major challenges that affect the tomato productivity worldwide. Tomato plants are susceptible to wide range of pests and pathogens including bacteria, fungi, oomycetes, nematodes, viruses and insects and this is the reason, tomato is considered as an excellent model for research and studying plant-pathogen interaction and helps to establish an effective control against these diseases to enhance productivity worldwide (Piquerez *et al.* 2014; Arie *et al.* 2007; Takahashi *et al.* 2005). Tomato provides a good example of how the use of biocontrol agents can be introduced into practice as an IPM strategy (Minuto *et al.* 2006).

The tomato diseases caused by soil-borne pathogens are widely distributed worldwide that include *Fusarium oxysporum f. sp. lycopersici*, *Verticillium* spp., *Ralstonia solanacearum* causing vascular wilt in tomato, *Pythium aphanidermatum and Rhizoctonia solani causing* Damping off, *Fusarium oxysporum f. sp. radicis-lycopersici* (fusarium crown rot), *Pyrenochaeta lycopersici* (corky root rot), *Colletotrichum coccodes* (Black dot root rot), *Clavibacter*

Mini Review

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Root colonization: imperative mechanism for efficient plant protection and growth

Abstract

Agriculture is important for food security, and is vulnerable to biotic and abiotic factors are responsible for lower agricultural services worldwide. To combat problems of pests and diseases occurrence and low soil productivity, farmers use conventional agrochemicals. Continuous and injudicious use of agrochemicals has detrimentally affected the soil, flora, fauna, wildlife, water bodies, air and human health. To overcome the challenges posed by ecological stresses sustainable approaches are required. Application of Agriculturally important microorganisms (AIMs) is considered as most eco–friendly approach. AIMs are naturally occurring beneficial microorganisms that sustainably protect plants from threats of pests and pesticides; promote plant growth and alleviate abiotic stresses. AIMs that reside in and around plant roots are termed as Rhizospheric microorganisms. Many rhizospheric microbes are known to support plant growth promotion and biocontrol in various economically important crops using plethora of mechanisms. Root colonization is considered as an important strategy of rhizospheric microbes for biocontrol and plant growth promotion. This review reveals the emerging insight on a process of root colo most crucial and prime step for biocontrol of plant diseases in addition to plant growth promotion; amelioration of abiotic stress and bioremediation nization displayed by rhizospheric microorganisms.

Keywords: agriculturally important microorganisms, agrochemicals, biocontrol, ecological stress, plant growth promotion

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Introduction

Agriculture is an important sector that determines the health and food security of a country during all the odds. Plants have suffered with productivity and yield losses and threats by several biotic and abiotic ecological factors, e.g. plant diseases, pest manifestation, flood, drought, heat waves, etc. These losses are gradually increasing due to rapid change in climatic odds and disasters due to high built up of greenhouse gases of anthropogenic origin mainly due to industrialization, and urbanization, etc. To produce safe food to feed increasing population, the agricultural practices have been entirely dependent on conventional means such as use of agrochemicals. Overuse of agrochemicals coupled with uncertainty in climatic behavior, resource depletion, land fragmentation and degradation, lack of awareness among farmers, increased incidence of abiotic stress and accelerated rate of pests and disease occurrence has challenged agricultural sustainability very seriously in the recent past.[1–4] Sustainable agriculture is an agricultural practice that maintains world's food security to feed growing population without disrupting ecological integrity. A paradigmatic shift is necessary to attain agricultural sustainability and providing food security to growing global population of 9–10 million by 2050 by doubling food production (70–100%).[5,6] Adoption of integrated pest and nutrient management methods, agroforestry, integrated waste management for livestock production, use of agriculturally important microorganisms (AIMs) present avenues for sustainable agriculture and reduces negative environmental externalities.[5,7]

Rhizosphere: a site for plant–microbe interaction

The soil has most influential effect on crop performance, preferably in "Rhizosphere", the zone surrounding plant roots, which is a tempting denizen for soil borne microorganisms that in order to sustain themselves display competition as well as co–operation with other existing living entities. Agriculturally important microorganisms (AIMs) show plant–microbe relationship and possess ability to alleviate ecological stresses. Many AIMs are rhizospheric inhabitants, endowed with some beneficial traits termed as Rhizospheric microorganisms. Beneficial rhizospheric microorganisms are saints in soil that nullifies the negative effects of other deleterious microorganisms and persisting hostile abiotic conditions, impeding on growth and productivity of plants by recruiting themselves in service of plants residing under same niche with their inherent multifarious valuable traits. Harnessing potential of such agriculturally important rhizospheric microorganisms can pave a way for sustainable agriculture. The plant growth promoting Rhizobacteria (PGPR) and plant growth promoting fungi (PGPF) are some AIMs that are generally found in rhizosphere (vicinity of roots) or associated with plant roots showing endophytic relationship.

Root colonization: most crucial mechanism for consistent beneficial performance

Root colonization by rhizospheric microorganisms is prior among all mechanisms that determine their efficiency to increase plant growth, biocontrol potential and ability to induce tolerance against abiotic stresses.[8,9]

The root colonization by microorganisms involves series of steps:

I. Recognition;

II. Adherence to the surface of root and penetration in case of endophytes;

III. Colonization in or around the roots by growth proliferation.[10] Plants initiate communication with microbes by releasing root exudates in rhizosphere.

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http://14.139.13.47:8080/jspui/handle/10603/203182
http://14.139.13.47:8080/jspui/handle/10603/199206
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Instances where selected sources appear:

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