Influence of endophytic bacteria on growth and tuber production in potato

Submitted in partial fulfilment of the requirements for the award of the Degree of

Master of Science in Biotechnology

SUBMITTED BY:

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UNDER THE SUPERVISION OF

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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in the dissertation entitled **" Influence of endophytic bacteria on growth and tuber production in potato"** in partial fulfillment of the requirement for the award of the degree of Masters in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is an authentic record of my own work during the period of six months from January 2011 to June 2011, under the supervision of Dr. M.S. Reddy, Professor & Dr. Anil Kumar, Assistant Professor Department of Biotechnology & Environmental Sciences, Thapar University, Patiala. The report has not been submitted for the award of any other degree or certificate in this or any other university.

Date:

Place: Patiala Alpi Bansal

CERTIFICATE

This is to certify that the thesis entitled "**Influence of endophytic bacteria on growth and tuber production in potato**" submitted by Ms. Alpi Bansal in partial fulfillment of the requirements for the award of Degree of Masters of Science in Biotechnology to Thapar University, Patiala, is a record of student's own work carried out by her under my supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other University or Institute.

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ABSTRACT:

Potato cultivar FC-3 cultures were showing the constant bacterial contamination which seems to be leaking from the internal tissues during multiplication cycles. Furthermore the cultures of cultivar FC-3 were underperforming in comparison to the other cultivars such as FC-1 and FC-4. In the present study, the effect of bacteria isolated from the the potato cultivar FC-3 was tested on the growth of potato cultivars. Bacteria from the FC-3 cultivar was isolated, characterized and inoculated into the three potato cultivars; FC-3, FC-4 and ATL. Both *in vitro* and *ex vitro* studies were carried out. The known growth promoting endophytes *Pseudomonas corrugata* (PC) and *Bacillus subtilis* (BS) were used as reference organisms to study the effect of inoculations with isolated endophytic bacteria from cultivar FC-3. FC-3 bacteria (FC-3 B) was found to be gram positive, cocci shaped, showed IAA production and it showed 98% similarity with *Paenibacillus* sp. In all the three cultivars, FC-3 bacteria was found to inhibit the plant growth in terms of height of plants, survival rate, weight of tubers, biomass increment as compared to plants in which no treatment was given. Whereas, P C and B S were able to show their growth promoting activity in all cultivars, but their activity was less observed in FC-3 cultivar as compared to FC-4 and ATL, due to the reason FC-3 cultivar found to inhabit FC-3 B, but P C and B S inoculated FC-3 plants showed better growth as compared to FC-3 B inoculated FC-3 plants.

ABBREVIATIONS

1.	cm	Centimetre
2.	rpm	Revolution per minute
3.	g	Gram
4.	mg	Milligram
5.	µg	Microgram
6.	L	Litre
7.	ml	Millilitre
8.	µl	Microlitre
9.	%	Percentage
10.	nm	Nanometre
11.	^{o}C	Degree centigrade
12.	M	Molar
13.	cfu	Colony formin units
14.	v/v	Volume by volume
15.	ppm	Parts per million
16.	IAA	Indole acetic acid
17.	PCR	Polymerase chain reaction

Chapter 1

INTRODUCTION

From its origins in the Andes, the cultivated potato has spread to most parts of the world in the last 400 years. It's now a major source of nutrition for people from a wide variety of cultural and ethnic backgrounds. In the developing countries of Asia and Africa, the rate of increase in potato production has been higher than that of most other crops and presently potato ranks fourth in terms of total global food production.

Potato belongs to the genus *Solanum* (family *Solanaceae*) that consists of about 2,000 species. The word potato may refer to the plant itself as well as the edible tuber. The potato tuber is a modified stem developed underground on a specialized plant part (subterranean stem) known as stolon. Potato tubers are usually oval to round in shape, although intermediate shapes are also frequently encountered. Tuber consists of an inner flesh color and an outer protective cover known as skin. There is a great variation in flesh color and skin finish. The eye shaped depressions on a potato tuber is known as its eyes and actually these are the dormant shoot buds, which give rise to new shoots under suitable conditions.

Tissue culture is a technique in which small pieces of living tissue (explants) are isolated from an explant and grown aseptically for indefinite period on a nutrient medium. For successful plant tissue culture it is best to start with an explant rich in undetermined cells i.e. those from cortex or meristem, because such cells are capable of rapid proliferation. The usual explants are buds, shoot tips, root tips, nodal segments or germinating seeds.

Under natural conditions, plants are constantly involved in interactions with a wide range of microorganisms including bacteria. Bacterial-host interactions can be harmful for the host (pathogenic interaction) or useful (symbiotic interaction). To have an optimal interaction, bacteria have to fine-tune itself with the biotic and abiotic environmental conditions of the host organism. This can be achieved via expression or repression of essential traits, such as host detection, motility toward the host, colonization, resistance to host defenses, growth and reproduction. The ability to quickly adapt to host environments is therefore critical for bacteria to have successful association with host.

These plant-associated bacteria colonize the rhizosphere (rhizobacteria), the phyllosphere (epiphytes) and inside of plant tissues (endophytes).

Endophyte (Gr. *endon*, within; *phyton*, plant) – the term was first coined by de Bary (de Bary, 1866) and has become deeply embedded in the literature. At present, endophytic organisms are defined as 'microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects' (Stone *et al*., 2000). Plant priming with non-pathogenic bacteria induces a faster defense reaction of the plant towards bacterial, fungal or viral infections and environmental stresses (Van Loon, 2007; Yang *et al*., 2009). A microorganism is considered as an endophyte based on its recovery from surface-disinfected plant material or extraction from within the plant and the essential feature that distinguishes endophytes from pathogens is the absence of any harmful effect on the host (Hallmann, 1997; Bacon *et al*. 2002). Typically, these are bacterial or fungal and may be of three types:

(1) Pathogens of another host that are nonpathogenic in their endophytic relationship;
(2) Nonpathogenic microbes;
(3) Pathogens that have been rendered nonpathogenic but still capable of colonizing host.

Endophytes are sheltered from environmental stresses and microbial competition by the host plant and they seem to be ubiquitous in plant tissues, having been isolated from flowers, fruits, leaves, stems, roots and seeds of various plant species (Kobayashi and Palumbo, 2000). Although bacterial endophytes are assumed to be derived from the rhizosphere of individual plants, the mechanisms are unknown. Possible processes are:

- Passive diffusion, wherein the most predominant members of the adjacent soil ecosystem will be seen as colonists of plants or
- Active selection, by which only particular bacterial species will successfully inhabit the plant interior.

Endophytic bacteria can actively or latently colonise plants locally or systematically. Endophytes are able to live inside plants without causing disease symptoms (Tervet and Hollis, 1948). Endophytic bacteria, co-evolved with plants, have been found in virtually every plant studied, where they colonize the internal tissues of their host plant and can form a range of different relationships including symbiotic, mutualistic, commensalistic and trophobiotic. Endophytic bacteria have been shown to promote growth in economically-important crops like potatoes (Conn *et al*., 1997; Frommel *et al*., 1991; Nowak, 1998; Sturz *et al*., 1998), tomatoes (Pillay and Nowak, 1997) and rice (Hurek *et al*., 1994).

Potato cultivar FC-3 cultures were showing the constant bacterial contamination which seems to be leaking from the internal tissues during multiplication cycles. These bacteria were slow growing on MS medium. Furthermore the cultures of cultivar FC-3 were underperforming in comparison to the other cultivars such as FC-1 and FC-4. In the present study, the effect of bacteria isolated from the potato cultivar FC-3 was tested on the growth of potato cultivars. Therefore, the present study was aimed at following objectives:

- Isolation of endophytic bacteria from microshoots of potato cultivar FC-3
- Physiological, biochemical and molecular characterization of isolated endophytic bacteria
- Influence of endophytic bacteria on growth and tuber production in potato

The known growth promoting endophytic bacteria namely *Pseudomonas corrugata* and *Bacillus subtilis* were used as reference organisms to study the effect of inoculations with isolated endophytic bacteria from cultivar FC-3.

Chapter 2

REVIEW OF LITERATURE

Endophytic bacteria, live in plant tissues without doing substantive harm to the plant or gaining any benefit other than residency (Kado, 1992). As cited extensively by Kobayashi and Palumbo (2000), endophytic bacteria exists in a variety of tissue types within numerous plant species, suggesting ubiquitous existence in most if not all higher plant species. Of nearly 300,000 plant species inhabiting, each individual plant is a host to several to hundreds of endophytes (Tan & Zou, 2001), creating an enormous biodiversity: a myriad of undescribed species, a rich source of novel natural products thereform. Mundt and Hinkle (1976) isolated bacteria from seeds and ovules of 27 different plant species. Moreover, endophytic bacteria have been isolated from both monocotyledonous and dicotyledonous plants ranging from woody tree species such as oak (Brooks *et al*., 1994) and pear (Whitesides and Spotts, 1991) to herbaceous crop plants such as sugar beets (Jacobs *et al*., 1985) and maize (Fischer *et al*., 1992; Lalande *et al*., 1989; Mclnroy and kloepper, 1995).

Endophytic bacteria have been isolated and identified from surface-disinfected tissues of non-leguminous crops. *Gluconacetobacter diazotrophicus* and *Herbaspirillum* spp. are reported endophytes of sugarcane, Cameroon grass, sweet potato, coffee, tea, banana and pineapple (Baldani *et al*., 1997; Cavalcante and Dobereiner, 1988). *Azoarcus* spp. have been isolated from Kallar grass (Hurek and Hurek, 1994) while *Burkholderia* spp., *Klebsiella* spp. and other organisms belonging to 36 genera are identified endophytes of cotton and sweet corn (Mclnroy and Kloepper, 1995). In Japan, Elbeltagy *et al*., (2001) also report the endophytic colonization and in planta N_2 fixation of *Herbaspirillum* spp. in wild rice. Abundant and diverse populations of bacterial endophytes were identified in potato (Garbeva *et al*., 2001; Sturz *et al*., 1999), maize (Fisher *et al*., 1992; Mclnroy and Kloepper, 1995), rice (Stoltzfus *et al*., 1998), cotton (Mclnroy and Kloepper, 1995) and cucumber (Mahafee and Kloepper, 1997). Bacterial endophytes have been observed in the tissues of woody plants, including gymnosperm and angiosperm species (Chanway, 1998).

Endophytes have been found associated with numerous plant species, most of these being members of common soil bacterial genera such as *Pseudomonas*, *Bacillus* and *Azospirillium*

(Chanway, 1996). Different bacterial species viz. *Alcali genes* spp. and *Kluyvera* spp. (Assis *et al*., 1998), *Pseudomonas fluorescens, P. alcaligenes, P. putida*, *Flavobacterium* spp., *Bacillus megaterium, B. pumilus* (Benhamou *et al*., 1998) and *Microbacterium* spp., *Clavibacter michiganensis*, *Curtobacterium* spp. and *B. subtilis* (Zinniel *et al*., 2002) have been reported as endophytes and were inhibitory to plant pathogens.

Endophytes enter plant tissue primarily through the root zone; however, aerial portions of plants, such as flowers, stems and cotyledons, may also be used for entry (Kobayashi and Palumbo, 2000). Specifically, the bacteria enter tissues via germinating radicles (Gagne *et al*., 1987), secondary roots (Aggarwal and Shende, 1987), stomates (Roos and Hatting, 1983) or as a result of foliar injury (Leben *et al*., 1968). Endophytes inside a plant may either become localized at the point of entry or spread throughout the plant (Hallmann *et al*., 1997). These microorganisms can reside within cells (Jacobs *et al*., 1985), in the intercellular spaces (Patriquin *et al*., 1978) or in the vascular system (Bell *et al*., 1995). Because of their systemic distribution throughout the plant via metabolic translocation, it has been postulated that plants can be defended from pathogens by manipulation of these naturally occurring microorganisms (Misaghi and Donndelinger, 1990). For example, endophytic bacteria can be genetically engineered for systemic delivery of antibiotics and biopesticides within the host plant tissues without genetic manipulation of the host genome. One of the important targets of such delivery system could be the maize stalk borer, *Chilopartellus*.

Significant variations in the populations of both indigenous and introduced endophytes have been reported. These variations are attributed to plant source, plant age, tissue type and environment. Generally, bacterial populations are larger in roots and decrease in the stems and leave (Lamb *et al*., 1996). Endophytic bacteria are good candidates for biofertilizers and biocontrol agents, as they are better protected from environmental stress as compared to rhizospheric bacteria and unlike rhizospheric bacteria, these can be transferred between plant generations (Rosenblueth and Martínez-Romero, 2006).

Eighty-seven culturable endophytic bacterial isolates belonging to 19 genera were obtained from coffee plants (Vega *et al*., 2005). Endophytes are a specific group of microorganisms (bacterial and fungal) that can be found residing within a wide variety of plant tissue types, including seeds and ovules (Mundt and Hinkle, 1976), fruit (Samish *et al*., 1961), stems (Misaghi and Donndelinger, 1990), roots (Germida *et al*., 1998; Jacobs *et al*., 1985) and

tubers (Hollis, 1951; Sturz *et al*., 1998). Since 1940, there have been numerous reports on indigenous endophytic bacteria inhabiting range of healthy tissues of plants such as tubers of potato (Garbeva *et al*., 2001; Sturz *et al*., 1999), fruits of tomato (Nejad and Johnson, 2000) and cucumber (Samish *et al*.,1961), roots of wheat (Coombs and Franco , 2003; Siciliano *et al*., 1998), oilseed rape (Germida *et al*., 1998) and legumes (Gagne *et al*., 1987), stems of Bermuda grass (Kostka *et al*., 1988), sweet corn and cotton (McInroy and Kloepper ,1995) and leaves of citrus plants (Araujo *et al*., 2001).

Classical studies on the diversity of bacterial endophytes have focused on characterization of isolates obtained from internal tissues following disinfection of plant surfaces with sodium hypochlorite or similar agents (Miche and Balandreau, 2001). Specific mechanisms of endophyte recognition by the plant, as well as reaction of the plant to colonization by endophytic bacteria may exist. These mechanisms and reactions could resemble the induced systemic resistance (ISR) promoted by rhizobacteria that is dependent on the plant growth regulators such as jasmonic acid (JA) and ethylene (ET) (Van Loon, 2007; Van Wees *et al*., 2008). However, involvement of direct antagonism and local plant reactions are possible in the case of endophytic bacteria, because they may colonize the entire plant internally (Podolich *et al*., 2009). Bacterial endophytes confront the plant defense system which they must overcome to colonize the host. Production of reactive oxygen species (ROS) is one of the primary events in the development of plant defense reaction while expression of defense genes is observed later (Iriti and Faoro, 2007). Therefore, the activity of enzymes responsible for ROS production and protection of the cells against the oxidative damage may change upon plant priming by endophytic bacteria. Such enzymes are superoxide dismutase (SOD) that produces hydrogen peroxide, which is reactive and a signal molecule of the plant defense reaction cascade and catalase that together with peroxidases neutralizes hydrogen peroxide. Both guaiacolperoxidase, which refers to unspecific peroxidases and produces phenolic compounds toxic for pathogens, and ascorbate peroxidase, which belongs to the ascorbate–glutathione cycle, act in protection of the plant cells against oxidative damage (Gechev *et al*., 2006).

Endophytic bacteria can not only promote plant growth and act as biocontrol agents, but also produce natural products to control plant diseases (Miller *et al*., 1998; Beck *et al*., 2003; Strobel *et al*., 2004; Guan *et al*., 2005). Bacterial endophytes can mediate *de novo* synthesis of novel antimicrobe compounds and antifungal metabolites, which has been accepted as a

potential fungicide to restrict the spread of plant disease (Sturz *et al*., 2000; Wellington and Maecella, 2004). Several strains are capable of inducing, both biotic (Chen *et al*., 1995; Liu *et al*., 1995; Sharma and Nowak, 1998) and abiotic stress resistance (Bensalim *et al*., 1998; Creus *et al*., 1998; Nowak, 1998) in inoculated plants.

Generally, researchers reported that endophytes promote plant growth by a number of similar mechanisms like soil plant growth promoting bacteria (PGPB), including phosphate solubilization activity (Verma *et al*., 2001; Wakeli *et al*., 2004), indole acetic acid (IAA) production (Lee *et al*., 2004) and production of siderophore (Costa and Loper, 1994). Endophytic organisms can also supply essential vitamins to plants (Pirttila¨*et al*., 2004). Moreover, a numberof other beneficial effects on plant growth have been attributed to osmotic adjustment, stomatal regulation, modification of root morphology, enhanced uptake of minerals and alteration of nitrogen accumulation and metabolism (Compant *et al*., 2005). In metal contaminated soil, plant associated bacteria, both rhizobacteria and endophyte, may play an important role in plant growth and metal accumulation (Rajkumar *et al*., 2009). Certain bacteria like *Pseudomonas* survive under stress conditions due to the production of exopolysaccharides (EPS), which protects microorganisms from hydric stress and fluctuations in water potential by enhancing water retention and regulating the diffusion of carbon sources in microbial environment (Sandhya *et al*., 2009). Exopolysaccharides possess unique water holding and cementing properties, thus play a vital role in the formation and stabilization of soil aggregates and regulation of nutrients and water flow across plant roots through biofilm formation (Roberson and Firestone, 1992; Tisdall and Oadea, 1982).

Endophyte communities have also been shown to ameliorate disease development (Benhamou *et al*., 1996; Sturz and Matheson, 1996) and in some instances, plant– endophyte relationships have been found to be tissue type and tissue site specific. For example, Sturz *et al*., (1999) found that anti-phytopathogenic activity of endophytes recovered from potato tuber peels were highest in the outermost layers of the peel and decreased progressively towards the centre of the tuber.

Investigations of biodiversity of endophyte strains for novel metabolites may identify new drugs for the treatment of human, plant and animal diseases (Strobel *et al*., 2004). Several bacterial endophytes have been shown to support plant growth and increase nutrient uptake by providing phytohormones (Jacobson *et al*., 1994), low molecular weight compounds

(Frommel *et al*., 1991), enzymes (Glick *et al*., 1998), antimicrobial substances like antibiotics (Bangera and Thomashow, 1996) and siderophores (O'Sullivan and O'Gara, 1992). Some endophytes offer increased resistance to pathogens thus making them ideal candidates for biological control (Madhaiyan *et al*., 2004). Other beneficial effects of endophytes to plants include nitrogen fixation (Barraquio *et al*., 1997), increased drought resistance (Nowak *et al*., 1998), thermal protection (Redman *et al*., 2002), survival under osmotic stress (Creus *et al*., 1998) and more recently, their potential for enhanced degradation of several pollutants has also been investigated (Doty, 2008). In recent years there has been an increasing interest in endophytic bacteria having potential applications in plant growth promotion or plant protection towards evolving "environment-friendly" technologies, thus opening newer areas in microbial exploitation (Hallmann, 1997; Compant *et al*., 2005; Rosenblueth and Martinez-Romero, 2006). Endophytes, recognized as the potential sources of novel natural products for exploitation in medicine, agriculture and industry with more bioactive natural products isolated from the microorganisms (Bacon and White, 2000; Strobel and Daisy,2004).

Tissue culture, wherein surface-sterilized tissue is used as the starting material for culture establishment, may offer a potentially useful system to recover endophytic bacteria associated with specific organs like shoot tips and the organisms that show active growth on tissue culture medium might be easily cultivable and exploitable. Tissue culture also offers controlled conditions for studying endophytes away from other interfering organisms (Nowak 1998; Hallmann 1997; Thomas 2004).

Some of the endophytes are the chemical synthesizers in inside the plants (Owen and Hundley, 2004). Many of them are capable of synthesizing bioactive compounds that can be used by plants for defense against human pathogens and some of these compounds have been proven useful for novel drug discovery. Recent studies have reported hundreds of natural products including substance of alkaloids, terpenoids, flavonoids, steroids, etc. from endophytes. Up to now, most of the natural products from endophytes are antibiotics, anticancer agents, biological control agents and other bioactive compounds by their different functional roles. Thus far, they have not been widely explored for therapeutic properties. A single endophyte may be able to produce not one but several bioactive metabolites. As a result, the role of endophytes in the production of novel structures for exploitation in medicine is receiving increased attention (Wang *et al*., 2000; Ezra *et al*., 2004a; Gunatilaka, 2006). Recent work has also investigated their potential for the enhanced biodegradation of pollutants in soil. Most of these studies have focused on bacteria from the rhizosphere of

plants (Lindow & Brandl, 2003; Berg *et al.*, 2005). Bacterial endophytes colonize an ecological niche similar to that of phytopathogens, which makes them suitable as biocontrol agents (Berg *et al.*, 2005). Indeed, numerous reports have shown that endophytic microorganisms can have the capacity to control plant pathogens (Sturz & Matheson, 1996; Duijff *et al.*, 1997), insects and nematodes (Hallmann *et al.*, 1997). In some cases, they can also accelerate seedling emergence, promote plant establishment under adverse conditions (Chanway, 1996) and enhance plant growth (Chanway, 1998). Along with the production of novel chemicals, many endophytes have shown a natural capacity for xenobiotic degradation or may act as vectors to introduced gradative traits. This natural ability to degrade these xenobiotics is being investigated with regard to improving phytoremediation (Siciliano *et al.*, 1998)

Chapter-3

MATERIALS AND METHODS

Chemicals and glassware

All routine chemicals were purchased from Himedia laboratories, Mumbai. Plant growth regulators were purchased from Sigma chemicals Co. St. Louis, USA. Unless otherwise mentioned all experiments were conducted in 300 ml culture bottles (Kasablanka, Mumbai).

Culture media

Murashige and skoog's medium (MS, 1962; detail in annexure) containing 3% (w/v) sucrose and 0.7% (w/v) agar was used as basal medium. Growth regulators 6-Benzylaminopurine (BAP) and 1-Naphthaleneacetic acid (NAA) were added to the basal medium.

The concentrated stock solutions of all ingredients were prepared and stored under refrigeration. Similarly stock solutions of growth regulators were also prepared. All plant growth regulators (PGR'S) were dissolved in few drops of potassium hydroxide (1N) or hydrochloric acid (1N) and made to desired volume (2.5 mM) with distilled water and stored at 4°C. Medium was prepared by adding required quantities of all the ingredients in the conical flask. After adding all the ingredients in required amount, final volume was made up with the help of distilled water .The pH of the medium was adjusted to 5.8 using 1N KOH or 1N HCL (Cyberscan 510, Eutech instruments, Singapore) and agar was added. After adjusting the pH, medium was poured in culture bottles and capped with plastic caps. It was then autoclaved (Equitron, Medical Instruments, India) at 121° C for 20 minutes.

Multiplication of aseptic culture

The laminar air flow chamber was set up by disinfecting the internal surfaces with alcohol just before work. The surgical tools (scalpel, glass plate and forceps) were washed with detergent, oven drying and subsequent autoclaved before use. Actively growing cultures were subcultured in the MS medium supplemented with 0.01 mg/l each of BAP and NAA. Each bottle was inoculated with 15 nodes and incubated at 25°C under the light intensity of 42µmol/m²/s inside the culture vessel.

Isolation of endophytic bacteria

As there was constant bacterial contamination leaking from the internal tissues of FC-3 cultures, this bacterial growth was taken with the help of sterile inoculating loop and spread on the Luria agar plates. After 48 hours of growth, single colony was picked and grown in Luria broth for further work.

Pseudomonas corrugata a*nd Bacillus subtilis* were kindly provided by Dr. Anita Pandey, G.B Pant Institute of Himalayan Environment and Development, Almora, Uttarakhand.

3 bacterial cultures were grown to the mid log phase, pelleted by centrifugation (3500 rpm for 10 minutes, 4°C) and suspended in distilled water

1. FC-3 Bacteria (bacteria isolated from FC-3 variety of potato)
2. *Pseudomonas corrugata*
3. *Bacillus subtilis*

Acclimatization and transfer of plantlets to soil

After 15 days of culture on MS media supplemented with 0.01 mg/l NAA and 0.01 mg/l BAP, plantlets were shifted to polythene bags filled with soil and farmyard manure (1:1 v/v) for their acclimatization. For acclimatization, plants with newly formed roots were carefully taken out from culture bottles with the help of forceps and dipped in warm water to remove the traces of agar. Then, plantlets were carefully planted in polythene bags.

Inoculation of plants with endophytic bacteria

Plantlets of three potato cultivars (FC-3, FC-4 and ATL) were inoculated with 500 µl each of 3 bacterial cultures (2.04 x 10^6cfu/ml) using sterile syringe. 25 plants were taken for each treatment and experiment was repeated five times.

Plants were thoroughly watered and kept under polyhouse having 80% humidity and 25-30°C temperature for 10 days. In between plants were thoroughly watered with the help of sprinkler to maintain required level of humidity. Then the plants were shifted to shade house with lower humidity level and indirect sunlight (Figure 2(a & b)).In shade house also plants were watered two times a day i.e. morning and evening to prevent wilting (if any).

Figure. 2. Plantlets of FC-3 cultivar (a) control (b) infected with FC-3 bacteria

In vitro inoculation experiment:

After growing the plantlets on MS medium supplemented with 0.01 mg/l each of BAP and NAA for 15 days, nodal segments were taken out with the help of sterile forcep and were dipped in bacterial suspension for half an hour. Then, plantlets (7-8) were cultured in the bottles containing agropeat and 10 ml of ½ MS medium (autoclaved at 121°C for 2 hours). All cultures were incubated under 16 h photoperiod, in a light intensity of 42µmol/m²/s (Provided by Polylux XL, GE Britain, 36 W) at 25± 1° C (Figure 3 (a,b,c&d)).

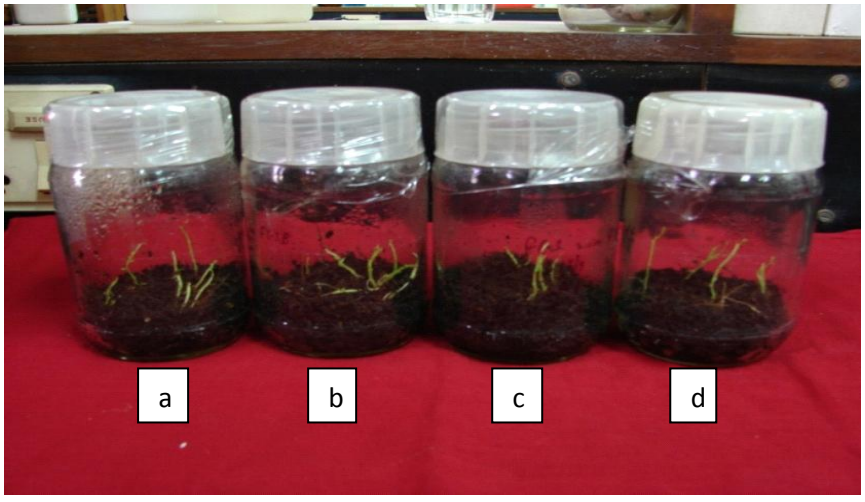

Figure 3: Plantlets of FC-3 cultivar inoculated with different bacterial cultures under *in vitro* conditions (a) control (b) inoculated with FC-3 bacteria (c) inoculated with *Pseudomonas corrugata*(d) inoculated with *Bacillus subtilis*.

Morphological, Biochemical and Physiological characterization:

To characterize all the bacterial isolates conventional physiological and biochemical characterization tests were carried out according to Bergey's Manual of Systematic Bacteriology (Holt *et al*., 1994).

Gram staining

Bacterial smear from actively growing cells were spread on a glass slide and heat fixed. Smear was flooded with crystal violet stain for 10 sec and then washed briefly in water to remove excess crystal violet. Later it was flooded with Gram's iodine for10 sec and washed briefly in water. Smear was decolourised with acetone until the moving dye front has passed the lower edge of the section and washed immediately in tap water. Counter staining was carried out with safranin for 15 sec and washed with water to remove the excessive stain. Finally slides were visualized under microscope at different magnification.

Cfu count

One ml bacterial culture was taken and was dissolved in 9 ml of distilled water and further dilutions were made up to 10^{-6}. 100µl of each dilution was plated on luria agar plates and plates were incubated for 24 hours and Colony count was taken.

Catalase test

Microorganisms were able to live in oxygenated environments, produce catalase which neutralizes toxic forms of oxygen. Catalase breaks hydrogen peroxide into water and molecular oxygen. A small amount of bacterial cells was placed onto a clean microscope slide and few drops of H_2O_2 (3%) were added. A rapid evolution of O_2 gas evidenced by bubbling supports positive result. A negative result was no bubbles or only a few scattered bubbles.

Oxidase test

One drop of reagent (N,N,N,N'-tetra-methyl-p-phenylenediamine dihydrochloride) was added onto the bacterial culture on an agar plate. Positive reactions turned the bacteria violet to purple immediately or within 10 to 30 seconds. Delayed reactions were ignored.

Nitrate reduction test

Nitrate broth is used to determine the ability of an organism to reduce nitrate (NO_3) to nitrite (NO_2) using the enzyme nitrate reductase. It also tests the ability of organisms to perform nitrification on nitrate and nitrite to produce molecular nitrogen.

Nitrate broth contained nutrients and potassium nitrate as a source of nitrogen. After incubating cultures in the nitrate broth, 2-3 drops of sulfanilic acid and α-naphthylamine were added. If the organism has reduced nitrate to nitrite, the nitrites in the medium will form nitrous acid. Sulfanilic acid was added; which reacts with the nitrous acid to produce diazotized sulfanilic acid. This reacts with the α-naphthylamine to form a red-colored compound. Therefore, if the medium turns red after the addition of the nitrate reagents, it was considered a positive result for nitrate reduction.

Fermentation of carbon substrate by bacterial isolates

Fermentation media consists of peptone,carbohydrate, sodium chloride , phenol red, distilled water, pH 7.3 . Three carbohydrate tests were performed with the isolated bacterial species. After inoculating the bacterial cultures in the fermentation media, tubes were incubated at 37° C for 24-48 hours and change in colour was observed.

Antibiotic profiling of bacterial isolates

Bacterial isolates were grown in luria broth until the absorbance reached to 1.0 at 600nm. The grown bacterial cells were spread on luria agar and antibiotic discs were kept on it. These plates were incubated at 37°C and the inhibition zone was noted. Ready precoated twelve antibiotic discs (Himedia Lab., India) were used to check the sensitivity of the bacterial isolates. These were: Vancomycin (10 µg), Gentamicin (10 µg), Ofloxacin (30 µg), Teicoplanin (75 µg), Ceftazidime (30 µg), Cephoxitin(30 µg), Penicillin G (30 µg), Oxacillin (30µg), Cephalothin(10 µg), Clindamycin (30 µg), Erythromycin(30µg), Amoxyclav(30µg)

IAA Estimation

- 100µl of log phase culture was inoculated in 20 ml of luria broth alone and luria broth medium amended with L-tryptophan (tryptophan works as a precursor for the production of IAA)

- The flasks were incubated at 30°C for 3 days on rotary shaker at 150 rpm
- Cultures were centrifuged at 10,000 rpm for 10 minutes and supernatant was collected in a test tube
- To 1 ml of supernatant, Salper's reagent (1 ml of 0.5 M $Fecl_3$ in 49 ml of 35% perchloric acid) was added dropwise but rapidly with continuous mixing in each tube
- The samples were incubated in dark for 30 minutes
- Develpoment of pink colour was assayed with spectrophotometer at 535 nm, the un-innoculated media was used as a blank

  The IAA was estimated from the standard curve of IAA (0-10 ppm)

Molecular characterization

Extraction of genomic DNA

Several protocols have been developed and described for the preparation of genomic DNA from microorganisms. Isolation of genomic DNA generally comprises chemical cell disruption by enzymic digestion and detergent lysis; extractions with organic solvents, and selective recovery of the DNA.

Isolation of genomic DNA

A single colony of bacterial isolate was inoculated into 25 ml of nutrient broth in a 250 ml flask and incubated for 14-18 hours at 37°C on shaker at 120 rpm. Liquid cultures (2.0 ml) were harvested and centrifuged (8,000 rpm, 10 min). The cell pellets were resuspended in 800 µl saline-EDTA and approximately 10 µl lysozyme were added. During incubation at 37°C for 30 min, the cell suspensions were mixed thoroughly by inverting the Eppendorf tube several times. To these, 200 µl SDS (10%) was added and cell suspensions were incubate at 65°C for 15 min. The cell suspension were extracted with organic solvents to remove proteins and cell debris: first, with an equal volume phenol:chloroform:isoamyl alcohol (25:24:1) solution and centrifuged 10 min at 12,000 rpm. The upper aqueous phase was then extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). To the aqueous phase,

0.7 volume of isopropanol was added and incubated for 15 minutes for DNA precipitation. Samples were centrifuged (12,000 rpm; 15 minutes) to pellet the DNA. The DNA pellets were washed with 750 µlethanol (70%) and microfuged another 10 min. Finally, the pellets were resuspended in 40 µl TE buffer/milliQ water and stored at 4°C.

Electrophoresis of DNA on agarose gels

DNA was loaded on agarose gels (0.7 % w/v) prepared in 1X TAE, pH 8.0 using a 6X loading dye. Ethidium bromide (0.5 µg/ml) was added to stain the gel prior to pouring. The nucleic acids were then electrophoresed at 3 volts/cm for 45-60 minutes and visualized on a U.V. transilluminator.

Spectrophotometric quantification of DNA

The concentration of extracted DNA in solution was estimated by spectrophotometric measurement at A_{260}. For double-stranded DNA suspensions, an OD of 1.0 at a wavelength of 260 nm and using a cuvette with 1 cm light path, is equal to a concentration of 50 µg/ml. The quality of the DNA was evaluated by measurement of the A_{260}/A_{280} and the A_{230}/A_{260} ratios. Ideally, the A_{260}/A_{280} ratio should be 1.8-2.0 while the A_{230}/A_{260} ratio should be 0.3-0.9. Ratios (A_{260}/A_{280}) less than 1.8 indicate protein or phenol contamination, while ratios greater than 2.0 indicate the presence of RNA.

Amplification of 16S rDNA and Purification of PCR products

The polymerase chain reaction (PCR) provides a rapid and highly sensitive method for the primer-mediated enzymatic amplification of specific target sequences in genomic DNA resulting in the exponential increase of target DNA copies.

For amplification of 16S rDNA gene the following primers were used: Forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 5'-ACGGGCGGTGTGTTC-3' (Weisberg *et al*., 1991). The amplification of 16S rDNA from isolates was carried out with a GenAmp thermocycler (Applied Biosystem, USA).The reaction mixture consisted of 40 ng of genomic DNA, 1.0 U Taq DNA polymerase (Larova, Teltow, Germany), 100 µm dNTPs mixture, 2.0 µl reaction buffer (10X), and 10 nmol primer, with Mill-Q water (Millipore India, Bangalore, India) was added to make up the volume to 20µl. Amplification conditions

were an initial denaturation of 94°C for 5 min, 36 cycles of 94°C for 1 min, 55°C for 30 sec and 72°C for 30 sec, with a final extinction at 72°C for 5 min. The amplified products were separated on a 1.2% (w/v) agarose gel and viewed under the UV transilluminator (Vilber Loumart , France) following ethidium bromide staining.

Amplified 16S rDNA was purified using the QIAquick PCR purification kit (Qiagen, USA), following the instructions of the manufacturer. Purified PCR products were eluted from the purification columns by the addition of 50 µl 10 mM Tris buffer (pH 8.0). PCR products, as they resulted from amplification of total DNA , were purified by agarose gel (0.8 %) electrophoresis. After staining with ethidium bromide, a defined band was visualized under UV irradiation and excised. Besides removing surplus primers, nucleotides, and salts, this method possessed the advantage that incomplete (shorter) amplification fragments are also removed prior to cloning. Subsequently, the DNA was extracted from the gel matrix material, using the QIAEX gel extraction kit (Qiagen), whereby the DNA is bound to silica gel particles, in the presence of high salt concentrations. Finally purified PCR products were eluted with 30 µl TE buffer (pH 8.0). In this manner, purified PCR products were applied directly for the cloning in T-vector.

Ligation of 16S rDNA in T-vectors

The 16S rDNA PCR products were cloned using the restriction independent InsTA Cloning Kit (TA cloning), following the manufacturer's protocol (Fermentas, USA). The 16S rDNA amplicon was ligated into pTZ57R/T vector. The reaction mixture was prepared as described below and incubated overnight at 4°C.

Plasmid pTZ57R/T (55ng/µl)	1.0 µl
Insert (75ng/µl)	1.5 µl
Buffer (5X)	1.0 µl
T4 Ligase	2.0 µl
H_2O	4.5µl

Genetic Transformation using $CaCl_2$

A single colony of *E. coli* DH5α from a freshly grown plate was inoculated into 25 ml of LB broth in a 250 ml flask and incubate for 16-20 hrs at 37°C on a shaker at 120 rpm.

Aseptically 200 µl of the above grown culture was transferred into 25 ml of fresh LB broth in a 250 ml flask. The culture was further incubated with vigorous shaking at 37°C for 2-3 hrs. To monitor the growth of the culture, the OD_{590} was determined at every one-hour (OD_{590} should be ~ 0.5). The above culture was transferred to sterile, disposable, ice-cold 50 ml polypropylene tubes. The culture was cooled to 0°C by storing the tubes on ice for 10 min. The cells were harvested by centrifugation at 8,000 rpm for 10 minutes at 4°C. The pellet was resuspend in 10 ml of ice-cold 0.1 M $CaCl_2$ and store on ice for 15 min. Further the cells were recovered by centrifugation at 8,000 rpm for 10 min at 4°C. The cell pellet was resuspended in 1 ml of ice-cold 0.1 M $CaCl_2$. The cells in this stage may be stored on ice for 12-24 hours. $CaCl_2$ treatment for 2 hours induces considerably a transient state of "competence" in the *E. coli* cells. One hundred micro liter of the suspension of competent cells was transferred to a sterile and prechilled microfuge tube (1.5 ml capacity). The plasmid DNA sample (~100 ng in a volume of 5 l or less) was added to each tube. The content of the tubes were mixed gently and stored the tubes on ice for 30 minutes. The tubes were incubated in a circulating water at 42°C for exactly 2 minutes without shaking. The tubes were rapidly transferred to an ice bath for 1-2 minutes. One ml of LB broth was added to each tube and incubated the cultures for 45-60 minutes at 37°C to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. One hundred micro liters of transformed cells was spreaded on each 90 mm Luria agar-Ampicillin-X-Gal-IPTG plates and incubated at 37°C. Transformed colonies should appear in 12-16 hours.

Blue/white screening for recombinant plasmids

After transformation of the ligated product, the *E. coli* DH5α (Lac Z^-) bacterial cells were plated on Luria agar medium containing 50 µg/ml ampicillin, for selection of transformants. X-Gal and IPTG were used to screen for colonies containing a recombinant plasmid. The cloning site in the pTZ57R/T vector is located in the multiple cloning site (mcs) of the plasmid's lacZα gene; if no insert is present, functional β-galactosidase is produced, and the transformed bacterial colony is blue. If the host cell receives a recombinant plasmid containing a 16S rDNA insert in the lacZα gene, the resulting transformant colony was white (Lac Z^-).

Isolation and purification of plasmid DNA from recombinant bacteria by alkaline lysis method

The plasmid DNA was isolated based on the alkaline lysis method. A single transformed *E. coli* white colony was transferred into 2 ml of Luria broth medium containing appropriate antibiotic (ampicillin, used in a final concentration of 50 g/ml) in a loosely capped 15-ml tube and incubated the culture overnight at 37°C with vigorous shaking. 1.5-2.0 ml of the above-saturated culture was poured into a microfuge tube and cells were harvested by centrifugation at 8,000 rpm for 1 min. The bacterial pellet was resuspended in 200 l of ice-cold Solution I by vigorous vortexing to ensure that the bacterial pellet is completely dispersed in this solution. Further 200 l of freshly prepared Solution II was added and the contents were mixed by gentle inversion of the tubes, five to ten times. Vortexed is avoided here. The tubes were stored on ice for 5 min. Finally 300 l of ice-cold Solution III was added and mixed by inversion to disperse Solution III through the viscous bacterial lysate. The tubes were stored on ice for 10 min. The tubes were centrifuged at 12,000 rpm for 10 min in a microfuge. The upper aqueous phase was then extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). To precipitate extracted plasmid DNA, 0.7 volumes isopropanol were added to the aqueous phase, followed by 10 min centrifugation at 12,000 rpm. The DNA pellets were washed with 750 µl ethanol (70%) and microfuged another 10 min. Finally, the pellets were resuspended in 40 µl TE buffer/milliQ water and stored at 4°C for further use.

Size screening for recombinant plasmids

Clones containing approximately 1.5-kb 16S rDNA inserts were identified by PCR screening, using the rapid protocol for preparation of template DNA from single bacterial colonies and M13-forward/M13-reverse plasmid primers. The amplification products were checked by agarose gel (0.8-1.0% w/v) electrophoresis.

Sequencing

The 16S rDNA inserts were sequenced for both strands using M13 forward and reverse primers, used for pTZ57R/T vectors. The sequence was generated by chain termination method (Sanger *et al*., 1977) using an Applied Biosystems automatic sequencer (DNA Sequencing Facility, Department of Biochemistry, South Campus, Delhi University, New Delhi, India).

Phylogenetic tree construction

The obtained sequences were subjected to BLAST analysis using bioinformatics tool, sequences were then aligned using MultAlin software. Phylogenetic analysis was carried out using Mega 4 software(Tamura *et al.*, 2007) by maximum parsimony method and phylogenetic tree was constructed.

Isolation of endophytic bacteria from the inoculated plants

Bacteria (from FC-3 cultivar) was isolated from the tubers as follows:

- Tubers washed with distilled water to remove soil, then with sterile distilled water
- Tubers were treated with 0.1% Bavistin (to avoid fungal contamination) for 15-20 minutes and allowed them to dry
- Surface disinfection was carried out with the help of 70% alcohol
- Tubers were then cut into two halves with the help of sterile blade
- With the help of cork borer, internal portion was taken out and homogenized in 2 ml of distilled water with the help of autoclaved pestle and mortar
- Macerated tissue was diluted into 10^{-1} dilution by adding 9 volumes of sterile distilled water
- Serial dilutions were made up to 10^{-6} dilution by taking 1 ml of well-shaken suspension and adding into 9 ml water blank tubes.

In the similar manner bacteria was isolated from *in vitro* raised plants, firstly the plantlets were surface disinfected with 70% ethanol for 15 seconds and were dipped in sterile distilled water. After pretreatment, the plantlets were crushed with sterile distilled water using sterile mortar and pestle. About 1 ml of crushed sample was serially diluted up to 10^{-6}. Luria agar medium was prepared and used for the isolation of endophytic bacteria. 100 µl of each dilution was spread on Luria agar

plate and incubated at 37°C (*Pseudomonas corrugata*) and 28°C respectively (*Bacillus subtilis* and FC-3 bacteria).

In both cases, colonies appear after 48 hours. These colonies were picked up with the help of sterile inoculating loop and were grown in luria broth.

RFLP (Restriction fragment length polymorphism)

Genomic DNA extraction was carried out from the isolated bacteria. Amplification of 16S rDNA was carried out by polymerase chain reaction. Amplified PCR products were digested with restriction endonuclease (*Mbo*1 and *Rsa*1) in 15 µl reaction volume by using the manufacturer's recommended buffer (1.5µl of 10 X) final volume of reaction was adjusted by adding water.

The digestion pattern was observed on agarose gel to confirm the isolated bacteria as an endophyte as positive control was also digested with the same enzymes

RESULTS

4.1 Micropropagation of potato:

Cultures of cultivars FC-3, FC-4 and ATL were multiplied on MS medium from previously maintained cultures. This process enabled us to maintain the germplasm in laboratory as well as for mass propagation of plantlets to be used as experimental material. Successful shoot multiplication was obtained on MS medium supplemented with 0.05 µM BAP and NAA as shown in (Figure 4.1 a & b)

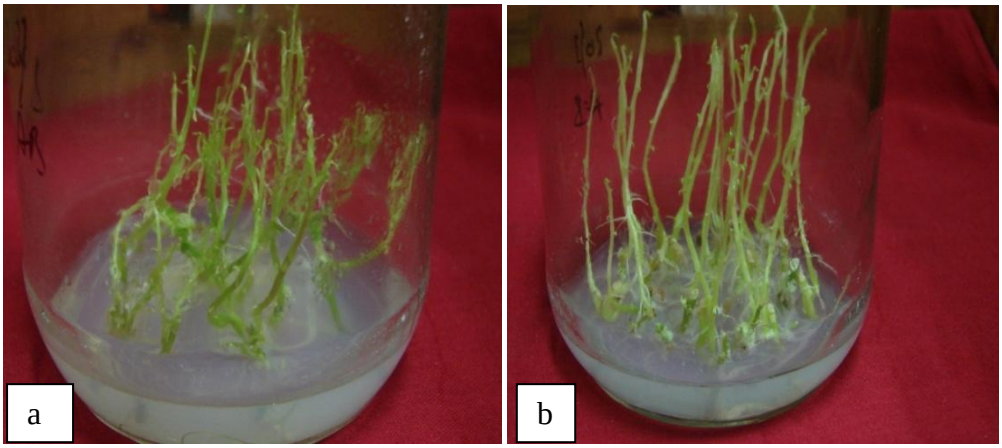

Figure. 4.1. Shoot multiplication of potato cultivars (a) FC-3 (b) FC-4

4.2 Isolation of endophytic bacteria

Bacteria were isolated from potato microtubers (FC-3 cultivar) using the protocol as described in materials and methods. On third day, colonies appeared creamish yellow,cocci shaped on the Luria agar medium. These colonies were picked and grown in Luria broth for further work.

4.3 Characterization of bacteria

a) Physiological and Biochemical characterization: To characterize all the bacterial isolates, various physiological and biochemical tests were carried out (Figure 4.2)

Figure 4.2: Biochemical and Physiological characterization of the three isolates

	FC – 3 Bacteria	*Pseudomonas corrugata*	*Bacillus subtilis*
Gram staining	+	-	+
Nitrate reductase	-	+	+
Oxidase	+	+	-
Urease	+	+	+
Antibiotic sensitivity	+	+	+
Cellulase	+	-	+
Fermentation of carbohydrates			
a.) Sucrose	+	+	-
b.) Mannitol	-	+	-
c.) Glucose	+	+	-

+: Positive reaction; -: Negative reaction

FC-3 bacteria found to be gram positive, giving positive results for oxidase, urease, cellulase and were able to ferment sucrose and glucose. This bacteria fails to ferment mannitol.

IAA estimation:

To see the growth related parameters, IAA estimation was done. IAA was estimated from the standard curve of IAA (0-10 ppm) drawn against O.D. IAA estimation was carried out in the presence and absence of tryptophan, a known precursor of IAA production. It was measured

on alternate days i.e. 3rd, 5th, 7th, 9th with the help of spectrophotometer. On 3rd day, IAA production was 0.51 ppm which increased to 1.30 ppm on 7th day in FC-3 bacteria (FC-3 B). Similarly for other two isolates, IAA production was maximum on seventh day and then on ninth day it decreased. *Pseudomonas corrugata* (PC) showed maximium production (1.68 ppm) of IAA on seventh day amongst three isolates (Figure 4.3).

In the presence of tryptophan, IAA production was increased. Amount of IAA was 1.30 ppm on 7th day in the absence of tryptophan, increased to 1.90 ppm in the presence of tryptophan in FC-3 B. Amongst all the three isolates, *Bacillus subtilis* (BS)showed maximum production (2.78 ppm) of IAA on 7th day (Figure 4.4). In both the cases, IAA production was lower in FC-3 B.

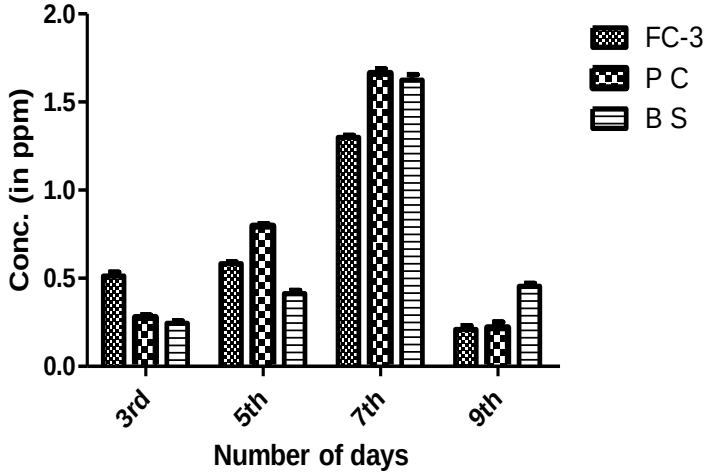

Figure 4.3: IAA production by three bacterial isolates in medium lacking tryptophan

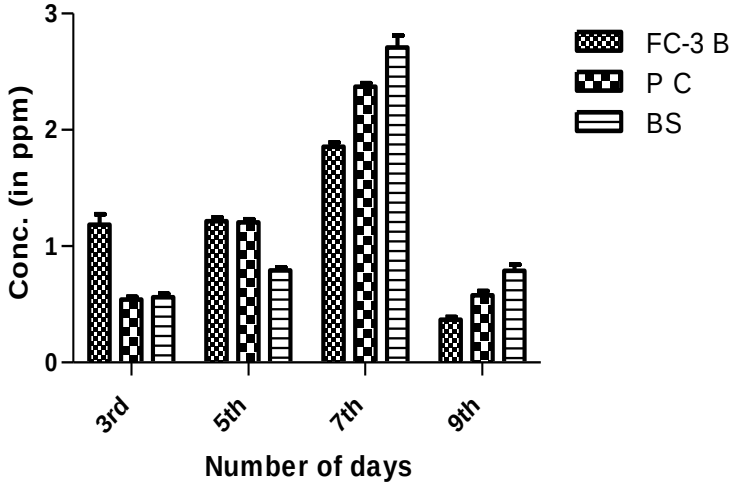

Figure4.4: IAA production by three bacterial isolates in medium supplemented with tryptophan

b) *Molecular characterization*:

G**e**nomic DNA was isolated and was subjected to 16S rDNA amplification using universal primers and about 1.5 Kb amplicon was observed in all isolates (Figure 4.5)

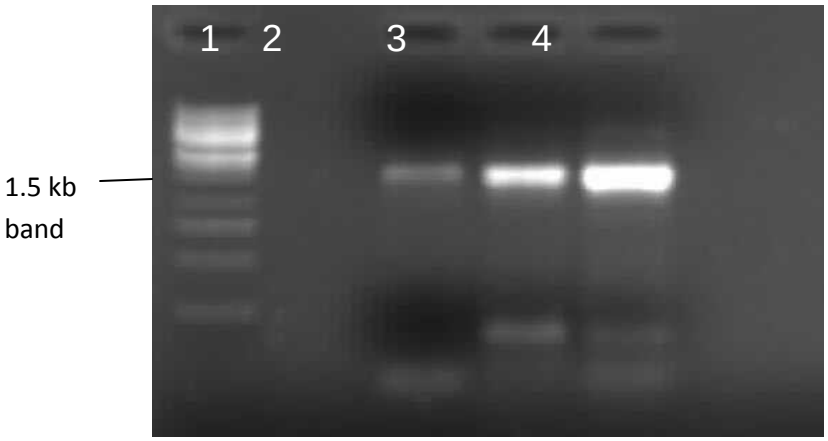

Figure 4.5: 16S rDNA Amplification of the three isolates. Lane 1, 1 kbladder ; Lane 2, FC-3 bacteria; Lane 3,*Pseudomonas corrugata;* Lane 4,*Bacillus subtilis*

The 1.5 kb fragment was cloned in the TA Cloning pTZ57R/T vector. The ligated product was transformed into competent cells of *E. coli* DH5α and plated on Luria agar plates containing 50 µg/µl of ampicillin. Positive colonies (white) were selected and plasmid DNA was isolated and amplified with M13-F and M13-R primers and send for sequencing.

The sequence thus obtained (Figure 4.6) was subjected to BLAST analysisusing bioinformatics tool, sequence was compared for the similarity in the GenBank DNA database using BlastN (NCBI) (Altschul *et al*., 1997).

Figure 4.6: The sequence of 16S rDNA fragment obtained

AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAG
CGGACTTGATGGAGAGCTTGCTCTCCTGAGAGTTAGCGGCGGACGGGTGAGTAACACGT
AGGCAACCTGCCTGCAAGACTGGGATAACTACCGGAAACGGTAGCTAATACCGGATACG
CAGTTTCCTCGCATGAGGGAGCTGGGAAAGACGGAGCAATCTGTCACTTGCGGATGGGC
CTGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCG
ACCTGAGAGGGTGAACGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG
CAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTG
ATGAAGGTTTTCGGATCGTAAAGCTCTGTTGCCAGGGAAGAACGTCGGGTAGAGTAACT
GCTATCCGAGTGACGGTACCTGAGAAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGC
GGTAATACGTAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCACGCG

GTCATGTAAGTCTGGTGTTTAACCCCGGGGCTCAACCCCGGGTCGCACTGGAAAACTGGG
TGACTTGAGTGCAGAAGAGGAAAGT

Figure 4.7: BLAST analysis of 16S rDNA sequence of FC-3 bacterial isolate

Accession number	Description	Maximum score	Total score	Query coverage	E value	Maximum identity
AB618501.1	*Paenibacillus* sp.	1153	1153	97%	0.0	98%
GU937424.1	*Paenibacillus favisporus* strain TG1R2	1068	1068	100%	0.0	95%
AY308758.1	*Paenibacillus favisporus* strain GMP03	1068	1068	100%	0.0	95%
HM627494.1	*Paenibacillus favisporus* strain BKB30	1062	1062	100%	0.0	95%
AY751754.1	*Paenibacillus rhizosphaerae* strain CECAP06	1051	1051	100%	0.0	94%
JF309261.1	*Paenibacillus* sp. 3492BRRJ	1050	1050	98%	0.0	95%
AY751755.1	*Paenibacillus rhizosphaerae* strain CECAP16	1046	1046	100%	0.0	94%
AF378699.1	*Paenibacillus turicensis*clone B2	1040	1040	100%	0.0	94%
GQ284356.1	*Paenibacillus barengoltzii* strain THWCS9	1035	1035	99%	0.0	94%
GQ284355.1	*Paenibacillus barengoltzii* strain THWCS8	1035	1035	99%	0.0	94%
FJ178001.2	*Paenibacillus*	1033	1033	97%	0.0	95%

	cellulositrophicus strain P2-1					
AF378700.1	*Paenibacillus turicensis* cloneB3	1035	1035	100%	0.0	94%

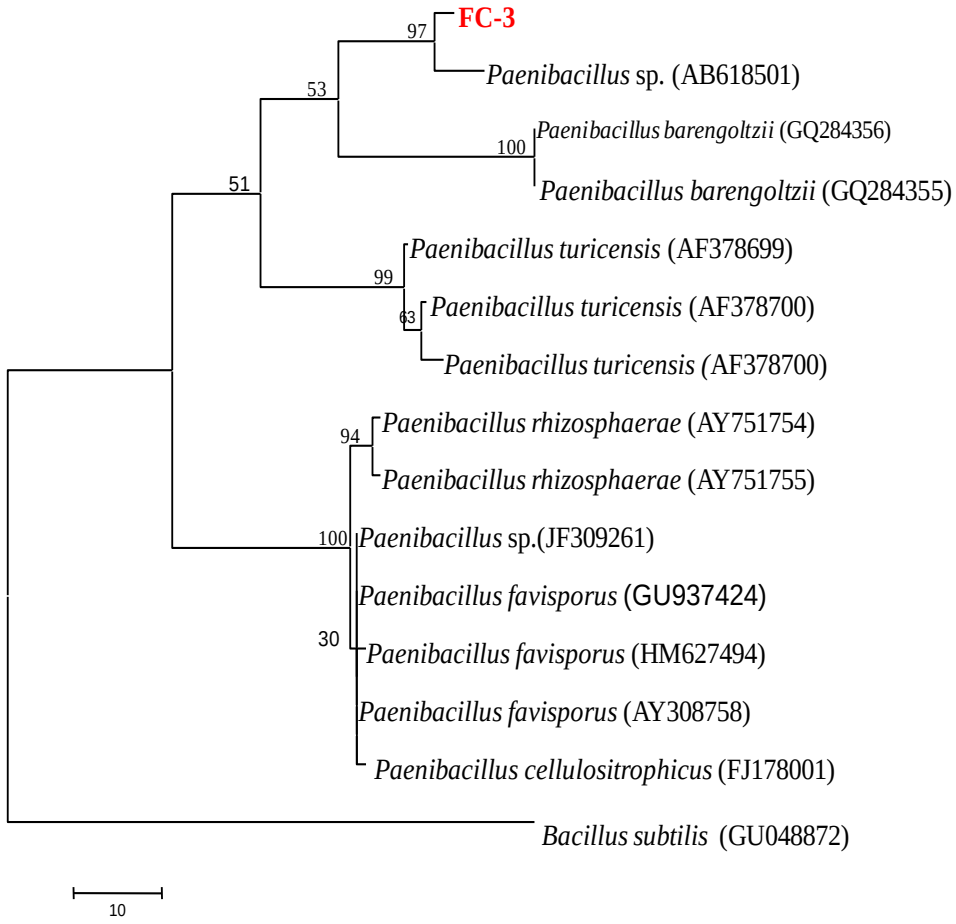

Figure 4.8: Maximium-Parsimony tree based on bacterial 16S rDNA sequence data from FC-3 bacterial isolate along with sequences available in GenBank database. Numerical values indicate bootstrap values.

The evolutionary history was inferred using the Maximum Parsimony method [1]. Tree (Figure 4.8) out of 44 most parsimonious trees (length = 189) is shown. The consistency index is (0.821138), the retention index is (0.908714), and the composite index is 0.802937 (0.746180) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown above the branches.. All positions containing gaps and missing data

were eliminated from the dataset. There were a total of 618 positions in the final dataset, out of which 73 were parsimony informative. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al*., 2007). *Bacillus subtilis* was used as an outgroup.

4.4 Influence of bacteria

A) Influence of bacteria on growth and tuber production in ex vitro plants

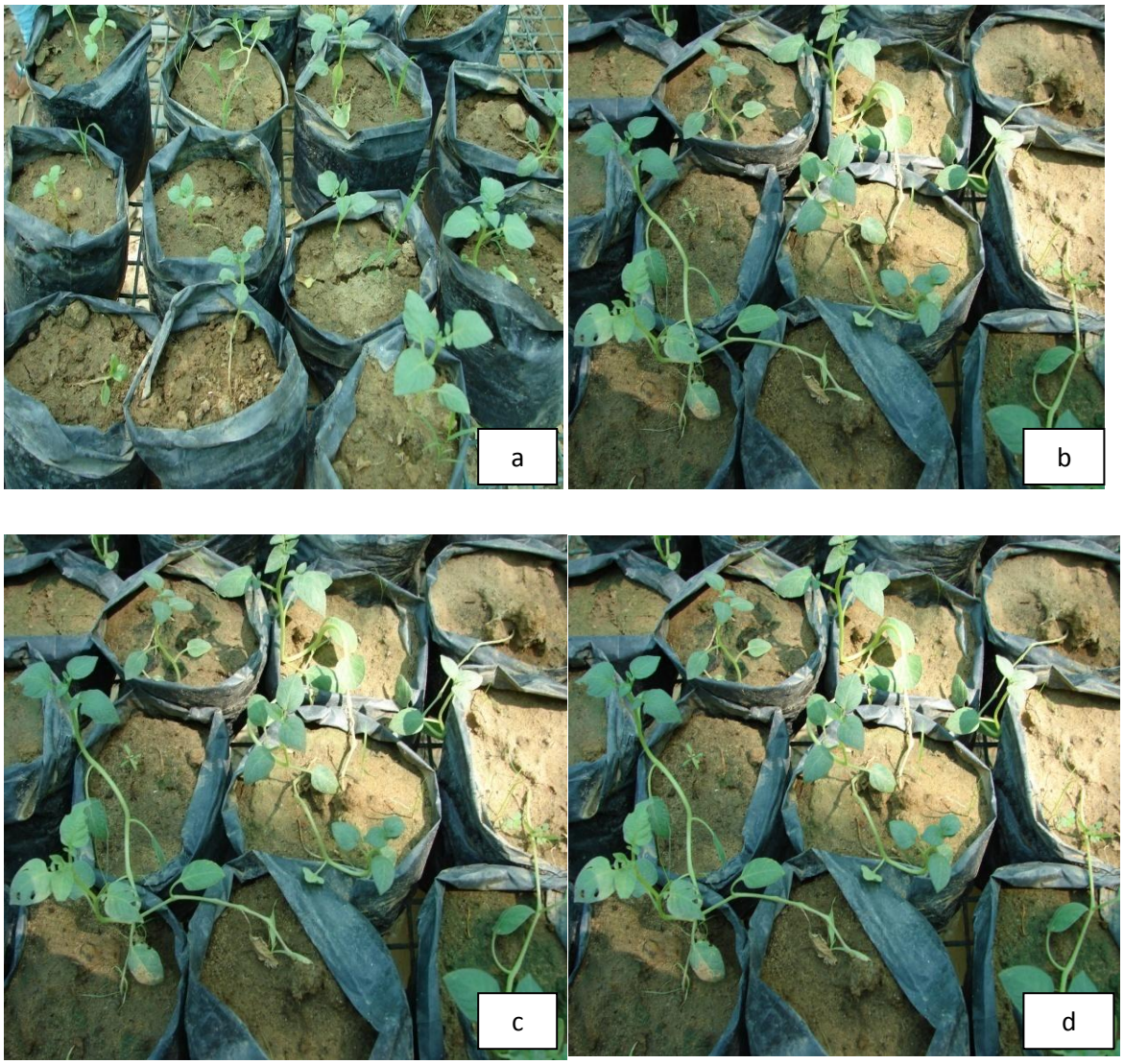

Figure 4.9 Plantlets of FC-3 cultivar (a) inoculated with FC-3 bacteria (b) control (c) inoculated with *Pseudomonas corrugata* (d) inoculated with *Bacillus subtilis*

a) Plant height

Prior to inoculation, plantlets having similar length (2.5 cm) were choosen for height analysis. It was observed that in FC-3 cultivar, maximum increase in plant height was shown by untreated (control) plants from 2.5 cm to 20.5 cm. Whereas, FC-3 bacterial (FC-3 B) inoculated FC-3 cultivar showed very less increase in plant height from 2.5 cm to 7.5 cm. *Pseudomonas corrugata* (PC) and *Bacillus subtilis* (BS) inoculated plantlets of FC-3 cultivar also showed less increase in plant height, but it was more than FC-3 bacterial inoculated plantlets. In the other two cultivars, maximum plant height was observed in which *Pseudomonas corrugata* and *Bacillus subtilis* were inoculated. Amongst all cultivars and treatments, ATL cultivar inoculated with *Bacillus subtilis* showed maximum increase in plant height from 2.5 cm to 27 cm. FC-3 bacterial treated plants again showed very less increase in plant height in these cultivars (Figure 4.10a).

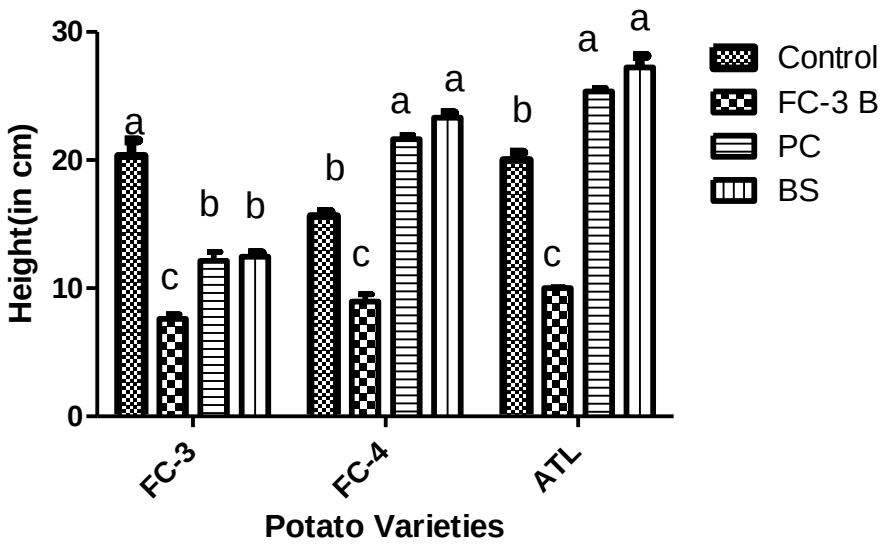

Figure 4.10a: Effect of bacterial isolates on the growth of micropropagated plantlets

b) Survival rate

In each set 100 plants were planted, 25 plants consisting of each inoculum. In FC-3 cultivar, survival rate was found to be maximum (50%) in control (without treatment), whereas FC-3 cultivar when inoculated with FC-3 bacteria survival rate was significantly reduced (34%) and FC-3 cultivar inoculated with *Pseudomonas corrugate* and *Bacillus subtilis* also showed

decreased survival rate, but it was more than the FC-3 bacterial inoculated plants. In other two cultivars, FC-4 and ATL which were inoculated with PC and BS showed maximum survival rate (99%). FC-3 bacterial inoculated cultivars showed very less survival rate (Figure 4.10b)

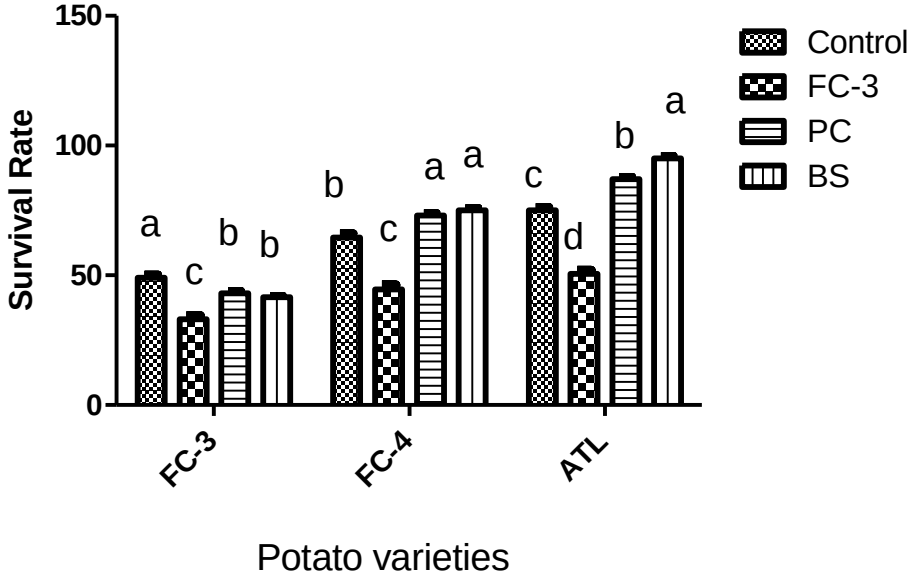

Figure 4.10b: Effect of bacterial isolates on the survival rate of micropropagated plants

c) Weight of tubers

After 5 months of growth, tubers were harvested and the weight of tubers was taken. FC-3 cultivar without any treatment (control), produced maximum number of tubers and weight of tubers was also higher as compared to treated one. FC-3 bacterial inoculated cultivars, produced less number of tubers and weight was also lower. In other two cultivars FC-4 and ATL, weight of tubers was much higher when inoculated with *Pseudomonas corrugata* and *Bacillus subtilis* as compared to FC-3 bacterial inoculated FC-4 and ATL cultivar (Figure 4.10c)

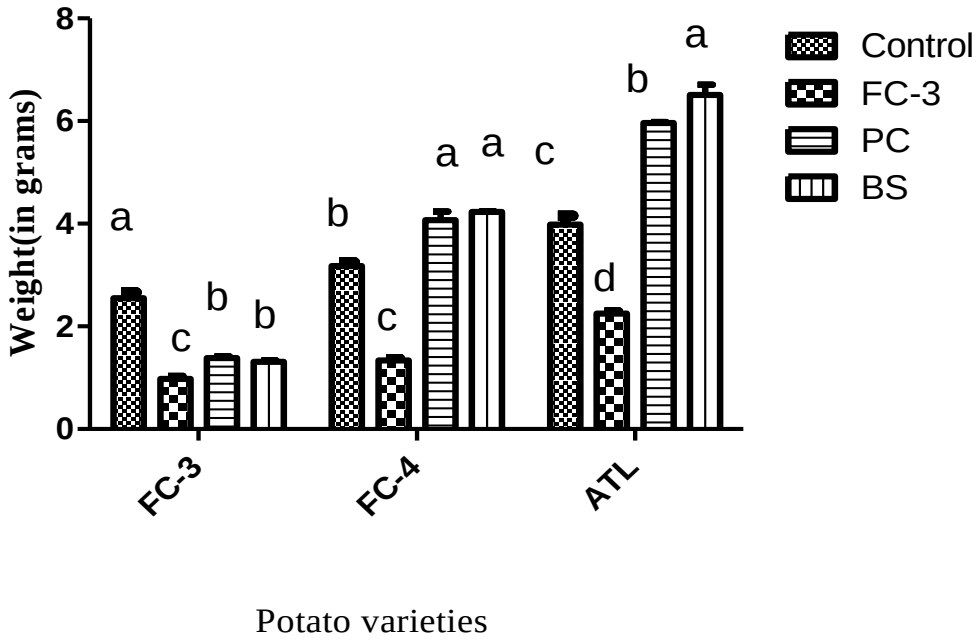

Figure 4.10 c: Effect of different bacterial inoculations on the weight of tubers produced by micropropagated plants

Influence of endophytic bacteria on in-vitro grown plants

Like *ex vitro* experiment, *in vitro* studies were also carried out to see the effect of bacterial isolates on micropropagated plants.

a) Plant growth

Prior to inoculation, plantlets of same length (1.5 cm) were choosen for growth studies. Maximum plant height (5.5 cm) was observed in control (without treatment) in case of FC-3 cultivar whereas in FC-3 B treated FC-3 cultivar, there was increase in plant height from 1.5 cm to 2.1 cm only. In other two cultivars, FC -4 and ATL; plant height was maximum in *Pseudomonas corrugata* (PC) and *Bacillus subtilis* (BS) inoculated plants. FC-3 B inoculated plants showed very less increase in plant height in the these two cultivars also (Figure 4.11 a)

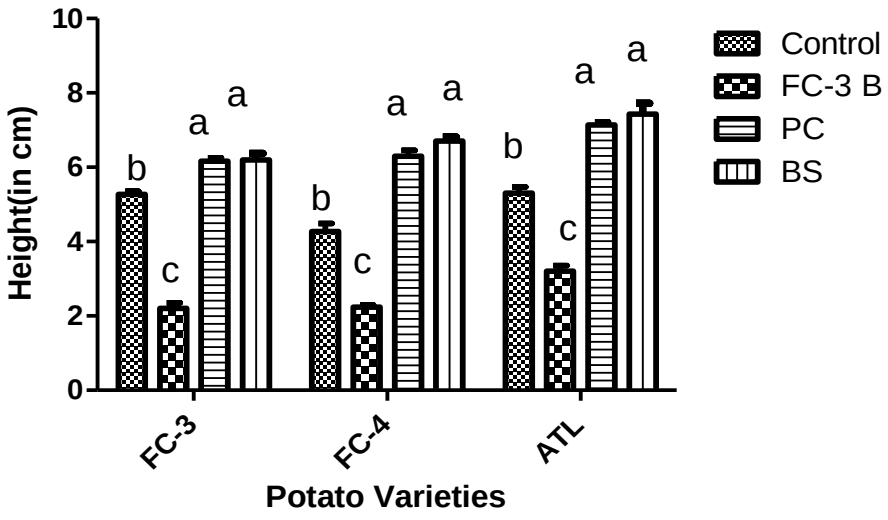

Figure 4.11a: Effect of endophytic bacteria on height of micropropagated plants

b) Biomass increment

As the survival rate of FC-3 bacterial inoculated plants was very less, biomass was observed to be decrease significantly in the FC-3 inoculated cultivars. In FC-3 cultivar, increase in biomass was maximum (3.2 g) in which no treatment was given (control). In other two cultivars, also FC-3 bacteria was seen to inhibit growth as the biomass increase was lower then control. *Pseudomonas corrugata* and *Bacillus subtilis* inoculated FC-4 and ATL cultivar showed maximum increase in biomass as both are growth promoting endophytic bacteria (Figure 4.11b)

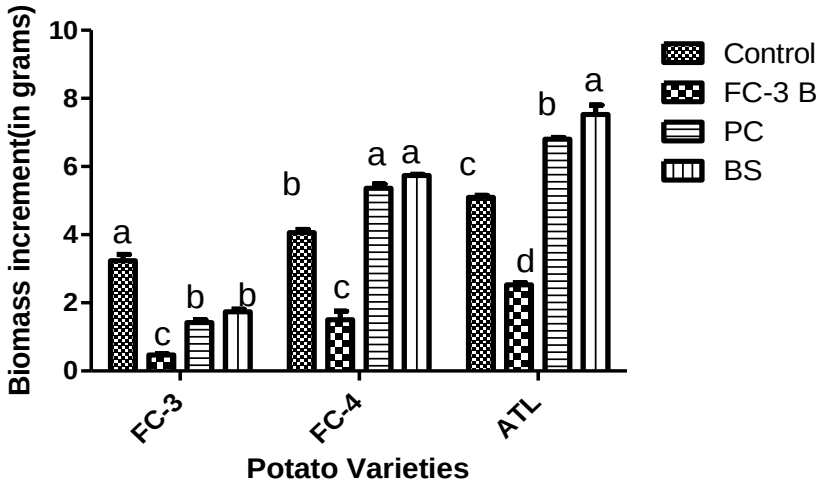

Figure 4.11b: Effect of endophytic bacteria on biomass increment

4.7 Isolation of endophytic bacteria from the inoculated plants

a) Endophytic bacterial populations recovered were $3x10^4$cfu/ml in FC-3 cultivar inoculated with FC-3 bacteria whereas 10^3 cfu/ml in FC-3 cultivar control from *in vitro* plants. In tubers numerous colonies were observed.

Bacteria was isolated from the tubers and from the *in vitro* plants by serial dilution method. DNA was extracted from the isolated bacteria and subjected to 16S rDNA amplification and approximate 1.5 kb amplicon was observed in all isolates (Figure 4.12)

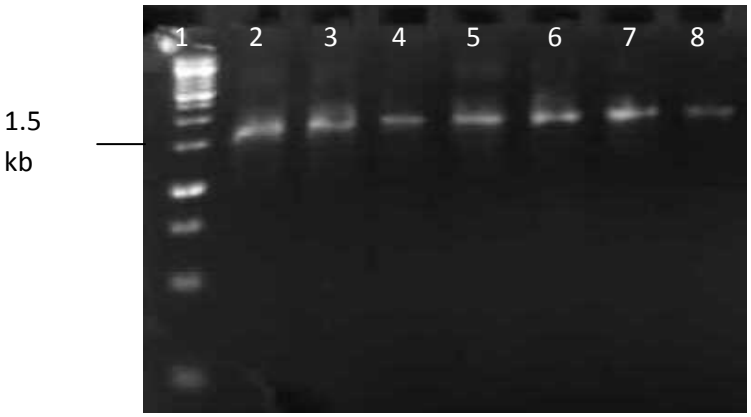

Figure 4.12: 16S rDNA amplification of the isolated endophytic bacteria. Lane 1: I kb ladder; Lane 2: FC-3 bacteria; Lane 3: FC-3 B isolated from FC-3 cultivar; Lane 4: FC-3 B isolated from FC-4 cultivar; Lane 5: FC-3 B isolated from ATL cultivar; Lane 6: FC-3 B isolated from *in vitro* FC-3 plants; Lane 7: FC-3 B isolated from *in vitro* FC-4 plants; Lane 8: FC-3 B isolated from *in vitro* ATL plants

b) RFLP analysis (Restriction fragment length polymorphism)

16S rDNA amplified product was then subjected to restriction digestion using two enzymes:*Rsa*1 (Figure 4.13)and *Mbo*1(Figure 4.14).Then banding profile was recorded. Banding profile of the bacteria isolated from the *in vitro* plants came out to be same with the positive control (FC-3 B), whereas banding profile of the bacteria isolated from tubers showed variation from the positive control. This might occurred due to the rich microflora present in the soil that may have isolated from the tubers along with the isolation of inoculated cultures and thus appeared on Luria agar plates.

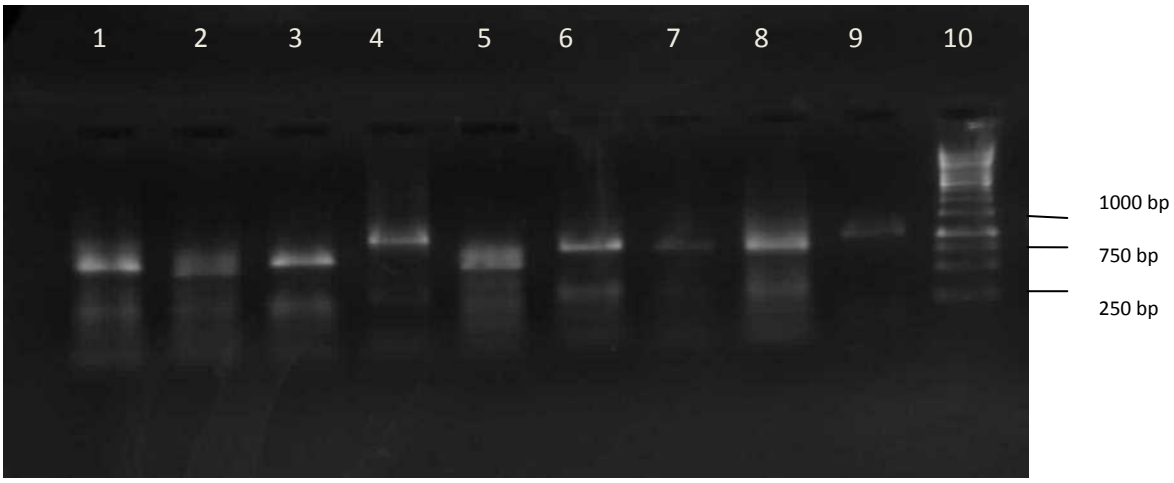

Figure 4.13; Restriction digestion pattern of amplified 16S rDNA fragment with *Mbo*1: Lane 1: Bacteria isolated from *in vitro* FC-3 cultivar; Lane 2: Bacteria isolated from *in vitro* from FC-4 cultivar; Lane 3: Bacteria isolated from*in vitro* ATL cultivar; Lane 4: *Bacillus subtilis*; Lane 5: FC-3 bacteria (positive control); Lane 6: Bacteria isolated from tubers ofFC-3 cultivar; Lane 7: Bacteria isolated fromtubers of FC-4 cultivar; Lane 8: Bacteria isolated from tubers of ATL cultivar; Lane 9: *Pseudomonas corrugata*; Lane 10: I kb ladder

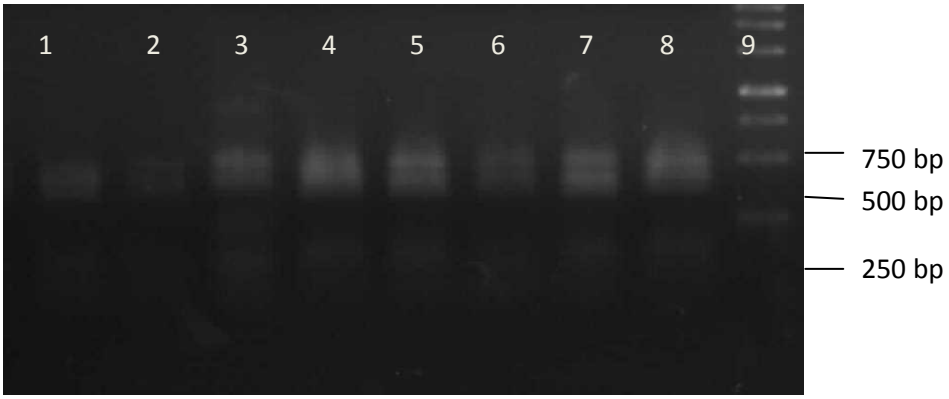

Figure 4.14;Restriction digestion pattern of amplified 16S rDNA fragment with *Rsa*1 Lane 1: *Pseudomonas corrugata;* Lane 2: Bacteria isolated from tubers of FC-3 cultivar*;* Lane 3: Bacteria isolated from FC-4 cultivar*;* Lane 4: Bacteria isolated from ATL cultivar*;* Lane 5: FC-3 bacteria*;* Lane 6: Bacteria isolated from *in vitro* FC-3 plants*;* Lane 7: Bacteria isolated from *in vitro* FC-4 plants*;* Lane 8: Bacteria isolated from *in vitro* ATL plants*;* Lane 9: I kb ladder

Chapter 5

DISCUSSION

Endophytes are microorganisms that reside within the inner parts of plants without causing any disease symptoms (Hallmann *et al*., 1997). Cultivable endophytic communities can be isolated after surface sterilization of the plant material. Endophytes exist in a range of tissue types within a broad range of plants, colonizing the plant systemically with bacterial colonies and biofilms, residing latently in intercellular spaces, inside the vascular tissue or within cells (Ulrich *et al*., 2008).

Potato cultivar FC-3 cultures were showing the constant bacterial contamination which seems to be leaking from the internal tissues. These bacteria were slow growing on MS medium. Furthermore the cultures of cultivar FC-3 were underperforming in comparison to the other cultivars such as FC-1 and FC-4. Therefore, the present study was aimed at isolation of endophytic bacteria from microshoots of potato cultivar FC-3. Further physiological and morphological characterization was also done. The strain was gram positive, cocci shaped. Earlier workers have reported a predominance of Gram negative bacteria in the tissues of various plants (Stoltzfus *et al*., 1997; Elbeltagy *et al*., 2000). However, Zinniel *et al*., (2002); reported an equal presence of Gram negative and Gram positive bacteria. IAA production was found to be 1.90 ppm in FC-3 B when medium was amended with tryptophan. Endophytes can also promote plant growth by producing the phytohormone IAA (Lee *et al*., 2004; Mendes *et al*., 2007). IAA increases root size and distribution, resulting in greater nutrient absorption from the soil.

Isolate was further characterized by partial sequencing of the 16S rDNA gene. Isolate showed 98% similarity with *Paenibacillus* sp. *Paenibacillus* sp.,previously reported as an endophytic bacteria in coffee (Jimenez- Salgado *et al.,* 1997, Sakiyama *et al.,* 2001) and was found to promote the growth.

Potato cultivars inoculated with FC-3 bacteria, showed less growth in terms of tuber weight, plant height, survival rate, increase in biomass and number of tubers thus concluding that it is a growth inhibitor rather than a growth promoter. FC-3 variety without treatment (control) showed maximum growth as compared to treated ones. In this study, *Pseudomonas corrugata*

and *Bacillus subtilis* were also used, as they are known growth promoting bacteria. FC-3 cultivar was also inoculated with these two endophytes, but they were not able to show their promoting activity on this cultivar, due to the reason FC-3 cultivar found to inhabit FC-3 bacteria. So the effect came out to be intermediate of the two. Also two other potato cultivars were used in this study, FC-4 and ATL, FC-3 bacteria was also found to inhibit in the same way as in FC-3 cultivar. But when these cultivars were inoculated with *Pseudomonas corrugata* and *Bacillus subtilis*, these bacteria showed their growth promoting activity in terms of increased plant height, more survival rate, more fresh weight and number of tubers. *Paenibacillus* sp. Reported endophyte of coffee, cause root rot disease in cotton seedlings. Pleban *et al*., 1997; isolated endophytic *Bacillus cereus* from mustard that produced an enzyme stable between pH 4 and 8.5 and significantly protected cotton seedlings from root rot disease caused by *Rhizoctonia solani*, *Paenibacillus amylolyticus*, an endophytic isolate from coffee cherries.

Micropropagated cultures of FC-3 cultivar also showed less growth, in every subculture, these were found to be associated with bacterial growth. Nevertheless, the presence of bacteria in micropropagated plants is commonly mentioned as microbial contamination, which must be prevented and eliminated (George *et al*., 2008). Only a few scientists consider these microorganisms endophytes present in the plant tissues. Recently, the presence of such bacteria was reported in peach palm plants (Almeida *et al*., 2009). But as surface sterilization was carefully carried out, still this bacteria was found to be present in potato tuber. It clearly indicated that it is an endophyte but a growth inhibitor.

Although plant growth promoting effects by endophytic bacteria can be cultivar specific (Pillay and Nowak, 1997. The majority of endophytic strains assayed that were growth promoting when inoculated into carrots (83%), were plant growth neutral (38%), plant growth promoting (33%) and plant growth inhibiting (29%) when inoculated into potatoes (Monique A *et al.*,2002). So the endophytic bacteria which is found to be growth inhibitor in potato in these cultivars, may help in promoting the activity in some other plant.

Chapter 6

CONCLUSION

Potato cultivar (FC-3) were showing the constant bacterial contamination which seems to be leaking from the internal tissues during multiplication cycles. This bacteria was isolated, characterized and then the potato cultivars were inoculated with this bacteria. FC-3 bacteria was found to inhibit plant growth in terms of plant height, weight of tubers, survival rate in the three cultivars (FC-3, FC-4, ATL) in both *in vitro* and *ex vitro* studies. FC-3 bacteria was found to be 98% identical with *Paenibacilus* species. The known growth promoting endophytic bacteria namely *Pseudomonas corrugata* (PC) and *Bacillus subtilis* (BS)were also used as reference organisms to study the effect of inoculations with isolated endophytic bacteria from cultivar FC-3. In FC-4 and ATL cultivar, PC and BS were found to enhance growth whereas in FC-3 cultivar, growth enhancement was less but was higher as compared to FC-3 bacterial inoculated FC-3 plants. So, effect of FC-3 bacteria in FC-3 cultivar can be supressed if known growth promoting endophytes are inoculated into FC-3 cultivar.

Chapter 7

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ANNEXURE

Media Composition
Murashige and Skoog (1962) Medium

1.	**Macronutrients (Hi Media)**	**mg/Lt**
	NH_4NO_3	1650
	KNO_3	1900
	$CaCl_2.2H_2O$	440
	$MgSO_4.7H_2O$	370
	KH_2PO_4	170
2.	**Micronutrients (Hi Media)**	
	$MnSO_4.4H_2O$	16.90
	$FeSO_4.7H_2O$	27.80
	$ZnSO_4.7H_2O$	8.60
	H_3BO_3	6.20
	KI	0.83
	$Na_2MoO_4.2H_2O$	0.25
	$CoCl_2.6H_2O$	0.025
	$CuSO_4.7H_2O$	0.025
	$Na_2EDTA.\ 2H_2O$	30.00
3.	**Vitamins (Hi Media)**	
	Myoinositol	100
	Glycine	2.0
	Nicotinic acid	0.5
	Pyridoxine HCL	0.5
	Thiamine HCL	0.1
	Sugar	3000
4.	**Agar**	7000