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ABSTRACT

Plant root growth is one of the most important factor in determining the plant health and increased crop production. Factors like root length, root hairs and number of secondary roots are crucial for the intake and availability of unreachable nutrients in soil. Plant roots are inhabited by large number of microbes called root microbiome. Utilizing beneficial root microorganism to promote plant growth can have lasting effect on plants ability to resist unfavorable environmental stresses and achieve optimized growth.

In this study known plant growth promoting rhizospheric and endophytic bacteria as well as newly isolated bacteria from tomato specie *Solanum pimpinellifolium* are tested on *Arabidopsis thaliana,* Gemini tomato plant and mutant *pin2*, *wei8/wei2*, *tir1* and *aux1-7* which are mutants defective in polar auxin transport and auxin signaling. The plant seedlings were grown on agar solidified 1/50 X Gamborg's B5 nutrient medium and also on 1 X Murashige and Skoog nutrient medium. The bacterial culture was inoculated to vertical agar plates under two different conditions: sucrose supplemented nutrient medium and without sucrose medium.

Roots of hydroponically growing *Solanum pimpinellifolium* tomato plants were utilized to isolate the root endophytes. 16S gene sequencing analysis revealed the identity of bacterial species. Three bacterial isolates recovered from the *S.pimpinellifolium* roots are *Micrococcus yunnanensis*, *Cohnella* spp. and *Bacillus flexis*. Newly isolated bacterial strains *Micrococcus yunnanensis* and *Cohnella* spp. were inoculated to the Gemini tomato plants in a pot experiment to check if they have any beneficial growth promoting traits.

During the course of this project we were able to find that bacterial strain 9 and 15 (*Pseudomonas* spp. & *Agrobacterium* spp.) received from Gennao Roberto Abbamondi, Hasselt University, Belgium are able to stimulate root hair development in Col-0 and also in mutants *pin2*, *wei8/wei2*, *tir1* and *aux1-7*. Among newly isolated strains *M.yunnanensis* is able to stimulate the primary root length to some extent, number of lateral roots and shoot weight in WT Col-0 in plants growing in 1 X MS medium supplemented with sucrose. *M.yunnanensis* is also shown to have some effect on shoot length and shoot weight of Gemini tomato plants.

Pseudomonas fluorescence (WCS417) is able to stimulate primary root length, number of lateral roots and shoot fresh weight in Col-0, *pin2* and also in *aux1-7* mutant.

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ABBREVIATIONS

ABA	Abscisic acid
ACC	1-aminocyclopropane-1-carboxylate
BRs	Brassinosteroids
CKs	Cytokinins
DNA	Deoxyribonucleic acid
ETHY	Ethylene
EDTA	Ethyldiaminetetraacetic acid
Fe-EDTA	Ferric-EDTA
GA	Gibberellins
KB	King's B medium
LR	Lateral roots
MS	Murashige and Skoog
MES	2-ethanesulfonic acid
OD	Optical density
PAT	Polar auxin transport
PCR	Polymerase chain reaction
pH	Potential of hydrogen
PGPB	Plant growth promoting bacteria
PGPR	Plant growth promoting rhizobacteria
PGP	Plant growth promoting
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SD	Standard deviation
TIR1	Transport inhibitor response
TAE	Tris-acetate-EDTA
UV	Ultraviolet
VAP	Vertical agar plate
WEI	Weakly ethylene insensitive

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1 INTRODUCTION

Better and large crop production and plant mass depend on the highly developed root structure of the plant. A primary limitation in nutrient uptake and crop productivity is nutrient availability. It is assumed that with suitable root traits, crop yield can be increased in infertile soils (Wang et al. 2016). Root proliferation is crucial in modifying the soil volume for better availability of mineral elements, and root length has been shown to be vital for less mobile nutrients (Barber, 1995; Lynch, 2013; White et al., 2013). Some factors involved in the root development includes plant perception to sense their environment to grow such as detecting light, air supply, the stock of nutrients and water (Carminati et al. 2009; Nakagawa et al. 2007).

One crucial factor in promoting plant growth is the phytohormones. Phytohormones are regarded as plant growth regulators and refer to the compounds biochemically synthesized by plants which can act locally or transport to different sites in the plant to mediate growth and development processes under ambient as well as stressful conditions. Hormones regulate the cellular process in targeted cells, control the formation of flowers, stem, leaves and also trigger ripening of fruits (Peleg and Blumwald 2011). The well-known hormones are abscisic acid (ABA), gibberellins (GA), ethylene (ETHY), auxins (IAA), cytokinins (CKs), and brassinosteroids (BRs), which are involved in controlling many physiological and biochemical processes (Iqbal et al. 2014).

Among these plant hormones, auxin, ethylene, and cytokinin play a considerable role in regulating cell development in root epidermis (Zhang et al. 2016). Auxins are characterized as root forming hormones of plants, and there is a strong link between this class of small molecules and root development. Effect of auxin can be well seen in auxin-associated phenotypes which result from dose-dependent increased in length of root hairs, effect of auxin concentration on primary root length, dose-dependent increase in number of lateral roots primordia and response to gravity (Overvoorde et al. 2010). Auxin is transported over long distances to sink tissues such as root tips, where cells are largely undifferentiated and where secondary cell wall does not inhibit auxin movement (Overvoorde et al. 2010). An important characteristic of auxin is the unequal distribution between cells, which is a prerequisite for cellular differentiation, signaling and cell division. This uneven distribution of auxin is based on polar auxin transport (PAT)

between cells and involves the action of auxin transporter. PAT is carried out by number of transporter proteins which include members of PIN protein family and also AUX family. The mutants in these proteins have abnormal auxin distribution and show strong phenotypes which underline the importance of these proteins in auxin distribution and plant development (Kuhn et al. 2017). Some of the mutant which are part of this study includes *pin2*, *aux1-7*, *tir1* and *wei8/wei2*.

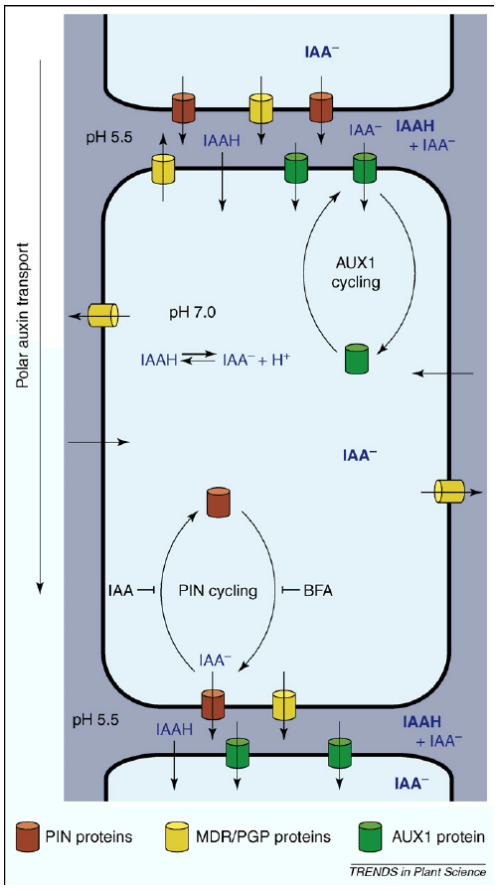


Figure 1-1-Schematic representation of polar auxin transport in root tip of Arabidopsis thaliana. Cell to cell auxin transport (Vieten, Sauer et al. 2007)

1.1 Auxin Mutants

1.1.1 *pin2* and *aux1* (efflux and influx carriers)

PINs are efflux auxin transporter which export auxin from cells and are essential for the reflux in the root apex resulting in vectorial auxin stream. Among the eight PINs in *Arabidopsis thaliana;* PIN1, PIN2, PIN3, PIN4 and PIN7 control auxin distribution in roots and are crucial for root

elongation. PIN2 protein functions mainly in epidermis and cortex in the root meristem, with an apical and subcellular localization on the plasma membrane. PIN2 is reported to be one of the most popular PIN for regulation of meristem enlargement in the root. The *pin2* mutant shows small roots and loss of gravitropic response in roots (Adamowski and Friml 2015; Blilou et al. 2005; Kleine-Vehn et al. 2008; Kuhn et al. 2017; Mravec et al. 2009). In contrast to PIN transporter protein, AUX1 is another important active auxin transporter in *A.thaliana*. AUX1 protein is auxin influx transporter which plays a role in the transport of auxin from the apoplast into the cytoplasm. It is a family of four protein involved in regulating the developmental processes such as lateral root formation and cotyledon vascular patterning (Grones and Friml 2015; Péret et al. 2012). Root hair tip and length of epidermal cells are sensitive to the different environmental stimulus, including plant hormones auxin and ethylene. Mutants defective in AUXIN RESISTANT 1 (*aux1*), are resistant to the inhibitory effects of exogenous and endogenous auxin on root elongation (Parry and Estelle 2006; Pickett et al. 1990).

1.1.2 Transport inhibitor response (TIR1)

Transport inhibitor response 1 protein (TIR1) is another protein of keen interest in auxin signaling pathway. It is an auxin receptor that mediates auxin protein proteasomal degradation and auxin-regulated transcription. TIR1 is involved in auxin signaling pathway which regulates root and hypocotyl growth, lateral root formation, cell elongation and gravitropism. The plants with the mutation in tir1 gene are deficient in many auxin-related growth processes mainly hypocotyl elongation and lateral root formation (Dharmasiri et al. 2005; Kepinski and Leyser 2005; Ruegger et al. 1998; Terrile et al. 2012).

1.1.3 Weakly ethylene insensitive (WEI8)

WEI8 gene is involved in the production of tryptophan aminoteransferase (TAA1) in indole 3 pyruvic acid branch of auxin biosynthetic pathway. Analysis of TAA1 has shown a link between local auxin production, tissue-specific auxin production and organ development. However roots of the plant mutant in *wei8* do not show proper gravitropism. It is also suggested that this gene is required for auxin signaling during lateral root and root meristem development (Stepanova et al. 2008).

The root architecture of plant and postembryonic development in roots is in large also affected by the levels and transportation of auxin between the cells. Three distinct processes mainly define the root architecture system: (1) Indeterminate growth of primary root; (2) lateral root formation and (3) root hair formation. Postembryonic development is mediated by cell division in the merismatic zone, cell expansion in elongation zone, and differentiation into the specialized type of cells in differentiation zone of the root. The auxin gradient such as those established by auxin transport proteins are of crucial importance for stem cell maintenance and function (Zamioudis et al. 2013).

1.2 Plant-bacteria Interaction

The plant is host to a diverse community of microorganism including fungi, archaea, and bacteria, in particular, the root system of the plant is energy rich habitat for plant microbiome. Among the soil bacteria present inside the thin layer of soil around the plant (rhizosphere) are Plant growth promoting rhizobacteria (PGPR). PGPR are characterized due to their ability to stimulate the growth of plants in several ways through solubilizing nutrients such as phosphorus and iron, fixing atmospheric nitrogen, and producing phytohormones when grown in association with plant roots.

Postembryonic root development in the plant is affected by PGPR because these bacteria alter the cell division and differentiation within the primary root as well as affecting root hair formation and lateral root development. The survival of these rhizospheric bacteria depends upon their ability to move towards the exudates secreted by the plant roots. Root exudates include sugars, amino acids, organic acids, fatty acids, phenolics, enzymes, and flavonoids. The microbes, in turn, can modify the exudate composition, increase growth, and induce systemic resistance to pathogen attack (Adesemoye et al. 2009; Badri and Vivanco 2009; Phillips et al. 2004; Verbon and Liberman 2016; Zamioudis et al. 2013). These beneficial association of microbes with plants play essential roles in agriculture, food safety, and contribute to the environmental equilibrium.

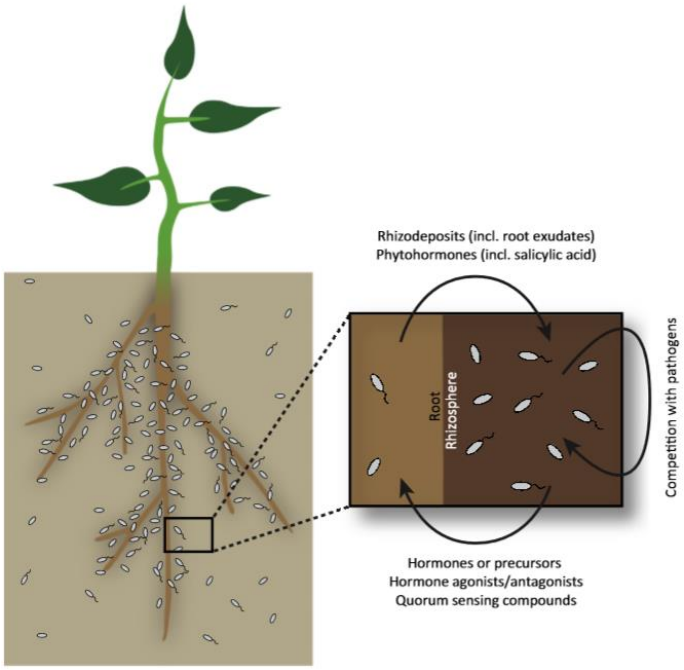


Figure 1-2-Effect of the plant on bacterial community within rhizosphere and the root (Verbon and Liberman 2016).

However, a clear line should be drawn between bacteria that reside in the rhizosphere; and bacteria that habitat inside the plant called endophytes. These endophytes live in plant tissue beneath epidermal cell layers and colonize the internal tissues and form a different kind of association with the host including symbiotic, mutualistic and trophobiotic. They are present everywhere and can colonize particular plants as host with highest densities in root and lowest densities from stem to leaves. Approximately all the endophytes have their origin from epiphytic bacterial communities in the rhizosphere, phyllosphere or other plant parts (Abbamondi et al. 2016; Bacon and Hinton 2007; Ma et al. 2016).

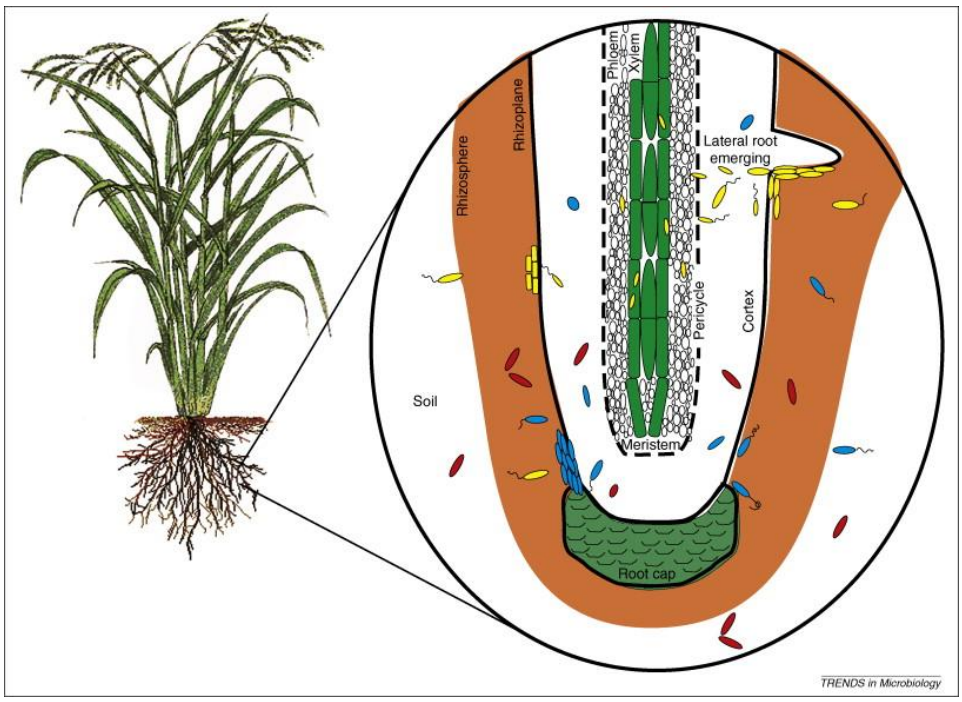


Figure 1-3- Different types of endophytic bacteria inside plant roots. Soil inhabiting bacteria which become endophyte through invasion are passenger endophytes (red cells) and are often restricted to the root cortex tissue. Opportunistic endophytes (blue cells) depict particular root colonization characteristics. However, opportunistic endophytes are restricted to particular plant tissues (e.g. the root cortex). Competent endophytes (yellow cells) have same properties as opportunistic endophytes and are well adapted to the plant environment (Hardoim et al. 2008).

Plant growth promoting bacteria stimulate the plant growth via different methods i.e. phytostimulation, biocontrol, and biofertilization (Bloemberg and Lugtenberg 2001). Phytostimulators enhance plant growth directly by producing phytohormones auxins, gibberellins, and cytokinins. The hormone indole-3-acetic acid (IAA) helps by directly promoting the growth. Similarly, synthesis of 1-aminocyclopropane-1-carboxylate (ACC) deaminase can be included in the same group: ACC deaminase breakdown ACC which is immediate precursor of ethylene and hence reduces its biosynthesis. Ethylene plays a role in inhibiting the growth of roots and shoots. Therefore, reduced ethylene levels contribute to plant growth promotion (Abbamondi et al. 2016).

Biocontrol is a method to protect plants against pathogenic infections by phytopathogens. Plant growth promoting bacteria which have the ability to produce siderophores are considered as biocontrol agent because iron chelation limits the amount of trace metal to plant pathogens.

Bio fertilizing bacteria harbor the potential to fix the atmospheric nitrogen and enhance the availability of iron and phosphorus to the soil (Jin et al. 2014).

1.3 Tomato associated endophytes

Tomato is one of the most commonly used daily vegetables around the world, and it contains nutritional value comprised of carbohydrates, amino acids, minerals, and vitamins. Use of inorganic fertilizers has a significant effect on yield and nutrient content of tomatoes. Recent studies (Abbamondi et al. 2016) have shown the presence of some PGP bacteria inside the roots of different cultivars of tomatoes. We also used a tomato cultivar in this study to isolate endophytes from roots of tomato.

1.4 16S ribosomal RNA (16S rRNA)

16S rRNA is the component of small ribosomal subunit 30S of prokaryotic ribosome. The gene which encodes for this is called 16S rRNA gene. The 16S rRNA gene is used in the study of phylogenies because this gene region is highly conserved between bacteria and archaea. It is also believed that multiple sequences of 16S gene may exist within a single bacterium. 16S rRNA gene have highly conserved binding sites for primers and, most commonly used primers for 16S rRNA gene are 27F and 1492R (Weisburg et al. 1991). 16S rRNA gene analysis is most helpful for the identification of bacteria and also for reclassifying the microbial species. This is because of hypervariability in 16S rRNA gene sequence which can give information about species-specific signature sequences for identification of bacteria (Lu et al. 2009; Pereira et al. 2010).

During this project 16S rRNA, PCR was used to identify the bacteria which were isolated from the roots of tomato cultivar namely *Solanum pimpinellifolium*.

1.5 *Pseudomonas fluorescence*

One of the highly abundant bacterial genera of root microbiome is *Pseudomonas* spp. It is well established that *Pseudomonas* helps in promoting plant growth. This bacteria is known to increase fresh shoot weight by 30% when co-cultivated in soil. Recent studies also suggest that *Pseudomonas* increases the growth of lateral roots and root hairs while inhibiting the primary root growth (Abbamondi et al. 2016; Wintermans et al. 2016). Therefore this bacteria is a

suitable candidate to study the effect of PGP on root architecture in wild-type *Arabidopsis* and the auxin impaired mutants (*aux1-7*, *pin2*,).

1.6 Aim of the study

The aim of this study was:

- To evaluate the effect of microbial inoculation on the root system of *Arabidopsis thaliana* and its mutant impaired in auxin transportation and signaling by using the vertical agar plates (VAP) method as employed by (Abbamondi et al. 2016).
- To isolate the endophytic bacteria from roots of a tomato plant (*Solanum pimpinellifolium*) growing under hydroponic conditions.
- To determine the effect of isolated bacterial strains on root growth of *Arabidopsis* and its mutant *pin*2 using VAP plate method. To check the effect of inoculated isolated strains on tomato plant growth when grown in pot experiment.
- To better understand the effect of plant growth promoting bacteria such as *Pseudomonas fluorescence* to enhance root growth of *Arabidopsis*, mutant *pin2* and *aux1-7* under two different MS media conditions (With sucrose, without sucrose).

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant Material

Arabidopsis thaliana is a commonly used model organism for laboratory work with plants. For this study, four types of *Arabidopsis* mutants were used; *pin2*, *tir1*, *aux1-7* and *wei8/wei2*. All work was done on plants of the species *A.thaliana*. Plants sown on soil were watered using 1 X Hoagland solution.

2.1.2 Mutant seeds

Mutant seeds of *A.thaliana* utilized for this study were received from Dr, Franck Ditennyou and Dr. Ivan Paponov at the Albert-Ludvig-Universitat Freiburg, Gemany. The seeds were mutant in auxin signal perception, auxin transportation and auxin synthesis (*tir1, pin2, aux1-7* and *wei8 wei2*). To determine the effect on root growth mutant seeds were inoculated with the bacterial strains (9, 15, 5, 6, 10, 16, 18 and CL8) known to have plant growth promoting properties namely indole acetic acid (IAA), ACC deaminase activity and siderophore production.

2.1.3 Bacterial Strains

A total of 8 bacterial strains isolated from tomato cultivars were received from Hasselt University, Environmental Biology, and Centre for Environmental Sciences, Belgium. The bacterial strains were described in the article (Abbamondi et al. 2016). One bacterial strain, *Pseudomonas fluorescence* (WCS417) was obtained from Come. MJ Pieterse at the Centre for BioSystems Genomics, 6700 AB Wageningen, the Netherlands. One bacterial strain CL8 was isolated in lab previously from tomato, *Solanum lycopersicum*, cv. Heinz. Also three bacterial strains isolated from roots of *Solanum pimpinellifolium* were tested on *Arabidopsis thaliana* (Col-0), mutant *pin2* and *aux1-7*. The bacterial strains are mentioned in table 2-1.

Table 2-1. Different bacterial strains utilized during the project.

Bacterial Strains (Abbamondi)	Bacterial strain (Netherlands)	Bacterial strains (Solanum penelli)	Bacterial Strain (Lillo's lab)
Pseudomonas spp. (9)	*Pseudomonas fluorescence* (WCS417)	*Micrococcus yunnanensis*	CL8
Agrobacterium spp. (18)		*Bacillus flexis*	
Rhizobium spp. (16)		*Cohnella* spp.	
Agrobacterium spp. (15)			
n.p* (5)			
n.p* (6)			
n.p* (10)			

n.p: Genotypic Characterization not performed, due to cultivation problem with standard media (LB with standard NaCl concentration and modified, NaCl 0.4% w/v) in (Abbamondi et al. 2016).

2.1.4 Plant Growth medium

The plants were grown on two different nutritional media during the project. During first five months (August-December 2016) plants were grown on 1/50 X Gamborg's B5 medium. Later the plants were grown on 1 X Murashige and Skoog agar solidified medium (February-April 2017). Plants growth was evaluated under two different conditions, growth media with sucrose and without sucrose

2.1.5 Gamborg B5 medium

1 X Gamborg stock solution (Table 2-2) was made and diluted 50 times to make nutrient media for plant growth. The media contained the following macronutrients: 0.5 mM KNO_3, 0.02 mM $MgSO_4.7H_2O$, 0.02 mM $CaCl_2.6H_2O$, 0.022 mM $NaH_2PO_4.H_2O$, 0.02 mM $(NH_4)_2SO_4$, Fe-EDTA and micronutrients as KI 90 nM, H_3BO_3 0.97 µM, $ZnSO_4.7H_2O$ 0.14 µM, $CuSO_4.5H_2O$ 2 nM, $CoCl_2$.6H2O, $Na_2MoO_4.2H_2O$ 20nM. To make 1/50 X Gamborg 20 ml from 1 X Gamborg stock solution was diluted in 980 ml of dH_2O. 0.78 g of 2.56 mM MES-buffer was added, and pH of the medium was adjusted to 5.7 with KOH. Media was divided into four bottles. Sucrose is added to only one bottle, and remaining bottles were left without sucrose. In all bottles, phyto-agar was added to solidify the medium.

Table 2-2. Chemicals used in making Gamborg B5 medium. Concentration of chemicals in 1 L.

Chemicals	Concentration in 1 X Gamborg (Stock)	Final Concentration (1/50x)
Macronutrients		
KNO_3	24.8 mM	0.5 mM
$CaCl_2.6H_2O$	1 mM	0.02 mM
$MgSO_4.7H_2O$	1 mM	0.02 mM
$(NH4)_2SO_4$	1 mM	0.02 mM
$NaH_2PO_4.H_2O$	1 mM	0.02 mM
Fe-EDTA	10 mM	0.2 µM
Micronutrients		
KI	0.45 mM	90 nM
H_3BO_3	4.85 mM	0.97 µM
$ZnSO_4.7H_2O$	0.7 mM	0.14 µM
$Na_2MoO4.2H_2O$	0.1 mM	20 nM
$CuSO_4.5H_2O$	0.01 mM	2 nM
$CoCl_2.6H_2O$	0.017 mM	2 nM

2.1.6 Murashige and Skoog (MS) medium

1 X MS medium solution was prepared from already available stock solutions A, B, C, D, E, Minor I and Minor II in Lab. In total 144 ml of these solutions and 1 ml of the vitamin (Sigma-Aldrich) was added to 400 ml of water and after mixing pH was adjusted by adding 5 M NaOH to 5.8. After adjusting the pH, dH_2O was added to make the total solution of 1 L. The solutions were added in the order explained in table 2-3. The solution was divided into four bottles, and 0.5% sucrose (5g/L) was added to one bottle and remaining three bottles were left without sucrose. Agar-Agar was added to all bottles to solidify the MS medium.

Table 2-3. Order of chemicals, their concentration in 1 l (Stock) and their volume used in making 1 X MS medium.

Order	Chemical	Concentration	Volume in 1 X MS
A	KNO_3	1 M	20 ml
B	HN_4NO_3	1.5 M	13 ml
C	$MgSO_4.7H_2O$	0.15 M	10 ml
D	KH_2PO_4	0.12 M	20 ml
E	$CaCl_2.2\ H_2O$	0.2 M	10 ml
Minor I			10 ml
Minor II			10 ml
Fe/EDTA	Fe/EDTA	1 mM	50 ml
Vitamin	Vitamin (Sigma)	1000X	1 ml

Table 2-4. Review of chemicals used for Minor I Solution, 1 l.

Chemical	Amount
$ZnSO_4.7H_2O$	0.920 g
H_3BO_3	0.620 g
$MnSO_4.4H_2O$	2.230 g

Table 2-5. Review of chemicals used for Minor II Solution, 1 l.

Chemical	Amount
$NaMoO_4.2H_2O$	0.025 g
$CuSO_4.5H_2O$	0.003 g
$CoCl_2.6H_2O$	0.003 g
KI	0.0083 g

2.1.7 King's B Agar medium

The recipe of the Kings B agar medium is shown in the table 2-6. The King's B agar was dissolved in distilled water and glycerol was added. The media was autoclaved properly, and when cooled down rifampicin (50 ug/mL) was added. In order to make 1M King's B agar medium, 33 g of King's Agar is dissolved in 990 ml of dH_2O with the addition of 10 ml of glycerol. Here is given the amount/volume of chemicals for making media in 400ml of H_2O (Table 2-6).

Table 2-6. Preparation of King's B Agar medium.

Chemical	Amount
King B Agar (Sigma-Aldrich)	13.2 g
Distilled water (dH_2O)	396 ml
Glycerol	4 ml
Rifampcin (Sigma-Aldrich)	400 ul

2.1.8 Hoagland plant nutrient solution

To prepare Hoagland solution, the chemicals of the micronutrient solution (Table 2-8) were dissolved in 1 l water. Water (2 l) was added to an empty bottle (5 l). The chemicals listed in table 2-7 were added in the following order; A, B, C, D, E, and micronutrients. Water was added to reach a total volume of 5 l, giving 10 X Hoagland solution. 50 ml of 10 X Hoagland was added to 950 ml of water for 0.5 X Hoagland solution.

Table 2-7. Chemicals used for Hoagland solution.

Order	Chemical	Amount	Nutrient concentration in 1x Hoagland
A	KH_2PO_4 (1M)	50 ml	1 m MPO_4-
B	KNO_3 (1M)	250 ml	5 mM NO_3-
C	$Ca(NO_3)_2.4H_2O$ (1M)	250 ml	10 mM NO_3-
D	$MgSO_4.7H_2O$ (1M)	100 ml	2 mM Mg++
E	Fe/EDTA, 1% (1M)	50 ml	-
Micronutrients		50 ml	-

Table 2-8. Chemicals used for 1 l. Micronutrient solution.

Chemical	Amount in one liter
H_3BO_3	2.86 g
$MnCl_2 \cdot 4\ H_2O$	1.81 g
$CuSO_4 \cdot 5\ H_2O$	0.089 g
$ZnSO_4 \cdot 7H_2O$	0.22 g
$H_2MoO_4 \cdot 1H_2O$	0.029 g

2.1.9 Luria Bertani low salt (Broth)

To grow overnight bacterial culture low salt LB broth was made in distilled water. To make LB media in 300 ml of dH_2O, 4.65 g of LB broth powder was added. The solution was mixed and autoclaved before use.

2.1.10 Ca-hypochlorite solution 1% (w/v)

The Ca-hypochlorite solution was made to sterilize the seeds. To make 1% Ca-hypochlorite solution, 25 ml of dH_2O was taken and 0.25 g of Ca-hypochlorite was added. One drop of Tween 20 was added and the solution was mixed and allowed to settle. 1 ml of this Ca-hypochlorite

solution was diluted in 9 ml of 95% ethanol. The formed solution was used to surface sterilize the seeds.

2.2 Methods

2.2.1 Experiments with strains used in Abbamondi's study

Effect of bacterial strains (see Table 1, section 2.1.3) received from Belgium was determined using vertical agar plate (VAP) assay as explained in (Abbamondi et al. 2016). To see the effect on root development of wild-type as well as the mutants, bacteria were inoculated on VAP.

2.2.2 Seed sterilization

Arabidopsis (Col-0) seeds and mutant *pin2*, *aux1-7*, *tir1*, and *wei8/wei2* were surface sterilized in 1% (w/v) Ca-hypochlorite solution. One drop of Tween 20 was added to the solution and allowed to settle for a few minutes. 1 ml of Ca-hypochlorite solution was mixed with 9 ml of 95% ethanol. Seeds were allowed to let stand in Ca-hypochlorite/ethanol solution for 3 minutes followed by three-time washing with 95% ethanol. Ethanol was removed from eppendorf tubes containing seeds and seeds were allowed to dry in laminar flow hood overnight.

2.2.3 Growing seeds on Gamborg B5 medium

Sterilized toothpick was utilized to sow the seeds on 1/50 X Gamborg B5 medium supplemented with 0.5% sucrose and later also on 1 X MS media supplemented with sucrose (1% sucrose). The vertical agar plates were kept at 4℃ in the dark. After a period of 3 days, plates were transferred to the growth chamber under 16 h light/8 h darkness period at 22 ℃ for 5 or 6 days for seeds to germinate. After 5/6 days, plantlets growing on the surface of agar nutrient media were transferred to fresh growth medium (Gamborg B5 medium without sucrose).

2.2.4 Bacterial Culture

During range of experiments, different bacterial cultures were made. Among the cultures used mostly in experiments included strain 9 and 15. Overall, bacterial culture of strains 5, 6, 9, 15, CL8, 10, 16 and 18 were made in LB broth (low salt). To make broth media, 4.65 g of LB low

salt broth powder (Sigma-Aldrich®) was dissolved in 300 ml of distilled H_2O and media was autoclaved. Under sterile conditions, bacterial colonies were added to the broth media in falcon tubes (8 ml) and incubated overnight (24 h) at 30℃ with shaking at 120 rpm.

2.2.5 Bacterial inoculation experiments (August-December 2016)

To observe the effect of bacterial inoculation on root development of *Arabidopsis thaliana* seeds (Col-0 type) and its mutants impaired in auxin transport (*pin2, aux107, tir1, and wei8/wei2*), bacterial strains were inoculated to different mutants in a number of experiments. Bacterial strains 5, 6, 9, 15, CL8, 10, 16 and 18 received from Hasselt University, Environmental Biology, and Centre for Environmental sciences, Belgium were tested. A vertical agar plate assay (VAP) was made of 50 folds dilution of Gamborg's B5 medium as explained in article (Abbamondi et al. 2016). Medium composition explained in table 2-2 section 2.1.5 of materials. The optical density of overnight grown culture was determined to be 0.5. The bacterial culture was centrifuged at 4000 rpm for 20 min, and the supernatant was discarded. Centrifugation followed by washing with 10 mM $MgSO_4$ twice at 4000 rpm for 5 min. The bacterial pellet was resuspended in 650 ul of 10 mM $MgSO_4$. 650ul of bacterial culture (in few experiments 1 ml) was inoculated to the Gamborg B5 medium plates without sucrose. Bacterial culture was spread all over the plates equally. Control plates were supplemented with 650 ul of 10 mM $MgSO_4$. The 5 d (or 6 d in few experiments) grown plantlets of *Arabidopsis* and its mutant were transferred to the VAP without sucrose. Plates were incubated at 22 ℃ vertically in a growth chamber with a 16-h light/8-h dark period for eight days.

2.2.6 Root growth analysis

After eight days of growth in a growth chamber, the plates were taken out and root growth was analyzed. Pictures of plates were taken using a digital camera and root hairs images were captured with the help of a microscope (Leica Microsytems). Number of lateral roots were counted and primary root length was measured using ImageJ software.

2.3 Bacterial Isolation from *Solanum pimpinellifolium*

2.3.1 Isolation of endophytic bacteria from roots

Roots of tomato cultivar *S.pimpinellifolium* growing in hydroponics in a growth chamber under light/dark 12 h condition were cut in the middle and taken in a 50 ml falcon tube. After weighing (0.5 g), 25 ml of sterilized phosphate buffer saline (PBS) (Per 400ml: 2.53g of $NaH_2PO_4.H_2O$, 6.6g of $Na_2HPO_4.7H_2O$; 80ul Tween 40) was added to falcon tube. The roots were washed five times with PBS buffer. Then roots were treated with 1% (w/v) Ca-hypochlorite solution for 3 min to remove surface microorganisms. Roots were washed then five times with distilled H_2O, and last wash was used to check the sterility of roots by spreading on LB agar media modified with low salt conditions. After rinsing, roots were cut with sterilizing razor blade into smaller root fragments. Fragmented roots were homogenized in 5 ml of 10 mM $MgSO_4$. The crude extract of roots was taken in a 15 ml falcon tube. 1 ml of crude extract taken in 1.5 ml Eppendorf tube and series dilution 5x and 10x were made. 100µl of crude extract, 5x and 10x dilutions were spread on separate LB agar low salt media plates. Plates were kept in an incubator at 30 ℃ for the overnight culture of colonies. After 7 days incubation single colonies were selected and further purified on the basis of morphological differences.

2.4 Genomic analysis of isolates

2.4.1 DNA purification

Isolated bacterial colonies from tomato roots were cultured in LB broth low salt overnight at 30 °C and 120 rpm. Before subjecting to DNA purification, the concentration of bacteria from overnight culture was measured using a spectrophotometer. The overnight culture had a concentration of 10^8 cells/ml. For genomic DNA purification, DNeasy^R 96 blood and tissue kit (Qiagen) was used and protocols for pretreatment for gram + and gram - bacteria were followed as instructed in DNeasy blood and tissue kit handbook with the exception RNase-free water instead of elution buffer was utilized for elution of DNA. A qualitative and quantitative analysis of extracted DNA was performed using Nanodrop ND-1000 spectrophotometer.

2.4.2 PCR

To amplify DNA from the template strand PCR reaction was performed. A thermocycler is utilized to make sure the sample will go through the correct amplification step at the correct time. During this study, DNA extracted from 3 morphologically different bacteria were subjected to 16S PCR. The 16S ribosomal coding region was amplified by using universal enzymes 26F (AGAGTTTCATCCTGGCTCAG) with 1520R (AAGGAGGTGATCCAGCCGGA) and also with 1492R (GGTTACCTTGTTACGACTT). One µl of particular sample was transferred to the particular PCR tube. PCR program was set and PCR was allowed to run for 30 cycles.

2.4.3 PCR reaction mixture

Table 2-9. The PCR reaction mixture consisted of following components.

Components	Volume (ul)
10x Dream Taq Buffer	5 µl
dNTP mix, 2mM each	5 µl
Forward Primer (10 µM)	2.5 µl
Reverse Primer (10 µM)	2.5 µl
Template DNA	1 µl
Dream Taq DNA polymerase	0.4 µl
Water, nuclease free	33.6 µl
Total	50 µl

2.4.4 PCR Profile

Table 2-10. Temperature and time conditions during PCR.

Step	Temperature	Time
1, Initial denaturation	95˚C	3 min
2, Denaturation*	95˚C	30 sec
3, Annealing*	60˚C	30 sec
4, Extension*	72˚C	1:30 min
5, Final extension	72˚C	10 min
6, Hold	4˚C	∞

**Step 2, 3 and 4 were repeated 30 times in sequence.*

2.4.5 Agarose gel electrophoresis protocol

Agarose, 0.5 g, was used to make the gel in 50 ml 1 X TAE buffer. The agarose in 1 X TAE buffer was heated for 1 min for the agarose to dissolve completely; the solution was poured into gel cast and allowed to solidify for 30 min. In each sample of PCR product (10 µl), 1.5 µl of loading buffer and 1.5 µl of gel red was mixed. 5 µl of Bioline Hyperladder 1 kb was mixed with 1.5 µl gel red to determine the size of PCR product. The gel was run for 30 min at 90 V and then analyzed using UV light to visualize the DNA bands.

2.4.6 PCR clean-up, Gel extraction

Nucleospin® Gel and PCR Clean-up kit (Macherey-Nagel) was used to isolate pure DNA from the PCR product in order to sequence the bacterial DNA. PCR clean-up, Gel extraction user manual was used for DNA extraction from agarose gels.

2.4.7 Agarose gel electrophoresis (Big gel)

Agarose 1 g was used to make the gel in 100 ml of 1 X TAE buffer. The solution of agarose and 1 X TAE buffer was heated up to 1 min to dissolve agarose. When cooled a bit, the gel was poured into the gel cast, the comb was fixed and the gel was allowed to solidify for 20 min. Each sample prepared for running on gel had a volume of 40 µl mixed with 3 µl of loading buffer and 3 µl of gel red. Hyperladder 3 µl mixed with 3 µl gel red was used to ascertain the size of band. The gel was allowed to run for 40 min at 90 V. The bands on gel were visualized and cut under dual UV transilluminator.

2.4.8 DNA extraction from gel

DNA was extracted from the gel by following the protocols from the user manual Nucleospin® Gel and PCR Clean-up kit (Macherey-Nagel). Briefly, DNA was excised under minimal UV exposure and taken into clean 1.5 ml tube. Buffer NTI was added to 1.5 ml tube, and samples were incubated for short period (5-10 min) at 50 °C until the gel slice completely dissolved. Between the incubation period samples were vortexed for the gel bands to dissolve completely. Gel clean-up column was placed in a collection tube, and solution was centrifuged at 11,000 x g. Silica membrane was washed by adding 700 µL buffer NT3 to Gel clean-up column and

centrifuging it at 11,000 x g. Again for drying silica membrane the column was centrifuged for 1 min at 11,000 x g. Complete ethanol removal was achieved by incubating the columns for 5-10 min at 70 °C. DNA was eluted by adding water free of RNase and protease to clean-up column and incubating it and centrifuging it. A qualitative and quantitative analysis of extracted DNA was performed using Nanodrop ND-1000 spectrophotometer.

2.4.9 Gene sequence analysis

The DNA of the isolated bacteria from *Solanum pimpinellifolium* was sent for gene sequence analysis to Seqlab-Sequence laboratories in Gottingen GmbH, Germany. The 16S rRNA sequence results received were compared with sequences in GenBank database using NCBI Blast program.

2.5 *Pseudomonas* Culture and inoculation experiments

2.5.1 Experiments with *Pseudomonas fluorescens* (February-April 2017)

Bacteria *Pseudomonas fluorescens* (WCS417) received from Come. MJ Pieterse, the Netherlands was also used to test on plants to see if it produces a significant difference in root development in *Arabidopsis* Col-0 type and also the mutant *pin2* and *aux1-7*. Previously in (Zamioudis, Mastranesti et al. 2013), it was well established that WCS417 contributed in the promotion of lateral roots and root hairs in wild-type Col-0 and also the mutant *aux1-7*. Prior it was also confirmed that bacteria WCS417 also inhibits primary root length of Wild-type plants (Zamioudis et al. 2013). Seeds of *A.thaliana* Col-0 type were used as wild-type plants. The *pin2* and *aux1-7* were the mutants tested during these experiments. Seeds were surface sterilized (See section 2.2.2) and sown on 1 X Murashige and Skoog agar-solidified medium which was supplemented with 0.5% of sucrose for the plants to germinate. After stratification at 4 ℃ for 3 days the MS media plates were placed vertically under artificial light at a 16 h day length (100 mmol $m^{-2}s^{-1}$) and 22ºC for seedlings to germinate.

2.5.2 *Pseudomonas fluorescens* Culture

P.fluorescens was cultured in King's B agar medium containing rifampicin 50 µg/ml at 28 ℃. The cells were mixed in 10 ml of 10 mM $MgSO_4$ and collected in 1.5 ml eppendorf tubes. The

cells were washed twice in 10 mM $MgSO_4$ at 5000 g for 5 min and resuspended in 10 mM $MgSO_4$ as explained in (Zamioudis et al. 2013). The O.D of culture was brought to 0.002 (10^6 cells/ml). 400 ul of WCS417 culture was inoculated on 1 X MS plates with no sucrose. In few experiments, a similar amount of culture was added to 1 X MS plates with sucrose (0.5 %). Control plates had 400 ul of 10 mM $MgSO_4$. The plantlets from 1 X MS media with sucrose were transferred to the plates without sucrose and also to the plates with sucrose. Agar solidified MS plates were then incubated at 22 ℃ in growth chamber under 16 h light/8 h dark period. After 8 d, plates were removed from growth chamber to analyze effect on root growth.

2.5.3 Root growth analysis

To measure primary root length digital images of seedlings of *Arabidopsis* were captured using a digital camera. Primary root length of at least 15 seedlings was measured by using software ImageJ. The total number of emerged lateral roots on each seedling was counted for at least 15 seedlings. To determine and analyze the growth effect of root hairs, seedlings were visualized under microscope (Leica) and pictures were taken. For shoot fresh weight measurements, seedling were cut at root-shoot junction, and 5 seedlings from a plate were immediately placed on an analytical balance to determine the weight.

2.6 Evaluation of effect of new isolates on root growth

2.6.1 Experiments with the isolates of *Solanum pimpinellifolium*

During this project, a total of 3 bacterial strains were isolated from roots of *S.pimpinellifolium* (see table 2.1.3). Similar experiment was performed with the new isolates, and their effect on *Arabidopsis* wild-type (Col-0) and its mutant *pin2* was determined. Seeds were grown on 1 X MS medium following similar methods and conditions as explained earlier in section 2.5.

2.6.2 Bacterial isolates (*Solanum pimpinellifolium*) culture and inoculation on 1 X MS medium

Two out of three bacterial strains namely *Micrococcus yunananensis*, *Cohnella* spp. were cultured in low salt LB broth media. LB broth, 5 ml was taken in small falcon tubes and in each

separate tube three of different bacterial colonies were inoculated with the help of sterilized loop. The culture was incubated at 28 ℃ and 120 rpm in the incubator overnight. Due to slow overnight growth, the bacteria *Cohnella* spp. was incubated separately at 30℃ and 150 rpm in the separate incubator. To spread bacteria on 1 X MS plates (without sucrose and also with sucrose), bacterial culture was washed in 10 mM MgSO4 twice at 5000 x g for 5 min. The pellet was resuspended in 1 ml 10 mM $MgSO_4$. O.D of culture was brought to 0.002 before inoculating on plates. 400 ul of culture was added to plates with sucrose (0.5%) and also plates without sucrose. Fresh 5 d grown plantlets of WT Col-0 and *pin2* were transferred to plates supplemented with bacteria. Control plates only had 400 ul of 10 mM MgSO4. All the Plates were kept in growth chamber 16-h light/8-h dark period and at 22 ℃ for 8 days.

2.6.3 Influence of bacterial isolates on Gemini tomatoes growth

To examine the effect of tomato (*Solanum pimpinellifolium*) isolated bacterial strains on the plant growth, the pot experiment was designed to inoculate Gemini tomatoes with the strains *M. yunnanensis* and *Cohnella* spp. Gemini tomato seeds were received from L.O.G. As (Oslo, Norway). In total 15 Gemini tomato plants growing on petri dishes were transferred to the pots containing vermiculite. Hoagland solution of 0.5 X concentration was given off to plants to maintain the nutrients for growth. After one week of growth, plants were inoculated with bacterial culture.

2.6.4 Bacterial treatment

To inoculate the plants, bacterial culture of *M.yunnanensis* and *Cohnella* spp. was made in LB low salt broth media and kept overnight in incubator at 30 ℃ and 120 rpm for *Micrococcus* and 150 rpm for *Cohnella.* The bacterial pellet was harvested by centrifuging the culture in falcon tubes at 5000 rcv for 2:30 min. Subsequently, the pellet was washed twice at 5000 rcv for 5 min and resuspended in 10 ml of 10 mM $MgSO_4$. The concentration of culture was brought at 10^8 cells/ml by dilution in 10 mM $MgSO_4$, and 100 ml of bacterial culture was added to each pot. In total, experiment contained 15 pots, 5 pots supplemented with *Micrococcus* and 5 pots with *Cohnella*. Each control pot had 100 ml of 10 mM $MgSO_4$.

2.6.5 Analyzing bacterial effect on Gemini growth

After the period of 4 weeks plants were taken out of plant room and pictures of plants were taken. Shoot length of all the plants was measured by using ruler. To measure the shoot fresh weight, shoots were cut at root and shoot junction and immediately weighed on analytical balance.

3 Results

3.1 Experiments with bacterial strains from Abbamondi's study

3.1.1 Inoculation of bacteria on *Arabidopsis* plants

Different bacterial strains employed by Abbamondi and collaborators in their study were tested during this experiment on *Arabidopsis* Col-0 type plant. During this experiment significant increase in primary root length was observed for bacterial strain CL8, 10 and 16 as compared to control (Figure 3-1, Table 3-1). Strain 9 and 15 show root length comparable and less than control but the data is not significant (Figure 3-1). All other strains shown to have more primary root length than control but not in significant way. The data for lateral roots show significant increase in number of lateral roots for strains CL8, 6 and 18. Strain 9 and 15 show number of lateral roots comparable and less than control (Figure 3-1, Table 3-2). The data for all the other strains was comparable to control but not significant for the increase in number of lateral roots. A striking effect was the increase in root hairs as observed under microscope. All the inoculated strains have affected the root hairs growth except strain 15 where the root hairs are more like control. Strain CL8, 6, 10 and 18 have more influence on growth of root hairs (Figure 3-2). The results were however only reproducible for strain 9 and 15. In one experiment (See appendix A-1) only strain 9 and 15 are shown to similar results as this experiment. However the data for these strains is not much significant statistically.

On the basis of reproducibility it is assumed the strain 9 and 15 (Figure 3-1 and Appendix A-1) have less effect on number or lateral roots and also the primary root length. It is assumed that strains 9 and 15 inhibits the root growth. Similar results for bacterial strain 9 and 15 were also observed by other two Master students (Iren & Samuel). In this experiment CL8 effected primary root length, number of lateral roots and root hairs we can say that CL8 increases the root growth of plant (Figure 3-1 and Figure 3-2). Results show that almost all the bacterial strains enhance root hair growth (Figure 3-2).

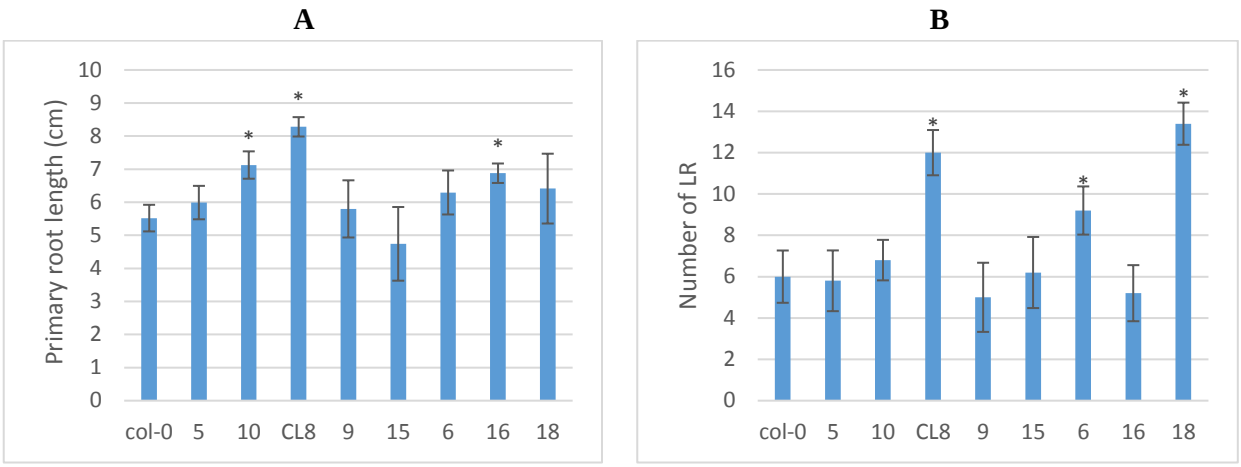


*Figure 3-1- Effect of bacterial inoculation on Arabidopsis Col-0 root development. **A** shows effect on primary root length, **B** shows number of lateral roots. The data is of the seedlings grown on 1/50x Gamborg's medium supplemented with 0.5% sucrose for 6 d, after 6 d seedlings were moved to plates without sucrose and 650 µl of bacterial culture was spread on whole plate. Plates were kept for 7 d in growth chamber. Error bars indicate ± Standard deviation (n=5). Asterisks indicate statistically significant differences compared with Control (Student's t test; P < 0.05)*

Table 3-1. VAP-test: Average primary root length. SD; Standard deviation.

Bacterial Type	No. of Seeds	Average primary root length (cm)	SD	P-value
Control	5	5.520686	0.402244	
5	5	5.98963455	0.505566	0.2
10	5	7.125221434	0.413786	0.0002*
CL8	5	8.281034884	0.292916	0.0001*
9	5	5.798751852	0.864793	0.5
15	5	4.742085004	1.114322	0.1877
6	5	6.295562273	0.665435	0.0513
16	5	6.877911157	0.294035	0.0003*
18	5	6.41248	1.055807	0.1090

*Statistical analysis; *Significant Values.*

Table 3-2. VAP-test: Number of lateral roots. SD; Standard deviation.

Bacteria type	No. of plants	Average Number of lateral roots	SD	P-value
Control	5	6	1.264911	
5	5	5.8	1.469694	0.8224
10	5	6.8	0.979796	0.2392
CL8	5	12	1.095445	0.0001*
9	5	5	1.67332	0.3042
15	5	6.2	1.720465	0.8391
6	5	9.2	1.16619	0.0031
16	5	5.2	1.356466	0.3611
18	5	13.4	1.019804	0.0001*

*Statistical analysis; *Significant Values.*

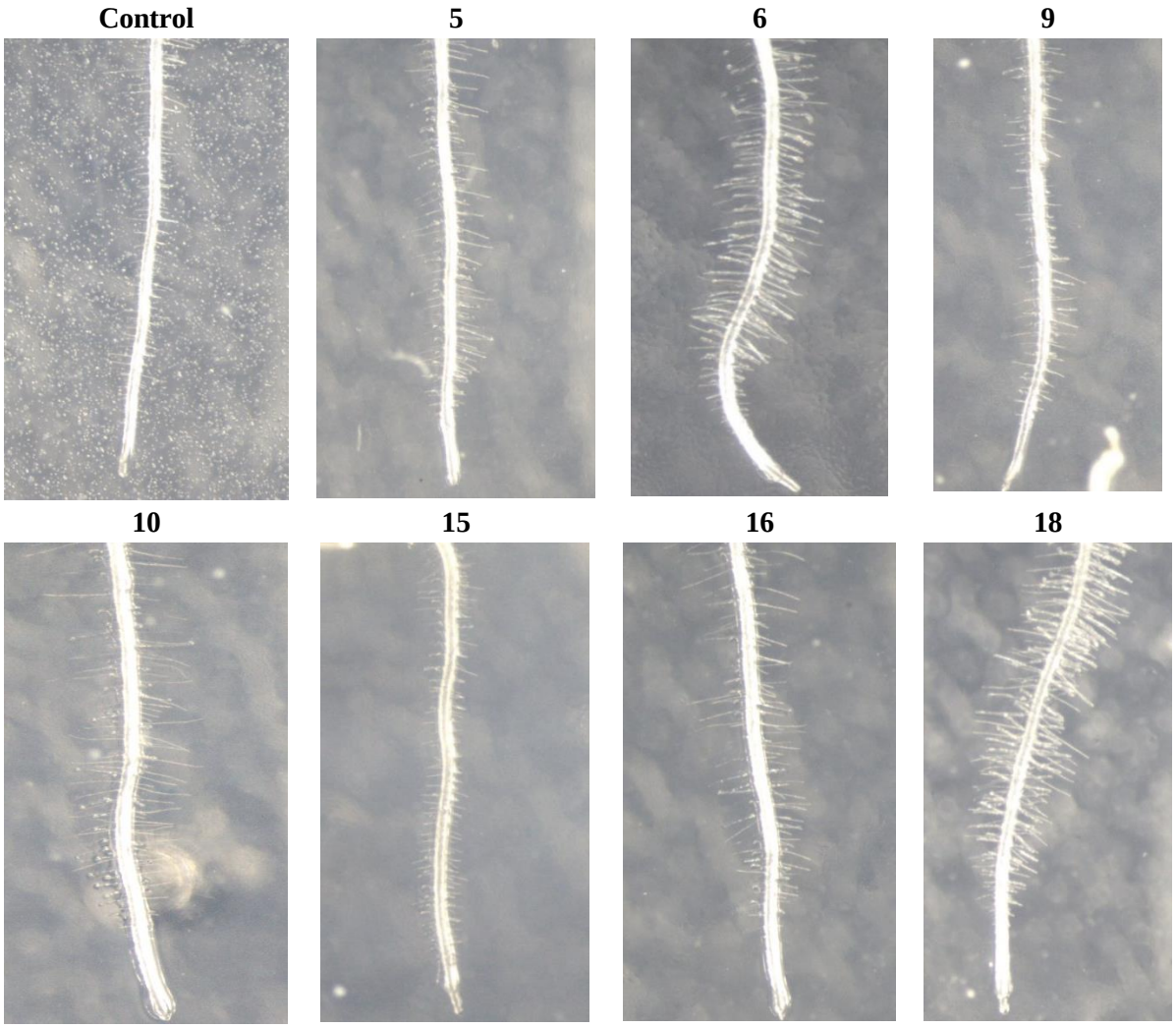


Figure 3-2- Vertical agar plate (VAP) test. Increase in root hairs due to inoculation of different bacterial strain as compared to the control.

3.1.2 Effect of bacterial inoculation on Col-0, *pin2* and *wei8/wei2*

In this experiment bacterial strain 9 and 15 were inoculated to Col-0, *pin2* and *wei8/wei2* to see effect of bacteria on root growth. The results show that after 8 days of inoculation, bacterial species inhibited primary root length in Col-0, *pin2* and *wei8/wei2* respectively (Figure 3-3). The data is significant for strain 9 when compared with control of wild type and also control of mutant *wei8/wei2.* The number of lateral roots were also reduced in bacterial treated seedlings in wild type as well mutant type. A significant reduction in number of lateral roots is observed in Col-0 plants when they are treated with bacteria 9 and 15 (Figure 3-3, Table 3-4). Figure 3-4 shows increase in amount of root hairs in all seedlings treated with the bacteria. This experiment showed the almost similar results for Col-0 plants as in previous experiment (See sub-section 3.1.1, Figure 3-1), that is inhibition of primary root length and reduction in number of lateral roots.

On the basis of this experiment it is assumed that strain 9 and 15 inhibit the primary root length and number of lateral roots but enhances root hairs growth in WT Col-0 and mutant *pin2* and *wei8/wei2.*

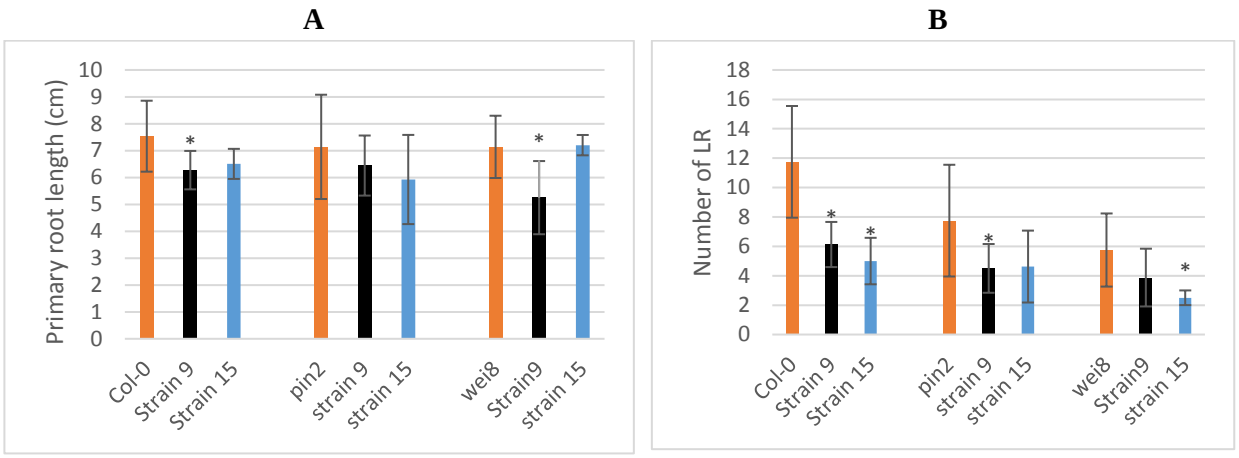


*Figure 3-3- Influence of bacteria 9 and 15 on root growth of Arabidopsis Col-0 type, mutant pin2 and wei8/wei2. **A** shows primary root length comparison between control and bacteria treated plants in wild type (Col-0) and pin2 and wei8. **B** shows average number of lateral roots per plant. Orange bars; Control group, Black bars; strain 9 treated and Blue bars; strain 15 treated seedlings. Seedlings were grown on 1/50x Gamborg medium supplemented with 0.5% sucrose for 6 d, after 6 d seedlings were moved to plates without sucrose and 650 µl of bacterial culture was spread on whole plate. Plates were kept for 7 d in growth chamber. Error bar indicate ± standard deviation (n=8). Asterisks indicate statistically significant differences compared with Control (Student's t test; $P < 0.05$).*

Table 3-3. VAP-test: Average primary root length. SD; Standard deviation.

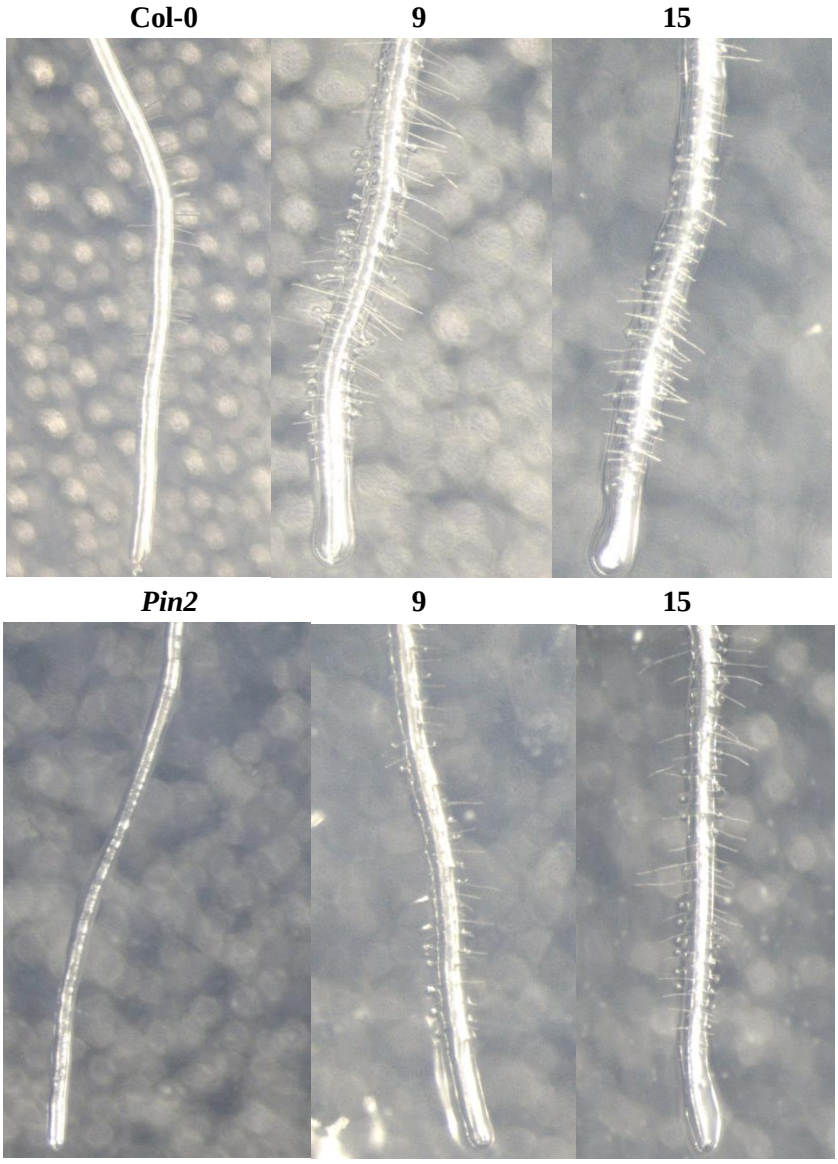
Bacterial Type	No. of Seeds	Average primary root length (cm)	SD	P-value
Col-0	8	7.536663	1.320247	
Strain 9	8	6.273765	0.714868	0.0259*
Strain 15	8	6.507206	0.559063	0.0617
pin2	8	7.139794	1.939847	
Strain 9	8	6.444402	1.11768	0.3824
Strain 15	8	5.925529	1.659197	0.1934
wei8	8	7.14026	1.15823	
Strain 9	8	5.250867	1.361283	0.0092*
Strain 15	8	7.203347	0.378229	0.8183

*Statistical analysis; *Significant Values.*

Table 3-4. VAP-test: Number of lateral roots. SD; Standard deviation.

Bacterial Type	No. of Seeds	Number of LR	SD	P-value
Col-0	8	11.75	3.799671	
Strain 9	8	6.125	1.536026	0.0016*
Strain 15	8	5	1.581139	0.0004*
pin2	8	7.75	3.799671	
Strain 9	8	4.5	1.658312	0.0423*
Strain 15	8	4.625	2.446298	0.0685
wei8	8	5.75	2.487469	
Strain 9	8	3.875	1.964529	0.0931
Strain 15	8	2.5	0.5	0.0022*

*Statistical analysis; *Significant Values.*



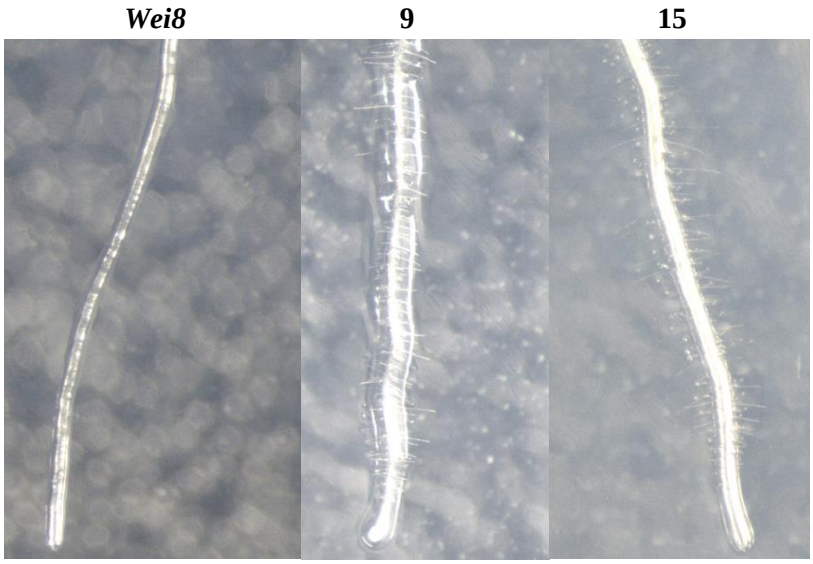


*Figure 3-4-**:** Effect of bacteria 9 and 15 on root hair growth of Col-0, pin2 and wei8 after 8 d of growth. From left is Control groups of Col-0, pin2 and wei8, middle; inoculation with strain 9 and right; inoculation with strain 15.*

3.1.3 Bacterial inoculation to Auxin mutants

During this experiment bacterial strain 9, 15 and also CL8 were tested on different mutant seeds. The effect on root growth was observed after 8 d growth in growth chamber. The primary root length of the wild type (Col-0) as well as the mutants was observed to be less or comparable with the control when inoculated with bacteria in most of cases (Figure 3-5). Only in *pin2* seedlings, when inoculated with the CL8 strain an increase in primary root length was observed. Similar results were obtained for lateral roots when treated with bacteria that is less number of lateral roots as compared to control groups. A significant difference in root hairs was observed because of treatment with bacteria in all seedlings as compared to their counterpart control group (Figure 3-6). With strain 9 and 15 this experiment showed the similar results for primary root length for Col-0, *pin2* and *wei8*/*wei2* as stated in previous experiment (see sub-section 3.1.2, Figure 3-3). Similar bacterial effect was observed on number of lateral roots in wild type and mutants with respect to the previous experiment (see sub-section 3.1.2, Figure 3-3). Only strain 9 showed an increase in number of lateral roots in Col-0 during this experiment.

According to the results of this experiment (Figure 3-5) it can be assumed that the bacterial inoculation did not have significant growth promoting effect on primary roots and number of lateral roots. The bacterial strain 9 and 15 showed overall decrease in primary root length and number of lateral roots in wild type and mutants. All the inoculated strains effected root hair growth in positive way in wild type and mutants but CL8 stimulated the root hair growth significantly as compared to the control plants. The number of plantlets varied among control and bacteria treated plates due to irregular growth and thus limiting the results.

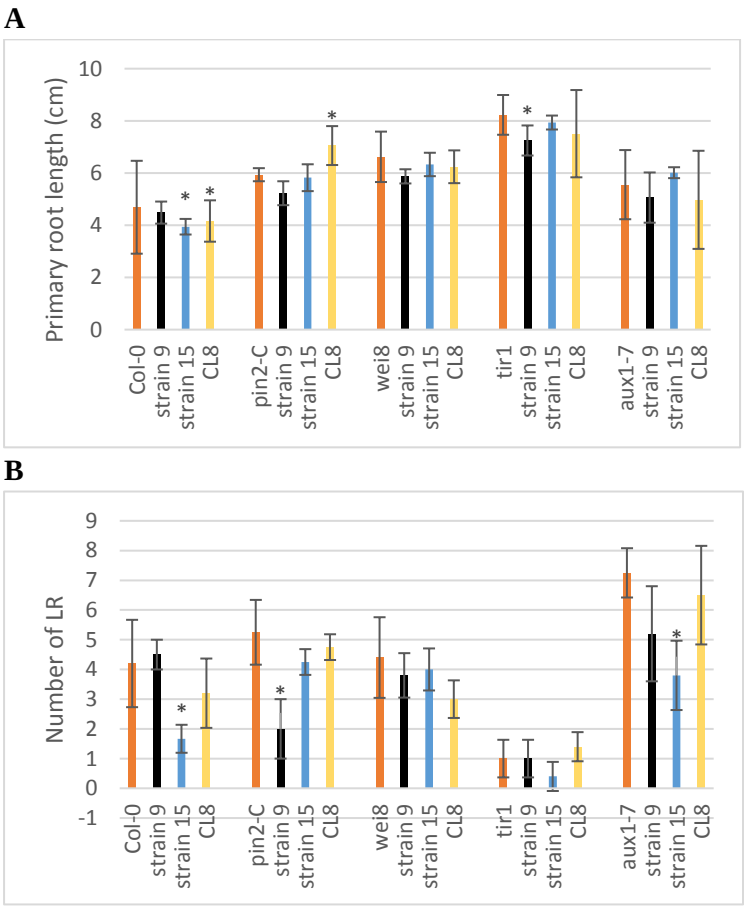


*Figure 3-5- Bacterial effect on root growth of Arabidopsis Col-0 type, mutant pin2, wei8, tir1 and aux1-7. **A** shows primary root length comparison between control and bacteria treated plants in wild type (Col-0), pin2, wei8, tir1 and aux1-7. **B** shows average number of lateral roots per plant. Orange bars; Control group, Black bars; strain 9 treated, Blue bars; strain 15 and yellow bars represent strain CL8 treated plants. Seedlings were grown on 1/50x Gamborg medium supplemented with 0.5% sucrose for 6 d, after 6 d seedlings were moved to plates without sucrose and 650 µl of bacterial culture was spread on whole plate. Plates were kept for 8 d in growth chamber. Error bar indicate standard deviation (n= Number of plants varied among different treatments because of irregular growth). Asterisks indicate statistically significant differences compared with Control (Student's t test; P < 0.05).*

Table 3-5. VAP test: Measurement of primary root lengths after 8 days in growth chamber. SD; Standard deviation.

Bacteria type	No. of Plants	Average primary root length (cm)	SD	P-value
Col-0 (Control)	4	5.5	0.54	
9	2	4.4	0.42	0.0684
15	3	3.9	0.29	0.0059*
CL8	4	4.4	0.45	0.0203 *
***pin2* (Control)**	4	5.8	0.25	
9	2	5.1	0.45	0.0608
15	4	6.1	0.51	0.3315
CL8	4	6.9	0.74	0.0305*
***tir1* (Control)**	5	8.2	0.76	
9	5	7.2	0.57	0.0464*
15	5	7.9	0.26	0.4279
CL8	4	8.3	0.27	0.8112
***aux1-7* (Control)**	4	5.5	1.47	
9	5	5.3	0.44	0.8321
15	5	5.9	0.20	0.5598
CL8	3	5.6	1.56	0.9341
***wei8* (Control)**	5	6.5	0.96	
9	5	5.8	0.27	0.1552
15	4	6.2	0.44	0.5847
CL8	4	6.2	0.62	0.6074

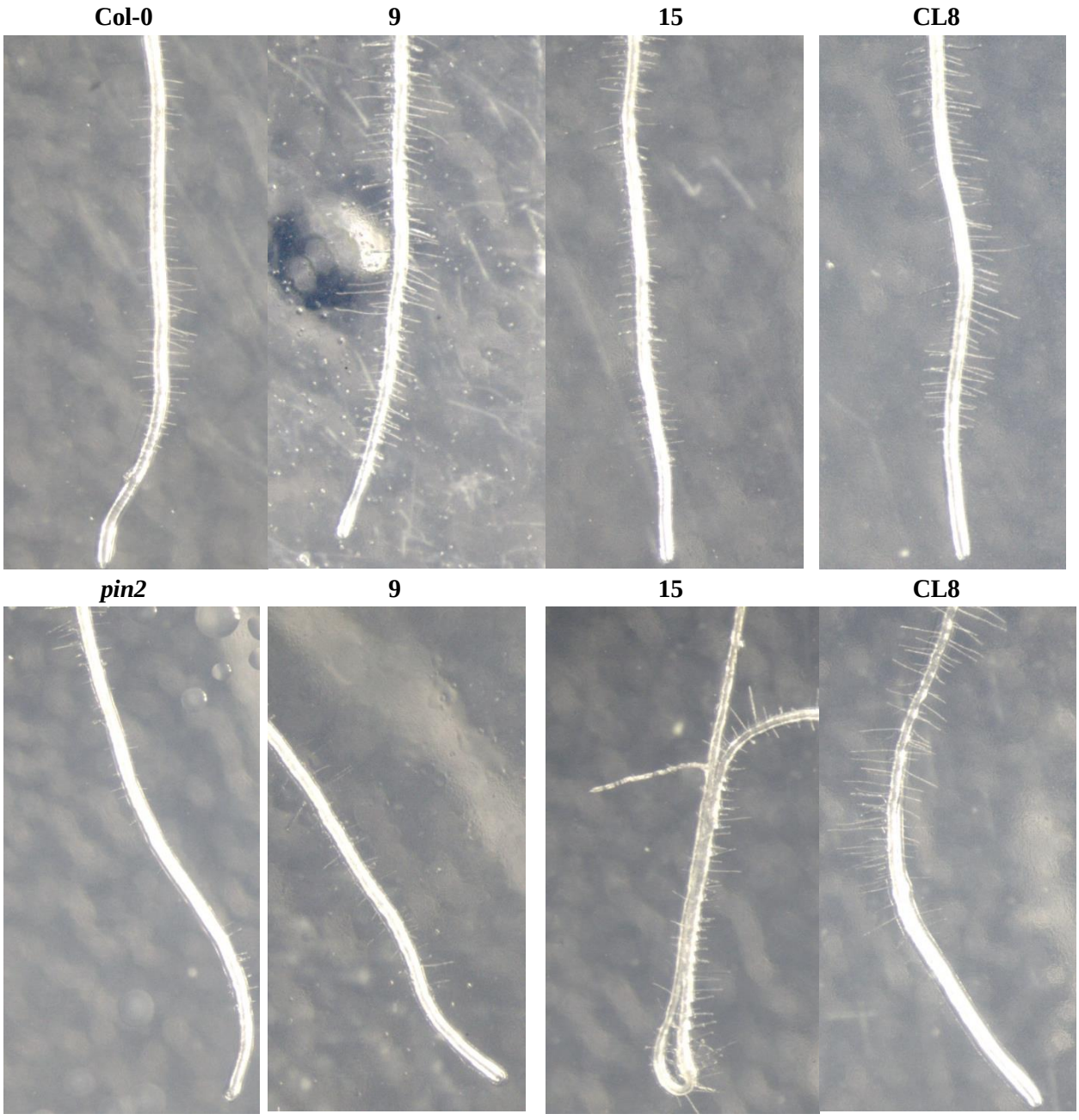
*Statistical analysis; *Significant Values.*

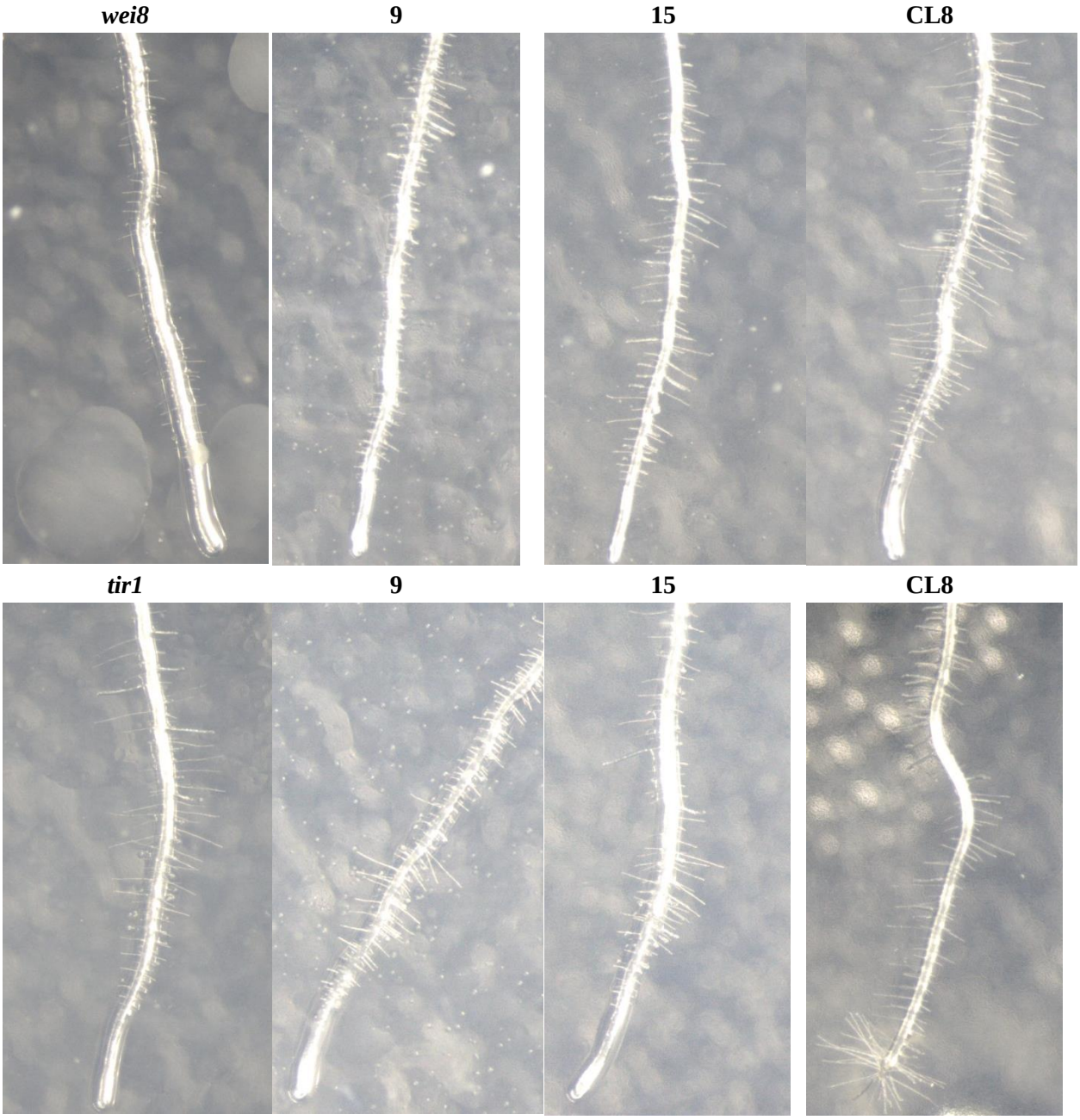
Table 3-6. VAP test: Average number of lateral roots counted after 8 days growth. SD; Standard deviation.

Bacteria type	No. of Plants/Plate	Average lateral roots/plant	SD	P-value
Col-0 (Control)	5	4.2	1.469694	
9	2	4.5	0.5	0.7975
15	3	1.666667	0.471405	0.0294*
CL8	5	3.2	1.16619	0.2657
***pin2* (Control)**	5	5.25	1.089725	
9	5	2	1	0.0012*
15	5	4.25	0.433013	0.0926
CL8	5	4.75	0.433013	0.3676
***tir1* (Control)**	4	1	0.632456	
9	3	1	0.632456	1.0000
15	4	0.4	0.489898	0.0157*
CL8	4	1.4	0.489898	0.3097
***aux1-7* (Control)**	4	7.25	0.829156	
9	5	5.2	1.6	0.0542

15	5	3.8	1.16619	0.0016*
CL8	4	6.5	1.658312	0.4467
***wei8* (Control)**	5	4.4	1.356466	
9	5	3.8	0.748331	0.4089
15	4	4	0.707107	0.6105
CL8	4	3	0.632456	0.0980

Note: *Plants which did not show proper growth patterns were not considered. Statistical analysis; *Significant Values.*




wei8
9
15
CL8
tir1
9
15
CL8

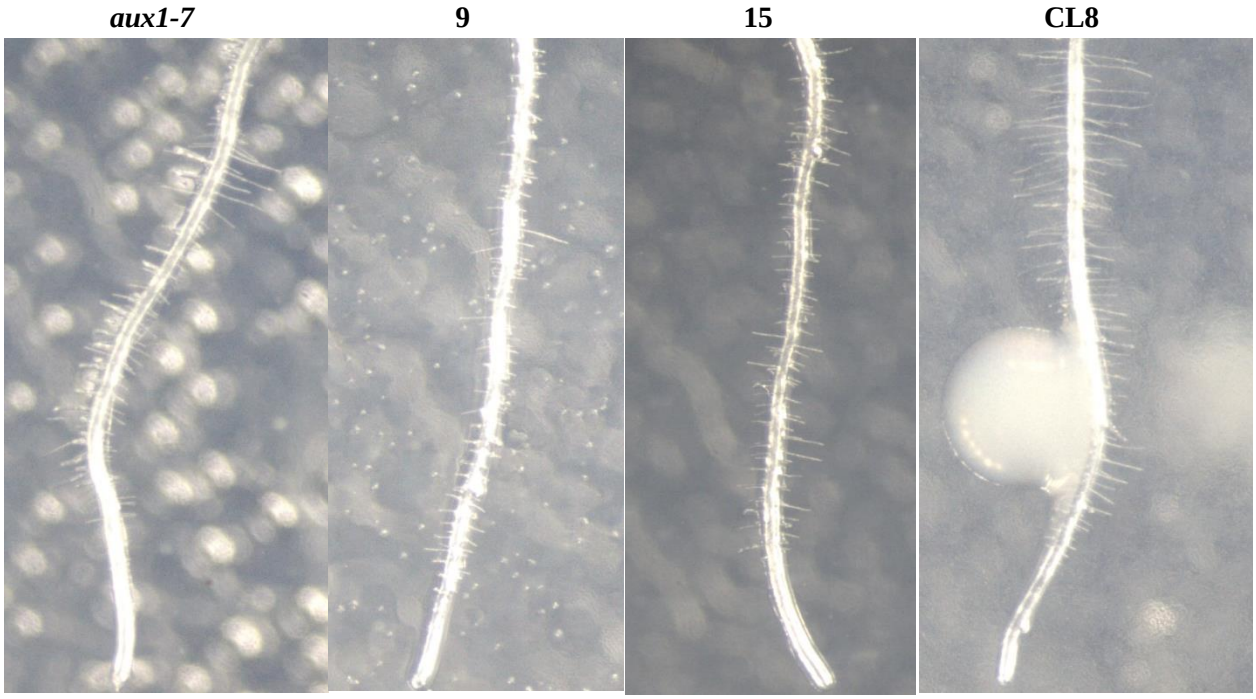


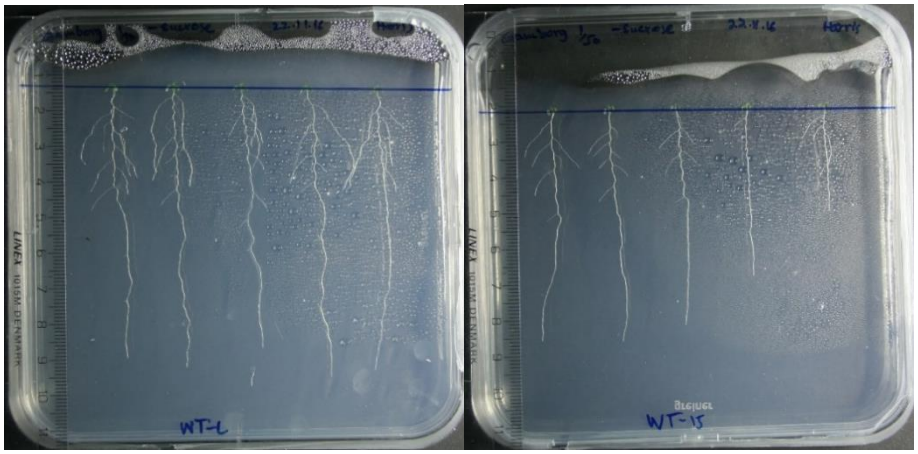
*Figure 3-6-**:** Effect of bacteria 9, 15 and CL8 on root hair growth of Col-0, pin2, wei8, tir1 and aux1-7 after 8 d of growth. From left is Control groups of Col-0, pin2, wei8, tir1 and aux1-7 and towards right are the inoculated strains in order 9, 15 and CL8.*

3.1.4 Inoculation on Arabidopsis WT and *pin2*

Experiment was performed with these petri dishes for each treatment. This experiment had large sample size. There were 15 seedlings for each treatment. Representative pictures are shown in (Figure 3-7). Primary root length of bacteria treated plates were not significantly different than control. The strain 15 treated plants have shorter primary root length than control. Similarly bacteria treated plants had no increase in number of lateral roots instead less number of lateral roots are seen in bacteria treated plants as compared to control (Figure 3-8). An increase in root hairs length are observed in Col-0 and *pin2* plants (Figure 3-9).

The bacterial strain 15 (*Agrobacterium* spp.) did not seem to have positive effect on primary root length and number of lateral roots. The strain 15 stimulated the root hairs growth in WT Col-0 and *pin2*.

A



B

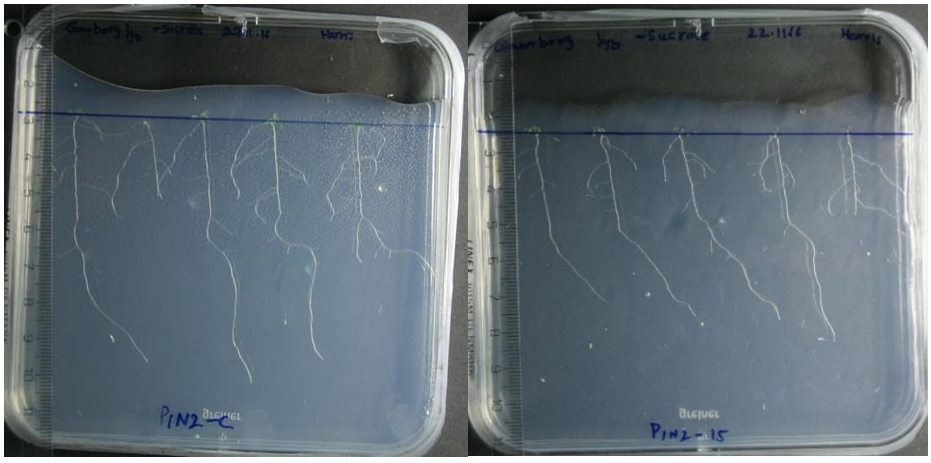


Figure 3-7- Effect of Agrobacterium spp. (strain 15) on root development of Arabidopsis Col-0 and mutant pin2 roots. ***A*** *part depicts Col-0 plants, Control (left) and bacteria induced (right).* ***B*** *shows mutant pin2 plants, Control (left) and bacteria induced (right). Seedlings were grown on 1/50x Gamborg medium supplemented with 0.5% sucrose for 6 d, after 6 d seedlings were moved to plates without sucrose and 650 µl of bacterial culture was spread on whole plate. Plates were kept for 7 d in growth chamber and after 7 d pictures were taken.*

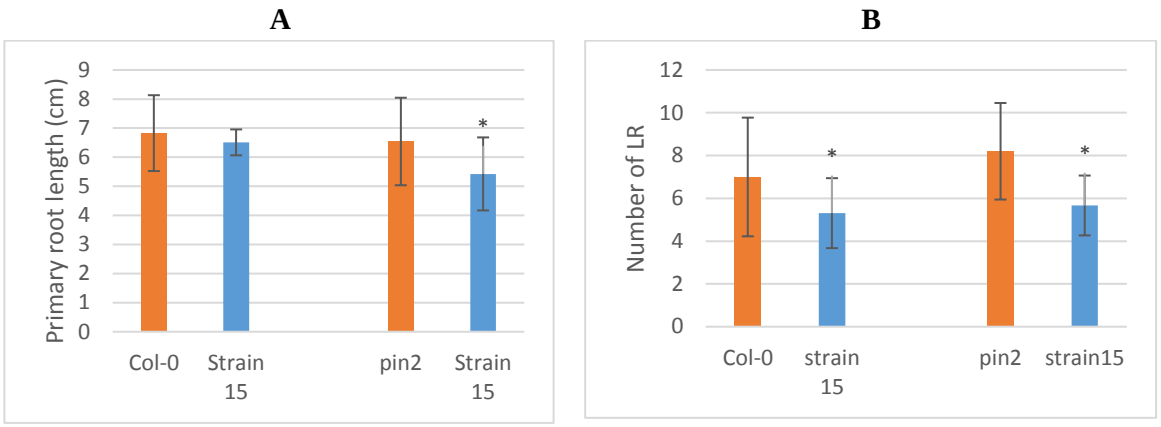


*Figure 3-8- Effect of strain 15 on root development of Arabidopsis Col-0 and mutant pin2. **A** shows primary root length, **B** shows average number of lateral roots. Orange bars indicate Control group (Col-0 and pin2), Blue bars indicate bacteria strain 15 in Col-0 and pin2 type. Error bars show ± SD (n=15). Experiment was repeated twice with similar results for primary root length. Asterisks indicate statistically significant differences compared with Control (Student's t test; $P < 0.05$).*

Table 3-7. VAP test- Average primary root length. SD; Standard deviation.

Treatment Type	No of Plants	Average Primary root length (cm)	SD	P-value
Col-0 (Control)	15	6.829373	1.30407	
Strain 15	15	6.409297	0.514237	0.2527
pin2 (Control)	15	6.541894	1.504032	
Strain 15	15	5.426331	1.255047	0.0349*

*Statistical analysis; *Significant Values.*

Table 3-8. VAP test- Number of lateral roots (LR). SD; Standard deviation.

Treatment	No of Plants	Number of LR	SD	P-value
Col-0	15	7	2.773501	
Strain15	15	5.066667	1.730767	0.0284*
pin2	15	8.2	2.256841	
Strain 15	15	5.666667	1.398412	0.0005*

*Statistical analysis; *Significant Values.*

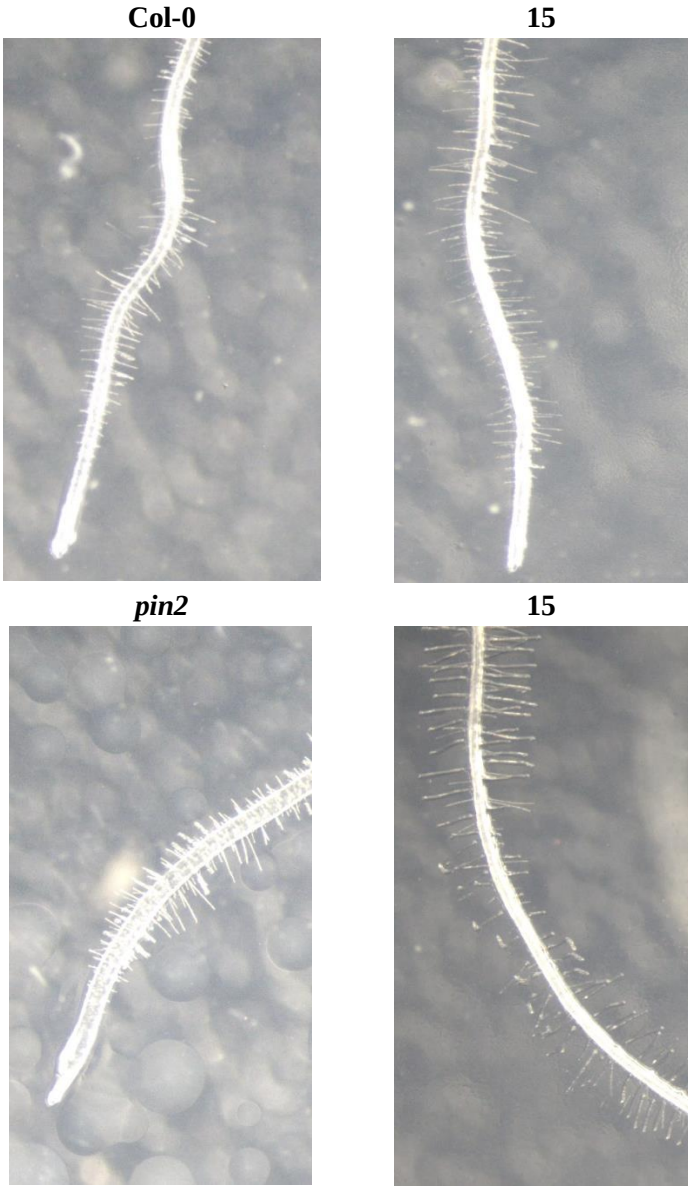


Figure 3-9- Root hairs comparison after 7 days in growth chamber. Col-0; Control, WT-15; Bacteria treated. pin2; Control, 15; strain 15.

3.2 Molecular identification of bacteria isolated from *Solanum pimpinellifolium*

3.2.1 16S PCR analysis

Molecular identification of the isolated bacteria was performed by amplifying and sequencing the 16S rRNA gene and comparing them to database of known 16S rRNA sequences. Forward primer 26F and two reverse primers R1520 and R1492 in combination with 26F (Separately)

targeted to conserved 16S rRNA gene were used for amplification. Visualization by agarose gel electrophoresis of the successfully amplified region of DNA is shown in Figure 10. It confirmed the presence of a PCR product.

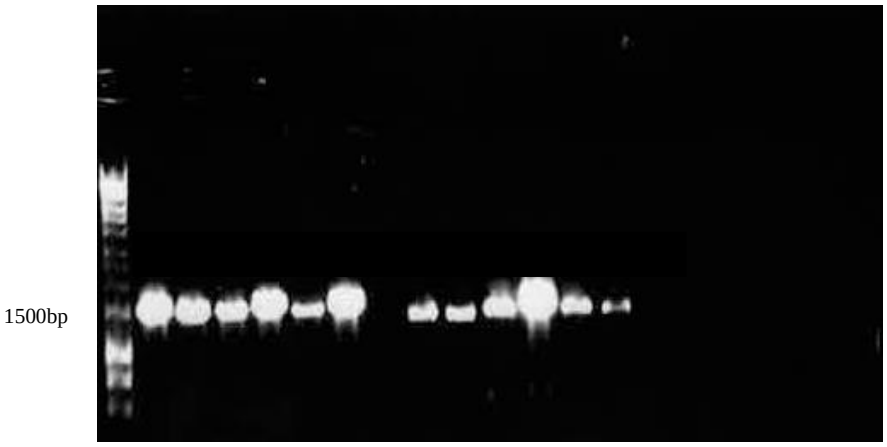


Figure 3-10- Amplification of 16S rRNA gene by PCR analysis.

All isolates were amplified at 1500bp. It confirms the presence of 16S rRNA gene in their genome.

Table 3-9. The table shows sequence of the PCR sample run on the gel. – Sign indicates gram negative bacterial sample and + sign indicate gram positive bacterial sample. R1520 are the samples in which reverse primer R1520 was used with 26F primer. R1492 shows sample with reverse primer R1492 used with 26F for PCR. Bacteria morphological appearance; Yellow, white and white slimy.

Sample No	With R1520	Sample No	With R1492
1	Yellow -	8	Yellow -
2	Yellow +	9	Yellow +
3	White -	10	White -
4	White +	11	White +
5	White slimy -	12	White slimy -
6	White slimy +	13	White slimy +
7	Control (Without DNA)	14	Control (Without DNA)

3.2.2 Endophytic bacteria inside *Solanum pimpinellifolium*

Three different bacterial strains were isolated from the roots of *S. pimpinellifolium* growing under hydroponic conditions. The genera included *Micrococcus, Cohnella* and *Bacillus* (Table 3-10). On the basis of the comparative analysis of sequences the strain white + and white – were found close to the genus *Bacillus* and showed 99% homology with *Bacillus flexus*, strain white slimy was found to be 98% similar to the genus *Cohnella* and strain yellow was 99% identical to Genus *Micrococcus*.

Table 3-10. The bacteria isolated from root interior (RI) of S. pimpinellifolium.

Sr. No.	Strain	Bacterial specie	Plant Component
1	Yellow	*Micrococcus yunnanensis*	RI
2	White	*Bacillus flexis*	RI
3	White slimy	*Cohnella* spp.	RI

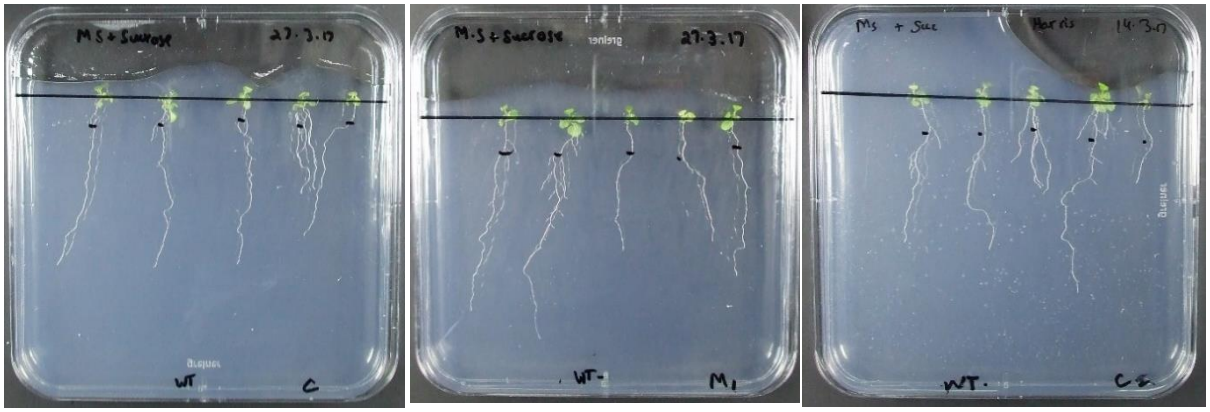
3.3 Effect of endophytic bacteria isolated from tomato on root growth of *Arabidopsis* WT and *pin2*

3.3.1 Experiment: Plants grown on 1 X MS medium supplemented with sucrose

To determine whether newly isolated bacteria make any difference in root development of plants, the bacteria *Micrococcus yunnanensis* and *Cohnella* spp. were inoculated to *Arabidopsis* Col-0 and mutant *pin2* plants growing in 1 X MS medium with 0.5% sucrose (Figure 3-11). The data shows statistically significant difference in primary root length between Col-0 control and *Micrococcus* treated plants. No significant difference in primary root length was observed in bacteria treated seedlings in *pin2* plants. *Micrococcus* treated Col-0 seedlings show increase in number of lateral roots as compared to control. The data show some importance for number of lateral roots in *Cohnella* treated seedlings as compared to control *pin2.* As compared to control groups graph show increase in shoot fresh weight in bacteria treated plantlets (Figure 3-13). No root hairs observed in control as well as bacteria treated plants (Figure 3-12).

The bacteria effected the fresh shoot weight of plants by increasing the shoot weight as compared to control. Not much reliable difference in primary root length seen by bacterial inoculation in *pin2* seedlings. *M.yunnanensis* stimulated the primary root length and lateral root formation in Col-0 seedlings. The number of plants in *Arabidopsis* Col-0 were less and limited the experiment results.

A



B

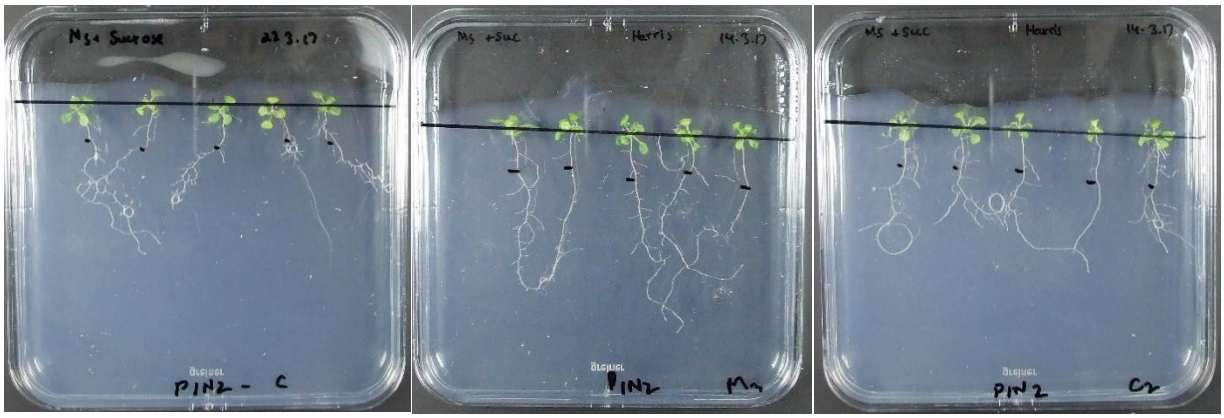


*Figure 3-11- The effect of Micrococcus yunnanensis and Cohnella spp. On root development of Arabidopsis Col-0 and mutant pin2. **A** shows wild type Col-0 (WT) control (upper left), M. yunnanensis treated (middle) and Cohnella treated plates (upper right) in Col-0. **B** shows control plants (lower left), M. yunnanensis treated (middle) and Cohnella treated plates (lower right) in mutant pin2. The plants were grown on 1 X Murashige and skoog agar solidified medium supplemented with 0.5% sucrose, after 5 d seedlings were moved to new plates with 0.5% sucrose and 400 ul of bacterial culture was spread on plates. Plates were kept for 8 d in growth chamber and photographs were taken after 8 d.*

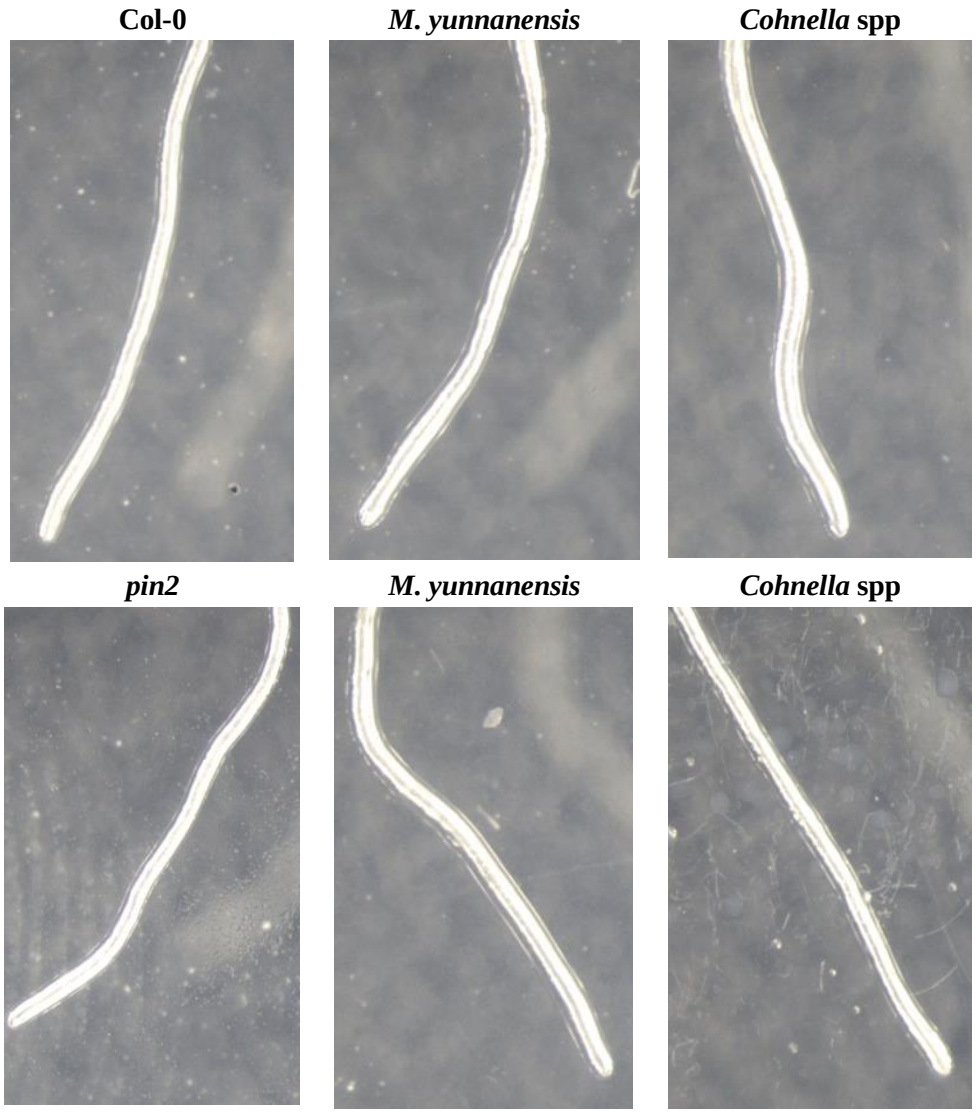


Figure 3-12- Effect of M. yunnanensis and Cohnella spp. on root hairs of Arabidopsis Col-0 and mutant pin2. Images of root hairs show Col-0 and pin2 as control on left side and toward right are bacterial treated roots. Data collected after 8 d of growth.

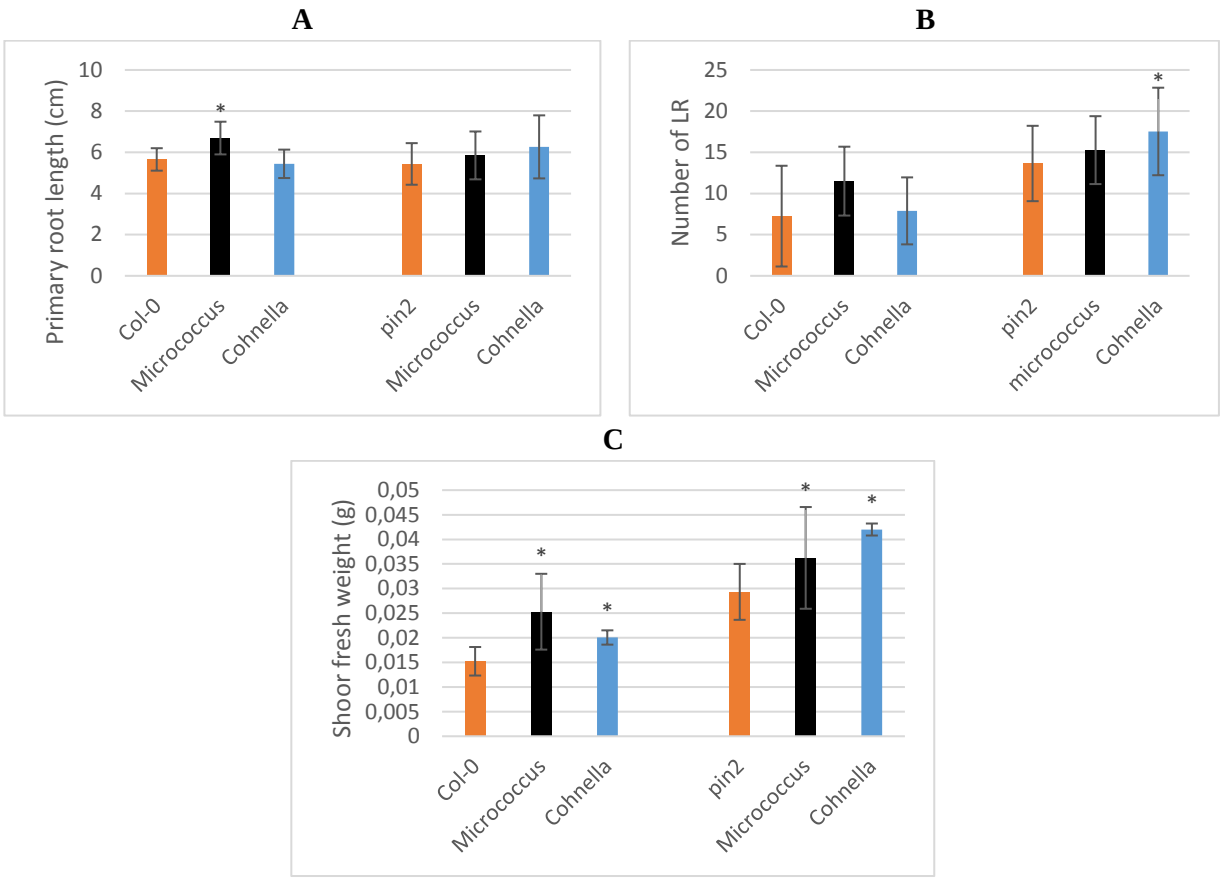


*Figure 3-13- Influence of isolated bacteria, M.yunnanensis and Cohnella spp. on root growth of Arabidopsis Col-0 type and mutant pin2. **A** shows primary root length comparison between control and bacteria treated plants in wild type (Col-0) and mutant pin2. **B** shows average number of lateral roots per plant and **C** shows the mean shoot fresh weight of seedlings. Orange bars; Control group, Black bars; M.yunnanensis treated and Blue bars; Cohnella treated seedlings. Error bar indicate ± standard deviation (n=8 in Arabidopsis Col-0, n=15 in mutant pin2). Asterisks indicate statistically significant differences compared with Control (Student's t test; P < 0.05).*

3.3.2 Experiment: Plants grown on 1 X Murashige and Skoog medium without sucrose

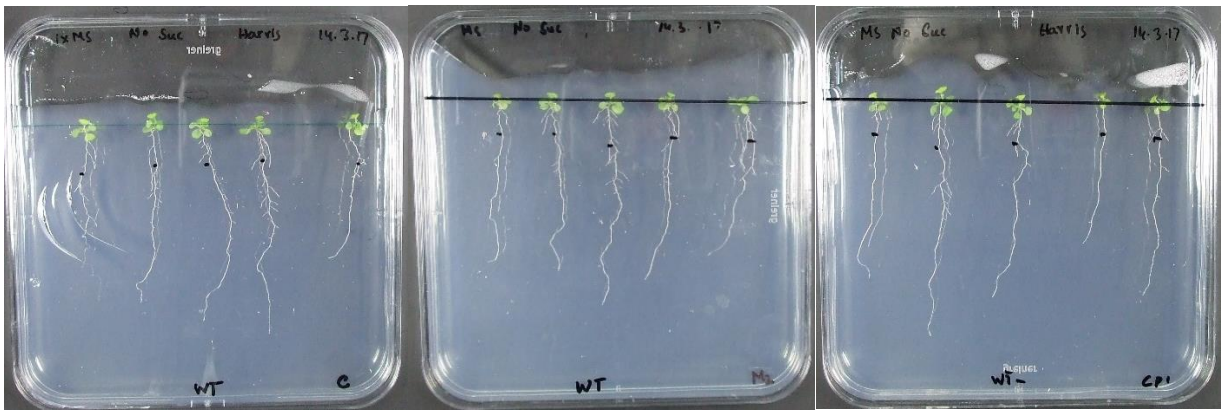
Each plate in the experiment represent separate treatment. Representative pictures of experiment are shown in figure 3-14. In this experiment results indicate that by inoculating bacterial species on seedlings growing on 1 X MS medium without sucrose, the primary root length remains unaffected as compared to control in WT Col-0 and *pin2*. (Figure 3-15). In Arabidopsis Col-0 seedlings the treatment with *Cohnella* spp. reduced the number of lateral roots whereas lateral root remain same for control and *Micrococcus* treated plants. In *pin2* mutant plants no significant increase in number of lateral roots observed between control and bacterial treated plants. The shoot fresh weight graph shows significant values for bacterial treated plants both in *Arabidopsis*

and *pin2* (Figure 3-15). The bacterial treatment seemed to increase the fresh shoot weight of plants. No root hairs were observed in control as well the bacterial treated seedlings (Figure 3-16).

The data shows big values for standard deviation in lateral roots graph it is because among seedlings the number of lateral roots were irregular. The high SD values in in Col-0 seedlings is also because of difference in number of seedlings while measuring shoot weight.

The bacterial inoculation of *M.yunnanensis* and *Cohnella* spp. enhanced the fresh shoot weight of plants but had no significant effect on primary root length, lateral roots and root hairs.

A



B

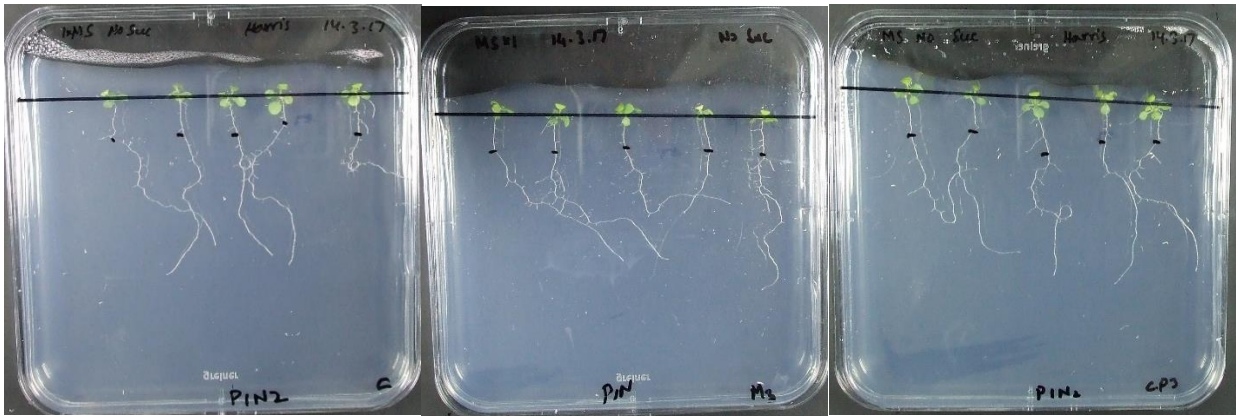


*Figure 3-14- The effect of M.yunnanensis and Cohnella spp. On root development of Arabidopsis Col-0 and mutant pin2. **A** shows wild type (WT) control (upper left), M. yunnanensis treated (middle) and Cohnella treated plates (upper right) in col-0. **B** shows control (lower left), M. yunnanensis treated (middle) and Cohnella treated plates (lower right) in mutant pin2. The plants were grown on 1 X Murashige and skoog agar solidified medium supplemented with 0.5% sucrose, after 5 d seedlings were moved to new plates without sucrose and 400 ul of bacterial culture was spread on plates. Plates were kept for 8 d in growth chamber and photographs were taken after 8 d*

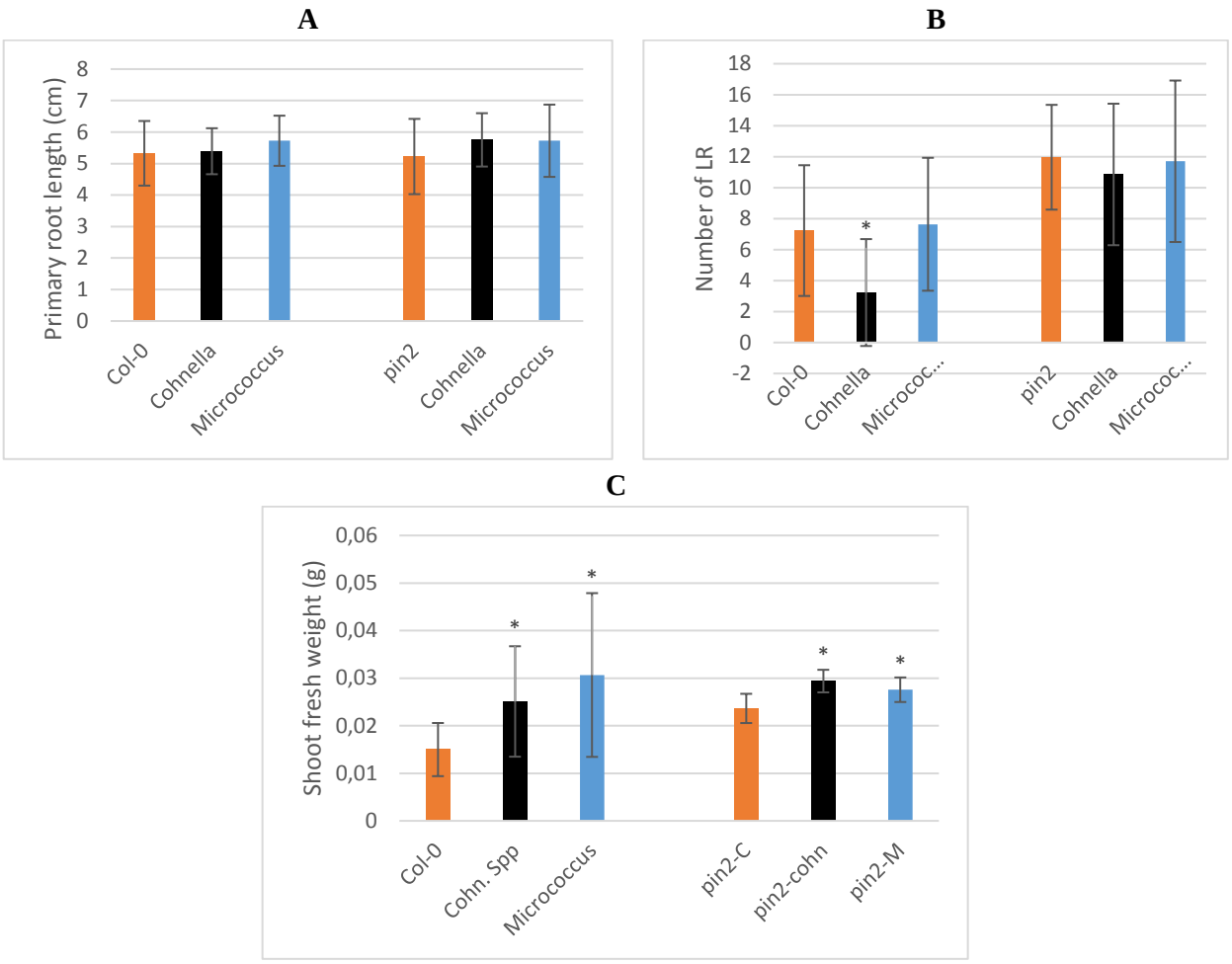


Figure 3-15- Effect of Micrococcus yunnanensis and Cohnella spp. on root system of Arabidopsis Col-0 and its mutant pin2 seedlings after 8 d of cocultivation. ***(A)*** *Primary root length,* ***(B)*** *Average number of lateral roots and* ***(C)*** *Shoot fresh weight of Control plants and bacterial treated plants after 8 d cultivation. Data represents the average of four group of seedlings each group containing 5 excised shoots in Col-0 and five group of seedlings each containing 5 excised shoots in pin2. Error bars indicate SD (n=20 in Arabidopsis and n=25 in mutant pin2). Orange bars; Control, black bars; Cohnella, and blue bars represent; Micrococcus treated plants. Asterisks indicate statistically significant differences compared with Control (Student's t test; $P < 0.05$). The data is combination of two independent experiments.*

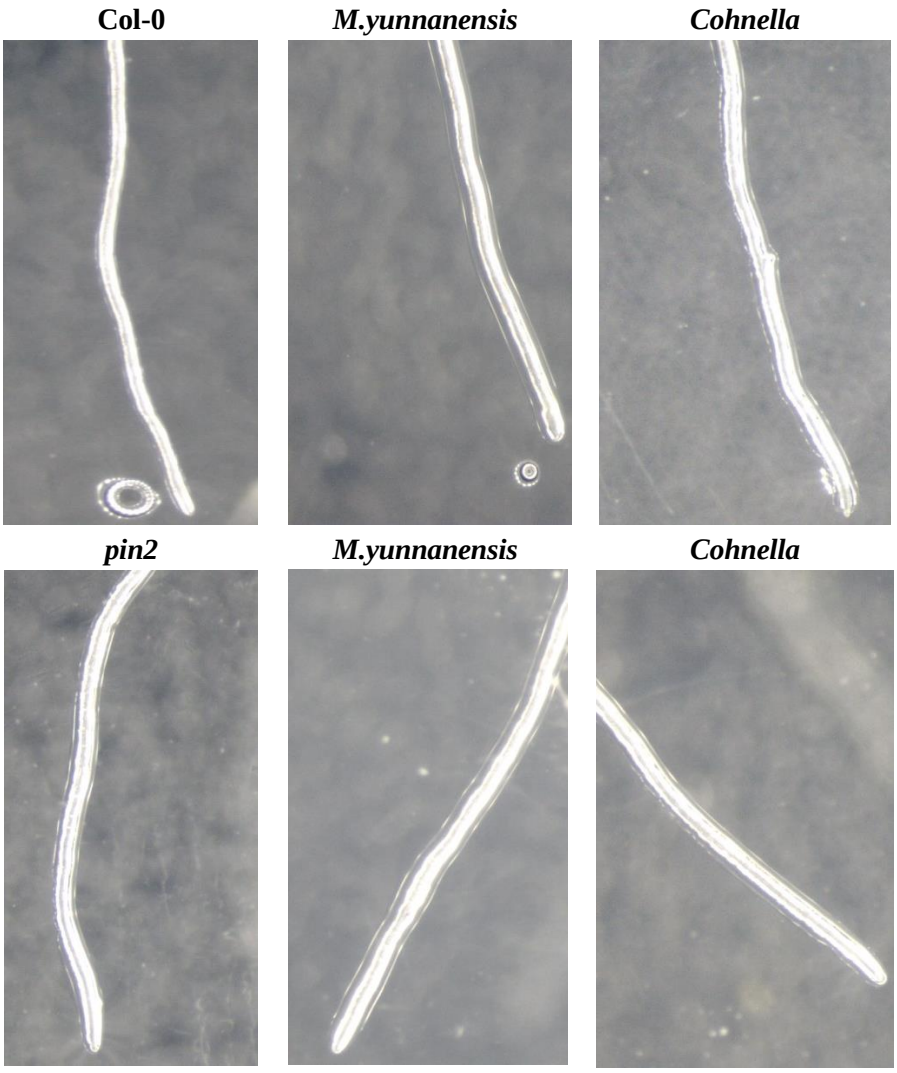


Figure 3-16-Effect of M. yunnanensis and Cohnella spp. on root hairs of Arabidopsis and mutant pin2. Images of root hairs show col-0 and pin2 as control on left side and toward right are bacterial treated roots. Data collected after 8 d of growth.

3.4 Effect of *Pseudomonas fluorescence* (WCS417) on root growth of Arabidopsis, *pin2* & *aux1-7*

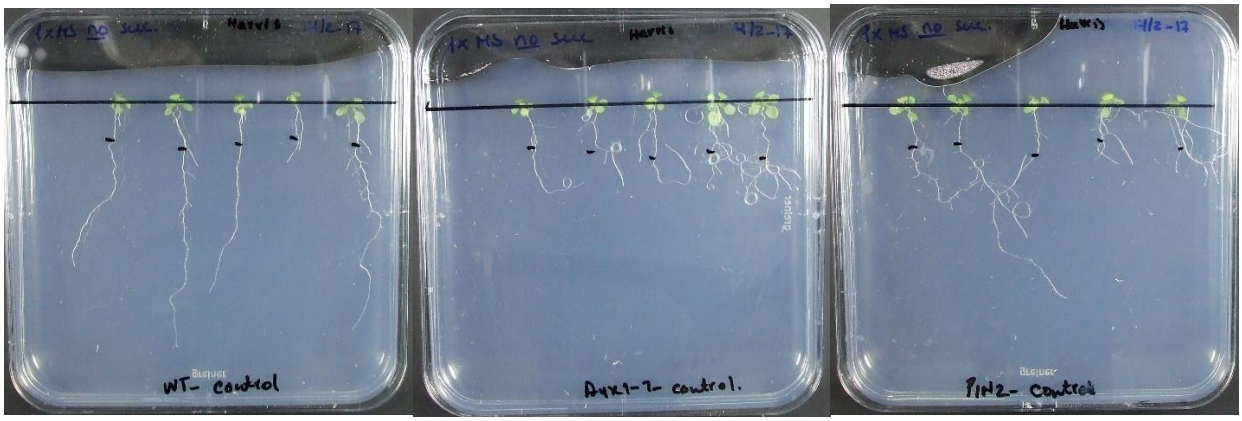
3.4.1 Experiment: Plants grown on MS media without sucrose

The effect of *Pseudomonas fluorescence* (WCS417) on root development of *Arabidopsis thaliana* Col-0 and its mutant impaired in auxin transport was determined. Representative picture are show in figure 3-17. In plants treated with WCS417 increase in primary root length was observed in both Col-0 and *pin2*. The data of primary root length depict statistically significant

difference for bacteria treated plants (Figure 3-19). Also number of lateral roots increased in plants in WCS417 treatment as compared without bacterial treatment (Figure 3-19). No significant effect on root hairs was observed except Col-0 type in which case treatment with bacteria resulted in slight increase of root hairs (Figure 3-18). Shoot fresh weight of bacterial inoculated treatment show significant increase in fresh weight in Col-0 and *aux1-7* as compared to their control groups (Figure 3-19). The primary root length of mutant *aux1-7* control type could not be measured because of curly roots and therefore cannot be compared with bacterial treated plants. However, when treated with bacteria the curls disappeared and roots bended in random positions.

According to results the data suggests that *Pseudomonas* stimulate primary root and lateral root growth and also positively effects the shoot weight of plants.

A



B

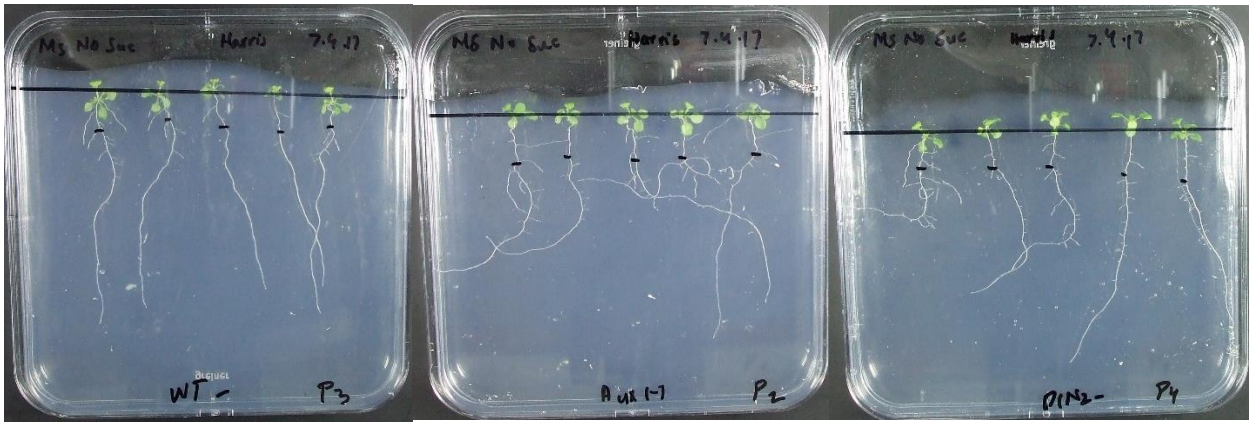
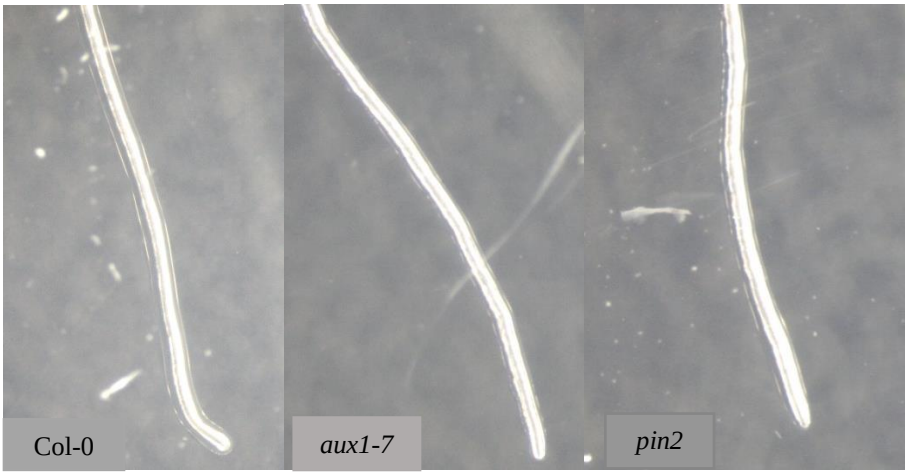


Figure 3-17- Effect of WCS417 bacteria on root growth in Arabidopsis and its mutant seedlings. ***(A)*** *Representative images of Control seedlings of Arabidopsis WT (Upper left), mutant aux1-7 (Middle) and mutant pin2 (Upper right) after 8 d of growth.* ***(B)*** *Effect of WCS417 on root development of WT (Lower left), mutant aux1-7 (Middle) and*

mutant pin2 (Lower right). The plants were grown on 1 X Murashige and skoog agar solidified medium supplemented with 0.5% sucrose, after 5 d seedlings were moved to new plates without sucrose and 400ul of bacterial culture was spread on plates. Plates were kept for 8 d in growth chamber and photographs were taken after 8 d.

A



B

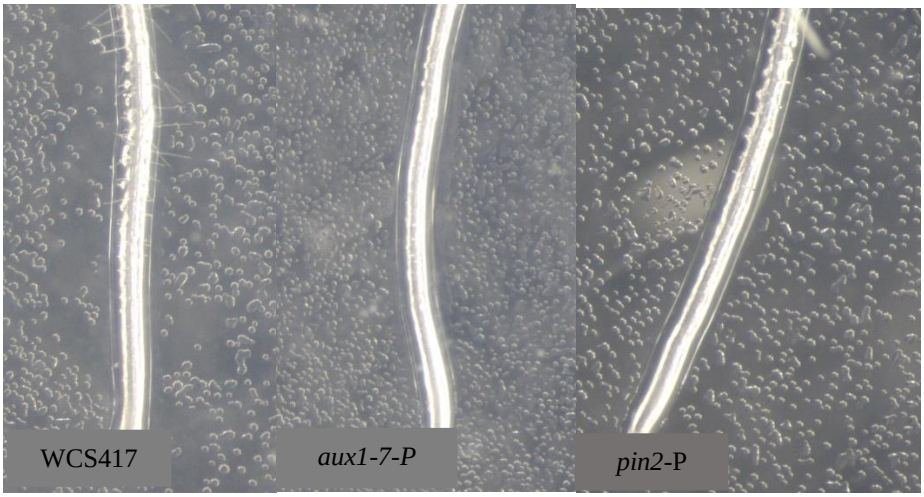


Figure 3-18- Effect of WCS417 on root hairs of Arabidopsis and its mutants. (A) All the images are of control (Without bacteria) treatment including Wild type Col-0, mutant aux1-7 and mutant pin2. (B) Images of seedlings treated with bacteria WCS417. WCS417 (Col-0), aux1-7-P (aux1-7 Pseudomonas) and pin2-P (pin2 Pseudomonas). Data collected after 8 d of growth.

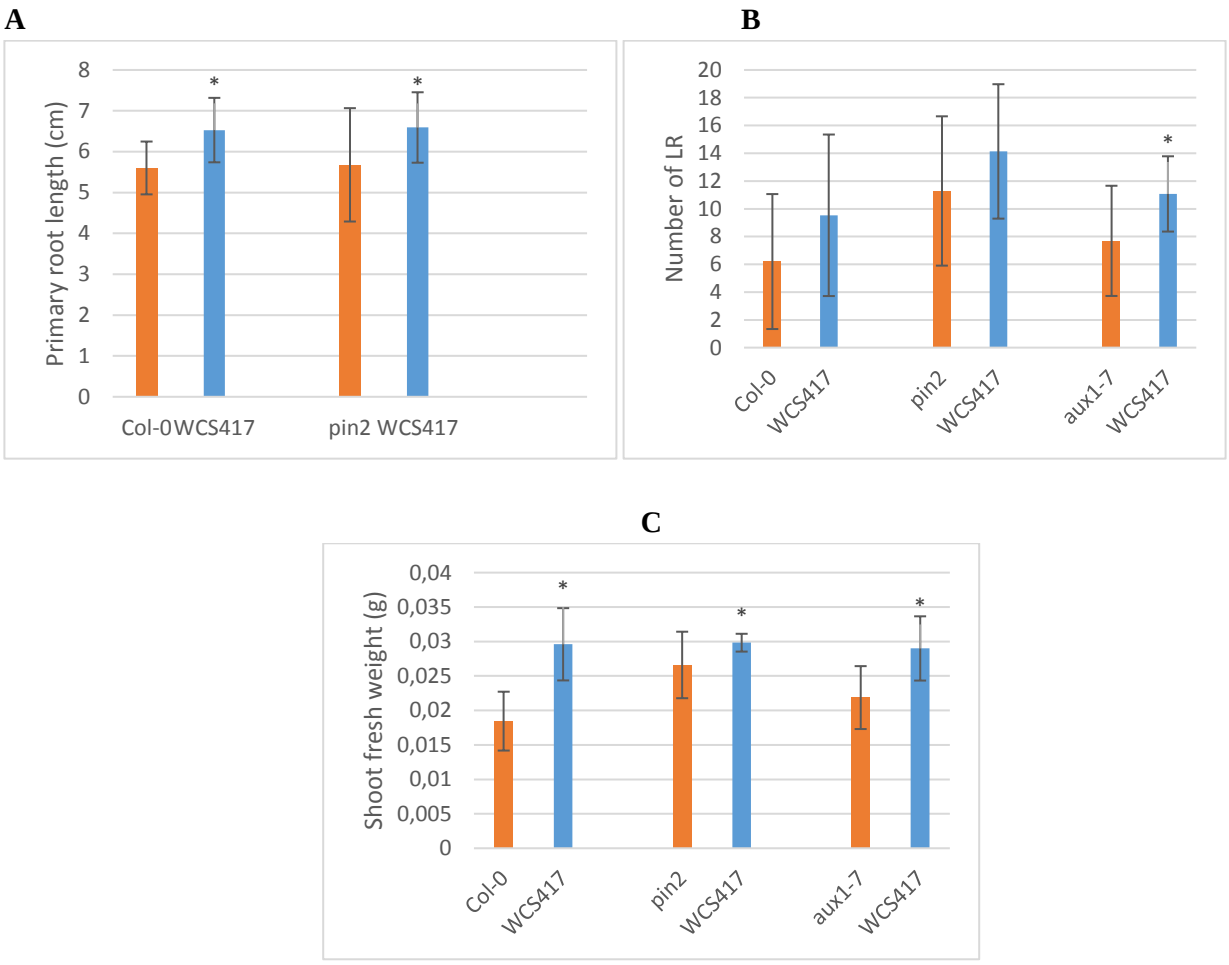


Figure 3-19- Effect of WCS417 on root system of A.thaliana seedlings and its mutant pin2 and aux1-7 after 8 d of cocultivation with WCS417. ***(A)*** *Primary root length,* ***(B)*** *Average number of lateral roots and* ***(C)*** *Shoot fresh weight of Control plants and bacterial treated plants after 8 d cultivation. Orange bars indicate Control treatments and blue bars show bacteria treated plants of Arabidopsis Col-0 type, mutant pin2 and mutant aux1-7. Data represents the average of three group of seedlings each group containing 5 excised shoots. Error bars indicate SD (n=15). This is the second experiment showing repetitions for Col-0 and pin2. Asterisks indicate statistically significant differences compared with Control (Student's t test; P < 0.05).*

3.4.2 Experiments: Plants grown on MS media with sucrose

The results show that by treating plants with the WCS417 strain, growth of primary root is significantly inhibited as shown in Figure 3-22. No inhibition of root length was observed in the previous experiment when plants were grown with WCS417 bacteria on 1 X MS media without sucrose (Figure 3-19). However bacteria affected the lateral root development with increase in lateral root numbers as compared to the non-bacterial treatment (Figure 3-22). Also increase in amount of root hairs observed when the plants, Arabidopsis Col-0 type and mutant *pin2*

inoculated with WCS417 strain (Figure 3-21). The graph for shoot fresh weight shows that WCS417 treated WT plants has decreased shoot fresh weight as compared to control. No difference in shoot weight for *pin2* was seen between control and bacterial treated samples.

This experiment explains that *Pseudomonas* WCS417 inhibits the primary root length and increases the number of lateral roots in WT Col-0 and *pin2*. The bacteria stimulate the root hair growth more in WT Col-0 but to some less extent in *pin2.*

A

B

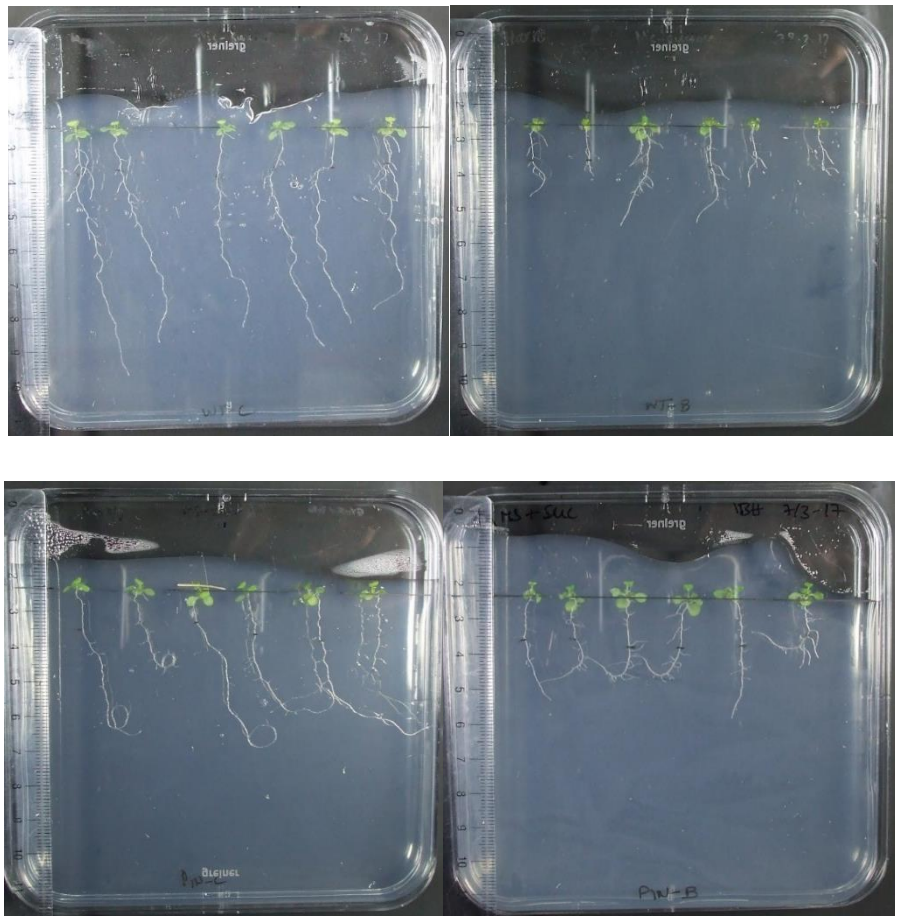


Figure 3-20- Effect of WCS417 on plant growth and root development of Arabidopsis Col-0 and mutant pin2 seedlings. A and B, representative images of seedlings growing on control plates (left) and plates containing WCS417 (right). The plants were grown on 1 X Murashige and skoog agar solidified medium supplemented with 0.5% sucrose, after 5 d seedlings were moved to new plates with 0.5% sucrose and 400 ul of bacterial culture was spread on plates. Plates were kept for 8 d in growth chamber and photographs were taken after 8 d.

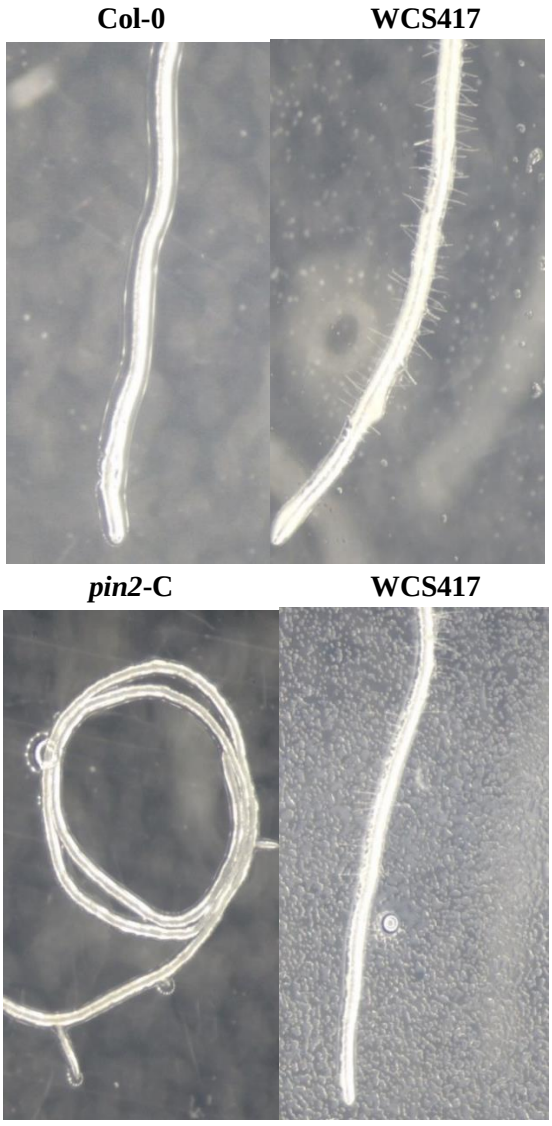


Figure 3-21- Effect of WCS417 on root hair growth of Arabidopsis Col-0 and pin2 after 8 d of growth. ***A*** *shows Arabidopsis Col-0, Control (left) and bacteria (right).* ***B*** *shows mutant pin2, control (left) and bacteria (right).*

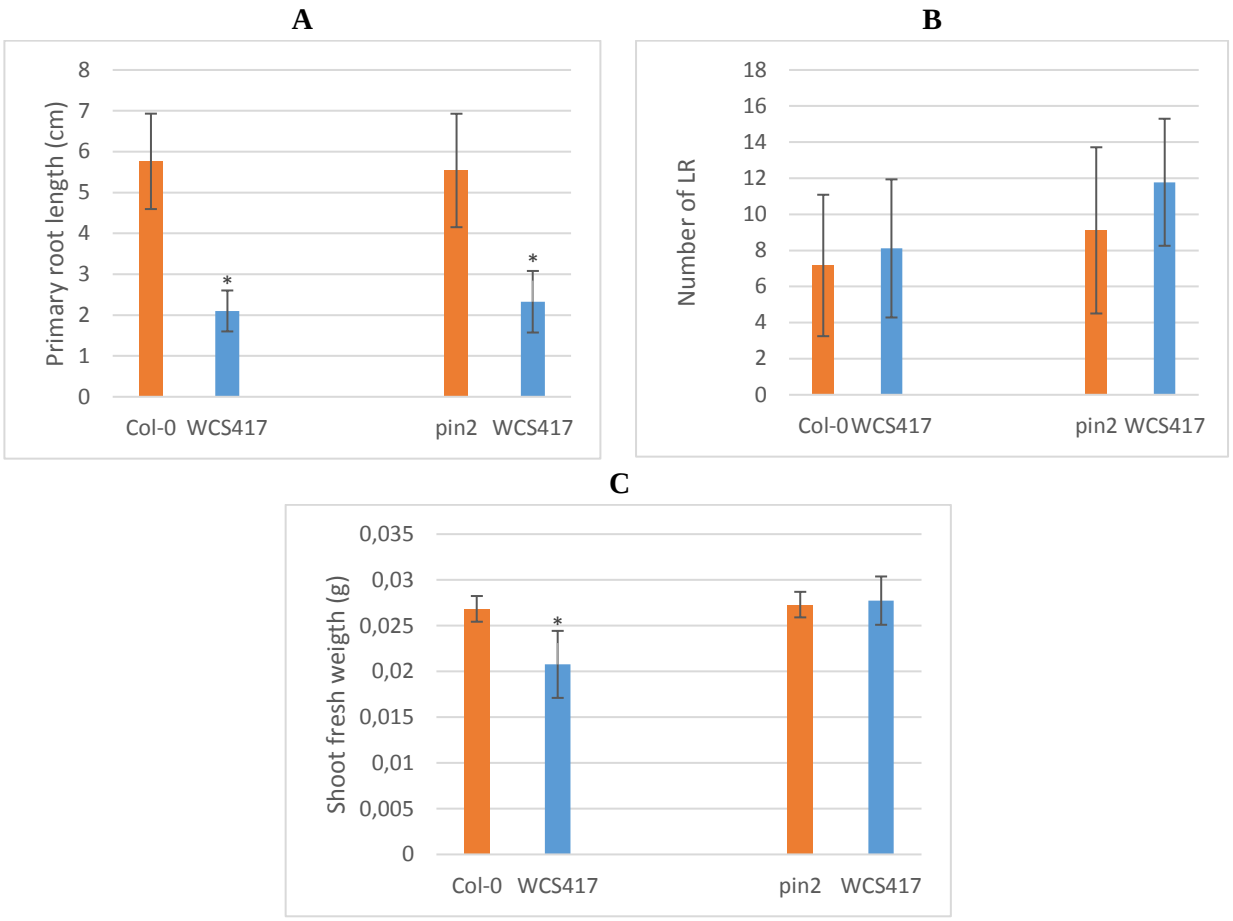


Figure 3-22- Effect of WCS417 on root system of A.thaliana seedlings and its mutant pin2 after 8 d of cocultivation with WCS417. ***(A)*** *Primary root length,* ***(B)*** *Average number of lateral roots and* ***(C)*** *Shoot fresh weight of Control plants and bacterial treated plants after 8 d cultivation. Orange bars indicate Control treatments and blue bars show bacteria treated plants of Arabidopsis Col-0 type and mutant pin2. Data represents the average of three group of seedlings each group containing 6 excised shoots. Error bars indicate ± SD (n=18). Asterisks indicate statistically significant differences compared with Control (Student's t test; P < 0.05).*

3.5 Effect of bacterial inoculation on growth of Gemini tomato plant

3.5.1 Pot Experiment

The effect of the isolated endophytic bacteria on Gemini tomato plants was evaluated in pot experiment. As compared to the Control the shoot length of the plants inoculated with bacteria was increased but this increase is not much significant as compared to control (Figure 3-27). The difference in shoot length at start and at the end of experiment was seen to be more in case of plants treated with the bacteria than plants without bacteria (Figure 3-27)**.** As for the shoot fresh weight a significant increase in weight of plants inoculated with *Micrococcus* strain was

observed. The difference of shoot weight between *Cohnella* spp. treated plants and Control plants was found to be less than the *Micrococcus* inoculated plants (Figure 3-28). Representative pictures of experiment are shown below.

The data from this experiment shows that inoculation with *Micrococcus* slightly increased the shoot length and show fresh weight of the plants. However further experimentation is with large sample size of plants is required to become certain about results.

Figure 3-23- Tomato plants (Gemini) at start of inoculation. The pots on right side are Control. The pots on left contain bacteria Micrococcus and middle one contains bacteria Cohnella spp.

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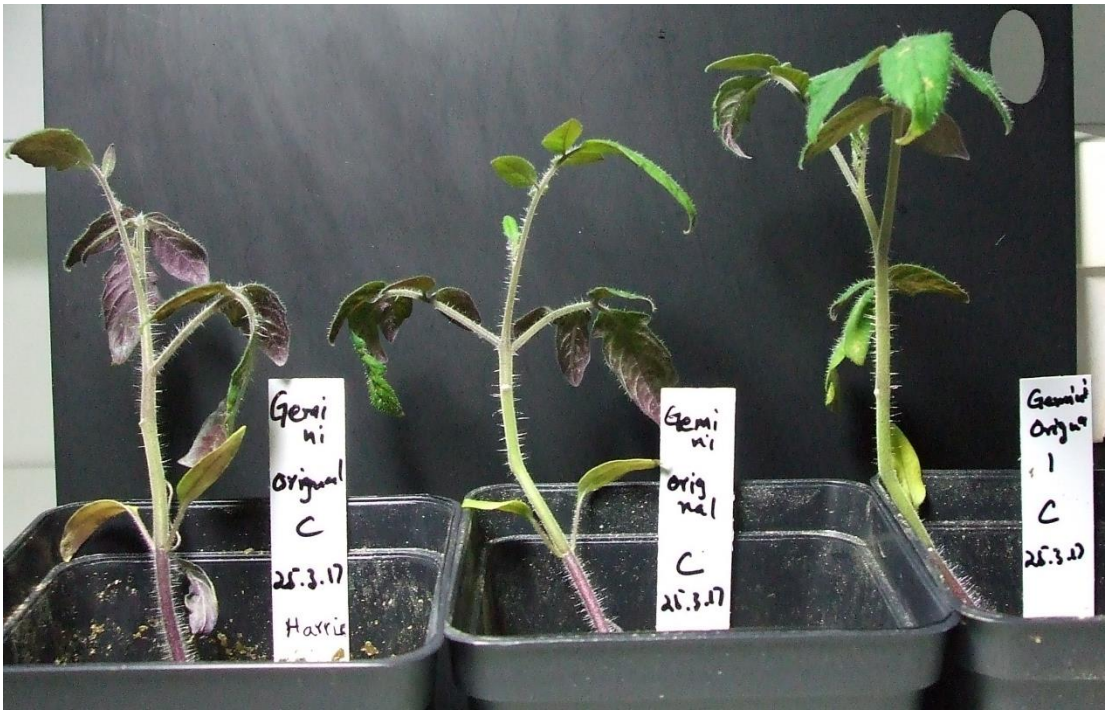

Figure 3-24- Control plants (Gemini) after 4 weeks growth in pots.

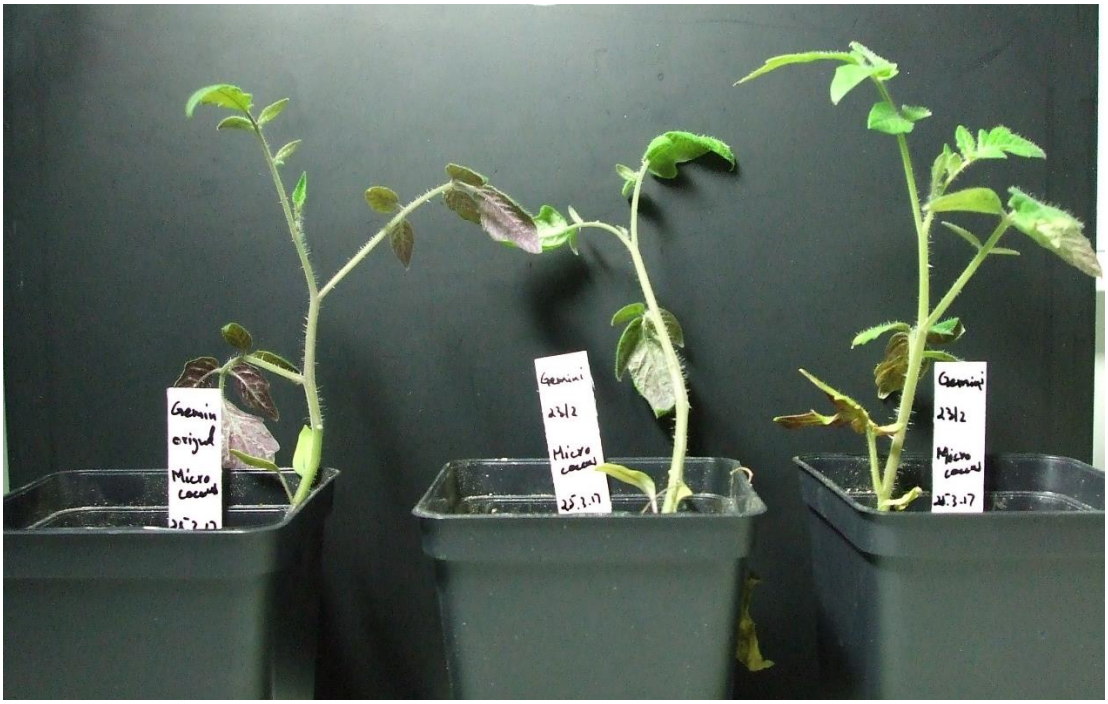

Figure 3-25- Gemini Plants inoculated with Micrococcus. Plant growth observation after 4 weeks.

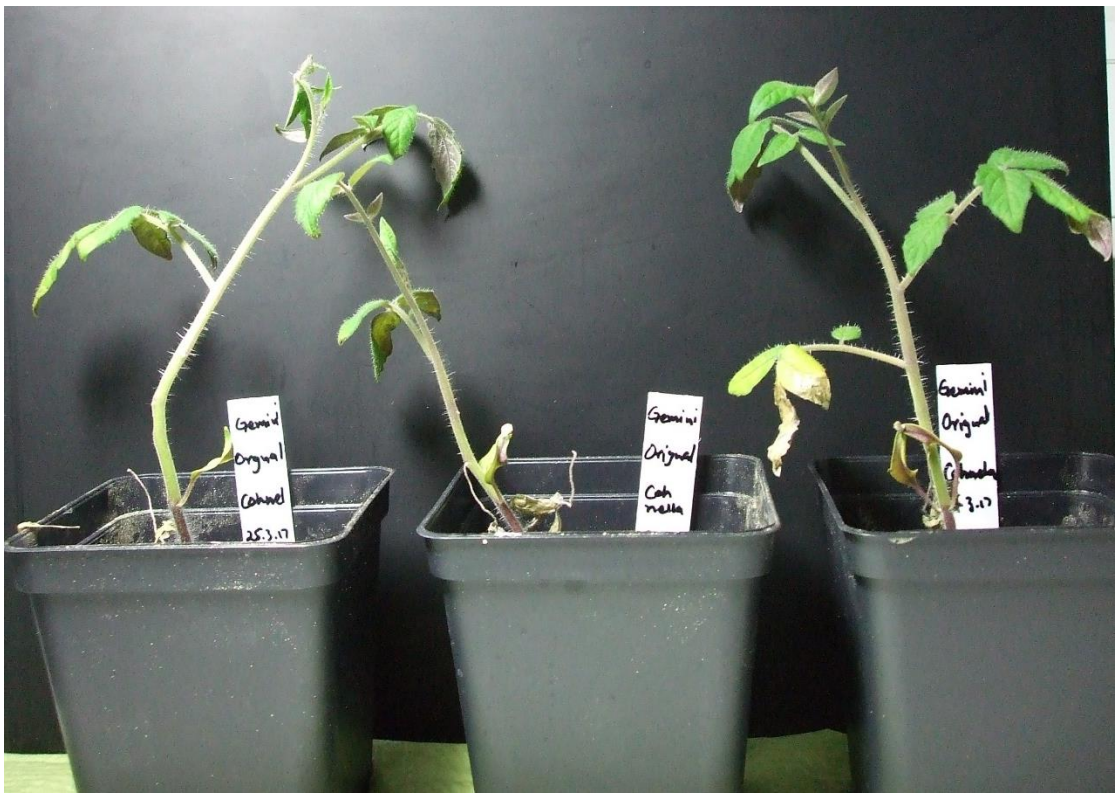

Figure 3-26- Gemini plants inoculated with Cohnella spp. Plant growth observation after 4 weeks.

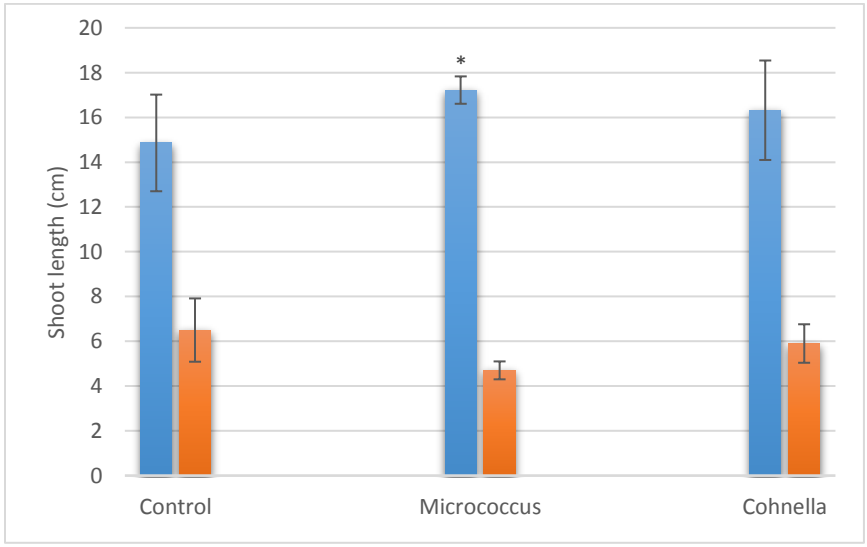

Figure 3-27- Shoot length of Gemini plant inoculated with bacteria Micrococcus and Cohnella for 4 weeks. Blue bars: Shoot length after 4 weeks. Orange Bars: Shoot length at start of experiment (Without bacteria). Figure shows control as Gemini and bacterial treated plants as Micrococcus and Cohnella. Error bars indicate standard deviation (n=5). Asterisks indicate statistically significant differences compared with Control (Student's t test; $P < 0.05$).

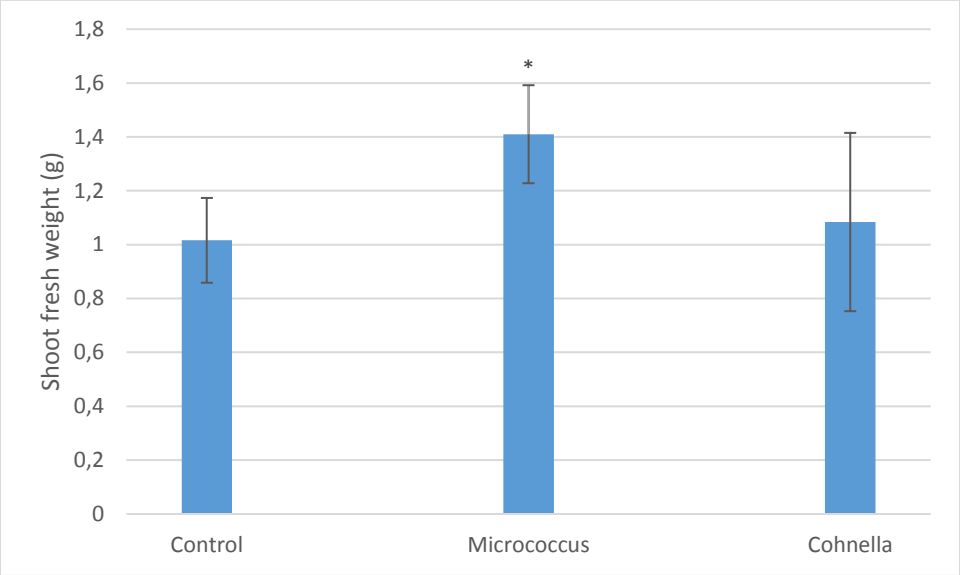

Figure 3-28- Shoot fresh weight of Gemini tomato plant inoculated with bacteria for 4 weeks. Blue bars represent a comparison between control and bacteria treated plants. Error bars indicate standard deviation ± (n=5). Asterisks indicate statistically significant differences compared with Control (Student's t test; P < 0.05).

4 Discussion

In this thesis we studied the effect of different plant growth promoting endophytic and rhizospheric strains obtained from the tomato cultivars by Abbamondi and collaborators. The main goal of the study was to see the role of these bacteria in root development of *Arabidopsis* and the mutant impaired in auxin transport and auxin signal reception. During the course, number of experiments were performed in which bacteria for their ability to promote root growth were tested. Although these bacterial strains were already tested by (Abbamondi et al. 2016) in his work on *Arabidopsis* but these plant growth promoting bacteria were not previously experimented with the *Arabidopsis* mutant seeds.

4.1 Bacterial effect on Wild type Col-0

In the start all the bacterial strains (5, 10, 9, 15, 6, 16, 18 and CL8) were inoculated to *A. thaliana* seedlings. The results show an increase in the primary root length for the bacteria 5, 6, 16, 10, 18 and CL8 and increase of lateral roots number only seen for the strains CL8, 6 and 18. But, the strain 9 and 15 showed primary root length that was comparable to control indeed the 15 strain show the inhibition of primary root length. Also number of lateral roots were more or less comparable to the control group for strain 9 and 15 (Figure 3-1). A significant increase in root hairs was observed during the experiment for all the inoculated bacteria. The bacteria CL8, 18, 6 and 10 stimulated root hair growth and longer root hairs as compared to control are seen in case of these bacteria (Figure 3-2).This was the second experiment and only similar results were found for the inoculated strain 9 and 15. In the previous experiment (not shown in results, in appendix A-1) all the bacterial strains showed decrease in primary root length as well as number of lateral roots as compared to control. This could be because of the difference in amount of the bacterial inoculation, during first experiment (not shown) the 1 ml bacterial culture was inoculated and during second experiment 650µl was inoculated. Also some smaller difference in bacterial culture O.D between two experiments can affect the growth of plants. However increase in amount of root hairs was consistent among the two experiments. Since reproducibility was seen only in case of strain 9 and 15, so these strains were selected for further experimentation.

4.2 Bacterial effect on *pin2* and *wei8/wei2*

Further experimentation is carried out with the two mutants, *pin2* and *wei8/wei2*. The *pin2* mutant seeds showed the curly roots, which explains the loss of gravitropic response because of mutation (Kleine-Vehn et al. 2008; Vieten et al. 2007). Also improper gravitropism is observed in *wei8/wei2* mutant roots (Stepanova et al. 2008). Inoculation with the bacterial strains 9 and 15 show significant inhibition of primary root length and reduced number of lateral roots in *Arabidopsis* Col-0 type. In the same manner *pin2* and *wei8/wei2* primary root and lateral roots are also inhibited when treated with the bacteria 9 and 15 (Figure 3-3). Upon inoculating with the bacteria the loss of gravitropic response is seen to reduce in *pin2* and *wei8* (See appendix A-2). It is known that by the application of the exogenous auxin the defective root phenotype can be rescued (Wu et al. 2007) , as (Abbamondi et al. 2016) explained in his study that strain 9 and 15 are known to produce IAA, this could be the reason that defect in gravitropism was reduced when mutants were treated with the bacteria. Also the effect of PGPB on root hairs is observed to be same as in (Abbamondi et al. 2016) study.

This experiment showed that strain 9 and 15 reduces the primary root length and lateral root number not only in Col-0 but also in the mutant *pin2* and *wei8/wei2*. These strains were found positive for IAA test in (Abbamondi et al. 2016) study. Research study has shown that high levels of auxin reduces the primary root length and stimulate the root hair development (Vacheron et al. 2013), a similarity which we also noticed in our experiments.

4.3 All mutant study

More experiments are conducted on all mutants with bacteria 9, 15 and CL8. The experiment indicates no significant growth of primary root and also the number of lateral roots in mutants *pin2*, *wei8*/*wei2*, *tir1* and *aux1-7* (Figure 3-5). Similar results for WT Col-0 mutant *pin2* and *wei8/wei2* have also reported in last experiment when inoculated with strain 9 and 15 (Figure 3-3). In this experiment the bacterial amount was 650 ul. Reduction in number of lateral roots also seen in the mutants when treated with the bacteria 9, 15 and CL8. However with strain 9 a slight increase in number of lateral roots observed in Col-0 type. This could be because of the difference in total number of plants between control and bacterial treatments (Table 3-5), the number of plantlets showing proper growth varied among control group and the bacteria treated

group so for results confirmation further repetitions are needed with all mutants. Though the decrease in primary root length and number of lateral roots of plants when treated with bacteria was consistent as above explained experiments (See subsection 4.2) but to be more accurate the number of seedlings in control and bacteria should be same to eventually say something about the observed effect. In *tir1* mutant almost no lateral root formation was observed. This is because of mutation in auxin reception that lateral root development is severely compromised and no effect of bacteria was observed in comparison to the control for lateral roots development. Also research studies has shown that plants with mutation in *tir1* gene are deficient in many auxin related growth processes including lateral root formation (Dharmasiri et al. 2005; Kepinski and Leyser 2005; Ruegger et al. 1998; Terrile et al. 2012). Significant increase in root hairs is seen as previously (Figure 3-1, Figure3-3) when plants are inoculated with the bacteria. Increase in root hairs by bacterial strain 9, 15 and CL8 indicates that bacteria stimulate root hair growth in WT Col-0 as well also in mutant *pin2*, *wei8/wei2*, *aux1-7* and *tir1*.

4.4 Inoculation of *pin2* with strain 15

Among the mutants, further experimentation involved *pin2*. Since *pin2* is responsible for auxin efflux and root development (Blilou et al. 2005) inoculation with bacteria to see any desired effect was to ensure whether a functional auxin transport machinery is required to promote the root growth. The data shows (Figure 3-8) that the strain 15 has no positive effect on primary root elongation and also no effect on lateral root numbers in Col-0 and *pin2* as compared to control treatment. The decrease in primary root length by number of these bacterial strains that have been used in different experiments may be because these strains produce high levels of IAA. The bacterial strain 15 (*Agrobacterium* spp.) is shown by (Abbamondi et al. 2016) to produce high levels of IAA. It is speculated that higher levels of IAA contribute to the reduction of primary root length, increases lateral root growth and increases root hairs (Vacheron et al. 2013).

Overall our findings suggest that endophytic strains 9 and 15 (*Pseudomonas* and *Agrobacterium* spp.) decreases primary root length and increase root hairs in wild type Col-0 and mutant *pin2*.

4.5 Effect of bacteria isolated from *S.pimpinellifolium*

Here we investigate the potential of bacteria isolated from roots of *S.pimpinellifolium* to promote the plant growth of *Arabidopsis* Col-0 and *pin2* in vertical agar plate assay. Also we assess the impact of these endophytic isolates on Gemini tomatoes when grown in pot experiment. Among three bacteria that we isolated only two strains are tested on plants. The bacterial strain *B.flexus* is not used during this study because of some previous research study showing it to have no plant growth promoting properties (Jasim et al. 2013). The *M.yunnanensis* is previously isolated from *Polyspora axillaris* roots (Zhao et al. 2009) and studies have shown that it has plant growth promoting properties (Siddikee et al. 2010). *Cohnella* spp. is found to be close to the species *Cohnella plantaginis* and *Cohnella ginsengisoli* with both having equal identity so it's not clear about the specie type. However, the genus confirmed is *Cohnella*. Recent researches has found *Cohnella* spp. in the rhizospheric soil and some researches also indicate that specie *C.plantaginis* has nitrogen fixing property (Kim et al. 2010; Wang et al. 2012).

In sucrose supplemented MS medium *M.yunnanensis* inoculation showed increase in primary root elongation, number of lateral roots and also the shoot fresh weight for WT Col-0 seedlings. Whereas *Cohnella* spp. did not seem to have growth promoting effect on WT Col-0 plants. In *pin2* mutant, the bacterial treatment produced little or no effect on primary root length and number of lateral roots. However, both bacterial strain *M.yunnanensis* and *Cohnella* spp. stimulated the shoot fresh weight of seedlings (Figure 3-13). Root hair development was not seen in Control as well as bacteria treated plants (Figure 3-12). Since no special growth stimulation is noticed in *pin2* mutant it could be because of auxin transport protein mutation. Also previous researches indicate that when *Arabidopsis* is co-cultured with *Laccaria bicolor* less stimulation of root growth was observed in *pin2* mutant line (Felten et al. 2009). In the earlier research studies it is shown that bacterial specie *M.yunnanensis* have root growth promoting effect in canola plants and promote root elongation (Siddikee et al. 2010).

In our study bacterial specie *M.yunnanensis* stimulate the root growth development in *Arabidopsis* Col-0 seedlings in MS media supplemented with sucrose. The total number of plants were less in Col-0 seedlings and experiment need to be further repeated in order to have statistically significant data for plants grown in MS media with sucrose.

Unexpectedly, no significant difference on growth was seen in plants grown on MS media without sucrose, the inoculated treatments did not show much significant effect on primary root length of Col-0 and *pin2* (See Figure 3-15). A difference in results for number of lateral roots was observed between plants growing in with sucrose and without sucrose media. Col-0 type seedlings in sucrose supplemented media show (Figure 3-13) increase in lateral roots upon inoculating with *Micrococcus* which is not seen in without sucrose treatments. Inhibition of lateral roots is observed for Col-0 plants treated with *Cohnella*. High standard deviation in *Cohnella* treated Col-0 seedlings are because of huge variation among lateral root number on different seedlings (Figure 3-15). No particular difference in lateral roots number is observed in case of *pin2* plants. No effect on root hairs is seen in inoculation with new strains (See Figure 3-12 and 3-16). However, an increase in shoot fresh weight is observed with inoculated plants as compared to the control types (See Figure 3-13 and 3-15).

Under the light of these results it can be said that bacterial species have their effect on shoot weight of *Arabidopsis* Col-0 and *pin2* both in sucrose supplemented and without sucrose medium. However bacteria seemed to effect root growth of Col-0 more in sucrose containing MS medium. The limiting factor in the experiment is sample population. In experiment with sucrose and with bacteria, the sample size was 8 plants (Figure 1-13) because of inability of Col-0 plants to show normal growth, the plantlets were discarded and only good ones are considered.

The results from pot experiment (See Figure 3-27) show the slight increase in shoot length of Gemini tomatoes also significant increase in shoot fresh weight (Figure 3-28) is seen in *Micrococcus* inoculated plants. The experiment needs to be repeated with large sample size to become certain about results. Further research is required on these endophytes to determine their plant growth promoting traits such as IAA production, ACC deaminase activity, siderophore production and organic acid production. More experiments including pot experiments and agar plate inoculating experiment with large sample size are required to confirm the conclusion.

4.6 *Pseudomonas fluorescence* effect on Col-0 and *pin2*

In this part of thesis we analyzed the effect of PGPB strain *Pseudomonas fluorescence* (WCS417) on root growth of Col-0, *pin2* and also *aux1-7*. Previously in research studies this strain WCS417 is shown to effect plant root development. It is shown that WCS417 suppresses

the primary root growth, enhances the lateral root growth and increase root hair formation in Col-0 plants (Zamioudis et al. 2013). Here, we used the bacteria WCS417 under two different growth media conditions; bacterial inoculation to plants (Col-0, *pin2*) growing in sucrose supplemented MS medium (Figure 3-22) and bacterial inoculation to plants growing in without sucrose MS medium (Figure 3-19). Our findings confirm the inhibition of primary root length in plants as explained in earlier studies. A general trend of increase in lateral roots and root hairs is also observed in Col-0 as well as mutant *pin2* (Figure 3-21 and 3-22). Data on shoot fresh weight does not seem to be according to the previous studies. A decline in shoot fresh weight seen in Col-0 inoculated plants whereas shoot fresh weight of *pin2* is comparable to control. More repetition of experiment is required to see the effect on lateral roots number and increase in shoot fresh weight. No difference in root growth trend was observed between *Arabidopsis* Col-0 and *pin2* and bacteria effected the *pin2* mutant in same manner as wild type. Previously it is reported by (Zamioudis et al. 2013) that WCS417 mediated lateral root development does not involve auxin efflux carrier protein PIN2 but other member of PIN family are involved in auxin efflux. According to results of our study we can say that WCS417 effected similarly to Col-0 and *pin2* so may be *pin2* mutation has less to do with the effect of PGPB bacteria. The bacterial inoculation also had its effect on the curling of main root in *pin2* with roots becoming less curly as compared to control (Figure 3-20).

An increase in primary root length, number of lateral roots and increase in shoot fresh weight is seen when WT Col-0 and mutant *pin2* and *aux1-7* plants are grown in without sucrose medium with bacterial inoculation (Figure 3-19). This finding contrast with the results of earlier studies (Zamioudis et al. 2013) and also the experiment carried out in lab where primary root length of Col-0 and *pin2* is inhibited by bacterial inoculation. Here it can be said that along with sucrose bacterial inoculation suppresses the primary root growth, it may be because of combined effect of sucrose and bacteria. The data for primary root length of *aux1-7* is not shown in figure 3-19, it is because control plants of *aux1-7* show curly roots in which case length cannot be measured using imageJ software. However by observing the plates (see Figure 3-17) it is evident that bacteria increased the primary root length. Also an interesting observation is that bacteria treated *aux1-7* seem to have less curly roots. On the basis of the results it can be concluded that PGPB *Pseudomonas* WCS417 stimulates the root growth of Col-0, and mutant *pin2* and *aux1-7*.

4.7 Conclusion

- The endophytic and rhizospheric bacterial strains from (Abbamondi et al. 2016) study promote the root hair growth in Col-0 seedlings. Bacteria 9 and 15 (*Pseudomonas* and *Agrobacterium* spp.) stimulates root hair growth in *pin2*, *wei8/wei2*, *tir1* and *aux 1-7* mutant. Bacterial strain CL8 significantly promoted the root hair growth in all above mentioned mutant and in wild type. In few experiments CL8 also stimulated primary root length growth and lateral root growth and needs further testing.
- Among newly isolated bacteria from *Solanum pimpinellifolium*, *M.yunnanensis* stimulated primary root growth, number of lateral roots and also fresh shoot weight in Col-0 in sucrose supplement 1 X MS medium. *M.yunnanensis* also increase the shoot fresh weight of *pin2* mutant. *M.yunnanensis* promotes shoot growth of Gemini tomato plants and increases the shoot fresh weight in Gemini tomato plant. The bacteria *Cohnella* spp. also enhance the shoot fresh weight in WT and *pin2*.
- *Pseudomonas fluorescence* (WCS417) promotes primary root growth, increase lateral root numbers and enhance fresh shoot weight in Col-0, *pin2* and *aux1-7* in 1 X MS medium without sucrose. In 1 X MS medium with sucrose WCS417 strongly inhibits the primary root growth and increases lateral root number to some extend in WT Col-0 and *pin2* mutant. Growth promotion by plant growth promoting bacteria is independent of mutation in auxin efflux protein PIN2 and auxin influx *aux1-7*.

4.8 Future research

Future research should target the determination of the plant growth promoting traits of the newly isolated bacterial strain *Micrococcus yunnanensis* and *Cohnella* spp. More experimentation under different bacterial concentration on Gemini tomato plants should be performed to better understand the bacterial effect on plant growth. As the bacterial strains CL8 was found to promote the root hair growth and in some instances primary root growth. The bacterial strain CL8 should be identified and plant growth promoting assays like IAA, ACC phosphate solubilization and siderophore production should be performed to become familiar with CL8 growth promoting traits. New vertical agar plate experiment in 1 X MS media with and without sucrose including wild type Col-0 and mutant *pin2*, *wei8/wei2*, *tir1* and *aux1-7* with CL8 should

be performed to get insights into the role of bacteria in modulating the growth of auxin mutant. The model bacteria *Pseudomonas fluorescence* should be experimented with the *tir1* and *wei8/wei2* and *aux1-7* mutant to understand the role of bacteria in promoting the root growth of mutants and to see if exogenously produced plant growth promoting compounds can have their effect on the mutant growth.

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Appendix A

Appendix A-1 Inoculation of bacteria to *Arabidopsis* plants

A **B**

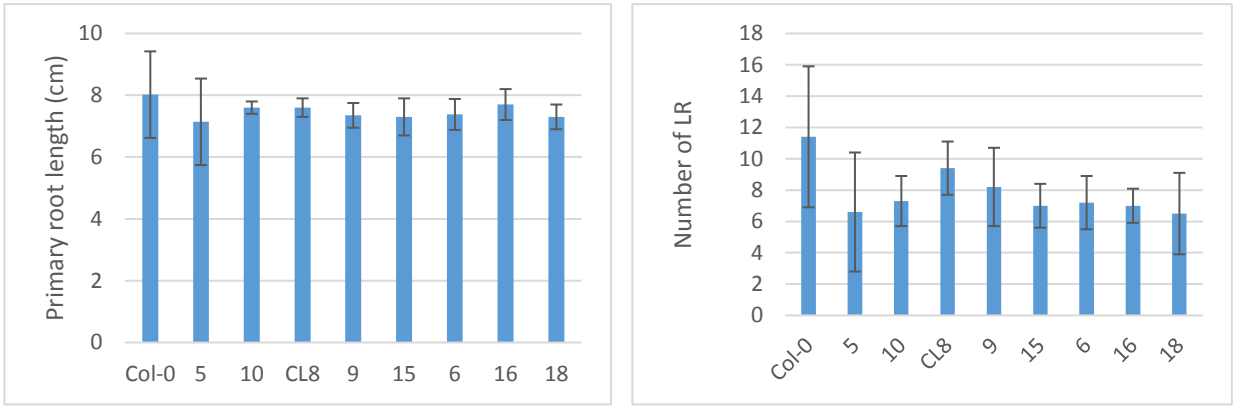
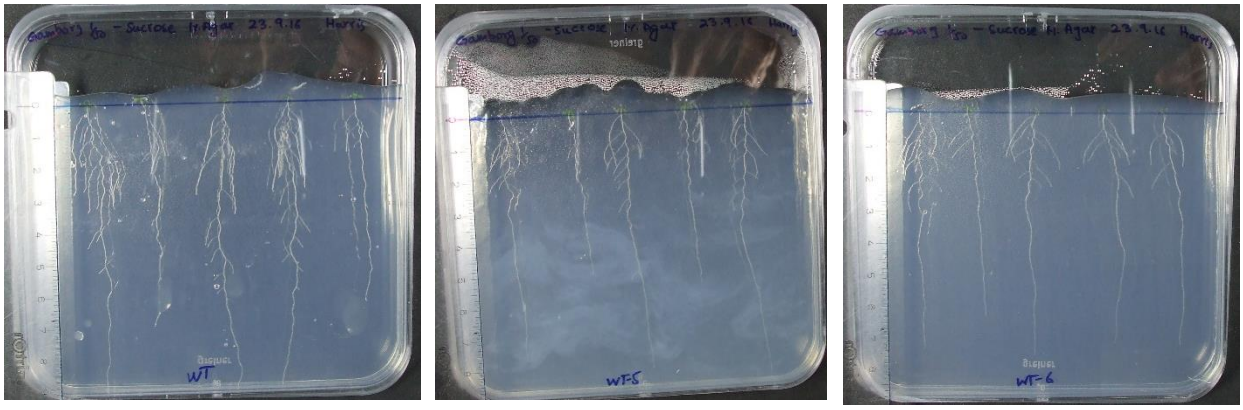


Figure A-1: *Effect of bacterial inoculation on Arabidopsis col-0 root development.* ***A*** *shows effect on primary root length,* ***B*** *shows number of lateral roots. Error bars indicate ± Standard deviation (n=5). Asterisks indicate statistically significant differences compared with Control (Student's t test; P < 0.05).*

Col-0 **5** **6**



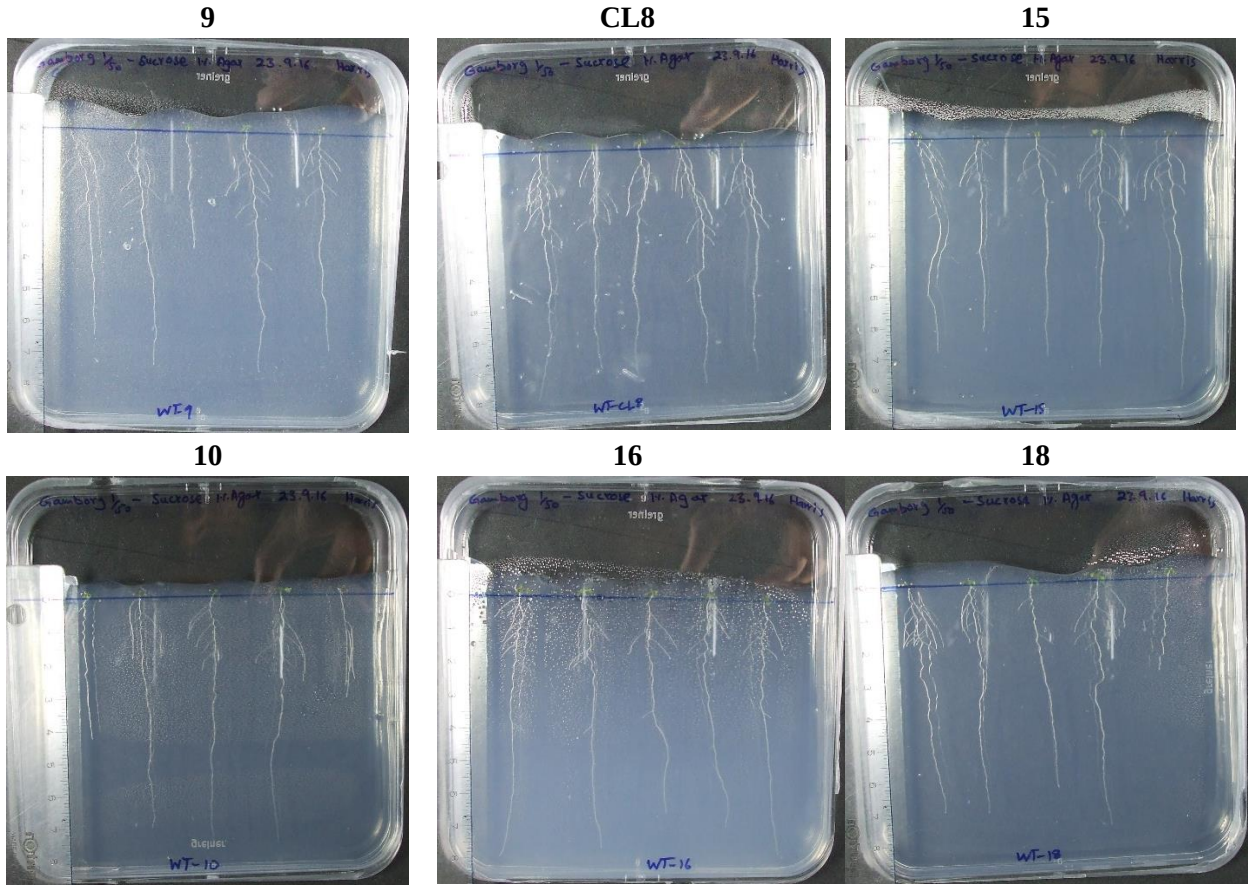
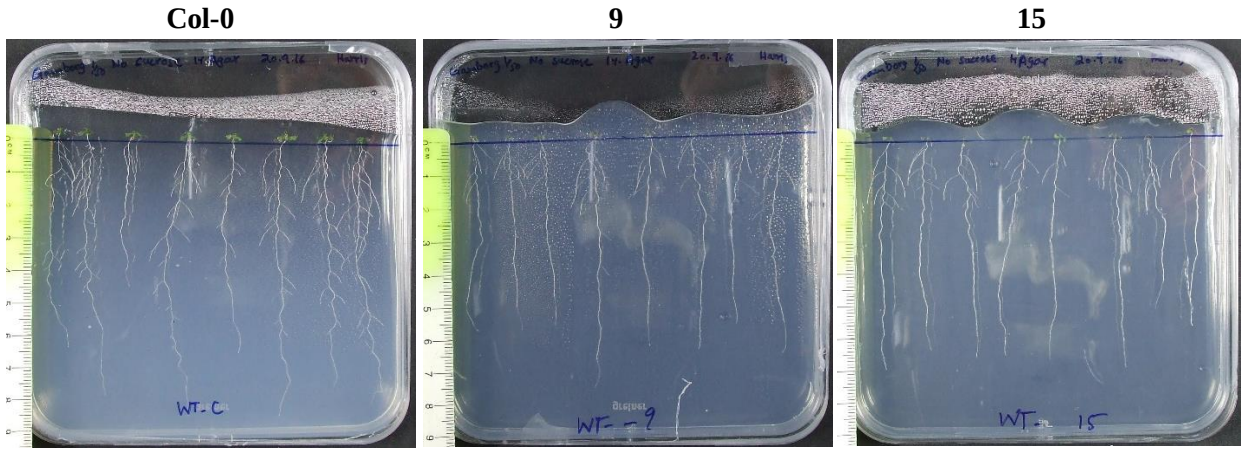


Figure A-2: *Effect of different endophytic bacteria on root development of Arabidopsis col-0. Seeds were grown in 1/50 X Gamborg's medium supplemented with 0.5% sucrose, , after 6 d seedlings were moved to plates without sucrose and 1 ml of bacterial culture was spread on whole plate. Plates were kept for 7 d in growth chamber.*

Appendix A-2 Bacterial inoculation to Col-0, *pin2* and *wei8/wei2*



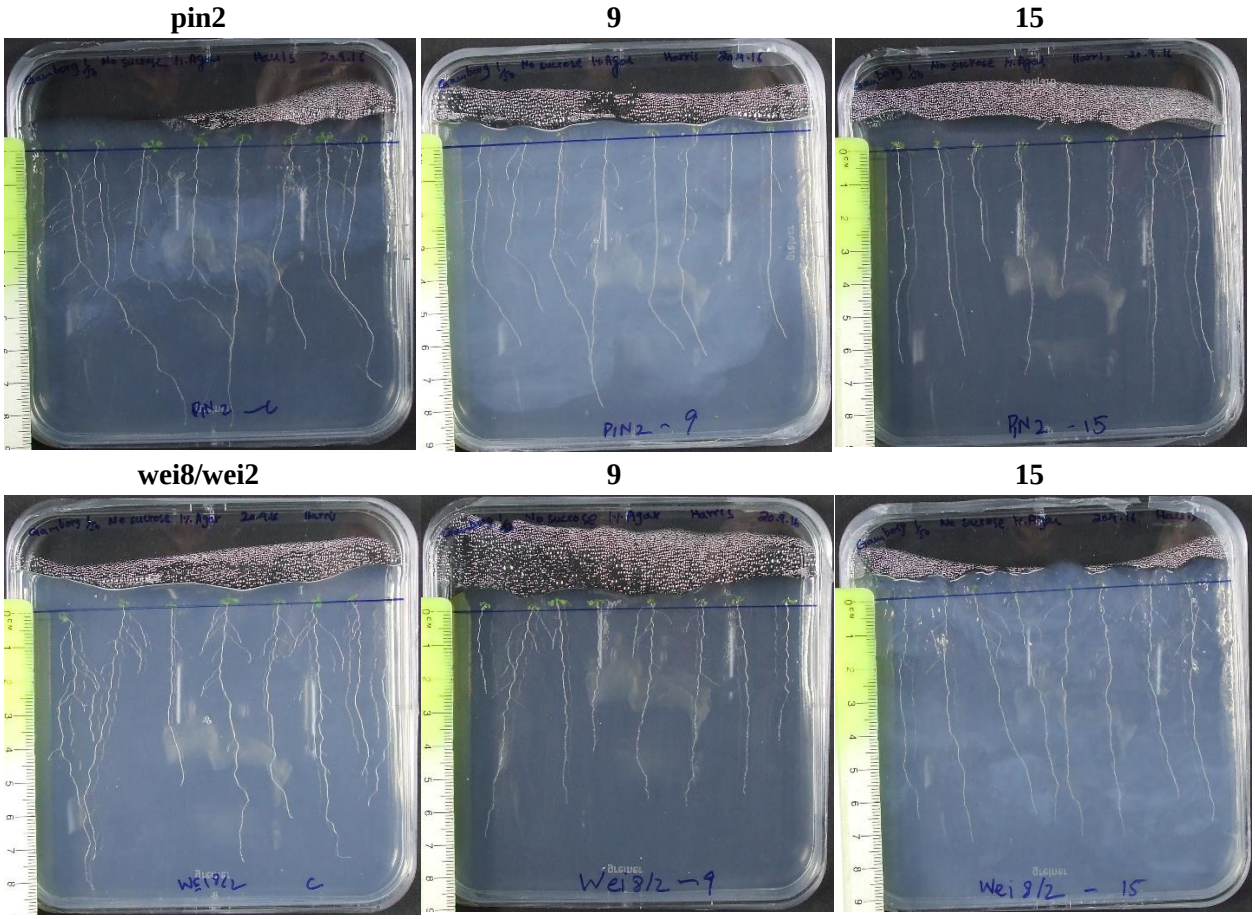
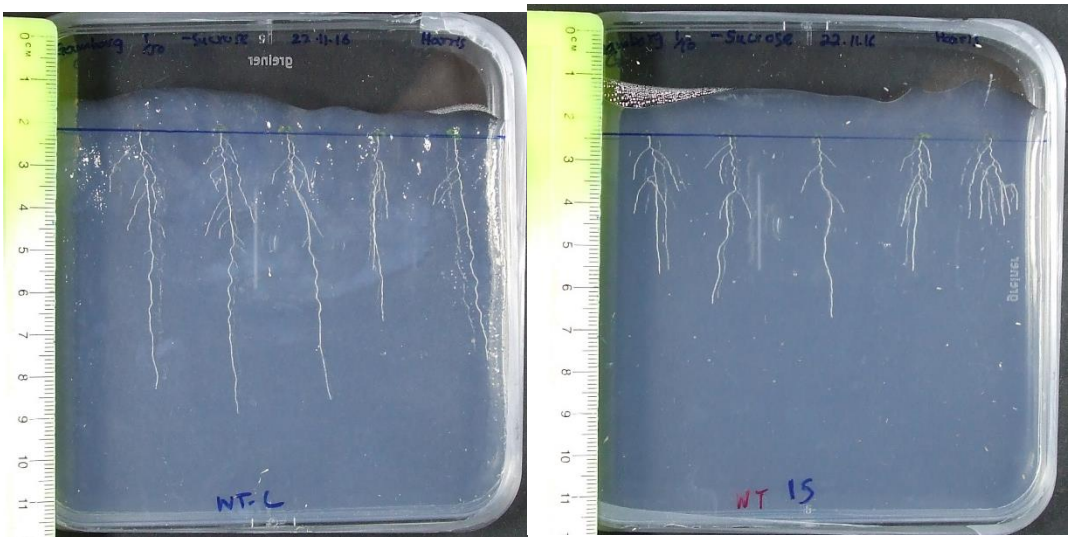


Figure A-3: *Influence of bacteria 9 and 15 on root growth of Arabidopsis col-0 type, mutant pin2 and wei8. Seedlings were sown on 1/50x Gamborg medium containing 0.5% sucrose. After 6 d post germination, 650 ul of bacterial culture was spread on plates. Photographs were taken after 8 d of cocultivation. From left is Control groups, middle is strain 9 treated and last 15 treated plants.*

Appendix A-3 Strain 15 inoculation to WT and *pin2*

A



B

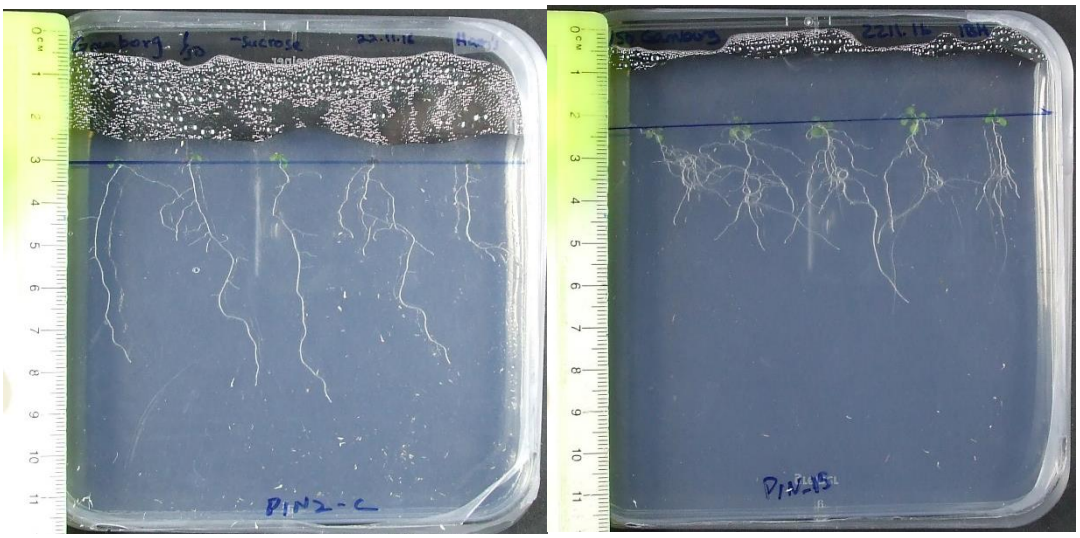


Figure A-4: *Effect of Agrobacterium spp. (strain 15) on root development of Arabidopsis col-0 and mutant pin2 roots. A part depicts Col-0 plants, Control (left) and bacteria induced (right). B shows mutant pin2 plants, Control (left) and bacteria induced (right). Seedlings were grown on 1/50x Gamborg medium supplemented with 0.5% sucrose for 6 d, after 6 d seedlings were moved to plates without sucrose and 650 µl of bacterial culture was spread on whole plate. Plates were kept for 7 d in growth chamber and after 7 d pictures were taken.*

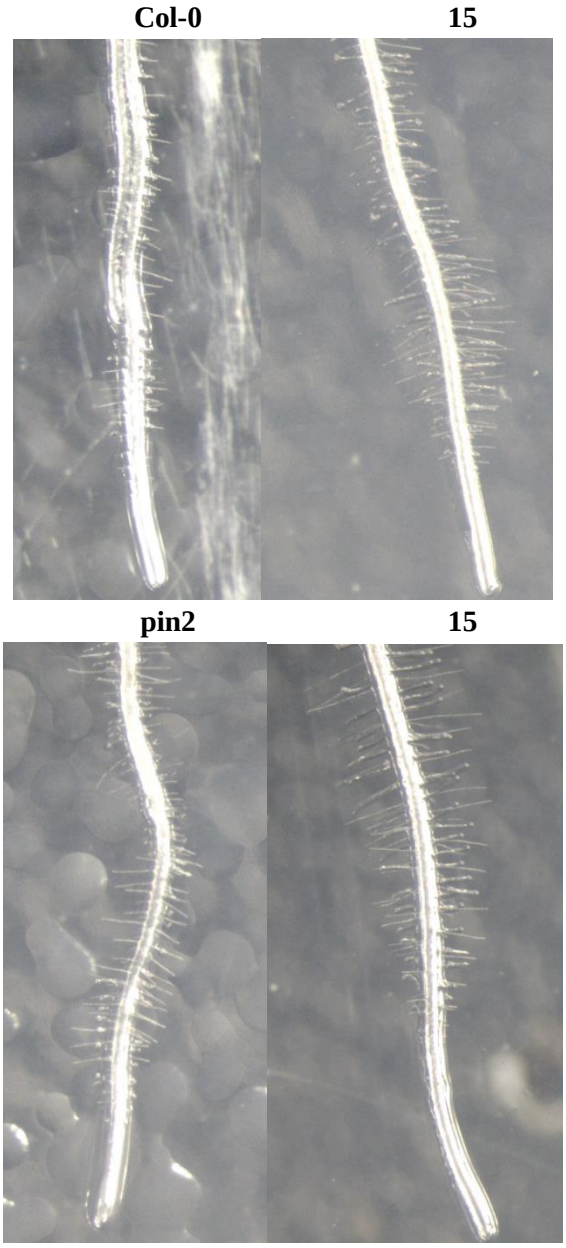


Figure A-5: *Root hairs growth comparison between Col-0 and 15, pin2 and 15 after 7 days of growth in growth chamber.*

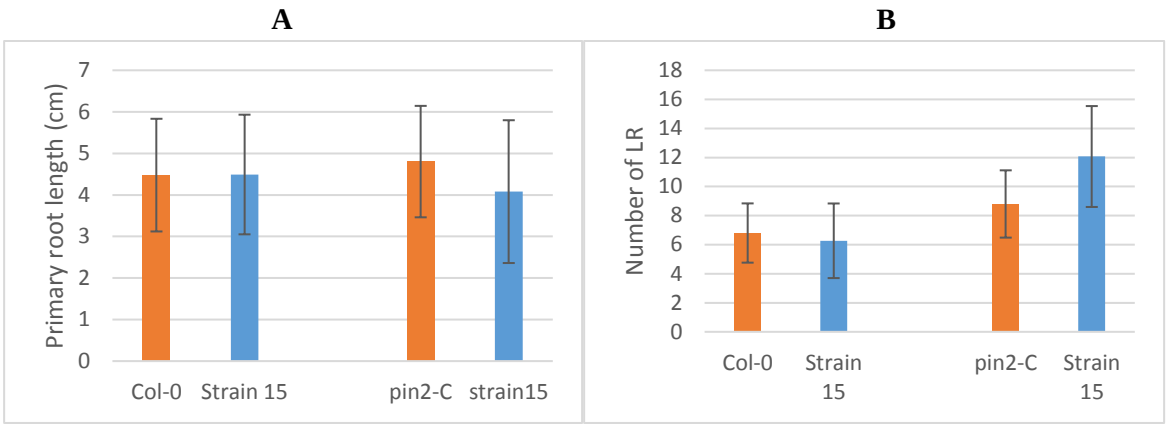


***Figure A-6**: Effect of strain 15 on root development of Arabidopsis Col-0 and mutant pin2. A shows primary root length, B shows average number of lateral roots. Orange bars indicate Control group (Col-0 and pin2), Blue bars indicate bacteria strain 15 in Col-0 and pin2 type. Error bars show ± SD (n=15).*

Appendix B

Appendix B-1 Plants inoculated with new isolates, 1 X MS medium with sucrose

***Table B-1**: VAP test-Measurement of Primary root lengths after 8 days in growth chamber. SD; standard deviation.*

Bacterial Type	No. of Seeds	Average primary root length (cm)	SD	P-value
Col-0	8	5.654164	0.545508	
Micrococcus	8	6.693018	0.794368	0.0082*
Cohnella	8	5.440296	0.690158	0.5089
pin2	15	5.435823	1.010438	
Micrococcus	15	5.84991	1.163878	0.3107
Cohnella	15	6.264637	1.531629	0.0905

***Table B-2**: VAP test- Average number of lateral roots (LR) after 8 d in growth chamber. SD; Standard deviation.*

Bacterial Type	No. of Seeds	Average number of LR	SD	P-value
Col-0	8	7.25	6.118619	
Micrococcus	8	11.5	4.1833	0.1246
Cohnella	8	7.888889	4.067334	0.8116
pin2	15	13.64286	4.576448	
Micrococcus	15	15.26667	4.122567	0.3166
Cohnella	15	17.53333	5.314968	0.0403*

Table B-3*: VAP test- Mean shoot fresh weight after 8 d in growth chamber. SD; Standard deviation*.

Bacterial Type	No. of Seeds	Mean Shoot Fresh weight (g)	SD	P-value
Col-0	8	0.015233	0.002899	
Micrococcus	8	0.0253	0.0077	0.0038*
Cohnella	8	0.020067	0.001452	0.0001*
pin2	15	0.029333	0.005681	
Micrococcus	15	0.036233	0.010336	0.0001*
Cohnella	15	0.042	0.001219	0.0001*

Appendix B-2 Plants inoculated with new isolates, 1 X MS medium without sucrose

Table B-4*: VAP test-Measurement of Primary root lengths after 8 days in growth chamber. SD; standard deviation.*

Bacterial Type	No. of Seeds	Average primary root length (cm)	SD	P-value
Col-0	20	5.326925	1.025848	
Cohnella	22	5.392597	0.730176	0.8167
Micrococcus	22	5.724934	0.796207	0.1781
pin2	25	5.224245	1.195126	
Cohnella	25	5.753193	0.846654	0.071
Micrococcus	25	5.725489	1.147593	0.12900

Table B-5*: VAP test- Average number of lateral roots (LR) after 8 d in growth chamber. SD; Standard deviation*.

Bacterial Type	No. of Seeds	Average number of LR	SD	P-value
Col-0	20	7.227273	4.220425	
Cohnella	22	3.227273	3.450057	0.00165*
Micrococcus	22	7.636364	4.291063	0.75698
pin2	25	11.96296	3.382761	
Cohnella	25	10.85185	4.568035	0.32351
Micrococcus	25	11.7037	5.212626	0.83236

***Table B-6**: VAP test- Mean shoot fresh weight after 8 d in growth chamber. SD; Standard deviation*.

Bacterial Type	No. of Seeds	Mean Shoot Fresh weight (g)	SD	P-value
Col-0	20	0.014983	0.005589	
Cohnella	22	0.0251	0.011611	0.0004*
Micrococcus	22	0.03066	0.017215	0.0010
pin2	25	0.023633	0.003072	
Cohnella	25	0.029383	0.002373	0.0001*
Micrococcus	25	0.027567	0.002582	0.0001*

Appendix B-3 WCS417 effect on root growth of plants in 1 X MS medium (No Sucrose)

***Table B-7**: VAP test-Measurement of Primary root lengths after 8 days in growth chamber. SD; standard deviation*

Bacterial Type	No. of Seeds	Average primary root length (cm)	SD	P-value
Col-0	15	5.601415	0.646635	
WCS417	15	6.528755	0.788738	0.0015*
pin2	15	5.67815	1.387422	
WCS417	15	6.592425	0.862335	0.0388

***Table B-8**: VAP test- Average number of lateral roots (LR) after 8 d in growth chamber. SD; Standard deviation.*

Bacterial Type	No. of Seeds	Number of LR	SD	P-value
Col-0	15	6.2	4.86072697	
WCS417	15	9.53333333	5.81797979	0.1006
pin2	15	11.2857143	5.37758006	
WCS417	15	14.1333333	4.84240528	0.1387
	15			
aux1-7	15	7.69230769	3.96881334	
WCS417	15	11.0714286	2.7114647	0.0110

Table B-9*: VAP test- Mean shoot fresh weight after 8 d in growth chamber. SD; Standard deviation*.

Bacterial Type	No. of Seeds	Shoot fresh weight (g)	SD	P-value
Col-0	15	0.01845	0.00427171	
WCS417	15	0.0296	0.00524277	0.0001*
pin2	15	0.0266	0.00482494	
WCS417	15	0.02983333	0.00129185	0.0183
aux1-7	15	0.02186667	0.00456387	
WCS417	15	0.029	0.00466119	0.0002*

Appendix B-4 WCS417 effect on root growth of plants growing in MS media with sucrose.

Table B-10*: VAP test-Measurement of Primary root lengths after 8 days in growth chamber. SD; standard deviation*

Bacterial Type	No. of Seeds	Average primary root length (cm)	SD	P-value
Col-0	18	5.76306674	1.16786144	
WCS417	18	2.10110617	0.50162664	0.0001*
pin2	18	5.54017643	1.38865426	
WCS417	18	2.32674597	0.75381249	0.0001*

Table B-11*: VAP test- Average number of lateral roots (LR) after 8 d in growth chamber. SD; Standard deviation*.

Bacterial Type	No. of Seeds	Number of LR	SD	P-value
Col-0	18	7.16666667	3.91932534	
WCS417	18	8.11111111	3.82809626	0.4696
pin2	18	9.11111111	4.60541817	
WCS417	18	11.7777778	3.52066212	0.0593

Table B-12*: VAP test- Mean shoot fresh weight after 8 d in growth chamber. SD; Standard deviation.*

Bacterial Type	No. of Seeds	Shoot fresh weight (g)	SD	P-value
Col-0	16	0.02683333	0.00140554	
WCS417	18	0.02076667	0.0036609	0.0001*
pin2	15	0.0273	0.00139523	
WCS417	18	0.02773333	0.00264113	0.5423