CHAPTER 2

Materials and Methods

2.1 Media and Culture conditions

The *E. coli* strains and *E. asburiae* PSI3 were cultured and maintained on Luria Agar (LA) (Hi-Media Laboratories, India). *E. coli* cultures were grown at 37°C. For growth in liquid medium, shaking was provided at the speed of 200rpm. The plasmid transformants of both *E. coli* and *E. asburiae* PSI3 were maintained using respective antibiotics at the final concentrations as mentioned in **Table 2.1** as and when applicable. Both *E. coli* and *E. asburiae* PSI3 wild type strains and plasmid transformants grown in 3ml Luria broth (LB) containing appropriate antibiotics were used to prepare glycerol stocks which were stored at -20°C.

Table2.1: Recommended dozes of antibiotics used in this study (Sambrook and Russell, 2001).

Antibiotics	Rich medium	Minimal medium
Kanamycin	50μg/ml	12.5μg/ml
Streptomycin	10μg/ml	2.5μg/ml
Trimethoprim	60μg/ml	15μg/ml
Ampicillin	50μg/ml	12.5μg/ml
Erythromycin	100μg/ml	25μg/ml
Gentamycin	20μg/ml	5μg/ml

The antibiotic dozes were maintained same for both *E. coli* and *E. asburiae* PSI3. All the antibiotics were prepared in sterile distilled water or recommended solvent at the stock concentrations of 1000x. The compositions of different minimal media used in

this study are as described below. Antibiotic concentrations in all the following minimal media were reduced to 1/4th of that used in the above mentioned rich media (**Table 2.1**).

2.1.1: Koser's Citrate medium

Composition of the Koser's Citrate medium included magnesium sulphate, 0.2g/L; monopotassium phosphate, 1.0g/L; sodium ammonium phosphate, 1.5g/L; sodium citrate, 3.0g/L and agar, 15g/L. The readymade media was obtained from Hi-Media Laboratories, India, and was used according to manufacturer's instructions.

2.1.2: M9 minimal medium

Composition of M9 minimal broth was according to Sambrook and Russell (2001) including Na_2HPO_4 $7H_2O$, 34g/L; KH_2PO_4, 15g/L; NH_4Cl, 5g/L; NaCl, 2.5g/L; 2mM $MgSO_4$; 0.1mM $CaCl_2$ and micronutrient cocktail. The micronutrient cocktail was constituted of $FeSO_4.7H_2O$, 3.5 mg/L; $ZnSO_4.7H_2O$, 0.16 mg/L; $CuSO_4.5H_2O$, 0.08 mg/L; H_3BO_3, 0.5 mg/L; $CaCl_2.2H_2O$, 0.03 mg/L and $MnSO_4.4H_2O$, 0.4 mg/L. Carbon sources used were glucose, xylose, arabinose, maltose, cellobios, mannose and sucrose as and when required. For solid media, 15g/L agar was added in addition to above constituents. 5X M9 salts, micronutrients (prepared at 1000X stock concentration) and carbon source (2M, 1M and 0.5M stock) were autoclaved separately. Fixed volumes of these were added aseptically into pre-autoclaved flasks containing distilled water to constitute the complete media with the desired final concentrations. Volume of water to be autoclaved per flask was calculated by subtracting the required volumes of each ingredient from the total volume of the media to be used.

2.1.3: Tris rock phosphate buffered medium

The media composition included Tris-Cl (pH=8.0), 100mM; NH_4Cl, 10mM; KCl, 10mM; $MgSO_4$, 2mM; $CaCl_2$, 0.1mM; micronutrient cocktail; Glucose, 50-100mM and phosphate (P) sources (Sharma et al., 2005). 1mg/ml Senegal Rock phosphate (RP) or KH_2PO_4 were used as insoluble and soluble P sources respectively. Each ingredient was separately autoclaved at a particular stock concentration and a fixed volume of each was added to pre-autoclaved flasks containing sterile distilled water (prepared as in Section 1.3) to constitute complete media.

2.1.4: Tris phytate buffered medium

Similar composition used in this medium as mention in Tris rock phosphate buffered medium. Rock phosphate replace with 0.3% Ca-phytate or Na- phyate.

2.1.5: Murashige-Skoog's Medium

Murashige-Skoog's (MS) medium was composed of macro elements and micro elements. The macro elements included $CaCl_2.2H_20$, 0.440g/L; KH_2PO_4, 0.17g/L; KNO_3, 1.9g/L; $MgSO_4.7H_2O$, 0.37g/L; NH_4NO_3, 1.65g/L and 10g/L agar. The micro elements included the essential trace elements. Micronutrients and desired carbon source (autoclaved separately), were added aseptically to reconstitute the complete media. The readymade micronutrient was obtained from Hi-Media Laboratories, India, and was used according to manufacturer's instructions while the macronutrients were reconstituted as and when required.

2.2: Molecular biology tools and techniques

2.2.1: Isolation of plasmid and genomic DNA

2.2.1.1: Plasmid DNA isolation from *E. coli* and *E. asburiae* PSI3

The plasmid DNA from *E. coli* and *E. asburiae* PSI3 was isolate using standard alkali lysis method (Sambrook and Russell, 2001).

2.2.1.2: Genomic DNA isolation from *Pseudomonas putida* KT2440

Fresh *Pseudomonas* culture obtained by growing a single colony inoculated in 3ml LB under shake conditions at 30°C was dispensed in 1.5ml sterile centrifuge tubes, pelleted at 9, 200x *g* and washed twice with sterile normal saline. Following this, the cells were used for genomic DNA isolation performed using standard genomic DNA extraction protocol for bacterial cell (Ausubel, M et al,2006). The DNA was finally re-suspended in 40µl of sterile double distilled water.

2.2.2: Transformation of plasmid DNA

2.2.2.1: Transformation of plasmid DNA in *E. coli*

The transformation of plasmids in *E. coli* using $MgCl_2$-$CaCl_2$ method and bluewhite selection of the transformants using IPTG and X-Gal (as and when applicable) was carried out according to Sambrook and Russell,(2001).

2.2.2.2: Transformation of plasmid DNA in *E. asburiae* PSI3

Plasmid transformation in *E. asburiae* PSI3 was done using the NaCl-$CaCl_2$ method (Cohen et al., 1972) with slight modifications which are as follows. *E. asburiae* PSI3 was grown at 37°C in LB broth to an O.D. 600 of 0.4-0.6. At this point, the cells were chilled for about 10minutes, centrifuged at 5000rpm for 5 minutes and washed once in 0.5 volume 10mM NaCl (chilled). After centrifugation (5000rpm for 5 minutes), bacteria were re-suspended in half the original volume of chilled 0.1M $CaC1_2$, incubated on ice-bath for 1hr, centrifuged (5000rpm for 5 minutes) and then resuspended in 1/10th of the original culture volume of chilled 0.1M $CaC1_2$. 0.2ml of competent cells treated with $CaCl_2$ was used per vial (microcentrifuge tube) to add DNA samples (minimum 0.8-1.0µg is required) and were further incubated on ice-bath for 1hr. Competent cells were then subjected to a heat shock at 42°C for 2 min to

enable DNA uptake, immediately chilled for 5 minutes and then were supplemented with 0.8ml of sterile LB broth followed by incubation for 1hour at 37°C under shake conditions. These cells were then centrifuged and plated on Luria Agar or Koser citrate agar plates containing appropriate antibiotics. The colonies obtained after overnight incubation of the plates at 37°C were then subjected to plasmid DNA isolation.

2.2.3: Transfer of plasmid DNA by conjugation

The plasmids were transformed in *E. coli* S17.1, for mediating the conjugal transfer and the resultant transformant strain was used as the donor strain. *E. coli* S17.1 harboring the plasmid and the recipient *E. asburiae* PSI3 were separately grown in 3ml LB broth with respective antibiotics at 37°C under shake conditions for approximately 16h. The freshly grown cultures of recipient and the donor strains were aseptically mixed in 1:1 ratio (v/v) in a sterile centrifuge tube and the cells were centrifuged at 5000rpm for 5 minutes. The media supernatant was discarded to remove the antibiotics and the pellet was re-suspended in 0.2ml of fresh sterile LB and the bacteria were allowed to mate at 37°C. After 16h, the bacterial culture mix was centrifuged at 5000rpm for 5 minutes and the resultant pellet was re-suspended in 0.05ml of sterile normal saline, this cell suspension were plated on Koser Citrate agar containing the appropriate antibiotics for selection (antibiotic dose was as described in **Table 1**) to obtain the transconjugants.

2.2.4: Agarose gel electrophoresis

The DNA samples were mixed with appropriate volume of 6X loading buffer (0.25% bromophenol blue, and 40% sucrose in water) and subjected to electrophoresis through 0.8% agarose (containing 1μg/ml ethidium bromide) gel in

Tris-acetate-EDTA (TAE) buffer at 5v/cm for 0.5-2h. The DNA bands were visualized by fluorescence under the UV-light using UV transilluminator.

2.2.5: Restriction enzyme digestion analysis

0.5-1.0μg DNA sample was used for each restriction enzyme digestion. 1-3U of the restriction endonuclease (RE) was used with the appropriate 10X buffers supplied by the manufacturer in a final reaction volume of 10μl. The reaction mixture was incubated overnight at 37°C. The DNA fragments were visualized by ethidium bromide staining after electrophoresis on 0.8% agarose gels and were subsequently photographed. In case of double digestion, a compatible buffer for the two REs was essentially checked. If not available, digestion with one enzyme is performed followed by purification and subsequent digestion with the other enzyme, using respective buffers. Restriction digestion was performed according to manufacturer instruction. Typical reaction follows

Components	**Quantity**
DNA (~300 ng/ μl)	3.0μl
Buffer (10X)	2.0μl
Restriction Enzyme (10U/μl)	0.3μl
Autoclaved D/W	14.7μl
Total	20 .0μl

Reaction mixtures were incubated for 4 hr at 37^0C. The samples ware analyze on 0.8% agarose

2.2.6: Polymerase Chain Reaction (PCR)

The PCR reaction set up was based on the guidelines given in Roche Laboratory Manual. The assay system and the temperature profile used are described as follows

PCR reaction mixture:

Forward primer (10pmol/ml)	**1.0 µl**
Reverse Primer (10pmol/ml)	**1.0 µl**
dNTPs (2.5mM)	**3.0 µl**
10X Pfu polymerase Buffer	**5.0 µl**
Pfu polymerase (3 units/µl)	**0.5 µl**
Template	**0.5 µl**
Nuclease free water	**39.0 µl**
Total system	**50.0 µl**

PCR amplifications were performed in DNA Engine thermal cycler (BioRad) or Variti thermocycler (Applied Biosystem). *Exact primer annealing temperature and primer extension time varied with primers (designed with respect to different templates) and has been specified in the text as and when applicable. Taq DNA polymerase, XT-20 polymerase, Pfu polymerase and Phusion polymerase used as when required mention in text. Polymerase, buffer, dNTPs and primers were obtained respectively from Bangalore Genei Pvt. Ltd., India, Sigma Chemicals Pvt. Ltd., Ocimum biosolutions, India, Integrated DNA Technology USA. The theoretical validation of the primers with respect to absence of intermolecular and intramolecular complementarities to avoid primer-primer annealing and hairpin structures and the appropriate %G-C was carried out with the help of online primer designing software Primer 3. The sequence, length and %G-C content of primers are subject to variation depending on the purpose of PCR and will be given as and when applicable in the

following chapters. The PCR products were analyzed on 0.8% agarose gel along with appropriate molecular weight markers.

PCR program		
Lid temperature	**105°C**	
Step -1 (Initial denaturation)	**94^0 C - 1 min cycle 1**	
Step - 2 (Denaturation)	**94^0 C - 30 sec**	
Step -3 (Annealing)	**52^0 C - 30 sec**	**30 cycle**
Step – 4 (Extension)	**72^0 C - 1 min 30 sec**	
Step -5 (Final extension)	**72^0 C – 5 min cycle 1**	
Hold: - 4^0C - 20 min		

2.2.7: Gel elution and purification

The DNA fragments of desired sizes were recovered from the gel by cutting the agarose gel slab around the DNA band. The agarose piece was weighed in a sterile microcentrifuge tube. Gel elution and purification was done by using PureLink Quick Gel Extractio Kit (Invitrogen) according to manufacturer protocol.

2.2.8: Ligation

The ligation reaction was usually done in 10µl volume containing the following constituents: Purified vector and insert DNA (volume varied depending on the respective concentrations); 10X T4 DNA Ligase buffer, 1µl; T4 DNA ligase (MBI Fermentas), 0.5- 1.0U and sterile double distilled water to make up the volume 10 µl. The cohesive end ligation reaction was carried out at 22°C for 1h. The vector to insert molar ratio (molar concentrations calculated by the under mentioned formula) of 1:4 was maintained, with a total of 50-100ng of DNA in each ligation system.

$$\text{pmoles of DNA} = \frac{\text{Amount of DNA (µg)} \times 1,515}{\text{Size of the DNA fragment (no. of base pairs)}}$$

2.2.9: SDS-PAGE

SDS-PAGE slab gel electrophoresis was carried out using 10-15% acrylamide gel by following the procedures described by Sambrook et al (2000).

Table 2.2: Composition of SDS-PAGE reagents (Sambrook and Russell, 2001)

(A) Monomer solution (30%) (Store at 4° C in dark)		**(B) Resolving gel buffer-1.5M Tris (pH 8.8)** Adjust pH with HCl		**(C) Stacking gel buffer 1.0M Tris (pH 6.8)** Adjust pH with HCl	
Acrylamide	14.6 gm	Tris base	9.1 gm	Tris	3.02 gm
Bisacrylamide	0.4 gm	SDS	0.2 gm	SDS	0.20 gm
D. H_20	Till 50 ml	D. H_20	Till 50 ml	D. H_20	Till 50ml
(D) Tank Buffer (pH 8.3)		**(E) Sample Loading buffer (2X)**		**(F) Other reagents**	
Tris base	6.0 gm	SDS	4%	APS	10% (fresh)
Glycine	28.8 gm	Glycerol	20%	TEMED	2-3 µl
SDS	2.0 gm	Tris-Cl (pH6.8)	0.125M	Water saturated n-butanol	
D. H_20	Till 2 L	Bromophenolblue	0.05%w/v	Sigma protein molecular weight marker SDS6H2-(30,000-200,000).Used 14µg total protein/well	
Adjust the pH with HCl		β-mercaptoethanol	10mM		
		D. H_20	Till 10ml		
(G) Separating Gel (8%, 10ml)		**(H) Stacking Gel (3.9%, 5ml)**		**(I) Staining Solution**	
30% Monomer	2.7 ml	30% Monomer	0.65 ml	0.025% Commassie Blue R-250 in 40% Methanol and 7% Acetic acid	
Separating gel buffer (pH 8.8)	2.5 ml	Stacking gel buffer (pH 6.8)	1.25 ml		
D. H_20	2.3 ml	D. H_20	3.05 ml	**(J) De-staining solution**	
10% APS	50 µl	10% APS	25 µl	(10% methanol and 10% Acetic acid)	
TEMED	2 µl	TEMED	3 µl		

After electrophoresis the gel was stained using the staining solution (**Table 2.2**) for approximately 1h and then de-stained with de-staining solution (**Table 2.2**) by incubating overnight under mild shaking conditions. Result was recorded by direct scanning of gel.

2.3: P-solubilization phenotype

P-solubilizing ability of the native as well as the transformant *E. asburiae* PSI3 was tested on Tris buffered RP-Methyl red (TRP) agar plates which represents a much more stringent condition of screening for PSMs (Gyaneshwar et al, 1998b). Liquid medium for RP solubilization is described in Section 2.1.3 with an addition of methyl Red as pH indicator dye and 1.5% agar for all the plate experiments. *E. asburiae* PSI3 cell suspension for these experiments was prepared as described in section 2.4.1 and 3µl of it was aseptically spotted on the above mentioned agar plates and was allowed to dry completely followed by incubation at 37°C for 5-7 days. P solubilization was determined by monitoring red zone on the TRP agar plates. Media acidification from pH=8.0 to pH<5 on TRP broth was used as an indicator for P-solubilization and organic acid secretion.

2.4: Physiological experiments

The physiological experiments were carried out using various WT and transformant *E. asburiae* PSI3 which included growth, pH profile and enzyme assays.

2.4.1: Inoculum preparation

The inoculum for M9 and Tris minimal media containing free Pi was prepared by growing the *E. asburiae* PSI3 cultures overnight at 37°C in 3ml LB broth. Cells

were harvested aseptically, washed twice by normal saline and finally re-suspended in 1ml normal saline under sterile conditions. Freshly prepared inoculum was used for all the experiments.

2.5.2: Growth characteristics and pH profile

Growth parameters and pH profile of the native as well as transformant *E. asburiae* PSI3 were determined using three different media conditions including TRP medium with RP as P source (Gyaneshwar et al, 1998).The media composition in this case was same as mentioned in Section 45- 100mM glucose or 50-100mM sucrose was used as the carbon source for all the experiments unless and until stated categorically. In 150ml conical flasks, 30ml of relevant minimal broth without free Pi was inoculated with cell suspensions to have 0.01-0.03 O.D600nm initially (0 hour O.D.).The batch culture studies were performed under aerobic conditions in Orbitek rotary shaker maintained at 37°C with agitation speed kept constant at 200rpm. 1ml samples were aseptically harvested at regular intervals (varying with every set of batch culture depending on media conditions) and were subjected to various analytical techniques.

2.4.3: Analytical techniques

The cell density determinations were done at 600nm as monitored spectrophotometrically. Change in absorbance was considered as the measure of growth and drop in pH of the media was taken as the measure of acid production. In all the cases, the observations were continued till the media pH reduced to less than 5. 1ml aliquots withdrawn aseptically at regular time intervals were immediately frozen at -20°C until further used for biochemical estimations. The stored samples were centrifuged at 9, 200x *g* for 1 min at 4°C and the culture supernatants derived were

used to estimate residual glucose and organic acid analysis using HPLC. For HPLC analysis, the culture supernatant was passed through 0.2μm nylon membranes (MDI advanced microdevices, India) and the secreted metabolites were quantified using RP-18 column or Luna column (Phenomenex). The column was operated at room temperature using mobile phase of 0.01M H_2SO_4 at a flow rate of 1.0 ml min 1 and the column effluents were monitored using a UV detector at 210 nm. Standards of organic acids were prepared in double distilled water, filtered using 0.2μm membranes and were subjected to chromatography for determining the individual retention time. Measurements of area under peak with an external standard were used for quantification.

2.5: Enzyme assays

2.5.1: Preparation of cells and cell free extracts

Glucose grown cells under above mentioned in TRP medium conditions till pH goes below 6.0. Cells were collected by centrifugation 9,200x *g* for 2 minutes at 4°C. The whole cell preparation for GDH assay was done by washing the harvested cells (mid-late log phase cultures) thrice with normal saline to remove the residual glucose of the medium and resuspending in 0.01M phosphate buffer (pH 6.0) with 5mM MgCl2

2.5.2: Enzyme Assay Protocols

2.5.2.1: Invertase assay

The bacterial isolate was grown overnight in M9 minimal medium with sucrose or glucose as the carbon source. Cells were harvested by centrifugation at 5000g for 10 min, then washed with and resuspended in sterile saline. An aliquot of the cells was used for toluenization to permeabilize the cells. Whole cells as well as

toluenized cells were used for enzyme assay. The assay system (2 ml) consisted of 0.01 M sucrose, 0.1 M buffer (acetate buffer, pH 5.0, phosphate buffer, pH 7.0, or Tris Cl buffer, pH 8.0), with an appropriate amount of cells as the source of enzyme. The reaction system was incubated at 37°C for 30 min and the reducing sugar produced was measured by the method of Miller (1959). One unit of activity is defined as the amount of enzyme that produced reducing sugar equivalent to 1 μmol of glucose per h. Specific activity is defined as units per milligram of protein.

2.5.2.2: GDH assay

GDH (D-glucose phenazine methosulphate oxidoreductase, (EC 1.1.5.2) was determined spectrophotometrically by following the coupled reduction of 2,6-dichlorophenolindophenol (DCIP) at 600nm (Quay et al., 1972). Molar absorbance of DCIP was taken as 15.1 mM-1 cm-1 at pH 8.75. The reaction mixture included: Tris-Cl buffer (pH 8.75), 16.66mM; D-glucose, 66mM; DCIP, sodium salt, 0.05mM; phenazine methosulfate, 0.66mM; sodium azide, 4mM; whole cells, and distilled water to 3.0ml.

2.5.2.3: GAD assay

Wild type and recombinant PSI3 were grown on RP containing minimal medium containing 75mM glucose and 100 mM Tris Cl (pH- 8.0) as carbon source. After pH drop below 5.5, cells were harvested (10000 g for 5 min) and washed with sterile saline and resuspend in 0.1M potassium phosphate buffer pH 6.0. Hole cells were used as GAD enzyme source and assay was performed according to Matsushita and Ameyama (1982). Units of activity of GAD that catalyze the oxidation of 1 micro mol of D- gluconate or reduction of 1 μmol of DCIP per minute at 25 C. Specific activity is defined as units per milligram of protein.

2.5.2.4: Phytase assay

Phytase and phosphatase activities were determined at acid (pH 4.5) and alkaline (pH 8.0) pH. Transformants were grown till stationary phase in 25 ml LB media or Minimal medium containing 50mM glucose as carbon source. Culture supernatants obtained by centrifuging culture suspensions at 4°C for 10 min at 12,000 × g were used for determining extracellular enzyme activities. For assaying cell associated enzyme activities, the cell pellets so obtained were washed two times with sterile 0.85 % NaCl solution, resuspended in 3 ml chilled sterile 0.85 % NaCl and subjected to sonication for 3.33 min at 9.9 sec on/off pulsed at 28 % amplitude by using Branson Sonifire ultrasonicator. Sonicated suspensions were centrifuged as above for 15 min to remove cell debris and supernatant was used for assaying cell associated enzyme activity.

Phytase activity was measured using 0.7 ml assay buffer consisting of 0.1 M Na-acetate pH 4.5 (for acid phytase) or 0.1 M of Tris-Cl pH 8.0 (for alkaline phytase), both containing 0.7 mM of Na-phytate and the total system was made up to 1.0 ml with enzyme aliquot and/or distilled water and incubated at 37°C for 30 min. Release of P from Na-phytate was estimated by Ames method (Ames, 1964).

Unit activity of phytase is defined as the amount of enzyme liberating 1μmol P/min.

2.5.2.5: Phosphatase assays

Phosphatase activity of the transformants was determined by using *p*-nitro phenyl phosphate (pNPP) as a substrate. For acid phosphatase, 0.5M Na-acetate buffer pH 4.5 and for alkaline phosphatase, 0.5M Tris-Cl (pH 8.0) were used. Different aliquots of the sample were incubated with 0.1 ml of 0.5 M pNPP along with 0.1 ml of 0.1M magnesium chloride ($MgCl_2$) and 0.1 ml of appropriate buffer.

Total system was made up to 2 ml by using distilled water and incubated in dark at 30° C for 30 min, after which the reaction was stopped by adding 2 ml of 2N NaOH. The reaction mixture was vortexed and the amount of p-nitro-phenolate (pNP) released was estimated by measuring absorbance at 405 nm. One unit of phosphatase is defined as the amount that releases 1 μmol of pNP/min.

2.6: Inoculation of mung beans (*Vigna radiata*)

Mung bean seeds were surface sterilized by treating with 0.1% (w/v) mercuric chloride for ~3-5 minutes followed by 5 rounds of thorough washing with 100ml freshly sterilized distilled water (Ramakrishna et al., 1991). These seeds were placed in autoclaved petri-dishes containing moist filter paper and were allowed to germinate in dark for 24h at room temperature. *E. asburiae* PSI3 was inoculated in 25 ml sterile LB broth and was allowed to grow at 37°C with overnight shaking. Fresh culture was harvested by centrifuging at 9, 200x *g* for 2 minutes; the pellet was aseptically washed twice with sterile normal saline and finally was re-suspended in 4 ml of normal saline (Ramos et al., 2000). Healthy germinated seeds were incubated with this cell suspension in a sterile petri-dish under aseptic conditions for 10-15 minutes. Root tips of hence obtained *E. asburiae* PSI3 coated mung bean seeds were implanted with the help of sterile forceps in autoclaved alkaline vertisol soil. Plants were allowed to grow at room temperature with sufficient light conditions for 15 days after which effect of *E. asburiae* PSI3 inoculation was monitored in terms of overall plant growth.

2.7: Root colonization study

Colonization of *E. asburiae* PSI3 on mung bean root was monitored by tagging with fluorescent plasmids. Plants were grown in hydroponics or in non sterile soil inoculated with tagged bacteria. In case of hydroponics system root colonization

was monitored at 5^{th} day of plant growth. In case of soil it was monitored on 5^{th} and 10^{th} day of plant growth. 10^6 to 10^8cfu/ml bacterial cells were used to soak the sterile condition germinated seed for 30 minute. Before doing confocal microscopy roots were washed twice with sterile saline and fixed on glass slide.