4 Spatiotemporal patterns of root colonization by *Ensifer* spp. individually and competitively on host plant

4.1 Introduction

Rhizobia can exist in a dual lifestyle; leading a saprophytic one in the bulk of soil, or in a mutualistic lifestyle within root nodules of specific hosts wherein they fix nitrogen. The transition from the free-living lifestyle to symbiotic one involves a complex but well-studied chemical dialogue between the two partners (Hirsch *et al.*, 2001). In field conditions, native rhizobia tend to evolve aggressively competitive traits for nodule development due to the fitness benefits provided by mutualistic association. The beginning of nodulation happens at the specific sites on the host root for a limited period of time, thus the rhizobial presence at the receptive location is essential (Poole *et al.*, 2018). A greater understanding of the rhizobial colonization in the presence (or absence) of a competing strain in the soil, in this regard can aid in better utilization of rhizobia for agricultural use. Ability of rhizobia to attach to and spread over the host root surface have been often described as determinants of nodulation competitiveness, thus assessing how these properties affect the colonization and if that is responsible for the nodulation competitiveness may be important (Archana, 2010). Most of the studies that revealing the colonization dynamics of rhizobia on the host roots are limited in the resolution and details due to the technological limitations; in this regard, recently, Massalha *et al.*, (2017) advocated using high resolution microscopy and microfluidics for plant-microbe interactions. Additionally, the colonization attributes of rhizobia also remain to be correlated with their ability ro attach and spread over host root surfaces. This part of the study, deals with mapping the colonization of rhizobial isolates –individually proficient in nodulation, over the roots of pigeon pea across

the length and breadth of primary, secondary and thin tertiary roots periodically up to five weeks. Their nodulation patterns are examined similarly. Further, their competitive colonization and nodulation with respect to each other when supplied differently has also been determined and correlated with their physiological attributes relevant to colonization. Electron microscopy and confocal laser scanning microscopy have been used to corroborate these findings. Finally, microfluidics coupled with high resolution confocal laser scanning microscopy was employed to visualize the rhizobial colonization of host root in real time.

4.2 Materials and Methods

4.2.1 Bacterial strains and culture conditions

Bacterial strains and plasmids are listed in Table 4.1. *Ensifer* HP113 and HP127 were transformed with pBBR1MCS2-eGFP or pBBR1MCS5 for imaging and/or discrimination for plate count (Figure S3 in supplementary section shows the plasmid maps). pBBR1MCS2 and pBBR1MCS5 confer resistance to Kanamycin and Gentamycin respectively and the strains containing these plasmids were cultured in their presence at 30 μg/ml and 40 μg/ml respectively. *Ensifer meliloti* 8530 was cultivated on Streptomycin at 100 μg/ml. All the strains were grown in TY broth [Tryptone (6 g/l), Yeast Extract (3 g/l) and calcium chloride (3 mM)] unless mentioned otherwise.

4.2.2 Construction of pBBR1MCS2-eGFP plasmid

Ensifer sp. HP113 as well as HP127 were electroporated with pBBR1MCS2-eGFP. The cloning of eGFP gene was performed using common cloning procedures as mentioned in Section 3.2.2. The gene was amplified from another vector gifted kindly by Prof. G. Nareshkumar, Department of Biochemistry, The M. S. University of Baroda, Vadodara, India. The primers used for the cloning are given in Table 4.1.

Ensifer sp. HP127 was also electroporated with pBBR1MCS5 (Gen^r) in order for it to be selectively plated when used with HP113-eGFP (Kana^r).

Strain name	Relevant characteristic	Source
Bacterial strains		
Ensifer sp. HP113	Nodualtes pigeon pea	This study
Ensifer sp. HP127	Nodulates pigeon pea	This study
Ensifer meliloti 8530	Nodulates *M. sativa*	Gift from Prof. Juan Gonzalez, UT-Dallas Described in Pellock *et al.* (2002)
Plasmids		
pBBR1MCS2-eGFP	eGFP cloned between *xbaI* and *sacI* of pBBR1MCS2	This study
pBBR1MCS5	Gen^r	Kind gift from Prof. Maravić-Vlahoviček Uni. of Zagreb, Croatia (Obranić *et al.*, 2013)
Primers (*5'→3' ; designed in this study*)		
eGFP Fwd	gc*TCTAGA***AGGAGGATAGTC**ATG CGTAAAGGAGAAGAACTTTTCACT	
eGFP Rev	tt*GAGCTC*TTATTTGTATAGTTCATCCATGCCATGTGTAAT	

Table 4.1 List of bacterial strains, plasmids and PCR primers used in this study. Italicized letters indicate the recognition site of restriction enzymes, boldface letters are the ribosome binding site, and underlined letters is the start codon. The "gc" at the 5' are added to facilitate the cleavage close to end by the enzyme.

4.2.3 Transformation of rhizobial strains by electroporation

The electroporation of rhizobia with required plasmids was performed as described by Garg *et al.*, (1999) with following details. Five hundred milliliters of TY broth was inoculated with desired rhizobial strain and allowed to grow up to the OD_{600} of 0.8. The cells were then given three successive washes with 10% glycerol as

described in the cited reference and the finally resuspended in 500 µl of 10% glycerol. These "electrocompetent" cells were used immediately or preserved at -80ºC for up to 6 months. The electroporation was performed with Gene Pulser Xcell electroporation system (BIO-RAD) using 2 mm gap cuvettes. Forty microliters aliquote of electrocompetent cells mixed with 1 µg of the prepared plasmid were incubated in ice for 15 min. This was added to the chilled cuvettes which were then maintained in ice until pulsed. A pulse at 2500 V, 25 µF and 200 Ω was given to the cells followed by immediate addition of TY broth at root temperature for outgrowth. The cells resuspended in 1 ml of TY broth were incubated in slow shaking at 30ºC for 3 h after which they were plated on TY agar plate containing respective antibiotic.

4.2.4 Surface sterilization and germination of seeds

Seeds of garden pigeon pea were surface sterilized using mercuric chloride as described in (Vincent, 1970) with following details. The seeds were dipped and swirled in sterile distilled water for 1 min followed by treatment with 70% v/v isopropanol for 30s which was followed by a 30s swirl in sterile distilled water. This ensured removal of surface contaminants and reduction of surface tension for better effectiveness of sterilizing agent. Subsequently, the seeds were treated with 0.1% mercuric chloride or 25% v/v sodium hypochlorite solution for 2 minutes with vigorous mixing followed by five washes with sterile distilled water. The seeds were then kept for germination in petri plates containing water-soaked filter paper for 48-72 h in dark at at 28º C while the plumule reached a length of 2-2.5 cm. The filter paper was re-moistened as required with sterile distilled water.

Alfalfa seeds were sterilized employing chlorine gas sterilization according to (Massalha *et al.*, 2017). The protocol is as follows. The seeds were filled inside a 2ml microcentrifuge tubes such that when the tube is kept horizontal, the seeds do not stack. The tube openings were sealed with parafilm bearing several holes to allow gas exchange and the caps were kept open. Several such tubes were placed inside a glass desiccator. Subsequently a flask containing 100 ml sodium hypochlorite was added with 6 ml hydrochloric acid (leading to immediate

emergence of chlorine gas) and placed in the desiccator which was then closed and sealed with parafilm. The treatment was stopped after 2h when the tubes containing seeds were capped. The seeds were then used for germination as described above or stored at RT in dryness.

4.2.5 Plant inoculation for colonization experiments

The pigeon pea seeds were inoculated by rhizobia either by coating on the germinated seeds or by mixing in the soil at different concentrations, or both- for dual inoculation experiments. The seedlings with the emergent root 2.5 +/- 0.5 cm were selected for the experiment. Coating of bacteria for seed-inoculation was performed as follows. Overnight grown culture was used for inoculation into 100 ml TY broth and allowed to grow up to OD_{600} 0.8 (corresponding to 2-2.5 x 10^8 cfu/ml cfu/ml) and the cells were harvested at 3000 rpm for 15 minutes at 25º C and given a wash with 0.85% saline in and pelleted with 300 rpm centrifuge without vigorously shaking. The cells were resuspended in20 ml of N-saline and the germinated seeds were immersed in them aseptically followed by an incubation for 4 hours in dark. The seeds were then removed and excess cell-suspension was allowed to be dripped off. In a pot containing 3 kg autoclaved and sieved soil, 2 seeds each were sown at the depth of 3 cm. Pots were maintained in the green house at 12 hr. light/dark cycles. Plants were watered with equal amounts of autoclaved deionized water in all the pots (10 ml for the first two weeks, 20 ml thereafter). Watering was always done by gentling pouring in from a 100 ml glass beaker at the stem origin. Pots were maintained this was for up to five weeks. Inoculation of rhizobia in the soil was done as follows. Soil-inoculation of rhizobia was done at two different concentrations of rhizobia, i.e. 10^4 and 10^6 cfu of the rhizobial strain per gram of soil. This was performed as follows. Rhizobial strains were grown to the OD_{600} 0.8 corresponding to 2-2.5 x 10^8 cfu/ml of culture. Appropriate amounts of rhizobial cultures were resuspended in 300 ml of sterile distilled water to correspond to 10^4 and 10^6 cfu per g of soil. For each pot, 3kg of soil was mixed with this 300 ml of the respective suspension containing either 10^4 or 10^6 cfu/g ($3x10^7$ or $3x10^9$ per pot respectively). This was done as six lots of 50 g

soil mixed with 50 ml of culture suspension in order to have homogeneity of rhizobia. The pots were then sown and maintained as described above.

4.2.7 Dual inoculations for competitive colonization

For assessment of competitive tendency of each strain when the other is present as a part of the bulk soil, they were applied dually. When one strain was coated on the root, the other was added to the soil (at two different loads) and vice versa (Above two sections describe the individual procedure for rhizobial inoculation). At the end of the experiment, the quantification of rhizobial cfu (see next section) was performed for each of the strains. Respective single inoculation counts served as controls to assess the effect of presence of the competing strain on colonization of the other strain.

4.2.8 Quantification of rhizobial colonization

After 5 weeks of growth, the plants were carefully removed from the soil. Root fragments of 1 cm length were cut from respective places of the root. The illustration in Figure 4.1 indicates different parts of the root considered for the counts and the notations used through the study. The pieces of roots were cleaned with 0.85% saline. The soil was removed by a brief 5s pulse in the sonic bath. The root piece was then transferred in a fresh tube which was placed in the sonic bath for two 10s pulses at an interval of 30s. This suspension of detached bacteria from the root was then used for serial dilution and plating. The root piece was then crushed and serially diluted and plated to assess the remaining rhizobial count and found that less than 10% of the total count would remaining undetached from the root. (Absence of endophytic colonization was confirmed by surface sterilizing the root piece before crushing and plating.). The sonic bath treatment was also found to cause no death of cells.

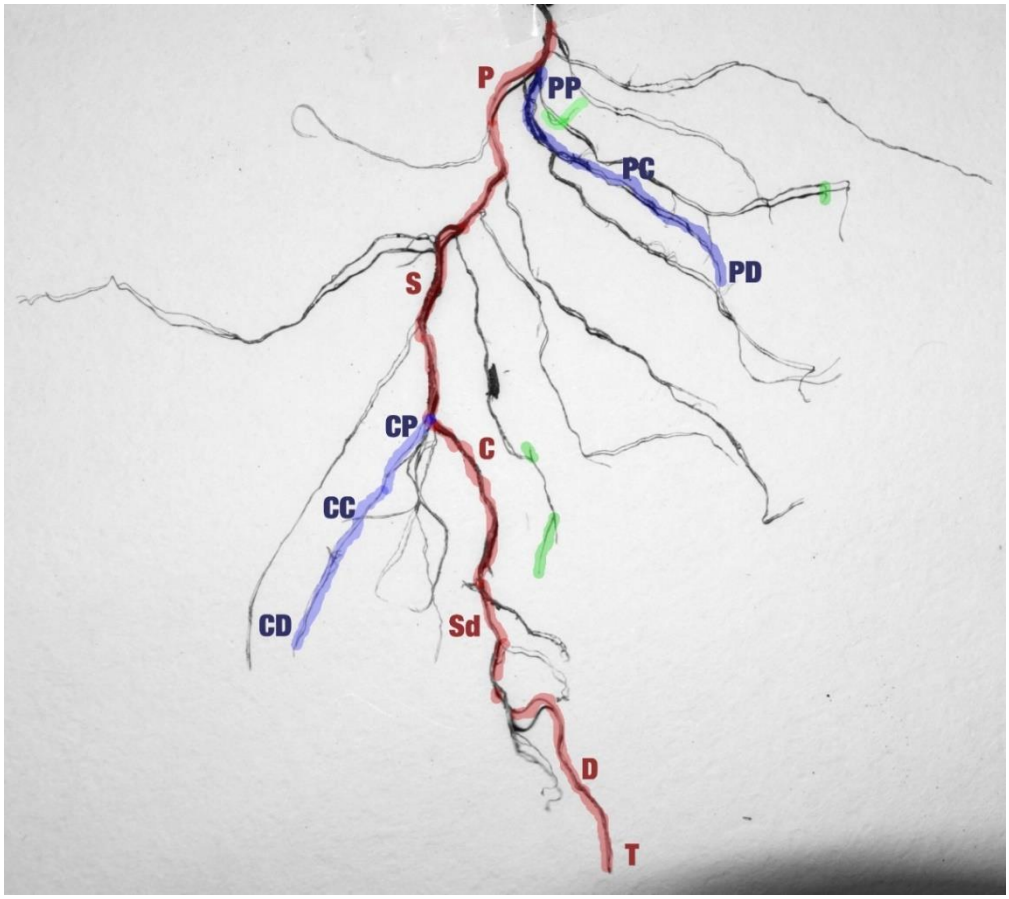

Figure 4.1: Notation styles used in the chapter to refer to different parts o the root. Images shows markup of different areas on the grayscaled pigeon pea root. For clarity, the primary root is traced with transparent red line while the secondary root is traced with blue line. The areas of primary root (with reference to the crown of the seed) i.e. Proximal, Subproximal, Central, Subdistal, Distal and Tip are marked as P, SP, C, sD, D and T respectively. The areas of secondary roots are marked as "YX", wherein "Y" indicates the location of origin of the secondary root while "X" denotes the particular zone of that secondary root; for instance, PP-proximal proximal stands for the proximal zone of the secondary root originating from the proximal part of the primary root, and CD means the distal zone of the secondary root that originated from the central region of the primary root. Similarly, DP would mean Distal Proximal (prox. Part of the secondary root originating from distal end of the primary root). Green tracing denotes the thin and newly emergent roots; they are referred to by the area of their origin.

4.2.9 Inoculation of rhizobia on pigeon pea seedlings and growth under hydroponic conditions

Pigeon pea seeds were surface sterilized and germinated as described in Section 2.2.3. Germinated seeds were coated with the respective rhizobial culture as described in Section 4.2.5**.** The coated seedlings were then transferred to the jars containing Hoagland's Nitrogen free minimal medium and incubated for 7d in 12h dark-light conditions at 30 ºC. The composition of medium is shown in Table 4.2. The values indicate their respective final concentration in mg/l.

Media component	**Final concentration (mg/l)**
Calcium chloride.2H2O	554.90
Potassium chloride	372.70
Monobasic potassium phosphate	136.03
Magnesium sulphate	240.33
Manganese chloride.4H2O	1.81
Boric acid	2.86
Ferric EDTA (-Na salt)	33.00
Zinc chloride	0.11
Copper chloride	0.045
Sodium molybdate	0.025

Table 4.2 Composition of Hoagland's minimal Nitrogen free medium.

4.2.10 Quantitation of exopolysaccharides production

Exopolysaccharide precipitation and quantification was performed as suggested in Janczarek and Skorupska, (2011). The *Ensifer* isolates were inoculated and grown in Tryptone yeast extract broth as well as Yeast extract mannitol broth (in g/L; mannitol 10.0, yeast extract 1.0, $MgSO_4.7H_2O$ 0.2, NaCl 0.1, K_2HPO_4 0.5) upto OD_{600} of each of the culture reached 1.5. The cultures were then harvested at 11000g for 15 minutes on centrifuge. The cell mass was dried overnight followed by measurement of dry biomass weight. The supernatant was added with double

the volume of isopropanol and precipitation of EPS was allowed for 45 min at room temperature. The precipitated EPS was then harvested by centrifugation at 12000rpm for 15 minutes. The tube was sealed with porous paper and the pellet was allowed to dry in hot air oven overnight. The dried EPS was suspended in 2ml of distilled water and the quantification was carried out by estimation of reducing sugars by dinitrosalicylic acid (DNSA) assay. 1ml of DNSA reagent was added to 1ml of EPS suspension and was heated at 100ºC for 15 minutes. The solution was cooled and 8ml of distilled water was added to make a 10ml system. The absorbance of the system was observed at OD540. The OD values were used in the linear regression equation obtained by standard curve of DNSA prepared using range of glucose concentrations. Final concentration of EPS was expressed as ratio of reducing sugars per µg of dry biomass.

4.2.11 Confocal laser scanning microscopy of *Ensifer* isolates on pigeon pea root

Attachment and biofilm formation by rhizobial isolates on pigeon pea roots was assessed by confocal laser scanning microscopy (CLSM). The analysis was done for immediate colonization and a long-term association assessment. For the immediate colonization study, the germinated seeds were coated with respective rhizobial culture (expressing e-GFP) as described in Section 2.3.2. At the end of the 4h coating time, the roots were removed from the rhizobial suspension and incubated in the moist chamber for three more hours at the end of which the seedlings were washed gently with PBS pH 7.2 and were ready for imaging. The long-term association was checked after 7d of rhizobial colonization on the pigeon pea roots grown under hydroponic conditions described in Section 4.2.9. Fragments from these roots were excised and washed with Phosphate buffer saline pH 7.2 (0.0666 M $Na_2HPO_4 \cdot 7H_2O$ + 0.0334 M $NaH_2PO_4 \cdot H_2O$) to remove loosely associated cells. If needed, a thick section was taken from the root and mounted considering the radial symmetry of the root. The samples were mounted in Dibutylphthalate Polystyrene Xylene (DPX) solution (Merck) and covered with thin cover slip before imaging. The Imaging was done on the LSM 700 microscope

(Carl Zeiss, GmbH) at Dr. Vikram Sarabhai Institute of Cell and Molecular Biology, MSU Baroda.

4.2.12 Scanning electron microscopy of *Ensifer* isoalates colonizing pigeon pea root

In order to visualize the architecture of the rhizobial colonies on the root surface, fragments of roots from a 7d old pigeon pea plant coated with *Ensifer* HP127 and *Ensifer* sp. HP113, and grown in hydroponics as described above were subjected to Scanning electron microscopy (SEM). A root fragment was excised from the root and immersed in and washed with 50 mM phosphate buffer saline pH 7.2 (PBS) prior to incubation in 2.5% v/v glutaraldehyde (diluted with PBS) for 30 min. The fragment was then washed once with PBS followed by a successive passage in 10%, 25%, 50%, 75% v/v ethanol in order to slowly dehydrate the tissue. Finally, it was immersed in the absolute ethanol and stored at -20 ºC until imaging. Just prior to the imaging, the tissue was briefly air- and infrared- dried followed by mounting on the metal stub using adhesive carbon tape and sputter-coated in vacuum with platinum using the coating device (JEOL-JFC-1600). The samples were examined at 10 kV under the scanning electron microscope (JEOL, JSM-7600F-FEG-SEM). Coating and microscopy was performed at Sophisticated Analytical Instrument Facility, IIT-Bombay, India.

4.2.13 Microfluidics for visualization of rhizobia colonization of host root in real time.

A real time colonization pattern of *Ensifer* sp. 8530 on the growing root of M. sativa (alfalfa) was investigated using Tracking Root Interaction System (TRIS), a device coupling microfluidics and confocal microscopy developed by Massalha *et al.* (2017). The imaging involves several coordinated separate processes which are detailed individually in following text.

Preparation of PDMS gel

The microfluidic chip was composed of polydimethylsiloxane (PDMS) gel. It is a gel that polymerizes to form a solid slab that can then be covalently bonded to glass surface. The gel solution was made by mixing the A and B components which are mixed well (10:1 proportion) and centrifuged at 2000 rpm for 10 min to remove gas bubbles. It was then poured into the mold which was etched by nanofacrication as described in Massalha *et al.* (2017) and was placed in the vacuum desiccator for 1h to remove all the bubbles prior to baking at 60ºC for 3h or overnight followed by a root temperature incubation for 10-12 h. The solidified gel slab was then lifted off the mold with a pair of tweezers and was sliced into individual chip using sharp scalpel. The holes for the entry of root and inoculation of bacteria are then made with biopsy puncher (1 mm diameter).

Bonding of chip slab to the glass surface

The chip slabs made as described above were housed in the 6-well plates which are then mounted on the microscope stage. The bottom of these 6-well plates are made up of very thin glass (<0.2 mm) allowing better usage with confocal microscopy. The bonding of gel slab and glass of the well involves treatment with plasma gun and is carried out in extremely clean dust-free environment as follows. The chip slab was stuck on the inside of the Scotch® tape, this prevents the contact with and removal of dust from the surface of the chip while not leaving any glue on it. The glass bottom and the etched side of the chip were then treated with plasma gun for 80 s and 30 s respectively and the treated surfaces are immediately met and gently pressed against each other (the glass bottom being very fragile, this step requires caution; the pressing should be homogenous and should leave no air gaps while gentle enough to not break the glass). This was then placed then at 90ºC for 1 h and then at RT for overnight before it can be used. This process leads to formation of covalent bonds between the gel slab and the glass surface.

Seed germination and placement in the gel slab

The seeds of alfalfa were surface sterilized with chlorine gas as described in Section 4.2.4. Meanwhile, the 200 µl microtips were filled with about 20 µl of half strength Murashige and Skoog (MS) basal salt mixture solidified with 1% agar and were subsequently cut short to the bottom cm. These cut-tips-of-tips were pierce-inserted in solidified half strength MS basal salt mixture agar contained in a large petri plate. Individual sterilized seeds were then placed in each of these tips and were allowed to germinate under 12h dark-light cycles while the root grew in the tip. Just before the root emerged out of the opening of the tip, the tip was placed in the dedicated hole made in the chip.

Rhizobial inoculation and imaging

A culture of *Ensifer meliloti* 8530 grown in Acid salt minimal medium (Poole *et al.*, 1994) of the OD_{600} 0.5 was taken and washed with half strength MS basal mixture. The cell suspension was then delivered into the chamber from its dedicated hole in the chip. This was then mounted on the Nikon A1 inverted confocal microscope (Nikon corporation). The real time imaging program was set up which would image the root growing in the chamber while the cells colonize newly forming root. The entire area of the chamber was scanned and imaged at 30-50 min intervals with set lasers (eGFP for bacteria, RFP for noise cancellation), while the software stiches each view to form the composite image of the entire chamber. At the end of 12 h (the total time of time lapse), the sequence of images were collected and analyzed with the software "NIS viewer v4.0" (Nikon corporation) or Fiji (Schindelin *et al.*, 2012), or were stacked in Adobe Photoshop (Adobe corporation).

4.3 RESULTS

4.3.1 Spatiotemporal colonization pattern of *Ensifer* spp. individually on pigeon pea root

Ensifer sp. HP113 and HP127 were best nodulating and host growth promoting strains of the nodule isolates of pigeon pea obtained in this study (see section 1.3.1). Their distribution pattern on the on the root of pigeon pea growing in soil was investigated when inoculated directly on to the roots of the germinated seedlings in comparison with when inoculated in the bulk soil but not on the seedling. Their abundance was quantified during the growth of the plantlet over different parts of the primary root, secondary roots and on the thin tertiary branches of the secondary roots as depicted in Figure 4.1. .

Table 4.3 gives the values of log (cfu/cm of root) for the isolates in space and time using the notation style for the different parts of the root as mentioned in Fig. 4.1.When the bacterial isolates were (separately, and in gnotobiotic conditions) applied coated on the germinated seedling, a distinct pattern of colonization was apparent in case of both the strains. On the primary root, both the isolates were found to colonize in large numbers near to the crown region (Primary- Proximal to central) of the root while their abundance decreased in a gradient going towards the tip (Primary-tip). In both the inoculations, the plant root growth was identical (20 ± 4 cm). *Ensifer* sp. HP127 was able reach in measurable numbers to a greater length of the root (Primary -subdistal and distal), while strain HP113 was found to be relatively restricted in its spread in those regions. On the secondary roots, both isolates colonized significantly the areas of the roots closer to the crown region i.e. relatively greater abundance in the areas of the roots closer to the primary root (For eg. Secondary –Prox, Prox) as compared to the farther areas on that root (Secondary Prox, Dist), and relatively greater abundance in the areas of secondary roots originating from relatively crown-proximal regions of the primary root (such as secondary- primary proximal) as compared to those originating from the farther areas of the primary root (such as Secondary- Central/Distal Proximal). *Ensifer* sp. HP113 was found to be less proficient in colonizing the farther areas (such as PD

or DP) as compared to HP127 as apparent from the order-of-magnitude level differences in many parts of the root, while some parts of the secondary root showed no significant difference in the relative abundance. While no thin nascent roots were observed in the Wk1 and 2, at Wk 3 and Wk5, some of the newly emergent thin roots were observed to bear the rhizobia. On such roots originating from the top $3/4^{th}$ of the primary root, HP127 was close to one order of magnitude more abundant than HP113. On the thin roots originating from the rest of the primary root, or from the secondary roots of different respective origin, HP113 was not recovered, while few cells of HP127 could be recovered from these areas as well. It is also observed that in the upper regions of the root (Primary proximal, Primary subproximal) the bacterial counts increase during the plant growth from week 1 to 5. On the contrary, this does not happen on the distal parts, wherein, in fact the bacterial counts reduced over the weeks for strain HP127 and were maintained at low counts for strain HP113. Such a distinct pattern could not be discerned for the secondary roots. Nonetheless, the secondary roots closer to the proximal part of the primary root retained high bacterial counts up to week 5 while measurable counts of bacteria appeared on the secondary roots emerging in the lower parts of the plant roots only at week 5. This indicates the proficiency of resident population in the zones of primary colonization to grow and multiply as well as ability to spread and colonize newly emerging distal roots over time.

In order to study the pattern of root colonization when the rhizobia are inoculated in the bulk soil, the cells were applied at 10^4 and 10^6 cfu/g of soil in separate sets. Table 4.4 shows the abundance of *Ensifer* sp. HP127 as well as HP113 at 10^4 cfu/g of soil in different areas of the roots at weeks 1, 2, 3, and 5. (The counts when inoculated at 10^6 cfu/g of soil are given as supplementary Table S4). Both rhizobial strains, at both the inoculum amounts, were found to colonize the primary root in high proportions along the length, with the highest counts recorded at different regions on different weeks. The absolute distal regions were found to be relatively less colonized as compared to the other regions. There was a significantly higher colonization of the strains in most areas of the primary root when they were inoculated at 10^6 cfu/g of soil as compared to when inoculated at 10^4 cfu/g of soil.

The strains were found to colonize relatively homogenously over the different areas of secondary roots of different origin. However, there was no significant difference or pattern between the counts at different soil inoculum levels. Similarly, the thin roots originating from various parts of the primary and secondary roots were seen to be bear abundant numbers of each strain. The results indicate that the isolates were able to colonize the root profusely and did not significantly differ in their root-colonization capabilities. In addition, the strains showed growth over time even at some of the distal regions (e.g. Secondary Cent. Dist. for HP117 and 1º Prox. in case of HP113). The colonization pattern of the strains when inoculated in the soil was thus quite different as compared to that when the strains were coated on the germinated seedlings.

4.3.2 Competitive colonization of *Ensifer* spp. on pigeon pea root

Competitive colonization experiments wherein one strain is coated on to the germinated seedling while the other is mixed with soil at two different loads (10^4 and 10^6 cfu/g of soil) were setup to understand the colonization ability of a seedling inoculated strain in face of a competent strain present in bulk soil The differential colonization of each strain in the presence of the other is hypothesized to mimic a situation similar to a field condition wherein the bioinoculant coated on the seed has to face competition with the native species present in bulk soil. Counts of seed-coated organism in the sterile soil and the counts of soil-inoculated organism in the absence of any seed-coated organism were used as controls respectively for the above sets. Each organism (*Ensifer* sp. HP113 and *Ensifer* sp. HP127) was tested as both, seed-coated as well as soil-inoculated strain with the other provided as vice versa. Each soil-inoculated set was performed at 10^4 as well as 10^6 cfu/g of soil of respective rhizobium in case of control or tests.

Table 4.5 details the counts of seedling-coated *Ensifer* sp. HP113 on the root of pigeon pea at different time and space in the presence (at 10^4 cfu/g soil) or absence of *Ensifer* sp. HP127 (referred as ‘test’ and ‘control’ in the Table 4.5 respectively). In parts of the root closer to the crown region (proximal, subdistal, PP etc.), the test set — i.e when root-coated HP113 is challenged by the soil-inoculated *Ensifer*

HP127 — relatively lower counts of *Ensifer* sp. HP113, were recovered as compared to the control — when the soil is sterile. This effect is seen in all the weeks, more significant in the later weeks. Interestingly, the relatively distal areas of the root which were observed to be meagerly colonized by *Ensifer* HP113 in the gnotobiotic soil, were colonized significantly greater in the presence of the soil-borne *Ensifer* sp. HP127. Although, the overall pattern of colonization of *Ensifer* HP113 in test was similar to control (decreasing count from the proximal to distal areas), the pattern of counts differed in the above mentioned manner. In some of the time points and regions *Ensifer* HP113 was isolated from farther-distanced secondary and thin roots when challenged with the soil-borne HP127 (Test) as compared to the control. Similar colonization patterns with enhanced magnitude in the difference in the counts were obtained when HP127 was present in the soil at 10^6 cfu/g. (Table S5; supplementary section)

In the same experiments, the counts of the soil-borne strain HP127 on the root parts yielded interesting observations (Table 4.6). The relatively proximal areas were colonized rather sparsely by HP127 in the presence of root-coated *Ensifer* HP113 but there was no significant difference in the colonization in distal areas between the Test (when the root was coated with HP113) and Control (no coated organism on root). Similar effects were seen when *Ensifer* HP127 was added to soil at 10^6 cfu/g of soil (Table S6 in Supplementary section).

In the other set of experiments the strains were swapped for their location, i.e. *Ensifer* sp. HP127 was coated on the germinated root while the *Ensifer* sp. HP113 inoculated in the soil (at 10^4 and 10^6 cfu/g soil). Table 4.7 shows counts of HP127 on root when the soil has10^4 cfu/g of HP113. As seen, the counts of *Ensifer* sp. HP127 on the root when challenged by the presence of HP113 in the soil were found to be reduced through the length of the root unlike how HP113 was found to be severely affected in the proximal regions but unaffected or enriched in the distal regions in the presence of soil-borne challenge (Table 4.5). Relatively greater diminution in the counts of HP127 on the roots was seen when HP113 was inoculated in the soil at 10^6 cfu/g of soil (Data in Table S7 of supplementary

section). While the counts varied, the pattern of the effect of HP113 on the colonization by HP127 was similar at all the weeks.

Counts of HP113 (when inoculated in the soil at 10^4 cfu/g of soil) over the root areas at different time points were taken as well (Table4.8). HP113 was found to be severely restricted in its ability to colonize the proximal areas of the root which housed greater density of HP127 (coated on the root). However, in the distal areas of the root the counts of HP113 were either unaffected or higher as compared to the control (where no organism is coated on the root). This pattern of colonization of HP113 is similar to the pattern of its colonization when it was coated on the root and challenged by the presence of HP127 in the soil (Table 4.5). Counts of set with 10^6 cfu of HP113/g of soil revealed similar pattern of colonization with different magnitude; those counts are detailed in TableS8 of supplementary section.

The dual inoculum experiments reveal that each of the two inoculated strain share some of the aspects of colonization whereas differ in some other.

4.3.3 Attachment attributes of rhizobial isolates

Owing to the differences observed in the colonization and noduation patterns,it was of interest to study the tendencies of rhizobia to attach on to solid surfaces were quantified. The results of biofilm formation assay measuring the biofilm formation capability of bacteria on ***polyvinyl carbonate*** surface are preented in Figure 4.2a in terms of absorbance by crystal violet staining the biofilm. While there was insignificant differences in the growth of the two rhizobia, Ensifer HP127 showed more than double the level of biofilm formation as compared to HP113. Rhizobia secrete large amounts of exopolysaccharides (EPS), many of which are components of the matrix of the biofilms. On quantitative asssessment of the differences in the amount of EPS produced by each of the strains, it was observed that *Ensifer* HP127 produced more than double the amount of EPS than *Ensifer* sp. HP113 (Figure 4.2b). These results indicate that the strains vary significantly in their attachment tendencies *in vitro*.

		Ensifer sp. HP127					*Ensifer* sp. HP113		
		Wk1	Wk2	Wk3	Wk5	Wk1	Wk2	Wk3	Wk5
Primary	Proximal	**5.06** (0.10)	**6.06** (0.10)	**7.39** (0.09)	**7.47** (0.07)	**5.56** (0.07)	**5.81** (0.12)	**6.42** (0.10)	**6.68** (0.15)
	Subprox.	**5.16** [a] (0.27)	**6.00** [b] (0.00)	**5.87** (0.09)	**5.10** (0.17)	**3.36** [A] (0.10)	**4.42** [B] (0.10)	**5.36** (0.10)	**5.35** (0.37)
	Central	**4.92** [a] (0.03)	**4.74** [b] (0.29)	**4.72** (0.06)	**4.34** (0.18)	**1.42** [A] (0.10)	**3.95** [B] (0.05)	**4.89** (0.11)	**4.13** (0.24)
	Subdistal	**4.88** [a] (0.06)	**2.47** [b] (0.07)	**3.00** [c] (0.18)	**2.32** [d] (0.15)	**1.10** [A] (0.17)	**1.10** [B] (0.17)	**1.87** [C] (0.11)	**1.81** [D*] (0.18)
	Distal	**4.76** [a] (0.15)	**2.32** [b] (0.15)	**2.02** [c] (0.14)	**2.10** (0.17)	**1.00** [A]	**1.00** [B] (0.00)	**1.10** [C] (0.17)	
	Tip	**3.06** (0.10)							
Secondary	Prox, Prox.	4.47 (0.07)	4.29 (0.11)	5.26 (0.07)	4.54 (0.06)	4.36 (0.10)	4.93 (0.08)	5.00 (0.68)	4.82 (0.11)
	Prox, Cent.	3.99 (0.04)	4.30 (0.00)	4.20 (0.17)	3.55 (0.15)	3.52 (0.07)	3.36 (0.10)	4.36 (0.10)	3.36 (0.10)
	Prox., Dist.	3.00 (0.00)	2.60 (0.21)	2.10 (0.17)		3.36 (0.10)	1.10 (0.17)	1.83 (0.16)	1.20 (0.17)
	Cent, Prox.	2.19 (0.19)	2.09 (0.18)	2.80 (0.13)	3.16 (0.10)	1.20 (0.17)	2.20 (0.17)	2.52 (0.46)	2.23 (0.40)
	Cent., Cent	1.60 (0.17)	1.63 (0.10)	2.02 (0.14)	2.65 (0.05)	1.10 (0.17)	1.36 (0.01)	2.36 (0.10)	2.36 (0.36)
	Cent., Dist.	1.43 (0.23)	1.20 (0.17)	1.19 (0.19)	1.36 (0.10)		1.10 (0.18)	1.65 (0.50)	
	Dist., Prox.				2.26 (0.07)			1.20 (0.17)	1.98 (0.32)
	Dist., Cent.				1.42 (0.10)			1.36 (0.10)	
	Dist., Dist								
Thin new roots from:	1º Prox.							1.77 (0.50)	
	1º Subprox.				2.36 (0.10)				1.44 (0.39)
	1º Central			2.54 (0.49)	2.26 (0.24)				
	1º Subdist.			1.56 (0.50)				1.48 (0.30)	1.52 (0.47)
	1º Distal			1.88 (0.13)	2.10 (0.17)				
	2º PP								
	2º PC								
	2º PD								
	2º CP				0.67 (0.58)				
	2º CC								
	2º CD				0.83 (0.76)				

Table 4.3: Spatiotemporal distribution pattern of *Ensifer* isolates coated on the germinated seeds of pigeon pea. The labels follow the same notation style as mentioned in Figure 4.1. The values refer to mean $\log_{10}$ (cfu/cm of root) of respective strains from different regions of the roots at week 1, 2, 3 and 5 (Wk1-5) (n=3). The colour gradation from Green to red (via yellow) indicates decreasing value bacterial count. Values in parenthesis indicate SD. Only the values in **boldface** are shown for statistical significance comparison. Values with same alphabet across the row are compared with each other. Only values with different case (lower and upper) of the same alphabet across the row are statistically significant. ($p \leq 0.001$ for all comparisons except * where $p \leq 0.05$). Cells highlighted in grey indicate that the roots hadn't emerged; cells highlighted in blue denote that no bacteria were recovered.

		Ensifer sp. HP127					*Ensifer* sp. HP113			
		Wk1	Wk2	Wk3	Wk5		Wk1	Wk2	Wk3	Wk5
Primary	Proximal	**3.26**^{a} (0.24)	**3.46**^{b} (0.15)	**4.26**^{c} (0.24)	**5.5**^{d} (0.21)		**3.57**^{a} (0.09)	**4.58**^{B**}(0.05)	**5.54**^{C*}(0.17)	**6.07**^{D*} (0.09)
	Subprox.	**4.26** ^{a} (0.24)	**3.42** ^{b} (0.10)	**6.07** ^{c} (0.21)	**5.52**^{d} (0.07)		**4.01**^{a} (0.15)	**5.44** ^{B**}(0.20)	**5.88**^{c} (0.08)	**5.69**^{d} (0.21)
	Central	**4.42**^{a} (0.10)	**5.36** ^{b} (0.10)	**4.20** ^{c} (0.17)	**4.26**^{d} (0.24)		**3.62**^{A**}(0.09)	**5.02**^{b} (0.35)	**3.74**^{C*}(0.15)	**4.54**^{d} (0.06)
	Subdistal	**4.46** ^{a} (0.15)	**3.42** ^{b} (0.10)	**5.10** ^{c} (0.17)	**5.42**^{d} (0.10)		**3.05**^{A**}(0.10)	**3.66**^{b} (0.05)	**4.46**^{C*}(0.18)	**3.98** ^{D**}(0.07)
	Distal	**3.36** ^{a} (0.10)	**3.26** ^{b} (0.24)	**3.20** ^{c} (0.17)	**2.69**^{d} (0.09)		**3.01**^{a} (0.05)	**3.22**^{b} (0.18)	**2.02**^{C*}(0.10)	**3.62** ^{D**}(0.12)
Secondary	Prox, Prox.	**3.20**^{a} (0.17)	**1.93**^{b} (0.13)	**4.36**^{c} (0.10)	**5.55**^{d} (0.13)		**3.05**^{a} (0.15)	**2.84**^{B**} (0.15)	**4.83**^{c} (0.04)	**4.87**^{D**} (0.17)
	Prox, Cent.	3.36 (0.10)	5.46 (0.15)	4.36 (0.32)	1.36 (0.10)		3.24 (0.06)	3.05 (0.19)	4.36 (0.07)	4.05 (0.17)
	Prox., Dist.	2.79 (0.44)	3.52 (0.07)	3.42 (0.10)	3.36 (0.10)		1.08 (0.04)		1.07 (0.07)	1.20 (0.04)
	Cent, Prox.	4.56 (0.07)	1.62 (0.15)	4.20 (0.17)	4.20 (0.17)		3.72 (0.15)	4.22 (0.20)	3.79 (0.24)	3.98 (0.11)
	Cent., Cent	3.36 (0.10)	2.09 (0.19)	3.56 (0.07)	4.36 (0.10)		3.05 (0.22)	3.55 (0.04)	3.40 (0.09)	3.05 (0.08)
	Cent., Dist.	1.92 (0.07)	3.20 (0.17)	3.36 (0.10)	3.46 (0.15)		1.08 (0.09)			1.02 (0.05)
	Dist., Prox.			3.93 (0.42)	4.20 (0.17)		2.55 (0.07)	2.08 (0.10)	1.40 (0.10)	3.54 (0.18)
	Dist., Cent.			3.26 (0.24)	2.36 (0.10)		2.05 (0.25)	1.05 (0.04)		2.74 (0.01)
	Dist., Dist			3.20 (0.17)	2.20 (0.17)					
Thin new roots from:	1º Prox.		6.10 (0.17)	3.85 (0.65)				1.50 (0.20)	3.21 (0.08)	4.88 (0.16)
	1º Subprox.		2.66 (0.10)	2.77 (0.21)	4.10 (0.17)				4.03 (0.08)	2.76 (0.10)
	1º Central		1.95 (0.09)	3.10 (0.17)	4.03 (0.48)					3.55 (0.06)
	1º Subdist.				3.46 (0.15)					3.81 (0.04)
	1º Distal				4.36 (0.10)			1.00 (0.07)		
	2º PP				**3.20** (0.17)				1.05 (0.05)	**3.95** (0.23)
	2º PC				4.10 (0.17)					2.05 (0.14)
	2º PD				2.98 (0.03)					1.16 (0.06)
	2º CP				**3.20** (0.17)					**3.58** (0.09)
	2º CC				**3.20** (0.17)					**3.00** (0.13)
	2º CD				**2.52** (0.07)					**2.07** (0.07)

Table 4.4: Spatiotemporal distribution pattern of *Ensifer* sp. HP127 and *Ensifer* sp. HP113 when inoculated in soil at 10^4 cfu/g of soil. The labels follow the same notation style as mentioned in Figure 4.1. The values refer to mean $\log_{10}$ (cfu/cm of root) of respective strains from different regions of the roots at week 1, 2, 3 and 5 (Wk1-5) (n=3). Values in parenthesis indicate SD. Only the values in **boldface** are shown for statistical significance comparison. Values with same alphabet across the row are compared with each other. Values with same case across the row are statistically non-significant whereas values with different case (lower and upper) across the row are statistically significant. (**p ≤ 0.01; *p≤0.05; n=3).

Cells highlighted in grey indicate the roots hadn't emerged, whereas cells highlighted in blue denote that no bacteria were recovered.

		Week1		Week2		Week3		Week5	
		Control	*Test*	*Control*	*Test*	*Control*	*Test*	*Control*	*Test*
Primary	P	5.56 (0.07)	4.20 (0.17)***	5.81 (0.12)	4.46 (0.15 ***	6.42 (0.10)	4.33 (0.37) ***	6.68 (0.15)	4.52 (0.07) ***
	SP	3.36 (0.10)	3.19 (0.15)^{ns}	4.42 (0.10)	3.80 (0.04)*	5.36 (0.10)	4.91 (0.12) ^{ns}	5.35 (0.37)	5.20 (0.17) ^{ns}
	C	1.42 (0.10)	2.42 (0.10)***	3.95 (0.05)	3.52 (0.07) ^{ns}	4.89 (0.11)	3.46 (0.15) **	4.13 (0.24)	3.42 (0.10) *
	SD	1.10 (0.17)	1.20 (0.17)^{ns}	1.10 (0.17)	3.20 (0.17) ***	1.87 (0.11)	3.42 (0.10) ***	1.81 (0.18)	3.42 (0.10) ***
	D	1.00 (0.00)	1.36 (0.10)^{ns}	1.00 (0.00)	3.20 (0.17) ***	1.10 (0.17)	2.10 (0.17) ***		2.10 (0.17)
Secondary	PP	4.36 (0.10)	4.67 (0.06)^{ns}	4.93 (0.08)	3.80 (0.04) ^{ns}	5.00 (0.68)	3.11 (0.16)***	4.82 (0.11)	3.36 (0.10) ^{ns}
	PC	3.52 (0.07)	2.36 (0.10)***	3.36 (0.10)	3.52 (0.07) ^{ns}	4.36 (0.10)	2.80 (0.14)***	3.36 (0.10)	2.20 (0.17)***
	PD	3.36 (0.10)	1.42 (0.10)***	1.10 (0.17)	1.56 (0.07) ^{ns}	1.83 (0.16)	2.36 (0.10)***	1.20 (0.17)	2.42 (0.10)***
	CP	1.20 (0.17)	1.43 (0.51)^{ns}	2.20 (0.17)	2.20 (0.17) ^{ns}	2.52 (0.46)	3.20 (0.17) ^{ns}	2.23 (0.40)	3.30 (0.00)***
	CC	1.10 (0.17)	1.20 (0.17)^{ns}	1.36 (0.01)	1.36 (0.10) ^{ns}	2.36 (0.10)		2.36 (0.36)	3.10 (0.17)*
	CD		1.49 (0.43)	1.10 (0.18)	1.42 (0.10) ^{ns}	1.65 (0.50)			1.84 (0.10)
	DP					1.20 (0.17)		1.98 (0.32)	1.59 (0.11) ^{ns}
	DC					1.36 (0.10)			
	DD								
Thin roots from:	1º P				2.75 (0.04)	1.77 (0.50)	3.20 (0.17) ***		4.36 (0.10)
	1º sp				2.75 (0.04)			1.44 (0.39)	3.20 (0.17)***
	1º C				3.36 (0.10)				2.40 (0.17)
	1º Sd				3.20 (0.17)	1.48 (0.30)	2.42 (0.10) ***	1.52 (0.47)	2.36 (0.10)*
	1º D						2.26 (0.24)		2.10 (0.17)
	2º PP								2.20 (0.17)
	2º PC								1.69 (0.09)
	2º PD								1.36 (0.10)
	2º CP								1.10 (0.17)
	2º CC								
	2º CD								

Table 4.5: Root colonization dynamics of seedling-coated *Ensifer* sp. HP113 in the presence (Test) or absence (Control) of bulk inoculation of *Ensifer* HP127 at 10^4 cfu/g of soil. The labels follow the same notation style as mentioned in Figure 4.1. The values refer to mean $\log_{10}$ (cfu/cm of root) of respective strains from different regions of the roots at week 1, 2, 3 and 5 (Wk1-5) (n=3). Values in parenthesis indicate SD. Values of *Control* are compared with those of the *Test* for statistical significance. (*p≤0.05, ** p≤0.01, *** p≤0.001. ns: non-significant.; n=3) Cells highlighted in grey indicate the roots hadn't emerged, whereas cells highlighted in blue denote that no bacteria were recovered.

		Week1		Week2		Week3		Week5	
		Control	*Test*	*Control*	*Test*	*Control*	*Test*	*Control*	*Test*
Primary	P	3.26 (0.24)	3.12 (0.10)^{ns}	3.46 (0.15)	3.42 (0.25)^{ns}	4.26 (0.24)	3.26 (0.24)***	5.53 (0.21)	4.77 (0.12)***
	SP	4.26 (0.24)	4.16 (0.28)^{ns}	3.42 (0.10)	2.72 (0.13)**	6.07 (0.21)	4.09 (0.35)***	5.52 (0.07)	4.42 (0.04)***
	C	4.42 (0.10)	3.20 (0.17)***	5.36 (0.10)	3.50 (0.17)***	4.20 (0.17)	4.20 (0.17)^{ns}	4.26 (0.24)	4.52 (0.13)^{ns}
	SD	4.46 (0.15)	3.93 (0.13)^{ns}	3.42 (0.10)	3.20 (0.17)^{ns}	5.10 (0.17)	4.70 (0.17)^{ns}	5.42 (0.10)	4.20 (0.05)***
	D	3.36 (0.10)	3.59 (0.36)^{ns}	3.26 (0.24)	4.20 (0.22)***	3.20 (0.17)	2.26 (0.24)**	2.69 (0.09)	3.42 (0.05)***
Secondary	PP	3.20 (0.17)	3.42 (0.10)^{ns}	1.93 (0.13)	3.36 (0.10)***	4.36 (0.10)	3.36 (0.10)**	5.55 (0.13)	4.69 (0.07)***
	PC	3.36 (0.10)	4.36 (0.10)***	5.46 (0.15)	3.26 (0.24)***	4.36 (0.32)	2.69 (0.27)***	1.36 (0.10)	2.66 (0.06)***
	PD	2.79 (0.44)	4.36 (0.10)***	3.52 (0.07)	4.10 (0.17)**	3.42 (0.10)	2.89 (0.36)^{ns}	3.36 (0.10)	2.75 (0.06)***
	CP	4.56 (0.07)	4.26 (0.24)^{ns}	1.62 (0.15)	4.20 (0.17)***	4.20 (0.17)	3.32 (0.27)*	4.20 (0.17)	3.20 (0.10)***
	CC	3.36 (0.10)	2.20 (0.17)***	2.09 (0.19)	4.42 (0.10)***	3.56 (0.07)	3.30 (0.30)^{ns}	4.36 (0.10)	4.42 (0.06)^{ns}
	CD	1.92 (0.07)	3.36 (0.12)***	3.20 (0.17)	3.20 (0.17)^{ns}	3.36 (0.10)	3.26 (0.24)^{ns}	3.46 (0.15)	3.50 (0.09)^{ns}
	DP					3.93 (0.42)	3.32 (0.27)^{ns}	4.20 (0.17)	3.52 (0.10)***
	DC					3.26 (0.24)	2.19 (0.36)**	2.36 (0.10)	3.10 (0.06)***
	DD					3.20 (0.17)	2.26 (0.24)**	2.20 (0.17)	3.20 (0.10)***
Thin roots from:	1º P			6.10 (0.17)	3.46 (0.18)***	3.85 (0.65)	3.36 (0.10)^{ns}		
	1º sp			2.66 (0.10)	3.36 (0.10)***	2.77 (0.21)	3.42 (0.10)^{ns}	4.10 (0.17)	2.59 (0.10)***
	1º C			1.95 (0.09)	4.10 (0.17)***	3.10 (0.17)	3.36 (0.10)**	4.03 (0.48)	2.42 (0.27)***
	1º Sd				4.26 (0.24)	4.26 (0.24)	3.16 (0.27)*	3.46 (0.15)	3.56 (0.08)^{ns}
	1º D					6.07 (0.21)	1.87 (0.51)***	4.36 (0.10)	3.75 (0.05)*
	2º PP					4.20 (0.17)		3.20 (0.17)	3.20 (0.10)^{ns}
	2º PC							4.10 (0.17)	3.20 (0.10)***
	2º PD							2.98 (0.03)	2.36 (0.02)**
	2º CP							3.20 (0.17)	3.56 (0.10)^{ns}
	2º CC							3.20 (0.17)	3.42 (0.10)^{ns}
	2º CD							2.20 (0.07)	3.36 (0.04)***

Table 4.6: Root Colonization dynamics of *Ensifer sp. HP127* on the root when originally inoculated in soil at 10^4 cfu/g of soil - in the presence (test) or absence (control) of seedling-coated *Ensifer sp. HP113*. The labels follow the same notation style as mentioned in Figure 4.1. The values refer to mean $\log_{10}$ (cfu/cm of root) of respective strains from different regions of the roots at week 1, 2, 3 and 5 (Wk1-5) (n=3). Values in parenthesis indicate SD. Values of Control are compared with those of the Test for statistical significance. (*p≤0.05, ** p≤0.01, *** p≤0.001. ns: non-significant.; n=3) Cells highlighted in grey indicate the roots hadn't emerged, whereas cells highlighted in blue denote that no bacteria were recovered.

		Week1		Week2		Week3		Week5	
		Control	*Test*	*Control*	*Test*	*Control*	*Test*	*Control*	*Test*
Primary	P	5.06 (0.10)	5.30 (0.18)^{ns}	6.06 (0.10)	5.88 (0.21)^{ns}	7.39 (0.09)	6.04 (0.21)***	7.47 (0.07)	6.54 (0.31)***
	SP	5.16 (0.27)	4.84 (0.11)^{ns}	6.00 (0.00)	5.44 (0.14)***	5.87 (0.09)	5.00 (0.11)***	5.10 (0.17)	5.00 (0.12)^{ns}
	C	4.92 (0.03)	4.08 (0.08)***	4.74 (0.29)	4.88 (0.21)^{ns}	4.72 (0.06)	3.97 (0.25)***	4.34 (0.18)	3.59 (0.15)***
	SD	4.88 (0.06)	3.66 (0.20)***	2.47 (0.07)	2.41 (0.07)^{ns}	3.00 (0.18)	2.49 (0.04)**	2.32 (0.15)	2.00 (0.05)^{ns}
	D	4.76 (0.15)	3.54 (0.17)***	2.32 (0.15)	1.48 (0.04)***	2.02 (0.14)	2.14 (0.13)^{ns}	2.10 (0.17)	1.05 (0.10)***
Secondary	PP	4.47 (0.07)	3.85 (0.28)**	4.29 (0.11)	3.04 (0.08)***	5.26 (0.07)	5.40 (0.21)^{ns}	4.54 (0.06)	3.47 (0.16)***
	PC	3.99 (0.04)	4.21 (0.21)^{ns}	4.30 (0.00)	3.40 (0.16)***	4.20 (0.17)	3.55 (0.09)*	3.55 (0.15)	2.40 (0.11)***
	PD	3.00 (0.00)	1.55 (0.08)***	2.60 (0.21)	2.02 (0.11)*	2.10 (0.17)			1.05 (0.05)
	CP	2.19 (0.19)	1.05 (0.20)***	2.09 (0.18)	1.45 (0.20)***	2.80 (0.13)	2.00 (0.04)**	3.16 (0.10)	1.44 (0.04)***
	CC	1.60 (0.17)		1.63 (0.10)		2.02 (0.14)	1.05 (0.21)***	2.65 (0.05)	1.70 (0.23)***
	CD	1.43 (0.23)		1.20 (0.17)		1.19 (0.19)		1.36 (0.10)	
	DP							2.26 (0.07)	1.09 (0.14)***
	DC							1.42 (0.10)	
	DD								
Thin roots from:	1º P						1.22 (0.20)		2.10 (0.06)
	1º sp							2.36 (0.10)	1.10 (0.11)***
	1º C					2.54 (0.49)		2.26 (0.24)	
	1º SD					1.56 (0.50)			
	1º D					1.88 (0.13)		2.10 (0.17)	
	2º PP								
	2º PC								
	2º PD								
	2º CP							0.67 (0.58)	
	2º CC								
	2º CD							0.83 (0.76)	

Table 4.7: Root colonization dynamics of seedling-coated *Ensifer* sp. HP127 in the presence (Test) or absence (Control) of bulk inoculation of *Ensifer* HP113 at 10^4 cfu/g of soil. The labels follow the same notation style as mentioned in Figure 4.1. The values refer to mean $\log_{10}$ (cfu/cm of root) of respective strains from different regions of the roots at week 1, 2, 3 and 5 (Wk1-5) (n=3). Values in parenthesis indicate SD. Values of Control are compared with those of the Test for statistical significance. (*p≤0.05, ** p≤0.01, *** p≤0.001. ns: non-significant.; n=3) Cells highlighted in grey indicate the roots hadn't emerged, whereas cells highlighted in blue denote that no bacteria were recovered.

		Week1		Week2		Week3		Week5	
		Control	*Test*	*Control*	*Test*	*Control*	*Test*	*Control*	*Test*
Primary	P	3.57 (0.15)	2.41 (0.05) ***	4.58 (0.23)	3.10 (0.14) ***	5.54 (0.18)	4.74 (0.22) **	6.07 (0.24)	4.83 (0.11) ***
	SP	4.01 (0.23)	3.88 (0.15) ns	5.44 (0.16)	3.77 (0.20) ***	5.88 (0.15)	5.04 (0.17) *	5.69 (0.14)	4.59 (0.13) ***
	C	3.62 (0.08)	3.50 (0.09) ns	5.02 (0.05)	4.19 (0.08) ***	3.74 (0.04)	3.14 (0.15) *	4.54 (0.06)	4.63 (0.21) ns
	SD	3.05 (0.08)	3.14 (0.06) ns	3.66 (0.11)	3.54 (0.16) ns	4.46 (0.27)	4.49 (0.14) ns	3.98 (0.18)	5.10 (0.34) ***
	D	3.01 (0.14)	3.57 (0.14) **	3.22 (0.06)	2.17 (0.16) ***	2.02 (0.08)	3.49 (0.17) ***	3.62 (0.13)	3.91 (0.14) ns
Secondary	PP	3.05 (0.16)	1.41 (0.13) ***	2.84 (0.5)	1.54 (0.10) ***	4.83 (0.16)	2.84 (0.21) ***	4.87 (0.11)	3.55 (0.30) ***
	PC	3.24 (0.05)	1.25 (0.07) ***	3.05 (0.07)	1.55 (0.05) ***	4.36 (0.09)	3.22 (0.20) ***	4.05 (0.17)	2.87 (0.16) ***
	PD	1.08 (0.21)	2.21 (0.17) ***		1.18 (0.06)	1.07 (0.04)		1.20 (0.17)	
	CP	3.72 (0.15)	3.45 (0.21) ns	4.22 (0.00)	3.81 (0.21) ns	3.79 (0.23)	3.55 (0.16) ns	3.98 (0.23)	4.25 (0.07) ns
	CC	3.05 (0.07)	3.11 (0.06) ns	3.55 (0.19)	3.88 (0.08) ***	3.40 (0.15)	3.21 (0.07) ns	3.05 (0.08)	4.54 (0.17) ***
	CD	1.08 (0.03)	2.22 (0.04) ***		1.34 (0.06)			1.02 (0.14)	1.47 (0.14) ns
	DP	2.55 (0.07)	2.84 (0.07) ***	2.08 (0.14)	3.41 (0.04) ***	1.40 (0.16)		3.54 (0.16)	4.26 (0.17) ns
	DC	2.05 (0.27)	1.44 (0.1) ***	1.05 (0.09)	1.51 (0.05) ns			2.74 (0.17)	3.44 (0.21) **
	DD		1.84 (0.19)						
Thin roots from:	1º P			1.50 (0.04)		3.21 (0.19)	1.44 (0.08) ***	4.88 (0.31)	2.55 (0.18) ***
	1º SP					4.03 (0.07)	2.91 (0.11) ***	2.76 (0.17)	2.59 (0.1) ns
	1º C							3.55 (0.16)	3.02 (0.1) *
	1º SD							3.81 (0.08)	1.44 (0.04) ***
	1º D			1.00 (0.07)					1.05 (0.06)
	2º PP					1.05 (0.21)		3.95 (0.35)	2.10 (0.16) ***
	2º PC						1.05 (0.16)	2.05 (0.04)	1.23 (0.14) ***
	2º PD							1.16 (0.14)	
	2º CP							3.58 (0.08)	1.33 (0.09) ***
	2º CC							3.00 (0.07)	
	2º CD							2.07 (0.31)	

Table 4.8: Colonization dynamics of *Ensifer sp.* HP113 on the root when inoculated in soil at 10^4 cfu/g of soil in the presence (test) or absence (control) of *Ensifer sp.* HP127 as the root-coated strain. The labels follow the same notation style as mentioned in Figure 4.1. The values refer to mean $\log_{10}$ (cfu/cm of root) of respective strains from different regions of the roots at week 1, 2, 3 and 5 (Wk1-5) (n=3). Values in parenthesis indicate SD. Values of Control are compared with those of the Test for statistical significance. (*p≤0.05, ** p≤0.01, *** p≤0.001. ns: non-significant.; n=3) Cells highlighted in grey indicate the roots hadn't emerged, whereas cells highlighted in blue denote that no bacteria were recovered.

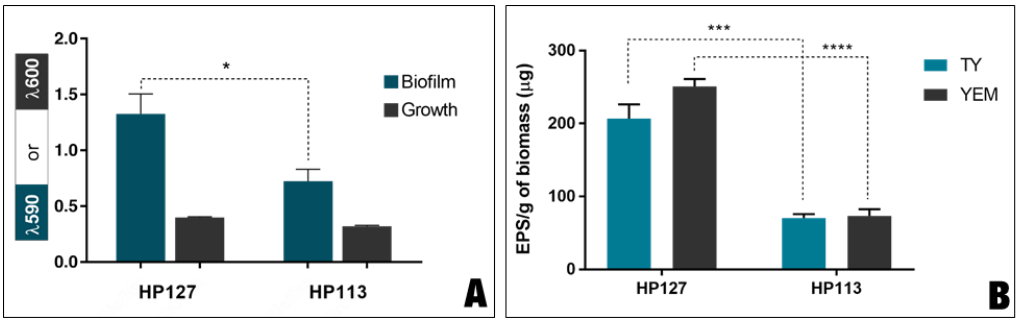

Figure 4.2: Biofilm formation and EPS production capabilities of *Ensifer* sp. HP127 and *Ensifer* sp. HP113. Y axis in (A) represents absorbance at different wavelengths λ_{590} denotes absorption by stained biofilms whereas the λ_{600} denotes the turbidometric assessment of growth prior to staining for biofilm. In (B), the Y-axis represents EPS produced in µg by the respective strains when grown in Tryptone yeast extract broth (TY) or when grown in Yeast extract mannitol broth (YEM). Error bars indicate standard error of means. n=3; *** $p \leq 0.001$, * $p \leq 0.05$.

4.3.4 Microscopic analysis of root colonization architecture of the rhizobial colonies on pigeon pea

The strains *Ensifer* spp. HP127 and HP113 were allowed to colonize the pigeon pea root under hydroponic conditions and imaged by confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) to assess their colony structure, architecture and extracellular matrix in which they are embedded while on the root surface. For CLSM the strains bearing pBBR1MCS2-eGFP were employed.

Colonization of *Ensifer* spp. HP127 and HP113 was assessed for early as well as late phase by CLSM. The images are shown in Figure 4.3. At the end of 4h of colonization (early phase), the cells of both isolates were observed to colonize the root area generally. Both the strains showed distinct foci of colonies beginning to form. *Ensifer* HP127 was found to contain many spots of aggregated colonies. In the late colonization phase (after 7d), *Ensifer* sp. HP113 was found to have mostly isolated and sparse cells and smaller cellular aggregates, whereas HP127 was largely present as denser and larger colonies often spread over large area of the root. Both the strains were found to colonize the root hair; the colonization over the root

hairs also followed the same pattern as other areas of the root where HP113 was found in smaller isolated colonies whereas HP127 covered large areas of root hairs with dense biofilms.

Upon examination by scanning electron microscopy, distinctive differences between the colonies of *Ensifer* spp. HP127 and HP113 were observed. HP127 was found to make very dense biofilms containing large aggregates of cells and copious amounts of extracellular matrix. These are evident in the panels A, C and E of the Figure 4.4. A matte of the HP127 biofilm covered in the matrix can be observed in the panel A; arrowheads mark the extended matrix in panel C. Zooming in reveals that the matrix is indeed bacterial origin and the cells are surrounded by thick coats of this material (panel E). *Ensifer* sp. HP113, conversely, did not make such biofilms on the root and was rarely found in small aggregate and instead colonized the root mostly in sparse cohorts. There was very little or no extracellular secretion observed.

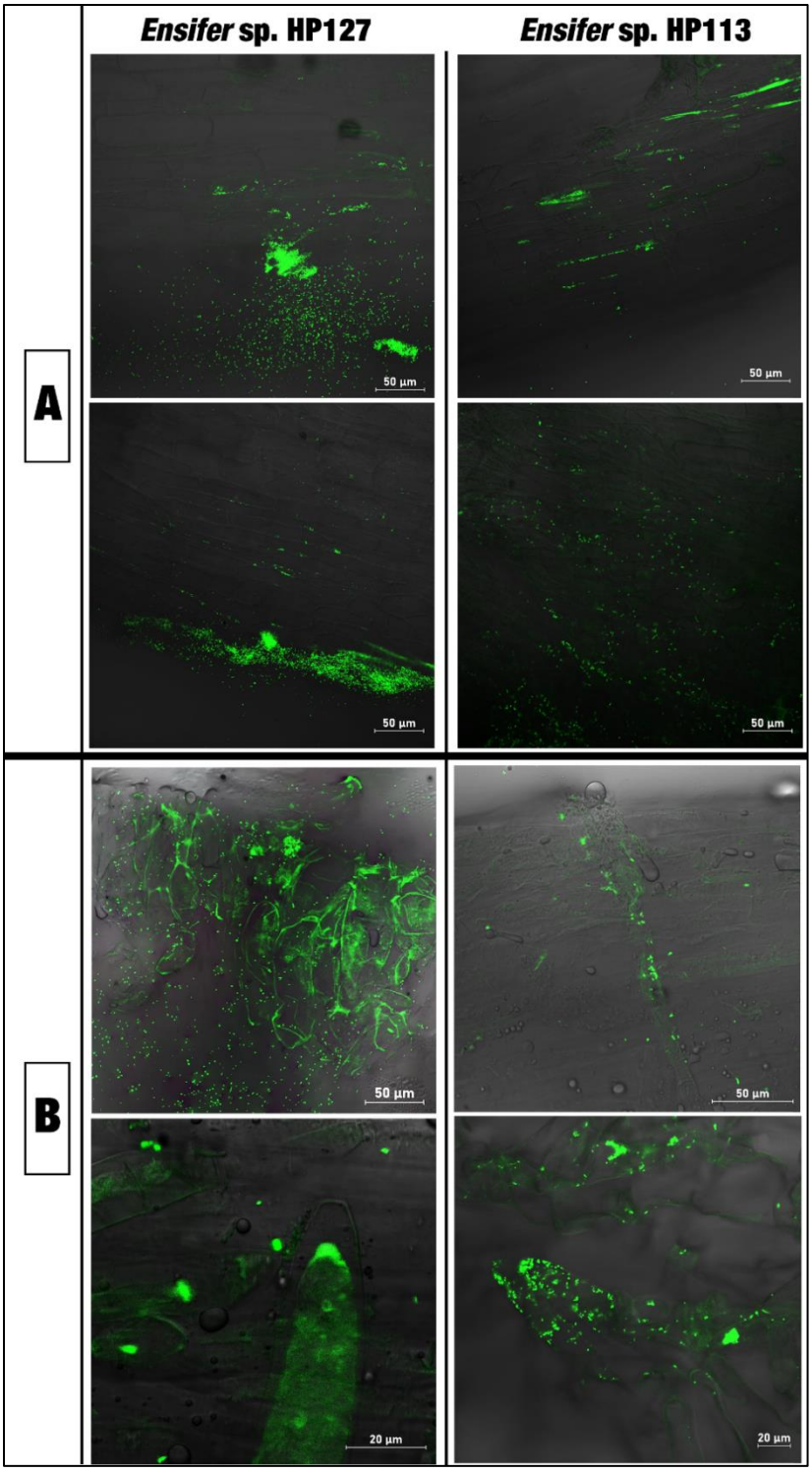

Figure 4.3 Confocal laser scanning microscopy of the *Ensifer* isolates on pigeon pea roots. (A) are from the 4h incubation experiment, and (B) are from the 7d incubation experiment (Section 4.2.11). Images are an overlap of Green channel for the eGFP-expressing strains and brightfiled micrographs for the root morphology. The Z-stacks have been merged for a 2D projection.

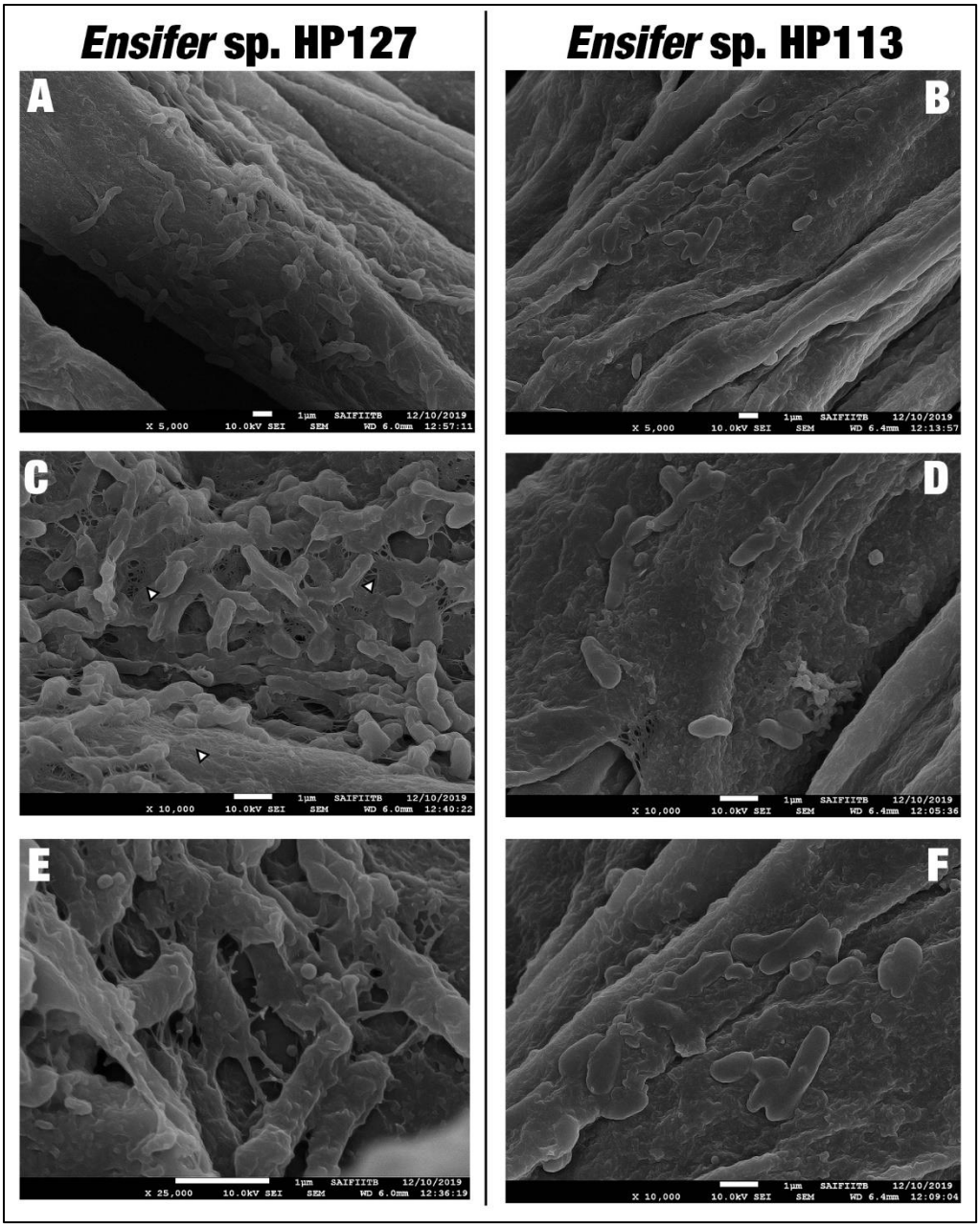

Figure 4.4 Scanning electron micrographs of the *Ensifer* isolates on pigeon pea root after 7d of colonization. The thick white bar at the bottom of each image represent 1 μm. Arrows in (C) indicate matrix of the biofilm.

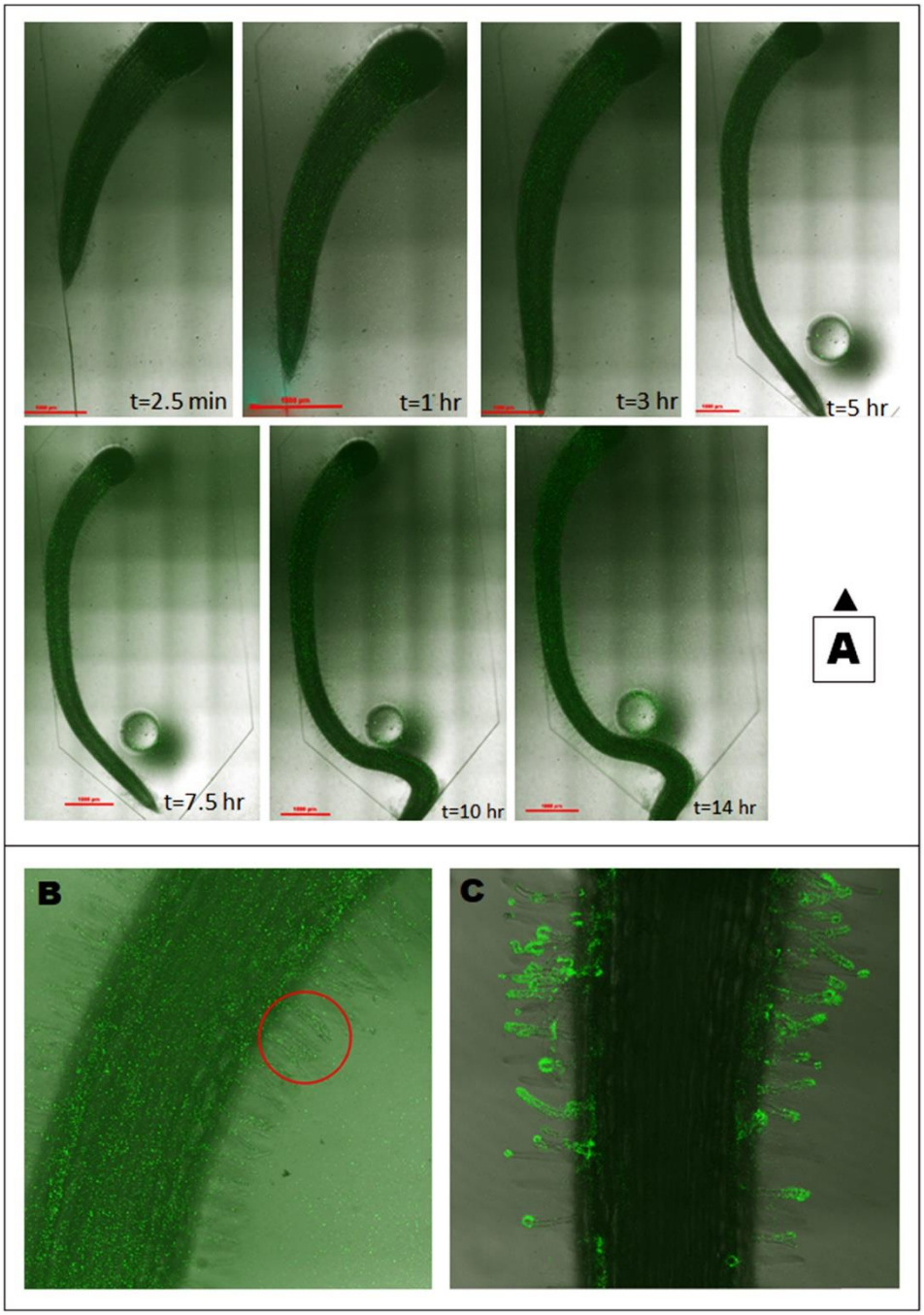

Figure 4.5 Time lapse imaging of alfalfa root growing in microfluidic chamber. Ensifer meliloti 8530 id tagged with eGFP and can be seen as green foci in the images. Panels in the part A of the figure show status of the root and bacterial colonization at the time mentioned at the bottom in each image. Panel B depicts a zoomed area showing root hair colonizatioon at 1h post inoculation. Panel C has been taken 20h post inoculation after purging the chamber with buffer to remove loosely bound cells showing biofilms across the areas of root and root caps.

4.3.5 Time-lapse imaging of root colonization by rhizobia

This work was done at Laboratory of Prof. Asaph Aharoni, Department of Plant and Environmental Sciences, Weizmann Institute of Science, Rehovot, Israel under the EMBO Short term fellowship. This part of the work involved using TRIS (Tracking Root Interaction System) (Massalha *et al.*, 2017) for real time visualization of bacterial colonization of root and required using a root with smaller diameter and thus alfalfa–*E. meliloti* host-rhizobium pair were used for this part of the work.

Ensifer meliloti strain 8530 was tracked for its colonization pattern at high spatiotemporal resolution on the roots of alfalfa using microfluidics and confocal microscopy in TRIS device. In the time lapse experiment, rhizobia were observed to begin colonization of the root immediately after the inoculation as seen in the image taken at 2 min 30 seconds (Figure 4.6, Panel A). Thereafter, although the rhizobia colonized all the zones of the root, they were relatively scarce in the zone of elongation whereas they proliferated and colonized the zones more proximal to the crown region in relatively greater abundance. A significant amount of colonization of rhizobia on the freshly emergent root hairs started before one hour from inoculation (Figure 4.5; Panel B). While the root hair colonization was observed through the length of the root, the root hairs of the proximal region were more profusely and sooner colonized by rhizobia. In order to get an insight into which interactions are better sustained with time, after 20 hours of incubation, the chamber was slowly flushed with normal saline in order to wash away the planktonic cells from the chamber. A significant amount of biofilm formation was seen on the root hairs whereas the epidermal cells generally did not harbor rhizobial cells. The quintessential root hair biofilm caps of the rhizobia were observed at this stage on most of the root hairs. The distribution of the biofilm formation on the root hairs also followed the same pattern as observed earlier with the general colonization of the rhizobial cells on the root. The results thus obtained reveal that even if the association of *Ensfier meliloti* 8530 with alfalfa begins nonspecifically wherein they rapidly colonize the nascent root, but as the root grown in a medium in the presence of rhizobia, the newly available regions are also colonized; however only the specific associations are sustained for long term.

4.4 Discussion

The mutualistic association between rhizobia and legume host has been well studied with respect to the signaling and the molecular events important to the process nodulation and N-fixation. However, the process of how rhizobia colonize the roots of host remain to be understood. (Gibson *et al.,* 2008; Rivilla *et al.,* 2017).

This part of the work deals with the investigating host root colonization patterns of nodule isolates of pigeon pea. The root nodule bacteria studied (*Ensifer* spp. HP127 and HP113) are local isolates which were characterized for their quorum sensing genes and molecules (Chapter 2). When the strains were coated on the germinated seed and tracked, both the strains were found to colonize in large numbers in the areas of the root proximal to the crown and their numbers decreased as the distance from the crown increased. Wadisirisuk *et al.,* (1989), in their experiments involving inoculation of soybean with *Bradyrhizobium* observed similar effect however, their findings were limited to primary roots unattributed. Such a pattern could arise due to the failure of rhizobia to reach the other areas of the root and may refelect upon the bacterial traits or due to the difference in the plant exudates along different parts of the root. In contrast with when the strains were coated on the roots when *Ensifer* spp. HP127 and HP113 were inoculated in the bulk soil, they were observed to colonize the entirety of the root effectively. Significant colonization was also found to take place on the secondary roots and the areas thereof which are distal from the crown of the seed. Even the newly emergent roots of distal origin were found to contain hundred to thousands of rhizobial cfu. . These results indicate that the different regions of the root are biologically receptive and conducive for the colonization under the experimental condition and it is the inability of the rhizobia coated on to the seeds to effectively colonized newer and emergent areas of the root, Thus the coating on the seed possibly restricts the spread of the rhizobia over the distal areas of the root. This is in accordance with the similar proposals about the rhizobial colonization made in case of soybean and *Bradyrhizobium* system (Brockwell *et al.,* 1987; Wadisirisuk *et al.,* 1989) and with the colonization pattern of rhizobia on rice roots in a recent work by Schmidt *et al.* (2018). It has also been

proposed that such patterns of colonization by rhizobia arise due to the fact that the epithelial cells of roots elongate and divide more rapidly than the motility/growth rate of bacteria can keep up with in order to cover the increasing area, however the motility may be required for the initial contact from the soil to the rhizoplane transition. (Caetano-Anollés *et al.*, 1992; Raina *et al.*, 2019). The effect of rhizobial properties such as surface or community structures have not been hitherto tested however. The observation that *Ensifer* sp. HP127 spanned greater area on the root than that covered by *Ensifer* sp. HP113 was interesting, and pointing to the fact that something inherent to the rhizobial physiology was also crucial to determining the colonization pattern. The studies mentioned earlier have not been able to attribute the colonization behavior to the rhizobial properties. In order to assess the effect of challenge of each of the strains on the other one, a dual inoculum experiment was set up involving coating one of the strains on the germinated seed of pigeon pea, while inoculating the soil with two different densities of the other strain (10^4 and 10^6 cfu/g). A challenge of this kind simulates the conditions in which the rhizobia face the competition. The counts of seed-coated or soil-inoculated strains were observed to be reduced in the proximal areas of the root in the presence of a competing strain as compared to the control (respective single inoculation). The counts of both the strains of rhizobia increased over the course of weeks, however the counts of any of the strains were significantly less (often 1-2 orders of magnitude) than the control. This could be due to the competition for the nutrient and real estate resources of that area of the root, given that the seed-coated organisms have a greatest presence in that area, and that the dominance of the soil-inoculated bacterial was relatively greater when present at 10^6 cfu/g as compared to when inoculated at 10^4 cfu/g.

With regard to colonization of distal areas, the counts of HP127 when inoculated on the roots in the presence of soil-HP113 were found to be less than the control i.e. in the absence of the soil challenger. This can be explained by that HP127 colonizes distal areas also prolifically, thus the control counts are relatively higher, and that in the presence of HP113, the homogenous of HP113 restricts the colonization by HP127. The counts of HP127 in the distal areas of the root, when

it was inoculated in the soil, in the presence of HP113 coated on root are largely unaffected or were reduced meagerly, indicating that the colonization of the soil-borne colonizer was not restricted in the areas where the root-origin strain was not abundantly present,

Interestingly, however, when *Ensifer* sp. HP113 was applied on root, its counts in the distal regions of root, were found to be significantly higher in the presence of HP127 in the soil as compared to the when the soil was sterile (Table 4.5). This was counter-intuitive because not only is *Ensifer* sp. HP113 a weak colonizer of the distal regions (Table4.3) , but also the root is also colonized by a soil-origin HP127. This unique pattern may be explained in light of the earlier observation that HP127 was capable of covering greater area of the root as compared to the HP113 when coated on the root, and that both phenomena could be the consequences of their differential capability to attach and interact with the root surface.

Rhizobial cells are believed to traverse the root surface by adhering tightly to the root surface and thus spreading with the rapidly expanding the root cell layer and also by moving along the percolating water down the root. (Benizri *et al.*, 2001; Caetano-Anollés *et al.*, 1992; Kuzyakov and Razavi, 2019). The strains were evaluated for the differences in the attachment to the root surface. *In vitro* analysis revealed that *Ensifer* sp. HP127 produced significantly greater amounts of EPS as compared to HP113 and also elicited relatively greater biofilm formation. EPS production and biofilm formation abilities are two of the most crucial properties of rhizobia that determine attachment and colonization of root. EPS produced by rhizobia are not only required for the recognition and signaling but also structure the colony architecture of the rhizobia on the root (Santaella *et al.*, 2008). EPS secreted by rhizobia has also been implicated in reshaping the soil aggregates around the root and facilitating the colonization (Alami *et al.*, 2000). Although, EPS plays an independent role in rhizobial root colonization, it is one of the most crucial components of rhizobial biofilms (Rinaudi and Giordano, 2010). This is also corroborated from the case of *E. meliloti* strain 8530 and 1021, where the former forms mucoid colonies and forms highly structured and architectural biofilm, as

opposed to the unstructured biofilms formed by the latter; the former produces a an-EPS II-glycan that the latter is incapable of (Rinaudi and Gonzalez, 2009). They also observed that the EPSII-deficient strain could only make biofilms as clusters of cells on the main tap root whereas the Em8530 was found to spread over the entire root in biofilms including the root hairs. Similar failure to produce biofilms in the absence of EPS production has also been observed in case of *Rhizobium leguminosarum* strains (Russo *et al.*, 2006).

The results of CLSM also revealed that *Ensifer* sp. HP127 colonized the root surface in large swarms and as layers whereas HP113 was observed as groups of distinct aggregates. SEM imaging confirmed the colony architectures and also showed the presence of copious amounts of typical exopolysaccharide surrounding the cells and embedding the biofilms of HP127 whereas HP113 cells had a smooth appearance and its colony aggregates were small in spread and had no matrix of EPS.

On the basis of these results, we believe the higher counts of HP113 in the distal areas of the root when HP127 was also colonizing the roots (as compared to control when HP113 colonized root alone) could be due to the large secretion of EPS and biofilm matrix by the latter which allowed a passive movement of HP113 along the greater areas of the root.

The phenomenon of root colonization was investigated at a higher resolution using microfluidics and confocal microscopy using TRIS device (Massalha *et al.*, 2017). Due to the size limitation of the chamber size, this analysis did not allow usage of a host with a thick root as that of pigeon pea, and thus was carried out with *Ensifer meliloti* 8530 (expressing eGFP) and alfalfa which has a sub-millimeter sized primary root. This analysis revealed that rhizobia are very quick to get attracted to a nearby root and colonize the root nonspecifically as soon as they come in the contact with it and that over the period of time, specific interactions persist and rhizobia form thick biofilms on the root and the root-hairs (the entry point for the infection thread formation) as soon as within 20 h post inoculation.

Failure of inoculated rhizobia to colonize the root in competition with the native strains is a problem largely unsolved. The exact way in which rhizobia lose to the native strains holds part of the solution (Poole *et al.*, 2018). This work highlights that the occupation of the root is limited by the contact between the root and the rhizobia to begin with. Earlier studies have shown that a rhizobial densities of 10^4 cfu or above pose as almost impregnable barrier to the inoculated strain (Meade *et al.*, 1985). The results of this work corroborate and correlate the observed patterns and the lower nodulation competitiveness. Our analysis further attributes some of these bottlenecks of colonization to physiological traits of the rhizobia, which may aid the engineering rhizobia for generation of better colonizaing strains for agricultural application. This information could be crucial to crop legumes such as pigeon pea which are of paramount economic importance to usually the poor farmers (Gates, 2014; Varshney *et al.*, 2012) especially as seed inoculation remains the most popular way of inoculation of rhizobia (Deaker *et al.*, 2004). In that sense a reletively new and popular way of inoculation may be more beneficial for the ioculants (López-García *et al.*, 2009) if it allows a greater and sooner access to the inoculated rhizobium of the host root. The study also reveals the contribution of attachment properties of rhizobia and the factors crucial for it in the colonization pattern of inoculated rhizobia on the host root. These findings indicate using strains that are better at attachment or engineering strains for better attachment may render them “better colonizers” and relieve the issue of nodulation competitiveness, alternatively inoculation approaches that allow a better contact opportunities for the inoculant with the host root. The work also finds an interesting inter-strain dynamics of rhizobia colonization where a strain capable of secreting large amount of matrix materials may inadvertently benefit the other strain. This information may be investigated further and may result into development of consortia where rhizobial or non-rhizobial “colonization helpers” that secrete large amounts of extracellular polysaccharides could be incorporated as potent plant growth promoting agents.