Chapter 2 : Influence of root exudates on chemotaxis and colonization of diverse plant growth promoting rhizobacteria in *Cajanus cajan – Zea mays* intercropping system

2.1 Introduction

Sustainable agricultural practices adopt intercropping for their high productivity, and the maintenance of ecosystem diversity with resource efficiency (Lithourgidis et al., 2011). Planting two different crops in each other's vicinity brings about physical proximity among the roots of the two plant species results in the variation in root architecture, root exudation patterns eventually leading to improved nutrient mobilization and nutrient transfer (Hauggaard-Nielsen and Jensen, 2005). A positive impact on soil structure as well as nutrient storage in high P occluded was mediated by physical root contact in the *Cajanus cajan – Zea mays* intercropping system (Garland et al., 2017).

Colonization of plant growth-promoting rhizobacteria (PGPR) strain onto the plant roots is the first and crucial step in the early growth stage of a crop and offers its beneficial functions to the hostthrough root exudates (Philippot et al., 2013). PGPR are free-living beneficial soil microbes that inhabit the rhizosphere. Plant growth in the presence of PGPR can reduce nearly 25% of the chemical fertilizer in the present agriculture system (Romero-perdomo et al., 2017). Moreover, a combination of growth-promoting bacteria [N_2-fixing, P-solubilizing, K-solubilizing, and indole acetic acid (IAA)-producing bacteria] can effectively improve N/P/K uptake and plant growth in monoculture wheat plants (Wang et al., 2020a). However, very few such studies deal with the usage of PGPR in an intercropping system. For instance, bio fertilization with PGPR such as *Pseudomonas* and *Bradyrhizobium* along with arbuscular mycorrhiza under autoclaved soil conditions, improved finger millet growth when co-cultivated with *C. cajan* plants (Saharan et al., 2018). These findings were further recently confirmed under field studies (Mathimaran et al., 2020). Also, the PGPR application serves as an effective treatment for enhancing the productivity and quality of fennel essential oil compared with the sole cropping (Rezaei-chiyaneh et al., 2019).

Root exudates are usually composed of compounds like amino acids, flavonoids, organic acids, etc. They have been shown to act as signaling molecules in the plant-to-plant interaction (Contreras et al., 2019) as well as plant-microbe interactions in intercropping systems (Duchene et al., 2017). Root exudates are also known to be closely linked with the rhizosphere microbiome where they directly affect different components of the rhizobiome, and vice versa (Mommer et al., 2016b). Thus, metabolites of the root exudates act as mediators for both plant-plant and plant-microbiome interactions. Chemotaxis of PGPR towards the many individual components of plant

root exudates, mainly primary metabolites, has been reported (Feng et al., 2018; Matilla and Krell, 2018). But still, how these microbes interact with plants in the intercropping system and their responses to altered plant root exudates is not known.

Primary metabolites (organic acids, sugars, and amino acids) are important components of root exudates are believed to be released passively lost from the root (Canarini et al., 2019). Plant roots usually have the total concentration of organic acids in roots of around 10-20 mM (1-4% (w/w) of total dry weight) in the rhizosphere and are key drivers in bacterial chemotaxis from bulk soil to the rhizosphere (Jones, 1998; Jones et al., 2004). Direct application of organic acids externally to soils enriches specific bacterial groups with known PGPR traits indicated the importance of low molecular weight organic acids in soil fertility (Macias-benitez et al., 2020). Therefore, the significance of the organic acids on inoculated PGPR in an intercropping system would be an interesting aspect.

To understand the dynamics of PGPR in the intercropped plants, the use of specific microbes as externally applied inoculants would help to study plant-microbe interactions in a well-defined manner. Therefore, we have used the well-studied model of *C. cajan – Z. mays* with three different PGPR strains that have been previously characterized in lab studies and well known legume symbiont. Further, we also addressed the question of whether the root exudates of the intercropping system could distinctly influence the physiology of the bacteria. Since organic acids are implicated in plant-microbe interaction, we analyzed the differences in major organic acids secreted in the root exudates of intercropped plants through LC/MS/MS.

2.2 Materials and Methods

2.2.1 PGPR strains used in this study

The microbes used in this study were *Enterobacter* sp. C1D (referred to as C1D) (GenBank accession no. JN936958.1), *Pseudomonas* sp. G22 (referred to as G22) (GenBank accession no. KY206885), *Rhizobium* sp. IC3109 (referred to as IC3109) (GenBank accession no. MW040081) and *Ensifer (Sinorhizobium) fredii* NGR234. Their plant growth-promoting (PGP) traits and other relevant information are described in Table 2.1. Two of these bacteria selected were the diazotrophic, *C. cajan* nodulating strain *Rhizobium* sp. IC3109 and broad host legume symbiont *Ensifer fredii* NGR234. Rhizobia are an important component of the legume-cereal intercropping system wherein efficient N management is brought about by increases in nodule biomass, nodule nitrogenase activity (Li et al., 2013a). On the other hand, phosphate mobilizing bacterium *Enterobacter* sp. C1D, which possesses high P-solubilizing activity and phosphatase activity is important in the facilitation of P uptake during intercropping (Schoebitz et al., 2020). The third strain G22, belonging to the genus *Pseudomonas*, displayed PGPR traits like siderophore, IAA production, and biocontrol ability (antibiotic production) against fungal plant pathogens (Patel and Archana, 2018).

2.2.2 Plant inoculation experiments

Surface sterilization of *C. cajan* (cultivar BDN- 2) and *Z. mays* (cultivar GM-6), as well as plant inoculation, was performed according to the protocol described by Gosai et al. (2019). Surface sterilized seeds of *C. cajan* and *Z. mays* were separately placed on 0.8% water agar for their germination at 30 °C for 3 d and 2 d respectively. Cultures of C1D and G22 were grown at 30 °C for 12-14 h in Luria-Bertani (LB) broth (HiMedia, India) while IC3109 and NGR234 were grown in Tryptone Yeast Extract (TY) broth (6 g/l tryptone type I, 3 g/l yeast extract) with added 3 mM $CaCl_2$ grown at 30 °C for 24 h. Seedlings of *C. cajan* and *Z. mays* plants were coated with cultures individually of 10^8 CFU ml^{-1} and were grown in pots containing 3 kg sterile coarse sand (which was priorly autoclaved at 121 °C, for 30 min for 3 consecutive days to eliminate spore formers). The plants were allowed to grow under greenhouse conditions (12-14 of photoperiod at 30 °C) and watered thrice a week with autoclaved, reverse osmosis (RO) water. Thereafter root and shoot (length and weight) were measured at 28 days after sowing (DAS). Fold change was measured in terms of the ratio of the growth parameter as inoculated/ un-inoculated (control)

plants. Ten biological replicates were considered for each of the monocropped *C. cajan* and *Z. mays* plants and were inoculated (individually with strains -C1D, G22, IC3109, NGR234) and uninoculated plants.

Table 2-1 Plant growth-promoting rhizobacteria used for this study

Name of the organism	Plant growth-promoting traits	Source	References
Enterobacter sp. C1D	Mineral Phosphate solubilization, ACC (1-aminocyclopropane-1-carboxylate) deaminase, Indole – 3 acetic acid (IAA) production, Siderophore production, Heavy metal tolerant (Cd, Cr)	Isolated from a sediment sample at an industrial waste effluent (IWE) dump site	Subrahmanyam et al., 2018; Sharma et al., 2019
Pseudomonas sp. G22	Antibiotic production (2,4-diacetylphloroglucinol) (DAPG), Siderophore production, IAA production	Isolated from groundnut rhizosphere	Patel and Archana, 2018
Rhizobium sp. IC3109	Efficient nitrogen fixer and nodulation proficient on *C. cajan*, siderophore production, IAA production	Isolated from nodules of *C. cajan* (Kind gift from Dr. A.K. Saxena, IARI, New Delhi, India)	Rajendran et al., 2007
Ensifer (Sinorhizobium) fredii NGR234	Nodulates and fixes nitrogen on more than 70 genera of legumes and non-legume *Parasponia andersoni*, siderophore production, IAA production	Originally isolated from *Lablab purpureas* (Procured from National Biological Resource Center, Japan)	Relic et al., 1993

2.2.3 Cross colonization studies of PGPR in intercropped plants

Seedlings inoculation protocol and plant growth duration were similar to those mentioned in Section 2.2.2. For monocropped plants, each pot received two seedlings of either *C. cajan* or *Z. mays* while for intercropped plants one seedling of each of the two plants (*C. cajan* and *Z. mays*) was sowed in the same pot with a distance of 10 cm between the two plants. For studying cross colonization in the inter-cropping system, seedlings of only one of the two plants were soaked with the bacterial suspension while seedlings of other plants were left uninoculated. Mesh barriers (MB) previously reported by Wang et al. (2007) were used with slight modifications. Here, seedlings of *C. cajan* and *Z. mays* were separated by an autoclaved MB (25 µm stainless steel) which was placed in the middle of the two sets of plants (Fig. 2.1). Plants were allowed to grow under similar conditions as mentioned in Section 2.2.2. After 28 DAS, the plants were gently uprooted and root-adhered bacteria were suspended in 0.85% NaCl solution (N-saline) appropriate serial dilutions of this suspension in N-saline were plated on LB or TY agar depending on the bacterium inoculated, and incubated at 30 °C for 2 d. Colony-forming units (CFU) per g of fresh root weight were recorded. Data represent the average of a total of six replicates of the experiment carried out two independent times (three replicates each time).

Fig. 2-1 Intercropped plants grown in the plastic bags in the presence of mesh barrier for cross colonization studies Mesh barrier was placed in between the two plants grown at a distance of 10cm

2.2.4 Colonization of NGR234 on *C. cajan* and *Z. mays* plants by Confocal laser scanning microscopy

2.2.4.1 Tagging of NGR234

The plasmid of pDsRed-Express- N1 (Reporter vector, encodes *dsred*, Km^R) was transformed into the *E. coli* strain S17-1/λ*pir*. Biparental spot-matings were carried out to transform plasmid into NGR234. For spot-matings *E. coli* and NGR234 strains were grown to stationary phase in Tryptone Yeast extract (TY) broth. Aliquots of each culture (30 µL) were dispensed together as a spot onto the surface of a 0.45 µm filter (Type HA, 47 mm, Millipore Corporation, USA) placed on a TY agar plate and incubated at 28°C overnight. The resultant bacterial growth was then streaked onto selective media containing rifampicin + kanamycin antibiotics.

2.2.4.2 Inoculation on to the C. cajan and Z. mays seedlings

Surface sterilized seedlings of both the plants were inoculated with NGR234 - DsRed-expressing cells for 23 DAS of *C. cajan* and 15 DAS of *Z. mays* plants in sterile sand. Plants were maintained and grown similar to the protocol mentioned in Section 2.2.2. Confocal laser scanning microscopy of NGR234 on the roots was performed using a dual channel Zeiss LSM 510 upright confocal microscope, with excitation 568 nm for DsRed and a BP505-530 emission filter an LP585 filter for DsRed. Images were recorded with LSM image browser software (Kelly et al., 2013).

2.2.5 Root exudates collection and organic acid analysis by LC/MS/MS

Uninoculated plants were grown as monocropped or intercropped for 28 d as depicted in Fig. 2.2 Fourteen seedlings of the same plant were grown individually in a pot (of diameter- 27 cm, length- 21cm) which was covered with a black plastic bag and were referred to as monocropped *C. cajan* or *Z. mays* plants, while 7 seedlings of both *C. cajan* and *Z. mays* were grown together in the same pot at a distance of 7 cm for intercropped plants. Pots were maintained in the greenhouse (12h of light and -12h of dark photoperiod at 30°C) and quarter strength Hoagland solution (Hoagland and Arnon, 1941) was used for watering (Huang et al., 2019). After harvesting the plants, root exudates were collected using a modified protocol based on that reported by Badri et al. (2012) and Zhang et al. (2014). Here 30 plants for each of the monocropped of *C. cajan* and *Z. mays* were planted separately in pots while 30 plants of each species in intercropped (30 plants *C. cajan* and 30 of *Z. mays*) that were grown together for 28 d.

(a)

Monocropped plants *C. cajan* (Left); intercropped plants *C. cajan* – maize (middle); monocropped plants maize (Right)

(b)

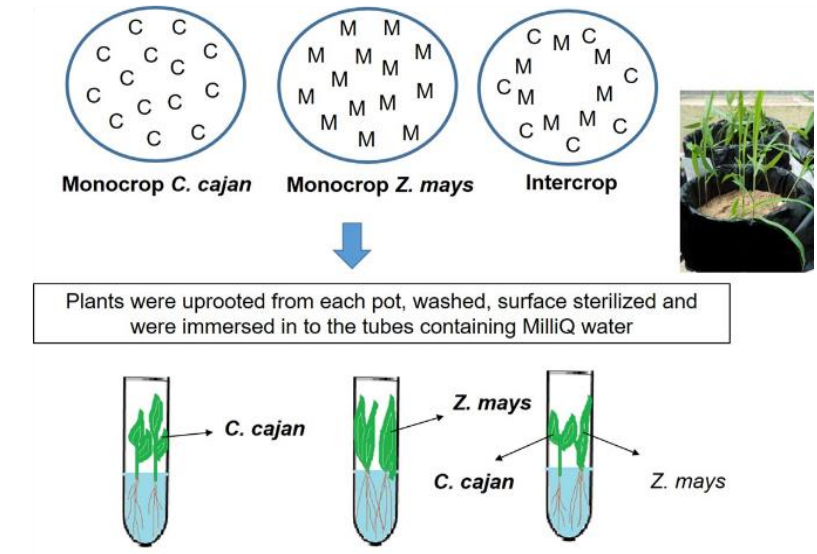


Fig. 2-2 Schematic for root exudates collection of *C. cajan* and *Z. mays* plants as monocrops and intercrops (a) Uninoculated plants grown for 28 DAS in individual pots as monocropped and intercropped plants, (b) Schematic of plants grown as monocropped or intercropped for root exudates collection.

Oburger et al. (2013) reported a step to minimize microbial interferences and to osmotically adjust root cells to the sampling conditions. Accordingly, roots were rinsed in autoclaved deionized water before being exposed to the sampling solution followed by chloramphenicol (30 µg ml^{-1} in st. water) treatment for 3 min to surface sterilize the roots as described by Wu et al. (2012). Thereafter, roots were washed twice with autoclaved MilliQ (AMQ) water, and plants were transferred into Borosil tubes (length-20 cm, diameter-35 mm) with 2 plant roots immersed in 40 ml AMQ water (Fig. 2b). Tubes were then covered with aluminum foil on the top while the bottom was covered with an opaque material (paper) to prevent light interference on the roots and was kept on a shaker for 6 h at 30 °C. To prevent reabsorption, root exudate samples of both moncropped and intercropped *C. cajan* and *Z. mays* were not kept for more than 6 h as mentioned by Carvalhais et al. (2011). Solutions containing the root exudates were pooled separately for monocropped and intercropped plants and filter sterilized through a 0.45µm nylon membrane. The solutions were lyophilized (Christ Lyophilizer Alpha 1-4, Germany) to a powdered form and preserved at -20 ºC until further use.

For LC/MS/MS analysis, a C8 column (120 × 4.6 mm, 5 µm) with a mobile phase of 0.5% formic acid in a gradient with methanol was used in Shimadzu model LCMS 2020 facility at the Dr. Vikram Sarabhai Institute of Cell and Molecular Biology (Faculty of Science) from M. S. University of Baroda. The identification protocol for organic acids from root exudates has been carried out according to Erro et al. (2009) using AB ScieX 3200 QTRAP in Multiple Reaction Monitoring (MRM).

2.2.6 Chemotaxis and Biofilm assay

To determine the capillary movement of bacterial strains towards root exudates the capillary method was performed with few modifications to the protocol (Gordillo et al., 2007). Bacterial cells were inoculated with 0.1 OD_{600} (optical density) into 50 ml broth and grown to a late log phase in a shaking condition in their respective minimal media (MM) to allow the flagella to develop completely. MM used were as follows: NGR234 and IC3109 were grown in glutamate supplemented rhizobium minimal medium (RMM) (Broughton et al., 1986; modified by replacing succinate) and C1D and G22, on M9 minimal medium (MM) with 0.5% glucose (Sambrook and Russel, 2001). Further, cultures were centrifuged at a low speed of 800 x *g* for 10 min to avoid the disruption of flagella and then washed with chemotaxis buffer and resuspended in the same (Darias

et al., 2014). The final density of the culture (aliquote) was set to (10^8 CFU ml^{-1}), taken in a 1.5 ml microfuge tube (Eppendorf), and a 2-cm 25-gauge needle used as the chemotaxis capillary was attached to a 1-ml tuberculin syringe (Dispovan) containing a 100 µl of the test solution (Fig. 2.3). Test solutions comprised of lyophilized and filter sterilized root exudates were taken at a final concentration of 1 mg ml^{-1} (dissolved in AMQ water) or organic acids (fumarate, malate, succinate, and citrate) which were filter sterilized with 0.2 µ nylon membrane filter and taken as standards at a final concentration of 50 µM. The control consisted of AMQ water. Assays were set up in triplicates for each test and control sample. After 90 min of incubation at 30 ºC, the needle and syringe were removed from the bacterial suspension and its contents were diluted into N- saline and plated on LB/TY plates. After incubation of 2 d at 30 ºC, CFU ml^{-1} of the bacterial count was determined in control and test samples. Fold difference in the capillary movement was calculated by comparing CFU ml $^{-1}$ of test and control (AMQ water) samples for each bacterial strain.

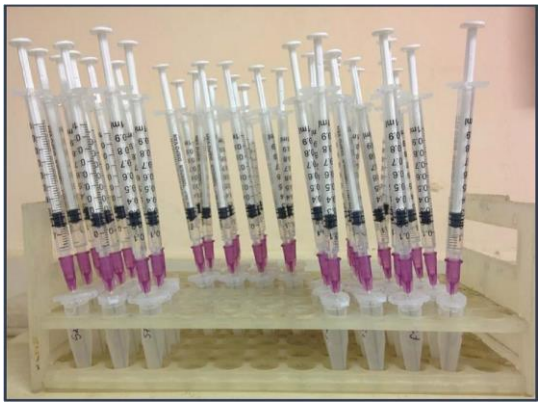


Fig. 2-3 Capillary assay set-up for studying chemotaxis of bacteria towards root exudates or metabolites

Further to examine biofilm formation in the presence of the root exudates, 150 µl suspensions (of 0.1 OD_{600}) of bacterial strains in the appropriate minimal medium with test samples (same as above) and control (untreated cells) were loaded into 96 wells of polystyrene microtiter plates. After 48 h of static incubation at 30 ^{o} C, the non-adherent cells were removed, wells were washed and the remaining cells adhered as biofilm were determined as mentioned by Lee et al. (2012). The absorbance is quantified at OD_{595} by the multimode plate reader (Synergy HT, BioTek, USA). Fold difference in the biofilm cells was calculated by comparing optical density (O.D.) of test and control (untreated cells) samples for each bacteria.

2.2.7 Statistical Analysis

Data were analyzed by applying one-way or two-way ANOVA followed by a Tukey post hoc test using Graph pad Prism software. Statistical significance was determined at the critical α-level of 0.05. Significance was represented as 'ns' if $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2.3 Results

2.3.1. Effect of PGPR on the monocropped *C. cajan* and *Z. mays* plants

Colonization of PGPR strains onto the monocropped pigeon pea and maize studies reflected that both C1D strain and G22 strain sustained efficiently on the roots of pigeon pea and maize with 10^7–10^8 CFU g^{-1} of root tissue (Fig. 2.4a). It was interesting to note that the rhizobial strain IC3109 which is a nodule isolate of pigeon pea plants colonized epiphytically on the roots of *Z.mays* with 10^5 CFU g^{-1} while NGR234 colonized at 10^{10} CFU g^{-1} of root tissue. Plant growth promotion by PGPR was seen on the pigeon pea and maize plants (Fig. 2.4b; 2.4c). Auxin levels of each bacteria estimated by the Salkowoski test were as follows: C1D strain (39 μg ml^{-1} ± 0.59), G22 strain (50 μg ml^{-1} ± 0.78), IC3109 strain (60 μg ml^{-1} ± 1.09), and NGR234 (70 μg ml^{-1} ± 0.89). Interestingly, the C1D strain contributed to the root length [1.52 fold change (FC)] and root weight (2.8 FC) while the NGR234 strain showed an increase in root length (2.2 FC) and root weight (2.5 FC) of maize plants. The C1D strain showed a positive effect on the shoot weight (8.3 FC) of pigeon pea as well compared to the other three strains. Strain G22 manifested its effect on the root weight (10.9 FC) of pigeon pea and IC3109 exhibited an effect on root length (1.35 FC) of both the plants. However, none of them had a prominent effect on the shoot length of either plant and shoot weight of maize plants as compared to uninoculated plants.

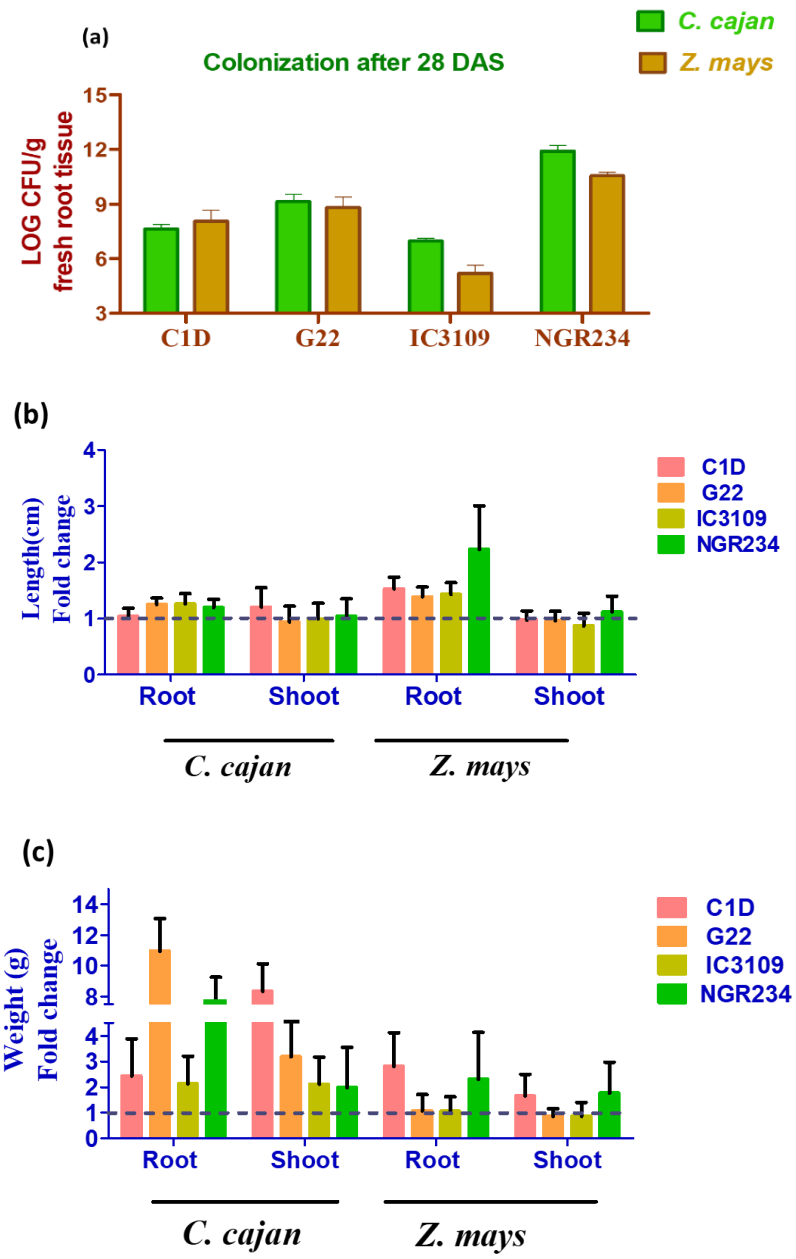


Fig. 2-4 Colonization and plant growth studies of PGPR on *C. cajan* and *Z. mays* plants Part (a) indicates Colonization, Part (b) Length, and (c) Weight of the monocropped roots of *C. cajan* and *Z. mays*. Fold change has been considered with respect to the ratio of the parameter for Inoculated/Uninoculated (Control) plants. Error bars indicate standard deviation with 3 replicates for colonization and 10 replicates for plant growth.

2.3.2. Study of cross colonization of PGPR in *C. cajan* – *Z. mays* intercropped plants

The intermingling of roots was observed between the legume (*C. cajan*) and cereal (*Z. mays*) plant roots when grown adjacently as intercropped plants (Fig. 2.5). Cross colonization experiments with a mesh barrier and without the barrier revealed that all PGPR strains cross colonized when the plants were placed at a distance of 10 cm. Mesh barrier studies with C1D strain (Fig. 2.6a; 2.7a) and IC3109 strain (Fig. 2.6c; 2.7c) indicated clearly that colonization was similar to that of no barrier pots. However, in the case of the G22 strain (Fig. 2.6b; 2.7b), it was distinguishably different with a significant reduction ($p < 0.01$) in colonization with a barrier condition compared to no barrier condition. When the colonization ability of PGPR strains on inoculated monocropped

Fig. 2-5 Facilitative interaction between roots of *C. cajan* and *Z. mays* plants Plants were grown in quarter strength Hoagland nutrient medium containing 0.4% phytagel for 10 days.

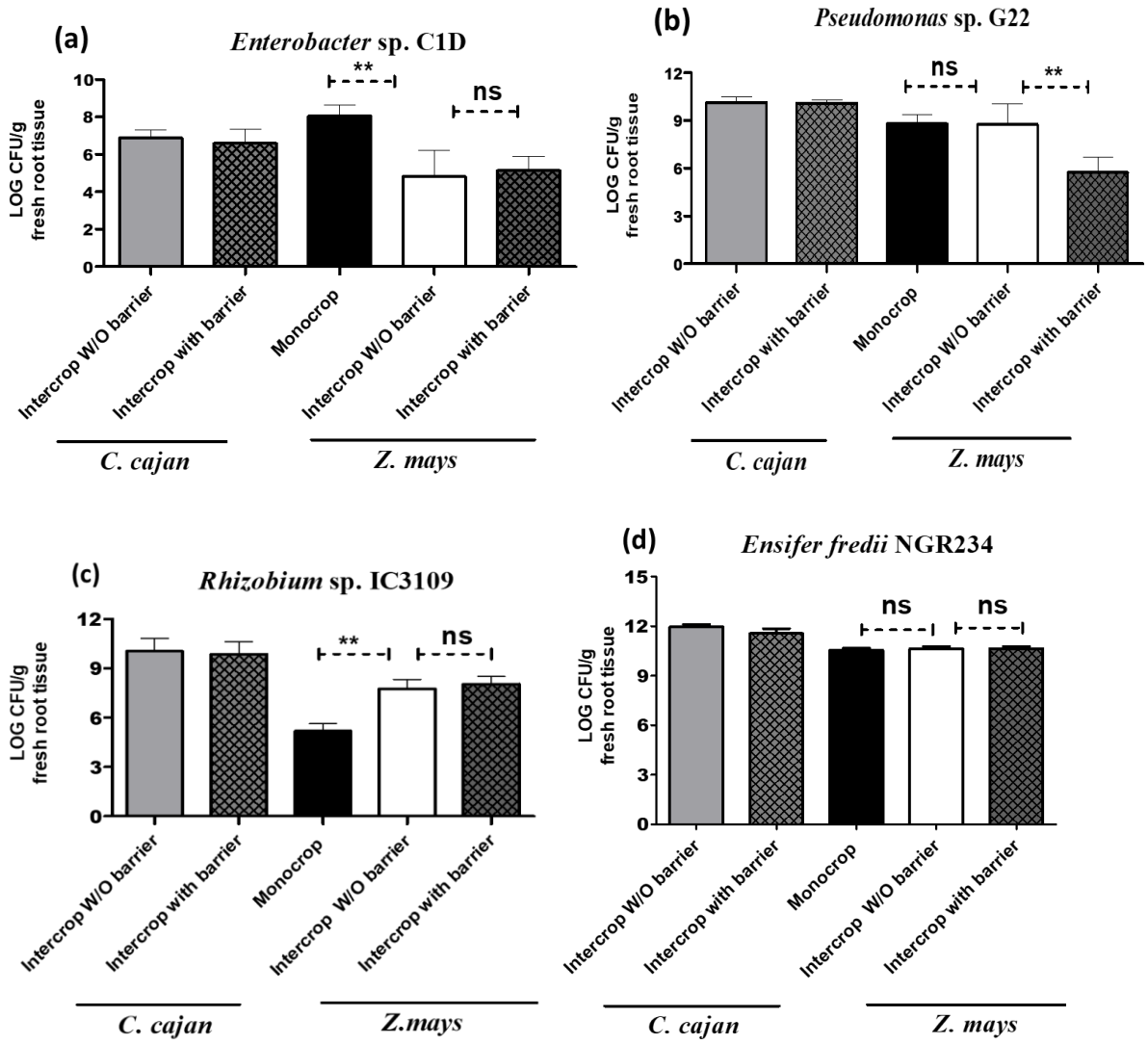


Fig. 2-6 Cross colonization of PGPR from *C. cajan* to *Z.* mays Grey bars represent *C. cajan* plants inoculated with the respective organism. White bars represent the cells on the roots of *Z. mays* plants when co-cultivated in the presence of inoculated *C. cajan* plants. Hatched bars indicate an experiment where mesh was used to separate the roots of the co-cultivated plants. Colonization data of control *Z. mays* plants, directly inoculated with the corresponding culture is shown as a reference for comparison (black bars). Error bars indicate standard deviation and the data were subjected to one-way ANOVA followed by post hoc Tukey's multiple comparisons test. 'ns' if non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. n=6.

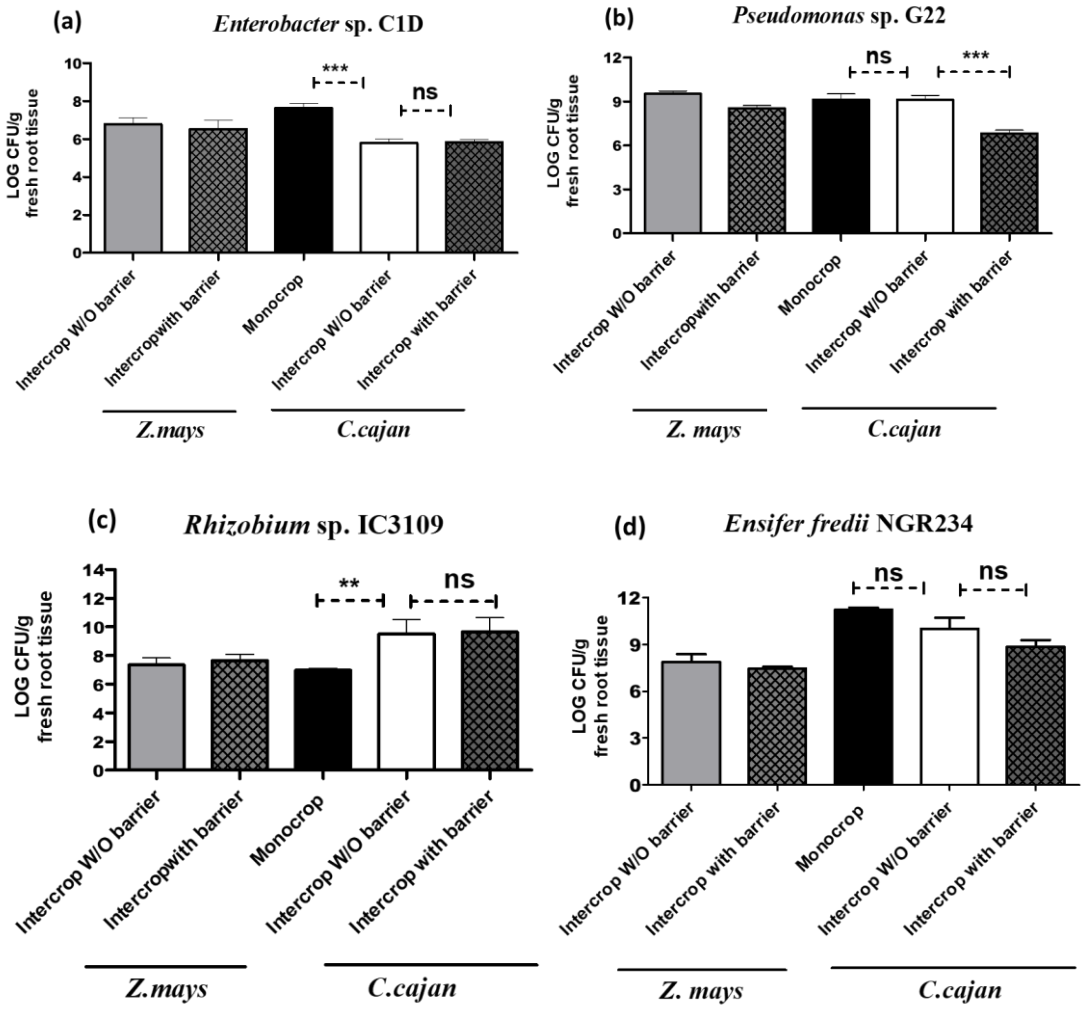


Fig. 2-7 Cross colonization of PGPR from *Z. mays* to *C. cajan* Grey bars represent *Z. mays* plants inoculated with the respective organism. White bars represent the cells on the roots of *C. cajan* plants when co-cultivated in the presence of inoculated *Z. mays* plants. Hatched bars indicate an experiment where mesh was used to separate the roots of the co-cultivated plants. Colonization data of control *C. cajan* plants, directly inoculated with the corresponding culture is shown as a reference for comparison (black bars). Error bars indicate standard deviation and the data were subjected to one-way ANOVA followed by post hoc Tukey's multiple comparisons test. 'ns' if non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. n=6.

2.3.3 Colonization of NGR234 on *C. cajan* and *Z. mays* plant roots

Colonization of NGR234 onto the roots of *C. cajan* and *Z. mays* plants was visualized through confocal laser scanning microscopy (CLSM). NGR234 cells showed colonization along the root hairs of *C. cajan* roots observed at 23DAS (Fig. 2.8) & root hairs of *Z. mays* roots at 15 DAS (Fig. 2.9).

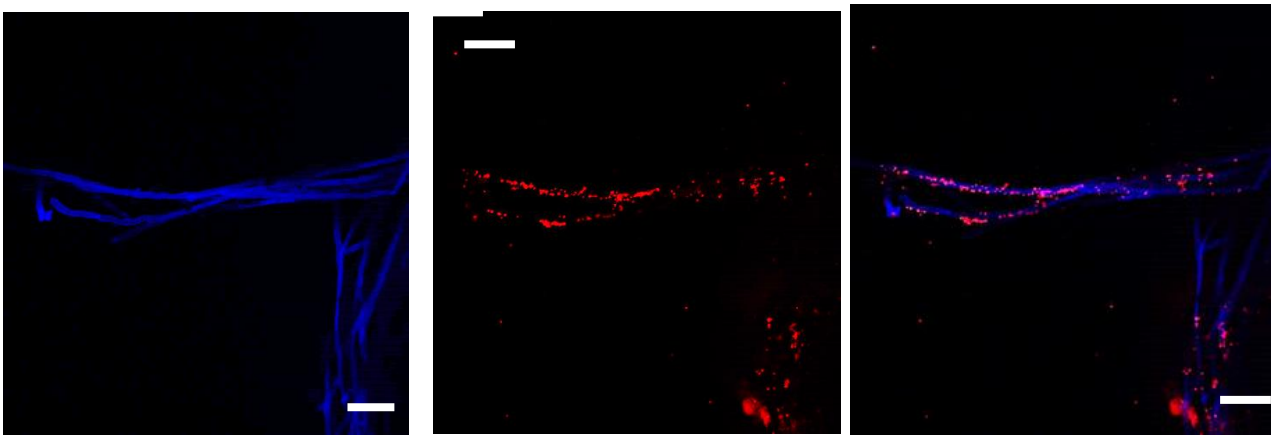
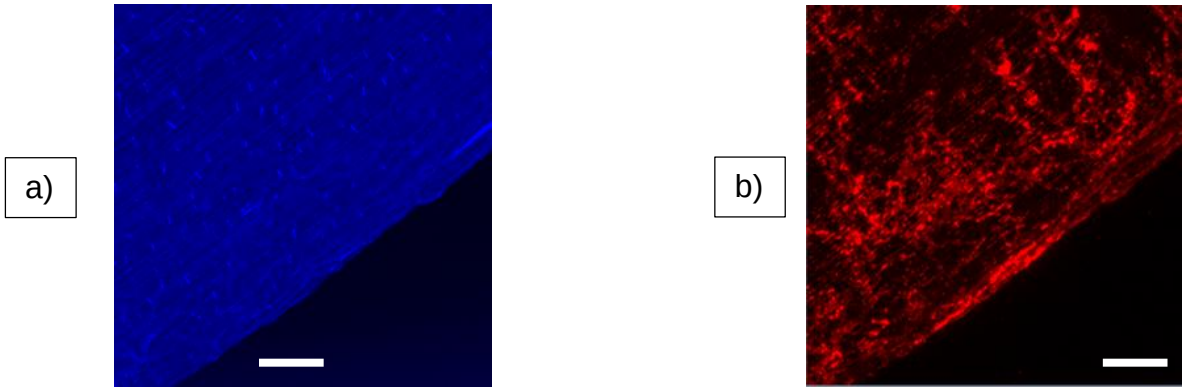


Fig. 2-8 Colonization of NGR234 on roots of *C. cajan* plants observed by CLSM NGR234 cells were tagged with *dsRed* plasmid. Dual laser CLSM images showing a) Excitation at 405nm, b) Excitation at 561nm, c) overlapped images. The image was observed on *C. cajan* plants at 23 DAS observed under the 20X objective of CLSM.



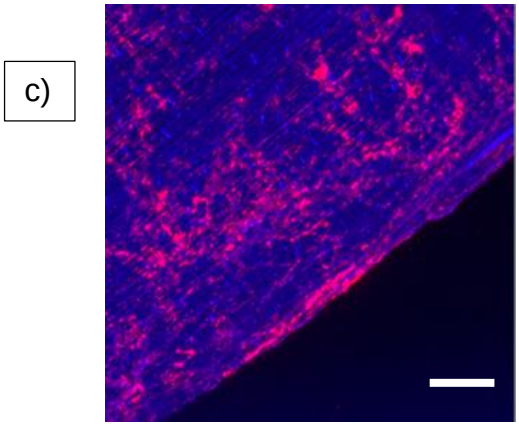


Fig. 2-9 Colonization of NGR234 on roots of *Z. mays* plants observed by CLSM NGR234 cells were tagged with *dsRed* plasmid. Dual laser CLSM images showing a) Excitation at 405nm, b) Excitation at 561nm, c) overlapped images. The image was observed on *Z. mays* plants at 15 DAS observed under the 20X objective of CLSM.

2.3.4. Assessment of the chemotactic response and biofilm formation of PGPR towards root exudates

The modified capillary assay measured quantitatively the chemotactic response of PGPR towards root exudates of monocropped and intercropped plants (Fig. 2.10 a). It was interesting to note that all PGPR were chemo-attracted to the root exudates of both plants under mono as well as co-cultivation conditions. However, there was a definite response towards each of them. Individually it was found that C1D strain showed specific and significant ($p < 0.05$) attraction towards root exudates of monocrop *Z. mays* and G22 strain migrated significantly ($p < 0.001$) more (3 fold high) towards intercropped and *Z. mays* monocropped root exudates as compared to *C. cajan* monocropped root exudates. The capillary movement of the IC3109 strain was 5 fold higher ascompared to control and significantly better migration towards intercropped when compared to monocropped *C. cajan* ($p < 0.001$) and *Z. mays* ($p < 0.001$).

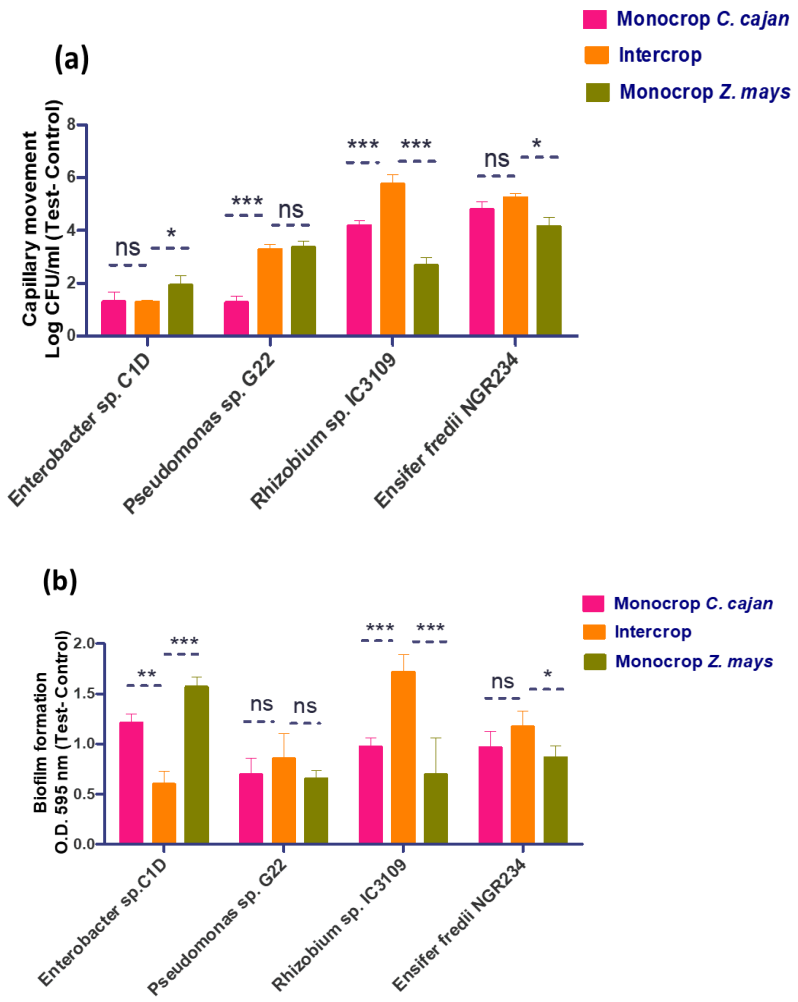


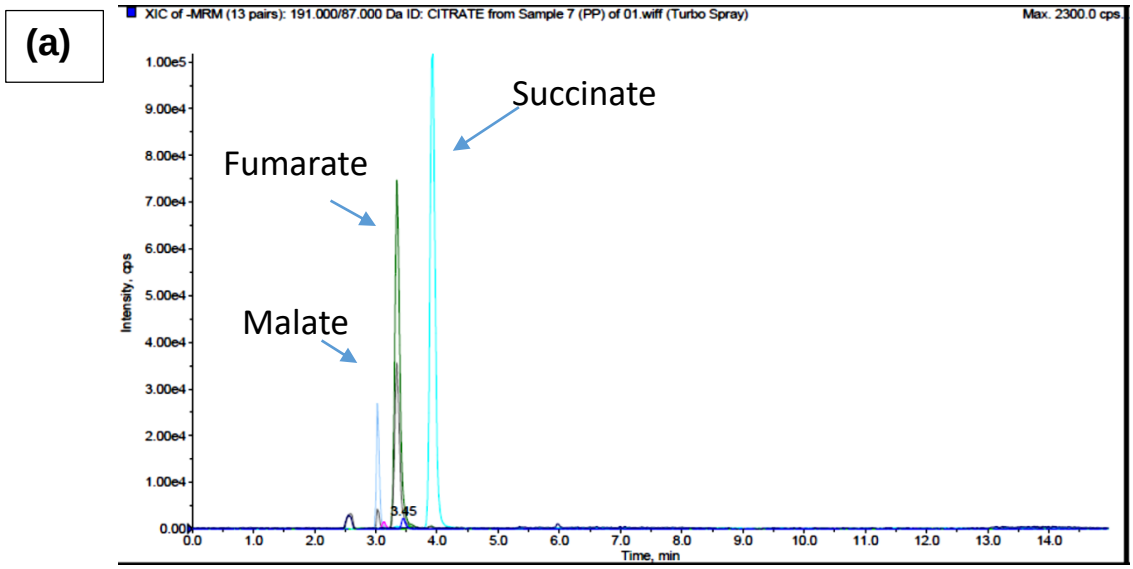
Fig. 2-10 Effect of root exudates of monocrop and intercrop plants on chemotaxis and biofilm formation by PGPR (A) Chemotaxis response towards the root exudates of monocropped and intercropped plants. The capillary movement was calculated by measuring the difference in no. of cells (CFU ml^{-1}) migrated in test samples to the respective organism's control (AMQ water). Part (B) Biofilm formation in the presence of root exudates of monocropped and intercropped plants. The biofilm measurement was done at 595 nm and the difference in the absorbance of the test with respect to their respective organism's control (untreated). Initial bacterial inoculum of 10^8 cells (CFU ml^{-1}) was used. The data were subjected to two way ANOVA followed by Tukey's multiple comparison post hoc test. 'ns' if non-significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $n = 3$.

In the case of biofilm studies (Fig. 2.10 b), C1D exhibited significantly higher biofilm-forming ability in presence of *Z. mays* monocropped root exudates ($p < 0.001$) and *C. cajan* monocropped root exudates ($p < 0.05$). IC3109 strain demonstrated a positive influence on biofilm

formation towards intercropped root exudates ($p < 0.001$). In response to intercropping root exudates, NGR234 strain showed a similar chemoattraction and biofilm formation to monocropped *C. cajan* while a significant ($p < 0.05$) difference was observed in comparison to monocropped *Z. mays*. On the contrary, strain G22 did not show any preference in the biofilm-forming ability in the presence of the root exudates of both conditions.

2.3.5. Analysis of organic acids in the root exudates of monocropped and intercropped plants

Among the 15 organic acids tested, we identified 7 common organic acids (Fig. 2.11; Table 2.2) prominently present in the root exudates of monocropped and intercropped plants. Organic acids like fumarate, malate, and succinate were released with a high fold change of 4.55, 6.44, and 6.08 in monocropped plants (cumulative effect) compared to intercropped plants while citrate was found to be similar in both conditions. Other organic acids like malonate and aconitate were found to be less in monocropped plants and more in intercropped plants, although their concentration was significantly low in both cases as compared to the major organic acids mentioned above.



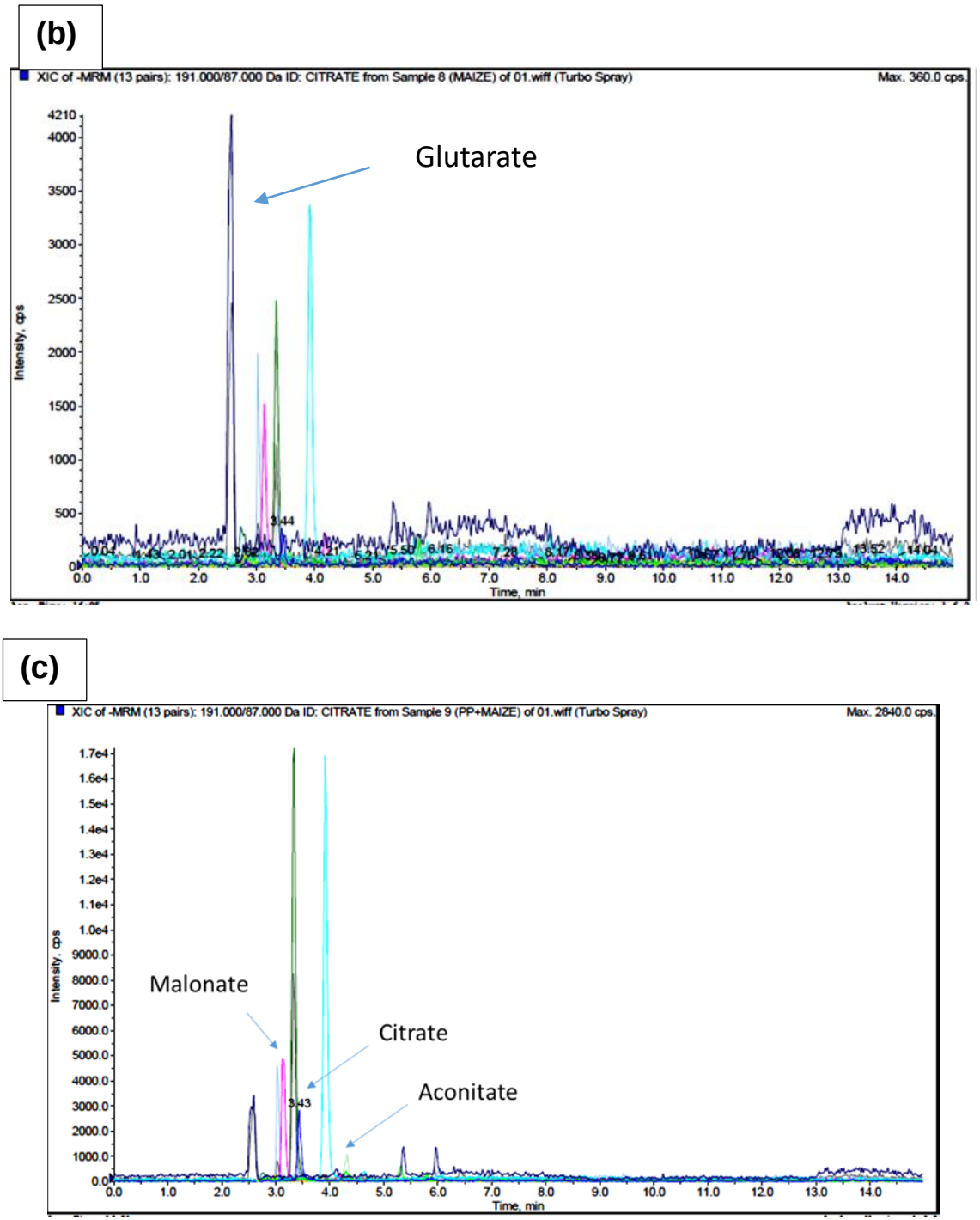


Fig. 2-11 Chromatogram of organic acids detected in the root exudates a) Monocrop *C. cajan*, b) Monocrop *Z. mays* and c) Intercrop (*C. cajan* + *Z. mays*)

Table 2-2 Identification of organic acids from the root exudates of monocropped and intercropped plants by LC/MS/MS (MRM mode)

Low molecular mass organic acids compounds	m/Z of the Q1 parent ion	m/Z of the Q3 fragment ion	Intensity (cps)			Monocrops Cumulative effect (A) (A1+A2)	Fold Change (A/B)
			C. cajan **(A1)**	***Z. mays*** **(A2)**	***C. cajan* + *Z. mays*** **(Intercrop) (B)**		
Fumarate	115	71	75000	2400	17000	77400	4.55
Malate	133	115	27000	1990	4500	28990	6.44
Succinate	117	73	100000	3370	17000	103370	6.08
Malonate	103	59	1590	1500	4870	3090	0.63
Citrate	191	111	2300	360	2800	2900	1.03
Aconitate	173	85	450	80	1090	530	0.49
Glutarate	131	87	2970	4210	3400	7180	2.11

2.3.6. Effect of organic acids on the chemotaxis and biofilm formation of PGPR

Chemotaxis studies (Fig. 2.12) with strain C1D and strain NGR234 revealed that chemo-attraction towards all 4 organic acids was similar, G22 strain migrated towards succinate, fumarate, and citrate with a 3 fold increase in response and IC3109 strain manifested 3 fold increased migration towards fumarate and to succinate with 2 fold increased. Biofilm formation in the presence of organic acids (Fig. 2.13) featured that IC3109 strain had a strong potential to form biofilm formation in presence of fumarate with a 1.2 fold increase while C1D strain, NGR234 strain, and G22 strain demonstrated comparatively less biofilm ability in presence of all four organic acids.

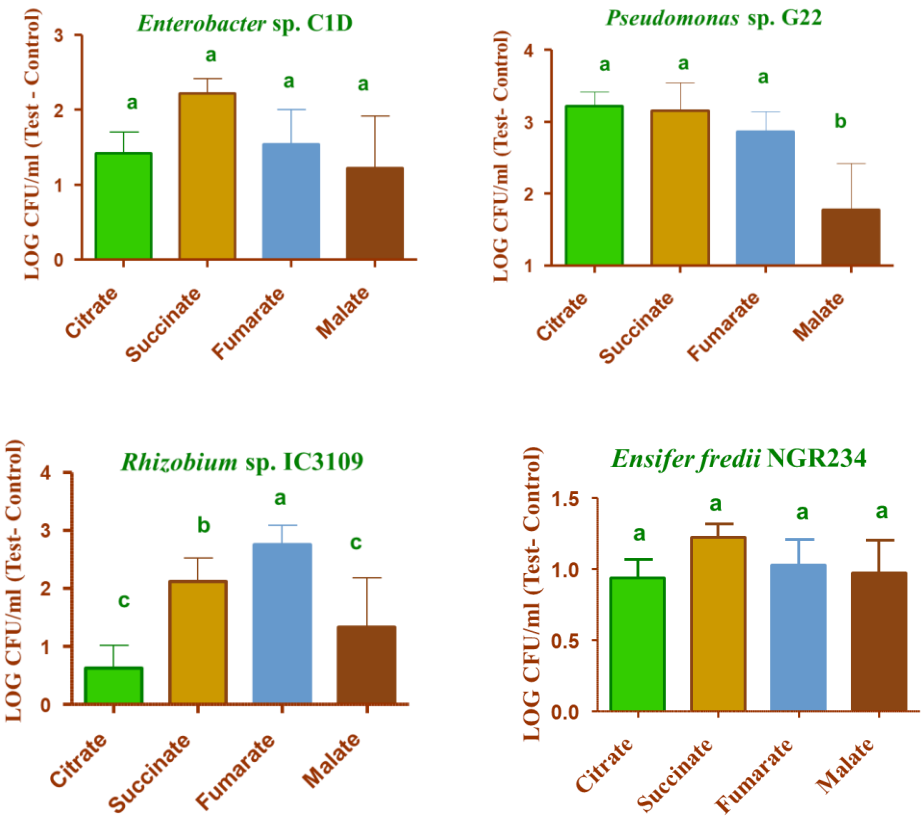


Fig. 2-12 Effect of organic acids on chemotaxis of PGPR Chemotaxis response towards selected organic acids at 50 µM concentration. The capillary movement was calculated as Log CFU ml^{-1} by measuring the difference in no. of cells migrated in test samples to the respective organism's control (sterile distilled water). Initial bacterial inoculum of 10^8 cells (CFU ml^{-1}) was used. Error ars represent standard deviation with three replicates and the data were subjected to one-way ANOVA followed by post hoc Tukey's multiple comparison test, n = 3.

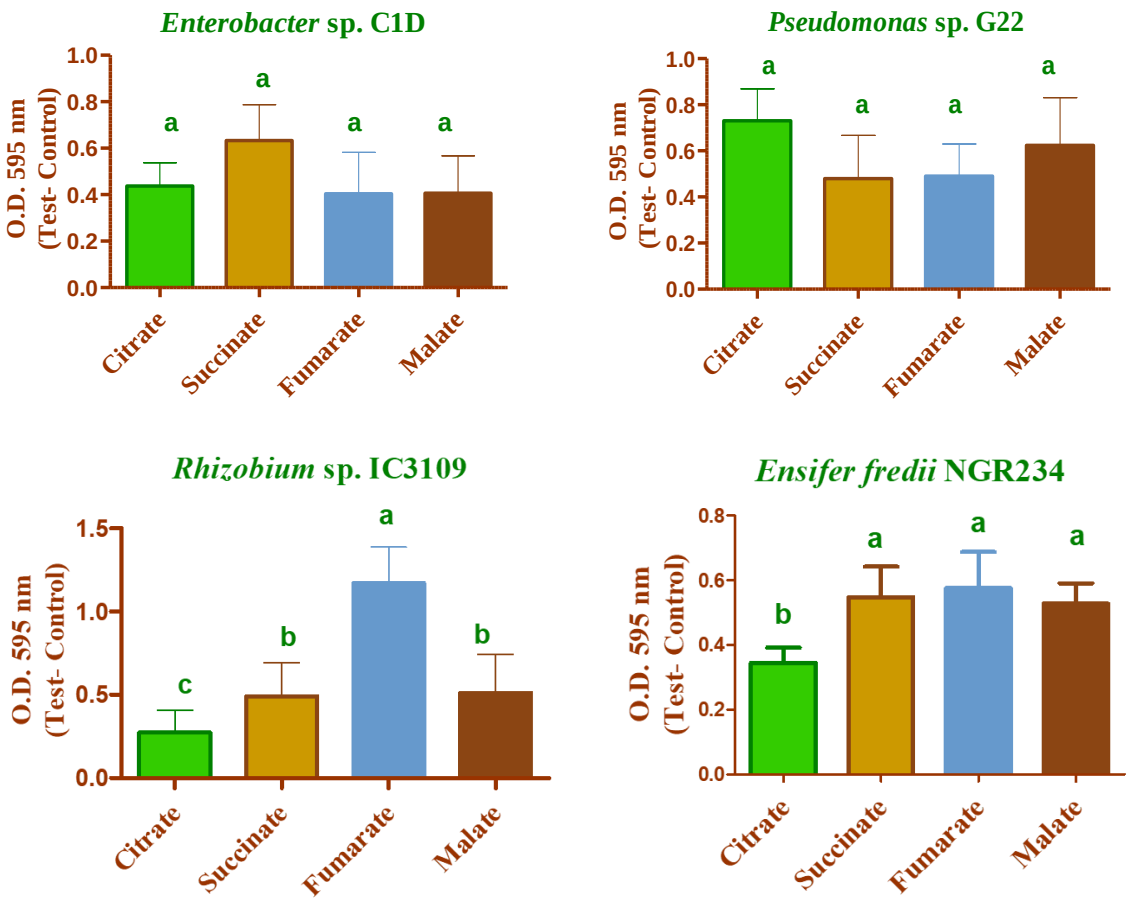


Fig. 2-13 Effect of organic acids on biofilm formation by PGPR: Biofilm formation was studied in the presence of organic acids at 50 µM concentration on 96 well polystyrene microtitre plates. The biofilm measurement was done at 595 nm and the difference in the absorbance of the test with respect to their respective organism's control (untreated) is reported. Initial bacterial inoculum of 10^8 cells (CFU ml^{-1}) was used. Error bars represent standard deviation with three replicates and the data were subjected to one-way ANOVA followed by Tukey's multiple comparison post hoc test, n = 3.

2.4 Discussion

Root exudates act as major ecological drivers of the microbial community by attracting specific microorganisms to colonize in the rhizosphere. In this work, we studied the effect of root exudates from an intercropping system consisting of a legume (*C. cajan*) and cereal (*Z. mays*), on the chemotaxis and root colonization behavior of specific beneficial bacterial strains. We used the sand-hydroponic hybrid method of root exudate sampling (Oburger and Jones, 2018). While this method may introduce certain biases in the sampling due to mechanical damage during uprooting and washing steps, yet this method has been considered to be more close to natural conditions (Oburger and Jones, 2018). Legume-cereal intercropping improves plant growth and the use of PGPR strains boosts the yield of intercropped plants which could be favorable in agricultural practice (Bechtaoui et al., 2019; Konkolewska et al., 2020). Moreover, for beneficial microbes to interact effectively with the plants, the PGPR strains need to move towards the rhizosphere of the plant and colonize subsequently on the surface or inside the root system (Backer et al., 2018). In the present study, we addressed plant-microbe interactions in a controlled intercropping setup with the application of only one microbial species at a time, eliminating the complexities of microbe-microbe interactions seen in natural settings. Our results demonstrate the differential behavioral response of individual PGPR towards root exudates of monocropped and intercropped plants and their degree of colonization in the *C. cajan* - *Z. mays* intercropped plants.

2.4.1. Colonization and plant growth promotion by PGPR on the monocropped plants

Enterobacter sp. C1D has been previously demonstrated to colonize *C. cajan* (Sharma et al., 2019) and mung bean (*Vigna radiata*) plants (Subrahmanyam et al., 2018). In this study, it was found to colonize *Z. mays* roots and promote plant growth possibly due to IAA production and other beneficial traits that it possesses (Table 2.1). Earlier, the endophytic *Enterobacter* strain FP17, possessing growth-promoting traits such as phosphate solubilization, auxin production, and ACC deaminase production, has been also shown to have a positive effect on maize plants (Naveed et al., 2013). *Pseudomonas* sp. G22, a plant beneficial isolate from the groundnut rhizosphere (Patel and Archana, 2018), showed colonization and growth promotion on both the *Z. mays* and *C. cajan* plants. Interestingly, both rhizobia, *Ensifer fredii* NGR234 showed better colonization on both host *C. cajan* and non-host *Z. mays* plants and the legume symbiont from *C. cajan*, *Rhizobium* sp. IC3109 (Rajendran et al., 2007) showed epiphytic colonization of maize. The colonization of NGR234 was also confirmed through CLSM (Fig. 2.8 & 2.9). Further, to know the colonization

pattern and molecular mechanism between legumes and non-legume plants, NGR234 was used as model organism in the following chapters. Also in the case of the broad host rhizobia NGR234, we could observe in the root biomass in both the plants which might be due to the flavonoids which can also induce IAA biosynthesis (Theunis M. et al., 2004). Recently, maize roots have been shown to naturally harbor efficient endophytic cowpea-nodulating rhizobial isolates (Cavalcanti et al., 2020). Similarly, a positive effect of rhizobial isolates from the legume *Desmodium incanum* upon inoculation on grasses such as oat and maize has been shown (Silva et al., 2020). With the rhizospheric isolates C1D, IC3109, NGR234, and G22 strains, our results additionally convey a broad range of colonization of these isolates on different plants.

2.4.2. Cross-colonization of PGPR in intercropped plants

During *C. cajan* - *Z. mays* intercropping there exists close physical proximity of the roots, such type of positive effect has been also observed when a legume *Medicago sativa* plant was introduced to influence the direction of succession in temperate grasslands (Sun et al., 2020). Therefore, these facilitative interactions can result in a mixing of microbial communities of the two plant species when intercropped (Rosenblueth et al., 2004). The present work demonstrated that cross-migration of bacteria from the roots of one plant to the other can occur and within a span of 28 DAS significant bacterial counts were found on the roots of the companion plant even though it was not inoculated. Interestingly, cross colonization ability from *C. cajan* to *Z. mays* and vice versa was demonstrated for all four bacterial strains with a slight difference in their efficiency. Additionally, a mesh barrier was used to discern the importance of physical contact between the roots for the exchange of microorganisms under study. Since the two bacterial strains (IC3109, NGR234 and C1D) showed cross-colonization proficiency irrespective of the barrier, it can be concluded that close physical proximity was not essential for the exchange of bacteria between the plant species, indicating active migration also played a role. On the other hand, the G22 strain exhibited a significant reduction in the case of barrier studies perhaps such type of tight adherence might be due to the specialized ability of *Pseudomonas* spp. to form biofilms in response to mucilaginous materials present at roots (Noirot-Gros et al., 2018). Besides among the rhizobial strains, after 28 DAS NGR234 showed better root colonization on *Z. mays* plants could be due to the release of flavonoids by *Z. mays* (Li et al., 2016).

To the best of our knowledge, similar phenomena have been reported for arbuscular mycorrhizal fungi in the intercropping system (Meng et al., 2015) but not for root-associated bacteria. Considering the possibility that root exudates might play a role in driving the bacterial migration towards the uncolonized roots, we tested the behavioral responses of bacteria towards the root exudates of monocropped and intercropped.

2.4.3. Root exudates of monocropped and intercropped plants differentially induced chemotaxis and biofilm formation of PGPR

Chemotaxis is an important mechanism that recruits motile soil bacteria to the roots of plants, and it is critical for the establishment of bacterial colonization on plant roots (Chagas et al., 2018). With the G22 strain, we couldn't observe any direct correlation for their colonization ability on plants and biofilm formation in the presence of low molecular weight root exudates. This might be because their ability of colonization is dependent more on mucilage (high molecular weight) present in root exudates as compared to the low molecular weight root exudates (Knee et al., 2001; Walker et al., 2003). Further, it was interesting to note that among all four organisms, the IC3109 strain prominently manifested significant (1.7 fold) chemotaxis towards intercropped root exudates consequently biofilm formation and colonization were higher on intercropped plants. This might be due to the higher release of specific flavonoids in intercropped plants released in both legume and cereal plants (Li et al., 2016; Liu et al., 2019). However, in the case of NGR234, the difference between monocropped *C. cajan* and intercropped plant root exudates was negligible, which could be due to the ability of its NodD1 response to a wide variety of flavonoids and related compounds such as vanillin (Le strange et al., 1990). Thus, these results suggest that the root exudates of intercropped plants can induce cross colonization and might facilitate the adaptation of PGPR onto the plant roots.

2.4.4. Identification of targeted organic acids from root exudates of monocropped and intercropped plants and their effects on PGPR

Typically plant roots contain many low molecular weight organic acids which are mainly intermediates of the TCA cycle (Jones, 1998). Out of these acids, particularly malic acid and citric acid have been associated with P mobilization and in the recruitment of bacteria to the plant roots (Ling et al., 2011; Hunter et al., 2014). Other than malate and citrate, which are the most prevalent and abundant organic acids detected in root exudates (Neumann and Romheld, 1999), we also

found fumarate and succinate in the root exudates of both conditions (Table 2.2). *C. cajan* when grown as monocrops has been reported to release malonate as a major component followed by oxalic and piscidic acid (Taylor et al., 1996; Krishnappa and Hussain, 2014), while *Z. mays* grown in isolation releases relatively more organic acids such as malate and citrate under nutrient deficiency (Jones and Darrah, 1995; Carvalhais et al., 2011). In the present study, there was an intense release in the fumarate, malate, and succinate in monocropped plants as compared to that in intercropped plants. Organic acids like malate, succinate, and citrate are known to be an important carbon source for the *Pseudomonas fluorescens* WCS365 strain (Kamilova et al., 2006).

To confirm their importance with the PGPR understudy, *in vitro* chemotactic studies of PGPR towards organic acids revealed that C1D showed a similar response (Fig. 2.12) towards all 4 organic acids, however, these organic acids did not have a strong impact on the biofilm formation (Fig. 2.13). This kind of disparity might be due to less efficiency for the catabolism of organic acids by this organism compared to sugars (Liu et al., 2007). On the other hand, G22 revealed a similar response in a capillary movement towards all four organic acids present in the root exudates, which was correlated to the findings observed by the *Pseudomonas mendocina* strain S5.2 (Chong et al., 2017). Fumarate, succinate, and malate were profoundly detected in root exudates of *C. cajan* which serve as carbon sources for the free-living rhizobia (Iyer et al., 2016). However, the only fumarate exhibited a remarkable increase in biofilm formation of the IC3109 strain. As *C. cajan* releases this compound, probably they are selecting the particular rhizobial IC3109 strain and not the NGR234. Therefore, these results demonstrated that PGPR strains exhibited a differential response towards organic acids which in turn may result in the variation in the colonization of monocropped and intercropped plants.