Chapter 3 : Identification of primary metabolites from the root exudates of *Cajanus cajan – Zea mays* intercropping system and their influence on *Ensifer fredii* NGR234

3.1 Introduction

Legume – cereal intercropping system facilitates complementarity and interspecific interactions through nutrient efficacy of nitrogen. It also increases the utilization of macro and micronutrients from the soil and promotes the intermingling of roots, their respective rhizosphere, and the dispersal area of root exudates (Xue et al., 2016; Glaze-corcoran et al., 2020). In this context, intercropping of *Z. mays* - *C. cajan* is known to significantly promote the formation of soil aggregation and organic phosphorus storage, primarily due to the root-root interactions (Garland et al., 2017).

Root exudates are one of the key factors influencing the rhizosphere bacterial community and often interact with the neighboring plant roots and microbiome (Dennis et al., 2010; Mommer et al., 2016b). They represent a significant source of soil organic carbon. Estimates show that about 50% of the C exported below ground is released into the soil (Kuzyakov and Schneckenberger, 2004). They are usually defined as plant-derived primary and secondary metabolites of both low molecular weight (MW; <1000 Da; e.g. sugars, organic acids, phenolics, etc.,) and high MW compounds (>1000 Da; e.g. enzymes and mucilage) (Oburger and Jones, 2018). An increase in the secretion of secondary metabolites like flavonoids genistein, hesperetin, and naringenin has been reported in faba beans when intercropped with wheat (Liu et al., 2019b). Non-leguminous plants like *Z. mays* have been shown to release from their roots flavonoids that influence the root nodulation and nitrogen fixation of legumes in the intercropping system (Li et al., 2016). Most recent studies highlight that secondary metabolites in root exudates influence the microbiome and also play a crucial role in enhancing arbuscular mycorrhizal fungal associations (Tian et al., 2021; Jacoby et al., 2021). Besides, the exudation of primary metabolites has strong effects on soil organic matter decomposition by soil microbes. The exudation patterns are linked to plant nutrient strategies, which can determine an ecosystem's performance through plant-soil feedback mechanisms (Canarini et al., 2019). Thus, root exudates facilitate interspecific and root-rhizobiome interaction in the soil. The role of secondary metabolites in a legume- cereal intercropping has been extensively examined, while the role of primary metabolites of the intercropping system in plant-bacteria interactions remains unclear.

Beneath the rhizosphere, the facilitative interactions between microbes and plants, and between legumes and cereals modulate the outcome of the intercropping system (Faget et al.,

2013). When legume and cereal are cultivated together in intercropping, intermingled roots facilitate the "sharing" of soil microbes, including symbiotic bacteria (Rosenblueth, 2004), and also increase the abundance of alpha proteobacteria, particularly nitrogen-fixing rhizobia (Chen et al., 2017; Solanki et al., 2020). Therefore, rhizobia are one of the predominant group of bacteria which can cross colonize between the plants and fixes nitrogen.

Metabolomics is becoming accepted as a tool for the (untargeted) analysis of primary or secondary metabolites in root exudates. With the recent advances in metabolomics, the untargeted approach allows us to detect and, to some extent, identifies a large number of the metabolites that are secreted by plants and the organisms interacting with them in the rhizosphere (Kuijken et al., 2015). Gas chromatography-mass spectrometry (GC–MS) and/or liquid chromatography-mass spectrometry (LC-MS) are widely applied for comprehensive and specified metabolomics studies in many different scientific areas (Lei et al., 2011). GC-MS is the platform of choice when studying rhizosphere interactions mediated by volatiles (boiling point 20–350° C) and primary metabolites after the derivatization step used to make them volatile (van Dam et al., 2016).

To gain insight into the eco-physiology of rhizobium under the influence of root exudates of *C. cajan- Z. mays* intercropping system, *Ensifer (Sinorhizobium) fredii* NGR234 (here called NGR234) was used as a model organism in this study. NGR234 has a broad host range nodulation potential with over 120 genera of legumes, as well as the non-legume *Parasponia andersonii* (Pueppke and Broughton, 1999). This chapter deals with the identification of primary metabolites in the root exudates of monocropped and intercropped *C. cajan* and *Z. mays* plants by the non-targeted metabolomics approach was undertaken. Further, the effect of these root exudates on the growth and biofilm formation of NGR234 have been reported.

3.2 Materials & Methods

3.2.1. Plant cultivation for root exudates collection

Plants were cultivated similarly as mentioned in Chapter 2- section 2.2.4. Multiple such sets of plants were grown to allow the collection of root exudates from a large number of plants. In total, 80 plants of *C. cajan* and 60 plants of *Z. mays* plants were grown in monocropping and intercropping conditions.

3.2.2. Preparation of root exudates from monocropped and intercropped plants

Plants were harvested by uprooting gently on the 28th day. The initial steps of washing and sterilizing the roots for root exudates collection were similar to a protocol mentioned in Chapter 2- section 2.2.5. Briefly, roots were rinsed with freshly autoclaved Milli Q (MQ) water in a laminar airflow hood, followed by chloramphenicol treatment (30 μg ml^{-1}) for 3 min to surface sterilize the roots and again washed with sterile MQ water. Thereafter, root exudates were collected by dipping roots of two plants into a Borosil tube (length-20 cm and diameter-35 mm) containing sterile MQ water to allow the entire root system to be immersed (30 ml for *C. cajan* and 40 ml for *Z. mays*) and incubated for 6h (Carvalhais et al., 2011) under shaking conditions at 30°C. The two plant species grown as intercrop were harvested and root exudates were collected separately from them. The root exudates of *C. cajan* grown along with *Z mays* were denoted as intercrop *C.cajan.* Similarly, root exudates of intercropped *Z. mays* (grown along with *C. cajan*) were designated as intercrop *Z. mays.* Plants grown separately in several individual pots were used for the collection of a pooled root exudate sample for each set of plants. The root exudates solutions obtained from 80 *C. cajan* and 60 *Z. mays* plants, were pooled to obtain 1.2 L of exudate solutions for four conditions of plant growth (*viz*. monocrop *C. cajan*, monocrop *Z. mays*, intercrop *C. cajan* and intercrop *Z.mays*). Samples were filtered (0.2 μm pore size), lyophilized to dryness using Christ Lyophilizer (Osterode, Germany), and recorded the dry weight. After root exudates were collected, the roots were separated from the shoots and dried using paper towels, and fresh weight was monitored (Appendix – Fig. A1).

The lyophilized root exudate samples were analyzed by GC-MS as described by Ankati & Podile, (2019). Briefly, 10 mg of the lyophilized crude root exudate was dissolved in 1 ml of 80% methanol along with 50 μL of 0.2 mg/ mL an internal standard arabitol (Sigma Aldrich, Germany) and incubated for 2 h on ice, centrifuged at 8,200 × *g* for 5 min at 4 °C. The pellet was discarded

and the supernatant containing the root exudate was again dried using a Savant DNA120 Speed Vac. Concentrator (Thermo Scientific, U.S.), lyophilized and stored at -20 °C till further use. Three technical replicates of each sample (10 mg/ml each) were used to perform GC-MS/MS analysis.

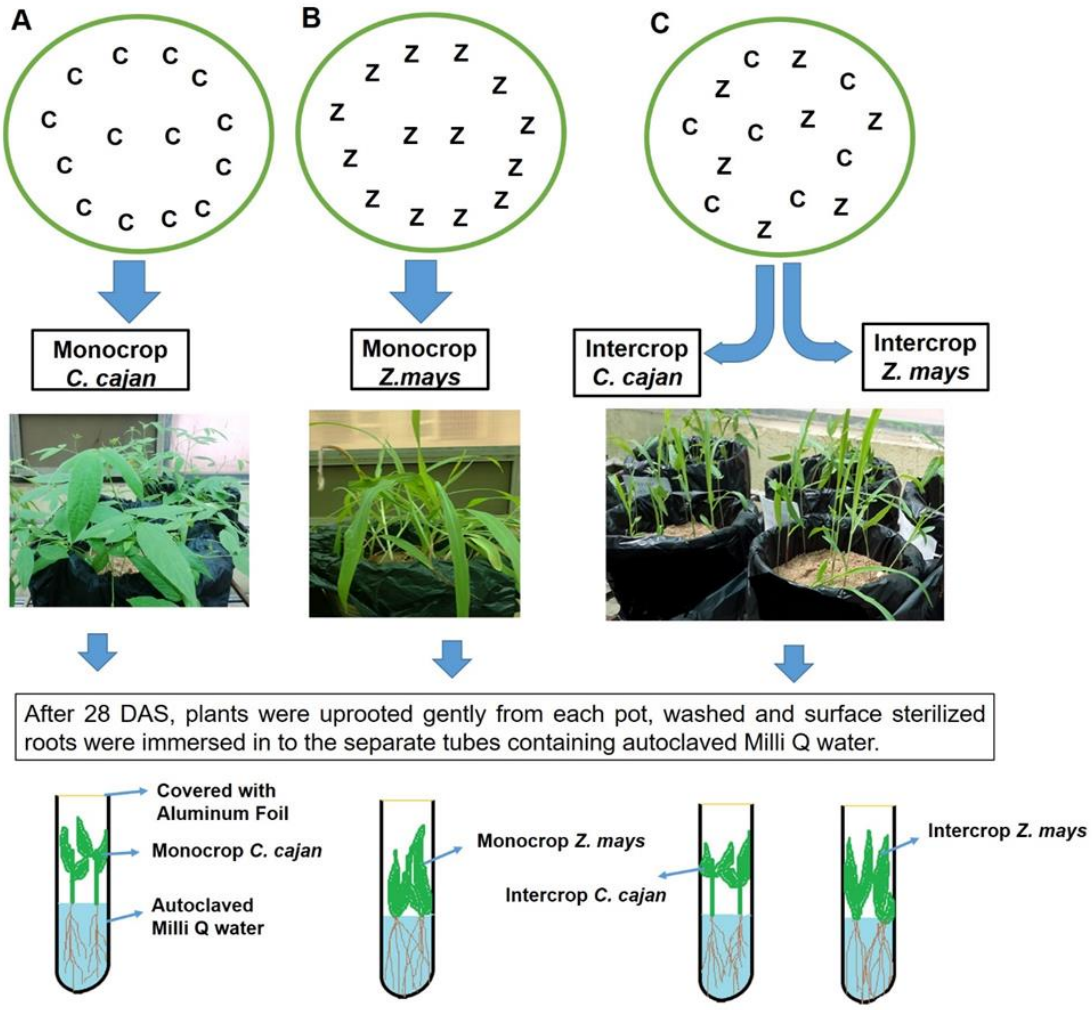


Fig. 3-1 Schematic overview of the experimental setup of monocropped and intercropped plants grown for collections of root exudates at 28 days after sowing (DAS) There were three types of pots and each pot consisted of 14 plants of either monocropped plants (A) & (B) (grown individually) or 14 intercropped plants (C) [7 C. cajan (C) + 7 Z. mays (Z)] plants placed at a distance of 7 cm.

3.2.3. Untargeted metabolite profiling of root exudates by GC-MS/MS

To analyze the root exudates,1 mg of processed sample was initially derivatized with 20 µL methoxyamine hydrochloride (20 mg ml^{-1} pyridine) for 90 min at 30 °C, followed by 20 µL N-methyl- N-(trimethylsilyl)-trifluoroacetamide for 30 min at 70 °C. Further, root exudates profiling was carried out using GC-MS/MS in three technical replicates with Agilent 7890 series gas chromatograph, USA. The following parameters were used for analyzing the root exudates samples. Electron Ionization (EI)-voltage of 70 eV with source temperature 230 °C; DB-1HT /IntegraGuard (Retek GmbH, Bad Homburg, Germany) column with 29.3 m x 0.25 mm dimensions having 0.1 µm film thickness to column (Agilent, Folsom, USA). Helium was used as the carrier gas at 1.5 mL/min constant flow with temp. Program of 70 °C (5 min), 70–300 °C, 300 °C (4 min) injection temperature: 240 °C, splitless manual injection, a mass range of m/z 35 to 1000. Further, data acquisition and evaluation were carried out using LECO-GCMS software. ChromaTOF software 4.44.0.0 chromatography version (LECO Corporation, USA) was used for processing the raw SMP files generated with a signal-to-noise ratio ≥ of 1. The compound hits were identified using NIST MS search v 2.0 software in the National Institute of Standard and Technology (NIST) library using the mass spectra extracted. The compound hits with > 700 similarities ± 30 RI (retention index) value deviation were considered to assign metabolite identity. The annotated metabolites were considered for analysis and unknowns were eliminated. Metabolites with different trimethylsilyl derivatives were combined as a single entity. The concentration of individual metabolites detected in the root exudates was calculated with the help of arabitol as an internal standard.

3.2.4. GC-MS/MS analysis

To identify the variations between *C. cajan* and *Z. mays* root exudates metabolites, in both intercropping and monocropping systems, a multivariate analysis was performed using the MetaboAnalyst 4.0 web tool (Xia and Wishart, 2016). An unsupervised principal component analysis (PCA), cluster analysis, and a supervised partial least square discriminant analysis (PLS-DA) were performed to look for the similarities and variations in the root exudates of intercrop and monocrop samples. Cross-validation of the PLS-DA method was done with the help of LOOCV and performance (Q_2) parameters. Key metabolites present in triplicates significantly (at $p<0.05$) in each sample were selected based on one paired t-test performed in the MetaboAnalyst 4.0, whereas, the concentration difference in key metabolites between monocrop and intercrop of

each plant was noted by an unpaired t-test between them. Venn diagram was prepared to represent the total number of metabolites detected in each system with respect to others by using the online software of *InteractiVenn* (Heberle et al., 2015). While hierarchical cluster analysis was performed by setting Euclidean distance as a similarity measure and Ward's linkage as the clustering algorithm.

3.2.5 Effect of root exudates and individual metabolites on the physiological response of NGR234

To understand the effects of key root exudate metabolites on NGR234, metabolites that significantly varied between intercrop and monocrop plants' root exudates, as detected through GC-MS/MS analysis, were used in their pure form. Among detected key metabolites, sugars (myo-inositol, galactose, mannose, and glycerol) and amino acids (proline and arginine) at 10 mM arbitrary concentration (based on literature) were considered to study the effect on chemotaxis, growth, and biofilm formation of NGR234. Solutions of root exudates in MQ water of individual monocrops (*C. cajan* and *Z. mays*), as well as their intercrops, were filter- sterilized using 0.2µm filters and used at a final concentration of 1 mg ml^{-1}.

3.2.5.1 Chemotaxis

The protocol for chemotaxis assay was similar to that mentioned in Chapter 2- section 2.2.6.

3.2.5.2 Growth pattern and Biofilm

NGR234 was grown at 30°C until it reached OD_{600} of 1.0 in the Rhizobium minimal medium (RMM) (Broughton *et al*. 1986) supplemented with 6.5 mM glutamate (Robertsen et al., 1981). Overnight grown culture of NGR234 in RMM broth was inoculated into fresh sterile RMM broth (absence of 55 mM mannitol and in the presence of 6.5mM glutamate as sole carbon source) to make up 0.1 OD_{600} and the 125 µl of inoculated broth was loaded into two separate 96 wells of polystyrene microtiter plates. One plate, which was used for recording growth, was incubated in the chamber of the microtiter plate reader (Synergy HT; BioTek, USA) with continuous shaking at 30°C throughout the study period. At every 2 h interval, absorbance was measured at 600nm on a microtiter plate reader for measuring cell density. The second microtiter plate was used to study the biofilm formation and was incubated under static conditions at 30°C for 72 h. Biofilm formation was quantified using a modified protocol of Lee et al., (2012). First, the OD_{600} of the static bacterial growth was recorded and then, the medium containing the planktonic cells was carefully removed aseptically by a multi-channel pipette. Each well was washed three times with

200 µl of sterile MQ water and the plate was air-dried aseptically for 15 min. Every well was stained with 150 µl of 0.1% (w/v) crystal violet (CV) for 45 min in the dark. The CV was removed by pipette, and each well was washed three times with 200 µl of sterile MQ water. To quantify the amount of biofilm, the CV was extracted with 200 µl of 100% methanol. The absorbance of 100 µl of the methanol solution was measured at 595 nm after transferring into a fresh microtiter plate. The experiment was carried out with 3 technical replicates for both growth and biofilm formation. The relative biofilm formed by NGR234 was calculated by taking the ratio of $OD_{595/600}$.

3.3. Results

3.3.1. Metabolite profiles of monocropped and intercropped plants root exudates

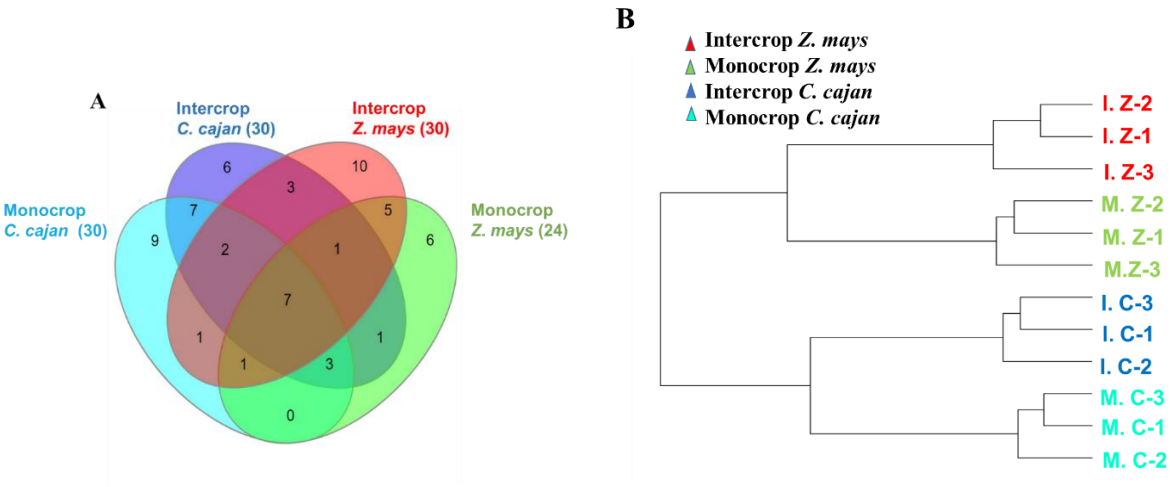
To understand compositional differences in a more comprehensive way, root exudates of both monocropped and intercropped *C. cajan* and *Z. mays* were analyzed through GC-MS/MS. Plants of *C. cajan* and *Z. mays* grown individually in the separate pot were considered as monocrop *C. cajan* and monocrop *Z. mays* respectively and together in the same pot were considered as intercrop *C. cajan* and intercrop *Z. mays*. After the analysis, we observed a clear and distinct difference between monocrop and intercrop root exudates metabolome for each plant. A total of 62 metabolites were identified putatively across four different samples present in at least 2 technical replicates as highlighted in the Venn diagram (Fig. 3.2A). There were 7 common metabolites in all the samples, while among unique metabolites intercrop *Z. mays* showed a maximum number of 10 metabolites, followed by 9 of monocrop *C. cajan*, 6 of intercrop *C. cajan*, and 6 monocrop *Z. mays.*

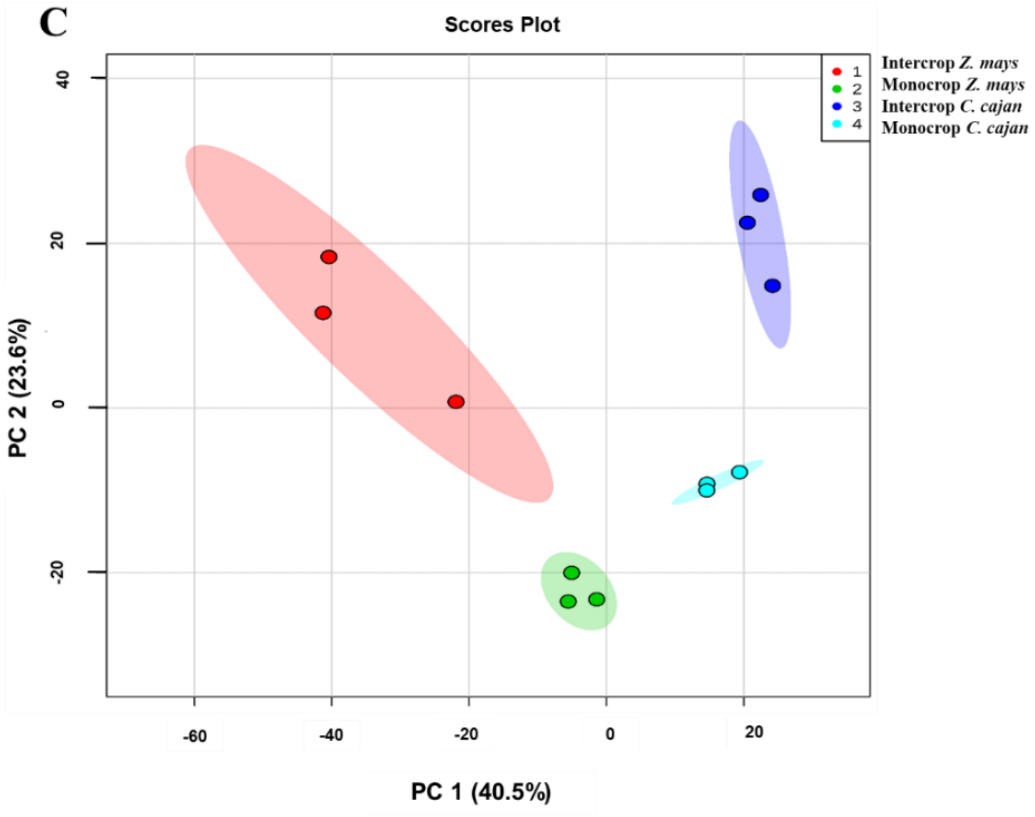
Cluster analysis with Euclidean correlation (Fig.3.2B) showed clear segregation between intercrop and monocrop root exudates of *C. cajan* and *Z. mays* plants. Further, multivariate data analysis with unsupervised PCA (Fig.3.2C) and supervised PLS-DA (Fig. 3.2D) showed a clear variation in the root exudates profiles. Here, each point served as a sample, and similar biochemical composition in root exudates samples grouped to be nearer, while dissimilar samples were grouped apart from each other. The unsupervised PCA plot depicts variation among root exudates profiles of monocrops and intercrops plants, where the first (PC1) and second (PC2) principal components of the plot showed a clear separation of intercrop *C. cajan* and intercrop *Z. mays* when compared to monocrop *C. cajan* and monocrop *Z. mays*, respectively. The PC1 of PCA showed a 40.5% crop-specific variation in root exudates profiles. Whereas, PC2 deciphered the intercropping

specific variations in root exudates with respect to crops by 23.6%. This variation in root exudates profiles due to intercropping was further prominently seen in supervised PLS-DA multivariate analysis. A correlation index R^2 of 0.989 and predictability variation Q^2 of 0.906 indicated higher segregation or variation in intercrop and monocrop root exudates profiles of *C. cajan* in PLS-DA over *Z. mays* (Fig. 3.2D). Therefore, the comprehensive analysis suggested that the root exudates of monocrops are different from intercrops for both plants.

Further, the identified 62 metabolites were categorized into different groups like amines, amino acids, fatty acids, hydrocarbons, sugars, sugar alcohols, organic acids, and others based on their chemical nature. Metabolites in each group are listed in (Appendix - Table A1) along with their relative abundance across the four samples. Among the 62 metabolites, 18 metabolites were significantly ($p < 0.05$) different in comparison to monocrop and intercrop root exudates of each plant (Table 3.1). There were 11 metabolites significantly present in the triplicates of both monocrops and intercrops root exudates. Some unique metabolites like eicosanoic acid and glycerol were present exclusively and significantly ($p <0.05$) in intercrop plants of *C. cajan* and *Z. mays* respectively. Besides, there were 7 common metabolites as shown in Fig. 3.2A, among them, myo-inositol was present at significantly higher concentration ($p <0.01$) in monocrop *Z. mays* and intercrop *C. cajan* compared to intercrop *Z. mays* and monocrop *C. cajan* plants root exudates respectively. When monocrop and intercrop plants of the same species were compared, it was interesting to observe that intercrop *C. cajan* showed release of long-chain organic acids- LCOA's or fatty acids such as heptadecanoic acid, hexadecanoic acid, octadecanoic acid, and oleic acid with the significance of ($p <0.01$) as well as 1, 2-benzene dicarboxylic acid, myo-inositol, mannose, turanose, and L-proline were significantly ($p <0.01$) more compared to monocrop *C. cajan*. In the case of intercrop *Z. mays* root exudates, metabolites like D-galactose, D-glucopyranoside, and L-arginine were increased. However, hexadecanoic acid, ribonic acid, and D-mannose showed no significant difference in the root exudates of *Z. mays*.

Metabolites like D-galactose ($p <0.05$), D-glucopyranoside ($p <0.05$), and L-arginine ($p <0.01$) increased while hexadecanoic acid, ribonic acid, D-mannose showed no significant difference in the root exudates of *Z. mays*. Although no significant metabolite was observed in monocrop *C. cajan*, monocrop *Z. mays* showed a significantly high content of D-turanose compared to other samples.


A
Intercrop
C. cajan (30)
Intercrop
Z. mays (30)
Monocrop
C. cajan (30)
Monocrop
Z. mays (24)
6
3
10
7
5
9
2
1
6
7
1
1
1
3
0
B
Intercrop Z. mays
Monocrop Z. mays
Intercrop C. cajan
Monocrop C. cajan
I. Z-2
I. Z-1
I. Z-3
M. Z-2
M. Z-1
M.Z-3
I. C-3
I. C-1
I. C-2
M. C-3
M. C-1
M. C-2


C
Scores Plot
1
2
3
4
Intercrop Z. mays
Monocrop Z. mays
Intercrop C. cajan
Monocrop C. cajan
40
20
0
-20
PC 2 (23.6%)
-60
-40
-20
0
20
PC 1 (40.5%)

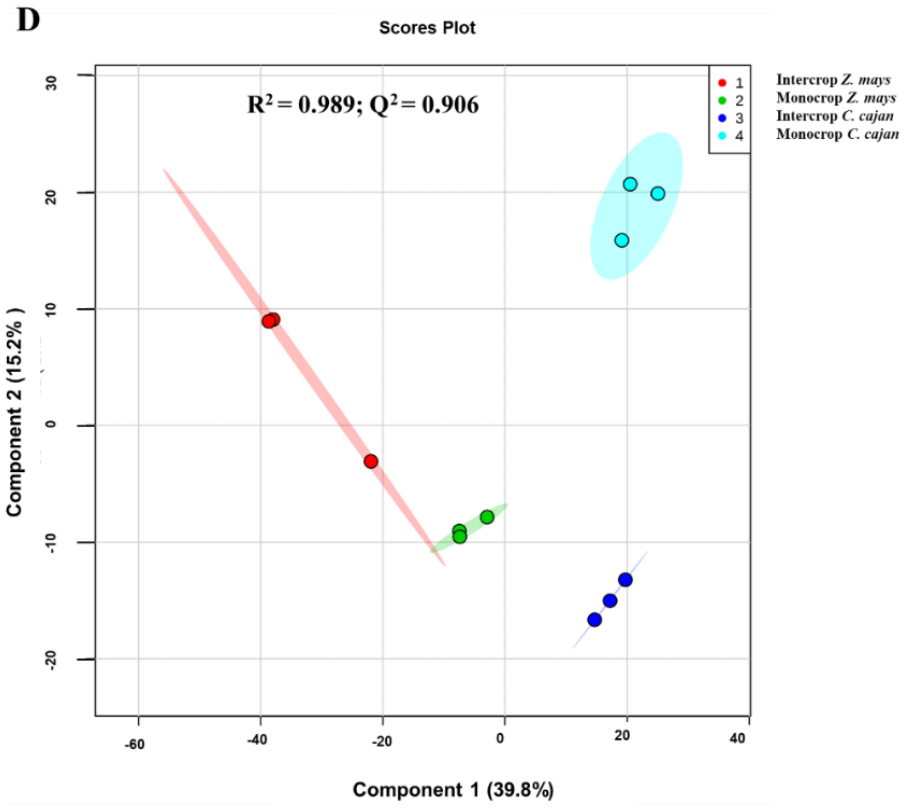


Fig. 3-2 Global differences in the metabolite profiles of *Z. mays* and *C. cajan* root exudates grown as monocropped or intercropped determined by GC-MS analysis A) Venn Diagram representing the total number of metabolites present in each group; B) Dendrogram of cluster analysis (Euclidean correlation); C) Unsupervised Principal Component Analysis (PCA) with percent of variation for each component represented as PC1, PC2; D) Supervised partial least squares discriminant analysis (PLS-DA). R^2 and Q^2 values indicate the correlation index and predictability variation respectively.

Table 3-1 List of key metabolites identified in *Cajanus cajan* and *Zea mays* root exudates of intercropped and monocropped plants by GC-MS/MS

Name of the metabolite	*Cajanus cajan*		*Zea mays*	
	Monocrop	**Intercrop**	**Monocrop**	**Intercrop**
(E)-1-Propene-1,2,3-tricarboxylic acid	ND	ND	0.55±0.07	ND
1,2-Benzenedicarboxylic acid	0.31± 0.2 #	1.12± 0.06** #	0.14±0.08	ND
Benzene acetic acid	ND	ND	0.26±0.06	ND
D-(+)-Turanose	0.08±0.03 #	2.19±0.63**#	22.64±5.64	ND
D-Galactose	ND	ND	0.16±0.09#	0.41±0.09 *#
D-glucopyranoside	ND	ND	0.16±0.07#	2.18±0.005*#
D-Mannose	ND	2.6± 0.72	1.26± 1.18#	0.91±0.5 ns #
Eicosanoic acid	ND	2.05±0.62** $	ND	ND
Glycerol	ND	ND	ND	2.05±0.58** $
Heptadecanoic acid	0.21±0.2#	5.51±1.78 **#	ND	0.69± 0.41
Hexadecanoic acid	1.21±0.37#	27.03±12.07**#	3.89±1.44#	5±1.41 ns#
Indole-7-carboxaldehyde	ND	0.2±0.03	0.06±0.014	ND
L-Arginine	ND	ND	0.065± 0.004#	0.58±0.05**#
L-Proline	1.14±0.95#	8.43±3.82**#	ND	ND
Myo-Inositol	0.41±0.11#	2.19±0.65**#	5.12±1.52 **#	0.67±0.2#
Octadecanoic acid	2.83±1.46#	70.2±36.5**#	6.03±0.99	ND

Oleic acid	$0.93\pm0.18^{\#}$	$6.85\pm2.44^{**\#}$	ND	ND
Ribonic acid	0.62 ± 0.31	ND	$0.55\pm0.2^{\#}$	$0.82\pm0.29^{ns\#}$

Metabolites with a significant difference between monocrop and intercrop (**p<0.01,*p<0.05, and ns-no significant change) or present only in a particular sample were calculated statistically using two paired and one paired t-test respectively. Symbol # indicates two paired t-test used to know the difference between monocrop and intercrop of each plant while symbol $ indicates one paired t-test used to know the significance of metabolite in that particular sample. Metabolites highlighted in bold were taken as pure standards to perform physiological assays**.** Three technical replicates were used for each sample. The relative abundance of the metabolites is expressed as µg/ml. ND means not detected in a particular sample.

3.3.2. Chemotaxis behavior of NGR234 towards root exudates of monocropped and intercropped *C. cajan* and *Z. mays* plants

NGR234 showed a significant ($p<0.001$) increase in the migration towards the root exudates of both monocrops and intercrops plants (Fig. 3.3). Metabolites like myo-inositol and proline also significantly contributed to chemotaxis ($p < 0.001$) and a significant difference towards glycerol was observed ($p< 0.01$) but not towards galactose and mannose.

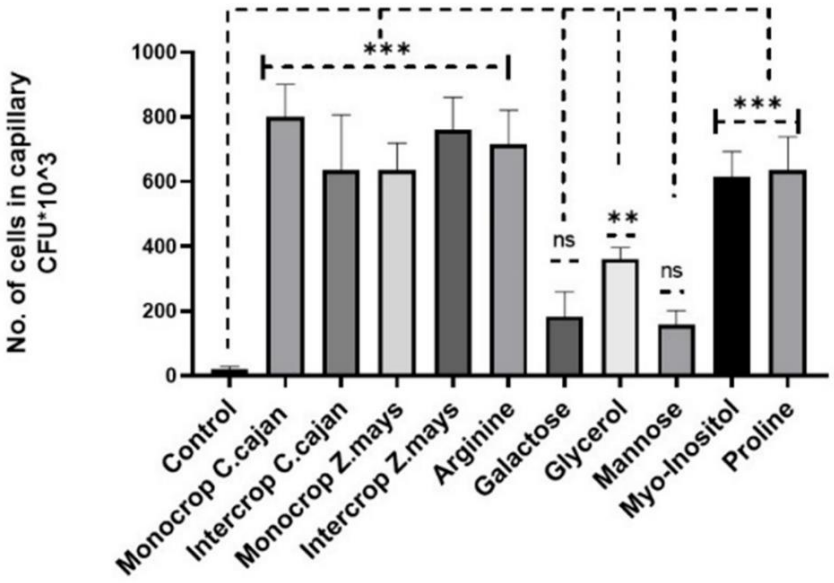


Fig. 3-3 Chemotaxis capillary assay of NGR234 with root exudates of monocropped and intercropped *C. cajan* and *Z. mays* plants and pure metabolites identified metabolites by GC-MS Error bars indicate standard deviation based on three independent values. The data were subjected to one-way ANOVA followed by Bonferroni's multiple comparison post hoc test. 'ns' if non-significant, * $p <0.05$,** $p <0.01$, ***$p <0.001$.

3.3.3. Growth and biofilm formation by NGR234 in presence of root exudates of monocropped and intercropped *C. cajan* and *Z. mays* plants

To determine the growth rate in the presence of root exudates of monocrops, intercrops, and selected metabolites, NGR234 was grown in a minimal medium without mannitol which supports the normal growth and supplemented with 1mg ml^{-1} of root exudates. NGR234 showed a longer log phase and significantly declined growth in presence of monocrop *C. cajan* root exudates ($p <0.01$) when compared to control (Fig. 3.4A), while intercrop and monocrop *C. cajan* showed differential growth patterns at 38 h (Fig. 3.5 A). On the other hand, a significant increase in the

growth was observed at 20 h post-inoculation in the presence of the intercrop ($p<0.001$) and monocrop ($p<0.01$) *Z. mays* root exudates as compared to the set without root exudates (Fig. 3.4B; 3.5 B). Further, to study whether the unique metabolites, D-galactose, D-mannose, L-proline, L-arginine, myo-inositol, and glycerol contribute to the growth, they were used at 10 mM (arbitrary concentration based on literature) (Fig. 3.4 C &D). As observed, there was a significant increase in growth ($p<0.001$) (Fig. 3.5 A) with D- mannose. Glycerol, present only in intercrop *Z. mays*, contributed to significant ($p < 0.001$) growth of NGR234 at 20 h; myo-inositol ($p<0.001$) and galactose ($p<0.001$) also accelerated growth significantly, while in the presence of arginine there was a significant ($p<0.01$) decrease in growth at 20h (Fig. 3.5 B).

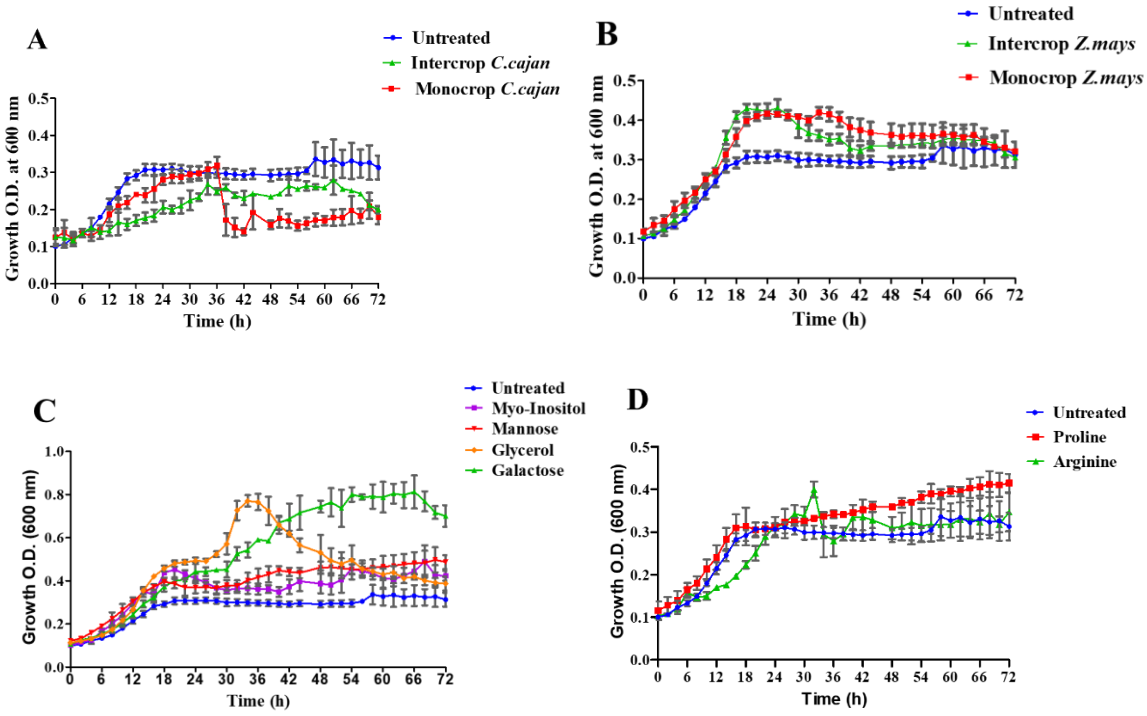


Fig. 3-4 Growth of NGR234 in RMM minimal medium in the presence of root exudates of *C. cajan* and *Z. mays* grown as monocrops or intercrops In the above figure, A) denotes growth on *C. cajan* root exudates, B) *Z. mays* root exudates, C) growth on pure sugar metabolites D) growth on pure amino acids. The growth was monitored at the optical density at $_{600nm}$. Error bars indicate the mean with a standard deviation of three technical replicates.

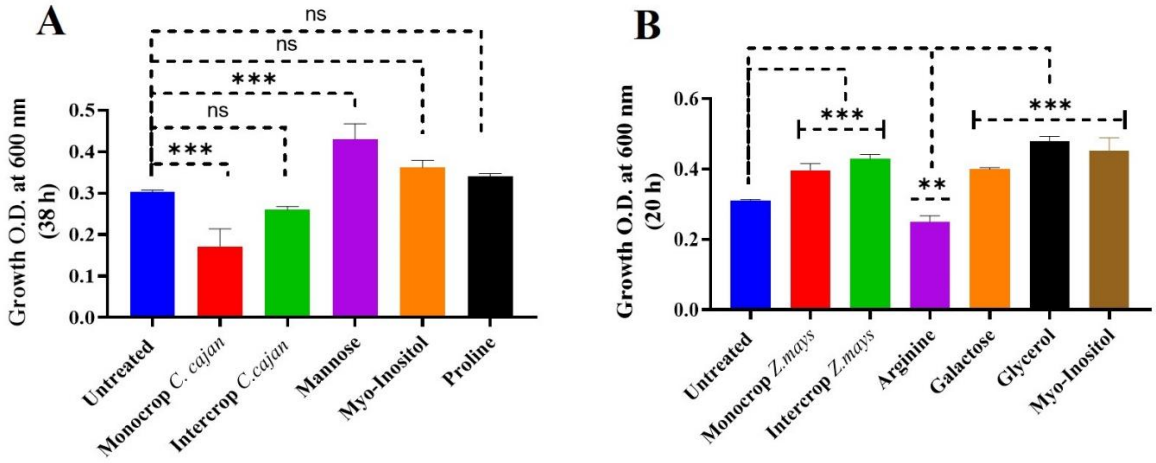


Fig. 3-5 Growth of NGR234 in minimal medium in the presence of *Z.mays* and *C.cajan* root exudates grown as monocrops or intercrops. A) growth of cells at 38 h in the presence of metabolites detected from *C. cajan* root exudates, B) growth of cells at 20 h in the presence of the *Z. mays* root exudates and metabolites. The growth was monitored at the optical density of 600nm under shaking conditions. Error bars indicate the mean with a standard deviation of three independent replicates. The data were subjected to one way ANOVA followed by Bonferroni's multiple comparisons post-hoc test. 'ns' if non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n=3.

Biofilm formation of NGR234 was assessed in the presence of the root exudates of monocrop, intercrop, and selected metabolites on a polystyrene 96 well microtiter well plate (Fig.3.6). Results revealed that NGR234, upon treatment of intercrop *C. cajan* RE showed a highly significant ($p<0.001$) biofilm formation compared to monocrop at 72 h. The *Z. mays* monocrop root exudates showed a significant increase ($p<0.01$) compared to intercrop root exudates in inducing biofilm formation of NGR234. It was interesting to note that biofilm formation in the presence of monocrop *Z. mays* and intercrop *C. cajan* was similar to control. In the case of pure metabolites, a significant increase in biofilm formation was observed with arginine ($p<0.001$), myo-inositol ($p<0.001$), and proline ($p<0.01$), whereas other metabolites like galactose, mannose, genistein, and glycerol showed no significant difference when compared to control.

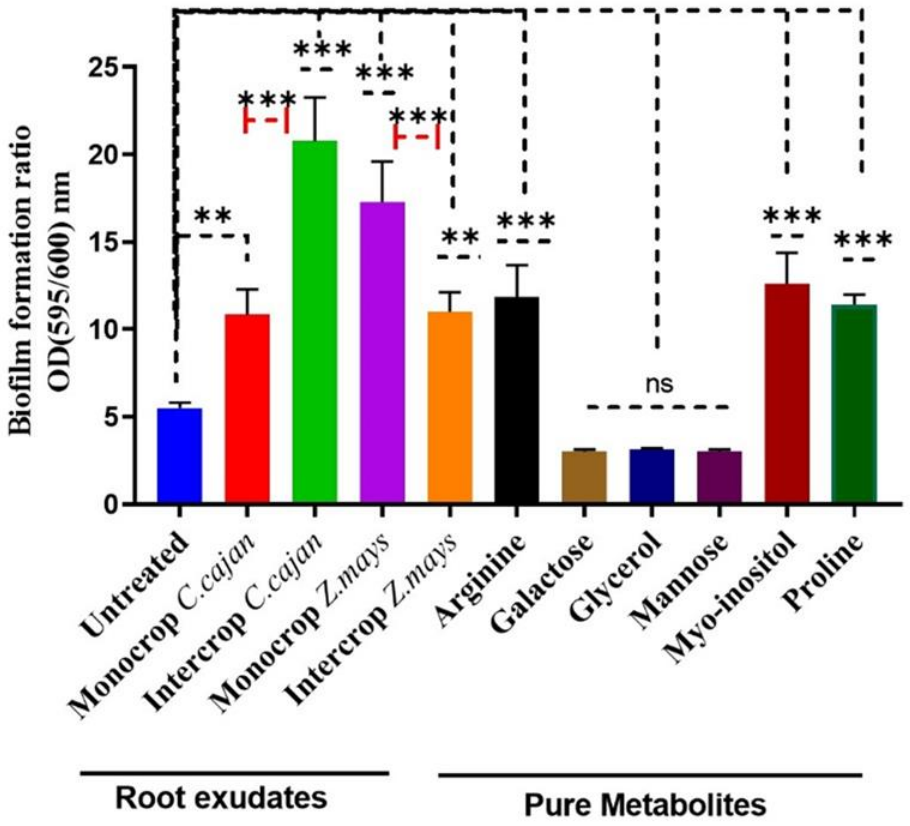


Fig. 3-6 Biofilm formation of NGR234 in the presence of root exudates of monocropped and intercropped *C. cajan* and *Z. mays* plants and the selected metabolites. Biofilm levels expressed as the ratio of OD $_{595nm}$ for crystal violet and overall growth measured as OD $_{600nm}$ compared to untreated conditions Error bars indicate standard deviation based on three independent values. The data were subjected to one-way ANOVA followed by Bonferroni's multiple comparison post hoc test. 'ns' if non-significant, * $p < 0.05$,** $p < 0.01$, ***$p < 0.001$.

3.4. Discussion

The rhizosphere is a highly complex and dynamic system, wherein root exudates facilitate and modulate both plant-plant (Li et al., 2016) as well as plant-microbe interactions (Pérez-Jaramillo et al., 2016). Attachment, infection, and nodulation by NGR234 (a broad host range rhizobium) is well studied in legume plants and is well characterized mechanistically (Kobayashi et al., 2004; Staehelin et al., 2006). Moreover, the type 3 effectors of NGR234 that play a critical role in the establishment of the host range (Khaosaad et al. 2010), help in the interactions not only in legumes but also in cereals (non-host plants). However, the detailed studies involving the attachment and colonization of rhizobia on cereal plants are not yet clear. Omics platforms were used to gain

insights into the complex metabolic and regulatory network in plant-microbe interactions (Ankati et al., 2019; Ramakrishna et al., 2019). The present work represents a comprehensive study to identify variations in the key primary metabolites in the root exudates of the legume (*C. cajan)* - cereal (*Z. mays*) intercropping system as well as their implications on the physiology of the multifaceted NGR234.

3.4.1. Identification of primary metabolites in the root exudates of *C. cajan – Z. mays* intercropped plants and their physiological effect on NGR234

Primary metabolites like sugars, amino acids, and organic acids are important components of root exudates that could play a role as chemoattractants or as nutrients and facilitate root colonization (Canarini et al., 2019). In our effort to understand the effect of intercropping on the primary metabolite exudation and its significance in rhizobia-plant interactions, 62 metabolites were identified in the root exudates from *C. cajan- Z. mays* in a comparison between the monocrop-intercrop systems (Fig. 3.2A). A distinct variation in the composition of the root exudates of both the plants was observed when grown individually and co-cultivated (Fig. 3.2B). Variation in the flavonoids content and proportion in the root exudates of faba bean was reported by Liu et al. (2019b) in faba bean and wheat intercropping. With an untargeted root exudates profiling, it was observed in the present work that intercropping distinctly modifies the exudation with respect to the plant. For example, the variation between intercrop and monocrop *C. cajan* root exudates was higher than in *Z. mays* (Fig. 3.2C and 3.2D). The carbohydrates detected in root exudates, like galactose, mannose, turanose, glycerol, and myo-inositol were significantly different between monocrop- intercrop systems. Galactose was significantly detected in intercrop *Z. mays* and mannose was detected in intercrop *C. cajan* plant root exudates. Our results are in agreement with the report (Bacic et al., 1986), wherein galactose was a major monosaccharide in the root slime layer and mannose was present as a minor component in *Z. mays*. Both galactose and mannose, in their pure form, enhanced the growth (Fig. 3.4) of NGR234 however chemotaxis (Fig. 3.3) and biofilm (Fig. 3.6) were significantly less. Interestingly, a prominent induction of NGR234 biofilm formation was noted in the presence of root exudates of intercrop *C. cajan,* over monocrop. This was in contrast to the case of *Z. mays* root exudates in inducing biofilm formation.

The growth curve experiment interestingly (Fig. 3.4A) showed a sharp and significant decline in the growth of NGR234 in presence of monocrop *C. cajan* root exudates at 38h. Such

contrast in the two conditions could be due to a difference in nutrient composition (Table 3.1), wherein the amino acid (proline) and sugar (myo-inositol) were found to be significantly lower in monocrop root exudates compared to intercrop *C. cajan* root exudates. Rinaudi et al., (2006) reported that the nutrient limiting condition leads to the transition of planktonic cells to sessile cells (biofilm) which can be further correlated well with mature biofilm formation at 72 h (Fig. 3.6). Besides, phenotypes related to growth rate and biofilm formation are controlled by quorum sensing systems located on a symbiotic plasmid (pSym) in NGR234 (He et al., 2003). These phenotypes are related to an optimal symbiotic performance with legume hosts to a broader extent (Calatrava-Morales et al., 2018). For example, myo-inositol, one of the abundant metabolites in root exudates of pea (Skøt & Egsgaard, 1984), and *Z. mays* (Naveed et al., 2017) were also one of the common metabolites present in all samples. Myo-inositol present in the rhizosphere was a preferred carbon source for *Rhizobium* (Poole et al., 1994), and is known to play a role in the early development of legume symbiosis, and also provides a competitive advantage to the strain of *R. leguminosarum* bv. viciae (Fry et al., 2001). The contribution of myo-inositol in the adhesion of rhizobia was reflected in our chemotaxis and biofilm studies (Fig. 3.3. & Fig. 3.6) as well. In addition, glycerol was found only in the root exudates of intercrop *Z. mays*. Glycerol contributed significantly to chemotaxis and growth of NGR234 (Fig. 3.4C; Fig. 3.5B), suggesting its role in competitive advantage in the rhizosphere. The report of Ding et al., (2012) suggested that the mutants defective in glycerol utilization were also deficient in competitiveness for nodulation of peas compared with the wild-type supports our observation. Overall, when compared with growth in the presence of pure compounds, there was a good correlation with the metabolites of glycerol, galactose, and myo-inositol (Fig. 3.4C & 3.4D) which are identified from *Z. mays* root exudates, suggesting their role in the growth promotion. Turanose, one of the non-metabolizable sucrose isomers was detected in root exudates monocrop *Z. mays*. Turanose was reported in leaf extracts of several *Poaceae* family plants and is a potent inhibitor of noctuid larva of cereal stemborer pest, *Busseola fusca* (Juma et al., 2013).

Interestingly, LCOAs present in the fatty acid group were also predominant in intercrop *C. cajan.* The release of LCOAs could provide a greater level of induced resistance against foliar pathogens due to microbiome-associated disease suppression (Yuan et al., 2018). Similarly, the intercrop *C. cajan* showed a significant increase in 1,2-benzene dicarboxylic acid, which was also reported to augment the resistance of tomato plants against *Alternaria alternate* (Ahmad et al.,

2019). Also similar to the previous reports of *Z. mays* root exudates identified through GC-MS/MS, our results confirm the presence of ribonic acid (organic acid) but with no significant difference (Canellas et al., 2019). Also, the D-glucopyranoside present at significant ($p < 0.05$) levels in intercrop *Z. mays* compared to monocrop *Z. mays*, present in root exudates of *Z. mays* can serve as a carbon source (da Silva Lima et al., 2014; Dall'Agnol et al., 2013).

Rhizobia depend on the availability of amino acids to build up the symbiosis with legumes. During rhizobium-legume symbiosis, some of these amino acids are synthesized by rhizobium, while others are acquired in a host-dependent manner (Lodwig et al., 2003). Amino acids like proline and arginine were significantly present in the root exudates of intercrop *C. cajan* and intercrop *Z. mays*, respectively compared to monocrop's root exudates. In this study, the presence of proline in intercrop *C. cajan* root exudates contributed to chemotaxis and biofilm formation in the NGR234 strain, similar to a report by Gosai et al. (2019) within different *Ensifer* spp. Proline may also serve as an energy source for rhizobia (King et al. 2000). Lv et al. (2020) showed that proline content was higher in intercrop faba beans as compared to monocrop plants. Arginine detected in intercrop *Z. mays* root exudates might be important in nitrogen fixation (Flores-tinoco et al., 2020) and contributes to resistance to abiotic stress (Kuznetsov et al., 1999). The enhanced amidation (synthesis of arginine), thus, could indeed be an economical system to salvage nitrogen in the rhizosphere (Dhont et al., 2006). Hence it can be speculated that the significant efflux of arginine in intercrop *Z. mays* helps to combat abiotic stress. It was even observed that there was an overall less efflux of amino acid in *Z. mays* root exudates. Thus, the metabolites like myo-inositol, glycerol, proline, and arginine present in the intercrop root exudates of both *C. cajan* and *Z. mays* act as a trigger for either nodulation or sustainable colonization on the roots.

Overall, this work highlights that NGR234 responds differentially with monocropped and intercropped plant root exudates. Also, their interactions with *Z. mays* plant root exudates at the physiological level were found to be different. Further, their molecular effects on this strain were studied in detail.