PLANT MICROBE INTERACTIONS

Alterations of Primary Metabolites in Root Exudates of Intercropped *Cajanus cajan–Zea mays* Modulate the Adaptation and Proteome of *Ensifer* (*Sinorhizobium*) *fredii* NGR234

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Abstract

Legume-cereal intercropping systems, in the context of diversity, ecological function, and better yield have been widely studied. Such systems enhance nutrient phytoavailability by balancing root-rhizosphere interactions. Root exudates (RE) play an important role in the rhizospheric interactions of plant-plant and/or plant-microbiome interaction. However, the influence of the primary metabolites of RE on plant-rhizobia interactions in a legume-cereal intercrop system is not known. To understand the plant communication with rhizobia, *Cajanus cajan-Zea mays* intercropped plants and the broad host range legume nodulating *Ensifer fredii* NGR234 as the model plants and rhizobium used respectively. A metabolomics-based approach revealed a clear separation between intercropped and monocropped RE of the two plants. Intercropped *C. cajan* showed an increase in the myo-inositol, and proline, while intercropped *Z. mays* showed enhanced galactose, D-glucopyranoside, and arginine in the RE. Physiological assays of NGR234 with the RE of intercropped *C. cajan* exhibited a significant enhancement in biofilm formation, while intercropped *Z. mays* RE accelerated the bacterial growth in the late log phase. Further, using label-free proteomics, we identified a total of 2570 proteins of NGR234 covering 50% annotated protein sequences upon exposure to *Z. mays* RE. Furthermore, intercropped *Z. mays* RE upregulated bacterioferritin comigratory protein (BCP), putative nitroreductase, IlvD, LeuC, D (branched-chain amino acid proteins), and chaperonin proteins GroEL2. Identification offered new insights into the metabolome of the legume-cereal intercrop and proteome of NGR234-*Z. mays* interactions that underline the new molecular candidates likely to be involved in the fitness of rhizobium in the intercropping system.

Keywords *Ensifer fredii* NGR234 · Root exudates (RE) · *Cajanus cajan* · *Zea mays* · Metabolomics · Proteomics

Introduction

To mitigate the problem of food security and nutrition, sustainable agriculture schemes need to be promoted, keeping in mind a balanced trade-off between productivity and ecosystem services [1]. One of the proposed approaches is to increase the production limits by utilizing the intercropping system that could optimally use the beneficial aspects of interactions between two plant species grown together in a common plot [2, 3] and contribute to sustainable agriculture.

The *Cajanus cajan-Zea mays* intercropping system, wherein the drought-tolerant, late-maturing *C. cajan* increases nitrogen through leaf litter and biological nitrogen fixation and thereby enhances soil fertility [4] is a classic example of legume-cereal intercrop. *Z. mays* is dominant as the cereal component in legume–cereal intercrops, mature early, and utilize nitrogen from the legume plant [5]. Intercropping provides niche complementarity and interspecific facilitation through nutrient efficacy of nitrogen. It also increases the acquisition of P, Fe, and Zn from the soil and is associated with the intermingling of roots, their respective rhizosphere, and the dispersal area of root exudates (RE) [6, 7]. The intercropping of maize with *C. cajan* also significantly promotes the formation of

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soil aggregation and organic phosphorus storage, primarily due to the root-root interactions [8].

The root exudates (RE) are key determinants of the rhizosphere microbial community structure and often provide detailed information to neighboring plant roots and rhizobiome of soil [9, 10]. The RE constitutes a significant source of organic carbon in the soil. Estimates show that about 50% of the C exported below ground is lost into the soil [11]. The exploitation of traits linked to RE of the crops' wild relatives appears promising to reduce the utilization of fertilizers and pesticides in agriculture [12]. An increase in the secretion of secondary metabolites like flavonoids genistein, hesperetin, and naringenin has been reported in faba beans when intercropped with wheat [13]. It is now known that maize releases the flavonoids that influence the root nodulation and nitrogen fixation in legumes in intercropping [14]. Most recent studies highlight that secondary metabolites of RE influence the microbiome and also play a crucial role in enhancing arbuscular mycorrhizal fungal associations [15, 16]. Thus, RE aid interspecific and root-rhizobium 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 RE in plant-bacteria interaction remains unclear.

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 [17]. Rhizobia are among the best-studied root-associated microbes, with a drastic lifestyle change from saprophytic to endosymbiotic bacteroids, by adopting an oligotrophic lifestyle inside the host plant [18]. Rhizobia-legume symbiosis is also one of the best-studied for the signal exchange process mediated through the flavonoids (secondary metabolites) released by the legume root [19]. Further, the primary metabolites in the RE serve as carbon, nitrogen, or sulfur sources for free-living and/or symbiotically associated rhizobia [20]. Thus, the role of primary metabolites in the RE of legume-cereal intercrop in the epiphytic interactions of rhizobia needs to be characterized.

RE 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) [21]. Rhizobia are also known to colonize the rhizosphere and promote the growth of non-legume plants such as maize and rice [22–24]. The carbohydrate-rich mucilage (high molecular weight RE) of the aerial roots of maize aided nitrogen fixation by the diazotrophic bacteria in Sierra Mixe, Mexico [25]. However, the signaling mechanism that facilitates the interaction of rhizobia with low molecular weight RE from maize and/or rice is not yet known.

Apart from significant plant-plant interactions, the facilitative interactions between microbes and plants, and between legumes and cereals can modulate the outcome of the intercropping system [26]. When legume and cereal are cultivated together in intercropping, intermingled roots facilitate the "sharing" of soil microbes, including symbiotic bacteria [22], and also increase the abundance of alpha proteobacteria, particularly nitrogen-fixing rhizobia [27, 28]. The contribution of different nitrogen-fixing bacteria to maize rootlets was revealed by metatranscriptomics [29], while the combination of metagenomics and metaproteomics enabled the identification of functional nitrogenase protein in the *Bradyrhizobium* found on the roots of field-grown sorghum [30]. But, more detailed studies on the interactions of rhizobia, with cereal plants intercropped with the legume plants, using an omics approach are not known.

Ensifer (Sinorhizobium) fredii NGR234 (here called NGR234) has a broad host range nodulation potential with more than 120 genera of legumes, and the non-legume *Parasponia andersonii* [31] was used as a model organism in this study. To gain insight into the eco-physiology of legume symbiont NGR234 under the influence of RE in *C. cajan-Z. mays* intercropping system, non-targeted metabolomics and a comparative proteomics approach were undertaken to identify key primary metabolites in the RE and the rhizobial proteins involved in the interaction with *Z. mays*. The present study identifies the REs specific primary metabolites and partly explains the adaptation and response of rhizobium during interaction with the RE in an intercropping setup.

Materials and methods

Bacterial strain and plant material

Ensifer fredii NGR234 procured from National Biological Resource Center (NBRC), Japan (Accession No. NBRC 101,917) was used throughout this study as a model organism. Seeds of *Cajanus cajan* (cultivar BDN-2) and *Zea mays* (cultivar GM-6) were procured from Pulse Research Station of Anand Agriculture University, Vadodara, Gujarat, India, and Maize Research Station of Anand Agriculture University, Godhra, Gujarat, India, respectively.

Plant cultivation

Surface sterilization of the seeds was carried out according to the protocol mentioned in Patel and Archana [32] with few modifications. Briefly, seeds were rinsed with 0.2% mercuric chloride for 3 min, followed by a rinse with 70% (v/v) ethanol, and then thoroughly washed 3 times with sterile distilled water. Germinated seedlings of *C. cajan* and *Z. mays* were placed on 0.8% water agar for germination at 30 °C for

3 days and 2 days, respectively. Surface sterilized seeds of *C. cajan* and *Z. mays* were grown in pots (diameter—27 cm, length—21 cm) containing 7 kg sterile coarse sand which was autoclaved at 121 °C, 15 psi for 30 min, in three rounds of autoclaving for 3 consecutive days to eliminate soil microbes and spore formers. For monocropped plant growth setup, 14 germinated seedlings of *C. cajan* were sown in one pot, and similarly, 14 *Z. mays* seedlings were grown in another pot. For the intercrop setup, 7 each of *C. cajan* and *Z. mays* plants were grown together in the same pot with a distance of 7 cm between the two plant species as shown in Supplementary Fig. 1. Multiple such sets of plants were grown to allow the collection of RE from a large number of plants. Pots were maintained in the greenhouse (12 h of light and -12 h of dark photoperiod at 30 °C) and quarter strength Hoagland solution [33] was used for watering [34].

Preparation of RE from monocrops and intercrops

Plants were harvested by uprooting gently on the 28th day. The initial steps of washing and sterilizing the roots for RE collection were similar to a protocol mentioned by Vora et al. [35]. Briefly, roots were rinsed with freshly autoclaved Milli Q (MQ) water in a laminar air flow 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, RE was 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 6 h [36] under shaking conditions at 30 °C. The two plant species grown as intercrop were harvested and RE was collected separately from them. The RE of *C. cajan* grown along with *Z mays* was denoted as intercrop *C. cajan*. Similarly, RE of intercropped *Z. mays* (grown along with *C. cajan*) was designated as intercrop *Z. mays*. Plants grown separately in several individual pots were used for the collection of a pooled RE sample for each set of plants. The RE 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 for the dry weight. After RE was collected, the roots were separated from the shoots and dried using paper towels, and fresh weight was monitored (Supplementary Fig. S2).

The lyophilized RE samples were analyzed by GC–MS as described by Ankati and Podile [37]. Briefly, 10 mg of the lyophilized crude RE 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 8200×*g* for 5 min at 4 °C. The pellet was discarded and the supernatant containing the RE was again dried using a Savant DNA120 Speed Vac. Concentrator (Thermo Scientific, USA), 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.

Metabolite profiling of RE by GC–MS/MS

To analyze the RE samples, 1 mg of the processed sample was 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, RE profiling was carried out using GC–MS/MS in three technical replicates with Agilent 7890 series gas chromatograph. The following parameters were used for analyzing the RE samples. EI-voltage of 70 eV with source temp. 230 °C; DB-1HT /IntegraGuard (Retek GmbH, Bad Homburg, Germany) column with 29.3 m × 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 NIST (National Institute of Standard and Technology) library using the mass spectra extracted. The compound hits with > 700 similarities ± 30 RI 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 RE was calculated with the help of arabitol as an internal standard. To identify the variations in the RE metabolites between intercropped and monocropped plants of both *C. cajan* and *Z. mays*, a multivariate analysis was performed using the MetaboAnalyst 4.0 web tool [38]. 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 RE of intercropped and monocropped 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 [39], while hierarchical cluster analysis was performed by setting Euclidean distance as a similarity measure and Ward's linkage as the clustering algorithm.

Effect of RE and individual metabolites on the physiological response of NGR234

To understand the effects of key RE metabolites on NGR234, metabolites that significantly varied between intercrop and monocrop plants' RE, 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 concentration [40] were considered to study the effect on chemotaxis (Supplementary Fig. S3), growth, and biofilm formation of NGR234. Solutions of RE in MQ water of individual monocrops (*C. cajan* and *Z. mays*), as well as their intercrops, were filter-sterilized using 0.2 μm filters and were streaked onto Luria agar plates to check purity and used at a final concentration of 1 mg ml^{-1}. The untreated cells (without root exudates) were taken as a negative control for the experiment. NGR234 was grown at 30 °C until it reached OD_{600} of 1.0 in the Rhizobium minimal medium (RMM) [41] by supplementing with 6.5 mM glutamate [42]. 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 6.5 mM 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 medium continuous shaking at 30 °C throughout the study period. At every 2-h interval, absorbance was measured at 600 nm 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. [43]. First, the OD_{600} of the static bacterial growth was recorded (Supplementary Fig. S5). 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 three technical replicates for both growth and biofilm formation. The relative biofilm-formed by NGR234 was calculated by taking the ratio of $OD_{595/600}$.

Colonization and cross colonization studies in an intercropping setup

To study the colonization of NGR234 on both *C. cajan* and *Z. mays*, surface-sterilized seedlings (approximately 3-cm radicle) were soaked for 4 h at 30° C in 10^8 CFU/ml growth phase NGR234 suspension which was prepared by taking overnight grown culture in Tryptone Yeast Extract Broth (TY) and cells were washed once with sterile 0.85% saline to remove the medium. After 4 h of incubation at 30° C, inoculated seedlings were placed separately in sterile sand, allowed to grow until 28 days under greenhouse conditions. The plants were allowed to grow under greenhouse conditions (12 h of light and 12 h of the dark of photoperiod at 30 °C) and watered thrice a week with autoclaved, reverse osmosis (RO) water. The CFU/g of the root was calculated for the bacterial count, wherein roots of both the plants were collected separately. The roots were rinsed with sterile water to remove adhered sand and the fresh weight of roots was taken individually by placing them in sterile Petri plates. Each root was then placed into glass vials to which 1.0 ml of sterile 0.85% saline was added and vortexed for 2 min. The suspension consisting of bacteria adhered to roots was then plated on TY agar plates. The number of colonies, after 2 days of incubation at 30° C, was noted to obtain CFU/ml and converted to CFU/g of fresh root weight.

For cross colonization studies, 5 seedlings of only *C. cajan* were soaked with the bacterial suspension (as above) and 5 seedlings of *Z. mays* were left uninoculated and placed as shown in Supplementary Fig. S6. Mesh barriers (MB) were used with slight modifications to the protocol by Wang et al. and Meng et al. [44, 45]. Here, seedlings of *C. cajan* coated with NGR234 and non-coated *Z. mays* were placed simultaneously at a vicinity of 10-cm distance in a pot. Seedlings were separated or not by an autoclaved mesh barrier (25 μm stainless steel) which was placed in the middle of the two sets of plants to prevent the root–root interaction. In the absence of MB, the root-root interactions take place in the pots, while the MB separates the roots and prevents direct physical interaction between roots. Plants were allowed to grow under similar conditions as mentioned above. The calculation of CFU/g of roots was similar to the monocrops colonization after 28 days after sowing (DAS). The experiment was done twice with three biological replicates.

Extraction of total proteins from NGR234

To know protein expression in the stationary phase, NGR234 was grown in 5.0 ml of RMM broth (~ 2.5 O.D$_{600}$) for 48 h at 30 °C in the presence of 1.0 mg ml^{-1} of RE collected from monocrop *Z. mays* and intercrop *Z. mays* individually for extraction of total proteins. The RMM broth without added RE served as control. Three biological replicates were taken for each sample. Total proteins of NGR234 were extracted [46] with minor modifications wherein cells were harvested aseptically by centrifugation in a 2.0 ml microcentrifuge tube at $14,000 \times g$ for 4 °C and the cell pellet was carefully washed with phosphate-buffered saline (3 mM KCl; 1.5 mM KH$_2$PO$_4$; 68 mM NaCl; and 9 mM NaH$_2$PO$_4$). Washed cells were resuspended in 500 µl lysis buffer (8 M urea; 2% CHAPS; 50 mM Tris–Cl; and 50 mM DTT) and lysed by using a sonicator (9.9 s on and off cycles, for 5 min at 35% amplitude). The lysates were separated from particulate material at $16,000 \times g$ for 15 min, at 4 °C. An additional step of concentration with phenol was done to remove any associated nucleic acids [47]. Briefly, aliquots (500 µl) of the lysates were mixed with a solution containing 0.8 ml of Tris-buffered phenol pH 8.0, and 0.8 ml of SDS buffer (0.1 M Tris–HCl pH 8.0; 2% SDS; 5% β-mercaptoethanol; 30% sucrose). The samples were mixed in the extraction buffer for 5 min and centrifuged at $16,000 \times g$ for 15 min at 4 °C, and the top phenol layer (500 µl) was transferred to a new tube. Proteins were precipitated for 1 h, at –20 °C with three volumes of pre-cooled 0.1 M ammonium acetate in absolute methanol and then centrifuged ($16,000 \times g$ for 15 min at 4 °C). The pellet was washed once with pre-cooled methanol and once with pre-cooled 80% v/v acetone. Samples were air-dried and stored at -20 °C till further use.

In-solution digestion of proteins

Trypsin digestion was carried out using 15 µg of NGR234 protein from each sample. Protein concentration was determined by Bradford's method [48]. Protein samples were reduced with 10 mM dithiothreitol (DTT) and incubated at 37 °C for 30 min followed by the addition of 30 mM iodoacetamide and incubated in the dark (30 °C, 30 min) to alkylate the free cysteine residues. The samples were diluted with 1 M urea and pH was adjusted to 8.0 before the addition of trypsin (1:50 (w/w)) and incubated at 37 °C for overnight digestion. The final concentration of 0.1% formic acid was used to quench the reaction. The digested peptides were dried under vacuum and desalted using a C-18 zip tip (Pierce C18 Tips, 10 µl bed). The eluted peptides were dried and re-dissolved in 2% acetonitrile/0.1% formic acid.

LC–MS/MS conditions and analysis

A digested peptide sample was injected into the Thermo Orbitrap fusion tribird mass spectrometer coupled with an EASY-nLC 1200 series system. The peptides were injected into reverse-phase C18 pre-column (Acclaim PepMap 100, C18, 3 µm, 75 µm × 2 cm nanoviper) and then separated on C18 analytical column (Easy spray Pepmap RSLC, C18, 2 µm, 15 cm × 75 µm) for a resolved separation. Peptides were eluted using a linear gradient for 168 min from 5 to 45% solvent B (80% acetonitrile in 0.1% formic acid) at a flow rate of 300 nL/min, 45% B to 98% B for 12 min and solvent A (0.1% formic acid in LC grade water). The mass spectrometer was operated with a positive ionization voltage of 1900 V and 273 °C temperature for the ion transfer tube. MS spectra were acquired in the orbitrap with a resolution of 120,000 over a mass range of 375–1700 m/z, automatic gain control (AGC) value was set to 4.0 e5 and a maximum injection time was kept as 50 ms. The 20 highly intense ions for fragmentation were selected by Top-Speed acquisition mode which was isolated by quadrupole with an isolation width of 1.2 Da. These ions with charge states ranging from 2 + to 7 + were fragmented by high energy collision-induced dissociation (HCD) with an optimized collision energy of 30% with step energy ± 5. The fragmented ion spectra were acquired by the ion trap in centroid mode, AGC value was set to 1e4 and a maximum injection time of 35 s was used. The data acquisition was done with Xcalibur software (Version- 4.1.31.9).

The MaxQuant software (v. 1.6.7.0) with the Andromeda search engine was used to analyze the LC–MS/MS data. The spectra were searched against the *Sinorhizobium_fredii* protein sequences which were downloaded from Uniprot in Aug 2018. A precursor mass tolerance of 10 ppm, MS/MS fragment ion tolerance of 0.6 Da was used for database search. Besides, enzyme specificity for trypsin (for up to two missed cleavages), methionine oxidation (M), and acetylation (protein N-term) were set as variable modifications while cysteine carbamidomethylation (C) was set as a fixed modification. Peptide identification was performed using the criteria that proteins should contain two unique proteins at a 1% false discovery rate (FDR). A 1% FDR was set for both peptide spectral match and protein identification. The Perseus software (version 1.6.2.3) was used to carry out the bioinformatics analysis. Proteins identified in at least two out of the three replicates and with at least 2 unique peptide hits were included for further peptide quantitation analysis. Differential expression analysis was performed using LFQ intensities. After Log$_2$ transformation of the intensities and normalization based on "with adjustment", a two-sample Student's T-test was used to determine differentially abundant proteins. The Log$_2$ FC values (Student's T-test difference between Log$_2$ intensities of treated (RE) and untreated

(Control). The following criteria was used to consider up-regulation (Log$_2$ FC $\geq$ 1.5 and p values $\leq$ 0.05) and down-regulation (Log$_2$ FC $\leq$ -1.0 and p-values $\leq$ 0.05).

Statistical analysis

Multiple mean comparisons were performed by using Bonferroni's post hoc tests through Graphpad Prism software. Statistical significance was determined at the critical α-level of 0.05. Significance was represented as 'ns' if $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Results

Variation in the RE profile of monocropped and intercropped plants

To understand compositional differences in a more comprehensive way, RE of both monocrops and intercrops were analyzed through GC–MS/MS. Plants of *C. cajan* and *Z. mays* grown individually in separate pots were considered as monocrop *C. cajan* and monocrop *Z. mays*, respectively and those grown together in the same pot were considered as intercrop *C. cajan* and intercrop *Z. mays* as shown in Supplementary Fig. S1. After the analysis, we observed a clear and distinct difference between monocrop and intercrop RE 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. 1A). 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. 1B) showed clear segregation between intercrop and monocrop RE of *C. cajan* and *Z. mays* plants. Further, multivariate data analysis with unsupervised PCA (Fig. 1C) and supervised PLS-DA (Fig. 1D) showed a clear variation in the RE profiles. Here, each point served as a sample, and similar biochemical composition in RE samples grouped to be nearer, while dissimilar samples were grouped apart from each other. The unsupervised PCA plot depicts variation among RE profiles of monocrop and intercrop 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 RE profiles, whereas, PC2 deciphered the intercropping specific variations in RE with respect to crops by 23.6%. This variation in RE 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 RE profiles of *C. cajan* in PLS-DA over *Z. mays* (Fig. 1D).

Fig. 1 Global differences in the metabolite profiles of root exudates of *Z. mays* and *C. cajan* grown as monocrops or intercrops as 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
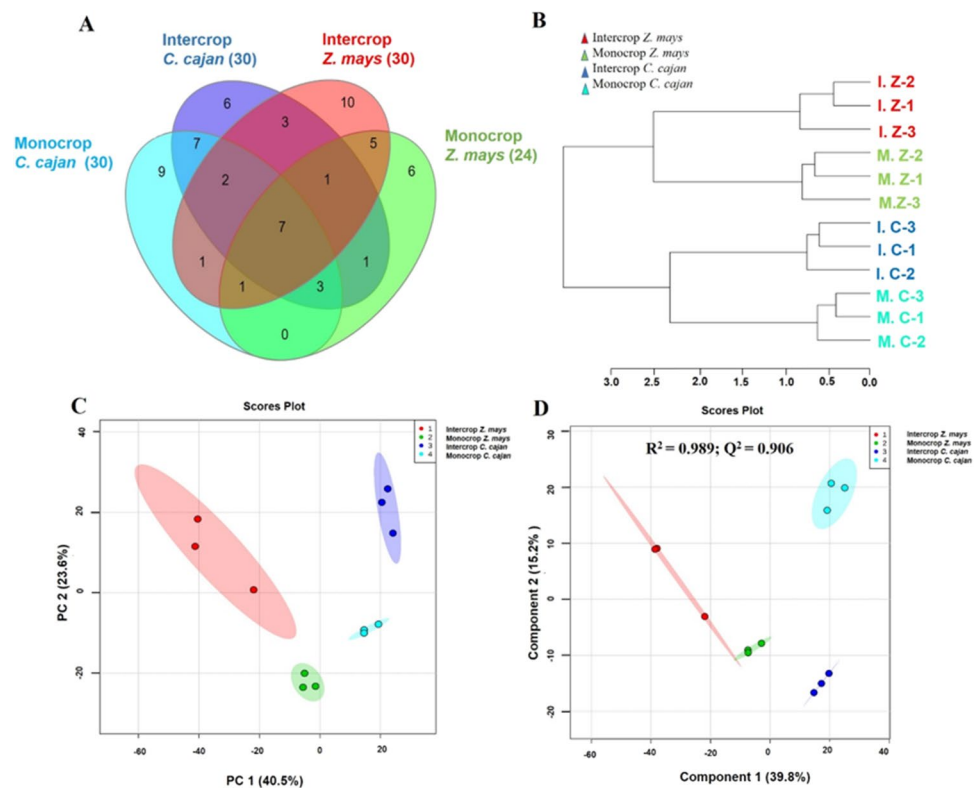
Therefore, comprehension analysis suggested that the RE of monocrop are different from intercrops for both plants.

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 Supplementary Table S1 along with their relative abundance across the four samples. Among the 62 metabolites, 18 metabolites were significantly ($p < 0.05$) different in a comparison between monocrop and intercrop RE of each plant (Table 1). There were 11 metabolites significantly present in the triplicates of both monocrops and intercrops RE. 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. 1A, among them, myo-inositol was present significantly at higher concentration ($p < 0.01$) in monocrop *Z. mays* and intercrop *C. cajan* compared to intercrop *Z. mays* and monocrop *C. cajan* plants RE, respectively. When monocrop and intercrop conditions were compared among the plant, it was interesting to observe that intercrop *C. cajan* released more of myo-inositol, mannose, and L-proline compared to monocrop *C. cajan*. In the case of intercrop *Z. mays*

RE, 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 RE of *Z. mays*.

RE contributes to the growth and biofilm formation of NGR234

To determine the growth differences in the presence of RE of monocrops, intercrops, and selected metabolites, NGR234 was grown in a minimal medium without mannitol which supports the normal growth, and supplemented with 1 mg ml^{-1} of RE. NGR234 showed a longer log phase and significantly declined growth in presence of monocrop *C. cajan* RE ($p < 0.01$) when compared to control (Fig. 2A), while intercrop and monocrop *C. cajan* showed differential growth patterns at 38 h (Supplementary Fig. S4-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* RE as compared to the set without RE (Fig. 2B; Supplementary Fig. S4-B). Further, to investigate the physiological significance of metabolites, D-galactose, D-mannose, L-proline, L-arginine, myo-inositol, and glycerol were used at 10 mM

Table 1 List of key metabolites identified in *Cajanus cajan* and *Zea mays* root exudates of intercropping and monocropping plants through 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 ± 0.18 [#]	6.85 ± 2.44** [#]	ND	ND
Ribonic acid	0.62 ± 0.31	ND	0.55 ± 0.2 [#]	0.82 ± 0.29 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
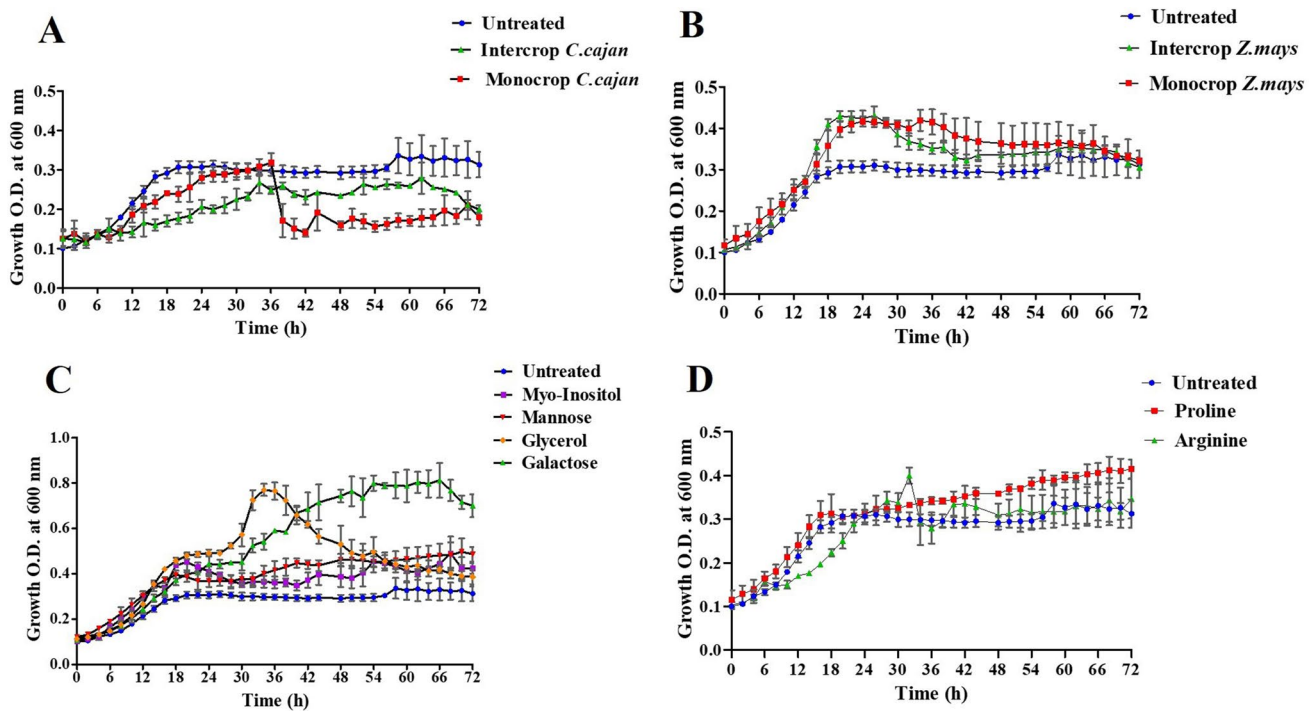
Fig. 2 Growth of NGR234 in RMG minimal medium in the presence of root exudates of *C. cajan* and *Z. mays* grown as monocrops or intercrops and on pure metabolites: (**A**) denotes growth on *C. cajan* root exudates, (**B**) *Z. mays* root exudates, (**C**) growth on pure sugar metabolites (**D**) growth on pure amino acids. The growth was monitored at the optical density at 600 nm. Error bars indicate the mean with a standard deviation of three technical replicates

(arbitrary) concentration in the interaction of RE with NGR234. The selected metabolites, present in the RE of *C. cajan* and *Z. mays*, were taken as standards and measured the growth rate in the presence of these metabolites, and results are presented in Fig. 2(C and D) . There was a significant increase in growth (p < 0.001) (Supplementary Fig. 4A) with D-mannose while in the presence of myo-inositol and proline the growth was similar to that of intercrop *C. cajan* RE at 38 h. 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 20 h (Supplementary Fig. 4B). Therefore, a differential pattern in their growth was exhibited upon exposure of *C. cajan* and *Z. mays* RE, observed at different time points.

Biofilm formation of NGR234 was assessed in the presence of the RE of monocrop, intercrop, and selected metabolites on a polystyrene 96 well microtiter well plates (Fig. 3). 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 RE showed a significant increase (p < 0.01) compared to intercrop RE 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* RE 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, and glycerol showed no significant difference when compared to control.

## NGR234 colonizes in a *C. cajan-Z. mays* intercropping system

We could see a clear difference (p < 0.01) between the colonization of NGR234 on monocrop *C. cajan* (host) and monocrop *Z. mays* (non-host) and that of the same in intercrop plants. During cross colonization studies, it was interesting to observe (Fig. 4) that NGR234 cross colonized from *C. cajan* to *Z. mays* plants even in the presence of mesh barriers. It was evident that NGR234 cross colonized in the presence of a barrier and sustained on both the plants after 28 DAS.

Differentially expressed NGR234 proteins in presence of monocrop and intercrop *Z. mays* RE

To unravel early molecular signaling of NGR234 initiated during colonization on roots of intercrop and monocrop

Fig. 3 Biofilm formation of NGR234 in the presence of root exudates and selected metabolites. Biofilm levels expressed as the ratio of OD of crystal violet at 595 nm and overall growth measured as OD 600 nm compared to untreated conditions. Error bars indicate standard deviation based on three technical replicates. The data were subjected to one-way ANOVA followed by Bonferroni's multiple comparison post hoc test. 'ns' if non-significant, $* p < 0.05,** p < 0.01$, $***p < 0.001$. Red dashed lines show the differences between monorop and intercrop of same plant
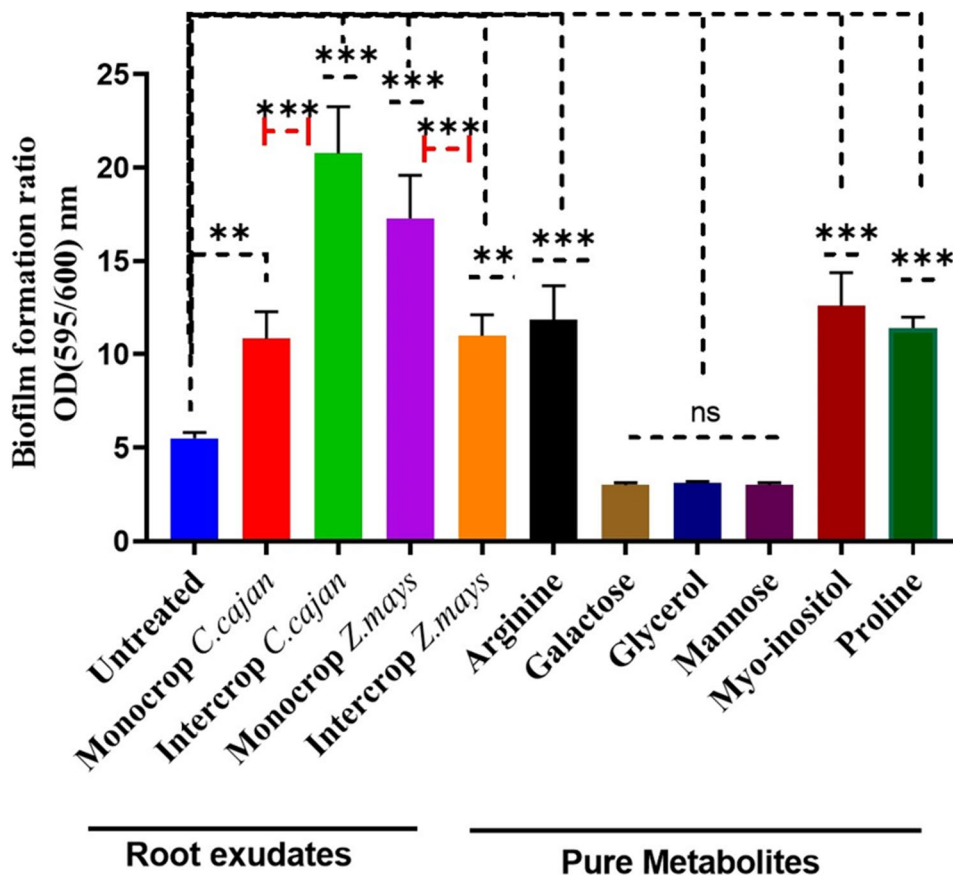
Fig. 4 Colonization of NGR234 on the roots of monocrops of *C. cajan* and *Z. mays* and cross colonization from inoculated *C. cajan* to uninoculated *Z. mays* at 28 DAS. Error bars indicate standard deviation of means with n = 6. The experiment was done twice with three biological replicates. The data were subjected to two-way ANOVA followed by Bonferroni post hoc test. 'ns' if non-significant, $** p < 0.01$
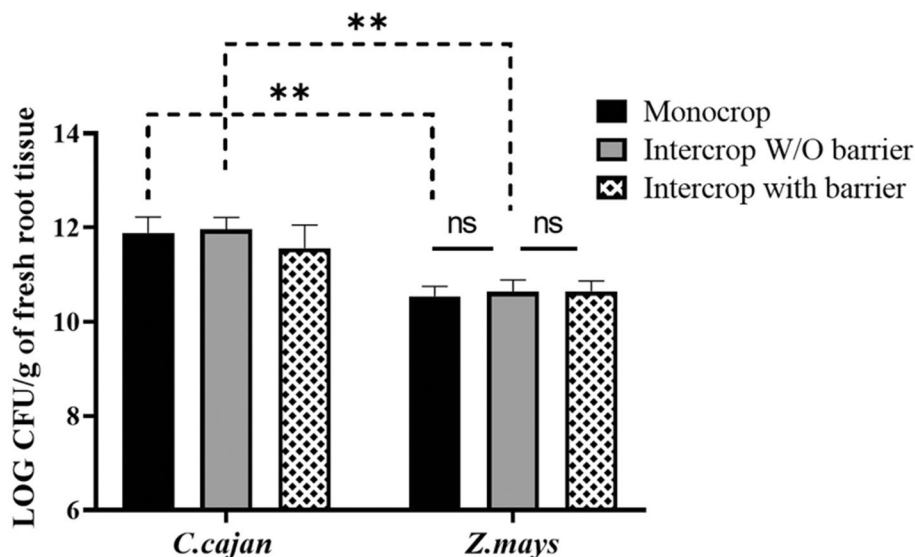
Z. mays, total proteins of NGR234 were isolated and were analyzed by a label-free quantitative proteomic approach. A total of 2570 proteins were identified across all three samples i.e. untreated cells (control), intercrop *Z. mays*, and monocrop *Z. mays* RE treated NGR234 cells as represented in the Venn diagram present in 2 or 3 biological replicates (Fig. 5A). There were around 50% of proteins in each sample that could be annotated in the UniProt database based on biological function. There were 50 unique proteins in control, 100 in intercrop *Z. mays,* and 51 in monocrop *Z. mays*. Functional classification based on gene ontology biological function (Fig. 5B) using Uniprot

Fig. 5 Proteome analysis of NGR234 treated with root exudates of monocrop *Z. mays* and intercrop *Z. mays* (with *C. cajan*) analyzed by LC/MS/MS among (**A**) Venn Diagram and (**B**) Classification of proteins done through Uniprot based on Gene Ontology biological classification
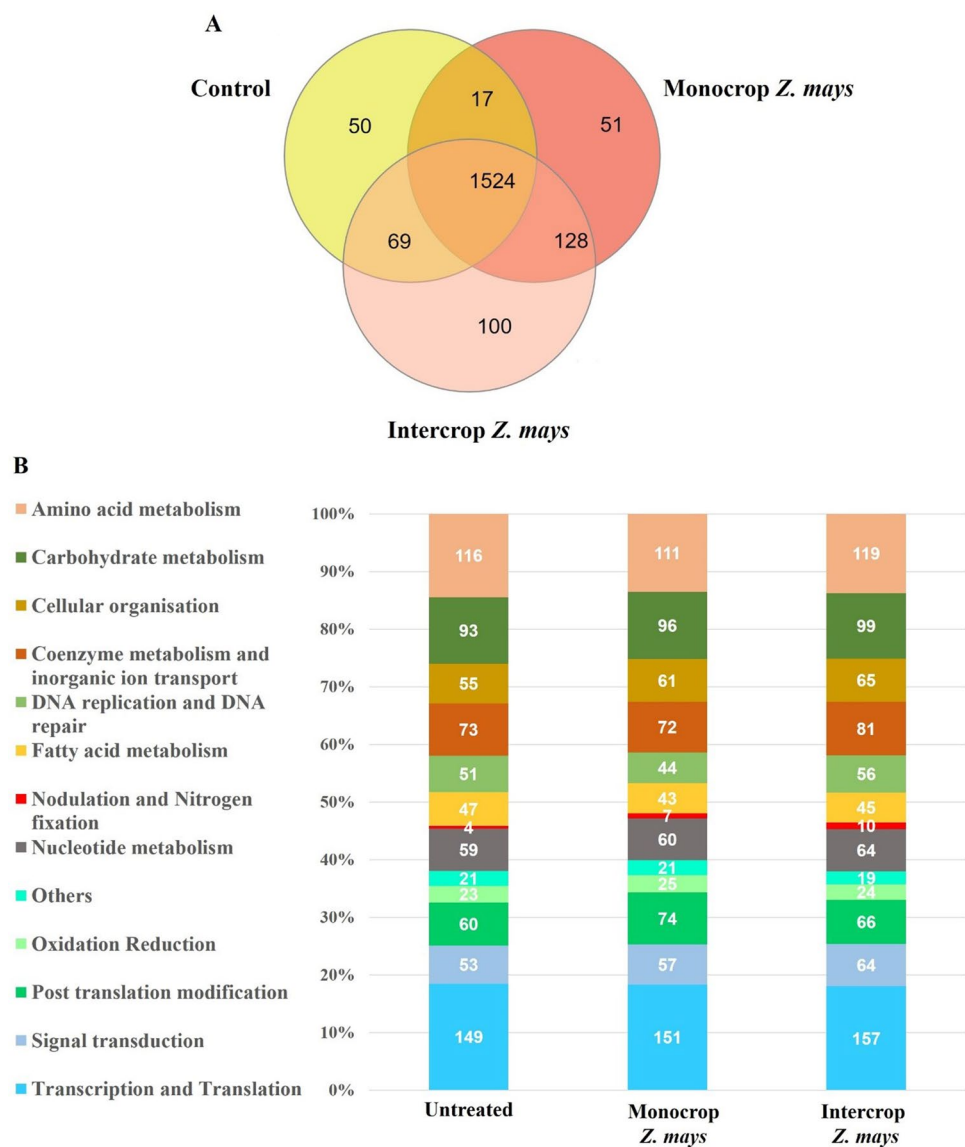

showed the highest number of proteins were uniquely present in NGR234 treated with intercrop *Z. mays* RE. The majority of them are related to signal transduction, nodulation, nitrogen fixation, and cellular organization. The cells treated with monocrop *Z. mays* RE indicated the presence of a higher number of proteins responsible for post-translational modification, while the DNA replication and repair proteins decreased compared to control. The proteins related to nodulation arranged in nod-boxes like NB-2(**NoeL**), NB-8(NodA, NodI, **NolO**, NoeI), NB-12 (**NodU**), and NB-19 (SyrM2) were notably found in treated NGR234 samples (Bold in parenthesis proteins were common in both RE treated samples; rest were specifically found by intercrop RE treated samples). It was also interesting to note that both GMD and FCL proteins, part of fucose biosynthesis on nod factors, were expressed in the presence of intercrop *Z. mays* RE in NGR234.

Quantitative expression of proteins in presence of monocrop and intercrop *Z. mays* RE

Unsupervised hierarchical cluster analysis of NGR234 expressed proteins demonstrated a clear separation between intercrop and monocrop *Z. mays* RE presence when compared to control. Out of 1519 proteins, 161 proteins showed differential expression across the three conditions which were represented in the heat map (Fig. 6) and listed in Supplementary Table S2. Overall, 18, 52, and 35 proteins of NGR234 were expressed (Log$_2$ fold increase > 1.5) in the case of control, and the presence of RE of intercrop *Z. mays*, and monocrop *Z. mays* RE, respectively. Whereas, the number of down-regulated (Log$_2$ fold decrease > 1.0) proteins were 24, 38, and 34, in the three treatments, respectively.

From the data analysis, it can be inferred that a total of 10 proteins are likely to participate in the adaptation

Fig. 6 Heat map of NGR234 proteins differentially expressed in presence of *Z. mays* root exudates (RE). NGR234 proteins expressed in presence of intercrop *Z. mays* (I-Z) and monocrop *Z. mays* (M-Z) RE were compared to the untreated cells (Control). Proteins identified in at least two of the three biological replicates were considered for the analysis. A heat map was generated using Unsupervised Hierarchical clustering with Perseus software (version 1.6.2.3). Asterisk marked (*) proteins are further discussed and details are mentioned in Table 2
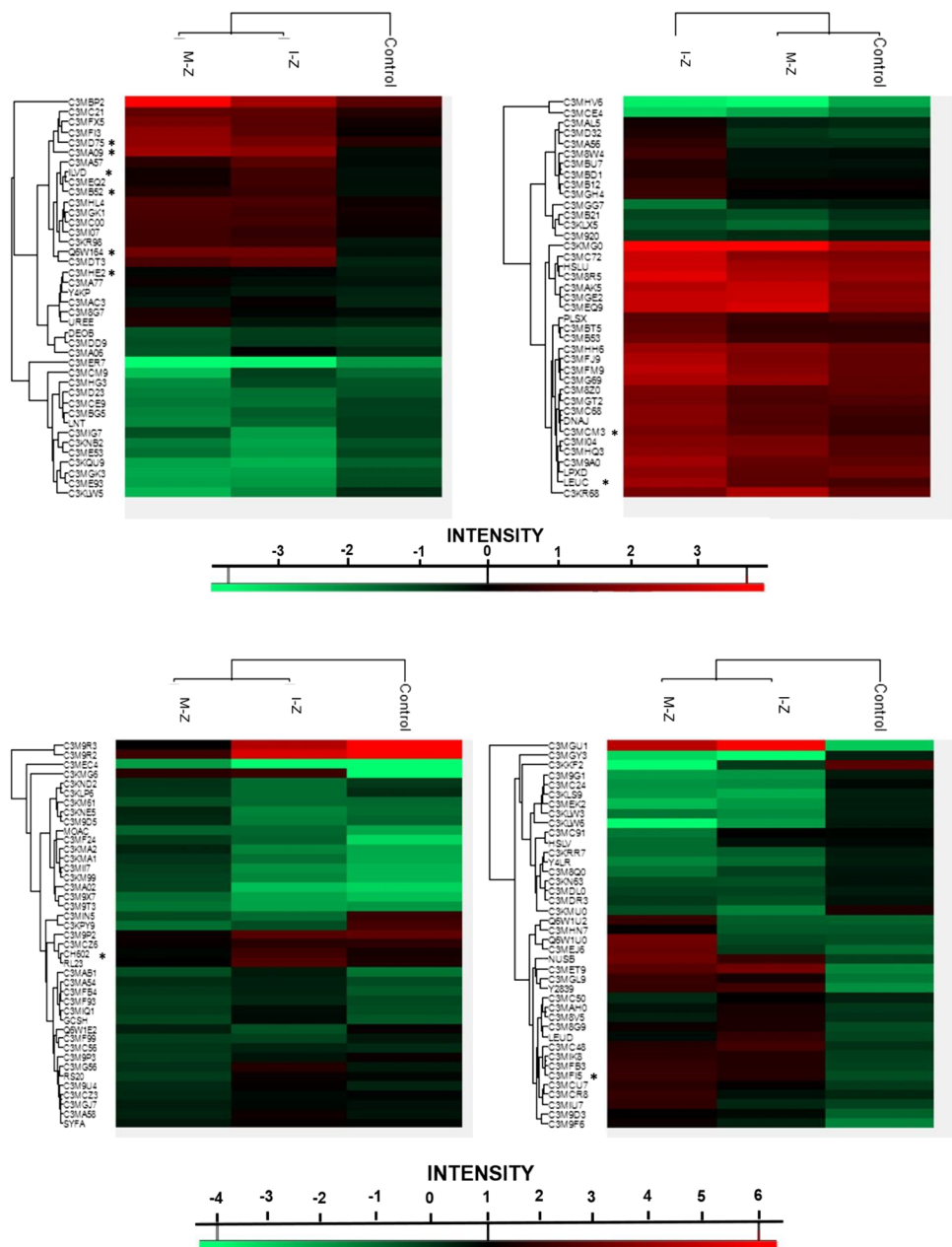
of rhizobia with the *Z. mays* plants at a molecular level (Table 2). Among them were 4 proteins as glutaredoxins are involved in redox reactions, Metallo beta-lactamase in quorum quenching of autoinducer molecules, pseudoazurins electron donor to copper-containing nitrite reductase, and GroES2 chaperonin proteins were found in the presence of both intercrop and monocrop *Z. mays*. Proteins like dihydroxy-acid dehydratase (IlvD), 3-isopropyl malate dehydratase large subunit (LeuC); 3-isopropyl malate dehydratase small subunit (LeuD) (Supplementary Fig. S7), putative nitroreductase, and bacterioferritin co-migratory protein (BCP) and GroEL- Cpn60 proteins were only up-regulated

in the presence of intercrop *Z. mays* RE. On the other hand, monocrop *Z. mays* induce the expression of PurD (Table 2). Proteins like AracA2 involved in the arginine deiminase pathway were significantly downregulated in both the treated samples, while HutU involved in histidine catabolism was downregulated in both treatments but significant only in the presence of monocrop *Z. mays* RE compared to control. NGR234 proteins like PheA and HisC expression were not significantly different from control. However, there was a significant upregulation ($p < 0.05$) by the RE of intercrop *Z. mays* compared to monocrop *Z. mays*. Nucleoside

Table 2 List of NGR234 proteins differentially expressed upon treatment with monocrop and intercrop root exudates of *Z. mays* identified by label free quantitative proteomics

Uniprot protein ID	Name of the protein	Average Fold change (Intercrop *Z. mays*/ Control)	p-value	Average Fold change (Monocrop *Z. mays*/ Control)	p-value
C3MD75	Glutaredoxin	1.9	0.042	2.3	0.043
C3MFI5	Metallo-beta-lactamase family protein	1.7	0.01	2.2	0.042
C3MA09	Pseudoazurin	6.3	0.01	7.0	0.002
Q6W164	10 kDa chaperonin (GroES protein—Cpn10)	6.6	0.02	6.6	0.01
CH602	60 kDa chaperonin 2 (GroEL protein 2- Cpn60)	1.5	0.03		
ILVD	Dihydroxy-acid dehydratase (ilvD)	4.1	0.01	-	-
C3MB52	Putative nitroreductase protein	3.8	0.043	-	-
LEUC	3-isopropylmalate dehydratase large subunit (leuC)	2.3	0.03		
C3MCM3	Bacterioferritin comigratory protein	2.2	0.043	-	-
C3MHE2	Phosphoribosylamine-glycine ligase (purD)	-	-	2.3	0.03

Proteins up-regulated with Log_2 fold change ≥ 1.5 and *p*-values ≤ 0.05 and expressed in three biological replicates were considered. Here "-'' denotes the expression of a protein with no significant fold change. A Student t-test was applied to know the significant fold change in NGR234 proteins between REs treated and control samples

diphosphate (NDK) protein, involved in quorum sensing, was upregulated by RE of *Z. mays* (Supplementary Fig. S7).

Discussion

The rhizosphere is a highly complex and dynamic system, wherein RE facilitates and modulates both plant-plant [14] as well as plant–microbe interactions [49]. Kerdraon et al. [50] recently reported that crop residues are a crucial ecological niche in the rhizosphere of wheat-oilseed rape rotation system and constitute a key fully-fledged microbial ecosystem. The monoculture studies of NGR234 (broad host range rhizobium) with legumes plants are well studied for attachment, infection, and nodulation and are well characterized mechanistically [51, 52]. Moreover, the type 3 effectors of NGR234 help in the interactions not only in cereals (nonhost plants) but also in legumes and play a critical role in the establishment of the host range [53]. However, the detailed mechanism involving the attachment and colonization of rhizobia on cereal plants is not yet clear.

Primary metabolites like sugars, amino acids, and organic acids are important components of RE that could play a role as chemoattractants or as nutrients and facilitate root colonization [17]. In our previous report, we highlighted the difference in the release of organic acids from root exudates of *C. cajan–Z. mays* intercropped plants [35]. Earlier, the variation in the secondary metabolites like flavonoids content in the RE of faba bean was also reported by Liu et al. [54] in the faba bean-wheat intercropping system. However, to know the untargeted profile of root exudates, omics platforms are used to gain insights into the complex metabolic and regulatory network in plant–microbe interactions [55–57]. The

present work represents a comprehensive study to identify variations in the key primary metabolites in the RE of the legume (*C. cajan*)-cereal (*Z. mays*) intercropping system as well as their implications on the physiology of the multifaceted NGR234.

In our efforts to understand the effect of intercropping on the primary metabolite exudation through untargeted analysis, a distinct variation in the composition of the RE of both the plants was observed when grown individually and co-cultivated (Fig. 1A; Fig. 1B). With an untargeted RE profiling, we observed that intercropping distinctly modifies the exudation with respect to the plant. For example, the variation between intercrop and monocrop *C. cajan* RE was higher than in *Z. mays* (Fig. 1C and 1D). Among the sugars, galactose was significantly higher in the RE intercrop *Z. mays* while mannose was detected in case of intercropped *C. cajan*. Our results are in agreement with the report by Bacic et al. [58], 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 of NGR234 and reduced the biofilm formation.

The growth curve experiment interestingly (Fig. 2A; Supplementary Fig. S4-A) showed a sharp and significant decline in the growth of NGR234 in presence of monocrop *C. cajan* RE at 38 h. Such contrast in the two conditions could be due to a difference in nutrient composition (Table 1), wherein the amino acid (proline) and sugar (myoinositol) were found to be significantly lower in monocrop RE compared to intercrop *C. cajan* RE. The nutrient limiting condition leads to the transition of planktonic cells to sessile cells (biofilm) [59] which can be further correlated well with mature biofilm formation at 72 h (Fig. 3). Besides,

phenotypes related to growth rate and biofilm formation are controlled by quorum sensing systems located on a symbiotic plasmid (pSym) in NGR234 [60]. These phenotypes are related to an optimal symbiotic performance with legume hosts to a broader extent [61]. For example, myo-inositol, one of the abundant metabolites in RE of pea [62] and maize [63] was also one of the common metabolites present in all samples and its contribution in the adhesion of rhizobia was reflected in our biofilm studies (Fig. 3) as well. Myo-inositol present in the rhizosphere was a preferred carbon source for *Rhizobium* [64], and 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 [65]. In addition, glycerol was found only in the RE of intercrop *Z. mays*. Glycerol contributed significantly to chemotaxis (Supplementary Fig. S3) and growth of NGR234 (Fig. 2C), suggesting its role in competitive advantage in the rhizosphere. The report of Ding et al. [66] 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, regarding growth in the presence of pure compounds, there was a good correlation with the metabolites of glycerol, galactose, and myo-inositol (Fig. 2C and 2D) which are identified from *Z. mays* RE, suggesting their role in growth promotion.

During rhizobium-legume symbiosis, some amino acids are acquired in a host-dependent manner [67]. Amino acids like proline and arginine were significantly present in the RE of intercrop *C. cajan* and intercrop *Z. mays,* respectively compared to monocrop's RE. Proline content was also found to be higher in intercropped faba beans as compared to monocrop plants [68]. In this study, NGR234 showed positive chemotaxis response towards proline (Supplementary Fig. S3) and biofilm formation (Fig. 3), similar to the report of Gosai et al. [69] with different *Ensifer* spp. Arginine detected in intercrop *Z. mays* RE might contribute to tolerance to abiotic stress [70]. The enhanced amidation (synthesis of arginine) could indeed be an economical system to salvage nitrogen in the rhizosphere [71]. Thus, the metabolites like myo-inositol, glycerol, proline, and arginine present in the intercrop RE of both *C. cajan* and *Z. mays* act as a trigger for either nodulation or sustainable colonization on the roots. In our study, NGR234 colonized (Fig. 4) both legume and non-legume plants. Notably, positive interactions between *C. cajan* and *Z. mays* plants and their influence on NGR234 led to the cross colonization from *C. cajan* to *Z. mays* plants. As the colonization was similar irrespective of the barrier, the RE may have a role in the bacterial cross migration during intercropping.

Mono-culture studies of legume–rhizobia symbiosis have been widely known; however, their interactions with non-legume plants have not been understood well. Liang et al. [72] highlighted the mechanism for perception and

response towards the Nod factor and effectively compared the associated consequences to legumes with non-legumes like *Arabidopsis*, tomato (a dicot), and corn (a monocot). However, molecular mechanisms that outline the fundamental basis of the initial interactions exhibited by free-living rhizobia with intercrop and monocrop of *Z. mays* plants are not known. Our proteomic analysis revealed that the RE of *Z. mays* induced the expression of nod-box (NB-2, NB-8, NB-12) proteins (Fig. 5). The gene expression of 18 of the 19 nod-boxes are known to be positively regulated in a flavonoid-dependent manner by the *nodD1* promoter, activated in the presence of flavonoids/phenolics released from both legumes and non-legume (wheat) plants [51, 73]. Besides, we could also find clear upregulation of GroES2 co-chaperone proteins by the RE of *Z. mays*, additionally, GroEL2 protein is also upregulated by RE of intercrop *Z. mays*. The GroEL/GroES chaperonins are involved in the folding and/or assembly of active NodD proteins to facilitate the regulation of nod gene expression [74]. Our findings are in concurrence with Liang et al. [72], wherein the perception and response of rhizobial nod factor towards non-legumes, including corn, suggest the possibility of nod factor-mediated signaling in the intercropping system.

Besides, it was interesting to note the expression of NodU by both monocrop and intercrop RE of *Z. mays*. In contrast, the RE from rice plants did not show the activation of the *nodA* of *R. leguminosarum* bv. *trifolii* and *nodSU* of *Rhizobium*. sp. NGR234 [75]. Overall our results with respect to nod box proteins imply that flavonoids present in RE of both monocrop and intercrop *Z. mays* [14] modulated *nodD1* gene-dependent nod boxes of NGR234. Furthermore, flavonoids are also known to regulate the quorum sensing (QS)-biofilm formation which leads to the development of the symbiotic biofilm on legume roots [76, 77]. A significant up-regulation of NDK protein (nucleoside diphosphate kinase) (Supplementary Fig. S7) associated with QS in the planktonic cells helps in the initial metabolic changes that occur before surface attachment [78]. Therefore, from our physiological studies and protein identification, we surmised that QS-biofilm in NGR234 allowed the formation of the symbiotic biofilm and successful root colonization (Fig. 3 and 4) on the *Z. mays* plant roots as well.

Further, our data indicates that against reactive oxygen species (ROS) stress, BCP was upregulated in NGR234 during interaction with intercrop *Z. mays* plant RE while glutaredoxin (Fig. 6 and Table 2), a part of the antioxidant defense mechanism was induced by both intercrop and monocrop *Z. mays* plant RE. It is known that during the legume-rhizobia association, ROS and antioxidant defense mechanisms play a crucial role in establishment of symbiosis with legumes [79, 80]. Also, to infect the plant successfully or to down-regulate the plant's ROS-producing systems, the bacteria depend on ROS scavenging enzymes [81]. The role of BCP

in the bacterial defense against H_2O_2 at the free-living stage of rhizobia [82]. Meanwhile, putative nitroreductase protein (known as oxidoreductase) was expressed upon treatment with RE of intercrop *Z. mays* in NGR234, is also possibly involved in the oxidative stress response [83]. Based on our results, it can be speculated that to maintain cellular redox homeostasis in bacteria, a similar mechanism exists while interacting with the non-legume, *Z. mays.*

Interestingly, we also noticed upregulation of key proteins of branched-chain amino acids like IlvD, LeuC, LeuD by intercrop *Z. mays* RE and IMP pathways like PurD by monocrop *Z. mays* RE in NGR234. The importance of these proteins has been described for the free-living growth of betarhizobia, as well as for their ability to form effective symbioses with their host plants [84, 85]. Recently, Li et al. [86] also highlighted the essential role of amino acids like leucine in *Xanthomonas oryzae* pv. *oryzae* pathogen and rice interaction, besides serving as a nutrient for growth. Apart from this, there was an activation of phenylalanine pathway proteins (PheA and HisC) (Supplementary Fig. S7) by RE of intercropped *Z. mays* which are reported to be induced in *R. etli* when infecting bean plants [87]. The catabolic pathway of histidine (HutU; urocanate hydratase) was found to be downregulated upon treatment RE as similarly observed in the case of *R. leguminosarum* bv. *viciae* 3841 in the rhizosphere of host or non-host plants [88].

Furthermore, our data reflected upregulation of putative beta-lactamase family protein in treated RE of both the plants, suggesting a probable role involved in fitness advantage on *Z. mays* plants as observed similarly on the cowpea roots [89]. Interestingly, pseudoazurins proteins, blue copper proteins served as an electron donor to nitrite reductase (NirK and NirS) present in the periplasmic region of the bacteria, found predominantly in case of exposure to both the intercrop and monocrop *Z.mays* plant RE. One of the pseudoazurins was also strongly up-regulated in *Leucaena leucocephala* nodules infected by NGR234 compared to the free-living condition [90]. Till now, its role has been presumed in the denitrification process and no direct association with symbiotic interaction.

To summarize, the proteome of NGR234 was largely similar in the case of treatment with both monocrop and intercrop *Z. mays* as compared to the untreated sample. However, notable variance in proteins are related to chaperonin proteins (GroES2 and GroEL2), and efficient symbiosis/interaction (BCP, IlvD, LeuC, LeuD) are expressed significantly by intercropping *Z. mays* RE, as evident from our studies. This work identified the variation in the key primary metabolites in the RE of a legume-cereal intercropping system, and the crucial proteins of rhizobia that could be involved in positive interactions with *Z. mays* (non-legume) plants in both monocrop and intercrop conditions.

Conclusion

The RE (root exudates) of the *C. cajan-Z. mays* intercropping system showed a distinct difference in the composition of intercropped plants when compared to monocrops of individually grown plants. In particular, intercrop *C. cajan* released important metabolites like proline, and intercrop *Z. mays* released arginine and glycerol in the RE that could enhance the growth of rhizobia on *Z. mays* plants.

Proteins of NGR234 identified through proteomics related to Nod boxes (NB-2, NB-8, NB-12, NB-19), and chaperonin proteins GroES2, GroEL2 were prominently present in NGR234 when exposed to RE of intercropped *Z. mays*. Major upregulated proteins like BCP, putative nitroreductase, branched-chain amino acids (LeuC, LeuD, and IlvD) indicated that a favorable condition exists for the rhizobia to colonize on *Z. mays* plants in *C. cajan–Z. mays* intercropping system. Therefore, the current study provides new lines of evidence on the contributions of the RE in the legume-cereal intercropping system and novel insight into the understanding of rhizobia-cereal interactions. Further studies on the role of rhizobia on growth and yield in legume-cereal intercropping under pot and field conditions in the presence of the selected metabolites from the RE are required to evaluate the potential future benefits.

Declarations

Ethics approval and consent to participate Not applicable, since this article does not involve any studies with human participants or animals by any of the authors.

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

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