Plant growth promoting properties of root microbiome and multispecies interactions in plant holobionts at the level of microbial communities and metabolites

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By

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BS-MS Dual degree program

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Certificate

This is to certify that this dissertation entitled “Plant growth promoting properties of root microbiome and multispecies interactions in plant holobionts at the level of microbial communities and metabolites” towards the partial fulfillment of the BS-MS dual degree program at the Indian Institute of Science Education and Research, Pune represents original research carried out by Abhishek Anand at National university of Singapore, Singapore under the supervision of Dr. Sanjay Swarup, during the academic year 2016-2017.

29/03/17

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Declaration

I hereby declare that the matter embodied in the report entitled “Plant growth promoting properties of root microbiome and multispecies interactions in plant holobionts at the level of microbial communities and metabolites” are the results of the investigations carried out by me at the Department of Biological sciences, National university of Singapore, Singapore, NUS Environmental Research Institute, Singapore Centre for Environmental Life Science Engineering (Nanyang Technological University) under the supervision of Dr. Sanjay Swarup and the same has not been submitted elsewhere for any other degree.

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Abstract

Plants host multispecies microbial communities in and around their roots, and these root-associated microbes are important for the growth and development of plants. Through exudation, plants release up to 40% of their photosynthetically fixed carbon along with other chemical compounds in their surrounding soil. These chemical compounds help in the selection of a distinct root microbial community. Certain members of this root microbial community are considered as plant growth promoting bacteria as they help plants in nutrient acquisition, disease suppression, stress tolerance, growth promotion. Hence studying these microbial communities are of great interest as the knowledge can help us develop novel agricultural tools. But it remains challenging to study the interaction between multispecies root microbial community and plants under controlled conditions. In this study, we have developed a plant holobionts assay with *Brassica oleracea* var. *alboglabra* and used multispecies root microbial community to show plant growth promoting properties under controlled conditions. We have also reported the presence of plant growth promoting metabolic pathways in case of plants grown with root associated microbiome through metabolic profiling and analysis. Our study showed that root associated microbiota helps plant in better growth (fresh weight gain, root elongation, and secondary root growth) and development under nutrient-poor conditions (0.8% w/v water agar) in the controlled environment. The developed plant holobionts assay has the potential to serve as an important platform to test different root microbial communities for their PGP properties under controlled lab conditions by allowing concurrent metabolomics and metagenomics analysis.

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Introduction:

Food security is a global challenge and with our current agricultural practices, it will be challenging to maintain a stable food supply to meet the increasing demand. Our food economy heavily relies on extensive land use, rigorous use of chemicals, energy and water which are all limited. Also, plants are prone to environmental stresses like diseases, lack of nutrients and drought. The environment is dynamic for plants to adapt to those changes quickly or for humans to develop stable long-term strategies. With the current technologies, we have a few strategies to get better and healthier crop yields but still we lose a significant portion of it (Christou and Twyman 2004, Strange and Scott 2005). New tools are coming up based on the Plant-microbes interaction. It is a core research area with the potential to answer many fundamental questions of biology and also to help us develop novel agricultural tools. Plant microbes interactome is a goldmine for bioactive compounds which can be used as additives or biocontrol but it is not very easy to study these (Ortiz-Castro, Contreras-Cornejo et al. 2009). An Interactome is classically defined as the whole set of molecular interactions within a biological system. The interactome depends on many factors; like temperature, pH, salinity, nutrients availability, soil type, plant species, microbiota and the databases are limited (H. 2009, Ortiz-Castro, Contreras-Cornejo et al. 2009). So there is a need to better understand this interaction. The current state of the art technology under development is using synthetic microbial consortia based on soft ecological principles as an additive in the soil for better and healthier plant growth(Niu, Paulson et al. 2017).

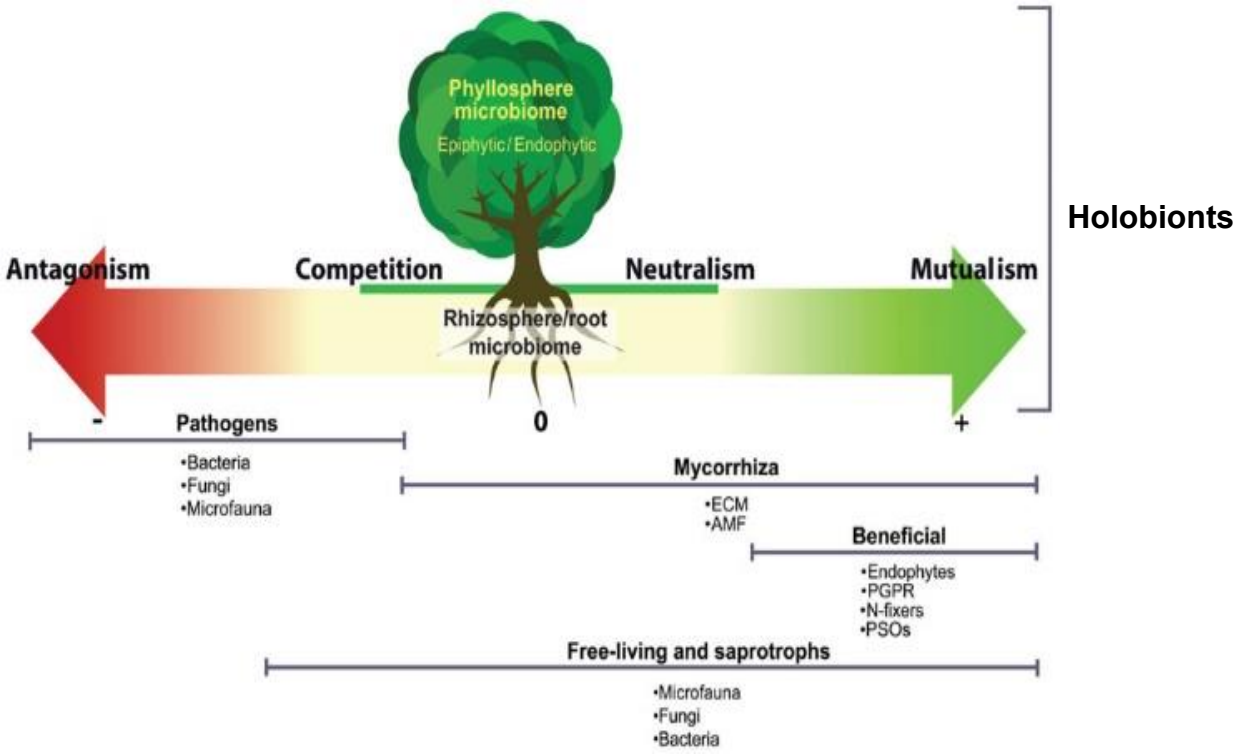

Figure 1: Plant-microbes interaction: various types of interactions are present in plant-microbiome interactions like pathogenic, beneficial, competition. Image source: (Quiza, St-Arnaud et al. 2015).

Losses to crops that threaten food security is heavily dependent on the environment. The environment introduces both biotic and abiotic stresses to plants. The survivability of plants depends on how quickly they adapt to these stresses and maintain their normal growth and development. Being sessile, plants must sense the changes in the environment and modulate their root exudates. Plants can release up to 40% of their photosynthetically fixed carbon along with other secondary metabolites to their immediate surrounding and this helps to select a distinct microbial community near the plant roots (Fig. 2)(Harsh Pal Bais 2004, Haichar, Marol et al. 2008, Ortiz-Castro, Contreras-Cornejo et al. 2009, Bulgarelli, Rott et al. 2012, Lundberg, Lebeis et al. 2012, Lebeis, Paredes et al. 2015). For better growth and development, plants require a considerable amount of nutrients and minerals which are not readily available for plants

to use. The selected microbial community helps plant in acquiring those as well as helps to counter pathogenic attacks on plants and also provide tolerance towards abiotic stresses(de Zelicourt 2013). This symbiotic relationship helps plant in better growth as well as microbes to get fixed carbon while excluding other microbes not associated with plants. Microbes of this category are commonly referred to as plant growth promoting bacteria or fungi (PGPB/PGPF). It is noted in the literature that these beneficial microbes activate common plant signalling pathways which help plants to better develop and grow (Fig. 3)(Corradi and Bonfante 2012). Studies suggest that plant growth promoting bacteria can gel with the plant innate immune system and provide protection from pathogens. Plant microbes interaction is a dynamic network of thousands of compounds released by plants and microbes depending upon their interaction and their immediate surroundings(Haichar, Marol et al. 2008). Hence, plant-associated microbes present a promising avenue to improve plant growth without additional nutrient inputs while mitigating losses due to environmental stress.

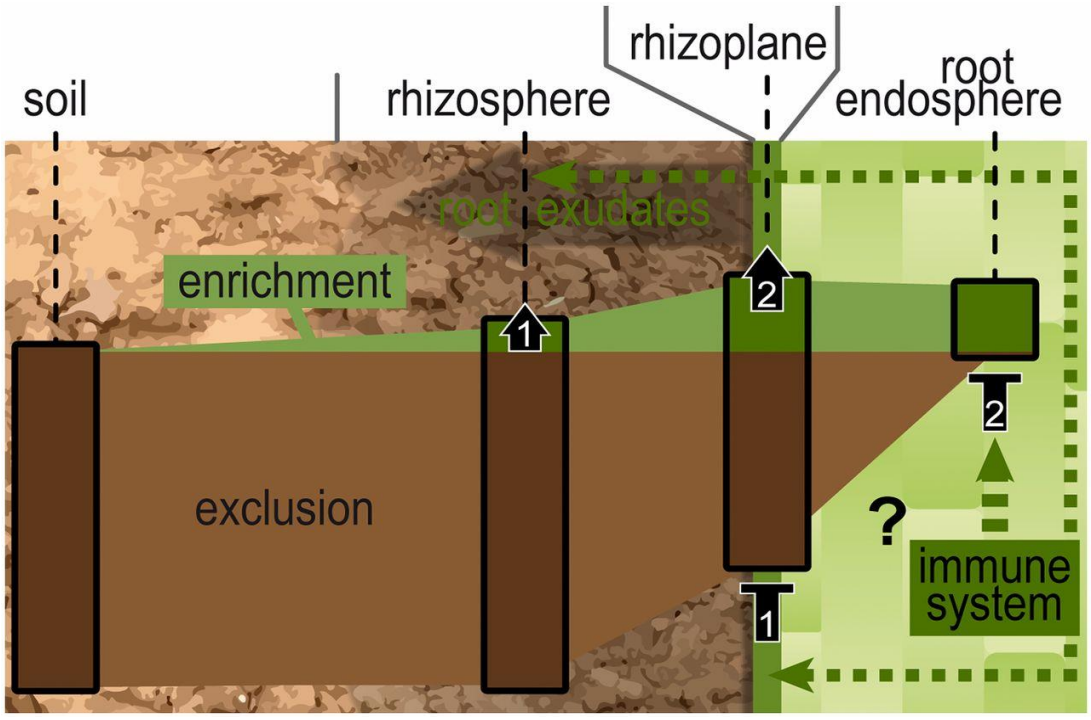

Figure 2: Recruitment of different root zone microbes from soil microbiota. Image source: (Schlaeppi 2015).

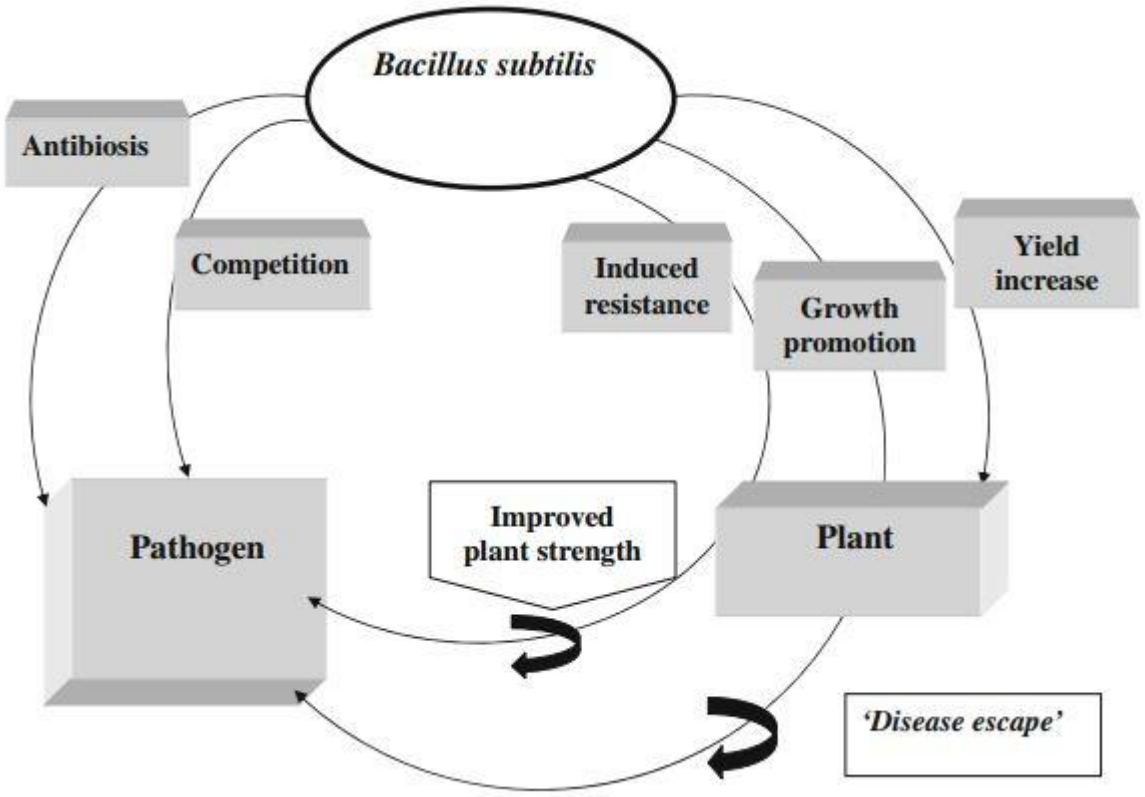

Figure 3: Mechanism of promoting plant growth by Bacillus subtilis. Image source: (Bhattacharyya and Jha 2012).

In order to better utilise plant-associated microbes, it is useful to view plants and their associated microbes as a holistic entity: a plant holobiont. A recent perspective of plant holobionts has tried to describe this interaction as a single biological unit rather than considering plant and microbes as different units (Vandenkoornhuyse, Quaiser et al. 2015, Hacquard 2016). Associated microbes are classically divided into three groups along the soil-root axis; microbes which are present in the soil surrounding the roots and influenced by root exudates are considered as rhizosphere microbes, microbes which are capable of long-term colonization of the root surface are rhizoplane microbes, and the microorganisms which are able to enter through the root tissues and inhabit inside them are known as endophytes(Edwards, Johnson et al. 2015). Many studies were able to demonstrate the beneficial effects of the specific and individual members of root

microbiota. Single species like *Pseudomonas fluorescen, Bacillus pumilus, Acinetobacter* were used to understand the reasons behind plant growth promotion and many of these were adapted for field trials(Loon 2007, de Zelicourt 2013). Although these studies were able to uncover some fundamental science behind plant microbes interaction but they were not very successful during field trials. One of the usual cause is the stable root microbiome outcompete an artificially introduced species. Introducing synthetic microbial consortia seems like another option as they provide a close to nature scenarios with dynamic network although it is hard to test this under controlled conditions and in a reproducible manner. A Recent paper by Ben Niu et al. has tried to develop a simplified and representative bacterial community to understand the beneficial effects of root microbiota on the host(Niu, Paulson et al. 2017). Hence, the field is advancing towards studying complex and multi-species model of plants and their associated microbes beyond simple pairs of plants and single microbial species.

The major research question for us is to understand how the root microbiome affects the plant growth and development under lab conditions. What are the chemical compounds involved in this multispecies interaction and what is the community level structure in case of root and bulk soil microbes? We can also understand the effect of microbiome on the root architecture or other plant phenotypes. Identifying the bacterial and plant genes that shape the functional output of the root microbiome can help us understand the molecular basis of this plant-microbes interaction. Understanding the stress adaptive regulatory network reprogrammed by beneficial microbes is one of the most important outcomes of understanding plant holobionts. Together these answers can help us develop novel agricultural tools and practices.

Arabidopsis is a common model plant for studies but it is difficult to use it for our plant holobionts assay due to its small size, technical difficulties and comparatively slow growth. We chose another model from the same Brassicaceae family of food crops. Kai-Lan (*Brassica oleracea* var. *alboglabra*) is an economically important vegetable (Fig. 4). It is used as a major food portion in Singapore (Scott, Galicia-Connolly et al. 2012). Brassica vegetables are commonly known for their high nutritional values like vitamin C, soluble fibre, multiple nutrients, and Indole-3-carbinol (Bradshaw 1983,

Mucha-Pelzer, Mewis et al. 2010). These vegetables are easy and fast to grow under laboratory conditions. Low-cost seeds are easily available. Larger seeds and seedlings size make it more suitable for us. Also due to the scarcity of land but high demand, Singapore has a major interest in increasing their yields. Hence we have used *Brassica oleracea* var. *alboglabra* (Kai-Lan) to develop our plant holobionts assay and study the plant growth promoting properties of root microbiome and multispecies interaction at the microbial community and metabolites level.

Figure 4: Kai-Lan seedlings grown at Kok-Fah farm, Singapore.

Materials and Methods:

Plant and soil materials:

The seeds of *B. oleracea* var. *alboglabra* (Kai-Lan) and *B. rapa* var*. parachinesis* (Choy-Sum) were obtained from Ban Lee Huat seed Private Limited and used for all the experiments. We have used potting soil mixture from Tref Universal potting mix from Far East Flora Holdings Pte Ltd (Singapore). Major constituents of the soil were white peat/black peat with a pH range of 5.0-6.0. Nitrogen (17), phosphorous (10) and potassium (14) content was 1.3kg/m3.

Seeds sterilisation and germination:

The seeds of *B. oleracea* var. *alboglabra* and *B*. *rapa* var. *parachinesis* were imbibed in sterile MilliQ (MQ) water for 60 minutes before surface sterilising. 50% bleach with 0.01% Tween® in a 2ml solution was used to surface sterilise seeds for 5 minutes. After incubation, six washes of sterile MQ water was given before spreading seeds on 1X MS (Murashige & Skoog) agar/ 0.8% w/v water agar plates (Table 1). The seeds were kept along the central horizontal line of the square plate to provide space for root and shoot growth (5 seeds per plate). The plated seeds were then transferred to growth chamber for required number of days (4-7 days).

Growth chamber parameters were 21°C± 2 with 16/8 hours of light/dark cycle. *B. oleracea* var. *alboglabra* and *B. rapa* var. *parachinesis* seeds were grown for 3-4 days before transplanting them to soil pots. After transplantation, the seedlings were grown in pots for 7 days in growth chamber.

Table 1: Media composition

Murashige and Skoog (MS) media	4.4 g/L MS salt, 0.5 g/L MES salt, and 5 g/L sucrose
Water agar media	8g/L Agarose

Microbiome harvesting

Four-day-old seedlings were transplanted to pots and grown for 7 days under controlled conditions in a growth chamber. Four seedlings were planted per pot (Fig. 6). After 7 days, the plants were carefully taken out from pots for microbiome retrieval. Using sterile tweezers, we removed as much soil as possible leaving around 1mm of soil around roots. For each sample around 200mg of roots was used and 2ml of 1X PBS was added. The samples were vortexed for 1 minute at full speed. Brief spin was given to settle soil and root. The supernatant was collected in a fresh 15ml falcon (Tube A). To collect rhizoplane bacteria, we added 5ml of 1X PBS to the previous tube and 5 sonication cycles (3/5 seconds on/off cycle with 21% amplitude) were given to detach the microbes(Edwards, Johnson et al. 2015). After vortexing for 1 min followed by brief spin, the supernatant was collected to tube A. To collect endosphere bacteria, we followed similar steps as above and added 5ml of 1X PBS to the previous tube and 5 sonication cycles (3/5 seconds on/off cycle with 21% amplitude) were given to detach the endophytes (Fig. 5) (Edwards, Johnson et al. 2015). After vortexing for 1 min and brief spin, the supernatant was collected to tube A (Edwards, Johnson et al. 2015). Tube A was left for 20 minutes in the hood to allow soil particles to settle, the supernatant was then transferred to new tubes and final spin of 10000g for 10 mins was given to collect the root microbes as the microbial inoculant.

To harvest bulk soil microbiome we transferred ~200mg of soil to a new 15ml tube with 2 ml 1X PBS. Vortex for 1 min and briefly spin down and transfer the supernatant to a new 15 ml tube. A similar treatment of sonication was given to soil as root microbes collection for procedural control. A final 10min 10000g spin was given to collect the soil microbes.

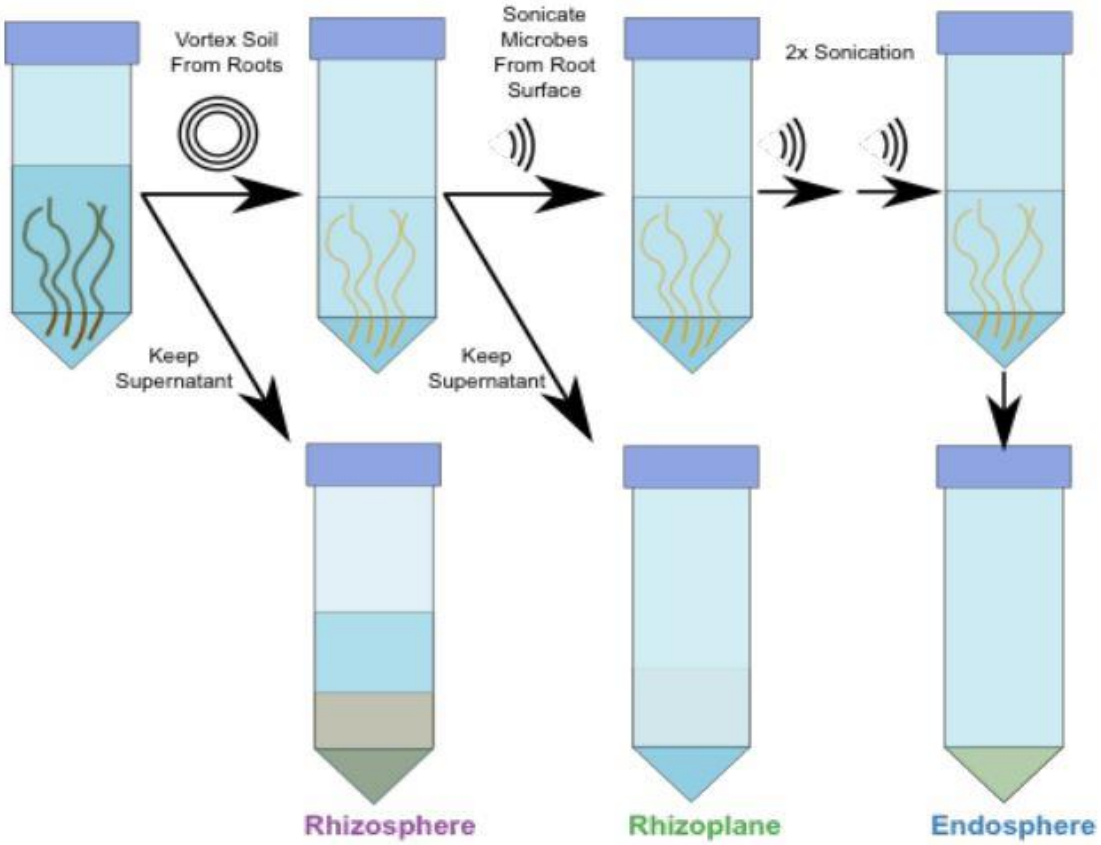

Figure 5: Protocol for harvesting microbes from different root compartments. Image source: (Edwards, Johnson et al. 2015).

Figure 6: Four days old seedlings in pots.

Plant holobionts assay

For the plant holobionts assay (PHA) (Fig. 8), we used freshly harvested soil and root microbiome. The bacterial pellet in tube A was resuspended in 5ml 1X PBS and100µl (10^5-10^6 cells) of this sample was used to inoculate on the water agar plates. The microbiome was then covered with a dialysis tubing (Sigma-Aldrich) with a molecular weight cut-off of 14kDa. Similar looking 3-4 days old seedlings of Kai-Lan were transplanted over the membrane. 1 seedling per sample was used. The plates were then transferred to the growth chamber for 7 days.

B. oleracea var. alboglabra	R	R	R	S	S	S	B	B	B
B. rapa var. parachinesis	R	R	R	S	S	S	B	B	B
R = root microbiome S = soil microbiome B = buffer control									

Figure 7: PHA sample matrix for one batch of the experiment. 1 assay represents 9 independent samples from one vegetable type.

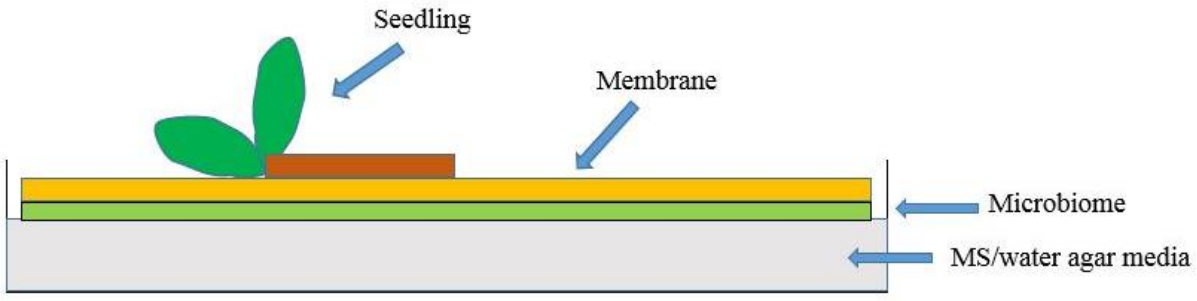

Figure 8: A representative image of 1 sample of PHA.

Plant phenotype analysis

The plant holobiont assay plates were scanned using Canon Scanner (CanoScan 8800F) at a resolution of 300dpi. The images were processed using ImageJ software(Schneider, Rasband et al. 2012) for all the root length measurements. A ruler was used with each scan as an external scale to set/calibrate software. Wiggle mode was used to follow the roots closely and do the final measurements. A dark background was used for better clarity.

On Day 0 of the assay, before transplanting seedlings on the membrane, an initial weight measurement was done using analytical weighing balance. After 7 days, we re-measured the plant's fresh weight. Percentage change in plant's fresh weight was calculated using the formula

$$\%\ change = \frac{final\ fresh\ weight - initial\ fresh\ weight}{\text{initial fresh weight}} * 100$$

Fold change was calculated using the formula

$$Fold\ change = \frac{final\ fresh\ weight - initial\ fresh\ weight}{\text{initial fresh weight}}$$

Flow cytometry

For flow cytometry, we used 500µl of the sample from root and soil microbiome from the 5ml stock. 0.75µl of SYTO9 (5µm stock) was used for staining the cells. The samples were incubated for 15 minutes in the dark, followed by analysis using FACS (AttuneTM NxT acoustic focusing cytometer, Invitrogen TM). One sample was kept unstained for each type as a control. A quality control sample was also prepared by pooling a small volume of all samples to account for autofluorescence and reduce noise in the data.

Metabolomics

Metabolites extractions

After plant holobionts assay (7 days), the plant roots were transferred to fresh 15ml falcon tubes with 5ml 1X PBS and put on a shaker for 120 minutes. The samples were briefly spun and the supernatant was collected in a fresh 15ml Falcon tube and kept overnight at -80 °C. The samples were then concentrated using Labconco lyophilizer.

Mass spectrometry of metabolites

The lyophilized samples were re-suspended in 0.1% formic acid and used for mass spectrometry after filter sterilising with a 0.2µm filter. A QC was prepared by pooling equal proportions from each sample. To achieve metabolites separation, a gradient cycle of 16 minutes with a flow rate of 300µl/min was used(Narasimhan, Basheer et al. 2003). Agilent C18 Eclipse 1.8u 2.1x100mm column was used for separating metabolites. An injection volume of 3µl was used. For mobile phase solvents, we used 0.1% formic acid and acetonitrile in 0.1% formic acid. Samples were eluted on a step gradient with 98% formic acid for 1 min, followed by an increasing concentration of solvent B to 98 % over 11 mins. This was held for 1 min followed by a drop to 2% solvent B within 0.5 min. Solvent B was held at 2% for 2.5 min before the end of the run. The total run time was 15 min. Instrument methods: Full scan at 60000 resolution on the FTMS to scan a mass range between 50 to 1500 m/z. The instrument was operated in the positive polarity mode and a Profile scan was captured.

Orbitrap Velos Pro (Thermo) was used for untargeted mass profiling of metabolites. We used HESI probe in positive mode of ionization. UHPLC system with column was connected with mass spectrometry. We had a sample run time of 15 min. The method parameters were 1) capillary temperature was set at 300°C, 2) heater temperature at 300ºC with flow rates of sheath gas and aux gas at 45 L/min and 15 L/mins respectively 3) Capillary voltage was set at 4 kV. Data acquisition was done under profile mode for MS scan with the mass range set to 50-1500 *m/z*. Three biological replicates were used for the experiment with two analytical replicates of each sample (Fig. 9).

Mass spectrometry data analysis

The raw files were processed using XCMS, XCMS online and METLIN (Tautenhahn, Patti et al. 2012, Zhu, Schultz et al. 2013). Features with p-value above 0.05, peak width below 1s and fold-change below 1.5 times were filtered off from the putative annotation. Raw data obtained after the mass spectrometry experiment was converted into a mzXML format using ProteoWizard Software. The files were then uploaded to Online XCMS server for analysis. The software version; XCMSOnline version 2.2.5, XCMS version 1.47.3, CAMERA version 1.26.0. The parameters used were retention time – in minutes, polarity – positive, Centwave method for feature detection with a minimum peak width of 10 and maximum peak width of 60. ANOVA statistical test was used followed by posthoc analysis with a p-value threshold of 0.05 and fold change of 1.5. Arabidopsis database was used for pathway analysis and metabolites annotation.

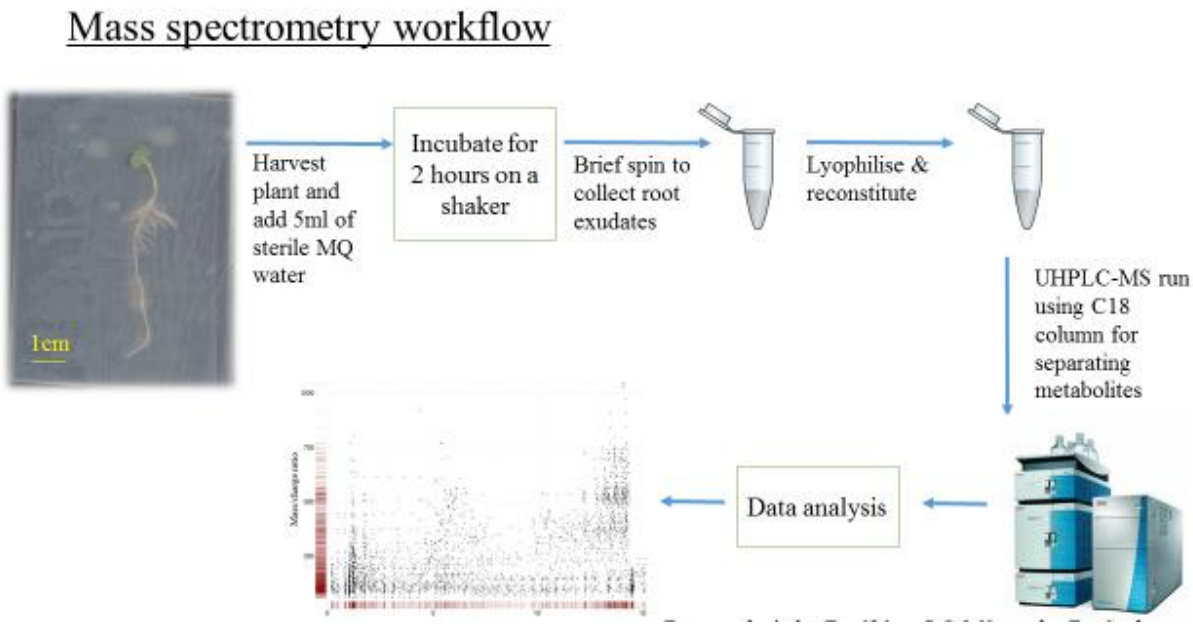

Figure 9: Mass spectrometry experiment workflow.

Microbiome composition analysis

Genomic DNA extraction

After removing plant and membrane from the assay plate, microbes were harvested by scrapping the agar plate surface with sterile inoculating loops and collected in 1X PBS. This was followed by gDNA extraction using ZR Soil Microbe DNA MiniPrep™ (Zymo Research). The gDNA concentration was analysed using the Qubit® 2.0 Fluorometer (Invitrogen) and Qubit® dsDNA HS Assay Kit.

16S ribosomal RNA gene amplicon sequencing

For 16S rRNA gene sequencing we used the following primers to amplify V7-8 region: Forward (5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-aaactYaaaKgaattgacgg-3') and reverse (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGacgggcggtgtgtRc-3'). We used 30ng of gDNA to do PCR amplification. PCR conditions were 95 °C for 10', 95 °C for 30'', 55 °C for 40', 72 °C for 40', 72°C for 5' and 10 °C for standby. We ran it for 34 cycles. To clean up amplified PCR products we used GeneJET PCR Purification Kit. Gel electrophoresis (1% agarose gel) was done to do a quality check of the amplified products (Fig. 10) and Qubit® 2.0 Fluorometer (Invitrogen) was used to quantitate the DNA concentrations. The samples were then submitted for Miseq sequencing (Illumina, SCELSE sequencing facility, NTU, Singapore).

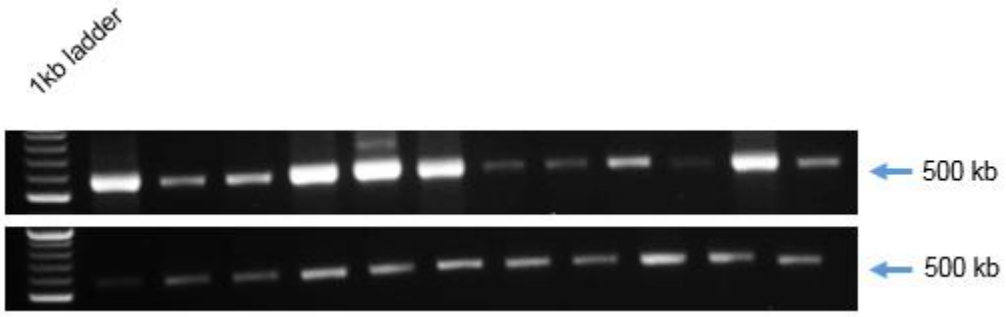

Figure 10: PCR amplified 16S rRNA gene V7-V8 region (500kb amplicons). Samples with above 5ng/µl concentration were sent for Illumina sequencing.

Results and discussion:

Plant holobionts assay

To understand the effects of root microbiome and soil microbiome on plant growth, we developed Plant holobionts assay (PHA) for vegetables. This assay can help us study plant phenotype changes, collect metabolites exchanged between plants and microbes and study the root and soil microbial community using imaging and multi-omics tools. We used 0.8% Agarose media as the platform to culture the complex plant-associated microbiome. Microbiome was covered using dialysis membrane which had a molecular cutoff of 14kDa. Single 3-4 days old seedling was transplanted over the membrane per sample (Fig. 8). In the final protocol, we used agarose media as we wanted to test the effects of root microbiome under nutrient-poor conditions. Also, we wanted to avoid the possibility of microbes or plants relying on growth media and to maintain a strict dependency on each other (plants & microbes). The membrane was introduced to avoid any direct contact between plant roots and microbes and only small metabolites exchange can happen. This also allowed us to do an easy collection of root associated metabolites. Lastly, plants and the cultured microbiome were grown in transparent Petri plates that allowed images can be taken at intervals and then analyzed for plant phenotype changes or to analyse growth rate profile.

Using this assay, we were able to show that under nutrient-poor conditions root microbiome supports plant growth and development when compared to soil microbiome (Fig. 15,16). We were also able to capture a number microbial taxa on the agar plates which were associated with plant growth promotion. This assay can be further used to test multiple questions like which are the keystone species in this community and what could be a simplified but representative root microbial community to enhance plant growth and development. Also, we could capture the metabolites exchanged between plant and roots under the influence of root and soil microbiota and understand the metabolites level interaction between microbes and plant under different microbiomes like root and soil microbiome.

A number of optimisations were done to develop PHA for vegetables. The assay is a 4 component system consisting of media, microbiome, membrane and seedling. We have tested this assay for MS media (Fig. 11,12), root exudates media (Fig. 13) and agarose media (Fig. 15,16,18). Also, we have tested rhizosphere (Fig. 18-a)), rhizoplane (Fig. 18-b)), endosphere (Fig. 18-c)) and root microbiome (by pooling rhizosphere, rhizoplane and endosphere microbes) as our microbiome inoculant (fig 13, 15, 16). We have also tested our hypothesis for vegetables at seed and seedlings stage (Fig 12). We kept membrane component constant across all our experiments. We could not see a significant plant growth enhancement in any case except when we used agarose as the platform and freshly harvested microbiome for inoculation.

Growth media played a major role in the outcome of this plant-microbiome co-culture assay. It is known that root exudates and plant microbes interaction depend on the environmental factors (Haichar, Marol et al. 2008, Luo, Zhao et al. 2012, Baetz and Martinoia 2014, Schreiter, Ding et al. 2014). Using full strength plant growth MS media leads to the overwhelming growth of microbes and it is possible that unwanted microbes have outcompeted the required, less abundant microbes before they could show any plant growth promoting effects. The vegetables are fast growing and the media is rich so it is possible that having symbiotic relationship with plants is not required anymore and hence no significant difference between plants grown with root microbiome and soil microbiome. One of the other possibilities that are relevant here is the enrichment of microbes using MS media, exudates media or PBS can affect the microbiome composition and the rhizobacteria are unable to stand that competition over the period of enrichment. Overall, plants-microbiome interactions were most evident in the nutrient-poor conditions, which likely fostered stronger plant-microbial symbiosis that resulted in plant growth promotion despite limiting nutrients. This further suggests a stress tolerance benefit from root-associated microbes compared to bulk soil microbes.

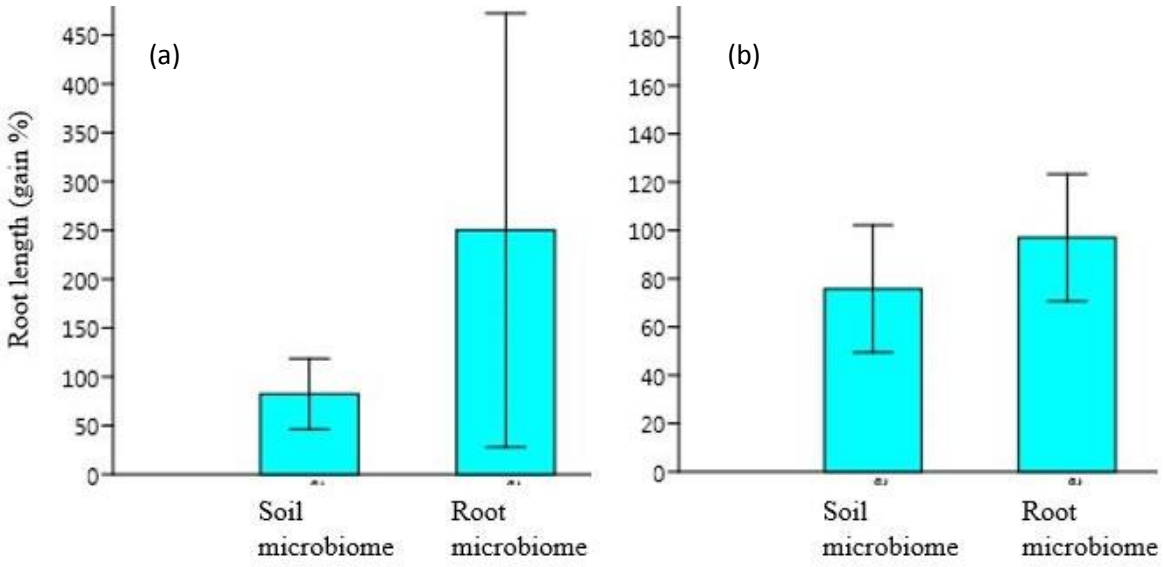

Figure 11: Root length analysis (a) *B. oleracea* var. *alboglabra* (b) *B. rapa* var. *parachinesis*. The seeds were kept for 3days at 4 °C in the dark before transferring them to growth chamber for 1 day. The four days old seedlings were then transplanted to soil pots and grown for 7 days. The microbes were then harvested for assay. The harvested microbiomes were incubated in MS broth for 3 days for enrichment before doing PHA. ANOVA (analysis of variance) statistical test was done to test the significance. Mean was plotted using the software Past 3.0. Error bars represent the standard error between samples. 3 experimental replicates with 2 biological replicates each.

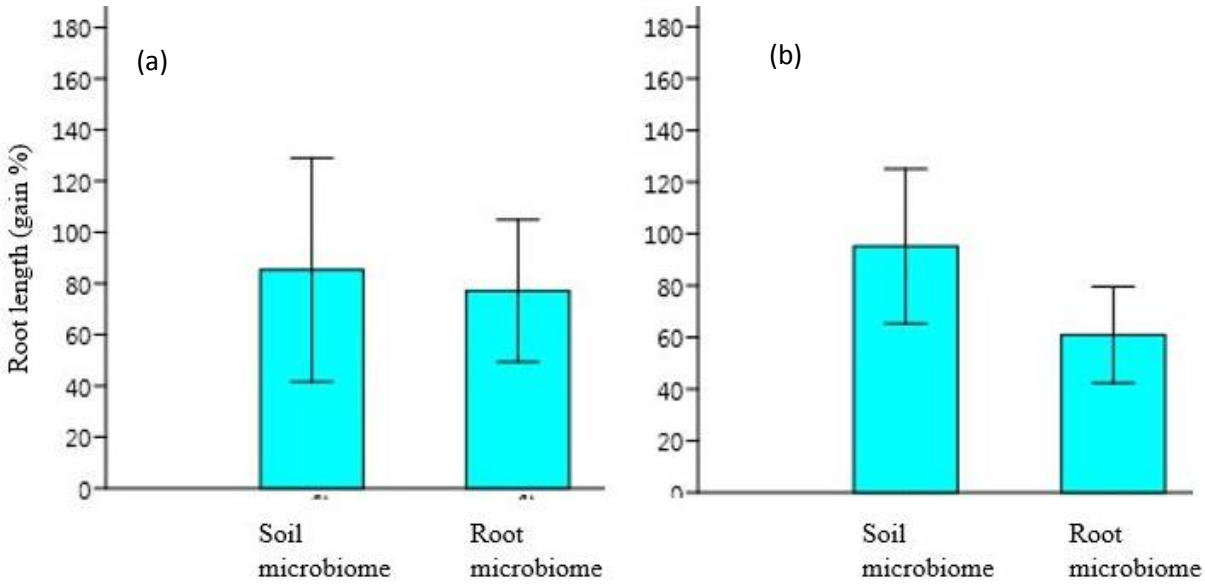

Figure 12: Root length analysis (a) *B. oleracea* var. *alboglabra* (b) *B. rapa* var. *parachinesis*. The seeds were grown directly in soil pots for 7days in growth chamber. 7 days old seedling were then used to harvest microbiome. Microbiome was incubated for 3 days in MS broth for enrichment before inoculating on MS media plates for PHA. ANOVA (analysis of variance) statistical test was done to test the significance. Mean was plotted using the software Past 3.0. Error bars represent the standard error between samples. 2 experimental replicates with 2 biological replicates each.

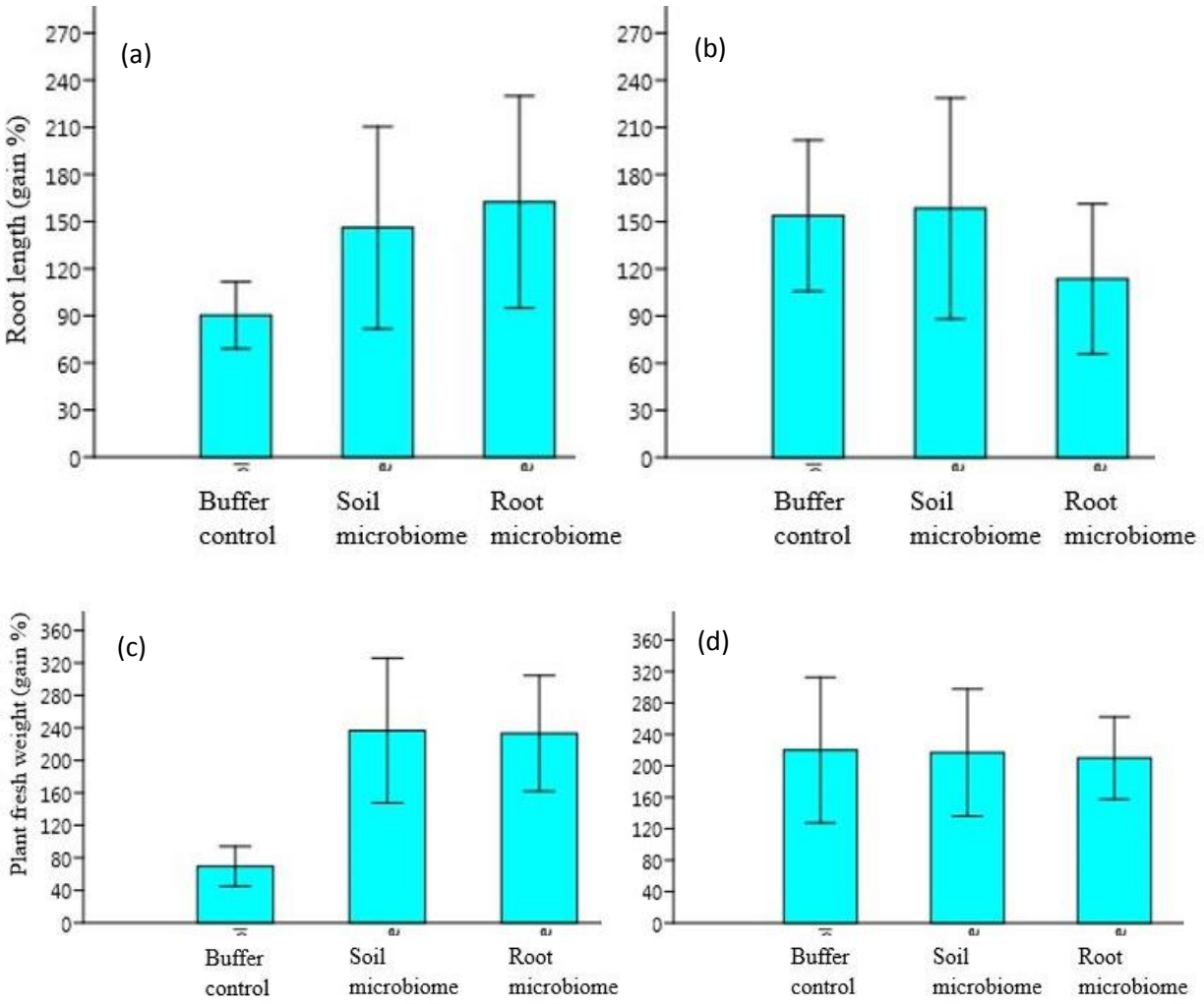

Figure 13: Root length analysis (a) *B. oleracea* var. *alboglabra* (b) *B. rapa* var. *parachinesis*. Plant's fresh weight analysis (c) *B. oleracea* var. *alboglabra* (d) *B. rapa* var. *parachinesis*. Plant holobionts assay using exudates as media component was done in triplicates. In this experiment, 0.8% agarose media with plant root exudates was used. Root exudates were harvested from vegetables grown for 7 days on circular dishes with agar media (60-70 seedlings per batch). The exudates were concentrated 50 times and then it was used as a nutrient source for the microbes in the PHA. Microbiome was incubated for 3 days in exudates media before inoculating on the media plates. ANOVA (analysis of variance) statistical test was done to test the significance. Mean was plotted using the software Past 3.0. Error bars represent the standard error between samples. 3 experimental replicates with 2 biological replicates each.

Having optimised the PHA, we used agarose media with freshly harvested microbes. Here we developed a plant holobionts assay for vegetables to study plant growth promoting effects of root microbiome and multispecies plant-microbes interaction at the level of microbial community and metabolites level (Fig. 14).

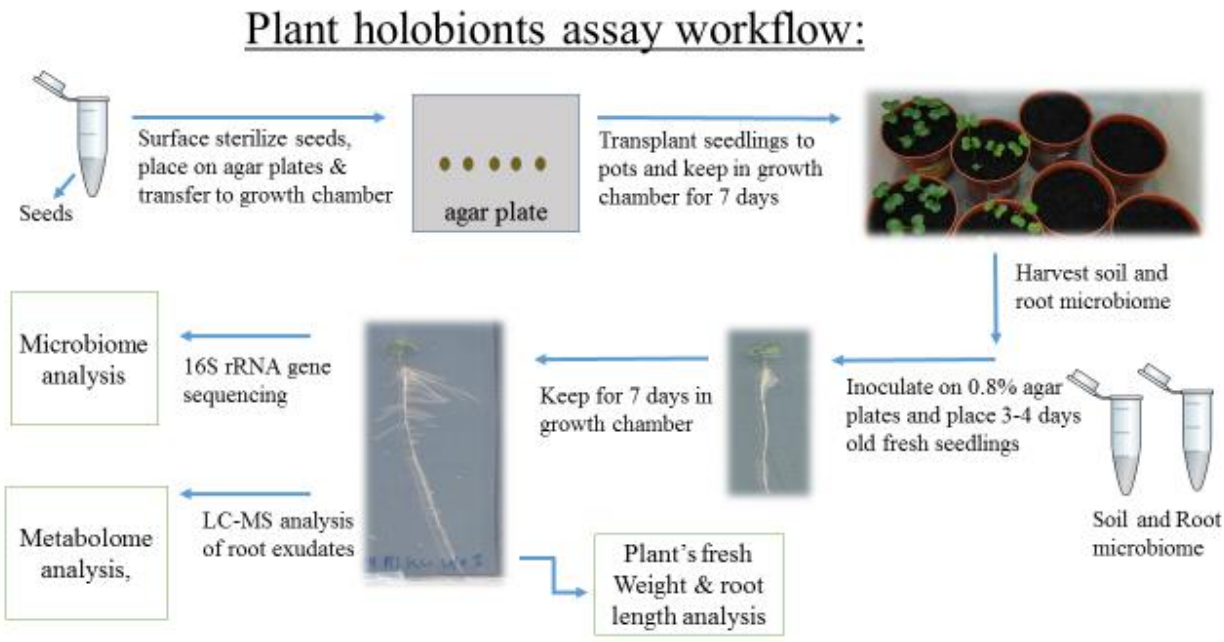

Figure 14: The workflow for plant holobionts assay.

Root-associated microbiota helps plant in better growth and development

To identify the effects of microbiome, we did plant holobionts assay with root microbiome, soil microbiome and buffer (control). We performed three experimental replicates. Experimental replicate (ER) 1 had 3 biological replicates, ER2 and ER3 had 6 biological replicates each. Microbiome treatment was given for 7 days in growth chamber followed by collecting samples for plant's fresh weight, plant root exudates, metabolites on the membrane and gDNA extraction from root and soil microbes (from assay plates).

Fresh weight analysis

The effect of root microbiome and soil microbiome on plant growth was calculated by measuring plant's fresh weight change (gain %) by comparing its initial and final weight. An initial plant weight was measured under sterile conditions before transplanting seedlings on the assay plates. After 7 days we re-measured the plant's fresh weight and used it to calculate the percentage change in plant's fresh weight. The data shows that plants grown with root microbiome have better growth and development under nutrient-poor conditions (*B. oleracea* var. *alboglabra*) when compared to the soil microbiome (Fig 15,16). Root microbiome has also shown better secondary root growth and greater gain in overall biomass (Fig. 17). Root microbiome has shown around 2-fold increase in plant's fresh weight as compared to 1.2-fold in soil microbiome. Data analysis was conducted using ANOVA followed by posthoc analysis. Previous studies have also shown better plant growth using single species (Bressan 2003, Bhattacharyya and Jha 2012, de Zelicourt 2013, Haney, Samuel et al. 2015). But here we were able to show plant growth promoting effects using multispecies root microbiome under lab conditions using *in-vitro* plant holobionts assay for vegetables. We were able to grow a complex microbial community on agarose plates (culturable species). Similarly, we tested this hypothesis for rhizosphere, rhizoplane and endosphere microbes. A similar PHA was done. The data shows that in the case of rhizosphere microbes, the plants have shown better growth (Fig. 18-a). Plants grown with rhizosphere microbes have shown 2.2-fold increase in their fresh weight as compared to 1.2-fold increase in plants grown with soil microbiome. We hypothesise that rhizosphere microbes have a greater number of plant growth-promoting microbes or they can produce/activate better plant growth promoting metabolites as compared to rhizoplane microbes and endophytes. One of the limitations of our system is using membrane as it does not allow direct contact of microbes with plants, hence rhizoplane and endosphere microbes cannot reach their normal habitat root zone and this might affect their functions.

Having shown that plants grow better when cultured with their associated microbes, we sought to understand how root microbiome assists in plant growth at the mechanistic level through studying their metabolites exchange, we harvested plant root exudates

and metabolites which were present on the membrane. The samples were then processed through our lab's metabolomics pipeline and analysed. Similarly to identify the microbial community structure which is associated with plant growth promoting properties we extracted gDNA from the microbes harvested from assay plates. The samples were then processed for 16S rRNA gene sequencing.

In summary, fresh weight analysis of plant holobionts assay suggests that plants grow significantly better in the presence of root-associated microbes compared to bulk soil microbes under lab conditions.

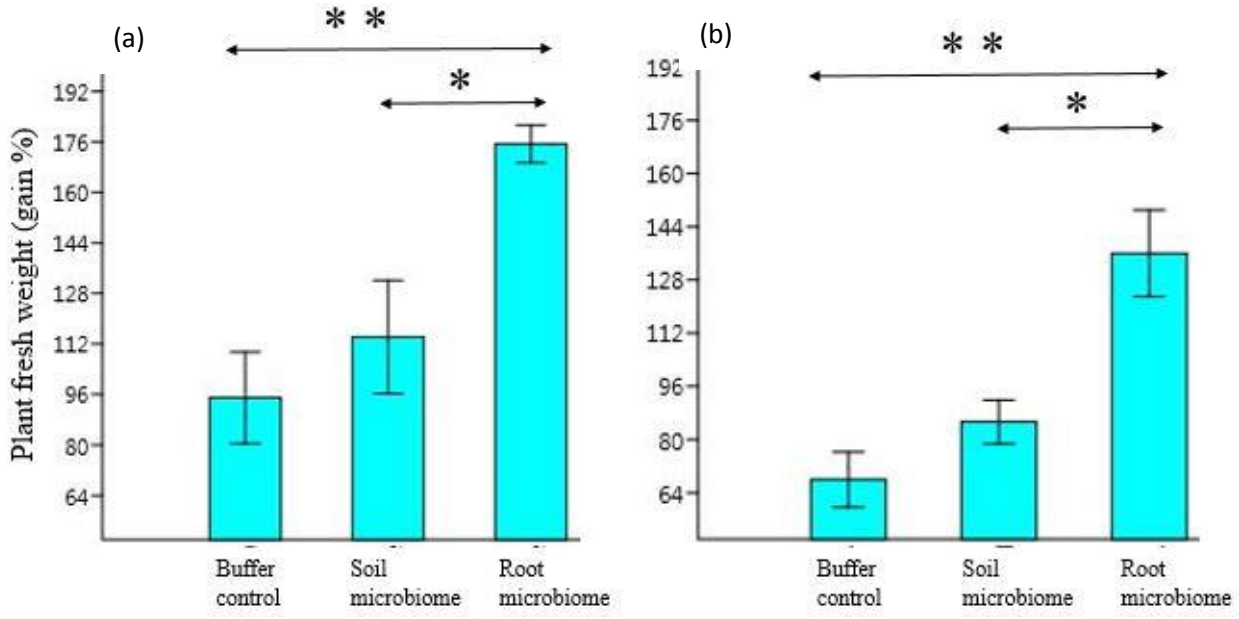

Figure 15: *B. oleracea* var. *alboglabra*'s fresh weight analysis for experimental replicates (ER), ER1 (a) and ER2 (b). Using root microbiome shows significantly better plant growth compared to soil microbiome. Freshly harvested root associated/ soil microbes (from seedlings grown in pot for 7 days, no microbiome incubation) were inoculated over 0.8% w/v agarose media and tested for their help in better plant growth. ANOVA (analysis of variance) statistical test was done followed by posthoc analysis using Tukey's pairwise test. Mean was plotted using the software Past 3.0. Error bars represent the standard error between samples. n = 3 (ER1) and n = 6 (ER2). p-value codes; * = p-value ≤ 0.05, ** = p-value ≤ 0.01, ***≤0.001

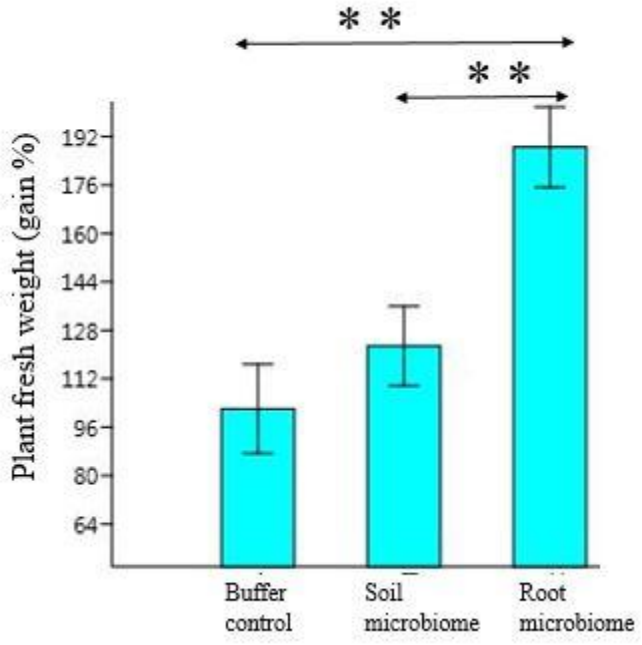

Figure 16: *B. oleracea* var. *alboglabra*'s fresh weight analysis for experimental replicates 3 (ER3). Using root microbiome shows significantly better plant growth compared to soil microbiome. Freshly harvested root associated/ soil microbes (from seedlings grown in pot for 7 days, no microbiome incubation) were inoculated over 0.8% w/v agarose media and tested for their help in better plant growth. ANOVA (analysis of variance) statistical test was done followed by posthoc analysis using Tukey's pairwise test. Mean was plotted using the software Past 3.0. Error bars represent the standard error between samples. n = 6 (ER3). P-value codes; * = p-value ≤ 0.05, ** = p-value ≤ 0.01, ***≤0.001

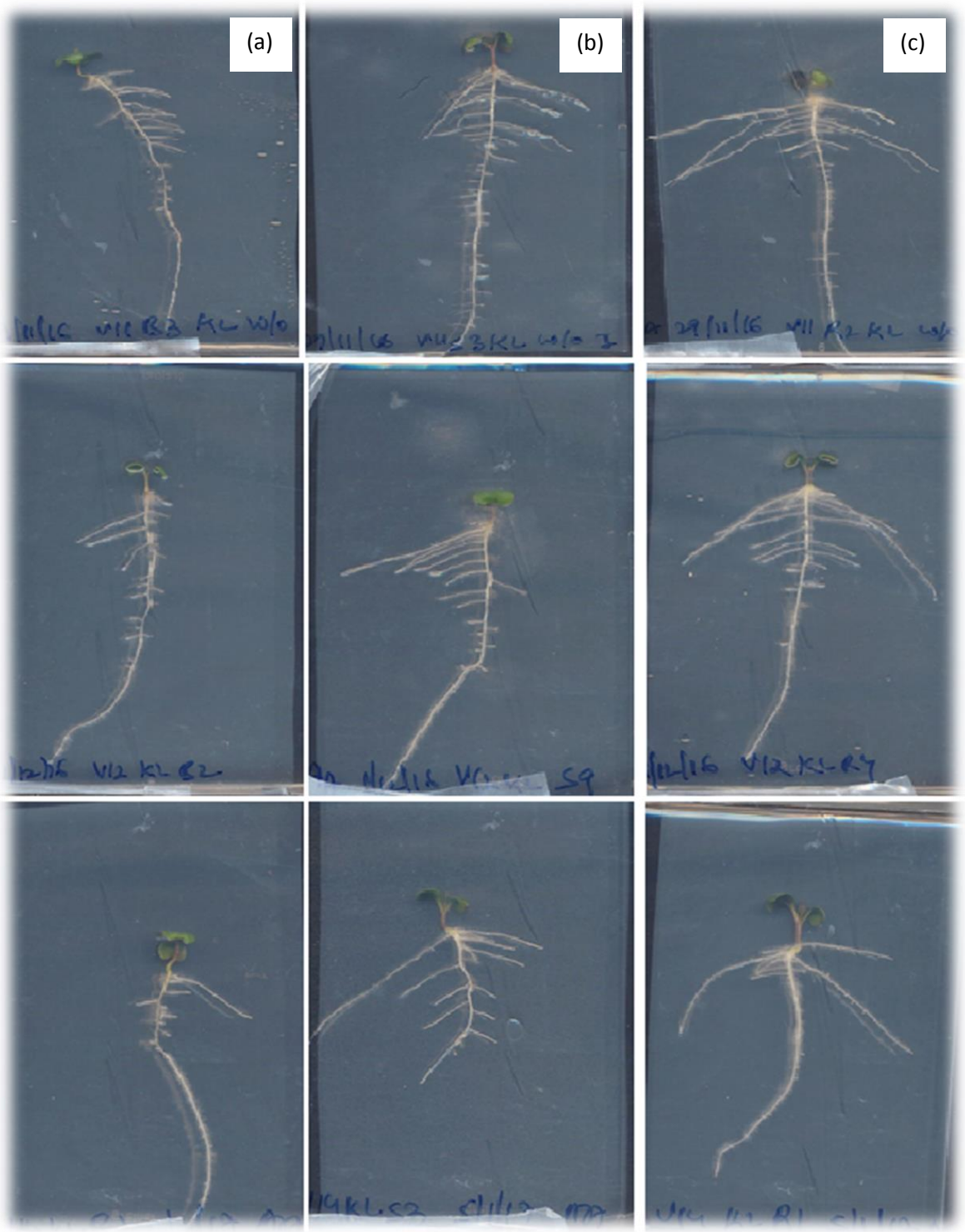

Figure 17: Plant holobionts assay images. Column (a) Plants grown with buffer, Column (b) Plants grown with soil microbiome, Column (c) Plants grown with root microbiome. The plants grown with root associated microbiome shows better secondary root growth as compared to plants grown with soil microbiome or buffer.

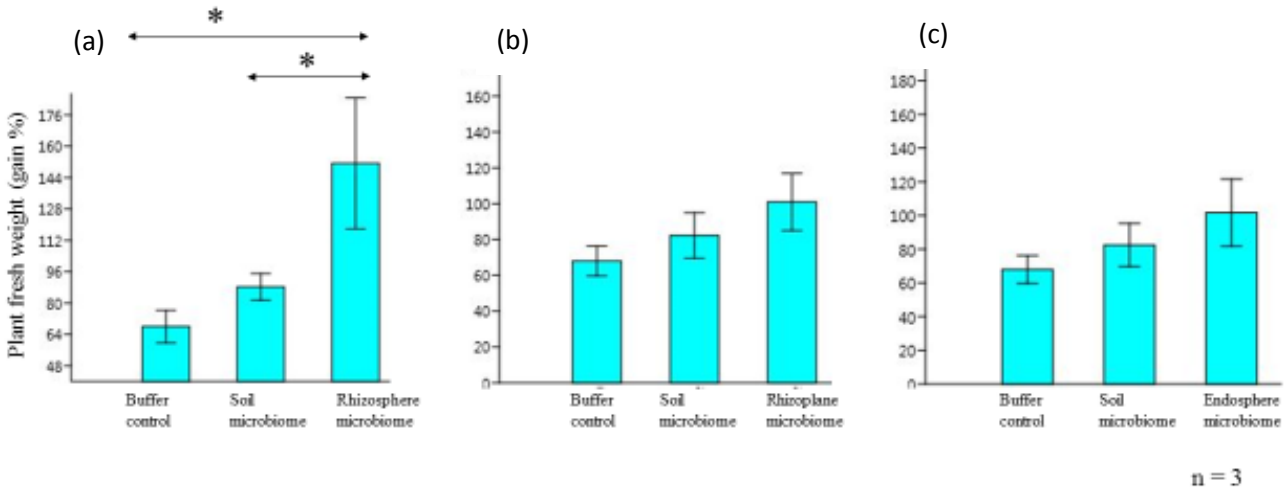

Figure 18: *B. oleracea* var. *alboglabra*'s fresh weight analysis for plants grown with rhizosphere (a), rhizoplane (b) and endosphere (c) microbes. Rhizosphere microbes have shown significantly better plant growth compared to soil microbiome. Freshly harvested root associated/ soil microbes were inoculated over 0.8% w/v agarose media and tested for their help in better plant growth. ANOVA (analysis of variance) statistical test was done followed by posthoc analysis using Tukey's pairwise test. Mean was plotted using the software Past 3.0. Error bars represent the standard error between samples. n = 3. p-value codes; * = p-value ≤ 0.05, ** = p-value ≤ 0.01, ***≤0.001

Plant-microbes interaction at the level of metabolites

To study the mechanisms behind plant-microbial interactions, we identified metabolites released by plants and microbes. We collected metabolites from plant roots under the influence of root microbiome, soil microbiome and buffer (control). We also collected metabolites from the membranes which serve as a communication mediator between plant and microbes. In our system, it is possible that the membrane can potentially act as a sink for metabolites exchanges between plants and microbes during the course of this assay. The metabolites were concentrated using Labconco freeze dryer and then analysed using UHPLC and Orbitrap Velos pro (Thermo) for untargeted metabolites profiling in a positive mode of ionisation. We ran a gradient condition mobile phase for even separation of metabolites using a mix of acetonitrile and formic acid. The raw data was converted into mzXML format and then analysed through XCMS Online excluding

features with p-value above 0.05, fold change less than 1.5 and peak width below 1s. Based on this analysis and parameters, we were able to identify 5921 features of the plant-microbiome interactome/ metabolites in total. Based on this data, a PCA plot was generated to visualise the variation among samples (Fig. 19). In the case of metabolites harvested from the plant roots, the PCA plot shows that the variation among the metabolites from plant - soil microbiome interaction is more dispersed and spread out across the ordination plot while the samples from plant and root microbiome interaction are mostly clustered together. It is interesting that the samples from plant grown with buffer and plants grown with root microbiome are similar to each other but they have significantly different plant phenotype. This might suggest that plants grow like buffered conditions with additional metabolites/pathway activation in case of root microbiome. These additional metabolites are either very low in concentration or could not be captured through this analysis with better signals as they are few in numbers. But we expect few PGP metabolites as we know that presence of PGP metabolites can result in the signal cascade within plant affecting its physiology and resulting in improved growth without outwardly modifying the plant's exudate profile(Berendsen, Pieterse et al. 2012). In the case of soil microbiome, the spread in PCA plot suggests that the plants undergo a more varied type of metabolites exchange scenarios; ranging from one extreme where metabolites were similar to root and buffer, yet also having metabolite profiles that were very different. One possibility is plants are trying to reconfigure the heterogeneous soil microbiome to select for beneficial microbes but the microbial diversity in case of soil microbiome is much greater than when compared to root microbiome and hence a diverse spread is observed. These plants may also require more than the 7 days of the experiment to recruit their associated microbes. Overall, plants grown with root microbiome were more similar in their metabolite profile. This provides evidence that a specific subset of soil microbes are associated with plant roots and their interactions have specific metabolites that were similar across sample replicates.

In the case of membrane metabolites, the interactome of plant - microbes' metabolites shows a different pattern. On the membrane, we expect metabolites from both plants and microbes. According to the PCA plot, the variation among the samples from the plants grown with root microbiome is low and they cluster together while the samples

from plant-soil microbiome interaction have some variation among them. In the case of buffer, the variation is much higher when compared to the previous cases. It is possible that the metabolites are very low in concentration on membranes and hence strong signals were not observed to clarify the situation. It is also valid to consider the membrane as an active place to exchange metabolites but also a stable place to accumulate unwanted metabolites and important metabolites are already taken up by plants quickly. Hence only a similar and not so important bunch of compounds are left on the membrane. These possible hypotheses were proposed to explain the PCA plot generated from the mass spectrometry data for plant holobionts assay. Nonetheless, the metabolite profiles of plants grown with root microbes tended to cluster together, both from the roots and from the membrane. This further supports the observation that plant-microbial interactions in the roots are highly specific in nature and could potentially be driven by only a small number of metabolites.

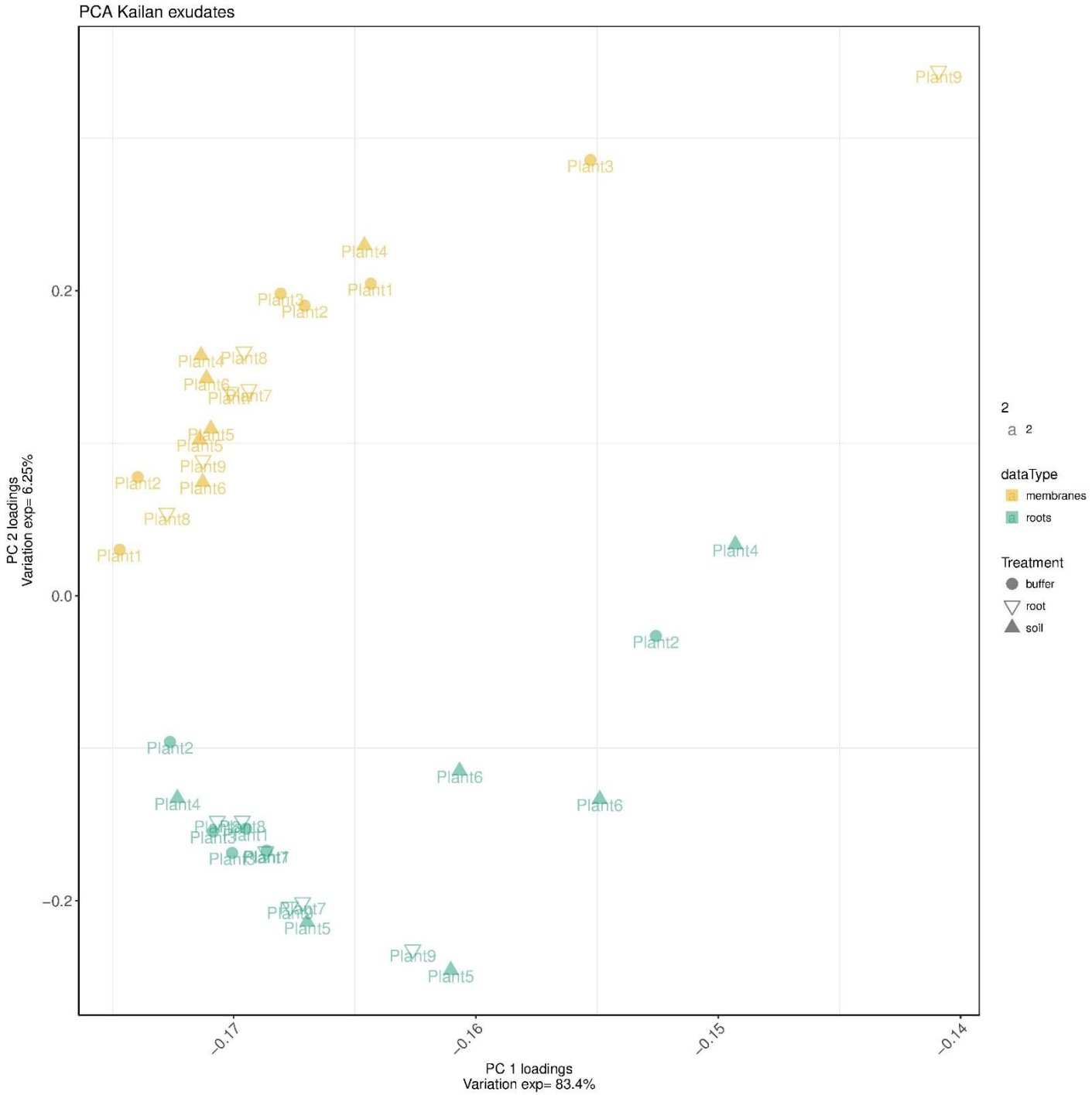

Figure 19: Principal component analysis (PCA) plot for samples under different microbiome conditions. Yellow spots represent metabolites extracted from the membranes between microbiome and plants. Cyan colour represents metabolites collected from root surface after microbiome treatment for 7 days.

We also performed a pairwise analysis between soil and root microbiome associated metabolites (Fig. 20). We were able to capture differential features with p-value less than 0.05 and fold change above 1.5. As we could observe significant difference in plant growth under the influence of root microbiome as compared to soil microbiome, we were expecting differential features to help us understand how root microbiome supports better plant growth at the metabolites level. We have used metabolites from one plant as one sample for metabolomics so it is possible that concentration of important metabolites are low and hence not many features were captured by this analysis since only 24 out of >5000 features showed differences in concentration. These few but specific number of metabolites appear sufficient to drive improved plant growth.

Using XCMS online, we were able to do annotation of the putative metabolites followed by pathway analysis using METLIN. When the metabolites of plants grown with soil microbiome were compared with metabolites from plants grown with root microbiome, XCMS predicted the putative presence of pathways associated with GA_{12} biosynthesis, NAD biosynthesis I (from aspartate), indole-3-acetyl-amide conjugate biosynthesis and coniferin metabolism in case of samples under the influence of root microbiome (Table 2). It was analysed based on the overlap between the presence of putative metabolites and known pathway metabolites (Table 2). It is known that these pathways help plants in better growth and development (Rogers 2004, Ortiz-Castro, Contreras-Cornejo et al. 2009). Their presence in case plants grown with root microbiome suggest how root microbes help plants achieve better growth and development. This suggests that root microbiome was associated with the release of phytohormones or small molecules to activate above-mentioned pathways. It is reported in the literature that beneficial microbes release a variety of compounds to successfully colonise the plant roots(Bulgarelli, Rott et al. 2012). These compounds either directly or indirectly affect the plants in beneficial ways like resistance to certain diseases or better plant growth and development(Ortiz-Castro, Contreras-Cornejo et al. 2009). Microbes in return get fixed carbon and other organic compounds from plants which microbes can use as a nutrient or for communication within microbial populations.

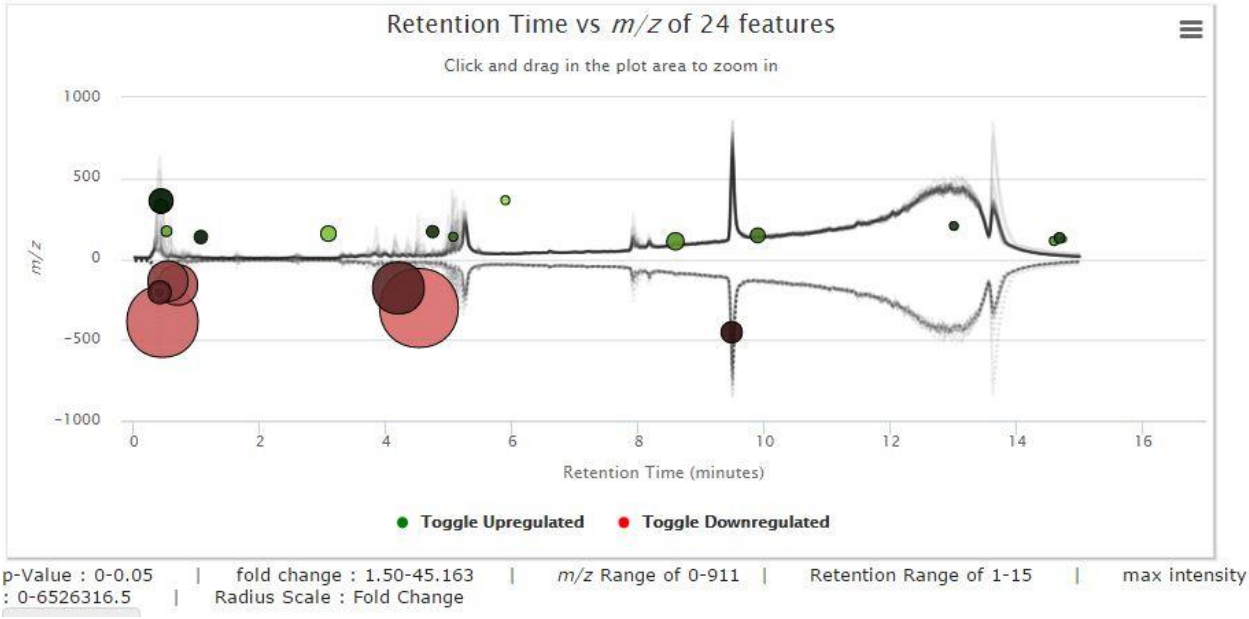

Figure 20: Cloud plot showing overall differential features between plants grown with soil microbiome (control) and root microbiome. Pairwise comparison of features was done. Upregulated features are shown in green and down-regulated features are shown in red. P-value is represented by colour intensity (more intense means lower p-value) and radius of each feature represents the fold-change. These features have p-value ≤ 0.05 and fold change above 1.5

Table 2: Metabolic pathways in case of differential analysis between soil microbiome (control) and root microbiome associated metabolites.

	Overlapping putative metabolites	All metabolites
GA12 biosynthesis	3	4
NAD biosynthesis I (from aspartate)	2	2
indole-3-acetyl-amide conjugate biosynthesis	2	2
coniferin metabolism	2	2
gibberellin inactivation II (methylation)	3	5

We have identified the possible presence of auxin biosynthesis pathway in case of root microbiome (based on XCMS Online analysis). Auxin levels are associated with better plant roots development and better and longer root structure helps plant in exploring possible nutrients in their surroundings. Many studies have shown positive correlation between plant growth promoting properties of beneficial microbes and auxin production (Khaliq 2002). Another important pathway observed in case of plants grown with root microbiome is GA_{12} biosynthesis pathway. It is associated with better plant growth and various developmental processes. Previous studies suggest that GAs are important phytohormones that help in biomass production, plant elongation and increased growth rate (Hedden and Phillips 2000, Olszewski, Sun et al. 2002). Similarly, coniferin metabolism pathway is associated with lignification (Förster, Pommer et al. 1999). Lignification is an important process which helps in plant support, water transport and disease resistance (C P Vance 1980, Anterola and Lewis 2002). In summary, root microbiome helps plant in better growth and development by either inducing or providing critical nutrients involved in plant defence or beneficial phytohormones which were not observed in case of plants grown with soil microbiome or buffer.

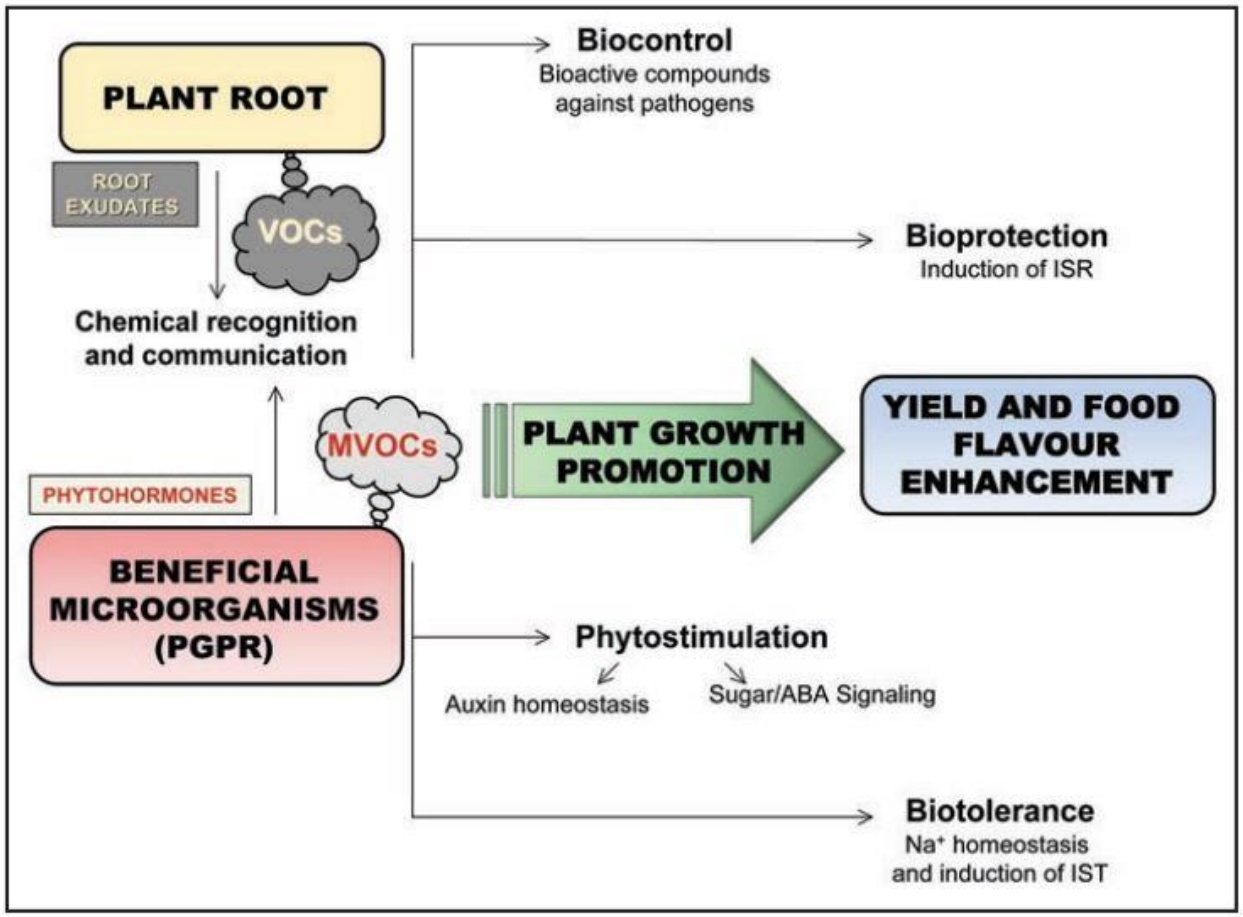

Figure 21: Plant-microbes interaction mechanisms and output. Image source: (Ortiz-Castro, Contreras-Cornejo et al. 2009)

Conclusion

Plants grown with root associated microbial community have shown better growth and development as compared to plants grown with bulk soil microbes under laboratory conditions on an *in-vitro* plant holobionts assay. We were able to grow multispecies root microbial community on assay plates and analyse the interaction between plants and microbes. It is shown that plants show 2 fold change in fresh weight gain in the presence of root microbiome compared to 1.2 fold change when soil microbiome is present. We also showed that rhizosphere microbes is particularly associated with better plant's fresh weight gain when compared to microbes from rhizoplane or endosphere. To identify the metabolites associated to this beneficial interaction we have done metabolites analysis. Putative metabolites annotation from the Plant-microbes interactome analysis has shown the presence of important plant growth promoting metabolites in the case of plants grown with root microbiome like GA12 biosynthesis pathway, NAD biosynthesis I (from aspartate), indole-3-acetyl-amide conjugate biosynthesis and coniferin metabolism. These pathways have important roles in overall plant growth and development. Further studies like metagenomics and metatranscriptomics can be used to understand the activities within the microbiome and do the functional analysis. Taxonomic analysis from this study can help in the development of synthetic microbial consortia which can serve as an agricultural tool for better and healthier plant growth.

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