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Construction of LC-MS maps of root exudates in cotton (*Gossypium hirsutum* L.) seedlings

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Abstract

Root exudates composition and pattern of biochemical expression is genotype specific and highly influenced by both by abiotic and biotic factors. During this investigation, various attempts made to standardize the techniques to construct LC-MS maps using cotton as a plant system. Construction of root exudates maps by LC-MS analysis found as very unique and having high utility in genotype identification through genotypic maps, detecting the presence/absence of specific chemicals of interest, and for rhizosphere engineering. As expected each sample (root exudates of a particular genotype) produced very distinct peaks-spectra. Each peak in the peak-spectral map (Y-axis) provides very useful information, the peak intensity (peak height), which represents the percent of each chemical/analyte present in the sample. The total number of peaks in each spectrum indicates the number of biochemicals present in that sample. The root exudates samples were probed in both positive and negative LC-MS mode, since some acidic compounds could not be detected in positive mode. The peaks displayed in the negative mode spectra maps indicates most of them are belong to the compounds in acidic groups. This distinction also provides additional chemical diversity and chemical specificity to include in the genotypic maps. By this way, the diversity present in all these parameters for each cotton genotype was included and the information presented was used to establish a very high-resolution maps. These peak spectral maps directly depend on the biochemicals produced by a specific genotype and genetically controlled; therefore, they can be called as genotypic maps or root exudates maps.

Keywords: Root Exudates, Silica sand, Liquid Chromatography mass spectroscopy (LC-MS)

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1. INTRODUCTION

The importance of rhizosphere in plant growth and development was first reported by Lorentz Hilter a century ago and it was redefined by Pinton as the zone that includes the soil influenced by the root along with root tissues colonized by microorganisms[6,16]. Roots are the major part of the plant through which plants derive nutrients and water from the soil. However, for higher efficiency of nutrient absorption and

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protection against abiotic and biotic stresses, plant roots establish sophisticated molecular signaling mechanisms with diverse soil flora and fauna. In the rhizosphere environment, the interaction between plant roots, soil and microbes occur in very complex ways and significantly effects on soil physical and chemical properties, which in turn modulate the microbial population in the rhizosphere[9,15]. Rhizodeposition refers to process of secreting organic carbon compounds such as sugars, amino acids in addition to more complex secondary metabolites from roots in the form of root exudates which are involved in nutritional, communication and regulatory functions[4,5,10,11,13]. The qualitative and quantitative composition of root exudates varies based on cultivar, plant species, plant developmental stage and various environmental factors, including soil type, pH, temperature, presence of microorganisms and in response to various predatory insects and herbivores [1,12]. These differences generate a unique microbial communities in the rhizosphere that have a certain degree of specificity for each plant species. The interactions between the plants and microbes *viz*., could be both positive and negative interactions. The positive interaction, for example, includes nitrogen fixation by *Rhizobium*, protection against harmful microbes, modulating soil structure, mineralization, *etc*. and negative interactions include attraction/activation of pathogens and parasitic weeds, deflation of nutrients disproportionately, allelopathic effect, *etc.*

Since root exudates can have diverse interactions with soil organisms, identifying a specific root exudates pattern that has positive effect appears to be a very efficient method for crop improvement. At present, lack of comprehensive knowledge on root exudates composition is another major barrier to understand rhizosphere complexicity [2,7,8,14]. Therefore, understanding of root exudates composition and their role in rhizosphere interactions for different genotypes may be a useful technique to identify a specific genotype having desirable rhizosphere interactions.

Although, investigation of root exudates composition and their role in individual crops have been reported for many crops, there are no studies that have systematically investigated on root exudates collection, detailed analysis of total and individual chemicals in the early stages of seedlings. No studies are available that uses root exudates pattern for screening of a large number of genotypes in short period of time in a controlled environment and with a minimal expenditure. Similarly no studies were found that explains collection of root exudates non-invasively and determine optimum number of seeds required and right stage of the seedlings to collect the root exudates. This information is very important for minimizing the seed resources when they are in scanty and reviving the selected seedlings based on root exudates analysis further extended in the field studies. Further no studies have reported establishing correlation between root exudates composition and exudates expression pattern of a specific genotype. Also construction of genotype maps based on root exudates composition and exudates pattern is not available. This information is very important for identifying the unique genotypes that are having desirable influence on rhizosphere activity, engineering of rhizosphere and utilization in crop improvement programme, which is a needed of the hour for achieving sustainable agriculture. Therefore, improvement of cotton crop through modification of rhizosphere by identifying genotypes with an unique root exudates composition have high potential to improve the productivity and decrease input cost.

2. MATERIALS AND METHODS

2.1 Collection of genotypes

Collection of suitable genotypes in cotton for root exudates studies is very important for generating reliable results. Different genotypes required for this research work was obtained from germplasm maintained by cotton research unit, UAS, Dharwad.

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Table1. List of cotton genotypes used along with their respective special characters

SNo.	Genotype Name	Special characters
1	Khandwa-2	Jassid resistant variety, drought tolerant variety.
2	L-761	High yielding, drought tolerant variety.
3	F-2226	Drought tolerant variety
4	JK-4	High yielding, drought tolerant variety
5	RAJ-2	Tolerant to sucking pests, drought tolerant variety.
6	AK-23	Drought tolerant variety
7	CCH1831	Drought tolerant variety.
8	543 3A2 A03 N83	Drought tolerant variety
9	MCU-5 (Susceptible)	Resistant to *Verticillum wilt*, Drought Susceptible variety
10	RHC-0811	Drought tolerant variety

2.2 Silica sand as medium of growth

White silica sand with 0.03mm diameter particle size (purchased from New water technologies, Coimbatore) was used as medium of growth. The sand was thoroughly washed several times with running tap water after which it was washed with 2 to 3 times with distilled water. After complete drying of the sand was spread on a plane paper for complete drying in room temperature for 48 hours. Washed and dried sand was sterilized in autoclave at 120 C for 30 minutes.

2.3 Sowing of seeds in cups filled with sterilized silica sand

Transparent plastic cups with diameter of – 7cm filled with sterilized sand up to one third volume of plastic cup height (40 grams of weight). Seeds were dibbled into half inch depth and watered. The numbers of seeds per cup sown were varied according to the experiment. The cups were watered two times a day with sterilized distilled water with enough to saturate the soil at the same time not to lose water by percolating out of the cup.

2.4 Experimental setup

The experiment was carried out in IABT glass house with dry and optimum temperature of 28 ± 2 °C. The cups were arranged in a rows and columns to accommodate replication and genotypes in a manner of experimental design.

2.5 Collection of the root exudates

At the time of collection (9th DAS four cotton seed in each cups as per standardization), the cups were watered to saturation point and allowed to release the exudates into the solution. About 30 minutes later, exudates were collected by washing off by adding 8 ml of water and collected the percolating solutions (water + root exudates) though the holes into sterile a 15 ml centrifuge tube. The centrifuge tubes with exudates were centrifuged at 9000 rpm for 10 min, to get rid of minute sand particles and any cell debris or sloughed off cells. After centrifugation, the samples were decanted into fresh 15 ml centrifuge tubes.

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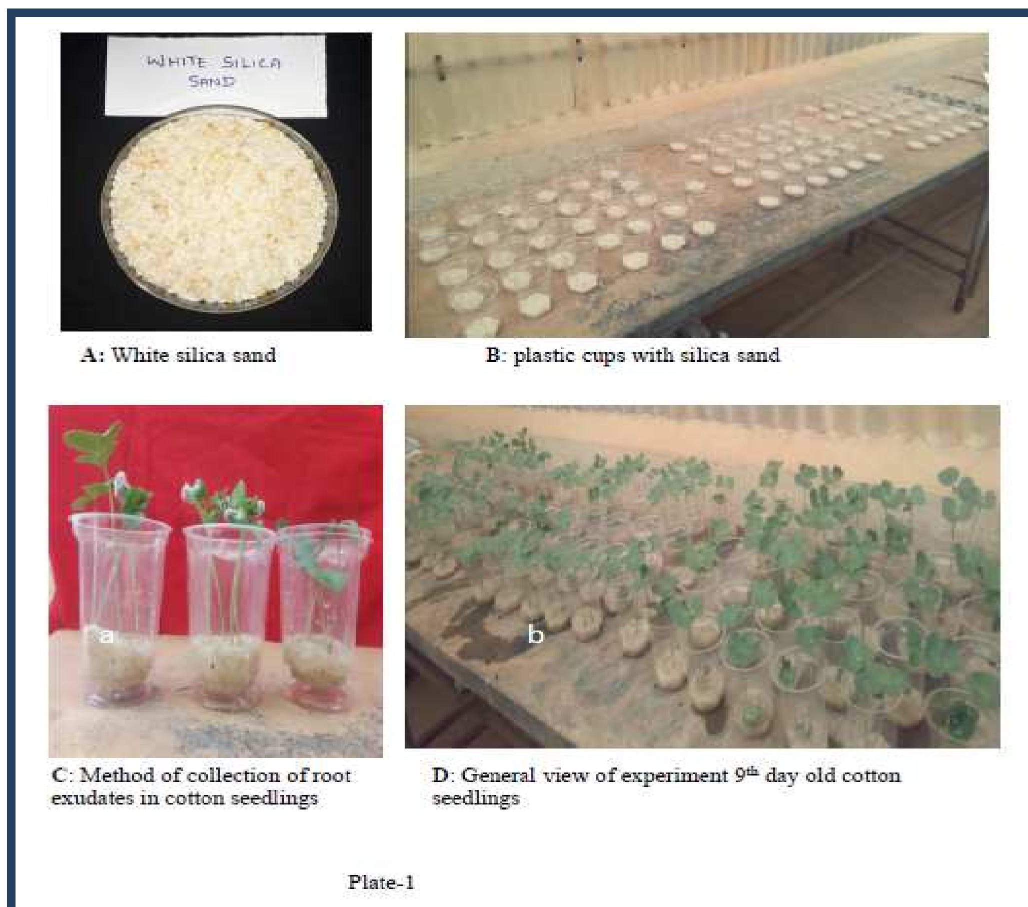

A: White silica sand

B: plastic cups with silica sand

C: Method of collection of root exudates in cotton seedlings

D: General view of experiment 9th day old cotton seedlings

Plate-1

2.6 Concentrating root exudates by lyophilization

The processed exudates were kept for pre–freezing which is most important process in freeze – drying .The pre-freezing step was carried out in two step involving a step-wise approach for freezing the samples. The first step involves freezing the samples in deep freezer (-20̊ C) in a slanting manner to increase the surface area of samples to be in contact with the vacuum in lyophilizer (Scan Vac model 110). After 5 hours of freezing, the samples were shiftled to ultra-freezer for overnight .The prefreezed samples were then kept in lyophilizer and allowed to run continuously till the samples get dried off completely.

2.7 Analysis with LC-MS

The apparatus consisted of a AQUITY H-class ultrahigh-performance liquid chromatography (UPLC)-MS/ MS (Xevo triple quadrupole (TQD) MS; Waters Corporation, Milford, Massachusetts, USA) consisted of a quaternary solvent manager (ACQUITY UPLC H-Class System; Waters Corporation, Milford, MA, USA), sample manager with flow through needle (ACQUITY UPLC H-Class System; Waters Corporation, Milford, MA, USA), nitrogen generator compressor (Peak Scientific, Milford, MA, USA), 2.1 mm × 50 mm C18-bonded silica column (1.7 µm ACQUITY UPLC BEH; Waters Corporation, Milford, MA, USA), mass analyzer (Xevo TQD Mass Spectrometry; Waters Corporation, Milford, MA, USA) with an ESI ionization source (Z-Spray; Milford, MA, USA), data analyzer software (Mass Lynx Software; Milford, MA, USA).

Chemicals: Methanol absolute ULC/MS grade from Biosolve; formic acid 98-100% AR from SDFCL HPLC grade water, prepared using Sartorius – Arium mini plus – ultrapure water system, and filtrated using 0.45 µm membrane filters.

2.8 Setup of the instrument

Chromatography was performed using a Waters Acquity UPLC system (Waters, Milford, MA, USA).Chromatographic analysis was performed using a Waters Acquity® UPLC bridged ethylene hybrid C18

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column of 1.7 µm particle size (2.1 × 50 mm) maintained at 35 °C.The injection volume of 20 µL was used. The mobile phase flow rate was 0.4 mL min^{-1} using a gradient elution method with 0.1% formic acid in water and methanol as the mobile phase as follows: Starting conditions were 90% A which was maintained for 0.5 min. This was then reduced to 30% A over 2.0 min and to 2% A over 3.0 min. Then returned to starting conditions in 3.5 min. Starting conditions were held for 1 min to allow re-equilibration. Total run time was 4.5min.

The UPLC system was coupled to a XevoTQD mass spectrometer with an orthogonal Z-spray–electrospray interface (Waters, Milford, MA, USA), equipped with an electrospray ionization source. The linkage between chromatographic system and the MS detector is via an interface. In LC (liquid chromatography) the role of this interface is to evaporate the solvents from the mobile phases and to transform in ions (positive or negative) the molecules of interest. Once the analyte is ionized it enters in the MS detector. During the chromatographic run, each peak is analyzed by selection on the basis of mass using the quadrupole of the MS detector and once separated.

2.9 Setup for MS analysis

The capillary voltage used was 2.5 kV. The capillary voltage of the ESI interface can have positive or negative polarity having the role to allow the ionization of the molecules during the desolvation process. Here the determination of acidic compounds was performed in negative ionisation mode and basic compounds were determined in positive mode with a scan range m/z 10–1,000 amu.

The instrumental was setup with Source temperature 150ºC and Dissovation temperature 500ºC. In ESI interface the liquid from the chromatographic system is entered in the capillary of the interface. The solvents and other additives that made the mobile phase have to be evaporated. This process required a higher temperature in the interface chamber. The appropriate value of this parameter is established based on the temperature of evaporation of the targeted molecules (as normally, the solvents and additives of the mobile phases are very volatiles molecules). The higher the temperature is, the higher the amount of analyte molecules transferred in gas state and prone to ionize will be. In the mean time, a higher temperature can promote a thermal fragmentation of the analyte molecules and as a consequence the yield of ions formed from the analyte molecules will decrease, giving a weaker signal.

Dissolvation gas (L/hr) 1000; The appropriate value depends on the chosen temperature of the source. This flow of nitrogen at high temperature promotes the desolvation and evaporation of the analytes molecules. In our experiments, considering the source temperature of 500 °C, the source gas flow was set at 1000 L/h. Nitrogen was used as the nebulising and desolvation gas.

Cone voltage (V) 30.0;The value of cone voltage has to be high enough to produce as many as possible molecular ions from the molecules of the analyte reaching the ESI interface, but also as low as possible to not fragment the molecular ions formed.

The values of the parameters considered optimal for the identification and confirmation presented in below table were used to further optimize the chromatographic conditions.

Capillary voltage: 2.5kV

Cone voltage (V): 30

Source temperature:150°C

Dissolvation temperature: 500°C

Dissolvation gas (L/hr): 1000

2.10 Preparation of samples for LC-MS analysis

The lyophilized samples of the genotype in table 1 were dissolved in 10 ml of Methanol and water mixture (80:20 v/v). totally 10 genotypes were used for LC/MS analysis .The samples were mixed thoroughly in sonicator for 15-20 min. and then filtered through 0.22 µm filter paper and kept ready for analysis.

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2.11 Loading of sample

The filtered samples 20ul were injected through column. Immediately after injecting the samples, the process was started to prevent errors. The TIC data was extracted for masses present in the sample.

2.12 Analysis of root exudates using LC-MS/MS

Out of curiosity to find individual compound in root exudates ,after analysis of root exudates using LC/MS the samples of MCU-5 at particular molecular mass were directly subjected to second phase of MS where the compound was compared with standards to find the original compound . The analysis was carried out for Organic acids ,flavanoids,alkaloids to find the specific compounds.

3. RESULTS AND DISCUSSIONS

3.1 Analysis by LC/MS

As the analysis of the root exudates by LC-MS provide enough information regarding the composition of root exudates, the samples were subjected to high sensitive LC/MS analysis . The sample was analyzed for total ion chromatogram (TIC). The ions were monitored for a dwell time of 0.1s. Data acquisition was performed using MassLynx 4.0 software with QuanLynx program (Water, Miliford, MA, USA). The selected precursor ions of the analytes were chosen for quantitative confirmation purpose, The results were obtained in the form of total molecular mass in X – axis with their ion intensity in Y axis with the scan range of 0 -1000m/z (Figure 1a to 10 b: go to supplementary files from website). The spectral peaks obtained between 0 -1000 m/z were selected as the molecular ion [M_H]- for ESI negative ion mode and [M_H]+ for ESI positive ion mode has been compared with the molecular weight of the compounds which has already been reported in cotton root exudates earlier with ESI ion source. The graphs were analysed by talking all the peaks and comparing with molecular mass of known compound which was collected from the internet. Compound with molecular masses which fall within the peak range studies were taken as probable compounds (Table 2 to 21: download supplementary files from website). This gave an overall view of probable compounds to be present in that particular molecular range.

3.2 Analysis by LC-MS/MS

The samples subjected to LC-MS/MS was analysed only for oxalic acid in case of organic acids and quercetin derivative in case of alkaloids and flavonoids (Table 27: download supplementary files from website). The results showed that the oxalic acid was found to be 15.03 mg/kg in MCU-5 comparing to the oxalic acids standard. The results of LC-MS/MS analysis of quercetin derivative showed that the level of quercetin was below 1µl of the detectable limit. The result of LC-MS/MS was on par with the result of LC-MS analysis of MCU-5 which also showed that the oxalic acid found to be 19 percent intensity.

DISSCUSSIONS

Cotton root exudates have mixture of very low molecular to high molecular weight compounds with varied physico-chemical properties. Because of this reason, it necessitated to go for high resolution (high sensitive) analytical techniques to separate and determine their molecular weights of different analytes present in root exudates. In particular, high-resolution analysis of crude root exudates (compound mixtures) collected from different cotton genotypes has become an essential factor in order to construct fine root exudates maps. Since, development of maps for genotypes and correlating specific biochemicals with different agronomic traits was one of the objectives of this investigation, thus high resolution analysis of root exudates was carried on by using LC-MS. For high resolution analysis, generally, the HPLC and recently the gas-liquid chromatographies (GLC) are used. These instruments are being high-end equipments, commonly used for analysis of various pharmaceutical, plant and environmental chemical mixtures. However, when compared to HPLC, the LC-MS technique has better sensitivity to analyze the chemical mixtures along with their molecular weights simultaneously. Therefore, analysis of root exudates by LC-MS, having high number of chemicals in a sample will not be a limiting factor.

The procedure for LC-MS analysis of root exudates for 10 cotton genotypes with different agronomic traits analyzed are as explained in the materials and methods. The peak spectral maps for

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different genotypes are given in the (Figures 1a-10b: download files from website). As expected each sample (root exudates of a particular genotype) produced very distinct peaks-spectra. Each peak in the peak-spectral map (Y-axis) provides very useful information, the peak intensity (peak height), which represents the percent of each chemical/analyte present in the sample. Thus, the height of the each peak is directly proportional to the amount of a compound present in the sample mixture. The total number of peaks in each spectrum indicates the number of biochemicals present in that sample. The number (integer) mentioned on the top of each peak indicates the molecular mass of a chemical (s). The root exudates samples were probed in both positive and negative LC-MS mode, since some acidic compounds could not be detected in positive mode. The peaks displayed in the negative mode spectra maps indicates most of them are belong to the compounds in acidic groups. This distinction also provides additional chemical diversity and chemical specificity to include in the genotypic maps. By this way, the diversity present in all these parameters for each cotton genotype was included and the information presented was used to establish a very high-resolution maps. These peak spectral maps directly depend on the biochemicals produced by a specific genotype and genetically controlled; therefore, they can be called as genotypic maps.

Further, by using these molecular mass numbers mentioned on top of each peak, all the probable chemicals present in that range have been name-listed with the help of Google search (or chemical database). This chemical list (Table 2 to 21: download files from website) includes both the chemicals identified by the scientists in cotton or reported for other crops and rest of them are other uncharacterized chemicals fall in this mass range (Table 2 to 21: download files from web). A number of uncharacterized chemicals (not confirmed by the experiments) can be a source material for further investigation to find out their possible role in the root exudates. However, in this investigation peak numbers (spectra) and their intensity (percentage) for both known and unknown compounds are included for constructing genotypic maps. Variation in the peak intensity, molecular mass (at each peak) and a number of peaks present in root exudates collected from different genotypes have become important analytical parameters, and used very effectively for construction of genotypic maps for differenting cotton genotypes. The application of peak spectral maps constructed by LC-MS analysis for root exudate of cotton genotypes provided a very fine biochemical maps. This type of investigation and the concept of constructing genotypic maps are first of its kind and not reported anywhere in the literature (Figures 1a-10 b: download files from web).

In most cases, the list of different compounds that fall in the range of each peak value (specific molecular mass) contained several compounds. However, very few chemicals are already been characterised and reported in some agricultural crops, in particular, root exudates of cotton (Sulochana 1962). The result of LC-MS analysis for amino acids in 10 cotton genotype showed that presence of Methionine, Phenylalanine, Valine, Histidine, Alanine, Asparagine, Aspartate, Cysteine, cystine, Glutamate, Serine, Tyrosine, proline, glycine, lysine, leucine, isoleucine ,glucose , galactose, sucrose, maltose are the probable compounds. Some other observations includes that the results of LC-MS analysis for phenols showed that in F-2226, RAJ-2, AK-23, CCH-1831, 5433A2A03N83, MCU-5 genotype, containing compounds like scopeletin and gossypol, which was also confirmed by Stephen *et al.,* 1974 in cotton genotype. other observations includes that the results of LC-MS analysis for organic acids showed that in L-761 ,RHC-0811, F-2226, RAJ-2, AK-23, CCH-1831, 5433A2A03N83, MCU-5 genotype, containing compounds like oxalic acid , maleic acid, succinic acid , citric acids and malonic acid which was also confirmed by Rakesh Kumar *et al*., 2007 in cotton genotype. Some other observations includes that the results of LC-MS analysis for vitamins showed that in L-761 ,RHC-0811, F-2226, RAJ-2, AK-23, CCH-1831, 5433A2A03N83, MCU-5 genotype, containing compounds like thiamine, biotin, pyridoxine,pyridoximine, and pyridoxal which was also confirmed by (Sulochana 1962) cotton genotype.

The genotypic maps developed by LC-MS analysis of biochemicals from root exudates can be applied to identify not only the genotypic differences between the various genotypes (germplasm or screening of segregating population), but it can also be applied to detect the genetic changes occur due to the affect of a specific endogenous or environmental factor (both abiotic or biotic factors). For example, differences found in the LC-MS peak spectral maps (number of peaks, peak intensity and molecular mass) of susceptible and resistant genotypes, can be used to identify a probable biochemical or gene product responsible for the resistance mechanism. In addition, a particular genotype can be challenged with a

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specific factor (temperature, moisture stress, pathogen, nutrients, and any soil factors), any changes in number of peaks, peak intensity and their molecular masses in the LC-MS spectral maps developed can be used to identified a unique biochemicals. In different situation, any deviation in treated plants, when compared to untreated, will help to identify the specific factor of interest and its role in the plant. These few examples clearly indicate potential of genotypic maps constructed based on LC-MS analysis application in crop improvement.

The methodologies developed during this investigation can be used for analysis of root exudates collected from any crop. The root exudates maps constructed by using LC-MS analysis can be used to identify/screen genotypes (germplasm or segregating material) that having novel characteristics. Large-scale screening of plant population can be taken up in the laboratory condition itself. Experiments conducted for identifying the effect of a specific factor (pathogen, insect pest, nutrient, temperature, etc.) on plant in the form of root exudates composition can be used as a means to identify a specific compound induced or suppressed in response to that factor by using genotypic maps.

4. CONCLUSIONS

The diversity present in all these parameters for each cotton genotype was included and the information presented was used to establish a very high-resolution maps. These peak spectral maps directly depend on the biochemicals produced by a specific genotype and genetically controlled; therefore, they can be called as genotypic maps or root exudates maps.

CONFLICT OF INTEREST

All the authors claim that there is no conflict of interest regarding the publication of this paper.

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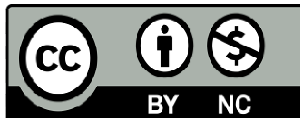

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