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Standardization of a novel method of root exudates collection and analysis of cotton root exudates by thin layer chromatography

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Abstract
Plant root exudates composition is genotype specific and its expression pattern helps to find the diversity and the role of exudates in plant growth and development. Study of exudates requires standard protocols. During this investigation established non-invasive method of root exudates collection by raising seedlings in a plastic cups filled with silica sand without addition of nutrients externally. Number of seedlings required (4/cup) and right growth stage of seedling to collect sufficient quantity of exudates (9th DAS) for analysis was experimentally determined Spectrometer analysis of root exudates (A280) was made to confirm the presence of biochemicals in a sample prior to undergoing for detailed analysis. For concentrating of root exudates, lyophilizer was found ideal. The possibility of reviving of seedlings whose exudates was analyzed by TLC.

Keywords: Thin layer chromatography, densitometer, silica sand, root exudates

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 (Darrah, 1993, Rathod and Channarayappa 2019) [2, 10]. Roots are the major part of the plant through which plants derive nutrients specially amino acids, protein, carbohydrate and organic acid and water from the soil (Balaji and Channarayappa 2019) [1]. However, for higher efficiency of nutrient absorption and 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 (Nihorimbere *et al.*, 2011) [7]. Recently, scientists have started realizing the importance of a specific rhizosphere interactions and its direct role in production of higher crop yield, while maintaining healthy soil conditions; which is the basis for sustainable agriculture and need of alternative approaches for overcoming some of the negative effects related to green-revolution, which includes overuse of chemical fertilizers, pesticides, reduced use of organic matter and poor nutrient management.
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.

2. Material 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

Table 1: List of cotton genotypes used along with their respective special characters

SL No.	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

For preliminary investigation, i.e., development of procedure for the collection of root exudates in situ was carried out with the popular Sunny NCS108 variety during 2017-18 at UAS, Dharwad.

2.2 Raising the seedlings

2.2.1. 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 (Plate 1 A). 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 120̊ C for 30 minutes.

2.2.2. 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. (Plate 1 A).

2.2.3 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.

3. Collection of the root exudates

At the time of collection (different intervals of time), 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 (Plate 1-C). 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.

3.1. Conformation of presence of root exudates by Spectophotometer analysis

The centrifuged samples were subjected to A280 spectrophotometer analysis (Eppendorf Biospetrometer Basic) as preliminary conformation to check the presence of the any compound in samples based on their absorbance values at 280 nm. The spectrophotometer blank set with control samples (collected from a cup without seed sown). Then the exudates samples were filled in a 2 ml cuvette and loaded to read in biospectophotometer. The absorbance maxima at 280 nm (generally used for detecting chemicals with amino group) was measured for all the genotypes and presented in both graphs and numerals.

3.2 Standardization of number of seeds for collection of root exudates

The plastic cups filled with silica sand prepared as mentioned above were used to sow the cotton seeds, 1,2,3,4 and 5 cups, in a CRBD design. Watering and collection root exudates were fallowed as mentioned above. The root exudates collected from cups with different number of seedlings were analyzed using spectrophotometer and Thin layer chromatography (TLC) technique. The sample that containing highest root exudates per cup were considered to be optimum and the seeds sown in that cup were used for all later experiment.

3.3. Standardization of number of days for collection of root exudates

Using the number of seeds per cup determined from experiment 3.5 was used to determine right seedlings growth stage. The exudates were collected in three days interval starting from the third day after sowing to 15th day after sowing. The experiment was replicated thrice. The day at which sufficient quantity of root exudates required for analysis was determined by collecting the root exudates and confirming the exudates quantity at all stages.

3.4. 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.

3.5. Analysis of root exudates by Thin layer Chromatography

3.5.1. Preparation of samples for TLC analysis

The lyophilized samples were dissolved in 50µl of autoclaved distilled water. In oder to dissolve completely, the sample were incubated at 60 ^{0}C in hot –water bath for 10 min. The samples were transferred to fresh 1.5 ml centrifuge tubes and stored in deep freezer.

Table 2: The chemicals used for TLC analysis and their concentrations

Component	Mobile phase	Detection reagent	Detection method	References
Amino acid	n-butyl alcohol, Acetic acid, Water (3:1:1) n-butyl alcohol: Acetic acid: Water (4:1:1)	Ninhydrin reagent (0.5g Ninhydrin + 0.5 ml Acetic acid + 100ml Acetone	TLC plates sprayed finely and heated at 100 ^{0}C for 5 min. to detect spots	Thimmaiah and Thimmaiah, 1999 [14]
Sugars	Ethylacetate-n-propanol-acetic-water (4:2:2:1)	Orcinal Reagent (250 mg orcinal,95ml ethanol,5ml H2So4)	TLC plates dipped in reagent and heated at 100 ^{0}C for 10 min. to detect spots	Waksmundzka-Hajnos *et al.*, 2008 [16]
Organic acid	Ethanol-butanol-ammonia-water (40:30:15:15)	UV light detection	Detection under UV	Tyman (2000) [15]
	Hexane –Acetic acid (96:4)	UV light detection	Detection under UV	Tyman (2000) [15]
Alkaloids	Chroform-Acetone-diethylamine (5:4:1)	Dragandraff reagent		Waldi *et al.*, 1961 [17]
	Methanol: Ammonia (100:1)	Dragendorf reagent	Dark orange colour spots in orange background	Kristanti and Tunjung, 2015 [5]
Phenols	Ethyl acetate-benzene (9:1)	Vanilin HCL (15 gm of vanillin in 250 ml of ethanol and 2.5 mil of Sulphuric acid		Thimmaiah 1999 [14]
lipid	Hexane-acetone (100:8)	Rhodamine-B (Dissolved 0.25gm of reagent in 100ml of ethanol		Georged *et al.*, 1970
	Petroleum ether (60-70^{0}C) : ethyl ether : glacial acetic acid (80:20:1)	0.5% Rhodamine B in ethanol	TLC plates were finely sprayed and orange florescence were observed under UV light	Thimmaiah and Thimmaiah, 1999 [14]

3.5.2. Protocol for Thin Layer Chromatography

The standard protocol for thin layer Chromatography was fallowed as mentioned by Fried and Sharma (1999) with small modification of using ready –made silica gel plates.

1. A thin line was drawn from a 2H pencil, 1 cm away from the bottom of TLC Silica gel edge, 60 F_{254} (Merck) plate to notify the basal line. In order to mark a boundary for the solvent front, another line with 1 cm thickness from top edge was marked in opposite end.
2. On the basal line, spots were marked in equal distances with pencil and the samples of 5µl were loaded.
3. Simultaneously, the mobile phase mixture was poured into the TLC chamber and left undisturbed for few hours to get the chamber saturated with the vapors of the mobile phase completely.
4. The spotted plates were dried completely in room temperature and then placed vertically in chamber such that the sample spots lies above the mobile phase. Immediately the chamber was closed with a lid.
5. The setup was left undisturbed for few hours until the mobile phase reaches the solvent front line. After reaching the solvent front, the plates were removed and allowed to dry in room temperature.
6. After complete drying of the mobile phase, the plates were sprayed or dipped with specific reagent (Table 2) and developed according to each compounds.
7. After developing spots with different development method (Table 2), the TLC plates were photographed and the spots were analyzed for Retention factor (Rf) which is the ratio of distance from the center of the spot for a given mixture component to the distance traveled by the mobile phase, also known as the solvent front. Retention factor (Rf) is calculated with the formula:

$$\text{Retention factor } (R_f) = \frac{\text{Distance travelled by the sample from origin}}{\text{Distance travelled by the solvent from origin}}$$

3.5.3 Analysis of TLC Plates by using Densitometer

The TLC plates were scanned in Densitometer (BIORAD GS 900TM Calibrated Densitometer) and then analyzed using Image Lab 6.0 software with reflective imaging and red CCD imaging technology for quantification of each spots compared to standards loaded.

4. Results

In this study, a novel method for extraction of root exudates was designed and a new method for construction of root exudates map /pattern using TLC and LC/MS was also being developed in cotton seedlings. With above novel method, genotypes with desirable root exudates pattern can be identified and used for constructing genotypic maps. This information is very useful for rhizosphere engineering in cotton. The results obtained during this study are presented in following sections.

4.1. Standardization of novel method for extraction of root exudates non-invasively

The root exudates were collected by means of non invasive method which refers to process of collection of exudates that has been secreted out of root in the ectorhizosphere. In this method, white silica sand was used to collect root exudates. The samples were collected by percolating the white silica sand with known quantity of water from the cup. There was no confirmation that, the samples collected from the seedlings by means of percolation were root exudates. In order to confirm the presence of root exudates in the samples, spectrophotometer analysis was done by A280 method of spectrophotometer analysis at 280nm. At 280nm, the

compounds with aromatic amino acids along with compounds containing disulphide bonds have their absorption maxima. Thus, the reading in spectrophotometer truly reflects the concentration of aromatic amino acids in the samples which confirms that the samples are root exudates.

4.1.1. Standardization of number of seeds and number of days required to get sufficient (~1ng) root exudates

The standardized procedure for extraction of root exudates using silica sand was used for standardizing the number of seeds and number of days required to get sufficient (~1ng) root exudates. The collected samples were subjected to A280 spectrophotometer analysis. The spectrophotometer readings obtained (Table 3, 4 and 5) are true representation of the concentration of protein / amino acids with aromatic side chain groups. Three replications were used to standardize the number of seeds and number of days required to get sufficient (~1ng) root exudates.

Investigation made for determining number of seedlings per cups showed that, four seedlings per cup were just enough to get sufficient root exudates for TLC and LC/MS analysis (Table 6). Plate 1 shows that level of variation in root exudates concentration as the number of seedling increases. This observation was helpful to reduce number of seeds of genotype which are very scanty. Fruther optimum (~1ng) quantity of root exudates production as earliest stage of seedlings growth was also determined (Table 6). Plate 2 shows the variation of root exudates concentration as number of days increases. Even though the concentration of root exudates at 3rd day sowing (DAS) was higher at earliest stage, it was not selected. The sample collected on 9th day after sowing (DAS) was finalized, as the concentration of root exudates produced were sufficient for TLC analysis. These results of number of day required to get sufficient (~1ng) root exudates were very useful because the genotypic characterization though root exudates analysis can be formed earliest stage of seedlings without supplement of any nutrient. This information also useful to conduct root exudates studies in large scale in laboratory condition within short period of time. These types of results are available for first time for cotton which is not recorded earlier.

4.2. Construction of TLC and LC/MS maps / pattern of root exudates in cotton

4.2.1. Analysis of root exudates by TLC

Thin layer chromatography (TLC) was preferred as it could separate the compounds from mixture of compound based on its affinity towards mobile and stationary phase. The TLC analysis was carried out for several compounds like amino acid, sugars, phenols, organic acids, lipids and alkaloids separately with their specific reagents as mention in (Table 2)

4.2.1.1 Analysis of Amino Acid by Thin-Layer Chromatography

For analysis of amino acids by TLC, two different mobile phase was used. The mobile phase with composition of n butanol: acetic acid : water (4:1:1) showed higher resolution compared to n butanol : acetic acid : water (3:1:1) as the farmer could resolve the standard with higher resolution. The result show that the analysis for amino acids developed some spots (Plate 2 B and Plate 3 A) except few genotypes MCU-5, 543 3A2 A03 N83, AK23,JK-4 and L-761 where there were no spote found in replication first (Plate 2 A). It may be due to lower quantity of amino acids in those genotype which couldn't detected by TLC. For the genotype which developed spots, Rf value was calculated and compared with the standard Rf value of each compound to detect amino acids to be present in individual genotypes. The results clearly showed that the number, the types and the quantity of each spots are very unique to each genotype and at same differ across genotype.

4.2.1.2. Analysis of sugars by TLC

For analysis of sugars by TLC, mobile phase was Ethylacetate-n-propanol-acetic-water (4:2:2:1) used. The result show that the analysis for sugars developed some spots (Plate 3 B and Plate 4 B) except few genotypes 543 3A2 A03 N83, JK-4, Khandwa-2, RHC0811 and L-761 where there were no spote found (Plate 4 B). It may be due to lower quantity of amino acids in those genotype which couldn't detected by TLC. for the genotype which developed spots,Rf value was calculated and compared with the standard Rf value of each compound to detect sugars to be present in individual genotypes. The results clearly showed that the number, the types and the quantity of each spots are very unique to each genotype and at same differ across genotype.

4.2.1.3. Analysis of other compound by TLC

Several mobile phase composition for separation of organic acids, alkaloids, phenols, lipids were used. The analysis resulted in no spots development in all genotypes. This may be due to the reason that all these compound in root exudates are very low quantity compared to amino acids and sugars which could be detected by the TLC. .

4.2.2. Densitometer analysis of TLC plates

The TLC plates obtained by amino acids (Plate 2 A, B and Plate 3A) and sugar (Plate 3B and A,B) analysis were subjected to densitometer analysis in oder to quantify the volume / intensity of each spot developed. The TLC plates were analysed in densitometer by reflective scan mode and red filter colour. As a result, the densitometer analysed TLC plates image high scan mode and red filter colour. As a result, densitometer analysed TLC plates image has high sensitive spectral image colour to visualize even the low concentration spots. By analysing in densitometer, the spot intensity between each spot within a genotype and across a genotype could be found. These data provides an overall view of either presence or absence of particular spots across the genotypes using their Rf values and also the differences in intensity between each genotype using volume of each spots (Table 8 and 9). The lane 1 and 2 in densitometer analysed spectral image TLC plates of amino acids (Plate 2 A,B and Plate 3A) and sugars (Plate 3B and A,B) are negative control and positive control respectively while all other genotypes were arranged in lane 3 to 13. The spectral image of TLC plate of amino acid clearly shows the presence of spot which is uncommon between genotypes these spots which are not common among other genotype provide an interesting part which could be studied in future to find its function and characteristics. (Table 8 and 9) shows the mean volume of each spots along with standard deviation (SD) of three replication there by providing the overall reliability of TLC analysis.

5. Discussion

Root exudates collection procedure is very complex and no single standard method that satisfies all the requirements is available. As stated by Phillips *et al.* (2008) [8], there are several significant difficulties in the process of collection of

root exudates. All those difficulties have to be crossed over to study the exact root exudates composition. For instance, usage of natural soil for collection of exudates is not advisable, as the use of heat-sterilized natural soil presents difficulties due to phytotoxins formed during sterilization (Rovira, 1959) [11]. The collection in normal soil will not give exact values of root exudates as there is possibility of contamination with soil microbes and nutrients from the soil. Henceforth, collection of organic root exudates should be carried out under axenic conditions to prevent losses and alterations by the microbial populations in the rhizosphere (Shay and Hale, 1973) [13]. Some other studies have used different approaches, which includes, some days after seedlings grown in soil for known period, they uprooted the plants and washed in running water and later with sterile water, then roots dipped in sterile distilled water for 24 hours to release exudates. The exudates released into water was concentrated and used for further analysis. However, this approach is not advisable since when roots are completely submerged in water, it creates anaerobic condition and the plant roots will not experience the sensation/stimulus, when they grown in natural soil conditions.

To overcome these and other problems new kind of experiments were designed, that addresses most of these issues in very simple and reliable way, though it may not be the best method. In this experiment clean and sterile white silica sand (~0.3 mm diameter particle size) was used as growing media, because silica sand doesn't contain any organic matter or microbial activity and very stable at the same time imitating the soil environment. The seedlings were not supplemented with any nutrients externally and only nutrients seedlings could use was from stored food material in the cotyledons. This way exudates getting contaminated with media components was avoided and also prevented microbial growth. This approach was found very simple, inexpensive and effective for rising cotton seedling for a short period.

The technique for root exudates collection needed to be standardized and has to be simple and efficient for conducting root exudates studies. At the same time, it necessitated to avoid some drawbacks associated for root exudates collection in other methods already reported. For instance, removal of plants from solid media into trap solutions as followed by others, inevitably causes damage to root systems by breakage of roots, rupture of root hairs and epidermal cells or by rapid changes in the environmental conditions (e.g. temperature, pH, oxygen availability) (Neumann and Römheld 2007) [6]. Moreover, the recovery of exudates by such approaches is generally compromised by various physiological effects on the plant and incomplete leaching or adsorption of exudates by the solid media (Gransee and Wittenmayer 2000; Neumann and Römheld 2007; Sandnes *et al*. 2005) [4, 6, 12]. These techniques are also not suitable for quantitative analysis of the exudation rates in relation to root properties (Gransee and Wittenmayer 2000) [4]. Because of the above said reasons, the process of using trap solution for collection of root exudates was not followed. All these above reports prompted to establish a new approach to collect the root exudates. Root exudates collected by flushing off roots *in situ* with 8 ml of distilled water without disturbing sand or roots, provided root exudates of desirable quality and quantity and are well suitable for all the biochemical analysis experiments conducted (Plate 1C). Because of these advantages, this novel approach only adopted for root exudates collection throughout this investigation.

Very often researchers encounter the scarcity of seeds due to un-availability or effect genetic factors or loss of viability due to influence of various environmental factors. This will be major issue, particularly, when a large number genotypes with diverse agronomic characters needed to screen. Therefore, an attempt was made to determine minimum number of seeds required to get sufficient quantity of root exudates in early developmental stage of a seedling. The results obtained (Plate 1) out of these experiments showed that four seeds per cup was enough to release sufficient quantity of root exudates for these experiments. Though single seed would provide the sufficient root exudates, to avoid problems associated with germination or dormancy, four seeds per cup was used. The root exudates released from four seedlings was good enough to perform all the analyses.

Seeds, when initiates germination process, release a considerable amount of amino acids and sugars within the first 72 hr of germination (Rajagopalan and Bhuvaneswari, 1964) [9]. However, the root exudates quantity and chemical composition keep changing with change of genetic, environmental conditions and developmental stages of the seedling/plants. Since one of the objectives was to analyse root exudates in early stage of the seedling growth, right stage of seedling to collect root exudates of was standardized. The root exudates collection and analysis performed in three days interval for total 15 DAS, revealed that root exudates collected on 3rd day after sowing (DAS) is also sufficient, but collection on 3rd day found too early, since not many roots by that time developed. Considering the amount of roots formed DAS, the decision was taken to collect the exudates on 9th DAS, during which most seedlings were germinated and good amount of roots formed. Root exudates collected at this stage will be mostly from the roots (Plate 2). To date no such studies are reported in the literature to confirm the accuracy of these results. Thus, these results considered as first time report.

5.1 Future line of work

The methodologies developed during this investigation can be used for analysis of root exudates collected from any crop. The genotypic maps constructed by using TLC/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

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Table 3: Concentration of amino acids/proteins at 280 nm in spectrophotometer for first replication samples

	3rd day	6th day	9th day	12th day	15th day
1 seedling	0.225	0.332	0.531	0.222	0.227
2 seedlings	0.264	0.378	0.597	0.359	0.281
3 seedlings	0.297	0.335	0.639	0.315	0.309
4 seedlings	0.306	0.392	0.722	0.373	0.365
5 seedlings	0.264	0.441	0.673	0.323	0.392

Table 4: Concentration of amino acids/proteins at 280 nm in spectrophotometer for second replication samples

	3rd day	6th day	9th day	12th day	15th day
1 seedling	0.268	0.284	0.559	0.358	0.284
2 seedlings	0.211	0.327	0.683	0.344	0.331
3 seedlings	0.265	0.356	0.624	0.398	0.255
4 seedlings	0.365	0.384	0.712	0.343	0.304
5 seedlings	0.267	0.669	0.632	0.336	0.263

Table 5: Concentration of amino acids/proteins at 280 nm in spectrophotometer for third replication samples

	3rd day	6th day	9th day	12th day	15th day
1 seedling	0.246	0.279	0.574	0.289	0.232
2 seedlings	0.337	0.269	0.514	0.274	0.278
3 seedlings	0.226	0.323	0.657	0.326	0.315
4 seedlings	0.225	0.319	0.774	0.363	0.362
5 seedlings	0.227	0.652	0.636	0.353	0.341

Table 6: Mean concentration of amino acids/protein at 280 nm in spectrophotometer

Seed No.	3rd day	6th day	9th day	12th day	15th day	Mean ± SD
1	0.246 ± 0.017	0.298 ± 0.023	0.554 ± 0.017	0.289 ± 0.055	0.247 ± 0.025	0.3268 ± 0.115
2	0.27 ± 0.51	0.324 ± 0.044	0.598 ± 0.068	0.327 ± 0.037	0.296 ± 0.024	0.363 ± 0.119
3	0.262 ± 0.029	0.338 ± 0.013	0.64 ± 0.013	0.346 ± 0.036	0.293 ± 0.026	0.3758 ± 0.135
4	0.298 ± 0.057	0.365 ± 0.032	0.736 ± 0.013	0.359 ± 0.012	0.343 ± 0.028	0.4202 ± 0.159
5	0.252 ± 0.018	0.587 ± 0.103	0.647 ± 0.027	0.337 ± 0.012	0.332 ± 0.053	0.431 ± 0.155
Mean ± SD	0.2656 ± 0.018	0.3824 ± 0.104	0.635 ± 0.060	0.3316 ± 0.023	0.3022 ± 0.033	

Table 8: Densitometer readings of TLC plates analysed for Amino acids

Rf value	- Control	+Control	Khandwa-2	L-761	F-2226	JK-4	Raj-2	AK-23	CCH-1831	5433A2A03 N83	MCU-5	RHC-0811
0.5			60.72 ± 85.87									
0.13			15.78 ± 22.32									
0.14		516.07 ± 579.87	21.21 ± 30.00			20.34 ± 28.76		496.88 ± 662.34		44.32 ± 32.58	23.93 ± 13.85	56.83 ± 50.14
0.20		12.67 ± 17.92	34.36 ± 48.59	46.66 ± 65.99	93.61 ± 76.37						27.32 ± 38.32	34.37 ± 48.61
0.24			28.45 ± 40.23	58.03 ± 50.37	44.21 ± 49.66	33.75 ± 25.78						
0.26												121.3 ± 112.87 ±
0.27					50.98 ± 38.81	108.61 ± 94.169	439.69 ± 502.25					
0.30		62.34 ± 40.54						30.24 ± 25.60			26.47 ± 27.75	
0.35		100.70 ± 42.51		43.79 ± 33.05			106.85 ± 34.997		42.45 ± 37.51			
0.38		101.78 ± 23.08			46.95 ± 44.70		28.07 ± 28.11				94.86 ± 35.57	
0.40		38.56 ± 27.78	154.91 ± 83.77	154.93 ± 83.77						53.21 ± 37.52		
0.43											107.79 ± 20.74	
0.45		90.37 ± 40.73					85.39 ± 46.59	557.70 ± 721.10				
0.55		77.68 ± 37.40	1265.69 ± 1671.72									

Table 9: Densitometer readings of TLC plates analysed for Sugars

Rf value	- Control	+Control	Khandwa-2	L-761	F-2226	JK-4	Raj-2	AK-23	CCH-1831	5433A2A03 N83	MCU-5	RHC-0811
0.4												
0.14						25.13 ± 35.53						
0.21		183.25 ± 152.51										
0.28				60.26 ± 6806					54.63 ± 73.76			
0.33		481.18 ± 612.95	41.89 ± 48.71	44.13 ± 49.80	44.13 ± 49.80					186.82 ± 166.30		
0.31				187.04 ± 201.26		24.10 ± 29.24					49.76 ± 46.44	
0.48		789.92 ± 956.33										
0.50							280.8 ± 272.13	297.07 ± 207.13				

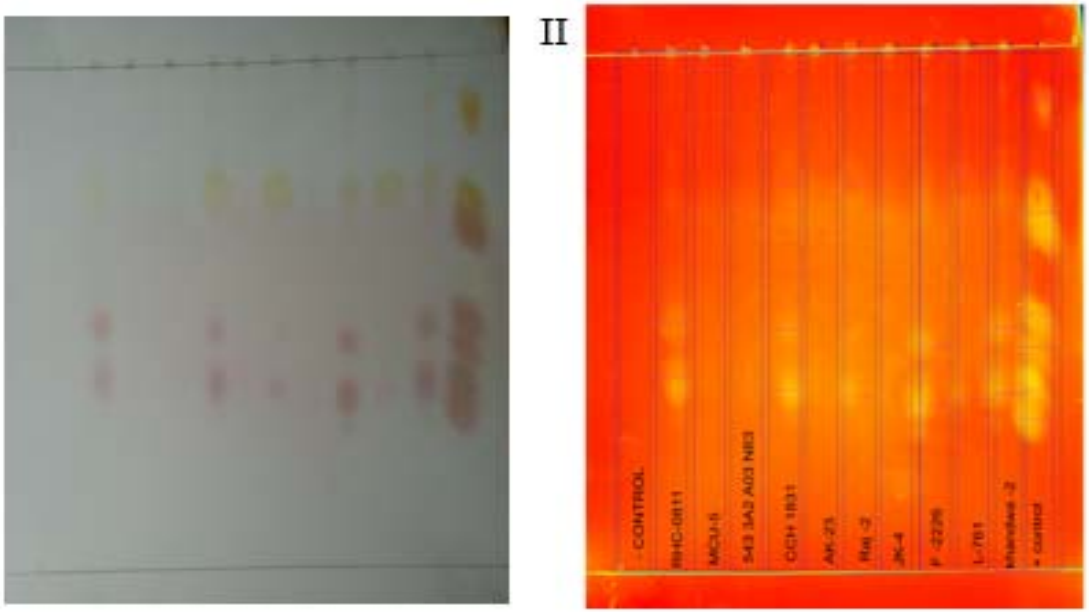
A: White silica sand

B: plastic cups with silica sand

C: Method of collection of root exudates in cotton seedlings

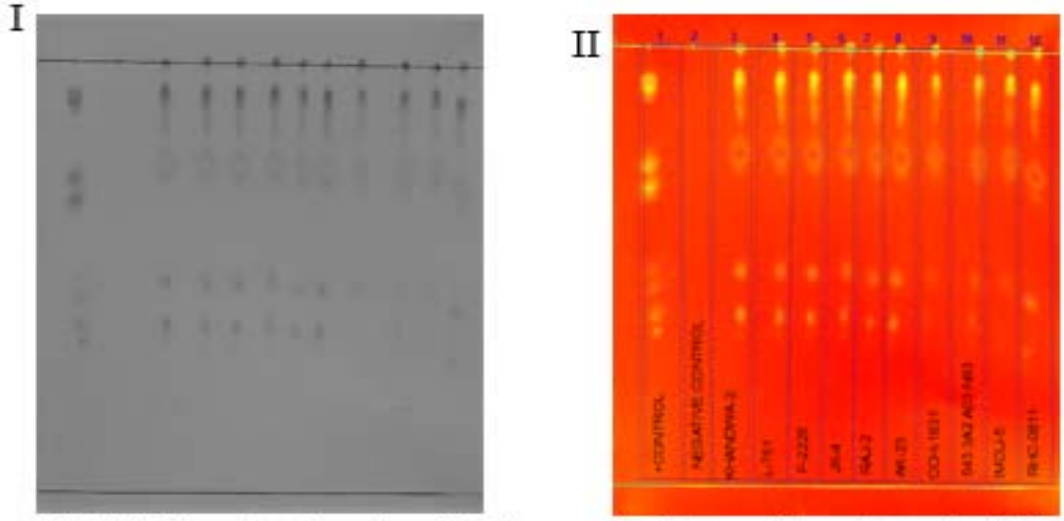
D: General view of experiment 9th day old cotton seedlings

Plate-1



A: TLC Plates of amino acid analysis for first rep I) normal view after analysis II) spectral view after densitometer analysis.

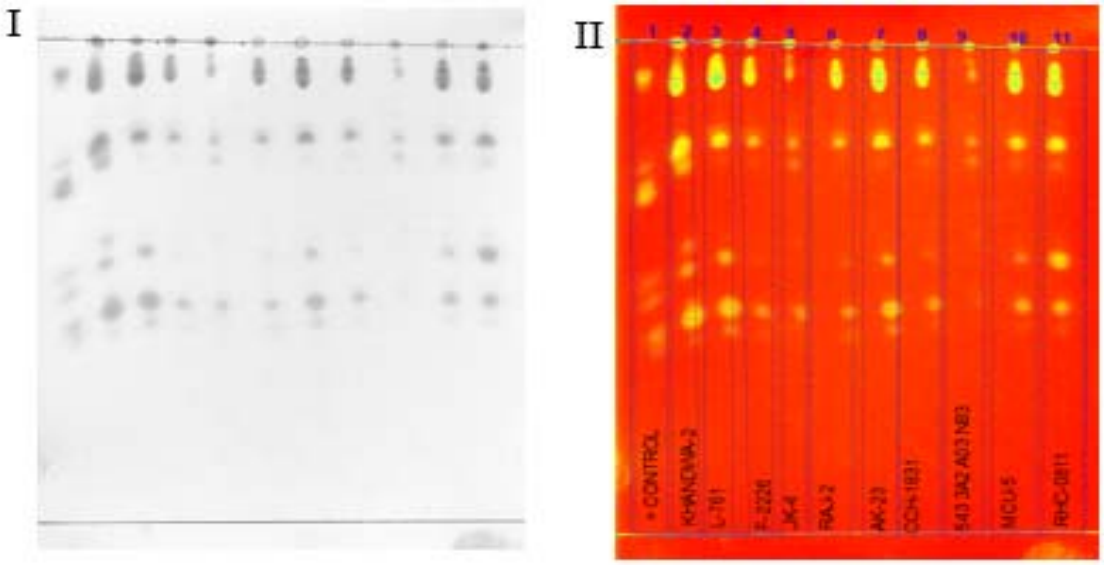
Lane 1- negative control, Lane 2- RHC-0811, Lane 3- MCU-5, Lane 4- 543 3A2 A03 N83, Lane 5- CCH1831, Lane 6- AK-23, Lane 7- RAJ -2 , Lane 8- JK-4, Lane 9- F-2226, Lane 10- 761, Lane 11- Khandwa-2, Lane 12- Positive Control



B : TLC Plates of amino acid analysis for secand rep I) normal view after analysis II) spectral view after densitometer analysis.

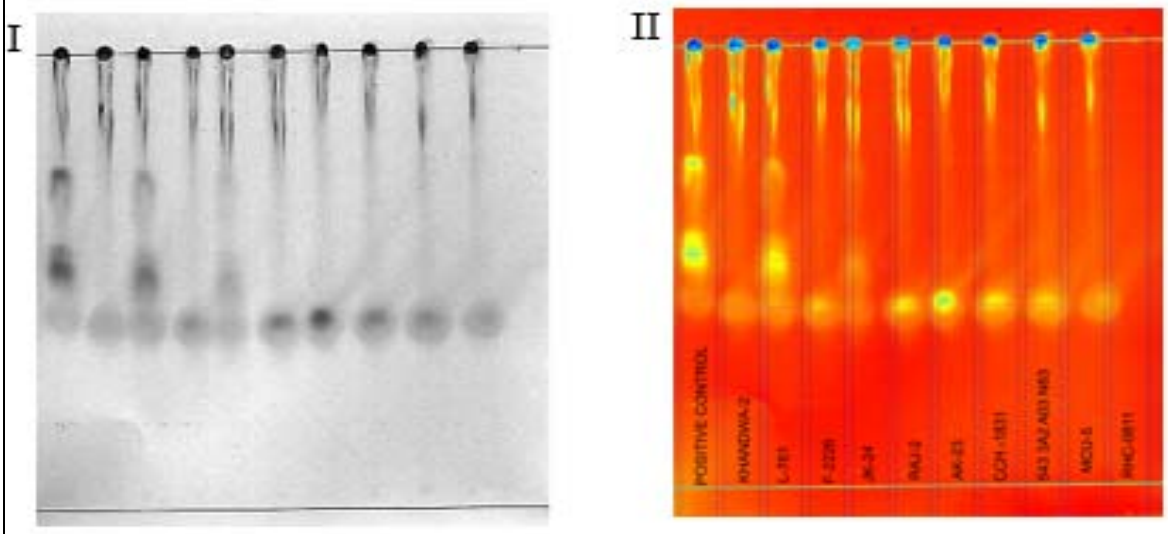
Lane 1- negative control, Lane 2- RHC-0811, Lane 3- MCU-5, Lane 4- 543 3A2 A03 N83, Lane 5- CCH1831, Lane 6- AK-23, Lane 7- RAJ -2 , Lane 8- JK-4, Lane 9- F-2226, Lane 10- 761, Lane 11- Khandwa-2, Lane 12- Positive Control

Plate-2



A : TLC Plates of amino acid analysis for third rep I) normal view after analysis II) spectral view after densitometer analysis.

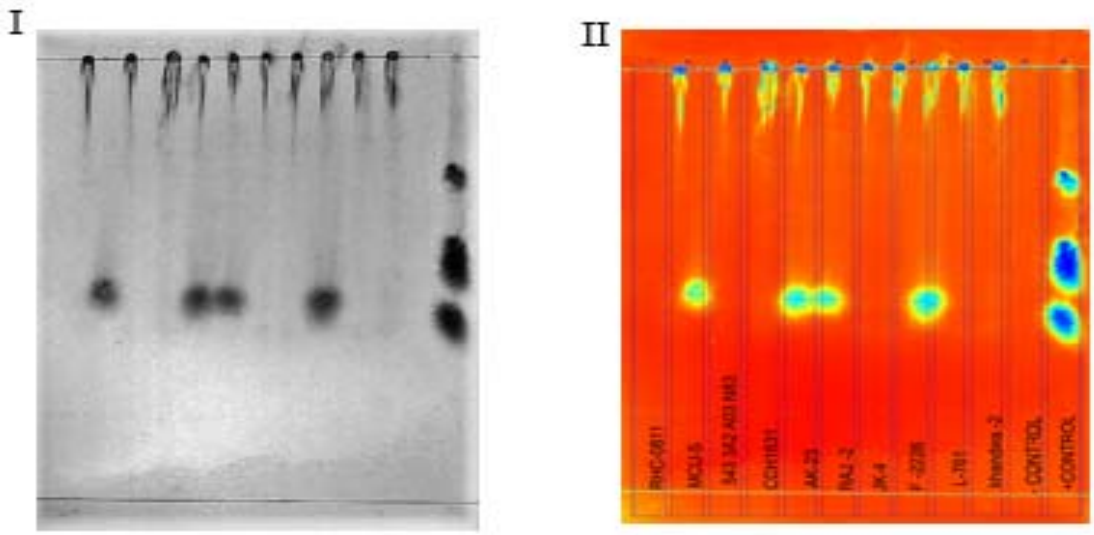
Lane 1- positive control, Lane 2 -Khandwa-2, Lane 3- 761, Lane 4- F-2226, Lane 5- JK-, Lane 6- RAJ -2 , Lane 7- AK-23, Lane 8-CCH-1831, Lane 9-5433A2A03N83, Lane 10- MCU-5, Lane 11- RHC-0811



B : TLC Plates of sugars analysis for first rep I) normal view after analysis II) spectral view after densitometer analysis.

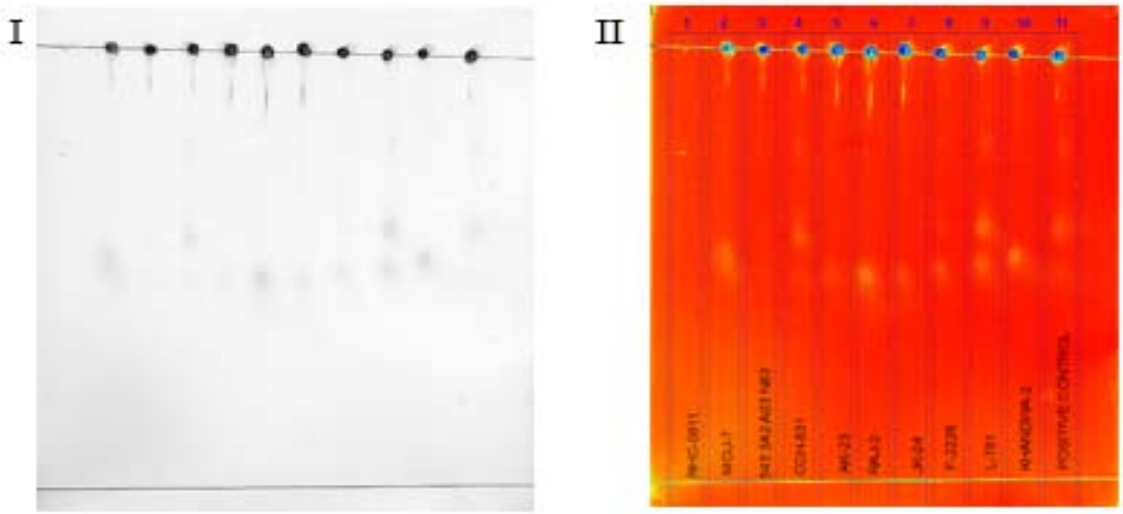
Lane 1- positive control, Lane 2 -Khandwa-2, Lane 3- 761, Lane 4- F-2226, Lane 5- JK-, Lane 6- RAJ -2 , Lane 7- AK-23, Lane 8-CCH-1831, Lane 9-5433A2A03N83, Lane 10- MCU-5, Lane 11- RHC-0811

Plate-3



A: TLC Plates of sugars analysis for secand rep I) normal view after analysis II) spectral view after densitometer analysis.

Lane 1- RHC-0811, Lane 2 -MCU-, Lane 3- 5433A2A03N83, Lane 4- CCH-1831, Lane 5- AK-23 , Lane 6- RAJ -2 , Lane 7-JK-4, Lane 8-F-2226, Lane 9-L-761, Lane 10- Khandwa-2, Lane 11- negative control, Lane 12 – positive control



B: TLC Plates of sugars analysis for first rep I) normal view after analysis II) spectral view after densitometer analysis.

Lane 1- RHC-0811, Lane 2 -MCU-, Lane 3- 5433A2A03N83, Lane 4- CCH-1831, Lane 5- AK-23 , Lane 6- RAJ -2 , Lane 7-JK-4, Lane 8-F-2226, Lane 9-L-761, Lane 10- Khandwa-2, Lane 11- positive control.

Plate–4

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