Plant Archives Vol. 24, No. 2, 2024 pp. 2037-2042 e-ISSN:2581-6063 (online), ISSN:0972-5210

Plant Archives

Journal homepage: http://www.plantarchives.org

DOI Url : https://doi.org/10.51470/PLANTARCHIVES.2024.v24.no.2.439

A NOVEL METHOD OF COLLECTION AND CHARACTERIZATION OF ROOT EXUDATES IN RICE SEEDLINGS

Paramesha H.T. and Channarayappa

Department of Biotechnology, College of Agriculture, UAS, Dharwad-580005, Karnataka, India.

*Corresponding author Email: paramesha3650@gmail.com

(Date of Receiving-29-06-2024; Date of Acceptance-11-09-2024)

ABSTRACT

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 was also being developed in rice. 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 root exudates. The experiments conducted to determine number of seeds and right stage of root exudation showed ten seeds (seedlings) per cup and 9th day after sowing is optimum. Thus, developing methods to isolate root exudates, analysis of root exudates composition is characterized with the help of TLC.

***Key words*:** Rice, Root exudates, thin layer chromatography, Spectrophotometer.

Introduction

Rice (*Oryza sativa* L.) belongs to family Poaceae, is one of the most important food crops of the world. There are 24 species out of which 22 are wild and two (*Oryza sativa* and *Oryza glaberrima*) are cultivated species. Rice being an important food crop, yield improvement and resistance to various biotic and abiotic stresses has become an important crop improvement program. Plants absorb minerals and water from soil and interact with various soil organisms. It has been well established that plants communicate with soil microorganisms through root exudes for various processes. Rice plants have been found to exude various compounds in genotype specific manner, such as sugars, amino acids in addition to more complex secondary metabolites. These involves in nutrient and water acquisition, plant defence and stimulatory or inhibitory interactions with other soil organisms. Understanding the root exudates composition and their role in rhizosphere is very important for the crop improvement. The genotypes having positive

interaction with growth promoting microorganisms based on root exudates composition can be effectively used for crop improvement, which helps to minimize the inputs and maximize the output, while retaining the soil health for sustainable agriculture.

Therefore, during this investigation, correlating root exudates composition with agronomic traits of different rice genotypes was made. This approach may help to identify genotypes with specific root exudates having positive interaction soil micro-flora and also to modify the rhizosphere suitably for better productivity.

Material and Methods

Collection of genotypes

Collection of suitable genotypes in rice for root exudates studies is very important for generating reliable results. Numbers of genotypes (at least 10 agronomically distinct genotypes) (Table 1) were obtained from a collection maintained by Dr. Prashanthi, S. K., Professor, Department of Biotechnology UAS, Dharwad. The standardization procedure for the collection of root exudates was carried out with the variety (JAYA) of rice.

Raising the seedlings

Silica sand as a medium of growth

White silica sand with ~0.3 mm diameter particle size (purchased from new water technologies,

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

Genotype	Germination Percentage	Duration of the crop (days)	Yield (q ha^{-1})	Special characters
INTAN	>90%	160-165	45-50	Grain type , medium slender
JAYA	>90%	130	50-60	Dwarf, susceptible to BLB, RTV and resistant to blast.
Abhilash	>90%	155-165	55-60	Long and bold seed, suitable for drill sowing.
Dodiga	>90%	130-145	-	Drought tolerant, good early vigor, well adapted for drill sowing
Antarsali	>80%	135-140	-	Drought tolerant, good early vigour
IMPROVED B P T 5204	>85%	150	60	Resistant to blast, medium slender.
NAVALI	>85%	140-150	-	Susceptible to leaf blast
BIDAR LOCAL 2	>90%	150	-	Susceptible to neck blast
MUGADSUGANDHA	90%	130-135	32-35	Moderate resistant to blast, moderate tolerant to LR.
MTU 1001	>85%	120-140	51	Tolerant to blast and BPH, medium slender.

Coimbatore) was used as a medium of growth (Fig. 1). The sand was thoroughly washed several times with running tap water after which it has been washed 2 to 3 times with distilled water. After complete drying of the sand in room temperature for 2 days, which was spread on plain paper. Washed and dried sand was sterilized by subjecting into autoclave at 120°C for 20 minutes.

Sowing of seeds in cups filled with sterilized silica sand

Transparent plastic cups with diameter of ~5 cm were purchased from market, filled with sterilized sand up to one-third of plastic cup height (~45 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 just enough to saturate the sand at the same time not to lose water by percolating out of the cup.

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 solution (water + root exudates) through the holes into sterile 15 ml

Fig. 1: White silica sand.

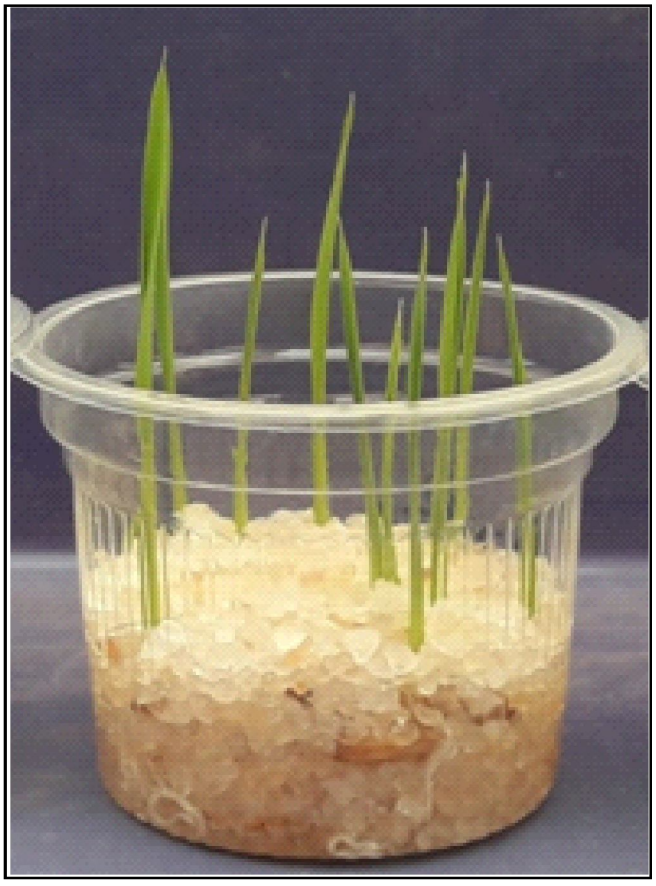


Fig. 2: Plastic cup with silica sand.

centrifuge tube (Fig. 3). 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.

Confirmation of presence of root exudates by reading the samples at A280 Spectrophotometer

The centrifuged samples were subjected to A280 spectrophotometer analysis (Eppendorf Bio-Spectrometer Basic) as preliminary confirmation to check the presence of the any compounds in samples based on their absorbance values at 280nm. The spectrophotometer blank-set (Distilled water) with control sample (collected from a cup without seed sown). Then the exudates samples were filled in a 2 ml cuvette and loaded to read in Bio-spectrophotometer (UVette). The absorbance maxima at 280 nm (generally used for detecting chemicals with amino-groups) were measured for all the genotypes and presented in both graphical and numerals.

Standardization of number of days required to get sufficient root exudates

Using the number of seeds per cup determined from the above experiment was used to determine the right seedling growth stage. The exudates were collected in three days interval starting from the third day after sowing to 21st 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.

Fig. 3: Method of collection of root exudates.

Concentrating root exudates by lyophilization

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

TLC analysis of rice root exudates for identifying different types of amino acids.

The lyophilized samples were dissolved in 50µl of autoclaved distilled water. In order to dissolve completely, the samples were incubated at 60^{o}C in hot-water bath for 10 min. The samples were transferred to fresh 1.5 ml centrifuge tubes and stored in deep freezer. The standard protocol for thin layer chromatography was followed as mentioned by Fried and Sherma, (1999) with the using ready-made silica gel plates (Merck). Development of the plate is done in development chambers with the mobile phase, which consisting combination of different solvents (n-butyl alcohol: Acetic acid: Water, 4:1:1) for analysis of amino acids. Visualization of the plate was done by spraying Ninhydrin reagent (0.3g Ninhydrin + 3ml Acetic acid + 100ml n-butanol).

After developing spots with development method, 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. Lee and Hoe (2001)

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

Analysis of TLC Plates by using Densitometer

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

Results and Discussion

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

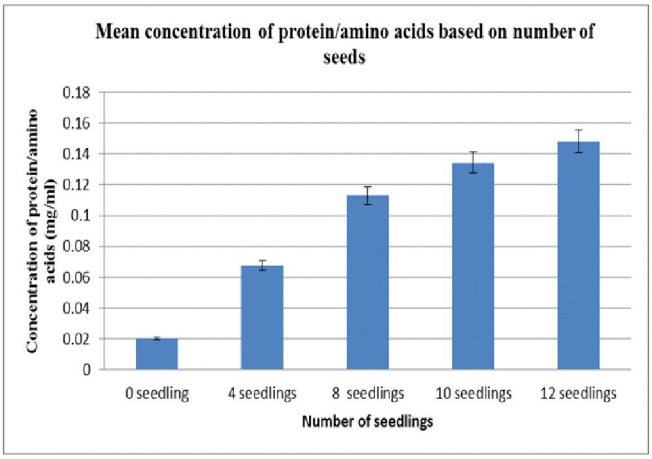
Root exudates collection procedure is very complex

Table 2: Mean concentration of amino acids/proteins at 280 nm in Spectrophotometer.

No. of days	0 seedling	4 seedling	8 seedling	10 seedling	12 seedling
3rd day	0.020	0.064	0.108	0.116	0.127
6th day	0.020	0.071	0.117	0.139	0.143
9th day	0.021	0.077	0.129	0.178	0.195
12th day	0.021	0.069	0.116	0.142	0.159
15th day	0.020	0.063	0.108	0.121	0.143
18th day	0.020	0.067	0.111	0.125	0.140
21st day	0.020	0.061	0.100	0.118	0.125
S.Em.	0.00036	0.00351	0.00577	0.01284	0.01430
C.D. @ 1 %	0.00146	0.01408	0.02323	0.05168	0.05756

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

To overcome these and other problems new kind of experiments were designed, that addresses most of these issues in very simple and reliable way. 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 rhizosphere. 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.



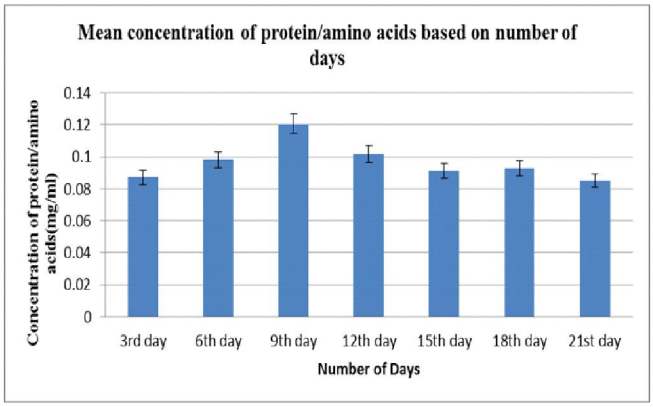
Graph 1: Mean concentration of protein/amino acids based on number of seedlings.

Standardization of number of seeds and number of days required to get sufficient (~1 ng) 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 (~1 ng) root exudates. The collected samples were subjected to A280 spectrophotometer analysis. The spectrophotometer readings obtained (Table 2) are true representations of the concentration of proteins/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 cup showed that, ten seedlings per cup were just enough to get sufficient root exudates for TLC analysis Table 2. Graph 1 shows that level of variation in root exudates concentration as the number of seedlings increases. This observation was helpful to reduce number of seeds of genotypes which were scanty. Further optimum (~1 ng) quantity of root exudates production at earliest stage of seedling growth was also determined Table 2. Graph 2 shows the variation of root exudates concentration as the number of days increases. The samples 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 days required to get sufficient (~1 ng) root exudates were very useful because the genotypic characterization through root exudates



Graph 2: Mean concentration of protein/amino acids based on number of days

analysis can be formed earliest stage of seedling 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 rice, which is not recorded earlier.

Analysis of root exudates by TLC

Root exudates being biochemical in nature, analysis of mixture of biochemicals require a very sensitive techniques that can separate and quantitate all possible components. TLC has been used as a very effective biochemical analysis technique for years. It is a very simple, cost effective, require a short time and can be applicable to analysis of most chemical compounds. Therefore, during these studies, TLC technique has been used to analyse root exudates of different genotypes. The root exudates pattern generated in the form of spots on the TLC plates for various rice genotypes used for constructing genotypic maps. Sahana *et al.,* (2011) reported that the results of TLC analysis of root exudates for various compounds showed that TLC analysis can be used to separate complex mixture of compounds into group of compounds. The results of Kavitha *et al*., (2014) showed that, root exudates having greater rhizosphere effect in cereal crops and it was assumed that this was due to greater amount of root exudates produced by them.

Thin Layer Chromatography (TLC) was preferred as it could separate the compounds from mixture of compounds based on its affinity towards mobile and stationary phase. For analysis of amino acids by TLC, the mobile phase with composition of n-butanol: acetic acid: water (4:1:1) showed higher resolution. The results

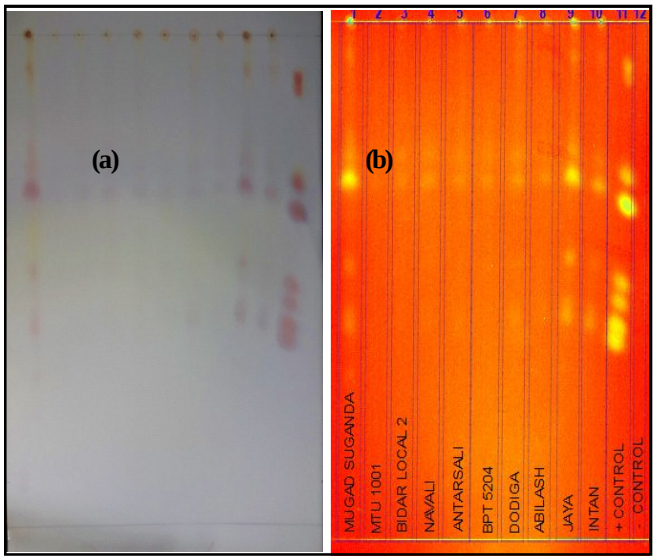


Fig. 4: TLC Plates of amino acid analysis for first rep (a) normal view after analysis; (b) spectral view after densitometer analysis.

showed that the analysis for amino acids developed some spots except in few genotypes (BPT 5204 and MTU 1001) where there were no spots were found (Fig. 4). It may be due to lower quantity of amino acids in those genotypes which couldn't be detected by TLC. For the genotypes with developed spots, R_f value was calculated and compared with the standard R_f 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 spot are very unique to each genotype and at same time differ across genotype.

Densitometer analysis of TLC plates

The TLC plates obtained by amino acids (Fig. 4a) analysis were subjected to densitometer analysis in order to quantify the volume/intensity of each spot developed. The TLC plates were analyzed in densitometer by reflective scan mode and red filter colour. As a result, the densitometer analysed TLC plates image has highly 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 provide an overall view of either presence or absence of particular spots across the genotypes using their R_f values and also the difference in intensity between each genotype using the volume of each spots. The lane 1 and 2 in densitometer analysed spectral image TLC plates of amino acids (Fig. 4b) are negative control and positive control respectively while all other genotypes were arranged in lane 3 to 12. The spectral image of TLC plate of amino acid clearly shows the presence of one spot which is uncommon between genotypes but found unique to Jaya and Mugad sugandha. The similarity between Jaya and Mugad sugandha is that, they both are moderate resistant to blast. 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 3 shows the mean volume of each spot along with standard deviation (SD) of three replications thereby providing the overall reliability of TLC analysis.

Conclusions

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 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.

References

Fried, B. and Sherma J. (1999). Thin-Layer Chromatography, revised and expanded, CRC Press, 98-111.

Kavitha, M.J. and Ilamurugu K. (2014). Metabolic profiling of rice root exudates and its impact on rhizosphere microbial dynamics under aerobic conditions. *Res. J. Agric. Sci.*, **5(4)**, 777-781.

Lee, K.Y., So J.S. and Heo T.R. (2001). Thin layer chromatographic determination of organic acids for rapid identification of bifidobacteria at genus level. *J. Microbiol. Methods.*, **45(1)**, 1-6.

Phillips, R.P., Erlitz Y., Bier R. and Bernhardt E.S. (2008). New approach for capturing soluble root exudates in forest soils. *Functl. Ecol*., **22(6)**, 990-999.

Sahana, A., Das S., Saha R., Gupta M., Laskar S. and Das D. (2011). Identification and interaction of amino acids with leucine-anthracene reagent by TLC and spectrophotometry: experimental and theoretical studies. *J. Chromatogr. Sci.*, **49(8)**, 652-656.

Shay, F.J. and Hale M.G. (1973). Effect of low levels of calcium on exudation of sugars and sugar derivatives from intact peanut roots under axenic conditions. *Plant Physiol.*, **51(6)**, 1061-1063.