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Effect of Inoculation on Root Exudates Carbon Sugar and Amino Acids Production of Different Rice Varieties

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Abstract: An experiment was conducted in axenic condition to study the effect of *Corynebacterium* sp. (Sb26) and *Rhizobium* sp. (Sb16) inoculation on the root exudates carbon sugars and amino acid production in three different rice (*Oryza sativa*) genotypes. A total of seven carbon sugars and 16 amino acids were determined from the Mahsuri, Mayang Segumpal and MR219 rice root exudates. The concentration of root exudate sugars, amino acids and its released pattern were significantly different with rice genotypes. Mahsuri released the highest sugar (25.73%) followed by MR219 and Mayang Segumpal (23.14% and 20.85% of plant dry wt.) rice, respectively. Inoculated plants produced different amount of sugar and amino acids in the presence of diazotrophs compared to non inoculated plants. Mahsuri rice inoculated with *Corynebacterium* sp. released the highest amount of fructose (791 $\mu\text{mol g}^{-1}$ root dry wt.) and arabinose (640 $\mu\text{mol g}^{-1}$ root dry wt.). Mayang Segumpal rice inoculated with *Rhizobium* sp. produced the highest amount of sucrose $\mu\text{mol g}^{-1}$ root dry wt in the root exudate. A significantly higher amount of glycine and isoleucine were detected in the inoculated root exudates of all rice varieties. However, inoculation enhanced production of sugars and amino acids in root exudates. In general rice genotypes inoculated with *Rhizobium* sp. produced higher amount of total sugars and amino acids in root exudates compared to that of *Corynebacterium* sp.

Key words: *Corynebacterium* sp., *Rhizobium* sp. rice genotypes, root exudates

INTRODUCTION

Plants naturally release carbon compounds across the root cell plasma membranes and any efflux is termed as exudation. A vast array of compounds in the rhizosphere is one of the most remarkable metabolic features of plant roots. These compounds include organic acids, amino acids and specific sugars. Carbohydrates and organic acids are the dominant component of root exudates and generally have a wide C/N ratio. The main components of root exudates in C_4 plant is malic acid and in C_3 plant are citric and oxalic acids. Plant exerts higher amount of carbon compounds during the first two weeks of growing periods. The composition and concentration of sugars and amino acids from rice root exudates, during the first month of life of the plants, exerts great attraction for the endophytic strains (Baciolo- Jamenez *et al.*, 2003).

Root exudates are readily available carbon source for microorganisms in the rhizosphere which participate in the early colonizing process. About 64 to 86% of the carbon released into the rhizosphere is used by microorganisms (Hutsch *et al.*, 2002). Root exudates stimulate the soil bacteria leading to an increase in population. Weller and Thomashow (1994) reported that about 10 to 100 fold

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microbial population size was found in the rhizosphere compared to the surrounding bulk soil. Microbial activity is higher in the vicinity of plant roots. Diazotrophs and plant growth promoting rhizobacteria utilized carbon compounds released from root and form an associative symbiotic relationship with the plant.

The amount and composition of root exudates entering the soil is variable. It depends on plant species and age of plants. Soil environment and microbial community of the rhizosphere also influenced the net root exudation. Amino acid exudation under normal conditions is a phenomenon that probably reflects both active manipulation and passive uptake by microorganisms (Phillips *et al.*, 2004). The concentrations of amino acids released by plants are considered to be insufficient sources of nitrogen in the rhizosphere (Simons *et al.*, 1997). Identifying the potential enhancers of root exudation and the most utilized carbon compounds by microbes may help to establish a better symbiotic association between diazotrophs and plants. The aim of this study was to determine the carbon sugars and amino acids in rice root exudates and the influences of two species of diazotrophs on root exudation during the first 20 days of the growing periods.

MATERIALS AND METHODS

The study was conducted at University Putra Malaysia in *in vitro* condition during March 2006. Three rice (*Oryza sativa*) cultivars; Mahsuri, Mayang Segumpal, MR219 and two diazotrophic strains *Corynebacterium* sp. (Sb26) and *Rhizobium* sp. (Sb16) were studied. The experiment was laid out in a factorial and complete randomized design (CRD) with three replications. The data were analyzed using the SAS (9.1 version) statistical software.

Seed Surface Sterilization

The seed surface sterilization method was modified from the report of Amin *et al.* (2004). Rice seeds were dehusked and shaken in 70% ethanol for 5 min. The ethanol was discarded and the seeds agitated in hypochlorite solution comprising 50% of Chlorox™ (2.6% NaOCl) and sterilized with a few drops (20) of distilled water for 5 seconds. After three rinses of sterile water the seeds were then rinsed with 2% sodium thiosulphate solution to neutralize chloramine residue on the seeds. Seeds were grown in Petri dishes lined with filter paper. The efficacy of sterilization was checked by germinating seeds on nutrient agar (NA) plates.

Preparation of Inoculation

Rhizobium and *Corynebacterium* strains were grown in ATCC broth for 48 h. The bacterial cells were harvested by centrifugation at 13500 rev min⁻¹ for 10 min in eppendorf tube and washed with 0.85% sterilized phosphate buffer saline. Optical density (OD₆₀₀) of washed cells were checked and adjusted accordingly. Approximately 10⁹ mL⁻¹ live bacterial cell were used to inoculate plants in each planting unit. The population was confirmed by cell enumeration in drop plate method on Nutrient Agar (NA).

In vitro Growth of Rice Seedlings

Plants were grown in glass tube (2 L × 5 cm). Each glass tubes were equipped with a stainless steel holder and sieve to support the seedlings. Eight axenic 5 days old rice seedlings were placed on the sieve surface of 50 mL nutrient solution. The seeds were placed in such a way that only the roots were in touch with nutrient solution. The plant nutrient, free of carbon and nitrogen was modified from Egener *et al.* (1999) contained in (L⁻¹): KH₂PO₄, 1.5, K₂HPO₄, 0.33, K₂SO₄ and 0.2 g, ferric citrate, 13 mg, CaCl₂·2H₂O, 0.4 g, MgCl₂, 0.4g Na₂MoO₄·2H₂O, 2 mg, H₃BO₃, 3 mg, MnSO₄·H₂O, 2 mg, ZnSO₄·7H₂O, 0.2 mg, CuSO₄·5H₂O, 0.1 mg was used as growing media. Prior to placement on the holder the seedlings were gently washed with sterile distilled water to discard

the plant metabolites formed during germination. Seedlings in each planting unit were subsequently inoculated with 5 mL of bacterial inoculums. Plants were grown for 20 days in growth chamber with 12 h light/dark cycle at 28°C.

Root Exudates Collection

The root exudates of non inoculated and inoculated plants were collected from the each planting unit at 5, 8, 14 and 20 days after transplanting. The growth culture solutions in all planting units were filtered through a 0.2 µm Millipore filter and were kept in screw-top glass bottles at -20°C for determination of sugars and amino acids.

Sugar Analysis

Sugars were determined using High Performance Liquid Chromatography (HPLC) with refractive index (RI) detector. Galactose, arabinose, xylose, fructose and sucrose were determined using NH₂-carbohydrate column. Acetonitrile (75%) was used as mobile phase with a flow rate of 1 mL min⁻¹. Mannose and glucose were determined by using Supel cogel column. Phosphoric acid (1%) was used as mobile phase at a flow rate of 0.8 mL min⁻¹.

Amino Acids Analysis

Amino acid concentrations were determined using HPLC by a modified method from Strydom and Cohen (1994) following pre-column derivatisation with AQC reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, Waters, USA). Tryptophan content was determined by alkaline hydrolysis. Cysteine and methionine were not determined.

Total Sugar and Amino Acids Production

The total sugar and amino acids were estimated as:

$$TP = TC + ER$$

where, TP is the total sugar and amino acids production by the plant, TC is the total sugar and amino acids consumption by the diazotrophs over period and ER is the extra sugar and amino acids remaining in the inoculated plant root exudates.

Total sugar consumption (TC) was determined as:

$$TC = S_t - S_{ti}$$

where, S_t is the total sugar production by the control plant over time and S_{ti} is the total sugar remaining in the culture solution by the inoculated plant over time.

Plant Biomass Production

Plant sample of each sampling period were dried in oven at 80°C. Total plant biomass recorded after constant weight was achieved.

RESULTS

Carbon Sugars and Amino Acids in the Root Exudates of non Inoculated Rice Genotypes

There were differences in concentrations and distribution of carbon sugars and amino acids in the root exudates of three rice varieties. Glucose, fructose, xylose, mannose arabinose, galactose and sucrose were determined from the root exudates of Mahsuri, Mayang Segumpal and MR219 rice varieties. Mahsuri rice released 25.73% of sugar at 8 days after transplanting (Table 1). Rapid release

Table 1: Percent root exudate values of three rice varieties represents the means of 3 replications

Days after transplanting	Percent sugar (RE) production by non inoculated plant dry weight		
	Mahsuri	Mayang segumpal	MR219
5	9.86 ^{bed}	20.85 ^{bed}	23.14 ^{ab}
8	25.73 ^a	0.8 ^{bed}	8.78 ^{bed}
14	6.34 ^{ed}	2.99 ^d	3.75 ^d
20	3.57 ^d	1.26 ^d	0.60 ^d

RE = root exudates. Different superscript letter(s) represent significant differences at the 5% level of significance

Table 2: Total amino acids production in the root exudates of three rice varieties. Value represents the means of 3 replications

Days after transplanting	Total amino acids production ($\mu\text{mol g}^{-1}$ root dry) by non inoculated rice varieties		
	Mahsuri	Mayang segumpal	MR219
5	259.41 ^a	16.39 ^e	63.98 ^e
8	97.58 ^e	43.6 ^f	104.02 ^b
14	72.62 ^d	44.43 ^f	7.90 ^h
20	50.12 ^f	35.96 ^f	7.45 ^h

RE = root exudates. Different superscript letter(s) represent significant differences at the 5% level of significance

of sugars was observed in Mayang and MR219 varieties. Mayang rice released 20.85% and MR219 rice released 23.14% of sugar in the root exudates at 5 days after transplanting.

A total of 16 amino acids were determined from root exudates of three rice varieties. The Mahsuri plant produced the highest amount (259.41 $\mu\text{mol g}^{-1}$ root dry wt.) of amino acids at 5 DAT. Mayang Segumpal and MR219 rice varieties produced the highest amount (43.60 and 104 $\mu\text{mol g}^{-1}$ root dry wt.) of amino acids at 8 d after transplanting, respectively (Table 2).

Effect of Inoculation on Carbon Sugar Production

The sugar concentrations were found to be differed among the rice varieties. Rice plant released different amount of sugars in the presence of diazotrophs. Inoculation with *Rhizobium* sp. enhanced the production of total sugars in all of three rice varieties (Fig. 1a-c). In Mahsuri rice, arabinose, galactose, fructose and sucrose production were enhanced by inoculation. Mahsuri rice inoculated with *Rhizobium* sp. produced additional amount of galactose and sucrose and inoculation with *Corynebacterium* sp. released highest amount of fructose (791 $\mu\text{mol g}^{-1}$ root dry wt.) and arabinose (640 $\mu\text{mol g}^{-1}$ root dry wt.) in the root exudates (Fig. 1a). However, there were no significant differences found in the glucose, mannose and xylose concentrations within inoculated and non inoculated Mahsuri rice. The non inoculated Mayang rice released mannose, xylose and arabinose where as, those of inoculated with *Rhizobium* sp. released extra amount of fructose, galactose and sucrose. Mayang rice inoculated with *Rhizobium* sp. produced the highest amount of sucrose 732 $\mu\text{mol g}^{-1}$ root dry wt. (Fig. 1b). There was no glucose detected in the root exudates of inoculated and non inoculated Mayang rice variety. MR219 rice inoculated with *Rhizobium* sp. produced extra amount of xylose and fructose in the root exudates (Fig. 1c) compared to that of non inoculated one.

Effect of Inoculation on Amino Acids Production

There were differences in amino acids production among three rice genotypes. Mahsuri rice that was inoculated with *Rhizobium* sp. released higher amounts of sixteen amino acids except tryptophan. The highest amount of glycine (133 $\mu\text{mol g}^{-1}$ root dry wt.) followed by isoleucine were found in the Mahsuri rice inoculated with *Rhizobium* sp. On the other hand, inoculation with *Corynebacterium* sp. enhanced the release of aspartic, glycine, histidine, proline, valine, leucine and phenylalanine in Mahsuri rice (Fig. 2a). However, Mayang rice inoculated with *Rhizobium* sp. enhanced all of the amino acids production except glutamine, glycine and tryptophan and inoculated with *Corynebacterium* sp.

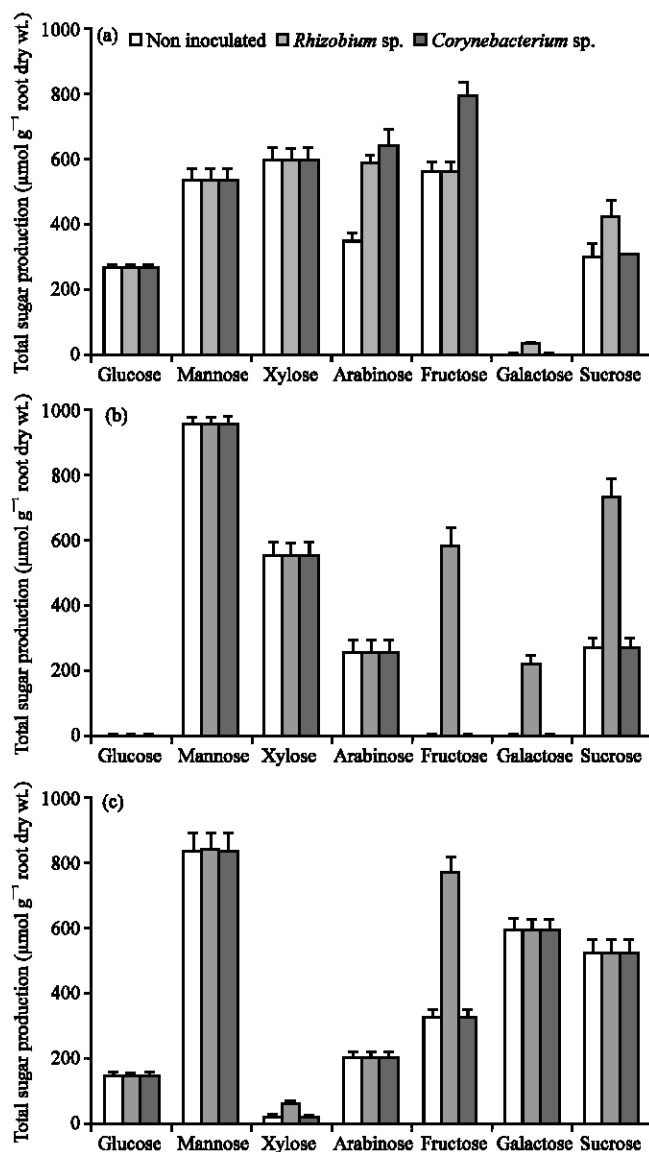


Fig. 1: Total carbon sugar production by inoculated and non inoculated, (a) Mahsuri rice, (b) Mayang segumpal rice, (c) MR219 rice varieties. (Bars indicates standard error, n = 3)

enhanced most of the amino acids production except tryptophan. In Mayang Segumpal rice root exudates *Corynebacterium* sp. produced the highest amount of glycine (36 μmol g⁻¹ root dry wt.) followed by isoleucine (Fig. 2b).

In MR219 rice *Corynebacterium* sp. released the highest amount of isoleucine (44 μmol g⁻¹ root dry wt.) followed by glycine. In general inoculation increased serine, glutamine, glycine, isoleucine and phenylalanine production in MR219 variety. Whereas, MR219 rice inoculated with *Rhizobium* sp. enhanced the amino acids of aspartic, serine, glutamine, histidine, proline, tyrosine, valine and phenylalanine. On the other hand, MR219 rice inoculated with *Corynebacterium* sp. enhanced higher amount of histidine, proline, tyrosine and isoleucine production (Fig. 2c).

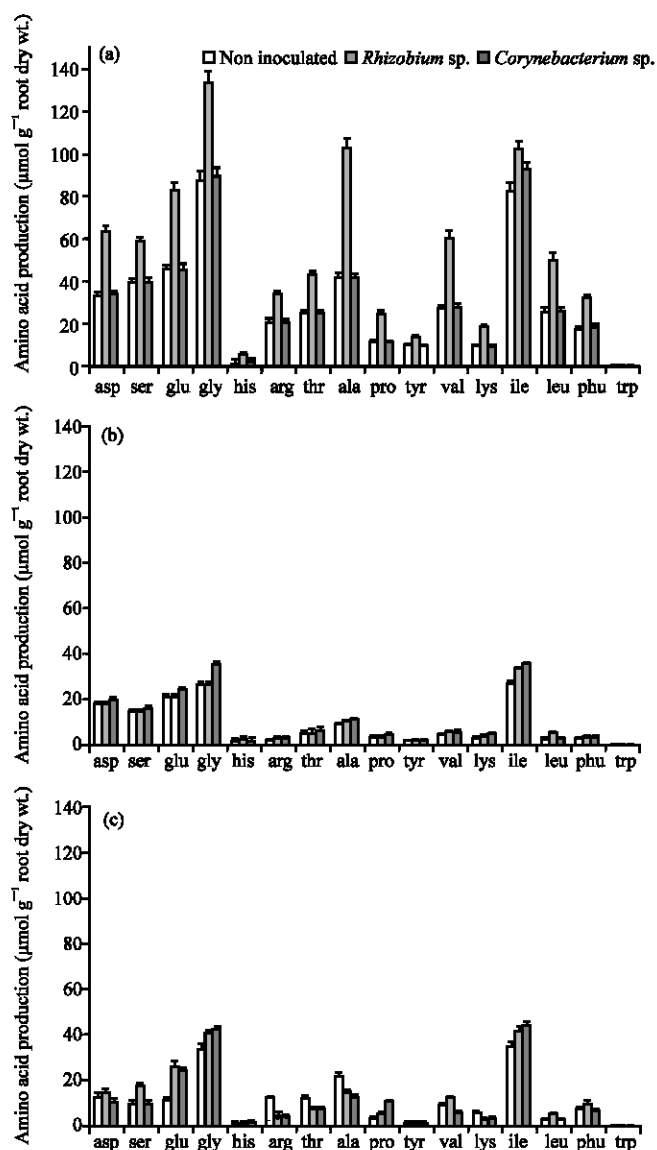


Fig. 2: Total amino acids production by inoculated and non inoculated (a) Mahsuri rice (b) Mayang segumpal rice (c) MR219 rice varieties. (Bars indicates standard error, n = 3)

DISCUSSION

The carbon sugars and amino acids concentration and their released pattern in rice root exudates are differed with the genotypes. Mahsuri and MR219 varieties released higher amounts of sugars and amino acids in root exudates that were regarded as higher yield producing rice compare to that of Mayang Segumpal which is local accession and regarded as lower yield producing variety. The present study showed that Mahsuri rice released the highest amount of sugars at 8 days after transplantation. Lynch and Whips (1990) reported that cereals were released from 4 to 29% of photosynthates as root

exudates. Most of the sugars and amino acids concentrations were found to be higher within the first two weeks of the growing periods. Similarly, Darrah (1993) also reported that the root exudates sugar accumulation was higher during the first two weeks of growth period in wheat seedlings. Mayang Segumpal and MR219 rice released higher amount of carbon sugars and earlier than Mahsuri rice. On the other hand, Mahsuri rice released the amino acids in the root exudates earlier than the other two varieties. The root exudates carbon sugars and amino acids concentrations showed a decreasing trend at 14 days after transplanting. Similar findings were observed in the rice root exudates grown in hydroponics condition (Bacilio-Jiménez *et al.*, 2003). The decrease rate in exudation of hydroponic culture systems was probably occurred by the accumulation of high levels of organic substances in the root environment that may repressing the release of more organic compounds (Jones and Darrah, 1993; Prickryl and Vancura, 1980) or by reabsorption of the organic compounds by the plants (Guckert *et al.*, 1991). In this study there was no glucose found in the Mayang rice root exudates and lower amount of glucose was detected in other two varieties, this may be due to the plants that were grown in strictly carbon free nutrient solution and faster reabsorption of this primary photosynthate product by the plant roots. Previous study revealed that rapid utilization of low molecular weight carbon substrates by plant roots enhances competition for labile carbon resource with microbial populations (Kuzakov and Jones, 2006). In the non sterile soil system at lower temperature some amount of glucose also could be converted into a water soluble intermediate such as maltose (Coddy *et al.*, 1986) that may be another cause of low glucose accumulation in the root exudates.

Bacterial inoculation enhanced sugar and amino acids released in the root exudates. In this study inoculated rice varieties produced additional sugar and amino acids compared to non inoculated plants. Previous results also showed that inoculation of plant roots in the culture solution increased root exudation (Beck and Gilmore, 1983; Lee and Gaskins, 1982; Prickryl and Vancura, 1980). Root exudates amino acids are actually a net release of both influx and efflux of components (Jones and Darrah, 1994). Relatively constant values obtained between efflux and influx rates or a pool of amino acids which is no longer influenced by the roots (Phillips *et al.*, 2004) and it can be measured. In the present study it was found that *Rhizobium* and *Corynebacterium* spp. produced additional amount of galactose, sucrose, fructose, arabinose, glycine and isoleucine in the root exudates. Higher amount of histidine and proline also detected in the inoculated plants root exudates compare to that of non inoculated rice varieties.

CONCLUSIONS

In this study seven sugars and sixteen amino acids were studied. To get a complete scenario in the root exudates, all of the carbohydrate components were needed to be determined. In the presence of microbes, exact determination of sugars in plant root environment need to be labeled the carbon study as both of the plant and microorganism were competed for low molecular weight carbon sources. Without labeling carbon, a frequent sampling can be generated as an assumption. However, this frequent and indirect determination of sugars and amino acids in the root exudates also can be produced the fundamental information for the released pattern, consumption and production in the presence of microorganisms.

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