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Physiological Responses of Rice Seedling (*Oryza sativa* L.) to Salt-stress Cultured under *in vitro* Photomixotrophic and Photoautotrophic Systems

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Abstract: Physiological responses of crop species to salt-stress in conventional *in vitro* culture may be found some errors due to sucrose as a main carbon source, causing to erratic data. Seven-day-old *in vitro* seedlings were aseptically transferred to culture under photoautotrophic and photomixotrophic systems for 13 days, subsequently adjusted to 0 and 342 mM NaCl. The pigment degradation of seedlings grown in 342 mM NaCl was positively related to NPR reduction in both the photoautotrophic (r=0.84) and the photomixotrophic system (r=0.95). This resulted in low growth, as measured by leaf expansion, shoot height, root length, fresh weight and dry weight. Moreover, the NPR reduction of seedlings cultured with 342 mM NaCl was positively related to the low survival percentage for both the photoautotrophic (r=0.96) and the photomixotrophic system (r=0.98). Moreover, the phenotypic responses of photoautotrophic seedlings to salt-stress expressed a more realistic phenotype than the photomixotrophic system. Besides, the phenotypic expression of seedlings cultured under photoautotrophic system responded more sensitively to salt-stress than those photomixotrophic system. Therefore, study of the phenotypic responses of seedlings to salt-stress would make use of the photoautotrophic system. This system should be a novel process for phenotypic expression of *in vitro* to salt stress.

Key words: Net photosynthetic rate, phenotypic expression, pigments, sodium chloride, survival percentage

INTRODUCTION

Sucrose plays a central role in the biosynthetic pathway of primary and secondary metabolites, serves as a building block for several plant macromolecules and is involved in the regulation of various developmental processes[1-3]. Heterotrophic and photomixotrophic *in vitro* culture systems generally use sucrose as their main carbon source. However, several reports have shown that the supplementation of sucrose within the culture medium has a negative effect on the photosynthesis of various plant species[4-6], which can lead to the misinterpretation of plant phenotypes[7]. Sucrose in the culture media can be taken up directly by plant cells, or it can be hydrolyzed extracellularly to glucose and fructose and then taken up in the form of hexose[8]. The sucrose is transported to sink organs as the end product of photosynthesis and causes the repression or feedback inhibition of Rubisco[9-11]. In addition, heterotrophic and photomixotrophic plantlets are potentially capable of developing a functional photosynthetic apparatus such as pigments, light harvesting complexes and electron transport protein complexes. On the contrary, sucrose stimulants affect the photosynthetic enzymes, such as PEP carboxylase, in plantlets *in vitro*[12-15]. However, study of the phenotypic expression in conventional *in vitro* culture is still limited because photomixotrophic plantlets showed the physiological, anatomical and morphological disorders[5,16,17]. There are many reports that generally used the photomixotrophic system as a prototype of plant responses to salt stress, as well as salt tolerance screening[18,19]. However, the phenotypic expression of plantlets cultured under photomixotrophic system should be found some errors, causing to erratic data.

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Photoautotrophic growth of *in vitro* plantlets is enhanced by photosynthesis, leading to stimulation of the physiological, anatomical and morphological characteristics[20]. Therefore, plantlets cultured in a photoautotrophic system should express phenotypic responses to certain abiotic stresses such as salt, drought and extreme temperature, which are more reflective of plant grown *ex vitro*. The photoautotrophic system has been successfully applied for salt stress responses in *Albizzia lebbek*[21] and salt tolerance screening of one hundred forest tree species[22]. The photoautotrophic system should be a better system for salt tolerant screening and for salt-tolerant ability testing than the conventional method. However, the convenient prototype of realistic phenotypic expression in both systems is still unclear.

In the present study, salt-stress was applied as the abiotic stressor of *in vitro* culture to evaluate phenotypic expression. This salt-stress directly causes reduction of photosynthetic ability such as pigments, light harvesting complexes and electron transport protein complexes, resulting in a low net-photosynthetic rate[23-26]. In this report we compared the physiological responses and growth ability of rice seedlings cultured photomixotrophically and photoautotrophically under either both with or without salt-stressed conditions.

MATERIALS AND METHODS

Plant materials: Seeds of jasmine rice (*Oryza sativa* L. ssp. *indica* cv. KDML 105) were obtained from the Pathumthani Rice Research Center (Rice Research Institute, Department of Agriculture, Ministry of Agriculture and Cooperative, Thailand). Seeds were dehusked by hand, sterilized once in 5% Clorox® (5.25% sodium hypochlorite, The Clorox Co, US) for 60 min, once in 30% Clorox® for 30 min and then rinsed three times with sterile distilled-water. Surface-sterilized seeds were germinated on 0.25% Phytagel®-solidified MS media[27] in a 250 mL glass jar vessel. The media were adjusted to pH 5.7 before autoclaving. Seedlings were cultured *in vitro* under condition of 25±2°C ambient temperature, 60±5% Relative Humidity (RH) and 60±5 μmol m^{-2} s^{-1} Photosynthetic Proton Flux (PPF). The PPF was provided by fluorescence lamps (TDL 36 W/84 Cool White 3350 Im, Philips, Thailand) with 16 h d^{-1} photoperiod. Seven-day-old rice seedlings were aseptically transferred to MS-liquid media under photoautotrophic (CO_2 as a carbon source) or photomixotrophic (sucrose and carbon dioxide as the carbon sources) systems, with vermiculite used as a supporting material. The photomixotrophic media contained 88 mM sucrose. The number of air-exchanges in the glass vessels of photoautotrophic and photomixotrophic systems was adjusted to 2.32 h^{-1} by punching a hole on plastic cap (⌀ 1 cm) and covering the hole with a microporous filter (0.20 μm of pore size). All seedlings were continuously cultured under the same conditions as during the seed germination process. After 13 days of growth, the NaCl concentration of the media were adjusted to 0 (control) or 342 mM NaCl (salt-stressed) and the cultures grown for an additional 0, 2, 4 and 8 days.

Pigments assay: Chlorophyll a (Chl_a), chlorophyll b (Chl_b) and total carotenoid (C_{x+c}) concentrations were analyzed following the methods of Shabala[24] and Lichtenthaler[28], respectively. The Ch_a and Ch_b concentrations were measured using an UV-visible spectrophotometer (DR/4000, HACH, USA) at wavelengths 662 and 644 nm. The C_{x+c} concentration was measured spectrophotometrically at 470 nm. A solution of 95.5% acetone was used as a blank. The Chl_a, Chl_b and C_{x+c} (μg g^{-1} FW) concentrations in the leaf tissues were calculated according to the following equations:

$$[Chl_a] = 9.784D_{662} - 0.99D_{644}$$

$$[Chl_b] = 21.42D_{664} - 4.65D_{662}$$

$$[C_{x+c}] = \frac{1000D_{470} - 1.90[Chl_a] - 63.14[Chl_b]}{214}$$

where, D_i is the optical density at wavelength I.

Flavonoid concentration was assayed according to Li[29]. The flavonoid concentration (μg g^{-1} FW) was measured spectrophotometrically at 330 nm. Likewise, anthocyanin concentration was assayed according to Bariola[30]. A milliliter of the aqueous/methanol phase extraction was used for measuring the anthocyanin concentration (μg g^{-1} FW) spectrophotometrically at 530 and 657 nm. The anthocyanin concentration was calculated according to the equation:

$$[\text{Anthocyanin}] = D_{530} - 0.24\,D_{657}$$

Net Photosynthetic Rate (NPR) analysis: Net Photosynthetic Rate (NPR) was calculated by measuring the difference in concentrations of carbon dioxide (CO_2) inside and outside of the glass vessel containing the seedlings. The CO_2 concentrations inside and outside the glass vessel (C_{in} and C_{out}) at steady state were measured by gas chromatography (GC; Model GC-17A, Shimadzu Co. Ltd., Japan). The NPR was calculated according to Fujiwara[31] and Kirdmanee[32] as follows:

$$[NPR] = K \bullet E \bullet V \bullet (C_{out}-C_{in})/L$$

where, K is the conversion factors converting the amount of CO_2 from volume to mole (40.9 mol m^{-3} at 28°C); E is the number of air exchanges per hour of the vessel (2.32 h^{-1}); V is the air volume of the glass vessel (0.0025 m^{-3}); C_{in} and C_{out} are CO_2 concentrations (μmol mol^{-1}) inside and outside the glass vessels at steady state condition, respectively; and L is the leaf area (m^2).

Growth measurement: The leaf area, shoot height, root length, fresh weight and dry weight of seedlings were measured as described by Lutts[19]. The seedlings were dried at 110°C in a hot-air oven (Memmert, Model 500, Germany) for 2 days and then incubated in a desiccator before measurement of dry weight. The leaf area was measured by a Leaf Area Meter DT-scan (Delta-Scan Version 2.03, Delta-T Devices, Ltd., UK).

Survival percentage assay: The survival percentage of seedlings was checked by the following growth on Phytagel®-solidified MS medium at day 0, 4, 8, 16 and 30 according to Gangopadhyay[33].

Experimental design: The experiment was designed as 2×2 factorials using a Completely Randomized Design with ten replicates and four plantlets per replicate. The mean values obtained were compared by Least Significant Difference and analyzed by SPSS software (SPSS for Windows, SPSS Inc., USA). The correlations between pigment and net photosynthetic rate, as well as net photosynthetic rate and survival percentage, were evaluated by Pearson's correlation coefficients.

RESULTS AND DISCUSSION

Thai jasmine rice seedlings grew well and exhibited 100% survival when cultured under either the photomixotrophic or photoautotrophic systems in the absence of salt-stress (0 mM NaCl). On the other hand, the survival percentage of seedlings in both systems gradually decreased with increasing exposure time in the presence of 342 mM NaCl. The survival percentage of *in vitro* seedlings cultured photoautotrophically with 342 mM NaCl was significantly lower than those cultured photomixotrophically (Fig. 1), indicating that seedlings cultured under the photomixotrophic system tolerated salt-stress better than those cultured under the photoautotrophic system.

When cultured without salt stress, *in vitro* rice seedlings significantly showed 1.6 fold higher concentrations of the major pigments (chlorophyll a,

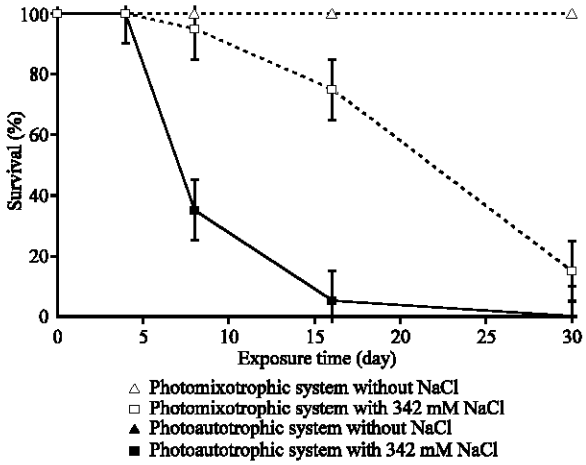

Fig. 1: Survival percentage of Thai jasmine rice seedlings cultured under photoautotrophic and photomixotrophic systems with 342 mM NaCl and without NaCl for 0, 4, 8, 16 and 30 days

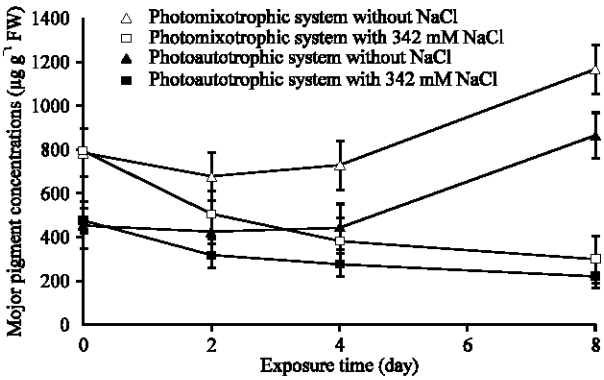

Fig. 2: Concentrations of major pigments (chlorophyll a, chlorophyll b and carotenoid) of Thai jasmine rice seedlings cultured under photoautotrophic and photomixotrophic systems with 342 mM NaCl and without NaCl for 0, 2, 4 and 8 days

chlorophyll b and total carotenoids) in photomixotrophic cultures compared to photoautotrophic cultures. Similarly, the major pigment concentrations, chlorophyll a, chlorophyll b and total carotenoid, of seedlings cultured photomixotrophically were 1.6, 1.4 and 1.3 times greater than those cultured photoautotrophically for 2, 4 or 6 days, respectively in the presence of 342 mM NaCl (Fig. 2). Likewise, in the absence of salt stress, the anthocyanin and flavonoid concentrations of seedlings grown under photomixotrophic systems were significantly accumulated higher than those grown photoautotrophic systems. The anthocyanin and flavonoid concentrations of *in vitro* seedlings cultured under either photomixotrophic or photoautotrophic growth conditions

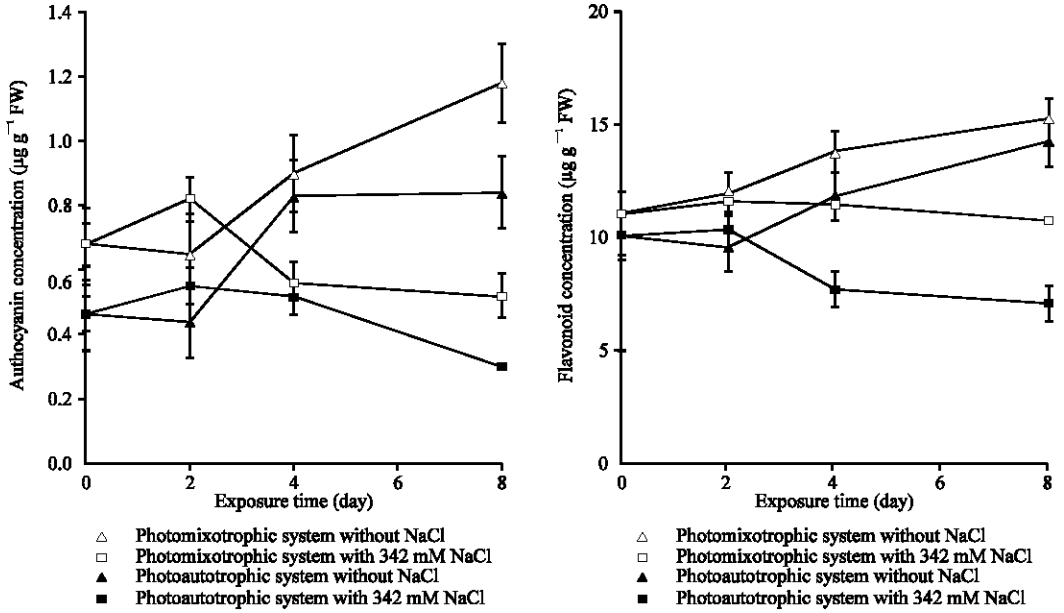

Fig. 3: Concentrations of minor pigments, anthocyanin (a) and flavonoid (b) of Thai jasmine rice seedlings cultured under photoautotrophic and photomixotrophic systems with 342 mM NaCl and without NaCl for 0, 2, 4 and 8 days

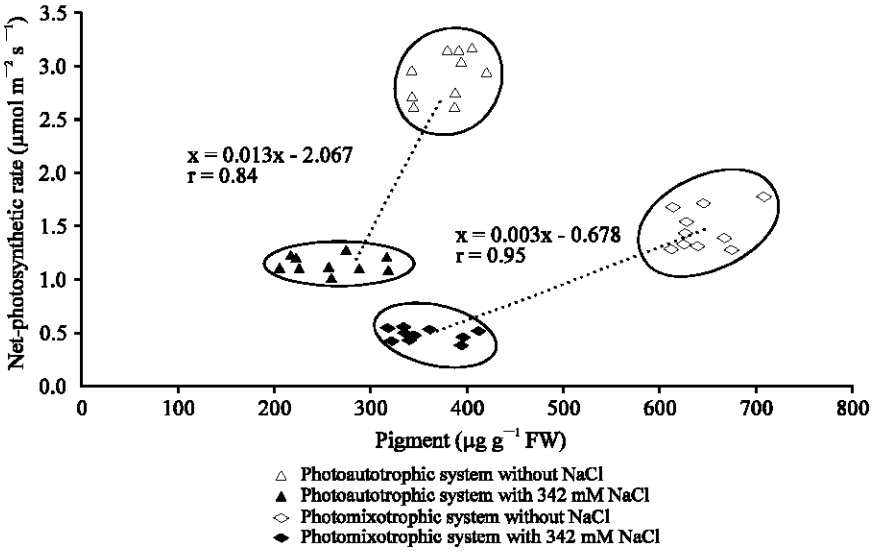

Fig. 4: Relationship between pigment and net-photosynthetic rate of Thai jasmine rice seedlings cultured under photoautotrophic and photomixotrophic systems with 342 mM NaCl and without NaCl for 4 days

increased slightly after 2 days exposure to 342 mM NaCl, but continuously decreased after extended exposure (4-8 days) (Fig. 3). After 4 days of salt stressed growth, the abundance anthocyanin and flavonoid pigments of *in vitro* seedlings were 1.1 and 1.5 folds lower respectively in photoautotrophic cultures than those in photomixotrophic cultures (Fig. 3).

Decreases in major and minor pigments of *in vitro* seedlings when cultured with 342 mM NaCl were positively related to NPR reduction for both photoautotrophic cultures (r = 0.84) (Fig. 4), as well as for photomixotrophic cultures (r = 0.95) (Fig. 4). The NPR of *in vitro* seedlings, with or without salt-stress, was significantly higher in photoautotrophic cultures

Table 1: Leaf area, shoot height, root length, shoot fresh weight, root fresh weight, shoot dry weight and root dry weight of Thai jasmine rice seedlings cultured under photoautotrophic and photomixotrophic systems with 342 mM NaCl and without NaCl for 4 days

Culture system	Leaf area (cm^2)	Shoot height (cm)	Root length (cm)	Fresh weight (mg)		Dry weight (mg)	
				Shoot	Root	Shoot	Root
Photoautotropic system without NaCl	4.32b	23.4a	6.7c	65b	26c	11c	3b
Photoautotropic system with 342 mM NaCl	2.01d	15.9c	5.7c	42b	21d	8d	3b
Photomixotropic system without NaCl	5.03a	24.3a	14.4a	68a	41a	17a	4a
Photomixotropic system with 342 mM NaCl	3.01c	19.5b	8.6bc	67ab	35b	16b	4a
Significance level							
Culture system	**	**	**	NS	**	**	**
NaCl	*	**	**	*	**	**	**
Culture system × NaCl	**	**	**	**	**	NS	**

Means within a row followed by the different letters in each column are significantly different at *p≤ 0.05 and **p≤ 0.01 by LSD test

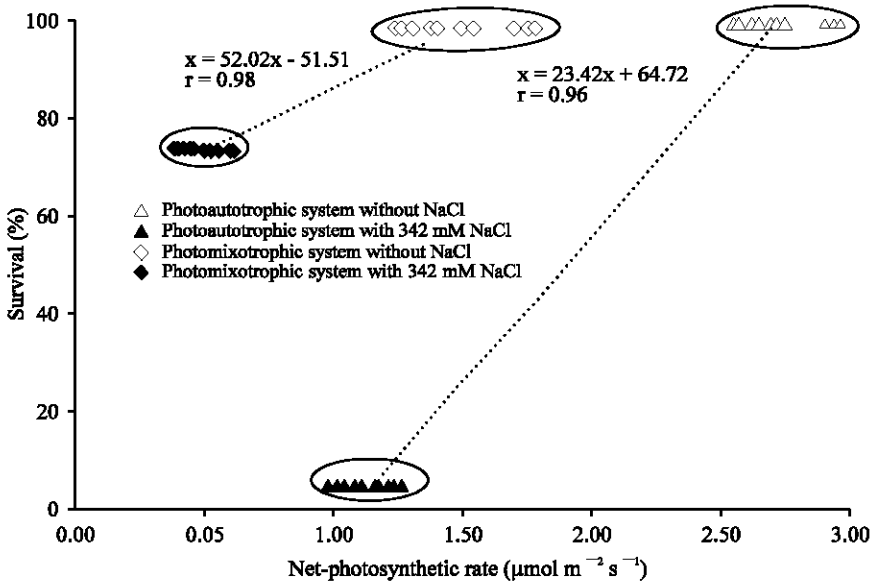

Fig. 5: Relationship between net-photosynthetic rate and survival percentage of Thai jasmine rice seedlings cultured under photoautotrophic and photomixotrophic systems with 342 mM NaCl and without NaCl for 4 days

compared to photomixotrophic cultures (Fig. 4). Decreases in the NPR of *in vitro* seedlings in the presence of 342 mM were positively related to survival percentage for both photoautotrophic cultures (r = 0.96) and photomixotrophic cultures (r = 0.98) (Fig. 5). This decreasing on NPR of seedlings cultured photoautotrophically with 342 mM NaCl likely contributes to the reduced growth (i.e. leaf area, shoot height, root length, fresh-weight and dry-weights) of these seedlings. The *in vitro* seedlings cultured photomixotrophically grew significantly better than those cultured photoautotrophically both under normal conditions and under salt stress (Table 1). Use of different culture systems (photomixotrophic or photoautotrophic) had a significant effect on leaf area and shoot and root fresh weights, shoot and root dry weights and also had a slight influence on shoot height. On the other hand, the salt-stress caused decreases in leaf area, shoot height, shoot and root fresh weights and shoot dry weights, but not root dry weight. Thus, both factors significantly affect the *in vitro* seedling growth.

Sucrose is generally used in the culture media of heterotrophic and photomixotrophic tissue cultures as a carbon source. It is directly uptaked by plant cells from the extracellular space through the plasma membrane, moving from cell to cell via plasmodesmata (symplastic loading). Frequently, the sucrose is mobilized from the apoplast into the phloem sieve elements for transport into sink organs (apoplastic loading)[34,35]. The function of sucrose in the expression of many plant gene(s) has been widely reported, indicating its involvement in many metabolic and developmental processes. The sucrose is also an important carbohydrate in respiratory metabolism and acts as a substrate for the biosynthesis of complex carbohydrates as starch and cellulose. Moreover, sucrose provides building blocks for the biosynthesis of primary

and secondary metabolites during all stages of plant growth and development[1,3,10,36,37]. In plant tissue culture, sucrose supplied in the culture medium is also taken up, translocated and accumulated in plant tissues as a defensive response to environmental stresses such as drought[38,39], high salinity[40-42] and low temperature[43] via an osmoregulation system. However, there are many reports that sucrose represses transcription of photosynthetic genes by feedback inhibition, leading to reduced rate of photosynthesis[9-11, 44,45].

Photoautotrophic culture systems have been successfully applied for phenotypic growth studying under the salt-stress[21,41] and for the screening of salt-tolerant plants[22]. Seedlings grown under photomixotrophic system showed higher salt tolerance than those grow under photoautotrophic system, as judged by pigment concentration and growth. The exogenous sucrose in the photomixotrophic system not only functions as an osmoregulant, but also plays a role in energy preservation, when exposed to salt-stress conditions[40-42]. On the other hand, the photoautotrophic system primarily uses carbon dioxide as the carbon source and is therefore more similar to the growth of plants under natural conditions.

In conclusion, present results have shown that rice seedlings culture *in vitro* under a photomixotrophic system exhibit better growth characteristics than those photoautotrophic seedlings which utilize CO_2 as their sole carbon source. However, seedlings grown in the presence of exogenously supplied sucrose also display a significant reduction in their sensitivity to salt stress. Since plants in soil typically do not grow in a sucrose-rich environment, the photoautotrophic system described in this study is more appropriate for the analysis of the phenotype responses to salt stress of plant grown under controlled conditions, as well as for the screening of plant with altered salt tolerance levels. This system would be applied as a tool for realistic phenotypic responses to salt stress nearby with plants grown in the natural conditions as well as for salt-tolerance screening program.

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