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Phenotypic Responses of Thai Jasmine Rice to Salt-stress under Environmental Control of *in vitro* Photoautotrophic System

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Abstract: Rice crop response to salt stress is well known to involve multifunction mechanisms, which is dramatically regulated by various environmental factors. To reduce the uncontrolled environmental signals, the phenotypic responses of rice crop to salt stress were investigated under an *in vitro* environmental engineering system. The chlorophyll a, chlorophyll b, carotenoid, flavonoid and anthocyanin concentrations of seedlings cultured under acidic pH and low Relative Humidity (RH) with salt-stress conditions were sharply reduced by the factors of 2.26, 2.04, 2.15, 1.60 and 1.49 folds, respectively when compared to those cultured under neutral pH and high RH without salt-stress conditions. Degradation on pigment concentrations of rice seedlings positively related to the net-photosynthetic rate reduction (r = 0.94), led to growth retardation i.e. leaf area, shoot height, fresh and dry weights. The environmental factors of *in vitro* culture system such as extreme pH and RH should exhibit their realistic phenotypic responses to salt-stress that further applied for salt-tolerant screening.

Key words: Growth, net-photosynthetic rate, phenotypic expression, pigment, pH, relative humidity

INTRODUCTION

The study on phenotypic expression of higher plants under extreme conditions as salt, drought, ultraviolet light, pH and temperature-stresses is still limited as it is controlled by both gene and environmental factors. The interaction between genotype and environment is generally used to select of superior genotypes form multi-environment trials, because of the difficulty of selecting test environments that adequately represent the entire target population of environments[1]. Such a situation is partially common in regions where the occurrence of stress is unpredictable, because the effect of stress on phenotypic expression depends on its timing[2]. Breeding programs targeting specific adaptation can only exploit genotype and environment interactions effectively, if the physiological causes of the interaction are well understood[1]. The phenotypic expression has been widely investigated using field trial or hydroponic system. There are found some errors due to uncontrolled environmental factors causing to erratic data[3]. Normally, most research works have been applied the *in vitro* culture as a tool for studying many aspects of selection of stress-tolerant clones, gene expression for stress resistance and the plant responses to extreme conditions. However, the exact conditions of natural environment are quite different from the conditions of conventional *in vitro* culture. Generally, conventional *in vitro* environments are composed of sugar as a carbon source, high Relative Humidity (RH), constant temperature, low Photosynthetic Proton Flux Density (PPF) and low carbon dioxide (CO_2) concentration[4]. In contrast, environmental control system such as CO_2 as a carbon source (photoautotrophic growth), low RH, temperature shift, high PPF, high number of air exchange and CO_2 concentrations have been applied for better growth, development and vigorous plantlet production[5,6]. The photoautotrophic plantlets cultured under environmental control system are expressed the physiological, anatomical and morphological characteristics closely with *ex vitro* conditions. This system has been used as a tool for study of salt-stress responses in *Albizzia lebbek*[7] and salt-tolerance screening of 100 forest tree species[8]. Therefore, the appropriate environment conditions of photoautotrophic growth would be necessarily established for realistic phenotypic responses, closely related to saline soil

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environments. The aim of this research was to investigate the *in vitro* environmental control system for phenotypic expression of rice seedlings to salt-stress.

MATERIALS AND METHODS

Plant materials: Seeds of Jasmine rice (*Oryza sativa* L. Cv. KDML105) were sterilized and then germinated on 0.25% Phytagel®-solidified MS media[9]. Seven-days-old rice seedlings were transferred to photoautotrophic (CO_2 as a carbon source) conditions. Vermiculite was used as a supporting material. The pH of culture media was adjusted to 4.5 (acidic pH), 7.0 (neutral pH) and 9.5 (basic pH) before autoclaving. The uncovered vessels containing photoautotrophic seedlings were transferred aseptically to culture chamber (Carry Box Model P-850, size 26×36×19 cm, Japan) controlled RH at 95±5% (high RH) by 1.5 L distilled-water and at 65±5% (low RH) by 1.5 L saturated-NaCl solution. Number of air exchange in the culture chambers was increased to 5.1±0.3 h^{-1} by punching the side of the plastic chambers with 32 holes and replacing with gas-permeable microporous polypropylene films (0.22 μm of pore size) over each hole. These chambers were incubated in Plant Growth Incubator (EYELA, Model EYELATRON FLI-301LH, Japan) under temperature shift 28±2/25±2°C (12 h photoperiod/12 h dark period), 500±100 μmol mol^{-1} carbondioxide concentration (CO_2), 60±5% RH, 120±5 m^{-2} s^{-1} PPF by fluorescence lamps for 13 days. The culture media were adjusted to 0 and 342 mM sodium chloride (NaCl) for 4 days.

Pigment assay: Chlorophyll a, chlorophyll b and carotenoid concentrations were analyzed following the methods of Shabala *et al.*[10] and Lichtenthaler [11], respectively. Chlorophyll a (Chl_a) and chlorophyll b (Chl_b) concentrations were measured using an UV-visible spectrophotometer (DR/4000, HACH, USA) at wavelengths 662 nm and 644 nm. Total carotenoid (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:

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

$$[Chl_b] = 21.42D_{644} - 4.65D_{662}$$

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

where, D_i is the optical density at wavelength I.

Flavonoid and anthocyanin concentrations were assayed according to Li *et al.*[12] and Bariola *et al.*[13]. The flavonoid concentration was measured spectrophotometrically at 330 nm and the anthocyanin concentration was measured spectrophotometrically at 530 and 657 nm, respectively. The anthocyanin concentration was calculated according to the equation:

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

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 *et al.*[14] and Kirdmanee *et al.*[15], 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 *et al.*[16]. 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).

Experimental design: The experiment was designed as 2×3×2 factorials in Completely Randomized Design (CRD) with ten replicates and four plantlets per replication. The mean of treatment was compared by Duncan Multiple Rang Test (DMRT) at $p \leq 0.01$ and analyzed by SPSS software (SPSS for Windows, SPSS Inc., USA). The correlation between pigment concentration and NPR was evaluated by Pearson's correlation coefficients.

RESULTS AND DISCUSSION

Major and minor pigment concentrations of rice seedlings cultured under salt stress (342 mM NaCl) were

Table 1: Concentrations of chlorophyll a, chlorophyll b, anthocyanin and flavonoid of KDML seedlings grown *in vitro* under photoautotrophic system with different NaCl, RH and pH conditions for 4 days

RH (%)	pH	NaCl (mM)	Chlorophyll a (μg g^{-1} FW)	Chlorophyll b (μg g^{-1} FW)	Anthocyanin (μg g^{-1} FW)	Flavonoid (μg g^{-1} FW)
65±5	4.5	0.0	1043.8c	400.00c	1.36a	13.43 a
		342.0	461.7f	196.20e	0.85def	9.03 g
	7.0	0.0	1175.9b	498.40b	0.90de	11.77cd
		342.0	519.8e	257.0de	0.76efg	10.02f
	9.5	0.0	1078.9c	474.10b	1.10b	12.47abc
		342.0	476.0f	228.1de	0.65gh	9.97fg
95±5	4.5	0.0	1107.7c	451.80bc	0.70fgh	11.44 de
		342.0	525.3e	237.4de	0.56h	9.94fg
	7.0	0.0	1353.5a	578.70a	1.05bc	12.95ab
		342.0	594.1d	288.8d	0.66gh	11.06de
	9.5	0.0	1217.3b	496.70b	0.95cd	12.42bc
		342.0	558.6de	267.8d	0.83def	10.59ef
Significant level						
RH			**	**	*	**
pH			**	**	*	NS
NaCl			**	**	**	**
RH×pH			*	NS	*	**
pH×NaCl			**	*	**	NS
RH×NaCl			*	NS	**	**
RH×pH×NaCl			NS	NS	**	**

The different letter (s) in each column are significantly different at p≤ 0.01 by DMRT

strongly reduced when compared with those grown under without salt stress (0 mM NaCl). The low relative humidity (65% RH) in the culture chamber under photoautotrophic condition significantly affected on chlorophyll a, chlorophyll b, total chlorophyll, anthocyanin and flavonoid degradations (Table 1). Factor of pH had also affected to most of pigments, except flavonoid. All pigments were dropped when the plants were exposed to salt stress and became more degraded when combined with low relative humidity (65% RH). Among various types of pigment, anthocyanin was significantly affected by all factors included salt stress, RH and pH. While, chlorophyll a and chlorophyll b were not greatly affect by different pH conditions, anthocyanin in dramatically displayed the significant level and flavonoid was responded slightly. The effect of RH and pH were evaluated in terms of major pigment (Fig. 1A) and minor pigment (Fig. 1B) concentrations. Low pH at 4.5 slightly showed its effect to decrease both pigments when plant cultures were subjected to salt stress, whereas neither low nor high RH had no obvious effect to chlorophyll but low RH slightly caused lower carotenoid concentration in seedling than under high RH. The combination of low RH, acidic pH conditions and salt-stress severely damaged all pigments concentrations. The concentration of total chlorophyll of seedlings cultured under low RH and acidic pH with salt stress conditions was strongly degraded for 2.9 folds when compared with those grown under high RH and neutral pH without salt-stress.

Pigment degradation of seedlings cultured under extreme environments was closely related to the NPR reduction as indicated by r = 0.94 (Fig. 2). The NPR of seedlings cultured under low RH and acidic pH with salt stress conditions was severely decreased for 2.4 folds when compared with those grown under high RH and neutral pH without salt stress conditions. Figure 3, leading on growth reduction. Leaf area, shoot height, fresh- and dry-weights of seedlings cultured on the

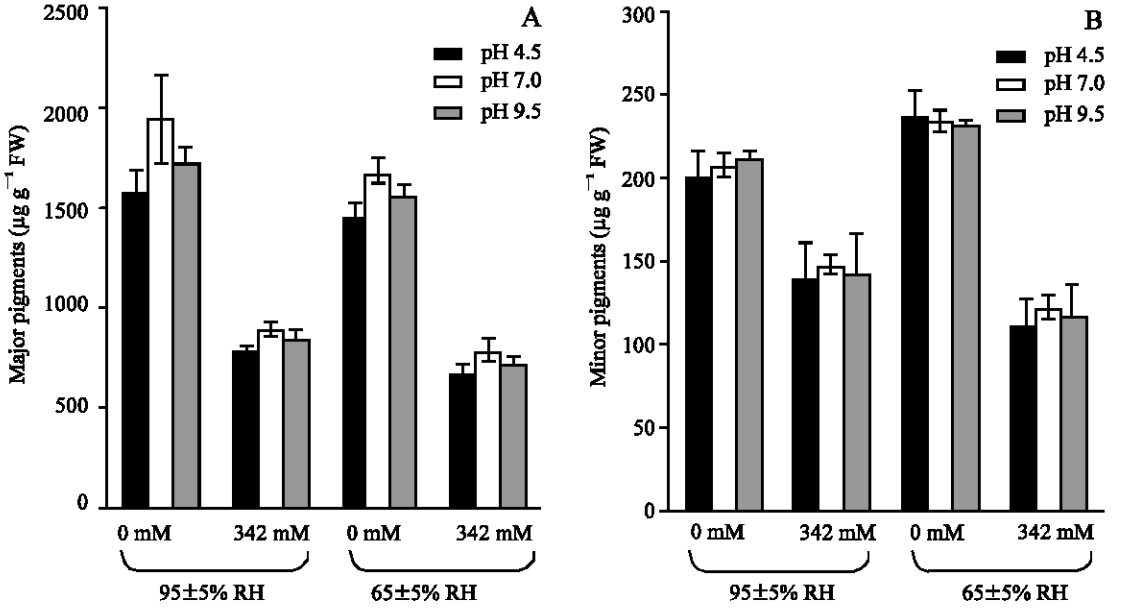

Fig. 1: Major pigments (A), chlorophyll a and chlorophyll b concentrations and minor pigments (B), carotenoid, anthocyanin and flavonoid concentrations, of Jasmine rice seedlings grown *in vitro* under photoautotrophic conditions with different salt-stress, RH and pH conditions for 4 days

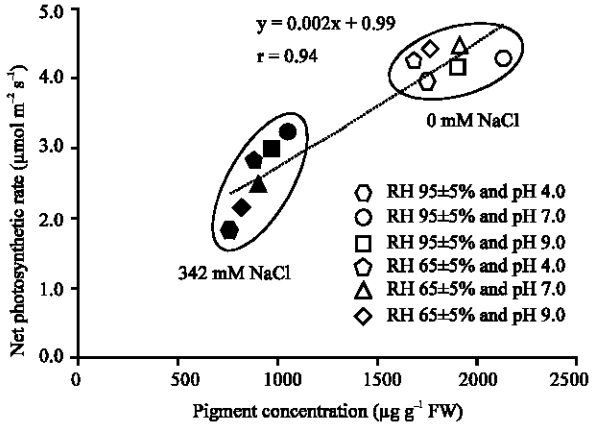

Fig. 2: Relation between pigment concentration and net-photosynthetic rate of Jasmine rice seedlings grown *in vitro* under photoautotrophic conditions with different salt-stress, RH and pH conditions for 4 days

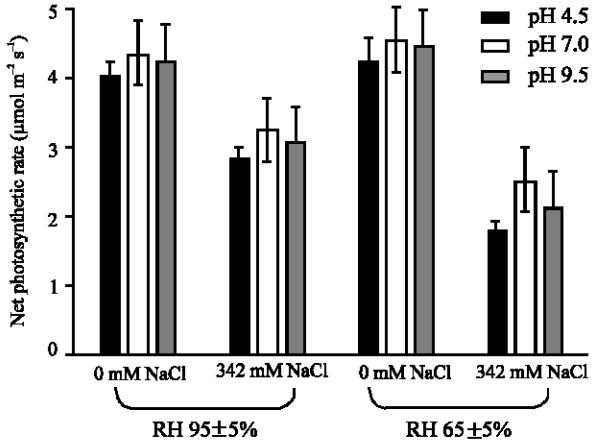

Fig. 3: Net-photosynthetic rate (NPR) of Jasmine rice seedlings grown *in vitro* under photoautotrophic conditions with different salt-stress, RH and pH conditions for 4 days

medium containing with salt-stress, low RH, low pH conditions decreased for 4.0, 1.6, 1.4 and 1.2 folds when compared with those cultured on the medium without salt-stress, medium pH and high RH conditions (Table 2).

The low RH condition in culture chamber using saturated-salt solution has been successfully applied for phenotypic expression[7] and salt-tolerant screening of one hundred forest tree species[8]. The RH in the culture vessel plays an important role on physiological and biochemical functions such as water relation, stomatal conductance, transpiration rate and water oxidation[17,18]. Generally, the RH in conventional *in vitro* culture is higher than 95%,

Table 2: Growth, shoot height, fresh weight, dry weight and leaf area, of Jasmine rice seedlings grown *in vitro* under photoautotrophic conditions with different salt-stress, RH and pH conditions for 4 days

RH (%)	pH	NaCl (mM)	Shoot height (cm)	Fresh weight (mg)	Dry weight (mg)	Leaf area (cm^{-2})
65±5	4.5	0.0	27.7bc	115.0a	18.0ab	6.7a
		342.0	20.0de	83.0de	15.0bc	1.7e
	7.0	0.0	28.8ab	113.0a	20.0a	7.2a
		342.0	21.3cde	82.0de	17.0abc	1.9de
	9.5	0.0	25.6c	98.0abcd	16.0bc	5.2b
		342.0	19.4e	73.0e	15.0bc	1.8e
95±5	4.5	0.0	27.2bc	92.0bcde	15.0bc	4.8b
		342.0	22.9d	88.0bcde	14.0c	2.4cde
	7.0	0.0	30.9a	112.0ab	18.0ab	6.8a
		342.0	22.3de	77.0de	14.0c	2.6c
	9.5	0.0	27.4bc	108.0abc	18.0abc	6.7a
		342.0	21.2def	82.0de	14.0c	2.5cd
Significant level						
RH			**	NS	*	*
pH			**	NS	**	**
NaCl			**	**	**	**
RH×pH			NS	*	*	**
pH×NaCl			NS	NS	NS	**
RH×NaCl			NS	NS	NS	**
RH×pH×NaCl			NS	NS	NS	**

The different letter (s) in each column are significantly different at $p \leq 0.01$ by DMRT

causing in disorder of the physiological, anatomical and morphological characteristics[19]. Reduction on RH of *in vitro* culture improved the growth, development and vigorous plantlet production[15]. In root zone environments, the pH condition of culture media mainly effect on the solubility and availability of mineral or nutrition[20,21]. Acidic pH or basic pH of culture media normally limited on availability of major and minor nutrients such as nitrogen (N), phosphorus (P) and molypdinum (Mo)[22]. Controlling the environmental factors, RH and pH, of photoautotrophic growth would exhibit the realistic phenotypic responses of rice to salt-stress closely with saline soil environments. The RH, pH and salt-stress factors normally damaged on pigments, function as photoreceptor and light harvesting complexes of photosystem II, causing on low net-photosynthetic rate[9,23,24]. In conclusion, the environmental factors of photoautotrophic growth would necessarily enhance the phenotypic expression in both of pigments, net-photosynthetic rate and growth characteristics to salt-stress. It should be noted that the realistic phenotypic expression of rice seedlings would intensively mimic environmental factors nearby with saline soil environments. The saline soil environments quite low RH with acidic RH or basic pH conditions should exhibit their realistic phenotypic responses to salt-stress. This investigation should be further applied as a model for *in vitro* salt-tolerant screening program.

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