RESEARCH ARTICLE
J. Crop Sci. Biotech. 10 (4) : 257 ~ 264

An Effective Defensive Response in Thai Aromatic Rice Varieties (*Oryza sativa* L. spp. *indica*) to Salinity

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Abstract

Rice is one of the world's staple crops and is a major source of carbohydrate. Rice is exported from several countries, providing a major source of income. There are many documents reporting that rice is a salt-sensitive crop in its developmental stages. The objective of this investigation is to evaluate the effective salt-tolerance defense mechanisms in aromatic rice varieties. Pathumthani 1 (PT1), Jasmine (KDML105), and Homjan (HJ) aromatic rice varieties were chosen as plant materials. Rice seedlings photoautotrophically grown *in-vitro* were treated with 0, 85, 171, 256, 342, and 427 mM NaCl in the media. Data, including sodium ion (Na^+) and potassium ion (K^+) accumulation, osmolarity, chlorophyll pigment concentration, and the fresh and dry weights of seedlings were collected after salt-treatment for 5 days. Na^+ in salt-stressed seedlings gradually accumulated, while K^+ decreased, especially in the 342-427 mM NaCl salt treatments. The Na^+ accumulation in both salt-stressed root and leaf tissues was positively related to osmolarity, leading to chlorophyll degradation. In the case of the different rice varieties, the results showed that the HJ variety was identified as being salt-tolerant, maintaining root and shoot osmolarities as well as pigment stabilization when exposed to salt stress or Na^+ enrichment in the cells. On the other hand, PT1 and KDML105 varieties were classified as salt-sensitive, determined by chlorophyll degradation using Hierarchical cluster analysis. In conclusion, the HJ-salt tolerant variety should be further utilized as a parental line or genetic resource in breeding programs because of the osmoregulation defensive response to salt-stress.

Key words: chlorophyll pigment, osmolarity, potassium ions, salt sensitive, salt tolerance, sodium ions

Introduction

Rice is a major crop which provides one-third of the total carbohydrate for the world's population, especially in Asian countries. It is the staple food for more than 3 billion people and provides 50 to 80% daily calorie intake (Khush 2005). The major limitation in rice crop production is abiotic stress caused by salinity, drought, extreme temperature, submergence, and ultraviolet irradiation. Saline soil is enriched with salts which are readily water-soluble i.e. sodium chloride (NaCl), sodium sulfate (Na_2SO_4), calcium chloride ($CaCl_2$), and magnesium chloride ($MgCl_2$). It is a major barrier to rice cultivation, reducing productivity (Shannon et al. 1998). Sodium chloride salt is a small molecule when oxidized to sodium ions (Na^+) and chloride ions (Cl^-), which is easily absorbed by root cells and transferred to the plant overall through its xylem vascular tissues (Maathuis and Amtmann 1999; Rodriguez-Navarro and Rubio 2006; Tester and Davenport 2003). Na^+ ions are well known as causing toxic damage to plant cells by both ionic and osmotic effects, causing growth retardation, low productivity, and eventually, cell death (Chinnusamy et al. 2005; Hasegawa et al. 2000; Mansour and Salama 2004). In the case of rice, it is reported as being salt-sensitive and displays the negative effects of salinity in its seedlings and reproductive stages (Zeng and Shannon 2000a; Zeng and Shannon 2000b; Zeng et al. 2003). There are many research groups investigating salt-tolerant rice from genetic resources or

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gene banks using multivariate screening systems in both growth and yield performances. Examples of salt-tolerant rice landrace, cultivars, and breeding lines are: Pokkali, Nona-Bokra, Agami, Daeyabyeo, GZ5310-20-2-1, GZ5310-20-3-2, GZ5310-20-3-3, and IR4630-22-2-2-5-1-3. These rice varieties have been used as the parental lines in salt-tolerant rice breeding programs worldwide. In addition, IR 26, M-104, M-202, M-205, L-205, S-102, GZ177, Sakha101, GZ5121-5-2-1, GZ5291-7-1-2, and IR63352-AC202 salt-sensitive cultivars and breeding lines are reported (Zeng et al. 2004; Zeng 2005). Aromatic rice varieties Jasmine (KDML105) and Pathumthani 1 (PT1) are popular in Thailand. They both have a distinctive aroma, delicate flavor, high cooking quality, long grains, high amylose content, and a soft texture. Because of this, they carry high export values (Ariyaphanphitak et al. 2005; Laohakunjit and Kerdchoechuen 2007). In addition, Homjan rice is a local variety that grows well in the salted rice fields near the seashore in the southern region of Thailand. This variety has been used as a resource for osmoregulation defense responses to salt-stress, namely glyciene betaine (Cha-um et al. 2007). The aim of this present study is to investigate salt-tolerance in aromatic rice varieties to provide an effective defense mechanism.

Materials and Methods

Plant materials

Pathumthani 1 (PT1), Jasmine (KDML105), and Homjan (HJ) rice seeds 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, Oakland, USA) for 60 min, once in 30% Clorox® for 30 min, and then rinsed three times using sterilized distilled-water. Surface-sterilized seeds were germinated on 0.25% Phytagel®-solidified MS media (Murashige and Skoog 1962) in a 250-mL glass vessel. The media were adjusted to pH 5.7 before autoclaving. Seedlings were cultured *in vitro* under conditions of 25 ± 2 ºC ambient temperature, 60 ± 5% relative humidity (RH), and 60 ± 5 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) provided by fluorescent lamps (TDL 36 W/84 Cool White 3350 Im, Philips, Thailand) with a 16 h d^{-1} photoperiod. Fourteen-day-old rice seedlings were aseptically transferred to MS sugar-free liquid media (photoautotrophic condition) using vermiculite as a supporting material. The number of air-exchanges in the glass vessels was adjusted to 2.32 h^{-1} by punching a hole in a plastic cap (Ø 1 cm) and covering the hole with a microporous filter (0.20 μm pore size). All seedlings were continuously cultured under the same conditions as during the seed germination. The culture media was adjusted to 0, 85, 171, 256, 342, and 427 mM NaCl using high NaCl concentration stock solutions for five days (Fig. 1). Sodium ions, potassium ions, osmolarity, chlorophyll pigments, and growth characters were measured.

Fig. 1. Scheme of the experiment on *in vitro* photomixotrophic germination for 14 days, photoautotrophic acclimatization for 7 days and subsequently exposed to 0, 85, 171, 256, 342, and 427 mM NaCl for 5 days.

Data collection

One hundred mg of whole plant materials were ground in liquid nitrogen. Sodium and potassium ions in plant materials were extracted by acidic methods (HNO_3 and $HClO_4$) and assayed according to Dionisio-Sese and Tobita (1998) using an Atomic Absorption Spectrophotometer (AA, Model M6, Thermo Elemental, MA, USA).

Root and leaf osmolarities of rice seedlings were measured, according to Lanfermeijer et al. (1991). One hundred mg of fresh root and leaf tissues were cut into small pieces, transferred to 1.5 mL micro tube, and then crushed by stirring with a glass rod. Twenty μL of extracted solution was dropped directly onto a disc-shaped filter paper in an osmometer chamber (Wescor, Utah, USA). The osmolarity was then measured.

Chlorophyll a (Chl_a), chlorophyll b (Chl_b), and total chlorophyll concentrations were analyzed following the methods of Shabala et al. (1998). The Cha and Ch_b concentrations were measured using an UV-visible spectrophotometer (DR/4000, HACH, Colorado, USA) at wavelengths 662 and 644 nm, respectively. A solution of 95.5% acetone was used as a blank. The Chl_a, Chl_b, and total chlorophyll (μg g^{-1} FW) concentrations in the leaf tissues were calculated according to the following equations:

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

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

$$\text{Total chlorophyll} = [Chl_a] + [Chl_b]$$

where D_i is the absorbance at wavelength i.

Experiment design

The experiment was designed as 3 × 6 factorial in a Completely Randomized Design (CRD) with six replicates and four plantlets per replication. The mean in each treatment was compared by Duncan's New Multiple Range Test (DMRT) at $P \leq 0.01$ and analyzed by SPSS software (SPSS for Windows, SPSS Inc., Chicago, USA). Total chlorophyll degradation in all rice varieties was input to classify group as salt-tolerance and salt-sensitivity using Hierarchical cluster analysis in SPSS software.

Results and Discussion

Sodium ions (Na^+) in salt-stressed rice seedlings were gradually accumulated in all varieties, linked to exogenous sodium chloride (NaCl) applied to the culture media (Fig. 2). The Na^+ in PT1, KDML105, and HJ salt-stressed seedlings (427 mM NaCl) reached its peak and was higher than in the control seedlings by 5.57, 5.50, and 5.39 times, respectively. This means that in the high salt treatments (342-427 mM NaCl), released Na^+ was quickly absorbed by the roots and was transferred to the whole plant in five days, detected by AA equipment. Moreover, the enriched Na^+ in PT1, KDML105 and HJ varieties was positively related to NaCl treatments (r^2 = 0.92, 0.92, and 0.92, respectively) (Fig. 2). On the other hand, potassium ions (K^+) in PT1, KDML105, and HJ salt-stressed seedlings slowly decreased and were inversely related to NaCl treatments (r^2 = 0.85, 0.96, and 0.92, respectively) (Fig. 3). In addition, the K^+ reduction in salt-stressed seedlings was inversely correlated with Na^+ accumulation (r^2 = 0.69, 0.83, and 0.88, respectively) (Fig. 4).

Na^+ in salt-stressed plants is taken up by the roots and is accumulated in the whole plant depending on: whether or not the cultivars are salt-tolerant or salt-sensitive (Alpaslan et al. 1999; Dionisio-Sese and Tobita 1998; Golldack et al. 2003; Lefevre et al. 2001; Walia et al. 2005), salt concentrations in the growing media (Basu et al. 2002; Lefevre et al. 2001; Ndayiragije and Lutts 2006), plant organelles (Shah et al. 2002; Ueda et al. 2006), exposure times (Lefevre et al. 2001; Mitsuya et al. 2003a; Parker et al. 2006; Ueda

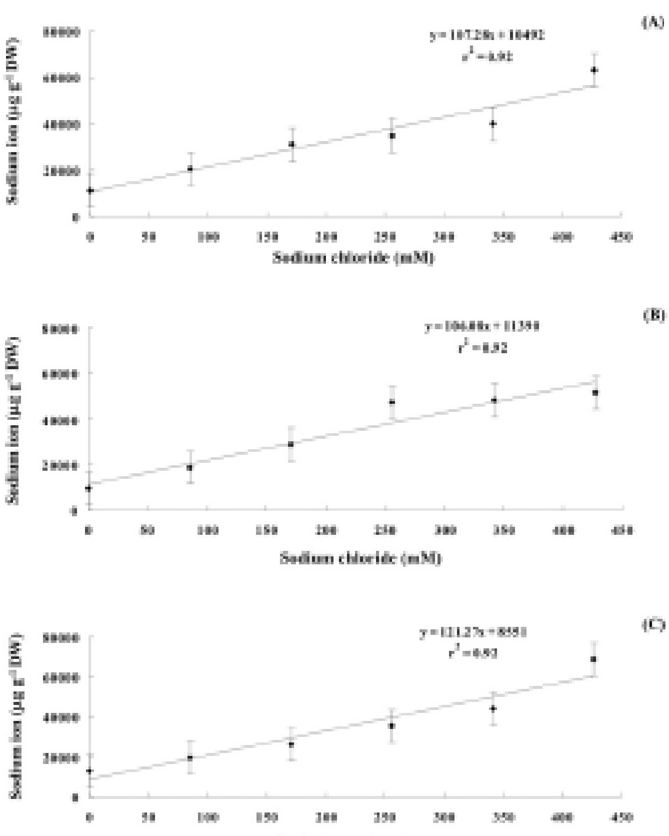

Fig. 2. Relationship between sodium chloride treatment and sodium ion in Pathumthani 1 (A), Jasmine (B), and Homjan (C) rice varieties grown in photoautotrophic culture supplemented with various sodium chloride (NaCl) concentrations for 5 days. Error bars represented by ± SE.

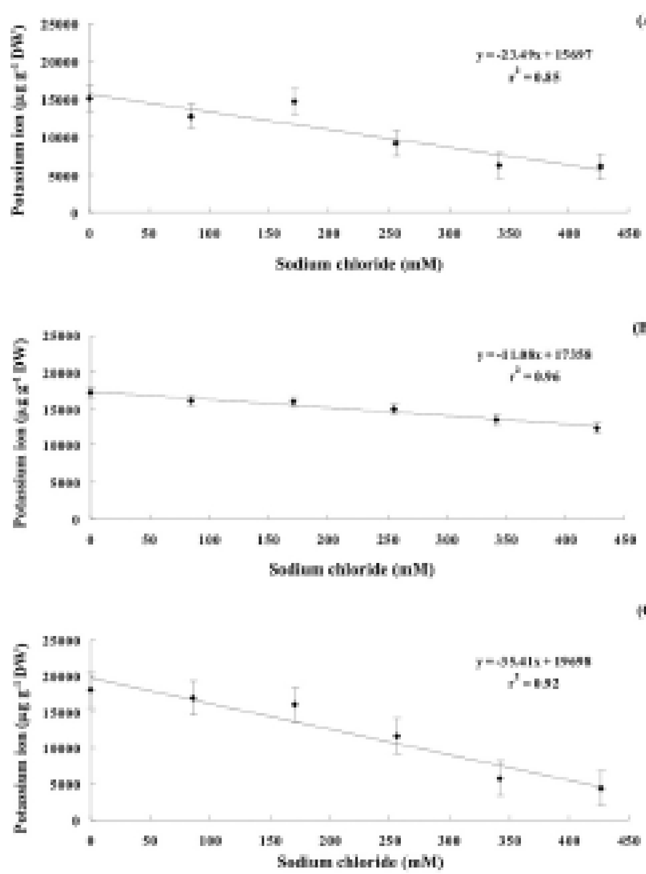

Fig. 3. Relationship between sodium chloride treatment and potassium ion in Pathumthani 1 (A), Jasmine (B), and Homjan (C) rice varieties grown in photoautotrophic culture supplemented with various sodium chloride (NaCl) concentrations for 5 days. Error bars represented by ± SE.

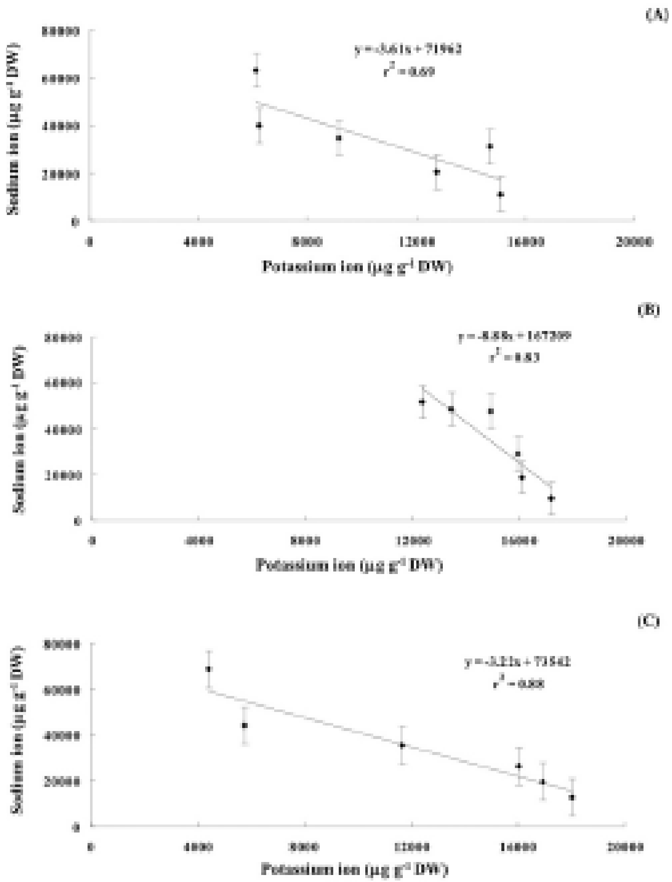

Fig. 4. Relationship between potassium ion and sodium ion in Pathumthani 1 (A), Jasmine (B), and Homjan (C) rice varieties grown in photoautotrophic culture supplemented with various sodium chloride (NaCl) concentrations for 5 days. Error bars represented by ± SE.

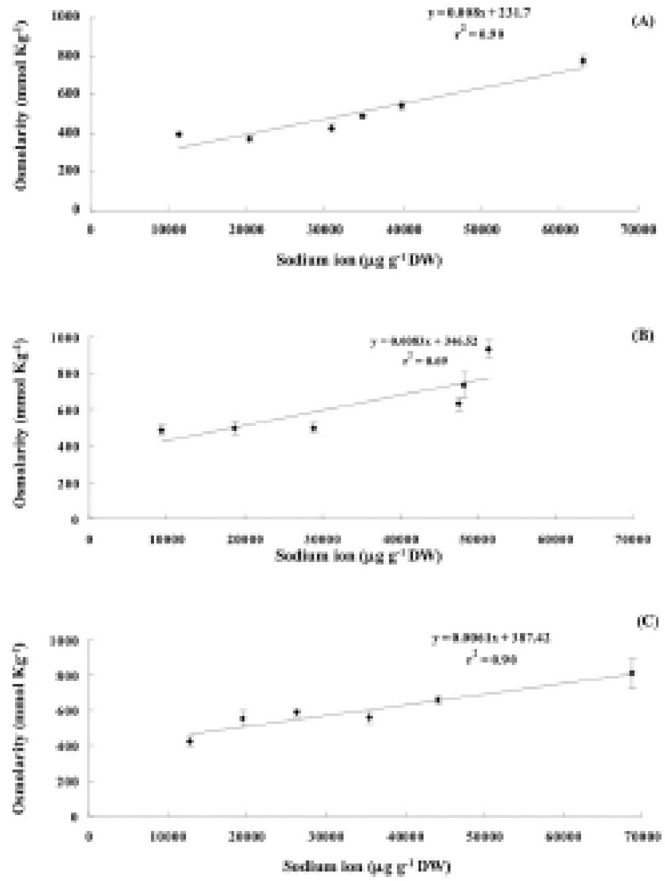

Fig. 5. Relationship between sodium ion and root osmolarity in Pathumthani 1 (A), Jasmine (B), and Homjan (C) rice varieties grown in photoautotrophic culture supplemented with various sodium chloride (NaCl) concentrations for 5 days. Error bars represented by ± SE.

et al. 2006), growing season (Asch et al. 1999), and if they are grown in conditions of light or darkness (Mitsuya et al. 2003b), while K^+ usually decreases. In this study, Na^+ accumulation and K^+ reduction in all rice cultivars showed a similar pattern related to NaCl concentration applications in the media. This means that the salt tolerant ability in aromatic rice varieties may not be identified by Na^+ accumulation. Conversely, there are some reports which indicate the low Na^+ accumulation via limited Na^+ absorption by H^+-ATPase (Roy et al. 2005; Zhang et al. 1999) and H^+-PPase activities (Liu et al. 2006), Na^+/H^+ antiporter in both plasma membranes and vacuolar membranes (Fukuda et al. 1999; Fukuda et al. 2004; Martinez-Atienza et al. 2007; Ohta et al. 2002; Zhao et al. 2006), leading to lower salt concentration in salt tolerant varieties than those in salt sensitive varieties. In addition, the Na^+ accumulation in rice crops has been effectively utilized to identify salt-tolerant or salt-sensitive varieties (Golldack et al. 2003; Zeng 2005; Zeng et al. 2004).

Osmolarity in both root and shoot organs of salt-stressed seedlings continuously increased, related to exogenous sodium chloride applied to the culture media (Table 1). The osmolarities in PT1, KDML105, and HJ salt-stressed roots (427 mM NaCl) reached their highest peak and were higher than those in the control seedlings by 1.98, 1.92, and 1.91 times, respectively. The results showed that osmolarities in both root and leaf tissues were related to the plant varieties, NaCl salt treatments, and their interactions. The Na^+ accumulation in salt-stressed seedlings was positively related to osmolarity (r^2 = 0.90, 0.69, and 0.90, respectively) (Fig. 5). The osmolarity in salt-stressed leaves (427 mM NaCl) reached its peak and was 2.39-3.03 times higher than in the leaves of the control. The high osmolarities in PT1, KDML105, and HJ varieties were connected to chlorophyll degradation (r^2 = 0.83, 0.88, and 0.81, respectively) (Fig. 6). Chlorophyll a, chlorophyll b, and total chlorophyll content in salt-stressed seedlings were similarly degraded when exposed to high sodium chloride treatments (342-427 mM NaCl) (Table 2). In addition, there are significant differences in the factors of rice varieties, salt treatments, and their interactions. The total chlorophyll content in PT1, KDML105, and HJ salt-stressed leaves (427 mM NaCl) was reduced by 76.95, 83.37, and 64.71%, respectively. The chlorophyll degradation percentage in salt stressed seedlings was effectively classified as salt tolerant and salt

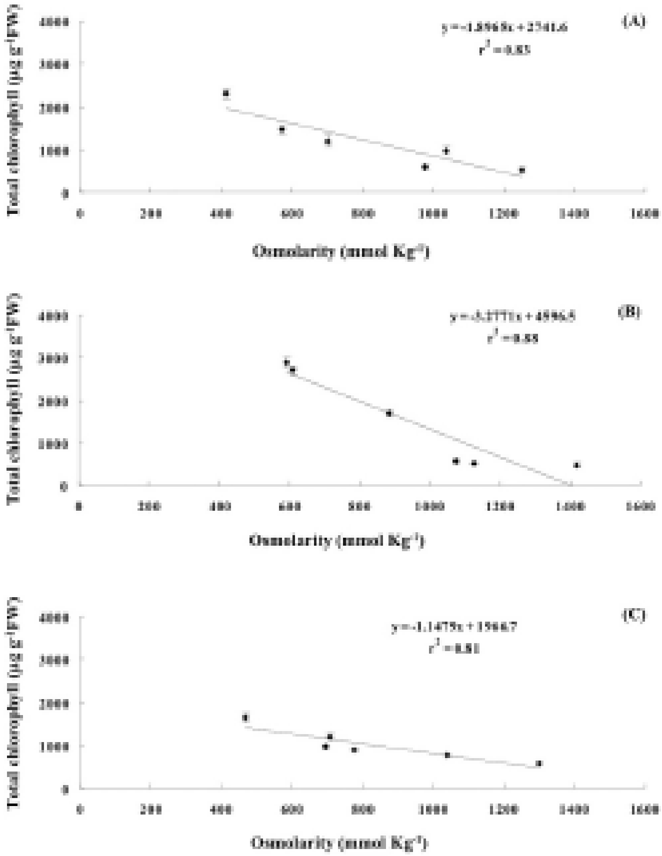

Fig. 6. Relationship between leaf osmolarity and total chlorophyll content in Pathumthani 1 (A), Jasmine (B), and Homjan (C) rice varieties grown in photoautotrophic culture supplemented with various sodium chloride (NaCl) concentrations for 5 days. Error bars represented by ± SE.

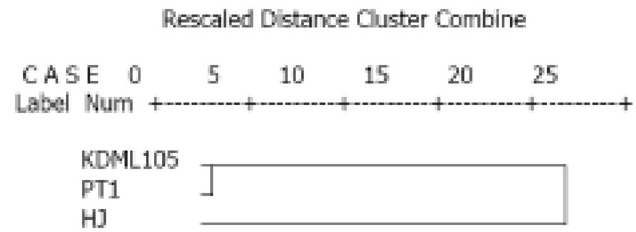

Fig. 7. Hierarchical Cluster analysis of aromatic rice varieties to classify as salt sensitivity, KDML105 and PT1, and salt tolerance, HJ, using total chlorophyll degradation by Hierarchical cluster analysis in SPSS software.

Table 1. Root and shoot osmolarity in Pathumthani 1 (PT1), Jasmine (KDML 105), and Homjan (HJ) rice varieties grown in photoautotrophic culture supplemented with various sodium chloride (NaCl) concentrations for 5 days

Rice varieties	NaCl (mM)	Root osmolarity (mmol Kg^{-1})	Leaf osmolarity (mmol Kg^{-1})
Pathumthani 1	0	392	413
	85	371	572
	171	427	703
	256	487	1036
	342	542	976
	427	777	1252
F-values		54.428	95.585
KDML 105	0	487	593
	85	498	611
	171	499	886
	256	630	1076
	342	735	1125
	427	933	1419
F-values		15.943	66.800
Homjan	0	423	470
	85	552	709
	171	587	697
	256	559	777
	342	659	1039
	427	809	1301
F-values		7.627	44.243
Significant level			
Variety		**	**
NaCl		**	**
Variety x NaCl		**	**

Highly significant is represented by **

sensitive varieties. This means that KDML105 and PT1 varieties were identified as salt-sensitive, while the HJ variety was classified as salt-tolerant using the chlorophyll degradation index (Fig. 7). As well as this, the chlorophyll degradation in salt-stressed seedlings directly affected plant growth, shown by the measurements of fresh weight and dry weight (Table 3).

There are many documents which otherwise mention the osmotic effects of salt stress conditions, leading to water deficit or drought in rice crops (Bahaji et al. 2002; Lefevre et al. 2001; Ueda et al. 2006). Similarly, the osmotic effects in both root and leaf tissues of salt-stressed aromatic rice progressively increased, related to exogenous NaCl salt concentrations in the media. An osmolarity or water deficit in either roots or leaves of rice crop grown under salt-stress conditions progressively increased, related to the cultivars used, salt concentrations, and exposure times (Lefevre et al. 2001; Ueda et al. 2006). In most cases, the osmolarity in the salt-stressed leaves was higher than that in the roots. High osmolarity in leaves is directly related to damage to photosynthetic systems in both light (pigments, electron transport machinery, water oxidation, photosystem II, and photosystem I) and dark reactions (CO_2 assimilation and Rubisco/PEPC activities), leading to inhibition of growth and decreased productivity (Bahaji et al. 2002; Mitsuya et al. 2003a). In the present study, pigment degradation in aromatic rice grown under high osmotic conditions derived from salt stress was represented in different patterns (Table 2), classified as salt tolerant, HJ, and salt sensitive, KDML105 and PT1. Chlorophyll degradation in the salt-stressed leaves of rice cultivars is a sensitive parameter which can be used to identify salt-tolerance or salt-sensitivity (Ali et al. 2004; Bahaji et al. 2002; Walia et al. 2005; Wanichananan et al. 2003). Additionally, rice genotypes have been identified into four clusters by Ward's minimum variance cluster analysis based on ion contents, ion selectivity, and growth performances (Zeng 2005).

Table 2. Chlorophyll a, chlorophyll b, and total chlorophyll in Pathumthani 1 (PT1), Jasmine (KDML 105), and Homjan (HJ) rice varieties grown in photoautotrophic culture supplemented with various sodium chloride (NaCl) concentrations for 5 days.

Rice varieties	NaCl (mM)	Chlorophyll a (μg g^{-1} FW)	Chlorophyll b (μg g^{-1} FW)	Total chlorophyll (μg g^{-1} FW)
Pathumthani 1	0	1740	559	2299
	85	1099	363	1462
	171	839	363	1202
	256	741	230	971
	342	453	138	591
	427	403	127	530
F-values		35.541	35.485	39.845
KDML 105	0	2098	794	2892
	85	2008	691	2698
	171	1297	399	1696
	256	431	152	583
	342	397	121	518
	427	378	103	481
F-values		253.538	68.820	201.760
Homjan	0	1198	454	1652
	85	765	437	1202
	171	646	324	970
	256	612	279	891
	342	583	187	770
	427	429	154	583
F-values		33.684	15.609	46.201
Significant level				
Variety		**	**	**
NaCl		**	**	**
Variety x NaCl		**	**	**

Highly significant is represented by **

Table 3. Fresh and dry weights in Pathumthani 1 (PT1), Jasmine (KDML 105), and Homjan (HJ) rice varieties grown in photoautotrophic culture supplemented with various sodium chloride (NaCl) concentrations for 5 days.

Rice varieties	NaCl (mM)	Fresh weight (mg)	Dry weight (mg)
Pathumthani 1	0	244	30
	85	195	24
	171	148	22
	256	127	26
	342	117	22
	427	113	23
F-values		6.778	4.156
KDML 105	0	455	57
	85	315	42
	171	269	34
	256	259	39
	342	253	46
	427	228	43
F-values		29.795	6.443
Homjan	0	204	26
	85	191	26
	171	194	23
	256	186	24
	342	184	23
	427	151	18
F-values		12.091	2.45
Significant level			
Varieties		**	**
NaCl		**	**
Varieties x NaCl		**	**

Highly significant is represented by **

The chlorophyll contents in salt-tolerant rice cultivars are generally stabilized by several osmolytes, i.e. proline (Basu et al. 2002; Demiral and Turkan 2005; Shah et al. 2002; Vaidyanathan et al. 2003), glycine betaine (Cha-um et al. 2007), sugar (Gupta and Kaur 2005; Morsy et al. 2007), and polyamine (Lefevre et al. 2001; Ndayiragije and Lutts 2006) when compared to salt-sensitive cultivars.

In conclusion, Na^+ accumulation and K^+ reduction in salt-stressed rice cultivars were positively related to NaCl salt concentration treatments in the culture media, causing high root and leaf osmolarity. The high osmolarity in the leaf tissues derived from salt stress, directly affecting chlorophyll degradation, leading to its application as an efficient indicator of salt-tolerance (HJ) or salt-sensitivity (KDML105 and PT1). The HJ salt-tolerant rice variety should be further used as a parental line in breeding programs for the improvement of salt-tolerance.

Acknowledgements

The authors are grateful to Dr. Teeraporn Busaya-angoon at Pathumthani Rice Research Center, for providing of Pathumthani 1, Jasmine, and Homjan rice seeds. This research is supported by the National Center for Genetic Engineering and Biotechnology (BIOTEC; Grant number BT-B-02-RG-BC-4905).

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