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# Salt stress induced ion accumulation, ion homeostasis, membrane injury and sugar contents in salt-sensitive rice (*Oryza sativa* L. spp. *indica*) roots under iso-osmotic conditions

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**Excess salt induced ionic and osmotic stresses that disturbed metabolism and led to reduction of plant development. Previous studies reported that sugars in stressed plants were involved in stress tolerance. However, the role of sugars in salt-stressed plants against only ionic effects is still unclear. The objective of this research was to investigate accumulation and homeostasis of ions, membrane injury, water content, growth characters and sugar contents in roots, in-response to salt stress under iso-osmotic conditions. Salt-sensitive rice, Pathumthani1 (PT1) was grown on MS culture medium for 7 days and was adjusted to salt stress under iso-osmotic conditions ( $-1.75 \pm 0.20$  MPa) by mannitol for 4 days. An increase in NaCl increased  $\text{Na}^+$  and  $\text{Na}^+:\text{K}^+$  in PT1 roots leading to increased membrane injury, while the water content was decreased. Additionally, growth characters, including number, length, fresh weight and dry weight of roots, were inhibited. Sugar accumulations in PT1 roots were enhanced by increases in NaCl. The increase in  $\text{Na}^+$  was positively related to total soluble sugars, resulting in an osmotic adjustment of the membrane that maintained water availability. The accumulation of sugars in PT1 roots may be a primary salt-defense mechanism and may function as an osmotic control.**

**Key words:** Mannitol, membrane injury, oligosaccharides, sodium ion, potassium ion, sodium chloride.

## INTRODUCTION

Salt-affected soil is one of the serious abiotic stresses that cause reduced plant growth, development and productivity worldwide (Qadir et al., 2008). In salt-affected soil, there are many salt contaminants, especially NaCl which readily dissolves in water to yield the toxic ions, sodium ion ( $\text{Na}^+$ ) and chloride ion ( $\text{Cl}^-$ ). Also, the water available in the salt-contaminated soil is restricted, inducing osmotic stress (Castillo et al., 2007; Pagter et al., 2009).  $\text{Na}^+$  is a small molecule that is easily absorbed into root tissues of higher plants and transported through-

out plant organs, leading to toxic ion damage, osmotic stress and nutritional imbalance (Cha-um et al., 2007; Siringam et al., 2009). Root tissues are the first barrier which not only select nutrient ions but also protect against toxic ions. Excess  $\text{Na}^+$  in plant cells directly damages membrane systems and organelles, resulting in plant growth reduction and abnormal development prior to plant death (Essah et al., 2003; Tester and Davenport, 2003; Davenport et al., 2005; Quintero et al., 2007). In halophyte species, there are many salt-defense mechanisms including ion homeostasis, osmoregulation, antioxidant and hormonal regulation (Hasegawa et al., 2000; Sairam and Tyagi, 2004). Sugars are compatible solutes which accumulate in plant tissues that are exposed to abiotic stresses, such as, water deficit, ex-

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treme temperatures and salt stress. The accumulation of sugars may play an important role in the plant defensive mechanisms of osmoregulation and energy preservation (Norwood et al., 2003; Minorsky, 2003; Morsy et al., 2007).

Rice (*Oryza sativa* L. spp. *indica*) is a top five world carbohydrate crop (Khush, 1997), especially in Asia. It has been previously classified as being salt-susceptible in both the vegetative and reproductive stages (Zeng et al., 2001; Moradi and Ismail, 2007), leading to a reduction in productivity of more than 50% when exposed to 6.65 dS m<sup>-1</sup> electrical conductivity (EC) of salinity (Zeng and Shannon, 2000). In Thailand, the Pathumthani1 (PT1) cultivar is an aromatic rice which has high cooking quality (long grain and soft texture) and high export value (Laohakunjit and Kerdchoechuen, 2007). It is a major cultivar widely grown in irrigated paddy fields and reported as being salt susceptible (Cha-um et al., 2007; Siringam et al., 2009). Previous studies showed that the response of rice included the effects of osmotic stress and ionic stress. However, the previous results could not separate osmotic effects from ionic effects (Radic et al., 2006; Ahmad et al., 2007). A rapid response in the root tissues of the salt-sensitive rice cultivar PT1 exposed to salt stress under iso-osmotic conditions is an attractive issue that needs to be clarified. Therefore, for this study we hypothesized that an increase of sugar contents could be a salt-defense mechanism. The objective of this research was to investigate the responses of physiological characteristics and sugar contents in PT1 salt-sensitive rice roots to salt stress under iso-osmotic conditions.

## MATERIALS AND METHODS

### Plant materials and salt stress treatments

Seeds of the PT1 rice cultivar (*O. sativa* L. spp. *indica*) were obtained from the Rice Research Institute, Pathumthani Rice Research Center, Pathumthani, Thailand. Rice seeds were dehusked, disinfected once in 5% (v/v) Clorox<sup>®</sup> [5.25% (w/v) sodium hypochlorite solution, Clorox Co. Ltd., USA] for 12 h, once in 25% (v/v) Clorox<sup>®</sup> for 30 min, and then rinsed with sterile distilled water. Surface-disinfected seeds were germinated on 25 ml 0.25% Phytigel<sup>®</sup>-solidified MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose (w/v) (photomixotrophic condition) in a 250 ml glass vessel. The medium pH was adjusted to 5.7 before autoclaving. *In vitro* rice seedlings were cultured in a culture room under conditions of 25 ± 2°C air temperature, 60 ± 5% relative humidity (RH), 60 ± 5 μmol m<sup>-2</sup> s<sup>-1</sup> photosynthetic photon flux (PPF) provided by fluorescent lamps (TLD 36 W/84 Cool White 3350 lm, Philips, Thailand) with a 16 h d<sup>-1</sup> photoperiod. Fourteen-day-old seedlings were aseptically transferred to MS sugar-free liquid medium (photoautotrophic condition) using vermiculite as a supporting material in NUAIRE<sup>™</sup> Biological Safety Cabinets (Model NU-440-400E Series 9, NuAire Inc., USA) and left on the shelf in the culture room for 7 days. Air-exchange in the glass vessels was adjusted to 2.32 μmol CO<sub>2</sub> h<sup>-1</sup> by punching a hole in the plastic cap (∅ 1 cm) and covering the hole with a gas-permeable microporous polypropylene film (0.22 μm pore size, Nihon Millipore Ltd., Japan). To adjust iso-osmotic conditions to -1.75 ± 0.20 MPa in the culture medium, 548.9, 329.4, 219.6, 109.8 or 0.0 mM mannitol were

added to 0.0, 85.5, 171.0, 256.5 or 342.0 mM NaCl, respectively and plants were cultured for 4 days. Sodium ions (Na<sup>+</sup>), potassium ions (K<sup>+</sup>), ion homeostasis (Na<sup>+</sup>:K<sup>+</sup>), membrane injury, water content, growth characters and sugar contents in PT1 rice roots were evaluated.

### Data measurements

#### Ion contents

One hundred milligrams of rice roots were ground in liquid nitrogen and extracted by the acidic method (Cha-um et al., 2007). Sodium ions (Na<sup>+</sup>) and potassium ions (K<sup>+</sup>) in the roots were determined according to Dionisio-Sese and Tobita (1998) by using an atomic absorption spectrophotometer (AA, Model M6, Thermo Elemental, MA, USA). In addition, Na<sup>+</sup>:K<sup>+</sup> was calculated following Lee et al. (2003).

#### Membrane injury

The membrane injury in roots was determined by the modified method of Shalata and Neumann (2001). The rice roots were cut into 5.0 ± 0.2 mm in lengths and placed in glass vessels (Opticlear<sup>®</sup>; KIMBLE, Vineland, New Jersey, USA) containing 10 ml deionized water. The glass vessels were capped and maintained at room temperature (25°C) for 2 h. The initial electrical conductivity (EC<sub>1</sub>) was measured by using an electrical conductivity meter (Model ID1010, INDEX, Kuala Lumpur, Malaysia). The root tissues were incubated at 100°C in a water bath for 30 min, cooled down to 25°C and then the electrical conductivity (EC<sub>2</sub>) was measured. The membrane injury was calculated following Shalata and Neumann (2001).

#### Growth parameters

Root number, root length and root fresh weight were measured after exposure to salt stress under iso-osmotic conditions for 4 days. The rice roots were dried in a hot-air oven (Memmert, Model 500, Germany) at 110°C for 48 h and then incubated in a desiccator before measuring the dry weight. Water content was calculated following Bonnet et al. (2000).

#### Sugar determinations

##### Sugar extractions

Sugar contents in rice roots were extracted by the modified method of Karkacier et al. (2003). Fifty-milligrams of rice roots were ground in liquid nitrogen with a pestle in a pre-cooled eppendorf tube. One milliliter nanopure water was added and then sonicated for 15 min. The aliquot was centrifuged at 12,000 rpm for 15 min. Supernatant was collected and filtered through a 0.45 μm millipore filter (VertiClean<sup>™</sup>; NYLON Syringe, Vertical Chromatography Co., Ltd., Thailand) and stored at -20°C prior to sugar content determinations.

##### Sugar analysis

Stachyose and raffinose were analyzed by high performance liquid chromatography (HPLC) integrated with a 410 differential refractometer (RI) detector that consisted of a Waters 600 gradient controller pump (Water, Milford, MA, USA) and on-line detection monitored by a RI detector. The stachyose and raffinose were analyzed by Empower software. Chromatography of stachyose and raffinose was performed by a VertiSep PRP-NH<sub>2</sub> column (4.6×250

**Table 1.** Sodium ions (Na<sup>+</sup>), potassium ions (K<sup>+</sup>), Na<sup>+</sup>:K<sup>+</sup> and membrane injury in fourteen-day-old PT1 salt-sensitive rice roots cultured on MS medium and subsequently exposed to salt stress under iso-osmotic conditions (-1.75 ± 0.2 MPa) for 4 days (n = 4).

NaCl (mM)	Mannitol (mM)	Na <sup>+</sup> (mg g <sup>-1</sup> FW)	K <sup>+</sup> (mg g <sup>-1</sup> FW)	Na <sup>+</sup> :K <sup>+</sup>	Membrane injury (%)
0.0	548.9	0.75 d	13.85	0.058 c	45.1 c
85.5	329.4	3.55 c	10.05	0.473 bc	45.2 c
171.0	219.6	11.29 b	15.13	0.749 ab	66.3 b
256.5	109.8	14.41 a	13.99	1.043 a	71.5 ab
342.0	0.0	13.38 a	11.80	1.138 a	79.2 a
ANOVA		**	ns	*	**

Means with different letters in the same column are significantly different at  $P \leq 0.01$  (\*\*),  $P \leq 0.05$  (\*) and non-significant (ns) by Duncan's new multiple range test. ANOVA, Analysis of variance.

mm) (Ligand Scientific Co., Ltd., Thailand) equipped with a guard column. Acetonitrile:nanopure water (75:25; v/v) was used as the mobile phase. An internal standard of stachyose and raffinose was added to the sample. The injection volume was 40  $\mu$ l and the flow rate was set at 1.0 ml min<sup>-1</sup>. In addition, sucrose, glucose and fructose were analyzed by Metacarb 87C (7.8×300 mm) (Varian Inc., USA) equipped with guard column. Nanopure water was used as the mobile phase. The injection volume was 40  $\mu$ l and the flow rate was set at 0.4 ml min<sup>-1</sup>. Quantification of sugar contents were performed by comparing the peak areas with the sugar standard solutions. Stachyose, raffinose, sucrose, glucose and fructose (Sigma, Germany) were used as standards and sugar contents were calculated using a standard curve equation.

#### Experimental design and data analysis

The experiment was arranged as a completely randomized design (CRD) with four replications per treatment, five seedlings per replicate. One-way analysis of variance (ANOVA) was performed using SPSS software. Mean values in each treatment were compared by Duncan's new multiple range test (DMRT).

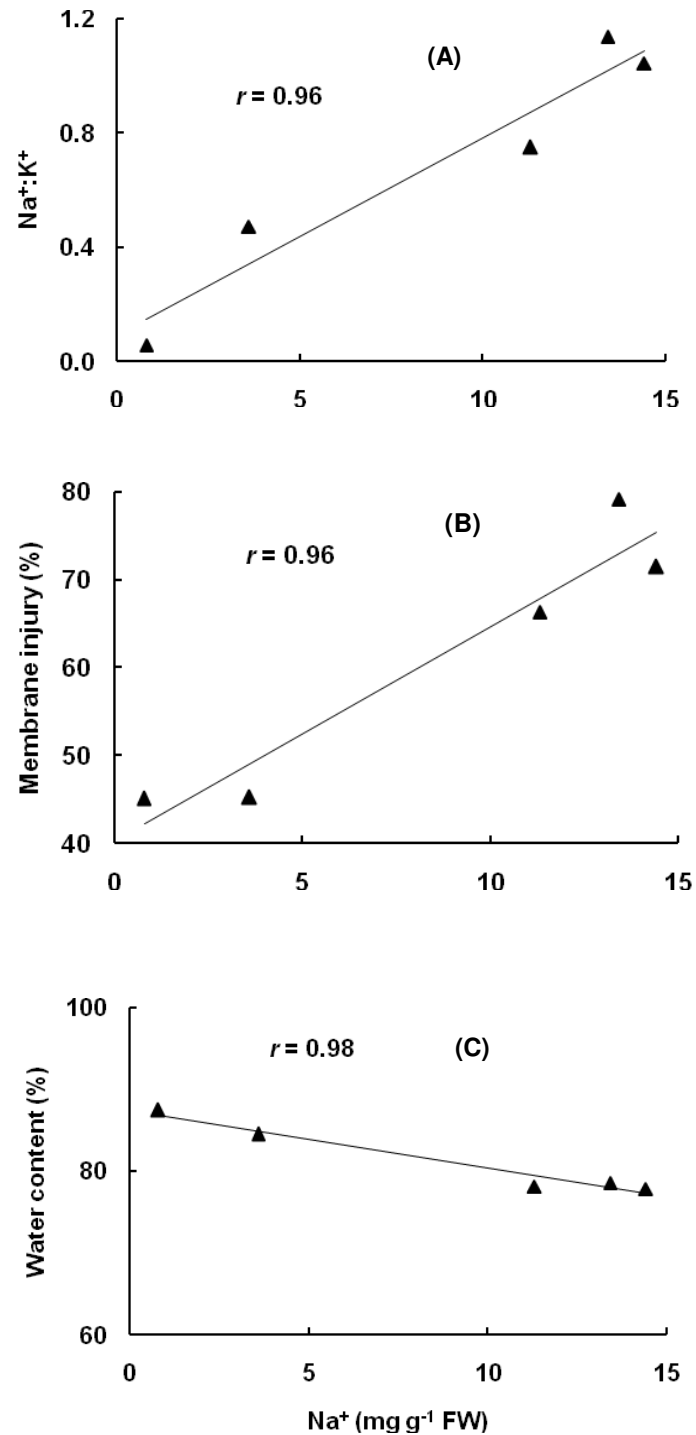
## RESULTS AND DISCUSSION

### Effect of NaCl on ion contents, ion homeostasis and membrane injury

Sodium ions (Na<sup>+</sup>), ion homeostasis (Na<sup>+</sup>:K<sup>+</sup>) and membrane injury in PT1 salt-sensitive rice roots progressively increased with increasing NaCl concentrations in the culture medium (Table 1) while potassium ion (K<sup>+</sup>) levels were not significantly related to increasing NaCl concentrations (Table 1). In this study, Na<sup>+</sup> accumulation in PT1 salt-sensitive rice roots showed a positive relationship to NaCl concentrations in the culture medium. The Na<sup>+</sup> in PT1 roots exposed to 85.5, 171.0, 256.5 and 342.0 mM NaCl accumulated 4.7, 15.0, 19.2 and 17.8 folds, respectively, when compared to the control (without salt-stress induction) (Table 1). In addition, the Na<sup>+</sup>:K<sup>+</sup> was enriched 8.2, 12.9, 18.0 and 19.6 folds, respectively, when compared to the control (Table 1). The membrane injury in PT1 roots was 1.0, 1.5, 1.6 and 1.8 folds at 85.5, 171.0, 256.5 and 342.0 mM NaCl, respectively, when

compared to the control (Table 1). The Na<sup>+</sup> in the salt-sensitive rice roots was increased, while the K<sup>+</sup> was unchanged, leading to an increased Na<sup>+</sup>:K<sup>+</sup> ( $P < 0.05$ ,  $r = 0.96$ ) (Figure 1A), leading to increased membrane injury ( $P < 0.05$ ,  $r = 0.96$ ) (Figure 1B), resulting in growth inhibition (Table 2). Moreover, the Na<sup>+</sup> was negatively related to the water content ( $P < 0.05$ ,  $r = 0.98$ ) (Figure 1C).

Generally, Na<sup>+</sup> is accumulated overall in plant tissues of salt-sensitive cultivars of rice such as IR 28 (Dionisio-Sese and Tobita, 1998; Nakamura et al., 2002), PT1 (Cha-um et al., 2007; Siringam et al., 2009), IR20 (Krishnamurthy et al., 2009), Khao Dawk Mali 105 (KDML105) (Summart et al., 2010) and I Kong Pao (IKP) (Lefevre et al., 2001), relative to salt stress levels. In this study, an increase in Na<sup>+</sup> in PT1 roots was related to increasing salt stress under iso-osmotic conditions (Table 1). Similar to Nakamura et al. (2002), who reported that in IR28 salt-sensitive rice cultivar, Na<sup>+</sup> was increased 3.0 folds over the control (0 mM NaCl) when exposed to 113 mM NaCl for 14 days. In addition, the Na<sup>+</sup> in PT1 leaves was increased 28.0 folds over the control when exposed to 342 mM NaCl for 4 days (Siringam et al., 2009). The Na<sup>+</sup> accumulation was increased while K<sup>+</sup> was unchanged. In contrast, the previous studies showed that the salt stress enhanced Na<sup>+</sup> accumulation while K<sup>+</sup> was decreased in salt-sensitive rice varieties such as IR28 (Dionisio-Sese and Tobita, 1998; Nakamura et al., 2002), IR20 (Krishnamurthy et al., 2009) and I Kong Pao (IKP) (Lefevre et al., 2001). It might be possible that the PT1 salt-sensitive rice variety exposed to NaCl may use other salt-defense mechanisms for survival. An increase in Na<sup>+</sup> in the salt-stressed rice roots directly increased the Na<sup>+</sup>:K<sup>+</sup>, especially in salt-sensitive cultivars. Damaging of cell membranes was identified by electrolyte leakage (Dionisio-Sese and Tobita, 1998) and water balance (Lefevre et al., 2001; Nakamura et al., 2002). At 342 mM NaCl, PT1 roots lost the membrane function of conserving ion homeostasis involved in controlling the Na<sup>+</sup> intake and K<sup>+</sup> conservation. An increase in Na<sup>+</sup>:K<sup>+</sup> in IR29, GZ5310-20-3-2, GZ177, Sakha101 and IR70074-AC14 rice cultivars cultured under 57 mM NaCl for 34



**Figure 1.** Na<sup>+</sup> induced Na<sup>+</sup>:K<sup>+</sup> (A) and membrane injury (B) while water content was decreased (C) in fourteen-day-old PT1 salt-sensitive rice roots cultured on MS medium and subsequently exposed to salt stress under iso-osmotic conditions ( $-1.75 \pm 0.2$  MPa) for 4 days ( $n = 4$ ).

days was reported (Zeng, 2005). Dionisio-Sese and Tobita (1998) reported that at 120 mM NaCl, the membrane injury was increased 8.7 and 12.0 folds in

Hitomebore and IR28 salt-sensitive cultivars, respectively in 7 days. In rice plants, the Na<sup>+</sup> accumulation, Na<sup>+</sup>:K<sup>+</sup> and membrane injury has been utilized to identify salt-

**Table 2.** Root number, root length, fresh weight (FW) and dry weight (DW) in fourteen-day-old PT1 salt-sensitive rice roots cultured on MS medium and subsequently exposed to salt stress under iso-osmotic conditions ( $-1.75 \pm 0.2$  MPa) for 4 days (n = 4).

NaCl (mM)	Mannitol (mM)	Root number	Root length (cm)	FW (mg)	DW (mg)
0.0	548.9	11 a	5.2 a	26.7 a	3.3 a
85.5	329.4	7 b	3.7 b	21.9 b	3.3 a
171.0	219.6	6 c	3.3 c	12.5 c	2.3 b
256.5	109.8	6 c	2.9 d	11.2 c	2.2 b
342.0	0.0	6 c	2.6 e	11.0 c	1.8 c
ANOVA		**	**	**	**

Means with different letters in the same column are significantly different at  $P \leq 0.01$  (\*\*) by Duncan's new multiple range test. ANOVA, Analysis of variance.

**Table 3.** Sugar contents in fourteen-day-old PT1 salt-sensitive rice roots cultured on MS medium and subsequently exposed to salt stress under iso-osmotic conditions ( $-1.75 \pm 0.2$  MPa) for 4 days. (n = 4).

NaCl (mM)	Mannitol (mM)	Contents ( $\mu\text{mol g}^{-1}$ FW)				
		Stachyose	Raffinose	Sucrose	Glucose	Fructose
0.0	548.9	7.8 d	6.0 c	37.8 e	94.1 e	-
85.5	329.4	19.5 c	14.5 c	39.2 d	98.1 d	-
171.0	219.6	46.8 a	42.7 a	48.4 a	119.3 a	-
256.5	109.8	39.7 b	28.0 b	43.4 b	110.0 b	-
342.0	0.0	38.9 b	33.1 b	43.1 c	109.0 c	-
ANOVA		**	**	**	**	ND

Means with different letters in the same column are significantly different at  $P \leq 0.01$  (\*\*) and non-detected (ND) by Duncan's new multiple range test. ANOVA, Analysis of variance.

tolerant or salt-sensitive varieties (Dionisio-Sese and Tobita, 1998; Zeng et al., 2004; Zeng, 2005).

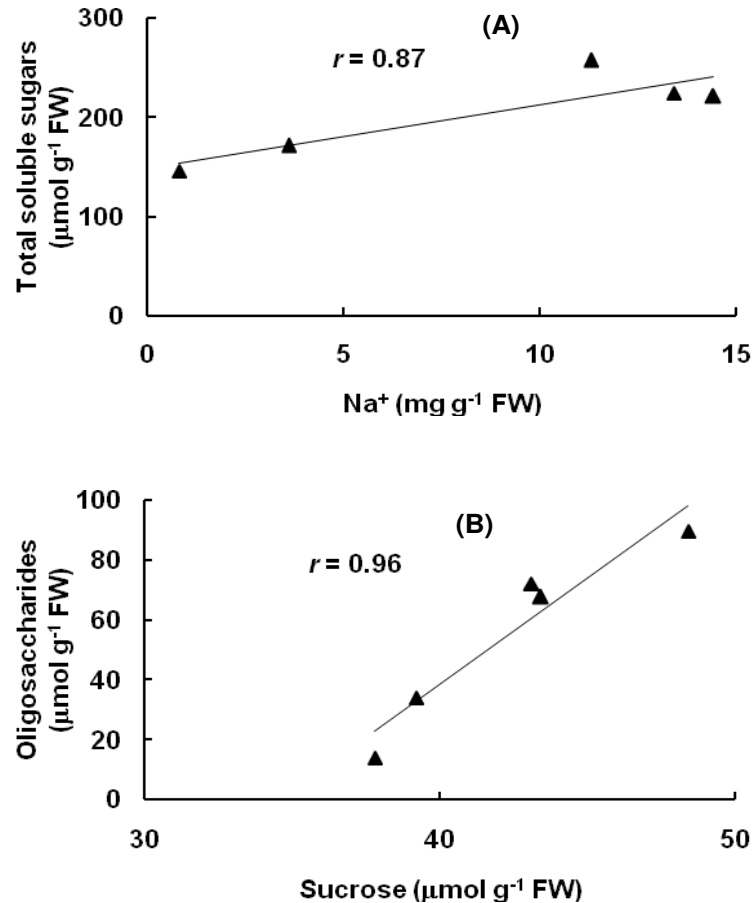
### Effect of NaCl on sugar accumulations

The sugar contents including stachyose, raffinose, sucrose and glucose in PT1 roots were enhanced by increasing NaCl concentrations, while a fructose profile did not appear (Table 3). At 171 mM NaCl, the stachyose, raffinose, sucrose and glucose in PT1 roots peaked at 46.8, 42.7, 48.4 and 119.3  $\mu\text{mol g}^{-1}$  FW, respectively and were 6.0, 7.1, 1.3 and 1.3 folds, respectively, when compared to the control (Table 3). The  $\text{Na}^+$  levels in PT1 roots were positively related to total soluble sugars ( $P < 0.05$ ,  $r = 0.87$ ) (Figure 2A). Furthermore, sucrose induced oligosaccharide (stachyose and raffinose) accumulations ( $P < 0.05$ ,  $r = 0.96$ ) (Figure 2B).

Sugar accumulations in salt-stressed plants have salt tolerant ability and play an important role in a salt-defense mechanism when plants are exposed to salt stress (Bohnert and Jensen, 1996). Salt stress induced sugar accumulations in many species, such as barley (Ahmad et al., 2006), sorghum (Almodares et al., 2008a), tomato (Chookhampaeng et al., 2008), rice (Pattanagul and Thitisaksakul, 2008) and eggplant (Abbas et al.,

2010). The increase of stachyose, raffinose, sucrose and glucose in PT1 roots (Table 3) may function as an osmotic adjustment to prevent water loss in the plant cells during salt stress (Cushman, 2001; Norwood et al., 2003; Sairam and Tyagi, 2004). This relationship was similar to *Arabidopsis* (Taji et al., 2002), algarrobo (Meloni et al., 2004), poplar (Jouve et al., 2004), tomato (Khelil et al., 2007), rice (Morsy et al., 2007; Cha-um et al., 2008), sweet sorghum (Almodares et al., 2008b) and maize (Hajlaoui et al., 2010). Furthermore, an increase in oligosaccharides (stachyose and raffinose) resulted from the increased sucrose content (Figure 2B). This result agreed with Karner et al. (2004) who reported that sucrose was the secondary substrate for raffinose, which played a key role in the raffinose family oligosaccharides (RFOs) biosynthesis. Therefore, variation of sucrose levels may affect the formation of RFOs which may play a crucial role in maintaining membrane stability *via* interaction with phospholipid headgroups and may scavenge reactive oxygen species when exposed to salt stress (Bohnert and Jensen, 1996; Bentsink et al., 2000; Roy et al., 2005).

In conclusion, the  $\text{Na}^+$  in PT1 salt-sensitive rice roots was directly increased with higher NaCl concentrations. The  $\text{Na}^+:\text{K}^+$  and membrane injury derived from  $\text{Na}^+$  toxicity was demonstrated, leading to lower water content



**Figure 2.** Total soluble sugars (A) and oligosaccharides (B) were induced by  $\text{Na}^+$  and sucrose, respectively, in fourteen-day-old PT1 salt-sensitive rice roots cultured on MS medium and subsequently exposed to salt stress under iso-osmotic conditions ( $-1.75 \pm 0.2$  MPa) for 4 days ( $n = 4$ ).

and growth inhibition. In addition, accumulation of soluble sugar contents was related to the increase of  $\text{Na}^+$  and may play a role as an osmotic adjustment to maintain the water use efficiency in the root cells when exposed to salt stress.

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