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Proline Profiles in Aromatic Rice Cultivars Photoautotrophically Grown in Responses to Salt Stress

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Abstract: The aim of this investigation is to monitor the proline profiles and green leaf percentage in Thai aromatic rice cultivars, relating to salt concentration and exposure times. Eleven aromatic, Pakkali (Pok) salt tolerant and IR29 salt sensitive rice cultivars were germinated and aseptically transferred to *in vitro* photoautotrophic conditions using vermiculite as supporting material for 7 days. Sodium chloride salts in the culture media were adjusted to 0, 171, 342, 512 or 684 mM for 4 days. Proline contents in the leaf tissues of salt-stressed seedlings were positively related to salt concentrations in the culture media. The high salt concentration progressively stimulated on proline accumulation in the leaf tissues. A unique proline profile in the aromatic rice cultivars was clearly classified into three classes, high, modulate and low accumulation classes using Hierarchical cluster analysis. The results evidently showed that the green leaf percentages in the high and modulate proline accumulation classes were higher than those in low proline class. The Pok and IR29 rice cultivars were defined as moderate and low proline accumulation classes, respectively. In addition to, the proline contents in salt stressed seedlings (342 mM NaCl) of all classes were positively correlated with salt exposure times. An increasing exposure period of salt stress directly enhanced the proline gathering. It should be demonstrated that the proline accumulation in Thai aromatic rice was depended on rice cultivars, salt concentrations and salt exposure time.

Key words: *Oryza sativa* L., cluster analysis, green leaf area, indica rice, proline accumulation, salt stress

INTRODUCTION

Proline is an amine amino acid, producing in higher plants and gathering in high amount quantities in responses to various kinds of abiotic stresses, especially salinity and water deficit (Hsu *et al*., 2003; Kishor *et al*., 2005; Ashraf and Foolad, 2007). It plays an important role as osmolyte in a neutral form to stabilize the organelles, proteins and membrane as well as energy preservation when plant species exposure to abiotic stresses (Hare and Cress, 1997; Hare *et al*., 1998). In case of salt stress inducible proline, there are many publications to mention on the proline biosynthesis and accumulation as rapid salt defense responses such as in rice (Hien *et al*., 2003; Hur *et al*., 2004), wheat (Wang *et al*., 2007), barley (Ueda *et al*., 2007), green gram (Misra and Gupta, 2005), foxtail millet (Veeranagamallaiah *et al*., 2007), *Vigna radiate* (Sumithra *et al*., 2006), sugar beet (Ghoulam *et al*., 2002), *Salsola* (Heidari-Sharifabad and Mirzaie-Nodoushan, 2006), sesame (Koca *et al*., 2007), parslane (Yazici *et al*., 2007), mulberry (Kumar *et al*., 2003), *Prosopis alba* (Meloni *et al*., 2004), *Medicago sativa* (Ehsanpour and Fatahian, 2003), tomato (Santa-Cruz *et al*., 1999; Amini and Ehsanpour, 2005), *Populus euphratica* (Watanabe *et al*., 2000) and *Catharanthus roseus* (Jaleel *et al*., 2007). An accumulation of proline contents is closely related to plant species, lethal doses, stressor types, plant organelles and salt exposure times (Hien *et al*., 2003; Koca *et al*., 2007; Ueda *et al*., 2007; Veeranagamallaiah *et al*., 2007). The proline biosynthesis partway in higher plants has been well established through glutamate as major route using Δ^1-pyrroline-5-carboxylate synthetase (P5CS) rate limiting enzyme (Igarashi *et al*., 1997), as well as ornithine using ornithine-δ-aminotransferase (δ-OAT) enzyme activity as alternative channel (Hare and Cress, 1997; Roosens *et al*., 1998; Kishor *et al*., 2005).

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Rice is a major crop to feed the world population as energy sources for 50 to 80% daily calorie intake (Khush, 2005). Sodium ion (Na^+) and chloride ions ($Cl^−$) are promptly absorbed by root cells and directly transferred to overall plant through xylem vascular tissues (Zhu, 2003; Tester and Davenport, 2003; Rodriguez-Navarro and Rubio, 2006). In addition, Na^+ ions are well known the toxic damages to plant cells in both ionic and osmotic effects, causing on growth retardants and low productivity prior to death (Hasegawa *et al*., 2000; Munns *et al*., 2002; Mansour and Salama, 2004; Chinnusamy *et al*., 2005). The rice crop has been reported as salt-sensitive and demonstrated the negative effects in seedling and reproductive stages (Zeng and Shannon, 2000a, b; Khan and Abdullah, 2003; Zeng *et al*., 2003). There are many documents to classify the salt-tolerant rice from the genetic resources using both growth and yield performances. However, the effective indices in term of biochemical, physiological and morphological characteristics are still need to investigate for salt tolerant screening in breeding program. In Thailand, there are many aromatic rice cultivars with aroma flavor, high cooking quality, long grain, high amylose content or soft texture and high exported values, especially jasmine rice (Ariyaphanphitak *et al*., 2005; Laohakunjit and Kerdchoechuen, 2007). The objective of this investigation is to monitor the proline profiles and green leaf percentage as indicators to set a group in Thai aromatic rice cultivars for salt tolerant classification, relating to salt concentration and exposure times.

MATERIALS AND METHODS

Plant materials: Seeds of eleven rice (*Oryza sativa* L. sp. *indica*) lines, including Hompaepalo (HPL; GS. No. 7672), Homsadung (HSD; GS. No. 9600), Homjan (HJ; GS. No. 4371), Homnangnuan (HNN; GS. No. 8266), Homtang (HT; GS. No. 1628), Hommaejun (HMJ; GS. No. 10473), Homphrae (HP; GS. No. 21681), Homthong (HTH; GS. No. 06823), Homjampa (HJP; GS. No. 01641), Hom (Hom; GS. No. 9862), Homdurian (HTR; GS. No. 231), Pokkali (Pok; GS. No. 17905) and IR29 (IR29; GS. No. 2818) were obtained from the Pathumthani Rice Research Center (Rice Research Institute, Department of Agriculture, Ministry of Agriculture and Cooperative, Thailand) in year 2006. Rice seeds were hand-dehusked, rinsed with 70% ethanol, surface-disinfected once in 5% (v/v) Clorox® (5.25% w/v sodium hypochlorite, Clorox Co, USA) for 12 h, once soaked in 25% Clorox® for 25 min and then rinsed thrice with sterile distilled water. Surface disinfected seeds were germinated on MS-solidified media (Murashige and Skoog, 1962). All seedlings were cultured under 25±2°C air-temperature, 60±5% Relative Humidity (RH) and 60±10 μmol m^{-2} sec^{-1} Photosynthetic Photon Flux Density (PPFD) with 16 h day^{-1} photoperiod provided by fluorescent lamps (TLD 36W/84, Cool White, Philips, Thailand). Fourteen-old rice seedlings were aseptically transferred to 50 mL liquid sugar-free MS media, supporting by 20 g vermiculite (photoautotrophic system) for 7 days (Fig. 1). An amount of air-exchange in the glass vessels was adjusted to 2.32 h^{-1}, punching a hole over the plastic cap (Ø 1 cm) and covering with a gas-permeable microporous polypropylene film (0.22 μm pore size, Nihon Millipore Ltd., Japan).

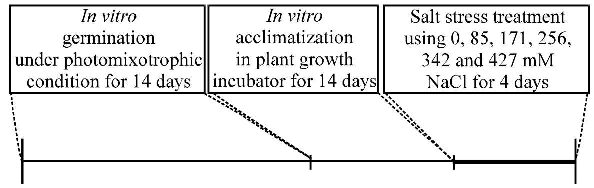

Fig. 1: Scheme of the experiment on *in vitro* photomixotrophic germination for 14 days, photoautotrophic acclimatization for 7 days and subsequently exposed to 0, 171, 342, 513 and 684 mM NaCl for 4 days

Effect of NaCl concentrations on proline accumulation: Sodium chloride (NaCl) in the culture media was adjusted to 0, 171, 342, 512 or 684 mM for 4 days (Fig. 1). The proline content in leave blades was extracted and determined with spectrophotometer by the modified method of Bates *et al.* (1973). Moreover, green leaf area was measured using a Leaf Area Meter DT-scan (Delta-Scan Version 2.03, Delta-T Devices, Ltd., UK). The proline accumulation and green leaf area (%) were calculated. The experiment was designed as Completely Randomized Design (CRD) with four replications and five plantlets per replicate. The correlation between proline content in each class and green leaf area (%) was evaluated by Pearson's correlation coefficients. The proline accumulation in salt stressed rice cultivars was subjected to classify group using Hierarchical cluster analysis in SPSS software.

Effect of NaCl exposure time on proline accumulation: Hompaepalo and Homthong, Homjan and Pokkali and Hom and IR29 cultivars in previous mention were classified as high, moderate and low proline accumulation classes, respectively. Sodium chloride (NaCl) in the culture media was adjusted to 0 (control) or 342 mM (salt stress) for 0, 1, 2, 3 and 4 days. The proline content in leave blades was extracted and determined according to the modified method of Bates *et al.* (1973). The experiment was designed as CRD with four replications and five plantlets per replicate.

Analysis of proline content: Fifty milligram fresh weights were grounded in a motar with liquid nitrogen. The homogenate powder was mixed with 1 mL aqueous sulfosalicylic acid (3% w/v) and filtered through filter paper (Whatman No. 1, England). Extracted solution was reacted with an equal volume of glacial acetic acid and ninhydrin reagent (1.25 mg ninhydrin in 30 mL of glacial acetic acid and 20 mL 6 M H_3PO_4) and incubated at 95°C for 1 h. The reaction was terminated placing on an ice bath. The reaction mixture was vigorously mixed with 2 mL toluene. After warming at 25°C, the chromophore was measured by Spectrophotometer DR/4000 (HACH, USA) at 520 nm as well as L-proline (Fluka, Switzerland) used as a standard.

RESULTS AND DISCUSSION

Proline content in leaf tissues of rice seedlings cultured under salt stress was accumulated, relating to the salt concentrations in the culture media. The proline-rich profiles in salt stressed seedlings were classified into three classes, high (HTH, HPL and HJP), modulate (HMJ, HNN, HT, Pok, HJ and HSD) and low (HTR, IR29, HP and Hom) accumulation classes using Hierarchical cluster analysis (Fig. 2). In high proline accumulation class, the results evidently showed that proline contents in HPL, HTH and HJP salt-stressed seedlings were rapidly increased and maintained in the high level (>200 µmol g^{-1} DW), relating to increase the salt concentrations in the culture media (Fig. 3A). In addition to, the proline concentrations in moderate class were gradually accumulated in the medium level (50-130 µmol g^{-1} DW) (Fig. 3B). On the other hand, the proline levels in low accumulation class were retained in a low content (<50 µmol g^{-1} DW) (Fig. 3C). The proline contents in high, moderate and low accumulation classes were represented in Table 1 and were positively related to salt concentration treatments with r^2 = 0.87, r^2 = 0.81 and r^2 = 0.96, respectively (Fig. 4). Whereas, the green leaf area in salt stressed rice seedlings was significantly decreased in a high salt concentration, especially in the low proline accumulation class (Table 1). Moreover, the green leaf percentage was negatively correlated with proline accumulation in high (r^2 = 0.92), modulate (r^2 = 0.86) and low classes (r^2 = 0.78) (Fig. 5). The results significantly showed that the green leaf percentage in the high proline class was maintained, while that in low proline class was damaged. It should be noted that the high proline

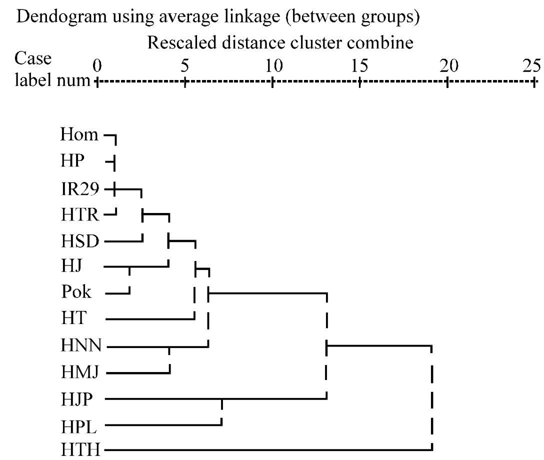

Fig. 2: Cluster analysis of 11 aromatic rice seedlings, Hompaepalo (HPL), Homsadung (HSD), Homjan (HJ), Homnangnuan (HNN), Homtang (HT), Hommaejun (HMJ), Homphrae (HP), Homthong (HTH), Homjampa (HJP), Hom (Hom), Homdurian (HTR) and IR29 as negative control as well as Pokkali (Pok) as positive control using proline accumulation by Hierarchical cluster analysis of SPSS software

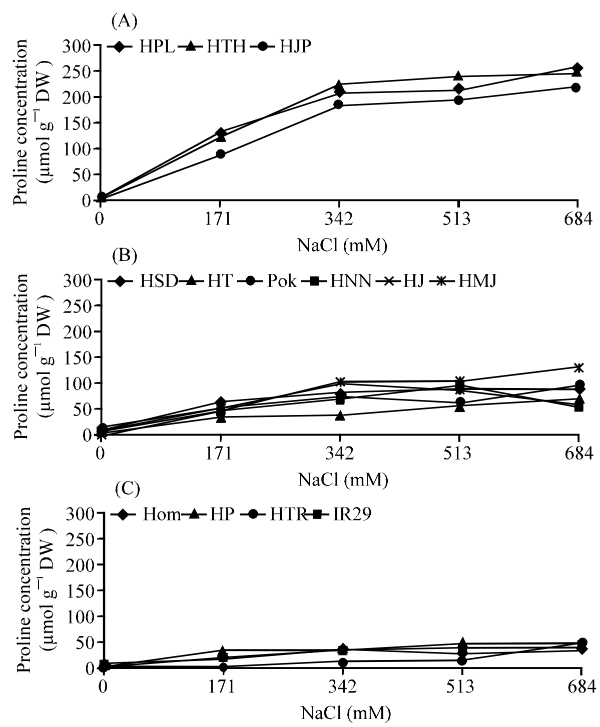

Fig. 3: Proline profiles in three classes, high (A), moderate (B) and low (C) proline accumulation of rice seedlings photoautotrophically grown under 0, 171, 342, 513 or 684 mM sodium chloride condition for 4 days

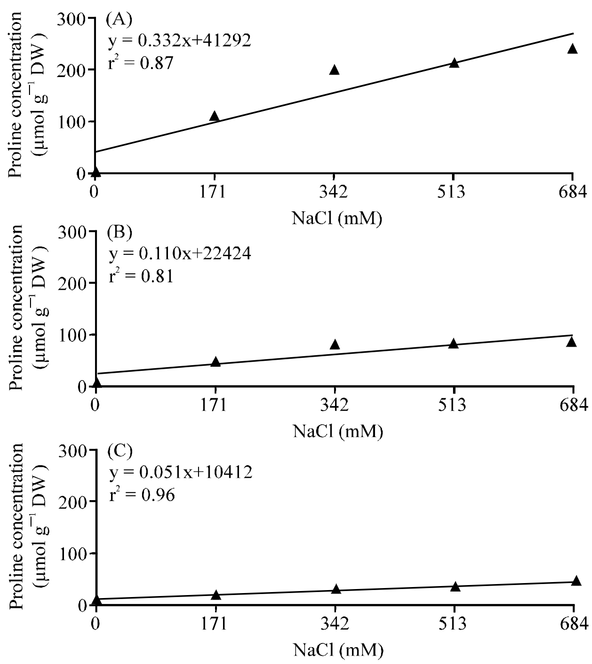

Fig. 4: Relationship between NaCl concentration and proline accumulation in three classes, high (A), medium (B) and low (C) proline accumulation of rice seedlings photoautotrophically grown under 0, 171, 342, 513 or 684 mM sodium chloride condition for 4 days

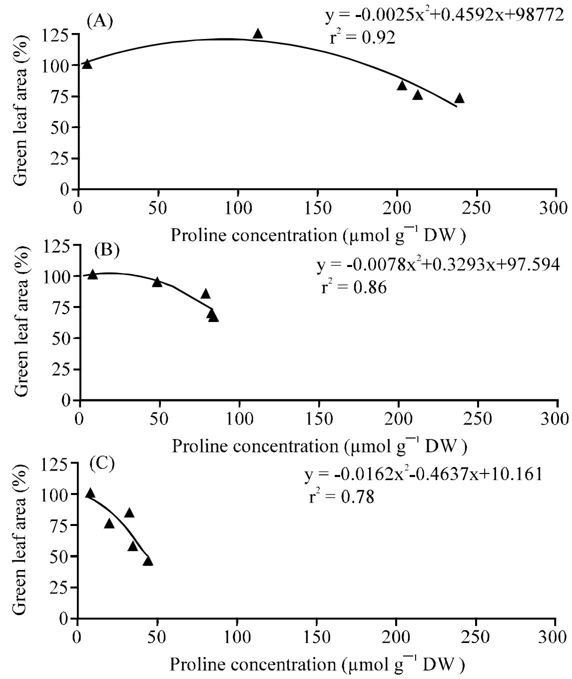

Fig. 5: Relationship between proline accumulation and green leaf area in three classes, high (A), medium (B) and low (C) proline accumulation of rice seedlings photoautotrophically grown under 0, 171, 342, 513 or 684 mM sodium chloride condition for 4 days

Table 1: Proline concentrations in three classes, high, moderate and low proline accumulation of rice seedlings photoautotrophically grown under 0, 171, 342, 513 or 684 mM sodium chloride condition for 0 and 4 days

Rice cultivars	Exposure time (days)	Proline concentration (μmol g^{-1} DW)	Green leaf area (mm^2)
High proline	0	5.60±0.30	72.56±4.95
	171	112.67±12.87	89.70±11.21
	342	203.20±10.45	61.21±1.54
	513	213.31±13.07	54.87±2.17
	684	238.88±11.43	52.91±1.22
Modulate proline	0	7.09±1.76	97.17±14.15
	171	48.41±3.95	90.28±11.74
	342	78.32±9.46	82.67±8.97
	513	82.56±8.11	66.35±8.40
	684	84.29±11.19	64.12±6.50
Low proline	0	8.31±2.78	91.35±9.82
	171	19.61±5.85	70.35±14.98
	342	32.54±5.35	66.98±11.97
	513	34.94±6.46	53.34±5.75
	684	44.63±3.25	42.66±6.03

Errors of mean represented by ±SE

accumulation should be functioned as osmoprotective agent to prevent the green leaf area, leading to enhance on salt tolerant ability when compared to those low proline group. In case of salt exposure time, the proline in all rice cultivars grown under control condition (0 mM NaCl) was demonstrated as very low level in all culture periods. In contrast, the proline content in salt stressed seedlings (342 mM NaCl) was dramatically increased, especially in high accumulation class (HTH and HPL) (Table 2). The proline accumulation in salt stressed seedlings was positively correlated with exposure time in high ($r^2 = 0.91$), modulate ($r^2 = 0.96$) and low ($r^2 = 0.95$) groups (Fig. 6).

Thai aromatic rice including HJP, HPL and HTH were classified as high proline accumulation class (Fig. 1A), leading to maintain the green leaf area (Table 1). There are many reports to investigate on proline accumulation in rice cultivars such as Taichung Native 1 (Lin and Kao, 1996), Taipei-309 (Shah *et al.*, 2002), CR203, Cuom, DR2 (Hien *et al.*, 2003), Anapurna, Keowha (Hoai *et al.*, 2003), SR-26B (Basu *et al.*, 2002) as early response to salt stress in both cell culture and whole plant systems. In Taichung Native 1, the proline contents in the root tissues of rice seedling grown under 50, 100 and 150 mM NaCl for 5 days are continuously accumulated, while those in leaves only in 150 mM NaCl are enhanced (Lin and Kao, 1996). It is similar to proline accumulation in the Taipei-309 rice cultivar, relating to salt concentrations (200, 250 and 300 mM NaCl) (Shah *et al.*, 2002). In addition, the

Table 2: Proline concentrations in three classes, high, moderate and low proline accumulation of rice seedlings photoautotrophically grown under 0 or 342 mM sodium chloride condition for 0, 1, 2, 3 and 4 days

Rice cultivars	Exposure time (days)	Proline concentration (μmol g^{-1} DW) 0 mM NaCl	342 mM NaCl
High proline			
Homthong	0	1.86±0.20	1.86±0.20
	1	1.84±0.28	13.64±0.71
	2	1.95±0.21	34.16±1.69
	3	1.45±0.06	83.72±6.60
	4	1.96±0.28	174.81±16.30
Hompaepalo	0	4.78±1.61	4.78±1.61
	1	7.20±0.42	4.83±0.42
	2	6.09±0.41	48.64±2.75
	3	5.07±0.41	121.64±3.61
	4	4.08±0.48	185.09±12.63
Moderate proline			
Homjan	0	1.37±0.06	1.37±0.06
	1	1.67±0.19	15.98±2.31
	2	0.59±0.14	41.31±2.18
	3	1.28±0.17	67.06±1.68
	4	0.61±0.14	99.27±3.24
Pokkali	0	1.07±0.09	1.07±0.09
	1	1.80±0.19	4.30±0.08
	2	1.56±0.21	43.60±1.79
	3	1.80±0.14	77.49±2.03
	4	0.34±0.03	67.45±0.57
Low proline			
Hom	0	0.33±0.04	0.33±0.04
	1	0.72±0.05	6.86±0.24
	2	1.28±0.14	11.55±0.64
	3	0.96±0.05	29.49±2.92
	4	0.81±0.05	48.97±2.96
IR29	0	0.26±0.03	0.26±0.03
	1	1.21±0.13	13.67±0.40
	2	1.63±0.33	28.13±3.37
	3	1.05±0.05	69.88±3.31
	4	3.26±0.46	74.46±4.89

Errors of mean represented by ±SE

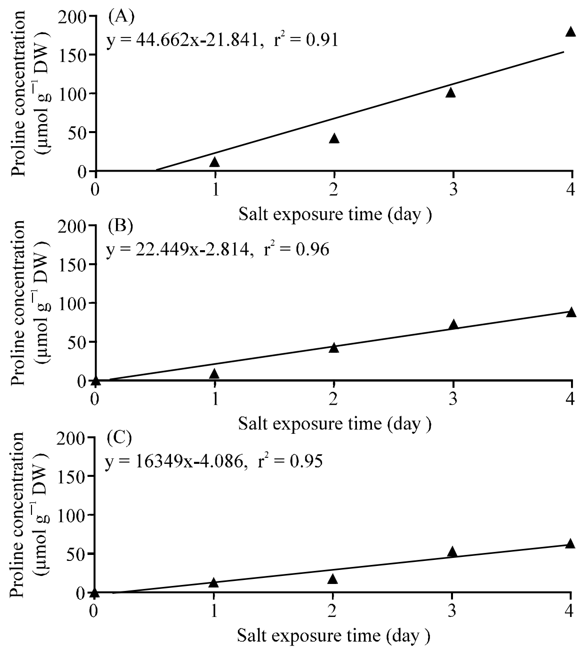

Fig. 6: Relationship between salt exposure time and proline accumulation a in three classes, high (Homthong and Hompaepalo) (A), moderate (Homjan and Pokkali) (B) and low proline accumulation (Hom and IR29) (C) of rice seedlings photoautotrophically grown under 342 mM sodium chloride condition for 0, 1, 2, 3 and 4 days

exposure time of salt stress is one of the most factors to stimulate on proline accumulation in rice (Lin and Kao, 1996; Hien *et al*., 2003), barley (Ueda *et al*., 2007), sesame (Koca *et al*., 2007), tomato (Santa-Cruz *et al*., 1999) and purslane (Yazici *et al*., 2007). In present study, the result indicated that IR29 (salt sensitivity), HP, Hom and HTR were defined as non-accumulative cultivars (Fig. 2C) and set as salt sensitivity, relating to the lowest green leaf percentage (<50%), especially in the condition of 684 mM NaCl. As well as, Pok cultivar is well known as salt tolerance, which is identified in a group of moderate proline accumulation (Fig. 2B). Similarly, proline contents in SR-26B salt tolerant rice grown under high salt stress (854.5 mM NaCl) are higher than those in Basmati 370 salt sensitive cultivar (Basu *et al*., 2002). Moreover, the proline concentrations in green gram cultivar T-44, foxtail millet cultivar Prasad and mulberry genotype S1 salt tolerance are expressed more than SML32 (Misra and Gupta, 2005), Lepakshi (Veeranagamallaiah *et al*., 2007) and ATP (Kumar *et al*., 2003) cultivars, respectively. In this case, the proline accumulation is functioned as osmolyte in salt-stressed plants to maintain the pigments, resulting in the green leaf maintenances when exposed to salt stress condition (Kumar *et al*., 2003; Misra and Gupta, 2005; Veeranagamallaiah *et al*., 2007; Wang *et al*., 2007). In contrast, the proline contents in Pok (salt tolerance) are unchanged, while those in IR28 are increased for 1.2 and 2.2 folds, respectively when exposed to 60 or 120 mol m^{-3} NaCl for 7 days (Demiral and Turkan, 2005). As well as, the proline contents in the flag leaf tissues of salt tolerant breeding lines, IR63295-AC209-7 and IR65192-4B-10-3, grown under control (0 dS m^{-1}) are accumulated higher than those of IR29 salt sensitivity for 2-3 folds. In salt stress treatment (6 dS m^{-1} NaCl), the proline contents in all rice lines are equally gathered for 22 μmol g^{-1} FW (Moradi and Ismail, 2007). In this issue, there are some confuses on the proline un-changes in sorghum salt-tolerant cultivars when exposed to salt stress condition. Whereas, the proline is more accumulate in a high level in salt sensitive varieties (De Lacerda *et al*., 2003, 2005).

CONCLUSION

Proline accumulation in Thai aromatic rice was depended on rice cultivars, salt concentrations and salt exposure time. The proline profiles in all rice cultivars responded to salt stress were identified the groups as high, moderate and low accumulation. The genetic resources of aromatic rice with enriched proline cultivars should be further investigated on genotypic expressions of proline biosynthesis pathway.

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