Effects of Salt Stress after Late Booting Stage on Yield and Antioxidant Capacity in Pigmented Rice Grains and Alleviation of the Salt-Induced Yield Reduction by Exogenous Spermidine

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Abstract: Pigmented rice is receiving much attention due to the large amounts of bioactive compounds and various health benefits. However, little information is available on its agronomic and physiological aspects. This research aimed to explore the effects of salinity on yield and grain quality of pigmented rice and the modulation of salinity responses by exogenous application of spermidine (Spd). Four cultivars of rice were grown in pots until the early booting stage. Thereafter, the plants were sprayed with 1 mM Spd for 7 successive days before being irrigated with 25 mM NaCl instead of tap water until maturity. Grain yield, yield components and harvest index in all rice cultivars were negatively affected by this salinity stress. Pretreatment with Spd dramatically improved yield and yield components of salt-treated plants, particularly the salt-sensitive cultivar. The major yield components which were improved and contributed most to the dramatic increase in seed yield were the number of filled grains per panicle and panicle fertility. Moreover, Spd pretreatment resulted in an increase in $\text{K}^+/\text{Na}^+$ ratio in rice grains. Salt stress increased nutritional quality of mature grains, i.e., total phenolic content, anthocyanins, proanthocyanins and antioxidant activities (evaluated by FRAP, DPPH and ABTS assays) in all rice cultivars. Furthermore, all aspects of health-promoting nutritional characters were further enhanced by Spd pretreatment. Thus foliar spraying of Spd to rice plants prior to salt application improved grain yield as well as nutritional quality of colored rice grains in relation to total phenolics, flavonoid pigments and antioxidant capacities.

Key words: Antioxidant activity, Pigmented rice, Salt stress, Spermidine.

Soil salinity is one of the most important abiotic stress factors affecting rice productivity in the arid and semi-arid regions of the world (Flowers, 2004). It is considered the most serious threat to agriculture and to the environment in many parts of the world (Parida and Das, 2005). Salinity stress causes a low soil water potential, accumulation of toxic sodium ions, nutritional imbalance and subsequently leads to an over-accumulation of reactive oxygen species (ROS) (Roychoudhury et al., 2008). Salinity results in growth reduction during the vegetative growth stage and yield reduction during the reproductive stage. Attempts to improve yield under stress conditions by genetic improvement have been difficult with limited success due to the complex polygenic nature of abiotic stress regulation. Recently, exogenous protectants such as osmoprotectants, plant hormones, antioxidants and polyamines have been found effective in mitigating the salt-induced damage in plants (Hamdia and Shaddad, 2010; Yusuf et al., 2012). Polyamines (PAs) are low molecular weight organic cations present in a wide range of organisms from bacteria to plants and animals (Bouchereau et al., 1999). The most common PAs are putrescine (Put), spermidine (Spd) and spermine (Spm) that are implicated in regulations of many physiological processes in plants (Alcázar et al., 2006). Mechanisms of action of PAs in protecting cells from abiotic stress damage have mainly been demonstrated during vegetative growth stages. Endogenous PAs which are induced by abiotic stresses play multifaceted roles in protecting plant cells against environmental stresses (Shahala et al., 2007; Farooq et al., 2009; Hussain et al., 2011). Synthesis of PAs during reproductive development has been demonstrated to be essential for normal seed development (Liang and Lur, 2002; Urano et al., 2005).
and their actions are antagonized by ethylene (Yang et al., 2006). Exogenous PAs have been shown to reduce salinity stress-induced damages in several plants including rice. Exogenous application of PAs either as seed priming, foliar spray or root soaking has proved beneficial for vegetative growth of rice under salt stress (Krishnamurthy and Bhagwat, 1989; Roychoudhury et al., 2011; Saleethong et al., 2011). During vegetative growth PAs have been shown to reduce the accumulation of Na⁺, alleviate the loss of K⁺, reduce ROS accumulation and enhance the activity of antioxidative enzymes and non-enzymatic antioxidants, alleviate chlorophyll loss and membrane leakage, and increase compatible osmolytes (Chattopadhayay et al., 2002; Roy et al., 2005; Ndayiragije and Lutts, 2006; Quinet et al., 2010; Roychoudhury et al., 2011; Saleethong et al., 2011). Exogenous application of PAs has also been reported to promote reproductive development and offers protection to reproductive structures leading to yield improvement under cold stress in chickpeas (Nayyar, 2005) and salt stress in rice (Ndayiragije and Lutts, 2007; Saleethong et al., 2013). However, the effects of exogenous PAs on the nutritional quality of harvested seeds were not addressed in previous studies. Relatively little information is available on the effects of exogenous PAs application on reproductive development and yield of rice exposed to salt stress (Ndayiragije and Lutts, 2007; Saleethong et al., 2013). Exogenous PA application has been suggested to be a convenient and effective approach for enhancing salinity tolerance of crops and eventually improving crop productivity under high salinity (Chattopadhayay et al., 2002; Verma and Mishra, 2005).

Red rice and black rice have become increasingly popular due to numerous health benefits because they contain large amounts of phenolic compounds, especially anthocyanins in pericarp (Abdel-Aal et al., 2006). However, little information exists on the effects of stresses on the production of beneficial pigments and antioxidant capacities of colored rice. The present study, therefore, aims to investigate the effects of salinity and exogenous Spd on yields as well as the grain quality in relation to antioxidant capacities of four cultivars of pigmented rice with different levels of salt tolerance. The information from the present investigation is expected to provide an insight into the mechanism of action and the potential application of PAs in modulating the reproductive development of rice under salt stress.

**Materials and Methods**

1. **Plant growth conditions**

   Seeds of three accessions of pigmented rice (*Oryza sativa* L.) were kindly provided by Faculty of Agriculture, Khon Kaen University and those of the cultivar Pokkali by Rice Gene Discovery Unit, Biotec, Thailand. Description of rice cultivars and their salt tolerance levels (Chunthaburee et al., 2012) are summarized in Table 1. The experiment was carried out in a net house (with walls of nylon net) during the months of June to November 2011. Plants were raised in black plastic pots (30 cm in height, 30 cm in diameter) containing 12 kg homogeneous mixture of 3:1 (v/v) soil and farmyard manure. Five seeds were planted in each pot (27 June 2011) and the plants were thinned to three per pot at 14 days after germination. The water level in each pot was maintained at 5 cm above the soil surface using tap water. At the early booting stage, the plants were divided into three groups, one control and two treatments including (1) NaCl and (2) NaCl + Spd. Six pots per treatment were arranged in a completely randomized design. For the NaCl + Spd group, the plants were treated with 1 mM Spd as a foliar spray between 1600 and 1700 for 7 successive days (28 September to 4 October 2011) at a volume of 50 mL per pot. The same amount of distilled water was sprayed in the control and the NaCl treatment groups. After the Spd treatment ended, salinity treatment was imposed as NaCl and NaCl + Spd treatments by adding 25 mM NaCl to the pots (5 October 2011 until harvest), while the control continued to be fed with tap water. The heading date of the plants was between 9 October and 11 October 2011. At maturity, the panicles were harvested and threshed manually. Yield and yield components were measured as number of panicles per plant, panicle length, number of filled grains per panicle, number of unfilled grains per panicle, 1000-grain weight, grain yield per plant and harvest index (%). The mean for each parameter was calculated from 6 repetitions, each was calculated as an average of three plants.

2. **Extraction procedure to determine the antioxidant properties**

   The method of extraction for determination of antioxidant properties of rice grains was adapted from
Sompong et al. (2011). De-hulled rice samples were finely ground and samples (1.5 g) were extracted with 85% aqueous ethanol for 30 min. The mixtures were centrifuged at 2,500 × g for 10 min and the supernatants were collected. The residues were re-extracted twice under the same conditions, resulting in a final volume of 50 mL crude extract and the supernatants were pooled. Extracts were used to determine total phenolic content (TPC) and antioxidant capacity and the measurements were replicated four times.

3. Determination of total phenolic content
TPC was assayed by Folin–Ciocalteu’s reagent method (Singleton et al., 1999). Briefly, 120 µL of extract was placed into test tubes and then 600 µL of freshly diluted 10-fold Folin–Ciocalteu’s reagent was added. After 2 min, 480 µL of sodium carbonate solution (75 g L⁻¹) was added. The mixtures were vigorously shaken and allowed to stand for 40 min. The absorbance of the resulting blue colour was measured at 760 nm. Gallic acid was used as standard and TPC was expressed as mg gallic acid equivalents (GAE) equivalent per g flour weight.

4. Determination of ferric reducing antioxidant power (FRAP)
The FRAP assay was adapted from Benzie and Strain (1999). Briefly, 200 µL of rice extract is mixed with 1.3 mL of the FRAP reagent. Absorption was measured at 595 nm using a spectrophotometer after 30 min incubation at 37°C. FRAP reagent consisting of 0.3 M acetate buffer (pH 3.6), 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) in 40 mM HCl and 20 mM FeCl₃ at a ratio of 10:1:1 (v/v/v) was freshly prepared as required. The FRAP value was expressed as milligrams of Trolox equivalents (TE) per g dry weight.

5. Determination of 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay
DPPH radical-scavenging ability of rice extracts was estimated according to the method of Brand-Williams et al. (1995). The reaction mixture contained 1.5 mL DPPH working solution (4.73 mg of DPPH in 100 mL ethanol HPLC-grade) and 100 µL rice extract. The mixture was shaken and incubated for 1 hr in the dark at room temperature. The absorbance was read at 515 nm relative to the control using a spectrophotometer. Trolox was used to develop standard curves. The percentage of inhibition was measured according to the equation:

\[
\% \text{ inhibition} = 100 \times \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}}
\]

6. Determination of 2,2′-azinobis (3-ethylbenzothiazoline 6-sulfonic acid) (ABTS) radical-scavenging assay
The ABTS radical cation scavenging assay was conducted by a modification of the method of Re et al. (1999). A stable stock solution of ABTS radicals was produced by adding potassium persulfate to a 7 mM aqueous solution of ABTS in the dark at room temperature and allowing to react for 12 – 16 hr before use. Rice extract (120 µL) was allowed to react with 1.5 mL of a diluted ABTS radical solution (absorbance of 0.70 ± 0.02 at 734 nm). The absorbance at 734 nm of the mixture was measured after 30 min reaction time. The percentage of inhibition was measured according to the equation:

\[
\% \text{ inhibition} = 100 \times \frac{(A_{\text{blank}} - A_{\text{sample}})}{A_{\text{blank}}}
\]

7. Total anthocyanins
Concentration of anthocyanins was determined according to the method of Abdel-Aal and Hucl (1999). Briefly, rice grains were finely ground with a mortar and pestle. Approximately 0.1 g of sample was soaked for 72 hr in 10 mL of acidified ethanol (ethanol and HCl 1.0 N, 85:15, v/v). The tube was centrifuged at 15,000 × g for 30 min. The suspension was filtered through Whatman No.1 filter paper (Sigma-Aldrich, St. Louis, MO, USA) and absorbance was measured at 535 nm.

8. Total proanthocyanidins
Rice grains were finely ground with a mortar and pestle. Rice flour (1 g) of each sample was extracted with 50 mL methanol for 24 hr followed by centrifugation at 15,000 × g for 15 min. The supernatant was collected for analysis. The total proanthocyanidins were measured using the modified vanillin assay described by Sun et al. (1998). In brief, the rice extract (0.5 mL) was mixed with 3 mL of 4% vanillin in methanol (w/v) solution and 1.5 mL hydrochloric acid. Absorption of the mixture was measured within 15 min at 500 nm. Total proanthocyanidins content was expressed as catechin equivalents (mg g⁻¹) using the equation based on the calibration curve.

9. Determination of the Na⁺ and K⁺ content
The grain husk was removed and the rice grains were ground with a mortar and pestle for analysis. Approximately 0.5 g powder of each dried sample was subjected to chemical analyses by digesting in 10 mL of nitric acid at 300°C, 5 mL perchloric acid at 200°C and 20 mL of 6 M hydrochloric acid at 200°C, for 30 min, respectively. The Na and K ions were measured using an atomic absorption spectrometer (Model GBC932AAA, England).

10. Statistical analysis of data
Analysis of variance was performed using SPSS 16.0 for Windows (SPSS, Inc., Chicago, IL). Significant difference between means among treatments was determined by Duncan’s multiple range test (DMRT). The p values smaller than 0.05 were considered as statistically significant.
Results

1. Yield and yield components

Salinity-induced changes in yield-related parameters of rice are given in Table 2. In general, rice exposed to salt stress from the late booting stage through to maturity showed a reduction in yield and all yield components. Panicle number per plant and panicle length decreased in all cultivars. Salinity caused a significant reduction in grain yield per plant in KKU-LLR-039 (−35%) and Niewdam Gs.no.00621 (−23%). Grain yield per plant for Pokkali and KKU-LLR-012 showed a reduction in yield and all yield components.

The pronounced effect of Spd was observed on the improvement of harvest index. The increase in harvest index was less severe in Pokkali and KKU-LLR-012. Treatment with Spd prior to salt stress showed a tendency to improve all yield-related characters. The number of panicles in all cultivars increased although the means were not significantly different. The length of panicles significantly increased from 27.97 cm in the salt-treated plants to 29.11 cm in the Spd-pretreated group in KKU-LLR-039 but was not significantly increased in KKU-LLR-012 or Niewdam Gs.no.00621. Grain yield per plant increased considerably in KKU-LLR-039 (+30%). The amount of increase in grain yield was less and non-significant in the remaining cultivars, i.e., +16%, +2% and +2% in Niewdam Gs.no.00621, KKU-LLR-012 and Pokkali, respectively. Spd treatment showed a tendency to increase the filled grain number in all cultivars, but the increase was significant only in KKU-LLR-039 (+22%). The 1000-grain weight and panicle fertility, on the other hand, were significantly increased in all cultivars by Spd treatment. The pronounced effect of Spd was observed on the improvement of harvest index. The increase in harvest index followed the same ranking as the increase in grain yield i.e. +33%, +14%, +8% and +8% for KKU-LLR-039, Niewdam Gs.no.00621, Pokkali and KKU-LLR-012 respectively.

The variations in yield and yield component were

| Cultivars | Treatment | Panicle number / plant | Panicle length (cm) | Filled grain number / panicle | Unfilled grain number / panicle | Total number of spikelet / panicle | 1000-grain weight (g) | Panicle fertility (%) | Grain yield / plant (g) | Harvest index (%) |
|-----------|-----------|------------------------|---------------------|-------------------------------|---------------------------------|-----------------------------------|----------------------|---------------------|----------------------|---------------------|
| Pokkali   | Control   | 7.67**                 | 30.02**             | 165.75**                      | 15.42**                         | 181.17**                         | 34.12**              | 91.41**             | 31.61**             | 60.28**             |
|           | NaCl      | 6.83**                 | 27.97**             | 149.92**                      | 24.92**                         | 174.85**                         | 33.54**              | 85.69**             | 27.60**             | 54.19**             |
|           | NaCl + Spd| 7.42**                 | 29.11**             | 155.42**                      | 21.67**                         | 177.08**                         | 33.94**              | 87.76**             | 29.29**             | 55.75**             |
|           | Control   | 9.89**                 | 27.03**             | 196.17**                      | 10.00a                          | 206.17**                         | 22.65**              | 95.15**             | 32.53**             | 59.30**             |
| KKU-LLR-012 | NaCl   | 8.67**                 | 25.88**             | 184.39**                      | 19.22de                         | 203.61**                         | 21.84**              | 90.66**             | 29.13**             | 53.27**             |
|           | NaCl + Spd| 9.28**                 | 26.58ab             | 191.61e                       | 14.33bc                         | 205.94c                          | 22.31b                | 93.23**             | 30.91**             | 57.40**             |
| Niewdam Gs.no.00621 | Control | 5.61ab                 | 28.16de             | 169.33cd                      | 11.33ab                         | 180.67b                          | 32.44f                | 93.708%            | 26.19f               | 51.44f              |
|           | NaCl      | 5.11a                  | 27.03bc             | 152.61bc                      | 21.06ef                         | 173.67b                          | 31.73e                | 87.78**             | 20.21ab              | 40.00b              |
|           | NaCl + Spd| 5.22a                  | 27.55cde            | 159.78bc                      | 16.50ed                         | 176.28c                          | 32.29f                | 90.46df             | 23.49bc              | 45.74**             |
|           | Control   | 6.60bcd                | 29.17f              | 166.27bc                      | 14.60bc                         | 180.87b                          | 25.89d                | 91.77def            | 26.06cd              | 52.63de             |
| KKU-LLR-039 | NaCl   | 5.73bc                 | 27.49cde            | 121.87a                       | 34.20bc                         | 156.07a                          | 24.82**               | 78.18a              | 17.05a               | 32.48a              |
|           | NaCl + Spd| 6.29bc                 | 28.37f              | 148.93b                       | 24.40f                          | 173.35b                          | 25.60d                | 85.84bc             | 22.31bc              | 43.04bc             |

| Cultivars (C) | Treatment (T) | Panicle number / plant | Panicle length (cm) | Filled grain number / panicle | Unfilled grain number / panicle | Total number of spikelet / panicle | 1000-grain weight (g) | Panicle fertility (%) | Grain yield / plant (g) | Harvest index (%) |
|---------------|----------------|------------------------|---------------------|-------------------------------|---------------------------------|-----------------------------------|----------------------|---------------------|----------------------|---------------------|
| Pokkali       | Control        | 155.7**                | 53.7*               | 40.0*                         | 951.0*                          | 12401.3*                         | 1414.0*               | 532.2*              | 902.5**             | 2334.5**            |
| Niewdam Gs.no.00621 | Treatment | 11.2*                  | 34.5*               | 7547.1*                       | 2213.0*                         | 1606.7ns                         | 10.0*                 | 854.1*              | 478.5*              | 2064.2*             |
| C × T         | 0.46 ns        | 0.76 ns                | 913.2 ns            | 100.4*                        | 429.6 ns                        | 0.18 ns                           | 69.0*                 | 25.6 ns             | 146.2*              | 13.28               |

*, significant at p < 0.05 level. ns, not significant. The values are the means of 6 replicates. Means with different letters are significantly different at p < 0.05 according to Duncan’s multiple range test.
number of spikelets per panicle. The interactions between cultivars and treatment (C × T) were significant for unfilled grain number per panicle, panicle fertility and harvest index, but not for the other traits were not significant.

2. TPC and antioxidant capacity

The amount of TPC (Fig. 1A) in the red-pericarp grains was 3- to 4-fold that in the black-pericarp grains. All cultivars tended to accumulate a larger amount of TPC by salinity treatment with further increase after Spd treatment. The increase of TPC by salt treatment was more pronounced in KKU-LLR-039 (39%) and Niewdam Gs. no.00621 (27%) which had black-pericarp than in Pokkali (12%) and KKU-LLR-012 (12%), which had red-pericarp. When salt stress was coupled with 1mM Spd treatment, the level of TPC increased significantly only in KKU-LLR-039 (17%) and KKU-LLR-012 (9%). However, the effect of Spd on enhancement of TPC was not significant in Pokkali (3.5%) or Niewdam Gs.no.00621 (11%).

The antioxidant activities of crude extracts from rice grains were determined by DPPH, ABTS and FRAP assays. Under salt stress, the FRAP values of all cultivars (except KKU-LLR-012) significantly increased as shown in Fig. 1B. The FRAP values in the black glutinous rice were 53% (KKU-LLR-039) and 40% (Niewdam Gs.no.00621) higher in salt-stressed conditions compared with the controls. On the other hand, the extent of salt-enhanced FRAP activity was much lower in the red rice (Pokkali; +15%; KKU-LLR-012; +13%). Similarly, after salinity stress DPPH radical scavenging activity significantly increased in all cultivars being more pronounced in the black than the red grains (Fig. 1C). The percentage increases were 14%, 11%, 8% and 7% in KKU-LLR-039, Niewdam Gs.no.00621, KKU-LLR-012 and Pokkali respectively. Salinity stress also increased the ABTS radical scavenging activity significantly in all cultivars (Fig. 1D), especially in KKU-LLR-039 which showed a 33% increase followed by Niewdam Gs.no.00621 (22%), Pokkali (13%) and KKU-LLR-012 (12%).

Exogenous Spd treatment increased the levels of FRAP in all cultivars but the effect was statistically significant only in KKU-LLR-039. Treatment with Spd before salt stress had no significant effects on DPPH activity in any cultivars. Spd tended to increase DPPH activity in the black rice but reduce it in the red rice. The ABTS activity showed similar patterns of response to Spd pretreatment, i.e., the activity further increased in the black rice but slightly decreased in the red rice. In addition, the extent of the increase in ABTS activities was most pronounced in KKU-LLR-039 (11%) followed by Niewdam Gs.no.00621 (8%).

3. Total anthocyanins and proanthocyanidins

Changes in anthocyanin content are presented in Fig. 2A. The black rice grains naturally contain more than 10

observed in Table 2. The main effect of cultivars (C) was highly significant and the interaction of treatments (T) was also significant for all yield parameters except the total

Fig. 1. Effects of salt stress without and with Spd pretreatment on total phenolic content (A) and antioxidant activities based on FRAP (B), DPPH (C) and ABTS (D) assays in mature grains of rice cvs. Pokkali, KKU-LLR-012, Niewdam Gs.no.00621 and KKU-LLR-039. Data are shown as means ± SD of four independent measurements. Values with different letters are significantly different at p < 0.05 according to Duncan’s multiple range test.
fold more anthocyanins than the red rice grains. Salinity stress tended to increase anthocyanin contents in all rice cultivars although not significantly except in KKU-LLR-039 (+13%). Spd treatment further increased anthocyanin content in all rice cultivars although not statistically significant. As shown in Fig. 2B, total proanthocyanidins markedly increased with salt stress in all cultivars. The most responsive cultivar was Pokkali (+36%) followed by Niewdam Gs.no.00621 (+31%), KKU-LLR-039 (+30%) and KKU-LLR-012 (+22%). Spd treatment significantly increased proanthocyanidin contents in the more tolerant cultivars (10 – 19%) but not in the sensitive KKU-LLR-039.

4. Ion concentration

Salinity stress reduced the K⁺ concentration in mature grains of all rice cultivars (Fig. 3A) and Spd application led to a non-significant increase. On the contrary, salinity increased the Na⁺ concentration (Fig. 3B). Spd pretreatment led to a slight non-significant reduction in all rice cultivars. The K⁺/Na⁺ ratio in mature grains decreased in all cultivars under salt stress. As shown in Fig. 3C, the K⁺/Na⁺ ratio was more affected by salt stress in KKU-LLR-039 than in other cultivars. In KKU-LLR-039 the ratio decreased from 4.41 in the control to 3.52 by salt stress (−20%) followed by −16%, −9% and −12% in Niewdam Gs.no.00621, KKU-LLR-012 and Pokkali, respectively. Pretreatment with Spd considerably raised the K⁺/Na⁺ ratio in all cultivars, especially in KKU-LLR-039 and Niewdam Gs.no.00621 where the ratios increased from 3.52 and 3.83, respectively, in the salt-treated plants to 3.85 and 4.17, respectively, in the Spd-pretreated plants (both +9%).

Discussion

The four rice cultivars showed different levels of sensitivity to salt stress. Lower percentages of reductions in yield and most yield components were noted in Pokkali and KKU-LLR-012 while KKU-LLR-039 showed the highest
The cultivars KUU-LLR-012 and Niewdan Gs.no.00621 which attained similar level of salt tolerance at the seedling stage in a previous study (Chunthaburee et al., 2012) displayed a wide difference in yield reduction; a non-significant 10% reduction in the former and a significant 23% reduction in the latter. Abdullah et al. (2005) also found that three rice inbred lines which showed similar levels of tolerance at the seedling stage (10 – 20% reduction in shoot growth) expressed a large difference in yield reduction (2 – 47%). However, Ali et al. (2004) reported that among ten rice cultivars tested salt tolerance scores based on visual symptoms at the seedling stage related well to grain yield under saline conditions. The observed varietal difference in salt-induced yield reduction hence depended on several factors including the intensity and duration of salt exposure as well as the developmental stage at which the plants were salinized (Asch and Wopereis, 2001; Zeng et al., 2001; Shereen et al., 2005).

In the present study, salt stress was initiated at the late booting stage and continued to maturity: therefore later formed yield components (filled grain number, unfilled grain number, 1000-grain weight and panicle fertility) were more severely affected than the earlier formed components (panicle length, tiller/panicle number per plant and total number of spikelets per panicle). However, an exception was noted in KUU-LLR-039 in which salt stress also negatively affected the total number of spikelets because reproductive development of this cultivar lagged behind the other cultivars for 3 – 4 days. This differential sensitivity to salt stress was most pronounced in the most sensitive cultivar KUU-LLR-039. The total number of spikelets was already fixed at panicle initiation (Hoshikawa, 1989) long before the commencement of salt stress and therefore was hardly affected by salinity whereas the panicle fertility and grain weight were more severely affected leading to high percentage increase in unfilled grain number and reduction in 1000-grain weight. Abdullah et al. (2001) reported multiple causes of sterility in seed set of rice under salinity including elevated Na+/K+ ratio in floral parts, reduction in pollen viability and stigma receptivity. Reduction in grain yield was also attributed to increased and decreased uptake of sodium and potassium ions respectively into rice panicles leading to reductions in K+ /Na+ ratio in mature grains (Fig. 3) which was most pronounced in the salt-sensitive KUU-LLR-039. The importance of ion imbalance in determining grain yield was previously illustrated that grain yield reduction was correlated with sodium concentration in developing panicles (Asch et al., 1999), Na+/K+ ratio in spikelets (Abdullah et al., 2001), young leaves at the late vegetative stages (Asch et al., 2000) and the shoots (Castillo et al., 2007). Reduction in grain weight could be due to limited soluble carbohydrate translocation from leaves to spikelets and inhibition of starch synthetase activity in developing rice grains (Abdullah et al., 2001). Final grain weight is also determined by grain filling rate which has been associated with ratio between ABA and ethylene in developing grains (Yang et al., 2006) and activity of sucrose synthase (Tang et al., 2009). In contrast to this study, when rice plants were exposed to salinity earlier in the vegetative stages, the reduction in tiller number per plant and total spikelet number per panicle were the major causes of yield loss (Zeng and Shannon, 2000; Zeng et al., 2001; Zeng et al., 2002). Similarly Asch and Wopereis (2001) concluded that the strongest salinity effects on yield were observed around panicle initiation and the most affected yield components were the total spikelet number per panicle.

The reversal of the inhibitory effects of salinity on yield by exogenous Spd was more pronounced in the sensitive cultivar than the more tolerant ones. Although Spd application significantly increased panicle fertility and 1000-grain weight in all four cultivars, significant improvement in grain yield per plant was observed only in KUU-LLR-039 (+31%). The large improvement in grain yield in KUU-LLR-039 resulted from significant increases in all later formed yield components including panicle length (3.23%), panicle fertility (+9.8%), 1000-grain weight (+3.13%) and harvest index (+32.51%). Similar results were obtained by Ndayiragije and Lutts (2007) who reported that the exogenous application of Put and Spd to salt-sensitive rice cv. I Kong Pao significantly improved yield in salt-treated plants. The beneficial effects of PAs were similarly related to an improvement in panicle number per plant and total number of spikelets per panicle. Put also had positive effects on seed set and 1000-grain weight but Spd did not. In that study, the number of panicles was improved because PAs were applied to the plants from early vegetative growth. The promotive effects of PAs on post-anthesis development of rice were demonstrated by Tan et al. (2009) who found that the grain filling rate and brown rice weight were highly correlated with endogenous Spd and Spm concentration in developing grains in six rice cultivars. Wang et al. (2012) also found that endogenous Spd and Spm concentrations in the developing panicles were positively correlated with the grain filling rate but negatively correlated with ethylene evolution rate. In developing maize grains, Cao et al. (2010) demonstrated that Spd concentration has much higher positive correlations with seed weight and quality than Put and Spm. Although the mechanism underlying salt stress alleviation by PAs has not yet been fully elucidated, several suggestions have been made including the reduced accumulation of toxic Na+ in leaves (Chattopadhayay et al., 2002; Quinet et al., 2010; Saleethong et al., 2011), shoots and roots (Ndayiragije and Lutts, 2007) and increased K+/Na+ ratio in grains (Saleethong et al., 2013). Shabala et al. (2007) suggested...
that PAs alleviated salinity effects by the reduction of Na\(^+\)/K\(^+\) uptake and K\(^+\) leakage from pea mesophyll cells by directly blocking the non-selective K\(^+\) and Na\(^+\) permeable cation channels. Evidence also pointed to an interplay between PAs and ROS generation and NO signaling in ABA-mediated stress responses leading to stomatal regulation and Na\(^+\)/K\(^+\) homeostasis (Alcázar et al., 2010).

The content of health promoting total phenolics in rice grains has been shown to be highly dependent on the bran color being several times higher in the red and purple than in the light brown (Goffman and Bergman, 2004) and white rice (Shen et al., 2009). Shen et al. (2009) and Sompong et al. (2011) found higher mean TPC in the black rice than in the red rice and very high within-color variations. However, the highest TPC value was found in the red variety (Shen et al., 2009). The red rice cultivars used in this study also contained higher TPC than the black ones. More importantly the antiradical efficiency showed a high positive correlation with TPC (Goffman and Bergman, 2004; Shen et al., 2009), suggesting that phenolics are the main compounds responsible for radical-scavenging activity. As expected, the red rice grains had higher antioxidant capacities as determined by the FRAP assay, ABTS radical cation scavenging activity and DPPH radical-scavenging ability. Although these assays detected the antioxidant capacity based on a similar mechanism, i.e., single electron transfer reactions (Prior et al., 2005), the FRAP assay was more responsive to Spd treatment than the others. Exogenous Spd may have stimulated the metabolic pathways leading to synthesis of certain groups of phenolic compounds responsible for the reducing ability in FRAP assays. This result confirmed earlier findings of greater antiradical ability in the red rice than in the black rice (Choi et al., 2007; Jun et al., 2012). Although the negative effects of salt stress on grain yield of rice have been widely reported, few studies have dealt with grain quality. To our knowledge, the present study demonstrated for the first time, the promotive effects of salinity on nutritional values of rice grains in terms of TPC and antioxidant activity.

The major types of flavonoids in seeds are flavonols, anthocyanins, phlobaphenes, isoflavones and proanthocyanidins (Lepimiec et al., 2006). Anthocyanins are primary pigments in the red and black rice grains (Abdel-Aal et al., 2006). Black rice had ten times higher anthocyanin contents than the red rice. On the contrary, the red rice had higher proanthocyanidin contents. Min et al. (2012) reported higher proanthocyanin content in red rice, and Furukawa et al. (2006) reported higher anthocyanin contents in black or purple rice. The anthocyanin and proanthocyanidin synthesis pathways share dihydroflavonols as precursor molecules. Cross talk between anthocyanin- and proanthocyanidin-specific branches existed where down-regulation of anthocyanidin reductase resulted in the accumulation of anthocyanins and inhibition of proanthocyanidin synthesis (Abrahams et al., 2002) while its overexpression led to down-regulation of anthocyanin and accumulation of proanthocyanidins (Xie et al., 2003). Regulation of flavonoid pathway branch is regulated by complex network of signals triggered by internal metabolic cues and external signals including abiotic stress factors (Abeynayake et al., 2012). Flavonoid pigments particularly anthocyanins and proanthocyanidins have been reported to be highly correlated with antioxidant capacities of colored rice grains (Finocchiaro et al., 2007; Min et al., 2012). Anthocyanin accumulation can be induced by biotic and abiotic stressors, such as UVB irradiation, drought, cold, toxic metals in soils and pathogens attack (Chalker-Scott, 1999). Salinity stress-induced accumulation of anthocyanins in leaves has been observed in several glycophytes. In rice, Roychoudhury et al. (2008) and Chutipajjit et al. (2011) reported that the level of anthocyanins in leaves of salt-tolerant rice was higher than in salt-sensitive rice under salinity stress. The enhancement of anthocyanins synthesis under salinity has been suggested to be important in the protection against salt-induced oxidative damages by binding the ions and thereby reduced toxicity on cytoplasmic structure (Wahid and Ghazanfar, 2006; Hichem et al., 2009). Furthermore, Roychoudhury et al. (2011) revealed that salinity-induced anthocyanin accumulation in seedlings of three rice cultivars was significantly enhanced by Spd application. Proanthocyanidins in the grains of all four rice cultivars were also significantly increased under salinity stress. It is interesting to note that the salt-enhanced increase in proanthocyanidins was more pronounced than that for anthocyanins. Moreover, the increase was greatest in the salt-tolerant red-grain cultivar (Pokkali). The enhanced level of proanthocyanidins under salt stress conditions has also been reported in seedlings of two maize varieties (Hichem et al., 2009) and salt-tolerant Acacia saligna (Elfeel and Backhashwain, 2012). Higher proanthocyanidins accumulation in the more salt-tolerant variety of maize was associated with greater antioxidant capacity (Hichem et al., 2009). The present study demonstrated for the first time in cereal grains that the concentration of anthocyanins was slightly increased and that of proanthocyanidins was significantly increased in response to salinity stress and that the accumulation of both pigments was further promoted significantly by exogenous Spd treatment. Although the mechanisms of action of Spd on modulation of flavonoid biosynthesis pathways leading to enhanced accumulation of anthocyanins and proanthocyanidins under salt stress is currently unknown, metabolic regulation by Spd has emerged as a promising approach relevant to practical application to improve rice grain nutritional qualities.

**Conclusions**

Salinity stress negatively affected yield and most yield
component parameters in four pigmented rice cultivars in a pot experiment and the extent of yield reduction was highest in the most sensitive cultivar. Foliar application of Spd prior to salt application resulted in an improvement in yield and yield components. Surprisingly, salt-stressed plants of all cultivars tended to produce grains with higher TPC, anthocyanins and proanthocyanidins resulting in enhanced antioxidant capacities. Exogenous Spd further increased all aforementioned health promoting characters. To our knowledge, this is the first report on the promotive effects of Spd on rice grain quality which warrants further in-depth research aimed at agricultural application as well as a better understanding of the physiological and molecular basis of Spd functions.

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