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Alleviation of salt stress effects on physiology of leaves in two cultivars of pigmented rice by application of spermidine

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Abstract

Treating plants with plant growth regulators to alleviate adverse effects of stresses such as drought and salinity has received growing attentions in recent years. In this study, the mitigation of salt stress effects induced by application of spermidine (Spd) in two indigenous pigmented rice cultivars, namely Niewdam Gs.no.00621 (salt-tolerant, having violet leaf color) and KKU-LLR-039 (salt-sensitive, having green leaf color) was investigated. Rice seedlings were grown in plastic pots filled with paddy soils mixed with farmyard manure (3:1) in a net-house under natural light conditions. Sixty days after planting the plants of each cultivar were divided into 3 groups: (1) control group; plants irrigated with tap water, (2) NaCl group; plants sprayed with distilled water for 7 days prior to being irrigated with 25 mM NaCl and (3) NaCl+Spd group; plants sprayed with 1 mM Spd for 7 days prior to being irrigated with 25 mM NaCl. The salt stress treatment was conducted for 20 days. Sampling of leaves was performed at 10-day intervals (days 0, 10 and 20) to determine changes in the physiological characteristics of the leaves. Salt stress triggered an increase in electrolyte leakage (EL), malondialdehyde (MDA), hydrogen peroxide (H_2O_2), proline, protein and Na^+ but caused a decrease in *Fv/Fm*, total chlorophyll and K^+/Na^+ ratio. Rice plants subjected to Spd pre-treatment showed, in comparison to the salt-stress treatment, a decrease in EL, MDA, H_2O_2, proline, protein and Na^+ but increase in *Fv/Fm*, total chlorophyll and K^+/Na^+ ratio. The alleviative effects of Spd were more prominent in the sensitive cultivar (KKU-LLR-039). Spd can improve salt tolerance of rice, particularly the salt-sensitive genotype, by enhancing several physiological mechanisms such as maintaining ion homeostasis, water balance and membrane integrity as well as protecting pigments and photosynthetic machinery.

Key words: Drought, Electrolyte leakage; Pigmented rice; Salt stress; Spermidine

1. Introduction

Saline soil is one of the serious abiotic stress factors negatively affecting development and productivity of food crops. High salt concentration in saline soils primarily causes water deficit which disrupts several physiological processes such as photosynthesis, respiration and carbohydrate metabolism [1, 2]. Moreover, accumulation of Na^+ and Cl^- ions in cytoplasm can cause an increase of reactive oxygen species (ROS) like superoxide anion (O^{2-}) and hydrogen peroxide (H_2O_2) which destroy the cell membrane, nucleic acids, proteins, lipid and photosynthetic pigments as well as disrupt the essential element uptake [3]. Salt stress also leads to an increase of malondialdehyde (MDA) which is a lipid peroxidation marker of abiotic stresses which readily damage cellular membranes [4]. In order to alleviate damages caused by oxidative stress, both antioxidative enzymes (superoxide dismutase: SOD, ascorbate peroxidase: APX, guaiacol peroxidase: POX) and non-enzymatic antioxidants including compatible solutes (proline, sugars, sugar alcohols and amines), phenolic compounds and anthocyanin are synthesized to reverse the inhibitory effects of salt stress [5, 6]. Polyamines

(PAs) act as plant growth regulators including spermine (Spm), putrescine (Put) and spermidine (Spd) known to influence several growth and development processes in plants [7]. PAs have also been documented to have multifaceted roles to alleviate negative effects of abiotic stresses such as drought [8] and salinity [9, 10]. When exposed to high salinity, plants accumulate increased amounts of PAs, which may contribute to increased stress tolerance [11]. Krishnamurthy and Bhagwat [12] reported that salt tolerant cultivars can effectively maintain higher concentration of Spd and Spm in the shoot system than salt sensitive cultivars. Previous reports indicated that PAs have important roles to achieve salt tolerance involving with numerous processes including detoxification, homeostasis and growth regulation [5, 9].

It has been demonstrated that treating plants with exogenous PAs is an effective alternative approach to improve productivity of salt-sensitive rice under salt stress [13]. PAs have been documented to alleviate chlorophyll loss and cell membrane leakage by inhibiting the accumulation of toxic ions, prevent the loss of K^+, decrease ROS through increasing production or activity of various antioxidative enzymes and non-enzymatic antioxidants. PAs have also been found to increase production of compatible solutes [6, 10, 14]. Exogenous application of PAs also led to yield increase as reported in salt-sensitive rice [13, 15]. Hence, increasing PAs levels in plants through exogenous Spd may help plants develop salt tolerance and increase yield of salt sensitive rice.

Rice (*Oryza sativa* L. spp. *indica*) is a glycophyte species sensitive to salt stress at both the seedling and reproductive stages [16]. Colored or pigmented rice is a type of cereal crop popularly consumed in Asia and Southeast Asia regions for a long time. Seeds of pigmented rice contain the important health-promoting anthocyanin pigment in different layers of the pericarp, seed coat and aleurone layer [17]. Moreover, pigmented rice grains have high nutritional advantages over white rice due to higher content of proteins, vitamins, minerals and polyphenols [18, 19]. Several reports showed that salt tolerance level of several rice cultivars could be enhanced by exogenous application of PAs for example in rice cultivars Nonabokra, Pokkali, M-1-48, IR8, I Kong Pao and KDML105 which are all white rice or the parental lines in breeding program [10 ,13]. However, very little is known about the beneficial effects of PAs in pigmented rice. Previous studies in our laboratory concluded that Spd can improve yield and yield components of salt-treated plants and nutritional quality of pigmented rice grains [15]. Nevertheless, role of exogenous Spd that induced physiological changes in leaves which actively supply photosynthate to developing grains leading to yield improvement of salt-stressed plants still remains unclear. Thus, the present study aims to investigate the effects of exogenous Spd on physiological responses of leaves in two cultivars of pigmented rice differing in salt tolerance level. This study may help to better understand the physiological mechanisms for Spd-mediated salt tolerance.

2. Materials and Methods

2.1 Plant materials and treatments

Seeds of two cultivars of black glutinous rice (*Oryza sativa* L. *indica*) including cv. Niewdam-Gs.no.00621 (salt-tolerant, having violet leaf color) and cv. KKU-LLR-039 (salt-sensitive, having green leaf color) were obtained from Department of Plant Science and Agricultural Resources, Faculty of Agriculture, Khon Kean University, Thailand. The experiments were carried out in the net-house at the Unit of Agronomy, Department of Plant Science and Agricultural Resources, Faculty of Agriculture, Khon Kean University, Thailand under natural illumination and temperature. Seeds were soaked in 5% sodium hypochlorite for 15 min and rinsed three times with distilled water, imbibed in distilled water for 6-8 h and placed on the moisten filter paper for three days. Five seedlings were transferred to plastic pots (30×30cm) containing 12 kg/pot. The soils are the homogeneous mixture of 3:1 (w/w) paddy soils and farmyard manure. Fourteen days after transplanting the plants were thinned to one per pot. The water level was maintained at approximately 5 cm above the soil surface and the chemical fertilizer (N:P:K; 15:15:15) was added (0.51 g/pot) at tillering stage. The pest and weed management was done following good practice of the Rice Department of Thailand. When the plants were 60-day-old, they were sprayed either with distilled water or with 1 mM Spd solution. The plants were sprayed until all leaves are thoroughly wetted (approximated 50 ml/pot). The treatment was given once a day between 1600-1700 pm for 7 successive days. Three treatments are set up as follows: (1) control group (plants sprayed with distilled water and irrigated normally with tap water), (2) salt stress group (plants sprayed with distilled water for 7 days then irrigated with 25 mM NaCl and (3) salt stress+Spd (plants sprayed with 1 mM Spd for 7 days then irrigated with 25 mM NaCl). The mature fully expanded leaf in each treatment was randomly harvested at 0, 10 and 20 days after salt-stress treatments to determine physiological parameters as described below. Soil salinity was monitored by measuring the electrical conductivity (ECe) of approximately 30 g of soils taken randomly from pots at the depth of 15 cm. The wet soil was dried at 120 °C for 3 days, homogenized and suspended in de-ionized water (5:1 v/w) and the EC was measured by portable EC meter (INDEX, Innovation Beyond 2000, USA, model ID1040). The mean EC values ranged from 1.13-1.99, 1.36-1.38 and 1.37-1.41 dS m^{-1} in control plant on days 0, 10, 20 after treatment, respectively. The means of EC in NaCl and NaCl+Spd

treatments ranged from 5.38-5.85 and 10.37-11.28 dS m^{-1} on days 10 and 20 days after treatment, respectively. The means of EC in within treatments groups did not differ significantly.

2.2 Determination of H_2O_2, malondialdehyde, electrolyte leakage and relative water content

The H_2O_2 content was determined following the method described by Velikova *et al*. [20]. Briefly, leaf sample (0.1 g) was homogenized using mortar and pestle with 1 ml of 0.1% (w/v) trichloroacetic acid (TCA) on ice and then centrifuged at 12,000×g for 15 min at 4 °C in a refrigerated centrifuge (Heraeus Sepatech refrigerated centrifuge Suprafuge 22. CA). The supernatant (0.5 ml) was added to 0.5 ml of 10 mM potassium phosphate buffer, pH 7.0 and finally 1 ml of 1 M potassium iodide. The absorbance was recorded at 390 nm and the amount of H_2O_2 was calculated from a standard curve. The malondialdehyde content (MDA) was determined by the thiobarbituric (TBA) reaction as described by modified procedure of Heath and Packer [21]. For each sample, 0.1 g leaf fresh weight was cut into smaller pieces and transferred to tube containing 1.5 ml of de-ionized water and added with 1.5 ml of 20% (w/v) of trichloroacetic acid (TCA) containing 0.5% (w/v) of TBA. The supernatant was centrifuged and heated at 100 °C for 15 min and then quickly cooled on ice. The samples were centrifuged at 5,000×g for 1 min and the absorbance of MDA was determined using spectrophotometer at 532 and 600 nm. The amount of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}. For electrolyte leakage (EL) measurement, about 0.1 g fresh leaf sample was cut into smaller pieces and then kept in de-ionized water in test tubes for 2 h at room temperature. After 2 h the EC was measured by a conductivity meter (E_1) and then the leaf samples were killed by autoclaving at 120 °C for 20 min. After the tubes were cooled to room temperature the final conductivity was measured (E_2) . The percentage of relative injury indicated by the electrolyte leakage rate was calculated following the method of Dionisio-Sese and Tobita [22]. The relative water content (RWC) was estimated according to the method given by Turner [23]. Approximately 0.1 g fresh leaf sample taken randomly from four plants in each treatment was cut into small pieces and quickly weighed to determine initial fresh weight (FW) and then leaf samples were floated in de-ionized water for 12 h to determine fully turgid weight (TW). The leaf sample was then oven-dried at 80°C for 3 days and to determine dry weight (DW). The relative water content (RWC) was determined using the following formula RWC = (FW-DW)/(TW-DW) × 100.

2.3 Determination of chlorophyll fluorescence, anthocyanin, total chlorophyll and total phenolic compounds

The maximum quantum yield of PSII photochemistry (*Fv/Fm*) was measured between 1600-1700 pm with chlorophyll fluorometer (Handy-PEA, Hansatech Instruments, UK) according to the manufacturer's instruction and modified procedure of Lutts *et al*. [24] and Moradi and Ismail [25]. Chlorophyll fluorescence was determined after a 30 min dark acclimation using a dark leaf clip. Anthocyanin content was extracted with acidified ethanol containing 95% ethanol and 1 N (M) hydrochloric acid (HCl) (85:15, v:v). Approximately 0.1 g of leaf sample was soaked for 48 h in the dark and then the absorbance of the solution was measured at 535 nm using acidified ethanol as blank. The anthocyanin content was calculated following the equation described by Abdel-Aal and Hucl [26]. The total chlorophyll was extracted with 80% acetone and the absorbance of the extract was measured at 645 and 663 nm using 80% acetone as blank. The equations reported by Arnon [27] were used for calculation. Total phenolic content was determined using the Folin-Ciocalteu assay according to Kähkönen *et al*. [28]. About 0.1 g was extracted with ethanol, samples (20 µl aliquot) were introduced into test tubes followed by 100 µl of Folin-Ciocalteu's reagent (10 times dilution) and 80 µl 7.5% (w/v) sodium carbonate and then allowed to stand for 30 min before the absorbance was determined at 765 nm. The total phenolic content was determined using gallic acid as standard.

2.4 Determination of proline content and total protein

Proline content was determined spectrophotometrically following the ninhydrin method described by modified procedure of Bates *et al*. [29] using pure proline as standard and toluene as the blank. Approximately about 0.1 g of fresh leaf was extracted with 5 ml of 3% aqueous sulfosalicylic acid. Two ml of extracted solution was reacted with 2 ml of acid ninhydrin and then 2 ml glacial acetic acid was added. The mixture was boiled in water bath at 100 °C for 1 h. The reaction was quickly stopped to 0 °C on ice. The reaction mixture was extracted with 4 ml of toluene and the absorbance was measured by spectrophotometer at 520 nm. Total protein was determined by the Bradford method [30]. Briefly, about 0.1 g leaf sample was homogenized in 1 ml of 10 mM potassium phosphate buffer, pH 7.0 containing 4% polyvinyl pyrrolidone (PVP) with liquid N_2. The homogenates were centrifuged at 12,000 × g at 4 °C for 15 min, then 20 µl of supernatant was mixed with 980 µl Bradford reagent (Bio-Rad, Richmond, CA) and the absorbance was monitored at 595 nm after 10 min incubation at room temperature. Protein concentration was calculated from a standard curve using bovine serum albumin.

2.5 Determination of Na^+ and K^+ ion content

To determine Na^+ and K^+, leaf sample was oven-dried at 80°C for 3 days. Approximately 0.1 g of each sample was subjected to chemical analyses by digesting in 10 ml of nitric acid (HNO_3) at 300 °C, 5 ml perchloric acid ($HClO_4$) at 200 °C and 2 ml of 6 M hydrochloric acid (HCl). The concentrations of Na^+ and K^+ were analyzed using a Flame photometer (Model M410 Sherwood) and calculated from a standard curve for each ion.

2.6 Experimental design and statistical analysis

The experiment was laid out as a completely randomized design with four replications. Data was analyzed by one-way ANOVA and Duncan's multiple range test (DMRT) was used for multiple mean comparisons ($p \leq 0.05$ was considered significantly different) by using SPSS version 16.0 (SPSS, Inc., Chicago, IL).

3. Results

3.1 Electrolyte leakage (EL), malondialdehyde (MDA), hydrogen peroxide (H_2O_2) and relative water content (RWC)

The percentage of EL from leaves of the control plants was stable during the 20 days of observation. However, salt stress for 10 days was causing the EL of KKU-LLR-039 to double (100% increase) in KKU-LLR-039 while that of Niewdam Gs.no.00621 it was increased by only 16% that of the control. In the plants pre-treated with exogenous Spd, EL increased by only 80% in KKU-LLR-039 and 10% in Niewdam Gs.no.00621. After 20 days of NaCl treatment, EL in the leaves was dramatically increased in KKU-LLR-039 (364%) and but much less increased in Niewdam Gs.no.00621 (184%). However in the NaCl+Spd groups the EL in KKU-LLR-039 and Niewdam Gs.no.00621 increased by only 248 and 145%, respectively. This result showed that Spd treatment reduced membrane damage in both cultivars especially in KKU-LLR-039 (Fig.1a).

The MDA content under the control condition was higher in KKU-LLR-039 (85.17 mmol g^{-1} FW) than in Niewdam Gs.no.00621 (65.56 mmol g^{-1} FW). Twenty-day exposure to salt stress did not have any effects on MDA content in the salt-tolerant Niewdam Gs.no.00621. However, when KKU-LLR-039 plants were exposed to salt stress significant increase in the MDA content was observed i.e. 9 and 18% increase after 10 and 20 days, respectively. Salt-stressed KKU-LLR-039 plants receiving Spd pre-treatment did not show an increase in MDA after 10 days and had a slight increase (5%) after 20 days. Interestingly, Spd treatment in Niewdam Gs.no.00621 resulted in a dramatic reduction in MDA content to the level lower than that of the controls. This result suggested that Spd could reduce lipid peroxidation during salt stress for both cultivars (Fig.1b).

Under control non-stress conditions the H_2O_2 level was higher in KKU-LLR-039 leaves than in Niewdam Gs.no.00621. Salt stress for 10 and 20 days triggered an increase in H_2O_2 production in both cultivars viz., 15 and 16% increase in Niewdam Gs.no.00621, and 14 and 7% increase in KKU-LLR-039, respectively. Interestingly, the application of Spd prior to salt stress maintained the H_2O_2 content near that of the control particularly in Niewdam Gs.no.00621 (Fig.1c).

The high values of RWC was observed in Niewdam Gs.no.00621 leaves of all treatments from the beginning to the end of experiment ranging from 81.99-89.01%, indicating that those leaves remained healthy. In contrast, RWC in KKU-LLR-039 leaves decreased from 86.42 to 74.34% and from 88.30 to 68.57% after 10 and 20 days of NaCl treatment, respectively. However, treatment with Spd before salt application significantly improved the RWC values in KKU-LLR-039 (Fig.1d).

3.2 Chlorophyll fluorescence, anthocyanin, total chlorophyll and total phenolic content

As shown in Fig. 2a, the *Fv/Fm* ratio (maximum quantum yield of PSII photochemistry) of the leaves in the control conditions was normal (more than 0.80) in both cultivars. However, after 10 and 20 days of salt stress the *Fv/Fm* ratios significantly reduced to 0.76 and 0.64 in Niewdam Gs.no.00621 and 0.71 and 0.63 in KKU-LLR-039. The exogenous Spd significantly reversed the declining of *Fv/Fm* ratio in both cultivars.

Total anthocyanin content was higher in Niewdam Gs.no.00621 (0.58 mg g^{-1}FW) than KKU-LLR-039 (0.33 mg g^{-1}FW) leaves. In Niewdam Gs.no.00621, anthocyanin was decreased by 14% after 10 days of salt stress but it was increased by 35% after 20 days whereas Spd supplement resulted in an increase in anthocyanin by 5 and 28% compared with the controls. In KKU-LLR-039, the anthocyanin content was slightly decreased for NaCl treatment at 10 days but dramatically increased (+21%) at 20 days. Supplement with Spd did not have significant effects on anthocyanin production in this cultivar (Fig.2b).

The total chlorophyll content in Niewdam Gs.no.00621 leaves under salt stress for 10 days was lower than that of the control but after 20 days it was slightly higher than the control. Treatment of Niewdam Gs.no.00621 with Spd did not have any beneficial effects on chlorophyll content. In contrast, salt stress significantly decreased chlorophyll content (54% reduction) in KKU-LLR-039 after 10 days of stress when compared with control while Spd effectively maintained the chlorophyll content under salt stress until 20 days after stress (Fig.2c).

Higher total phenolic content was observed in Niewdam Gs.no.00621. Surprisingly, at 10 days of NaCl and NaCl+Spd treatments significant increase in phenolic compounds was observed in Niewdam Gs.no.00621 but not in KKU-LLR-039. At 20 days, phenolic contents were not significantly different in all treatments of Niewdam Gs.no.00621 whereas in KKU-LLR-039 lower content was observed in the NaCl+Spd group (Fig.2d).

3.3 Proline and total protein content

As shown in Fig. 3a, neither NaCl nor Spd treatments had any effects on proline production of Niewdam Gs.no.00621. For KKU-LLR-039, proline content was significantly increased in NaCl (30%) and NaCl+Spd (19%) after 10 days while at 20 days it was intensely increased i.e. 351% and 49% increase in NaCl and NaCl+Spd treatment, respectively. This result indicates that proline accumulation in leaves was associated with prolonged salt stress and the exogenous Spd reduced proline buildup in the salt-sensitive cultivar.

The protein content was higher in KKU-LLR-039 than Niewdam Gs.no.00621 at the beginning. On day 10 after salt treatment, no significant changes due to NaCl or Spd were observed in Niewdam Gs.no.00621. However, in KKU-LLR-039, protein content was significantly increased (58%) when exposed with NaCl for 10 day, and but it remained unchanged for NaCl+Spd treatment compared with the control. The protein content of both cultivars at 20 days was much reduced and no effects of NaCl or NaCl+Spd were detected (Fig.3b).

3.4 Na^+ content (A), K^+ content (B) and K^+/Na^+ ratio

As in Fig.4a, the Na^+ content of rice leaves was higher in KKU-LLR-039 than in Niewdam Gs.no.00621. Salt stress caused a significant rise Na^+ in Niewdam Gs.no.00621 and a slight non-significant increase in KKU-LLR-039 after 10 days of treatments. Exogenous Spd decreased Na^+ content particularly in Niewdam Gs.no.00621. At 20 days, salt stress significantly increased the Na^+ predominantly in KKU-LLR-039. Interestingly, the application of Spd effectively inhibited the Na^+ accumulation in KKU-LLR-039 but not in Niewdam Gs.no.00621. For Niewdam Gs.no.00621 NaCl stress did not have any effects on K^+ level and exogenous Spd caused a slight increase. However, for KKU-LLR-039 treated with NaCl for 20 days K^+ level significantly decreased but then significantly increased by the action of exogenous Spd. Surprisingly, on day 20 exogenous Spd increased the K^+ content (compared with the controls) by 18% (Niewdam Gs.no.00621) and 5% (KKU-LLR-039) (Fig. 4b). However, both NaCl and NaCl combined with Spd had no effects on the K^+/Na^+ratio. The exception was seen in KKU-LLR-039 after 20 days of stress in which K^+/Na^+ ratio significantly decreased by NaCl stress but dramatically improved by pre-treatment with Spd (Fig. 4c).

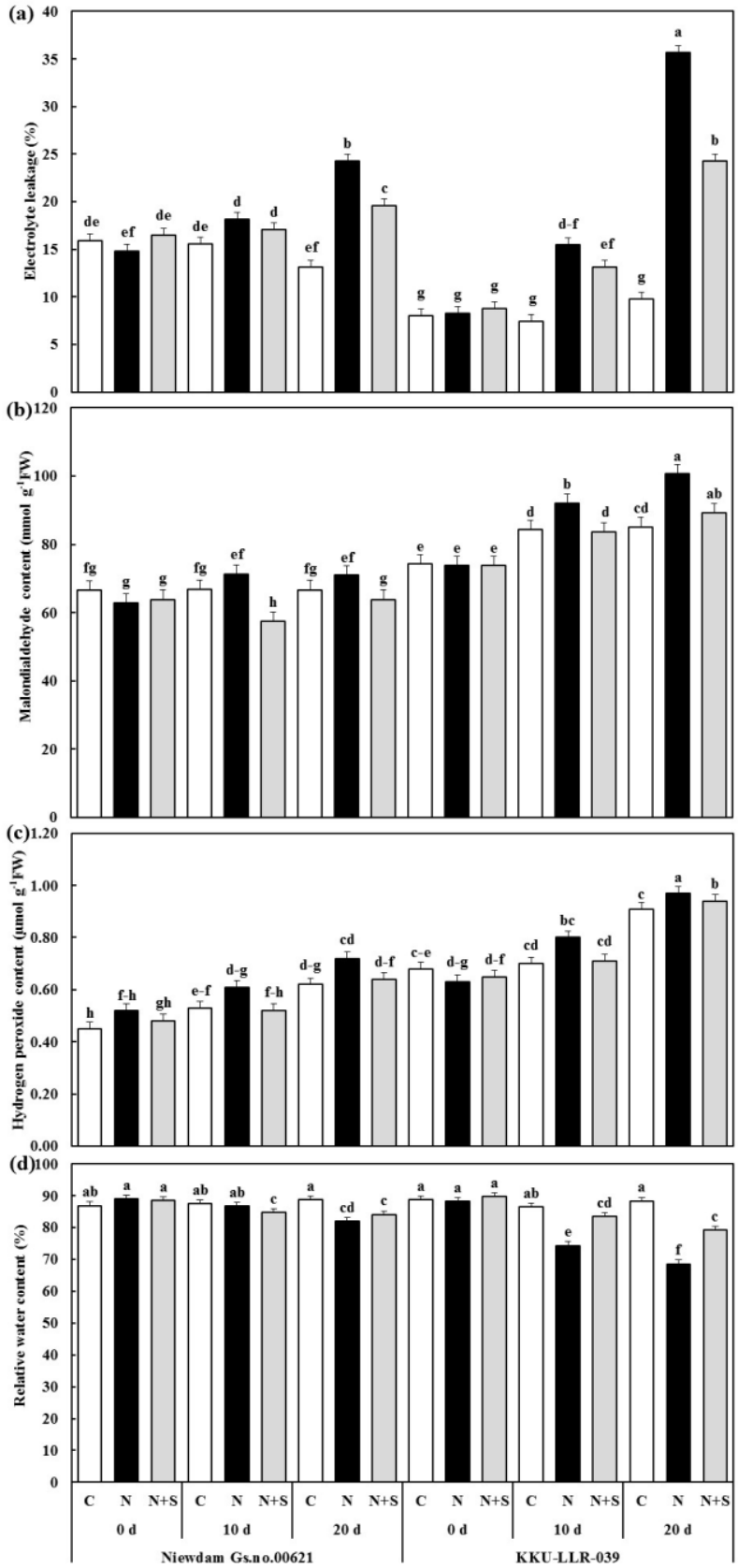

Figure 1 The electrolyte leakage (a), malondialdehyde content (b), hydrogen peroxide (c) and relative water content (d) in the leaves of two pigmented rice cultivars at 0, 10 and 20 days after treatment with NaCl. C, control; N, salt stress; N+S, salt stress plus 1 mM Spd. Values represent mean ± standard error (n=4); means with different letters are significantly different at ($p \leq 0.05$)

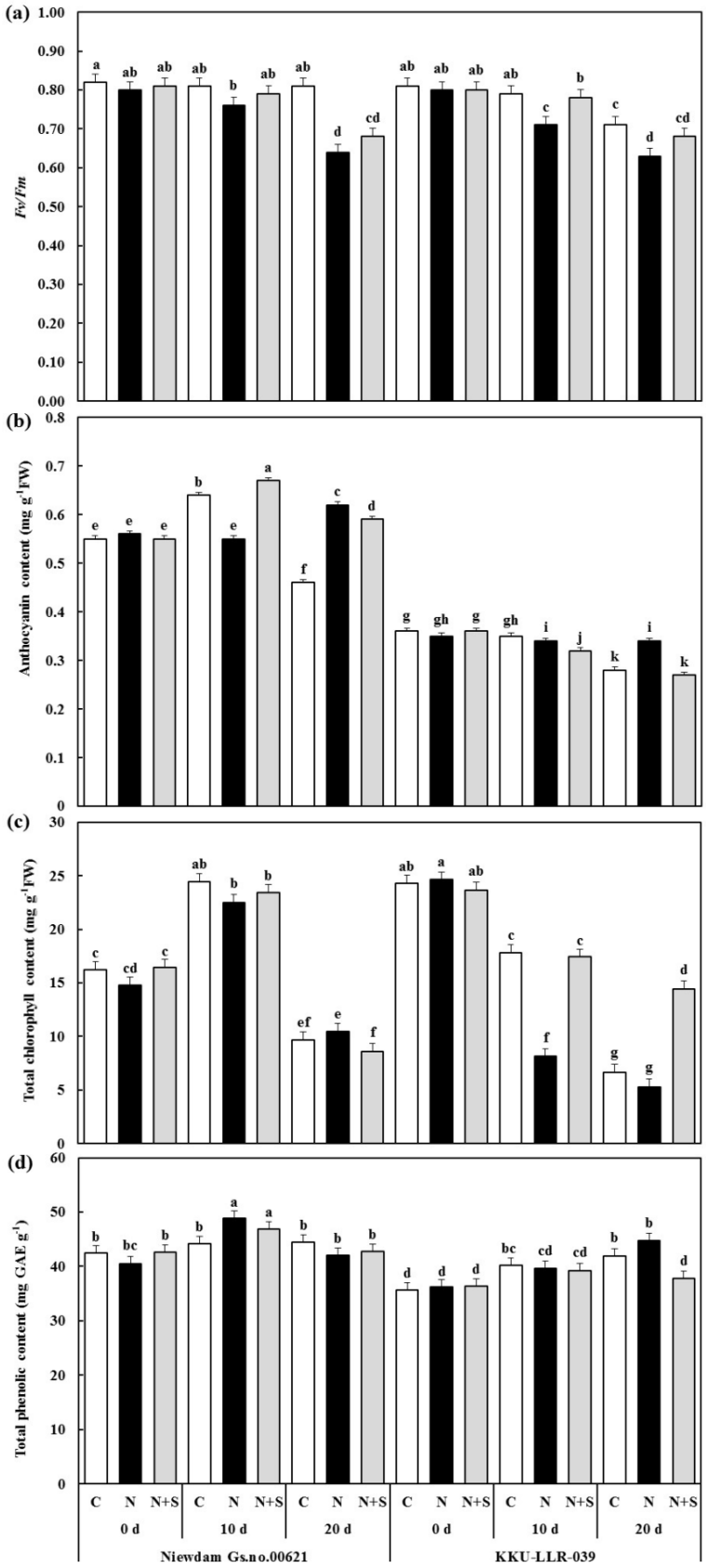

Figure 2 The F_v/F_m (a), anthocyanin content (b), total chlorophyll content (c) and total phenolic content (d) in the leaves of two pigmented rice cultivars at 0, 10 and 20 days after treatment with NaCl. C, control; N, salt stress; N+S, salt stress plus 1 mM Spd. Values represent mean ± standard error (n=4); means with different letters are significantly different at ($p \leq 0.05$)

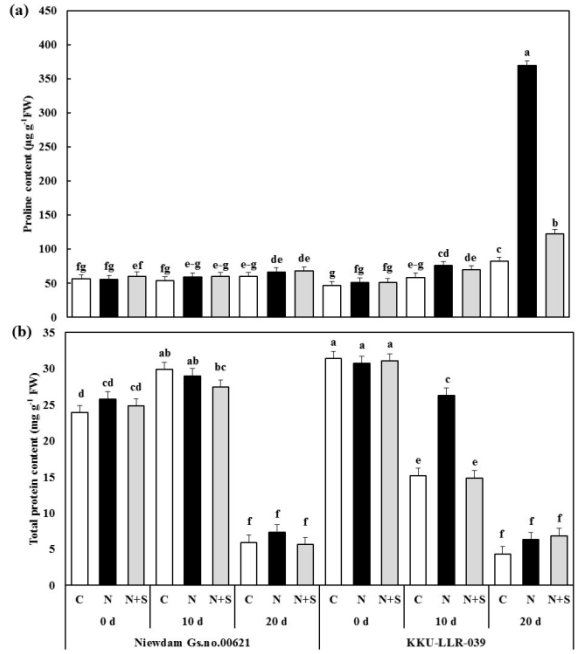

Figure 3 The proline content (a) and total protein content (b) in the leaves of two pigmented rice cultivars at 0, 10 and 20 days after treatment with NaCl. C, control; N, salt stress; N+S, salt stress plus 1 mM Spd. Values represent mean ± standard error (n=4); means with different letters are significantly different at ($p \leq 0.05$)

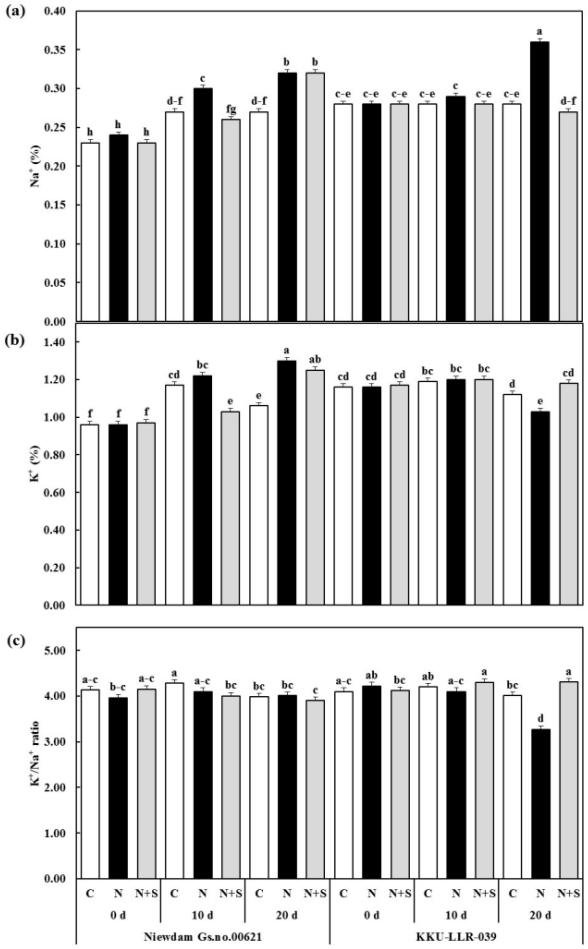

Figure 4 The Na^+ content (a), K^+ content (b) and K^+/Na^+ ratio (c) in the leaves of two pigmented rice cultivars at 0, 10 and 20 days after treatment with NaCl. C, control; N, salt stress; N+S, salt stress plus 1 mM Spd. Values represent mean ± standard error (n=4), means with different letters are significantly different at ($p \leq 0.05$)

4. Discussion

In the current study, salinity stress imposed by adding NaCl to the soils caused prominent disturbances in several physiological traits of rice leaves leading to cellular damage and loss of function, and exogenously applied Spd efficiently alleviated those damages. Salt stress causes an accumulation of toxic Na^+ and reduction in uptake of essential K^+ leading to lowered K^+/Na^+ ratio. Na^+ is toxic to cell membrane as well as causing the production of ROS while K^+ has various roles such as maintaining the osmotic balance, regulating opening and closing of stomata, phloem solute transport, and acting as co-factors for numerous enzymes involved the protein synthesis and photosynthesis [31].Thus, the plants with higher level of salt tolerance can maintain higher K^+/Na^+ ratio than those with lower tolerant level [32]. In this work, we found that the application of Spd led to increase of the K^+/Na^+ ratio (Fig. 4c) of salt-stressed plants particularly in the salt-sensitive KKU-LLR-039 because Spd inhibited the Na^+ accumulation and improved the K^+ content in leaves (Figs. 4a and b). This beneficial effect of Spd was similar to those reported by Saleethong *et al*. [10] that the alleviative effects of Spd on ion homeostasis was more prominent in the salt-sensitive cv. KDML105 than in the salt-tolerant Pokkali. In cucumber [33], Spd increased the K^+/Na^+ ratio in both salt-sensitive (Jinchun No.2) and salt-tolerant cultivar (Changchum Mici). Furthermore, Spd also improved the K^+/Na^+ ratio in roots and shoots [13] and rice grains [15, 34] under salt stress. It was suggested that Spd may act by reinforcing the Casparian bands in roots, resulting the K^+ and Na^+ accumulation which was highly detected in the exodermal intercellular space and cortical cells [35]. Exogenous Spd also increased the activity of the H^+-ATPase in plasma membrane and tonoplast as well as that of H^+-PPase and Na^+/H^+ transporter [36, 37]. Moreover, application Spd may induce the production of other PAs (putrescine and spermine) or itself may be present in the free, soluble conjugated and insoluble bound forms [38].

Modifications the cell membrane structure and mitigation of the ROS are important biochemical strategies of salt tolerance [6]. The electrolyte leakage is widely used as a test for the degree of cell membrane injury induced by salt stress [22]. Currently, numerous reports believe that maintenance ability of cell membranes or stability of phospholipids on plasma membrane under salt stress conditions is a valuable criterion for salt and drought tolerant in plants [39, 40]. Moreover, the electrolyte leakage may be correlated with those aspects causing oxidative stress such as molondialdehyde (MDA) which is a product of lipid peroxidation [41] and H_2O_2 [42] damaging the cell membrane structures when plants are exposed to salt stress [3, 4]. In this study, salt stress induced the electrolyte leakage (Fig. 1a), increased MDA (Fig.1b) and H_2O_2 (Fig. 1c) in leaves. More intense electrolyte leakage and higher concentrations of MDA and H_2O_2 occurred in KKU-LLR-039 than in Niewdam Gs.no.00621. These results confirmed the previous report by Chunthaburee *et al*. [43]. Exogenous Spd could alleviate those physiological aspects, particularly for the salt-sensitive KKU-LLR-039. These results are similar to that of Roychoudhury *et al*. [5] who reported that exogenous Spd/Spm can mitigate damaging effects of salts on MDA and H_2O_2 accumulation in sensitive rice varieties. Ndayiragije and Lutts [13] reported that exogenous application of Spd reduced MDA in shoots of a salt-sensitive rice (I Kong Pao) when exposed to 30 mM NaCl for 70 days. Duan and colleagues [38] reported that, in cucumber, exogenous Spd alleviated the cell membrane damage because it induced the endogenous PAs, antioxidant enzymes and osmotica which can effectively scavenge the free radicals and stabilizing the membranes. Moreover, PAs can bind to DNA, RNA, protein, cell wall and phospholipids group in cell membranes and maintain cellular pH and ion homeostasis, thus help maintain the stability and permeability of cell membrane [44].

The relative water content is an excellent characteristic used for evaluation of water status in drought- and salt-treated plants [45]. The water imbalance between root uptake and leaf transpiration would start when plants close the stomata to avoid water stress [46]. In the present study, salt stress decreased the RWC in only KKU-LLR-039, but not in Niewdam Gs.no.00621. Belkheiri and Mulas [47] suggested that *Atriplex halimus* clone which accumulated less Na^+ can better maintain plant water content under increasing NaCl salinity. Supplementation of Spd increased the RWC of both cultivars (Fig. 1d), indicating that Spd improved the water balance in leaves. Based on previous reports, PAs improved the water content by binding with aquaporins and help improve water uptake [46], protecting the xylem vessel elements [48] and inducing the compatible solutes and antioxidant enzymes for osmotic adjustment [14].

Chlorophylls and anthocyanins are the major pigments present in leaves of rice cultivars under study. Chlorophylls are the major photosynthetic pigments while the anthocyanin helps to prevent chlorophyll molecules from photooxidation, photoinhibition and stressful environments [49]. Generally, salt stress inhibited the chlorophyll synthesis due to degradation of chlorophyllase enzyme [50]. In the present study, salt stress decreased the chlorophyll pigments (only in KKU-LLR-039) while increased the anthocyanin content in both cultivars after 20 days of salt stress (Figs. 2b and c). Similar results were described in a previous study of Chanthaburee *et al*. [43] who found that the reduction of chlorophyll content was noted in salt-sensitive while anthocyanin accumulation was found in rice cultivars having violet leaf color. Chutipaijit *et al*. [51] concluded that salt-tolerant genotypes showed higher content of anthocyanin than salt-sensitive genotypes under salt stress. In this study, exogenous application was found to prevent the chlorophyll loss in KKU-LLR-039 whereas it further increased anthocyanin in Niewdam Gs.no.00621 particularly at 10 days after stress. The exogenous Spd

can protect the chlorophyll molecules through maintaining K^+/Na^+ homeostasis [52] and maintaining cellular ROS homeostasis [53].

The chlorophyll fluorescence parameters particularly the reduction in maximum quantum yield of PSII photochemistry (*Fv/Fm*) is used as selection criterion to study stress-induced damage in PSII [54]. The exogenous application of Spd improved the *Fv/Fm* ratio in leaves of both cultivars (Fig. 2a) in the present study. Similarly Shu *et al.* [55] noted that exogenous Spm increased *Fv/Fm* ratio in cucumber seedlings exposed to NaCl stress due to an increase in antioxidant enzymes and levels of antioxidants in the chloroplasts and a reduction in the aberrations in thylakoid membranes. Chattopadhayay *et al.* [14] reported that exogenous Spm and Spd induced the PSII activity and prevented the chlorophyll loss in salt-stressed rice. PAs can bind the light-harvesting complex (LHC) and D1 and D2 protein and protect the PSII from stress-induced damage [56].

The non-enzymatic antioxidant, phenolic compounds, is another defense mechanism to destroy ROS [57]. In our study, the total phenolic content increased in Niewdam Gs.no.00621 after 10 days of salt stress and in KKU-LLR-039 after 20 days while significant decrease was found in KKU-LLR-039 after 20 days of stress in Spd+NaCl group when compared with control (Fig. 2d). Many reports showed that the increase of phenolic content under salt stress may play a role as ROS scavengers [58]. In contrast, Yuan *et al.* [59] found that total phenolic content in radish sprouts declined after treated with 10 and 50 mM of NaCl for 5 and 7 days. It was reported that exogenous Spd maintained phenolic compounds in banana peels under low temperature storage by inhibiting the activity of polyphenol oxidase and peroxidase [60]. However, the information on roles of Spd on phenolic compounds under salt stress is still lacking and inconclusive.

The accumulation of osmoprotectants i.e., proteins, sugars and amino acid is another mechanism to protect damaging effects from salt stress [61]. Among the osmoprotectants investigated in this study, changes in proline and protein content was prominent only in KKU-LLR-039 after 20 and 10 days, respectively after salt stress (Figs. 3a and b). Many authors suggested that proline accumulation was positively related to the degree of salt tolerance [62]. However, Yamamoto *et al.* [63] reported that the accumulation of proline was more noted in salt-sensitive cultivar NERICA2 than in salt-tolerant cultivar NERICA1. Some authors suggested that over-accumulation of proline is a symptom of salt stress injury in salt-sensitive genotypes rather than an indicator of salt resistance [64]. Over-accumulation of proline in KKU-LLR-039 after 20 days of salt stress (Fig. 3a) co-inciding with dramatic reduction of total protein (Fig. 3b) could indicate salt-induced perturbation of nitrogen metabolism. The reduction in free proline by Spd supplement (Fig. 3a) could be related to its action on inhibition of protein degradation [65].

5. Conclusion

Spd can enhance the salt tolerance of pigmented rice, particularly the salt-sensitive cultivar, through multi-mechanisms such as improving the K^+/Na^+ ratio, maintaining water status and preventing the chlorophyll loss in leaves. Moreover, Spd can help maintain cell membrane integrity and mitigated the production and action of ROS.

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7. References

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