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DIFFERENTIAL ACCUMULATIONS OF PROLINE AND FLAVONOIDS IN INDICA RICE VARIETIES AGAINST SALINITY

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Abstract

Four varieties of *indica* rice differing in salt sensitivity were used for comparative study of defense systems in response to salinity. Rice seedlings were transferred into the photoautotrophic system containing NB medium with 100 mM NaCl as a salt stress treatment for 4 days. The physiological responses of jasmine rice (KDML105) and Sangyod (SY) varieties showed the better tolerance to salinity than those of Pathumthani 1 (PT1) and Black Sticky (BS) varieties. In salt stress treatment, the relative water content (RWC) of rice seedlings was decreased when compared to untreated seedlings. The RWC of salt-tolerant seedlings (KDML105 and SY) were decreased (0.33-0.53%) less than those of salt-sensitive seedlings (PT1 and BS), which exhibited 1.18-2.63% reductions. The salt-tolerant KDML105 and SY showed the lesser extent of membrane damage (lipid peroxidation; 7.94-19.26%) while enhanced the proline level (56.56-78.56%) and the flavonoid level (1.72-3.48%) more than those of the sensitive PT1 and BS varieties. Moreover, the positive correlation between proline or flavonoid accumulations and RWC was observed whereas the correlation between proline or flavonoid accumulations and MDA content was negative. These results suggest that high proline and flavonoids levels accumulated in each varieties of rice might be closely related to tolerant abilities indicating by physiological performances.

Introduction

Salinity is a harsh environmental factor that has the major effect on plant quantity and quality (Zhu, 2002). In order to survive in salt stress condition, plants develop the network responses of physiological and biochemical defense mechanisms to protect themselves against stress. Salt stress composes of ion toxicity and water stress. Water stress is the evident effects of salinity; therefore the determination of water contents in plants is critical for the study of plant tolerant efficiency (Parida & Das, 2005; Qasim & Ashraf, 2006). Under stress conditions, plant cells have the ability to prevent water loss and to maintain the continuous growth. Plants commonly react to these stresses by accumulation of compatible solutes, such as proline, in cells which results in the improvement of environmental stress tolerance (Hong *et al.*, 2000; Ramajulu & Sudhakar, 2001; Ashraf & Foolad, 2007). These solutes can be accumulated in high concentrations without impairing plant metabolisms. Over accumulation of these osmolytes may help plants to tolerate against stress by improving their ability to maintain osmotic balance within the cell (Hasegawa *et al.*, 2000; Apse & Blumwald, 2002). The maintenance of turgor by osmotic adjustment is an importance of physiological adaptations for minimizing the detrimental effects of salt stress (Munns, 2002).

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In many plant species, free proline accumulates in response to a wide range of stresses, such as drought and salinity (Nanjo *et al.*, 1999; Jain *et al.*, 2001). However, the relationship between the proline accumulation and stress resistance is much argument. The high level of proline may be protecting plants from stress conditions (Kumar *et al.*, 2003). In spite of many studies in plants, the roles of proline in osmotolerance in plants remain controversialism and there are few concrete demonstrations of the proline functions and action mechanisms through out plant growth (Fedina *et al.*, 2002).

In addition, the high level of Na^+ also causes the secondary responses in plants; consequently the oxidative stress is occurred leading to cellular damages in the plant cells (Apel & Hirt, 2004). This stress can induce production of reactive oxygen species (ROSs) (Jaleel *et al.*, 2007; Ashraf, 2009). ROSs derived from molecular oxygen can accumulate in the plant cell and cause oxidative damages in cellular components, including proteins, chlorophylls and lipids by lipid peroxidation. Reaction of lipid peroxidation can degrade polyunsaturated fatty acids in cell membranes, which impart the membrane functions. Therefore this system is easily approved to oxidative damage and also commonly measured (Lin & Kao, 2000).

To prevent the potential cytotoxic effects of ROSs, the stimulation of antioxidant systems can assist in plant protection from oxidative stress. Plants have developed antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase, glutathione reductase, catalase, peroxidase and non-enzymatic scavengers like glutathione, ascorbic acid, carotenoids and flavonoids which regularly maintain ROS balances within the cell (Vranova *et al.*, 2002; Dalmia & Sawhney, 2004). Flavonoids are reported as antioxidant agents by scavenging ROSs, which are functioned by virtue of the number and arrangement of their hydroxyl groups attaches to ring structures. Their ability to act as antioxidants depends on the reduction potentials of their radicals and accessibility of the radicals (Rice-Evans, 2001; Heim *et al.*, 2002). A correlation between the antioxidant enzyme activities and salt tolerance has been demonstrated in several plant species (Dionisio-Sese & Tobita, 1998; Benavides *et al.*, 2000; Ashraf & Harris, 2004). The objective of this research was to investigate the possible salt defense mechanism using proline and flavonoids to maintain the relative water content (RWC) and lipid peroxidation in *indica* rice seedlings (*Oryza sativa* L. spp. *indica*).

Materials and Methods

Plant materials and salt stress treatments: Seeds from four varieties of rice [*Oryza sativa* L. spp. *indica* cv. jasmine rice (KDML105) and Sangyod (SY)-salt tolerant varieties, Pathumthani 1 (PT1) and Black Sticky (BS)-salt sensitive varieties] (Sompornpailin *et al.,* 2007) were obtained from the Pathumthani and Sakolnakorn Rice Research Center (Rice Research Institute, Department of Agriculture, Ministry of Agriculture and Coorperative, Thailand). Seeds were hand-dehusked, sterilized by 70% ethanol, once by 5% Clorox® (5.25% sodium hypochlorite, The Clorox Co., USA) for 40 min, once by 30% Clorox® for 30 min., and washed extensively with distilled water. Surface-disinfected seeds were germinated on NB medium (Li *et al.*, 1993) containing 3% (w/v) sucrose and 0.8% (w/v) agar and grown under 25±5°C air temperature, 85-90% relative humidity (RH) and 60±5 μmol m^{-2} s^{-1} photosynthetic photon flux (PPF) with 16 h d^{-1} photoperiod. Seven-day-old rice seedlings were aseptically transferred to NB-liquid medium under the photoautotrophic system (CO_2 as a carbon source) using vermiculite as a supporting material. The number of air-exchanges of the photoautotrophic system was adjusted to 2.32 h^{-1} by punching a hole on plastic cap (Ø 1 cm) and covering the hole with a microporus filter (0.20 μm in pore size) (Cha-um & Kirdmanee, 2008). After 7 days of growth under this system, the NaCl concentration of the medium was adjusted to 100 mM NaCl or without NaCl (control). After 4 days, rice seedlings were harvested, frozen in liquid nitrogen and stored at -80°C prior to analysis.

Relative water content: Relative water content (RWC) as percentage of fresh weight was calculated using the formula following Sumithra *et al.* (2006) method:

$$\text{RWC } (\%) = [(\text{FW-DW}) \times 100] / \text{FW}$$

Lipid peroxidation: The levels of lipid peroxidation in rice seedling sample were determined in terms of malondialdehyde (MDA) content according to the method of Hodges *et al*., (1999).

Proline content: Free proline content was determined according to Gilmour *et al*., (2000). Seedling samples from each variety was homogenized in 3% (w/v) Sulphosalicylic acid 1 mL at room temperature and then stored at 4°C over night. The supernatant was added with acid ninhydrin and glacial acetic acid. The mixture was heated at 100°C for 45 min in a water bath. Reaction was then stopped by using an ice bath. The mixtures were extracted with toluene and measured using an UV-visible spectrophotometer (Thermo Electron, Model Bio Mate 3, Massachusetts, USA) at wavelength 519 nm. Proline concentration was determined using calibration curve and expressed as mg proline g^{-1} FW.

Total flavonoid levels: Total flavonoid contents were determined according to Harborne (1998). Rice seedlings were ground in liquid nitrogen and extracted with 1% (w/v) HCl in methanol for 2 h at room temperature and subsequently added 1 mL chloroform. The supernatant was collected and assayed by an UV-visible spectrophotometer at wavelength 300 nm for total flavonoids.

Statistical analysis: The experiment was arranged as a completely randomized design (CRD) with five replicates (n=5). The mean values were compared by *t*-test and analyzed by SPSS software (SPSS for Windows version 15, SPSS Inc., Chicago, USA).

Results and Discussion

In this experiment, the loss of turgor, indicated by a considerable decrease in RWC occurred in all seedlings treated with 100 mM NaCl for 4 days. Varieties of rice seedlings exposed to salinity revealed the difference in RWC (Fig. 1). The reduction of RWC was 0.28 and 0.45% in KDML105 and SY (salt-tolerant varieties), respectively lower than those in BS and PT1 (salt-sensitive varieties; 1.02 and 2.27%, respectively) when compared to their controls. Salt-stressed seedlings showed turgor loss in salt-sensitive varieties more than salt-tolerant varieties, indicated by a considerable decrease in RWC. Under salt condition, although these seedlings showed a slight decrease in water potential the salt-tolerant seedlings could maintain normal growth performance due to their osmotic adjustment. The reduction of RWC in the stressed-plant resulted from high salt concentration in the external media, which caused osmotic potential and dehydration at cellular level (Liu & van Staden, 2001; Stepien & Klobus, 2006). Therefore plant must have osmotic adjustment inside the cell, since turgor maintenance is required for cell expansion and the biochemical, physiological and developmental processes (Kameli & Losel, 1993; Flowers, 2004).

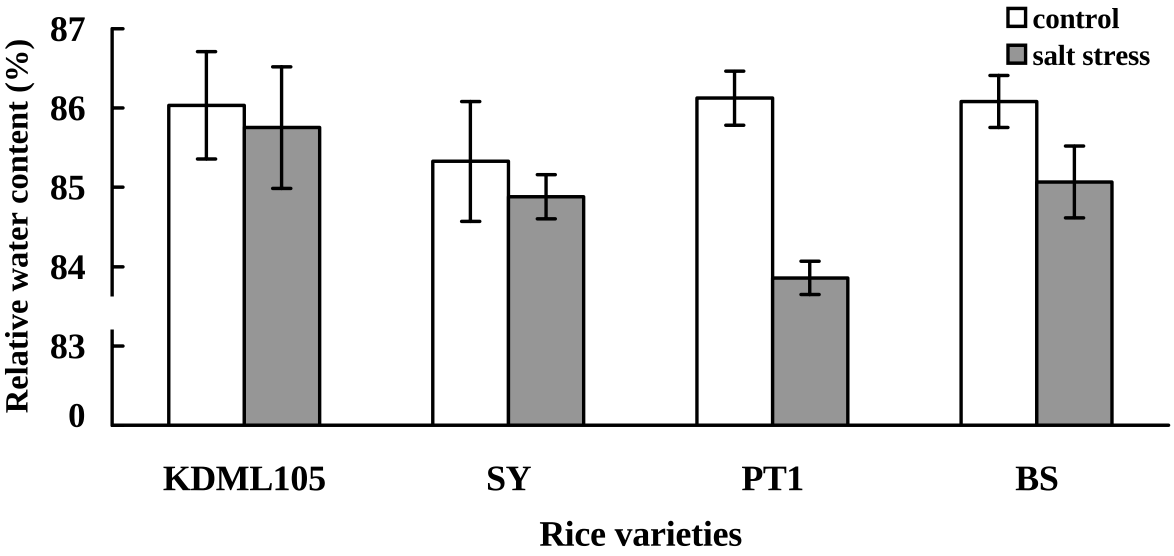

Fig. 1. Relative water content (RWC) of rice seedlings exposed to 100 m*M* NaCl in NB media for 4 days. Error bars are represented by ± SE (n=5).

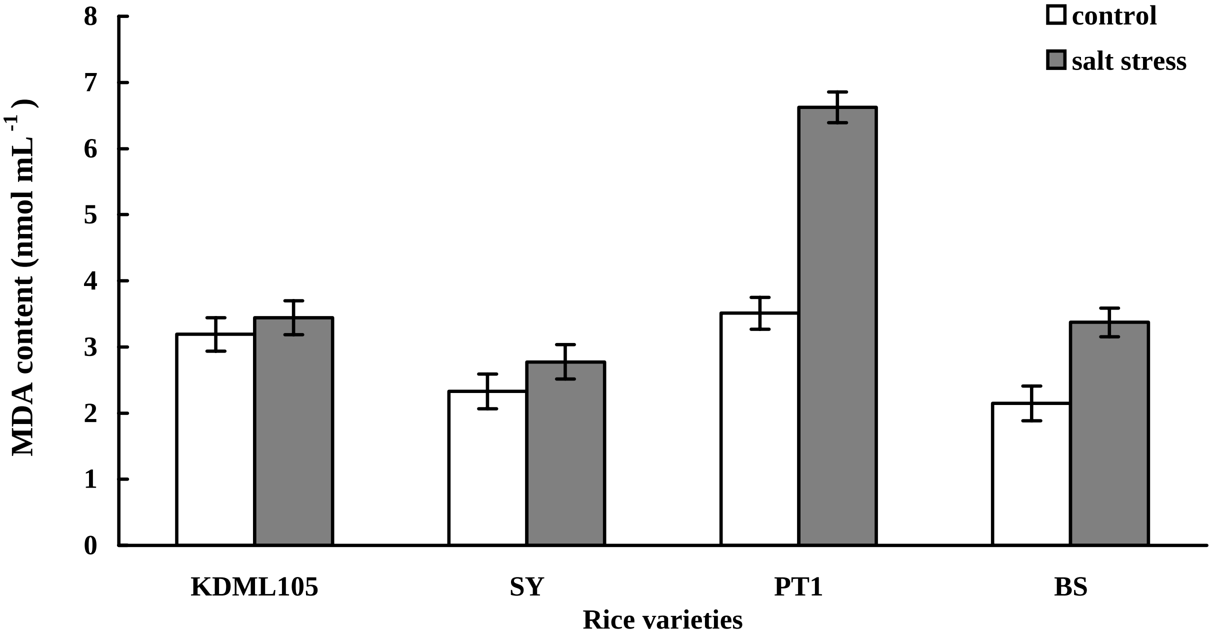

Fig. 2. Malondialdehyde content in rice seedlings subjected to 0 or 100 mM NaCl grown under photoautotrophic condition for 4 days. Error bars are represented by ± SE (n=5).

Salinity induced increase in lipid peroxidation and ROSs accumulation has been reported earlier in various plant species including rice especially in sensitive varieties (Dionisio-Sese & Tobita, 1998; Hernandez *et al*., 2000; Blokhina *et al.,* 2003). Massive ROSs generated during stress conditions may have the deleterious effects in plant tissue by the peroxidation reaction of membrane lipids (Sairam *et al*., 1998; Jain *et al*., 2001; Ratnayaka *et al*., 2003). MDA, produced by lipid peroxidation of cell membrane, is often used as an indicator of salt and oxidative damages (Mandhania *et al*., 2006). MDA concentrations of salt-stressed seedlings in all varieties were increased when compared to that in the control treatment (Fig. 2). KDML105 and SY had significantly less level of MDA (7.94 and 19.26%, respectively) than those of PT1 and BS (88.71 and 57.02%, respectively). The results indicated that KDML105 and SY seedlings were better efficiency to endure the damage of cellular membranes than PT1 and BS seedlings.

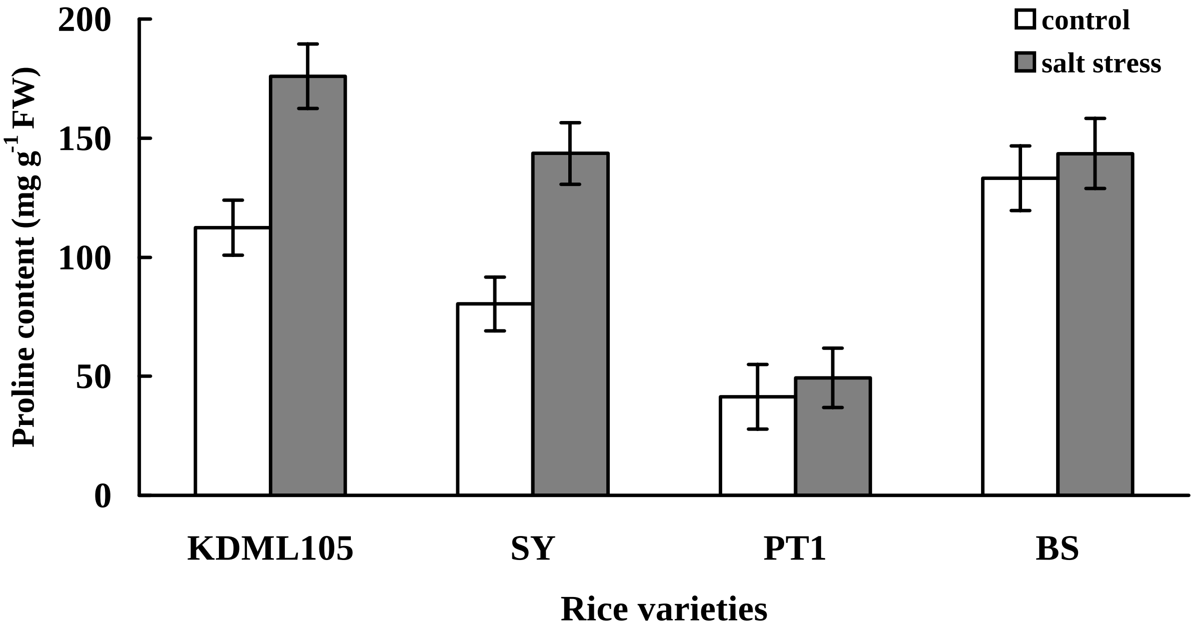

Fig. 3. Proline content in rice seedlings subjected to 0 or 100 mM NaCl grown under photoautotrophic condition for 4 days. Error bars are represented by ± SE (n=5).

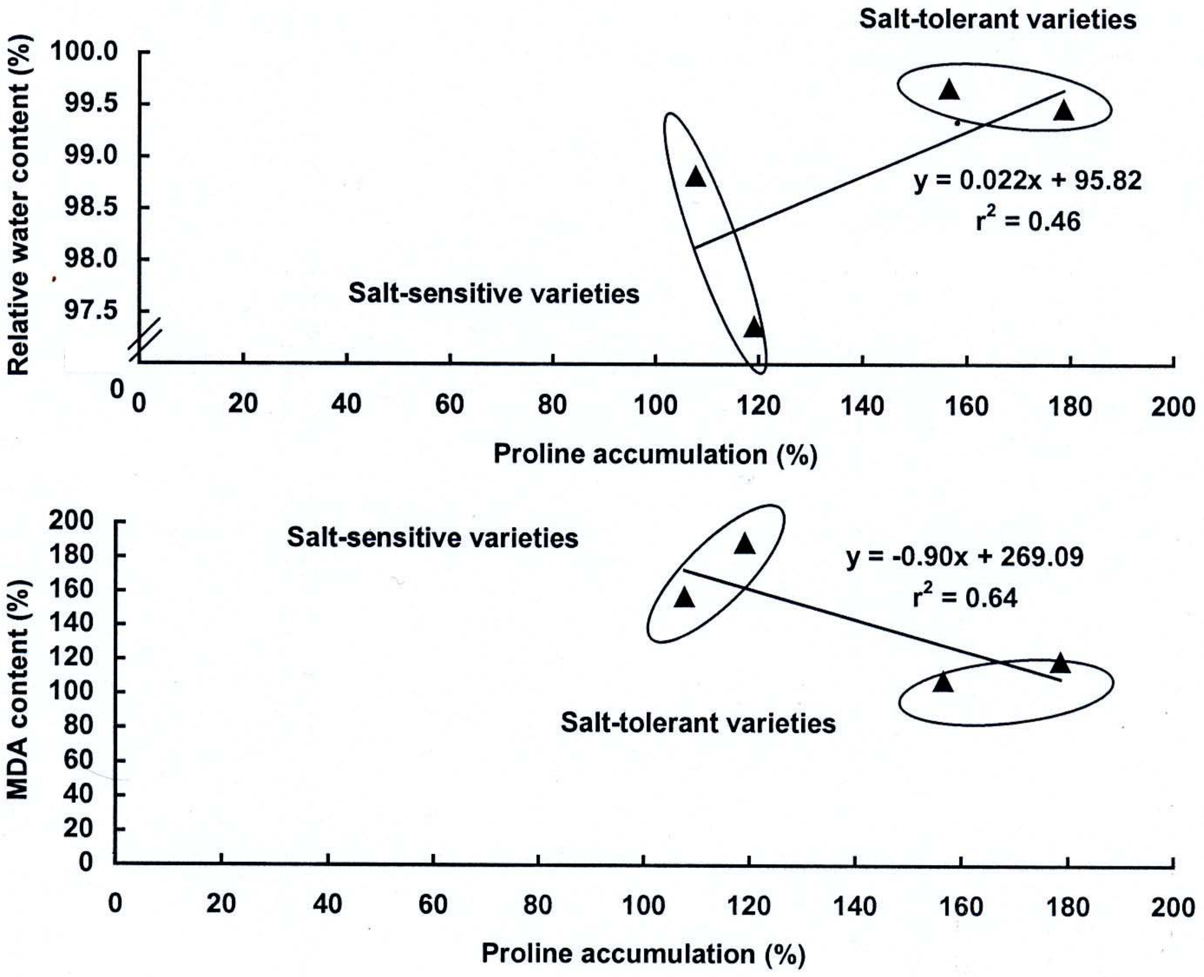

Fig. 4. Relationship between relative water content and proline accumulation (A), MDA content and proline accumulation (B) in salt-tolerant and salt–sensitive varieties subjected to 100 mM NaCl grown under photoautotrophic condition for 4 days.

One of the important mechanisms exerted by higher plants under abiotic stress is the accumulation of compatible solutes, such as proline. In this experiment, free proline content in the stressed-seedlings of rice varieties was increased (Fig. 3). Proline contents in salt-tolerant varieties (KDML105 and SY) were increased by 56.56-78.56% and higher than those in salt-sensitive varieties (PT1 and BS). Moreover the positive correlation between proline accumulation and RWC was observed (Fig. 4A) whereas the correlation between proline accumulation and MDA content was negative (Fig. 4B). Proline accumulation in different genotypes during salt stress has been noticed depending on a variety of crop species. Proline concentration has been shown to be generally higher in stress-tolerant than in stress-sensitive plants under stress in many plant species such as rice (Shereen *et al*., 2007), alfalfa (Petrusa & Winicov, 1997), maize (Sharp *et al*., 1994; Cha-um & Kirdmanee, 2009), pigeon pea (Waheed *et al*., 2006) and potato (Rahnama & Ebrahimzadeh, 2004). Proline accumulation in the cytosol might be enough for osmotic adjustment under stress condition. Proline has been reported to activate other mechanisms, such as the formations of strong H-bonded water around protein for protecting protein structures and scavenger of free radicals (Rascio *et al*., 1994; Alia *et al*., 2001; Ghoulam *et al*., 2002).

Total flavonoid contents in salt-stressed seedlings of rice varieties were increased. These substances presented in the stressed seedling of salt-tolerant varieties KDML105 and SY (7.31 and 6.34%, respectively) higher than those of salt-sensitive varieties PT1 and BS (1.72 and 3.48%, respectively) when compared to control (Fig. 5). The flavonoid accumulations and RWC in stressed seedling of salt-tolerance were higher than those of salt-sensitive varieties and showed the positive correlation (Fig. 6A). While the results of correlation between flavonoids accumulation and MDA content showed negative correlation (Fig. 6B). Thus flavonoids may have protective role under stress condition in similarity to proline compound. Flavonoids are frequently induced by abiotic stress and promote roles in plant protection (Dixon & Paiva, 1995; Grace & Logan, 2000). These compounds accumulated in plant tissue could help to protect themselves from damaging effects by act as a free radical scavenger because the hydroxyl groups present in their structure. Moreover the modifications of flavonoid structure i.e., glycosylation, prenylation and methylation could affect their antioxidant properties, thus they may help inhibit lipid peroxidation in stressed-plants (Caturla *et al*., 2003; Potapovich & Kostyuk, 2003).

Conclusions

The different changes of RWC, MDA, proline and total flavonoids observed in *indica* rice varieties response to salt stress, enabled to distinguish the metabolic events caused by ionic or osmotic components of salinity. Moreover, the metabolic pathways of proline and total flavonoids appeared to be strongly co-ordinated in salt-tolerant varieties more than salt-sensitive varieties, and their synchronous changes seem to be morphological and physiological traits associated with plant tolerant to salt stress.

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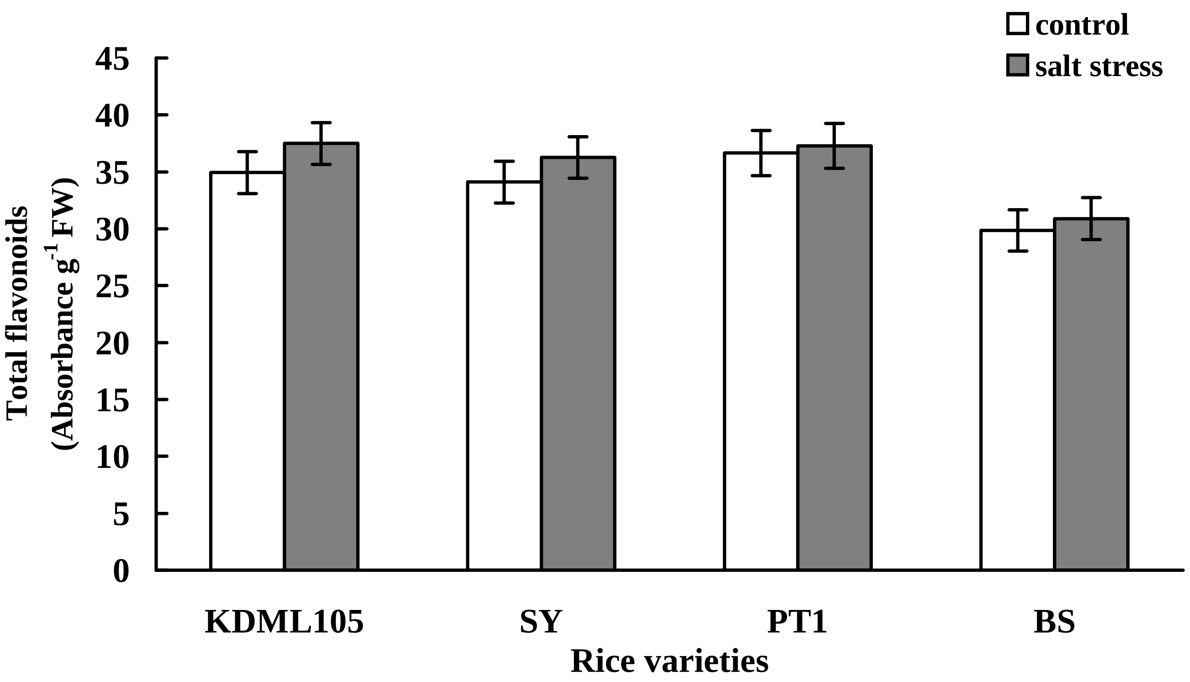

Fig. 5. Total flavonoid contents in rice seedlings subjected to 0 or 100 mM NaCl grown under photoautotrophic condition for 4 days. Error bars are represented by ± SE (n=5).

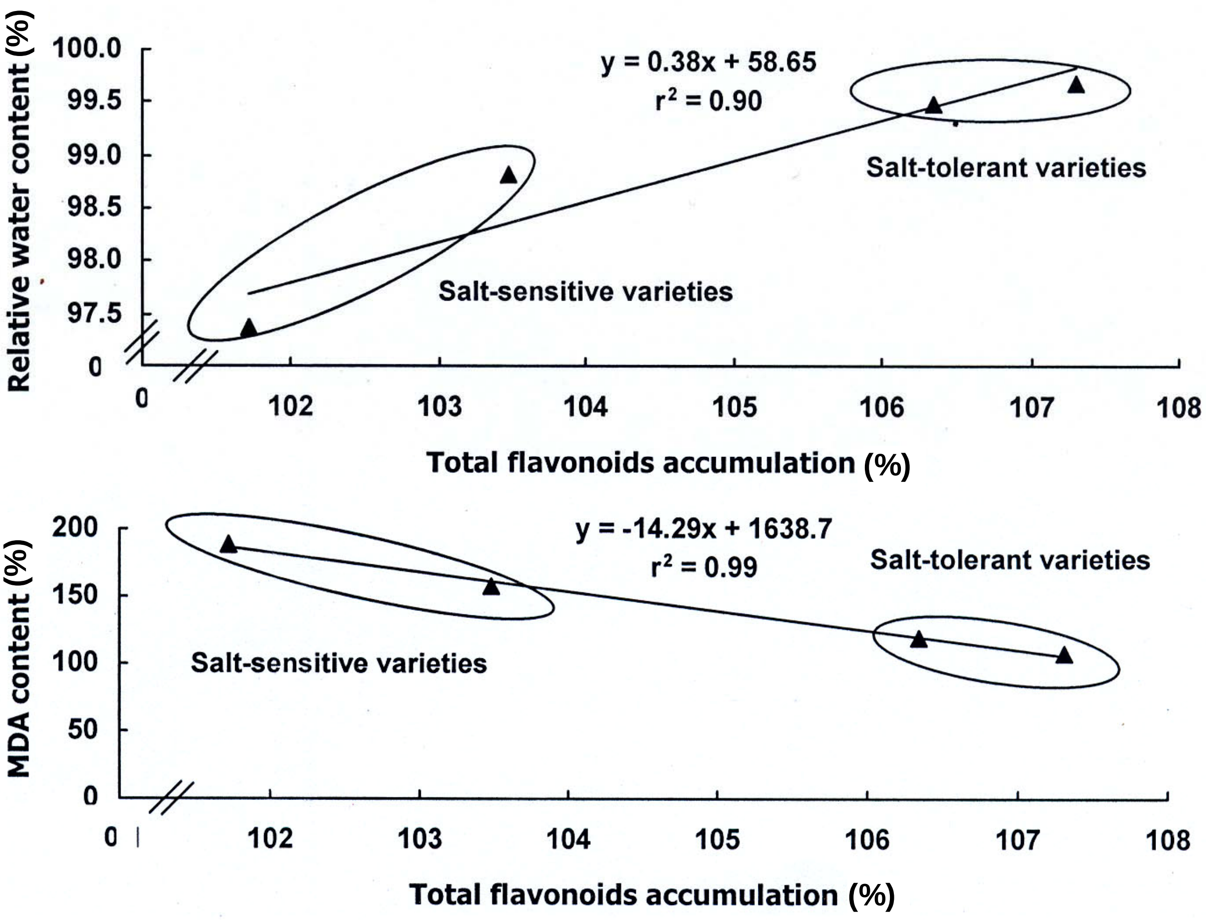

Fig. 6. Relationship between relative water content and total flavonoid accumulation (A), MDA content and total flavonoid accumulation (B) in salt-tolerant and salt–sensitive varieties subjected to 100 mM NaCl grown under photoautotrophic condition for 4 days.

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