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Full Length Research Paper

Effect of salt stress on growth, inorganic ion and proline accumulation in Thai aromatic rice, Khao Dawk Mali 105, callus culture

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The inhibitory effect of salt stress in rice is complex and is one of the main reasons for reduction of plant growth and crop productivity. In the present study, the response of rice callus cultivar Khao Dawk Mali 105 (KDML105), commonly known as Thai jasmine rice, to salt stress was examined. Callus cultures of KDML105 rice were exposed to salt stress by placing on Murashige and Skoog (MS) medium containing 250 mM NaCl. Growth, water content, proline and inorganic ion content in rice cells were measured during stress treatment for 8 - 10 days. After prolonged exposure to salt stress, growth and water content of rice cells were progressively decreased. Rice cells accumulated high level of Na^+ during stress, whereas the accumulation of K^+ and Ca^{2+} was decreased. High level of Na^+ inside the cells inhibited the K^+ uptake resulted in increase level of the Na^+/K^+ ratio. In addition, salt stress also caused an increase in the accumulation of proline. This result suggested that proline may play a crucial role in protecting the KDML105 rice cells under salt stress.

Key word: Aromatic rice, osmoprotectant, callus culture, salt stress.

INTRODUCTION

Saline soils are one of the major biotic stresses that adversely affect the overall metabolic activities and cause plant demise (Roychoudury et al., 2008). It has been estimated that over 2 million acres of agricultural land is lost from production each year due to the occurrence of high Na^+ and Cl^- levels in soils, so called salinization.

Salt stress leads to the suppression of plant growth and development, membrane leakage, ion imbalance or disequilibrium, enhanced lipid peroxidation and increased production of reactive oxygen species like superoxide radicals, hydrogen peroxide and hydroxy radicals, which are scavenged by both enzymatic and non-enzymatic reactions (Roychoudury et al., 2008).

In order to maintain homeostasis during stress condition, plants need to have special mechanisms for adjusting internal osmotic conditions and changing of osmotic pressure inside the cell, a process called osmotic adjustment (OA). Stressed plants diminish osmotic potential by accumulating low molecular weight, osmotically active compounds called osmolytes.

These compounds, which include simple or complex sugars, sugar alcohols, polyols, inositols, quarternary amino compounds like glycine-betaine, proline and higher polyamines (PAs), serve as osmoprotectants under stress conditions, maintain membrane structure and act as free-radical scavengers preventing lipid per oxidation or as regulators of K^+ channels in stomata (Hasegawa et al., 2000).

A wide variety of species also synthesize the phytohormone abscisic acid (ABA) as an adaptive response to reduce transpiration via stomatal closure (Finkelstein et al., 2002) and express common array of genes and similar specific proteins such as late embryogenesis abundant

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(LEA) (Goyal et al., 2005) or salt protein (Claes et al., 1990) which has several proposed protective functions.

The tolerance ability of rice (*Oryza sativa* L.) to salt stress depends on plant genotypes. For example, the indica varieties Pokkali and Nonabokra, having high endogenous ABA levels during stress conditions, are classified as highly salt-tolerant ecotypes, while the majority of high yielding cultivars such as M-1 - 48, IR - 29, IR - 36 and IR72 are salt-sensitive (Moons et al., 1995).

Khao Dawk Mali 105 (KDML105), commonly known in food markets as jasmine rice or Thai Hom Mali rice, is one of the indica-type rice. It is the most popular aromatic rice variety grown in Thailand. It is a photo-sensitive variety which could be grown only one a year (Pongtongkam et al., 2004) and is also known as a salt-sensitive, giving low yield and poor grain milling quality when it was grown under salinity soils. Over the past few years, much attention has been concentrated on physiological and biochemical responses of non-aromatic and salt-tolerant rice line such as Pokkali and Nonabokra to salt stress (Wanlchananan et al., 2003).

Reports of high-quality aromatic rice linked to salt stress are very few. Thus, the effects of high salinity on total fresh and dry weight, relative water content, proline content, Na^+, K^+, Ca^+ and Na^+/K^+ ratio in callus of aromatic rice KDML105 were investigated in this study.

MATERIALS AND METHODS

This study was conducted in the Plant Cell Laboratory, Department of Biotechnology, Faculty of Technology, Khon Kaen University, Thailand.

Plant material

Seeds of *O. sativa* L. cv. KDML105 used in this study were kindly provided by The International Training Center for Agricultural Development, Khon Kaen, Thailand.

Sterilization of rice seeds

KDML105 seeds were carefully dehusked, washed with detergent and rinsed thoroughly with tap water. They were surface sterilized in 95% ethanol for 3 min, 15% (v/v) sodium hypochlorite (Clorox®) solution containing Tween® 20 as wetting agent for 15 min and 30% Clorox® solution containing Tween® 20 for 15 min. The seeds were rinsed three times with sterile double distilled water and were used for callus induction.

Callus induction

Induction of KDML105 rice callus was performed by placing sterilized seeds on MS medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose and supplemented with 2 mg/l 2, 4-D (2, 4-dichlorophenoxyacetic acid) and 3 mM MES (2-(*N*-morpholino)-ethane -sulfonic acid). The seeds were incubated at 25 ± 2°C with a 16/8 h light/dark cycle (light intensity 20 µmole m^{-2} s^{-1}, cool white fluorescent TLD Philips 18W/33). Calli (1 g) formed from scutellar region were transferred onto new fresh media and sub culturing was performed at every 4 weeks intervals. The fast-growing callus lines were selected and were used in the stress experiments.

Stress treatments

7-day-old calli were used in this experiment. NaCl was added to the MS medium at a final concentration of 250 mM. The controls received no NaCl additions. After addition of NaCl, cells were incubated at 25 ± 2°C with a 16/8 h light/dark cycle (light intensity 20 µmole m^{-2} s^{-1}, cool white fluorescent TLD Philips 18W/33) for the desired period and samples were taken for biochemical assay.

Determination of cells growth

Growth of callus was determined by measuring the increase in the cell fresh weight (FW) and cell dry weight (DW). For cell dry weight, calli were taken and placed on a Petri dish, and then they were dried at 50°C in an oven to a constant weight. Results were expressed as FW (g) and DW (g).

Determination of water content

Callus samples of known fresh weight were dried to constant weight at 50°C in an oven. The water content was expressed as g water per g dry weight of cells. The water content of cells was calculated as (Fresh weight - Dry weight) / Fresh weight x 100 (Lai and Lui, 1988).

Determination of free proline content

Proline was extracted from stressed and non-stressed calli. 250 mg of fresh calli were ground in a mortar with liquid nitrogen, mixed with 5 ml aqueous sulfosalicylic acid (3% w/v) and filtered through Whatman®#1, 110 mm diameter filter paper (Whatman, England). The filtrate, 1 ml was reacted with an equal volume of glacial acetic acid and ninhydrin reagent (1.25 g of ninhydrin, 30 ml of glacial acetic acid and 20 ml of 6 M H_3PO_4) and incubated for 1 h at 100°C in boiling water.

The reaction was terminated by placing the reaction tubes in an ice bath. The reaction mixture was vigorously mixed with 2 ml toluene. After warming at 25°C, the chromophore was measured for proline at 520 nm with a DR/4000 Spectrophotometer (HACH, USA).

Determination of Na^+, K^+ and Ca^{2+} content

Calli were ground to a fine powder and 0.2 g of the cell powder was ashed in a muffle furnace at 500°C for 3 h. The ashes were digested with 5 ml of 7N nitric acid (HNO_3). After appropriate dilutions, the filtrate was assayed for Na^+, K^+ and Ca^{2+} using atomic flame emission spectrometry.

Statistical analysis

All the measurements were repeated on two set of calli with showed similar results. Data presented therefore are obtained from

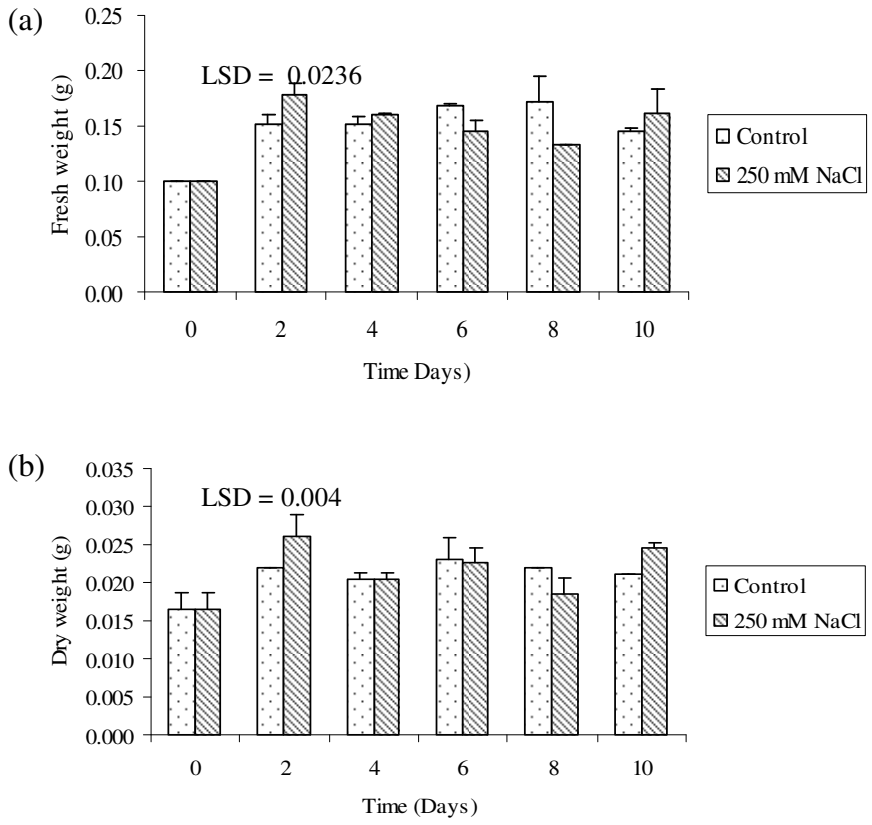

Figure 1. Growth of the KDML105 rice callus under salt stress. Cells were grown at 25 ± 2°C for 10 days on MS medium without (control) and with 250 mM NaCl supplementation. Data represent mean of three replicates and vertical bars represent ± standard error. (a) represents fresh weight and (b) represents dry weight of the rice callus.

the single experiment. Each value is presented in the form of mean ± standard error and lowest standard deviations (LSD) of mean with a reading of at least three samples per treatment. The analysis of the main effects of the stress was based on the analysis of variance (ANOVA).

RESULTS

Effect of salt stress on growth and water content in KDML105 rice callus

The effect of salt stress on growth of callus was determined by adding NaCl to the MS medium at a final concentration of 250 mM. The results revealed that growth of the callus as measured by fresh and dry weight was not significantly different between salt and non salt-stressed or control cells during the first 4 days of exposure to stress treatment. However, after 6 days of exposure to stress growth of the salt-stressed cell was slightly decreased as compared to the control cells (Figure 1). The water content of the salt-stressed cells was also slightly declined after 6 days of exposure to salt stress (Figure 2).

Effect of salt stress on proline content in KDML105 rice callus

Proline contents of the salt-stressed and control callus are illustrated in Figure 3. The proline content in the salt-stressed cells was approximately 2.0 - 2.5 times higher than that in the control cells. The highest proline content in the salt-stressed cells was about 246 µg/g FW at day 10.

Effect of salt stress on ion content in KDML105 rice callus

The level of Na^+ content in the control cells was constant throughout the period of stressed, however it was significantly increased in the salt-stressed cells, about 68 - 100 times higher than that in the control cells (Figure 4). Figure 5 showed the level of K^+ content in stressed and non-stressed rice callus. The level of K^+ content was gradually decreased in the salt-stressed cells, but slightly increased in the control cells.

The K^+ content of the salt-stressed cells at day 8 was

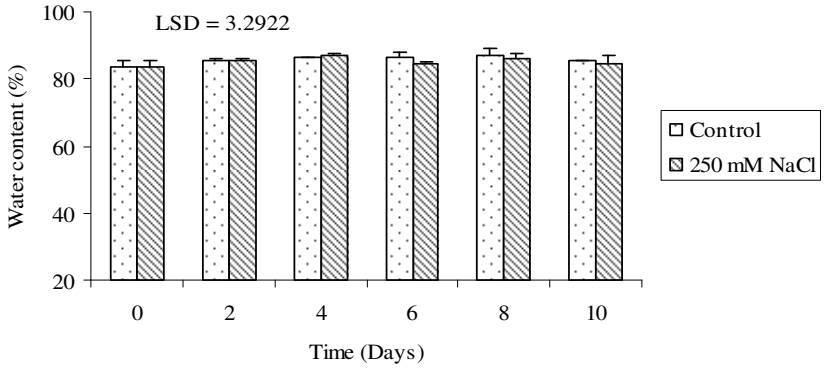

Figure 2. Changes in water content of the KDML105 rice callus under salt stress. Cells were grown at 25 ± 2°C for 10 days on MS medium without (control) and with 250 mM NaCl supplementation. Data represent mean of three replicates and vertical bars represent ± standard error.

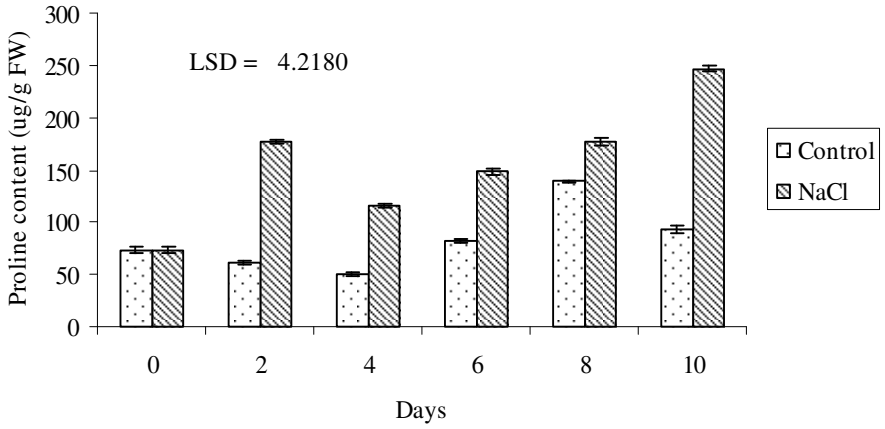

Figure 3. The accumulation of proline in the KDML105 rice callus under salt stress. Cells were grown at 25 ± 2°C for 10 days on MS medium without (control) and with 250 mM NaCl supplementation. Data represent mean of three replicates and vertical bars represent ± standard error.

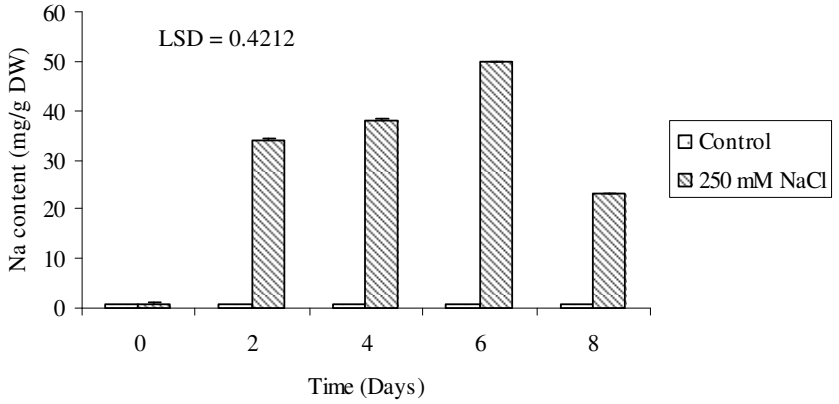

Figure 4. The accumulation of Na^+ in the KDML105 rice callus under salt stress. Cells were grown at 25 ± 2°C for 8 days on MS medium without (control) and with 250 mM NaCl supplementation. Data represent mean of three replicates and vertical bars represent ± standard error.

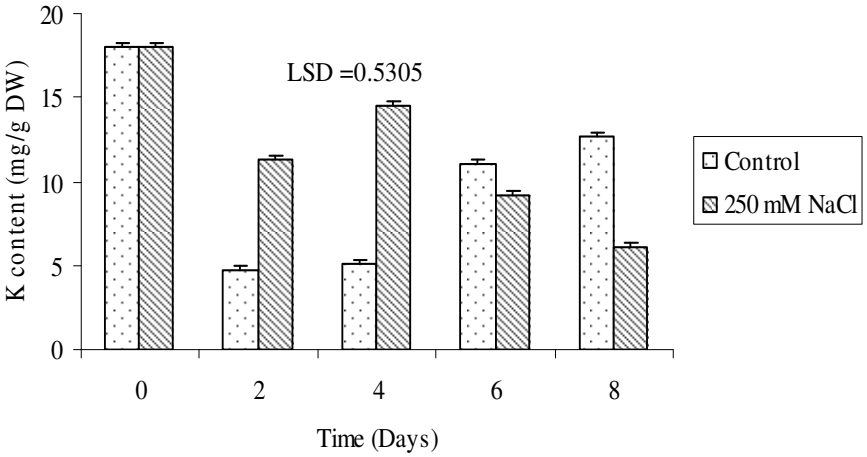

Figure 5. Changes in K^+ content of the KDML105 rice callus under salt stress. Cells were grown at 25 ± 2°C for 8 days on MS medium without (control) and with 250 mM NaCl supplementation. Data represent mean of three replicates and vertical bars represent ± standard error.

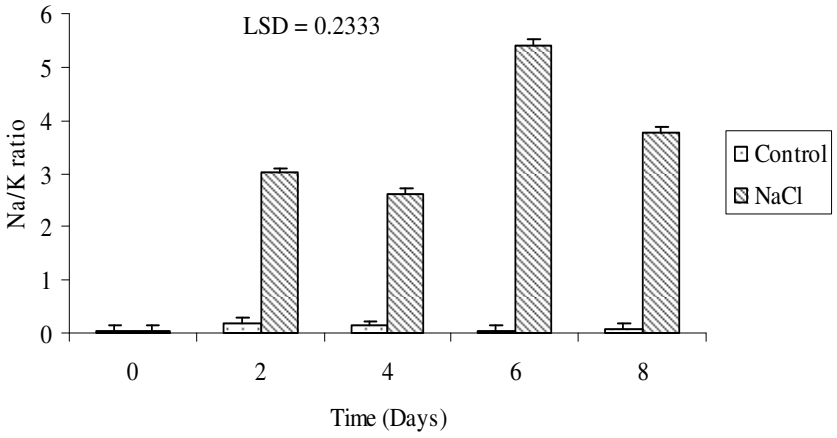

Figure 6. Changes in the Na^+/K^+ ratio of the KDML105 rice callus under salt stress. Cells were grown at 25 ± 2°C for 8 days on MS medium without (control) and with 250 mM NaCl supplementation. Data represent mean of three replicates and vertical bars represent ± standard error.

approximately 50% lower than that of the control cells. Considering the Na^+/K^+ ratio, the level of this ratio in the control cells was approximately 19 times lower than that of the salt-stressed cells (Figure 6). Salt stress also caused decreased in the level of Ca^{2+} content particularly after 6 days of exposure to stress treatment (Figure 7).

Although the maximum Ca^{2+} content in the salt-stressed cells was found at about 3.1 mg/g DW at day 2, this was gradually decreased and was lower than that found in the control cells on day 6 and 8. These findings indicated that an ion transport mechanism exists for Na^+ and against K^+ and Ca^{2+} accumulation under salt stress in the rice cells.

DISCUSSION

In order to overcome the detrimental effect of soil salinity, one of the most obstructive impacts on crop production, studying on the physiological response in the cellular level is the principle prerequisite before generating a salt tolerant line. Since KDML105 is one of the most widely consumed Thai aromatic indica rice, a study on its response to salinity stress and evaluation of its performance would enable us to ensure its improvement against salt injury.

In the present study, salinity caused slightly reduction in cells growth and water content of KDML 105 rice after

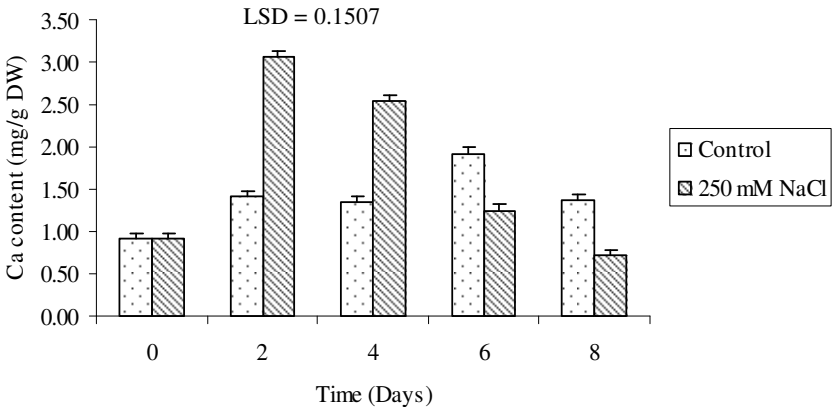

Figure 7. Changes in Ca^{2+} content of the KDML105 rice callus under salt stress. Cells were grown at 25 ± 2°C for 8 days on MS medium without (control) and with 250 mM NaCl supplementation. Data represent mean of three replicates and vertical bars represent ± standard error.

prolonged exposure to stress treatment at 250 mM NaCl (Figures 1 and 2). The negative effect of salinity on plant growth and water content may be due to the occurring of defect metabolism in plant cells. Since high osmotic pressure resulted from high salinity restricted plant cells to uptake water and some mineral nutrients dissolved in the culture medium (Cicek and Cakirlar, 2002).

Rhodes and Samaras (1994) described that growth inhibition under osmotic condition might be mainly due to the reduction in cytoplasmic volume and the loss of cell turgor as result of osmotic outflow of intracellular water. Reduction in plant growth and water content as a result of salinity has also been reported in several other plant species such as *Zea mays* (Ashraf and McNeilly, 1990), *O. sativa* japonica type (Lutts et al., 1996), *Triticum durum* (Ashraf and O'Leary, 1997; Lutts et al., 2004), *Tagetes minuta* (Mohamed et al., 2000) and *Saccharum officinarum* (Errabii et al., 2006).

In *S. officinarum*, water content of the callus was decreased significantly and proportionally to the stress intensity in the medium. At the highest osmotic pressure, water content declined to about 45 - 48% of the control cells.

Proline accumulation in response to environmental stresses has been considered by a number of investigators as an adaptive trait concerned with stress tolerance (Rhodes and Hanson, 1993). It is generally assumed that proline is acting as a compatible solute in osmotic adjustment (Larher et al., 1993; Perez-Alfocea et al., 1993). It may act as an enzyme protectant, stabilizes membranes and cellular structures during hostile conditions, detoxifies free radicals by forming long-lived adducts with them and affects solubility of various proteins by interacting with their hydrophobic residues (Delauney and Verma, 1993; Hare and Cress, 1997; Hong et al., 2000). Proline may also serve as an organic nitrogen reservoir ready to be used after stress relief to sustain both amino acid and protein synthesis (Trotel et al., 1996; Sairam and Tygai, 2004).

In this study, KDML105 cells accumulated high level of proline under salt stress as compared to the control condition (Figure 3). The increase in the proline content under stress condition may be due to breakdown of proline rich protein or *de novo* synthesis of proline (Tewari and Singh, 1991). It could also be due to prevention of feedback inhibition of the biosynthetic enzyme caused by sequestering proline away from its site of synthesis or by relaxed feedback inhibition of the regulatory step enzyme or by decreased activity of enzymes involved in degradation of proline such as proline dehydrogenase and proline oxidase (Girija et al., 2002). In order to clarify these possibilities, further study on activity of enzymes involved in proline synthesis and degradation should be carried out.

Proline accumulation and aroma generation in the aromatic rice have been reported by a number of authors. For example, Yoshihashi et al. (2002) demonstrated that the accumulation of proline as osmoregulator is positively correlated with aroma generation in seeds of KDML105 rice grown in the regions of dry and increased salt conditions. Suprasanna et al. (1998) reported that the supplementation of L-proline into the culture medium can yield in increase in aroma production in callus cultures of aromatic rice PB1 and Basmati. We did not determine the production of aroma in the callus culture of KDML105 in this study, therefore further investigation is needed.

K^+ and Ca^{2+} have been reported to be the major cautions in cell organization as well as the major contributors to osmotic adjustment under stress conditions in several plant species (Santos-Diaz and Alejo-Ochoa, 1994; Hirschi, 2004). In the present study, the level of K^+ and Ca^{2+} in the salt-stressed rice cells gradually decreased (Figures 5 and 7) while that of Na^+ was dramatically increased (Figure 4). The decrease in K^+ and Ca^{2+} content

under stress condition has been previously reported in other species particularly in the salt-sensitive lines (Lutts et al., 1996, 2004).

According to Weimberg (1987), high levels of Na^+ inside the cells inhibit the K^+ uptake and as a result it causes an increase in the Na^+/K^+ ratio. The increase in the Na^+/K^+ ratio might be attributed to the fact that Na^+ causes a disturbance in the ion balance in plant by an increase in the Na^+ uptake (Cicek and Cakirlar, 2002). Many of the deleterious effects of Na^+ seem to be related to the structural and functional integrity of membranes (Kurth et al., 1986).

It is generally known that the maintenance of low cytosolic Na^+ concentration and Na^+/K^+ homeostasis is an important aspect of salinity tolerance and that the salt-tolerant lines show lower Na^+/K^+ levels (Chattopadhyay et al., 2002). Based on the Na^+/K^+ ratio in this study, the KDML105 is classified as a salt-sensitive line.

Taking the above into consideration, in response to salt stress which adversely effects plant growth, the aromatic KDML105 rice cells accumulate high level of proline, suggesting that proline plays a crucial role in plant cells during stress treatment. The KDML105 rice is a salt sensitive. In order to increase its general tolerance mechanism against salt injury, future study using new technologies such as the over expression of the genes involved in salt tolerance may be needed for crop improvement program.

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