INTRODUCTION

Rice (Oryza sativa L.) is a main staple crop around the world, feeding millions of people and providing the necessary daily calories to more than half the world’s population (Khush, 1997; Khush, 2005). It is one of the most widely grown crops in coastal areas inundated with sea water during high tidal period; although it is usually considered moderately susceptible to salinity (Akbar and Yabuno, 1972; Korbe and Abdel-Aal, 1974; Mori and Kinoshita, 1987; Hong et al., 2007), it is salt sensitive (Maas and Hoffman, 1977). Salinity of soil and water resources is the most basic and oldest environmental problems that it can limit crop production in many parts of the world and is considered as serious danger for agriculture (Abrolet al., 1988; Munns, 2002; Netondo et al., 2004; Haq et al., 2010). It has been predicted that the demand for rice in the world will increase to 780 million tons by the year 2020 (Shabbir et al., 2001). Major environmental limitations on rice production are salinity and drought (Toennissen, 1995). According to reports, about 900 million hectares of lands are affected by salinity in the world that is nearly 20% of the world’s cultivated area and about half of the world’s total irrigated lands (Munns, 2002; FAO, 2007). Soil salinity is a complex effect causing disturbance to membrane integrity, nutrient imbalance and disturbances on general metabolic activities. Accumulation of salts in the soils of arid and semi-arid regions is a continuing threat to crop production. A possible alternative is the introduction of crop species/cultivars capable of tolerating higher soil salinities with moderate economic yield (Yeo and Flowers, 1986). Two types of plant responses to salinity have been distinguished: pre-existing resistance mechanisms and adaptation or acquired tolerance (Amzallag et al., 1990). Response of plants to any stress agent is particularly of adaptive nature when the stress is sublethal. On the other hand, response shown may be biased towards cell death if the stress is lethal (Groover et al., 2001). Salinity can limit plant growth and yield by reducing osmotic potential, ion toxicity creation, uptake disarrangement and ion imbalance and can cause disorders in enzyme activities and membrane and metabolic activities in plants (Marschner, 1986; Gorham, 1993; Hasegawa et al., 2000; Basu et al., 2002; Murphy et al., 2003; Islam et al., 2008). These processes could affect morphological parameters and plant growth and will reduce vegetative growth (Linghe and Shannon, 2000; Sairam and Tyagi, 2004; Rogers et al., 2009), active leaf area, chlorophyll content (Netondo et al., 2004, Cha-um et al., 2007, Saleh and Mafioun (2008) and chlorosis is a common morphological and physiological characteristic in response to salt stress (Harinasut et al., 2000). Chlorophyll content of salt stressed rice can be described as a function of the leaf sodium content (Yeo and Flowers, 1983). The response of plants to excess NaCl is complex and involves changes in their morphology, physiology and metabolism (Hilal et al., 1998; Djianaguiraman et al., 2003; Rahmanet al., 2008) and consequently reducing plant dry weight (Zeng and Shannon, 2000; Pesqueira et al., 2003; Razzakuet al., 2009; Rogers et al., 2009) and dry matter production (Mansour and Salah, 2007) and ultimately crop yield (Shannon et al., 1998; Zeng and
Salinity is rapidly increasing on a global scale causing decline in average yield for most of the major crop plants (Bray et al., 2000). Due to a number of environmental factors the coastal soils are slightly to moderately saline on the surface and highly saline in sub-surface layers and substrata. Saline soil contains an excess of soluble salts, especially sodium chloride. In other words, soil salinity develops under the influence of the electrolytes of sodium salts, with a nearly neutral reaction. Salinity imposes both ionic and osmotic stresses on plants (Munns et al., 2006) and salt exclusion from photosynthetic tissues is considered an important mechanism associated with salt tolerance in monocots (Yeo et al., 1990; Moradi et al., 2003; Davenport et al., 2005). Soil salinity is one of the major constraints of arid and semi-arid regions, where soluble salts are frequently high in the soil or in irrigation water. It adversely affects the growth of most agricultural crops through its influence on certain aspects of plant metabolism such as osmotic adjustment (Bernstein, 1963; Yokoi et al., 2002; Zeng et al., 2003), the uptake of certain essential nutrients (Greenway et al., 1996), photosynthesis (Downton, 1977) and enzyme activity (Weinberg, 1970) as well as causes hormonal imbalance (Shah and Loomis, 1965). Chlorophyll fluorescence is a rapid and non-intrusive tool used to screen varieties for salinity tolerance (Maxwell and Johnson, 2000, Ashraf 2010). Some toxic effects of salt stress include decreased germination and seedling growth (Heenan et al., 1988; Zeng and Shannon, 2000).

The effects of salt stress on rice are highly dependent on plant phenology: young seedlings and plants at the flowering stage appear to be the most sensitive while tillering plants are less sensitive (Sahu and Mishra, 1987; Lutts et al., 1995). Salinity applied at the seedling stage frequently induces premature senescence of leaves (Kura-Hotta et al., 1987; Yeo et al., 1991). Leaf senescence is most often quantified by decrease in protein or chlorophyll concentration (Dhindsa et al., 1981; Hashimoto et al., 1989; Chen and Kao, 1991; Chen et al., 1991) and by increase in membrane permeability (Björkman, 1987). Chlorophyll fluorescence kinetics, and more especially the ratio of the maximal variable fluorescence to the maximum level of chlorophyll fluorescence (Fv/Fm), which is directly related to PS II photochemical efficiency, may also be modified during ageing although the relationship between chlorophyll fluorescence and naturally occurring senescence processes is rarely considered (Bohra and Dörffling, 1993). In rice, it is found that NaCl salinity lowered the Fv/Fm chlorophyll fluorescence ratio after 47 day of stress and also at the flowering stage (Bohra et al., 1995; Pu and Gong, 2000). Chlrophyll, the green pigment common to all photosynthetic cells, absorbs all wave lengths of visible light except green. All photosynthetic organisms have chlorophyll a. Chlorophyll a absorbs its energy from the violet-blue and reddish orange-red wavelengths, and little from the intermediate (green-yellow-orange) wavelengths. Due to chlorophyll absorption, the visible region of green plants shows a maximum reflectance at approximately 550 nm and lower reflectance in blue (450 nm) and red (680 nm) (Bohnert and Jensen, 1996).

Sugar may play key role in salt defence mechanisms, including membrane stability, via interaction with phospholipid head groups and reactive oxygen species detoxification (Bentsink et al., 2000; Smeekens, 2000; Roy et al., 2005). Moreover, soluble sugar, produced by photosynthesis in higher plants and a major energy source, also plays a critical role in signal transduction in primary and secondary metabolites, including the building blocks of macromolecules in the developmental processes of plants (Price et al., 2004; Yamane et al., 2008). However, photosynthetic pigment degradation, chloroplast destruction, chlorophyll fluorescence diminution and net photosynthetic rate reduction in salt stressed plants have been reported (Asch et al., 2000; Cha-um et al., 2004; Cha-um et al., 2009).

The effects of salinity on chlorophyll synthesis and integrity seem to vary with the level of salt stress (Santo, 2004; Rout et al., 1997). However, significant differences between genotypes were sometimes observed regarding the effects of salt stress on chlorophyll concentration in leaves (Sies and Stahl, 1995; Datta et al., 2009). Chlorophyll concentration usually is a good indicator of plant nutrient stress, photosynthesis and growing periods, the content of chlorophyll in the plant leaves indicates the growth status of the crops, also it is the important condition for exchange of mass and energy from the outside world and therefore real-time monitoring of the content of chlorophyll is a key step to complete crop monitoring and yield estimation (Canfield et al., 1993; Rao et al., 2007; Costache et al., 2012). The presence of pigments in plant tissues gives colour to leaf, vegetables and fruit, which is different depending on variety and species. Pigments are substances with very different chemical structure; they are present in the form of porphyrin pigments, carotenoids, anthocyanin and flavones. The main porphyrin pigments found in vegetables are chlorophyll a, b and c. Chlorophyll a, the main pigment in plants, converts light energy into chemical energy through photosynthetic process. The content of chlorophyll pigments varies by species (Richardson et al., 2002). Carotenoid pigments can be located in chromoplasts, contributing to the colour of vegetables and fruits, or in chloroplasts together with chlorophylls. Among carotenoid pigments in vegetables and fruits, β-carotene is the most popular and widespread.

In photosynthesis, antenna pigments in leaf chloroplasts absorb solar radiation, and through resonance transfer the resulting excitation is channelled to the reaction centre pigments, which release electrons and set in motion the photochemical process. The chlorophylls, Chl a and Chl b, are the most important of these pigments, and are thus virtually essential for the oxygencconversion of light energy to the stored chemical energy that powers the biosphere (Groff et al., 1995). Carotenoids constitute a family of pigmented lipophilic compounds that are widely distributed in biological systems. They are synthesized by plants and microorganisms but not animals. They are yellow to red pigments and include non-polar hydrocarbons, carotenoids (α- carotene, β-carotene, lycopene) and their oxygenated derivatives, xanthophylls (Petoet al., 1981). They are highly physiologically important and protect plants and microorganisms against excessive irradiation. Also, some carotenoids possess provitamin A activity. Carotenoids interact with reactive oxygen species and thus act as free radical quenchers, singlet oxygen scavengers and lipid antioxidants.

The present experiment was carried out so as to study the effect of salt stress on the variation in chlorophyll content and total carotenoid content in native rice cultivars collected from
traditional saline and non saline rice tracts of Kerala State of India. Progressive salinity stress starting from 43rd day of planting was applied in this case so as to mimic the situation in the saline fields of the geographical area where the fields are almost free from salinity in the early stages of plant development thanks to high rainfall from south-west monsoon and salinity increases as the monsoon recedes.

MATERIALS AND METHODS

Germination of seeds and transfer to the experiment site

The experiment was conducted in the experimental rainout poly house of Department of Botany, University of Calicut, Kerala, India located at 11°35’N latitude and 75°48’E longitude in the first crop season of 2013. Seven native cultivars of rice (Oryza sativa), including five cultivars namely Orthadian, Orkazhama, Kathiru, Kattusan and Chovvarian collected from one of the saline rice tracts of Kerala and two native rice cultivars namely Kunhutty and Veliyan collected from one of the non-saline rice tracts of Kerala were used for the study. Enough numbers of healthy mature caryopses from a single plant were used. The seeds were washed in running tap water to remove infected, unfilled grains and dust particles, soaked in distilled water and allowed to germinate in 10cm diameter Petri dishes (Borosil) covered with lid under room temperature. The water was changed every day. The seeds started to germinate from the third day.

Plant materials and treatments

On the 10th day, required numbers of the germinated seedlings were transferred to coloured plastic pots of 25cm diameter filled with paddy soil mixed with enriched compost in 3:1 ratio. Two seedlings were initially planted per pot and after establishment of the seedlings the smaller among the two were removed. The plants were maintained in the experimental poly house under wetland conditions, always maintaining 3cm of water above the soil level. The soil was fertilized with 1g of N: P: K = 18: 18: 18 per pot at fortnightly intervals starting from the 30th day. Weeding was done manually whenever required. Plants were grown in Randomized Block Design with three replications.

Experimental treatments and observations

The experimental treatment was started from the 45th day onwards starting from 10mM (0.91dSm⁻¹) to 200mM (18.26 dSm⁻¹) aqueous solution of sodium chloride as detailed in Table 1.

Estimation of pigment composition of leaf

Estimation of the pigments was done according to the protocol advocated by Arnon (1949). Fresh leaves of control as well as experimental plants were collected for analysis on 90th day, washed with water and blotted between sheets of filter paper. To estimate chlorophyll and carotenoids, chilled 80% acetone was used as the extraction medium. Enough precautions were taken to avoid any exposure of the extract to light. 0.1g of fresh leaf sample was weighed in an electronic balance (Sartorius). It was then powdered using liquid nitrogen, crushed with the help of mortar and pestle in 20ml of 80% acetone (v/v) (Merck, India). Then the homogenate was centrifuged at 5,000 rpm for 10 minutes in a cooling centrifuge at 4°C (Sigma, Germany) and the supernatant was collected in a polypropylene tube (Tarsons, India). The residue was again washed with 80% acetone and centrifuged again. The process was repeated till the pellet became colour less.

<table>
<thead>
<tr>
<th>Treatment No.</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>Control</td>
</tr>
<tr>
<td>T2</td>
<td>10mM (0.91dSm⁻¹) on 45th day</td>
</tr>
<tr>
<td>T3</td>
<td>10mM (0.91dSm⁻¹) on 45th day &amp; 30mM (2.74 dSm⁻¹) on 53rd day</td>
</tr>
<tr>
<td>T4</td>
<td>10mM (0.91dSm⁻¹) on 45th day &amp; 30mM (2.74 dSm⁻¹) on 53rd day</td>
</tr>
<tr>
<td>T5</td>
<td>10mM (0.91dSm⁻¹) on 45th day &amp; 30mM (2.74 dSm⁻¹) on 53rd day</td>
</tr>
<tr>
<td>T6</td>
<td>10mM (0.91dSm⁻¹) on 45th day &amp; 30mM (2.74 dSm⁻¹) on 53rd day</td>
</tr>
<tr>
<td>T7</td>
<td>10mM (0.91dSm⁻¹) on 45th day &amp; 30mM (2.74 dSm⁻¹) on 53rd day</td>
</tr>
</tbody>
</table>

The final volume of the pooled supernatant was noted. The absorbance was read at 663nm, 646nm, 750nm and 470nm against the solvent blank (80% acetone) using a UV visible spectrophotometer (Systronics, India). Then the amount of chlorophyll and carotenoids present in the extract was calculated using the following formulae adopted from Arnon (1949), Manuela et al., (2005), Molazem et al., (2010) and Khaleghi et al., (2012). The concentration of chlorophyll and carotenoids are expressed in mg/g fresh weight of the leaf tissue.

Chlorophyll a (mg/g) = [12.7 x (A663) - 2.69 x (A645)] x V / (1000 x W)

Chlorophyll b (mg/g) = [22.9 x (A645) - 4.68 x (A663)] x V / (1000 x W)

Total Chlorophyll (mg/g) = [20.2 x (A645) + 8.02 x (A663)] x V / (1000 x W)

Carotenoids total (mg/g) = [1000 x (A470) + 3.27 x (chl a - chl b)] x V / W x (229 x 1000)

Where, W is the fresh mass of the leaf sample taken and V is the total volume of the sample solution.

Statistical analysis

Differences between the values for control and salt stressed plants were analysed by one-way ANOVA taking P < 0.05 as significance level. Data are shown as mean ± standard error (SE).

RESULTS

The photosynthetic pigments Chl a, Chl b and carotenoids showed significant reduction in rice plants when subjected to incremental dozes of salinity treatments (Table 2, Figs. 1 & 2).
Table 2 Details of variation in Chlorophyll a, Chlorophyll b, Total Chlorophyll, Chlorophyll a/Chlorophyll b ratio and carotenoids as affected by salt stress in the rice cultivars studied

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Chlorophyll a</th>
<th>Chlorophyll b</th>
<th>Total Chlorophyll</th>
<th>Chlorophyll a/b</th>
<th>Carotenoids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.72 ± 0.003</td>
<td>0.497 ± 0.003</td>
<td>2.220 ± 0.002</td>
<td>1.77 ± 0.001</td>
<td>0.143 ± 0.001</td>
</tr>
<tr>
<td>10mM</td>
<td>1.69 ± 0.03</td>
<td>0.494 ± 0.004*</td>
<td>2.160 ± 0.001*</td>
<td>2.7 ± 0.003</td>
<td>3.92 ± 0.003*</td>
</tr>
<tr>
<td>30mM</td>
<td>1.60 ± 0.02</td>
<td>0.407 ± 0.004*</td>
<td>2.012 ± 0.002*</td>
<td>9.37 ± 0.035*</td>
<td>13.92 ± 0.101*</td>
</tr>
<tr>
<td>50mM</td>
<td>1.62 ± 0.02</td>
<td>0.405 ± 0.004*</td>
<td>2.007 ± 0.003*</td>
<td>9.59 ± 0.035*</td>
<td>14.41 ± 0.071*</td>
</tr>
<tr>
<td>100mM</td>
<td>1.34 ± 0.03</td>
<td>0.364 ± 0.006</td>
<td>1.759 ± 0.007*</td>
<td>24.41 ± 4.003*</td>
<td>30.35 ± 0.662*</td>
</tr>
<tr>
<td>100mC</td>
<td>1.29 ± 0.03</td>
<td>0.273 ± 0.004*</td>
<td>1.563 ± 0.002*</td>
<td>29.59 ± 4.740*</td>
<td>36.60 ± 0.026*</td>
</tr>
<tr>
<td>200mC</td>
<td>1.25 ± 0.01*</td>
<td>0.263 ± 0.002*</td>
<td>1.517 ± 0.003*</td>
<td>31.67 ± 4.763*</td>
<td>37.26 ± 0.014*</td>
</tr>
</tbody>
</table>

among the cultivars collected from the saline rice tract, *Orthadian* showed the maximum reduction (38.95%) among the cultivars collected from the saline tract and *Kuthiru* showed the minimum in the total carotenoid content among the cultivars collected from the saline rice tract. *Kunhutty* showed the highest reduction (38.95%) in the case of *Chovvvarian* (79.97%). In the case of Total Chl content, *Kuttusen* showed the maximum reduction (38.95%) among the cultivars collected from the saline rice tract and *Kuthiru* showed the minimum reduction (22.65%) among the highest salt concentration when compared to the control plants. While considering the cultivars collected from the non saline rice tract, *Kunhutty* showed the highest reduction (28.48%) in Chl a content at the highest salt level. In the case of Chl b content, *Kuttusen* showed the maximum percentage of reduction (73.09%) followed by *Chovvvarian* (63.62%) among the cultivars collected from the saline rice tract. Minimum reduction was shown by *Kuthiru* (40.55%) at the highest salt level. Among the cultivars collected from the saline rice tract, *Veyilan* showed the maximum reduction in the case of Chl b (79.97%). In the case of Total Chl content,
place due to the differential variation in Chl $a$ and Chl $b$ content.

**DISCUSSION**

The present study shows that in the case of the rice cultivars collected from the saline tract and the non saline tract, progressive increase in salinity resulted in reduction of chlorophyll production. Among the cultivars studied, *Kuthira* showed the minimum reduction in Chl $a$ content (22.65%), Chl $b$ content (40.55%) and Total Chl content (25.75%).

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**Fig. 1** Variation in Chlorophyll $a$, Chlorophyll $b$, Total chlorophyll, Chlorophyll $a$/Chlorophyll $b$ ratio and carotenoids as affected by salt stress in the rice cultivars studied.
under salinity stress followed by Chovvarian and Orkazhama. The trend shown by Chl $a$/Chl $b$ ratio indicated that Chl $b$ content exhibited higher reduction due to salt stress when compared to reduction in Chl $a$ content and Total Chl content. Moreover, the percentage of reduction was in proportion to relative increase in salinity.

Relative percentage of reduction was the highest in the case of Chl$b$ content when compared to Chl $a$ content and Total Chl content. However, the reduction in Total Chl content was the maximum among the cultivars collected from the non saline tract. Cultivars collected from the saline tract showed comparatively lesser reduction in chlorophyll content when compared to others. More over, the results showed that reduction in chlorophyll content and carotenoid content were cultivar specific. This difference in the variation in chlorophyll content manifests one major adaptation of salinity tolerant rice cultivars making them capable of growing under saline conditions. Earlier workers have reported that rice cultivars such as Kuthiru and Orkazhama performed comparatively well under conditions of moderate salt stress (Chandramohan and Mohanan, 2012). Reports have established that salt stress causes reduction in leaf surface expansion ratio, leading to cessation of expansion as salt concentrations increase (Wang and Nil, 2000). Srivastava et al. (1988) have reported chlorophyll content as one of the parameters of salt tolerance in crop plants. In the present study, the salt treatments significantly decreased chlorophyll and carotenoid contents in the salt treated plants when
compared to control plants both in the case of the rice cultivars collected from the saline tract and the non saline tract. However, the reduction was higher in the case of the plants collected from the non saline tract.

High soil salinity is one of the important environmental factors that limit distribution and productivity of major crops. NaCl stress decreases chlorophyll content even at the lowest concentration (Santo, 2004). Rice is a major grain crop and carbohydrate source, supplying the necessary daily calories for more than half the world’s population (Dubey and Singh, 1999; Khush, 2005). It has been predicted that the demand for rice in the world will increase to 780 million tons by the year 2020 (Shabbir et al., 2001). However, environmental stress is a serious issue confronting rice production, especially the problem of salinity (Yokoi et al., 2002; Zeng et al., 2003).

Chlorophyll is an important part of chlorophyll protein complexes on the thylakoid membranes. It is the key photosynthetic pigment and its content directly reflects the photosynthetic efficiency and assimilation capacity. As a result, chlorophyll content is an important index in determining salt stress level (Munnis, 1993). Considering that Chl a is the main photosynthetic pigment (Dai et al., 2002; Santo, 2004) reduction in its quantity could probably be one of the main reasons for reduced photosynthesis under salt stress (Moradi and Ismail, 2007). Significant differences in chlorophyll concentrations under salt stress have been observed between genotypes, with the tolerant genotypes having higher Chl a, but lower Chl b, resulting in substantially higher chlorophyll a/b ratio than the moderately tolerant genotypes. Ability of the tolerant genotypes to maintain higher concentration of Chl a is probably one of the important mechanisms contributing to salinity tolerance in this genotype, which could consequently result in higher photosynthetic capacity and carbohydrate formation (Moradi and Ismail, 2007; Rout et al., 1997; Datta et al., 2009). The structural integrity of chloroplasts is also affected by salt stress (Yang et al., 2008). Decrease in total chlorophyll content may occur due to ion accumulation and functional disorders observed during stoma opening and closing under salinity stress (Seemann and Critchley, 1985; Aranda and Syvertsen, 1996; Khalehi et al., 2012; Nawaz et al., 2010). Another reason for the decrease of chlorophyll content under salt conditions is stated to be the rapid maturing of leaves (Yeo et al., 1991). Decrease in chlorophyll content under salinity stress is observed more in salt sensitive genotypes in comparison to cultivars with low tolerance (Khan et al., 2009). The observed reduction of chlorophyll in water stressed plants may be due to a reduction in the lamellar content of the light harvesting chlorophyll a/b protein (Randall et al., 1977). The efficiency of light captured to drive photosynthesis is directly correlated to the chlorophyll concentration in the leaf (Netondo et al., 2004).

CONCLUSION

Significant reduction in the concentration of Chl a, Chl b Total Chl and total carotenoids has been caused by salt stress in the case of all the rice cultivars studied presently. Reduction in Chl b content is higher when compared to reduction in Chl a content and Total Chl content. Chl a/b ratio has also been altered. The rate of reduction is proportionate to the rate of increase in salt stress. Cultivars collected from saline rice tracts show comparatively lesser reduction in pigment content when compared to the cultivars collected from non saline rice tracts. This reduction presently observed may be probably due to the inhibitory effect of the accumulated ions on the biosynthesis of the different chlorophyll fractions. As the chloroplast is membrane bound its stability is dependent on membrane stability which under high salinity condition seldom remains intact. Salt tolerance is not a function of single organ or plant attribute, but it is the product of all the plant attributes. Therefore a genotype exhibiting relative salt tolerance for all the plant attributes may be the ideal one.

References


