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ASSESSMENT OF SALT TOLERANCE IN *EUCALYPTUS*, RAIN TREE AND THAI NEEM UNDER LABORATORY AND THE FIELD CONDITIONS

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Abstract

The aim of this investigation was to discover an effective index for salt-tolerant selection in plant tissue culture system and to assay the physiological responses in a salinity field trial. Net-photosynthetic rate (NPR) in salt-tolerant clones of *Eucalyptus* (*Eucalyptus camaldulensis* Dehnh.), Rain tree (*Samanea saman* Merr.) and Thai neem (*Azadirachta siamensis* Val.) was gradually reduced when exposed to 0.17, 0.34, 0.68 or 1.02 *M* NaCl salts contained in the culture media, while that in salt-sensitive clones was sharply decreased. The reduction percentage of NPR in salt-tolerant species grown under salt stress was lower than that in salt-sensitive species by a factor of 2-3 folds. The NPR reduction in plant species grown under salt-stress was evidently investigated as effective index for salt-tolerance. In addition, physiological characteristics, chlorophyll content and maximum quantum yield of PSII (F_v/F_m), in salt-tolerant clones were significantly adapted to salinity field trial at Mahasarakham province, Northeastern region of Thailand, leading to high survival percentage and grew well when compared to the salt-sensitive clones. The salt-tolerant clones of forest tree species can be further used for salinity phytoremediation and ecological succession.

Introduction

Saline affected areas are dividing into inland and coastal salinity areas, which are a serious problem in many regions of the world *i.e* Africa, Asia, Europe, Latin America, Near East, Australia and North America. The salinity areas are widely spread over 397 million hectares worldwide, which are three times larger than agricultural area (Anon., 2000). The inland salinity area is one of the major problems which is continuously expanding, depending on natural underground salt, unsustainable agricultural cultivation, low quality irrigation, industrial waste and human-induced salinization (Pitman & Läuchli, 2002). Saline soil is an area comprising of various mineral salts in both cations, Na^+, Ca^{2+}, Mg^{2+}, and K^+, and anions, Cl^-, SO_4^{2-}, HCO^{3-}, CO_3^{2-}, and NO_3^- (Tanji, 2002). The ions directly affect plant growth and development causing either osmotic effect or ionic effect (Neumann, 1997; Mansour & Salama, 2004; Parida & Das, 2005; Chinnusamy *et al*., 2005; Läuchli & Grattan, 2007). A primary response of plants exposed to salt stress is a decrease in plant water potential, resulting in a detrimental of water use efficiency (Glenn & Brown, 1998). The defense response of higher plants to salt stress is a complex system, which depends on a particular stage of morphological and developmental processes, salt tolerant ability (halophyte or glycolphyte) and environmental effects (Ashraf & Harris, 2004). The responses of each genotype are displayed as the cascades of their genetic background and phenotypic expressions. Salt-tolerant or halophytic species seem to lack the unique metabolic machinery, which is

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sensitive to or activated by high toxic-ions, especially Na^+ and Cl^-. Defense responses of halophilic species comprise of many mechanisms such as osmoregulation, ion-homeostasis, antioxidant and hormonal systems (Hasegawa *et al*., 2000; Ashraf & Foolad, 2007). Therefore, there are many reports that depict that salt-tolerant species can be categorized using physiological criteria such as chlorophyll content and chlorophyll fluorescence (Percival & Fraser, 2001). In that case, net-photosynthetic rate (NPR) is one of the candidate physiological parameters, which is simple, rapid, and sensitive method to establish the salt-tolerance index (Ashraf, 2004).

A major cause of salinity problem is a human activity i.e., deforestation, waste releasing, poor water irrigation, enriched fertilizer etc., (Pannell &d Ewing, 2006). Reforestation is one of the most practical and effective strategies to solve the saline soil problems as phytoremediation. Trees cause remediation of saline soil in terms of lowering saline water table, using underground water and decreasing evaporation rate (Barrett-Lennard, 2002). However, the lack of salt-tolerant species is an important barrier to solve this problem. Screening for salt tolerance has been investigated in woody plant species such as Thai neem (Cha-um *et al*., 2004), olive (Melgar *et al*., 2008; Marin *et al*., 1995), *Eucalyptus* (Marcar *et al*., 2002), acacia (Nguyen *et al*., 2004), pine (Khasa *et al*., 2002), toothbrush tree (Ramoliya *et al*., 2004) and mulberry (Tewary, 2000). Photoautotrophic growth of *In vitro* plantlets is one of fruitful technology to grow the plant with enhanced photosynthesis, leading to stimulation of the physiological, anatomical and morphological characteristics (Kozai *et al*., 1997). The plantlets cultured under a photoautotrophic system should express phenotypic responses to salt stress, which are more reflective mimic to those grown *ex-vitro*. The photoautotrophic system has been successfully applied to study the salt stress responses in *Albizzia lebbek* (Kirdmanee *et al*., 1997) and screening for salt tolerance (Kirdmanee & Mosaleeyanon, 2000; Cha-um *et al*., 2004). Nevertheless, the *In vitro* environments are quite different from *ex-vitro* conditions, causing on erratic evaluation of salt-tolerant selections. Field trial of salt-tolerant lines from *In vitro* selections is necessarily required to confirm the adaptation and degree of salt-tolerance. In this study, *Eucalyptus*, Rain tree and Thai neem were chosen as the model plant species, which are widely distributed in all regions of Thailand and dominated in conditions of nutrient deficiency, drought or salinity (Koul *et al*., 1990; Akilan *et al*., 1997; Staples & Elevitch, 2005). The objective of this investigation was to discover an effective index for *In vitro* salt-tolerant selection and to assay the physiological responses in a salinity field trial.

Materials and Methods

I. Investigation of salinity tolerant index: Salt-tolerant and salt-sensitive clones of *Eucalyptus*, Rain tree, and Thai neem from photoautotrophic *In vitro* selection were photoautotrophically cultured on liquid-MS media, following the procedure of Cha-um *et al*., (2003). After 42 days culturing, the culture media were adjusted to 0, 0.17, 0.34, 0.68 and 1.02 *M* NaCl. Carbon dioxide (CO_2) inside and outside culture vessel containing plantlets was measured using a Gas Chromatograph (GC; Model GC-17A, Shimadzu Co. Ltd., Japan) and net photosynthetic rate (NPR) was calculated according to Fujiwara *et al*., (1987). The ratio of NPR values of plantlets cultured on MS medium supplemented with 1.02 *M* NaCl (salt stress) to 0 *M* NaCl (control) were investigated as salt tolerance index (STI). The STI in each clone was calculated according to the following equation.

$$STI = \frac{\text{NPR at } 1.02\ M \text{ NaCl}}{\text{NPR at } 0\ M \text{ NaCl}}$$

The experiment was arranged in a Completely Randomized Design (CRD) with 10 replications and 4 plantlets per replication. Analysis of variance (ANOVA) was analyzed using the SPSS software (SPSS for Windows, SPSS Inc., USA) as well as mean values in each treatment were compared by Duncan's New Multiple Range Test (DMRT).

II. Physiological responses to salinity in field trials: The selected salt-tolerant and salt-sensitive clones of *Eucalyptus*, Rain tree and Thai neem from laboratory were photoautotrophically acclimatized, according to Cha-um *et al*., (2003). The plantlets were transplanted to open plastic bags (size 10W×10L×20H cm), containing a mixture of two parts of soil and one part of vermiculite. The transplanted plantlets were incubated in a glasshouse at 30±2°C ambient temperature, 75±5% relative humidity (RH) and 500±100 μmol m^{-2} s^{-1} photosynthetic proton flux density (PPFD) by natural light intensity at plant level with 10 h d^{-1} photoperiod. Six-month-old transplanting plantlets were acclimatized by irrigation with 50 m*M* NaCl under 50% shading natural light intensity for 7 days and then directly planted in either a container (0.5 m in diameter and 1.0 m in height) or salinity field at Mahasarakham, Northeastern region of Thailand. Total chlorophyll content, maximum quantum yield of PSII (F_v/F_m), plant height and survival percentage in all clones were measured in rainy, winter and summer seasons. Chlorophyll content in the third leaf of plant from shoot tip was measured using a Chlorophyll Meter SPAD-502 (Minolta, Minolta Co, Ltd, UK) as described by Hussain *et al*., (2000). The F_v/F_m of adaxial side of the leaf was monitored using a Plant Efficiency Analyser (PEA version 2.05; Hansatech Instruments Ltd., UK) in the pulse amplitude modulation mode, as previously described by Loggini *et al*., (1999).

The experiment was arranged in a Completely Randomized Design (CRD) with 10 replications and 4 plants per replication. Analysis of variance (ANOVA) was computed using the SPSS software (SPSS for Windows, SPSS Inc., USA) as well as mean values in each treatment were compared by the *t*-test. The correlation between chlorophyll content and F_v/F_m was evaluated by Pearson's correlation coefficients.

Results and Discussion

I. Investigation of salinity tolerant index: Net photosynthetic rate (NPR) of Rain tree, *Eucalyptus* and Thai neem grown under photoautotrophic *In vitro* displayed as a similar pattern that reduced with increasing the NaCl concentration in the culture media. The NPR reduction percentage of salt-tolerant clones gradually increased, while the salt-sensitive clone was sharply augmented, relating to NaCl concentrations (Fig. 1). At 1.02 *M* NaCl, the NPR of the salt-tolerant clones of *Eucalyptus*, Rain tree and Thai neem remained higher than those of salt-sensitive clones by 1.17, 1.54 and 1.45 folds, respectively (Table 1). The NPR of salt-tolerant and salt-sensitive plantlets was dramatically inhibited by high salt concentrations (0.68-1.02 M NaCl). In addition, the NPR reduction of salt-sensitive plantlets grown under salt stress quickly declined when compared to salt-tolerant plantlets. The ratio of NPR of salt-stress (1.02 M NaCl) and control plantlets was directly applied to sensitive indicator as salt-tolerant index (STI) for rapid salt-tolerant screening (Table 1).

Table 1. Net-photosynthetic rate of salt-tolerant (ST) and salt-sensitive (SS) clones of *Eucalyptus* (EU), Rain tree (RT) and Thai neem (TN) grown under varying various concentrations of sodium chloride (0-1.02 *M* NaCl) under *In vitro* photoautotrophic system for a day. Salt-tolerance index (STI) is represented by the ratio of NPR at 1.02 *M* NaCl and NPR at 0 *M* NaCl.

NaCl	Net-photosynthetic rate (μmol m^{-2} s^{-1})					
(M)	ST-EU	SS-EU	ST-RT	SS-RT	ST-TN	SS-TN
0	13.99a	13.91a	16.97a	16.62a	11.39a	11.06a
0.17	13.57ab	13.11ab	16.77a	16.47a	11.25a	10.14b
0.34	13.47ab	12.97b	16.43a	15.28a	10.76b	9.76b
0.68	12.87b	11.55b	15.29b	13.03b	10.43b	8.46c
1.02	11.69c	9.96c	14.26c	9.28c	9.81c	6.77d
STI	0.84	0.72	0.84	0.56	0.86	0.61

The different letters in each column are significantly different at $P \leq 0.01$ by New Duncan's Multiple Range test (DMRT)

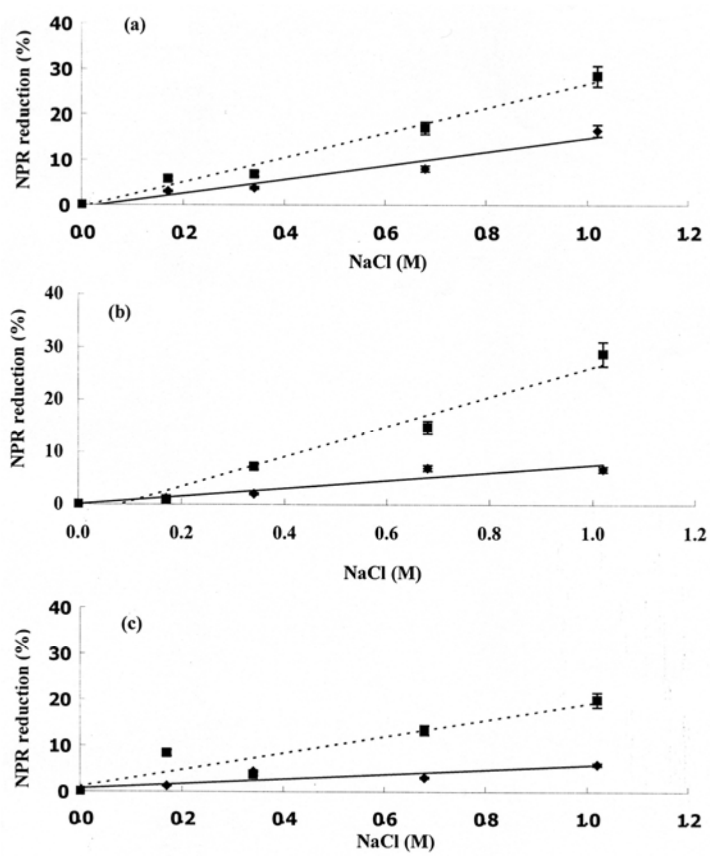

Fig. 1. Net photosynthetic rate (NPR) reduction percentage of salt-tolerant (dark-line) and salt-sensitive (dot-line) clones of *Eucalyptus* (a), Rain tree (b) and Thai neem (c) photoautotrophically grown under 0, 0.17, 0.32, 0.68 or 1.02 M NaCl in the culture medium for a day treatment.

There are many documents on NPR reduction of tree species, growing in the salinity condition such as *Populus* species (Sixto *et al*., 2005), tamarind (Gebauer *et al*., 2004), Thai neem (Cha-um *et al*., 2004), *Citrus* (Torrecillas *et al*., 2003) and olive tree (Chartzoulakis *et al*., 2002). Salt stress is directly inhibited either on light reaction, pigment degradation, water oxidation and electron transportation, or dark reaction, Rubisco enzyme, stomatal conductance, CO_2-assimilation and transpiration rate (Chartzoulakis *et al*., 2002; Meloni *et al*., 2003; Tezara *et al*., 2003). Moreover, the NPR reduction in plant cultured under photoautotrophic salt-stress condition had been successfully applied to use as effective index, simple method, and rapid evaluation for salt-tolerant selection (Kirdmanee & Mosaleeyanon, 2000; Cha-um *et al*., 2004). The STI of forest species, *Eucalyptus*, Rain tree and Thai neem, should be played as an effective index for salt-tolerant selection in breeding program of forest tree species.

II. Physiological responses to salinity in the field trials: Selected clones of salt-tolerant and salt-sensitive *Eucalyptus*, Rain tree and Thai neem from laboratory classification were intensively pretreated with salt-solution in the field trial environments of the salinity field. These plants were greatly adapted to those conditions in both salinity field and container of the rainy season in May-October 2004 because of high precipitation rate at 100-250 mm per month with high relative humidity (RH). Plant height of salt-tolerant clones in *Eucalyptus*, Rain tree and Thai neem was significantly higher than those salt-sensitive clones in both salinity field and container. In addition, the plant height of forest species grown in the container exhibited more than those grown on the salinity field trial in all seasons (Table 2). In the winter season of salinity field, plant height of salt-tolerant clones in *Eucalyptus*, Rain tree and Thai neem was better than those salt sensitive clones by the factors of 1.14, 1.54 and 1.65 folds, respectively. In rainy season, survival percentage of salt-sensitive clones of *Eucalyptus* and Rain tree was 100%, while Thai neem was gradually reduced to 80-90%. On the other hand, the survival percentage of salt-sensitive clones of forest species grown on both salinity field and container continuously decreased higher than those salt-tolerant clones, especially in the winter and summer seasons. As well as, the salt sensitive clone of Thai neem absolutely died in the salinity field of summer season (Fig. 2). In all seasons, chlorophyll pigment concentrations in the leaf tissues of salt-sensitive clones were significantly dropped higher than those salt-tolerant species when grown in both salinity field and container. The chlorophyll concentration in salt-tolerant clones of *Eucalyptus*, Rain tree and Thai neem was higher than those salt-sensitive clones by the factors of 1.10, 1.24 and 1.28 folds, respectively in the salinity field of rainy season. The chlorophyll contents in both salt-tolerant and salt-sensitive species were gradually reduced in the winter season and then increased in the summer season, except salt-sensitive clone of Thai neem died already in the salinity field (Fig. 3). In the similar pattern, maximum quantum yield (F_v/F_m) of salt-sensitive forest species was significantly reduced lower than those salt-tolerant species, especially in the winter season (Fig. 4). Moreover, the chlorophyll concentrations in the leaf tissues of *Eucalyptus*, Rain tree and Thai neem were positively related to F_v/F_m (r = 0.83, r = 0.85 and r = 0.88, respectively) (Fig. 5). It means that the adaptation ability of salt-tolerant species in salinity field was better than those salt-sensitive species, identifying by chlorophyll content and F_v/F_m, leading to high survival percentage and growth performance in salinity field.

Table 2. Plant height (cm) of salt-tolerant (ST) and salt-sensitive (SS) clones of Eucalyptus (EU), Rain tree (RT) and Thai neem (TN) grown in a salinity field in the rainy, winter and summer seasons.

Season	**Plant height (cm)**					
	ST-EU	**SS-EU**	**ST-RT**	**SS-RT**	**ST-TN**	**SS-TN**
Rainy						
Container	83.0a	62.9b	52.1a	43.9b	18.3a	13.6b
Salinity field	55.0a	43.1b	47.0a	40.4b	18.4a	13.5b
Winter						
Container	158.4a	125.0b	83.0a	62.9b	45.2a	31.9b
Salinity field	145.4a	120.5b	66.2a	43.1b	45.0a	27.3b
Summer						
Container	168.4a	132.1b	95.4a	80.7b	52.5a	36.1b
Salinity field	165.6a	127.3b	77.0a	58.8b	52.9	*

The different letters in each column are significantly different at $P \leq 0.01$ by *t*-test
* The plants absolutely died.

The conduction of field trial in a salinity area is one of the most important practices to evaluate the salt-tolerant ability of many plant species such as poplar (Sixto *et al*., 2005), desert plants (Arndt *et al*., 2004), *Eucalyptus* (Mahmood *et al*., 2003; Morris *et al*., 2006), tree species (Tomar *et al*., 2003), deciduous tree species (Paludan-Muller *et al*., 2002), and pannonian endemism (Mile *et al*., 2002). The salt-tolerant species are significantly adapted to salinity by several defense mechanisms, limited-ions absorption and translocation (Chartzoulakis *et al*., 2002; Paludan-Muller *et al*., 2002; Fernandez-Ballester *et al*., 2003; Estan, *et al*., 2005), osmoregulation *i.e.* proline (de Lacerda *et al*., 2005; Misra & Gupta, 2005), soluble carbohydrate (de Lacerda *et al*., 2005), and glycine betaine (Khan *et al*., 1998), and antioxidation (Vaidyanathan *et al*., 2003) with regular biochemical and physiological characteristics *i.e*., chlorophyll stabilization (Misra & Gupta, 2005), water use efficiency (Tezara *et al*., 2003), chlorophyll fluorescence (Percival & Fraser, 2001), and net-photosynthetic rate (Tezara *et al*., 2003), leading to growth stimulation as well as high survival percentage (Chartzoulakis *et al*., 2002; Torrecillas *et al*., 2003; Nasim *et al*., 2008). The ability of salt-tolerance depends on various factors such as salt-concentration (Chartzoulakis *et al*., 2002; Gebauer *et al*., 2004), plant species (Percival & Fraser, 2001; Sixto *et al*., 2005; Nasim *et al*., 2008), and plant varieties (Chartzoulakis *et al*., 2002; Mahmood *et al*., 2003; Torrecillas *et al*., 2003; Sixto *et al*., 2005). In our results of the present study show that the salt-tolerant species of forest tree species significantly adapted to realistic saline soil and environments in terms of physiological adaptations, resulted in high survival percentage when compared to the sensitive species. In addition, the survival percentage of forest species was positively related to winter and summer seasons because of the low relative humidity with high light intensity and high temperature in the field trial. It means that the environmental conditions of a salinity field trial, especially summer season should be essentially considered while classifying plants for salt tolerance.

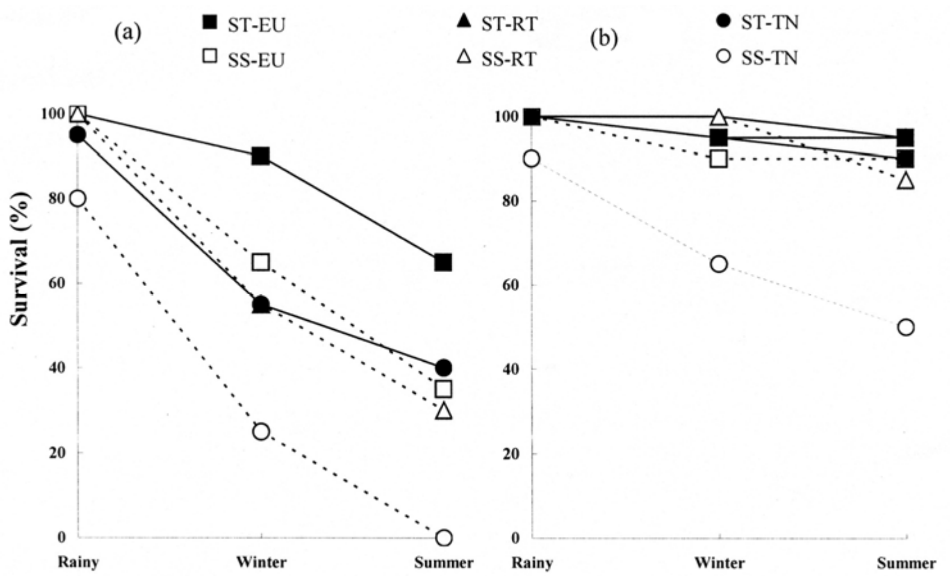

Fig. 2. Survival percentage of salt-tolerant (ST) and salt-sensitive (SS) clones of *Eucalyptus* (EU), Rain tree (RT) and Thai neem (TN) grown in a salinity field (a) and in a container (b) in the rainy, winter and summer seasons.

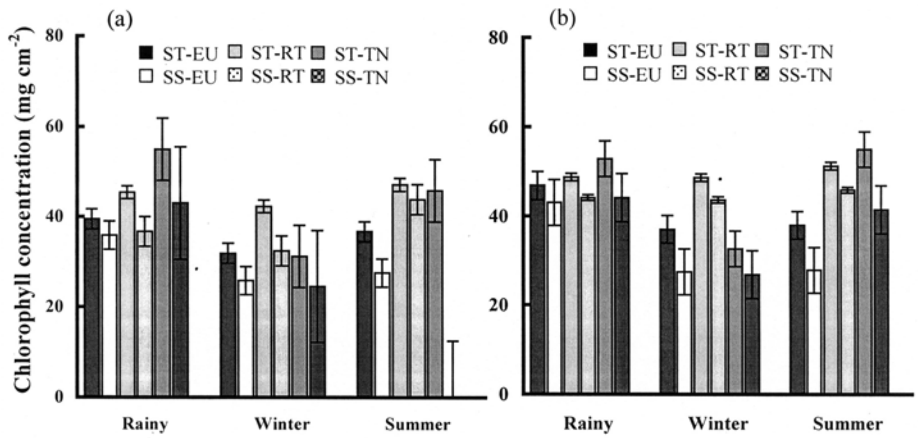

Fig. 3. Chlorophyll concentration in the leaf tissues of salt-tolerant (ST) and salt-sensitive (SS) clones of *Eucalyptus* (EU), Rain tree (RT) and Thai neem (TN) grown in a salinity field (a) and in a container (b) in the rainy, winter and summer seasons.

Conclusion

Salt-tolerant index (STI) of *In vitro* photoautotrophic plantlets was a more rapid indicator for selection for salt-tolerance. The salt-tolerant species of forest trees, *Eucalyptus*, Rain tree, and Neem tree, identified by STI were greatly acclimatized to salinity field in terms of chlorophyll stabilization and water oxidation, resulting in high survival percentage with growth stimulation. The salt-tolerant forest species should be further reforested in salinity areas as an efficient phytoremediation.

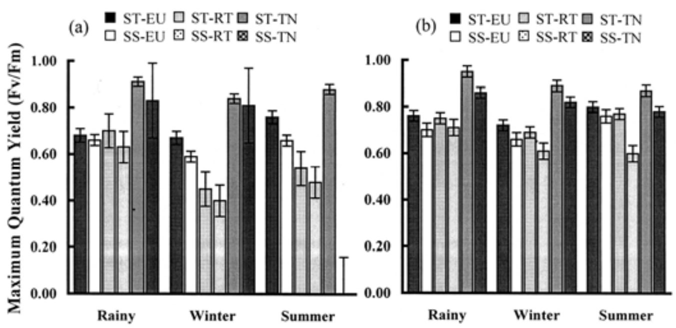

Fig. 4. Maximum quantum yield (F_v/F_m) in the leaf tissues of salt-tolerant (ST) and salt-sensitive (SS) clones of *Eucalyptus* (EU), Rain tree (RT) and Thai neem (TN) grown in a salinity field (a) and a container (b) in the rainy, winter and summer seasons.

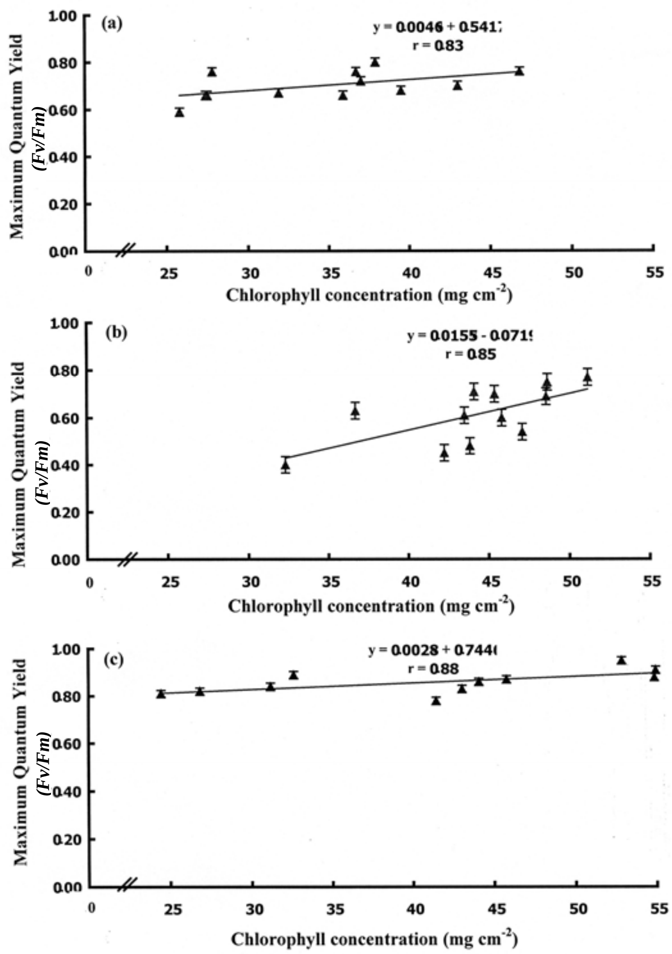

Fig. 5. Relationship between chlorophyll concentration and maximum quantum yield (F_v/F_m) of *Eucalyptus* (a), Rain tree (b) and Thai neem (c) grown in a salinity field.

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