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Submergence-tolerant rice withstands complete submergence even in saline water: Probing through chlorophyll *a* fluorescence induction O-J-I-P transients

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Abstract

Plants experience multiple abiotic stresses during the same growing season. The implications of submergence with and without saline water on growth and survival were investigated using four contrasting rice cultivars, FR13A (submergence-tolerant, salinity-susceptible), IR42 (susceptible to salinity and submergence), and Rashpanjor and AC39416 (salinity-tolerant, submergence-susceptible). Though both FR13A and IR42 showed sensitivity to salinity, FR13A exhibited higher initial biomass as well as maintained greater dry mass under saline condition. Greater reduction of chlorophyll (Chl) contents due to salinity was observed in the susceptible cultivars, including FR13A, compared to the salinity-tolerant cultivars. Exposure of plants to salinity before submergence decreased the survival chance under submergence. Yet, survival percentage under submergence was greater in FR13A compared to other cultivars. Generally, the reduction in the Chl content and damage to PSII were higher under the submergence compared to salinity conditions. The submergence-tolerant cultivar, FR13A, maintained greater quantities of Chl during submergence compared to other cultivars. Quantification of the Chl *a* fluorescence transients (JIP-test) revealed large cultivar differences in the response of PSII to submergence in saline and nonsaline water. The submergence-tolerant cultivar maintained greater chloroplast structural integrity and functional ability irrespective of the quality of flooding water.

Additional key words: energy flux; *Oryza sativa*; performance index; saline flooding water; stress tolerance.

Introduction

To keep pace with the ever growing global population, progressive and sustained increase in rice production is necessary, especially in areas with extremely variable climatic conditions as in eastern Indian deltaic plains. Rice production in most coastal deltas is adversely affected by numerous abiotic stresses, such as submergence, water-logging, drought, and salinity, which normally prevail through part or most of the growth season (Wassmann *et al*. 2009). The impact is anticipated to be greater due to climate change (Wassmann *et al*. 2009). The sea level along the Indian coast has been rising at an average rate of about 1.3 mm per year and the trend is likely to continue in the future. There is possibility of an increase in flooding by about 10–30% over the existing magnitudes (World Bank

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Abbreviations: ABS/RC – quantum of light absorption per active reaction centre [$M_0 \times (1/V_J) \times (F_m/F_v)$]; Chl – chlorophyll; C-SU-NW – normal growth, submergence with nonsaline water; C-SU-SW – normal growth, submergence with 12 dS m^{-1} saline water; ET_0/ABS – electron transport per quantum of absorption of light [$(F_v/F_m) \times (1 - V_J)$]; ET_0/RC – electron transport per active reaction centre [$M_0 \times (1/V_J) \times (1 - V_J)$]; F_0 – minimal fluorescence; F_m – maximal fluorescence; F_v – variable fluorescence ($F_m - F_0$); F_v/F_m – maximum photochemical efficiency of PSII; $F_{50\mu s}$, $F_{150\mu s}$, $F_{300\mu s}$, and F_{2ms} – fluorescence intensity at 50, 150, or 300 μs, and 2 ms, respectively; M_0 – initial slope of relative variable chlorophyll fluorescence [$4 (F_{300\mu s} - F_{50\mu s})/F_v$]; OEC – oxygen evolving complex; PI_{ABS} – performance index on the basis of utilization of absorbed energy; RC/CS_0 – number of reaction centres per excited cross-section [$F_v/F_m \times (V_J/M_0) \times F_0$]; S-SU-NW – 12 dS m^{-1} saline treatment before submergence, submergence with nonsaline water; S-SU-SW – 12 dS m^{-1} saline treatment before submergence, submergence with 12 dS m^{-1} saline water; TR_0/RC – quantum of light utilized per reaction centre [$M_0 \times (1/V_J)$]; V_I – relative variable fluorescence at time 30 ms (I-step) after start of actinic light pulse [$(F_{30ms} - F_{50\mu s})/(F_m - F_{50\mu s})$]; V_J – relative variable fluorescence at time 2 ms (J-step) after start of actinic light pulse [$(F_{2ms} - F_0)/(F_m - F_0)$]; ΔV_{I-P} – amplitude of the IP-phase [$(1 - V_I) = (F_m - F_{30ms})/(F_m - F_{50\mu s})$].

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Report 2008). It is estimated that the flood-affected area has more than doubled in size from about 5% (19 million ha) to about 12% (40 million ha) of India's geographical area in the past five decades (World Bank Report 2008). A possible sea-level rise from 15 to 38 cm by the 2050s (Sarwar and Khan 2007, Douglas 2009) would cause saline water to penetrate further inland and ultimately displace *ca*. 35 million people around the Bay of Bengal, and change the conditions in other deltas and coastal plains on a similar scale (Douglas 2009, Wassmann *et al*. 2009). Analysis of rainfall pattern of Paradeep, Odisha, India, during 2005–2012 revealed that heavy rainfall events contributed nearly 40 to 50% of total rainfall during monsoon and post-monsoon periods in most of the years (unpublished, National Initiative of Climate Resilient Agriculture Annual Workshop, July 3–5, 2014). Consequences of such erratic rainfalls were that rice crop in the same season experienced drought, submergence, waterlogging, apart from salinity stress. Addressing multiple abiotic stress tolerance, as well as the consequences of one stress followed by another stress could broaden knowledge related to adaptation of crop under variable climatic conditions.

Submergence similarly as salt stress reduces plant growth and productivity. In India, out of 8.1 million ha of the salt-affected area, nearly 5.0 million ha are inland and 3.1 million ha occur along the east and west coasts (Singh and Sarkar 2014). In coastal areas, depending on rainfall pattern and tide, initial salt injury followed by submergence with or without saline water is common. (Mahata *et al*. 2010). Most of the salt-affected lands in coastal regions are monocropped with rice during the wet season. Well-adapted rice cultivars are mostly traditional landraces that are photoperiod-sensitive, less responsive to fertilizers, and prone to lodging due to tallness, with a limited yield potential and poor grain type (Sarkar and Bhattacharjee 2011). A combination of flooding and salinity is detrimental to most plants (Teakle *et al*. 2014, Duarte *et al*. 2014). The studies have been initiated to examine combined effects of salinity and waterlogging/ submergence in common reed and other uncultivated plants in relation to growth and survival (Bennett *et al*. 2009, Gorai *et al*. 2010, Rogers *et al*. 2011, Song *et al*. 2011, Chen *et al*. 2013). In contrast, literature is lacking on crop plants grown in coastal zones in relation to multiple abiotic stresses (Yan *et al*. 2013).

Changes in Chl *a* fluorescence emission arising mainly from PSII provide information on almost all aspects of photosynthetic activity (Stirbet and Govindjee 2011, Kalaji *et al*. 2011, Breštić *et al*. 2012). Photosynthetic energy conversion may be judged based on PSII activity. The sensitivity of PSII activity in response to environmental changes has made this a key technique to understand the vitality of photosynthetic apparatus of a plant (Lazár and Schansker 2009). Chl fluorescence and its association with submergence tolerance in rice has been reported earlier (Panda *et al*. 2006, Sarkar and Panda 2009), however, no information is available on the submergence damage of photosynthetic apparatus in rice, which experienced salinity stress followed by complete submergence with saline water, that is so crucial for survival and higher productivity of this crop in coastal zones (Singh *et al*. 2010). The present study characterizes the submergence sensitivity in rice which was exposed to salinity before complete submergence. Structural and functional alterations of PSII were studied by Chl *a* fluorescence emission. Plant responses to single abiotic stress (either salt or submergence) differ greatly from multistress response. Therefore, the main goal of the current investigation was to find out the extent of adaptive mechanisms to salinity followed by submergence stress with and without saline water in rice. Another objective was to elucidate whether submergence-tolerant type was equally capable to counteract the submergence effect with saline water or not.

Materials and methods

Experiment 1

Plants, salinity treatment, and growth conditions: The study was performed with four rice cultivars (*Oryza sativa* L.), namely FR13A (submergence-tolerant but susceptible to salinity), IR42 (susceptible to both submergence and salinity), Rashpanjor (a non-Pokkali type, salinity-tolerant cultivar), and Pokkali (Accession No. 39416, highly tolerant to salinity, but susceptible to submergence). Rapid screening method for evaluation of responses in genotypes to salinity stress at a seedling stage was carried out (Sarkar *et al*. 2013). Seeds were placed in an oven for five days at 50°C to break the dormancy and ensure uniform germination. Seeds were then surface sterilized with 0.1% $HgCl_2$ solution for 5 min and thoroughly washed with sterilized distilled water. Sterilized seeds were placed in Petri dishes with moisten filter paper and incubated at 30°C for 48 h for germination. Two pregerminated seeds were placed in each hole on the styrofoam seedling float suspended on plastic trays (37.5 × 35.0 × 13.0 cm) filled with the Yoshida culture solution (Yoshida *et al*. 1976). The solution was changed every fifth day and the pH was maintained at 5.0 daily. Each time, seedlings along with the styrofoam were transferred into new trays containing solution of a desired salt concentration. The pH was maintained with a portable pH meter (*model 250A, Orion*, UK) using either 1.0 N NaOH or HCl solutions. After five days of growth in the normal Yoshida culture solution, the seedlings were transferred to the solution containing NaCl (EC of 6 dS m^{-1}) for hardening. Salt stress was raised to EC of 12 dSm^{-1} after further 5 days of growth at EC of 6 dSm^{-1}. After 7 days of growth at EC of 12 dSm^{-1} plants were harvested to determine growth

parameters and a nonstructural carbohydrate content. After harvesting, the submergence treatment was provided on the rest of the salt-treated plants. A control set was kept without any addition of salt in the Yoshida culture solution. Average maximum and minimum temperatures during the study period were 31.2 and 25.5°C, respectively. Relative humidity was moderate to high (76–94%). The experiment was conducted using a factorial randomized complete block design with three replications. The light intensity during the period of experiment varied from 720 to 1,050 µmol(photon) m^{-2} s^{-1}.

Cultivars, treatments, numbers of plants, and trays used in each experiment. #Survival count was taken after 7 d of withdrawal of submergence.

Cultivar	Treatment	Description	No. of plants	Tray No.	Duration of experiment [d]
Experiment 1					
FR13A, IR42, Rashpanjor, Pokkali	C	Control (without any stress)	40	6	19
	S	Salt stress 7 d at EC 12 dS m^{-1}			
Experiment 2					
FR13A, IR42, AC39416	C	Control (without any stress)	60	18	C, C-SU-NW, S-SU-NW, C-SU-SW, S-SU-SW: 26# S: 19
	S	Salt stress for 7 d at EC 12 dS m^{-1}			
	C-SU-NW	Normal growth, submergence with nonsaline water			
	S-SU-NW	12 dS m^{-1} saline treatment before submergence, submergence with nonsaline water			
	C-SU-SW	Normal growth, submergence with 12 dS m^{-1} saline water			
	S-SU-SW	12 dS m^{-1} saline treatment before submergence, submergence with 12 dS m^{-1} saline water			

Dry mass (DM) determination: After 7 d of the salt treatment, shoot portions combined of both leaves and stems from each treatment were harvested, oven-dried at 65°C for three days, and the biomass was determined. Ten plants per treatment in each replication were used for this analysis.

Nonstructural carbohydrate estimation: Soluble sugar and starch contents of the shoots were estimated after 7 d of the salinity (S) treatment (EC of 12 dS m^{-1} ≈ 103 mM NaCl) and respective non-saline control (C) plants following the procedure of Yoshida *et al*. (1976) with three replications. Briefly, for each measurement, shoot samples of ten plants were oven-dried and ground to a fine powder and extracted using 80% ethanol (v/v). The extract was then used for soluble sugar analysis after addition of the anthrone reagent, followed by a measurement of absorbance at 630 nm using a spectrophotometer (*SL 164 double beam*, *ELICO*, Hyderabad, India). The residue remaining after the soluble sugar extraction was dried and extracted using perchloric acid and then analyzed for starch (as glucose equivalent) using the same anthrone reagent as for soluble sugars.

Experiment 2

Plant survival under complete submergence: Seedlings were raised in the same way as in the experiment 1. After 7 d of growth under salt stress (EC of 12 dS m^{-1}), the cultivation solution was decanted and the plastic trays with seedlings were immediately submerged under 80 cm of water for another 7 d under saline (EC of 12 dS m^{-1}) and nonsaline water, respectively. The plants remained completely under water during the period of submergence (SU plants). After 7 d of submergence, the plants were re-exposed to air for 7 d under normal conditions in which rice was cultivated, *i.e*. with 2 cm of stagnant water above the base of the plant, to determine the survival percentage. The characteristics of the floodwater in terms of light availability were measured at 11:30 h (*LI-189*, *LI-COR*, Lincoln, USA) and water temperature and oxygen concentration were determined at 06:00 and 17:30 h (*Simplair-F-5*, *Syland Scientific*, Heppenheim, Germany). Light intensity at 60 cm water depth or at the vicinity of a canopy level was 259 ± 16 (mean ± SD) µmol(photon) m^{-2} s^{-1}, whereas it was 1,764 ± 59 µmol(photon) m^{-2} s^{-1} above the water surface. The oxygen concentrations at the same water depth were 5.11–5.22 (5.16 ± 0.03) mg L^{-1} and 5.95–6.14 (6.08 ± 0.06) mg L^{-1} whereas pH of floodwater ranged from 7.81 to 8.25 (8.02 ± 0.17) and 8.96 to 9.10 (9.04 ± 0.04) at 06:00 and 17:30 h, respectively. The floodwater temperatures varied from 27.3–29.5°C (28.4 ± 0.9) throughout the experiment.

Chl *a* fluorescence was measured on the fully expanded leaf (the second leaf from the top) using three rice cultivars namely, FR13A, IR42, and AC39416, 1 h after

recovery from submergence using a plant efficiency analyzer, *Handy PEA* (*Hansatech Instruments*, Norfolk, UK) and recorded from 0.01 ms up to 1 s, with a data acquisition every 0.01 ms for the first 0.30 ms, then every 0.10 ms up to 3.0 ms, every 1 ms up to 30 ms, every 10 ms up to 300 ms, and after that every 100 ms. During the 1-s time period, 118 data points were obtained. The signal resolution was 12 bits (0–4,000). For each treatment, the Chl *a* fluorescence transients of nine individual leaves were measured on control plants (C), on salinity-treated plants (S), and plants grown under control and salinity conditions followed by submergence under saline and nonsaline flooding water. Leaves were maintained in darkness for 20 min before recording the Chl fluorescence data. The maximal intensity of the light source, providing a saturating pulse of 3,000 μmol(photon) m^{-2} s^{-1}, was used. From the fast O-J-I-P transients, several bioenergetic parameters were derived according to the equations of the JIP-test using the program *BIOLYSER* (*Bioenergetic Laboratory*, University of Geneva, Switzerland).

To visualize the L-band between O- and J-step, Chl *a* fluorescence transient of each cultivar were double normalized between F_0 (0.05 ms) and F_K (0.30 ms) as follows: $(F_{100\mu s} - F_0)/(F_{300\mu s} - F_0)$ (Oukkarroum *et al*. 2007, Desotgiu *et al*. 2012). Further, in order to compare the amplitude of the K-band between O- and J-step, the transients of each cultivar were double normalized at F_0 and F_J: $(F_{300\mu s} - F_0)/(F_J - F_0)$ (Lazár 1999, 2006; Jiang *et al*. 2006). In both cases, the control transients were then subtracted from the transients of the treated leaves. The resulting difference transients were multiplied by ten for clear visualization.

Chl estimation: After measuring the Chl fluorescence characteristics, the same leaves were used for the measurement of the total Chl content, which comprised both Chl *a* and Chl *b*. Finely chopped fresh leaves (100 mg) were placed in a capped measuring tube containing 25 mL of 80% acetone, and placed inside a refrigerator (4°C) for 48 h. The Chl was measured spectrophotometrically (*model SL 164, Elico*, India) following Porra (2002).

Statistical analysis: Differences between shoot DM, nonstructural carbohydrates, and Chl contents and various Chl fluorescence parameters were compared by analysis of variance (*ANOVA*) using *CropStat* (*International Rice Research Institute*, Philippines) software at least significant difference (LSD, $p<0.05$), provided the *F*-test was significant. Separate analysis was done for two experiments based on a randomized complete block design model with three replications in each experiment. In the case of the experiment 1, the number of treatments was two, whereas in the case of the experiment 2, six treatments were analysed. The p ($LSD_{*p<0.05}$) of the overall *ANOVA* for a cultivar, treatment, and cultivar × treatment interaction for different Chl fluorescence parameter are presented in Table 1. *Tukey*'s test was done using the *ASSISTAT version beta 7.6* (www.assistat.com).

Results

Experiment 1

Shoot DM and nonstructural carbohydrates: Significant genotypic differences were observed in shoot DM accumulation under both C and S conditions (Fig. 1*A*). In general, shoot DM was greater under the C as compared with the S conditions. In both treatments, shoot DM was significantly lower in IR42 than in the other three cultivars. The reduction in shoot DM due to salt stress was more than 38% in IR42. In other three tested cultivars, the reduction of shoot DM was 15.0, 12.4, and 6.8% in

Table 1. The p ($LSD_{*p<0.05}$) of the overall *ANOVA* for the cultivar (FR13A, IR42, and AC39416), treatment, and cultivar × treatment interaction for different chlorophyll (Chl) fluorescence parameters. F_0 – minimal fluorescence; F_m – maximal fluorescence; F_v/F_m – maximum photochemical efficiency of PSII; RC/CS_0 – number of reaction centres per excited cross-section; ABS/RC – quantum of light absorption per active reaction centre; ET_0/ABS – electron transport per quantum of absorption of light; ET_0/RC – electron transport per active reaction centre; TR_0/RC – quantum of light utilized per reaction centre; $(1 - V_J)$ – the efficiency with which the trapped excitons move an electron further than Q_A^-; ΔV_{I-P} – amplitude of the IP-phase; PI_{ABS} – performance index on the basis of utilization of absorbed energy. ns – not significant.

Chl fluorescence parameter	Cultivar	Treatment	Cultivar × treatment
F_0	ns	31	54
F_m	ns	166	288
F_v/F_m	0.012	0.017	0.030
RC/CS_0	7.68	10.87	18.82
ABS/RC	0.083	0.117	0.204
ET_0/ABS	0.021	0.030	0.052
ET_0/RC	0.050	0.071	0.123
TR_0/RC	0.086	0.114	0.202
$1 - V_J$	0.027	0.039	0.067
ΔV_{I-P}	0.019	0.026	0.046
PI_{ABS}	1.761	2.491	4.315

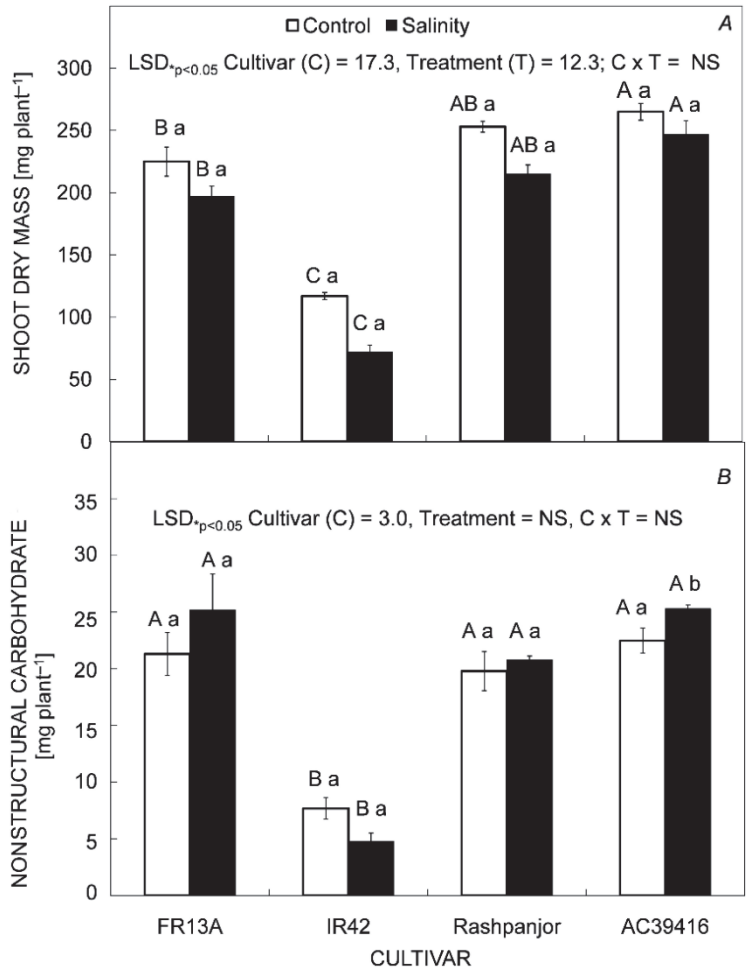

Fig. 1. Shoot dry mass (*A*) and nonstructural carbohydrate content (*B*) before submergence and after 7 d of 12 dS m^{-1} saline treatment. Experiment 1. C – control; S – 12 dS m^{-1} saline treatment. n = 3. Bars represent standard errors. The *Tukey*'s test at 5% probability level was applied. Cultivars followed by the same *uppercase letter* across control or saline treatment do not differ statistically. Cultivar × treatment interaction followed by the same *lowercase letter* does not differ statistically. LSD – least significant difference; NS – not significant.

Rashpanjor, FR13A, and AC39416, respectively, under S compared with C conditions. Interestingly, the genotypes with greater shoot DM under the C conditions maintained higher DM under the S conditions. Unlike shoot DM, the nonstructural carbohydrate content under S either increased or decreased in different rice cultivars (Fig. 1*B*). The nonstructural carbohydrate content decreased in IR42, whereas it increased in other three cultivars under S compared with C (Fig. 1*B*). Significant genotypic differences were observed in the nonstructural carbohydrate content under the C condition only.

Experiment 2

Survival percentage due to complete submergence (SU) was greater in tolerant cultivar FR13A irrespective of the quality of the flooding water (Fig. 2). The survival of FR13A, however, varied from 47 to 100% depending on salinity of the flooding water and exposure to S stress before SU. The impact of SU with saline water (SW) was greater when plants experienced S stress before SU. The survival percentage was merely 47% in the SU-tolerant cultivar, FR13A. SW of flooding water had little or no effect on the SU-tolerant plants, which were grown under normal condition. Among the four cultivars, IR42 and Rashpanjor were found to be highly susceptible, whereas AC39416 was intermediate type.

Chl content: Large genotypic differences were found in the Chl content under S as well as SU stress (Fig. 3). The values of the Chl content under S were greater in AC39416 compared to FR13A and IR42. The Chl content did not decrease significantly in the S-tolerant cultivar, AC39416, due to S stress, whereas the values greatly decreased in the S-susceptible cultivars FR13A and IR42. The reduction was greater in IR42, the cultivar susceptible to both S and SU compared to FR13A (SU-tolerant) and AC39416 (S-tolerant). The decrease in the content of Chl under SU was greater if plants were S-stressed before SU. The reduction of Chl was greater under flooding with SW compared to NW. Yet, the SU-tolerant cultivar FR13A maintained greater quantities of Chl under SU compared to AC39416 and IR42, irrespective of the quality of flooding water.

Analysis of Chl *a* fast fluorescence transients: The present investigation characterized the main effect of S and SU on the function of PSII in different rice cultivars as observed by the Chl *a* fluorescence induction kinetics

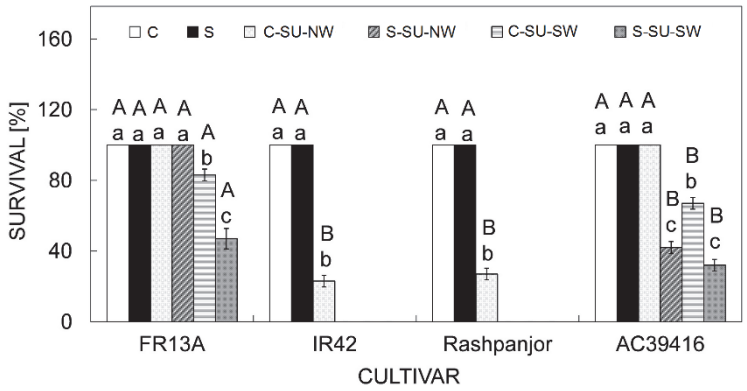

Fig. 2. Survival after 7 d of complete submergence under nonsaline and saline (12 dS m^{-1}) water. Experiment 1. C – control; S – 12 dS m^{-1} saline treatment; C-SU-NW – normally grown, submergence with nonsaline water; C-SU-SW – normally grown, submergence with 12 dS m^{-1} saline water; S-SU-NW – 12 dS m^{-1} saline treatment before submergence, submergence with nonsaline water; S-SU-SW – 12 dS m^{-1} saline treatment before submergence, submergence with 12 dS m^{-1} saline water. n = 3. Bars represent standard errors. The *Tukey*'s test at 5% probability level was applied. Cultivars followed by the same *uppercase letter* across the treatment do not differ statistically. Cultivar × treatment interaction followed by the same *lowercase letter* does not differ statistically.

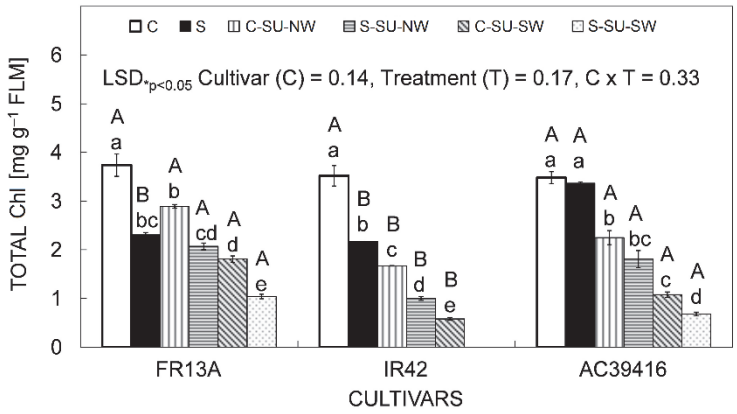

Fig. 3. Reduction of the chlorophyll content due to salt and submergence treatment with nonsaline and saline water. Experiment 2. C – control; S – 12 dS m^{-1} saline treatment; C-SU-NW – normally grown, submergence with nonsaline water; S-SU-NW – 12 dS m^{-1} saline treatment before submergence, submergence with nonsaline water; C-SU-SW – normally grown, submergence with 12 dS m^{-1} saline water; S-SU-SW – 12 dS m^{-1} saline treatment before submergence, submergence with 12 dS m^{-1} saline water. n = 3. Bars represent standard errors. The *Tukey*'s test at 5% probability level was applied. Cultivars followed by the same *uppercase letter* across the treatment do not differ statistically. Cultivar × treatment interaction followed by the same *lowercase letter* does not differ statistically.

(Fig. 4). The magnitude of fluorescence signal rose from the initial fluorescence level (0 ≈ F_0) to the maximum level (P ≈ F_m) with intermediate steps J and I. Distinct treatment effects were observed in the rise of F_0 to F_m. The relative values of P were greater in C followed by S plants. However, normalization of data at F_0 and F_m level showed that compared with C there was a rise of fluorescence due to stress. The rise was greater in the SU samples compared to S samples. Under S stress, the rise was higher in FR13A, followed by IR42 and AC39416, whereas under SU, the rise was greater in AC39416, followed by IR42.

The appearance of the L-band was either in a negative or positive direction from the C condition depending on the nature of stress and cultivar × environment interaction. L-band did not appear on a positive side compared with C under salt stress both in the S-sensitive and tolerant cultivars (Fig. 5*A,B,C*). Variations in the appearance of the L-band occurred under SU in different rice cultivars. In FR13A, the SU-tolerant cultivar, none of positive deviations from C occurred, whereas in IR42, the SU-susceptible cultivar, the positive deviation from C happened under SU with SW. In case of AC39416 (SU-susceptible and S-tolerant), the distinct positive deviation occurred under SU. The appearance of the K-band was more prominent. The deviation was in a positive direction from C (Fig. 5*D,E,F*). The deviation was more positive under SU compared to S. Comparison of the deviations among three cultivars under SU revealed that in the SU-tolerant cultivar, FR13A, the deviations were lesser compared to intolerant cultivars. SU with SW showed greater deviations of the K-band compared to SU with NW in the intolerant cultivars (Fig. 5*E,F*).

Chl fluorescence parameters: The PSII activity was studied by measuring different Chl fluorescence parameters in a dark-adapted leaf. The parameters changed significantly due to treatments (Tables 1, 1S – *supplement available online*). The cultivar × treatment interaction was also significant. Cultivar differences for the parameters F_0 and F_m, however, were not significant. Values of the fluorescence parameters, such as F_0, F_m, F_v/F_m, RC/CS_0, ABS/RC, ET_0/ABS, ET_0/RC, $1 - V_J$, ΔV_{I-P}, and overall performance index (PI_{ABS}) changed due to S stress and subsequent period of SU with and without SW compared with the C (Fig. 6, Table 1S). The F_v/F_m values decreased, whereas the values of F_0 increased due to S stress in the S-susceptible cultivars, FR13A and IR42, compared with C. Under SU, the values of F_v/F_m decreased greatly compared with C, however, no such trend was observed in the case of F_0 values. FR13A maintained, in general, greater values of

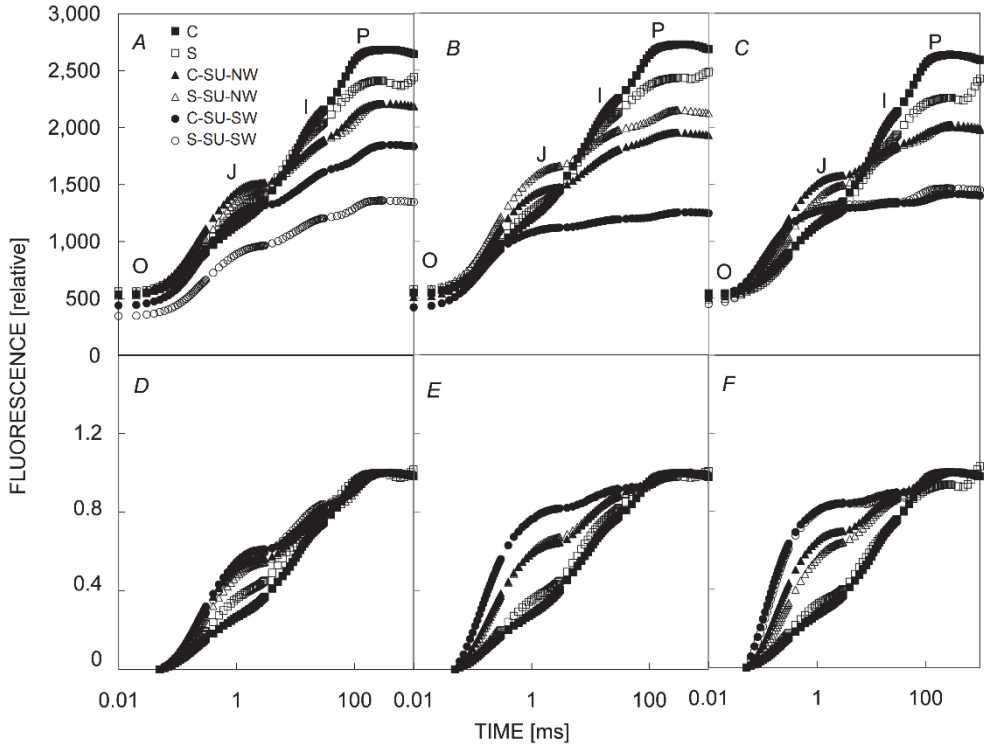

Fig. 4. The fast fluorescence rise in rice leaves subjected to salinity, submergence with nonsaline and saline water of three cultivars namely, FR13A (submergence-tolerant, susceptible to salinity), IR42 (susceptible to both salinity and submergence), and AC39416 (salinity-tolerant, susceptible to submergence). *A,D* – FR13A; *B,E* – IR42; *C,F* – AC39416. The measurements were carried out on fully expanded mature leaves, 2nd from top. Each curve was based on average data of nine measurements. A typical fluorescence transient (O-J-I-P) exhibited upon illumination of a 20 min dark-adapted leaf both in control and stress plant with greater reduction of maximal fluorescence ($P \approx F_m$) in stressed plant (*A,B,C*). The fluorescence at 50 μs, 2 ms, and 30 ms are known as O-, J-, and I-phases, respectively. The highest peak in the curve was designated as P. Normalization at F_0 and F_m revealed that a positive deviation of fluorescence rise from control to stress occurred (*D,E,F*), signifying the damage of PSII. Experiment 2. C – control; S – 12 dS m^{-1} saline treatment; C-SU-NW – normally grown, submergence with nonsaline water; S-SU-NW – 12 dS m^{-1} saline treatment before submergence, submergence with nonsaline water; C-SU-SW – normally grown, submergence with 12 dS m^{-1} saline water; S-SU-SW – 12 dS m^{-1} saline treatment before submergence, submergence with 12 dS m^{-1} saline water.

F_v/F_m compared to other two cultivars under SU stress. The changes in the values of ET_0/ABS, ET_0/RC, $1 - V_J$ (ET_0/TR_0), ΔV_{I-P}, and PI_{ABS} under S and SU stress were quite distinct among the S- and SU-tolerant and sensitive cultivars. In general, under S stress, the values of these parameters decreased significantly over the C plants in the S-susceptible cultivars FR13A and IR42. The differences of the parameters $1 - V_J$, ET_0/ABS, ET_0/RC, and ΔV_{I-P} between C and S were not significant in AC39416. Though the values of PI_{ABS} were significantly reduced under S stress in the S-tolerant cultivar AC39416, yet the magnitude of the decrease was far lesser compared to other two cultivars. The differences of ABS/RC, TR_0/RC, and RC/CS_0 under S stress compared with C were insignificant in all types of cultivars. The values of ABS/RC and TR_0/RC further increased under SU, whereas the values of RC/CS_0 decreased. The degree of the increase was greater in the SU-sensitive cultivars compared to the SU-tolerant cultivar. The SU-tolerant cultivar FR13A maintained greater values of RC/CS_0, ET_0/RC, $1 - V_J$, ET_0/ABS, ΔV_{I-P}, and PI_{ABS} and lower values of ABS/RC and TR_0/RC under SU, irrespective of the quality of flooding water, compared to the SU-sensitive cultivars IR42 and AC39416. Comparison of different Chl fluorescence parameters under SU revealed that the deviation from C was higher under SU with SW compared to NW.

Discussion

Geographically, rice is being grown in lands as far as 50°N (Aiwei, China) to 30°S (New South Wales, Australia). It is grown at altitudes ranging from below sea level (Kerela, India) to 2,761 m a.s.l. (Jumulla valley, Nepal), thus making its presence in all the environments and all continents of the world (Chang 2000). This rice adaptation to extremely variable conditions offers a hope to combat the current challenges caused by variable abiotic stresses, as well as

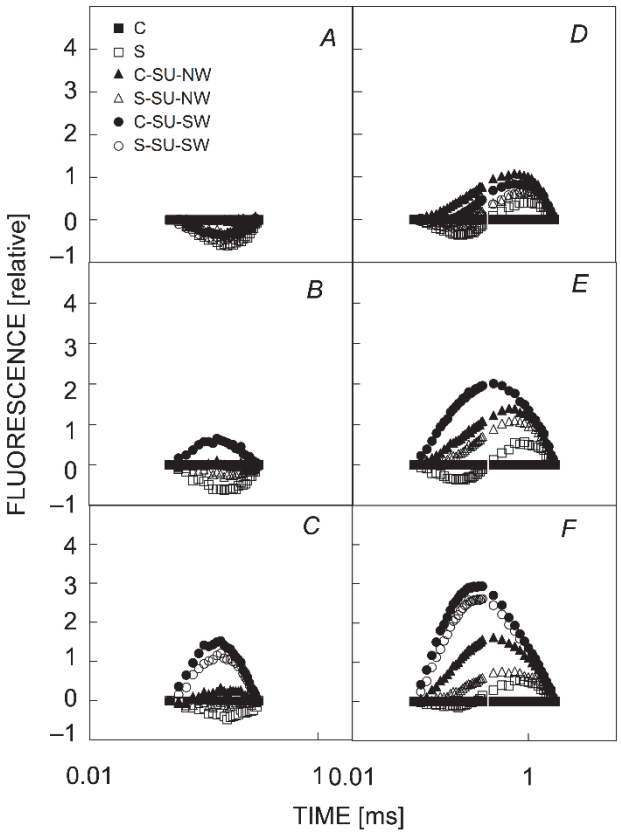

Fig. 5. Chlorophyll *a* fluorescence measured with 20 min dark-adapted rice leaves. *A*,*D* – FR13A; *B*,*E* – IR42; *C*,*F* – AC39416. The measurements were carried out on a fully expanded mature leaf, 2nd from top. Each curve was prepared based on average data of nine measurements. The fluorescence curves were normalized between 0.05 and 0.30 ms for visualization of the L-band. The fluorescence rise at 0.05–0.30 ms above the control designated as the L-band (*A,B,C*). In order to visualize the K-band, the fluorescence curves were normalized between F_0 and F_J. The fluorescence rise at O-J phase above the control was designated as the K-band (*D,E,F*). Experiment 2. C – control; S – 12 dS m^{-1} saline treatment; C-SU-NW – normally grown, submergence with nonsaline water; S-SU-NW – 12 dS m^{-1} saline treatment before submergence, submergence with nonsaline water; C-SU-SW – normally grown, submergence with 12 dS m^{-1} saline water; S-SU-SW – 12 dS m^{-1} saline treatment before submergence, submergence with 12 dS m^{-1} saline water.

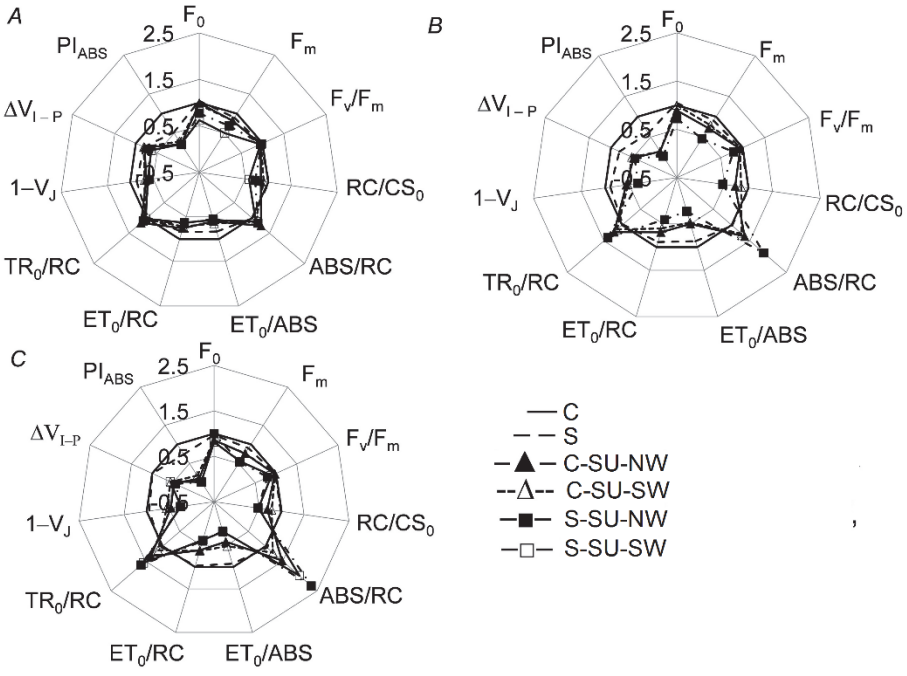

Fig. 6. Selected JIP-test parameters quantifying the behaviour of PSII subjected to salinity, submergence under nonsaline and saline water were presented as relative to that of control (*i.e.*, stress/control). *A* – FR13A; *B* – IR42; *C* – AC39416. The value of the control sample was set to unity. Each curve was based on average data of nine measurements. Experiment 2. C – control; S – 12 dS m^{-1} saline treatment; C-SU-NW – normally grown, submergence with nonsaline water; S-SU-NW – 12 dS m^{-1} saline treatment before submergence, submergence with nonsaline water; C-SU-SW – normally grown, submergence with 12 dS m^{-1} saline water; S-SU-SW – 12 dS m^{-1} saline treatment before submergence, submergence with 12 dS m^{-1} saline water.

means to cope with adverse effects of climate change, to secure food and livelihood. Coastal areas are more vulnerable than inland. Multiple abiotic stresses are common in the same growing season depending on the rainfall pattern, high wind, and higher tidal wave. Intrusion of salt water/deficit rain causes initial salt injury. Later on, due to other adverse factors, submergence with or without saline water might also damage rice crops. Rice is the only crop that could be grown in these hostile environments when all other crops are damaged completely (Mahata *et al*.

2010). The salt-tolerant germplasm lines, *e.g.*, Rashpanjor and AC39416, also showed susceptibility to submergence (Fig. 1*A*). Imposition of complete submergence stress with saline water was more deleterious for these S-tolerant cultivars compared to the SU-tolerant cultivar FR13A (Fig. 1*A*). Improving SU tolerance of the S-tolerant cultivars could help in their use in coastal areas where both stresses are common within the same season. Before SU, if plants experienced S stress, their growth decreased (Figs. 1*B*, 2) and they were more vulnerable to SU stress.

A reduction in shoot DM due to salinity at early seedling stages is a common feature, yet the cultivar with greater initial vigour and SU-tolerance ability had some advantage of preserving the shoot biomass and nonstructural carbohydrate accumulation under S stress (Fig. 1). FR13A, the S-susceptible genotype, accumulated the similar content of nonstructural carbohydrates in shoots as the S-tolerant AC39416 after 7 d of 12 dS m^{-1} NaCl treatment. Irrespective of the floodwater conditions, survival of FR13A was the highest, followed by AC39416 (Fig. 2). The contents of nonstructural carbohydrates were comparable among three cultivars, *e.g.*, FR13A, Rashpanjor, and AC39416 under control, and FR13A and AC39416 under salt stress. Nonstructural carbohydrates are important for providing energy needed for maintenance of metabolism during SU and recovery after SU (Panda and Sarkar 2012, Qin *et al*. 2013, Luo *et al*. 2014). If the initial content of the nonstructural carbohydrates is low, plants are more vulnerable under SU as it was found in IR42, however, it is not the sole criterion in classifying the SU-tolerant cultivar. Das *et al*. (2005, 2009) reported that differences in tolerance of complete submergence were not necessarily associated with an initial carbohydrate status before submergence, but rather with the ability to maintain their high contents after submergence.

Diminution of the Chl content due to salt (Kalaji and Pietkiewicz 1993, Sarkar *et al.* 2013, Singh and Sarkar 2014) as well as submergence stress is common in rice (Sarkar and Panda 2009). Maintenance of the higher Chl content might be essential for survival or, in other words, degradation of Chl means the loss of basic functions in plants. The tolerant cultivar maintained greater quantities of Chl so that basic functions of the plant prevailed for a longer time (Fig. 3). Compared to salt injury, SU stress caused more damage to plants. Both the stresses acted in concert and the damage was more pronounced in the cultivar which was susceptible to both SU and S. In general, if a plant gets shock by a single abiotic stress during the growing season, the plant of the cultivar tolerant to one particular stress may withstand this type of stress very efficiently, whereas it fails under multiple stresses or another type of stress (Figs. 2, 3). FR13A even after receiving salinity shock before SU, showed the greater survival rate under SU compared to any other cultivars. The cultivars tolerant to both S and SU are, no doubt, more suitable for flood-prone coastal areas.

The progressive reduction of the acceptor side in PSII leads to three distinct major phases of fluorescence rise from O to P with two intermediate steps, J and I (Strasser and Govindjee 1992, Kalaji *et al*. 2012). All the curves had a typical O-J-I-P shape with varying maximum fluorescence (Fig. 4*A,B,C*). The shape of the O-J-I-P transient is very sensitive to stress caused by changes in different environmental conditions, *e.g*. irradiance, temperature, drought, ozone elevation, flooding, *etc.* (Sarkar and Panda 2009, Perboni *et al*. 2012, Breštić *et al.* 2012, Fini *et al*. 2013, Lee *et al*. 2014, Xing and Wu 2014). The analysis of fluorescence curves revealed that maximum fluorescence decreased greatly due to S as well as due to SU. SU stress caused greater damage compared to S stress. The damage of S stress was greater in the S-sensitive cultivar, whereas damage by SU was greater in the SU-sensitive cultivars (Panda *et al*. 2008b, Sarkar *et al*. 2013, Singh and Sarkar 2014). Under stressless conditions, the O-J-I-P shape did not differ between the cultivars (Fig. 4). The normal shape of O-J-I-P changed due to stress as evident by the positive deviation of the curve from C when normalization at O and P were done (Fig. 4*D,E,F*). Under certain conditions, additional steps, the L, K, H, and G could appear in the fluorescence rise (Lazár 2006, Lazár and Schansker 2009). The two steps, H and G are found mainly in lichens or certain other lower forms of plant life (Lazár 2006). The appearance of the L-band indicates a decrease in energetic connectivity due to the decrease of grouping of PSII units (Jiang *et al*. 2006). The greater is grouping of PSII, the higher is the energetic connectivity (Strasser and Stirbet 1998). This grouping is known to be sensitive to thylakoids stacking and destacking (Kalaji *et al.* 2014). Therefore, the higher is the energetic connectivity, the greater is the use of excitation energy, and thus greater is the system stability (Lazár 2015). Our data suggest that all cultivars maintained energetic connectivity under S stress (Fig. 5*A,B,C*). Probably the S stress applied to plant was not so severe to cause expression of the L-band. The appearance of the L-band could mean that FR13A did not loose energetic connectivity even under SW submergence, whereas loss of energetic connectivity was greater in AC39416, followed by IR42 (Fig. 5*A,B,C*). The source of the K-band is usually hidden in nonstressed samples and for dynamic reasons, it does not appear as a clear rise in the fluorescence rise (Lazár 2003). The appearance of the K-band is generally influenced by factors, such as partial disconnection of the oxygen evolving complex (OEC) on the Mn-side, the acceptor side of PSII, and the connectivity among PSII units (Lazár 2006, Jiang *et al*. 2006). Normalization at F_0 and F_J excluded the effect of the acceptor side in PSII (Jiang *et al*. 2006). Thus, the appearance of the K-step is attributed to the partial inactivation of OEC and loss of connectivity among PSII (Oukarroum *et al.* 2014). Unlike the L-band, the K-band appeared due to both S and SU stress. The magnitude of the K-band due to S stress was almost similar in three rice cultivars (Fig. 5), whereas the magnitude of the K-band under SU was high in AC39416

followed by IR42 and FR13A which indicated that inactivation of OEC and damage of thylakoid structures were lesser in FR13A compared to the other two cultivars. The damage was greater under SW submergence compared to NW submergence.

The JIP-test, developed by Strasser and Strasser (1995), is used to translate several biophysical expressions that quantify PSII function. F_v/F_m, the maximum quantum yield of primary PSII photochemistry, is a robust parameter and is mainly used to state the primary health of a plant (Stirbet and Govindjee 2011, Murchie and Lawson 2013, Chen *et al*. 2013, Xing and Wu 2014). The existence of any type of stress that results in inactivation of PSII is often referred as photoinhibition (Long *et al.* 1994) or the induction of sustained quenching (Demmig-Adams and Adams 2006) and results in a lowering of F_v/F_m (Fig. 6). The F_v/F_m ratio decreased significantly in FR13A and IR42 due to S stress compared with C (Table 1S, *supplement available online*). The values of F_v/F_m under SU were significantly lower in all cultivars compared with C. It is generally stated that the F_v/F_m ratio varies between 0.78 and 0.83 in a healthy leaf (Björkman and Demmig 1987, Stirbet and Govindjee 2011, Murchie and Lawson 2013). The values of F_v/F_m ratio more than 0.78 was obtained in FR13A and AC39416 in certain cases though the damage was apparent. This type of discrepancy was also reported earlier (Strasser *et al*. 2004, Sarkar and Panda 2009, Xing and Wu 2014). Values of F_0 increased due to S stress in FR13A and IR42 (Fig. 6, Table 1S). An increase in fluorescence at F_0 is either one of the most direct signs of photoinhibition (Aro *et al.* 1993) or damage of the acceptor side of PSII (Kalaji *et al*. 2014). However, under SU, the values of F_0 either decreased or not (Fig. 6). As F_v/F_m is a ratio $(1 - F_0/F_m)$, any alteration in both the parameters in the same direction would not change the ratio even though there was great damage to the PSII activities (Panda *et al*. 2008a). Greater structural damage of reaction centres per cross section or per unit of leaf area (RC/CS_0) might be responsible for decreasing the values of F_0 under SU (Fig. 6). The decline of the values of F_m and F_v/F_m indicates the decreasing ability of PSII to reduce the primary acceptor Q_A. As other abiotic stresses, both S and SU stress with and without saline water affected the photosynthetic apparatus and therefore, a decrease in the values of F_m and F_v/F_m was observed. FR13A, in general, maintained greater values of F_m and F_v/F_m ratio compared to other two cultivars under SU (Fig. 6).

The Chl fluorescence parameters ABS/RC (effective antennae size), $1 - V_J$ (the efficiency with which the trapped excitons move an electron further than Q_A^-), and ET_0/ABS (probability that an absorbed photon will move an electron into electron transport chain) are sensitive indicators of SU stress in rice (Panda *et al*. 2008a). Average absorption per active reaction centre (ABS/RC) increases due to the inactivation of some RCs, *i.e*., the increase in ABS/RC is due to a decrease in active Q_A reducing reaction centres (Strasser and Stirbet 1998). Non-Q_A reducing reaction centres exist always in light-grown plants acting as heat sink or silent RCs. This function acts as heat radiators and protects the plant from high temperature and high light (Živčák *et al*. 2014). Under SU, though the temperature and light intensity was not high, the damage of photosynthetic apparatus occurred. The values in ABS/RC did not change much under S stress (Fig. 6*A,C,E*), however, under SU, the increase was many fold higher in the SU-susceptible cultivars compared to C. It suggested that the tolerant cultivars were able to regulate better the amount of energy reaching the RC (Strasser *et al*. 2004). S stress caused a decrease in $1 - V_J$ and ET_0/ABS mainly in the S-susceptible cultivars (Fig. 6). The values decreased further under SU. The rate of decrease, however, was greater in the SU-susceptible cultivars. Quality of flooding water had little impact on the change of these parameters. The flux of trapping energy per reaction centre (TR_0/RC) increased greatly under SU only in SU-susceptible cultivars compared with C (Fig. 6). The greater the value of TR_0/RC, the greater the inhibition of reoxidation of Q_A^- to Q_A (Strasser *et al*. 2000). If TR_0/RC becomes greater, ET_0/RC (electron transport per reaction centre) decreases (Fig. 6), resulting in lesser electron transport per trapping $[(ET_0/RC)/(TR_0/RC) = ET_0/TR_0$ or $1 - V_J]$. ET_0/RC increases under high temperature stress due to a thermal activation of dark reaction (Strasser *et al*. 2000). Under SU, however, ET0/RC decreased more in the SU-susceptible cultivars compared to SU-tolerant cultivar. (Fig. 6). Density of active RC per cross section or per unit of leaf area (RC/CS_0) decreased mainly due to SU stress. ΔV_{I-P}, amplitude of the IP-phase, which indicates the efficiency of electron transport around the PSI to reduce the final acceptors of the electron transport chain, *i.e*., ferredoxin and NADP, decreased under S stress in both S-susceptible cultivars FR13A and IR42 and under SU in both SU-susceptible cultivars IR42 and AC39416. The parameter was found to be highly sensitive in distinguishing the tolerance level of the cultivars (Salvatori *et al*. 2013). PI_{ABS} is a key Chl fluorescence parameter that provides useful and quantitative information about the plant vitality (Strasser *et al*. 2000, Perboni *et al*. 2012, Singh and Sarkar 2014). The PI_{ABS} is a combination of three partial components that favour photosynthetic performances (Strasser *et al*. 2004). Our data showed that the PI_{ABS} was a highly sensitive parameter and changed significantly under both S and SU stress in all cultivars (Table 1). However, the reduction of PI_{ABS} was higher in the S-sensitive cultivars compared to the S-tolerant cultivar under S stress. Likely, reduction of PI_{ABS} was greater in SU-sensitive cultivars compared to the SU-tolerant cultivar under SU stress. SU with SW showed greater reduction of PI_{ABS}, especially, in the SU-susceptible cultivars. It showed that under stress, the cultivar is able to maintain a greater number of active RCs with the capacity to trap and transport more energy

per unit of excited leaf area in order to counteract adverse situations efficiently. Chl fluorescence studies revealed that FR13A, the SU-tolerant cultivar, maintained greater chloroplast structural integrity and functional capacity under SU, irrespective of the quality of flooding water. Winkel *et al*. (2014) reported that FR13A maintained superior photosynthetic capacity under SU due to the retention of leaf gas films that aid O_2 and CO_2 exchange under water. This superior photosynthetic performance of FR13A was not evident in Swarna-Sub1 (a SU-tolerant cultivar carrying the *SUB1* QTL). The underwater photosynthesis of Swarna-Sub1 was equal to that of IR42. Teakle *et al*. (2014) reported that leaf gas films delayed the entry of salt into leaves of *Melilotus siculus* under saline submergence. FR13A, probably due to the superior capacity to maintain gas films under SU, also maintained the structural and functional integrity of chloroplasts and continued photosynthesis even under saline submergence.

Our results showed that the fast Chl *a* fluorescence transient measurement provided a noninvasive and rapid method for investigating stress effects on PSII. Since salinity and submergence induce structural disorganization, a general trend in the disorganization of the photosynthetic apparatus during salinity/submergence could be visualized. Exposure to salinity before submergence was found to be more deleterious. The effect was more harmful in the submergence-sensitive cultivars. The submergence-tolerant cultivar maintained chloroplast structure and functional capacity better compared to the salinity-tolerant cultivars even under complete submergence with saline water. In wet season, when flooding is more prevalent in coastal flood-prone areas, growing of submergence-tolerant rice is more judicious than just growing salinity-tolerant cultivars for better survival of the plants. Testing of submergence-tolerant cultivars under saline submergence would add more resources to genetic pool and it will be useful in developing high-yielding rice cultivars tolerant to multiple abiotic stresses.

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