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WATER DEFICIT STRESS IN THE REPRODUCTIVE STAGE OF FOUR INDICA RICE (*ORYZA SATIVA* L.) GENOTYPES

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Abstract

Relative water content (RWC) in the leaf of different rice cultivars dropped significantly in relation to reduced soil water content (SWC), especially in PT1 and IR20. In contrast, the proline content in the leaf-blade and leaf-sheath increased when plants were subjected to 7% SWC. The RWC was positively related to chlorophyll degradation. Chlorophyll a, chlorophyll b, total chlorophyll, total carotenoids, maximum quantum yield of PSII, stomatal conductance and water use efficiency in rice grown under water-deficit conditions declined significantly in comparison to the control group, leading to a reduction in net-photosynthetic rate. In addition, when exposed to water-deficit, panicle length and fertile grains in KDML105 and NSG19 were stabilized, leading to greater productivity than in PT1 and IR20. These data were utilized as effective criteria for the classification of water-deficit tolerance. From the results, KDML105 and NSG19 were identified as water deficit-tolerant, and PT1 and IR20 as water deficit-susceptible.

Introduction

Water deficit is a major problem for crop production worldwide, limiting the growth and productivity of many crop species, especially in rain-fed agricultural areas (>1.2 billion hectares) (Chaves & Oliveira, 2004; Passioura, 2007). Biochemical changes, such as decreased Rubisco (ribulose-1,5-bisphosphatase carboxyase/oxygenase) activity, reduced photochemical efficiency, enhanced accumulation of stress metabolites (proline, glycinebetaine, polyamine, glutathione, polyamines, sugars, sugar alcohols and α-tocolpherol), increased antioxidant enzymes (superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase) are generally the responses to water deficit in higher plants. Physiological changes include reduced relative water content, pigment degradation, decreased stomatal conductance, reduced internal CO_2 concentration, net photosynthetic rate (P_n) reduction and growth inhibition prior to plant death (Chaves & Oliveira, 2004; Cattivelli *et al*., 2008; Tuna *et al*., 2010). Some parameters in crop species in response to water deficit stress have been investigated as criteria for improving water deficit tolerance through breeding programs (Ashraf, 2010). It has also been established that yield traits are the most important criteria for water-deficit tolerance screening (Yang *et al*., 2001; Pantuwan *et al*., 2002; Kumar *et al*., 2008).

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Rice is a major food crop in many regions of the world, especially Asian countries. It is a staple food, feeding more than 3 billion people and providing 50-80% of their daily calorie intake (Khush, 2005). Rice has been identified as water deficit susceptible, showing negative effects especially in the reproductive stages. In rain fed paddy fields, water deficit has been reported as being a serious issue, especially in the booting stage, during which plants are particularly susceptible, leading to low crop productivity (Pantuwan *et al*., 2002). In Thailand, KDML105 is the dominant rice variety, widely planted in rain fed lowlands (non-irrigated zone) in the north and northeastern regions. Both KDML105 and Pathumthani 1 (PT1) which is a water-deficit susceptible variety (irrigated zone), have been identified using multivariate parameters (Cha-um *et al*., 2010). NSG19 is a positive check of water-deficit tolerance, which is utilised in drought tolerance screening, whereas IR20 is a negative check (Pantuwan *et al*., 2002; Uyprasert *et al*., 2004; Kumar *et al*., 2006). However, multivariate parameters are required as water-deficit selection criteria in rice breeding programs.

Materials and Methods

Plant materials and water deficit treatments: Seeds of four rice cultivars viz., Khao Dawk Mali 105 (KDML105), Pathumthani 1 (PT1), Nam Sa-Gui 19 (NSG19) and IR20 provided by Pathumthani Rice Research Center, (Rice Research Institute, Department of Agriculture, Ministry of Agriculture and Cooperative, Thailand) were germinated and transplanted to 72 pots containing clay soil (EC = 2.687 dS m^{-1}; pH = 5.5; organic matter = 10.36%; total nitrogen = 0.17%; total phosphorus = 0.07%; total potassium = 1.19%) in 50% shading (acclimatization) light intensity and grown on for 2 weeks. The pots were arranged on plastic trays (30 × 45 cm). Water irrigation was supplied using a moisture spray. One hundred acclimatized plants were transferred directly to water-flooded pots (15 cm in diameter × 30 cm in height) containing clay soil. The experiment was located at the Thailand Science Park, Pathumthani, Thailand (Latitude 14°01'12"N and Longitude 100°31'12"E) and conducted between August and November 2009. In the booting stage [85 days after sowing (DAS)], soil water content (SWC) was adjusted to 56% (full irrigation or well watering), 25% (7 days withholding irrigation or mild water-deficit), 7% (14 days withholding irrigation or severe water-deficit) and re-watering 3 days prior to grain harvesting. The SWC was calculated using the weight fraction: SWC (%) = [(FW-DW)/DW] × 100, where FW was the fresh weight of a soil portion of the internal area of each pot and DW was the dry weight of the soil portion after drying in a hot air oven at 85°C for 4 days (Coombs *et al*., 1987). Relative water content (RWC), proline content in the leaf blade and leaf sheath, photosynthetic pigments, chlorophyll fluorescence, net-photosynthetic rate (P_n), transpiration rate (E), stomatal conductance (g_s) in flag leaf and panicle traits in rice plants were measured.

Data collection: Relative water content (RWC) was calculated according to Bonnet *et al*., (2000). This parameter was calculated from fresh weight (FW) dry weight (DW) and turgid weight (TW) following the equation:

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

Proline in the root and leaf tissues was extracted and analyzed according to the method of Bates *et al*., (1973). Fifty milligrams of fresh material was ground with liquid nitrogen in a mortar. The homogenate powder was mixed with 1 mL aqueous sulfosalicylic acid (3% w/v) and filtered through filter paper (Whatman #1, England).

The extracted solution was reacted with an equal volume of glacial acetic acid and ninhydrin reagent (1.25 mg ninhydrin in 30 mL glacial acetic acid and 20 mL 6 M H_3PO_4) and incubated at 95°C for 1 h. The reaction was terminated by placing the container in an ice bath. The reaction mixture was mixed vigorously with 2 mL toluene. After cooling to 25°C, the chromophore was measured by spectrophotometer (HACH DR/4000; Model 48000, HACH Company, Loveland, Colorado, USA) at 520 nm using L-proline as a standard.

Chlorophyll a (Chl_a), chlorophyll b (Chl_b) and total chlorophyll (TC) content, were analyzed following the methods of Shabala *et al*., (1998) and total carotenoid (C_{x+c}) concentrations were assayed according to Lichtenthaler (1987). One hundred milligrams of leaf material was collected and placed in a 25 mL glass vial along with 10 mL 95.5% acetone, and blended using a homogenizer. The glass vials were sealed with parafilm to prevent evaporation, and then stored at 4°C for 48 h. Chl_a and Chl_b concentrations were measured using a UV-visible spectrophotometer at 662 nm and 644 nm wavelengths. The C_{x+c} concentration was also measured by spectrophotometer (HACH DR/4000; Model 48000, HACH Company, Loveland, Colorado, USA) at 470 nm. A solution of 95.5% acetone was used as a blank.

Chlorophyll fluorescence emission from the adaxial surface on the leaf was measured using a fluorescence monitoring system (FMS 2; Hansatech Instruments Ltd., Norfolk, UK) in the pulse amplitude modulation mode, as previously described by Loggini *et al*., (1999). A leaf, adapted to dark conditions for 30 min using leaf-clips, was initially exposed to the modulated measuring beam of far-red light (LED source with typical peak at wavelength 735nm). Original (F_0) and maximum (F_m) fluorescence yields were measured under weak modulated red light (<0.5µmol m^{-2} s^{-1}) with 1.6 s pulses of saturating light (>6.8µmol m^{-2} s^{-1} PAR) and calculated using FMS software for Windows®. The variable fluorescence yield (F_v) was calculated by the equation of F_m–F_0. The ratio of variable to maximum fluorescence (F_v/F_m) was calculated as maximum quantum yield of PSII photochemistry.

Net photosynthetic rate (P_n; µmol m^{-2} s^{-1}), transpiration rate (E; mmol m^{-2} s^{-1}), stomatal conductance (g_s; mmol H_2O m^{-2} s^{-1}) and water use efficiency (WUE; %) were measured using a Portable Photosynthesis System (Model LI 6400, LI-COR® Inc, Lincoln, Nebraska, USA) with an Infra-red Gas Analyser following Cha-um *et al*., (2007). WUE was calculated according to equation: WUE (%) = $[P_n/E] \times 100$. Panicle length, fertile grains, sterile grains, total grains and one-hundred seed weight per panicle in the well-watering or severe water deficit (14 days water withholding) were measured. Yield loss, RWC and WUE abilities, proline accumulation, P_n reduction, F_v/F_m diminution, and pigment degradation were calculated following Cha-um *et al*., (2009).

Experiment design and statistical analysis: The experiment was arranged as 4 × 4 factorials in Completely Randomized Block Design (CRBD) with eight replicates (n=8). The mean values obtained were compared using Duncan's New Multiple Range Test (DMRT) and analyzed with SPSS software. The correlations between physiological and biochemical characters were evaluated using Pearson's correlation coefficients. Yield loss, RWC and WUE abilities, proline accumulation, P_n reduction, F_v/F_m diminution, and pigment degradation in the flag leaves of rice grown under severe water deficit stress were assessed in order to classify cultivars as either tolerant and susceptible using Ward's method of Hierarchical cluster analysis in SPSS software.

Table 1. Relative water content (RWC), chlorophyll a (Chl_a), chlorophyll b (Chl_b), total chlorophyll (TC) and total carotenoids (C_{x+c}) content in the flag leaf of different rice genotypes grown under water varying degrees of deficit stress and re-watering.

Rice genotypes	Soil water content (%)	RWC (%)	Chl_a ($\mu g\ g^{-1}FW$)	Chl_b ($\mu g\ g^{-1}FW$)	TC ($\mu g\ g^{-1}FW$)	C_{x+c} ($\mu g\ g^{-1}FW$)
KDML105	56	92.67a	84.45c	56.57ab	141.02c	3.80a
	25	89.57ab	75.38e	53.59bcd	128.97ef	3.34ab
	7	84.12b	69.58f	44.86g	114.44h	2.63c
	Re-watering	93.77a	67.88f	50.59de	118.47gh	3.28ab
PT1	56	93.83a	98.95a	55.19abc	154.14a	3.85a
	25	84.72b	94.78ab	52.27cd	147.05b	3.28ab
	7	63.23d	47.09h	34.14h	81.23j	1.78d
	Re-watering	73.21c	83.09cd	48.64ef	131.73de	3.72a
NSG19	56	94.60a	91.45b	56.27ab	147.72b	3.81a
	25	90.88a	83.79cd	52.88bcd	136.67cd	3.55a
	7	83.60b	76.92e	46.54fg	123.46fg	2.74bc
	Re-watering	91.89a	85.52c	55.14abc	140.66c	3.44a
IR20	56	93.58a	94.88ab	58.56a	153.44a	3.51a
	25	72.49c	78.92de	44.99g	123.91fg	2.61c
	7	60.41d	43.82h	27.45i	71.27k	1.48d
	Re-watering	74.50c	61.61g	44.61g	106.22i	2.70bc

Different letters in each column show significant difference at $p \leq 0.01$ by Duncan's New Multiple Range Test (DMRT).

Results and Discussion

Relative water content (RWC) in the flag leaf of PT1 (lowland irrigated cultivar) and IR20 (negative check) rice cultivars (water-deficit susceptible) dropped significantly in plants exposed to mild water-deficit with 25% soil water content (SWC) and RWC recovery was delayed during re-watering. The RWC in both KDML105 (moderately water-deficit tolerant) and NSG19 (positive check) was maintained in mild water-deficit but decreased significantly when plants were exposed to severe water shortage (7% SWC) (Table 1) and increased quickly after re-watering. A positive correlation between SWC and RWC was demonstrated in each cultivar (Fig. 1). The gradient of the SWC and RWC relationship in the water-deficit susceptible cultivars IR20 and PT1 was greater than that of the water-deficit tolerant cultivars, NSG19 and KDML105. It should be noted that the reduction of RWC was progressively related to SWC, especially in the water-deficit sensitive cultivars (Fig 1B and 1D). Data presented in this investigation demonstrates that RWC decreased upon the reduction of SWC in the pot culture. In wheat, SWC is set at 82% (well watering), 31% (withholding water for 4 days) and 18% (withholding water for 7 days) which reduces the leaf RWC by 72.6%, 74.3% and 63.9%, respectively (Nunes *et al*., 2008). In three rice cultivars, Gangyon 527, Yixiangyou 9 and Gangyou 188, the RWC shows similarly trend, decreasing when plants are exposed to water stress for 10 and 20 days (Wang *et al*., 2010). The reduction of RWC in 80 double haploid rice genotypes is closely correlated with grain yield and has been investigated as a water-deficit tolerance indicator in rice breeding programs (Lafitte, 2002).

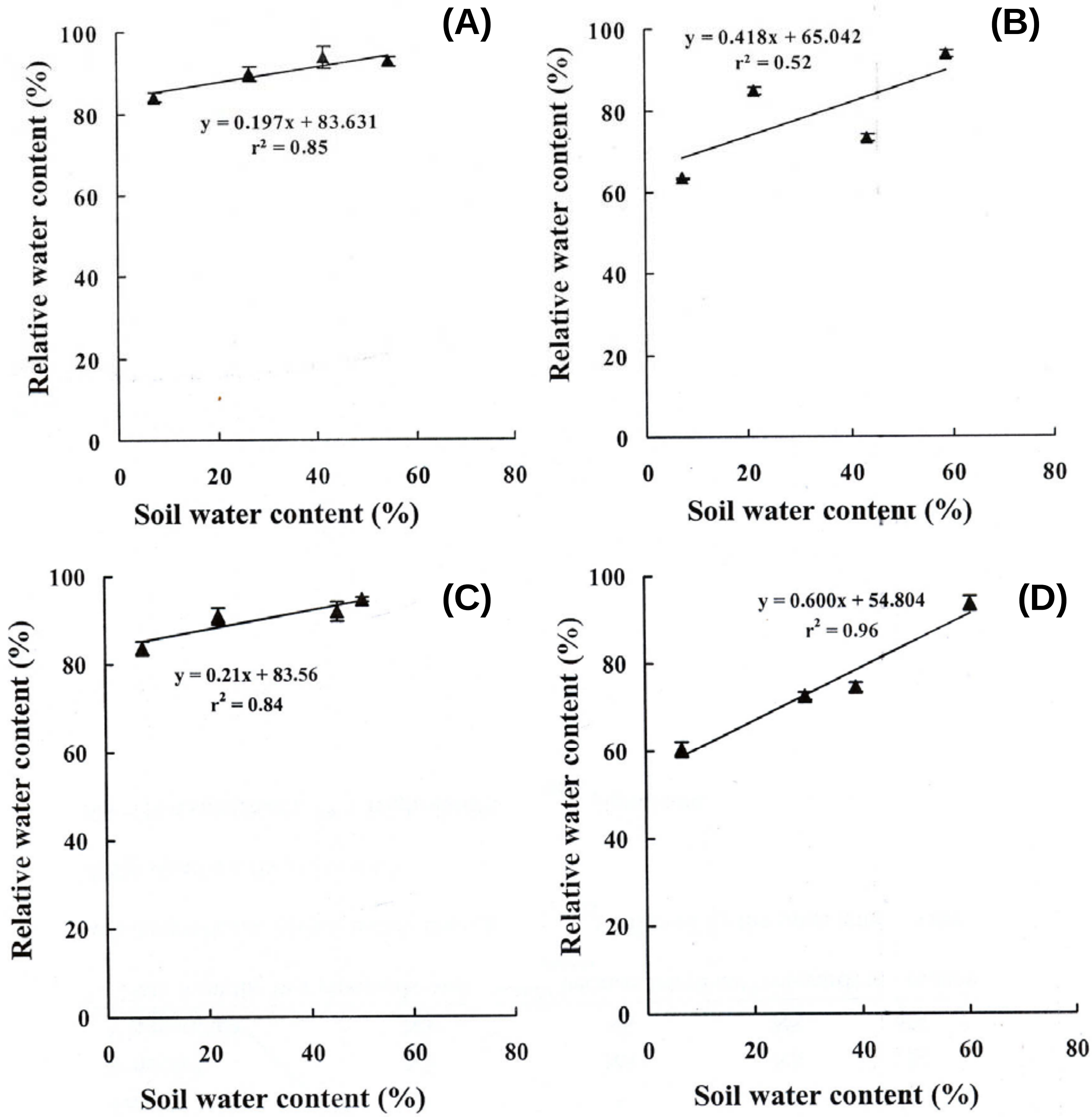

Fig 1. Relationship between soil water content (%) and relative water content in rice genotypes, KDML105 (A), PT1 (B), NSG19 (C) and IR20 (D) grown under varying degrees of water deficit stress and re-watering. Error bars represented by ± SE.

In contrast, the proline content in flag leaf blade and leaf sheath increased, depending on the degree of water deficit stress and the cultivar (Fig. 2). The proline content in leaf blade and leaf sheath of PT1 and IR20 rice subjected to both mild and severe water-deficit stress increased (Fig. 2B and 2D) and accumulated to a higher level than in KDML105 and NSG19 (Fig. 2). A positive relationship between proline levels in the leaf blade and leaf sheath was found (Fig. 3A). Proline accumulation in the leaves of water-deficit stressed plants may play a role as a stress indicator, mainly in salt sensitive cultivars. Proline content in the leaf blade and leaf sheath increased depending on the reduction of RWC in the leaf tissues. Proline accumulation in crop species has been established as a good indicator of responses to water stress. In wheat, proline levels in the leaf blade and leaf sheath increase in response to water shortage, and perform a role as a drought resistance indicator (Karamanos, 1995). In the present study, proline content in PT1 and IR20 (water deficit susceptible) peaked in plants subjected to severe water deficit (7% SWC) and was higher than in the tolerant cultivars (KDML105 and NSG19). Similar results have been obtained from investigations into IR20, KDML105 and NSG19 which are used as standard check genotypes (Uyprasert *et al*., 2004). Proline accumulation in the leaf tissues of drought susceptible CR203 (3.12 times) is higher than in drought tolerant DR2 (1.41 times) and Cuom (2.06 times) when plants are subjected to mannitol-induced water stress (Hien *et al*., 2003). In contrast, the proline content in drought-tolerant rice cultivars, Monohar Sail and Ranjit is at its highest (37.5-46.1%) in plants exposed to PEG-6000 (polyethylene glycol)-induced water deficit (Roy *et al*., 2009).

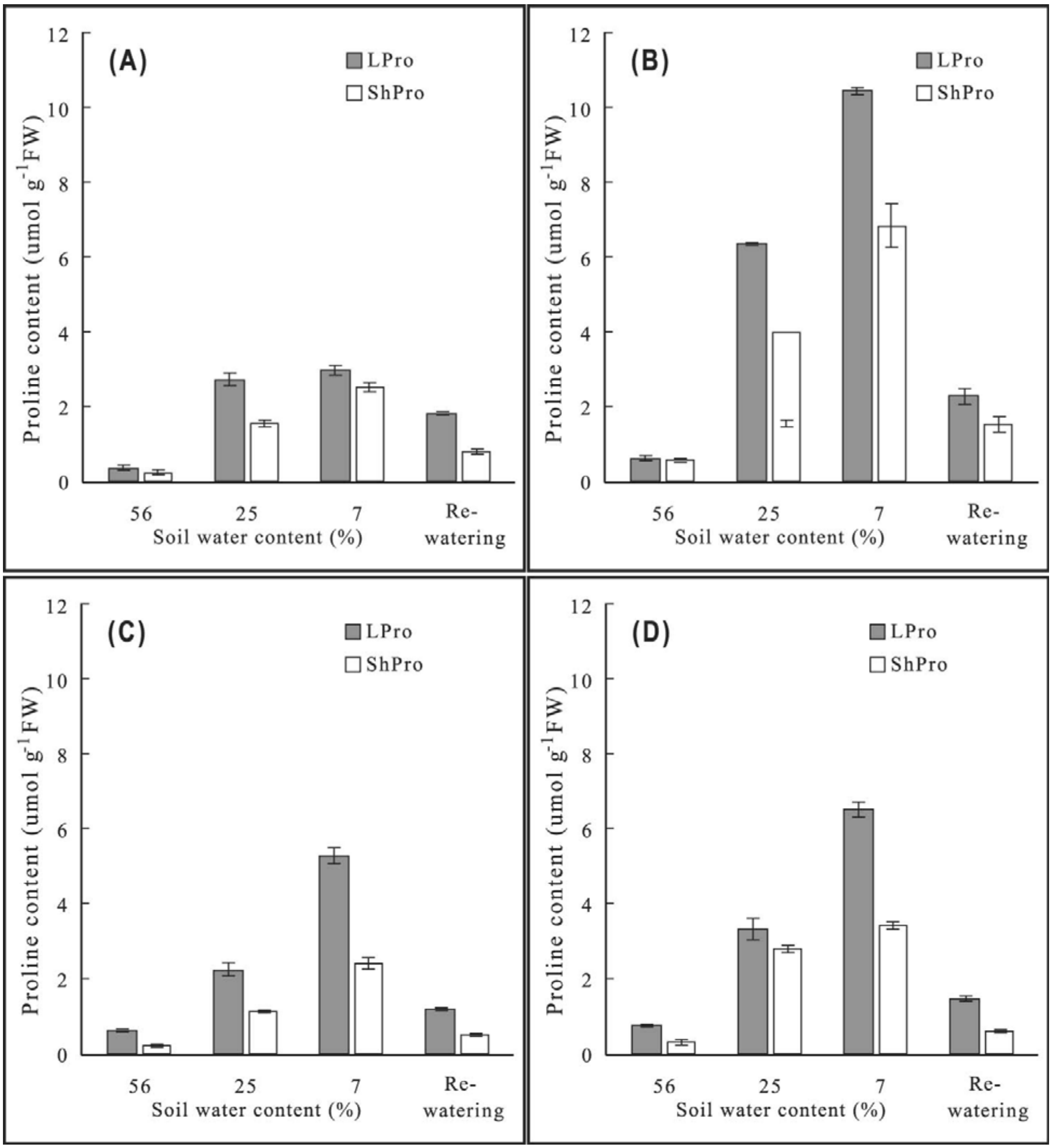

Fig 2. Proline content in the leaf blade and leaf sheath of rice genotypes, KDML105 (A), PT1 (B), NSG19 (C) and IR20 (D) grown under varying degrees of water deficit stress and re-watering. Error bars represented by ± SE.

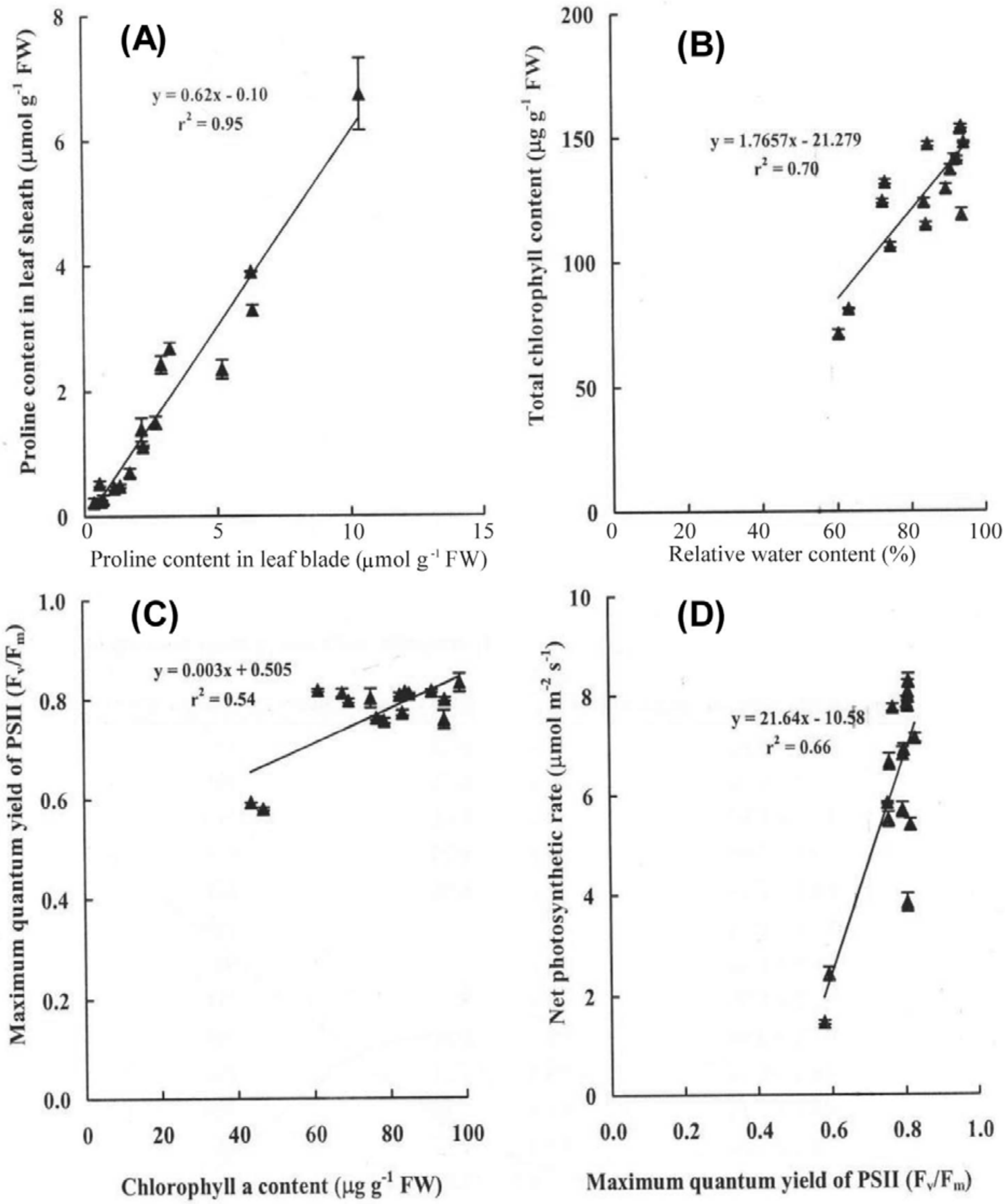

Fig 3. Relationships between proline content in the leaf blade and leaf sheath (A), relative water content and total chlorophyll content (B), chlorophyll a content and maximum quantum yield of PSII (F_v/F_m) (C), maximum quantum yield of PSII (F_v/F_m) and net photosynthetic rate (P_n) (D) in rice genotypes grown under varying degrees of water deficit stress and re-watering. Error bars represented by ± SE.

Moreover, reduction of RWC in the flag leaf was positively correlated with total chlorophyll (TC) content (Fig. 3B). Chlorophyll a (Chl_a), chlorophyll b (Chl_b) TC, and total carotenoids (C_{x+c}) in all rice cultivars were drastically degraded when subjected to severe water deficit (Table 1). The degradation percentage of those pigments in KDML105 and NSG19 plants subjected to water-deficit was lower than in PT1 and IR29 and the recovery percentage in the re-watering process greatly improved. Degradation of Chl_a pigments was positively related to maximum quantum yield of PSII (F_v/F_m) (Fig. 3C), leading to reduced net photosynthetic rate (P_n) (Fig. 3D). F_v/F_m in all rice cultivars grown in severe water-deficit conditions was severely diminished, whereas F_v/F_m in KDML 105 was maintained when plants were subjected to mild water-deficit (Table 2). Also, P_n reduction was demonstrated in all rice cultivars when subjected to severe water shortage. In mild water-deficit, P_n was maintained better in NSG19 than the other cultivars (Table 2). Stomatal conductance (g_s) in PT1 and IR20 cultivated in mild water-deficit stress dropped significantly, whereas it was stable in KDML105 and NSG19 (Table 2). Water use efficiency (WUE) in rice subjected to water deficit declined significantly. In contrast, transpiration rate (E) was inversely related to the other parameters, and increased relative to the degree of water deficit stress (Table 2). The reduction of RWC in the flag leaf was a primary effect, damaging photosynthetic pigments including Chl_a, Chl_b, TC and C_{x+c}, and diminishing F_v/F_m, leading to decreased P_n and WUE. Photosynthetic pigment degradation in rice exposed to water deficit is well established in indica rice (Basu *et al*., 2010; Cha-um *et al*., 2010; Wang *et al*., 2010) and japonica rice (Yang *et al*., 2001). In addition, the diminution of F_v/F_m and reduction of P_n depended on the degree of water deficit stress (including mannitol-induced stress (Cha-um *et al*., 2010), the sprinkler system (Centritto *et al*., 2009) and the number of days after the withholding of water (Yang *et al*., 2001). The F_v/F_m and P_n values in the water-deficit tolerant cultivars, Homjan (HJ) and RD6 were maintained better than in water-deficit susceptible PT1 (Cha-um *et al*., 2010). Transpiration rate (E) in rice cultivars subjected to water deficit has been considered as an indicator for water deficit tolerance classification (Cabuslay *et al*., 2002). Moreover, yield traits, such as grain yield, productivity and grain sterility are the most popular parameters used to identify water deficit tolerance in rice breeding programs (Yang *et al*., 2001; Pantuwan *et al*., 2002; Kumar *et al*. ,2008; Venuprasad *et al*., 2008; Wang *et al*., 2010).

The parameters RWC, Chl_a, Chl_b, C_{x+c}, F_v/F_m, P_n and WUE were positively correlated at $p \leq 0.01$, but proline content was negatively related (Table 3). Panicle traits, including panicle length, in KDML105 and NSG19 subjected to severe water deficit, were better, while the number of sterile grains was less than in PT1 and IR20 (Table 4). The number of fertile grains, total grains and hundred grain weights decreased significantly when plants were subjected to both mild and severe water-deficit conditions. In addition, yield failure, RWC and WUE abilities, proline accumulation, P_n reduction, F_v/F_m diminution, and pigment degradation data were applied to classify members of the group as water deficit tolerant, KDML105, NSG19 and water deficit susceptible, PT1 and IR20 using Ward's method (Fig. 4). The flowering stage of rice has been reported as being sensitive to stress which can lead to disruption of developmental processes and pollen and ovule abortion, causing sterility of spikelets and reduction of yield (Kato *et al*., 2008). However, multivariate indices have been recommended for water deficit tolerance classification, especially in rice (Cubusley *et al*., 2002; Cha-um *et al*., 2010). In this study, multivariate parameters, RWC and WUE, proline accumulation, pigment degradation, F_v/F_m diminution, P_n reduction and yield loss, in response to water deficit stress were developed as effective indices for water-deficit tolerance classification in rice genotypes.

Table 2. Maximum quantum yield of PSII (F_v/F_m), net-photosynthetic rate (P_n) transpiration rate (E), stomatal conductance (g_s) and water use efficiency (WUE) in the flag leaf of different rice genotypes grown under varying degrees of water deficit stress and re-watering.

Rice genotypes	Soil water content (%)	F_v/F_m	P_n (μmol m^{-2} s^{-1})	E (mmol m^{-2} s^{-1})	g_s (mmol H_2O m^{-2} s^{-1})	WUE (%)
	56	0.814a	8.07a	0.99g	45ef	8.13d
KDML105	25	0.802ab	6.91b	1.29ef	38fg	5.45e
	7	0.797bc	5.70c	2.44b	26gh	2.37h
	Re-watering	0.812a	7.79a	0.78gh	42ef	9.98c
	56	0.832a	7.15b	1.02g	72c	7.02d
PT1	25	0.758d	5.50c	1.52cd	47ef	3.64fg
	7	0.580e	1.47f	3.43a	24h	0.43h
	Re-watering	0.808ab	3.85d	1.43de	43ef	2.81fg
	56	0.815a	8.29a	0.44i	93ab	18.78a
NSG19	25	0.772cd	7.76a	1.10fg	83b	7.07d
	7	0.762cd	6.67b	1.73cd	67c	3.91f
	Re-watering	0.810a	7.86a	0.55i	104a	14.56b
	56	0.800ab	6.83b	0.98g	91a	7.04d
IR20	25	0.756d	5.84c	1.84c	38fg	3.18fg
	7	0.593e	2.44e	3.65a	34fg	0.67h
	Re-watering	0.818a	5.40c	0.56h	56d	8.34d

Different letters in each column show significant difference at $p \leq 0.01$ by Duncan's New Multiple Range Test (DMRT).

Table 3. Relationship between physiological and biochemical parameters in rice genotypes grown under varying degrees of water deficit stress and re-watering.

Parameters	PRO	RWC	Chl_a	Chl_b	C_{x+c}	F_v/F_m	P_n	WUE
PRO	-	-	-	-	-	-	-	-
RWC	-0.590**	-	-	-	-	-	-	-
hl_a	-0.530**	0.737**	-	-	-	-	-	-
Chl_b	-0.642**	0.890**	0.867**	-	-	-	-	-
C_{x+c}	-0.635**	0.768**	0.811**	0.870**	-	-	-	-
F_v/F_m	-0.790**	0.747**	0.706**	0.810**	0.781**	-	-	-
P_n	-0.688**	0.902**	0.690**	0.836**	0.728**	0.789**	-	-
WUE	-0.606**	0.662**	0.459**	0.624**	0.567**	0.565**	0.737**	-

Highly significant level at $p \leq 0.01$ is represented by ** using Pearson's correlation coefficients

Table 4. Yield traits, panicle length (PL), fertile grains (FG), sterile grains (SG) total grains (TG) per panicle and 100-grain weight (HGW) in rice genotypes grown under well watering and water deficit stress.

Rice genotypes	Water treatment	PL (cm)	FG	SG	TG	HGW (mg)
KDML105	Well watering	26.0a	62.0b	11.0d	73.0c	2.69a
	Water deficit	24.7a	59.0b	11.7d	70.7c	2.51b
PT1	Well watering	21.2b	71.3a	21.0c	92.3b	2.45b
	Water deficit	14.8d	61.0b	43.3a	104.3a	2.27c
NSG19	Well watering	18.0c	44.3c	9.0d	53.3d	2.06d
	Water deficit	17.8c	32.0d	10.7d	42.7e	1.73e
IR20	Well watering	21.2b	40.3c	19.3c	59.6d	2.05d
	Water deficit	14.0d	24.7e	33.0b	57.7d	1.34f

Different letters in each column show significant difference at $p \leq 0.01$ by Duncan's New Multiple Range Test (DMRT).

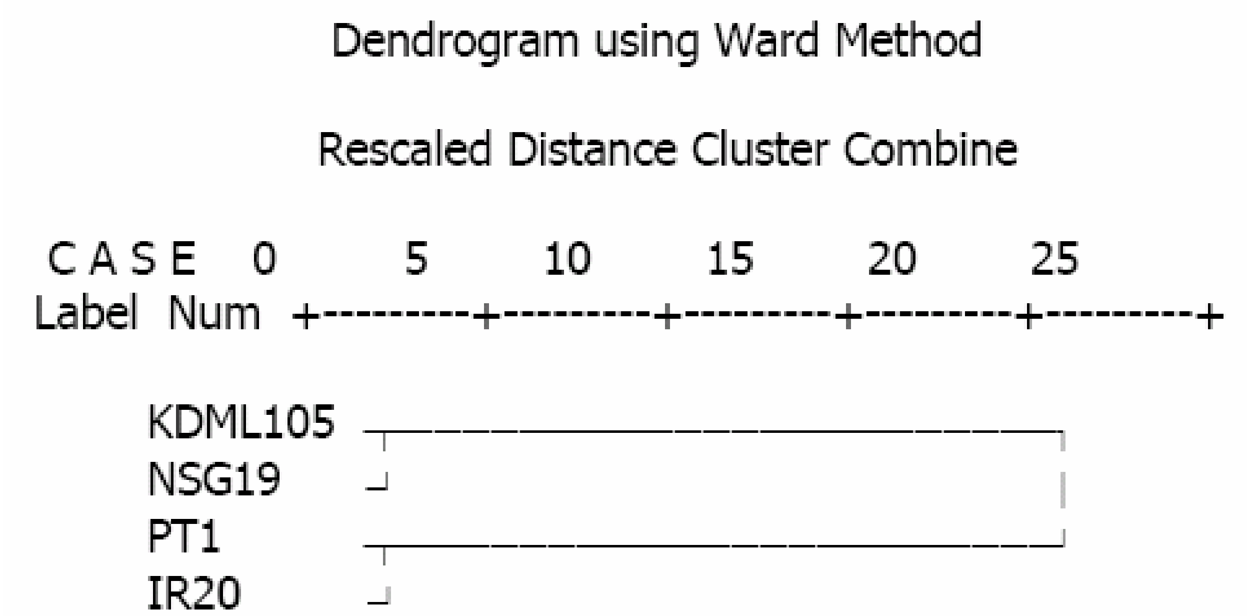

Fig. 4. Ward's dendrogram for four rice cultivars to classify them as water-deficit susceptible, PT1 and IR20 and water-deficit tolerant, KDML105 and NSG19, using yield loss, RWC and WUE abilities, proline accumulation, P_n reduction, F_v/F_m diminution, and pigment degradation.

Conclusion

Biochemical (proline accumulation) and physiological characters (RWC, WUE and photosynthetic abilities) and panicle traits of rice genotypes in response to varying degrees of water deficit stress were developed as multivariate criteria for water deficit tolerance screening. The basic knowledge gained from this investigation should be further applied for water deficit tolerance screening of rice genetic resources i.e. inbred lines, double haploid lines, hybrid population and wild rice species.

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