

## Physiochemical Traits as Potential Indicators for Determining Drought Tolerance during Active Tillering Stage in Rice (*Oryza sativa* L.)

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### ABSTRACT

It is well known that water scarcity limits crop production and further expansion of agriculture. In order to combat the adverse effect, plants have developed various morphological, physiological, and biochemical responses. Several studies were carried out separately, but there have been limited reports which explored on the combined effects of stress factors. Therefore, the present study was attempted to relate physiochemical traits under water deficit condition and explore the possibility of utilizing it for further crop improvement in rice. Five commercial rice varieties, namely MR-37, MR-84, MR-219, MR-220 and MR-232, were grown in randomized block design replicated thrice. At active tillering stage (i.e. 45 days after sowing), drought was induced by withholding water for a period of 7-10 days. Drought symptom was noticed by wilting of leaves. At the time of wilting, physiological parameters like relative water content (RWC), total chlorophyll content, chlorophyll stability Index (CSI), proline accumulation and protein content were estimated for both stress induced and control plants. Statistical analyses were performed to determine the effect of drought stress on the rice varieties. Significant differences were noticed for the physicochemical traits studied under drought condition. Mean performance of the genotypes also showed a sharp decline in RWC (%), total chlorophyll content, CSI (%), and protein. Meanwhile, free proline accumulation was found to be more in water deficit condition. Correlation coefficient ( $r$ ) revealed a significant negative association of RWC (%) with total chlorophyll content, chlorophyll stability index (CSI %), proline and protein with, while total chlorophyll content itself had significant positive relation with CSI (%) under water deficit condition. Correlation coefficient clearly demonstrated that reduction in RWC would affect the osmotic adjustment, cell membrane stability, and photosynthetic machinery in crop plants. Estimates of genotypic variance, board sense heritability, and genetic advance revealed moderate to high estimates for RWC (%), CSI (%), and proline. These high estimates indicated that the rice varieties had inherent potential for drought improvement program. Moreover, the physiochemical parameters involved in the study are fair enough to distinguish between tolerance and susceptible genotypes. Therefore, these parameters could be utilized as key indicators for laboratory screening in determining drought tolerance in rice plants.

**Keywords:** Drought, rice, physiochemical parameters, correlation coefficient, heritability, genetic advance

### INTRODUCTION

Water scarcity limits crop production and further expansion of agriculture in the world. As water resources for agronomic use are becoming

scarce, development of drought tolerant lines has been considered as a valid breeding target to mitigate yield loss. Several efforts have been taken to improve rice production under water limiting conditions through conventional

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breeding techniques. However, progress in traditional breeding approach has been slow due to limited knowledge on genetics of drought tolerance and involvement of several complex tolerance mechanisms (Yeo and Flower, 1986). Moreover, inadequate screening techniques and low screening efficiency (Gregorio *et al.*, 1997; Fukai *et al.*, 1999) have also limited the progress in breeding drought tolerance plants.

It is recognized that plants under stress have developed various physiological and biochemical adaptive responses like accumulation of sugars, amino acids, organic acids, and inorganic compounds (Morgan, 1984; Sairam *et al.*, 2002). These biochemical compounds play a key role in preventing membrane disintegration and provide tolerance against drought and cellular dehydration (Hanson and Hitz, 1982; Bohnert and Jensen, 1996; Mahajan and Tuteja, 2005). Although many studies were carried out separately on physiological and biochemical basis of drought tolerance, there have been limited studies exploring the combined effects of the stress factors (Tal, 1985). Therefore, physiochemical parameters associated with drought tolerance should be evaluated extensively for screening drought tolerance in rice. As mechanism of responses to drought stress varies with genotypes and growth stages of its life cycle (Ashraf and Harris, 2004), it would be much more valuable if biochemical indicators are specified for individual crop species. Knowledge on heritability, genetic advance and inter-relationship, among various physiochemical dehydration responses, will offer an insight for developing practicable strategies to improve drought stress tolerance in rice. In the present study, drought induced physiochemical responses were monitored in five commercial rice varieties using parameters like total chlorophyll content, chlorophyll stability index (CSI), relative water content (RWC), proline accumulation and total soluble protein, and responses of individual varieties are correlated using statistical tools.

## MATERIALS AND METHODS

Seeds of five commercial rice varieties (MR 232, MR 37, MR 87, MR 219, and MR 220) were grown in 30cm x 30 cm plastic pots filled with clay loam soil. Thinning was done on the 15<sup>th</sup> day after sowing (DAS) and only five plants were retained per pot. A slow release commercial fertilizer (15% N, 15% P, and 15% K) of 7g was added to each pot to maintain healthy crop stand. Randomized block design was followed with three replications, while two sets of experimental materials were maintained - one was kept as a control and the others for stress induction. A gradual dehydration condition was applied on 45 DAS by withholding water for a period of 7-10 days. The response was noticed by wilting. At the time of wilting, the leaf samples were collected from both the control and stress plants and analyzed for their physiochemical parameters.

### *Determination of Relative Water Content*

Leaf relative water content (RWC) was measured according to the method of Weatherly (1950); four leaf samples were collected and weighed to determine their fresh weight (FW). The leaves were rehydrated by placing them in distilled water for 12 h at 25 °C in order to obtain turgid weight (TW), followed by oven drying at 80 °C for 48 h and reweighed the leaf samples to get their dry weight (DW). Meanwhile, relative water content was calculated using the following formula:

$$\text{RWC (\%)} = \frac{\text{Fresh weight (FW)} - \text{Dry weight (DW)}}{\text{Turgid weight (TW)} - \text{Dry weight (DW)}} \times 100$$

### *Determination of the Total Chlorophyll Content*

Chlorophyll content was determined using the methods proposed by Harbone (1984). 500 mg of the leaf tissues was homogenized in 80 % acetone at 4 °C. The supernatant was taken for

determination of photosynthetic pigments using the following formula:

$$\begin{aligned} \text{Total chlorophyll content (mgL}^{-1}\text{)} &= 17.3 A_{646} + 7.18 A_{663} \\ \text{Chlorophyll } a \text{ (mgL}^{-1}\text{)} &= 12.21 A_{663} - 2.81 A_{646} \\ \text{Chlorophyll } b \text{ (mgL}^{-1}\text{)} &= 20.13 A_{646} - 5.03 A_{663} \end{aligned}$$

#### *Estimation of Chlorophyll Stability Index (CSI)*

CSI in the leaves was estimated using a UV spectrometer following the method of Koleyoreas (1958). Two leaf samples of 250 mg each were put in two test tubes containing 10 ml of distilled water. One of the test tubes was placed in a water bath and heated to 65 °C for 30 minutes, while the other was kept as a control. Then total chlorophyll content was estimated using a spectrophotometer at 652 nm. CSI was calculated using the following formula:

$$\text{CSI (\%)} = \frac{\text{Total chlorophyll content (heated)}}{\text{Total chlorophyll content (control)}} \times 100$$

#### *Estimation of Free Proline Content*

Free proline content in the leaves was determined using the method described by Bates *et al.* (1973). The leaf tissue of 0.5g was homogenized in 3% of aqueous sulphosalicylic acid using mortar and pestle. The homogenate was centrifuged at 9000 rpm. The reaction mixture consisted of 2 ml of supernatant, 2 ml of ninhydrin, and 2 ml of glacial acetic acid. The reaction mixture was incubated in a water bath at 100 °C for 1 h, and then allowed to cool to room temperature. After cooling the reaction mixture, about 2 ml of toluene was added and mixed on a vortex mixture for 20s in a fume hood. The test tube was allowed to stand for 5 min to allow the separation of toluene and aqueous phases. The absorbance of toluene phase was read at 520 nm in the spectrophotometer against toluene blank. Meanwhile, the concentration of proline was estimated by referring to a standard curve of proline and expressed as mg/g fresh weight.

#### *Protein Extraction and SDS-PAGE Analysis*

About 1.0 g of the leaf tissues was ground in a cold mortar. The grinding medium (4-6 ml/g fresh mass) consists of 50mM Tris-HCL buffer (pH 8.0), 1mM PMSF, 10% (v/v) glycerol and homogenizing beads. The homogenate was filtered through four layers of cheesecloth and centrifuged at 12000 rpm for 20 minutes at 4 °C, and the supernatant was taken after that. An aliquot of the extract was used for protein concentration following the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

SDS-PAGE was performed according to Laemmli (1970) with 12.5% acrylamide gels. Prior to electrophoresis, an equal volume of loading buffer [100 mM Tris-HCL, pH – 8.0, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, 0.025% (w/v) bromophenol blue] was added to the protein sample and boiled at 100 °C for 2 minutes. Electrophoresis was carried out at a constant current of 30mA per plate towards the cathode for 2 h. Gels were stained with 0.03% Coomassie Brilliant Blue R-250.

### **STATISTICAL ANALYSIS**

The experiment was performed using a randomized block design with three replications. Differences among the stress induced, as well as control, were tested using the SPSS software (Version 11.) Statistical variance analysis of the data was performed using ANOVA. From the ANOVA table, genotypic variance, broadsense heritability and genetic gain were estimated. The correlation coefficient analysis was performed and compared with the least significant differences at 5% level.

### **RESULTS AND DISCUSSION**

#### *Effect of Drought on RWC (%)*

Plant water stress was measured in terms of leaf water potential or leaf relative water content. Fig. 1(a) depicts the performance of genotypes for RWC (%), under water deficit condition, in

which a sharp decline in RWC was recorded among the genotypes. From *Fig. 1*, it is evident that the genotypes MR 220 and MR 219 showed a decrease in RWC (%). This genotypic variation might be attributed to the variations in stomatal control of transpiration (Dingkuhn *et al.*, 1989), water extraction ability (Lilley and Fukai, 1994), and variation in the canopy size at the onset of stress (Mitchell *et al.*, 1998). Progressive decline in RWC was reported by Silva *et al.* (2007) in sugarcane. Some previous studies (e.g. Jamaux *et al.*, 1997; Altinkut *et al.*, 2001; Colom and Vazzana, 2003) have shown that maintenance of a relatively high RWC during mild drought is an indicative of drought tolerance.

#### *Effect of Drought on Total Chlorophyll Content*

Total chlorophyll was found to decrease with the severity of stress. Under controlled condition, the amount of total chlorophyll ranged from  $3.72 \pm 0.05$  to  $20.201 \pm 1.034$  mg/g FW. On the other hand, stressed plants revealed total chlorophyll between  $0.58 \pm 0.02$  and  $17.78 \pm 0.29$  mg/g FW, and the extent of this decrease was strongly cultivar-dependent. In both the conditions, the highest mean for this particular trait was obtained in MR219 and the lowest in MR37 (*Fig. 1(b)*). Chlorophyll degradation is considered as one of the consequences of drought stress which has resulted from sustained photo-inhibition and photo bleaching (Long *et al.*, 1994). Meanwhile, the decrease in chlorophyll is associated with a reduction in the flux of nitrogen into the tissue, as well as alteration in activity of enzyme systems such as nitrate reductase (Begaum and Paul, 1993).

#### *Effect of Drought on CSI (%)*

Wider variation was noticed between the control and drought stress plants for chlorophyll stability index (*Fig. 1(c)*). Under a normal growth condition, a slight variation for CSI (%) was seen among the genotypes. In more specific, significant differences were noticed when the same genotypes were exposed to water deficit

condition. CSI (%) was found to range from  $47.79 \pm 4.67$  to  $91.62 \pm 3.13$ . Based on the result obtained for CSI (%), MR232 had a high mean value and it also tended to be stable, while MR37 was found to be sensitive to water deficit stress. Meanwhile, higher CSI indicates the level of polyunsaturated lipids stabilizes chloroplast membrane and increases adaptive response to tolerance under water stress condition.

#### *Effect of Drought on Proline*

Accumulation of proline is one of the common characteristics in many monocotyledons under stress (Wyn Jones, 1981; Asraf, 1994; Mansour, 2000). From *Fig. 1(d)*, two to three-folds of increase in the accumulation of proline were observed among the genotypes. In particular, MR220 exhibited a higher accumulation of proline ( $67.13 \pm 0.245$ ), whereas MR232 showed a minimal value for this trait ( $17.57 \pm 1.02$ ). The synthesis of proline helps the cell to maintain their hydrated state and therefore functions to provide resistance against drought and cellular hydration (Hockstra *et al.*, 2001; Ramanjulu and Bartels, 2002). Similarly, Maggiao *et al.* (2002) suggested that proline might act either as a signalling or regulatory molecule which is capable of activating multiple responses which are component of the adaptation process.

#### *Effect of Drought on Protein*

Like other physiochemical traits, water stress condition has also been known to alter protein metabolism. Total soluble protein was found to respond to stress either by increasing or decreasing its amount. Four genotypes, namely MR232, MR37, MR84, and MR220, showed a decreasing trend, whereas MR219 alone revealed an increasing trend (*Fig. 1(e)*). The increase and decrease in the total soluble proteins under drought stress were found to be consistent with the findings of some other researchers (e.g. Riccardi *et al.*, 1998; Ti da *et al.*, 2006; Nayer and Reza, 2008). Proteins synthesize *de novo* in response to stress and increase when plants are exposed to stress (Pareek *et al.*, 1997; Ashraf

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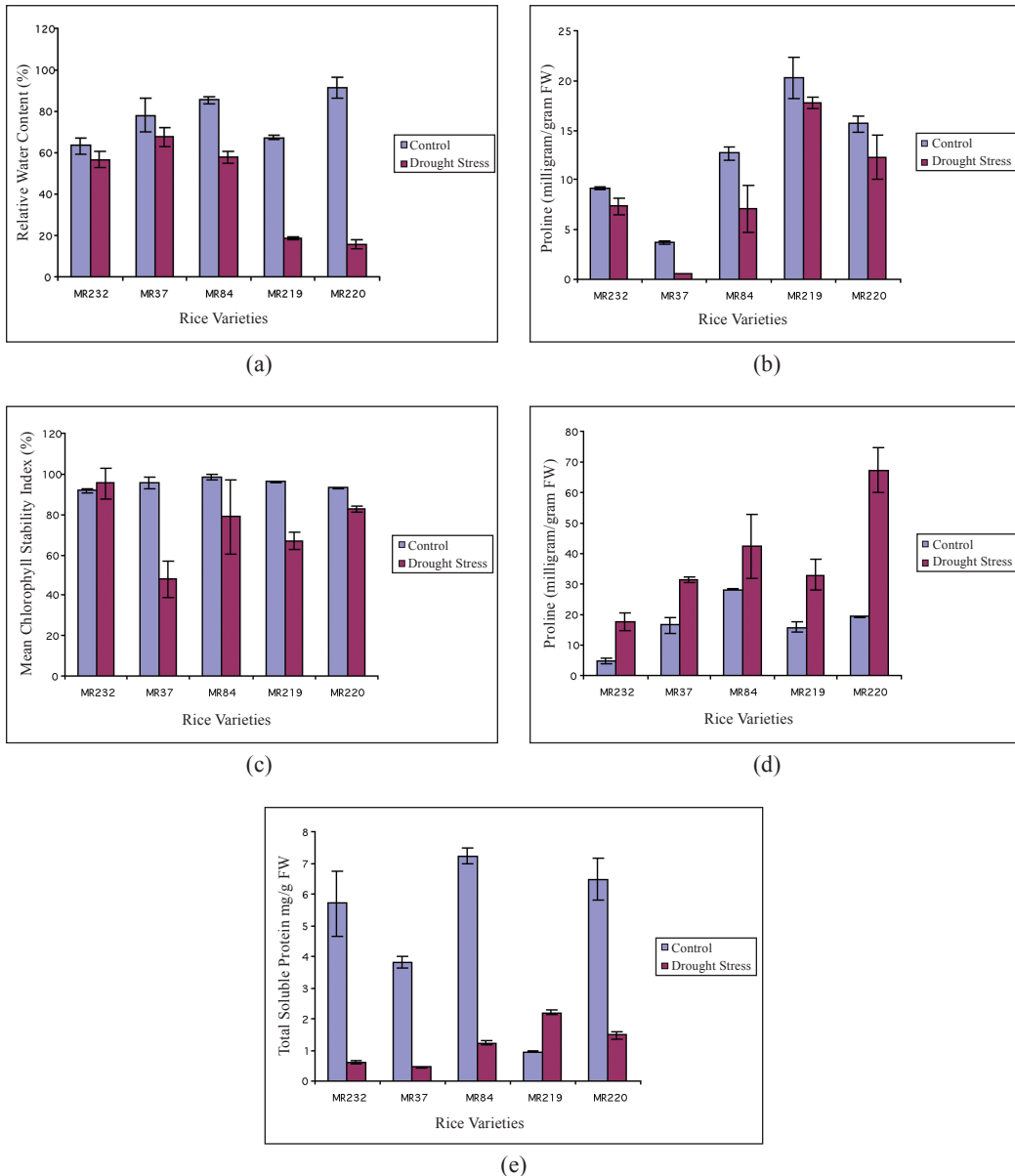


Fig. 1: Effect of drought stress on (a) RWC (%), (b) Total chlorophyll, (c) CSI (%), (d) Proline and (e) Total soluble proteins. Values shown are the average of five plants in each replication (mean  $\pm$  S.E). All the treatments were significantly different from the control ( $p < 0.05$ )

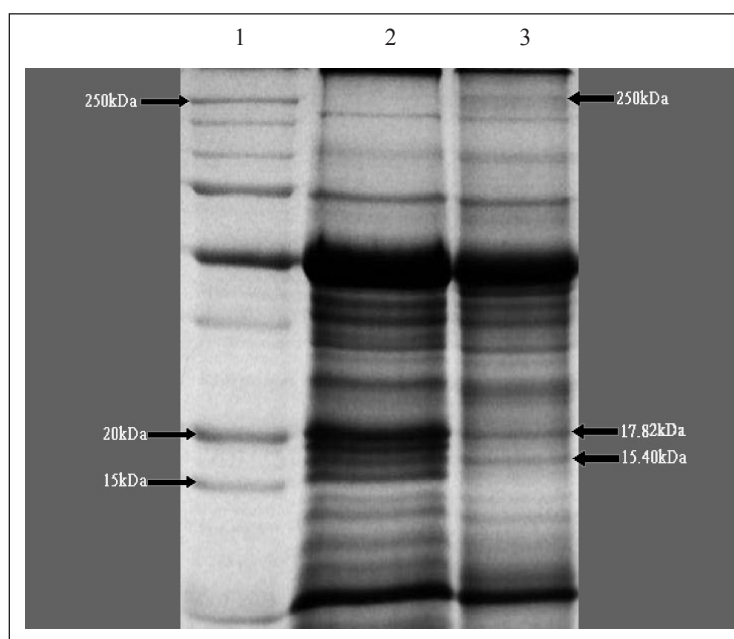
and Harris, 2003). Proteins which accumulate in plants, which are grown under stress conditions, may provide a storage form of nitrogen that is re-utilized when the stress is over (Singh *et al.*, 1987) and it may also play a role in

osmotic adjustment. In general, drought induces metabolic changes related to protein turnover. This is probably due to the alteration in protein synthesis, and maintenance the level of the same proteins or protein degradation.

The leaf tissues of both the control and stress genotype of MR219 were analyzed for protein using one-dimensional SDS-PAGE. The SDS gel electrophoresis revealed a considerable difference in the banding pattern. A gel comparing control and stressed proteins from the leaf tissue is shown in *Fig. 2*. Meanwhile, low (~ 15.5 –18.0 kDa) and high molecular weights (~ 250 kDa) of protein bands were also detected. In response to drought, protein synthesis exhibits a wide range in terms of size, i.e. from 9 to 200 kDa (Close, 1996). These proteins are called dehydrins and belong to group II of the late embryogenesis abundant protein (LEA). The results obtained in the study are similar to the findings of Mohammadkhani and Heidari (2008). They reported a leaf protein accumulations of 15, 17, 20, 27, 30, 33, 37 kDa in wheat in progressive water deficit condition.

#### *Estimates of Correlation Coefficient*

Correlation coefficient ( $r$ ) of physiochemical parameters was estimated for both the controlled and water deficit conditions and the results obtained are presented in Table 1. In this investigation, an interesting association was found among the various physiochemical parameters towards drought. The correlation coefficient ( $r$ ) in Table 1 illustrates that the total chlorophyll has a negative significant correlation with RWC (%) ( $r = -0.872, p < 0.05$ ), while a strong positive significant relation was noticed with CSI (%) ( $r = 0.603, p < 0.05$ ) and protein ( $r = 0.899, p < 0.05$ ). The magnitude of association between the two traits was found to be more in stressed plants than in well-watered plants. The findings of this study are in agreement with the findings of Thornber *et al.* (1991).



*Fig. 2: SDS PAGE profile indicating the changes in protein expression in both stress-induced and control plants. The lane on the left side indicates the molecular weight of standard protein (Precision plus protein # 161-0373 was used as a molecular marker). Lane 1: molecular marker; Lane 2: Proteins obtained from control plants, and Lane 3: Proteins obtained from water deficit plant. The arrow head indicates ~ 15- 18kDa proteins that are responsive for drought tolerance*



On the other hand, under water deficit condition, RWC had significant negative correlation with the total chlorophyll content, proline ( $r = -0.569$ ,  $p < 0.05$ ) and protein biosynthesis ( $r = -0.830$ ,  $p < 0.05$ ). Meanwhile, the negative interrelationship of RWC with proline is presumed because under a normal condition, RWC and proline are in equilibrium and when the plants are exposed to stress, the amount of RWC tends to decrease while proline accumulates. Hein *et al.* (2003) observed that the accumulation of proline was inversely correlated to the P<sub>5cs</sub> mRNA detected, and further suggested that a short-term accumulation might be a good indicator of rice osmotic stress tolerance. On the contrary, a decline in the accumulations of RWC and proline indicated the severity of dehydration, whereas osmotic adjustment might fail to maintain the turgor in the affected tissues. Out of several biochemical indices of water deficit injury, the accumulation of proline and the decline in protein synthesis in plants have been widely reported (Irigoyen *et al.*, 1992). In more specific, drought stress was found to disturb protein metabolism and production pathway, and hence was substituted by the accumulation of proline. Hence, under a water deficit condition, the synthesis of

proline was found to be associated with protein hydrolysis. The results of the present study are in accordance with the findings of Irigoyen *et al.* (1992), as well as Ashraf and Iram (2005). Meanwhile, protein profile of SDS-PAGE revealed the presence of low molecular protein (~ 15.5 – 18.0 kDa) and high molecular protein (~ 250 kDa).

A protective role of exogenously supplied proline in relation to cell membrane stability and mechanism of its action were documented in many papers (Blum, 1981; Rudolph *et al.*, 1986; Smirnov and Cumbes, 1989; Bandurska, 1998; and Schurr *et al.*, 2000).

As presented in Table 1, the negative association obtained for RWC with the total chlorophyll content ( $r = -0.872$ ,  $p < 0.05$ ), chlorophyll stability index ( $r = -0.222$ ,  $p < 0.05$ ), proline ( $r = -0.569$ ,  $p < 0.05$ ) and protein ( $r = -0.830$ ,  $p < 0.05$ ) indicated that the expression of RWC in the plant tissue was determined by several characteristics such as osmotic adjustment, cell membrane stability, and accumulation of compatible solutes like proline. Meanwhile, the reduction in RWC, due to drought stress, was found to affect photosynthetic machinery, cell membrane stability, and proline synthesis.

TABLE 1  
Pooled phenotypic correlation coefficient (r) among physiochemical traits for the controlled and drought stress conditions in rice plants

	TC	CSI (%)	RWC (%)	Proline (mg/g) FW	Protein (mg/g) FW
TC	1.000	0.150	0.22	0.202	-0.275
		<b>0.603**</b>	<b>-0.872**</b>	0.259	<b>0.899**</b>
CSI (%)		1.000	-0.276	0.018	0.099
			-0.222	0.135	0.086
RWC (%)			1.000	0.735*	0.543*
				<b>-0.569**</b>	<b>-0.830**</b>
Proline				1.000	0.273
					0.341
Protein					1.000

- Drought stress values are indicated in bold letter
- Correlation coefficients significance at \*p = 0.05 level and \*\* p = 0.01 level

TABLE 2  
Genetic variation, Heritability (%) and Genetic advance of physiochemical parameters under controlled and drought stressed conditions in rice

Parameters	Genetic variance		Heritability (%)		Genetic advance	
	Control	Stress	Control	Stress	Control	Stress
Relative water content (%)	130.32	585.44	28.29	97.66	3.96	49.26
Total chlorophyll (mg/ml)	38.96	44.35	97.13	89.99	12.67	13.01
Chlorophyll stability index	3.28	263.79	57.6	73.12	2.83	28.61
Proline content (mg/g) FW	70.96	89.20	96.91	89.20	17.08	35.38
Protein content (mg/g) FW	6.18	0.50	92.56	98.21	4.93	1.44

#### *Estimates of Genetic Variance, Heritability and Genetic Advance*

Estimates of genotypic variance offer scope for crop improvement, while heritability and genetic advance enable us to make prediction about the progress of selection. Determination of genotypic variance, broad sense heritability and genetic advance (Table 2) indicated that under water stress condition, RWC % (585.44) and CSI % (263.79) had a very high genotypic variance, followed by proline (89.20) which was moderate. These variations could effectively be utilized for screening drought tolerance at an early stage in rice. The effectiveness of selection is in turn dependent on the percentage of broad sense heritability. A high heritability was noticed for all the physiochemical traits involved in the study, indicating that the physiochemical traits possess inherent potential for drought improvement. Progress in selection is therefore determined by high estimates of genetic advance. In the current study, a moderate genetic gain was recorded for RWC (%), CSI (%) and proline content, respectively.

From the foregoing results, it is obvious that the physiochemical traits undertaken in this study were affected, either positively or negatively, by water deficit condition. A sharp decline in RWC (%), total chlorophyll content, CSI (%) and protein was noticed, whereas the accumulation of proline was more prominent. The correlation coefficient analysis revealed the presence of interrelationship between RWC (%), Proline and Protein, as well as the total chlorophyll content and CSI (%) under a water deficit condition. All

the physiochemical parameters involved in the study had high heritability but moderate genetic advance which indicate the scope for further selection and improvement of rice for drought tolerance. Therefore, the parameters viz., total chlorophyll content, chlorophyll stability Index, relative water content, proline accumulation, and protein synthesis are sufficient enough to provide reliable laboratory screening indicators to screen for drought tolerance in rice breeding programmes. Moreover, these parameters are fair enough to distinguish between tolerance and susceptible, and these parameters could therefore be used as a selection criterion for drought stress improvement.

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