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INCREASED ANTIOXIDANTS AND PROLINE ACCUMULATION CORRELATES WITH ACQUIRED THERMOTOLERANCE IN RICE

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ABSTRACT

Rice (*Oryza sativa* L.) occupies the enviable prime place among the food crops cultivated around the world and remain as the most important food crop in Asia. Most of rice is currently cultivated in the regions where temperature is close to optimal or above for growth. Plants have both inherent ability to survive at high temperature and ability to acquire tolerance to lethal temperatures. Thermotolerance can also be induced by gradual increase in temperature from normal to lethal temperature as would be experienced in natural environment. We have measured cellular level tolerance of eleven rice genotypes using temperature induction response (TIR) technique and clustered them into different groups based on survival per cent and growth reduction. This resulted in identification of five rice tolerant genotypes *viz.,* ADT 43, Apo, N22, TKM9 and white ponni. Further, we demonstrated that tolerant genotypes showed higher catalase and peroxidase activity and proline accumulation. In case of antioxidants, significant negative correlation was found between catalase activity and growth reduction of seedlings of acclimated rice genotypes indicating the major role of the enzyme in temperature acclimation process.

KEY WORDS
Thermotolerance
acclimation
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INTRODUCTION

By the end of the 21st century, the earth's climate is predicted to warm by an average 1.8 to 4.0 ºC due to both anthropogenic and natural factors (Eitzinger *et al*., 2010). Most of rice is currently cultivated in the regions where temperature is close to optimal or above for growth. Therefore, any further increase in mean temperature would certainly reduce the rice yield. While some countries in temperate zone may reap some benefit from climate change, many countries in the tropical and subtropical zones appear more vulnerable to the potential impacts of global warming.

Plants overcome high temperature stress by adapting several physiological and biochemical mechanisms (Srikanthbabu *et al*., 2002). Thermotolerance can also be induced by gradual increase in temperature to normally lethal temperature as would be experienced in natural environment (Hikosaka *et al.,* 2006). Plants growing in their natural habitat at regular temperature range may experience high temperature that would be lethal in the absence of acclimation and hence ability to acquire thermotolerance is of significant importance to plants.

Many studies have shown that genetic variability was seen only upon acclimation treatment prior to severe stress (Srikanthbabu *et al.,* 2002) and the variability observed when seedlings were exposed to severe stress directly is marginal. Since the genetic variability is seen only upon acclimation, assessing stress responses on exposure to acclimation treatment could be a potential tool to screen for thermotolerance. Such a selection procedure is referred to as the temperature induction response (TIR) based screening technique (Senthil Kumar *et al*., 2003). Such a response was demonstrated across the genotypes of pea, wheat, rice and sunflower (Srikanthbabu *et al.,* 2002, Venkatesh babu *et al.,* 2013).

Although rice has been used as a model plant for many years, the responses of rice genotypes to high temperature stress is still poorly understood. Developing high temperature tolerant rice genotypes becomes a feasible option that would require screening of genotypes for thermotolerance. This study was conducted to screen the rice genotypes for thermotolerance and physiological and biochemical traits associated with acclimated tolerance rice genotypes mainly proline content and antioxidants activity.

MATERIALS AND METHODS

Measurement of cellular level tolerance using temperature induction response
The experiment was conducted with eleven genotypes including popular rice varieties of Tamil Nadu, land races and genotypes known for high temperature tolerance and susceptibility. In this method, the seedlings were exposed to standardised induction temperature of 36-44 ºC for 5 h initially immediately

followed by lethal temperature of 50 ºC for 3 h. After lethal temperature, the seedlings were kept for recovery at 30 ºC for 3 days. The seedling growth measured as lengths of root and shoot. The per cent reduction in induced seedling growth over absolute control were calculated using method adopted by Senthil Kumar *et al.*, 2003.

Measurement of proline content of rice seedlings exposed to TIR technique

The method for the estimation of proline content was adopted from Bates *et al*. (1973) with slight modifications. The treated seedlings (1g) were homogenized with 10 ml of 3 % sulphosalicylic acid and centrifuged at 3000 rpm for 10 minutes. Two ml of the supernatant and 2 ml of glacial acetic acid and 2 ml of acid ninhydrin mixture were added. The contents were allowed to react at 100 ºC for 1 h and then it is incubated on ice for 10 min to terminate the reaction. The reaction mixture was mixed vigorously with 4 ml toluene for 15-20 seconds.

The chromophore containing toluene was separated from the aqueous phase, brought to room temperature and optical density was read at 520 nm. The proline content was determined from the standard graph prepared using commercially available proline in the concentration range of 20-100 μg.

Estimation of catalase and peroxidase in acclimated and non-acclimated rice seedlings

Peroxidase activity (change in OD value at 430 nm g^{-1} $min^{-1)}$ was determined according to Angelini *et al*. (1990). One gram of fresh leaves was extracted using 0.1M phosphate buffer (pH 7.0) and a known volume of the extract was added to a cuvette containing 3 ml phosphate buffer and 3 ml pyrogallol and the increase in absorbance at 430 nm was recorded. The change in absorbance in minutes was used to calculate the enzyme activity.

Catalase activity was assayed from the rate of H_2O_2 decomposition extinction coefficient of 39.4 mmol as measured by the decrease in the absorbance at 240 nm, following the procedure of Aebi, (1974). The reaction mixture contains 50 mmol potassium phosphate buffer (pH 7.0) and the appropriate volume of extract. The reaction was initiated by adding 10 mmol of H_2O_2. One unit of catalase is defined as the amount of enzyme that liberated half of the peroxide oxygen from 10 mmol H_2O_2 solutions in 100 sec at 25ºC.

RESULTS AND DISCUSSION

Prevalence of high temperature is the major limitation for the cultivation of crops in tropical conditions. The effect of high temperature can be seen at cellular and whole plant level affecting growth, reproduction and productivity of crop plants. Moisture stress coupled with high temperature is known to adversely affect the growth and development in rice, ultimately resulting in low yield.

Plants overcome high temperature stress by adapting several physiological and biochemical mechanisms (Srikanthbabu *et al.*, 2002). Thermotolerance can also be induced by gradual increase in temperature to normally lethal temperature as would be experienced in natural environment (Hikosaka *et al.*, 2006). Plants growing in their natural habitat at regular temperature range may experience high temperature that

would be lethal in the absence of acclimation and hence ability to acquire thermotolerance is of significant importance to plants.

Identification of temperature tolerant genotypes using TIR

Several studies have shown that plants develop the ability to withstand severe temperature stress if it is exposed to mild temperature stress previously. Earlier reports (Fender and O'Connell, 1989) suggest that genetic variability could not be visualized when plants were directly exposed to severe stress. The altered metabolism in the acclimated plants is due to expression of different stress responsive genes which imparts tolerance to subsequent severe stress.

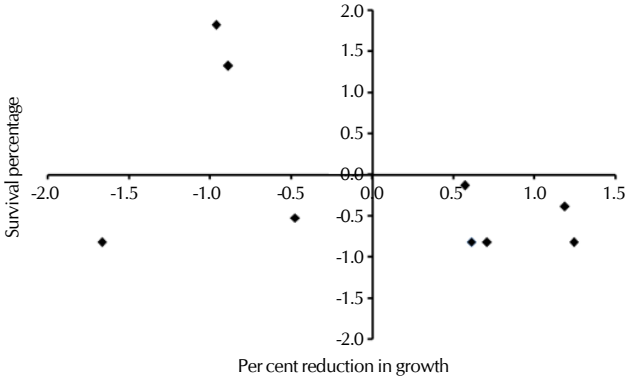
Several thermotolerant traits like cell membrane stability (Manohar ram *et al.*, 2014), cell viability and chlorophyll stability can be considered as potential parameters to screen for tolerance. However, selection based on survival and recovery growth on exposure to severe temperature is a desirable option, as it reflects the sum of variation in intrinsic tolerance brought about by different tolerance mechanisms.

Table 1: Measurement of cellular level tolerance of rice genotypes using Temperature Induction Response technique (TIR)

Genotypes	Root Length (cm)		Shoot Length (cm)		Seedling growth (RL + SL) (cm)		Survival per cent		Per cent growth
	Induced	Control	Induced	Control	Induced	Control	reduction	Induced	Control
ADT43	8	5.9	7.5	5.4	15.5	11.3	100	82.6	27.4
Apo	5.3	4.3	3.8	2.3	9.1	6.7	100	100	26.6
IR64	6.4	3.1	5.7	3	12.1	6.1	100	45.9	49.1
Kallurundaikar	6.5	3.4	6.1	3.9	12.6	7.2	100	92.5	42.9
Moroberekan	6.5	3.2	5.9	1.2	12.5	4.4	100	100	66.4
N22	4.5	3.5	5.5	4.7	10	8.2	100	100	17.4
Nootripathu	6.4	3.1	5.7	3	12.1	6.1	100	45.8	49.1
Norungan	8	5.9	7.5	5.4	15.5	11.3	100	82.6	27.4
TKM9	5.4	2.8	4.4	4.5	9.9	7.3	100	100	25.4
Varapukkudanchan	6.8	4.5	6	2.8	12.7	6.3	3	33.3	50.2
White Ponni	3.8	3.2	5.3	4.3	9.2	7.5	100	92	18.2
Mean	6.2	3.9	5.8	3.7	11.9	7.5	99.4	79.5	36.4
G	T	G x T	G	T	G x T	G	T	G x T	G	T	G x T
SEd 0.39	0.16	0.5	0.25	0.1	0.36	0.49	0.21	0.69	2.08	0.88	2.95
CD (0.05) 0.79*	0.33*	1.1*	0.5*	0.2*	0.73*	0.99*	0.42*	1.40*	4.20*	1.79*	5.94*
CV (%) 6.54				10.4		5.77	5.11				

Table 2: Effect of temperature induction on Proline Content, Peroxidase activity, Catalase activity

Genotypes	Proline(μmole g^{-1})			Peroxidase Activity(Ä 430 nm g^{-1} min^{-1})			Catalase Activity(μg H_2O_2 min^{-1}g^{-1})		
	Control	Induced	Non-induced	Control	Induced	Non-induced	Control	Induced	Non-induced
ADT43	1.51	2.65	1.6	1.5	1.96	1.72	2.83	5.41	3.47
Apo	1.19	1.96	1.27	1.48	3.3	2.3	2.59	5.3	3.26
IR64	1.14	1.65	1.22	1.52	3.95	3.05	1.41	2.29	1.68
Kallurundaikar	1.09	1.61	1.18	1.49	2.47	1.88	0.4	1.24	0.49
Morobereken	0.94	1.27	1.2	2.55	2.88	2.69	1.76	2.13	2.3
N22	1.13	2.03	1.25	1.58	3.47	2.69	0.76	1.88	1.26
Nootripathu	0.96	1.39	1.06	2.77	3.45	2.87	1.68	2.2	1.85
Norungan	1.02	1.4	1.09	1.58	3.04	2.64	4.27	6.69	5.06
TKM9	1.07	2.03	1.21	1.29	3.16	2.48	3.42	6.75	4.14
Varappukudanchan	0.91	1.25	1.02	1.84	1.93	2.01	2.63	3.15	2.94
White Ponni	1.05	1.47	1.1	2.55	3.67	2.72	3.67	5.91	4.86
	G	T	G X T	G	T	G X T	G	T	G X T
SEd	0.07	0.04	0.12	0.15	0.08	0.26	0.36	0.19	0.62
CD (0.05)	0.15	0.08	0.25	0.3	0.16	0.52	0.72	0.37	1.24
CV (%)	11.05		6.13			9.4			



I - Lower per cent reduction in growth and lower survival per cent
II - High per cent reduction in growth and lower survival per cent
III - High per cent reduction in growth and high survival per cent
IV - Lower per cent reduction in growth and high survival per cent

Figure 1: Clustering of genotypes for thermo tolerance based on normal 'Z' distribution

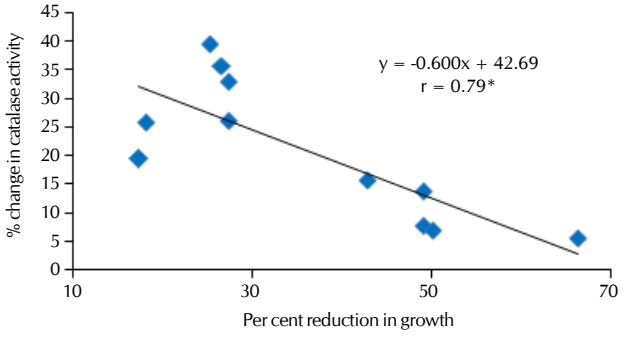


Figure 2: Correlation between percent reduction in growth and catalase activity

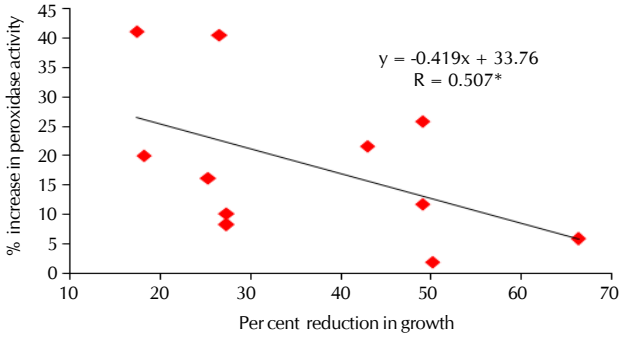


Figure 3: Correlation between percent reduction in growth and peroxidase activity

Such a selection procedure is referred to as the temperature induction response (TIR)-based screening technique and it has been effective in identifying thermotolerant genotypes in many crops (Venkatesh babu *et al.,* 2013, Srikanthbabu *et al.,* 2002, Senthil- Kumar *et al.,* 2003).

Observations from Temperature Induction Response technique revealed significant differences among the rice genotypes for cellular level tolerance. Growth reduction was observed sin all the induced genotypes over control (Table 1). In control condition, the genotypes ADT43 and Norungan exhibited longer root length (8.0) and shoot length (7.5) while White ponni and Apo recorded the shorter root (3.8) and shoot length (3.8) respectively. The survival per cent in all the genotypes was 100 per cent except Varapukkudanchan which showed 93 per cent.

Under induced conditions, ADT43 and Norungan exhibited longer root length (5.9) and shoot length (5.4) and minimum values were recorded in TKM9 (2.8) and Moroberekan (1.2) respectively. The higher total growth was recorded in the genotype ADT43 (11.3) while Moroberekan showed lower (4.4). The highest survival percentage (100) was exhibited in Apo, Moroberekan, N22, Nootripathu and TKM9 while lower value was recorded in Nootripathu (45.8). The maximum per cent growth reduction was recorded in Moroberekan (66.4) while minimum was recorded in N22 (17.4).

A statistical tool termed as Normal Z distribution (Fig. 1) was used to cluster the genotypes into different groups based on survival percent and reduction in growth. The third quarter comprises of genotypes ADT43, Apo, N22, TKM9 and White ponni which showed lower percent reduction in growth and higher survival percent classified as tolerant genotypes. It is noteworthy to mention that N22 and TKM9 which emerged as tolerant genotypes at plant level through open top chambers studies (data not shown) were among the genotypes with higher acquired tolerance at seedling level.

Increased proline content associated with acquired thermotolerance

The observed higher growth in acclimated plants was due to alternation in the metabolism. Various studies showed that protein synthesis was maintained significantly higher in acclimated seedlings especially heat shock proteins compared to non-acclimated seedlings. In this study, data on the proline content showed significant differences among the genotypes under the induced and non-induced conditions (Table 2). The induced seedlings of all the genotypes showed higher proline content compared to control and non-induced seedlings. The proline content in non-induced of all the genotypes was higher than control but lower than the induced seedlings. The genotype ADT43 showed high proline content under control (1.51 μmole g^{-1}), induced (2.65 μmole g^{-1}) and non-induced (1.60 μmole g^{-1}) while Varapukkudanchan recorded lower value under control (0.91), induced (1.25) and Non-induced conditions (1.02).

Catalase and peroxidase correlates with acquired thermotolerance

The analyzed data on catalase and peroxidase activity revealed significant correlation with the acquired tolerance. Between these two enzymes, the greater role of the catalase is observed through stronger correlation with acquired tolerance (Fig 2 and 3). This is based on fact that the primary responses of plants to high temperature is enhanced synthesis of reactive oxygen species ROS (Sung *et al*., 2003), the role of antioxidants in improving the ability of plants to acquire thermotolerance has been demonstrated in this study.

It is therefore conceived that antioxidants are not only important for protection against oxidative stress, but also for the acquired thermotolerance. Taken together, these data suggest that the acquisition of thermotolerance involves induction of number of parallel systems which has to act simultaneously to bring out acquired thermotolerance at whole plant level. Further, the plants typically experience diurnal temperature fluctuations, the acquisition of thermotolerance may reflect a general mechanism that contributes to homeostasis of metabolism. Therefore, it is very relevant in the context of field level thermotolerance.

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