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Physiological inquiry into the acquired thermotolerance of minor millets using Temperature Induction Response (TIR) technique

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Abstract
Minor millets are important staple foods in India. The global rise in temperature has increased the severity of other environmental stresses and markedly reduced the area of minor millet cultivation. Since minor millets are usually grown in marginal lands prone to drought and high temperature there is a need to identify minor millets tolerant to high temperature stresses. To assess temperature stress tolerance, it is necessary to expose the plants to an induction stress before exposing them to the severe stress. Hence an efficient screening protocol referred to as the Temperature Induction Response (TIR) technique has been developed to identify thermo tolerant lines in minor millets viz. Thenai, Kudiraivalli, Varagu, Panivaragu and Samai. Using this standardized TIR protocol, lethal temperature for minor millets was found to be 56ºC for 3 hours and the sub – lethal temperature was standardized at 46 to 54ºC for 1,2 and 3 hours respectively. Among the minor millets, Samai (CO4) and Thenai (CO6) showed higher levels of intrinsic cell tolerance. Samai CO4 showed highest thermotolerance in terms of high seedling survival (94%) and less reduction in root and shoot growth followed by Thenai CO6. The physiological basis of tolerance revealed that, Samai (CO4) was able to maintain higher chlorophyll stability coupled with higher chlorophyll fluorescence values. It also recorded high photosynthetic rates and stomatal conductances at flowering stage compared to other minor millets under stress conditions. The physiological traits recorded under stress conditions in Thenai (CO6) were also comparable with Samai (CO4). Hence, these genotypes with high level of intrinsic temperature tolerance can be explored as novel donor sources in breeding programmes aimed for developing high temperature stress tolerant minor millets.

Keywords: Minor millet, Temperature induction response, Photosynthetic rate, Stomatal conductance, Transpiration rate, Chlorophyll index

Introduction
India is the largest producer of many kinds of millets, which are often referred to as coarse cereals. Minor millets are one among the important staple foods in India. Minor millets are highly nutritious and even superior to rice and wheat in certain constituents. However, realizing the nutrient composition of these grains and their importance in human diets, they are now considered as nutri - cereals (Chandel *et al.*, 2014) [3].

Production of minor millets, the world's most important crop for ensuring nutritional security and addressing poverty is mostly grown in marginal lands and in areas prone to temperature and drought stress. Climate change scenarios poses serious threat to growing minor millets. Hence, there is a need to develop a technique to screen a large number of minor millet genotypes for high temperature tolerance. Plants adapt to high temperature stress by inherent basal level tolerance as well as acquired tolerance to severe temperature stress. Acquired thermotolerance is quite rapid and has been shown to be induced during cell acclimation to moderately high temperature periods (Hikosaka *et al.*, 2006; Larkindale *et al*., 2005) [5, 15].

Temperature affects a broad spectrum of cellular components and metabolism, and temperature extremes impose stresses of variable severity that depend on the rate of temperature change, intensity, and duration. The ability to withstand and to acclimate to supra-optimal temperatures results from both prevention of heat damage and repair of heat-sensitive components (Sung *et al.*, 2003; Senthi *et al.*, 2006) [22, 18]. Therefore, to assess stress tolerance, it is necessary to expose the plants to an induction stress before exposing them to the severe stress. Based on preliminary studies, an efficient screening technique referred to as the temperature induction response (TIR) technique has been developed to identify thermo tolerant lines in minor millets.

According to this technique, the seedlings are exposed to an optimum induction temperature before being exposed to a severe challenging temperature and subsequently allowed to recover at room temperature. The surviving seedlings at the end of the recovery period are selected as thermo tolerant lines (Renuka *et al*., 2013) [17].

High temperature stresses bring significant physiological alterations. Heat stress caused a net photosynthetic rate reduction due to stomatal and non-stomatal limitations, photoinhibition increase, and Rubisco activity reduction (Camejo *et al.*, 2005) [2]. High temperature stress modified PSII functionality in leaves of the tomato plants manifested by lower variable chlorophyll fluorescence yield (Fv), maximum photochemical efficiency of photosystem II in dark adapted leaves (Fv/Fm) in the heat shocked tomato cultivars (Camejo *et al.*, 2005) [2]. Heat stress led to reductions in the chlorophyll a+b and chlorophyll/carotenoid ratios for the stressed maize cultivars (Sinsawat *et al.*, 2005) [19].

Till date, there are no rapid, reliable and reproducing protocols to screen minor millets for high temperature stresses using physiological traits. Hence, the present study was aimed to (i) Standardize the challenging temperature and TIR protocols to screen the minor millet genotypes for acquired thermo tolerance (ii) Study the morphological and physiological changes in minor millets under high temperature condition (iii) To understand the physiological bases of thermo tolerance in minor millets by understanding the PSII efficiency and related gas exchange parameters.

Materials and Methods

Plant Material

The seeds of minor millets namely *Setaria italica* **(**Thenai CO6), *Echinochloa colona* (Kudiraivalli CO2), *Paspalum scrobiculatum* (Varagu CO3), *Panicum miliaceum* (Panivaragu CO5) and *Panicum sumatrense* (Samai CO4) were procured from Department of Millets, Tamil Nadu Agricultural University, and Coimbatore, India. About 7-days-old seedlings germinated on filter paper in Petri dishes and 30-d-old plants raised in pots (50 kg soil) were used for the experiments I and II respectively.

Experiment 1: Designing the TIR protocol for minor millets

Standardized of challenging temperature

Setaria italica **(**Thenai CO6) was used to identify the Challenging temperature. Uniform 7 days old seedlings were taken and it was exposed to different temperatures (52, 54, 56, 58 and 60°C) for 1, 2 and 3 hr without prior induction and these seedlings were immediately allowed to recover at 30°C for 72 h in an incubator. At the end of the recovery period the temperature treatment at which 90% mortality of the seedlings occurred was taken as the challenging temperature in order to assess the genetic variability for seedling survival. The temperature treatment at which 50% reduction in seedling growth occurred was considered as the challenging temperature to assess differences in recovery growth.

Determination of optimum induction and non-induction treatments for seedlings

Seedlings of minor millets (7-d-old) were subjected to different induction temperature treatments following which they were transferred to a defined challenging temperature. The seedlings were exposed to an induction temperature (46°C to 54°C for 3 hrs) before being exposed to a severe challenging temperature (56°C) and other set of seedlings directly exposed to challenging temperature (non-induced). The method was standardized as given in Fig.1

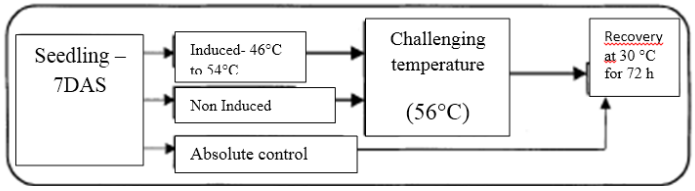

Fig 1: The general protocol to study the induction response of minor millets seedlings and plants. Induced: Seedlings or Plants subjected to an induction temperature treatment (46ºC-54ºC for 3 hrs) and immediately transferred to the challenging temperature (56ºC for 3 hrs). Non-induced: temperature maintained at 30 °C then exposed to the challenge temperature (56ºC for 3 hrs). Absolute control: Temperature maintained at 30 °C for 72 hrs throughout the experiment.

Cell viability of the seedlings during recovery from high temperature stress: After subjecting the seedlings to challenging temperature, seedlings were allowed to recover at 30 °C for 72 h. At the end of recovery, the percentage survival and root and shoot growth of the surviving seedlings were recorded. The root and shoot length (cm) was measured to arrive at total seedling growth. The seedlings which were maintained at 30°C (normal temperature) throughout the experimental period were taken as absolute controls.

The following parameters were recorded from the seedlings

a) Per cent reduction in root growth = [Actual root growth of control seedlings – (Actual root growth of treated seedlings /Actual root growth of control seedlings)] x 100
b) Per cent reduction in shoot growth = [Actual shoot growth of control seedlings – (Actual shoot growth of treated seedlings /Actual shoot growth of control seedlings)] x 100
c) Percent survival of seedlings = [No. of seedlings survived at the end of recovery/Total number of seedlings] x 100

Experiment – II: Understanding the physiological basis of thermotolerance in minor millets

Pot culture experiment was designed using the same set of minor millets. Each genotype was grown in six pots, maintaining 3 plants / pot normally until anthesis. Then one set of pots (3 pots/ genotype) were pushed into the Open Top Chambers (OTC's) for a period of 14 days (anthesis to flowering). During the flowering stage, plants were exposed to 38ºC for 4 hrs (9.00 am to 1.00 pm) in OTC. Changes in plant height, chlorophyll content, chlorophyll fluorescence, photosynthetic rate, transpiration rate and stomatal conductance at flowering stage, was recorded in control and stressed plants.

Plant Height

Plant height was measured from the ground level to the tip of the growing point and expressed in cm.

Chlorophyll Index

SPAD chlorophyll meter (Minolta model 502, Japan) was used to measure SPAD values. The measurements were taken from physiologically fully matured leaf from five plants in each replication and the mean values were computed using the method described by Peng *et al*. (1993) [16].

Chlorophyll Fluorescence

The chlorophyll florescence was measured using the Fluorescence meter (Plant PAM-210 (Teaching PAM), Heinz Walz, Germany). The key fluorescence parameters *viz*., $F_{o,}$ (initial fluorescence), F_v (variable fluorescence), F_m (maximal fluorescence) and the ratio of F_v/F_m were measured using this instrument.

$$\frac{Fv}{Fm} = \frac{\text{Variable fluorescence}}{\text{Maximum fluorescence}}$$

Fv/Fm is a useful ratio that depicts the proportion of quantum yield relation to a high degree of photosynthesis.

Leaf gas exchange parameters

Gas exchange parameters *viz*., photosynthetic rate, transpiration rate and stomatal conductance were recorded using an advanced portable photosynthesis system (LI-6400 XT, Licor Inc, Nebraska, USA). The readings were recorded from 10.00 am to 12.00 noon on a clear sunny day when the photo synthetically active radiation was more than 1000 µmol photons m^{-2} s^{-1}. Fully expanded leaf from the top was clamped inside the leaf chamber and held perpendicular to incident light and computed values were recorded. The instrument maintained a constant CO_2 flux to leaf chamber, which was maintained at ambient concentration. Relative humidity was maintained at a steady level equal to the ambient relative humidity to simulate a condition similar to that of ambient air. Photosynthetic rate was expressed as µmol CO_2 m^{-2} s^{-1}, stomatal conductance was expressed as mol H_2O m^{-2} $s^{-1}$and transpiration rate was expressed as mmol H_2O m^{-2} s^{-1}.

Results and Discussion

Experiment I: Designing the TIR protocol for minor millets

Standardization of challenging and induction temperature to identify thermotolerant minor millet genotypes

The experiment was carried out to screen the highly thermotolerant genotypes of minor millets. A seedling of Thenai (CO6) was used to find out the challenging temperature. The seedlings of Thenai CO6 were exposed to different challenging temperatures and after recovery, seedling growth and seedling mortality was assessed. At the end of recovery period the temperature at which 90% mortality of the seedlings occurred was taken as the challenging temperature in order to assess the genetic variability for seedling survival. A lethal temperature of 56ºC for 3 hours was fixed best lethal temperatures for screening of minor millets seedlings for intrinsic heat tolerance at cellular level (Table 1).

The optimum induction temperature was arrived at by exposing the seedlings to a defined lethal temperature following different induction treatments. Seedlings were exposed to the gradual induction temperature. This involved subjecting seedlings to a gradual temperature increase from 46°C to 54°C for three hours (induction treatment), immediately followed by challenging at 56 °C for 3 h. Subsequently, seedlings were allowed to recover at room temperature (30ºC and 60% RH) for 48 h. Thus, an induction treatment from 46 - 54ºC for three hours was standardized using TIR (Thermo Induction Response). Similar method of standardizing the lethal and induction temperature was adopted in screening cotton (Khier *et al.* 2012) [10] and ragi lines (Venkatesh babu *et al.*, 2013) [23]. The threshold and induction temperature for tolerance differs among species as studied in sunflower (Senthil *et al.* 2006) [18] and groundnut (Lokesh *et al.* 2004) [14].

Temperature stress response in different minor millet genotypes

The effect of TIR on acquired tolerance of minor millet genotypes revealed variable results. These results indicated that the induction response was seen both in recovery growth and also in seedling survival. At the end of recovery, different minor millet genotypes differed significantly in seedling mortality and recovery growth. The genotypes also recorded a significant reduction in the root and shoot growth when subjected directly to challenging temperatures, compared to the control and induced seedlings. Several studies showed that an acclimated plant survives when exposed to a temperature that would be lethal to a non-acclimated plant (Key *et al*. 1981; Hong *et al.* 2003) [9,6]. This phenomenon is the major aspect of acclimation response termed as acquired thermotolerance. A large variation in seedling survival among genotypes was noticed only in induced treatment, where the survival percentage ranged from 58% in Panivaragu to 94% in Samai. A similar trend of variation in recovery growth of seedlings was noticed in induced seedlings compared to non induced seedlings. The observed higher recovery growth of induced seedlings is mainly because of altered metabolism in response to acclimation as seen in cotton (Kheir *et al.* 2012) [10]; sunflower (Kumar *et al.* 1999; Senthil *et al*. 2006) [11, 18]; sorghum and pearl millet (Howarth *et al.*, 1997) [7]; beans (Keeler *et al.* 2000) [8]; wheat (Burke 1998) and groundnut (Srikanthbabu *et al.* 2002) [21].

The minor millets seedlings were exposed to an induction temperature, non-induction temperature and absolute control to observe the seedling mortality (%) (Fig.2). Maximum seedling mortality (%) was observed in Panivaragu (42%) induced treatment and non-induced (79%). Very low seedling mortality per cent was observed in Samai (6 %) in induced treatment and 0% was observed in absolute control followed by Thenai CO 6 which recorded 13% in induced; 41% in non-induced and 4% in absolute control. Hence, among all the minor millets Samai (CO4) exhibited lesser seedling mortality percentage and could be considered as thermotolerant. Earlier studies in ragi indicate that, the thermotolerant genotypes could be selected based on seedling mortality (%) (Venkatesh babu *et. al*. 2007) [23].

Genetic variation in minor millet genotypes on per cent survival of seedlings, per cent reduction in root growth and per cent reduction in shoot growth were observed after recovery period (Table 2). Among the genotypes, Samai CO4 showed highest thermotolerance in terms of seedlings survival (94%) followed by Thenai (CO6) with higher thermotolerance of 87 per cent seedlings survival in induced treatment. Highest reduction in root and shoot per cent was observed in Panivaragu (50.7%) and lowest per cent reduction in growth over control was observed in Samai under induced treatment (4.2%). This gradual progression results in the exposure of plants to milder stress before plants experience severe stress. Increased tolerance to otherwise lethal stress in plants exposed to induction stress is referred to as acquired tolerance (Senthilkumar *et. al.,* 2003). This indicates that the thermotolerant genotypes selected based on TIR technique at the seedling level are intrinsically tolerant at the plant level (Srikanthbabu *et. al.* 2002). Results of this study indicated that the effect of TIR on minor millet revealed variable results. The study showed that Samai (CO4) recorded higher

levels of intrinsic tolerance to high temperature stress compared to other minor millets. Use of TIR to identify the tolerant and susceptible genotypes based on seedling mortality was reported by Venkatesh babu *et al.* 2013 [23].

Experiment II: Assessment of physiological basis of high temperature stress tolerance/susceptibility in rice.

Genotypes

The Pot culture experimental data recorded showed contrast values for plant height, chlorophyll content, chlorophyll fluorescence, photosynthetic rate, transpiration rate and stomatal conductance at flowering stage stress (Table 3). The data on plant height representing the growth of minor millets plants at flowering stage showed that with an increase in the temperature stress there has been decrease in plant height (Fig.3). It was observed that in Samai CO4 the plant height showed a reduction percent (2.99 %) under stress condition compared to other minor millets. Higher chlorophyll index was recorded in Samai CO4 compared to other minor millets (Fig. 4). High temperature stress treatments, decreased chlorophyll index values when compared to untreated plants. These temperature stress induced the oxidative injuries could enhance Chlorophyll degradation or the inhibition of its biosynthesis (Papadakis *et al.,* 2004) [15].

The study of data on chlorophyll fluorescence showed that the fv/fm values declined under stress induced at flowering phenophases in minor millet (Table 3). Among the minor millets, Samai CO4 (0.68) followed by Thenai CO6 (0.60) maintained higher chlorophyll fluorescence values even under high temperature stresses whereas, Panivragu CO5 (0.51), Varagu CO3 (0.52) and Kudiraivalli CO2 (0.58) recorded lower fluorescence values when subjected to high temperature treatment, wherein their PSII might have been liable for high temperature stress damage. Temperature stress modified PSII functionality in leaves of the plants, manifested by lower variable chlorophyll fluorescence yield (Fv), maximum photochemical efficiency of photosystem II in dark adapted leaves (Fv/Fm), and efficiency of the open reaction centre in light (PSIIopen) in the heat shocked cultivars of rice (Langjun *et al.,* 2005) [12].

The data pertaining to photosynthesis is presented in Table 3 and it showed significant differences between the genotypes under stress condition at flowering stage. It was observed that photosynthesis was highest in Samai CO4 (37.81 µmol CO_2 m^{-2} s^{-1}) at flowering stage compared to other minor millets both under stress and control conditions. Dekov *et al.* (2000) [4] reported that the temperature stress caused a net photosynthetic rate reduction in the plants due to stomatal and non-stomatal limitations, photo inhibition increase and Rubisco activity reduction. Higher photosynthetic rates were also coupled with high stomatal conductance (0.73 mmol H_2O m^{-2} s^{-1}) and transpiration rate (7.04 mmol H_2O m^{-2} s^{-1}) in Samai (CO4) compared to other minor millets. Temperature stress induce an array of physiological process like the oxidative injuries, enhance chlorophyll degradation or the inhibition of its biosynthesis, damage PSII components, inactivate many chloroplast enzymes especially those participating in CO_2 assimilation (Souza *et al.,* 2004) [20] and could further explain the reductions in photosynthetic rate, Fv/Fm, stomatal conductance, and leaf chlorophyll content. These findings are in the line with the above results which clearly explains the physiological basis of tolerance. Hence it is clearly understand that Samai (CO4) and Thenai (CO6) overcome high temperature stress by adopting several physiological and biochemical mechanisms including morphological short-term avoidance (Venkatesh babu *et al.,* 2013) [23] acclimation mechanisms.

Conclusion

The above results suggest that the TIR technique can be used as a powerful and non – destructive technique to identify genetic variability in high temperature tolerance in minor millets within a short period of time. This can be used as a rapid and reliable technique for screening large number of genotypes. Using this technique, it was clearly demonstrated that there is sufficient genetic variability among the minor millets. Samai (CO4) and Thenai (CO6) were found to be high temperature tolerant genotypes. The identified high adaptation variety of minor millets Samai (CO4) and Thenai (CO6) can be used as donor source for developing high temperature tolerant minor millets to resist the global rise in temperatures. Using this stable technique, Institutes having large germplasm collection of Samai and Thenai could go in for screening the entries for different physiological traits and use them in Crop improvement programmes aimed at evolving high temperature stress tolerant genotype in minor millet.

Table 1: Per cent mortality of Thenai (CO6) seedlings at different lethal temperatures

S No	Temp (C)	Per cent mortality of seedlings at different lethal temperature		
		Duration of temperature treatment		
		1 hour	2 hours	3 hours
1	52	0	0	25
2	54	0	32	55
3	56	35	66	91
4	58	48	94	100
5	60	55	100	100

Table 2: Screening of thermotolerant minor millets genotypes through TIR technique

Genotypes	% Survival of seedlings			% Reduction in root			% Reduction in shoot			% Reduction in growth over control	
	Absolute control	Induced	Non Induced	Absolute control	Induced	Non Induced	Absolute control	Induced	Non Induced	Induced	Non Induced
Thenai (CO6)	96	87	59	0.0	11.6	21.1	0.0	9.5	18.3	10.6	20.1
Kudiraivalli (CO2)	96	76	34	0.0	23.8	39.0	0.0	25.9	41.3	22.8	38.0
Varagu (CO3)	92	73	25	1.0	45.9	53.8	0.0	37.5	57.0	44.9	52.8
Panivaragu (CO5)	89	58	17	1.0	51.7	59.0	1.0	50.7	65.9	50.7	58.0
Samai(CO4)	100	94	63	0.0	5.2	16.3	0.0	6.4	15.7	4.2	15.3
Mean	94.6	77.6	39.6	0.4	27.7	37.8	0.2	26.0	39.6	26.7	36.8
SEd	0.251	0.237	0.548	0.150	0.264	0.373	0.169	0.267	0.336	0.159	0.277
CD (0.05)	0.516	0.487	0.267	0.308	0.541	0.765	0.346	0.548	0.689	0.326	0.555

Table 3: Changes in leaf gas exchange parameters in minor millets at during flowering stage under ambient and high temperature stresses

Crop	Photosynthetic rate (µmol CO_2 m^{-2} s^{-1})		Transpiration rate (mmol H_2O m^{-2} s^{-1})		Stomatal conductance (mmol H_2O m^{-2} s^{-1})		Chlorophyll fluorescence (Fv/Fm)	
	Control	Stress	Control	Stress	Control	Stress	Control	Stress
Thenai CO6	36.58	35.78	6.88	6.41	0.71	0.68	0.70	0.60
Kudiraivall CO2	36.60	34.68	6.65	6.07	0.70	0.57	0.62	0.58
Varagu CO3	34.80	30.56	6.38	5.75	0.67	0.53	0.68	0.52
Panivaragu CO5	34.06	27.29	6.18	5.59	0.65	0.60	0.63	0.53
Samai CO4	38.78	37.81	7.04	6.88	0.78	0.73	0.73	0.68
Mean	36.16	33.22	6.63	6.14	0.70	0.62	0.67	0.58
SEd	0.3224	0.2814	0.0607	0.0560	0.0063	0.0056	0.0062	0.0057
CD (0.05)	0.6871	0.5997	0.1295	0.1195	0.0134	0.0119	0.0132	0.0122

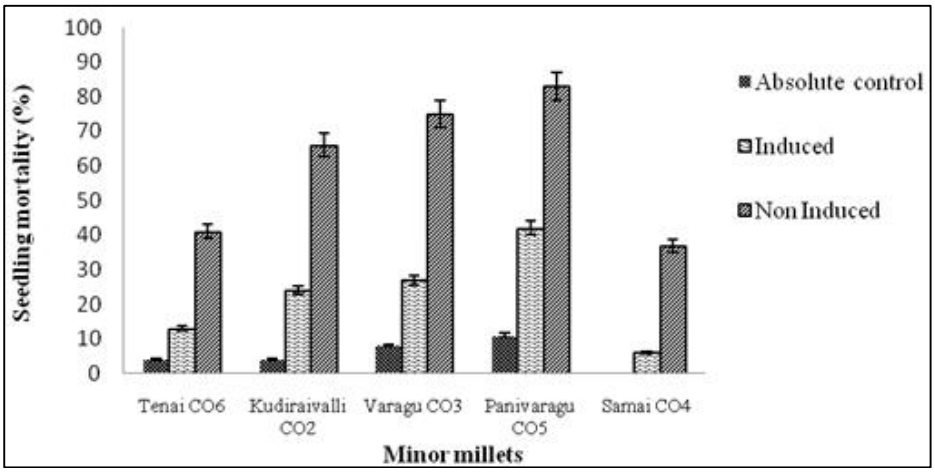

Fig 2: Variation in thermotolerance of different minor millet genotypes in terms of percent seedling mortality

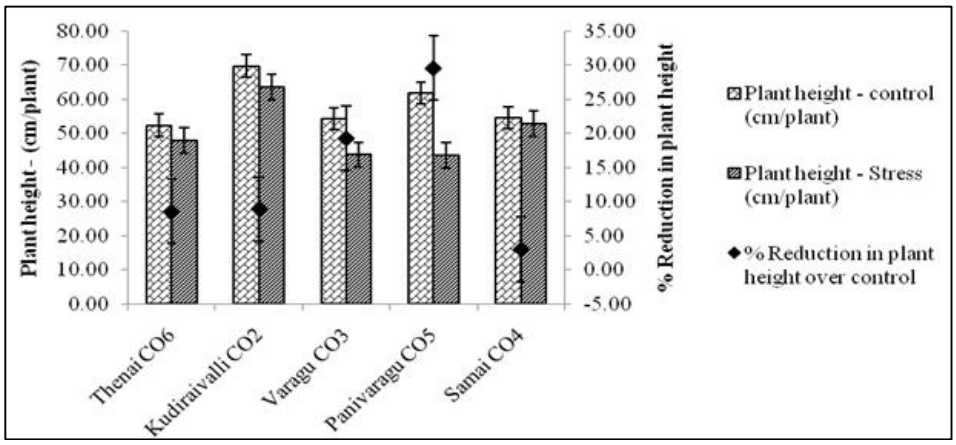

Fig 3: Effect of stress on Plant height at flowering stages of minor millets

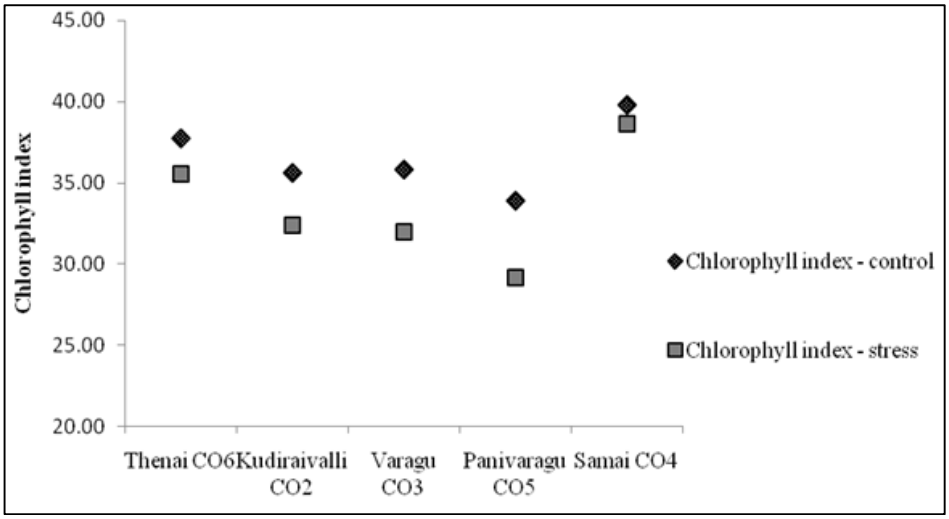

Fig 4: Effect of stress on Chlorophyll index at flowering stages of minor millets

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