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Evaluation of Finger millet as Heat Tolerant Crop using Physiological and Biochemical Assays

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ABSTRACT

Under changing climatic conditions, high temperature stress is the most severe problem for the whole agriculture. Identification and utilization of crop plants which can sustain and yield better under high temperature conditions is need of the day. In this study, we established finger millet as thermotolerant crop. For this, we characterized thermotolerant cotton, thermosensitive wheat along with finger millet by MDA accumulation after heat stress and shown that finger millet is even better than cotton. Further, using seed germination test and growing seedlings at higher temperature, it was observed that finger millet was least affected at 42 °Cwhereas germination percent and fresh weight reduced at 47 °C. With biochemical assay, it was shown that finger millet had very less difference at 42 °C as compared to 37 °C, however there is significant reduction at 47 °C in chlorophyll and carotenoid content and relative water content (RWC) percent whereas increase in electrolyte leakage (%) and H_2O_2 and O_2 concentration. Still finger millet plants can tolerate temperature of 47 °C. Overall, the present study strongly identified finger millet as thermotolerant crop and can be utilized for allele mining of known genes and prospecting of novel genes for crop improvement for high temperature stress.

Keywords: Finger millet, thermo-tolerance, physiological assay, biochemical assay

INTRODUCTION

Finger millet (*Eleusinecoracana*) is an annual tetraploid; a small millet known to have tolerance to various biotic and abiotic stresses, including drought, salt, cold, high temperatures and blast disease (Muthamilarasan and Prasad 2015; Rehman *et al*., 2016; Sen*et al*., 2016; Singh et al., 2016; Singh et al., 2014 and Ramakrishna *et al*., 2018). It is a rich source of phytochemicals such as polyphenols and dietary fiber. It has high levels of mineral like calcium and amino acids, lysine and methionine (Mbithi-Mwikya *et al*., 2000; Devi *et al*., 2014; Gull *et al*., 2016).

Abiotic stresses are serious threat to the global food security as it is badly affecting the crop productivity causing major economic loss. Tolerance levels of crops to these stresses differs from species to species (Dida and Devos, 2006). Among all abiotic stresses, high temperature is one of the severe stress which is adversely affecting plant growth as well as its yield. Particularly when the global temperature will likely increase by 2-4 °C at the end of this century (Houghton *et al*. 2001 and IPCC 2007). High temperatures can cause scorching, including sunburn of stem, branches and leaves, leaf abscission, inhibition of shoot and root growth, significant pre and post-harvest damages and reduced yield in plants (Stone and Basra, 2000; Vollenweider and Gunthardt Goerg, 2005 and Wahid *et al*., 2007).

In agriculture, the negative impact of heat stress can dramatically reduce the crop yield. Therefore, knowledge of the complex system involved in plant tolerance to heat stress is very important. Ion imbalance and hyperosmotic stress are main effects of heat stress. The direct result of these effects is enhanced production of ROS species such as hydroxyl radical (OH), superoxide radical (O_{2^-}), hydrogen peroxide (H_2O_2), and single oxygen (O_2).

These are harmful to plant cell at elevated concentration and disturbs aerobic metabolism (Tuteja *et al*. 2009; Mittler,2002). Nonetheless, plants developed protective mechanisms to moderate and restore the damage cause by ROS. The ROS-scavenging mechanisms consist of enzymatic system, catalase, peroxidase, superoxide dismutase and non-enzymatic system, which include of glutathione ascorbic acid and ascorbic acid etc. Earlier studies showed that antioxidant enzyme provided tolerance to high temperature stress (Almeselmani *et al*., 2006).

There are many commonly used assays of heat tolerant in plants related to membrane-based processes such as chlorophyll, carotenoid assay, electrolyte leakage, relative water control and photosynthesis related parameters (Blum, 1988 and Maestri *et al*., 2002). Finger millet is an invaluable genetic resource, to discover the molecular mechanisms for stress tolerance in monocots, it alleles may play vital role in enhancing and improving tolerance to multiple abiotic stresses in economically significant crops.

In this study, we established finger millet as a thermotolerant crop plant. In order to completely understand the heat stress tolerance mechanism, we did physiological and biochemical analysis to uncover the genetic potential of finger millet under heat. In future we can exploit finger millet for gene prospecting and increasing resilience in other cereal crops

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and exaggerate their productivity under adverse growth conditions.

MATERIALS AND METHODS

Plant material and treatments

Finger millet genotype MR1 is used in this study. Wheat variety Raj 3765 and cotton variety Bikaneri Narma were also germinated in soilrite and grown along with finger millet and stress was imposed at 37 °C, 42 °C and 47 °C for different time period. Finger millet seedlings were grown in pots containing soilrite and on petri dish with 1/2 X MS media. A growth chamber maintained at 24°C with 16h light/ 8h dark of photoperiod at 400 μM m^{-2} s^{-1}along with 60% relative humidity. Fourteen days old healthy seedlings were exposed to three different temperatures: 37°C, 42°C and 47°C. For control samples, plants were grown at 24±1 °C. Whole seedlings were sampled at normal condition (24°C) and at intervals of 2, 4, 8 and 10h of heat treatments. Samples were immediately frozen in liquid nitrogen and stored at –80°C prior to biochemical and physiological analysis. In another experiment, to check effect of for longer time, seedlings were subjected to heat stress at 42°C up to 7 days and control samples were kept at 24±1 °C.

Seed germination under heat stress

For germination analysis under heat stress, seeds were sterilized with 70% ethanol and 2% sodium hypochlorite, washed with double distilled water and placed on half strength MS (Murashige and Skoog) medium. Plates carrying sterilized seeds were kept at 37°C, 42°C and 47°C along with optimal temperature for germination. The germination rate and fresh weight was observed after 3 days. Same experiment was repeated on soilrite and kept at different temperature, and fresh weight, shoot and root length was recorded after 7 days.

Electrolyte leakage (EL)

For estimation of electrolyte leakage, treated and untreated samples were cleaned with sterile water for removal of electrolytes present on the surface. Leaf samples were transferred to closed vials containing deionized water and incubated overnight on a rotary shaker. Afterward, electrical conductivity was recorded (L_t) by conductivity meter. Later, samples were autoclaved for 15 min at 120°C, samples were cooled at 25°C and electrical conductivity (L_0) was again measured (Lutts *et al*., 1996). Following formula was used to calculate the percent electrolyte leakage -

$EL(\%)=100\times L_t/L_0$

Relative Water Content (RWC)

Fresh weight from heat treated and untreated finger millet leaves were taken and further dipped it into deionized water overnight. After determination of turgid weight, all samples were dried at high temperature and dry weight was taken. Following formula was used to calculate the relative water content (RWC %):

RWC (%)=100 × (Fresh weight – Dry weight)/ (Turgid weight – Dry weight)

Estimation of chlorophyll and carotenoid content

For this experiment fourteen days old seedling treated with heat were taken. Total chlorophyll and carotenoid contents were estimated from leaves and determined as per gram fresh weight of tissue described as Arnon (1949).

Quantification of O_2, H_2O_2, and lipid peroxidation

O_2 content and H_2O_2 content was estimated from treated and untreated finger millet using methods described by Ke *et al.* (2002)and Patterson *et al.* (1984), respectively. Absorbance was measured at 530 and 415 nm for determining O_2 production and H_2O_2 content, respectively. Estimation of lipid peroxidation was done by calculating the MDA (malondialdehyde) produced by TBA (thiobarbituric acid) reaction (Draper and Hadley, 1990) from all finger millet, wheat and cotton crops.

RESULTS AND DISCUSSIONS

Under changing climatic conditions, increase in global temperature is the major problem for crop plants. Crops suffer heat stress through various stages of their life cycle (Stone, 2000). Day by day agricultural productivity, quality and productive area are decreasing due to severe heat stress. As world population is growing faster, there is need to improve present and future varieties ready for warmer climate conditions. For developing thermotolerant crops, first we need to identify crop plants tolerant to heat stress and then prospect novel genes and alleles from it. In this study we established finger millet as thermotolerant crop and plausible source of genes to develop thermotolerant crops.

Comparison for Finger millet with cotton and wheat for thermotolerance using MDA

Thermotolerance of finger millet was compared with cotton and wheat plants using estimation of MDA concentration (Fig. 1). MDA is observed to be increase in cotton and wheat under 37 °C from 2h to 10h, did not increased in finger millet. At 42 °C, it increased gradually in all three crops; however change in increase was very high in wheat followed by cotton and very less in finger millet. Same thing was observed at 47 °C temperature; however rate of increase at every time-point is almost doubled as compared to 42 °C. At 47 °C, the increase in MDA level was four-fold (12.26 μmol g^{-1} FW) in finger millet at 10h compared to 0h, whereas it is five-fold in both wheat and cotton. in Thus, in cotton MDA was more than wheat, however in wheat, it was doubled than finger millet at 47 °C.

Thus, we established finger millet as thermotolerant crop comparing with heat tolerant cotton crop (Rehman *et al*., 2004 and Wahid *et al*., 2007) and sensitive crop wheat (Nagai and Makino, 2009) using MDA estimation. An increase in the amount of MDA is directly related to oxidative stress or oxidative damage (Ozden *et al*., 2009 and Gur*et al*., 2010). We observed that MDA accumulation was less in finger millet, comparatively more in cotton and maximum in wheat, suggesting finger millet as even more tolerant than cotton under high temperature conditions.

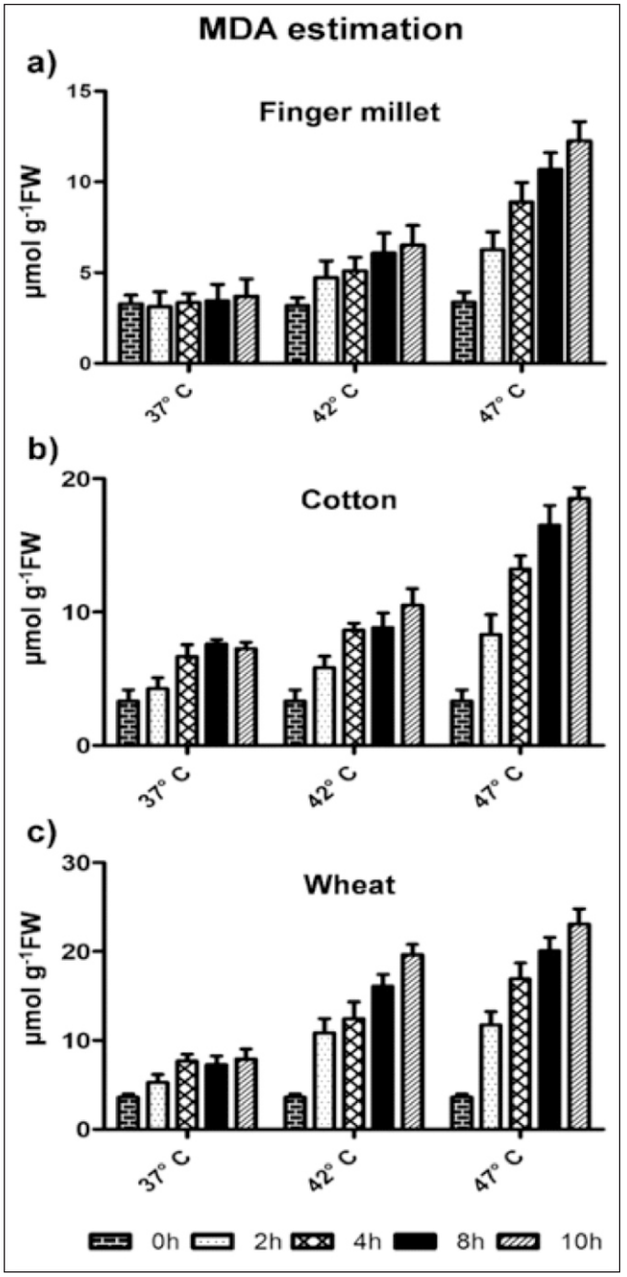

Fig. 1: MDA estimation in finger millet, cotton and wheat

Germination of finger millet under heat stress

Germination of finger millet seeds was conducted at 37°C, 42°C and 47°C on MS agar plates. The effect of temperatures on germination percentages is measured after 3 days and shown in Table 1. There are marked differences between all treated temperatures. At 37 °C, seeds developed into proper seedlings and did not influence percentage germination and seedling weight was slightly higher as compared to higher as well as lower temperatures. Seeds which were grown at 42°C treatments showed significantly lower germination values (Fig. 2a). At 47°C, no germination was observed. In case of soilrite, at 37°C germination was equal or better than control conditions (Fig. 2b), at 42°C germination was there but plants were having less shoot, root and weight (Table 1). At 47°C no germination or very less germination was observed.

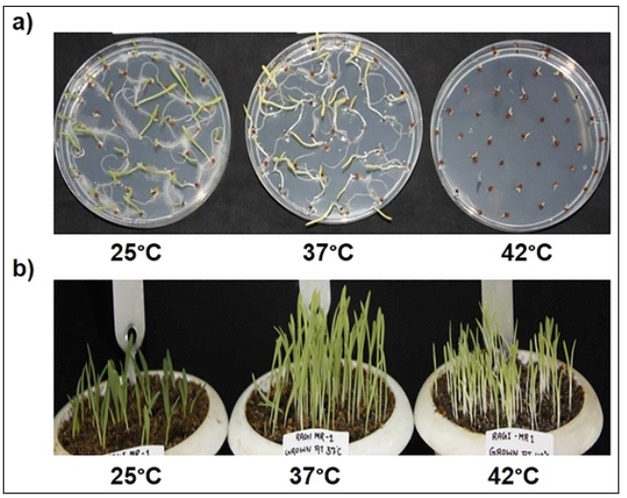

Fig. 2: Germination assay of finger millet seedlings at 25, 37 and 42 °C

Table 1: Estimation of germination percent of finger millet at ½ MS medium and in soilrite grown at different temperature conditions.

Germination in ½ MS				
	At 25°C	At 37°C	At 42°C	At 47°C
Germination rate	97.52	96.79	72.33	No
Fresh weight (gm)	0.257	0.163	0.058	No
Germination in soilrite				
Germination rate	100%	100%	98%	No
Fresh weight (gm)	0.208	0.24	0.16	No
Avg, root length (cm)	3.94	4.57	2.19	No
Avg, root length (cm)	2.37	2.87	2.27	No

Physiological properties of finger millet under heat treatment

Physiological characteristics of finger millet seedlings like shoot length, root length; fresh weight and dry weight were affected by heat stress. Morphologically, plants of finger millet exposed to heat stress up to 3 days, no leaf wilting was observed. On fifth day leaf wilting was observed but no severe injury was seen. After seventh day of heat stress, the seedlings were exhibited leaf wilting, chlorosis of mature leaves, and stunted growth was observed (Fig. 3a). Seedling shoot and root length and fresh weight decreased with an increase in temperature (Fig. 3, Table 2). In terms of shoot and root length, plant treated with 42°C were less affected on forth day but shoot and root stopped growing after 5 days. Same results were obtained in the case of fresh weight, after 7 days of heat stress fresh weight of plants were more than half at 42°C than those produced under controlled conditions. Seedling length and weight were not significantly affected at 42°C in starting period of stress but was reduced at by 4d. After 7 days of heat

stress at 42°C plants were kept at normal temperature (25°C) for acclimatization. Thus, we checked seed germination under higher temperatures and observed that finger millet seeds can germinate at 42 °C at petri plate and in soilrite with slight reduction in germination per cent. We also shown that these seedlings can sustained in prolonged heat stress up to seven days with some reduction in shoot and root length and also in fresh weight.

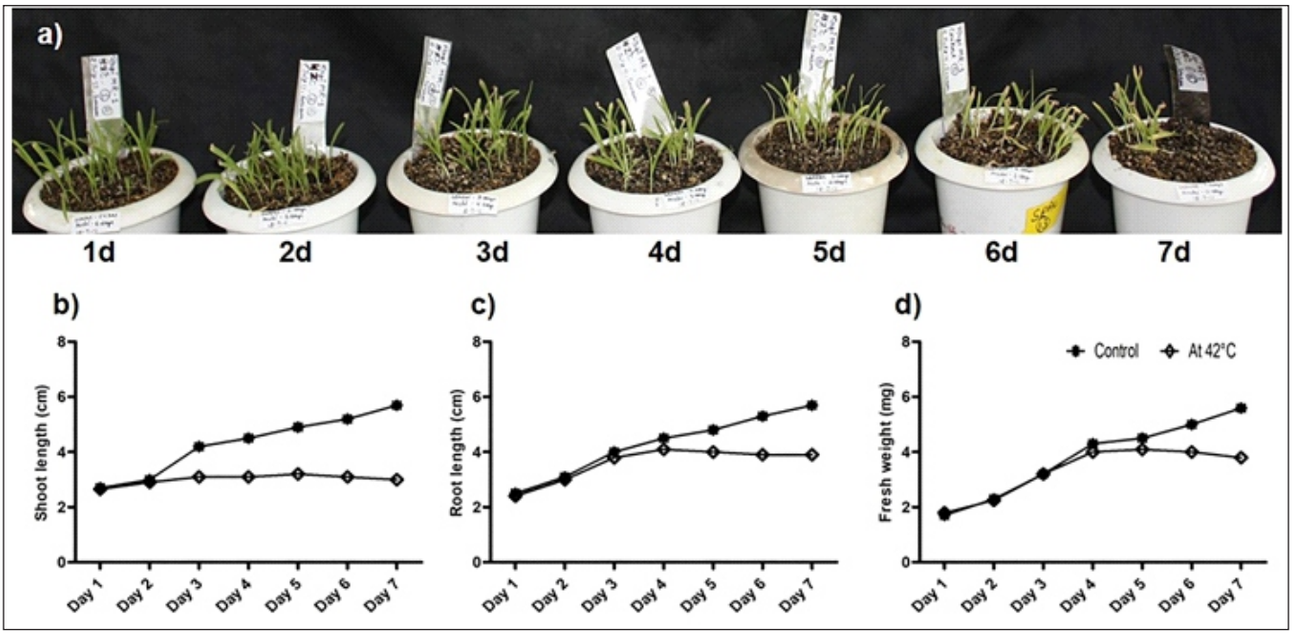

Fig. 3: High temperature stress to 15 days old seedlings for seven days and estimation of shoot length, root length and fresh weight

Table 2: Effect of temperature (42 °C) on seedling fresh weight, root length and shoot length during 7 days of stress period.

	Fresh weight (mg)		Root length (cm)		Shoot length (cm)	
	Control	At 42°C	Control	At 42°C	Control	At 42°C
Day 1	1.7	1.8	2.5	2.4	2.7	2.7
Day 2	2.3	2.3	3.1	3.0	3.0	2.9
Day 3	3.2	3.2	4.0	3.8	4.2	3.1
Day 4	4.3	4.0	4.5	4.1	4.5	3.1
Day 5	4.5	4.1	4.8	4.0	4.9	3.2
Day 6	5.0	4.0	5.3	3.9	5.2	3.1
Day 7	5.6	3.8	2.7	3.9	5.7	3.0

Electrolyte leakage (EL) and Relative water control (RWC) under heat treatment

Electrolyte leakage was unaltered at 37 °C through all the time period whereas at 42 °C very trivial increase was observed after 6 h (28%). However, at 47 °C during 6 h electrolyte leakage is equal to control but 8 h onwards its increased quickly about 92% (Fig 4a). Relative water content was unchanged at 37 °C and 42 °C but gradually decline (30%) at 47°C (Fig 4b, Table 3).

Chlorophyll and carotenoid content under heat treatment

Changes in chlorophyll and carotenoid contents under heat stress isshown in Fig. 4a and 4b. Chlorophyll content was slightly higher after first 2h of heat stress than control samples

Table 3: Physiological assays for analysis of temperature stress in finger millet seedlings.

Assay	Time point	Temperature		
		37°C	42°C	47°C
Electrolyte Leakage (%)	Con	24.25	24.98	24.34
	2h	24.13	26.48	29.24
	4h	24.28	28.05	30.52
	8h	24.18	30.22	41.48
	10h	25.00	32.11	46.84
Relative Water Content (%)	Con	73.57	72.04	72.78
	2h	73.17	70.53	65.79
	4h	72.62	68.93	61.81
	8h	71.12	61.07	55.84
	10h	70.34	57.23	50.01
Chlorophyll Content (mg g^{-1} FW)	Con	4.19	4.01	4.16
	2h	4.14	4.08	3.65
	4h	4.05	3.84	2.73
	8h	3.99	3.04	2.01
	10h	4.06	2.56	1.65
Carotenoid Content (mg g^{-1} FW)	Con	6.52	6.70	6.47
	2h	6.62	6.43	5.90
	4h	6.50	6.03	5.03
	8h	6.03	5.67	3.95
	10h	5.64	5.23	3.55

at 37°C and 42°C. At 37°C chlorophyll content was unaffected during treatment. At 42°C up to 4h chlorophyll content was unaffected, and at 8h and 10h, it dropped down. However, at 47°C, it was declined gradually (Fig 4c, Table 3). Carotenoid content was mostly unchanged compared to the control at 37°C and 42°C in starting period of stress but decreasing slightly at 10h at both the temperatures. However, at 47°C, the decrease in carotenoid was visible even at 2h and after 6h rapidly and gradually declined by 45% (Fig 4d, Table 3).

H_2O_2 and O_2- production rate and concentration under heat treatment

The alteration in O_2- production rate and H_2O_2 concentration is shown in Fig. 4e,4fand Table 4. The pattern of H_2O_2 concentration at 37 °C and 42°C was almost similar with very slight increase up to 10 h compared with untreated samples. However, at 47°C the gradual increase in H_2O_2 concentration was observed from 2h and at 10h of heat treatment, concentration increased by around 25% (Fig. 4e). O_2- production rate increased slightly at 42 °C, with gradual exposure. At 47°C, O_2- production rate enhanced actively and at the 10h heat treatments, it was about 50% higher than the control samples (Fig. 4f, Table 4).

Table 4: Biochemical assays for analysis of temperature stress in finger millet seedlings.

Assay	Time point	Temperature		
		37°C	**42°C**	**47°C**
H_2O_2 (μmol g^{-1} FW)	Con	40.48	41.00	40.81
	2h	41.32	43.96	43.23
	4h	40.96	42.57	46.13
	8h	42.29	44.25	52.08
	10h	43.27	44.61	54.74
O_2 (μmol min^{-1} g^{-1} FW)	Con	7.38	7.53	7.47
	2h	7.96	8.02	8.46
	4h	7.65	8.61	9.70
	8h	8.05	8.49	10.79
	10h	8.30	9.42	12.56
MDA (μmol g^{-1} FW)	Con	3.28	3.18	3.17
	2h	3.12	4.72	6.26
	4h	3.34	5.11	8.90
	8h	3.44	6.09	10.69
	10h	3.70	6.52	12.26

In high temperature, membrane functions modify due to change in membrane fluidity. In plants cells for membrane-based progression like respiration and photosynthesis is vitally essential. It is well known and accepted fact that high temperature acclimation comes from an intricate process associated with the number of biochemical and physiological changes, together with membrane function and structure, global gene expression, tissue water content, lipid, protein and secondary metabolite composition (Wahid *et al.*, 2007andJha*et al.*, 2014). Just after exposure to high temperatures and sensing signals, changes happen at the molecular level which alter the gene expression and accumulation of transcripts, which initiate the synthesis of stress related proteins (Iba, 2002). Here with the help of physiological and biochemical assays, we have shown that heat stress caused significant declines in RWC, Chlorophyll content, and carotenoid content and increase in electrolyte leakage and production of H_2O_2 and O_2 at 47°C. Changes in photosynthetic characteristics of the plants like electron transport and oxygen evolution are related with the chlorophyll and carotenoid loss (Havaux and Tardy, 1999). However, these changes were very small at 42 °C of heat stress. In fact, there is no significant H_2O_2 and O_2 production at 42 °Csuggesting very little effect on growth. The detrimental effects were more pronounced for the 47°C of heat stress.

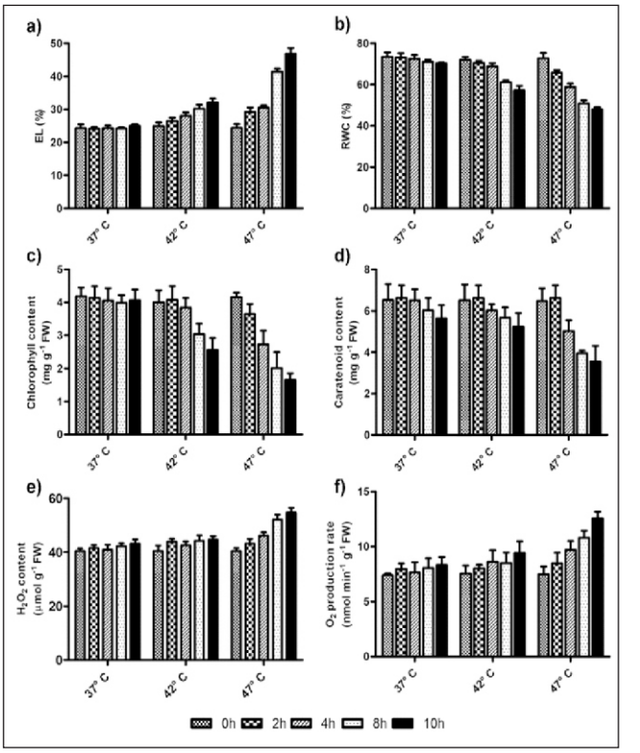

Fig. 4: Analysis of physiological and biochemical parameter of finger millet seedlings grown at 37, 42 and 47 °C. a) electrolyte leakage (%); b) relative water content (RWC %); c) chlorophyll content; d) carotenoid content; e) H_2O_2 content and f) O_2 production rate.

CONCLUSION

Global climate change is increasing earth's temperature by this century. Identification and characterization of crops which can sustain in higher temperature can help to keep the need of increasing population. In this study we characterize the climate resilient crop Finger millet under high temperature stress. Our data for membrane thermostability, seed germination, physiological and biochemical parameters like, EL, RWC, Chlorophyll and carotenoid content and production of H_2O_2 and O_2 suggested that finger millet is thermotolerant crop and can be used for gene prospecting for stability of cell under high temperature stress.

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