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Full Length Article

Possible Involvement of Secondary Metabolites in the Thermotolerance of Maize Seedlings

Saqib Mahmood[1*], Abida Parveen[1], Iqbal Hussain[1*], Sadia Javed[2] and Muhammad Iqbal[1]
[1]*Department of Botany, Government College University, Faisalabad-38000, Pakistan*
[2]*Department of Chemistry, Government College University, Faisalabad-38000, Pakistan*
[*]For correspondence: drsaqibm@yahoo.com; iqbalbotanist1@yahoo.com

Abstract

Heat stress is a factual menace to our food production. Rising populace urge for highr crop yield that is linked with better understanding of thermotolerance. Contribution of secondary metabolites towards heat stress tolerance at seedling stage and the consequent variant distribution in the shoots and roots of two differentially heat tolerant varieties of maize was assessed. Seedlings of a heat-tolerant (Sultan) and heat-sensitive (S-2002) maize variety were subjected to heat stress (40±2°C) for 2, 4 and 6 days. The determinations were made for total soluble phenolic, flavonoids, tannins, anthocyanins, total alkaloids and saponins. Moreover, to explore antioxidant potential of these metabolites, and their correlation with H_2O_2 and membrane permeability were determined. A positive correlation between heat tolerance and enhanced synthesis of total soluble phenolics and saponins was found. Negative relation of secondary metabolites with H_2O_2 and membrane permeability indicated their alleviative role against oxidative damages at cellular level. Nevertheless, both cultivars responded differently to high temperature in terms of secondary metabolite synthesis with better accumulation in resistant variety. The maize variety Sultan was well equipped with secondary metabolites in terms of total soluble phenolics, flavonoids, tannins, anthocyanins, and saponins as free radical quenching system and thus showed better thermotolerance.

Keywords: Secondary metabolites; Maize; Seedling; Antioxidant; Thermotolerance

Introduction

Elevated temperature prompts strain within stressed cells that lowers quality and yield of various crop species (Maestri *et al.,* 2002; Anderson-Teixeira *et al.,* 2012). Disturbing the photosynthetic activities (52%) resulted in 33% decrease in root weight and 28% of whole plants weight (Jochum *et al.,* 2007). In general, heat stress induces the production of reactive oxygen species (ROS) in plants. Overproduction of ROS such as superoxide (O^{2-}), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and singlet oxygen that damage the membrane lipids, nucleic acids, structural proteins, photosynthetic pigments and enzymes (Ozkur *et al.,* 2009; Nawaz *et al.,* 2013), and consequently disturb the normal metabolic activities of plants. Plant metabolism contributes towards heat stress tolerance by providing the energy and metabolites essential for cellular homeostasis, or via production of protective osmolytes like sucrose, fructose and myo-inositol in excess. The strength and recovery of these systems under heat stress significantly rely upon the plant metabolism and its products (Gu *et al.,* 2012; Bartwal et al., 2014). As a strategic adaptation, to cope with the environmental challenges, plants modulate the production and accumulation of a number of enzymatic (Xu *et al.,* 2011; Ahmad *et al.,* 2013) and non-enzymatic (Gu *et al.,* 2012; Mahmood *et al.,* 2012) antioxidants. These antioxidants scavenge the heat stress triggered ROS.

Plants overproduce secondary metabolites under heat stress as one of the adaptive strategeis (Jochum *et al.,* 2007; Tanveer *et al.,* 2012). They have diverse protective functions ranging from toxicity and light/UV shielding to signal transduction. Their importance in plant stress physiology is mediated by structural and functional modifications, diversity of chemical types and their interactions. They possibly allow the resumption of normal cellular and physiological activities (Vasconsuelo and Boland, 2007). Secondary metabolites interact with phosphoric acid residues in DNA and uronic acid residues in cell wall matrix. They possibly stabilize the plant cell structures with the help of their electrostatic interactions, thereby mediating the defensive adaptations in heat stressed plants (Edreva *et al.,* 2008).

Heat stress affects the whole life cycle of plants as it exaggerates the effects of other abiotic stresses. A minor variation in ambient temperature directly upsets the plant growth and development. It considerably alters the physiological settings of the plants, particularly at seedling stage. There are a number of studies that assessed the physiological, molecular and genetic basis of temperature tolerance in different crops (Wang *et al.,* 2003). However, the effects of heat stress upon secondary plant metabolism are still not fully unveiled. Furthermore, the comparative

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data with respect to thermotolerance and thermosensitivity mediated by secondary metabolites in maize seedlings is scarce. Maize (*Zea mays* L.), being a C_4 plant exhibits a characteristic physiology under heat stress. This study was planned to determine the contribution of metabolic adjustments mediated by modulation of secondary metabolites in maize cultivars differing in thermotolerance.

Materials and Methods

General Experimental Details

A series of laboratory experiments were conducted to compare the variations in total soluble phenolics, flavonoids, tannins, anthocyanins, alkaloids and saponins at seedling stage in a heat tolerant (Sultan) and heat sensitive (S-2002) varieties of maize (*Zea mays* L.) (Mahmood *et al.,* 2010). The seeds were surface sterilized with 0.1% $HgCl_2$ for 3 min and washed several times with sterilized distilled water before sowing in pots containing 2 kg of washed and dried river sand. The plants were supplemented with Hoagland and Arnon (1950) nutrient solution at interval of two days. Six day old seedlings were divided into two sets; one set placed in a growth chamber (FLI, Eyelatron, Rikkakai, Japan) at 27/22°C±2 (light/darkness period 14/10 h, control) whereas the second set was placed in another same type growth chamber at 42/37°C±2 (light/darkness period 14/10 h, heat stress). The experiment was laid out in a completely randomized design (CRD) with three replications. Seedlings were harvested at 2, 4 and 6 day after heat treatment. For biochemical estimations, the shoots were cut from its attachment with the root, while the roots were washed, blotted and dried and then used for different measurements.

Preparation of Fat Free Sample

Secondary metabolites were determined as per standard procedures from defatted samples prepared following AOAC (1996).

Analysis of Secondary Metabolites

Total soluble phenolics: Total phenolics content was determined using Folin-Ciocalteu reagent method (Julkenen-Titto, 1985). Dilutions were made to ensure the oxidation of 1 mL of Folin-Ciocalteu reagent with 200 µL, neutralized with 2 mL of 7.5% sodium carbonate (w/v). Volume was made up to 7 mL. After incubation for 2 h in the dark at room temperature, the resulting blue color was measured at 765 nm using spectrophotometer. Tannic acid was used as a standard for making the calibration curve.

Flavonoids content: Total flavonoids were measured with minor modifications of the colorimetric assay (Zhishen *et al.,* 1999). Based on this method, each sample (0.5 mL) was mixed with 2 mL of distilled water and subsequently with 0.15 mL of a $NaNO_2$ solution (15%). After 6 min, 0.15 mL of $AlCl_3$ solution (10%) was added and allowed to stand for 6 min, and then 2 mL of NaOH solution (4%) was added to the mixture. The water was added immediately to bring the final volume up to 5 mL followed by thorough mixing. After 15 min incubation, absorbance was determined at 510 nm versus the water blank. Results were expressed as rutin trihydrate equivalents (mg rutin trihydrate/g dried extract).

$$Y = 0.0205X + 1494; r = 0.9992$$

Where, Y is the absorbance, and X is the flavonoid content in µg g^{-1}.

Anthocyanins: They were determined with the method of Stark and Wray (1989). For anthocyanins extraction, 0.1 g of fresh leaf material was added to 1 mL of acidified methanol (1% HCl v/v) in a microfuge tube, macerated with a glass rod and heated at 50°C for 1 h. Centrifuged and supernatant was used to quantify the anthocyanins at 535 nm using a spectrophotometer. The background of the spectrophotometer was set using acidified methanol. The quantity of anthocyanins was expressed as A_{535}.

Tannins: Tannins determination was done according to the method of Van-Burden and Robinson (1981) with some modifications. A 0.5 g of fat free sample was added to 50 mL of distilled water in a 500 mL flask, kept on a shaker at 100 rpm for 1 h, filtered into a 50 mL volumetric flask and made the volume up to the mark. Then 5 mL of the filtrate was pipetted out into a test tube and mixed with 2 mL of 10 fold diluted of 0.1 M $FeCl_3$ in 0.1 N HCl and 0.008 M potassium ferrocyanide and kept at room temperature. The absorbance was measured at 605 nm within 10 min. Standard curve was prepared using 10, 20, 30, 40 and 50 µg/mL tannic acid to quantitatively determine the tannins in the samples.

Alkaloids: Alkaloids in the fat free samples were estimated following Harborne (1973). To 0.5 g of fat free samples, 20 mL of 10% acetic acid in ethanol was added, covered and allowed to stand for 4 h and then filtered. The filtrate was concentrated on a water bath at 90°C till one-quarter of the original volume of the extract was attained. Concentrated ammonium hydroxide was added drop by drop to the concentrated extract until the completion of precipitation. The precipitates were collected and washed with dilute ammonium hydroxide followed by refilteration. The resultant residue was weighed after oven drying to express as the final quantity of alkaloids.

Saponin: Saponins were determined according to the method of Chapagain and Wiesman (2005). Defatted residue (0.1 g) was dried for 24 h in a screw capped test tube and 30 mL of methanol were poured into it. The test tubes were kept over a shaker at 100 rpm for two days and then centrifuged. Three consecutive extractions were carried out followed by pooling of three resultant extractions with methanol. The extract was evaporated on a rotary evaporator. Final yellowish crystalline powder was carefully weighed to express tissue saponin concentrations.

Statistical Analysis

The analysis of variance (ANOVA) was done using computer software, MSTAT-C. Correlations between different attributes was determined by using Costat (CoHort 6.4) software. The means were compared using Duncan's new multiple range (DMR) test at $P \leq 0.05$. and MS-Excel was used for graphical presentation of the data

Results

Secondary Metabolites

Results regarding shoot phenolics revealed significant (*P<0.01*) differences in the varieties, temperature treatments and harvests with significant (*P<0.01*) interactions among these factors. Under control condition, the varietal difference was least. In stressed seedlings, maximum accumulation was exhibited at first harvest. Furthermore, both the varieties differed significantly with a better accumulation in Sultan (Fig. 1a-b). Varietal difference in both parts was greatest at final harvest under heat stress.

For flavonoid content there was a significant (*P<0.01*) difference in both the varieties, temperature treatments and harvests along with significant (*P<0.01*) interactions of these factors. S-2002 displayed a reducing trend in control but maintained the level at all harvests in stress. On the other hand, it was higher at final harvests of control in Sultan (Fig. 1c). As regards root flavonoids, under control condition, overall declining trend was observed in S-2002 but a higher level was displayed by Sultan (Fig. 1d). However, under heat stress at day-4 sharp increase followed by fall was noted at final harvest. Varietal difference was variable at all harvests. Sultan displayed superior status at final harvest.

The shoot tannins data revealed the significant (*P<0.01*) differences in heat stress treatments and harvests but not the varieties, with significant (*P<0.01*) interactions. Under control condition, the shoot tannins remained fairly steady with time, while S-2002 indicated a consistent decline. However, stressed Sultan indicated increased shoot tannins at all harvests but S-2002 indicated a consistent decline (Fig. 1e). The root tannins exhibited significant (*P<0.01*) difference in the varieties, heat stress treatments and harvests, with significant (*P<0.01*) interactions. Under control condition, roots of both varieties showed declining trend with lower accumulation in Sultan. Under heat stress, root tannins increased in both the varieties at final harvests yet this increase was greater in S-2002 (Fig. 1f).

Data pertaining to shoot anthocyanins revealed significant (*P<0.01*) differences in the heat stress treatments, but non-significant difference in the varieties and harvests. However, there were significant (*P<0.01*) interactions among all three factors. Under control condition, both the varieties indicated higher shoot anthocyanins contents at day-2, which reduced at day-4 and then increased at day-6 (Fig. 2a). However, under heat stress Sultan showed higher shoot anthocyanins at day-4, while S-2002 indicated reduced shoot anthocyanins at day-6. Unlike shoot anthocyanins, root anthocyanins indicated significant (*P<0.01*) difference in the varieties, heat stress treatments and harvests. Under control condition, Sultan indicated a decline in the root anthocyanins at day-6, while S-2002 indicated a decline at day-4 and then an increase at day-6 (Fig. 2b).

There were significant (*P<0.01*) differences in the varieties, heat treatments and harvests along with significant (*P<0.01*) interactions among these factors except a non-significant interaction of varieties × harvests for shoot alkaloids. Under control condition, the shoot tannins increased in both the varieties at all harvests although this increase in Sultan was lower than S-2002 at day-2, higher at day-4 and equal at day-6. Under heat stress, however, S-2002 indicated increased shoot alkaloids at day-2, which declined at day-4 and then increased at day-6; nonetheless, Sultan indicated lower value of this attribute at all harvests (Fig. 2c). Conversely for root alkaloids, statistical analysis of data showed significant (*P<0.01*) difference in the heat stress treatments and harvests, while all interactions among these factors were significant (*P<0.01*) except a non-significant varieties × heat stress interaction. Under control condition, S-2002 manifested greater alkaloids contents than Sultan. However, under heat stress Sultan displayed higher root alkaloids at later harvests (Fig. 2d).

The data regarding shoot saponin manifested non-significant (*P>0.05*) differences in the varieties, temperature treatments but a significant one (*P<0.01*) in the harvests. However, all possible interactions among these factors were significant (*P<0.01*). Under control condition, although saponin was higher at day-2, declined at day-4 and then increased at day-6. However, under heat stress both the varieties indicated increased shoot saponin at day-2, which declined at day-4 and day-6; nonetheless, Sultan indicated greater value of this attribute at all harvests (Fig. 2e). As regards root saponin, data revealed significant (*P<0.01*) difference in the varieties, heat stress treatments and harvests, while all possible interactions among these factors were significant (*P<0.01*). Under control condition, both the varieties indicated a decrease in root saponin at day-4 and then an increase at day-6. S-2002 indicated much higher quantities at all harvests. However, under heat stress, Sultan displayed the greatest saponin at day-2, which substantially declined at day-4 and day-6, while reverse was true for S-2002 at all harvests (Fig. 2f).

Correlation of Metabolites with Oxidative Damage Parameters

Correlation between secondary metabolites and oxidative damage characteristics was almost non-significant under normal environmental conditions. However, there was a significant correlation under heat stress. In both maize

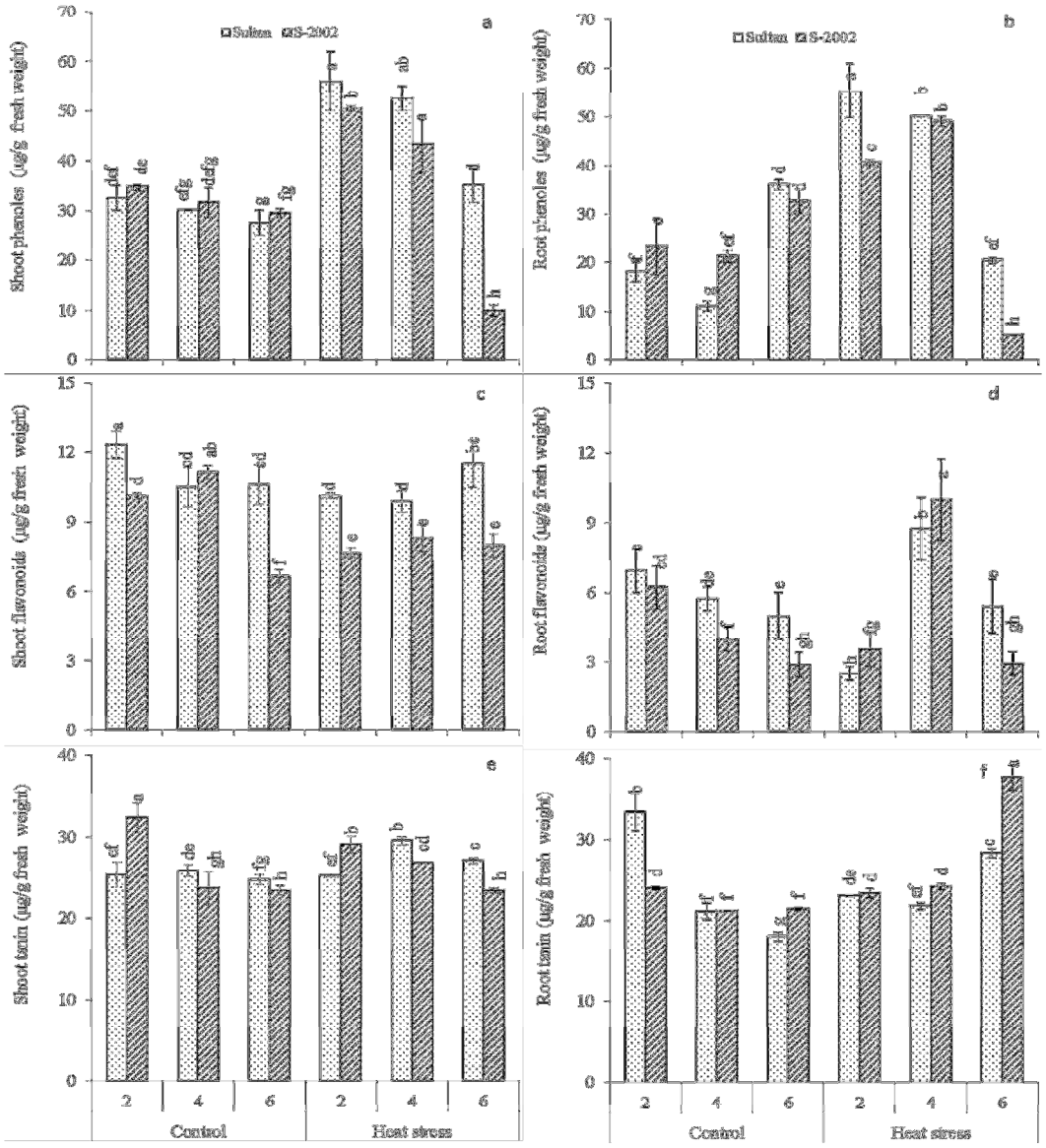

Fig. 1: Time course accumulation of secondary metabolites in shoots and roots of differentially heat tolerant maize varieties subjected to heat stress (40±2°C) for 2, 4 and 6 days. Different alphabets on columns are significant at P<0.05

varieties, soluble phenolics showed negative correlation with H_2O_2 and membrane permeability both in the roots and shoots. For other metabolites, both varieties showed differential correlation patterns. Stressed seedlings of the sensitive variety exhibited significant correlation for root saponins (r = 0.99**), root alkaloids (r = -0.99**), shoot tanins (r = -0.99**), and shoot anthocyanins (r = -0.900**) with membrane permeability. Moreover, there was also significant correlation with H_2O_2 for shoot tanins (r = -0.99**), and shoot anthocyanins (r = -0.70**). Resistant variety (Sultan) showed significance of correlation only for root saponins (-0.99**) with H_2O_2.

Discussion

Under stressful circumstances, plants cope with strain by modulating their metabolism to synthesize different enzymatic and non-enzymatic ROS scavengers (Mahmood

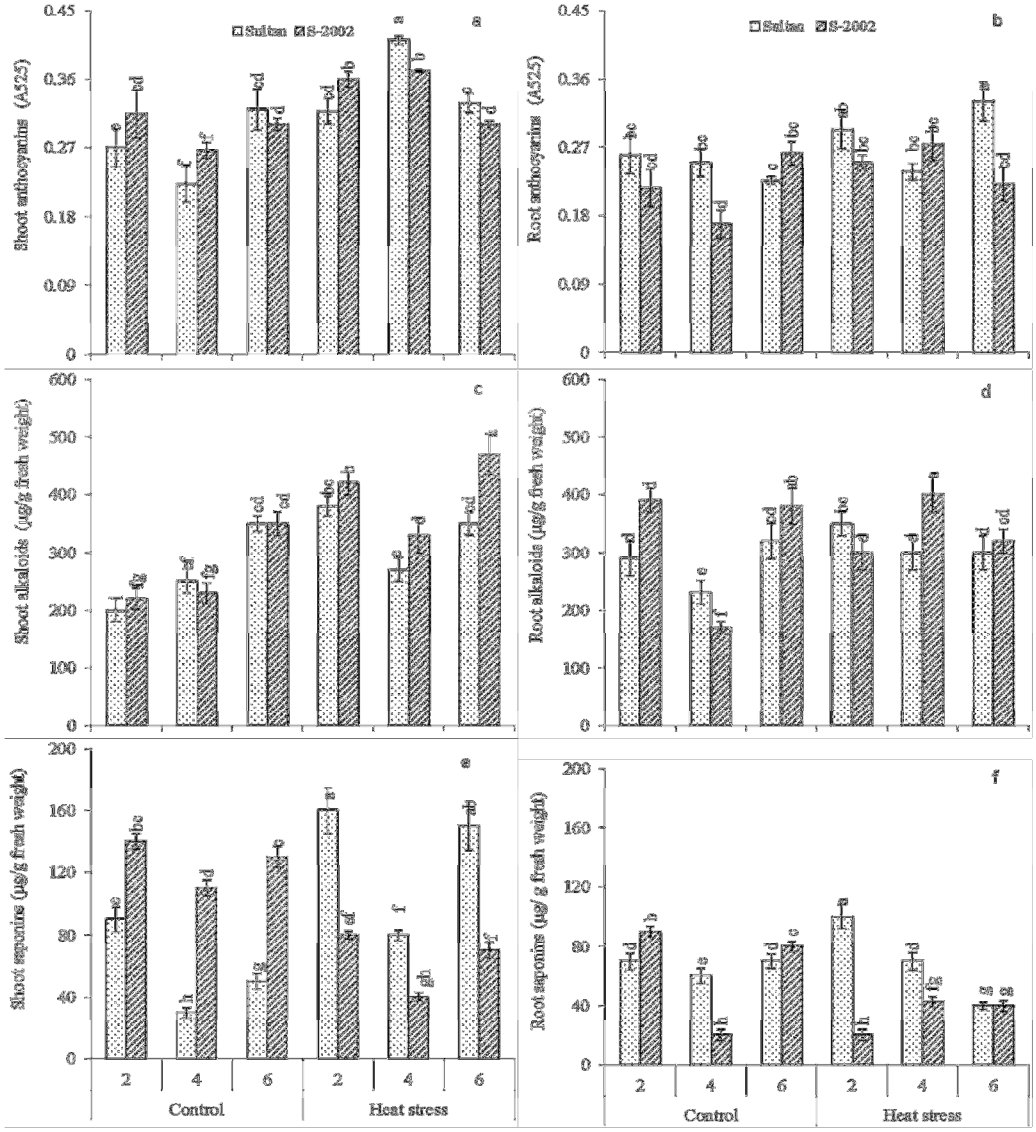

Fig. 2: Time course accumulation of secondary metabolites in shoots and roots of differentially heat tolerant maize varieties subjected to heat stress (40±2°C) for 2, 4 and 6 days. Different alphabets on columns are significant at P<0.05

et al., 2012; Wang *et al.,* 2012). Secondary metabolites are one of these scavengers (Wahid, 2007; Wahid *et al.,* 2008). From previous experimental findings, it is known that H_2O_2 is a toxic compound, the overproduction of which results in lipid peroxidation leading to damaged cell membranes (Adachi *et al.*, 2009). Therefore, relative membrane permeability and H_2O_2 are considered good measures of antioxidant potential of a plant variety (Moussa and Abdel-Aziz, 2008; Mahmood *et al.,* 2012). Secondary metabolites did not show correlation with oxidative damage characteristics (membrane permeability and H_2O_2) under controlled conditions. However, under stress, there was significant relation among them (Table 1), which confirmed incidental production of secondary metabolites under stressful circumstance. Secondary metabolites indisputably play central roles in plant physiology; ranging from toxicity and light/UV shielding to signal transduction (Vasconsuelo and Boland, 2007).

Table 1: Correlation matrix between secondary metabolites and oxidative damage characteristics in maize seedlings

Metabolite	Varieties	Treatments	Shoot		Root	
			Membrane permeability	H_2O_2	Membrane permeability	H_2O_2
Anthocyanin	Sultan	Control	0.20^{ns}	0.60^{ns}	0.20^{ns}	0.10^{ns}
		Heat stress	0.20^{ns}	0.40^{ns}	0.70*	0.40^{ns}
	S-2002	Control	-0.60^{ns}	0.04^{ns}	0.50^{ns}	0.50^{ns}
		Heat stress	-0.90**	-0.70*	0.02^{ns}	-0.50^{ns}
Flavonoid	Sultan	Control	-0.10^{ns}	-0.03^{ns}	0.10^{ns}	-0.05^{ns}
		Heat stress	0.60^{ns}	0.40^{ns}	-0.40^{ns}	0.40^{ns}
	S-2002	Control	-0.10^{ns}	0.10^{ns}	-0.40^{ns}	-0.10^{ns}
		Heat stress	0.20^{ns}	0.30^{ns}	0.40^{ns}	-0.40^{ns}
Tanin	Sultan	Control	-0.30^{ns}	-0.40^{ns}	0.09^{ns}	-0.10^{ns}
		Heat stress	-0.50^{ns}	-0.70*	0.80*	0.80*
	S-2002	Control	-0.50^{ns}	0.30^{ns}	-0.30^{ns}	0.10^{ns}
		Heat stress	-0.99**	-0.99**	0.50^{ns}	0.40^{ns}
Phenoles	Sultan	Control	0.30^{ns}	-0.40^{ns}	-0.30^{ns}	-0.70*
		Heat stress	-0.90**	-0.70*	-0.04^{ns}	-0.9**
	S-2002	Control	-0.50^{ns}	0.30^{ns}	0.30^{ns}	0.40^{ns}
		Heat stress	-0.99***	-0.99**	-0.30^{ns}	-0.50^{ns}
Saponin	Sultan	Control	0.10^{ns}	0.40^{ns}	0.30^{ns}	-0.80**
		Heat stress	-0.20^{ns}	-0.10^{ns}	-0.40^{ns}	-0.99**
	S-2002	Control	-0.30^{ns}	0.50^{ns}	0.10^{ns}	0.50^{ns}
		Heat stress	0.10^{ns}	0.40^{ns}	0.99**	0.02^{ns}
Alkaloid	Sultan	Control	0.030^{ns}	0.10^{ns}	0.30^{ns}	-0.8**
		Heat stress	-0.30^{ns}	-0.60^{ns}	0.80*	0.50^{ns}
	S-2002	Control	0.30^{ns}	-0.40^{ns}	0.20^{ns}	-0.30^{ns}
		Heat stress	0.60^{ns}	0.30^{ns}	-0.99**	-0.01^{ns}

Values with asterisk indicate significant coefficients of correlation; *, $P < 0.05$; **, $P<0.01$ and ns, non-significant

Heat stress substantially increased the soluble phenolics in maize seedlings. This increase was greatest with the onset of heat period and declined later (Fig. 1a-b). It indicated that maize seedlings responded immediately to heat stress by synthesizing higher total soluble phenolics. Moreover, negative correlation of H_2O_2 and membrane permeability with total soluble phenolics has confirmed their contribution in alleviation of oxidative injuries at cellular level. Better accumulation of phenolics in tolerant variety (Sultan) may be related with better protection against oxidative damage, screening of harmful radiations, stabilization of sub-cellular structures and improvement in cell water balance as previously reported (Wahid *et al.,* 2008; Fardus et al., 2014).

Soluble phenolics are a big class of compounds (Winkel-Shirley, 2002). So the determinations of its subgroups individually may be important for assessment of their possible antioxidative contribution under heat stress. Amongst phenolics, flavonoid biosynthesis contributes in the protection against abiotic stress tolerance (Winkel-Shirley, 2002) as they have the potential to maintain osmotic activity of the cell (Chalker-Scott, 1999; Wahid *et al.,* 2007, 2008). In present research, lower accumulation of this metabolite in sensitive variety under heat stress showed some relevance with former outcomes (Fig. 1c-d).

Tannins are another class of phenolics that have been reported for their contribution in plant stress tolerance (Lees *et al.,* 1994), which are distributed in plants in two forms i.e., condensed and hydrolysable (Buchanan *et al.,* 2000). Since shoots were directly under the exposure of stress, the varietal difference was more pronounced (Fig. 1e-f). Lower tannins concentration in sensitive variety and significant negative correlation with oxidative damage characteristics indicated their possible involvement in thermotolerance.

Anthocyanins are considered a part of efficient antioxidant system (Chutipaijit *et al.,* 2008). They donate hydrogen to scavenge ROS (Rice-Evans, 2001). Likewise, in the present study, anthocyanins showed lowered accumulation in the sensitive maize (Fig. 2a-b). Moreover, its negative correlation with membrane characteristics indicated relatively greater oxidative damage in the sensitive variety (Table 1).

Saponins content is generally influenced under abiotic factors of the environment (Szakiel *et al.,* 2010). They have been acknowledged as contributor of chemical protection of the cell against various environmental stresses (Lin *et al.,* 2009). In the present study, a negative correlation of shoot saponins with H_2O_2 and the membrane permeability has confirmed their involvement in ROS management (Table 1). Furthermore, this indicated that as a tolerance strategy, saponins also contributed in the metabolic adjustments of maize seedlings. Nonetheless, the exact mechanism of their involvement in the membrane protection and down regulation of ROS under heat stress needs to be explored.

Alkaloids have undoubted role in plant tolerance against biotic stresses, but a little is known about their contribution against abiotic stresses. A promotion of alkaloids in *Catharanthus roseus* has been noted under high temperature (Guo *et al.,* 2007). Similarly, the accumulation of some alkaloids had been found correlated with tolerance in plant seedlings against heat (Zu *et al.,* 2003) and drought

(Liu, 2000). In the present study, a negative correlation of akaloids in the sensitive variety along with its lower accumulation in it as compared with the resistant one pointed out towards their possible contribution in thermotolerance. Moreover, a negative correlation of H_2O_2 and the membrane permeability with total soluble phenolics has confirmed the contribution of soluble phenolics in the alleviation of oxidative injuries at the cellular level. For other metabolites, both varieties showed extremely different pattern of correlations.

In conclusion, the tolerance ability of the maize to heat stress was partly related to the increased synthesis of phenolics (anthocyanins and tannins) and alkaloids (saponins). A direct exposure of shoots to heat might have caused greater injuries in this part that triggered the synthesis of secondary metabolites as an adaptive strategy for thermo-tolerance in maize varieties.

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