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Evaluation of Screening Techniques for Heat Tolerance in Wheat

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Abstract: Cell membrane thermal stability, antioxidant activity, phenolics content, Paraquat tolerance and kernel weight were compared for their ability to identify heat tolerant genotypes. Four wheat (*Triticum aestivum* L.) genotypes, Kauz, MTRWA116, Opata and W7984 were used in this study, Kauz and MTRWA116 being thermotolerant and thermosensitive, respectively. Plants were exposed to high temperatures of 39 and 35°C and then their measurements form different techniques were compared to each other and to the controls. The experiments were run several times to measure the repeatability of the measurements. Although the amount of phenolic compounds increased under stress condition, there was no significant difference between tolerant and susceptible varieties. Membrane thermal stability, antioxidant activity, phenolics content and Paraquat tolerance did not provide repeatable data. Nor did they discriminate among tolerant and susceptible genotypes. kernel weight, however, varied between tolerant and susceptible genotypes. The results indicate that kernel weight is more suited for heat stress screening than other physioloical techniques evaluated in this study.

Key words: *Triticum aestivum*, heat tolerance, screening techniques, kernel weight

INTRODUCTION

Heat stress is a major limitation to wheat (*Triticum aestivum* L.) productivity in arid, semiarid, tropical and subtropical regions of the world (Ashraf and Harris, 2005). Consequently, development of heat-tolerant cultivars is of major concern in wheat breeding programs. A detailed understanding of the genetics and physiology of heat tolerance as well as the use of the proper germplasm and selection methods will facilitate the development of heat tolerant cultivars.

Exposure to higher than optimal temperatures reduces yield and decreases quality of cereals (Fokar *et al*., 1998; Maestri *et al*., 2002; Wardlaw *et al*., 2002). Decreased yield may be due to a wide range of interrelated processes, including accelerated development (Al-Khatib and Paulsen, 1984); reduced photosynthesis, either via damage to photosystem II (Paulsen, 1994) or inhibition of Rubisco activase (Law and Crafts-Brandner, 1999) increased respiration (Berry and Bjorkman, 1980) or disruptions to the respiratory mechanism (Lin and Markhart, 1990) and decreased starch synthesis in developing grain (Bhullar and Jenner, 1985). The nature and severity of the yield reduction depends on the developmental stage at which the stress occurs (Acevedo *et al*., 1991; Paulsen, 1994). High temperatures during floral initiation and spikelet development (a period of several weeks preceding anthesis) reduce the potential number of grains, thus determining maximum yield potential. Heat stress during the post-anthesis grain-filling stage affects availability and translocation of photosynthates to the developing kernel and starch synthesis and deposition within the kernel, thus resulting in lower grain weight and altered grain quality (Bhullar and Jenner, 1985; Mohammadi *et al*., 2004). Evaluating grain yield under heat stress has long been practiced by breeders to identify genotypes better adapted to hot conditions. This approach has the advantage of combining the effects of many different factors without having to know the relative importance or the physiological basis of each factor. Screening in the target environment has also the advantage of using the relevant stress levels. However, there are many difficulties with respect to field screeing, particularly in relation to abiotic stress: The occurrence of stress is unpredictable as the natural climate is variable both day to day and year to year; screeing is often limited to a small portions of the year; the target environment is not always conveniently situated; selection for yield in a limited number of environments might ignore the effects of processes that contribute to stress tolerance over a wider range of environments; the heritability of grain yield is very low; growing yield trials, which require large plot sizes grown in multiple replications and locations are expensive. Moreover, in the field, the interactions of water stress, high temperature and other adaphic constraints on plant

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responses complicate the assessment. So plant breeders are searching for laboratory screeining techniques which are not affected by these problems and can be conducted under controlled condition (Howarth, 2005).

Cell membrane stability after a prehardening treatment has been suggested for estimating cellular thermotolerance of plants (Sullivan *et al*., 1977; Blum and Ebercon, 1981; Reynolds *et al*., 1994; Fokar *et al*., 1998; Ibrahim and Quick, 2001). Exposure of plant cells to heat causes cellular membrane disruptions that are apparently related to temperature-specific phase changes in the membrane lipid bilayer (Suss and Yordanov, 1986). The basic mechanism of cellular membrane disruption under heat stress can impact photosynthetic or mitochondrial activity or even decrease the ability of the plasmalemma to retain solutes (Lin *et al*., 1985). The cellular Membrane Thermal Stability (MTS) test estimates the amount of electrolyte leakage from heat-stressed tissues *in vitro* using a simple conductometric technique. Although MTS has been suggested for heat screening, the repeatability of the technique has not been well studied.

A well-known consequence of elevated temperatures in plants is oxidative damage caused by a heat-induced imbalance of photosynthesis and respiration (Fitter and Hay, 1987). Limitation of photosynthesis can increase the rate of active oxygen formation in chloroplasts or hinder the activity of antioxidant defenses (Smirnoff and Colombe, 1998). Active oxygen species (e.g., O_2^-) attack a variety of cellular components. They cause lipid peroxidation and consequently membrane injury, protein degradation, enzyme inactivation, pigment bleaching and disruption of DNA strands (Sairam *et al*., 2000). The antioxidant defense mechanism is part of heat stress adaptation and its strength is correlated with acquisition of thermotolerance (Maestr *et al*., 2002). Antioxidant capacity can be measured by ABTS radical scavenging capacity (RiceEvans and Miller, 1997). ABTS is the acronym for the radical 2, 2'-Azino-*bis* (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt. It is proved that antioxidative effect is mainly due to phenolic compounds (Ossawa 1994). Phenolic compounds are frequently up-regulated in response to stress episodes. Some of these are protective because of their antioxidant properties and are highly correlated with free radical scavenging capacity (Javanmardi *et al*. 2003). Total phenolics content is measured by using a straightforward spectrophotometric assay. An attempt was made in our study to check whether antioxidant capacity or phenolics content can be used for measuring heat tolerance.

Paraquat is a bypridlium herbiside that causes oxidative stress in plants by producing highly toxic free radicals (Dodge, 1971). Enhanced tolerance to paraquat has showed correlation with water stress tolerance (Altikut *et al*., 2001). So a close correlation is expected between the plants tolerance to stresses imposed by heat and paraquat. One of the purposes of this research was to verify whether the conclusions of Altikut *et al*. (2001) apply to heat tolerance.

Photosynthetic rate, stomatal conductance as well as canopy temperature depression are other heat tolerance measurements which have shown correlation with field performance (Reynolds *et al*., 1994). Photosynthetic rate is hard to measure and does not seem to be able to differentiate the tolerant genotypes under moderate heat stress. Single leaf readings of stomatal conductance always have associated errors that may be caused by environmental fluxes, leaf position and the fact that leaves may show diurnal and cylindrical patterns in stomatal behavior (Reynolds *et al*., 2001). Canopy temperature depression is not a useful selection trait in generally cool and/or humid conditions and is quite sensitive to environmental fluxes because the trait is best expressed at high vapor pressure deficit condition associated with low relative humidity and warm air temperature (Amani *et al*., 1996). In addition, Photosynthetic rate, stomatal conductance and canopy temperature depression have the disadvantage of being measured in the field.

To our knowledge, to date, there is no report on using antioxidant activity, total phenolics or paraquat tolerance for heat tolerance. There are some literature on MTS, but its repeatability has not been tested. The objectives of this study were: to asses the suitability of MTS, antioxidant activity, total phenolics and paraquat techniques for screening heat tolerant genotypes and to evaluate the parents of two wheat mapping populations for heat tolerance. We would ultimately evaluate the populations for repeatable tests capable of discriminating among heat tolerant and heat susceptible genotypes.

MATERIALS AND METHODS

Plant material: Four wheat genotypes, Kauz, MTRWA116, Opata and W7984 were used in this study. Kauz has been developed in CIMMYT, Mexico and is known as tolerant to high temperature. MTRWA116 is an unrealized experimental line from Montana State University (USA) and is considered as thermosensitive (Butler, 2002 ; Ibrahim and Quick, 2001). Opata is a spring wheat variety and W7984 is a synthetic hexaploid wheat. Kauz × MTRWA116 and Opata × W7984 crosses have been used to develop wheat mapping populations by Dr. Patrick F. Byrne (Colorado State University, USA) and International Triticeae Mapping Initiative (ITMI).

Membrane thermal stability: Seedlings were germinated and grown in vermiculite in green house at 20-25°C. The experimental design was completely randomized with four replications. Ten days after emergence, the green seedlings were transferred to an environmental chamber for heat acclimation at 34/29°C and 50/70% humidity day/night for 24 h. Three leaves (4.2 cm form the tip) from each pot were excised and put in a 17 mL test tube containing 10 mL deionized water after being washed three times. The same was done for three replications as control. Treatment tubes were placed in a water bath at 52°C for an hour, while control tubes remained in room temperature. All tubes were kept at room temperature for 24 h. Conductivity of solutions, then, was measured with a conductivity meter (Electroanalyzer 4400, Markson Science Inc., Del Mar, CA). All tubes were autoclaved at 120°C and 0.1 MPa for 20 min and then conductivity was measured the next day. Membrane thermal stability (%) was calculated as the reciprocal of relative leakage: MTS = (1-T1/T2)×100

Where T1 and T2 are the conductivity readings before and after autoclaving, respectively. The entire assay was done two times (assay 1 and 2). MTS was also measured on 4-5 leaf stage plants in two other assays (assay 3 and 4). Analysis of variance (ANOVA) was performed for controls to make sure that there was not significant difference among control entries. Otherwise, an adjustment should have been made to the formula.

Paraquat tolerance: Seedlings were germinated and grown like the MTS experiment for three weeks (4-5 leaf stage). Removing the first 2 cm of the last fully expanded leaf with collar, the next 5 cm was excised to form experimental unit. Leaf samples were weighed and placed in 10 mL vials. The treatment vials were filled up with 10 um paraquat with 0.5% TWEEN20 and were placed in chamber at full light and 25°C for 14 h. The leaves, then, were removed from vials, rinsed with distilled water and placed in 15 mL tubes. The samples were left in freezer (-80°C) overnight and then grinded in 10 mL Buffered acetone. Ground samples were refrigerated for 1 h and then centrifuged at maximum speed for 10 min. The absorbance of supernatant was measured spectrophotometrically at 647 and 664 nm. Leaf cholorophyll content was then calculated according to the Arnon's (1949) formula. The chlorophyll loss in response to paraquat was determined by comparing the treated and control samples. The assay was repeated four times.

Phenolics content: Plants were grown for about 4 weeks (5-6 leaf stage) in green house at 25/20°C and then moved into the growth chamber with 335 μmol m^{-2} S^{-2} light and 39/34°C for 24 h day/night. Leaves were dissected and frozen in liquid nitrogen and kept at -80°C and freeze-dried. Leaves were then ground and 1/10 w/v solution of leaf samples were prepared using acetone (80%). Sample extracts were rotated overnight in the dark and centrifuged at 3500 rpm for 4 min. The amount of total phenolics in the supernatant was determined with the Folin-Ciocalteu reagent according to the method of Spanos and Wrolstad (1990) using gallic acid as a standard. 2.5 mL 1/10 dilution of Folin-Ciocalteu's reagent and 2 mL of Na_2CO_3 7.5% (w/v) were added to 50 μL of each sample (three replicates) and incubated at 30°C for 1.5 h. The absorbance of all samples was measured at 765 nm using the SPECTRAmax-PLUS384 UV-Vis spectrophotometer. Results were expressed as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/gdw). The second assay was the same except that the green house temperature was 35°C.

Antioxidant capacity: The leaf samples were made in the same way as mentioned for phenolics content. The total antioxidant activity was measured by the trolox equivalent antioxidant activity (Miller and Rice-Evans, 1996). The relative capacity of antioxidants to scavenge the ABTS was measured in comparison with the antioxidant potency of Trolox (water soluble vitamin E) as standard. The absorbance at 734 nm was measured by spectrophotometer after 1 min. Measured values of each sample, were calibrated to the results obtained from Trolox standards (20-100 μM), which were plotted as a calibration curve. The antioxidant activity of samples was measured against a Trolox standard and expressed as trolox equivalent antioxidant activity. The assay was done twice similar to the phenolics content.

Kernel weight: Seedlings were germinated and grown in Metro-Mix2000® growing medium in greenhouse at 20-25°C in Conetainer® pots called. The experimental design was completely randomized with twenty replications. Plants were watered as needed and fertilized with a complete solution of PETER-Professional®. One week after the first anther extrusion was observed, the pots of ten replications, each containing one seedling, were transferred to a controlled environment chamber for heat shock. Chamber was set at 35/30°C and 14/10 h day/night, 50/70% relative humidity and illumination of 335 μmol m^{-2} S^{-2}. Plants were exposed to this high temperature for three days and then moved back to the greenhouse. Since the lines varied in their anthesis-date, they were transferred to chamber in different days. When the color of peduncle turned to yellow (physiological maturity)plant heads were excised and incubated in 40°C for three days. kernel weight were, then, measured.

Statistical analysis: Analysis of variance was conducted for each of the measurements and means were compared by Least Significant Difference (LSD). t-test was performed to compare the two parents and also the two conditions.

RESULTS AND DISCUSSION

Membrane thermal stability: Analysis of variance showed significant difference among varieties in assay 3 (Table 1). MTRWA116 and Opata had significantly higher MTS (more tolerant) than Kauz and W7984 in assay 3. Varieties indicated no significant difference for MTS in assays 1,2 or 4. There is a high variation among the values of assays. For instance, the mean MTS of Kauz was 15.24, 5.32, 33.54 in assay 1, 2, 3 and 33.09 in assay 4 (Table 2). MTS of Kauz has been reported as 76.4 in Ibrahim and Quick's study (2001). In addition, MTS of Kauz was significantly lower than MTRWA116 in assays 2 and 3. Kauz, however, is a thermotolerant variety and is expected to have higher cell membrane thermal stability. MTS, therefore, does not seem to be a repeatable and reliable technique. This is in agreement with Reynolds *et al.* (1994) that conclude a day- to- day variability change in MTS values.

The data demonstrate that MTS rises when plant grows to higher stages as all the values for assay 3 and 4 are higher than those for assay 1 and 2 (Table 2). This agrees with the previous studies (Reynolds *et al.*, 2001).

Paraquat tolerance: The amount of chlorophyll loss was extremely changing from one assay to another (Fig. 1). For example, the range of chlorophyll loss of Kauz was from -2.7 to 43.2%. The Praquat tolerance does not seem to be a powerful technique for heat tolerance screening. Although Altikut *et al.* (2001) reported a correlation between tolerance to paraquat and water stress tolerance, they did not prove the repeatability of their data.

Table 1: Mean square of ANOVA for cell membrane thermal stability, phenolics content and antioxidant activity in wheat varieties

Measurement	Assay 1	Assay 2	Assay 3	Assay 4
Membrane thermal stability	42.02	23.24	2046.99*	357.82
Phenolics content	0.057*	0.139		
Antioxidant activity	5202707**	9549264**		

*, **, Significant at 5 and 1% level, respectively

Table 2: Mean cell membrane thermal stability of varieties in four assays

Variety	Assay 1	Assay 2	Assay 3	Assay 4
Kauz	15.24	5.32	33.54^{b†}	33.09
MTRWA116	11.81	10.48	60.86^{a}	32.67
Opata	9.77	5.44	68.31^{a}	43.16
W7984	7.07	6.83	32.54^{b}	24.29

† Values followed by the same letter(s) are not significantly different according to LSD (p = 0.05). Kauz was compared to MTRWA116 and Opata to W7984

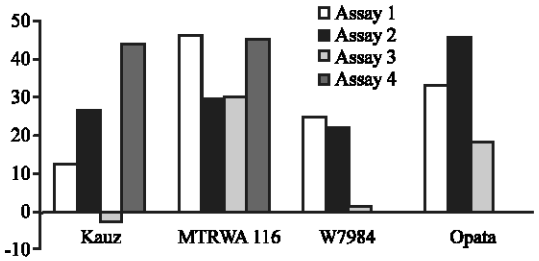

Fig. 1: Chlorophyll loss (%) of varieties in response to paraquat in four assays

Table 3: Mean phenolics content (mg/g dw) and antioxidant activity (mM/g dw) of varieties under heat stress in two assays

	Phenolics content		Antioxidant activity	
Variety	Assay 1	Assay 2	Assay 1	Assay 2
Kauz	22.868^{a†}	17.198^{a}	2826.725^{b}	2890.14^{b}
MTRWA116	23.951^{a}	16.679^{a}	3245.488^{a}	3274.59^{a}
Opata	21.005^{a}	23.990^{a}	3987.367^{a}	4326.53^{a}
W7984	20.247^{a}	15.833^{a}	2856.136^{b}	2692.32^{b}

† Values followed by the same letter(s) are not significantly different according to LSD (p = 0.05). Kauz was compared to MTRWA116 and Opata to W7984

Table 4: Mean kernel weight of varieties (g) in control and heat stress conditions

Activity	Kauz	MTRWA116	Opata	W7984
Control	1.074	1.206	0.973	1.126
Heat stress	0.797	0.612	0.868	1.016

Phenolics content: The analysis of variance (Table 1) showed that there was a significant difference among varieties for phenolics content at 39°C (assay 1), but not at 35°C (assay 2). On the other hand, the t-test showed that phenolics content of varieties at 35°C was not significantly different than the control condition. This indicates that moderate heat stress does not considerably change the phenolics content, while heat shock stress arising from higher temperatures does have a significant effect on phenolics content of plant.

Although the amount of phenolic compounds increased in plants under heat stress, there was no significant difference either between Kauz and MTRWA116 or between Opata and W7984 (Table 3). So, the data do not support the idea that there is a relationship between phenolics content and tolerance to heat stress.

Antioxidant capacity: The ANOVA (Table 1) showed a very significant difference among varieties for antioxidant activity at the stress conditions of 39°C (Assay 1) and 35°C (assay 2). The t-test demonstrated unexpectedly that MTRWA116 under stress condition had a significantly higher antioxidant activity than Kauz both in assay 3 and 4. The antioxidant activity of Opata under heat stress

condition was more than W7984 in both assays (Table 3). As shown in the Table 3 the antioxidant activity in control condition was sometimes higher than heat stress condition. The results showed that there was no association between antioxidant activity of plant and heat tolerance using the genotypes evaluated in this study.

Kernel weight: Comparison of means (Table 4) by t-test demonstrated that Kauz and MTRWA116 were different in their response to heat stress for kernel weight of MTRWA116 decreased more than Kauz under heat stress. Opata and W7984, however, were not significantly different for their kernel weight under control or heat stress conditions.

CONCLUSION

We conclude that kernel weight under high temperature stress is a better measurement for heat screening than physioloical techniques such as cell membrane thermal stability, antioxidant activity, phenolics content, or paraquat tolerance. Because these techniques could not identify the tolerant genotypes and the data were not consistent enough to be used as a screening technique. Kernel weight is still preferred for breeding purposes as it has the advantage of combining the effects of many different factors without having to know the relative importance or the physiological basis of each factor. Kernel weight, however, is time consuming and difficult to measure. we should, therefore, look for faster and more convenient approaches.

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