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Screening of advanced breeding lines for high temperature tolerance using biochemical parameters in Indian mustard (*Brassica juncea*)

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ABSTRACT

A set of 30 advanced breeding lines of *Brassica juncea* were screened for heat tolerance in terms of biochemical parameters in field condition at ICAR-DRMR. The selection was based on (1) early sowing (ES) (September) when average soil temperature was 41°C and atmospheric temperature was around 35ºC so that heat stress coincided with seedling growth and (2) normal sown (NS) (mid October) where soil temperature was 34.2°C so that seedling growth did not coincide with any stress. Various biochemical parameters like total chlorophyll, total carotenoid content, total antioxidant capacity, radical scavenging activity, lipid peroxidation and proline content were measured in leaves at flowering stage to evaluate the variability among the genotypes and comparison between ES and NS was done. Stress susceptibility index (SSI) categorized genotype NPJ-124 and DRMR-1165-40 to be highly tolerant. Correlation analysis among all the traits showed total antioxidant capacity to be significantly correlated to carotenoids and chlorophyll pigment levels showing the importance of these parameters as indices for screening.

Key words: Antioxidant capacity, Chlorophyll, Heat stress, Indian mustard, Lipid peroxidation, Proline, Radical scavenging activity

Elevating atmospheric temperature due to global climate change has become a major problem that is affecting crop yield of Indian mustard (*Brassica juncea*) and farm income of farmers of the country. IPCC (Inter governmental panel on climate change, 2018 https://archive.ipcc.ch/) reported the impact of global warming has led to an increase of 1.5°C above pre-industrial level due to greenhouse gas emissions worldwide. This prompted the researchers in agricultural sector to identify lines that can withstand higher temperature. The effect of heat stress has been well studied in Indian mustard (Azharudheen *et al*. 2013, Wilson *et al*. 2014, Sharma and Sardana 2016, Ram *et al.* 2016, Ram *et al.* 2017).

Oilseed brassica is a major oilseed crop covering across continents stand next to soybean in terms of area and production. Among the brassica species grown in India, 90% is shared by Indian mustard (Shekhawat *et al.* 2014). It is a cool season crop; hence high temperatures has a detrimental effect on its growth, development and in turn its yield. The optimum temperature of 25^{0}C is required for seedling establishment (Lallu and Dixit 2008). But due to the changing climate the soil temperature rises to about 40-42^{0}C in the month of September especially in hotter mustard growing areas like Rajasthan (Azharudhen *et al*. 2013).

Heat stress leads to an array of physiological, biochemical and molecular changes. During high temperature stress oxidative burst leads to an increase in ROS (Reactive oxygen species) like hydrogen peroxides which was also observed in mustard seedlings after heat treatment (Dat *et al*. 1998). The ROS are highly toxic and can lead to oxidative destruction in the cell. The consequences of ROS depend upon the intensity of stress and on the physiochemical conditions in the cell. Excessive generation of ROS produced as a result of heat stress, induces cell membrane injury, causes damage to the PS-II oxygen evolving complex and thus influence the protein synthesis (Sairam *et al*. 2000, Wahid and Close 2007). Cell membranes are most affected by high temperature due to lipid peroxidation with increased level of ROS products like malondialdehyde (MDA). The content of MDA depends upon the level of stress injury. Plants adapt to stress by naturally evolved defense mechanism to maintain cell

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homeostasis between the ROS production and the capacity to scavenge the toxic free radicals by cellular antioxidants. Under heat stress the release of ROS not only lead to lipid peroxidation, membrane damage, leakage of cellular content, protein degradation, but also pigment bleaching (Sharma *et al*. 2012). Therefore estimation of chlorophyll and its pigments is an important parameter for identifying the status of plant stress. Chlorophyll absorbs sunlight and uses its energy to synthesize carbohydrates from carbon dioxide (CO_2) and water. The change in chlorophyll content depends upon stress intensities thus, making the concentration of chlorophyll a marker of photosynthetic stability (Singh *et al.* 2019). Plants also adapt to stress by naturally evolved mechanism at cellular level by maintaining its homeostasis through the production of compatible solutes like proline. The increase in proline concentration influences the retention of water and maintains the normal membrane function of the plant. Apart from maintaining cellular balance, proline is also known to act as hydroxyl radical scavenger (Smirnoff and Cumbes 1989).

One of the ways to study heat stress during seedling establishment in field conditions is to do early sowing of the seeds during the month of September when soil temperature is above 40^0C which exposes the seedling to soil temperature above 25^0C. To have a better understanding of plant response to high temperature stress it is important to identifying genotypes that can adapt to high temperature. Keeping this in mind our objective was screening heat tolerant lines from advanced breeding materials with the help of biochemical markers and to identify and classify those lines based on their tolerance level for use in future breeding programmes.

MATERIALS AND METHODS

Experimental site and design

A total of 30 advanced high temperature tolerant breeding lines were selected for this experiment. Two hundred fifty seeds of each selected lines were sown under heat stress condition (maximum temperature 41°C at 0-10 cm soil depth on seeding date on September 28, 2015) and normal conditions (maximum temperature 34.2°C at 0-10 cm depth on seeding date on October 24, 2015) in randomized complete block design with three replications at ICAR-DRMR, Bharatpur (77.270 E longitude; 27.120 N latitude and 178.37 m above mean sea level), India. The soil of the experimental site was sandy loam with EC 1.5 dSm^{-1}, low organic carbon (0.25-0.30%), poor available N (125-135 kg/ha), medium P (20-22 kg/ha), and available K of 240-260 kg/ha and a pH 8.1. The Indian mustard crop was raised strictly under conserved moisture conditions. All the selected breeding lines were grown in three rows of five meter length. The distance between row to row and plant to plant was 45 cm and 15 cm, respectively.

Biochemical analysis

Selected advanced breeding heat tolerant lines (Table 1a,b) were evaluated for various biochemical parameters to identify the most heat tolerant lines. Leaf samples during the flowering stage were taken from ES and NS for evaluating various biochemical parameters.

Chlorophyll estimation

Chlorophyll estimation was done in fresh leaf by a common method (Hiscox and Israelstam 1979) with the following formula for deriving Chlorophyll *a* (Chl *a*), Chlorophyll *b* (Chl *b*), Total chlorophyll (Chl_{total}) and Total carotenoids content.

Chl a (mg/g FW) = [(12.7 × A663) – (2.69 × A645)] × V/1000 × W

Chl b (mg/g FW) = [(22.9 × A645) – (4.68 × A663)] × V/1000 × W

Carotenoids (mg/g FW) = [(1000 × A470) – (3.29 × Chl a) – (104 × Chl b)]/198

where, V-volume of DMSO added, W-weight of sample taken, FW- fresh weight.

Proline estimation

Fresh leaves were used for estimation according to the method described by Bates *et al.* (1973) using proline (Himedia) as the standard. Proline content was expressed in µmole/g FW.

Total antioxidant capacity

Total antioxidant capacity (TAC) was determined as per Prieto *et al*. (1999). The 100 mg of leaf sample was homogenised in 2ml of 80 % methanol, and kept overnight. The supernatant was collected after centrifuging at 4000 rpm for 10 min and final volume was raised to 2 ml. Reduction of Mo (VI) to Mo (V) and the subsequent formation of green colour complex was measured by spectrophotometer (Labomed UV-VIS Double beam UVD-3500) at 695 nm, using ascorbic acid as standard. The TAC was expressed as ascorbic acid equivalent (AAE).

Radical scavenging activity (RSA)

The same methanolic extract used for TAC was used for determining the potential antioxidant properties by determining the scavenging of 1,1- diphenylpicryl hydrazyl and employing the following formula according to Mellors and Tappel (1996)

$$\text{RSA (\%)} = \frac{\text{OD of control-OD of sample)}}{\text{OD of control}} \times 100$$

where, OD –Optical density at 517 nm

Lipid peroxidation

MDA content was determined by the thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968); Hagege *et al*. (1990); Hodges *et al*. (1999) with slight modifications. The 250 mg of leaf sample was homogenized with 5 ml of 1% Trichloro acetic acid (TCA) followed by centrifugation at 8500 rpm for 10 min. After centrifugation, 1ml of the supernatant was mixed with 4ml of TCA/TBA

Table 1 a and b Biochemical analysis of advanced breeding lines in Indian mustard under early sown and normal sown

(a) For each parameter, means with the same letter are not significantly different

Genotype	Chlorophyll a (mg/g FW)			Chlorophyll b (mg/g FW)			Carotenoid content (mg/g FW)			Total chlorophyll content (mg/g FW)		
	Early	Normal	% increase (+)/ decrease (-)	Early	Normal	% increase (+)/ decrease (-)	Early	Normal	% increase (+)/ decrease (-)	Early	Normal	% increase (+)/decrease (-)
Urvashi	0.70^{a}	$1.44^{nopqrstuv}$	-106.22	0.19^{stu}	$0.27^{lmnopqrs}$	-47.44	2.60^{u}	$5.42^{klmnopq}$	-108.01	0.88^{b}	1.72^{rstuvw}	-93.91
DRMR-541-44	1.11^{wxy}	$1.62^{hijklmnopqr}$	-46.62	0.02^{v}	$0.37^{cdefghijklmn}$	-1455.87	4.59^{rs}	$5.56^{jklmnop}$	-21.21	1.13^{zab}	$1.99^{klmnopq}$	-76.19
RH-119	$1.54^{jklmnopqrst}$	1.40^{qrstuv}	9.02	$0.36^{jklmnopqr}$	$0.34^{ghijklmnop}$	4.94	$5.62^{ijklmnop}$	4.61^{rs}	17.84	$1.85^{nopqrstu}$	$1.74^{qrstuvw}$	5.94
RH-406	0.84^{yza}	$1.55^{jklmnopqrs}$	-85.61	0.12^{tuv}	$0.36^{defghijklmno}$	-199.33	2.90^{ut}	5.20^{opqr}	-79.45	0.96^{b}	$1.92^{lmnopqrs}$	-100.02
DRMR-HT-13-20	$1.77^{efghijklm}$	$1.49^{lmnopqrstuv}$	15.42	0.51^{a}	$0.25^{noppqrs}$	50.74	6.03^{fghijk}	4.61^{rs}	23.54	2.28^{efghij}	$1.75^{qrstuvw}$	23.39
DRMR-HT-13-13	$1.42^{pqrstuv}$	$1.91^{bcdefgh}$	-34.37	$0.35^{ghijklmnop}$	0.47^{abcdef}	-35.09	5.08^{opqr}	6.31^{efgh}	-24.11	$1.77^{pqrstuvw}$	2.38^{cdefgh}	-34.52
DRMR-HT-13-7	0.81^{za}	$1.88^{cdefghi}$	-132.39	0.21^{qrstu}	$0.46^{abcdefg}$	-112.95	3.15^{ut}	6.09^{efghij}	-93.21	1.02^{ab}	2.34^{defghi}	-128.31
DRMR-HT-13-28	1.00^{xyz}	$1.79^{efghijk}$	-79.99	0.26^{nopqrs}	$0.34^{ghijklmnop}$	-33.92	3.35^{t}	6.12^{efghij}	-82.94	1.25^{zya}	$2.14^{ghijklm}$	-70.58
DRMR-HT-729	$1.70^{hijklmnop}$	$1.63^{hijklmnopqr}$	4.22	$0.27^{mnopqrs}$	$0.36^{defghijklmno}$	-35.62	5.88^{hijklm}	$5.63^{ijklmno}$	4.22	$1.97^{klmnopqr}$	$1.99^{klmnopq}$	-1.23
BPR-543-2	$1.67^{hijklmnopqr}$	1.89^{cdefgh}	-13.54	$0.35^{fghijklmnop}$	$0.46^{abcdefg}$	-28.87	6.06^{efghij}	6.41^{defgh}	-5.78	$2.02^{jklmnop}$	$2.35^{cdefghi}$	-16.22
BPR-349-9	$1.49^{lmnopqrstuv}$	$2.01^{abcdefg}$	-34.63	0.19^{rstu}	$0.45^{abcdefgh}$	-137.67	4.87^{qr}	6.53^{cdefg}	-33.87	1.68^{stuvw}	2.46^{abcdef}	-46.34
BPR-549-9	1.26^{tuvwx}	$1.53^{jklmnopqrst}$	-21.68	0.12^{uv}	$0.40^{abcdefghijkl}$	-240.30	4.19^{t}	5.00^{pqr}	-19.56	1.37^{xyz}	$1.92^{lmnopqrs}$	-40.17
BPR-540-6	$1.43^{opqrstuv}$	1.86^{efghi}	-30.11	$0.45^{abcdefghi}$	0.48^{abcd}	-7.57	6.21^{efghi}	6.17^{efghij}	0.69	$1.88^{mnopqrst}$	2.34^{defghi}	-24.70
BPR-541-4	$1.48^{mnopqrstuv}$	$1.60^{ijklmnopqrs}$	-8.15	$0.30^{jklmnopqrs}$	$0.36^{efghijklmno}$	-18.84	4.60^{rs}	5.15^{opqr}	-12.13	$1.78^{pqrstuvw}$	$1.96^{klmnopqr}$	-9.96
NPJ-124	$1.89^{cdefghi}$	$1.51^{klmnopqrstu}$	20.05	0.51^{ab}	$0.39^{cdefghijklm}$	24.35	6.62^{cdef}	4.92^{qr}	25.64	$2.40^{abcdefg}$	$1.90^{mnopqrs}$	20.97
RH-555	$1.54^{jklmnopqrst}$	0.89^{yza}	42.44	$0.44^{abcdefghi}$	0.22^{qrstu}	50.65	6.27^{lmnopq}	3.08^{ut}	50.79	$1.98^{klmnopqr}$	1.11^{zab}	44.26
DRMR-1672-2	$1.74^{fghijklm}$	$1.56^{jklmnopqrs}$	10.37	0.24^{opqrst}	$0.39^{cdefghijklm}$	-59.76	6.00^{fghijk}	5.24^{nopq}	12.72	$1.98^{klmnopqr}$	$1.94^{klmnopqrs}$	1.76
JN-032	$1.78^{efghijkl}$	$1.69^{hijklmnopq}$	4.92	$0.31^{jklmnopqr}$	$0.35^{fghijklmnop}$	-13.39	6.09^{efghij}	$5.57^{jklmnop}$	8.45	$2.09^{ijklmno}$	$2.04^{jklmnop}$	2.19
DRMR-1617-45	$1.72^{ghijklmn}$	$1.73^{fghijklm}$	-0.74	$0.32^{jklmnopq}$	$0.30^{jklmnopqrs}$	3.68	5.95^{ghijkl}	$5.68^{ijklmno}$	4.54	$2.04^{jklmnop}$	$2.04^{jklmnop}$	-0.05
DRMR-1165-40	2.23^{a}	1.87^{defghi}	16.10	$0.39^{bcdefghijkl}$	$0.31^{jklmnopqr}$	21.27	7.58^{a}	$5.86^{hijklmn}$	22.73	2.62^{abc}	2.18^{ghijkl}	16.87
DRMR-1191-2	2.04^{abcde}	1.23^{unwx}	39.85	$0.34^{ghijklmnop}$	$0.29^{klmnopqrs}$	16.08	7.00^{abcd}	4.09^{s}	41.52	$2.38^{bcdefgh}$	1.52^{wxy}	36.43

Cond.

Table 1 a and b (*Continued*)

Genotype	Chlorophyll a (mg/g FW)			Chlorophyll b (mg/g FW)			Carotenoid content (mg/g FW)			Total chlorophyll content (mg/g FW)		
	Early	Normal	% increase (+)/ decrease (-)	Early	Normal	% increase (+)/ decrease (-)	Early	Normal	% increase (+)/ decrease (-)	Early	Normal	% increase (+)/decrease (-)
NRCDR-601	2.16^{abc}	2.20^{ab}	-1.44	$0.42^{abcdefghij}$	$0.40^{abcdefghijk}$	4.61	7.25^{ab}	7.12^{abc}	1.75	2.58^{abcd}	2.59^{abcd}	-0.47
Varuna	$1.51^{klmnopqrstu}$	1.32^{stuvw}	12.12	$0.33^{ijklmnopq}$	$0.29^{klmnopqrs}$	13.24	0.15^{v}	4.13^{s}	-2583.50	$1.84^{opqrstuv}$	1.61^{tuvwx}	12.33
NRCHB-101	2.03^{abcde}	1.90^{cdefgh}	6.30	$0.46^{abcdefg}$	0.48^{abcde}	-3.88	0.13^{v}	6.43^{defgh}	-4740.66	2.48^{abcde}	2.37^{cdefgh}	4.42
DRMR1187-55	1.86^{defghi}	2.15^{abcd}	-15.51	$0.42^{abcdefghij}$	$0.38^{cdefghijklm}$	8.56	0.19^{v}	6.67^{bcde}	-3479.37	2.28^{efghij}	2.53^{abcde}	-11.12
DRMR-1616-47	2.20^{ab}	$1.89^{cdefghi}$	13.97	0.51^{ab}	$0.31^{jklmnopqr}$	39.29	0.12^{v}	6.06^{efghij}	-5044.02	2.71^{a}	2.20^{fghijk}	18.74
DRMR-1187-71	1.80^{efghij}	1.33^{stuvw}	26.56	0.49^{abc}	0.25^{nopqrs}	49.09	0.17^{v}	4.16^{s}	-2352.85	2.29^{efghij}	1.57^{vwx}	31.35
GM-2	1.21^{vwx}	2.21^{a}	-83.19	$0.39^{bcdefghijkl}$	$0.44^{abcdefghi}$	-12.82	0.17^{v}	7.36^{a}	-4328.77	1.60^{uvwx}	2.65^{ab}	-65.97
DRMR-64	1.39^{rstuvw}	$1.50^{lmnopqrstuv}$	-7.91	$0.34^{ghijklmnop}$	0.24^{pqrstu}	31.30	0.12^{v}	5.00^{pqr}	-4198.85	$1.73^{qrstuvw}$	$1.73^{qrstuvw}$	-0.15
NPJ-113 (Pusa Mustard 26)	$1.72^{hijklmno}$	$1.65^{hijklmnopqr}$	3.70	$0.40^{abcdefghijk}$	$0.33^{hijklmnopq}$	16.04	0.19^{v}	5.32^{mnopq}	-2761.40	$2.11^{hijklmn}$	$1.99^{klmnopqr}$	6.03

(b) For each parameter, means with the same letter are not significantly different

Genotype	Proline (μmole/g FW)			Total antioxidant capacity (mg/g AAE)			RSA (%)			Lipid peroxide (nmole/g FW)		
	Early	Normal	% increase (+)/decrease (-)	Early	Normal	% increase (+)/decrease (-)	Early	Normal	% increase (+)/decrease (-)	Early	Normal	% increase (+)/decrease (-)
Urvashi	$2.70^{mnopqrstuv}$	0.47^{yza}	82.48	29.60^{no}	21.35^{r}	27.87	47.22^{mno}	77.50^{b}	-64.12	3.01^{lmnop}	2.36^{stuv}	21.46
DRMR-541-44	$1.63^{tuvwxyza}$	$2.76^{mnopqrstu}$	-68.90	31.35^{klm}	26.10^{p}	16.75	41.33^{qp}	61.37^{fgh}	-48.47	2.90^{lmnop}	2.58^{qrs}	15.12
RH-119	$2.04^{qrstuvwx}$	$1.37^{tuvwxyza}$	32.94	31.52^{kl}	8.10^{s}	74.30	53.62^{ijk}	77.50^{b}	-44.53	4.36^{bc}	2.32^{stuv}	46.75
RH-406	0.45^{yza}	$1.70^{stuvwxyz}$	-278.21	90.60^{a}	9.60^{s}	89.40	25.40^{tuv}	42.86^{pq}	-68.75	2.32^{stuv}	2.13^{tuvw}	8.33
DRMR-HT-13-20	$1.70^{stuvwxyz}$	9.00^{ef}	-429.41	46.10^{d}	39.85^{f}	13.56	41.07^{pq}	69.05^{cd}	-68.12	1.94^{wx}	0.36^{c}	81.33
DRMR-HT-13-13	0.44^{yza}	$2.32^{nopqrstuvw}$	-421.74	30.35^{lmn}	29.10^{no}	4.12	12.79^{bc}	12.50^{bcd}	2.27	5.29^{a}	3.21^{jklm}	39.27
DRMR-HT-13-7	$2.35^{nopqrstuvw}$	7.17^{ghi}	-204.92	32.60^{ijk}	23.60^{q}	27.61	16.28^{ab}	15.28^{bc}	6.15	3.66^{efghi}	1.54^{yz}	58.10
DRMR-HT-13-28	0.68^{wxyza}	10.71^{d}	-1478.20	30.60^{lmn}	22.85^{qr}	25.33	11.63^{cd}	23.61^{vwxy}	-103.06	2.95^{mnopq}	2.13^{tuvw}	27.95

Cond.

Table 1 a and b. (*Concluded*)

Genotype	Proline (µmole/g FW)			Total antioxidant capacity (mg/g AAE)			RSA (%)			Lipid peroxide (nmole/g FW)		
	Early	Normal	% increase (+)/decrease (-)	Early	Normal	% increase (+)/decrease (-)	Early	Normal	% increase (+)/decrease (-)	Early	Normal	% increase (+)/decrease (-)
DRMR-HT-729	19.23^{b}	4.76^{jk}	75.23	52.60^{c}	43.35^{e}	17.59	24.42^{tuvw}	22.22^{wxyz}	8.99	3.02^{lmnop}	1.87^{wxy}	38.03
BPR-543-2	16.37^{c}	$3.23^{lmnopqr}$	80.26	33.85^{hij}	29.35^{no}	13.29	20.93^{uvwxy}	20.83^{yza}	0.46	4.45^{b}	3.12^{klmno}	29.86
BPR-349-9	$1.93^{rstuvwxy}$	5.89^{ij}	-205.51	34.10^{hi}	30.60^{lmn}	10.26	24.42^{uvwx}	27.78^{tu}	-13.76	3.10^{klmno}	1.21^{za}	60.83
BPR-549-9	$1.40^{tuvwxyza}$	$2.18^{pqrstuvwx}$	-55.50	36.10^{g}	31.85^{kl}	11.77	23.26^{uvwxy}	26.39^{tuvw}	-13.47	3.42^{ijk}	0.97^{ab}	71.70
BPR-540-6	3.90^{klm}	3.71^{klmno}	4.75	36.10^{g}	31.35^{klm}	13.16	58.00^{ghij}	51.07^{nlm}	11.96	2.80^{opqr}	0.98^{ab}	64.98
BPR-541-4	$3.20^{lmnopqrs}$	7.33^{ghi}	-129.38	40.10^{f}	32.35^{jk}	19.33	6.98^{d}	20.83^{xyza}	-198.61	3.35^{ijkl}	1.14^{a}	66.15
NPJ-124	4.10^{klm}	3.67^{klmnop}	10.33	35.35^{gh}	32.85^{ijk}	7.07	26.19^{ut}	65.12^{def}	-148.63	2.58^{rs}	3.51^{hij}	-36.20
RH-555	4.89^{jk}	20.92^{a}	-328.32	31.85^{kl}	28.35^{o}	10.99	53.62^{ijk}	61.37^{gf}	-14.44	3.66^{efghi}	2.04^{uvwx}	44.37
DRMR-1672-2	9.45^{de}	7.55^{fgh}	20.10	1.85^{vwxy}	1.18^{wxyz}	36.04	35.90^{r}	54.35^{jkl}	-51.40	4.92^{a}	2.39^{stu}	51.44
JN-032	3.81^{klmn}	$1.29^{tuvwxyza}$	66.02	3.35^{tuv}	1.10^{wxyz}	67.16	21.54^{wxyz}	42.86^{pq}	-98.98	4.23^{bcd}	1.99^{vxw}	53.05
DRMR-1617-45	15.23^{c}	15.91^{c}	-4.46	4.85^{t}	0.60^{yz}	87.63	31.67^{sr}	40.35^{q}	-27.42	2.98^{lmnop}	2.57^{rs}	13.85
DRMR-1165-40	6.43^{hi}	$2.77^{mnopqrst}$	56.93	34.10^{hi}	29.10^{no}	14.66	50.35^{klm}	26.79^{uvwx}	46.80	3.91^{defg}	2.95^{mnopq}	24.42
DRMR-1191-2	7.04^{hi}	$2.78^{mnopqrst}$	60.46	77.60^{b}	29.10^{no}	62.50	56.10^{hij}	42.59^{opq}	24.07	4.34^{bc}	2.50^{rst}	42.26
NRCDR-601	7.90^{fgh}	$3.54^{klmnopq}$	55.25	1.68^{wxy}	0.10^{z}	94.06	45.45^{nop}	83.34^{a}	-83.34	2.97^{mnop}	1.79^{wxy}	39.57
Varuna	$2.04^{qrstuvwx}$	$1.74^{rstuvwxy}$	14.83	2.18^{uvwx}	0.85^{xyz}	61.07	33.90^{rs}	33.33^{rs}	1.67	4.01^{cdef}	0.65^{bc}	83.92
NRCHB-101	$2.23^{opqrstuvwx}$	$2.25^{opqrstuvwx}$	-0.90	2.35^{uvwx}	1.52^{wxyz}	35.46	42.22^{opq}	42.59^{opq}	-0.88	3.94^{defg}	3.64^{fghi}	7.54
DRMR1187-55	0.17^{a}	8.60^{efg}	-5040.38	2.52^{uvw}	1.85^{vwxy}	26.49	52.38^{kj}	60.42^{ghi}	-15.34	2.70^{pqrs}	2.70^{pqrs}	-
DRMR-1616-47	$1.74^{rstuvwxy}$	0.76^{xyza}	56.11	4.52^{t}	2.52^{uvw}	44.28	16.28^{zab}	25.00^{uvwx}	-53.57	3.83^{efgh}	3.10^{klmno}	19.19
DRMR-1187-71	1.20^{vwxyza}	$1.50^{tuvwxyza}$	-24.66	8.52^{s}	1.10^{wxyz}	87.08	64.10^{def}	30.51^{st}	52.40	1.70^{xy}	1.81^{wxy}	-6.06
GM-2	0.47^{yza}	$1.65^{tuvwxyza}$	-250.34	29.85^{mno}	28.35^{o}	5.03	68.42^{cd}	67.39^{cde}	1.50	2.99^{lmnop}	2.83^{nopqr}	5.60
DRMR-64	$1.26^{uvwxyza}$	1.24^{vwxyza}	1.58	3.68^{tu}	1.60^{wxyz}	56.56	42.22^{opq}	63.83^{efg}	-51.18	3.68^{efghi}	3.20^{jklmn}	12.98
NPJ-113 (Pusa Mustard 26)	4.36^{kl}	0.20^{za}	95.35	28.60^{o}	32.60^{ijk}	-13.99	39.13^{q}	71.11^{c}	-81.73	4.03^{cde}	3.57^{ghij}	11.22

T comparison lines for least Squares means of genotypes sowing. The alphabets represent an interaction studies between early sown and Normal sown.

(0.5% TBA in 20%TCA) reagent. For control, 1 ml of 1% TCA plant extract was incubated in hot water (95°C) for 30 min. Thereafter, it was cooled immediately on ice to stop the reaction and centrifuged at 10000 rpm for 10 min at 4°C. Absorbance was measured at 535 and 600 nm using a spectrophotometer (Labomed UV-VIS Double beam UVD-3500), and MDA concentration was estimated by subtracting the non-specific absorption at 600 nm from the specific absorption at 535 nm. The absorbance coefficient of extinction is 155 mM^{-1} cm^{-1}.

MDA (nmol/gFW) = (A535 – A600) V/€ d FW

where, A-Absorbance, ε - malondialdehyde (MDA) molar extinction coefficient at 532nm [155mM^{-1} cm^{-1}], d- optical distance (width of cuvette) (1cm), V-volume of sample (L), FW-fresh weight equivalent in the sample (g).

Stress susceptibility index

The stress susceptibility index (SSI) was determined, by using the mean of different traits under normal (Non-stress sown) and early sown (Stress). Fischer and Maurer (1978) method of SSI was employed for calculation. Differences in the results obtained for stress (early sown) and non-stress (normal) conditions were employed to calculate SSI by using the following equations:

$$SSI = \frac{\left[1 - \frac{Y_p}{Y_s}\right]}{SI}$$

$$\text{Stress Intensity (SI)} = 1 \quad \frac{MY_s}{MY_p}$$

In the above equations, Y_P is the mean value for the investigated trait under non-stress conditions, Y_s is mean trait value under stress conditions, MY_P is mean trait value of all 30 genotypes under non-stress conditions and MY_s is mean trait value under stress conditions.

Statistical analysis

The data obtained for different treatments with respect to various parameters under consideration were subjected to Analysis of Variance (ANOVA) using SAS 9.4 software package available at ICAR-IASRI, New Delhi, India. Pair-wise comparisons of the least square means (LSMEANS) were performed using the Tukey's honest significant difference (HSD) test. Further, SSI was also calculated for each genotype employing the formula given by Fischer and Maurer (1978) to identify the genotypes with high temperature tolerance.

RESULTS AND DISCUSSION

Chlorophyll content

Study in Indian mustard explains the importance of sink to source translocation for positive improvement of harvest index and consequently seed yield (Kumar and Srivastava 2003). High temperature causes poor translocation of photosynthates by both upper and lower pods of Indian mustard (Subrahmanyam and Rathore 1994) which was also observed under stress. This has been documented by reduction of photosynthetic pigments (chlorophyll *a*, *b*, total chlorophyll and carotenoid) in the leaves of various crops (Yordanov *et al*. 2000, Montagu and Woo 1999, Nilsen and Qrcutt 1996, Kumar *et al*. 2013). This indicates the importance of leaf pigments like chlorophyll as they have role in fixation of CO_2 and harvesting of energy required for photosynthesis leading to higher yield and harvest index. Kumar *et al*. (2013) reported the importance of chlorophyll and carotenoid content as heat tolerant indices that help to identify heat tolerant genotypes. It is well known that photosynthetic efficiency is dependent on pigments like chlorophyll which absorbs sunlight and uses its energy to synthesize carbohydrates from carbon dioxide and water. The change in chlorophyll content depends upon stress intensities. In this study the total chlorophyll content under NS condition ranged from 1.11 mg/g FW (RH-555) to 2.65 mg/g FW (GM-2). In ES condition the total chlorophyll content ranged from 0.88 mg/g FW (Urvashi) to 2.71 mg/g FW (DRMR-1616-47) (Table 1a). There is one report in Indian mustard (Kumar *et al*. 2013) under three different dates of sowing 15th October, 1st November and 15th November in which the total chlorophyll content ranged from 1.45 mg/g FW (EJ-15) to 2.1 mg/g FW (CS-52); 1.36 mg/g FW (EJ-15) to 1.91 mg/g FW (Proagro) and 0.89 mg/g FW (EJ-15) to 1.67 mg/g FW (CS-52) respectively which is in agreement with the present report.

Photosynthetic function has been recognized as indicator of heat stress. Photosynthetic dysfunction happens as a result of the loss of pigments like chlorophyll that causes disruption of electron flow, thermos-liability of photosystem II, carbon fixation and assimilation reduction (Sinsawat *et al*. 2004). There are many reports that suggest stress leads to reduction of chlorophyll content (Yordanov *et al*. 2000, Montagu and Woo 1999, Nilsen and Qrcutt 1996, Kumar *et al*. 2013). In this study about 57% of the genotypes showed reduction in total chlorophyll by 0.1% (DRMR-64, DRMR-1617-45) to 128.3% (DRMR-13-7) compared to NS, while, 43% of the genotypes showed increase in total chlorophyll content under early sown by 1.8% (DRMR-1672-2) to 44.3% (RH-555) compared to NS (Table 1a). These genotypes that can maintain chlorophyll content under ES could adapt to heat stress.

The ability of plants to absorb light is governed by chlorophyll which is composed of two pigments chlorophyll *a* and chlorophyll *b*. Chlorophyll *a* act as the pigment which is required for the light reactions of photosynthesis. Chlorophyll *b* act as the accessory pigment that function indirectly in photosynthesis by transferring the energy to chlorophyll *a* (Soengas *et al*. 2018).

In advanced breeding lines we observed Chlorophyll *a* content ranged from 0.89 mg/g FW (RH-555) to 2.21 mg/g FW (GM-2) under NS condition. In ES condition the chlorophyll *a* content ranged from 0.70 mg/g FW (Urvashi) to 2.23 mg/g FW (DRMR-1165-40) (Table 1a). Chlorophyll *b* content under NS condition ranged from

0.22 mg/g FW (RH-555) to 0.48 mg/g FW (BPR-540-6, NRCHB-101). For ES condition the chlorophyll *b* content ranged from 0.02 mg/g FW (DRMR-541-44) to 0.51 mg/g FW (DRMR-HT-13-20, NPJ-124, DRMR-1616-47) (Table 1a). A study in Indian mustard variety Varuna and RH-30 showed chlorophyll *a* content of 1.11 to 1.27 mg/g FW and chlorophyll *b* content of 0.53 to 59 mg/g FW (Mobin and Khan 2007) which is also similar to the present study.

Under ES the chlorophyll *a* content decreased by 0.7% (DRMR-1617-45) to 106.2% (Urvashi) over 53% genotype in NS. While in rest 47% it increased by 3.7% (NPJ-113 (PM-26)) to 42.4% (RH-555) (Table 1a). For, chlorophyll *b,* we observed similar trend where there was a reduction under ES by 7.57% (BPR-54-06) to 1456% (DRMR-541-44) over NS (Table 1a). In previous reports on heavy metal cadmium (Cd) stress in Indian mustard (Mobin and Khan 2006) and heat stress in *B. oleracea* (Soengas *et al*. 2018) it was observed that chlorophyll *b* showed more reduction in comparison to chlorophyll *a*, which according to Cui *et al*. (2006) may be due to faster degradation of chlorophyll *b*. The variation in these pigment content with reduction of chlorophyll *b* showed higher chlorophyll *a*:*b* ratio is linked to lowering of light harvesting chlorophyll proteins (LHCPs) (Loggini *et al*. 1999). It was proposed that the reduction in LHCPs play adaptive role or defense mechanism of the plant against adverse stress conditions (Asada *et al*. 1998).

Apprehending the factors regulating the chlorophyll metabolism during heat stress could give more insight into the development of tolerant genotypes with stay-green traits either through marker assisted selection or transgenic approach (Jespersen *et al*. 2016).

Carotenoids

Carotenoids apart from functioning as accessory pigments in photosynthesis they also play a role in preventing the oxidative stress by acting as antioxidants. They safeguard the photosystem by scavenging the ROS produced during the photo oxidative stress by quenching both the triplet chlorophyll ($^3Chl^*$) and singlet oxygen ($^1O_2$) (Edge *et al.* 1997; Triantaphylides and Havaux 2009). The presence of singlet oxygen, the main ROS, is detrimental to the plants (Triantaphylides and Havaux 2009), which make carotenoid estimation, an important parameter to screen heat tolerant genotypes.

The carotenoid content under NS condition ranged from 3.08 mg/g FW (RH-555) to 7.36 mg/g FW(GM-2) and for ES condition the carotenoid content ranged from 0.12 mg/g FW (DRMR-1616-47& DRMR-64) to 7.58 mg/g FW (DRMR-1165-40) (Table 1a). A report in Indian mustard (Kumar *et al*. 2013) under three different date of sowing 15 October, 1 November and 15 November, the total carotenoid content ranged from 0.38 mg/g FW (EJ-15) to 0.49 mg/g FW (CS-52, NDR 8801), 0.33 mg/g FW (EJ-15) to 0.48 mg/g FW (CS-52) and 0.26 mg/g FW (Pusa Agrani) to 0.42 mg/g FW (EJ-15) which is in tune with this experiment. During ES, the total carotenoid content decreased by 5.8% (BPR-543-2) to 5044.0 % (DRMR-161647) in about 60% of genotypes compared to NS, while in rest of the genotypes there was an increase of total carotenoid content by 0.7% (BPR-5406) to 50.8% (RH-555) (Table 1a). The genotypes that are less affected in terms of reduction in chlorophyll and carotenoids content can be grouped as heat tolerant.

Proline

Plants being static under environmental stress have to adapt themselves in order to survive. One of the mechanisms at cellular levels that defend them is the accumulation of electroneutral molecules also known as osmolytes like proline. Proline is widely studied and is known to have diverse roles like shielding proteins against elevated concentration of inorganic ions and extreme temperature (Singh *et al.* 2017), stabilizing membranes and sub-cellular structures and also protecting cells from reactive oxygen species (Singh *et al*. 2017). Accumulation of proline under stress acts as a defense mechanism to maintain cellular redox state during stress (Singh *et al*. 2017). Hence, the estimation of proline helps in the selection of heat tolerant genotypes. In this experiment it was observed that proline content under NS condition ranged from 0.20 µmole/g FW (NPJ-113 (Pusa Mustard 26)) to 20.9 µmole/g FW (RH-555). For ES condition it ranged from 0.17 µmole/g FW (DRMR1187-55) to 19.23 µmole/g FW (DRMR-HT-729) (Table 1b).

Under ES condition about 47% of the genotypes showed reduction in proline content by 0.9% (NRCHB-101) to 5040.4% (DRMR-1187-55) while, 53% showed increase in proline content by 1.6% (DRMR-64) to 95.4% (NPJ-113 (PM-26)) (Table 1b). The increase in proline content indicates that those genotypes are capable of adapting against heat stress. The genotypic variations in proline content under ES have been reported in sunflower as well (Amutha *et al*. 2007). However, in their report it was concluded that the increase in proline is not to be associated with stress tolerance. Proline content could be a mere indicator of plant water status (Amutha *et al*. 2007). Another report on moth bean where inspite of increase in proline under stress it was not qualified as heat tolerant (Harsh *et al*. 2016). Similarly, in this experiment genotypes like Urvashi, BPR-543-2 that had a high increase in proline content (>80%) at ES condition was not qualified as highly heat tolerant.

Lipid peroxidation

Under stress, membrane damage is often caused by MDA the product of lipid peroxidation of unsaturated fatty acid in membrane phospholipids (Da Costa and Huang 2007). The intensity of membrane damage depends upon the rise in concentration of MDA content. Under NS the MDA content ranged from 0.36 nmole/g FW (DRMR-HT-13-20) to 3.64 nmole/g FW (NRCHB-101). Under ES it ranged from 1.70 nmole/g FW (DRMR-1187-71) to 5.29 nmole/g FW (DRMR-HT-13-20) (Table 1b). In one report under controlled condition, seedling stage of Indian mustard the MDA content was reported as 4.66 MDA g/ FW in tolerant and 7.44 MDA g/FW in susceptible variety

(Wilson *et al*. 2013). In this study, almost all the genotypes showed increase in MDA content by 5.6% (GM-2) to 81.3% (DRMR-HT-13-20) over that of NS (Table 1b). This indicates the membrane damage during stress. However, there are only two genotypes that showed less effect on lipid membrane damage where there was 6.1% (DRMR-118-7-7) to 36.2% (NPJ-124) reduction in MDA content compared to NS condition and in one genotype (DRMR-1187-71) it did not change at all under ES. This could be due to the ability of these genotypes to have protective antioxidant system to scavenge the ROS preventing lipid peroxidation.

Total antioxidant capacity and radical scavenging activity

Plants are able to naturally adapt to change in climatic conditions because of the presence of antioxidant molecules that enable them to regulate cellular metabolism under temperature stress. The damage on leaves intensify upon stress if the defense mechanism like antioxidants are reduced. Thus measuring the antioxidant capacity can help in identifying plants that can withstand stress. In this experiment the Total antioxidant capacity (TAC) under NS condition ranged from 0.10 mg/g AAE (NRCDR-601) to 43.35 mg/g AAE (DRMR-HT-729). In ES condition the TAC ranged from 1.6 mg/g AAE (NRCDR-601) to 90.60 mg/g AAE (RH-406) (Table 1b). Radical scavenging activity (RSA) under NS condition ranged from 12.50 % (DRMR-HT-13-13) to 83.34% (NRCDR-601) and under ES condition it was found to range from 6.98% (BPR-541-4) to 68.42% (GM2).

Surprisingly, under ES all the genotypes showed increase in TAC from 4% (DRMR-HT-13-13) to 94.1% (NRCDR-601) except for one genotype NPJ-113 (PM-26) which can be expected as the increase in MDA content almost all the genotypes in was accompanied by parallel increase in TAC. This is so to allow plants to combat the stress, but the capacity to scavenge radicals depends upon the genotype's ability to withstand stress as observed with the variation in TAC among genotypes during ES and NS (Table 1b). In case of RSA only 33% of the genotypes showed increase in its capacity to scavenge the radicals by 0.5% (BPR-543-2) to 52.4% (DRMR-1187-71) (Table 1b). The increase in antioxidant properties was also observed by Rani *et al*. (2016) in five day old seedlings at high temperature and which was significantly higher in tolerant lines, as observed in this tune with our experiment where TAC increased in almost all genotypes under stress (ES). A comparative study of antioxidant properties among various vegetables has also concluded the difficulty to compare antioxidant properties verses assay methods (Rameh *et al*. 2011). Therefore, it cannot be an index and can only rank the genotypes based on the antioxidant properties.

Correlation analysis

To understand the relationship between the parameters under ES and NS a correlation analysis was performed. It was observed that during early sown, TAC had significant correlation with carotenoids (Table 2), while RSA did not show any significant correlation. In case of RSA, we observed that only 33% of the genotypes showed increase

Table 2 Correlation analysis of biochemical traits under early sown (ES) and normal sown (NS)

Parameter	Environments	Chl *a*	Chl *b*	Total chlorophyll	Total carotenoid	TAC	RSA	Lipid peroxide	Proline
Chl *a*	ES	1							
	NS								
Chl *b*	ES	0.68**	1						
	NS	0.69**							
Total chlorophyll	ES	0.98**	0.80**	1					
	NS	0.99**	0.80**						
Total carotenoid	ES	0.19	-0.08	0.13	1				
	NS	0.98**	0.72**	0.97**					
TAC	ES	-0.33*	-0.31*	-0.34*	0.34*				
	NS	-0.07	0.17	-0.02	-0.04		1		
RSA	ES	0.19	0.33*	0.23	-0.11	-0.08			
	NS	-0.12	-0.26	-0.16	-0.1	-0.16			
Lipid peroxide	ES	0.16	-0.07	0.11	0.14	-0.17	-0.24	1	
	NS	0.2	0.09	0.19	0.25	-0.16	0.14		
Proline	ES	0.32*	0.01	0.26	0.49**	0.04	-0.11	0.17	1
	NS	-0.21	-0.22	-0.22	-0.22	0.09	-0.04	-0.21	

** Sig nificant at 1% level, *significant at 5% level

Table 3 Classification of genotypes based on SSI in 30 advanced breeding lines of Indian mustard. HT-Highly tolerant, T-Tolerant, MT-Moderately tolerant, S- Susceptible, HS- Highly susceptible

Advanced breeding lines	Stress susceptibility Index (SSI)								Number of Parameters showing less effect on Stress	Total parameters analysed	Classification
	Chl *a*	Chl *b*	Total chlorophyll	Total carotenoid	TAC	RSA	Lipid peroxide	Proline			
Urvashi	8.01	4.75	-7.37	1.85	0.86	1.95	0.51	-75.53	4	8	MT
DRMR-541-44	4.94	13.82	6.58	0.62	0.45	1.63	0.33	6.55	3	8	S
RH-119	-1.54	-0.77	-0.96	-0.77	6.46	1.54	1.64	-7.88	5	8	MT
RH-406	7.17	9.84	7.61	1.58	18.84	2.03	0.17	11.80	1	8	HS
DRMR-HT-13-20	-2.83	-15.22	-4.65	-1.10	0.35	2.02	8.12	13.01	5	8	MT
DRMR-HT-13-13	3.98	3.84	3.91	0.69	0.10	-0.12	1.21	12.97	3	8	S
DRMR-HT-13-7	8.86	7.84	8.56	1.72	0.85	-0.33	2.58	10.78	2	8	S
DRMR-HT-13-28	6.91	3.74	6.30	1.62	0.76	2.53	0.72	15.03	2	8	S
DRMR-HT-729	-0.68	3.88	0.18	-0.16	0.48	-0.49	1.14	-48.72	6	8	T
BPR-543-2	1.85	3.31	2.12	0.19	0.34	-0.02	0.79	-65.22	5	8	MT
BPR-349-9	4.00	8.56	4.82	0.90	0.26	0.60	2.89	10.79	3	8	S
BPR-549-9	2.77	10.43	4.36	0.58	0.30	0.59	4.72	-5.73	4	8	MT
BPR-540-6	3.60	1.04	3.02	-0.02	0.34	-0.68	3.46	-0.80	4	8	MT
BPR-541-4	-1.17	2.34	-1.38	0.39	0.53	3.31	3.64	9.05	4	8	MT
NPJ-124	-3.90	-4.75	-4.04	-1.23	0.17	2.98	-0.50	-1.85	7	8	HT
RH-555	-11.46	-15.16	-12.09	-3.68	0.28	0.63	1.49	12.30	6	8	T
DRMR-1672-2	-1.80	5.53	-0.27	-0.52	1.26	1.69	1.97	-4.04	4	8	MT
JN-032	-0.80	1.74	-0.34	-0.33	4.57	2.48	2.11	-31.17	4	8	MT
DRMR-1617-45	0.11	-0.56	0.01	-0.17	15.82	1.07	0.30	0.69	6	8	T
DRMR-1165-40	-2.98	-3.99	-3.09	-1.05	0.38	-4.38	0.60	-21.21	8	8	HT
DRMR-1191-2	-10.30	-2.83	-8.72	-2.53	3.72	-1.58	1.36	-24.54	6	8	T
NRCDR-601	0.22	-0.71	0.07	-0.06	35.35	2.26	1.22	-19.81	5	8	MT
Varuna	-2.14	-2.25	-2.14	3.43	3.50	-0.08	9.73	-2.79	5	8	MT
NRCHB-101	-1.05	0.55	-0.70	3.49	1.23	0.04	0.15	0.14	5	8	MT
DRMR1187-55	2.09	-1.38	1.52	3.47	0.80	0.66	0.00	15.73	4	8	MT
DRMR-1616-47	-2.52	-9.56	-3.51	3.50	1.77	1.74	0.44	-20.51	5	8	MT
DRMR-1187-71	-5.62	-14.24	-6.95	3.42	15.05	-5.49	-0.11	3.17	5	8	MT
GM-2	7.06	1.68	6.05	3.49	0.12	-0.08	0.11	11.46	3	8	S
DRMR-64	1.14	-6.73	0.02	3.48	2.91	1.69	0.28	-0.26	3	8	S
NPJ-113 (Pusa Mustard 26)	-0.60	-2.82	-0.98	3.44	-0.27	2.24	0.24	-329.04	6	8	T

in RSA under ES over that of NS. The strong positive correlation of total carotenoids with TAC at ES (Table 2) indicates that the carotenoids act as antioxidants to scavenge the radicals produced under stress. Studies in chickpea on exogenous application of proline showed less injury to membranes and improved water and pigment associated photosynthesis (Kaushal *et al*. 2011) which we also observed with the significant correlation of carotenoids with proline. According to Hasanuzzaman *et al*. (2013) the strong association with antioxidant capacity implies the genotypes tolerance to heat stress. Hence, carotenoids could be an important indicator for heat stress tolerance.

Under NS the TAC had no significant correlation with any of the parameters (Table 2), while under ES condition it was observed that the TAC was negatively correlated to chlorophyll *a*, chlorophyll *b*, total Chlorophyll and in turn the carotenoid had significant positive correlation to TAC (Table 2) indicating its role as antioxidants to protect the photosystem from dysfunction by quenching triplet chlorophyll ($^3Chl^*$) and singlet oxygen ($^1O_2$) as reported by Mitchel Havaux (2013). The significant correlation only during stress condition points us to the importance of these parameters for screening of heat tolerant lines.

Selection of genotypes

Heat tolerant lines were identified based on the percentage of increase or decrease in each parameter under ES over that of NS and based on individual SSI of each parameter analysed. Using SSI, comparative analysis between tolerance and susceptibility of genotype(s) to heat stress was screened. When, SSI is less the tolerance of genotypes will be higher. SSI less than one considering all the parameters analysed was identified as tolerant. The cumulative SSI of individual parameter helped in classifying the genotypes into highly tolerant, tolerant, moderately tolerant, susceptible and highly susceptible as shown in table below (Table 3). The negative SSI indicates tolerance and intensive synthesis or accumulation of compounds that help in combating oxidative stress as also reported in previous work in tomatoes (Zdravkovic *et al*. 2013).

Conclusion

The study showed variation in most of the biochemical traits which can be exploited in various breeding programmes. The genotypes classified in this study as tolerant should be further evaluated at different developmental stages in field conditions. The best among the lines identified in this study are NPJ-124 and DRMR-1165-40 which could serve as potential source in breeding programmes for high temperature tolerance.

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