Agric Res (March 2021) 10(1):15–20
https://doi.org/10.1007/s40003-020-00486-z

FULL-LENGTH RESEARCH ARTICLE

Comparison of Methods of Pollen Selection for Heat Tolerance and Their Effect in Segregating Population of Maize (*Zea mays*)

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Received: 18 March 2019 / Accepted: 31 August 2020 / Published online: 2 November 2020

Abstract The reproductive stage in many crops including Maize (*Zea mays* L.) is most sensitive to heat stress. Due to limitations in sporophytic selection, an alternative pollen selection approach opens a way for breeders to develop heat stress resilient lines. In present study, the efficiency of different methods of pollen selection for heat stress tolerance was evaluated. The effect of selection in F_1 generation in terms of the frequency of heat-tolerant plants in F_2 generation was assessed. The heat susceptible line BTM4 was crossed to heat-tolerant BTM6, and the true F_1 plants were identified using two SSR markers. Three different methods of pollen selection for heat tolerance, *viz.* incubating the freshly dehisced pollen grains of F_1 plants at 36 °C for 3 h in growth chamber (GRC), dry bath (DB) before selfing and growing F_1 plants in summer at ARS, Bheemarayanagudi (BGD). Thus, selected F_2 from different treatments was produced. The control F_2 was also produced without heat treatment of pollen grains. The control and selected F_2 population were compared for heat tolerance by selfing the F_2 plants with heat-stressed pollen grains. The selected F_2 (GRC) showed superiority over other methods for seed yield traits and its component traits and also positive effect of pollen selection for heat tolerance.

Keywords Generation · Susceptible · Treatment · Tolerant · Selection · Pollen grains

Introduction

Climate change impacts cereal production mainly through heat and water stress [22]. The productivity of wheat, maize and rice is predicted to decrease in both tropical and temperate regions [2]. The model shows that 1 °C increase in global mean temperature declines the grain yields by 7.4% in maize which is highest reduction among cereals [33].

Among the developmental stages, the reproductive stage is the most sensitive to heat stress which leads to delay in the flowering, increase in days to anthesis and silking interval (ASI) [16] and increase pollen sterility in maize [30]. Though a wide range of screenable traits are reported which allow successful selection for heat tolerance in different crops in distinct field conditions [10, 23], the methods are not very effective for screening as heat stress is a complex quantitative trait. The lack of comprehensive screening method for heat tolerance at sporophytic level is the major challenge in identification and development of heat stress-tolerant lines.

The pollen has been frequently used as an indicator of performance of a plant genotype for resistance to biotic and abiotic stress tolerance [4]. A simple but effective technique of screening the genotypes based on the performance of their pollen grains is emerging as a viable alternative to sporophytic screening [25–27]. The basis of pollen selection strategy is that, from the heterogeneous pollen mixture, it is possible to select stress-tolerant pollen grains by increasing the fitness of the pollens by stressing them and improve the progeny performance under stress condition.

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There are only few studies on pollen selection strategy that indicate the tolerance and persistence for a particular stress over the generations [24, 31].

Despite the success of pollen selection strategy in many crops, one of the most challenging problems is the methods used to screen pollen grains under stress condition for effective pollen selection. In present study, three different methods of pollen exposure heat stress were compared and identified one of the effective methods. The effect of pollen selections was also studied in segregating F_2 population.

Materials and Methods

Treatments, Pollen Selection and Yield Parameters

The heat susceptible inbred line BTM4 was crossed to tolerant line BTM6 to produce F_1 seeds [27, 28].

Total 60 F_1 plants were grown in the field, and hybridity of each plant was tested using two polymorphic markers (umc1144 and phi077). At flowering stage, the pollen grains from each F_1 plant were collected and immediately brought to laboratory separately and following heat stress treatments were given along with control before selfing for pollen selection.

a. *Using growth chamber (GRC)*: The pollen grains were uniformly spread in a petriplates, and the petriplates were kept in a growth chamber maintained at 36 °C temperature for 3 h and RH 70%. After the treatment, pollen grains were used to self the same F_1 plant to produce selected F_2 (GRC) seeds.
b. *Using dry bath (DB)*: The pollen grains were transferred to 0.5 ml PCR tubes in the laboratory and the tubes were incubated in dry bath at 36 °C for 3 h to produce selected F_2 plants (DB).
c. *In vivo at Bheemarayanagudi (BGD)*: The hybrids plants were grown at ARS (agriculture research station) Bheemarayanagudi during late summer season and selfed to produce selected F_2 (BGD) seeds. The average day and night temperature was 43.5 °C/ 30.5 °C during the reproductive growth and flowering.
d. *Control (RT)*: The pollen grains were incubated at room temperature (22 °C) for 3 h (as long as the heat treatment was going on for other treatments) before selfing to produce control F_2 (RT).
e. *Control*: The F_1 plants were self-pollinated immediately after collection of pollen grains in the field to produce control F_2.

For all the treatments except pollen selection (in vivo), equal quantity of pollen grains by volume was used for selfing and one cob per plant was maintained. The selfed cobs were harvested separately.

The following important yield and yield-related parameters were recorded.

Cob length (cm): The length of the matured, main cob was measured in centimeters from the base to the tip.

Cob diameter (cm): Cob girth at the middle of the main ear was measured and recorded in centimeters.

Number of seeds per cob: The number of seeds obtained from the main cob was counted and recorded as number of seeds per cob.

Seed yield per plant (g): Seed weight per plant expressed in grams was recorded by weighing the seeds obtained after shelling of the total cob from individual plant.

Test weight (g): One hundred well-filled seeds were counted randomly, and the weight was recorded in grams as test weight. If the seed number was less than 100 in the cob, then the weight of all the seeds was taken and converted to 100 seed weight.

Evaluation of F_2 Populations for Heat Tolerance

Five F_2 populations [control F_2, control F_2 (RT), selected F_2 (GRC), selected F_2 (DB) and selected F_2 (BGD)] of cross BTM4 × BTM6 were grown during kharif season of 2016 at GKVK, Bengaluru, and all the agronomic practices followed to raise a good crop. Fifty plants from each F_2 were selected; the pollen grains were collected and selfed immediately. Another set of 50 plants were selected from each F_2 population, and the pollen grains of these plants were collected in the morning hours and brought in laboratory immediately. The pollen grains in the heating blocks of PCR machine were exposed to 36 °C for 3 h. The logic behind to use of heating block of PCR machine was to maintain uniform temperature for exposure of pollen grains. For all plants, the equal quantity of pollen grains was used for selfing. The yield and yield-related (*viz*. cob length, cob diameter, number of seeds per cob and seed yield per plants) traits were recorded as given earlier.

Statistical Analysis

The mean values of yield and yield-related parameters of five F_2 populations were compared by one-way ANOVA with post hoc LSD (least significance difference) test. The pairwise comparison of selected F_2 populations was done with control F_2 (RT) population using two sample KS (Kolmogorov–Smirnov) test [1].

Results and Discussion

Heat stress is a complex trait, and few studies proposed different genes and QTLs for heat tolerance in maize [5, 6, 17, 29, 32]. Gametophytic selection for heat stress tolerance in maize increased the ability of progeny to synthesize heat shock proteins [8, 9]. Because of the overlap of transcriptomes, selecting for gametophytes with greater or more efficient HSP response to heat stress can positively affect the heat stress response of the resulting sporophyte. So, using male gametophtyic selection in the improvement of thermotolerance can be an efficient way to augment plant breeding programs [7]. Only few studies are available on pollen selection strategy, and it can be used as an effective tool for heat tolerance and crop improvement.

In the present study, the seed yield per plant and number of seeds per cob has significantly reduced in F_1 plants when heat-stressed pollen grains were used for self pollination compared with control plants where pollen grains were not treated with heat stress. The highest number of seeds per cob (385.20) and seed yield per plant (127.75 g) was observed in control treatment followed by control (RT), and the lowest number of seeds per cob and seed yield per plant was observed in plants selfed using heat-stressed pollen grains (Table 1). Two controls (control and control RT) did not significantly differ to each other for seed yield per plant. The results indicated that the incubation of pollen grains at room temperature for 3 h does not affect the viability, vigor and fertility of pollen grains. Our results corroborate the finding that maize pollen grains remain viable under room temperature up to 48 h [11]. It is also indicates an adverse effect of high-temperature stress on pollen development showing a positive relationship with seed yield per plant.

Further to evaluate the effect of different methods of pollen selection in F_1 generation on the performance of F_2 population for seed yield and its related parameters, all the five F_2 populations were evaluated under heat stress (pollen stressed) and control (no stress) condition at GKVK, Bengaluru. The analysis of variance revealed that, under no stress pollinations, all the five F_2 population significantly differed to each other for the cob length and seed yield per plant. Similarly, all the five F_2 population under stress condition cob length, cob diameter, number of seeds per cob and seed yield per plant showed significant difference. It was also observed that the mean values for recorded traits of all the five F_2 populations selfed under heat stress were significantly lower than the mean values of respective F_2 populations under no stress conditions (Table 2).

The mean values control F_2 and control F_2 (RT) for cob length, cob diameter, number of seeds per cob and seed yield per plant were not significantly different, whereas the incubating pollen grains at room temperature for 3 h did not alter the fitness of the pollen grains. So, all the population were compared with the control F_2 (RT). The selected F_2 (GRC) recorded the higher mean values for cob length (11.20 cm), cob diameter (1.82 cm), number of seeds per cobs (46.92) and seed yield per plant (15.98 g) for in comparison with all other F_2 populations.

The differences between the mean values of control F_2 (RT), the selected F_2 (DB), and selected F_2 (BGD) were not significant. However, the mean values of selected F_2 (GRC) showed significant difference with the mean value of control F_2 (RT) for cob diameter, number of seeds per cob and seed yield per plant, suggesting that the pollen selection for heat tolerance using growth chamber is the best method for pollen selection for heat tolerance (Table 3).

Noticeably, the F_1 plants grown under high-temperature condition at ARS Bheemarayanagudi where temperature recorded was 43.5 °C/30.5 °C (day/night) showed lack of response to a gametophytic selection. This is because the seed yield is a complex trait affected by various environmental factors acting throughout the whole sporophytic phase. The duration of sporophytic stage is much longer than that of the gametophytic phase [9]. Therefore, under field conditions at high temperature, significant yield reduction was observed. It is therefore reasonable to presume that a conspicuous number of environmental effects influencing the yield performance do not involve the short gametophytic phase [21]. The F_1 plants although grown

Table 1 Effect of different pollen selection treatments on seed set of F_1 plants in maize

Treatment	Number of plant	Cob length (cm)	Cob diameter (cm)	Number of seeds per cob	Seed yield per plant (g)	Test weight (g)
Control	10	14.70 ± 0.37	3.64 ± 0.13	385.20 ± 20.49	127.75 ± 6.38	28.42 ± 1.04
Control (RT)	10	14.50 ± 0.45	3.78 ± 0.20	356.0 ± 3.20	126.70 ± 5.57	27.88 ± 1.02
Heat-stressed pollen grain (GRC)	12	12.04 ± 0.25	2.50 ± 0.16	62.70 ± 11.67	11.50 ± 3.98	33.25 ± 1.23
Heat-stressed pollen grain (DB)	12	11.5 ± 0.30	2.45 ± 0.15	40.2 ± 7.76	10.80 ± 3	32.12 ± 1.20

RT = Selfed by using incubated pollen grains at room temperature for 3 h

GRC = Selfed by using pollen grains which were kept in growth chamber at temperature 36 °C for 3 h

DB = Selfed by using pollen grains were incubated in dry bath at 36 °C for 3 h

Table 2 Mean sum of square of six quantitative characters from five F_2 populations evaluated under stress (heat-stressed pollen grains) and no stress (no stressed pollen grains) conditions

Traits	MSS	
	No stress pollination	Heat stress pollination
Cob length	26.49*	39.47*
Cob diameter	3.30	2.22**
Number of seeds per cob	22,812.43	7411.07**
Seeds yield per plant	5333.28**	739.95*

*Significant at $P < 0.05$, **significant at $P < 0.01$

No stress pollination: F_2 plants selfed without pollen selection

Heat stress pollination: F_2 plants selfed with heat-stressed pollen grain

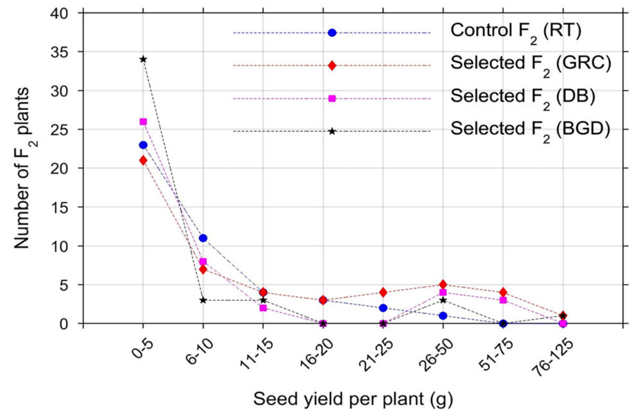

Fig. 1 Frequency distribution of four F_2 population for seed yield per plant selfed after heat stress pollen grains

under high temperature and under well-irrigated conditions at ARS Bheemarayanagudi, it is possible that the reproductive stage and the sensitive tissues were not exposed sufficiently to high-temperature stress. Therefore, the resultant F_2 progeny (BGD) has not produced higher frequency of tolerant plants as compared with other methods. Similarly, response was reported in many crops [13]. Like, pollen selection for cold tolerance in tomato did not show positive response [3]. This was probably because the heat sensitive tissue of pollen did not exposed for heat stress [7]. So, better method of pollen selection required.

The pollen selection using dry bath (DB) was found not to be very successful. In this technique, the pollen grains were filled in Eppendorf tubes and tubes were exposed to high temperature in dry bath, maintaining the temperature of tube not the pollen grains inside the tube. Further, the temperature inside the tubes may not be uniform and all the pollen grains in the tubes were not exposed uniformly to high-temperature stress for the selection of tolerant pollens. Hence, it is not a reliable method to impose heat stress on pollen grains for selection.

On the other hand, in growth chamber (GRC) the pollen grains were spread on petriplates uniformly and exposed to high temperature. Therefore, all the pollen grains were uniformly exposed to high temperature for selection to happen. The increased response obtained for improvement in seed yield under stress could be the result of intense gametophytic selection in growth chamber. The similar results were found in different crops by following different selection procedures. They have also observed different pollen selection procedure producing different effects on the progeny [18].

The distribution pattern of the five populations revealed that the populations differ significantly for many of the traits under heat stress conditions. The selected F_2 (GRC) population differed significantly from control F_2 (RT) for traits, *viz.* cob diameter, number of seeds per cob and seed yield per plant. The other populations did not differ significantly from control F_2 (RT) for majority of traits (Fig. 1). The selected F_2 (GRC) recorded more frequency of plants with higher values for seed yield per plant than other methods of pollen selection (Table 5).

The results clearly showed that the effect of pollen selection for heat tolerance using growth chamber (GRC) increases the tolerance of resultant sporophyte. The pollen selection effect was also observed [15, 19] for pollen tube growth rate through kernel weight relationship in maize. These results support the contention that gamete selection

Table 3 Mean performances of control and selected F_2 populations for important seed yield parameters under heat stress environment (selfed with heat-stressed pollen grains) at GKVK, Bengaluru

Traits	Control F_2 (T_1)	Control (RT) (T_2)	Selected F_2 (GRC) (T_3)	Selected F_2 (DB) (T_4)	Selected F_2 (BGD) (T_5)	T_2–T_1	T_3–T_2	T_4–T_2	T_5–T_2
Cob length (cm)	9.36 ± 0.55	10.83 ± 0.53	11.20 ± 0.46	11.64 ± 0.53	9.77 ± 0.53	1.47	0.37	0.81	− 1.06
Cob diameter (cm)	1.56 ± 0.10	1.82 ± 0.11	2.17 ± 0.11	1.98 ± 0.09	1.91 ± 0.13	0.26	0.35*	0.16	0.09
Number of seeds per cob	18.25 ± 3.20	18.95 ± 3.50	46.92 ± 8.65	28.70 ± 7.50	17.23 ± 5.42	0.70	27.97**	9.75	− 1.72
Seed yield per plant (g)	7.24 ± 1.40	6.77 ± 1.14	15.98 ± 3.06	11.11 ± 2.86	6.98 ± 2.31	− 0.47	9.21**	4.34	0.21

*Significant at $P < 0.05$ and **significant at $P < 0.01$

Table 4 Mean performances of control and selected F_2 populations for important seed yield parameters under no heat stress (selfed with control pollen grains) environment at GKVK, Bengaluru

Traits	Control F_2 (T_1)	Control F_2 (RT) (T_2)	Selected F_2 (GRC) (T_3)	Selected F_2 (DB) (T_4)	Selected F_2 (BGD) (T_5)	T_2–T_1	T_3–T_2	T_4–T_2	T_5–T_2
Cob length (cm)	13.70 ± 0.35	14.58 ± 0.26	14.80 ± 0.28	14.25 ± 0.43	14.51 ± 0.35	0.88	0.22	− 0.33	− 0.07
Cob diameter (cm)	2.99 ± 0.07	3.18 ± 0.05	3.27 ± 0.08	3.60 ± 0.48	3.30 ± 0.07	0.19	0.09	0.42	0.42
Number of seeds per cob	309.64 ± 13.68	326.16 ± 11.78	347.48 ± 12.33	322.27 ± 15.66	358.96 ± 14.55	16.52	21.32	− 3.89	19.79
Seed yield per plant (g)	89.91 ± 4.28	101.35 ± 3.69	106.15 ± 3.26	96.76 ± 4.55	115.17 ± 4.73	11.44	4.80	− 4.59	13.82*

*Significant at $P < 0.05$ and **significant at $P < 0.01$

Table 5 Comparison of distribution of five F_2 populations using KS test

Trait	No pollen selection (No heat stress to pollen grains)				Pollen selection (heat stress to pollen grain)			
	Control F_2 (RT)- Control F_2	Selected F_2 (GRC)- Control F_2 (RT)	Selected F_2 (DB)- Control F_2 (RT)	Selected F_2 (BGD)- Control F_2 (RT)	Control F_2(RT)- Control F_2	Selected F_2 (GRC)- Control F_2 (RT)	Selected F_2 (DB)- Control F_2 (RT)	Selected F_2 (BGD)- Control F_2 (RT)
Cob length	NS	NS	S	NS	S	NS	NS	S
Cob diameter	NS	S	NS	NS	NS	S	NS	NS
Number of seeds per cob	NS	NS	NS	S	NS	S	NS	S
Seed yield per plant	S	NS	NS	S	NS	S	NS	S

S = KS test significance; NS = KS test non-significance

can enrich the frequency of genes/alleles associated with useful agronomic traits such as yield [14]. It shifts allelic frequencies in desirable directions such that large numbers of haploid genotypes can be screened during pollination resulting in a non-random population of progenies for further evaluation [20].

The F_1 plants of a cross between heat susceptible and resistant parent produce haploid pollen grains containing different alleles for heat tolerance. By subjecting the heterogeneous haploid pollen grains to heat stress, the fitness of pollen grains carrying alleles for susceptibility in fertilization would be reduced before pollination. It is presumed that only the pollen grains carrying alleles for tolerance of heat stress are able to fertilize thus increasing the frequency of tolerant plants in F_2. The results from this study also demonstrated that exposure to elevated temperature during gamete function can influence the heat stress tolerance of plants in the following F_2 generation.

The comparison of mean performance of the five F_2 populations under non-stress (selfed with no stress pollen grains) conditions for important yield contributing traits was also compared and the mean values showed no significant differences between populations for all the traits studied except between control F_2 (RT) and selected F_2 (BGD) for number of seed rows per cob and seed yield per plant (Table 4).

The KS test also confirmed that the pollen selection for heat tolerance has not altered the population structure of selected F_2 populations. This is in terms of altering the loci/ and mechanisms contributing to yield adversely (Table 5). Hence, the gametophytic selection had very limited or no adverse effect on the other sporophytic traits and it could be used for selectively improvement of trait of interest which is supports findings [12] where gametophytic selection showed limited effects on non-targeted sporophytic traits. Therefore, pollen selection or gametophytic selection can be combined with conventional recurrent selection program in crop improvement to target traits as an efficient tool to supplement sporophytic selection.

Acknowledgements The first author acknowledges DBT (Department of Biotechnology), New Delhi, for fellowship during the study. Authors also thanks to DBT-FIST funded Department of Plant Biotechnology, UAS, GKVK Campus, Bengaluru and Agriculture Research Station, Bheemarayanagudi for proving necessary facilities to carry out research work.

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