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Genetic evidence of pollen selection mediated phenotypic changes in maize conferring transgenerational heat-stress tolerance

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Abstract

Structural genes of pollen are expressed in both sporophytic and gametophytic generations. This genetic overlap makes possible superior pollen genotype selection. Pollen selection is more effective than sporophytic selection since more pollen grains can be exposed to selection pressure at the haploid level. In this study, selection pressure was applied in the F_1 generation at the pollen level for heat tolerance. The frequencies of heat-tolerant plants were studied for seed yield in F_2 and F_3 generations and for seedling heat tolerance in F_4 generations. The heat-susceptible inbred line BTM4 was crossed to heat-tolerant inbred line BTM6 of maize (*Zea mays* L.). In response to heat stress, we compared F_2 plants produced by selfing of heat-stressed pollen grains and without heat-stressed pollen grains. The resulting F_2 plants from heat-stressed pollen grains showed significantly higher seed yield per plant (5.41 ± 0.31 g) than control F_2 (2.90 ± 0.19 g) populations under stress. The selected and control F_2 plants were also subjected to genotyping using simple sequence repeat (SSR) primers. We observed that the frequency of alleles from tolerant parents was higher in selected F_2 populations, providing genetic evidence for positive effect of pollen selection. The heat tolerance of F_4 generation progenies of the same cross suggested that the cyclic pollen selection for heat tolerance in F_1, F_2, and F_3 generations has significantly improved the tolerance of progenies. The results from this study demonstrate that the feasibility of this approach seems to be promising for hastening the incorporation of desirable alleles in a short time.

1 | INTRODUCTION

Biotic and abiotic stresses are the major causes for low productivity of maize in India and worldwide (Lobell et al., 2014). Among abiotic stresses, maize is more susceptible to heat stress (Begcy et al., 2019). Climate models predict that the global mean temperature will increase by 1–4 °C by the end of the 21st century (Hansen et al., 2015). A rise of 1 °C in global mean temperature would, on average, reduce global maize yields by ~7.4% globally, which is highest among the cereals (Zhao et al., 2017).

Abbreviations: ARSB, Agriculture Research Station, Bheemarayanagudi, Gulburga, Karnataka, India; SSR, simple sequence repeat; UASB, University of Agricultural Sciences, GKVK, Bengaluru, Karnataka, India.

Heat stress affects all stages of maize crop but the reproductive stage is the most sensitive stage (Cairns, Sanchez, Vargas, & Araus, 2012; Rattalino Edreira, Mayer, & Otegui, 2014). The male gamete of maize is more sensitive than female gamete for heat stress (De Storme & Geelen, 2014; Rieu, Twell, & Firon, 2017). It has been shown that slight fluctuations in temperature reduced the fertility and volume of pollen shed accompanied by dramatically reduced viability (Hoegemeyer, 2011). Pollen grains are generated during microsporogenesis and microgametogenesis. During microsporogenesis, meiosis I and II take place, leading to the formation of four haploid microspores from spore mother cells (meiocytes). Several reports have pointed out that stress exposure during microgametogenesis leads to microspore abortion and associated male sterility, reduction to grain number per cob, and yield reduction (Begcy & Dresselhaus, 2018; De Storme & Geelen, 2014; Rieu et al., 2017). This can be attributed to lower ability of pollen to germinate and the rate of pollen tube growth (Yang et al., 2013).

Therefore, it is important to understand the effect of heat stress on pollen viability and ability to fertilize under heat-stress treatment. Consequently, pollen selection is a simple, effective, and emerging technique of screening the genotypes based on the performance of their pollen grains as a viable alternative to sporophytic screening (Hormaza & Herrero, 1996; Ravikumar & Patil, 2004, Clarke, Mur, Wood, & Scott, 2004). It is possible to select tolerant pollen grains from the heterogeneous pollen mixture by increasing the fitness of pollen grains by subjecting the pollen grains to stress. The precise control of the gametophytic phase of the life cycle made possible through in vitro pollination fertilization may represent a powerful experimental system for identifying differences in gamete vigor (Domínguez, Cuartero, & Fernández-Muñoz, 2005; Kakani et al., 2005; Koval, 2000).

A combination of pollen selection and conventional sporophytic selection could be an effective tool for crop improvement under heat stress. The selection of rare favorable allelic combinations have been used in population improvement programs to achieve higher levels of tolerance in a relatively short time (Landi, Frascaroli, Tuberosa, & Conti, 1989). Selection at the pollen level has been proposed as a strategy to enrich the frequencies of genes associated with useful agronomic traits (Zamir & Vallejos, 1983). The effect of selection pressure under various biotic and abiotic stresses during the gametophytic generation was reported in several studies (Chi, Löffler, & Van Tuyl, 1999; Clarke et al., 2004; Domınguez et al., 2005; Frova, Portaluppi, Villa, & Goria, 1995; Maisonneuve, Hogenboom, & Den Nijs, 1986; Mandhu, Cresti, & Shivanna, 1992; Zamir, Tanksley, & Jones, 1982; Totsky & Lyakh, 2015) and has proved successful in increasing the frequency of tolerant progeny (Hormaza & Herrero, 1996; Ravikumar & Patil, 2002). Further, it is revealed that plants cope with heavy metal stress by making heritable changes in gene expression (Cong et al., 2019).

To date, no investigations have clearly demonstrated that the pollen selection have persistence of positive response for heat tolerance in the succeeding generations. Hence, in the present study, an attempt was made to study the effect of pollen selection in the improvement of thermotolerance level in F_2, F_3, and F_4 generations along with genetic evidence as an effective way to augment plant breeding programs in maize.

2 | MATERIAL AND METHODS

2.1 | Experimental material

Maize inbred lines were developed from drought-tolerant populations and lines available at our station by selfing the selected plants for six generations. During the process of development of inbred lines, the selected plants were grown during summer season, and the plants were subjected to heat stress at flowering. Further, these lines were tested for heat tolerance under field conditions during summer at Agriculture Research Station Bheemarayanagudi, Gulburga, Karnataka, India (ARSB), which is a known station for testing heat tolerance. The inbred lines showed high variability for heat tolerance from high susceptibility to high tolerance under field conditions, making them ideal for gametophtyic heat-tolerance studies. Based on our previous screening, a heat-stress susceptible inbred line BTM4 and heat-stress tolerant inbred line BTM6 (Singh, Ravikumar, & Jingade, 2016; Singh and Ravikumar, 2017) were crossed to produce F_1, F_2, F_3, and F_4 seeds (Figure 1).

2.2 | Experimental procedure

2.2.1 | Experiment I—Evaluation of F_2 progenies

The F_1 plants were grown during rainy season 2016 (July–November) at the experimental fields of University of Agricultural Sciences, GKVK, Bengaluru, Karnataka, India (UASB) (12°58′ N, 77°35′ E, 930 m asl). The temperature was between 25 and 30 °C during the growth period (Supplemental Table S1a). The F_1 plants were grown by following all standard practices for fertilizers, fungicidal, and insecticidal sprays under well-irrigated conditions. The hybridity of F_1 plants was confirmed using polymorphic SSR markers for the parent, that is umc1144 and

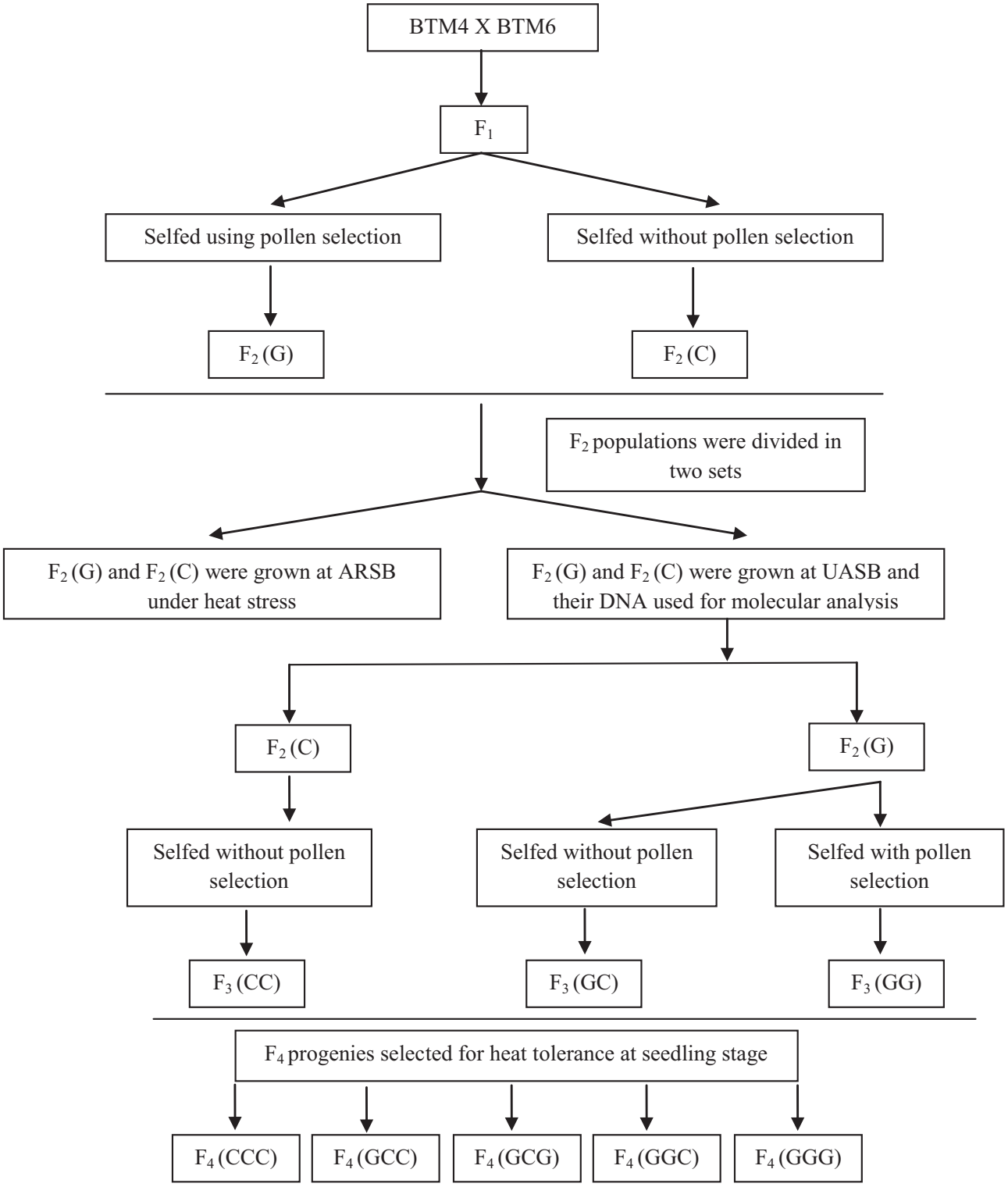

FIGURE 1 Flow chart of different cycles of pollen selection for production of different populations for heat tolerance

phi077. Thirty F_1 plants were selfed by heat-stressed pollen grains at 36 °C for 3 h (termed as pollen selection) and the rest without any heat-stress treatment (termed as no pollen selection or control). The temperature and duration for heat-stress treatment to pollen grains followed in this study was standardized in our previous work (Singh et al., 2016). For pollen heat-stress treatment, the freshly dehisced pollen grains were collected from the field-grown F_1 plants at 9:00 a.m. and transferred to 0.5-ml microfuge tubes. A set of four microfuge tubes from each F_1 plant were incubated at 36 °C for 3 h in a heating block of thermocycler for pollen heat-stress treatment. Another set of

four microfuge tubes were incubated at room temperature at 22 °C for 3 h (control condition). After incubation, pollen grains were used for selfing of field-grown F_1 plants (Singh & Ravikumar, 2017; Singh et al., 2016). The resulting F_2 populations from treated pollens grains and nontreated pollen grains were referred to as pollen selected F_2 (G) and control F_2 (C) populations, respectively.

The control (C) and selected (G) F_2 populations with the parents were evaluated simultaneously for heat-stress tolerance at two different locations—UASB and ARSB—during summer 2017 (March–June). At the experimental fields of UASB, ~250 plants each from control (C) and selected (G) F_2 populations were grown in two alternative blocks (125 plants in each block) by following recommended practices with respect to fertilizers, regular irrigation, fungicidal, and insecticidal sprays. A spacing of 60 by 30 cm (row by plant) was maintained. The F_2 population evaluated at UASB was naturally not exposed to heat stress. We also recorded the daily temperature (°C, min and max) during crop growth period (30–35 °C) (Supplemental Table S1b). One set of 100 plants from control (C) and selected (G) F_2 populations were randomly selected, and the plants were selfed without pollen selection. Another set of 100 plants were randomly selected from both the F_2 populations and selfed using heat-stressed pollen grains as mentioned earlier. As a measure of heat tolerance of F_2 plants, the following observations were recorded: plant height at maturity (cm), membrane leakage percentage, anthesis to silking interval, cob length (cm), cob diameter (cm), number of seeds per row of cob, number of seed rows per cob, number of seeds per cob, and seed yield per plant (g). The observation on membrane leakage was recorded at the flowering stage from leaf samples of individual F_2 plants 65 d after sowing, where plants were self-pollinated either by heat-stressed or nonstressed pollen grains (Leopold, Musgrave, & Williams, 1981). The details of each recorded trait are given in Table 1.

The other set of the same F_2 population was evaluated for seed yield at ARSB (16°43′ N, 76°51′ E, 411.75 m asl), which is also recognized by CYMMIT for the heat-stress tolerance screening in maize. A total of 250 plants from selected F_2 (G) and control F_2 (C) populations were grown in two blocks in summer 2017 (March–June) under irrigated condition. The sowing date was adjusted to expose the crop to heat stress during the flowering stage. The day temperature during flowering stage (April and May) was 41–44 °C (Figure 2; Supplemental Table S1c), suggesting the F_2 plants were naturally exposed to heat stress during preflowering and flowering stage. Observations on seed-yield-contributing and heat-stress-tolerance-related traits were recorded on control (C) and selected (G) F_2 populations.

The number of pollen grains per anther and pollen sterility percentage was estimated by using 1% Tween 20 (Sigma–Aldrich) detergent methods. The third spikelet from the top of the tassel was collected just prior to anthesis, and the spikelets were oven-dried at 65 °C for 48 h. These three anthers—one each from top, middle, and bottom flowers of the spikelets—were selected and carefully removed (Supplemental Figure S1a). The anthers were transferred to 1.5-ml tubes containing 1 ml of 1% (v/v) Tween 20 solution. One anther per tube was maintained. Tubes were sonicated for 5 min to release the pollen grains from anthers. The pollen grains were thoroughly mixed by shaking the tubes. Five microliters of sample were drawn from each tube and all the pollen grains present in 5 µl were counted under a microscope. Three samples of 5 µl were used from each tube to count the pollen grains. The irregular shaped and transparent pollen grains were considered as sterile pollen grains, and the pollen grains that were fully circular were considered as fertile pollen grains (Supplemental Figure S1b). Fertile and sterile pollen grains per anther was calculated as total number of pollen grains per anther. The data on the number of pollen grains per anther and percentage pollen sterility were recorded for both the control (C) and selected (G) F_2 populations. The pollen sterility percentage was calculated using following formula for each F_2 population:

$$\text{Polen sterility} = \frac{\text{Number of sterile pollen grains}}{\text{Total number of pollen grains}} \times 100$$

2.3 | Experiment II—Evaluation of F_3 progenies

The 12 F_3 lines from each group of the following F_3 progenies were selected based on the performance for seed yield in F_2 generation for evaluation of seed yield under field condition at UASB: (a) GG, pollen selection in F_1 and F_2 generations; (b) GC, pollen selection only in F_1 and no pollen selection in the F_2 generation; and (c) CC, no pollen selection in the F_1 and F_2 generations.

All 36 lines were grown in randomized complete block design with three replications during rainy season 2017 (August–November). Each F_3 line was grown in a single row of 3.0 m with the spacing 60 by 30 cm (row by plant), thus 10 plants in each row. The plants were grown by following the recommended practices for fertilizers, fungicidal and insecticidal sprays to raise a good crop, and under well-watered condition. The temperature during crop growth period was 26–32 °C (Supplemental Table S1d). Six plants were randomly chosen from each F_3 line per replication to record the observation on yield parameters. Out of six, three plants were used for selfing with

TABLE 1 Description of heat-stress tolerance measures in Maize

Trait	Abbreviation	Description
Plant height (cm)	PH	Plant height was measured in centimeters from ground level to the tip of the central tassel spikelet.
Anthesis to silking interval	ASI	Anthesis dates were recorded when 50% of the plants had shed pollen, whereas silking date was recorded when 50% of the plants had extruded silks. The ASI was calculated as the difference between the recorded anthesis and silking dates
Membrane leakage (%)	ML	The membrane leakage was recorded at flowering stage 65 d after sowing (DAS). The cell membrane integrity was tested by exposing leaves to high temperature and computing relative injury to the membranes in terms of electrolyte leakage. The fully opened, third leaf from the top was used and 0.1 g of fresh leaf sample was collected from each F_2 plant and incubated in 10 ml of distilled water in a beaker. The beakers were continuously shaken using a platform shaker for 3 h. The light absorbance values of the water in the beaker was measured at 273 nm (initial absorbance, Ia) using UV 1800 visible spectrophotometer. The beakers were again transferred to hot water bath (90 °C) for 30 min and the final absorbance value of the water was recorded at 273 nm (final absorbance, Fa) using a spectrophotometer. The cell content leak was calculated by following formula: Percentage leakage (%) = (Ia/Fa) × 100
Cob length (cm)	CL	Length of the cob was measured from the base to the tip of the cob and recorded in centimeters at the time of harvest as cob length.
Cob diameter (cm)	CD	Cob girth at the middle of the main cob was measured and recorded in centimeters after harvest.
No. of seeds per row of cob	S/RC	The number of seeds per row in the cob was counted and recorded as number of seeds per row.
No. of seed rows per cob	SR/C	The number of seed rows in the cob was counted and recorded as number of seed rows per cob.
No. of seeds per cob	S/C	Total number of seeds per cob was counted.
Seed yield per plant (g)	SY/P	Cobs were harvested at physiological maturity stage and dried in optimum sunlight for 3 d. Seed yield per plant expressed in grams was recorded by weighing the seeds obtained after shelling the cobs from individual plant.
SPAD Chlorophyll Meter Reading	SCMR	The SCMR values were recorded using the SPAD-502 (Soil Plant Analytical Development) meter from fully expanded fourth leaf from top. The SCMR is an indication of the light-transmittance characteristics of the leaf, which is dependent on the leaf chlorophyll content (Renuka et al., 2013). The SCMR value was recorded at flowering stage (65 DAS) and at maturity stage (85 DAS).

heat-stressed pollen grains at 36 °C for 3 h, as mentioned earlier, and the remaining three plants were selfed without pollen selection. The physiological and yield attributing traits were recorded for both pollen heat-stress tolerance and control conditions, where pollen grains were not heat-stress treated.

2.4 | Experiment III—Molecular comparison of selected and control F_2 populations

The genomic DNA from young leaves of 85 plants from selected F_2 (G) and control F_2 (C) populations along with two parents were extracted by modified CTAB method (Doyle & Doyle, 1987). One hundred eighty-five SSR primers were used to screen the parental lines to identify polymorphic markers between the parents, out of which, the identified 20 polymorphic primers were used for genotyping individual F_2 plants of selected and control populations (Supplemental Table S6).

2.5 | Experiment IV—Evalution of F_4 progenies for seedling heat-stress tolerance

The following F_4 generation progenies obtained from one, two, and three cycles of pollen selection for heat-stress

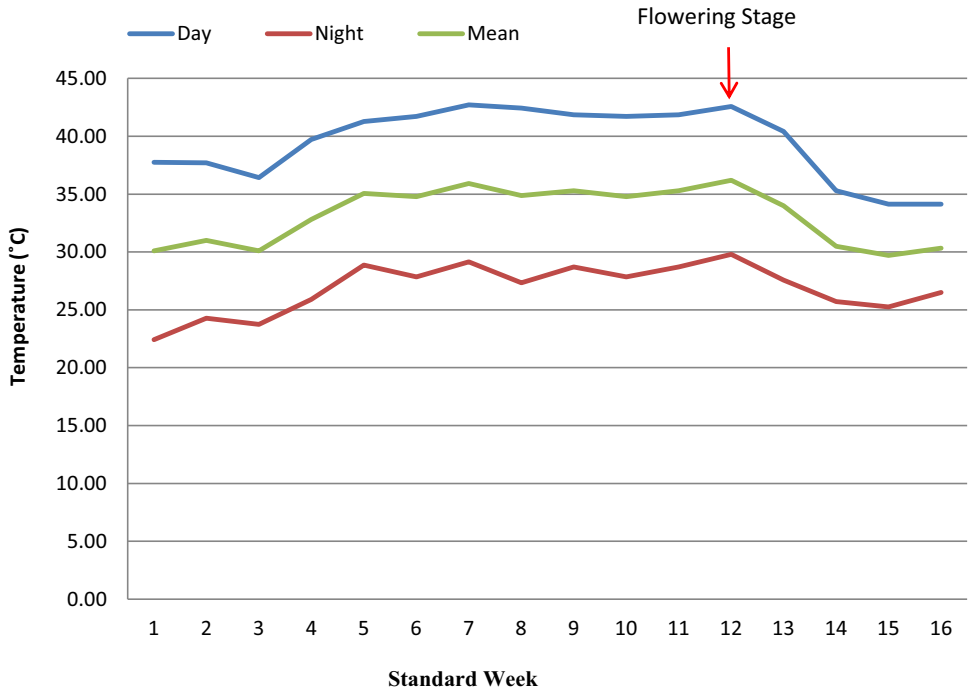

FIGURE 2 Standard week-wise temperature data during the crop period (March–June, 2017) at Agriculture Research Station, Bheemarayanagudi, Gulburga, Karnataka, India

tolerance were selected based on yield performance in the F_3 generation for seedling heat tolerance: (a) GGG, pollen selection in F_1, F_2, and F_3 generations; (b) GCG, pollen selection in F_1 and F_3, but no pollen selection in the F_2 generation; (c) GGC, pollen selection in F_1 and F_2, but no pollen selection in the F_3 generation; (d) GCC, pollen selection only in F_1, and no pollen selection in the F_2 and F_3 generations; and (e) CCC, no pollen selection in the F_1, F_2, and F_3 generations. From each group, 10 progenies were selected.

3 | Procedure of seedling heat-stress tolerance

The five groups of F_4 generation lines (GGG, GCG, GGC, GCC, and CCC) were screened for heat-stress tolerance (50 °C for 1 h) at seedling stage using temperature induction response (Nieto-Sotelo et al., 2002). The experiments were performed in three replications. Fifty maize seeds for each replication of the each selected lines were surface-sterilized in 2% Bavistin (Biostadt India, Ltd.) and rinsed in sterile water two or three times. The seeds were soaked in sterile water for 24 h before keeping them for germination. Seed germination and seedling growth were performed under aseptic conditions on enamel trays containing blotting paper saturated with 0.1-mM $CaCI_2$ and wrapped in aluminum foil. The trays were incubated in the dark at 28 °C in growth chambers.

Three-day-old germinated seedlings with root length of 1.0–1.5 cm were selected for heat treatment. Germinated seedlings were transferred to sterile flasks containing 80 ml of 0.1-mM $CaCI_2$. After 1 h of preincubation at 28 °C on a rotary shaker (60 rpm) then flasks were transferred to a water bath. Induced thermotolerance treatment was performed by incubating these flasks in a water bath for 1 h at 40 °C. This was followed by incubation for 1 h at 28 °C on a rotary shaker (60 rpm). A second heat-shock treatment of 50 °C for 1 h was given again in a water bath. Soon after the last heat-shock treatment, the seedlings were placed carefully on trays for recovery for 3 d at 28 °C. After 3 d of recovery, the seedlings were transferred to a tray containing sand in the green house for the establishment and allowed to grow for 6 d. The percentage establishment was recorded at 9 d after treatment. The shoot length and root lengths were also measured by uprooting the seedlings. The seedlings were kept at 28 °C during the entire period of experiment and were considered as control.

4 | Statistical analysis

The physiological and yield attribute traits of control (C) and selected (G) F_2 populations were compared by *t*-test. Chi-square (χ^2) test was used to check the segregation of individual molecular markers for goodness of fit to the expected Mendelian monogenic ratio of 1:2:1 for both the populations independently. The mean values of three F_3

TABLE 2 Mean sum of squares (MSS) and mean values of control F_2 (C) and selected F_2 (G) populations for important traits evaluated under pollen heat stress at University of Agricultural Sciences, GKVK, Bengaluru, Karnataka, India, during summer 2017

Trait	MSS		Mean value		Kolmogorov–Smirnov test
	Control F_2 (C)	Selected F_2 (G)	Control F_2 (C)	Selected F_2 (G)[a]	
Plant height at maturity (cm)	1399.77	898.36*	196.69 ± 4.06	221.40 ± 3.25** (12.56)	S[b]
Membrane leakage (%)	21.05	10.30**	10.36 ± 0.50	8.39 ± 0.35** (−19.02)	S
Anthesis–silking interval	1.38	0.71**	2.45 ± 0.13	1.66 ± 0.09** (−32.24)	S
Cob length (cm)	5.23	9.52*	13.15 ± 0.31	14.52 ± 0.40** (10.42)	S
Cob diam. (cm)	0.22	0.27*	1.60 ± 0.10	2.07 ± 0.45** (29.38)	S
No. seeds per row of cob	17.05	48.41*	2.00 ± 0.40	3.69 ± 0.48** (84.50)	S
No. of seed rows per cob	3.79	3.79**	2.00 ± 0.4	3.69 ± 0.48** (84.50)	S
No. seeds per plant	6936.94	14,683.84**	3.51 ± 0.82	18.00 ± 1.23** (412.82)	S
Seed yield per plant (g)	563.67	1,363.34*	2.90 ± 0.19	5.41 ± 0.31** (86.55)	S

[a]Values in parentheses are percentages of change in pollen selected F_2 (G) vs. control F_2 (C) population.
[b]S, significant.
*Significant at the .05 probability level.
**Significant at the .01 probability level.

and five F_4 progenies were compared using one-way analysis of variance (ANOVA), and least significant difference (LSD) were computed to compare these groups of F_3 and F_4 progenies separately. The Kolmogorov–Smirnov test was employed to compare one-dimensional distribution of F_2, F_3, and F_4 progenies.

5 | RESULTS

The seed yield has significantly reduced in F_1 (BTM4 × BTM6) plants when the heat-stressed (36 °C for 3 h) pollen grains were used for self-pollination vs. self-pollinated with control pollen grains. This confirmed the adverse effect of heat stress on pollen germination and fertilization ability (Supplemental Table S2).

5.1 | Evaluation of control and selected F_2 populations

The results of the F_2 population produced by selfing the F_1 (BTM4 × BTM6) plants with heat-stress pollen grains (G) and control pollen grains (C) at UASB indicated significant differences for plant height, membrane leaching, anthesis to silking interval, cob length, cob diameter, number of seed rows per cob, number of seeds per row, number of seeds per cob, and seed yield per plant between two F_2 populations (Table 2). The mean values of anthesis to silking interval of control F_2 population (2.45 ± 0.13) were significantly higher than the selected F_2 population (1.66 ± 0.09). The results also showed the selected F_2 population had lower membrane leakage (8.39 ± 0.35%) than the control F_2 population (10.36 ± 0.5%). This clearly suggested the selected F_2 population is more resistant to membrane leakage of solute, which is an important parameter for measuring heat-stress tolerance. The plant height was significantly taller in the selected F_2 population (221.10 ± 3.25 cm) than in the control F_2 population (196.69 ± 4.06 cm). The mean values for traits cob length, cob diameter, number of seed rows per cob, number of seeds per row, number of seeds per cob, and seed yield per plant were higher (10.42, 29.38, 84.50, 84.50, 412.82, and 86.25%, respectively) in the selected F_2 population than the control F_2 population (Table 2). The Kolmogorov–Smirnov test also showed significant difference of one-dimensional distribution between selected (G) and control (C) F_2 populations for all the recorded parameters, indicating that both the F_2 populations were distinct (Table 2; Supplemental Figure S2). The effect of pollen selection for heat tolerance on the per se performance of progenies were also determined in the absence of pollen heat-stress treatment by selfing the plants at the field of UASB. The mean performance of the selected F_2 (G) population for yield and its component traits were higher than control F_2 (C) but were not significant (Supplemental Table S3).

The similar set of derived control (C) and selected (G) F_2 populations were evaluated at ARSB. The pollen sterility of the control F_2 (15.24 ± 1.51%) population was significantly higher than the selected F_2 (7.22 ± 1.04%) populations (Supplemental Figure S5). Similarly, plant height was higher in the selected F_2 population (174.10 ± 2.25) than the control F_2 population (162.30 ± 1.94). The difference in F_2 population for plant height was observed when grown under two different environmental

TABLE 3 Mean sum of squares (MSS) and mean values of control F_2 (C) and selected F_2 (G) populations for seed yield and yield components under natural heat stress during flowering stage at Agriculture Research Station, Bheemarayanagudi, Gulburga, Karnataka, India, during summer 2017

Trait	MSS		Mean value		Kolmogorov–Smirnov test
	Control F_2 (C)	Selected F_2 (G)	Control F_2 (C)	Selected F_2 (G)[a]	
Plant height at maturity (cm)	195.78	153.94*	162.30 ± 1.94	174.10 ± 2.25** (7.27)	S[b]
Anthesis–silking interval	2.55	1.46*	1.92 ± 0.22	1.81 ± 0.22 (−5.73)	S
No. pollen grains per anther	441959.60	550373.49*	1126.00 ± 94.50	1243.20 ± 105.45 (10.41)	S
Pollen sterility (%)	113.57	53.66**	15.24 ± 1.51	7.22 ± 1.04** (−52.62)	S
Cob length (cm)	18.72	7.41**	9.81 ± 0.60	12.39 ± 0.49** (26.30)	S
No. seed rows per cob	39.82	51.84	4.52 ± 0.91	6.45 ± 1.31(42.70)	S
No. seeds per plant	9618.03	18543.98	133.58 ± 19.35	168.90 ± 25.07 (26.44)	S
Seed yield per plant (g)	446.41	923.45	29.77 ± 4.10	36.78 ± 5.52 (23.55)	S

[a]Values in parentheses are percentages of change in pollen selected F_2 (G) vs. control F_2 (C) population.
[b]S, significant.
*Significant at the .05 probability level.
**Significant at the .01 probability level.

conditions, namely UASB and ARSB. The anthesis to silking interval of the selected F_2 population was less (1.81 ± 0.22) than the control F_2 population. Similarly, for seed-yield-contributing traits, that is, number of seed rows per cob, number of seeds per plant, and seed yield per plant, the selected F_2 population produced higher yield (42.70, 26.44, and 23.55%, respectively) than the control F_2 population (Table 3; Supplemental Figure S4).

5.2 | Evaluation of F_3 progenies derived through different cycles of pollen selection

Twelve F_3 progeny lines, each from CC, GC and GG, were evaluated for yield attribute traits and pollen heat-stress tolerance under heat-stress conditions at UASB. The ANOVA indicated significant differences among three F_3 groups of progenies for SPAD chlorophyll meter reading value, plant height at flowering, membrane leakage, anthesis to silking interval, cob length, cob diameter, seed rows per cob, seeds per row of cob, seeds per cob, and seed yield per plant (Table 4). There was no definite trend observed with respect to effect of pollen selection for heat tolerance on plant height at maturity in F_3 progenies. The yield parameters among three groups of F_3 progenies suggested the GG group had significantly higher mean value for cob diameter, seed rows per cob, seeds per row of cob, seeds per cob, and seed yield per plant than GC (one cycle of pollen selection) and control (CC) progenies (Figure 3 and 4). The number of seeds per cob of GG progenies was 122.60% higher than CC progenies and 86.29% higher than the GC progenies. Similarly, seed yield per plant of GG progenies was 137.89% higher than CC progenies and 72.84% higher than the GC progenies. Though one cycle of pollen selection (GC progenies) recorded a higher value than CC progenies for many traits, but it was not significant (Table 4).

The F_3 progenies obtained from different cycles of pollen selection for heat-stress tolerance were also evaluated under no pollen stress (control) conditions. The results of this study demonstrated that pollen selection for heat tolerance has no negative effect on seed yield and yield-related parameters. The progenies derived through different cycles of pollen selection either performed significantly better than the progenies derived without pollen selection or on par with CC progenies for seed yield and yield-related parameters (Supplemental Table S4).

The Kolmogorov–Smirnov test also showed significant difference for distribution pattern between two cycles of selected F_3 (GG) and one cycle of selected F_3 (GC) populations for traits like plant height at maturity, SPAD chlorophyll meter reading value (85 DAS), membrane leakage, and anthesis to silking interval. The result also showed the significant differences for plant height at flowering, plant height at maturity, and anthesis to silking interval were observed between selected F_3 (GC) and control F_3 (CC) populations under pollen heat stress (Table 4). The mean values of plant height at maturity and the SPAD chlorophyll meter reading was observed more in inbred lines BTM6 (236.58 ± 1.48 cm and 59.82 ± 0.14, respectively) than BTM4 (212.75 ± 1.89 cm and 52.36 ± 0.17, respectively) inbred line. The seed yield per plant for BTM6 (101.08 ± 2.96 g) was significantly higher than for BTM4 (41.15 ± 2.16 g) under control condition (Supplemental Table S3). The comparison of population distribution pattern of GG, GC, and CC populations confirmed the fact that pollen selection has no negative effect on the genotypic

TABLE 4 One-way ANOVA mean sum of squares (MSS), mean values, and least significant difference (LSD) between means of three groups of F_3 progenies (GG, GC, and CC) under pollen heat stress for physiological and seed yield parameters evaluated at experimental field of University of Agricultural Sciences, GKVK, Bengaluru, Karnataka, India, during rainy season 2017

		Mean value			Mean difference[a]			Kolmogorov–Smirnov test[b]		
Trait	MSS	GG	GC	CC	GG–CC	GG–GC	GC–CC	GG–CC	GG–GC	GC–CC
Plant height at flowering (45 d after sowing [DAS])	4152.22**	121.78 ± 1.40	120.64 ± 1.80	112.94 ± 1.31	8.84** (7.80)	0.86 (0.90)	7.70** (6.80)	S	NS	S
Plant height at maturity (cm)	2436.59	249.50 ± 1.71	257.67 ± 1.62	252.72 ± 1.40	3.25 (−1.30)	8.17 (3.20)	4.95 (2.00)	S	S	S
SPAD chlorophyll meter reading (45 DAS)	48.92**	55.55 ± 0.18	54.91 ± 0.23	54.39 ± 0.24	1.16** (2.10)	0.63* (−1.20)	0.52 (1.00)	NS	NS	NS
SPAD chlorophyll meter reading (65 DAS)	401.56**	63.07 ± 0.15	59.96 ± 0.17	60.45 ± 0.18	2.62** (4.30)	3.10** (−5.20)	0.49* (−0.80)	S	S	NS
Membrane leakage (%)	0.01*	8.73 ± 0.34	10.11 ± 0.39	10.22 ± 1.37	1.49** (−14.60)	1.38** (−13.60)	0.11 (−1.10)	S	S	NS
Anthesis–silking interval	6.77**	2.45 ± 0.051	2.76 ± 0.05	2.87 ± 0.07	0.42** (−14.60)	0.31** (−11.20)	0.10 (−3.80)	S	S	NS
Cob length (cm)	33.43**	13.16 ± 0.22	12.71 ± 0.27	11.76 ± 0.19	1.39** (11.90)	0.45 (3.50)	0.95** (8.10)	S	S	S
Cob diam. (cm)	3.32**	1.45 ± 0.04	1.14 ± 0.03	1.02 ± 0.03	0.43** (42.20)	0.31** (27.20)	0.12* (11.80)	S	S	S
No. seeds per row of cob	9.22*	1.80 ± 0.24	1.24 ± 0.15	1.08 ± 0.13	0.71** (66.70)	0.55* (45.20)	0.16 (14.80)	NS	NS	NS
No. of seed rows per cob	75.86*	4.98 ± 0.60	3.28 ± 0.47	3.00 ± 0.47	1.98** (66.00)	1.70* (51.80)	0.28 (9.30)	S	S	NS
No. of seeds per cob	1340.67**	15.28 ± 2.77	8.20 ± 1.75	6.86 ± 1.29	8.41** (122.70)	7.07* (86.30)	1.33 (19.50)	S	S	NS
Seed yield per plant (g)	171.30*	5.40 ± 1.07	3.13 ± 0.68	2.27 ± 0.45	3.13** (137.90)	2.28* (72.50)	0.86 (37.90)	S	S	NS

[a]Values in parentheses are percentages of change in pollen selected F_3 (GG) vs. F_3 (GC) and control F_3 (CC) population.

[b]S, significant; NS, not significant.

*Significant at the .05 probability level.

**Significant at the .01 probability level.

FIGURE 3 Cob development and seed set after the heat stress treatment to pollen grains in F_3 progenies of maize

performance under no-stress environments. In fact, pollen selection for heat tolerance has resulted in the improvement of quantitative traits like seed yield and its component traits even under control conditions (Supplemental Table S5).

5.3 | Comparison of F_2 population obtained with and without pollen selection for heat tolerance using molecular markers

Twenty polymorphic markers between parents BTM4 and BTM6 were used to screen the selected and control F_2 populations on agarose gel (Supplemental Table S5). The segregation of individual markers was tested for the expected monogenic 1:2:1 ratio in the F_2 population using χ^2 test. The χ^2 test of control F_2 (C) was nonsignificant for 19 out of 20 markers, suggesting that no segregation distortion from the expected ratio (1:2:1), and only one marker showed significant deviation from the expected ratio (Supplemental Plate S1). The normal Mendelian segregation of SSR markers indicated that the parental lines and F_2 plants tested in this study were ideal. On the other hand, the selected F_2 (G) population showed significant deviation from Mendelian monohybrid ratio. Eighteen out of 20 markers recorded significant deviation from the 1:2:1 ratio, and in all the markers, the number of individuals showing homozygous male parent alleles and heterozygotes were significantly more than the expected number (Supplemental Plate S2). The results showed that the selected F_2 population skewed toward the alleles that were contributed by the male parent for which the selection was made (Table 5).

5.4 | Assessment of F_4 progenies derived through pollen selection for seedling heat stress tolerance

The selected five groups of F_4 progenies (GGG, GGC, GCG, GCC, and CCC) were used to assess the heat-stress tolerance at seedling stage with three replications at 40 °C for 1 h followed by 50 °C for 1 h. One-way analysis of variance suggested significant effect of temperature treatment at seedling stage among the selected five groups of F_4 progenies for seedlings establishment and root and shoot length (Table 6). The mean values were found significantly higher in the F_4 GGG progenies for seedling establishment and root and shoot length among the five sets of F_4 progenies under heat-stress condition (Table 6). The performance of these groups of F_4 progenies were also tested under non-stress conditions, which showed significant differences for shoot and root growth. The F_4 progenies derived from three cycles of pollen selection (GGG) recorded the highest mean for root and shoot length and the least was observed in CCC progenies (Table 6). The frequency distribution Kolmogorov–Smirnov test of different groups of F_4 progenies clearly showed that the GGG group had a significantly higher number of seedlings with more root and shoot growth than the CCC progenies and progenies under heat stress treated and control conditions (Figure 6 and 7).

6 | DISCUSSION

The data of our study showed that exposure of pollen grains to heat stress under in vitro conditions not only affected the potential for the pollen grains to grow and fertilize on the stigmatic surface but also reduced seed yield and yield parameters in the F_1 generation. The differences in pollen tube growth in vivo under stress lead to pollen selection. Thus the effect of pollen selection for competitive ability of the male gametophyte in modifying pollen quality is based solely on the choice of seed set (Edreira, Mayer, & Otegui, 2014; Landi et al., 1989; Ottaviano, Gorla, & Pe, 1982, 1986; Singh et al., 2016).

The selected (G) and control (C) F_2 populations with parents evaluated under pollen heat stress at UASB clearly indicated that the pollen selection for heat stress in F_1 has resulted in increased frequency of tolerant plants in selected F_2 populations vs. no pollen selection in F_1 of the same cross with respect to heat-stress-tolerant-related traits. Such positive influence of pollen selection for heat stresses from the differential response of the pollen grains with reference to fertilization could be due to allelic differences in the pollen grains (Ravikumar, Patil, Soregaon, & Hegde, 2007). The effect of pollen selection for biotic and abiotic stress tolerance is clearly visible at the sporophytic

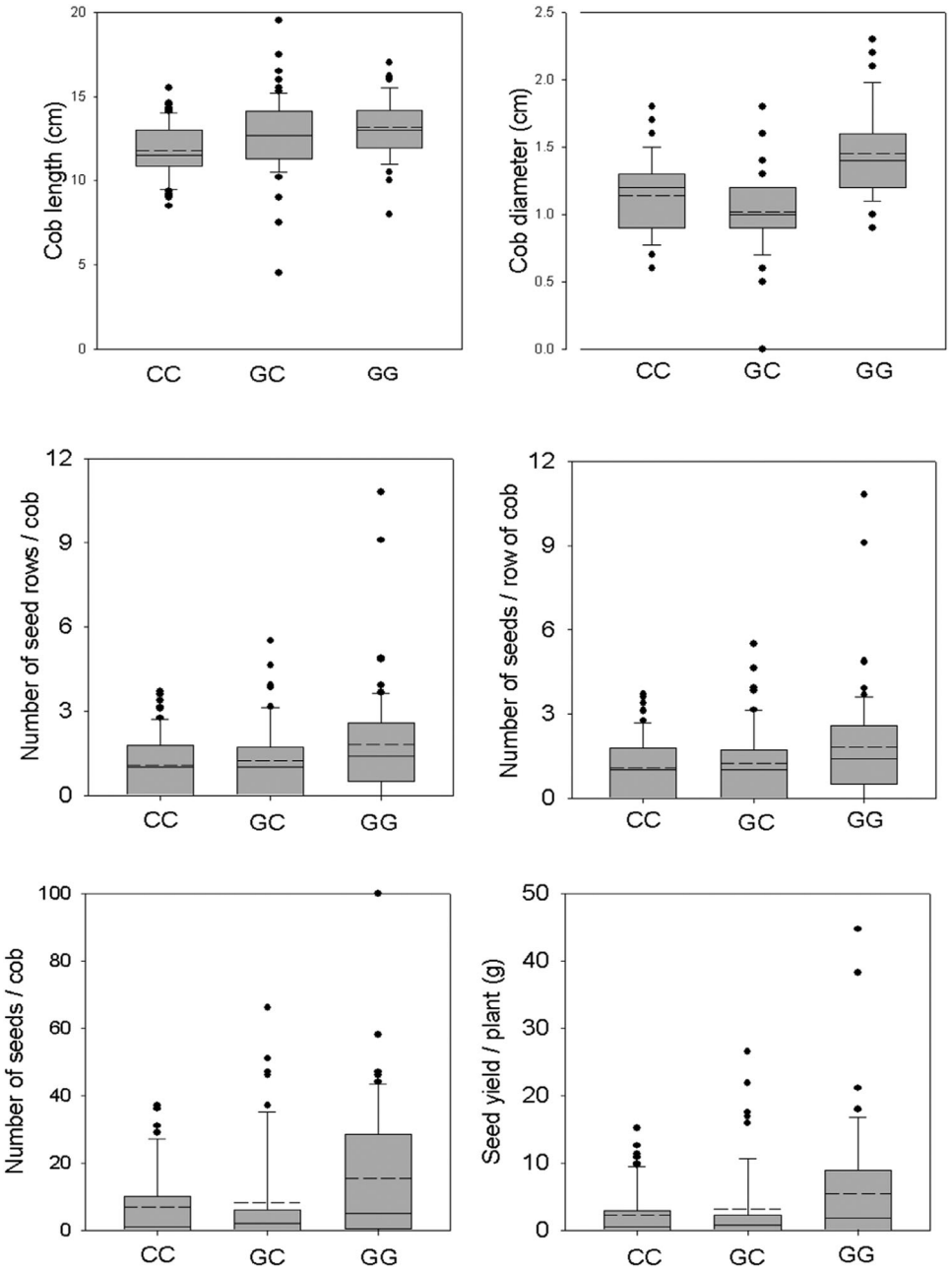

FIGURE 4 Distribution pattern of three F_3 populations (GG, GC, and CC) of maize for seed yield and their components under stress condition (dash line represent the mean values, dots represent the outliers)

stage of plants (Ravikumar et al., 2007; Ravikumar, Patil, & Salimath, 2003; Sacher, Mulcahy, & Staples, 1983; Totsky & Lyakh, 2015).

Simultaneously, the selected and control F_2 populations were also compared for their performance under no-stress conditions where the pollen grains, as well as crop, were not exposed to heat stress before selfing the F_2 plants at UASB. As the result suggested, tolerant parent BTM6 showed significantly more yield and yield-related traits than susceptible parent BTM4. The selected population skewed toward the male parent for which selection was made. Thus the selected F_2 population showed more seed yield than the control F_2 population even under control conditions (Supplemental Table S3). It is also confirmed that pollen selection has no negative effect on the yield performance of selected and control F_2 populations under no-stress environments. Therefore, pollen selection can be used for selective improvement of specific traits. Hence, the pollen selection had no effect on the nontarget sporophytic traits under nonstressed conditions (Landi et al., 1989; Palmer & Zimmerman, 1994).

The selected (G) and control (C) F_2 populations showed differences for various traits at ARSB under natural heat-stress environments during flowering stage in summer

TABLE 5 Chi square (χ^2) test for segregation of simple sequence repeat markers in control and selected F_2 populations of maize

Serial No.	Primer name	Control F_2 BTM4 allele	Hybrid allele	BTM6 allele	χ^2 (1:2:1)	Selected F_2 BTM4 allele	Hybrid allele	BTM6 allele	χ^2 (1:2:1)
1	umc1894	20 (21.25)	48 (42.50)	17 (21.25)	NS[a]	13 (21.25)	40 (42.5)	32 (21.25)	S[b]
2	bnlg1662	22 (21.25)	42 (42.50)	21 (21.25)	NS	32 (21.25)	40 (42.5)	13 (21.25)	S
3	umc1894	20 (21.25)	48 (42.50)	17 (21.25)	NS	13 (21.25)	40 (42.5)	32 (21.25)	S
4	bnlg1063	18 (21.25)	40 (42.50)	27 (21.25)	NS	18 (21.25)	53 (42.5)	14 (21.25)	S
5	umc2225	21 (21.25)	37 (42.50)	22 (21.25)	NS	12 (21.25)	51 (42.5)	22 (21.25)	NS
6	bngl1144	25 (21.25)	40 (42.50)	20 (21.25)	NS	18 (21.25)	30 (42.5)	39 (21.25)	S
7	umc2408	19 (21.25)	41 (42.50)	25 (21.25)	NS	11 (21.25)	32 (42.5)	42 (21.25)	S
8	umc1113	18 (21.25)	45 (42.50)	22 (21.25)	NS	12 (21.25)	43 (42.5)	30 (21.25)	NS
9	bngl1350	24 (21.25)	40 (42.50)	17 (21.25)	NS	19 (21.25)	31 (42.5)	33 (21.25)	S
10	umc2362	24 (21.25)	38 (42.50)	23 (21.25)	NS	18 (21.25)	27 (42.5)	40 (21.25)	S
11	mmc041	26 (21.25)	38 (42.50)	21 (21.25)	NS	15 (21.5)	29 (42.5)	41 (21.5)	S
12	umc1299	32 (21.25)	32 (42.50)	14 (21.25)	S	17 (21.25)	32 (42.5)	36 (21.25)	S
13	bngl1017	24 (21.25)	42 (42.50)	19 (21.25)	NS	19 (21.25)	53 (42.5)	13 (21.25)	S
14	bngl127	23 (21.25)	43 (42.50)	19 (21.25)	NS	8 (21.25)	49 (42.5)	28 (21.25)	S
15	Phi077	21 (21.25)	45 (42.50)	19 (21.25)	NS	10 (21.25)	50 (42.5)	25 (21.25)	S
16	umc1144	24 (21.25)	42 (42.50)	19 (21.25)	NS	25 (21.25)	50 (42.5)	10 (21.25)	S
17	um1040	26 (21.25)	40 (42.50)	19 (21.25)	NS	16 (21.25)	38 (42.5)	31 (21.25)	S
18	umc1736	23 (21.25)	37 (42.50)	25 (21.25)	NS	9 (21.25)	60 (42.5)	16 (21.25)	S
19	mmc0471	26 (21.25)	40 (42.50)	19 (21.25)	NS	18 (21.25)	58 (42.5)	9 (21.25)	S
20	bngl1371	17 (21.25)	45 (42.50)	23 (21.25)	NS	18 (21.25)	55 (42.5)	12 (21.25)	S
Total		451 (425)	823 (850)	410 (425)	–	314 (425)	868 (850)	518 (425)	–
Mean		22.55 (21.25)	41.15 (42.50)	20.5 (21.25)	–	15.7 (21.25)	43.4 (42.50)	25.9 (21.25)	–

Note: Value in parentheses is expected number of alleles.

[a]NS, not significant.

[b]S, significant.

2017, indicating the selected (G) F_2 population was more vigorous and recorded significantly higher seed yield as than the control (C) F_2 population. Plant height is also influenced by the interaction of environmental conditions and genetic constitution of the plant. Plant height was found to be significantly correlated with seed yield under heat stress in maize (Khodarahmpour, 2012). Increase in temperature affects the plant growth, which ultimately influences the plant height (Shrestha, Gurung, & Dhital, 2014). It is also reported that plant height contributes significantly toward total genetic diversity under heat stress (Rani, Nirala, Kumari, Singh, & Kumari, 2018). The reduced anthesis to silking interval, along with high-yielding hybrids proved effective in selection for tolerant hybrids under heat stress (Mhike, Magorokosho, & Ndlela, 2012). Traits like anthesis to silking interval, pollen viability, and chlorophyll contents are indicative of reproductive success and can be used along with grain yield in selection of suitable germplasm for heat-stress tolerance (Alam et al., 2017). The phenological traits affected by stress have an effect on seed yield as well as other traits such as cob length, cob diameter, number of seed rows, and number of seeds per cob. Maize loses a significant amount of dry weight from the stem and husk during grain filling, and the taller plants are most effective in maintaining cob and seed weight when stress occurs during flowering and grain filling. Increased rates of cob growth under stress results in higher rate of reproductive success and increased seed yield and harvest index under stress (Edmeades, Chapman, & Lafitte, 1999). Therefore the cob parameters, such as seed numbers and seed weight per cob, are the most preferred way of estimation of stress tolerance through remobilization of nutrients and the keys to yield stability under stress (Araus, Serret, & Edmeades, 2012). The heat stress led to pollen sterility during the early reproductive stages, whereas ovary fertility remained unaffected (Ji et al., 2010). When silks begin to desiccate, they lose their capacity for pollen tube growth and fertilization (Madhiyazhagan, 2005). Further, high temperature also reduces the pollen shedding, production, and fertility (Hoegemeyer, 2011). It

TABLE 6 Mean sum of squares (MSS), mean values, and least significant difference (LSD) between means of five groups of F_4 progenies (CCC, GCC, GCG, GGC, and GGG) of maize under seedling heat stress and no stress (control) condition at seedling stage

		Mean value					LSD (mean difference)[a]				Kolmogorov–Smirnov test[b]			
Trait	MSS	GGG	GGC	GCG	GCC	CCC	GGG–CCC	GGG–GCC	GGG–GGC	GGG–GCG	GGG–CCC	GGG–GCC	GGG–GGC	GGG–GCG
Heat stress														
Root length (cm)	7.59**	2.32 ± 0.14	1.29 ± 0.11	1.83 ± 0.20	1.93 ± 0.31	1.34 ± 0.36	0.98** (73.13)	0.39 (20.21)	1.03** (79.84)	0.49 (26.78)	S	S	S	S
Shoot length (cm)	30.32**	4.81 ± 0.28	3.11 ± 0.27	3.80 ± 0.39	3.27 ± 0.37	2.44 ± 0.24	2.37** (97.13)	1.54** (47.09)	1.70** (54.66)	1.01** (26.58)	S	S	S	S
Establishment (%)	3441.65**	58.11 ± 4.20	23.15 ± 3.46	23.97 ± 4.07	14.12 ± 2.62	12.11 ± 3.27	45.90** (379.85)	43.90** (311.54)	34.90** (151.02)	34.10** (142.43)	–	–	–	–
No stress (control)														
Root length (cm)	68.16**	12.41 ± 0.39	9.70 ± 0.49	10.68 ± 0.53	9.23 ± 0.42	8.12 ± 0.56	2.29** (52.83)	1.18 (34.45)	2.71** (27.94)	1.73** (16.20)	S	S	S	S
Shoot length (cm)	83.45*	12.57 ± 0.32	12.47 ± 1.25	12.04 ± 0.32	12.28 ± 0.32	9.88 ± 0.32	2.70** (27.23)	0.30 (2.36)	0.10 (0.80)	1.53 (4.40)	S	NS	S	S
Establishment (%)	–	100	100	100	100	100	0	0	0	0	–	–	–	–

[a]Values in parentheses are percentages of change in pollen selected F_4 (GGG) vs. F_4 (GGC), F_4 (GCG), F_4 (GCC), and control F_4 (CCC) population.

[b]S, significant; NS, not significant.

*Significant at the .05 probability level.

**Significant at the .01 probability level.

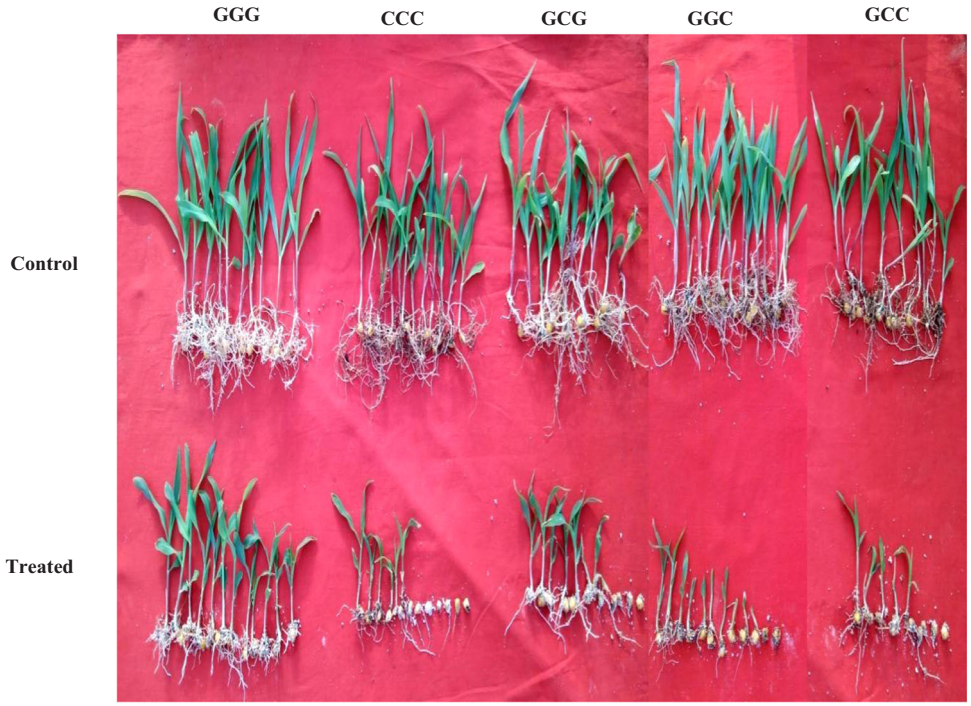

FIGURE 5 Seedling establishment after 9 d of heat stress treatment and control condition in F_4 progenies of maize

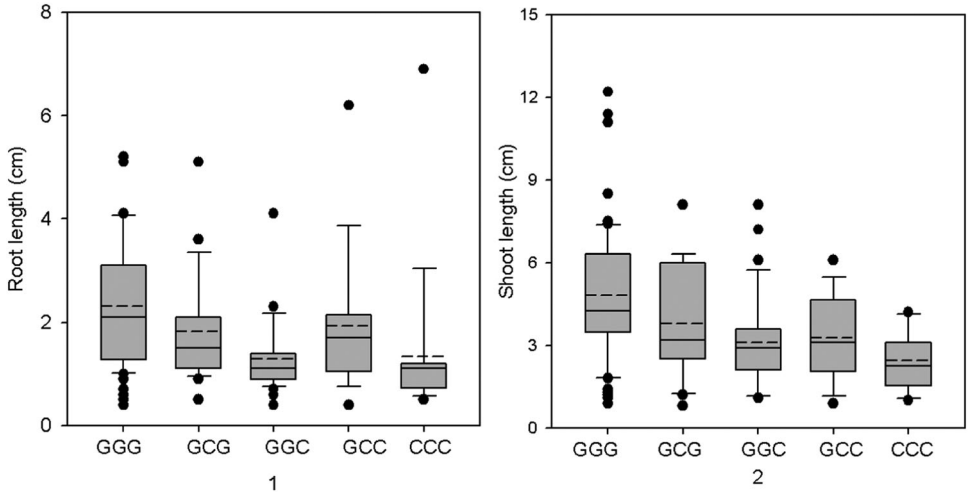

FIGURE 6 Distribution pattern of five selected F_4 progenies (GGG, GGC, GCG, GCC, and CCC) of maize for root and shoot length of seedlings under heat stress (dash line represent the mean values, dots represent the outliers)

has a very significant effect on pollen grains and it reduces the fertilization rate and seed set (Young, Wilen, & Bonham Smith, 2004; Zinn, Tunc-Ozdemir, & Harper, 2010). First, temperature >35 °C depresses pollen production. Even continuous heat over several days before and during pollen shed results in formation of a fraction of fertile pollens because of the availability of reduced sugar. In addition, high temperature reduces the period of pollen viability to a couple of hours (or even less). So the total number of pollen grains per anther and sterility or fertility is an important parameter that is affected because of high temperature, leading to decreased number of pollen grains per anther as well as increased number of sterile pollen grains. In the present study, at higher temperature, the plants of control F_2 populations produced more sterile pollen grains and fewer total pollen grains per anther than plants of selected F_2 populations. These results support the contention that gamete selection for a specific trait

may also enrich the frequency of genes associated with useful agronomic traits (Kyung, Gill, Ghosh, & Casella, 2010; Zamir, 1983).

The results of three groups of F_3 populations derived through different cycles of pollen selection evaluated at UASB clearly showed that the progenies derived through two cycles of pollen selection (GG) showed significantly improved performance for seed yield and heat-stress-related traits compared with progenies obtained from one cycle of pollen selection (GC) and progenies derived without pollen selection (CC). The results also indicated that the increased number of cycles of pollen selection improve the quantitative traits associated with seed yield per plant and heat tolerance. It is also noted that pollen selection can be more effective when carried out in more adverse environments (Landi et al., 1989). Further, it was notable that discontinuous pollen selection in each generation affected the performance of progenies in succeeding generation under heat stress as well as under nonstressed conditions. Heat stressing pollen grains and pollinating the plants allowed us to select genetically tolerant pollen grains for successful fertilization. The subsequent progeny will be more tolerant to heat stress (Singh et al., 2016). The inheritance of traits selected through gametophytic tolerance has been demonstrated in chickpea (*Cicer arietinum* L.) against Fusarium wilt and cold tolerance (Clarke et al., 2004; Kron & Husband, 2006; Ravikumar et al., 2007). The evaluation of the correlated response to the gametophytic selection has allowed the detection of significant variation for many sporophytic traits and the variation transmitted from generation to generation (Landi et al., 1989). Ottaviano et al. (1982, 1986) found that the intense pollen selections were characterized by higher seedling dry weight, longer roots, and heavier kernels in maize. The agronomic performance of the gametophytically selected progenies was found to be superior as evidenced by higher grain yield, greater seedling vigor, and reduced stalk and root lodging (Petolino, Cowen, Thompson, & Mitchell, 1990). The pollen selection resulted in the improvement of heat tolerance and also productivity-related traits in the progenies of continuous selection over generations under pollen heat-stress conditions.

The 20 polymorphic SSR markers were used to differentiate control and selected F_2 population. The segregation of individual markers was tested for the expected monogenic 1:2:1 ratio in the F_2 using χ^2 test. The χ^2 test of control F_2 was nonsignificant for 19 out of 20 markers, suggesting no segregation distortion from the expected ratio (1:2:1). The result showed the deviation from normal Mendelian segregation in the selected F_2 plants for the DNA markers provides strong evidence that pollen selection increases the frequency of heat-tolerant alleles in the progeny. Therefore, it is possible that the increased frequency of heat-tolerant plants observed in the selected F_2 progenies following pollen selection could be due to increased number of pollen grains with the heat-tolerant alleles fertilizing the ovules, a result of selection of zygotes containing the heat-tolerant alleles, or both. However, the deviation observed in the selected F_2 progenies is a clear indication of selection occurring at the male gametophytic level, as only pollen that survived the heat stress were used to generate F_2 individuals. The deviation from normal Mendelian segregation in selected F_2 populations and skewness toward the alleles selected from male parent provides strong, molecular genetic evidence that pollen selection increases the frequency of heat-tolerant alleles in the progeny. Thus pollen selection can be a useful means of shifting allelic frequencies in the desirable directions so that large numbers of haploid genotypes can be screened during pollination resulting in a nonrandom population of progenies for further evaluation (Ottaviano, Sari-Gorla, & Villa, 1988). The results indicated that pollen selection was able to favor those gametes containing alleles for heat tolerance at the DNA level, thus emphasizing the potential of this method. The effectiveness of gamete selection for simply inherited traits was also revealed in tobacco (*Nicotiana tabacum* L.) (Touraev, Fink, Stöger, & Heberle-Bors, 1995), maize (Frascaroli et al., 2001), and chickpea (Ravikumar et al., 2007). The feasibility of this approach seems to be promising for hastening the incorporation of desirable alleles in a short time particularly for complex polygenic trait like heat tolerance in maize.

The temperature induction response technique can be used to select the maize inbred lines for heat tolerance, which has been validated in many crops (Selvaraj et al., 2011; Srikanthbabu, Krishnaprasad, Gopalakrishna, Savitha, & Udayaku, 2002; Sudhakar, Latha, Babu, Sujatha, & Reddy, 2012; Venkateshbabu et al., 2013). Hence the effect of pollen selection for heat tolerance in maize observed at seedling stage in the temperature induction response technique is highly dependable. The seedlings of five groups of F_4 progenies were screened under heat stress. Three cycles of pollen selection (in three generations) were found superior over two or one cycle of pollen selection in terms of seedling establishment and growth, suggesting that the heat tolerance is a complex trait requiring cyclic selection over generations to accumulate alleles for heat tolerance. With heat tolerance being a polygenetic trait, recurrent selection programs have been suggested for improvement (Landi et al., 1989). The seedling evaluation in F_4 progenies under heat stress confirmed that the progenies selected through three cycles of pollen selection lead to more established and vigorous progenies in succeeding generations. The combination of pollen and sporophytic selection over many cycles has the potential to increase the heat tolerance quickly in maize.

7 | CONCLUSION

Heat stress had an effect on pollen germination, tube growth, and fertilization, which directly resulted in seed set. We found that the pollen selection in F_1 populations and subsequent generations increase the number of heat-tolerant plants under natural heat-stress conditions. The pollen-selected progenies showed improvement in the pollen quality and quantity, which resulted in higher seed yield per plant than nonpollen selected progenies under heat-stress conditions. Molecular marker results confirmed the positive effect of pollen selection on parental allele frequencies for heat stress, as the population skewed toward the heat-stress-tolerant male alleles. The genetic effect of pollen selection in the F_1 generation has resulted in the improved tolerance of F_3 and F_4 progenies. In addition, the continuous pollen selection for heat-stress tolerance in the early generations has resulted in the improved tolerance of F_4 progenies. Thus we can combine this pollen (gametophyte) selection strategy with sporophytic selection for improvement of heat-stress tolerance in crops.

AUTHOR CONTRIBUTIONS

A. Singh and Suresh. H. Antre performed the experiments, analyzed the data and wrote the manuscript; R.L. Ravikumar, P.H. Kuchanur, and H.C. Lohithaswa designed the experiments and discussed the results. All authors reviewed and approved the final manuscript.

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