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Effect of gamete selection in improving of heat tolerance as demonstrated by shift in allele frequency in maize (*Zea maize* L.)

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Abstract Pollen genotype selection for genes expressed in both the haploid and diploid phases of the plant life cycle can lead to correlated responses detectable in the sporophyte. Here, Impact of pollen selection for heat tolerance was assessed in segregating population of maize. Two subsets of F_1 were made among which one was self-fertilized with heat treated pollen grains while other set by normal pollen where no such selection was made that resulted in two F_2 populations TF_2 and CF_2, respectively. The TF_2 population recorded significantly higher mean values for pollen per anther (4646.78 ± 176.70) than that for control F_2 population (3321.89 ± 164.91). In addition, the test F_2 population recorded significantly higher seed yield per plant (2.02 ± 0.72 g) than that of test F_2 population (1.38 ± 0.08 g) under heat stress. These two populations were also evaluated under normal condition to verify whether the TF_2 population showed heat tolerance improvement has any impact on yield attributes. The result showed non-negative impact of pollen selection on performance of TF_2 under normal condition. Thus, pollen selection could be used to selectively improve the trait of interest. The segregation analysis of SSR markers followed the expected Mendelian distribution in CF_2 population whereas a significant deviation in allelic frequency was observed in TF_2 population. These results clearly evidence that the gametophytic selection for heat tolerance in F_1 generation had positive effect towards increasing the resistant alleles in F_2 population of maize.

Keywords Genotype selection · Haploid · Heat stress · Gametophytic selection

Introduction

Maize is often referred to as 'Queen of Cereals' owing to its wider adaptability in varied agro climatic condition. In India, maize is grown in a wide range of climate extending from semi-arid to sub-humid to temperate conditions. At present, about 80% of the maize cultivated area is confined to rainfed condition and hence, it often suffers from vivid abiotic stresses. This is one of the major reasons that lower productivity of maize in India (Dass et al. 2010). Among the abiotic stresses, moisture and temperature stress are

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two most critical stresses that limit productivity of crops worldwide (Fahad et al. 2017).

It has been observed that the crops like wheat, rice, soybean and maize witness drastic reduction in yield with every degree rise in and the reduction is highest for the maize (Zhao et al. 2017). An increase in temperature of 2 °C reduced maize yield by 13% (Cairns et al. 2013). Traditionally, selection is focused on the sporophytic phase of crop plants. But sporophytic selection has many limitations. It requires large population size and consumes larger space in order to obtain the combination of desirable attributes needed to improve a crop and hence is labor intensive and time consuming. To overcome these bottlenecks gametophytic selection can be capitalized in maize that offers the opportunity to introduce early selection in breeding programs (Singh et al. 2017).

In diploid angiosperms, haploid gametophytes, such as pollen and ovules, are derived from diploid sporophytic generation. The male gamete is particularly vulnerable to high temperature at all the stages of development compared to female gametophyte (Hedhly et al. 2009). Male sterility and the impairment of pollen development have been the main factors for reduced seed set and productivity under high temperature stress (Sakata and Higashitani 2008; Burke and Chen 2015). The haploid gametophytic phase has been shown to be relatively more responsive to selection and considered ideal, under certain circumstances, to exercise selection for desirable alleles for crop improvement (Clegg et al. 1978; Hormaza and Herrero 1996; Ravikumar et al. 2007). Many evidences support the gametophytic selection for both biotic and abiotic stress tolerance (Laughnan and Gabay 1973; Zamir et al. 1982; Searcy and Mulcahy 1985; Clarke et al. 2004; Dominguez et al. 2005; Ravikumar et al. 2003). For example selection pressure enforced at gametophytic stage improves sporophytic performance.

Applying environmental stresses to developing gamete is a possible means to alter allele frequency in order to select for resistance plant (Ottaviano et al. 1991). A transgenic reconstructive approach using tobacco pollen provides direct proof that selection acting on male gamete development can alter the allele frequency of the sporophyte (Touraev et al. 1995). Direct selection on gametophytes avoids dominance issues of sporophytes. Selection pressure for temperature has been applied during the gametophytic generation in several studies (Chi et al. 1999; Clarke et al. 2004; Dominguez et al. 2005; Frova et al. 1995; Maisonneuve and Den Nijs 1984; Bajaj et al. 1992; Zamir et al. 1982) and proved successful for increasing the frequency of tolerant progeny (Hormaza and Herrero 1996; Ravikumar and Patil 2004). Frascaroli and Songstad (2001) observed a consistent and marked improvement in maize for chlorosulphuron resistance at the end of second sporophytic generation. Similarly, Chikkodi and Ravikumar (2000) proved that pollen selection was effective in increasing the resistant individuals in sunflower for *Alternaria* leaf and stem blight resistance after two generations of selection. In fact, so far there is no investigations have clearly demonstrated that the gamete selection have persistence of positive response for heat tolerance in the succeeding generations.

Hence, the major objective was to study the effect of pollen selection for heat tolerance in F_1 generation on the phenotype and genotype of F_2 populations.

Materials and methods

Experimental material

The heat susceptible inbred line BTM14 was crossed to heat tolerant BTM6 to produce hybrid seeds (Singh and Ravikumar 2017). The hybrid seeds were identified using the molecular markers and selfed to produce two sets of F_2 populations.

The F_1 seeds were grown in field during kharif, 2016 and selfed using two different methods as given below to produce CF_2 and TF_2 populations (Fig. 1).

CF_2: The F_1 plants were grown in the field and the pollen grains dehisced on the same day were collected in the morning hours (8.00–9.00 A.M.) from each plant and brought to laboratory immediately and transferred to 0.5 ml PCR tubes. The PCR tubes were incubated at 25 °C temperature for 3 h and used for selfing the F_1 plants to produce seeds of control F_2 and labelled as CF_2 population.

TF_2: The F_1 plants were grown in the field and the pollen grains dehisced on the same day were collected in the morning hours (8.00–9.00 A.M.) from each plant and brought to laboratory immediately and transferred to PCR tubes. The PCR tubes were incubated at 36 °C for 3 h in thermocycler and used for selfing the F_1 plants to produce seeds of tested F_2 and labelled as TF_2 population.

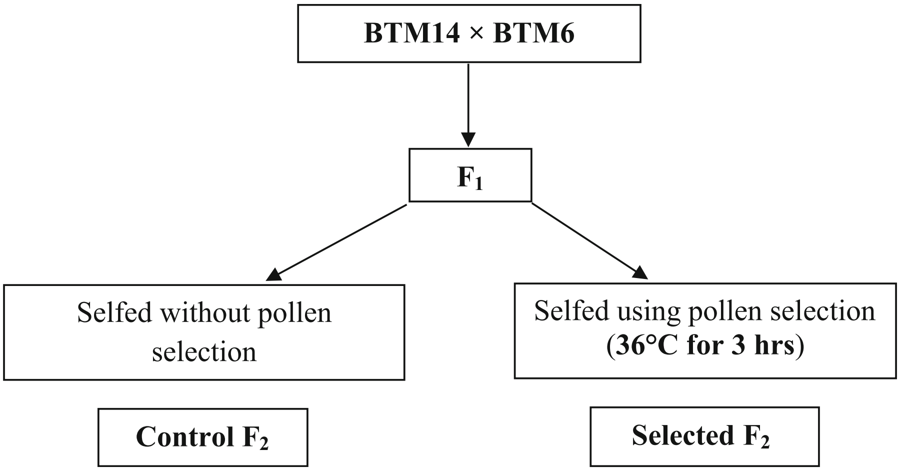

Fig. 1 Development of control and selected F_2 populations

Yield performance of CF_2 and TF_2 population

The CF_2 and TF_2 were grown in summer 2017 at the farm of GKVK, Bengaluru. The plants were grown in 3 replications of 8 rows each alternating CF_2 and TF_2 and each row containing 15 plants. The two border rows and border plants in each row were planted with bulk hybrid seeds to reduce the border effect. All the agronomic practices were followed to raise a good crop. From each F_2 population 100 plants were selected from all the replications randomly and allowed for open pollination.

Another sets of 150 plants were selected randomly each from CF_2 and TF_2 populations to study the pollen heat tolerance. At anthesis the freshly dehisced pollen grains from each of the F_2 plants were collected in the morning hour (8.00–9.00 A.M.) and immediately brought to the laboratory and transferred to 0.5 ml PCR tubes. For each F_2 plant two tubes were prepared and incubated at 36 °C for 3 h in PCR. The heat stressed pollen grains were used for self-pollination of the same F_2 plant to determine the pollen tolerance to heat stress indirectly assessing the heat tolerance of the plant producing pollen grains. Equal quantity by volume of the heat treated pollen grains was used to self-pollinate the plant. The observations on the following traits were recorded on all the individual F_2 plants.

The following observations were recorded

1. *Plant height (cm)*: Plant height was measured on individual plants in centimeters as height between the base of a plant to the tip of the top most leaf before tasseling and to the tip of the tassel after tasseling of a plant at maturity.
2. *Anthesis silking interval (ASI)*: ASI was calculated as the difference between the anthesis and silking dates (Bolanos and Edmeades 1993.
3. *SCMR values*: SCMR values (SPAD Chlorophyll Meter Reading) was recorded from three positions of fourth leaf from top using a SPAD 502 chlorophyll meter. The average value of these three positions was taken as chlorophyll content. The chlorophyll content was measured on the same leaf on 45th, 65th and 85th day after sowing.
4. *Membrane leaching*: Cell membrane stability was tested by exposing the leaf bits to high temperature and computing relative injury to the membranes in terms of electrolyte leakage. Relative injury or membrane leaching per cent was measured using third leaf from top as given below.

   Hundred mili gram of fresh leaf discs was collected from all the randomly tested F_2 (both control F_2 and tested F_2) plants and incubated in 10 ml of distilled water (known volume) filled in a tube and kept for mild shaking for 3 h. The supernatant was decanted to cuvette and the light absorbance values were recorded at 273 nm (initial absorbance, I), using UV 1800 visible spectrophotometer. The beakers were refilled with same supernatant and transferred to hot water bath (90 °C) for 30 min. Final absorbance in decanted water was recorded at 273 nm (final absorbance, F) using spectrophotometer and the cell content leak was calculated by following formula (Leopold et al. 1981).

$$\text{Membrane Leaching } (\%) = (\text{ML}) = \left(\frac{\text{I}}{\text{F}}\right) \times 100$$

where I, initial absorbance; F, final absorbance.

5. *Number of pollen grains per anther*: The unopened spikelets (last spikelet from the bottom) which were about to dehisce the next day were collected from 100 plants each from CF_2 and TF_2 populations and kept for incubation at 65 °C for 48 h in an oven. From three positions of spikelets the anthers were carefully removed. The anthers were transferred to 1.5 ml tubes containing 1 ml of tween-20 (5% v/v). One anther per tube was maintained. The tubes were sonicated several times to release all the pollen grains into the solution. The pollen grains were thoroughly mixed by shaking the tubes. From each tube five micro litre of samples were pipetted out and poured on slide for observation. Three replications of 5 µl for each tube were used to count all the pollen grains. The total numbers of pollen grains were recorded under projection microscope EUEOMEX-HOLLAND, Model-CMEX DC.300X at magnification of 400×. The irregular shaped and transparent pollens were considered as sterile and the fully circular pollen grains were considered as fertile pollen grains. The sum of sterile and fertile pollen grains was considered as total number of pollen grains per anther.
6. *Cob length (cm)*: The cob length was measured in centimeters from the base to the tip of the cob at the time of harvest.
7. *Cob diameter (cm)*: Cob girth at the middle of the cob was measured and recorded in centimeters after harvest.
8. *Number of seed rows per cob*: The number of seed rows in each cob was counted and recorded as number of rows per cob.
9. *Number of seeds per row*: The number of seeds in the longest row of cob was counted and recorded as number of seeds per row.
10. *Number of seeds per cob*: The total number of seeds of cob was counted and recorded after harvest.
11. *Seed yield per plant (g)*: The weight of total seeds obtained from all the cobs was measured in grams as seed yield per plant.

Allelic diversity analysis of control and tested F_2 populations

One hundred F_2 plants each from CF_2 and TF_2 population along with parental lines were chosen for isolation of DNA. The DNA was isolated by modified CTAB method (Doyle and Doyle 1987). The DNA of parental lines were screened with hundred SSR primers out of which 10 are polymorphic. Four of the ten identified polymorphic primers were used to genotype the control and tested F_2 populations to compare the allelic distribution.

Statistical analysis

The mean value of all the traits of CF_2 and TF_2 populations were compared by using *t* test. The comparison of these two F_2 populations for distribution pattern was done using two sample KS test (Chakravarti 1967). Chi square (χ^2) test 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.

Result and discussion

Evaluation of control and tested F_2 populations for heat tolerance

The pollen grains of F_1 were selfed after exposing the pollen grains to high temperature stress in heating block of PCR machine. The seed yield attributes were significantly reduced in F_1 plants when the heat stressed pollen grains were used for pollination confirming the effect of heat stress on pollen quality and fertilization ability (data is not given).

High temperature affects maize through its negative effects at critical phenological stages in the crop growth and development. Stage-specific sensitivity and significant genotypic variability were reported mainly at flowering stage (Cicchino et al. 2010b). Flowering dynamics, plant growth rate and biomass partitioning to reproductive organs are mainly affected under pre-antheis stress leading to barrenness in plants, while abortion of fertilized structures and reduced ear growth rate lead to reduction in seed number, thereby affecting yield (Cicchino et al. 2010a). In this background CF_2 and TF_2 populations were evaluated for seed yield and yield related traits under stress (pollen stressed) and control (no stress) environments.

The CF_2 and TF_2 populations were compared for growth and physiological traits before giving the heat stress to the plants. The mean plant height at harvesting stage was 181.90 ± 3.54 cm in TF_2 population while 166.70 ± 4.12 cm in CF_2 population. This result indicated that TF_2 population has more vigorous growth than that of TF_2 population. Similarly the mean SCMR values (55.03 ± 0.54, 60.26 ± 0.36 and 49.27 ± 0.68) at all the three stages (45, 65 and 85 DAS) were significantly higher in TF_2 population compared to CF_2 population (Table 1). The SCMR values represent the greenness of plant and indirect related with chlorophyll content. In the heat stress tolerant plants less cholorophyllase activity is the reason behind the higher chlorophyll content. The higher chlorophyll content is an indicator of plant stress tolerance (Kebede et al. 2012). It is reported that various antioxidant enzymes showed positive correlation with chlorophyll content (Almeselmani 2006). Our results showed that gamete selection in F_1 has improved greenness and vigour of F_2 population. The membrane leaching the TF_2 population had significantly lower membrane leachates (11.57 ± 0.46%) compared to CF_2 population (13.19 ± 0.31%) (Table 1). It means TF_2 population is more resistant for leakage of solute. Sung et al. (2003) reported that thermostability of plant cellular membrane should be a target trait for improving the high temperature tolerance in maize. The maintenance of cellular membrane function under high temperature stress is essential for a sustained photosynthetic and respiratory performance (Chen et al. 2014).

Earlier studies in maize reported that heat stress leads to pollen sterility during the early reproductive stages such as YM (Young Microspore stage), 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 et al. 2004). Heat also affects pollen production and viability. Even continuous heat, over several days before and during pollen-shed, results in only a fraction of fertile pollen being formed, probably because of the reduced sugar available. In addition, high temperature reduces the period of pollen viability to a couple hours (or even less). Further, high temperature also reduces the pollen shedding (Tom 2011). So the total number of pollen grains per anther is an important parameter that is affected due to high temperature leading to decreased number of pollen grains per anther. The TF_2 population recorded significantly higher pollen number (4646.78 ± 264.91) than CF_2 population (Table 1). Our result showed that the TF_2 population had the significantly higher pollen grains per anther suggesting its ability to produce more number of pollen grain per anther.

The CF_2 and TF_2 plants were randomly chosen to selfed with heat stressed pollen grains to study the effect of pollen selection. The mean values were significantly reduced for seed yield and yield components of populations of both plants (Fig. 2). The mean value of cob diameter is significantly higher in TF_2 population (1.53 ± 0.04 cm) as against CF_2 population (1.32 ± 0.03 cm). The TF_2 population had significantly higher mean values for total number of seeds

Table 1 Mean values of growth, physiological and pollen parameters of CF_2 and TF_2 population in field condition

Trait	Mean values		KS test
	CF_2	TF_2	
Plant height	166.7 ± 4.12	181.9 ± 3.54**	NS
SCMR (45 DAS)	52.43 ± 0.56	55.03 ± 0.54**	S
SCMR (65 DAS)	58.27 ± 0.39	60.26 ± 0.36**	S
SCMR (85 DAS)	45.96 ± 0.79	49.27 ± 0.68**	S
Membrane leaching (%)	13.19 ± 0.31	11.57 ± 0.46**	S
Number of pollen grains/anther	3321.89 ± 176.70	4646.78 ± 264.91**	S

NS non significant, *S* significant

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

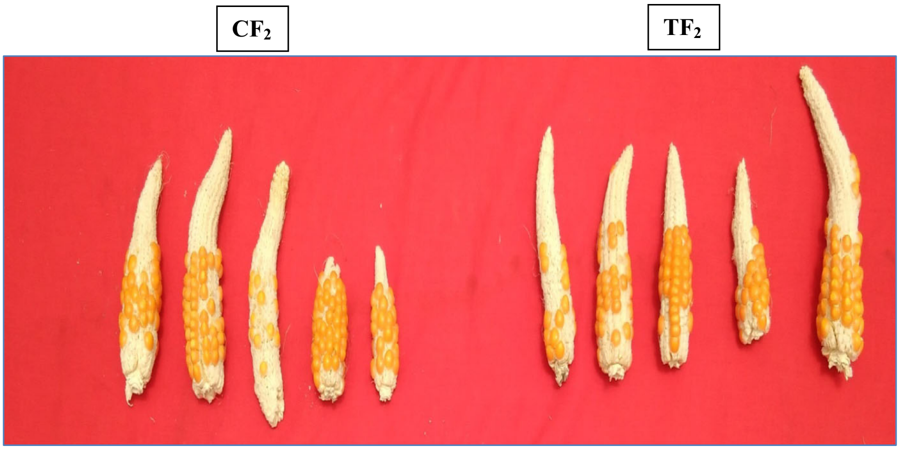

Fig. 2 Effect of heat stress on maize cobs of both CF$_2$ and TF$_2$ populations

per cob (8.55 ± 2.66) and seed yield per plant (2.02 ± 0.72 g) as compared to CF$_2$ population (Table 2). A number of reports support this result, for instance. The agronomic performance of the selected progenies was found to be superior as evidenced by higher grain yield, greater seedling vigor, and reduced stalk and root lodging. These results demonstrated that exposure to elevated temperature during gamete function can influence the heat-stress tolerance of pollen in the following generation. The overall increased seedling vigour, higher seed yield along with increased pollen tolerance to high temperature of TF$_2$ population suggest the overlap of gametophytic and sporophytic heat stress tolerance. So, the gamete selection improves the frequency of genes associated with useful agronomic traits (Zamir 1983).

The KS test also suggested that the distribution of CF$_2$ and TF$_2$ populations were significant different for cob diameter, number of seeds per row and seed yield per plant (Tables 1, 2, 3). This indicates that the TF$_2$ population had comparatively more number of plants having higher cob diameter, number of seeds per row, seed row length and seed yield per plant than CF$_2$ plants. The means of yield and yield attributes of TF$_2$ population is shifting toward higher yield through channelizing genes for heat stress (Fig. 3).

The CF$_2$ and TF$_2$ populations were also used to compare under no stress condition for yield parameters. The mean cob diameter of TF$_2$ population (2.29 ± 0.04 cm) is significantly higher than the CF$_2$ population (1.98 ± 0.05 cm). Also the TF$_2$ population had significantly higher cob length (14.76 ± 0.35 cm), number of seeds per row (23.76 ± 0.74), seeds per cob (265.35 ± 12.57), test weight (23.81 ± 0.69 g) and total seed yield per plant (59.53 ± 0.69 g) (Table 2). Landi et al. (1989) observed a good seed set in pollen selection derived maize lines under favorable growing condition and also similarly, Mulcahy et al. (1978) noticed the effect of gamete selection applied in the F$_1$ generation changing the genetic composition of F$_2$ generation.

Table 2 Mean values of seed yield and yield components of CF$_2$ and TF$_2$ populations under no stress conditions in maize

Traits	Mean		
	CF$_2$	TF$_2$	KS test
Cob Diameter (cm)	1.98 ± 0.05	2.29 ± 0.04**	S
Cob length (cm)	12.28 ± 0.36	14.76 ± 0.35**	S
Number of seeds per row	20.65 ± 0.75	23.76 ± 0.74*	S
Number of seeds per cob	229.57 ± 12.93	265.35 ± 12.57*	NS
Test weight (g) (100 seed weight)	22.13 ± 0.63	23.81 ± 0.69*	NS
Seed yield per plant (g)	50.88 ± 3.09	59.53 ± 0.69*	S

NS non significant, *S* significant

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

Table 3 Mean values seed yield and yield components of control and tested F_2 populations under stress condition in maize

Trait	Mean		
	CF_2	TF_2	KS test
Cob diameter (cm)	1.32 ± 0.03	1.53 ± 0.04**	S
Number of seeds per cob	2.69 ± 1.02	8.55 ± 2.66*	NS
Seed yield per plant(g)	0.38 ± 0.08	2.02 ± 0.72*	S

NS non significant, *S* significant

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

The results of present study showed that gamete selection can modify the genetic composition of the sporophytic generation and improve many quantitative traits associated with vigour of the plants and also productivity. Ottaviano et al. (1982) and Ottaviano and Mulcahy (1986) compared the progenies derived from moderate or intense gametophytic selection in maize and found that the intense gametophytic selections resulted in higher seedling weight, longer roots and heavier kernels. The results under normal condition of two population shows the non-negative impact of pollen selection on the non-target traits and it could be selectively use to improve our trait of interest.

Allelic diversity analysis of CF_2 and TF_2 populations using molecular markers

Four polymorphic markers were used to screen the CF_2 and TF_2 populations of the same cross BTM14 × BTM6. The polymorphic markers showed segregation in both control and tested F_2 populations (Supplementary plate 1). 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 CF_2 was non-significant for all four markers, suggesting that no segregation distortion from the expected (1:2:1) ratio. The normal Mendelian segregation of SSR markers indicate that the parental lines and F_2 genotypes tested for this study is ideal. On the other hand the TF_2 population showed significant deviation from Mendelian monohybrid ratio (1:2:1). All the four markers showed the more number of homozygote male parent alleles are significantly more than the expected number. In none of the markers, the frequency of homozygote individual with female alleles was more than expected. The results clearly indicate that in TF_2, the population skewed towards the alleles that are contributed by the male parent for which the selection was made (Table 4). The deviation from normal mendelian segregation and skewness towards the alleles selected from male parent provide a strong, molecular genetic evidence that gamete selection increase the frequency of favored alleles in the progeny. The gamete selection can be a useful means of shifting allelic frequencies in the 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 (Ottaviano et al. 1988).

The results clearly indicate that the gamete selection was able to favor those gametes containing alleles for heat tolerance, at the DNA level thus emphasizing the potentiality of this method. It could permit rapid transfer of an allele conferring heat tolerance into elite germplasm by subjecting the heterozygous plants to gamete selection. The effectiveness of gamete selection for simply inherited traits was also revealed in tomato (Zamir and Gadish 1987) tobacco (Touraev et al. 1995), maize (Frascaroli and Songstad 2001) sorghum (Ravikumar et al. 2003) and chickpea (Ravikumar et al. 2007). Most recently Ravikumar et al. (2013) observed the deviation in expected segregation ratio in gamete selection derived progenies demonstrating the utility of gamete selection for biotic stress in increasing the frequency of resistant plants and resistant alleles in the progeny of next generation. The feasibility of this approach seems to be promising for hastening the incorporation of desirable alleles or QTLs in a short time for complex polygenic trait also, like heat tolerance in maize.

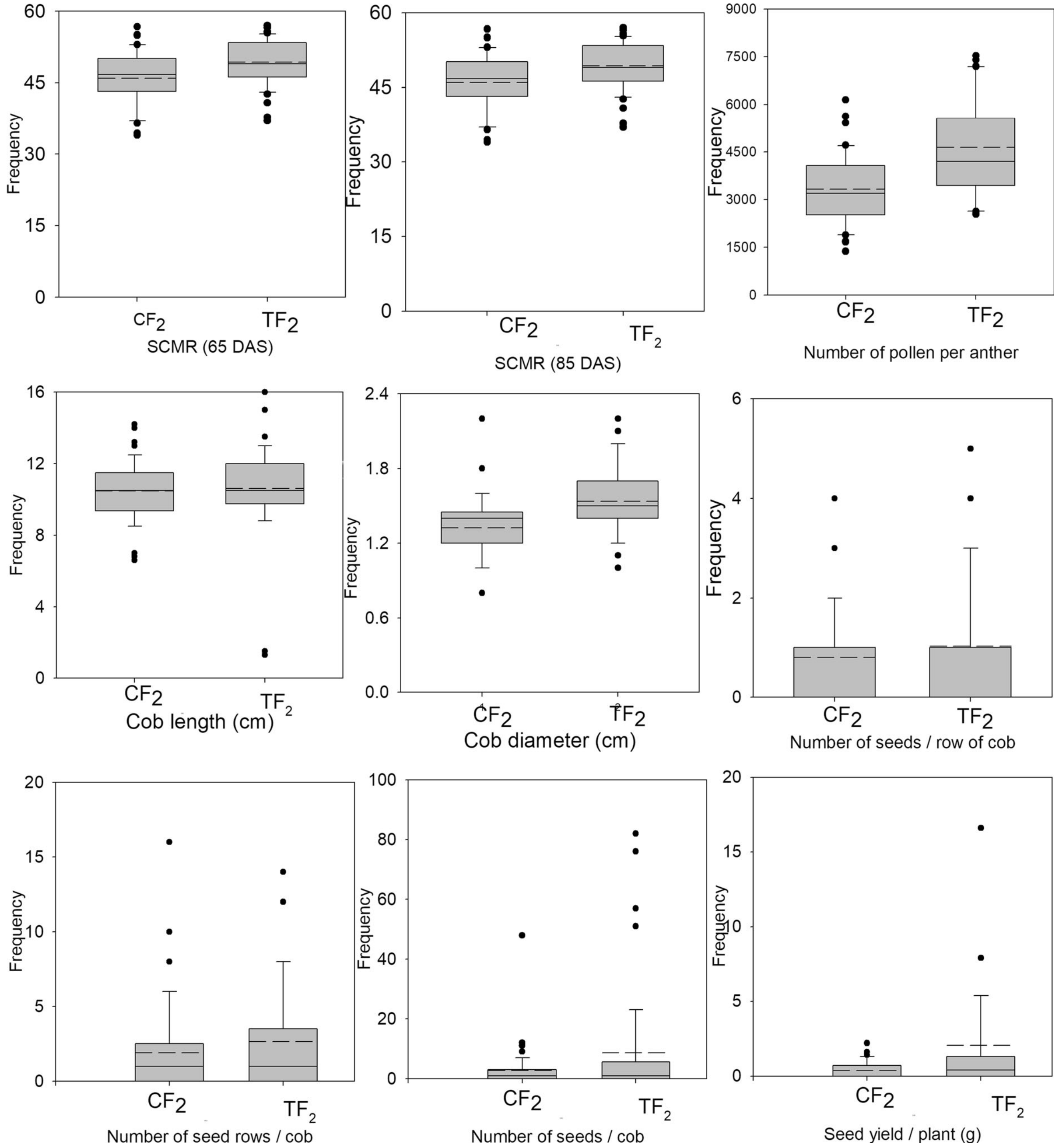

Fig. 3 Distribution pattern of physiological parameters, pollen parameters and seed yield of CF_2 and TF_2 populations under stress condition in maize

Conclusion

We can conclude that the effectiveness of selection for agronomic traits at the gamete level could be a function of the genotypes used. The evaluation of the correlated response to the gamete selection has allowed the detection of significant variations for many sporophytic traits; in particular, an increased adaptability of the plants to heat stress in less and also the gamete selection channelized the variability with non-negative impact on other traits.

Table 4 Segregation of SSR markers in control and tested F_2 populations of maize

Primer name	CF_2				TF_2			
	BTM 14 allele	Hybrid allele	BTM 6 allele	χ^2 (5%) 1:2:1	BTM 14 allele	Hybrid allele	BTM 6 allele	χ^2 (5%) 1:2:1
mmc 0401	27 (25)	54 (50)	19 (25)	NS	18 (25)	44 (50)	38 (25)	S
umc2362	30 (25)	45 (50)	25 (25)	NS	17 (25)	45 (50)	38 (25)	S
umc2315	20 (25)	46 (50)	34 (25)	NS	17 (25)	46 (50)	37 (25)	S
bnlg1887	23 (25)	56 (50)	21 (25)	NS	18 (25)	37 (50)	45 (25)	S
Total	100	201	99	NS	70	172	158	S
Mean	25	50.25	24.75	NS	15	43.25	41.75	S

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