

Article

Reproductive Cold Stress Tolerance in Sorghum F₁ Hybrids is a Heterotic Trait

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Abstract: The sensitivity of sorghum to pre-flowering cold stress, resulting in reduced pollen viability and poor seed set, is a major constraint for expanding growing areas into higher altitudes and latitudes. Nevertheless, compared to juvenile cold tolerance, reproductive cold tolerance in sorghum has received much less attention so far, and very little is known about its inheritance in F₁-hybrids. We have composed a representative factorial ($n = 49$ experimental F₁-hybrids) for a comprehensive study on heterosis and combining ability for crucial tolerance traits as spikelet fertility (*panicle harvest index*), seed yield and pollen viability, using field trials in stress- and control environments in Germany and Mexico as well as climate chamber experiments. Our results indicate a heterotic and rather dominant inheritance of reproductive cold tolerance in sorghum, with strong effects of female general combining ability (GCA) on F₁-hybrid performance in our material. These findings, together with the comparatively low contribution of specific combining ability (SCA) effects and high heritability estimates, suggest that robust and efficient enhancement of reproductive cold tolerance is feasible via hybrid breeding.

Keywords: reproductive cold tolerance; sorghum hybrid breeding; combining ability; heterosis; pollen fertility; spikelet fertility; panicle harvest index

1. Introduction

The sensitivity of sorghum (*Sorghum bicolor*(L.) Moench) as an originally tropical C₄-plant to temperatures below 15 °C substantially obstructs its successful implementation into both high-latitude temperate climates and tropical high-altitude areas [1]. Early juvenile development [2] and pre-flowering reproductive stage [3] are considered the most critical growth stages. While several recent studies have targeted enhancements of juvenile cold tolerance (e.g., [3,4]) and shown its heterotic character [5,6], comparably little research has focused on reproductive cold tolerance of sorghum to date. However, for adaption of sorghum into temperate climates such as Central Europe, this trait is at least equally important [7]. While farmers can opt for later sowings to avoid juvenile cold stress (even though on the expense of the available growth period and yield potential), there is no escape strategy for cold spells during reproductive stage, which can induce male sterility leading to reduced or even no seed set and grain yield.

Downes and Marshall [8] firstly described, in search of a new crossing method, the occurrence of male sterility in sorghum after a cold treatment (13 °C). Problems of meiosis in motherspore cells were described as a possible reason for this phenomenon [3]. Singh [1] measured reproductive chilling tolerance in a set of 380 accessions, and identified tolerance sources originating mainly from Ethiopia, Uganda, the USA and China. Furthermore, this was also the first study giving some information on the inheritance of this trait using factorial F₁ hybrids. Several Mexican studies [9–13] deal with

the breeding of cold tolerant grain sorghum hybrids for the Mexican High Valleys (>2000 m a.s.l.), where cold nights prevail throughout the season. In this context, Leon Velasco et al. [12] described heterosis for the traits grain yield, seed weight and seed number in sorghum F₁ hybrids under cold stress. Krishnamurthy et al. [14] evaluated the reproductive cold tolerance of sorghum under the Indian post-rainy season and suggested the trait *Panicle Harvest Index* (PHI), which represents the level of seed set per panicle, calculated as ratio between seed yield (after threshing) and whole panicle weight (before threshing) as an efficient score for spikelet fertility.

The production of a high pollen number with sufficient vitality also at lower temperature is considered to be the underlying physiological process to ensure seed set under cold stress in sorghum [11] and other tropical crops like chickpea [15] and rice [16,17]. While in the past the screening of pollen vitality traits based on staining techniques was labor-intensive and tedious, nowadays, the impedance flow cytometry [18] provides an efficient novel tool for a fast screening of a higher number of genotypes.

Since the discovery of a cytoplasmic male sterility (CMS) system and corresponding fertility restorers [19], commercial sorghum breeding is mainly focused on the development of F₁-hybrid varieties. Heterosis in sorghum is not only expressed for yield, but also for maturity [20] and abiotic stress tolerance, including juvenile cold tolerance [5,6,21]. The goal of this study is to assess whether breeding for reproductive cold tolerance can also rely on heterosis, aiming at the design of efficient hybrid breeding strategies to enhance this trait. In this regard, a better understanding of combining ability and the relation between *per se* and hybrid performance is of special interest.

2. Material and Methods

2.1. Germplasm

The plant material utilized in this study comprised a 7 × 7 factorial (half-diallele mating scheme), consisting of seven seed parent lines (*females*), seven restorer (pollinator) lines (*males*) and their 49 factorial F₁-hybrids. Regarding the females, the sterile A-lines (CMS-lines with A₁-cytoplasm) were only used to produce the F₁-hybrid seeds in hand-crosses, while their corresponding isogenic fertile B-lines (*maintainer*) were measured in the experiments. These parental lines have diverse pedigrees and are grain or dual-purpose types (100–160 cm tall) originating from a running breeding program of Norddeutsche Pflanzenzucht Hans-Georg Lembke KG (NPZ) and Deutsche Saatveredelung AG (DSV). They were selected for this study due to (i) their different levels of reproductive cold tolerance shown in former experiments, aiming at a diverse, representative selection; (ii) their similar maturity, to minimize the influence of different weather conditions at critical growth stages distorting the results; (iii) their similar panicle architecture, being all of the *Sorghum bicolor*(L.) Moench subspecies *caudatum* and *kafir* or their intermediate types with semi-compact panicles, which can possibly reduce the impact of different panicle compactness on PHI values under control conditions (for this reason, no material with extremely open or compact panicles, like *Sorghum bicolor*(L.) Moench subspecies *guinea* or *durra*, was utilized). However, due to the limited seed availability of some hybrids, the factorial was not complete in all environments.

2.2. Field Trials

Field trials were conducted at six locations (four in Germany and two in Mexico, Table 1) which represent different mega-environments. Among the German locations, Asendorf (located in NW-Germany) and Poel (a small island in the Baltic Sea) have a cool maritime climate, whereas Rauischholzhausen (located in a low mountain range landscape in Hesse) is characterized by a more continentally influenced climate with tendency to cold nights in late summer. Usually, all these locations provide harsh conditions for sorghum. However, the summer of 2018 was unusually hot in Central Europe, so that no cold stress was observed in the experiments of Poel (2nd year) and Rauischholzhausen. Gross-Gerau, the fourth German location, is located in the Upper Rhine Valley

and characterized by a warm and sunny climate, being a suitable control environment without cold stress. The locations in Mexico represent very contrasting environments to Germany, having shorter days during the growing season, but a much stronger radiation. While San Juan del Río (1920 m, federal state Querétaro) is considered to be at the altitude limit for commercial sorghum cultivation in Mexico, Texcoco (2250 m, federal state México) is a tropical high-altitude stress environment for sorghum, providing the lowest minimum temperatures of all locations.

Table 1. Overview and climate data of the different environments during the duration of the experiments (from sowing until harvest of the panicles, i.e., May until September or October).

Site	Coordinates	Altitude	Soil Type	Year	Mean Temp. (°C)	Mean Max. Temp. (°C)	Mean Min. Temp. (°C)	Absolute Max. and Min. Temp. (°C)	Precipitation (mm)
Poel (PO)	53°99' N, 11°47' E	19 m	Loamy sand	2017	16.7	20.2	13.1	29.5/6.6	301
				2018	18.7	23.2	14.2	35.6/6.4	118
Rauischholz-Hausen (RH)	50°46' N, 8°53' E	270 m	Loam	2018	18.7	26.5	10.9	37.9/−0.3	174
Asendorf (AS)	52°46' N, 9°01' E	49 m	Loamy sand	2017	16.1	21.0	11.6	31.7/5.1	611
Gross-Gerau (GG)	49°55' N, 8°29' E	90 m	Sand	2018	21.3	28.9	13.7	38.8/5.3	94 (+150 irrigation)
San Juan del Río (SJR)	20°25' N, 99°56' W	1920 m	Loam	2017	23.0	31.4	14.7	36.6/11.6	326
Texcoco (TEX)	19°31' N, 98°51' W	2250 m	Loam	2017	16.4	24	8.7	27.0/−2.4	360

At all sites, the groups' parental and hybrids were grown in adjacent, but separate blocks to avoid shading of the shorter parental by the taller hybrids. Within the groups, an unreplicated randomized complete block design (RCBD) was used. Entries were grown in single rows (2.5 × 0.7 m) at Gross-Gerau, and in double rows (2.5 × 1.4 m) at all other sites, with 0.7 m row spacing and a plant density of approx. 20 plants/m². Plant protection and fertilizer application were executed following good agronomical practice. Per entry, the primary panicles of five healthy plants were covered before anthesis with a transparent Cryovac[®] bag (330 mm × 750 mm, 15 µm) (Sealed Air[®], Charlotte, NC, USA) to avoid cross pollination. These five self-pollinated panicles were considered as biological replications for further analyses. At maturity, they were harvested with secateurs and dried. The peduncles of each panicle were cut just below the first branches before determining the panicle weight. Subsequently, the *Panicle Harvest Index* (PHI) was calculated according to Krishnamurthy et al. [14]:

$$\text{PHI} = \text{grain dry weight (i.e., seed yield per panicle)} / \text{panicle dry weight (before threshing)} \quad (1)$$

Consequently, a PHI value of 0 implies absolutely no seed set, while values close to 1 indicate a high seed set. However, even assuming complete spikelet fertility, PHI will be < 1, due to the panicle raw weight. In addition, grain number was measured using seed-counter Contador (Pfeuffer GmbH, Kitzingen, Germany).

2.3. Climate Chamber Experiments

Climate chamber experiments were carried out at the IFZ Research Centre for Biosystems at the Justus Liebig University Giessen. Five representative hybrids (out of the factorial $n = 49$) with different cold tolerance and their parental lines (three females and four males) were tested under controlled cold stress conditions during the reproductive phase. Temperature (24 °C day/7 °C night) and light (13 h day/11 h night, Table 2) settings of the stress treatment were chosen to resemble cold spell scenarios during late summer. Per entry, ten plants were grown in adequate-sized pots (15 × 15 × 20 cm, one plant per pot) filled with high quality soil (Fruhsdorfer soil type NTM). After cultivation at optimal temperature conditions (30/24 °C) during vegetative growth, one half of the

plants was transferred to a separate chamber and exposed to cold stress from flag-leaf-stage (BBCH 39, described by Brooking [3] as most sensitive stage) until the start of grain filling stage (BBCH 71), while the other half of the plants remained at 30/24 °C (control treatment). Before anthesis, panicles were covered with a transparent Cryovac® bag (330 mm × 750 mm, 15 µm) to avoid cross pollination. In addition, here, the panicles of individual plants (five per treatment) were considered as biological replications. Under both treatments, plants were sufficiently watered and fertilized to exclude any other than thermal stress.

Table 2. Settings of the climate chamber experiments.

	Temperature in Celsius	Day/Night Cycle in Hours	Rel. Air Humidity
Control Treatment	30/24	16/8	60%
Stress Treatment	24/7	13/11	60%

At seed maturity, panicles were harvested and dried to score PHI and grain number per panicle as previously described.

2.4. Pollen Analyses via Amphasys® Impedance Flow Cytometry

To quantify the amount and the viability of the pollen on both stressed and not stressed plants, we used the impedance flow cytometry (IFC), manufactured by Amphasys® AG (Amphasys® AG, Root, Switzerland), as a fast and non-invasive technique. It measures electrical capacity (and hence viability) of a cell, utilizing a small microfluidic chip where the pollen grains flow through and the electric charge of the cells is measured via different radio frequencies [18].

For the field trials in Gross-Gerau and Rauischholzhausen, 21 factorial hybrids and their parental lines (three females and seven males) were analyzed by IFC, while in the climate chamber experiments, five hybrids and their parental lines (as previously described) were measured for pollen traits. Per genotype, four plants (i.e., replications) were sampled in the field trials, while in the climate chamber experiments ten plants were analyzed (five under the control- and five under the stress treatment). For sample preparation, three florets from each plant were taken in both field and climate chamber experiments. As anthesis in sorghum panicles proceeds from the tip downwards within several days, for sample collection, the panicle region just below the currently flowering florets was chosen. The florets were cut with a scissor and kept cool until IFC measurements were started shortly afterwards. Subsequently, the anthers were gently squeezed from the florets with a tweezer and transferred into a 2 mL-tube filled with 1 mL AF6-buffer. After crushing the anthers with a pipette-tip, the tube was shaken and the solution was filtered (using 100 µm filters) into a fresh 2 mL-tube. For the IFC measurements, the recommended default settings from Amphasys® based on the average sorghum pollen size were utilized, analyzing samples at 2 and 12 Mhz. However, since measurement results at both frequencies were extremely similar, only the results obtained at 2 Mhz are regarded in this manuscript. To determine the pollen amount, i.e., the total cell number including both dead and alive cells, the whole sample volume was soaked into the device and the concentration (cells/mL) was recorded until there was no change in cell concentration anymore (approx. 90 s). Furthermore, the viable pollen number was also analyzed. For this purpose, a negative control was used as dead-sample, in which the pollen had been devitalized by exposure to 95 °C for 5 min in a Thermomixer (Eppendorf Thermomixer comfort, Eppendorf AG, Hamburg, Germany). Results were analyzed with Amphasoft 2.0 (Amphasys® AG, Root, Switzerland) using the gating adjustment method, in which the negative control was used as a marker for the gating threshold. With this setting, all measuring points to the left hand of the gating threshold are recognized as dead and all dots to the right-hand site are recognized as alive.

2.5. Statistical Analyses

For statistical analyses, a general linear model was used, in which genotypes, females, males and environments (combination of location and year) were considered as fixed and replications (individual plants) as random effects. Interaction between females and males was used to compute the specific combining ability (SCA) variance [5].

The heritability was calculated as proposed by Piepho and Möhring (2007) [22] using the following formula:

$$H^2 = \frac{\sigma_G^2}{\sigma_G^2 + \frac{1}{2}vd} \quad (2)$$

where H^2 represents broad-sense heritability, σ_G^2 is the genotypic variance calculated by a random effect model considering genotype and environment as random factors, and vd is the average variance of the difference between two means.

Analysis of Variances (ANOVA) of lines vs. hybrids was utilized to test for significance of mid-parent heterosis (MPH). A Student-Newman-Keuls Test (SNK) was applied to determine genotype subsets being significantly different from one another, identifying cases of significant high-parent heterosis (HPH). The general combining ability (GCA) was calculated according to established methods [23]. The prediction accuracy of GCA for hybrid performance was described by the coefficient of determination (r^2) between the sum of parental GCA effects and actual hybrid performance [6,24]. Statistical analyses were conducted using IBM SPSS Statistics version 23 (IBM Software, Armonk, NY, USA).

3. Results

3.1. Variation for SeedSet in Field Trials

The temperature conditions and consequently the level of cold stress differed strongly among the field environments (see climate data in Table 1 and Figure 1), which is also demonstrated by the highly significant environment effect on the traits (Table 3). The environments of Texcoco, Asendorf and Poel 2017 showed a strong reduction of the average PHI compared to environments without thermal stress as Gross-Gerau, San Juan del Río and Poel 2018 (Figure 2, Tables S1 and S5). However, even under these harsh conditions, some males and hybrids still attained high PHI values. Consequently, the environments of Asendorf, Texcoco and Poel 2017 were considered as stress environments for further analyses, while Gross-Gerau, San Juan del Río and Poel 2018 were regarded as control environments.

Highly significant differences among the entries for the measured seed set traits were observed in both environmental groups. Considering lines and hybrids separately to exclude possible masking heterotic effects, differences still remained highly significant. Under stress conditions, males as a group performed significantly better than females (Table 3, Figure 2). As expected, the coefficient of variation was higher for the group stress environments (Table 3). Genotype \times environment interactions were significant for all groups of entries (lines, males, females and hybrids) and environments (stress and control field environments). For the group stress environments, genotypic variance was much higher than genotype \times environment variance, leading to high heritability estimates for all traits ($H^2 = 0.83$ in average for both lines and hybrids). In comparison, the relative impact of genotype \times environment interaction was higher for the group control field environments, and heritability was consequently somewhat lower.

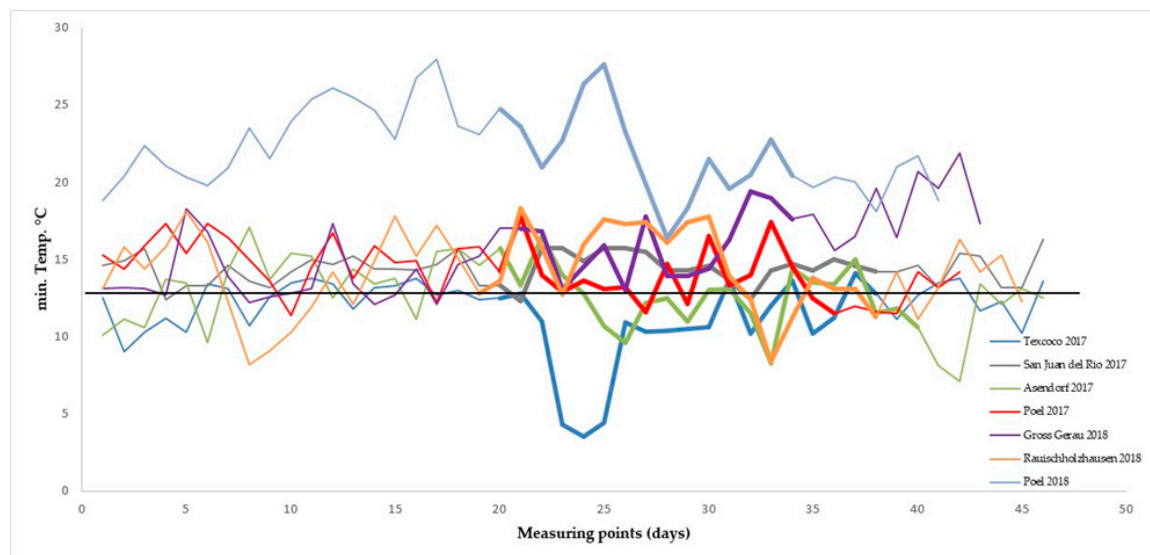


Figure 1. Course of minimum temperatures at the different environments, shown for the time period between 20 days before anthesis of the earliest entry and seven days after anthesis of the latest-flowering entry. The duration of anthesis at the respective locations is indicated by bold graphs. The black horizontal line indicates 13 °C.

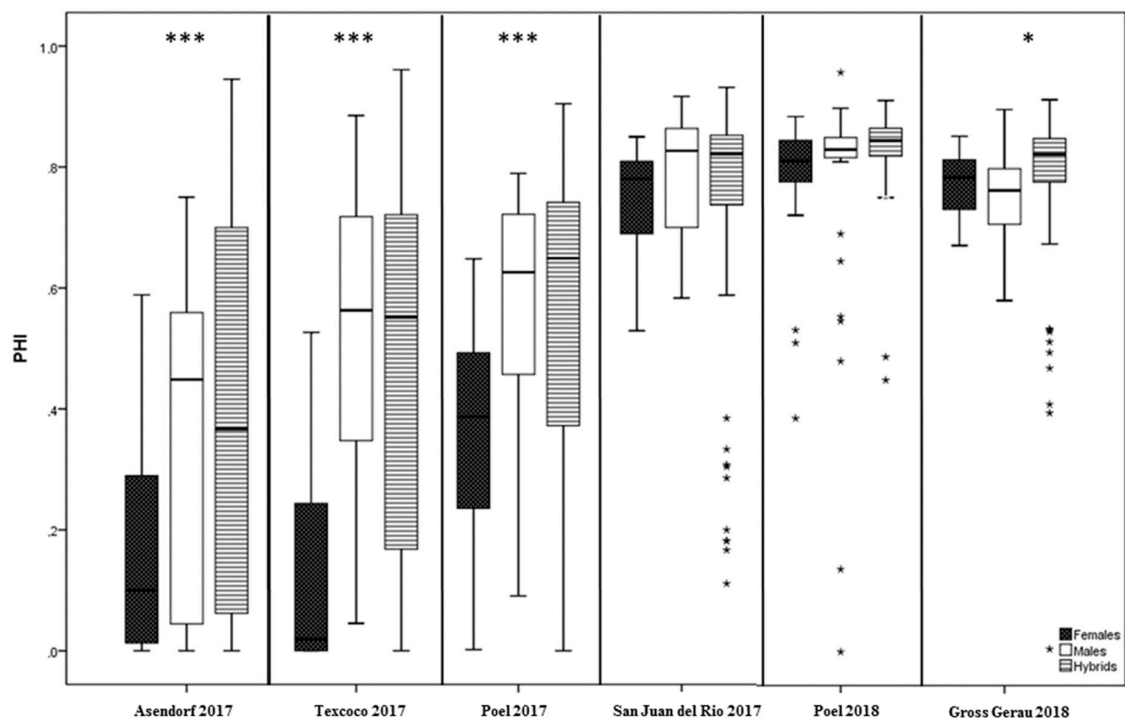


Figure 2. Boxplots showing the panicle harvest index (PHI) of female lines, male lines and their hybrids at the six field environments. * Significant at 0.05 level; ** Significant at 0.01 level; *** Significant at 0.001 level. Significance levels refer to differences between the group hybrids and lines (females and males).

Table 3. Genotypic variances (mean squares) and their environmental interaction for the traits seed yield per panicle (g), seed number and panicle harvest index (PHI). (Stress environments: Asendorf, Poel 2017, Texcoco; control environments: Gross-Gerau, Poel 2018, San Juan del Río).

Items	All Environments				Stress Environments				Control Environments			
	d.f.	Seed Yield Per Panicle (g)	Seed Number	PHI	d.f.	Seed Yield Per Panicle (g)	Seed Number	PHI	d.f.	Seed Yield Per Panicle (g)	Seed Number	PHI
Entries	61	4263.81 ***	5,520,078.53 ***	0.42 ***	54	1995.35 ***	3,475,582.26 ***	0.70 ***	61	3534.64 ***	3,700,130.29 ***	0.06 ***
CV Entries		2.18	1.93	1.05		3.15	2.80	1.95		1.37	1.15	0.31
Environments (Env)	5	59,090.35 ***	79,477,724.73 ***	9.26 ***	2	7634.70 ***	21,494,186.41 ***	1.91 ***	2	2724.82 ***	16,577,269.0 ***	0.23 ***
Entries × Env	259	689.00 ***	859,084.66 ***	0.1 ***	100	234.20 ***	459,181.84 ***	0.10 ***	110	950.32 ***	1,032,507.28 ***	0.03 ***
Error	1158	123.6	157,059.12	0.01	521	46.44	77,405.71	0.01	661	179.93	213,954.88	0.01
Heritability (H^2)		0.83	0.84	0.75		0.87	0.86	0.85		0.72	0.7	0.57
Lines	13	1159.35 ***	1,972,584.96 ***	0.36 ***	13	492.79 ***	943,314.11 ***	0.66 ***	13	1415.42 ***	1,970,893.33 ***	0.06 ***
CV Lines		1.72	1.57	1.07		2.97	2.38	2.32		1.20	1.05	0.32
Environments (Env)	5	11,348.65 ***	18,701,493.15 ***	3.04 ***	2	927.90 ***	3,878,005.31 ***	0.69 ***	2	2658.51 ***	75,731,121.65 ***	0.01
Lines × Env	61	384.38 ***	572,938.83 ***	0.08 ***	25	65.17 ***	170,723.20 ***	0.09 ***	26	519.92 ***	813,648.57 ***	0.03 ***
Error	295	64.69	118,969.66	0.01	148	14.05	33,708.51	0.01	163	104.32	184,820.95	0.01
H^2		0.65	0.71	0.77		0.85	0.8	0.85		0.85	0.8	0.85
Males	6	1501.12 ***	2,098,936.97 ***	0.49 ***	6	633.35 ***	1,132,878.41 ***	0.62 ***	6	1457.84 ***	1,646,223.05 ***	0.08 ***
CV Males		1.94	1.63	1.13		2.41	2.10	1.64		1.30	1.02	0.37
Environments (Env)	5	4172.49 ***	7,700,134.83 ***	0.92 ***	2	802.86 ***	2,288,173.25 ***	0.29 ***	2	513.53 **	3,346,600.43 ***	0.02
Males × Env	30	418.34 ***	587,861.50 ***	0.08 ***	12	85.62 ***	218,893.87 ***	0.08 ***	12	678.15 ***	933,770.85 ***	0.04 ***
Error	157	63.49	87,720.17	0.01	78	17.82	34,379.41	0.01	81	105.9	136,920.52	0.01
H^2		0.73	0.73	0.81		0.88	0.79	0.87		0.58	0.47	0.53
Females	6	964.95 ***	2,102,749.73 ***	0.17 ***	6	134.0 ***	607,000.85 ***	0.32 ***	6	1485.72 ***	2,377,031.89 ***	0.04 ***
CV Females		1.60	1.61	0.81		2.72	2.62	2.57		1.16	1.08	0.26
Environments (Env)	5	7849.99 ***	12,241,455.85 ***	2.31 ***	2	230.84 ***	1,659,332.93 ***	0.51 ***	2	3022.14 ***	6,286,193.59 ***	0.01
Females × Env	26	283.93 ***	427,133.83 ***	0.06 ***	11	36.99 ***	131,383.11 ***	0.08 ***	12	310.17 **	494,212.78 *	0.02 ***
Error	138	66.059	154,521.623	0.007	70	9.86	32,960.94	0.01	82	102.76	232,137.23	0.003
H^2		0.65	0.71	0.77		0.47	0.76	0.66		0.79	0.8	0.57
F vs. M		17.330	14,409.571	1.232 ***	1	1885.67 ***	2,060,061.57 ***	3.19 ***	1	747.27	1,455,133.58	0

Table 3. Cont.

Items		All Environments			Stress Environments			Control Environments				
Hybrids (H)	47	4312.99 ***	5,845,056.24 ***	0.44 ***	40	2228.03 ***	3,977,574.78 ***	0.70 ***	47	3411.13 ***	3,723,235.56 ***	0.06 ***
CV Hybrids		1.96	1.82	1.02		2.83	2.61	1.81		1.24	1.09	0.31
Environments (Env)	5	49,111.22 ***	62,037,528.31 ***	6.30 ***	2	6901.22 ***	17,764,234.16 ***	1.27 ***	2	2522.91 ***	12,040,802.69 ***	0.25 ***
Hybrids × Env	193	760.60 ***	938,533.24 ***	0.10 ***	73	290.83 ***	562,860.35 ***	0.11 ***	82	1047.37 ***	1,057,291.96 ***	0.03 ***
GCA (F)	6	12,602.34 ***	23,111,079.88 ***	1.89 ***	6	4359.36 ***	10,771,964.45 ***	2.62 ***	6	14,651.5 ***	19,470,675.5 ***	0.34 ***
GCA (M)	6	8273.90 ***	7,254,811.86 ***	0.30 ***	6	5752.50 ***	7,628,863.68 ***	0.64 ***	6	4299.45 ***	1,739,162.29 ***	0.02 ***
Ratio GCA F: M		1.52	3.18	6.3		0.76	1.41	4.09		3.41	11.20	14.78
SCA (F × M)	35	1015.06 ***	1,152,137.36 ***	0.07 ***	28	380.65 ***	613,548.49 ***	0.11 ***	35	1284.52 ***	1,277,323.82 ***	0.02 ***
Env × GCA (F)	30	1862.58 ***	1,507,934.05 ***	0.28 ***	12	356.82 ***	455,922.27 ***	0.18 ***	12	2804.65 ***	1,886,401.36 ***	0.09 ***
Env × GCA (M)	30	424.13 ***	1,101,327.01 ***	0.11 ***	12	232.56 ***	731,861.38 ***	0.11 ***	12	576.51 ***	836,901.45 ***	0.01
Env × SCA	133	554.90 ***	729,432.51 ***	0.05 ***	49	276.75 ***	498,152.1 ***	0.08 ***	58	733.09 ***	886,435.04 ***	0.02 ***
Error	863	143.74	170,079.27	0.01	373	59.29	94,744.02	0.01	498	204.68	223,490.68	0.01
H ²		0.8	0.81	0.74		0.83	0.81	0.85		0.67	0.69	0.58
L vs. H	1	51,085.44 ***	46,330,361.32 ***	1.1 ***	1	12,047.69 ***	17,365,106.10 ***	1.53 ***	1	38,709.27 ***	28,897,161.25 ***	0.15 ***

* Significant at 0.05 level; ** Significant at 0.01 level; *** Significant at 0.001 level.

3.2. Variation for PHI and Pollen Traits in Climate Chamber Experiments

Under climate chamber conditions, the stress treatment caused a highly significant reduction of the PHI as compared to the control treatment (0.56 vs. 0.80 in average, Table 4, Table S6), while surprisingly no reduction of pollen amount and viable pollen due to cold was detectable. Nevertheless, highly significant differences among the entries were observed for PHI and the measured pollen traits as well. However, for the control treatment, differences were only significant among the lines. For the stress treatment, variance for pollen traits was again much higher for the lines than for the hybrids, but, surprisingly, for PHI, it was vice versa (Figure 3). Genotype \times treatment interaction was significant and even higher than genotypic variance for the hybrids. Interestingly, the heritability was higher for pollen amount ($H^2 = 0.83$) than for viable pollen and PHI ($H^2 = 0.25$ and 0.40 , respectively).

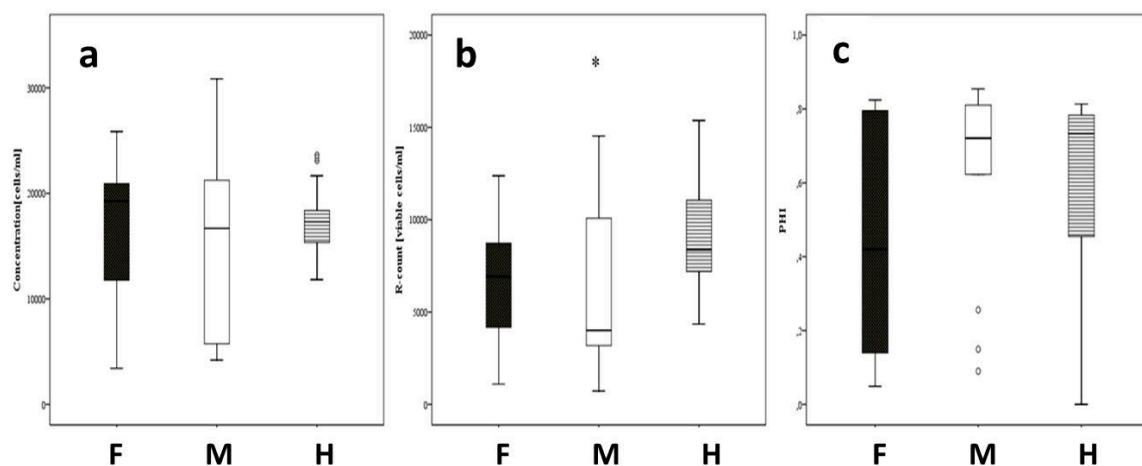


Figure 3. Boxplots showing the pollen amount (cells/mL) (a), viable pollen number (cells/mL) (b), and panicle harvest index (c) for female lines, male lines and their hybrids for the climate chamber stress treatment the ratio between PHI stress / PHI control is 0.29 for females, 0.6 for males and 0.7 for hybrids. * Significant at 0.05 level; ** Significant at 0.01 level; *** Significant at 0.001 level. Significance levels refer to differences between the groups hybrids and lines (females and males). F = female lines; M = male lines; H = hybrids

3.3. Analysis of Pollen Traits via IFC for the Field Trials in Gross-Gerau and Rauischholzhausen

Pollen traits were also scored in the field experiments of Gross-Gerau and Rauischholzhausen 2018. Since no cold stress occurred during this season, these experiments are regarded as 'control field environments as previously outlined. Nevertheless, the entries ($n = 31$) showed highly significant differences for the scored traits pollen amount and viable pollen within all groups (lines, females, males and hybrids, Table 5, Table S3). Vice versa to the climate chamber experiments, under field conditions, the heritability was higher for viable pollen than for pollen amount (Table 5).

Table 4. Genotypic variances (mean squares) and their treatment interaction for the traits pollen amount (cells/mL), viable pollen number (cells/mL) and panicle harvest index (PHI) for the climate chamber experiments.

Items	d.f.	Both Treatments			d.f.	Stress Treatment			d.f.	Control Treatment		
		Pollen Amount (Cells/mL)	Viable Pollen (Cells/mL)	PHI		Pollen Amount (Cells/mL)	Viable Pollen (Cells/mL)	PHI		Pollen Amount (Cells/mL)	Viable Pollen (Cells/mL)	PHI
Entries	11	189 mio ***	54 mio ***	0.21 ***	11	137 mio ***	41 mio ***	0.294 ***	11	87 mio ***	55 mio ***	0.034 ***
Treatment	1	70 mio *	2 mio	1.63 ***								
Entry × Treatment	11	34 mio *	36 mio ***	0.124 ***								
Error	89	15 mio	7 mio	0.016	42	10 mio	6 mio	0.026	47	20 mio	8 mio	0.007
H ²		0.83	0.25	0.40								
Lines	6	340 mio ***	83 mio ***	0.23 ***	6	232 mio ***	57 mio ***	0.27 ***	6	154 mio ***	92 mio ***	0.06 ***
Lines × Treatment	6	58 mio *	54 mio ***	0.072 **								
Error	49	20 mio	7 mio	0.022	21	114 mio	4 mio	0.029	27	27 mio	10 mio	0.01
H ²		0.83	0.27	0.66								
Males	3	519 mio ***	91 mio ***	0.104 **	3	318 mio ***	83 mio ***	0.17 *	3	270 mio **	92 mio **	0.003
Males × Treatment	3	81 mio *	75 mio **	0.1 **								
Error	26	26 mio	11 mio	0.016	10	12 mio	4 mio	0.037	16	35 mio	16 mio	0.002
Females	2	242 mio ***	77 mio ***	0.41 ***	2	2158 mio	48 mio	0.484	2	43 mio	32 mio ***	0.09
Females × Treatment	2	42 mio	3 mio	0.062								
Error	23	13 mio	3 mio	0.029	11	9 mio	5 mio	0.021	11	17 mio	1 mio	0.02
F vs. M	1	157,570	129 mio **	0.44 **	1	7 mio	257,664	0.16	1	26 mio	216 mio **	0.185 ***
Hybrids	4	8 mio	12 mio	0.22 ***	4	5 mio ***	9 mio **	0.41 ***	4	8 mio	13 mio	0.001
Hybrids × Treatment	1	69 mio *	31 mio	0.23 ***								
Error	40	10 mio	8 mio	0.009	19	11 mio	9 mio	0.013	20	9 mio	6 mio	0.005
H ²		0.83	0.25	0.40								
L vs. H	1	2 mio	23 mio	0.004	1	17 mio	70 mio *	0	1	6 mio	2 mio	0.001

* Significant at 0.05 level; ** Significant at 0.01 level; *** Significant at 0.001 level.

Table 5. Genotypic variances (mean squares) and their interactions for the traits pollen amount (cells/mL) and viable pollen number (cells/mL) across the environments of Gross-Gerau and Rauischholzhausen.

Items	d.f.	Pollen Amount (Cells/mL)	Viable Pollen (Cells/mL)
Genotype	30	57 mio ***	24 mio ***
Environment (Env)	1	82 mio ***	25 mio ***
Genotype × Environment	27	31 mio ***	9.4 mio ***
Error	144	7.8 mio	3.6 mio
Heritability (H^2)		0.41	0.6
Lines	9	22 mio ***	19 mio ***
Environment (Env)	1	96 mio ***	23 mio **
Genotype × Environment	9	14 mio **	9.5 mio ***
Error	45	4.5 mio	2.5 mio
Heritability (H^2)		0.31	0.47
Males	6	25 mio **	26 mio ***
Environment	1	49 mio **	21 mio *
Males × Env	6	8.9 mio	10 mio *
Error	30	5.8 mio	3.2 mio
Females	2	10 mio *	7.6 mio **
Environment	1	55 mio ***	2.7 mio
Females × Env	2	36 mio ***	8.7 mio **
Error	15	2 mio	1 mio
F vs. M	1	6.7 m	0.3 m
Hybrids	20	70 mio ***	27 mio ***
Environment (Env)	1	836 mio ***	6.7 mio
Genotype × Env	17	37 mio ***	9.1 mio **
GCA (F)	2	350 mio ***	176 mio ***
GCA (M)	6	49 mio ***	10 mio *
Ratio GCA F: M		7.1	17.2
Environment (Env)	1	845 mio *	6.5 mio
SCA (F *M)	12	46 mio ***	11 mio **
GCA (F) *Env	2	176 mio ***	27 mio **
GCA (M) *Env	6	22 mio *	6 mio
SCA × Env	10	19 mio *	5 mio
Error	99	9.3 mio	4 mio
Heritability (H^2)		0.41	0.6
L vs. H	1	125 mio *	17 mio

* Significant at 0.05 level; ** Significant at 0.01 level; *** Significant at 0.001 level.

3.4. Heterosis

Under field environments, hybrids as a group performed significantly better for seed set traits than lines in average (as shown by the significant line vs. hybrid effects in Table 3), indicating significant midparent-heterosis (MPH). However, the magnitude of MPH differed strongly among the environments and was much higher under stress conditions (Table 6, Table S2). Looking at the most important trait PHI in detail, an increase in stress intensity implied a higher degree of MPH, ranging from 18.3% MPH in Poel 2017 over 27.2% in Texcoco to 43.4% in Asendorf (the environment with the lowest PHI in average). Selecting the two most tolerant and the two most susceptible females and males (based on their PHI *per se* across all stress environments) and looking at the average MPH of their hybrids, susceptible × susceptible combinations showed by far the highest MPH (205.3%), followed by combinations of 'tolerant female × susceptible male (125.0%), while MPH expressed in combinations of susceptible female × tolerant male (37.4%) and 'tolerant × tolerant (31.9%) was similar to the average MPH of all factorial hybrids (31.4%).

Table 6. Heterosis, GCA prediction accuracy and Pearson's correlation (r) between line *per se* and hybrid performance for the field trials (stress environments: Asendorf 2017, Poel 2017 and Texcoco 2017; control environments: Gross-Gerau 2018, Poel 2018, San Juan del Río 2017).

Items	n	All Environments			Stress Environments			Control Environments		
		Seed Yield Per Panicle	Seed Number	PHI	Seed Yield Per Panicle	Seed Number	PHI	Seed Yield Per Panicle	Seed Number	PHI
[%] of Hybrids with Sign. HPH	47	37.5	35.4	0	18.3	14.3	4.1	18.3	6.1	0
Average MPH [%] (all MPV-Hybrid Comparison)	47	79.0 ***	55.7 ***	10.9 ***	123.3 ***	77.0 ***	31.4 ***	50.4 ***	32.2 ***	3.9 ***
GCA Prediction Accuracy (r^2)	47	0.71 ***	0.70 ***	0.73 ***	0.85 ***	0.86 ***	0.86 ***	0.75 **	0.74 ***	0.8 ***
Correlation(r) <i>per se</i> M: GCA M	7	0.57	0.36	0.66	0.83 *	0.69	0.73	0.14	−0.3	0.35
Correlation (r) <i>per se</i> F: GCA F	7	0.67	0.71	0.6	0.27	0.54	0.24	0.87 **	0.89 **	0.035
Correlation (r) MPV: Hybrid Performance	47	0.52 ***	0.47 ***	0.49 ***	0.65 ***	0.61 ***	0.45 **	0.50 ***	0.51 ***	0.075

* Significant at 0.05 level; ** Significant at 0.01 level; *** Significant at 0.001 level.

In contrast, MPH for PHI was weak, but still significant for the control environments of Gross-Gerau and Poel 2018 (4.9% and 6.5%, respectively), while, in San Juan del Río, there were even no differences between lines and hybrids (Table S2). High-parent heterosis (HPH) for PHI was observed in only a few cases and limited to the stress-environments. For the traits, seed yield per panicle and seed number, higher levels of MPH were observed, and also HPH occurred more frequently. All the same, the expression of heterosis was stronger in the stress environments (Table 6).

Under field environments, hybrids as a group performed significantly better for seed set traits than lines in average (as shown by the significant line vs. hybrid effects in Table 3), indicating significant midparent-heterosis (MPH). However, the magnitude of MPH differed strongly among the environments and was much higher under stress conditions (Table 6, Table S2). Looking at the most important trait PHI in detail, an increase in stress intensity implied a higher degree of MPH, ranging from 18.3% MPH in Poel 2017 over 27.2% in Texcoco to 43.4% in Asendorf (the environment with the lowest PHI in average). Selecting the two most tolerant and the two most susceptible females and males (based on their PHI *per se* across all stress environments) and looking at the average MPH of their hybrids, susceptible \times susceptible combinations showed by far the highest MPH (205.3%), followed by combinations of tolerant female \times susceptible male (125.0%), while MPH expressed in combinations of susceptible female \times tolerant male (37.4%) and 'tolerant \times tolerant (31.9%) was similar to the average MPH of all factorial hybrids (31.4%).

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Analyzing the pollen traits under field conditions, the expression of MPH differed from case to case (Table S4). For pollen amount, significant ($\alpha = 0.05$) line vs. hybrids effects were observed for the mean of both environments and for Rauischholzhausen solely, but not for Gross-Gerau. In contrast, for the viable pollen number, the result was vice versa, with significant MPH at Gross-Gerau but not at Rauischholzhausen. HPH for pollen traits under field conditions only occurred exceptionally (Table S4).

For the climate chamber experiments, significant ($\alpha = 0.05$) MPH was only observed for viable pollen under stress conditions. Surprisingly and in contrast to field experiments, PHI showed no significant difference in line vs. hybrid performance.

3.5. Combining Ability for Seed Set Traits

Under both stress and control field environments, all seed set traits of the factorial F_1 -hybrids were strongly influenced by general combining ability (GCA) effects (Table 3). In most cases, the female impact was stronger than the male impact, with the magnitude of female predominance being higher for the group of control environments. Only for seed yield per panicle in stress environments were male GCA effects prevalent. GCA \times environment interaction was significant in most cases, but much lower than variance explained by GCA. Effects of specific combining ability (SCA) were much weaker than GCA effects, but still highly significant, with the relative importance of SCA being higher for the group of control environments. However, SCA \times environment interaction was comparatively strong. Altogether, GCA prediction accuracy for seed set traits was high, especially for the stress environments (0.85–0.86), but still satisfying also for the control environments (0.74–0.80) (Table 6).

3.6. Combining Ability for Pollen Traits

Pollen traits of the F_1 -hybrids measured via IFC in the field environments of Gross-Gerau and Rauischholzhausen were strongly influenced by female GCA effects as well (Table 5), and the predominance of female over male GCA effects (seven-fold for pollen amount and 17-fold for viable

pollen) was even higher than for seed traits. The extent of GCA (F) \times environment interaction was much higher for pollen amount than for viable pollen. SCA effects were significant and, in their magnitude, comparable with male GCA effects. As could be expected, the GCA prediction accuracy (approx. 0.75 for both pollen amount and pollen number, Table S4) was also satisfying for pollen traits.

3.7. Relationship between *Line per se* and Hybrid Performance

The results regarding the correlation between mid-parent value (MPV) and hybrid performance, as well as between line *per se* performance and GCA, diverged among the different environments. The correlation between MPV and hybrid performance for seed set traits was higher for the group of stress environments (Table 6), especially for PHI, where no correlation was observed under control environments. The relationship between *per se* and GCA is more difficult to summarize. For the group stress environments, the correlation was much higher for the males, particularly for seed yield per panicle ($r = 0.83^*$). In contrast, for the group control environments, female *per se* and GCA were highly correlated for seed yield per panicle ($r = 0.87^{**}$) and seed number ($r = 0.89^{**}$), while no relationship was observed on the male side. Looking at the trait PHI, no significant correlation between *per se* and GCA was found across the environments. However, a high correlation ($r = 0.78^*$) between female *per se* and GCA was observed for the most stress-intense environment of Asendorf (Table S2).

Regarding the pollen traits scored under field conditions, a significant correlation between MPV and hybrid performance was only observed for viable pollen at Rauischholzhausen ($r = 0.47^*$). Interestingly, this trait also showed a high correlation between female *per se* and GCA ($r = 0.82$, Table S4).

3.8. Correlations between the Different Experiments

Pearson's correlations among the measured seed set traits and groups of field environments are shown in Figure 4, separately for lines and hybrids to avoid overestimations due to heterosis.

In most cases, there was a medium to high correlation between both environmental groups (stress and control environments) for the particular traits, with higher correlations observed for hybrids than for lines. Furthermore, the different seed set traits were highly correlated with each other within the groups as was to be expected. Looking at the locations in detail, correlations among the different stress environments (Asendorf, Texcoco, Poel 2017) were generally high, whereas correlations among the single control environments (Gross-Gerau, San Juan del Río, Poel 2018) were lower and less consistent (Figure S1). This finding coincides with the relatively higher amount of genotype \times environment interaction observed for the group of control environments. Regarding the correlation for PHI between field and climate chamber experiments, a high and significant correlation was observed ($r = 0.87^{***}$) between field stress environments and climate chamber stress treatment, and only a weak and insignificant ($r = 0.13$) for the controls. Furthermore, a significant and medium high correlation ($r = 0.58^*$) was also found between viable pollen under the climate chamber stress treatment and PHI under field stress environments (Table S7).

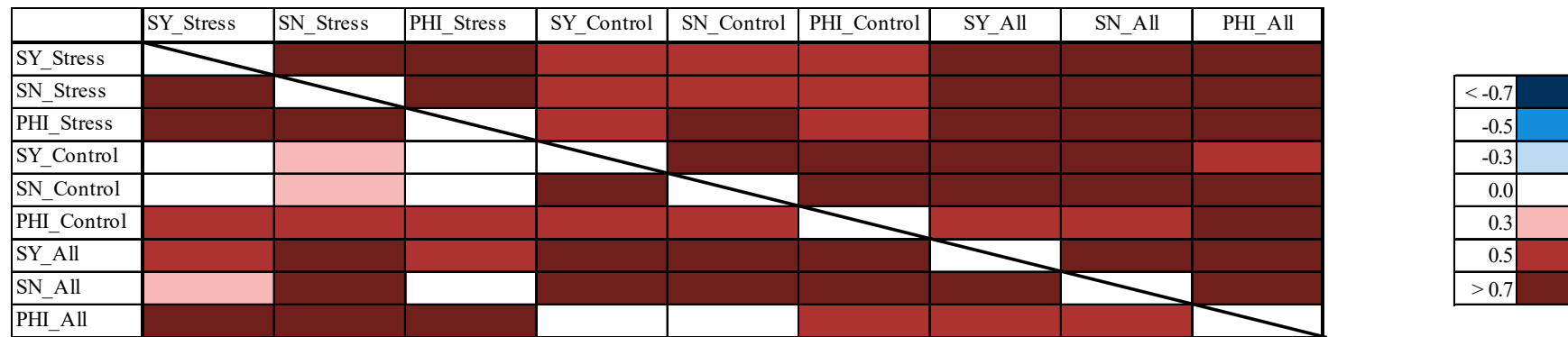


Figure 4. Heat map showing Pearson's correlation for the measured traits for lines (below the diagonal) and hybrids (above the diagonal). SY = Seed yield per panicle; SN = seed number; PHI = panicle harvest index.

4. Discussion

4.1. Heterosis for Reproductive Cold Tolerance

The results of this study show the heterotic character of reproductive cold tolerance in sorghum using a broad data set spanning seed set traits of covered panicles to avoid distortions by cross pollination and including the use of impedance flow cytometry for analysis of pollen traits. To our knowledge, it is only the second study tackling this topic (after [12]), limiting comparison of results. However, heterosis for reproductive cold tolerance has been shown for the related crop rice as well [25], suggesting the same response also for sorghum. A profound dissection of genetic causes underlying heterosis for abiotic stress tolerance is beyond the scope of this study. However, the higher levels of mid-parent heterosis observed in hybrids with susceptible parents than in tolerant \times tolerant combinations coincide with dominance theory. Nevertheless, overdominance theory, stating that heterozygosity is generally advantageous, allowing for a better adaptability to different environments and a more efficient protein metabolism [26], can still play a role and explain the few cases of high-parent heterosis. Since hybrids generally have bigger organs compared to lines [27,28], it could be speculated that this may also apply for anther size, facilitating more pollen production.

Our results confirm the findings of León-Velasco et al. [12] regarding a general heterosis for seed yield under cold stress. However, the level of mid parent-heterosis (MPH) for seed yield (47% and 29%, depending on the set of hybrids) reported in their study was much lower than the magnitude of MPH observed in our stress environments (123.3% in average). Besides differences in experimental set-up (they measured open-pollinated panicles), these divergent levels may be explained by the different background of parental lines. While the authors in [12] utilized only lines adapted to the cold Mexican highlands, our study covered the diversity for cold tolerance in the parental lines, and the inclusion of susceptible lines facilitated a higher expression of MPH due to dominance effects. The higher MPH observed in cold stress environments than in control environments (MPH of 123.3% vs. 50.4% for seed yield and 77.0% vs. 32.2% for seed number) results from the coaction of two heterotic traits, (i) spikelet fertility and (ii) higher number of spikelets. MPH for spikelet fertility, which is most accurately described by the trait PHI, was low in control environments (3.9%), while it reached 31.4% in the group 'stress environments' in average. Hence, heterosis for seed yield and seed number in control environments relied principally on bigger panicles (Figure 5) with a higher number of florets, while, under cold stress, spikelet fertility enhanced the heterotic potential of panicle size. Physiologically, the better spikelet fertility (PHI) of hybrids can be explained by heterosis for viable pollen number, as shown in the climate chamber stress treatment via impedance flow cytometry (IFC). Even though the receptivity of the pistil can also suffer from severe cold stress [13], there is consensus that a high amount of viable pollen guarantees high seed set in sorghum [11] and rice [17].

However, from a breeder's point of view, hybrid performance as sum of mid-parent value and heterosis is decisive and high-parent heterosis (HPH) of more interest than MPH. In contrast to MPH, HPH was less commonly expressed in our study, and for seed yield, the percentage of hybrids expressing significant HPH was the same for stress and control environments (18.3%). Probably, expression of HPH under cold stress was limited by too strong differences between the respective parents, and, in general, a lower performance of the females (Table 3).

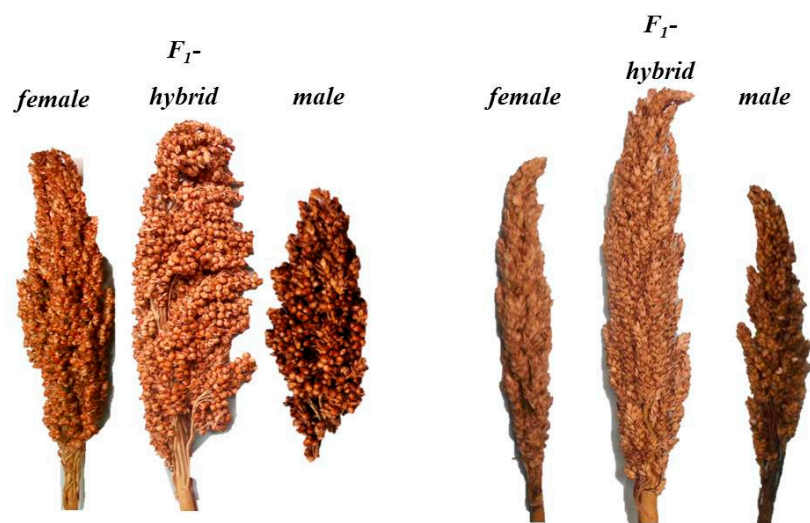


Figure 5. Panicles before threshing from the environment of Texcoco, showing a cold tolerant (left) and a cold sensitive hybrid (right) and their respective parental lines.

4.2. Combining Ability for Reproductive Cold Tolerance

Both seed set and pollen traits were predominantly influenced by female general combining ability (GCA) effects, while male GCA and specific combining ability (SCA) effects played only a minor role. Prevalence of female impacts in F_1 -hybrids were higher for spikelet fertility (PHI) than seed yield traits, suggesting female determination of pollen fertility regardless temperature conditions, while the seed yield potential (spikelet number) is also influenced by the male parent. The 17-fold higher female GCA effect on viable pollen number observed in this study (Table 5) clearly confirms this assumption.

Unfortunately, the literature on combining ability for spikelet or pollen fertility is very limited. While Dane et al. [29] found pollen fertility in tomato hybrids under heat stress to be mainly determined by GCA effects, a strong influence of SCA effects on pollen and spikelet fertility in rice hybrids was reported in some studies (e. g. [30]), contrasting the results of our experiments. However, the GCA/SCA ratio is also known to depend on the genetic diversity of the parental lines, with a high genetic distance between the female and male group enhancing GCA effects [31], and Ram et al. [32] described also the predominance of GCA effects for reproductive cold tolerance in rice.

4.3. General Mode of Inheritance for Reproductive Cold Tolerance

All in all, our results point at a rather dominant inheritance of reproductive cold tolerance in sorghum hybrids. This finding is supported by the observed mid-parent heterosis for PHI and viable pollen number under cold stress, with comparatively higher levels of MPH in crosses including susceptible parents, and the only medium correlation between mid-parent value and hybrid performance. In addition, Singh [1] reported reproductive cold tolerance as a dominant trait but admitted a wide range of variation of cold tolerance in the offspring of a susceptible \times tolerant cross. On the other hand, the strong predominance of GCA over SCA also indicates some additive gene action in our material. However, as previously outlined, this might mainly reflect a high genetic distance between the female and male pool [31] and cannot be generalized in consequence.

4.4. Implications for Hybrid Breeding on Reproductive Cold Tolerance

The strong prevalence of GCA effects on reproductive cold tolerance in sorghum hybrids is good news for breeders, since it facilitates the identification of suitable parents and reduces the amount of necessary crosses to identify hybrids with superior tolerance [31]. Further positive aspects include the environmental stability of GCA (as shown by the comparatively low GCA \times environment interaction,

Table 3) and the high heritability estimates ($H^2 > 0.85$ for hybrids), altogether suggesting the feasibility of a robust breeding progress.

The predominance of female impacts obviously suggests giving priority to enhancements of the female pool. Unfortunately, the development of new female lines tends to be neglected in sorghum breeding, since the backcrossing of new material with maintainer-reaction into existing sterile seed parent lines is time-consuming and of uncertain outcome, due to frequently occurring issues regarding CMS-stability [33]. Hence, the variation in breeding programs is usually much higher among the restorers [34], and many of the currently used females trace back to US *kafir* germplasm [34] with limited general cold tolerance [35]. In addition, in our study, the level of tolerance observed among the females was inferior to the males. Sources of reproductive cold tolerance can be mainly found in tropical highland accessions [1], but obviously these landraces would need conversion to photo insensitivity and improvements in important agronomic traits before using them as hybrid parents in temperate breeding programs. The results regarding correlation between female *per se* and GCA were not concordant over the environments in our study, so that we cannot provide a clear recommendation on how strict breeders should select for *per se* performance prior to conducting GCA tests. However, when evaluating new maintainer lines, practical breeders will need to pre-select anyway based on *per se* before initiating backcrossing and conducting first GCA tests several cycles (years) afterwards. Supporting the efficiency of this procedure, Mendoza-Onofre [9] reported a high correlation ($r = 0.68^{**}$) between female *per se* and GCA for grain yield under cold.

In spite of the higher female impact observed in this study, the restorer pool should not be completely disregarded, since expression of the desirable high-parent heterosis seems only possible when the differences in stress tolerance between the parentals are not too extreme. Moreover, the yield potential of sorghum hybrids is not only determined by the female-influenced spikelet fertility, but also by spikelet number. For seed yield as the most important trait agronomically, the male impact under stress was even slightly higher (Table 3), and the high correlation between male *per se* and GCA ($r = 0.83^*$, Table 6) facilitates selection for suitable restorers.

4.5. Suitable Screening Methods for Reproductive Cold Tolerance

Regarding screening methods, the PHI is the most suitable and reliable trait for the observation of reproductive cold tolerance in sorghum [14], providing the best approximation for spikelet fertility [36,37] by reducing the effect of different spikelet numbers. While spikelet fertility is the crucial trait when evaluating lines seeking for tolerance sources, for hybrid evaluation, the seed yield also obviously needs to be measured, since the PHI indicates pollen fertility, but not the yield potential which is also determined by spikelet number. For a proper evaluation of cold tolerance in a nursery or trial, where lots of genotypes with different tolerance levels and flowering times are grown, the covering of panicles before anthesis is mandatory to avoid overestimations by cross pollination. Impedance flow cytometry (IFC) facilitates the analysis of pollen traits, allowing for a better understanding of the physiological backgrounds. In this regard, an improved knowledge about the most sensitive growth stage would be beneficial for an adequate timing of the stress treatments. As for all studies on abiotic stress tolerance, the choice of adequate selection environments is essential. Under Central European conditions, a sequence of cold nights during the critical pre-flowering stages can induce severe yield losses on sensitive genotypes. However, usually these stress conditions do not occur there steadily, and are preceded and followed by intervals of warmer weather, which complicates the comparability of tolerance levels among genotypes with different flowering times. Hence, tropical highland areas with more constant night temperatures are interesting selection environments, which, in contrast to climate chamber experiments, allow for screening of a high number of genotypes under natural conditions. One important aspect to be taken into account is that there is no clear temperature threshold for pollen sterility induction. Brooking [38] observed a linear decrease of pollen fertility from 14 to 5 °C, describing pollen sterility induction as a quantitative response and not a qualitative one occurring below a specific temperature threshold. Downes and Marshall [8] used 13 °C night

temperature in their phytotron experiment, while, for our material, 7 °C night temperature in climate chamber experiments provided a satisfying variation. In our field experiments, an average night temperature of approx. 13 °C during the critical phase as in the environment of Asendorf was sufficient to induce severe stress reactions on the same material, underlining the well-known problems in the comparison of field and controlled climate chamber experiments, since problems in the seed set can also be enhanced by other factors under natural conditions. Among our field environments, Texcoco in the Mexican highlands had the lowest minimum temperatures (Figure 1). Nevertheless, PHI was lowest in average in Asendorf, showing that other factors than only minimum temperatures play a role. For the maritime high-latitude environments of Asendorf and Poel 2017, lack of radiation and suboptimal daily temperatures of frequently < 20 °C induced constant stress conditions, while radiation was not limiting in the tropical highland environment of Texcoco, and the daily temperatures were also higher there. However, in spite of these climatic differences, the genotype × environment interaction observed among the stress environments in our study was surprisingly low, and the high heritability estimates suggest good prospects for breeding of hybrids with stable reproductive cold stress tolerance.

5. Conclusions

Enhancements in reproductive cold tolerance are essential for a successful adaption of sorghum into both tropical highland areas and temperate climates. Our study indicates a heterotic and rather dominant inheritance of this complex trait. Along with the strong GCA effects and high heritability estimates, this finding suggests that efficient hybrid breeding can enable a robust breeding progress.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4395/9/9/508/s1>. Table S1. Genotypic variances (mean squares) of the measured traits separately for each field environment. Table S2. Heterosis, GCA prediction accuracy and Pearson's correlation between line *per se* and hybrid performance separately for each field environment. Table S3. Genotypic variances (mean squares) for the traits pollen amount (cells/mL) and viable pollen number (cells/mL), separately for the environments of Gross-Gerau and Rauischholzhausen. Table S4. GCA prediction accuracy and Pearson's correlation (r) between line *per se* and hybrid performance for pollen amount and viable pollen number for the field experiments of Gross-Gerau and Rauischholzhausen. Table S5. Descriptive statistical data for the field experiments. Table S6. Descriptive statistical data for the climate chamber experiments, and the scored pollen traits in the field experiments of Gross-Gerau and Rauischholzhausen. Table S7. Correlation between the field trials and the climate chamber experiments. Figure S1. Heat map showing Pearson's correlation for the reported traits for lines (below the diagonal) and hybrids (above the diagonal). SY = seed yield per panicle (g); SN = grain number; PHI = panicle harvest index; AS = Asendorf; PO = Poel; SJR = San Juan del Rio (MX); TEX = Texcoco (MX); GG = Gross Gerau.

Author Contributions: A.S. and S.W. contributed equally to this manuscript and are both listed as first authors in consequence. Devised the study, B.W. and S.W. received the funding, B.W. and R.S.; planned and oversaw the field trials and climate chamber experiments, and conducted the data analysis, A.S.; interpreted the results and wrote the manuscript, A.S. and S.W.

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