Male parent effects on stigma receptivity and seed set of sorghum A-lines under chilling field temperatures

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With 3 tables

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Abstract
Sorghum hybrid seed production involves restorer (R) and maintainer (B) lines as pollen donors. In the Mexican Highlands, R-lines produce more viable pollen than B-lines. The influence of this characteristic on stigma receptivity and seed set (seeds/flowers) of A-lines is unknown. Six pairs of A/B lines and four R-lines were sown. In the A-lines, length of stigma, style and ovary, stigma receptivity and seed set were registered. In the B- and R-lines, pollen traits (production, diameter and viability) were measured. Crosses A × R were made, and the A × B isogenic crosses served as controls. There were differences (P ≤ 0.05) among genotypes for all variables. Stigma receptivity traits averaged over the A × R crosses were lower than those of the A × B crosses. Seed production traits of the A-lines varied depending upon the R-line crossed to, but the highest seed set was 0.37. Pollen characteristics of the B- or R-lines did not correlate with stigma receptivity and neither with seed production nor with seed set of the A-lines.

Key words: cold tolerance — hybrid parental lines — seed set — stigma receptivity — Sorghum bicolor

The reproductive systems of sorghum (Sorghum bicolor L. Moench) include self-pollination, open pollination, cytoplasmic male sterility and even apomixes, which contribute to the adaptation of this species to a wide range of environments (Rao et al. 2002). The ecological male sterility is widely present, and it is a generalized phenomenon in sorghum, because temperatures below 12°C decrease pollen production and viability, especially in cold-sensitive genotypes (Wang et al. 2000, Osuna-Ortega et al. 2003). According to McLaren (1997), pollen viability of cold-sensitive genotypes is significantly reduced by preflowering chilling temperatures (≤16°C). In contrast, cold-tolerant (CT) genotypes adapted to the Mexican Highlands where chilling temperatures (8–12°C) prevail during microsporogenesis can produce up to 90% of viable pollen (Mendoza-Onofre et al. 2006). Stigma receptivity depends on the state of maturity of the flower (Weigang et al. 2006) to allow pollen germination (Shivanna and Sastrı 1981, Dafni et al. 2005). On this regard, seed set (harvested seeds number/flowers number) summarize the final reproductive success as a result of the combination of stigma receptivity, pollen viability and pollen quantity traits.

Germination failures of viable pollen in sorghum could be due to a fast senescence of the stigma (Sun et al. 1991). Problems in pollen–pistil compatibility can also cause the death of the embryo (Wilcock and Neiland 2002, Hodnett et al. 2005). Even in the presence of normal female organs, poor pollen viability decreases pollen germination and the growth of pollen tubes, so seed number per panicle may be reduced (Marshall and Diggle 2001, Cisneros-López et al. 2010). Therefore, a practical indicator to measure pollen–pistil compatibility problems is also a seed set trait.

Reproducer lines (R-lines) and maintainer lines (B-lines) are involved as male parents of the male sterile line (A-line, female parent) in the production of hybrid sorghum seeds. The development of the Mexican CT sorghum lines (León-Velasco et al. 2009) was started in 1980, when a set of 250 lines derived from accessions of India and Africa (donated by ICRI SAT at CIMMYT to the Colegio de Postgraduados) were crossed to cytoplasmic male sterile and cold-sensitive A-lines having the CMS-A1 system (Milo-Kafir) (donated by the University of Nebraska, USA). Only five of these lines showed a clear B CT response at the Mexican Highlands, because they produced more than 70% of grain in selfed panicles under field conditions, so their corresponding A isogenic lines were then developed. From all possible crosses among the five B-lines carried out in 1986, individual selection (pedigree) was applied based on amount of grain in selfed panicles, plant height, panicle size, exertion length, grain colour, grain size and earliness. In every cycle, the selected plants were backcrossed to A-plants from the previous cycle, until a set of 40 new A and B CT elite lines with better agronomic traits than their ancestors were defined in 1992. At the same time, the 50 R-lines were evaluated in field trials until 20 of them were selected with the same criteria used for the B-lines.

The CT R-lines adapted to the Mexican Highlands are more vigorous than the B-lines (León-Velasco et al. 2009), and they also produce a greater amount of pollen (Cisneros-López et al. 2009). However, the influence of the cold tolerance trait on stigma receptivity, number of seeds per panicle and on seed set, when crossed to A-lines, is unknown.

In this study, hand pollinations were made under field conditions, to (i) evaluate the effect of the type of male parental lines (B or R male fertile lines) on stigma receptivity, seeds per panicle and seed set of A-lines; and (ii) relate these variables with pistil phenotypic traits of the female line (A-lines) and with pollen traits of the male lines (B- and R-lines).

Materials and Methods
Six isogenic A/B pairs (A1/B1, A2/B2, A3/B3, A5/B5, A6/B6 and A9/B9) and four restorer lines (R14, R17, R19 and R22) were sown in
Montecillo, State of México (19º29’N, 98º54’W, 2240 m altitude), under irrigated field conditions. These lines are considered representative of the B- and R-lines in our Sorghum Breeding Programme. Sowing was done in May and harvest in October 2006. Each line was sown in plots of five rows, 5 m long and 0.92 m wide, with one plant for every 15 cm along a row. At heading, the panicles of 30 healthy, uniform and fully competitive plants per plot of the three types of lines (A, B and R) were covered with paper pollination bags. Once all the stigmas of each A-line had been exposed (end of flowering), 20 plant to plant crosses were carried out between the A- and R-lines coinciding in flowering. Two crossing groups resulted: Group I (An × R; n = A1, A2, A3, A5, A6 and A9), in which the six A-lines were crossed to two R-lines (R14 and R17); and Group II (An × R; n = A5 and A9), where two A-lines (A5 and A9) were crossed to the four R-lines. The same number of plant to plant crosses (A × B) was carried out in each isogenic A/B pair, which was used as control. Temperature and relative humidity were recorded at a weather station located 150 m from the experimental site.

**Pistil traits in A-lines and pollen characteristics in B- and R-lines.** The methods used to determine the following variables are described in detail in Cisneros-López et al. (2009). A brief description follows: At the end of flowering of the male sterile lines, 10 pistils were taken from the central branch of each panicle, and the length (mm) of pistil (PsL), stigma (StL), ovary (OvL) and style (StL) was measured. From the male fertile pollinator lines (B- and R-lines), at the onset of anthesis a branch from the middle of the panicle of five plants was excised and fixed in FAA (3.6% formaldehyde, 5% acetic acid and 50% ethanol in distilled water); pollen was extracted and dyed with aniline blue. Pollen grain diameter was measured (Gpd, μm) in a Zeiss® Axioskop 2 plus; Carl Zeiss SMT AG Co., Oberkochen, Germany) coupled with an AxiosVision 4.4 image processor. Viability of pollen (VP, %) was measured in 10 anthers per branch (average of five optical fields of 20 grains per anther) by counting pollen grains showing normal spherical shape and a cytoplasm density over 75%. The total pollen production per panicle (PP, mg) was measured in individual panicles of five plants of the male fertile lines during the flowering period.

**Effect of the male parent on stigma receptivity, seeds per panicle and seed set of male sterile lines.** A full description of these traits assessment can be found in Cisneros-López et al. (2010). A summary follows: Three panicles were selected from each A × B and A × R crosses. A central branch of each panicle was cut 18 h after pollination and fixed in a Farmer solution (3 : 1). Stigma receptivity traits were evaluated in 10 complete pistils through pollen germination and pollen tube growth in vivo, applying the ‘squashed, aniline blue and epifluorescence’ method. The fixed pistils were observed under a Zeiss® AxioCam MRC5 Axioskop 2 plus; Carl Zeiss SMT AG Co., Oberkochen, Germany) coupled with an AxiosVision filter for epifluorescence. Digital images were analysed with the image processor already mentioned. Callose of pollen tubes was observed in blue-green fluorescence. Pollen grains adhered to the stigma and pollen tubes growing in the stigma and in the ovary were counted. A pollen grain was considered germinated when the pollen tube length was equal to or greater than the diameter of the corresponding pollen grain adhered to the stigma. Each individual pistil was considered a single repetition.

At the end of flowering, five panicles of the three types of lines (A, B and R) were sampled and the number of fertile flowers per panicle (FFP, sessiles) was counted. The length of rachis (LR) was measured, and panicle density (PD = FFP/LR) was estimated in the B- and R-lines. The PD was classified into very dense, dense, medium, scarce and very scarce, according to a visual scale for sorghum descriptors (UPOV (International Union for the Protection of New Varieties of Plants) 1989). Once physiological seed maturity (presence of the black layer) (Eastin et al. 1973) was reached, five panicles per each female line were harvested from each cross and the number of seeds per panicle (SP) was registered; then the seed set was calculated (SS = SP/FFP).

**Statistical analysis.** The statistical package sas 9.1 (SAS Institute 2002) was used for data analysis. Variance analysis was conducted in a completely randomized design of treatments to (a) detect differences in the pistil and the number of flowers among the six male sterile (female parental lines); (b) contrast the pollen traits among the six maintainer lines and the four restorer lines (male parental lines); and (c) compare the pollen germination variables and pollen tube growth, seed yield, seeds number per panicle and seed set among crosses with the same or with different male parent. Variables expressed in percentages were transformed by arcsine [Yi] ± ½, before the variance analysis. Mean comparisons were made with the Tukey’s test (P ≤ 0.05). Pearson’s correlations were calculated: (i) among the pollen traits and those of stigma receptivity and seed production, and (ii) among FFP, PD, PP, Gpd and VP within each group of male parental lines.

**Results**

**Weather conditions**

At the experimental site, the annual mean temperature and rainfall were 15.9°C and 686 mm in 2006. In the last 30 years (from 1982 to 2010), maximum, minimum and medium temperature averages were 23.3, 9.9 and 16.6°C, and 483 mm of rainfall. In 2006, the monthly temperatures during the growing cycle (May–October) were 30.0, 6.7 and 14.8°C, with relative humidity averages of 99.2%, 43.3% and 71.3%, and 455 mm of accumulated rainfall.

**Pistil traits in A-lines and pollen characteristics in B- and R-lines**

The analyses of variance (data not shown) indicated an effect of the genotype (P < 0.01) on the number of flowers per panicle and on all traits related with pistil morphology in the male sterile lines, and also on pollen traits of the male fertile lines.

Among the male sterile lines, line A9 stands out for its greater (P < 0.05) number of flowers per panicle (2376), twice the mean number (1302) of lines A3, A5 and A6 (Table 1). No A-line was superior in all variables regarding pistil characteristics. In addition, correlations (data not shown) among pistil traits and number of flowers were not significant; for example, line A9 has small pistils, StLs and stigma, but it has large ovaries. In contrast, line A2 has large pistils combined with medium-sized ovaries and an average number of flowers.

Regarding the average number of flowers per panicle of the A/B isogenic lines and PD of the B-lines, A1/B1 and A9/B9 had dense panicles (2000–2400 flowers and 81–96 flowers per cm) while A2/B2, A3/B3, A5/B5 and A6/B6 showed medium dense panicles (1200–1700 flowers and 54–70 flowers per cm) (Table 1). The group of R-lines showed more flowers per panicle than the group of B-lines (2141 vs. 1595) with more dense panicles (86 vs. 72 flowers per cm).

Between the two types of male parental lines, the amount of pollen produced per panicle by the R-lines was almost twofold that of the B-lines (886 vs. 459 mg). The pollen grain size averaged over the four R-lines was smaller (P < 0.05) and with greater viability (P < 0.05) than the corresponding averages of the six B-lines group; nonetheless, these differences were minimal (42.4 vs. 43.9 μm for size and 81.0% vs. 79.5% for viability). Lines B9 and B1 produced three times more pollen (600 mg per panicle) than the least productive line B3 did (179 mg per panicle), but lines R14 and R22 produced almost double as much pollen (1187 mg per panicle) than the average of the best two B-lines (Table 1).
Correlations among FFP, PD, PP, GpD and VP (data not shown) varied depending upon the group of male lines. Pollen production in B-lines was associated with flowers per panicle \(\text{r} = 0.83^*\), PD \(\text{r} = 0.88^*\) and pollen grain diameter \(\text{r} = 0.89^*\), while in the R-lines, pollen production was only associated with flowers per panicle \(\text{r} = 0.97^*\) but not with PD \(\text{r} = 0.85\) nor with pollen size \(\text{r} = 0.53\). In both groups, the number of flowers per panicle was associated with PD \(\text{r} = 0.97^*\) (average), and pollen viability was not associated with flowers per panicle, PD, pollen size or pollen quantity in any group.

Effect of the male parent on stigma receptivity, seeds per panicle and seed set of male sterile lines

Averaged over the six A-lines, the male fertile maintainer B-lines were better pollinators than the male fertile restorer R14 and R17-lines for traits involved from the initial contact and adhesion of pollen grains on the stigma (26 vs. 13 vs. 7) through the final number of seeds per panicle (511 vs. 331 vs. 270) and seed set (0.31 vs. 0.20 vs. 0.16) (Table 2). However, when the number of R-lines was expanded, R19 and R22-lines performance in pollen tubes in the ovary, seeds per panicle and seed set was better than that of R14 and R 17-lines and as better as that of the corresponding controls (A \(\times\) B crosses) (Table 2).

Among controls (A \(\times\) B crosses), there was a wide variation \(P < 0.05\) in the degree of initial contact and adhesion of pollen grains from the maintainer lines on the stigma of their respective isogenic male sterile lines (Table 2); that is, the number of pollen grains adhered to the stigma in the A9 \(\times\) B9 cross (81) was five times greater than the mean of the other isogenic crosses (15). As pollen grains germinated and the pollen tubes grew, the differences among the A \(\times\) B crosses decreased. These wide variations also occurred within each group of A \(\times\) R crosses.

Discussion

Weather conditions

Temperature during the growing season of 2006 was 2–6°C cooler than the historical climatic data of the site. Despite the chilling temperatures prevailing during microsporogenesis, pollen viability averaged more than 80%. Therefore, the fertile B and R sorghum lines used in this study can be considered CT lines.

Pistil traits in A-lines and pollen characteristics in B- and R-lines

Ergot \((Claviceps africana\) Frederickson, Mantle & de Milliano) is a disease that infects non-pollinated pistils. This disease has induced the study of flower behaviour as well as of the morphological and physiological characteristics of pollen and stigma in sorghum. The significant effect of the genotype \(P < 0.01\) on the number of flowers per panicle and on all traits related with pistil morphology in the male sterile lines confirms that sorghum plants possess a wide genotypic
It is common that sorghum breeders have their B- and R-line development programmes separated, and once new potential lines are developed, their combining ability performance is tested (Menz et al. 2004). In this experiment, we found significant differences (P ≤ 0.05) between the two groups of fertile lines in reproductive traits (Table 1). As an average, the group of R-lines exceeded the group of B-lines in flowers per panicle, PD, pollen production and pollen viability (Table 1), possibly because R-lines are selected as pollen donors for hybrid seed production and so they should produce abundant fertile pollen grains. These results might anticipate a better performance of the R-lines as pollinators than that of B-lines. Sorghum pollen diameter has been reported varying from 20 to 45 μm (Chaturvedi et al. 1994, Ryley 2005); therefore, the pollen size in our male fertile lines (≥40 μm) can be regarded as a large one.

Variability within each group of male fertile lines differed according to the phenotypic trait. Less variability was found within the B- and R-lines in size of pollen grain (43.9 ± 4.7 and 42.4 ± 4.5) and VP (79.5 ± 8 and 81.0 ± 9) than in flowers per panicle (1595 ± 484 and 2141 ± 218), PD (72 ± 14; 86 ± 9) and pollen production (459 ± 167 and 886 ± 364). When B vs. R groups were contrasted, a similar variability was found for all traits, except for flowers per panicle in which B-lines showed more variability than R-lines (484/1595 = 30% vs. 218/2141 = 10%). Our results contrast with those of Menz et al. (2004) in which lesser diversity was found among elite B-lines than in R-lines. Sorghum breeders know that B-line development is more restrictive, so the incorporation of new B-lines in a breeding programme is slower than for R-lines. In addition, stable and restorable CMS response is a rare character (Rao et al. 1999), so the genetic base of male sterile A-lines is quite narrow compared to the R-lines.

Effect of the male parent on stigma receptivity, seeds per panicle and seed set of male sterile lines

Studies on the effect of the male fertile pollinator genotype on stigma receptivity and number of seeds per panicle of male sterile lines, even under manual pollination, are scarce and complex. The environmental effect on these reproductive variables depends on the crop phenological growth stage. For example, the photoperiod during the vegetative stage affects floral differentiation (Clerget et al. 2004), while chilling temperatures cause male sterility only during microsporogenesis (Brooking 1976). During the flowering period, daytime temperature and relative humidity affect the amount of released pollen (Ryley 2005), so the hour of the day should be critical when hand pollination is carried out. During the grain filling period, frosts can decrease seed number and seed size.

With the aim of attenuating the effect of some of above-mentioned factors, A × B crosses were used as control in this study, under the assumption that after eight backcrossing cycles each isogenic pair represents the nearest ideal condition of flower synchronicity and pistil–pollen compatibility of the respective A-line. Likewise, both A × R groups involved only parental lines whose flowerings were coincident, so that pollen collection and pollination of these A × R crosses could be performed at the same hour time and same days as they were performed on the A × B crosses (controls). During the pollination period, the mean relative humidity during most part of the day was 70%, thus allowing keeping the pollen grains hydrated. The first frost occurred after harvest.

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Table 2: Stigma receptivity and pollen production traits of six A-lines crossed to two R-lines (Group I), considering the corresponding A × B crosses as controls

<table>
<thead>
<tr>
<th>A-lines</th>
<th>B (n)</th>
<th>R14</th>
<th>R17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pollen grains adhered to the stigma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1 ×</td>
<td>13 b, b</td>
<td>29 a, a</td>
<td>7 ab, c</td>
</tr>
<tr>
<td>A2 ×</td>
<td>21 b, b</td>
<td>16 b, b</td>
<td>8 a, c</td>
</tr>
<tr>
<td>A3 ×</td>
<td>7 c, ab</td>
<td>8 c, a</td>
<td>4 bc, b</td>
</tr>
<tr>
<td>A5 ×</td>
<td>14 b, a</td>
<td>6 c, b</td>
<td>8 a, b</td>
</tr>
<tr>
<td>A6 ×</td>
<td>21 b, a</td>
<td>3 c, c</td>
<td>8 a, b</td>
</tr>
<tr>
<td>A9 ×</td>
<td>81 a, a</td>
<td>16 b, b</td>
<td>7 ab, c</td>
</tr>
<tr>
<td>Mean</td>
<td>26 A</td>
<td>13 B</td>
<td>7 B</td>
</tr>
</tbody>
</table>

| Pollen tubes in the stigma | | | |
| A1 × | 7 b, b | 29 a, a | 7 a, b |
| A2 × | 8 b, a | 8 b, a | 5 a, b |
| A3 × | 6 b, a | 4 b, ab | 2 b, b |
| A5 × | 6 b, a | 4 b, ab | 2 b, b |
| A6 × | 11 b, a | 2 b, b | 5 a, b |
| A9 × | 20 a, a | 4 b, b | 2 b, b |
| Mean | 10 A | 9 A | 4 B |

| Pollen tubes in the ovary | | | |
| A1 × | 2 b, a | 3 a, a | 2 ab, a |
| A2 × | 2 b, a | 3 a, a | 3 a, a |
| A3 × | 2 b, ab | 3 a, a | 1 b, b |
| A5 × | 2 b, a | 1 b, a | 1 b, a |
| A6 × | 4 a, a | 1 b, b | 1 b, b |
| A9 × | 4 a, a | 3 a, a | 1 b, c |
| Mean | 3 A | 2 B | 2 B |

| Seeds per panicle | | | |
| A1 × | 570 a, a | 272 c, b | 195 b, b |
| A2 × | 532 b, a | 580 a, a | 607 a, a |
| A3 × | 442 c, a | 396 b, a | 201 b, b |
| A5 × | 473 c, a | 393 b, a | 205 b, b |
| A6 × | 518 b, a | 76 d, c | 197 b, b |
| A9 × | 550 a, a | 270 c, b | 215 b, b |
| Mean | 511 A | 331 B | 270 B |

In each pair of lower case letters accompanying each value, the first letter serves to compare between crosses with the same male parent and different A-lines, and the second letter compares crosses with different male parent in the same A-line. The upper case letter corresponds to the mean comparison of the means of each group of crosses. Values with different letters are different (Tukey, P = 0.05).

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Assuming that seed production is an effective way to measure stigma receptivity (Dafni et al. 2005) and pollination success (Westgate et al. 2003), this group of A/B lines proved to have reproductive failures because the mean seed set was only 0.31 (Table 2), even when the male sterile lines had 1730 flowers per panicle (Table 1). In an in vitro pollen germination study, the low seed set of hybrid C212/DeKalb 28E/C213 grown under a controlled temperature of 32/22°C was attributed to a decrease in pollen production and germination (Prasad et al. 2006), but the receptiveness of the stigma was not elucidated in this study.

As we anticipated, the group of R-lines was better pollinator than the group of B-lines. Nevertheless, our results clearly indicate that the pollination success depends upon the specific R-line used in the cross. For example, when the six A-lines were crossed to R14 and R17-lines (Table 2), the average magnitude of all the measured variables was lower (P ≤ 0.05) than in the A × B crosses. However, among the four R-lines (Table 3), R19 and R22 showed a better performance as pollinators than R14 and R17-lines, because the first two lines produced a higher number of pollen tubes growing both in stigma and in ovary, as well as a higher number of seeds per panicle and seed set, than the last two lines. Despite the fact that all sampled ovaries had at least one pollen tube growing inside, the number of seeds per panicle depended on the restorer line used for crossing, and the highest seed set was below 0.40. The particular effect of chilling temperatures on stigma receptivity deserves further studies.

These results suggest the existence of interactions between gametophyte tissues (pollen and embryo sack) and sporophyte tissues (tissues of the ovary), previous to fertilization (Weternings and Russell 2004). The proportion of developed seeds with regard to the number of flowers will depend of the results of these interactions (Edlund et al. 2004), without considering the effect of low temperatures (below 12°C) on stigma receptivity, which still has to be elucidated in sorghum.

**Conclusions**

Significant differences (P ≤ 0.05) were found among A-lines for length of stigma, StL and ovary, as well as for stigma receptivity (number of pollen grains adhered and germinated in the stigma, and pollen tubes growing in the StL and ovary 18 h after pollination), number of fertile flowers, seeds and seed set per panicle. Significant differences (P ≤ 0.05) were also found among B- and R-lines for PP, and pollen diameter and viability. The A × R crosses had lower stigma receptivity traits than the A × B crosses, when compared as groups. Seed production traits in A-lines varied depending upon the male R-line that provided the pollen. Pollen characteristics of the B- or R-lines did not correlate with stigma receptivity, seed production or seed set in the A-lines.

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