Incidence of male fertility in haploid elite dent maize germplasm

Geiger HH, Schönleben M

University of Hohenheim, Stuttgart, GERMANY

In vivo haploid induction presently is a widely used tool in maize research and breeding (Geiger 2009, pp. 641-657 *in* Handbook of Maize: Genetics and Genomics, Springer). However, virtually all haploid plants are male sterile and therefore cannot be selfed. In contrast many haploids display a certain degree of female fertility if pollinated by diploids (Chalyk 1994, Euphytica 79:13-18; Geiger *et al* 2006, MNL 80:28-29). To fully restore male and female fertility, the chromosome set of haploids needs to be doubled by mitosis inhibiting substances like colchicine. But artificial chromosome doubling is laborious and colchicine is extremely toxic. Spontaneous chromosome doubling occurs only at a rate of 1-10% (Chase 1949, Proc Iowa Acad Sci 56:113-115; Beckert, 1994, pp.201-213 *in* Biotechnology in Agriculture and Forestry, Springer). In the present newsletter we are reporting about two experiments in which we determined the male fertility of haploids in a genetically broad collection of actual elite maize breeding materials. One experiment was conducted in the field (Exp. I) and one in the greenhouse (Exp. II).

Experiment I: Materials were comprised of 23 haploid (H) lines which had been development from 22 dent doubled haploid (DH) lines and 1 flint DH-line by *in vivo* haploid induction. The H-lines were as uniform as their parental DH lines but smaller and less vigorous. All H-lines had been preselected for female fertility (Geiger et al. 2006, *l.c.*). The experiment was laid out in a randomized block design with 2 replicates on single-row plots of 2.5m length at Stuttgart-Hohenheim in 2007 (Fig.2). Up to 20 seeds were drilled per row. Ten lines could not be grown in the 2nd replicate due to lack of seed. No particular stress occurred during the growing season. Male fertility was assessed on a 1-4 scale defined as follows:

Score 1: One or very few extruding anthers, unbranched tassel.

Score 2: Several extruding anthers at the central spike; few lateral branches but without extruding anthers (Fig.3).

Score 3: Multiple extruding anthers at the central spike and at most branches but no pollen release visible when touching the anthers.

Score 4: Many pollen-shedding anthers at all branches.

To avoid uncontrolled pollination, all ears were bagged before anthesis. Selfing was tried whenever a plant displayed any degree of male fertility. For this purpose, we bagged the tassel in the morning and pollinated the respective plant about four hours later. Before removing the bag from the tassel, the bagged tassel was carefully shaken to excite pollen release. No attempts were made to squeeze out pollen grains from closed anthers.

Experiment II: Materials consisted of H-plants developed by *in vivo* haploid induction from each of 70 single, three-way, double, or multiple crosses between inbred lines of the dent gene pools Iowa Stiff Stalk, Iodent, Lancaster, Longdent, and Danube. The crosses represented actual elite dent breeding material. Six H-plants per cross (420 in total) were raised in a greenhouse at Hohenheim in 10-liter pots during the summer in 2007. The experiment was laid out as a lattice design with six replicates. Forty-nine plants were removed before anthesis because they looked like heterozygous diploids. Optimal maize growing conditions (temperature, water supply, light, fertilizer) prevailed throughout the season. This was reflected in a faster growth and a taller and more vigorous stature than in the field. Male fertility assessment and selfing were conducted as in Exp. I.

To confirm haploidy, one plant per H-line in Exp. 1 and all plants in Exp. 2 were analyzed by flow cytometry (PARTEC instrument CA-II) For this purpose, tissue was taken from the youngest leaf in the 4-leaf stage. A second analysis was conducted during anthesis of all greenhouse plants with anther score 4. Tissue was taken from the top leaf. Microsatellite markers phi011, phi072, umc1153, umc1887, phi032, and phi041, located on chromosomes 1, 4, 5, 6, 9, and 10, respectively (http://maize gdb.org), were used to check the DH offspring of each successfully selfed H-plant for genetic uniformity. DNA extraction and marker assessment were performed according to standard protocols. An ALFexpress sequencer was used for visualizing the marker genotypes.

Results: In the field, 412 plants displaying the typical morphology of haploids were inspected for extruding anthers. About half of them (212 plants) conformed to anther scores 1,2, or 3. They were found in 10 of the 23 tested H-lines. We attempted to self these plants repeatedly over two or three successive days. Twenty-nine of them (7.0% of all 412 plants) belonging to 9 lines set one or more seeds per ear (Table 1). By far the highest seed set occurred in line 20, in which 10 out of 19 plants could successfully be selfed. Seed set ranged among these plants from 1 to 12 (85 seeds in total). In the other 8 lines only 1 to 2 plants per line set seed. A moderate correlation existed between anther score and seed set ($r = 0.56$, $P < 0.01$).

In the greenhouse, 248 out of 371 inspected haploid plants displayed some degree of male fertility. In all these cases, selfing was attempted in the same way as in the field experiment. Twenty-seven plants (7.3% of all tested plants) showed seed set. With three of these plants, the number of kernels per ear amounted to 23, 16, and 11, respectively, and 9 plants had 5 to 9 seeds per ear (Table 2, Figs.1 and 4). The successfully selfed plants originate from 21 parental crosses comprising all tested gene pool combinations. The correlation between anther score and seed set was only weak ($r = 0.30$ $P < 0.01$).

All visually classified haploids were confirmed by flow cytometry, and in Exp.II molecular marker analysis attested the genetic uniformity of all progenies resulting from self-pollination.

Discussion: Results confirm the pioneering work of Chase (1952, pp. *in* Heterosis. Iowa State College Press) and later reports of Zabirova *et al. (*1993, MNL 67:67), Chalyk (1994 *l.c.*) and others. Beyond that, our flow cytometry and molecular marker studies for the first time proved that the selfed plants actually were haploid and gave rise to genetically uniform progenies. It remains unclear whether the functional male and female gametes arose by meiotic restitution (Ramana and Jacobsen 2003, Euphytica 133, 3-18) or by chromosome doubling due to endomitosis in meristematic cells of the reproductive tissue leading to diploid sectors in the gametophyte (Chase 1952, *l.c.*).

In both of our experiments, we found significant genetic variation for male fertility and selfing success in haploids. Recurrent selection (RS) for these characteristics therefore seems promising. This is supported by results of Zabirova *et al.* (1993, *l.c.*) who obtained a remarkable increase of male fertility after four generations of selection for male fertility in a Russian dent population. Thus breeders might not need to go back to accessions from the world collection to improve their elite gene pools in this regard.

In conclusion, our studies indicate that in the long run breeders might be able to improve the degree of male fertility in haploids to an extent that would allow them to eventually abolish the laborious and hazardous artificial chromosome doubling step of the present maize DH technology.

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Table 1. Number of successfully selfed plants and means across these plants for anther score and number of kernels per ear in nine haploid dent lines; Exp.I, Hohenheim 2007

Line	Number of plants		Means across success-fully selfed plants		
	Total	Successfully selfed	Anther score (1 - 4)[a]	No. of kernels per ear	Plant height (cm)
20	19	10	3,6	8,5	92
2	11	1	1,6	2,0	93
8	12	1	2,8	2,0	92
14	10	2	1,3	2,0	63
22	23	1	-[b]	2,0	92
1	10	2	1,0	1,0	66
7	11	1	1,0	1,0	87
18	19	2	1,1	1,0	93
21	11	2	1,0	1,0	109

[a] For score definitions see text.
[b] Missing value.

Table 2. Anther score, number of kernels per ear, and plant height of successfully selfed haploid plants yielding at least five kernels per ear; Exp.II, Hohenheim 2007

Plant no.	Origin[a]	Anther score (1 - 4)[b]	No. of kernels per ear	Plant height (cm)
1	Da/La	3	23	162
2	Io/La	3	16	172
3	Ld/SS	3	11	160
4	Ld/SS	4	9	133
5	Ld/Io	4	8	173
6	Ld/Io	2	6	113
7	NN/SS	1	6	138
8	La	2	6	183
9	SS/Ld	2	6	125
10	Io/La	1	5	123
11	Io/La	3	5	151
12	Io/La	1	5	180

[a] Da = Danube, Io = Iodent, La = Lancaster, Ld = Longdent, SS = Iowa Stiff Stalk, NN = Unknown.
[b] For score definitions see text.

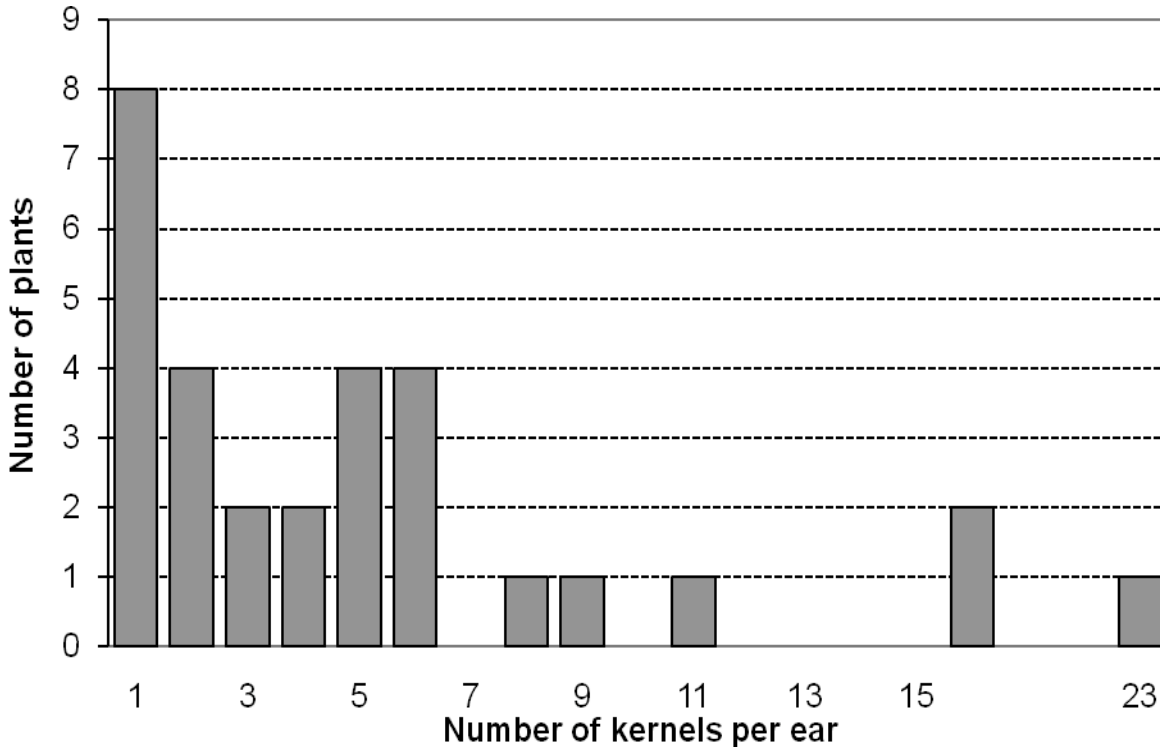
Figure captions

Figure 1. Frequency distribution of the number of kernels per ear obtained from the successfully selfed plants in Exp.2; greenhouse, Hohenheim 2007

Figure 2. Haploid lines in the field compared with a vigorous hybrid; Exp.I, Hohenheim 2007

Figure 3. Detail of a tassel of a haploid plant with anther score 2; greenhouse, Hohenheim 2007

Figure 4. Ears of two selfed haploid plants (no. 1 and 2 in Table 2); Exp.II, Hohenheim 2007



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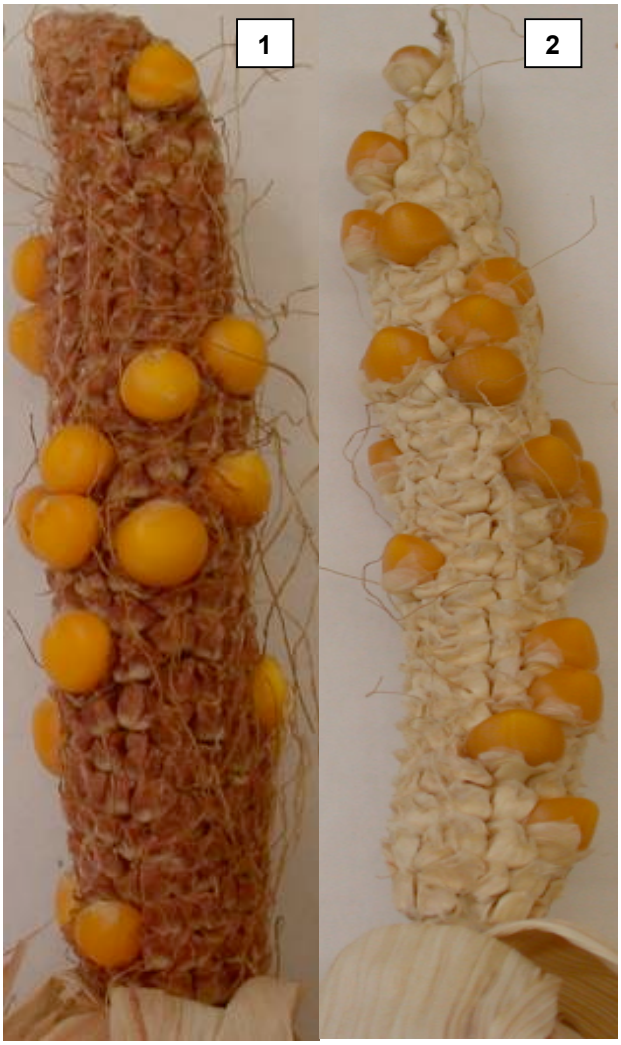

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