Reproductive Efficiency in Reciprocal Crosses of *Arachis duranensis* and *A. stenosperma* with *A. hypogaea* cv. NC 6[1]

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ABSTRACT

The wild species germplasm resources of *Arachis* are potentially valuable for improving disease and insect resistance in *A. hypogaea* L. Improving cultivars through interspecific hybridization is restricted because of reproductive barriers and/or genetic incompatibility with many *Arachis* spp. A description of reproductive efficiency in reciprocal crosses between wild and cultivated *Arachis* species is needed to clarify potentials for germplasm utilization. This study documents reproductive efficiency using the diploid species *A. duranensis* (K 7988) and *A. stenosperma* (HLK 410) in reciprocal crosses with *A. hypogaea* cv. NC 6. A significant parental effect was observed among crosses and NC 6 was more successful when used as the female parent. Differences in total reproductive efficiency were not observed between the two wild diploid species. However, when *A. duranensis* was used as a female parent embryos aborted at a high frequency. In contrast, the reduced efficiency observed with *A. stenosperma* was due to lower fertilization. As attempts are made to utilize the genetic resources of *Arachis*, different approaches will be needed to overcome reproductive barriers which restrict introgression of potentially desirable traits.

Key Words: Interspecific hybridization, fertilization timing, abortion, peanut, groundnut

Arachis hypogaea L. is the only member of the genus *Arachis* which is cultivated to any appreciable extent. Improvement of this species through interspecific hybridization and subsequent introgression of potentially useful genes from wild *Arachis* species is severely impeded because of reproductive barriers and/or genetic incompatibility. Several species have been hybridized with *A. hypogaea* (see Stalker and Moss (27) for review), but most are incompatible with the cultivated peanut (6). Our understanding of the reproductive barriers and genetic incompatibilities in interspecific crosses of *Arachis* is limited. In *Arachis*, compatible crosses include interspecific hybrids between closely related species which are at the same ploidy level. An example is *A. hypogaea* X *A. monticola*. Marginally-compatible crosses include most intrasectional crosses where a limited number

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of hybrids can be obtained (20, 23, 27). An example of marginal crosses is *A. hypogaea* by most diploid species of section *Arachis*. Crosses between *A. hypogaea* and the 40 or more species outside section *Arachis* are completely incompatible.

Significant differences between cultivated and wild species (15, Pattee and Stalker, unpub. data) have been observed for ovary component structure, onset and rate of peg growth, and presence of starch grains in the embryo sac. In addition, embryology and associated anatomical changes in *A. hypogaea* have been extensively investigated by Reed (18), Banerji (1), Smith (21, 22, 23), Conagin (4), Gerassimova-Navashina (5), Periasamy and Sampoornam (17) and Pattee and Mohapatra (13). Embryo development in other *Arachis* species has been reported by Halward and Stalker (7) and Pattee et al. (15), who found differences in growth rates between cultivated and wild taxa; but Bharathi and Murty (3) found no significant differences. Although information on embryo development in hybrids between cultivated and wild taxa has appeared in the literature (8, 9, 12), a description of reproductive efficiency, which we define as the percentage of growing or viable reproductive tissues, and embryo abortion has not been published.

This study documents reproductive efficiency using *A. duranensis* and *A. stenosperma* in reciprocal crosses with *A. hypogaea* cv. NC 6. A comparative basis for evaluating reproductive efficiency in marginally-compatible crosses between *Arachis* species where egg apparatus-fertilization failure or embryo abortion occur in the ovary is thus provided.

Materials and Methods

Two diploid ($2n=2x=20$) species, *A. duranensis* Krap. et Greg. *nom. nud.* (K 7988, PI 219823) and *A. stenosperma* Greg. et Greg. *nom. nud.* (HLK 410, PI 338280), were used to make reciprocal crosses to the tetraploid ($2n=4x=40$) *A. hypogaea* L. cv. NC 6, a large-seeded virginia type peanut. *Arachis duranensis* is an annual and was originally collected in northern Argentina. *Arachis stenosperma* is a perennial and was originally collected on the eastern coast of Brazil. Both wild species have a similar A genome whereas *A. hypogaea* has AB genomes. Plants were grown in a greenhouse at North Carolina State University, Raleigh, NC, from May through July, 1989 and April through July, 1990 using boxes filled with a growth medium of one part sand, one part commercial potting mixture, and one part top soil. The plants were fertilized regularly with a soluble nitrogen-phosphorus-potassium (20-20-20) fertilizer. Landplaster was applied as a source of calcium, which is necessary for embryo development.

Control flowers were tagged with numbered bands and allowed to self-pollinate. Inflorescences were than collected 5 and 10 days after anthesis. Flowers to be crossed were emasculated ca. 18 h before anthesis and hand-pollinated between 8:00 A.M. and 8:30 A.M. the morning of anthesis. Thirteen flowers were hand-pollinated and tagged with numbered bands for each of the 10 sampling stages. Anthesis stage (A) samples were collected immediately after pollination. The nine additional stages were collected at anthesis plus 15 h (A+15), and 1, 1.5, 2, 2.5, 3, 4, 5, and 10 days after anthesis (D1, D1.5, D2, D2.5, D3, D4, D5, and D10, respectively). All harvested samples were fixed in FAA (9 pts 70% EtOH: 0.5 pt glacial acetic acid: 0.5 pt formalin) for 72 h and then stored at 5 C in 70% EtOH until they were processed for light microscopy. Specimens were dehydrated and paraffin embedded according to Berlyn and Miksche (2). Paraffin embedded tissues were sectioned at 7 µm thickness and stained with safranin-fast green. The standards used for normal *Arachis* embryo development were those described by Pattee and coworkers (13, 15). The zygote, proembryo, or embryo was classified as aborted if they showed cellular disorganization and /or collapsing and disintegration of the cell mass.

Peg length was determined from photographs taken before tissue processing. Growing and non-growing designations were made for pegs sampled between D1.5 and D10. Reproductive observations were made on six representative samples at anthesis, A+15, and D1 stages, respectively. Three to six representative samples from the designated no peg growth or developing peg groups were observed across the remaining seven sample collection times for each cross; however, in three cross by collection-time designations only one or two no-growth pegs were available. In addition, no-growth pegs were not available at D10 in the *A. duranensis*, *A. stenosperma*, and *A. hypogaea* selfs. At D10 an additional growth stage was also designated as aerial to differentiate the elongating pegs that had not entered the soil as opposed to ones under the soil.

Chi-square analysis was performed to determine statistical significance. Information on the application of Chi-square analysis as used in this study may be found in Snedecor and Cochran, Chapter 11, Section 10 (24).

Results

Fertilization and Peg-Growth Onset Visual observations indicated an immediate separation of specimens into two categories - developing peg and no peg growth. Average category length is given in Table 1 for selected developmental stages of the reciprocal crosses, and specimens collected at D4 are illustrative of each category (Fig. 1). Light microscopy observations indicated that fertilization in *A. duranensis* had commenced prior to the D1 stage, while in NC 6 x *A.*

Table 1. Observations on peg growth of *A. duranensis* and *A. stenosperma* in reciprocal crosses with *A. hypogaea* cv. NC 6.

Growth Stage	A. duranensis X NC 6	A. duranensis Self	A. stenosperma X NC 6	A. stenosperma Self	NC 6 X A. duran[a]	NC 6 X A.steno[b]	NC 6 Self
			Avg. Length (mm)[c]				
A - Ovary	1.6		1.5		1.2	1.3	
D1.5 - Developing Peg	2.6		1.7		1.8	1.5	
- No Peg Growth	1.4		(1)1.3		1.5	1.2	
D3 - Developing Peg	5.8		2.1		2.1	1.8	
- No Peg Growth	1.6		1.6		1.4	1.4	
D4 - Developing Peg	4.1		2.2		2.6	2.2	
- No Peg Growth	1.7		1.6		1.6	1.4	
D5 - Developing Peg	11.1	15.2	3.2	3.3	3.3	3.2	4.1
- No Peg Growth	1.6	1.8	1.5	1.8	1.6	2.1	1.6
D10 - Developing Peg							
Aerial	29.9	51.5	30.8	37.6	19.9	31.4	33.6
In Soil	49.0	56.1	57.3	42.2	32.3	30.8	39.2
- No Peg Growth	1.5	-[d]	1.8	-[d]	-[d]	1.4	-[d]

[a] A. duran = A. duranensis

[b] A. steno = A. stenosperma

[c] Number of observations per average is between 4 and 18 for developing peg and 1 and 18 for no peg growth.

[d] No specimen obtained

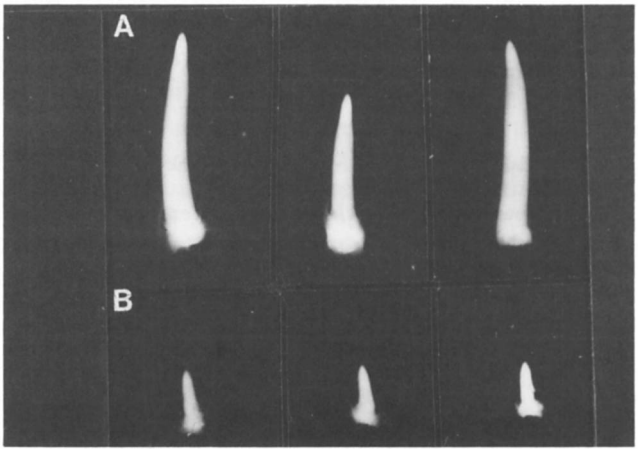

Fig. 1. Comparison of (A) normally developing peanut pegs versus (B) no growth peanut pegs four days after anthesis from the cross *A. duranensis* X *A. hypogaea* cv NC 6. Magnified 3.4 times.

duranensis fertilization commences after D1 (Table 2). In the reciprocal crosses between *A. stenosperma* and NC 6, fertilization commenced after D1.5 and was usually completed during the D2 to D2.5 period. Although the timing of fertilization is different in each of the reciprocal crosses, by the D3-D5 stages the proportion of ovules which had been fertilized are nearly equivalent among all crosses.

Table 2. Observations on syngamy timing for *A. duranensis* and *A. stenosperma* by *A. hypogaea* cv. NC 6 crosses, in reciprocal, as measured by the number of fertilized-normal plus aborted embryos at selected growth stages.

Growth Stage	A. duranensis X NC 6	A. stenosperma X NC 6	NC 6 X A.duran[a]	NC 6 X A.steno[b]
	[(Fertilized-Normal) + (Aborted Embryos)]/Ovules			
Stage D1	3+0/12	0+0/12	0+0/12	0+0/14
Stage D1.5				
Developing Pegs	4+1/ 8	0+0/ 6	10+0/10	0+0/ 7
No Peg Growth	1+2/ 6	0+0/ 4	2+0/ 6	0+0/ 7
Stages D2 - D2.5				
Developing Pegs	11+0/20	4+2/19	18+0/20	12+0/16
No Peg Growth	1+1/14	1+0/15	2+0/15	5+2/12
Stages D3 - D5				
Developing Pegs	13+4/28	13+3/28	34+1/49	23+1/28
No Peg Growth	3+3/29	1+1/18	2+1/34	3+0/16
Stage D10				
Aerial	4+6/12	3+3/10	2+0/ 4	8+0/ 9
In Soil	0+6/10	4+1/ 6	12+0/12	12+0/12
No Peg Growth	0+6/ 7	0+0/ 6	--	0+0/ 6

[a] A. duran = A. duranensis

[b] A. steno = A. stenosperma

The subjective demarcation between developing and no-growth pegs has limitations, but in the no-peg-growth category many fertilized ovules aborted their zygote before cell division could be initiated. Comparison of the average peg length data for the various growth stages (Table 1) also illustrates the difficulty of making a subjective demarcation in the growth stages up to D3.

Reproductive Efficiency General reproductive efficiency can be estimated by observing the number of developing pegs per total pegs collected from each cross because peg development suggests that the ovary has at least one fertilized ovule. Using this estimate, reproductive efficiency in reciprocal crosses of *A. duranensis* and NC 6 is lower than that of *A. stenosperma* and NC 6 (Table 3) for stages D1.5 to D5.

Table 3. Observations on reproductive efficiency of *A. duranensis* and *A. stenosperma* by *A. hypogaea* cv. NC 6 crosses, in reciprocal, as measured by number of developing pegs.

Growth Stage	A. duranensis X NC 6		A. stenosperma X NC 6		NC 6 X A.duran[a]	NC 6 X A.steno[b]	
	Developing Pegs/Total Pegs Collected						
Stages D1.5 - D5	55/124		43/64		48/89	44/77	
Stage D10							
Aerial	8/21	14/25	10/24	8/18	2/12	6/23	4/15
In Soil	5/21	7/25	4/24	10/18	9/12	12/23	11/15

[a] A. duran = A. duranensis

[b] A. steno = A. stenosperma

At stage D10 each cross has a different efficiency which appears to be conditioned by the female parent. Of primary interest is the number of pegs which obtain sufficient length to penetrate the soil because this indicates the potential to recover viable embryos directly or via embryo rescue procedures. When the wild species are used as a female parent, the data suggest a poor potential to recover viable embryos. In contrast, when NC 6 is used as the female, an efficiency value higher than 50% was observed. This suggests a moderate potential for recovering interspecific hybrid plants.

The undeterminable factors relating to recovering viable embryos by using number of developing pegs as a measure of reproductive efficiency are the timing and proportion of embryos which abort. This information can only be obtained by microscopic observation. The percentage of ovules which are unfertilized, which have aborted embryos, or ones containing developing embryos within (a) the developing peg or (b) no-peg-growth categories can provide the necessary information (Table 4). When *A. duranensis* was the female parent, only 50% of the ovules had developing embryos in the D1.5 to D5 stages. This percentage decreased to 0% by D10. Thus abortion becomes a significant factor by D10 for *A. duranensis* at which time approximately 60% of the ovules observed contained an aborted embryo.

Arachis stenosperma female parent crosses presented a different reproductive pattern than *A. duranensis*. However, the data for percent developing embryos within developing pegs across the D1.5 to D5 stages are comparatively lower because *A. stenosperma* has a delayed fertilization and does not reach its full reproductive efficiency potential until D3. This is reflected in the increased percent developing embryos in developing pegs in the soil at D10. In this cross, few pegs

Table 4. Observations on reproductive efficiency of *A. duranensis* and *A. stenosperma* by *A. hypogaea* cv. NC 6 crosses, in reciprocal, as measured by embryo status in fertilized and unfertilized ovules.

Growth stage	A. duranensis X NC 6	Self	A. stenosperma X NC 6	Self	NC 6 X A. duran[a]	NC 6 X A. steno[b]	Self
	Percent						
Stages	D1.5 - D5		D1.5 - D5		D1.5 - D5	D1.5 - D5	
Developing Pegs							
Developing Embryos	50	100	32	100	79	69	95
Aborting Embryos	9	0	9	0	1	2	0
Unfertilized Ovules	41	0	59	0	20	29	5
No Peg Growth							
Developing Embryos	12	0	5	17	10	23	0
Aborting Embryos	10	100	3	50	2	6	0
Unfertilized Ovules	78	0	92	33	87	71	100
Stage	D10	D10	D10	D10	D10	D10	D10
Developing Pegs In Soil							
Developing Embryos	0	77	66	83	100	100	92
Aborting Embryos	60	0	17	0	0	0	0
Unfertilized Ovules	40	23	17	17	0	0	8
Aerial							
Developing Embryos	33	50	30	77	50	89	86
Aborting Embryos	50	50	30	8	0	0	14
Unfertilized Ovules	17	0	40	15	50	11	0
No Peg Growth							
Developing Embryos	0	-[d]	0	-[d]	-[c]	0	-[d]
Aborting Embryos	46	-[d]	0	-[d]	-[c]	0	-[d]
Unfertilized Ovules	54	-[d]	100	-[d]	-[c]	100	-[d]

[a] A. duran = A. duranensis

[b] A. steno = A. stenosperma

[c] Single specimen obtained

[d] No specimen obtained

elongate to the point of entering the soil, but approximately 50% of the more advanced pegs provided a developing embryo of a size for possible culturing and hybrid recovery.

The use of NC 6 as the female parent had a significant impact on the reproductive efficiency of the crosses with *A. duranensis* and *A. stenosperma*. High percentages of developing embryos in developing pegs were observed in both crosses in the D1.5 to D5 stages (Table 4). By D10, 100% of the ovules in pegs which had entered the soil contained a developing embryo. Abortion was not a significant factor at any stage when NC 6 was the female parent.

Since *A. stenosperma* exhibited delayed fertilization, the counts of unfertilized ovules, aborting embryos, and developing embryos for the two sets of reciprocal crosses, observed at the two developmental stage groups (D1 and D1.5 vs D2-D10) are shown separately in Tables 5A and 5B. The two tables show distinctly different patterns. The two x two sub-table, (NC 6 as male parent with *A. duranensis* and *A. duranensis* as male parent with NC 6) by unfertilized ovules and developing embryos from Table 5A, gave a highly significant chi-square test for lack of independence ($\chi^2_1 = 34.59$, $p < .001$). The data in Table 5B allows a more extensive, but also highly significant test for lack of independence, based on the whole four x three table ($\chi^2_6 = 69.35$, $p < .001$). Tables 6A and 6B show the same data, but collapsed into two columns, NC 6 used as female parent and NC 6 used as male parent. The chi-squared test for independence of mating type and development status based on these tables are quite different. The test in Table 6A, based on only two rows (row two was deleted because there were too few aborting embryos) is borderline for significance ($\chi^2_1 = 3.88$, $p = .049$). The corresponding test in Table 6B (based on all three rows) is highly significant ($\chi^2_2 = 64.45$, $p < .001$). The alternative analysis, pooling the unfertilized ovule and aborted embryo counts, yields $\chi^2_1 = .38$, $p = .54$ and $\chi^2_1 = 9.25$, $p = .002$, respectively. Clearly reproductive efficiency as determined at stages D1 and D1.5 is not effected by whether NC 6 is used as male or female parent. Alternatively, if the determination is at a later stage (stages D2-D10), using NC 6 as a female parent gives a much higher reproductive efficiency. The two x two, unfertilized or aborting embryo vs NC 6 as male or female, show that part of this difference is due to the higher abortion rate when *A. duranensis* and *A. stenosperma* were used as female parents ($\chi^2_1 = 11.68$, $p < .001$).

A slightly different picture emerges if one collapses the four columns in Tables 5A and 5B into two columns, *A. duranensis* as a parent and *A. stenosperma* as a parent. Now the D1 and D1.5 data yields a statistically significant chi-squared value and the D2 to D10 data is not significant ($\chi^2_1 = 27.82$, $p < .001$ and $\chi^2_2 = 3.26$, $p = .20$, respectively), a consequence of the delayed fertilization in *A. stenosperma* crosses relative to *A. duranensis* crosses. The three x two sub-table; unfertilized, aborting embryo, or developing embryo vs *A. duranensis* or *A. stenosperma* as female parent at the D2-D10 stages, indicated no difference in the reproductive performance of the two diploid species as female parent ($\chi^2_2 = 5.30$, $p = .07$).

Table 5. Sum of observations for reciprocal crosses to *A. hypogaea* cv. NC 6 by *A. duranensis* and *A. stenosperma* across unfertilized ovules, aborting embryos, and developing embryos.

Table 5A. Observations for developmental stages D1 and D1.5.

Ovule (Embryo)	NC 6 x A. duran[a]	A. duran x NC 6	NC 6 x A. steno[b]	A. steno x NC 6
Unfert	16 (57)[c]	15 (58)	28 (100)	22 (100)
Aborting	0 (0)	3 (12)	0 (0)	0 (0)
Developing	12 (43)	8 (31)	0 (0)	0 (0)

[a] A. duran = A. duranensis
[b] A. steno = A. stenosperma
[c] Values in parenthesis are percentages. Columns sum to 100.

Table 5B. Observations for developmental stages D2 to D10.

Ovule (Embryo)	NC 6 x A. duran[a]	A. duran x NC 6	NC 6 x A. steno[b]	A. steno x NC 6
Unfert	62 (46)	68 (53)	33 (33)	66 (65)
Aborting	2 (2)	26 (21)	3 (3)	10 (10)
Developing	70 (52)	32 (25)	63 (64)	26 (25)

[a] A. duran = A. duranensis
[b] A. steno = A. stenosperma
[c] Values in parenthesis are percentages. Columns sum to 100.

Table 6. Summary of observed counts of unfertilized ovules, aborting embryos and developing embryos for crosses using a *A. hypogaea* cv NC 6 as female and male parent.

Table 6A. Observations for developmental stages D1 and D1.5.

Ovule (Embryo)	NC 6 as female	NC 6 as male
Unfert	44	37
Aborting	0	3
Developing	12	8

Table 6B. Observations for developmental stages D2 to D10.

Ovule (Embryo)	NC 6 as female	NC 6 as male
Unfert	95	134
Aborting	5	36
Developing	133	58

Discussion

Reproductive efficiency in the *Arachis* species includes both the ability to produce elongating pegs and the development of viable embryos. Recovery of viable embryos can be accomplished by *in vitro* embryo culture if the embryo has reached the heart-stage of development (10, 26). This usually corresponds to an age ranging from 20 to 30 days after fertilization in *Arachis*. Many highly desirable

interspecific hybrids, however, abort before embryos reach the heart-stage. For example, Johansen and Smith (9) reported embryo abortion in crosses between *A. hypogaea* and *A. diogoi* Hoehne)(not true *A. diogoi*, see Gregory and Gregory (6)) at 10-12 days after fertilization. Early embryo abortion also occurred after crosses between *A. hypogaea* and *A. glabrata* Benth (11). Halward and Stalker (8) reported even earlier embryo abortion in diploid by hexaploid interspecific crosses. Thus, in considering reproductive efficiency, one must take into account both abortion frequency and timing. In this study, abortion is a major factor in the reproductive efficiency of *A. duranensis* when it is used as a female parent. The timing of most abortion events appears to be during soil penetration by the peg. Because embryo growth goes into a quiescent phase during peg elongation, and upon soil penetration this quiescent phase is broken with embryo cell division being reinitiated, embryo growth during initial soil penetration has been proposed to be a critical developmental point by Pattee and coworkers (13, 15, 16). Failure to reinitiate embryo cell division would lead to embryo abortion. Previous work has cited failure to reinitiate peg development after soil penetration (8, 9, 19) as a possible reason for *Arachis* interspecific cross failures. Because the concept of the embryo quiescent phase was not fully recognized at that time, the lack of hybrids was attributed to a failure to reinitiate peg development rather than a failure to reinitiate embryo cell division. It is not possible to ascertain from these cited studies whether the peg development failure was a result of embryo abortion at a time previous to or at the initiation of soil penetration. In the present study with *A. duranensis* microscopic observations indicate a failure to reinitiate embryo cell division during initial soil penetration as the cause for abortion in this cross.

The observation of delayed fertilization in *A. stenosperma* has not been previously reported. This delay has several consequences when using *A. stenosperma* for interspecific hybridization programs. First, evaluation of fertilization should be done after 72 hrs following pollination. Second, to prevent collection of a high percentage of unfertilized ovules, peg tips younger than D2 should not be used for embryo culture.

When selecting *Arachis* species for hybridization, one must consider the timing of fertilization in reciprocal crosses. For example, *A. duranensis* is fertilized within 24 hr after pollination, however, *A. stenosperma* is delayed for more than two days. Further, the data suggests that when *A. duranensis* is the female parent peg growth commences earlier than in crosses involving either *A. stenosperma* or *A. hypogaea* cv. NC 6 as the female parent. Apparent elongation rates from D1.5 to D3 indicates that elongation is also more rapid in *A. duranensis*. This observation is in agreement with recently published comparative-peg-growth onset data for these species (15). It is thought that peg-growth onset is triggered by fertilization. Subjective observations on reproductive efficiency as judged by peg production by selfed flowers have suggested that such factors as day length and air temperature may effect anthesis timing (Pattee and Stalker, unpublished data). The observations on fertilization timing for *A. stenosperma* and *A. duranensis* crosses could also be affected by these same factors and studies are in progress to investigate such possibilities.

Stalker et al. (25) generally obtained the largest number of F_1 hybrids from reciprocal crosses using *A. hypogaea* and section *Arachis* accessions when *A. hypogaea* was the female parent. Our data confirm these observations and further show that choice of the maternal parent is a highly significant consideration when making crosses in *Arachis*. When *A. hypogaea* subsp. *hypogaea* is used and the number of F_1 hybrids obtained in a crossing program is of primary interest, the crossing effort should solely utilize *A. hypogaea* as the female parent. The specific cause of this parental factor affect is not known, but comparative observations of the embryo sac of *A. hypogaea* cvs. NC 6 and Argentine and *A. duranensis* and *A. stenosperma* have shown significant differences in starch content (14). Differences in starch content may effect proembryo growth by supplying energy during syngamy and immediately afterwards during development. Using wild species as the female parent may be desirable, for example to create cytoplasmic lines, and methods to circumvent incompatibilities will be needed to recover the large number of plants required for introgression experiments.

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