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Wide hybridization between Brazilian soybean cultivars and wild perennial relatives

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Abstract Employing a different culture strategy, we obtained a greatly improved frequency of embryo rescue in intersubgeneric soybean hybrids. Successful crosses were obtained in 31 different genotype combinations between nine Brazilian soybean lines as the female parents and 12 accessions from *Glycine canescens*, *G. microphylla*, *G. tabacina* and *G. tomentella*. The hybrid pod retention rate dropped to about 10% during the first 8 days after pollination and stayed largely unchanged up to the 20th day. Immature harvested seeds fell into three size groups: Group 1, smaller than 1.3 mm (mostly empty seed coats); Group 2, 1.9–5.0 mm; Group 3, larger than 5 mm (from selfing). A total of 90 putative hybrid embryos were rescued using a highly enriched B5 medium to nourish the newly dissected embryos. The growing embryos were then placed in a high osmotic, modified B5 medium to induce maturation and dormancy. Schenk and Hildebrandt medium was used to germinate the dormant, partially dehydrated, physiologically mature embryos. Approximately 37% of the rescued embryos developed into plantlets *in vitro*, and approximately 8% grew into mature plants in the greenhouse. Morphological, cytological and isoenzyme patterns confirmed the hybrid status of all seven mature plants, all of which were generated using *G. tomentella* G 9943 as the paternal parent. It was observed that all soybean lines crossed with G 9943 were capable of producing mature hybrid plants. There was no correlation between the initial size of Group 2 seeds and plant survival rate. The hybrids were cloned by grafting and treated with colchicine. One of the treated plants displayed chromosome doubling.

Key words Embryo rescue · *Glycine* spp. · Intersubgeneric hybrids · Soybean · Wide hybridization

Introduction

Brazil is the world's second largest soybean-producing and -exporting nation. Although participants in the Brazilian soybean breeding program are aware of the ever-changing needs of agriculture, the improvement program is severely handicapped due to the cultivated Brazilian soybean gene pool being extremely limited (Hiromoto and Vello 1986; Abdelnoor et al. 1995). Such breeding difficulties are further compounded by the semitropical Brazilian climate; cultivated soybeans were originally introduced from countries with temperate climate.

One well-recognized means by which to increase the soybean gene pool is to introduce genes from wild perennial relatives, principally the subgenus *Glycine*. Members of this subgenus possess many identified agronomically favorable characteristics not readily found in the soybean. The principal desirable traits are resistance to pathogens, such as viruses, bacteria, fungi and nematodes; tolerance to stress, such as saline, drought, heat, cold; and resistance to herbicides (see Newell et al. 1987; Shoemaker et al. 1990; Coble and Schapaugh 1990 for the listings of specific references). All the perennial soybean relatives are wild plants of tropical origin and it is highly likely that these plants have evolved numerous traits specific for tropical environments that will be of great use in the semitropical Brazilian soybean improvement program.

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The difficulty in transferring genes from the subgenus *Glycine* to the cultivated soybean gene pool is due to recalcitrant sexual incompatibility of the post-fertilization type. The pollen tube grows normally and enters the ovule within 24 h after pollination in both reciprocal crosses of wild perennial *Glycine* × *G. max* (Singh and Hymowitz 1987), but the hybrid embryos are aborted at an early developmental stage due to the degeneration of the endosperm (Palmer and Hadley 1968; Ladizinsky et al. 1979). By means of histological examinations, Sakai and Kaizuma (1985) found that some of the late-aborting hybrid pods contained embryos well into the heart stage that had the potential to develop further but at very low growth rates.

The standard rescue procedure for post-fertilization-incompatible hybrids is to excise the immature embryos (or the ovules) and culture them *in vitro* (Hu and Zanettini 1995). The use of embryo and ovule cultures to rescue incompatible soybean intersubgeneric crosses has been investigated world-wide. Out of the 15 known species in the subgenus *Glycine*, rescue has been reported in crosses between soybean and the only 2 known *Glycine* tetraploid species, *G. tabacina* and *G. tomentella*, and 2 diploid species, *G. canescens* and *G. clandestina*. Most of such hybridization attempts have been carried out in the USA (Newell and Hymowitz 1982; Newell et al. 1987; Singh and Hymowitz 1987; Singh et al. 1987; Shoemaker et al. 1990; Coble and Schapaugh 1990). Successful hybrid rescue has also been reported for the Australian (Broue et al. 1982) and Korean (Chung and Kim 1990) soybeans. Japanese workers (Sakai and Kaizuma 1985) made a large number of crosses for use in histological examinations but did not attempt embryo rescue. Our laboratory is concentrating on the improvement of Brazilian soybeans, and here we report our initial success in the wide crosses between Brazilian soybean cultivars and the subgenus *Glycine*.

Materials and methods

Plant material

Plant material used in this study were soybean strains from the subgenus *Soja* (Moench) F. J. Herm. and wild perennial species from the subgenus *Glycine* Willd. The soybeans (*Glycine max*, L. Merr; 2n = 2x = 40) were Brazilian cultivars and breeding lines CEP-10, CEP-12-Cambará, CEP-20-Guajuvira, CEP-26-Umbu, CEP-7403, COBB, IAS-5, PRATA and RS-7-Jacuí. The wild perennial species were *G. canescens* F. J. Herm. (2n = 2x = 40; G 2528, G 9937 and PI 440151), *G. microphylla* (2n = 2x = 40; PI 509488), *G. tabacina* (Labill.) Benth. (2n = 4x = 80, PI 339661, PI 505197, PI 509495, PI 509496 and PI 509498) and *G. tomentella* Hayata (2n = 4x = 78; G 9941, G 9943 and PI 509501). Seeds of wild perennial species were obtained from CSIRO, Canberra, Australia, and The Asian Vegetable Research and Development Center-AVRDC, Shanhua, Tainan, Republic of China. Soybeans and wild perennials used in the crossing program were grown in a temperature-regulated (27° ± 2 °C) greenhouse at FUNDACEP-FECOTRIGO, Cruz Alta, RS, Brazil.

Crosses

Crosses were made in 1993 and 1994 with soybeans as the female parents. The young buds were emasculated 2 or 3 days before anthesis and immediately pollinated with pollen from the newly opened flowers of the perennial *Glycine* species. Only three or four buds from each soybean raceme were used for hybridization. To encourage the retention and growth of the pods, the hybridized gynoecia were sprayed daily with gibberellic acid (100 mg/l) for 20 days (Singh and Hymowitz 1987; Chung and Kim 1990).

Hybrid rescue

Immature putative hybrid pods were harvested between 20–30 days after pollination (DAP) in 1993 and on the 20th DAP in 1994. Pods were surface-disinfected with 70% ethanol for 1 min followed by a 20-min soaking in 1% sodium hypochlorite with a trace amount of Tween. After being rinsed with three changes of sterile, distilled water, the pods were dissected under a stereo-microscope and the embryos were excised and cultured. The seed and embryo sizes were measured during dissection. The culturing procedure was divided into the following three stages. (I) embryonic development, (II) maturation and dormancy and (III) germination and seedling development. The cultures were maintained at 25° ± 1 °C under a 16-h photoperiod of approximately 2.0 klx fluorescent light for all three stages.

The newly excised embryos were placed in 15 × 40-mm glass tubes containing 2 ml of liquid Stage I Embryonic Development Medium (EDM). The EDM consisted of B5 Long medium formulation [an organic-enriched B5 medium (Gamborg et al. 1968); Carolina Biological Supply Co, Burlington, N.C., see Hu et al. 1995 for the organic ingredients] supplemented with Yeung's amino acids (Yeung and Sussex 1979; 500 mg/l glutamine, 100 mg/l serine, 100 mg/l asparagine and 250 mg/l casein hydrolysate), 1 μ*M* BAP, 0.1 μ*M* NAA and 4% sucrose.

After 22 (for larger embryos) to 61 (for smaller embryos) days, the embryos were transferred to Stage II Maturation and Dormancy Medium (MDM) and incubated under reduced light. The MDM consisted of B5 Long medium with 10% sucrose, 0.5% activated charcoal and 1% agar.

After 31–56 days incubation, the mature, dormant embryos were transferred onto Stage III Germination and Seedling Development Medium (GSM) consisting of SH medium (Schenk and Hildebrandt 1972) with 1% sucrose and 0.6% agar. After 13–67 days, plantlets with well-developed root systems were transplanted to 250-ml plastic pots containing a 3:1 mixture of peat and carbonized rice hulls.

Hybrid identification

Root-tip mitosis, microspore meiosis and leaf isoenzyme analysis were used to identify the hybrid status of the resultant plants.

Root tips were harvested from the putative hybrid plantlets before they were transplanted *ex vitro*. Root-tip squash and chromosome counts were carried out according to Palmer and Heer (1973). Floral buds which were harvested from greenhouse-grown hybrid plants were fixed (absolute ethanol: acetic acid = 3:1) for 24 h and stored in 70% ethanol. Anthers were squashed in 0.6% propionic carmine for analysis of the meiotic chromosome behavior in the pollen mother cells.

Leaves from 2 greenhouse-grown adult putative hybrid plants were assayed for their isoenzyme patterns. Isoenzyme analysis of glutamate oxalacetate transaminase (GOT), peroxidases (PER), superoxide dismutases (SOD), malate dehydrogenases (MDH), amylases (AMY) and esterases (EST) were carried out by horizontal polyacrylamide gel electrophoresis. The migration conditions utilized for each enzyme system were: 7% gel and Brown's (1983) buffers for GOT; 7% gel and Scandalios' (1969) buffers for PER, SOD and AMY; 6% gel and Roose and Gottlieb's (1976) buffers for MDH; 8% gel and Scandalios' (1969) buffers for EST. The gels were run at 10 V/cm and stained as described by the following authors: Vallejos (1983) with modifications for GOT; Gottlieb (1973) for PER; Brewer (1970) for MDH; Scandalios (1969) for EST; Chao and Scandalios (1972) with modifications for AMY. SOD was stained using the methods of Brewer (1970) for glutamate dehydrogenase and the staining mixture incubated under illumination.

Grafting and chromosome doubling

To increase the plant number, we cloned the confirmed hybrid plants using the grafting procedure described by Newell and Hymowitz (1979). Buds from both the original and the grafted clonal hybrid plants were treated with colchicine (0.1% or 0.2%) to induce chromosome doubling as described by Cheng and Hadley (1983).

Results

Crosses and hybrid rescue

Five perennial *Glycine* accessions comprising three species, and seven lines of *G. max* were used as parent lines in 1993 (Table 1A). From approximately 400 crosses, 19 putative hybrid pods were harvested and 23 embryos cultured. Eleven embryos survived the initial Stage I culture, developing to the plantlet stage and successfully transplanted *ex vitro*. Two of these hybrid plants, both with *G. tomentella* G 9943 as the paternal parent, survived to maturity. The maternal parents were soybean strains CEP-12 and CEP-7403.

Nine wild perennial *Glycine* accessions representing four species and four Brazilian soybean cultivars were used as parent lines in 1994 (Table 1B). The pod retention rates up to 20 DAP are presented in Fig. 1. Nearly identical trends on the retention curves were observed regardless of parental genotypes. In general, the pod retention rates dropped quickly and steadily immediately after pollination until the 8th day and stabilized at approximately 10% up to 20 DAP (= the pod harvesting day). From 678 crosses, 62 putative hybrid pods were harvested.

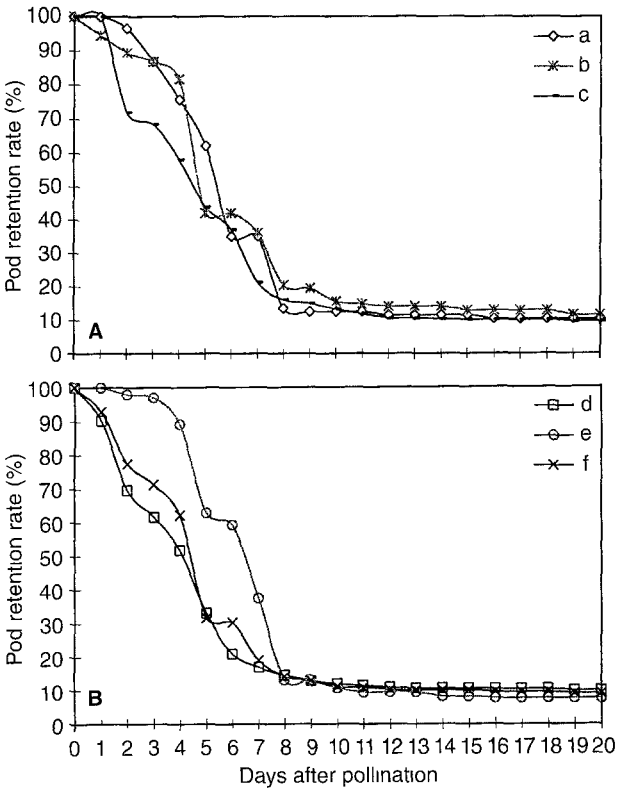


Fig. 1A, B Pod retention rates up to 20 days after 1994 crosses between four Brazilian soybean strains and nine perennial accessions representing four *Glycine* species. **A** Rates based on the maternal parents, **B** rates based on the paternal parents *a* CEP-12 × *G.* spp., *b* CEP-26 × *G.* spp., *c* IAS-5 × *G.* spp., *d G. max* × *G tomentella*, *e G. max* × *G. tabacina*, *f G. max* × *G.* spp.

Table 1 Hybrid embryo rescue in intersubgeneric crosses between Brazilian soybean (*G. max*) cultivars and wild perennial relatives (*G. can G. canescens*, *G mic G. microphylla*, *G. tab G. tabacina*, *G. tom G. tomentella*)

A) 1993 crosses

Combination	Pod set	Number of embryos or plants				
		Stage 1	Stage 2	Stage 3	*Ex vitro*	Adult
G. max (2)[a] × *G. can* (2)[b]	3	3	2	2	2	0
G. max (3) × *G tab* (1)	3	5	1	1	1	0
G. max (1) × *G. tom* AVRDC-G9941	2	2	2	2	2	0
G. max (2) × *G. tom* AVRDC-G9943	11	13	6	6	6	2
G max × *G.* spp.	19	23	11	11	11	2

B) 1994 crosses

Combination	Number of crosses	Pod Set	Seed[c]		Number of embryos or plants					
			T	SG2	Stage 1	Stage 2	Stage 3	*Ex vitro*	GH[d]	Adult
G. max (1)[a] × *G. can* (1)[b]	16	1	0	0	0	0	0	0	0	0
G max (1) × *G mic* (1)	13	1	1	0	0	0	0	0	0	0
G. max (4) × *G. tab* (4)	211	16	32	16	16	10	10	1	0	0
G max (4) × *G. tom* AVRDC-G9941	115	7	16	7	7	5	5	1	0	0
G. max (3) × *G. tom* AVRDC-G9943	208	30	61	33	33	28	28	12	5	5
G max (4) × *G. tom* PI509501	115	7	14	11	11	11	11	8	2	0
G. max × *G.* spp.	678	62	124	67	67	54	54	22	7	5

[a] Number of soybean cultivars used in the crosses
[b] Number of wild perennial accessions used in the crosses
[c] Seed: T, total; SG2, seed size group 2
[d] GH, Plants transferred to the greenhouse

Three distinct seed-size groups were observed from the 124 immature seeds obtained in 1994. The 52 Group 1 seeds were less than 1.3 mm in length, and we were not confident that the minute structures dissected from them were in fact embryos because none of them showed embryonic growth in culture. It is highly probable that most of them were empty seed coats. Therefore, we excluded this seed group from our statistics. The 67 Group 2 seeds ranged in sizes from 1.9 to 5.0 mm (average length = 3.05 mm) with all containing small, shrunken embryos. Group 3 consisted of 5 seeds larger than 5.0 mm containing apparently healthy embryos, probably resulting from self-pollination.

Sixty-seven embryos were dissected and cultured from Group 2 seeds; 54 survived the initial culture and progressed through the maturation and germination stages with 22 of these forming plantlets that were transplanted *ex vitro*. All but 1 of these plantlets had *G. tomentella* as the paternal parent. It appeared that there was no correlation between the initial seed size and the embryo survival rate among the surviving embryos excised from the Group 2 seeds.

Seven plantlets, with *G. tomentella* as the paternal parents, outlived *ex vitro* shock and were transplanted into a greenhouse. Of these 7, 5, all with accession G 9943 as the paternal parent, reached maturity. The initial size of these 5 was: 1.9, 2.8, 3.0, 3.5 and 4.2 mm. The maternal parents of these 5 mature plants included three soybean cultivars.

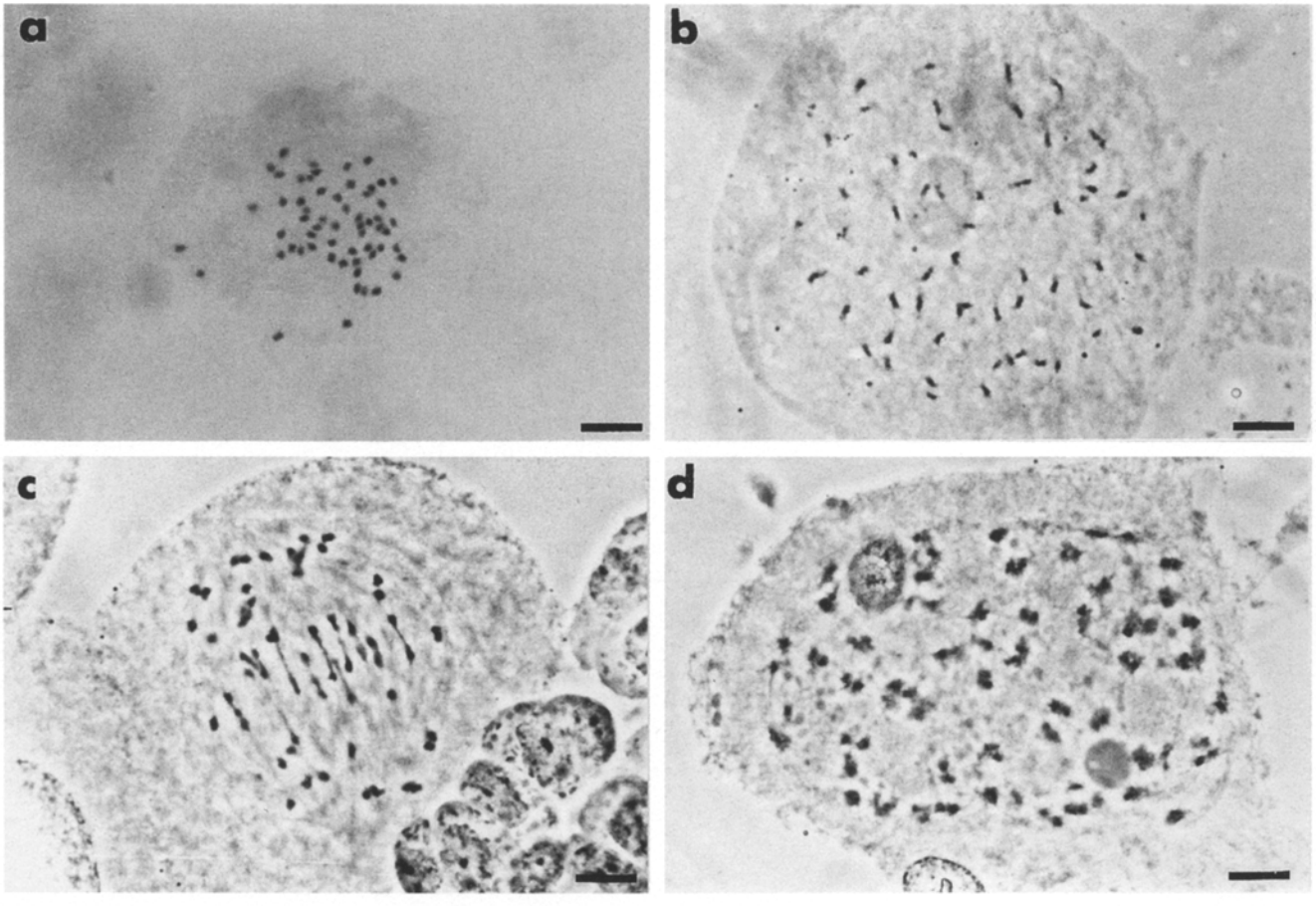
Five embryos were excised from Group 3 seeds and cultured; all them produced plantlets that were transferred *ex vitro*.

Hybrid identification

Both mature plants from 1993 crosses and all 22 *ex vitro* plantlets derived from Group 2 medium-sized seeds of the 1994 crosses expressed hybrid morphology as described by Newell and Hymowitz (1982). All 5 plantlets derived from Group 3 large seeds showed maternal soybean phenotypes.

Root mitotic metaphase chromosome examination confirmed the hybrid status of both of the mature 1993 plants. From the 22 Group 2 plantlets (1994) we attempted chromosome analysis on 15, of which 3 gave unreadable chromosome spreads. All of the 12 analyzed plantlets were hybrids with chromosome numbers 2n = 59 (Fig. 2a). We were not able to count the chromosomes from 2 plantlets derived from Group 3 seeds with the other three given their chromosome numbers of

Fig. 2a–d Cytological figures in F_1 (**a–c**) and colchicine-doubled (**d**) interspecific hybrids between Brazilian soybean cultivars (2n = 40) and *G. tomentella* G9943 (2n = 78). **a** Mitotic metaphase chromosomes (2n = 59), **b** diakinesis (2n = 59) with 55 I + 2 II, **c** metaphase I (2n = 59) showing 33 I + 13 II, **d** diakinesis (2n = 118) showing 16 I + 51 II Bar: 10 μm



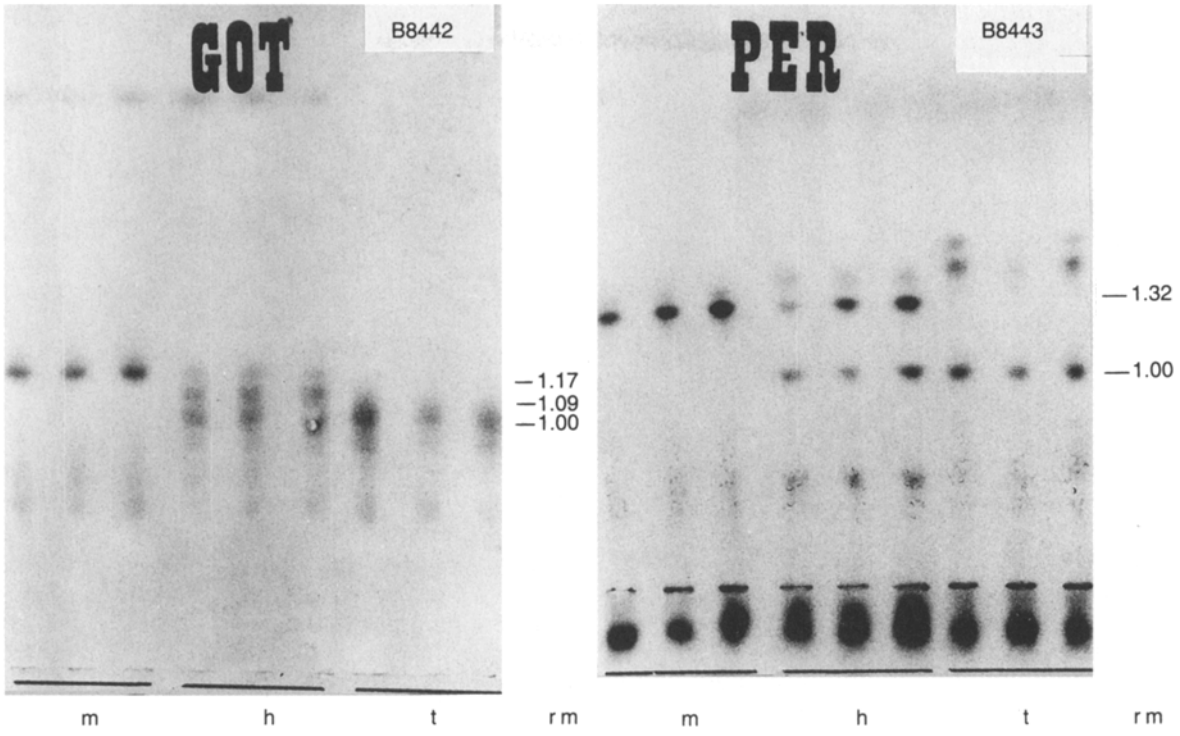


Fig. 3 Isoenzyme patterns. Gels showing peroxidase (PER) and glutamate oxalacetate transaminase (GOT) patterns of *G. max* (*m*), *G. tomentella* (*t*) and their hybrids (*h*)

2n = 40. On the basis of their phenotypes and chromosome counts, they must be the selfing products of the maternal soybean parents.

G. max and *G. tomentella* parents showed different isoenzyme patterns and specific bands in all of the systems analyzed. Hybrid status was confirmed by the presence of additive isoenzyme patterns in the putative hybrid plants (Fig. 3). For GOT, two isoenzymes were specific for the two species: the band of RM = 1.17 only occurred in *G. max* (m) and that of RM = 1.00, only in *G. tomentella* (t). The hybrids (h) presented both bands as well as a hybrid band with intermediate mobility (RM = 1.09), which could be interpreted as an heteromer formed by the polypeptide subunits from bands 1.17 and 1.00. For PER, *G. max* showed an isoenzyme with RM = 1.32, and *G. tomentella* presented another band with RM = 1.00, with the hybrids presenting both bands. All other systems showed similar profiles, thereby confirming their hybrid status.

Chromosome doubling

Colchicine (0.1% or 0.2%) treatments were performed on 5 mature hybrid plants and their grafted clones. The 5 hybrid plants (with the maternal origin and number of clonal plants treated in parenthesis) were: Hyb 5 (CEP-

Table 2 Average chromosome configurations in F_1 and colchicine-doubled interspecific hybrids between Brazilian soybean (2n = 40) cultivars (female) × *G. tomentella* G9943 (2n = 78) (male)

Soybean cultivars used as female	Total PMCs	2n	Chromosome configurations			
			I	II	III	IV
CEP-12 (2)[a]	118	59	43.30 (27–57)	7.85 (1–17)		
CEP-26 (1)	40	59	44.40 (33–55)	7.30 (2–13)		
	10	118	20.70 (4–42)	46.90 (37–57)	0.90 (0–4)	0.20 (0–1)
CEP-7403 (1)	30	59	43.17 (33–55)	7.90 (2–13)		0.06 (0–1)
IAS-5 (2)	41	59	44.49 (29–53)	7.24 (3–15)	0.03 (0–1)	

[a] Number of plants from separate fertilization events

12; 3), Hyb 20 (CEP-7403; 8), H 3 2A (IAS-5; 7). H 5 3B (CEP-12; 5) and H 9 3B (CEP-26; 4). One of the four clonal plants of H 9 3B showed chromosome doubling having 118 chromosomes at meiosis (Table 2).

Meiotic chromosomal behaviour of the hybrids

Pollen mother cell meiotic chromosomal configurations of the confirmed hybrids are shown in Table 2 and Fig. 2. The overall average of univalents, bivalents, trivalents and quadrivalents for the 6 F_1 hybrids analyzed was 43.71, 7.64, 0.01 and 0.01, respectively. The same values for the colchicine-doubled hybrid were 20.7, 46.9, 0.9 and 0.2, respectively.

Discussion

Because hybrid embryos between soybean and perennial *Glycine* are feeble, poorly developed and at very early developmental stages when excised, the success rate for *in vitro* rescuing is usually low. From 1,534 hybrid embryos, Newell et al. (1987) obtained 22 (1.4%) *in vitro*-germinated plantlets; 16 (1.04%) of these survived *ex vitro* transfer, greenhouse conditions and reached maturity. Chung and Kim (1990) rescued 45 hybrid embryos with 17.8% reaching *in vitro* germination. However, the authors did not present data on the number of plants that reached maturity. Their higher success rate was partially due to the fact that only *G. tomentella* was used in their crosses. This species appears to be more cross-compatible with soybean than the other *Glycine* species (Newell et al. 1987). The highest rate of hybrid embryo rescue has been reported by Coble and Schapaugh (1990). From six excised hybrid embryos from a *G. max* × *G. tomentella* cross, four *in vitro* plantlets were obtained on B5 salts with Williams' (1978) vitamins and 3% sucrose. Again, no mention was made of the number of plants that survived *ex vitro* transplanting.

In order to improve the rate of rescuing, we have developed a different culturing strategy. In addition to using a very rich medium to nurse the newly dissected embryos, we exposed the *in vitro*-growing embryos to a maturation and dormancy period using a high osmotic medium (Ranch et al. 1985). It is known that when soybean embryos are partially dehydrated and dormant, a higher percentage of them will germinate *in vitro* (Buchheim et al. 1989). With our protocol, from 23 rescued putative hybrid embryos in 1993, we obtained 11 (47.8%) *in vitro*-germinated plantlets and 2 (8.7%) mature hybrid plants. In 1994, from 67 putative hybrid Group 2 seeds we obtained 22 (32.8%) *in vitro*-regenerated plantlets and 5 (7.5%) greenhouse-matured plants. The pooled data, including the putative hybrid embryos obtained in 1993 and 1994, gives an average of 36.7% regenerated plantlets and 7.8% adult plants. Clearly we have greatly improved the hybrid embryo rescuing percentage for soybean with our culture strategy.

All of the 7 mature plants possessed the expected hybrid chromosome number of 59. The meiotic chromosome configurations observed by us (Table 2) were similar to those recorded by Newell and Hymowitz (1982) but they had on average higher numbers of bivalents than those reported by Newell et al. (1987) on *G. max* × *G. tomentella* crosses. Although a portion of the bivalents and multivalents might result from chromosome interactions within the *G. tomentella* (n = 2X) or soybean (n = X; Crane et al. 1982) genomes, some of them should result from chromosome interactions between the two genomes and provide an opportunity for genomic exchanges.

We have obtained 1 chromosome-doubled plant from our F_1 hybrid clones. Newell et al. (1987) obtained chromosome doubling in 6 of their 7 *G. max* × *G. tomentella* F_1 hybrid clones. Since the F_1 hybrids are perennial in nature and vigorous in growth, an unlimited number of clonal plants via grafting can be obtained from each F_1 genotype. Fertile progenies can be expected to be obtained from them after a sufficient number of colchicine treatments are performed. Shoemaker et al. (1990) obtained fertile F_2 and F_3 plants with exclusively a 2n = 40 soybean genome from 1 of the colchicine-doubled F_1 hybrids. An analysis of isoenzymes indicated that regions of the *G. tomentella* genome were retained. This fact suggests that the complete *G. tomentella* chromosome complement from the F_1 hybrid might automatically be eliminated after genetic exchange had taken place. Preferential elimination of one parental genome is common in human-mouse somatic hybrid cells (Cowell 1992). It is also well-documented in crosses between *Hordeum vulgare* and *H. bulbosum* (Kasha and Kao 1970) and *Triticum aestivum* with *Hordeum bulbosum* (Barclay 1975).

All 7 F_1 hybrids were obtained using *G. tomentella* G 9943 as the sole paternal parent. It appears that all of the Brazilian soybean strains that we tested were capable of producing mature F_1 plants when crossed with this accession. We observed no correlation between the initial seed size at dissection and the hybrid survival rate, but the genotype and the physiological condition of the excised embryo probably play roles in determining rescue success. Figure 1 shows that hybrid pod-retaining rates dropped quickly to about 10% during the first 8 days, similar to that observed by Chung and Kim (1990). Although the pod number stayed largely unchanged from the 9th to the 20th day, the physiological condition of the embryos would be expected to deteriorate during this period. Therefore, it would be advantageous to develop strategies to rescue embryos prior to 8 DAP. Obviously, the path ahead of us is to obtain F_1 plants from a wider range of accessions and species of the perennial *Glycine*.

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