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Effect of genotype on somatic embryogenesis from immature cotyledons of soybean

W.A. PARROTT[1], E.G. WILLIAMS*, D.F. HILDEBRAND & G.B. COLLINS

Dept of Agronomy, University of Kentucky, Lexington, KY 40546-0091, USA
*([1]present address: Dept of Agronomy, University of Georgia, Athens, GA 30602, USA; *requests for offprints)*

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Abstract. Genotype has a large effect on the ability of immature soybean cotyledons to undergo auxin-stimulated somatic embryogenesis. Among 33 soybean lines, all those showing good regeneration were found to have in their pedigrees one or both of the highly regenerative ancestral lines, 'Manchu' or 'A.K. Harrow'. When 'Manchu' was crossed with 'Shiro', a genotype showing extremely poor regeneration, F_1 hybrid cotyledons showed intermediate regeneration capacity.

Introduction

The soybean, *Glycine max* (L.) Merr., is an important oil and protein crop for which in vitro technologies have considerable potential [1]. Induction of adventive or de novo regeneration from non-meristematic cells of soybean occurs through auxin-stimulated somatic embryogenesis from immature zygotic embryos, explanted either whole or with the embryonic axes removed. Lippman [2] obtained somatic embryos on immature cotyledons exposed to 2,4-D, but were unable to obtain plants. Plants have subsequently been obtained from somatic embryos induced by 2,4-D [3–8, 9] or NAA [5, 6, 9, 10, 11, 12].

All genotypes tested have regenerated via somatic embryogenesis, but the frequency of response varies [3, 4, 11]. Attempts have been made to find indicators of a genotype's ability to produce high numbers of somatic embryos, but no relationships have been found with maturity group, seed coat color, flower color, and disease susceptibility or resistance [3, 10]. Here we show that certain predictions of regenerative capacity can be made on the basis of pedigree.

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Materials and methods

Immature cotyledons from 33 soybean genotypes were evaluated for their ability to regenerate via somatic embryogenesis. These genotypes (Table 1) were selected according to four criteria:

- they were adapted to Kentucky growing conditions,
- they had been reported in the literature as being susceptible to some degree to infection by *Agrobacterium tumefaciens* [13],
- they had been reported as being capable of regeneration from hypocotyl tissue [1], or
- were ancestral to currently growth North-American cultivars [15].

Plants were grown in a greenhouse with supplementary heating and cooling as required to maintain approximately 27 °C, and supplementary lighting (high-pressure sodium) as required to maintain a 14-h photoperiod. Plants were grown, 5 per ten-inch pot, using a 2:2:1 mixture of sand:soil (Maury Silt Loam):Promix (Premier Brands, New Rochelle, NY). Fertilizer was applied weekly (20-20-20; Peter's; Grace and Co., Fogelsville, PA).

Pods containing immature seeds 3–5 mm in length were harvested, and surface-sterilized by immersion in 70% 2-propanol for 30 s, followed by immersion in 1.3% sodium hypochlorite (prepared as 25% commercial chlorine bleach) for 12 min. They were then rinsed three times in sterile de-ionized water. The immature seeds were aseptically removed from the pods, and the end containing the embryonic axis cut away and discarded. The two cotyledons were pushed out of the seed coat and placed abaxial side down on N10 medium (MS salts, B5 vitamins, 1.5% sucrose, pH adjusted to 5.8, and 0.2% Gelrite (Scott Laboratories Inc., Fiskeville, RI) [10]. The medium was autoclaved for 20 min at 120 °C and 15 psi (104 kPa), and dispensed into 100 × 20 mm disposable Petri dishes in 35-ml aliquots. Twenty cotyledons were placed on each plate. At least 200 cotyledons per genotype were cultured, where possible.

Cotyledons were cultured at 25 °C with a 23-h photoperiod provided by cool-white fluorescent tubes supplemented with 50 Watt incandescent bulbs (approx. 10 $\mu Em^{-2}s^{-1}$). The 23-h photoperiod was effective in preventing premature floral induction of the soybean regenerants. At the end of one month, the numbers of somatic embryos forming on each cotyledon were counted, and the following parameters were calculated:

- The percent of explanted cotyledons that formed somatic embryos.
- The average number of somatic embryos that formed on cotyledons that underwent embryogenesis.
- The average number of somatic embryos per explanted cotyledon.

The last parameter was analysed with a one-way analysis of variance, and

Table 1. Regeneration capacity of 33 strains of soybean. Genotypes are sorted by frequency of somatic embryos per explanted cotyledon.

Genotype	% Cots responding	Embryos per responding cot		Embryos per explanted cot		Total cots
		Mean	SE	Mean	SE	
Manchu	66.49	3.14	0.16	2.09	0.15	188
Century	76.33	2.08	0.09	1.59	0.09	300
Williams 82	65.81	1.95	0.10	1.28	0.10	155
J103	56.50	2.04	0.11	1.16	0.10	200
A.K. Harrow	50.50	2.28	0.16	1.15	0.11	200
Clark r	45.30	2.47	0.14	1.12	0.10	298
P.I. 283332	44.67	2.22	0.17	0.99	0.12	150
Harosoy	42.00	2.24	0.44	0.94	0.21	100
McCall	39.07	2.00	0.17	0.78	0.10	151
Wilson 5	34.50	1.54	0.09	0.69	0.07	200
Forrest	36.97	1.60	0.10	0.59	0.06	238
Wayne	30.42	1.81	0.15	0.55	0.07	240
Douglas	32.50	1.51	0.11	0.49	0.06	200
Kent	31.12	1.49	0.14	0.46	0.07	196
Heilongjiang 26	25.91	1.65	0.13	0.43	0.06	220
Elf	28.65	1.45	0.12	0.42	0.06	171
Stafford	22.54	1.67	0.11	0.38	0.06	173
Essex	22.62	1.66	0.15	0.38	0.06	168
Ripley	22.73	1.63	0.18	0.37	0.07	132
Heilongjiang 10	27.50	1.29	0.08	0.36	0.05	200
Mandarin	15.50	1.97	0.27	0.32	0.07	200
Peking	16.50	1.76	0.21	0.30	0.06	200
Jilin 5	16.67	1.63	0.13	0.27	0.05	180
Manitoba Brown	19.57	1.33	0.12	0.26	0.05	138
Pennyrile	11.43	1.69	0.25	0.19	0.54	140
P.I. 420338	12.50	1.33	0.13	0.17	0.04	120
P.I. 423897	9.29	1.85	0.34	0.17	0.06	140
Sooty	15.79	1.00	0.00	0.16	0.04	76
Shiro	6.96	1.50	0.50	0.10	0.05	115
Lee	5.00	1.50	0.50	0.08	0.04	160
Richland	5.00	1.14	0.14	0.05	0.02	160
Cobb	2.15	1.25	0.25	0.03	0.01	186
Columbia	2.00	1.00	0.00	0.02	0.01	200

Fisher's $LSD_{0.05}$ = 0.91 for somatic embryos per explanted cotyledon.

Fisher's Least Significant Difference (LSD) was calculated.

The pedigree of each genotype was examined [16, 17] in an effort to explain regeneration capacity based on pedigree relationships. Any ancestral genotype common to genotypes with high regeneration potential was examined for its own regeneration capacity (Table 2). Finally, hand crosses were made by Dr Todd Pfeiffer, University of Kentucky, between 'Shiro', one of

Table 2. Presence of ancestral strains in the pedigrees of cultivars evaluated during the genotype screen. Ancestral genotypes are listed on the left, and current genotypes are on the top row. The numbers of somatic embryos formed per explanted cotyledon (SE/COT) are provided if the genotype was evaluated for regeneration. An asterisk (*) indicates the presence of the ancestral genotype in the pedigree of a soybean genotype.

Ancestral strain		Derived cultivars														
		Century (1.59)	Williams 82 (1.28)	J103 (1.16)	Clark (1.12)	Harosoy (0.94)	McCall (0.78)	Forrest (0.59)	Wayne (0.55)	Douglas (0.49)	Kent (0.46)	Elf (0.42)	Essex (0.38)	Pennyrile (0.19)	Lee (0.08)	Cobb (0.02)
Mandarin	(0.32)	*	*	*	*	*	*		*	*	*	*		*		
Manchu	(2.09)	*	*	*	*		*		*	*	*	*		*		
Richland	(0.05)		*	*	*		*		*	*		*		*		
A.K. Harrow	(1.15)	*	*	*		*	*	*		*		*	*	*	*	*
CNS[a]	(0.06)		*					*	*	*		*	*	*	*	*
Mukden[a]	(0.53)						*									
Strain 171				*												
Tokyo		*								*	*	*				*
PI 5460		*								*	*	*				*
Roanoke[a]	(0.14)											*				
PI 240664																
Palmetto[a]	(0.08)															*

CNS, Mukden, Palmetto and Roanoke were evaluated separately, and consequently were not included in Table 1.

the poorest regenerators, and 'Manchu', the best regenerator of the genotypes evaluated. Parental plants were grown in the field at the University of Kentucky's Agriculture Experimental Farm in Lexington. Immature cotyledons from self-pollinated parental plants were compared in culture with F_1 hybrid cotyledons as described above.

Results and discussion

The results of the genotype evaluations are presented in Table 1. Genotypes have been ranked according to the number of somatic embryos formed per explanted cotyledon (SE/COT). This statistic contains two parameters, the percent of explanted cotyledons that formed somatic embryos, and the number of somatic embryos formed by these responding cotyledons. In no case did all explanted cotyledons respond. 'Century' had the highest response rate, with 76% of cotyledons forming somatic embryos. The percent of explanted cotyledons that formed somatic embryos is significantly correlated ($r = 0.71$; d.f. = 30) with the number of somatic embryos formed on each responding cotyledon. That is, genotypes with the highest percentage of responding cotyledons tend to also form more somatic embryos per responding cotyledon.

The number of somatic embryos obtained per explanted cotyledon (SE/COT) is of most importance to the user. Several genotypes with excellent regeneration potential were identified, including 'Manchu', which averaged 2.09 SE/COT, 'Century' (1.59 SE/COT), and 'Williams 82' (1.28 SE/COT). There were significant differences in SE/COT between genotypes, as determined by analysis of variance. Regeneration response within any genotype was highly variable, and consequently, two genotypes were required to differ by almost 1 SE/COT for the difference to be statistically significant, as determined by LSD at the 5% level.

North-American soybean germplasm is derived from a very small number of ancestral genotypes [15]. Those ancestral lines which contributed to the tested cultivars (Table 2) were included in the genotype evaluation in an attempt to identify any that may have been a source of a high regeneration potential. Two of these genotypes, 'Manchu' and 'A.K. Harrow', were identified as having high regeneration capacity. All other genotypes with high regeneration capacity had 'Manchu' and/or 'A.K. Harrow' in their background. All genotypes examined which did not have either 'Manchu' or 'A.K. Harrow' in their background were very poor regenerators (overall average of 12 genotypes = 0.33 SE/COT). However, there were genotypes, such as 'Pennyrile' and 'Wayne', that were poor regenerators despite having

'Manchu' in their background. Thus, having ancestral genotypes with a high regeneration capacity is a necessary but not sufficient condition for regeneration capacity. Presumably, if genetic factors are responsible for regenerative capacity, these may have segregated out during the production of pure lines after crossing.

To further test the heritability of high regeneration capacity, cross pollinations were made between 'Shiro' (0.10 SE/COT) as the seed parent and 'Manchu' (2.09 SE/COT) as the pollen parent. The regeneration capacity of F_1 hybrid cotyledons was 0.81 SE/COT. This is higher than that of 'Shiro', but lower than that of the midparent value (1.1 SE/COT), suggesting that at least some of the regeneration capacity of the pollen parent was expressed in the F_1 progeny. A final determination of the heritability of this trait will be made once pure lines are derived from this cross and its reciprocal.

Because both 'Manchu' and 'A.K. Harrow' are ancestral to most northern soybean genotypes [15], most northern soybeans should be amenable to current in vitro technology. Genotypic specificity for regeneration capacity in legumes is well-documented, occurring, for example, in alfalfa [18] and white clover [19, 20]. Genotype-specific capacity for regeneration has been exploited to breed alfalfa with a high regeneration capacity [21]. This approach could potentially be applied to improve regeneration in soybeans, as the regeneration capacity of the best genotype identified so far is still lower than that of many other species.

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References

1. Goodman RM, Kiser J (1985) Biotechnology and its impact on the future developments in soybean production and use. In: Shibles R (Ed) World Soybean Research Conference III: Proceedings pp 261–271. Westview Press, Boulder and London
2. Lippmann, B, Lippmann G (1984) Induction of somatic embryos in cotyledonary tissue of soybean, *Glycine max*. Plant Cell Rep 3: 215–218
3. Ranch JP, Oglesby L, Zielinski AC (1985) Plant regeneration from embryo-derived tissue cultures of soybeans. In Vitro Cell Dev Biol 21: 653–657

4. Ranch JP, Oglesby L, Zielinski AC (1986) Plant regeneration from tissue culture of soybean by somatic embryogenesis. Cell Culture and Somatic Cell Genetics of Plants 3: 97–110.
5. Lazzeri PA, Hildebrand DF, Collins GB (1985) A procedure for plant regeneration from immature cotyledon tissue of soybean. Plant Mol Biol Rep 3: 160–167
6. Lazzeri PA, Hildebrand DF, Collins GB (1987a) Soybean somatic embryogenesis: effects of hormones and culture manipulations. Plant Cell Tiss Org Cult 10: 197–208
7. Ghazi, TD, Cheema HV, Nabors MW (1986) Somatic embryogenesis and plant regeneration from embryogenic callus of soybean, *Glycine max* L. Plant Cell Rep 5: 452–456
8. Hammatt N, Davey MR (1987) Somatic embryogenesis and plant regeneration from cultured zygotic embryos of soybean (*Glycine max* L.). J Plant Physiol 128: 219–226
9. Hartweck LM, Lazzeri PA, Cui D, Collins GB, Williams EG (1988) Auxin-orientation effects on somatic embryogenesis from immature soybean cotyledons. In Vitro Cell Dev Biol 24: 821–828
10. Lazzeri PA, Hildebrand DF, Collins GB (1987b) Soybean somatic embryogenesis: effects of nutritional, physical and chemical factors. Plant Cell Tiss Org Cult 10: 209–220
11. Barwale UB, Kerns HR, Widholm JM (1986) Plant regeneration from callus cultures of several soybean genotypes via embryogenesis and organogenesis. Planta 167: 473–481
12. Tetu T, Sangwan-Norreel B, Sangwan R (1987) Embryogenese somatique et regeneration in vitro chez trois varietés prococes de soja. CR Acad Sci Paris (Series III) 305: 613–617
13. Owens LD, Cress DE (1985) Genotypic variability of soybean response to *Agrobacterium* strains harboring the Ti and Ri plasmids. Plant Physiol 77: 87–94
14. Research Group of the Soya Bean Tissue Culture (1976) Successful induction of the plantlets from the callus culture of soya hypocotyl. Acta Bot Sin 18: 258–262
15. Delannay X, Rodgers DM, Palmer RG (1983) Relative genetic contributions among ancestral lines to North American soybean cultivars. Crop Sci 23: 944–949
16. Hymowitz T, Newell CA, Carmer SG (1977) Pedigrees of soybean cultivars released in the United States and Canada INTSOY. Series Nr 13
17. St Martin SK, Bernard RL (1982) Strain index for uniform regional soybean tests 00 to IV, 1967–1980. Research Bulletin 1147. The Ohio State University, Wooster, Ohio
18. Chen THH, Marowitch J (1987) Screening of *Medicago falcata* germplasm for in vitro regeneration. J Plant Physiol 128: 271–277
19. Mohapatra SS, Gresshoff PM (1982) Ecotypic variation of in vitro plantlet formation in white clover (*Trifolium repens*). Plant Cell Rep 1: 189–192
20. Bhojwani SS, Mullins K, Cohen D (1984) Intra-varietal variation for in vitro plant regeneration in the genus *Trifolium*. Euphytica 33: 915–921
21. Bingham ET, Hurley LV, Kaatz DM, Saunders JW (1975) Breeding alfalfa which regenerates from callus tissue in culture. Crop Sci 15: 719–721