Plant Science, 83 (1992) 103–111
Elsevier Scientific Publishers Ireland Ltd.

Somatic embryogenesis in *Arachis hypogaea* L.: genotype comparison

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(Received November 20th, 1991; revision received January 24th, 1992; accepted January 28th, 1992)

Somatic embryogenesis and embryogenic callus formation occurred from immature cotyledon and embryo axis explants of seven peanut (*Arachis hypogaea* L.) genotypes including one valencia, three Spanish and three Virginia botanical types. The initiation medium consisted of Murashige and Skoog's salts and vitamins with 3% sucrose and 0.5 mg/l picloram. There were significant differences among genotypes for somatic embryo formation, subculture capacity and plant regeneration using a single media sequence. Somatic embryo formation from cotyledons showed better correlation with plant regeneration than somatic embryo formation from the embryo axis. Maturity of the embryo explant, as estimated by cotyledon length, did not show a significant effect on response, but there was a trend toward reduced somatic embryo formation from older cotyledons. Relatively few plants were regenerated from the valencia genotype. All peanut genotypes tested thus far are competent for somatic embryogenesis and in vitro response could probably be improved by media manipulation.

Key words: Arachis; groundnut; peanut; somatic embryogenesis; tissue culture

Introduction

For successful foreign gene transfer, it is necessary to select a tissue culture system with a high capacity for plant regeneration. Different genotypes of the same crop species often do not respond identically in tissue culture. A continuum from no to high response has been observed; however, low response occasionally can be modified by media manipulation so that the degree of difference among genotypes can be narrowed. Genotype or genotype × medium interactions in a number of crop plants have been observed for regeneration via somatic embryogenesis. In cotton, some genotypes did not form somatic embryos while others were highly embryogenic [1]. Altering the medium did not change the relative levels of response. Gawel and Robacker [2] observed a genotype × medium effect in cotton resulting from intra-varietal variation, even though cotton is primarily an inbred crop.

Among forage legumes, intra-varietal variation for in vitro regeneration capacity is prominent within outcrossing species such as *Medicago sativa* L. (alfalfa) and *Trifolium* species. Much of the tissue culture research conducted with alfalfa has utilized lines bred from several highly regenerable clones [3]. It is possible, however, to select regenerable genotypes from other cultivars of regional interest [4]. A similar variation in regeneration potential is seen in white clover (*Trifolium repens* L., Ref. 5) and red clover (*Trifolium pratense* L., Ref. 6). Keyes et al. [6] did not find a significant change in embryogenic potential to occur with different media. The success of other in vitro procedures, e.g. suspension or protoplast culture, compounded with regeneration ability from primary cultures or callus, can further limit the number of useful genotypes. This is illustrated with red clover where 14 out of 64 genotypes could be regenerated from callus, but only 3 could be regenerated from protoplasts [7].

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Grain legumes are largely inbreeding and are not subject to as much intra-varietal variation as outcrossing forage legumes. Comparisons among cultivars have shown considerable variation for embryogenic competence in soybean (*Glycine max* (L.) Merrill, Refs. 8,9) and pea (*Pisum sativum* L., Ref. 10), although within cultivar response may be relatively uniform. In most genotypes of soybean and pea, embryogenic competence is not totally lacking, but cannot be increased substantially by manipulating the medium.

Somatic embryogenesis and plant regeneration in peanut (*Arachis hypogaea* L.) have been achieved from immature cotyledon explants [11], immature embryo axes [12] and whole immature embryos [13]. Using whole immature embryos 2–5 mm in length, Sellars et al. [13] tested the ability of cultivars from each of three botanical types (NC-7, virginia; McRan, valencia; Comet, spanish) to form somatic embryos and found that significant effects could be attributed to genotype. However, embryogenesis does not necessarily correlate with regeneration ability. The present study was designed to test the effect of peanut genotype (three virginia, one valencia and three Spanish types) on the ability of immature embryo axes and cotyledons to produce somatic embryos, the capacity for further propagation of somatic embryos and embryogenic tissue over a single subculture, and the potential of these cultures for plant regeneration.

Materials and Methods

Plant material

Seven genotypes were selected for their genetic diversity based on common parentage estimates [14]. These cultivars included *A. hypogaea* ssp. *hypogaea* var. *hypogaea* Tifrun, Sunrunner, Virginia Runner G-26; *A. hypogaea* ssp. *fastigiata* var. *vulgaris* Toalson, Dixie Spanish, Spancross; and *A. hypogaea* ssp. *fastigiata* var. *fastigiata* Georgia Red. Peanut plants were grown in pots in the greenhouse and immature pods were harvested from various plants between March and October, 1990. Date of pod harvest was recorded and converted to a numerical value with the equation, (month × 30) + day, for statistical analysis.

Tissue culture

Immature seeds were removed from pods and surface sterilized according to the method of Ozias-Akins et al. [15]. Briefly, after removal of surface debris on pods by washing, seeds were excised and treated sequentially in 70% (v/v) ethanol (1 min), 20% (v/v) commercial bleach (Clorox) (20 min) and sterile deionized H_2O (four rinses). Immature embryos at various stages of development ranging from early cotyledonary to morphologically mature, corresponding to stages 4–7 of Pattee et al. [16], were excised from the seed and each embryo was separated into three explants, two cotyledons and the embryo axis. Cotyledons were oriented with the abaxial side in contact with the medium and two embryos were cultured per 100 × 15 mm plastic Petri dish. The initial culture medium consisted of Murashige and Skoog's [17] salts and vitamins (MS), 3% (w/v) sucrose, 0.5 mg/l picloram (Dow/Elanco) and 0.8% (w/v) agar, autoclaved for 15 min at 121°C, 15 psi. After 4 weeks in the dark at 28°C, the number of somatic embryos from each explant type was recorded and embryogenic tissues from embryo axis and cotyledons were combined and transferred to medium of the same composition for one additional 4-week period, subsequently referred to as the subculture period. Further culture of embryogenic tissues was according to a protocol for embryo maturation and conversion [15] that had been determined from 10-month-old embryogenic cultures of Florunner maintained by monthly transfers on a medium containing 0.5 mg/l picloram. Briefly, somatic embryos were subcultured for one passage on 0.05 mg/l picloram (dark), transferred to MS plus 10% (w/v) sucrose and 0.5% (w/v) charcoal for 4–5 weeks (dark), followed by MS plus 3% (w/v) sucrose and 25 mg/l benzylaminopurine (BAP) for 3 weeks (light), then basal MS medium for 6 weeks (light), at which time the cultures were scored for shoot formation. Conditions for light incubation were 16/8 h (light/dark) cycle at an average photon flux density of 50 $\mu E\ s^{-1}\ m^{-2}$ provided by cool white fluorescent lamps.

Data collection and statistical analysis

One 'culture' hereafter refers to one original, seed-derived embryo. The length of cotyledons

from each embryo was measured as an estimate of embryo maturity. Cotyledon length was converted to a percentage value that could be compared across genotypes by dividing length by the average length of 50 mature seeds from each respective genotype and multiplying by 100. Data from the primary culture period were used to calculate the percentage of cultured cotyledons that gave rise to somatic embryos, the number of somatic embryos formed per cultured cotyledon, the percentage of cultured embryo axes that gave rise to somatic embryos, and the number of somatic embryos formed per cultured embryo axis. At the end of the first subculture period, both the number of somatic embryos and the presence of embryogenic callus were recorded. Data from the first subculture period were used to calculate the percentage of cultures that formed somatic embryos during subculture, the number of somatic embryos formed per culture during the subculture period and the percentage of cultures that formed embryogenic callus during the subculture period. Cultures were scored for plant regeneration only if a tetrafoliolate leaf was observed. Data on shoot formation were used to

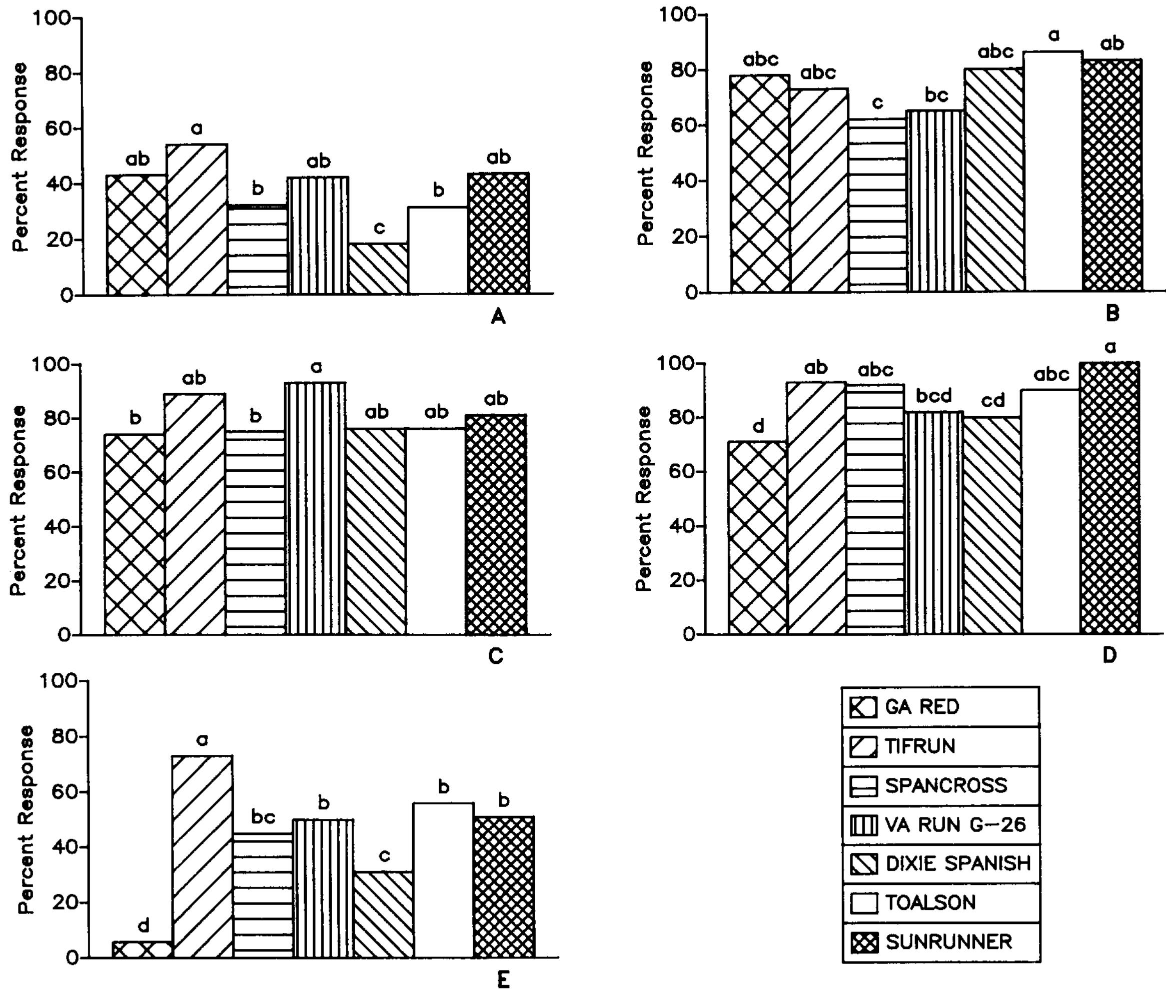

Fig. 1. Percent of cultures forming somatic embryos from the cotyledons (A) and embryo axis (B) at the end of the primary culture period; percent of cultures forming somatic embryos (C) and embryogenic callus (D) at the end of the first subculture period; and percent of cultures forming shoots (E) at the end of the regeneration period. Mean separation was performed by the Waller procedure, $P \leq 0.05$. Bars with different letters are significantly different.

calculate the percentage of cultures producing shoots and the number of shoots per culture. Characters calculated as described above were analyzed using the general linear models (GLM) program of SAS [18]. Mean separation analysis was performed using the Waller procedure. Product-moment correlation (Pearson) coefficients were calculated among genotypes for all traits and within genotypes for the number of shoots formed with all other traits.

Results

Both cotyledon and embryo axis explants formed somatic embryos after 4 weeks on medium containing picloram. The percentage of cultures producing somatic embryos (Fig. 1A,B) and the number of somatic embryos per responding explant (Fig. 2A,B; Table I) were higher for all genotypes with embryo axis than with cotyledon explants. However, only clearly identifiable somatic embryos were scored and cotyledons frequently formed proliferating embryogenic tissue that could be subcultured but was not classified as a somatic embryo. For explant type, the percentage and number of somatic embryos at the end of one subculture were significantly correlated only with the number of somatic embryos on the cotyledon and not the embryo axis (Table II). Embryo axes tended to produce friable callus in addition to somatic embryos (Fig. 3A). Significant differences among genotypes existed for both frequency and magnitude of primary and secondary responses (Table III). At the end of one subculture period, the genotypic differences on somatic embryo and embryogenic callus production remained significant (Figs. 1C,D,2C). Georgia Red, the only valencia cultivar we tested, gave an intermediate response for culture initiation but was the poorest for culture maintenance (performance during subculture) and shoot formation.

Thirty-seven percent (35 out of 95 somatic em-

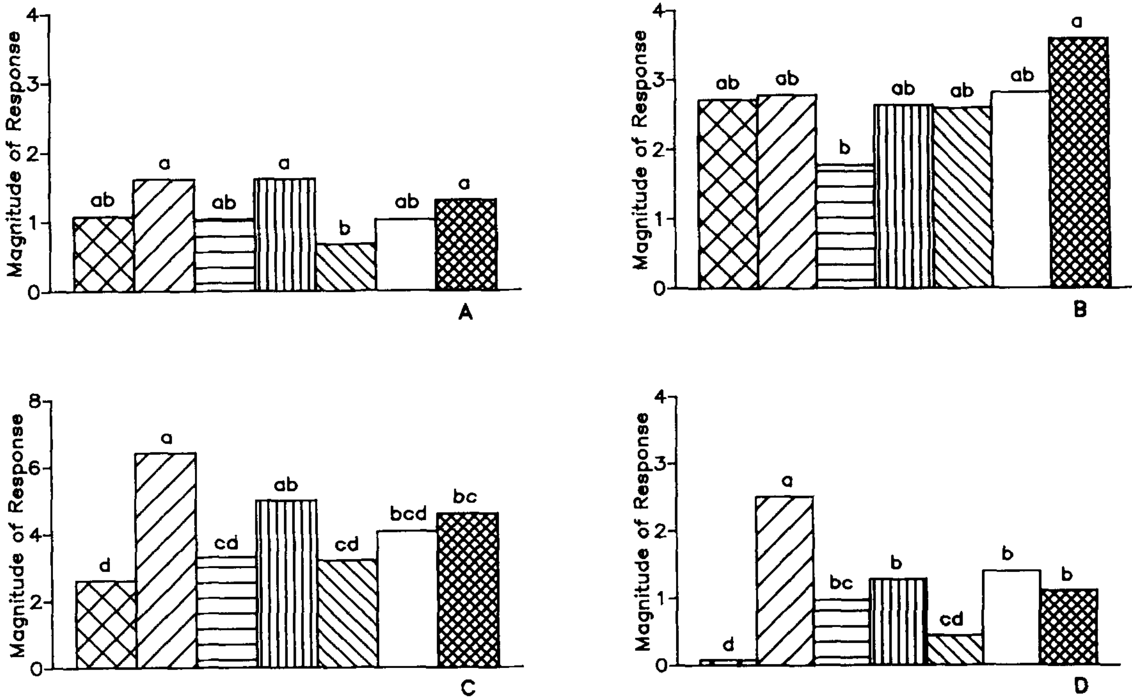

Fig. 2. Number of somatic embryos (magnitude of response) formed per cotyledon (A) and embryo axis (B) at the end of the primary culture period; number of somatic embryos formed from combined explants at the end of the first subculture period (C); and number of shoots formed per culture (D) at the end of the regeneration period. Mean separation and legend for genotypes as in Fig. 1. Bars with different letters are significantly different.

Table I. Range of data collected for measured and calculated characters by genotype. The number of somatic embryos after subculture refers to combined embryo axis and cotyledon embryogenic tissues at the end of the first subculture period.

	Genotype						
	GA Red	Tifrun	Spancross	VA Runner	Dixie Span.	Toalson	Sunrunner
Date	104–261	103–291	111–303	112–323	113–303	95–255	102–315
Maturity (%)	31–85	28–107	36–127	12–112	30–140	42–108	27–93
Number somatic embryos per cotyledon	0–4	0–5.5	0–8	0–9	0–4	0–5	0–6
Number somatic embryos per embryo axis	0–15	0–10	0–12	0–14	0–9	0–10	0–13
Number somatic embryos after subculture	0–7	0–15	0–18	0–17	0–12	0–18	0–21
Number shoots per explanted embryo	0–2	0–12	0–7	0–9	0–2	0–11	0–8
Number cultured embryos	35	55	60	60	54	63	53

bryos on 11 culture plates) of Florunner somatic embryos obtained from 10-month-old cultures formed plants on basal medium after maturation using a previously published protocol [15]. The same protocol was used to evaluate the ability of different genotypes to form plants. Mature somatic embryos either were clearly dicotyledonous (Fig. 3B) or composed of multiple, fasciated cotyledons and an expanded shoot apical region (Fig. 3C). Plant formation could occur from both types of somatic embryos. Root and hypocotyl elongation and/or hypertrophy occurred more frequently than shoot elongation. Presence of tetrafoliolate leaves, not root or hypocotyl elongation, was the criterion for evaluating embryo conversion (Fig. 3D). The percentage of cultures forming shoots ranged from 5% for Georgia Red to 71% for Tifrun (Fig. 1E). The other cultivars were significantly different from the two extremes at $P \leq 0.05$. The average number of shoots per responding culture reflected the same trend as the percentage of cultures forming shoots, i.e., Georgia Red

Table II. Pearson's correlation coefficients for calculated characters obtained by combining data from all genotypes. A, percent of cotyledons forming somatic embryos; B, number of somatic embryos per cotyledon; C, percent of embryo axes forming somatic embryos; D, number of somatic embryos per embryo axis; E, percent of cultures forming somatic embryos after subculture; F, Number of somatic embryos per culture after subculture; G, percent of cultures forming shoots; H, percent of cultures forming embryogenic callus; I, number of shoots per culture; J, cultured embryo maturity.

	A	B	C	D	E	F	G	H	I	J
A	1.00	0.88**	−0.18	0.45	0.45	0.62	0.34	0.09	0.57	−0.69
B		1.00	−0.33	0.36	0.79*	0.80*	0.56	0.19	0.69	−0.65
C			1.00	0.69	−0.26	−0.00	0.38	0.21	−0.01	0.09
D				1.00	0.28	0.45	0.27	0.29	0.30	−0.45
E					1.00	0.88**	0.74	0.33	0.72	−0.23
F						1.00	0.89**	0.51	0.94**	−0.16
G							1.00	0.75	0.93**	0.11
H								1.00	0.55	0.16
I									1.00	0.00
J										1.00

*,**Significant at $P \leq 0.05$ and 0.01, respectively.

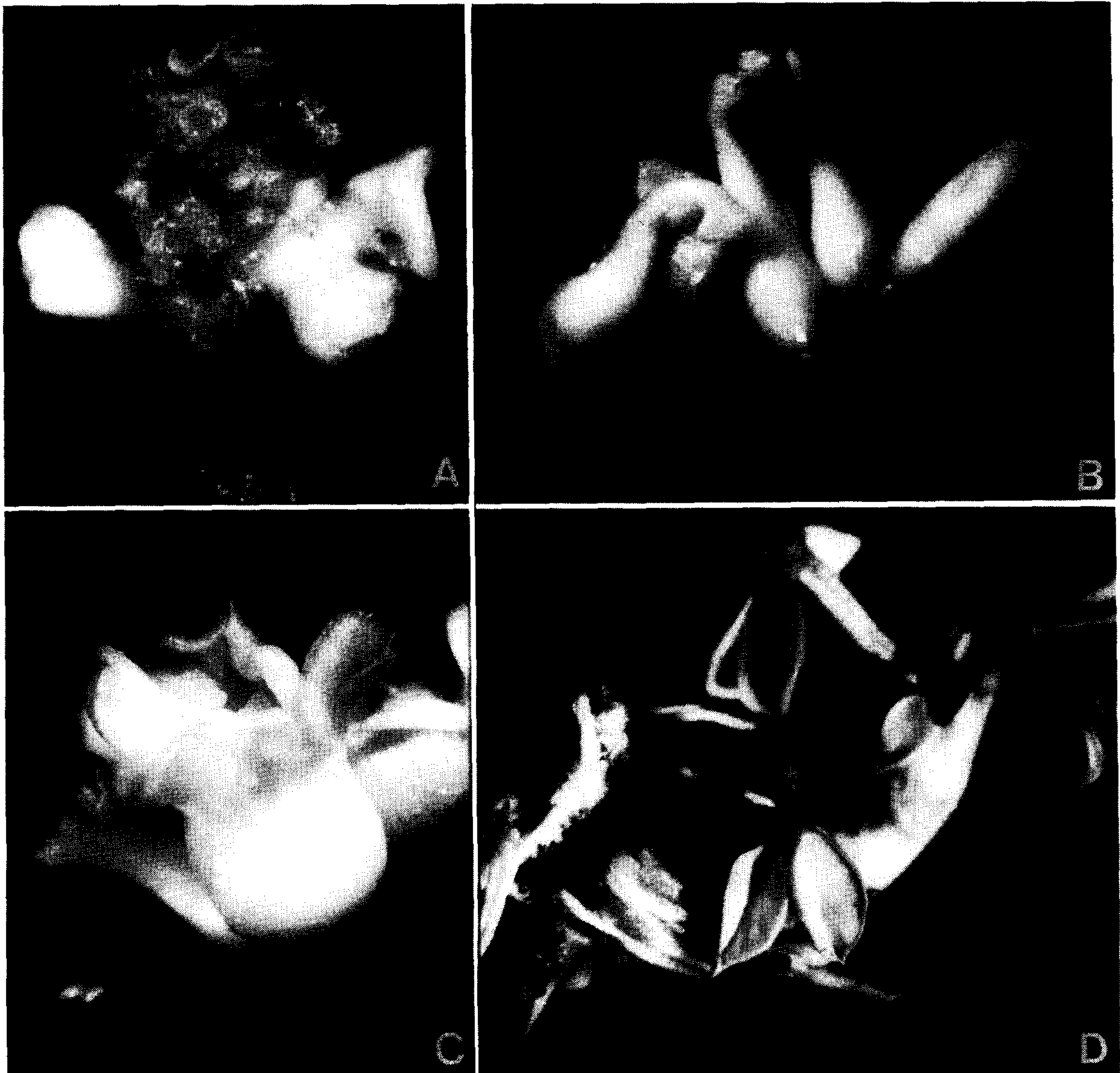

Fig 3. (A) Somatic embryos and friable callus developing from a cultured immature embryo axis (× 14). (B) Dicotyledonous somatic embryos on maturation medium (× 15). (C) A fasciated somatic embryo (× 18). (D) A germinating somatic embryo (× 7.5).

formed the least and Tifrun formed the most shoots under our culture conditions (Fig. 2D). Shoot formation from certain genotypes was negatively correlated with date of culture, but there was no correlation with embryo maturity (Table IV). In 5 out of 7 genotypes, the number of shoots was significantly correlated with the number of somatic embryos formed on cotyledons (Table IV). In all genotypes, the correlation between number of shoots and percentage of cultures forming shoots was highly significant.

Discussion

Somatic embryos have been observed to arise from cotyledon [11] and embryo axis [12] explants

Table III. Mean squares and errors for effect of genotype on tissue culture characters examined.

	Genotype	Error
Frequency of cotyledons forming somatic embryos	0.74***	0.12
Number of somatic embryos per cotyledon	6.55**	2.12
Frequency of embryo axes forming somatic embryos	0.48*	0.18
Number of somatic embryos per embryo axis	15.94	7.86
Frequency of cultures forming somatic embryos after subculture	0.32*	0.15
Number of somatic embryos per culture after subculture	83.75***	15.84
Frequency of cultures forming embryogenic callus	0.43***	0.10
Frequency of cultures forming shoots	1.92***	0.22
Number of shoots per culture	29.14***	3.35

*,**,***Significant at $P \leq 0.05$, 0.01 and 0.001, respectively.

of peanut. In soybean, apparently only the cotyledons are competent for somatic embryogenesis [8,13] and only within a relatively narrow range of development [8]. Our results with peanut show no significant differences in embryogenic response across a wide range of developmental stages designated as percent mature seed size. Although the differences in maturity did not significantly affect response, there was a trend towards reduced somatic embryo formation from older cotyledons. Sellars et al. [13] also did not observe a significant effect of explant stage; however, they only cultured whole embryos 2 to 5 mm long. The percentage and number of somatic embryos formed from the cultured embryo axes were consistently higher than from the cotyledons; however, statistically the magnitude of somatic embryo formation from the cotyledons and not the embryo axes, was correlated with shoot formation for most genotypes. This might partially be explained by the large number of axis-derived structures that were scored as somatic embryos but which appeared to develop

Table IV. Correlation coefficients for the number of shoots per culture and other calculated characters by genotype.

	Genotype						
	GA Red	Tifrun	Spancross	VA Runner	Dixie Span.	Toalson	Sunrunner
Date	−0.01	−0.26	−0.34**	−0.38**	−0.05	−0.44***	−0.34*
Maturity	−0.25	−0.02	0.07	0.05	−0.07	0.17	0.14
Percent cotyledons forming somatic embryos	0.37*	0.33*	0.14	0.17	0.08	0.04	0.17
Number somatic embryos per cotyledon	0.48**	0.34*	0.26*	0.33**	0.17	0.04	0.34*
Percent embryo axes forming somatic embyos	0.13	0.15	0.09	0.07	0.12	0.08	0.21
Number somatic embyos per embryo axis	−0.05	0.30*	0.05	0.22	−0.16	0.03	0.07
Percent cultures forming somatic embryos after subculture	0.14	0.24	−0.09	0.10	0.05	−0.00	0.12
Number somatic embryos formed after subculture	−0.06	0.37**	−0.04	0.20	−0.05	−0.00	−0.02
Percent cultures forming shoots	0.95***	0.57***	0.68***	0.65***	0.92***	0.59***	0.65***
Percent cultures forming embryogenic callus	0.15	0.19	0.11	0.26*	0.06	0.16	0.00

*,**,***Significant at $P \leq 0.05$, 0.01 and 0.001, respectively.

from trichomes and had a persistent trichome-like projection at the distal end that preempted shoot formation.

We observed highly significant differences among genotypes for most of the characters examined. Some spanish and virginia genotypes showed good proliferation during subculture and moderate plant regeneration capacity. The one valencia genotype used in our study did not regenerate well with the culture protocol used. This probably is not a common feature of valencia types since Sellars et al. [13] obtained a good response with McRan. From our experience thus far, it seems likely that most peanut genotypes are competent for somatic embryogenesis but may vary in their optimal culture requirements. The ability to maintain repetitive embryogenesis and regeneration of shoots is the most important aspect of a tissue culture system that is to be used for transformation. Genotypes with the highest competence for somatic embryo production do not necessarily give the highest rate of embryo conversion to plants [19]. In our study, the percentage and number of shoots formed were highly correlated with each other in all genotypes as were the percentage and number of somatic embryos formed from the cotyledons. Parrott et al. [9] observed the same relationship between percentage and number of somatic embryos formed on cotyledons of soybean.

The ability of most genotypes of peanut to produce somatic embryos or embryogenic callus at a reasonable frequency and magnitude and to regenerate plants using a single protocol is encouraging for transformation of elite genotypes. Determining the factors that reduce anomalous embryo development and altering the media sequence for plant regeneration with or without a desiccation step should increase the frequency of embryo conversion above the 30–40% we routinely achieve from long-term cultures.

Acknowledgements

The technical assistance of Anne Bell is gratefully acknowledged. We thank Drs. David Knauft, Jennifer Schnall and Phil Bruckner for critical reading of the manuscript. This work was partially supported by grants from the National Peanut Foundation and the Georgia Agricultural Commodity Commission for Peanuts.

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