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The effect of parental genotype on initiation of embryogenic callus from elite maize (*Zea mays* L.) germplasm

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Summary. Embryogenic callus consisting of both Type 1, firm, compact, translucent, relatively slow growing callus and Type 2, highly friable, rapidly growing callus with well-formed somatic embryos, were observed in elite maize germplasm, notably B73 and hybrids with B73. Parental genotype is very important in the ability to identify and isolate embryogenic callus after 14 and 28 days in culture. A partial diallel analysis revealed that a large proportion of the genotypic variation was of the additive type although heterosis did positively increase culture response in most cases. A significant negative maternal effect for culture response was noted for inbred B73 from Reid-type germplasm while four lines sampled from Lancaster germplasm showed similar response whether used as male or female. Although significant media differences were observed in some genotypes, culture media did not preclude observation of Type 1 or Type 2 embryogenic cultures in this study after 14 and 28 days. Plants could be regenerated from all genotypes in this study after 14-days of culture, but not all genotypes were capable of sustained subculture and plant regeneration. Plant regeneration from Type 2 cultures of B73 and B73 hybrids has been obtained up to a year after initiation.

Key words: Somatic embryogenesis – Plant regeneration – In vitro culture – Genetic variance

Introduction

Tissue and cell culture is a tool which potentially can be used for propagation, selection at the callus level and as a vehicle for recovery of complete plants from genetic transformation of individual cells such as protoplasts. Efficient use of this technology in the major cereal crops including maize has been hampered by the inability to initiate and establish stable cell cultures capable of efficient in vitro manipulation including plant regeneration (Tomes and Swanson 1982).

Embryogenic callus cultures have been described in maize inbred A188 by Green (1982) and by Vasil et al. (1984) in the hybrid cultivar 'DeKalb™ XL82'. Several factors are important in initiating embryogenic cultures of maize including the explant (Green 1982), culture medium (Lu et al. 1983; Green et al. 1983) and genotype (Armstrong 1984; Bartkowiak 1982; Beckert and Qing 1984; Neštický et al. 1983).

The ability to regenerate plants from callus cultures has a large genotypic component in other species such as alfalfa (Bingham et al. 1975; Brown and Atanassov 1984), red clover (Keyes et al. 1980), birdsfoot trefoil (Glover and Tomes 1980), and tomato (Frankenberger et al. 1981). Neštický et al. (1983) observed a clear genotypic response for callus growth from mature embryos from a diallel analysis of six inbred lines of maize while Tabata and Motoyoshi (1965) made similar observations for endosperm callus growth. Regeneration ability was not assessed in these studies. Bartkowiak (1982) observed both genetic and environmentally-induced differences in ability to regenerate plants among a series of inbred maize lines. Beckert and Qing (1984) measured callus growth, shoot and root forming capacity among eight inbred maize lines, and observed a "considerable" amount of genetical variation for these traits. Lu et al. (1983) found variation among several commercial maize hybrids in their study, but, because of lack of pollen control, could not demonstrate the relative magnitude of genetic and nongenetic components of their tissue culture response.

The objectives of this research were to isolate embryogenic cultures capable of plant regeneration from a series of elite maize inbreds and hybrids and to determine the relative contribution of genotype in establishing stable embryogenic cultures.

Materials and methods

The maize inbred lines used in the present studies are shown in Table 1. Each inbred is used as a parent of at least one

Table 1. Germplasm background of maize inbred lines used for partial diallel analysis of embryogenic callus growth from immature embryos

Inbred	Germplasm (%)		
	Reid[a]	Lancaster type[b]	Other[c]
B73	100	–	–
G39*	75	–	25
G50*		50	50
B76*		50	50
G35*		75	25

* Proprietary inbreds
[a] Iowa Stiff Stalk Synthetic (BSSS), Sprague (1946). B73 released from Iowa State University, 1972
[b] Includes Iodent, Minn 13, Ohio (Illinois Long Ear)
[c] Complex pedigree and/or exotic germplasm

commercial maize hybrid in the United States. In addition, inbred A188 (Minnesota Agricultural Experiment Station, 1948) was used for some studies. Genetic identity was assured by hand pollination. Embryo size was measured at the time of culture to assure a size range between 0.9–2.1 mm in length. Embryos from A188 and B73 were sampled from greenhouse and field grown plants in 1983 and 1984. Plants were grown under environmental conditions which promoted excellent, vigorous plant growth prior to the dissection of immature embryos for culture. A partial diallel cross from field grown plants in the summer of 1983 which included self-pollinations and reciprocals was made between many combinations of the lines in Table 1. For these studies, the size of each embryo was recorded at the time of culture with only embryos between 0.9–2.1 mm used for analysis. At least two plants were sampled from each genotype with a minimum of 100 embryos cultured unless otherwise noted.

Approximately equal numbers of embryos were cultured on a Murashige and Skoog (MS) mineral salts medium with 1 mM asparagine (Green and Phillips 1975) and on N6 salts with 6 mM proline (Green et al. 1983). In both culture media, 2,4-D was used at 0.75 mg/l and the culture medium was solidified with Gelrite™ at 3 g/l. Unless otherwise noted, the results from the two culture media were combined for data analysis.

Embryos were classified into three categories after 14 and 28 days in culture. Embryos which produced nonembryogenic callus or germinated precociously were classified as Type 0 and discarded after 14 days in culture. Type 1 response consisted of the proliferation of a translucent, convoluted, and compact callus similar to that described as organogenic by Green (1982) and Armstrong and Green (1984) and embryogenic by Lu et al. (1983). Type 2 response was characterized as friable and fast growing with well-defined somatic embryos with suspensor-like structures as described by Green (1982); Armstrong and Green (1984), and Vasil et al. (1984). Both Type 1 and Type 2 are considered an "embryogenic" response. Embryos classified as Type 1 or Type 2 after 14 days were transferred to fresh medium and again evaluated after a further 14 days in culture. Embryos which became contaminated or were damaged by the dissection process were excluded from the data.

The proportion of responsive (i.e., embryogenic response) embryos (Type 1 plus Type 2) was the trait analyzed in the partial diallel analysis. The data are presented as percent responsive embryos. Ear and culture media effects were considered as random effects while genotypic effects were fixed. The data were analyzed using the following linear model:

$$Y_{ij} = u + m_i + a_i + a_j + Sh + e_{ij}$$

where u is the overall mean,

m_i is the maternal effect of the i^{th} parent when used as a female,
a_i and a_j are the additive effects of the i^{th} and j^{th} parents,
S is equal to 0 for $i = j$ and 1 otherwise,
h is the average heterosis effect, and
e_{ij} is the interaction effect of the i^{th} and j^{th} gametes.

The analysis of variance was partitioned into additive, nonadditive, and maternal effects. The linear model was used to estimate additive effects of each of the inbreds in this study, maternal effects of B73 and G39, and an estimate for heterosis.

Results

A consistent genotypic difference in response was observed between A188 (high responder) and B73 (low responder) sampled at four different times (Table 2). In all cases, plant growth was vigorous and plant condition was healthy. A188 plants grown under more adverse conditions such as low light and high plant populations had a very low response (data not shown). The effect of different environmental conditions is suggested by the relatively different response from different sampling times. Both A188 and B73 had some embryos which produced Type 2 cultures, but not under every environment tested.

All of the inbreds and hybrids in this study had immature embryos which were capable of an embryogenic response after 14 days in culture. Inbred G35 and hybrids between G35 and B73 also produced Type 2, friable callus at a low frequency after 14 and 28 days.

Table 2. Percent embryos producing embryogenic callus after 14 days from maize inbreds A188 and B73 sampled in different environments

Time	Location	Genotype	*N*	% embryo response	
				Total	Type 2
5/83	GH[a]	A188	751	14.0	4.0
7/83	F	A188	90	77.0	+
12/83	GH	A188	85	38.0	7.0
5/84	GH	A188	890	30.0	18.0
5/83	GH	B73	858	0.6	0.6
7/83	F	B73	469	0.0	0.0
4/84	GH	B73	805	0.0	0.0
6/84	GH/F	B73	2,247	0.4	0.2

[a] GH = greenhouse, F = field
+ = Type 2 embryogenic callus not recorded

B73 was a very low responder (< 1%) while G35 had a very high response (> 50%) (Table 3). Among intermediate responding inbreds, inbred G39 response declined between 14 and 28 days because plants were formed with little or no subsequent callus growth while inbred B76 produced only nonembryogenic callus during the second subculture period.

Hybrids between most lines had midparent values or above after both 14 and 28 days. A notable exception to midparent values was observed in crosses of B73 and G39 with inbred G35. When G35 was the maternal parent, near midparent values were observed in the G35 × B73 hybrids while the B73 × G35 hybrid had a significantly lower response (Tables 3 and 4). A similar though less dramatic response was also noted with G39 × G35 reciprocal crosses (Table 3). As a group, the Reid germplasm (Iowa Stiff Stalk Synthetic, BSSS) was less responsive in both inbred and hybrid combinations than the Lancaster-type inbreds sampled in this experiment (Tables 1 and 3).

Highly responsive lines such as G35 had a consistent response throughout the 1–2 mm range while B73 gave a nil response in all but the 2 mm size. Hybrid G35 × B73 had a response pattern similar to G35 while the B73 × G35 hybrid responded best at the 1.7 mm size (Table 4).

Further analysis of the diallel cross revealed that a large proportion (70%) of the observed genotypic variation was additive at both 14 and 28 days (Table 5). Maternal effects and heterosis also accounted for a significant portion of the genetic variation observed. Reciprocal effects were nonsignificant except for specific maternal effects (see below). The genotypic estimates were used to calculate a predicted response using the linear model (Table 6). These estimates indicate a wide range in the inherent response among the inbred lines sampled in this study (i.e., B73 versus G35). These estimates also indicated that the lines sampled from Reid germplasm (i.e., BSSS) have a lower response when used as a maternal parent than when used as a male parent while Lancaster germplasm had nonsignificant maternal effects. Heterosis was also shown to enhance the level of in vitro response in a positive fashion.

F_1 and F_2 embryos with the same genotypic background performed similarly in the two combinations tested (Table 7). Specific genotypes responded to MS and N6 media formulations similarly in some pedigrees (B73, B73 × G35) while others showed an increased response to MS such as G35 and G35 × B73 (Table 8). Although statistically significant differences were often observed, the media formulations did not result in a complete inhibition of response, nor preclude observation of the Type 2 cultures within pedigrees in which they were observed (Tables 2 and 3).

Table 3. Proportion (%) immature embryos which produced embryogenic callus after 14 and 28 days from partial diallel cross of maize lines in 1983

♀/♂	Days	B73	G39*	G50*	B76*	G35*
B73	14	0.4	5			5[a]
	28	0.0	3			3[a]
G39*	14	4	10		42	30
	28	2	0		20	15
G50*	14			17		54[b]
	28			12		45
B76*	14		40		16[b]	
	28		21		0	
G35*	14	25[a]	48	44		58[a]
	28	18[a]	40	37		50[a]

* Proprietary inbreds
[a] Produced Type 2 friable embryogenic callus
[b] No. of embryos is 65 and 44 for G50/G35 and B76, respectively
Least significant difference at the 0.05 probability level = 7.5

Table 4. Proportion (%) immature embryos of different sizes from four genotypes of maize which produced embryogenic callus after 14 and 28 days

Embryo size (mm)	Percent response			
	B73 14–28 days	G35* 14–28 days	G35* × B73 14–28 days	B73 × G35* 14–28 days
0.9	0–0	33–22	7– 6	0–0
1.1	0–0	56–56	16–16	4–4
1.2	0–0	48–33	28–19	7–0
1.4	0–0	57–45	16–12	5–0
1.5	0–0	60–53	33–18	4–4
1.7	0–0	55–45	33–17	14–5
1.8	0–0	78–71	40–30	0–0
2.0	2–0	69–69	29–14	–
2.1	0–0	–	–	–

* Proprietary inbreds

Table 5. Partitioning of genotypic variation from the analysis of variance of embryogenic callus formation after 14 and 28 days of a partial diallel cross in maize

Source	DF	14 days			28 days		
		SS	MS	%	SS	MS	%
Additive	4	0.388	0.097	70	0.301	0.075	70
Maternal	1	0.054	0.054	10	0.040	0.040	9
Heterosis	1	0.018	0.018	3	0.010	0.010	2
Residual	8	0.095	0.012	17	0.077	0.010	18
Total	14	0.556			0.428		

Discussion

Our results indicate that elite maize germplasm has the necessary genetic potential to give rise to both Type 1 and Type 2 embryogenic callus (Tables 2 and 3). Highly embryogenic (Type 2) callus has been observed not only in A188 (Green 1982; Armstrong 1984), but also in B73 in contrast to the results of Armstrong (1984). The importance of genotype is clear for Type 1 embryogenic response and is implied for the highly embryogenic (Type 2) response observed in B73 × G35 crosses (Table 3) and in A188 × B73 segregants (Armstrong 1984).

Our inheritance data indicate a higher proportion of in vitro response determined by parental genotype than studies of Beckert and Qing (1984). Our measurements of embryo response were made at 14 and 28 days of culture while those of Beckert and Qing (1984) were made after one to three subcultures of 28 days each. Our inheritance data are more similar to Neštický et al. (1983) and Tabata and Motoyoshi (1965) who looked at nonembryogenic callus from mature embryo and endosperm tissue, respectively. Since plants have been regenerated from all of the genotypes studied following at least two subcultures, early classification of embryo response appears to be an accurate method to identify responsive genotypes at an early stage of culture. In addition, Type 2 callus from B73 retained the ability to regenerate plants for over a year while Type 1 cultures rapidly lost the ability to regenerate shoots in most pedigrees. The potential number of plants per unit of callus does differ among genotypes which is in agreement with Beckert and Qing (1984). Our results confirm those of Vasil et al. (1984) and Green et al. (1983) in which stabilizing highly embryogenic cultures depends on both genotype and the culture environment following initial observation. Stable Type 2 cultures have been repeatedly established from B73 (Table 2) and from B73 hybrids (Table 3). Further genetic analysis in which different environments are sampled may also clarify the extent of the genetic contribution and better describe the environmental conditions which enhance in vitro response.

Our genetic analysis shows a strong negative maternal effect for embryogenic callus production for maize inbred B73 and to a lesser extent in G39.

Similar maternal effects have been noted for callus growth per se from mature embryos (Neštický et al. 1983), for endosperm callus growth (Tabata and Motoyoshi 1965), and

Table 6. Estimates of genotypic response for proportion (%) embryogenic callus response after 14 and 28 days based on analysis of variance of a partial diallel cross of maize inbreds

Parameter	Estimate	
	14 days	28 days
Intercept	20.3	12.5
Additive		
B73	−13.9	− 9.0
G39	− 1.8	− 2.5
G35	13.8	13.6
B76	2.7	− 3.0
G50	− 0.8	0.9
Maternal		
B73	−21.5	−17.7
G39	− 8.7	− 9.9
Heterosis	14.3	11.8

Prediction equation: $\hat{y} = \mu + \text{maternal} + \text{additive male} + \text{additive female} + \text{heterosis}$

Table 7. Comparison of F_1 and F_2 embryo response for proportion (%) embryogenic callus production from two maize hybrid combinations after 14 days in culture

Pedigree	Embryo generation	*N*	%
G39* × B76*	F_1	156	42 a**
(G39 × B76)F_2	F_2	829	39 a
G39* × G35*	F_1	204	30 b
(G39 × G35)F_2	F_2	411	32 b

* Proprietary inbreds

** Different letter in a column denotes means are significantly different at the 0.05 probability level, Duncan's Multiple Range Test

Table 8. Comparison of MS and N6 media formulation on proportion (%) of embryogenic callus formation after 14 days from three maize genotypes

Culture media	Pedigree							
	N	B73	*N*	G35*	*N*	G35* × B73 *N*		B73 × G35*
MS	253	0.4 a**	147	65 a	83	34 a	74	5 a
N6	216	0.5 a	159	59 b	106	21 b	75	5 a

* Proprietary inbred

** Different letter in a column denotes means are significantly different at the 0.05 probability level, Duncan's Multiple Range Test

for other culture characteristics (Beckert and Qing 1984). Our small sample suggests that this response may be characteristic of BSSS and perhaps other Reid-type germplasm. The similarity of the lines used in our studies and those in other work is not known and thus prevents direct comparison of the results. A strong relationship between germplasm source and in vitro response has been noted in alfalfa (Brown and Atanassov 1984).

The underlying genetic basis of the reciprocal differences for in vitro response could be cytoplasmic factors (e.g., mtDNA), physiological characteristics of maternal plants, or segregation of nuclear factors in which a maternal parent of high responsiveness would have an advantage in vitro because of the immature embryo explant tissue. Although cytoplasmic factors have been hypothesized (Beckert and Qing 1984; Neštický et al. 1983; Tabata and Motoyoshi 1965), an unequivocal determination of the basis of maternal effects depends on genetic tests with different cytoplasms (i.e., mtDNA), backcrosses of F_1 with parental genotypes, and observations in F_2 generations.

Both culture media used in this experiment were broadly supportive of both Type 1 and Type 2 embryogenic callus growth from a range of maize genotypes. A differential response to N6 culture media has been observed in A188 (Armstrong 1984) and in other genotypes an enhanced response to MS (Table 8). Specific culture media used did enhance total response in some genotypes, but was not a necessity for either Type 1 or Type 2 cultures (Tables 2 and 3). The highly friable culture response within the first 14 days has been very low in our germplasm sample, but has been repeatable in several trials (data not shown). Further media experiments such as those described for A188 by Armstrong (1984) and Green et al. (1983) which enhance the highly friable response are needed for elite germplasm. The similarity in F_1 and F_2 embryo (Table 7) performance suggests that commercial maize hybrid cultivars, which are generally more responsive, with appropriate pollen control, might be used for critical media experiments in order to determine specific media changes which enhance the highly friable response.

The genetic data indicate that elite inbred germplasm can be used directly to establish callus and cell lines for further in vitro manipulations [B73 (Table 2), G39, G50 (Table 3)]. Inbreds which are especially difficult to maintain in culture could be used in specific hybrid combinations [e.g., G35 × G39 (Table 3)]. The high heritability for in vitro culture response also suggests that selection could be carried out rapidly in elite germplasm to maximize in vitro response (see Beckert and Qing 1984). The use of elite germplasm for both in vitro selection and genetic transformation has obvious advantages if the eventual objective is the commercialization of improved lines (Tomes and Swanson 1982).

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