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Improved efficiency of somatic embryogenesis and plant regeneration in tissue cultures of maize (*Zea mays* L.)*

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Summary. Immature embryos of eleven cultivars of hybrid maize (*Zea mays* L.), cultured on 2,4-D-containing nutrient media, showed rapid proliferation of the scutellum and improved efficiency in the formation of embryogenic callus and somatic embryos. High concentrations of sucrose were found to be most favorable for the formation of the embryogenic callus. Embryoids obtained in cultures of all eleven cultivars germinated in vitro and produced normal green plants which were grown to maturity in soil. The effect of genotype and other factors on somatic embryogenesis in maize are discussed.

Key words: *Zea mays* L. – Cereals – Maize – Somatic embryogenesis – Tissue culture

Introduction

Regeneration of plants has been obtained from tissue cultures of maize initiated from immature embryos (Green and Phillips 1975; Freeling et al. 1976; Torne et al. 1980). Regeneration was found to be restricted to a few highly inbred lines, and was believed to take place by the organization of shoot meristems (Springer et al. 1979). Compact, white structures described as scutellar bodies, were observed by some authors who noted that plants generally developed near these structures (Green and Phillips 1975; Freeling et al. 1976). In an earlier report we described the formation of embryogenic callus tissues from cultured immature embryos of 12 commercially used cultivars of maize, and the regeneration of plants by somatic embryogenesis in the cultivar 'Silver Queen' (Lu et al. 1982). These preliminary results have now been extended to all the cultivars tested, accompanied by improved efficiency of the formation of embryogenic callus, embryoids and plants.

Materials and methods

Eleven cultivars of hybrid maize ('Asgrow Rx112', 'Coker 16', 'Coker 22', 'Dekalb XL80', 'Dekalb XL82', 'Florida Stay Sweet', 'Funk G4507A', 'Funk G4864', 'Pioneer 3030', 'Pioneer 3320', 'Silver Queen') were grown in the field at Gainesville, Fla. Three plantings were done at two week intervals, beginning March 29, 1982. Whole cobs were removed following open pollination, and were sterilized with 70% ethanol for 1 min followed by 20% Clorox for 20 min. After three rinses with sterilized water, the immature embryos (ca 1–1.5 mm) were dissected out from the kernels and placed on the agar nutrient medium with the embryo axis in contact with the medium, and the scutellum exposed. Forty-two embryos of each cultivar were cultured on Murashige and Skoog's (1962) medium, supplemented with different concentrations of 2,4-D and sucrose (Table 1). The pH of the medium was adjusted to 5.8 with NaOH and the medium was sterilized by autoclaving for 17 min at 121 °C, 15 psi. The cultures were incubated in the dark at 27 °C. Results obtained from embryos of the first planting only are presented. Embryos from second and third planting were found to be of unsatisfactory quality.

Table 1. MS medium, with different concentrations and combinations of sucrose and 2,4-D, used for the culture of immature embryos of maize

2,4-D sucrose	0.25 mg/l	0.5 mg/l	1.0 mg/l	2.0 mg/l
3%	Z1	Z4	Z7	Z10
6%	Z2	Z5	Z8	Z11
12%	Z3	Z6	Z9	Z12

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Table 2. Response of immature embryos of maize cultured in different media. All figures are percentages. Forty-two embryos were cultured on each medium and observations were made 10 – 12 days after culture. (*C* = elongation of coleoptile, *R* = formation of roots, *Sc* = formation of soft callus, *Ec* = formation of embryogenic callus)

Cultivar		Medium											
		Z1	Z2	Z3	Z4	Z5	Z6	Z7	Z8	Z9	Z10	Z11	Z12
'Asgrow	C	92	95	33	86	95	39	100	77	57	81	71	40
Rx 112'	R	27	17	7	29	23	12	20	12	12	23	22	19
	Sc	32	15	8	24	19	8	23	14	4	37	21	17
	Ec	20	34	55	22	36	51	24	34	55	27	44	49
'Coker 16'	C	100	85	36	98	94	36	92	94	42	89	94	43
	R	14	39	15	14	23	5	13	14	5	10	3	0
	Sc	22	12	7	37	17	10	19	17	14	30	21	23
	Ec	20	36	50	29	35	61	37	50	55	12	25	50
'Coker 22'	C	98	86	46	90	88	20	94	67	27	81	83	14
	R	21	19	10	2	14	2	5	0	2	2	2	0
	Sc	12	5	12	19	8	5	15	12	11	24	20	16
	Ec	28	46	56	24	50	69	28	57	79	29	49	53
'Dekalb	C	98	81	10	100	93	21	100	86	21	100	95	52
XL 80'	R	7	10	0	7	10	0	7	5	2	0	2	2
	Sc	10	5	0	14	12	0	19	5	5	26	10	10
	Ec	81	69	95	55	83	98	40	69	95	45	64	79
'Dekalb	C	84	95	7	90	80	5	93	89	23	98	80	23
XL 82'	R	7	17	5	7	6	0	12	15	2	19	16	2
	Sc	18	10	7	27	14	7	38	16	11	24	20	19
	Ec	39	52	65	31	51	67	24	37	45	24	32	31
'Florida Stay	C	93	73	36	98	86	34	95	96	88	100	95	40
Sweet'	R	63	33	20	19	28	7	9	51	9	7	12	13
	Sc	27	10	2	17	2	0	20	14	2	20	16	12
	Ec	20	25	47	25	35	46	25	36	53	18	7	40
'Funk	C	80	78	14	78	92	14	85	78	23	80	75	38
G4507A'	R	14	11	0	11	25	0	20	5	2	26	16	5
	Sc	42	17	12	22	28	18	34	23	12	21	29	19
	Ec	18	43	54	33	45	68	27	48	72	30	37	50
'Funk G4864'	C	95	95	5	95	93	17	98	98	36	98	88	45
	R	19	31	2	24	26	2	10	26	7	10	17	12
	Sc	24	17	2	31	7	10	31	26	7	38	26	17
	Ec	57	62	83	38	81	90	48	71	81	33	69	76
'Pioneer	C	100	79	17	95	74	24	88	98	7	98	98	33
3030'	R	21	31	2	14	19	0	7	12	0	10	17	5
	Sc	20	29	5	12	17	5	27	17	0	21	24	10
	Ec	21	14	48	21	36	45	31	26	93	26	24	83
'Pioneer	C	62	48	33	74	52	21	60	60	10	67	60	12
3320'	R	7	7	0	12	12	5	5	12	2	2	2	2
	Sc	2	2	0	12	2	2	10	5	2	12	10	5
	Ec	57	93	95	38	81	93	43	86	95	40	67	95
'Silver	C	93	88	38	95	81	48	100	98	71	100	95	71
Queen'	R	38	31	5	48	24	0	21	29	0	21	33	2
	Sc	38	14	2	48	7	7	24	12	5	36	12	10
	Ec	43	74	88	33	67	88	40	62	90	14	52	79

For scanning electron microscopy cultured embryos and associated proliferations or embryoids were fixed in 2% glutaraldehyde for 2 h, and post-fixed in 1% osmium tetroxide overnight. After ethanol dehydration, the samples were critical point dried and coated with gold. The specimens were examined and photographed in a Hitachi S-450 scanning electron microscope at 20 KV.

Results

Initiation of callus from immature embryos

At the time of culture the immature embryos showed a well-defined shoot-root axis and a scutellum that was approximately twice the length of the embryo axis (Fig. 1). Within a few days after culture the scutellum increased in size, became opaque and showed early signs of cell proliferation at its periphery (Fig. 2). After 8–10 days a compact, opaque, white tissue, designated as embryogenic callus, was formed at the coleorhizal end of the scutellum. Occasionally callus was also seen over the entire upper (abaxial) surface of the scutellum.

The concentration of 2,4-D in the medium (0.25–2.0 mg/l) did not have as much influence on the quality and quantity of embryogenic callus formed as did the amount of sucrose (Table 2). The embryogenic callus formed in media containing 3% sucrose was less compact and opaque than that formed at higher concentrations of sucrose (6 and 12%). The callus was produced only in localized areas of the scutellum while other parts of it either became leafy or formed some soft callus. In some instances the leafy structures appeared to be arising from the embryogenic callus which itself was surrounded by a softer callus. These white and compact embryogenic calli dispersed amongst the soft callus are probably what Freeling et al. (1976) referred to as scutellar bodies. They have a smooth surface and show some organization. Higher concentrations of sucrose (6 and 12%) caused an increase in the frequency of embryogenic callus formation (Table 2). Improved organization of embryoids was observed at 6% sucrose. The soft callus produced was either negligible or absent.

Formation of somatic embryos

By the eighth to the ninth day of culture, organized structures similar to the early scutellar stage of zygotic embryos were observed on the underside (adaxial surface) of the scutellar proliferation. The appearance of these early stages of somatic embryos was soon followed by the formation of coleoptiles (Fig. 3). By 11–12 days, 5–14 well differentiated coleoptiles which either showed a terminal pore or a longitudinal slit were seen along the periphery of the scutellar proliferation. Generally each coleoptile was enveloped by a single well-developed scutellum. However, sometimes several coleoptiles were enclosed by a common scutellum, or the scutelli of the adjoining embryoids were fused (Figs. 4–6). In most cases each coleoptile contained one shoot meristem, but the presence of multiple shoot meristems was also noticed (Vasil and Vasil 1982a, b; Wang and Vasil 1982; Ozias-Akins and Vasil 1982). In three week old cultures the embryoids appeared to be present on the exposed (abaxial) surface of the scutellum. This was due to the continued growth and folding of the scutellar proliferation which pushed the embryoids upwards and outwards.

The coleoptile continued to elongate in the 2,4-D medium. In light both the coleoptile and the scutellum occasionally turned purple owing to the development of anthocyanin.

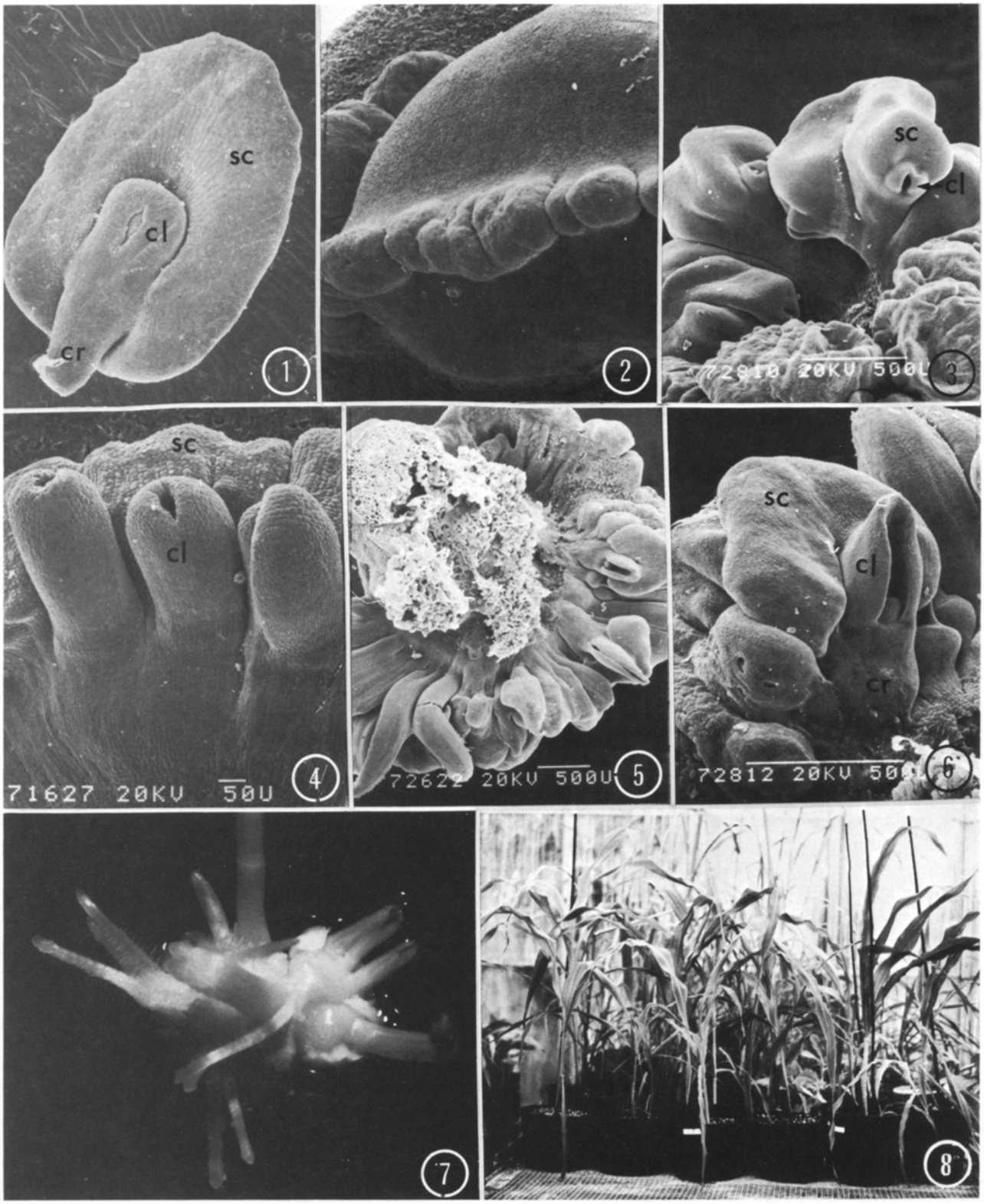
Regeneration of plants from somatic embryos

Roots emerged rapidly from 90 of the 100 somatic embryos – representing every cultivar used – that were transferred to half-strength MS medium with 2% sucrose, or basic MS medium with 3% sucrose, or MS medium with 1 mg/l gibberellic acid and 3% sucrose (Fig. 7). Of these, 75 plantlets were placed on half-strength MS medium in a culture tube. Finally, 50 plants were transplanted to soil, acclimatized in a growth chamber for two weeks and then moved to the greenhouse (Fig. 8). Each of the transplanted plants was grown to maturity.

Discussion

In maize as well as in other plants, genotype has often been considered to be an important factor in determining the response in vitro. Our results, based on four experiments carried out during two years with 14 cultivars of maize, do not entirely support this view (Lu et al. 1982 and present study). All the cultivars used showed more or less a similar response both to the amounts of sucrose and 2,4-D in the medium. The initiation and nature of the embryogenic callus formed, the site and manner of formation of somatic embryos, and the ability of the somatic embryos to develop into normal maize plants were also similar. Whether these results are due to the use of hybrid cultivars in our experiments as opposed to the inbred lines used by other investigators remains to be determined.

The concentration of sucrose in the medium had a marked effect on the nature and efficiency of callus formation, the number of embryoids which differentiated, and the germination of the cultured zygotic embryos. The frequency of embryogenic callus formation was up to 65% higher in media containing 12% sucrose than in the same medium with 3% sucrose. High concentration of sucrose also inhibited the germination of cultured embryos. Other factors that affected the



Figs. 1 – 8. Initiation of embryogenic callus, formation of somatic embryos and regeneration of plants (*cl* = coleoptile, *cr* = coleorhiza, *sc* = scutellum). **Fig. 1.** Immature embryo at the time of culture (×49). **Fig. 2.** Compact and white embryogenic callus tissue being formed at the coleorhizal end of the scutellum five days after culture (×50). **Fig. 3.** Early stages of somatic embryo formation showing the initiation of coleoptiles. **Figs. 4 and 5.** Somatic embryos formed on the adaxial surface of the scutellar proliferation. In Fig. 4. coleoptiles with either a terminal pore or a lateral slit can be seen. The demarcation between each embryoid is not clear probably because of the fusion of adjoining scutella. **Fig. 6.** Enlarged view of a single embryoid from Fig. 5 showing embryo axis enclosed by a single scutellum. **Fig. 7.** Root formation in several somatic embryos after transfer to MS medium (3% sucrose) without 2,4-D (×8). **Fig. 8.** Normal green plants from somatic embryos of various cultivars in the greenhouse

formation of embryogenic callus were the developmental and physiological state of the embryos at the time of excision and culture, and the concentration of 2,4-D in the medium. No embryogenic callus was formed if the embryos were not at the right stage of development (ca 1–1.5 mm in size, when the embryo axis is about half the length of the scutellum that is rather opaque and contains some starch). Older embryos (10 embryos of each cultivar larger than 2 mm were used) germinated, while the younger embryos (5 embryos of each cultivar less than 1 mm in length and transluscent owing to the absence of storage starch were used) showed no response. There was no significant difference in the quality and quantity of embryogenic callus produced in media containing 0.25–2.0 mg/l 2,4-D. Higher concentrations were less effective, and no embryogenic callus was formed at 4–5 mg/l 2,4-D (Lu et al. 1982). These observations lead to the conclusion that the developmental and physiological state of the embryos at the time of culture, and sucrose and 2,4-D concentrations were the most critical factors in eliciting a favorable response for the formation of embryogenic callus from immature embryos of maize.

All the cultivars used in the present study showed a higher frequency of embryogenic callus formation than in our earlier experiments (Lu et al. 1982). For example, 16.7% of the embryos from 'Funk G4864' produced embryogenic callus in the earlier study. This was increased to 81% in the present experiments. Similar improvement was seen in all other cultivars. This is due to selection of embryos at the most suitable developmental stage for culture. The conditions in the field – fertilizer, irrigation water, rainfall, temperature, photoperiod – prevailing during the different periods of growth affected the physiological state of the embryos which in turn controlled the amount and nature of the embryogenic callus formed. Embryos obtained from a given cultivar that had been grown under different environmental conditions gave different responses. There was more variability in the response of embryos from the same cultivar grown at different times than amongst embryos obtained from different cultivars of a single planting. For example, plants of the second and third plantings were not robust, formed fewer and smaller ears, and embryos obtained from such ears were transluscent owing to lack of storage starch as compared to the rather whitish embryos from the first planting. Comparison of growing conditions during the three plantings indicates that these differences may be related to insufficient irrigation, low rainfall and other environmental factors. There was reduced or delayed storage of food reserves in the scutellum of such translucent embryos which produced 40–50% less embryogenic calli, that were less compact and produced fewer somatic embryos. It may be more advantageous, therefore, to grow the experimental plants under controlled greenhouse conditions. It should be noted, however, that seed set in maize under greenhouse conditions is less than ideal.

Green and Phillips (1975) obtained regenerable callus from 54–80% of immature embryos of the inbred line A188 of maize. After transplantation to soil, 10–15% of the plants survived and grew normally. These results were confirmed by Freeling et al. (1976). Torne et al. (1980) obtained 9 plantlets from mesocotyl of 25 immature embryos but only one produced flowers and developed a normal ear. Lu et al. (1982) described the formation of embryogenic callus in 12 commercial hybrid cultivars of maize. The efficiency of embryogenic callus formation in the various cultivars ranged from 0–40%. In pearl millet, 80% of the cultured immature embryos produced embryogenic callus (Vasil and Vasil 1981). In wheat, the frequency of embryogenic callus formation was increased from about 9% to at least 30% on media with double the concentration of MS inorganic salts (Ozias-Akins and Vasil 1982, 1983).

In the present study the efficiency of embryogenic callus formation was upto 98%, and all the plants regenerated from somatic embryos of each of the cultivars used and transferred to soil were grown to maturity.

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