In Vitro Cell.Dev.Biol.—Plant (2007) 43:215–224
DOI 10.1007/s11627-007-9042-9

DEVELOPMENTAL BIOLOGY/MORPHOGENESIS

In vitro plant regeneration from quality protein maize (QPM)

Gerardo Armando Aguado-Santacruz · Edmundo García-Moya · José Luis Aguilar-Acuña · Blanca Moreno-Gómez · Ernesto Solís-Moya · Ernesto Ricardo Preciado-Ortiz · Juan Francisco Jiménez-Bremont · Quintín Rascón-Cruz

Received: 30 June 2006 / Accepted: 1 February 2007 / Published online: 31 March 2007 / Editor: P. Ozias-Akins
© The Society for In Vitro Biology 2007

Abstract Breeding efforts to obtain more nutritious maize materials aimed at alleviating dietary deficiencies in developing countries have resulted in an improved maize germplasm known as quality protein maize (QPM). Quality protein maize has higher contents of tryptophan, lysine, and leucine than common maize, but suffers from some major agronomic drawbacks found in common inbred maize lines, such as susceptibility to insect pests and fungal and bacterial diseases and herbicide sensitivity. The development of a reproducible and efficient protocol for tissue culture of QPM is expected to solve some of these deficiencies. In this work, we have evaluated different formulations for *in vitro* induction of morphogenic responses in three QPM lines developed by the International Maize and Wheat Improvement Center (CIMMYT): CML (CIMMYT maize line)-145, CML-176, and CML-186. Only CML-176 and CML-186 have proven to be responsive to the *in vitro* conditions considered in this work, with CML-176 showing the highest efficiency in regenerable callus formation and growth. N6C1 medium was found to be efficient for *in vitro* culture of QPM, whereas no plants could be regenerated by using MPC medium. From CML-176 embyogenic calli cultured on N6C1 medium, we were able to regenerate up to 0.3 plants per 500 mg fresh weight (FW) callus. Further modifications in this experimental protocol, including the replacement of 3,6-dichloro-o-anisic acid with 2,4-dichlorophenoxyacetic acid and modification of the N6C1 vitamin balance, significantly increased the regeneration response of the induced calli, with up to 16.8 and 9.3 plants recovered per 500 mg FW callus for CML-176 and CML-186, respectively.

Keywords *Zea mays* · Somatic Embryogenesis · Organogenesis · Tissue Culture · Immature Embryos · Genetic Transformation

G. A. Aguado-Santacruz (✉) · B. Moreno-Gómez
Unidad de Biotecnología, Campo Experimental Bajío, Instituto Nacional de Investigaciones Forestales y Agropecuarias,
Km 6.5 Carretera Celaya-San Miguel de Allende,
Celaya, Guanajuato 38110, Mexico
e-mail: gaguado@prodigy.net.mx

E. García-Moya
Postgrado en Botánica, Colegio de Postgraduados,
km 36.5 Carretera México-Texcoco, Montecillo,
Texcoco, Edo, Mexico 56230, Mexico

J. L. Aguilar-Acuña · E. Solís-Moya
Campo Experimental Bajío, Instituto Nacional de Investigaciones Forestales y Agropecuarias,
Km 6.5 Carretera Celaya-San Miguel de Allende,
Celaya 38110, Mexico

E. R. Preciado-Ortiz
Programa de Mejoramiento Genético de Maíz, Campo Experimental Bajío, Instituto Nacional de Investigaciones Forestales y Agropecuarias,
Km 6.5 Carretera Celaya-San Miguel de Allende,
Celaya 38110, Mexico

J. F. Jiménez-Bremont
Departamento de Biología Molecular, Instituto Potosino de Investigacíon Científica y Tecnológica, A.C.,
Camino a la Presa San José 2055, Lomas 4[a] Sección,
San Luís Potosí, S.L.P. 78216, México

Q. Rascón-Cruz
Facultad de Ciencias Químicas,
Universidad Autónoma de Chihuahua,
Avenida Universidad s/n, Colonia Universidad, Chihuahua,
Chih 31170, Mexico

Introduction

Maize (*Zea mays* L.) is an important crop for human consumption, particularly in developing countries, where this cereal can represent up to 65% of the total calories and 53% of the protein intake (Bressani 1991). Although the protein content of maize is relatively high (9% on average), its quality is poor due to an imbalance in three essential amino acids, in which the contents of tryptophan and lysine are low, whereas that of leucine is high. This amino acid balance ratio is not enough to satisfy the FAO requirements for human nutrition, especially for children (Hamaker and Rahmanifar 1994). At the beginning of the 1960s, low levels of zein, and high levels of lysine and tryptophan, were discovered in opaque-2 maize (Mertz et al. 1964), which, on the other hand, displayed some undesirable agronomic traits, such as chalky soft kernel, low yields, lower resistance to fungi and insects, and longer drying time (Mertz 1994). The superior nutritional qualities of opaque-2 maize motivated maize researchers to overcome these problems. These efforts resulted in the discovery of modifiers of the opaque-2 maize trait, which conferred to it a normal, hard, and vitreous endosperm. The conversion of opaque-2 maize to dent- and flint-type maize gave rise to quality protein maize (QPM), which is, today, practically interchangeable with common maize in cultivation and kernel characteristics. Quality protein maize was developed by the International Maize and Wheat Improvement Center (CIMMYT) in Mexico and contains 55% more tryptophan, 30% more lysine, and 38% less leucine than common maize (Bressani 1992). Therefore, this high-quality maize is expected to alleviate some of the problems associated with malnutrition in countries where this cereal provides a high percentage of the total protein intake (Hamaker and Rahmanifar 1994). Efforts in several countries of eastern and southern Africa, Asia, and Latin America are presently devoted to incorporating the QPM characteristic into locally adapted germplasm by production of hybrids.

Because of their superior combining ability, Quality Protein, CIMMYT maize lines (CML) 176 and 186 (CML-176 and CML-186) were utilized as parents of several hybrids released in Mexico by the National Institute for Forestry, Agriculture, and Livestock Research (INIFAP) to increase the grain nutritional quality of local maize materials. However, these and other QPM lines suffer from some major deficiencies found in common maize inbred lines, such as susceptibility to diseases or herbicides (CIMMYT 1999). Thus, breeders involved in QPM improvement not only have to face the challenges met by common maize breeders, but also have to preserve the lysine and tryptophan levels in endosperm, and, consequently, the conditions required for breeding and germplasm improvement of QPM are usually more complex than those for common maize.

Although the possibility for introducing transgenes with agronomic importance into elite maize or QPM materials by backcrossing to transgenic lines exists, this process can be costly and time- and labor-consuming. Additionally, the recovery of progeny possessing both the transgenic trait and suitable agronomic traits is often difficult due to incompatible heterotic groups and reduced combining ability. Therefore, genetic engineering can probably be more fruitfully applied to maize improvement by introducing agronomically useful genes directly into the genome of the selected maize lines (O'Kennedy et al. 2001). Today, the possibilities of *in vitro* plant technology (genetic transformation, recovery of somaclonal variants or haploid plants, somatic hybridization, and micropropagation, among others) have rapidly expanded in such a way that it is practically, or at least theoretically, possible to manipulate any aspect of plant performance, from the modification of food quality to increased tolerance of plants to environmental stresses, biotic or abiotic. As a first step to developing the required technological background to overcome some of the deficiencies of QPM maize lines or their hybrids through molecular tools, we have developed a reproducible and efficient protocol for tissue culture of two QPM lines.

Materials and Methods

Plant materials. CML-176, CML-186, and CML-145 are QPM lines, which were used as donor plants of the explants to be tested for morphogenic response under *in vitro* culture conditions. CML-145 is a late, tropical QPM line, tolerant to *Helminthosporium maydis* and *Puccinia polysora*, which is characterized by white, dent grains, 9.1% total protein, and 0.87% tryptophan content. Because of its high susceptibility to herbicides, this inbred line was excluded from the INIFAP's QPM breeding program. CML-176 and CML-186 are late, subtropical maize lines tolerant to *Exserohilum turcicum* that have white and flint grains, and contain 7.4 and 8.2% total protein and 1.05 and 1.00% tryptophan, respectively (CIMMYT 1999).

Description of media utilized for induction of morphogenic responses in QPM. As a first step to designing a QPM tissue culture protocol, we initially tested two different media reported as being highly effective for induction of regenerable calli in maize: MPC (O'Connor-Sánchez et al. 2002) and N6C1 (Bohorova et al. 1995) media. MPC medium has also been successfully utilized for generating the first embryogenic chlorophyllic cell line in Poaceae (Aguado-Santacruz et al. 2001b).

The N6C1 medium contains the Chu N6 basal salts and vitamins (Chu et al. 1975), 2.3 g l^{-1} L-proline, 0.2 g l^{-1} casein hydrolyzate, 2 mg l^{-1} 3,6-dichloro-o-anisic acid

(Dicamba), and 30 g l^{-1} sucrose. The MPC medium contains the basal salts and vitamins of Murashige and Skoog (MS) medium (Murashige and Skoog 1962), 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 2 mg l^{-1} N6-benzylaminopurine (BAP), 40 mg l^{-1} adenine, and 30 g l^{-1} sucrose. Basic constituents of the respective media were dissolved in deionized water, mixed with 2.5 g l^{-1} Gelrite (MPC medium) or 8 g l^{-1} Bacto agar (N6C1 medium), and adjusted to pH 5.8, and the resultant mixture was sterilized at 120°C/15 PSI for 15 min. The above-mentioned amounts of respective growth regulators were always added to the basic media after the sterilization step, when the temperature was around 50°C. MS salts, N6C1 salts, L-proline, growth regulators, casein hydrolyzate, and Gelrite were acquired from Sigma (St. Louis, MO, USA). Bacto agar and sucrose were acquired from Becton and Dickinson (Franklin Lakes, NJ).

Although shoot tips and immature embryos were respectively utilized as explants for development of the original MPC (O'Connor-Sánchez et al. 2002) and N6C1 (Bohorova et al. 1995) protocols, in this investigation, we tested the response of both kinds of explants to culture on N6C1 and MPC media. Because of the relatively higher efficiency of the N6C1 medium for induction of regenerable calli in QPM (as shown in the "Results" section), further experiments only analyzed the effect of varying the composition of this medium (Table 1) using immature embryos as the starting material. The effect of these modifications (media 2 to 9) was compared to the original N6C1 formulation (medium 1). These modifications to the original N6C1 formulation resulted in nine different media formulations to be evaluated in QPM (Table 1). Media 1 to 5 contained Dicamba (2 mg l^{-1}), media 6 to 9 utilized 2,4-D (2 mg l^{-1}) as the callus promoter auxin, and L-proline (2.3 g l^{-1}) was omitted from media 3, 4, 8, and 9. Media 1, 3, 6, and 8 considered the "D"vitamin balance (myo-inositol-devoid vitamin balance; Table 1), whereas media 2, 4, 5, 7, and 9 considered the "M" vitamin balance (myo-inositol vitamin balance). Because the use of 40 mg l^{-1} adenine has been shown to increase the *in vitro* regenerative capacity of maize (O'Connor-Sánchez et al. 2002), the effect of adding this compound to the medium considering the "M" vitamin balance was also evaluated (medium 5).

Table 1 Media tested for induction of *In vitro* morphogenic responses in three QPM maize lines

Medium	Auxin (2 mg l^{-1})	Proline (2.3 g l^{-1})	Vitamin balance[z]	Adenine (40 mg l^{-1})
1[y]	Dicamba	Added	D	–
2	Dicamba	Added	M	–
3	Dicamba	–	D	–
4	Dicamba	–	M	–
5	Dicamba	Added	M	Added
6	2,4 D	Added	D	–
7	2,4 D	Added	M	–
8	2,4 D	–	D	–
9	2,4 D	–	M	–

[z] Vitamin balance "D" contained 0.5 mg l^{-1} nicotinic acid, 0.5 mg l^{-1} thiamin-Cl, and 1.0 mg l^{-1} pyridoxine-Cl, whereas vitamin balance "M" included 0.5 mg l^{-1} nicotinic acid, 0.1 mg l^{-1} thiamin-Cl, 0.5 mg l^{-1} pyridoxine-Cl, and 0.1 mg l^{-1} myo-inositol

[y] Original N6C1 formulation according to Bohorova et al. (1995)

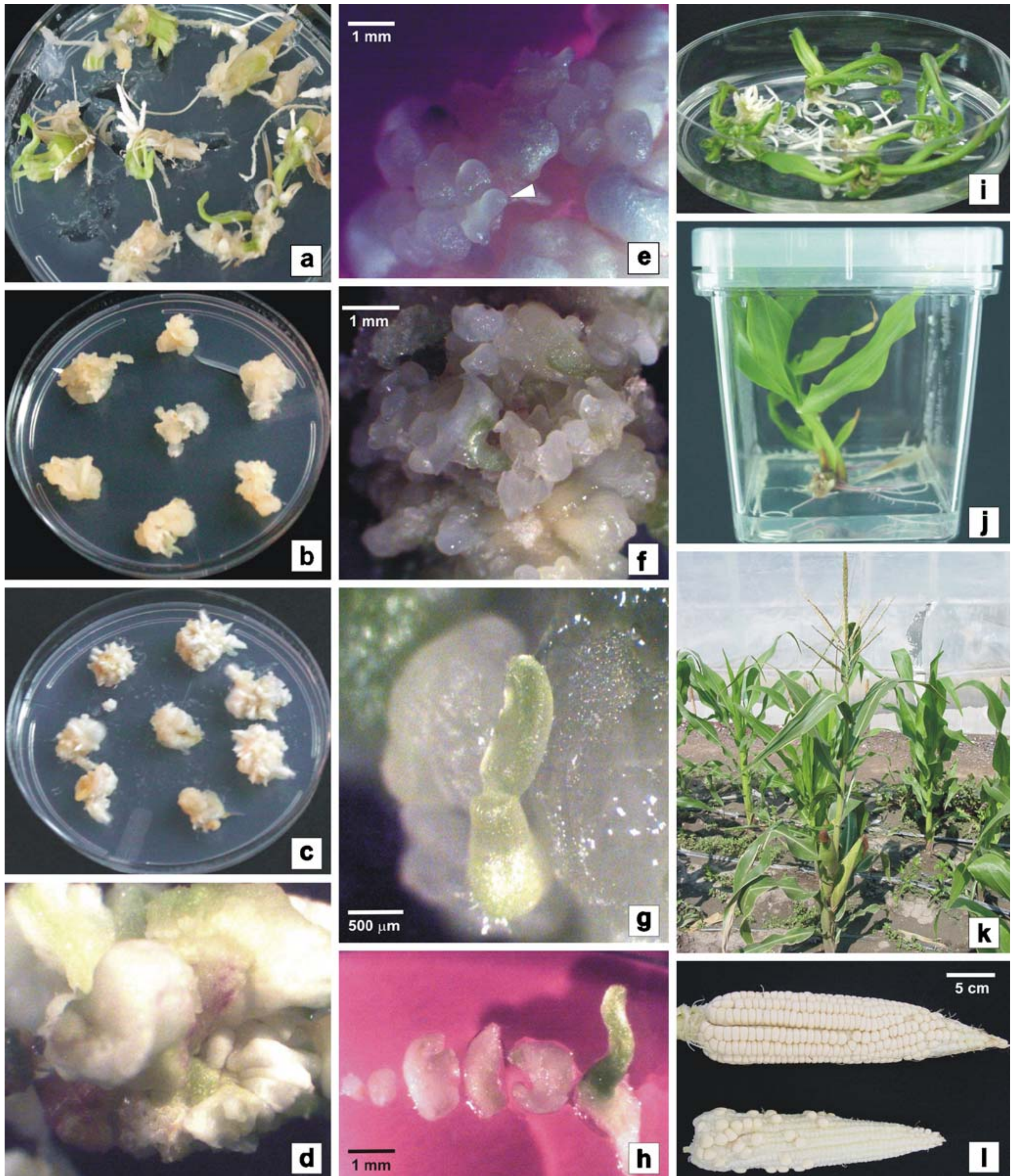
Isolation and sterilization of explants. Immature embryos were isolated from ears collected 12–15 d after pollination in field-grown QPM plants. Ears were surface-sterilized by submersion in 70% (v/v) ethanol for 5 min and then in 20% (v/v) NaClO solution for 20 min, and were finally rinsed five times in sterile distilled water. Grains were detached from the ears using a sharp knife and then the zygotic immature embryos (1.5–2.0 mm long) were extracted under a stereomicroscope using a dissection needle and forceps. Embryos were placed with the embryonary axis face down, in contact with the respective induction medium and incubated in a growth room at 30°C under dark conditions for three consecutive subcultures carried out every 20 d.

For isolation of shoot apices, mature seeds were surface-sterilized with 70% (v/v) ethanol for 5 min and 0.02% (w/v) mercuric chloride ($HgCl_2$) for 20 min, and then these explants were rinsed five times with sterile distilled water. Disinfected seeds were soaked in water for 3 h and then transferred to Petri dishes containing full-strength MS medium and 2.5 g l^{-1} Gelrite. For initiating germination, the Petri dishes containing the seeds were incubated in a growth room (30±1°C) under dark conditions. Ten-millimeter-long tubular sections (containing the shoot apices) from 1-wk-old dark-grown seedlings were obtained by cutting 5 mm above and below the subcoleoptilar node. These segments were placed on the respective induction media and cultured in the dark in a growth room maintained at 30±1°C. Subculturing of these explants was carried out every 20 d until callus formation was evident.

Fifty Petri dishes containing eight explants (shoot tips or immature embryos) each were evaluated for every QPM line/medium studied. We tried to reduce the ear-to-ear effect on culture initiation and maintenance by choosing only 10 immature embryos from every ear until completing the required amount of explants per treatment.

Maintenance of calli and regeneration of plants. Once the dedifferentiation processes leading to callus formation were initiated, germinal lines derived from single embryos or shoot tips were labeled and treated as individual genotypes. After dissecting and propagating the individualized germinal lines through five consecutive

subcultures (carried out every 20 d with the same media and growth conditions considered for callus initiation), part of this material was transferred to Petri dishes containing hormone-free, full-strength MS and 30% sucrose. After maintaining the calli on this medium for 1 mo., an evaluation of the regeneration capacity of the induced callous masses was carried out. The regenerative callus induction efficiency (RCE) was calculated as the



number of regenerable calli/number of induced calli× 100. Plant regeneration efficiency (PRE) was calculated as the number of plants regenerated from 500 mg fresh weight (FW) callus (20 replicates per QPM callus line generated).

Once the plantlets completed their development within the Petri dishes, they were moved into 400-ml GA 7 Magenta boxes containing the same medium until they acquired a vigorous and profuse root system; this ensured that all regenerated plants survived the transfer process to greenhouse conditions. *In vitro* regenerative and developmental responses were always induced in a growth room equipped with cool fluorescent light (80 μmol s^{-1} m^{-2}; 30±1°C).

One-way analysis of variance was utilized to test significant differences in RCE and PRE among the different tested media. Means were separated using Tukey's test (Zar 1974).

Acclimation and hardening of regenerated plants in the greenhouse. *In vitro* 6–7-cm-long regenerated plantlets possessing at least four to five well-developed roots (2–3.5 cm long) were washed with distilled water to eliminate excess agar, transferred to ½-l pots containing a sterilized mixture of peat moss and sand (50:50), covered with transparent plastic bags, and then placed in a greenhouse (maximum temperature=38°C, minimum temperature=10°C). After 15 d, the plastic bags were removed and the aciclimatized plants were transferred to 20-l pots containing the same soil mixture plus 10 g of a 17–17–17 chemical fertilizer and watered to field capacity twice a wk. When they reached a height of 20–30 cm, the maize plants were transferred to the field, hand-pollinated, and grown up to seed production.

Results

Morphogenic response of explants to MPC medium. Shoot apices cultured on MPC medium formed small calli on the apical ends of the explants. These calli were slow-growing and not able to regenerate into complete plants. On the other hand, all immature embryos cultured on MPC medium turned black 20 d after culture and died. Thus, no plants could be regenerated from MPC medium independently of the QPM line, explant, or growth conditions considered.

Morphogenic response to auxin source, L-proline, adenine, and vitamin balance in N6C1 basal medium. Shoot tips from all QPM lines cultured on N6C1 initially formed small areas of callus masses around the shoot tips, but after 20–30 d, a great proliferation of roots occurred from these calli (Fig. 1*a*). After transfer to MS medium, these soft, root-forming calli were not capable of regenerating into whole plants. Therefore, in further experiments evaluating modifications to the original N6C1 medium, we only used immature embryos as the starting material.

Practically all immature embryos from CML-145, CML-176, and CML-186 QPM lines cultured on N6C1 media, or its variations, produced calli within the first 7–10 d of culture; averaged over all QPM lines, the callus induction ranged from 90.5% on media 8 and 9 to 100% on media 6 and 7. These calli acquired a "popcorn" appearance after 15–20 d of culture (Fig. 1*b*). These calli were subcultured every 20–25 d, except those formed by CML-145 embryos, which did not continue being propagated after the second subculture because they started forming roots (Fig. 1*c*) or turned brown at the region in direct contact with the induction medium; after transfer to regeneration medium, these root-forming calli were unable to regenerate into whole plants, so this QPM line was excluded from additional experiments.

Regenerable calli developed white, compact (type 1 calli; Fig. 1*d*), and friable, pale yellow or white clear areas (type 2 calli; Fig. 1*e*, *f*) and never initiated the differentiation processes conducive to plant regeneration as long as they were maintained on fresh media, even in the light. Embryogenic calli were commonly immersed within a mucilaginous matrix, similar to that observed by Ahn et al. (1987) in bermudagrass (*Cynodon dactylon*) and Aguado-Santacruz et al. (2001a) in blue grama grass (*Bouteloua gracilis*). Well-developed coleoptiles (Fig. 1*g*), such as those

◀ **Figure 1.** Plant regeneration in QPM. (*a*) Nonregenerable calli obtained by the culture of shoot tips on N6C1 medium. (*b*) Highly regenerable QPM calli developed 20 d after initial culture of immature embryos on N6C1 medium. (*c*) Rooty, nonregenerable calli derived from the culture of immature embryos on N6C1 medium. (*d*) Compact (type 1) callus from regenerable tissues of QPM. (*e*) Friable, highly embryogenic (type 2) callus derived from immature embryos cultured on medium 6 containing N6 basal salts, 2,4-D, L-proline, and myo-inositol-devoid vitamin balance. *Arrow* indicates an embryo with a short suspensor-like structure. (*f*) Multiple regenerating embryogenic calli from CML-186 10 d after transfer to light on hormone-free MS medium. (*g*) Coleoptile formed in regenerating calli from CML-176. (*h*) Bipolar development of QPM plantlets. (*i*) Development of complete QPM plantlets on hormone free-MS medium. (*j*) Finalization of QPM plantlet development in GA 7 Magenta boxes. (*k*) Mature QPM plants established on the field that were obtained by *in vitro* culture of immature embryos on medium 7 incorporating N6 basal salts, 2,4-D, L-proline, and myo-inositol-containing vitamin balance. (*l*) Ears produced by an *in vitro* regenerated QPM plant grown in the field.

observed by Armstrong and Green (1985), as well as bipolar development (Fig. 1*h*), also characterized the embryogenic cultures of QPM lines. Conversely, the white and compact calli regenerated plants from preformed structures.

Some of these regenerable calli were maintained in the dark and the rest were transferred to light conditions. The compact calli transferred to light conditions turned green, whereas the friable areas either retained their consistency and color or turned light green. Exposure of the calli to light increased the compact areas relative to the friable portion. Therefore, the calli were always maintained in the dark. A light-dependent interconversion between maize organogenic and embryogenic calli has been previously reported (Armstrong and Green 1985; Lowe et al. 1985; O'Connor-Sánchez et al. 2002).

Media 2 (basic N6 medium salts plus proline and Dicamba with "M" vitamin balance), 6, and 7 (basic N6 medium salts plus proline and 2,4-D with "D" or "M" vitamin balance; Table 1) were found to be the most efficient formulations for induction of regenerable calli in both CML-176 and CML-186 QPM lines, with approximately 15 from 100 calli becoming regenerative using these media (Table 2).

In this study, a strong dependence on L-proline for regenerative callus formation was detected as immature embryos from both QPM lines were unable to develop regenerable calli on media 3, 4, 8, and 9 (Table 2), from which this amino acid was omitted (Table 1).

After establishing the type of calli induced in CML-176 and CML-186, the growth rate of the resultant material was determined for media resulting in regenerative calli, i.e., 1, 2, 5, 6, and 7. All callus lines possessing regeneration capacity survived the subculturing process. As can be observed in Fig. 2, trends in FW gain on all formulations were similar for both maize lines, although CML-176, in general, exhibited faster growth than CML-186, especially in treatments 1 and 6, where differences in this variable were 28.2 and 19.0%, respectively. Fresh weight increase in both QPM lines was greater on media 6 containing 2,4-D, L-proline, and "D" vitamin balance (63 and 44% for CML-176 and CML-186, respectively) and lower on medium 7, which incorporated 2,4-D, L-proline, and "M" vitamin balance (10.9 and 3% for CML-176 and CML-186, respectively; Fig. 2). Calli from both maize lines produced on the original N6C1 formulation (medium 1) showed a growth rate intermediate to these latter media, but superior to CML-176 calli grown on media containing Dicamba, L-proline, and "M" vitamin balance with (medium 5) or without (medium 2) adenine added.

In relation to the effects of adenine, it was observed that the inclusion of this additive to the media mixtures reduced twofold the induction of regenerable calli of CML-176, but had no effect on this variable for CML-186 (medium 2 vs medium 5; Table 2). Callus growth rate was, on the other hand, enhanced in both QPM lines (1.2-fold increase) in comparison to its adenine-free counterpart (medium 2 vs medium 5; Fig. 2).

Table 2 *In vitro* regenerative response of immature embryos from two QPM maize lines to nine media formulations

Medium	RCE		PRE	
	176	186	176	186
1	7.2[b]	3.0[b]	0.30[b]	0.20[b]
2	15.4[c]	3.6[b]	1.50[c]	0.60[c]
3	0.0[a]	0.0[a]	0.00[a]	0.00[a]
4	0.0[a]	0.0[a]	0.00[a]	0.00[a]
5	7.7[b]	4.3[b]	5.90[d]	2.20[d]
6	14.8[c]	8.1[c]	16.80[f]	9.30[e]
7	14.3[c]	8.4[c]	7.10[e]	2.40[d]
8	0.0[a]	0.0[a]	0.00[a]	0.00[a]
9	0.0[a]	0.0[a]	0.00[a]	0.00[a]

RCE is no. of regenerative calli/no. of induced calli × 100; PRE is no. of plants regenerated per 500 mg FW callus. Means followed by different *letters* are significantly different ($P \leq 0.05$) among media formulations tested. Within each QPM line, RCE and PRE values were averaged over all genotypes (individual callus lines derived from single explants). Each value represents the mean from approx. 400 explants. Experiments were performed at least twice

Plant regeneration. Plant regeneration occurred after the regenerable calli were transferred to Petri dishes containing hormone-free MS medium under light conditions. Once the plantlets completed their development in these containers (approx. 1 mo.; Fig. 1*i*), they were moved into 400 ml GA 7 Magenta boxes for another 20–30 d until a well-developed root system was observed (Fig. 1*j*). Regenerated plantlets were subsequently transferred to a greenhouse, fertilized once, and watered twice a wk to field capacity. When plants were 20–30 cm tall, they were finally established in the field (Fig. 1*k*), hand-pollinated, and grown up to seed production (Fig. 1*l*).

The highest plant regeneration potential was observed in calli cultured on formulations 5, 6, and 7 (Table 2). Utilizing these formulations, in CML-176 we were able to regenerate 5.9, 16.8, and 7.1 plants per 500 mg FW callus, respectively, whereas these values in CML-186 were 2.2, 9.3, and 2.4 plants per 500 mg FW callus (Table 2). Calli induced on media 6 were clearly the most efficient for QPM plant regeneration.

Addition of adenine resulted in an improved response of QPM measured as plant regeneration potential; an almost fourfold increase in PRE was observed in both maize lines (medium 2 vs medium 5; Table 2). Independent of the formulation utilized for induction of the regenerative calli, fertile plants were regenerated from both QPM lines throughout a year without evident abnormalities or reduction in their ability to set seed.

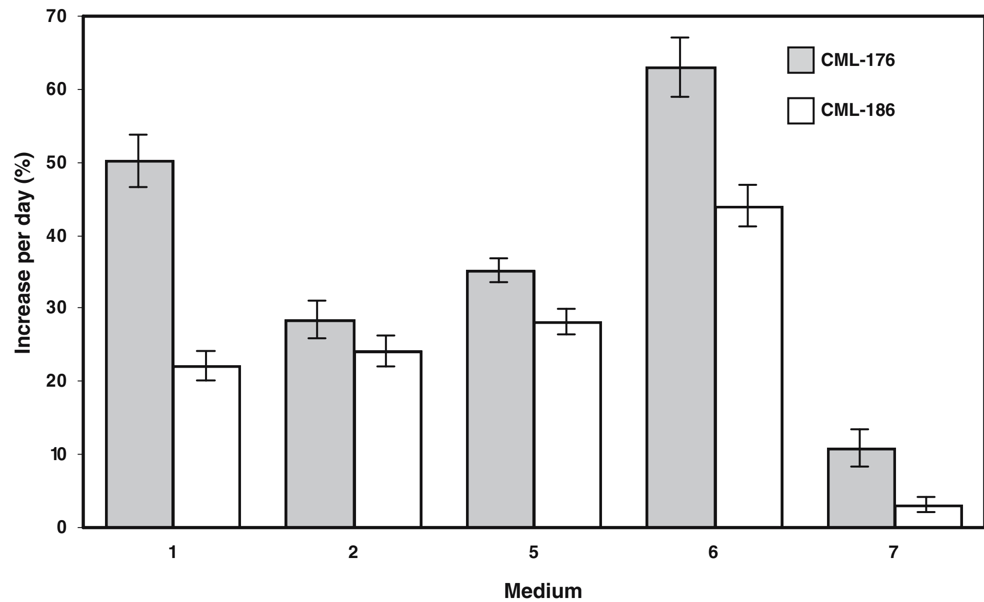


Figure 2. Daily averaged FW increase in regenerable calli derived from culturing two QPM lines on five formulations.

Discussion

Recognition of the determinant influence of plant genotype, type and physiological and developmental stage of explant, growth regulator combination, nutritional and vitamin balance, and general environmental growth conditions on the regenerative response of plants has played a fundamental role in overcoming the well-known recalcitrance of Poaceae to be grown under *in vitro* conditions. Within the last 10 yr, great advances have been achieved in the research of *in vitro* culture and genetic manipulation of cereals.

Since the pioneering work of Green and Phillips (1975), several protocols for *in vitro* culture of maize have been developed (e.g., Rice et al. 1978; Springer et al. 1979; Torne et al. 1980; Ting et al. 1981; Armstrong and Green 1982; Green 1982; Lu et al. 1982, 1983; Rhodes et al. 1982, 1986; Sachs et al. 1982; Santos et al. 1984; Suprasanna et al. 1986; Conger et al. 1987; Pareddy and Petolino 1990; Ray and Ghosh 1990; Songstad et al. 1992; Zhong et al. 1992; O'Connor-Sánchez et al. 2002; Zhang et al. 2002; Huang and Wei 2004). However, because profound differences exist in the potential of maize lines for *in vitro* culture (Armstrong and Green 1985) with only a small number of maize genotypes possessing regenerative capacity (such as A188), it is important to analyze the response of particular maize materials to *in vitro* culture to define the specific growth conditions required for generating the totipotent material across which to exploit the potential tools of the *in vitro* technology, such as genetic transformation, somaclonal variants recovery, somatic hybridization, and molecular farming, among others.

Quality protein maize is the successful result of conventional breeding efforts focused on increasing the nourishing quality of this cereal to alleviate some of the alimentary problems in developing countries. Extensive and detailed supplementary research has permitted elucidation of the molecular basis of QPM origins, and led to the development of dominant opaque and nutritionally superior maize variants by a transgenic approach (Segal et al. 2003). Despite these advances in the research of QPM, there are no published reports on tissue culture of this improved crop that permit to apply the *in vitro* culture technological tools.

In this work we have identified critical components for *in vitro* induction of morphogenic responses in two QPM lines, CML-176- and CML-186, from which we were able to recover whole plants with high efficiency and repeatability. To develop more reproducible and effective protocols for *in vitro* culture of plants, criteria such as RCE, callus growth rate, and PRE should be taken into account. Evaluation of these variables showed MPC medium (O'Connor-Sánchez et al. 2002) to be an ineffective method for *in vitro* culture of QPM, whether using the shoot apices or immature embryos as the starting material. Conversely, the original N6C1 medium resulted in a high percentage of immature embryos producing calli, from which a fraction became regenerative when an amenable genetic background was available (CML-176 or CML-186). Hence, this research confirms the high potential of immature embryos for generating material relatively more competent for reproducible and efficient regeneration of maize plants (Huang and Wei 2004; Vasil 2005), as well as the importance of the genetic background for the successful *in vitro* culture of maize.

From our results it is evident that the final outcome of the media mixture in terms of morphogenic and growth responses of the QPM lines tested was dependent on the components

added to the N6 basal medium. This is in agreement, for example, with the results of Armstrong and Green (1985), who found that the nitrogen composition of the basal growth medium has a dramatic effect on embryoid and embryogenic callus formation in maize when L-proline and 2,4-D were added to the medium, with N6 basal medium generating better results than MS medium. We observed, on the other hand, a strict requirement of L-proline, not only for maintenance, but also for induction of QPM regenerable calli lines. This finding is consistent with previous evidence showing a positive effect of this amino acid on the embryogenesis of maize (Armstrong and Green 1985), triticale (Sozinov et al. 1981), and alfalfa (Stuart and Strickland 1984a, b). Armstrong and Green (1985) reported a statistically significant correlation between L-proline concentration (up to 25 mM) and embryoid formation. Evidence presented by these authors additionally supports a role for L-proline in the regenerative response of maize more as a compatible solute than as a nitrogen source, or energy and reducing power supply. Thus, because we used this amino acid in a lower concentration (20 mM), it would be interesting to test the effect of L-proline at higher concentrations on the embryogenic response of QPM.

The effects of the vitamin balance tested in this work depended on the growth regulator utilized in combination with the N6 mixture. When Dicamba was used, the RCE and plant regeneration potential of both QPM lines were increased, and callus growth reduced (Fig. 2) in the medium containing the "M" vitamin balance (medium 2) relative to that containing the "D" vitamin balance (medium 1; Table 2). When 2,4-D was employed in conjunction with "M" vitamin balance (medium 7), an improvement in the regenerative response of both maize lines was not observed in relation to the medium having the "D" vitamin mixture (medium 6; Table 2). Likewise, results were unfavorable for CML-176 and CML-186 lines in terms of regeneration capacity (2.4- and 3.9-fold reductions, respectively; Table 2) and callus growth (5.8- and 14.7-fold reductions, respectively; Fig. 2).

The "M" vitamin balance utilized in this work was characterized by five- and twofold reductions in thiamin and pyridoxine concentrations, respectively, and the addition of myo-inositol (at 0.1 mg l^{-1}; Table 1). Although myo-inositol is included in most plant tissue culture media, the basis for its requirement is unknown (Hrib et al. 1997). In maize, Green et al. (1974) found that callus growth of a single cross (Oh51A × Os420) was slightly improved (17%) when myo-inositol was added to the maintenance medium at a concentration of 400 mg l^{-1}. Evidence related to the action mechanism of this vitamin on the regenerative response of maize was not provided by these authors. Interestingly, in addition to its participation in cell signaling and membrane biogenesis, a role of myo-inositol as a compatible solute has been proposed (Nelson et al. 1999), which would be in agreement with the above-mentioned role of L-proline as a compatible solute; osmolytes may help buffer the likely suboptimal growing conditions associated with the *in vitro* culture of maize (Armstrong and Green 1985).

All callus variables tested in this work are important for defining the most efficient formulation to be utilized for the successful *in vitro* culture of maize. Adenine is an additive that has been recently shown to increase the regenerative capacity of shoot-tip-derived calli (O'Connor-Sánchez et al. 2002). Our work confirms the positive effect of this compound on callus growth and regeneration efficiency, but not in RCE of QPM. For many practical applications of *in vitro* technology, it is convenient to generate fast-growing and highly regenerable (embryogenic) tissue cultures of maize. In particular, callus growth rate is a useful criterion for defining plant lines particularly amenable to genetic transformation; fast-growing cells are more easily transformed than cells with a low mitotic activity (Lida et al. 1991; Hazel et al. 1998). Because regenerative response can be complemented to some extent by callus growth rate, the detrimental effect of adenine addition observed in the regenerative response of CML-176 might be alleviated by obtaining fewer but faster-growing and highly regenerable tissues, with the same final result in terms of amount of highly regenerable material generated within a defined period of time. Because preliminary experiments revealed a significant effect of the "M" vitamin balance on the regenerable response of CML-176, we only tested the effect of supplemental adenine in a medium containing this vitamin balance. Therefore, in future work it will be important to evaluate the effects and probable interactions among adenine, myo-inositol (and the complete vitamin balance), and type of auxin or auxin-analog employed in the media for induction of morphogenic responses in maize.

Other components known to determine the morphogenic response of maize should be adjusted or tested. In this context, it would be relevant, for example, to evaluate higher concentrations of 2,4-D. An enhanced induction of primary calli (derived from mature embryos) was observed in two maize elite inbred lines when the concentration of this auxin (up to 5 mg l^{-1}) was augmented in the induction medium (Huang and Wei 2004). Likewise, the effect of adding ethylene antagonists (such as silver nitrate) would be important because, from the time of the first evaluation of $AgNO_3$ on the morphogenic response of maize by Songstad et al. (1988), increasing evidence have come to support a dramatic effect of this compound on embryogenic response and regeneration capacity of maize tissue cultures (Vain et al. 1989a,b; Songstad et al. 1991, 1992; Carvalho et al. 1997; Huang and Wei 2004).

In this research, we have developed an efficient protocol for *in vitro* culture and regeneration of two QPM lines. We

considered this work to be an initial approximation for designing highly efficient and reproducible protocols for tissue culture of QPM. As indicated by the variables tested in this research, CML-176 was found to be a QPM line more responsive to the *in vitro* culture conditions tested in this work than CML-186 (Table 2 and Fig. 2). Under our culture conditions, CML-145 was a non-*in vitro*-cultured maize line. The potential of CML-176 for *in vitro* culture growth was improved by using the medium formulation that included the N6C1 salts and vitamins, 2,4-D, and L-proline. Some authors (Armstrong and Green 1985; Huang and Wei 2004; Armstrong 1994) have found 2,4-D to be a suitable growth regulator for replacing Dicamba in the N6C1 medium. That 2,4-D can be successfully replaced by Dicamba in the N6C1 medium means a great reduction in the costs of the media required for initiation and maintenance of QPM embryogenic tissues. It is therefore important to mention that, although we successfully utilized 2,4-D or Dicamba for induction of regenerable calli in QPM lines, these growth regulators were not interchangeable for subculture of calli already formed. That is, calli induced on media containing Dicamba could not be subcultured on media containing 2,4-D, and vice versa.

Acknowledgements This research was supported by funds from CONACyT-SAGARPA (project no. 2004-C01-17), Fondos Mixtos CONACyT-Guanajuato (project no. 06-24-A-033), and CONCyTEG (project no. 04-24-K117-042).

References

Aguado-Santacruz, G.A.; Cabrera-Ponce, J.L.; Olalde-Portugal, V.; Sánchez-González, M.R.; Márquez-Guzmán, J.; Herrera-Estrella, L. Tissue culture and plant regeneration of blue grama grass, *Bouteloua gracilis* (H.B.K.) Lag. ex Steud. In Vitro Cell. Dev. Biol., Plant 37:182–189; 2001a.

Aguado-Santacruz, G.A.; Cabrera-Ponce, J.L.; Ramírez-Chávez, E.; Rascón-Cruz, Q.; Herrera-Estrella, L.; Olalde-Portugal, V. Establishment, characterization and plant regeneration from highly chlorophyllous embryogenic cell cultures of blue grama grass, *Bouteloua gracilis* (H.B.K.) Lag. ex. Steud. Plant Cell Rep. 20:131–136; 2001b.

Ahn, B.J.; Huang, F.H.; King, J.W. Regeneration of bermudagrass cultivars and evidence of somatic embryogenesis. Crop Sci. 27:594–597; 1987.

Armstrong, C.L. Regeneration of plants from somatic cells cultures: applications for *in vitro* genetic manipulation. In: Freeling, M.; Walbot, V., eds. The Maize Handbook. New York: Springer-Verlag; 1994:663–671.

Armstrong, C.L.; Green, C.E. Initiation of friable, embryogenic maize callus: the role of L-proline. In: Agronomy Abstracts of the 74th Annual Meeting of the American Society of Agronomy; 1982:89.

Armstrong, C.L.; Green, C.E. Establishment and maintenance of friable, embryogenic maize callus and the involvement of L-proline. Planta 164:207–214; 1985.

Bohorova, N.E.; Luna, B.; Briton, R.M.; Huerta, L.D.; Hoisington, D.A. Regeneration potential of tropical, and subtropical, midaltitude, and highland maize inbreds. Maydica 40:275–281; 1995.

Bressani, R. Protein quality of high-lysine maize for humans. Cereal Foods World 36:806–811; 1991.

Bressani, R. Nutritional value of high-lysine maize in humans. In: Mertz, E.T., ed. Quality Protein Maize. St. Paul: American Association of Cereal Chemists; 1992:205–225.

Carvalho, C.H.S.; Bohorova, N.; Bordallo, P.N.; Abreu, L.L.; Valicentle, F.H.; Bressan, W.; Paiva, E. Type II callus production and plant regeneration in tropical maize genotypes. Plant Cell Rep. 17:73–76; 1997.

Chu, C.C.; Wang, C.C.; Sun, S.C.; Hsu, C.; Yin, K.C.; Chu, C.Y.; Bi, F.Y. Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci. Sin. 18:659–668; 1975.

CIMMYT. Maize inbred lines released by CIMMYT. A compilation of 424 CIMMYT maize lines (CMLs). CIMMYT First draft. Texcoco: CIMMYT; 1999.

Conger, B.V.; Novak, F.J.; Afza, R.; Erdelsky, K.E. Somatic embryogenesis from cultured leaf segments of *Zea mays*. Plant Cell Rep. 6:345–347; 1987.

Green, C.E. Somatic embryogenesis and plant regeneration from the friable callus of *Zea mays*. In: Fujiwara, A., ed. Plant Tissue Culture. Proceedings of the 5th International Congress Plant, Tissue and Cell Culture. Tokyo, Japan; 1982:107–108.

Green, C.E.; Phillips, R.L. Plant regeneration from tissue culture of maize. Crop Sci. 15: 417–421; 1975.

Green, C.E.; Phillips, R.L.; Kleese, R.A. Tissue cultures of maize (*Zea mays* L.): Initiation, maintenance, and organic growth factors. Crop Sci. 14:54–58; 1974.

Hamaker, B.R.; Rahmanifar, A. QPM and nutritional needs of children in poor communities. In: Larkins, B.A.; Mertz, E.T., eds. Quality Protein Maize: 1964–1994. Proceedings of the International Symposium on Quality Protein Maize. EMBRAPA/CNPMS. Sete Lagoas, MG, Brazil; 1994:27–39.

Hazel, C.B.; Klein, T.M.; Anis, M.; Wilde, H.D.; Parrot, W.A. Growth characteristics and transformability of soybean embryogenic cultures. Plant Cell Rep. 17:765–772; 1998.

Hrib, J.; Vookova, B.; Kormutak, A. Biochemical differences between normal callus and embryogenic suspensor mass of silver fir. Biol. Plant. 39:507–513; 1997.

Huang, X.Q.; Wei, Z.M. High-frequency plant regeneration through callus initiation from mature embryos of maize (*Zea mays* L.). Plant Cell Rep. 22:793–800; 2004.

Lida, A.; Yamashita, T.; Yamada, Y.; Morikawa, H. Efficiency of particle-bombardment-mediated transformation is influenced by cell cycle stage in synchronized cultured cells of tobacco. Plant Physiol. 97:1585–1587; 1991.

Lowe, K.; Taylor, D.B.; Ryan, P.K.; Paterson, E. Plant regeneration via organogenesis and embryogenesis in the maize inbred line B73. Plant Sci. 41:125–132; 1985.

Lu, C.; Vasil, I.K.; Ozias-Akins, P. Somatic embryogenesis in *Zea mays* L. Theor. Appl. Genet. 62:109–112; 1982.

Lu, C.; Vasil, V.; Vasil, I.K. Improved efficiency of somatic embryogenesis and plant regeneration in tissue cultures of maize (*Zea mays* L.). Theor. Appl. Genet. 66:285–289; 1983.

Mertz, E.T. Thirty years of opaque-2 maize. In: Larkins, B.A.; Mertz E.T., eds. Quality Protein Maize: 1964–1994. Proceedings of the International Symposium on Quality Protein Maize. EMBRAPA/CNPMS. Sete Lagoas, MG, Brazil; 1994:1–9.

Mertz, E.T.; Bates, L.S.; Nelson, O.E. Mutant gene that changes protein composition and increases lysine content of maize endosperm. Science 145:279–280; 1964.

Murashige, T.; Skoog, F.A. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–497; 1962.

Nelson, D.E.; Koukoumanos, M.; Bohnert, H.J. Myo-inositol-dependent sodium uptake in ice plant. Plant Physiol. 119:165–172; 1999.

O'Connor-Sánchez, A.; Cabrera-Ponce, J.L.; Valdez-Melara, M.; Téllez-Rodríguez, P.; Pons-Hernández, J.L.; Herrera-Estrella, L. Transgenic maize plants of tropical and subtropical genotypes obtained from calluses containing organogenic and embryogenic-like structures derived from shoot tips. Plant Cell Rep. 21:302–312; 2002.

O'Kennedy, M.M.; Burger, J.T.; Berger, D.K. Transformation of elite white maize using the particle inflow gun and detailed analysis of a low-copy integration event. Plant Cell Rep. 20:721–730; 2001.

Pareddy, D.R.; Petolino, J.F. Somatic embryogenesis and plant regeneration from immature inflorescences of several elite inbreds of maize. Plant Sci. 67:211–219; 1990.

Ray, D.S.; Ghosh, P.D. Somatic embryogenesis and plant regeneration from cultured leaf explants of *Zea mays*. Ann. Bot. 66:497–500; 1990.

Rhodes, C.A.; Green, C.E.; Phillips R.L. Regenerable maize tissue cultures derived from immature tassels. Maize Genet. Coop. Newsl. 56:148–149; 1982.

Rhodes, C.A.; Green, C.E.; Phillips, R.L. Factors affecting tissue culture initiation from maize tassels. Plant Sci. 46:225–232; 1986.

Rice, T.B.; Reid, R.K.; Gordon, P.N. Morphogenesis in field crops. In: Hughes, K.W.; Henke, R.; Constantin, M., eds. Propagation of Higher Plants Through Tissue Culture. Springfield: National Technical Information Service; 1978:262–277.

Sachs, M.M.; Lorz, H.; Dennis, E.S.; Elizur, A.; Ferl, R.J.; Gerlach, W.L.; Pryor, A.J.; Peacock, W.J. Molecular genetic analysis of the maize anaerobic response. In: Sheridan, W., ed. Maize for Biological Research. Charlottesville: Plant Molecular Biology Association; 1982:139–144.

Santos, M.A.; Torne, J.M.; Blanco, J.L. Methods of obtaining maize totipotent tissue. I. Seedling segments culture. Plant Sci. Lett. 33:309–315; 1984.

Segal, G.; Song, R.; Messing, J. A new opaque variant of maize by a single dominant RNA-interference-inducing transgene. Genetics 165:387–397; 2003.

Songstad, D.; Duncan, D.; Widholm, J. Effect of 1-aminocyclopropane-1-carboxylic acid, silver nitrate, and norbornadiene on plant regeneration from maize callus cultures. Plant Cell Rep. 7:262–265; 1988.

Songstad, D.D.; Armstrong, C.L.; Peterson, W.L. Silver nitrate increases type II callus production from immature embryos of maize inbred B73 and its derivatives. Plant Cell Rep. 9:699–702; 1991.

Songstad, D.D.; Peterson, W.L.; Armstrong, C.L. Establishment of friable embryogenic (type II) callus from immature tassels of *Zea mays* (Poaceae). Am. J. Bot. 79:761–764; 1992.

Sozinov, A.; Lukjanjuk, S.; Ignatova, S. Anther cultivation and induction of haploid plants in triticale. Z. Pflanzenzücht. 86:272–285; 1981.

Springer, W.D.; Green, C.E.; Kohn, K.A. A histological examination of tissue culture initiation from immature embryos of maize. Protoplasma 101:269–281; 1979.

Stuart, D.A.; Strickland, S.G. Somatic embryogenesis from cells cultures of *Medicago sativa* L. I. The role of amino acid additions to the regeneration medium. Plant Sci. Lett. 34:165–174; 1984a.

Stuart, D.A.; Strickland, S.G. Somatic embryogenesis from cells cultures of *Medicago sativa* L. II. The interaction of amino acids with ammonium. Plant Sci. Lett. 34:175–181; 1984b.

Suprasanna, P.; Rao, K.V.; Reddy, G.M. Plantlet regeneration from glume calli of maize (*Zea mays* L.). Theor. Appl. Genet. 72:120–122; 1986.

Ting, Y.C.; Yu, M.; Zheng, W.Z. Improved anther culture of maize. Plant Sci. Lett. 23:139–145; 1981.

Torne, J.M.; Santos, M.A.; Pons, A.; Blanco, M. Regeneration of plants from mesocotyl tissue cultures of immature embryos of *Zea mays* L. Plant Sci. Lett. 17:339–344; 1980.

Vain, P.; Flament, P.; Soudain, P. Role of ethylene in embryogenic callus initiation and regeneration in *Zea mays* L. J. Plant. Physiol. 135:537–540; 1989a.

Vain, P.; Yean, H.; Flament, P. Enhancement of production and regeneration of embryogenic type II callus in *Zea mays* L. by AgNO3. Plant Cell Tiss. Org. Cult. 18:143–151; 1989b.

Vasil, I.K. Tissue cultures of maize. Maydica 50:361–365; 2005.

Zar, J.H. Biostatistical analysis. Englewood Cliffs: Prentice-Hall; 1974.

Zhang, S.; Williams-Carrier, R.; Lemaux, P.G. Transformation of recalcitrant maize elite inbreds using *in vitro* shoot meristematic cultures induced from germinated seedlings. Plant Cell Rep. 21:263–270; 2002.

Zhong, H.; Srinivasan, C.; Sticklen, M.B. *In-vitro* morphogenesis of corn (*Zea mays* L.). I. Differentiation of multiple shoot clumps and somatic embryos from shoots tips. Planta 187:483–489; 1992.