Current Topics in Plant Biology
Vol. 13, 2012

Original Communication

Plant regeneration via somatic embryogenesis in a Mexican landrace of maize (*Zea mays* L.)

Martín Vargas-Suárez[1,#], Cristina García-Flores[1,#,$], María de Jesús Jiménez-Villalobos[1], Octavio Guerrero-Andrade[1,$], Teresa de Jesús Olivera-Flores[1], Judith Márquez-Guzmán[2] and Estela Sánchez-de-Jiménez[1,*]

[1]Facultad de Química, Departamento de Bioquímica, Universidad Nacional Autónoma de México, México DF 04510, [2]Facultad de Ciencias, Departamento de Biología, Universidad Nacional Autónoma de México, México DF 04510, México

ABSTRACT

Tuxpeño is a Mexican maize landrace adapted to a wide range of environments and is agriculturally and economically relevant worldwide. To contribute to the preservation of the Tuxpeño germplasm and to develop a platform that allows performing biotechnological improvements of its traits for human benefit, this study aims to explore the potential of Tuxpeño maize to produce *in vitro* embryogenic callus tissue with plant regeneration capability via somatic embryogenesis. When compared to CML137, a maize line well known for its considerable plant regeneration capability, the immature Tuxpeño embryos produced embryogenic calli that efficiently proliferated and regenerated plantlets through somatic embryogenesis. Importantly, a microscopic analysis of the somatic embryogenesis process mirrored the major histological events of zygotic embryogenesis from a unicellular origin. The amount of Tuxpeño callus tissue generated on N6 medium, the number of regenerated plants from these cultures and the plant regeneration percentage were not affected by silver nitrate ($AgNO_3$), and these parameters were found to be comparable between the Tuxpeño and CML137 cultures. The callus yield, callus proliferation, and number of regenerated plants from the Tuxpeño embryogenic cultures were enhanced with cocktail 20 (ck20) and 2,4-dichlorophenoxyacetic acid (2,4-D) but not with 3,6-dichloro-2-methoxybenzoic acid (Dicamba) or 2-(2 methyl, 4-chlorophenoxy propionic acid (MCPP). Therefore, we established the conditions for successful plant regeneration via somatic embryogenesis from a callus culture of the maize landrace Tuxpeño. This achievement offers the opportunity to preserve the great genetic resource represented by the Tuxpeño germplasm and to improve the genotype through molecular biotechnology tools for human benefit.

KEYWORDS: somatic embryogenesis, landraces, maize diversity, Tuxpeño, *Zea mays*

ABBREVIATIONS

$AgNO_3$: silver nitrate; Dicamba: 3,6-dichloro-2-methoxybenzoic acid; MCPP: 2-(2 methyl, 4-chlorophenoxy propionic acid; 2,4-D: 2,4-dichlorophenoxyacetic acid

*Corresponding author
estelas@unam.mx
[#]These authors contributed equally to this work.
[$]Present addresses:
Cristina García-Flores: Instituto Mexicano de la Propiedad Industrial, Arenal 550. Col. Pueblo Santa María Tepepan, Xochimilco, México DF 16020, México.
Octavio Guerrero-Andrade: Departamento de Producción Agrícola y Animal, División de Ciencias Biológicas y de la Salud, Universidad Autónoma Metropolitana, Unidad Xochimilco, México DF 04960, México.

INTRODUCTION

Cereals are important crops in world agriculture due to their relevance for human and animal use as foodstuffs, providing more than half of the food consumed by humans. In particular, maize represents the basic nutrient supply for a large part of the world's population. Through continuous divergent selection, farmers in Mexico have diversified maize into domesticated landraces, 60 of which still persist and fulfill their cultural and agronomic needs [1, 2, 3]. These Mexican maize landraces represent improved germplasms with unique characteristics that have produced over 300 varieties [4], which have been spread throughout the world by the green revolution. To maintain this wide phenotypic diversity of agronomic traits, it is important to develop suitable strategies to conserve and optimize this genetic resource. Occupying an important place among the most relevant Mexican landraces cultivated at present, Tuxpeño [3] is characterized by its height, resistance to ear rots and foliar diseases, and inherent response to fertility conditions with a high yield production [4]. The Tuxpeño race is widely distributed from northeast Mexico to the Yucatán Peninsula and to other world regions, such as China, India, Africa, and Latin American countries [3, 5].

The traditional techniques for cereal improvement constitute a slow process that has strong biological and practical limitations [6]. Tissue culture alone or combined with molecular biotechnology techniques [7-9] has opened new opportunities for the preservation or improvement of useful agronomic traits and for specific genetic transformation via plant regeneration through somatic embryogenesis [10-14]. In addition, unlike traditional agricultural systems, one embryo in plant tissue culture can give rise to many regenerated plants carrying the same genotype through somatic embryogenesis. Indeed, embryogenic cultures have become the preferred method for the regeneration of most of the commercially cultivated transgenic crops [12]. However, somatic embryogenesis does not occur in all plants or tissue sources because it is influenced by several factors, such as developmental stage, explant type, culture medium, hormonal content, and, in particular, genotype [15-18].

Somatic embryogenesis is a complex process that is particularly difficult to accomplish in monocotyledonous plants. Specifically, plant regeneration in maize has been proven to be highly dependent on the genotype [18] because only relatively few genotypes, mainly inbreeds and hybrids, have been found to be successful in producing somatic embryos, such as the immature embryo-derived calli of CML137 [19-21]. To contribute to the preservation of the Tuxpeño germplasm and to develop a platform that allows biotechnological improvements for human benefit, this study aims to explore the potential of Tuxpeño maize to produce *in vitro* embryogenic callus tissue with plant regeneration capability via somatic embryogenesis. In this report, evidence is presented that immature Tuxpeño embryos successfully gave rise to embryogenic calli that regenerated complete plantlets via somatic embryogenesis of a single-cell origin. Furthermore, this high-yield plant regeneration led to seed production.

MATERIALS AND METHODS

Plant material

The *in vitro* callus cultures were induced from immature embryos of subtropical Mexican maize (*Zea mays* L.), the Tuxpeño landrace; immature embryos from the maize line CML137 [19] were also used for comparison. To establish the conditions for the embryogenic cultures, donor plants of immature embryos were grown in a greenhouse in CIMMYT (Centro Internacional de Mejoramiento de Maíz y Trigo) at El Batán, Mexico, a zone with a temperate climate. To improve the proliferation of the embryogenic cultures, donor plants were grown under subtropical conditions at INIFAP's (Instituto Nacional de Investigaciones Forestales y Agropecuarias) field station in Zacatepec, Morelos, Mexico. Whole ears of Tuxpeño or CML137, containing immature embryos at 15 days after self-pollination, were harvested. The ears were surface-sterilized with 70% ethanol for 1 min, followed by 20% (v/v) sodium hypochlorite containing 10 drops/l Tween-80 for 30 min and then rinsed three times with sterile deionized water [19]. Immature embryos of 1.5-2.0 mm were manually and aseptically dissected from the kernels and placed on the induction medium with the flat-side down and the scutellum facing up.

Induction and maintenance media and culture conditions

To induce the callus cultures, batches of 800 immature embryos of either Tuxpeño or CML137 were used. The embryos were cultured on induction medium N6C1SN [19]. This medium contained the inorganic components of N6 medium [22] supplemented with MS vitamins [23], 200 mg/l casein hydrolysate, 2.302 g/l L-proline, 30 g/l sucrose, 2 mg/l 3,6-dichloro-2-methoxybenzoic acid (Dicamba), 15.3 mg/l silver nitrate ($AgNO_3$), and 2.3 g/l phytagel, adjusted to pH 5.7. The effect of $AgNO_3$ on callus generation was evaluated using N6C1SN lacking $AgNO_3$ (N6C1) [19]. Here, this second medium is referred to as N6-DiMS medium, for N6-Dicamba with MS vitamins. Subculturing was performed on the same corresponding induction medium.

The effects of the types of vitamins and auxins on callus yield and proliferation on N6-DiMS medium were then evaluated in another set of cultures using the following media that included cocktail 20 (ck20, a mixture of amino acids, vitamins, and nitrogen compounds) instead of MS vitamins [24]: N6-Dicamba (N6-Dick20); N6-2-(2 methyl, 4-chlorophenoxy propionic acid (MCPP) (N6-Mck20); and N6-2,4-dichlorophenoxyacetic acid (2,4-D) (N6-Dck20). Unless otherwise stated, all of the callus cultures were subcultured twice every 14 days and were maintained in darkness at 25 ± 2ºC.

Somatic embryo germination and plant regeneration

The plantlet regeneration capability of the callus tissue subcultured on both N6C1SN and N6-DiMS was induced upon transfer of the calli onto MSR medium: basal MS medium [23] plus 20 g/l sucrose, 0.5 mg/l indol-3-acetic acid, and 1 mg/l 6-benzylaminopurine, pH 5.7. To induce root development, the plantlets were transferred to modified MS medium containing 1 mg/l naphthaleneacetic acid. The plantlets were later transplanted to pots containing a sterilized mixture of peat moss with leaf spoil (1:1), covered with transparent plastic bags and incubated for one week in a growth chamber before being moved into the greenhouse [19]. The plantlet regeneration of the calli grown on the N6-DiMS, N6-Di, N6-M, and N6-D media was promoted by reducing the casein hydrolysate, L-proline, and growth regulator concentrations to one-half at the third subculture, to one-quarter at the fourth subculture, and by completely eliminating them at the fifth subculture when the visible germination of somatic embryos was apparent. The germinating embryos were then transferred to MS medium containing 3% sucrose and no growth regulators. The regenerating cultures were incubated at 25 ± 2°C under a 16:8 h light:dark photoperiod at a 100 $\mu E/m^2/s$ light intensity. The regenerated plantlets were transferred to the greenhouse when they were 10 cm tall.

Histological analysis

Histological samples were prepared from the Tuxpeño and CML137 immature embryo-derived calli subcultured on N6-DiMS and then on MSR medium for 2, 5, 15, and 21 days. The samples were first fixed in formaldehyde:acetic acid:ethanol:water (2:1:10:7) and were then dehydrated in a series of different ethanol:water mixtures (30, 50, 70, 85, 96, and 100%) for 30 min each. Lastly, the tissues were embedded in Paraplast to prepare the samples for microscopy. The slides for the microscopic analysis were produced with 9 µm thick tissue sections generated with a rotary microtome and stained using the Johansen's safranin O-fast green protocol [25]. The histological samples were observed using an optical microscope (Olympus).

RESULTS

Callus induction

The immature embryos from both Tuxpeño and CML137 maize, cultured on N6-DiMS medium, exhibited major visible changes within the scutellum since the first week of culture. The primary callus-growing region was located near the basal end of the embryo, a site where the cells proliferated and grew rapidly (Figure 1a). The primary callus tissue was yellowish and friable (Figure 1b). The nascent calli were then subcultured on N6-DiMS medium. After two subcultures, the calli differentiated into typical, compact, nodular embryogenic type II calli (Figure 1c).

Histological analysis

To evaluate whether the Tuxpeño callus actually regenerates plants via somatic embryogenesis, a

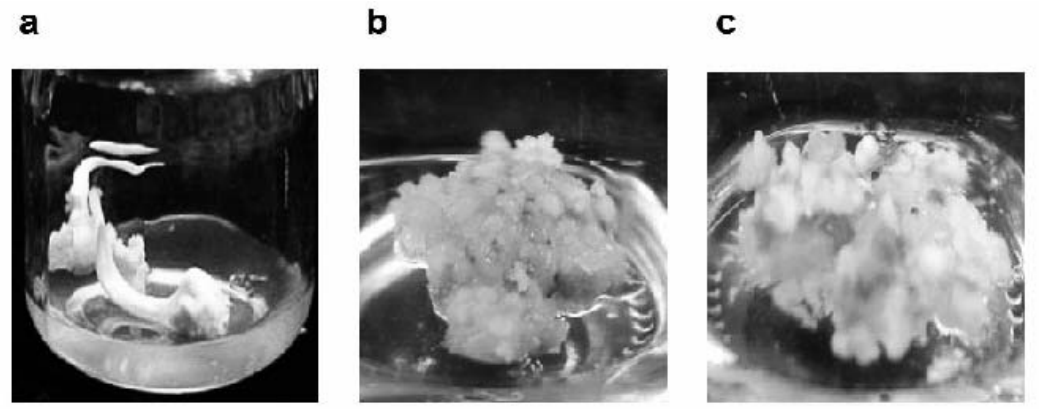

Figure 1. Callus induction from Tuxpeño maize immature embryos. (a) Primary callus induction at the basal end of the embryo. (b) Proliferation of friable primary callus. (c) Embryogenic type II callus after two subcultures on N6-DiMS medium.

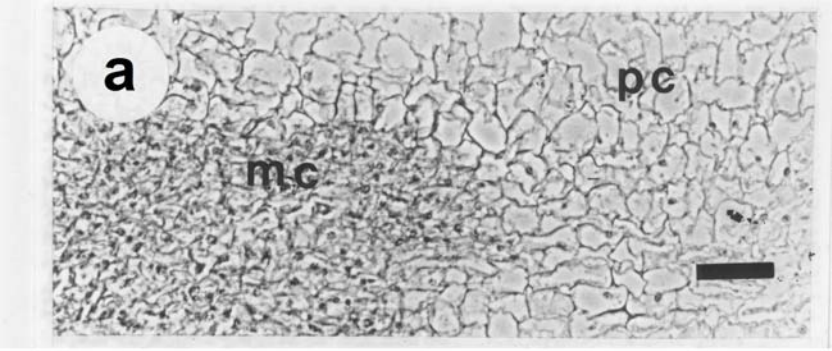

Figure 2. Histological analysis of Tuxpeño immature embryo-derived callus. Histological preparations from embryogenic callus subcultured twice on N6-DiMS maintenance medium. Meristematic centers (mc) surrounded by parenchymal cells (pc). Bar: 2.6 µm.

histological examination of the callus cultures generated and subcultured on N6C1SN was performed. Callus tissue from the CML137 maize line was also analyzed for comparison. The histological analysis of the Tuxpeño callus from the immature embryos revealed meristematic centers surrounded by parenchymal cells after the second subculture on the maintenance medium (Figure 2). After two subcultures on the maintenance medium, the calli were transferred onto MSR medium, and evaluations of the morphogenetic process were performed over the next 21 days. On this medium, the callus tissue started to differentiate, exhibiting abundant and prominent meristematic zones surrounded by parenchymal cells (Figure 3a). On day 5 of the incubation, abundant meristematic cells appeared, showing periclinal cell divisions that produced bi-, tri-, and tetra-cellular proembryos (Figure 3b). After 15 days of incubation, these initial proembryos developed into globular somatic embryos (Figure 3c) with homogenous cell populations within their central regions (Figure 3d). By day 21, the parenchymal cells of the somatic embryos were followed by a suspensor-like structure composed of meristematic cells (Figure 3e) connected to the embryonic structure. Similar events have been considered to represent the first steps of the embryogenic process [26, 27]. The somatic embryos in the more advanced developmental stages had high-cell-density regions, indicating that these cells have a higher mitotic index than those from the rest of the structure (Figure 3f). This difference in cell division speed corresponds to the embryonic cell population

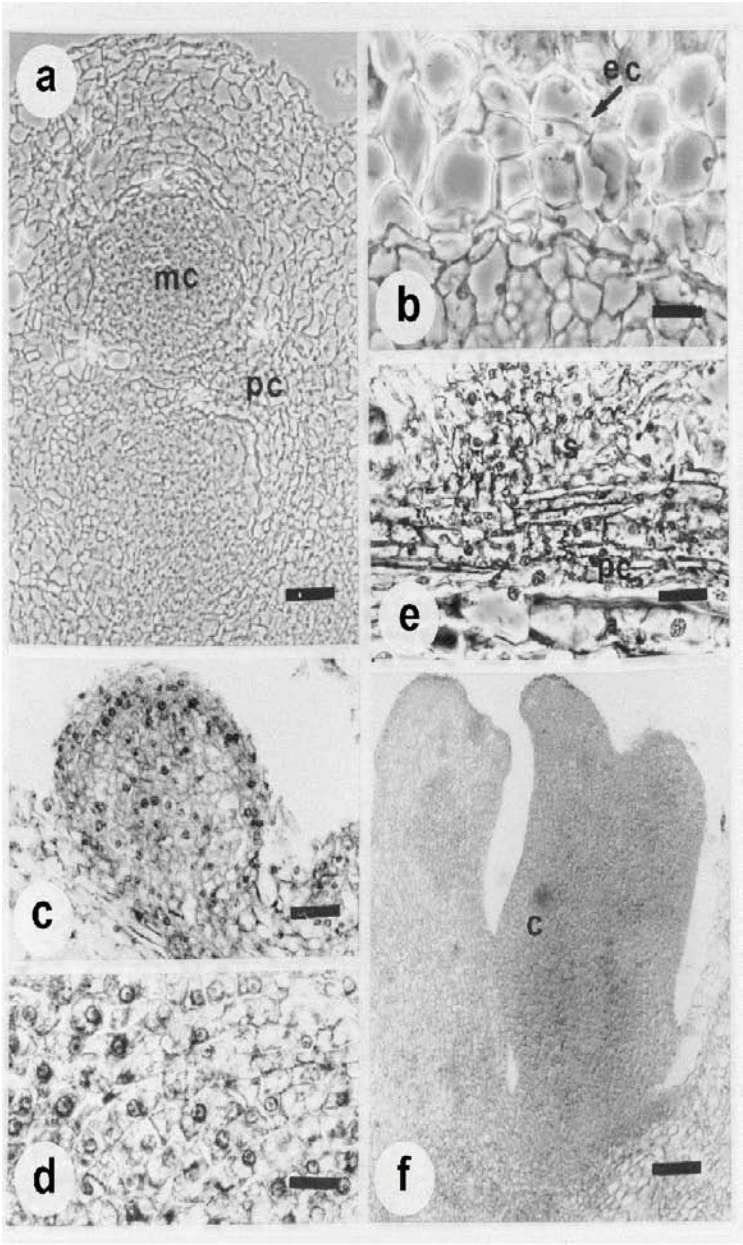

Figure 3. Histological development during plant regeneration process. Pieces of embryogenic callus cultured for various periods on MSR regeneration medium were prepared for histology. (a) Two days: Parenchymal cells (pc) around a meristematic center (mc). Bar: 2.4 µm. (b) Five days: Group of conspicuous embryogenic cells (ec). Bar: 1.8 µm. (c) Fifteen days: Somatic embryo at the globular stage. Bar: 5.5 µm. (d) Homogeneous meristematic cells in a body somatic embryo. Bar: 1.2 µm. (e) Twenty-one days: The suspensor (s) of a somatic embryo showing parenchymal cells (pc). Bar: 2.6 µm. (f) Somatic embryos with developing cotyledon (c). Bar: 6.7 µm.

structure. From this stage, the somatic embryos developed very rapidly, reaching normal plant regeneration 25 days later. In addition to these structures, the remainder of the callus contained parenchymal cells throughout this process. Vascular tissue in the inner region of the callus was observed and cells containing plastids were found on its surface, accounting for the green

color developed by the calli (data not shown). Altogether, these results indicate that somatic embryogenesis recapitulated zygotic embryogenesis and point to a unicellular origin of the somatic embryos. The morphogenetic process for CML137 followed essentially the same pattern as that of Tuxpeño, albeit faster, as evidenced by the anticipated appearance of globular somatic embryos and plant regeneration, which occurred between days 10 and 21.

The somatic embryos produced from the Tuxpeño immature embryo-derived callus tissue rapidly grew and became greenish in color upon transfer to regeneration medium MSR and illumination. The evident germination of the embryos with the development of typical structures was observed by day 10 (Figure 4A); 5 days later, complete plantlets with incipient roots were formed (Figure 4B). For the CML137 cultures, this response was similar, though it occurred slightly earlier, after only 11 days. Well-developed roots appeared approximately 2 weeks later in both genotypes (Figure 4C). The rooted plantlets transplanted to soil and transferred to a greenhouse grew into morphologically and reproductively normal adult plants that produced seeds. Qualitatively, both the Tuxpeño and CML137 embryogenic calli presented normal plant developmental and phenotypic patterns. Typical regenerated Tuxpeño plants are shown in Figure 4D. The histological and morphological analyses definitively demonstrated that Tuxpeño plant regeneration occurred via somatic embryogenesis and not organogenesis.

Callus proliferation and plant regeneration

As the next step in the characterization of Tuxpeño maize *in vitro* cultures, the effects of $AgNO_3$ on the callus generation and plant regeneration were evaluated. The amount of callus generated per embryo after induction on either N6C1SN or N6-DiMS medium was the same. In addition, the number of regenerated plants per g FW callus and the plant regeneration percentage were similar in the calli from both media: 10 (with $AgNO_3$) vs. 8 (without $AgNO_3$) and 27 (with $AgNO_3$) vs.

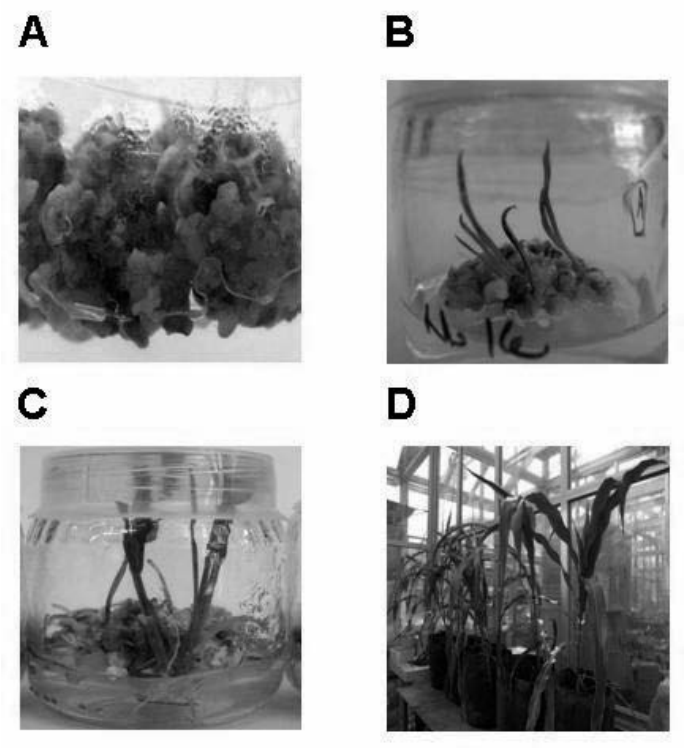

Figure 4. Plant regeneration from Tuxpeño maize callus. (A) Germinating somatic embryos and nascent shoots in MSR medium. (B) Plantlets with emergent roots on rooting medium. (C) Abundant developing roots from plantlets. (D) Normal regenerated adult plants growing in greenhouse.

30 (without $AgNO_3$), respectively (Table 1). These results indicate a non-significant effect of $AgNO_3$ on the Tuxpeño maize cultures under the tested conditions. When comparing these results to the response of the CML137 maize cultures, it was observed that the amount of generated calli per embryo after induction on N6C1SN medium was 1.4-fold higher for CML137 than for Tuxpeño. However, the number of regenerated plants per g FW callus and the plant regeneration percentage were similar for both genotypes: 12 (CML137) vs. 10 (Tuxpeño) and 32 (CML137) vs. 27 (Tuxpeño), respectively (Table 1). These results indicate that the Tuxpeño genotype has almost the same plant regeneration capability as the cell line used as the control.

In an attempt to optimize the Tuxpeño callus cultures, the effects of two components of the culture medium, the types of vitamins and auxins, on callus generation and plant regeneration capability were analyzed. To this end, the embryogenic callus cultures were induced and subcultured on N6-DiMS or N6-Dick20 medium, both of which contained Dicamba as the auxin and either MS vitamins or ck20 vitamins, respectively. The results revealed no differences in either callus yield or the number of regenerated plants (Table 2). Next, N6-Mck20 (containing the auxin MCPP) and N6-Dck20 (containing the auxin 2,4-D) media were evaluated and compared with the callus tissue generated on N6-Dick20 as a control. The results indicated that the percentage of explants producing embryogenic calli was very similar for the three media, ranging between 94 and 98% (Table 2). The results were similar when the generation of primary embryogenic calli, the production of subcultured calli, and the number of regenerated plants were evaluated and compared between the N6-Dick20, N6-Mck20, and N6-Dck20

Table 1. Callus generation and plant regeneration from immature embryos of two maize genotypes.

Genotype[a]	Tuxpeño		CML137
Medium	N6C1SN	N6C1 (=N6DiMS)	N6C1SN
Yield[b] (g FW callus/explant)	0.60	0.61	0.85
Regenerated plants/g FW callus[c]	10	8	12
Plant regeneration pecentage[d]	27	30	32

[a]Batches of 800 embryos were used as starting material. [b]The mass produced after callus induction. [c]Counts of the callus tissue induced during 3 weeks, subcultured twice on N6-DiMS medium, and later incubated for 3 weeks on MSR medium. [d]The fraction of embryos that regenerated at least three plants out of the total number of embryos plated, multiplied by 100.

Table 2. Effects of vitamins and auxins in the culture medium on Tuxpeño embryogenic callus generation and plant regeneration.

Medium	N6-DiMS	N6-Dick20	N6-Mck20	N6-Dck20
Percentage of explants with embryogenic callus[a]	97	98	96	94
Yield (g FW callus/explant)[b]	0.5 ± 0.14	0.6 ± 0.12	0.7 ± 0.12	0.9 ± 0.18
Total callus (g)[c]	0.50 ± 0.15	0.53 ± 0.05*	0.60 ±0.10	0.73 ± 0.06*
Regenerated plants/g FW callus[d]	8 ± 2	12 ± 2	14 ± 2	16 ± 3

[a]The fraction of embryos that produced embryogenic calli out of the total number of embryos that generated any callus type, multiplied by 100. [b]The mass produced after callus induction on the different media. [c]Fresh weight of callus mass at the third subculture using a 0.3-g inoculum. [d]Counts of the callus tissue incubated on MS medium without growth regulators. *Significant difference at p=0.5.

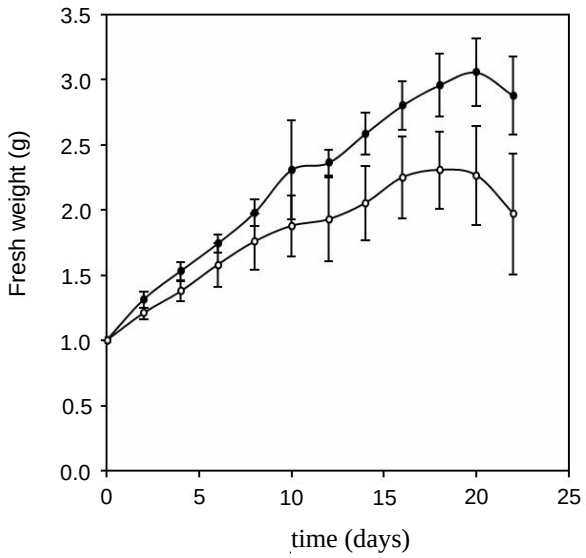

Figure 5. Effect of vitamins composition on growth rate of Tuxpeño immature-embryo-derived callus. Cell growth was measured for 3 weeks after the third subculture. One gram of callus piece was the inoculum. Tissues were directly weighted without previous treatment at the indicated times. Each value represents the average of four replicates (p=0.05 from 0 to 12 days; p=0.01 from 14 to 21 days). Closed circles: N6-Dick20; open circles: N6-DiMS.

media, with the exception of a significant increase (p=0.5) in the production of subcultured calli on N6-Dck20 compared to N6-Dick20 (Table 2). These findings suggest that positive effects can be induced under specific combinations of types of auxins and vitamins in the culture medium. To further support this interpretation and considering that 2,4-D was the best auxin to enhance callus growth, the proliferation capability of the Tuxpeño embryogenic callus, as affected by vitamins, was analyzed on the N6-DMS (N6-2,4-D with MS vitamins) and N6-Dck20 media. As shown in Figure 5, the gain in the fresh weight of the callus on N6-Dck20 during a three week growth period was higher than that on N6-DMS, with maximum values of 3.1 and 2.4 g, respectively. In addition, the callus cultured on the N6-Dck20 medium appeared to be healthier, indicating that the substitution of MS vitamins with ck20 has effects on both the qualitative and quantitative aspects of the callus in the presence of 2,4-D but not in the presence of Dicamba (compare Figure 5 with Table 2).

DISCUSSION

Several plant regeneration systems using somatic embryogenesis have been developed for different inbred and hybrid maize lines [19, 20, 28-32], including some of nutritional relevance, such as quality protein maize lines [21]. However, most of these maize lines are not readily available: they are not widely commercialized because they do not necessarily display agronomically important traits or exhibit flexible adaptations to different ecosystems. To the best of our knowledge, the results generated in this study with Tuxpeño maize, which is readily available, extensively commercialized and has great adaptability, constitute the first case of embryogenic callus culture establishment from a maize landrace with plant regeneration capability. This goal was accomplished using immature embryos, the most efficient maize explant to achieve plant regeneration via somatic embryogenesis [18]. Although maize seed banks are a conventional germplasm source, plant tissue cultures in general, including maize cultures, offer an excellent alternative to traditional agricultural practices with regard to solving the problems encountered with seed germination and plantlet establishment. Furthermore, as opposed to one plant emerging from a seed via traditional sowing, the Tuxpeño embryogenic cultures had the advantage of exhibiting a high plant regeneration percentage that was similar to that of the CML137 line (Table 1), producing up to 16 plants per g FW callus (Table 2).

However, as the maize embryos used in this investigation are a landrace that consists of a mixture of genetic components, variability could be expected in the initiation of embryogenic cultures and plant regeneration. Nevertheless, among the five batches of embryos we analyzed, the frequency of embryogenic callus-producing immature embryos, the number of plants regenerated and the percentage of plant regeneration remained fairly constant; this was further supported by our findings that a high potential of regeneration capability was maintained over 3 years in the *in vitro* callus cultures (data not shown), a potential that is known to be lost over time in maize cultures [33]. Thus, the Tuxpeño landrace represents a solid genetic background for regenerating plants by *in vitro* cultures through somatic embryogenesis. As we assessed the responses of Tuxpeño under specific

culture conditions, which were similar to those that allowed callus growth and the development of somatic globular embryos of different inbred maize lines [19], these results strongly suggest that the molecular basis controlling somatic embryo development and plant regeneration in the Tuxpeño landrace are similar to those experimentally established for maize germplasms from regenerable inbred and hybrid lines [20, 21, 28-32]. Most importantly, our results indicate that this genotype can be exploited in a reliable and suitable *in vitro* system for maize plant regeneration.

Tuxpeño plant regeneration represents a way to preserve a vast genetic wealth that is a part of the great maize diversity in Mexico, helping to guarantee the possibility of continuously cultivating Tuxpeño-derived varieties in their own centers of origin [34] and in other regions of the world. This opportunity might also be relevant for a large number of other landraces that are currently widely cultivated in Mexico. Furthermore, because our plant regeneration system occurred via somatic embryogenesis with a unicellular origin, as supported by our histological analysis (Figure 3), it offers a great opportunity to generate transgenic maize landraces that would not present mosaicism, in contrast to what can be anticipated when regenerated plants arise by organogenesis. Recent biotechnological progress either by Agrobacterium-mediated transformation or particle bombardment has revealed the current need to improve different traits in maize, including herbicide tolerance, primary biomass feedstock, prevention of insect development that damages grains during storage, starch content, seed weight, and grains used as biofactories for industrial enzymes or high-value molecules [10, 13, 14, 35, 36, 37]. Interestingly, our encouraging results suggest that plant regeneration capability via somatic embryogenesis is not limited to the Tuxpeño landrace because the immature embryos of its derived varieties, Chalqueño and Costeño, are also capable of forming embryogenic cultures (data not shown). Our *in vitro* system represents an excellent tool for solving the issues of both germplasm preservation and transformation in maize.

CONCLUSIONS

We have established the conditions for the successful *in vitro* establishment of regenerable embryogenic cultures from immature embryos of the Tuxpeño maize, a landrace that has a tremendous economic and agronomical impact worldwide and is able to perform well in a wide range of environments. These features, along with the ready availability of the Tuxpeño genotype, make it an ideal target for applied biotechnological purposes with a high impact for human benefit.

ACKNOWLEDGEMENTS

We thank Dr. N. Bohorova for kindly providing the CML137 line material used in this study and for allowing some of the experiments to be performed at CIMMYT (Centro Internacional de Mejoramiento de Maíz y Trigo). We also thank Dr. A. Trujillo Campos for providing the Tuxpeño embryos from Zacatepec and A. Zavaleta Fernández de Córdova for performing the statistical analysis. We acknowledge Dr. S. Sánchez-Nieto and Dr. V. Chávez-Ávila for their critical reading of this manuscript. This work was partly supported by grants from CONACYT No.101327 and PAIP 4290-10, Facultad de Química, UNAM.

REFERENCES

1. Hernández, E. 1985, Econ. Bot., 39, 416.
2. Pressoir, G. and Berthaud, J. 2004, Heredity, 92, 95.
3. Kato, T. A., Mapes, L. M., Mera, L. M., Serratos, J. A. and Bye, R. A. 2009, Origen y diversificación del maíz: una revisión analítica (Universidad Autónoma de México, Comisión Nacional para el Conocimiento y Uso de la Biodiversidad), México, D.F.
4. CIMMYT. 1992, 1991/92 World maize, facts and trends, Mexico City.
5. Song, Y. and Manicad, G. 2000, Revista de agroecología, 15, 18.
6. Reyes, C. P. 1990, El maíz y su cultivo, AGT Editor SA, México.
7. Altpeter, F., Baisakh, N., Beachy, R., Bock, R., Capell, T., Christou, P., Daniell, H., Datta, K., Datta, S., Dix, P. J., Fauquet, C., Huang, N., Kohli, A., Mooibroek, H., Nicholson, L., Nguyen, T. T., Nugent, G., Raemakers, K., Romano, A., Somers, D. A., Stoger. E., Taylor, N. and Visser, R. 2005, Mol. Breed., 15, 305.
8. Halpin, C. 2005, Plant Biotechnol., 3, 141.

9. Frame, B., Main, M., Shick, R. and Wang, K. 2011, Methods Mol. Biol., 710, 327.
10. Kramer, K. J., Morgan, T. D., Throne, J. E., Dowell, F. E., Bailey, M. and Howard, J. A. 2000, Nat. Biotechnol., 18, 670.
11. Guerrero-Andrade, O., Loza-Rubio, E., Olivera-Flores, T., Fehérvári-Bone, T. and Gómez-Lim, M. A. 2006, Transgenic Res., 15, 455.
12. Vasil, I. K. 2008, Plant Cell Rep., 27, 1423.
13. Li, N., Zhang, S., Zhao, Y., Li, B. and Zhang, J. 2011, Planta, 233, 241.
14. Naqvi, S., Ramessar, K., Farré, G., Sabalza, M., Miralpeix, B., Twyman, R. M., Capell, T., Zhu. C. and Christou, P. 2011, Biotechnol. Adv., 29, 40.
15. Emons, C. M., Droppers, S. A. and Tomm, V. C. 1993, J. Plant Physiol., 124, 597.
16. Jiménez, V. M. and Bangerth, F. 2001, Plant Cell Tiss. Org. Cult., 67, 37.
17. Jiménez, V. M. and Bangerth, F. 2001, Plant Sci., 160, 247.
18. Fontanet, P. and Vicient, C. M. 2008, Methods Mol. Biol., 427, 17.
19. Bohorova, E. N., Luna, B., Brito, R. M., Huerta, L. D. and Hoisington, D. A. 1995, Maydica, 40, 275.
20. Che, P., Love, T. M., Frame, B. R., Wang, K., Carriquiry, A. L. and Howell, S. H. 2006, Plant Mol. Biol., 62, 1.
21. Aguado-Santacruz, G. A., García-Moya, E., Aguilar-Acuña, J. L., Moreno-Gómez, B., Solís-Moya, E., Preciado-Ortíz, E. R., Jiménez-Bremont, J. F. and Rascón-Cruz, Q. 2007, In Vitro Cell Dev. Biol. Plant, 43, 215.
22. Chu, C. C., Wang, C. C., Sun, C. S., Hsu, C., Yin, K. C., Chu, C. Y. and Bi, F. Y. 1975, Sci. Sin., 18, 659.
23. Murashige, T. and Skoog, F. 1962, Physiol. Plant., 15, 473.
24. Rosales-Mendoza, S., Soria-Guerra, R., Olivera-Flores, M. T., López-Revilla, R., Argüello-Astorga, G. R., Jiménez-Bremont, J. F., García-de la Cruz, R. F., Loyola-Rodríguez, J. P. and Alpuche-Solís, A. G. 2007, Plant Cell Rep., 26, 969.
25. Johansen, D. A. 1940, Plant microtechnique, McGraw-Hill Book Co, New York.
26. Fransz, P. F. and Schel, J. H. N. 1991, Can. J. Bot., 69, 26.
27. Fransz, P. F. and Schel, J. H. N. 1991, Can. J. Bot., 69, 858.
28. El-Itriby, H. A., Assem, S. K., Hussein, E. H. A., Abdel-Calil, F. M. and Madkour, M. A. 2003, In Vitro Cell Dev. Biol. Plant, 39, 524.
29. Huang, X. Q. and Wei, Z. M. 2004, Plant Cell Rep., 22, 793.
30. Ahmadabadi, M., Ruf, S. and Bock, R. 2007, Transg. Res., 16, 437.
31. Hernández-García, C. M., López-Peralta, C., Buenrostro-Nava, M. T., Cárdenas-Soriano, E. and Pellegrineschi, A. 2007, Agrociencia, 41, 743.
32. Sidorov, V., Gilbertson, L., Addae, P. and Duncan, D. 2006, Plant Cell Rep., 25, 320.
33. Lozovaya, V., Ulanov, A., Lygin, A., Duncan, D. and Widholm, J. 2006, Planta, 224, 1385.
34. Acevedo, F., Huerta, E., Burgeff, C., Koleff, P. and Sarukhán, J. 2011, Nature Biotechnol., 29, 23.
35. Cao, M., Sato, S. J., Behrens, M., Jiang, W. Z., Clemente, T. E. and Weeks, D. P. 2011, J. Agric. Food Chem., 59, 5830.
36. Gray, B. N., Bougri, O., Carlson, A. R., Meissner, J., Pan, S., Parker, M. H., Zhang, D., Samoylov, V., Ekborg, N. A. and Michael Raab, R. 2011, Plant Biotechnol. J., 9, 1100.
37. Hood, E. E., Devaiah, S. P., Fake, G., Egelkrout, E., Teoh, K. T., Requesens, D. V., Hayden, C., Hood, K. R., Pappu, K. M., Carroll, J. and Howard, J. A. 2011, Plant Biotechnol. J., 10, 20.