Emir. J. Food Agric. 2014. 26 (6): 552-557
doi: 10.9755/ejfa.v26i6.18021
http://www.ejfa.info/

Regular Article

PLANT TISSUE CULTURE

Induction of somatic embryogenesis in *Phytolacca tetramera*, medicinal species of Argentina

M. A. Basiglio Cordal*, M. Adema, V. Briones, B. Villarreal, M. H. Panarisi, W. Abedini and S. Sharry

Centro Experimental de Propagación Vegetativa (C.E.Pro.Ve), Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, CICPBA- CONICET, Diagonal 113 N° 469 (1900), La Plata, Buenos Aires, Argentina

Abstract

Phytolacca tetramera Hauman is an endemic species of the Province of Buenos Aires (Argentina), in danger of extinction. This species has active fungicides. The methanol extract of its berries possess antifungal activity against opportunistic fungal pathogens. The somatic embryogenesis technique relies on the formation of an embryo from a somatic cell, that is, without the need for the fusion of gametes (Tisserat et al, 1979), which facilitates mass production of in vitro plants. Obtaining somatic embryos gives us the ability to automate the production process in short periods of time and in a confined space. The technique is a necessary step to generate synthetic seeds. In order to adjust a protocol of somatic embryogenesis sections young leaves of Phytolacca tetramera obtained in vitro were placed in a Murashige and Skoog medium supplemented with 2, 4-dichlorophenoxyacetic acid at different concentrations. Somatic embryos were obtained directly from the cutting edge of the blade and embryogenic callus (indirect way) from the midrib.

Key words: Somatic embryogenesis, Endemism, Ombusillo, *In vitro* culture, Germplasm

Introduction

The Family Phytolaccaceae includes 6 subfamilies, 18 genera and 70 species. It is widespread in tropical and temperate regions, especially in the Neotropics and S. Africa. All species of this family are widely used in folk medicine for the treatment of diseases such as edema, rheumatism and dermatitis (Gattuso, Zacchino, 1998).

Phytolacca tetrámera Hauman, commonly known as "Ombusillo", is a dioecious species, belonging to the family Phytollacaceae, up to 1.5 meters high. Is an endemic NE Bs. As geophyte bush, whose distribution starts near the city of La Plata to Ensenada Samborombón, and around, forming a fundamental part and endemism of the flora characteristic of the Parque Costero Del Sur (36° lat. S-. 57° 30'long OR) area declared by UNESCO, "World Biosphere Reserve", since 1984. Regarding compounds and properties, *P. tetramera*, is a natural source of active principles and fungicides (Escalante, 2003) and probably antiviral, antitumor and antibacterial compounds (Abedini et al., 1997). The methanol extract of the berries, showed antifungal activity against opportunistic fungal pathogens. Fractionation of the butanol extract, allowed the isolation of three triterpenoid saponins monodesmosidics: phytolaccosides B [3-O-β-d-xylopiranosyl-phytolaccagenin], E [3-O-β-d-glucopyranosyl-(1 → 4) - β -d-xylopiranosyl-phytolaccagenin] and F [3-O-α-l-rhamnopyranosyl-(1 → 2) - β-d-glucopyranosyl-(1 → 2) - acid β-d-xylopyranosyl-phytolaccagenic] (Escalante et al., 2008).

The first of these compounds was the most active and also showed the broad spectrum of action, resulting in defects of the hyphae and the fungal membrane thickening in *Saccharomyces cerevisiae* (Escalante et al., 2003).

The agar dilution method showed minimum inhibitory concentrations (MICs) between 25 and 250 mg/ml, *Trichophyton mentagrophytes* being the most susceptible species. The structures of the three saponins were elucidated by spectroscopic methods mainly 1D and 2D NMR (Escalante et al., 2003).

Received 30 December 2013; Revised 04 February 2014; Accepted 05 February 2013; Published Online 25 March 2014

*Corresponding Author

M. A. Basiglio Cordal
Centro Experimental de Propagación Vegetativa (C.E.Pro.Ve), Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, CICPBA- CONICET, Diagonal 113 N° 469 (1900), La Plata, Buenos Aires, Argentina

Email: maribasiglio@hotmail.com

Escalante et al. (2008) has shown that the Phytolaccosido B, alters the morphology of fungi and yeasts. The defects were similar to those produced by enfumafungin, a known inhibitor of (1 -> 3) - beta - D-glucan synthase, an enzyme that catalyzes the synthesis of (1 -> 3) - beta-D - glucan, one of the main polymers of the fungal cell wall. However, tests revealed that this enzyme does not inhibit phytolaccoside (1 -> 3)-beta-D-glucan synthase, but produced a marked improvement in the activity of chitin synthase and, concomitantly, an increase of chitin, another important polymer cell walls of fungi. This finding was confirmed by fluorescence microscopy and also by measuring the chitin (Escalante et al., 2008). Medicinal plants as an alternative to traditional crops are revealed as an important source for the development of new activities.

To resolve the problem of extraction of forest plants, one option is asexual or vegetative propagation of this species, which would generate a large number of individuals with the same genetic identity. In vitro culture is an alternative for the breeding and conservation of species difficult to cultivate. The tissue culture techniques have been used routinely for over four decades (Murashige and Skoog, 1962).

In vitro culture conditions, somatic embryos or cells can regenerate shoots, roots and / or flowers in response to given stimulus (Radice, 2004). Somatic embryogenesis is based on the formation of an embryo from a somatic cell, that is, without the need for the fusion of gametes (Tisserat et al., 1979), which facilitates mass production of in vitro plants. This technique has emerged as a new way of propagation and is a tool for *in vitro* conservation of germplasm (Griga, 2000) and breeding (Das et al., 2002). Used for increasing the multiplication coefficients, lower production costs and gives the possibility to automate the production process using bioreactors (De Feria, 2000).

There are two types *in vitro* somatic embryogenesis, direct and indirect. In direct somatic embryogenesis, embryos appear directly over the original explant. In indirect somatic embryogenesis, embryos originating from callus or from embryogenic cell suspension (Perez Molphe et al., 1999). The process of somatic embryogenesis involves different steps (Thorpe, 1995), starting from the induction, followed by histo-differentiation, maturation and subsequent germination, concluding with the conversion of the embryo plant. The technique of somatic embryogenesis is a necessary step to generate synthetic seeds. Synthetic seed described generally to an encapsulated somatic embryo with an artificial covering and can be a technology that enables the extensive scaling required for commercial production of species of interest, even with high germination (http://www.cyd.conacyt.gob.mx/258/articulos/semillassinteticas.html).

In *Phytolacca tetramera*, somatic embryogenesis, would be a vital process because, the species is known for being critically endangered threat, and being a natural source of active compounds.

The objective of this work was to achieve the induction of somatic embryogenesis in *Phytolacca tetrámera* Hauman.

Materials and Methods

The mother plants were obtained by *in vitro* micropropagation from nodal and internodal sections, seeded in a basal culture medium of Murashige and Skoog (1962) (MS) supplemented with 3% sucrose and without the addition of plant growth regulators to induce the process of organogenesis adventitia. The leaf sections 3-5 cm were used as explants for the induction of somatic embryogenesis. The sheets were cut into 1.5 cm sections, retaining the midrib, and were placed on the abaxial side of a 5 explants per container in the culture medium containing Murashige and Skoog (MS) complete, supplemented con 2, 4 D (2, 4 - dichlorophenoxyacetic acid) at different concentrations (0.5, 1 and 1.5 ppm) with the corresponding Witness in the absence of 2, 4 D. As gelling agent Difco Bacto agar was used in a range of 0.6 to 0.7% and the pH was adjusted between 5.8 to 6.2.

All media were autoclaved at 1 atm pressure and a temperature of 120°C for 20 minutes. The containers used were Petri dishes with 15 ml culture medium c / u. The explants were incubated in the dark at 25 + / -2°C temperature.

Results

Experiments followed for 20 days to evaluate the effect of different concentrations of 2, 4 D showed qualitative and quantitative differences. The response of nodal sections placed in culture medium containing different concentrations of 2, 4-D are shown in Table 1. The differentiation began at 6 days of seeding explants, showing the winding sections of leaves. At 20 days of culture, callus formation was observed in both the cut surface of the sections at the level of the main rib. No callus formation in the control treatment was observed. However, root formation ranging between 2 to 4 per explant, and 3 to 5 cm in length was observed. The calluses formed were rib level and white

friable, embryogenic callus typical characteristics. In the same could be identified in globular stage embryos. They subcultured in liquid medium continued development.

The callus formed on the edges of the explant were disintegrable, and they could be observed in different stages of somatic embryos (globular and torpedo).

Figure 1. *Phytolacca tetramera in vitro*.

Figure 2. Foliar Sections of *Phytolacca tetramera* in induction medium.

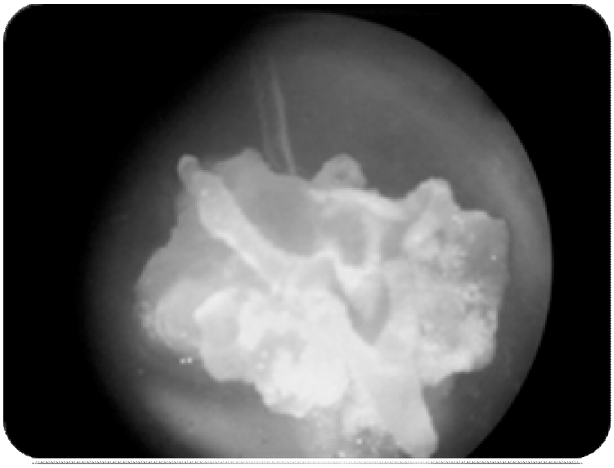

Figure 3. Cotyledon embryo stage, located in the midrib.

Table 1. Effect of the concentration of 2, 4 D in the induction of callus formation from leaf segments *Phytolacca tetramera* Hauman 30 days of cultivation.

Culture Medium	Concentration of growth regulator 2,4-D (ppm)	Number of explants with callus formation	Percentage of explants with callus formation (%)
MS	0.5	20	100%
MS	1.0	10	50%
MS	1.5	17	85%

Discussion

In this paper, the indirect somatic embryogenesis was induced from leaf sections of *P. tetramer*a. The similar results were obtained in different plant species previously (Stamp and Henshaw, 1987; Bespalhok Filho et al., 1993; Robacker 1993; Jayasree 2001; Devaraju and Reddy 2013).

In the present study, it was found that the incubation conditions and culture medium influences the development of embryogenic callus. The concentration of 2,4-D and the area of the explant were decisive for the induction of embryogenic callus and embryos, which is consistent with the reports by Raghavan (2004). He found that in embryos from *Arabidopsis*, cultured in a medium containing 2, 4-D, following a brief period of growth by cell expansion, divisions was initiated in the procambial cells facing the adaxial side at the base of the cotyledons. Cell division activity later spread to almost the entire length of the leaf cotyledons to form a callus on which globular and heart-shaped embryos appeared in about 10 d after culture. In the present investigation, the results on somatic embryogenesis have shown that auxin such as 2,4-D are essential for induction of somatic embryogenesis from leaf explants of *P. tetramera,* where in the growth regulators play a primary role in the medium for induction of somatic embryogenesis in nature. The type of auxin or auxin in combination with cytokinin used in the medium can greatly influence somatic embryo frequency. Proliferated embryogenetic suspension cultures were established in this species. Reddy and Reddy (1983) reported the improved response of auxin like 2,4-D in a single treatment for the induction of somatic embryogenesis compared to combination of 2,4-D and cytokines in *Arachis hypogea*. Somatic embryogenesis is generally believed to be triggered by an auxin and for many plants, 2, 4-D has been widely regarded to be effective for somatic embryogenesis (Ammirato 1983; Finer, 1988). In this study, callus formation was observed after 20 days in culture. Bespalhok et al. (1993) reported that somatic embryos were first observed 15 days after culture initiation. The majority of the somatic embryos appeared to form directly without intermediate callus formation. In fact due to the extremely high pressure, the species *P. tetramera* has been declared as endangered (Delluchi, 2006). In recent years, several groups have addressed the need to conserve native medicinal species and to explore the possibilities of identifying high-yielding individuals or populations for the development of *in vitro* production systems (Ved, 1997; Ciddi, 2000; Cantelmo et al., 2013). The present work on the regenerated plants were morphologically similar to seed-derived plants. Khadke and Kuvalekar (2013) reported similar results with the medicinal plant *Nothapodytes foetida,* a critically endangered plant species, and suggest that this system may prove to be beneficial for *ex situ* conservation as well as for amenability of this endangered plant for genetic manipulation.

Conclusion

Reliable protocols useful for *P. tetramera* mass propagation through callus culture and somatic embryos formation had been established and verified. Plant regeneration through somatic embryogenesis has several advantages over other routes to *in vitro* plant production and appears that most promising area of research for large scale production, rapid plant propagation and support of conservation strategies. This system is potentially useful for the micropropagation of this species, as well as for the production of substances with pharmacological interest, such as phytolaccoside B [3-O-β-d-xylopiranosyl-phytolaccagenin]. The results obtained in this study could have enormous significance, since this is the first report of somatic embryogenesis for this medicinal important and to the whole genus *Phytolacca* species. However, these studies should continue to improve embryo conversion and complete plant regeneration. This technique will allow us to have a lot of plants to introduce and domesticate wild species to establish crop field or restore populations in natural ecosystems.

References

Abedini, W. and S. Sharry. 1997. Aplicación del cultivo de tejidos para la obtención de productos medicinales. VI Congreso Italo Latinoamericano de Etnomedicina "Alejandro Malaspina. Libro de Resúmenes: Octubre 1997, Guatemala.

Ammirato, P. V. 1983. The regulation of somatic embryos development in plant cell cultures, suspension cultures technique and hormone requirements. Biol. Technol. 1:68-74.

Bespalhok Filho, J. C., J. M. Hashimoto and G. L. Esteves Vieira. 1993. Induction of somatic embryogenesis from leaf explants of *Stevia rebaudiana*. R. Bras. Fisiol. Veg. 5:51-53.

Cantelmo, L., B. O. Soares, L. P. Rocha, J. A. Pettinelli, C. H. Callado, E. Mansur, A. Castellar and R. F. Gagliardi. 2013. Repetitive somatic embryogenesis from leaves of the medicinal plant *Petiveria alliacea* L. Plant Cell Tiss. Organ Cult. 115(3):385-393.

Ciddi, V. and M. L. Shuler. 2000. Camptothecin from callus cultures of *Nothapodytes foetida*. Biotech. Lett. 22:129–32.

Das, D., M. Reddy, K. Upadhyaya and S. K. Sopory. 2002. An efficient leaf-disc culture method for the regeneration via somatic embryogenesis and transformation of grape (*Vitis vinifera* L.). Plant Cell Rep. 20:991-1005.

De Feria, M. 2000. Empleo de biorreactores para la multiplicación y diferenciación de suspensiones celulares embriogénicas de *Coffea arabica* L. cv. Catimor 9722. Tesis presentada en opción al grado científico de Doctor en Ciencias Agrícolas. Instituto de Biotecnología de las Plantas. UCLV. Santa Clara.

Delucchi, G. 2006. Las especies amenazadas de la provincia de Buenos Aires: una actualización. Boletín científico APRONA XIV (39):19-31.

Escalante, A., C. Santecchia, M. González Sierra, M. Gattuso and S. Zacchino. 2003. Estudio Morfoanatómico de *Phytolacca tetrámera* y localización Histoquímica de sus saponinas antifúngicas. Río de Janeiro, Brasil. - Río de Janeiro, Brasil. Congreso. XII Congreso Italo-Latinoamericano de Etnomedicina "Nuno Alvares Pereira".

Escalante, A., M. Gattuso, P. Pérez, S. Zacchino. 2008. Evidence for the mechanism of action of the antifungal phytolaccoside B from *Phytolacca tetramera*. J. Nat. Prod. 71:1720-1725.

Finer, J. J. 1988. Apical proliferation of embryogenic tissue of soyabean (*Glycine max* L.) Plant Cell Rep. 7:238-241.

Gattuso, M., G. Rodríguez, C. Santecchia, S. López, E. Martínez and S. Zacchino. 1998. Estudios *in vitro* actividad antifúngica de Rizomas y frutos de *Phytolacca tetrámera* Hauman. VI Simposio Argentino de Farmacobotánica, Posadas, p. 25.

Griga, M. 2000. Morphological alterations in sterile mutant of *Pisum sativum* obtained via somatic embriogenesis. Biol. Plant. 43(2):161-165.

Jayasree, T., U. Pavan, M. Ramesh, A. V. Rao, K. Jagan Mohan Reddy and A. Sadanandam. 2001. Somatic embryogenesis from leaf cultures of potato. Plant Cell Tiss. Organ Cult. 64:13-17.

Khadke, S. and A. Kuvalekar. 2013. Direct somatic embryogenesis and plant regeneration from leaf and stem explants of *Nothapodytes foetida*: a critically endangered plant species. Internat. J. Plant Anim. Env. Sci. 3(1):257-264.

Perez Molphe, E., M. R. Ramírez, N. H. Y. Gordon and A. N. Ochoa. 1999. Introducción al cultivo de tejidos vegetales. Univ. Autónoma de Aguascalientes. México: p.180.

Radice, S. 2004. Morfogénesis *in vitro*. En: Biotecnología y mejoramiento vegetal. Ediciones Instituto Nacional de Tecnología Agropecuaria. Editores: Dra. Viviana Echenique, Dra. Clara Rubinstein, Ing. Agr. Luis Mroginski. Parte II, Capitulo I. pp. 26-33.

Raghavan Val. 2004. Role of 2,4-dichlorophenoxyacetic acid (2,4-D) in somatic embryogenesis on cultured zygotic embryos of *Arabidopsis*: cell expansion, cell cycling, and morphogenesis during continuous exposure of embryos to 2,4-D. Am. J. Bot. 91(11):1743-1756.

Reddy, P. and S. Reddy. 1983. Scope for Production rise. The Hindu Survey of Indian Agriculture.

Robacker, C. 1993. Somatic embryogenesis and plant regeneration from Muscadine Grape leaf explants. Hortsci. 28(1):53-55.

Santecchia, C, A. Escalante, M. Gattuso, M. Zacchino, A. Gutierrez Ravelo, F. Delle Monache, G. Sierra. 2002. *Phytolacca tetrámera*, una fuente de saponinas triterpenoides. Revista Latinoamericana de Química. Suplemento especial nº 28, 246-247. México.

Stamp, J. A. and G. G. Henshaw. 1987. Somatic embryogenesis from clonal leaf tissues of cassava. Ann. Bot. 59(4):445-450.

Thorpe, T. A. 1995. *In vitro* Embryogenesis in Plants. Springer, 558 páginas.

Tisserat, B., E. Esan and T. Murashige. 1979. Somatic embryogenesis in angiosperms. Hort. Rev. 1:1-78.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15(3):473-497.

Ved, D. K. 1997. Trade in medicinal plants – the state of our ignorance. Amruth 1:2-8.

Devaraju, V. S. and K. Jaganmohan Reddy. 2013. Study of somatic embryogenesis in leaf explants of *Sapindus emarginatus*. Agric. Forest. Fish. 2(1):33-37.

Zacchino, S. 2000. Compuestos Antifúngicos naturales con actividad antifúngica promisoria. Farmacognosia, Facultad de Ciencias Bioquímicas y Farm. Universidad Nacional de Rosario. J. Ethnopharm. 1:29-34.