ΦYTON
REVISTA INTERNACIONAL DE BOTÁNICA EXPERIMENTAL
INTERNATIONAL JOURNAL OF EXPERIMENTAL BOTANY
FUNDACION ROMULO RAGGIO
Gaspar Campos 861, 1638 Vicente López (BA), Argentina
www.revistaphyton.fund-romuloraggio.org.ar

Plant regeneration of Mullein Nightshade (*Solanum donianum* Walp.) from leaf explants

Regeneración de plantas de Mullein Nightshade (*Solanum donianum* Walp.) a partir de explantes de hoja

O'Connor-Sánchez[1] A, AV Domínguez-May[1], MA Keb-Llanes[1], YJ Peña-Ramírez[2], VA Herrera-Valencia[1]

Abstract. A reliable protocol for plant regeneration of Mullein Nightshade (*Solanum donianum* Walp.) was developed from *in vitro* leaf explants. They were cultured on Murashige and Skoog semisolid medium, supplemented with several combinations of zeatin riboside and 1-naphthaleneacetic acid for shoot regeneration. The formulation that originated most shoots per explant was 8.5 μM zeatin riboside in absence of 1-naphthalene acetic acid. Explants with shoots were transferred to Murashige and Skoog medium, with half the normal salt concentration and without plant growth regulators for elongation. Elongated shoots were individualized, and they rooted readily in half-strength Murashige and Skoog medium without plant growth regulators. The regenerated plants, which were transferred to soil in a greenhouse, followed a similar phenological pattern to plants grown from seed.

Abbreviations: *BAP*: 6-benzyl amino purine; *MS*: Murashige and Skoog medium; *NAA*: 1-naphthalene acetic acid; *ZR*: zeatin riboside.

Keywords: Morphogenesis; Organogenesis; Drought-tolerant species; Zeatin riboside.

Resumen. Se desarrolló un protocolo confiable para la regeneración *in vitro* de plantas de Mullein Nightshade (*Solanum donianum* Walp.) a partir de explantes de hojas. Para la regeneración de brotes, los explantes fueron cultivados en medio semisólido Murashige y Skoog complementado con varias combinaciones de ribósido de zeatina y ácido 1-naftalén acético. La formulación que originó más brotes fue 8,5 μM ribósido de zeatina en ausencia de ácido 1-naftalenacético. Para la elongación, los explantes con brotes fueron transferidos a medio Murashige y Skoog sin reguladores de crecimiento vegetal. Los brotes elongados fueron individualizados y enraizados eficientemente en medio Murashige y Skoog con la mitad de la concentración normal de sales y sin reguladores de crecimiento vegetal. Las plantas regeneradas transferidas a suelo en el invernadero siguieron un patrón fenológico similar al de las plantas derivadas de semillas.

Abreviaturas: *BAP*: 6-bencil amino purina, *MS*: medio Murashige y Skoog; *NAA*: ácido 1-naftalén acético; *ZR*: ribósido de zeatina.

Palabras clave: Morfogénesis; Organogénesis; Especie tolerante a sequía; Ribósido de zeatina.

[1] Centro de Investigación Científica de Yucatán, A.C. Unidad de Biotecnología. Calle 43 No. 130, Col. Chuburná de Hidalgo. C.P. 97200, Mérida, Yucatán, México.
[2] Instituto Tecnológico Superior de Acayucan. Unidad de Investigación en Biotecnología Vegetal. Carretera Costera del Golfo Km 216.4, Col. Agrícola Michapa. C. P. 96100, Acayucan, Veracruz, México.
Address Correspondence to: Aileen O'Connor-Sánchez. Centro de Investigación Científica de Yucatán, A.C. Unidad de Biotecnología. Calle 43 No. 130, Col. Chuburná de Hidalgo. C.P. 97200, Mérida, Yucatán, México.
Phone: +52(999) 942 83 30 ext 205. Fax: +52(999) 981 39 00. *e-mail*: aileen@cicy.mx
Recibido / Received 7.IX.2009. Aceptado / Accepted 18.XI.2009.

INTRODUCTION

In worldwide economic terms, Solanaceae is the third most important plant family, and the most valuable among the vegetable crops. It contains not only several major food plants, but also fruits, spices, and stimulants, as well as plants with chemical compounds which are important in medicine, pharmacology, and drug therapy (Hawkes, 1999). Evolution, and the exceptionally high level of conservation of genome organization at the macro and micro levels in the Solanaceae family, makes it a unique subject for exploring the basis of phenotypic diversity and adaptation to natural and agricultural environments (The International Solanaceae Project, http://sgn.cornell.edu/solanaceae-project/).

With about 1400 species, *Solanum* is the largest genus in the Solanaceae, and one of the largest genera of flowering plants. *Solanum* species are adapted to some of the most diverse and extreme habitats on earth, from some of the wettest forests in the world to the driest deserts, and are found throughout a huge altitudinal range, from sea level to over 4500 m. The genus *Solanum* is of worldwide economic importance, including some major crop species such as potato (*S. tuberosum*), tomato (*S. lycopersicum*), and eggplant (*S. melongena*). There are also a number of species cultivated on a smaller scale (1) for their edible fruits, tubers, or leaves, and (2) as sources of medicinally valuable alkaloids. Some examples of less well known fruit crops include the tree tomato (*S. betaceum*), naranjilla (*S. quitoense*), and pepino (*S. muricatum*) from the "New world", and the scarlet and gboma eggplants (*S. aethiopicum* and *S. macrocarpon*, respectively) from the "Old world" (Natural History Museum, http://www.nhm.ac.uk/research-curation/research/projects/solanaceaesource/solanum/).

The potential use of wild species of *Solanum* for the genetic improvement of domesticated species through modern gene technology is a promising field of research. Nevertheless, to have the possibility to do this, it is first necessary to find out and study the genes involved in the interesting wild plant properties. *Solanum donianum* Walp., also called *Solanum blodgettii* (Gentry et al., 1974), is a very poorly studied perennial shrub species growing wild on the dunes of the northern coast of Yucatán, Mexico. Its presence has also been reported in Honduras, in the south of Florida and in the Bahamas. It grows on the sand of the beach, near to the mangrove zone, and is able to tolerate drought, high insolation, temperatures above 40 °C, and flooding by sea water. To perform studies on genes involved in its capability to tolerate these conditions, it would be useful to generate transgenic plants; to achieve this, an efficient plant regeneration system is a prerequisite. There are no reports for regeneration of *S. donianum.* However, given their (1) worldwide economic importance, (2) value in medicine, and (3) tolerance to various biotic and abiotic stresses, several closely related *Solanum* species have been regenerated *in vitro*. Some recent examples are *S. trilobatum* L. (Chakravarthi-Dhavala, et al., 2009), *S. sessiliflorum* (Schuelter, 2009), *S. dulcamara* L. (Mutlu & Turker, 2008), *S. phureja* (Diazgranados & Chaparro, 2007), *S. virginianum* L. (Borgato et al., 2007), *S. aethiopicum,* and *S. macrocarpon* (Gisbert et al., 2006). In the present work, we report a reliable method for direct shoot regeneration of *Solanum donianum* Walp. from *in vitro* leaf explants.

MATERIALS AND METHODS

Plant material. Seeds of *S. donianum* were handpicked from fruits collected from morphologically identified wild plants growing on the north coast of Yucatán, Mexico (21° 18' 48.58" N; 89° 21' 14.37" W), in a location named Xcambo. These seeds were transferred to the laboratory, washed in sterile water, and surface-sterilized during 15 min by dipping them in 10% (v/v) commercial bleach [*Clorox* (6% free chlorine)] solution containing 1 μL/L of polyoxyethylene sorbitan monolaurate (Tween 20®). This was followed by rinsing seeds five times with sterile water. The sterilized seeds were blot-dried with sterile paper towels, and germinated in Magenta vessels containing 40 mL of semisolid basal Murashige and Skoog (MS) medium (Murashige & Skoog, 1962). It was (1) supplemented with 58.5 mM sucrose, and (2) solidified with 2 g/L Gel-Rite®, pH 5.7. All cultures were incubated in a growth chamber at 25 ± 2 °C with a 16 h light / 8 h dark photoperiod (cool-white fluorescent lights, 60 μmol/m²/s) during 3 weeks.

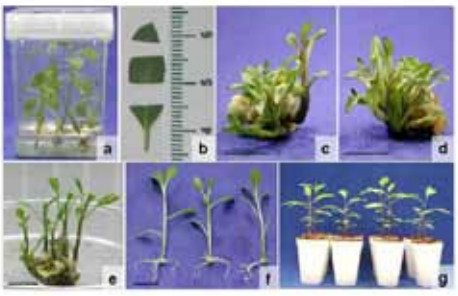
Micropropagation from nodal axillary buds. Starting from a population of seedlings of *S. donianum* (coming from *in vitro* germination), the most vigorous and fastest growing plant was selected. It was dissected in 9-mm-long nodal stem segments, each of them containing at least one axillary bud. These segments were placed vertically in Magenta vessels containing 40 mL of basal MS medium to induce bud proliferation. This medium was supplemented with 87.6 mM sucrose, and solidified with 2 g/L Gel-Rite®, pH 5.7. Subsequently, shoots derived from buds were employed as a source of new nodal stem segments for further micropropagation rounds.

Shoot regeneration. Fully expanded leaves from 6-week-old micropropagated plants (Fig 1a) were excised, and 2-3 mm length was cut off and discarded from both the distal and proximal ends (Fig 1b). Leaf explants were placed with the adaxial side upwards in plastic 1 × 9 cm (height × diameter) Petri dishes containing basal MS medium solidified with 2 g/L Gel-Rite®, pH 5.7. Basal medium was supplemented with 87.6 mM sucrose, and 30 different combinations of the plant growth regulators (PGR): (1) zeatin riboside (ZR) (0.0, 1.4, 2.8, 5.7, 8.5, and 14.2 μM) and (2) naphthalene acetic acid (NAA) (0.0, 0.11, 0.27, 0.54, and 1.08 μM); or (3) 8.5 μM benzyl amino purine (BAP) in absence of NAA. During the first week, cultures were kept in the dark, and afterwards under a 16 h light / 8 h dark photoperiod (cool-white fluorescent

lights, 60 μmol/m²/s). The results represent the average of 6 replicates repeated twice.

Fig 1. Steps in plant regeneration from leaf explants of *Solanum donianum*. (a) Plants propagated via germination of nodal stem segments after 6 weeks in propagation medium; (b) leaf explants prepared for regeneration; (c and d) regenerated shoots after 4 weeks in regeneration medium; (e) elongated shoots after 2 weeks in elongation medium, (f) rooted shoots after 4 weeks in rooting medium; (g) regenerated plants growing in a glasshouse.
Scale bar: 1 cm.

Fig 1. Etapas en la regeneración de plantas a partir de explantes de hoja de *Solanum donianum*. (a) Plantas propagadas mediante la germinación de segmentos nodales después de 6 semanas en medio de propagación; (b) explantes de hoja preparados para la regeneración; (c y d) brotes regenerados después de 4 semanas en medio de regeneración; (e) brotes elongados después de 2 semanas en medio de elongación, (f) brotes enraizados después de 4 semanas en medio de enraizamiento; (g) plantas regeneradas que crecieron en un invernadero.
Barra de escala: 1 cm.



Shoot elongation. After 28 days in shoot-induction medium, explants with shoots were transferred to Magenta vessels containing 40 mL of MS medium solidified with 2 g/L Gel-Rite®, pH 5.7, supplemented with 87.6 mM sucrose, without PGR. Only shoots longer than 10 mm were considered for evaluation, which were transferred to rooting medium.

Rooting. After 1-2 weeks in the elongation medium, regenerated shoots were excised and transferred for rooting to Magenta vessels containing 60 mL of half-strength MS basal medium supplemented with 58.5 mM sucrose, solidified with 2 g/L Gel-Rite®, pH 5.7.

Glasshouse cultivation. After 4 weeks, well rooted plants were washed with tap water and transferred to plastic cups containing soil (obtained from a regional supplier), agrolite (*Dicamex*, supplied by Dicalite de México S.A. de C.V.), and peat-moss (Premier Horticulture Inc.) in a proportion of 1:1:1. These plants were irrigated with water under glasshouse conditions. Each cup was covered with a plastic bag, and plants were hardened for 10 days by gradually reducing the humidity by making holes in the bag. Plants of *S. donianum* derived from seeds germinated *in vitro* were treated with the same procedure and cultured under similar conditions to be used as controls.

Experimental design and statistical analysis. All experiments were repeated a minimum of three times (except the soil establishment experiment) following a completely randomized block design. Data were analyzed using standard ANOVA procedures. Differences between means were calculated using the Fisher´s least significant different test (LSD) with assistance of the Statistica® Software (StatSoft, Tulsa OK).

RESULTS AND DISCUSSION

Shoots propagation from nodal stem segments. A micropropagation system from nodal axillary buds of a *S. donianum* clonal line was established to generate enough plant material for regeneration experiments. After four micropropagation rounds, 165 nodal stem segments were generated. One hundred and fifty three of them (92.7%) developed into rooted plants with leaves after 6 weeks of culture (Fig 1a). This micropropagation system was very efficient; however, it would be unsuitable for further transformation experiments because of the multicellular origin of the axillary buds, which has been correlated to the production of chimeric plants. In contrast, organogenic shoot regeneration has been reported to be mainly a single-cell originated event, and therefore useful to generate solid transgenic plants.

Organogenic shoot induction and elongation using ZR and NAA. A matrix experiment was designed to evaluate the effect of different combinations of ZR and NAA to induce shoot organogenic regeneration from leaf explants. After 4 weeks, explants on shoot induction media with concentrations above 2.8 μM ZR and below 0.27 μM NAA formed several primordial shoots (Fig 1c-d and Fig 2). Shoots were formed mainly at the cut edges in contact with the medium, without callus formation (data not shown). In the media containing ZR at less than 2.8 μM, there was very slight shoot formation, and at concentrations above 0.11 μM NAA there was only formation of roots or compact, necrotic, dark calli. Table 1 shows the number of well developed elongated shoots derived from each induction media after the elongation step (Fig 1e). The medium that produced the highest frequency of shoot induction contained 8.5 μM ZR without NAA, followed by the media that contained 5.7 μM ZR without NAA.

When leaves of plants derived from seeds (n=5) were treated with the same procedure, using medium containing 8.5 μM ZR and no NAA for shoot induction, the means showed no significant differences ($p \leq 0.05$) from those already described (data not shown). This implies that our methodology is reliable and does not depend on a specific cloned line.

Organogenic shoot regeneration using BAP. As ZR is an expensive PGR, BAP was tried as a substitute. When induction media containing equimolar concentrations of ZR and BAP were compared, those with BAP formed significantly fewer and smaller shoots (Fig. 3), with more callus formation. A mean of 6.0 (±1.7) shoots per explant were obtained with 8.5 μM ZR in absence of NAA. At the same time, only 1.0 (±0.6) shoots per explant were regenerated with 8.5 μM BAP in absence of NAA.

Rooting of elongated shoots. Twenty elongated shoots derived from medium with 8.5 µM ZR and no NAA were excised and transferred to rooting medium. After 3 weeks, 85% (± 2.3%) of them formed roots (Fig 1f). This was repeated twice with similar results.

Table 1. Mean number of shoots per leaf explant of *Solanum donianum* regenerated on the different combinations of NAA and ZR. Each value is the average of 6 replicates repeated 3 times. Different letters indicate significant differences at $p \leq 0.05$.
Tabla 1. Número promedio de brotes por explante de hoja de *Solanum donianum* regenerados en las distintas combinaciones de NAA y ZR. Cada valor es el promedio de 6 réplicas repetidas 3 veces. Letras diferentes indican diferencias significativas a $p \leq 0,05$.

NAA (µM)	*ZR (µM)*	Number of shoots / explant *($\bar{x}$ ± SD)*
0.00	0.0	0.00 ± 0.00 a
0.00	1.4	1.00 ± 0.50 abc
0.00	2.8	2.00 ± 0.87 c
0.00	5.7	4.67 ± 1.59 d
0.00	8.5	6.17 ± 1.74 e
0.00	14.2	4.50 ± 1.15 d
0.11	0.0	0.00 ± 0.00 a
0.11	1.4	0.00 ± 0.00 a
0.11	2.8	0.17 ± 0.17 a
0.11	5.7	1.83 ± 0.83 bc
0.11	8.5	1.83 ± 0.67 bc
0.11	14.2	1.00 ± 0.50 abc
0.27	0.0	0.00 ± 0.00 a
0.27	1.4	0.00 ± 0.00 a
0.27	2.8	0.67 ± 0.44 abc
0.27	5.7	0.67 ± 0.17 abc
0.27	8.5	1.83 ± 0.88 bc
0.27	14.2	1.33 ± 0.17 abc
0.54	0.0	0.00 ± 0.00 a
0.54	1.4	0.00 ± 0.00 a
0.54	2.8	0.17 ± 0.17 a
0.54	5.7	0.33 ± 0.33 a
0.54	8.5	0.50 ± 0.29 ab
0.54	14.2	0.33 ± 0.17 a
1.08	0.0	0.00 ± 0.00 a
1.08	1.4	0.00 ± 0.00 a
1.08	2.8	0.00 ± 0.00 a
1.08	5.7	0.00 ± 0.00 a
1.08	8.5	0.00 ± 0.00 a
1.08	14.2	0.00 ± 0.00 a

Fig. 2. Effect of different combinations of ZR and NAA on shoot induction of *S. donianum*. Leaf explants after 4 weeks in the different tested regeneration media.
Fig. 2. Efecto de diferentes combinaciones de ZR y NAA en la inducción de brotes de *S. donianum*. Explantes foliares después de 4 semanas en los distintos medios de regeneración probados.

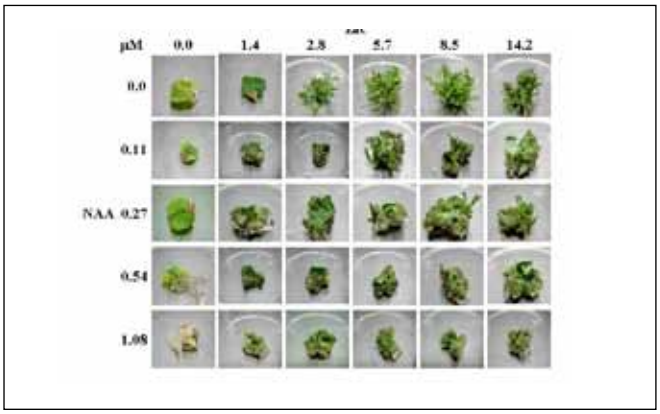
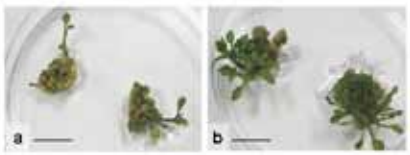


Fig. 3. Effect of BAP (a) or ZR (b), at the same molar concentration (5.7 µM), on shoot induction of *S. donianum* leaf explants. Scale bar: 1 cm.
Fig. 3. Efecto de la BAP (a) o del ZR (b), a la misma concentración molar (5.7 µM), sobre la inducción de brotes en explantes de hoja de *S. donianum*. Barra de escala: 1 cm.



Glasshouse cultivation. Three groups of 17 rooted plants each were transferred to glasshouse conditions. One hundred per cent of them were successfully acclimatized (Fig 1g). They developed a similar phenological pattern (growth rate, rate of leaf appearance, flowering, and final plant size) to plants grown from seeds.

In agreement with studies reported on other *Solanum* species, our results showed that leaf explants usually had a good response for *in vitro* shoot formation, and that the number of shoots per explant was quite similar for most of them, even when using different methodologies. Zeatin riboside is not commonly used; however, it could increase the efficiency of regeneration and the quality of the regenerated shoots. It is advisable to include it when trying to establish a protocol for regeneration of a *Solanum* species that has not been previously reported.

We were able to develop a reliable method for organogenic shoot regeneration from *in vitro* leaf explants of *S. donianum*. This is very important because it opens the way to either perform further experiments for obtaining transgenic plants of this species or making developments on some other biotechnological approaches.

ACKNOWLEDGEMENTS

This work was funded by CICY institutional resources. The authors are grateful to Filogonio May-Pat, Francisco Chi-May and Ivón M Ramírez-Morillo for plant collection and taxonomic identification, to Ileana C Borges Argaez for giving ideas and technical assistance, and to Reynaldo C Pless for reviewing the manuscript.

REFERENCES

Borgato, L., F. Pisani & A. Furini (2007). Plant regeneration from leaf protoplasts of *Solanum virginianum* L. (Solanaceae). *Plant Cell Tiss Org Cult* 88: 247-252.

Chakravarthi-Dhavala, V.N., R.D. Tejeswara, V.R. Yechuri & K. Prabavathi (2009). Effect of explant age, hormones on somatic embryogenesis and production of multiple shoots from cotyledonary leaf explants of *Solanum trilobatum L. African Journal of Biotechnology* 8: 630-634.

Diazgranados, C. & A. Chaparro (2007). Development of a regeneration system in Papa criolla (*Solanum phureja* Juz et Buck) var. Yema de huevo clon 1. *Agronomía Colombiana* 25: 7-15.

Gentry, J.L. Jr. & P.C. Standley (1974). Flora de Guatemala. Ed. Fieldiana. 107-108 p.

Gisbert, C., J. Prohens & F. Nuez (2006). Efficient regeneration in two potential new crops for subtropical climates, the scarlet (*Solanum aethiopicum*) and gboma (*S. macrocarpon*) eggplants. *New Zealand Journal of Crop and Horticultural Science* 34: 55-62.

Hawkes, J.G. (1999). The economic importance of the family Solanaceae. Solanaceae IV (Advances in Biology and Utilization). Published by The Royal Botanical Gardens, Kew: 1. ISBN 1 900347 90 3.

Mutlu, E.C. & A.U. Turker (2008). Efficient plant regeneration of bittersweet (*Solanum dulcamara* L.) a medicinal plant. *Acta Soc Bota Pol* 77: 275-280.

Schuelter, A.R., A.K. Grunvald, A.T. Amaral Jr., C.L. da Luz, C.L. Luz, L.M. Gonçalves, S. Stefanello & C.A. Scapim (2009). *In vitro* regeneration of cocona (*Solanum sessiliflorum*, Solanaceae) cultivars for commercial production. *Genetics and Molecular Research* 8: 963-75.