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Full Length Research Paper

Efficient plant regeneration from leaf explants of *Solanum americanum*

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A very efficient system for direct plant regeneration from *in vitro*–derived leaf explants of *Solanum americanum* was developed. *S. americanum* is a tropical plant with important medical properties. The *in vitro* procedure that was established consists of (i) induction of shoots from leaf tissue, (ii) elongation of shoots, and (iii) rooting of plantlets. The induction of shoots was achieved on Murashige and Skoog solid medium supplemented with different combinations of zeatin riboside and 1–naphthalene acetic acid or 6–benzylaminopurine and 1–naphthalene acetic acid. The best combination for plant regeneration was MS with 5.7 µM zeatin riboside and 0.11 µM 1–naphthalene acetic acid. In the second step, the shoot clumps were transferred to MS basal medium without plant growth regulators, resulting in the differentiation of most of the shoot initials into well developed shoots. In the third stage, plantlets were efficiently rooted on half-strength MS basal medium supplemented with 58.5 mM sucrose. The rooted plants were established in soil with a 100% success rate. This system can be useful to perform further experiments to obtain transgenic plants of this species as well as for other biotechnological approaches.

Key words: American nightshade, organogenesis, shoots regeneration, *in vitro* culture, zeatin riboside, 1–naphthalene acetic acid, medicinal plant species.

INTRODUCTION

The genus *Solanum* is of worldwide economic importance, including major crop species such as potato (*S. tuberosum*), tomato (*S. lycopersicum*), and eggplant (*S. melongena*). *Solanum americanum* is a diploid wild herbaceous flowering plant native to the Americas that is widespread in tropical and subtropical areas throughout the world (Schilling et al., 1992). Its common name in English is American nightshade; in Spanish it is known as "Hierba Mora". It grows up to 1-1.5 m tall and is an annual or short-lived perennial plant. As for its use in traditional medicine, it has been reported in Hawaii for asthma (Hope et al., 1993), in Puerto Rico for gastrointestinal disorders (Hernández et al., 1984), in Guatemala for dermatophytoses and for protozoal infections (Cáceres et al., 1991; 1998), as a molluscicidal (Meléndez and Capriles, 2002), and in Brazil for cutaneous leishmaniasis (França et al., 1996).

To study genes involved in some of these medicinal properties it would be useful to have a method for genetic transformation, for which an efficient plant regeneration system is required. The objective of this work was to establish a system for *in vitro* regeneration of *S. americanum* that allowed its further transformation. Work on *S. americanum* transgenic plants has recently been reported (Sin et al., 2006); however, the conditions used to regenerate the plants were not described.

Because of the convenience of using *in vitro* systems to study their properties, plant regeneration has been reported in recent years for several *Solanum* species, like *S. aethiopicum* and *S. macrocarpon* (Gisbert et al., 2006),

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Abbreviations: BAP, 6-Benzylaminopurine; **MS,** murashige and Skoog medium; **NAA**, 1-naphthalene acetic acid; **ZR,** zeatin riboside.

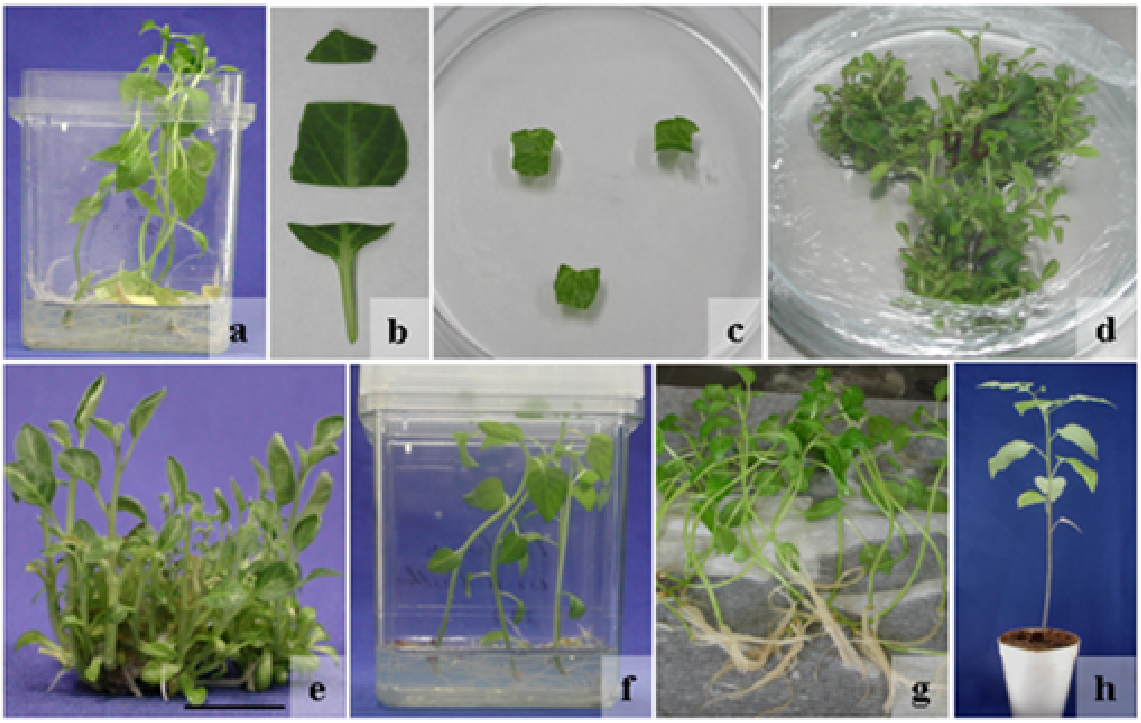

Figure 1. Several steps in plant regeneration from leaf explants of *S. americanum*. (a) Plants propagated by nodal stem segments, after 3 weeks in propagation medium; (b and c) leaf explants prepared for regeneration; (d) regenerated shoots after 3 - 4 weeks in regeneration medium; (e) elongated shoots after 1 week in elongation medium, (f) rooted shoots after 3 weeks in rooting medium; (g) regenerated plants ready to be transferred to soil; and (h) regenerated plat growing in a glasshouse. Scale bar: 1 cm.

S. betacea (Patiño-Torres et al., 2007), *S. dulcamara* L. (Mutlu and Turker, 2008), *S. nigrum (*Xu et al., 2009), *S. phureja* (Diazgranados and Chaparro, 2007), *S. surattense* (Pawar et al., 2002), *S. trilobatum L* (Alagumanian et al., 2004 and Chakravarthi–Dhavala et al., 2009), *S. sessiliflorum* (Medina–Rivas et al., 2008), and *S. virginianum* L. (Borgato et al., 2007). Recently, in our group, we established a protocol for *S. donianum* regeneration (O'Connor-Sánchez et al. in press). Nevertheless, it does not exist as a common methodology that can be applied to regenerate any *Solanum* species, and therefore it is necessary to look for a suitable one in each new case. In the present work, we report an efficient method for direct shoot organogenesis from leaf explants of *S. americanum*.

MATERIALS AND METHODS

Plant material

Seeds of *S. americanum* were obtained 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). Seeds were handpicked from fruits, washed in sterile water, and surface-sterilized by dipping in 10% (v/v) commercial bleach (*Clorox* [6% free chlorine]) solution containing 100 µL of Tween 20® (polyoxyethylene sorbitan monolaurate) (all chemicals were purchased from Sigma, St. Louis MO) per 100 mL of solution for 15 min, followed by rinsing five times with sterile water. The sterilized seeds were blot–dried with sterile paper towels and germinated *in vitro* in Magenta® vessels containing 40 mL of basal Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) supplemented with 58.5 mM sucrose and solidified with 2 g·L^{-1} Gel-Rite®, pH 5.7. All cultures were incubated in a growth chamber at 25 ± 2 ℃ with a 16 h light / 8 h dark photoperiod (cool-white fluorescent lights at 60 □mol·m^{-2}·s^{-1} photon flux).

Propagation by adventitious shoots from nodal stem segments

The most vigorous and fast growing plant from a population of *in vitro* germinated seedlings of *S. americanum*, was selected and 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 supplemented with 87.6 mM sucrose and solidified with 2 g·L^{-1} Gel-Rite®, pH 5.7 to induce bud proliferation. Subsequently, shoots derived from buds were employed as a source of new nodal stem segments for further micropropagation rounds.

Shoot induction

Fully expanded leaves from 21–day–old plants (derived from nodal segments, Figure 1a) were excised, and, from both the distal and proximal ends, 2 – 3 mm lengths were cut off and discarded (Figure 1b). Leaf explants were placed adaxial side upwards (Figure 1c) in plastic 1 × 9 cm (height × diameter) Petri dishes containing 25 mL of basal MS medium solidified with 2 gL^{-1} Gel-Rite®, pH 5.7,

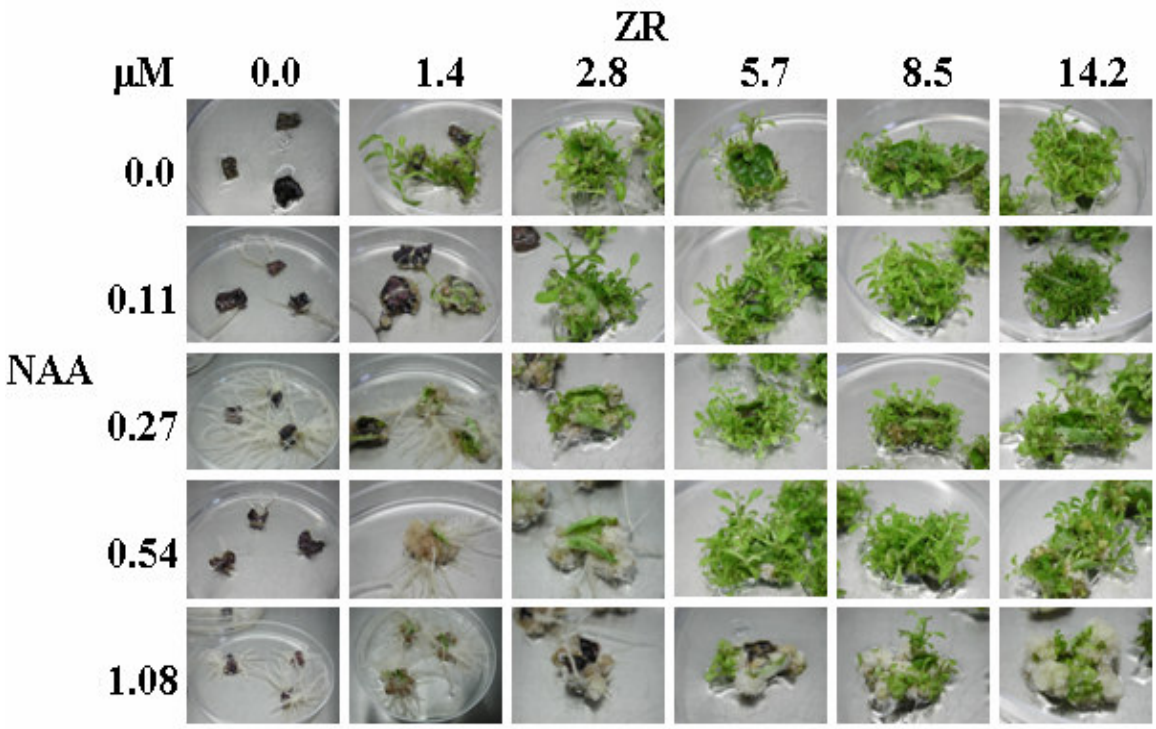

Figure 2. Effect of different combinations of ZR and NAA on shoot induction of *S. americanum*. Leaf explants after 3 weeks in the different regeneration media

supplemented with 87.6 mM sucrose and 30 different combinations of the plant growth regulators (PGR): ZR (0.0, 1.4, 2.8, 5.7, 8.5, and 14.2 µM) and NAA (0.0, 0.11, 0.27, 0.54, and 1.08 µM) or 5.7 µM BAP and 0.11 µM NAA.

Shoot elongation

After 21 days in shoot induction medium, explants with shoots were transferred to Magenta vessels containing MS medium solidified with 2 g·L^{-1} Gel-Rite®, pH 5.7, supplemented with 87.6 mM sucrose, without growth regulators.

Rooting

After one week in elongation medium, shoots were excised and transferred for rooting to Magenta vessels containing half-strength MS basal medium supplemented with 87.6 mM sucrose, solidified with 2 g·L^{-1} Gel-Rite®, pH 5.7. Only shoots longer than 10 mm were considered for evaluation and transferred to rooting medium.

Glasshouse cultivation

After 3 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, irrigated with water, under glasshouse conditions. Each cup was covered with a plastic bag and the plants were hardened for 10 days by gradually reducing the humidity by making holes in the bag. Seed derived plants were germinated *in vitro* and transferred to the glasshouse following the same procedure 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. The data were analyzed using standard ANOVA procedures. Differences between means were calculated using the Fisher's least significant difference test (LSD) with assistance of the Statistica® software package v 6.0 (StatSoft, Tulsa OK).

RESULTS AND DISCUSSION

Propagation by adventitious shoots from nodal stem segments

200 nodal stem segments were derived from the plant chosen for propagation. One hundred and ninety of them (95%) developed into rooted plants with leaves after 21 days (Figure 1a).

Shoot induction and elongation using ZR and NAA

After 3- weeks on shoot induction media, most explants had formed a large number of shoots (Figures 1d and 2). Shoots were formed predominantly at the cut edges in contact with the medium, without callus formation (data not shown). In the media without ZR, there was no shoot formation, and at concentrations above 0.11 µM NAA, there was formation of roots or of compact necrotic

Table 1. Mean number of shoots *per* leaf explant of *S. americanum* regenerated on the different combinations of NAA and ZR tested. Each value is the average of 6 replicates repeated 3 times.

NAA (µM)	ZR (µM)	Number of shoots / explants ($\bar{x}$ ± SD)
0.00	0.0	00.0 ± 0.0 [a]
0.00	1.4	10.7 ± 2.1 [efg]
0.00	2.8	20.0 ± 1.8 [k]
0.00	5.7	16.0 ± 2.4 [ij]
0.00	8.5	11.9 ± 1.4 [fg]
0.00	14.2	08.2 ± 2.8 [de]
0.11	0.0	00.0 ± 0.0 [a]
0.11	1.4	09.0 ± 2.0 [ef]
0.11	2.8	07.9 ± 2.1 [cde]
0.11	5.7	12.9 ± 4.9 [gh]
0.11	8.5	15.3 ± 2.9 [hi]
0.11	14.2	18.6 ± 3.8 [jk]
0.27	0.0	00.0 ± 0.0 [a]
0.27	1.4	00.0 ± 0.0 [a]
0.27	2.8	00.1 ± 0.1 [a]
0.27	5.7	00.8 ± 0.6 [a]
0.27	8.5	11.9 ± 1.9 [fg]
0.27	14.2	07.7 ± 2.5 [cde]
0.54	0.0	00.0 ± 0.0 [a]
0.54	1.4	00.0 ± 0.0 [a]
0.54	2.8	00.0 ± 0.0 [a]
0.54	5.7	04.9 ± 1.5 [bc]
0.54	8.5	05.6 ± 1.5 [cd]
0.54	14.2	10.7 ± 2.1 [efg]
1.08	0.0	00.0 ± 0.0 [a]
1.08	1.4	00.0 ± 0.0 [a]
1.08	2.8	00.0 ± 0.0 [a]
1.08	5.7	00.1 ± 0.1 [a]
1.08	8.5	02.2 ± 1.1 [ab]
1.08	14.2	01.7 ± 0.7 [a]

Letters next to the values correspond to the significance level by Fisher's LSD test at $p \leq 0.05$.

dark calli. The best shoot formation was on media containing ZR at concentrations above 2.8 µM, and NAA at below 0.54 µM, where several dozen shoots per explant were obtained. Table 1 shows the number of well developed elongated shoots derived from each induction media after the elongation step (Figure 1e). The media that produced most shoots per explant contained 2.8 µM ZR and no NAA. Nevertheless, shoots that arose on medium with 5.7 µM ZR and 0.11 µM NAA were harder, bigger, and still were produced in large numbers. Considering quality and quantity together, this was clearly the best media for shoot production.

When leaves of plants derived from seeds of 5 different wild plants were treated with the same procedure, using medium containing 5.7 µM ZR and 0.11 µM NAA for shoot induction, the results showed no significant difference ($P \leq 0.05$) from those already described, showing that the methodology that was established does not depend on the cloned line (data not shown).

Shoot induction using BAP and NAA

As ZR is an expensive growth regulator, BAP was tried as a substitute. As can be seen in Figure 3, when induction media with the same molar concentrations of ZR and BAP were compared, those with BAP formed significantly fewer shoots and showed vitrification and callus formation: with 5.7 µM ZR / 0.11 µM NAA a mean of 18.6 shoots per explant were formed, while with 5.7 µM BAP / 0.11 µM NAA only 7.4 shoots per explant were obtained.

Rooting

30 plantlets coming from medium 5.7 µM ZR and 0.11 µM NAA were excised and transferred to rooting medium. After 3 weeks, all of them formed roots (Figure 1f). This was repeated twice with the same results.

Glasshouse cultivation

Three batches of 30 rooted plants each (Figure 1g) were transferred to glasshouse conditions. All of them were successfully acclimatized (Figure 1h) and developed following a similar phenological pattern than seed derived plants.

When the number of regenerated plants per explant obtained in this work (up to 20) was compared with reports of some other *Solanum* species, where protocols considered as very efficient were developed using other methodologies, like *S. dulcamara* L. (Mutlu and Turker, 2008); *S. betaceae* (Patiño–Torres et al., 2007), *S. aethiopicum* and *S. macrocarpon* (Gisbert et al., 2006), and *S. trilobatum* L. (Alagumanian et al., 2004), it was clear that the method developed in this work for shoot regeneration from *in vitro* leaf explants of *S. americanum* produced a quite similar amount of shoots per explant, and therefore can be considered very efficient as well.

Additionally, as the system developed in this work was planed to be used for further transformation experiments, only the elongated shoots that were clearly independent from each other were cut and considered for evaluation (to prevent having more than one plant coming from the same transformation event in the future). Nevertheless, it is worth highlighting that, as can be seen in Figure 2, the number of shoots' primodia that arose in most induction media was very much higher than that in well formed shoots reported in Table 1, and therefore, if the shoot clumps stayed longer than one week in elongation medium,

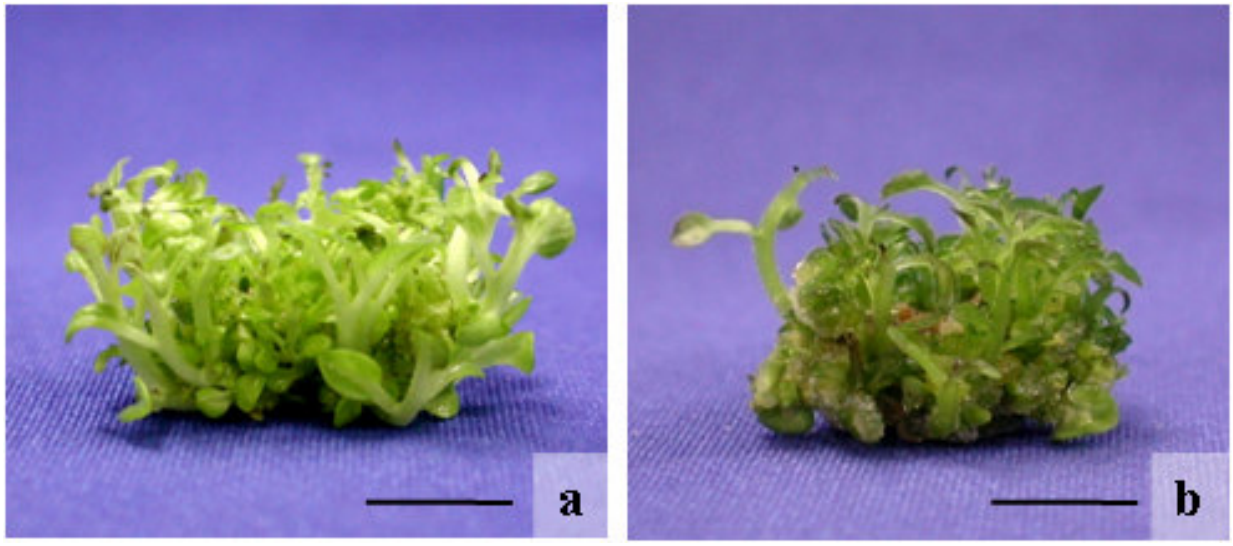

Figure 3. Effect of ZR (a) or BAP (b), at the same molar concentration (5.7 µM), on shoot induction of *S. americanum* leaf explants. Scale bar: 1 cm.

many more elongated shoots could be recuperated. This observation may be useful if the protocol is intended for some other applications, like plant micro-propagation or basic studies on plant regeneration.

On the other hand, in most reports zeatin riboside is not used, although possibly it could increase the efficiency of regeneration and the quality of the regenerated shoots in some cases. On the basis of the present work, it would be advisable to include it as a growth regulator when trying to establish a regeneration system for a new *Solanum* species, because although it is more expensive, it can lead to much better results.

Based on the results obtained in this work, it can be concluded that an efficient method for shoot regeneration from *in vitro* explants of *S. americanum* has been developed. It can be used to perform further experiments to obtain transgenic plants of this species as well as for other biotechnological or agricultural approaches.

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