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Regeneración de plantas via embriogénesis somática en *Phaseolus acutifolius* A. Gray

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ABSTRACT
Plant regeneration protocols are a requirement to develop plants transformation systems. The capacity of *Phaseolus acutifolius* A. Gray to regenerate plants from embryogenic callus formation was investigated. Two explants were used to form calli on a culture medium containing thidiazuron and indole-3-acetic acid. The embryonic axes showed better capacity than cotyledons to form embryos. Solar light was the most favourable to develop the *in vitro* plants. The 32% of somatic embryos with complete germination was achieved. These results indicate that somatic embryos formation and their germination in *Phaseolus acutifolius* A. Gray depended on the explant, culture medium and illumination conditions. *In vitro*-germinated plantlets were established in the greenhouse.

Key words: bean, callus formation, plant regeneration, tissue culture

RESUMEN
Para desarrollar un sistema de transformación genética un requisito fundamental es contar con un protocolo de regeneración de plantas. En el trabajo se investigó la capacidad de *Phaseolus acutifolius* para regenerar plantas a partir de callos con estructuras embriogénicas. Se utilizaron dos tipos de explantes para la formación de callos en un medio de cultivo que contenía thidiazuron y ácido indol-acético. Los ejes embrionarios mostraron una mejor capacidad que los cotiledones para la formación de embriones somáticos. La luz solar resultó ser la más favorable para el desarrollo de las plantas *in vitro.* En estas condiciones se alcanzó el 32% de los embriones somáticos con germinación completa. Estos resultados indicanron que la formación de embriones somáticos y su germinación en *Phaseolus acutifolius* dependieron del explante, el medio de cultivo y las condiciones de iluminación. Las plantas germinadas *in vitro*, pudieron ser aclimatizadas en el invernadero.

Palabras clave: cultivo de tejidos, formación de callos, frijol, regeneración de plantas

Abbreviations: BAP 6- Benzylaminopurine, TDZ Thidiazuron, IAA indole-3-acetic acid, GA_3 gibberellic acid, $AgNO_3$ silver nitrate

INTRODUCTION

Tepary bean (*Phaseolus acutifolius* A. Gray), one of the five cultivated species of the genus *Phaseolus*, is an annual legume adapted to arid and semi-arid regions (Zambre *et al*., 2005). There is an interest in *Phaseolus acutifolius* stems due to its value as a source of several desirable genetic traits, such as resistance to pests, diseases, high temperature and drought, which could be incorporated in *P. vulgaris* (Singh, 1999).

Phaseolus is still considered recalcitrant to *in vitro* culture (Varisai Mohamed *et al*., 2006, Kawapata *et al*., 2010). Although organogenesis had been reported using different explants (Veltcheva *et al*., 2005; Varisai Mohamed *et al*., 2006), *Phaseolus* species regenerated with limited success. Most of the efforts has been concentrated on *P. vulgaris* (Veltcheva *et al*., 2005; Delgado-Sánchez *et al*., 2006; Gatica Arias *et al*., 2010; Kawapata *et al*., 2010, Quintero-Jiménez *et al*., 2010), *P. aureus* (Malik and Saxena 1992), *P. coccineus* (Santalla *et al*., 1998), *P. polyanthus* (Zambre *et al*., 2001), *P. angularis* (Varisai Mohamed *et al*., ab), *P. lunatus* (Chidananda and Massimo, 2008) and *P. acutifolius* (Zambre *et al*., 1998).

On the other hand, the induction of somatic embryogenesis has been informed on common bean (Saunders *et al*., 1987), *P. cocineus* (Nagl *et al*., 1997), *P. coccineus* and *P. acutifolius* (Malik and Saxena, 1992). Although several combinations of growth regulators, explants and culture conditions were used to induce callus and cell suspensions to obtain somatic embryos, no plantlets were regenerated successfully. Somatic embryogenesis was described in *P. angularis* (Varisai Mohamed *et al*., 2006 b) and *P. vulgaris* (Kawapata *et al*., 2010).

Currently, plant regeneration via organogenesis through green nodular callus is the only way used to obtain *Agrobacterium*-mediated transgenic plants in *P. acutifolius* (Dillen *et al*., 1997; De Clercq *et al*., 2002; Zambre *et al*., 2005). A plant regeneration system via somatic embryogenesis for these purposes was not described in this specie.

The present research fucussed on the regeneration of plants from somatic embryos in Tepary bean, taking into account the potentialities of somatic embryogenesis for plants regeneration.

MATERIALS AND METHODS

Plant material

Seeds of *P. acutifolius* a Gray var. TB1 were disinfected and germinated *in vitro* as described before (Dillen *et al*., 1995).

Culture media

The basal medium (BM) contained Murashige and Skoog salts (Murashige and Skoog, 1962) and vitamins (Heinz and Mee, 1969) with 20 g l^{-1} sucrose, and 2 g l^{-1} Gelrite® (SIGMA). Medium pH was adjusted to 5.7 before adding gelling agent and autoclaving at 121ºC for 20 min. Culture medium was dispensed into 250 ml glass flasks (30 ml per flask).

Statistical analysis

For each experiment, 80 explants were used and each experiment was repeated four times to assess the reproducibility. A completely randomized design was used in all experiments. The variability among treatments to determine the level of nodular callus formation in the explants was achieved using Kruskal-Wallis test. For other indicators, analysis of variance and mean separation were carried out using Tukey´s Multiple Range Test and the significance was determinated at 5% level.

Callus formation

After 3 days of incubation under dark conditions, the seed coat was removed and the cotyledons were gently pulled apart from the embryonic axes. Both parts were cultured.

Four callus induction media (CIM) were used: CIM1: (BM with 0.05 mg l^{-1} IAA), CIM2 (BM with 0.05 mg l^{-1} TDZ and 0.05 mg l^{-1} IAA), CIM3: (BM with 0.1 mg l^{-1} TDZ and 0.05 mg l^{-1} IAA), CIM4 (BM with 0.2 mg l^{-1} TDZ and 0.05 mg l^{-1} IAA) and CIM5: (BM with 0.5 mg l^{-1} TDZ and 0.05 mg l^{-1} IAA).

Explants were incubated in darkness at 26±2ºC. All cultures were subcultured every 25 days. The meristematic tissues originated, following the first subculture, were cut out together with some of the original tissue.

The indicators evaluated during the callus formation stage were: callus coloration, consistency, number of explants with callus formation at 50 days of culture, level of nodular callus formation (Schumann *et al*., 1995) in the explants according to the scale described in table 1.

Somatic embryos induction

After two subcultures on CIM, the callus cultures were transferred twice to somatic embryos induction media (SEIM). These contained SEIM1(BM with 0.2 mg l^{-1} TDZ), SEIM2: (BM with 0.2 mg l^{-1} TDZ and 0.05 mg l^{-1} IAA), SEIM3: (BM with 0.2 mg l^{-1} TDZ and 0.2 mg l^{-1} IAA), SEIM4: (BM with 0.2 mg l^{-1} TDZ and 0.5 mg l^{-1} IAA), SEIM5: (BM with 0.2 mg l^{-1} TDZ and 1.0 mg l^{-1} IAA), SEIM6: (BM with 0.2 mg l^{-1} TDZ and 3 mg l^{-1} IAA), SEIM7: (BM with 0.2 mg l^{-1} TDZ and 5.0 mg l^{-1} IAA), and SEIM8: (BM with 0.2 mg l^{-1} TDZ and 7.0 mg l^{-1} IAA).

Explants were incubated in darkness at 26±2ºC. All cultures were subcultured every 25 days.

The indicators evaluated during the somatic embryos induction were: number of explants with somatic embryos formation (%) and explants area with somatic embryos formation.

Table 1. Scale for the evaluation of nodular callus formation level in *P. acutifolius* cv. TB1 explants.

Escale	Callus formation
Level - 1	Dead explants
Level - 2	Alive explants but without callus formation
Level - 3	Alive explants with small areas of nodular callus formation
Level - 4	Explants with 50% of nodular callus formation
Level - 5	Explants with 100% of nodular callus formation

Somatic embryos germination and convertion

The somatic embryos germination medium (SEGM) contained BM with 0.09 mg l^{-1} IAA, 2.0 mg l^{-1} Zeatin, 0.01 mg l^{-1} GA_3 and 100 mgl^{-1} myo-inositol. Somatic embryos were separated in clusters of few embryos. These were placed on SEGM for their germination. Five subcultures, every 25 days, were carried out.

Two treatments were conformed for somatic embryos germination and plantlet development. The first one, CC1: solar light, 12:12 h (light/dark) photoperiod with 48.0 - 62.5 µmol m^{-2} s^{-1} at 26±2ºC. The second, CC2: Fluorescent light with 62 - 68 µmol $m^{-2}s^{-1}$ at 24±2ºC and 16h light.

The number of somatic embryos, with partial or complete germination, was quantified in this stage. Number of leaves per plant and leaves coloration were also decribed.

Plant conversion

In vitro plants regenerated were transplanted into pots containing a zeolita and organic matter mixture (20:80) with basal nutrients (1.25 $kg.m^{-3}$ N-P-K 14:16:18 percentages). After month of transplantion, these were fertilized with urea. A second fertilization was carried out after the flowering induction. Irrigation was applied five times a day during 2 minutes each.

RESULTS

Callus formation

Explants in all the treatments maintained a greenish coloration and increased their volume during the first 10 days. Brown and compact callus formation with small whitish areas of dry appearance were observed after this time. In the cotyledons, calli were formed on the surface of the explants in contac with the culture medium (Figure 1 A), while in the embryonic axes these structures appeared in the bud area (Figure1 B).

Most embryonic axis explants tested produced brown callus. The same was obtained from a limited number of the tested cotyledon explants (80.5%). Calli were morphologically similar to the ones obtained from cotyledons or embryonic axes.

Embryonic axes reached the bigger levels of growth followed by the cotyledonal section in each treatment (Table 2). In both explants, the best culture medium for nodular callus formation (Figure 1C) was either with 0.20 or 0.50 $mg.l^{-1}$ TDZ (CIM4, CIM5).

Explants inoculated on media with all the concentrations produced calli except on TDZ-free medium. However, calli formation (%) was higher in the embryonic axes (Table 2).

Somatic embryos induction

Callus cultured on SEIM proliferated vigorously. At this stage, nodular callus was pooled for further subculturing. It continued proliferating into sectors of glossy nodular brown and white-yellow callus. Embryos-like structures were obtained on some calli during the second passage on SEIM (Figure1D).

The appearance of embryogenic masses was only observed in the nodular calli from embryonic axes when these were placed in the culture medium SEIM (Figure1E). The calli obtained from cotyledon explants were not able to form these structures.

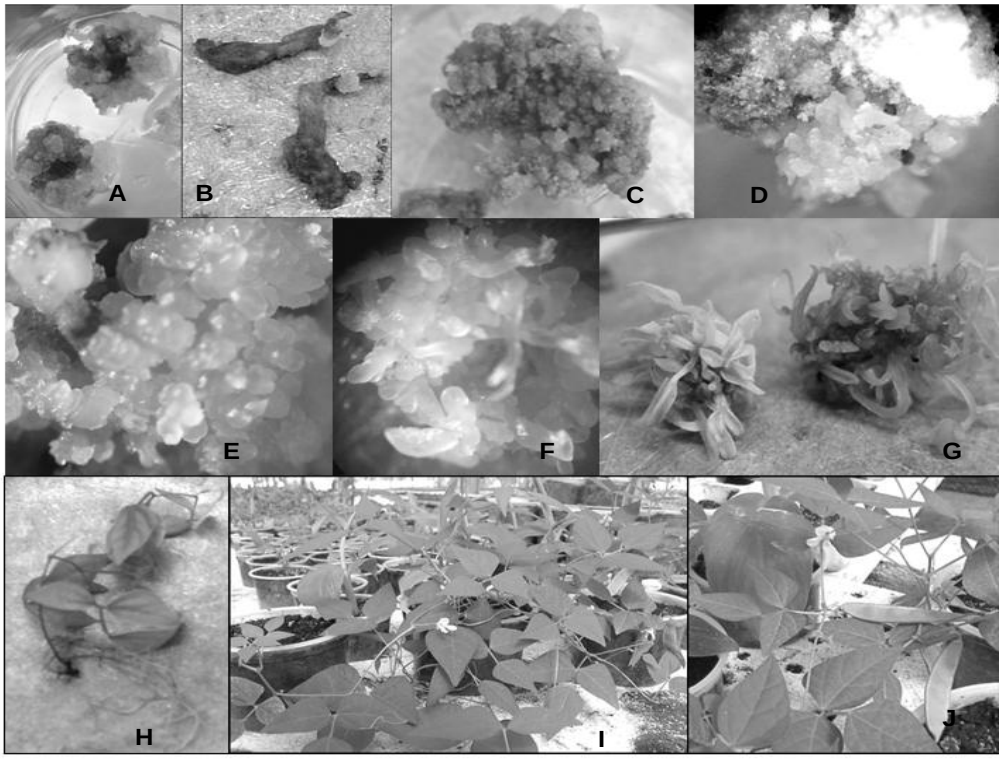

Figure 1. Plant regeneration from somatic embryos in *Phaseolus acutifolius* A. Gray var. TB1 (A) Brown coloration callus formed starting from cotyledon sections Bar= 5 mm (B) Callus developed in embryonic axes, in the buds zone. Bar= 5 mm (C) Nodular callus from embryonic axes after 40 days of cultivation (arrows) Bar= 5 mm (D) embryogenic callus from embryonic axes placed in the culture medium SEIM6 Bar= 2 mm (E) Embryogenic mass formation after 40 days of cultivation in medium SEIM6 Bar= 0.5 mm (F) Begining of the germination process after embryos were placed in the medium SEGM Bar= 5 mm (G) germinated shoots from somatic embryos (left : artificial light, right: solar light) Bar= 5 mm (H) Mature plants regenerated from somatic embryos Bar= 15 mm (I) Greenhouse-grown regenerants with normal phenotypically appearance (J) Plants flowered and with seeds.

Table 2. Influence of TDZ on callus formation in *P. acutifolius* var. TB1 after 50 days of culture.

Medium	Callus formation level in the explant *		Callus formation (%) **	
	Cotyledon	Embryonic axe	Cotyledon	Embryonic axe
CIM1	1.50 d	1.84 c	0	0
CIM2	2.42 cd	3.00 b	77.0±0.686 c	97.0±3.029 b
CIM3	2.97 bc	3.07 b	70.0±0.759 d	98.9±2.031 a
CIM4	3.44 ab	3.50 a	85.0±0.669 b	99.8±0.646 a
CIM5	3.64 a	3.75 a	90.0±0.621 a	99.8±0.460 a

** Means within a column followed by the same letter are not significantly different by Kruskal-Wallis test.*
*** The data were represented as means ±S.E. with significance being determined at P<0.05 level using Tukey*

CIM1: BM with 0.05 mg l^{-1} IAA. CIM2: BM with 0.05 mg l^{-1} TDZ and 0.05 mg l^{-1} IAA. CIM3: BM with 0.1 mg l^{-1} TDZ and 0.05 mg l^{-1} IAA. CIM4: BM with 0.2 mg l^{-1} TDZ and 0.05 mg l^{-1} IAA. CIM5: BM with 0.5 mg l^{-1} TDZ and 0.05 mg l^{-1} IAA.

Figure 2 shows the embryogenic structure formation percentage proportionally to the concentrations of IAA evaluated. It demonstrated the funtion of this growth regulator in the induction of somatic embryogenesis in this genotype. A 20% of embryogenic masses formation was achieved using 3 mg.l^{-1} and 5 mg.l^{-1} IAA. All treatments with IAA, were superior to the control.

When the somatic embryos were placed on SEGM, in the first week of culture, these became white-yellowish color to green and then began the germination process under both light regimes (Figure1F). During this phase, 100% of somatic embryos germinated, with shoot development (partial germination) or shoot and root development (complete germination), under both illumination conditions.

Somatic embryos germination and plant conversion

The light conditions influenced notably in the somatic embryos germination. The differences were marked in the shoot coloration and its later growth (Fig. 1G). The solar light produced high values of somatic embryos germination and formed plants with regard to those obtained under fluorescent light conditions. The frequency of explants regeneration with complete germination markedly increased when cultures were place to illumination with solar light reaching values of 32%. Whereas, when cultures were cultured in the artificial light this variable was reduced to 14%. Higher elongation was also observed in plants incubated at solar light (6.75 cm with seven leaves) (Figure 1H).

Plant conversion

The *in vitro* regenerated plants were phenotypically similar to the ones decribed before for this species. Shoots featured one or trifoliate leaves and had a typically elongated morphology reminiscent of normal phaseolus plants (Figure 1I). All established plants flowered and produced seeds (Figure 1J).

DISCUSSION

Lately, many researches have been involved in establishing reliable regeneration procedures for agronomically important plants. This is the first step to facilitate plant transformation and improvement of the crop.

Different tissues can respond in different ways during the *in vitro* culture process (Jiménez, 2001). In the present work, the best results to obtain embryogenic callus were achieved using embryonic axes. The presence of a meristem allows the formation of embryo-like structures. Other authors such as Dillen *et al*. (1996); Zambre *et al*. (1998) and Eissa *et al*. (2002) demonstrated that the explants with meristematic tissue induced the calli formation obtaining similar results.

Regeneration-competent callus have been obtained from pedicels of two genotypes of *P. vulgaris* using two growth regulators combinations (0.5 mg l^{-1} TDZ and 0.25 mg l^{-1} IAA; 1.0 mg l^{-1} TDZ and 0.5 mg l^{-1} IAA (Mohamed *et al*., 1993). It was subsequently demonstrated that through a similar approach, regeneration by organogenesis could be obtained in Tepary bean (Dillen *et al*., 1996) and *P. polyanthus* (Zambre *et al*., 2001). Zambre *et al.* (1998) working with mature cotyledons of *P. acutifolius* and *P. vulgaris* evidenced the formation of brown and compact callus with 0.1 mg.l^{-1} TDZ. The best results were obtained in this investigation using concentrations of 0.2 mg.l^{-1} TDZ and 0.05 mg.l^{-1} IAA.

Table 3. Influence of IAA on the embryogenic masses formation from *P. acutifolius* cv. TB1 formed calli.

	1	2	3	4	5	6	7	8
Culture medium	ESIM1	ESIM2	ESIM3	ESIM4	ESIM5	ESIM6	ESIM7	ESIM8
Embryo-like structure (%)	0	0	7±0.656	12±0.566	16±0.852	20±0.777	20±0.907	16±0.807

*** The data were represented as means ±S.E. with significance being determined at P<0.05 level using Tukey*

Results confirmed the first observations of Veltcheva *et al.* (2005) using initial dark incubation period to improve regeneration efficiency. In this work the callu formation and embryos induction were developed under darkness conditions which influenced favorably in the formation of somatic embryos. Varisai Mohamed *et al*. (2006 a) optimized the photoperiod conditions on shoot bud induction. They found that frequency of regenerated explants markedly increased when cultures of all the cultivars were pre-cultured in the dark.

The best results to obtain embryogenic callus were reached using embryonic axes. The presence of a meristem favors the formation of embryo-like structures. Picciarelli *et al.* (2005) studied the auxins endogenous content and the somatic embryogenesis in *P. coccineus* seeds. Results demonstrated that the endogenous levels of free IAA are the distinctive characteristic for the establishment of calli lines with embryogenic potential.

The simultaneous occurrence of embryogenic and organogenic formations in some of the explants was an interesting aspect observed. Kwapata *et al*. (2010) reported similar results in *P. coccineus* and *Phaseolus vulgaris* by Malik and Saxena (1992).

In this investigation, the 32% of complete germination was obtained. It suggests the necessity to study several components in the culture medium to improve this indicator.

P. acutifolius one of the few species in which the number of transformed plants generated is not an enough amount to enable the application of transgenic techniques (Dillen *et al*., 2000; Broughton *et al*., 2003; Zambre *et al*., 2005). Nevertheless, the development of new methods for the *in vitro* regeneration from somatic embryos could improve the plants transformation efficiency in this specie.

In conclusion, the results demonstrated the possibility to generate somatic embryos in *P. acutifolius* A. Gray cv. TB1 and it opens the way for new investigations that allow using the somatic embriogenesis to obtain transgenic plants.

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