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RESEARCH ARTICLE

In vitro plant regeneration system for common bean (*Phaseolus vulgaris*): effect of N^6-benzylaminopurine and adenine sulphate

Andrés M. Gatica Arias*
Escuela de Biología
Universidad de Costa Rica
P.O. Box 2060, San Pedro, Costa Rica
Tel: 506 2075091
Fax: 506 2074216
E-mail: agatica@biologia.ucr.ac.cr

Jenny Muñoz Valverde
Escuela de Biología
Universidad de Costa Rica
P.O. Box 2060, San Pedro, Costa Rica

Pilar Ramírez Fonseca
Escuela de Biología
Universidad de Costa Rica
P.O. Box 2060, San Pedro, Costa Rica

Marta Valdez Melara
Escuela de Biología
Universidad de Costa Rica
P.O. Box 2060, San Pedro, Costa Rica

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Keywords: common bean, *in vitro* culture, morphogenesis, *Phaseolus vulgaris* L., plant growth regulators.

Abbreviations: AS: Adenine sulphate
BAP: N^6-Benzylaminopurine
MS: Murashige and Skoog (1962) medium

A method for regeneration of the commercially important common bean (*Phaseolus vulgaris*) using N^6-benzylaminopurine(BAP) and adenine sulphate (AS) was established. Embryogenic axes of the Costa Rican common bean cultivars Bribrí, Brunca, Guaymí, Huetar and Telire were cultured on Murashige and Skoog medium supplemented with 100 mgl^{-1} myo-inositol, 1 mgl^{-1} thiamine, 30 gl^{-1} sucrose, BAP (0, 5 and 10 mgl^{-1}), AS (0, 20 and 40 mgl^{-1}) and 8 gl^{-1} agar. Regardless of the concentration of BAP and AS in the induction medium, the number of shoots and leaves differed significantly among the common bean cultivars evaluated. The higher average of shoots was obtained for Brunca > Telire > Bribrí > Guaymí > Huetar. Moreover, independently of the cultivar, the induction medium supplemented with 5 mgl^{-1} BAP and 20 or 40 mgl^{-1} AS resulted in the higher average of shoots formation. Culture of Bribrí, Brunca, Guaymí, Huetar and Telire embryogenic axes on induction medium supplemented with different BAP and AS resulted in a differential response. Successful acclimatization of common bean *in vitro* plants were achieved in the greenhouse, and plants appeared morphologically normal. The regeneration system developed in this investigation for this important crop could be a useful tool for the genetic modification through mutagenesis or genetic transformation.

Common bean (*Phaseolus vulgaris*) is an economic important crop and one of the major grain legumes for human consumption in Latin America, Africa and Asia (Delgado-Sanchez et al. 2006; Varisai Mohamed et al. 2006). Despite its importance, production growth rates are limited by viral, fungal and bacterial pathogens, insects, lack of drought tolerances and nutritional deficiencies (Aragão et al. 1996). Therefore, there is considerable interest in the development of new bean cultivars with useful agronomical traits (Aragão et al. 1996).

Plant biotechnology, together with conventional breeding methods, could facilitate bean improvement since resistance or tolerance to biotic and abiotic stress could be increased and seed quality, plant architecture and reproduction modes could be altered (Veltcheva et al. 2005). Nevertheless, a reliable and efficient *in vitro* culture

*Corresponding author

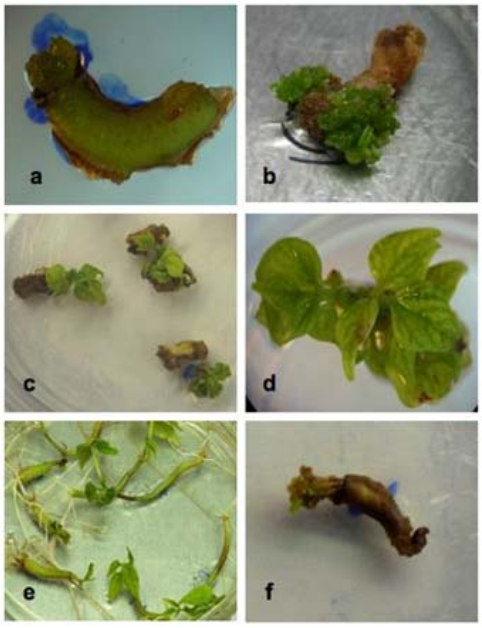

Figure 1. Apical bud cluster induction from embryogenic axesofcommon bean (*Phaseolus vulgaris* L.).
(a) embryogenic axes cultured on induction medium supplemented with BAP (0, 5 and 10 mgl^{-1}) and AS (0, 20 and 40 mgl^{-1}).
(b) Bud clusters formed after 30 days in induction medium.
(c) Bud cluster propagation.
(d) Bean plant developed from bud clusters.
(e) Roots of germinated embryogenic axes.
(f) Phenolic oxidation observed on embryogenic axes.

system that results in efficient differentiation, shoot development and whole plant regeneration is an essential requirement for improvement of common bean through genetic transformation or mutagenesis (Svetleva et al. 2003; Varisai Mohamed et al. 2006). In addition to genetic improvement, *in vitro* culture is an important tool for the recovery, conservation of germplasm and embryo rescue (Delgado-Sanchez et al. 2006).

In vitro plant regeneration of *Phaseolus* has been reported by organogenesis (Malik and Saxena, 1991; Malik and Saxena, 1992; Ahmed et al. 2002; Veltcheva and Svetleva, 2005; Delgado-Sanchez et al. 2006; Varisai Mohamed et al. 2006) or through somatic embryogenesis (Zambre et al. 1998; Schryer et al. 2005). Although several protocols have been described in the literature for bean regeneration, development of an optimal *in vitro* culture system still remains a major challenge since this and other species from the *Phaseolus*genus, are recalcitrant for *in vitro* regeneration (Veltcheva et al. 2005).

Therefore, the objective of the present study was to optimize an *in vitro* plant regeneration system for five commercial Costa Rican *Phaseolus vulgaris* cultivars by studying the influence of N^6-benzylaminopurine and adenine sulphate, as a prerequisite for improvement of common bean through genetic transformation or mutagenesis.

MATERIALS AND METHODS

Plant material and explant preparation

Five Costa Rican common bean (*Phaseolus vulgaris*) cultivars -Bribrí, Brunca, Guaymí, Huetar and Telire- were obtained from Fabio Baudrit Moreno Experimental Station, University of Costa Rica. Seeds were washed with running tap water and soaked for 4 min in 70% ethanol, disinfected with 20% (v/v) sodium hypochlorite with two drops of Tween 20 solution for 20 min and rinsed three times with sterile distilled water. Then, the seeds were immersed in 0.01% Benomyl (Piscis, Costa Rica) solution for 4 min and rinsed three times with sterile distilled water. The seeds were soaked in sterile distilled water overnight at 26 ± 2ºC to soften the seed coat. The embryogenic axes were excised from the seeds and cotyledons and root meristems were removed.

Induction and plant regeneration medium

The embryogenic axes were cultured on Petri dishes containing 20 ml of induction medium which consisted of MS mineral salts (Murashige and Skoog, 1962) supplemented with 100 mgl^{-1} myo-inositol, 1 mgl^{-1} thiamine, 30 gl^{-1} sucrose and 8 gl^{-1} agar. The induction medium was supplemented with a combination of BAP (0, 5 and 10 mgl^{-1}) and AS (0, 20 and 40 mgl^{-1}) to comprise 9 treatments (Delgado-Sanchez et al. 2006). Experimental treatments consisted of three replicates of three Petri dishes with 10 explants in each one. All medium pH was adjusted to 5.6 before autoclaving for 21 min at 121ºC and 1.07 kg cm^{-2}.

Table 1. Effect of genotype on the average number of shoots and leaves induced from embryogenic axesofcommon bean (*Phaseolus vulgaris* L.) regardless of the BAP and AS concentration. Data were recorded after 30 days of culture on induction medium supplemented with different BAP and AS concentrations.

Genotype	Shoots[1]	Leaves[1]
Bribrí	0.24 ± 0.04[2] b	0.29 ± 0.03 b c
Brunca	0.57 ± 0.06 a	0.42 ± 0.05 b
Guaymí	0.06 ± 0.01 c	0.34 ± 0.04 b c
Huetar	0.02 ± 0.01 c	0.21 ± 0.03 c
Telire	0.38 ± 0.04 b	0.96 ± 0.08 a

[1]Values represent the average shoot and leaves s formation per genotype in 9 Petri dishes with 10 explants and three replicates.
[2]Mean ± SE
Same letters within columns denote statistically equal means with the Tukey Unequal N HSD test ($P < 0.05$).

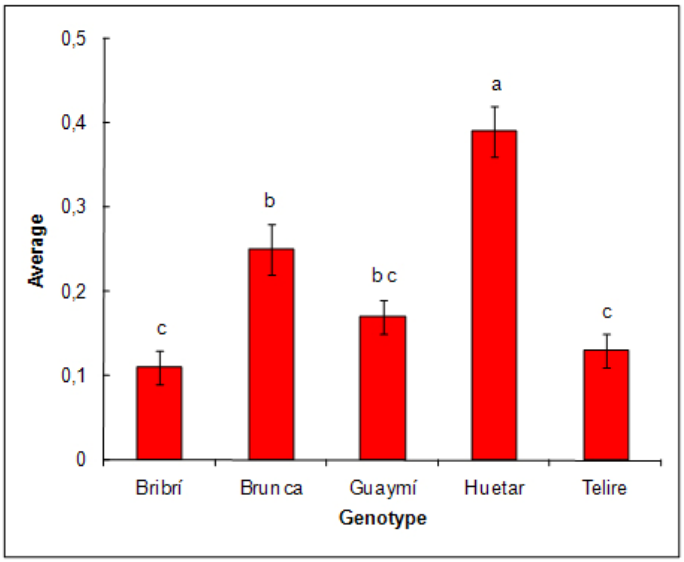

Figure 2. Effect of genotype on phenolic oxidation of embryogenic axesofcommon bean (*Phaseolus vulgaris* L. cvs. Bribrí, Brunca, Guaymí, Huetar and Telire) regardless of the BAP and AS concentration. Mean ± SE. Values within columns followed by the same letter are not significantly different with the Tukey Unequal N HSD test ($P < 0.05$).

The shoots regenerated from bud clusters were excised from the original explant and transferred to baby food jars, closed with polyethylene food wrap (Glad, Costa Rica), containing 20 ml of induction medium to promote elongation and root formation. The *in vitro* cultures were maintained at 26 ± 2ºC under a 16 hrs light photoperiod (30 µmol m^{-2} s^{-1}). The average number (mean ± SE) of shoots, leaves and roots per explant were estimated after 30 days of culture on each induction medium. The efficiency of the treatments [(Number of embryogenic axes with de *novo* shoots/total of embryogenic axes) x 100] were calculated.

Acclimatization and field transfer

Regenerated shoots (approximately 3 cm long) with well developed leaves and roots were transferred to pots containing Floragard Blumenerde potting soil (Floragard products, Germany), covered with plastic bags and maintained in the greenhouse under 12 hrs light photoperiod at 26 ± 2ºC. After one week the plastic bags were removed. The plantlets were watered twice a week.

Statistical analysis

The statistical analysis was performed using one-way ANOVA and the significance of differences among treatment means were contrasted with Tukey's Honestly Significant Difference Test (HSD) at $P < 0.05$. The program STATISTICA (StatSoft, Tulsa, OK, USA)version 6.0 was used.

RESULTS

Multiple shoot formation began with the swelling of embryogenic axes (Figure 1a), and apical bud clusters formed after 30 days of culture (Figure 1b). The apical bud clusters were multiplied by culturing them in the induction medium (Figure 1c) until shoots were developed (Figure 1d).

The Table 1 show the effect of genotype on the average number of shoots, leaves and roots induced from embryogenic axes of common bean (*Phaseolus vulgaris*) regardless of the BAP and AS concentration. The average number of shoots and leaves differed significantly among the common bean cultivars evaluated. The higher multiple shoot formation was obtained using Brunca > Telire > Bribrí > Guaymí > Huetar. Moreover, the higher average of leaves was obtained using Telire > Brunca > Guaymí > Bribrí > Huetar. On the other hand, no significant differences were observed in the number of roots formed from embryogenic axes cultured on induction medium supplemented with different BAP and AS concentrations (data not shown).

The effect of BAP and AS concentration on phenolic oxidation and average number of shoots, roots and leaves induced from embryogenic axesofcommon bean (*Phaseolus vulgaris*) regardless of the genotype is shown in the Table 2. Independently of the cultivar, significant differences were observed in the number of roots formed among the treatments evaluated. The higher number of roots per explant was obtained in the induction medium depleted of BAP and AS (Figure 1e). It was observed that as the BAP and AS concentrations increased, the number of roots per explant decreased (Table 2).

Moreover, regardless of the cultivar, the induction medium supplemented with 5 mgl^{-1} BAP and 20 and 40 mgl^{-1}AS resulted in a higher average number of shoots (Table 2). On

Table 2. Effect of BAP and AS concentration on phenolic oxidation and average number of shoots, roots and leaves induced from embryogenic axesofcommon bean (*Phaseolus vulgaris* L.) regardless of the genotype. Data were recorded after 30 days of culture on induction medium supplemented with different BAP and AS concentrations.

Treatments					
BAP (mgl^{-1})	AS (mgl^{-1})	Shoots[1]	Leaves[1]	Roots[1]	Phenolic oxidation[1]
0	0	0.00 ± 0.00[2] d	0.89 ± 0.05 a	3.53 ± 0.18 a	0.00 ± 0.00 e
0	20	0.00 ± 0.00 d	0.34 ± 0.04 d	1.85 ± 0.16 b	0.19 ± 0.03 c d
0	40	0.37 ± 0.04 b c	0.81 ± 0.07 a b	0.65 ± 0.05 c	0.45 ± 0.04 a
5	0	0.09 ± 0.04 d	0.00 ± 0.00 e	0.09 ± 0.04 d	0.00 ± 0.00 e
5	20	0.56 ± 0.08 a b	0.70 ± 0.13 a b c	0.01 ± 0.01 d	0.31 ± 0.04 b c
5	40	0.66 ± 0.09 a	0.49 ± 0.09 c d	0.00 ± 0.00 d	0.05 ± 0.02 d e
10	0	0.01 ± 0.01 d	0.00 ± 0.00 e	0.00 ± 0.00 d	0.31 ± 0.04 b c
10	20	0.20 ± 0.06 c d	0.25 ± 0.06 d e	0.31 ± 0.08 c d	0.43 ± 0.04 a b
10	40	0.40 ± 0.05 b c	0.51 ± 0.08 b c d	0.00 ± 0.00 d	0.15 ± 0.02 d

[1] Values represent the average shoot, leaves and roots formation per treatment in 15 Petri dishes with 10 explants and three replicates.
[2] Mean ± SE
Same letters within columns denote statistically equal means with the Tukey Unequal N HSD test ($P< 0.05$).

the other hand, no shoots were observed in the induction medium supplemented with 0 mgl^{-1} BAP and 0 and 20 mgl^{-1}AS. It is important to mentioned that the leaves and roots formed with 0 mgl^{-1} BAP and 0 and 20 mgl^{-1}AS correspond to the germinated embryogenic axes and not from developed shoots (Table 2).

Table 3 shows the effect of BAP and AS on the number of shoots induced from embryogenic axes of *Phaseolus vulgaris*. Cultures of Bribrí, Brunca, Guaymí, Huetar and Telire embryogenic axes on induction medium supplemented with different BAP and AS resulted in a differential response. In all the cultivars evaluated, no organogenic bud clusters were induced using 0 mgl^{-1} BAP and 0 and 20 mgl^{-1} AS after 30 days of culture. Addition of 40 mgl^{-1} AS, combined with 0 mgl^{-1} BAP, improved multiple shoot bud formation in Bribrí, Brunca, Huetar and Telire. In the Bribrí and Brunca cultivars, the higher average of shoots and the higher efficiency percentage were obtained using 5 mgl^{-1} BAP combined with 20 or 40 mgl^{-1} AS. For Guaymí and Huetar cultivars, no significant differences were observed among treatments; nevertheless, the best results were obtained using 0 mgl^{-1} BAP with 40 mgl^{-1} AS, 5 mgl^{-1} BAP with 20 and 40 mgl^{-1} AS or 10 mgl^{-1} BAP with 20 and 40 mgl^{-1} AS. In the Telire cultivar, the higher average number of shoots was obtained using 5 mgl^{-1} BAP combined with 20 AS or 10 mgl^{-1} BAP with 40 mgl^{-1} AS. Nevertheless, the higher efficiency percentage was obtained using 0 mgl^{-1} BAP combined with 40 AS (Table 3).

Common bean *in vitro* plant regeneration is limited by phenolic oxidation (Figure 1f). The higher average of phenolic oxidation was observed using Huetar (0.39 ± 0.03), Brunca (0.25 ± 0.03), Guaymí (0.17 ± 0.02), Telire (0.13 ± 0.02) and Bribrí (0.11 ± 0.02), regardless of the concentration of BAP and AS (Figure 2). On the other hand, independently of the cultivar, no phenolic oxidation was observed in the explants cultured on the induction medium depleted of BAP and AS; whereas the higher average of phenolic oxidation was obtained using the induction medium supplemented with 0 mgl^{-1} BAP and 40 mgl^{-1} AS, 5 mgl^{-1} BAP with 20 mgl^{-1} AS and 10 mgl^{-1} BAP and 0 and 20 mgl^{-1} AS (Table 2).

The shoots regenerated from the apical bud clusters (Figure 1d) and developed into plants with leaves and roots (Figure 3a). The *in vitro* plants were successfully acclimated with 100% survival (Figure 3b). The entire procedure starting from apical bud cluster induction to establishing a plant under greenhouse conditions took approximately 3 months.

DISCUSSION

Regeneration protocols using different explants of *Phaseolus* spp. (Malik and Saxena, 1991, Malik and

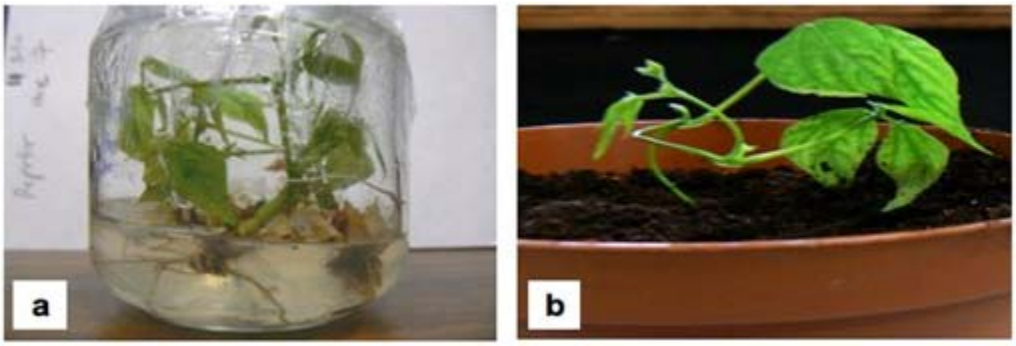

Figure. 3. (a) Bean plants regenerated from bud clusters after 60 days on induction medium. (b) Bean plants acclimated in the greenhouse.

Saxena, 1992; Ahmed et al. 2002; Veltcheva and Svetleva, 2005; Delgado-Sanchez et al. 2006; Varisai Mohamed et al. 2006; Arellano et al. 2009) have been described. This process in *Phaseolus vulgaris* has been histologically demonstrated by Malik and Saxena (1992) and Arellano et al. (2009). In the present study an efficient and reproducible method for regeneration of the commercially important Costa Rican common bean using BAP and AS was established.

Many factors induce development of new structures via organogenesis, but it has been demonstrated that regenerative capacity and response to growth conditions is species and genotype dependent as reported in *Helianthus annuus* (Deglene et al. 1997) and *Solanum melongena*. (Sharma and Rajam, 1995). Our results confirmed earlier observations that the genotype influences the regeneration process via organogenesis in the bean (Santalla et al. 1998; Veltcheva et al. 2005; Delgado-Sanchez et al. 2006; Varisai Mohamed et al. 2006). The genotype effects suggest that genetic factors are important in the response to *in vitro* tissue culture (Santalla et al. 1998). In this sense, due to the reduced genetic variability in modern bean cultivars, the screening of a large number of cultivars may be useful in the attempt to achieve plant regeneration (Svetleva et al. 2003; Veltcheva et al. 2005).

The composition of the shoot induction medium is important in the regeneration process through organogenesis since in this medium a higher number of buds and shoots are formed (Santalla et al. 1998). In this study, BAP and AS played an important role in the induction of organogenic structures in the Costa Rican bean cultivars evaluated. It is well known that cytokinins stimulate plant cell division and participate in the release of lateral bud dormancy, in the induction of adventitious bud formation, in the growth of lateral buds and in the cell cycle control (Gaspar et al. 1996; Gaspar et al 2003). Malik and Saxena (1991) reported that 5 µM BAP had a favorable effect on shoot formation from cultured leaf explants of *P. vulgaris* compared to the explants cultured on MS medium depleted of BAP. Moreover, Varisai Mohamed et al. (2006) indicated that BAP and thidiazuron at 5 µM was the best concentration for shoot formation in *P. angularis* (binomial name *Vigna angularis*) cvs. KS-6, KS-7 and KS-8 (azuki bean). In the absence of BAP, no shoot bud development was found in *P. angularis*. (Varisai Mohamed et al. 2006). Delgado-Sanchez et al. (2006) observed that 22.2 or 44 µM BAP induced the formation of buds in two Mexican *P. vulgaris* cultivars. BAP has also been reported to be effective for shoot induction in *P. vulgaris* and *P. coccineus* (Malik and Saxena 1992; Santalla et al. 1998). The beneficial effects of BAP on adventitious shoot induction were also observed in other legumes such as *Vigna angularis* (Avenido and Hattori, 2000) and *V. mungo* (Saini and Jaiwal, 2002).

It has been demonstrated that adenine, adenosine and adelynic acid have cytokinin activity and they are added to the culture medium in order to improve growth or to reinforce the response normally attributed to cytokinin action. In this sense, adenine stimulates somatic embryogenesis and caulogenesis, enhances the growth of isolated meristem tips, induces the proliferation of axillary shoots in shoot cultures and promotes adventitious shoot formation indirectly from calli or directly from explants (van Staden et al. 2008).

The benefits of adenine are often only noticed when it is associated together with ammonium nitrate or with cytokinins such as BAP or kinetin (van Staden et al. 2008). Our results suggest that BAP, in combination with AS, improves the process of organogenesis. Several explanations for this are possible. One possibility is that adenine acts as a precursor for natural cytokinin synthesis or enhances natural cytokinin biosynthesis; the compounds produced could be more effective in causing the physiological response than the cytokinins added to the culture medium. Another possibility is that adenine acts as a synergist of cytokinins such as kinetin and zeatin. The natural and synthetic purine cytokinins are degraded to adenine and related nucleotides; therefore, the addition of adenine to the medium may retard the degradation of cytokinins by feed-back inhibition or by competing for the

Table 3. The effect of BAP and AS on the number of shoots induced from embryogenic axes of *Phaseolus vulgaris* L. after 30 days of culture on induction medium supplemented with different BAP and AS concentrations.

Treatments		Shoots[1] (efficiency %)[2]				
BAP (mgl^{-1})	AS (mgl^{-1})	Bribrí	Brunca	Guaymí	Huetar	Telire
0	0	0 ± 0[3] c (0)	0 ± 0 c (0)	0 ± 0 a (0)	0 ± 0 a (0)	0 ± 0 c (0)
0	20	0 ± 0 c (0)	0 ± 0 c (0)	0 ± 0 a (0)	0 ± 0 a (0)	0 ± 0 c (0)
0	40	0.03 ± 0.03 c (3)	0.93 ± 0.05 b (93)	0 ± 0 a (0)	0.10 ± 0.06 a (10)	**0.80 ± 0.07 ab (80)**
5	0	0 ± 0 c (0)	0.47 ± 0.18 b c (21)	0 ± 0 a (0)	0 ± 0 a (0)	0 ± 0 c (0)
5	20	**1.20 ± 0.22 a (53)**	0.53 ± 0.20 b (24)	**0.13 ± 0.06 a (10)**	0 ± 0 a (0)	**0.93 ± 0.15 a (67)**
5	40	0.17 ± 0.07 bc (17)	**2.43 ± 0.17 a (96)**	**0.17 ± 0.07 a (17)**	**0.07 ± 0.05 a (7)**	0.45 ± 0.11 abc (60)
10	0	0.07 ± 0.07 bc (7)	0 ± 0 c (0)	0 ± 0 a (0)	0 ± 0 a (0)	0 ± 0 c (0)
10	20	0.50 ± 0.17 b (20)	0 ± 0 c (0)	0.13 ± 0.06 a (13)	0 ± 0 a (0)	0.37 ± 0.25 bc (10)
10	40	0.20 ± 0.07 bc (20)	0.77 ± 0.14 b (57)	0.13 ± 0.06 a (13)	0 ± 0 a (0)	**0.90 ± 0.13 a (70)**

[1] Values represent the average shoot formation in three Petri dishes with 10 explants and three replicates.
[2] [(Number of embryogenic axes with *novo* shoots/total of embryogenic axes) x 100]
[3] Mean ± SE
Same letters within columns denote statistically equal means with the Tukey Unequal N HSD test ($P < 0.05$). Treatments that gave the best results of shoot formation are highlighted in bold.

enzyme systems involved in cytokinin metabolism (van Staden et al. 2008).

Adenine sulphate has been used for *in vitro* multiplication of *Carica papaya* (Saha et al. 2004; Schmildt et al. 2007), *Musa* sp. (Schmildt et al. 2007) and *Uraria picta* (Anand et al. 1998). Moreover, Nandagopal and Ranjitha Kumari (2006) used AS for shoot organogenesis induction in *Cychorium intybus* cv. Focus. Deshpande et al. (1998) indicated that a low concentration of cytokinins in combination with AS favors multiple shoot induction in *Ficus religiosa*. In black pepper (*Piper nigrum*), Philip et al. (1992) reported that AS increased the number of shoot buds per regenerating explant.

In contrast to our results, Delgado-Sanchez et al. (2006) reported that the application of AS at different concentrations did not produce a relevant increase in bud cluster formation in two Mexican common bean cultivars. Such divergent or contrasting results could be due to differences in cultivars, physiological state of explant and the season of explant collection.

On the other hand, higher concentration of cytokinin and AS generally delays root formation (van Staden et al. 2008) as seen in the present study (Table 2). In this sense, Delgado-Sanchez et al. (2006) reported that low BAP concentration (0 and 0.1 mgl^{-1}) induced only root formation and stem elongation.

The browning of the explant and surrounding culture media limit the establishment of *in vitro* cultures in several species, especially woody plant species. Browning is caused by the oxidation of phenolic compounds, released from the cut ends of the explants, by polyphenoloxidases, peroxidases or air (Bhat and Chandel, 1991; Laukkanen et al. 1999). In this sense, browning hampers the establishment of tissue culture in *Aloe* (Abrie and van Staden, 2001), *Dioscorea alata* (Bhat and Chandel, 1991), *Musa* spp. (Titov et al. 2006) and *Pinus sylvestris* (Laukkanen et al. 1999). Moreover, some plant growth regulators such as BAP, kinetin, TDZ, IAA alone or in combination with 2,4-D were reported to increase browning in *Aloe* species (Natali et al. 1990; Meyer and van Staden, 1991). Asahira and Nitsch (1969) reported that the addition of kinetin to the culture medium intensified the extend of browning in *D. batatas* and *D. japonica*. The application of BAP and kinetin enhanced biosynthesis of phenolic compounds in hairy roots of *Panax ginseng* (red ginseng). In contrast, the addition of auxins did not show an increase

in the contents of total phenolic compounds in hairy roots of *P. ginseng* when no gibberellic acid or cytokinins were present (Jeong et al. 2007). Cytokinins are known to stimulate the synthesis of phenolic compounds (Asahira and Nitsch, 1969). Schnablova et al. (2006) reported that transgenic tobacco overproducing endogenous cytokinins accumulates more phenolic compounds during *in vitro* growth. (Reword: too many "overs"). Therefore, understanding the oxidation of phenols and how these can be minimized is critical for successful *in vitro* culture.

The *in vitro* protocol reported in this study could be used for clonal propagation of common bean and to obtain competent target tissue for genetic modification through in order to generate plants resistant to diseases and pests.

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