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In vitro Plants of Common Bean (*Phaseolus vulgaris* L.) Obtained by Direct Organogenesis

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Abstract

Common bean crops are staple food of great economic importance in developing countries and the most important source of plant protein around the world. Nevertheless, an efficient and reliable regeneration method is still lacking for this crop. Here it is described two economically important bean varieties, Bayomex (BAY) and Flower of May 199 (FM199), that yielded 84 and 78% explants with shoots and developed 3.6 and 2.61 shoots per explant respectively, in MS culture media with B5 vitamins (MSB5) and 10 µM benzylaminopurine (BAP). Multiple shoots of BAY and FM199 were promoted in MSB5 with BAP or thidiazuron (TDZ). Using 4.54 µM TDZ induced 2.5 and 2.9 shoots per explant and at 4.44 µM BAP 1.7 and 2.2 shoots per explant were formed in BAY and FM199, respectively. In the hypocotyl zone, rooting was induced with TDZ between 0.41 and 1.73 µM and with BAP between 0.44 and 1.68 µM. Callus was induced with 2.72 and 4.54 µM TDZ, adding 2.66 and 4.44 µM BAP. The FM199 shoots obtained with 2.72 and 4.54 µM TDZ and subsequently cultured in media with 2.72 µM TDZ promoted plant development and regeneration. There was an increase a 4.44 shoots per explant using the BAY variety with TDZ and subsequently grown with 4.44 µM BAP. Rooting of shoots was promoted in the media with 0.44 µM BAP or without phytoregulators. Regenerated plants were acclimatized in soil, grew normally and developed seeds.

Keywords: beans, recalcitrant, multiple shoots, cytokinin

1. Introduction

Common beans are one of the major grain legumes for human consumption in Latin America, Africa and Asia, and represent an important source of protein and minerals such as iron and zinc, and certain vitamins (Gepts et al., 2008). However, bean plant development and its seed production are limited by pathogens, insects, drought and nutritional deficiencies. The use of tissue culture for plant regeneration and the introduction of foreign genes that confer pathogens resistance or drought tolerance are a good option for bean cultivation.

Most of the published protocols are based on direct organogenesis or shoot development from meristematic cells (Arellano et al., 2009). Some recent examples of direct organogenesis may be found in the literature (Ahmed et al., 2002; Albino et al., 2005; Quintero-Jiménez et al., 2010). Until now several types of cells, tissues and organs (cotyledonary nodes, embryonic axes, auxiliary shoots, cotyledon with split embryo axis, internodes, hypocotyls, leaves, leaf petioles or intact seedlings) have been used to induce the regeneration pathways (Albino et al., 2005; Delgado-Sánchez et al., 2006; Mahamune et al., 2011; Thảo et al., 2013; Hnatuszko-Konka et al., 2014). However, it should be remarked that these protocols did not always yield regeneration of the whole *P. vulgaris* plants.

Different explants from embryonic axis cultivated with the transverse thin cell layer of cells (tTCL) protocol were *in vitro* grown from epicotyl, hypocotyl, cotyledons and roots from two weeks old seedlings (Cruz-Carvalho et al., 2000). Regenerated plants were obtained from embryonic axis of mature seeds (Delgado-Sánchez et al., 2006; Kwapata et al., 2010; Quintero-Jiménez et al., 2010). Cotyledons with part of the embryonic axis as explant have been used to regenerate whole plants (Dang & Wei, 2009). These protocols are very efficient.

It has been reported the regeneration of black bean (*Phaseolus vulgaris* L.) from Flower of May and Flower of June via organogenesis where fully regenerated plants were obtained from axillary or apical meristems (Delgado-Sánchez et al., 2006). However, the disadvantage of this protocol is its genotype-specific dependence; therefore its use must be adapted to different varieties to achieve an efficient regeneration. For Flower of May 199 (FM199) and Bayomex (BAY) varieties such protocols have not yet been established.

BAP has been the cytokinin of preference to induce multiple bean shoots at high concentrations between 22.2 and 88.8 μM. TDZ has a cytokinin-like effect that was exploited for the regeneration of *Phaseolus acutifolius* (Zambre et al., 1998; Cruz-Carvalho et al., 2000; Zambre et al., 2001). Combinations of TDZ and indoleacetic acid (IAA) have also been used for obtaining plants from callus of cv. Xan 159 and GN-Tara hybrids, both genotypes obtained from interspecific crosses between *P. vulgaris* with *P. acutifolius* (Mohamed et al., 1993; Zambre et al., 1998).

The type of plant growth regulators (PGR) is a key element to determine the morphogenetic pathway for the *in vitro* regeneration of black beans. This has been observed for the organogenesis promoted by BAP or TDZ (Kwapata et al., 2010). TDZ has also been used in beans to promote indirect organogenesis from leaf petioles (De Clercq et al., 2002; Veltcheva & Svetleva, 2005); direct organogenesis from cotyledonary knots (Dang & Wei, 2009) and to induce morphogenesis and regeneration from embryonic axis (Kwapata et al., 2010).

The concentration of PGR is also very important for regeneration of beans; BAP has been used to induce multiple bean shoots at high concentrations (22.2 to 88.8 μM). TDZ has proved to be a very active compound at low concentrations (0.1 to 10 μM), and in some bean varieties 10 μM TDZ induces a greater number of shoots whereas 80 μM BAP is required to achieve the same effect. However, the induction of high amounts of callus at the wound site of hypocotyl, on the presence of different cytokinin concentrations (4.44 and 88.8 μM), blocks the root formation and increases the production of phenolic compounds, which causes tissue death due to oxidation (Arnaldos et al., 2001). Therefore, the presence of callus and phenolic compounds are limiting factors for tissue development, viability and rooting emergence of bean regeneration.

The aim of the present study was to assess the effect of BAP and TDZ on shoot development of embryonic axis from commercial bean varieties, in order to develop an *in vitro* regeneration system that allows induction of multiple shoots in the absence of callus at wound sites and reduce the possible formation of phenolic compounds.

2. Method

Seeds from bean varieties were incubated in a solution of Triton-X-100 during two min, placed in 70% ethanol for an additional two min, 5% sodium hypochlorite (NaOCl) for 15 min, washed three times with distilled sterile water and kept on soaked in water during 24 h. Subsequently, seeds were dissected on a flow hood, to eliminate the head, embryonic axis, plumule and radicle.

2.1 Stage I. Shoot Inductions

Bean seeds were disinfected and preincubated on MS salts with B5 vitamins MSB5 (MS salts supplemented with B5 vitamins) liquid medium (Murashige & Skoog, 1962; Gamborg et al., 1968) with 1 μM BAP for 24 h. Embryonic axis were dissected and transferred to Petri dishes containing MSB5 medium with BAP 10 μM and incubated 16/8 h light/dark photoperiod at 26 ± 2 °C during four weeks to determine the explant varieties that display the greatest sprouting percentage. In addition, embryonic axes of the different varieties were placed on MSB5 medium with 10 μM BAP and incubated for the same period to determine the bean variety that yields more shoots per explant on the apical region.

To establish the optimal concentration of PGR that determines the limit between the formation of root or callus at the wound site, embryonic axes were placed on Petri dishes with MSB5 medium at different BAP concentrations (0.09, 0.40, 1.69, 4.44, 18.8 and 79.84 μM). The dishes were incubated for four weeks under 16/8 h light/dark photoperiod at 24 ± 2 °C. The material was evaluated considering the number of shoots per explant, explant length and root or callus emergence in the hypocotyl cutting area.

Once the hormonal treatment conditions were determined, the embryonic axes were cultivated in MSB5 medium containing 3% sucrose, 100 μM of silver nitrate ($AgNO_3$) and 0.7% agar at pH 5.8. Phase I treatments consisted of six BAP (0.40, 0.66, 1.00, 1.69, 2.66, and 4.44 μM) and six TDZ (0.41, 0.68, 1.02, 1.73, 2.72 and 4.54 μM) concentrations.

Embryonic axes were placed on Petri dishes with the MSB5 media supplemented with either BAP or TDZ and incubated at 26 ± 2 °C under cool-white illumination (20 $\mu E \cdot m^{-2} \cdot s^{-1}$) with 16/8 h light/dark photoperiod during five weeks to induce bud formation or stem primordia and shoots. Ten embryonic axes at each concentration of

BAP or TDZ and a randomized experimental design with three replicates were used.

The cotyledon shoots per apex and shoots per knot, and total shoots per explant were evaluated. Likewise, the presence of root or callus on the cutting area of explant hypocotyls and the apical zone length from the cotyledon knot of the apical meristem were determined. Buds or bud clusters were not recorded.

2.2 Stage II. Development of Shoots

To promote shoot obtained in Stage I, callus or root explants were removed before subculturing. For the FM199 variety, explants from shoot induction or TDZ treatments of Stage I or shoot induction were subcultured on MSB5 media with TDZ or BAP on each of the six different concentrations of Stage I experiments, to test the effect of TDZ on explants on the two regeneration stages. For BAP treatments explants were transferred to MSB5 media with BAP only for an additional period of four weeks.

2.3 Stage III. Rooting

To promote rooting, well-developed shoots (3-5 cm in length) from Stage II, were dissected from explants and subcultured on media without PGR (MS0), MSB5 with 0.4 μM BAP or MSB5 with 0.62 μM NAA (naphthaleneacetic acid).

2.4 Stage IV. Acclimatization

Complete rooted young plants (plantlets) were acclimated in mixture of peat moss ("Premier 3.0 cu." FT. Peat Moss") and covered with plastic bags for two weeks (Figure 2, Stage IV a) Plants were watered daily with a 0.5X MS diluted solution during two weeks. Subsequently, the plants were transferred to pots with a mixture of 80% of clay soil and 20% perlite irrigated with 0.5 X MS solution and watered every other day, during four weeks.

2.5 Statistical Analysis

To compare the effect of BAP and TDZ on plant regeneration, as well as the bean varieties in terms of numbers of shoots per explant, the number of shoots per apex and cotyledonary knots, the Tuckey range test was applied (Kruskal & Wallis, 1952), which establishes if the differences between the BAP and TDZ treatments in embryonic axes of two varieties are statistically significant. A variance analysis and comparison of means of Tuckey for apex lengths was applied. For explants with callus or root frequency null hypothesis tests were performed to compare the proportions of two populations with binomial distribution ($p = 0.05$) and establish statistical differences among means.

3. Results and Discussion

3.1 Stage I (SI). Induction of Stem Primordia and Buds

The embryonic axis response was 84 and 78% explants with shoots in the varieties BAY and FM199, respectively. In addition, the response of multiple budding was 3.6 and 2.61 shoots per explant with 10 μM BAP in BAY and FM199, respectively. The two varieties Flower of May and Flower of June were selected to develop an efficient regeneration protocol (Delgado-Sánchez et al., 2006), in particular it was tested the FM199 variety which is related to the variety Flower of May because this latter had the best shooting percentage. Therefore, it was considered to carry on the regeneration experiments with only BAY and FM199 varieties because these varieties had the highest percentage of shooting and the greatest number of shoots per explant (data not shown).

Organogenesis results with the BAY variety showed shoot induction in the embryonic axes with a concentration of 18.8 μM and showed a higher number of shoots per explant, namely 8.03 ± 0.65 (Table 1). At higher concentrations the number of shoots per explant decreased to 3.87 ± 0.25. Similarly, the bud per explant values varied from 1.63 to 0.09 and 2.82 to 4.44 μM BAP on MSB5. The response of root induction in hypocotyl cutting was observed between 0.09 to 4.44 μM. In addition, callus formation was observed between 18.8 and 79.84 μM BAP, corresponding to 100% explants with callus but without roots (Table 1).

The above results showed that root formation (morphogenesis) and callus development (undifferentiation) is the consequence of the increase of exogenous cytokinin concentration and thus it is probably altering the balance of endogenous auxins and cytokinins. This enabled a test that could make more efficient the regeneration protocol. As far as we know, this type of effects has not been described before.

Table 1. Multiple shoot induction *via* direct organogenesis, epicotyl length and root development (morphogenesis) or callus (undifferentiation) in hypocotyl of embryonic axes of BAY variety with different concentrations of BAP during four weeks incubation

Stage I BAP (μM)	† Shoots/explant	†† Epicotyl length (cm)	Hipocotyl	
			% roots	% callus
0.09	1.63 ± 0.32^{cd}	1.76 ± 0.10^{a}	100	0
0.44	1.78 ± 0.16^{cd}	0.99 ± 0.60^{ab}		
1.69	1.32 ± 0.50^{d}	0.53 ± 0.38^{b}	100	50¤
4.44	2.82 ± 0.83^{bc}	0.37 ± 0.045^{b}		
18.8	8.03 ± 0.65^{a}	0.31 ± 0.13^{b}		
79.84	3.87 ± 0.25^{b}	0.18 ± 0.03^{b}		

Note. ANOVA † ($F = 73.69$ vs $F_{0.01} = 5.06$) SD** y †† ($F = 11.86$ vs $F_{0.01} = 5.06$) SD**.

Tuckey test. Root primordia or small callus.

Another element that has not been correlated with PGR concentration is the length of the apical region. The largest epicotyl length (1.76 cm) was obtained with 0.09 μM BAP, which suggested that in the absence or at low concentrations of cytokinins the apical region enlarges too probably promoted by auxins. In contrast, the shortest length occurred at the highest BAP concentrations (79.84 μM) (Table 1). As the number of sprouts increased, the length of the apical region is reduced. This response was related to the high cytokinin concentration that promoted bud formation from explant sprouts. However, it is probably acting antagonistically to endogenous auxins and does not promote apical elongation. This is important because on a significant length of the apical region, the stem thickness was reduced and formed very thin seedlings with little chance of further development.

On the next step it was necessary to reverse the elongation inhibition promoted by the high cytokinin concentration for the sprout development. The tested treatments induced the formation of a large number of stem buds, also known as shoot meristem or bud clusters, as well as multiple shoots where shoots had a higher level of development, and started to differentiate to stem, leaf primordial and apical bud. Table 2 shows the numbers of shoots per explant, per apex and per cotyledonal node at the different hormonal treatments. In the FM199 variety, when the medium contained 4.44 μM BAP, the largest number of shoots per explant and shoots per apex were obtained (2.27 and 2.0, respectively). The largest number of shoots per cotyledonary node, were obtained on 0.46 with 1.68 μM BAP. The response with 4.54 μM TDZ in FM199 variety generated the highest bud induction per explant and per apex (2.55 and 2.05, respectively).

For the BAY regeneration, the frequency of multiple shoot induction was 67 and 90%, respectively (Table 2), when the medium contained BAP or TDZ. The response of BAY to 2.72 μM TDZ, led 4.15 and 2.85 shoots per explant and per apex, respectively, although it was not significantly different to using other TDZ concentration (Table 2). The greater amount of shoots and buds were observed with this variety using TDZ.

The results are consistent with the idea that TDZ promotes better morphogenetic responses than BAP and exerts an influence by modifying the metabolism of endogenous cytokinins (Dang & Wei, 2009). In the present study, TDZ has induced more shoots at 10 μM than 80 μM BAP, but the shoot viability is lower probably because there are more bud clusters in meristematic tissue and callus in cutting areas (Kwapata et al., 2010). Therefore, TDZ promoted better shooting response in both FM199 and BAY varieties in contrast with results obtained with BAP (Table 2).

Comparing both phytoregulators, the shoots induction response was lower at low concentrations of cytokinins. Kwapata et al. (2010) reported up to 20 buds per explant, depending on the bean cultivar, but the 'Olathe' and 'Sedona' varieties formed 12 buds per explant with 10 μM BAP and 0.5 μM AIA.

Additionally, embryonic axes of mature seeds of Flower of May 'Anita' and Flower of June 'Marcela' varieties showed an 88% regeneration efficiency with 44.4 μM BAP (Delgado-Sánchez et al., 2006), whose May Flower variety is different from the tested in the present work. This confirms that the morphogenetic responses are both genotype-specific and cytokinin- and concentration-dependent. However, it is important to note that at higher BAP or TDZ concentration, explants become necrotic and the wound sites develop greater amount of callus, which potentially can be induced with exogenous auxins to form buds but that makes the process more long and laborious.

Table 2. Comparison of BAP and TDZ treatments on embryonic axes bean *P. vulgaris* "Bayomex" (BAY) and "Flower of May 199 "(FM199)

Plant growth regulators	(μM)	BAY			FM199		
		Shoots/ explant [y]	Shoots/ apex [y]	Shoots/ node [y]	Shoots/ explant [y]	Shoots/ apex [y]	Shoots/ node [y]
TDZ	4.54	2.95 b [z]	1.62 c	1.33 a	2.55 a	2.05 a	0.50 a
	2.72	4.15 a	2.85 a	1.30 a	1.85 b	1.45 ab	0.40 ab
	1.73	2.91 b	2.00 ab	0.91 bc	1.33 b	1.23 bc	0.10 bc
	1.02	3.44 ab	2.15 ab	1.29 a	1.39 bc	1.04 cd	0.35 ab
	0.68	3.25 ab	2.05 ab	1.20 b	1.15 c	1.00 d	0.15 bc
	0.41	2.41 b	1.81 b	0.60 c	1.19 bc	1.04 cd	0.15 bc
	0.00	1.00 c	0.83 d	0.17 d	1.23 bc	0.88 d	0.35 ab
BAP	4.44	1.70 ab	0.96 b	0.74 a	2.27 a	2.00 a	0.27 ab
	2.66	1.85 a	1.27 a	0.58 a	1.70 b	1.35 b	0.35 ab
	1.69	1.78 a	1.19 a	0.59 a	1.50 bc	1.04 c	0.46 a
	1.00	1.81 a	1.00 ab	0.81 a	1.33 bcd	1.00 c	0.33 abc
	0.66	1.72 a	1.24 a	0.48 a	1.20 bcd	1.10 c	0.10 bc
	0.44	1.40 b	1.03 ab	0.37 a	1.20 bcd	1.03 c	0.17 abc
	0.00	1.50 ab	1.00 ab	0.50 a	1.00 cd	1.00 c	0.00 bc

Note. [y] Kruskal-Wallis test for several independent samples (design completely at random) and comparisons of mean ranks (p = 0.05); [z] Rankes with the same letter are not significantly different.

The hormonal treatments described in the present work are similar to other reports, where 2 to 5 times more shoots were obtained in cultured explants in the presence of 0.25 or 1.0 μM Forchlorfenuron (CPPU) or TDZ than with 5 μM BAP (Mohamed et al., 1992). Therefore, our results showed that with low concentration of BAP, the number of shoots on BAY is 2-4, but the efficiency of shoots induction or buds per explant was 95% which allowed an increased regeneration, especially with a further exposure to BAP of explants in stage II (development of buds or shoots). This could be due to a reduction in the induction of callus and a lower synthesis of phenolic compounds (Kwapata et al., 2010), replacing this response with a response morphogenetic response (root primordial), necrosis reduction on wound sites and therefore a better bud development.

The induction of stem primordia and shoots in cotyledonary nodes on BAY and FM199 varieties was 1.3 with 4.54 μM TDZ (Table 2), possibly due to a competition between the cotyledonary nodes with the apical area. In this work we observed that TDZ produced more buds on bean tissue than BAP, when used at equal concentration (Table 2). Similarly, it was found that BAP and TDZ in the germination medium enhance the induction of buds in bean tissue (Dang & Wei, 2009).

3.2 Induction of Roots and Callus

The hormonal treatments established in the present study, showed two important results at the hypocotyl level. Firstly, the formation of root when the concentration of cytokinins in the medium was reduced or there was absence of phytoregulators; and on the other hand, the formation of callus when cytokinin concentration increased. It is likely that root formation limits the callus formation and promotes shoot development. On the contrary, the callus formation and the production of phenols resulting from cell death in this tissue greatly limits the explant development and stem primordia (Kwapata et al., 2010).

Root induction in FM199 displayed a maximum response of 94% with 0.41 μM TDZ. The response of the other treatments (0.68, 1.02, 1.73, 2.72 and 4.54 μM) showed a tendency to decrease the root formation up to 47.5% as TDZ concentration increases. In a similar manner, the BAY variety showed a 44% root induction with 0.41 μM TDZ, and the effect of the other treatments (0.68, 1.02, 1.73 and 2.72 μM) decreased down to 10% with 4.54 μM TDZ. This result established a negative correlation between root formation and cytokinins as is shown in Figure 1A.

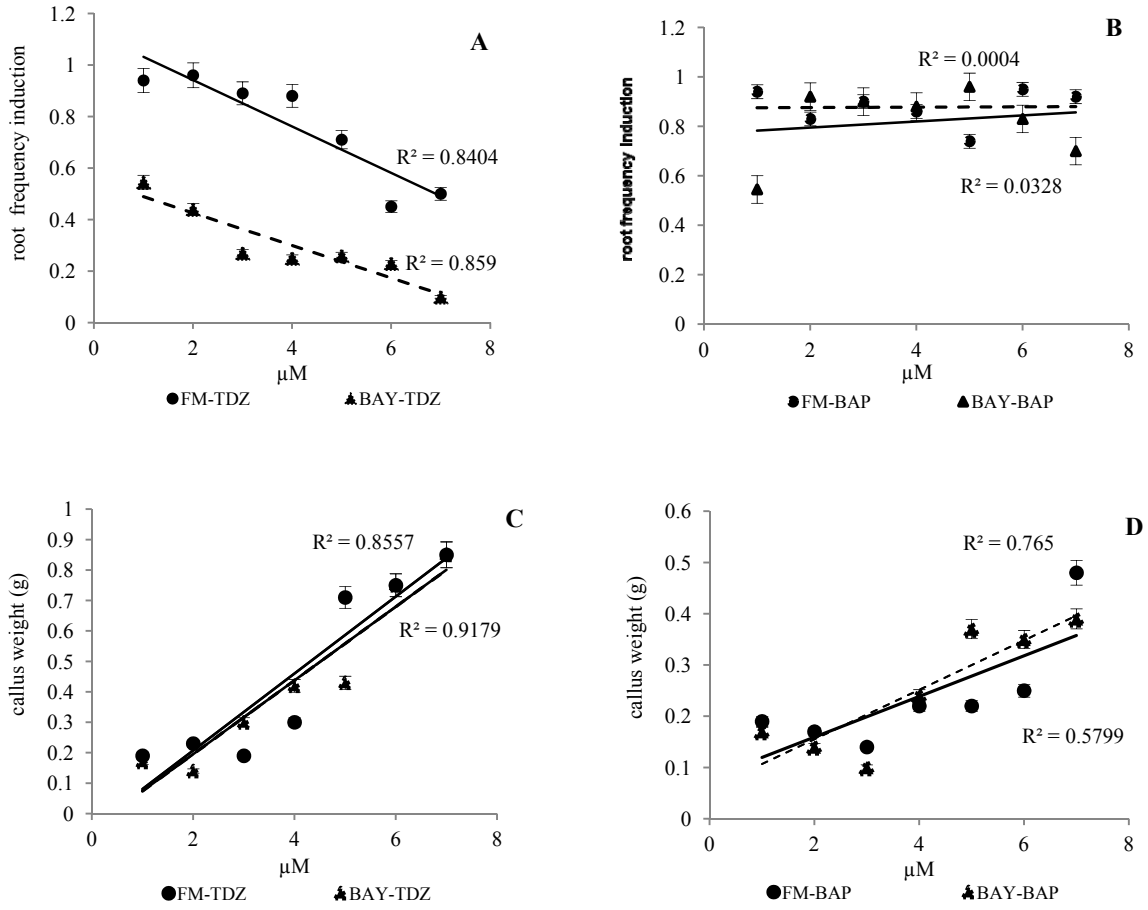

Figure 1. Response of root induction (A and B) and callus weight (C and D) in stage I to different concentrations of TDZ and BAP in embryonic axes of the two bean varieties

The root induction in FM199 displayed a maximum response of 95% with 2.66 µM BAP and 75% with 1.68 µM BAP, suggesting that there is a trend to reduce the root induction. In a similar way, root induction of the BAY variety was only 54.5% on 0.40 µM BAP. However, in other treatments (0.66, 1.00, 1.69, 2.66, and 4.44 µM) the root induction rose from 70 to 100%. There was no variation on this parameter with BAP (Figure 1B).

The frequency of callus induction in FM199 was 85% in the presence of 4.54 µM TDZ. The response rose in accordance with the increase of cytokinin concentration (Figure 1C). The frequency of callus induction for BAY variety was equal to FM199, thus in both cases the response was an increase of cytokinin to promote callus induction (Figure 1C).

The frequency of callus induction in FM199 had a maximum of 48% in the presence of 4.54 µM BAP, the response rose in accordance with an increase of cytokinin concentration. In the case of BAY variety, it had 39%, increase in the callus formation under the increased BAP concentration. The trend of callus induction with BAP was not as dramatic as with TDZ, that promotes a higher frequency of callus induction (Figure 1 D).

The best response of callus induction was with 4.54 µM TDZ from 1 µM in the BAY variety. It is important to take into account that at higher concentration of TDZ the shoot induction is directly proportional to the callus induction, limiting the shoot development in the following stages.

3.3 Elongation of the Shoots

It must be remarked that in this work the embryonic axes length was measured, since so far it has not been correlated this response to regeneration (Kwapata et al., 2010). In general, it has been observed that at zero or low concentration of cytokinins in Stage I, the shoots lengthen as a response to the low concentration of cytokinins and this affects the subsequent shoot development, resulting in very long and weak seedlings (data not shown).

The shoot length was 3.71 cm for the FM199 variety in media without cytokinins (MS0), however significant differences were observed after BAP and TDZ treatments. In the presence of BAP, a reduction tendency was visible along the apical region of FM199 shoots, which consisted of 1.86 cm with 0.40 to 1.0 cm with 4.44 µM BAP. The trend for shoot reduction was similar in the presence of TDZ averaging 1.85 cm length with 0.41 µM and 0.83 cm with 1.73 µM (Table 3).

Table 3. Effects of different treatments of BAP and TDZ in the length of the apical region of the embryonic axes in two varieties of beans

Plant growth regulators (µM)		FM	BAY
		Average (cm) †	Average (cm) †
Control	0.0	3.71 a ††	2.14 bc
BAP	0.40	1.86 bc	2.52 ab
	0.66	1.70 bcd	2.06 bc
	1.00	1.39 bcd	1.55 bcd
	1.68	1.49 bcd	1.28 bcd
	2.66	1.95 bc	1.17 cd
	4.44	1.00 cd	0.48 d
TDZ	0.41	1.85 b	0.72 de
	9.68	1.45 bc	0.63 de
	1.02	1.16 cd	0.74 de
	1.73	0.83 cde	0.48 e
	2.72	0.85 cde	0.48 e
	4.54	0.93 cde	0.36 e

Note. †: Comparison of Tuckey means (P = 0.05); ††: Stockings with the same letter in columns are not significantly different.

The observed negative correlation between the effects of cytokinins over the apical region length of the embryonic axes on the FM199 variety agrees with previous reports in beans, where the developmental response was similar for both cytokinins (Mohamed et al., 1992). However, the effect found in the present study was different with the BAY variety, which suggests that there is a differential response among genotypes. Therefore, we can conclude from our data that when cytokinins concentration increased the apical shoot decreased.

This observation is very important for the later regeneration stages where it is required to formulate media that promote lengthening of the apical region of the embryonic axes by decreasing the concentration of cytokinins, and likewise with the addition of auxins or GA_3. Internode elongation is promoted without PGR, with low concentration of BAP (2 µM) or with addition of 2 to 4 µM gibberellic acid (GA_3) (Mohamed et al., 1993).

In addition, increase of the apical region length of the embryonic axes was reported on media with inorganic MS salts only MS supplemented with 22.2 µM BAP and 0.057 µM AIA. The recovery of plants was on MS medium with 4.44 µM BAP and 0.58 µM GA3 (De Clercq et al., 2002; Veltcheva & Svetleva, 2005). The GA_3 also plays an important role in increasing the apical region length of the embryonic axes, more than on the shoot formation (Dang & Wei, 2009). In this work, it was not necessary the incorporation of auxins or gibberellins to increase the length of the apical region of the embryonic axes. Therefore, the effect of high cytokinin concentration for reducing the apical region length, can be reversed by decreasing its concentration on the next developmental stage.

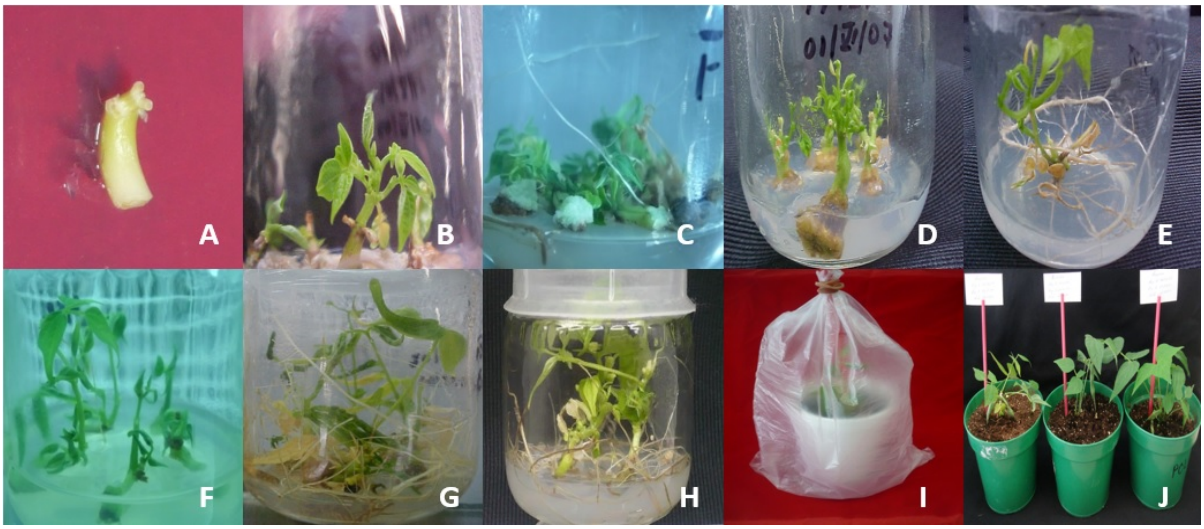

Figure 2. Developmental stages of *in vitro* propagation of bean by direct organogenesis from embryonic axis of mature seed.

Note. A) Embryonic axis used as explant; B) embryonic axis developed; C) explants with elongated shoots and necrotic wound (Stage I-bud and shoot induction of common bean using 2.66 and 4.44 µM BAP or 2.72 and 4.52 TDZ with BAY and FM199 varieties, respectively, after 35 days); D) explants with callus formed on the wound site and stem primordia and buds (Stage II-shoot development at 28 days); E) explants with roots formed at the site of the wound; F) explants with shoots; G) explants with developed shoot whit root (Stage III-rooting on MSB5 containing 0.44 µM BAP and MSB5 without phytoregulators, after 15 days); H) plantlets previously to acclimatization; I) plant acclimatization (Stage IV-*ex vitro* plants with peat-moss, after 15 days); and J) plants with pods and seeds (growth in pots with soil and perlite after four weeks).

3.4 Stage II (IBD). Bud Development

From shoots of Stage I, a length reduction was promoted in the apical zone when the concentration of TDZ increased. However, when shoots of Stage I were induced with 0.41 or 2.72 µM TDZ and were transferred to media with 2.72 µM TDZ, only 20% shoots survived. On the other hand, when buds were induced with 0.41 µM TDZ and were transferred to media with BAP, the response of shoot development was higher with 60% survival with 1.73 or 1.02 µM TDZ. The other treatments did not affect shoot development, but survival declined (data not shown). There was not direct and systematic evidence of TDZ effecting bud induction at low cytokinins concentration and its further development, or if it affects the plantlet regeneration obtained in later stages.

The plant regeneration response *in vitro* was better when TDZ (Stage I)-TDZ (Stage II) combinations were used at low concentrations, TDZ (Stage I)-BAP (Stage II) and BAP (Stage I)-BAP (Stage II), with the latter combination the percentage of survival was 90% (Figure 2F, Stage II).

The results mentioned above have been previously observed in bean where TDZ effect is better on the initial stage (Stage I and germination) (Dang & Wei, 2009). Thus, it has been recommended the presence of BAP in Stages II or III, and it is also possible the continued presence of TDZ for shoot development.

3.5 Stage III (CIRT). Rooting

Shoots obtained in Stages I and II (Figure 2) were transferred to the rooting medium. The MSB5 with 0.62 µM NAA gave best results in the root induction but low acclimatization. However, MSB5 with BAP 0.44 µM and the MS0 medium (MSB5 without the addition of phytoregulators) also promoted rooting (Table 4 and Figure 2, Stage III). The induction of primary root explants, once the buds are developed significantly helps the regeneration process. However, it is necessary to eliminate the root or callus formed in Stage I to induce new roots connected to the shoot vascular system. This is consistent with a report where individual shoots were transferred to medium without cytokinins, which formed new roots and these rooted more efficiently. The bud proliferation and simultaneous elongation was observed in medium with 0.44 or 1.0 µM BAP were shoots formed roots easily after 7 to 10 days in medium without cytokinins (Table 4). However, MSB5 containing 0.44 µM BAP led to 23% rooting frequency. BAP and GA_3 also promoted internodes elongation in beans, but the subsequent formation of root was limited mostly due to shoots of more than two weeks (Mohamed et al., 1993).

The most limiting factor for common bean plants shooting and rooting *in vitro* in is the high callus proliferation that blocks root formation and generate phenolic compounds that cause death due to oxidation (Arnaldos et al., 2001). This has been a challenge in some studies with beans, since compounds that cause darken oxidized tissue and cell death prevented or significantly reduced rooting (Kwapata et al., 2010). Therefore, it is very important for bean tissue culture to use media with regulators at low concentrations. For example, 1 μM IAA or NAA promote rooting in bean shoots after 5 weeks, but it was more effective to immerse explants for 30 seconds in 5 μM indolebutyric acid (IBA) prior to transferring to a medium without auxins (Kwapata et al., 2010). Additionally, 3 μM IBA and 0.08 μM BAP, led to 84.3% rooting frequency (Dang & Wei, 2009). These conditions significantly reduce callus production and phenolic compounds.

Table 4. Percentage of rooted plants on Bayomex variety and of acclimatized rooted plants

Plant growth regulator (μM)	Stages I-II (μM)	Stage III-BAP (μM)	% Rooted plants	% Acclimatized rooted plants
0.0	0.0	4.44	3.3	100 *
BAP	0.44	0.44	23.3	57 *
	0.66	0.44	10.0	100 *
	1.00	0.44	20	50 *
	1.68	0.44	10	30 **
	2.66	0.44	3.3	100 **
	4.44	0.44	3.3	100 *
	0.40	0.0	13.3	100 *
	0.66	0.0	3.3	0
	1.00	0.0	6.6	100 *
TDZ	0.41	0.44	20	100 *
	1.02	1.68	10	100 *

Note. * (with mature seeds); ** (seedless).

3.6 Stage IV (EIV). Acclimatization

Complete plants were acclimated and transferred to the greenhouse for two months before flowering and seed formation was observed (Figure 2, Stage IV). The obtained plants with 0.44 μM BAP in Stages I and II of both BAY and FM199 varieties and rooting on medium without regulators were those that acclimated and bloomed in less time, about 45 days and with 100% of acclimatization, similar to the others regeneration protocols. However, the regeneration protocol that gave plants with higher seed number was obtained with 0.44 μM BAP in Stages I-II-III, although the percentage of acclimatization was lower. TDZ regeneration presented two pathways with the same behavior as BAP affected only a little rooting percentage when its concentration increased (Table 4).

4. Conclusions

Shoot induction of embryonic axes is genotype-dependent, since there were different responses among varieties. The BAY variety presented the best response in number of shoots per explant compared to FM199. Likewise, TDZ and BAP promoted a great number of shoots per explant in the apical zone. In Stage I or shoot induction, the largest number of shoots per explant were grown in 18.8 μM BAP, but the explant induced more callus and buds. The root induction in the hypocotyl of embryonic axes size arose using 4.44 μM BAP with little callus formation. Further induction of shoot development on media with high concentration of cytokinins was limited by the presence of callus. The apical zone length was significantly reduced and concomitantly the shoot development in TDZ as BAP concentration increased for both BAY and FM199 varieties. In Stage II, or bud development, the best response to cytokinins was when shoots were transferred to MSB5 supplemented with BAP and not with TDZ. In Stage III or shoot rooting, acclimatization was better in MS with 0.44 μM BAP than MSB5 without growth regulators. Finally, fully regenerated plants formed mature seeds.

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References

Ahmed, E. E., Bisztray, G., & Velich, I. (2002). Plant regeneration from seedling explants of common bean (*Phaseolus vulgaris* L.). *Acta Biologica Szegediensis, 46*, 27-8.

Albino, M. M. C., Vianna, G. R., Falcao, R., & Aragao, F. J. L. (2005). *De novo* regeneration of fertile common bean (*Phaseolus vulgaris* L.) plants. *Journal of Plant Biotechnology, 7*(4), 267-72. http://dx.doi.org/10.1034/j.1399-3054.2001.1130303.x

Arellano, J., Fuentes, S. I., Castillo, E. P., & Hernández, G. (2009). Regeneration of different cultivars of common bean (*Phaseolus vulgaris* L.) via indirect organogenesis. *Plant Cell Tissue and Organ Culture, 96*, 1-11. http://dx.doi.org/10.1007/s11240-008-9454-1

Arnaldos, T. L., Muñoz, R., Ferrer, M. A., & Calderon, A. A. (2001). Changes in phenol content during strawberry (*Fragaria x ananasa*, cv. Chandler) callus culture. *Plant Physiology, 113*, 315-322. http://dx.doi.org/10.1034/j.1399-3054.2001.1130303.x

Cruz de Carvalho, M. H., Van-Le, B., Zuily-Fodil, Y., Pham-Thi, A., T., & Thanh-Van, K. T. (2000). Efficient whole plant regeneration of common bean (*Phaseolus vulgaris* L.) using thin-cell-layer culture and silver nitrate. *Plant Science, 159*, 223-232. http://dx.doi.org/10.1016/S0168-9452(00)00346-0

Dang, W., & Wei, Z. M. (2009). High frequency plant regeneration from the cotyledonary node of common bean. *Biologia Plantarum, 53*(2), 312-316. http://dx.doi.org/10.1007/s10535-009-0056-5

De Clercq, J., Zambre, M., Van Montagu, M., Dillen, W., & Angenon, G. (2002). An optimized Agrobacterium-mediated transformation procedure for Phaseolus acutifolius A. *Gray. Plant Cell Reports, 21*, 333-40. http://dx.doi.org/10.1007/s00299-002-0518-0

Delgado-Sánchez, P., Saucedo-Ruiz, M., Guzmán-Maldonado, S. H., Villordo-Pineda, E., González-Chavira, M., Fraire-Velásquez, S., & Mora-Avilés, A. (2006). A. An organogenic plant regeneration system for common bean (*Phaseolus vulgaris* L.). *Plant Science, 170*, 822-827. http://dx.doi.org/10.1016/j.plantsci.2005.11.015

Gamborg, O. L., Miller, R. A., & Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research, 50*, 151-158. http://dx.doi.org/10.1016/0014-4827(68)90403-5

Gepts, P., Aragão, F. J. L., De-Barros, E., Blair, M. W., Brondani, R., Broughton, W., Yu, K. (2008). Genomics of Phaseolus beans, a major source of dietary protein and micronutrients in the tropics. In P. Moore & R. Ming (Eds.), *Genomics of Tropical Crop Plants* (pp. 113-143). Berlin: Springer. http://dx.doi.org/10.1007/978-0-387-71219-2_5

Hnatuszko-Konka, K., Kowalczyk, T., Gerszberg, A., Wiktorek-Smagur, A., Kononowicz, A. K. (2014). *Phaseolus vulgaris*-Recalcitrant potential. *Biotechnology Advances, 32*, 1205-1215.

Kruskal, W. H., & Wallis, W. A. (2011). Use of Ranks in One-Criterion Variance Analysis. *Journal of the American Statistical Association, 47*(260), 583-621. http://dx.doi.org/10.1080/01621459.1952.10483441

Kwapata, K., Sabzikar, R., Sticklen, M. B., & Kelly, J. D. (2010). *In vitro* regeneration and morphogenesis studies in common bean. *Plant Cell Tissue and Organ Culture, 100*, 97-105. http://dx.doi.org/10.1155/2012/198960

Mahamune, S. E., Bansode, R. P., Sangle, S. M., Waghmare, V. A., Pandhure, N. B., & Kothekar, V. S. (2011). Callus induction from various explants of French bean (*Phaseolus vulgaris* L.). *Journal of Experimental Science, 2*, 15-6.

Mohamed, M. F., Coyne, D. P., & Read, P. E. (1993). Shoot organogenesis in callus induced from pedicel explants of common bean (*Phaseolus vulgaris* L.). *Journal America Society Horticultural Science, 118*, 158-162.

Mohamed, M. F., Read, P. E., & Coyne, D. P. (1992). Dark preconditioning, CPPU, and thidiazuron promote shoot organogenesis on seedling node explants of common and faba beans. *Journal of the American Society of Horticultural Science, 117*(4), 668-672.

Murashige, T., & SKoog, F. (1962). A revised medium for rapid growth bioassays with tobacco tissue cultures.

Physiologia Plantarum, 15(3), 473-493. http://dx.doi.org/10.1111/j.1399-3054.1962.tb08052.x

Quintero, J. A., Espinosa, H. E., Acosta, J. A., Guzmán, H. S., & Mora, M. A. (2010) An improved method for *In vitro* regeneration of common bean (*Phaseolus vulgaris* L.). *Agrociencia, 44*, 57-64.

Thảo, N. T., Thảo, N. T. P., Hassan, F., & Jacobsen, H. J. (2013). *In vitro* propagation of common bean (*Phaseolus vulgaris* L.). *Journal of Science and Development, 11*, 868-76.

Veltcheva, M. R., & Svetleva, D. L. (2005). In vitro regeneration of *Phaseolus vulgaris* L. via organogenesis from petiole explants. *Journal Central European Agriculture, 6*, 53-58.