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Influence of 24- epibrassinolide on *in vitro* shootlets regeneration *via* direct organogenesis of *Phaseolus vulgaris* L.

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This study aimed to optimize an *in vitro* shootlets regeneration system via direct organogenesis for *Phaseolus vulgaris* L. cv. Brunca using 24- epibrassinolide (24-Epi). The best medium for shootlets proliferation as well as growth parameters was MS supplemented with 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi. Stem explants recorded the best results of shootlets proliferation percentage compared with leaf or root explants, respectively. MS medium supplemented with 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi gave the best results of recovered shootlets (%); photosynthetic pigments, number of leaves, number of shootlets and shootlet length (cm). From the obtained results it can be recognized that, the behavior and trends of 24-Epi on *in vitro P. vulgaris* cultures media may be oriented and arranged to the behavior of auxins in the nutrient medium for roots formation and as a cytokinin in shootlets proliferation.

Key words: *Phaseolus vulgaris* L., 24- epibrassinolide, shootlets regeneration, direct organogenesis.

INTRODUCTION

Common bean (*Phaseolus vulgaris*) is one of several crop species belong to the Fabaceae family, commonly known as grain legumes or pulses. In total, there are about 650 genera and 18,000 species in the legume family (Hymowitz, 1990). Common bean is a very important source of vegetable protein, especially in those regions of the world in which animal and fish protein is scarce. Common bean satisfy 22% of the total protein requirement worldwide (Delgado-Sanchez et al., 2006) and account for over 50% of all legumes consumed globally (Blair et al., 2006). Like most grain legumes, common bean is rich in the essential amino acid lysine (Babaoglu et al., 2000; Popelka et al., 2004).

Plant biotechnology, together with conventional breeding methods, could facilitate bean improvement since resistance or tolerance to biotic and abiotic stress and could increase the seed quality, plant architecture and reproduction modes (Veltcheva et al., 2005). Nevertheless, a reliable and efficient *in vitro* culture 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 (Varisai Mohamed et al., 2006).

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A general feature of common bean genotypes is their recalcitrance to regenerate *in vitro*. This is because they produce significant amounts of phenolic compounds *in vitro* which inhibit their regeneration. Regeneration of many legumes has been successfully obtained by means of somatic embryogenesis and organogenesis and has been reported by numerous researchers. However, the regeneration of common bean by somatic embryogenesis has been unsuccessful because of the recalcitrance of this species and the inability to advance the development of embryos past the globular stages. Similarly, organogenesis has been successful in very few occasions (Carvalho et al., 2000; Guidolin, 2003). This limits the possibilities of genetic transformation of common bean. In order to overcome this difficulty, it is necessary to analyze and determine the chemical conditions and the type of explant that allow a reliable regeneration of plants by both, somatic embryogenesis and organogenesis.

Brassinosteroids (BRs) are naturally occurring plant growth regulators, which exhibit structural similarities to animal steroid hormones (Mandava, 1988). BRs affect a variety of physiological processes, including stem elongation, pollen tube growth, leaf bending and epinasty, root inhibition, fruit development, ethylene biosynthesis, proton pump activity, xylem differentiation, photosynthesis, and gene expression (Dhaubhadel et al., 1999; 2002; Singh and Shono, 2005). Moreover, BRs can induce plant tolerance to a variety of biotic and abiotic stresses (Xia et al., 2009). BRs were also found to have an activity *in vitro*. They were reported to increase the rate of cell division and colony formation of Chinese cabbage mesophyll protoplasts (Nakajima et al., 1996) and *Petunia hybrida* protoplasts (Oh and Clouse, 1998). BRs were also found to be essential for the differentiation of isolated *Zinnia* mesophyll cells into tracheary elements (Iwasaki and Shibaoka, 1991) and in the morphogenesis of *Arabidopsis* (Li et al., 1996). Moreover, BRs promoted adventitious shoot regeneration from segments of cauliflower hypocotyls (Sasaki, 2002).

The main objectives of the present study are to investigate the efficiency of 24- epibrassinolide (24-Epi) on *in vitro* shootlets regeneration *via* direct organogenesis as well as study the interaction between 24-Epi and IAA on rootlets formation of *Phaseolus vulgaris* L. cv. Brunca.

MATERIALS AND METHODS

This study was carried out in Plant Biotechnology Department, Genetic Engineering Division, National Research Centre, Dokki, Cairo, Egypt and Botany Department, Faculty of Women for Arts, Science and Education, Ain Shams University, Cairo, Egypt.

Sterilization and *in vitro* germination

Seeds of *P. vulgaris* L. cv. Brunca were obtained from Faculty of Agriculture, Cairo University, Egypt. The process of sterilization and *in vitro* germination of *P. vulgaris* seeds was carried out according to the method described by Nafie et al. (2013).

Plant culture and media composition

Murashige and Skoog, 1962 salts medium (MS) including vitamins, glycine and supplemented with 30 g L^{-1} sucrose, and solidified with 7 g L^{-1} agar was used in this study. The pH of the pre-agar MS medium was adjusted to 5.8 using 0.1 M NaOH before autoclaving. Cultures were incubated in illumination light condition at 26 ± 1°C and sufficient fluorescent light of 1500 lux for 16/8 h (light/dark) photoperiod for shootlets regeneration and 1000 lux for rootlets formation after incubation in complete darkness for three days.

Growth regulators used in this study

24-Epibrassinolide (24-Epi) was first dissolved in dimethyl sulfoxide (DMSO) before treatment according to Shujie et al. (2013), 2,4-dichlorophenoxy acetic acid (2,4-D). 3-indole acetic acid (IAA), thidiazuron (TDZ) were purchased from Sigma company. IAA was filtered through 0.2 µm pore size in Laminar Air flow cabinet**.**

Shootlets regeneration

For achievement of direct shootlet regeneration, different explants excised from three weeks old *in vitro* growing seedlings were cut into convenient sizes using scalpel blade, aseptically 3-4 mm length from stem, root and 4 mm^2 size from leaf. Aseptically explants were cultured on MS medium supplemented with 24-Epi alone or in combination with thidiazuran (TDZ) as follow: S_0 = MS free growth regulators; S_1 = MS + 0.025 mg L^{-1} 24-Epi; S_2 = MS + 0.05 mg L^{-1} 24-Epi; S_3 = MS + 0.1 mg L^{-1} 24-Epi; S_4 = MS + 2 mg L^{-1} TDZ; S_5 = MS + 2 mg L^{-1} TDZ + 0.025 mg L^{-1} 24-Epi; S_6 = MS + 2 mg L^{-1} TDZ + 0.05 mg L^{-1} 24-Epi; S_7 = MS + 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi.

Each treatment consisted of 5 replicates (jars) and each replicate contained three explants. Cultures were maintained by sub-culturing using the same medium at three weeks intervals from incubation. After 4 weeks from incubation, frequency of recovered shootlets from direct organogenesis (%), photosynthetic pigments (mg g^{-1} FW**)**, shootlet length (cm), number of shootlets/explant, number of leaves and shootlets fresh/dry weights (g/jar) were recorded.

Roots formation

The healthy regenerated shootlets with 6-7 cm length which resulted from S7 medium were transferred to MS roots formation medium which supplemented with different concentrations of 24-Epi (0.025, 0.05 and 0.1 mg L^{-1}) alone or in combinations with 0.5 mg L^{-1} IAA as follow: R_0 = MS free growth regulators; R_1 = MS + 0.025 mg L^{-1} 24-Epi ; R_2 = MS + 0.05 mg L^{-1} 24-Epi; R_3 = MS + 0.1 mg L^{-1} 24-Epi; R_4 = MS + 0.5 mg L^{-1} IAA; R_5 = MS + 0.5 mg L^{-1} IAA + 0.025 mg L^{-1} 24-Epi ; R_6 = MS + 0.5 mg L^{-1} IAA + 0.05 mg L^{-1} 24-Epi; R_7 = MS + 0.5 mg L^{-1} IAA + 0.1 mg L^{-1} 24-Epi.

Each treatment consisted of 5 replicates (jars) and each replicate contained three shootlets. The cultures were incubated in a controlled growth chamber in complete darkness for 3 days at 26 ± 1°C then transferred to light condition (1000 Lux for 16/8 h light/dark). Frequency of shootlet producing root (%); number of roots; root length (cm) and roots dry weight (g/jar) were recorded after four weeks of cultivation.

Table 1. Effect of interaction between media composition and explant types on frequency of direct shootlets regeneration, number of shootlets/explant, number of leaves/shoot , shootlet length and shootlet fresh/ dry weights of *P. vulgaris* L. cv. Brunca.

Culture media composition	Shootlets (%)	Number of shootlets/explant	Number of leaves/shoot	Shootlet length (cm)	F.W (g/jar)	D.W (g/jar)
Leaf explants						
S_0 = MS free growth regulators	-	-	-	-	-	-
S_1= MS + 0.025 mg L^{-1} 24-Epi	-	-	-	-	-	-
S_2= MS + 0.05 mg L^{-1} 24-Epi	-	-	-	-	-	-
S_3= MS + 0.1 mg L^{-1} 24-Epi	-	-	-	-	-	-
S_4= MS + 2 mg L^{-1} TDZ	50^{cd}	6.5^{d}	12.5^{f}	6.2^{f}	0.607^{e}	0.02^{d}
S_5= MS + 2 mg L^{-1} TDZ + 0.025 mg L^{-1} 24-Epi	50^{cd}	7^{d}	16^{d}	6.5^{f}	0.944^{c}	0.03^{cd}
S_6= MS + 2 mg L^{-1} TDZ + 0.05 mg L^{-1} 24-Epi	83^{ab}	6.5^{d}	17^{cd}	7.3^{f}	1.109^{b}	0.05^{bc}
S_7= MS + 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi	83^{ab}	7^{d}	21^{b}	7.2^{bc}	1.249^{a}	0.05^{abc}
Stem explants						
S_0 = MS free growth regulators	-	-	-	-	-	-
S_1= MS + 0.025 mg L^{-1} 24-Epi	-	-	-	-	-	-
S_2= MS + 0.05 mg L^{-1} 24-Epi	-	-	-	-	-	-
S_3= MS + 0.1 mg L^{-1} 24-Epi	-	-	-	-	-	-
S_4= MS + 2 mg L^{-1} TDZ	67^{bc}	7^{d}	14.5^{e}	6.2^{g}	0.41^{f}	0.03^{d}
S_5= MS + 2 mg L^{-1} TDZ + 0.025 mg L^{-1} 24-Epi	67^{bc}	8^{c}	$17.^{cd}$	7^{ef}	0.743^{d}	0.06^{ab}
	83^{ab}	8.5^{bc}	17.5^{cd}	9^{ab}	0.929^{c}	0.06^{ab}
	90^{a}	12^{a}	23.5^{a}	9^{ab}	0.928^{c}	0.07^{a}

Means having the same letters in a column were not significantly different 0.05 level.

Photosynthetic pigments extraction and estimation

Photosynthetic pigments (chlorophyll a, b and carotenoids) were estimated according to the method of Lichtenthaler and Wellburn (1987). Definite weight from each treatment (0.2 g) were homogenized in acetone 85% (v/v), filtered and made up to a final volume of 4 ml. Pigments at each maximum absorption wave length were estimated using (JENWAY 6305 UV-VIS) Spectrophotometer apparatus and absorbance was recorded at 646 and 663 nm for chlorophyll assay and 470 nm for carotenoids. Calculated using the formula given below:

Chl *a* mg/g F.W = (12.25 A_{663} - 2.79 A_{646}) × V/W
Chl *b* mg/g F.W = (21.21 A_{646} - 5.1 A_{663}) × V/W
Car mg/g F.W = (1000 A_{470} - 1.8 Chl *a* - 85.02 Chl *b*)/198

Where, A = Absorbance at 663, 646 and 470 nm. V = Final volume of chlorophyll extract in 85% acetone (ml). W = Fresh weight of the leaf tissue (g).

Statistical analysis

The test of least significant difference (LSD) at the level of 0.05% significance was used to examine differences among treatment means and interactions. Data were statistically analyzed using MSTAT-C software package according to the method described by Freed et al. (1989).

RESULTS

Effect of media composition and type of explants on efficiency of shootlets regeneration

Data in Table 1 represent the effect of cultures media and type of explants on shootlets regeneration *via* organogenesis. Data clearly showed significant differences between treatments concerning the frequency of direct shootlets regeneration (%), number of shootlets, number of leaves, shootlet length (cm) and fresh/dry weights of *P. vulgaris* L. cv Brunca after 4 weeks of culturing on MS medium supplemented with 2 mg L^{-1} TDZ and different concentrations of 24-Epi. In this regard, MS medium supplemented with 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi significantly gave the best results of shootlets regeneration compared with other treatments. On the other hand, among the seven tested media; S_4, S_5, S_6 and S_7 only induced shootlets regeneration using leaf and stem explants compared with S_0 (free growth regulators), S_1, S_2 and S_3 which recorded no responses to shootlets regeneration. The obtained results indicated that stem explant produced the highest frequency of shootlets (90%) followed by leaf explant (83%) on MS medium

Figure 1. Regenerated shootlets from stem (A) and leaf (B) segment of *P. vulgaris* after the formation ofcallus around the explants on MS medium supplemented with 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi. (5×).

supplemented with 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi, respectively (Figure 1A, B). Furthermore, the obtained results clearly indicated that there were no responses to shootlet regeneration on MS medium supplemented with 24-Epi alone. Whereas, adding TDZ (2 mg L^{-1}) alone or in combinations with the three different concentrations of 24-Epi induced shootlets regeneration from either leaf or and stem explants.

Moreover, the frequency of direct regenerated shootlets produced from either leaf or stem explants was increased with graded increasing of 24-Epi concentrations. Where it recorded the maximum values (83 and 90%) with MS medium supplemented with 2 mg L^{-1} TDZ + 0.05 or 0.1 mg L^{-1} 24-Epi for leaf and stem explant, respectively. In addition, the maximum number of shootlets (12) was produced from stem explants cultured on MS medium supplemented with 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi.

However, there were insignificant differences among S_4, S_5 and S_6, which represented the lowest rates of regeneration at the same time. Also, the maximum number of leaves/shoot (23.5, 21) were recorded with MS medium supplemented with 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi for stem and leaf explants, respectively. While, there were insignificant differences between S_5, S_6 and S_4, S_5 for leaf and stem explants, respectively. Whereas, S_4 produced the minimum number of leaves/shoot (12.5). Moreover, the longest shootlet (9 cm) was produced on MS medium supplemented with 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi from stem explants. Whereas, 2 mg L^{-1} TDZ represents the shortest shootlet (6.20 cm) for stem explant.

In general, collected data pointed out that addition of 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi to MS medium greatly improved most vegetative growth criteria concerned to shootlets regeneration of *P. vulgaris* L. *via* direct organogenesis during the whole growth period in particularly for stem explants.

Effect of media composition and type of explants on accumulation of photosynthetic pigments in regenerated shootlets

The photosynthetic system is often highly sensitive to exogenous plant growth regulators. Therefore, the impact of MS medium fortified with different concentrations 0.025, 0.05 and 0.1 (mg L^{-1}) of 24-Epi alone or in combinations with 2 mg L^{-1} TDZ (regeneration media) on photosynthetic pigments content was examined. Where it had been evaluated the response of shootlets produced pigments from leaf and stem explants after exogenous of 24-Epi and TDZ applications. Chlorophyll *a*, *b* and carotenoids (mg/g FW) show significant differences between treatments, as shown in Table 2. MS medium supplemented with 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi induced highly significant increase in chlorophyll *a* content which recorded maximum value (311 mg/g FW) for shootlet produced from leaf explants. Moreover, the obtained results clearly indicated that, chlorophyll *a* content in shootlets produced from leaf and stem explants was increased with graded increasing of 24-Epi concentrations. Whereas, minimum value of chlorophyll *a* content (91 mg/g FW) was observed with MS medium supplemented with 2 mg L^{-1} TDZ for shootlets produced from leaf explant. However, shootlets produced from stem explants recorded the maximum increase in chlorophyll *b* content (575 mg/g FW) with MS supplemented with 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi. As well as, chlorophyll *b* content in shootlets derived leaf and stem explants was increased with increasing 24-Epi concentrations. Whereas, minimum value of chlorophyll *b* content (84 mg/g FW) observed with MS medium supplemented with 2 mg L^{-1} TDZ for shootlets produced from leaf explants. In addition, 24-Epi at the 0.025, 0.05 and 0.1 mg L^{-1} concentration significantly stimulated carotenoids. Where MS supplemented with 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi induced highly significant increase in carotenoids content which recorded the maximum value (996 mg/g FW). Furthermore, carotenoids content in shootlets produced from both leaf and stem explant was increased with graded increasing of 24-Epi concentrations. Whereas, minimum value of carotenoids content (372 mg/g FW) recorded with MS medium supplemented with 2 mg L^{-1} TDZ for shootlets produced from stem explants.

Effect of culture media composition on rooting of shootlets

The effect of MS medium supplemented with different concentrations of 24-Epi (0.025, 0.05 and 0.1 mg L^{-1}) alone or in combination with 0.5 mg L^{-1} IAA on rooting (%), number of roots, root length (cm) and roots dry weight (g/jar) of shootlets derived leaf and stem explants was investigated.

Table 3 clearly shows that MS medium supplemented with 0.5 mg L^{-1} IAA + 0.05 mg L^{-1} 24-Epi gave the maximum value for rooting percentage (71%) in case of shootlets produced from leaf explant. However, there were insignificant differences among R_3, R_6 and R_7. While, there were no responses to rooting on MS medium

Table 2. Effect of interaction between media composition and explants types on chlorophyll (a, b) and carotenoids contents of *P. vulgaris* L. cv. Brunca.

Culture media composition	Chlorophyll (a) mg/g FW	Chlorophyll (b) mg/g FW	Carotenoids mg/g FW
Shootlets of leaf explant			
S_0 = MS free growth regulators	-	-	-
S_1= MS + 0.025 mg L^{-1} 24-Epi	-	-	-
S_2= MS + 0.05 mg L^{-1} 24-Epi	-	-	-
S_3= MS + 0.1 mg L^{-1} 24-Epi	-	-	-
S_4= MS + 2 mg L^{-1} TDZ	91^{h}	84^{h}	421^{g}
S_5= MS + 2 mg L^{-1} TDZ + 0.025 mg L^{-1} 24-Epi	248^{e}	213^{e}	763^{d}
S_6= MS + 2 mg L^{-1} TDZ + 0.05 mg L^{-1} 24-Epi	300^{c}	298^{c}	815^{b}
S_7= MS + 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi	311^{a}	405^{b}	996^{a}
Shootlets of stem explant			
S_0 = MS free growth regulators	-	-	-
S_1= MS + 0.025 mg L^{-1} 24-Epi	-	-	-
S_2= MS + 0.05 mg L^{-1} 24-Epi	-	-	-
S_3= MS + 0.1 mg L^{-1} 24-Epi	-	-	-
S_4= MS + 2 mg L^{-1} TDZ	138^{g}	144^{g}	372^{h}
S_5= MS + 2 mg L^{-1} TDZ + 0.025 mg L^{-1} 24-Epi	198^{f}	185^{f}	461^{f}
S_6= MS + 2 mg L^{-1} TDZ + 0.05 mg L^{-1} 24-Epi	260^{d}	292^{d}	652^{e}
S_7= MS + 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi	310^{b}	575^{a}	678^{c}

(-) no shoots. Means having the same letters in a column were not significantly different at 0.05 level.

supplemented with 0.025 or 0.05 mg L^{-1} 24-Epi. In addition, there were insignificant differences among R_0, R_4 and R_5 that recorded the minimum value for rooting percentage (33%). On the other hand, concerning to shootlets resulted from stem explants, the best medium that produced the maximum rooting percentage (88%) was MS supplemented with 0.5 mg L^{-1} IAA + 0.1 mg L^{-1}24-Epi. As well as shootlets failed to roots formation on MS medium supplemented with 0.025 or 0.05 mg L^{-1} 24-Epi. Moreover, there were insignificant differences among R_3, R_4, R_5, R_6 and R_7. Furthermore, the minimum value of rooting percentage (36%) was obtained with R_0. The results demonstrated that MS medium supplemented with 0.5 mg L^{-1} IAA + 0.1 mg L^{-1}24-Epi was the most suitable medium for rootlets formation on shootlets of *P. vulgaris* L. cv. Brunca compared to all tested composition media (Figure 2A and B). Moreover, shootlets produced from stem explant recorded high response to rooting media compared to shootlets produced from leaf explant, where it recorded maximum rooting percentage (88%).

About number of roots and concerning to shootlets produced from leaf explant, there were insignificant differences among R_3, R_4, R_5, R_6 and R_7. Whereas R_3, R_6 and R_7 recorded the maximum number of roots formation (7.3). While R_0 gave the lowest number of roots formation (4.1) followed by R_4 (6.2). On the other hand, concerning to shootlets produced from stem explant, the best medium that produced the maximum number of roots (8.5) was R_7. Furthermore, there were insignificant differences among R_3, R_4, R_5 and R_6. Whereas, the minimum number of roots (5) was obtained with R_0.

Concerning the shootlets produced from leaf explant, there were insignificant differences among R_3, R_4, R_5, R_6 and R_7 that recorded the maximum root length (6.4 – 7 cm). While, the shortest root (3.2 cm) obtained with R_0. On the other hand, concerning to shootlets produced from stem explant, the longest root (9.4 cm) recorded with R_7 followed by R_3, which recorded (7.4 cm). Furthermore, there were insignificant differences among R_4, R_5 and R_6. Whereas, the shortest root (4.3 cm) was obtained with R_0 (MS free growth regulators).

In general, as mentioned before it can be concluded that shootlets derived stem explants showed a highly response to rooting media compared to shootlets derived leaf explants. Culturing of shootlets derived stem explants on R_7 medium recorded the highest values (88, 8.5 and 9.4) of rooting percentage, number of roots and roots length, respectively.

DISCUSSION

Legumes are not easily amenable to stable genetic transformation and hence, protocols for high throughput generation of transgenic legume plants are not available. In general, the difficulty for achieving efficient genetic transformation of legumes is related to their low responsiveness for *in vitro* regeneration (Arellano et al.,

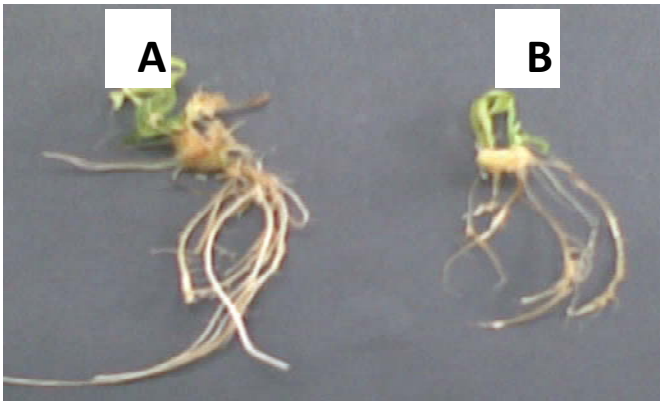

Figure 2. Rooting of regenerated shootlets derived from stem (A) and leaf (B) explants on MS medium supplemented with 0.5 mg L^{-1} IAA + 0.1 mg L^{-1} 24-Epi (5×).

Table 3. Effect of interaction between media composition and explants types on rooting, number of roots, root length and root dry weight of *P. vulgaris* L. cv. Brunca.

Culture media composition	Rooting (%)	Number of roots	Root length (cm)	Root dry weight (g/jar)
Shootlets derived leaf explant				
R_0= MS free growth regulators	33.3^{b}	4.1^{d}	3.2^{e}	0.03^{a}
R_1 = MS + 0.025 mg L^{-1} 24-Epi	-	-	-	-
R_2= MS + 0.05 mg L^{-1} 24-Epi	-	-	-	-
R_3= MS + 0.1 mg L^{-1} 24-Epi	70^{ab}	7.3^{b}	6.4^{c}	0.09^{a}
R_4 = MS + 0.5 mg L^{-1} IAA	33.3^{b}	6.2^{b}	6.4^{c}	0.07^{a}
R_5 = MS + 0.5 mg L^{-1} IAA+ 0.025 mg L^{-1}24-Epi	33.3^{b}	7.2^{b}	6.7^{c}	0.07^{a}
R_6 = MS + 0.5 mg L^{-1} IAA+ 0.05 mg L^{-1}24-Epi	71^{a}	7.3^{b}	6.4^{c}	0.09^{a}
R_7 = MS + 0.5 mg L^{-1} IAA+ 0.1 mg L^{-1}24-Epi	67^{a}	7.3^{b}	7^{c}	0.07^{a}
Shootlets derived stem explant				
R_0= MS free growth regulators	36.3^{b}	5^{c}	4.3^{d}	0.03^{a}
R_1 = MS + 0.025 mg L^{-1} 24-Epi	-	-	-	-
R_2= MS + 0.05 mg L^{-1} 24-Epi	-	-	-	-
R_3= MS + 0.1 mg L^{-1} 24-Epi	87^{a}	7.5^{b}	7.4^{b}	0.09^{a}
R_4 = MS + 0.5 mg L^{-1} IAA	68^{ab}	6.2^{b}	4^{c}	0.02^{a}
R_5 = MS + 0.5 mg L^{-1} IAA+ 0.025 mg L^{-1}24-Epi	70^{ab}	8^{b}	7^{c}	0.06^{a}
R_6 = MS + 0.5 mg L^{-1} IAA+ 0.05 mg L^{-1}24-Epi	70^{ab}	7.5^{b}	7^{c}	0.09^{a}
R_7 = MS + 0.5 mg L^{-1} IAA+ 0.1 mg L^{-1}24-Epi	88^{a}	8.5^{a}	9.4^{a}	0.07^{a}

Means having the same letters in a column were not significantly different at 0.05 level.

2009). Thus, in recent years, many research groups have been involved in establishing reliable regeneration procedures for economically important legumes, because it would be a primary step to facilitate gene introduction and improvement of the crop.

In vitro regeneration of *Phaseolus* spp. had been reported by organogenesis (Varisai Mohamed et al. 2006) or through somatic embryogenesis (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 *P. vulgaris* and other species from the *Phaseolus* genus are recalcitrant for *in vitro* regeneration (Quintero-Jiménez et al., 2010). However, earlier studies on shootlet regeneration via direct organogenesis of *P. vulgaris* L. have been reported by several workers (Ahmed et al., 2002).

This study focused on the effects of 24- Epi alone or in combinations with TDZ on *in vitro* shootlets regeneration in *P. vulgaris* L. cv. Brunca. The naturally occuring

Brassinosteroids (BRs) are a new group of plant hormones with significant growth-promoting activity. Application of BRs significantly stimulated adventitious bud formation from hypocotyl segments of cauliflower cultured *in vitro* in the light**.** Additive effect of BRs with cytokinins was also demonstrated on shoot regeneration from cauliflower hypocotyl segments (Sasaki, 2002). BRs affect regeneration potentials positively and their applications could be useful for regeneration of recalcitrant genotypes (Lu et al., 2003).

EBR (24-epibrassinolide) was suggested to be able to induce the activity of cytokinin, BRs have also been reported to be involved on branching responses and changing endogenous cytokinin levels in various plant species (Pereira-Netto et al., 2003). The efficacy TDZ on direct multiple shoot formation from *P. vulgaris* L, which is attributed that TDZ mimics the effects of cytokinins on organogenesis. Furthermore, TDZ may be involved in the synthesis and/or accumulation of cytokinins in plant tissue culture (Carvalho et al., 2000). In this regard, TDZ is considered one of the most promising cytokinin for shoot induction in legumes (Jayanand et al., 2003; Tewari et al., 2004; Kiran et al., 2005; Anwar et al., 2008, 2010). TDZ was most effective inducing healthier shoots when used at lower concentration (~2.2 mg L^{-1}) compared to higher concentrations (Jiang et al., 2005; Anwar et al., 2008, 2010).

In the present study, the obtained results clearly demonstrated that 24-Epi (at the different concentrations used) induced shootlets regeneration via direct organogenesis of *P. vulgaris* L. cv. Brunca when combined with 2 mg L^{-1} TDZ. The highest response was recorded with 2 mg L^{-1} TDZ + 0.1 mg L^{-1} 24-Epi. These findings are similar to those of Anwar et al. (2008 and 2010) and Jiang et al. (2005). Concerning regeneration from root explants, it is observed that root explants completely failed to shootlets regeneration. Therefore, Yoshida (2002) observed that too much contact of the explants into the medium may inhibit adventitious shoot formation in soybean. This may suggest that there is a considerable variation existed between the different types of explants in their ability to form shoots.

Furthermore, 24-Epi enhanced the accumulation of photosynthetic pigments content (chlorophyll *a*, *b* and carotenoids) in the regenerated shootlets. These results are in agreement with the results of Janeczko et al. (2007) and Wang and Zeng (1993). In addition, 24-Epi promoted rooting of *in vitro* regenerated shootlets. These results coincided with the results of Vardhini and Rao (2003), Kartal et al. (2009) and Vardhini et al. (2012).

Conclusion

It could be concluded that a simple, reliable and efficient regeneration system of *P. vulgaris* from stem explants was established. Thus, shoot regeneration via direct organogenesis is likely to be good for genetic transformation of this crop. The procedure presented in this study yields efficient adventitious shoots regeneration from stem-segment explants. The high frequency of shootlets (90%) may be very advantageous for a variety of purposes, including both classical and molecular breeding of *P. vulgaris*.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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