IN VITRO REGENERATION OF PHASEOLUS VULGARIS L. VIA ORGANOGENESIS FROM PETIOLE EXPLANTS

IN VITRO РЕГЕНЕРАЦИЯ ОТ ЕКСПЛАНТИ ОТ ЛИСТНИ ДРЪЖКИ НА PHASEOLUS VULGARIS L. ЧРЕЗ ОРГАНОГЕНЕЗИС

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ABSTRACT

A system for somatic organogenesis in common bean (Phaseolus vulgaris L.) was developed. Precultivation of seeds on different media was investigated for the ability to influence the process of in vitro regeneration. Leave petioles excised from in vitro derived seedlings at different ages (7 and 14 days-old), were used as primary explants. Precultivation of the seedlings on medium MS-BAP 1 μM for 7 days and dark cultivation of their leave petioles on medium MSI (2 μM TDZ, 0.6 μM NAA and 2 μM paclobutrazol) benefit the process of shoot initiation in all investigated genotypes. Shoot elongation took place on MSE (22.2 μM BAP and 0.057 μM IAA) medium. Plant recovery was established on MSG_3 (4.44 μM BAP and 0.58 μM GA_3) medium.

KEY WORDS: Common bean, in vitro regeneration, precultivation.

Abbreviations: ABA – abscisic acid; BAP 6-Benzyl-Amino-Purine; CPPU - N-(2-chloro-4-pyridyl)-N'-phenylurea (forchlorfenuron); IAA Indole Acetic Acid; IBA Indole-Butiric-Acide; GA3 Gibberellic Acid; NAA α-Naphtalene Acetic Acid; TDZ N-phenyl-N'-1, 2,3-thiadiazol-5-urea [thidiazuron].

РЕЗЮМЕ

Разработена е система за соматичен органогенезис при фасул (Phaseolus vulgaris L.). Изследвано е предкултивирането на семена върху различни среди за способността да се повлияе върху процеса на in vitro регенерацията. Листни дръжки нарязани от in vitro развити прорастъци на различна възраст (7 и 14 дневни) са използвани като първични експланти. Предкултивирането на прорастъците върху среда MS-BAP 1 μM за 7 дни и култивиране на тъмно на експлантите от техните листни дръжки върху среда MSI (2 μM TDZ, 0.6 μM NAA и 2 μM паклобутразол) подобрява процеса на иницииране на прорастъци от тях при всички изследвани генотипи. Удължаването на прорастъците се проявява на среда MSE (22.2 μM BAP и 0.057 μM IAA). Растенийца се развиват на среда MSG_3 (4.44 μM BAP и 0.58 μM GA_3).

KEY WORDS: Фасул, in vitro регенерация, предкултивиране.

Abbreviations: ABA – абсцизиева киселина; BAP 6-бензил-амино пурин; CPPU - N-(2-хлоро-4-пиридил)-N'-фенил карбамид (форхлорфенурон); IAA индол оцетна киселина; IBA индол-бутирова киселина; GA3 гиберелова киселина; NAA α-нафтален-оцетна киселина; TDZ N-фенил-N'-1, 2,3-тидиазол-5-карбамид [тидиазурон].

DETAILED ABSTRACT

Full success of in vitro cultivation and regeneration can be achieved in plant experiments with continuous repeats, i.e. when a good regeneration system is created.

Es a result from our investigations a good system for somatic organogenesis in common bean Bulgarian varieties Plovdiv 10, Plovdiv 11M and Dobroudjanski 7 was developed. Studied varieties have good regeneration capacity. They showed genotype dependent reactions. Best results were obtained with variety Plovdiv 11M.

Precultivation of seeds on BAP, CPPU, IAA, ABA and Fluridone containing media was investigated for the ability to influence the process of in vitro regeneration. Leave petioles excised from in vitro derived seedlings at different ages (7 and 14 days-old), were used as primary explants. Different concentrations of TDZ, NAA and Paclobutrazol applied separately or in combinations to MS media were evaluated to induce in vitro bean regeneration. Precultivation of the seedlings on medium MS-BAP 1 μM for 7 days and dark cultivation of their leave petioles on medium MSI (2 μM TDZ, 0.6 μM NAA and 2 μM paclobutrazol) benefit the process of shoot initiation in all investigated genotypes. Our data have shown that precultivation on BAP - contained medium is essential for improvement of regeneration ability. BAP might be not the factor determining the process of regeneration. It could be presumed that BAP increase regeneration ability through stimulation of the competent cells division in the tissues. Regeneration potential can be increased if more competent cells are produced (multiplied) in one tissue. By precultivation of seedlings on MS medium supplemented with 1μM BAP an significant increase (5-7 times) of the regenerant's number per explant was determined for all studied varieties, comparing to the control medium - MSO, without hormones.

Shoot elongation took place on MSE (22.2 μM BAP and 0.057 μM IAA) medium. Plant recovery was established on MSG_3 (4.44 μM BAP and 0.58 μM GA_3) medium.

INTRODUCTION

Common bean is among of the most cultivated species in family Leguminosae. Classical breeding is the basic approach for production of the widespread varieties. Some problems based on the less genetic variations, low surviving ability of the interspecific hybrids, specific inheritances of some value characteristics as yield, disease and pests' resistance, harvesting characteristics, etc. are difficult or time and labor cost to be resolve by the conventional techniques. Plant biotechnology offers different strategies to overcome these difficulties.

With some exemptions, species belonging to Leguminosae are difficult to regenerate in vitro. Grain legumes have less regeneration potential compared to the forages one. Regeneration ability depends on the genotype, physiological state of the explant, tissue and cell specialization of the culture and the cultivation conditions [2, 5, 13].

Several systems for bean regeneration were published [5, 12]. However, the regeneration protocols are with low repeatability and due to the specific individual genotypes. Towards to increase the regeneration efficiency has to be concentrated on the control of the competence of the explants ("time window" - 2). Modification of the physiological state of the initial plants is one of the approaches able to alter in vitro answer [5]. Theoretical expectation of a precultivation of parent plants on phytohormonal's medium is modification of in vitro answer as a result of break of endogenous hormonal ratio, induction of other physiological changes or genetics changes.

A protocol for direct somatic embryogenesis in dry bean was developed based on precultivation of parent plants on the medium supplemented with CPPU [5] and BAP [7]. Zhang et al. [13] also used BAP precultivation for development of efficient system for regeneration.

Aim of this investigation was:

i) to develop a system for in vitro regeneration in common bean;

ii) to determine the factors influencing the process of in vitro regeneration;

MATERIAL AND METHODS

Initial material and explant preparing

Three different genotypes were investigated for their in vitro ability to regenerate plants. Seeds from Bulgarian common bean varieties - Plovdiv 10, Plovdiv 11M and Dobroudjanski 7 were sterilized by routine procedure [10]. Sterile seeds were cultivated on MS media differing in their hormonal compositions. Six media were investigated for possibility to alter in vitro regeneration - MS-BAP 1 μM, MS-BAP 100 μM, MS-IAA 1 μM, MS-CPPU 1 μM, MS-ABA 1 μM, MS-Fluridone 1 μM (Fig. 1). Leave petioles excised from in vitro plants at different age (7- and 14-days old) were used as primary explants. Precultivation of the seedlings were carried out in growth chamber at 16/8 hours photoperiod, 2500 Lx light intensity, 24° C temperature and 70% humidity.

Induction of organogenesis from petiole explants in common bean

Petiole explants, coming from seedlings germinated on

control variant - medium MSO, were dark cultivated on MS medium supplemented with different concentrations of TDZ, NAA and Paclobutrazol, applied alone or in combinations (Table 1).

Table 1: Investigated combinations of TDZ, NAA and Paclobutrazol on the process of somatic organogenesis in common bean

Componentes **Media**	TDZ	NAA	Paclobutrazol
MSI1	2 µM	0.3 µM	-
MSI2	2 µM	0.6 µM	2 µM
MSI3	4 µM	0.3 µM	2 µM

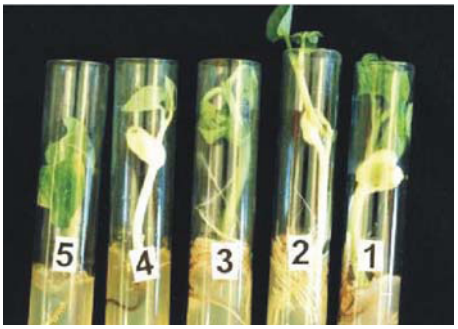

Figure 1: Precultivation of seeds from Bulgarian common bean variety Plovdiv 11 M
on media supplemented with different hormones:
1- CPPU; 2 - ABA; 3 - BAP_1; 4 - Fluridone ; 5 – BAP_{100}

Ten explants per petri dish in 5 replicates were designed for all variants. Shoot initiation was carried out on dark, at 26° C temperature for two transfers (two fresh media changes) of four weeks each one. The best variant for shoot induction was chosen to reveal the influence of the precultivation of seeds on in vitro answer.

Shoot elongation and plant recovery

Initiated shoots were elongated on MSE media - MS basal enriched with 5 mg/l BAP and 0.001 mg/l IAA. That medium was estimated as optimal in our previous experiments (unpublished data). Shoots were cultivated in jars (volume 200 cm^3) in average of 5 initiated explants per container for four weeks in the standard conditions of the growth chamber - 24° C temperature, 70% humidity, 16/8 hours photoperiod and 2500 Lx light intensity.

Elongated shoots were detached from the primary explants and transferred to the conversion medium – MSG_3 - MS supplemented with 1 mg/l BAP and 0.2 mg/l GA_3 where they rooted.

Histological analysis

Pieces of initiated explants were fixed temporary in mixture of Chamberlain (5 vol. formaline : 90 vol. C_2H_5OH : 5 vol. acetic acid) in intervals of one week. Paraphine preparatus were prepared, colored by shematoxiline of Gomory [8].

Results and discussion

Analysis of the results concerning optimization of the process of organogenesis induction by the explant age revealed considerable role of this factor. It was estimated that in all genotypes, explants arising from 7-days old plants are competent for in vitro regeneration, while explants from older plants (14-days old) are non-regenerable at the investigated conditions. A lot of authors confirm the determinative role of explant´s type and age in this process [5; 9].

It was found that organogenesis is limited around the embryonic tissues - pedicells from flowering buds [5], cotyledons [7; 11], embryonic axis [7] etc. It could be assumed that the cells of young petioles (7-days after germination) are still competent for regeneration and after this period the cells loose the regeneration ability.

We investigated (data are not shown) different cytokinins, suggested in already published protocols for bean regeneration - CPPU [5], BAP [3; 11], kinetine [1], 2iP [4] etc. Our preliminary results indicated that TDZ benefits the process of organogenesis. Four weeks after the initiation of the explants on MSI media started callus formation. Type of the callus differs according to the type

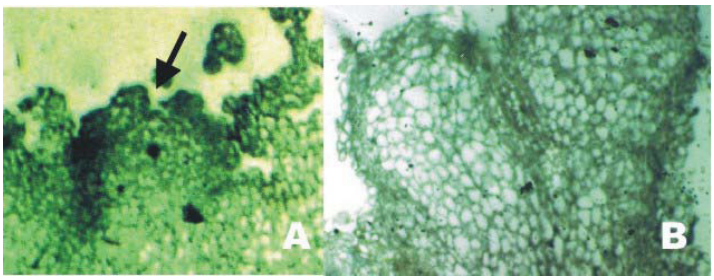

Figure 2: Histological cuttings of regenerable callus:
A- formation of granulated callus; B – shoot's formation

and concentration of applied growth regulators.

Regenerable callus was estimated to be brown-green, with granulated structures. Histological analysis exhibits that such callus is consisted from developing clusters of shoots instead of unorganized callus cells (Fig. 2 A-B).

The highest total number of shoots was induced when 2 μM TDZ in combination with 0.6 μM NAA and 2 μM Paclobutrazol (MSI2) were applied (Table 2). A best result on the same medium according to the total number of shoots per explant was observed when variety Plovdiv 11M was utilized as a donor of explants (3.11 shoots per explant). Most of the initiated shoots proliferated lather callus cells on their surface or got necroses and degenerated after transfer to new medium. Shoot elongation was inhibited. Paclobutrazol stimulated callus proliferation, however, shoot induction was suppressed when MSI 3 medium was used (Table 2).

Only 10.34 % of the explants give rise of shoots and only 6.89% of the initiated shoots regenerated plants from all varieties.

To modify physiological state of the explant and to find some determinative factors affecting the process of regeneration a precultivation of the initial seedlings on different hormonal media was carried out (Fig. 3, A-D).

Morphological differences were observed during the precultivation of the initial seedlings on media supplemented with different hormones. Seedlings developed on medium MS-BAP 100 μM were greener with shorter internodes and more fate leaves and steams. Precultivation of the seedlings on MS supplemented with 1 μM ABA or 1μM BAP stimulated the process of somatic organogenesis in all investigated varieties (Fig. 3, A-C). Precultivation of the seedlings from variety Plovdiv 10 on 1 μM BAP containing medium increased 3.3 times total number of shoots and 3 times shoots per explant. Similar results were also found for the other varieties – Plovdiv 11M and Dobroudjanski 7. According to the total number of initiated shoots and shoots per explant, no big differences were observed when seedlings from all varieties were precultivated on MS-BAP 1μM or MS-ABA medium (Fig. 3, A-B).

Precultivation on BAP - contained medium was essential for improvement of regeneration ability (Fig. 3, C). It could be presumed that BAP is not the factor determining the process of regeneration. BAP increase regeneration ability through stimulation of the competent cells division in tissues. As more competent cells are produced in one tissue, so the regeneration potential will be higher.

Total number of shoots was decreased after precultivation on Fluridone containing medium. Comparing to the control (MSO), no differences were observed in regenerants per explant (Fig. 3, C) when precultivation was carried out in media supplemented with CPPU or Fluridone. The process of shoot elongation was inhibited and induced shoots either developed abnormal roots or produced callus on the surface of the upper part, or died soon after transferring on the elongation media when seedlings were precultivated on medium supplemented with Fluridone or CPPU (Fig. 3, D).

Successful elongation of initiated shoots was released from BAP 1μM (or ABA) precultivation variants on BAP and IAA containing (MSE) medium. Establishment of the factors controlling the process of elongation was provided in separate experiment. Shoot elongation was stimulated on MSE medium supplemented with 5 mg/l BAP and 0.001 mg/l IAA.

Plant recovery was easily established on MSG_3 medium, where elongated shoots formed first leaves and rooted also. The highest total number of regenerants was produced after 1 μM BAP precultivation of the initial plants. ABA - precultivation affected significant plant conversion. An average of 4 regenerated plants per explant for all studied varieties was observed after precultivation on ABA containing medium (Fig. 3, C).

Callus, shoots and regenerants are presented on figure 4, A-F.

Table 2: Influence of induction medium on in vitro regeneration of three different Bulgarian common bean varieties

Varieties	Total number of shoots			Shoots per explant		
	MSI 1	MSI 2	MSI 3	MSI 1	MSI 2	MSI 3
Dobroudjanski 7	7.2	26	0	0	1	0
Plovdiv 11M	8.7	29	0	0	3.11	0
Plovdiv 10	8.1	27	0	0	2	0
	Total number of regenerants			Regenerants per explant		
Dobroudjanski 7	0	2	0	0	1	0
Plovdiv 11M	0	1	0	0	1	0
Plovdiv 10	0	2	0	0	1	0

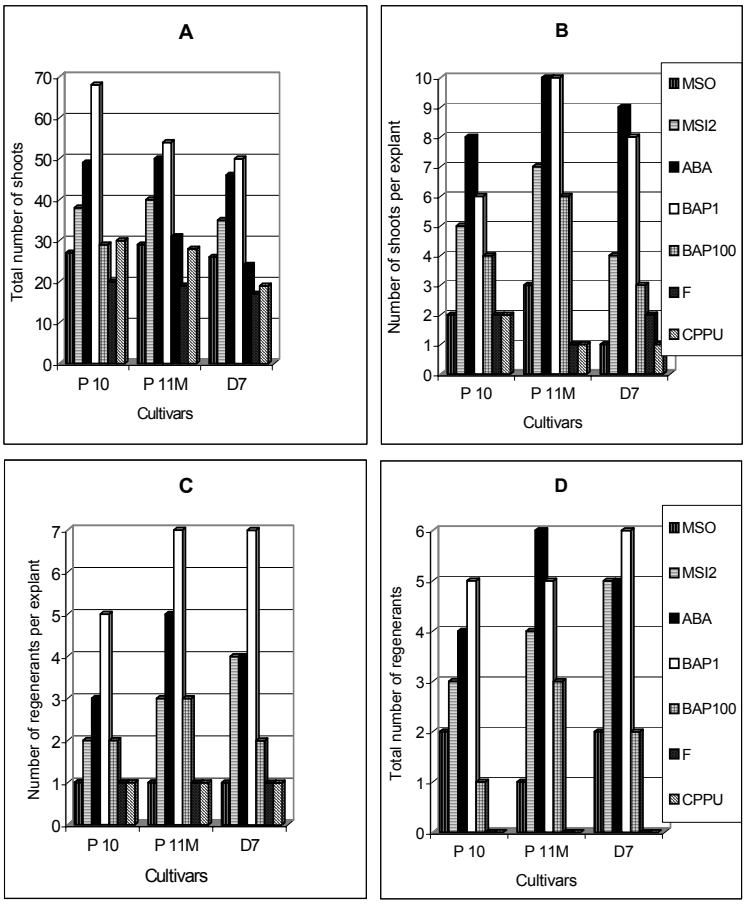

Figure 3: Regeneration possibilities of Bulgarian common bean cultivars: Plovdiv 10 (P 10); Plovdiv 11M (P 11M) and Dobroudjanski 7 (D 7).
A – total number of shoots; B – number of shoots per explant;
C – number of regenerants per explant; D – total number of regenerants.

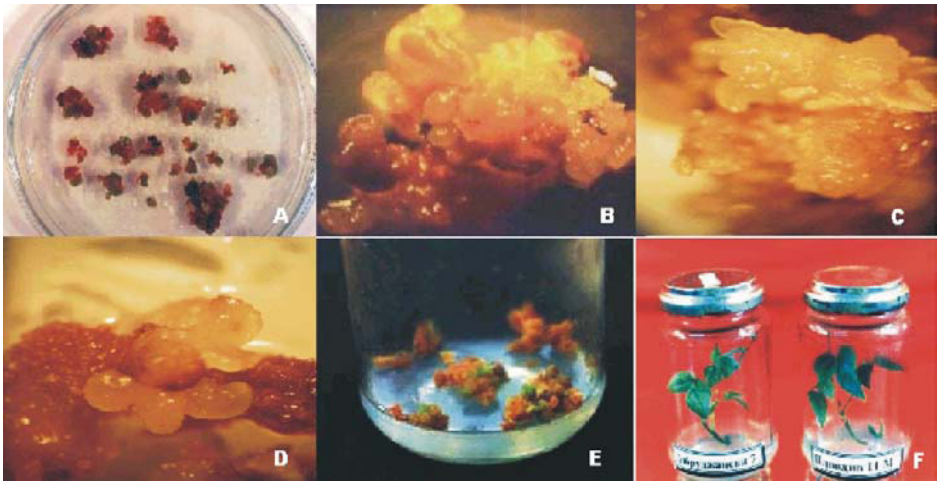

Figure 4: Regeneration of common bean from callus culture:
A – E callus and induced shoots; F – regenerants ready for adaptation

CONCLUSIONS

On the base of conducted investigations can be concluded that:

1. A system for somatic organogenesis was developed which included next steps:

- precultivation of seedlings on medium MS-BAP 1μM for 7 days;
- dark cultivation of leave petiole explants on MSI 2 medium for four weeks (two transfers on fresh medium);
- shoot elongation on medium MSE for four weeks (two transfers on fresh medium);
- plant recovery on medium MSG 3 for four weeks (two transfers on fresh medium).

2. Studied Bulgarian common bean varieties Plovdiv 10, Plovdiv 11M and Dobroudjanski 7 have good regeneration capacity. They showed genotype dependent reactions. Best results were obtained with variety Plovdiv 11M.

3. Precultivation of seedlings on MS medium supplemented with 1μM BAP, comparing to the medium without hormones (MSO) increased 5-7 times the number of regenerants per explant for all studied varieties.

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