RESEARCH ARTICLE

Georgina P. Kosturkova [1]
Margarita S. Dimitrova [1]
Rositsa V. Todorova [2]
Krasimira N. Tasheva [1]
Peter I. Petrov [1]
Shashank Tidke [3]
Ravishankar A. Gokare [3]

Authors' addresses:
[1] Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Sofia, Bulgaria.
[2] Soybean Experimental Station, Agricultural Academy, Pavlikeni, Bulgaria.
[3] Dayananda Sagar Institutes, Bangalore, India.

Correspondence:
Georgina P. Kosturkova
Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Sofia, Bulgaria.
Email: georgina_kosturkova@abv.bg

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Soybean long-term callus cultures – potential for biotransformation and nutraceutical production

ABSTRACT

Soybean (*Glycine max* (L.) Merrill.) is the world leading cultivated pulse crop as a source of protein, oil and nutraceuticals. Recently, alternative approaches for the synthesis of new products gain bigger interest. Biotransformation based on *in vitro* cultures is well-established technology for such alternative production. In this respect initiation of callus and long-term cultures were established from four Bulgarian soybean varieties. The potential for callus development of cotyledons and cotyledonary nodes plated on media containing various combinations of kinetin (0.1 – 1.0 mg/l) and 2,4-D (0.5 – 1.0 mg/l) was tested. The optimal concentrations for initiation and maintaining of nondifferentiated growth for most of the varieties were twice higher levels of the cytokinin to the auxin. Two of the Bulgarian genotypes (Rossa and Srebrina) revealed better potentials for callogenesis and long-term growth. These are promising for the development of suitable procedures for biotransformation.

Key words: Soybean, *Glycine max*, callus, long-term cultures, biotransformation

Introduction

Grain legumes have been substantial part of the mankind development with evidence for their use since neolithic epoch. However, in the middles of the last century, these crops have become less attractive, especially in Europe and North America, due to increased consumption of meat and promotion of cereal monocultures (Hedley, 2001). Recently growing interest in more healthy diets has turned the focus to grain legumes providing wholesome food. Nowadays grain legumes cultivation attracts more interest due to their potential to solve some environmental problems providing new ecology friendly materials and technologies. Among this group of plants, soybean is distinguished as the most important representative. *Glycine max* (L.) Merrill.) is an annual species widely cultivated in the hot regions of all continents. It is the most valued grain legume in the world with multiple uses as human food and animal feed, as a natural fertilizer and as a raw material for the industry for the production of fuel, fibers, carbohydrates, clues, etc. (Kosturkova & Mehandjiev, 2002; Kosturkova, 2003; Krezhova, 2011).

Soya is considered to be the most important edible plant providing more than 50 % of the phytoprotein and 30 % of phytooils for human consumption and 70 % protein for animal meals (Georgiev, 2005, Todorova & Kosturkova, 2010). High levels of isoflavones, folic acid, vitamins and minerals make the soybean seeds preferable component for wholesome food and health-based nutrition programs, and increase its nutraceutical potential (Sakthivelu et al., 2008a; Shashank et al., 2015; Kuligowski et al., 2017).

Recently, alternative approaches for biosynthesis of new products gain bigger interest, too. Plant *in vitro* cultures are a well-established technology for such alternative production of new substances, especially for those with complex structures (Guen & Knorr, 2011). Biotransformation and elicitation make the possible modification of chemical compounds by isolated plant cells and tissues. The development of powerful new "omics" technologies has extended the potential of the plant cell cultures to be obtained

high-value-novel metabolites. The advanced knowledge of secondary metabolism pathways opened the possibility of transforming inexpensive, available and plentiful products into rare and expensive ones (Ochoa-Villarreal, 2016). In addition, bioconversion capacity of cell cultures could be improved by genetic engineering like transfer of genes and different genetic manipulations like mutagenesis and cell selection.

Plant cell and tissue cultures, and various in vitro manipulations, are the basis for nowadays plant biotechnologies. No matter how sophisticated might be the upper level techniques like genetic transformation, cell and mutagenesis selection, biotransformation or alternative reactor biosynthesis, the first step is to obtain cell division leading to sustainable callus growth or organogenic tissues formation for regeneration of new plants. No matter that successful *in vitro* cultures and the first plant regeneration were achieved in the middle 50s of XXth century, there is no universal culture protocol. *Glycine max* is one of the species which gained interest in the form of plant biotechnologies. Since the pioneer work for initiation of cell and callus culture (Gamborg et al., 1968), embryolike structures (Beversdorf & Bingham, 1977), shoot formation (Cheng et al., 1980), and morphogenesis at different level from cell, anther and protoplast cultures (Barwal & Widholm, 1990) a lot of progress has been done exploiting one of the most modern technique e.g. genetic transformation (Zia, 2011, Muthukrishnan et al. 2016). Production of valuable metabolites by soybean callus suspension cultures were reported, too (Gueven & Knorr, 2011). Despite development of new protocols for callus cultures and plant regeneration (Radhakrishnan & Ranjithakumari, 2007; Akita Devi et al, 2012; Rathod et al., 2017) search for reliable protocol is constant process, as far as, *in vitro* processes depend on culture medium, growth regulators, explant type and age, genotype and environmental conditions (Kosturkova, 2000).

In this respect initiation of callus and long-term cultures optimization were carried out. Objectives of the presented research were to study the potential of Bulgarian soybean varieties for callus formation and further maintaining of nondifferentiated cell division for the establishment of long-term callus cultures which could be a basis for experiments in biotransformation for production of nutraceuticals and other valuable substances.

Materials and Methods

Plant material

Bulgarian soybean (*Glycine max* L.) varieties were developed in the Experimental Station of Soybean, Pavlikeni. The object of the present investigations cv "Rossa", cv "Daniela", cv "Richy" and cv "Srebrina" were obtained by induced mutagenesis and/or hybridization (Todorova & Goranova, 2009). The American variety Hodgson and the Bulgarian variety Daniela were used as world and national standards.

In vitro experiments

Seeds were washed with liquid soap surface disinfected by dipping in 70% v/v ethanol for 1 min, followed by 30% v/v commercial bleach ACE and rinsed three times in autoclaved distilled water. Seeds were germinated on solid basic MS medium (Murashige & Skoog, 1962) without plant growth regulators.

Explants from cotyledons and cotyledonary nodes from seedlings obtained from mature soybean seeds grown in vitro were used. Explants were excised from seedlings 6th to 8th days after initiation of in vitro cultivation on basic MS medium (Fig 1). Cotyledon explants were plated with the basal side down in contact with the medium surface. The seedling cotyledonary nodes (after removal of the cotyledons and the stem apex) were dipped 3-5 mm into the medium.

Figure 1. *Explant excision.*

Three culture media were used for induction and maintaining of callogenesis. All media contained MS macrosalts, microsalts and vitamins, 20 g/l sucrose and 7 g/l agar-agar. Media were supplemented with different concentrations of the cytokinin kinetin (N6-furfuryladenine) and the auxin 2,4-D (2,4-Dichlorophenoxyacetic acid) as follows: 1 mg/l kinetin and 0.5 mg/l 2.4-D, designated as Ca1 medium; 0.5 mg/l kinetin and 1 mg/l 2,4-D, designated as

Ca2; 0.1 mg/l kinetin and 0.5 mg/l 2,4-D, designated as Ca3. Thirty to fifty explants/calli were tested in each experimental set. Pieces of 0.3 g callus tissue were used in the tests for sustainable growth. The growth rate was calculated as the ratio of the fresh weight of callus obtained after one month subculturing to the initial fresh callus weight. Results were scored after one month of tissue development and were subjected to statistical analysis using Sigma plot.

All cultures were maintained at 25±1 ^{0}C under 16 h illumination (40 μmol$m^{-2}s^{-1}$).

Histological experiments

Callus pieces were fixed in 3 % glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) and embedded in low viscosity Spurr's epoxy-resin. Semi-thin cross sections cut on an ultramicrotome Tesla BS 490 (Czech Republic) were stained with 0.01 % (w/v) toluidine blue and observed under a light microscope Carl Zeiss, Jena (Germany). Microscopic images were captured and saved on a digital image processor (International Micro-Vision Inc., Redwood City, CA, USA).

Results

Explants from all tested genotypes after being plated on the culture media grew in size up to 4-5 folds before formation of callus (Fig. 2a). New nondifferentiated tissue appeared usually at the cutting surface (Fig. 2b). Small clusters (about 1-2 mm^3) of white cells could be seen on the surface of the cotyledons, more often of cv "Srebrina" (Fig. 2c). However, these formations did not grow further developing necrosis. In the case of cotyledonary nodes, callus appeared on the basal side of the stem cutting and spread to the nearby surface tissues. Callus was formed from all genotypes with different frequency and efficiency. The potential of the both examined explants to callogenesis on the first tested medium Ca1 is presented in Table 1. Formation of callus from cotyledons ranged from 35 % to 90 %, while those from cotyledonary nodes ranged from 50 % to 100 %, depending on the variety with highest values for cv "Rossa", "Richy" and "Srebrina". Callus growth was explant and genotype dependent, too. Fresh tissue weight from cotyledons reached values of 1.15 - 2.5 g within a month of cultivation while these values for cotyledonary nodes were from 0.22 g to 0.43 g. Varieties "Srebrina", "Rossa" and "Daniela" had a better growth rate.

The characteristics of callus obtained from the both explants showed differences concerning structure, texture and colour. Cotyledon callus was more compact, greenish to pale (Fig 3a), while that one of cotyledonary nodes was grained, fragile and pale to brownish (Fig 3b).

A B C

Figure 2. *Induction of callus on: cotyledon cutting edge (A); cotyledonary node cutting edges (B); cotyledon surface (C).*

A B

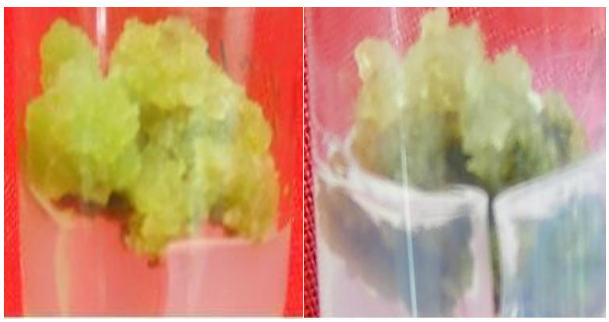
Figure 3. *Callus obtained from: cotyledons (A) and cotyledonary nodes (B).*

Results presented above demonstrated callogenesis potential on medium Ca1 where the ratio of cytokinin to auxin is 2:1. In the next series of experiments, the medium for callus induction contained the opposite ratio 1:2 of e.g 0.5 mg/l kinetin and 1 mg/l 2,4-D. The latter medium was not efficient like the first one – Ca1 (Table 2) neither for initiation of callus (20-50 %) nor for its growth (0.5 -0.8 g for cotyledons, and 0.10-0.22 g for cotyledonary nodes). Better results for callus induction and growth were not obtained on medium Ca3 (data not shown).

These observations were confirmed in the parallel experiment when pieces of calli developed on the best induction medium Ca1 were transferred to the three culture media (Table 3). The growth of calli from the four tested varieties was the most vigorous on Ca1 medium (Fig. 4a) followed by Ca2 medium (Fig. 4b). On these media, cell mass increased 6-8 folds and 4-7 folds, respectively, while mass accumulation on Ca3 medium was only 2-3 folds. Comparing potential for callus production varieties could be ranged as follows: "Srebrina", "Rossa", "Daniela" and "Richy".

Further growth of callus was studied after one month subculture of obtained calli on their initiation media (Table 4). Fresh weight from cotyledon callus increased from 1.2

folds to 9.1 fold on Ca1 medium, and 2.1 to 5.4 folds on Ca2 medium, and 1.5 to 5.1 on Ca3 medium. Growth response among varieties was best for "Rossa", "Srebrina" and "Daniela". Values for cotyledonary nodes were lower than those of cotyledons but demonstrated similar trends of performance of the media and the varieties.



A **B**

Figure 4. Vigorous callus growth from cotyledons on media: Ca1 (A) and Ca2 (B).

Table 1. *Callogenesis induction on Ca1 medium from explants excised from soybean in seedlings cultured vitro.*

Variety	**Callogenesis on the 30th day of cultivation of:**		**Mean fresh weight of callus from:**	
	Cotyledons [%]	Cotyledonary nodes [%]	Cotyledons [g ± SD]	Cotyledonary nodes [g ± SD]
Hodgeson	50	80	1.20 ± 0.14	0.310 ± 0.04
Daniela	35	50	1.25 ± 0.13	0.250 ± 0.03
Srebrina	70	100	2.50 ± 0.28	0.435 ± 0.05
Rossa	90	100	1.15 ± 0.13	0.280 ±0.04
Richy	80	100	1.30 ± 0.12	0.220 ± 0.03

Table 2. *Callogenesis induction on Ca2 medium from explants excised from soybean seedlings cultured in vitro.*

Variety	**Callogenesis on the 30th day of cultivation of:**		**Mean fresh weight of callus from:**	
	Cotyledons [%]	**Cotyledonary nodes [%]**	**Cotyledons [g ± SD]**	**Cotyledonary nodes [g ± SD]**
Hodgeson	25	40	0.65 ± 0.12	0.10 ± 0.01
Daniela	20	35	0.52 ± 0.10	0.15 ± 0.02
Srebrina	45	50	0.73 ± 0.14	0.22 ± 0.03
Rossa	25	50	0.80 ± 0.12	0.12 ± 0.01
Richy	33	33	0.58 ± 0.10	0.20 ±0.02

Table 3. *Growth of callus obtained on Ca1 medium and transferred on three media for nondifferentiated cell division for one month*

Variety	**Mean fresh weight [g ± SD] of callus grown on:**		
	Ca1 medium	**Ca2 medium**	**Ca3 medium**
Daniela	2.524 ± 0.30	1.670 ± 0.14	0.660 ± 0.09
Srebrina	2.687 ± 0.35	2.256 ± 0.24	0.758 ± 0.11
Rossa	2.725 ± 0.42	1.830 ± 0.22	0.880 ± 0.10
Richy	1.804 ± 0.24	1.250 ± 0.15	0.540 ± 0.09

Table 4. *Callus growth rate after a month of cultivation on the same medium for initiation of callus.*

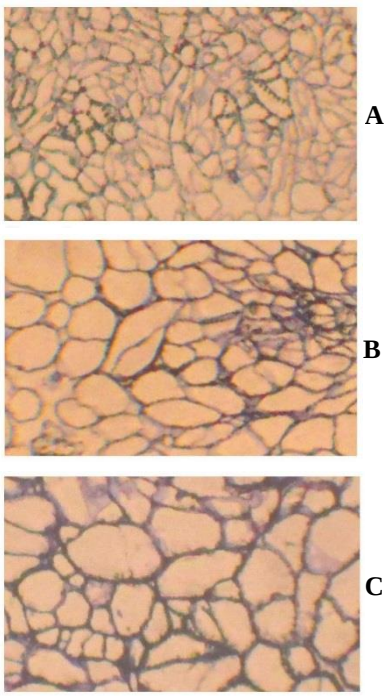
	Callus growth rate from cotyledons [min-max values] on			Callus growth rate from cotyledonary nodes [min-max values] on		
	Medium Ca1	**Medium Ca2**	**Medium Ca3**	**Medium Ca1**	**Medium Ca2**	**Medium Ca3**
Daniela	3.5 – 5.6	2.5 - 4.4	1.5 – 2.3	2.2 – 4.6	2.5 – 4.1	2.0 – 4.5
Srebrina	2.3 – 6.2	2.7 – 5.4	2.2 – 4.2	3.4 – 5.1	2.8 – 4.5	3.1 – 5.2
Rossa	3.2 – 9.1	2.1 – 4.5	1.8 – 5.1	3.2 – 5.8	2.8 – 5.4	4.0 – 6.4
Richy	1.2 – 7.8	2-2 – 4-8	1.5 – 2.5	2.2 – 3.8	2.1 – 4.5	2.4 – 4.4

Nondifferentiated cell division was monitored monthly for several passages on medium Ca1. During the 3rd passage, cotyledonary callus increased its mass up to 6 folds reaching 1.840 g, 1.860 g, 2,480 g and 2.200 g for "Daniela" "Srebrina", "Rossa", and "Richy", respectively. During the 4th passage, these values were 2.890 g, 3.250 g, 3.620 and 2.980 g respectively. The growth rate was monitored up to the 10th passage and calli clones with a fast accumulation of mass (15 - 20 folds) were identified (Fig. 5). Thus, long-tern callus cultures were obtained which have been maintained for more than two years.

A **B**

Figure 5. *Long-term callus cultures (5th passage on Ca1 medium) initiated from cotyledons of varieties: "Rossa" (A) and "Srebrina" (B).*

Histological analysis of callus obtained on different media showed that primordia like structure could be seen even after a month of cultivation of nondifferentiated tissue originated from cotyledons on Ca1 medium (Fig 6a). Clusters of meristematic cells were observed in callus tissues growing on Ca2 medium (Fig. 6b). Less small cells, amenable to division, were recognized after the longer period of six weeks of cultivation on medium Ca1 (Fig. 6c). This suggested that the shorter period of subculture could be more appropriate if the differentiated processes are desired. However, a period of thirty to forty-five days is convenient for transfer to fresh medium.



A

B

C

Figure 6. *Cell morphology of callus formed on media: Ca1 (A) and Ca2 (B) after four weeks of subculture and medium Ca1 (C) after six weeks subculture.*

Discussions

In vitro response revealing callogenesis potential depends on many factors but, explant type, culture media and genotype are substantial. Various plant and seed parts were used to initiate in vitro cultures but seedling cotyledons and cotyledonary nodes were the most exploited and reported for high efficiency for callogenesis organogenesis (Kosturkova, 2000, Akita Devi et al., 2012, Rathod et al., 2017, Zia 2011). In the present study, cotyledonary node response to nondifferentiated tissue development was higher (50-100 %) compared to that of cotyledons (35-90 %). However, the growth of cotyledon callus was faster reaching 1.15 - 2.5 g of fresh weight vs 0.22 - 0.43 g callus from cotyledonary nodes. Variety "Srebrina" demonstrated highest potentials

for callogenesis compared to the other four varieties.

The efficiency of callus induction depended not only on explant type but on the various plant growth regulators and their concentrations in the media. Silva et al. (2003) report that the use of 2,4-D 1.0 mg/l and kinetin 0.1 mg/l provided the highest average weight of cotyledons calli fresh matter, whereas the use of 2,4-D 2.0 mg/l and kinetin 0.1 mg/l provided the highest average weight of hypocotyl calli fresh matter. Sakthivelu et al. (2008 b) induced formation of callus from hypocotyl explants on MS basal medium supplemented with 1 mg/l 2,4-D and 0.5 mg/l kinetin. Radhakrishnan & Ranjithakumari (2007) observed callus initiation from half seed explants on B5 medium supplemented with kinetin (4.7 µM to 23.5 µM), 6-benzyladenine (4.4 µM to 22.2 µM), naphthaleneacetic acid (5.4µ M to 27.0 µM), indolebutyric acid (4.9 µM to 24.5 µM) and 2,4- dichlorophenoxyacetic acid (4.5 µM to 22.5 µM) but the highest cell proliferation was obtained in the presence of 13.3 µM BAP and 13.5 µM 2,4-D. Joyner et al. (2010) obtained abundant amounts of calli from cotyledons on media enriched either with 2,4-D or with 2,4-D and NAA combined.

Results presented here coincide with the results of other authors for the role of auxins and cytokinins for initiation and proliferation of callus. Our data demonstrated that callogenesis potential was best revealed on medium Ca1 where the concentration of the cytokinin to the auxin was twice higher than that of the auxin. This medium was more efficient for maintaining cell division and accumulation of biomass thus answering to one of the goals of the experiments e.g to study the possibility to obtain a sustainable division of nondifferentiated cells and long-term callus cultures.

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