IN VITRO REGENERATION POTENTIAL OF SEVEN COMMERCIAL SOYBEAN CULTIVARS (*Glycine max* L.) FOR USE IN BIOTECHNOLOGY

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Abstract: This work is aimed to evaluate *in vitro* regeneration potential of seven commercial soybean varieties Bohemians, Cardiff, Gallec, Merlin, Moravians, Naya and Silensia (*Glycine max* L.) cultivated in Central Europe. Our results showed the half-seeds could be effectively used as an explant source for all tested cultivars. The regeneration was initiated on the media containing growth regulators 1.67 $mg.l^{-1}$ BAP and 0.25 $mg.l^{-1}$ GA_3. Within the first five days culture, green chlorophyll-containing explants were observed with frequency from 18.3% to 55.9%. Two weeks later, the explants responded by production of calli with the efficiency up to 83.0%. First shoots appeared after 2-3 weeks of subculture on the media. The soybean regeneration showed to be genotype-dependent with variable efficiencies from 5.7% (cv. Naya) to 37.7% (cv. Gallec). The cultivars Cardiff, Merlin and Gallec appear to be the most promising candidates for further biotechnological use. Application of antioxidants such as L-cysteine, dithiothreitol and sodium thiosulfate does not have effect on the explant regeneration for the first five days.

Key words: antioxidants, half-seeds, chlorophyll fluorescence, in vitro, soybean

1. Introduction

Soybean (*Glycine max* (L.) Merrill) is one of the major legume crops in the world. The seeds are important source of protein and edible vegetable oil for livestock, human consumption and industrial sector. Soybean is one of the few plants that contain all eight amino acids essential for human health. The consumption of soybean reduces cancer, cholesterol, osteoporosis and heart diseases (BIRT *et al*., 2004). Because soybean has been growing for many centuries around the world under

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different climatic conditions, there is a wide range of soybean varieties available. The major producers are the United States, Brazil and Argentina.

The first genetically modified (GM) Roundup Ready soybeans were planted in the USA in 1996. In 2011-2012 soybeans were grown on about 30 million hectares in the USA, with Roundup Ready GM soy contributing 93–94% of the production (BØHN *et al*., 2014).

The successful application of genetic engineering in plant improvement is dependent on availability of the efficient *in vitro* regeneration protocol. The regeneration of soybean has been reported using immature embryos (BARWALE *et al*., 1986), immature cotyledons (ISHIMOTO *et al*., 2010), hypocotyls (WANG and XU, 2008), mature cotyledonary nodes (LIU *et al*., 2008) or half-seeds (PAZ *et al.,* 2006). However, the regeneration efficiency was variable depending on the genotype and the explant type used (reviewed by VERMA *et al*., 2014).

The half-seeds represent alternative cotyledonary explants derived from mature soybean seeds. The half-seed system has been developed as a demand for elimination of deliberate tissue wounding prior *Agrobacterium* infection (PAZ *et al*., 2006). Using the half-seeds as an explant source, the regeneration efficiency and the number of obtained transgenic plants were 1.5-fold higher (PAZ *et al*. 2006) compared to the cotyledonary node method (PAZ *et al*. 2004).

Many plant tissue cultures respond to the culture manipulation by oxidative browning and necrosis that typically result in poor regeneration (reviewed by DAN *et al*. 2008). Beside, tissue browning is associated with plant defence response to infection with *Agrobacterium* (KUTA and TRIPATHI, 2005). Several studies reported that adding antioxidants into the culture media can considerably improve plant regeneration (reviewed by VERMA *et al*., 2014). Adding of L-cysteine and other thiol compounds sufficiently inhibited wound- and *Agrobacterium*-induced responses of soybean cotyledonary node cells and increased the number of regenerated transgenic plants (OLHOFT and SOMERS, 2001; OLHOFT *et al*., 2001). Therefore, thiol compounds are common part of the regeneration media in soybean cotyledonary node and half-seed transformation and regeneration systems (PAZ *et al*., 2004; PAZ *et al*., 2006; WANG and XU, 2008; KIM *et al*., 2012).

In this work, seven commercial soybean varieties Bohemians, Cardiff, Gallec, Merlin, Moravians, Naya and Silensia were studied for their ability to regenerate *in vitro.* The half-seeds were used as the explant source. The regeneration approach designed here led to obtaining of plants from each of tested cultivars, however, with variable efficiencies. This is (up to our knowledge) the first report on *in vitro* regeneration of these commercial soybean cultivars.

2. Material and methods

2.1 Plant material and explant preparation

The soybean cultivars (*Glycine max* (L). Merrill) Bohemians, Cardiff, Gallec, Merlin, Moravians, Naya and Silensia were obtained from the company Matex s.r.o (Veškovce, Slovak Republic). Mature seeds were surface-sterilized for 20 hours

using chlorine gas produced by mixing 3.5 ml 12 $mol.l^{-1}$ with 100 ml commercial bleach (SAVO) described by DI *et al.* (1996). One day prior experiments, sterilised seeds were soaked in sterile water for about 20 hours in Petri dishes covered with aluminium foil. The cotyledons were separated by longitudinal cut along hilum. The half-seed explants were obtained by the excision of the embryogenic axis.

2.2 Plant regeneration

The half-seed explants were cultivated on callus induction medium (CIMA, Table 1) lined with sterile filter paper. The explants were placed adaxial side (flat side) down on the medium. Following 5 days, the explants were transferred on the shoot-induction medium (SIM, Table 1) so the nodal end of the half-seed explant was imbedded into the media. After 14 days, the explants were transferred to the fresh SIM medium. After 5 weeks of culture, the explants were transferred on the shoot elongation medium (SEM, Table 1).

The explants were regenerated at 24°C and 16 h/ 8 h light/dark photoperiod under 50 $\mu E\ m^{-2}\ s^{-1}$ light intensity.

2.3 Chlorophyll fluorescence measurement

Chlorophyll fluorescence was measured using portable fluorometer Handy FluorCam FC 1000-H (PSI s.r.o. Czech Republic). Measurement was performed on Petri dishes containing half-seed explants (10 explants per Petri dish). Petri dishes containing explants were placed in the dark for 30 min prior to the measurement. The chlorophyll fluorescence was measured directly from the top of dishes with removed lids according to the protocol "short" Kautsky effect in continuous light. We measured minimum chlorophyll fluorescence (F_0), variable fluorescence ($Fv = Fm - F_0$) and quantum efficiency of open PSII centers in a dark-adapted state ($QYmax = F_v/F_m$).

2.4 Histochemical staining

The cell viability was determined after staining with a 0.25 % (w/v) Evans blue for 30 min at room temperature and subsequent washing three times with distilled water, for 10 min each according to TAMAS *et al*. (2008).

Lipid peroxidation was detected using Schiff´s reagent for 60 min as described previously POMPELLA *et al*. (1987)

2.5 Explant sampling and statistical analyses

The sets of analysed explants for *in vitro* regeneration potential were subjected to analyses at (1) 5^{th} day and (2) 5^{th} weeks culture.

For chlorophyll fluorescence measurement, the explants were analysed at 5^{th} day-culture on the callus-induction media with or without antioxidants (CIMA or CIMB, respectively).

The cell viability and lipid peroxidation was detected on explants immediately after separation of cotyledons and excision of embryogenic axis. Data are presented as the means of three replications. Statistical significance of the experimental results was evaluated by Duncan's test with help of STATISTICA_version 7.1.

Table 1. Composition of media used in regeneration experiments.

CIMA	0.32 $g.l^{-1}$ Gambor B5 including vitamins (Duchefa), 2.78 $mg.l^{-1}$ $FeSO_4.7H_2O$, 3.72 $mg.l^{-1}$ NaEDTA, , 4.26 $mg.l^{-1}$ MES, 30 $g.l^{-1}$ sacharose, 1.67 $mg.l^{-1}$ BAP, 0.25 $mg.l^{-1}$ GA_3, 5 $g.l^{-1}$ agar pH 5.4, 400 $mg.l^{-1}$ L-cysteine, 0.154 $g.l^{-1}$ DTT, 0.158 $mg.l^{-1}$ STS and 5 $g.l^{-1}$ $AgNO_3$.
CIMB	0.32 $g.l^{-1}$Gambor B5 including vitamins (Duchefa), 2.78 $mg.l^{-1}$ $FeSO_4.7H_2O$, 3.72 $mg.l^{-1}$ NaEDTA, 4.26 $mg.l^{-1}$ MES, 30 $g.l^{-1}$ sacharose, 1.67 $mg.l^{-1}$ BAP, 0.25 $mg.l^{-1}$ GA_3, 5 $g.l^{-1}$ agar pH 5.4
SIM	3.2 $g.l^{-1}$ Gambor B5 including vitamins (Duchefa), 278 $mg.l^{-1}$ $FeSO_4.7H_2O$, 372 $mg.l^{-1}$ NaEDTA, , 0.64 $mg.l^{-1}$ MES, 30 $g.l^{-1}$ sacharose, 1.67 $mg.l^{-1}$ BAP, 5 $g.l^{-1}$ $AgNO_3$, 7 $g.l^{-1}$ agar pH 5.7
SEM	3.2 $g.l^{-1}$ Gambor B5 including vitamins (Duchefa), 278 $mg.l^{-1}$ $FeSO_4.7H_2O$, 372 $mg.l^{-1}$ NaEDTA, 0.64 $mg.l^{-1}$ MES, 20 $g.l^{-1}$ sacharose, 0.5 $mg.l^{-1}$ GA_3, 0.1 $mg.l^{-1}$ IAA, 1 $mg.l^{-1}$ zeatín, 50 $mg.l^{-1}$ asparagine, 7 $g.l^{-1}$ agar pH 5.7

CIMA - callus induction medium, SIM - shoot induction medium, SEM - shoot elongation medium, MES - 2-(N-Morpholino) ethanesulfonic acid, BAP- benzylaminopurine, GA_3 – gibberellic acid, DTT – 1,4 dithiothreitol, STS – sodium thiosulfate, IAA – indolyl acetic acid

3. Results and discussion

Soybean is one of the most important crops worldwide. Presently, biotechnology offers soybean traits that provide healthier ingredients for our diets and the diets of farm animals, as well as increased disease and insect resistance. However, soybean is considered particularly difficult to transform due to different factors, including regeneration efficiency *in vitro*. Here we studied *in vitro* regeneration potential of selected soybean cultivars, yet not tested elsewhere, using the half-seeds as an explant source (Fig 1a). This type of the explant has been previously shown to be a convenient material not only for soybean regeneration itself (JANANI *et al.*, 2013) but also for genetic modification via *A. tumefaciens* (PAZ *et al.,* 2006; KIM *et al.* 2012). The regeneration efficiency was genotype dependent and ranged from 1.4% to 8.7%.

The half-seed explants were prepared by splitting of imbibed seeds and excision of embryogenic axis. Such manipulation is very often accompanied with browning and necrosis of the affected tissue that can lead to poor *in vitro* regeneration.

Generally, mechanical wounding is associated with production of reactive oxygen species (ROS), common components of plant defence response against various stresses (OROZCO-CÁRDENAS *et al.*, 2001; DEMIDCHIK *et al*., 2015). However, ROS at low level regulate numerous plant biological processes (DEL RÍO *et al.,* 2015). For example, soybean seed germination is regulated through ethylene production in response to ROS (ISHIBASHI *et al.,* 2013). Contrary, high level of ROS promotes oxidative stress through oxidation of the cell compounds (DEMIDCHIK *et al*., 2015) and often leads to loss of cell function and apoptosis (MARNETT 2000).

Fig. 1. Representative photos of *in vitro* regeneration process of the cv. Merlin. **a)** The half-seeds were prepared by splitting of imbibed seeds and excision of embryogenic axis and placed on the CIMA medium; **b)** The half seeds after five days culture on the CIMA medium; **c)** First shoots after two weeks subculture on the SIM medium; **d)** Shoots after five weeks subculture on the SIM medium. Bars 500 µm.

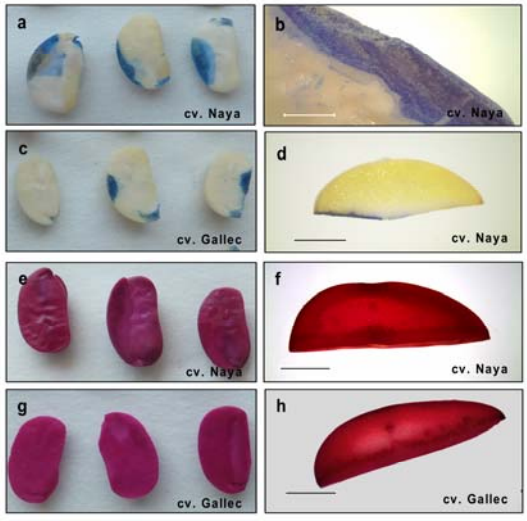

Fig. 2. Detection of cell viability (**a,b,c, d**) and membrane lipid peroxidation (**e, f, g, h**) in the half-seeds after splitting and excision of the embryogenic axis. The cell death was determined using staining with Evans blue. The membrane lipid peroxidation was stained using Schiff´s reagent. **a, c)** histochemical staining with Evans blue in the explants of the cvs. Naya and Gallec (respectively); **b, d)** cross sections of the explants of the cv. Naya indicating cell death at the side of the embryogenic axis removing; **e, g)** histochemical staining with Schiff´s reagent in the explants of the cvs. Naya and Gallec (respectively); **f, h)** cross sections of the explants of the cv. Naya and Gallec (respectively) indicating the extent of the lipid peroxidation. Bars 500 µm.

In our experiments, histochemical staining proved peroxidation of membrane lipids all over the surface of the half-seeds immediately after explant preparation (Fig. 2a, 2b). Besides, the stain was detected in the cross section of the half-seed explants (Fig. 2c, Fig 2d). The sides of wounding areas of death cells were detected histochemically as a result of the separation of cotyledons (Fig. 2e, 2f) and at the site of excision of embryogenic axis (Fig 2g, 2h). Similar histochemical patterns were observed for all cultivars.

Several studies reported the inclusion of antioxidants in the culture media can prevent necrosis and consequently improve plant *in vitro* regeneration (reviewed by DAN 2008). Moreover, antioxidants can reduce cell damage following *Agrobacterium*-mediated transformation by inhibiting of wound- and plant pathogen-induced responses (OLHOFT *et al.,* 2001, OLHOFT and SOMERS, 2001). In cotyledonary node transformation of soybean, adding a mixture of thiol compounds such as L-cysteine, dithiothreitol and sodium thiosulfate into the co-cultivation medium improved regeneration potential of transformed cells (OLHOFT *et al.,* 2003; PAZ *et al.*, 2004; WANG and XU, 2008; KIM *et al*., 2012). However, a high level of thiol compounds might also have a negative effect on *in vitro* regeneration. For example, the application of L-cysteine at concentration higher than 600 $mg.l^{-1}$ resulted in reduced number of transformed soybean shoots (LIU *et al.*, 2008). Thus, above mentioned thiol compounds were applied at concentrations (Table 1) recommended by others (OLHOFT *et al.,* 2003; PAZ *et al.*, 2004; KIM *et al*., 2012). The half-seeds were cultivated on the CIM medium supplemented with (CIMA) or without (CIMB) antioxidants. After 5 days culture on the media (Fig 1b), the chlorophyll fluorescence of the explants (QYmax) as an indicator of regeneration was measured (Fig. 3). The results showed the chlorophyll content significantly varied with genotype (at $p \leq 0.001$) (Table 2). We did not observe any negative effects of these compounds on the half-seed regeneration. However, the effect of thiols as protective agents against ROS produced by wounding was not statistically significant (Table 2). Nevertheless, considering that promising soybean cultivars are expected to be subjected to genetic modification via *Agrobacterium*, these thiols (Table 1) were also applied in our further regeneration experiments.

It is well known the regeneration is essential requirement for transformation. Moreover, regeneration potential of transformed cells can be dramatically decreased also for plant genotypes that are routinely regenerated. For example, regeneration efficiency of 50% was reduced to 1.3% after *Agrobacterium*-mediated transformation of cotyledonary petiole explants of oilseed rape cultivar Campino (BOSZORADOVA *et al.*, 2011). Thus, identifying genotypes with high regeneration potential represents one of the prerequisites for successful transformation experiments.

Table 2. Variance analyses.

	Source [a]	**F empirical** [b]
Photosynthetic activity	(1)	16.75 ***
	(2)	1.81 [ns]
	(1) × (2)	1.06 [ns]
Regeneration efficiency	(1)	52.27 ***

[a] Effect of (1) genotype, (2) treatment with or without antioxidants
Statistical significance at ***$p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns – not significant

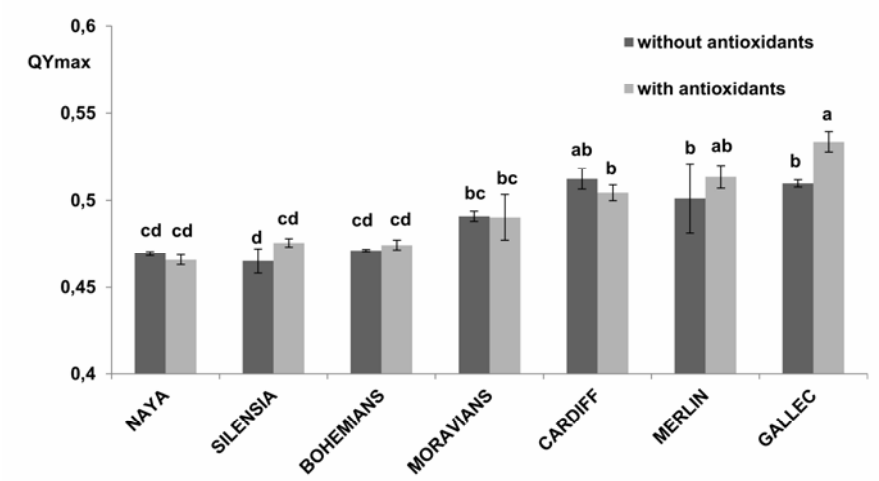

Fig. 3. Effect of antioxidants on the maximum quantum yield of PSII (QYmax = Fv/Fm). The Chlorophyll fluorescence was measured in the half-seed explants after 5th days culture on the media with (CIMA) or without (CIMB) antioxidants. Bars represents means ± standard deviations of three replications. Distinct letters denote statistically significant differences with Duncan's tests.

The half seed explants (Fig 1a) were cultured on the callus inducing (CIMA) media supplemented with the plant hormones 1.67 $mg.l^{-1}$ BAP and 0.25 $mg.l^{-1}$ GA_3. Such combination of phytohormones has been successfully applied for regeneration of transformed cotyledonary node (OLHOFT *et al.,* 2003; PAZ *et al.*, 2004) and half-seed (PAZ *et al*., 2006) explants of different soybean varieties. During the first five days, the most of explants positively responded by producing of green chlorophyll. (Fig. 1b). The number of green explants varied from 18.3% (cv. Naya) to 55.9% (cv. Gallec) (Table 3). Besides, direct regeneration of shoots from cotyledonary explants and/or from shoot apical meristem was observed (Fig. 1b). Following five days, the explants were sub cultured on the shoot-inducing (SIM) medium (Fig. 1c). The calli and the shoots were appeared after 2-3 weeks culture on the SIM medium. The first larger shoots were excised and discarded. Treatment with BAP breaks down apical dominance and triggers adventitious shoot formation (WRIGHT *et al.,* 1986). As was noticed by others (SAIRAM *et al.,* 2003), only the callus induced from the nodal region gave rise to shoots. Healthy shoots (Fig. 1d) were excised, transferred to the shoot-elongation (SEM) medium. The efficiency of regeneration of cultivars was evaluated five weeks after subculture on the SIM medium. It was calculated as percentage of the number of explants producing at least one shoot to the total number of explant used. Data are given in Fig. 4. The best regeneration responses were observed for the cultivars Merlin (36.6%) and Gallec (37.7%) while the lowest was found for the cultivars Naya (5.7%) and Silensia (8.0%). The regeneration was clearly genotype dependent ($p \leq 0{,}001$) (Table 2). Based on the data obtained (Table 3), the response of the explants to the culture media for the first five days seems to define the regeneration potential. Generally, low regeneration potential is associated with sensitivity and strong plant stress responses to the *in vitro* manipulation (BENSON,

2000). However, histochemical staining of some parameters of stress (Fig. 2) did not reveal obvious differences between individual genotypes, probably because points on the surface layer of epidermal cells that are usually most damaged. We suppose that lower regeneration efficiencies achieved for some cultivars as Naya and Silensia might coincide rather with the composition of the regeneration media. Optimizing of the exogenous hormone levels in media might lead to increased regeneration efficiencies of these genotypes, albeit this would require much loads of time and other inputs thus are not usable for routine screenings. Nevertheless, the half-seed regeneration system appears to be suitable for regeneration of soybean plants from all tested genotypes.

Table 3. Results from in *vitro* regeneration of soybean cultivars

		Number of responded explants					
	No. Expl.[a]	**Green[b]**	**[%][c]**	**Calli[d]**	**[%][e]**	**Calli and shoots[f]**	**[%][g]**
Bohemians	103	30	29.1	69	67.0	24	34.8
Cardiff	111	46	41.4	91	82.0	36	39.6
Gallec	104	55	55.9	86	82.7	39	37.2
Merlin	106	50	47.2	88	83.0	39	44.4
Moravians	113	42	37.2	68	60.2	34	50.0
Naya	109	20	18.3	52	47.7	6	11.5
Silensia	136	28	20.6	68	50.0	11	16.2

[a] Total number of explants used in experiments
[b] The number of green chlorophyll containing explants after 5th day culture on the CIMA medium
[c] The number of green chlorophyll containing explants as a percentage of the total number of explants used
[d] The number of explants producing calli
[e] The number of callus-producing explants as a percentage of the total number of explants used.
[f] The number of callus- and shoot -producing explants after 5th weeks culture on the media
[g] The number of callus- and shoot -producing explants after 5th weeks culture on the media as a percentage of the number of callus-producing explants.

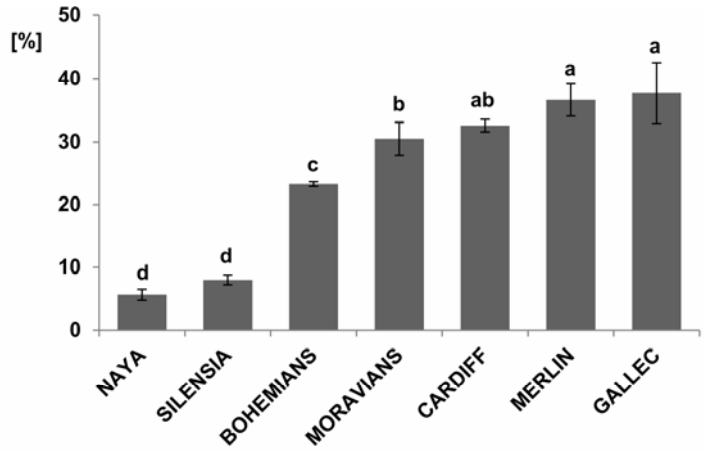

Fig. 4. Regeneration efficiencies of soybean cultivars. Regeneration efficiency was calculated as percentage of the number of explants producing at least one shoot to the total number of explant used. Bars represents means ± standard deviations of three replications. Distinct letters denote statistically significant differences with Duncan's tests.

4. Conclusions

In vitro regeneration potential of commercially important soybean cultivars Bohemians, Cardiff, Gallec, Merlin, Moravians, Naya and Silensia was studied. Half-seeds as an explant source were used. Within five weeks subculture on the regeneration media, shoots were generated from the explants of all cultivars. Adding the given antioxidants into the callus-induction medium had no significant effect on the cell regeneration. Regeneration efficiency was genotype dependent and varied between 5.7% and 37.7%. We concluded that the genotypes Naya and Silensia are likely to appear as recalcitrant for regeneration after transformation with *Agrobacteria*. On the other hand, the cultivars with the high regeneration potential such as Cardiff (32.6%), Merlin (36.6%) and Gallec (37.7%) appear to be more promising for further biotechnological applications via genetic transformations.

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