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In vitro regeneration of *Brassica napus* L. shoots from hypocotyls and stem segments

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Abstract

In order to use genetic transformation to enrich *Brassica napus* L. genetic resources, an efficient method of shoot regeneration *in vitro* is required. In this assay ten commercially important winter rapeseed cultivars were tested for the regeneration capacity through shoot organogenesis from hypocotyls and stem segments. Results show that shoot regeneration intensity depends on the genotype used. Hypocotyls of cultivar 'Valesca' show the highest overall capacity to produce shoots among all genotypes tested, while the higher overall regeneration frequency from stem segments was obtained for the cultivars 'Insider', 'Siska' and 'Kazimir H'. Shoot regeneration efficiency strongly correlated with the explant type used: regeneration frequencies from stem segments were by 25.78% ('Casino') to 85.11% ('Libea') higher than those from hypocotyls. Exogenous growth regulators promoted shoot formation from both hypocotyls and stem segments, but they had variable effect on different genotypes. Thus, the composition of growth regulators must be optimized for every *Brassica napus* L. genotype and explant type.

Key words: adventitious shoots, *Brassica napus* L., explants, genotype, growth regulators.

Introduction

Rapeseed (*Brassica napus* L.) is one of the most important vegetable oil and protein-rich meal crops in the world. Its cultivation has increased tremendously during the last decade and, by now, it is the second largest contributor to the world supply of vegetable oil /Ben Ghnaya et al., 2008/. Conventional methods of plant breeding and, in present years, modern biotechnology have evolved into powerful tools for developing improved crop species and novel superior cultivars. The creation of novel germplasm through techniques of tissue culture and gene transfer holds great potential for improving the quality and agronomic traits of rapeseed. There are several reports on rapeseed transformation with respect to the introduction of various new traits such as modified oil composition /Knutzon et al., 1992/, herbicide tolerance /De Block et al., 1989/, altered

protein composition /Altenbach et al., 1992/ and insect resistance /Stewart et al., 1996/. Transformation has been carried out using various explants such as stem internodes /Fry et al., 1987/, stem segments /Pua et al., 1987/, cotyledonary petioles /Moloney et al., 1989/, hypocotyls /Cardoza, Stewart, 2003; Sonntag, 2007/ and mesophyll protoplasts /Wang et al., 2005/. However, transformation has been limited to certain cultivars, and the frequency is not necessarily high /Akasaka-Kennedy et al., 2005/. The generation of transgenic plants is an integrated process, which involves many different factors such as plant regeneration, the choice of regenerable explant culture conditions and transformation techniques. Therefore, selection of genotypes with higher overall regeneration rates will help improve the efficiency of genetic transformation. Moreover, explant types and growth regulators have a strong influence on the regeneration frequencies of various tissue culture techniques /Ovesna et al., 1993; Jonoubi et al., 2004; Akasaka-Kennedy et al., 2005; Kamal et al., 2007; Ben Ghnaya et al., 2008/.

The present paper presents the investigation of the factors influencing shoot regeneration from hypocotyls and stem segments in winter rapeseed. The effect of genotype, explant type and growth regulators is studied to establish an efficient regeneration system.

Materials and methods

Investigation was carried out in 2007 and 2008 at the Agrobiotechnology Laboratory, Lithuanian University of Agriculture and Lithuanian Institute of Horticulture. Ten winter rapeseed varieties – 'Casino', 'Kasimir H', 'Libea', 'Liprima', 'Silvia', 'Siska', 'SW Celsius', 'Valesca', 'Banjo H', 'Insider' were taken for analysis. Seeds were surface sterilized with 10% sodium hypochlorite for 10 min, washed with sterile water and placed for germination and growth *in vitro* on basal MS medium /Murashige, Skoog, 1962/ without growth regulators, supplemented with 10.0 g l^{-1} sucrose and 8.0 g l^{-1} agar. Seeds were incubated at +22 ± 2ºC temperature, under illumination 50 µmol m^{-2} s^{-1}, photoperiod 16/8 h (day/night). Hypocotyls were excised from 4–5-day old seedlings. Explants were placed on MS medium with three variants of phytohormone additions: without growth regulators (A), supplemented with 6-benzylaminopurine (BAP) (4.0 mg l^{-1}) (B) and BAP in combination with 1-naphthylacetic acid (NAA) (0.05 mg l^{-1}) (C), 30.0 g l^{-1} sucrose and 8.0 g l^{-1} agar. Media adjusted to pH 5.5 prior to autoclaving at +115ºC for 30 min. Culture media (20 ml) were dispensed into 90 mm diameter Petri dishes and sealed with parafilm. Sterilization of explants and transfer of the culture were carried out under aseptic conditions. Explants were cultivated at +22 ± 2ºC temperature, under illumination 50 µmol m^{-2} s^{-1}, photoperiod 16/8 h (day/night).

Proliferated shoots were carefully excised from the explants and transferred for rooting on MS medium containing 0.1 mg l^{-1} NAA and 10.0 g l^{-1} sucrose. Medium was solidified with 8.0 g l^{-1} agar, pH 5.7. Rooted plantlets were transferred to plastic pots containing soil:vermiculite (1:1) mixture and were placed in a greenhouse.

Experiments were set up in a completely randomized design and three replicates per treatment with 50 explants for each replicate were used. The percentage of bud regeneration [(number of explants with buds/total number of explants) x 100%] and the number of shoots per explant (number of shoots/number of explants forming shoots) were calculated for the explants that had been cultured for 4 weeks. The least significant differences of the results were computed using the software *Anova* and *Stat* /Tarakanovas, Raudonius, 2003/. Mean value and standard error (SE) for each genotype were calculated based on the number of independent replications.

Results and discussion

Adventitious shoots regeneration from hypocotyls. Hypocotyls of rapeseed grown on MS medium with and without growth regulators had swollen and formed callus after 7–10 days culture and the first shoots were observed after 14 days. Shoot regeneration frequency varied with the genotype and medium composition. Results of the effect of growth regulators on percentages of responding explants and number of shoots per explant are summarized in Figure 1.

Having placed rapeseed hypocotyls explants onto the medium without growth regulators, shoots regeneration frequency ranged from 0 to 7.33% with an average of 3.33% (Fig. 1 A). On this medium hypocotyls of cultivar ʻValescaʼ and ʻSilviaʼ showed higher shoot regeneration frequency in comparison with other tested cultivars. Callus of cultivars ʻLibeaʼ, ʻLiprimaʼ and ʻBanjo Hʼ did not show any organogenic response even after 28 days of culture and, subsequently, became necrotic. The number of shoots per explant ranged from 1.0 in ʻKasimir Hʼ and ʻSiskaʼ to 1.8 in ʻSW Celsiusʼ.

Explants cultured on the media fortified with cytokinins alone induced shoots at a higher frequency compared to the explants on the medium without growth regulators. Shoot formation frequency varied from 0 to 34.67% with an average 14.33% (Fig. 1 B). Among the genotypes examined, the cultivars ʻSW Celsiusʼ and ʻValescaʼ showed the highest shoot regeneration frequency and high number of shoots per explant, while cultivar ʻLibeaʼ did not regenerate any shoots.

The use of NAA in combination with BAP resulted in significant increase ($P < 0.05$) in shoot formation frequency for cultivars ʻCasinoʼ, ʻKasimir Hʼ, ʻValescaʼ and ʻInsiderʼ, but significantly reduced ($P < 0.01$) shoot formation in ʻSW Celsiusʼ (Fig. 1 C). Callus of cultivar ʻLibeaʼ did not show any organogenic response. The number of shoots per explant varied from 1.44 in ʻKasimir Hʼ to 4.11 in ʻValescaʼ.

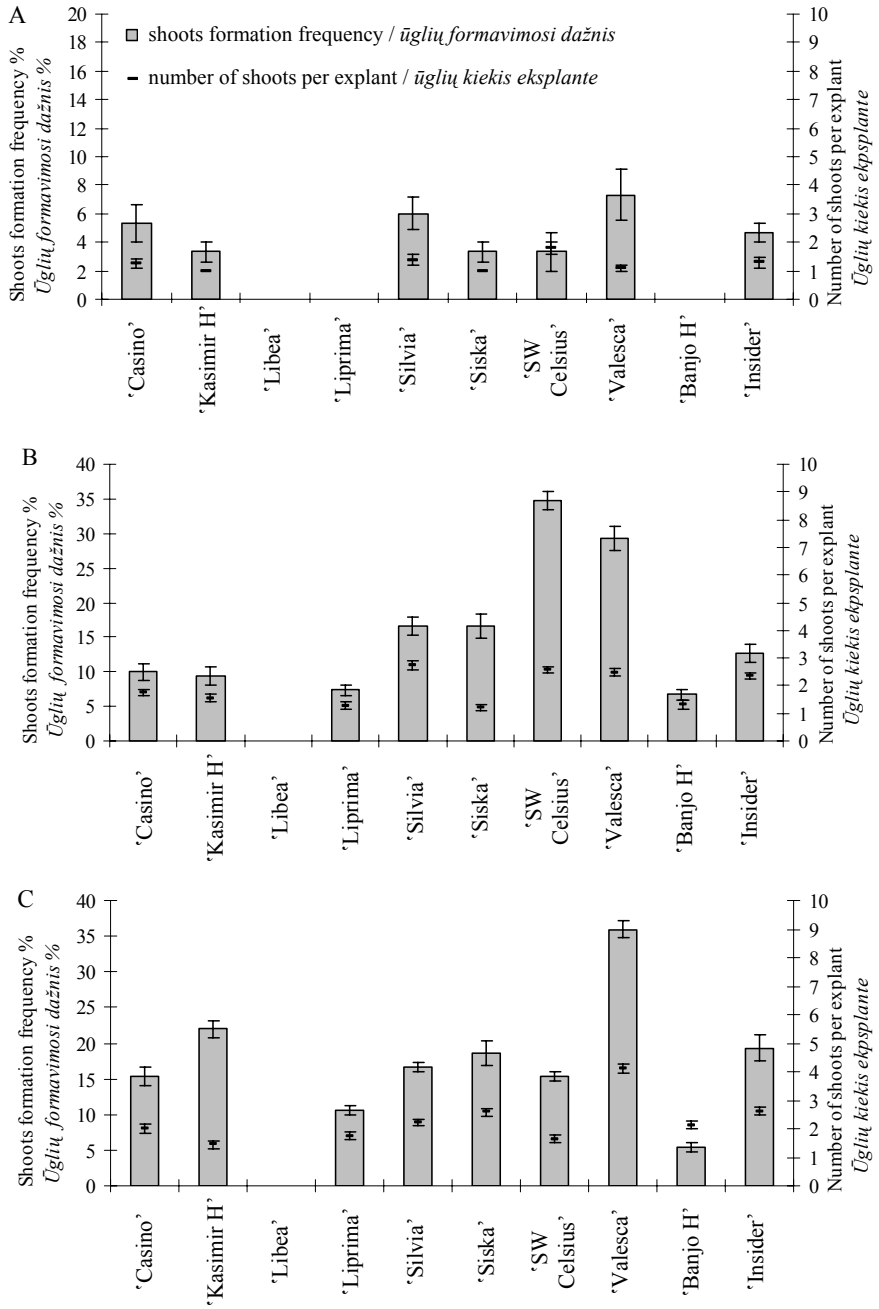

Figure 1. Effect of growth regulators on shoot regeneration frequency and number of shoots per explant from winter rapeseed hypocotyls: A – medium without phytohormons, B – with BAP (4.0 mg l^{-1}), C – with BAP (4.0 mg l^{-1}) and NAA (0.05 mg l^{-1})

1 paveikslas. *Augimo reguliatorių poveikis ūglių formavimosi dažniui ir jų kiekiui eksplante žieminių rapsų hipokotilių eksplantuose: A – terpė be fitohormonų, B – su BAP (4,0 mg l^{-1}), C – su BAP (4,0 mg l^{-1}) ir NAR (0,05 mg l^{-1})*

Shoot regeneration from stem segments. All the media induced shoot regeneration via direct organogenesis without forming callus. Regeneration potential of stem segments was explored on MS medium without and with various plant growth regulators and results are summarized in Figure 2.

On the media without growth regulators shoot formation frequency ranged from 25.33 to 87.33% with an average 62.20% (Fig. 2 A). The cultivars 'Siska' and 'Insider' showed the highest shoot regeneration frequency, 87.33 and 86.67% respectively. The number of shoots per explant varied from 1.1 for cultivars 'Liprima' and 'Silvia' to 2.0 for cultivar 'Banjo H'.

In the presence of cytokinin only the highest bud formation frequency was achieved for 'Kasimir H' (100%), and 'Libea' (100%) followed by 'Silvia' (98.67%), 'Siska' (96.67%) and 'Insider' (96.00%). The addition of BAP was especially beneficial for cultivar 'Libea', shoot regeneration frequency increased by 44.67% compared with that on the medium without growth regulators (Fig. 2 B). However, BAP significantly reduced ($P < 0.01$) shoot formation in 'Casino' and 'SW Celsius'. Shoot regeneration frequency of these genotypes decreased by 16.00 and 24.00%, respectively, in comparison with the variant on hormone-free medium. Stem segments of cultivars 'Casino', 'SW Celsius' and 'Valesca' produced the highest shoot number per explant, 3.68, 3.33 and 3.74, respectively.

The explants of cultivars 'Casino' and 'Liprima' appeared to be more responsive on the medium containing BAP in combination with NAA than those on the media with cytokinin only. On this medium shoot regeneration frequency increased by 28.00 and 40.66%, respectively (Fig. 2 C). On the other hand, the use of the auxin/cytokinin combination led to similar or lower regeneration efficiency for other tested cultivars. The number of shoots per explant ranged from 1.58 for cultivar 'Kasimir H' to 4.21 for cultivar 'SW Celsius'. The ability of *in vitro* regeneration can vary considerably among various species of a genus or among cultivars within the same species. In the genus *Brassica* most of the published reports noted the strong relationship between genotype and response to any given tissue culture system /Takasaki et al., 1997; Zhang et al., 1998; Ono et al., 2000; Tang et al., 2003; Burbulis, Kuprienė, 2005/. The capacity of hypocotyls and stem segments to regenerate whole plants was examined. Genotype variability in shoot formation was evident for both hypocotyls and stem segments regeneration. Having used hypocotyls as initial source for shoot regeneration, cultivar 'Valesca' demonstrated the highest overall regeneration efficiency.

On the other hand, having used stem segments as the explant, cultivars 'Insider', 'Siska' and 'Kazimir H' had a higher overall efficiency of regeneration than others. Such genotypic variability indicates the genetic control of shoot regeneration ability. In this respect, a strong genotypic effect was previously reported in *Brassica napus* tissue cultures /Akasaka-Kennedy et al., 2005; Ben Ghnaya et al., 2008; Burbulis et al., 2008; Mashayekhi et al., 2008/. Ono et al. (2000) suggested that the regenerability from cotyledonary explants of *Brassica napus* was controlled genetically, with both additive and dominant effects being significant.

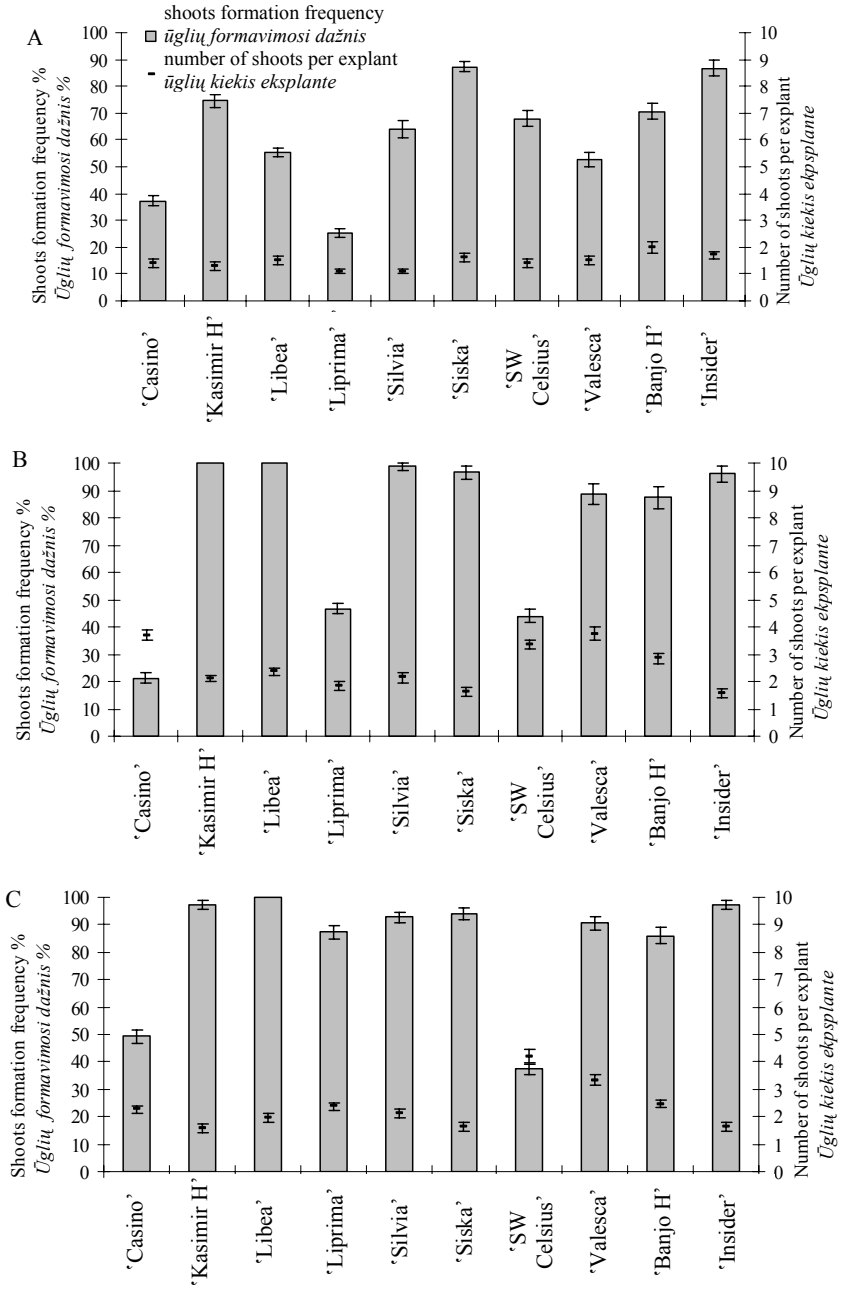

Figure 2. Effect of growth regulators on shoot regeneration frequency and number of shoots per explant from winter rapeseed stem segments: A – medium without phytohormons, B – with BAP (4.0 mg l^{-1}), C – with BAP (4.0 mg l^{-1}) and NAA (0.05 mg l^{-1})

2 paveikslas. *Augimo reguliatorių poveikis ūglių formavimosi dažniui ir jų kiekiui eksplante žieminių rapsų stiebo segmentų eksplantuose: A – terpė be fitohormonų, B – su BAP (4,0 mg l^{-1}), C – su BAP (4,0 mg l^{-1}) ir NAR (0,05 mg l^{-1})*

However, in this study there were no relationships between frequency of shoot formation from hypocotyls and from stem segment explants. This may be due to the difference of the genes or their activity, controlling direct and callus mediated regeneration system.

Among the investigated genotypes, the hypocotyls of cultivar 'Valesca' showed the highest capacity to produce shoots, while 'Libea' did not regenerate any shoots on all tested media. The use of these two genotypes, favourable and recalcitrant, within the same genus, may be a useful approach to conduct a genetic analysis of shoot regeneration. The origin of explants also affected shoot regeneration: stem segments responded better than hypocotyls for all the genotypes tested. Use of stem segments instead of hypocotyls increased the overall regeneration rate by 25.78% ('Casino') to 85.11% ('Libea').

Despite the fact that in this study regeneration from stem segments was more efficient, cotyledon and hypocotyl have been widely suggested as the best explants in regeneration and transformation experiments /Zihang, Bhalla, 2004, Mashayekhi et al., 2008/. Direct shoot regeneration – bud and shoot formation – is speculated to involve more cells of the explant. This increases possibility to obtain false transformants or chimeras /Zaccai et al., 2007/. Therefore, higher number of regenerants does not always mean higher transformation efficiency. Those aspects require more detailed research.

Plant growth regulators play the key role in controlling the differentiation process required for regeneration. BAP has been widely employed for the *in vitro* culture of rapeseed. It plays an important part in both the induction and regeneration of *Brassica napus* via direct shoot organogenesis, somatic embryogenesis or through a callus phase /Koh, Loh, 2000; Kupriene et al., 2004; Burbulis et al., 2007; Kamal et al., 2007/. The present work indicates that variability in growth regulators necessary for plant regeneration can reflect different endogenous hormone levels within different genotypes.

Normal plantlets were regenerated from induced shoots, transferred to soil: vermiculite mixture and placed in a greenhouse. These regenerated plants had a well-developed root system and developed normally. The plants produced flowers and were fertile. The regenerated plants were also identical with the source plants and true-to-type.

Conclusion

Significant genotypic variability in shoot formation was evident for both explant sources – hypocotyls and stem segments. Among the investigated genotypes, the hypocotyls of cultivar 'Valesca' showed the highest capacity to produce shoots (regeneration rate 34.67%), while 'Libea' did not regenerate any shoots on all tested media. 100% regeneration frequency from stem segments was obtained for cultivars 'Insider', 'Siska' and 'Kazimir H'. The overall regeneration efficiency was by 25.78% ('Casino') to 85.11% ('Libea') higher when using stem segments instead of hypocotyls. Citokinine BAP induced efficient shoot regeneration from hypocotyls of cultivars 'SW Celsius', 'Silvia', 'Siska' and from stem segments of seven from the ten tested cultivars. Combination of BAP and NAA significantly improved shoot formation frequency from hypocotyls of 'Insider', 'Kasimir', 'Valesca', 'Casino' and from stem segment of cultivars 'Casino' and 'Liprima'.

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Brassica napus L. ūglių regeneracija *in vitro* iš hipokotilių ir stiebo segmentų kultūrų

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Santrauka

Augalų genetinės transformacijos metodus siekiant panaudoti rapso genofondui gausinti, būtina optimizuoti ūglių regeneracijos procesą *in vitro*. Tirtas žieminio rapso 10 veislių organogenezės procesas hipokotilių ir stiebo segmentų kultūrose *in vitro*. Nustatyta, kad tirtų eksplantų ūglių regeneravimo dažnis priklausė nuo augalo genotipo. Iš tirtų veislių vidutiniškai didžiausiu dažniu ūglius regeneravo veislės 'Valesca' hipokotiliai. Vidutiniškai didžiausias ūglių formavimosi dažnis iš stiebo segmentų gautas auginant veislių 'Insider', 'Siska' ir 'Kazimir H' eksplantus. Nustatytas ūglių regeneracijos dažnio priklausomumas nuo eksplanto tipo: stiebo segmentai ūglius regeneravo nuo 25,78 % ('Casino') iki 85,11 % ('Libea') didesniu dažniu nei hipokotiliai. Egzogeniniai augimo reguliatoriai skatino ūglių formavimąsi iš hipokotilių ir stiebo segmentų *in vitro* kultūroje. Tačiau augimo reguliatorių poveikis priklausė nuo genotipo, todėl jų sudėtis maitinamojoje terpėje turi būti parenkama konkrečiam *Brassica napus* L. genotipui ir eksplanto tipui.

Reikšminiai žodžiai: pridėtiniai ūgliai, *Brassica napus* L., eksplantai, genotipas, augimo reguliatoriai.