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Callus induction and plant regeneration from somatic tissue in spring rapeseed (*Brassica napus* L.)

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The effect of culture media, explants and genotypes on adventitious shoot regeneration in spring rapeseed (*Brassica napus* L.) was examined. Hypocotyls and cotyledons of the doubled haploid lines NL-611, NL-662, NL-685 were induced to form callus by culturing on the Murashige–Skoog medium supplemented with different concentrations of 6-benzylaminopurine (BAP) and α-naphtylacetic acid (NAA) or zeatin and 2,4-dichlorphenoxyacetic acid (2,4-D). Adventitious buds were regenerated from the organogenic callus on the same medium. A large variation of shoot regenerability was observed, ranging within 0–37.5% for the frequency of bud formation and within 0–3.8 for the number of buds per explant. Generally, cotyledon-derived callus exhibited a higher bud regeneration frequency than hypocotyls, however, hypocotyl-derived callus developed a higher number of buds per explant. The maximum number of adventitious buds from hypocotyl-derived callus was obtained on a medium supplemented with 4.0 mg l^{-1} BAP and 0.05 mg l^{-1} NAA. Regenerated shoots were rooted in MS medium containing 0.1 mg l^{-1} NAA. Well rooted plantlets were acclimatized and subsequently established in soil.

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

INTRODUCTION

Rapeseed (*Brassica napus* L.) is the third (after soybean and palm) most important sources of edible vegetable oil, industrially used oil and protein-rich products in the world and one of most important crops in Lithuania. In the past years, researchers made great efforts in developing biotechnological methods to facilitate rapeseed breeding. Several shoot regeneration protocols have been reported for rapeseed tissue culture. These include the regeneration of shoots either directly from the explants through pre-formed or newly formed meristems, or also indirectly via a callus phase followed by the regeneration of shoot buds or somatic embryos [1–4]. Biotechnological approaches combined with classical breeding schemes offer a wide spectrum of methods, which are altogether involved in developing improved basic stocks and cultivars possessing novel desirable traits. However, further adjustments of rapeseed quality will not be realized satisfactorily without the assistance of genetic engineering. A considerable amount of research has already been undertaken in this direction, and rapeseed has been exploited for genetic engineering purposes [5]. Of various systems used for rapeseed transformation, *Agrobacterium*-mediated transformation using hypocotyls and cotyledons have been commonly employed owing to the high regeneration ability of these explants [6, 7]. However, transformation has been limited to certain cultivars, and the frequency was not sufficiently high [8, 9]. Therefore, selection of genotypes with higher overall regeneration rates will help improve the efficiency of genetic transformation. Moreover, shoot regeneration largely depends on explant types and the composition of phytohormones [10].

In this investigation, the effect of growth regulator combinations and explant types on the shoot regeneration of newly selected yellow-seeded spring rapeseed doubled haploid (DH) lines was studied.

MATERIALS AND METHODS

The novel yellow-seeded spring rapeseed DH lines NL-611, NL-662, and NL-685, recently generated following the Guelph doubled haploid production method [11], were used in the present study. 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 [12] without growth regulators, solidified with 8.0 g l^{-1} agar. Seeds were incubated at a temperature of 22 ± 2 °C under illumination of 50 μmol m^{-2} s^{-1}, photoperiod 16/8 h (day / night). Cotyledons and hypocotyls were excised from 4–5-day-old seedling. Cotyledons were carefully separated from hypocotyls and shoot meristems. Explants were placed on MS medium supplemented with 2.5 mg l^{-1} silver nitrate ($AgNO_3$), 30.0 g l^{-1} sucrose and 8.0 g l^{-1} agar. The medium was supplemented with different combinations of BAP (2.0–4.0 mg l^{-1}) and NAA (0.05 mg l^{-1}), zeatin (2.0–6.0 mg l^{-1}) and 2,4-D (0.1–0.15 mg l^{-1}). The media were adjusted to pH 5.5 prior to autoclaving at 115 °C for 30 min.

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Culture media (20 ml) were dispensed into 90 mm diameter Petri dishes and sealed with parafilm. Explants were cultivated at 22 ± 2 °C temperature, illumination 50 μmol m^{-2} s^{-1}, photoperiod 16/8 h (day / night). Proliferated adventitious shoots were carefully excised from the callus and transferred for rooting into MS medium containing 0.1 mg l^{-1} a-naphthylacetic acid (NAA) and 10.0 g l^{-1} sucrose. The medium was solidified with 8.0 g l^{-1} agar, pH 5.7. Rooted plantlets were transferred to plastic pots containing a 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 20 explants for each replicate were used. The percentage of bud regeneration [(the number of explants with adventitious buds / total number of explants) × 100%] and the number of buds per explant (the number of adventitious buds / the number of explants forming adventitious buds) were calculated for the explants that had been cultured for 4 weeks. The significance of differences among means was determined using Duncan's multiple range test at $P \leq 0.05$. The mean value and SE for each genotype were calculated based on the number of independent replications.

RESULTS

Hypocotyls and cotyledons of rapeseed grown on MS medium containing different plant growth regulators swelled and formed callus within 7–10 days of culture. There were no differences between hypocotyl-derived and cotyledon-derived callus in terms of colour and texture. Variable callogenic responses were expressed by all genotypes tested on different media. The percentage of responding hypocotyls varied from 32.3% to 100% (Table 1). The effect a combination of growth regulators on callus induction was genotype-dependent. Genotype NL-611 showed a high value of responding hypocotyls on all the growth regulator combinations tested, except 6.0 mg l^{-1} zeatin + 0.1 mg l^{-1} 2,4-D. Hypocotyls of NL-662 showed the best response on medium supplemented with 3.0 mg l^{-1} BAP + 0.1 mg l^{-1} NAA; 4.0 mg l^{-1} zeatin + 0.15 mg l^{-1} 2,4-D and 2.0 mg l^{-1} zeatin + 0.15 mg l^{-1} 2,4-D. All test combinations of zeatin and 2,4-D promoted a high callus induction frequency from hypocotyls of NL-685, whereas callus formation in this genotype was strongly reduced by BAP and NAA combinations. Generally, hypocotyls of the test genotypes showed a higher callogenic potential than cotyledons.

The percentage of cotyledons that developed callus ranged from 13.6% to 100%. A combination of growth regulators containing 2.0 mg l^{-1} zeatin + 0.15 mg l^{-1} 2,4-D was most suitable for callus induction in all genotypes. A combination of 4.0 mg l^{-1} zeatin + 0.15 mg l^{-1} 2,4-D promoted callus development from NL-685 cotyledons, but significantly reduced the number of cotyledons producing callus in NL-611 and NL-662.

Callus was initiated within 10 days, with adventitious bud formation occurring shortly thereafter (Figure, A). Bud regeneration frequency varied with the genotype, explant type and medium composition. Data on the effect of growth regulator combinations on the percentage of responding explants are summarized in Table 2.

The results showed that hypocotyl-derived callus grown in the presence of 4.0 mg l^{-1} BAP and 0.05 mg l^{-1} NAA gave rise to buds at the highest frequency. Bud regeneration from explants

Table 1. **Effect of growth regulators on callus induction from different explants of spring rapeseed**

Genotype	Growth regulators (mg l^{-1})				Callus induction (%)	
	BAP	NAA	Zeatin	2,4-D	Hypocotyls	Cotyledons
NL-611	0	0	0	0	50.1 ± 1.19c	49.9 ± 1.34d
	4.0	0.05	0	0	100h	20.5 ± 0.68b
	3.0	0.1	0	0	100h	16.7 ± 0.43a
	2.0	0.15	0	0	100h	13.6 ± 0.18a
			6.0	0.1	89.4 ± 1.26f	25.2 ± 0.27b
			4.0	0.15	100h	37.5 ± 0.41c
			2.0	0.15	100h	100i
NL-662	0	0	0	0	32.3 ± 0.19a	54.5 ± 1.34e
	4.0	0.05	0	0	94.4 ± 1.05g	65.2 ± 1.42f
	3.0	0.1	0	0	100h	51.2 ± 0.67e
	2.0	0.15	0	0	95.7 ± 1.12g	31.7 ± 0.39c
			6.0	0.1	95.8 ± 1.27g	55.7 ± 0.46e
			4.0	0.15	100h	35.1 ± 0.28c
			2.0	0.15	100h	84.4 ± 1.33h
NL-685	0	0	0	0	40.2 ± 0.78b	39.1 ± 0.67c
	4.0	0.05	0	0	77.8 ± 0.55e	67.3 ± 0.98f
	3.0	0.1	0	0	60.4 ± 0.82d	45.7 ± 0.75d
	2.0	0.15	0	0	62.5 ± 0.61d	31.6 ± 0.29c
			6.0	0.1	100h	55.9 ± 0.35e
			4.0	0.15	100h	100i
			2.0	0.15	100h	100i

Data are means ± SE within four weeks of culture. Means within a column followed by the same letter are not significantly different from those indicated by Duncan's multiple-range test ($P \leq 0.05$).

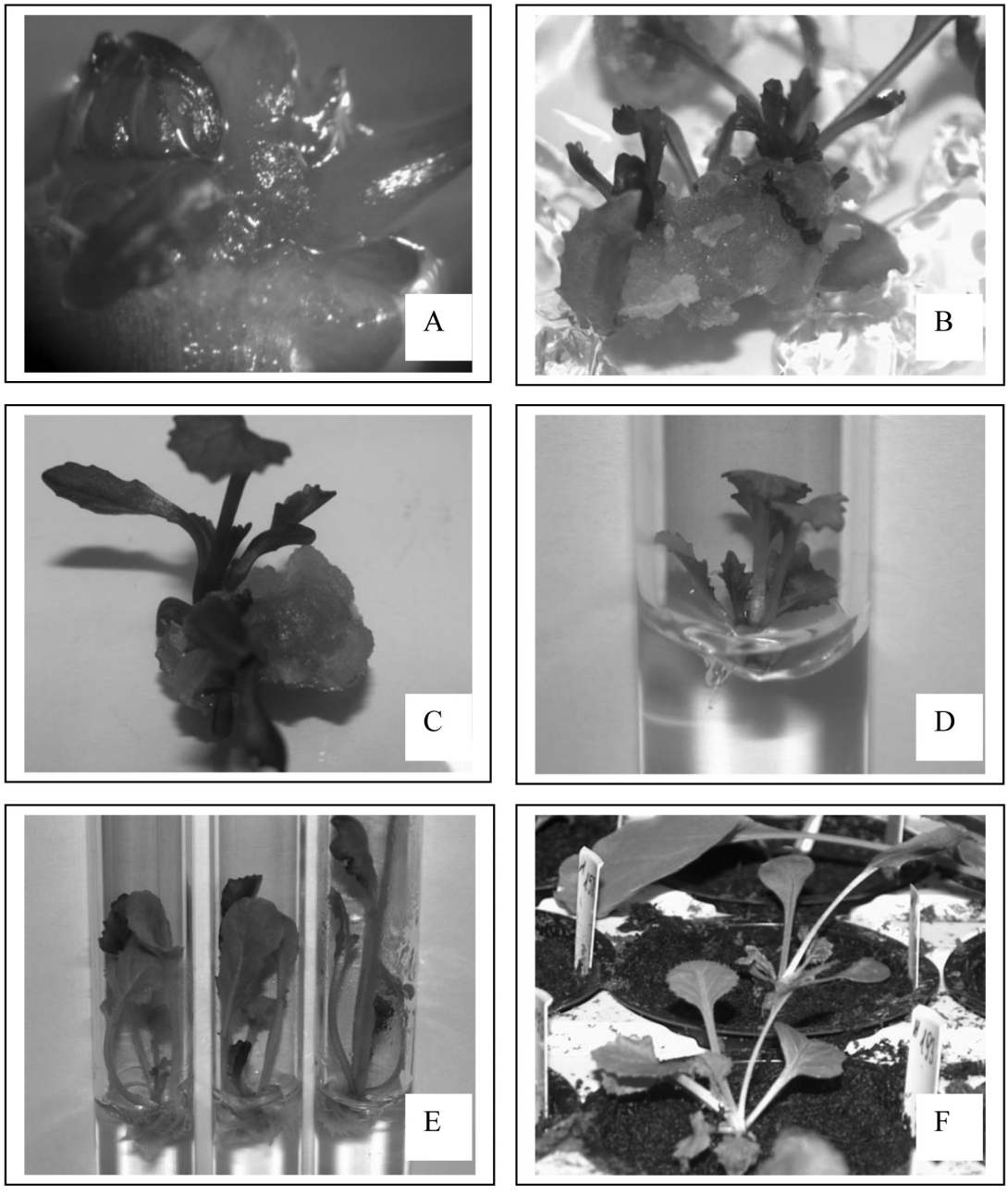

Figure. Plant regeneration from hypocotyls of spring rapeseed doubled haploid line NL-662. (A) Adventitious bud formation after two weeks on MS medium containing 4.0 mg l^{-1} BAP and 0.05 mg l^{-1} NAA. (B) Shoots developed from adventitious buds after three weeks on MS medium containing 4.0 mg l^{-1} BAP and 0.05 mg l^{-1} NAA. (C) Elongated shoot after 30 days on MS medium containing 4.0 mg l^{-1} BAP and 0.05 mg l^{-1} NAA. (D) Shoot transferred on rooting medium supplemented with 0.1 mg l^{-1} NAA. (E) Rooted plantlets after two weeks of cultivation on rooting medium. (F) Regenerated plants transplanted to pots for one week

was strongly related to genotypes. On a medium supplemented with 4.0 mg l^{-1} BAP and 0.05 mg l^{-1} NAA, the percentage of bud regeneration from hypocotyl-derived callus of NL-611, NL-662 and NL-685 was 21.2%, 25.6% and 13.1%, respectively. The results indicate that shoot regeneration ability is strongly influenced by the genotype.

Cotyledonary derived callus of NL-611 and NL-662 showed the highest bud regeneration frequency in a medium supplemented with 6.0 mg l^{-1} zeatin + 0.1 mg l^{-1} 2,4-D and 4.0 mg l^{-1} zeatin + 0.15 2,4-D, respectively, while 4.0 mg l^{-1} BAP + 0.05 mg l^{-1} NAA combinations were more suitable for bud regeneration from cotyledons of NL-685.

When explants were placed onto medium without growth regulators, the frequency of explants producing buds varied depending on the genotype and explant type, reaching 5.7% for hypocotyls (NL-685) and 31.8% for cotyledons (NL-662 and NL-685). This was an indication that high levels of endogenous growth regulators might be present in the explant source.

In terms of the number of buds produced per explant, the test genotypes showed a high variation. Significant differences

Table 2. **Effect of growth regulators on adventitious bud regeneration frequency**

Genotype	Growth regulators (mg l^{-1})				Explants forming buds (%)	
	BAP	NAA	Zeatin	2,4-D	Hypocotyls	Cotyledons
NL-611	0	0	0	0	4.3 ± 0.08bc	12.5 ± 0.22d
	4.0	0.05	0	0	21.2 ± 0.65h	8.2 ± 0.11b
	3.0	0.1	0	0	12.3 ± 0.28g	9.1 ± 0.16c
	2.0	0.15	0	0	8.1 ± 0.53e	10.3 ± 0.23c
			6.0	0.1	5.1 ± 0.27c	18.8 ± 0.17f
			4.0	0.15	4.4 ± 0.11bc	9.7 ± 0.09c
			2.0	0.15	3.8 ± 0.06b	7.1 ± 0.6b
NL-662	0	0	0	0	0a	31.8 ± 0.19h
	4.0	0.05	0	0	25.6 ± 0.72h	5.1 ± 0.04a
	3.0	0.1	0	0	10.9 ± 0.74f	22.4 ± 0.13fg
	2.0	0.15	0	0	6.7 ± 0.37d	15.6 ± 0.21e
			6.0	0.1	4.2 ± 0.09b	24.3 ± 0.17g
			4.0	0.15	0a	35.2 ± 0.14i
			2.0	0.15	0a	31.8 ± 0.11h
NL-685	0	0	0	0	5.7 ± 0.12c	31.8 ± 0.15h
	4.0	0.05	0	0	13.1 ± 0.39g	37.5 ± 0.10i
	3.0	0.1	0	0	8.1 ± 0.27e	29.3 ± 0.09h
	2.0	0.15	0	0	6.5 ± 0.32d	10.1 ± 0.12c
			6.0	0.1	10.3 ± 0.11f	12.5 ± 0.13d
			4.0	0.15	7.9 ± 0.07e	16.7 ± 0.17e
			2.0	0.15	6.1 ± 0.11cd	8.3 ± 0.07b

Data are means ± SE within four weeks of culture. Means within a column followed by the same letter are not significantly different from those indicated by Duncan's multiple-range test ($P \leq 0.05$).

Table 3. **Effect of growth regulators on bud number per explant–derived callus of spring rapeseed**

Genotype	Growth regulators (mg l^{-1})				Number of buds / explant	
	BAP	NAA	Zeatin	2,4-D	Hypocotyls	Cotyledons
NL-611	0	0	0	0	1.0 ± 0.04b	1.0 ± 0.02a
	4.0	0.05	0	0	3.7 ± 0.18e	1.2 ± 0.04a
	3.0	0.1	0	0	3.3 ± 0.15d	1.1 ± 0.02a
	2.0	0.15	0	0	2.1 ± 0.10c	1.1 ± 0.01a
			6.0	0.1	1.1 ± 0.04b	1.5 ± 0.07b
			4.0	0.15	1.2 ± 0.05b	0.9 ± 0.01a
			2.0	0.15	1.1 ± 0.02b	1.1 ± 0.01a
NL-662	0	0	0	0	0a	1.5 ± 0.03b
	4.0	0.05	0	0	3.1 ± 0.16d	1.4 ± 0.06ab
	3.0	0.1	0	0	3.0 ± 0.11d	1.3 ± 0.04ab
	2.0	0.15	0	0	2.9 ± 0.09d	1.5 ± 0.05b
			6.0	0.1	2.0 ± 0.10c	1.2 ± 0.02a
			4.0	0.15	0a	1.5 ± 0.03b
			2.0	0.15	0a	1.1 ± 0.02a
NL-685	0	0	0	0	2.5 ± 0.08cd	1.5 ± 0.04b
	4.0	0.05	0	0	3.8 ± 0.17e	2.0 ± 0.09c
	3.0	0.1	0	0	3.0 ± 0.13d	2.0 ± 0.06c
	2.0	0.15	0	0	1.1 ± 0.01b	1.5 ± 0.03b
			6.0	0.1	2.5 ± 0.08cd	1.0 ± 0.01a
			4.0	0.15	1.2 ± 0.05b	1.5 ± 0.04b
			2.0	0.15	0.8 ± 0.02b	1.0 ± 0.01a

Data are means ± SE within four weeks of culture. Means within a column followed by the same letter are not significantly different from those indicated by Duncan's multiple-range test ($P \leq 0.05$).

were observed in the mean level of bud formation on responsive culture media. The maximum number of buds per explant from hypocotyl-derived callus was obtained in a medium supplemented with 4.0 mg l^{-1} BAP and 0.05 mg l^{-1} NAA (Table 3). On this medium, each explant regenerated 3.1 (NL-662) to 3.8 (NL-685) buds. A combination of zeatin and 2,4-D also induced bud formation from NL-611 and NL-685 hypocotyl-derived callus, but the bud number per explant was significantly lower.

The mean number of adventitious buds from cotyledonary derived callus varied within 0.9 to 2.0 per explant. In most cases,

cotyledons developed a lower number of buds in comparison to hypocotyls.

Adventitious buds develop further into shoots after growing for another 2–4 weeks on the same medium (Figure, B). For root induction, elongated shoots (Figure, C) were transferred to MS medium supplemented with 0.1 mg l^{-1} NAA (Figure, D). Root differentiation on elongated shoots occurred over a period of 1–3 weeks (Figure, E). No phenotypic variation in the regenerated acclimatized plants was observed (Figure, F).

DISCUSSION

The use of hypocotyls and / or cotyledons as explants for *in vitro* plant regeneration has received considerable attention [13, 14]. A large number of explants can be obtained by growing seeds under sterile conditions over a short period of time all year around. Moreover, these explants possess a relatively high ability of shoot organogenesis, somatic embryogenesis and protoplast culture [15]. The capacity of rapeseed hypocotyls and cotyledons to regenerate whole plants via indirect organogenesis was examined. Generally, there was no difficulty in callus induction, with hypocotyls producing more callus than cotyledons. Although regeneration of buds or shoots from different explants (i. e. hypocotyls, cotyledons, stem segments) has been reported, the regeneration frequencies vary dramatically depending on the explant type of given genotypes. It has been documented that in canola somatic tissue culture the highest regeneration rate was obtained from cotyledons (60.9%) and lower for hypocotyls (27.1%) and roots (5.7%) [16]. In the present investigation, both test explants showed the development of adventitious buds, but bud regeneration frequency and the number of buds per explant varied with the explant. Generally, cotyledons-derived callus exhibited a higher bud regeneration frequency than hypocotyls, however, hypocotyl-derived callus developed a higher number of buds per explant. According to Ono et al. [3], the ability of shoot regeneration from cotyledons is mainly controlled by dominant nuclear genes, and such genes can be accumulated in agronomically important cultivars.

Among the many factors that may affect plant tissue culture responses, especially regeneration ability, genotypic difference is a primary one. A great genetic diversity in tissue culture responses has been reported among the cultivars of many crops, such as *Helianthus annuus* [17], *Crataegus pinnatifida* [18], *Rubus* spp. [19]. In the present study, significant differences in callus induction and plant regeneration were observed among three DH lines; for example, hypocotyls of NL-611 and NL-662 showed a greater capacity to produce shoots on a medium containing 4.0 mg l^{-1} BAP and 0.05 mg l^{-1} NAA than did NL-685. Our data confirmed previous results on *Brassica napus* [16, 20, 21] concerning a strong genotypic effect on shoot regeneration. Similar conclusions have also been drawn for other *Brassica* species, namely *B. campestris* [22], *B. oleraceae* [23], *B. rapa* [24].

Several biochemical processes and cellular signaling are required for differentiation during shoot morphogenesis in plants. Plant growth regulators play a key role in controlling the differentiation process required for regeneration. The frequency of adventitious bud formation from cultured explants is affected by the growth regulators in the medium. Our results have shown that hypocotyls grown in the presence of BAP and NAA gave rise to buds at the highest frequency, while the highest frequency of bud formation from cotyledons in two of the three test genotypes was observed in a medium containing zeatin and 2,4-D. In spite of the variability in response, the different growth regulator requirements necessary for each explant could suggest a different endogenous hormone balance of the explants.

All *in vitro* regenerated plantlets appeared normal without any morphological variations. The regenerated plants were also identical with the source plants and true-to-type. Our study has shown that seedling explants of newly selected yellow-seeded spring rapeseed DH lines are amenable to multiple shoot formation with high regeneration frequencies and could be used for genetic transformation experiments.

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VASARINIŲ RAPSŲ (*BRASSICA NAPUS* L.) KALIAUS INDUKCIJA IR AUGALŲ REGENERACIJA SOMATINIŲ AUDINIŲ KULTŪROJE

Santrauka

Tirtas maitinamosios terpės, eksplanto ir genotipo poveikis vasarinių rapsų (*Brassica napus* L.) pridėtinių ūglių formavimuisi. Dvigubų haploidų linijų NL-611, NL-662 ir NL-685 hipokotilių ir skilčialapių audiniai formavo kalių MS maitinamojoje terpėje, papildytoje skirtingomis 6-benzilaminopurino (BAP) ir α-naftilacto rūgšties (NAR) bei zeatino ir 2,4-dichlorfenoksiacto rūgšties (2,4-D) koncentracijomis. Pridėtinių pumpurų regeneracija vyko iš organogeninio kaliaus toje pačioje maitinamojoje terpėje. Ūglių susiformavimo dažnumas varijavo nuo 0 iki 37,5%, o pumpurų kiekis iš eksplanto – nuo 0 iki 3,8 vnt. Skilčialapių audinių suformuotas kalius pumpurus formavo dažniau nei hipokotilių suformuotas kalius, tačiau didesnis pumpurų kiekis vienam eksplantui gautas auginant hipokotilių suformuotą kalių. Didžiausias pumpurų susiformavimo iš hipokotilių audinių suformuoto kaliaus dažnis nustatytas terpėje su 4,0 mg l^{-1} BAP ir 0,05 mg l^{-1} NAR. Regeneravusių ūglių rizogenezė indukuota MS terpėje, papildytoje 0,1 mg l^{-1} NAR. Augalai regenerantai su gerai išsivysčiusiomis šaknimis adaptuoti auginimo kambaryje.