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IN VITRO SHOOT REGENERATION FROM SEEDLING EXPLANTS IN *BRASSICA* VEGETABLES: RED CABBAGE, BROCCOLI, SAVOY CABBAGE AND CAULIFLOWER

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Abstract - *Brassica oleracea* varieties (red cabbage, broccoli, Savoy cabbage and cauliflower) were tested for their ability to regenerate shoots *in vitro*. Cotyledon, hypocotyl and root explants of 7 day-old seedlings were incubated on Murashige and Skoog's (MS) medium supplemented with 1 mg l^{-1} 6-benzyladenine (BA) or 6-furfurylaminopurine (KIN) in combination with 0, 0.1, and 0.2 mg l^{-1} indole-3-butyric acid (IBA). Hypocotyls showed the best explants in almost all varieties tested with a minimum regeneration potential of 75% and producing 3.5-7.4 shoots per explant. The BA-supplemented media were optimal for both shoot regeneration and multiplication. Shoots rooted maximally (100%) on plant growth regulator-free MS medium containing 2% or 4% sucrose. Increased sucrose content improved plant acclimation in the greenhouse.

Keywords: Brassica oleracea, regeneration, seedling explants, cytokinins, shoot multiplication, plant acclimation

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INTRODUCTION

Brassica vegetables are an economically important and highly diversified group of crops belonging to the family Brassicaceae. *Brassica oleracea* is one of the major species of this group which includes many distinct vegetable and fodder varieties, such as cabbage, broccoli, Brussels sprouts, cauliflower, collards, Savoy cabbage, kohlrabi, rutabaga, and turnip. They are consumed worldwide as food of high nutritional value. *Brassica* vegetables contain little fat and are a source of vitamins, minerals, fiber, important proteins, and new phytochemicals that could be beneficial in the prevention of tumors.

Because of their significant importance *Brassica* vegetables are the objects of many breeding programs with the aimed at additionally improving the agronomic and nutritional performances of the existing genotypes. In conventional breeding hybridization with wild *Brassica* species was frequently used to improve disease resistance and environmental tolerance (Sretenović-Rajičić, 2000). The breeding of *Brassica* is complicated due to its two year head-seed-head cycle and a problem with sporophytic incompatibility.

Nowadays, many breeders attempt to improve *Brassica* crops by employing the biotechnological and genetic transformation approaches, in addition to the classic ones (reviewed by Vinterhalter et al., 2007). The successful application of these approaches requires an efficient and reliable tissue culture regeneration system. Plant regeneration systems for commercial micropropagation and disease-free plants production have been developed for many *Brassica* vegetables. Shoot regeneration was achieved from various tissues and organs including hypocotyls, cotyledons, roots, leaves, peduncle segments, callus and cell cultures, thin cell layers and protoplasts (reviewed by Cardoza and Stewart, 2004). Regeneration in *B. oleracea* has been reported from leaf and root segments (Lazzeri and Dunwell, 1986), hypocotyls (Lillo and Shanin, 1986), and cotyledons (Dale and Ball, 1991). However, considerable variation has been observed by diffe-

rent groups, even when working with the same species or variety.

Since genotypic specificity for regeneration is very high, the development of a suitable regeneration protocol for each genotype is necessary. From the many *B. oleracea* varieties and lines selected in Serbia, only a few have been investigated with respect to their tissue culture response and genetic transformation (Sretenović-Rajičić et al., 2004, 2006, and 2007). As part of a long-term project on improvements of *B. oleracea* varieties at the Institute for Vegetable Crops in Smederevska Palanka, we found it necessary to investigate the shoot regeneration ability in four varieties that represent prospective material for further breeding.

We studied the regeneration ability in red cabbage (*Brassica oleracea* var. *capitata*), broccoli (*Brassica oleracea* var. *italica*), Savoy cabbage (*Brassica oleracea* var. *sabauda*) and cauliflower (*Brassica oleracea* var. *botrytis*). The use of different explants and culture media formulations was studied.

MATERIALS AND METHODS

Plant material

Four *B. oleracea* varieties were used in this study, including red cabbage (*B. oleracea* var. *capitata*, cv. Rubin) and cauliflower (*Brassica oleracea* var. *botrytis*, cv. Rasa), both selected at the Institute for Vegetable Crops in Smederevska Palanka, along with open-pollinated cultivars of broccoli (*Brassica oleracea* var. *italica*, cv. Korvet), and Savoy cabbage (*Brassica oleracea* var. *sabauda*, cv. Vertus). Mature seeds were rinsed in 70% (v/v) ethanol for 1 min, the surface sterilized in 20% commercial bleach (8% NaOCl) for 20 min, and then rinsed five times with sterile distilled water. The surface-sterilized seeds were germinated in 90 mm Petri dishes (14-17 seeds per dish) with 20 ml of a plant growth regulator (PGR)-free MS (Murashige and Skoog, 1962) medium containing 2% (w/v) sucrose, and 0.7% (w/v) agar (Institute for Virusology, Torlak, Belgrade, Serbia).

Media composition and culture conditions

Cotyledon, hypocotyl and root explants were aseptically excised from 7 day-old seedlings and cultured on a MS solid shoot regenerating medium supplemented with 1 mg l^{-1} 6-benzyladenine (BA, Sigma Co., USA) or 1 mg l^{-1} 6-furfurylaminopurine (kinetin, KIN, Sigma Co., USA) in combination with 0, 0.1 or 0.2 mg l^{-1} indole-3-butyric acid (IBA, Sigma Co., USA). The pH of the media was adjusted to 5.8 prior to autoclaving at 117°C for 25 min. The cultures were maintained in a growth room under white fluorescent tubes with a photon flux density of 47 μmol m^{-2} s^{-1}, and a 16 h day length, at 23 ± 2 °C.

After four weeks of culture on the shoot regenerating medium, single regenerated shoots were transferred on a MS shoot multiplication medium supplemented with 1.0 mg l^{-1} BAP + 0.2 mg l^{-1} IBA, 0.5 mg l^{-1} BAP + 0.1 mg l^{-1} IBA, and 0.5 mg l^{-1} KIN + 0.1 mg l^{-1} IBA.

The multiplied shoots reached 3 cm or more in height and were cultured for four weeks on an MS medium containing 2 or 4% sucrose and supplemented with 0, 1, 2 or 4 mg l^{-1} IBA for rooting.

Rooted shoots with three to five leaves were transplanted to pots containing soil for acclimation and cultured in a growth chamber with high relative humidity (80%) for 3-4 weeks before being moved to the greenhouse for further growth.

Data recording and statistical analysis

For plant regeneration (the percentage of shoot forming explants and the average number of shoots per explant) 18 treatments were tested for each variety, 3 explant types and six media formulations. Each treatment consisted of 22-35 explants with two replicates. The regeneration rate was recorded after four weeks of culture on a shoot regenerating medium. For shoot multiplication three treatments were tested (1.0 mg l^{-1} BAP + 0.2 mg l^{-1} IBA, 0.5 mg l^{-1} BAP + 0.1 mg l^{-1} IBA, and 0.5 mg l^{-1} KIN + 0.1 mg l^{-1} IBA). The multiplication index was calculated as

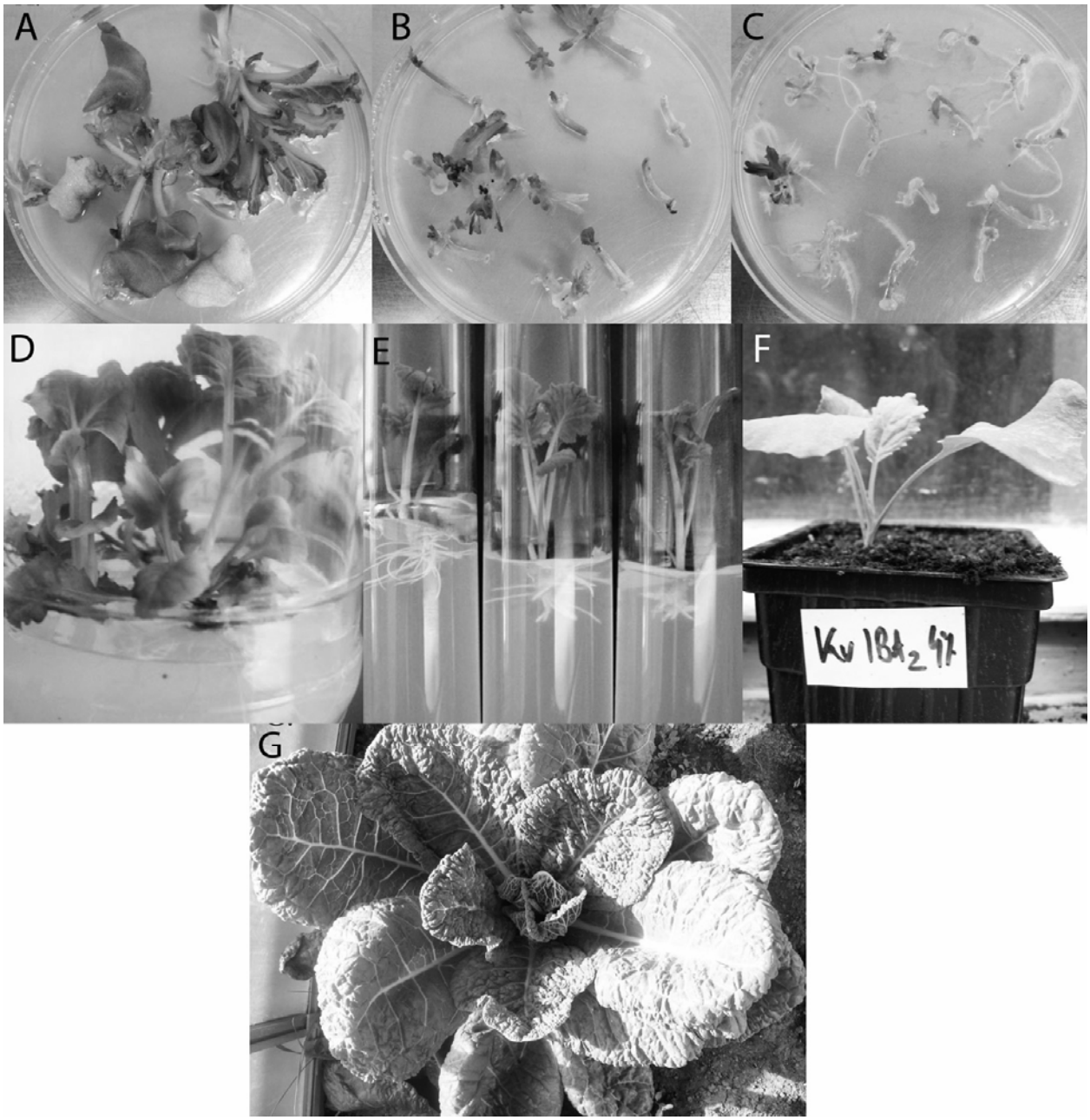

Fig. 1. Different stages of Savoy cabbage during *in vitro* regeneration. Shoots were induced from cotyledon (A), hypocotyl (B), and root explants (C) on MS medium with 1mg l^{-1} BA after culture for 4 weeks. Shoot multiplication on MS medium containing 0.5 mg l^{-1} BA + 0.1mg l^{-1} IBA (D). Rooted shoots after 4 weeks of rooting on a medium with 1, 2, and 4 mg l^{-1} IBA (from left to right) and 4% sucrose (E). Growth chamber (F) and greenhouse (G) grown acclimated plants produced from shoots rooted on a medium containing 4% sucrose.

the mean number of shoots per explant after four weeks of culture on the multiplication media.

We also evaluated the effect of IBA (1, 2 or 4 mgl^{-1} IBA) and sucrose-supplemented media (2 or 4%) on rooting.

The data were subjected to standard analysis of variance (ANOVA) and the means were separated using the LSD test at $P \leq 0.05$.

The survival rate of the acclimated plants was recorded for three weeks after transplantation.

RESULTS AND DISCUSSION

The seeds of all the *B. oleracea* varieties were germinated on a solid plant growth regulator-free MS medium. After 7 days the percentage of germination was highest in broccoli (97%), and followed by Savoy cabbage, cauliflower and red cabbage (88%, 84%, and 81%, respectively). Contamination in the seeds varied from 0.5% to 3.9%. Within 7 days of germination, the seedlings reached about 3-4 cm with expanded cotyledons and considerably elongated hypocotyls and roots.

For regeneration the cotyledon, hypocotyl and root segments were excised from the seedlings and cultured on MS medium containing either BA or KIN, alone or in combination with IBA.

On the regenerating media the explants expanded in size and become swollen. After four weeks the formation of calli and shoots was observed on swollen cut edges and/or in the middle part of explants (Fig. 1A-C). The shoot regeneration pattern was the same in all the varieties tested, occurring via adventitious shoot organogenesis. The percentage of explants regenerating shoots and the average number of shoots per explant on six media formulations are presented in Table 1. The dominant factor on the percentage of regenerating explants was the choice of explant type. In general, in all the *B. oleracea* varieties the hypocotyls showed the highest percentage of shoot formation ranging from 75% in red cabbage to 92% in Savoy cabbage (Table 1). The regeneration response of cotyledonary explants varied significantly (0-85%), depending on the variety and culture media used. Roots were poorly regenerated in the explants of broccoli and red cabbage. In cauliflower on the BA-containing media as well as in Savoy cabbage these explants displayed a satisfactory regeneration response of about 50% (Table 1).

We also compared the regeneration potential based on the average number of shoots per explant (Table 1). In broccoli, the average number of shoots from hypocotyl explants was 3.9- and 7.4-fold higher than from cotyledon and root explants, respectively. In red cabbage, the hypocotyls regenerated two times more shoots than the cotyledon and root explants. There were insignificant differences between cotyledon and hypocotyl explants cultured on the medium containing 1.0 mg l^{-1} BA with respect to the number of shoots per explant in Savoy cabbage (5.4 and 5.2, respectively) (Table 1, Fig. 1 A and B), while root explants produced fewer shoots (Fig. 1C). In cauliflower, the root explants cultured on the medium with 1.0 mg l^{-1} BA and cotyledon explants cultured on the medium containing 1.0 mg l^{-1} KIN displayed the highest rate of shoot formation (5.2 and 5.5, respectively).

Seedling hypocotyls were preferred for regeneration (Lazzeri and Dunwell, 1986; Yang et al., 1991; Fuller et al., 1994; Cardoza and Stuart, 2004) and transformation of Brassicas (Metz et al., 1995; Puddephat et al., 2001).

Overall, the medium containing BA was optimal for shoot regeneration and multiplication in all the investigated varieties (Table 1). There are significant differences in the frequencies of explants with shoots when BA was applied alone or in combination with IBA. The addition of 0.1 mg l^{-1} or 0.2 mg l^{-1} IBA in many cases influenced the highest percentage of regenerating explants, except in the case of the hypocotyl segments in red cabbage. On the other hand, the application of BA or KIN alone was satisfactory with respect to the number of shoots regenerated per explant, except in the hypocotyl explants in red cabbage, where the com

Table 1. The effect of media and explant type on adventitious shoot regeneration in four *B. oleracea* varieties. *Different explants on the same medium were compared. Values with different letters are statistically different (P ≤ 0.05) with the LSD test, (n=22-35).

Hormones (mg l^{-1})	Frequency of explants with shoots (%)			No. of shoots per explant		
	Cotyledon	Hypocotyl	Root	Cotyledon	Hypocotyl	Root
Broccoli						
BA 1.0	10	57	0	1.9 ± 0.1b*	7.4 ± 0.3c	0.0a
BA 1.0 + IBA 0.1	5	84	0	1.8 ± 0.1b	5.2 ± 0.6c	0.0a
BA 1.0 + IBA 0.2	20	68	0	1.9 ± 0.1b	6.3 ± 0.1c	0.0a
KIN 1.0	32	48	0	1.3 ± 0.0b	2.1 ± 0.1b	0.0a
KIN 1.0 + IBA 0.1	12	61	5	1.0 ± 0.1a	5.0 ± 0.4b	1 ± 0.0a
KIN 1.0 + IBA 0.2	5	50	17	1.9 ± 0.1b	1.8 ± 0.1b	1.0 ± 0.1a
Red cabbage						
BA 1.0	29	75	0	1.6 ± 0.1b	3.8 ± 0.04c	0.0a
BA 1.0 + IBA 0.1	41	54	14	2.0 ± 0.1a	5.3 ± 0.02b	2.3 ± 0.3a
BA 1.0 + IBA 0.2	5	70	0	2.1 ± 0.3b	5.0 ± 0.1c	0.0a
KIN 1.0	5	38	0	2.0 ± 0.1b	2.2 ± 0.2b	0.0a
KIN 1.0 + IBA 0.1	0	67	0	0.0a	3.1 ± 0.1b	0.0a
KIN 1.0 + IBA 0.2	15	32	47	1.7 ± 0.3a	2.6 ± 0.06b	2.4 ± 0.1ab
Cauliflower						
BA 1.0	74	59	22	3.4 ± 0.1a	3.6 ± 0.3a	5.2 ± 0.1b
BA 1.0 + IBA 0.1	72	72	42	4.2 ± 0.03b	2.1 ± 0.06a	4.3 ± 0.1b
BA 1.0 + IBA 0.2	47	77	57	3.4 ± 0.1c	3.1 ± 0.1b	2.0 ± 0.0a
KIN 1.0	85	51	0	5.5 ± 0.1c	2.1 ± 0.1b	0.0a
KIN 1.0 + IBA 0.1	54	54	0	3.1 ± 0.06c	2.9 ± 0.0b	0.0a
KIN 1.0 + IBA 0.2	75	56	15	2.3 ± 0.04a	2.2 ± 0.1a	2.5 ± 0.5a
Savoy cabbage						
BA 1.0	64	71	37	5.4 ± 0.1b	5.2 ± 0.04b	2.5 ± 0.1a
BA 1.0 + IBA 0.1	68	92	50	2.5 ± 0.5a	4.9 ± 0.01b	2.9 ± 0.1a
BA 1.0 + IBA 0.2	21	86	50	3.5 ± 0.04a	4.8 ± 0.01b	3.6 ± 0.05a
KIN 1.0	43	46	29	2.1 ± 0.1a	1.8 ± 0.3a	2.4 ± 0.1a
KIN 1.0 + IBA 0.1	24	62	22	3.5 ± 0.3a	3.2 ± 0.1a	3.1 ± 0.06a
KIN 1.0 + IBA 0.2	31	71	55	1.2 ± 0.3ab	2.0 ± 0.1b	1.1 ± 0.3a

bination of BA with IBA had a positive effect on shoot regeneration. The highest number of shoots regenerated per explant (7.4 ± 0.3) was obtained in broccoli from hypocotyl explants cultured on the medium with 1.0 mg l^{-1} BA (Table 1).

The positive effects of BA on regeneration from wide range of explants were reported in different *Brassica* species. A high frequency of regenerated shoots (100%) from hypocotyls using a BA and NAA combination was achieved in *B. carinata*

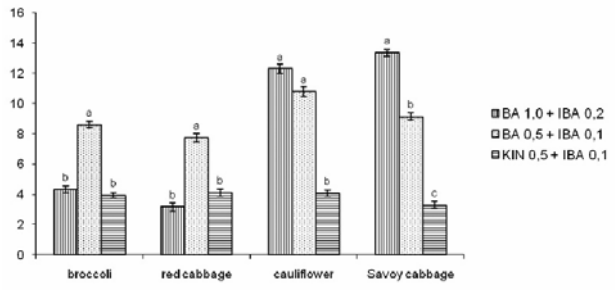

Fig. 2. I*n vitro* shoot multiplication performance of four *B. oleracea* varieties. Differences between media are significant at the level of $P \leq 0.05$.

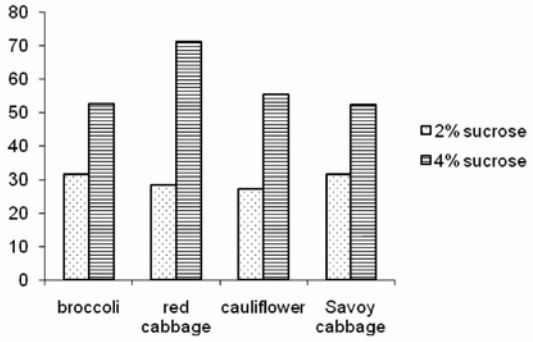

Fig. 3. Percentages of acclimated plants rooted on a medium with 2% and 4% sucrose.

(Yang et al., 1991). Without the addition of the auxin NAA, 4.44 μM BA was the optimum concentration for shoot regeneration in *B. juncea* var. *tsatsai* (Guo et al., 2005). Regeneration frequencies of up to 98% have been reported for broccoli peduncle explants cultured on media supplemented with 1 mg l^{-1} BA (Christey and Earle, 1991). The presence of BA in the medium markedly increased the number of shoots produced per explant in rapid-cycling *B. oleracea in vitro* (Cheng et al., 2001). Furthermore, in this group of plants shoot regeneration occurred nearly 4 days earlier in hypocotyls explants in the presence of BA.

On the other hand, we observed that the BA-containing media caused a high percentage of hyperhydricity in the shoots, especially in the cauliflower and Savoy cabbage cultures (over 50%). Several factors have been ascribed as being responsible for this hyperhydricity (Debergh et al., 1992). Li et al., (2003) proposed that an excess of cytokinins along with the high water potential of the medium were the major reasons for the vitrification of shoots. Vandemoortele et al., (2001) reported a simple procedure of shoot propagation of cauliflower cv. Commandeur without apparent hyperhydric symptoms using an osmotic pretreatment by soaking the explant in sucrose (- 2 MPa for 24 h) before culture on a PGR-free medium. In our experiment this problem was overcome by the substitution of BA with KIN in the media, which satisfactory regeneration results in these plants, but reduced the appearance of vitrified shoots.

The induced shoots were placed on another three media containing BA (0.5-1.0 mg l^{-1}) or KIN (0.5 mg l^{1}) in combination with IBA (0.1-0.2 mg l^{-1}) for multiplication (Fig. 2). In this step the combinations containing BA were also more effective. For cauliflower and Savoy cabbage the most favorable medium for the growth, maintenance and multiplication of developed shoots was 1.0 mg l^{-1} BAP + 0.2 mg l^{-1} IBA (Fig. 1D), while for red cabbage and broccoli the most favorable medium contained the same hormone combination but in reduced concentrations (Fig. 2). The highest multiplication rate ranged from 8.7 in broccoli to 13.4 in Savoy cabbage.

For root establishment well-developed shoots approximately 3 cm in height were transferred onto a PGR-free MS medium as well as onto media with three different concentrations of IBA (1, 2, and 4 mg l^{-1}) that contained 2% or 4% sucrose. Rooting was 100% on the PGR-free media containing 2% sucrose, except in cauliflower where 100% rooting was achieved on a PGR-free medium with a higher, 4% sucrose concentration (Table 2). The addition of IBA increased the number of roots produced per one shoot while at the same time the average root length was decreased (Fig. 1E, Table 2).

One of the major problems in our experiment was the acclimation of the propagated plants. It appears that good rooting does not always coincide with adequate acclimation. Previous results have shown that an important factor in acclimation could be the sucrose content in the rooting

Table 2. Frequency of rooting, number of roots per rooted shoot and average root lengths of regenerated shoots of four *B. oleracea* varieties. *Value represents the mean ± standard error.

Sucrose % + IBA mg l^{-1}		**Broccoli**			**Red cabbage**			**Cauliflower**			**Savoy cabbage**	
		Rooting, %	No. of roots ± SE*	Length of root ± SE, mm	Rooting, %	No. of roots ± SE	Length of root ± SE, mm	Rooting, %	No. of roots ± SE	Length of root ± SE, mm	Rooting, %	No. of roots ± SE
2%	0	100	12.0±6.3	35.4±3.4	100	24.0±7.0	60.5±3.5	75	18.7±4.1	35.0±1.4	100	9.4±5.2
	1	100	17.8±7.1	34.2±4.1	100	26.0±2.5	36.5±6.5	100	17.0±1.2	32.0±7.7	100	4.6±1.9
	2	100	24.8±8.3	24.2±3.8	100	12.0±1.0	24.5±2.5	100	21.5±2.5	37.0±2.3	100	10.0±5.7
	4	80	25.2±9.8	19.8±7.6	50	16.0±1.0	19.5±2.5	75	10.0±4.3	38.0±2.4	80	9.0±3.9
4%	0	100	13.8±6.1	50.0±9.3	100	28.5±4.5	48.5±1.5	100	16.7±2.2	26.5±6.9	83.3	8.8±3.7
	1	100	17.8±6.2	32.8±8.8	100	16.0±1.0	47.0±7.0	100	34.5±1.5	33.5±4.5	60	8.7±4.9
	2	80	19.0±4.2	39.2±9.9	100	9.5±3.5	32.5±9.5	100	33.0±4.0	24.0±1.0	83.3	7.6±3.6
	4	100	21.4±6.5	26.8±8.3	50	28.5±2.5	41.0±3.0	60	33.0±2.0	25.0±3.0	80	8.7±3.8

medium, and that it might even be more important than the hormone content (Sretenović-Rajičić et al., 2002). Our results confirmed the beneficial effect of a higher sucrose content in the rooting medium on the acclimation of plantlets when an average of 58% of the plants rooted on a medium with 4% sucrose acclimated successfully (Fig. 1F and G, Fig. 3) in comparison to 30% of survived plants rooted on a medium with 2% sucrose. Further experiments are required to clarify this point.

In conclusion, our results show a satisfactory frequency of shoot regeneration from hypocotyl explants and multiplication of shoots on media containing 1 mg l^{-1} BA alone or in combination with IBA in the four investigated *B. oleracea* varieties. In cauliflower and Savoy cabbage the replacement of BA for KIN was less favorable for shoot vitrification. Using the protocol presented here, plantlets ready for transfer to soil were obtained in 12 weeks. An increased sucrose content in the rooting medium can improve the percentage of plant acclimation. An efficient *in vitro* plant regeneration, rooting, and acclimation protocol may be useful in breeding processes used in developing new lines and cultivars for shorter time, and in genetic improvement by using biotechnological approaches. We are currently using this protocol for generating *B. oleracea* varieties with an improved tolerance to biotic and abiotic factors.

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РЕГЕНЕРАЦИЈА ПУПОЉАКА *IN VITRO* ИЗ ЕКСПЛАНТАТА КЛИЈАНАЦА КОД ПОВРТАРСКИХ КУЛТУРА РОДА *BRASSICA*: ЦРВЕНОГ КУПУСА, БРОКОЛИЈА, КЕЉА И КАРФИОЛА

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Испитивана је способност четири варијетета *Brassica oleracea* (црвени купус, броколи, кељ и карфиол) да регенеришу пупољке *in vitro*. Експлантати котиледона, хипокотила и коренова, узетих са 7 дана старих клијанаца, су гајени на Murashige и Skoog (MS) хранљивој подлози са додатком 1 mg l^{-1} 6-бензиладенина (BA) или 6-фурфуриламинопурина (KIN) у комбинацији са 0, 0.1, и 0.2 mg l^{-1} индол-3-бутиричне киселине (IBA). Експлантати хипокотила су се показали као најбољи за регенерацију код скоро свих тестираних варијетета са минималним регенеративним потенцијалом од 75% и са продукцијом 3.5-7.4 пупољака по експлантату. Подлоге које су садржале BA су биле оптималне, како за регенераију пупољака, тако и за њихову каснију мултипликацију. Максималан проценат оживљавања изданака (100%) је постигнут на MS медијуму без додатих регулатора растења, а који је садржао 2% или 4% сахарозу. Повећан садржај сахарозе у медијуму за ожиљавање утицао је на побољшану аклиматизацију биљака у стакленику.