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-furfurylamino-purine (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

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 spe-
cies or variety.

Since genotypic specificity for regeneration is
very high, the development of a suitable regene-
ration protocol for each genotype is necessary.
From the many *B. oleracea* varieties and lines selec-
ted 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 Insti-
tute for Vegetable Crops in Smederevska Palanka,
we found it necessary to investigate the shoot rege-
neration 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
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-butryic 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 supple-
mented 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 mg l\(^{-1}\) 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 ≤ 0.05.

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

RESULTS AND DISCUSSION

The seeds of all the \textit{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 \textit{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).

<table>
<thead>
<tr>
<th>Hormones (mg l⁻¹)</th>
<th>Frequency of explants with shoots (%)</th>
<th>No. of shoots per explant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cotyledon</td>
<td>Hypocotyl</td>
</tr>
<tr>
<td><strong>Broccoli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BA 1.0</td>
<td>10</td>
<td>57</td>
</tr>
<tr>
<td>BA 1.0 + IBA 0.1</td>
<td>5</td>
<td>84</td>
</tr>
<tr>
<td>BA 1.0 + IBA 0.2</td>
<td>20</td>
<td>68</td>
</tr>
<tr>
<td>KIN 1.0</td>
<td>32</td>
<td>48</td>
</tr>
<tr>
<td>KIN 1.0 + IBA 0.1</td>
<td>12</td>
<td>61</td>
</tr>
<tr>
<td>KIN 1.0 + IBA 0.2</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td><strong>Red cabbage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BA 1.0</td>
<td>29</td>
<td>75</td>
</tr>
<tr>
<td>BA 1.0 + IBA 0.1</td>
<td>41</td>
<td>54</td>
</tr>
<tr>
<td>BA 1.0 + IBA 0.2</td>
<td>5</td>
<td>70</td>
</tr>
<tr>
<td>KIN 1.0</td>
<td>5</td>
<td>38</td>
</tr>
<tr>
<td>KIN 1.0 + IBA 0.1</td>
<td>0</td>
<td>67</td>
</tr>
<tr>
<td>KIN 1.0 + IBA 0.2</td>
<td>15</td>
<td>32</td>
</tr>
<tr>
<td><strong>Cauliflower</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BA 1.0</td>
<td>74</td>
<td>59</td>
</tr>
<tr>
<td>BA 1.0 + IBA 0.1</td>
<td>72</td>
<td>72</td>
</tr>
<tr>
<td>BA 1.0 + IBA 0.2</td>
<td>47</td>
<td>77</td>
</tr>
<tr>
<td>KIN 1.0</td>
<td>85</td>
<td>51</td>
</tr>
<tr>
<td>KIN 1.0 + IBA 0.1</td>
<td>54</td>
<td>54</td>
</tr>
<tr>
<td>KIN 1.0 + IBA 0.2</td>
<td>75</td>
<td>56</td>
</tr>
<tr>
<td><strong>Savoy cabbage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BA 1.0</td>
<td>64</td>
<td>71</td>
</tr>
<tr>
<td>BA 1.0 + IBA 0.1</td>
<td>68</td>
<td>92</td>
</tr>
<tr>
<td>BA 1.0 + IBA 0.2</td>
<td>21</td>
<td>86</td>
</tr>
<tr>
<td>KIN 1.0</td>
<td>43</td>
<td>46</td>
</tr>
<tr>
<td>KIN 1.0 + IBA 0.1</td>
<td>24</td>
<td>62</td>
</tr>
<tr>
<td>KIN 1.0 + IBA 0.2</td>
<td>31</td>
<td>71</td>
</tr>
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</table>

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

![Fig. 2.](image)

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

![Fig. 3.](image)

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 ($\sim 2$ 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.
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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REFERENCES


IN VITRO SHOOT REGENERATION FROM SEEDLING EXPLANTS IN BRASSICA VEGETABLES

REGENERACIJA PUPOЉAKA IN VITRO IЗ ЕКСПЛАНТАТА КЛИЈАНАЦА КОД ПОВРТАРСКИХ КУЛТУРА РОДА BRASSICA: ЦРВЕНОГ КУПУСА, БРОКОЛИЈА, КЕЉА И КАРФИОЛА

СУЗАНА ПАВЛОВИЋ1, БРАНКА ВИНЕРХАЛТЕР2, НЕВЕНА МИТИЋ2, С. АЏИЋ1, Н. ПАВЛОВИЋ1, М. ЗДРАВКОВИЋ1 и Д. ВИНЕРХАЛТЕР2

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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 (МS) 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 тес- тираних варијетета са минимальним регенеративним потенцијалом од 75% и са продукцијом 3.5-7.4 пупољака по експлантату. Подлоге које су садржали BA су биле оптималне, како за регенерацију пупољака, тако и за њихову каснију мултипликацију. Максималан процент 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 оживљавање утицао је на побољшану аклиматизацију биљака у стакленику.