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MICROPROPAGATION OF ORNAMENTAL SPECIES *BRASSICA OLERACEA* CV. KYOTO RED GIVEN AND *AGERATUM* SP.

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Abstract

Ornamental red cabbage (*Brassica oleracea* cv. Kyoto red given) and ageratum (*Ageratum* sp.) are important ornamental plants which are typically grown in balconies, yards, parks and other open area spaces during the summer and winter period. Approximately, about 156 ornamental species/from different genera/ are produced via *in vitro* culture in different commercial laboratories worldwide. *In vitro* culture of plants is a key tool in plant biotechnology which utilizes the plant cell totipotency.

In this research the effects of different concentrations and combinations of BA, GA_3, IAA, and NAA on meristem buds and cotyledons as starting explants of ornamental red cabbage and ageratum were studied. The highest percentage of frequency of shoot formation from meristem buds was obtained on MS + 2 mg/l BA + 0.1 mg/l IAA + 0.1 mg/l GA_3 and MS + 2 mg/l BA for the ornamental red cabbage. On the contrary, the meristem buds of ageratum showed the best percentage of frequency of shoot formation on MS + 2 mg/l BA + 0.1 mg/l NAA. Cotyledons from ornamental red cabbage gave the highest frequency (56%) of shoot formation when initially cultivated on MS + 2 mg/l BA + 0.1 mg/l NAA, while the ageratum cotyledons responded with necrosis on all media utilized in the research. This research is a basis for further study about the enhancement of regeneration of different plant explants from different, economically important, ornamental species.

Key words: *ornamental red cabbage (Brassica oleracea cv. Kyoto red given), ageratum (Ageratum sp.), in vitro, growth regulators, meristem bud, cotyledon, shoot*

Abbreviations: BA (6-Benzylaminopurine), GA_3 (Gibberellic acid), IAA (Indole-3-acetic acid), NAA (alpha-Naphthaleneacetic acid)

INTRODUCTION

The commercial production of ornamental plants is growing worldwide. Its monetary value has significantly increased over the last two decades and there is a great potential for continued further growth in both domestic and international markets (Jain, 2002). Major pot plants such as Begonia, Ficus, Anthurium, Chrysanthemum, Rosa, Saintpaulia, and Spathiphyllum are being produced in the developed countries. About 212.5 million plants including 157 million ornamental plants amounting to 78% of the total production were reported (Rout et al., 2006). Ornamental industry has applied immensely *in vitro* propagation approach for large-scale plant multiplication of elite superior varieties. As a result, hundreds of plant tissue culture laboratories have come up worldwide, especially in the developing countries due to cheap labour costs. However, micropropagation technology is more costly than conventional propagation methods, and unit cost per plant becomes unaffordable compelling to adopt strategies to cut down the production cost for lowering the cost per plant (IAEA-TECDOC-1384, 2004).

Ornamental red cabbage (*Brassica oleracea* cv. Kyoto red given), also known as 'flowering' cabbage, is round shaped cabbage characterized by blooming in red and white colors during autumn period which makes it a desirable plant for different floristic decorations. It is plant of cold weather and it needs low temperatures to give the best leaf colors. As it is grown in late summer and early autumn, this species has less diseases and pests compared to spring species (Bajaj and Nietsch, 1975).

The name of the genus Ageratim (*Ageratum* sp.) is of antique Greek origin (ageratos = forever young) which is related to the blooming duration. The flowers of this species are mainly in shades of blue, but pink, lavender and white variations can be also found. The ageratum flowers are fluffy with nice fragrance. Each flower group is composed of 5 to 15 flowers. It blooms from late spring to early frost. The ageratum needs well drainage soil, but dry conditions are adverse for its growth. It does not have serious disease or pest problems, although mites can cause problems, particularly during dry and hot weather conditions (Stephens, 2007).

Shoot regeneration of different Brassica species was achieved from various tissues and organs including hypocotyls, cotyledons, roots, leaves, peduncle segments, callus and cell cultures, thin cell layers and protoplasts (Cardoza and Stewart, 2004). However, there are almost no literature sources on micropropagation of ageratum, which, beside ornamental, owns great medicinal values (Stephens, 2007).

MATERIALS AND METHODS

The research described in this paper was conducted at the Department of Plant Biotechnology, Faculty of Agriculture, Goce Delcev University – Stip. As starting explants were used meristem buds and cotyledons of commercially genotypes of ornamental red cabbage (*Brassica oleracea* cv. Kyoto red given) and ageratum (*Ageratum* sp.).

Obtaining of starting material for *in vitro* propagation

The seeds of commercial genotypes of both species after sterilization were inoculated on basal medium (1/2 MS solution, 3% sucrose, free of growth regulators) as 10 seeds in 10 Erlenmeyer flasks, or in total 100 seeds of each species. After seed germination, the meristem buds and cotyledons were isolated and they were used as starting explants in the research.

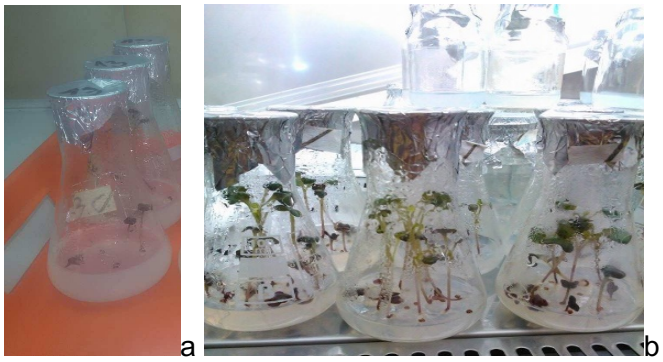


Figure 1. a) Seed germination of ornamental red cabbage.
b) Fully developed seedlings of ornamental red cabbage before isolation of meristem buds and cotyledons.

Sterilization of seeds

The seeds were surface sterilized with:

- Submersing in 70% C_2H_5OH for 3 minutes,
- Submersing in 1,5% Izosan G for 10 minutes,
- Afterwards they were washed (x3 times) in sterile distilled water.

Regeneration of meristem buds and cotyledons on MS medium with different growth regulators

The meristem buds and cotyledons as starting explants of ornamental red cabbage and ageratum were inoculated on MS medium (Murashige and Skoog, 1962), supplemented with certain concentration of BA, GA_3, IAA and NAA. The development of the explants was followed during the experiment.

Ornamental red cabbage bud meristem and cotyledons were initially cultivated on MS medium enriched with the following growth regulators:

A: MS + 2 mg/l BA +0.1 mg/l IAA + 0.1 mg/l GA_3

B: MS + 2 mg/l BA +0.1 mg/l NAA

C: MS + 2 mg/l BA

D: MS + 5 mg/l BA + 5 mg/l NAA

Ageratum bud meristem and cotyledons were initially cultivated on MS medium enriched with the following growth regulators:

A: MS + 2 mg/l BA +0.1 mg/l IAA + 0.1 mg/l GA_3

B: MS + 2 mg/l BA +0.1 mg/l NAA

E: MS + 5 mg/l BA

F: MS + 3 mg/l BA + 1.5 mg/l NAA

Obtained shoots from the both species under study were subcultured on rooting medium MS + 0.5 mg/l IAA + 2.5 mg/l IBA.

The starting explants which after one month of cultivation did not resulted in formation of regenerant were subcultured on fresh MS medium supplemented with the same combination and concentration of growth regulators as in the starting cultivation.

Obtained shoots from the both species under study were subcultured on rooting medium MS + 0.5 mg/l IAA + 2.5 mg/l IBA.

Statistical analysis

Statistical analysis of variance was applied for the evaluation of each of the parameter among all tested combinations of growth regulators (One-Way ANOVA test) with IBM SPSP Statistics Software 19.0.

For evaluation of the difference between tested combinations of growth regulators Duncan's multiple range test is utilized for each trait in each experimental year on 0.05%

RESULTS AND DISCUSSION

In Table 1. the number and size of the meristem bud and cotyledons of ornamental red cabbage on MS medium supplemented with different growth regulators are presented. On the medium MS + 2 mg/l BA + 0.1 mg/l IAA + 0.1 mg/l GA_3 initially were cultivated 40 meristem buds with average width of 3.5 mm and average height of 8.8 mm and 48 cotyledons with average width 1.2 mm and length 2.5 mm. On MS + 2 mg/l BA + 0.1 mg/l NAA were inoculated 40 meristem buds with average width 4.7 mm and height 9.2 mm, and 55 cotyledons with average width 1.9 mm and length 2.1 mm. On MS + 2mg/l BA were initially cultivated 31 meristem buds with average width 1.15 mm and average height of 3.8 mm, and 47 cotyledons with average width 3.2 mm and length 1.4 mm. On MS + 5 mg/l BA + 5 mg/l NAA were inoculated 50 meristem buds with average width 2.8 mm and average height of 9.5 mm, and 50 cotyledons with average width 1.0 mm and average length 2.4 mm.

Very often the cells react differently in different developmental phases, when interaction between signal paths of auxins and cytokinins can occur (Shi et al., 1994). BA alone or in combination with auxin is proven as optimal for regeneration and multiplication of different Brassica species (Metz et al., 1995; Munshi et al., 2007). Gerszberg et al. (2015) tested cotyledon and hypocotyl of eight Brassica cultivars on five types of media, where MS + 8.88 μM 6-benzyloaminopurine (BAP) + 0.53 μM α-naphthylacetic acid (NAA) have been identified as most effective for shoot regeneration. Cogbill et al. (2010) reported that *Brassica rapa* L. five-day-old cotyledonary explants produced shoots on a MS medium containing 1.5 mg/L thiadiazuron (TDZ) and 0.5 mg/l 1-naphthaleneacetic acid (NAA) at a mean rate of 8.8%. This rate was increased to 14.8% in explants placed on 1.5 mg/L TDZ and 0.5 mg/l NAA medium supplemented with 5.0 mg/l silver nitrate ($AgNO_3$).

Table 1. Initial cultivation of meristem buds and cotyledons from ornamental red cabbage (*Brassica oleracea* cv. Kyoto red given) on MS medium supplemented with different combination and concentrations of growth regulators.

Medium + growth regulators	Number of meristem buds	Width (mm)	Height (mm)	Number of cotyledons	Width (mm)	Length (mm)
A	40.0	3.5a	8.8a	48.0	1.2b	2.5a
B	40.0	4.7b	9.2a	55.0	1.9b	2.1ab
C	31.0	1.15c	3.8b	47.0	3.2a	1.4b
D	50.0	2.8a	9.5a	50.0	1.0c	2.4a

Means within each column having different letters are significantly different according to Duncan's test at $p<0,05$.

In Table 2. the number and size of the meristem bud and cotyledons of ageratum on MS medium supplemented with different growth regulators are presented. On the medium MS + 2 mg/l BA + 0.1 mg/l IAA + 0.1 mg/l GA_3 were inoculated 6 meristem buds with average width and height of 1.7 mm and 12 cotyledons with average width 2.2 mm and length 1.4 mm. On MS + 2 mg/l BA + 0.1 mg/l NAA were cultured 6 meristem buds with average width 1.2 mm and height 1.0 mm, and 12 cotyledons with average width 1.6 mm and length 1.1 mm. On MS + 5 mg/l BAP were inoculated 6 meristem buds with average width 1.3 mm and average height of 1.2 mm, and 12 cotyledons with average width 2.2 mm and length 1.0 mm. On MS + 3 mg/l BA + 1.5 mg/l NAA were cultured 6 meristem buds with average width 1.7 mm and average height 1.8 mm, and 12 cotyledons with average width 1.4 mm and average length 1.2 mm.

Table 2. Initial cultivation of meristem buds from ageratum *(Ageratum* sp.) on MS medium supplemented with different combination and concentrations of growth regulators.

Medium + growth regulators	Number of meristem buds	Width (mm)	Height (mm)	Number of cotyledons	Width (mm)	Length (mm)
A	6.0	1.7a	1.7a	12.0	2.2a	1.4a
B	6.0	1.2a	1.0a	12.0	1.6a	1.1a
E	6.0	1.3a	1.2a	12.0	2.2a	1.0a
F	6.0	1.7a	1.8a	12.0	1.4a	1.2a

Means within each column having different letters are significantly different according to Duncan's test at $p<0,05$.

Meristem buds and cotyledons from ornamental red cabbage resulted in shoot formation without exception, but different combination of growth regulators stimulated different frequency of shoot formation (Figure 2).

The percentage of shoot formation from meristem buds stimulated by the growth regulators ranged from the lowest frequency of 64% (MS + 5 mg/l BA + 5 mg/l NAA) to the highest 70% (MS + 2 mg/l BA +0.1 mg/l IAA + 0.1 mg/l GA_3 and MS + 2 mg/l BA) (Table 3, Figure 3a).

After one month of cultivation of cotyledons, the medium MS + 2 mg/l BA + 0.1 mg/l NAA showed the best stimulation effect with significantly the highest 56% shoot formation, while MS + 2 mg/l BA +0.1 mg/l IAA + 0.1 mg/l GA_3 and MS +2 mg/l BA gave significantly the lowest frequency of shoot formation from cotyledons. The cotyledons of the fourth used combination of growth regulators were lost due to narcotization in the course of the experiment, thus, without response to the applied growth regulators in one month period (Table 3, Figure 3b).

The presence of BAP in the medium significantly increase the number of produced explants in *in vitro* cultures of *Brassica oleracea* L. (Sretenović-Rajičić et al., 2007). Genus *Brassica* has shown increased regeneration of meristem buds in media supplemented with BAP and NAA. In different *Brassica* species, the regeneration depends on explants age, where younger explants always give better regeneration ratio (Maheshwari et al., 2011). Cheng et al. (2001) reported a high-frequency shoot regeneration obtained with BA or TZD (thidiazuron)-supplemented media. Pavlović et al. (2010) results showed a satisfactory frequency of shoot regeneration from hypocotyl explants and multiplication of shoots on media containing 1 mgl^{-1} BA alone or in combination with IBA in the four investigated *B. oleracea* varieties.

Table 3. Effects of MS medium and different growth regulators on meristem buds and cotyledons of ornamental red cabbage one month after the initial cultivation.

Medium + growth regulators	Number of meristem buds	Width (mm)	Height (mm)	Regenerants	% of frequency of shoot formation	Number of cotyledons	Width (mm)	Height (mm)	% of frequency of shoot formation	Regenerants
A	18	4.1c	1.9a	shoot	70a	18	10.0b	6.0a	37.5b	shoot
B	26	3.1b	2.5a	shoot	65b	31	9.6b	17.0a	56.0a	shoot
C	21	4.4c	2.5a	shoot	70a	31	17.5b	13.1b	38.0b	shoot
D	32	1.3a	2.2a	shoot	64b	Cotyledons lost due to necrotization				shoot

Means within each column having different letters are significantly different according to Duncan's test at $p<0,05$.

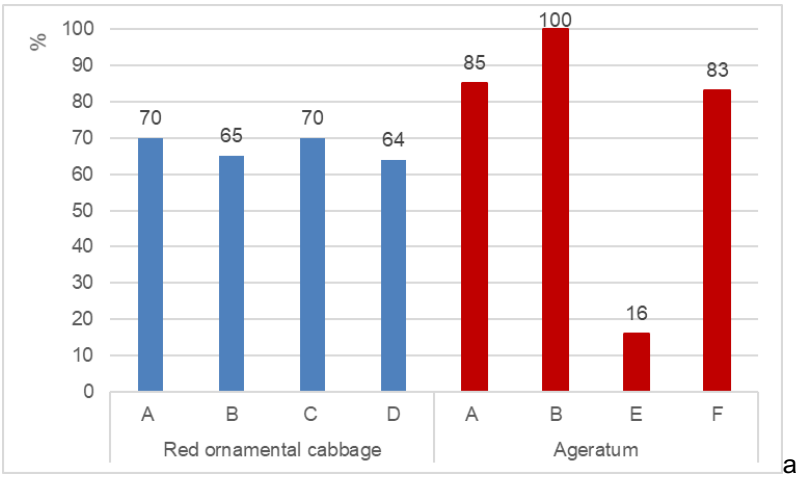
Figure 2. Shoot regenerants as a result of the effect of MS medium and different growth regulators on meristem buds of ornamental red cabbage one month after the initial cultivation.

Ageratum meristem buds on all MS media supplied with certain growth combinations resulted in shoot formation. MS suppled with 2 mg/l BA + 0.1 mg/l NAA was significantly the best combination for initiation of ageratum meristem buds in shoots, therefore 100% of the meristem buds responded with shoot formation. Meristem buds cultivated on MS supplied with 5 mg/l BA resulted in significantly the lowest shoot formation frequency (16%). One month after the initial cultivation, the ageratum cotyledons on all media under research resulted in narcotization, thus, no response to the applied growth regulators in one month period (Table 4, Figure 3a).

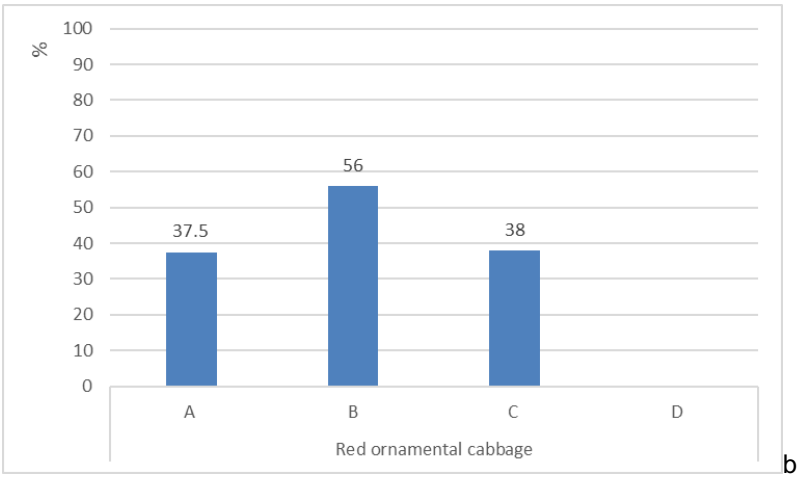
Table 4. Effects of MS medium and different growth regulators on meristem buds and cotyledons of ageratum one month after the initial cultivation.

Medium + growth regulators	Number of meristem buds	Width (mm)	Height (mm)	Regenerants	% of frequency of shoot formation	Number of cotyledons
A	7	11.2ab	19.4a	Shoot	85b	Cotyledons lost due to narcotization
B	6	12.0ab	9.6a	Shoot	100a	
E	2	5.0b	10.0a	Shoot	16c	
F	6	13.1a	14.2a	Shoot	83b	

Means within each column having different letters are significantly different according to Duncan's test at $p<0,05$.



a



b

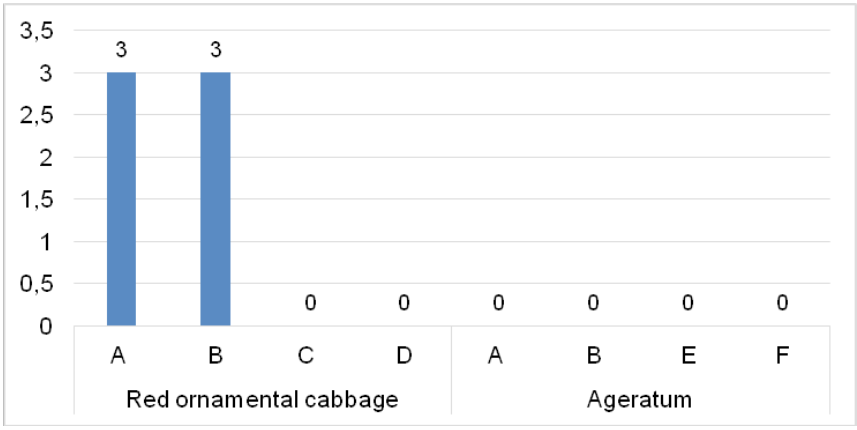
Legend:
A: MS + 2 mg/l BA + 0.1 mg/l IAA + 0.1 mg/l GA_3
B: MS + 2 mg/l BA + 0.1 mg/l NAA
C: MS + 2 mg/l BA
D: MS + 5 mg/l BA + 5 mg/l NAA
E: MS + 5 mg/l BA
F: MS + 3 mg/l BA + 1.5 mg/l NAA

Figure 3. a) Frequency of shoot formation from meristem buds of ornamental red cabbage and ageratum on MS medium supplied different growth regulators one month from the initial cultivation.

b) Frequency of shoot formation from cotyledons of ornamental red cabbage on MS medium supplied different growth regulators one month from the initial cultivation.

From the available literature, little-to-none report on tissue culture research has been done on ageratum. Certainly, it is not enough to produce a regeneration method or transformation protocol. If and when genetic engineering is successful with ageratum, caution would be warranted because *A. houstonianum* readily establishes itself as a weed and transgenic races could easily escape cultivation, especially in the tropics (Stephens, 2007). According to Laxmikant (2008) maximum number of multiple shoots of ageratum propagated *in vitro* were developed in plant medium fortified with 3.0 mg/l concentration of IAA-BAP combinations. In this study, IAA-BAP combinations proved as best effective for inducing multiple shooting and roots were formed at 2.0 mg/l and 3.0 mg/l rather than IAA-KN combinations.

The starting explants which after one month of cultivation did not resulted in formation of regenerant were subcultured on fresh MS medium supplemented with the same combination and concentration of growth regulators as in the starting cultivation. After one month of the subculturing, there was root formation on few initial explants. Three initial explants of ornamental red cabbage resulted in formation of roots on MS + 2 mg/l BA +0.1 mg/l IAA + 0.1 mg/l GA_3 and 3 on MS + 2 mg/l BA + mg/l 0.1 NAA (Figure 4). The ageratum initial explants did not respond in root formation on media under this research.



Legend:
A: MS + 2 mg/l BA + 0.1 mg/l IAA + 0.1 mg/l GA_3
B: MS + 2 mg/l BA + 0.1 mg/l NAA
C: MS + 2 mg/l BA
D: MS + 5 mg/l BA + 5 mg/l NAA
E: MS + 5 mg/l BA
F: MS + 3 mg/l BA + 1.5 mg/l NAA

Figure 4. The effect of MS medium and different growth regulators on root formation on meristem buds from ornamental red cabbage and ageratum two months after the initial cultivation.

CONCLUDING REMARKS

In this research the influence of different combination of BA, GA_3, IAA and NAA on meristem buds and cotyledons of ornamental red cabbage and ageratum was studied. For ornamental red cabbage, the highest percentage of frequency of shoot formation was obtained on MS + 2 mg/l BA + 0.1 mg/l IAA + 0.1 mg/l GA_3 and MS + 2 mg/l BA. On the contrary, the meristem buds of Ageratum showed the best percentage of frequency of shoot formation on MS + 2 mg/l BA + 0.1 mg/l NAA. Cotyledons from ornamental red cabbage gave the highest frequency (56%) of shoot formation when initially cultivated on MS + 2 mg/l BA + 0.1 mg/l NAA, while the ageratum cotyledons responded with necrosis on all media utilized in the research.

Future micropropagation research extension for these two species will be focused on combination of growth regulators suitable for rooting of regenerants, which will lead to the development of complete protocol from micropropagation of ornamental red cabbage and ageratum in future.

Finally, the results presented in this paper are contribution to the limited research data conceding micropropagation of ornamental red cabbage and particularly ageratum.

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МИКРОПРОПАГАЦИЈА НА УКРАСНИТЕ ВИДОВИ *BRASSICA OLERACEA* CV. KYOTO RED GIVEN И *AGERATUM* SP.

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Резиме

Украсната црвена зелка (Brassica oleracea cv. Kyoto red given) и агератумот (Ageratum sp.) се важни украсни растенија кои вообичаено се одгледуваат на балкони, дворови, паркови и други места на отворено во летниот и во зимскиот период од годината. Околку 156 украсни видови се добиваат со in vitro култури во различни комерцијални лаборатории ширум светот. In vitro култура на растенија е една клучна алатка во растителната биотехнологија што ја користи тотипотентноста на растителната клетка.

Во ова истражување беа проучувани влијанијата на различните концентрации и комбинации на BA, GA3, IAA и NAA на меристемски пупки и котиледони како почетни експлантанти од украсна црвена зелка и агератум. Кај украсната црвена зелка највисокиот процент на честота на формирање изданоци од меритемиски пупки беше добиен на MS + 2 mg/l BA + 0.1 mg/l IAA + 0.1 mg/l GA3 и MS + 2 mg/l BA. Спротивно, меристемските пупки од агератум покажаа најдобар процент на честота на формирање на изданоци на MS + 2 mg/l BA + 0.1 mg/l NAA. Котиледоните од украсната црвена зелка покажаа најголема честота (56%) на формирање изданоци кога почетно беа култивирани на MS + 2 mg/l BA + 0.1 mg/l NAA, додека котиледоните од агератум реагираа со некроза на сите медиуми користени во текот на истражувањето. Ова истражување е основа за понатамошно проучување за подобрување на регенерацијата на различни експлантанти од различни економски важни растителни видови.

Клучни зборови: *украсна црвена зелка (Brassica oleracea cv. Kyoto red given), агератум (Ageratum sp.), in vitro, регулатори на раст, меристемска пупка, котиледон, изданок*