

**GOCE DELCEV UNIVERSITY - STIP  
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## CONTENT

<b>Emilija Arsov, Galina Ivanova, Sasa Mitrev,</b> Multigene characterization of ' <i>Candidatus phytoplasma solani</i> ' in pepper and tomato plants in the Republic of Macedonia .....	7
<b>Biljana Balabanova, Trajče Stafilov, Robert Šajn, Claudiu Tănăselia</b> Bioindication ability of <i>Hypnum cupressiforme</i> and <i>Homolothecium lutescens</i> for determination of arsenic distribution in environment .....	15
<b>Olivera Bicikliski, Krste Tashev, Fidanka Trajkova, Ljupco Mihajlov, Liljana Koleva Gudeva</b> Comparative analysis of capsaicin content in peppers ( <i>Capsicum annum</i> L.) grown in conventional and organic agricultural systems .....	27
<b>Zoran Dimitrovski</b> Inspection of pesticide application equipment .....	37
<b>Zoran Dimitrovski, Dimitrov Sasko, Kukutanov Risto</b> Condition of air assisted sprayers in Shtip region and possibility of applying European standard EN 13790 .....	45
<b>Violeta Dimovska, Fidanka Ilieva, Sanja Kostadinovic, Ljupco Mihajlov</b> Physical and chemical characteristics of pomegranate fruit ( <i>Punica granatum</i> L.), of cv. Karamustafa .....	53
<b>Sanja Filipovska, Darko Andronikov, Aco Kuzelov</b> Chemical and fatty acid composition in meat of young chickens different hybrid lines .....	61
<b>Natasa Gunova, Dusan Spasov, Biljana Atanasova, Dragica Spasova, Mite Ilievski</b> Correlation between population dynamics of <i>Tuta absoluta</i> (Lepidoptera: Gelechiidae) and climate, at tomato in protected area .....	69
<b>Verica Ilieva, Natalija Markova Ruzdik, Ilija Karov, Ljupco Mihajlov, Mite Ilievski, Biljana Kovacevik</b> Genetic variability for yield and some yield-related traits in rice ( <i>Oryza sativa</i> L.) .....	75
<b>Dijana Indzhelieva, Katja Velkova-Jorgova, Darko Andronikov, Aco Kuzelov</b> The influence of starter culture of lactic- acid bacteria and bifid bacteria over the sanitary- hygienic, sensor and physical – chemical indicators on the re – boiled – smoked durable sausage .....	81
<b>Viktorija Maksimova, Liljana Koleva Gudeva, Rubin Gulaboski, Maja Shishovska, Zorica Arsova Sarafinovska</b> Capsaicin and dihydrocapsaicin variability in <i>Capsicum</i> sp. cultivars from Republic of Macedonia revealed by validated HPLC method .....	89
<b>Ivana Velesanova, Fidanka Trajkova, Liljana Koleva Gudeva</b> Micropropagation of ornamental species <i>Brassica oleracea</i> cv. Kyoto red given and <i>Ageratum</i> sp. ....	97



## 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<sub>3</sub>, 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<sub>3</sub> 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<sub>3</sub> (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 *Ageratum* (*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<sub>3</sub>

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 SPSS 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<sub>3</sub> 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<sub>3</sub>).

**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<sub>3</sub> 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<sub>3</sub> 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<sub>3</sub> 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).

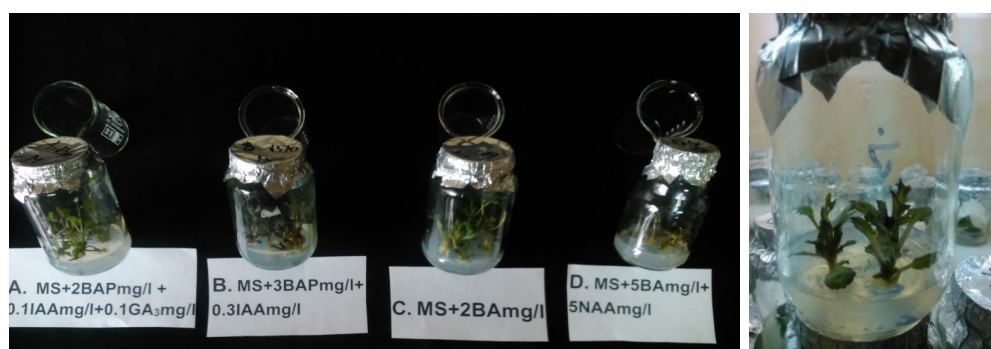
The presence of BAP in the medium significantly increase the number of produced explants in *in vitro* cultures of *Brassica oleracea* L. (Sretenović-Rajičić et al., 2007). Genus *Brassica* has shown increased regeneration of meristem buds in media supplemented with BAP and NAA. In different *Brassica* 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 mg<sup>-1</sup> 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 supplied 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

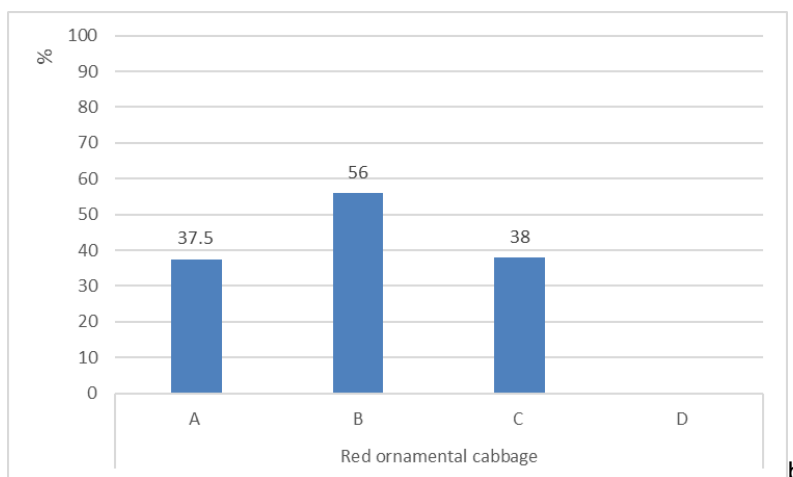
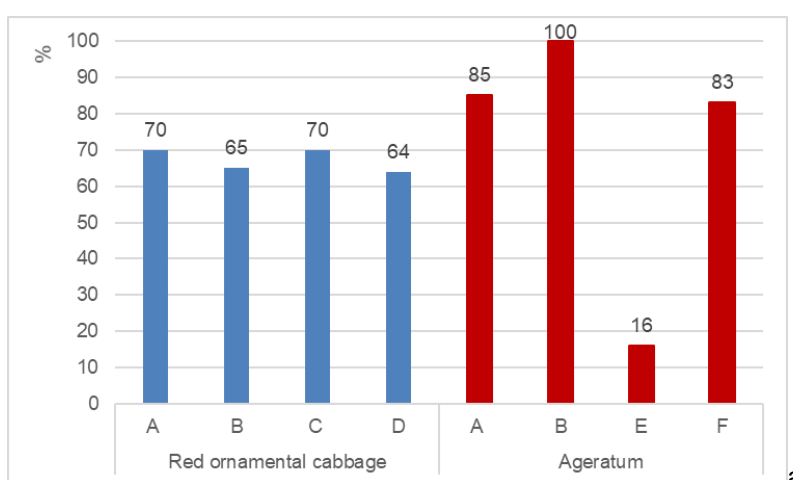
supplied with 5 mg/l BA resulted in significantly the lowest shoot formation frequency (16%). One month after the initial cultivation, the ageratum cotyledons on all media under research resulted in narcotization, thus, no response to the applied growth regulators in one month period (Table 4, Figure 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  $p < 0,05$ .



Legend:

- A: MS + 2 mg/l BA + 0.1 mg/l IAA + 0.1 mg/l GA<sub>3</sub>
- 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

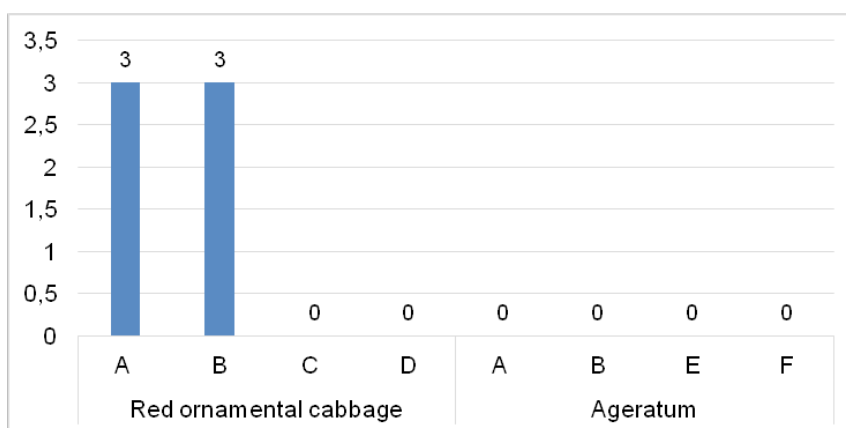
**Figure 3. a)** Frequency of shoot formation from meristem buds of ornamental red cabbage and ageratum on MS medium supplied different growth regulators one month from the initial cultivation.

**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<sub>3</sub> 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<sub>3</sub>

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<sub>3</sub>, 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<sub>3</sub> 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 меристемски 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