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IN VITRO PLANT RECOVERY FROM ALGINATE-ENCAPSULATED *CHRYSANTHEMUM* × *GRANDIFLORUM* (RAMAT.) KITAM. SHOOT TIPS

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Abstract

Shoot tips obtained from bi-weekly *in vitro* cultures of four cultivars of *Chrysanthemum* × *grandiflorum* (Ramat.) Kitam. were encapsulated in 3% calcium alginate hydrogel, containing Murashige and Skoog medium components. Survival and possibilities of further growth were estimated after 7, 30, and 60-day storage on medium with BAP, kinetin or plant growth regulator-free control at room temperature. Afterwards the capsules were transferred to an IAA-enriched medium for another 30 days. Morphogenic response (formation of shoots and roots, as well as their weight and length) of encapsulated buds to variants of the recovery medium was evaluated. The highest recovery rate after 90 days of culture reached 90%. Even though regrowth of shoots was recorded on the control medium, the presence of cytokinins significantly increased its efficiency. BAP stimulated activation of more axillary bud primordia than kinetin, but at the same time inhibited rooting. The obtained results suggest that synthetic seeds technology can be valuable in storage of chrysanthemums.

Key words: caulogenesis, cytokinins, germination, rhizogenesis, synthetic seeds

INTRODUCTION

Chrysanthemum × *grandiflorum* (Ramat.) Kitam. (Asteraceae) is one of the most important ornamental plants on the market. Today, novel biotechnological tools are applied in the production of this beautiful species (Rout and Das 1997). The development of mutation breeding and possibility of stimulating organogenesis *in vitro* allows producing hundreds of new cultivars every year (Zalewska et al. 2007, 2011). However, field and greenhouse collections are exposed to natural disasters, attack by pests, and pathogens. Moreover, labour costs and the requirement for technical staff are very high (Bekheet 2006). Therefore it is important to develop more efficient methods for storage of these valuable genetic resources. Synthetic seeds technology (i.e. explants encapsulated in a nutrient alginate-based matrix) is considered as an efficient alternative technique for production and germplasm conservation of commercially important plants that had problem in seed propagation and plants that produced non-viable seeds or without seeds (Daud et al. 2008, Ozden-Tokatli et al. 2008, Sharma et al. 2012).

Originally the term 'synseeds' (also 'artificial-' or 'manufactured seeds') referred to encapsulated somatic embryos (Gray and Purohit 1991). Today, its meaning has broadened to practically any plant organ (or its part), which can guarantee the recovery of an entire organism (Sharma et al. 2012). Production of synthetic seeds by encapsulating vegetative parts (e.g. shoot tips or axillary buds) is an even more suitable alternative to somatic embryos, which often demonstrate problems with conversion into plants (Lema-Rumińska and Kulus 2012, 2014), as shown with some chrysanthemum cultivars (Mani and Senthil 2011). Furthermore, because the species has high spontaneous mutation rates, meristem explants are more preferred to get true-to-type plants (Pinker and Abdel-Rahman 2005). Artificial seeds can be considered as a possible solution for solving problems with propagation of chrysanthemums which, due to a high level of ploidy and self-incompatibility, produce few, tiny, difficult-to-collect seeds without well-developed endosperms (Zalewska et al. 2007).

There are various advantages of synseeds such as: clonal plants of high quality can be produced and more consistent and synchronized harvesting of economically important crops could be realized (Ravi and Anand 2012). The technology facilitates storage, handling, transport, and delivery of plants. After introducing a

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dehydration step, it can also be applied in low-cost cryoconservation of biological material at the temperature of liquid nitrogen (-196°C) (Zalewska and Kulus 2013). Nowadays, the encapsulation-based cryopreservation techniques are the most frequently used ones with ornamental species (Pawłowska 2008, Kulus et al. 2013, Kulus and Zalewska 2014). However, the presence of the capsule decreases the availability of nutrients and physically inhibits the explant growth, possibly resulting in a low survival and/or regrowth rate (Kulus and Zalewska 2014). Therefore, it is recommended to supply *in vitro* recovery medium used after encapsulation with plant growth regulators (PGRs). However, previous studies with some chrysanthemums revealed the formation of callus tissue and the regeneration of vitrified, deformed shoots after improper selection of PGRs (Halmagyi et al. 2004, Osorio-Saenz et al. 2011, Zalewska and Kulus unpublished). This is especially unwanted with chrysanthemum cultivars since many of them (about 50%) are genetically unstable chimeras (Zalewska et al. 2007).

In the past many authors focused on the encapsulation procedure mainly (sodium alginate and calcium chloride concentration and time of exposition) (Rout and Das 1997, Nagananda et al. 2011) but few papers investigated the effect of recovery medium composition (Pandey and Chand 2005, Tsvetkov et al. 2007, Zalewska and Kulus 2013). Presumably, the use of proper recovery medium may greatly improve synseeds germination rates, the condition of produced microshoots and consequently, expand the effectiveness of encapsulation-based storage techniques.

In 2005 Pinker and Abdel-Rahman developed an encapsulation method of chrysanthemum nodal segments of that can be used for non-sterile sowing. The use of nodal explants, however, has a limited use for the purpose of cryoconservation. Moreover, other PGRs and their effect on the development of shoots and roots should be included.

The aim of our study was to investigate the effect of different plant growth regulators (both cytokinins and auxin) and time of culture on the survival, further growth/proliferation as well as organogeneic (caulogenesis and rhizogenesis) potential of encapsulated shoot tips from four chrysanthemum cultivars for the purpose of conservation and further recovery *in vitro*.

MATERIALS AND METHODS

The bi-weekly shoot tips from *in vitro* grown *Chrysanthemum* × *grandiflorum* (Ramat.) Kitam. cultivars ('Lady Pink', 'Lady Vitroflora', 'Lady White' and 'Lady Yellow'), 1-2 mm in length, with one or two leaf primordia, isolated under a binocular microscope, were used in the experiment. The experiment was conducted during the period September-December.

To stimulate the production of axillary shoot tips, single-node explants of *Chrysanthemum* × *grandiflorum* with cut-off leaves (Fig. 1A,B) were cultured for two weeks on modified MS (Murashige and Skoog 1962) medium supplemented with 0.09 M (standard 3% w/v) sucrose, and 330 mg l^{-1} $CaCl_2 \cdot 6H_2O$, 13.9 mg l^{-1} $FeSO_4 \cdot 7H_2O$ and 20.6 mg l^{-1} $Na_2EDTA \cdot 2H_2O$, and solidified with 8 g l^{-1} agar. The nutrient medium was poured out in 350 ml glass jars (40 ml of medium, 10 explants per jar – a single replication) and pH was adjusted to 5.8 prior autoclaving.

The isolated shoot tips had been incubated for 10 min in 3% (w/v) sodium alginate solution based on the modified (as above) MS nutrients without $CaCl_2$, supplemented with 0.09 M sucrose and then dipped with an automatic pipette into 0.1 M $CaCl_2$ solution for 30 min. The formed beads (4 mm in diameter) were further rinsed three times with distilled sterile water to stop the alginate polymerization. The beads produced were with firm coats, round shaped uniform size and in good shape for handling (Fig. 1C).

Then, the capsules were transferred on a recovery, solid modified MS medium (40 ml, 10 beads per jar) supplemented with 0.09 M sucrose and 0.1 mg l^{-1} 6-benzylaminopurine (BAP); 0.1 mg l^{-1} kinetin (N^6-furfuryladenine, KIN); or PGRs-free control (MS0) and cultured (stored) under initial temperature and lighting conditions for two months (Fig. 1D).

Subsequently, the beads with microshoots were transferred to a rooting MS medium with 2 mg l^{-1} indole-3-acetic acid (IAA) for another 30 days.

All the explants were grown in a growth room at 24 ± 2°C, with 16 h of cool white flourescent light at photosynthetic photon flux density 30.86 µmol m^{-2} s^{-1} daily.

To assess the effect of various PGRs, the regrowth potential (% of explants which remained green and showed signs of further development) was estimated visually 7, 30, 60, and 90 days after inoculation on the variants of the recovery medium. The percent of explants forming roots was recorded prior, to and after transferring to the rooting medium (60 and 90 days of culture, respectively), number (per explant), fresh weight (mg) and length (mm) of all shoots and roots produced were recorded after 90 days from the beginning of whole experiment (Fig. 1E).

The percentage data were arcsine transformed according to Freeman-Tukey procedure before analysis. The results were subjected to analysis of variance (ANOVA; all effects were considered random) and the comparisons of means were done according to Tukey's Multiple Comparison Test ($p \leq 0.05$) by using Statistica 10.0 PL and ANALWAR-5.2-FR tools. Within the experiment 12 factorial combinations were studied: 3 variants of the recovery medium × 4 cultivars in 4 replications, 10 shoot tips in each replication (for each cultivar a grand total of 120 beads). As for recovery and rhizogenic efficiency, time-factor was also considered.

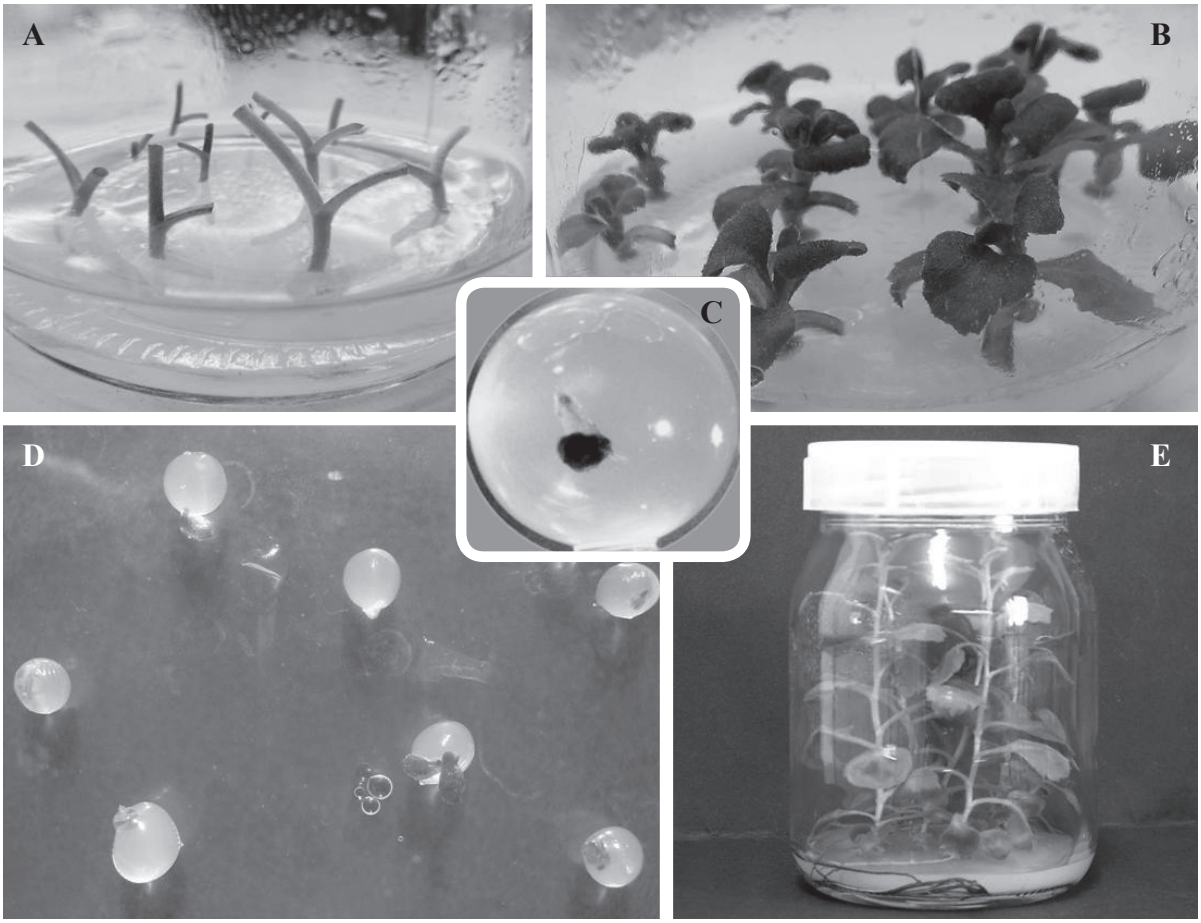

Fig. 1. Selected experiment stages. A) Single node explants of chrysanthemum (*Chrysanthemum* × *grandiflorum*), B) Fourteen-day old axillary shoot tips prior to isolation, C) Encapsulated shoot tips, D) Germinating synthetic seeds from benzylaminoprine-supplemented recovery medium, E) Microshoots of chrysanthemum produced from synseeds after 90-day culture on the benzylaminopurine-supplemented medium.

RESULTS AND DISCUSSION

Survival and capability of explants for further growth are the most important parameters determining the efficiency of the encapsulation and recovery protocols.

In the present experiment, similar to *Rauvolfia tetraphylla* L. (Faisal et al. 2006) germination began after about 10-14 days (Fig. 1D). One week after inoculation, all explants showed similar survival regardless of the medium composition (93-96%, $F = 0.33$, $p = 0.7179$, Table 1); with a significant cultivar peculiarity (88-98%, $F = 4.73$, $p = 0.007$, Fig. 2). However, after longer observation (60 and 90 days, $F = 17.24$, $p < 0.0001$) of the chrysanthemums, a significant survival decrease was recorded on the control medium ($F = 26.294$, $p < 0.0001$), especially in 'Lady Vitroflora' ($F = 10.683$, $p < 0.0001$, Tables 1 and 2).

Similarly to chrysanthemum, *Rhodiola kirilowii* Rgl. ex Maxim and *Olea europaea* L. demonstrated a significant decrease in synthetic seed germination capacity after a 60-day culture (Zych et al. 2005, Ikhlaq et al. 2011). This, however, was not noticed with encapsulated protocorm-like bodies (PLBs) of *Vanda coerulea* Grifft. ex. Lindl. (Sarmah et al. 2010). During the entire experiment 'Lady Yellow' proved to be the most stable in showing recovery capabilities and further growth (99/90% survival after 7 and 90 days, respectively, Table 2, Figs 2 and 3). Therefore, it should be assumed that various cultivars might have different requirements – presumably naturally slow growing ones require the addition of cytokinins into the medium.

In general, after 90 days of culture, a mean of 82% of shoot tips grew into microshoots (Tables 1 and 2). Similar high values were reported about other commercial cultivars of chrysanthemum (Pinker and Abdel-Rahman 2005) and *Saintpaulia ionantha* Wendl. microshoots (Daud et al. 2008). In comparison, encapsulated embryoids of *Glossocardia bosvallea* (L.f.) DC showed about 67% germination. However, storage of capsules beyond 25 days decreased the viability of the encapsulated embryos and the germination was 0 after 30 days of storage (Geetha and Gopal 2009).

Table 1. Medium composition and time effects on the alginate-encapsulated chrysanthemum (*Chrysanthemum* × *grandiflorum*) shoot-tips recovery potential (%).

Recovery medium	Time of culture (days)			
	7	30	60	90
MS0	92.88 ± 3.48 a A	86.58 ± 4.68 b AB	72.95 ± 6.59 b BC	69.38 ± 6.92 b C
KIN	95.72 ± 1.70 a A	96.63 ± 1.98 a A	89.38 ± 3.22 a A	88.59 ± 3.22 a A
BAP	95.92 ± 1.37 a A	97.34 ± 1.20 a A	90.94 ± 2.04 a A	89.38 ± 3.01 a A

The means (M) ± standard error (SE) within a column followed by the same small letter and in the rows followed by the same capital letter are not significantly different at $p \leq 0.05$ estimated by One-Way ANOVA followed by Tukey's Multiple Comparison Test.

Table 2. Medium and cultivar effects on the alginate-encapsulated chrysanthemum (*Chrysanthemum* × *grandiflorum*) shoot-tips recovery after 90 days of culture (%).

Cultivar	Medium		
	MS0	KIN	BAP
'Lady Pink'	87.50 ± 4.79 a A	82.50 ± 10.31 a A	82.50 ± 6.29 a A
'Lady Vitroflora'	32.50 ± 14.36 b B	87.50 ± 4.79 a A	82.50 ± 8.54 a A
'Lady White'	75.00 ± 6.45 a A	97.50 ± 2.50 a A	91.88 ± 2.77 a A
'Lady Yellow'	82.50 ± 7.50 a A	90.00 ± 5.77 a A	97.50 ± 2.50 a A

The means (M) ± standard error (SE) within a column followed by the same small letter and in the rows followed by the same capital letter are not significantly different at $p \leq 0.05$ estimated by One-Way ANOVA followed by Tukey's Multiple Comparison Test.

Somatic embryos of *Eustoma grandiflorum* (Raf.) Shinn. and *Genista monospermia* Lam. showed low (30%) germination rates as well (Ruffoni et al. 1994). Also, embryos of the tested chrysanthemum Lady group presented serious problems with conversion (Lema-Rumińska personal communication). This may confirm the suggestion that vegetative tissues are more efficient explants for encapsulation than somatic embryos. It should be underlined that there were no morphological disorders observed within the four tested cultivars. In comparison, encapsulated in 3% Na-alginate shoot tips of *Gypsophila paniculata* L. produced over 80% of vitrified shoots (Rady and Hanafy 2004).

It should be noted that unlike *Rauvolfia tetraphylla* (Faisal et al. 2006) chrysanthemums synseeds were able to grow on a PGRs-free medium, even though these microshoots together with those from the BAP-supplemented medium were the shortest ones (means: 35 mm and 30 mm, respectively) after 90-day culture ($F = 20.16$, $p < 0.0001$, Table 3). The longest micro-

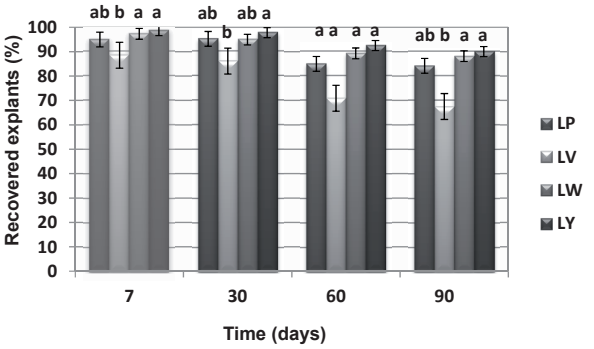

Fig. 2. Effect of cultivar on the alginate-encapsulated chrysanthemum (*Chrysanthemum* × *grandiflorum*) shoot-tips recovery, regardles of medium composition.
The means (M) ± standard error (SE) marked with the same letter are not significantly different at $p \leq 0.05$ estimated by One-Way ANOVA followed by Tukey's Multiple Comparison Test; LP - 'Lady Pink', LV - 'Lady Vitroflora', LW - 'Lady White', LY - 'Lady Yellow'.

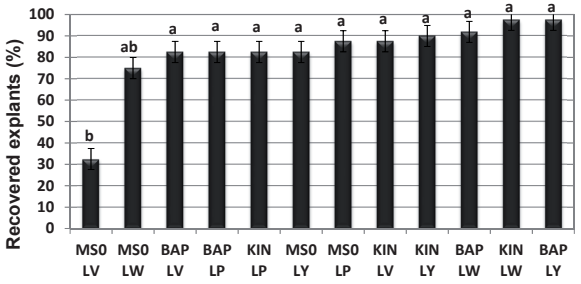

Fig. 3. Medium composition and cultivar effects on the alginate-encapsulated chrysanthemum (*Chrysanthemum* × *grandiflorum*) shoot-tips recovery after 90-day culture.
The means (M) ± standard error (SE) marked with the same letter are not significantly different at $p \leq 0.05$ estimated by One-Way ANOVA and Tukey's Multiple Comparison Test; LP - 'Lady Pink', LV - 'Lady Vitroflora', LW - 'Lady White', LY - 'Lady Yellow'.

Table 3. Effect of cultivar on shoot/root length and fresh weight of *Chrysanthemum* × *grandiflorum* plants recovered from alginate-encapsulated shoot tips after 90-day culture.

Characteristic	Cultivar	MS0	KIN	BAP
Shoot length (mm)	'Lady Pink'	31.98 ± 2.52 a A	29.82 ± 3.55 b A	28.81 ± 1.54 ab A
	'Lady Vitroflora'	39.04 ± 5.08 a B	58.85 ± 3.53 a A	39.21 ± 1.57 a B
	'Lady White'	36.34 ± 1.86 a A	43.10 ± 3.56 ab A	20.94 ± 1.30 b B
	'Lady Yellow'	37.44 ± 1.45 a A	52.08 ± 3.75 a A	39.22 ± 2.74 a A
Shoot weight (mg)	'Lady Pink'	124.28 ± 25.62 a A	182.00 ± 64.89 a A	112.08 ± 19.09 a A
	'Lady Vitroflora'	229.35 ± 47.93 a A	171.35 ± 13.82 a A	153.57 ± 15.05 a A
	'Lady White'	209.84 ± 35.08 a A	123.65 ± 19.64 a A	88.25 ± 16.93 a A
	'Lady Yellow'	143.50 ± 17.31 a A	130.22 ± 15.04 a A	120.92 ± 17.80 a A
Root length (mm)	'Lady Pink'	34.58 ± 3.53 a A	40.29 ± 7.49 a A	23.02 ± 3.26 a A
	'Lady Vitroflora'	29.93 ± 1.72 a A	32.32 ± 3.05 a A	21.20 ± 1.61 a A
	'Lady White'	30.32 ± 4.55 a A	28.98 ± 1.09 a A	17.14 ± 1.40 a B
	'Lady Yellow'	29.09 ± 3.09 a A	31.47 ± 2.96 a A	28.42 ± 1.96 a A
Root weight (mg)	'Lady Pink'	17.67 ± 4.53 a A	29.97 ± 6.33 a A	20.89 ± 2.57 a A
	'Lady Vitroflora'	32.97 ± 7.49 a A	14.60 ± 1.22 a A	15.04 ± 1.44 a A
	'Lady White'	31.69 ± 7.31 a A	15.81 ± 2.86 a B	17.85 ± 5.88 a B
	'Lady Yellow'	25.06 ± 6.54 a A	20.54 ± 1.94 a A	25.96 ± 2.38 a A

The means (M) ± standard error (SE) within a column followed by the same small letter and in the rows followed by the same capital letter are not significantly different at $p \leq 0.05$ estimated by One-Way ANOVA followed by Tukey's Multiple Comparison Test.

shoots were produced in the presence of KIN (mean: 45 mm). In comparison, 42-day old plantlets from non-encapsulated shoot tips of chrysanthemum produced *in vitro* 20 mm long shoots on the PGRs-free medium (Waseem et al. 2011a) while a 56-day culture of nodal segments resulted in 21 mm long microshoots (Waseem et al. 2011b). Therefore it can be assumed that the presence of the capsule did not inhibit the regrowth pace of the tested cultivars. The greatest mean weight of shoots (176.74 mg) was registered on the PGRs-free control, whereas the smallest weight (118.71 mg) was recorded in the presence of BAP ($F = 4.91$, $p = 0.0131$). 'Lady Pink' and 'Lady White' produced shorter shoots (mean: 33 mm; $F = 16.9$, $p < 0.0001$) of smaller weight (140 mg; $F = 5.43$, $p = 0.0034$) than 'Lady Vitroflora' (43 mm, 185 mg, Table 3).

It was also recorded that BAP stimulated formation of axillary shoots at a higher frequency (32% of explants, $F = 34.821$, $p < 0.0001$), which additionally increased the proliferation rate and productivity of the culture, as compared to both KIN-supplemented variant and the control (4.3 and 0.7% explants, respectively, Figs 4 and 5). With *Dendrobium wardianum* Warner, the PLBs formation of multiple plants were recorded on both control and BAP-supplemented medium (5 mg l^{-1}), although the latter one inhibited their further development (Sharma et al. 1992). The production of multiple shoots is probably a result of activation of axillary bud primordia, present in shoot tips (inactive due to apical dominance) rather than a response to meristems injury and adventitious organogenesis, which would lead to additional formation of callus tissue and numerous deformed shoots of short internodes. Noticeably during the entire experiment, no presence of callus was recorded even though its formation was present after inoculating cryopreserved encapsulated chrysanthemum explants on a BAP-enriched medium (Zalewska and Kulus unpublished).

It was also recorded that rooting was inhibited by the presence of capsule, since only 52% shoots formed roots in the best conditions ('Lady Vitroflora' on the medium with KIN) after 60 days of culture (Fig. 6), whereas without encapsulation a majority of chrysanthemum microshoots often produce roots much faster

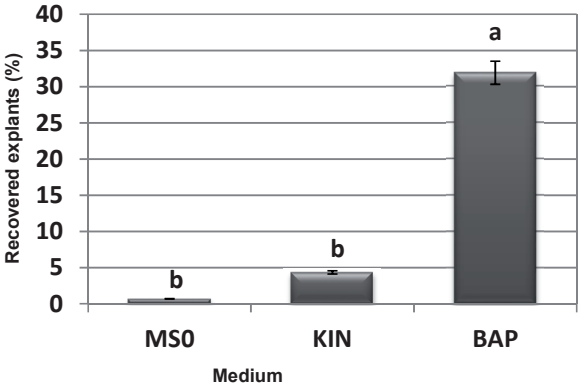

Fig. 4. Effect of medium composition on the percentage of chrysanthemum (*Chrysanthemum* × *grandiflorum*) explants forming multiple shoots after 90-day culture.
The means (M) ± standard error (SE) marked with the same letter are not significantly different at $p \leq 0.05$ estimated by One-Way ANOVA and Tukey's Multiple Comparison Test.

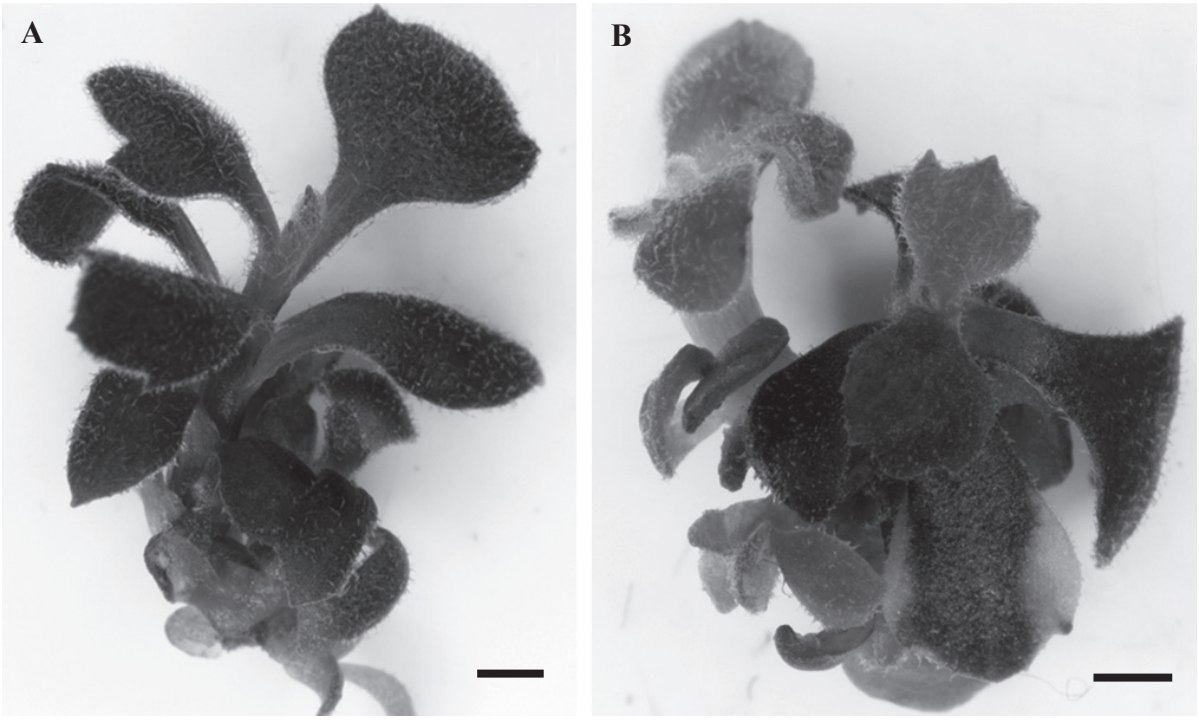

Fig. 5. Regrowth of single (A) and multiple shoots (B) of encapsulated chrysanthemum (*Chrysanthemum* × *grandiflorum*) shoot tips from kinetin-supplemented recovery medium after 30-day culture, bar = 1 mm.

without any PGRs (Teixeira da Silva 2003, Hodson de Jaramillo et al. 2008, Zalewska et al. 2010). Also Pinker and Abdel-Rahman (2005) recorded 50% regeneration of roots *in vitro* in encapsulated nodal segments of chrysanthemum, without the addition of auxins. In the present experiment, explants cultured in the presence of BAP for 60 days produced less roots (9%), than explants on the control medium or in the presence of KIN (24-35%) ($F = 7.547$, $p = 0.001$, Table 4). It was recorded that the efficiency of rhizogenesis was also cultivar-specific ($F = 2.745$, $p = 0.0492$) since 'Lady Yellow' produced more roots (34%) than 'Lady Pink' or 'Lady White' (18-19%, Table 5). Moreover, the roots produced had less developed root hairs. Piccioni (1997) suggested that encapsulation inhibits the oxygen supply of the explants and therefore suppresses root induction. Rady and Hanafy (2004) described in *Gypsophila paniculata* that encapsulated shoot tips rooting efficiency was significantly higher after preparing the beads in sterile distilled water (60-65%) rather than in MS nutrients (40-50%), which may also be helpful with chrysanthemum.

In the present experiment, significant increase in rooting efficiency was recorded only after transferring the synseeds to medium supplemented with IAA ($F = 328.47$, $p < 0.0001$), regardless of the cultivar used (80-93%, $F = 1.32$, $p = 0.2749$) (Table 5), which was also described by Pinker and Abdel-Rahman (2005). Also Mohanraj et al. (2009) and Waseem et al. (2007, 2008) recorded that IAA was the most beneficial auxin for stimulating germination of *Coelogyne breviscapa* Lindl. synseeds and chrysanthemum shoot tip/nodal segments culture. On the other hand, Rout and Das (1997) described the best response for germination of encapsulated chrysanthemum shoot tips after applying 0.1 mg l^{-1} indole-3-butyric acid (IBA).

Previous 60-day culture on variants of the medium with KIN and MS0 stimulated the growth of longer roots (33 and 30 mm, respectively) as compared to

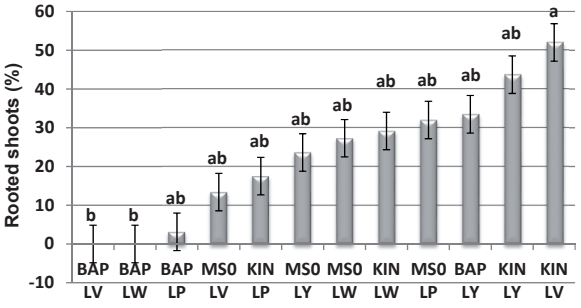

Fig. 6. Medium composition and cultivar effects on chrysanthemum cultivars rhizogenesis efficiency after 60-day culture. The means (M) ± standard error (SE) marked with the same letter are not significantly different at $p \leq 0.05$ estimated by One-Way ANOVA and Tukey's Multiple Comparison Test; LP - 'Lady Pink', LV - 'Lady Vitroflora', LW - 'Lady White', LY - 'Lady Yellow'.

Table 4. Effect of medium composition on chrysanthemum (*Chrysanthemum* × *grandiflorum*) rhizogenesis efficiency (%) before and after transferring to rooting medium (60- and 90-day culture, respectively).

Recovery medium	Time of culture (days)	
	60	90
MS0	24.07 ± 3.32 a B	75.21 ± 4.57 b A
KIN	35.59 ± 4.97 a B	88.79 ± 2.19 a A
BAP	9.15 ± 4.24 b B	89.75 ± 3.85 a A

The means (M) ± standard error (SE) within a column followed by the same small letter and in the rows followed by the same capital letter are not significantly different at $p \leq 0.05$ estimated by One-Way ANOVA followed by Tukey's Multiple Comparison Test.

BAP (22 mm, Table 3) after transferring the explants to rooting medium ($F = 17.46$, $p < 0.0001$). Therefore, the latter cytokinin is less favorable for chrysanthemum *in vitro* culture. The influence of cultivar effect was also recorded (Table 3, $F = 3.81$, $p = 0.018$). 'Lady Pink' produced the longest roots (33 mm), while 'Lady Vitroflora' and 'Lady White' - the shortest ones (26 and 25 mm, respectively). Problems with organogenesis may also suggest lower acclimatization efficiency of the latter cultivars. In comparison Kereša et al. (2012) after a 30-day culture of 1 cm long 'Palisade White' shoots on rooting medium (2 mg l^{-1} IAA) gained similar root lengths (22.4 mm) and number of roots per shoot (4.1). On the other hand, according to Waseem et al. (2011a) plantlets from non-encapsulated shoot tips of chrysanthemum produced *in vitro* 62-90 mm long roots. In the present experiment, microshoots from the control medium produced heavier roots (26.85 mg), than these from other variants of the medium (20 mg; $F = 5.057$, $p = 0.0116$). The cultivar effect, however, had no influence on this parameter (approx. 22 mg, $F = 2.63$, $p = 0.0977$, Table 3).

Table 5. Effect of cultivar on chrysanthemum (*Chrysanthemum* × *grandiflorum*) rhizogenesis efficiency (%) before and after transferring to rooting medium (60- and 90-day culture, respectively).

Cultivar	Time of culture (days)	
	60	90
'Lady Pink'	17.54 ± 5.16 b	80.67 ± 3.16 a
'Lady Vitroflora'	21.80 ± 7.19 ab	93.37 ± 2.55 a
'Lady White'	18.82 ± 4.88 b	81.01 ± 6.43 a
'Lady Yellow'	33.60 ± 4.83 a	83.29 ± 4.70 a

The means (M) ± standard error (SE) within a column followed by the same letter are not significantly different estimated by One-Way ANOVA and Tukey's Multiple Comparison Test at $p \leq 0.05$.

In the present study, explants cultured previously on the control medium formed less roots (75%) than those cultivated on cytokinine-supplemented ones (89-90%) after transferring to rooting medium (Table 4). This may be somewhat surprising for it is believed that cytokinins (alternatively to auxins) inhibit rhizogenesis initiation, promoting growth of the shoot instead (Müller and Sheen 2008). The observed phenomenon could be explained by the model proposed by Jones et al. (2010), according to which cytokinins function as a positive regulator of auxin biosynthesis. The pool of the latter PGRs could get activated after transferring the explants to a cytokinin-free medium. One should keep in mind though that auxins (IAA especially) at higher concentrations are known to stimulate the production of ethylene, which causes the inhibition of growth (Waseem et al. 2008). Therefore, inoculating the beads on the recovery medium supplemented with both cytokinin and auxin could be beneficial to stimulate efficient root formation as well as shoot elongation, without the need of subsequent transfers as shown with *Begonia* × *hiemalis* Fotch (Awal et al. 2007) and *Rauvolfia tetraphylla* (Alatar and Faisal 2012).

In general the greatest mean number of shoots (1.5; maximum 2.1) after a 90-day culture was recorded on the medium with BAP ($F = 57.046$, $p < 0.0001$), while the lowest one was observed in the presence of KIN and on the control variant (1.0; maximum 1.2, Fig. 7). On the other hand, both PGRs-free and kinetin-supplemented variants of the medium stimulated better rhizogenesis (5.4 and 4.1 roots per explant, respectively, Fig. 7) than BAP (3 roots/explant, $F = 6.529$, $p = 0.0038$). In comparison, 42-day old plantlets from non-encapsulated shoot tips of chrysanthemum, after transferring to a rooting medium with auxins, produced 9.3-14.3 roots per explant and 1.5/7.7 shoots/roots per explant on the MS0 medium (Waseem et al. 2011a).

Conventional encapsulation is a very labour intensive process (Sharma et al. 2012). An interesting alternative, considering the usage of electrostatic droplet generator (electrostatic extrusion directly from a plastic syringe or a needle of various diameter), allowing for the automation of the synseeds production was introduced by Al-Hajry et al. (1999) for *Saintpaulia ionantha* callus. Applying this approach, the positive electrode wire is connected to the needle and the ground wire is attached to the $CaCl_2$ collecting solution. As the alginate is forced out of the end of the needle by the syringe pump or the air pressure from air tank, the droplets are pulled off by the action of electrostatic forces.

The results of the work with *Feijoa sellowiana* Berg., showed that the germination of encapsulated explants was facilitated by pre-treatment of the synthetic seeds with solution of 100 mM KNO_3 (Guerra et al. 2001). The K^+ ions from KNO_3 replace the Ca^{+2} cations of the calcium alginate capsule allowing softening of

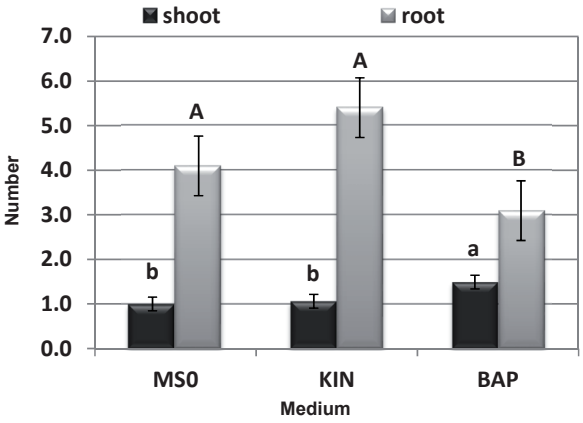

Fig. 7. Effect of medium composition on the mean number of shoots and roots after 90-day culture of chrysanthemum. The means (M) ± standard error (SE) marked with the same letter are not significantly different at $p \leq 0.05$ estimated by One-Way ANOVA and Tukey's Multiple Comparison Test. Lower-case letters refer to shoots and upper-case letters refer to roots.

the synseeds, thus making easier subsequent germination of the explants. This approach could be useful in further attempts to facilitate the access to nutrients and stimulate both rhizogenesis and caulogenesis. The preparation of bead matrix based on more nutritive compounds than alginate (e.g. polysaccharides) or the addition of active charcoal could be beneficial as well (Sorvari et al. 1997).

The presented results suggest opportunities for further development towards selecting propagules excised directly from *in vivo* cultivated plants and/or adding antimicrobial agents and PGRs to the capsule content and further transfer of beads (directly or after storage) to field conditions, as it was done with *Cymbidium giganteum* Wall. (Corrie and Tandon 1993).

The present research confirmed that the addition of PGRs is beneficial for chrysanthemum synthetic seeds germination. However, different cytokinins provoked various reactions in encapsulated shoot tips. Kinetin seems more preferable for chrysanthemums, as it stimulated the formation of longer shoots of greater weight, without their proliferation. Unlike BAP it also did not inhibit root formation. Apical shoot tips are unipolar without root meristems. Moreover, the presence of the bead decreased rhizogenesis efficiency and therefore, an addition of auxins for that purpose is also necessary. It was recorded that some cultivars possessed lower germination ability than others, which decreased additionally with time. Still, the successful results suggest that the production of uniform beads with high germination frequency without callus formation would be useful for clonal multiplication and long-term maintenance by applying encapsulation-based cryopreservation techniques of valuable genetic resources of chrysanthemum.

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