Botanica SERBICA 35 (1): (2011) 61-66

Original Scientific Paper

In vitro conservation of micro-propagated *Ruscus aculeatus* L. (*Liliaceae*) plants

Teodora Ivanova*, Chavdar Gussev, Yulia Bosseva and Tatyana Stoeva

Department of Applied Botany, Institute of Botany, Bulgarian Academy of Sciences, 23 Acad. G. Bonchev Str.,1113 Sofia, Bulgaria

ABSTRACT: *In vitro* conservation of micropropagated *Ruscus aculeatus* L. (*Liliaceae*) cultures was studied. Plants were maintained without transfer on fresh media more than a year. Efficient medium-term storage in two-phase culture at room temperature was proposed. Recovery of the cultures was investigated on media supplied with various plant growth regulators. Two-phase cultivation allowed effortless storage for 16 months. The best regeneration rates in the post-storage cultivation were obtained on media with thidiazuron or kinetin.

Key words: *Ruscus aculeatus*, conservation, thidiazuron, micropropagation.

Received 10 May 2010 Revision accepted 02 December 2010

UDK 582.711.16:581.19

INTRODUCTION

Ruscus aculeatus L. (Liliaceae) is a small evergreen perennial used both as a medicinal and ornamental plant. Steroid saponins extracted from rhizomes possess anti-inflamatory and vasoprotective properties and are used both in conventional and traditional medicine (Bouskela & Cyrino 1994). The rigid, evergreen branches make the species attractive as a cut foliage and garden plant. However market relies on the natural resources. Foliage and rhizomes are taken from the wild and excessive utilization already affected some natural populations (Tansi *et al.* 2009). In Bulgaria, *R. aculeatus* is under regulated regime of gathering (Anex 4 of the Biodiversity Act 2002). Slow growth determines the limited field cultivation of the species due to long production cycle of the planting material and specific requirements for shading (D'Antuono & Lovato 2003; Zistler *et al.* 2008). *In vitro* propagation was proposed as a method for multiplication and conservation of *R. aculeatus.* Choice of suitable primary explants, regeneration rates and characteristics of the cultures were discussed in the recent years (Moyano *et al.* 2006; Ivanova *et al.* 2008; Banciu *et al.* 2009). However, there is not enough data or protocol about *in vitro* conservation and storage conditions.

In vitro cultivation for conservation purposes is recognized as efficient technique complementary to *in situ* practices for preservation of valuable plant species (Dodds 1991; Sarasan *et al.* 2006). Nonetheless, maintenance of cultures *in vitro* for a long period could be efficient only by reduction of costs and labor. Different strategies are applied to minimize growth and to reduce frequent transfers on fresh media. It is beneficiary that the slow-growing species could be conserved at standard conditions (Engelmann 2009). Unfortunately, the existing procedures are not universal and often a specific protocol has to be developed for each species or variety (Withers 1986).

The present work highlights the opportunities for *in vitro* conservation of *R. aculeatus* shoot cultures. An easy-to-maintain two-phase culture is proposed for medium-term storage at room temperature and recovery of the cultures on media supplemented with various growth regulators was studied.

*correspondence: tai@bio.bas.bg

MATERIALS AND METHODS

Clusters of 2-4 shoots were obtained *in vitro* from *R. aculeatus* rhizome explants on agar solidified MS medium (Murashige & Skoog 1962) with modified vitamins (10 fold increased thiamine) and supplied with 1 mg/L 6-benzylaminopurine (BAP) and 0.5 mg/L α-naphthaleneacetic acid (NAA) as previously described by Ivanova *et al.* (2008). Shoot clusters were stored in two-phase (agar-liquid) culture at 24±1°C. Plant material was fixed on a plain agar (6.5 mg/L Plant agar, Dushefa, NL) used as a support. Liquid MS medium (20 ml) with 60 g/L sucrose and 2 mg/L NAA was added above the agar (125ml) without covering the plants. Reference cultures on agar-solidified MS media without growth regulators were kept at 15±1°C and 24±1°C. All cultures were placed in Vitro Vent containers (Dushefa, NL). All media were autoclaved at 121°C and 1atm for 20min; pH was adjusted to 5.75 prior to sterilization. Cultures were kept at 16/8 h photoperiod (40.5 μmol $m^{-2}s^{-1}$). Five repetitions with 36 clusters were incubated. Vitality of the cultures was checked monthly for 16 months without transfer on fresh media. Recovery of the two-phase cultures was performed on MS media with 30 g/L sucrose, supplied with NAA, BAP, 2,4-Dichlorophenoxyacetic acid (2,4-D), thidiazuron (TDZ), kinetin (Kin) and indol-3-butyric acid (IBA)

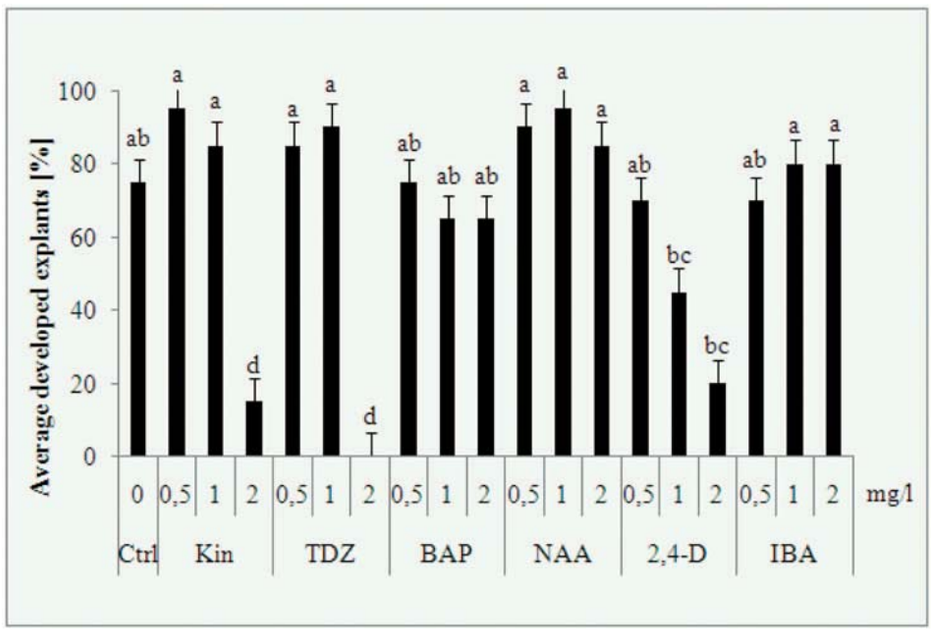

Fig. 1. Regeneration capacity of *R. aculeatus* cultures after 16-month storage in two-phase system. Values are mean ± SE. Bars labeled with same letters are not significantly different at 0.05% level (Duncan's multiple range test)

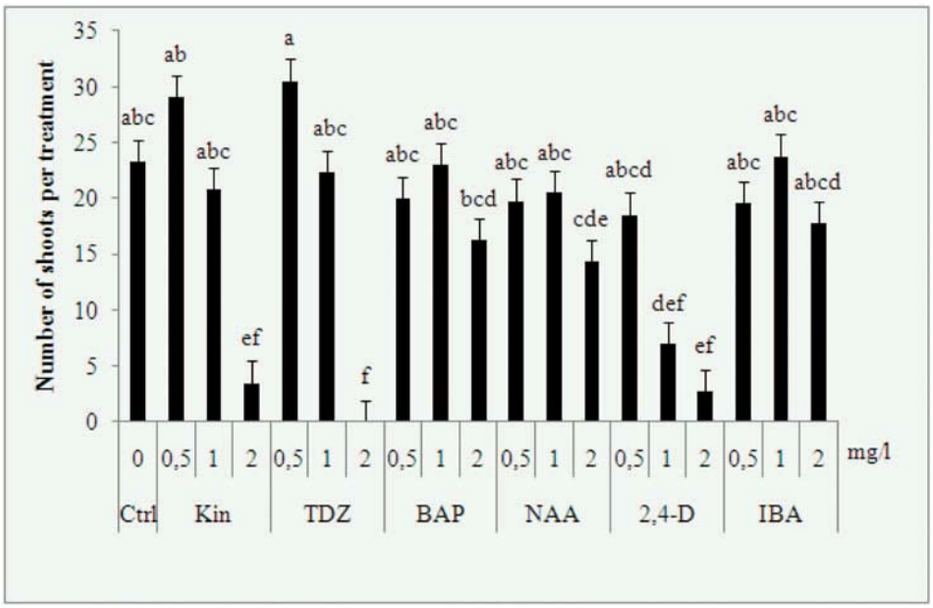

Fig. 2. Regeneration rate of *R. aculeatus* cultures after 16-month storage in two-phase system. Values are mean ± SE. Bars labeled with same letters are not significantly different at 0.05% level (Duncan's multiple range test)

Fig. 3. Development of clusters within one month on control medium without growth regulators, with kinetin (0.5 mg/l) and thidiazuron (0.5 mg/l) – left to right.

at concentrations 0.5, 1, 2 mg/L. All media (60ml) were dispensed in Magenta® GA7-3 boxes. The regeneration capacity of the stored cultures (percentage of clusters where regeneration occurred), the regenerated rate (number of regenerated shoots) and the shoot length were recorded after one month. Four repetitions of one vessel with 5 clusters per vessel for each treatment were tested. Data was statistically analyzed and mean values were compared for similarity using Duncan's multiple range test.

RESULTS AND DISCUSSION

Two-phase cultivation resulted in 91% survival of the cultures for 16 months without transfer on fresh media. Damages due to contamination or loss of vigor were not observed. Long incubation period in liquid media were reported to cause culture decline and low bud proliferation in *R. aculeatus* and *R. hipophyllum* L. cultures (Ziv 1983; Ivanova *et al.* 2008). Such effect was not observed in the two-phase culture. This could be explained by the fact that the clusters were only partially submerged which allowed normal development of plantlets. The maintenance of two-phase cultures was easier than the agar ones. After full consumption of the liquid phase it could be simply replenished. Maximum storability of *R. aculeatus* cultures on agar at 24±1°C was determined to be six months. Prolongation of incubation period at these conditions led to rapid decay of all cultures. The limiting factor was drying of the agar medium at ambient temperature. Successful *in vitro* conservation on agar media without transfer for 12 to18 months at room temperature was reported for other species (Divakaran *et al.* 2006; Tyagi *et al.* 2006, 2009). However, possibility for expanding the storage period depends greatly of the plant species as well as of whole complex of chosen conditions. Lowering the incubation temperature is one of the frequently used methods for reduction of the number of transfers during the storage (Ng & Ng 1991). At lower temperature (15°C) the decline of the *R. aculeatus* cultures was detected after the eighth month of storage. Average survival of the clusters reached 80%. The decline and low remaining number of the clusters on agar did not allowed further work and experiments with these cultures.

Regeneration capacity of the cultures stored for 16 months in two-phase system was estimated on different media in order to investigate the culture response after storage (Fig. 1). It ranged between 0 and 100% depending on the used medium. Generally multiplication rates were lower than those reported for *R. aculeatus in vitro* cultures without storage, although not significantly (Ivanova *et al.* 2008). The number of obtained shoots with cytokinin treatments was higher compared to those with auxins (Fig. 2). Moreover visible shoots on the cytokinin containing media were developed at the second week of cultivation and quality of shoots was better as well. Thidiazuron or kinetin ensured highest number of regenerated shoots within one month (Fig. 3). Capelle *et al.* (1983) and Cruz de Carvalho *et al.* (2000) assumed involvement of TDZ in the synthesis and/or accumulation of cytokinins in plant tissue cultures. It was recently used for induction of shoot- and organogenesis in many species (Murthy *et al.* 1998; Faisal & Anis 2006; Xu *et al.* 2009). Kinetin was also

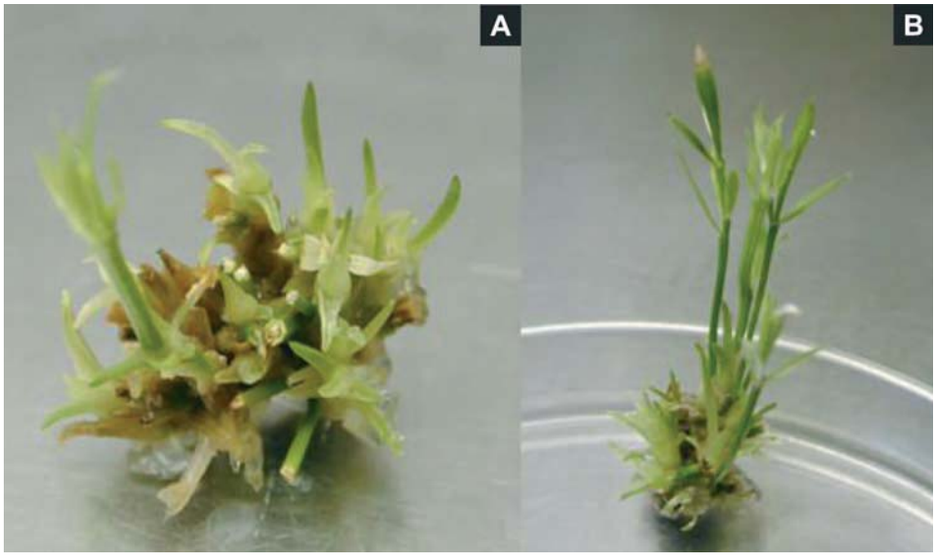

Fig. 4. Changes in morphology caused by NAA – **A** and 2,4-D – **B**.

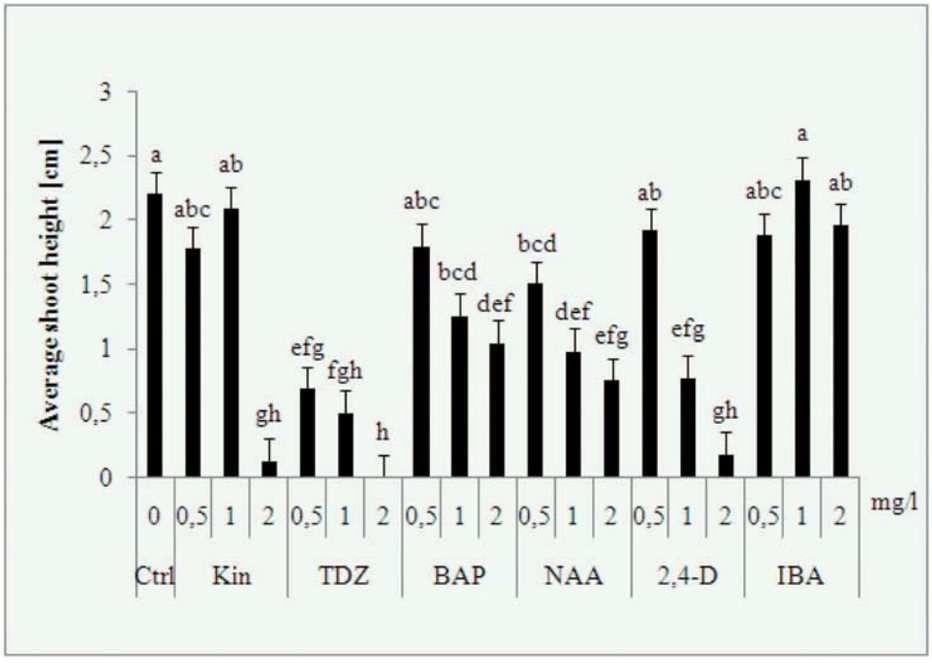

Fig. 5. Shoot height of regenerated *R. aculeatus* cultures after 16-month storage in two-phase system. Values are mean ± SE. Bars labeled with same letters are not significantly different at 0.05% level (Duncan's multiple range test)

reported to have shoot promoting effect in *Ruscus* cultures (Jha & Sen 1983; Moyano *et al.* 2006). Nevertheless, higher TDZ and kinetin concentrations reduced shoot regeneration and caused decay of the cultures. Similarly Abou Dahab *et al.* (2005) observed the same effect in *R. hypoglossum* L. cultures.

Auxins (2,4-D, NAA) were recommended as a shoot and callus promoting regulators for *R. aculeatus* and *R. hypophyllum* (Ziv 1983; Moyano *et al.* 2006; Ivanova *et al.* 2008). NAA that was previously used to increase shoot production had no such effect on stored cultures. Adversely, the obtained shoots were thinner and of low quality (Fig. 4a). Addition of 2,4-D resulted in shoots with needle-shaped phylloclades (Fig. 4b). Moreover, the regeneration rate of the cultures dropped with the increase of 2,4-D concentration. These effects could be due to alteration of the culture response to growth regulators. Accordingly Hamad & Taha (2008) reported that prolonged incubation on same media could modify the regeneration performance in *Ananas comosus* (L.) Merr. cultures. The culture age was also proved to play role in induction of organogenesis (Routh *et al.* 2001).

Overall root formation after storage was very poor compared to abundant rooting of *R. aculeatus* cultures on

media without growth regulators or with NAA (Ivanova *et al.* 2008). Spontaneous rooting was observed only in IBA treatments.

Shoots from all treatments were morphologically typical: 3-4 phylloclades on an unbranched stem. Shoot height was significantly influenced by both type of regulator and its concentration (Fig. 5). Generally, the increase of concentration of BAP, NAA and 2,4-D shortened the shoots. IBA had no effect on the shoot height and obtained shoots were as tall as in the control. Kinetin also induced regeneration of tall shoots only at 0.5 and 1 mg/L. Shortest shoots with reduced stems and phylloclades were observed in TDZ treatments. Reduction of shoot height caused by TDZ could be attributed to its strong cytokinin-like activity. TDZ was shown to stimulate rather shoot proliferation than elongation (Huetteman & Preece1993, Lincy & Sasikumar 2010).

CONCLUSION

R. aculeatus could be successfully conserved *in vitro* at ambient temperature using two-phase culture. The method is effective allowing effortless storage for 16 months without transfer on fresh media. Best regeneration rate in the post-storage cultivation was achieved on MS media with TDZ or Kinetin at concentrations lower than 2 mg/L. Additionally TDZ induced considerable reduction of the shoot height. This effect was favorable to save space for keeping the *in vitro* collections.

Acknowledgements – Authors are grateful to Bulgarian Ministry of education, youth and science for the financial support (OP HRD, Project №: BG051PO001/07/3.3-02/70).

REFERENCES

Abou Dahab AM, Habib A, Hosni Y & Gabr A. 2005. Effect of some sterilization treatments and growth regulators on *Ruscus hypoglossum* L. *Arab. J. of Biotech.* **8 (1)**: 127-140.

Banciu C, Mitoi ME & Brezeanu A. 2009. Biochemical peculiarity of in vitro morphogenesis under conservation strategy of *Ruscus aculeatus* L. *Ann. For. Res.* **52**: 109-116.

Bouskela E & Cyrino FZGA. 1994. Possible mechanisms for the effects of *Ruscus* extract on microvascular permeability and diameter. *Clinical Hemorheology* **14**: 23-36.

Capelle SC, Mok DWS & Turner JE. 1983. Effects of thidiazuron on cytokinin autonomy and the metabolism of N6-(D2-isopentenyl) [8-14C] adenosine in callus cultures of *Phaseolus lunatus* L. *Plant Physiol.* **73**: 796–802.

Cruz de Carvalho MH, Van Le B, Zuily-Fodil Y, Pham Thi AT & Thanh Van KT. 2000. Efficient whole plant regeneration of common bean (*Phaseolus vulgaris* L.) using thin-cell-layer culture and silver nitrate. *Plant Sci.* **159**:223–232.

D'Antuono LF & Lovato A. 2003. Germination trials and domestication potential of three native species with edible sprouts: *Ruscus aculeatus* L., *Tamus communis* L. and *Smilax aspera* L. *Acta Hort.* **598**: 211-218.

Divakaran M, Babu KN & Peter KV. 2006. Conservation of *Vanilla* species, in vitro. *Sci. Hort.* **110**: 175-180.

Dodds JH (ed.). 1991. *In Vitro* Methods for Conservation of Plant Genetic Resources,. Chapman and Hall London.

Engelmann F. 2009. Use of biotechnologies for conserving plant biodiversity. *Acta Hort.* **812**: 63-82.

Faisal M & Anis M. 2006. Thidiazuron induced high frequency axillary shoot multiplication in *Psoralea corylifolia*. *Biol. Plant.* **50**: 437–440.

Hamad AM & Taha RM. 2008. Effect of sequential subcultures on *in vitro* proliferation capacity and shoot formations pattern of pineapple (*Ananas comosus* (L.) Merr.) over different incubation periods. *Sci. Hort.* **117**: 329–334.

Huetteman C & Preece J. 1993. Thidiazuron: a potent cytokinin for woody plant tissue culture. *Plant Cell Tiss. Org. Cult.* **33**: 105-119.

Ivanova T, Gussev C, Bosseva Y, Stanilova M & Stoeva T. 2008. *In vitro* regeneration of *Ruscus aculeatus* L. – effective micropropagation by shoot cultures. Propagation of Ornamental plants. **8**: 39-41.

Jha S & Sen S. 1985. *In vitro* regeneration of *Ruscus hypophyllum* L. plants. *Plant Cell Tiss. Org. Cult.* **5**(1): 79-87.

Lincy AK, Remashree AB & Sasikumar B. 2010. Enhanced adventitious shoot regeneration from aerial stem explants of ginger using TDZ and its histological studies. *Turk. J. Bot.* **34**: 21-29.

Moyano E, Montero M,. Bonfill M, Cusidó RM, Palazón J, Piñol MT. 2006. *In vitro* micropropagation of *Ruscus aculeatus*. *Biol. Plant.* **50**: 441-443.

Murashige T & Skoog F 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Phisiol. Plant.* **15**: 473-497.

Murthy BNS, Murch SJ & Saxena PK. 1998. Thidiazuron: A potent regulator of *in vitro* plant morphogenesis. *In vitro Cell. & Dev. Biol. Plant* **34**: 267–275.

Ng SYC & Ng NQ. 1991. Reduced-growth storage of germplasm. In: Dodds JH (ed.). *In Vitro* Methods for Conservation of Plant Genetic Resources. pp. 11–39. Chapman and Hall London.

Rout GR, Palai SK & Das P. 2001. Onset of *in vitro* rhizogenesis response and peroxidase activity in *Zingiber officinale* (Zingiberaceae) *Rev. Biol. Trop*.**49** (3-4): 965-71.

Sarasan VA,Cripps R, Ramsay MM, Atherton C, Mcmichen M, Prendergast G & Rowntree JK. 2006. Conservation *in vitro* of threatened plants – progress in the past decade. In Vitro Cell. Dev. Biol. Plant. **42**: 206–214.

Tansi S, Karaman S & Toncer O. 2009. Ecological and morphological variation in wild *Ruscus aculeatus* from Mediterranean region of southern Turkey. *Acta Hort.* **862**: 175-183.

Tyagi RK, Agrawal A & Yusuf A. 2006. Conservation of *Zingiber* germplasm through in vitro rhizome formation. *Sci. Hort.* **108**: 210–219.

Tyagi RK, Goswani R, Sanayaima R, Singh R, Tandon R & Agrawal A. 2009. Micropropagation and slow growth conservation of cardamom (*Elattaria cardamomum* Maton). *In Vitro Cell. Dev. Biol. Plant*. **45**: 721-729.

Xu L, Ma F & Liang D. 2009. Plant regeneration from *in vitro* cultured leaves of Lanzhou lily (*Lilium davidii* var. *unicolor*). *Sci. Hort.* **119**(4): 458-461.

Withers LA. 1986. *In vitro* approaches to the conservation of plant genetic resources. In: Withers LA & Alderson PG (eds.). Plant tissue culture and its agricultural applications, pp 219–238. Butterworths, London.

Zistler C, Krafka O, Kranvogel A & Sonnenschein M. 2008. Cultivation experiments with butcher's broom (*Ruscus aculeatus* L.). *Z. Arz. & Gewuerzpflanzen* **13**(3): 114-116.

Ziv M. 1983. The stimulatory effect of liquid induction medium on shoot proliferation of *Ruscus hipophyllum* L. *Sci. Hort*. **19**: 387-394.

REZIME

In vitro konzervacija mikropropagiranih biljaka *Ruscus aculeatus* L. (Liliaceae)

Teodora Ivanova, Chavdar Gussev, Yulia Bosseva, Tatyana Stoeva

Izučavana je *in vitro* konzervacija kultura mikro-propagiranog *Ruscus aculeatus* L. (*Liliaceae*). Biljke su držane bez prebacivanja na svež medijum više od godinu dana. Navodi se efikasan srednje vremenski process za čuvanje ovih kultura na sobnoj temperature. Izučavano je obnavljanje biljaka nakon ovog perioda sa različitim kombinacijama regulatora rastenja dodatim u medijum. Dvofazna kultivacija omogućava čuvanje u periodu do 16 meseci. Najbolja stopa obnavljanja postignuta je dodatkom tidiurazona ili kinetina u medijum.

Ključne reči: *Ruscus aculeatus*, conservation, thidiazuron, micropropagation.