## International Scientific Conference

# FORESTS IN THE FUTURE
# Sustainable Use, Risks and Challenges

# PROCEEDINGS

Institute of Forestry
Belgrade Serbia


International Union of
Forest Research
Organizations

European Forest Institute


International Centre of
Forestry and Forest
Industries

Faculty of Applied
Ecology FUTURA
Belgrade, Serbia

4-5 October 2012

Belgrade

Republic of Serbia

**Citation**

*International Scientific Conference* ***Forests in the Future – Sustainable Use, Risks and Challenges,***
*4-5 October 2012, Belgrade, Republic of Serbia*

**Publisher**
Institute of Forestry, Belgrade, Serbia

**Editor**
Ph.D. Ljubinko Rakonjac, director

**Technical Editor and Layout**
M.Sc. Tatjana Ćirković-Mitrović

**Cover Design and Cover Page**
Suzana Mitrović, B.Sc.
Author of the photo Ph.D. Milorad Veselinović

**Printed in**
200 copies

**Printed by**
Klik tim DOO, Belgrade



Belgrade, September 2012

**Address of the Organizer**
Institute of Forestry
Kneza Višeslava 3
Belgrade, Serbia
*www.forest.org.rs*

CIP - Каталогизација у публикацији
Народна библиотека Србије, Београд

630(082)(0.034.2)

INTERNATIONAL Scientific Conference Forests in Future - Sustainable Use, Risks and Challenges (2012 ; Beograd)
Proceedings / International Scientific Conference Forests in Future - Sustainable Use, Risks and Challenges, 4th-5th October 2012, Belgrade, Republic of Serbia ; [organized by] Institute of Forestry, Belgrade ... [et al.] ; [editor Ljubinko Rakonjac]. - Belgrade : Institute of Forestry, 2012 (Belgrade : Klik tim DOO). - 1 elektronski optički disk (CD-ROM) : tekst ; 12 cm

Sistemski zahtevi: Nisu navedeni. - Nasl. sa nasl. ekrana. - Tiraž 200. - Bibliografija uz svaki rad.

ISBN 978-86-80439-33-4
1. Institut za šumarstvo (Beograd)
a) Шумарство - Зборници
COBISS.SR-ID 195909644

## SCIENTIFIC COMMITEE

PhD Alexander Buck, IUFRO Executive Director-Chairman, Vienna Austria

PhD Margarida Tome, IUFRO Coordinator Division 4, Vice Chairman, Lisbon, Portugal

PhD Dragana Dražić, Institute of Forestry, Belgrade, Serbia, Chairman of Scientific Committee

PhD Ljubinko Rakonjac, Director, Institute of Forestry, Belgrade, Serbia

PhD Mara Tabaković-Tošić, Institute of Forestry, Belgrade, Serbia

PhD Radovan Nevenić, Institute of Forestry, Belgrade, Serbia

PhD Miloš Koprivica, Institute of Forestry, Belgrade, Serbia

PhD Snežana Rajković, Institute of Forestry, Belgrade, Serbia

PhD Mihailo Ratknić, Institute of Forestry, Belgrade, Serbia

PhD Zoran Miletić, Institute of Forestry, Belgrade, Serbia

PhD Milorad Veselinović, Institute of Forestry, Belgrade, Serbia

PhD Biljana Nikolić, Institute of Forestry, Belgrade, Serbia

PhD Vera Lavadinović, Institute of Forestry, Belgrade, Serbia

PhD Miroslava Markovic, Institute of Forestry, Belgrade, Serbia

PhD Gordana Dražić, Dean, Faculty of Applied Ecology, Serbia

PhD Alexander S. Alekseev, 1st Vice Rector, St. Petersburg State Forest- Technical Academy, Russia

PhD Ioan Abrudan, Dean, Transylvania University of Brasov, Romania

PhD Paolo Cherubini, WSL, Swiss Federal Institute, Switzerland

PhD Ahmet Yesil, Dean, Istanbul University, Turkey

PhD Urmas Peterson, Estonian University of Life sciences, Tartu, Estonia

PhD Ulrich Schraml, University of Freiburg, Germany

PhD Jan Tucek, Faculty of Forestry, Technical University, Zvolen, Slovakia

PhD Roman Longauer, National Forest Centre, Zvolen, Slovakia

PhD Hristo I. Tsakov Forest Research Institute, Sofia, Bulgaria

PhD Georgi Georgiev, Institute for Forestry, Sofia, Bulgaria

PhD Plamen Mirchev, Institute for Forestry, Sofia, Bulgaria

PhD Margaret Shannon, European Forest Institute, USA

PhD Zaika Andrii, National University of Life and Environmental Sciences, Ukraine

PhD Maziar Razavi, Branch of Science and Research, Islamic Azad University. Tehran, Iran

PhD Zoran Popovic, Faculty of Agryculture, Beograd

PhD Kalliopi Radoglou, Department of Forestry and Management of Environmental and Natural Resources, University of Thrace, Greece

PhD Oguzhan Sarikaya, Faculty of Forestry, Department of Forest Engineering, Suleyman Demirel University, Turkey

PhD Iskra Vasileva, University of Forestry, Sofia, Bulgaria

PhD Małgorzata Gajos, Faculty of Informatics and of Materials Sciences University of Silesia, Poland

PhD Maria Martinova Ivanova, Южный федеральный университет, Rostov on Donu, Russia

PhD Maja Jurc, Biotechnical Faculty, Ljubljana, Slovenia

**International Scientific Conference**
**FORESTS IN THE FUTURE – SUSTAINABLE USE, RISKS AND CHALLENGES**
**4-5 October 2012, Institute of Forestry, Belgrade, Republic of Serbia**

# ROOTING AND PREVENTING SHOOT-TIP NECROSIS OF *IN VITRO* CULTURED HORSE CHESTNUT SHOOTS

Snežana ZDRAVKOVIĆ-KORAĆ[1], Ljiljana TUBIĆ[1], Jelena MILOJEVIĆ[1], Nina DEVRNJA[1], Igor KOSTIĆ[2], Dušica ĆALIĆ[1]

**Abstract:** *Efficient bud regeneration was achieved from germinating horse chestnut (Aesculus hippocastanum L.) somatic embryos cultivated on 1-10 μM benzyladenine (BA). Adventitious buds were detached from the mother tissue and used to establish permanent shoot cultures on 0-20 μM BA. Secondary buds were regenerated from the shoot base of the explants. Bud multiplication was very poor (1.9) and shoot-tip necrosis was very high (100%) on plant growth regulator (PGR)-free medium. The highest multiplication was achieved on 5 and 10 μM BA (16.8 and 18.7, respectively), with no shoot-tip necrosis, while hyperhydration was rather frequent on shoots cultivated on BA above 5 μM. Individual shoots were elongated on medium with 1 μM BA and 500 mg/l polyvinylpyrrolidone (PVP MW 40 000) for 4 weeks. However, it was necessary to reduce BA level below 1 μM for shoot rooting and that caused mass shoot-tip necrosis. As classical rooting methods failed, the basal part of each elongated shoot was first wounded by cutting with a sterile blade and then dipped into a 0, 5 or 10 mM indole-3-butyric acid solution for 1 min and cultivated on solid half-strength MS PGR-free medium with 0.02% activated charcoal for 2-3 weeks. To prevent shoot tip necrosis during this phase, a BA solution was applied directly on apical meristem. Shoot-tip necrosis was completely eliminated by weekly application of 10 μl of 1 μM BA. As soon as the root initials were observed, the shoots were transferred to MS medium supplemented with 500 mg/l PVP and 5 μM BA. The frequency of rooting was 23%, and further optimisation of root-inducing phase is needed.*

**Key words:** *Aesculus hippocastanum*, rooting, shoots-tip necrosis

## INTRODUCTION

Horse chestnut is one of the most popular ornamental trees, widely distributed throughout the temperate parts of the northern hemisphere, especially in the urban areas, as it withstands high pollution. However, conventional propagation of horse chestnut through cuttings and seedlings is rather inefficient (Radojević 1991). As the use of adult specimens for clonal propagation of woody plants is preferable to juvenile individuals because of its known genetic development potential (Bonga *et al.* 2010), seedlings are not suitable, since they represent genotypes of unknown performance. In line with this, cloning by the means of tissue culture is widely recognised technique for vegetative propagation of woody plants.

Modern biotechnological techniques are increasingly employed in the propagation of woody plants, with shoot culture being a convenient method for mass clonal propagation of elite specimens. However, despite its great potential, the application of *in vitro* propagation is often limited in some plant species due to the presence of physiological disorders, like shoot-tip

[1] University of Belgrade, Institute Biological Research "Siniša Stanković", Despot Stefan Boulevard 142, 11060 Belgrade, Serbia
[2] University of Belgrade, Faculty of Agriculture, Nemanjina 6, 11080 Belgrade - Zemun, Serbia

necrosis (reviewed in Bairu *et al.* 2009a). This is especially the case in woody plant species, including *Castanea sativa* (Piagnani *et al.* 1996, Vieitez *et al.* 1989), *C. dentata* (Xing *et al.* 1997), *Quercus rubber* (Vieitez *et al.* 1989), and fruit trees like pear (Grigoriadou *et al.* 2000), apple (Kataeva *et al.* 1991), apricot (Perez-Tornero & Burgos 2000), grape (Thomas 2000) and banana (Martin *et al.* 2007).

Shoot-tip necrosis (browning of the apical shoot) could be caused by a variety of factors, including plant growth regulators (PGR), calcium and boron content, and culture environment (Bairu *et al.* 2009a). In fact, this is a rather complex phenomenon, which is the most probably caused by a synergistic action of multiple factors.

One of possible causes of shoot-tip necrosis is suboptimal level of cytokinins in the shoot apex due to decreased availability of cytokinins. This is because the level of endogenous cytokinins is low due to the absence of roots (the main site of their synthesis) and exogenously supplied cytokinins are generally excluded or kept at low level during root-inducing phase. Optimisation of cytokinin type and concentration in growth medium was essential for the control of shoot-tip necrosis in *Q. rubber* (Grigoriadou *et al.* 2000) and *Harpagophytum procumbens* (Bairu *et al.* 2009b, 2011), and the application of cytokinins directly to the shoot apex was helpful in preventing shoot-tip necrosis in some species, like apricot (Perez- Tornero & Burgos 2000) and *C. sativa* (Piagnani *et al.* 1996).

Bairu *et al.* (2011) demonstrated that changes in cytokinins availability, rather than their absence, may be related to shoot-tip necrosis in *H. procumbens*. They found higher levels of total cytokinins in all parts of the necrotic shoots comparing to normal shoots, and in both necrotic and normal shoots the level of cytokinins decreased acropetally. Further structural analysis of cytokinin compounds showed accumulation of inactive and toxic cytokinin-9-glucosides and lower levels of storage cytokinin-O-glucosides in necrotic shoots than in normal shoots (Bairu *et al.* 2011). The authors assumed that shoot-tip necrosis might be a consequence of the conversion of active cytokinin forms to inactive and toxic 9-glucosides.

Calcium and boron deficiencies may also cause shoot-tip necrosis. Calcium strongly affects plant growth and development participating in a number of physiological functions, including modulation of cytokinin activity (Hepler 2005, Hirschi 2004). Higher $Ca^{2+}$ concentration in culture medium, its better uptake and efficient translocation decreased shoot-tip necrosis syndrome in certain plant species (Piagnani *et al.* 1996; Bairu *et al.* 2009b), although there are opposite reports (Grigoriadou *et al.* 2000). Boron deficiency also inhibits growth of shoot and root meristems (Hu & Brown 1994), and affects levels of auxins and cytokinins (Wang *et al.* 2006).

This study attempted to evaluate the capacity of horse chestnut shoots for *in vitro* multiplication and to find factors that can help to control necrosis of the shoot apex.

## MATERIAL AND METHODS

### *Basal medium*

The basal medium contained Murashige & Skoog (MS) mineral solution (Murashige & Skoog 1962), 2% sucrose, 0.7% agar, 100 mg/l myo-inositol, 200 mg/l casein hydrolysate, 2 mg/l thiamine, 2 mg/l adenine, 5 mg/l nicotinic acid, 10 mg/l panthotenic acid. pH of the media was adjusted to 5.6 using a pH-meter and the media were sterilized by autoclaving at 114°C for 25 min.

### *Bud induction and multiplication*

Shoot buds were induced from germinating horse chestnut (*Aesculus hippocastanum* L.) somatic embryos (3 cm) cultivated on solid MS medium supplemented with 1-10 μM benzyladenine (BA) (Zdravković-Korać *et al.* 2008). Bunches of adventitious buds were

detached from the mother tissue and subcultivated on solid MS medium with 5 μM BA until they were 1 cm high. These shoots (hereinafter referred to as the primary shoots) were split and the solitary shoots were cultivated on solid MS medium with 0-20 μM BA during four weeks, for secondary shoot induction.

***Shoot elongation, rooting and preventing shoot-tip necrosis***

For elongation and rooting of shoots, we used a procedure developed by Xing *et al.* (1997) for *C. dentata*. Briefly, individual shoots were elongated on solid MS medium with 1 μM BA and 500 mg/l polyvinylpyrrolidone (PVP, MW 40 000) for 4 weeks, and then the basal part of elongated shoots was wounded by cutting with a sterile blade, dipped into an indole-3-butyric acid (IBA) solution at 0, 5 or 10 mM for 1 min, and placed on half-strength MS PGR-free solid medium supplemented with 0.02% activated charcoal for 2-3 weeks. To prevent shoot tip necrosis during this phase, in another experiment, 10 μl of 0, 1, 5 or 10 μM BA solution was applied directly on apical meristem of 10 mM IBA-treated shoots by using a pipetman. As soon as the root initials were observed, the shoots were transferred to MS medium supplemented with 500 mg/l PVP and 5 μM BA.

***Culture conditions***

All cultures were maintained under cool white fluorescent light with a photosynthetic photon flux density of approximately 50 μmol $m^{-2}$ $s^{-1}$ (as measured by an LI-1400 DataLogger equipped with an LI-190SA Quantum sensor, LI-COR Biosciences) for 16 h per day at 25 ± 2 °C.

***Recordings and statistical analysis***

A completely randomised design was used for culture placement in all experiments. Experiments were performed in two replicates, each with three samples (Erlenmeyer flasks) and five subsamples (shoot buds) for each treatment (n=30).

The number of shoot buds per somatic embryo and the number of secondary buds per primary shoot were counted with the aid of a stereomicroscope after 4 weeks of culture. The number of roots was recorded 4 weeks following the root-inducing treatment, and the presence of shoot-tip necrosis was evaluated at the end of bud multiplication experiment and 4 weeks following root-inducing treatment.

Statistical differences among treatments were tested using standard analysis of variance (ANOVA). The means were separated using Duncan's *post-hoc* test for $P \leq 0.05$. Percentage data were subjected to angular transformation and bud number data were subjected to square root transformation prior to analysis, followed by inverse transformation for presentation. For bud induction, results were expressed as the frequency of regenerating somatic embryos, the mean number of buds per somatic embryo, and an index of bud-forming capacity (BFC), which was used to evaluate the cumulative effect of the two aforementioned variables, calculated as follows: BFC = (mean bud number per explant) × (% of regenerating explants)/100. Bud multiplication was evaluated through the mean number of secondary buds per primary shoot and the fidelity of explants was evaluated by the frequency of shoot-tip necrosis. Finally, rooting was evaluated by the frequency of rooting.

## RESULTS

***Bud induction and multiplication***

Shoots buds were induced from hypocotyls, cotyledons, apices and roots of germinating horse chestnut somatic embryos cultivated on media with BA (Fig. 1a). ANOVA indicated

significant effect of BA concentration on both the frequency of bud regeneration, the mean number of shoot buds per somatic embryo and BFC (not shown).

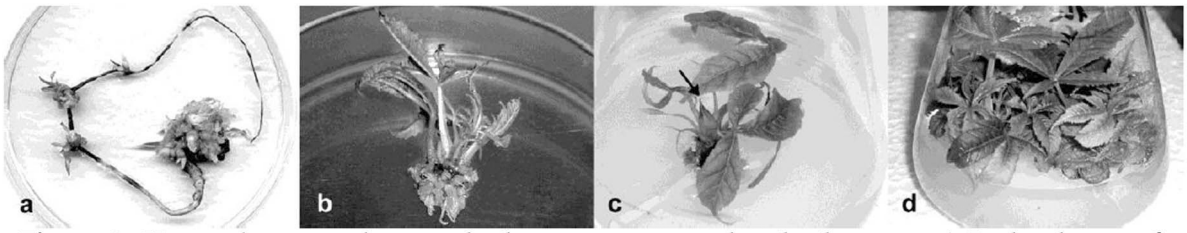


Figure 1. *Horse chestnut adventive bud regeneration and multiplication. a) Bud induction from somatic embryo cultivated on MS medium with 5 μM BA. b) Secondary bud regeneration from the primary shoot base cultivated on MS medium with 5 μM BA for four weeks. c) Shoot apex necrosis (arrow) of the shoot cultivated on MS medium with 1 μM BA. d) Shoot culture on MS medium with 5 μM BA.*

The highest values for all the three variables tested were achieved with 10 μM BA, although the frequency of regeneration and BFC index was not significantly different comparing to 5 μM BA (Tab. 1). As hyperhydration was frequently observed in shoot buds induced on 10 μM BA, 5 μM BA was the most appropriate treatment for bud induction.

Table 1. *Bud induction from germinated horse chestnut somatic embryos (3 cm) cultivated on solid MS medium with 0, 1, 5 or 10 μM BA for 4 weeks.*

| BA concentration μM | Frequency of regeneration | Bud mean number | Bud forming capacity |
|---|---|---|---|
| 0 | 3.55 ± 0.46 c | 0.35 ± 0.03 d | 0.42 ± 0.17 b |
| 1 | 74.33 ± 1.25 b | 2.49 ± 0.05 c | 3.45 ± 0.96 b |
| 5 | 94.82 ± 2.08 ab | 12.33 ± 0.18 b | 15.67 ± 4.84 a |
| 10 | 100 a | 20.27 ± 0.14 a | 23.27 ± 3.59 a |

Data represent mean values. Two replicates, each with three samples and five subsamples (n=30), were used per treatment. Treatments denoted by the same letter in a column were not significantly different ($P \leq 0.05$) according to Duncan's test.

Secondary buds were regenerated from the base of the primary shoots (Fig. 1b). ANOVA showed that BA concentration significantly affected both secondary bud number and the frequency of shoot-tip necrosis (not shown). Although they formed spontaneously (1.9 ± 0.3 on average), the addition of BA in culture medium significantly increased the number of secondary buds (Fig.2). The highest shoot number was achieved with 10 μM BA, although it was not significantly different from 5 μM BA. Necrosis of the shoot apices (Fig. 1c) occurred very frequently (76-100%) with BA levels up to 2.5 μM, while it was not observed with BA at 5 μM or higher (Fig. 2). However, hyperhydration was rather frequent in shoots cultivated on BA above 5 μM. Therefore, BA at 5 μM was the most appropriate for development of healthy secondary buds. The shoots formed rosette-like habitus (Fig. 1d), therefore they had to be subjected to a shoot elongation treatment.

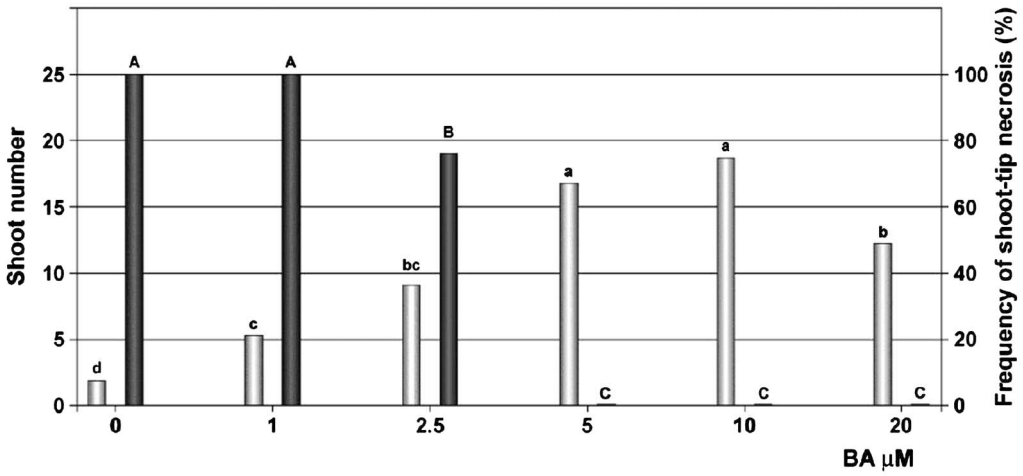


Figure 2. *Secondary bud formation from germinated horse chestnut somatic embryos cultivated on solid MS medium with 0-20 µM BA (white bars) and the frequency of shoot-tip necrosis (black bars). Data represent mean values. Two replicates, each with three samples and five subsamples (n=30), were used per treatment. Treatments denoted by the same letter were not significantly different (P ≤ 0.05) according to Duncan's test.*

***Shoot elongation, rooting and preventing shoot-tip necrosis***

Individual shoots elongated (Fig. 3a) at relatively high frequency (63%), and those treated with IBA developed root initials within 2-3 weeks (Fig. 3b). According to ANOVA, IBA significantly affected the frequency of root induction. Spontaneous rooting (in shoots dipped in $dH_2O$) was not observed, and the highest rooting rate was observed in shoots treated with 10 mM IBA (23%), whereas it was 8.3% with 5 mM IBA. Shoots developed a very good root system, with main root of 10-12 cm and many laterals within 4 weeks following the root inducing treatment (Fig. 3c). Despite well developed root system, the majority of these explants suffered from shoot-tip necrosis, and this problem was overcome by the application of BA directly to the shoot apex. Shoot-tip necrosis was completely cured by the application of 1µM BA (Fig. 3d). Higher levels of BA or shorter intervals of BA application caused hypertrophy of the shoot tip, which was even 7 mm in diameter in some shoots treated with 10 µM BA (not shown). Therefore, the weekly application of 1µM BA was optimal treatment.

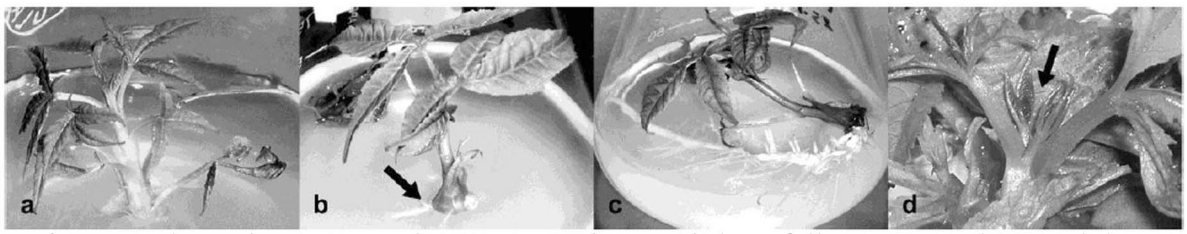


Figure 3. *Shoot elongation and rooting. a) Elongated shoot following 4 weeks on solid MS medium with 1 µM BA and 500 mg/l PVP. b) A root initial emergence (arrow) following the treatment with 10 mM IBA for 1 min and 2 weeks on half-strength MS PGR-free medium. c) The same plantlet after an additional 2 weeks. d) Healthy shoot apex (arrow) treated by weekly application of 10 µl of 1 µM BA.*

## DISCUSSION

We reported here on efficient bud induction from all organs of germinated somatic embryos of horse chestnut. For this purpose, we used 3 cm long somatic embryos, as it was

shown in a previous study that red horse chestnut somatic embryos of this size predominantly regenerate shoot buds (Zdravković-Korać *et al.* 2008), in contrast to smaller somatic embryos (≤ 1 cm) of horse chestnut (Kiss *et al.* 1992) and red horse chestnut (Zdravković-Korać *et al.* 2008), which almost exclusively regenerate secondary somatic embryos.

Somatic embryogenesis, an alternative method for *in vitro* clonal propagation, was previously achieved in horse chestnut (Dameri *et al.* 1986, Radojević 1988, Jörgensen 1989, Profumo *et al.* 1991, Gastaldo *et al.* 1994, 1996). Although it could be additionally amplified through highly efficient secondary somatic embryogenesis (Ćalić *et al.* 2005), a serious obstacle of this approach is the absence of an efficient protocol for conversion of somatic embryos to healthy plantlets and their successful acclimatization (Capuana & Debergh 1997, Troch *et al.* 2009). For this reason, we tried to develop an alternative protocol for mass *in vitro* vegetative propagation of this species. A protocol for *in vitro* propagation by meristem culture derived from shoot apex of both androgenic and somatic embryos, and dormant buds of horse chestnut is available (Radojević *et al.* 1987). However, with that protocol shoot multiplication occurred predominantly through less efficient axillary branching and adventitious buds were only seldom observed.

Efficient secondary regeneration from the shoot base enabled further multiplication of shoots in the present study and led to the establishment of a permanent shoot culture. We found 5 μM BA being an optimal for both healthy bud induction and multiplication, since higher BA levels provoked hyperhydricity. Increased hyperhydricity with higher cytokinin concentration is a common phenomenon (Ivanova *et al.* 2006).

It is well known that cytokinins inhibit root initiation, so it was necessary to reduce BA level for shoot elongation and rooting and that caused mass shoot-tip necrosis. These two phenomena, rooting and shoot-tip necrosis, have opposite requirements for growth regulators. Cytokinins are needed for the maintenance of the shoot apex and in the same time they inhibit rooting. This problem could be overcome by local BA application directly to the shoot-tip, as was described in *C. sativa* (Vieitez *et al.* 1989; Piagnani *et al.* 1996). In the present study, shoot-tip necrosis was completely eliminated by weekly application of 10 μl of 1 μM BA during root-inducing phase. Actually, it is important to maintain shoot-tip vigour only until root system develops. In this study week intervals for BA application was an optimal period. Initiation of roots, which are the main site of cytokinin synthesis, restores the development of shoot apex, as was seen in *C. sativa*, *Q. rubber* (Vieitez *et al.* 1989) and *H. procumbens* (Bairu *et al.* 2009b). Alternatively, development of axillary shoots, which is delayed comparing to apical shoot, may circumvent the time of root initiation (Thomas 2000).

Cytokinin application following the emergence of root initials was beneficial in decreasing shoot-tip necrosis in *C. dentata* (Xing *et al.* 1997). Despite cytokinins inhibit root initiation, they do not interfere with root growth if they are applied following root initiation. According to this, in the present study shoots were transferred to medium supplemented with 5 μM BA as soon as the root initials were observed.

A classical rooting method proposed by Radojević *et al.* (1987), which included a 10-day-dark treatment and rooting on solid medium with 2 mg/l IBA for a few weeks, failed in the present study, so we adopted a method of Xing *et al.* (1997). This method includes quick-deep of shoot base in a high-concentration-IBA solution, following a few weeks on a half-strength PGR-free medium and then subcultivation on cytokinin-containing medium. Although the method is rather laborious, it was very efficient in *C. dentata*, allowing rooting of 14-77% of shoots with 25-67% shoot-tip necrosis. Without the postrooting BA treatment, these authors found shoot-tip necrosis in 86% of shoots. Similarly, 77% shoots of *C. sativa* explants exhibited shoot-tip necrosis without postrooting treatment (Piagnani & Eccher 1988).

The frequency of rooting obtained in this study is better than by classical rooting (23% vs. 0%), but it is still unsatisfactory. In a previous study, we were able to increase the rooting

rate of horse chestnut plantlets transformed with *Agrobacterium rhizogenes* strain A4GUS using the same rooting procedure (Zdravković-Korać *et al.* 2004). However, these plants have changed habitus due to the expression of the *rol* genes and could be used only for specific horticultural purposes. Generally, rooting of woody plants is rather inefficient, and poor results were obtained in many studies. Using a quick-deep rooting method, rooting was achieved only in approximately 20% of *C. sativa* explants (Piagnani & Eccher 1988), while Radojević *et al.* (1987) achieved rooting rate of 7-24% in horse chestnut shoots using 2 mg/l IBA.

In the present study, 10 mM IBA was the best rooting treatment. By contrast, Xing *et al.* (1997) found 5 mM IBA to be better than 10 mM IBA, in terms of both higher rooting rate and less shoot-tip necrosis. The authors concluded that IBA level should be adjusted for each genotype, and that is in accordance with Radojević et al. (1987), who found a rather high variability in rooting rates (7-24%) among different clones.

In conclusion, we described here a very efficient protocol for bud induction from somatic tissue of an elite horse chestnut specimen. In addition to an efficient shoot multiplication, this protocol made a significant contribution to mass vegetative propagation of this species. However, the inefficiency of rooting and necrosis of the shoot apex has limited our success. Although some solutions that may help to overcome these problems are proposed in the present study, further optimisation is needed and these experiments are underway in our laboratory.

**Acknowledgement**

*The Ministry of Education and Science of the Republic of Serbia, Contract No. 173015, supported this work.*

## REFERENCES

Bairu MW, Stirk WA, Van Staden J (2009a) Factors contributing to *in vitro* shoot-tip necrosis and their physiological interactions. Plant Cell Tiss Organ Cult 98:239–248.

Bairu MW, Jain N, Stirk WA, Dolezal K, van Staden J (2009b) Solving the problem of shoot-tip necrosis in *Harpagophytum procumbens* by changing the cytokinin types, calcium and boron concentrations in the medium. S Afr J Bot 75:122–127.

Bairu MW, Novák O, Doležal K, Van Staden J (2011) Changes in endogenous cytokinin pofiles in micropropagated *Harpagophytum procumbens* in relation to shoot-tip necrosis and cytokinin treatments. Plant Growth Regul 63:105–114.

Bonga JM, Klimaszewska KK, Von Aderkas P (2010) Recalcitrance in clonal propagation, in particular of conifers. Plant Cell Tiss Org Cult 100:241–254.

Capuana M, Debergh PC (1997) Improvement of the maturation and germination of horse chestnut somatic embryos. Plant Cell Tiss Org Cult 48: 23-29.

Ćalić D, Zdravković-Korać S, Radojević Lj (2005) Secondary embryogenesis in androgenic embryo cultures of *Aesculus hippocastanum*. Biol Plant 49: 435–438.

Dameri RM, Caffaro I, Gastaldo P, Profumo P (1986) Callus formation and embryogenesis with leaf explants of *Aesculus hippocastanum* L. J Plant Physiol 126: 93–96.

Gastaldo P, Carli S, Profumo P (1994) Somatic embryogenesis from stem explants of *Aesculus hippocastanum*. Plant Cell Tiss Org Cult 39: 97-99.

Gastaldo P, Caviglia AM, Carli S, Profumo P (1996) Somatic embryogenesis and esculin formation in calli and embryoids from bark explants of *Aesculus hippocastanum* L. Plant Sci 119: 157–162.

Grigoriadou K, Leventakis N, Vasilakakis M (2000) Effects of various culture conditions on proliferation and shoot tip necrosis in the pear cultivars 'William's' and 'Highland' grown *in vitro*. Acta Hortic 520:103–108.

Hepler PK (2005) Calcium: a central regulator of plant growth and development. Plant Cell 17:2142–2155.

Hirschi KD (2004) The calcium conundrum. Both versatile nutrient and specific signal. Plant Physiol 136:2438–2442.

Hu H, Brown PH (1994) Localization of boron in cell walls of squash and tobacco and its association with pectin: evidence for a structural role of boron in the cell wall. Plant Physiol 105:681–689.

Ivanova M, Novák O, Strnad M, Van Staden J (2006) Endogenous cytokinin in shoots of *Aloe polyphylla* cultured *in vitro* in relation to hyperhydricity, exogenous cytokinins and gelling agents. Plant Growth Regul 50:219–230.

Jörgensen J (1989) Somatic embryogenesis in *Aesculus hippocastanum* L. by culture of filament callus. J Plant Physiol 135: 240–241.

Kataeva NV, Alexandrova IG, Butenko RG, Dragavtceva EV (1991) Effect of applied and internal hormones on vitrification and apical necrosis of different plants cultured *in vitro*. Plant Cell Tiss Org Cult 27:149–154.

Kiss J, Heszky LE, Kiss E, Gyulai G (1992) High efficiency adventive embryogenesis on somatic embryos of anther, filament and immature proembryo origin in horse-chestnut (*Aesculus hippocastanum* L.) tissue culture. Plant Cell Tiss Org Cult 30:59–64.

Martin KP, Zhang C-L, Slater A, Madasser YJ (2007) Control of shoot necrosis and plant death during micropropagation of banana and plantains (*Musa spp.*). Plant Cell Tiss Organ Cult 88:51–59.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15: 473–497.

Perez-Tornero O, Burgos L (2000) Different media requirements for micropropagation of apricot cultivars. Plant Cell Tiss Org Cult 63:133–141.

Piagnani C, Zocchi G, Mignani I (1996) Influence of $Ca2^{+}$ and 6-benzyladenine on chestnut (*Castanea sativa* Mill.) *in vitro* shoot-tip necrosis. Plant Sci 118:89–95.

Piagnani C, Eccher T (1988) Factors affecting the proliferation and rooting of chestnut *in vitro*. Acta Hortic 227:384–386.

Profumo P, Gastaldo P, Bevilacqua L, Carli S (1991) Plant regeneration from cotyledonary explants of *Aesculus hippocastanum* L. Plant Sci 76: 139–142.

Radojević Lj, Druart P, Boxus P (1987) Vegetative propagation of androgenous embryos of horse chestnut by meristem culture *in vitro*. Acta Hortic 212:531–537.

Radojević Lj (1988) Plant regeneration of *Aesculus hippocastanum* L. (horse chestnut) through somatic embryogenesis. J Plant Physiol 132: 322–326.

Radojević Lj (1991) Horse chestnut (*Aesculus* spp.). In: Bajaj YPS (Ed.) Biotechnology in agriculture and forestry. 1 st ed. Berlin-Heidelberg, Springer-Verlag, pp. 111–141.

Thomas P (2000) Microcutting leaf area, weight and position on stock shoot influence root vigour, shoot growth and incidence of shoot tip necrosis in grape plants *in vitro*. Plant Cell Tiss Org Cult 61:189–198.

Troch V, Werbrouck S, Geelen D, Van Labeke MC (2009) Optimization of horse chestnut (*Aesculus hippocastanum* L.) somatic embryo conversion. Plant Cell Tiss Org Cult 98: 115–123.

Vieitez AM, Sanchez C, San-Jose C (1989) Prevention of shoot tip necrosis in shoot cultures of chestnut and oak. Sci Hortic 41:101–109.

Wang G, Römheld V, Li C, Bangerth F (2006) Involvement of auxin and CKs in boron deficiency induced changes in apical dominance of pea plants (*Pisum sativum* L.). J Plant Physiol 163:591–600.

Xing Z, Satchwell MF, Powell WA, Maynard C (1997) Micropropagation of American chestnut: increasing rooting rate and preventing shoot-tip necrosis. In Vitro Cell Dev Biol 33:43–48.

Zdravković-Korać S, Ćalić-Dragosavac D, Uzelac B, Janošević D, Budimir S, Vinterhalter B, Vinterhalter D (2008) Secondary somatic embryogenesis versus caulogenesis from somatic embryos of *Aesculus carnea* Hayne.: developmental stage impact. Plant Cell Tiss Org Cult 94:225–231.

Zdravković-Korać S, Muhovski Y, Druart Ph, Ćalić D, Radojević Lj. (2004) *Agrobacterium rhizogenes*-mediated DNA transfer to *Aesculus hippocastanum* L. and the regeneration of transformed plants. Plant Cell Rep 22:698–704.