ISBN 978-86-80439-21-1
ISBN 978-86-90439-23-5 (VOL. 2)

INTERNATIONAL SCIENTIFIC CONFERENCE

FOREST ECOSYSTEMS AND CLIMATE CHANGES

MARCH 9-10TH, 2010
BELGRADE, SERBIA

PROCEEDINGS
VOLUME 2

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INSTITUTE OF FORESTRY - BELGRADE

INTERNATIONAL UNION OF FOREST RESEARCH ORGANIZATIONS

EFI ASSOCIATED EVENT

International Scientific Conference

FOREST ECOSYSTEMS AND CLIMATE CHANGES

PROCEEDINGS
Volume 2

Organised by:
INSTITUTE OF FORESTRY, BELGRADE

IN COOPERATION WITH

IUFRO
EFI
MINISTRY OF SCIENCE AND TECHNOLOGICAL DEVELOPMENT, REPUBLIC OF SERBIA
MINISTRY OF AGRICULTURE, FORESTRY AND WATER MANAGEMENT – DIRECTORATE OF FORESTRY, REPUBLIC OF SERBIA
MINISTRY OF ENVIRONMENT AND SPATIAL PLANNING, REPUBLIC OF SERBIA
PE „SRBIJAŠUME“
SOCIETY OF FORESTRY ENGINEERS AND TECHNICIANS OF SERBIA

Belgrade
March 9-10th, 2010.

Citation
International Scientific Conference
Forest ecosystems and climate changes

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Publisher
Institute of Forestry
Belgrade, Serbia

Chief Editor
Ph.D. Ljubinko Rakonjac

Technical Editor and layout
M.Sc. Tatjana Ćirković-Mitrović

Cover design
Nevena Čule, B.Sc.
Suzana Mitrović, B.Sc.

Printed in
200 copies

Printing
KLIK PRINT, Belgrade

Belgrade
March, 2010

Address of the Organizer
Institute of Forestry
Kneza Višeslava 3
Belgrade, Serbia
www.inforserb.org

Complete set of two volumes: ISBN 978-86-80439-21-1
Volume 1: ISBN 978-86-80439-22-8
Volume 2: ISBN 978-86-90439-23-5

CIP - Каталогизација у публикацији
Народна библиотека Србије, Београд

630*42(082)
551.583(082)
504.7

INTERNATIONAL Scientific Conference Forest Ecosystems and Climate Changes (2010 ; Beograd)
Proceedings. #Vol. #2 / International Scientific Conference Forest Ecosystems and Climate Changes, Belgrade, March 9-10th, 2010 ; organized by Institute of Forestry, Belgrade ... [et al.] ; [chief editor Ljubinko Rakonjac]. - Belgrade : Institute of Forestry, 2010 (Belgrade : Klik print). - 342 str. : ilustr. ; 30 cm

Tiraž 200. - Napomene uz tekst. - Bibliografija uz svaki rad. - Rezimei.

ISBN 978-86-80439-23-5
1. Institut za šumarstvo (Beograd)
a) Шуме - Климатски утицаји - Зборници b) Климатске промене - Зборници
COBISS.SR-ID 176326156

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MATURATION OF *AESCULUS FLAVA* (MARSHALL) ANDROGENIC EMBRYOS

Dušica ĆALIĆ-DRAGOSAVAC[1], Snežana ZDRAVKOVIĆ-KORAĆ[1], Nina DEVRNJA[1], Ljiljana RADOJEVIĆ[1], Branka VINTERHALTER[1]

Abstract: *Nevertheless, cultivated A. flava tree, about 15 years old, growing in the Botanical Garden "Jevremovac" of the Belgrade University. Closed flower buds (4-5 mm long) with premature anthers were harvested from A. flava tree. Androgenesis was induced in anther culture on solid MS (Murashige and Skoog, 1962) medium with 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (1.0 mg $l^{-1}$of each). Globular androgenic embryos were appeared after two months. However, after globular embryos, heart, torpedo-like and cotyledonal embryos were appeared.*

Feature development and multiplication of androgenic embryos proceed on a solid medium with reduced concentration of 2,4-D (0.01 mg l^{-1}) and same concentration of kinetin. After medium for multiplication embryos were cultured on media for embryo maturation supplemented with various concentrations (0.1, 0.5, 1, 2 and 3 %) of activated charcoal.

The effect of activated charcoal has been attributed to the absorption of inhibitory substances, such as phenolic compounds which are produced embryos, from the medium.

The best results of germination and maturation of A. flava androgenic embryos were obtained on medium supplemented with 1 % activated charcoal. Also, the greatest number of secondary somatic embryos and the lowest number of albino embryos were noticed on the same medium.

This is the first report about induction and maturation androgenic embryos originating from A. flava anther culture.

Key words: anther culture, androgenesis, germination, maturation, yellow buckeye

1. INTRODUCTION

Aesculus flava (syn. *A. octandra*) is a species of buckeye native to eastern North America. *A. flava* is, as most of trees, characterised by a long reproductive cycle and a high level of heterozygosity. This makes genetic improvement by classical breeding difficult. The production of homozygous (doubled-haploid) plants *via* androgenesis *in vitro* can contribute to more efficient breeding. Not only can large, uniform embryo populations be generated, but these are for the most part free of genetic anomalies since embryos of tree species develop directly from microspores without an intermediate callus phase (Radojević 1991, Germana *et al*. 1994, Capuana and Deberg, 1997, Radojević *et al*. 1998, Ćalić *et al*. 2003).

Haploid plants are increasingly used in the breeding of many crop species to obtain isogenic lines in a faster and reliable way as an alternative to the classic genetic crosses. Woody species in general, and forest trees in particular, have shown to be extremely recalcitrant in anther cultures, and only few examples of successful regeneration of plantlets, from confirmed microspore origin have been reported (Höfer *et al*. 2002). The induction of haploid embryos in anther cultures, from corkoak (*Quercus suber* L.), 20–30 days after application of specific stress conditions has been reported by Bueno *et al.* (1997). However, some embryos are formed up to 10–12 months later, with diploid genomes. These facts raised the question of the cellular origin of the embryos and their *in vitro* development as the occurrence of diploid clonal embryos could limit in some way the feasibility of the method for use in regeneration and breeding programs.

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2. MATERIAL AND METHODS

Inflorescences were harvested from of *A. flava* tree (about 15 years old) growing in the Botanical garden ″Jevremovac″ of Belgrade University. Anthers were excised from closed flower buds (about 4-5 mm). In fact, a high correlation has been shown between flower lenght and phase of pollen development. In buds of 4-5 mm microspores were at the uninucleate stage of development. Determining the correct developmental stage of the microspores, were performed with 0.1 mg l^{-1} 4',6-diamidino-2-phenylindole (DAPI). Pollen dimorphism determined with aceto-carmine while microspore viability with fluorescein diacetate (FDA).

The selected buds from three segments (*A*-female; *B*-bisexual and *C*-male flowers) of inflorescence was surface sterilized with 95 % ethanol and 70 % ethanol for about 5 min, followed by three rinses in sterile distilled water. Induction of androgenic embryos of *A. flava* was achieved on solid MS medium contained Murashige and Skoog's (1962) mineral salts, 2 % sucrose, 0.7 % agar, and was supplemented with the following (mg L^{-1}): panthotehenic acid 10.0, nicotinic acid 5.0, vitamin B_1 2.0, adenine sulphate 2.0, myo-inositol 100, casein-hydrolysate 200, 2,4-dichlorophenoxyacetic acid (2,4-D) 1.0 and kinetin (Kin) 1.0. The MS_1 medium for embryo multiplication consisted reduced concentration of 2,4-D (0.01 mg L^{-1}) and the same concentration of Kin (1.0 mg L^{-1}).

Anthers were inoculated in each culture tube containing 8 cm^3 of the MS induction medium. Embryo development and multiplication of androgenic embryos from anther culture proceeded on MS_1. After medium for multiplication embryos were cultured on A_0 media without hormone, as well as A_1-A_5 media with 0.1, 0.5, 1, 2 and 3 % of activated charcoal (AC). Filter sterilized L-glutamine (Glu) was added 400 mg l^{-1} to improve embryo maturation in later stages of their development.

3. RESULTS

Microspores isolated from flower buds (Table 1, Fig.1) after aceto-orceine (Table 1, Fig.2), and fluorescein-diacetate (Table 1, Fig.3-5) stained showed different size. However, DAPI treated microspores had uninuclear stage of development (Table 1, Fig.6).

The development of androgenic embryos was asynchronous, so that embryos at their globular, heart-shaped, torpedo-like and cotyledon stages were observed in the same culture, on MS_1 medium (Figs. 1-2). Rapid differentiation of androgenic embryos was obtained in *in vitro* culture over the second months, producing embryos with different cotyledon numbers. Great numbers of these embryos were irregular, hypertrophy, with abnormal cotyledons or without hypocotyls.

After medium for multiplication embryos were cultured on hormone-free medium, as well as on maturation media supplemented with various concentrations (0.1, 0.5, 1, 2 and 3 %) of activated charcoal.

The effect of activated charcoal has been attributed to the absorption of inhibitory substances (abscisic acid, phenolics) from the medium.

Percentage germination of androgenic embryos was followed after 30, 60 and 90 days of growing on different maturation media.

The best results of androgenic embryos germination on media supplemented with 1 % AC (His. 1). Also, androgenic embryos on media with 1, 2 and 3 % AC showed a rapid development of green embryos (Table 2, Fig.1) in the cotyledonary stage, the greatest number of secondary somatic embryos (Figs.7-11) and lower percentage of albino embryos (Table 1, Table 2, Fig.3). Himeric androgenic embryos were only appeared on hormone-free medium (Table 1, Table 2, Fig.2).

Secondary embryos appeared on the radiculi of androgenic embryos grown on the MS hormone-free medium and maturation media (Table 1, Figs.7-12). Some embryos formed root and epicotyls (Table 1, Fig.12).

The largest number of *A. flava* embryos per anther formed from female buds (segment A). These results are in agreement with previous reports on the induction in anther culture of horse chestnut (Radojević *et al*. 2000) and *A. flava* (Ćalić *et al*. 2005). The goal of the work was mass production of haploid androgenic embryos from anther culture and induction of secondary somatic embryogenesis and plantlets formation of *A. flava* in order to develop efficient *in vitro* regeneration methods to be used in genetic transformation experiments applicable in the pharmaceutical industry.

4. DISCUSSION

The smaller *A. flava* microspores (Ćalić-Dragosavac *et al*. 2008, 2009) in uninucleate stage are known to be crucial for androgenesis as well as in all other trees (Ćalić *et al.* 2003).

Establishment of anther culture in woody plants is generally rather complicated. This is the first reported succesful plant maturation in anther culture of yellow buck. The described protocol for an efficient haploid induction in anther culture of *A. flava* can be used in genetic manipulation to secondary metabolism.

Acknowledgments: *This work was supported by the Ministry of Science and Environmental Protection of Serbia, grant* N_0*. 143026.*

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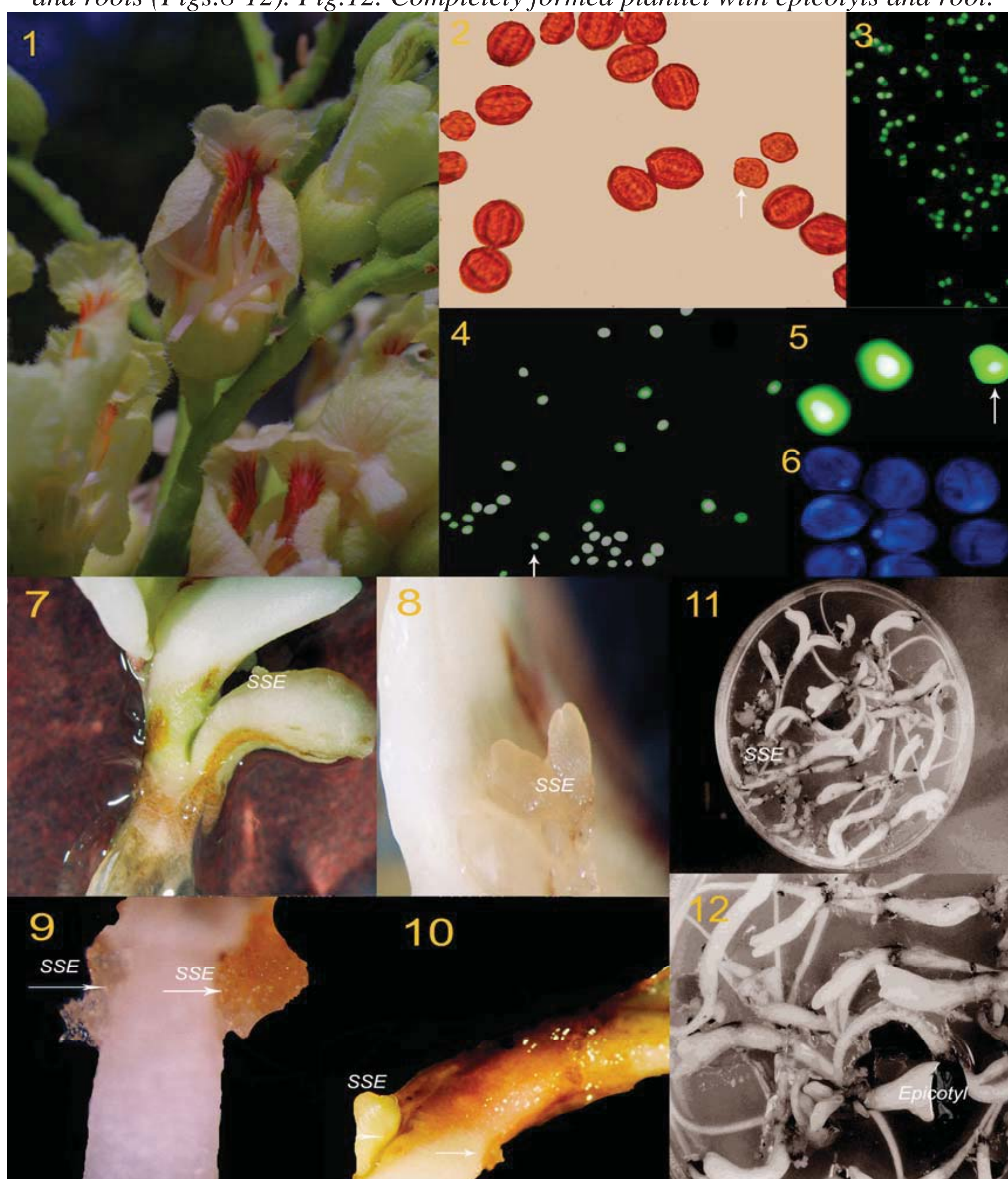
Table 1. *Percentage of green, albino and himeric androgenic embryos on different maturation media.*

Medium	Type of embryos (%)		
	Green	Albino	Himeric
A_0=MS	87	12	1
$A_1=A_0$ + AC 0.1 %	90	10	0
$A_2= A_0$ + AC 0.5 %	93	7	0
$A_3= A_0$ + AC 1 %	95	5	0
$A_4= A_0$ + AC 2 %	96	4	0
$A_5= A_0$ + AC 3 %	96	4	0

Table 1. **Figures 1-12.** *Flower buds (Fig.1); aceto-orcein (Fig.2.), fluorescein diacetate (Figs.3-5), DAPI (Fig.6) treated microspores; secondary somatic embryos (SSE) on cotyledon (Fig.7) and roots (Figs.8-12). Fig.12. Completely formed plantlet with epicotyls and root.*



Histogram 1. *Influence of AC on androgenic embryo germination, after 3, 6 and 9 weeks.*

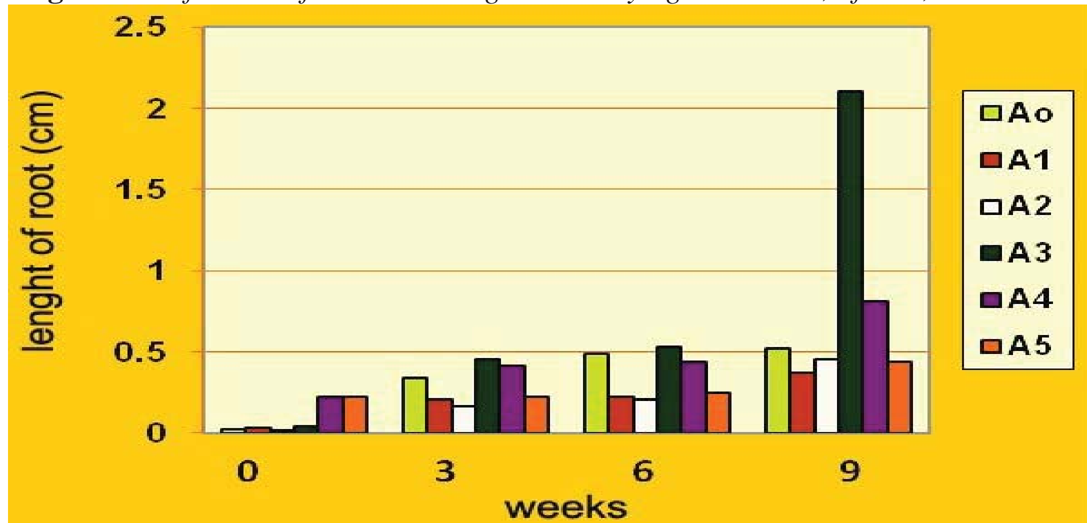


Table 2. Figures 1-3. *Green Fig.1), himeric (Fig.2) and albino (Fig.3) Aesculus flava embryos.*