Journal of Horticultural Research 2014, vol. 22(1): 49-61
DOI: 10.2478/johr-2014-0006

PHYSIOLOGICAL RESPONSE OF *IN VITRO* CULTURED *MAGNOLIA* SP. TO NUTRIENT MEDIUM COMPOSITION

Rossen S. SOKOLOV, Bistra Y. ATANASSOVA, Elena T. IAKIMOVA*
Institute of Ornamental Plants
1222 Negovan, Sofia, Bulgaria

Received: March 16, 2014; Accepted: April 14, 2014

ABSTRACT

The objective of this study was to assess the regeneration response of *in vitro* cultured *Magnolia* × *soulangeana* 'Alexandrina' and *Magnolia liliiflora* 'Nigra' to nutrient medium composition. In the primary culture (initiated from dormant axillary buds) combinations of Murashige and Skoog (MS) basal salts with 6-benzylaminopurine and α-naphthaleneacetic acid were tested. The primary explants of cv. 'Alexandrina' expressed higher regeneration rate than cv. 'Nigra'. For both species, the regeneration was most strongly potentiated at addition of 0.25 mg·dm^{-3} of the cytokinin alone. The auxin exerted undesirable effects. Several basal salts media were applied in proliferation stage and their physiological effects were evaluated in reference to traditionally used MS. At culturing on Chée & Pool C2d Vitis Medium (VM) that is for the first time introduced to magnolia and on MS, *M. liliiflora* formed more but less elongated shoots than *M. soulangeana*. However, on VM, substantial increase (25-30%) of the number of axillary shoots and leaves, shoot length and fresh and dry weights over MS was established for both species. This suggested VM as promising composition of nutrients in multiplication stage. Microshoots obtained on MS, VM, Rugini Olive Medium and DKW Juglans Medium were successfully rooted *in vitro* and subsequently established *ex vitro*. The findings expand the information on magnolia response to culture conditions and contribute to elaboration of innovative elements of protocols for establishing tissue cultures with high regeneration capacity.

Key words: *Magnolia* sp., basal medium, plant growth regulators, primary culture, rooting, shoot proliferation

INTRODUCTION

The plants from family Magnoliaceae, genus *Magnolia* L. (consisting of over 250 species) are flowering deciduous or evergreen trees and shrubs cultivated in sub-tropical and temperate climates (Callaway 1994; Figlar & Nooteboom 2004). For their attractive ornamental qualities magnolias are highly demanded for landscaping purposes. Conventionally, magnolia is propagated through seeds, grafting and rooting of stem cuttings but the germination rate of the seeds is relatively low and the cuttings often express poor rooting ability (Callaway 1994; Ming & Huan-Cheng 2003). For obtaining large amount of disease-free and genetically identical planting material, clonal micropropagation has been introduced as appropriate approach. Among the genotypes of commercial interest, the research has addressed *in vitro* growth response mainly of saucer magnolia (*Magnolia* × *soulangeana*), a hybrid of *M. denudata* × *M. liliiflora*, star magnolia (*Magnolia stellata*), hybrids from the series of *Magnolia* 'Little Girl' (*M. liliiflora* × *M. stellata*), and the yellow-blooming magnolias (crosses between *M. accuminata* and *M. heptapeta*) (Biedermann 1987; Callaway 1994; Tubesing 1998; Kamenická & Lanaková 2000; Parris et al. 2012; Radomir 2012). More broadly studied are *Magnolia* sp. with pharmacological properties and serious effort is paid to *in vitro* conservation of the gene pool of rare and endangered genotypes (Merkle & Wiecko 1990; Callaway 1994; Parris et al. 2012 and references therein).

*Corresponding author:
e-mail: elena_iakimova@abv.bg

Different morphogenetic capacity has been established for primary explants (apical or axillary buds) sampled from *M.* × *soulangeana*, *M. stellata* and yellow-flowering hybrids (Biedermann 1987; Kamenická & Lanaková 2000; Tubesing 1998; Parris et al. 2012; Radomir 2012). It has also been demonstrated that *in vitro* growth and development of magnolias is dependent on exogenous supply of plant growth regulators (PGRs) and the requirements for the type, concentration and ratio of the hormones vary between the stages of initiation, multiplication and rooting (Biedermann 1987; Gabryszewska 1997; Kamenická & Valka 1997; Kamenická 1998; Kamenická & Lanaková 2000; Podwyszyńska et al. 2000; Kamenická et al. 2001; Zefang et al. 2003; Marinescu 2008; Radomir & Radu 2008, Parris et al. 2012; Radomir 2012). For example, purine and non-purine cytokinins have been shown to exert different physiological effects. Gabryszewska (1997) reported that 6-benzylaminopurine (BAP) supplemented to Murashige and Skoog (MS) basal salts mixture stimulated the formation of axillary shoots in *M.* × *soulangeana* whereas kinetin, 6-(γ,γ-dimethylallylamino) purine (2iP) and phenylurea cytokinin thidiazuron [TDZ, N-phenyl-NO-(1,2,3-thidiazol-5-yl) urea] did not promote shoot regeneration. Similar influence of these cytokinins has been established on *M. stellata* and *Magnolia* 'Ann' (*M. liliiflora* 'Nigra' × *M. stellata* 'Rosea') (Parris et al. 2012; Radomir et al. 2012). Microcuttings of *M.* × *soulangeana* produced on MS medium containing meta-topolin [6-(3-hydroxybenzylamino) purine] – a non-purine aromatic compound with cytokinin activity have been found to express better rooting potential and higher survival rate *ex vitro* in comparison to rooted explants derived from MS supplemented with equimolar concentration of BAP (Podwyszyńska et al. 2000). Improvement of proliferation rate has been reported in complex media comprising combinations of BAP, α-naphthalene-acetic acid (NAA) or indole-3-acetic acid (IAA), gibberellin (GA_3), ascorbic acid and 2-(N-morpholino) ethanesulfonic acid (MES) or NaFeEDTA (Gabryszewska 1997; Podwyszyńska et al. 2000; Parris et al. 2012; Radomir 2012). In rooting stage, genotype-specific response to the concentration of indole-3-butyric acid (IBA) has been determined (Kamenická & Lanaková 2000; Podwyszyńska et al. 2000; Parris et al. 2012; Radomir 2012).

The physiological performance of *in vitro* cultured magnolia is suggested to be influenced by basal salts medium composition. Biedermann (1987) has determined higher proliferation rate and better overall quality of microplants of *M.* 'Elizabeth', *M.* 'Yellow Bird', *M.* 'Cultivar # 149' and *M. stellata* grown on MS in comparison to culturing on Anderson's Rhododendron Medium (RM). Kamenická & Lanaková (2000) reported that the number of microshoots developed on *M.* × *soulangeana* propagated on modified S-medium (Standardi & Catalano 1985) and ½ (half strength) S-medium was almost doubled compared to McCown Woody Plant Medium (WPM) and ½ WPM. Different growth response of *M.* 'Ann' has been observed on basal MS, ½ MS, WPM, Blaydes Modified Basal Salts Medium (Blaydes) (Blaydes 1966) and Driver & Kuniyuki basal salts mixture (DKW) (Parris et al. 2012). Lepoivre basal medium (Quoirin & Lepoivre 1977) has been reported as efficient for micropropagation of *M.* × *soulangeana* (De Proftet al. 1985). For *in vitro* culturing of shoot apexes of *M. obovata* Nakamura et al. (1995) tested MS, Linsmaier and Skoog, WPM and broad-leaved tree (BT) basal media and their modifications in a primary culture of shoot apex and found that WPM and BT were not effective for the flushing of shoot apex whereas it was strongly promoted on medium containing one fourth nitrate of ½ MS (¼ -½ MS).

Taken together, the studies indicate that for establishing of magnolia tissue cultures with high regeneration capacity, the culture conditions need to be optimised for the particular species and cultivar and stage of culture development.

The aim of this work was to assess the physiological performance of *in vitro* cultured *M. liliiflora* 'Nigra' and *M.* × *soulangeana* 'Alexandrina' in dependence on the components of nutrient medium and to select appropriate compositions for establishing tissue cultures with increased regeneration capacity. To achieve the goal, in initiation stage, the effect of combinations of MS with BAP and NAA on regeneration of primary explants (dormant axillary buds) was assessed. In multiplication stage the

influence of several basal salts media, some of which for the first time tested for magnolia micropropagation was evaluated in reference to the traditionally used MS. The regeneration response of microplants propagated on MS, WPM, RM, Driver & Kuniyuki Juglans medium (DKW), Chée & Pool C2d Vitis Medium (VM), Olive Medium (OM), Gamborg B5 Medium (B5), Westvaco medium (WV3) and MS was characterized by shoot and leaf development and, accumulation of FW and DW. The processes of *in vitro* rooting and *ex vitro* acclimatization of plantlets were also addressed.

MATERIALS AND METHODS

Primary explants preparation

The experiments were undertaken with *M.* × *soulangeana* Soul.-Bod. cv. 'Alexandrina' and *M. liliiflora* Desr., cv. 'Nigra'. *In vitro* culture was initiated from dormant axillary buds sampled in the month of February from 15-years-old *M.* × *soulangeana* 'Alexandrina' and from 50-years-old *M. liliiflora* 'Nigra', growing in urban areas in cities in Middle South Bulgaria. One-year old, about 20 cm long apical shoots (Fig. 1a), were collected from the plants and transferred to laboratory. For removing rough contaminants, the shoots were three times rinsed with distilled water, dried on a filter paper and transferred to sterile conditions in laminar flow cabinet. The buds (Fig. 1b) were separated from the branches by using a scalpel and surface sterilized with immersing in 0.1% $HgCl_2$ for 3 min followed by triple rinsing with sterile distilled water for 10 min as described by Kamenická & Lanaková (2000) with slight modification. Thereafter the buds were left to dry on sterile filter paper in the flow cabinet. Subsequently, with the aid of forceps and scalpel, the external wrapping of the buds and residues of wood tissue at the base of explants were removed. The in this way prepared primary explants (Fig. 1c) were placed onto introductory medium for regeneration.

Establishment of primary culture

To select appropriate medium for culture initiation, combinations of 0.25, 0.5 and 1.0 $mg \cdot dm^{-3}$ BAP and 0.25 $mg \cdot dm^{-3}$ NAA supplemented to MS basal salts with vitamins were tested. The media were enriched with 30 $g \cdot dm^{-3}$ sucrose, solidified with 7 $g \cdot dm^{-3}$ agar and pH was adjusted to 5.8 with 0.1 N NaOH or 0.1 M HCl. The media were then autoclaved at 121 °C at 1.04 $kg \cdot cm^{-2}$ pressure for 20 min. Single explants (altogether 100 = 50 buds per variant in 2 replicates for each variant of the medium) were placed in individual sterile glass culture tubes covered with aluminium foil and the experiments were repeated two times. The tubes were maintained under room temperature 25 ± 2 °C, photoperiod day/night 16/8 and 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity provided by 40 W cool white fluorescent tubes (Philips, Bulgaria). Regeneration rate was scored by the number of explants that developed stems and leaves and is presented in average percentage regenerated explants of 200 individual primary explants in total.

Shoot proliferation

To study the effect of basal salts medium on the process of multiplication, 60 days after culture initiation, nodal segments approximately 4 to 5 mm long with one leaf were separated from microshoots regenerated on MS supplemented with 0.25 $mg \cdot dm^{-3}$ BAP and transferred onto multiplication media containing different basal salts. Segments with apical bud were not used. Eight commercially available (Duchefa, The Netherlands) basal salts media with vitamins (Table 1) were tested: MS (Murashige & Skoog 1962), RM (Anderson 1980), B5 (Gamborg et al. 1968), VM (Chée & Pool 1987), WPM (Lloyd and McCown 1980), DKW (Driver and Kuniyuki 1984), OM (Rugini 1984) and WV3 (Patent, Coke 1996).

The amounts of basal medium in nutrient mixtures were as recommended in manufacturer manuals: 4.4 $g \cdot dm^{-3}$ MS, 2 $g \cdot dm^{-3}$ RM, 3.2 $g \cdot dm^{-3}$ B5, 4.5 $g \cdot dm^{-3}$ VM, 2.5 $g \cdot dm^{-3}$ WPM, 5.6 $g \cdot dm^{-3}$ DKW, 4.2 $g \cdot dm^{-3}$ OM and 4.3 $g \cdot dm^{-3}$ WV3. All basal media were supplemented with 0.5 $mg \cdot dm^{-3}$ BAP, 0.25 $mg \cdot dm^{-3}$ NAA and 30 $g \cdot dm^{-3}$ (determined in a separate experiment), sucrose and solidified with 7 $g \cdot dm^{-3}$ agar. Media pH, autoclaving and environmental conditions were as described above for culture initiation. The explants were grown in 400 ml glass vessels in which the medium quantity was equalized to 60 ml by using automatic Ismatec ISM404B MCP Pump (ISMATEC, Switzerland).

The vessels were tightly covered with metal screw cups. For each separate variant of the basal medium, 50 nodal segments, distributed in 10 replicates, each replicate containing 5 explants per vessel were used. The experiment was repeated three times. The subculturing was performed every 60 days onto the same media and by using the same type of nodal segments.

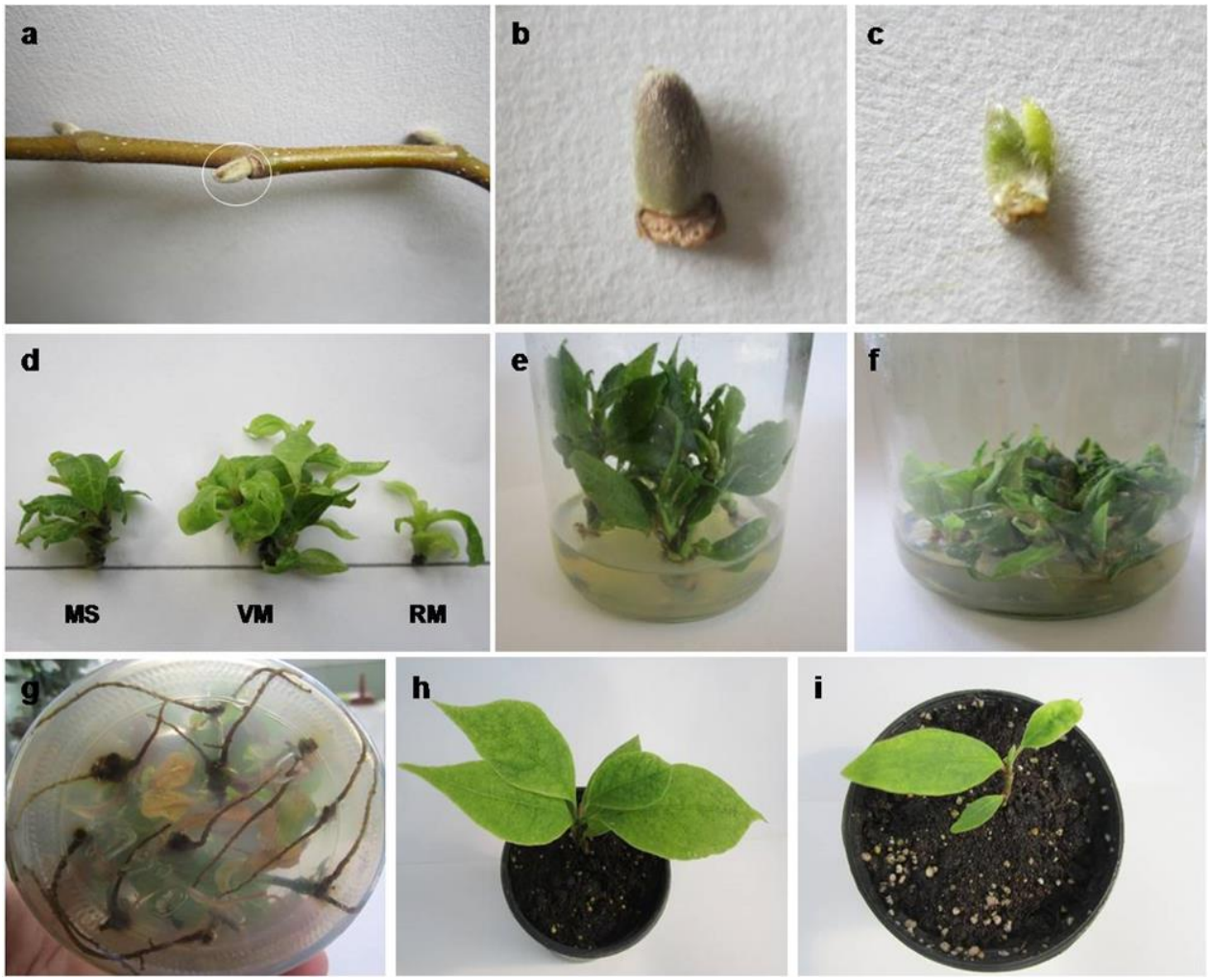

Fig. 1. Representative images of consecutive stages of *in vitro* propagation of magnolia

a. Axillary buds of *M.* × *soulangeana* 'Alexandrina' attached to apical shoot; **b**. isolated axillary bud of *M. soulangeana*; **c**. primary explants of *M. soulangeana* with removed wrapping, Note: the axillary buds and the primary explants of *M. soulangeana* and *M. liliiflora* look almost identically; **d**. sixty-days-old individual explants of *M. soulangeana* propagated on MS, VM and RM; **e**. sixty-days-old culture of *M.* × *soulangeana* and **f**. *M. liliiflora* grown on VM medium; **g**. *In vitro* rooted explants of *M.* × *soulangeana* after 60 days in rooting medium, Note: the rooted explants of *M. liliiflora* appear morphologically similar to *M. soulangeana* and are not shown; **h**. sixty-days-old acclimatized plants derived from *in vitro* culture of *M.* × *soulangeana*; **i**. *M. Liliiflora*. MS = Murashige & Skoog medium; VM = Chée & Pool C2d Vitis Medium; RM = Anderson's Rhododendron Medium.

Table 1. Compositions of basal salts media

Compound	MS	RM	B5	VM	WPM	DKW	OM	WV3
Macroelements (mM)								
$CaCl_2$	2.99	2.99	1.02		0.65	1.01	2.99	4.08
KNO_3	18.79	4.75	24.73	18.79			10.88	9.00
K_2SO_4					5.68	8.95		
KCl							6.71	8.81
KH_2PO_4	1.25			1.25	1.25	1.95	2.50	1.98
NaH_2PO_4		2.75	1.09					
$MgSO_4$	1.50	1.50	1.01	1.5	1.50	3.00	6.09	7.51
NH_4NO_3	20.61	5.00		20.61	5.00	17.70	5.15	
$Ca(NO_3)_2$ $2H_2O$				2.99	2.35	8.30	2.54	
$(NH_4)_2SO_4$			1.01					
Microelements (µM)								
$CoCl_2$ $6H_2O$	0.11	0.11	0.11	0.11			0.11	0.11
$CuSO_4$ 5H2O	0.10	0.10	0.10	0.10	1.00	1.00	1.00	1.00
H_3BO_3	100	100	48.52	100	100	77.63	200	500
KI	5.00	1.81	4.52				5	5
$MnSO_4$ H_2O	100.0	100.0	59.16	5.00	130.0	200.0	100.0	90.0
Na_2MoO_4 $2H_2O$	1.03	1.03	1.03	1.03	1.03	1.61	1.03	1.03
$ZnSO_4$ $7H_2O$	29.91	29.91	6.96	29.91	29.91	72.19	49.75	30.0
FeNa EDTA	100.0	200.0	100.0	100.0	100.0	112.0	100.0	100.0
Vitamins (µM)								
Adenine sulphate		430.0						
Myoinositol	556.0	556.0	556.0	55.60	556.0	556.0	556.0	5560.0
Thiamine hydrochloride	0.30	1.19	29.65	3.00	2.96	5.93	1.48	1.19
Nicotinic acid	4.10		8.12	8.12	4.06	8.12	40.94	
Glycine	26.64				26.64	26.64	26.64	
Biotin							0.20	
Folic acid							1.13	
Pyridoxine hydrochloride	2.43		4.86		2.43		2.43	

The compounds in the nutrient media are presented in final concentrations. Media full names and abbreviations: Murashige & Skoog (MS), Anderson's Rhododendron Medium (RM), Gamborg B5 Medium (B5), Chée & Pool C2D Vitis Medium (VM), McCown Woody Plant Medium (WPM), DKW Juglans medium (JM), Rugini Olive Medium (OM), Westvaco WV3 medium (WV3).

Characterization of growth and development of explants

Growth and development of explants during proliferation stage were evaluated after the second sub-culture. The number of lateral shoots and number of leaves produced from single explant and its length (from the level of the media to the tip of the longest shoot) were recorded. The effect of basal medium on the physiological status of microplants was also estimated by FW and DW. DW was determined after drying the samples at 80 °C for 24 h, then the samples were cooled, placed in a dessicator for 2 h and measured; thereafter transferred back to the oven for additional 24 h and measured again followed by subsequent measurements at intervals of 2 h until reaching constant weight.

In vitro rooting and *ex vitro* acclimatization

Microshoots derived from cultures propagated on media containing MS, VM, OM or DKW basal salts were placed for rooting onto MS supplemented

with 2 mg·dm^{-3} NAA. The experiments were performed with 30 microplants from each basal medium (3 repetitions, each consisting of 10 microplants per Petri dish) and the whole set of experiments was repeated three times. After 60 days, explants with well-developed roots were used for acclimatization. The roots were washed for removing the traces of agar and the microplants transferred for acclimatization into plastic containers (200 ml volume) on substrate peat : perlite 4:1. For providing proper nutrient supply, commercial peat enriched with N, P, K and microelements (Fe, Mn, Co, B, Mg, Zn); pH 6.0 (DURPETA, Lithuania) was used. The containers were plastic covered and maintained for 20 days at temperature 24-26 °C overnight, 90% relative humidity, photoperiod day/night 14/10 h, light intensity 50 μmol·m^{-2}·s^{-1}. The cover was regularly opened once per day for 2 h. After the cover was removed, the plants were left for 60 days at above conditions with a difference that air humidity and temperature were reduced to 70% and 22-24 °C respectively. At day 60, the percentage of rooted explants was determined. Thereafter the plants were established in pots (diameter 9 cm) on the same substrate and grown in a greenhouse under the above conditions. For preventing rotting the plants were treated with 0.1% solution of Proplant 722 SL (Chimac Agriphar S.A., Belgium). Survival rate of acclimated plants was determined 60 days after potting.

Data analysis

Data were subjected to one-way analysis of variance (ANOVA) and Duncan's multiple range test at probability level $p < 0.05$ (SPSS, IBM) was used to separate treatment means. The presented values are means of all experiments ± SEM (n–1).

RESULTS

Effect of BAP and NAA on regeneration of primary explants

On hormone-free MS medium (control), *M.* × *soulangeana* 'Alexandrina' expressed higher regeneration rate than *M. liliiflora* 'Nigra'. Same trend of differences between the genotypes was pronounced in presence of 0.25 and 0.5 mg·dm^{-3} BAP and the combination of these BAP concentrations with 0.25 mg·dm^{-3} NAA. The highest number (approximately 30 and 40% over hormone-free medium) of buds from both genotypes regenerated in presence of 0.25 mg·dm^{-3} BAP alone (Table 2). In comparison to hormone-free medium, the addition of 0.5 mg·dm^{-3} BAP + NAA stimulated the regeneration of *M.* × *soulangeana* whereas for *M. liliiflora* this composition did not promote satisfactory effect. At amounts of BAP higher than 0.25 mg·dm^{-3} and in presence of NAA the quality of explants of both studied genotypes was reduced due to occurrence of leaf deformations, blackening, callus formation, shortening of the shoots and symptoms of hyperhydricity. NAA alone entirely suppressed the regeneration and resulted in mass callus formation (not subjected to a statistical calculation). BAP at 1.0 mg·dm^{-3} + NAA did not cause an increase of regeneration rate over the control. The observations determined 0.25 mg·dm^{-3} BAP as most efficient for stimulating the regeneration of the used primary explants of both cultivars (Table 2).

Table 2. Effect of BAP and NAA on *in vitro* regeneration of primary explants of *Magnolia* sp.

MS basal salts mixture BAP and NAA in mg·dm^{-3}	Regeneration (%)	
	M. × *soulangeana* 'Alexandrina'	*M. liliiflora* 'Nigra'
Control (growth regulator-free)	45.0±1.6b	35.2±1.7b
0.25 BAP	75.0±5.0e	60.0±2.3e
0.5 BAP	55.4±1.9c	43.3±1.3c
1.0 BAP	30.3±3.2a	33.2±2.0b
0.25 BAP+0.25 NAA	60±1.2d	53.3±1.2d
0.5 BAP+0.25 NAA	50.2±1.9c	30.1±2.6b
1.0 BAP+0.25 NAA	25.4±2.7a	30.2±4.9b

Data are means ± SEM (n-1). Values with different letters in the same column are significantly different from each other at $p < 0.05$.

Effect of basal salts media on growth and development in multiplication stage

For *M.* × *soulangeana* 'Alexandrina' the largest number of shoots developed when the explants

were grown on VM and DKW media, exceeding the control MS with 44 and 28%, respectively (Table 3, Fig. 1d & e). Lower number of shoots was recorded on media B5, RM, WPM and WV3 (Table 3). The effect of OM was commensurable to MS. For both genotypes cultured on VM basal salts, the number of axillary shoots exceeded MS with 30% (Fig. 1d). For *M. liliiflora* 'Nigra' DKW and OM-promoted shoot formation to extent similar to MS whereas on B5, WPM and WV3 media the shoots were 15-25% less than on MS (Table 4). On RM basal salts the number of shoots of both cv. 'Alexandrina' and cv. 'Nigra' was reduced (approximately 36% less in comparison to MS). The effect of RM on shoot number of *M. soulangeana* is illustrated on Fig. 1d.

M. soulangeana 'Alexandrina' formed more elongated shoots in comparison to *M. liliiflora* 'Nigra' (Fig. 1e & f). For both genotypes the longest shoots developed on VM, approximately 35–36% over MS (Tables 3 & 4, Fig. 1d). Shoot elongation on RM, B5 and WV3 media was suppressed up to 50% in comparison to MS (Tables 3 & 4, Fig. 1d). WPM and DKW media appeared not to stimulate the elongation. In comparison to MS, on WV3 and RM 31–37% inhibition of shoot growth of both genotypes was observed. On OM the shoot length of *M.* × *soulangeana* was similar to MS (Table 3) whereas for *M. liliiflora* it slightly exceeded the control (Table 4).

The number of leaves developed on both *Magnolia* genotypes was most positively influenced by VM basal salts – approximately 35% over MS and did not substantially differ between the genotypes. For explants cultured on DKW the leaf number was similar to MS. RM, B5 and WV3 media did not stimulate leaf development – the leaf number was 25-50% of that on MS medium (Tables 3 & 4, Fig. 1d). For cv. 'Nigra' slight stimulation of leaf growth over the control was observed on OM whereas for cv. 'Alexandrina' this medium appeared to inhibit the leaf development (Tables 3 & 4). Comparative evaluation of the species response, especially to MS and VM revealed that *M.* × *soulangeana* formed lower number but longer shoots than *M. liliiflora* (Tables 3 & 4, Fig. 1d, e & f).

These results showed that VM basal salts most strongly affected the shoot and leaf growth and shoot elongation of both studied magnolia genotypes. The data also pointed to genotype specificity of the response to basal medium composition.

Table 3. Effect of basal salts media on growth and development in the multiplication phase of *in vitro* cultured *M.* × *soulangeana* 'Alexandrina'

Basal salts medium	Shoot number	Length of longest shoot (mm)	Leaf number
MS	2.9±0.1b	32.9±0.6d	13.7±0.4d
RM	1.9±0.1a	13.9±0.4a	7.4±0.3a
B5	1.7±0.1a	18.3±0.7b	6.8±0.2a
VM	4.2±0.2d	44.3±0.8e	18.4±0.5e
WPM	2.1±0.1a	241±0.7c	9.2±0.3b
DKW	3.7±0.2c	25.7±0.4c	13.1±0.4d
OM	2.5±0.1b	34.5±0.8d	11.1±0.4c
WV3	1.9±0.1a	20.3±0.7b	6.5±0.3a

For media full names, please, refer to Table 1. Data are means ± SEM (n-1). Values with different letters in the same column are significantly different from each other at $p < 0.05$.

Table 4. Effect of basal salts media on growth and development in the multiplication phase of *in vitro* cultured *M. liliiflora* 'Nigra'

Basal salts medium	Shoot number	Length of longest shoot (mm)	Leaf number
MS	4.6±0.1c	18.6±0.3c	12.7±0.2c
RM	3.0±0.1a	12.9±0.3a	9.5±0.2b
B5	3.9±0.1b	16.3±0.24b	9.6±0.21b
VM	6.1±0.2d	25.4±0.4e	17.2±0.3e
WPM	4.0±0.1b	17.4±0.2c	10.9±0.3b
DKW	4.5±0.2c	17.4±0.2c	12.3±0.2c
OM	4.4±0.1c	22.7±0.3d	13.7±0.3d
WV3	3.5±0.1b	11.8±0.4a	6.6±0.2a

For media full names, please, refer to Table 1. Data are means ± SEM (n-1). Values with different letters in the same column are significantly different from each other at $p < 0.05$.

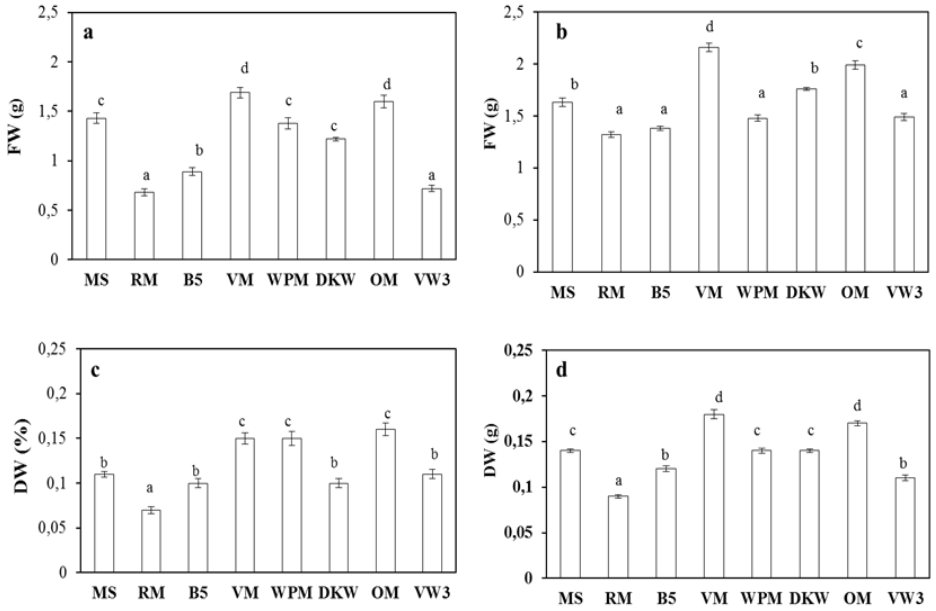

Fig. 2. Effect of basal salts media on fresh (FW) and dry (DW) weight of *in vitro* cultured *M.* × *soulangeana* 'Alexandrina' and *M. liliiflora* 'Nigra'

a. FW of *M.* × *soulangeana*; **b**. FW of *M. liliiflora*; **c**. DW of *M.* × *soulangeana*; **d**. DW of *M. liliiflora*.
For media full names, please, refer to Table 1. Bars represent the mean values. Error bars indicate ± SEM (n–1). Significant differences at $p < 0.05$ are indicated with different letters. FW, fresh weight; DW, dry weight

Effect of basal media on FW and DW

Highest FW of the explants from *M.* × *soulangeana* 'Alexandrina' was recorded at culturing on VM and OM media (18% and 11% more than on MS). Similar FW to that of explants propagated on MS was obtained on WPM and DKW. Media RM, B5 and WV3 did not potentiate the accumulation of FW which remained up to approximately 50% from that in the control (Fig. 2a). For *M. liliiflora* 'Nigra', FW of the explants was increased on VM, exceeding the MS control with 32% and on OM with app. 22%. DKW medium exerted same effect as MS. More stimulating influence of OM on FW of *M. liliiflora* 'Nigra' as compared to *M.* × *soulangeana* 'Alexandrina' was observed. The explants of *M. liliiflora* 'Nigra' grown on RM, B5, WPM and WV3 had significantly lower FW than those grown on MS (Fig. 2b).

An increase of DW was established when *M.* × *soulangeana* 'Alexandrina' explants were cultured on OM, VM and WPM media; DW of explants grown on DKW, B5 and WV3 did not differ from MS; the explants propagated on RM accumulated significantly less dry mass than the explants on MS (Fig. 2c). Increased DW of *M. liliiflora* 'Nigra' was measured on VM and OM, respectively, 27% and 20% more than the control. On WPM and DKW media DW was commensurable to MS. On RM, B5 and WV3 a decrease of DW was detected (Fig. 2d).

These results indicated that in reference to MS, VM and OM media stimulated the accumulation of fresh and dry mass of the explants from both genotypes whereas the other tested media provided none or inhibitory effect.

***In vitro* rooting and *ex vitro* acclimatisation**

The first roots appeared after 20 days. Sixty days after the transfer, 84% of *M.* × *soulangeana* 'Alexandrina' and 98% of *M. liliiflora* 'Nigra' formed well-microshoots developed roots (Fig. 1g). No difference in the rooting potential of explants derived from different basal media was observed. From *in vitro* rooted plants of both genotypes 97% were successfully acclimatised and further established in pots in greenhouse conditions (Fig. 1h & i).

DISCUSSION

Regeneration response of primary explants is genotype specific and is modified by BAP and NAA

Studies on the effects of PGRs on proliferation and rooting of *in vitro* cultured magnolias have pointed to genotype-specific requirements (Biedermann 1987; Gabryszewska 1997; Kamenická & Valka 1997; Kamenická 1998; Kamenická & Lanaková 2000; Podwyszyńska et al. 2000; Kamenická et al. 2001; Zefang et al. 2003; Marinescu 2008; Radomir & Radu 2008; Parris et al. 2012). However, few works have addressed the genotype specificity of the performance of primary explants in initiation stage. Radomir (2012) has found that independently on the type, concentration and ratio of PGRs in introductory medium, dormant axillary buds of *M. stellata* expressed higher regeneration rate in comparison to *M.* × *soulangeana*. Our observations showed that the regeneration potential of primary explants of *M. soulangeana* and *M. liliiflora* also differed between the genotypes: cv. 'Alexandrina' produced more regenerated buds than cv. 'Nigra'. The response was modified by BAP and NAA with the highest regeneration rate in the presence of 0.25 mg·dm^{-3} BAP. NAA induced undesirable callus formation. The established effects of these two hormones are to some extent in line with the findings of Nakamura et al. (1995). These authors tested various concentrations of BAP, NAA and IBA supplemented to ¼ N – ½ MS used for establishing of primary culture of shoot apex of *M. obovata*. They reported that 1-5 µmol BAP (0.25-1.25 mg·dm^{-3}) in combination with 0.1 µmol IBA (approximately 0.02 mg·dm^{-3}) most efficiently stimulated the shoot elongation after bud flushing whereas BAP alone induced bud flushing but not shoot elongation. Higher concentrations of 10 µmol BAP (2.5 mg·dm^{-3}) caused browning. Additionally, they showed that NAA even at low concentrations caused browning of the explants and callus formation. We found that in the initiation stage of culturing only 0.25 mg·dm^{-3} BAP (1 µmol) is sufficient for promoting the formation of stems and leaves from nodal buds of both magnolia genotypes.

Composition of basal salts medium affects growth performance of explants during proliferation stage

In an effort to elaborate improved protocols for establishing cultures with high potential for *in vitro* proliferation, we have tested the physiological effects of several basal salts media including such that have not been earlier applied to *Magnolia* sp. In previously reported works, some of the effects of basal salts mixtures MS, WPM, DKW and RM on *in vitro* performance of magnolia have been described (Biedermann 1987; Kamenická & Lanaková 2000; Parris et al. 2012; Radomir 2012), but to the best of our knowledge, in the reported study, VM, OM, B5 and WV3 were introduced for the first time. Kamenická & Lanaková (2000) have observed morphological differences of microplants grown on WPM and S-medium. On WPM, the leaves were smaller and the stems shorter with increased number of nodes; on S- medium more shoots with elongated internodes were formed and the leaves appeared larger and more succulent. By comparing MS and DKW media Parris et al. (2012) showed that the highest number of shoots developed on full strength MS whereas DKW-simulated shoot elongation; fresh and dry weights of explants increased at culturing on ½ MS.

The studies have also indicated that various basal salts media can be successfully administrated for micropropagation of species taxonomically distant from the ones for which the medium has been originally elaborated (de Oliveira et al. 2011; Đurković 2003; Fernándes-Lorenzo et al. 2000; Fett-Neto et al. 1993; Harada & Murai 1996; Orlikowska & Gabryszewska 1995; Phelan et al. 2009; Shanjani 2003; Zhang et al. 2000). Therefore, we hypothesize that media non-traditional for magnolia could be appropriate and might improve the regeneration performance of *in vitro* grown magnolia.

It has been shown that independently on basal medium composition, for efficient proliferation of *Magnolia* sp. cytokinins and auxins are strongly required (Biedermann 1987, Kamenická & Lanaková 2000; Parris et al. 2012; Radomir 2012). We have also established that when the media were not supplemented with BAP and NAA the regeneration in multiplication stage was prevented (data not

shown). An interesting observation was that whereas in initiation stage only 0.25 mg·dm^{-3} BAP was sufficient for the growth of primary explants, in multiplication stage higher concentration of BAP and compulsory the addition of NAA were required. In order to distinguish the influence of basal medium by possibly ignoring the effects exerted by exogenously added PGRs, the media were supplemented with same concentrations of BAP and NAA.

MS basal salts medium has been commonly used for *in vitro* propagation of various magnolia genotypes (Biedermann 1987; Kamenická & Lanaková 2000; Podwyszyńska et al. 2000; Parris et al. 2012; Radomir 2012). For this reason, in our experiments MS was selected as control basal medium. Comparative assessment of magnolia growth response to MS, DKW, WPM, Blaydes medium and RM has shown highest efficiency of MS (Biedermann 1987; Parris et al. 2012). In line with these finding, our data also show satisfactory proliferation rate on MS. In addition, we determined an advantage of MS over WPM and RM and over the non-traditional for magnolia B5 and WV3 media.

VM basal salts most substantially stimulated the growth and development of both *M.* × *soulangeana* 'Alexandrina' and *M. liliiflora* 'Nigra' including the formation of axillary shoots and leaves, shoot elongation and augmentation of FW and DW. On MS, multiplication rate and FW and DW were in general 30% lower than at culturing on VM. VM is routinely used for micropropagation of grape varieties (Chée & Pool 1987; Gray & Benton 1991; Dutt et al. 2007) but is also reported as appropriate for *in vitro* culturing of plum, apricot and other fruit trees (Burgos & Ledbetter 1993; Emershad & Ramming 1994). Here we demonstrate that VM can be administrated for micropropagation of *M.* × *soulangeana* 'Alexandrina' and *M. liliiflora* 'Nigra'. The obtained results give us a reason to propose VM as potential novel composition of nutrient medium for in vitro multiplication stage of magnolia. In our laboratory, evaluation of the applicability of VM to *in vitro* cultures of other magnolia genotypes is in progress.

DKW is known for *in vitro* propagation of walnuts, ash, elm, hazel bush, olive tree and hornbeam (Driver & Kuniyuki 1984; Chalupa 1990; Hammatt & Ridout 1992; Fenning et al. 1993; Yu & Reed 1993; Tang et al. 2000; Santos et al. 2003). According to Parris et al. (2012), DKW was less effective than MS for *in vitro* propagation of *M.* 'Ann'. Our data indicated that for both studied genotypes, DKW promoted shoot and leaf formation and accumulation of FW and DW to extent commensurable to MS whereas the shoot elongation of *M.* × *soulangeana* was suppressed. OM basal salts have been initially developed for micropropagation of olive tree (Rugini 1984; Rugini & Caricato 1995). In our experiments it was for the first time tested for magnolia culturing. We have established that OM positively affected the growth and development in similar to MS manner. Moreover, on OM, FW and DW of explants were comparable to VM.

Basal media RM, WV3, WPM and B5 appeared inappropriate for studied genotypes. Our results for WPM and RM substantiate the literature data that these basal mixtures are not advantageous over MS for other magnolia cultivars (Biedermann 1987; Kamenická & Lanaková 2000; Parris et al. 2012). B5 has been elaborated for suspension cultures of soybean root cells (Gamborg et al. 1968) and WV3 has been elaborated for *in vitro* culturing of loblolly pine (Coke 1996). No information for testing B5 and WV3 media on magnolia could be found. Our data indicate that they are inefficient for improving the *in vitro* proliferation of studied genotypes.

The presented results clearly demonstrate that the composition of basal salts medium is an important factor affecting the physiological performance of *in vitro* cultured magnolia. The applied media differ in the type of salts and concentrations of macro- and microelements, and vitamins. Limited studies are available on the influence of individual macro- and microelements and vitamins on micropropagation of *Magnolia* sp. Biedermann (1987) has established that 120 mg·dm^{-3} KNO_3 added to MS-promoted optimum rate of multiplication of *M.* 'Yellow Bird' and *M.* 'Cultivar #149' whereas 240-480 mg·dm^{-3} were necessary for *M.* 'Elizabeth' and 480 mg·dm^{-3} KNO_3 were required for *M. stellata*. Higher amount of 1900 mg·dm^{-3} KNO_3 has been found toxic. Analysis of accumulation of K, Ca, Na, Zn, Mn, Fe and Cu in *M.* × *soulangeana* explants cultured on S-medium

have shown that the content of these elements dynamically changes in the course of explants development. Essential role of Ca and K for growth stimulation has been suggested (Valova et al. 1996). In addition to Murashige & Skoog vitamins, other compositions such as Linsmaier & Skoog vitamins (Linsmaier & Skoog 1965), Miller vitamins (Miller 1978) have been used in conjunction with MS or RM basal salts (Beidermann 1987; Radomir 2012) but the specific effects have not been described.

Our findings provide novel information on magnolia growth response to non-traditional for these species basal salts and pose further questions on the impact of individual components of basal media, their uptake and utilization in plant tissues.

In vitro rooting and acclimatization *ex vitro*

Our data showed that on MS with 2 $mg \cdot dm^{-3}$ NAA the rooting capacity of explants derived from cultures propagated on media containing MS, VM, DKW or OM basal salts did not differ. This suggests that the rooting potential of explants was probably not affected by basal salts composition. During acclimatisation stage and after establishment in pots, a large proportion of *in vitro* derived rooted microplants survived and grew well. This demonstrates that the suggested novel nutrient media are applicable for producing high-quality planting material of the studied magnolia genotypes.

The reported findings expand the information on the physiological response of studied magnolia genotypes to *in vitro* culture conditions and contribute to elaboration of protocols for establishment of highly productive tissue cultures.

CONCLUSIONS

In summary, the results obtained from this study show that:

1. Regeneration response of *M.* × *soulangeana* 'Alexandrina' and *M. liliiflora* 'Nigra' to *in vitro* culture conditions in initiation and multiplication stages occurs in genotype-specific manner.
2. The physiological performance of studied magnolia species was affected by basal salts media composition.
3. The non-traditional for magnolia basal medium VM substantially improved the growth and development resulting in increased proliferation rate and overall better quality of the microplants in comparison to conventionally used MS basal salts. Therefore VM can be proposed as efficient component of proliferation medium.
4. DKW and OM basal salts exerted similar effects as MS and are also promising for introduction in multiplication stage.

Acknowledgement

This work was partially supported by PhD project 'Application of biotechnological methods for micro-propagation of valuable ornamental trees", Ministry of Education, Youth and Science of Bulgaria and project No. 88 "Investigation of adaptive potential and application of advanced scientific approach for improving the performance of ornamental plants", Agricultural Academy, Bulgaria.

REFERENCES

Anderson W.C. 1980. Tissue culture propagation of red and black raspberries, *Rubus idaeus* and *R. occidentalis*. Acta Hort. 112: 13-20. http://www.actahort.org/books/112/112_1.htm.

Biedermann I.E.G. 1987. Factors affecting establishment and development of *Magnolia* hybrids *in vitro*. Acta Hort. 212: 625-629. http://www.actahort.org/books/212/212_104.htm.

Blaydes O.F. 1966. Interaction of kinetin and various inhibitors in the growth of soybean tissue. Physiol. Plant. 19: 748-753. DOI: 10.1111/j.1399-3054.1966.tb07060.x.

Burgos L., Ledbetter C.A. 1993. Improved efficiency in apricot breeding: Effects of embryo development and nutrient media on *in vitro* germination and seedling establishment. Plant Cell Tiss. Org. 35: 217-222. DOI: 10.1007/BF00037273.

Chalupa V. 1990. Micropropagation of hornbeam (*Carpinus betulus* L.) and ash (*Fraxinus excelsior* L.). Biol. Plant. 32: 332-338. DOI: 10.1007/BF02898493.

Callaway D.J. 1994. The world of Magnolias. Timber Press, Portland, Oregon, USA, 260 p.

Chée R., Pool R.M. 1987. Improved inorganic media constituents for *in vitro* shoot multiplication of *Vitis vinifera*. Sci. Hort. 32: 85-95. http://dx.doi.org/10.1016/0304-4238(87)90019-7.

Coke J.E. 1996. Basal nutrient medium for *in vitro* cultures of loblolly pines. US Patent: 5, 534, 434.

de Oliveira L.F., Ribas L.L.F., Quoirin M., Koehler H.S., Higa A.R. 2011. Micropropagation of *Pinus taeda* L. via axillary buds. BMC Proc. 5 (Suppl. 7): 144. DOI: 10.1186/1753-6561-5-S7-P144.

De Proft M.P., Maene L.J., Debergh P.C. 1985. Carbon dioxide and ethylene evolution in the culture atmosphere of *Magnolia* cultured *in vitro*. Physiol. Plant. 65: 375-379. DOI: 10.1111/j.1399-3054.1985.tb08660.x.

Driver J.A., Kuniyuki A.H. 1984. *In vitro* propagation of Paradox walnut rootstock [*Juglans hindsii* × *Juglans regia*, tissue culture]. HortScience 19: 507-509.

Ďurković J. 2003. Regeneration of *Acer caudatifolium* Hayata plantlets from juvenile explants. Plant Cell Rep. 21: 1060-1064. DOI: 10.1007/s00299-003-0634-5.

Dutt M., Li Z.T., Dhekney S.A., Gray D.J. 2007. Transgenic plants from shoot apical meristems of *Vitis vinifera* L. "Thompson Seedless" via Agrobacterium-mediated transformation. Plant Cell Rep. 26: 2101-2110. DOI: 10.1007/s00299-007-0424-6.

Emershad R.L., Ramming D.W. 1994. Effects of media on embryo enlargement, germination and plant development in early-ripening genotypes of *Prunus* grown *in vitro*. Plant Cell Tiss. Org. 37: 55-59. DOI: 10.1007/BF00048117.

Fenning T.M., Gartland K.M.A., Brasier C.M. 1993. Micropropagation and regeneration of English Elm, Ulmus procera. J. Exp. Bot. 44: 1211-1217. DOI: 10.1093/jxb/44.7.1211.

Fernándes-Lorenzo J.L., Iglesias-Díaz M.I., Gutiérrez-Araujo O. 2000. Micropropagation of a selected rootstock of *Acer palmatum*. Acta Hort. 536: 347-353. http://www.actahort.org/books/536/536_40.htm.

Fett-Neto A.G., Melanson S.J., Sakata K., DiCosmo F. 1993. Improved growth and taxol yield in developing calli of *Taxus cuspidata* by medium composition modification. Nat. Biotechnol. 11: 731-734. DOI: 10.1038/nbt0693-731.

Figlar R.B., Nooteboom H.P. 2004. Notes on Magnoliaceae IV. Blumea 49: 87-100. http://dx.doi.org/10.3767/000651904X486214.

Gabryszewska E. 1997. Wpływ tidiazuronu i cytokinin na wzrost I rozwój pędów *Magnolia* × *soulangeana* 'Alexandrina' *in vitro*. In: Dubert F., Skoczowski A. (Eds.), Zastosowanie kultur *in vitro* w fizjologii roślin. PAN, Kraków, pp. 79-82. [in Polish]

Gamborg O.L., Miller R.A., Ojima K. 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50: 151-158. http://dx.doi.org/10.1016/0014-4827(68)90403-5.

Gray D.J., Benton C.M. 1991. *In vitro* micropropagation and plant establishment of muscadine grape cultivars (*Vitis rotundifolia*). Plant Cell Tiss. Org. 27: 7-14. DOI: 10.1007/BF00048199.

Hammatt N., Ridout M.S. 1992. Micropropagation of common ash (*Fraxinus excelsior*). Plant Cell Tiss. Org. 31: 67-74. DOI: 10.1007/BF00043477.

Harada H., Murai Y. 1996. Micropropagation of *Prunus mume*. Plant Cell Tiss. Org. 46: 265-261.

Kamenická A. 1998. Influence of selected carbohydrates on rhizogenesis of shoots saucer magnolia *in vitro*. Acta Physiol. Plant. 20: 425-429. DOI: 10.1007/s11738-998-0030-4.

Kamenická A., Lanaková M. 2000. Effect of medium composition and type of vessel closure on axillary shoot production of magnolia *in vitro*. Acta Physiol. Plant. 22: 129-134. DOI: 10.1007/s11738-000-0067-5.

Kamenická A., Valka J. 1997. Cultivation and propagation of magnolias. Technical University Publishers, Zvolen, Slovakia, pp. 42-82.

Kamenická A., Kormuťák A., Lanaková M. 2001. Establishing micropropagation conditions for three Magnolia species. Propag. Ornam. Plants 1: 41-45.

Linsmaier E.M., Skoog F. 1965. Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 18: 100-127. DOI: 10.1111/j.1399-3054.1965.tb06874.x.

Lloyd G., McCown B. 1980. Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Comb. Proc. Int. Plant Prop. Soc. 30: 421-427.

Marinescu L., Radomir A.M., Radu T., Teodorescu A., Fleancu M., Popescu C. 2008. Preliminary results regarding the influence of cytokinin on the micropropagation of *Magnolia soulangiana* Soul. Lucrări Ştiinţifice, Seria B, Horticultură 51: 601-607.

Merkle S.A., Wiecko A.T. 1990. Somatic embryogenesis in three magnolia species. J. Amer. Soc. Hort. Sci. 115: 858-860.

Miller C.O. 1978. Cytokinin modification of metabolism of p-coumaric acid by a cell suspension of soybean (*Glycine max* (L.) Merrill). Planta 140: 193-199. DOI: 10.1007/BF00390248

Ming L., Huan-Cheng M.A. 2003. The review of the asexual propagation on Magnoliaceae. J. Southwest Forestry College 23: 92-96.

Murashige T., Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497. DOI: 10.1111/j.1399-3054.1962.tb08052.x.

Nakamura K., Wakita Y., Yokota S., Yoshizawa N., Idei T. 1995. Induction of multiple shoots by shoot apex culture in *Magnolia obovata* Thunb. Plant Tiss. Cult. Lett. 12(1): 34-40.

Orlikowska T., Gabryszewska E. 1995. *In vitro* propagation of *Acer rubrum* cv. Red Sunset. J. Fruit Ornam. Plant Res. 3: 195-204.

Parris J.K., Touchell D.H., Ranney T.G., Adelberg J. 2012. Basal salt composition, cytokinins, and phenolic binding agents influence *in vitro* growth and *ex vitro* establishment of Magnolia 'Ann'. HortScience 47: 1625-1629.

Phelan S., Hunter A., Douglas G.C. 2009. Effect of explants source on shoot proliferation and adventitious regeneration in 10 Buddleia. Sci. Hortic. 120: 518-524. http://dx.doi.org/10.1016/j.scienta.2008.11.009.

Podwyszyńska M., Wojtania A., Gabryszewska E. 2000. Application of m-topolin for plant micropropagation. Zesz. Nauk. Inst. Sadow. Kwiac. 7: 173-180. [in Polish with English abstract]

Quoirin M., Lepoivre E. 1977. Improved media for *in vitro* culture of Prunus sp. Acta Hort. 78: 437-442.

Radomir A.-M. 2012. Comparative study on the *in vitro* multiplication potential of *Magnolia stellata* and *Magnolia* × *soulangiana* species. J. Hort. Forest Biotechnol. 16: 39-44.

Radomir A.-M., Radu C.M. 2008. Research on behavior of *Magnolia soulangeana* in the multiplication stage of '*in vitro'* culture. Lucrări Ştiinţifice, Seria B, Horticultură 51: 258-261.

Rugini E.1984. *In vitro* propagation of some olive (*Olea europaea sativa* L.) cultivars with different rootability, and medium development using analytical data from developing shoots and embryos. Sci. Hortic. 24: 123-134. http://dx.doi.org/10.1016/0304-4238(84)90143-2.

Rugini E., Caricato G. 1995. Somatic embryogenesis and plant recovery from mature tissues of olive cultivars (*Olea europaea* L.) 'Canino' and 'Moraiolo'. Plant Cell Rep. 14: 257-260. DOI: 10.1007/BF00233645.

Santos C.V., Brito G., Pinto G., Fonseca H.M.A.C. 2003. *In vitro* plantlet regeneration of *Olea europaea* ssp. Maderensis. Sci. Hortic. 97: 83-87. http://dx.doi.org/10.1016/S0304-4238(02)00148-6.

Shanjani P.S. 2003. Nitrogen effect on callus induction and plant regeneration of *Juniperus excelsa*. Int. J. Agr. Biol. 5: 419-422.

Standardi A., Catalano F. 1985. Tissue culture propagation of kiwi fruit. Comb. Proc. Int. Plant Prop. Soc. 34: 236-243.

Tang H., Ren Z., Krczal G. 2000. Improvement of English walnut somatic embryo germination and conversion by desiccation treatments and plantlet development by lower medium salts. *In Vitro* Cell. Dev. – Pl. 36: 47-50. DOI: 10.1007/s11627-000-0011-9.

Tubesing Ch. E. 1998. Magnolias with a future: Propagation and nursery culture. In: Hunt D. (Ed.), Magnolias and their allies. International Dendrology Society, Milborne Port, pp. 193-200.

Valova M., Krajcova D., Kamenická A. 1996. Changes of mineral elements in explant of *Magnolia* × *soulangiana* Soul.-Bod. during the culturing *in vitro*. Folia Dendrol. 21-22: 331-339.

Yu X., Reed B.M. 1993. Improved shoot multiplication of mature hazelnut (*Corylus avellana* L.) *in vitro* using glucose as a carbon source. Plant Cell Rep. 12: 256-259. DOI: 10.1007/BF00237130.

Zefang T., Yahui H., Chao H. 2003. *In vitro* culture of *Magnolia grandiflora*. J. Hunan Agr. Univ. 29: 478-480.

Zhang C.H., Mei X.G., Liu L., Yu L.J. 2000. Enhanced paclitaxel production induced by the combination of elicitors in cell suspension cultures of *Taxus chinensis*. Biotechnol. Lett. 22: 1561-1564.