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EFFECTIVENESS OF *Daphne* L. (Thymelaeaceae) *IN VITRO* PROPAGATION, ROOTING OF MICROSHOOTS AND ACCLIMATIZATION OF PLANTS

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Abstract

In this study, an attempt was made to investigate *in vitro* morphogenetic competence of three shrub species from the Thymelaeaceae family. The studied plant material originated from Russia, Greece and China, and the effectiveness of *in vitro* shoot formation and rhizogenesis of *Daphne caucasica, D. jasminea*, and *D. tangutica* was verified. The multiplication coefficient was compared for different propagation media. Medium composed of WPM mineral salts, MS microelements and a set of vitamins, supplemented with 1.0 mg dm^{-3} 2iP, 0.1 mg dm^{-3} NAA, and 0.65 g dm^{-3} calcium gluconate, was appropriate for micropropagation of the tested genotypes. Shoot propagation in medium containing B_5 vitamins and microelements was not as effective as on WPM/MS medium. The rooting phase, especially in *D. tangutica,* needs further optimization in order to reduce the costs associated with acclimatization of microplantlets obtained to *in vivo* conditions. After stabilization, the plants were successfully cultivated under greenhouse conditions.

Key words: regeneration capacity, micropropagation, rooting efficacy, acclimatization, *Daphne* sp.

INTRODUCTION

Thymelaeaceae is a family of flowering plants of cosmopolitan distribution, distinguished in the eighteenth century by Antoine Laurent de Jussieu, which includes genera representing mostly shrubs or trees, and considerably less frequently vines and herbaceous plants. As far as the systematic position of the family is concerned, according to the APG classification Thymelaeaceae are assigned to Malvales, and currently several genera of economic importance are known within the family, among them numerous *Daphne* species (van der Bank, 2002; Herber, 2003). Such species are exploited in a diverse manner, for example in Japan and in Nepal particular species from the genus (*D. bhoula, D. papyracea*) have been used in writing paper production because of their exceptionally long fibres of secondary phloem. The fibres obtained from inner bark of *Daphne bhoula* are also utilized to prepare ropes. Hippocrates was the first to make use of *Daphne* extracts as a remedy, and up to now extracts have been continually made from some species. Especially *D. genkwa, D. guidum, D. laureola,* and *D. mezereum* are used as a valuable therapeutic agent (Wasicky, 1932; Volák and Stodola, 1992; Okunishi et al. 2001; Okunishi et al. 2002; Wu et al. 2009). Species and cultivars belonging to this genus are frequently used as valuable ornamental plants which are more and more willingly used in European green areas due to attractive foliage as well as the tint of flowers and fruits. Individual species represent an extensive colour range. The extraordinary fragrance of their flowers certainly increases their aesthetic value, thus, aromatherapy is yet another contemporary application of *Daphne* species. In spite of this, all parts of those excellent plants, and especially fruits, may be poisonous to mammals (Reichholf and Steinbach, 1995; Brickell and White, 2000; Latocha, 2006; Seneta and Dolatowski, 2009).

Daphne specimens can be multiplied by means of both generative and vegetative propagation. In nature melliferous flowers are pollinated by insects, mainly bumble-bees and butterflies. Seeds can be transmitted by birds (Alonso and Herrera, 2001; Seneta and Dolatowski, 2009). In nursery pro-

duction, it is preferred to obtain offspring vegetatively, even though in such a case efficient propagation is rather difficult. It is not easy to meet the requirements of young shoots being rooted as far as conditions of air temperature regime, relative humidity, acid or alkaline reaction of medium, moisture, and other characteristics are concerned, so usually the rooting phase have only limited capacity (Bärtels, 1982; Hrynkiewicz-Sudnik et al. 2001; Noshad et al. 2009). That is the main reason why attempts are made to obtain a significant increase in the multiplication coefficient via micropropagation technique (Malá and Bylinský, 2004; Noshad et al. 2009). For the above-mentioned reasons, the objective of the present experiments was to test the influence of medium mineral composition on the effectiveness of *Daphne jasminea*, *D. caucasica,* and *D. tangutica* multiplication and rooting in order to improve efficiency of *in vitro* technique used for propagation of this valuable plant material.

MATERIALS AND METHODS

To start *in vitro* experiments, buds taken from young *Daphne jasminea* Sibth. & Sm., *D. caucasica* Pall. and *D. tangutica* Maxim (Thymelaeaceae Juss.) plants growing in a greenhouse were used as primary explants. The studied plant material originated from Russia (*D. caucasica)*, Greece (*D. jasminea)*, and China (*D. tangutica*). Collected buds were surface sterilized with 0.1% v/v solution of mercuric chloride for one minute, several times rinsed repeatedly with sterile distilled water, and afterwards placed under aseptic conditions on 25 ml of solidified MS (Murashige and Skoog, 1962) medium poured to a 100 cm^3 Erlenmeyer flask. For evaluation of micropropagation abilities, apical explants, about 20 mm in length, were taken from the obtained shoot cultures of the respective genotype as starting material. Shoot explants were placed on medium consisting of macroelements from woody plant medium (WPM) (Lloyd and McCown, 1981), a mixture of micronutrients and vitamins, either from MS or B5 medium (Murashige and Skoog, 1962; Gamborg et al. 1968), supplemented with 20 g×dm^{-3} sucrose, 0.6 g×dm^{-3} activated charcoal, 0.65 g×dm^{-3} calcium gluconate, 1.0 mg×dm^{-3} 2-isopentenyladenine (2iP), 0.1 mg×dm^{-3} 1-naphthaleneacetic acid (NAA), and solidified with 8 g×dm^{-3} Difco Bacto agar. The pH of media was adjusted to 5.6. Culture flasks, 350 cm^3 capacity, containing 50 cm^3 of medium, were used in this stage of micropropagation. After two passages onto fresh medium of the same composition every four weeks, cultures were treated as stabilized, so from now on their regenerative potential was assessed during two subsequent passages. The multiplication coefficient was assessed at the end of each passage along with microscopic analysis of representative samples. Macroscopic observations were done every second day during the course of the whole experimental arrangement. The culture environment was maintained at 24°±C2°C, 80 mmol×m^{-2}×s^{-1} of PAR, photoperiod 16/8 h. As a light source, cool white fluorescent lamps were used. Afterwards, the shoots obtained were aseptically rooted in perlite moistened with liquid WPM medium diluted to 1/3 contents of macro- and microelements, additionally supplemented with different indole-3-butyric acid (IBA) concentrations (3-, and 6 mg dm^{-3} IBA, respectively). The number of regenerated roots, their length, and the frequency of rooted shoots were evaluated after ten weeks, that is, at the end of the rooting phase. The culture environment during the rooting phase was changed only as far as light intensity is concerned (30 µmol×m^{-2}×s^{-1}).

Following the rooting phase, the material obtained was acclimatized to *ex vitro* conditions. Initially, hardened rooted plantlets were transplanted to an autoclaved potting mixture of sand, perlite, peat moss and horticultural soil (1 : 1 : 1 : 1 v/v) and were watered for two weeks with WPM medium diluted to 1/3 content of macro- and microelements, and were kept in a controlled growth chamber under the same lighting regime as during micropropagation, at 22±4°C and 50% relative humidity. After stabilization, the plants were grown in an isolated greenhouse section and irrigated every two days.

In every replication of the multiplication phase, six shoot explants of *D. caucasica* and *D. tangutica*, as well as ten explants of *Daphne jasminea* were put into a single container. Sixty explants of each species were evaluated per experiment, and the experiments were repeated thrice. Afterwards, fifty shoots obtained in every experiment were rooted. The results were analyzed by one-factorial design, using ANOVA. Tukey's test was used to assess differences between the means with the significance level α=0.05.

RESULTS AND DISCUSSION

In vitro cultures of the studied genotypes were obtained. Following a four-week establishment period, buds transplanted into fresh medium of the same composition gradually developed into shoots, which served as secondary explants to set up the first stage of micropropagation experiments. Shoots explanted from stabilized cultures were placed vertically onto medium designed for propagation assay. During two successive 28-day-long passages on modified WPM medium containing 2 % sucrose, 1.0 mg×dm^{-3} 2iP, 0.1 mg×dm^{-3} NAA, and 0.8% agar, shoot cultures were vital and produced adventitious buds, which showed a satisfactory rate of

development (Fig. 1A-C). In general, irrespective of the genotype evaluated, in the treatment with the MS mixture of micronutrients and vitamins we obtained a higher multiplication coefficient (MC), that is, from 1.5 in *D. caucasica* to 2.8 in *D. jasminea*, whereas the treatment with the mixture of micronutrients and vitamins from B_5 medium proved to be less efficient, especially in the case of *Daphne caucasica* (MC – 1.4) (Table 1). Nevertheless, in every genotype tested, we obtained numerous shoots suitable to be used without the elongation stage in the rooting phase of micropropagation (Fig. 1D,E). Thus, both medium and physical conditions used in the course of the experiment proved to be appropriate to multiply the tested *Daphne* species. Under the experimental conditions, *Daphne jasminea* demonstrated the highest regenerative potential, while *Daphne caucasica* the lowest. In sum, the multiplication phase protocol was elaborated, even if it is still possible to increase its efficiency. The results obtained during the rooting phase are presented in Table 2. Early rhizogenesis was recognized by the emergence of small protuberances on the shoot basis submerged in the rooting medium. In this respect, we did not obtain unequivocally satisfactory results for all tested genotypes when they were recorded after seventy days of culture on rooting medium. In the control treatment, the percentage of rooted shoots reached thirty three percent in *Daphne caucasica* and *Daphne jasminea*. The addition of 6 mg×dm^{-3} IBA resulted in developing adventitious roots about 37 millimetres long in over 80% of *D. caucasica* shoots verified. Cultures of *Daphne tangutica* were more recalcitrant to the rhizogenic stimuli, but we obtained still a rather satisfactory rooting percentage, reaching more than 50% at the higher dose of IBA (6 mg×dm^{-3}).

After potting, all plantlets kept in the controlled growth chamber, under the same lighting regime as during the micropropagation stage and watered with WPM medium, remained alive for at least two weeks of *ex vitro* growth. Following introductory stabilization, when the acclimatized material was cultivated under greenhouse conditions in the first growing season, the plants did not grow much in height. Notwithstanding this, *Daphne jasminea* specimens produced numerous new shoots. Eight weeks after transplanting *Daphne jasminea* plants showed a survival of 58%, whereas those of *Daphne caucasica* 63% (Fig. 2A, B).

By using suitable culture techniques, appropriate plant material can be multiplied *in vitro* with the aim of secondary metabolites production, and *in vitro* culture is applied more and more widely in both industry and health protection (Okunishi et al. 2001; Okunishi et al. 2002; Pietrosiuk and Furmanowa, 2006; Wu et al. 2009). On the other hand, to fulfill the demand for large scale commercial uses, induction of adventitious organogenesis on shoots developing in a large number from axillary buds of primary nodal explants is widely used. It is possible to obtain, in a few or several months depending on taxonomical affiliation, thousands of rooted microshoots commercially treated as seedlings. Micropropagation ought to ensure genotype stability of plant material (Bach and Pawłowska, 2009; Andrzejewska-Golec and Makowczyńska, 2010). Under the conditions of the present experiments, we obtained proliferative cultures of three species belonging to the *Daphne* genus (*D. caucasica, D. jasminea, D. tangutica*), but unfortunately we failed to improve distinctly the phase of shoot multiplication in comparison with the effects obtained by Noshad et al. (2009). The next objective was to verify whether the composition of proliferation medium influence rooting in *in vitro* conditions, and the rooting efficiency of isolated *Daphne* spp. microshoots was tested with routinely used auxin type and treatment. Nutrient salts in the medium influence the rooting percentage and root number per microshoot (Preece, 1995), so indirectly the mineral composition of medium determine the duration of the rooting period. The rooting phase of *D. caucasica, D. jasminea,* and especially of *D. tangutica*, needs to be significantly shorten, so it needs further optimization. Marks and Simpson (2000) underlined that a factor of great importance is the orientation of explants both in induction and in the rooting medium. Apart from the single auxin treatment, also other factors affecting *in vitro* rhizogenesis should be taken into consideration in the genus *Daphne*. These could be an interaction between sucrose and auxin concentration (Calamar and de Clerk, 2002), a considerable change in plant growth regulators used (Ronsch et al. 1993), or supplementing the medium with various bioactive compounds (Orlikowska, 1992; Hausman et al. 1994). Another point is that for the rooting experiment explants were excised from *in vitro* shoots cultured with cytokinin, without transfer into cytokinin-free medium, which possibly could be beneficial.

Nutrient effects are often modified by light. An increased level of endogenous auxin is thought to occur under reduced light, thus enhancing rooting, whereas much higher light levels usually contribute to a higher acclimatization percentage. Another area of concern is the study of plantlets obtained via standard micropropagation protocols and in the typical culture environment, which influences epicuticular wax production, functioning of stomata, photosynthetic effectiveness of plantlets, and sometimes even mesophyll anatomy (Pospišilova et al. 1999; Debnath, 2005; Siddique and Anis, 2008). Further studies concerning optimization of conditions for more efficient micropropagation, especially as far as rooting and acclimatization stages are concerned, will soon be undertaken.

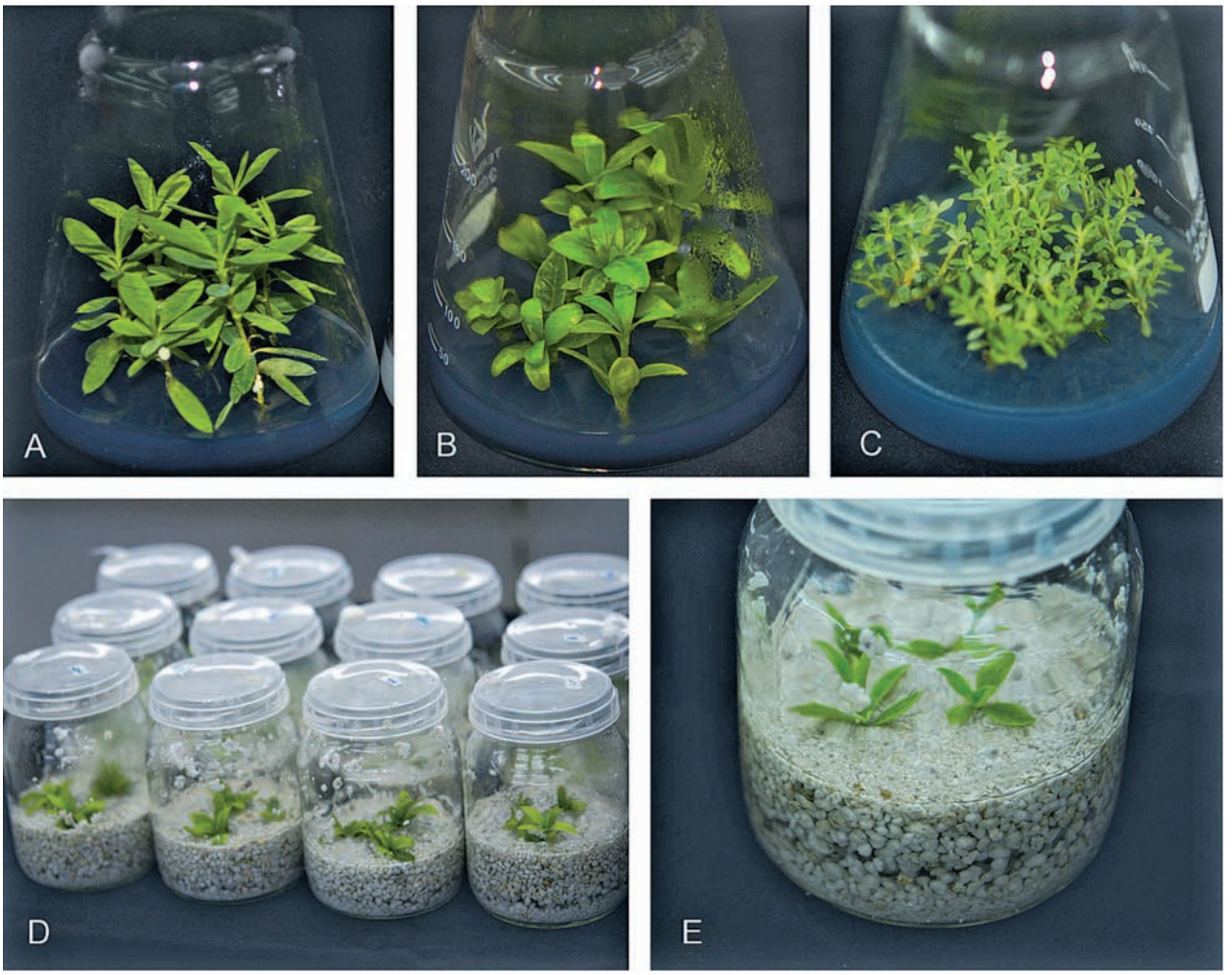

Fig. 1. Micropropagation of *Daphne* species. Shoot cultures of A) *D. caucasica*, B) *D. tangutica*, and C) *D. jasminea* on WPM medium supplemented with 1.0 mg×dm^{-3} 2iP and 0.1 mg×dm^{-3} NAA, D-E) rooting phase on 1/3 WPM medium supplemented with 3 and 6 mg×dm^{-3} IBA.

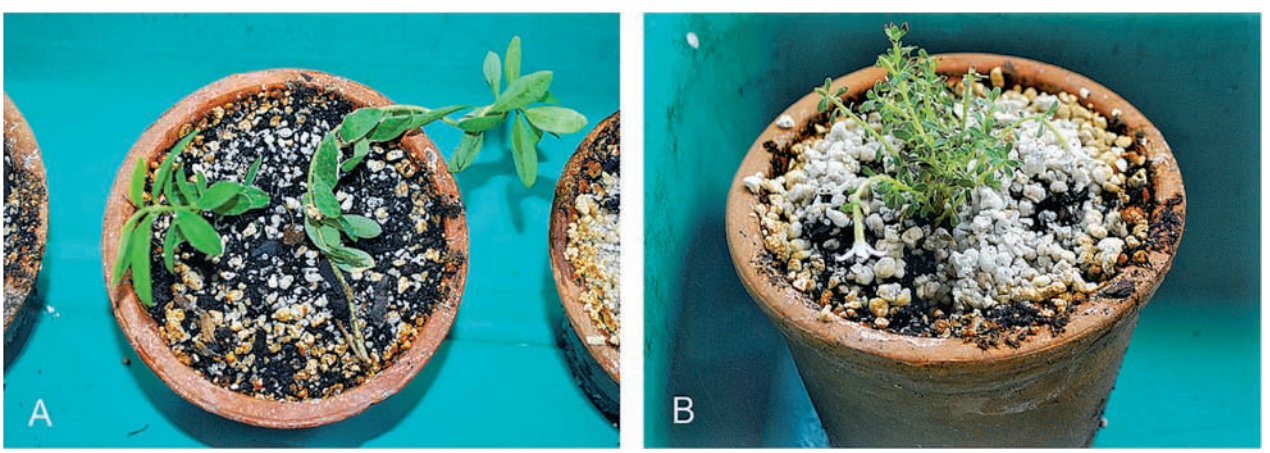

Fig. 2. Acclimatized plants of A) *D. caucasica* and B) *D. jasminea* after eight weeks of *ex vitro* growth.

Table 1.
Regenerative potential of *Daphne* shoot cultures on propagation medium[1] supplemented with 1.0 mg×dm^{-3} 2-isopentenyladenine, 0.1 mg×dm^{-3} 1-naphthaleneacetic acid

Species	Origin	Microelement & vitamin mixture	Number of shoots obtained [2]	Multiplication coefficient
Daphne caucasica	Russia	MS	90/270	1.5 ± 0.52[3] ab[4]
		B_5	84/252	1.4 ± 0.51 a
Daphne jasminea	Greece	MS	168/504	2.8 ± 1.22 d
		B_5	132/396	2.2 ± 0.78 bcd
Daphne tangutica	China	MS	138/414	2.3 ± 0.82 cd
		B_5	108/324	1.8 ± 0.63 abc

[1] according to Lloyd and McCown (1981)
[2] from 60/120 explants
[3] standard deviation
[4] values followed by the same letter are not significantly different for $p < 0.05$

Table 2.
Rooting efficiency of *Daphne* microshoots in perlite moistened with 1/3 WPM supplemented with 3 and 6 mg×dm^{-3} indole-3-butyric acid (IBA) doses

Species	Treatment	Number of regenerated roots[1]	Root length [mm]	Rooted shoots [%]
D. caucasica	Control – no IBA	0.33 ± 021[2] a[3]	16.6 ± 0.74 a	33.3
	3 mg dm^{-3} IBA	0.83 ± 030 a	30.8 ± 1.05 ab	66.7
	6 mg dm^{-3} IBA	2.33 ± 0,33 b	37.0 ± 0.52 ab	83.3
D. jasminea	Control – no IBA	0.83 ± 0,54 a	18.3 ± 0.85 a	33.3
	3 mg dm^{-3} IBA	0.84 ± 0,47 a	20.5 ± 0.95 a	50.0
	6 mg dm^{-3} IBA	1.33 ± 0.42 ab	61.6 ± 2.21 b	83.3
D. tangutica	Control – no IBA	0.33 ± 0,51 a	16.6 ± 1.05 a	66.7
	3 mg dm^{-3} IBA	0.50 ± 0.22 a	16.7 ± 0.84 a	50.0
	6 mg dm^{-3} IBA	0.83 ±0.31 a	25.5 ± 0.86 a	66.7

[1] from 50 explants
[2] standard deviation
[3] values followed by the same letter are not significantly different for $p < 0.05$

CONCLUSIONS

1. Micropropagation should be applied in production of valuable *Daphne* species which are recalcitrant during traditional vegetative propagation. Acceptable rooting and acclimatization conditions were elaborated for *Daphne caucasica* and *Daphne jasminea*.
2. Among numerous known *in vitro* techniques, adventitious organogenesis can be exploited in *Daphne* germplasm multiplication for commercial, medical and experimental usage.
3. The proposed techniques are recommended as an important method to protect natural local European and Asian populations by making use of tissue culture material for production of secondary metabolites.

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Efektywność rozmnażania *in vitro* *Daphne* L. (Thymelaeaceae), ukorzeniania mikrosadzonek i aklimatyzacji roślin

Streszczenie

Badano kompetencję morfogenetyczną trzech gatunków roślin krzewiastych z rodziny wawrzynkowatych. Materiał roślinny pochodził z Rosji, Grecji i Chin. Regenerowano *in vitro* pędy i korzenie przybyszowe u wawrzynka kaukaskiego (*Daphne caucasica*), wawrzynka jaśminowego (*D. jasminea*) oraz wawrzynka tanguckiego (*D. tangutica*). Pożywka składająca się z zestawu soli mineralnych dla roślin drzewiastych (WPM), mikroelementów i witamin według Murashige i Skooga (MS), wzbogacona o 1.0 mg×dm^{-3} 2iP, 0.1 mg×dm^{-3} NAA i 0.65 g×dm^{-3} glukonianu wapnia okazała się odpowiednia do mikrorozmnażania badanych genotypów. Namnażanie pędów na pożywce zawierającej zestaw witamin według Gamborga i współpracowników (B_5) było mniej efektywne niż na pożywce WPM/MS. Etap ukorzeniania, szczególnie dla wawrzynka tanguckiego, wymaga dalszej optymalizacji w celu zmniejszenia kosztów ponoszonych w trakcie uzyskiwania materiału przydatnego do uprawy. Otrzymane drogą rozmnażania *in vitro* rośliny przystosowano do uprawy w warunkach szklarniowych.