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Danuta Kulpa, Marcin Kubus

Micropropagation of *Calycanthus fertilis*

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Abstract: *Calycanthus fertilis* Walt. is a shrub belonging to the family *Calycanthaceae,* has great potential as ornamental. In the literature there are no reports on method propagation of this shrub in *in vitro* cultures. Therefore, the aim of this study was a development of the method micropropagation of *Calycanthus fertilis* Walter. Shoot explants of the size 1 cm, with an apex or node with lateral meristems were placed on the media with mineral composition according to MS and WPM supplemented with BAP (from 0.5 to 2.0 mg·dm^{-3}) and TDZ (from 0.1 to 0.5 mg·dm^{-3}). BAP turned out to significantly increase initiation frequency whereas TDZ inhibited the formation of adventitious shoots and caused explant death. The highest percentage of initiated explants were found in shoot fragments placed on WPM medium supplemented with 1.0 mg·dm^{-3} BAP. Primary explants which initiated growth were transferred on the proliferation media containing WPM macro- and microelement, with the addition of different cytokinin: BAP (from 0.5 to 2.5 mg·dm^{-3}), KIN (1.0 to 5.0 mg·dm^{-3}) or TDZ (from 0.1 to 0.5 mg·dm^{-3}). *Calycanthus* multiplication *in vitro* should be conducted on WPM media with 1.0 mg·dm^{-3} BAP. Proliferated shoots were placed on the WPM rooting medium supplemented with auxins: IBA, IAA or NAA at the concentration from 0.1 to 2.0 mg·dm^{-3}. Maximum rooting was obtained on WPM medium supplemented with 0.5 mg·dm^{-3} IBA. To sum up, it should be stated that an efficient method of micropropagation of *Calycanthus fertilis* Walt. has been developed.

Additional key words: *in vitro* cultures, multiplication, plant growth regulators

Address: D. Kulpa, Department of Genetics, Plant Breeding and Biotechnology, West Pomeranian University of Technology, Szczecin, Poland, e-mail: Danuta.Kulpa@zut.edu.pl

Introduction

Calycanthus fertilis Walt is a shrub belonging to the family *Calycanthaceae*, growing in natural habitat in the south-eastern USA (Chant 1978). The plants of the genus *Calycanthus* (4 species) are phylogenetically old and as far as certain traits are concerned, (e.g., the flower structure – there is no differentiation between corolla and calyx in perianth) they are very similar to the genus *Magnolia* (Renner 1999, Goremykin et al. 2003). The plant was introduced to Europe (Great Britain) in 1806, and to Poland, to the arboretum in Niedźwiedz in 1820 (Seneta 1996). The main decorative values of the shrub are its effective, abundant and long flowering, from June to August, under Polish climatic conditions as well as the change of leaf colour in autumn. Flowers are dark cherry or purple-brown, with or without a delicate fragrance (Phot. 1). According to Seneta (1996), a glabrous, mat and blue leaf underside is a good diagnostic trait of the species. Specimens growing in Poland undergo the full cycle of generative development and produce seeds with germination capacity.

However, this is not a very popular shrub in Poland, can be usually seen in botanic gardens, arboreta and is offered only by a few nurseries. Krüssmann (1985) says that this plant is often mistaken for *Calycanthus floridus* L which is less often cultivated and has poorer fruiting. In the arboretum in Glinna near Szczecin (north-western Poland) *Calycanthus fertilis*, from which the samples for this research were collected thrives. No frost damage was recorded during severe winter

2005/06, at absolute minimum temperature –26°C (Nowakowska and Baran 2007). Also, in the same winter, in the SGGW arboretum in Rogowo (central Poland), at extreme temperature –31°C, there was no frost damage in n *Calycanthus fertilis*, *C. fertilis* var. *laevigatus* (Willd.) Bean and *C. fertilis* 'Lusławice'. The above characteristics show that *Calycanthus fertilis* is a valuable ornamental shrub worth being recommended for planting around Poland.

The most popular methods of propagation are: sowing seeds, cuttings and layering (Krüssmann 1985). Their weak points are: the lack of permanent desired traits, very slow growth and a small number of obtained progeny plants, respectively.

An alternative to conventional methods of tree and shrub propagation is the method of cultures *in vitro*, which allows to obtain a large number of pathogen- and virus-free plantlets in a short time. In the literature there are no reports on this way of propagation of this shrub. Therefore, the aim of this study was a preliminary development of the method for shoot proliferation of *Calycanthus fertilis* Walter *in vitro*.

Material and methods

Culture initiation

Study material – this year shoots, were collected from four 28-year-old shrubs from Arboretum in Glinna near Szczecin (north-western Poland). The seeds from which the shrubs were propagated came from the Arboretum Borres from Nogent sur Vernisson (central France). The shoots were collected in April 2009.

The leaves were removed prior to soaking the shoots for 15 minutes in water with a detergent, at the temperature 40°C. Next, they were rinsed several times under running water and immersed for 30 secs in 70% ethanol solution. After initial disinfection they were shaken in 7.5% solution of natrium hypochlorite (NaClO) for 15 minutes.

Shoot fragments of the size 1 cm, with an apex or node with lateral meristems were used as primary explants for *in vitro* culture. They were placed on the media containing macro- and microelements according to MS (Murashige and Skoog, 1962) or WPM (Woody Plant Media, Lloyd and McCown, 1980) supplemented with thidiazuron – TDZ (from 0.1 to 0.5 $mg \cdot dm^{-3}$) or 6-Benzylaminopurine – BAP (from 0.5 to 2.0 $mg \cdot dm^{-3}$), 25 explants per each medium. At this stage and throughout the following ones the cultures were maintained in a phytotrone, at the temperature 24±1°C, light intensity 40 $mE \cdot m^{-2} \cdot s^{-1}$ and 16h photoperiod. Initiation stage lasted 8 weeks.

Table 1. Percent (%) of initiated explants of *Calycathus fertilis* depending on type of medium (WPM and MS) and its composition

Plant growth regulators [$mg \cdot dm^{-3}$]		Percentage of responding shoot apex explants	
		WPM	MS
control	0.0	8	4
TDZ	0.1	64	10
	0.2	80	14
	0.5	42	4
BAP	0.5	80	46
	1.0	92	38
	2.0	60	20

Multiplication stage

Primary explants which initiated growth were transferred on the proliferation media containing WPM macro- and microelement, with the addition of different cytokinin: BAP (from 0.5 to 2.5 $mg \cdot dm^{-3}$), kinetin – KIN (1.0 to 5.0 $mg \cdot dm^{-3}$) or TDZ (from 0.1 to 0.5 $mg \cdot dm^{-3}$), 25 explants per each medium. Multiplication stage lasted 6 weeks.

Rooting stage

Proliferated shoots were placed on the WPM rooting medium supplemented with auxins: indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) or 1-naphthaleneacetic acid (NAA) at the concentration from 0.1 to 2.0 $mg \cdot dm^{-3}$, 25 explants per each medium. Rooting stage lasted 6 weeks.

At all stages of experiments the media were supplemented with 8 $g \cdot dm^{-3}$ agar and 30 $g \cdot dm^{-3}$ sucrose and 100 $mg \cdot dm^{-3}$ inositol and their pH was adjusted to 5.7 using the solutions: 0.1M NaOH and HCl. After adding growth regulators, the media were autoclaved for 20 minutes at the temperature 121°C. The plants on MS media without growth regulators constituted control at all stages.

Statistical analysis

This experiment was established in one-factor completely randomised design. Significance of differences was determined by analysis of variance and the Tukey t-test at $P=0.05$. Homogenous groups between analysed combinations were labelled with successive letters of alphabet.

Results and discussion

Observed number of infections confirmed the effectiveness of chosen disinfection method – there were few infections – only 2%. Such low amount may be connected with appropriate time of explant collection – initiation was conducted in April, at the beginning of vegetation period and only very young, healthy, shoot fragments, were used.

In the case of herbaceous plants culture initiation *in vitro* usually takes place on the media with mineral content according to Murashige and Skoog (1962). In

Table 2. Mean values of morphological traits *Calycantus fertilis* propagated on WPM medium with different concentrations of cytokinins

Cytokinins [mg·dm^{-3}]		Plant height [cm]	Number of multiplied shoots	Plant weight[g]	Leaf number [cm]
BAP	0.5	3.13 c	2.34 ef	0.28 ab	8.4 de
	1.0	4.65 b	4.72 ab	0.37 ab	20.1 a
	2.5	1.42 e	5.43 a	0.32 ab	18.6 ab
KIN	1.0	4.32 b	1.45 f	0.18 b	11.6 cd
	2.5	6.53 a	2.47 de	0.31 ab	14.5 bc
	5.0	3.32 c	3.34 cd	0.24 b	10.9 cd
2iP	2,0	2.46 d	2.22 ef	0.19 b	8.9 de
	5,0	4.76 b	3.16 cde	0.24 b	10.9 cd
	7.5	3.56 c	3.99 bc	0.21 b	11.2 cd
control	0.0	2.43 d	1.15 g	0.19 b	6.2
Mean		3.66	3.03	0.25	12.13
NIR $_{0,05}$		0.56	0.89	0.11	5.3

the case of trees and shrubs mineral composition must be modified since MS medium contains too many macroelements. The medium with lower content of NH_4NO_3 and KNO_3 was used for the proliferation of avocado *Persea americana* Mill, belonging like *Calycanthus* to the order *Laurales,* by Witjaksono et al. (1999). The application of complete MS medium, in the case of avocado, caused the reduction of leaf size, numerous necroses and dying of apical meristem (Witjaksono 1991). In the studies on avocado, Barcelo-Mu oz (1999) used the medium containing microelements according to MS with macroelements reduced by half for the micropropagation of this species. We have found a negative effect of MS medium on the *Calycanthus fertilis* explants. On this medium, irrespective of the applied concentration of growth regulators, explants developed properly at the beginning but after 2 weeks necrotic changes on leaves and dying of growing tips were observed. Explants which were cultured on WPM developed properly.

The highest percentage of initiated explants were found in shoot fragments placed on WPM medium supplemented with 1.0 mg·dm^{-3} BAP. Application of 2.0 mg·dm^{-3} BAP or 0.5 mg·dm^{-3} TDZ resulted in the occurrence of hyperhydricity. This finding is in agreement with the studies of Huang et al. (1998), who initiating shoot cultures of *Cinnamomum camphora* discovered that 0.3 mg·dm^{-3} BAP added to initiation media brings about hyperhydricity and with the increase of the dose to 10 mg·dm^{-3} the frequency of the plants affected by the disease amounts even to 80%.

Explant multiplication is usually conducted on the media with cytokinin as the only PGR or together with auxins. Shao et al. (2006) multiplied the shoots of *Calycanthus chinensis* on MS medium supplemented both with 2.0 mg·dm^{-3} BAP and 0.1 mg·dm^{-3} NAA. As a result of this kind of proliferation the obtained rate of regeneration was 4.06. In our studies initiated explants multiplied most intensively on the media with the addition of 1.0 mg·dm^{-3} BAP, producing numerous new shoots and leaves (Table 2, Fig. 1). In addition, the shoot length (4.65) afforded to divide them into smaller fragments. The plants from this medium had healthy leaves without necrosis and discolouration, devoid of hyperhydricity. Explants from the medium with kinetin formed a smaller number of shoots and leaves. It is in line with the results presented by Babu et al. (2003) who multiplying *Cinnamomum camphora* on the WPM medium with cytokinin addition, found considerably lower usefulness of kinetin application at this stage.

Our studies show that shoots of *Calycanthus* easily forming a roots in *in vitro* culture. The root production was observed on all media used in the experiment (Table 3). The highest frequency was observed in the case of shoots placed on WPM media with 0.5 and 1.0 mg·dm^{-3} IBA – all explants produced long and numerous roots. Shi et al. (2010) rooted the shoots of *Cinnamomum camphora* on similar media – half MS medium with 0.5 mg·dm^{-3} IBA. The roots, formed on the media supplemented with IAA, were longer in comparison with those rooted on the media with IBA, but less abundant. The plant rooting frequency was also lower. In our studies the poorest rhizogenesis was noted in the case of plants rooted on the media with NAA – there was a low frequency of rooted plantlets (from 16 to 32%), a small number of rather short roots. At the same time, in all explants on the media supplemented with NAA, callus tissue, whose presence at this stage is not desired, was formed.

To sum up, it should be stated that an efficient method of micropropagation of *Calycanthus fertilis* Walt. has been developed. Micropropagation should be conducted on WPM media with the addition of 1.0 mg·dm^{-3} BAP, at the stage of initiation and proliferation, and – 0.5 mg·dm^{-3} IBA at the stage of rooting.

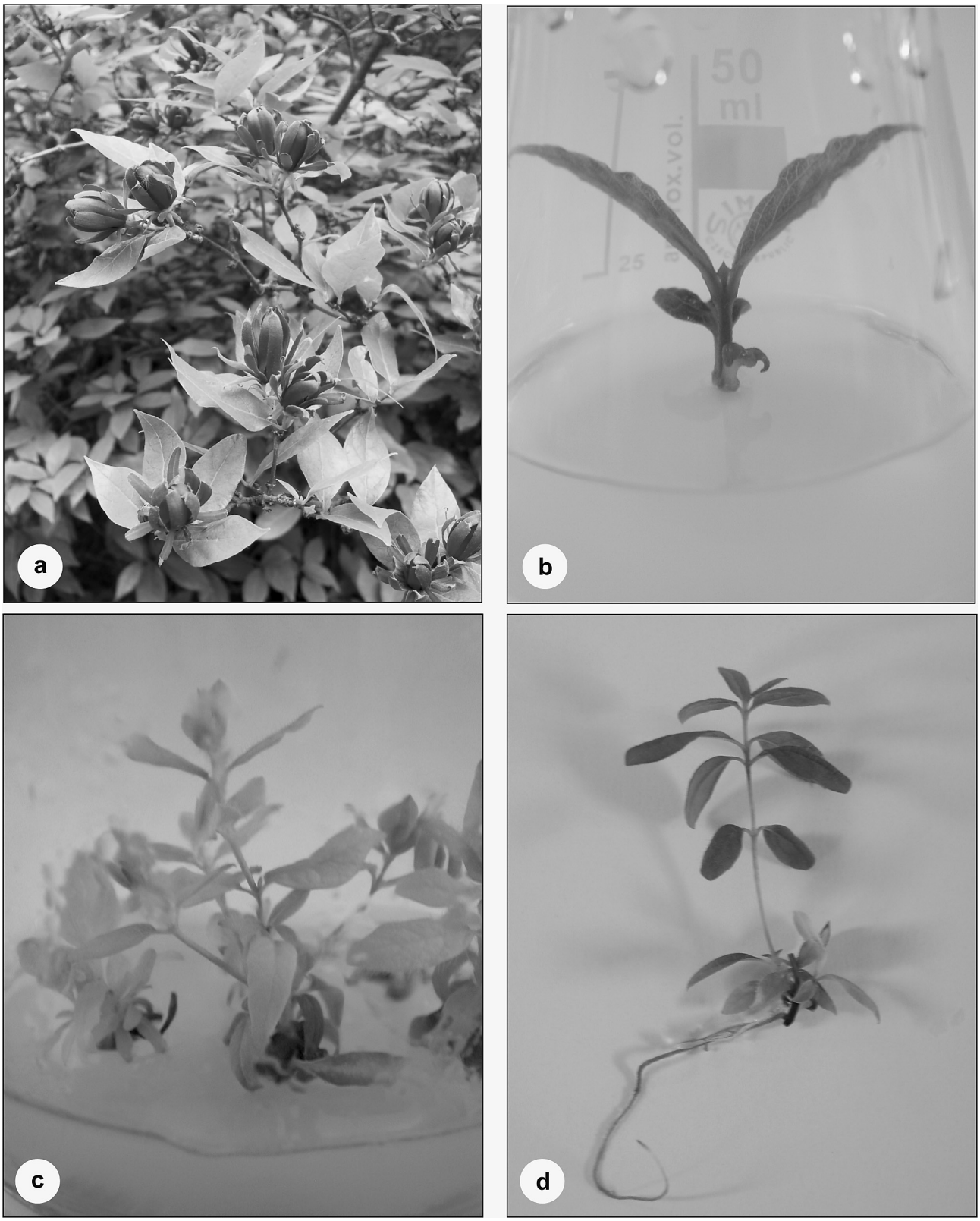

Fig. 1. Micropropagation of *Calycanthus fertilis* a) shrub from which shoots were collected at flowering b) apical meristem initiating growth on WPM medium with 1.0 BAP c) explant at proliferation stage on WPM medium with 1.0 mg·dm^{-3} BAP d) shoot rooted on the medium with 0.5 mg·dm^{-3} IBA

Table 3. Mean values of morphological traits of *Calycanthus fertilis* rooted on WPM medium with different concentrations of auxins

Auxins [mg·dm^{-3}]		% of plant rooted	Plant height [cm]	Roots length [cm]	Roots number	Plant weight [g]
IBA	0.1	76	1.21 f	3.21 a	1.23 e	0.11 bc
	0.5	100	2.21 e	4.15 a	3.15 a	0.12 bc
	1.0	100	2.34 e	2.98 ab	2.45 ab	0.09 bc
	2.0	84	3.52 bc	2.65 abc	1.98 bcd	0.23 a
IAA	0.1	60	1.80 f	2.15 cd	1.25 cde	0.18 ab
	0.5	90	3.21 cd	2.56 bc	1.36 cde	0.15 abc
	1.0	88	4.21 a	1.98 de	1.21 de	0.13 bc
	2.0	72	3.96 ab	1.99 de	1.14 e	0,24 a
NAA	0.1	16	2.04 e	1.65 de	2.15 bc	0.18 ab
	0.5	24	2.98 d	1.52 e	2.14 bc	0.09 bc
	1.0	32	2.65 de	1.62 de	2.65 ab	0.07 c
	2.0	16	3.15 cd	0.99 f	2.05 bcd	0.12 bc
Control	0.0	4	1.32 f	1.30 f	1.00 e	0.13 bc
Mean		59.3	2.66	2.21	1.83	0.14
NIR $_{0,05}$			0.65	0.58	0.83	0.09

a, b, ... – mean values followed by the same letters are not significantly different at $P = 0.05$

References

Babu K.N., Sajina A., Minoo D., John C.Z., Mini P.M., Tushar K.V., Rema J., Ravindran P.N. 2003. Micropropagation of camphor tree (*Cinnamomum camphora*). Plant Cell, Tissue and Organ Culture 74: 179–183.

Barcelo-Mu oz A., Encina C.L., Simon-Perez E., Pliego-Alfaro F. 1999. Micropropagation of adult avocado. Plant Cell, Tissue and Organ Culture 58: 11–17.

Chant S.R. 1978. *Calycanthaceae*. In: Heywood V. H (ed.) Flowering Plants of the World. Elsevier, Oxford, pp. 35–36.

Goremykin V., Hirsch-Ernst K.I., Wolfl S., Hellwig F.H. 2003. The chloroplast genome of the ''basal'' angiosperm *Calycanthus fertilis* – structural and phylogenetic analyses. Plant Systematics and Evolution 242: 119–135.

Huang L.-Ch., Huang B-L., Murashige T. 1998. A micropropagation protocol for C*innamomum camphora. In Vitro* Cellular & Developmental Biology – Plant 34:141–146.

Krüssmann G. 1985. Manual of Cultivated Conifers, 2nd ed. Timber Press, Portland, Oregon.

Lloyd GB., McCown B. 1980. Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Combined Proceedings – International Plant Propagators' Society 30: 421–427.

Murashige T., Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15: 473–479.

Nowakowska M., Baran J. 2007. Uszkodzenia mrozowe drzew i krzewów rosnących w Ogrodzie Dendrologicznym w Glinnej podczas zimy 2005/2006. Roczniki Dendrologiczne 55: 129–140.

Renner S.S. 1999. Circumscription and phylogeny of the *Laurales*: evidence from molecular and morphological data. American Journal of Botany 86: 1301–1315.

Seneta W. 1996. Drzewa i krzewy liściaste. Tom II Callicarpa-Cytisus. Wydawnictwo Naukowe PWN: 16–21.

Shao G., Cai R., Wang L., Liang G.-L. 2006. Study on Tissue Culture of *Calycanthus chinensis*. Journal of Zhejiang Forestry Science and Technology 2006: 05. http://en.cnki.com.cn/Article_en/CJFDTOTAL-ZJLK200605008.htm.

Shi X.P., Dai XG., Liu G.F., Zhang J.W., Ning G.G., Bao M.Z 2010. Cyclic secondary somatic embryogenesis and efficient plant regeneration in camphor tree (*Cinnamomum camphora* L.). *In Vitro* Cellular & Developmental Biology – Plant 46: 117–125.

Witjaksono, Schaffer B.A., Colls A.M., Litz R.E., Moon P.A. 1999. Avocado shoot culture, plantlet development and net CO_2 assimilation in an ambient and CO_2 enhanced environment. *In Vitro* Cellular & Developmental Biology – Plant 35: 238–244.

Witjaksono. 1991. Tissue culture medium for 'Pinkerton' avocado (*Persea Americana* Mill.). In: Proceedings of the Seminar and National Congress on Biology X, Bogor: Indonesian Biological Society and IUC Life Science, IPB; pp. 411–417.