BIOLOGIA PLANTARUM 48 (4): 633-636, 2004

BRIEF COMMUNICATION

Micropropagation of endangered species *Daphne cneorum*

J. MALÁ*[1] and V. BYLINSKÝ**

*Forestry and Game Management Research Institute, Jíloviště - Strnady, CZ-15604 Prague 5, Czech Republic**
*Agency for Protection of Nature and Countryside, Řetězová 22/3, Prague 1, Czech Republic***

Abstract

A new protocol for micropropagation of endangered *Daphne cneorum* through multiple shoot formation has been developed. Two different types of explants (dormant apical buds and *in vitro* seed-derived young seedlings) from plants in two different localities were used for the initiation of multiple shoots on agar woody plant medium (WPM) with 0.2 mg dm^{-3} benzylaminopurine (BAP), 0.1 mg dm^{-3} β-indolebutyric acid (IBA), 200 mg dm^{-3} glutamine, and 200 mg dm^{-3} casein hydrolysate. From 10 seeds only one germinated and the multi-apex culture bearing 12 shoots sprouted out from *in vitro* seed-derived young seedling. After 6-month cultivation 35 multi-apex cultures were achieved from *in vitro* seed-derived young seedling. On 1/3 strength WPM medium supplemented with 2.83 mg dm^{-3} IBA 50 % of cultures (clusters of 3 - 5 shoots) rooted but no rooting occurred in the presence of α-naphthaleneacetic acid (NAA). The rooted plantlets were acclimatized for 4 weeks in the greenhouse and then transferred into natural conditions. The plants successfully survived the winter and flowered.

Additional key words: plantlet regeneration, organogenesis, growth regulators.

The *Daphne cneorum* is an evergreen low shrub originally occurring in thermophyte and in the warm parts of mesophyte regions of the Czech Republic. The plant gradually disappeared during the last few decades and nowadays it occurs only in two localities in Central Bohemia. Daphne could be propagated by means of cuttings, however, the collecting of plant material decreases the viability of donor plants and threatens the survival of remnant daphne individuals in their natural stand. The micropropagation of different species or varieties of daphne has been rarely mentioned (Cohen 1975, Cohen and Le Gal 1976, Marks and Myers 1992, 1994). Although *D. cneorum* was firstly considered as irresponsible to induction of organogenesis (Cohen 1975), its successful micropropagation was described (Christie and Brascamp 1989, Wei *et al*. 1992). We showed recently (Malá 2000) the possibility of daphne micropropagation by technology, which was successfully applied for different woody plant regeneration (*e.g*. elm, oak and beach).

The objective of this study was to establish an efficient protocol for micropropagation of *D. cneorum* and optimize conditions for acclimatization and transfer into natural conditions.

Two localities were selected as a source of *Daphne cneorum* L. explants: the locality A (25 km west of Prague) and the locality B (45 km north of Prague). The shoots 3 cm long bearing an apical dormant bud were collected in February 2000 from both localities. Ten seeds (about 4 mm in size) were collected in the locality A at the end of May 2000**.** The shoots and the seeds were surface-sterilized in 0.1 % (m/v) $HgCl_2$ by vigorous shaking for 20 min. After three rinsing in sterile distilled water, the shoots and seeds were placed into agar medium in 100 cm^3 Erlenmeyer flasks.

Organogenesis both in the meristematic tissue of apical dormant buds and *in vitro* seed-derived young seedling was induced on 6 % agar woody plant medium (WPM, Lloyd and McCown 1980) containing: 200 mg dm^{-3} of glutamine, 200 mg dm^{-3} of casein-

Received 25 August 2003, *accepted* 28 May 2004.
Abbreviations: BAP - benzylaminopurine; IBA - β-indolebutyric acid; NAA - α-naphthaleneacetic acid; WPM - woody plant medium; MS medium - Murashige and Skoog medium.
Acknowledgements: We are grateful to Dr. M. Cvikrová for critical reading of the manuscript.
[1] Corresponding author, e-mail: mala@vulhm.cz

hydrolysate, 30 g dm^{-3} of sucrose, 0.2 mg dm^{-3} of benzylaminopurine (BAP), and 0.1 mg dm^{-3} of β-indole-butyric acid (IBA). Multiplication of *in vitro* formed shoots and growth of multi-apex cultures were achieved on the WPM agar medium of the same composition as used for the induction of organogenesis.

Cultures were maintained at 25 °C and the irradiance of 30 µmol m^{-2} s^{-1} (*Philips Master Super 80*, *TDL 36W/83*, Rosendaal, The Netherlands) for a 16-h photoperiod. Cultures were regularly transferred onto fresh medium every 4 weeks. The number of shoots per culture was counted at the end of subculture intervals.

The 25 cultures (clusters of 3 - 5 shoots) each from the localities A and B and from the seed-derived cultures were selected for rooting and acclimatization. Multi-apex cultures (3 - 5 shoots, 2 - 3 cm long) were transferred onto 1/3 strenth WPM medium supplemented with 2.83 mg dm^{-3} of IBA and placed in the darkness for 7 d. After this period the cultures were transferred to light and replanted to the same medium without IBA. Simultaneously, the influence of NAA on rooting was studied. The IBA in the 1/3 strength WPM medium was substituted for 7.1 mg dm^{-3} of NAA. The 25 multi-apex cultures each from the localities A and B, and from the seed-derived cultures were used in the study of NAA effect on the induction of rhizogenesis.

The rooted plantlets were replanted into the *Perlite* and maintained in constant cultivation conditions, watered once a day with the MS medium (Murashige and Skoog 1962) diluted 1:10 with distilled water and cultivated under continuous light (30 µmol m^{-2} s^{-1}) at 20 °C for 2 weeks. The plantlets with well-developed root system were transplanted into the peat substrate. They were acclimatized to ambient humidity for 4 weeks in the greenhouse. After this period acclimatized plants were transferred into outdoor beds.

One-way analysis of variance was used for the statistical evaluation of results.

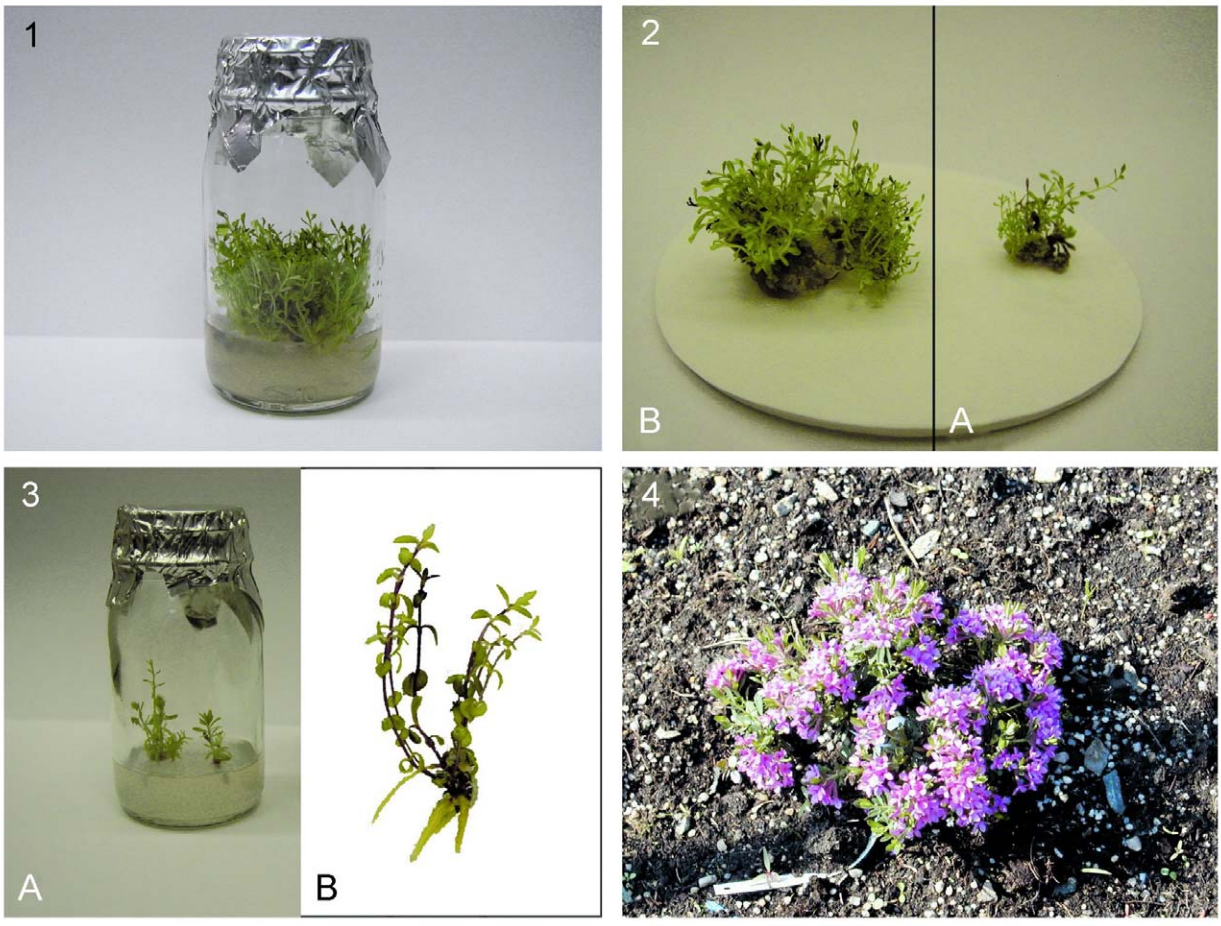

Fig. 1. Multi- apex cultures of *D. cneorum.*
Fig. 2. Differences in multiplication rate between the explants of *D. cneorum* from the two studied localities.
Fig. 3. Multi-apex cultures of *D. cneorum* cultivated on rooting medium (*A*) and the detail of rooted multi-apex culture (*B*).
Fig. 4. Flowering regenerant *D. cneorum* in outdoor beds.

Table 1. The growth of explants of *D. cneorum*. Numbers of total multi-apex cultures were counted at the end of the sixth subculture.

Locality		Number of explants	Responded explants	Shoots [primary expl.$^{-1}$] in subcultures 1	3	6	Total multi-apex cultures
A	shoots	20	16	2.8 ± 1.2	20.2 ± 5.3		344
	seeds	10	1	12.0		26.7 ± 8.2	35
B	shoots	10	6	4.5 ± 2.6	38.3 ± 12.2		236

The meristematic tissue of apical dormant buds developed into microshoots (approximately of the length 2.5 ± 0.6 cm) from 16 primary explants of locality A and 6 explants of locality B in the course of 4 weeks (Table 1). These *in vitro* formed microshoots were used for the further micropropagation and in average 2.8 ± 1.2 (locality A) and 4.5 ± 2.6 (locality B) of adventitious shoots was obtained in 4-week subculture interval. These shoots were used for second-set multiplication in which multi-apex cultures with a remarkably increased numbers of shoots (Fig. 1) developed during 3 subcultures over the period of 3 months. Differences in multiplication rate between the explants from two studied localities were observed. Higher number of adventitious shoots per multi-apex culture (38.3 ± 12.2) occurred in cultures originating from explants of locality B than in cultures derived from explants of A locality (20.2 ± 5.3) (Fig. 2). Total numbers of multi-apex cultures obtained after 6 months of cultivation from 16 (locality A) and 6 shoots (locality B) were 344 and 236, respectively (Table 1). When considering the proliferation activity of explants, the clones used in our experiment showed higher regeneration capability than that observed in nanoid cultivar of *D. cneorum* grown in a greenhouse. Marks and Myers (1992, 1994) reported that after the induction of organogenesis in the shoots from proximal and distal stem segments of this cultivar only 7.3 ± 0.67 shoots were obtained during the cultivation.

Only one seed (from 10 collected seeds) germinated and grew into young seedling in 3 weeks. The induction of organogenesis from this *in vitro* seed-derived young seedling resulted in the formation of 12 shoots after 21 d of cultivation. These shoots were maintained as an individual clone. The regeneration potential of this seed-derived clone was rather high and 26.7 ± 8.2 of adventitious shoots per a multi-apex culture was obtained. The total number of produced multi-apex cultures of this clone was 35 within 6 months (Table 1).

Rooting was successfully induced within two weeks in 52 and 68 % of cultures originating from A and B localities, respectively (Fig. 3, Table 2). However, total losses during rooting amounted to 49 % due to the low percentage of rooting (32 %) of seed-derived cultures (Table 2). The influence of various auxins (IBA and NAA) was tested for induction and promotion of rooting.

The rooting of *D. cneorum* multi-apex cultures was achieved only on the WPM medium supplemented with IBA. Our study showed the necessity of auxin IBA for the induction of rhizogenesis in agar substrate. Similar observation, confirming the essential role of IBA for root induction of *D. cneorum* explants was published by Marks and Simpson (2000). In contrast, Cohen (1977) reported that the successful rooting of *D. cneorum* occurred in the medium with auxin NAA. The use of the combination of IBA and NAA for stimulation of *D. cneorum* rooting in a peat substrate was recommended by Christie and Brascamp (1989).

Table 2. Number and percentage (in parentheses) of rooting of multi-apex cultures (clusters of 3 - 5 shoots) and of acclimatized plantlets of *D. cneorum*.

Locality		Multi-apex cultures	Rooted plantlets	Acclimatized plantlets
A	shoots	25	18 (52)	10 (77)
	seeds	25	8 (32)	7 (87)
B	shoots	25	17 (68)	16 (94)

We suppose that the observed differences in multiplication rate and rooting capacity between individual clones (localities A and B and *in vitro* seed-derived) might be determined genetically, and/or interpreted as a result of the influence of specific local chemical and microbial flora compositions of soil (data not published).

The rooted plantlets were replanted into the *Perlite* where well-developed root system was formed during two weeks. Total losses during acclimatization (4 weeks) in the greenhouse amounted to 13 % (Table 2). Acclimatized regenerants were transferred into the outdoor beds, where they survived the winter period without any losses and flowered (Fig. 4).

Daphne cneorum belongs to endangered plant species in the Czech Republic. With respect to the fact that daphne plants do not propagate generatively in natural conditions due to low fertility and decreased seed germination the protocol of *Daphne cneorum* micropropagation represents an important information for preservation of *D. cneorum* shrubs in their last natural refuges.

References

Christie, C.B., Brascamp, W.: Exflasking high health *Daphne* plantlets. - Int. Plant Propag. Soc. Proc. **38**: 394-398, 1989.

Cohen, D.: Plant tissue culture – possible applications in the New Zealand nursery industry. - Int. Plant Propag. Soc. Proc**. 25**: 310-315, 1975.

Cohen, D.: Thermotherapy and meristem-tip culture of some ornamental plants. - Acta Hort. **78**: 381-388, 1977.

Cohen, D., Le Gal, P.M.: Micropropagation of *Daphne* × *burkwoodii* Turill. - Int. Plant Propag. Soc. Proc. **26**: 330-333, 1976.

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

Malá, J.: Micropropagation of mature elm trees *in vitro.* - J. Forest Sci. **46**: 2260-264, 2000.

Marks, T.R., Myers, P.E.: Effect of explant location upon early culture development *in vitro.* - J. hort. Sci. **67**: 583-591, 1992.

Marks, T.R., Myers, P.: Physiological variability arising from *in vitro* culture is induced by shoot selection and manipulation strategies. - J. hort. Sci. **69**: 1-9, 1994.

Marks, T.R., Simpson, S.E.: Rhizogenesis in *Forsythia* × *intermedia* and *Syringa vulgaris*; application of a simple internode experimental system. - Plant Cell Rep. **19**: 1171-1176, 2000.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassay with tobacco tissue cultures. - Physiol. Plant. **15**: 473-797, 1962.

Wei, C., Green, M.J., Gotkin, S.E., Monette, P.L.: Use of *in vitro* cultures of *Daphne cneorum* L. for western detection of *Daphne* virus X. - J. environ. Hort. **10**: 153-156, 1992.