MICROPROPAGATION OF CLODBERRY (*RUBUS CHAMAEMORUS* L.) BY INITIATION OF AXILLARY SHOOTS

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ABSTRACT

A method for micropropagation of the endangered *Rubus chamaemorus* L. (Rosaceae) from shoot tips was developed. Murashige and Skoog (MS) medium supplemented with BA and IBA in different concentrations was used for axillary shoot development. The most effective was MS medium containing 0.88 μM BA and 0.49 μM IBA with value of pH = 4.0. Cultures of microshoots (up to 12 shoots from a single explant) developed into regular shoots on the same medium or after transferring them onto MS media with 0.44 μM BA, 0.49 μM IBA and 434 μM SA. The rooting of initiated axillary shoots proved extremely difficult. A two-step procedure for root induction was adopted. Auxin-treated shoots were put to medium devoid of plant growth regulators. Rooted shoots were then transferred to pots and grown in the greenhouse until planted in the ground of the Botanical Garden.

KEY WORDS: *Rubus chamaemorus* L., endangered species, micropropagation, shoot tips, axillary shoots.

INTRODUCTION

Cloudberry (*Rubus chamaemorus* L.) also known as bakeapple, belongs to *Rubus* genus (Rosaceae). *R. chamaemorus* is the only representative of *Chamaemorus* (Hill.) Focke subgenera (Heslop-Harrison 1968). Cloudberry has a boreal, circumpolar distribution (Fægri 1958). The plant is common in peat bogs of the northern hemisphere of Europe and North America. The naturally growing plants are of economic importance, especially in Scandinavia.

In Poland it only occurs in a few habitats as a glacial relict under strict protection. *R. chamaemorus* is placed in the list of rare and endangered species (Żukowski and Jackowiak 1995) and listed in the Polish Red Data Book of Plants (Kruszelnicki and Fabiszewski 1993).

Cloudberry is a herbaceous, octoploid, dioecious, 5-30 cm high plant. The edible fruits, large, globular, amber or yellowish in colour, are composed of a few soft drupelets (Fig. 1). They contain a lot of ascorbic acid (Ryvarden 1993) and in northern areas are used fresh or frozen or serve as a material for syrups, confitures, wines, liqueurs and sweets.

The berries contain: mineral elements (Kuhnlein et al. 1994), vanillin and its derivatives (Pyysalo and Honkanen 1977), essential oils (Honkanen and Pyysalo 1976) and benzoic acid as a natural preservative. From the seeds oil was separated and many

triacylglycerols were identified in it (Johansson et al. 1997). Flavonoids and polyphenolic acids are present in cloudberry fruits, where ellagic acid is the main compound (Törrönen et al. 1997; Häkkinen et al. 1998). Many studies support the hypothesis of antioxidative and anticarcinogenic properties of these compounds (e.g. Tanaka et al. 1993; Knekt et al. 1997).

Rubus chamaemorus has not been studied in propagation in vitro so far. The aim of this study was to develop a micropropagation method for *Rubus chamaemorus* L. by using shoot tips and to observe whether the applied method of clonal propagation can be used to preserve the endangered species in Poland.

MATERIAL AND METHODS

Plant material

Ripe fruits were collected on natural habitats in the mountains of the Hade Land – southern province of Norway (600 m above sea-level) (August 1995, 1997, 1998). Voucher specimens are deposited in the Department of Pharmaceutical Botany Herbarium, University of Medical Sciences in Poznań. The pyrenes were cleaned of the mesocarp and stored at 4°C for 3 months. The term „pyrene” refers to the hard endocarp and the entire seed (Naess et al. 1993). The pyrenes were rinsed with 70% ethanol and then in sterile distilled water, in which they were kept for two days. Sterilization was successively carried out with 20% water solution of a common household bleach „Clorox” with two drops of Tween 80 for 20 min. Next, pyrenes were kept in liquid medium MS (Murashige, Skoog, 1962) containing 2.9 μM GA₃ at 4°C for 5 days to allow imbibition and

Abbreviations:

MS – Murashige, Skoog, BA – 6-benzyladenine, IBA – indole-3-butyric acid, IAA – indole – 3-acetic acid, kin-kinetin, NAA- α – naphthaleneacetic acid, SA – adenine sulfate, GA₃ – gibberellic acid

Fig. 1. Cloudberry *Rubus chamaemorus* L. in the Słowiński National Park. Photo by J. Herbich.

endocarp and seed coat softening (Fiola et al. 1990). Following incubation the pyrenes were aseptically removed from endocarp and seed coat, as described by Galleta et al. (1986). Embryo excision was executed in sterile conditions under binocular microscope. Embryos were inoculated in the glass test tubes containing 10 ml solidified half-strength MS medium and with an addition of 2.9 μM GA_3 .

Axillary shoot development

Shoot tips (ca 5 mm long) from 4-week-old sterile seedlings were used as initial explants. Consequently, they were transferred to Erlenmeyer flasks with 20 ml shoot multiplication medium and growth regulators as indicated in Table 1, supplemented with 434 μM SA. Microshoots difficult to separate were transferred to MS medium with 2.9 μM GA_3 or 2.45 μM IBA for elongation and further development. Every eight weeks the microshoots were transferred to fresh medium. Rates of shoot multiplication were determined by counting the number of shoots minimum 3 mm in length, produced per explant after an 8 week culture.

Rooting of shoots

Multiple shoots were dissected into individual stems (> 1 cm) after 6-8 weeks and cultured in half-strength MS rooting medi-

TABLE 1. Media used in successive stages of *Rubus chamaemorus* micropropagation (phytohormons concentration in μM).

MS + GA_3 (2.9)	seedling development
1/2 MS + BA (0.88) + IBA (0.49)	shoot multiplication
MS + BA (4.44) + IBA (0.49)	
MS + BA (8.88) + IBA (0.49)	
MS + BA (8.88) + IBA (2.45)	
MS + GA_3 (2.9)	shoot elongation
MS + BA (0.44) + IBA (0.49) + SA (434)	
1/2 MS + IBA (4.9) + BA (0.98)	rooting
MS + IBA (4.9) + IAA (0.57) + kin (0.92)	
two-step procedure – see table 2	

um with auxin (in several concentrations) and kinetin (Table 2). In some experiments a two-step procedure for root induction was done. In the first step, shoots were kept in MS liquid medium supplemented with high concentration of auxin (571 or 1142 μM IAA) for 1 hour or shoots were inoculated in half-strength MS liquid medium containing 24.5, 49.0, 73.5 and 98.0 μM IBA for 2-4 days. In the second step, auxin-treated shoots were planted on agar-solidified half-strength growth regulator-free MS medium for root initiation. Rooting percentage was evaluated 8 weeks later. One part of unrooted shoots, previously treated with powdered commercial product („Ukorzeniacz B” produced by P.P.H. „Himal” Łódź, Poland) containing 0.1% IBA, was transferred directly to pots filled with peat moss.

Culture media and conditions

MS medium supplemented with combinations of plant growth regulators was used for multiple shoot cultures (with BA, IBA) and for rooting (IBA, IAA, NAA, BA, kin). The pH values (from 3.5-5.6) of media were also tested. Subsequently, the

TABLE 2. Effect of auxins (μM) on rooting of *in vitro* initiated shoots of *Rubus chamaemorus* after 8 weeks of culture.

Medium	Rooting (%)		
agar-solidified			
1/2 MS			0
MS			0
MS + IBA (1.0) + IAA (0.1) + kin (0.2)			26
two-step procedure			
liquid		agar-solidified	
MS + IAA (571)	– 1 h	1/2 MS	12
MS + IAA (1142)	– 1 h	1/2 MS	37
1/2 MS + IBA (24.5)	– 48 hrs	1/2 MS	58
1/2 MS + IBA (49.0)	– 48 hrs	1/2 MS	51
1/2 MS + IBA (73.5)	– 96 hrs	1/2 MS	45
1/2 MS + IBA (98.0)	– 96 hrs	1/2 MS	33
rooting directly in the peat moss after treatment with powdered 0.1% IBA „Ukorzeniacz B”			39

shoot tips were cultured on pre-selected, defined media with chosen concentration of growth regulators (Table 1).

In the following experiments the used nutrient media, containing 3% sucrose, were solidified with 0.8% Difco Bacto Agar. The pH medium was adjusted to the value of 4.0 before autoclaving at 121°C for 20 min. Cultures were maintained in the growth chamber under a 16-h photoperiod (cool-white fluorescent light lamps, $60 \mu\text{mol m}^{-2} \text{s}^{-1}$) at $24^\circ \pm 2^\circ\text{C}$.

Rooted shoots 1.5 cm or longer were washed in water and transferred to pots with a mixture of sterilized sand and peat moss (1:5; pH = 4.5). The plantlets continued to grow under artificial light in the growth chamber. Glass covers were used to ensure high humidity around the plants. Plantlets were watered every other day with quarter-strength MS mineral salts. They were cultured a few months *ex vitro* in the growth chamber and in the greenhouse before transferring them to the ground in the Botanical Garden.

All the experiments concerning shoot multiplication and rooting were repeated three times. Each treatment consisted of minimum ten replicated cultures.

Long term cold storage of micropropagated shoots

Regenerated cloudberry shoots were cultured in 200 ml jars, each containing 50 ml MS medium with $0.88 \mu\text{M}$ BA and $0.49 \mu\text{M}$ IBA, 3% sucrose and $434 \mu\text{M}$ SA like above. Eight – week old shoot cultures were transferred to cold chamber at 4°C , under the light intensity of $10 \mu\text{mol m}^{-2} \text{s}^{-1}$. After six month the shoots were cultured on the fresh medium and kept again at 24°C under the light intensity of $60 \mu\text{mol m}^{-2} \text{s}^{-1}$.

RESULTS AND DISCUSSION

Data obtained from ample literature on micropropagation by shoot tips of other species of *Rubus* genus helped to choose and

Fig. 2-5. Micropropagation of *Rubus chamaemorus* L. from shoot tip explants. Photo by B. Thiem.

Fig. 2. Axillary shoot development after 8 weeks on MS medium containing $0.8 \mu\text{M}$ BA and $0.49 \mu\text{M}$ IBA.

Fig. 3. Multiple shoot formation after 12 weeks on MS medium supplemented with $4.44 \mu\text{M}$ BA and $0.49 \mu\text{M}$ IBA.

Fig. 4. Plantlet rooted *in vitro*.

Fig. 5. Plant adopted to growth in a pot after 1 month of acclimatization.

work out the composition of numerous media used for multiplication and rooting of *R. chamaemorus* shoots (e.g. Snir 1981, 1988; Mc Pheeters et al. 1988; Hoepfner and Nestby 1991). The reports show that different varieties or genotypes belonging to *Rubus idaeus* or other *Rubus* species differ significantly in their ability to regenerate and to perform rhizogenesis (e.g. Anderson 1980; Hoepfner and Nestby 1991). The review of the tissue culture of *Rubus* species was reported by Donnelly and Daubeney (1986).

Obtaining seedlings from poorly germinating *R. chamaemorus* seeds proved very difficult. Despite the application of suitable procedures, i.e. stratification and scarification (Peacock, Hummer 1996) germination rates varied between 10–16% depending on the year. Therefore, to ensure better effects, the hard endocarp and seed coat were removed. In natural habitats *Rubus chamaemorus* reproduces both vegetatively and sexually, although vegetative reproduction by rhizomes is the most effective. Germination rates in experimental conditions varied from 0–31% (Warr et al. 1979; Korpelainen 1994). It seems that the seed covering (seed coat and endocarp) of cloudberry, similar to that of blackberry and raspberry, forms a physical barrier for seed germination that prevents germination in vitro (Mian et al. 1995).

The present study and preliminary experiments showed that the germination of seeds create serious problems, therefore excised embryos were used as initial explants.

Most (> 95%) shoot tip explants (from seedlings) formed multiple shoots from axillary shoot formation within 6–8 weeks when cultured on MS medium containing 0.88–8.88 μM BA and 0.49–2.45 μM IBA (Fig. 2). A high frequency of responding explants (ca. 80%) was found on these media. Continued culture on the same media resulted in formation of normal shoots. Some microshoots were transferred to MS medium with 2.9 μM GA₃ or 0.44 μM BA and 0.49 μM IBA for elongation and further development. New shoots on that media (Table 1) grew vigorously and attained a mean length of 1 cm within 8 weeks. The addition of SA (434 μM) like in experiments of Anderson (1980) showed a positive effect on growth. Media of a very low pH = 4.0 value used in experiments was optimum for the micropropagated plants. The common habitat for cloudberry are sphagnum bogs and bog pine-woods which are generally acidic and poor in nutrients. Such a poor habitat has a pH of 2.5–4.5 (Warr et al. 1979). However, the plant is known to show greater productivity on fertilized soil (Taylor 1971). The obtained shoots were used as subculture material. For each subsequent subculture the procedure was repeated as above at 8-week intervals. The highest multiplication rate of cloudberry shoots was achieved on MS medium supplemented with 0.88 μM BA and 0.49 μM IBA. On average 10–12 shoots per explant were produced, so by this practice a large number of shoots was obtained (Fig. 3).

The vigorous shoots, longer than 1.5 cm, were used for rooting experiments. The excised shoots were individually transferred to rooting medium (Table 2). Root initiation was not observed on MS or 1/2 MS medium devoid of growth regulators. As a pretreatment to provide shoot elongation for the rooting trial, shoots were subcultured on a medium with 0.44 μM BA, 0.49 μM IBA and 434 μM SA.

Difficulties in root induction on shoots of *Rubus* genus, reported by many authors (e.g. Anderson 1980; Sobczykiewicz 1980; Snir 1981; Hoepfner and Nestby 1991), induced multidirectional experiments with *R. chamaemorus* in vitro cultures. The attempts to root the shoots in vitro were performed on both

agar and liquid media. Media with auxins were used (IBA, NAA or IAA), sometimes supplemented with a small amount of BA or kin. The 25 modifications of media were studied of which only a few were chosen (Table 2). But rooting in vitro was still not successful (26%), so a two-step procedure for root induction was adopted, as suggested by Siril and Dhar 1997. Auxin-treated shoots planted on growth-regulator-free, half-strength MS medium produced adventitious roots within 2 weeks. IBA treatment significantly improved the rooting percentage. Optimal rooting (58%) was obtained by a 48h treatment of shoots in 98 μM IBA (Table 2). The shoots produced on average 5 roots (Fig. 4). Control shoots cultured on auxin-free medium did not root. *Rubus chamaemorus* require media with auxins for root initiation. Similarly, IBA was essential for obtaining of good rooting percentage and plantlet growth of *Rubus occidentalis* L., however *Rubus idaeus* L. did not require addition of auxin, whereas IBA was beneficial for plantlet growth (Anderson 1980).

Rooting in vitro of *R. chamaemorus* is still problematic because of the rapid browning and/or necrosis of explants. These problems are at least partly caused by polyphenols which are abundant in cloudberry (Thiem 1998) or fitoalexins produced in in vitro cultures as a response to abiotic elicitors. The autoinhibitory response in *R. chamaemorus* cultures was slightly reduced by culturing of explants on fresh media every 5 days after inoculation.

Some attempts were also made to root the shoots of cloudberry ex vitro, directly in the moss after dipping them in a commercial rooting preparation containing 0.1% IBA. Plantlets resumed growth and were transferred to growth chamber after potting (Fig. 5). Nearly 85% of the rooted plantlets survived their transfer to pots. Five month-old plants were fully acclimatized (Thiem 2000). Some acclimatized plantlets were cultivated in an experimental plot in the Botanical Garden of Adam Mickiewicz University in Poznań, where field performance of plants is being studied.

The method described above is reproducible. It can be applied for clonal propagation of the selected genotype and preservation in Poland of endangered *Rubus chamaemorus* germplasm. The obtained plantlets appeared morphologically uniform with natural forms. Micropropagation of plants through axillary shoot formation reduces or eliminates the possibility of abnormalities occurring with use of other methods (Bajaj et al. 1988). In the *Rubus* genus, DNA fingerprinting has been used successfully to distinguish genotypes. Genetic stability of some clones of raspberries micropropagated by shoot tip cultures yielded identical DNA fragment profiles (Hoepfner et al. 1993).

The introduction of regenerated plantlets of *Rubus chamaemorus* in the Botanical Garden allows to present this endangered species to visitors and work out the right method of its cultivation. Protection of *R. chamaemorus* can also be obtained by storing in vitro cultures in a temperature of 4°C and in low light. The storage of cultures can be performed for a prolonged time – from a few to several months – without the need of their passaging. Preliminary studies on long-term cold storage of micropropagated shoots was done as reported (Verma et al. 1998). The results indicate that rooting was essential for long-term cold storage of in vitro-grown cloudberry shoots which seemed to store sufficient nutrients for survival and retaining their shoot formation capacity similar to the control.

The performed studies may become a way for active protection of other endangered wild species from extinction. Plant

biotechnology has taken more and more significance in protection of rare and endangered species of the Polish flora (Pawłowska and Bach 1998; Kromer and Nowak, 1999).

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MIKROROZMNAŻANIE MALINY MOROSZKI (*RUBUS CHAMAEMORUS* L.)
POPRAZEC INICJACJĘ PĄKÓW PACHWINOWYCH

STRESZCZENIE

Opracowano metodę mikrorozmnażania in vitro ginącego gatunku *Rubus chamaemorus* L. (Rosaceae) ze szczytów pędów. Zastosowano pożywkę Murashige-Skooga (MS) wzbogaconą BA i IBA w różnych stężeniach. Najkorzystniejszą dla rozwoju pąków pachwinowych okazała się pożywka MS zawierająca 0.88 μM BA i 0.49 μM IBA. Kultury mikropędów (do 12 pędów z pojedynczego eksplantatu) rozwijały się w regularne pędy na tej samej pożywce lub po ich przeniesieniu na pożywkę MS z 0.44 μM BA, 0.49 μM IBA i 434 μM SA. Ukorzenienie rozwijających się pędów sprawiało dużo trudności. Zastosowano dwustopniową metodę indukcji korzeni. Pędy traktowane auksynami przenoszono na pożywkę MS pozbawioną roślinnych regulatorów wzrostu. Ukorzenione pędy przenoszone były do doniczek. Roślinki rosły dalej w szklarni aż do przesadzenia ich do gruntu w Ogrodzie Botanicznym.

SŁOWA KLUCZOWE: *Rubus chamaemorus* L., ginący gatunek, mikrorozmnażanie, szczyty pędów, pąki pachwinowe.