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Propagation of adult curly-birch succeeds with tissue culture

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TIIVISTELMÄ: VISAKOIVUN LISÄYS SOLUKKOVILJELYN AVULLA

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Plantlets were produced from adult curly-birch. Murashige and Skoog's medium was used as the culture medium. Growth was initiated on a medium containing 1 mg/l BAP. Bud formation was induced using a medium containing 10 mg/l BAP and 0.2 mg/l NAA. Development of shoots was achieved on a medium containing 1/2 × Murashige and Skoog's macrominerals and sucrose, 1/1 × Murashige and Skoog's microminerals and vitamins, and 0.5 mg/l BAP and 0.5 mg/l IAA. The medium used for inducing root formation was the same as the above, but without any growth regulators.

Aikuinen visakoivu lähtömateriaalina on tuotettu solukkotaimia. Elatusalustana käytettiin Murashige-Skoogin alustaa. Kasvun aloitus tapahtui alustalla, johon oli lisätty BAP:ia 1 mg/l. Silmujen indusointi tapahtui alustalla, jossa oli BAP:ia 10 mg/l ja NAA:ta 0.2 mg/l. Versot saatiin kehittymään alustalla, jossa oli 1/2 × Murashige-Skoogin makromineraalit ja sakkaroosi sekä 1/1 × Murashige-Skoogin mikromineraalit ja vitamiinit sekä BAP:ia 0.5 mg/l ja IAA:ta 0.5 mg/l. Versojen juurtumiseen käytetty alusta oli sama kuin edellinen, mutta ilman kasvunsäätely-aineita.

Key words: *Betula pendula* var. *carelica*, cell differentiation, clonal propagation, clonal variegation, plantlet
ODC 176.1 *Betula pendula* var. *carelica* + 165.44
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1. Introduction

Curly-birch (*Betula pendula* var *carelica* Mercklin) is considered to be a special form of *B. pendula* Roth, occurring throughout Northern Europe and parts of Central and Eastern Europe. Its most important characteristic is the unusual structure of the wood (e.g. Heikinheimo 1951, Raulo 1980). As the wood is strong and decorative, it is in great demand for making wooden ornaments. Curly-birch wood is sold according to weight, and its price makes it much more valuable than ordinary birch. The curly-grained trait is inheritable, although its genetic background has not yet been determined. How-

ever, it is presumably not a question of only one Mendelian gene since, for instance, there are a number of different types of curly-birch. Four main structural types of curly-birch have been distinguished: trees with protuberances (type P), necks (K), stripes (J) and rings (R) (Saarnio 1976). The P-type is the most common, occurring in its pure form in around 60 % of the curly-birches in recearch stands. If mixed forms dominated by the P-type are taken into account, then the proportion of P-type curly-birch is as high as 81 % (Saarnio 1976). Furthermore, the P-type curly-birch also contains the greatest amount of curly-grained wood, and hence its proportion should be favoured in breeding work.

There are as many explanations for the formation of curly-grained wood as there are experts in this field. One or more recessive genes has been proposed as the causal agent (Johnsson 1951). It has been suspected that the property is homozygotic lethal (Ruden 1954), or assumed to be heritable only through the mother tree (Ruden 1954). Valanne (1972) produced curly-birch type branching in seedlings of ordinary *B. pendula* by artificially changing the number of chromosomes. On the other hand, it has been suggested that curly-birch is a genetical disease of birch, supposedly caused by an abnormal physiology of the under-bark tissue of the tree (Jevdokimov 1984) or a micro-organism such as a plasmid or, according to a number of researchers, a virus (e.g. Atanasoff 1967).

Curly-birch is strongly selfsterile, and the progeny obtained in controlled crossings between two curly-birch individuals do not all possess the curly-grained trait. A maximum of 80 % of the progeny can be curly-birch (Sarvas 1966, Jevdokimov 1984). Only about 50 % of the free-pollinated seed collected from managed curly-birch stands develop into curly-birches (Raulo 1980). It takes about 10 years before it is possible to tell whether an individual will become a curly-birch. This makes establishing curly-birch plantations a rather uncertain process. Neither the proportion of curly-grained individuals nor the precise form which they will develop into are known at the establishment stage. The normal birches have to be removed as the stand grows up because their faster rate of growth would result in the curly-birches becoming suppressed. The vegetative propagation of adult curly-birches of the desired type would radically change the cultivation of curly-birch.

The propagation of curly-birch from cuttings was first attempted in summer 1940 in Finland. About 12 % of the cuttings representing different types of curly-birch developed roots, and after one year only 3 % of the cuttings were still viable (Pohjanheimo 1980). One major problem in the production of cuttings is their poor winter resistance. Furthermore, the bushy curly-birches with short stems are the best ones to produce those cuttings which root (Jevdokimov 1984). This goes against the aim of curly-birch breeding, which is to produce trees with as much stemwood as possible.

The aim of this study has been to develop a tissue culture technique for propagating curly-birches with the optimum stem form, including triploid curly-birches of the P-type.

2. Material and methods

Five curly-birches were used in the experiment: J-type curly-birch E8469, N-type curly-birch E8999, P-type curly-birch E9000, P-type curly-birch E9141 and Olli curly-birch E1092, which is triploid. E8469, E8999, E9000 and E9141 were planted in the Punkaharju clone collection in 1960 using curly-birch seedlings of Punkaharju origin. The curly-birch stand where E1092 is growing was established in 1932 using curly-birch seedlings originating from Aulanko. Branches were collected from the trees at the end of the growing season and stored in the dark at +2°C. The water was changed twice a week, a piece of branch about 1 cm long being cut from the base of the branches at the same time. Before starting to make the cultures, the branches were forced for about three weeks either on a window ledge at room temperature, or in a growth chamber where the temperature during the ten daylight (350 $mEm^{-2}s^{-1}$) hours was 23°C, and the ten dark hours +9°C. There was a two-hour twilight period (175 $mEm^{-2}s^{-1}$) between the dark and light periods in both the morning and evening. Tissue cultivation was carried out at room temperature (about 23°C). The length of the daylight period (300−350 $mEm^{-2}s^{-1}$) was 16 h. Both apical and axillary buds were used in the cultures. Each bud and a section of stem about 1 cm long were sterilised by immersing in 70 % ethanol for one minute, after which the growing point was exposed. A small piece of stem (2−5 mm) and exposed growing point, or a piece of stem and two of the innermost leaves and a growing point, were transferred to the culture medium.

Growth initiation: Different variations of two basic culture media were used as the initiation medium; 1) A so-called wood medium, containing macrominerals "N_7" according to Chu et al. (1975), and microminerals and vitamins according to Murashige and Skoog (1962), with additional components – 20 g/l sucrose, 165 mg/l $CaCl_2x$ $2H_2O$, 1 g/l casein hydrolyzate and 0.5 g/l of glutamine. The agar content was 0.6 % and the pH 5.6. The following growth regulators were tested: wood medium 2: 0.5 mg/l kinetin and 2 mg/l 2,4-D. Wood medium 3: 10 mg/l i^6Ade and 10 mg/l IAA. Wood medium 32: 0.2 mg/l BAP and 2 mg/l NAA. 2) The medium of Murashige and Skoog (1962), containing as growth regulators in the 1A medium: 2 mg/l zeatine and 0.2 mg/l NAA, and in the 1B medium 1 mg/l BAP.

Induction of bud formation: The explants were transferred from the initiation medium to the bud-inducing medium. Murashige and Skoog's medium, containing 10 mg/l BAP and 0.2 mg/l NAA, was used as the bud-inducing medium.

Shoot elongation: Explants which had developed bud primordia were transferred either whole or cut up into pieces to the so-called shoot elongation medium. The medium comprised 1/2 × Murashige and Skoog's macrominerals and sucrose, 1/1 × Murashige and Skoog's microminerals and vitamins, and 0.5 mg/l BAP and 0.5 mg/l IAA. While part of the new shoots were transferred to the rooting treatment, the remaining shoots and bud primordia were again divided up and cultured in fresh elongation medium. This gave a continuous supply of new shoots.

Root formation: The optimum shoot length for transferring the shoots to the rooting medium was 3−5 cm. The medium used for rooting was the same as for shoot elongation, except that it did not contain the growth regulators.

Cultivation of the plantlets: After the roots had reached a length of 2−5 cm, the agar was removed by rinsing with distilled water and the plantlets transferred to pots containing a peat/soil mixture. The pots were either moved directly to the greenhouse, or kept for some time in a propagator in a growth chamber. The plantlets were tended using normal greenhouse routines.

3. Results and discussion

All the wood media and the Murashige and Skoog 1A medium gave the same results when used as the initiation medium (Table 1). Abundant callus developed on all the medium. The callus turned green as it aged. Although the callus developed red spots on some of the media, there were no signs that the organs would have become differentiated. The result is rather different to that obtained by Simola (1985) with leaf callus from young *Betula pendula* f. *purpurea*. Simola used a medium corresponding to the wood 2 medium to produce callus, but on the other media corresponding to those we used either roots or shoots were formed on the callus. Huhtinen and Yahyaoglu (1974) have also obtained corresponding differentiation of roots and shoots on cambium callus from young *B. pendula*. They also mentioned that it is possible to produce callus tissue from old birch individuals, but they did not make any speculations about possible differentiation of this sort of callus culture *in vitro*. It is propably that the age of the starting material

Table 1. The results of cultures made using different initiation media.

Medium	E 8469			E 8999			E 9141			E 9000			E 1092		
	No. of cultures	Explant apical/ axillary bud	Callus	No. of cultures	Explant apical/ axillary bud	Callus	No. of cultures	Explant apical/ axillary bud	Callus	No. of cultures	Explant apical/ axillary bud	Callus	No. of cultures	Explant apical/ axillary bud	Callus
Wood 1	3	ap./ax.	–	3	ap.	3	–	–	–	3	ap./ax.	–	–	–	–
Wood 2	7	ap./ax.	5	3	ap.	1	8	ap./ax.	–	10	ap./ax.	6	11	ap./ax.	4
Wood 3	2	ap./ax.	1	3	ap.	–	3	ap./ax.	–	3	ap./ax.	–	4	ap./ax.	3
Wood 32	3	ap./ax.	1	3	ap.	–	–	–	–	3	ap./ax.	3	–	–	–
MS-1A	6	ap./ax.	5	9	ap./ax.	8	4	ap.	3	6	ap./ax.	5	4	ap.	3

used in the study in hand inhibits organogenesis in the callus culture.

The best growth was initiated on the Murashige and Skoog 1B medium when the explant contained, in addition to the growing point, also a small piece of shoot and two of the innermost leaves from around the growing point. Explants of this sort grew in size on the Murashige and Skoog 1B medium, but there was no development of callus or bud primordia proper (Fig. 1). When an enlarged explant in satisfactory condition was then transferred to the bud-inducing medium within about two weeks after the culture had been started, bud induction took place and lasted for 2–3weeks (Fig. 2). However, the buds did not develop into shoots on this substrate, and attempts to produce roots on the buds alone was not successful. In order to overcome this problem, an intermediate stage involving the so-called elongation medium was developed for use between the induction medium and the rooting medium. The largest buds grew into shoots capable of root formation on this medium in about four weeks. Rooting was not successful when the shoots were less than 2.5 cm long – the shoots merely turned yellow and gradually died, starting from the base. The greater the number of seedlings required, the more the tissue was divided up and transferred to new elongation medium as the largest shoots were transferred to the rooting medium (Fig. 3 and 4). It was also possible to suspend culture development at this stage by transferring the tissue to storage at +4°C in dim light. The longest period when the tissue was kept in this state was 4 months, after which the work was continued normally. The rooting stage lasted for about 4 weeks, after which the plantlets were potted in peat (Fig. 5 and 6).

The first plantlets were produced at Punkaharju in spring 1985 from plustrees E8999 and E9141. 318 plantlets from the same clones were left outside to overwinter in the autumn. Restricting the number of plantlets to only a few hundred was not a direct result of the number of induced buds, but merely to the shortage of working facilities and the fact that producing a larger number of plantlets was not considered to be necessary at this stage. Thousands of buds/shoots were produced from, for instance, E8999. The plantlets were inventoried in spring 1986. A total

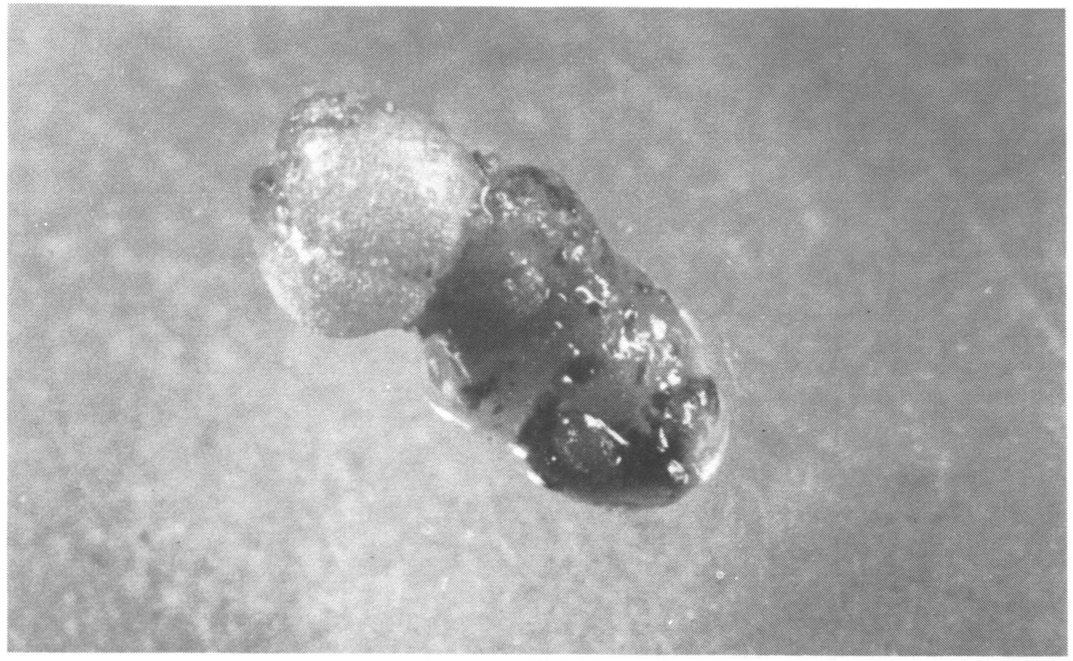

Fig. 1. An explant on the initiation medium. The explant has enlarged. ×25.

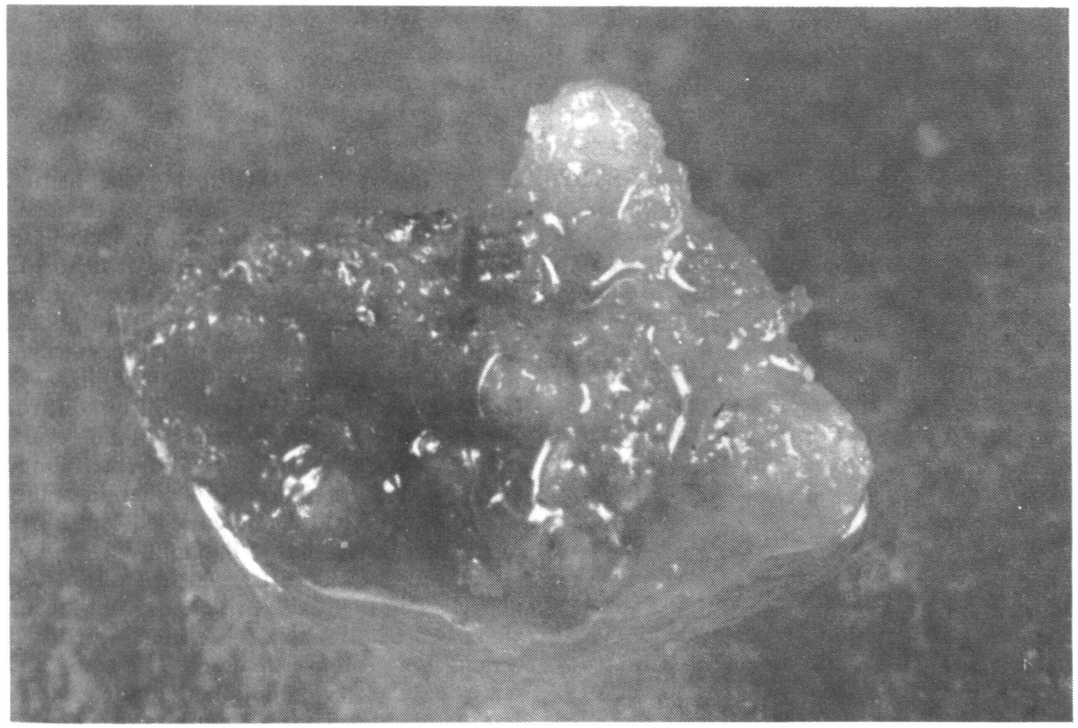

Fig. 2. An explant on the bud-inducing medium. Roundish swellings, bud primordia, are visible on the originally smooth surface. ×24.

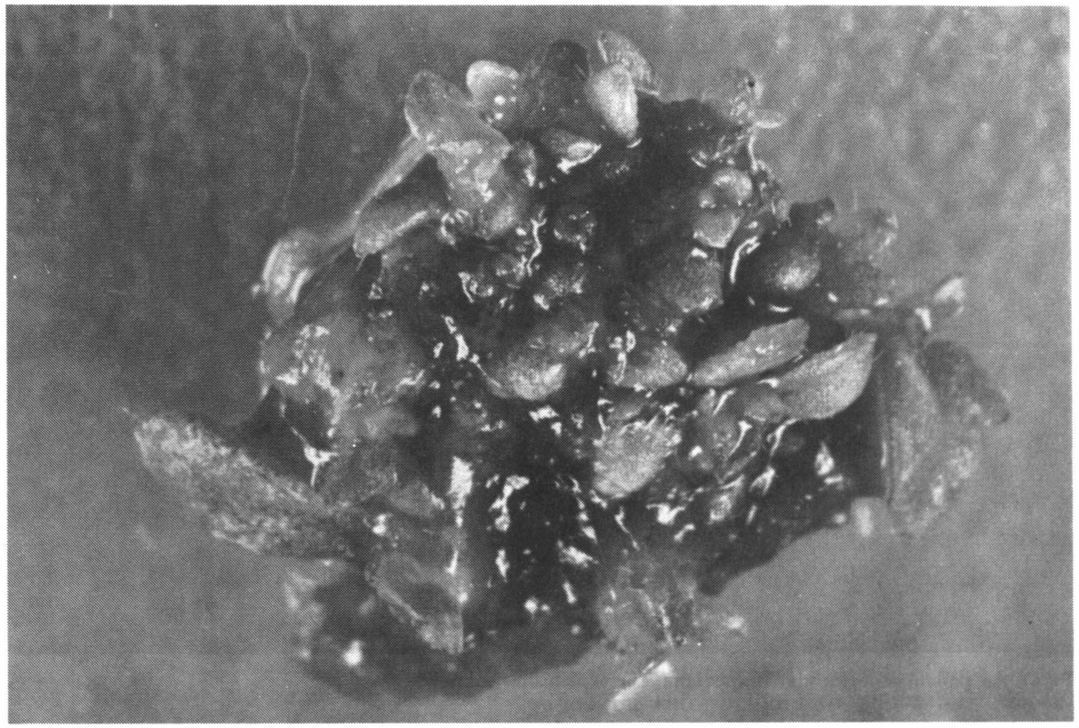

Fig. 3. Tissue on the elongation medium. A large number of bud primordia/buds are visible in the tissue. Growing shoots covered by leaves are distinguishable. ×18.

Table 2. Propagation of curly-birch by tissue culture. Situation per 28. 1.1986.

plustree	No. of cultures	plantlets	stage 2–4	bact. inf.	dead or rejected
E 8469	30	–	–	14 48 %	16 53 %
E 8999	23	10 44 %	–	2 9 %	11 47 %
E 9000	53	–	32 60 %	11 21 %	10 19 %
E 9141	57	1 2 %	22 39 %	8 14 %	26 45 %
E 1092	60	–	28 47 %	10 17 %	22 36 %

of 9 plantlets had died during the winter. Four of the dead seedlings were potted in soil on 8. 8. 1985, and five on 20. 8. 1985. The height of these plantlets varied from 5–12 cm. The plantlets were taken outdoors only a couple of days after being potted in the peat in order to give them time to winter hardening, but this obviously did not take place.

Work was restarted on curly-birch towards the end of 1985. A summary of the results obtained so far, covering the abovementioned plantlets and the cultures in different stages of development (all inventoried on 28. 1. 1986), are presented in Table 2.

Although the plustrees selected for this study are individuals with a good stem form, there were a few 2–3 stemmed seedlings among the group of plantlets. Huhtinen and Yahyaoglu (1974) mention that their plantlets were bushy. However, in this case we would consider that stem forking is one typical external trait of curly-birch, especially

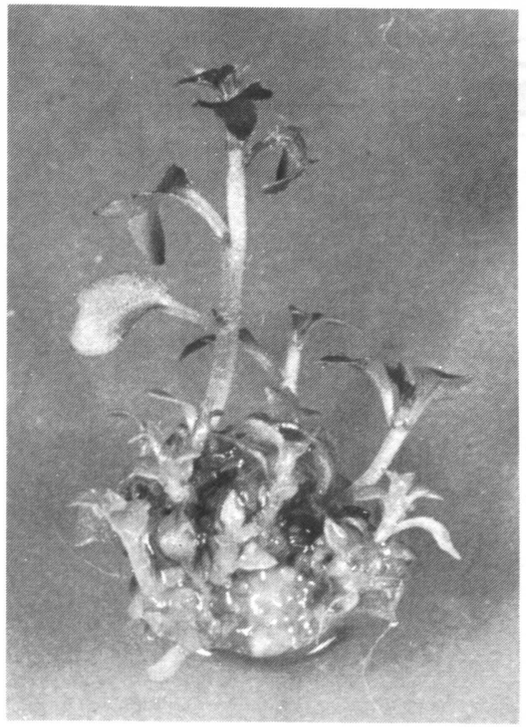

Fig. 4. Tissue transferred back to the elongation medium. Small shoots on the upper part, buds/bud primordia on the lower part. ×5.

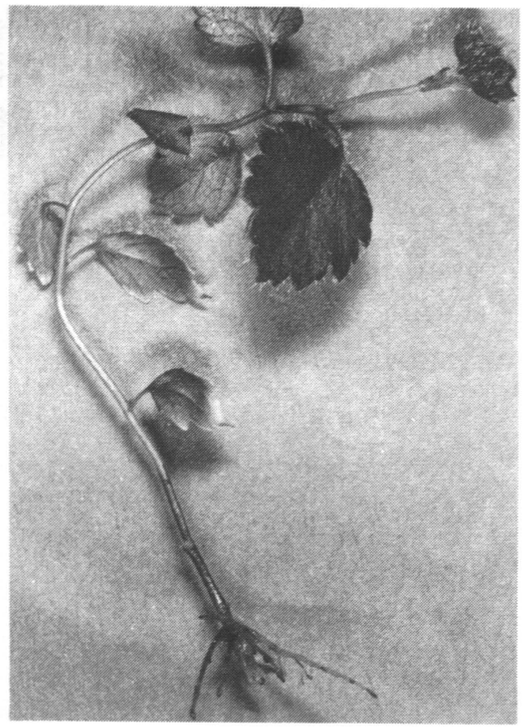

Fig. 5. A plantlet ready to be potted in peat. ×2.

when hundreds of the *B pendula* plantlets produced using the same method have perfect stem form.

More explants were taken from the apical buds for culture because they were easier to prepare, and also because it was considered that this would avoid the inhibiting effect of apical dominance on the growth of the explants from the axillary buds. However, it became apparent when the results were calculated that most of the explants which started to differentiate had originated from axillary buds.

There is no mention in the literature of successful propagation of adult birch by tissue culture, even though attempts have been made with ordinary *B. pendula* at least (Cameron and Matthews 1981). However, it has succeeded with many other adult deciduous species (e.g. Chalupa 1979, Tricoli et al. 1985). Although the techniques they used differ rather radically from the one we have developed now, it should be remembered that the starting material used in tissue culture in studies on adult deciduous trees, e.g. many different species of *Ulmus* and *Populus, Quercus, Fagus* and *Prunus,* was axillary buds. Ahuja (1983) differentiated hundreds of tissue seedlings from adult *Populus* species, *P. tremula, P. tremuloides* and their hybrids. He used meristematic tissue from the buds as the starting material. Ahuja considers that mass cloning for specific genotypes presupposes that the explants can be induced to proliferate buds without almost any visible formation of callus. Ahuja cultured 48 clones of both young cuttings and adult trees in his study. He succeeded in producing plantlets from 22 of them, which fairly closely matches our results. It is obvious that the regeneration capacity of an adult tree is not only dependent on the genotype, but also on the culturing conditions and the physiological state of the tree at the time of the year when the

Fig. 6. Tissue culture derived curly-birch plants.

explants are taken. The results presented on this paper indicate that adult deciduous trees can be best propagated through tissue culture when the least differentiated cells, i.e. the initial cells of the promeristem, are used as the starting material. The axillary buds provide easily available study material which can be prepared with little difficulty and are continuously renewed.

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Seloste

VISAKOIVUN LISÄYS SOLUKKOVILJELYN AVULLA

Koivun visautuminen on ominaisuus, joka näkyy vasta yli 10 vuotiaissa puissa. Visautuneisuus on lisäksi ominaisuus, joka suvullisessa lisäyksessä ei missään olosuhteissa periydy 100 prosenttisesti. Hoidetusta visametsiköstä kerätty siemen tuottaa jälkeläistöjä, joista vain n. 50 % visautuu. Täten visakoivuviljelykset joudutaan siementaimilla istuttamaan ikäänkuin umpimähkään. Visautuneiden puiden osuutta ei tiedetä metsikön perustamisvaiheessa. Lisäksi metsikön vanhetessa täytyy normaalit rauduskoivut poistaa, koska ne nopeampikasvuisina tukahduttaisivat visautuvat puut. Aikuisten haluttua tyyppiä olevien visakoivujen kasvullinen lisääminen muuttaisi visakoivun viljelyn perinpohjaisesti.

Tämän tutkimuksen tarkoituksena onkin ollut löytää menetelmä, jolla solukkoviljelyn avulla saataisiin monistettua parhaita runkomaisia visoja. Lähtömateriaaliksi valittiin aikuisen visakoivun kärki- tai sivusilmun kasvupiste. Elatusalustana käytettiin Murashige-Skoogin alustaa ja prosessi jakautui neljään vaiheeseen. Kasvun aloitus tapahtui alustalla, johon oli lisätty BAP:ia 1 mg/l. Silmujen indusointi tapahtui alustalla, jossa sytokiniinina oli BAP:ia 10 mg/l ja auksiinina NAA:ta 0,2 mg/l. Silmujen puhkeaminen versoiksi saatiin aikaan siirtämällä silmuaiheet alustalle, jossa oli 1/2 × Murashige-Skoogin makromineraalit ja sakkaroosi sekä 1/1 × Mureashige-Skoogin mikromineraalit ja vitamiinit sekä BAP:ia 0,5 mg/l ja IAA:ta 0,5 mg/l. Versojen juurtumiseen käytetty alusta oli sama kuin edellinen, mutta ilman kasvunsäätelyaineita. Syntyneet solukkotaimet siirrettiin turvemultaan kasvihuoneeseen, josta ne 10–20 cm:n pituisina siirrettiin ulos. Syksyllä 1985 jäi ulos talvehtimaan yli 300 visakoivun solukkotainta.