METSÄNTUTKIMUSLAITOKSEN TIEDONANTOJA 728, 1999

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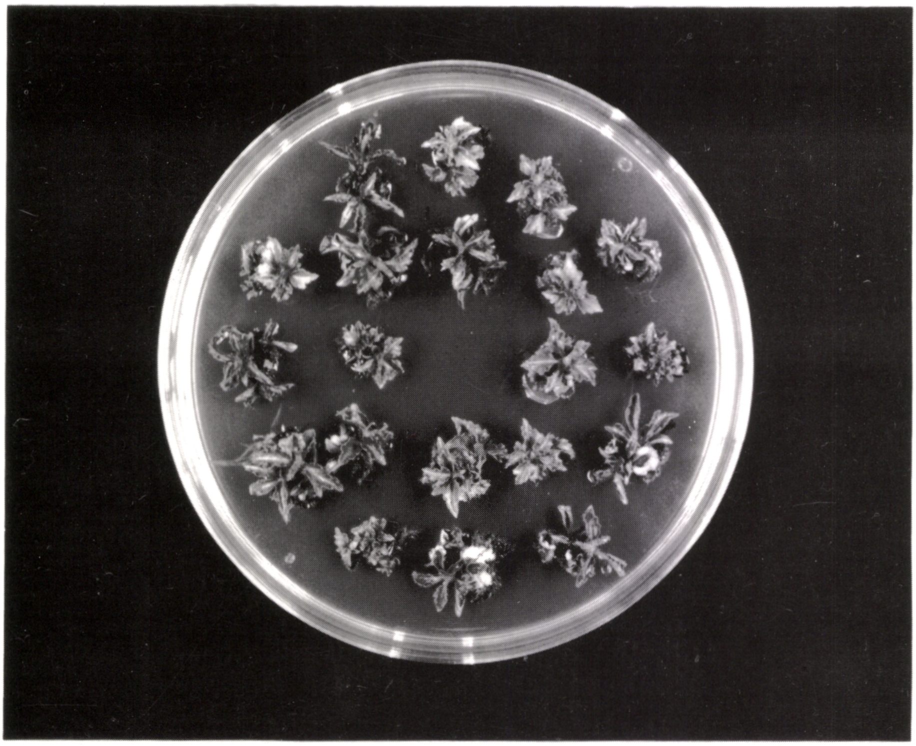

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Cover illustration: Regeneration of the cryopreserved *in vitro* shoot tips of *B. pendula*. Photo by Jouko Lehto.

30.04.99

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Academic Dissertation to be presented, with the permission of the Faculty of Science of the University of Helsinki, for public criticism in the Auditorium of the Division of Systematic Biology, Unioninkatu 44, Helsinki, on May 27th, 1999, at 12 o'clock noon.

University of Helsinki
Department of Biosciences
Division of Plant Physiology

and

Finnish Forest Research Institute
Punkaharju Research Station

1999

Ryynänen, Leena. 1999.
Cryopreservation of buds and *in vitro* shoot tips of *Betula pendula.*
Metsäntutkimuslaitoksen tiedonantoja 728 -
The Finnish Forest Research Institute,
Research Papers 728.
67 p + 5 original papers.
ISBN 951-40-1675-0, ISSN 0358-4283

Publisher:
Finnish Forest Research Institute,
Punkaharju Research Station

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To Päivi and Juhana

ABSTRACT

Ryynänen, L. 1999. Cryopreservation of buds and *in vitro* shoot tips of *Betula pendula*. Metsäntutkimuslaitoksen tiedonantoja 728 - The Finnish Forest Research Institute, Research Papers 728. 67 p + 5 original papers. ISBN 951-40-1675-0, ISSN 0358-4283.

The aim of the present study was to develop cryopreservation methods for both *in vivo* buds and shoot tips derived from *in vitro* shoot cultures of *Betula pendula* Roth. The *in vivo* buds were collected in March and April from five *B. pendula* genotypes of local origin. The *in vitro* shoot cultures were derived from the genotypes collected in March.

According to the results of the study, the prerequisites for successful cryopreservation of both *in vivo* and *in vitro B. pendula* material were the meristematic nature of the explant, cold hardening, slow cooling, and fast thawing. Vegetative buds cold hardened in nature were used as explants for developing an *in vivo* cryopreservation protocol for *B. pendula.* The buds were frozen slowly to a temperature of –38°C before immersion in liquid nitrogen. After fast thawing in a water bath, the buds were cultured according to the normal tissue culture protocol. The successful regrowth of *in vivo* buds was dependent on the correct timing of bud collection in midwinter and the absence of a catkin in the bud. When this protocol was used, there was no significant decrease in the regrowth of *in vivo* vegetative buds after 8 days', 6 months', 1, 3, and 5 years' cryostorage compared to unfrozen buds. *In vitro* shoots were cold hardened under a short day photoperiod and low temperature on a medium containing abscisic acid (ABA) and no ammonium. After cold hardening the shoots were decapitated. The dissected shoot tips were precultured with 5 % dimethylsulphoxide (DMSO) (v/v) and treated with a cryoprotective mixture (PGD) containing 10 % polyethylene glycol (w/v), 10 % glucose (w/v), and 10 % DMSO (v/v) in water and then immersed in liquid nitrogen following slow cooling. After 8 days' storage the shoot tips were thawed rapidly and the cultures were started on ammonium-free medium. On the third day of culture they were returned to ammonium-rich medium. Young shoot cultures with shoot tips containing meristematic cells gave the best survival. Using the protocol the average regeneration of the shoot tips of all genotypes of *B. pendula* was over 58 %. However, the genotypical differences were great, ranging from 15 to 90 %. *B. pendula* plants regenerated after cryopreservation are at present growing in the greenhouse. Leaves for monitoring the genetic stability will be collected during the next growing season and field experiments will be established.

The results show that cryopreservation of *in vivo* and *in vitro* material of *B. pendula* with concomitant regeneration of the plants is possible. The developed cryopreservation method, especially the *in vivo* method, is a complementary technique for other gene conservation techniques of *B. pendula*, and has the advantages of minimum space and maintenance requirements. The *in vitro* application makes it possible to maintain the juvenility of *in vitro* grown material being used for research and breeding programs. With both *in vivo* and *in vitro* applications the regenerated plantlets are juvenile and it takes a number of years before mature, seed producing trees become available.

Keywords: ABA, BA, *Betula pendula* Roth, cold hardening, cryoprotectants, liquid nitrogen, low temperature, regeneration, short photoperiod, slow cooling, substitution of ammonium, silver birch, tissue culture, WPM.

PREFACE

This study was carried out at the Punkaharju Research Station of the Finnish Forest Research Institute. It started as a part of the project "Application of biotechnology to forest tree breeding", and finished in the project "Application of biotechnology for genetic research and gene conservation of forest trees", under the leadership of Docent Hely Häggman, Ph.D. She proposed the theme of this study, and was my co-author in the last paper of the thesis. She was also an excellent "local" supervisor during the course of writing this thesis. I am deeply grateful to her for all her help and encouragement.

My special thanks go to my teacher Professor Liisa Simola for her critical comments on the manuscript and the fact that she patiently encouraged me, even after years of silence from my side, to carry out this thesis.

My sincere thanks go to my colleague Docent Tuija Aronen, D.For., who always had the time to comment on my papers and who, as a former debater at our station, advised me in so many practical matters. I would also thank Docent Aune Koponen, Ph.D., for helping me to determine the natural geographical zones of woody plants. My special thanks go to Ms. Airi Huttunen for her skillful and flexible technical assistance during the laboratory work. So many times we worked in the laboratory from sunrise to sunset, and arranged our holidays in accordance with the time schedules of the experiments. Further thanks are due to Mrs. Rauni Ritola, Mrs. Paula Matikainen, Ms. Aila Viinanen, Ms. Taina Paukkunen, Mr. Jouko Lehto, Mr. Pentti Manninen, Mr. Esko Oksa and all the other persons working at the Punkaharju Research Station.

I would like to express my thanks to the two reviewers, Prof. Olavi Junttila and Prof. Veikko Koski, for their valuable comments on the manuscript. I also thank Mr. John Derome, For. Lic., for revising the English language of the thesis.

I wish to express my warmest thanks to my children, Päivi and Juhana, for their love and support while writing this thesis.

The present study was supported by the Finnish Forest Research Institute, the Regional Fund of Mikkeli of the Finnish Cultural Foundation, and The Niemi Foundation.

Punkaharju, March 1999

Leena Ryynänen

LIST OF ORIGINAL PAPERS

This thesis is based on the following papers and manuscripts, which are referred to in the text by their Roman numerals. All the papers are printed with the permission of the publishers.

I Ryynänen, L. 1996. Survival and regeneration of dormant silver birch buds stored at super-low temperatures. Can. J. For. Res. 26: 617-623.

II Ryynänen, L. 1999. Effect of early spring birch bud type on post-thaw regrowth after prolonged cryostorage. Can. J. For. Res. 29: 47-52.

III Ryynänen, L. 1996. Cold hardening and slow cooling: tools for successful cryopreservation and recovery of *in vitro* shoot tips of silver birch. Can. J. For. Res. 26: 2015-2022.

IV Ryynänen, L. 1998. Effect of abscisic acid, cold hardening, and photoperiod on recovery of cryopreserved *in vitro* shoot tips of silver birch. Cryobiol. 36: 32-39.

V Ryynänen, L. and Häggman, H. 1999. Substitution of ammonium ions during cold hardening and post-thaw cultivation enhances recovery of cryopreserved shoot tips of *Betula pendula* Roth. J. Plant Physiol. In press.

CONTENTS

ABREVIATIONS

ABA	abscisic acid
BA	6-benzylaminopurine
2,4-D	2,4-dichlorophenoxyacetic acid
DMSO	dimethylsulphoxide
FDA	fluorescein diacetate
GA	gibberellic acid
GDH	glutamate dehydrogenase
GOGAT	glutamate synthase
IBA	indole-3-butyric acid;
IPGRI	The International Plant Genetic Resources Institute
2iP	2-isopentenyl adenine
LD	long daylength, a 16/8 light/dark photoperiod
LEA proteins	late embryogenesis abundant proteins
NAA	naphthaleneacetic acid
PEG	polyethylene glycol
PGD	a mixture of 10 % polyethylene glycol (w/v), 10 % glucose (w/v), and 10 % DMSO (v/v) in water
PVP	polyvinylpyrrolidone
PVS2	a mixture of 30 % glycerol (v/v), 15 % ethylene glycol (v/v), and 15 % DMSO (v/v) in the liquid medium with 0.4 M sucrose
RAPD	the random amplified polymorphic DNA
SD	short daylength, an 8/16 light/dark photoperiod
TTC	2,3,5-triphenyl tetrazolium cloride
WPM	woody plant medium

INTRODUCTION

1.1. *Betula pendula*

B. pendula Roth, silver birch, is a wide-ranging woody species that grows throughout the boreal zone of Eurasia (Hämet-Ahti et al. 1989). *B. pendula* is an important raw material for the mechanical and chemical forest industries. Planting of *B. pendula* and *B. pubescens* has increased during the last decades, accounting for 10 to 20 % of the annual area of artificial regeneration during the 1990's (Sevola 1998).

B. pendula has a sympodial, monocasic growth habit: an abscission layer is formed just above the uppermost axillary bud of the long shoot, the shoot tip drops during late spring or summer, and the function of the terminal bud is overtaken by that of the uppermost axillary bud (Romberger 1963, Kalela 1963). Axillary buds develop on the main axis above the leaf primordia of long shoots, and terminally on short shoots (Kalela 1963). A bud is an unextended shoot in which the apical meristem is covered by leaf primordia and two brown scales, formed from rudimentary leaves i.e. stipules, that overlap it (Wareing and Phillips 1975).

For the genus *Betula*, as well as other tree species in the boreal zone, adaptation to the annual cycles in climatic conditions has had an effect on the pattern of the annual rhythm, including an active growing period and different stages of dormancy. In order to unify the terminology of dormancy, it is recommended that the terms para-, endo-, and ecodormancy be used to describe the different stages of the suppressed growth and dormancy of buds (Lang et al. 1987). Paradormancy means the period of inhibited bud growth after the spring burst. The suppression of bud growth moves from the apex (apical dominance) to the growing and mature leaves (foliar inhibition), and then to the axis of the lateral buds before reaching the immediate vicinity of the meristems within the lateral buds themselves (Crabbé and Barnola 1996). Endodormancy has been initiated at the end of this process. Ecodormancy covers the period from the end of endodormancy to bud burst, during which growth is suppressed by environmental conditions.

According to Rinne and co-workers (1994), the embryonic foliage leaves of *B. pendula* for the next growing season develop during the spring and summer, and reach their maximum size in the late summer during summer dormancy. Initiation of the scales has also been observed in late summer at the time when the abscisic acid (ABA) concentration reaches a maximum. The water content of the new buds (ca. 60 % in July) decreases to 37 % in September, and remains at that level until one month before bud burst in spring. The decrease in water content correlates with the increase in the ABA concentration, dry weight, and starch accumulation. The ABA concentration remains at a maximum level from August to November. The bud burst ability of terminal buds remains dormant from July until October, thereafter it increases gradually and reaches a maximum in March-April. The basal buds, however, lack or have weak dormancy (Rinne et al. 1994).

External factors are the primary causes of growth suppression and enhancement in all the stages of dormancy. A short day length, interacting with low night temperatures, mainly induces the cessation of elongation growth and development of the dormancy of *B. pendula* (Håbjørg 1972, Junttila 1980). Non-freezing, chilling temperatures from 0 to 12°C result in increased dormancy during the period of decreased growth ability of the buds in the beginning of dormancy. The effect of chilling is cumulative, and long-term exposure (up to 105 days) to chilling temperatures will gradually release the dormancy in *Betula* species (Hänninen 1990, Myking and Heide 1995, Rinne et al. 1997). Apart from during the initiation of dormancy, night length is assumed to play a subordinate role in the release of dormancy in boreal trees (Hänninen 1990, Myking and Heide 1995). According to the latest results, however, lengthening of the photoperiod has been found to enhance the bud burst of *B. pendula* and *Picea abies* (Häkkinen et al. 1998, Partanen et al. 1998). The bud ontogenesis of *B. pendula* starts after a signal from the light conditions (Häkkinen et al. 1998). Freezing-induced release is not involved in overcoming the bud dormancy of *B. pendula* in nature (Rinne et al. 1997). However, bud burst is not possible after dormancy release until sufficient heat sum accumulation and water supply have been reached.

The cold tolerance of trees fluctuates annually: it is minimal or non-existent in spring and summer. The cold tolerance starts to increase in August – September followed by a sharp increase later in September – November. The maximum hardiness of *Betula, Pinus sylvestris* and *P. abies* is obtained in midwinter, from December to February (Koski 1985, Stushnoff and Junttila 1986). Hardening and dehardening are slow processes producing an increase or decrease in cold hardiness of a maximum of 1°C per day (Beck et al. 1995). Dormancy and cold hardiness tend to overlap in woody plants, although they are regulated somewhat independently (Fuchigami et al. 1982). In addition to the increasing effect of photoperiod and chilling, the timing of hardening correlates positively with the heat sum accumulation (Koski 1985). On the other hand, cold tolerance, as indicated by the presence of putative cryoprotectant LEA proteins in buds of *Betula*, exists in the tissues long after endodormancy had been broken (Rinne et al. 1998). The capability of trees to undergo a change in cold tolerance is genetically controlled. Crossing experiments with *Pinus* species indicate polygenic inheritance of cold tolerance (Rudolph and Nienstaedt 1962, Norell et al. 1986). Reciprocal crossings with *Malus* cultivars indicate the possibility of maternal inheritance of cold tolerance (Harris 1965, Wilner 1965).

Freezing injury in hardy *Betula* species is rare in midwinter. The cold tolerance of native tree species in the boreal zone is based on the freezing tolerance of the tissues, i.e. hardy woody plant cells survive the presence of extracellular ice. In nature, the air temperature seldom falls faster than one or two degrees Celsius an hour, and ice is first formed extracellularly and in the large, non-living tracheids where the water is purest, and then proceeds extracellularly between the cells throughout the tree (Asahina 1956).

1.2. Conventional gene conservation

During the past few decades, the need for germplasm conservation in forest trees has been emphasized for several reasons. The effect of the predicted global warming on forests and their genetic composition has been considered. In the geological past, changes in the composition and even extinction of forests have occurred over the course of thousands of years due to natural changes in climate. During the next 50 to 100 years the predicted change is expected to be relatively rapid. This is because the climate change, the so-called greenhouse effect, will take place faster than species are able to migrate to new habitats (Peters 1990). It is expected that within a few decades the concentration of carbon dioxide in the atmosphere will double, and this will be sufficient to increase the mean global temperature by as much as 5°C by the end of the next century (Schneider 1995). A rise of 1°C in temperature translates into a northward shift of 100 km. According to fossil evidence, however, the migration rate of trees is only between 10 and 45 km per century (Roberts 1989). In addition, the influence of human activity on the genetic diversity of trees has also been more direct. Over-grazing, agriculture and industry have destroyed forests and drastically changed the use of forest land. Forest management has also changed, more forest being regenerated with bred seed and cloned material, foreign provenances and exotic tree species (Gene Resources of ... 1992).

One of the duties of forest tree breeding is to preserve biodiversity within the species also in changing environments. The importance of the evaluation of gene resources and gene conservation has been recognized. Strategies for the management and conservation of the genetic resources of forest trees have been created all over the world. Several papers on the current situation and management guidelines of national and international organizations have been published during the past few years (Falk 1990, Dvorak 1990, Gene Resources of ... 1992, Koski 1996, Engelman 1997).

The “Nordisk ministerråd” appointed a working group in 1990 to evaluate the needs, prerequisites and costs of efforts in the gene conservation of forest trees both in the Nordic countries and internationally. According to the working group, conservation of the main tree species of the Nordic countries, *P. sylvestris*, *P. abies,* and *B. pendula*, is being carried out at the national level by means of natural conservation areas, clone archives, seed orchards, seed and pollen storage. All of these methods were already to some extent being performed in practice in the countries. In addition, the working group recommended establishing 20 – 30 new *in situ* gene reserve forests of ca. 100 ha for autochthonous populations of each of the main species. The group also wanted to strengthen the collaboration between the Nordic countries, especially in the conservation of commercially unimportant, broad-leaved species. The Nordic countries could make the system known internationally and assist other countries with their knowhow (Gene Resources of ... 1992).

Conservation of *Betula* species is a good example of conventional gene conservation methods for forest trees in Finland. Both *Betula* species, *B. pendula* and *B. pubescens*, are within the sphere of gene conservation. In Finland the species are growing in nature conservation areas, ca. 1070 trees are grafted in clonal archives for research and breeding purposes, and over 500 are grafted in seed orchards for the mass production of genetically improved seed. According to the guidelines of the Nordic working group, 4 gene reserve forests have been established for *Betula* in North Finland since 1993 (Yrjänä-Ketola and Karvinen 1997). In these *in situ* and *ex situ* gene conservation methods, the germplasm is exposed to the changing environmental conditions and natural selection and mutations that will change the gene frequencies over the course of time. During seed storage the germplasm is more protected, but the maintenance of clonal integrity is not possible by storing seed and, although the seed of *B. pendula* is orthodox, seed viability decreases during storage. In Finland the birch seed which is currently collected is primarily stored as short- or medium-term material for practical silviculture.

1.3. Biotechnological applications

Rapid progress was made in tissue culture after the finding of Skoog and Miller (1957) that it is the quantitative interaction of auxin and cytokinin that regulates growth and differentiation in tissue culture. Up to now, tissue culture methods have been developed for more than 1000 species (Villalobos and Engelman 1995), including several hundred woody plants. Differentiation in tissue culture progresses in two ways: via organogenesis and via somatic embryogenesis. In organogenesis the culture conditions are adjusted to favour first shoot formation, and the root starts to grow when this stage is finished. In somatic embryogenesis, an embryo with shoot and root primordia develops, and this embryo may germinate further to form a plant. In general, hardwoods differentiate most readily via organogenesis, and conifers via somatic embryogenesis.

Initiation for the organogenetic micropropagation of juvenile genotypes of the genus *Betula* is successful from cambium cells and buds, and that of mature genotypes only from buds. Initiation from intact buds is possible throughout the year under culture conditions with a long day length and a medium containing cytokinins. However, initiation from mature trees is more successful in March – April, since the bud burst ability of buds chilled in nature has reached its maximum (Rinne et al. 1994). It is least successful in May–June, due to damage to the developing tissues during transfer from the field to the laboratory (Huhtinen, pers. com.). Buds obtained from short-day treated seedlings are easier to establish than buds from mature *Betula* trees. They have the highest content of starch and nitrogen, and lower content of phenolics, resulting in more rapid growth in tissue culture (Welander 1988).

After an *in vivo* bud is transferred to tissue culture it will elongate. The number of axillary shoots regenerated from the parental bud is low, ranging from 0 to 3 shoots (Welander 1988). Axillary shoots are vigorous with short internodes and large leaf laminae. Multiplication of the parental bud takes place more easily via adventitious buds, which differentiate from the basal callus of the parental bud. Adventitious buds will develop without a rest period into shoots with long internodes and small leaves (Welander 1988). The vascular connections between developing shoots and the parent structure are formed simultaneously with the formation of the first leaves in adventitious shoots (Romberger 1963). The sympodial growth habit of *Betula* is also seen in tissue culture. Independent of the number and duration of the subcultures, the shoots formed from the parental bud will die after a weeks cultivation (Welander 1988). Adventitious shoots transferred to new medium behave in a similar fashion; they produce from 5 to 8 new adventitious shoots, and start to dry 3 – 4 weeks after transfer (Ryynänen 1993). Drying starts from the terminal bud and progresses basipetally through the shoot. Axillary buds with transparent, pale green scales in the drying adventitious shoot only occasionally form new shoots. The appearance of the axillary shoots is identical to adventitious shoot with long internodes and small leaves. *In vitro* shoot cultures of *Fraxinus excelsior* showed similar changes in growth and dormancy as the tree in nature (Nougarède et al. 1996). However, *Fraxinus* has a monopodial growth habit and it is insensitive to photoperiod. It is possible that the development of axillary buds on the adventitious shoots of microcultured *Betula* is incomplete in the absence of a short day length and low temperature in the tissue culture. This results in the bursting of weak (if there are any at all) axillary shoots incapable of taking over the function of the terminal bud. Otherwise, the miniaturized size of the shoots in tissue culture is dependent on both the decreased number of cells and their smaller size, meaning that the anatomical structure of the tissues, such as the vascular system and lamina parenchyma, are poorly developed (Smith et al. 1986). Nevertheless, genotypical traits such as the size and shape of leaves, as well as the multiplication rate, remain stable from one subculture to another (Jokinen et al. 1991). The rooted shoots recover the normal shape and size of leaves and an appearance similar to that of seedlings soon after transfer to the greenhouse (Viherä and Ryynänen 1994, 1995).

Organogenetic micropropagation of juvenile *B. pendula* succeeded for the first time in 1974 (Huhtinen and Yahyaglu 1974). In Finland, the micropropagation of *B. pendula* was started by Prof. Liisa Simola, who propagated plantlets from the leaf callus of juvenile *B. pendula* f. *purpurea* in the beginning of the 80's (Simola 1985a). The micropropagation of mature silver birch started soon after that by the propagation of *B. pendula* var. *carelica* (Ryynänen and Ryynänen 1986), followed by *B. pendula* and *B.pubescens* (Ryynänen 1988). In Finland, *B. pendula* plants have been commercially micropropagated for silviculture since 1988, a total of about 830 000 plants (Martimo, pers. com.) being produced in co-operation between Enso Gutzeit, Kemira and Hortus. The economic slump at the beginning of the 1990's ended this activity, and since then only *B. pendula* var. *carelica* and some other special forms have been commercially propagated by private nurseries, mostly for landscaping and ornamental purposes. However, a large number of plants of *Betula* species are micropropagated each year for research purposes in the universities and research institutes in Finland (Häggmann 1991). It is also possible to propagate *B pendula* via somatic embryogenesis (Srivastava and Steinhauer 1981, Kurtén et al. 1990), even from mature *B. pendula* (Jokinen et al. 1991). Compared to micropropagation via organogenesis, however, multiplication rate is very low and of no commercial importance.

In vitro conservation has proceeded simultaneously with the development of tissue culture techniques with a high multiplication rate. *In vitro* conservation is based on the technique of restricted growth, in which growth reduction and an increase in the time intervals between subcultures is achieved by modifying the growth conditions, such as lowered temperature, lowered oxygen level, and modified medium (Grout 1995). *In vitro* conservation is routinely used as short-and medium-term storage for the germplasm of some tropical woody plants, e.g. *Coffea arabica* (Kartha et al. 1981) and *Actinidia chinensis* (Monette 1986), the recalcitrant seed of which cannot be stored for long periods. One special form of *in vitro* conservation is encapsulation of the material in alginate beads. This method facilitates the easy transport of material. The encapsulation method for *in vitro* conservation has been used e.g. for the shoot buds of *Morus indica* (Bapat et al. 1987) and the shoot tips of the important tropical forest trees, *Cedrela odorata*, *Guazuma crinita* and *Jacaranda mimosaefolia* (Maruyama et al. 1997a). The *in vitro* conservation technique is not used on a large scale for trees in Finland, but only for storing research material and some special genotypes for shorter periods. As an example, a triploid genotype of *B. pendula* var. *carelica,* E1092, has been stored *in vitro* under slightly modified temperature and light conditions at the Punkaharju Research Station since 1984. The most recommendable biotechnological technique for germplasm conservation is, however, cryopreservation.

1.4. Cryopreservation

Cryopreservation means the storage of material at a very low temperature, usually in liquid nitrogen at a temperature of – 196°C. Most of the information that we have today about cryopreservation comes from work on animal cells and tissues. The landmark paper of cryobiology was that of Polge, Smith and Parkes (1949), which showed that the spermatozoa of a cock survived prolonged freezing at –80°C in 10 - 20 % glycerol. Other classical studies with applications to higher animals are those of Lovelock and Bishop (1959) as well as Mazur (1963). Lovelock and Bishop (1959) studied the effectiveness of cryoprotectants (dimethylsulphoxide (DMSO) and glycerol) in modulating the rise in salt concentration during freezing. Mazur (1963) concentrated on the effect of cooling rate and the probability of intracellular freezing. Cryopreservation studies on plant material started already in the 1960's (Sakai 1960, 1965). Sakai studied the cryotolerance of twigs of woody plants. Survival, in the period before tissue culture, was estimated by planting the twigs in water or sand in a greenhouse and observing budding and rooting of the twigs.

Cryopreservation is based on the reduction and subsequent standstill of the metabolic activities of cells at the temperature of liquid nitrogen. Based on the super-low temperature, one could postulate that biological material could be indefinitely preserved in such a state in liquid nitrogen (Kartha 1985). However, the most long-lasting empirical longevity and stability results for woody plants cover a period of no more than 3.5 -5 years of cryostorage (Niino et al. 1993, 1995, Forsline et al. 1998). According to Niino and coworkers (1993, 1995), no changes were observed at the morphological or cellular level of *in vivo* bud material of *Morus* *bombycis* cryostored for an extended period. In the sense of maintaining the clonal integrity of tree species, the cryopreservation technique has several advantages over conventional conservation methods. Cryopreservation retains the juvenility of stored material and, during cryopreservation, environmental effects such as climate change, natural selection, and the effects of different kinds of stress, which are threatening conserved material under conventional methods, are avoided. One special advantage of *in vitro* cryopreservation is that the explant is already miniaturized and may also be pathogen free. The practical advantages of cryopreservation are the minimum demand for space and maintenance; A liquid nitrogen storage container is passive in nature, and only requires that the liquid nitrogen level is checked regularly and liquid nitrogen added. The material, e.g. ca. 20 dormant buds per se or ca. 25 shoot tips of *B. pendula* with 1.25 mL medium and cryoprotectants, is plugged in a 2 mL cryotube, which is stored in the vapour phase or in the liquid nitrogen of the container. Cryopreservation is economically cost-effective. According to Forsline and coworkers (1998), field maintenance costs of clonally propagated *Malus* trees at the same site were ca. \$75/accession/year. Estimated one-year processing costs, including a desiccation period at –4°C for some weeks, per accession for cryostorage were about \$70. Because cryostorage containers were already available, the only additional maintenance costs, including labour and operating expenses in connection with checking and refilling the liquid nitrogen of an accession, were ca. \$1 per each of the following years.

However, the use of cryopreservation of plant species has its limitations, because e.g. information is needed about the method to be used in regenerating the cryopreserved material. This is because regeneration of the cryopreserved material takes place via tissue culture. On the other hand, this means that all the material needed for the cryopreservation of a selected genotype is a piece of meristematic tissue. Overall, meristematic tissues are most suitable for both freezing and regeneration through tissue culture (Engelmann 1991). As a matter of fact, when cryopreservation is to be used for real long-term storage (one rotation age) of germplasm (Bonner 1990), the requirement of a small piece of tissue only is an advantage. One exception to micropropagation regeneration is certain fruit trees e.g. *Malus*, whose buds are grafted to rootstocks in order to retain the narrower shape of grafts and to prevent delayed flowering, both of which are important aspects in commercial fruit orchards (Zimmerman and Miller 1991, Larsen and Higgins 1993).

The possibility of cryopreserving orthodox seed and pollen instead of storing at temperatures of –20°C has also been studied. The relative reliability and ease of use of liquid nitrogen storage compared to other systems has been considered as an advantage of the cryopreservation of seed. However, temperatures of between –20°C, and –196°C, do not guarantee significantly better seed germination (Stanwood 1985). The germination percentage of seed of *P. abies* after storage at –3°C was equal to that stored in liquid nitrogen for 2 years. However, the rate of germination of the seed was higher after cryostorage (Chmielarz 1998). According to Towill (1985), the storage of pollen at –20°C is sufficient to retain the viability for crossing purposes, but lower temperatures are recommended for germplasm preservation. The same conclusion was drawn by Lanteri and coworkers (1992), although the longevity of the pollen of *P. abies* and a number of *Pinus* species was dependent on the species and not on the storage temperature between –18°C and –196°C. Germination of the pollen of *P. abies* and *Pinus nigra* did not show any decrease, while that of the other *Pinus* species decreased to various levels during storage for 2 years (Lanteri et al. 1993). In any case, irrespective of the storage temperature, the exact genetic integrity of seed and pollen material is unknown.

1.4.1. Freezing of *in vivo* material

The most feasible method for germplasm conservation is the cryopreservation of *in vivo* material. Avoidance of the tissue culture phase before freezing makes the method very fast and simple. In addition, artificial hardening and the use of chemical cryoprotectants which may have toxic effects prior to freezing, are not needed.

Among the first woody plants for which an *in vivo* cryopreservation protocol for gene conservation was developed, were several tropical species of great economical importance that have recalcitrant seed, e.g. *Elaesis guinensis* (Grout et al. 1983) and *Camellia sinensis* (Chaudry et al. 1991). Their germplasm has been conserved through direct immersion of the desiccated embryos or their segments in liquid nitrogen, and regrowth achieved by means of tissue culture.

Another application of this method is based on the early findings of Sakai (1960, 1965), who found that the cryopreservation of the twigs of tree species cold hardened in nature was possible when the twigs were slowly frozen to about –30°C in order to withdraw water out off the cells by means of extracellular freezing without injuring the cells, even when immersed in liquid nitrogen. Since then cryostorage of the buds of several hardwood trees has been performed successfully (Sakai and Nishiyama 1978, Katano et al. 1983, Tyler and Stushnoff 1988a, 1988b, Yakuwa and Oka 1988, Oka et al. 1991, Niino et al. 1993, 1995, Forsline et al. 1998) (Appendix).

1.4.2. Freezing of *in vitro* material

Research on cryopreservation increased during the 1980's when tissue culture methods were applied for several plants, and when *in vitro* conservation had proved to be too laborious. In the case of germplasm conservation, meristems and somatic embryos are most appropriate for cryopreservation. Depending on the species, cold hardening of the donor material can be performed before freezing the explants. Cold hardening prior to cryopreservation of the meristems takes place by cultivating the donor material at low temperature under a normal photoperiod for the species, as with shoot cultures of *Pyrus* (Scottez et al. 1992) and *Prunus* species (Brison et al. 1995), or under a short photoperiod in the case of photoperiodic species such as *Rubus* (Reed 1988,1990) and *Ribes* species (Reed and Yu 1995). In the case of embryogenic tissues, cold hardening can also be achieved by growing the tissue at a low temperature in the dark as in *P. sylvestris* (Häggman et al. 1998) and *Abies cephalonica* (Aronen et al. 1999). ABA can be used to enhance the effect of low temperature and short photoperiod, as for the cold hardening of *Rubus* meristems (Reed 1993), or per se as for the preculture of shoot primordia of *Vanda pumila* (Na and Kondo 1996).

Successful cryopreservation of *in vitro* material can be achieved using actively growing meristematic tissue and either slow cooling or vitrification (Debergh et al. 1992) (fast cooling) methods. The two cooling techniques differ in how the material is pretreated before immersion in liquid nitrogen and in the rate of cooling. The cryoprotectants used for the pretreatment of plant material are mainly the same as those used successfully with animal cells. They can be divided into two categories: those permeating the plasmalemma, e.g. DMSO used for the first time in the plant kingdom in 1968 (Quatrano 1968) and glycerol, and the non-permeating cryoprotectants. The non-permeating additives, most of which act as osmotic agents, are sugars (e.g. sucrose and glucose) sugar alcohols (e.g. sorbitol) and high molecular weight compounds such as polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP) (Chen and Kartha 1987) (See 4.3). Most of the cryoprotectants are toxic at higher concentrations, but they are used at less toxic concentrations (Chen and Kartha 1987) and are often also combined in mixtures (Finkle and Ulrich 1970, Ulrich et al. 1979). A cryoprotectant has a colligative or antifreezing effect; it reduces the amount of extracellular ice formed at a specific temperature. As a consequence of this, the extraxellular salt will concentrate at a slower rate, thus reducing the degree of osmotic cell water loss (Meryman and Williams 1985).

In both the slow-cooling technique and vitrification the explant is first cultured for 2 to 3 days with a cryoprotective agent, and after that shortly incubated and then frozen in a cryoprotectant solution. In the slow-cooling method the samples are frozen slowly at a defined cooling rate to a terminal temperature between –30 to –40°C before immersion in liquid nitrogen. Slow cooling allows cryodehydration of the cells; water is removed from the cells in which the solutions are concentrated to the point where they can be cryostored without intracellular freezing. In vitrification, no ice crystals are formed either intracellularly or extracellularly when the samples loaded with cryoprotectants are passed rapidly through the temperatures where ice crystal growth occurs, by immersing them directly in liquid nitrogen.

The slow-cooling technique has been used for the cryopreservation of meristematic tissues of woody dicotyledons (Kuo and Lineberger 1985, Reed 1988, 1989, 1990, 1993, Brison et al. 1995, Reed and Yu 1995). It has also been used for the cryopreservation of embryogenic tissues of hardwoods (Tisserat et al. 1981, Marin and Duran-Vila 1988, Bertrand-Desbrunais et al. 1988, Marin et al. 1993, Pérez et al. 1997) and conifers (Gupta et al. 1987, Kartha et al. 1988, Galerne et al. 1992, Klimaszewska et al. 1992, Lainé et al. 1992, Find et al. 1993, Nørgaard et al. 1993a and 1993b, Häggman et al.1998, Aronen et al. 1999) (Appendix).

The first plant tissues to be vitrified were nucellar cells of *Citrus sinensis* (Sakai and Kobayashi 1990, Sakai et al. 1990). Since then the vitrification technique has been also applied for the meristems of commercially important woody dicotyledons (Niino et al. 1992, 1997, Brison et al. 1995, Reed and Yu 1995, Kuranuki and Sakai 1995, Maruyama et al. 1997b) (Appendix). The so-called encapsulation-dehydration technique has also been developed simultaneously with vitrification (Fabre and Dereuddre 1990). In this technique the explants are encapsulated in alginate beads and air-dried prior to vitrification. The encapsulation-dehydration technique has been used for the cryopreservation of the shoot tips of woody dicotyledons (Niino and Sakai 1992, Scottez et al. 1992, Plessis et al. 1993, Mari et al. 1995) (Appendix). The benefit of the encapsulation-dehydration technique is that controlled cooling and cryoprotectants are not necessarily needed, but the technique is time consuming because the beads must be handled individually and the encapsulation of shoot tips results in large beads. The future of this technique lies in the cryopreservation of small tissues e.g. somatic embryos of about equal size as a storage phase for artificial seeds.

1.4.3. Thawing, regeneration, survival, and genetic stability

Rapid thawing in a water bath is an appropriate method for thawing most *in vivo* material and tissue cultured cells and meristems. In some cases slow thawing on ice at room temperature has been used as the only thawing method, e.g. for the thawing of cryopreserved *in vivo* material of hardy fruit trees (Sakai and Nishiyama 1978, Tyler and Stushnoff 1988a and 1988b). For *Morus* (Yakuwa and Oka 1988) and *Malus* (Katano et al. 1983) both thawing methods are applicable. Washing with culture medium is recommended to reduce the exposure of cells to toxic cryoprotectant concentrations after thawing (Duran-Vila 1995). However, post-thaw washing has been shown to have a detrimental effect (Withers and King 1979). In connection with vitrification, transfer of the material to progressively less concentrated media is used as well, or instead of washing.

With the exception of some fruit trees such as *Malus* (Tyler and Stushnoff 1988a and 1998b, Forsline 1998), the *in vivo* buds of which are grafted to rootstocks after thawing, cryopreserved material is nowadays recovered by *in vitro* culture with tissue culture medium and conditions specific for the species in question. In some cases, recovery of cryo-damaged tissues has been improved by a decreased light intensity (Henshaw et al. 1985, Benson et al. 1989), or by elimination of ammonium ions (Kuriyama et al. 1989, 96, 97) during the first days of post-thaw cultivation. The latter modification has also been used for the recovery of *in vitro*-grown shoot tips of *Malus* and *Pyrus* (Niino et al. 1992).

The cell death that occours during cryopreservation is associated with the formation of ice, not the low temperature per se (Mazur 1963, Weiser 1970). According to Mazur (1963), water nucleation in the cell is not probable during *optimal cooling rates* due to the lack of nuclei in the protoplasma. This means that ice is formed extracellularly, and the ice crystals protrude into the cells by piercing the plasmalemma and continue their formation intracellularly. However, it is probable that this phenomenon is not the cause of injury but a result of damaged plasmalemma (Kartha 1985, Meryman and Williams 1985). Intracellular ice formation is possible during *supraoptimal cooling rates*, when water is trapped in the protoplasma during freezing. Water retained in the cell will become supercooled along with the decreasing temperature. In such a case a sudden flash of intracellular freezing, nucleated by extracellular ice throughout the cell, is possible (Mazur 1963). The latter case raises the question of how the protoplasma survives intracellular ice. The cells containing ice crystals visible through a light microscope are destroyed at the moment of freezing (Asahina 1956, Levitt 1980). Whether the cells containing thousands of tiny ice crystals can survive is unsure. This may be possible under artificial conditions, but not in nature (Levitt 1980). In any case, slow thawing would be fatal to them: during slow thawing there is a transition of tiny ice crystals, both intra- and extracellular ones, to form larger disruptive ones (Luyet 1937). The most probably injury caused by ice crystals to the protoplasma is mechanical stress and dehydration of the cellular macromolecular structure. One of the most apparent manifestations of these events and the most obvious reason for cell death is membrane destruction (Sakai and Yoshida 1968). This results in the breakdown of cellular compartmentalization, with the consequence that liberated enzymes cause damage in the injured cells (Burke et al. 1976). The cold hardiness of plants such as *Betula*, which tolerate extracellular freezing, means that they can also tolerate drought, because the removal of water from the cells produces a desiccation stress in the protoplasma. Although cell dehydration is suggested to play a primary role in cell death during *suboptimal cooling rates*, desiccation cannot be the sole reason for damage. Cell death is more likely due to interaction of the presence of ice crystals in the tissue, desiccation, and other effects of low temperature e.g. changes in the lipid phase (Burke et al. 1976, Meryman and Williams 1985). The effect of desiccation is due to damage to proteins, which play a crucial role in cell structure and function. As a consequence of desiccation, proteins are denatured e.g. due to the loss of bound water (Lovelock 1957), oxidation of sulphhydryl groups (Levitt 1962), increased solute concentration and changes in pH (Mazur 1963).

The survival of thawed cells can be determined by different techniques depending on the type of tissue. The viability of embryogenic cell and callus cultures with cell typing can easily be assessed by fluorescein diacetate (FDA) (Widholm 1972) or 2,3,5-triphenyl tetrazolium cloride (TTC) vital stains (Steponkus and Lanphear 1967). Viability staining methods cannot usually be reliably employed to determine the survival of shoot tips and meristems. Visual estimation of the regrowth is the only applicable method. On the other hand, when the purpose of the cryopreservation method is to ensure conservation and regeneration of the material for future use, measuring the regrowth is the only reliable evaluation method available.

Totipotency, the ability of a single cell to regenerate into a whole plant, makes successful cryopreservation of some plant cells easy, but it has also disadvantages; the risk of somaclonal variation manifested as variation in morphology, chromosome number, changes in DNA sequences, and levels of gene expression. Using meristems instead of cell and callus cultures for cryopreservation may, however, decrease this risk (Kartha 1985). So far, cryopreservation studies on plants, especially woody plants, have concentrated on the development of cryopreservation techniques, and less attention has been drawn to the assessment of genetic stability. The genetic stability of cryopreserved woody plants has been controlled in only a few cases. No changes in the morphology or chromosome number were found in regenerated *Morus* after long-term storage of *in vivo* buds (Niino et al. 1993). Neither was there any dissimilarity in zymograms for peroxidase isoenzymes or in the banding pattern of leaf proteins compared to untreated *Morus* (Niino et al. 1995). Molecular markers such as the random amplified polymorphic DNA (RAPD) assays can detect genetic changes that are not readily expressed as morphological or physiological variations of the phenotype. RAPD analysis have been performed after cryoprotectant treatment and cryostorage of embryogenic cultures of *P. sylvestris*, and has given no evidence of genetic changes (Häggman et al. 1998), and after the corresponding study of *A. cephalonica* suggesting genetic instability (Aronen et al. 1999). There are only a few reports in the literature of field tests made with cryopreserved material. Plants regenerated from cryopreserved excised embryos of *Elaesis* (Grout et al. 1983), and from embryogenic cultures of *Larix* x *eurolepis* and *Picea mariana* (Klimaszewska et al. 1992), have been established in field trials. The RAPD analysis was performed, and phenotypic traits of cryopreserved and non-cryopreserved *Lolium perenne* and *Festuca rubra* were observed in the field tests (Stadelmann et al. 1998).

1.5. Aims

The aim of the study was to develop cryopreservation methods for *in vivo* and *in vitro* explants of *B. pendula* to serve as complementary and/or alternative methods for the existing *in situ* and *ex situ* gene conservation methods. The possibility of cryostoring material for research or during e.g. long-term progeny testing, was also investigated.

These results have been or will be published in the five original papers included in this thesis. The material and methods of the different papers are listed and the results are summarized and discussed.

2. MATERIAL AND METHODS

2.1. Material

The first experiment presented in paper I was performed with dormant buds of *Betula pendula* Roth, and that in paper II with early spring buds. Twigs bearing vegetative and flower buds of *B. pendula* were collected, in March and April 1993, from five plus trees (E1987, E5201, E5382, E5387, and E5398) growing in a silver birch stand at Punkaharju (61°49'N; 29°18'E; 90 m above sea level). The stand had been established by planting with 1-year-old seedlings of local origin in 1932. The twigs (about 30 cm) were collected from the uppermost third of the tree crown. The average height of the trees was 27 m. Buds with scales and a short piece of attached twig (1 cm) were used as explants for *in vivo* cryopreservation.

The experiments presented in papers III, IV and V were performed with the shoot cultures derived from the same genotypes used in paper I (March collection). The shoot cultures were subcultured every third week and maintained on woody plant medium (WPM) (Lloyd and McCown 1980) containing 4.4 μM 6-benzylaminopurine (BA) in glass jars. The growth conditions for the shoot cultures were a 16/8 hour light/dark photoperiod (LD) with a light intensity of 85 – 114 $\mu Em^{-2}s^{-1}$ at +22°C. The *in vitro* shoot cultures had been cultivated from 20 to 60 subcultures before starting the experiments (III, IV and V). Shoot tips, about 0.3 – 0.4 cm in length and including 4 – 5 leaves with axillary buds from cold- and non-hardened subcultures of shoots (later called donor shoots) of *B. pendula*, were used as material for *in vitro* cryopreservation.

2.2. Factors affecting cold hardening

The *in vivo* material in papers I and II was cold hardened in nature. For the *in vitro* material, the physical cold hardening conditions performed for actively growing donor shoot cultures cultivated on WPM containing 4.4 μM BA were a low temperature and short photoperiod. The temperature used was +5°C and the photoperiod was an 8/16 hour light/dark cycle (SD) with a light intensity of 23 $\mu Em^{-2}s^{-1}$.The effect of both low temperature and SD together were studied for 21 days (Table 1 in III) and for 14 and 28 days (IV). The effect of either low temperature or SD was studied for 14, 21, and 28 days (IV). The effect of cold hardening on cryopreservation was studied using the slow-cooling method with PGD and vitrification with PVS2 (Table 1 in III).

The effect of abscisic acid (ABA) on the cold hardening and regrowth of the *in vitro* shoot tips of *B. pendula* was studied using three different ABA concentrations instead of 4.4 μM BA. In order to find the most effective ABA concentrations, the donor shoots were cultivated on WPM containing 1, 10, or 100 μM ABA at +5°C under SD for 14, 21, and 28 days (IV). The possibility of replacing either SD or low temperature by ABA was studied with 100 µM ABA. The donor shoots were cultivated at +5°C under LD to replace SD, or at +22°C under SD to replace low temperature for 14, 21, and 28 days (IV). The possibility of replacing both SD and low temperature by ABA was studied by culturing the donor shoots at +22°C under LD for 21 days on WPM containing 1, 10, or 100 µM ABA (IV). The effect of ABA was studied using the slow-cooling method with PGD as a cryoprotectant mixture.

The effect of ammonium substitution on cold hardening was performed by cultivating the donor shoots on the WPM in which 5 mM NH_4NO_3 was A) totally eliminated from cold hardening until the first three days of post-thaw culture, after which the material was transferred to medium containing NH_4NO_3, B) the post-thaw culture was started on medium containing NH_4NO_3, or C) NH_4NO_3 was eliminated only during cold hardening, and in the liquid freezing medium (Table 1 in V). NH_4NO_3 was substituted by 10 mM KNO_3 or by 5 mM L-glutamine. Alternatively both 5 mM NH_4NO_3 and 2mM $Ca(NO_3)_2$ were omitted and the nitrogen of the NH_4NO_3 and $Ca(NO_3)_2$ was substituted by 10 mM KNO_3 (i.e. total N = 10 mM) or by 7 mM L-glutamine (i.e. total N = 15 mM). In the latter case, 1.4 mM $Ca(Cl)_2 \times 2\ H_2O$ was included in the media to replace half of the omitted calcium, $Ca(NO_3)_2$. Cold hardening during substitution of ammonium took place on WPM containing 100 µM ABA under SD at +5°C for 28 days (Table 1 in V). Cryopreservation was done using the slow-cooling method with PGD as a cryoprotectant mixture.

2.3. Cooling rates and pretreatments

Both types of material were frozen using two different cooling rates. In the slow-cooling method the samples were stored at ±0°C for 36 hours (dormant *in vivo* buds), or the samples were treated according to the schedule of slow cooling (*in vitro* shoot tips) after which they were frozen slowly (10°C/h) down to –38°C in a programmable controlled-temperature cooling chamber (Lauda Ultra-Kryomat RUK60). After reaching the terminal temperature of –38°C, the samples were kept at this stage for 24 h before immersion in liquid nitrogen (dormant *in vivo* buds) (Table 1 in I, II), or immersion took place immediately when the temperature was reached (*in vitro* shoot tips) (Table 1 in III, IV, V). In vitrification, the material was transferred from approx. ±0°C *(in vivo* buds) (Table 1 in I) or from room temperature (*in vitro* shoot tips) (Table 1 in III) directly into liquid nitrogen.

No precultivation or cryoprotectant treatments were used for *in vivo* buds hardened in nature (I, II), whereas the cryoprotectants used for *in vitro*-grown material depended on the cooling rate.

In slow cooling with PGD, either the cold-hardened or the non-hardened donor shoots were decapitated. The dissected shoot tips were precultured on WPM containing 5 % dimethylsulphoxide (DMSO) (v/v) for 72 hours under the same conditions as during the foregoing subculture. After precultivation, the shoot tips were transferred into 0.25 mL of the liquid WPM without growth regulators in 2 mL cryotubes on ice. 1 mL of PGD cryoprotectant (10 % polyethylene glycol (w/v), 10

% glucose (w/v), and 10 % DMSO (v/v) in water) was added dropwise over a period of 30 minutes. The samples were let to stand for 30 in ±0°C before slow cooling (III, IV, and V).

In vitrification, the dissected shoot tips were precultured on WPM containing 0.7 M sucrose for 72 hours under the same conditions as during the foregoing subculture. After precultivation the material was transferred into a cryotube containing 1 mL of PVS2-cryoprotectant (30 % glycerol (v/v), 15 % ethylene glycol (v/v), and 15 % DMSO (v/v) in the liquid medium with 0.4 M sucrose) at room temperature. The cryoprotectant (1 mL) was changed twice at intervals of 6 minutes. After the third change using 0.6 mL of PVS2, the samples were immediately immersed in liquid nitrogen (III).

2.4. Liquid nitrogen storage

The material was stored in the liquid phase in a liquid nitrogen container for 8 days (I, II, III, IV, V). The effect of the long-term storage period on the survival of the early spring buds of *B. pendula* was observed after 6 and 12 months (Table 1 in I, II), as well as after three and five years (II).

2.5. Factors affecting thawing and regeneration

Two different thawing methods were tested for dormant buds (I). In the fast-thawing method the samples were thawed in a water bath at +37°C for max. 5 min, and in the slow-thawing method they were thawed on ice for 3 h (Table 1 in I). After thawing the *in vivo* buds were surface-sterilized with 70 % ethanol for 1.5 min. The cultures were started according to the published protocol for the micropropagation of *B. pendula* (Ryynänen and Ryynänen 1986) on WPM containing 4.4 µM BA and 2 % sucrose solidified with 0.8 % agar in test tubes, one bud per tube (I, II).

The fast-thawing method was the only method used for the *in vitro*-grown material (III, IV, V). After thawing, the cryoprotectant was drained from the cryotubes and replaced with liquid WPM (after PGD) (III, IV, V) or with liquid WPM containing 1.2 M sucrose (after PVS2) (III) for 30 minutes at +22°C. After changing the medium, the tubes were emptied onto sterile filter paper in a petri dish, from where the shoot tips were subsequently plated on WPM containing 4.4 µM BA and 2 % sucrose solidified with 1 % agar on petri dishes (III, IV, V), 20 shoot tips per dish. Post-thaw cultivation took place under the same culture conditions as shoot cultures under LD with a light intensity of 85 – 114 $\mu Em^{-2}s^{-1}$ at +22°C.

The effect of elimination of ammonium during post-thaw culture was studied by substituting NH_4NO_3 in the liquid freezing and washing media and in the solid post-thaw medium by A) 10 mM KNO_3, B) 2.5 mM L-glutamine and 5 mM KNO_3, or C) 5 mM L-glutamine (Table 1 in V). In these treatments the $Ca(NO_3)_2$ concentration of

WPM remained unchanged. The post-thaw culture was started both on medium containing NH_4NO_3 and on NH_4NO_3-free medium. The material was transferred from the NH_4NO_3-free medium onto WPM containing NH_4NO_3 after 3 or 7 days of culture. Cold hardening of the donor shoots took place on WPM containing 100 μM ABA under SD at +5°C for 28 days. Substitution of ammonium during regeneration was studied with *in vitro* shoot tips of *B. pendula*. Cryopreservation was carried out using the slow-cooling method with PGD as a cryoprotectant mixture (Table 1 in V).

The survival of the material was estimated visually after 2 and 4 (I, II) and after 2, 4 and 6 (III, IV, V) weeks of post-thaw culture. Buds and shoot tips, which regenerated directly or formed adventitious shoots from callus, were classified as regrowing ones. Regrowing buds and shoot tips, and those forming undifferentiated callus or remaining green, were considered to be viable. The regrowth rate was calculated as a percentage of the regrowing *in vivo* buds after 2 weeks of post-thaw culture (II). In the cryopreservation studies with *in vivo* material, 20 buds per genotype were used for each experiment with three replicates in the March collection (I). In the April collection, 10 buds per genotype were used for each experiment with three replicates, except for the 8 days of storage that was performed only once, and for the 5 years storage period when all the buds that were left (122 in total) were used (II). In the cryopreservation studies with *in vitro* material, in the cold hardening experiments 10 (IV) and in the cold hardening and cooling experiments, 60 (III) shoot tips per genotype were used for each experiment with three replicates. In the experiments on the effect of ABA (IV) and substitution of ammonium ions (V) on cold hardening and recovery, 20 shoot tips per genotype were used for each experiment with three replicates.

2.6. Controls

The controls for the *in vivo* material were untreated dormant buds, the *in vitro* culture of which started at the same time as cryopreservation (I, II). The number of control buds in the March collection was 20 per genotype with three replicates (I). In the April collection the number of unfrozen control buds was 10 per genotype without any replicates (II). In the cold-hardening experiments with the *in vitro* material, the control shoots were placed on WPM at the same time as the pretreatments for the other material were started. In the slow-cooling and vitrification experiments, the controls were treated in the same way as the other material, except that they were not frozen. The number of control shoot tips in the experiments 15 per genotype with three replicates (III). In the ABA experiments the controls were cold hardened in the same way as the other material, but without the ABA treatments. The number of control shoot tips was 10 per genotype with three replicates (IV). In the ammonium substitution experiments, the control shoots were cultivated on WPM in the same way as the other material, but without ammonium substitution. The number of control shoot tips was 20 per genotype with three replicates (V).

2.7. Statistical analysis

Statistical comparisons between the treatments were performed using one- and two-way analysis of variance, and the means were compared using Tukey´s test (I, III) or the Student-Newman-Keuls multiple range test (II, IV, V) (BMDP/DYNAMIC program).

3. RESULTS

3.1. Cryopreservation of *in vivo* buds

Collecting the buds in midwinter and slow cooling were the prerequisites for successful cryopreservation of *in vivo* buds (I, II). The survival was zero after the fast-cooling treatment. The mean regrowth of the *in vivo* buds of *B. pendula* after March collection, stored for 8 days in liquid nitrogen, was 78 % for fast-thawed buds and 82 % for slowly-thawed buds (Fig. 1 in I). The difference between the fast- and slow-thawing methods was not significant. Moreover, there were no significant differences between the average regeneration after cryopreservation and the unfrozen control. The regrowth of different genotypes after cryopreservation did not differ from that of the corresponding unfrozen controls, although genotype E5382 had a significantly lower regrowth percentage than the best regenerating genotypes (Table 2 in I). The post-thaw regrowth of the buds started without any lag phase.

3.1.1. Long-term cryostorage

The mean regrowth of *in vivo* buds which were collected in April was 65 % after 8 days of storage. The regrowth was lower than the corresponding result after March collection, but not significantly. The viability of the buds remained unchanged after five years of cryostorage (99 %). The regrowth of the buds decreased during the same storage period from 86 % of the non-cryopreserved controls to an average of 47 % of the cryopreserved buds. However, the decrease in regrowth was not significant (Fig. 2 in II). The regrowth ability of the buds was dependent on the presence or absence of a female catkin in the bud. The regrowth of the vegetative buds without a female catkin was good after five years of cryostorage (66 %), but there was a significant decrease in the regrowth ability of the buds growing in the axil of a female catkin (26 %) compared to the corresponding unfrozen controls (72 %) (Fig. 3 in II). The regrowth rate, as reflected by the percentage of regrowth after two weeks, decreased significantly both with and without a catkin within the five-year period in liquid nitrogen (Fig. 4 in II).

3.1.2. Protocol for the cryopreservation of *in vivo* buds of *B. pendula*

Based on the above results, the optimized protocol for cryopreservation of *in vivo* buds of *B. pendula* is as follows. Vegetative buds without a female catkin, which are cold hardened in nature, are stored at ±0°C for 36 hours, after which they are frozen slowly at a cooling rate of 10°C/h to a terminal temperature of –38°C. After about 24 hours at this temperature the buds are immersed in liquid nitrogen for the desired storage period. After fast thawing (max. 5 min in water bath at +37°C) the buds are surface sterilized and cultured according to the normal tissue culture protocol (Fig. 1).

3.2. Cryopreservation of *in vitro* shoot tips

The prerequisites for the successful cryopreservation of the *in vitro* material were cold hardening and slow cooling. Cryopreservation of non-hardened material and/or using vitrification resulted in minimal survival. When cold hardening was obtained by culturing the donor shoots at +5°C under SD for at least 21 days on the culture medium containing 4.4 μM BA, the mean regrowth of the buds was 22.6 % (Table 2, Fig. 2 in III). Either low temperature or SD alone did not induce cold hardening. The cold-hardened donor shoots were shorter and thicker than the non-hardened ones. The leaves were yellow, in contrast to the green leaves of non-hardened donor shoots (Fig. 3 in IV). In all cases the regeneration of cryopreserved shoot tips was delayed compared to the unfrozen controls. When cold hardening took place using BA as a growth regulator, no differentiated tissues survived cryostorage. The regeneration of new shoots and leaves took place during the entire post-thaw culture period. New shoots always grew from the axillary buds in the basal part of the shoot tips without any callus formation (III). There were differences between the genotypes, the regrowth of genotype E5387 (58.9 %) being significantly better than that of the others (Fig. 2 in III).

3.2.1. Effect of ABA on cold hardening

When 100 μM ABA was used as a growth regulator and the donor shoots were cultured at +5°C under SD for 28 days, the regrowth of the shoot tips doubled (42.5 %) compared to that when the donor shoots were cold hardened on the medium containing BA under the same conditions for 28 days (17.4 %) (IV). Lower ABA concentrations and/or shorter cold hardening periods did not increase the regrowth significantly (Fig. 1 in IV). When the donor shoots were cold hardened at +5°C under LD on the medium containing 100 μM ABA for 14, 21, or 28 days, the mean regrowth percentages increased with the increasing cold hardening periods, but not significantly (Fig. 2 in IV). Replacing low temperature or low temperature and SD together was not successful by using ABA (IV).

When cryopreservation took place using ABA instead of BA as a growth regulator during cold hardening, the morphology of the donor shoots differed in the colour and abscission of the leaves; more yellow, shed leaves were found on the medium with ABA (Fig. 3 in IV). Some differentiated leaves survived cryostorage. During the post-thaw culture they remained green and became relatively hard and thick. In addition, some callus formed from the first meristems, which started to regenerate, and several new shoots and leaves grew from some of them (Fig. 3 in IV). The shoot tips, which started to regenerate later, had less or no callus formation. The concentration of ABA (100 μM) was the most effective concentration for all the other

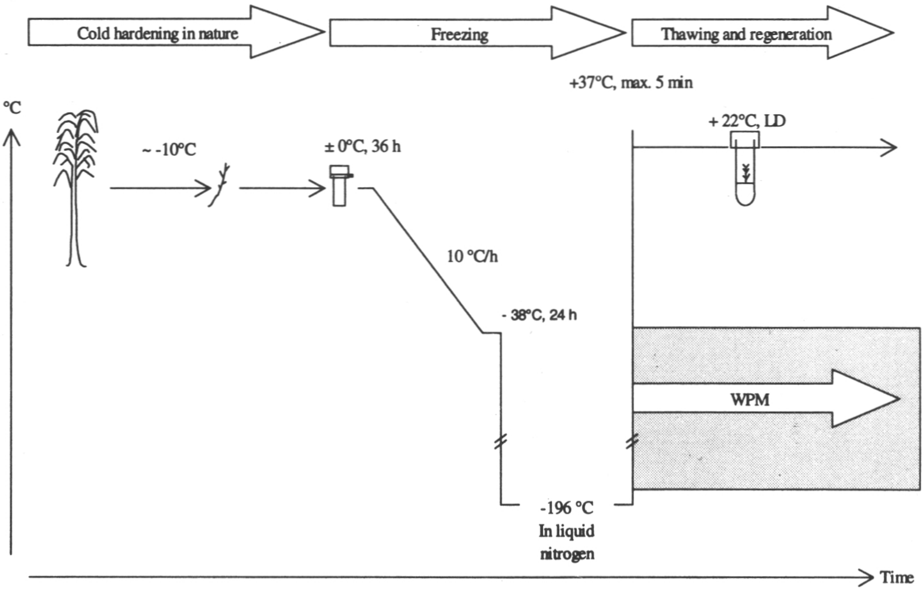

Fig. 1. Cryopreservation protocol developed for dormant in vivo buds of B. pendula (Design of the figure is modified from Helliot and de Boucaud 1997).

genotypes except E5387, which regenerated better after cold hardening on the medium containing 10 μM ABA (Fig.1 in IV).

3.2.2. Effect of ammonium substitution during cold hardening

The regrowth of the cryopreserved shoot tips cold hardened on the medium containing 5 mM NH_4NO_3 substituted by inorganic nitrogen or glutamine was increased by all the substituents. The average regrowth of all genotypes using the treatment, in which all inorganic nitrogen (5 mM NH_4NO_3 and 2 mM $Ca(NO_3)_2$) of the medium was substituted by KNO_3 (10 mM) in the solid cold hardening medium, in the liquid freezing medium, in the liquid washing medium, and in the solid post-thaw medium for the first three days before transferring to the ammonium-rich culture medium, was the only one which differed significantly from the regrowth of the control. The average regrowth after this treatment was 58.3 %, i.e. almost threefold higher than that of the control, 22.7 %. In all genotypes the average recovery increased as a consequence of ammonium substitution, but genotypical differences in the recovery of the cryopreserved *in vitro* shoot tips were still obvious, the best regrowth of E5387 being 88.3 % and the lowest one 15 % in the case of E5382. However, regrowth after all treatments with ammonium substitution throughout cold hardening was higher than that in the control or in the treatments in which ammonium was substituted after the cold hardening phase. However, there were no significant differences between the substituents used or the treatments within each substituent (Fig 2 in V).

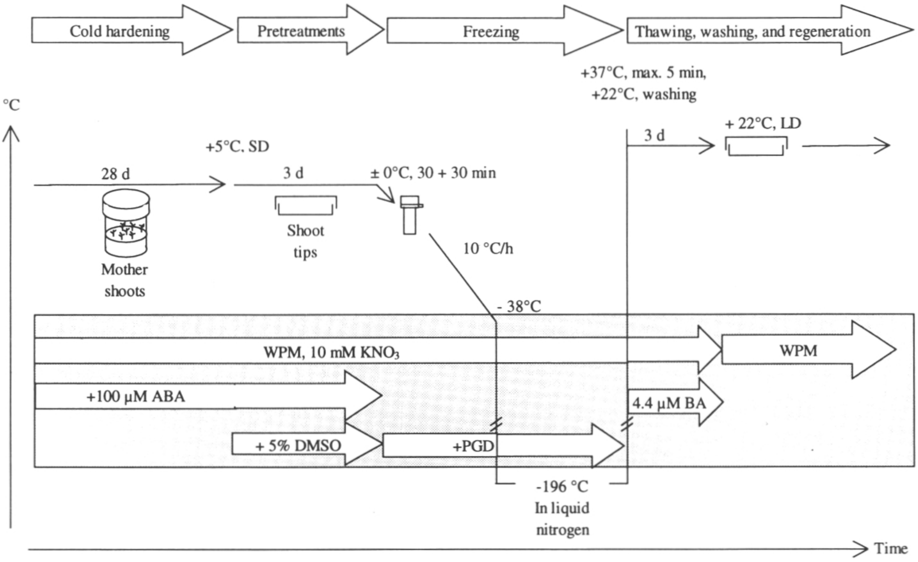

Fig. 2. Cryopreservation protocol developed for in vitro shoot tips of B. pendula (Design of the figure is modified from Helliot and de Boucaud 1997).

The morphological appearance of the regenerating shoot tips after cold hardening on the medium containing inorganic substituents was excellent; the shoots were straight and vigorous without callused leaves, but smaller than normally on the medium with a reduced nitrogen concentration (Fig. 3 in V). When the ammonium ions or the total nitrogen amount of medium were substituted by glutamine 5 mM or 7 mM, respectively, hard callused, brown leaves were formed, and numerous leaves and shoots regenerated on their edges.

3.2.3. Effect of post-thaw ammonium substitution

The regrowth of the frozen shoot tips was increased when NH_4NO_3 (5 mM) in the post-thaw culture medium containing 4.4 µM BA was substituted by KNO_3 (10 mM) or by L-glutamine (5 mM). When NH_4NO_3 was substituted by a combination of KNO_3 (5 mM) and L-glutamine (2.5 mM), the regrowth was decreased (Fig. 1 in V). The average growth percentage of the shoot tips (46.1 %) in the treatment where NH_4NO_3 was substituted by L-glutamine in the liquid freezing medium, in the liquid washing medium, and in the solid post-thaw medium for the first three days of the post-thaw culture before transferring to the ammonium-rich culture medium, was the only one that differed significantly from that of the control (22.7 %) (Fig. 1 in V). Elimination of ammonium in the post-thaw medium had no effect on the morphology of the recovering shoot tips (V).

3.2.4. Protocol for the cryopreservation of *in vitro* shoot tips of *B. pendula*

Based on the above results, the optimized protocol for cryopreservation of *in vitro* shoot tips of *B. pendula* is as follows. Donor shoots are cold hardened for 28 days at +5°C under SD on medium containing 100 µM ABA and all the inorganic nitrogen of the medium substituted by 10 mM KNO_3. The same nitrogen composition of the medium is used in the following liquid freezing and washing media, and in the post-thaw medium during the first free days. Donor shoots are decapitated after cold hardening, and dissected shoot tips are precultured under the same conditions for 72 hours on the same medium containing additionally 5 % DMSO. The material is then transferred to cryotubes containing 0.25 mL growth regulator-free medium on ice, and 1 mL of a mixture of PGD is added dropwise over a period of 30 minutes. The samples are let to stand for 30 minutes at ±0°C before freezing. The material is slowly cooled to −38°C and immersed in liquid nitrogen immediately after reaching the terminal temperature. After fast thawing, max. 5 min in a water bath at +37°C and washing at +22°C, the culture is started on the ammonium-free medium, from which the shoot tips are transferred to ammonium-rich WPM after three days of culture (Fig. 2).

4. DISCUSSION

In this work functional cryopreservation methods have been developed for both *in vivo* and *in vitro* explants of *B. pendula*. The prerequisites for successful cryopreservation were the meristematic nature of the explant, cold hardening, slow cooling, and fast thawing.

4.1. Meristematic nature of the explant

In the case of *in vivo* material, a bud is the only type of the explant that can be used (I, II). This is because, after thawing, a bud is the explant to be regenerated. Mature *B. pendula* can only be propagated through tissue culture when the undifferentiated cells, i. e. the initial cells of the promeristems, are used (Ryynänen and Ryynänen 1986). On the other hand, meristematic cells most likely withstand freezing; they are small, contain only a few vacuoles (i.e. only a small amount of water), their cytoplasm is dense, and their nucleo-cytoplasmic balance is high (Engelmann 1991).

The buds of *B. pendula* in this study were frozen with scales and a piece of attached twig. A study on the cryopreservation of *Morus* (Yakuwa and Oka 1988) showed that the survival of cryopreserved *in vivo* buds was dependent on the presence or absence of surrounding tissues like scales and a segment of the twig. The scales protecting the bud were the most important factor. In addition to the scales, inclusion of a segment of the twig reduced injury to the meristems in cryopreserved *Morus*. The hardiness of the *Morus* twigs was not measured (Yakuwa and Oka 1988), but twigs of maximally hardened *Betula* and *Salix* were hardy down to –80°C, which was about 20°C lower than the temperature that the buds alone could stand (Stushnoff and Junttila 1986). In the present study on *B. pendula*, the presence of a catkin in the bud drastically decreased the regrowth ability and regrowth rate after long-term cryostorage (II). The smaller size and different site of the vegetative bud in the axil of the female catkin may be one reason for the decline in regrowth. Another reason could be growth inhibition induced by apical dominance from a female catkin. The different tolerance of the two bud types of *B. pendula* to cryostorage is in accordance with the tolerance of apical and axillary shoot tips of *Dianthus caryophyllus* to freezing in liquid nitrogen. The regrowth of apical shoot tips of *Dianthus* is close to 100 %, whereas that of axillary shoot tips decreases with increasing distance from the apical shoot tip during one week of cryostorage (Deuddre et al 1988).

Although *B. pendula* is rejuvenated during tissue culture (Viherä-Aarnio and Ryynänen, 1994, 1995), the regeneration of cryopreserved *in vitro* shoot tips starts from the less differentiated tissues; regrowth of shoot tips cold hardened on the medium containing BA is possible only from axial meristems derived from the basal part of the shoot tips which were in connection to medium (III). Callus production of material cold hardened on the medium containing ABA makes the observation of the initially regenerating cells more unsure (IV). The importance of the meristematic nature of an *in vitro* explant has often been proved by the cryopreservation of different plant species. Most of the surviving cells of shoot apical meristems of *Pisum sativum* were located in primordial leaf tissue and lateral areas of the dome (Haskins and Kartha 1980). Only meristematic tissues of the shoot apex survived freezing in the cryopreservation of *Dianthus* (Seibert and Wetherbee 1977). Suspension-cultured cells of *Acer pseudoplatanus* (Sugawara and Sakai 1974) survived freezing better during the late lag or early cell division stage, when the cells were small and dense. Head cells of the embryonic cultures of conifers survived cryopreservation e.g. *Pinus* spp. (Gupta et al. 1987, Lainé et al. 1992, Häggman et al. 1998) and *Picea* spp. (Gupta et al. 1987, Kartha et al. 1988, Klimaszewska et al. 1992, Find et al. 1993). On the other hand, when the donor shoots of *B. pendula* were cold hardened and regenerated on the medium containing L-glutamine, leaves and shoots regenerated from the leaf tissues. They were initiated on the edges of callused leaf blades, which were in contact with the medium (V). Valobra and James (1990) and Leege and Tripepi (1993) have also reported the effect of medium contact on the initiation of shoots from the leaf blades of *B. pendula*.

When dissected shoot tips of *B. pendula* were transferred onto the preculture medium, they were about 0.3 - 0.4 cm in length and included 4 - 5 leaves with axillary buds. The regrowth of cryopreserved *in vivo* buds is dependent on the presence or absence of surrounding tissues, and the same effect has been reported for the *in vitro* shoot tips. The number of leaf promordia sets in the explant influenced the survival of *Dianthus* (Seibert and Wetherbee 1977). Maximal survival rates occurred with shoot tips containing two or three sets of leaf primordia or more. Shoot tips of *Prunus* spp. with a size of 1.5 to 2 mm survived freezing and regrew easier and faster than the smaller ones (Brison et al. 1995).

In the present study, a loss of cryopreservability was seen in the regrowth of the shoot tips frozen with ABA after 60 subcultures (22.7 %) compared to those frozen with ABA after over 20 subcultures (42,5 %). It is obvious that the long-term *in vitro* culture before cryopreservation reduced the ability of the shoot tips to survive cryopreservation. This is in accordance with the cryopreservation results for *Solanum tuberosum* cultivars (Harding et al. 1991). The regrowth of *Solanum* shoot tips was significantly reduced when cryopreserved after three years' *in vitro* culture (ca. 5 %) compared to that after some weeks' culture (ca. 40 %).

4.2. Cold hardening at low temperature and under a short photoperiod

At the cellular level, the accumulation and synthesis of compounds with a possible cryoprotective effect, such as sugars (Sakai and Yoshida 1968, Guy et al. 1980, Koster et al. 1989, Koster and Lynch 1992, Hansen and Beck 1994) and proteins (Sakai and Yoshida 1968, Craker et al. 1969, Pomeroy et al. 1970, Kushad and Yelenosky 1987), takes place during cold hardening. Biochemical changes in proteins are often associated with protein changes in plasma membranes (Yoshida 1984). The synthesis of low-temperature, stress-related proteins not present in non-hardened tissues has recently also been detected in cold-hardened trees including *B. pubescens*

(Guy et al. 1988, Wiesniewski et al. 1996, Rinne et al. 1998) (See 4.2.1. and 4.3). On the other hand, studies on enzymes from trees subjected to cold hardening and dehardening have demonstrated changes in enzyme activity, isoenzymic variation, and freeze stability (McCown et al. 1969, Hall et al. 1970, Beck et al. 1995).

In the present study, the *in vivo* buds hardened in nature were collected in March (I) and later in April (II). Collection in March, at the time when *Betula* possesses hardiness varying from 20 to –40°C at Punkaharju (Koski 1985) resulted in successful regeneration, but collecting the twigs in April was probably too late for long-term cryostorage because *B. pendula* had obviously already begun to loose its hardiness. A decrease in regrowth (65 %) of *B. pendula* was seen already in the April material (II) cryostored for 8 days compared to the average regrowth percentage (78 %) for March collection (I). This is in accordance with the result for *B. pubescens* reported by Rinne and coworkers (1994), indicating that the first anatomical changes take place about one month before bud burst in April. The loss of cold tolerance in *P. sylvestris* after the middle of April in Finland, and its effect on the cryopreservation of *P. sylvestris* buds, was even more pronounced. The viability of the buds cryopreserved in the middle of April was 27 %, while that of the buds cryopreserved in the beginning of April was 100 % (Kuoksa and Hohtola 1991). In the present study, collection in March was selected because bud burst ability reaches a maximum in March-April (Rinne et al. 1994). The selected time also falls within the period of maximum cold tolerance of *Betula* measured at Punkaharju (Koski 1985), although the timing of maximum annual hardiness varies according to heat sum accumulation, which also varies from year to year (Koski 1985). On the other hand, temperatures lower than –40°C were not tested at Punkaharju. According to Stushnoff and Junttila (1986), buds of *Betula* can tolerate the temperatures of –60°C even.

The improved regrowth of the *in vitro* material in the present study resulting from cold hardening at the temperature of +5°C was expected, although the ability to cold harden in the field does not necessary relate to the ability of apical meristems of *in vitro* plants to cold harden and survive liquid nitrogen (Reed 1990). There was no correlation between the cryotolerance of the embryogenic cultures of *P. abies* and the cold hardiness of the progeny trials of the same parents in the field (Nørgaard et al. 1993b). However, the survival of cryopreserved *in vitro* shoot tips of even temperate and subtropical tree species has been improved by cold hardening (Reed 1988, 1990, Scottez et al. 1992, Brison et al. 1995, Helliot and de Boucaud 1997). It is obvious that the influence of low temperatures on trees from warmer geographical zones is due to changes in the physical stage of the membranes and the rate of enzymatic reactions (Crabbé and Barnola 1996). During cold hardening, the temperature of the donor material has been continuously lowered to near 0ºC in *Pyrus* species (Nino et al. 1992, Scottez et al. 1992), has been lowered more (10/5°C) during the dark period in *Camellia* (Kuranuki and Sakai 1995), or has been lowered only during the dark period (22-25/–1°C) e.g. in several berry bushes (Reed 1988, 1990, 1993, Reed and Yu 1995) and *Prunus* (Brison et al. 1995). The cold hardening time used for woody plants varied from 24 hours at +4°C in *Prunus* (Helliot and de Boucaud 1997) to 8 – 12 weeks at 0°C in *Pyrus* (Scottez et al. 1992). In the present study, the regrowth of *in vitro* shoot tips of *B. pendula* was inferior, and varied greatly depending on the genotype, compared to that of the buds hardened in nature of the same genotypes. Cold hardening of genotypes under periods shorter than 3 weeks resulted in insufficient cold hardening. Prolonging the hardening time to 4 weeks resulted, on the other hand, in a slightly decreased cryopreservability. It is evident that in an artificial hardening method based, in this case on physical conditions i.e. the length of the period at low temperature and under a short photoperiod, different exposure durations, chilling temperatures, and photoperiods should be used for different genotypes. Studies on *B. pubescens* indicate that there may be considerable variation in the photoperiodic response within a population (Håbjørg 1978). The genotype-dependent ability to cold harden has been reported in cryopreservation studies on different genotypes of *Rubus* species (Reed 1993) and *Lilium* sp. (Matsumoto et al. 1995).

4.2.1. Improvement of cold hardening by ABA

ABA is usually considered to be a growth inhibitor that is involved in dormancy induction in plants, although it can also stimulate callus and adventitious bud formation in dicotyledons (Heide 1968, Altman and Goren 1974). ABA may be responsible for the timing of cold hardening in photoperiodic-sensitive trees. The ABA level of *B. pubescens* was increased under natural SD conditions before hardening. Desiccation, osmotic adjustment and accumulation of ABA responsive LEA (RAB) proteins accompanied cold hardening in *B. pubescens* (Rinne et al. 1998).

According to the results of the present study, ABA cannot be used to replace SD, low temperature or both, for the cold hardening of *B. pendula* meristems. It has, however, been possible to compensate physical cold acclimation by means of ABA, e.g. in cell suspension cultures of *Triticum aestivum*, *Secale cereale* and *Bromo inermis* (Chen and Gusta 1983). When ABA was used instead of SD in the present study, it increased the degree of cold hardiness of *B. pendula* to some extent, but not significantly. The result is similar to that obtained with seedlings of *B. pubescens*. Freezing tolerance estimated for the bud apical meristems of seedlings of *B. pubescens* grown under LD conditions was increased after exogenous ABA application for 3 weeks, but was significantly less than that in seedlings under SD (Welling et al. 1997). For dormancy induction in the buds of *B. pendula* and *B. pubescens*, exogenous ABA was less effective than SD or water-stress treatment (Rinne et al. 1997). The finding that ABA is effective only in combination with low temperature and SD, is in accordance with the result for *Rubus* (Reed 1993); ABA was effective in increasing cold hardiness when combinated with a low temperature compared to a low temperature alone. The survival rate of V*anda* shoot primordia precultured in a medium containing ABA before desiccation was high (65 %), while those without the ABA treatment did not withstand freezing (Na and Kondo 1996). The genotypical differences in *Rubus* species with respect to their ability to cold harden at a low temperature, were also evident when ABA was included in the cold-hardening treatment (Reed 1993). In the present study, the ABA supplement in the cold hardening medium doubled the average regrowth of the shoot tips of *B. pendula*, but the genotypical differences did not diminish. An ABA-deficient *B. pubescens* f. *hibernifolia* showed reduced water loss and osmoregulation, absence of inducible

LEA proteins and delayed or reduced cold hardiness after cultivation under SD conditions (Rinne et al. 1998). ABA applications to ABA-deficient *B. pubescens* f. *hibernifolia* did not lead to the accumulation of LEA proteins under LD conditions (Rinne et al. 1998). In the present study, cold hardening of *B. pendula* with ABA under LD increased the cryopreservability of the genotypes to different levels, except for E5382, which did not survive at all.

In the present study, the preferred ABA concentration, 100 μM, was rather high compared to that used for other *in vitro* cultured meristems (IV); concentrations of 50 μM ABA used for *Rubus* meristems (Reed 1993) and 4 μM for shoot primordia of *Vanda* (Na and Kondo 1996). Also, the effective cold-hardening period needed for *B. pendula* was long compared to the 3 days used for *Vanda* (Na and Kondo 1996) and 7 days for *Rubus* meristems (Reed 1993). Extending the cold hardening period to over 7 days, which was required for maximal hardiness with 10 μM ABA in cell suspension cultures of *Betula papyrifera*, resulted in the loss of freezing tolerance (Tremblay et al. 1992). In the present study, ABA enhanced cryopreservability only when combined with low temperature and SD. ABA did not increase the rate of cold hardening, but the results indicate that ABA prolonged the capacity for cold hardening. Thus, by using exogenous ABA, it was possible to continue cold hardening and to reach better cryopreservability than without ABA. The high exogenous ABA concentration needed in the present study might be partly degraded in the medium due to the effect of light as well as partly in the cells (Guy 1990), during the long exposure time.

The effect of ABA in combination with a low temperature and SD caused morphologically detectable caracteristics such as more yellow, shed leaves, expressing the better hardiness of the *B. pendula* donor shoots and the formation of callus during the regeneration of the shoot tips (Fig. 3 in IV). Callus formation in shoot tips of *B. pendula* may reflect the increased cryotolerance of explants; less or only partially injured meristems formed callus, and some of them were capable of organogenesis. Callus formation from shoot tips after thawing is suggested to be an indication of cryoinjury (Kuo and Lineberger 1985, Reed 1993). Unlike *B. pendula*, callus formed in cryopreserved *Malus* shoot tips (Kuo and Lineberger 1985) did not regenerate. The result was the same with the meristems of *Rubus* species (Reed 1993), even though they were cold hardened with ABA. The decreasing callus formation during the course of post-thaw regeneration of *B. pendula* may also indicate that callus formation by regrowing shoot tips is an aftereffect of ABA, since ABA can increase callus formation in woody species, e.g. *Populus tremuloides* (Schier 1973) and *Citrus* (Altman and Goren 1974). ABA also has the ability to stimulate adventitious bud formation (Heide 1968) but, taking into account the high regenerability of the callus of *B. pendula* originating in bud explants, which is also seen after cryopreservation of *in vivo* material (II), such an effect of ABA on *B. pendula* is not probable. Although the regeneration of some genotypes of *B. pendula* increases due to callus formation after cryopreservation, callus formation per se is not favored because of the increased probability of somaclonal variation in the regenerated material (Lester and Berbee 1977, Cameron 1990).

Although the SD conditions, low temperature and the role of ABA used in inducting the cold tolerance of *B. pendula* in this study are similar to those prevailing in nature, the actual physiological state of the buds is different during cryostorage and under a fluctuating winter temperature. Cold tolerance of cryopreserved buds is required during the slow cooling before cryostorage, after which the buds are held at a stable, super-low temperature with the reduction and subsequent standstill of metabolism (Kartha 1985). In contrast, buds in nature maintain their metabolic activity at a basal level even at low temperatures (Guy 1990). The extent to which natural cryoprotectants and the changed metabolism of cold-hardened *in vitro* shoot tips affects the cryopreservability during slow cooling is not known. Dehydration of the buds already during cold hardening, and the almost free movement of water out of hardened cells, however, may result in a more appropriate cryodehydration and concomitant increase in cryopreservability. The unsuccessful cryopreservation by vitrification of shoot tips loaded with cryoprotectants confirms this assumption.

4.2.2. Improvement of cold hardening by ammonium substitution

The treatment in which all the inorganic nitrogen of the medium was substituted by 10 mM KNO_3, starting from cold hardening up until the third day of post-thaw culture, gave the highest regrowth, which was almost threefold that of the control (V). One explanation for the increase in regrowth after ammonium elimination could be that exclusion of ammonium at the time of low temperature decreased its cytotoxic effect. Ammonium ions, which are toxic to plant cells, are usually rapidly incorporated into organic compounds without accumulation in plant cells (Kirby et al. 1987, Bonga and von Aderkas 1992). When the metabolic activity slows down during low temperatures the functioning of the key enzymes of ammonia-nitrogen assimilation, glutamate dehydrogenase (GDH) and /or glutamate synthase (GOGAT), may cease or be retarded leading to the accumulation of toxic levels of ammonium. Qualitative utilization of inorganic nitrogen sources has been demonstrated in other *Betula* species also at normal temperatures. Under normal culture conditions, the ability of shoot tips of *B. papyrifera* to use NH_4^+ or NO_3^- as the sole nitrogen source is dependent on the age of the donor plant from which the shoot tips were sampled. The shoot tips taken from young seedlings grew equally well on medium containing either ammonium or nitrate as the sole nitrogen source, while the shoot tips from older seedling always required nitrate for growth and development (Minocha 1982). However, the callus tissue of *Betula alleghaniensis* grew best on medium containing a combination of ammonium and nitrate, but was unable to grow on medium containing ammonium as the sole nitrogen source (Minocha 1982). With *B. pendula* seedlings, the NH_4^+ to NO_3^- ratio in the nutrient solution has no effect on seedling growth rate (Ingestad 1971).

In the present study with *B. pendula*, substitution of ammonium by L-glutamine during cold hardening enhanced the regrowth, but resulted in an inferior morphology. This may by connected to the role played by glutamine in accumulation in spring buds, and in improving the growth of callus and cell cultures of several conifers (See 4.2).

There are no references in the literature of studies in which the nitrogen constituents of the cold-hardening medium have been changed to improve the regrowth of cryopreserved plants. However, in cold-hardening studies with grasses, the concentration and type of nitrogen source have also proved to be important factors with respect to cold hardiness. The cold tolerance of seedlings of *Phleum pratense* was reduced significantly more by ammonium than by nitrate, while the cold tolerance of seedlings of *Trifolium repens* was reduced only in treatments where the ammonium concentrations had toxic effects (Junttila et al. 1995). Heavy nitrogen fertilization under growth chamber conditions led to less hardy seedlings of *P. sylvestris* with a high nitrogen content and decreased cold hardiness (Hellergren 1981).

The effect of a lowered nitrogen concentration on the regrowth of *B. pendula* after cryopreservation is uncertain. The nitrogen concentration of the medium (14.4 mM) was only slightly decreased (to 12 mM) when all the inorganic nitrogen in the medium was substituted by 10 mM KNO_3, and there were no significant differences in regrowth compared to that on medium containing the normal nitrogen concentration and NH_4NO_3 substituted by KNO_3 and $Ca(NO_3)_2$ (Fig. 2 in V). When nitrate was used as the sole nitrogen source, concentrations within the range of 0.4 to 40 mM did not significantly affect the growth of shoot tips of *B. papyrifera* (Minocha 1982). The leaf callus cultures of *B. pendula* grew well and differentiated on the medium containing nitrate as a sole nitrogen source at concentrations ranging from 7 to 14 mM (Simola 1985b). According to Ingestad (1971), the optimal nitrogen concentration for seedlings of *B. pendula* varies between 4 and 8 mM.

4.3. Slow cooling

The cryopreservation of both *in vivo* and *in vitro* material was possible only by using slow cooling. Slow cooling is the only method used in the cryopreservation of in-nature-hardened material of tree species e.g. buds of *Malus* (Sakai and Nishiyama 1978, Tyler and Stuschnoff 1988a and 1988b) and *Morus* (Yakuwa and Oka 1988). The apical meristems of woody dicotyledons and embryogenic cultures of conifers have usually been cryopreserved using the slow-cooling method. An exception to this is the encapsulation-dehydration method, in which the beads are usually frozen by vitrification. In some cases, e.g. shoot tips of *Malus* and *Pyrus* species and cultivars (Niino et al. 1992) and the apical meristems of *Ribes* species (Reed and Yu 1995), both cooling rates were used at the same time with no significant differences.

The prefreezing temperature and fine adjustment of the cooling rate also have a considerable influence on regrowth. The terminal temperature used for *B. pendula in vivo* and *in vitro* material is in the range of –37°C to –40°C, which is also the most commonly used temperature range for woody plants such as *Malus* (Katano et al. 1983, Kuo and Lineberger 1985), *P. sylvestris* (Kuoksa and Hohtola 1991), and *Prunus* (Brison et al. 1095, Helliot and de Boucaud 1997). The amount of liquid water in the cells decreases when the terminal temperature is lowered, thus increasing the cold hardiness (Sakai 1965, Tyler et al. 1988). The lowest temperatures are close to –40°C, which is approximately the temperature to which water can be supercooled (Meryman and Williams 1985).

Although the prefreezing temperature has a greater influence on the dehydrating of the cells than the duration of prefreezing (Tyler et al. 1988), the cooling rate is also one of the key factors (Mazur 1963). The cooling rate used for both types of *B. pendula* material, 10°C/hour (0.17°C/min), was as fast as possible with respect to the linear decrease in the temperature from ±0°C to –38°C. For *in vivo* cold-hardened material this was faster than the naturally occurring rate in regions where *B. pendula* is a native tree species. However, it is within the cooling rate range used for *in vivo* material of other tree species, ranging from 10°C/min used for excised embryogenic axes of *Camellia* (Wesley et al. 1992) to 10°C/day for dormant buds of *Morus* (Yakuwa and Oka 1988).

The cooling rate used for the *in vitro* shoot tips of *B. pendula* was slower than that normally used in the slow cooling of *in vitro* meristems, the fastest rate being up to 1 °C/min for *Prunus* (Brison et al. 1995). It is possible that the regrowth of *in vitro* material in the present study might have been increased by using faster cooling rates, but this would have required a more specialized freezing technique. A cooling rate of 0.5°C/min was significantly better for *Prunus* shoot tips than higher or lower rates (Helliot and de Boucaud 1997). However, the meristems of *Pyrus* (Reed 1990) and *Vaccinium* species (Reed 1989) survived significantly better when cooled at a rate of 0.1°C/min than at a rate of 0.5°C/min. It is obvious that the cooling rates used for embryogenic cell cultures of conifers exert an effect in the lag phase at the beginning of regeneration, and have less effect on the survival. There was a minimal lag phase in the beginning of the cell cultures of *Picea glauca* when a cooling rate of 0.3°C/min was used, but with other cooling rates of 0.1 and 0.5°C/min the regrowth of the culture was identical to that recorded for 0.3°C/min after 34 days of culture (Kartha et al. 1988). Different cooling rates ranging from 0.1 to 1.0°C/min were studied for the cryopreservation of *Picea sitchensis*. No lag phase in the beginning of regeneration was observed when an optimum cooling rate of 0.5°C/min was used (Find et al. 1993).

The PGD cryoprotectant used for the shoot tips of *B. pendula* is the same as that used for woody plant meristems e.g. *Pyrus*, *Rubus*, *Vaccinium* (Reed 1988, 1989, 1990, 1993), and *Ribes* species (Reed and Yu 1995). Since the development of the PGD mixture for cryopreservation of cell cultures of *Saccharum* sp. (Ulrich et al. 1979), it has also been used for embryogenic cultures of *Phoenix dactylifera* (Tisserat et al. 1981), *P. abies* and *Pinus taeda* (Gupta et al. 1987), *P. sylvestris* (Häggman et al. 1998), and *A. cephalonica* (Aronen et al. 1999). It gives better survival for cultures of conifers than the more widely used DMSO with either sorbitol or sucrose as an osmoticum. The superiority of PGD over DMSO alone may be dependent on the fact that polyethylene glycol in the presence of DMSO penetrates the cells and helps to maintain the membrane structure systems (Zavala and Finkle1981). The non-penetrating cryoprotectants, on the other hand, have the ability to increase the extracellular solute viscosity, thus slowing down the movement of water out of the cells to form extracellular ice (Meryman and Williams 1985, Strauss and Hauser 1986). The PVS2 cryoprotectant mixture developed for vitrification has used together with slow cooling for *Prunus* shoot tips (Brison et al. 1995, Helliot and de Boucaud 1997). The use of DMSO alone as cryoprotectant was ineffective for shoot tips of *Malus* (Kuo and Lineberger 1985).

Compatible solutes of the most common natural cryoprotectants observed in plants include sugars, sugar alcohols, amino acids and polyamines. These compounds, which contains H-bonding groups, act by stabilizing the membranes and proteins in cells (Sakai and Yoshida 1968, Meryman and Williams 1985). The most effective solutes in preventing freezing injury are sugars, which are present in the extra-vacuolar compartments of cells during cold hardiness (Koster et al. 1989, Koster and Lynch 1992). Sucrose is supposed to form a substitute for water at the membrane surface during desiccation (Caffrey et al. 1988). In one of the first studies on the role of sugars in cold tolerance, an intimate correlation was found between the sugar concentration and the cold tolerance in the cortical tissues of *Morus* twigs and in the stem bark cells of *Robinia* (Sakai and Yoshida 1968). Since then, the accumulation of different sugars has been found to be closely correlated with the onset of cold hardening of trees e.g. sucrose in *Citrus* seedlings (Cuy et al. 1880), sucrose, raffinose and stachyose in the needles and other tissues of *P. sylvestris* (Hansen and Beck 1994), fructose and raffinose in the leaves of tolerant *Eucalyptus* sp. (Beck et al. 1995). Sucrose has a tendency to crystallize during desiccation. The role of raffinose as a cryoprotectant may be that it protects the membranes from desiccation by restricting or preventing the crystallisation of sucrose (Caffrey et al. 1988). Proline has been observed to accumulate in the leaves of *Citrus* cultivars during cold hardening. The absolute amount of proline was more important than the relative increase in the proline level (Kushad and Yelenosky 1987). Of the polyamines, only the level of spermidine increased significantly in the leaves of *Citrus* cultivars during cold hardening. The increase resulted from a shift in the ratio of bound to free spermidine and possible also from the stimulation of spermidine synthetase (Kushad and Yelenosky 1987). In the case of deciduous trees, the accumulation of solutes, e.g. proteins in the cortical cells of living bark in autumn, may be more related to storage processes or other physiological changes and only partly involved in the cold tolerance mechanism (Sakai and Yoshida 1968, Craker et al. 1969, Guy 1990). Some of the protein changes in the bark cells of *Morus* were related to growth changes and defoliation. However, phospholipid enrichment in the plasma membranes seemed to be related to cold hardening (Yoshida 1984). In evergreen trees, as in the needles and living bark tissues of conifers, seasonal fluctuations in soluble proteins are more closely correlated with changes in hardiness (Pomeroy et al. 1970). If the proteins that accumulate during cold hardening are only partly involved in cold tolerance, the proteins that are synthesized following changes in gene expression in response to a lowering temperature probably have a greater cryoprotective activity (Guy et al. 1988, Wiesniewski et al. 1996, Rinne et al. 1998). A putative cryoprotectant protein, CAPs 160, has been detected in *Citrus* seedlings (Guy et al. 1988), and a representative of Group 2 LEA (RAB) proteins has, so far, been demonstrated in the tissues of some woody plants such as *Prunus, Malus, Populus* and *Salix* (Wiesniewski et al. 1996) and *B. pubescens* (Rinne et al. 1998). Like other cryoprotective proteins, LEA proteins are probably noncolligative and may affect specific loci of the membranes (Vogel and Heber 1975), or they may prevent cytotoxicity by binding excess ions during desiccation (Baker et al. 1988). The above-mentioned natural cryoprotectants have been detected in evergreen leaves and in the cortical parenchyma ray cells of trees. The extent to which these solutes, in addition to LEA proteins, are involved in the tissues of deciduous *Betula* during cold hardiness has not been studied.

The fast temperature changes that occur during cryopreservation, in addition to the super-low storage temperature, require protection that is not necessary in nature. Only a few natural cryoprotectants have been used as artificial additives during cryopreservation, and even in these cases in high concentrations compared to the concentrations present during cold hardening in nature. The most widely used are sugars, which act as nonpenetrating osmotica during cryopreservation. However, sugars such as sucrose and raffinose have been observed to have the same effect on plasma membrane stabilisation, when used as extracellular cryopretectants during freezing, as when acting as natural intracellular cryoprotectants (Sakai and Yoshida 1968, Koster and Lynch 1992). The same intra- and extracellular stabilizing effect of sucrose has also been demonstrated with animal cells (Strauss and Hauser 1986, Crowe et al. 1987). Proline has improved the recovery of cryopreserved *Zea mays* cells compared to DMSO and glycerol (Withers and King 1979). Proline has also been used as a pretreatment with DMSO for *Prunus* shoot tips (Brison et al. 1995, Helliot and de Boucaud 1997). The success of proline as a supplemantary cryoprotectant is probably largely due to its small molecular weight, which enables it to penetrate rapidly into the cells (Meryman and Williams 1985). Pretreatment of shoot tips of *Ribes* before vitrification, with proline, with an extract of crude RAB or with bovine serum albumin in sucrose, improved regeneration to the same extent compared to pretreatment with DMSO and sorbitol (Luo and Reed 1997).

4.4. Fast thawing

There was no difference in the regrowth of *in vivo* buds of *B. pendula* after slow or fast thawing (I). Slow thawing on ice at room temperature has been used for the thawing of cryopreserved *in vivo* material of *Malus* (Sakai and Nishiyama 1978, Tyler and Stushnoff 1988a, 1988b, Tyler et al. 1988, Forsline et al. 1998). In all these cases the cryopreserved twigs were rather long, from 2.5 cm to 15 cm. Fast thawing of long twigs proved to be unsatisfactory because of bark shattering (Tyler and Stushnoff 1988a). According to studies with buds containing shorter sections of twig, e.g. *Malus* (Katano et al. 1983), *Morus* (Yakuwa and Oka 1988) and *Prunus* (Oka et al. 1991), both thawing methods were usable if the optimal terminal temperature was used during prefreezing. During relatively slow thawing there is a transition of small extracellular ice crystals to larger ones. This transition can be avoided by increasing the thawing rate of the material during the critical temperatures from ca. –196°C to –40°C for the formation of disruptive ice crystals (Luyet 1937). Slow thawing of somatic embryos of *Citrus* resulted in a recovery of 4 % (Marin and Duran-Vila 1988), but after fast thawing a survival of 30 % was achieved (Marin et al. 1993).

Subsequent rapid thawing in a water bath is a convenient, safe, and nowadays the only method used for thawing cultured cells and meristems. Washing shoot tips of

B. pendula after thawing results in better regrowth than without washing (unpublished data). The addition of PGD at ±0°C instead of +22°C, and post-thaw washing out at +22°C, improved the recovery of cells of *Saccharum* and callus cultures of *Oryza* mutants, but the washing of cells at ±0°C proved to be harmful (Finkle and Ulrich 1982). Careful washing with culture medium is also recommended to reduce the exposure of cells to cryoprotectant toxicity in the case of embryogenic cells of *Citrus* (Duran-Vila 1995). On the other hand, post-thaw washing has proved to be detrimental perhaps due to deplasmolysis (Withers 1985) or the depletion of essential factors released into the medium during freezing and thawing. Washing the cells delayed the recovery of *Zea mays* cells treated with proline (Withers and King 1979).

4.5. Substitution of ammonium in post-thaw culture

The post-thaw culture of *in vivo* buds (I, II) and *in vitro* shoot tips in papers III and IV took place on standard WPM under LD at 22°C. When the cryopreserved meristems have remained uninjured, no problems have been reported in connection with regeneration using standard medium and respective culture conditions for the species. However, not only dead meristems but also cryoinjured tissues decrease the regeneration of thawed material. The recovery of cryodamaged *Solanum* meristems has been increased by a lowered photointensity (Henshaw et al. 1985, Benson et al. 1989). The same physical method has also been used during the regeneration of *in vitro* shoot tips of *Prunus* (Brison et al. 1995, Helliot and de Boudaud 1997). The elimination of NH_4NO_3 from the post-thaw medium has been studied as a chemical method for curing cryodamaged cells. NH_4NO_3-free recovery medium was used successfully in the case of *Malus* shoot tips (Niino et al. 1992), even though no comparison was made between the medium with or without ammonium. The recovery of cryodamaged cells of *Oryza* was inhibited by the presence of ammonium in the media but, after recovering from injury, ammonium was necessary to achieve a high growth rate of the cells (Kuriyama et al. 1989). Not only substitution of ammonium ions by other nitrogen sources, but even adsorption of ammonium by activated charcoal, reduced the post-thaw viability loss of *Lavandula* cells (Kuriyama et al. 1990, 1996). Substitution of NH_4NO_3 in the post-thaw culture medium by KNO_3 or L-glutamine in this study resulted in increased regrowth, but to a lesser extent than if the substitution was started already at the beginning of cold hardening (V). The duration of ammonium-free post-thaw culture was not significant for *B. pendula*, even the use of the ammonium-free freezing and washing media increased the regrowth (V). *Lavandula* cells recovered from freezing injury during one day's culture on ammonium-free medium (Kuriyama et al. 1996). Obviously the first 24 – 48 post-thaw hours are critical; during this period the cells that have suffered sublethal damage recover, whereas those that have suffered severe injury during cryopreservation will die (Day et al. 1997).

In this study with *B. pendula*, the best substitution for ammonium in the post-thaw medium appeared to be L-glutamine. One explanation for the effectiveness of L-glutamine in the regeneration in the beginning of post-thaw culture could be that glutamine is readily assimilated and an energetically inexpensive source of nitrogen during the short period when nitrate and ammonium assimilation is not fully operative (Kirby et al. 1987). Glutamine promotes cell growth in several conifers. With glutamine as a source of nitrogen, cell multiplication in spruce (*P. glauca*?) occured in the form of masses of small, compact, meristematic cells, while ammonium reduced meristematic activity and promoted earlier differentiation with substances characteristic of aging (White and Gilbey 1966). High concentrations of glutamine decreased the lag phase and stimulated the growth of richly cytoplasmic, dense cells in suspension cultures of *Pseudotsuga menziesii* (Kirby 1982).

In this study with *B. pendula*, the substitution of ammonium by a mixture of KNO_3 and L-glutamine resulted in decreased regrowth. The regrowth of *Lavandula* cells was increased when NH_4NO_3 was substituted by alanine or glutamine in the medium that contained KNO_3 as the principal source of nitrogen (Kuriyama et al. 1997). On the other hand, elimination of ammonium ions did not increase the viability of the embryogenic cell cultures of *Oryza.* Cell growth was optimal using the same post-thaw medium containing glycine as nitrogen source in which cell suspension was normally maintained (Lynch et al. 1994).

The glutamine concentration increases dramatically in the vegetative buds of *P. glauca* at the beginning of the growing season just prior to active cell division (Durzan 1968). Unfortunately there was not enough material remaining after five years' storage of the *in vivo* buds of *B. pendula* to study the effect of substitution of ammonium in the post-thaw medium. The substitution of ammonium by L-glutamine at the beginning of post-thaw culture may have reduced to some extent the decrease in regrowth.

4.6. Genotypical differences

The essential prerequisites for the cryopreservation of *B. pendula* were common to all genotypes. Although additional treatments, i.e. the application of ABA in cold hardening and the substitution of ammonium during cold hardening and/or regeneration, increased the regrowth of *in vitro* shoot tips, the genotypical differences did not diminish. As a matter of fact, after all these studies, more attention should be paid to the genotypical differences in the regrowth of *in vivo* buds. Although there were no significant differences in the regrowth of different genotypes after 8 days of cryostorage compared to the corresponding unfrozen controls, the regrowth of genotype E5382 was significantly decreased compared to that of genotype E5387. This difference was seen throughout all the studies. Later on, in the studies with *in vitro* shoot tips, genotype E5387 reacted to all the improvement treatments with increased regrowth, while genotype E5382 was almost recalcitrant. There were no treatment-dependent differences in the plants, but genotypical differences were obvious in plantlets growing in the greenhouse in the middle of October 1998. Genotype E5387 was already without leaves, the uppermost leaves of E5382 were still both green and yellow and the rest of the leaves yellow, and the other three genotypes had yellow leaves. The donor trees from which the twigs were collected in the silver birch stand show the same variation in coloration. Studies with *B. pubescens* have shown that the variation in photoperiodic response within a population may vary considerably (Håbjørg 1978). According to Nørgaard et al. (1993b), there was no correlation between the cryotolerance of the embryogenic cultures of *P. abies* and the cold hardiness of the progeny trials of the same parents in the field. According to this study, the cryotolerance of *B. pendula* may be dependent on the ability of the donor trees to cold harden in nature (see 4.2.1.)

4.7. Long-term storage in liquid nitrogen

The regrowth ability of the *in vivo* buds of *B. pendula* in the present study remained unchanged after five years' storage, except for that of the buds in the axil of a catkin. The reports on the longevity of cryopreserved dormant buds of woody dicotyledons e.g. *Morus* (Niino et al. 1993, 1995) and *Malus* (Forsline et al. 1998), show good viability after up to five years' storage in liquid nitrogen. However, considering the small amount of space needed for the cryostorage of a great number of accessions bearing dormant buds of *Malus*, a viability standard of 40 % has been concluded to provide an acceptable probability level for a sample (Forsline et al. 1998). Strictly speaking, the genetic integrity of a genotype can be preserved in only one bud cabable of regenerating (Forsline et al. 1998). In the cryopreservation of *B. pendula* the regrowth results exceeded this probability level. Only the storage of buds with a catkin for five years resulted in a decrease in the regrowth of *B. pendula*. In addition to the presence of a female catkin, the decrease depended on the developmental phase of the buds due to the late collection time. Damage may also occur as a result of repeated removal of cryotubes from the containers. Temperatures of up to –112°C, where the formation of ice crystals is theoretically possible, were observed in tubes during removal from liquid nitrogen (Day et al. 1997). This phenomenon could be one explanation for the total loss of viability of *Euglena gracilis* after 22 years' storage in liquid nitrogen. At the end of the storage period the viability of eight eukaryotic algae, except for *Euglena*, was unchanged (Day et al. 1997). The maintenance of a stable storage regime must be considered when material is stored for longer periods especially. Some genotypes might even require modified methods for more successful long-term cryostorage. In the present study, the regrowth rate of both bud types of *B. pendula*, with and without a catkin, had decreased significantly after long-term cryostorage. The finding that a decrease in the longevity of cryopreserved buds of *B. pendula* can be seen as a lowered regrowth rate before it is expressed in the regrowth ability of the buds, is in accordance with the results achieved with seeds of *P. abies* (Chmielarz 1998). The germination percentage of seeds stored for two years at liquid nitrogen was equal to that stored at –3°C, but the germination rate of the seeds stored at –196°C was higher. The rate of germination is a trait used in standardized seed testing to predict possible changes in seed germinability (International Rules for seed... 1976, Ryynänen 1980).

In the future, the long-term cryostorage of *in vitro* material will be more important e.g. for retaining the juvenility of micropropagated genotypes during the required field testing of up to 15 years (Grossnickle et al. 1996, Walter and Smith 1995) or for research purposes. In the case of dicotyledons, the regeneration of embryogenic callus cultures of *Citrus* cryostored for two years was excellent (100 %) (Pérez et al. 1997), but up to six months' cryostorage of *in vitro* meristems has not been very successful. According to Kartha and coworkers (1979), the plant regeneration of frozen *in vitro* meristems of *Pisum* decreased from 72 % when thawed after one hour, to 60% when thawed after six months' storage in liquid nitrogen. The decrease in the regrowth of *Fragaria* x *ananassa* was similar but faster; the plant regeneration of frozen *in vitro* meristems of *Fragaria* decreased from 95 % when thawed after one week, to 56 % when thawed after eight weeks' storage in liquid nitrogen (Kartha et al. 1980). Although there were no differences in the regrowth percentages of meristems of tissue culture plantlets of *Rubus spectabilis* when they were cryostored for one day or six months, the regrowth of another cultivar, *Rubus* spp. 'Merton Thornless', decreased from 60 % to 20 % during the same cryostorage period (Reed 1988).

In the sense of germplasm conservation, in addition to the longevity of cryostored material, attention has to be paid to monitoring the genetic fidelity as well as observing morphological and physiological characteristics of cryopreserved material in field tests. No changes in the morphological or cellular level of *in vivo* material cryostored for extended period have been reported. The morphology and chromosome number of regenerated *in vivo* buds of *Morus* were stable after storage at –196°C or –135°C for 3.5 years (Niino et al. 1993). No dissimilarities were found in zymograms for peroxidase isoenzymes or in the banding pattern of leaf proteins from untreated *Morus* buds and those cryostored for five years (Niino et al. 1995). The importance of studying the genetic stability is especially true in the case of cryopreservation of *in vitro* material using cryoprotectants, especially DMSO (Hervás and Giménez-Martín 1973, Friend and Freedman 1978, Fukaï and Oe 1990). The plants obtained from embryogenic tissues of *Larix* and *P. mariana* have not displayed any abnormalities in field tests (Klimaszewskaja et al. 1992). There are only a few reports concerning the genetic stability of cryopreserved woody plants. DMSO-dependent genetic instability has been found with cell lines of embryogenic cultures of *A. cephalonica* interspecific hybrids (Aronen et al. 1999). No evidence of genetic changes due to cryoprotectant treatments was found in a corresponding study with embryogenic cultures of *P. sylvestris* (Häggman et al. 1998). Variations in the ribosomal RNA genes have been observed in plants regenerated from callus after cryopreservation of the shoot tips of *Solanum* with 10 % DMSO as cryoprotectant (Harding 1997). The phenotypical deviations observed in field tests established with cryopreserved and non-cryopreserved material of *Lolium* and *Festuca* were partly reflected in the results of the RAPD analysis. However, the differences detected in RAPDs were between different somaclones and not affected by cryopreservation (Stadelmann et al. 1998). In the present study, the cryopreserved and regenerated plants of *B. pendula* are growing in the greenhouse. Leaves for monitoring the genetic stability will be collected during the next growing season and field experiments will be established. General rules for placement, maintenance, determination of genetic stability, and field testing of cryopreserved material are needed for cryopreservation in the sense of germplasm conservation. Guidelines for the management of germplasm collections, including cryopreservation, are in preparation by The International Plant Genetic Resources Institute (IPGRI) (Engelmann 1997).

5. CONCLUSIONS

The importance of the evaluation of gene resources and germplasm conservation has been recognized. Gene conservation is an activity designed to ensure genetic resources and make provision for its multiplication. In Finland the conservation of germplasm of *B. pendula* is possible by traditional *in situ* and *ex situ* methods; *B. pendula* is growing in nature conservation areas and gene reserve forests, and it is also grafted in clone archives and seed orchards. Cryopreservation is an option of germplasm preservation, in particular for certain genotypes, i.e. individuals with a specific combination on several desirable traits. Although it is not a universal method that meets answering all the requirements of conservation, it is a tool that can be used together with conventional conservation methods. The cryopreservation technique has several advantages compared to other conservation methods for maintaining the clonal integrity of tree species. Cryopreservation retains the juvenility of stored material and, during cryopreservation, environmental effects such as climate change, natural selection, and the effects of different kinds of stress, which are threatening material conserved by conventional methods, are avoided. One special advantage of *in vitro* cryopreservation is that the explant is already miniaturized and may also be pathogen free. Compared to storage in long-term tissue culture, it is less laborious and the risk of superficial contamination is minimized.

During cryopreservation, the metabolic activity of biological material is restricted and subsequently arrested at the temperature of liquid nitrogen. One could postulate, based on the super-low temperature, that material could be stored indefinitely in such a state in liquid nitrogen. Cryopreservation is a new approach for the conservation of material, which means that empirical results covering decades or even longer-lasting cryopreservation experiments with true-to type analyses or large-scale field tests are missing. So far, only preliminary results of long-term cryopreservation experiments, including those of *B. pendula* in the present study, support this hypothesis. However, the risk of losing biological material during long-term cryopreservation can be reduced using method similar to that used in conventional gene conservation; samples from material in cryostorage have to be regularly evaluated. In addition to estimating the regeneration of cryopreserved material, the regrowth rate should be measured regularly. As shown by the results of the long-term cryostorage of *in vivo* buds of *B. pendula*, a decreased regrowth rate indicates a reduced survival of the cryopreserved material in the phase not yet expressed in regrowth ability.

As the results of this study show, the cryopreservation of *in vivo* buds and *in vitro*-grown shoot tips of *B. pendula* with concomitant regeneration of plants is possible, at least when the cryostorage period lasts for a few years. The cryopreservation of buds cold-hardened in nature (*in vivo* application) provides several advantages as a method of germplasm conservation: the cryopreservation method is more feasible, and cold hardening and the use of chemical cryoprotectants is not required, thus making the method inexpensive and simple. It is most suitable for use together with *ex situ* clone archives for selected genotypes of *B. pendula*. In addition to its use in germplasm conservation, the cryopreservation of *in vivo* buds can also be used as an aid when organizing the time schedule for the micropropagation of *B. pendula* using buds as the starting material. Although the initiation of micropropagation from buds is possible throughout the year, even the short-term cryopreservation of buds collected in March or April for later use is worth doing due to their more successful initiation ability.

Most of the genotypes of *B. pendula* can be micropropagated using the routine laboratory protocol for *Betula* tissue culture. However, in order to ensure that a genotype of *B. pendula* can be regenerated through tissue culture, micropropagation of the genotype has to be confirmed before cryopreservation. Thus the genotype can be stored either by cryopreservation of *in vivo* buds or by cryopreservation of *in vitro* shoot tips. The cryopreservation of *in vitro* shoot tips (*in vitro* application) maintains the juvenility of genotypes already growing in tissue culture. The *in vitro* protocol may be applied e.g. for research purposes or as a means of storage during progeny testing. It is also possible that the *in vitro* cryopreservation of *B. pendula* will increase in the future; *B. pendula* is an important hardwood in molecular biology studies.

With both *in vivo* and *in vitro* applications of *B. pendula* the regenerated plantlets are juvenile and it takes a number of years before mature, seed producing trees become available. Before cryopreserved plants can be used for breeding or practical silviculture, genetical stability analysis and large-scale field tests will have to be carried out. The plants of *B. pendula* regenerated using different treatments in this study are now growing in the greenhouse. Leaves will be collected for monitoring the genetic stability during the next growing season, and field experiments will be established for measuring the morphological and physiological variations of the phenotypes.

Another study to be continued is the effect of ammonium on the cold hardening and/or post-thaw regeneration of *B. pendula*. The physiological mechanism behind the elimination and substitution of ammonium ions deserves more attention. A better understanding of this mechanism will increase the cryopreservability of *in vitro* material, and in the regeneration of long-term *in vivo* material it may play a key role. After having optimized the timing, selection, cooling, and stable storage of the buds, as presented in this study, the conditions during post-thaw culture are the only possible means of affecting the regeneration of buds. The use of the medium with a modified nitrogen source could be one way of improving the survival of injured buds.

The cryopreservation of woody plants is a new and intensively studied field of research. There are some common features in the cryopreservation of deciduous trees i.e. the demand for a micropropagation protocol, and slow cooling as well as fast thawing methods. On the other hand, not only species but also genotypes and tissues behave differently and require more specific research. This means that, for certain types of explant, basic research is needed before practical applications are possible. In all these respects cryopreservation is like that of micropropagation, which is nowadays an essential part of daily life in agriculture, horticulture, and forestry.

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APPENDIX: Cryopreservation protocols for woody plants referred to in the Introduction.

Species/ Explant	Geographical zone	Pretreatment: Additives in medium/ time/ temperature, light	Cryogenic protocol/ cryoprotectant/ storage time	Recovery medium + growth regulator/ temperature, light	Recovery	Regeneration	Reference
Gymnosperms							
Coniferales							
Pinaceae							
Abies cephalonica							
Embryogenic culture/ 8 cell lines	Europe, temperate	0.2 M sucrose / 24 h and 0.4 M sucrose/ 24 hours/ 5°C, dark	Slow cooling/ PGD/ 7 days	MS, SH + 4.4 µM BA/ 24°C, dark	87 % of cell lines survived. Average growth rate was improved	No maturation experiments	Aronen et al. 1999
A. nordmanniana							
Embryogenic culture/ 5 genotypes	Asia, temperate	0.2 M sorbitol/ 24 hour and 0.4 M sorbitol/ 24 hours/ 24°C, dark	Slow cooling/ 0.4 M sorbitol and 5 % DMSO/ 2 hours	PMI-S1 + 10 µM BA/ 24°C, dark	All cultures survived. Lag phase 3.5 weeks. Average growth rate was decreased	No maturation experiments	Nørgaard et al. 1993a
Picea abies							
Embryogenic culture	Europe, boreal	–	Slow ooling/ PGD/ 10 min	BM_1 + 5 µM 2,4-D, 2 µM kinetin, and 2 µM BA 23°C, n.m.	Average growth rate was decreased	Mature somatic embryos obtained 65 % of the controls	Gupta et al. 1987

Embryogenic culture		0.5 M sucrose/ –/ 25°C, dark	Slow cooling/ 0.5 M sucrose and 5 % DMSO/ n.m.	GD + 5 µM 2,4-D, 2 µM kinetin, and 2 µM BA/ 25°C, dark	Lag phase shorter that 3 days. Average growth rate was improved	Plants	Galerne et al. 1992
Embryogenic culture 81 cell lines		0.4 M sorbitol/ 48 hours/ 20°C, dark	Slow cooling/ 0.4 M sorbitol and 5 % DMSO/ 2 hours	BMI-S1 + 10 µM BA/ 20°C, 24°C, dark	96 % of cultures survived. Average growth rate was decreased	Mature somatic embryos	Nørgaard et al. 1993b
P. glauca							
Embryogenic culture, 1 cell line	North-America, boreal	0.2 M sorbitol/ 24 hours and 0.4 M sorbitol/ 24 hours/ 20°C, low light intensity	Slow cooling/ 0.4 M sorbitol and 5 % DMSO/ 1 hour, 1 year	LP, GMD + 0.1 µM 2,4-D and 5 µM BA/ 26°C, LD, low light intensity	Maximal growth rate was 95 % of the control	Plants	Kartha et al. 1988
P. mariana							
Embryogenic culture	North-America, boreal	0.4 M sorbitol/ 24 hours/ 25°C, continuous dim light	Slow cooling/ 0.4 M sorbitol and 5 % DMSO/ 30 min	LM + 9 µM 2,4 D and 2.2 µM BA/ 25°C, continuous dim light	Fast recovery after a short lag phase	Plants. Field trials established	Klimaszewska et al. 1992
P. sitchensis							
Embryogenic culture	North-America, boreal–temperate	0.3 M sorbitol/ 24 hours and 0.4 M sorbitol / 24 hours/ 24°C, dark	Slow cooling/ 0.4 M sorbitol a and 5 % DMSO/ 1 week	BMI-S1 + 10 µM BA/ 24°C, dark	Fast recovery without any lag phase	Plants	Find et al. 1993

Larix x *eurolepis*							
Embryogenic culture	Europe, boreal–temperate	0.4 M sorbitol/ 24 hours/ 25°C, continuous dim light	Slow cooling/ 0.4 M sorbitol and 10 % DMSO/ 30 min	MSG, + 9 µM 2,4 D and 2,2 µM BA/ 25°C, continuous dim light	Fast recovery after a short lag phase	Plants. Field trials established	Klimaszewska et al. 1992
Pinus caribaea							
Embryogenic culture	Central-America, subtropical–tropical	0.4 M sucrose/ 24 hours/ 20°C, LD	Slow cooling/ 0.4 M sucrose and 10 % DMSO/ 30 min, 4 months	MM + 5 µM 2,4-D and 2 µM BA/ 22°C, dark	Embryogenic potential similar to controls	Plants	Lainé et al. 1992
P. sylvestris							
Winter buds	Eurasia, boreal–temperate	–	Slow cooling/ – / 10 min	MS + 0.9 µM 2,4-D and 0.44 µM BA/ 26/23°C, LD	100 – 7 % depending on collection time	–	Kuoksa & Hohtola 1991
Embryogenic Culture 9 cell lines		0.2 M sucrose / 24 hours and 0.4 M sucrose/ 24 hours/ 5°C, dark	Slow cooling/ PGD/ 1 day 24 hours	DCR + 9.1 or 13.6 µM 2,4-D and 2.2 µM BA/ 25°C, dark	78 % of cell lines survived. Average growth rate was improved	No maturation experiments	Häggman et al. 1998
P. taeda							
Embryogenic culture	North-America, temperate	–	Slow cooling/ PGD/ 10 min	MS, + 5 µM 2,4 D, 2 µM kinetin, and 2 µM BA/ 23°C, n.m.	Average growth rate was decreased	Mature somatic embryos obtained 50% of the control	Gupta et al.1987

Angiosperms **Dicotyledons** Moraceae *Morus bombycis*						
Winter buds	Asia, temperate	–	Slow cooling/ –/ 1 day	MS + 4.4 µM BA/ 25°C, LD	67 % of buds formed shoots	Plants	Yakuwa & Oka 1988
Winter buds 12 genotypes		–	at –135°C 3.5 years	MS + 4.4 µM BA/ 25°C, LD	Average shoot formation rate was 72 %	Plants	Niino et al. 1993
Winter buds 376 genotypes		–	at –135°C 5 years	MS + 4.4 µM BA/ 25°C, LD	Average shoot formation rate was 80%.	Plants	Niino et al. 1995
					Recovery of grafted buds was 35 %	Grafts	
Grossulariaceae *Ribes* spp							
In vitro apical meristems 3 genotypes.	Northern boreal–temperate	5 % DMSO/ 2 days/ 22°C/–1°C, SD	Slow cooling/ PGD/ n.m.	NCGR-Ribes medium + 0.4 µM BA and 0.6 µM GA_3/ 25°C, LD	Low – good recovery. No genotypical differences	n.m.	Reed & Yu 1995
		5 % DMSO/ and 1.2 M sorbitol/ 2 days/ 22°C/–1°C, SD	Vitrification/ PVS2/ n.m.	NCGR-Ribes medium + 0.4 µM BA and 0.6 µM GA_3/ 25°C, LD	Great genotypical differences in recovery	n.m.	

		Desiccation/ 0.75 M sucrose/ 18 hours followed by sterile air flow for 2 - 3 hours	Encapsulation-dehydration/ n.m.	NCGR-Ribes medium + 0.4 µM BA and 0.6 µM GA_3/ 25°C, LD	Most successful of the three tested methods. Great genotypical differences	n.m.	
Rosaceae *Malus* spp.							
Winter buds	Northern temperate	–	Slow cooling/ – / up to 23 months	–	Recovery of grafted buds was 77 %	Grafts	Sakai & Nishiyama 1978
Winter buds 15 genotypes		–	24 hours	–	Average recovery of grafted buds was 82 %	Grafts	Tyler & Stushnoff 1988a
Winter buds 64 genotypes		–	4 years	–	Average recovery of grafted buds was 66 %	Grafts	Forsline et al. 1998
Excised shoot tips of winter buds		–	Slow cooling/ – / n.m.	MS + 4.4 µM BA/ 25°C, continuous light	100 % survival	Shoot multiplication	Katano et al. 1983
In vitro shoot tips		–	Slow cooling/ glycerol/ 15 min	MS +11.1 µM BA and 0.5 µM NAA/ 22 – 24°C, LD	72 % of shoot tips produced callus	No shoot formation	Kuo & Lineberger 1985
In vitro shoot tips 5 genotypes		0.7 M sucrose/ 1-2 days/ 5°D, SD	Vitrification/ PVS2/ 1 day	MS, NH_4NO_3 eliminated, + 4.4 µM BA/ 25°C, LD	63 % of shoot tips regenerated	Plants	Niino et al. 1992

In vitro shoot tips		0.1, 0.4, and 0,7 M sucrose/ 1+1+1 day/ 5°C, SD	Encapsulation-dehydration/ 3 days – 5 months	MS + 1.5 µM BA/ 25°C, LD	80 % of shoot tips regenerated	Plants	Niino & Sakai 1992
Prunus spp.							
In vitro shoot tips 2 genotypes	Northern boreal–temperate	5 % DMSO and 2 % proline/ 24 hours/ 23°C/4°C, LD	Slow cooling/ PVS2/ 1 – 5 days	MS + 4.4 µM BA, 0.25 µM IBA, and 0.29 µM GA_3/ 23°C, in dark (2 days), then under low illumination until regrowth, then LD	71 % if shoot tips regenerated	Plants	Brison et al. 1995
In vitro shoot tips		5 % DMSO and 20 % proline/ 24 hours/ 4°C, dark	Slow cooling/ PVS2 + proline/ n.m.	MS + 4.4 µM BA and 4.9 nM IBA 23/18°C, 2-3 days in the dark then LD	Survival of shoot tips 50 – 60 %	n.m.	Helliot & de Boudaud 1997
In vitro shoot tips		0.7 M sucrose/ 1 day/ 5°C, SD	Vitrification/ PVS2/ 1 day – 10 months	MS 4.4 µM BA and 0.5 µM IBA/ 25°C, LD	80 % of shoot tips regenerated	Plants	Niino et al. 1997
Pyrus spp.							
Winter buds	Northern temperate–subtropical	–	Slow cooling/ – / 1 day	MS, WPM + 4.4 µM BA/ 25°C, LD	50 % of buds formed shoots	Plants	Oka et al. 1991

In vitro apical meristems 8 genotypes		5 % DMSO/ 48 hours/ 22°C/–1°C, SD	Slow cooling/ PGD/ 1 hour	Cheng + 8.8 µM BA/ 25°C, LD	Total survival (shoots and callus) 95 %	n.m.	Reed 1990
In vitro shoot tips 8 genotypes		0.7 M sucrose/ 1-2 days/ 5°D, SD	Vitrification/ PVS2/ 1 day	MS, NH_4NO_3 eliminated, + 4.4 µM BA/ 25°C, LD	60 % of shoot tips regenerated	Plants	Niino et al. 1992
In vitro shoot tips		0.75 M sucrose/ 18 hours followed by sterile air flow for 4 hours	Encapsulation – dehydration/ 1 hour	MS + 2.2 µM BA/ 23°C, LD	80 % of shoot tips formed shoots	n.m.	Scottez et al. 1992
Rubus spp. *In vitro* apical meristems	Northern and southern temperate	5 % DMSO/ 48 hours/ 25°C/–1°C, SD	Slow cooling/ PGD/ 1 hour	MS + 43 µM adenine sulfate, 10 µM BA, and 2 µM IBA 25°C, LD	60 % of meristems regenerated	n.m.	Reed 1988,
In vitro apical meristems 10 genotypes		50 µM ABA, 5 % DMSO/ 48 hours/ 22°C/–1°C, SD	Slow cooling/ PGD/ 1 hour	MS + 0.4 µM BA, 0.25 µM IBA, and 0.03 µM GA_3/ 25°C, LD	Total survival (shoots and callus) 71%. No regeneration from callus	n.m.	Reed 1993
Rutaceae *Citrus spp.* Somatic embryos	Asia, subtropical	–	Slow cooling/ 10 % DMSO/ 5 min	MS – / 27°C, LD	4 % of somatic embryos survived (Slow thawing)	Plants	Marin & Duran-Vila 1988

Somatic embryos		–	Slow cooling/ 10 % DMSO/ 5 min	MS – / 27°C, LD	30 % of somatic embryos survived (Fast thawing)	Plants	Marin et al. 1993
Nucellar cells		–	Vitrification/ PVS2/ 30 min	MT – / –	80 – 90 % of cell survived (FDA-staining)	Plants	Sakai & Kobayashi 1990
Nucellar cells		–	Vitrification/ PVS2/ 30 min	MT 44.4 µM BA/ 25°C, LD –	Survival 65 %	Plants	Sakai et al. 1990
Embryogenic callus. Several species and cultivars		–	Slow cooling/ 10 % DMSO/ 1 day – 2 years	BS –/ 27°C, LD	Survival 100 %	Plants	Pérez et al. 1997
Vitaceae *Vitis vinifera*							
In vitro shoot tips	Eurasia, temperate	1 M sucrose/ 2 days/ 23/21°C/LD	Encapsulation-dehydration/ 12 hours	BS + 1 % fetal calf serum/ 23/21°C, LD	30 % of shoot tips formed shoots without callus formation	n.m.	Plessis et al. 1993
Sterculiaceae *Guazuma crinita*							
Bud cluster segments	South-America, tropical	–	Vitrification/ PVS2/ 1 hour	WPM + 10 µM zeatin/ 25 – 30 °C, LD	80 % of bud clusters survived	Plants	Maruyama et al. 1997

Theaceae *Camellia sinensis*							
Embryogenic axes	Asia, temperate–subtropical	Desiccation in sterile air flow/ 3 hours	Fast cooling/ –	Nitsch & Nitsch/ n.m. 25°C, LD	93 % of axes germinated	Seedlings	Chaudry et al. 1991
In vitro shoot tips		0.2 M sucrose/ 2 days/ 5°, SD	Vitrification/ PVS2/ 1 hour	MS + 4.4 µM BA, 0.05 µM IBA, and 3 µM GA/ 25°C, LD	60 % of shoot tips formed shoots	Plants	Kuranuki & Sakai 1995
Ericaceae *Vaccinium* spp.							
In vitro apical meristems 8 genotypes	Northern arctic–boreal–temperate	5 % DMSO/ 48 hours/ 22°C/–1°C, SD	Slow cooling/ PGD/ 1 hour	WPM + 24.6 µM 2iP 25°C, LD	58 % of apical meristems formed shoots	n.m.	Reed 1989
Rubiaceae *Coffea* spp.							
Embryogenic culture	Africa, tropical	0.75 % sucrose/ overnight/ 27°C	Slow cooling/ 0.75 M sucrose and 5 % DMSO/ 1 hour	MS + 4.4 µM BA/ 27°C, lowered light	Recovery 50 % of control. Lag phase 5 weeks	Plants	Bertrand-Desbrunais et al. 1988
In vitro shoot tips		0.1 - 0.75 M sucrose/ 1 - 10 days/ 27°C, 12 h light/12 h dark	Encapsulation-dehydration/ 25 min	MS + 1.3 µM BA/ 27°C, 12 h light/12 h dark	20 % of shoot tips developed directly, 20 % formed only callus	n.m.	Mari et al. 1995

Monocotyledons							
Palmae							
Arecaceae							
Elaesis guinensis							
Excised embryos	West-Africa, tropical	Desiccation in sterile air flow	Fast cooling/ –/ 1 and 8 months	MS + 2.9 µM IAA and 0.5 µM kinetin/ 28°C, LD	No loss of viability after both storage times	Seedlings. Field trials established	Grout et al. 1983
Phoenix dactylifera							
Embryogenic culture	Afroasia, subtropical	–	Slow cooling/ PGD/ 3 months	MS + 14.8 µM 2iP/ 25°C, LD	Fast recovery after lag phase of 2 – 4 weeks	Plants	Tisserat et al. 1981

I

Survival and regeneration of dormant silver birch buds stored at super-low temperatures

Leena Ryynänen

Abstract: Cryopreservation was developed for the storage of in vivo buds of silver birch (*Betula pendula* Roth). The principles of the optimized method were the use of buds already acclimated in nature and slow freezing with a cooling velocity of 10°C/h down to a terminal temperature of −38°C. After 24 h at this temperature the buds were immersed in liquid nitrogen at −196°C for 8 days, 6 months, or 12 months. After fast thawing, 5 min in a water bath at 37°C, the buds were surface sterilized and cultured according to the normal laboratory routine. The buds were examined after 2 and 4 weeks of cultivation. There were no significant differences in survival and growth between the unfrozen controls and buds stored in liquid nitrogen for different times. The growth percentage of buds without a female catkin was double that of buds with a catkin. These results indicate that cryopreservation would be an ideal method for the ex situ gene conservation of birch and retention of the juvenility of adult genotypes.

Résumé : Une méthode de cryopréservation a été développée pour l'entreposage in vivo de bourgeons de bouleau pleureur (*Betula pendula* Roth.). Cette méthode consiste à utiliser des bourgeons déjà acclimatés en nature et refroidis, par la suite, à une vitesse de 10°C/h jusqu'à une température de −38°C. Après 24 h à cette température, les bourgeons ont été immergés dans l'azote liquide à −196°C pour 8 jours, 6 mois et 12 mois. À la suite d'un réchauffement rapide de 5 min dans une eau à 37°C, les bourgeons ont été stérilisés en surface et cultivés selon une procédure courante en laboratoire. Les bourgeons ont été examinés après 2 ou 4 semaines de culture. Aucune différence significative n'a été observée dans la survie ni dans la croissance entre les bourgeons témoins et ceux entreposés dans l'azote liquide. Le pourcentage de croissance des bourgeons sans chaton femelle était le double de ceux avec chaton. Ces résultats montrent que la cryopréservation serait une méthode idéale pour la conservation ex situ des gènes de bouleau et pour le maintien de la juvénilité des génotypes adultes.

[Traduit par la Rédaction]

Introduction

Gene conservation is an activity designed to ensure genetic diversity and provides for its multiplication. In Finland the conservation of tree genes is possible by traditional in situ and ex situ methods; birch is growing in nature conservation areas, gene reservation forests have been established for silver birch (*Betula pendula* Roth) since 1993, and both silver birch and pubescent birch (*Betula pubescens* Ehrh.) are grafted in clonal archives and seed orchards (Nordic Council of Ministers 1992). Although there is plenty of land available for the in situ and ex situ gene conservation of trees in the Nordic countries, the germ plasm is still exposed to changing environmental conditions. At seed storage the germ plasm is more protected, but the minimum time for gene reservation purposes must exceed the interval between germination and seed production for the next generation (Bonner 1990). The seed of birch is orthodox, but seed viability decreases during storage. The birch seed that is currently collected is primarily stored as short- or medium-term material for afforestation and reforestation (Nordic Council of Ministers 1992). The micropropagation technique successful in silver birch, on the other hand, is a laborious in vitro method as a means of gene conservation.

Cryopreservation, the storage of material at a very low temperature, is usually viewed as a backup technique to complement other methods of germ plasm conservation, not the sole means of conserving a species or genotype. It is a method used for storing material without alteration and modification for an unlimited period of time. However, information is needed about the method to be used in regenerating the cryopreserved material. The use of meristematic tissue as a material for cryopreservation is recommended; it is the tissue that best survives freezing and regeneration through tissue culture. The tissues suitable for cryopreserving in vivo material are embryos and vegetative buds. Cryopreservation of excised embryos has been studied with tropical crops, the seeds of which are unsuited to conventional seed storage (e.g., Grout et al. 1983; Chaudhury et al. 1991; Wesley-Smith et al. 1992). In vivo dormant vegetative buds of some hardy deciduous trees have successfully survived immersion in liquid nitrogen after prefreezing. When apple (*Malus domestica* Borkh.) buds prefrozen at −30 to −40°C before immersion in liquid nitrogen were grafted onto rootstocks, most of them retained the ability to grow (Sakai and Nishiyama

Received May 11, 1995. Accepted October 12, 1995.

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Table 1. Experimental design to optimize the cryopreservation procedure of silver birch in vivo buds.

Treatment	Cooling	Storage		Thawing
		Temperature	Time	
		March collection		
A	Fast	LN	8 days	Fast
B	Fast	LN	8 days	Slow
C	Slow	LN	8 days	Fast
D	Slow	LN	8 days	Slow
Control A–D	—	—	—	—
		April collection		
E	Slow	LN	8 days	Fast
F	Slow	LN	8 days	Slow
G	Slow	LN	6 months	Fast
H	Slow	LN	12 months	Fast
I	Slow	−70°C	8 days	Fast
J	Slow	−70°C	8 days	Slow
K	Slow	−70°C	6 months	Fast
L	Slow	−70°C	12 months	Fast
M*	Slow	LN(−70°C)	8 days (6 months)	Fast
N*	Slow	LN(−70°C)	8 days (12 months)	Fast
Control E–N	—	—	—	—

Note: LN, liquid nitrogen, −196°C.

*Liquid nitrogen was first used for 8 days. The buds were then switched to −70°C for the amount of time given in parentheses.

1978; Tyler and Stushnoff 1988). Apple shoots subjected to the same pretreatment and stored for 1 year in liquid nitrogen sprouted in water after thawing (Sakai and Nishiyama 1978). Intact vegetative buds of mulberry (*Morus bombycis* Koidz.) have been successfully micropropagated after cryopreservation (Yakuwa and Oka 1988).

The aim of the present study was to develop a cryopreservation method for in vivo buds of silver birch. It was done to retain the juvenility of adult, elite genotypes with minimum space and maintenance requirements. The possibility of starting the micropropagation of this species using dormant buds at any time of the year was also examined.

Material and methods

Twigs with vegetative and flower buds of silver birch were collected from five clones, E1987, E5201, E5382, E5387, and E5398, in Kokonniemi stand, at Punkaharju (61°49′N, 29°18′E; 90 m above sea level). The stand had been planted with 1-year-old seedlings of local origin in 1932. The average height of the trees was 27 m, the lowest living branches of the crown growing at 11 m. The twigs were collected from the highest third of the crown. Twigs (about 30 cm) were collected for the first time at the beginning of March 1993 and for the second time from the same trees in the middle of April 1993.

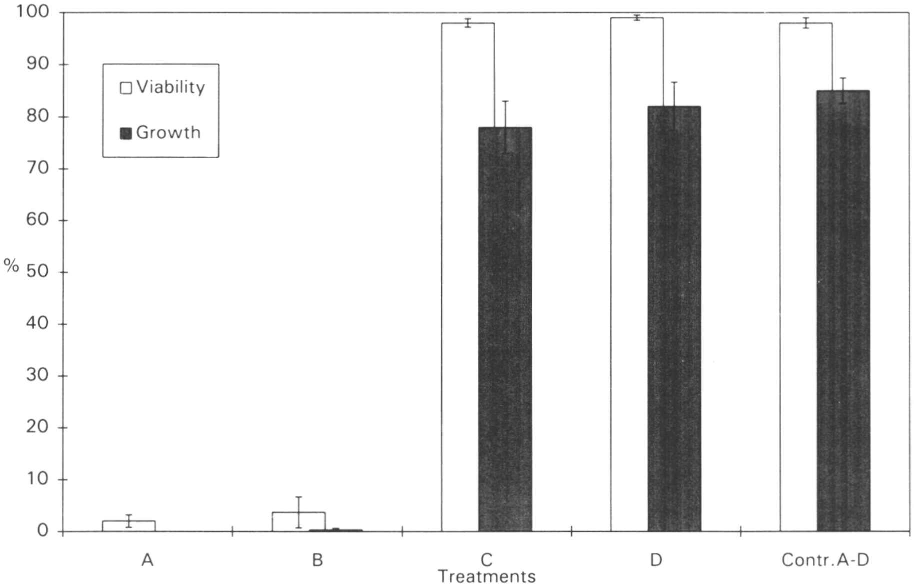
The Punkaharju area has a rather continental climate, with a mean January temperature during 1961–1990 of −10.4°C and the corresponding February temperature of −9.6°C. In 1993 the average temperature during the week before the March collection was −8°C and that of the April collection was −2.5°C. The collected material was stored in a cold room at −5°C from 1 to 4 weeks before cryopreservation. The bases of the twigs were sealed in plastic bags, filled with snow. Both axillary and apical buds of the twigs were used for cryopreservation. The buds with scales and a short piece of attached twig (1 cm) were sealed in 2-mL plastic ampoules. The ampoules were kept at approx. 0°C for 36 h until the procedure started.

The March buds were cooled and thawed according to the programs in Table 1. In fast cooling the samples were transferred from approx. 0°C straight into liquid nitrogen and kept there for 8 days. In slow cooling the samples were first frozen slowly from approx. 0°C down (10°C/h) to −38°C in a programmable controlled-temperature cooling chamber (Lauda Ultra-Kryomat RUK60). The decrease in temperature was followed by means of 0.5-mm copper–constantin thermocouples connected to Acurex Autodata/30. The samples were kept at the terminal prefreezing temperature of −38°C for 24 h and then immersed in liquid nitrogen for 8 days. In fast thawing the samples were thawed in a water bath at 37°C for 5 min, and in slow thawing, on ice for 3 h. The cultivation of unfrozen control buds was started at the same time as the cooling of other treatments. In the March collection 20 buds per clone were used each time, the test being repeated three times at intervals of 1 week.

All the April buds were cooled slowly, while storage temperature, storage time, and the thawing varied as presented in Table 1. In the April collection there were 10 buds per clone in each replication, and all three replications in each treatment were carried out at the same time. An exception to the procedure was protocol M, in which only two clones, E1987 and E5201, and two replications were used.

After thawing the buds were surface sterilized with 70% ethanol for 1.5 min. Cultivation was started according to the routine for micropropagation (Ryynänen and Ryynänen 1986) in test tubes, one bud per tube. WPM medium (Lloyd and

Fig. 1. Viability and growth percentage (±SE) of all buds collected in March after 8 days of storage in liquid nitrogen. Treatments: A, fast cooling and fast thawing; B, fast cooling and slow thawing; C, slow cooling and fast thawing; D, slow cooling and slow thawing; Contr. A–D, no cryopreservation. The viability and growth percentages in treatments A and B are significantly different from those in treatments C and D and control ($P < 0.01$).



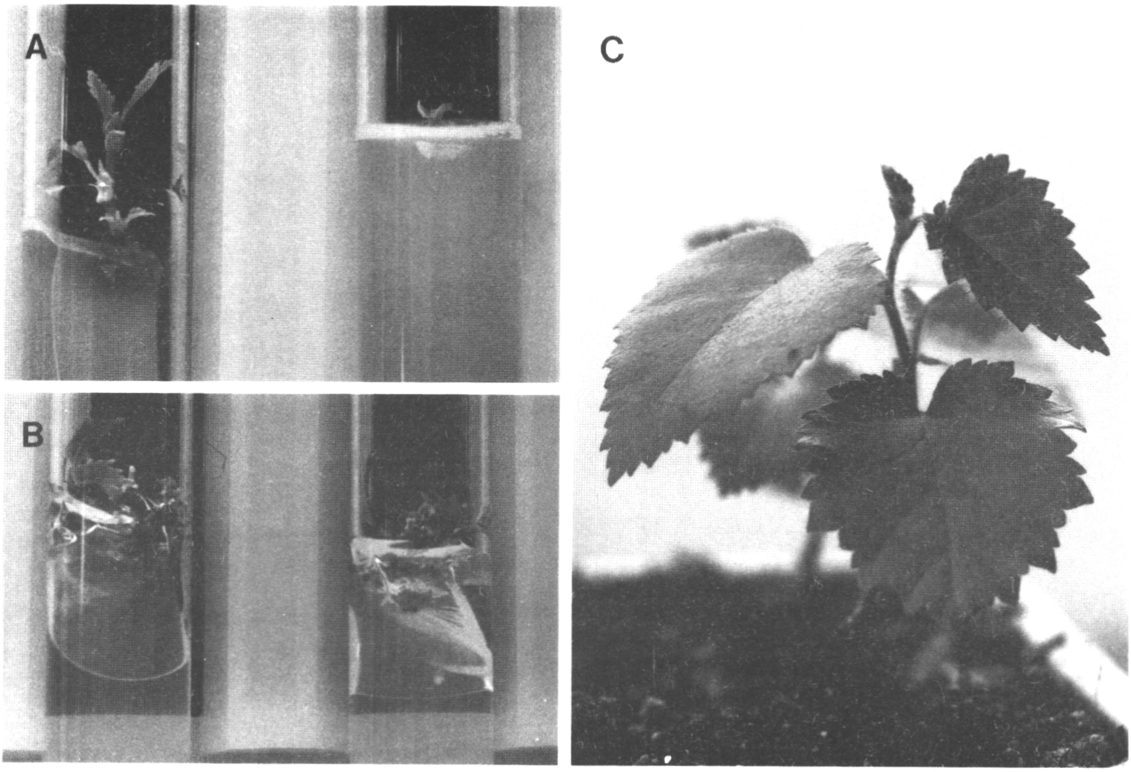
McCown 1980) with 4.4 μM BAP (6-benzylaminopurine) and 2% sucrose solidified with 0.8% agar was used for cultivation. Survival of the bud material was estimated visually after 2 and 4 weeks in the culture. Growth percentage includes both the buds that developed into a shoot directly and those that developed nodular callus and then spontaneously formed adventitious shoots within 4 weeks. All growing buds, buds staying green and (or) forming only callus were considered to be viable. When plantlets were micropropagated the material was transferred after the second estimation on the same medium solidified with 1% agar in baby food jars. The rooting of shoots took place in the same WPM medium with 1% sucrose and without any growth regulators. The rooted shoots were potted in peat—vermiculite (1:1), and plantlets were grown for 2 weeks in propagators in decreasing relative air humidity before transferring to the greenhouse.

One- and two-way analyses of variance were calculated, and the differences between the treatments were tested using Tukey's test (BMDP/DYNAMIC program).

Results

The slow-cooling program was essential for the survival of cryopreserved buds. In the March collection the survival of buds cryopreserved using a fast-cooling program combined with fast or slow thawing (treatments A and B) was negligible. There was no significant difference between bud survival in the slow-cooling programs, irrespective of the thawing treatment (C and D), and the control. In this first experiment the survival of slowly cooled buds in treatments C and D was almost identical with that of the controls. After 2 weeks of cultivation the growing percentage of treatment C was 69 ± 5.8%, and of treatment D, 71 ± 5.1%. The value for the corresponding control was 72 ± 4.7%. After 4 weeks the growing percentages were 78 ± 5.0% for C, 82 ± 4.6% for D, and 85 ± 2.4% for the control. The viability percentages after 2 weeks of cultivation were the same as after 4 weeks of cultivation: in treatment C, 98 ± 0.8%, in D, 99 ± 0.5%, and in the control, 98 ± 1.0% (Fig. 1). The growth of the cryopreserved buds started without any lag phase, as was the case for the control buds. There was no difference in the growth rate, size, or colour of the frozen buds and plantlets in the greenhouse compared with the unfrozen control (Fig. 2C). The growth percentage after 2 weeks of cultivation indicates the development of axillary and apical buds directly into a shoot. The increased growth percentage after 4 weeks of cultivation is dependent both on the retarded development of these buds (clones E1987 and E5398) and partially on the development of adventitious shoots (other clones). Adventitious shoot development of clone E5387 was strong regardless of the development of axillary and apical buds (Fig. 2B). One-half of the buds not developing shoots but considered viable stayed unchanged through the entire 4 weeks of cultivation. Another half of the undeveloped buds produced callus after 2 weeks of cultivation. In the rapidly cooled buds, tissue browning started immediately after thawing. Survival was zero in the first and second replications, and in the third replication the viability percentage of treatment A was 6.0 ± 3.0% (clones E5382 and E5398) and that of treatment B was 11 ± 8.7% (clone E5398). There were no significant differences between the

Fig. 2. Regeneration of silver birch buds stored for 8 days in liquid nitrogen. (A) A vegetative bud on the left and a vegetative bud from the axil of a female catkin on the right after 2 weeks of cultivation. (B) An axillary bud developing into a shoot (two bigger leaves) and developing adventitious shoots on the left. Adventitious shoots are developing from callus surrounding the undeveloped axillary bud on the right within 4 weeks of cultivation. (C) Silver birch plantlet in peat at the age of 8 weeks.



clones in the experiment, except for the growth percentage of E5382, which in treatment C was significantly different ($P < 0.05$) from the growth of E5387, and in treatment D from that of clones E1987 and E5387 ($P < 0.05$) (Table 2).

In the April collection the slow-cooling program was combined with different storage temperatures and storage times. After storage for 8 days, bud viability in treatments E and F (storage in liquid nitrogen, fast or slow thawing), in treatment J (storage at −70°C, slow thawing), as well as in the control was 100%. The growth of all buds in the treatments varied from 82% for the control to 55.2% for treatment F (Table 3). The overall viability of cryopreserved buds in the experiment, independent of the storage temperature and storage time, was almost 100%. The growth of all the buds subjected to long-term cryopreservation varied between 88% and 55%, but there was no significant difference between the growth of the buds cryopreserved in liquid nitrogen or at −70°C (Table 3). More detailed analysis of these growth results suggests that the absence of a female catkin had an effect on the growth of the vegetative bud (Fig. 2A). The growth percentage in buds with a female catkin was lower than that of the vegetative buds. When the buds with a female catkin were excluded, the growth percentage in the April collection varied from 100% to 72.0% (Table 3, Fig. 3). The corresponding maximum and minimum values for catkin buds were 88.4% and 40.0%. The average growth of buds without a catkin was 86.6% and that of buds with a catkin was 58.6%. The difference between these two groups was significant ($P < 0.001$). Owing to freezing damage no results were obtained for the long-term storage of buds at −70°C (treatments K and L). After storage at −70°C for 2 months, the freezing equipment broke down and the ampoules were transferred to −20°C, where they were retained for 10 months. Storage at this temperature resulted in zero survival.

Discussion

In the present work the acclimated silver birch buds were collected in March, and in April, when birch buds possess hardiness (Stushnoff and Junttila 1986) as well as good viability in in vitro cultivation (Ryynänen and Ryynänen 1986; Jansson and Wellander 1990). Acclimated tissues have shown to have a lower water content or higher solute concentration, and slow cooling to about −30°C promotes extracellular ice formation and further reduces the amount

Table 2. Influence of different treatments on the mean growth percentage (±SE) of five different silver birch clones stored for 8 days in liquid nitrogen, after 4 weeks of cultivation.

	Fast cooling		Slow cooling		
Clone	Fast thawing (A)	Slow thawing (B)	Fast thawing (C)	Slow thawing (D)	Control A–D
E1987	0	0	83.3±7.3	95.0±5.0*c*	77.0±6.0
E5201	0	0	90.0±7.7	88.3±9.3	86.7±3.3
E5382	0	0	58.3±8.8*b*	60.0±8.7*d*	86.7±4.4
E5387	0	0	91.7±1.7*a*	93.3±3.3*c*	95.0±2.9
E5398	0	1.7±1.7	68.3±15.9	75.0±10.0	78.3±3.3

Note: Values in a column and followed by different letters are significantly different ($P < 0.05$).

Table 3. Regeneration of cryopreserved silver birch buds collected in April, after 4 weeks of cultivation.

		Growth, %		
Treatment	Viability, %	All buds	Buds without a female catkin	Buds with a female catkin
E	100±0	66.8±6.6	72.0±8.8	58.5±21.0
F	100±0	55.2±5.0	nc	nc
G	94.0±1.9	88.0±2.0	94.4±3.3	88.4±3.0
H	100±0	55.3±5.8	83.3±8.2	44.0±5.8
I	99.3±0.7	69.9±5.6	93.4±3.8	51.3±6.7
J	100±0	72.7±4.5	nc	nc
K	—	—	—	—
L	—	—	—	—
M	97.5±2.5	77.5±10.3	80.0±20.0	40.0±0
N	100±0	72.3±6.8	85.0±8.4	63.9±7.7
Control E–N	100±0	82.0±9.2	100±0	61.4±21.9
Mean	99	72.2	86.6	58.6

Note: Values are means ± SE. nc, not counted.

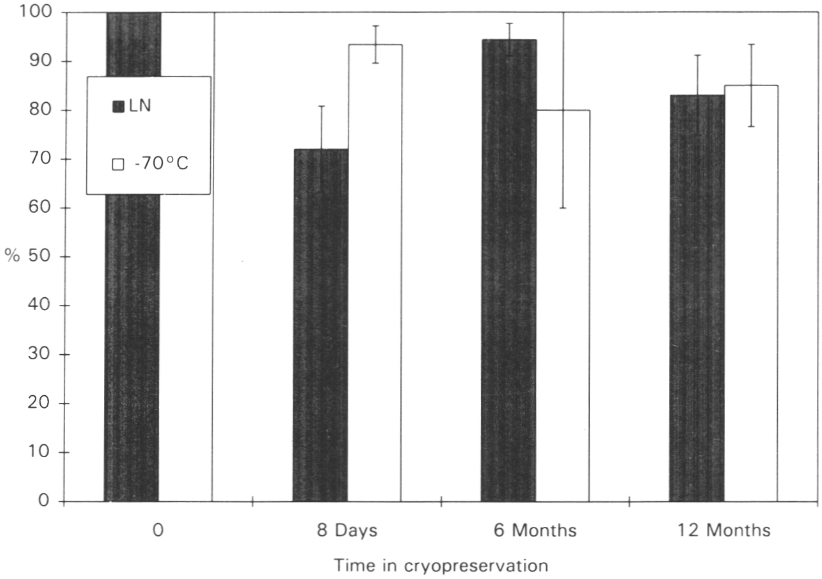
of liquid water within the cells (Sakai 1965; Tyler and Stushnoff 1988; Tyler et al. 1988). Hardiness is a state where the freezing of water no longer desiccates tissues to damaging levels (Vertucci and Stuchnoff 1992). Several cooling rates have been used during slow cooling when freezing in vivo material, ranging from 10°C/min (Wesley-Smith et al. 1992) to 10°C/day (Yakuwa and Oka 1988); the slow cooling of silver birch material is within these ranges.

In the present study the survival of cryopreserved buds after fast cooling was negligible. In fast cooling using high cooling rates, the water can be vitrified into an amorphous state, while using lower rates results in the formation of small intracellular ice crystals. During relatively slow warming there is a transition of small ice crystals to larger ones, resulting in extensive damage to cell membranes. Such damage can be avoided by increasing the cooling or warming rate and (or) rapidly drying the material (Wesley-Smith et al. 1992). According to Sakai (1965), all the woody twigs prefrozen at −5°C for 1 h to 5 days were destroyed by immersion in liquid nitrogen. The result was the same as that obtained in the present study except for the third replication, where some viability was observed. This may be due to the storage conditions; the twig bases were sealed in plastic bags, and the upper part of the twigs were exposed to the air current of the cooling system. Thus the twigs were slowly dehydrated and perhaps lost some vitality at the same time.

In the present study there was no significant difference in the regeneration of cryopreserved material when using storage temperatures of −70°C and −196°C for 8 days, 6 months, or 12 months. Storage in liquid nitrogen is preferred to that at −70°C, because no special equipment or electricity is required that would make storage uncertain. After 10 months at −20°C the silver birch buds were no longer viable. Desiccated embryos of oil palm stored at −18°C for 1 month completely lost their viability, but storage for 8 months in liquid nitrogen caused no loss of viability (Grout et al. 1983).

In the present study there was no significant difference between the slow- and fast-thawing methods, while conflicting results have been obtained with other plants. Sakai and Nishiyama (1978) reported that the rapid cooling and

Fig. 3. Growth of vegetative buds (slow cooled and fast thawed) after storage in liquid nitrogen and at −70°C for 8 days, 6 months, and 12 months. Growth percentage (±SE) was measured after 4 weeks of cultivation. Differences in growth between temperature and time are not significant.



rewarming method is not suitable for the shoots and buds of woody plants. The thawing of apple buds after exposure to different prefreezing temperatures prior to cryopreservation was carried out by the slow-thawing method only (Tyler et al. 1988). The studies of Yakuwa and Oka (1988) with buds of mulberry and those of Katano et al. (1983) with apple shoots indicate that the explants survive if an optimal prefrozen temperature is used, regardless of the thawing temperature. In several other tree species fast thawing is the only method used (Grout et al. 1983; Kuoksa and Hohtola 1991; Chaudhury et al. 1991; Wesley-Smith et al. 1992).

Survival of cryopreserved in vivo buds is not only dependent on the method used, but also on the presence or absence of surrounding tissues like scales. The scales protecting the bud are the most important. Inclusion of bud scales and a segment of the twig reduced injury to the meristems in cryopreserved mulberry (Yakuwa and Oka 1988). The beneficial influence of accompanying shoot segments on the survival of buds may be explained by the fact that the cortex of dormant shoots is very hardy against frost injury. The cortex of dormant apple shoots survived low temperatures at least as well as the buds (Sakai and Nishiyama 1978). According to Stushnoff and Junttila (1986), the twigs of maximumly hardened *Betula* and *Salix* were hardy to −80°C, which was about 20°C lower than the temperature that the buds could stand.

The flowering of birch was very abundant during the year when the present study was carried out, so that over half of the buds in both collections included a female catkin. The size of the vegetative bud in the axil of the female catkin was considerably smaller than that of pure vegetative buds. The small size of these buds may be one reason for the decline in their survival after cryopreservation.

In the present study the growth rate of the cryopreserved birch buds did not differ from that of the unfrozen controls; growth started immediately without any browning after culture. The initial growth of cryopreserved, in vivo mulberry buds (Yakuwa and Oka 1988) was extremely slow compared with the unfrozen ones. Some of the mulberry explants in which the apical meristem had deteriorated produced shoots. In the present study new shoots were also regenerated from the callus of cryopreserved birch, not only from callus with destroyed apical buds, but also from the basal callus of the growing buds. Callus produced from the in vitro grown apical meristems of *Pyrus* after cryopreservation did not redifferentiate into shoots (Reed 1990).

In conclusion, when slow cooling is used, the survival of the cold-acclimatized, cryopreserved buds of silver birch is excellent and there is no decrease in the success of micropropagation compared with fresh material. Using material acclimated in nature avoids the need for cryoprotectants for preconditioning, and their toxic influence on tissue culture and regeneration is therefore avoided. Cryopreservation of germ plasm has its advantages. Although it is not a universal method that meets all the needs of conservation purposes, it is a new tool that can be used together with other methods to achieve sufficient gene conservation. As well as for gene conservation, this method is also of assistance in organizing the time schedule of silver birch micropropagation using dormant buds as the starting material.

Acknowledgements

I thank Dr. Hely Häggman for critical reading of the manuscript and Ms. Airi Huttunen and Mr. Jouko Lehto for technical assistance.

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II

Effect of early spring birch bud type on post-thaw regrowth after prolonged cryostorage

Leena Ryynänen

Abstract: Early spring buds of silver birch (*Betula pendula* Roth), collected with and without a female catkin in the middle of April, were cryopreserved using slow cooling followed by immersion in liquid nitrogen at –196°C for 8 days, 6 months, 1 year, 3 years, or 5 years. After fast thawing the buds were cultured in vitro according to the published protocol. The regrowth ability of the two types of buds was different. The regrowth of vegetative buds without a female catkin was good after all the cryopreservation times. There was a significant decrease in the regrowth ability of buds growing in the axil of a female catkin compared with the corresponding unfrozen controls after 1 year in cryostorage. In addition to the effect of the presence of a catkin on the regrowth ability, the late collecting time of the buds also probably decreased the regrowth and regrowth rates of both types of bud. The regrowth rates of buds without a catkin were 66, 67 and 24% after 1, 3, and 5 years of cryostorage, respectively, while those of buds with a catkin were 13.5, 32, and 2.6%, respectively.

Résumé : Des bourgeons végétatifs de bouleau verruqueux (*Betula pendula* Roth) accompagnés ou non d'un chaton pistillé ont été récoltés à la mi-avril et maintenus en cryoconservation en procédant à un refroidissement lent suivi d'une immersion dans l'azote liquide à –196°C pour 8 jours, 6 mois, 1 an, 3 ans et 5 ans. À la suite d'un dégel rapide, les bourgeons ont été cultivés in vitro en suivant le protocole publié. La capacité de reprise de croissance des deux types de bourgeons était différente. La reprise de croissance était bonne pour les bourgeons végétatifs sans chaton pistillé pour toutes les périodes de cryoconservation étudiées. Comparativement aux bourgeons témoins conservés non gelés, on a observé une diminution significative de la capacité de reprise de croissance des bourgeons situés à l'aisselle de chatons pistillés à la suite d'une année de cryoconservation. En plus de l'effet de la présence d'un chaton sur la capacité de reprise de croissance, la date de récolte tardive des bourgeons a probablement diminué la reprise de croissance et le taux de reprise de croissance des deux types de bourgeons. Les taux de reprise de croissance des bourgeons sans chaton atteignaient respectivement 66, 67 et 24% après 1, 3 et 5 ans de cryoconservation tandis que celui des bourgeons avec chaton était de 13,5, 32 et 2,6%.

[Traduit par la rédaction]

Introduction

The cryopreservation protocol for in vivo material was first developed for tropical woody plants of great economical importance that produce recalcitrant seed that cannot be stored for long periods, e.g., oil palm (*Elaesis guinensis* L.) (Grout et al. 1983), coconut (*Cocos nucifera* L.) (Bajaj 1984), and tea (*Camellia sinensis* (L.) O. Kuntze) (Chaudhury et al. 1991). The germplasm of these plants has been conserved by means of direct immersion of the desiccated embryos or their segments in liquid nitrogen, and regrowth has been performed by culturing in a nutrient medium. Another application of this method is the cryopreservation of dormant vegetative buds of species acclimated in nature. The cryostorage of buds of several hardwoods has been successful, e.g., apple (*Malus* sp.) (Sakai and Nishiyama 1978; Katano et al. 1983; Tyler and Stushnoff 1988), mulberry (*Morus bombycis* Koidz.) (Yakuwa and Oka 1988), and silver birch (*Betula pendula* Roth) (Ryynänen 1996).

Although the details of the cryopreservation protocols for the above tree species differ to some extent, the main recommendations concerning the quality and treatment of the bud material before immersion in liquid nitrogen are the same. Dormant twigs are cut into 1- (Ryynänen 1996) to 15-cm (Sakai and Nishiyama 1978) pieces, each containing at least one bud, prefrozen slowly up to –40°C, after which they are immersed in liquid nitrogen without any cryoprotectants. After being cryostored for periods ranging from 15 min (Katano et al. 1983) to 8 days (Ryynänen 1996), both fast and slow thawing have been tested on all the samples. In addition to visual estimation (greening or browning) of viabilty, the post-thaw regrowth ability has been tested by grafting the buds (Sakai and Nishiyama 1978; Tyler and Stushnoff 1988) or by means of tissue culture (Katano et al. 1983; Yakuwa and Oka 1988; Ryynänen 1996). When using tissue culture in the regeneration of cryopreserved buds, about 50% of the buds of mulberry (Yakuwa and Oka 1988) that were prefrozen to –10°C and immersed in liquid nitrogen for 24 h formed shoots. The regrowth percentage of silver birch buds prefrozen to –38°C and cryostored in liquid nitrogen for 8 days was about 80% (Ryynänen 1996). Although the first cryopreservation studies with in vivo bud material were performed already two decades ago, no results have been published about the survival in cryostorage for more than 1 or 2 years. Winter vegetative buds of apple (Sakai and Nishiyama 1978) sprouted normally when grafted after 1 year of cryostorage, and were still alive after

Received May 27, 1998. Accepted November 3, 1998.

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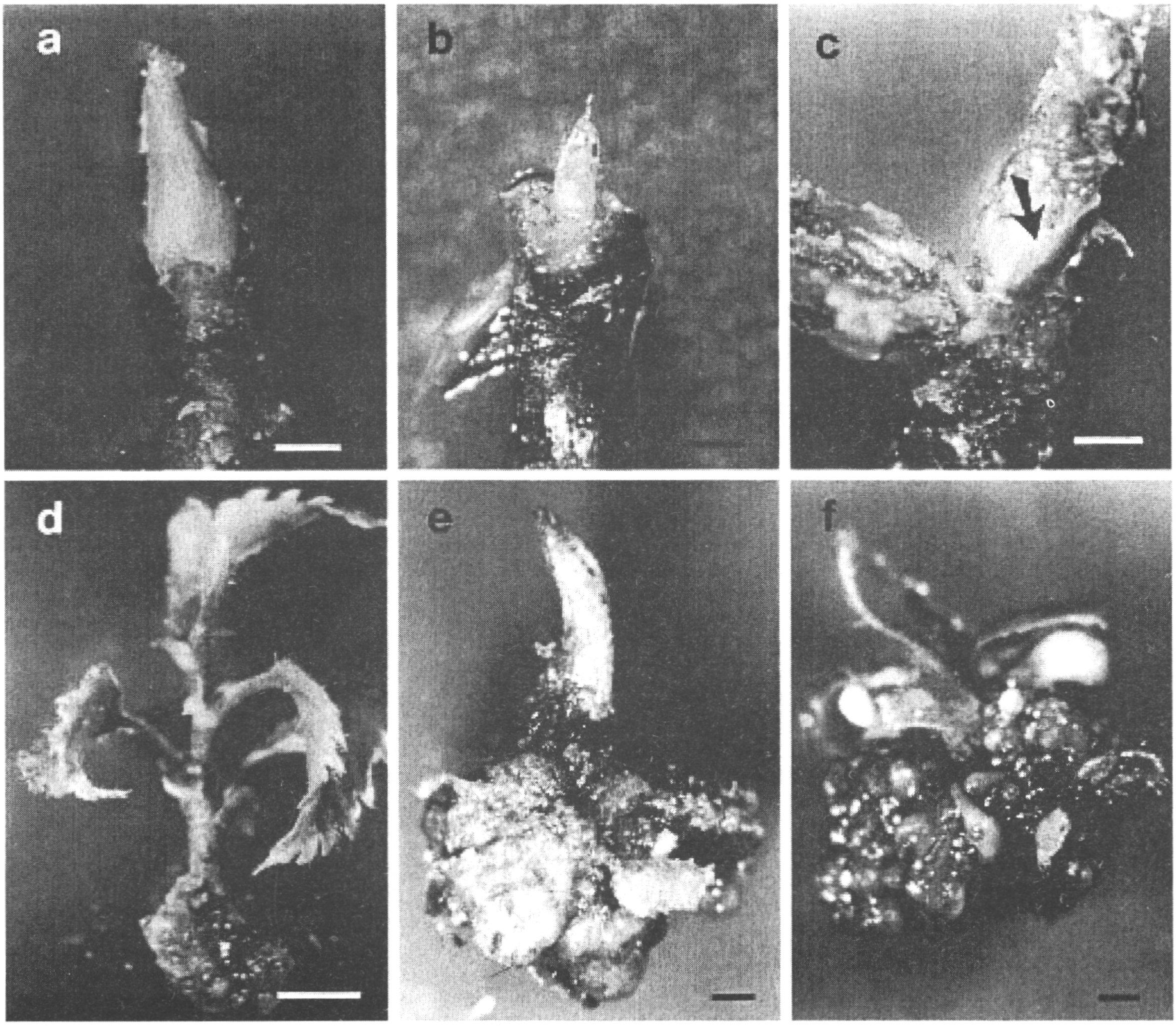
Fig. 1. Different types of cryopreserved bud after thawing. **Figs. 1*a*–1*c*.** Buds before cultivation. (*a*) A large, vegetative bud. (*b*) A vegetative bud of average size. (*c*) A bud (arrow) of above average size in the axil of a female catkin. The leaf on the left and the female catkin on the right were cut off before cultivation. Scale bars in Figs. 1*a*, 1*b*, and 1*c* = 500 µm. **Figs. 1*d*–1*f*.** Buds in culture. (*d*) A vegetative bud after 2 weeks of culture. Scale bar = 250 µm. (*e*) Viable, non-regrowing bud from the axil of a female catkin after 4 weeks of culture. (*f*) A regenerating bud from the axil of a female catkin 13 weeks after thawing that was estimated to be viable, but nonregrowing at the estimation time after 4 weeks of culture. Scale bars in Figs. 1*e* and 1*f* = 500 µm.



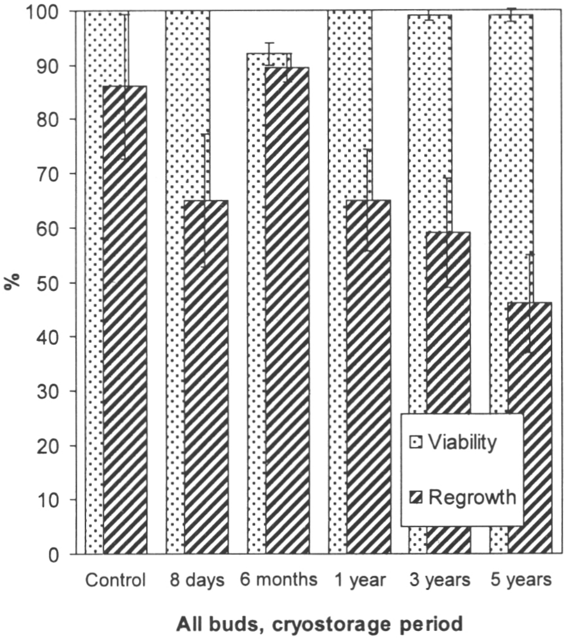
storage for 23 months in liquid nitrogen. The regrowth percentage of silver birch buds stored in liquid nitrogen for 6 or 12 months remained unchanged compared to unfrozen controls (Ryynänen 1996).

Cryopreservation has some advantages as a method for the germplasm preservation of forest trees: it retains the juvenility of the material and has minimum space and maintenance requirements. The basic demands for material and maintenance must, however, be known before applying the cryopreservation of dormant buds to the germplasm preservation of forest trees. The aim of the present study was to investigate the effect of two types of silver birch buds collected during early spring on their post-thaw viability and regrowth ability after being cryopreserved for 8 days to 5 years.

Materials and methods

Twigs bearing vegetative and flower buds of silver birch (*Betula pendula* Roth) were collected in the middle of April 1993 from five plus trees (E1987, E5201, E5382, E5387, and E5398) growing in a silver birch stand at Punkaharju (61°49′N, 29°18′E, 90 m above sea level). The stand had been established by planting with 1-year-old seedlings of local origin in 1932. The twigs (about 30 cm) were collected from the crown of the trees, which had an average height of 27 m.

Fig. 2. Viability and regrowth percentages (±SE) of all buds after 8 days, 6 months, 1 year, 3 years, and 5 years of storage in liquid nitrogen estimated after 4 weeks of post-thaw culture.



The Punkaharju area has a continental climate, the mean January temperature during 1961–1990 being –10.4°C and the corresponding February, March, and April temperatures –9.6, –4.3, and 1.6°C. In 1993 the average temperature during the week before twig collection was –2.5°C. The collected material was stored in a cold room at –5°C an average for 10 days before cryopreservation. During storage in cold room the twigs were sealed in plastic bags with their bases in snow. As the flowering of birch was very abundant in 1993, over half of the buds included a female catkin. The presence or absence of a female catkin in a bud could not be determined without peeling open the bud, so all the buds on the twigs, both axial and apical ones, were cryopreserved.

The buds with scales and a short piece of attached twig (1 cm) were sealed in 2-mL cryotubes. The cryotubes were kept at approximately 0°C for 36 h and then slowly frozen down (10°C/h) to –38°C in a programmable controlled-temperature cooling chamber (Lauda Ultra-Kryomat RUK60). The decrease in chamber temperature was followed by means of 0.5-mm copper–constantin thermocouples connected to an Acurex Autodata/30. The samples were kept at the terminal temperature of –38°C for 24 h and then immersed in liquid phase in liquid nitrogen container. (Ryynänen 1996). The samples were thawed in a water bath at 37°C after 8 days, 6 months, 1 year, 3 years, or 5 years of cryostorage.

After thawing, the buds were surface sterilized with 70% ethanol for 1.5 min. The buds were peeled, and the presence or absence of a female catkin as well as any marked differences in the size or morphology of the vegetative bud were noted (Figs. 1*a*–1*c*). Bud culture was carried out according to the standard protocol for the micropropagation of silver birch (Ryynänen and Ryynänen 1986) in 13 × 100 mm test tubes, one bud per tube. The growth conditions in the growing room were a 16 h light : 8 h dark photoperiod with the light intensity of 85–114 $\mu E \cdot m^{-2} \cdot s^{-1}$ at 22°C. WPM (woody plant medium) (Lloyd and McCown 1980) with 4.4 μM BAP (6-benzylaminopurine) and 2% sucrose solidified with 0.8% agar was used for cultivation. The material was not transferred onto new media during the 4 weeks of post-thaw culture.

Viability and regrowth of the bud material was estimated visually after 2 and 4 weeks of post-thaw culture. The regrowth percentage includes both buds that developed into a shoot directly and those that formed adventitious shoots (early shoot formation) from nodular callus that developed in the basal part of buds. All regrowing buds and the ones that remained green and (or) formed only callus were considered to be viable. Viability and regrowth percentages presented in results and, in Figs. 2 and 3, were estimated after 4 weeks of post-thaw culture. The regrowth rate was calculated as a percentage of the regrowing buds after 2 weeks of post-thaw culture. For plantlet propagation from the cryopreserved buds, the material was transferred after 4 weeks of post-thaw culture into glass jars containing medium of the same composition but solidified with 1% agar. Shoot rooting took place in WPM with 1% sucrose and no growth regulators. The rooted shoots were potted in peat–perlite (1:1), and the plantlets were grown for 2 weeks in propagators in a decreasing relative air humidity before transfer to the greenhouse.

A total of 150 buds were used for each cryostorage period (10 buds each from five trees with three replicates), except for the 5 years storage period when all the buds that were left, 122 in total (10–43 buds per tree) were used. The culture of unfrozen control buds was started at the same time as the freezing for cryostorage. The number of unfrozen control buds was 50. Cultivation of the control buds and those after 8 days of cryostorage was performed only once.

Statistical comparisons between the two bud types and cryostorage periods were made using one- and two-way analysis of variance, and the means were compared using the a Student–Newman–Keuls multiple range test (BMDP/DYNAMIC 7.0 program).

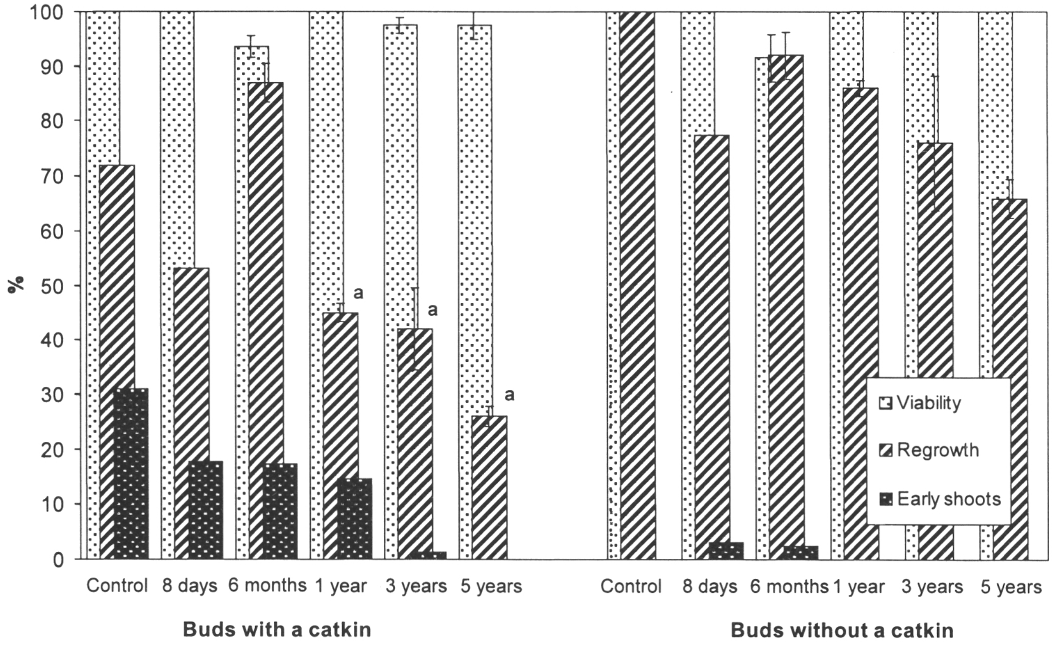
Results

Prolonging the cryopreservation period of early spring birch buds for up to 5 years did not decrease the viability of the buds as compared with the viability of noncryopreserved control (100%) when the viability of all buds was considered (Fig. 2). The viability of buds cryostored for 5 years was still 99 ± 1.0% (mean ± SE). During the same storage period the average regrowth of the buds decreased from 86 ± 14% of the noncryopreserved controls to 46 ± 9.0% of the buds stored for 5 years. However, the decrease in regrowth was not significant (Fig. 2).

The viability of buds without a female catkin was 100%, irrespective of the length of the cryostorage period (Fig. 3). The growth percentage of the noncryopreserved control buds without a catkin was also 100%. Although there was a slight tendency for decreased regrowth with increasing storage time, the regrowth percentage of buds without a catkin cryostored for 5 years (66 ± 3.5%) did not differ significantly from that of the control (Fig 3). Neither were there any significant differences in the viability of buds with a female catkin. However, the regrowth of buds with a female catkin after 1 year (45 ± 1.6%), 3 years (42 ± 7.5%), and 5 years (26 ± 1.8%) of cryostorage differed significantly ($P < 0.05$) from the corresponding control (72%) (Fig. 3).

Although the regrowth of buds without a female catkin remained unchanged after storage for 5 years in liquid nitrogen, the regrowth rate of the buds (24 ± 13%) had decreased significantly ($P < 0.05$) compared with the control (100%) during the storage period (Fig. 4). The regrowth of buds

Fig. 3. Viability and regrowth percentages (±SE) of buds with and without a female catkin and percentages of those forming early shoots after 8 days, 6 months, 1 year, 3 years, and 5 years of storage in liquid nitrogen. *a*, regrowth significantly ($P < 0.05$) lower than the corresponding control. Estimation after 4 weeks of post-thaw culture.



with a catkin decreased already after 1 year of cryostorage. This was also reflected in a lower regrowth rate that was significantly ($P < 0.05$) different after 1 year (13 ± 1.6%) and after 5 years of storage (2.6 ± 2.6%) in liquid nitrogen compared with that of the corresponding control (62%) (Figs. 1*d* and 4).

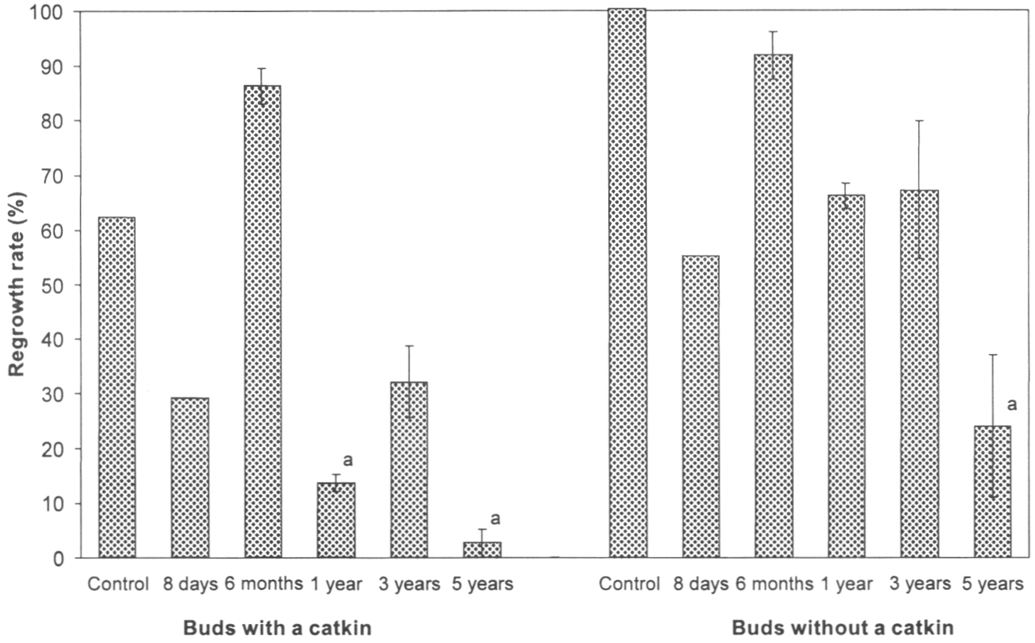
A change in bud vigour was also seen in the varying ability of callus to form spontaneously adventitious shoots estimated after 4 weeks of culture. Nearly all the buds with early shoot formation were those bearing a female catkin collected from two of the five plus trees (E5201 and E5387). The percentage of buds with a catkin forming early shoots was 17.6% after 8 days of cryostorage, 17.3% after 6 months of storage, 14.6% after 1 year of storage, and 1.3% after 3 years of storage, while that of the non-cryopreserved control buds with a catkin was 31.0%. No early shoot formation was observed from the buds after 5 years of storage in liquid nitrogen. However, after the material was transferred for micropropagation some days after 4 weeks estimation, multiplication of the plantlets through adventitious shoots also occurred from those buds that were estimated to be viable and observed to produce callus at the estimation after four weeks of culture (Figs. 1*e* and 1*f*). The multiplication and rooting of shoots during tissue culture and the growth of potted plantlets were equal to controls. A number of propagated plants (about 100) was limited to that needed for further studies.

Discussion

Visual estimation of viability based on green colour and callus formation, in addition to the regrowth of a cryopreserved bud, gave high viability percentages for all the cryostorage periods. Compared with regrowth and regrowth rate percentages, both of which decreased with increasing storage time, the viability remained unchanged. The result is similar to that for the viability of mulberry buds, which remained unchanged (100%) even though the shoot formation capacity of the buds changed as a result of different prefreezing temperature and thawing methods (Yakuwa and Oka 1988). Greening and callus formation of cryopreserved material could mean that it is viable but injured during cryostorage and lost ability to differentiate (Reed 1993). This is true in most cases, e.g., in the regeneration of organized shoots in the callus of cryopreserved shoot tips of apple (*Malus domestica* "Jonathan") (Kuo and Lineberger 1985) or in the regeneration of cryopreserved meristems of pear (*Pyrus* sp.) (Reed 1990). In this respect, silver birch seems to be an exception; callus formed after cryopreservation still retained some ability to differentiate. This is possible for callus formed after the cryopreservation of in vivo buds, as in the present study, as well as for callus formed after the cryopreservation of in vitro shoot tips of silver birch cultivated in the presence of abscisic acid (ABA) (Ryynänen 1998). However, when the purpose of the cryopreservation method is to ensure conservation and regeneration of the material for future use, measuring the regrowth is the only reliable evaluation method available.

With regard to the regrowth percentages obtained with prolonged cryostorage periods, there was a significant decline in the regrowth of buds in the axil of a female catkin compared with the unfrozen controls. Dormant buds of apple sprouted after 12 months cryostorage in liquid nitrogen as well as after 2 h storage and remained alive when stored for

Fig. 4. Regrowth rates (regrowth percentages (±SE) 2 weeks after thawing) of buds with and without a female catkin. Buds thawed after 8 days, 6 months, 1 year, 3 years, and 5 years of storage in liquid nitrogen. a, Regrowth rate was significantly lower ($P < 0.05$) than in the corresponding control.



23 months in liquid nitrogen (Sakai and Nishiyama 1978). According to Kartha et al. (1979), the plant regeneration of frozen in vitro meristems of pea (*Pisum sativum* L.) decreased from 72% when thawed after 1 h to 60% when thawed after 6 months storage in liquid nitrogen. There were no differences in the regrowth percentages of meristems of tissue culture plantlets of salmonberry (*Rubus spectabilis* Pursh.) cryostored for 1 day or 6 months while the regrowth of *Rubus* spp. "Merton Thornless" decreased from 60 to 20% during the same cryostorage period (Reed 1988).

The result that the regrowth of birch buds with a catkin decreased with increased cryostorage time was not expected considering the temperature (–196°C) of liquid nitrogen. According to studies performed with animal cells, some alterations in the distribution of isoenzymes of LDH occurred in the primary monkey kidney cells during cryostorage lasting for a few months. Alterations were similar to those detected in cell culture growing under anaerobic conditions (Peterson and Stulberg 1964). Thus a decrease in survival during prolonged cryostorage is possible, and differences in the anatomical or physiological state of cryopreserved material may result in differences in survival during storage.

The presence of a catkin significantly affected the regrowth ability of the buds. In addition to lower regrowth of the controls, the regrowth ability and regrowth rate of buds with a catkin decreased faster during cryostorage (Figs. 3 and 4). The smaller size and different site of the vegetative bud in the axil of the female catkin may be one reason for the decline. The different tolerance of the two types of birch bud to cryostorage is in accordance with the tolerance of apical and axillary shoot tips of carnation (*Dianthus caryophyllus* L. var. Eolo) to freezing in liquid nitrogen. The regrowth of apical shoot tips of carnation was close to 100%, whereas that of axillary shoot tips decreased with increased distance from the apical shoot tip during 1 week of cryostorage (Deuddre et al. 1988). As suggested in the carnation studies, growth inhibition induced by apical dominance, in the case of silver birch by a female catkin could explain the decreased regrowth of buds with a catkin.

The average weekly temperature in April in 1993 before the buds were collected was –2.5°C, which was already considerably warmer than during midwinter (January–February), when the mean temperature remained at about –10°C. Collecting the twigs at this time was probably too late for long-term cryostorage. Although the morphological appearance of the buds remained unchanged, the physiological cessation of dormancy had probably already started. Compared with the regrowth percentage (78%) of all the buds cryostored for 8 days after collection in the beginning of March (Ryynänen 1996), a decrease in regrowth (65%) was already seen in the present material cryostored for the same time. The different developmental state of the buds collected early in the winter and those in April also had an effect on the ability of buds to form early adventitious shoots. There were no differences in the ability of different types of bud to form early shoots after the winter collection, while in this study the buds with a catkin formed early shoots. The cessation of dormancy after the middle of April in Finland was even more evident in the decreased viability of cryopreserved buds of Scots pine (*Pinus sylvestris* L.). When Scots pine twigs were collected in the beginning of April the viability after quick immersion in liquid nitrogen was 100%, but when collection took place in the middle of April, the viability fell to 27% (Kuoksa and Hohtola 1991). According to Rinne et al. (1994), the first anatomical changes associated with the cessation of dormancy in birch are also seen in April, for instance, the amount of starch in the terminal buds of *Betula pubescens* Ehrh. increased considerably in the material collected from central Finland.

Conclusions

The results of this study show that the cryopreservation of silver birch buds with concomitant regeneration of plants is possible for a few years at least. However, during prolonged storage, up to 5 years, the regrowth ability of the cryopreserved buds decreased with the passage of time. The regrowth of cryostored buds was dependent on both the developmental state of the buds, on the presence or absence of a female catkin, and on the timing of bud collection. All these factors have to be carefully considered before the cryopreservation of dormant silver birch buds can be safely used for gene conservation.

Acknowledgments

The author thanks Dr. Hely Häggman and Dr. Tuija Aronen for critical reading of the manuscript and Airi Huttunen and Jouko Lehto for their technical help. The present study was supported by the Regional Fund of Mikkeli of the Finnish Cultural Foundation.

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III

Cold hardening and slow cooling: tools for successful cryopreservation and recovery of in vitro shoot tips of silver birch

Leena Ryynänen

Abstract: Shoot tips of silver birch (*Betula pendula* Roth) derived from in vitro cultures originating from mature trees were used as explant material. Several pretreatments prior to cryopreservation were studied in order to enhance shoot tip recovery after storage in liquid nitrogen. Cold acclimation and the classical slow freezing procedure proved to be essential for successful survival, while both nonhardening and vitrification resulted in minimal survival. The optimum procedure was the following: Shoots were cold hardened (5°C on a 8 h : 16 h (light:dark) cycle) for 3 weeks, and dissected shoot tips were then precultivated on woody plant medium with 5% dimethyl sulfoxide for 72 h. The material was transferred to cryotubes, and a mixture of 10% polyethylene glycol, 10% glucose, and 10% dimethyl sulfoxide was used as cryoprotectant. The cryotubes were slowly cooled to −38°C before immersion in liquid nitrogen for 8 d. After fast thawing, axillary buds formed shoots without callus formation, but the growth of the new shoots was delayed from a few days to several weeks. Variability in regrowth was considerable as a result of genotype differences.

Résumé : Des apex de rameaux de bouleau verruqueux (*Betula pendula* Roth) obtenus en culture in vitro à partir d'arbres matures ont été utilisés comme explants. Plusieurs traitements préparatoires à la cryopréservation furent étudiés dans le but d'améliorer la récupération des apex de rameaux après les avoir conservés dans l'azote liquide. L'acclimatation au froid et la procédure classique de gel progressif se sont avérés nécessaires pour obtenir un taux de survie adéquat tandis que l'absence d'endurcissement et la vitrification entraînaient un minimum de survie. La procédure optimale était la suivante : les rameaux étaient endurcis (5°C et photopériode de 8 h) pendant 3 semaines, les apex des rameaux étaient disséqués et pré-cultivés sur un milieu pour plantes ligneuses additionné de 5% de diméthyl sulfoxide pendant 72 h. Le matériel était transféré dans des tubes de congélation et un mélange de 10% de polyéthylène glycol, 10% de glucose et 10% de diméthyl sulfoxide était utilisé comme cryoprotectant. Les tubes étaient refroidis lentement jusqu'à −38°C avant d'être immergés dans l'azote liquide pour 8 jours. Après un dégel rapide, les bourgeons axillaires formaient des rameaux sans qu'il y ait formation de cal mais la croissance des nouveaux rameaux était retardée de quelques jours à plusieurs semaines. La reprise de croissance était très variable à cause des différences génétiques.

[Traduit par la Rédaction]

Introduction

During the last 2 decades in vitro propagation techniques have been developed for several commercially important tree species. In addition to the multiplication of selected genotypes, these techniques have been applied to the gene conservation of woody plants (Kartha et al. 1981; Monette 1986; Dorion et al. 1993). In vitro storage is suitable for short- and medium-term conservation, since it extends the subculture intervals of micropropagated material. For long-term conservation of in vitro material cryopreservation is the method of choice. Cryopreservation of germ plasm has the advantages of minimum space and maintenance requirements. It also provides protection against contamination and retains the juvenility of adult genotypes. Although it is not a universal method answering all the conservation needs, it is a tool that can be used together with other methods or for special applications to achieve sufficient gene conservation.

Successful regeneration of plantlets following cryopreservation and genetic stability are prerequisites for applying the method in germ-plasm conservation. In in vitro cultivation the risk of somaclonal variation is much lower when using shoot apical and axillary meristems than with other systems such as suspension and callus cultures (Villalobos and Engelmann 1995). The cloning of silver birch (*Betula pendula* Roth) takes place through organogenesis in vitro via axial and adventitious shoot formation (Ryynänen and Ryynänen 1986), and silver birch has been micropropagated for both research and breeding purposes. Storage of germ plasm during long-duration laboratory testing and field trials is a problem. In vitro storage is laborious, and although cryopreservation of in vivo buds of silver birch is successful (Ryynänen 1996), development of cryopreservation protocol for in vitro material would be preferred.

Unlike dormant in vivo buds, plant material cultivated in vitro is actively growing and thus easily damaged during cryopreservation. However, application of different

Received February 1, 1996. Accepted June 6, 1996.

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Table 1. Experimental design to optimize the cryopreservation procedure of silver birch in vitro shoot tips.

		Hardening		Precultivation on WPM		
Treatment	No. of shoot tips	None or cold	Time, d	Compound added	Time, h	Cryo-protectant
First experiment						
A	900	None	—	5% DMSO	72	PGD
B	900	None	—	0.7 M sucrose	72	PVS2
C	900	Cold	21	5% DMSO	72	PGD
D	900	Cold	21	0.7 M sucrose	72	PVS2
AC	75	None	—	5% DMSO	72	PGD
BC	75	None	—	0.7 M sucrose	72	PVS2
CC	75	Cold	21	5% DMSO	72	PGD
DC	75	Cold	21	0.7 M sucrose	72	PVS2
0-control						
22°C, 16-h day	50	None	—	No precultivation	—	None
0-control						
5°C, 8-h day	50	Cold	21	No precultivation	—	None
Second experiment						
C/2	180	Cold	21	5% DMSO	72	PGD
E	300	Cold	21	—	72	PGD
F	300	Cold	24	No precultivation	—	PGD
C/2C	50	Cold	21	5% DMSO	72	PGD
FC	50	Cold	24	No precultivation	—	PGD
0-control						
5°C, 8-h day	50	Cold	21	No precultivation	—	None

Note: Cold-hardening conditions were an 8 h : 16 h (light:dark) cycle at 5°C. DMSO, dimethylsulfoxide; PGD, 10% polyethylene glycol, 10% glucose, and 10% DMSO in water; PVS2, 30% glycerol, 15% ethylene glycol, and 15% DMSO in WPM with 0.4 M sucrose; A–C, treatments of the first experiment; C/2–F, treatments of the second experiment; AC–DC, C/2C, FC, corresponding unfrozen control treatments; 0-controls, controls for none or cold hardening.

physical and chemical dehydrative treatments has made the in vitro cryopreservation possible for many plant species. The regeneration of cryopreserved in vitro tissue derived from a woody species was first reported by Ulrich et al. (1984), who cryopreserved callus cultures established from the hypocotyls of seedlings of *Ulmus americana* L. after slow cooling with dimethylsulfoxide (DMSO). Shoot tips of *Malus domestica* 'Jonathan' were the first ones cryopreserved by slow cooling (Kuo and Lineberger 1985). Apical meristems of *Rubus* (Reed 1988, 1990, 1993), *Pyrus* (Reed 1990), and *Ribes* (Brison et al. 1995; Reed and Yu 1995) have been cryopreserved with the same method. Regeneration of cryopreserved embryogenic cultures using slow cooling has also been successful for several conifers, e.g., *Picea glauca* (Moench) Voss (Kartha et al. 1988), *Picea abies* (L.) Karst. (Galerne and Dereuddre 1988), *Larix ×eurolepis* and *Picea mariana* (Mill.) BSP (Klimaszewska et al. 1992), *Pinus caribaea* Mor. var. *hondurensis* (Lainé et al. 1992), and *Picea sitchensis* (Bong.) Carrière (Find et al. 1993).

Vitrification, a fast cooling process that solidifies aqueous solutions into amorphous glass without crystallization (Debergh et al. 1992), was first developed for cryopreservation of nucellar cells of *Citrus sinensis* var. *brasiliensis* Tanaka (Sakai and Kobayashi 1990). Since then it has also been applied to the cryopreservation of apical meristems of several perennials (e.g., *Wasabia japonica* Matsumara (Matsumoto et al. 1994) and *Lilium japonicum* Thunb. (Matsumoto et al. 1995)), woody dicots (e.g., *Malus* and *Pyrus* species and cultivars (Niino et al. 1992)), and *Ribes* spp. (Reed and Yu 1995).

Cold hardening by cultivation of the donor shoot cultures at lowered temperature and in shorter daylight time has often been successfully used to improve the regeneration of cryopreserved apical meristems. Cold-hardening temperature has been constantly lowered near to 0°C (e.g., Seibert and Wetherbee 1977; Niino et al. 1992; Scottez et al. 1992) or has been lowered only during the dark period, which is usually 16 h (e.g., Reed 1988, 1990, 1993; Reed and Yu 1995). The most effective cold-hardening time varies with species, from 3 d for *Dianthus* sp. (Seibert and Wetherbee 1977) to 12 weeks for *Pyrus* sp. (Scottez et al. 1992).

The aim of this study was to develop a cryopreservation method for organogenetically micropropagated silver birch. Successful cryopreservation of in vitro shoot tips of silver birch could be used for the preservation of germ plasm as well as to replace in vitro storage of valuable research and breeding material.

Material and methods

Material

Twigs with dormant buds of silver birch were collected from five clones (E1987, E5201, E5382, E5387, and E5398) in Kokonniemi stand, at Punkaharju, Finland (61°49′N, 29°18′E; 90 m above sea level). The stand had been planted with 1-year-old seedlings of local origin in 1932. Shoot cultures were initiated from apical and axillary in vivo buds on WPM (Lloyd and McCown 1980) containing 4.4 μM BAP (6-benzylaminopurine) according to Ryynänen (1993). The shoot cultures were subcultured every third week and maintained on the same medium in baby food jars. Three lines of shoot cultures from the five clones were used. Line 1 was regenerated from slowly cooled and fast thawed buds, which after having acclimated in nature, were cryopreserved for 8 d in liquid nitrogen. Line 2 was regenerated from slowly cooled and slowly thawed buds (Ryynänen 1996). Line 3 was initiated from unfrozen and untreated buds. The growth conditions for the shoot cultures were a 16 h : 8 h (light:dark) cycle with a light intensity of 85–114 $\mu E \cdot m^{-2} \cdot s^{-1}$ at 22°C. The shoot cultures had been cultivated for about 10 months before starting the experiment.

Methods

The first experiment

In the first experiment (Table 1) the survival of shoot tips cryopreserved by a slow cooling method and vitrification was studied with and without cold hardening of the material. During the last subculture before the cryopreservation, the shoot cultures to be cold hardened were cultured on WPM under an 8 h : 16 h (light:dark) cycle with a light intensity of 23 $\mu E \cdot m^{-2} \cdot s^{-1}$ at 5°C. The nonhardened shoots were cultured under the same culture conditions as during the foregoing subcultures. The duration of the non- or cold-hardening subculturing was 21 d.

After cold hardening, all shoots (including those not hardened) were decapitated. Dissected shoot tips, about 0.3–0.4 cm in length, including four or five leaves with axillary buds, were transferred on Petri dishes and their bases were submerged in the preculture medium. In the slow cooling method with PGD (10% polyethylene glycol, 10 % glucose, and 10 % DMSO in water), the shoot tips were precultured on WPM with 0.5% DMSO for 72 h under the same non- or cold-hardening conditions as during the foregoing subculture (treatments A and C of the first experiment) (Table 1). After precultivation the shoot tips were transferred into 0.25 mL of liquid WPM in 2-mL plastic ampoules on ice. PGD was added dropwise to fill the ampoule over a period of 30 min. The ampoules stood for 30 min on ice before cooling. The samples were frozen at a rate of 10°C/h to the prefreezing temperature of −38°C in a programmable controlled-temperature cooling chamber (Lauda Ultra-Kryomat RUK60). After reaching the terminal temperature, the ampoules were immediately immersed in liquid nitrogen for 8 d. After storage the samples were thawed in a 37°C water bath for 5 min. The cryoprotectant was drained from the ampoules and replaced with liquid WPM for 30 min. The liquid medium was then changed, and the ampoules were emptied onto sterile filter paper in a Petri dish, and from there the shoot tips were plated on WPM in another Petri dish, 20 shoot tips per Petri dish.

In the vitrification (treatments B and D of the first experiment) (Table 1) the shoot tips were also precultured on WPM with 0.7 M sucrose for 72 h in the same non- or cold-hardening conditions as during the foregoing subculture. After precultivation the material was transferred to an ampoule containing 1 mL of PVS2 (30% glycerol, 15% ethylene glycol, and 15% DMSO in liquid medium with 0.4 M sucrose) at room temperature. The cryoprotectant (1 mL) was changed twice at intervals of 6 min. After the third change using 0.6 mL of PVS2, the ampoules were immediately immersed in liquid nitrogen. After storage the ampoules were thawed in a 37°C water bath for 5 min. The cryoprotectant was drained from the ampoules and replaced for 30 min with liquid WPM containing 1.2 M sucrose. The liquid medium was then changed, and the ampoules were emptied onto sterile filter paper in a Petri dish, from where the shoot tips were plated on WPM in a Petri dish as in the previous treatments.

The treatments of the first experiment described above (A–D), slow cooling and vitrification with non- and cold-hardened material, were performed on all three lines of all five clones. The number of replicate shoot tips per line was 20. The test was repeated three times. The control treatments (AC–DC) were treated in the same way as treatments A–D before freezing and after thawing, except that they were not frozen. Number of shoot tips in control treatments was five per line per clone. For the 0-controls, immediately after cold-hardening all shoot tips (including those not cold hardened) were cultivated in Petri dishes without any precultivation or cryoprotectant treatment. Number of shoot tips in 0-controls was 10 per clone (Table 1).

The second experiment

The second experiment (Table 1) involving modification of the precultivation stage in the slow cooling cryopreservation method was carried out only with cold-hardened material. In this experiment the precultivation of shoot tips on WPM containing 5% DMSO for 72 h (treatment C/2) was compared with the precultivation of shoot tips on WPM without DMSO (treatment E) and with the treatment in which cold hardening was lengthened to 24 d and no precultivation took place (treatment F). This experiment was performed using only line 3 from each clone. The experimental design was as follows: 20 shoot tips per Petri dish per clone. The experiment was repeated three times, except in treatment C/2, in which most of the clones were tested twice and clone E5398 was tested only once. As in the first experiment the controls (C/2C and FC) were treated in the same way as treatments C/2 and F before freezing and after thawing, except that they were not frozen. 0-control for cold-hardened material was equal to that in the first experiment. Number of shoot tips for C/2C, FC, and 0-control was 10 per clone. No controls for treatment E were done (Table 1).

Regeneration and anatomical studies

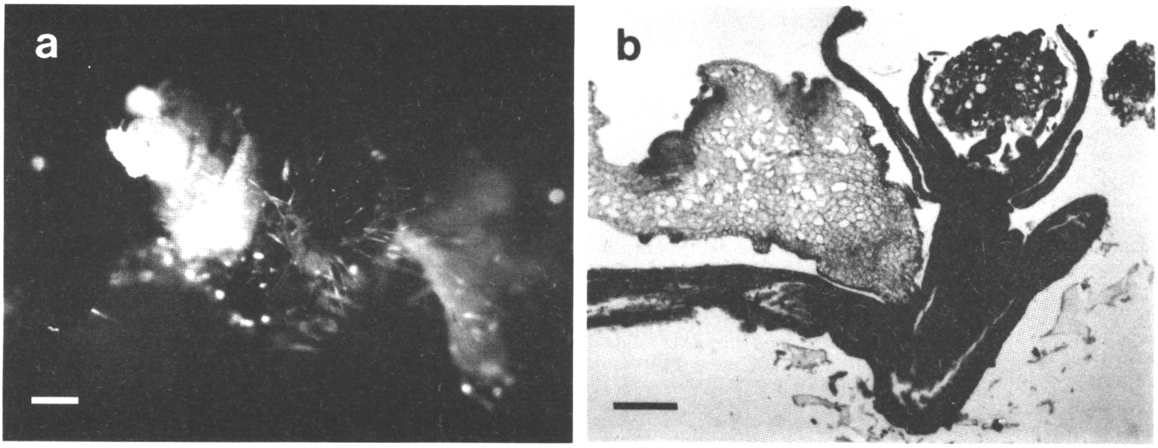
After thawing the shoot tips were cultured on WPM solidified with 1% agar in Petri dishes in the same culture conditions as shoot cultures; under a 16 h : 8 h (light:dark) cycle with a light intensity of 85–114 $\mu E \cdot m^{-2} \cdot s^{-1}$ at 22°C. The survival of the material was estimated visually after 2, 4, and 6 weeks of cultivation. Shoot tips that regenerated new shoots and leaves were classified as regrowing.

The anatomical structure of the regenerating tissues in the shoot tips was examined under a microscope (Zeiss Universal R). The shoot tips were fixed in FAA (formalin – acetic acid – 95% ethanol, 10:5:85, by vol.), and embedded in paraffin. Sections 15 μm thick were stained with safranin-fast green (Gerlach 1984).

Statistical analysis

Comparisons between different treatments were made by ANOVA. Means were compared by Tukey's test (BMDP/DYNAMIC program).

Fig. 1. Regeneration of cold-hardened in vitro shoot tip of silver birch: clone E5387 cryopreserved by slow cooling after WMP precultivation, 6 weeks after thawing. (*a*) A new shoot is growing from the axillary bud of the leaf on the left. Both old leaves and the old bud in the centre are dead. Bar = 500 µm. (*b*) Longitudinal section: the new shoot growing in the axil of the leaf on the left, all old parts are dead. Bar = 250 µm.



Results and discussion

Morphological observations

The cold-hardened shoots were shorter and thicker than the nonhardened ones. Apical buds, with pale green scales, were occasionally detectable by the naked eye in the cold-hardened material. The growth of all the controls started immediately after thawing without any lag phase or browning of the tissue. The regeneration of cryopreserved shoot tips was delayed compared with the unfrozen controls. The cryopreserved shoot tips turned brown within 1–2 days after thawing. When estimating the survival after 2 weeks of cultivation, the first signs of regeneration were seen: callusing of the uppermost leaves and growth of roundish axillary buds in the basal part of the shoot tips. In some cases the regeneration of leaves and scales surrounding the bud was also observed. More callus formation or organogenesis was not later observed in yellow-brown leaf callus. Further regeneration of the axillary buds took place during the entire cultivation time of 6 weeks. Regeneration of new shoots and leaves always started from the axillary buds in the basal part of the shoot tips without any callus formation. The developing axillary buds spontaneously detached from the leaf axil of the dead shoot tips (Figs. 1*a* and 1*b*). All the growing cold-hardened material, including controls, were red coloured during the first subculture. The regrowing buds and newly regenerating leaves and stems were initially pink, but the colour changed to green, starting from the tip of the leaves, during further development of the organs. Except for the colouring induced by cold hardening, no differences were detected in the delay or regeneration of shoot tips between the different treatments. The cryopreservation methods used had no effect on the regenerated plantlets in the greenhouse.

The delayed recovery of shoot tips in the present study indicates that the differentiated tissues had been cryodamaged, and that the new growth leading to organogenesis was initiated only from the undifferentiated primary meristems of the shoot tip. Callusing of the uppermost leaves at the beginning of the cultivation indicates that meristems in the upper part of shoot tips probably have also survived, but further development of them was unsuccessful because of a failure in nutrient supply through injured tissues. Organogenesis took place only in meristems that had a direct connection to medium in the bases of shoot tips. Survival of meristems and death of differentiated tissues observed in this study is in agreement with early reports. Only meristematic tissues of the shoot apex survived freezing in the cryopreservation of *Dianthus caryophyllus* L. (Seibert and Wetherbee 1977), and only embryonic head cells could be recovered after cryopreservation of several conifers (e.g., Gupta et al. 1987; Kartha et al. 1988; Klimaszewska et al. 1992; Lainé et al. 1992).

Previously cryopreserved material

The previous in vivo cryopreservation of the material had no aftereffect on the in vitro cryopreservation of the shoot tips. There were no significant differences between the three lines within each clone with respect to cold hardening or regrowth after in vitro cryopreservation using either the slow cooling method or vitrification. Average growth percentages of different lines within each treatments are shown in Table 2.

Cryopreservation of nonhardened material

Cryopreservation of the nonhardened shoot tips was not very successful in spite of the cryopreservation method used (Table 2, Fig. 2). No significant differences existed between the methods. The PGD cryoprotectant solution used in slow cooling proved to be nontoxic to silver birch. PGD is the cryoprotectant most widely used with successful recovery of shoot tips of woody dicots (Reed 1988, 1990, 1993; Reed and Yu 1995). On the other hand, the regrowth percentage of the nonhardened vitrification controls indicated some cytotoxity. This was more obvious when the PVS2 cryoprotectant was changed at intervals of 10 min; there was only a small degree of callus formation in the vitrification controls and no survival after vitrification was detected.

Cryopreservation of cold-hardened material

Slow cooling method and precultivation with 5% DMSO

Cold hardening at 5°C in 8 h daylight for 21 d considerably improved the regeneration of the material cryopreserved by the slow cooling method studied in the first experiment. The regrowth was over 20-fold more than that of nonhardened material (Table 2, Fig. 2). After 2 weeks of cultivation the average regrowth was 2.6 ± 0.9%, after 4 weeks it was 18.0 ± 3.5%, and after 6 weeks it was 22.6 ± 3.5%. With the slow cooling method the differences between the survival of nonhardened (A) and cold-hardened (C) material were statistically significant ($p < 0.0001$). The shorter cold hardening time of 1 week had no hardening effect on clones E1987 and E5382, which were cryopreserved by the slow cooling method.

The improvement of survival using cold hardening was expected; in its natural range silver birch can tolerate low temperatures during winter, and even the survival of cryopreserved in vitro shoot tips of temperate and subtropical tree species has been improved by cold hardening (Reed 1988, 1990; Scottez et al. 1992; Brison et al. 1995). Anyhow, the regeneration of in vitro material is inferior compared with that of silver birch buds acclimated in nature, which can be cryopreserved by slow cooling following storage at −196°C without losing their viability (Ryynänen 1996). According to Reed (1990), the ability to cold harden in the field does not necessarily relate to the ability of apical meristems of in vitro plants to cold harden and survive liquid nitrogen.

The cooling rate was the same as that which was successfully used in the cryopreservation of in vivo buds of silver birch (Ryynänen 1996). During slow cooling water will flow out of the cells and crystallize extracellularly, at the same time dehydrating the cells. This concentrates the cell sap and thus minimizes the formation of intracellular ice during subsequent fast cooling from −38°C to −196°C. The cooling rate used was slower than that normally used in the slow cooling of in vitro material, changing from 0.3°C·min^{-1} to 1°C·min^{-1} (e.g., Kartha et al. 1988; Brison et al. 1995). However, it is much faster than that occurring naturally in regions where silver birch is a native tree species.

Table 2. Percent survival ± SE for silver birch shoot tips after cryopreservation and for controls.

Cryopreservation method (treatment) and line	No hardening	Cold hardening
	% regrowth	% regrowth
Slow cooling (A, C)		
Line 1	1.0±0.7	27.4±7.7
Line 2	1.0±1.0	21.0±5.7
Line 3	0.7±0.5	19.3±4.4
Mean	*a* 0.9±0.4 *A*	*a* 22.6±3.5 *B*
Vitrification (B, D)		
Line1	1.3±1.3	0
Line2	0	0.7±0.5
Line3	0	1.3±0.8
Mean	*a* 0.4±0.4 *A*	*c* 0.7±0.3 *A*
Slow-cooling control (AC, CC)		
Line 1	100	100
Line 2	100	100
Line 3	100	100
Mean	*b* 100 *A*	*b* 100 *A*
Vitrification control (BC, DC)		
Line 1	90.7±3.9	100
Line 2	85.3±6.3	100
Line 3	96.0±2.1	100
Mean	*c* 90.7±2.6 *A*	*b* 100 *B*
0-control		
Line 1	100	100
Line 2	100	100
Line 3	100	100
Mean	*b* 100 *A*	*b* 100 *A*

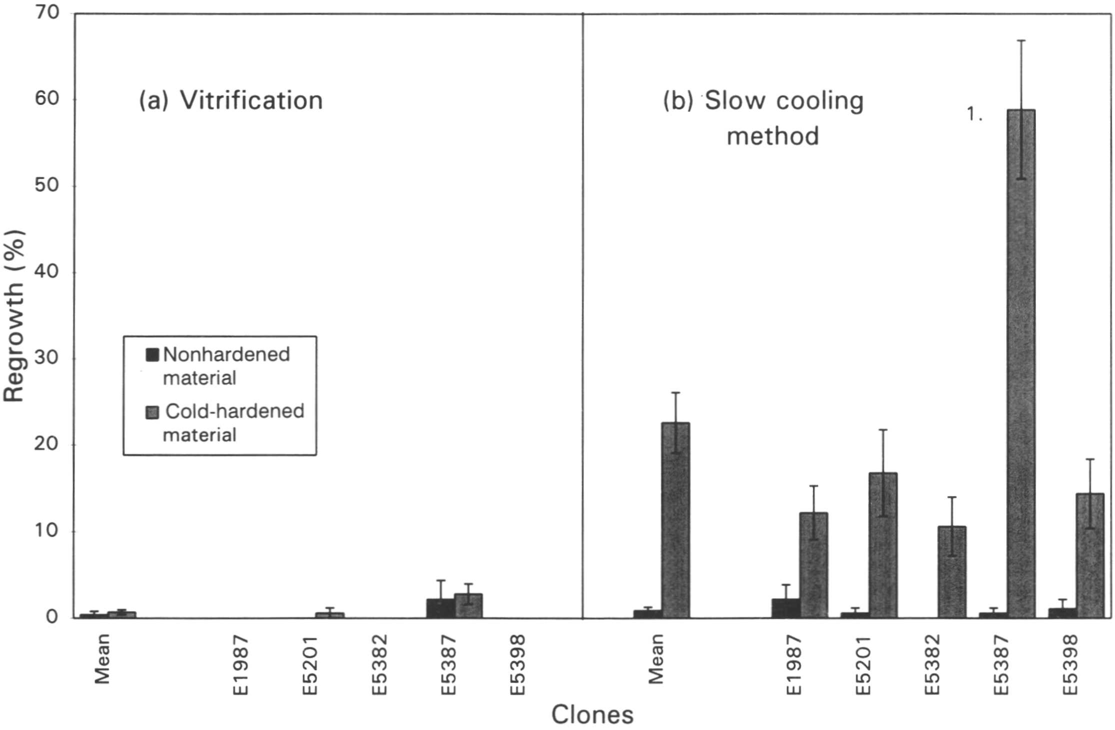
Note: There were no significant differences between lines 1 and 2, regenerated from previously in vivo cryopreserved material, and line 3, initiated from nonfrozen material, in respect to cold hardening or survival after in vitro cryopreservation. Different lowercase letters before mean regrowth percentages indicate differences affected by cryopreservation method compared with non- or cold-hardened material. Different capital letters after the percentages indicate differences between non- or cold-hardened material ($p < 0.01$).

Testing of other precultivations

In the second experiment different modifications of precultivation were tested with the slow cooling method after cold hardening. The regrowth for shoot tips that underwent the slow cooling method following precultivation with 5% DMSO (C/2), which was repeated with fewer shoot tips, was 24.7 ± 6.6%. That of shoot tips precultivated on WPM without the addition of protective or dehydrative compounds was 21.4 ± 9.3%. The corresponding survival for the material with a prolonged cold-hardening time instead of the precultivation of dissected shoot tips was 8.0 ± 4.4%. However, there were no statistical differences between these three treatments, the genotypically within-group variation being high. In the treatment where shoot tips were precultivated with DMSO, 90% of the axillary buds, which had started to grow, regenerated new shoots and leaves. The corresponding value for shoot tips precultivated on WPM without the addition of DMSO was 70%, and for those with no precultivation, it was only 65%.

In the present study the survival of shoot tips was lowest after cryopreservation without any precultivation. It is obvious that precultivation helps the shoot tips to recover from the dissection stress. Reports on the morphological regeneration of new shoots draw attention to the superiority of DMSO precultivation compared with other ones.

Fig. 2. Percent regrowth (±SE) of non- or cold-hardened in vitro shoot tips of different silver birch clones in the first experiment. (*a*) Cryopreservation by vitrification. (*b*) Cryopreservation by the slow cooling method. 1, regrowth of clone E5387 was significantly higher ($p < 0.01$) than that of the others.



Precultivation with 5 or 10% DMSO has been used successfully for the cryopreservation of shoot tips of woody dicots, *Rubus* (Reed 1988), *Pyrus* (Reed 1990), *Prunus* (Brison et al. 1995), and *Ribes* (Reed and Yu 1995). Only in a few cases has the cryopreservation of woody species succeeded without any special precultivation procedures, such as in *Ulmus* (Ulrich et al. 1984) and *Citrus* (Marin and Duran-Vila 1988).

Vitrification

Compared with the nonhardened material, cold hardening had no significant effect on the survival of the material frozen by vitrification using PVS2 as a cryoprotectant (Table 2, Fig. 2). Vitrification has been studied rather extensively for the crypreservation of in vitro shoot tips of perennials especially (e.g., Harding et al. 1991; Matsumoto et al. 1994, 1995) and as well trees of the temperate zone (e.g., Niino et al. 1992). When cold hardening was used before vitrification, the regeneration of shoot tips was improved significantly compared with nonhardened material (Harding et al. 1991; Niino et al. 1992). When comparing slow cooling and vitrification of the shoot tip material, the results vary according to the plants studied. In *Solanum*, recovery is possible after vitrification but not after slow cooling (Harding et al. 1991). Nearly the same excellent survival rate was obtained by these two protocols for *Wasabia* (Matsumoto et al. 1994). The survival of cryopreserved shoot tips of *Prunus* (Brison et al. 1995) and *Ribes* (Reed and Yu 1995) was higher after slow cooling than after vitrification.

Clonal differences

There were no significant differences in the survival of different clones when the nonhardened shoot tips were cryopreserved using the slow cooling method or vitrification (Fig. 2). In the case of cold hardening, the survival of the shoot tips varied depending on their genotypes; the same clones were successful and others recalcitrant independent of the pretreatment or cryoprotectant used. The percent regrowth of clone E5387 cryopreserved by the slow cooling method was significantly better ($p < 0.01$) than that of the other clones of the same treatment (Fig. 2). However, all the genotypes were of local origin, and regeneration of in vivo buds acclimated in nature was uniform when cryopreserved in vivo (Ryynänen 1996). Furthermore, callus cultures derived from the basal callus of the micropropagated shoots of the same clones could not be cryopreserved using cold hardening and the same slow cooling methods as in this study (L. Ryynänen, unpublished). It is evident that the acclimation method, i.e., the duration of acclimation and the temperature and photoperiod needed to achieve maximum hardening, should be different not only for each species but also for different genotypes and perhaps also for different types of tissue. The genotype-dependent ability to cold acclimate has been reported in studies on different genotypes of *Rubus* sp. (Reed 1993) and *Lilium* sp. (Matsumoto et al. 1995).

According to Reed (1990), different vigor of the genotypes in tissue culture could be one explanation for the different survival rates after cryopreservation. As well as decreasing growth, the lack of vigor can lower the tolerance to dehydration and cooling. In the present study the vigor of the clones cultured on WPM is different, e.g., the average weight of one shoot of clone E1987 after 4 weeks of cultivation was only 66% of that of clone E5387 (Ryynänen 1993). Because the vigor and the multiplication rate of silver birch in vitro are dependent on the composition of the medium used (Ryynänen 1993), the survival of cryopreserved birch could be improved by changing the medium.

Conclusions

In the present study the cryopreservation of in vitro silver birch material was shown to be possible. The slow cooling method seems to be the best technique for this species. Cryopreservation of in vitro shoot tips is a potential germ plasm conservation method for silver birch. Further studies will be focused on the use of physical and chemical acclimation and recovery medium to increase the survival of all genotypes of silver birch sufficient for standardized germ plasm conservation.

Acknowledgments

The author is grateful to Dr. Hely Häggman and Dr. Tuija Aronen for a critical reading of the manuscript. I also thank Airi Huttunen, Rauni Ritola, and Jouko Lehto for technical assistance during the work.

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IV

CRYOBIOLOGY **36,** 32–39 (1998)
ARTICLE NO. CY972067

Effect of Abscisic Acid, Cold Hardening, and Photoperiod on Recovery of Cryopreserved *in Vitro* Shoot Tips of Silver Birch

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Recovery of cryopreserved *in vitro* shoot tips of silver birch (*Betula pendula* Roth) was doubled by incorporating abscisic acid (ABA) in the culture medium during cold hardening of the mother shoots. The average recovery of shoot tips was over 40% after cold hardening for 28 days at +5°C under an 8/16 light/dark photoperiod on medium containing 10^{-4} M ABA. ABA was effective in combination with low temperature and short daylength only, although large genotypical differences were noted. ABA had two different effects: it enhanced cold hardening and increased callus formation during regeneration of cryopreserved shoot tips.

Key Words: Betula pendula Roth; cryopreservation; cold hardening; short daylength; long daylength; abscisic acid.

For conservation of silver birch germplasm, cryopreservation is a new tool to be used to complement the traditional conservation methods, i.e., *in situ* gene reservation forest and *ex situ* clone collection. The regenerability of the *in vivo* buds acclimated in nature is excellent in all silver birch clones investigated (17). Cryopreservation of *in vitro* shoot tips of cold acclimated silver birch cultures is also possible using slow cooling and PGD (a solution of polyethylenglycol, glucose, and dimethylsulfoxide) as cryoprotectant (18). However, for the same silver birch clones regeneration of *in vitro* shoot tips is considerably lower than that of *in vivo* buds, and genotypic differences are considerable. According to Chen and Gusta (4), cold hardening can be improved by abscisic acid (ABA) in species capable of hardening by low temperature. ABA-induced cold hardening has been studied in tissue cultures of several plants (3, 4, 9, 14) including some deciduous trees, such as *Betula papyrifera* March and *Alnus incana* (L.) Moench (21). ABA has also been used to improve tolerance to cryopreservation, e.g., with meristems of *Rubus* spp. (16), zygotic embryos of *Triticum aestivum* L. (10), and suspension cells of *Bromus inermis* Leyss and *Medicago sativa* L. (15). Species react differently to ABA: In most cases, e.g., *Solanum* spp. (3), *Lotus* spp. (9), and *B. papyrifera* Marsh (21), it is even possible to replace cold treatment by ABA. In other cases, e.g., for cold hardening of meristems of *Rubus* spp. (16) and suspension cells of *Medicago* spp. (14), ABA is effective in combination with cold treatment only and increases the degree of cold hardening.

In the case of silver birch, cold hardening is affected by both short daylength and low temperature (6, 8) and both elements have been used for cold hardening of *in vitro* shoot tips before cryopreservation with recovery of about 20% (18). The aim of the present study was to investigate the influence of the application of exogenous ABA on cold hardening of the *in vitro* shoot tips of silver birch, to observe if it is possible to replace both short daylength and low temperature or one of them with ABA or if ABA only increases the degree of cold hardening when used in combination with the other factors. In addition, attention was paid to the effect of ABA on the morphology and anatomy of the cold hardened mother shoots and on the recovery pattern of cryopreserved shoot tips.

MATERIALS AND METHODS

Plant Material

Twigs with dormant buds of silver birch (*Betula pendula* Roth) were collected during winter from five plus trees, E1987, E5201, E5382, E5387, and E5398, growing in the silver birch

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Received September 2, 1997; accepted November 11, 1997.

0011-2240/98 $25.00

stand at Punkaharju, southeastern Finland (61°49′N; 29°18′E; 90 m above sea level), planted with seedlings of local origin in 1932.

Culture Conditions

The shoot cultures established from the buds as described by Ryynänen (17) were subcultured every 3 weeks and maintained in glass jars on WPM (woody plant medium, 12) containing 4.4×10^{-6} M BA (6-benzylaminopurine), later called standard medium. The growth conditions of shoot cultures were a 16/8 light/dark photoperiod with the light intensity of 85–114 $\mu Em^{-2}\ s^{-1}$ at +22°C. The cultures had been cultivated *in vitro* for 2 years and they had undergone over 20 subcultures before the experiments started.

ABA Treatments and Cold Hardening

The first experiment investigated the influence of three different ABA concentrations on the cold hardening and recovery of silver birch shoot tips. Mother shoots, about 1 cm in length, were transferred on WPM containing 10^{-6}, 10^{-5}, or 10^{-4} M ABA and cultivated at +5°C under short daylength (SD) (an 8/16 light/dark photoperiod) for 14, 21, and 28 days.

The possibility of replacing either SD or low temperature or both by ABA was studied in the following experiments. The replacement of SD by ABA was studied in the second experiment, during which mother shoots were cultivated at +5°C under long daylength (LD) (a 16/8 light/dark photoperiod) for 14, 21, and 28 days on WPM containing 10^{-4} M ABA. Correspondingly the possibility of replacing low temperature by ABA was studied in the third experiment, during which mother shoots were cultivated at +22°C under SD for 14, 21, and 28 days on WPM containing 10^{-4} M ABA. The possibility of replacing both SD and low temperature by ABA was studied in the fourth experiment, during which mother shoots were cultivated at +22°C under LD for 21 days on WPM containing 10^{-6}, 10^{-5}, or 10^{-4} M ABA.

Twenty shoot tips per clone were used for each treatment, with three replicates, except for the treatment where shoots were cultivated at +22°C under LD for 21 days with ABA, which was performed only once. The cytotoxic effects of ABA and cryoprotectants were determined by transferring 10 shoot tips per clone on standard recovery medium after treatment, without freezing. In control treatments the mother shoots were cultivated under the same conditions on standard medium. The number of shoot tips was 10 per clone in each control treatment and the treatments were repeated three times.

Freezing

Before slow cooling with PGD (10% (w/v) polyethylenglycol, 10% (w/v) glucose, and 10% (v/v) M_2SO (dimethylsulfoxide) in water), the dissected shoot tips were precultured for 72 h under the same conditions as during the foregoing subculture on medium containing 10^{-6}, 10^{-5}, or 10^{-4} M ABA and 0.5% (v/v) M_2SO. After preculture the shoot tips were treated according to Ryynänen (18). They were transferred into 0.25 ml of hormone free liquid WPM in 2-ml cryo tubes on ice. One milliliter of PGD was added dropwise over a period of 30 min. The tubes were let to stand on ice for 30 min before freezing. The samples were then frozen at a rate of 0.17°C/min to the prefreezing temperature of −38°C in a programmable controlled temperature cooling chamber (Lauda Ultra-Kryomat RUK60). After reaching the terminal temperature, the tubes were immediately immersed in liquid nitrogen and stored for 8 days at −196°C.

Thawing and Regeneration

The samples were thawed in a +37°C water bath. The cryoprotectant was drained from the tubes and replaced with liquid standard WPM for 30 min. The liquid medium was changed once and the content of the tubes poured on sterile filter paper. The shoot tips were subsequently plated onto the standard medium solidified with 1% (w/v) agar in a Petri dish. Apices were transferred onto fresh medium after 3 weeks of cultivation.

Survival of the shoot tips was estimated visually after 2, 4, and 6 weeks of cultivation. Shoot tips with new shoots and leaves growing

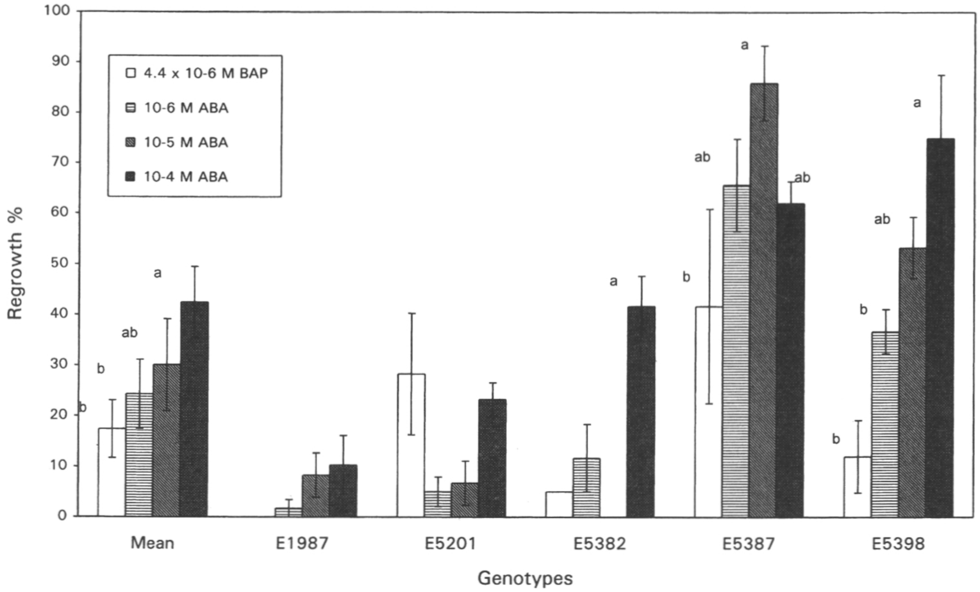


FIG. 1. Regrowth percentages (±SE) of cryopreserved shoot tips of silver birch genotypes cold hardened in the presence of 4.4 × 10^{-6} M BA or 10^{-6}, 10^{-5}, or 10^{-4} M ABA at +5°C under SD (an 8/16 light/dark photoperiod) for 28 days. Different letters indicate significant differences ($P < 0.05$) among treatments.

straight from meristems or from callus were classified as regrowing. After 6 weeks of cultivation, growing shoots were transferred on the same medium in glass jars. Rooting of shoots took place on WPM with 1% (w/v) sucrose and without any growth regulators. Rooted shoots were potted in peat:perlite (1:1), and plantlets were grown for 2 weeks in propagators under decreasing relative air humidity before transfer to the greenhouse.

Anatomical Observations

The anatomical structure of the cold hardened mother shoots before cryopreservation and of the regenerating cryopreserved tissues was examined under a microscope (Olympus CK2). The material was fixed with FAA (formalin:acetic acid:95% ethanol, 10:5:85, by vol.) and embedded in paraffin. Fifteen-micrometer-thick sections were stained with safranin-fast green (5).

Statistical Analysis

Statistical comparisons among treatments were made by one- and two-way analysis of variance, and means were compared by a Student–Newman–Keuls multiple range test (BMDP/DYNAMIC 7.0 program).

RESULTS AND DISCUSSION

ABA Concentration and Precultivation Time

After 28 days of cold hardening at +5°C under SD, the mean regrowth percentages of all ABA concentrations investigated were higher than after the treatment on standard medium, although regrowth percentages varied greatly among different genotypes (Fig. 1). Shorter cold hardening periods on medium with ABA had no effect on regrowth percentages compared to standard medium. The highest mean regrowth percentage (42.5%) was achieved on the medium with 10^{-4} M ABA but it did not differ significantly from that (30.1%) on the medium with the 10^{-5} M ABA. Only the mean regrowth percentage on medium with 10^{-4} M ABA was significantly better ($P < 0.05$) than cultivation on standard medium. For genotype E5387, a concentration of 10^{-5} M ABA was more efficient since regrowth reached 86.0% (Fig. 1).

The ABA concentrations used for cold hardening of silver birch are in the range of concentrations used for successful cold acclimation of other *in vitro* cultured plants, in spite of the type of tissue cultured. A total of 5 × 10^{-5} M ABA has been used for meristems of *Rubus* spp. (16) and 4 × 10^{-6} M for shoot primordia of *Vanda pumila* Hook.f. (13). The 2 × 10^{-6} M ABA concentration was most successful for cold hardening of zygotic embryos of *T. aestivum* L. (10). Concentrations used for cell suspension cultures are, e.g., 5 × 10^{-5} M and 10^{-4} M for *M. sativa* L (14, 15) and 7.5 × 10^{-5} M for *B. inermis* Leyss (4, 15). The effective cold hardening period used for silver birch meristem is, however, long compared to the periods most often used for cell cultures, varying from 3 days (13) to 10 days (10). Extending the cold hardening period over 7 days, which was requested for maximal hardiness in cell suspensions of another *Betula* species, *B. papyrifera,* resulted in the loss of freezing tolerance (21).

Using ABA Instead of SD and Low Temperature

When LD was used instead of SD for cold hardening of mother shoot at +5°C, the regrowth percentages of shoot tips were very low under standard conditions. When 10^{-4} M ABA was used instead of BA, the regrowth percentages varied among genotypes, but the mean percentage increased with increasing cold hardening durations, although not significantly (Fig. 2). Using 10^{-4} M ABA instead of BA at +22°C under SD or LD had no effect on the recovery of silver birch shoot tips. Recovery rates were close to nil under all conditions. According to these results, ABA cannot be used to replace SD, low temperature, or both for cold hardening of silver birch meristems.

The effect of using ABA instead of SD for cold hardening of silver birch is in accordance with the results obtained with seedlings of another photoperiodic birch, *Betula pubescens* Ehrh. Freezing tolerance estimated for the bud apical meristems of pubescent birch seedlings grown under LD conditions was increased after exogenous ABA application for 3 weeks, but was significantly less than in seedlings under SD (22). With cold hardening of stem cultures of *Solanum commersonii* ABA was substituted for low temperature, while for *S. tuberosum* ABA was effective with low temperature only, increasing frost hardiness (3). With cold hardening before cryopreservation of meristems, ABA has been used only twice earlier. The result that ABA is efficient in combination with low temperature only and increases the degree of cold hardiness compared to cold hardiness caused by low temperature alone has been demonstrated with meristem cultures of *Rubus* spp. (16). As with silver birch, genotype had a great influence on the survival of cryopreserved *Rubus* meristems (16). Shoot apices of cultured protocorms in *V. pumila* were successfully cryopreserved after ABA preculture and desiccation (13).

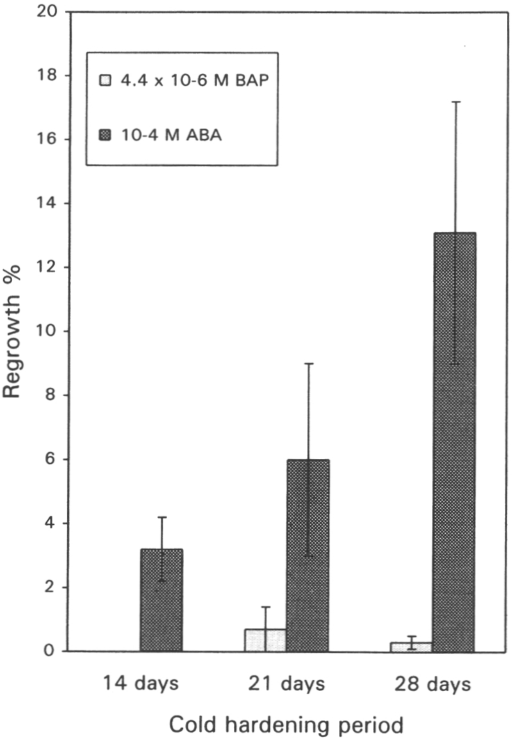


FIG. 2. Regrowth percentages (±SE) of cryopreserved shoot tips of silver birch cold hardened in the presence of 4.4 × 10^{-6} M BA or 10^{-4} M ABA at +5°C under LD (a 16/8 light/dark photoperiod) for 14, 21, and 28 days.

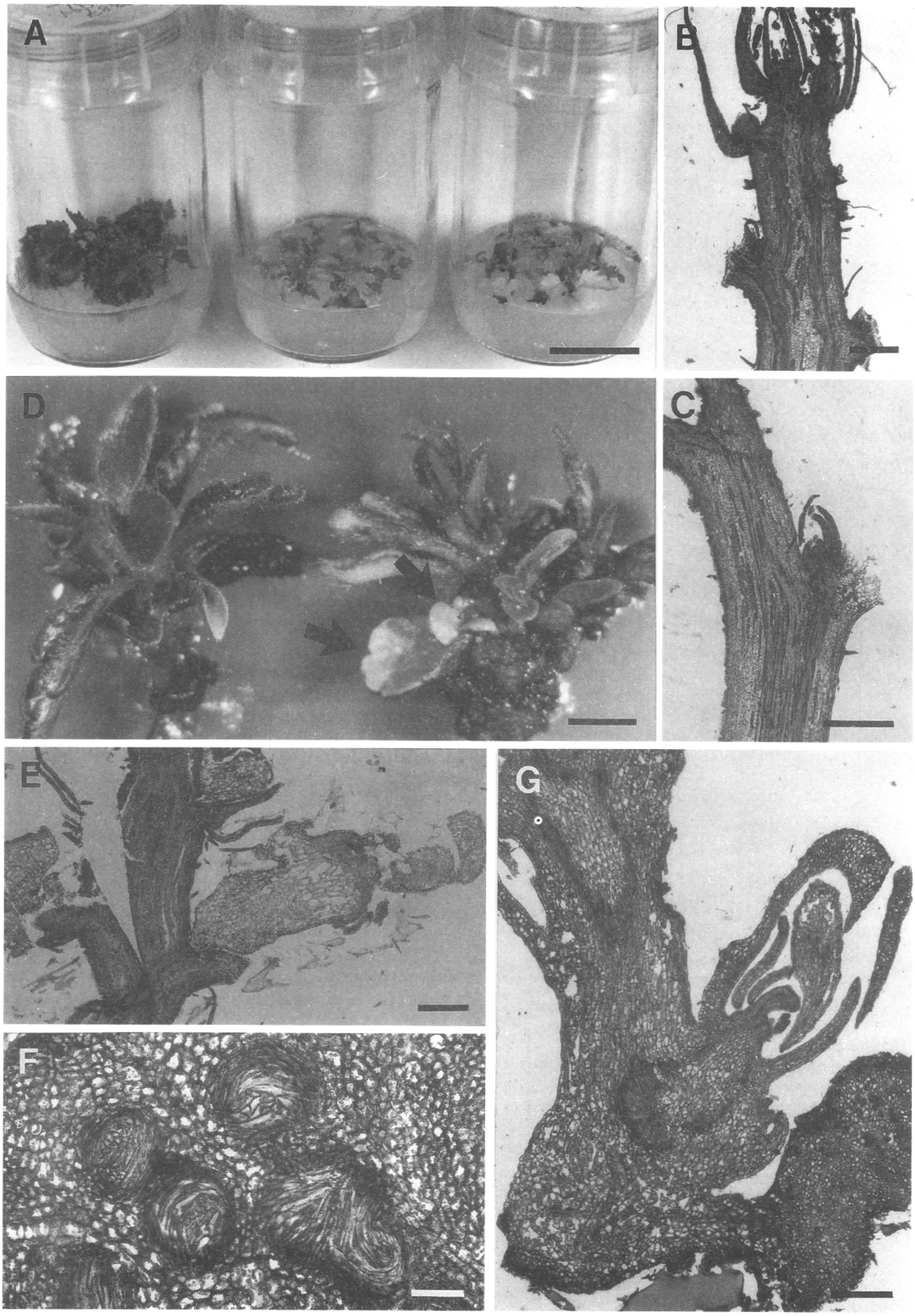
The response of cell cultures differs remarkably from the effect of ABA on meristems. In most cases the effect of ABA has been independent of the temperature, e.g., with suspension cultures of *T. aestivum* L. cv Norstar, *Secale cereale* L. cv Cougar, and *B. inermis* Leyss (4), in callus cultures of *Lotus corniculatus* L (9), and with suspension cultures of *B. papyrifera* March and *A. incana* (L.) Moench (21). Only a slight increase in hardiness was observed with *Lotus* (9), *Betula,* and *Alnus* (21) grown at low temperature without ABA. However, ABA was effective when used in combination with low temperature only and increased cold hardening with cell suspension cultures of *M. sativa* L. (14).

Morphology

The morphology of silver birch mother shoots cold hardened on medium with or without ABA for 4 weeks differed in the color and abscission of leaves only: more yellow and shed leaves were observed on medium with ABA (Fig. 3A). Anatomically, this difference was seen as formation of more abundant abscission zones. There were no other anatomical differences between shoots cold hardened with or without ABA (Figs. 3B and 3C). There are no earlier reports of the effect of ABA on the anatomy of *in vitro* meristems. Increased sugar content and cell wall augmentation of suspension cells of *B. inermis* Leyss caused by ABA treatment were, however, found similar to changes induced by cold acclimation (20).

The increased cold hardening in the presence of ABA was also detected through the appearance of regenerating shoot tips. Compared to shoot tips cold hardened without ABA (Figs. 3D and 3E) not only more meristems but even some differentiated tissues survived after cryopreservation, including some leaves of the regenerating shoot tips that remained green and became relatively hard and thick (Fig. 3D). In addition, some callus formed from the first meristems which started to regenerate (Fig. 3F), and several new leaves and shoots grew from some of them (Figs. 3D and 3G). Less or no callus formation of recovering meristems was found in shoot tips that started to regenerate later. Differences between genotypes were considerable; callus formed mostly in the best regenerating genotypes cold hardened for 21 and 24 days. Later on, *in vitro* and *ex vitro,* no differences could be detected between plantlets regenerated from callus or straight from meristems of shoots cold hardened with ABA or between plantlets regenerated from shoots cold hardened with or without ABA. Total survival, including shoot and callus, of shoots cold hardened with 10^{-4} M ABA was 56.7%. Thus the percentage of shoot tips producing callus without further development of new leaves and shoots was 14%. In callus formed in shoot tips cold hardened on standard medium no organogenesis was observed and the corresponding percentage of callus producing shoot tips was 11%. Callus formation of shoot tips cold hardened with ABA may reflect the increased survival of meristems; less or only partially injured meristems formed callus and some of them were capable of organogenesis, when more injured meristems cold

FIG. 3. Effect of ABA on the morphology and anatomy of mother shoots and regenerating shoot tips of silver birch. (A) Mother shoots cultivated with BA at +22°C under LD for 28 days (on the left), with BA at +5°C under SD for 28 days (in the middle), and with 10^{-4} M ABA at +5°C under SD for 28 days (on the right). Bar, 2 cm. (B and C) Longitudinal sections of cold hardened mother shoots on medium with BA (B) or on medium with ABA (C) from which leaves have shed during processing. (D–G) Regrowing shoot tips 6 weeks after thawing. (D) A shoot growing from a mother shoot cold hardened on medium with BA (on the left) and several shoots with callus proliferation, a mother shoot of which cold hardened on medium with ABA (on the right). Arrows indicate the two viable leaves differentiated already before cryopreservation. Bar, 2 mm. (E) Longitudinal section of a shoot tip cold hardened with BA. All differentiated tissues are dead, axillary bud growing without any callus formation. (F and G) Longitudinal sections of shoot tips cold hardened with ABA. (F) Bundles of vascular tissues in differentiating callus. (G) Shoots differentiating from callus. (A–F) genotype E5387; (G) genotype E5398. Bars in B, C, E, and F, 400 μm; in G, 100 μm.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hardened without ABA formed only callus. Callus formation from shoot tips after thawing is suggested to be an indication of cryoinjury (16). The decreasing callus formation in the course of postthaw growth can also indicate that callus formation of regrowing shoot tips is an aftereffect of ABA, since ABA can increase callus formation in woody species (1, 19). ABA also has the ability to stimulate adventitious bud formation (7), but taking into account the high regenerability of silver birch callus on standard medium, the latter effect of ABA on silver birch is not probable. Callus formation may increase the recovery of some silver birch genotypes after cryopreservation, but this is not favored due to increased probability of somaclonal variation in the regenerated material (2). Unlike silver birch, callus formed in cryopreserved *Rubus* meristems after cold hardening with ABA did not regenerate (16).

CONCLUSIONS

It is possible to increase the recovery of cryopreserved shoot tips of silver birch by cold hard ening mother shoots at +5°C under SD with the addition of ABA in the culture medium. This treatment doubles the recovery compared to cold hardening with low temperature and SD. ABA has two different effects: it enhances cold hardening and increases callus formation during regeneration of cryopreserved shoot tips, which both lead to better recovery. Improvement of the cryopreservation method of *in vitro* shoot tips of silver birch is still needed to reduce the great genotypical differences and to standardize the method for germplasm conservation.

ACKNOWLEDGMENTS

The author thanks Dr. Hely Häggman and Dr. Tuija Aronen for critical reading of the manuscript and Airi Huttunen and Jouko Lehto for technical help.

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Leena Ryynänen and Hely Häggman

J. Plant Physiol. Vol. . pp. – (1999)
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JOURNAL OF Plant Physiology

Substitution of Ammonium Ions during Cold Hardening and Post-thaw Cultivation Enhances Recovery of Cryopreserved Shoot Tips of *Betula pendula*

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Received September 29, 1998 · Accepted November 9, 1998

Summary

Silver birch shoot tips were cryopreserved after cold hardening of the mother shoots for 28 days at +5 °C under an 8/16 light/dark photoperiod on medium containing 100 µmol/L ABA. The aim of the present study was to investigate the effect of the elimination of ammonium ions on the recovery of cryopreserved silver birch shoot tips. Ammonium was substituted by different nitrogen sources; with KNO_3 or L-glutamine already during cold hardening of the mother shoots, or with KNO_3, L-glutamine, or a mixture of the two, during post-thaw cultivation of the shoot tips. According to the results, the substitution of ammonium nitrogen improved the recovery of the cryopreserved and regenerated silver birch shoots. The highest recovery and improved morphological appearance of the regenerated shoots were achieved when ammonium was substituted by KNO_3 already during the cold hardening phase.

Key words: Betula pendula, ammonium, cryopreservation, cold hardening, nitrate, potassium.

Abbreviations: ABA = abscisic acid; BA = 6-benzylaminopurine; PGD = a mixture of 10 % polyethylene glycol, 10 % glucose, and 10 % dimethylsulfoxide (DMSO) in water; WPM = woody-plant medium.

Introduction

The cryogenic storage of silver birch (*Betula pendula* Roth) meristems is a new tool that complements the traditional conservation methods i.e. *in situ* gene reserve forest and *ex situ* clone collection. The cryopreservation of silver birch is possible using both *in vivo* and *in vitro* meristems. Using *in vivo* buds acclimated in nature avoids the need for other preconditioning treatments (Ryynänen, 1996 a). Successful cryopreservation of *in vitro* shoot tips is dependent on cold acclimation, on the growth regulator in the cold hardening medium, on the cooling rate and on the use of cryoprotectants (Ryynänen, 1996 b, 1998). However, there are considerable genotypical differences in the recovery of cryopreserved *in vitro* shoot tips compared to that of *in vivo* buds of the same genotypes. The actively growing plant tissue cultivated *in vitro* is physiologically and morphologically very different from dormant *in vivo* material, and is thus easily damaged during cryopreservation. The use of cold hardening medium that promotes more profitable cold acclimation and post-thaw medium that promotes healing of the injured tissues might enhance the recovery of more recalcitrant genotypes after cryopreservation.

Nitrogen affects both freezing tolerance and the recovery of cells from freezing injury. A high nitrogen content can decrease the frost hardiness of plants by increasing cell size and vacuolization (Levitt, 1980). In forest tree nurseries in Finland, heavy nitrogen fertilization is used in the beginning of the growing season, after which the nitrogen fertilizer dose is gradually reduced to nil toward the end of the growing season (Rikala and Westman, 1978). Heavy nitrogen fertilization decreases the winter survival of several perennial grasses (Huokuna and Hiivola, 1974; Jung and Kocher, 1974), as well as the frost hardiness of winter wheat (*Triticum aestivum* L.) (Tyler et al., 1981). Even the type of nitrogen can affect the development of hardiness. High ammonium level reduced

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the frost resistance of timothy (*Phleum pratense* L.) significantly more than high levels of nitrate (Junttila et al., 1995).

In cryopreservation, ammonium in the post-thaw medium is reported to be deleterious to rice cultures (*Oryza sativa* L. cv. Nihonbare) derived from mature embryos (Kuriyama et al., 1989) and to non-embryogenic *Lavandula vera* DC. cells (Kuriyama et al., 1996), inhibiting the recovery from cryoinjury. The viability of cryopreserved *Lavandula vera* cells decreased with increasing ammonium concentrations in the post-thaw medium (Kuriyama et al., 1996). The cells were sensitive to ammonium ions only a few days after thawing, after which the application of ammonium was needed to promote cell growth. The toxic effect of ammonium on cryodamaged cells was successfully reduced e.g. by the addition of activated charcoal to the recovery medium to absorb ammonium (Kuriyama et al., 1990) or by excluding ammonium from the recovery medium (Kuriyama et al., 1989, 1996). A post-thaw medium lacking ammonium was also favourable for the recovery of shoot tips of apples (*Malus domestica* Borkh. cv. Fuji) (Niino et al., 1992).

According to our earlier studies, cryopreservation of silver birch is possible if the mother shoots are cold hardened before cryopreservation (Ryynänen, 1996 b). The aim of this study was to investigate the effect of eliminating ammonium ions on the recovery of cryopreserved silver birch shoot tips. Ammonium was substituted by different nitrogen sources; with KNO_3 or L-glutamine already during cold hardening of the mother shoots, or by KNO_3, L-glutamine, or a mixture of the two, during post-thaw cultivation of the shoot tips.

Materials and Methods

Twigs bearing dormant buds of silver birch (*Betula pendula* Roth) were collected from five 63-year-old plus trees (E1987, E5201, E5382, E5387 and E5398) growing in a silver birch stand at Punkaharju, Finland (61° 49′ N; 29° 18′ E; 90 m above sea level). The shoot cultures established from the buds, as described by Ryynänen (1996 a), were subcultured every third week and maintained in glass jars on WPM (Lloyd and McCown, 1980) containing 4.4 µmol/L BA. The growth conditions of the shoot cultures were a 16/8 h light/dark photoperiod with light intensity of 85–114 $\mu mol\ m^{-2}\ s^{-1}$ at +22 °C. The shoot cultures had undergone about 60 subcultures before the experiments started.

The mother shoot cultures were cold hardened according to Ryynänen (1998) on WPM containing 100 µmol/L ABA for 28 days at +5 °C under an 8/16 h light/dark photoperiod, following precultivation of the dissected shoot tips on medium containing 100 µmol/L ABA and 0.5 % DMSO for 72 h under the same conditions. After precultivation, the dissected shoot tips were transferred into 0.25 mL of liquid WPM containing no growth regulators in 2 mL cryotubes on ice (later called the liquid freezing medium). One mL of PGD was added dropwise over a period of 30 min. The tubes were let to stand on ice for 30 min before freezing. The samples were then frozen at a rate of 10 °C/h to the prefreezing temperature of −38 °C. After reaching the terminal temperature, the tubes were immediately immersed in liquid nitrogen and stored for 8 days at −196 °C. The samples were thawed in a +37 °C water bath. The cryoprotectant was drained from the cryotubes and replaced with liquid WPM with 4.4 µmol/L BA for 30 min (later called the liquid washing medium). The liquid medium was changed once and the tubes poured onto sterile filter paper. The shoot tips were subsequently plated on WPM solidified with 1% agar in a Petri dish. All apices were transferred onto fresh medium after 3 weeks of cultivation.

Substitution of ammonium nitrogen

The main nitrogen sources of the WPM medium are inorganic NH_4NO_3 (5 mmol/L) and $Ca(NO_3)_2$ (2 mmol/L), and the minor sources are the organic compounds thiamin HCl (3 µmol/L), pyridoxine HCl (2 µmol/L), nicotine acid (4 µmol/L), and glycine (27 µmol/L). The total nitrogen concentration in the medium is 14.4 mmol/L.

In the first experiment NH_4NO_3 in the liquid freezing and washing medium and in the solid post-thaw medium was substituted by 1) 10 mmol/L KNO_3, *2)* 2.5 mmol/L L-glutamine and 5 mmol/L KNO_3, or *3)* 5 mmol/L L-glutamine as summarized in Table 1. In these treatments the $Ca(NO_3)_2$ concentration of WPM remained unchanged. The post-thaw cultivation was started both on medium containing NH_4NO_3 (treatments *c*) and on NH_4NO_3-free medium (treatments *a* and *b*). The material was transferred from the NH_4NO_3-free medium onto WPM medium containing NH_4NO_3 after 3 (treatments *a*) or 7 (treatments *b*) days of cultivation.

In the second experiment ammonium nitrogen was either totally eliminated from cold hardening up until the first three days of post-thaw cultivation, after which the material was transferred to medium containing NH_4NO_3 (treatments *d*), or post-thaw cultivation was started only on medium containing NH_4NO_3 (treatments *e*), or after cryopreservation NH_4NO_3 was included both in the liquid washing medium and post-thaw cultivation medium (treatments *f*). The different treatments are summarized in Table 1. NH_4NO_3 was substituted by 10 mmol/L KNO_3 (treatments *4*) or 5 mmol/L L-glutamine (treatments *5*). In treatments *6*, 10 mmol/L KNO_3 (i.e. total N = 10 mmol/L) was used, and both NH_4NO_3 and $Ca(NO_3)_2$ were omitted. In treatments *7* the nitrogen of the NH_4NO_3 and $Ca(NO_3)_2$ was substituted by 7 mmol/L L-glutamine (i.e. total N = 15 mmol/L). As a result of $Ca(NO_3)_2$ omission, 1.4 mmol/L $Ca(Cl)_2 \times 2\,H_2O$ was included in the media to replace half of the omitted calcium (treatments *7*).

In the control treatments, WPM containing NH_4NO_3 was used for the cold hardening of the mother shoots and for the freezing, thawing and cultivation of the shoot tips. A total of 300 shoot tips, twenty shoot tips per clone with three replicates, were used in each treatment and control (Table 1).

The survival of the bud material was estimated visually after 2, 4, and 6 weeks of cultivation. Shoot tips with regenerating shoots and leaves were classified as regrowing. All regrowing shoot tips, and those that remained green and (or) only produced callus, were considered to be viable. After six weeks of cultivation the proliferating shoots were transferred onto the same WPM in glass jars. Rooting took place in WPM with no growth regulators. The rooted shoots were potted in peat : perlite (1 : 1), and the plantlets were grown for two weeks in propagators with decreasing relative air humidity before transfer to the greenhouse.

Statistical comparisons between the treatments were performed using one- and two-way analysis of variance, and the means were compared using the Student-Newman-Keuls multiple range test (BMDP/DYNAMIC 7.0 program).

Results

In the first experiment the effects of eliminating ammonium on post-thaw recovery varied depending on the substitute. When NH_4NO_3 (5 mmol/L) was substituted either by KNO_3 (10 mmol/L) (Treatment *1*) or by L-glutamine (5 mmol/L) (Treatment *3*) the post-thaw recovery increased.

Table 1: Experimental design to substitute NH_4NO_3 (5 mmol/L) by ammonium-free nitrogen sources. * = both NH_4NO_3 and $Ca(NO_3)_2$ (2 mmol/L) are omitted, total N = 12.0 mmol/L. ** = both NH_4NO_3 and $Ca(NO_3)_2$ are omitted, total N = 14.4 mmol/L. $CaCl_2 \times 2\ H_20$ (1.4 mmol/L) included to substitute half of the omitted Ca^{2+}-ions.

Treatment	WPM containing NH_4NO_3 or substituted by other nitrogen sources				Post-thaw cultivation on NH_4NO_3-free medium for days
	Cold hardening medium	Liquid freezing medium	Liquid washing medium	Solid post-thaw medium	
First Experiment					
1a	NH_4NO_3	KNO_3	KNO_3	KNO_3	3
1b	NH_4NO_3	KNO_3	KNO_3	KNO_3	7
1c	NH_4NO_3	KNO_3	KNO_3	NH_4NO_3	
2a	NH_4NO_3	gln + KNO_3	gln + KNO_3	gln + KNO_3	3
2b	NH_4NO_3	gln + KNO_3	gln + KNO_3	gln + KNO_3	7
2c	NH_4NO_3	gln + KNO_3	gln + KNO_3	NH_4NO_3	
3a	NH_4NO_3	gln	gln	gln	3
3b	NH_4NO_3	gln	gln	gln	7
3c	NH_4NO_3	gln	gln	NH_4NO_3	
Second Experiment					
4d	KNO_3	KNO_3	KNO_3	KNO_3	3
4e	KNO_3	KNO_3	KNO_3	NH_4NO_3	
4f	KNO_3	KNO_3	NH_4NO_3	NH_4NO_3	
5d	gln	gln	gln	gln	3
5e	gln	gln	gln	NH_4NO_3	
5f	gln	gln	NH_4NO_3	NH_4NO_3	
6d	KNO_3*	KNO_3*	KNO_3*	KNO_3*	3
6e	KNO_3*	KNO_3*	KNO_3*	NH_4NO_3	
6f	KNO_3*	KNO_3*	NH_4NO_3	NH_4NO_3	
7d	gln**	gln**	gln**	gln**	3
7e	gln**	gln**	gln**	NH_4NO_3	
7f	gln**	gln**	NH_4NO_3	NH_4NO_3	
Control	NH_4NO_3	NH_4NO_3	NH_4NO_3	NH_4NO_3	

When NH_4NO_3 was substituted by a combination of KNO_3 (5 mmol/L) and L-glutamine (2.5 mmol/L) (Treatment *2*) the post-thaw recovery decreased (Fig. 1). Treatment *3a*, (regrowth 46.1 %) in which NH_4NO_3 was substituted by L-glutamine, and freezing, washing and cultivation of the shoot tips took place on the medium with glutamine for the first three days after thawing, differed significantly from the control (regrowth 22.7 %). Treatment *3a* was the best for all genotypes except E1987, which grew better (regrowth 41.7 %) on the medium in which NH_4NO_3 was substituted by KNO_3 (Treatment *1a*), but it did not differ significantly from treatment *3a* (regrowth 18.3 %) of the same genotype. However, there were no significant differences in the recovery of the growing shoots between the treatments (*a, b,* or *c*) within each nitrogen source (*1, 2,* or *3*) (Fig. 1). The morphological appearance of the shoots was similar between the treatments within each nitrogen source.

In the second experiment, in which ammonium was eliminated already during the cold hardening period, the regrowth percentages after all treatments and of all substitutes were better than that of the control, and also better than in the first experiment in which ammonium was substituted after the cold hardening period. However, only treatment *6d* (regrowth 58.3 %) in which KNO_3 (10 mmol/L) was used and NH_4NO_3 and $Ca(NO_3)_2$ were omitted grew significantly better than the control (Fig. 2). There were no significant differences in regrowth percentages between the different nitrogen sources (Treatments *4, 5, 6,* or *7*) or between treatments (*d, e,* or *f*) in each nitrogen source. Genotypes E1987, E5201, E5382 and E5387 grew best on the medium containing KNO_3 (Treatment *6d*) with regrowth percentages of 51.7, 76.7, 15, and 88.3, respectively. A combination of KNO_3 and $Ca(NO_3)_2$ (Treatment *4d*) was the best substitute (regrowth 70 %) for genotype E5398, with no significant difference to treatment *6d* (regrowth 60 %) of the same genotype.

The morphological appearance of the developing shoots that were cold hardened and cultivated on the medium containing inorganic nitrogen sources other than ammonium (Treatments *4* and *6*) (Fig. 3 A–D) was superior compared to those cultivated on the medium containing glutamine (Fig. 3 E) or control (Fig. 3 F). They were characterized by a vigorous, straight shoot and the absence of callusing leaves. The morphological differences between the regenerating shoots of the three treatments using WPM with KNO_3 and $Ca(NO_3)_2$ (Treatments *4d, 4e,* or *4f*) were more marked than the differences between the regrowth percentages, e.g. only part of the regenerating shoots in treatment *4e* had a good morphology (Fig. 3 A and 3 B). The size of the growing shoots cultivated

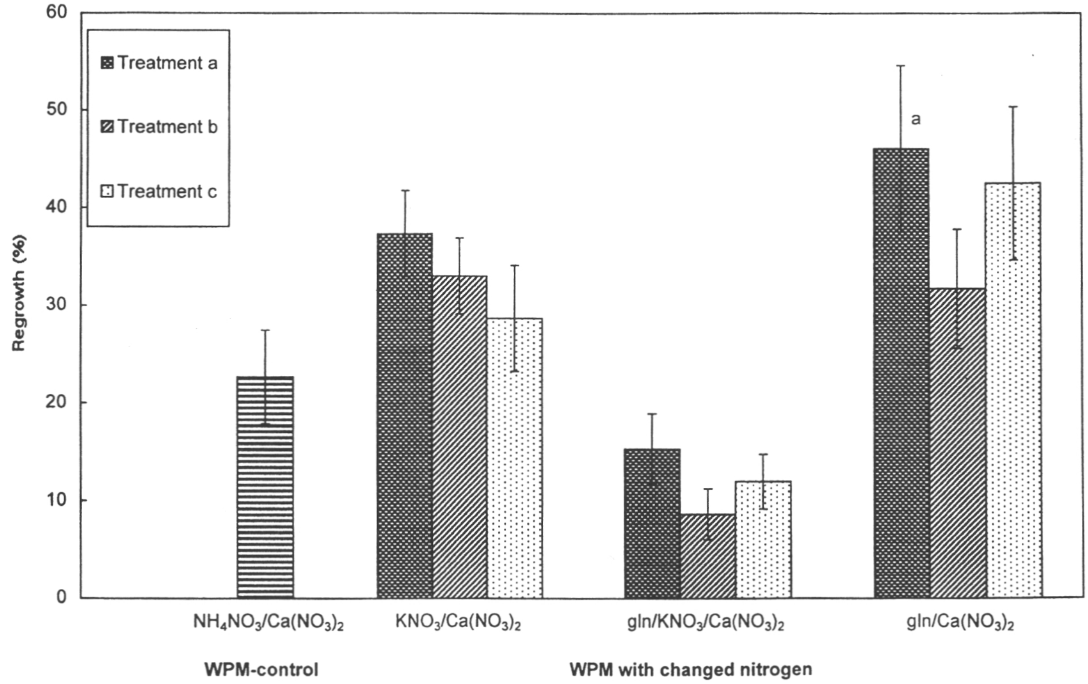

Fig. 1: Average regrowth percentages (± SE) of silver birch shoot tips of all genotypes frozen and washed with medium containing KNO_3 (10 mmol/L), L-glutamine (2.5 mmol/L) and KNO_3 (5 mmol/L), or L-glutamine (5 mmol/L) instead of NH_4NO_3 (5 mmol/L). Post-thaw cultivation of the shoot tips took place on medium containing the same nitrogen sources for three days (Treatment *a*) or seven days (Treatment *b*), after which cultivation was continued (Treatments *a* and *b*) or the material was transferred (Treatment *c*) to WPM containing NH_4NO_3. Regrowth in a column marked with the letter «a» was significantly increased ($P<0.05$) compared to the WPM control.

on the medium with ammonium substituted by KNO_3 (Treatment *6*) was smaller than that of those cultivated on the medium containing KNO_3 and $Ca(NO_3)_2$ (Treatment *4*). However, the different treatments (*6d, 6e,* or *6f*) had only a marginal effect on the morphology of the regenerating shoots (Fig. 3 C, D). When the shoot tips were cold hardened and cultivated on the medium containing glutamine or glutamine and $Ca(NO_3)_2$ yellow, brown, or even black coloured leaves with hard calli were commonly observed (Fig. 3 E). The regeneration of numerous leaves and shoots on the edges of the leaf blades was a characteristic feature of the cases where glutamine and $Ca(NO_3)_2$ (Treatment *5*) were used as a nitrogen source.

Discussion

Cold hardening, ABA supplement in the cold hardening medium, cooling rate and the use of cryoprotectant mixture are needed for the successful cryopreservation of *in vitro* silver birch shoot tips (Ryynänen, 1996 b, 1998). In the present study, substitution of the ammonium nitrogen in the media by either inorganic nitrate or organic L-glutamine proved to be necessary for improved recovery and/or good morphological appearance of the regenerated shoots. Genotypical differences in the recovery of cryopreserved *in vitro* shoot tips found in the earlier studies (Ryynänen, 1996 b, 1998) were still obvious in the present study, but in all genotypes the average recovery increased as a consequence of ammonium substitution.

Both the source and amount of nitrogen are of importance for the growth and development of plant tissues *in vitro*. The WPM medium employed in the present study and which has been successfully used in silver birch tissue culture, includes nitrogen mainly in the form of inorganic ammonium or nitrate as well as a small amount of nitrogen as the organic supplements glycine, pyridoxine, nicotinic acid and thiamin. Generally, nitrate and ammonium are both included in the media because a proper balance between these two nitrogen sources stimulates morphogenesis and embryogenesis in several species, and the pH of the media can also be more easily 4 shoot tips taken from young seedlings grew equally well on medium containing either ammonium or nitrate as the sole nitrogen source, while the shoot tips from older seedling always required nitrate for growth and development (Minocha, 1982). The callus tissue of *B. alleghaniensis* Britt. grew best

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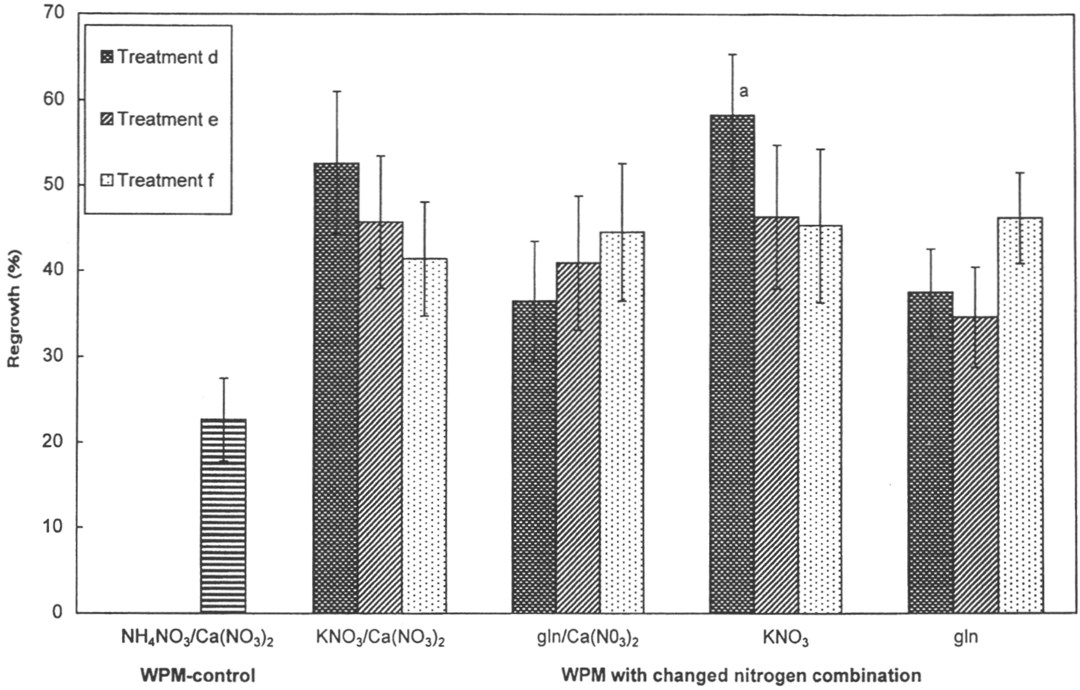
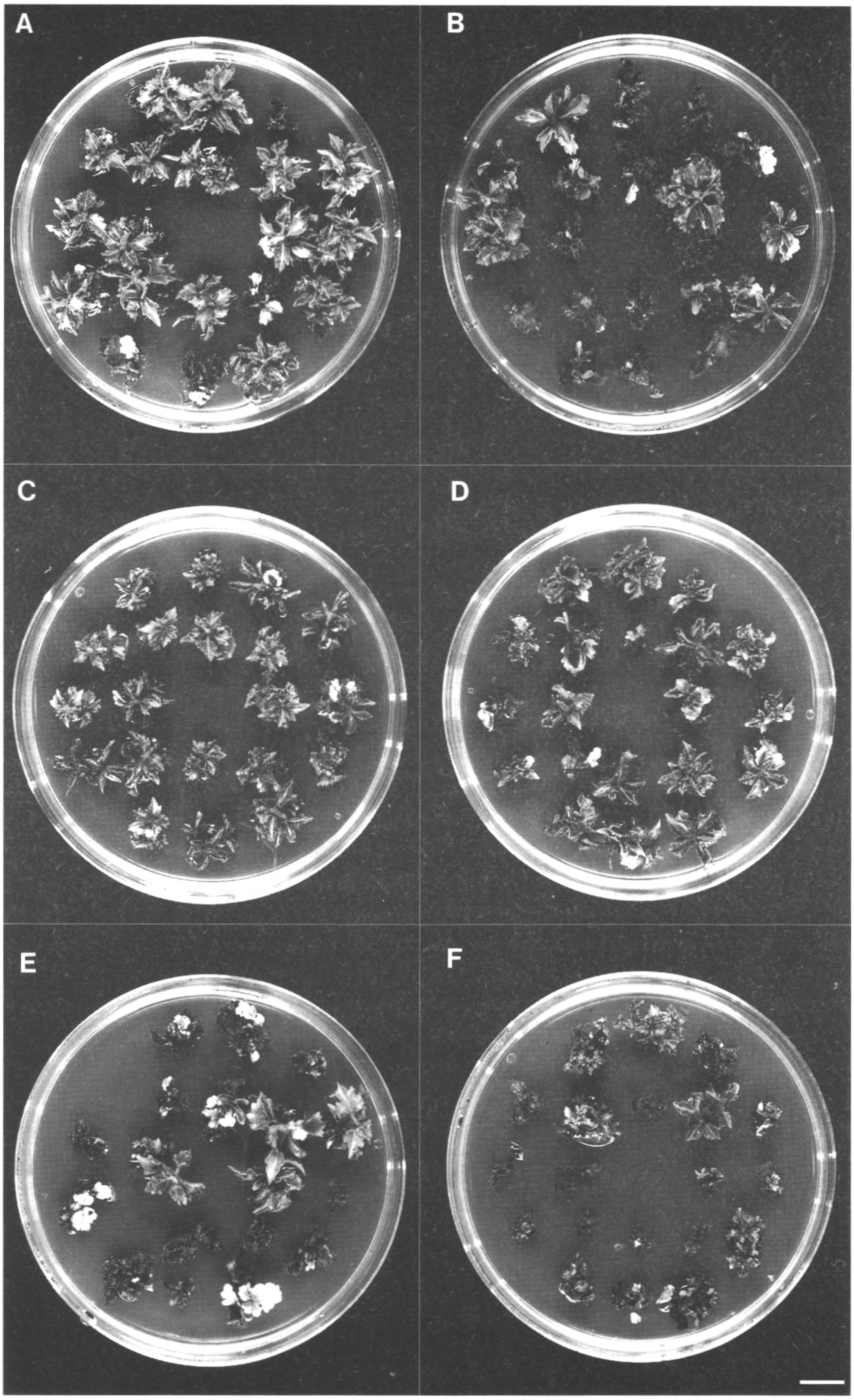


Fig. 2: Average regrowth percentages (± SE) of silver birch shoot tips of all genotypes cold hardened on medium containing KNO_3 (10 mmol/L), or L-glutamine (5 mmol/L) instead of NH_4NO_3 (5 mmol/L) or KNO_3 (10 mmol/L), or L-glutamine (7 mmol/L) and $Ca(Cl)_2 \times H_2O$ (1.4 mmol/L) instead of NH_4NO_3 and $Ca(NO_3)_2$ (2 mmol/L). Post-thaw cultivation of the shoot tips on the same medium for three days, after which cultivation was continued on WPM containing NH_4NO_3 (Treatment *d*) or post-thaw cultivation was started on WPM containing NH_4NO_3 (Treatment *e*), or after cryopreservation NH_4NO_3 was included both in the liquid washing medium and post-thaw cultivation medium (Treatment *f*). Regrowth in a column marked with the letter «a» was significantly increased ($P < 0.05$) compared to the WPM control.

on medium containing a combination of ammonium and nitrate, but was unable to grow on medium containing ammonium as the sole nitrogen source (Minocha, 1982).

Of the amino acid supplements used in tissue culture media, L. glutamine is the most popular even though asparagine, arginine and glycine are still often used as well (Bonga and von Aderkas, 1992). Energetically speaking, nitrate reduction is an expensive process due to the resulting increased activity of the pentose phosphate pathway, while ammonium can be assimilated directly into amino acids especially if α-keto acids are available (Kirby et al., 1987; Bonga and von Aderkas, 1992). Glutamine, as a source of reduced nitrogen, is energetically the less costly alternative (Leustek and Kirby, 1988).

In cryopreservation studies the elimination of NH_4NO_3 from the post-thaw medium has increased recovery. NH_4NO_3-free recovery medium was used successfully in the case of apple (*Malus domestica* Borkh cv. Fuji) shoot tips (Niino et al., 1992), even though no comparison was performed between the media with or without ammonium. The recovery of cryodamaged cells of rice (*Oryza sativa* L. cv. Nihonbare) was inhibited by the presence of ammonium in the media, but after recovering from injury, ammonium was necessary to achieve a high growth rate of the cells (Kuriyama et al., 1989). In the case of *Lavandula vera* cell culture, the recovery from cryopreservation was increased when ammonium in the media containing KNO_3 as a principal nitrogen source was substituted by alanine or glutamine (Kuriyama et al., 1997). The same phenomenon was also found in the present study, in which substitution of ammonium by either inorganic nitrogen or glutamine but not by a combination of them increased the recovery of the silver birch shoot tips. However, some conflicting results have also been published. The recovery of embryogenic cell cultures of rice *Oryza sativa* L. cv. Taipei 309) was optimal using the same post-thaw medium containing glycine as a nitrogen source in which cell suspension was normally maintained. In this case, the presence or absence of ammonium in the post-thaw medium had no significant effect on cell recovery (Lynch et al., 1994).


A
B
C
D
E
F

It would appear that the length of the post-thaw culture period without ammonium is not significant from the point of view of recovery. In the present study with silver birch shoot tips, there were no significant differences in the recovery percentages between three or seven days post-thaw culture on ammonium-free medium, which is in agreement with the results for *Lavandula vera* cell cultures (Kuriyama et al., 1996). In addition to that, the elimination of ammonium in the liquid freezing medium shortly before freezing and in the liquid washing medium after thawing followed by starting the cultivation of silver birch shoot tips on medium containing NH_4NO_3 increased the recovery.

The substitution of ammonium by either inorganic nitrate or organic L-glutamine during cryopreservation and post-thaw cultivation had no effect on the morphological properties of the regenerating shoots. As has been the case in studies with suspension cultures of rice (Kuriyama et al., 1989) and *Lavandula vera* cells (Kuriyama et al., 1996, 1997), the effect was seen only as increased recovery. On the other hand, substitution of ammonium already during cold hardening by inorganic nitrogen resulted in both improved morphology and increased recovery, while the use of glutamine resulted in an inferior morphology combined with increased recovery.

Interestingly, the results of the present study show that the substitution of ammonium by either inorganic nitrate or organic L-glutamine already in the beginning of the cold hardening phase was more effective than substitution in the post-thaw medium. The best shoot tip recovery resulted when the ammonium in NH_4NO_3 was substituted with KNO_3, leading to a slightly lowered total nitrogen concentration (from 14.4 mmol/L to 12.0 mmol/L). According to the literature, 12 mmol/L of nitrogen is not too low for silver birch, since the optimal nitrogen concentration for seedlings varied between 4 and 8 mmol/L (Ingestad, 1971), and the leaf callus cultures grew well and were able to differentiate on medium containing nitrogen as nitrate at concentrations ranging from 7 to 14 mmol/L (Simola, 1985). According to Minocha (1982), when nitrate was used as the sole source of nitrogen, concentrations within the range of 0.4 to 40 mmol/L did not significantly affect the growth of shoot tips of *B. papyrifera*.

In cold acclimation studies with seedlings, the nitrogen concentration and type of nitrogen source have also proved to be important factors in regard to frost hardiness. Heavy nitrogen fertilization led to less hardy Scots pine seedlings with a high nitrogen content and decreased frost hardiness (Hellergren, 1981). The frost resistance of timothy was reduced by ammonium significantly more than by nitrate, while the frost resistance of white clover was reduced only by the highest level of ammonium (Junttila et al., 1995).

Substituting NH_4NO_3 with KNO_3 increased the total potassium concentration in the medium (from 12.3 mmol/L to 22.3 mmol/L). An increased potassium concentration should also be favourable for tissue culture because the concentration of potassium in the most commonly used woody plant tissue culture media varies between 5 and 25 mmol/L (McCown and Sellmer, 1987). Different potassium levels did not affect the growth response of shoot tips of *B. papyrifera* to nitrate or ammonium (Minocha, 1982). Studies on the effect of potassium on the frost hardiness of silver birch seedlings have shown that lower levels of potassium fertilization increased their tolerance to freezing (Jozefek, 1989). In the case of another tree, Scots pine, potassium fertilization had no effect on the frost resistance of seedlings (Christersson, 1975; Sarjala et al., 1997), and for hardened seedlings and adult trees potassium had a negative correlation with frost resistance (Sarjala et al., 1997).

Thus it appears that ammonium itself is harmful for plant tissues exposed to cold or freezing temperatures, causing a decrease in frost resistance or in recovery after cryopreservation. Ammonium ions, which are toxic to plant cells, are usually rapidly incorporated into organic compounds without accumulation in plant cells (Kirby et al., 1987; Bonga and von Aderkas, 1992). It is known that metabolic activity slows down with decreasing temperatures, and the functioning of the key enzymes of ammonia-nitrogen assimilation, glutamate dehydrogenase (GDH) and/or glutamate synthase (GOGAT), may cease or slow down leading to ammonium accumulation. The results of the present study clearly demonstrated the effect of ammonium substitution on the improved recovery of the silver birch shoots. The recovery even increased when ammonium was substituted already in the beginning of the cold hardening phase. The physiological mechanisms behind ammonium elimination/substitution, however, deserve more attention in future work.

Acknowledgements

The authors are grateful to Prof. Liisa Simola and Dr. Tuija Aronen for critical reading of the manuscript and Airi Huttunen and Jouko Lehto for their technical help. The present study was supported by the Regional Fund of Mikkeli of the Finnish Cultural Foundation.

Fig. 3: Effect of different NH_4NO_3-free treatments on the regrowth and morphological appearance of silver birch shoot tips after six weeks of post-thaw cultivation. 3 A and 3 B. Ammonium substituted by KNO_3 (10 mmol/L) in WPM with normal $Ca(NO_3)_2$ (2 mmol/L). 3 A. Cold hardening, freezing, washing and cultivation for three days using medium containing KNO_3. Regrowth percentage/viability percentage of silver birch shoots on the Petri dish (R/V) = 90/100 %. 3 B. Cold hardening, freezing, and washing using medium containing KNO_3, cultivation on medium containing NH_4NO_3. R/V = 60/90. 3 C and 3 D. KNO_3 (10 mmol/L) as inorganic nitrogen source. 3 C. Cold hardening, freezing, washing and cultivation for three days using medium containing KNO_3 as nitrogen source, after which transferred to WPM containing NH_4NO_3. R/V = 100/100 %. 3 D. Cold hardening and freezing using medium containing KNO_3, washing and cultivation using WPM containing NH_4NO_3. R/V = 95/100 %. Small shoots were typical of both treatments using KNO_3 as the only inorganic nitrogen source. 3 E. Ammonium nitrate substituted by L-glutamine (5 mmol/L); cold hardening, freezing, washing and cultivation for three days using medium containing L-glutamine. R/V = 72/94 %. Typical hard, yellow and black leaves were seen in almost every explant. 3 F. WPM control, cold hardening, freezing, washing, and cultivation on WPM containing NH_4NO_3. R/V = 70/85 %. Genotype E5387, bar = 1 cm.

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controlled (Kirby et al., 1987; Bonga and von Aderkas, 1992). The ratio of NH_4^+ and NO_3^- in the nutrient solution has no effect on the growth rate of silver birch seedlings (Ingestad, 1971), nor in the medium for megagametophyte and embryo callus lines of *Picea abies* (L.) Karst. (Simola and Santanen, 1990). On the other hand, the ability of shoot tips of *Betula papyrifera* Marsh. to use NH_4^+ or NO_3^- as the sole nitrogen source is dependent on the age of the donor plant from which the shoot tips have been taken. The

KT-Paino, Punkaharju 1999

ISBN 951-40-1675-0
ISSN 0358-4283