IN -VITRO PROPAGATION AND CONSERVATION OF *Betula utilis*

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TABLE OF CONTENTS

Chapter No.	Topic	Page No.
	Certificate from the supervisor	i
	Acknowledgement	ii
	Summary	iii
	List of tables	iv
	List of abbreviations	v
CHAPTER-1	**Introduction**	1
CHAPTER-2	**Review of Literature**	8
CHAPTER-3	**Materials and Methods**	14
	3.1 Collection of seeds	15
	3.2 Germination of seeds	15
	3.3 Media Preparation	16
	3.4 Surface Sterilization	16
	3.5 Culturing of seeds	16
	3.6 Culture conditions	17
	3.7 *In vitro* seedling culturing on prepared MS media	17
	3.8 *In vitro* callus induction on prepared MS media	17
	3.9 In vitro callus regeneration on prepared MS media	17
CHAPTER-4	**Results and Discussions**	**18**
	4.1 Surface sterilization of seeds and seedlings	19

4.2 Seed germination and culturing 20

4.3 In virto callus induction on prepared MS media 22

4.4 In vitro callus regeneration on prepared MS media 24

CONCLUSION 28

REFERENCES 29

ANNEXURE 36

CERTIFICATE

This is to certify that the work titled " **IN -VITRO PROPAGATION AND CONSERVATION OF *Betula utilis*** " submitted by " **PARAS PIRTA** " in partial fulfillment for the award of degree of B.Tech of Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Signature of Supervisor .....

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Date 27/5/2013

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SUMMARY

Betula utilis (Himalayan birch, *bhojpatra*, Sanskrit: Bhûrja) is a birch tree native to the Himalayas, growing at elevations up to 4,500 m (14,800 ft). The specific epithet, *utilis*, extensively used for firewood, paper production, furniture ,traditional ayurvedic medicines,etc. It act as an indicator of climatic shift and containing significant amount of betulin (anti-tumor activity) in its bark. It has poor seed viability and low regeneration rate in its natural habitat has brought its population near to extinction. It has been listed as critically endangered by IUCN so micropropagation provides immediate rescue and alternative for its large scale production. In present research we explored all the culture conditions for carrying out its micropropagation along with callus induction and regeneration. Seeds were cultured on different Murashige and Skoog media supplemented with different plant growth hormones. MS medium containing BAP(2mg/l)+KN(2mg/l)+sucrose(30g/l)+ agar(9g/l) gave best germination (75%) of seeds within 20 days of inoculation. Germinated seedlings were cultured and shoot were obtained and further subcultured on MS medium containing BAP(2mg/l)+GA3(2mg/l)+sucrose(30g/l)+ agar(9g/l) . Callus was obtained within 4 weeks of culturing of seedlings on MS medium containing 2,4-D(2mg/L)+ IBA(1mg/L) sucrose(30g/l)+ agar(9g/l) and subcultured on MS medium containing BAP(2mg/L) + NAA(0.5mg/L) + sucrose(30g/L) + agar(9g/L) for best growth. By far now these culture conditions were optimized for the first time, and which developed as an effective alternative technology for its conservation and commercialization.

Paras Pirta

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Dr. Hemant Sood

Date : 29/5/13

List of Tables: **Page no.**

Table 1: Effect of the concentration of bavistin and Hgcl2 on surface sterilization of seedlings:- 19

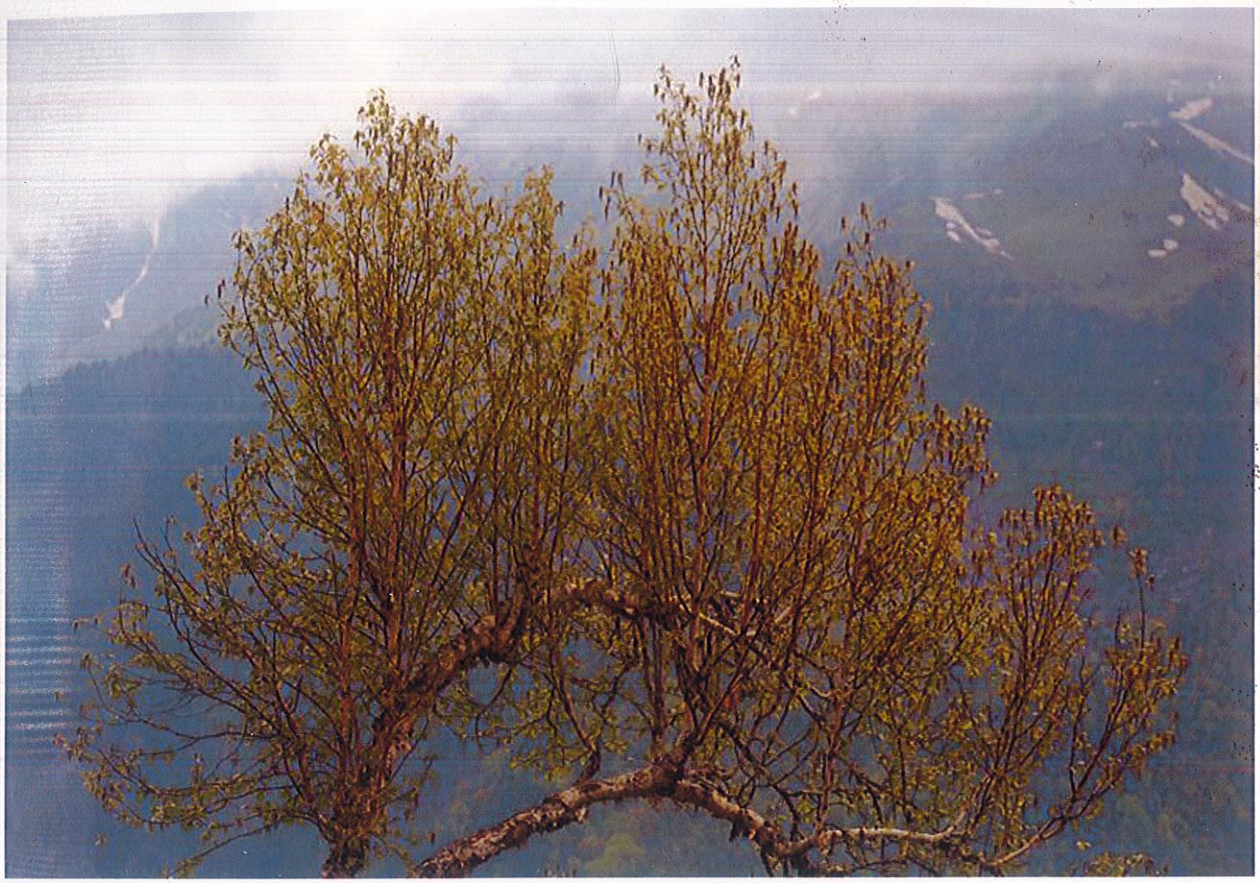
Table2: *In vitro* shoot multiplication on different combinations and concentrations of cytokinins and auxins at 25°C 20

Table 3: *In vitro* callus induction on different combinations and concentrations of cytokinins and auxins at 25°C 22

Table 4: Growth of callus on MS media having different plant hormones 24

List of abbreviations:

Abbreviations	Full forms
KN	Kinetin
IBA	Indol-3- butyric acid
GA_3	Gibberellic acid
BAP	6-Benzylaminopurine
NAA	Naphthalene acetic acid
2,4-D	2, 4 dichlorphenoxyacetic acid
IAA	3-indoleacetic acid
MS	Murashige and Skoog



Betula utilis

INTRODUCTION

Introduction

Trees constitute an important component of the ecosystem maintaining the ecological balance at regional as well as at global level. It is an admitted fact that trees are the invariable resources for providing food, fuel, timber, medicines, gums, resins and other items of daily life. Since there are natural and anthropogenic pressures on these bio-resources, which have pleaded their depletion from the nature at an unprecedented rate, thus making them threatened.(Mehbooba Zaki et .al 2011)

The major causes that have threatened the existence of plant species are(Mehbooba Zaki et .al 2011) :-

- Plants are threatened due to natural phenomena such as land up-heavals, volcanic eruptions, glaciation, heavy rains or drought, expansion of deserts, forest fire etc.

- In certain other cases species are said to have become very old or scenescent thus becoming unable to adapt to the new environment and eventually leading to their extremely confined distribution. Apart from these, affect of plant pests and diseases have also threatened several economically important plants.

- Generally the threatened species are almost always exposed to UV radiations and face drastic climatic conditions. So their reproductive capacity, natural regeneration and proliferation is reduced. All such conditions leading to genetic erosion as a result of which native variability is greatly threatened.

- As such natural processes no doubt led to the extinction of plants, the resulting new environmental conditions have also resulted in the evolution, speciation and migration of pants.

- One of the major causes for the loss of bio-resources has been the depletion of vegetative cover in order to expand agriculture. Since most of the biodiversity rich forests also contain the maximum mineral wealth and are also the best sites for water-impoundment, mining and developmental projects, which has often led to the destruction of habitats in such areas.

- Economically important plants are over-exploited to meet the demand of growing population throughout the globe that has resulted in drastic decline in the size of their populations. Some important plant species have already become extinct and there are many facing danger of extinction.

- The other main causes of loss of plant resources are conversion of forest areas for settlements, roads, development of projects, shifting cultivation, conversion to monoculture, grazing beyond carrying capacity, firewood collection, introduction of exotics and pollution. Threats to species and genetic diversity are directly linked to threats to the ecosystem.

These threatened species can be conserved either by *in-situ* methods or by *ex-situ* methods :-

In-situ conservation

In situ conservation include conservation of plants in their native ecosystem or even in manmade ecosystem, where they naturally occur. This type of conservation applies only to wild flora and not to the domesticated plants. *In-situ* conservation includes a system of protected areas of different categories e.g. National Parks, Sanctuaries, National Monument, Cultural Landscapes, Biosphere Reserves etc.

Approximately 4.2% of the total geographical area of India has been enmarked for extensive *in-situ* conservation of habitats and a protected area network of 8 Biosphere Reserves, 85 National Parks and 448 Wildlife Sanctuaries has been created (Asija and Kumar, 2007). The Indian Council of Forest Research and Education (ICFRE) has identified 309 forest preservation plots of representative forest types for conservation of variable and representative areas of biodiversity. 187 of these plots are natural

forests covering a total area of 8,500 hectares (Asija and Kumar, 2007).

Ex-Situ Conservation

Ex-situ conservation includes conservation of samples of genetic diversity (particularly representing endangered species) away from their field habitats.

Ex-situ conservation, is done through establishment of gene banks, which include genetic resource centres, zoo"s, botanical gardens, tissue culture techniques, collections etc. Thus *ex-situ* conservation is the chief mode for preservation of genetic resources, which may include both

cultivated and wild material. Some of the important modes of *ex-situ* conservation are discussed below:-

In vitro conservation

Progress in the development of plant cell and tissue culture techniques for long term germplasm conservation has been quite significant. The techniques of cell culture in particular have been so refined that only a few species are likely to be recalcitrant to known procedures.
In biotechnology laboratories, freeze preservation and storage of plant material can be of enormous value in maintenance of stock cultures and samples. Almost an indefinite number of replicates from germplasm of large number of plant species can be stored *in vitro* using little space in contrast to the thousands of hectares of land that would be required for the same number of plants if maintained in-situ. The material stored in vitro may be protoplasts, isolated cells grown in suspension or on semi-solid medium, meristem cultures at various stages of development or organized plantlets.

Cold storage

Here the germplasm conservation is made by storing plant material in cultures at low and non freezing temperature. By this technique the ageing of plant material is slowed down but not completely stopped as in freeze preservation.

In vitro techniques are being increasingly applied for large scale micropropagation to supplement the conventional methods. The tissue culture techniques offer potential means not only for rapid mass multiplication of existing stocks but also for the conservation of important, elite and rare plants.(Mehbooba Zaki et .al 2011)

Betula utilis (Himalayan birch, *bhojpatra*, Sanskrit: Bhûrja) is a birch tree native to the Himalayas, growing at elevations up to 4,500 m (14,800 ft). The specific epithet, *utilis*, refers to the many uses of the different parts of the tree. The white, paper-like bark of the tree was used in ancient times for writing Sanskrit scriptures and texts. It is still used as paper for the writing of sacred mantras, with the bark placed in an amulet and worn for protection. Selected varieties are used for landscaping throughout the world. Some areas of its native habitat are being lost due to overuse of the tree, so there is a need for its conservation.

Division- plantae

Class - Gymnospermeae

Sub class -Dicotyledoneae

Order- Fagales

Family- Betulaceae

Genus -*Betula*

Species -*utilis*

Birches are versatile trees. The sap, bark, leaves, wood, twigs and roots are used for food, construction materials, medicine, lubricants and other applications.

Bark is used in the treatment of hysteria and Jaundice. It is applied as drops to relieve ear ache. A paste made from the bark is used as a poultice on cuts, wounds and burns (Manandhar, 2002).

Paper is made from the inner bark (Polunin and Stainton et al 1984).

Outer bark can be carefully peeled off and used as paper (Gamburg and Philip, 1996).

The outer bark can also be used as water proofing material and for roofing houses.(Nanda and Kochhar, 1988).

The birch species are used in folk remedies for abdominal and mammary cancers, (Duke and Wain, 1981).

Birch leaves are used to make a diuaritic tea for colds, dysentery, milky urine and stomach ailments (Besendorfer, 1991).

Extracts of birch are used for flavouring and in cosmetics such as soaps and shampoo (Kajaba, et al 1996).

In northern latitudes pollens of birch tree are however, considered to be the most allergic tree pollens. (Mehbooba Zaki et .al 2011)

Morphology

In its native habitat, *B. utilis* tends to form forests, growing as a shrub or tree reaching up to 20 m (66 ft) tall. The tree depends on moisture from snowmelt, rather than from the monsoon rains. They often have very bent growth due to the pressure of the deep winter snow in the Himalaya.

Leaves are ovate, 5 to 10 cm (2.0 to 3.9 in) long, with serrated margins, and slightly hairy. Flowering occurs from May–July, with only a few male catkins, and short, single (sometimes paired) female catkins. The perianth has four parts in male flowers, and is absent in the female flowers. Fruits ripen in September–October.

The thin, papery bark is very shiny, reddish brown, reddish white, or white, with horizontal lenticels. The bark peels off in broad, horizontal belts, making it very usable for creating even large pages for texts. A fungal growth, locally called *bhurja-granthi*, forms black lumps on the tree weighing up to 1 kg.

The wood is very hard and heavy, and quite brittle. The heartwood is pink or light reddish brown.

Trees are distributed mostly in northern temperate climatic regions including East Asia (Afghanistan, Pakistan, and India) to South China. Leaves are simple, alternate, toothed or lobbed, deciduous and stipulate. . It grows well in heavy clay soils and dislikes wet soils. The tree is shade loving and wind tolerant. Birches are monoecious. There are 2-4 catkins together at the ends of small shoots. It hybridizes freely with other members of its genus.

The tree is distinguished by its white-brownish bark, which is peeled off in very thin pale almost transparent horizontal strips. In ancient times these bark strips were used as paper for manuscript. . (Mehbooba Zaki et .al 2011)

Objective:-

Its bark contains significant amount of betulin which is an anti-tumor agent. It is a good indicator of climate shift. As it is the national heritage of the country and the tree falls in the criteria A of critically endangered of the IUCN , and only one such report is available on in vitro micropropagtion of *B. utilis,* so the present study is carried out by having the following objectives :-

(i) To optimize the culture conditions for the in vitro propagation of *B.utilis*

(a) To optimize conditions for callus induction and regeneration

(ii) To optimize the hardening of in vitro grown plantlets for field transfer.

REVIEW OF LITERATURE

Not much work has been carried out on the *in vitro* propagation of *Betula* utilis. Literature related to *betula* species is as follows:

Zaki Mehbooba *et al.*,2011 gave a reproducible protocol for raising plants from leaf segments excised from mature plants. They achieved callusing from leaf explants of *Betula utilis* on MS medium supplemented with 2, 4-D (2.5mg/l). The callus was friable, nodular, having numerous embryoids which exhibited organogenesis on subculturing. Production of multiple shoots was achieved on MS medium supplemented with BAP (1.5mg/l).
Rooting from these shoots was obtained within 10 days of inoculation on MS medium fortified with NAA (1.0mg/l). A combination of BAP (2.0mg/l) + NAA (0.5mg/l) proved to be the best auxin-cytokinin combination in which explants exhibited best morphogenetic potential in terms of development of shoots along with roots within 8-10 days of inoculation. The maximum frequency of root differentiation (8.2) roots was achieved on MS basal medium supplemented with NAA (1.0mg/l) after 10 days of inoculations in 80% cultures. The complete plantlets were produced which showed enhanced growth after transferring in the same media composition.

Clonal propagation of birch (*Betula spp.*) via tissue culture has been attempted since 1970"s. The first micropropagated birches that were established in soil substrate was *B. pendula* (Huhtinen and Yahyaogly 1974).

The first step towards the large scale production of micropropagated plants of *Betula* was reported by McCown and Amos (1979). They produced several hundred plants from juvenile branches of *Betula platyphylla* var *Szechuanica*.

McCown and Amos (1979) compared the growth of seed raised *Betula* plants and micropropagated birches *Betula platyphylla* in the field and found that both had identical growth rates in the spring and summer, but the micropropagated plants stopped growth one month earlier than the seedlings. This resulted in the micropropagated plants having a smaller size than the seedlings. They attributed the earlier cessation of growth in the miropropagated material to the genetic differences.

Lee *et al.* (1986) reported *in vitro* propagation of *B. schmidtii*. They succeeded in the plant regeneration from shoot tips and axillary buds taken from a 4 year old seedling. They also succeeded in obtaining a clone by successive subculturing of tissues of Japanese white birch (*Beula*

platyphylla var japonica) in MS medium supplemented with (GA3) and found that GA3 help in sprouting of adventitious buds and multiple shoot induction. This experiment was intended to clarify the effects of GA3 concentrations on the culture of the germinated seedling of *B. schmidtii*. They established *in vitro* propagation method for mass propagation of this birch from germinating seedlings.

Ryynanen and Ryynanen (1986) reported micropropoagation of *Betuala pendula* var carelica from mature explants.

Elisa Sarkihita (1988) regenerated tetrapolid plantlets from cultured apical and auxiliary buds of 2-3 year old (colchine polypolid and mutant) *Betula pendulla* Roothi. Bud explants were grown on modified MS medium supplemented with BAP (2.0 mg/l and NAA (0.01mg/l). The medium allowed both induction of adventitious buds and development of shoots. The micropropagated shoots were rooted on MS medium containing NAA (0.1 mg/l). Plantlets regenerated were transferred to a peat/ soil mixture (1:1) in green house. Plantlets had a haploid chromosome set (4n=55) and an altered leaf morphology. The mutant nature of the parent tree was also evident in light green colour of the leaves of plantlets.

Cesar *et.al.*, 1988 successfully regenerated plantlets from shoot segments of *Betula cetibercia* excised from seedlings. Initiation and elongation of multiple shoot buds was achieved after 20 days culture in MS medium supplemented with BAP (0.6 mg/l) followed by 20 day culture in the same medium in the presence of a reduced BAP concentration (0.1mg/l). Rooting was achieved 7 days after having transplanted the isolated shoots to fresh medium supplemented with IBA (0.2 mg/l).

Valobra & James (1990) observed the effect of Zeatin, NAA, (α- naphthaleneacetic acid), putrescine and cefotaxime on the frequency of shoot regeneration from *Betula pendula* leaf discs. They observed that about 80% of leaf discs formed adventitious shoots when the culture medium contained (45.6 µmol 1-1) Zeatin and (0.1 mmol 1-1) cefotaxine. The addition of NAA to Zeatin-containing media prevented shoot regeneration but stimulated root development directly from leaf tissues. Putrescine (0.1mmol 1-1) and cefotaxime (0.1mmol 1-1) could both significantly increase the percentage of leaf discs regenerating shoots on optimal zeatin containing media and increase the number of shoots per regenerating disc.

John and Paul (1991) while studying the rooting behaviour in five varieties of birches reported that optimum concentration for inducing higher rooting percentage (88.6%) was 6000 ppm IBA.

Meier-Dinkel (1992) was the first to report significant differences between different clones of *B. pubescens* in field trials with regard to their growth. He observed that the growth of micropropagated plants of *Betula pubescens* (three clones) in the lowlands was good. In another trial at higher elevation the growth of micropropagated plants of *B. pendula* and *B. pubescens* was much slower, which was explained by poorer growth conditions. In the same study, hybrid birches (*B. platyphylla var. japonica X B pendula*) propagated *in vitro* from mature genotypes, showed a vigorous orthotrophic growth typical to seedlings.

Ide.Y and Nishikawa.H (1993) achieved plantlet regeneration in *Betula schmidtii* when they cultured cotyledonal nodes of seedlings on ½ MS medium containing (0.8 mg/l) of NAA and (10 mg/l) of GA3. On GA3 free medium, these explants did not produced multiple shoots. For the rooting ½ MS medium containing (0.02 ml/l) of NAA and (0.5 mg/l) of IBA was appropriate. The regenerated plantlets were successfully acclimatized on soil mixture.

Vihera, Aarnio and Ryynanen (1995) compared silver birch seedlings, grafts and micropropagated plants with regard to their growth, crown structure, flowering and seed production during the first four years in polythene greenhouse experiment with ten different genotypes. At the age of two years, the growth of the seedlings was the most vigorous and that of the grafts the lowest, the mictopropagated plants being intermediate. The difference between the seed-born and the micropropagated plants was, however, not significantly higher in number of branches than the micropropagated plants, whereas the differences in branch length, branch thickness and seed production between these two groups were not significant. The closer similarity between the micrtopropagated plants and the seedlings suggests that the micropropagated material had been rejuvenated. They found, that micropropagated plants were closer to the grafts than the seedlings with respect to the male flowering, indicating that all features of the micropropagated trees may not be juvenile.

Jones et al. (1996) compared the field performance of silver birch trees produced by micropropagation with that of seedlings. The study was carried out over a period of seven years. Material for micropropagation was collected from a 20-years-old tree, which in turn had been produced by grafting a shoot from a 40- year-old tree. In their study, micropropgated trees grew at

similar rate to seedling trees and no obvious mutant types were observed. The micropropagated trees were more uniform in height and trunk girth than seedlings.

Jones *et al.,*1996 compared the growth of trees produced by micropropogation from nodal stem sections or callus tissue of a 20 years old silver birch (*Betula pendula*) tree with that of seedlings; growth was monitored for 17 months in pots followed by six years in the field. Micropropogated trees from both nodal stem sections and callus tissue grew at similar rate to seedling trees and no obvious mutant types were observed. However, micropropogated trees were more uniform in height and trunk girth than seedling trees and more than 80% flowered within three years of planting, whereas only 39% of seedling trees flowered within this time.

Joressia,*et.al.,*1998 developed a method of propagation through nodal culture for *verginia round* leaf birch *Betula uber* Ashi. Nodal cultures of *verginia round* leaf birch had the greatest shoot elongation on woody plant medium supplemented with BAP (7.5Mm) and GA3 (2.9Mm). Shoot elongation was significantly greater when BAP was combined with GA. Propagation through nodal culture provides a feasible method by which *verginia round* leaf birch can be propagated and maintained. The same technique may be applicable to other endangered hard woody species.

Zong *et al.,*2000 developed a micropropogation system for mass propagation of „Fargo" a newly released cultivator of Asian white birch (*Betula platphylla).* Shoot tips from the mature, 7-year-old tree were established on 75% strength Murashige and Skoog medium supplemented with (0.1 µM) benzyladenine (BA), solidified with (6.5 g/liter) agar, and cultured at 24 oC. Microshoots were rooted *in vitro* or *ex vitro* followed by establishment in the greenhouse. A system to regenerate plantlets from leaves of aseptically cultured shoots was also developed. The generated shoots proliferated on the micropropagation medium were divided and the resulting shoots were rooted *ex vitro* and acclimated in greenhouse conditions.

Vihera et al., 2001 micropropagated seed born silver birches (*Betula pendula Roth*) for survival, height, growth and tolerance against biotic damage (voles, mooses, stem lesions and cankers) in field trails in southern Finland. The material consisted of 11 clones and 10 different lots of seedlings growing in 10 field trails, established in clear cut forest cultivation areas. The plants were 6-7 years old. The micropropagated and seed-born material types did not significantly differ from each other as regards survival, height, growth and frequencies of damage between single clones. These workers recommended careful selection and testing of birch clones in field conditions, before wide-scale commercial micropropagation and practical forest cultivation takes place.

Jansson, E. and Welander, M. (1990) *In vitro* cultures were established by inducing adventitious shoots from dormant buds or actively growing shoot tips on a medium with N6 macronutrients, MS micronutrients and vitamins supplemented with 0.5-1.0 mg BA and 0-0.001 mg NAA/litre. During shoot multiplication, axillary shoot tips or nodal segments were subcultured on a basal woody plant medium (WPM) containing 0.5 mg BA and 0.001 mg NAA/litre. After the first subculture, multiplication declined sharply, due to a successive shift from adventitious to axillary shoot formation. Shoots were rooted on basal WPM with 0.1 mg IBA/litre and the plantlets were transferred to a 1:1 mixture of peat and perlite in a greenhouse. The 2 basal nutrient media N6 and WPM had marked effects on root and shoot morphology. When N6 medium was used in the establishment phase more explants survived and formed adventitious buds than with WPM. However, a greater number of shoots longer than 1 cm formed on WPM, which also favoured the development of long roots with numerous laterals. Plantlets were regenerated successfully from shoots of mature trees of *B.albosinensis, B.costata, B. ermanii* cv. Mount Apoi and *B. jacquemontii.*

Cesar Perez and Paloma Pestigo :Micropropagation of *Betula celtiberica* (1988) Plantlets were successfully regenerated from shoot segments of *Betula celtiberica* excised from young seedlings. Initiation and elongation of multiple shoot-buds were obtained after 20 d culture in MS-modified medium plus BAP 0.6 mg l^{-1} followed by 20 d culture in the same medium in the presence of a reduced BAP concentration (0.1 mg l^{-1}). Rooting was achieved 7 d after having transplanted the isolated shoots to fresh medium, supplemented with IBA (0.2 mg l^{-1}).

Lu-Min Vaario et al., 1995 differentiated adventitious shoots on IS medium supplemented with BAP and NAA from root explants. The highest differentiation rate was attained on the medium containing 0.8mg/l BAP and 0.03mg/l NAA.

MATERIALS AND METHODS

Materials and Methods

The present experiments were done to check the effect of MS media having different hormones concentrations and compositions on germinated seeds.

Micropropagation of *betula utilis*

3.1.Collection of seeds

3.2.Germination of seeds

3.3. Media Preparation

3.4. Surface Sterilization

3.5. Culturing of seeds

3.6. Culture conditions

3.7. *In vitro* seedling culturing on prepared MS media

3.8. *In vitro* callus induction on prepared MS media

3.9. In vitro callus regeneration on prepared MS media

3.1. Collection of seeds

The sseds were collected from the trees growing in Kaza, Himachal Pradesh by HFRI (Himalayan Forest Research Institute) Panthaghati, Shimla, H.P.

The seeds were brought to this institute and stored in dark place in enclosed glass jars.

3.2. Germination of seeds

The seeds were separated from husks manually. A petri-plate was taken and autoclaved. Blotting sheets were taken and folded four times as the size of petri-plate, so that four layers of the blotting sheets could be fixed at the base of the petri-plate. The seeds were placed on it and another layer of blotting sheet was kept on it.The plate was then kept inside the culture room at twenty five degree celcius. Distilled water was regularly sprinkled on it till germination appeared.

3.3. Media Preparation

The composition of Media was based on Murashige & Skoog (1962) medium divided into different stocks .The Stock Solutions were prepared and kept in the refrigerator. Each salt of the stock solution was dissolved separately in distilled water so as to avoid precipitation. The Chemicals used were of reliable grade and were obtained from reliable firms like Sigma Aldrich. Each Stock solution of MS Media was added one by one to prepare the desired medium. After addition of sucrose (30g/l), growth hormones, desired volume was made with distilled water and the pH of the medium was adjusted to 5.6-5.7 by using pH meter. Then, agar-agar (9g/l) was added and dissolved by boiling the medium till it became homogenous. The medium was finally dispensed in 250 ml culture jars for culturing. These were closed with the jar caps and autoclaved at a pressure of 15 psi, 121 °C for 20 minutes for proper sterilization of culture medium. This medium was allowed to solidify and was used after 2 days of preparation.

3.4. Surface sterilization

Surface Sterilization of the germinated seeds was carried out by using different surface sterlants for different time intervals. The different sterlants used were as follows

i) Bavistin - 0.5 %
ii) Mercuric chloride - 0.1 %

The surface sterilization was carried out in Laminar air hood.

i) The germinated seeds were then treated with Bavistin (0.5 %) for 4 minutes. Subsequently, the germinated seeds were washed with autoclaved distilled water 1-2 times.
ii) After being treated with Bavistin, the seedlings were treated with $HgCl_2$ (0.1%) for 1.5-2 minutes. Finally, the seedlings were washed with distilled water 3-4 times.

3.5. Culturing of seeds

After surface sterilization the seedling are cultured (in laminar air flow chamber) with the help of forceps in various flasks having MS media (different hormone conc.).

3.6. Culture Conditions

All the operations were carried out aseptically in a Laminar Air Hood filled with UV Light. The Laminar Flow Chamber was thoroughly wiped with rectified spirit prior to use and the UV Light was kept on for 15-20 minutes in Laminar Flow Chamber. Thereafter, the airflow was allowed to run and the UV Light was switched off. Before starting with aseptic manipulations, hands and arms were washed with soap and water. Hands were frequently wiped during manipulations with rectified spirit which was allowed to evaporate. All the metallic tools like scalpel blade, forceps and needles were first autoclaved and wiped with rectified spirit and flame sterilized at the time of use. The rims of tubes and jars were quickly flame sterilized before and after inoculations. Subsequently, the jars were kept in trolleys in culture room at 25 ± 2 °C under 16 hours photoperiod and 3000 flux light intensity.

3.7. *In vitro* seedling culturing on prepared MS media

The germinated seedlings were further cultured on MS media supplemented with different plant growth hormones.

The seedlings were subcultured on same media after four weeks.

3.8. *In vitro* callus induction on prepared MS media

The plantlets (one to two month old) grown from the cultured seedlings were inoculated on MS media supplemented with different plant growth hormones for the induction of callus.

3.9. *In vitro* callus regeneration on prepared MS media

The calli obtained were inoculated on MS media having various cytokinins and auxins like BAP, BA, KN, NAA, and IAA.

RESULTS AND OBSERVATIONS

Results and Observations

4.1 Surface sterelization of seeds and seedlings

4.2 Seed germination and culturing

4.3 *In vitro* callus induction on prepared MS media

4.4 *In vitro* callus regeneration on prepared MS media

4.1 Surface sterelization of seeds and seedlings

Before culturing in suitable MS media the seeds and the ex – vitro grown seedlings were treated with various concentrations of Bavistin and $Hgcl_2$ and for various time intervals.

However treatment with 0.5 % bavistin for 3 minutes followed by treatment with 0.1% $Hgcl_2$ for 1.5 minutes gave the desirable results. (Table 1)

Table 1: Effect of the concentration of bavistin and Hgcl2 on surface sterilization of seedlings:-

Concentration (%)		**Percentage of successful sterilization**
Bavistin	**$Hgcl_2$**	
.4	**.1**	**60%**
.5	**.1**	**85%**
.6	**.1**	**85%**

4.2 Seed germination and culturing

The seeds germinated ex-vitro showed germination at the fifth day and about 2cm long seedlings were observable after 8 days.(Figure 1 a)

MS medium containing BAP(2mg/l)+KN(2mg/l)+sucrose(30g/l)+ agar(9g/l) gave best germination (75%) of seeds within 20 days of inoculation.

The germinated seedlings were surface sterilized by giving treatments of Bavistin and Mercuric Chloride (Table 1) and then cultured in MS media with different growth hormones and the growth parameters were regularly observed. (Table 2) (Figure 1 b)

The plantlets were subcultured on the same medium after every four to five weeks.(Figure 1c)

Best shoot elongation and growth was observed on MS medium containing BAP(2mg/L) and GA_3 (2mg/L).

Table2: . *In vitro* shoot multiplication on different combinations and concentrations of cytokinins and auxins at 25°C

MS + Growth Regulators (mg/L)				Rate of leaf formation
KN	BAP	IBA	GA_3	(after 20 days)
3	0	1	0	10%
2	0	0	1	12%
2	2	0	0	5%
0	2	0	2	15%
0	0	0	0	11%

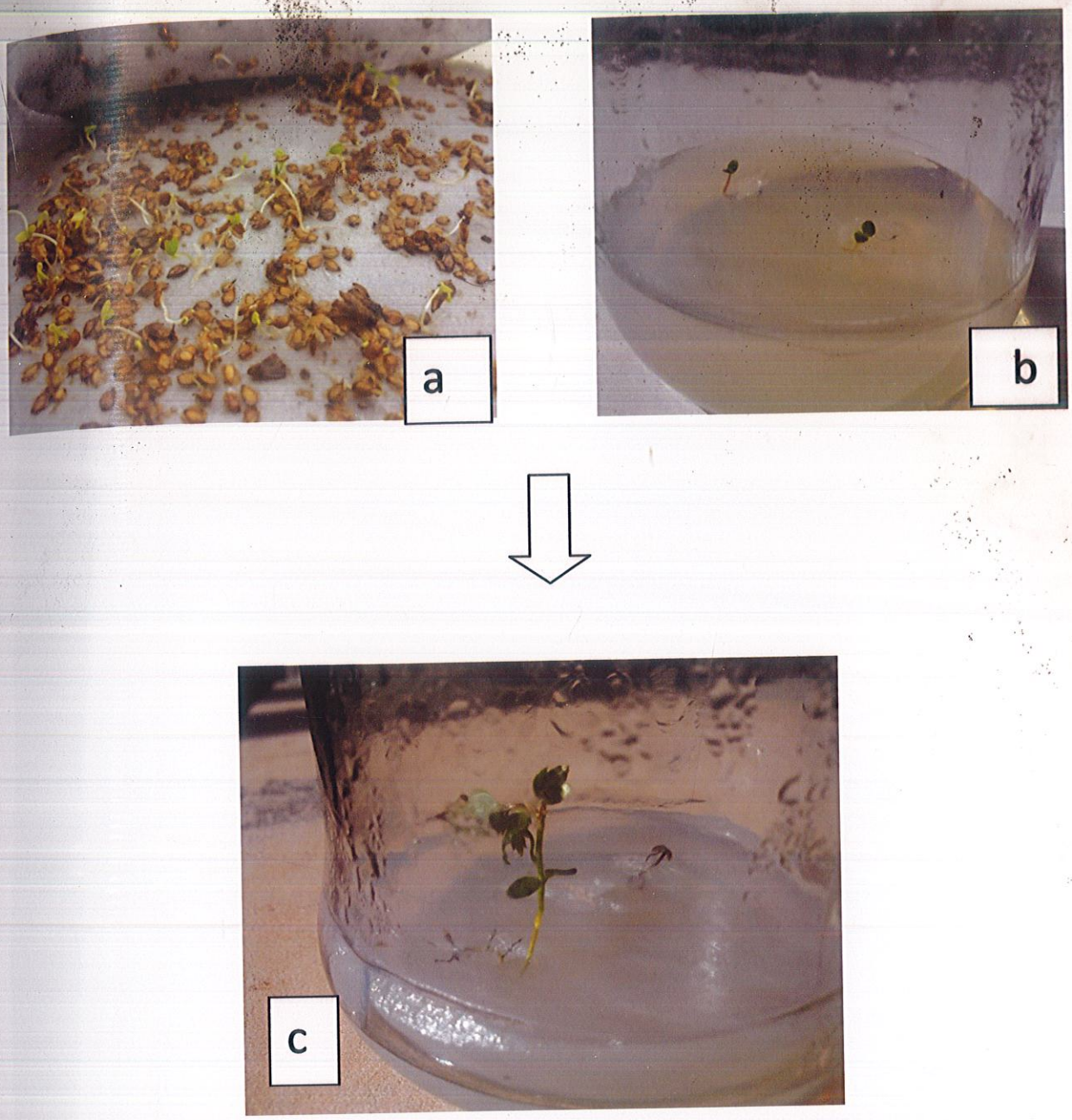


FIGURE 1 :

a) Ex- vitro germinated seedlings – after 8th day of inoculation.
b) In – vitro germinated seedlings – after 20th day of inoculation.

c) A seedling subcultured on MS medium

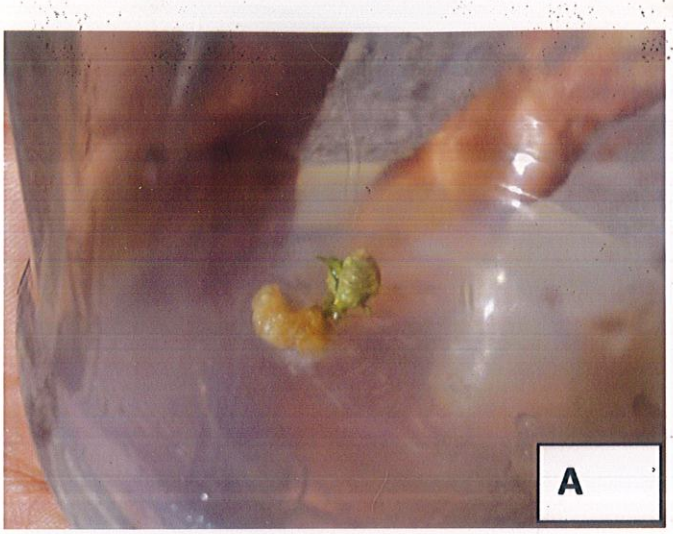


FIGURE 2:-

Callus induced on MS medium containing 2,4-D (2mg/L) and IBA (1mg/L) after 20 days of inoculation.

4.4 *In vitro* callus regeneration on prepared MS media

The calli obtained were subcultured on MS media having different plant hormones. (Table 4)

The calli subcultured on MS medium containing BAP(2mg/L) and NAA(0.5mg/L) showed fastest and best growth in terms of size.(Figure 3)

The greenest calli were observable on MS medium containing BAP(2mg/L) and GA_3(2mg/L) ; and on medium containing KN(2mg/L) and GA_3(1mg/L).(Figure 4)

The formation of shoots were very negligible in almost all these media compositions (Figure 5), but results on various other combinations are awaited.

Table 4: Growth of callus on MS media having different plant hormones

MS + Growth Regulators (mg/L)						Increase in size of callus
BAP	KN	GA3	NAA	BA	IAA	After 25 days
2	0	2	0	0	0	4 fold
0	2	1	0	0	0	3 fold
2	0	0	0.5	0	0	5-6 fold
1.5	0	0	0	0	0	4 fold
2.5	0	0	0	0	0	4 fold
0	0	0	0	3	0	3 fold
0	0	0	0	2	1	3 fold

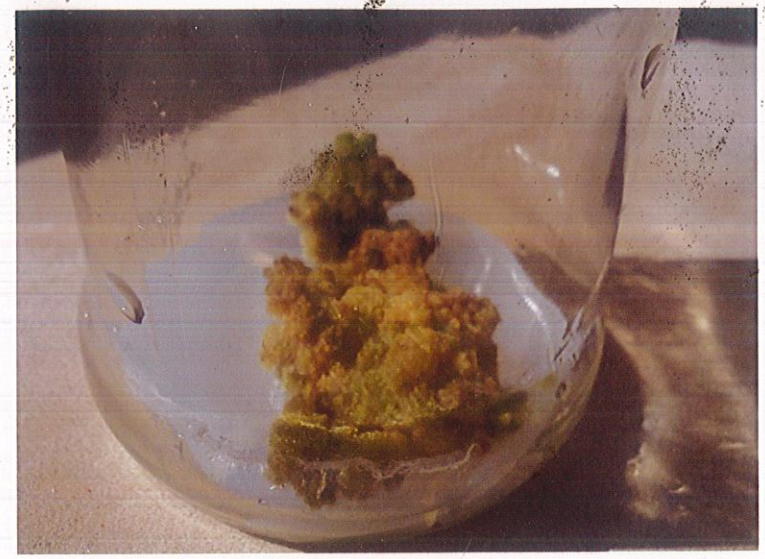
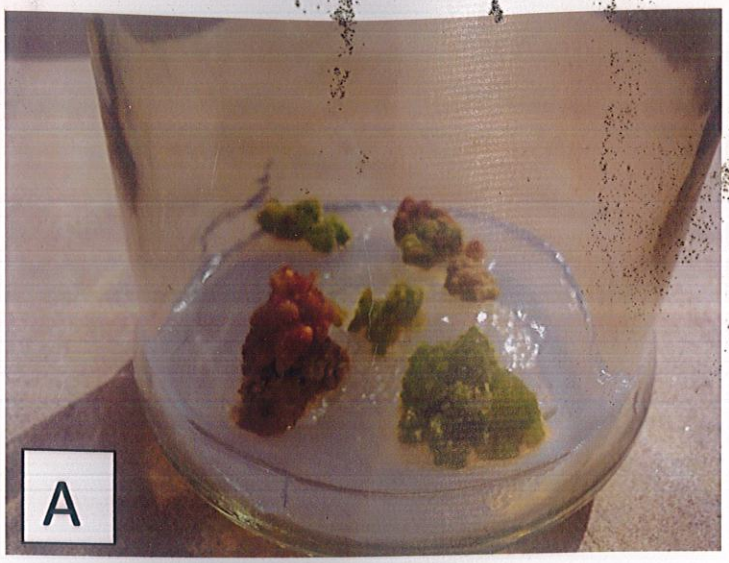


FIGURE 3:-

Callus obtained on MS medium containing BAP(2mg/L) and NAA(0.5mg/L) after one month of subculturing.



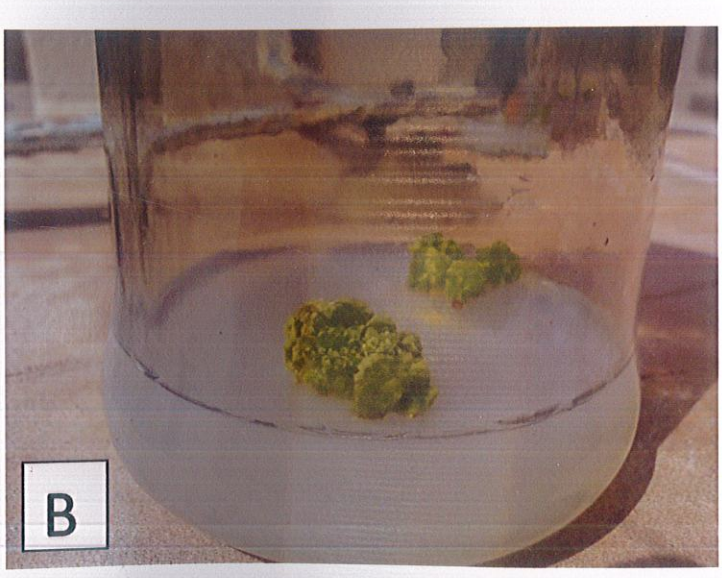


FIGURE 4:-

A. Callus obtained on MS medium containing KN (2mg/L) + GA_3 (1mg/L).
B. Callus obtained on MS medium containing BAP(2mg/L) and GA_3(2mg/L) after twenty days of subculturing.

FIGURE 5:-Micro shoot regenerated from shoot regeneration media

Conclusions:

Since the plant *Betula utilis* is very tedious to grow and very less work has been done in the field of its conservation through plant tissue culture, it is important to optimize the strategies of its micropropagation for its conservation.

- This study gives a protocol for in-vitro and ex-vitro seed germination of *Betula utilis* which is being reported for the first time in this species with a germination rate of 30 – 40%.

- A successful protocol for obtaining substantial amount of callus from seedlings was also achieved by subculturing the callus (which was induced on MS medium containing 2,4-D(2mg/L) + IBA(1mg/L) sucrose(30g/L) + agar(9g/L)) obtainred from the nodal segment of the seedling on MS medium containing BAP(2mg/L) + NAA(0.5mg/L) + sucrose(30g/L) + agar(9g/L).This protocol hold potential for the production of important metabolite betulin under cell culture conditions .

- As there are very less reports on obtaining shoots by callus regeneration even in the related species of *B.utilis* and we have archivedthat but not of the significant numbers. Efforts are still under process to generate large number of shoots and plantlets so that it can be utilized for large scale production of plants as well as metabolites.

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ANNEXURE

Composition of used Murashige and Skoog (1962) basal medium (MS Medium)

STOCKS	CHEMICALS	ORIGINAL STRENGTH (mg/l)	STOCK (g/l)	FINAL VOLUME
A - 10X	KNO_3	1900	19	}
	$MgSO_4 .7H_2O$	370	3.7	} 100ml/l
	KH_2PO_4	170	1.70	
B - 20X	NH_4NO_3	1650	33	50ml/l
C - 100X	$CaCl_2 .2H_2O$	440	44	10 ml/l
D - 100X	Na_2EDTA	37.26	3.72	} 10 ml/l
	$FeSO_4 .7H_2O$	27.85	2.28	
E - 100X	KI	0.83	0.083	100 ml/l
F - 100X	H_3BO_3	6.2	0.62	}
	$CoCl_2 .6H_2O$	0.025	0.0025	}
	$ZnSO_4 .7H_2O$	8.6	0.86	}
	$CuSO_4 .5H_2O$	0.025	0.0025	10 ml/l
	$MnSO_4 .4H_2O$	22.3	2.23	
	$Na_2MbO_4 .2H_2O$	0.25	0.025	

G - 100X	m-INOSITOL	100	10	} 10ml/l
	GLYCINE	2	0.2	
H - 100X	PYRIDOXINE-HCl	0.5	0.05	}
	NICOTINE ACID	0.5	0.05	} 10 ml/l
	THIAMINE	0.1	0.01	

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