Micropropagation of Threatened Betula Species for *in vitro* Conservation

Mariam Gaidamashvili*, Eka Khurtsidze, and Tinatin Barblishvili

***Abstract*—** The development of reliable *in vitro* protocols are of great importance for conservation of rare and threatened plant species by virtue of producing uniform planting material for offsetting the pressure on the natural populations and providing accelerated growth of biomass.

The present study summarizes the reported propagation techniques for genus *Betula* and describes probability of micropropagation from callus tissue of threatened birch species *Betula medwedewii, Betula megrelica* and *Betula raddeana*. The feasibility of large-scale propagation is discussed for efficient conservation of genetic resources of forest ecosystems.

***Keywords*—** Birch, Tissue culture, Red list, *in vitro* propagation, conservation**.**

I. Introduction

THE forest ecosystems are of special significance for conservation of biodiversity of Georgia. The forest covers 39.9% of the country territory the greater percentage (98%) of which is presented by mountainous forests. The natural forest habitats are frequently presented by relict, endemic and endangered woody species. As of the modern condition, many unique representatives of Georgian flora are threatened by extinction and/or genetic pollution due to negative impact of various environmental and anthropogenic factors.

Birch is one of the important forest tree species in Georgia. Timberline of mixed forests is mainly created with crooked-steam forests/elfin woods of birch species (*Betula litwinowii, Betula raddeana,* etc.) in humid parts and with woodlands of oriental oak and Caucasian pine (*Pinus kochiana*) in drier parts – 1800 (2000)m -2600 m. Birches are fast growing and pioneer species, able to establish cover on bare and devastated lands. However, birches are not long-lived trees and they are gradually replaced by other species, such as oaks, spruces or beeches that have grown up in their shelter. Among the woody threatened species of the Red list of Georgia genus *Betula* is represented by three species *Betula medwedewii* Regel, *Betula megrelica* Sosn., *Betula raddeana* Trautv. All three species are stated as vulnerable and were included in the Red List of Georgia, issued in 2006 on the basis of assessments made earlier and conferring to the requirements of IUCN Red List Categories and Criteria, Version 3.1, 2001, and IUCN Guidelines for National and Regional Red Lists, 2003 [1-2].

The natural range of *Betula* spp. is small fragmented distribution which is named as a reason for including these species in the Red List. The following factors are considered to be responsible for declining population of these three species: for *Betula medwedewii* - forest logging, grazing, which damages seedlings; for *Betula megrelica* - its small stands are heavily threatened by excess grazing, habitat destruction; for *Betula raddeana* - cutting of subalpine birch-woods; use of twigs for feeding the livestock, mostly horses.

Betula raddeana Trautv. (Famiy Betulaceae) –Radde's birch. In the Red List 2006 *Betula raddeana* was assessed as category VU, on the basis of the following IUCN criteria - B2a(i)b - relic species, Endemic of Caucasus, occurring in Azerbaijan, Georgia and Russia. In Georgia *Betula raddeana* occurs in Shida Kartli, Mtskheta-Mtianeti, Kazbegi Municipality and Tusheti Pshav-Khevsureti. Geographic range by area of occupancy estimated to be less than 20,000 km^2, and is severely fragmented, or known to exist at no more than 10 locations. The overall population of this species is assumed to be large, but scattered. Many subpopulations are reported to exist, but some populations are scattered and many are thought to contain a low number of individuals. *B. raddeana* is described as a rare species and little is known about it's overall population status.

Betula megrelica Sosn. (Family Betulaceae) - Megrelian birch. *Betula megrelica* is an extremely rare birch known only from collections made many years ago from Mt. Migaria in Georgia [3]. In the Red List 2006, *Beula megrelica* was assessed as IUCN category VU on the basis of the following IUCN criteria - B1a. *Betula megrelica* is endemic of Georgia (Colchis), very rare species, local endemic of the west Georgia, gathered on Migaria Mountain and was often regarded as synonym of *Betula medwedewii*. According to the Flora of Georgia, there is small, but steady difference between birches, grown in Samegrelo Mountains and Ajara-Guria range, which is evident on a big material. These two populations are sharply delimited by the deep and wide depression of Colchis lowland. This disjunction is the oldest and populations being separated for a long time now belong to two disjunctive vicarious geographical races [4].

Betula medwedewii Regel (Family Betulaceae) – Medwedew's birch. In the Red List *Betula medwedewii* was assessed as category VU, on the basis of the IUCN criteria -

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B1b (i,ii,iii). In Georgia *Betula medwedewii* distribution area extended in Guria and Ajara regions; Mount Mtirala; Mount Tbeti; in West part of Ajara, in upper and subalpine belt at 1900-2250 m altitude ASL, in some cases reaching 2350 m height. Grows in small groups and creates peculiar complexes with Pontic oak.

Dwarf birches of Caucasus and North-East Turkey high mountains sharply differ from other birch species. Caucasian populations are tertiary relicts, which survived and evolved in isolation in the region for around 15 million years, similar fossils of this age being known from Iceland [4-5]. Because of small wild populations in the Caucasus all taxons of genus Betula are of considerable interest for conservation.

The *ex situ* conservation has received the international recognition after its inclusion in the "Convention on biological diversity" [6]. The *in vitro* conservation or the technology of plant tissue culture is considered as one of the most efficient methods of *ex situ* conservation and used when the reproduction of plants growing in wild nature impossible by traditional methods, seeds of plants are "recalcitrant", does not store in dry form or low temperature conditions. The *in vitro* reproduction of rare and endangered species for conservation of gene pool and also with the purpose of accelerated growth of biomass is used especially, when the population number of species is critically low in wilderness, or when populations of rare plants decrease as a result of their unreasonable harvesting and intensive exploitation of natural resources. The technology of plant tissue culture or *in vitro* propagation is an efficient way for avoiding of problems related to traditional reproduction of plants. The method has prospects for obtaining *in vitro* conditions of genetically pure elite populations, which are free from systematic bacterial, fungal and viral diseases and guarantee emergence of quality specimens. At the same time it provides the large-scale production of young seedlings within short period and limited space. For many rare species the method of tissue culture is the more reliable process in comparison with the vegetative reproduction or reproduction by seed.

The aim of this study was to summarize the reported propagation techniques for some species of Betulaceae family and develop feasible *in vitro* methods for propagation of endangered species such as *B.megrelica*, *B. medwedewii* and *B. raddeana* which could be applied to several birch genotypes. An important target was also the evaluation of the physiological viability of seeds as a source for large-scale propagation.

II. Materials and Methods

A. Materials

The seeds of *Betula megrelica* were collected from the locality situated on NW slope of Migaria massif in September. *Betula medwedewii* seeds were collected from the Colchis section of the Batumi Botanical Garden in September. *Betula raddeana* seeds were obtained from Kazbegi Municipality in September.

B. Production of axenic seedlings and culture conditions

Seeds were soaked in 1% H_2O_2 for 48 h at 4°C, followed by sterilization with 30% H_2O_2 for 15 min. After being rinsed in sterile distilled water, they were germinated aseptically on 0.8% agar medium in growth chamber at 24±0.5°C under 16h light/8 h dark photoperiod with an irradiance 20-30 μmol m^{-2} s^{-1} from cool-white fluorescent tubes. Two week old seedlings were transferred at MS-modified culture medium containing 2.5, 7.5, or 10 μM N^6-benziladenine (BA) and 1 μM gibberellin. The medium was adjusted to pH 5.7 before being autoclaved. Cultures were grown in growth chamber at 24±0.5°C in a 16-h photoperiod with an irradiance of 20-30 μmol m^{-2} s^{-1}. After four weeks of culture remained sterile explants were produced two or three shoots 2-3 cm in length. A mass of callus tissue was produced at the base of each shoot. Callus mass was excised and shoots were placed in rooting medium.

Rooting was carried under the same culture conditions as described for shoot proliferation. The modified MS medium was used as the rooting medium except that the macromolecules were reduced to half concentration and indole-3-butyric acid (IBA) was used as the growth regulator at 1μM concentration.

III. Results And Discussion

As a general rule, trees are produced by seed or selected cultivars grafted onto seedling rootstocks. There are exceptions, such as tree species that can be micropropagated. Propagation methods vary depending on the species. Birch propagates by seed and vegetatively and considered to be difficult to propagate by cuttings. Cuttings root only with difficulty. Cultivars of *Betula* species need special measures to root successfully, but leafy semi-hardwood cuttings root under mist if taken in midsummer [7]. Several *Betula* species grow most reliably from seed; Seeds will germinate without pretreatment. In the presence of light seeds germinate readily; however, stratification period varying from one to several months is required for germination when seed is maintained in dark [8].

Seedlings of *B.megrelica*, *B. medwedewii* and *B. raddeana* were obtained by germinating surface sterilized seeds on agar plates (0.8% agar in water). Approximately 50% of the seeds germinated within two weeks. The seedlings were used at an age of about 10-12 days, when the first foliage leaf had emerged. From each species 20-30 seedlings were used. Germination rate showed differences among species indicating the differences in viability between genotypes. The germination rate was 43-50% for *Betula megrelica*; 15% for *Betula raddeana* and 4-5% for *Betula medwedewii*, Seeds from all taxonomic groups were collected at the same period, namely in September and germinated in the same culture conditions. Obviously, genotype affected more the success of germination rate then culture condition. Genetic polymorphism occurs in birch and different forms of it have

been recognized. One reason of it may be the hybridization between birch specimens that is well-documented within the genus *Betula*. Natural and artificial hybrids between different species have been described [9-10].

As reviewed by Meier-Dinkel (1992), research on tissue culture has been done with a number of species and varieties of birches [11]. Various protocols were reported for *in vitro* propagation using either different mature parts of the plant or seedlings, via somatic embryogenesis and direct or indirect organogenesis. Table I shows some *Betulaceae* species for which in vitro propagation has been done.

TABLE I
IN VITRO PROPAGATION OF SOME BETULACEAE SPECIES

Botanical name	Explant used	References
B. pendula Roth.	Seeds, seedlings, leave, bud (somatic embryos, callus cultures)	12, 13,14,15,16,
B. platyphylla	Shoot, leaf	17
B. uber (Ashe) Fernald.	Shoot (nodal culture)	18
B. celtiberica	Seedlings	19

The proliferation experiments were started after sufficient number and length (1-1.5 cm) of seedlings were available (Fig. 2A). BA concentration had a significant effect on shoot proliferation (Fig. 1) Explants exhibit greatest elongation on modified MS supplemented with 7.5 μM BA for all taxons. The fastest shoot growth response was achieved for the *Betula megrelica* in 4 week period with an average of 3-4 shoots greater than 2.5-3 cm. long enough to transfer to *in vitro* in the rooting medium (Fig. 2B). At the lover and highest level of BA tested (2.5 μM and 10 μM respectively) shoots were inconsistent in size. The lowest response to proliferation showed *B. medwedewii* that was in accordance with the germination rate (5%). Micro shoots rooted *in vitro* with an overall rooting rate 75%. Rooting was carried under the same culture conditions as described for shoot proliferation. Shoots excised from callus were placed on rooting medium supplemented with indole-3-butyric acid (IBA). Roots started appeared after 10-14 days of culture.

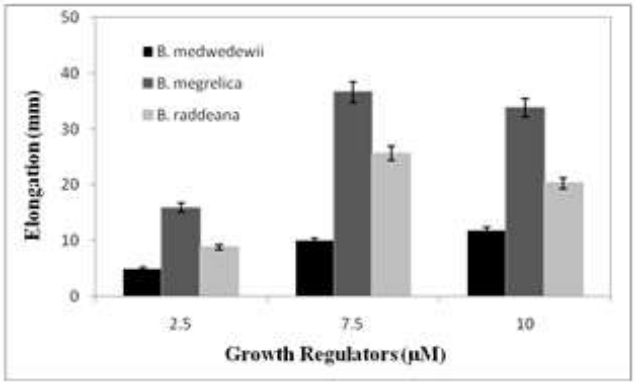

Fig.1 Comparison of multiple shoot formation and elongation of *Betula* species from callus tissue at 4 wk stimulated by BA and gibberellin at different concentrations. Bars indicate mean±S.d.; (ANOVA, n=20, p<0.05; Student's t-test)

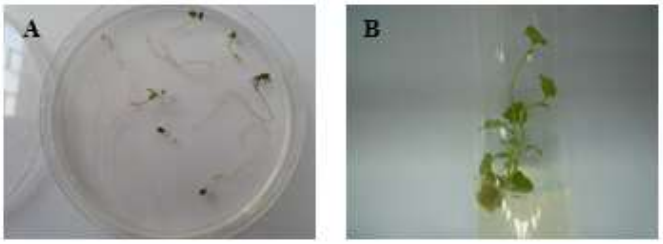

Fig.2 Germination (A) and multiple shoot formation (B) from 12 days old seedling of *B. megrelica* produced on MS-modified medium supplemented with 7.5 μM BA and 1 μM gibberellin.

Results demonstrated that threatened birch species can be effectively propagated *in vitro* using viable seedlings as explant source. Propagation through seedling culture provides a feasible method by which *Betula* spp. can be propagated and maintained. The same technique may be applicable to other endangered hardwood species for producing uniform planting material, providing accelerated growth of biomass for efficient conservation of genetic resources of forest ecosystems.

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