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Efficient Technique for Long-Term *in Vitro* Storage of Transgenic Aspen Genotypes

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Abstract

In vitro culture of isolated cells from tissues and organs is sometimes used to preserve and reproduce unique genotypes of woody plants. The technique, however, requires regular subculturing which raises storage costs and creates risks for contamination and accumulation of somaclonal variations. We examined the effects of sugar composition of culture medium, the length of photoperiod, light intensity, and ambient temperature on the survival of plant material *in vitro*. The study was performed on 49 genotypes of *Populus tremula* (46 transgenic genotypes carrying *GFP*-, *Xeg*- and *Gus*-genes, and 3 control (wild-type) genotypes). It was shown that effective storage of plants was achieved through optimization of the combined effects of all storage parameters under study. Based on the experimental data, we developed a protocol for long-term *in vitro* storage of desirable genotypes without subculture and with a survival rate of up to 98%. The best results were obtained when the plant material was pre-cultured on a WPM medium containing 15 g/L sucrose, 7.5 g/L sorbitol and 7.5 g/L mannitol, and then stored at +4˚C under a 24-hour light day cycle with only 8 hours of light per day and maximum light intensity of 2000 lux. Post-storage recovery was done by culturing on a medium containing 1 mg/L gibberellic acid. The developed method can be used for effective *in vitro* storage of the studied genotypes for up to 24 months without subculture.

Keywords

Populus tremula, Long-Term Storage, *In Vitro* Culture, Osmolytics, Transgenic Genotypes, Microshoots

1. Introduction

One of the currently most promising methods for storage and recovery of plant

material of valuable genotypes is *in vitro* culture of cells isolated from tissues and organs. The method can be used for long-term aseptic maintenance of plant material of certain genotypes and varieties, and thus can contribute to plant breeding and preservation of rare and endangered species. There are a lot of specialized *in vitro* collections of plants in the world, e.g. the *in vitro* Collection of Potatoes at NordGen (Nordic Genetic Resource Center, Sweden, Norway), *in vitro* Collection of Clones of Valuable Genotypes of Deciduous Woody Plants (All-Russian Research Institute of Forest Genetics, Breeding and Biotechnology, Russia), *in vitro* Collection of the Royal Botanical Gardens (Royal Botanic Gardens, Kew, UK), *in vitro* Collection of Selectively Bred Varieties of Aspen *Populus tremula* from Palencia province (Spain) and others. Each of the collections serves the aim of preserving valuable plant genotypes. However, *in vitro* maintenance of plants is quite costly and associated with the risk of somaclonal variations [1], therefore for most collections the researchers are looking for optimal conditions and develop protocols for longer *in vitro* storage of plants in order to reduce the costs and better preserve the genotypes [2] [3] [4] [5].

The choice of a storage method depends on the intended storage period. For short- and medium-term storage, growth inhibition is used to increase the subculture intervals. It is the simplest way to limit the growth of plant material *in vitro*. Culture growth can be slowed down in several ways, including by keeping the culture at low temperatures [6] [7] in the presence of growth regulators such as abscisic acid (ABA), by reducing sucrose levels or adding osmotically active substances (e.g., 3% mannitol) to the culture medium [8] [9]. Exposure to low temperatures results in increased accumulation of unsaturated lipids on cell membranes and hence their thickening and slowing down of cell division and elongation. Cold storage of some species was done either in low light or in complete dark [2] [9]. In the simplest case, reducing the culture temperature below levels needed for active growth is used to significantly increase subculture intervals. However, one should avoid low temperatures that can cause cold damage [10] or denaturation of active macromolecules [11].

At near-zero temperatures plants can be stored *in vitro* for years without subcultures. This storage method is suitable for many plants of temperate climate including berry and fruit trees [12] [13] [14] [15]. Temperatures of 0˚C - 5˚C were shown to suit well for cold-resistant species such as apple (*Malus domestica*), plum (*Prunus*), and strawberry (*Fragaria* × *ananassa*) [16] [17]. For example, the first reported successful cold storage of apple explants was that of the "Golden Delicious" variety. Its microshoots survived after one-year *in vitro* storage in the dark at 1˚C - 4˚C, with some losses for contamination [18]. Such protocols, however, are not suitable for longer than a 12-month *in vitro* storage of aspen, a species often used in biotechnological manipulations [19].

Aspen (*Populus tremula*) and its hybrids have long been widely used in biotechnology as a model for studying various aspects of forest woody plant genetics. This is due to a number of reasons: a wide distribution area, a relatively small and effectively transformable genome, rapid growth, easy clonal micro-

propagation and *in vitro* cultivation. A topical problem of today is to preserve a large panel of selectively bred and transgenic lines, each having its unique characteristics. These lines may be used as parents, donors of new features, or in fundamental research [20]. Long-term *in vitro* storage of cultures without subculturing and creation of a genotype bank are viewed as a way to preserve plants with valuable properties.

2. Materials and Methods

2.1. Plant Materials

In-vitro cultures of aspen *Populus tremula* of the non-transgenic genotypes Pt, F2 and PtV22 (kindly provided by the Forest Institute of the National Academy of Sciences of Belarus, the Republic of Belarus), and 46 genotypes/lines of transformed plants carrying the genes *GFP*, *Xeg* or *Gus* and obtained from the Forest Biotechnology Group at the Branch of the Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences (**Table 1**).

All selected plant lines were cultured *in vitro* on a WPM medium [21] without growth regulators, with the addition of 30 g/L sucrose, 9 g/L agar, and MS vitamins, and at pH of 5.6 - 5.8. After all the ingredients were added, the medium was autoclaved for 15 minutes at 1 atm and 120˚C. The plants were cultured under optimal growth conditions: at a temperature of +22˚C - 24˚C and on a 16-hour light/8-hour dark cycle with a light intensity of 2500 - 3000 lux.

2.2. Storage at Low Positive Temperatures

Four alternative sets and control set of long-term storage conditions and medium composition were tested to choose the optimal one (**Table 2**). Variants of medium composition were compiled on the basis of literature data [9] [22]. Each set of conditions was tested on 60 microshoots of each genotype. The microshoots were planted in 330-mL jars, 15 per jar. Each jar contained 50 mL of medium. On average, about 2950 microplants took part in each storage condition.

The plants were cultured for 3 weeks on a 16-hour light: 8-hour dark cycle with a light intensity of 2000 - 2500 lux. After a 3-week growth, after root formation in microplants, the jars were placed into a refrigerator at + 4˚C, with a short photoperiod and a low light intensity. Explant survival was assessed at 6, 12 and 24 months. The obtained data were analyzed by ANOVA-1 using the Statistics 7.0 software.

2.3. Culture Recovery after Long-Term Storage

After 6, 12 or 24 months of storage shoot tips were excised and transferred onto a recovery WPM medium supplemented with 30 g/L sucrose, 9 g/L agar-agar, MS vitamins, 1 mg/L gibberellic acid (GA). Microshoots were planted into 330-mL jars containing 50 mL of the medium each, 15 shoots per jar. The plants were cultivated for 4 weeks with a photoperiodic lighting at 2000 - 2500 lux, 16 hours of light:8 hours of dark. Upon completion of the 4-week period, cutting for multiplication was done.

Table 1. List of transgenic aspen lines used in the study.

Name of recombinant gene	Line name	Target effect of recombinant gene
GFP, gene encoding fluorescent protein (green fluorescent protein)	Pt III GFP 2b, Pt III GFP 3b, Pt III GFP 3c, Pt III GFP 5b, Pt III GFP 5c, Pt III GFP 6a, Pt III GFP 6c	Reporter gene
Gus, gene encoding *β*-glucuronidase	f2 VII Gus 1a, f2 VII Gus 1b, f2 VII Gus 1c, f2 VII Gus 3a, f2 VII Gus 4a, Pt I Gus 1b, Pt I Gus 5a, Pt II Gus 1c, Pt II Gus 3a, Pt V Gus 2c, Pt V22IIGus 1a, Pt V22II Gus 1b, Pt V22II Gus 1c, Pt V22V Gus 14a	Reporter gene
Xeg, gene of xyloglucanase *sp-Xeg* from *Penicillium canescens*	Pt XIV Xeg 1a, Pt XIV Xeg 1b, Pt XIV Xeg 1c, Pt XIV Xeg 4a, Pt XV Xeg 1a, Pt XV Xeg 1b, Pt XV Xeg 1c, Pt XV Xeg 2a, Pt XV Xeg 2b, Pt XV Xeg 2c, Pt XV Xeg 3a, Pt XV Xeg 3b, Pt XV Xeg 3c, Pt XV Xeg 4a, Pt XV Xeg 4b, Pt XV Xeg 4c, Pt XV Xeg 5a, Pt XV Xeg 5b, Pt XV Xeg 5c, Pt XVI Xeg 1a, Pt XVI Xeg 1b, Pt XVI Xeg1c, Pt XVI Xeg 5c, Pt XVI Xeg 8a, Pt XVI Xeg 8b	Changing the carbohydrate composition of wood, increasing biomass

Table 2. Tests of alternative conditions for long-term storage of aspen explants without subculture.

Test	WMP sugar content (g/L)	Light intensity (lux)	Light/dark cycle
Control	Sucrose, 30	4000	16/8
1	Sucrose, 15 Sorbitol, 7.5 Mannitol, 7.5	4000	16/8
2	Sucrose, 15 Sorbitol, 7.5 Mannitol, 7.5	2000	16/8
3	Sucrose, 15 Sorbitol, 7.5 Mannitol, 7.5	2000	12/12
4	Sucrose, 15 Sorbitol, 7.5 Mannitol, 7.5	2000	8/16

3. Results and Discussion

After the long-term storage without subculture, all studied aspen genotypes, both transformed and wild-type, showed similar survival rates under similar conditions. However, test 4 was the only one where plants survived the 24-month storage (**Table 3**). In all other tests the 24-month storage survival was zero.

As seen during the tests, exposure to the temperature of +4˚C slowed down the growth, caused fall of leaves, with the apical bud alone remaining in the

Table 3. Survival (mean ± SD) of aspen plants of all studied genotypes under different storage conditions at +4˚C.

Test	The number of survived plants (survival rate, %)					
	6 months		12 months		24 months	
	Wild-type	Transgenic	Wild-type	Transgenic	Wild-type	Transgenic
Control	95 ± 4 (53)	1434 ± 23 (52)	0	0	0	0
1	119 ± 5* (66)	1821 ± 34* (66)	0	0	0	0
2	132 ± 4* (73)	2017 ± 43* (73)	42 ± 2# (23)	631 ± 16# (23)	0	0
3	178 ± 5* (99)	2760 ± 11* (100)	104 ± 7# (58)	1572 ± 20# (57)	0	0
4	180 ± 7* (100)	2760 ± 15* (100)	180 ± 4# (100)	2940 ± 11# (100)	176 ± 7$ (98)	2706 ± 37$ (98)

*,#,$Statistically significant difference from the results of control according to ANOVA-1, $p \leq 0.05$ for all studied storage periods.

vegetative state. Judging by their appearance, the plants went dormant (**Figure 1**).

After 6, 12, 24 months of storage microshoots were cut and thansferred onto a recovery medium, and the culture jars were exposed to optimal growth conditions. Within a week, 100% of the explants produced green growing shoots from the apical and lateral buds. The leaves that remained after the storage period were not viable and died off. Upon further cultivation, the explants formed well-developed microplants (**Figure 2**).

According to the obtained results, the 4th set of storage conditions (Test 4) was chosen as the most suitable for *in vitro* storage of aspen explants and was used as a basis for the development of a protocol of long-term *in vitro* storage of aspen culture without subculture.

Our findings demonstrate the significance of the intergrated effect of ambient parameters and growth medium on the efficiency of long-term *in vitro* storage of aspen microplants at low-positive temperatures without subculture. They also show that a change in even one parameter can notably affect microplant survival during their long-term storage for up to 24 months without subculture (**Table 3**). Noteworthy is that earlier methods of long-term storage mostly modified the composition of culture medium [4] [9] [19] [23]. Yet, according to our findings, changing the culture medium composition alone is not enough for aspen plants. The life cycle of aspen, as well as of any temperate-climate perennial, consists of alternating periods of active vegetation and rest at low temperatures. Therefore, one might expect that lighting may also play an important role. The study has demonstrated that light intensity and cycle are also essential factors for successful long-term storage without subculture. Going dormant is associated with changes in the hormonal composition and properties of cell membranes, cell division rate and other physiological processes that allow plants successfully survive adverse conditions. In aspen, processes leading to dormancy are triggered

Figure 1. Aspen plantlets of the Pt XV Xeg 1b genotype on a WPM medium supplemented with 15 g/L sucrose, 7.5 g/L sorbitol, and 7.5 g/L mannitol after 24-month storage at +4˚C and a photoperiodic lighting (8 h of light:16 h of dark) with a light intensity ca. 2000 lux.

Figure 2. Aspen plantlets of the PtV22IIGus1a genotype at Week 3 of recovery phase, formed on microshoots after 24-month storage at +4˚C (on a WPM medium supplemented with 15 g/L sucrose, 7.5 g/L sorbitol, 7.5 g/L mannitol, exposed to photoperiodic lighting (8 h of light:16 h of dark) with a light intensity ca. 2000 lux). The pale chlorotic leaves persist after the storage phase.

by changes in the composition of incoming substances, light exposure, and effect of low temperatures [24]. Therefore, the conditions of Test 4, *i.e.* reduced content of easily metabolizable sugars in the medium, low light intensity and short photoperiod (**Table 2**), proved to be most suitable for long-term *in vitro* storage of aspen explants without subculture.

Based on the results of the tests we have developed an effective method for long-term *in vitro* storage of aspen explants for up to 24 months without subculture and with high survival. The most suitable conditions were as follows: culture on a WPM medium supplemented with 15 g/L sucrose, 7.5 g/L sorbitol, and 7.5 g/L mannitol; 8:16 hours light:dark cycle with light intensity ca. 2000 lux. When transferred to recovery conditions after such storage, the survived explants developed into viable plants without abnormalities within 3 weeks. This method can be used for long-term *in vitro* storage of both transformed and

non-transformed aspen genotypes.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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