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Effects of Temperature and Season on *In Vitro* Establishment and Shoot Multiplication of *Picea abies* (L.) H. Karst

Mehrnaz Zarei, Hassan Salehi*, Abolfazl Jowkar

Department of Horticultural Science, Faculty of Agriculture, Shiraz University, Shiraz, Iran.

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Abstract
Plant propagation originated from the mature tissues is the most desirable method in producing clonal plants, however microbial contamination usually is the main concern during regeneration processes. Moreover, commercial production of plants using clonal propagation is considered as high throughput method due to the removal of seasonal barriers, lower cost of production with higher yield when compared to the conventional propagation methods. In this research the regulatory roles of 2-iso-pentenyl adenine (2iP) and Zeatin (Z) along with two temperature regimes, 22 and 28 °C, on mature explants of *Picea abies* (L.) H. Karst was evaluated for adventitious shoot regeneration. Norway spruce micro-cuttings considerably benefited from Ridomil and Benomyl treatments for fungal decontamination in explants taken in spring or fall. The highest percentage of regenerated explants (68.75%) as well as the highest numbers of adventitious shoots per explant were obtained in MS media supplemented with 4 µM Z. Furthermore, the highest length of proliferated shoot (1.17 cm) was achieved at 3.2 µM 2iP treatment. However, there were no significant differences in the length of adventitious shoots using different concentrations of 2iP. Incubating explants at 28˚C resulted in null or deformed regeneration in Norway spruce.

Keywords: clonal propagation, decontamination, micro-cutting, Norway spruce, Zeatin.

Introduction

Norway spruce [*P. abies* (L.) H. Karst] is an evergreen and fast growing conifer which grows up to 40 m (Warren, 1982). The Norway spruce forests have been used for almost 2000 years as a source of timber, paper, fiber, fuel, etc. In addition, its aesthetic value has made it an ideal option as the main Christmas tree around the world and its usage in landscape designs (Carlsson, 2012). However, by increasing demands for conifers' timber products and wood, the rate of harvest surpasses the rate of plant regeneration and restoration. Therefore, there is an urgent need for clonal propagation to produce large number of elite and fast-growing trees to replace the harvested plants. Intensive breeding programs and reforestation are based on clonal production of elite trees (Sutton, 2002; Thorpe et al., 1991). Norway spruce is propagated by seeds in fall. Alternatively stem cutting is used for its clonal propagation. Cuttings should be selected from apical branches to inhibit asymmetrical pattern in ramets (Hartmann et al., 2002). Rooted plants derived from plagiotropic shoots will represent an unusual growth habit unable to grow upright

* Corresponding Author, Email: hsalehi@shirazu.ac.ir

(Hartmann and Kester, 2011). Clonal propagation of mature Norway spruce by using of stem cuttings is negatively influenced by maternal age which result in a lower rooting percentage (Girouard, 1974). Lack of propagules and low rate of growth are other disadvantages in cutting stem method (Libby, 1974; Shelbourne and Thulin, 1974; Sweet and Wells, 1974). In recent years, plant tissue culture has been applied to accelerate the propagation of selected cultivars. Therefore plant tissue culture has become an indispensable method in forest tree breeding programs (Murashige, 1974; Von Arnold and Eriksson, 1979).

Using of immature tissue of embryo, cotyledons and seedlings in *Pinus radiata* to regenerate adventitious root, shoot and buds has been reported frequently (Stange et al., 1999). Successful *in vitro* propagation of *P. abies* has been reported via somatic embryogenesis from germinating seeds (Hakman et al., 1985; Verhagen and Wann, 1989). However, adventitious shoot propagation of aerial parts (shoots) of Norway spruce as an explant source has not been reported so far. This can imply that proper offsprings by seeds and embryos tissue culture will not be reproducible. The objectives of the current research are i) thet establishment of an effective decontamination procedure in tissue culture of mature shoots of *P. abies* and ii) investigation the effectiveness of cytokinin in shoot proliferation of the explant source.

Materials and Methods

Plant materials and surface sterilization

Ten to fifteen years old *P. abies* (L.) H. Karst grown at School of Agriculture, Shiraz University, Shiraz, Iran (52°28-E and 29°45-N, 1810 m.a.s.l.) were chosen as a source of explants for this study. Micro-cuttings (2-4 cm) were selected from new apical shoots (upper half) of branches grown at spring and fall. To reduce the contamination, the explants were kept under running tap water for 2 h before transferring to the antifungal solutions on a shaker set at 150 rpm for up to 6 h. The concentrations of antifungal solutions were 0, 4, 8 and 16 g L^{-1} of Ridomil (Bahavar Shimi Company, Iran) and Benomyl (Agro & Chemie, Hungary). The explants were transferred to laminar flow Cabinet and submerged into 70% ethanol for 60 s and rinsed quickly with sterilized distilled water. These explants were treated with 0, 5, 10 and 15% Clorox (5.25 % sodium hypochlorite) solution containing 0.1% Tween-20 for 5, 10 and 15 min and rinsed six times with sterilized distilled water. Explants were finally transferred to potato dextrose agar (PDA) media (300 gr potato, 30 gr dextrose and 8 g agar).

Proliferation treatments

In one experiment, 2iP (0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, and 12.8 µM) and Zeatin (0, 2, 4 and 8 µM) along with 1 g L^{-1} MES (2-(N-morpholino) ethanesulfonic acid) in MS media were used to evaluate explants proliferation rate. The pH of the media was adjusted to 5.8 by 0.1 N HCl and 0.1 N NaOH before autoclaving for 15 min at 121 °C and 103 kPa. Zeatin was added to the MS media after autoclaving when the media temperature reached to 55 ˚C. In another experiment, shoot induction was investigated at 22 and 28 ˚C. After 3 weeks, explants were transferred to MS media without plant growth regulators. Cultures were kept under cool-white fluorescent light (30 µmol m^{-2} s^{-1}) with 16-hour photoperiod. Each experiment was carried out as a completely randomized design with 4 replications and 5 explants in each replication. Zeatin, 2iP and MES used in this study were purchased from Sigma-Aldrich (St. Louis, Missouri, U.S.A.).

Data recording and statistical analysis

Decontamination rate was recorded after 2 weeks and further proliferation percentages, number of proliferated shoots and mean length of shoots were measured after 6 weeks. The statistical analysis was done using SAS (9.0 version) statistical software and means were compared with LSD test ($p \leq 0.01$).

Results and discussion

Surface sterilization

Significant differences were not observed in the spring micro-cuttings between 8 g L^{-1} of Ridomil and Benomyl (87.50%) and 16 g L^{-1} of Ridomil and Benomyl (93.75%). However, 16 g L^{-1} of Ridomil and Benomyl was the most effective treatment in removing the fungal contamination (81.25%) in micro-cuttings collected in fall (Table 1).

Table 2 shows that 10% Clorox for 10 min resulted in highest rate of contamination control (80 and 75% at spring and fall, respectively) without having negative effect on explants' quality. Although the longer time and higher concentration of treatments lead to higher reduction in contamination, the explants were severally damaged and became necrotic.

Unexpected internal and external microbial contamination is the main concern for micropropagation of perennial woody plants. Type and concentration of antimicrobial agents as well as their exposure time on explants play important role in explant contamination. It has been frequently shown that antimicrobial agents are incapable of removing contaminations and potentially result in unexpected tissue damages. Therefore, it is important to determine a proper concentration of biocidal agents based on the tissue type, age and condition. Notably some antimicrobial agents like Hg_2Cl_2 are highly effective, their toxicity and environmental hazards has made them redundant (Chawla, 2000; Hartmann and Kester, 2011).

The small scale-like and compact spirally arranged leaves of Norway spruce explants can harbor high fungal and bacterial contaminants. Our results show that Norway spruce tissues are susceptible to surface sterilization, therefore applying proper antifungal agents like Ridomil and Benomyl at appropriate concentration play pivotal role in microbial decontamination of this plant. Antifungal agents by the concentration of 8 g L^{-1} effectively reduced contamination in those explants harvested in spring. This could be because of the fact that the Norway spruce leaves were not completely mature at spring therefore the populations of microorganisms on the plants were lower. However, for the explants that were collected in fall, 16 g L^{-1} of antifungal agent was effective possibly because the fall explants were fully matured and contained higher number of microorganisms. In second round of surface sterilization in Laminar Flow Cabinet, 15% Clorox for 10 and 15 min were effective treatments. However, at this concentration of Clorox and these durations tissue damage was observed. The 10% concentration of Clorox for 10 min was the most effective treatment in controlling the contaminant without adversely affecting the quality of the explants.

Proliferation

Murashige and Skoog (MS) media (1962) supplemented with 1.6 µM 2iP resulted in 68.75% proliferation rate at 22 ˚C which is significantly higher than the other treatments (Table 3 and Fig. 1). The aforementioned concentration of 2iP resulted in the highest number of adventitious shoots on the micro-cuttings. The proliferation rate of explants declined as the concentration of 2iP raised above 6.4 µM. Furthermore, at high concentration of 2iP (6.4 and 12.8 µM) the induced shoots showed necrotic spots. Significant differences were not observed between induced adventitious shoots length at different concentrations of 2iP (Table 3).

Explants grown on MS media supplemented with 4 µM Zeatin at 22 ˚C had the highest rate of proliferation, shoot length and shoot number (Table 4). Generally, explants grown at 28 ˚C did not properly proliferate at any concentrations of PGRs used and when they got proliferate the resultant shoots were malformed.

Table 1. Effect of Benomyl and Ridomil on *P. abies* (L.) H. Karst trees explants collected in spring and fall.

Benomyl + Ridomil (g L^{-1})	Fungal decontamination (%)	
	Spring	Fall
0	$0^{d\dagger}$	0^{d}
4	40.62^{c}	18.75^{c}
8	87.50^{a}	43.75^{b}
16	93.75^{a}	81.25^{a}

†Means with the same letters are not significantly different at $p \leq 0.01$ by LSD test.

Table 2. The effects of time and concentration of Clorox treatments on fungal decontamination of *P. abies'* explants.

Clorox Concentration (%)	Time of treatment (min)	Spring		Fall	
		Necrotic spots (%)	Inhibition of fungal contamination (%)	Necrotic spots (%)	Inhibition of fungal contamination (%)
0	0	$0^{e\dagger}$	0^{a}	0^{d}	0^{a}
5	5	0^{e}	0^{a}	0^{d}	0^{a}
5	10	0^{e}	0^{a}	0^{d}	0^{a}
5	15	0^{e}	5^{a}	0^{d}	0^{a}
10	5	0^{e}	40^{b}	0^{d}	20^{b}
10	10	0^{e}	80^{c}	0^{d}	75^{d}
10	15	20^{d}	90^{d}	10^{dc}	85^{d}
15	5	30^{c}	55^{c}	15^{c}	55^{c}
15	10	65^{b}	90^{d}	50^{b}	80^{d}
15	15	90^{a}	100^{d}	70^{a}	90^{d}

†In each column means with the same letters are not significantly different at $p \leq 0.01$ by LSD test.

Table 3. Regulatory role of 2iP on mean length and number of shoots per explant and proliferation rate of *P. abies* micro-cuttings.

2iP (µM)	Proliferated explants (%)	Shoot length (cm)	Number of shoots per explant
0	$0^{d\dagger}$	0^{c}	0^{d}
0.2	25^{bc}	0.84^{a}	1^{c}
0.4	12.5^{dc}	0.7^{a}	0.5^{dc}
0.8	37.5^{b}	0.81^{a}	1.25^{bc}
1.6	68.75^{a}	0.68^{ab}	3.25^{a}
3.2	27.68^{bc}	1.17^{a}	0.97^{c}
6.4	25^{bc}	0.93^{a}	2^{b}
12.8	12.5^{dc}	0.2^{bc}	0.75^{cd}

† In each column means with the same letters are not significantly different at $p \leq 0.01$, according to LSD test.

Table 4. Regulatory role of Zeatin on mean length and number of shoots per explant and proliferation rate of *P. abies* micro-cuttings.

Zeatin (µM)	Proliferated explants (%)	Shoot length (cm)	Number of shoots per explant
0	$0^{b\dagger}$	0^{c}	0^{b}
2	0^{b}	0^{c}	0^{b}
4	68.75^{a}	0.26^{a}	6.25^{a}
8	12.5^{b}	0.1^{b}	0.5^{b}

† In each column means with the same letters are not significantly different at $p \leq 0.01$, according to LSD test.

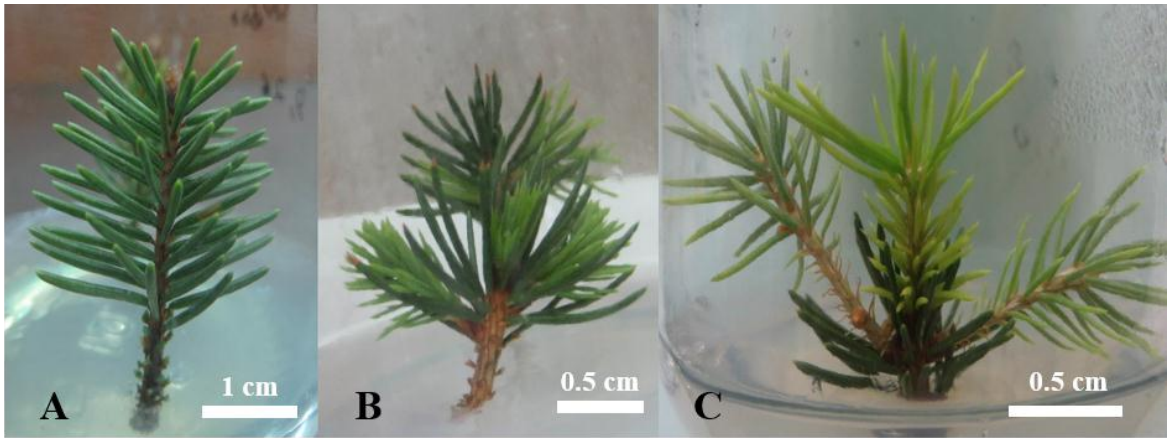

Fig. 1. Shoot proliferation of *P. abies* micro-cuttings in MS media. Control (A), supplemented with Zeatin (B) and with 2iP (C).

In a report on *P. abies* (L.) H. Karst, shoots-derived embryos showed malformation however the main reason of this phenomenon is still unknown. It is assumed that most probably the high relative humidity of *in vitro* culture caused cuticle damage to the regenerated shoots (Von Arnold, 1982). Reliable progresses have been done by previous studies on adventitious shoots induction from hypocotyl, embryo and needle-like leaves at temperature around 20 ˚C (Von Arnold, 1982; Von Arnold and Eriksson, 1979). In contrast to 20 ˚C, 28 ˚C resulted in a higher proliferation rate in *Cedrus libani* and *C. atlantica* (Piola and Rohr, 1996).

In a previous research, adventitious shoot induction in Norway spruce explants positively responded to Zeatin and kinetin compared to 2iP treatment (Von Arnold, 1982). However in contrast to the report of von Arnold (1982), we observed 2iP as an effective cytokinin in our system. Variation between explant sources in different origins of two experiments is most likely the reason for different responses. Moreover, in consistent with other researches on conifers' micropropagation, using sole cytokinins is enough for adventitious shoot induction (Gómez and Segura, 1995). Our results suggest that transferring of the detached proliferated shoots to half strength MS medium without plant growth regulators induce stem elongation. Full strength MS media and/or in combination with auxin and cytokinin, can cause unfavorable callus induction at the proximal end of micro-cuttings (Von Arnold and Eriksson, 1979).

Our research provides an alternative method for clonal multiplication of mature trees of *P. abies* (L.) H. Karst. The explants proliferate higher and faster than the other seed based conventional propagation methods. The current protocol developed in this research shortened the production time to 4-5 months, compared to 7 months of conventional method of propagation implying that that reforestation program is capable of the producing of the elite trees in shorter time. In addition, we established an effective decontamination method for Norway spruce at spring and fall, because mature vegetative tissues' contaminations restrict the aseptic *in vitro* establishment of this coniferous plant.

Research on adventitious shoots and their rooting competency as well as use of regenerated shoots as a main explants' source would be of great interest and importance for future research on this valuable coniferous species.

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