STUDY REGARDING THE CAPACITY OF REGENERATION AND MULTIPLICATION *IN VITRO* OF DIFFERENT TYPES OF EXPLANTS OF *PICEA ABIES* (L) KARST

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Abstract

The programmes of the tree melioration were not based on what is genetically more efficient but rather on what is genetically possible. Even starting with the '80s, the researchers stated that the classical melioration strategies can exploit only one part of the genetic variability, asserting that the vegetative propagation is a good way to exploit this genetic variability of the woody species, establishing almost 20 advantages for the method of multiplication in vitro. (3) The present paper presents some aspects regarding the capacity of regeneration and multiplication of different types of spruce explants in vitro: meristem, apex, vegetative point, as well as the achievement of callus from mature embryos. This paper has in view the mass of callus, the diameter, colour, consistency and the presence of the meristemoids, as well as, the stages necessary for the acclimatization ex vitro of the spruce plants achieved in vitro.

Key words: *Picea abies* L., multiplication *in vitro*, regeneration, explants: meristem, apex, vegetative point, mature embryo

INTRODUCTION

The classical melioration strategies that were used (e.g. mass selection) with all the remarkable results and with their large-scale implications in the production area, cannot exploit only a part of the genetic variability of the species. On the other hand, the wood consumption recorded a significant increase, on a global and national level, altogether with the needs for a "sustainable forestry" to amplify the social and protective functions of the forest ecosystem (4). The objectives of the tree melioration remained the same but there is a need of "redesigning the melioration strategies" (3) by taking into account mainly the particular biological features of the woody species with the purpose of optimizing the main functions of the forest, in accordance with the demands of this century, especially in accordance with the demographic increase.

Regarding the genetic diversity, the trends for the European Union are (5):

- the extension of the natural regeneration with natural works, using the reproduction material based only on the local proveniences;
- the conservation of the eco-types in the conservation system of the genetic resources.

The vegetative multiplication was seen as being a good way to use the genetic variability of the vegetal species in general and of the woody species in particular. Since the ‘80s Libby, M makes known the advantages of the multiplication and propagation *in vitro*, a period associated with the search for new solutions that were more efficient, adequate to the future demands (7). If the propagation and multiplication *in vitro* have in view the redesign of the melioration strategies, the culture of callus and cells *in vitro* are the basis of the somatic hybridisation

Our research had in the view the regeneration capacity of the spruce species and the differences with respect to the type of spruce explant and the culture medium, in achieving some more rapid propagation techniques and of a callus mass with superior features for the somatic embryogenesis.

MATERIAL AND METHODS

Seeds and explants from two-year old samples have been picked up from a nursery. Plantlets have been obtained from the spruce seeds germinated *in vitro* from which a meristem of about 0.1 – 0.2 mm and an apex of 0.4 mm has been detached. A vegetative point of about 0.5 mm has been detached from the plants in the nursery. Some embryos have been detached from the sterile seeds and they were cultivated on the mediums to obtain the callus.

The period or season of inoculation plays an essential part. The explants from the nursery were inoculated at the beginning of May and the seeds for the germination *in vitro* were inoculated in March, in the periods when the moon phases were increasing, fact that determined as the germination of the spruce seeds and the formation of the plantlets *in vitro* to take place two weeks earlier than the previous experiments when the moon phases were not taken into account. The seeds germinated after about two months and not after four months as in the previous experiments (9).

The *seeds* after the well-known classical sterilization of the vegetal material and of the seeds (1,2,3) were cultivated on some germination mediums by using three basic mediums: Murashige –Skoog (MS), Schenk-Hildebrandt (SH) and Evens (E), with halved micro and macro-elements and with an addition of charcoal 5g/l. The germination percent and the height of the plantlets achieved from the seeds were monitored (table 1).

After the germination of seeds *in vitro*, after two months from the germination, the detachment of the *meristem* and *apex* that was cultivated on the mediums in table 2 was accomplished. The vegetative point sampled from the sprouts in the nursery (of 2-3 years old) was inoculated on the same variants of medium. SH was used as a basic medium, proving to have a superior regeneration capacity in the case of the woody species and even

in the case of the gymnosperms (9).

Table 1

Basic mediums used for the spruce seed germination

Variant	Basic medium	Germinated seeds (%)	Plantlet height (cm)	Root system no/length	Rating
V_1	MS 1/2	50	0,5	-	xx
V_2	MSC 1/2	52	1,0	-	xx
V_3	SH 1/2	75	0,8	-	xxxx
V_4	SHC 1/2	75	1,8	1-2 de 1 cm	xxxx
V_5	E 1/2	28	0,5	-	x
V_6	EC 1/2	32	0,7	-	x

MSC 1/2 = Murashige-Skoog + 5g/l charcoal; SHC1/2 = Schenk- Hildebrandt (1972)+5g/l coal; EC 1/2= Evens + 5g/l coal; Rating: x = poor, xx = satisfactory; xxx= good; xxxx = very good; xxxxx = exceptional

Schenk-Hidebrabdt was further used as a basic medium that proved to be in many experiments for woody species as being the best (6) with different combinations and hormonal balances (table 2). At about 2.5 months from the inoculation *in vitro* some observations have been made regarding the percent of regenerated explants, the number of plantlets/explants, their length, the number and length of the roots.

Table 2

Culture mediums used for the inoculation of the spruce explants

Variant	Explant	Basic medium	Z mg/l	BA mg/l	AIB Mg/l
V_0	Meristem, apex, Vegetative point	SH	-	-	-
V_1		SH	1,0	-	0,5
V_2		SH	2,0	-	0,5
V_3		SH	3,0	-	0,5
V_4		SH	-	1,0	0,5
V_5		SH	-	2,0	0,5
V_6		SH	-	5,0	0,5

SH = Schenk-Hildebrandt(1972) ; Z =zeatine; BA = benziladenine; AIB = indolil butyric acid

The hormonal balances that were used have had certain reasoning. The cytochinines Z and BA proved to be the best for both woody species and gymnosperms, especially in concentrations of 1 – 2 mg/l (10, 11), and among auxines, AIB generated the most complex root system.

For the achievement of mature callus, the embryos detached from the seeds of about maximum 2 years old, after sterilization, were inoculated on the variants in table 3. For these combinations, we took into account the results of the previous experiments, that is: high concentrations of 2,4D (2-4mg/l), moderate concentrations of cytochinine (1-2mg/l) and low concentrations (0,5mg/l) of ANA were used.

Table 3

Culture medium of the mature embryos to achieve the callus

Variant	Basic medium	2,4D mg/l	Z mg/l	BA mg/l	ANA mg/l
V_1	MS	2,0	1,0	-	0,5
V_2	MS	4,0	1,0	-	0,5
V_3	MS	2,0	-	1,0	0,5
V_4	MS	4,0	-	2,0	0,5

MS =Murashige-Skoog; 2,4D = DTT; Z=zeatine; BA = benziladenine; ANA = naphthyl acetic acid

RESULTS AND DISCUSSION

The first observations that were made after 2.5 months but less than 3 months and the aspects had in view after this period from the inoculation are presented in the medium values in table 4.

Table 4

The regeneration capacity of the spruce explants cultivated *in vitro*

Var.	Explant	Regeneration percent (%)	No. of plants	Plant length (cm)	No. of roots	Root length (cm)	% of necrosed explants
V_0	meristem	56	1	**3,0**	1	0,5	44
V_1		50	2	1,5	**10**	2,0	**50**
V_2		58	4	1,0	4	0,8	42
V_3		**70**	**10**	0,8	4	0,5	30
V_4		60	2	1,0	8	**2,4**	40
V_5		65	2	1,2	5	0,5	45
V_6		64	8	1,2	2	0,5	36
V_0	apex	62	1	**3,0**	1	0,2	38
V_1		80	4	**1,2**	**6**	1,0	20
V_2		80	4	0,7	3	0,8	20
V_3		**85**	**14**	0,7	3	0,5	15
V_4		70	4	**1,2**	5	1,2	30
V_5		72	5	0,7	3	0,7	28
V_6		**83**	**12**	0,5	3	0,7	17
V_0	vegetative point	60	1	3,5	2	0,5	40
V_1		65	3	1,0	**15**	0,5	35
V_2		65	3	0,8	12	**0,8**	35
V_3		**80**	**5**	0,8	0,8	0,4	20
V_4		60	3	0,8	**18**	0,4	40
V_5		67	3	1,0	8	0,5	33
V_6		75	**7**	1,0	8	0,5	25

In the case of the spruce *meristem*, the Zeatine determines the highest percent of regeneration and the highest number of plants (V_3). In small doses Z and AIB stimulate the formation of the root system, obtaining

about 10 roots of 2 cm. Looking at table 4, we notice that benziladenine offers inferior results at all the parameters of the meristem, only the root formation is superior at a low concentration (V_4).

The apex makes the best difference for a medium with a high dosage of Z *in vitro* (V_3). The greatest number of plants *in vitro* is also obtained on the mediums with a high dosage of cytochinine. For low doses and supply of AIB we obtain an adequate root system (V_1 and V_4). The vegetative point has a better regenerative capacity in comparison with the spruce apex on the same variants V_3 and V_6 up to 80% of the explants. The number of the plants is of 3-5-7 that of the new plants is of 1.0 – 0.8 cm with a good root system.

2,4D has a major implication in the formation of the callus at spruce, as well as, for all the woody species cultivated *in vitro*. (7). There is the problem of the adequate concentration and combination. From the achieved results, the explants have generated a callus of consistent origin and variable behaviour. The callus is friable with necrosis traces in the case of higher concentrations of Z and BA. The callus release phenols in the medium and after about one month are necrosed in the case of a medium with a high dose of Z.

Table 5

The aspect of callus formed *in vitro* from mature embryos

Var.	Hormones	Regeneration (%)	Callus diameter (cm)	Colour	Consistency	Embrionary Value (%)
V_1	2,4D-1mg/l +Z-1mg/l+ ANA-0,5mg/l	90	1,2	olive green	Hard with embryoids	70
V_2	2,4D-2mg/l +Z-2mg/l+ ANA-0,5mg/l	56	0,8	green with brown traces	Friable, necrose traces, phenols	5
V_3	2,4D-2mg/l +BA-1mg/l+ ANA-0,5mg/l	98	2,5	intense green	Good, hard. good proliferation capacity	88
V_4	2,4D-4mg/l +BA-2mg/l+ ANA-0,5mg/l	72	1,5	light green with some traces of necrosis	Reduced proliferation capacity	42

The evolution of callus is superior having an intense green colour with a rapid proliferation capacity, fact that was proved in the previous experiments on a medium with 1mg/l BA(V_3) (8). The callus was mashed on fresh mediums after about 20 days, and after each mashing, it increases in diameter with about 0.8cm. After a month of subculture, the callus mass is doubled and obvious intergrown globular cones with meristematic cells of intense green colour with white cones, fact that indicates that we have an

embryonary callus, fact revealed by the cytological studies.

Study originality. By a large variety of experimental variants, all the stages of the micro-propagation *in vitro* were accomplished, till the achievement of the rooted new plants, including the stage of autotrophic nutrition, by observing the evolution of the spruce new plants with respect to their development.

CONCLUSIONS

1. The novel element was represented by the *inoculation season* chosen according to the Moon development phases, the results being superior in comparison with the previous experiments when this detail was not taken into account.

2. The best percent of germinated seeds *in vitro* was accomplished on SH ½ of about 75% and on SHC ½ by obtaining the highest new plantlets (of about 1.8 cm) with 1-2 roots of about 1 cm.

3. All the types of explants on the medium V_0 (SHC with 5 g/l of charcoal) have presented a regenerative capacity between 56-60%, obtaining just one high new plantlet with 1-2 roots of 0.5 cm.

4. The *meristem* have presented the regenerative capacity and the greatest number of plants per medium V_3 with the highest concentration of Zeatine, of about 7-%, and about 10 new plantlets, completely organized but with inferior values with respect to the other explants. The cause could be the size of the meristem. It seems that a meristem of 0.1 cm is too small for the woody species.

5. BA, regardless of the concentration in the meristem, reaches a regeneration percent of 60 – 65% with the highest number of new plantlets on V_6. The lowest concentration of BA stimulates the number and length of the roots.

6. The *apex* has a superior evolution with respect to the other tissues and the regeneration percent is of 75-85%. The root system is superior on the mediums with moderate doses.

7. The *vegetative point* has a superior evolution with respect to the meristem but inferior to that of the apex; the regeneration capacity is of 75 – 80% on the variants with high concentration of cytochinine and the root system is the best on the mediums with low doses (15-18 roots/explant).

8. The acclimatisation of the new plants of spruce in vitro was accomplished successfully (48%) on a mixture of perlite with peat (1:1) after about 2 weeks of protection under a glass bell and moderate moistening.

9**.** After the cytological test, the callus on the medium with 2 mg/l - 2,4D + BA – 1mg/l + ANA – 0.5 mg/l ($V_{3)}$ forms some globular pro-

embryoids with a particular structure, containing a mass of meristematic cells.

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