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Studies concerning the shooting capacity of black pine (*Pinus nigra* Arn.) „in vitro" cultures

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Abstract The aim of the study was to establish the best hormonal combinations for in vitro shooting capacity in pine. Four population of black pine were studied, two from Romania, two from Greece. The initiation of in vitro culture was started from mature pine embryos obtained from mature seeds. The base culture medium contained 0.5% Murashige-Skoog added with 10 g/l agar and various hormonal balances. As vegetable hormones were used: cytokines (6-benzylaminopurine (BAP) and kinetin (KIN)), and auxins (indole-3-acetic acid (IAA), indolebutyric acid (IBA) and alpha-naphthalene acid (NAA)) and gereberellins (gereberlic acid (GA3). Between the four studied populations, there are statistically assured differences in terms of shooting capacity. The shooting capacity was present in all populations, the average of shoot number per explant being from 1.25 in the population of Kerasia Evia, up to 5.59 in the population of Ano Polidrosos. The Romanian populations have a higher shooting capacity than the Kerasia Evia population. *In vitro* regeneration can become a way to multiply pine.

Key words

black pine, "in vitro" culture, shooting capacity

Micropropagation in forest species is an activity that can increase the capacity of propagation. Besides obtaining a large number of plants, their quality is superior to those obtained by conventional multiplication.

Different working protocols have been aplied to different pine species. In the *Pinus taeda* L. species, WV5 media supplemented with 44 pM 6-benzylaminopurine (BAP) and 0.05 pM α-naphthaleneacetic acid (NAA) were tested for 14 days, followed by subcultures on medium without growth regulator. On such mediums, were tested different lengths of explants, but also concentrations of BAP. In order to obtain a large number of axillary shoots, are recommended the explants of 1 cm from nodal segments. For the apical shoots, 0.5 cm explants are more favorable. The average number of shoots per explant depends on the genotype, but is influenced by the presence of BAP 2.5 μM. The best rooting rate was obtained when using medium with 2.68 μM NAA and 0.44 μM BAP for nine days [8].

In *Pinus nigra*, somatic embryogenesis was also attempted. Immature zygotic embryos were used to initiate embryogenic tissue. The embryo cultures initiated were maintained on solid DCR culture medium supplemented with 9 μM 2,4-D and 2.2 μM BA. The use of abscisic acid results in the maturation of somatic embryos. It may also contribute to the carbohydrate content or higher concentration of gelling agent in the aging medium. Germination of cotyledonous somatic embryos occurred on a hormone-free medium and ended with seedling regeneration. Were also performed the cryopreservation tests of embryogenic tissue for long-term maintenance. Good results are obtained by slow freezing. Tissue regeneration during the thaw period was good, with mature somatic embryos being produced and subsequently plantlets. The embryogenic tissues can be used in genetic transformation, by biolitic methods or by the Agrobacterium method [16].

In micropropagation at *Pinus tecunumanii* we evaluated the effect of 2 or 4 pM BA (6-benzyl adenine) on in vitro regeneration on three subcultures. For elongation 1.5 g of L-1 activated carbon was used, and for rooting 2.68 pM Naphthalene-acetic acid and 0.44 pM BA. After the third subculture on medium supplemented with 2 μM BA, over 70% of the explants presented new shoots, with an average of 3.7 new shoots /explant [18].

Cytokines are involved in the cellular cycle, determining the cells to divide synchronously, but to high concentrations, as those of 9 μM BA, from the variant V2, could determine unexpanded shoots or high mortality in some genotypes. Individuals of Kerasia Evia population proved to be refractory to tissue culture; this ability is also genetic determined and the reactions are thus different from one genotype to another [4, 7].

The commonly used explants in conifers are constituted by embryos, especially by mature embryos

or cotyledons for the induction of shoots *in vitro* regeneration [12]. The most efficient cytokinin in the induction of adventitious shoots is N6 – Benzylaminopurine (BAP) [14]. The shoots are produced directly on the surface of each cotyledon between 6 and 10 weeks after setting up of the culture and the number of regenerated shoots/cotyledon varies with the species. The highest number of shoots/cotyledon was obtained for Douglas-fir and was 264[12]. High numbers of shoots/cotyledon were obtained in other coniferous species as 180 for radiata pine [1], 50–215 for stonepine (*Pinus pinea*) [11] or 20 for Chilgoza pine (*Pinus gerardiana*) [12].

Gibberellins are also useful in tissue culture, in all the species tested and their contribution to pine cells and tissues proliferation was proved by the increased number of shoots regenerated by all the black pine individuals tested on the hormonal variant V2 that contains 1 μM GA3 [2].

Auxins contribute more to cell division and proliferation of apical dominance, being very important in embryogenesis and shoots formation and together with cytokines help in plants proliferation both *in vivo* (the natural phytohormones produced by the plant itself) and *in vitro* (synthetic or natural phytohormones added in culture media) by stimulating the shoots production and growth [2, 4, 9].

Material and Method

The aim of the study was to establish the best hormonal combinations for *in vitro* shooting capacity in pine.

The biologic material was constituted of blak pine (*Pinus nigra)* population: two from Greece (Ano Polidrosos and Kerasia Evia), and two from Romania (Herculane and Svinita).

For *in vitro* culture initiation, the micromultiplication was started from mature pine embryos derived from mature seeds. We have chosen these types of explants for the reason that they presented the best results [9].

Seed sterilization was done after prolonged washing, immersed in 70% ethanol for 10 seconds, sterilized for 1-2 minutes by immersion in mercuric chloride solution, 4-6 minutes in calcium hypochlorite solution. Post-sterilization was achieved by successive rinses in sterile distilled water.

Basal culture medium was constituted by salts of 0.5% MS [10] added with 10g/l agar and different hormonal balances. For micropropagation of regenerated shoots different types of plant hormones have been used: cytokines (6-benzylaminopurine (BAP) and kinetin (KIN)), and auxins (indole-3-acetic acid (IAA), indolebutyric acid (IBA) and alpha-naphthalene acid (NAA)) and gibberellins (gibberellic acid (GA3)) (table 1).

Table 1

Hormonal balances used for black pine micropropagation, according with literature

Hormonal variants	IAA	IBA	NAA	GA_3	KIN	BAP
V 1	0	0	12	0.1	0	0
V 2	0	2	0	1	3	9
V 3	2	0	0	0.2	0	1

Data obtained from the biometric measurements were statistically processed, determining the estimates of mean, the standard deviation of the mean and coefficient of variationn [3]. To determine the significance of differences between genotypes studied, experimental data processing was performed by analysis of variance and t test for two-way experiences [3].

Rezults and Discussions

The seeds of gymnosperms have a variable number of cotyledons from 2 to 24, forming a spiral on top hypocotyls (embryonic stem) surrounding the plumule. Black pine has 5-9 cotyledons, and the ability of micropropagation, by multiple axillary shooting is determined by this feature [9]. The success of an optimal micropropagation, also depends on genotype and chronological and physiological age of the donor plant [15]. In our case, starting from seeds, the germ cells, even if derived from mature embryos are in juvenile stage, donor age is not so significant, the differences being due to only the donor genotype and its origin.

Refering to the effect of genotype on shoots number/seed in black pine populations (Table 2), amplitude of variation of 3,03 was correlated with a very high variability among (44,40 %) with limits from 1,24 in case of population from Kerasia Evia until 4,27 in population of Ano Polidrosos.

Considering the average values of this character for the four populations on the entire experiment, we noticed that there are statistically insured differences between them. The population Ano Polidrosos achieved a significantly higher number of shoots compared with the other populations with increases ranging from 46.23% as against population from Sviniţa to 244.35% from Kerasia Evia. Also, the Romanian populations Herculane and Sviniţa showed a close proliferation capacity and also a significantly higher number of shoots/ seed comparing with the population Kerasia Evia.

Data presented in the Table 2 emphasize that the average number of shoots regenerated from the two Romanian populations had the highest values in population from Sviniţa (x=2.92), but differences between then are not statistically significant. This could mean the common origin of both populations. It

can be assumed that they arise from an ancestral genotype of *Pinus nigra*, and were separated by distances and differentiated by naturalization in the variety Banatica [13]. After leaving the seeds, cotyledons were passed on different culture media for micropropagation, yielding an average 2.74 shoots per cotyledon (Herculane) and 2.92 shoots per cotyledon (Svinita) and almost a double number of shoots per cotyledon was obtained by the Greek genotypes of Ano Polidrosos (x=4.27). These differences might be due to genotype structure and its ability to regenerate in artificial condition, given that there are genotypes refractory to in vitro cultures [9]. Optimal hormonal balance to regenerate the largest number of shoots per cotyledon (between 4.32 in Herculane population and 8.22 by population from Ano Polidrosos) was set on the culture variant V2, containing 2 μM IBA, 3 μM KIN and 9 μM BA (Table 2).

Table 2

The effect of genotype on shoots number/seed in black pine populations

Population	Average		Relative values (%)	Difference/ Significance
Svinita -Herculane	2.92	2.74	106.57	0.18
Kerasia Evia - Herculane	1.24	2.74	45.26	-1.50^{000}
Ano Polidrosos - Herculane	4.27	2.74	155.84	1.53***
Kerasia Evia - Svinita	1.24	2.92	42.47	-1.68^{000}
Ano Polidrosos - Svinita	4.27	2.92	146.23	1.35***
Ano Polidrosos–Kerasia Evia	4.27	1.24	344.35	3.03***

$LSD_{5\%} = 0.42$; $LSD_{1\%} = 0.64$; $LSD_{0,1\%} = 1.02$.

Taking into account the unilateral effect of hormonal variant, data from Table 3 show that the number of shoots/seed presented a high variability (of 39.83%) among populations with values between 3.86 in V2 to 1.64 V1. Amid this variability is noted that conditions in the V2 version has given rise to a very significant number of shoots superior to the other two hormonal variants, with increases of 34-135%. Using the V3 hormonal variant resulted in an increase in the average number of shoots by 75% compared to V1.

The influence of different hormonal variants on the number of shoots/ seed regenerated by each population (Table 4, Figure 1) presented the highest amplitude of variation (3.02) in Ano Polidrosos population, while in Kerasia Evia population amplitude was significantly reduced (0.48).

Table 3

The effect of variant on shoot number/seed in black pine populations

Variant	Average		Relative values (%)	Difference/ Significance
V2-V1	3.86	1.64	235.37	2.22***
V3-V1	2.88	1.64	175.61	1.24***
V3-V2	2.88	3.86	74.61	-0.98***

$LSD_{5\%} = 0.30$; $LSD_{1\%} = 0.42$; $LSD_{0,1\%} = 0.58$

Table 4

The effect of genotype and variant on shoot number/seed in black pine populations

Population	Variant			$\bar{x} \pm s_{\bar{x}}$	$s_{\%}$
	V1	V2	V3		
Herculane	z1.52b	x4.33b	y2.35c	2.74±0.43	46.76
Svinita	z1.46b	x4.25b	y3.05b	2.92±0.42	42.68
Kerasia Evia	x1.00b	x1.25c	x1.48d	1.24±0.11	27.56
Ano Polidrosos	z2.57a	x5.59a	y4.66a	4.27±0.46	32.61
$\bar{x} \pm s_{\bar{x}}$	1.64±0.18	3.86±0.49	2.88±0.37	2.79±0.26	
$S_{\%}$	38.73	43.93	44.03	55.29	

Populations $LSD_{5\%} = 0.60$; $LSD_{1\%} = 0.82$; $LSD_{0,1\%} = 1.11$

Variants $LSD_{5\%} = 0.61$; $LSD_{1\%} = 0.84$; $LSD_{0,1\%} = 1.15$

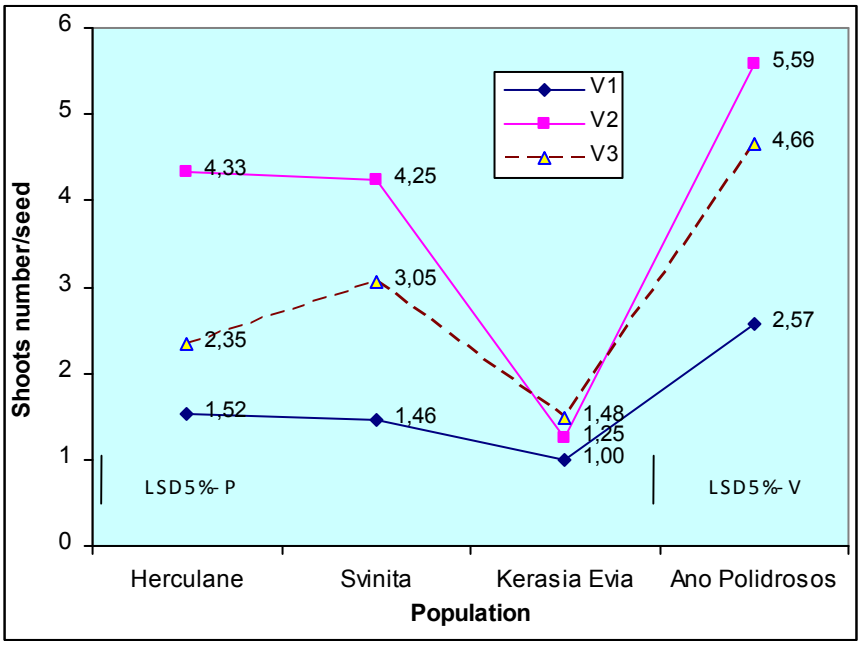

Figure 1 Shoot number/seed in black pine populations on different variants

Mean number of shoots recorded of the four populations in the hormonal variant V1 (Table 5) ranged from 1.00 in Kerasia Evia to 2.57 in Ano Polidrosos, amid a variability within population of 38.73%. Ano Polidrosos population achieved in these culture conditions averages of this character significantly superior to other populations with increases ranging from 69% compared to Herculane and 157% compared to Kerasia Evia. Greek populations and that of Sviniţa raised similar results in shoots proliferation on culture variant V1.

Hormonal variant V2 was optimal for shoots regeneration, all populations giving the best results with values from 1.25 shoots/seed in Kerasia Evia to 5.59 shoots/seed in Ano Polidrosos. Greek populations represented the minimal and the maximal response to the *in vitro* regeneration from seeds and the Romanian populations showed similar responses to artificial conditions of culture. Ano Polidrosos used more efficiently the V2 variant conditions recording significant increases in the number of shoots than on other genotypes, between 29 and 347%.

Number of shoots in the conditions of V3 hormonal variant ranged from 1.48 in Kerasia Evia to 4.66 in Ano Polidrosos population, due to a high variability (46.67%). Ano Polidrosos made for this version of proliferation capacity of shoots significantly superior to other populations, with increases ranging from 53% to Sviniţa population and 315% to Kerasia Evia. Also Sviniţa population has used a significantly higher level of these variant conditions, achieving a higher number of shoots compared to populations Herculane and Kerasia Evia.

The effect of different hormonal variants on the number of shoots / seed for each population showed a high variability of 46.76% and important variation amplitude of 2.81 in population of Herculane(Table 6). The conditions offered by V2 variant had a significantly better effect on the formation of shoots, recording increases ranging from 84% of V3 to 185% of V1. Depending on the hormonal variant used in the culture media, population of Sviniţa recorded a high variability of 42.68%; the number of shoots regenerated per seed varied from 1.46 on the hormonal variant V1 and 4.25 on the variant V2. In these case, depending on the hormonal balance used, they gave similar answers to the individuals of Herculane. In this respect could be noticed that, Sviniţa population showed increases in the number of shoots/seed from 39% higher than V3 and 191% higher than V1.

Population of Ano Polidrosos regenerated the highest number of shoots/seed, the number of cotyledons generated by a species in vivo being directly correlated with the number of shoots regenerated in vitro [9]. Best results were registered on culture hormonal variant V2, with an average of 5.59 shoots/seed, and an production gain of 20% comparing with V3 results and of 117% than V1, minimal values being recorded on V1 hormonal variant. The inner variability was intermediary to the other populations.

In case of Kerasia Evia genotypes, the number of shoots/seed registered values between 1.00 on V1 variant of medium and 1.48 on V3, the highest number of shoots being regenerated on this hormonal variant. It could be observed that Kerasia Evia individuals reacted different than the other populations, best results being registered on a different hormonal variant, the culture variant V3 with equilibrated values both auxins (2µM IAA) and cytokines (1 µM BA) and a minimal concentration of gibberellins (0.2 µM GA3).

Table 5

The effect of genotype on shoot number/seed for each hormonal variant

Population x V1	Average		Relative values (%)	Difference/ Significance
Svinita -Herculane	1.46	1.52	96.05	-0.06
Kerasia Evia - Herculane	1.00	1.52	65.79	-0.52
Ano Polidrosos - Herculane	2.57	1.52	169.08	1.05**
Kerasia Evia - Svinita	1.00	1.46	68.49	-0.46
Ano Polidrosos - Svinita	2.57	1.46	176.03	1.11***
Ano Polidrosos–Kerasia Evia	2.57	1.00	257.00	1.57***
Population x V2	Average		Relative values (%)	Difference/ Significance
Svinita -Herculane	4.25	4.33	98.15	-0.08
Kerasia Evia - Herculane	1.25	4.33	28.87	-3.08^{000}
Ano Polidrosos - Herculane	5.59	4.33	129.10	1.26***
Kerasia Evia - Svinita	1.25	4.25	29.41	-3.00^{000}
Ano Polidrosos - Svinita	5.59	4.25	131.53	1.34***
Ano Polidrosos–Kerasia Evia	5.59	1.25	447.20	4.34***
Population x V3	Average		Relative values (%)	Difference/ Significance
Svinita -Herculane	3.05	2.35	129.79	0.70*
Kerasia Evia - Herculane	1.48	2.35	62.98	-0.87^{0}
Ano Polidrosos - Herculane	4.66	2.35	198.30	2.31***
Kerasia Evia - Svinita	1.48	3.05	48.52	-1.57^{000}
Ano Polidrosos - Svinita	4.66	3.05	152.79	1.61***
Ano Polidrosos–Kerasia Evia	4.66	1.48	314.86	3.18***

$LSD_{5\%} = 0.60$; $LSD_{1\%} = 0.82$; $LSD_{0,1\%} = 1.11$

Table 6

The effect of variant on shoot number/seed for each genotype

Variant x Herculane	Average		Relative values (%)	Difference/ Significance
V2-V1	4.33	1.52	284.87	2.81***
V3-V1	2.35	1.52	154.61	0.83*
V3-V2	2.35	4.33	54.27	-1.98^{000}
Variant x Svinita	Average		Relative values (%)	Difference/ Significance
V2-V1	4.25	1.46	291.10	2.79***
V3-V1	3.05	1.46	208.90	1.59***
V3-V2	3.05	4.25	71.76	-1.20^{000}
Variant x Kerasia Evia	Average		Relative values (%)	Difference/ Significance
V2-V1	1.25	1.00	125.00	0.25
V3-V1	1.48	1.00	148.00	0.48
V3-V2	1.48	1.25	118.40	0.23
Variant x Ano Polidrosos	Average		Relative values (%)	Difference/ Significance
V2-V1	5.59	2.57	217.51	3.02***
V3-V1	4.66	2.57	181.32	2.09***
V3-V2	4.66	5.59	83.36	-0.93^{00}

$LSD_{5\%} = 0.61$; $LSD_{1\%} = 0.84$; $LSD_{0,1\%} = 1.15$

It seems that this mix of cytokines, involved in the stimulation of bud organogenesis from immature embryos by multiple axillary shooting, and determination of a greater number of shoots, with auxins that have a role in differentiation and cell proliferation, was the best solution for micropropagation of black pine. The lowest number of shoots was regenerated on hormone on variant V1, which has additional components constituted of gibberellic acid (GA_3 0.1 μM) and naphthalene acetic acid (NAA 12 μM) [17].

Germinating cotyledons from mature embryos were directly obtained when excised from seeds before inoculation and placed horizontally on ½ DCR medium, which were added various concentrations of phytohormones (2 or 10 μM BAP plus 0, 1 μM NAA) [5]. For shoot elongation of *Pinus armandii* Franchi var. *amamiana* obtained from culture of cotyledons, cotyledons were transferred on the same type of medium added with charcoal and a hormonal supplement (IAA) for root formation [6].

Conclusions

Between the four populations studied there are statistically assured differences in terms of shooting

capacity. The population of Ano Polidrosos produced a significantly higher number of shoots compared to the other populations, with increases between 46.23% compared to the population of Svinița and up to 244.35% compared to the population of Kerasia Evia. The Romanian populations of Herculane and Svinița showed a more limited proliferation capacity, but a significantly higher number of shoots / seeds compared to the population of Kerasia Evia.

The average number of regenerated shoots in the Romanian populations had the highest values in the population of Svinița (2.92 shoots), but the differences between the two populations are not statistically significant. This could mean the common origin of both populations. The optimal hormonal balance for the regeneration of the largest number of shoots per cotyledon (between 4.32 in the Herculane population and 8.22 in the population of Ano Polidrosos) was established on the culture variant containing 2 µM indolebutyric acid, 3 µM kinetin and 9 µM 6-benzylaminopurine. This hormonal variant was optimal for the regeneration of shoots, all populations offering the best results with values from 1.25 shoots / seeds in the population of Kerasia Evia, up to 5.59 shoots / seeds in the population of Ano Polidrosos.

In vitro regeneration can become a way to multiply pine.

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