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INITIATION OF *IN VITRO* CULTURES OF *PINUS SYLVESTRIS* L.

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Abstract. The aim of this study was to determine the effects of medium composition and the amount of plant growth regulators essential for shoot initiation in the mature seeds culture of *Pinus sylvestris* L. For seed germination the following media were used: White's medium, Murashige and Skoog's medium [MS], a half of MS nutrients, B5 [according to Gamborg et al. 1968], Tisserat's medium [cited in Bhojwani and Razdan 1990] and DCR [Gupta i Durzan 1985] marked by the symbols A-N. The above mentioned media combinations differed in the amount of cytokinin BAP (0.25-4.0 mg·dm^{-3}) and tidiazuron – TDZ (0.05-1.0 mg·dm^{-3}) and auxin IBA (0.5 mg·dm^{-3}). Pine shoots on DCR medium without growth regulators were the control sample. It was found that for *Pine sylvestris*, the Tisserat's medium [cited in Bhojwani and Razdan 1990] without growth regulators is the most useful medium for shoots initiation during a germination test.
Abbreviations: **BAP** – 6 benzylaminopurine, **IBA** – 3-indolylbutyric acid, **MS** – Murashige and Skoog's medium, **NAA** – 1-naphtaleneacetic acid, **TDZ** – thidiazuron [1--phenyl-3-(1,2,3-thiadiazol-5-yl)]urea, **DCR medium** – Douglas for cotyledon revised medium.

Key words: *Pinus sylvestris*, *in vitro* culture, mature seeds

INTRODUCTION

According to Partanen-Hertell et al. [1999] the Baltic Sea is the dirtiest sea in the world. That is why the degradation of the Baltic Sea and its coastal biotope has recently become a popular area of academic research in European countries. It has been registered that the area of the occurrence of certain species is being reduced and some species are even disappearing from the quality structure of coastal ecosystems [Chojnacki and Bracki 1999]. In order to preserve this area in the present state and prevent from further degradation "the protected areas of the Baltic Sea" were established including coastal sand dunes covered with *Pinus sylvestris*.

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As a contribution to the above mentioned activities research was undertaken aiming at determining pine diversity growing on the dunes of western coast and selecting the genotypes tolerant to pollutants and periodical flooding with the salty Baltic Sea water. This kind of research may be possible using *in vitro* culture and DNA analysis [Goto et al. 1998, Tang et al. 2001]. The present paper is an introduction to the above specified research programme. The aim of the study was to develop the methods of initiation of *in vitro* cultures for the needs of future selection and genetic studies of *Pinus sylvestris* genotypes with tolerance to salt and chemical pollution of the Baltic Sea water.

Table 1. Media composition initiating pine seed germination
Tabela 1. Skład pożywek zastosowanych do inicjacji kultur

Symbol	Growth regulators Regulatory wzrostu $mg \cdot dm^{-3}$	Macro- and microelemnts according to: Makro- i mikroelementy według:	Organic elements Składniki organiczne $mg \cdot dm^{-3}$	
a	–	White'a	thiamine	0.01
b	TDZ 0.05	[Bhojwani and Razdan 1990]	pyridoxine	0.01
c	BAP 0.25		niacin	0.05
			glicyne	3.00
			sucrose	30 000.00
d	–	MS	inozytol	100.00
e	BAP 0.25		thiamine	0.10
f	BAP 4.00		pyridoxine	0.50
g	BAP 4.00 + IBA 0.50		niacin	0.50
H	TDZ 0.05		glicyne	2.00
			sucrose	30 000.00
I	–	1/2 MS	inozytol	50.00
J	TDZ 0.05		thiamine	0.05
K	BAP 0.25		pyridoxine	0.25
			niacin	0.25
			glicyne	1.00
			sucrose	15 000.00
L	–	B_5	inozytol	100.00
Ł	TDZ 0.05		thiamine	10.00
M	BAP 0.25		pyridoxine	1.00
			niacin	1.00
			sucrose	30 000.00
N	–	Tisserat	thiamine	0.40
			glicyne	2.00
			sucrose	30 000.00
			activatedcarbon	3 000.00
O	–	DCR	inozytol	100.00
P	BAP 0.50		thiamine	0.10
R	BAP 2.00		pyridoxine	0.50
S	TDZ 0.50		niacin	0.50
T	TDZ 1.00		glicyne	2.00
			sucrose	30 000.00

MATERIAL AND METHODS

Mature seeds of *Pinus sylvestris* L. harvested in Międzyzdroje in the winter 2000 were used in this study. Primary explants were prepared in the following way. 4000 pine seeds were disinfected in 0.5% sulphuric acid (15 min) and after washing three times in 7% sodium hypochlorite. Next seeds were washed again with sterile water and left in it for 24 hours. After that seeds were disinfected again in 10% (2 min) and washed with sterile water under aseptic conditions. Prepared in this way seeds were transferred to a pre-sterilized petri-dishes (10 seeds on one petri-dish), containing 20 ml of various media (Table 1). Cultures were maintained for 4 weeks at 20±1ºC temperature under a 16 h photo period with cool white fluorescent light 40 PAR ($\mu Em^{-2} \cdot s^{-1}$). Each experimental combination (media), completely randomized, was represented by 200 pine seeds.

After four weeks of culture the obtained pine plants were evaluated. Their morphological traits were analyzed by analysis of variance ("version 1.0" of IUNG, Puławy). The significance of differences was determined using Tukey test at $\alpha = 0.05$.

RESULTS

In general, the medium composition had a significant influence on germination ability of pine seeds *in vitro* conditions (Fig. 1), which ranged from 0-36%. The greatest amount of germinated seeds in a sample (36%) was observed on Tisserat's medium supplemented with activated carbon (medium N; Fig. 1, Phot. 1). The second group of the media named A, B, C and E (Table 1, Fig. 1) and the third one included the media

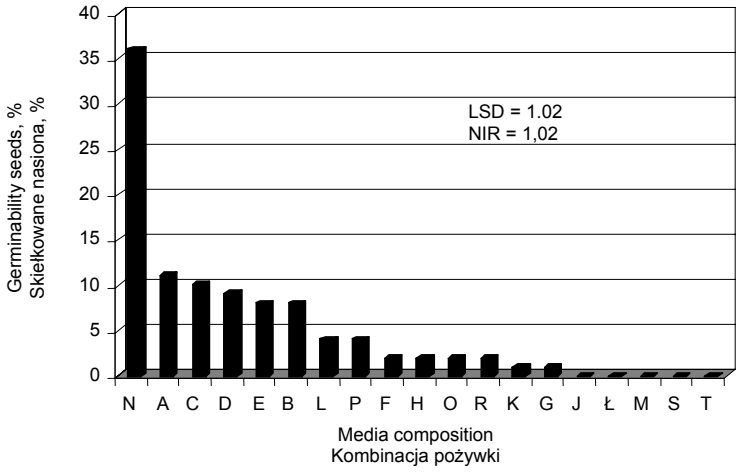

Fig. 1. Percentage of germinability pine seeds depending on media combination
Rys. 1. Zdolność kiełkowania nasion sosny pospolitej w zależności od zastosowanej pożywki wyrażona w procentach

Phot. 1. Germinating pine seeds on initiation medium N (photo by D. Kulpa)
Fot. 1. Nasiona sosny kiełkujące na pożywce N (fot. D. Kulpa)

H, O, R and I and K with germination ability 1-2%. The lack of shoots initiation was found on the media G, J, Ł and S. There was a clear relationship between the length of time necessary for pine seed germination and the medium composition (Fig. 2). The earliest, after 5 days, germination was observed on medium C and the latest on E.

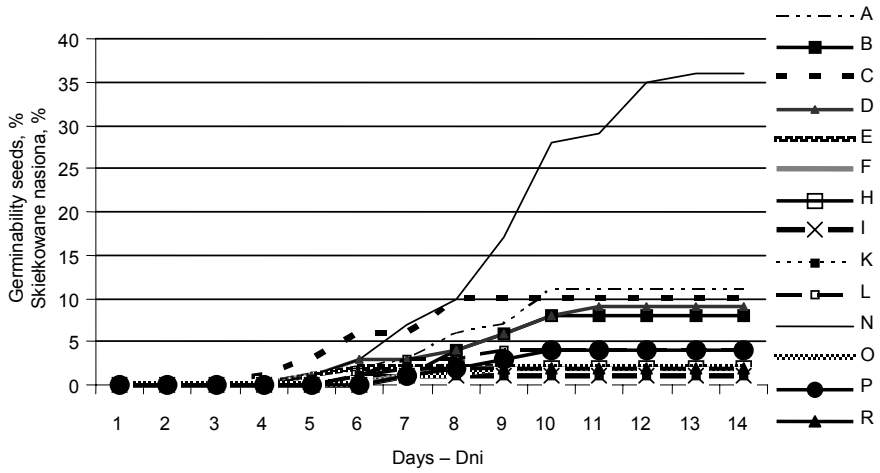

Fig. 2. Percentage of germinability pine seeds up to first 14 days, depending on media combination
Rys. 2. Procent skiełkowanych nasion w terminie do 14 dni od nastawienia na kiełkowanie w poszczególnych kombinacjach pożywek

In general, no influence of medium composition was noted on the explants infection of *P. sylvestris*. The greatest number of infected seeds was observed on G, F and T media and the smallest – on the medium named N.

DISCUSSION

In the literature some reports may be found on propagation of the following pine species *Pinus wallichiana* A.A. Jacks, [Mathur and Nadgauda 1999], *P. eldarica* Med. [Gladfelter and Philips 1987], *P. sylvestris* L., *P. niore* Arn., *P. strobus* Sweet [Bara 1990]. These reports showed that pine belongs to tree plants which are difficult to be regenerated *in vitro* conditions and successful culture depends both on the primary explants and the choice of appropriate medium composition [Perez-Bermudes and Sommer 1987, McKellar et al. 1994, Pulido et al. 1994]. Different explants were used: pine seeds [Chang et al. 1991, Harry and Thorpe 1994] mature and immature germs [Gracia-
-Ferriz et al. 1994] megagametophytes and buds [Bara 1990]. However, BAP and TDZ were found to be especially useful for stimulating organogenesis in pine [Gonzalez et al. 1998, Salajowa et al. 1999].

The study results presented in this paper support the opinion of the above mentioned authors concerning the difficulty in pine *in vitro* culture initiation and propagation. In the studies on *P. sylvestris* the initial material *for in vitro* culture consisted of 4000 mature seeds, six media varying in the amount of mineral and organic elements, the content of BAP, TDZ and auxin IBA. Only 100 seeds were initiated and the obtained plantlets that survived propagation stage made up nearly 38% of the whole population.

Mathur and Nadgauda [1999] examined the germination ability of *P. wallichiana* (A.B. Jacks), using two kinds of media: WPM [Lloyd and McCown 1980] for propagation of forest plants described by Lloyd and Mc Cown [1980] and DCR medium [Gupta and Durzan 1985] with 0.5 $mg \cdot dm^{-3}$ BAP. The latter one proved to be the most suitable for germination initiation in the case of the pine they studied.

Among the media combinations for *P. sylvestris* L. applied to seeds germination in our studies Tisserat's medium [cited in Bhojwani and Razdan 1990] with activated carbon, without growth regulators was considered to be the most optimal one for pine *in vitro* culture, whereas only 4% seeds were initiated on DCR with 0.5 $mg \cdot dm^{-3}$ BAP.

Positive effect of activated carbon on initiating pine seed germination [*P. elliotti* and *P. ayacahuitie*] was recognized in earlier studies by Burns et al. [1991] and Saborio et al. [1997] – in *P. ayacahuitie* studies. According to Fridborg et al. [1978] it probably results from absorbing phytohormones and phenol substances decreasing morphogenesis by activated carbon.

The results of the quoted authors and our own prove that plant genotype has undoubtedly the greatest effect on the initiation and course of *in vitro* culture, not only in the case of pine [Garin et al. 1998, Tang et al. 2001].

CONCLUSIONS

1. The seeds of *P. sylvestris* L. are sufficiently good initial material for *in vitro* culture for this species. The culture success depends on the basal medium composition, the type of growth regulators and their proportion in the media.

2. The studies show that Tisserat's medium without growth regulators may be used for shoot initiation during germination test and DCR medium without growth regulators – for the propagation of juvenile plants of *P. sylvestris* L.

3. The cytokinin – TDZ was found to be the least favourable growth regulator for initiating seed germination in pine culture.

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INICJACJA KULTUR *IN VITRO* SOSNY ZWYCZAJNEJ

Streszczenie. Celem badań było określenie wpływu składu pożywki i ilości roślinnych regulatorów wzrostu niezbędnych do inicjacji kiełkowania dojrzałych nasion *Pinus sylvestris* L. i namnażania w kulturach *in vitro*. Do inicjacji kiełkowania zastosowano pożywki: White'a, MS, połowę składników pokarmowych MS, B_5 (według Gamborga), Tisserata oraz DCR. Wymienione kombinacje pożywek różniły się ilością cytokinin – BAP (0,25-
-4,0 mg·dm^{-3}) i tidiazuronu – TDZ (0,05-1,0 mg·dm^{-3}) oraz auksyny – IBA (0,5 mg·dm^{-3}). Namnażanie zainicjonowanych do wzrostu siewek sosny prowadzono na pożywce DCR z różną ilością BAP (0,5 i 2 mg·dm^{-3}) i TDZ (0,5 i 1 mg·dm^{-3}). Próbą kontrolną były siewki sosny z pożywki DCR bez regulatorów wzrostu. Na podstawie otrzymanych wyników badań stwierdzono, że *P. sylvestris*, podobnie jak i inne gatunki sosny, jest obiektem trudnym do prowadzenia w kulturach *in vitro*. Najbardziej przydatną do inicjacji kiełkowania nasion sosny okazała się pożywka opracowana przez Tisserata bez regulatorów wzrostu.

Słowa kluczowe: *Pinus sylvestris*, kultury *in vitro*, dojrzałe nasiona, organogeneza, regulatory wzrostu

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