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MICROPROPAGATION OF OAKS FROM SEEDLING FRAGMENTS

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Abstract. Studies were carried out to work out a protocol for *in vitro* propagation of the Krotoszyn oak tree using fragments of 3-month old seedlings. The focus of the study was to select the best medium and the concentration of growth hormones for oak multiplication. The highest average multiplication rates were observed on A2, WPM and Pin media supplemented with 2.0-3.0 $mg \cdot dm^{-3}$ BA + 0.01 $mg \cdot dm^{-3}$ NAA.

Key words: *Quercus*, oak, propagation, *in vitro*

INTRODUCTION

Generative multiplication of long-lived forest trees, such as oaks, is very difficult due to the long period required to achieve their physiological maturity and irregular occurrence of seed years. The above-mentioned problems encourage scientists to look for vegetative methods of oak propagation which would allow obtaining progeny from plus trees. Traditional methods of vegetative multiplication, such as grafting and shoot rooting, are very difficult and, moreover, require huge quantities of maternal material. An alternative to these methods is micropropagation which allows production of large numbers of identical plants in a short period of time.

The purpose of this study was to investigate which medium and hormonal level can guarantee successful *in vitro* cultivation when initial explants are fragments of seedlings.

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MATERIAL AND METHODS

The plant material used for the experiments were 3-month old fragments of Krotoszyn oak seedlings (WDN 90f) obtained by sowing acorns, earlier subjected to thermo therapy, then growing in a culturing chamber with a 16 hour photoperiod: day – 16 hours (Philips – 60 μM/m^2s) and night – 8 hour and temperatures: 25°C during the day and 19°C at night. Fragments of seedlings were about 1-1.5 cm long and had at least one lateral bud.

The following media were used in the experiments:

- WPM (Woody Plant Medium – Lloyd and McCown 1981),
- A2, A3, S4 – authors' own modification of Anderson medium (1980) (Table 1)
- Pin (Pinus) – authors' own modification of ½ QL (Quoirin and Lepoivre 1977) (Table 2)

The employed media were supplemented with:

– 6 g glucose,
– 7 g agar,
– 0.5 ml/l PPM (Plant Preservative Mixture),

Table 1. Comparison of medium Anderson (1980) and media usage in experiments
Tabela 1. Porównanie pożywki Anderson (1980) ze stosowanymi w doświadczeniach

Components Składniki	Media – Pożywki			
	Anderson, mg/l	S4+*, mg/l	A2+*, mg/l	A3+*, mg/l
$CaCl_2 \times 2H_2O$	332.02	332.02	332.02	332.02
KNO_3	480.00	400	500	500
$MgSO_4 \times 7H_2O$	370.60	100	200	250
NaH_2PO_4	330.60	330.60	330.60	330.60
NH_4NO_3	400.00	300	300	400
$CoCl_2 \times 6H_2O$	0.025	0.020	0.020	0.020
$CuSO_4 \times 5H_2O$	0.025	0.050	0.050	0.050
FeNaEDTA	73.40	–	–	–
FeEDDHA	–	17	20	25
H_3BO_3	6.20	3.00	3.00	3.00
KI	0.30	0.50	0.50	0.50
$MnSO_4 \times H_2O$	16.90	16.90	16.90	16.90
$Na_2MoO_4 \times 2H_2O$	0.25	0.10	0.10	0.10
$ZnSO_4 \times 7H_2O$	8.60	–	–	–
Zn Na_2EDTA	–	2.00	2.00	2.00
Adenine hemisulfate	80.00	15.00	20.00	20.00
Myo-inositol	100.00	25	50	50
Thiamine HCL	0.40	0.50	0.50	0.50
Ala-Gln	–	100	100	100
pH	5.4	5.2	5.2	5.2

Table 2. Comparison of medium QL (Quoirin and Lepoivre 1977) as well as medium Pinus
Tabela 2. Skład pożywki QL (Quoirin i Lepoivre 1977) i pożywki Pinus

Components Składniki	Media – Pożywki	
	QL, mg/l	Pinus, mg/l
KNO_3	1800.00	500.00
$Ca(NO_3)\times4H_2O$	833.77	700.00
$MgSO_4\times7H_2O$	175.79	250.00
NH_4NO_3	400.00	400.00
KH_2PO_4	270.00	100.00
FeEDDHA	–	30.00
$CuSO_4\times5H_2O$	0.025	0.05
$MnSO_4\times H_2O$	0.76	7.00
$Na_2MoO_4\times2H_2O$	0.25	0.10
$ZnNa_2EDTA$	–	2.00
H_3BO_3	6.20	3.00
KI	0.08	0.25
$CoCl_2\times6H_2O$	0.025	0.02
$NiCl_2\times6H_2O$	–	0.01
Thiamine HCl B_1-tiamina (HCI)	0.40	1.00
Pantothenic acid B_2-kwas pantotenowy	–	2.00
Pyridoxine-HCl B_6-pirydoksyna	–	1.00
Biotin H-biotyna	–	0.02
Nicotinic acid PP-kwas nikotynowy	–	2.00
Ascorbic acid C-kwas askorbinowy	–	50.00
Glycine Glicyna	–	2.00
Myo-inositol *Myo*-inositol	100.00	50.00
L-Glutamine Glutamina	–	200.00
PEG 6000	–	250.00
Casein hydrolysate Hydrolizat kazeiny	–	400.00
pH	4.9	4.9

Table 3. Composition of features used to evaluation of degree of development of plants
Tabela 3. Zestawienie cech używanych do oceny stopnia rozwoju roślin

Degree of development Ocena stopnia rozwoju	Group of note Grupa ocen	Guilds influencing explant onto opinion, measured after 8 weeks of cultivation Cechy eksplantatu wpływające na ocenę, mierzone po 8 tygodniach hodowli
0	weak słaba	lack of explant development or his death brak rozwoju eksplantatu lub jego śmierć
1		development of explant limited of survivals or producing of one new shoot below 0.5 cm length or swelling of buds rozwój eksplantatu ograniczony do jego przeżycia lub wytworzenia jednego nowego pędu poniżej 0,5 cm długości lub nabrzmienie pąków
2	average średnia	producing one or two new shoots about lengths above 0.5 cm by every explant, at least 1 to 3 leaves, with including necrosis no more than 50% of surface of leaf wytworzenie jednego lub dwóch nowych pędów o długości powyżej 0,5 cm przez każdy eksplantat, przynajmniej 1 do 3 liści, z nekrozą obejmującą najwyżej 50% powierzchni liścia
3		producing three new shoot about lengths above 0.5 cm, orginated at least four new leaves, including necrosis covering no more than 30% of leaf surface wytworzenie do trzech nowych pędów o długości powyżej 0,5 cm każdy, powstanie przynajmniej czterech nowych liści, z nekrozą zajmującą najwyżej 30% powierzchni liścia
4	high wysoka	producing up to five new shoots, every above 0.5 cm long. Rise of at least 5 leaves, including necrosis covering no more than 30% of leaf surface wytworzenie do pięciu nowych pędów, każdy o długości powyżej 0,5 cm. Powstanie przynajmniej 5 liści, z nekrozą zajmującą najwyżej 30% powierzchni liścia
5		producing over five new shoots, every each one over 0.5 cm long. Rise at least 5 leaves, including necrosis covering no more than 10% of leaf surface powstanie powyżej pięciu nowych pędów powyżej 0,5 cm długości oraz powyżej pięciu liści, z nekrozą zajmującą najwyżej 10% powierzchni liścia

– appropriate combinations of plant hormones:
A – (control) – 0.0 mg·dm^{-3}BA + 0.0 mg·dm^{-3} NAA
B – 0.2 mg·dm^{-3}BA + 0.01 mg·dm^{-3} NAA
C – 0.3 mg·dm^{-3} BA + 0.01 mg·dm^{-3} NAA
D – 2 mg·dm^{-3}BA + 0.01 mg·dm^{-3} NAA
E – 3 mg·dm^{-3}BA + 0.01 mg·dm^{-3} NAA

The pH of each medium was maintained at – 5.2; then autoclaved at the temperature of 121°C, for 20 minutes.

Sterilization of plant material:

• preliminary stage

– cutting seedlings into 1-1.5 cm fragments,

– soaking seedling fragments in distilled water supplemented with Tween 20 (1 drop/100 ml) for one hour,

– single rinsing in distilled water,

– washing with 70% ethyl alcohol for 30 seconds,

– single rinsing in distilled water,

• proper sterilisation

– sterilization in 0.2% $HgCl_2$ for 3.5 – 4 minutes,
– threefold rinsing in sterile water.

Explants were placed in jars, one explant in each jar. Experiments using each hormonal treatment were carried out in 6 series, 7 jars in each. Explants were cultured for 8 weeks, passaging them onto fresh medium every 14 days and maintaining the same condition as in the cultivation of seedlings. The survival rate and degree of development was assessed separately for each explant in the 8th week of culturing. For this purpose, a special scoring system was developed which is shown in Table 3.

RESULTS

The performed analyses examined the influence of 5 types of media on the development of 3-month old fragments of oak seedlings. 186 experiments were carried out for each type of medium. Samples which did not grow into pants (lack of explant growth) c ures. The total of 9

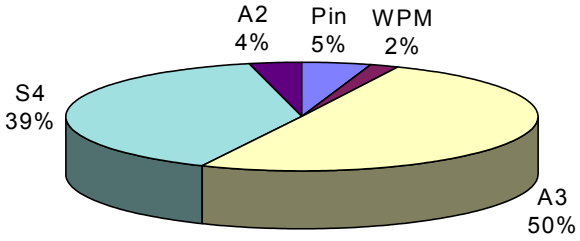

Fig. 1. Proportional part of unsuccessful tests onto individual medium
Rys. 1. Procentowy udział wszystkich nieudanych prób po użyciu poszczególnych pożywek

In the course of experiments, the survival rate of explants on tested media after 4 and 8 weeks of *in vitro* culturing was determined (Fig. 2-6). The highest survival rates after 8 weeks of culturing were recorded in the case of media: A2 and WPM.

Taking into consideration only successful samples, regeneration capacities expressed by the frequency of occurrence of scores from 0 to 5 for each of the examined 5 media with different concentrations of the BA hormone were ascertained. The obtained results are presented in Fig. 7-11.

High initiation performances and, later, culturing were observed on WPM, A2 and Pin media on which failures did not exceed 5%. Poor development, frequently confined only to explant survival, was observed on the above-mentioned media.

The WPM medium was characterized by moderate regeneration capacity and explants often produced 3 new shoots – scores 2 and 3 (Fig. 7). However, many explants did not show regeneration (47% for the A hormonal combination; 46% – for C and 44% – for B). It should be stressed that score "4" occurred rarely (only for variant C and D), while score "5" did not occur at all.

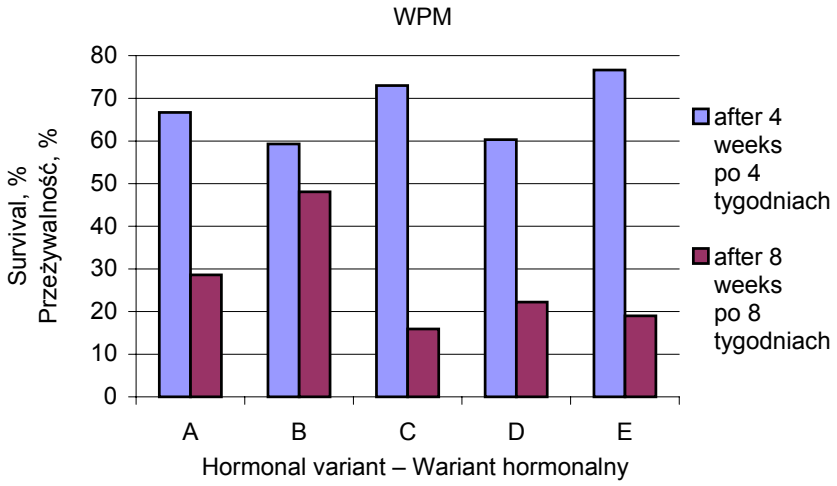

Fig. 2. Survival of explants on WPM on five hormonal variants
Rys. 2. Przeżywalność eksplantatów na pożywce WPM w pięciu wariantach hormonalnych

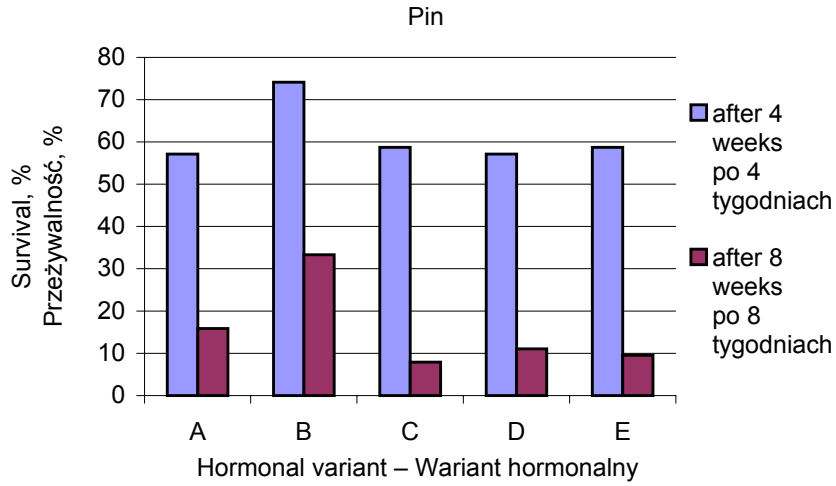

Fig. 3. Survival of explants on Pin medium on five hormonal variants
Rys. 3. Przeżywalność eksplantatów na pożywce Pin w pięciu wariantach hormonalnych

The A2 medium (Fig. 8) supported poorer multiplication than the WPM medium. This medium was also characterized by rare cases of explants dying out – only 5% as well as a frequent development of 3 new shoots on them (score "3") (67% for the control hormonal treatment; 46% for the B combination, 35% – for C and 32% – for D). In the case of the A2 medium, similarly to WPM, a high degree of regeneration, ranging from 0.1 to 0.15%, was assessed at score "4".

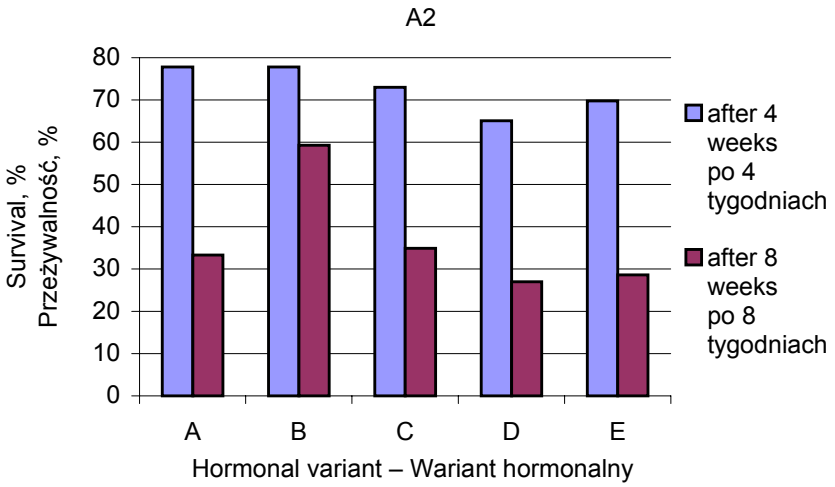

Fig. 4. Survival of explants on A2 medium on five hormonal variants
Rys. 4. Przeżywalność eksplantatów na pożywce A2 w pięciu wariantach hormonalnych

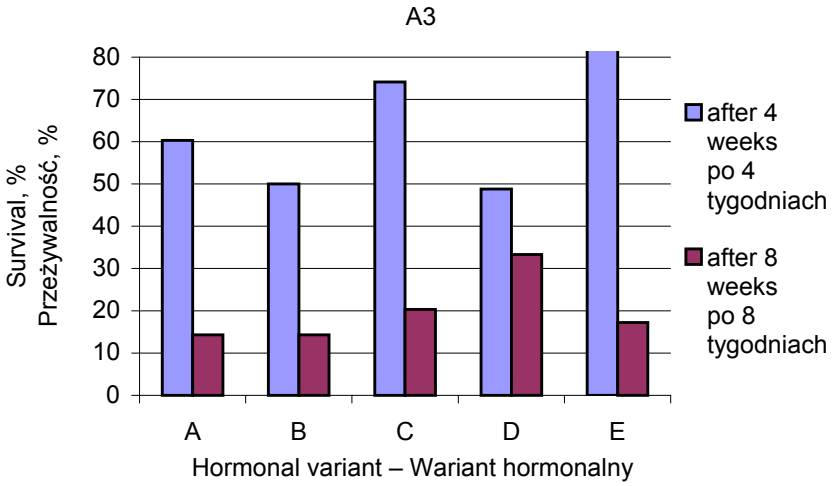

Fig. 5. Survival of explants on A3 mediun on five hormonal variants.
Rys. 5. Przeżywalność eksplantatów na pożywce A3 w pięciu wariantach hormonalnych.

The development of explants on the Pin medium was confined only to the survival for each combination of growth regulators (from 33% – for A to 75% – for E) (Fig. 9). No "4" and "5" scores, referring to strong explant regeneration, were allocated in the case of this medium (Table 4).

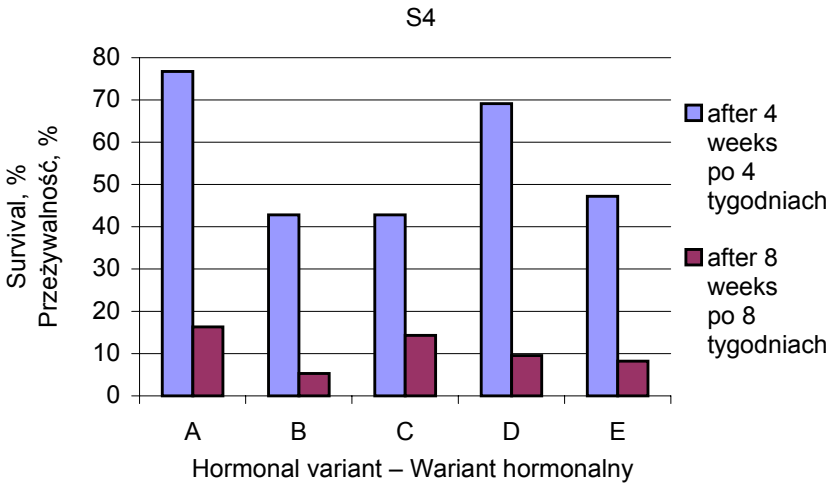

Fig. 6. Survival of explants on S4 medium on five hormonal variants
Rys. 6. Przeżywalność eksplantatów na pożywce S4 w pięciu wariantach hormonalnych.

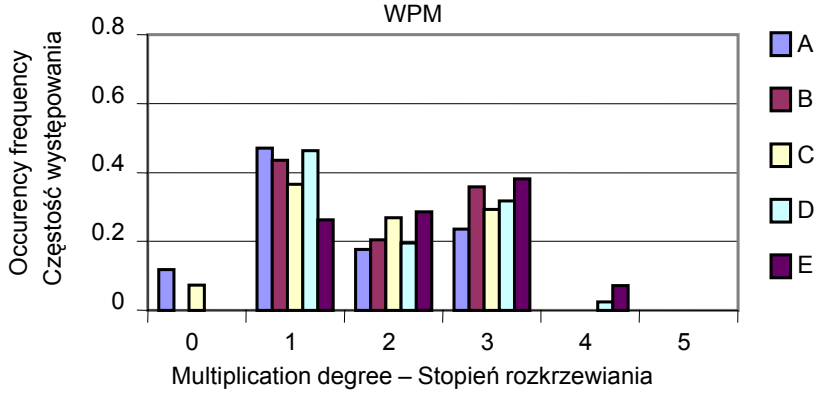

Fig. 7. Degree of multiplication expressed by frequency of occurrence of opinions from 0 to 5 on WPM
Rys. 7. Stopień rozkrzewiania wyrażony częstością występowania ocen od 0 do 5 na pożywce WPM

Both initiation and development were worse on the S4 and A3 media (Fig. 10 and 11). These media were characterized by considerable percentage shares of unsuccessful samples – A3 – 50%, S4 – 39% (Fig. 1).

In cases when the medium did not contain cytokinins and auxins, a significant correlation was found between the score referring to the degree of plant development after 8 weeks of culturing and the kind of medium used (Table 5). Highly significant correlations were also found when 3.0 mg·dm^{-3} BA + 0,01 mg·dm^{-3} NAA were used in the medium.

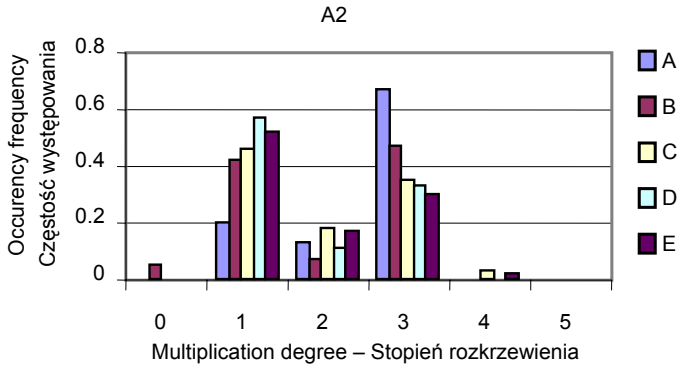

Fig. 8. Degree of multiplication expressed by frequency of occurrence of opinions from 0 to 5 on A2 medium
Rys. 8. Stopień rozkrzewiania wyrażony częstością występowania ocen od 0 do 5 na pożywce A2

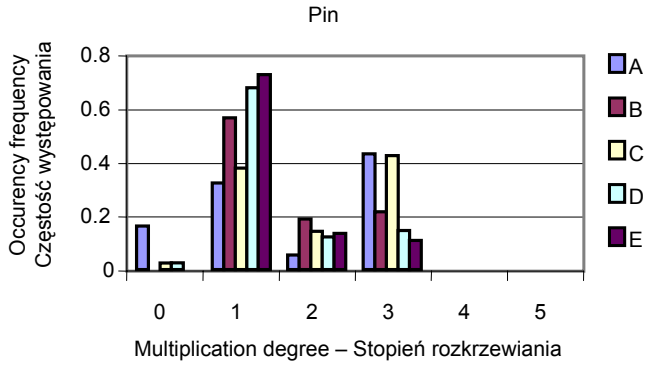

Fig. 9. Degree of multiplication expressed by frequency of occurrence of opinions from 0 to 5 on Pin medium
Rys. 9. Stopień rozkrzewiania wyrażony częstością występowania ocen od 0 do 5 na pożywce Pin

Table 4. Value of statistics χ^2 for test of independence between hormone concentration and degree of development of plant after 8 weeks of cultivating
Tabela 4. Wartość statystyki χ^2 dla testu niezależności między stężeniem hormonów i stopniem rozwoju rośliny po 8 tygodniach hodowli

Medium – Pożywka	Value χ^2 – Wartość χ^2
Pin	12.7*
WPM	12.3 ns
S4	2.3 ns
A2	8.6 ns

* Important result at level $\alpha = 0.05$. ns – result negligible statistically.
* Wynik istotny na poziomie $\alpha = 0{,}05$. ns – wyniki nieistotne statystycznie.

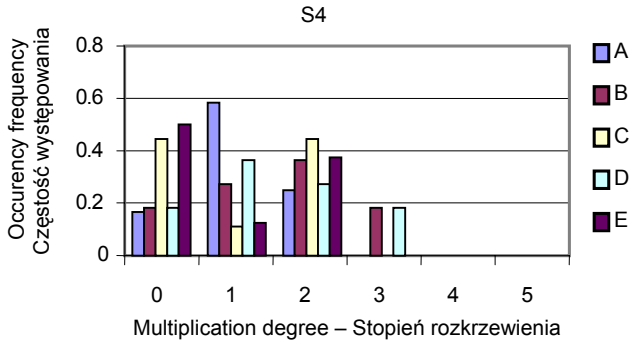

Fig. 10. Degree of multiplication expressed by frequency of occurrence of opinions from 0 to 5 on S4 medium
Rys. 10. Stopień rozkrzewiania wyrażony częstością występowania ocen od 0 do 5 na pożywce S4

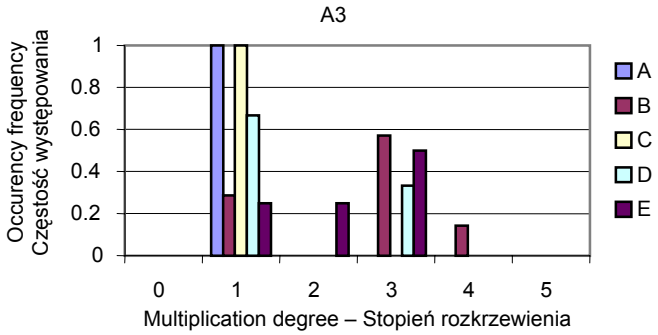

Fig. 11. Degree of multiplication expressed by frequency of occurrence of opinions from 0 to 5 on A3 medium
Rys. 11. Stopień rozkrzewiania wyrażony częstością występowania ocen od 0 do 5 na pożywce A3

Table 5. Value of statistics χ^2 for test of independence between kind of medium and hormonal variant and degree of development plant after 8 weeks of cultivating
Tabela 5. Wartość statystyki χ^2 dla testu niezależności między rodzajem pożywki i wariantem hormonalnym a stopniem rozwoju rośliny po 8 tygodniach hodowli

Medium – Pożywka	Value χ^2 – Wartość χ^2
A	12.7*
B	4.6
C	23.2**
D	6.0 ns
E	3.0 ns

* Important result at level $\alpha = 0.05$. ** Important result at level $\alpha = 0.01$. ns – result negligible statistically.
* Wynik istotny na poziomie $\alpha = 0{,}05$. ** Wynik istotny na poziomie $\alpha = 0{,}01$. ns – wyniki nieistotne statystycznie.

DISCUSSION

The fundamental problem in plant tissue culture is the application of appropriate, for each species and type of explant, culturing medium. The applied media ought to supply explants with indispensable, essential nutrients and create suitable conditions for their regeneration.

From among components used in media, on which explant regeneration occurs, the most important are plant growth regulators. With regard to the experimental combinations with different contents of cytokinins studied in the described investigations, the poorest effect on shoot regeneration was observed when 0.2 $mg \cdot dm^{-3}$ BAP and 0.01 $mg \cdot dm^{-3}$ NAA concentrations were applied.

The strongest regeneration occurred at concentrations 2 and 3.0 $mg \cdot dm^{-3}$ BA (where, in both cases, the content of NAA was 0.01 $mg \cdot dm^{-3}$). As shown by the performed investigations, the best results of explant development after 8 weeks of culturing (from 4-6 passages) were achieved on WPM. This refers to all four tested hormonal treatments (0.2; 0.3; 2; 3 $mg \cdot dm^{-3}$ BAP and 0.01 $mg \cdot dm^{-3}$ NAA). Satisfactory increase development of shoots, at similar cytokinin and auxin concentrations, was reported by Juncker and Favre [1989] as well as by Romano et al. [1992]. In the research on phenomena associated with morphogenesis in *in vitro* cultures, the cooperative effect of auxins with cytokinins is well known, and this effect was also confirmed in our investigations as well as in experiments carried out on, for example, *Quercus alba* and *Quercus rubra* by Schwarz and Schlarbaum [1993]. The highest regeneration indices were recorded for WPM and for the following cytokinin concentrations: 3.0 and 2.0 $mg \cdot dm^{-3}$ BAP + 0.01 $mg \cdot dm^{-3}$ NAA. In these treatments, up to 20 shoots per one explant were obtained. Similar results indicating cytokinin and auxin interactions for *Quercus suber* were reported by Romano et al. [1992]. In addition, they reported a positive influence of higher concentrations of BAP (1-2 $mg \cdot dm^{-3}$) on the development of leaves, whereas lower concentrations led to frequent leaf chlorosis.

In the above-presented experiments, the poorest results of oak regeneration were recorded on S4 and A3 media. The microelement composition in those media was balanced in such a way that the pH value declined with the progress of the culturing process. They were also poor media – the total amount of microelements ($g \cdot dm^{-3}$) constituted about 25% of the complete MS medium.

The investigations carried out so far [Chalupa 1988, Vieitez et al. 1993, Tóth at el. 1994] indicate WPM as the medium which appears most suitable for *in vitro* culturing of trees. Results obtained in our research confirm this view as evidenced by the fact that average lengths of shoots were longer in comparison with those cultured on the remaining experimental media. Similarly good results for this medium were reported in research carried out by Romano et al. [1992], who, additionally, observed a considerably lower number of vitrified tissues in plants growing on WPM, than for example, on the MS medium.

CONCLUSIONS

1. WPM, Pin and A2 media exert a significant influence on the development of new shoots. The remaining tested media failed to show a significant effect on shoot numbers developed after 8 weeks of culturing.

2. A significant effect was observed of the hormonal concentration on the development new shoots on microplants. The hormonal treatment, which guaranteed the highest degree of development was the one which contained 2.0-3.0 $mg \cdot dm^{-3}$ BA + 0.01 $mg \cdot dm^{-3}$ NAA.

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MIKROROZMNAŻANIE DĘBÓW Z FRAGMENTÓW SADZONEK

Streszczenie. Celem pracy było opracowanie metodyki rozmnażania *in vitro* fragmentów 3-miesięcznych sadzonek dębu krotoszyńskiego. W badaniach skoncentrowano się na doborze najkorzystniejszej pożywki hodowlanej oraz układu hormonalnego. Najwyższy stopień rozkrzewiania uzyskano na pożywkach A2, WPM oraz Pin z dodatkiem 2,0-3,0 $mg \cdot dm^{-3}$BA+ 0,01 $mg \cdot dm^{-3}$ NAA.

Słowa kluczowe: *Quercus*, dąb, mikrorozmnażanie, *in vitro*

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