Bulletin of the *Transilvania* University of Braşov
Series II: Forestry • Wood Industry • Agricultural Food Engineering • Vol. 14(63) No. 2 – 2021
https://doi.org/10.31926/but.fwiafe.2021.14.63.2.2

PRESERVATION OF *QUERCUS ROBUR* AND *QUERCUS PETRAEA* GENETIC RESOURCES THROUGH *IN VITRO* CULTURE

Lucia IONIȚĂ[1] Ionel MIRANCEA [1] Ecaterina N. APOSTOL[1]
Marius BUDEANU[2] Emanuel BESLIU[2]

Abstract: *In this paper, the effect of various factors (clone, initial explant, sterilization method, and culture medium) on the in vitro multiplication of pedunculate and sessile oak species was tested. No influence of clone was found and a 44-83% survival rate was found for different types of explants, the best result being that of using mature acorns germinated in vitro. The most efficient sterilization method was: mercuric chloride 0.2 mg/l, ascorbic acid 1 mg/l, for 30 min., with or without antibiotic. The best results were obtained with GD medium complemented with 0.5 mg/l BAP and 0.1 mg/l IBA, which allowed obtaining of a high rooting percentage. The regenerated plants were transferred in vivo, in a climate controlled greenhouse.*

Key words: *afforestation, biotechnology, breeding strategy, ex situ conservation.*

1. Introduction

In vitro somatic micropropagation allows the clonal multiplication of trees and also their breeding through the possibility of superior genotypic trees reproduction, resulting in descendants with the same characteristics. This method allows to obtain seedlings for species with major flaws of seed production, such as the oak species [13], [37]. The main methods for *ex situ* preservation through biotechnology are crioconservation and *in vitro* storage, according to which it is possible to preserve

[1] National Institute for Research and Development in Forestry "Marin Drăcea", 128 Eroilor Blvd., 077190, Voluntari, Ilfov county, Romania;
[2] National Institute for Research and Development in Forestry "Marin Drăcea", Brașov Research Station, 13 Cloșca street, 500040, Brașov, Romania;
Correspondence: Marius Budeanu; email: marius.budeanu@icas.ro.

meristems, callus, and cells suspensions [15-16].

In vitro obtained plants should be truthful copies of the trees from which they derive, but it was observed that some variation appeared (morphological, physiological, etc.) and this variability was called ”somaclonal variability” [1]. This may cancel an important flaw of this regeneration method, namely the reduction of genetic diversity. What remains is only the inconvenience of the high cost in comparison with the classical methods of artificial regeneration. *In vitro* multiplication of oaks through organogenesis or somatic embryogenesis was the subject of numerous studies [7-8], [10], [14], [20], [25], [36]. So far, the used explants were particularly the intermodal fragments or acorn embryos [2], [19], [27-29], [33], [38], [40].

The possibility to obtain seedlings from all parts of a plant, from somatic and genetic cells, enables *in vitro* preservation of genotypes [16]. The use of this method has many benefits: a small space for the storage of a big number of multiplicated clonal plants, the plants are protected against pathogens and viruses in special preservation conditions, the plants do not need special care during storage, and the material has a suitable form for creating nuclear stocks which makes possible a fast propagation of a large number of plants [9].

Ex situ preservation of forest genetic resources through biotechnological methods is a part of the global objective of gene preservation and maintaining of genetic diversity [17]. Preservation of germinative plasma through *ex situ* methods is an efficient method for the species which cannot be multiplicated through vegetative propagation, which have long life cycles and a very slow seed production, for the species with ”doubtful quality of seeds” that die when dehydrated, for individuals with particular qualities, for the propagules harvested outside the seed dissemination season, and for the species which need disease eradication in order to assure good future preservation and multiplication [19].

The newest biotechnologies, i.e., CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats), can modify the genomes of tree species at very precise locations, thus creating the necessary conditions to induce climate change adaptation [3], [39]. But the cultivation of genetically modified plants is not allowed according to the E.U. legislation.

The objective of this research was to establish a working protocol for *in vitro* multiplication of the main oak species, pedunculate oak (*Quercus robur* L.) and sessile oak (*Quercus petraea* (Matt.) Liebl.). The influence of clone, type of plant material, sterilization method, and culture medium composition to the *in vitro* micropropagation were tested. Also, we tested the influence of the same factors on the rooting of the *in vitro* plants, and a protocol for *in vivo* transfer was established.

2. Materials and Methods

The working protocol for *in vitro* somatic micropropagation requires the following steps [18]:

- Identifying the plus-trees inside of the genetically improved populations (forest genetic resources (FGR), seed orchards, seed stands) and collecting the plant materials;
- Identifying the optimal explant and the most suitable sterilization method;
- Establishing the optimal hormonal

balances and the culture medium in order to obtain high percentages of reactivity to *in vitro* culture conditions;

- Identifying the appropriate culture medium for *in vitro* micropropagation and rooting;
- Creating a protocol for *in vivo* transfer.

The research was carried out for the main oak species, namely pedunculate oak (*Quercus robur* L.) and sessile oak (*Quercus petraea* (Matt.) Liebl.). For culture initiation we used 9 clones of pedunculate oak, which were located in four populations, and 9 clones of sessile oak, sampled from only one FGR population. For pedunculate oak, the plant material was harvested from:

- Fundeanu arboretum, located in Galați county (45˚58ˈ north latitude, 27˚42ˈ east longitude, 260 m altitude, a.s.l.), managed by Griviţa forest district and located in the management unit III, compartments 18, 19A, B, C, 20, and 21);
- Mândrești seed orchard, located in Galați county (45˚53ˈ north latitude, 27˚41ˈ east longitude, 170 m altitude), managed by Tecuci forest district and located in the management unit V, compartments 104 A, B, code: PS-ST-GL85 [30];
- Câmpina seed stand, located in Prahova county (45˚10ˈ north latitude, 25˚45ˈ east longitude, 630 m altitude), managed by Câmpina forest district and located in the management unit II, compartments 50E, J, code: GO-B250-5 [30];
- A tree located in the National Institute for Research and Development in Forestry (henceforth INCDS) "Marin Drăcea" park (44˚30ˈ north latitude, 26˚11ˈ east longitude, 70 m altitude).

The following pedunculate oak clones were used:

1. Clone 240, tree 299, Mândrești seed orchard;
2. Clone 178, tree 180, Mândrești seed orchard;
3. Clone 51, Fundeanu;
4. Clone 175, tree 181, Mândrești seed orchard;
5. Clone 258, tree 296, Mândrești seed orchard;
6. Plus-tree from Câmpina seed stand;
7. Plus-tree from NIRDF "Marin Drăcea" park;
8. Clone 267, Fundeanu arboretum;
9. Clone 158, tree 296, Mândrești seed orchard.

The plant material for sessile oak was harvested from genetic resources located in a forest from Brașov county (45˚45ˈ north latitude, 25˚25ˈ east longitude, 590 m altitude), managed by Codrii Cetăţilor forest district and located in the management unit II, compartment 26, code: RG-GO/FR, FA, ST-B150-5 [31]. Nine plus-trees were selected, located at a minimum distance of 50 m among each other, from which acorns were collected. After the initial evaluations, it was found that the clone factor did not show a significant effect, so investigations continued by considering the whole material as belonging to a single clone (Codrii Cetăților sessile oak provenance).

For the initiation of *in vitro* cultures of pedunculate oak, three types of plant material were tested: nodal segments from acorns germinated *in vivo* and immature and mature acorns germinated *in vitro*. For sessile oak, we used only mature acorns germinated *in vivo* and *in vitro*.

For *in vivo* germination of acorns we used a mixture of sand and soil, the germination taking place in the growing chamber, in controlled environmental conditions. *In vitro* germination was done

on sterile culture media and it also took place in the growing chamber in controlled environmental conditions.

Sterilization method: We tested six sterilization methods for sessile oak and four for pedunculate oak (1, 2, 5, and 6), as follows:

- S1: mercuric chloride 0.2%, ascorbic acid 1 g/l, for 30 min., ethanol 70%, for 1 min.;
- S2: mercuric chloride 0.2%, for 35 min.;
- S3: ethanol 70%, for 1 min., followed by pericarp removal, mercuric chloride 0.2 mg/l, ascorbic acid 1 g/l, for 30 min., ethanol 70%, for 1 min.;
- S4: ethanol 70%, for 1 min., followed by pericarp removal, mercuric chloride 0.2 mg/l, ascorbic acid 1 g/l, fluconazole 150 mg for 30 min., ethanol 70%, for 1 min.;
- S5: mercuric chloride 0.2%, ascorbic acid 1 g/l, fluconazole 150 mg, for 30 min., ethanol 70%, for 1 min.;
- S6: mercuric chloride 0.2%, for 50 min.

In the next step, the explants were washed three times with sterile water. Study was conducted such as the effect of the sterilization method on the survival and reaction capacity for the *in vitro* culture conditions of the initial explants.

Culture medium: For the initiation of the *in vitro* cultures we used GD medium [12] and MS1 medium, which is the Murashige-Skoog medium [26], modified by Chalupa [7], supplemented with different hormonal balances which were auxins and cytokine's; and the effect of medium composition on the initiation of the *in vitro* cultures for this species was tested. All the media contained BAP 0.1-1 mg/l (6-benzylaminopurine), with or without either IBA 0.05-0.1 mg/l (indole-3-butyricacid) and NAA 0.05-0.1 mg/l (α-naphthaleneacetic acid) and in one of the media hydrolysed casein 500 mg/l was added. For sessile oak the WPM medium was also used [24].

The following hormonal balances were used:

a) For pedunculate oak:

GD1: BAP 0.2 mg/l;
GD2: BAP 0.5 mg/l;
GD3: BAP 1 mg/l, IBA 0.05 mg/l, NAA 0.05 mg/l;
GD4: BAP 0.8 mg/l, IBA 0.05 mg/l, NAA 0.05 mg/l;
GD5: BAP 0.4 mg/l, IBA 0.05 mg/l.

b) For sessile oak:

GD1: BAP 0.1 mg/l, NAA 0.01 mg/l; GD2: BAP 0.2 mg/l, IBA 0.1 mg/l; GD3: BAP 0.2 mg/l; GD5: BAP 1 mg/l;
MS14: BAP 0.2 mg/l; MS16: BAP 0.8 mg/l, IBA 0.05 mg/l, hydrolysed casein 500 mg/l;
WPM7: BAP 1 mg/l, IBA 0.05 mg/l, NAA 0.05 mg/l, hydrolysed casein 500 mg/l; WPM8: BAP 0.8 mg/l, IBA 0.05 mg/l, NAA 0.05 mg/l, hydrolysed casein 500 mg/l; WPM9: BAP 0.8 mg/l, IBA 0.05 mg/l, NAA 0.05 mg/l, hydrolysed casein 500 mg/l.

The explants were preserved on this culture medium for 4 to 6 weeks until the first leaves appeared.

Culture condition: the cultures were kept at a temperature of 25°C±1°C, 70% relative humidity, and a 16 h photoperiod, for the entire duration of the experiment.

Culture initiation: tests were done regarding the influence of different factors on culture initiation for this forest species, which were: clone, type of initial explant, sterilization method, and culture medium.

The data were processing using STATISTICA 10.0 software.

3. Results

3.1. *In Vitro* Culture Initiation

For pedunculate oak, the variance analysis (the ANOVA test) indicated that the explants' survival depends significantly on the utilized clone and the explant reactivity depends significantly on the type of explant. The sterilization method and the culture medium did not have a significant influence on the survival and reactivity of the plants. For sessile oak, ANOVA showed that the survival of the explants depends significantly on the type of explant and the sterilization method, while the explants reactivity was not significantly influenced by any of the factors (Table 1).

ANOVA for initiation of pedunculate and sessile oaks in vitro cultures Table 1

Factors			Pedunculate oak		Sessile oak	
			MS	F	MS	F
Clone	Survival	Between groups	15193	2.287*	x	x
		Intra-group	28238		x	x
	Reactivity	Between groups	14128	1.487	x	x
		Intra-group	40368		x	x
Type of explant	Survival	Between groups	2284	1.110	3057	5.603*
		Intra-group	41148		8730	
	Reactivity	Between groups	7766	3.324*	260	0.315
		Intra-group	46731		13178	
Sterilization method	Survival	Between groups	1197	0.369	8411	5.979**
		Intra-group	42234		3376	
	Reactivity	Between groups	9114	2.611	1950	0.407
		Intra-group	45383		11488	
Culture medium	Survival	Between groups	12821	1.047	3938	0.564
		Intra-group	30611		7849	
	Reactivity	Between groups	11636	0.679	7648	1.486
		Intra-group	42861		5790	

MS=mean squares, F=*Fisher* test, significant (*) at $p < 0.05$ and distinctly significant (**) at $p < 0.01$

All types of pedunculate oak explants reacted well to the *in vitro* culture conditions, with the survival percentage ranging between 63 and 83%. As far as the sessile oak is concerned, from the last two chosen explants, only one presented a 70% survival rate, the others presenting only a 44% survival rate (Figure 1).

Although all the types of initial pedunculate oak explants had survived well to the *in vitro* culture conditions, their reactivity was different. The nodal segments from the acorn germinated *in vivo* and the immature acorn germinated *in vitro* reacted better, their reactivity being an average of 44 and 42%, respectively (Figure 1).

For sessile oak, both types of explants reacted well, 61% for the mature acorns germinated *in vitro* and 54% for the nodal segments of the acorns germinated *in vivo,* respectively (Figure 1).

For pedunculate oak, the analysis of the sterilization method showed that the best results for the survival of the initial explants were obtained when sterilization method S1 was used. For this method, even if the survival percent was

only 66%, the reactivity was very good, with a percent of 58%, which is high percentage for oaks (Figure 2).

This method of sterilization was the only one which contained ascorbic acid, so this was essential for obtaining good survival and reactivity rates.

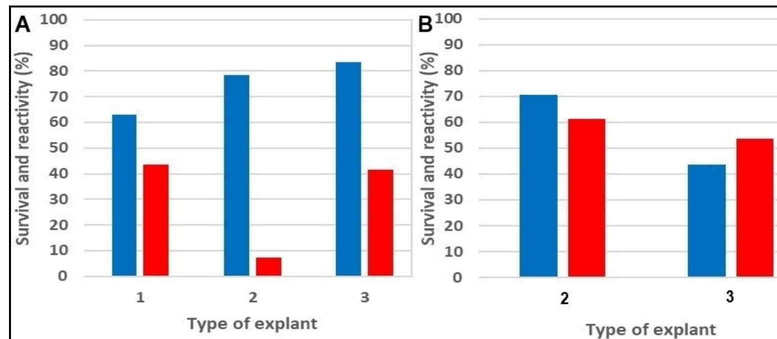

Fig. 1. *The effect of explant type on the survival (blue) and reactivity (red) of the initial explants of pedunculate (A) and sessile oak (B)*

The time of sterilization was of 30 minutes, a longer time leading to the death of the explants.

For sessile oak, the best results for the survival of the initial explants (83%) and their reactivity (75%) were obtained when sterilization method S5 (Figure 2) was used. Good results were also obtained for sterilization methods S4 and S6 (Figure 2) for which we obtained the reactivity percentages of 62.5% and 61%, respectively (Figure 2).

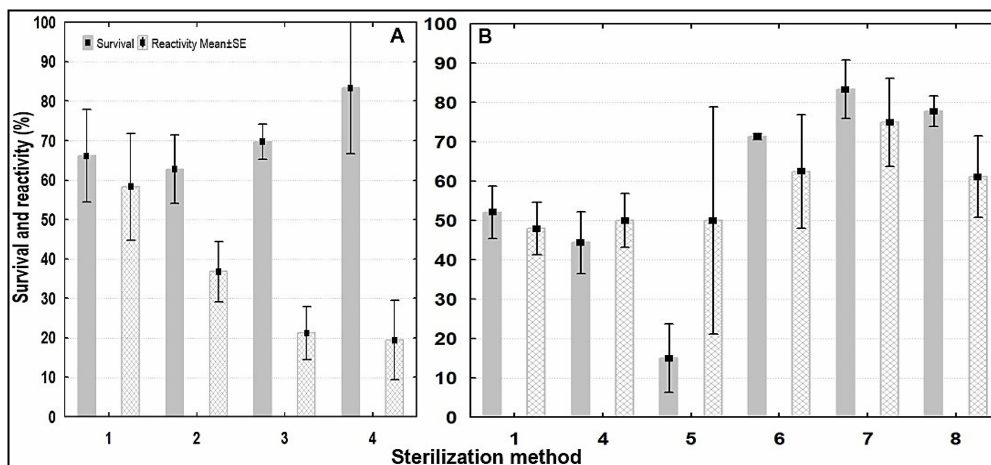

Fig. 2. *The effect of the sterilization method on the survival and reactivity of the initial explants of pedunculate (A) and sessile oak (B)*

In regard to the culture medium, it was observed that GD_3 (3) and MS_1 (10) media gave the best results regarding both the survival and the reactivity of the pedunculate oak plants (Figure 3). If we analyse the hormonal balances, we

observe that both media contained BAP 0.2 mg/l, and adding NAA in the case of the GD_3 medium was not essential for obtaining a high reactivity percentage. For sessile oak, GD_3 (8) and WPM_9 (15) media gave the best results, for which the reactivity percentage was 84% and 83%, respectively (Figure 3). High reactivity percentages were also obtained for the GD_1 (1) medium (75%).

The reactivity of pedunculate oak clones to the *in vitro* culture conditions was very different. Thus, clone 240 (1, in Figure 4) reacted the best, the reactivity percentage being an average of 64%.

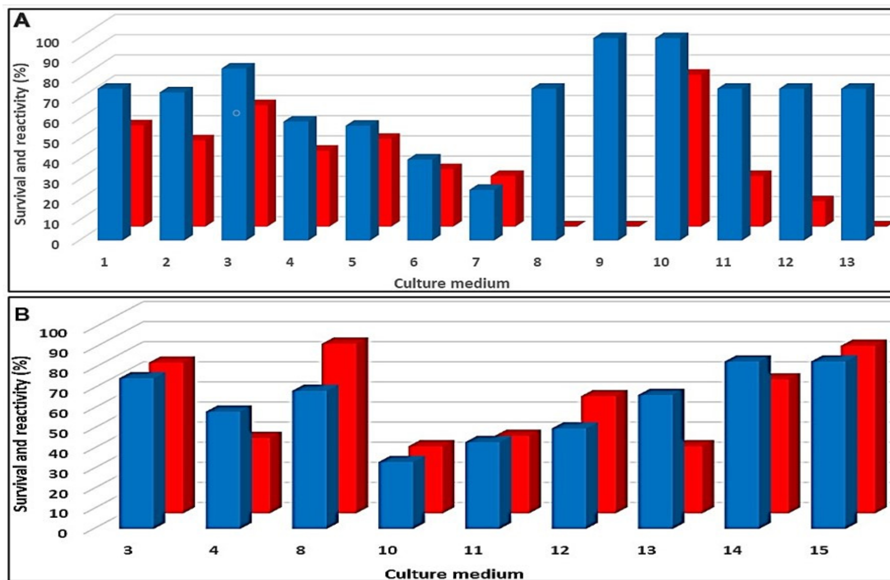

Fig. 3. *The effect of culture medium on the survival (blue) and reactivity (red) of the initial explants of pedunculate (A) and sessile oak (B)*

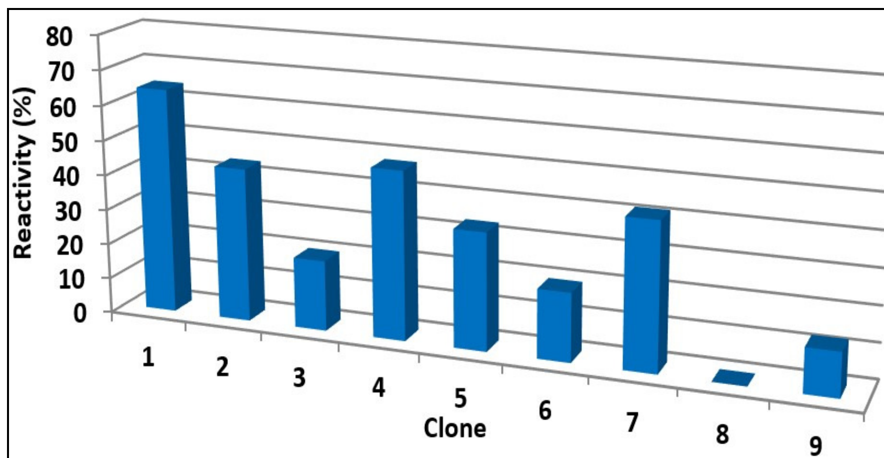

Fig. 4. *The effect of clone on the reactivity of the initial pedunculate oak explants*

Good results were also obtained for clones 178, 175, as well as for the NIRDF clone (7), for which reactivity percentages between 40% and 48% were obtained. The poorest results were obtained for clone 267, for which it was not possible to obtain *in vitro* plants, and for clone 158, with a reactivity of 12.5% (Figure 4).

3.2. Multiplication of *in Vitro* Cultures

The micropropagation of *in vitro* cultures was achieved through elongation, and from the plants obtained we cut nodal segments with buds which were placed on the multiplication medium. The multiplication medium was GD, improved with BAP 0.2−1 mg/l to which we added auxins: IBA and NAA. Only for sessile oak the MS1 medium (BAP 0.2 mg/l) was also tested. The plants were transferred on fresh medium every 4 weeks. The influence of clone, type of explant, and culture medium on the *in vitro* cultures micropropagation were tested (Table 2).

Regarding the clone influence for pedunculate oak, it was observed that the best multiplication capacity was obtained for the pedunculate oak clones 178 and 240, which also had the highest reactivity to the *in vitro* culture conditions. Relatively good results were obtained for clone 175 as well, which had a high reactivity for the initiation of the *in vitro* cultures (Table 2).

The analysis of the influence of the culture medium on the multiplication of the *in vitro* cultures led to the conclusion that the highest multiplication coefficients were obtained when we utilized GD2 and GD3 media (Table 2), so a higher concentration of BAP led to a more efficient multiplication of the *in vitro* plants (Figure 5).

Table 2

Variation of the multiplication rate of oaks on "in vitro" cultures

Pedunculate oak			
Clone	**Medium**	**Buds/ explant**	**Multip. coeff.**
240	GD_1	2.8	2.3
	GD_2	3.2	3.2
178	GD_1	1.8	1.8
	GD_2	3.0	3.5
	GD_3	1.5	2.5
51	GD_1	1.8	1.5
	GD_2	2.5	1.8
175	GD_1	1.5	1.5
	GD_2	2.5	2.0
	GD_3	3.2	2.8
INCDS park	GD_1	1.5	1.2
	GD_3	1.2	1.5
	GD_4	2.0	1.5
	GD_5	1.8	2.0
Sessile oak			
Explant	**Medium**	**Buds/ explant**	**Multip. coeff.**
M.a.g. *in vitro*	GD_1	3.2	3.5
	GD_2	2.8	2.8
N.s.g. *in vivo*	GD_1	2.5	2.0
	GD_2	1.8	1.5
	MS_1	2.8	2.5

GD_1- GD_5 and MS_1 medium compositions are described in methodology. Multip. coeff. = Multiplication coefficient. M.a.g. = Mature acorn germinated. N.s.g. = Nodal segments from acorn germinated *in vivo*.

For sessile oak, the analysis of the culture medium effect on the multiplication of the *in vitro* cultures led to the conclusion that the highest multiplication coefficients were obtained in the GD1 medium, so a lower concentration of BAP led to a more efficient multiplication of the *in vitro* plants, in contrast to the result recorded for pedunculate oak.

Fig. 5. *Multiplication of* the *in vitro cultures of pedunculate oak*

3.3. Rooting of the *in vitro* Plants

The multiplicated plants were transferred on a rooting medium, which was GD medium and MS1 medium with the concentration reduced by half (MS1½), and supplemented with different growing hormones which were auxins as well as cytokinin's. In what concerns the pedunculate oak clones, the highest rooting percentage was obtained for clones 178 and 175, which were 70% and 65%, respectively. Clone 240 also had relatively good results, with a rooting percentage of 50% (Table 3), all in the GD medium. So, the best medium was GD, on which we obtained a very high rooting percentage of 70%, this result being considered very good for oaks (Figure 6). This result can be improved for obtaining a more efficient method for the ex situ conservation of genetic resources.

For sessile oak, the mature acorn germinated *in vitro* was the best explant (Table 3).

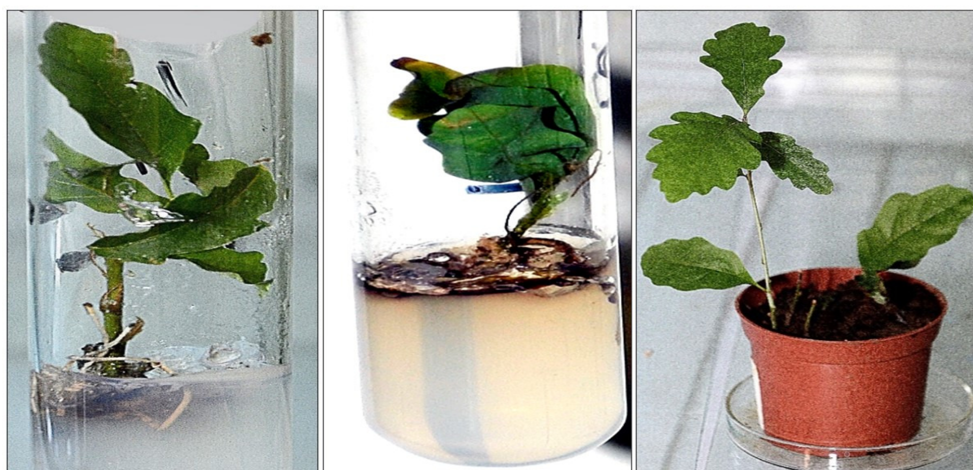

Fig. 6. *Rooting of pedunculate (left) and sessile (centre) oaks in vitro plants and in vivo acclimatization (right)*

Table 3
Rooting of pedunculate and sessile oaks on "in vitro" cultures

Pedunculate oak			
Clone	**Culture medium**	**Regenerated plants (%)**	**Rooting (%)**
240	GD	50	50
	$MS_1$1/2	70	30
178	GD	65	70
	$MS_1$1/2	50	35
175	GD	80	65
	$MS_1$1/2	50	40
Sessile oak			
Explant	**Culture medium**	**Regenerated plants (%)**	**Rooting (%)**
M.a.g. *in vitro*	GD_1	70	65
	GD_2	80	70
N.s.g. *in vivo*	GD_1	50	35
	GD_2	65	40

GD: BAP 0.5 mg/l, IBA 0.1 mg/l; MS_1: ½-IBA 0.3 mg/l, NAA 0.1 mg/l. M.a.g. = Mature acorn germinated. N.s.g. = Nodal segments from acorn germinated *in vivo*.

3.4. Acclimatization of *in Vitro* Plants

In vitro plants were transferred *in vivo*, in pots with a mixture of soil, sand, and peat, which was sterilized in the oven for 6 h at 120°C. The plants were preserved in the growing chamber with controlled light and temperature for 2 months. After this period, the plants will be moved to an air-conditioned greenhouse that is also equipped with an installation for artificial fog.

4. Discussion

The working protocol for *in vitro* somatic micropropagation begins with harvesting the plant material from genetically improved trees and populations, followed by identifying the optimal types of explants and the most efficient method for sterilization; then the optimal hormonal balances and the culture medium for *in vitro* micropropagation and rooting must be established, while the last step involves establishing a protocol for *in vivo* transfer [18].

Regarding the type of plant material, the sterilization method, and the culture medium, similar results were previously obtained for grayish oak [17], which indicates the possibility of applying the same working protocol for all species of the genus Quercus in Romania. This study is consistent with findings of previous research indicating that the tissue culture of mature-phase oak material is difficult [21, 22].

Concerning the type of explant, Pandey and Tamta [29] reported similar results by using *in vitro* seed germination to obtain aseptic seedlings for *Quercus serrata*. Other authors used acorns from *Quercus robur* cultured in mould and the seedlings were used as source of explants [11].

Regarding the sterilization method, the same authors [29] used a lower concentration of mercuric chloride, but first they added a fungicide for 30 minutes. Other authors also used a fungicide, but in addition to free chlorine for the sterilization of mature holm acorns [6].

In what concerns the culture medium, the GD was the more favourable in our research, as previously reported by Romano *et al*. [35]. Different results were found in North America (USA and Mexico), where the reactivity and survival were higher for the explants grown on the WPM medium than in GD [4]. Similarly, in India [29], WPM (with 4.44 µM BAP) was the best medium for culture multiplication.

The rooting process needed the presence of an auxin [34], and IBA was more efficient than NAA for pedunculate oak, as suggested by Puddephat *et al*. [32]. Other authors used both IBA and NAA in the rooting process of holm oak to obtain better results [6].

Concerning the *in vivo* transfer, similar results were obtained by Pandey and Tamta [29], who kept the rooted plants in the growing chamber, but for a longer period of time. Liao and Chuang [23] used peat, vermiculite, and perlite as potting media for the acclimatization of *in vitro* plants and kept them in the growing chamber for 30 days.

5. Conclusions

For the initiation of *in vitro* cultures of the two oak species, the best type of explant was the mature acorn germinated *in vitro*. The most efficient sterilization method was mercuric chloride 0.2 mg/l, ascorbic acid 1 mg/l, for 30 min., with or without antibiotic. The best results for the initiation of *in vitro* cultures were obtained with GD medium supplemented with different concentration of BAP, with or without auxins, regarding the utilized species.

This medium (GD) was also the best one for both the multiplication and the rooting steps. The maximum multiplication rate was obtained when we used a higher concentration of BAP for pedunculate oak and a smaller one for sessile oak. The maximum rooting percentage was obtained when we used the GD medium increased with 0.5 mg/l BAP and 0.1 mg/l IBA.

Preservation of tree genetic resources through *in vitro* culture for oak species is a modern alternative for restoring and conserving biodiversity in relation to the problem of seed production and the phenomenon of oak tree mortality.

Acknowledgments

The research was financed by the Romanian Ministry of Research, Innovation and Digitalization, within the framework of the 'Nucleu' Programme contracted with the National Institute for Research and Development in Forestry "Marin Drăcea" (Project PN19070301). We wish to thank our devoted colleagues: Dan Pepelea, Gabriela Grosu, Cristiana Ciuvăț, and Pătruță Roxana for their help in the collection of biological material as well as in carrying out the laboratory activities.

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