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A PROTOCOL FOR *IN VITRO* PROPAGATION OF *QUERCUS PUBESCENS* WILLD.

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Abstract: *In this study, a protocol was developed for the in vitro propagation of Quercus pubescens Willd. Through this research, we aimed to determine how in vitro cultivation conditions, culture medium, and the sterilisation method influence in vitro regeneration response. Explants aseptically disinfected in sodium hypochlorite solution, Benzalkonium chloride, and ethanol were inoculated onto two types of medium (WPM and GD), to which different combinations of plant growth regulators were added. Sterilisation treatment consisting of benzalkonium chloride 1%, 3 minutes + Domestos 20%, 3 minutes + Ethanol 70%, 1 minute led to cultures free of microbial contamination and the highest percentage of viable explants (80%). After one month from the initiation of the cultures, measurements were made regarding microshoots height, number and length of the leaves. The WPM medium with gibberellin 0.25 mg/l and BAP 1 mg/l positively influenced the growth of microshoots and leaf size, leading to the best results in terms of shoot height (3.27 cm) and leaf length (0.62 cm). On the GD medium, the number of leaves was slightly higher (2.82), but their length was shorter (0.52 cm). The rooted microshoots were acclimatised in the greenhouse, and the survival rate was 84.62%.*

Key words: *micropropagation, pubescent oak, growth regulators, internode culture, plant regeneration.*

1. Introduction

The genus *Quercus* represents one of the most significant groups of woody angiosperms in the Northern Hemisphere, notable for its high species richness, ecological prominence, and economic importance [12, 36, 40]. In Europe, *Quercus* species are important because they are ecologically dominant, biodiversity-rich, long-lived trees that maintain forest structure, provide key ecosystem services, and have significant economic value. The genus *Quercus* plays a key ecological role in forest ecosystems by supporting biodiversity and wildlife, protecting soil

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and watersheds, providing valuable timber and commercial products, improving environmental quality, and offering medicinal resources [8, 28, 36, 48, 49]. Holocene pollen records from Romania show that *Quercus* has remained highly abundant and resilient, recovering from declines and demonstrating long-term ecological stability in Eastern European forests [14].

Among European oaks, pubescent oak (*Quercus pubescens* Willd.) is particularly notable for its drought- and heat-resistance. Although currently more restricted than sessile and pedunculate oaks, the ecological and societal significance of pubescent oak is expected to increase under ongoing climate warming [44]. *Quercus pubescens* (Willd.) exhibits greater tolerance to rising temperatures than *Q. robur* (L.), indicating a potential competitive advantage under future climate conditions [47], and projections suggest that its range may expand in Romania, driven by climate-induced shifts, including both altitudinal and latitudinal migration [13, 22]. Pubescent oak, a Mediterranean deciduous broadleaf species, is well adapted to survive dry and drought-prone conditions [16, 53].

Quercus pubescens has evolved several strategies to cope with drought, including conservative water use through gradual stomatal closure and a marked decline in water potential. Under intense drought, it significantly reduces photosynthetic efficiency while maintaining effective protection against excess light [24]. Drought tolerance is further supported by a balance between water loss and uptake, aided by high root and stem hydraulic conductivity, while its hydraulic structure – the highest resistance in current-year stems – boosts tolerance to seasonal drought [27, 35]. Severe drought, intensified by higher temperatures, increasingly threatens forest ecosystems by disrupting water and carbon fluxes, reducing productivity, and raising pest and disease risks. Plant species respond differently, using structural and physiological strategies of resistance, avoidance or tolerance [2, 5, 53].

Climate change is projected to increase the frequency and severity of drought events in many regions, particularly across the Northern Hemisphere. Over the twenty-first century, global average surface temperatures are expected to rise by 2-4.5°C, accompanied by shifts in precipitation patterns in Western Europe, including heavier winter rainfall and more pronounced summer water deficits [5, 7, 19, 23]. Severe, repeated droughts drive widespread tree decline and mortality across Europe, with diffuse patterns shaped by soil water retention and genetic differences among and within species [5, 7, 32, 54].

Despite its ecological and economic value, *Quercus pubescens* is difficult to propagate traditionally due to irregular seed yield and limited vegetative regeneration, making *in vitro* propagation critical for conservation, breeding, and large-scale multiplication of superior genotypes [17, 18, 46]. Southern Moldavian populations of *Quercus pubescens* (pubescent oak, downy oak) have declined due to long-term coppicing, aging stands, and low seed production, resulting in reduced genetic diversity and poor natural regeneration [11].

The advanced propagation technologies (*in vitro* preservation, cryopreservation, and tissue culture), which are now applicable to a wide range of taxa, ensure the production of plant material without

harming wild populations. The *in vitro* micropropagation allows fast, large-scale multiplication, and reduces the risk of disease transfer. Also, due to controlled environments (greenhouses and growth chambers), it supports the cultivation of slow-growing perennial species [1, 3, 10, 29, 41]. Limited by recalcitrant, short-lived, and vulnerable acorns, *Quercus pubescens* relies on biotechnological methods, such as *in vitro* germination and somatic embryogenesis, for rapid multiplication of elite genotypes, supporting forest restoration, and the maintenance of local genetic diversity [11]. Limited sexual reproduction, obstructed by long juvenility, irregular mast production, and short-lived seeds makes biotechnological interventions essential [38].

In vitro micropropagation of *Quercus* spp. through organogenesis or somatic embryogenesis has been the subject of numerous studies [9, 21, 33, 34, 45]. The most used explants have been the internodal fragments and acorn embryos [4, 20, 37-39, 41, 42, 52, 56]. This research aims to establish a working protocol for *in vitro* multiplication of pubescent oak, by testing the influence of explant, the sterilisation method, and culture medium composition on the *in vitro* micropropagation and rooting of the *in vitro* plants.

2. Materials and Methods

2.1. Plant Material

Q. pubescens young shoots of 20-25 cm length, actively growing, came from mother plants cultivated in the NIRDS "Marin Drăcea" nursery, Brasov. The introduction of biological material from the *in vivo* environment into sterile conditions involves certain stages, and the success of the *in vitro* cultivation depends on their compliance.

2.2. Explants Sterilisation

The pre-sterilisation stage consisted of the following steps (Figure 1):

- detaching the shoots and removing the leaves;
- rinsing them under running water for 45 minutes;
- immersing the shoots for 30 seconds in sterile distilled water to which 2 drops of Tween 80 were added (under sterile conditions).

Sterilisation of biological material (Figure 2) is the stage that precedes the inoculation of nodal explants on the culture medium. The sterilising agent, its concentration and the time of exposure of the plant tissue to the disinfectant solution must be chosen in such a way as to prevent the occurrence of microbial infections, but at the same time as not to affect the viability of the explants so that new plants can be regenerated from them.

Three variants of sterilisation were tested, as follows:

- V1:
    - Benzalkonium chloride 1%, 3 minutes;
    - Domestos 20%, 3 minutes;
    - Ethanol 70%, 1 minute;
- V2:
    - Domestos 20%, 5 minutes;
    - Ethanol 70%, 1 minute;
- V3:
    - Domestos 20%, 10 minutes;
    - Ethanol 70%, 1 minute.

Fig. 1. *Pre-sterilisation stage of pubescent oak shoots*

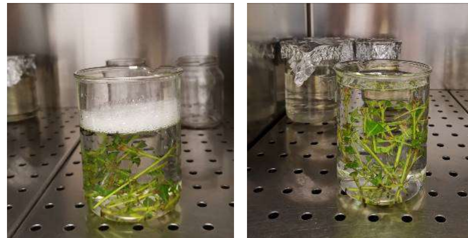

Fig. 2. *Aspects of the sterilisation stage of pubescent oak shoots*

After exposure of the shoots to the sterilizing agents, they were rinsed with sterile distilled water, in three rounds of 5 minutes each. All these steps were carried out in the laminar airflow hood, previously sterilised by irradiation with an ultraviolet lamp, for 30 minutes.

2.3. Culture Media Preparation and Inoculation of Explants

After the sterilisation stage, the explants were prepared (sized) and inoculated onto the culture medium. Explant preparation refers to removing the leaf and a portion of the petiole (the latter having the role of protecting the bud during sterilisation) and sizing the explants. Fragments of approximately 1.5-2 cm were inoculated onto the nutrient media, ensuring polarity. The nodal fragments contain both axillary and apical buds. The inoculation of the nodal explants onto the nutrient medium was performed in a horizontal laminar flow cabinet and using sterile instruments on a surface that was previously disinfected with 70° alcohol (Figure 3).

Two basal medium variants were used in order to induce shoot formation from the internodes: Woody Plant Medium (WPM – [26]) and Gresshoff-Doy (GD – [15]). To solidify the medium, agar was added to its composition, and before autoclaving, the pH was adjusted to 5.7 (Table 1). The nutrient medium was distributed in test tubes and sealed with aluminium foil. The sterilisation of the test tubes was carried out in an oven for 2 hours at 180°C, and the culture medium was sterilised in an autoclave for 20 minutes at 120°C.

Fig. 3. *Inoculation of cuttings from shoots onto the culture medium*

Composition of medium variants used for inoculation of nodal explants Table 1

V1		V2	
Components	Quantity/1 l medium	Components	Quantity/1 l medium
WPM	2.46 g	GD	2.8 g
Gibberellin	0.25 mg	BAP	0.2 mg
BAP*	1 mg	NAA*	0.01 mg
Activated charcoal	0.5 g	Sucrose	30 g
Vitamin C	20 mg	Agar	9 g
Sucrose	30 g		
Agar	9 g		

Note: *BAP – 6-benzyl-aminopurine; NAA – α-naphthyl acetic acid.

2.4. Incubation of Cultures in the Growth Chamber

The *in vitro* cultures were incubated in the growth chamber, further monitoring the evolution of the explants (Figure 4). Controlled conditions of light (photoperiod of 16 hours light/8 hours dark), temperature (23±2°C), and humidity (75-80%) were ensured.

2.5. Rooting of *IN VITRO* Shoots

Shoots developed from buds that reached a height of at least 2 cm were transferred to another type of medium (Table 2), which would stimulate root formation. Before incubation in the growth chamber, the explants were kept in the dark for five days [46] (Figure 5). Explants with viable buds, but not yet developed, were passaged monthly on fresh medium until they reached a size of 2-3 cm.

Fig. 4. *Cultures incubation under controlled conditions (growth chamber)*

Fig. 5. *Incubation of pubescent oak microshoots in dark conditions for 5 days to stimulate rooting*

2.6. Acclimatisation

The rooted microshoots were transferred in pots containing a mixture of sterilised peat (50%), perlite (30%) and vermiculite (20%) for the acclimatisation stage (Figure 6). The substrate was sterilised in an oven at a temperature of 85°C for 60 minutes. The

transfer of microshoots into the substrate was carried out by following certain steps: removing the medium from the roots; moistening the substrate; covering the culture vessels to maintain high humidity.

Before transferring to the substrate, the roots were immersed in a solution of Accudo® (1 ml/l) for 15 minutes. Accudo® is a biostimulant that stimulates root branching and increases root surface area, promotes rooting and development, increases water use efficiency, and improves nutrient absorption. Accudo® was also applied to the substrate at transplantation and one week after transfer.

Rooting medium composition Table 2

Components	Quantity/1 l medium
WPM	2.46 g
IBA*	1 mg
Activated charcoal	0.5 g
Vitamin C	20 mg
Sucrose	30 g
Agar	9 g

Note: *IBA – indole-3-butyric acid.

Fig. 6. *Transfer of microshoots rooted "in vitro" to the substrate for the acclimatisation stage*

3. Results and Discussion

Young, actively growing shoots constituted a viable source of explants that were used to initiate *in vitro* cultures.

After one month of cultures incubation in the growth chamber, they were evaluated in terms of shoot regeneration and percentage of microbial contamination, depending on the sterilisation treatment (Table 3).

The best results were obtained after applying the V1 (benzalkonium chloride 1%, 3 minutes + Domestos 20%, 3 minutes + Ethanol 70%, 1 minute) sterilisation treatment. This led to cultures free of microbial contamination and the highest percentage of viable explants (80%).

After sterilisation, a similar survival rate of explants cultured *in vitro* was also obtained by other researchers: between 63 and 83% [18], from 65 to 73% [57], between 44 and 83% [38]. For surface sterilisation of plant tissue, they used different sterilising solutions: mercuric chloride, ethanol, fluconazole, commercial bleach, to which they added ascorbic acid. The most effective treatments consisted of using mercuric chloride 0.2%, ascorbic acid 1 g/l, for 30 minutes, ethanol 70%, for 1 minute [18]; a 1:10 dilution of commercial bleach with 0.05% Tween 20 [57]; 70% ethanol for 10 minutes followed by 15–20 minutes treatment in 0.1% solution of mercuric chloride [38].

Percentage of viable explants and infection rate after 30 days from initiation Table 3

Sterilisation variant	Viable explants [%]	Infection rate [%]
V1	80	0
V2	75	6.25
V3	70	16.7

The evolution of the explants on the two medium variants was monitored, and after one month from the initiation of the cultures, measurements were made regarding the height of the cuttings, the number of leaves, and the length of the leaves (Figure 7).

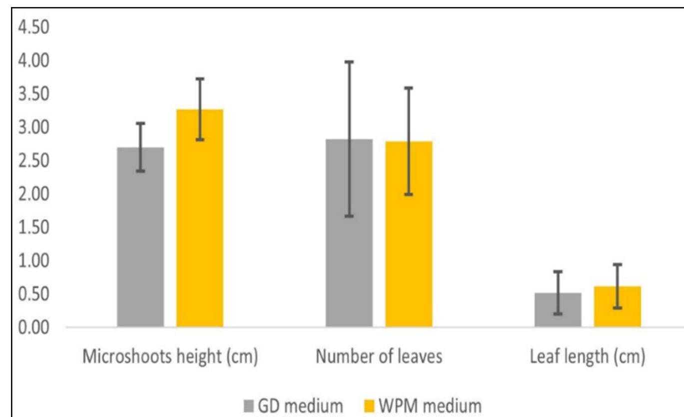

Fig. 7. *Evolution of pubescent oak microshoots after one month from the initiation of in vitro cultures*

The results presented in Figure 7 show that the WPM medium positively influenced the growth of microshoots and leaf size, leading to the best results in terms of explant height (3.27 cm) and leaf length (0.62 cm). On the GD medium, the number of leaves was slightly higher (2.82), but their length was shorter (0.52 cm). However, the differences between WPM and GD were statistically not significant ($p > 0.05$) for all three analysed traits. Also, [45] obtained the best results regarding the height of pubescent oak microshoots on the WPM culture medium, compared to the GD and

MS (Murashige -Skoog) medium. In a recent publication of Wang et al. [54], the use of the WPM medium appears specified along with growth regulators. Wang et al. [55] established that optimal results were obtained for shoot proliferation by using 0.3 mg/L 6-BA and 0.4 mg/L KT, achieving a mean proliferation coefficient of 5.22. For another species, *Quercus arbutifolia* Hickel & A. Camus, Li et al. [25] studied a protocol for multiplying. Woody plant medium (WPM) with 0.5 mg/L BA was optimal for budding initiation to produce initial shoots for further subculture of proliferation. WPM with 0.5 mg/L BA and 0.02 mg/L IBA is suitable for subculture that can vigorously induce shoot regeneration. The appearance of the shoots one month after inoculation on the growth medium is presented in Figure 8.

Fig. 8. *Appearance of shoots on the two medium variants, after one month from the initiation of cultures (A - WPM medium; B - GD medium)*

After two months from the initiation of the "in vitro" cultures, ensuring controlled temperature and photoperiod conditions, as well as a specific nutrient medium, the pubescent oak microshoots formed roots (Figure 9). The rooting percentage was 65%. Hormonal balance and the addition of activated charcoal to the culture medium positively influenced rhizogenesis. Using IBA (1 mg/l) in the rooting stage led to favourable results in terms of shoot rooting.

Studies conducted by other researchers led to the following results: the percentage of *in vitro* rooting of explants varies between 11 and 89% [9]; depending on the auxin (IBA) concentration in the culture medium, the rooting response ranged between 30 and 50% [51]. In the article by [29], the rooting rate is around 80%, depending on the concentration of exogenous auxin (IAA or IBA) and activated charcoal level.

Fig. 9. *In vitro rooting of pubescent oak microshoots*

Activated charcoal (AC) is often used in plant tissue culture to improve cell growth and development [30]. The addition of AC to media is a recognised practice and its influence on growth and development may be attributed mainly to the adsorption of inhibitory substances in the culture medium, drastic decrease in the phenolic oxidation or brown exudate accumulation, alteration of medium pH to an optimum level for morphogenesis and establishment of a darkened environment in medium, and thus simulating soil conditions [50].

Sánchez et al. [43] used shoots excised from *in vitro* cultures of mature *Quercus robur* (L.) and *Quercus rubra* (L.). Treatment with IBA 25 mg L^{-1} for 24 h followed by transfer to auxin-free medium + 1% activated carbon was suitable for rooting.

The transplanting of rooted microshoots for acclimatisation was successful. The survival rate was 84.62%.

Meier-Dinkel et al. [31] studied survival after *ex vitro* transfer. Microcuttings were rooted *ex vitro* after treatment with rooting powder containing 0.5% indole-3-butyric acid or 1.0% indole acetic acid. Shoots derived from subcultured shoot tips and nodal segments had a low rooting and survival rate (21%) after four months. 56% of shoots derived from subcultured basal segments with a callus rooted and survived.

The main objective of this study was the establishment of a working protocol for *in vitro* micropropagation of *Quercus pubescens*. This consisted of going through the following basic steps: selecting donor plants, identifying an optimal treatment for sterilising explants, preparing nutrient medium recipes specific to each growth stage (initiation, elongation/multiplication, rooting), and finally, the *in vivo* transfer of microshoots obtained *in vitro*.

Periodic transfer of cultures to fresh medium had a favourable effect, stimulating the growth in length of microshoots. Also, monthly passage of explants on fresh medium is recommended to prevent the inhibitory effect of phenolic substances secreted during the growth stage, a phenomenon encountered in *in vitro* micropropagation of species from the *Fagaceae* family [46].

Regarding the culture medium, the GD

was more favourable in studies carried out by Ioniță et al. [18] and Romano and Martins-Louzao [42]. Different results were found in the study and in others [6, 39].

4. Conclusions

The initiation of *in vitro* cultures starting from nodal stem fragments, containing an axillary or apical bud, taken from young, actively growing shoots, was successfully achieved. The sterilisation treatment consisted in a combination of benzalkonium chloride 1%, 3 minutes + Domestos 20%, 3 minutes + Ethanol 70%, 1 minute, it was the most effective, leading to the highest survival rate (80%) and no microbial contamination rate (0%). The WPM medium positively influenced the growth of microshoots and leaf size, leading to the best results in terms of explant height and leaf length. The addition of IBA (1 mg/l) and activated charcoal (0.5 g/l) to the culture medium positively influenced the "in vitro" formation of roots. The use of a substrate consisting of peat, perlite, and vermiculite, the addition of Accudo®, and maintaining high humidity in culture pots were factors that contributed to the successful acclimatisation of pubescent oak microshoots (84.62% survival rate).

In vitro conservation of forest species plays a crucial role in preserving genetic resources, providing pathogen-free and high-quality planting material, maintaining forest biodiversity, supporting *ex situ* conservation of endangered species, and contributing to adaptive strategies for climate change mitigation.

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