© by PSP Volume 29 – No. 11/2020 pages 10287-10293 Fresenius Environmental Bulletin

# ENHANCEMENT OF SPROUTING AND ROOTING OF *QUERCUS PUBESCENS* BY BENZYLAMINOPURINE AND INDOLE-BUTYRIC ACID IN MICROPROPAGATION

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## ABSTRACT

Micropropagation has the advantage of genetic homogeneity and reduces difficulties related to obtaining pathogen-free plant material. However, some plant species present challenging problems when used for *in vitro* culture. This research work dealt with the micropropagation of *Quercus pubescens* Willd. with the aim of enhancing the percentage proliferation and rooting from micro-cuttings. Micro-cuttings, containing apical or axillary buds, were taken from two year-old branches using a clone of *Q. pubescens* grown at the Forest Nursery of Sant'Angelo in Vado, Pesaro-Urbino, Marche, Italy. A preliminary screening of the proliferation was performed using increasing concentration of benzylaminopurine (BA) in the culture medium (0.25; 0.5; 0.75; 1 mg/l) in order to select the best hormones to use in the experiment. From the results it was possible to deduce that BA plays a key role in influencing shoot proliferation, leading to the formation of a callus and to the production of new shoots. Moreover, we observed that, especially higher BA concentrations promoted the production of a larger number of shorter shoots compared to low concentrations, that produced the small number of longer shoots. Furthermore, these trials have shown that, in order to have a large number of seedlings with an optimal shoot length, it is necessary to divide the process of propagation in two phases. The first phase involves a high concentration of BA, with the aim of increasing the number of new micro-cuttings; the second phase, with lower levels of BA, to increase the length of the shoots. With regards to root induction, the hormone selected was indole-butyric acid (IBA). Results showed that micro-cutting exposed for 24h to higher hormone concentration gave the best results for root induction and root length. The study developed an efficient method for the micropropagation of *Q. pubescens* for the selection of genotypes in *in vitro* and sterile (e.g., free microorganisms and insects) conditions. It is envisaged that the development of this protocol paves the way for further utilisation of micro-propagated *Q. pubescens* in new areas of research, such as *in vitro* mycorrhization.

**KEYWORDS**:
Micropropagation, *Quercus pubescens,* Benzylaminopurine, Indole-butyric acid.

## INTRODUCTION

Over thousands of years agricultural practices and breeding techniques have permitted the selection of plant material with useful growth characteristics and enhanced properties, such as increased yield quality and resistance to disease [1]. Cloning is extremely useful in fruit, vegetable, floriculture and ornamental plant production, as well as for the conservation of highly heterozygous genotype of many cultivars [2]. Plant propagation through the micropropagation enhances the totipotency of plant cells in order to differentiate and to produce new and structurally complete plants in a relatively short time [3]. Moreover, micropropagation allows the differentiation and the multiplication of cells of different tissues in order to trigger the production of new meristematic tissues and plants [3].

Micropropagation has been applied since the mid-30s, though with several limitations when it comes to the propagation of woody plants. Furthermore, literature on this topic is dated. Generally, the micropropagation of woody species is performed on dormant buds, that however show a strong resistance to initiate growth [4]. The growth of woody species is characterised by a strong growth sprout at the start of the growing season (bud burst) followed by a slow period of growth. The development of buds is very slow especially under conditions of *in vitro* culture, where they tend to alternate between dormant and vegetative periods [5]. In particular, slow-growing trees, such as oak (*Quercus*), walnuts (*Juglans*), spruce (*Picea*) and fir (*Abies*), have rarely been successfully micro-propagated when starting from two years old plants [6]. On the other hand, fast-growing trees have shown successful results in microculture, as in the case of cottonwood (*Populus*), elm (*Ulmus*), eucalyptus (*Eucalyptus*) and birch (*Betula*).

The protocols of micropropagation of woody angiosperm species were successfully developed in

the 1980s, by breaking the inhibition of bud dormancy, as shown in *Prunus* [7], *Malus* [8], *Erica* [9], and, likewise, in conifers, such as *Pinus* [10] and *Picea* [11]. The micropropagation of *Quercus robur* was achieved a few years later [12, 13, 14] . The micropropagation of recalcitrant and old tree species requires tissue rejuvenation with a series of grafts or pruning cycles in order to obtain micro-cuttings [15, 16, 17, 18]. The micro-cuttings retain the functionality of the original plant, that drives the plagiotropic growth or the total absence of rooting [19, 17, 20].

For *in vitro* conditions, rooting requires the rejuvenation of the plant material [21, 22, 23], through hormone treatment [24], or even using activated charcoal [14, 25]. Whereas, the rooting of explants from 2-3 years old saplings can be successfully induced in *ex vitro* conditions through the exposure to high humidity [26]. Traditionally, oak seedlings used for the reforestation have been obtained from seeds and rarely through plant tissue cultures. However, in past years, protocols of the micropropagation of oaks have been developed and improved in different *Quercus* species [27, 28, 29, 30]. Oaks are highly interesting due to the tolerance to various environmental stresses and their cultivation in urban landscapes. However, the *in vitro* propagation of *Quercus pubescens* is poorly developed.

The aim of the study was to develop an efficient method for the micropropagation of *Q. pubescens* for the selection of pathogen-free genotypes. The study was performed by testing micro-cuttings treated with benzylaminopurine (BA) and indole-butyric acid (IBA) to optimise the micropropagation of oak species in order to obtain genetically homogeneous and pathogen-free certified plants. The hypothesis of the study is that the percentage of sprouting and rooting of tree cuttings in micropropagation can be enhanced through hormonal treatment.

## MATERIALS AND METHODS

**Plant material.** Micro-cuttings, containing apical and axillary buds, were taken from branches of *Quercus pubescens* Willd., grown in the Forest Nursery of Sant'Angelo in Vado (Pesaro-Urbino, Marche, Italy). Two years old plants were used, since the explants taken on older plants are usually refractory to treatment [31]. The plant species was chosen due to the potential to establish mycorrhizal symbiosis with fungi especially belonging to the genus *Tuber*, for the truffles production. Branches of the mother plant were dissected in order to obtain micro-cuttings with one bud each. The incision at the bottom of the micro-cuttings was performed at 45° in order to increase the absorbent surface area and to promote the production of stem cells; whereas the upper part was cut at 90°, to decrease the water loss surface area from vascular tissue.

**Micro-cuttings sterilization.** All the operations of *in vitro* micropropagation were carried out under sterile technique using laminar hood, with surfaces sterilized with 70% ethanol. Micro-cuttings were treated with 200 ml of deionized sterile water solution, containing 1 ml of Tween 80, for 15 minutes. Micro-cuttings were subsequently removed from the detergent solution and then placed in a 2% solution of sodium hypochlorite at for 20 minutes. Subsequently, micro-cuttings were rinsed three times in sterile distilled water for 10 minutes and then dried (under a laminar flow hood).

**Micropropagation.** The sterilized micro-cuttings were individually placed in glass tubes (25mm x 200mm) containing approximately 20 ml of culture medium, previously autoclaved at 121°C for 20 minutes. The culture medium contained: 7 g $L^{-1}$ plant agar, (Duchefa Biochemie), 2.4 g $L^{-1}$ vitamins-enriched Woody Plant Medium (WPM) (Duchefa, Biochemie, The Netherlands), 30 g $L^{-1}$ sucrose (Sigma Aldrich), at pH 5.6. The culture medium was supplemented with 6-benzilaminopurine (BA, Sigma). Four different concentrations of BA were prepared namely 0.25 mg $L^{-1}$; 0.5 mg $L^{-1}$; 0.75 mg $L^{-1}$; 1 mg $L^{-1}$. For each BA concentration, 45 micro-shoots were used. Each treatment had 3 replicates, Magenta vessel GA-7 (Sigma Aldrich, Germany) had 9 explants for a total of 675 micro-shoots.

The micro-cuttings were placed in the incubator (Angelantoni, Perugia, Italy) with controlled conditions namely, air temperature: 24 ± 2°C; photoperiod: 16 hours light – 8 hours dark; light intensity: 3000 lux; 80% RH. Those conditions were kept throughout all the phases of the experiment. The micro-cuttings were left in the incubator for 20 days and were checked daily in order to control if any biological contaminants such as fungal or bacterial were present. Once micro-cuttings apical and lateral buds had consistent elongation of the stems, the micro-shoots were placed in a Petri dish using sterile technique and the callus was cut off. Then micro-shoots were placed in Magenta vessel with fresh media.

**Rooting.** After reaching a substantial number of micro-cuttings through the micropropagation phase, micro-cuttings were subsequently exposed to IBA to induce rooting. The micro-cuttings were removed from the Magenta vessel and placed in sterile glass Petri dish. Then the callus from the micro-cuttings was excised and planted in the culture medium enriched with two different concentrations of indole-3-butyric acid (IBA), 10 and 20 mg $L^{-1}$, in order to verify the optimal concentration for root induction. Additionally, two different time exposure treatments were performed, namely, 24h at 10 and 20 mg $L^{-1}$, and 48h at 10 and 20 mg $L^{-1}$. Each treatment was carried out three times and each Magenta contained 9

shoots (n=135). For each different treatment 9 seedlings were used as a control. After 24 and 48 hrs of exposure to IBA medium, the seedlings were transplanted into plant-growth-regulator-free medium containing agar 6 g $L^{-1}$, WPM 1.2 g $L^{-1}$, sucrose 20 g$L^{-1}$, charcoal 1 g $L^{-1}$, at pH 5.6. After 20 days in incubator, the rooting was assessed. Figure 2 is shows micro-cuttings with root system fully developed.

**Acclimatization.** Rooted micro-cuttings were acclimatized for 4 weeks in a Magenta vessel GA-7 (16 cm x 8 cm) (Sigma Aldrich,), containing peat and perlite (3: 1, v / v), with a plastic cap, provided with filters to allow the air exchange. The acclimatization phase reduces shock that plants suffer due to the drastic changes of environmental conditions and nutritional availability. After one month, the plants were transferred to larger pots (18 x 18 cm), containing peat. The plants were protected from direct light for further 4 weeks, and subsequently exposed to full light in greenhouse followed by open field culture at the Regional Nursery in Campochiaro (Molise, Italy).

**Biometric analysis.** Biometrical traits were measured at shoot level for BA treatment and at root level for IBA treatment by counting the number of shoot and root and by measuring the length of shoot and root, respectively. The measurements carried out at root level were performed considering the time exposure to IBA.

**Statistical analysis.** Descriptive statistics (means, standard errors) were carried out for all measured parameters. Analysis of variance (ANOVA) was applied to test the effect of BA (one-way analysis) and IBA (two-way analysis, considering also the effect of time exposure) on seedlings. A LSD post-hoc test was applied to assess for significantly different means among treatments ($p < 0.05$ level). All statistical analyses were carried out with Origin Pro 8 program (Origin Lab, Northampton, MA, USA).

## RESULTS AND DISCUSSION

The sterilization protocol used was efficient allowing the germination of the buds under sterile conditions. The conditions of acclimatization were optimal for stabilization of almost all the seedlings. The acclimation of seedlings took place at 24 ± 1 °C and 70% relative humidity. Shoot regeneration was recorded after 20 days, and after 30 days, micro-cuttings had developed a good root system these results are illustrated in Figures 1 and Figures 2 below. Figure 1 shows plating of micro-cuttings and transplanting in BA medium. Figure 2 shows fully developed root system.

The BA treatment significantly affected the mean number of shoots as well as the mean length of the micro-cuttings and the length of the longer micro-cutting (Table 1). The increasing of BA concentration in culture medium induced the increase of the sprouting (mean values of shoots per plants 1.6, 1.7, 2.7 and 1.6), and the reduction of the shoot length (mean values 2.2, 2.0, 1.9 and 1.6 cm) by increasing BA concentration at 0.25, 0.5, 0.75 and 1 mg $L^{-1}$, respectively (Table 1). The highest number of plants per explant, the highest rate of proliferation and cell multiplication were recorded at the 0.75 mg $L^{-1}$ concentration of BA in comparison to the other concentrations. However, the highest length of the seedlings was reached at low BA concentration, our results coincide with [30] . The BA treatments induced sprouting in *Q. pubescens*, providing indication to address the multiplication phase.

Treatments for root induction showed significant effects of time exposure to IBA concentration in rooting time, root number, and root length. The highest rooting percentage of seedlings (86.7%) was obtained at 20 mg $L^{-1}$ concentration of IBA for 24 h (mean root length 3.4 cm), whereas the exposure for 48h induced the rooting in just 36.7% of the seedlings (mean length 1.9 cm). The IBA concentration of 10 mg $L^{-1}$ induced rooting in 63.3% of roots (mean length 3.0 cm) and in 50.0% (mean length 2.8 cm) after 24 and 48 hours of exposure, respectively.

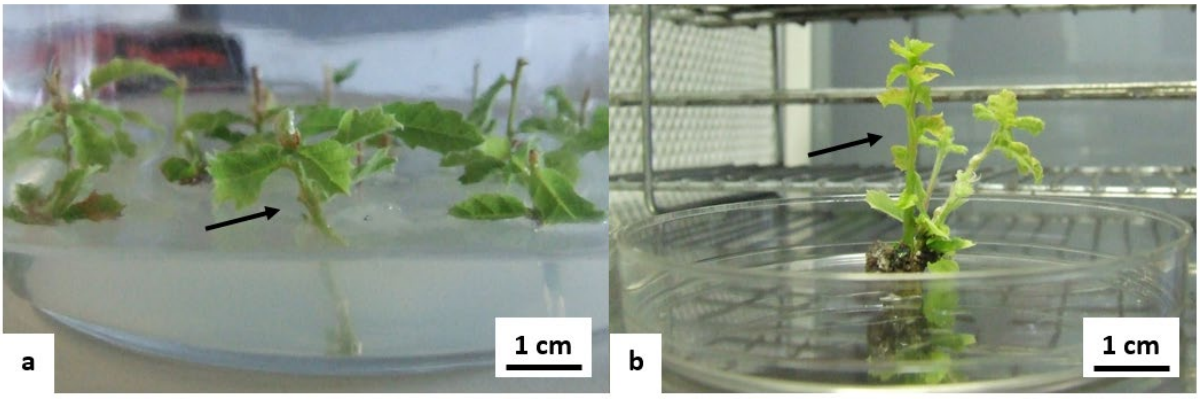


**FIGURE 1**
**Propagation phase of *Quercus pubescens*, micro-cuttings: (a) micro-cuttings transplanted in BA medium; (b) micro-shoots after 4 weeks.**

**TABLE 1**

**Data for shoot regeneration were recorded after 20 days. All values are an average of 45 explants; individual treatments consisted of 3 replicates, 9 explants for Magenta (n=675).**

| BA treatment (mg $L^{-1}$) | shoots per explant (n) | shoot length (cm) | length of longest shoot (cm) |
|---|---|---|---|
| **0** | 0 ***c*** | 0 ***c*** | 0 ***c*** |
| **0.25** | 1.6 ± 0.17 ***b*** | 2.2 ± 0.15 ***a*** | 4.1 ± 0.13 ***a*** |
| **0.50** | 1.7 ± 0.19 ***b*** | 2.0 ± 0.13 ***a*** | 3.6 ± 0.19 ***ab*** |
| **0.75** | 2.7 ± 0.24 ***a*** | 1.9 ± 0.11 ***a*** | 3.2 ± 0.07 ***ab*** |
| **1** | 1.6 ± 0.17 ***b*** | 1.6 ± 0.12 ***b*** | 3.0 ± 0.11 ***b*** |
| ANOVA | | | |
| p-level (F-value) | 0.000 (32.2) | 0.000 (61.5) | 0.000 (16.6) |

Number of shoots per explant, average shoot length, length of longest shoot of explant exposed to BA (6-benzylaminopurine) treatment, namely 0.25 mg $L^{-1}$, 0.5 mg $L^{-1}$, 0.75 mg $L^{-1}$, 1 mg $L^{-1}$. The values are the mean ± SD (n = 5). One-way ANOVA was applied to determine significant differences between treatments (p-level and F-value are given). Different letters correspond to statistical differences between treatments (LSD post-hoc test, $p < 0.05$ level).

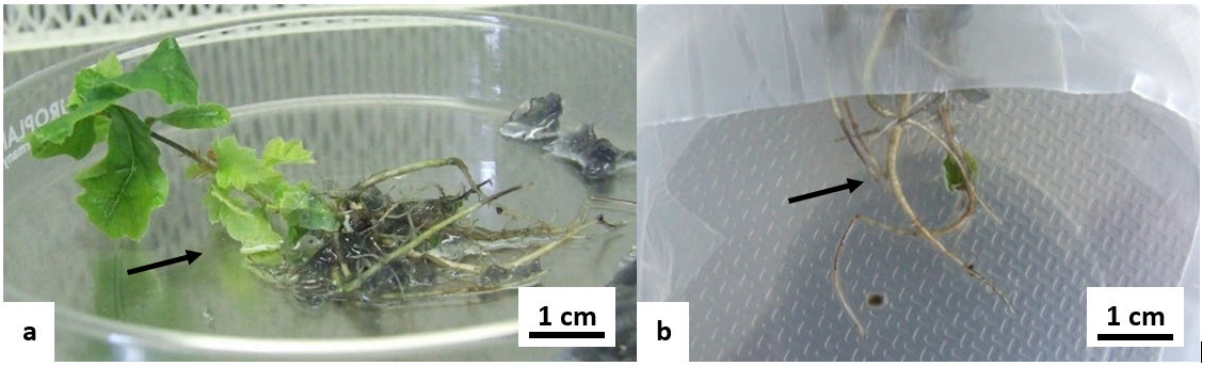


**FIGURE 2**

**Rooting phase of *Quercus pubescens*, micro-cuttings: (a) micro-cutting with root-system taken out from in activated charcoal medium; (b) root-system.**

The micropropagation system used in this study using proper hormone treatment, has been found suitable for the propagation of *Q. pubescens*. The protocols of sterilization, proliferation, rooting and acclimatization developed gave optimal results. Micropropagation techniques represent a valid tool to increase the commercial potential of the species, ensuring the production of a high number of homogeneous plants, as well as healthy certified plants. However, the efficiency of micropropagation is frequently influenced by the plant death during the later stages of acclimatization [32]. The micro-propagated plants are highly vulnerable to water stress, due to the low absorption capacity of roots, and the stomata's limited ability to regulate water use [33, 34]. The acclimatization of micro-cuttings corresponds to a transition period in which the roots are adapting to a substrate where nutrients are less available, and to an autotrophic mode of life. Moreover, in the acclimatization phase, it hypothesised that the presence of mycorrhizae could increase the availability of limiting nutrients, such as phosphorus and nitrogen, facilitating the absorption [32]. The mycorrhization of micro-propagated plants, prior to acclimatization has been shown to increase the survival, enhance the functionality of the root system and the mineral nutrition of the plant [10, 32, 35, 36, 37, 39, 40, 41, 42, 43]. Similarly, the *in vitro* mycorrhization of micro-propagated plants increase the survival and growth potential during the *ex vitro* weaning [44].

The development of these protocols using hormone treatment allow us to perform the micropropagation species with recalcitrance traits such as *Q. pubescens.*

## CONCLUSIONS

This study confirms that *Q. pubescens* can be micropropagated *in* vitro even though it is considered a recalcitrant species. Using the developed protocols, it is possible to produce large number of micro plants. In order to maximise the propagation as well as the rooting two phases were identified. The propagation phase involved the use of BA 0.75 mg $L^{-1}$ which provided the higher number of shoots followed by a

**TABLE 2**
**Micro-cuttings were subjected to a 24-h or 48-h pulse treatment with concentrations of IBA (indole-3-butyric acid) namely 10 and 20 mg L-1, before subculture to plant-growth-regulator-free Woody Plant Medium.**

| IBA treatment (mg $L^{-1}$) | roots per shoot (n) | rooting (%) | root length (cm) |
|---|---|---|---|
| **0** | 0 ***c*** | 0 | 0 ***d*** |
| **10 (24 h)** | 2.6 ± 0.32 ***b*** | 63.3 ± 0.57 | 3.0 ± 0.65 ***ab*** |
| **10 (48 h)** | 3.7 ± 0.41 ***a*** | 50.0 ± 0.37 | 2.8 ± 1.00 ***b*** |
| **20 (24 h)** | 3.7 ± 0.44 ***a*** | 86.7 ± 0.32 | 3.4 ± 1.00 ***a*** |
| **20 (48 h)** | 4.3 ± 0.60 ***a*** | 36.7 ± 0.11 | 1.9 ± 0.59 ***c*** |
| | ANOVA | | |
| p-level (F-value) | | | |
| IBA | 0.000 (38.2) | 0.000 (30.2) | 0.000 (144.5) |
| exposure | 0.095 (3.3) | 0.013 (8.6) | 0.001 (17.1) |
| IBA x exposure | 0.339 (1.2) | 0.039 (4.3) | 0.006 (8.2) |

Root formation was scored 3 weeks later. Each treatment was carried out in triplicate and each Magenta contained 9 shoots (n=135). Number of shoots per shoot explant, average shoot length, length of longest shoot root of shoot, seedlings exposed to IBA (indole-3-butyric acid) treatment, namely 10 and 20 mg $L^{-1}$ for 24 and 48 hours. The values are the mean ± SD (n = 5). Two-way ANOVA was applied to determine significant differences between treatments and time exposure to IBA (p-level and F-value are given). Different letters correspond to statistical differences between treatments (LSD post-hoc test, $p < 0.05$ level).

rooting phase with IBA 20 mg $L^{-1}$ for 24 h, which induced the production of high number of roots for shoot. The development of this protocol could improve in *in vitro* studies of *Q. pubescens* in various areas of research, such in implementation of *in vitro* mycorrhization of the genus *Tuber spp.*.

## REFERENCES


[1] Andersen, M.M., Landes, X., Xiang, W., Anyshchenko, A., Falhof, J., Østerberg, J.T., Olsen, L.I., Edenbrandt, A.K., Vedel, S.E., Thorsen, B.J., Sandøe, P., Gamborg, C., Kappel, K., Palmgren, M.G. (2015) Feasibility of new breeding techniques for organic farming. Trends in Plant Science. 20(7), 426-434.

[2] Lütken, H., Clarke, J.L., Müller, R. (2012) Genetic engineering and sustainable production of ornamentals: current status and future directions. Plant Cell Rep. 31, 1141-1157.

[3] Fletcher, J.C., Meyerowitz, E.M. (2000) "Cell Signaling Within the Shoot Meristem." Current Opinions in Plant Biology. 3(2000), 23–30.

[4] Kärkönen, A., Simola, L., & Koponen, T. (1999) Micropropagation of several Japanese woody plants for horticultural purposes. Annales Botanici Fennici. 36(1), 21-31.

[5] Kumar, N., Reddy, M.P. (2011) In vitro Plant Propagation: A Review. Journal of Forest Science. 27, 61-72.

[6] Herrmann, S., Buscot, F. (2008) Why and How Using Micro-propagated Trees rather than Germinations for Controlled Synthesis of Ectomycorrhizal Associations? A. Varma (ed.) Mycorrhiza, Springer-Verlag Berlin Heidelberg. 2008, 439-465.

[7] Riffaud, J.L., Cornu, D., Capelli, P. (1981) Use of in vitro culture for propagation of adult cherry trees (Prunus avium L.) selected in the forest. Agronomie, EDP Sciences. 1(8), 633-640 (in French).

[8] Druart, P. (1997) Optimization of culture media for in vitro rooting of Malus domestica Borkh. cv. Compact Spartan. Biologia Plantarum. 39, 67–77.

[9] Beaujard, F., Astié, M. (1983) Les Bruyères in vitro. I. In vitro culture of Erica x darleyensis and evidence of increased rhythmic growth of orthotropic twigs. Can. J. Bot. 61, 3533–3535 (in French).

[10] Rancillac, M. (1982) Vegattive multiplication in vitro and mycorrhizal synthesis: maritime pine, Hebeloma, Pisolithus. Les colloques de l´ÌNRA. 13, 351-355 (in French).

[11] Misson, J.P., Coumans, M., Giot-Wirgot, P., Gaspar, T. (1982) Induction of adventitious buds in Picea pungens in in vitro culture. Z Pflanzenphysiol 107, 161–167 (in French).

[12] Chalupa, V. (1984) In vitro propagation of oak (Quercus robur L.) and linden (Tilia cordata MILL.). Biol. Plant 26, 374–377.

[13] Vieitez, A.M., San-Jose, M.C., Vieitez, E. (1985) In vitro plantlet regeneration from juvenile and mature Quercus robur, L. J. Hortic. Sci. 60, 99–106. 58
[14] Favre, J.M., Juncker, B. (1987) In vitro growth of buds taken from seedlings and adult plant material in Quercus robur L. Plant Cell Tissue Organ Cult. 8, 49–60.
[15] Evers, P., Vermeer, E., Van Eeden, S. (1993) Rejuvenation of Quercus robur. Ann. Sci. Forestière. 50, 330–335.
[16] Greenwood, M.S. (1987) Rejuvenation of forest trees. Plant Growth Regul. 6, 1–12.
[17] Vermeer, E. (1991) Rejuvenation, micropropagation and field testing of Quercus robur. Acta Hortic. 289, 324–325.
[18] Vieitez, A.M., Sanchez, M., Amo-Marco, J., Ballester, A. (1994) Forced flushing of branch segments as a method for obtaining reactive explants of mature Quercus robur trees for micropropagation. Plant Cell Tissue Organ Cult. 37, 287–295.
[19] Franclet, A., Boulay, M., Ekkaoui, F., Fouret, Y., Verschoore-Martouzet, B., Walker, N. (1987) Rejuvenation. In: Bonga M, Durzan DJ (eds) Cell and tissue culture in forestry, vol. 1 General principles and biotechnology. Nijhoff, Dordrecht, pp 232–248.
[20] Pierik, R.L.M., Oosterkamp, J., Ebbing, M.A.C. (1997) Factors controlling adventitious root formation of explants from juvenile and adult Quercus robur fastigiata. Sci Hortic 71:87–92.
[21] Hackett, W.P. (1985) Juvenility, maturation, and rejuvenation in woody plants. Horticultural Reviews. 7, 109-155.
[22] McCown, B.H. (2000) Special symposium: in vitro plant recalcitrance. Recalcitrance of woody and herbaceous perennial plants dealing with genetic predeterminism. In Vitro Cell Dev Biol Plant. 36, 149–154.
[23] Chabukswar, M.A., Deodhar, M.A. (2006) Restoration of rooting competence in a mature plant of Garcinia indica through serial shoot tip grafting in vitro. Sci Hortic 108, 194–199.
[24] Monteuuis, O., Bon, M.C. (2000) Influence of auxins and darkness on in vitro rooting of micropropagated shoots from mature and juvenile Acacia mangium. Plant Cell Tissue Organ Cult. 63, 173–177.
[25] Dumas, E., Monteuuis, O. (1995) In vitro rooting of micro-propagated shoots juvenile and mature Pinus pinaster explants: influence of activated charcoal. Plant Cell Tissue Organ Cult. 40, 231–235.
[26] Meier-Dinkel, A., Becker, B., Duckstein, D. (1993) Micropropagation and ex vitro rooting of several clones of late-flushing Quercus robur L. Ann. Sci. For. 50, 319–322.
[27] Bellarosa, R. (1988) In vitro propagation of oaks (Q.suber, Q.pubescens, Q.cerris). Acta Hortic. 227, 433-435.
[28] Ostrolucká, M.G., Gajdošová, A., Libiaková, G. (2007) Protocol for micropropagation of Quercus spp.. In: Mohan Jainne S. and Häggman H. (eds.) Protocols for micropropagation of woody trees and fruits, Springer Verlag. 85-91.
[29] Di Santo, P., Cocozza, C., Tognetti, R., Palumbo, G., Di Iorio, E., Paura, B. (2016) A quick screening to assess the phytoextraction potential of cadmium and copper in Quercus pubescens plantlets. iForest - Biogeosciences and Forestry, 10(1), 93-98.
[30] Sezgin, M. (2018) In vitro propagation of Quercus pubescens Willd. (downy oak) via organogenesis from internodes. Fresen. Environ. Bull. 27, 5163-5172.
[31] Bonga, J.M. (1983) Vegetative propagation in relation to juvenility, maturity, and rejuvenation. - In Bonga J.M. Durzan D.J. –Tissue Culture in Forestry. 1982, 93-108
[32] Martins, A. (2008) In vitro mycorrhization of micro-propagated plants: studies on Castanea sativa Mill. Chapter 14 in Siddiqui, Akhtar and Futai (eds.), Mycorrhizae: Sustainable Agriculture and Forestry, Springer, Dordrecht, The Netherlands 319-334.
[33] Bonga, J.M. (1977) Applications of tissue culture in Forestry. In: Plant Cell Tissue and Organ Culture. eds. J. Reinert and Y. P. S. Bajaj, Springer-Verlag, New York pp 93-107.
[34] Flick, C.E., Evans, D.A., Sharp, W.R. (1983) Organogenesis. In: Handbook of Plant Cell Culture. Vol. I-Techniques for Propagation and Breeding. eds. D. A. Evans, W. R., Sharp, P. V., Ammirato, Y., Yamada. McMillan Publishing Co. New York pp. 13-81.
[35] Grellier, B., Letouzé, R., Strullu, D.G. (1984) Micropropagation of birch and mycorrhizal formation in vitro. New Phytol. 97, 591-599.
[36] Heslin, M.C., Douglas, G.C., (1986) Effects of ectomycorrhizal fungi on growth and development of poplar plants derived from tissue culture. Sci. Hort. 30, 143-149.
[37] Poissonier, M. (1986) In vitro mycorrhization of eucalyptus clones. Laboratory note, Research annals in Silviculture v.1985, 81-93 (in French).
[38] Tonkin, C.M., Malajczuk, N., Mc Comb, J.A. (1989) Ectomycorrhizal formation by micropropagated clones of Eucalyptus marginata inoculated with isolates of Pisolithus tinctorius. New Phytol. 111, 209-214.
[39] Martins, A. (2004) Controlled mycorrhization of Castanea sativa Mill.: physiological aspects of mycorrhization in vitro and ex vitro. PhD thesis in Plant Biology/ Plant Biotechnology. Faculdade de Ciências de Lisboa. Universidade Clássica de Lisboa. pp. 506 (in Portuguese).

[40] Martins, A., Barroso, J., Pais, M.S., (1996) Effect of ectomycorrhizal fungi on survival and growth of micro-propagated plants and seedlings of Castanea sativa Mill. Mycorrhiza. 6, 265-270.
[41] Martins, A., Pais, M.S. (2005) Mycorrhizal inoculation of Castanea sativa Mill. micro-propagated Plants: Effect of mycorrhization on growth. Acta Hortic. 693, 209-217.
[42] Herrmann, S., Munch, J.C., Buscot, F. (1998) A gnotobiotic culture system with oak micro-cuttings to study specific effects of mycobionts on plant morphology before and in the early phase of ectomycorrhiza formation by Paxillus involutus and Piloderma croceum. New Phytol. 138, 203-212.
[43] Díez, J., Manjón, J.L., Kovács, G.M., Celestino, C., Toribio, M. (2000) Mycorrhization of vitroplants raised from somatic embryos of cork oak (Quercus suber L.) Appl. Soil Ecol. 15, 119-123.
[44] Nowak, J. (1998) Benefits of in vitro "biotization" of plant tissue cultures with microbial inoculants. In vitro Cell. Dev. Biol. Plant. 34, 122-130.

**Received:** **08.08.2020**
**Accepted:** **16.08.2020**

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