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# Forced flushing of branch segments and induction of primary somatic embryogenesis on mature Moroccan Cork Oak (*Quercus suber* L.) tree

**Safaâ Rahmouni[1*], Lamarti Ahmed[2]**

**[1]Laboratory of plant biotechnology, Department of Biology, Faculty of Sciences, Abdelmalek Essaadi University, Tetouan, Morocco**
**[2]AVAPAM, 93030 Tetouan, Morocco**

***Corresponding author: safaarahmouni@hotmail.fr**

**Abstract:** The present work aimed to study the induction of primary somatic embryogenesis in Moroccan cork oak (*Quercus suber* L.) using expanding leaves from epicormic shoots derived from crown branch segments. Branches were cut to obtain cuttings 20 cm in length. Cuttings were classified into five classes according to their diameter and cork thickness and were cultivated under controlled conditions to promote budbreak and epicormic shoot development. Budbreak occurred after 13 days and lasted 32 days, with cuttings longevity of 45 days (from the first day of cuttings cultivation until the last day of budbreak). Cuttings diameter influenced the percentage of budbreak, the mean number of buds per cutting, the mean length of shoots, the mean number of leaves per shoot, and the mean number of leaves per cutting. In general, large diameter cuttings (14.21 and 74.58 mm) with cork provided the best results. Primary somatic embryogenesis was successfully induced; however, the frequency of responsive leaves was generally low. This limitation was overcome by establishing secondary somatic embryogenesis, resulting in continuous and unlimited embryo production. These results highlight expanding leaves of epicormic shoots derived from cuttings as a promising explant source for somatic embryogenesis and vegetative propagation strategies in cork oak.

**Keywords:** Cork oak, *Quercus suber* L, cutting, budbreak, somatic embryogenesis.
**Abbreviations:** SE_Somatic Embryogenesis; PGRs_plant growth regulators; BAP_6-Benzylaminopurine; NAA_1-Naphthalene Acetic Acid; MS_Murashig and Skoog.

## Introduction

Cork oak (*Quercus suber* L.) is an evergreen oak species native to many countries of the Mediterranean basin. It belongs to the family *Fagaceae*, section *Suber* of the subgenus *Cerris*, genus *Quercus* (Ceballos and Ruiz de la torre, 1979). Cork oak forests cover 2.1 million hectares of the world forest surface (Mechergui et al., 2024; Leite et al., 2020) and play important ecological, economic, and social roles. Nevertheless, cork oak populations are declining in many regions and are considered endangered due to several factors, including the absence of natural regeneration, overexploitation and overgrazing (Capote et al., 2019; Catry et al., 2009). To overcome these problems, new vegetative propagation techniques, such as organogenesis and somatic embryogenesis, have been used in order to preserve this valuable ecological heritage.
Somatic embryogenesis (SE) is considered one of the most powerful techniques for clonal multiplication, synthetic seed production, germplasm conservation, cryopreservation, and genetic improvement (Gang et al., 2025; Martínez et al., 2024; Guan et al., 2016). This approach has been successfully applied to several forest species (Ben Ali et al., 2025; Bradaï and Sánchez-Romero, 2021; Martínez et al., 2017a; Rathore et al., 2012). SE allows the regeneration of trees from asexual embryos that arise from cells of a tissue or an organ (Pires et al., 2025). Moreover, the process of SE enables the production of a large number of transgenic plants over a short period of time (Giridhar et al., 2004). SE can occur either indirectly through an intermediate callus phase or directly from explants under specific culture conditions, without an intermediate callus phase (Martínez et al., 2024; Guan et al., 2016; Yang and Zhang, 2010).
The induction of somatic embryo can be influenced by the physiological or developmental stage of the donor tree from which the initial explants are directly or indirectly obtained, and also by tissue collection time (whether during the dormant season or not) (Valladares et al., 2006; Bonga, 2004; Toribio et al., 2004). Somatic embryos are morphologically similar to zygotic embryos. They are bipolar and contain typical embryonic organs, including the radicle, hypocotyl, and cotyledons. However, they develop through a different developmental pathway (Von Arnold et al., 2002).
The induction of primary somatic embryogenesis from cuttings is widely used in cork oak (Toribio et al., 2005; Hernandez et al., 2003; Pinto et al., 2002). In the absence of mature material with juvenile physiological characteristics for

micropropagation, cuttings have been induced to form epicormic shoots, which are more responsive explant sources for the induction of primary somatic embryogenesis and the propagation of mature trees (Martínez et al., 2017b; Martínez et al., 2012). This is probably due to culture conditions where controlled temperature and light are more suitable for shoot growth (Gomes and Canhoto, 2009). The ability of oak species to form epicormic shoots varies according to genotype, collection date, and cutting diameter (Valladares et al., 2006; Toribio et al., 2004).

Unlike primary somatic embryogenesis induced from explant cells, secondary somatic embryogenesis allows the production of new somatic embryos from pre-existing somatic embryos (Bogdanović and ćukovic, 2025; Guan et al., 2016). It occurs continuously on media supplemented with low concentrations of cytokinins, auxins, or both. (Bogdanović and ćukovic, 2025; Mallón et al., 2013; Wilhelm, 2000), or even in the absence of growth regulators, resulting in a recurring process that can be maintained for several years without any apparent decrease in multiplication capacity, simply through monthly subculturing onto fresh medium (Toribio et al., 2005). This process confers on somatic embryogenesis its potential for clonal mass propagation.

In this work, we evaluated the effect of cuttings diameter, taken from cork oak tree of the Bouhachem-Chefchaouen region, on the percentage of budbreak, the mean number of buds per cutting, the mean length of shoots, the mean number of leaves per shoot, and the mean number of leaves per cutting. In addition, we investigated the possibility of inducing primary somatic embryogenesis from the expanding leaves of epicormic shoots obtained from the cuttings.

## Results

### *Culturing and budbreak*

Cuttings placed in the growth chamber were irrigated daily with tap water. The results presented in Table 1 indicate the budbreak (day of appearance of the first bud), the budbreak period and the longevity of the cuttings (from the first day of cuttings cultivation until the last day of budbreak).

Budbreak began after 13 days of culturing. The budbreak period lasted 32 days, and the longevity of cuttings was 45 days (Table 1). After bud appearance, epicormic shoots developed, followed by leaf formation (Fig. 1).

**Table 1.** Budbreak, budbreak period and longevity of cuttings from *Quercus suber* L..

| Cork oak | Month | Budbreak (days) | Budbreak period (days) | Longevity of cuttings (days)* |
|---|---|---|---|---|
| Bouhachem-Chefchaouen | April | 13 | 32 | 45 |

*Longevity of cuttings (days) = budbreak + budbreak period.

**Figure 1.** Appearance of buds, epicormic shoots and leaves on *Quercus suber* L. cuttings.

### *Effect of cuttings diameter on the percentage of budbreak and the mean number of buds per cutting*

The percentage of budbreak and the mean number of buds per cutting varied according to cuttings diameter. In general, cuttings of the first class (diameter < 12.59 mm and without cork) showed the lowest percentage of budbreak (Fig. 2). Cuttings of class 3 (28.51-42.15 mm) and class 5 (57.88-74.58 mm) showed the highest budbreak percentages (100%) (Fig. 2). Cuttings belonging to classes 2, 3, 4 and 5 exhibited a higher mean number of buds per cutting. The recorded values were 11.49±0.64a; 11.91±0.57a; 9.94±0.78ab and 11.14±0.64a, respectively (Fig. 3). No correlation was observed between the increase in cuttings diameter, cork thickness, the percentage of budbreak, and the mean number of buds per cutting.

Effect of cuttings diameter on the mean length of shoots, the mean number of leaves per shoot and the mean number of leaves per cutting.

The highest mean length of shoots (20.92±3.04a) was obtained from cuttings of class 3 (28.51-42.15 mm). The values recorded for the other cutting classes were all less than 14 mm (Fig. 4). The mean number of leaves per shoot obtained from

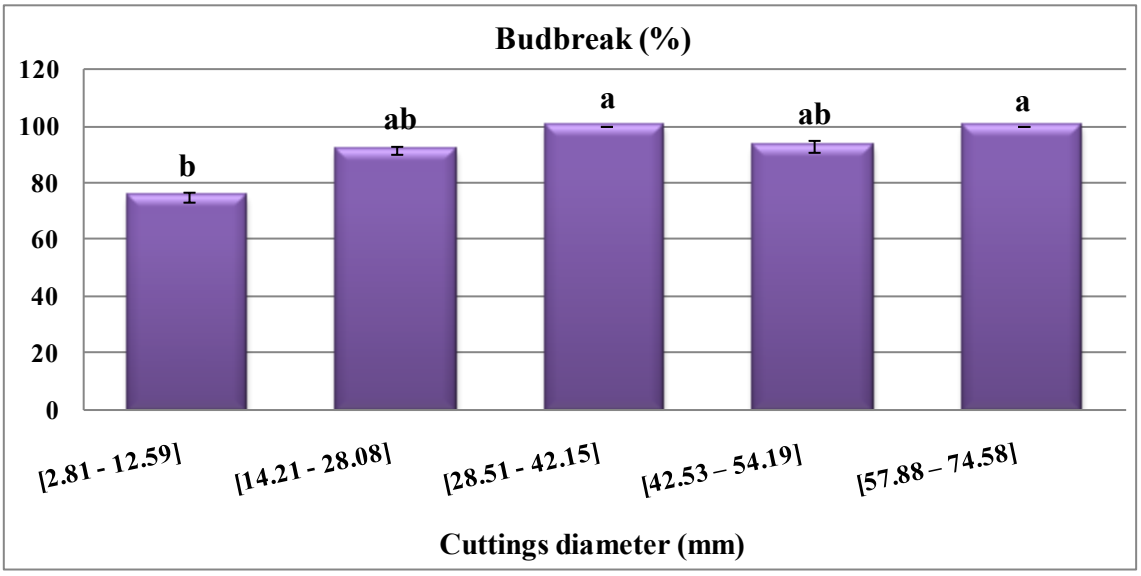


**Figure 2.** Effect of cuttings diameter on budbreak in *Quercus suber* L..

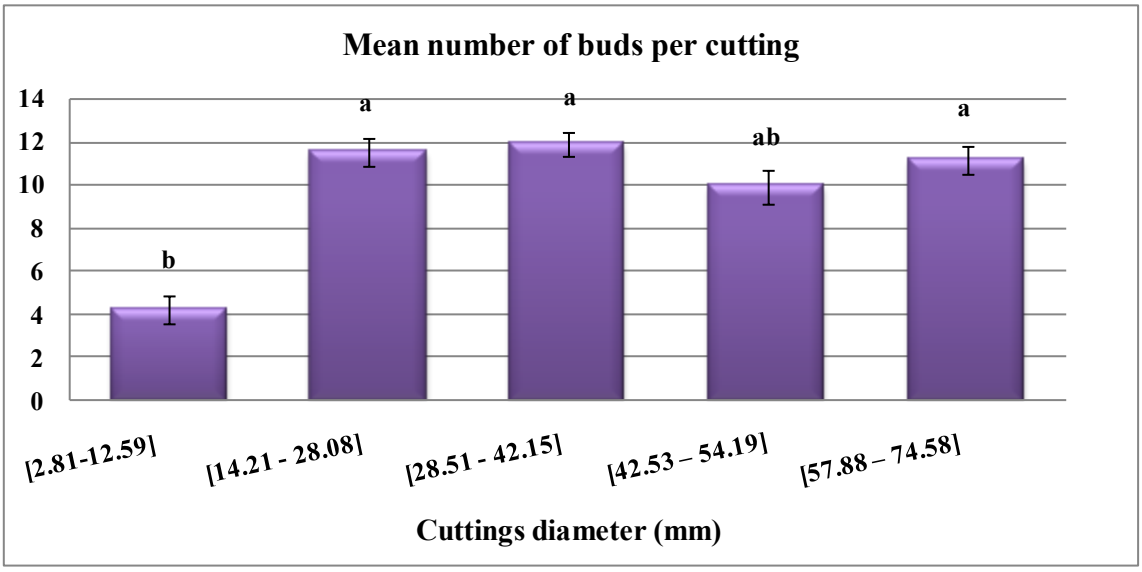


**Figure 3.** Effect of cuttings diameter on the mean number of buds per cutting in *Quercus suber* L..

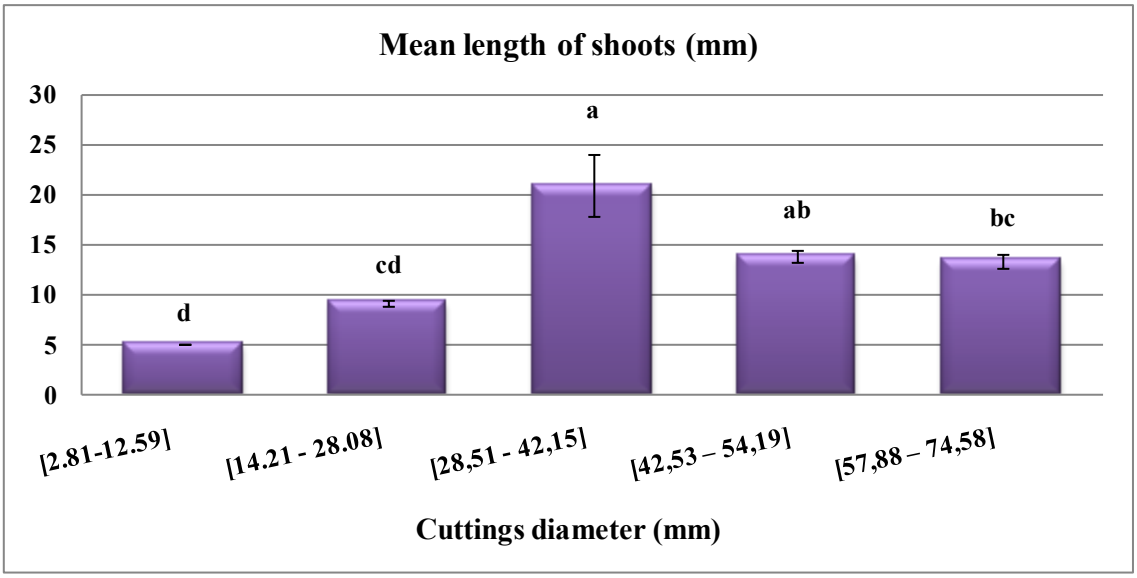


**Figure 4.** Effect of cuttings diameter on the mean length of shoots in *Quercus suber* L..

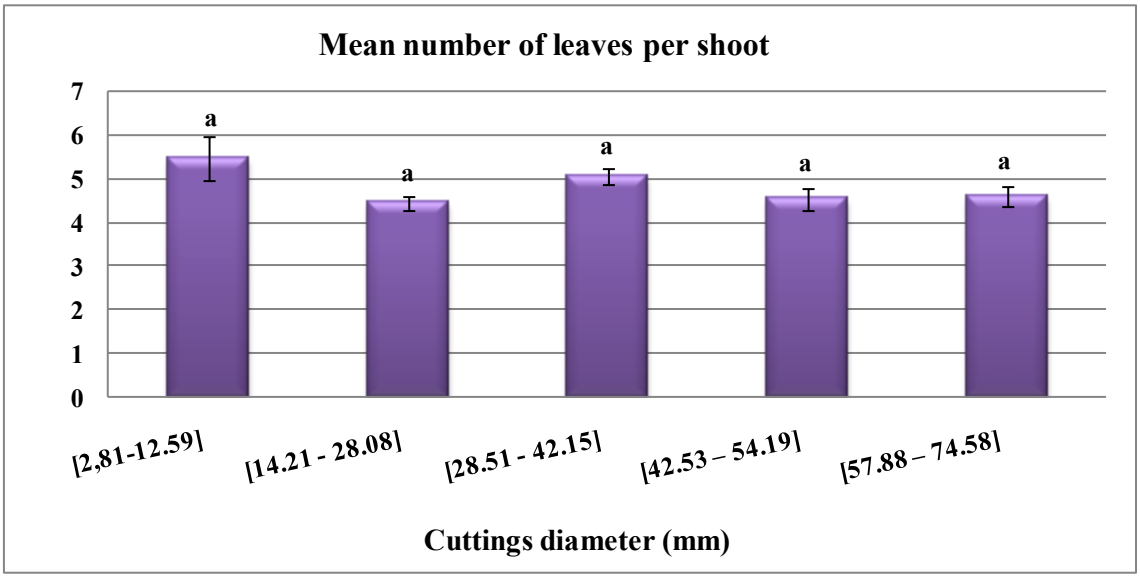


**Figure 5.** Effect of cuttings diameter on the mean number of leaves per shoot in *Quercus suber* L..

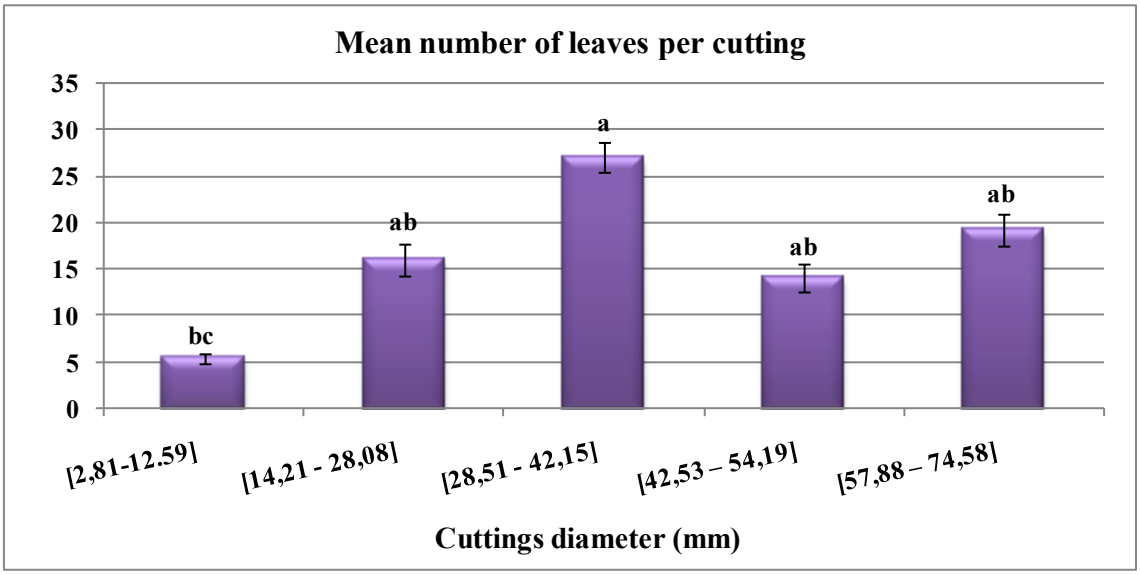


**Figure 6.** Effect of cuttings diameter on the mean number of leaves per cutting in *Quercus suber* L..

**Figure 7.** *Quercus suber* L. leaves after a few days of culture on the preconditioning medium.

the different cutting classes was greater than four leaves (Fig. 5). The maximum mean number of leaves per cutting was recorded for cuttings of class 3 (27.1 ± 1.54a) (Fig. 6).

***Induction of somatic embryogenesis***

Culture on the preconditioning medium promoted the release of phenolic compounds from the leaves and allowed the elimination of contaminated explants (Fig. 7). Leaves showed different degrees of proliferation and necrosis. Some became

**Table 2.** Different cutting classes from *Quercus suber* L. tree collected in the Bouhachem-Chefchaouen region.

| Origin of cuttings | Cuttings diameter (mm) | | Cork thickness (mm) |
|---|---|---|---|
| Bouhachem-Chefchaouen | Classe 1 | [2.81 – 12.59] | - |
| | Classe 2 | [14.21 – 28.08] | [0.46 – 3.63] |
| | Classe 3 | [28,51 – 42.15] | [0.84 – 5.48] |
| | Classe 4 | [42.53 – 54.19] | [2.89 – 6.88] |
| | Classe 5 | [57.88 – 74.58] | [5.96 – 9.54] |

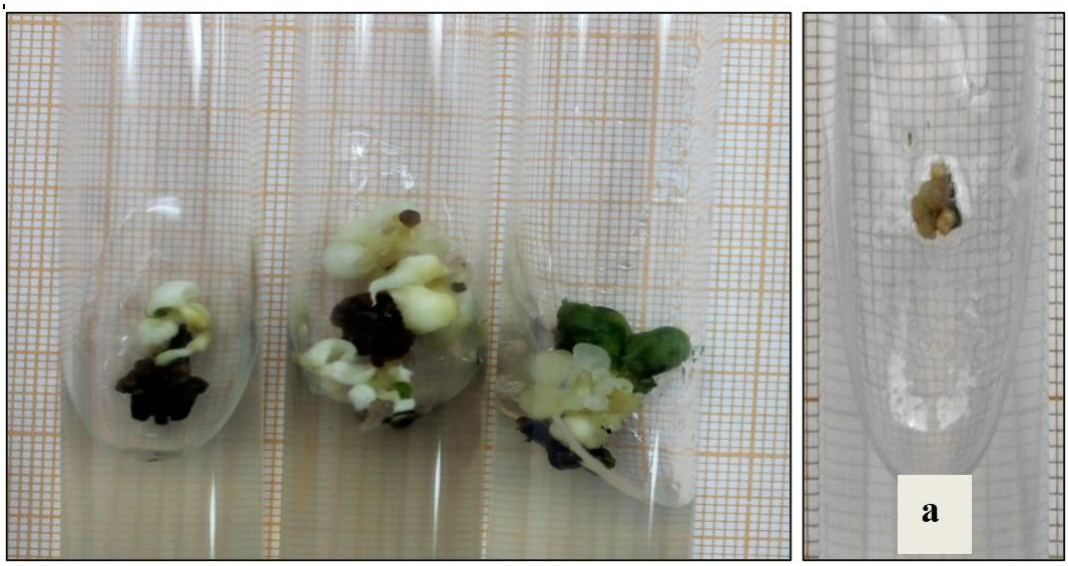


**Figure 8.** Somatic embryos (a) and callus (b) formed on the leaves of *Quercus suber* L..

necrotic a few days after culture on the preconditioning medium, whereas other explants gave rise to callus formation. Some leaves produced somatic embryos at the end of the secondary induction phase. These somatic embryos were localized at the base of the leaves and were characterized by the presence of two cotyledons and roots attached to the leaves from which they originated (Fig. 8).

Overall, the percentage of leaves producing somatic embryos was low. This situation was compensated by regular subculturing (every two months at 25°C and in the dark) of somatic embryos on a medium containing the macronutrients of Margara (1978, $N_{30}K$), micronutrients, vitamins and Fe-EDTA of MS, supplemented with 3 % glucose and 0.7 % agar (bacteriological type E). The choice of the culture medium and the conditions was based on the study conducted by Ben Ali and Lamarti (2014).

When isolated and subcultured onto fresh medium, embryos gave rise to secondary somatic embryos. They were essentially formed on the root of the primary somatic embryo (Fig. 9). This process of secondary somatic embryogenesis stopped when the somatic embryos became older, matured, and turned green.

**Discussion**

An alternative procedure can be used for micropropagation in the absence of mature material with juvenile physiological characteristics. This approach is based on the induction of branch segments to form epicormic shoots, which constitute a source of more reactive and rejuvenated explants (Martínez et al., 2012; Martínez et al., 2017b). Gomes and Canhoto (2009) showed that epicormic shoots derived from branches of adult trees represent a good source of explants for the propagation of mature trees. This response is probably due to the culture conditions, in which controlled temperature and light are more favorable for shoot growth.

In the present study on Moroccan cork oak, we evaluated the effect of cuttings diameter on the percentage of budbreak, the mean number of buds per cutting, the mean length of shoots, the mean number of leaves per shoot, and the mean number of leaves per cutting. The first buds appeared after 13 days of culture. In contrast, in *Quercus robur*, bud emergence on branch segments was observed after 6-8 days, regardless of the branch collection date (Valladares et al., 2006). In *Arbutus unedo*, cuttings with diameter ranging from 0.4 to 2.5 cm exhibited epicormic shoot development after 15 days of culture (Gomes and Canhoto, 2009).

The use of cuttings collected at different periods of the year has also been reported to induce the formation of epicormic shoots in *Quercus robur*, providing an abundant source of expanding leaves for the induction of embryogenic cultures (Valladares et al., 2006). In our study, epicormic shoots were obtained from cork oak cuttings collected in April. Similarly, Chmielarz et al. (2023) used branches of *Quercus robur* L. collected in late April, with diameters of approximately 2-4 cm and lengths of 30-40 cm. They indicated that the mean length of epicormic shoots was strongly influenced by the age of the donor trees. Similar shoot lengths were observed in material derived from younger trees (approximately 20–200 years old), whereas greater variability was recorded in shoots originating from older trees (approximately 300–800 years). Woody shoots (40-50 cm in length and 3-5 cm in diameter) from tow strawberry tree genotypes were collected during September

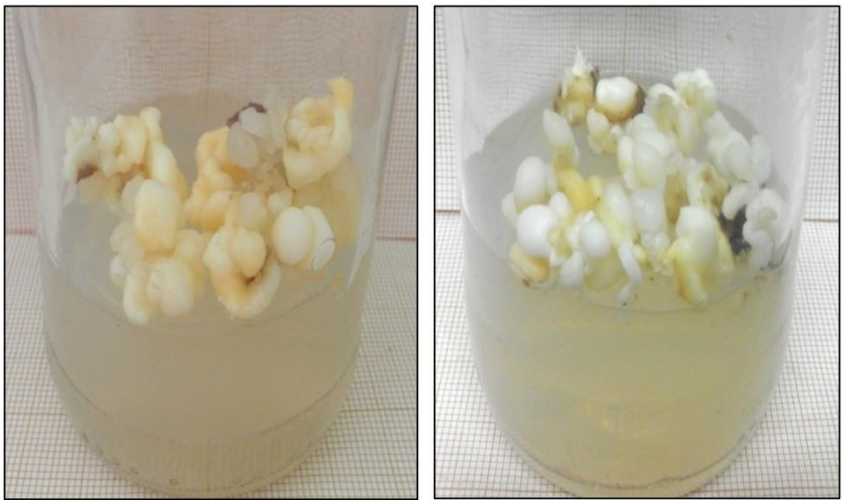

**Figure 9.** Subculture of *Quercus suber* L. somatic embryos on $N_{30}K$ medium for multiplication.

and October to induce the sprouting of epicormic shoots (Sulusoglu Durul and Memis, 2022). Van Sambeek et al. (2002) reported that branches with diameters ranging from 20 to 100 mm, collected in February, March and April from ten hardwood species, produced higher numbers of buds and shoots. Valladares et al. (2006) obtained the highest embryogenic rate from explants derived from *Quercus robur* branches collected in November, at the onset of dormancy. Epicormic shoots of 5-10 cm in length were obtained from branches (10-25 mm in diameter) of *Schinopsis balansae* (Anacardiaceae) collected between November and December (Sansberro et al., 2003). Brennan et al. (2017) induced leaf formation in cuttings of twelve oak species collected between February and May and maintained in a growth chamber under favorable conditions. Sabja et al. (2008) used shoots formed on branches collected in spring for the in vitro culture of *Nothofagus alpina*.

Our study indicates that cuttings diameter affected budbreak percentage, the number of buds per cutting, shoot length, and leaf production. In general, cuttings with diameters between 10.1 and 74.58 mm produced the best responses, whereas those with diameters less than 10.1 mm and without cork tissue presented the lowest performance. Similarly, Martinez et al. (2017b) showed that the mean length of shoots is influenced by branch diameter, with Thicker segments producing longer shoots than thiner ones. Valladares et al. (2006) evaluated the effects of collection period and cuttings diameter in *Quercus robur* and reported that the mean number of shoots increased with increasing cutting diameter; notably, all cuttings with a diameter greater than 20 mm and collected in March produced shoots. This response may be related to the physiological status of the cuttings, associated with the seasonal mobilization of hormones and nutrients. In *Toona ciliata*, branches of three diameter classes (20-30; 30-100 and 100-200 mm) were used to induce the formation of epicormic shoots, which began to appear on all branches after 5 to 10 days of culture. A greater development of epicormic shoots, exceeding 2 cm in length, was observed in segments with diameters less than 100 mm (Mroginski et al., 2003). Cuttings with diameters ranging from 10 to 40 mm were also succefully used to induce epicormic shoots in *Quercus suber* L. (Hernandez et al., 2009; Hernandez et al., 2008), *Quercus ilex* L. (Blasco et al., 2013), *Quercus alba* L. (Corredoira et al., 2012; Vieitez et al., 2009), *Quercus bicolor* Willd., and *Quercus rubra* L. (Vieitez et al., 2009; Toribio et al., 2004). In *Alnus glutinosa*, cutting thickness also affected the percentage of shoot development, with the best results obtained from cuttings measuring 0.5 to 2 cm in diameter (San José et al., 2013). In *Balanites aegyptiaca,* the percentage of budbreak was higher in large-diameter cuttings (30.8%) than in small-diameter ones (12.6%), which was attributed to their higher content of meristematic tissues (Massaoudou et al., 2022).

The ability of oaks to form epicormic shoots varies according to genotype (Valladares et al., 2006). Using branches of *Quercus lusitanica* (0.5-1.5 cm in diameter) from two genotypes, San José et al. (2017) reported that one genotype produced a significantly higher number of shoots than the other. Genotype also influenced both the total number of shoots and the mean shoot length formed on branches of *Quercus ilex* (2-4 cm in diameter) (Martinez et al., 2017b). Similarly, in *Quercus robur*, genotype and the season of cutting collection affected the frequency of somatic embryos formation (Toribio et al., 2004). San José et al. (2013) showed that shoots appeared in branch segments of all evaluated genotypes of *Alnus glutinosa* after 10-15 days of culture. Shoot development was influenced by both the collection date and genotype. Van Sambeek et al. (2002) demonstrated that the use of branches from older trees reduced the number of shoots.

To promote the development of epicormic shoots in *Arbutus unedo*, Gomes and Canhoto (2009) tested the effect of BAP on uncovered and covered branches (with transparent polythene plastic). They demonstrated that the number of shoots per branch was not affected by the applied treatment; however, the combined use of BAP and branch covering resulted in longer shoots compared with uncovered branches treated with BAP. Brhadda et al. (2003) showed that the culture medium influences the outgrowth of axillary buds as well as the number and length of shoots derived from microcutting in Moroccan olive trees. Van Sambeek and preece (1999) evaluated the effect of environmental conditions on epicormic shoot production from cuttings taken from *Juglans nigra*, *Fraxinus americana*, *Quercus alba* and *Quercus rubra*. They found that water-treated cuttings were more effective in inducing epicormic shoots across all four species.

Chalupa (1993) used cuttings of *Quercus robur* L. and *Quercus petraea* (Matt.) Liebl., collected between May and July, from six-year-old hedged stock plants as well as from seedlings and trees of different ages for rooting. The author reported that the capacity of cuttings to form adventitious roots decreased rapidly with increasing plant age. Similarly, Kour et al. (2022)


**Figure 10.** *Quercus suber* L. cuttings of different sizes. Selected tree (a), cuttings before treatment (b and c) and after treatment (d and e).

used cuttings 20-22 cm in length and 6-8 cm in diameter to evaluate the effect of different concentrations of Indole-3-butyric acid and various growth conditions on adventitious root formation in rough lemon stem cuttings. Metaxas et al. (2004) used cuttings taken from eight phenotypically different *Arbutus unedo* plants growing in the field and seven phenotypically different *Taxus baccata* plants grown in the greenhouse under controlled environmental conditions to evaluate the effect of genotype and growth regulators on rooting. They observed that genotype had significant effects on the number and length of roots as well as on the rooting percentage.

Induction of primary somatic embryogenesis from different explants is possible in cork oak (Nejjar El Ansari et al., 2021; Testillano et al., 2018; Pérez et al., 2015; Jiménez et al., 2013; Pintos et al., 2010; Toribio et al., 2005). However, the frequency of obtaining embryogenic lines is generally low. This limitation can be compensated by the establishment of secondary somatic embryogenesis. In our study, primary somatic embryogenesis was induced from young apical leaves excised from epicormic shoots. Primary somatic embryos were obtained by transferring the leaves to culture media according to a specific protocol. Similarly, several studies have reported the induction of somatic embryogenesis from expanding leaves of epicormic shoots forced to sprout from branch segments collected from 60-year-old and 100-year-old cork oak trees (Ben Ali, 2014; Jiménez et al., 2013; Loureiro et al., 2005; Toribio et al., 2005; Valladares et al., 2004; Hernandez et al., 2003; Pinto et al., 2002). Induction of primary somatic embryogenesis in woody species is also realized from different explants (Yan et al., 2025; Fei et al., 2024; Gomez-Garay et al., 2018; Martinez et al., 2015; Barra-Jimenez et al., 2014; Mallón et al., 2013; Corredoira et al., 2012; Pena-Ramirez et al., 2011).

## Materials and methods

### *Collection of cuttings*

Crown branches of different sizes were collected in April 2014 from cork oak tree (*Quercus suber* L.) located in the Bouhachem-Chefchaouen region (Western Rif I2, Morocco; latitude N 35°18'520'' and longitude W 005°40'622''). The

branches were cut into 20 cm long segments (cuttings) and classified into five classes according to their diameter and cork thickness. Cuttings without lateral branches or leaves were treated with 10% (v / v) commercial bleach for 30 min. After two successive rinses in water for 15 min each, they were left to dry for one day (Table 2 and Figure 10).

***Culturing of cuttings***

After disinfection, the cuttings were planted in pots containing peat and placed in a growth chamber to induce the formation of epicormic shoots. The culture was maintained at 25 ° C and 80–90% relative humidity under a 16-h photoperiod, with a light intensity of 90–100 µmol $m^{-2}$ $s^{-1}$ provided by cool-white fluorescent lamps. Cuttings were watered daily and treated with bleach when required (appearance of fungi). As soon as the first bud appeared, daily observations were carried out. For each cutting class, the percentage of budbreak, the mean number of buds per cutting, the mean length of shoots, the mean number of leaves per shoot, and the mean number of leaves per cutting were recorded.

***Sterilization of leaves***

Previous studies have shown that only small leaves (0.5-1.5 cm) are capable of producing somatic embryos (Hernandez et al., 2003). Expanding leaves (approximately 0.5 cm in length, measured from petiole to tip) were excised from the growing epicormic shoots. Leaves were surface-sterilized by vigorous shaking in 70% ethanol for 30 s, followed by immersion in a 10% (w/v) calcium hypochlorite solution ($Ca(ClO)_2$) supplemented with three drops of Tween 80 for 30 min. This step was followed by immersion in 10% (v/v) Mercryl foaming solution supplemented with three drops of Tween 80 for 10 min. Finally, the leaves were rinsed three times with sterile distilled water for 5, 5 and 10 min, respectively.

***Culture of leaves***

After sterilization, leaves were cultured following the protocol proposed by Hernandez (2007) for the induction of primary somatic embryogenesis, with a modification in the agar concentration (Ben Ali, 2014). The induction protocol consisted of four phases. The culture medium used during the pre-conditioning phase consisted of Gamborg's (1966, B5) macronutrients, Murashig and Skoog (1962, MS) micronutrients, vitamins and Fe-EDTA, and was supplemented with 1% sucrose and 0.7% agar (bacteriological type E). The primary induction medium consisted of Schenk and Hildebrandt (1972, SH) macronutrients, MS micronutrients, vitamins and Fe-EDTA, supplemented with 3% sucrose and 0.7% agar, and contained 10 µM NAA and 10 µM BAP. The secondary induction medium had the same composition as the primary induction medium, but the concentrations of plant growth regulators (PGRs) were reduced to 0.5 µM NAA and 0.5 µM BAP. The culture medium for the proliferation phase had the same components but was free of PGRs. Surface-sterilized leaves were cultured on the pre-conditioning medium in the dark at 25±2°C for 8 days. They were then transferred to the primary induction medium and cultured in the dark at 25±2°C for 30 days. Subsequently, the explants were subcultured onto the secondary induction medium and placed in a growth chamber under a 16 h photoperiod (4000 lux (100 µmol $m^{-2}s^{-1}$)) at 25±2°C for 30 days. Leaves were then transferred to the proliferation medium under the same conditions (16 h photoperiod / 25 ± 2°C) for 45 days. All media were adjusted to a pH of 5.7-5.8 prior to autoclaving at 121°C for 20 min.

***Statistical analysis***

For each cutting class, the experiment was carried out with two replicates of 24 explants. Results were analysed using one-way analysis of variance (ANOVA), and means were compared using Duncan's multiple range test at $p \leq 0.05$.

## Conclusion

Cork oak (*Quercus suber* L.) is one of the most important Mediterranean tree species. However, it is under serious threat, highlighting the urgent need to develop efficient vegetative propagation techniques to ensure its conservation and improvement. In the present study, we demonstrated the feasibility of obtaining epicormic shoots from Moroccan cork oak cuttings of different diameters. Cuttings with a diameter ranging from 10.1 to 74.58 mm produced the best results in terms of budbreak percentage, mean number of buds per cutting, mean length of shoots, mean number of leaves per shoot, and mean number of leaves per cutting. The young apical leaves obtained from the epicormic shoots were succefully used to induce primary somatic embryogenesis. Primary somatic embryos were obtained after a series of leaf transfers onto culture media according to a specific protocol. They were then multiplied through the establishment of secondary somatic embryogenesis.

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