A Cork Cell Wall Approach To Swelling And Boiling

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Abstract

The bark of cork oak (*Quercus suber L.)* is mostly used for cork stopper production, whereas bark is undergoing a series of industrial procedures, boiling usually leading to changes in the characteristics of the tissue. Trees are traditionally grown under natural conditions; however, irrigation is now being used in plantations. These permanent water availability affects cork-oak development, while its effects on industrial procedures is unknown. This study provides a first insight into the behaviour of the cell walls of cork during the process of swelling and boiling when trees have been grown under irrigation, subject to a specific water regime. Cork tissue was analysed using environmental and scanning electron microscopy under three regimes: raw conditions; following immersion in water; and after boiling. Additionally, the radial expansion of samples was determined. The results showed greater cell-wall expansion in cork from the irrigated site than cork from the traditional rainfed plot, when hydrated for 24h. After boiling, the cell walls of the rainfed site were thinner than in the raw stage, in contrast to the irrigated cork. This study suggests that irrigation during cork-oak growth produces a higher capacity for adsorption, increasing cell-wall thickness from the raw stage to the boiling stage.

Introduction

The cork industry is one of the major forestry sectors that contributes to Portuguese Gross Domestic Product. It accounts for 1.2% of total employment in the Portuguese manufacturing sector[1] and sustains the economy in inland areas.

A range of activities fall under the umbrella of the cork industry: cork preparation, the manufacturing of cork stoppers and other cork products, the wholesale trade, and the production of other cork products like agglomerates. Cork exports account for 2% of all Portuguese manufactured products, while 2% of all companies in Portugal are involved in the trade. However, there has been a reduction of 2.7% in the volume of cork production over the last 10 years[1]. Levels of forestry productivity have decreased as a consequence of cork-oak decline in current areas of implantation, associated with several factors, in which climate change – associated with increasingly frequent or severe drought events and heatwaves[2] – plays a role. Climate change due to greenhouse gas emissions associated with heat stress has also impacted other species, leading to the mortality of species and the vulnerability of forest ecosystems.[3] In order to promote tree vitality, tree growth and cork production, a new silviculture modelof fertirrigation in stands where water is available has been introduced (the Regasuber, Irricork and GO-Regacork projects) and is currently the subject of a number of studies in progress.

Cork from *Quercus suber* L. is a sustainable, renewable material produced by means of the phellogen of cork cambium, which is most active during two periods: spring and autumn. Through periclines divisions, phellogen produces an interior live tissue called phelloderm and an exterior group of dead cells called phellem[4]. Cork rings present different cell dimensions and cell-wall characteristics along the extension of the annual cork-ring[5]. Cells produced during the autumn growth period (late cork) are smaller in size than cells produced during the spring growth period (early cork). Cell rows growth in the radial direction after the differentiation of cork cells, with the appearance of layers of bricks, in the radial and transverse directions[6]. Spring growth represents 90 to 95% of the total volume and accounts mainly for cork cell characteristics[6]. These cell-wall features and biochemical properties of cell walls have an influence on some cork characteristics[6]. During cell growth there is a thickening of the cellular membranes which is the result of the deposition of some cell-wall layers with different structures and a different chemical composition[7]. Pereira[8] determined the chemical composition of cork throughout Portugal as 42.8% suberin, 22% lignin and 16.2% extractives. According to Sen et al.[9], compounds differ in accordance with regional location: Turkish and Bulgarian cork contains higher levels of lignin and lower levels of extractives than Portuguese cork. Inside the cell walls, plasmodesmatas may be observed, which are responsible for cell connections by crossing cell walls at the sub-microscopic level[6]. Plasmodesmata are intercellular organelles found in plants consisting of pores and channels, lying between individual plant cells and connect cells. Cell size depends on seasonal growth. Spring cells are larger and show a greater range in size (30 to 40 µm) while cell-wall thickness varies from 1 to 1.5 µm, whereas the height of autumn cells is 10 µm less and cell walls are twice as thick[10]. Such characteristics are important for the type of cork goods produced and are often studied by means of microscopic approaches, such as scanning electron microscopic (SEM) approach[6,7]. The use of environmental scanning electron microscopy (ESEM) enables in addition the dynamic process of water interaction and the thermodynamic stability of

moist[11] samples to be analysed without specific sample preparation[12]. Although this method is not often used in the cork tissue analysis, it is used in wood research with great potential[11]. ESEM can preserve samples as hydrated due to its saturated water vapour environment within the analytical chamber[13], so it was used to analyse cork cells after 24h hydration.

At the end of each cork-growth cycle, the cork harvest is obtained by means of the physical rupture of the phellogen cells when they are most active, from May to August. After that, a new traumatic phellogen is formed by means of a meristematic activation[7].

After harvesting, there are several stages in the industrial preparation of cork such as waiting and boiling procedures. First of all, it needs to be stabilized in order for some tangential tensions to be released and a reduction in moisture content to be achieved; the second stage is the boiling process, used to remove some residues and to increase thickness by 15%. After two days' steady, planks are ready for the stopper manufacturing process to begin. In accordance with the literature, the boiling procedure produces changes in some cork characteristics, such as cork thickness, porosity and density[7]. Cork is a poor water conductor due to the lack of intercellular spaces and the presence of gas in the cells; nevertheless it can absorb water up to 10% w/w over sorption[14], causing cell-wall swelling. Water diffuses throughout the cell walls until saturation is reached and it penetrates the cells. Through a process of evapotranspiration and condensation, water can penetrate the cell walls[15]. The aim of this study was to evaluate the cell-wall thickness of cork from two study sites under three set of conditions: under raw conditions, after immersion in water and after boiling. The cork expansion observed in macro samples were also analysed, simulating industrial procedures. Our hypothesis is that, due to cork from trees subjected to fertirrigation having thinner cell walls[16], a different behaviour when swelling and boiling will occur. Such information would contribute to providing an understanding of the behaviour of cork grown under a different water regime on the industrial boiling procedure. SEM under high vacuum conditions was used for raw conditions and after boiling, while ESEM was used on samples after swelling following 24h hydration.

Results

Cork from the fertirrigated site showed raw cell walls measuring 1.10 ± 0.30 µm, hydrated cell walls measuring 1.44 ± 0.40 µm and cell walls after boiling measuring 1.24 ± 0.30 µm, while cork from the rainfed site presented raw cell walls measuring 1.38 ± 0.34 µm, hydrated cell walls measuring 1.42 ± 0.33 µm and cell walls after boiling measuring 1.30 ± 0.26 µm (Fig. 1). While the cell walls of cork from the fertirrigated plot are thinner than those of cork from the rainfed plot under raw conditions[16], the former expanded to a greater degree during hydration (Fig. 1). After boiling, both treatments presented a decrease in thickness compared with the hydration stage, but cell walls from the irrigated site showed a gain in thickness as compared with cork under raw conditions.

The relationships between cell-wall thickness at the different stages (raw conditions, hydrated conditions and after the boiling stage) and *Type of treatment*, *Sample within treatment* and *Residual* were established by means of a general linear model.

Table 1
Analysis of variance for Type of treatment, Sample within treatment and Residual for cell walls, with regard to raw conditions, hydrated conditions and after the boiled stage

		Raw conditions			**Hydrated conditions**			**After the boiled stage**		
Source	DF	F	*p*-Value	VE (%)	F	*p*-Value	VE (%)	*F*	*p*-Value	VE (%)
Treatment	1	59.334	0.002	86.7	0.040	0.851	0	2.583	0.183	36.9
Sample/Treatment	4	3.741	0.005	9.7	39.087	0.0001	92.7	6.478	0.0001	40.7
Repetition/Sample/Treatment (Residual)	1194			3.6			7.3			22.4

The source of variation designated as *Treatment* provided a high contribution to the variation in raw cell-wall thickness (p = 0.002), demonstrating the significant influence of irrigation on this characteristic, accounting for 86.7% of total variation (Table

1). However, the treatment effect did not lead to significant differences in cell-wall thickness in the hydrated stage. This means that the cell walls of cork from the fertirrigated plot adsorb large amounts of water, reaching a cell-wall thickness statistically equal to those of cork from the rainfed site (there was a difference of only 0.02 µm between the two). After the boiling procedure no significant differences were found between the cell-wall thickness of cork from the two treatments (p = 0.183). Nevertheless, the boiling procedure accounted for 36.9% of the variation in cell-wall thickness (Table 1). *Sample within treatment*, referring to the sample variability of the cork in addition to the treatment, proved to provide a high contribution in cell-wall thickness, accounting for 92.7% of the variation when hydrated and 40.7% of the variation after boiling (Table 1). This features the high variability within the cork samples. A gain in cell-wall thickness after boiling was observed in cork from the fertirrigated plot, when compared to raw conditions.

Regarding cork thickness after the boiling procedure observed in macro samples (on the basis of three radial measurements of each 10-cm strip), a mean ± std. deviation for the expansion of 2.29 ± 1.06 cm for cork from the rainfed plot and 2.17 ± 1.02 cm for cork from the irrigated plot were recorded. Figures for expansion after boiling were 6.5% for cork from the irrigated plot and 7.6% in cork from the rainfed plot. Analysis of variance showed that there was no significant contribution of *Treatment* to expansion: p = 0.822 and F = 0.058, or any significant contribution of *Sample within Treatment*: p = 0.387 and F = 1.132. However, *Residual*, designated as *Measurements within each Sample*, accounted for the main source of variation: 96% of variation.

Discussion

Cork from the fertirrigated plot, with a higher rate of growth, presented a lower density than cork from the traditional rainfed site[16], which affects the cell wall thickness. The results found in Poeiras et al.[16], as cork from the traditional plot showed greater cell wall thickness under raw conditions, and also present in Fig. 1, was the starting point. This is in accordance with Nativdade[5], who found a greater cell wall thickness in cork from sites in Algeria and Morocco than cork from Portugal, where it is comparatively slow growing, associated with the local climatic conditions. Although cork is a poor water conductor due to a lack of intercellular spaces and the presence of gas in the cells, cork from the fertirrigated plot presented a higher level of water sorption, which may also be related to cell-wall structure and composition. Despite cork tissue not having intercellular voids[6], some channels called plasmodesmatas with a cross-sectional diameter of approximately 100nm may appear to cross cell walls[6]. Their presence may provide an explanation for the increased cell-wall thickness of cork from the irrigated site, when hydrated. Teixeira and Pereira[17] stated that plasmodesmatas are present during suberization, which is a rapid process. Furthermore, these authors[17] found that cell walls were thinner in areas where plasmodesmatas were found, which is in accordance with the thinner cell walls found in cork from the irrigated site. Cork from both treatments may present different chemical composition content as observed by Sen et al.[9] in cork from different sites with regard to lignin and the amount of extractives. Pereira[6] found differences in chemical composition between cork from 29 locations, with a range of variations and differences in structural components. Therefore, different cell-wall behaviour under hydration conditions could also be explained by a variation in chemical composition, such as suberin and lignin content, differentiated due to growth conditions such as for example fertirrigation. Suberin is a structural component of cork which confers integrity on cork tissue[6] and whose deposition starts during cell formation. Lignin is likewise a structural cell-wall component. The behaviour of higher grow rate corks (with a higher level of water sorption) may suggest a lower content of these components. Following the boiling procedure, the level of tension and wrinkle in cell walls decreased, producing a loss in cell-wall thickness after hydration, in both treatments. However, cork from the fertirrigated plot showed an increase in thickness as compared with raw conditions. Despite cork from the fertirrigated site showing a greater porosity coefficient[16], analysis of variance with regard to cork expansion of mac-o sized samples did not reveal significant influence of treatments (p = 0.822). As regards cork expansion, an important aspect of industrial procedure, the findings of this study demonstrated no differences between cork from the fertirrigated site and cork form the rainfed site. Furthermore, fertirrigation showed no significant changes in expansion rates after the boiling procedure, on the large scale. In addition, a slight negative correlation was found between the expansion of cell walls when boiled and the expansion of macro samples thickness (p = 0.963; Pearson correlation = -0.025). However, on the small scale, hydration and

boiling procedures had significant effects on cell walls, which raises the question of possible differences in chemical composition content between the treatments, leading to some changes in structure.

Conclusions

The aim of this study was to gauge the effect of two different tree-growth regimes on cork cell walls in regard to swelling and boiling and the differences on the expansion of cork thickness. For the purpose of cell analysis, SEM under high vacuum and under environmental conditions after hydration were used. As cork from the specific fertirrigated site showed a lower density[16], it was expected that the increase in thickness of macro samples would be higher as compared with the rainfed plot. However, no statistical significance was found. At the cellular level, after 24h hydration, differences in thickness were greater for cork from the fertirrigated site than cork from the traditional rainfed plot. The findings of this study suggest that the cell walls of cork from fertirrigated cork oaks display a greater capacity for adsorbing water, which could be associated with the fact that their cell walls are thinner – the consequence of water being available during all periods of cork growth. A decrease in the thickness of the cork cell walls from the rainfed site was found between the raw stage and the boiling stage, in contrast to cork from the irrigated site, where an increase in cell-wall thickness was observed. These findings are helpful in clarifying the effects of sorption on cell structure and provide the basis for further analysis of cork structures deriving from the application of different water regimes, including chemical composition and presence of ultrastructural channels.

Material And Methods

Study sites

Irrigated site: Tthe fertirrigated site (39º2’49.77”N, 7º57’32.08”W, UTM coordinates) was installed in 2003 near an intensive olive plantation with some centenary cork oaks scattered throughout the stand, located 35 km from the rainfed site. The fertirrigation system is coupled to the olive plantation. From 2003, the cork oaks were irrigated during the summer, usually for four months. Average watering figures from the cork formation period were 1928.6 m^3 ha^{-1} (2011-2017) and annual precipitation for the same period was 452.19 mm. The plot is located on a Luvisol with 164 trees per hectare, a basal area of 16.93 m^2, and an average stem perimeter at breast height of 81.2 ± 40.28 cm. Trees on the plot present a mean ± SD of 8.50 ± 1.76 m of tree height and no decline symptoms. Tree age (years) is 18y + 13 centenary trees.

Rainfed site: A set of permanent plots was installed in 1995 in cork oak forests in the centre of Portugal (39º05’54.93”N, 8º21’26.23”W UTM coordinates). Centenary cork oaks were systematically monitored for tree growth and cork production[18]. The study site presented an annual precipitation of 400.93 mm for the period 2008-2017 and is located on a Cambisol with no limitations on cork oak growth. The site has around of 150 trees per hectare, a basal area of 6.94 m^2 and a stem perimeter at breast height (mean ± SD) of 134.6 ± 37.20 cm. The trees showed no decline symptoms and tree height (mean ± SD) is 10.33 ± 2.01 m.

Sampling

Cork was harvested in 2017 on the two sites. Before harvesting, a 20x20 cm square sample was taken from each truck at a height of 130 cm. The six samples selected for the analysis were from centenary trees: three from each plot. The height (mean ± SD) of the study trees was 11.23 ± 1.68 m for the irrigated plot and 9.93 ± 1.90 m for the rainfed plot. Stem perimeter at breast height (mean ± SD) was 214.3 ± 15.28 cm for the irrigated plot trees and 139.5 ± 45.89 cm for the rainfed plot trees. The harvested cork weight (mean ± SD) was 51.73 ± 7.93 Kg for the irrigated site and 24.9 ± 12.36 Kg for the rainfed site.

Cell Walls - Raw Samples Measurements

Samples prepared for SEM analysis were cut with a movable blade microtome (Reichert, with Jung blades) in the transverse plane (cross section) with 1 mm thickness[16]. Samples were fixed on aluminium specimen holders using conductive double-

sided adhesive carbon tabs and coated with 40nm carbon using the EMITECH K905 Carbon Coater (Emitech Ltd, Ashford, Ken, UK). On each raw sample, one image was gathered using MAPS software (MAPS version 2.1.38.1199, Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 10kV beam energy and a spot size of 2.5 under high vacuum conditions using SEM (Quanta FEG 650, Thermo Fisher Scientific, Waltham, Massachusetts, USA). For each sample (6), an image with 168 to 224 subframes was taken with high magnification (Fig. 2). Subframes were stitched together using the MAPS software for image analysis. Cell-wall thickness was measured using ImageJ 1.52a Program (Wayne Rasband, National Institutes of Health, USA). 200 measurements per image were done.

Cell walls - 24h water immersion and 98% humidity measurements

Samples prepared for raw measurement were immersed for 24 hours in cold water and one image was gathered per sample (3 samples per treatment) with MAPS software using ESEM (Quanta FEG 650, Thermo Fisher Scientific, Waltham, Massachusetts, USA) (Fig. 2C an D). Cell-wall swelling was analysed under 98% humidity at 10kV beam energy and a spot size of 2.5 under 98% humidity (800Pa; 4-6°C and working distance of 6,5) within the same area as raw sample measurements. For each sample acquired, 200 measurements were performed using ImageJ 1.52a Program (Wayne Rasband, National Institutes of Health, USA).

Cell walls - after boiling measurements – 1h at 100ºC

Following the cork industrial processing, previous hydrated samples were boiled in water (100 ºC) for one hour and dried under environmental conditions for two days. One image per sample (3 samples per treatment) was gathered using MAPS software in SEM (Fig. 2E and F). 200 measurements were performed using ImageJ, as in previous analyses.

The measurements, in every condition (raw, hydrated and boiled) were done on the same samples and same sample' spots.

Radial Macro Samples - Boiling Procedure

From the original harvested cork samples, pieces of around 10 cm long and 3 cm thick were cut and scanned using Epson Scan-Expression 11000XL. For each sample, three random lines (Fig. 3) along the radial length were tagged with a permanent marker pen and measured using ImageJ. Samples were boiled at 100^{o} C for one hour, simulating the industrial procedure. After boiling, samples were scanned and radial swelling was measured at the same spots using ImageJ.

All experiments were performed in accordance with relevant named guidelines and regulations. Cork samples and research plots are maintained by University of Évora.

Declarations

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Figures

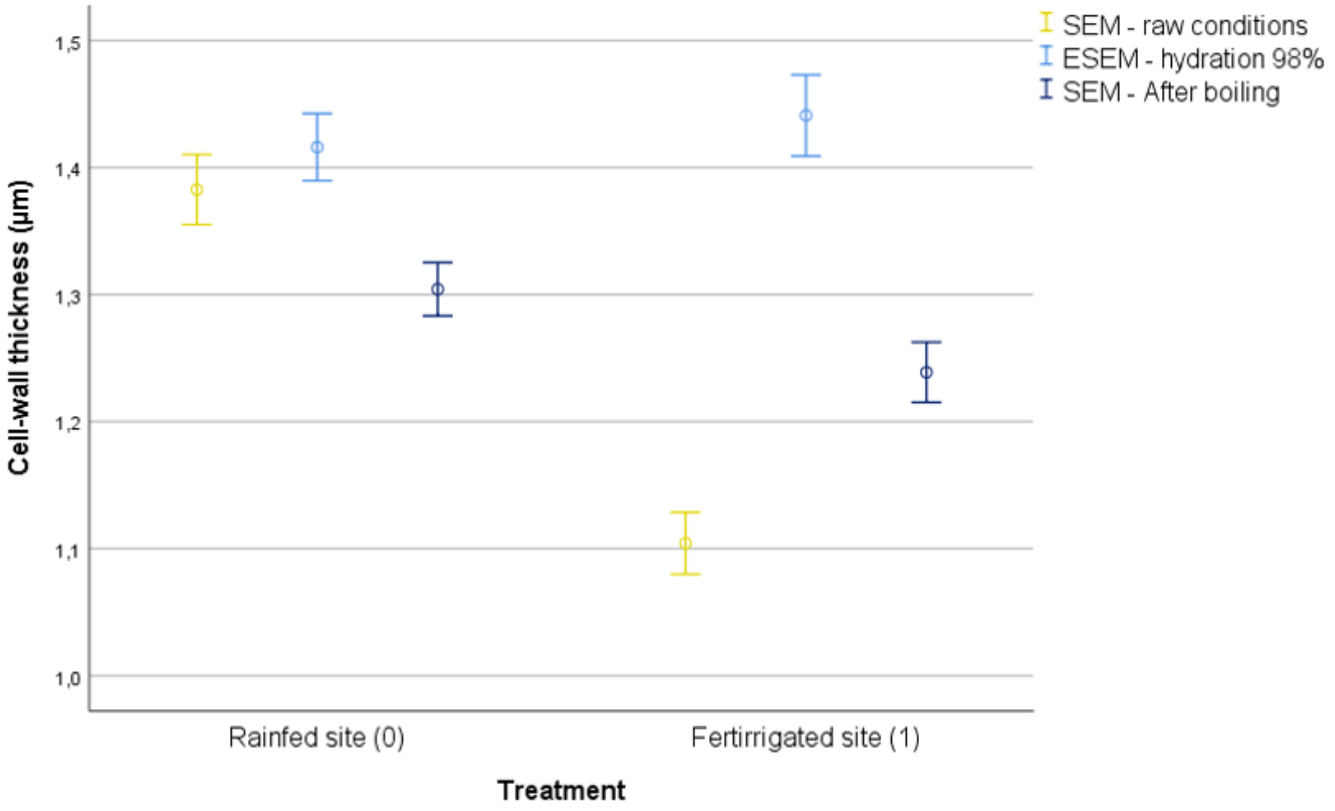

Figure 1

Mean ± Standard deviation of cell-wall thickness with regard to raw conditions, hydrated conditions and after the boiling stage, for cork samples from the rainfed site (0) and the fertirrigated site (1)

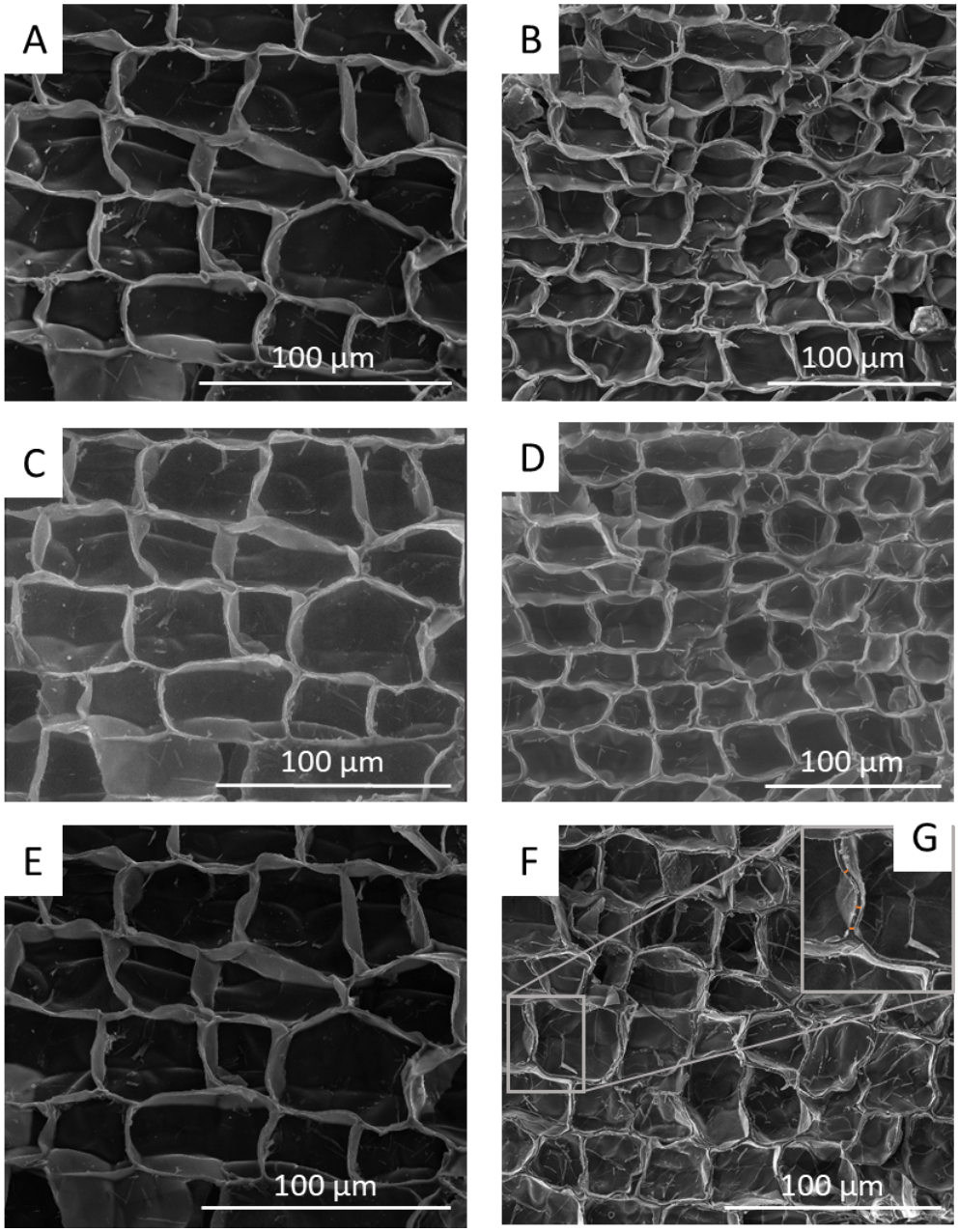

Figure 2

Microphotographs of the cork cell structure of a transverse section of a cork sample from the fertirrigated plot (A, C, E) and a cork sample from the rainfed plot (B, D, F): A and B were obtained using SEM on raw cork; C and D were obtained using ESEM after 24h immersion in cold water; E and F were obtained with SEM, after boiling and two days of drying. G: Yellow marks represent an example of a cell wall thickness measurement.

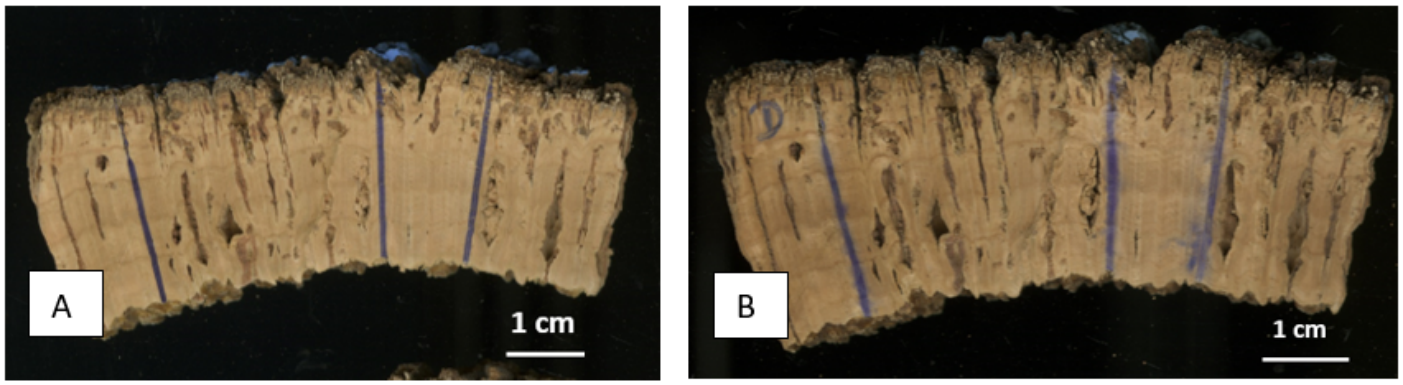

Figure 3

Example of a macro sample from the irrigated plot: A – before the boiling procedure; B – after the boiling procedure