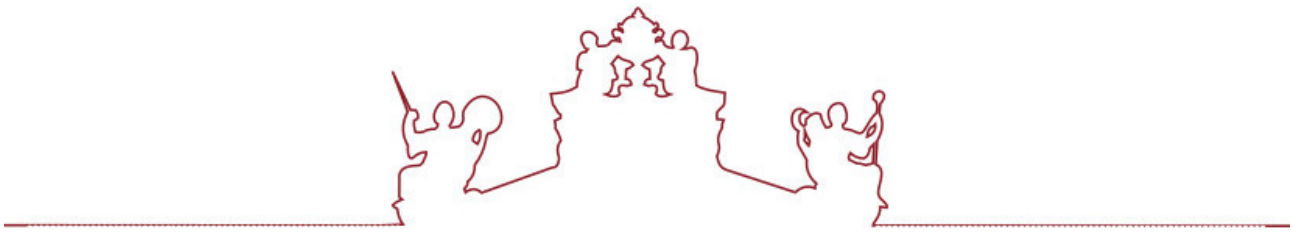

Universidade de Évora - Instituto de Investigação e Formação Avançada
Universidade do Algarve - Faculdade de Ciências e Tecnologia

Programa de Doutoramento em Ciências Agrárias e Ambientais

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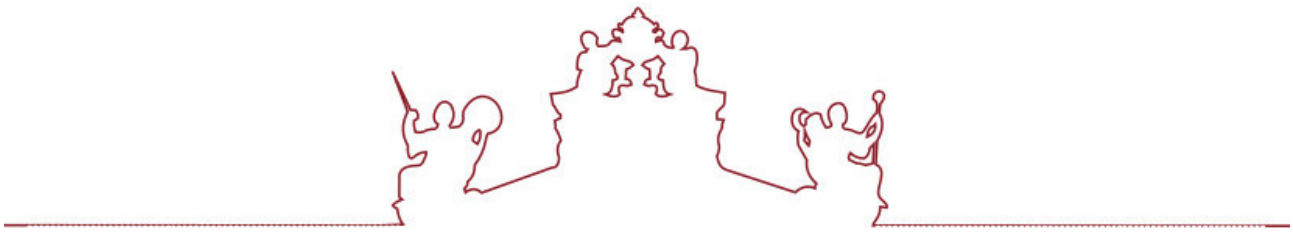

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Évora 2022

To the curious reader.

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Agradecimentos | Acknowledgments

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—

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Abstract

Cork (from *Quercus suber* L.) is one of the most important non-wood forest products in the Mediterranean basis, especially in Portugal. However, according to the latest Portuguese national forest inventory, cork oaks areas are decreasing, and consequently cork production is falling. This reduction is due to several reasons, such as climate changes and some management practices. The aim of this study is to enable to understanding the response of cork formation (at macro and cellular level), characteristics and quality to some implemented silviculture models. It intends to examining the evolution of cork quality over consecutive harvests at a specific site with a traditional silviculture model of a pure cork oak stand to cork and silvopastoril production, analysing the influence of site quality, climate and intraspecific competition indices on cork thickness and density; and to analyse the response of cork growth at a specific site with a silviculture model of irrigation, part of an on-going study. X-ray image analysis techniques (microdensitometry and micro-computed tomography) were used, as well as scanning and environmental scanning electron microscopy, and a number of image analysis programs were used to analyse cork tissue. As results of this study: cork thickness has been decreasing over time and cork density increasing. At the dynamics stand level, Hd2 and Hh3 competition and search indices showed high significance on cork thickness and density, respectively. It was proved a cork density control through management actions once competition is related to the light cone. It was also demonstrated a cork density control through the intensity of debark. As a response to the silviculture model of irrigation cork presented a faster-growth rate, greater thickness, higher coefficient of porosity and lower density. However, through the results of Hd2 and Hh3 competition indices on cork density and thickness, it is possible to increase cork density and quality through high density stands. Cork cells and cell walls showed different characteristics, such as thinner cell walls, which show greater expansion when hydrated. Cork formation response to some silviculture models continue to be studied.

Key-Words: *Quercus suber* L., field conditions, forest management, image analysis, cork characteristics, cellular tissue

Resumo

Análise espacial da qualidade da cortiça em descortiçamentos consecutivos: do microsite a condições regionais

A cortiça é um dos principais produtos florestais não lenhosos obtidos na bacia do mediterrâneo e especialmente em Portugal. Contudo, a produção de sobreiro (*Quercus suber* L.) e consequentemente a produção de cortiça estão a diminuir em Portugal, de acordo com o último inventário florestal nacional, devido a vários fatores, entre os quais alterações climáticas e algumas ações de gestão. Este trabalho visa compreender a resposta da formação, características e qualidade da cortiça (a nível macrométrico e celular) a alguns modelos de gestão aplicados. O trabalho pretende estudar a evolução das características da cortiça ao longo de descortiçamentos consecutivos num local com um modelo de silvicultura para povoamentos puros de sobreiro para produção de cortiça e silvopastorícia, analisando a influência da qualidade da estação, clima e índices de competição na densidade e espessura da cortiça; e pretende analisar as características da cortiça de um local com um modelo de silvicultura de irrigação, cujo faz parte de projetos em desenvolvimento. Foram utilizadas técnicas de raios-X tais como microdensitometria e microtomografia computorizada, bem como técnicas de microscopia eletrónica e outros programas de análise de imagem. Como resultados: ao longo das extrações consecutivas analisadas verificou-se uma perda na espessura e um ligeiro ganho de densidade da cortiça; ao nível da dinâmica do povoamento, os índices de competição e pesquisa Hd2 e Hh3 mostraram grande significância na densidade e espessura da cortiça, respetivamente. Demonstrou-se, assim, ser possível controlar a densidade da cortiça através da gestão, nomeadamente da manutenção de povoamentos com densidades mais elevadas, estando relacionada com a competição de árvores próximas, pelo cone de luz. Foi também demonstrado a possibilidade de controlo da densidade e crescimento através da intensidade de descortiçamento. A resposta da formação da cortiça ao modelo de silvicultura baseado em irrigação centra-se num rápido crescimento, maior espessura, maior porosidade e menor densidade. No entanto, através dos resultados da aplicação dos índices de competição Hd2 e Hh3 mostrou-se ser possível fazer aumentar a densidade da cortiça, através de compassos mais apertados e maior área basal. As células e as paredes celulares mostraram diferentes características, tais como espessura da parede menor, a qual mostra maior expansão quando

hidratada. As respostas da formação da cortiça aos vários modelos de gestão continuam em desenvolvimento.

Palavras-chave: *Quercus suber* L., condições de campo, gestão florestal, análise de imagem, características da cortiça, tecido celular

Foreword

Most of the work involved in this study was carried out at the University of Évora, as a member of the Pró-Flormed team (supervised by Professor Nuno de Almeida Ribeiro), the Czech University of Live Sciences Prague (Internship) supervised by Professor Peter Surový, and the University of Trás-os-Montes e Alto Douro (UTAD) supervised by Professor Maria Emília Silva. A collaborative work with the University of Évora Department of Veterinary Science was guided by Professor Joana Reis and Dr. Teresa Oliveira, and the work carried out at the Technical University of Dresden (Erasmus+) was guided by Dr. Cordula Vogel and Dr. Björn Günther.

This dissertation takes the form of six chapters, four of which are based on scientific articles. Chapter 1 provides a brief state of the art and describes the objectives and overall aims of the study, as well as a short description of study sites. Chapters 2 to 5 comprise scientific articles produced during the PhD work, with the aim of attempting to understand the response of cork formation (at a macro and cellular level), cork characteristics and cork quality, with reference to some implemented silviculture models.

- Poeiras A. P., Silva M. E., Günther B., Vogel C., Surový P., Ribeiro N.A., Cork influenced by a specific water regime – macro and microstructure characterization – the first approach. (2021). *Wood Science and Technology* (Published at 08.10.2021, DOI - 10.1007/s00226-021-01334-1, Chapter 2)

- Poeiras A. P., Vogel C., Günther B., Camilo-Alves C, Surový P., Silva M. E., and Ribeiro N.A., A cork cell wall approach to swelling and boiling. Submitted to *Nature Scientific Reports* at 24.11.2021 (Chapter 3)

- Poeiras A. P., Oliveira T., Reis J., Surový P., Silva M.E., Ribeiro N.A., Influence of water supply on cork increment and quality in *Quercus suber* L. *Central European Forestry Journal* (Published at 24.11.2021, DOI: 10.2478/forj-2021-0024, Chapter 4)

- The influence of site quality in consecutive harvests for a specific Portuguese site - density and growth analysis (in preparation for submission to Forest Ecology and Management, Chapter 5)

The last chapter includes the final remarks about the work developed and future perspectives.

The candidate worked on Prodehesa Montado Project (Ref: 0276_PRODEHESA_MONTADO_6), Biotrans Project (Ref: 0620_BIOTRANS_4_E) and RegaCork-Trade (Ref: ALT20-03-0246-FEDER-000061) during the period of the PhD research. The research was also supported by Medida 4.1—Cooperação para a Inovação/ProDeR 52131 and 52132", by PDR2020-101-FEADER-031427 "GO-RegaCork"; by Czech University of Life Sciences Prague, Department of Forest Management, and the Erasmus+ Programme during the period in TUDresden.

Chapter 1 - Introduction

1.1 A brief state of the art

According to the latest National Forest Inventory in Portugal, cork oak (*Quercus suber* L.) stands occupy an area of 719,900 hectares. However, 1,300 ha were lost between 2005 and 2015 (ICNF, 2020), possibly jeopardising cork-oak surveillance in the future. The cork-oak forest is a multifunctional system which plays an important socio-economic, environmental and heritage role in the life of the country. Trees are the basis of this ecosystem, which has an important influence on local climate, soils and the safeguarding of water resources, acts as a carbon sink and protects biodiversity, promotes ecosystems services and safeguards the environmental heritage. From an economic perspective, cork-oak forests and cork production contribute to employment and the development of inland regions, contributing to economic growth. Average export figures for cork over the last five years are about 188.8 m t per annum, worth 1012.82 m euros per annum, while imports are worth 189.7 m euros per annum, thus producing a positive trade balance. Natural cork, either processed or raw, waste cork, and cork agglomerate are the most commonly imported types of cork products. Principal exports are cork stoppers of several different types (an average of 50.7 m t per annum over the last five years), building materials (128.68 m t); raw cork (6.86 m t); and other cork products (2.6 m t).

Cork from *Quercus suber* L. is produced by the phellogen of cork cambium through periclines divisions. During the most active growth periods – spring and autumn – phellogen produces an interior live tissue called pheloderm and an exterior group of dead cells called phellem or cork (Meyer et al., 1970; Pereira, 2015). The number of phellem cells produced is usually greater than the pheloderm cells (Fortes et al., 2004). Cell meristematic activity decreases over the lifespan of the tree, as well as the number of cells produced. With regard to cork production during the cork growth period (nine years according to official regulations) there is a decreasing on the cork growth trend, which means the initial rings produced after debarking are larger than the later rings due to pressure caused by the older rings (Natividade, 1934; Fortes et al, 2004; Ghalem et al, 2016), in contrast to the density trend. Cork structure is similar to a honeycomb pattern, hexagonally shaped with no intracellular spaces. At the nanoscale level some plasmodesmatas may be observed, which are channels between cork cells that demonstrate

active intercellular communication (Teixeira and Pereira, 2009). Cell walls are comprised of a first, second and third wall with different chemical compositions of cellulose, lignin and suberin (Fortes et al., 2004) and there is a medium lamella between two cells which is probably high in lignin content. The cell-wall properties of cork, widely described in the literature (Knapic et al., 2016; Pereira, 2007; Silva et al., 2005; Fortes et al., 2004), make it an important sustainable and renewable material with a positive environmental footprint.

Cork characteristics, such as porosity, density and thickness are widely described in the next chapters.

Silviculture models and silviculture practices are integrated to ensure sustainable management and cork oaks vitality, over the stand lifepath (Ribeiro et al, 2021). Scrub control, formation pruning, plantation and the natural regeneration conservation are aimed at maintaining continuous crown cover and continuous cork production, thereby ensuring the ecological and economic sustainability of the system (Ribeiro et al., 2021). The aim of silviculture models is to apply the sequence of forest operations to achieve established site goals. Each forest stand goes through several physiological stages throughout its lifetime and in perpetuity. In this study cork from silviculture models of a *pure cork oak stand for cork and silvopastoril production* and a *silviculture model of irrigation for pure cork oak stand* were used. Silviculture models of irrigation have been introduced in underuse marginal areas of irrigation perimeters as providing a good opportunity for increasing cork-oak forest areas and cork production; and are part of on-going projects (Go-Regacork, Irricork, Regasuber).

Cork oaks may form part of the “montado” system, in which an agro-silvo-pastoral system characterized by open forest stands with dispersed trees associated to cattle pastoralism and agronomical exploration; and can integrate a markedly forest exploration aimed to achieving high level of cork production and sometimes silvopastoril as well, associated to greater stand density, without cattle or agronomical exploration. This thesis aims to approach the cork oak forest stands to cork production.

Each silviculture model involves the same physiological stages in the growth of trees: the initiation stage, the juvenile stage, the mature stage and equilibrium stage (PROF, ICNF, 2019).

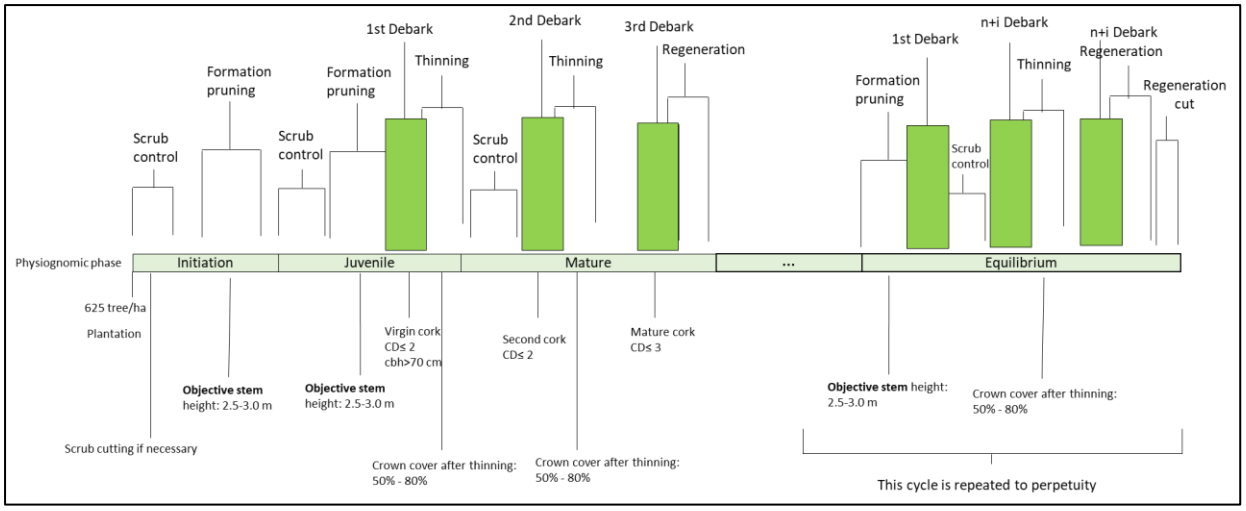

Fig. 1 Silviculture model of a *pure cork oak stand for cork production* (PROF, ICNF 2019 in Ribeiro et al, 2021)

The silviculture model of a pure cork oak stand for cork production and the silviculture practices applied are represented in the scheme (Fig.1). Debarkings may begin at the end of the juvenile stage, following some specific guidelines (as an example: trees must be >70 cm perimeter at breast high). Some cares about the procedure, such as involving the disinfection of tools, cautions with trees injuries, may be important to trees health and cork value. Formation pruning in the first years of cork-oak development is also important: the choose may be done from top to bottom in order to achieve a straight trunk with an average height of 3 meters (Reis, 2004). To silvo-pastoral exploitation, natural pasture improvement could be done after the 1st debarking.

Some authors refer to decreasing cork growth due to some climatic events, in the Mediterranean basin. According to Fernández-Montes et al. (2016) in Lionello et al. (2017), a scenario of intensification of negative precipitation-temperature in the Iberian Peninsula may will occurred in the future, as a result of the intensification of warm-dry and cold-wet periods. Such climate changes associated with periods of drought affect cork-ring development (Leite et al, 2019). Some projections for climate change predict a decrease in precipitation events and an increasing in the dry season, which may impact trees vitality and consequently cork production. PROF, ICNF (2019) also predict 3 scenarios, the first of which is in accordance with current climate conditions, the second in accordance with scenario RCP 4.5 and the third in accordance with scenario RCP 8.5. Site quality for the silviculture model of *pure cork oak stand for cork production* was calculated for the 3 periods of consecutive harvests, in a soil and climatic approach, as described in Chapter 5 of this study. Besides climate and soil conditions, stand dynamics also plays an important role: competition for water, nutrients and sun light may affect

stands structure. Competition indices provides spatial or non-spatial information, which means a dependency or independency of trees distance.

There are several spatial indices (Ribeiro, 2006; Table 1) that calculate the significance of each stand' tree as a competitor (j) of a certain tree (i). Several variables are applied to their calculation, such as: d – trunk diameter at breast height (at 130 cm); h – total height; $dist_{ij}$ - distance between i and j; n – number of competitors, r - radius of an isolated tree; a_{ij} - overlap area , A_i – horizontal projection area of an isolated tree with an equal diameter of i; θ_{ij} – competitor influence angle; VR – radicular volume, CR – radicular competition component, VSR - insertion volume; CC – crown competition component; AC – sectional crown area; VC – crown volume above several height; ASC – crown surface above several height; β_{ij} – angle between the vertical light cone of search competitors and the competitor tree top as > ±60˚.

Table 1 Competition indices (Ribeiro, 2006)

ID	Mathematical formula	Method
H	$\sum_{j=1}^{n} \frac{d_j}{d_i} * \frac{1}{dist_{ij}}$ (Hegyi, 1974)	**Based on dimension ratios**
D	$\sum_{j=1}^{n} \left(\frac{d_j}{d_i}\right)^2 * \frac{1}{dist_{ij}}$ (Daniels, 1976)	
ME	$\sum_{j=1}^{n} \left(\frac{d_j}{d_i}\right) * e^{\frac{-16*dist_{ij}}{d_i+d_j}}$ (Martin and Ek, 1984)	
ME1	$\sum_{j=1}^{n} \left(\frac{d_j}{d_i}\right) * e^{\frac{-c*dist_{ij}}{RC_i+RC_j}}$ (Monserud and Ek,1977)	
ME2	$\sum_{j=1}^{n} \frac{d_j}{d_i} * \frac{1}{dist_{ij}+1}$ (Martin e Ek, 1984)	
C	$\sum_{j=1}^{n} \frac{AC_j}{AC_i} * \frac{1}{(dist_{ij}+1)}$ (Biging and Dobbertin, 1992)	
V	$\sum_{j=1}^{n} \frac{VC_j}{VC_i} * \frac{1}{(dist_{ij}+1)}$ (Biging and Dobbertin, 1992)	
M	$\sum_{j=1}^{n} \frac{ASC_j}{ASC_i} * \frac{1}{(dist_{ij})}$ (Biging and Dobbertin, 1992)	
VU	$\sum_{j=1}^{n} \frac{VC_j}{VC_i}$ (Biging and Dobbertin, 1992)	
MU	$\sum_{j=1}^{n} \frac{ASC_j}{ASC_i}$ (Biging and Dobbertin, 1992)	
MDRF	$\sum_{j}^{n_1} \frac{d_j}{d_i} F^{123} - \sum_{j}^{n_2} \frac{d_j}{d_i} F^{123}$ (Tomé and Burkhart, 1989)	
DDF	$(d_j - d_i) * F^{123}$ (Tomé and Burkhart, 1989)	

G	$\frac{1}{A_i} * \sum_{j=1}^{n} a_{ij}$ (Gerrard, 1969)	**Based on overlapping influence areas**
B	$\sum_{j=1}^{n} \left(\frac{a_{ij}}{A_i} * \left(\frac{d_j}{d_i} \right)^{\exp} \right)$ (Bella, 197)	
Ar	$\frac{\sum_{j=1}^{n} a_{ij} + A_i}{A_i} * 100$ (Arney, 1972)	
EM	$\frac{\sum_{j=1}^{n} \left(a_{ij} * \frac{r_j * h_j}{r_i * h_i} \right)}{A_i} * 100$ (Ek and Monserud, 1974)	
HR	$\sum_{j=1}^{n} \frac{VSR_{ij}}{VR_i}$ (Holmes and Reed, 1991)	
P	$\sum_{j=1}^{n} \beta_{ij} * \left(\frac{AC_j}{AC_i} \right)$ (Pretzsch, 1995)	
HR1	$\sum_{j=1}^{n} \left(\frac{a_{ij} * FP_{ij}}{A_i} \right)$ (Holmes and Reed, 1991)	
MAOF	$\sum_{j}^{n_1} \frac{a_{ij}}{A_i} F^{123} - \sum_{j}^{n_2} \frac{a_{ij}}{A_i} F^{123}$ (Tomé and Burkhart, 1989)	
A	$n \Big/ \sum_{j=1}^{n} \left\{ \pi * \left(\frac{dist_{ij} * d_i}{dist_{ij} + d_j} \right)^2 * \frac{\frac{d_j}{dist_{ij}}}{\sum_{j=1}^{n} \left(\frac{d_j}{dist_{ij}} \right)} \right\}$ (Alemdag, 1978)	**Based on growth space**
Do	$\sum_{j=1}^{n} \left[\frac{\theta_{ij}}{360} * \left(\frac{d_i * dist_i * CR_i}{d_i + d_j} \right)^2 \right]$ (Doyle, 1983)	
APA	Available Potential Area (Brown, 1965; Moore et al., 1973; Pelz et al., 1978 and Nance et al, 1987)	

Several competitors search algorithms (Table 2) were used to reach the competitors, based on the canopy overlapping method (Fabrika and Pretzsch, 2013) and basal area method:

Table 2 Search algorithms to search competitors (Ribeiro, 2006)

ID	Mathematical formula	Method
D1	$dist_{ij} \langle \frac{d_i + d_j}{8}$	**Basal area method**
D2	$dist_{ij} \langle 0.33 * d_j$	
D3	$dist_{ij} \langle 0.25 * d_j$	
D4	$dist_{ij} \langle \frac{d_i + d_j}{6}$	
D5	$dist_{ij} \langle \frac{d_i + d_j}{8}$	

Da	$dist_{ij} < a * d_j$ a=0.15, 0.25, 0.35, 0.50	
H4	$dist_{ij} < \frac{HT_j - HCB_j}{(50 - HCB_i)} * 30$	
H1	$dist_{ij} < \frac{HT_j}{1.73}$	**Canopy Overlapping method**
H2	$dist_{ij} < \frac{HT_j}{1.19}$	
H3	$dist_{ij} < \frac{HT_j - HCB_j}{1.19}$	
CW1	$dist_{ij} < (CW_i - CW_j)$	
CW2	$dist_{ij} < (CW_i - CW_j) * 2$	
HT – total height; HCB – canopy basis height; CW – canopy deep degree		

1.2 Image analysis

Density, coefficient of porosity, intern porosity and cork thickness (as well as cork-ring width) were the principal characteristics studied in this thesis, described in detail in the following chapters. Cork samples were analysed by means of several different technologies of image analysis:

- X-Ray Microdensitometer
- X-Ray Micro computed Tomography
- Scanning Electron Microscope and Environmental Scanning Electron Microscope

For each of these, a specific sample shape was prepared, as explained below, in the specific chapters dealing with image analysis.

The X-Ray Microdensitometer - QTRS-01X Tree Ring Analyser (Quintek Measurement Systems Inc., Knoxville, TN, USA) - was used to gauge total density and cork thickness, and was also used on the analysis of cork-ring width. Measurements were obtained automatically using QTRS-01X software. Operational principles of working were based on the absorption of radiation from a collimated beam of X-rays and the density calculation at each measurement point is performed from the ratio of the measured attenuation and the beam intensity: $\frac{\mathbf{I}}{\mathbf{I_o}} = \boldsymbol{e}^{-\boldsymbol{\mu l}^{\boldsymbol{t}}}$ as described below, where **I** is radiation beam intensity after passing through the sample, **Io** is radiation beam intensity not passing through the sample (from bark to pith), $\boldsymbol{\mu l}$ is the sample linear attenuation coefficient, and **t** is sample thickness. The mass attenuation coefficient is dependent on the material and the energy of the incident radiation. X-Ray micro computed

tomography (SkyScan1174v2, Bruker microCT, Belgium, version 1.1) was used for the analysis of internal porosity and volume fraction of lenticels. CTan version 1.14.4.1+ (64-bit) was used for quantification and CTVox version 3.0.0r1114 (64-bit) was used to perform some cork sample reconstructions. Micro-computed tomography (μCT) works by means of the action of an attenuated X-ray beam that passes through the sample, producing a reconstruction sample, which is obteined automatically using mathematical algorithms through a set of cross-sections (Elliott et al. 1990; Rajczakowska et al. 2015). Environment Scanning Electron Microscopy was used for cell-wall expansion analysis due to the dynamic process of water interaction and the thermodynamic stability of moisture (Fránková et al., 2018). ImageJ and Image ProPlus were used especially to the cell structure measurements – areas, diameter, number of cells, and cell-wall thickness – and to the number of pores and pore area.

1.3 Objectives

The present study intends to:

- Analyse the response of cork formation, characteristics and quality to some silviculture models: cork from a silviculture model of fertirrigation; and cork from a traditional *pure cork oak stand for cork production*, during consecutive harvests, observing the impact of site quality, climate and competition on it;

- Examine the impact of climate changes on the Mediterranean system, at a specific site;

- Use a set of (new) methods to analyse cork tissue, some of them innovative in the analysis of this material, such as Environmental Scanning Electron Microscopy used in cell-wall expansion analysis.

1.4 Study sites

1.4.1 Machoqueira do Grou

Situated in Tagus River (39º07’07.80’’N, 8º21’33.78’’W UTM coordinates), Machoqueira do Grou has been the site of several studies, since the 1990´s (Ribeiro, 2004, 2006; 2010, 2011). A group of 60 plots was implemented and several data collected. It was the basis of spatial

models of trees' development. The site is separated by groups of different years of harvest (Fig. 2), from 1996 until nowadays. The sequence of harvests from 1996 to 2017 was considered in this analysis, while the sequences 2000-2009-2018 and 2001-2010-2019 were not taken in account, but will play a role in further analysis. The site is under its natural environmental condition, water supply by rainfed. Soil varying between Cambisols, Arenosols, Regosols and Podzols. Cambisols are a sandy loam with fine subsurface horizons, presents rapid rates of permeability, which derive from the mother rock intensively changed and are commonly used for agriculture and forestry (PROF, ICNF 2019); Arenosols are sandy-textured with no significant soil profile development, presenting excessive permeability; Podzols are clayey soils and Regosols present unconsolidated parent material and are of alluvial origin. Soil quality is calculated in regards to their limitation for trees growth. In accordance with the PROF (2019) soils were classified as: *Good* (3) with no limitation on root growth (especially Cambisols), *Regular* (2) soils with a low capacity for water retention (especially Arenosols) and *Poor* (1) hydrophilic soils (especially Regosols and Podzols).

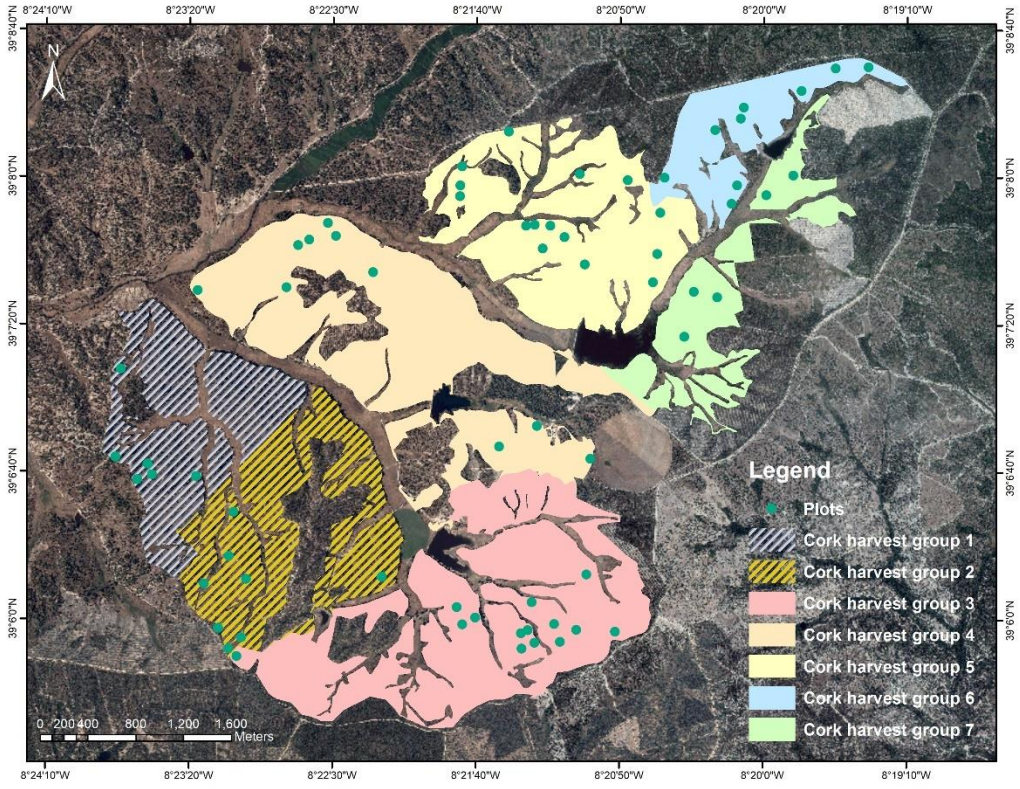

Fig. 2 Harvest groups in Machoqueira do Grou. Cork extracted from the zebra groups were not considered for the analysis.

The site presents a typical Mediterranean climate with hot dry summers and mild winters (Fig. 3). Minimum temperatures (˚C) were recorded in January and maximum temperatures in

August, for all the periods of cork growth. Total annual precipitation (mm.year $^{-1}$) figures decreased from the 1st to the 3rd decade of cork growth (from 636.81 ± 194.50 to 421.04 ± 141.86). Climatic parameters, such as monthly average maximum and minimum temperature is described in the Chapter 5, and were used for the calculation of Climatic Indices (Termicidity, Ombrothermic and Continentality Indices), for cork-ring growth period for the range of harvests.

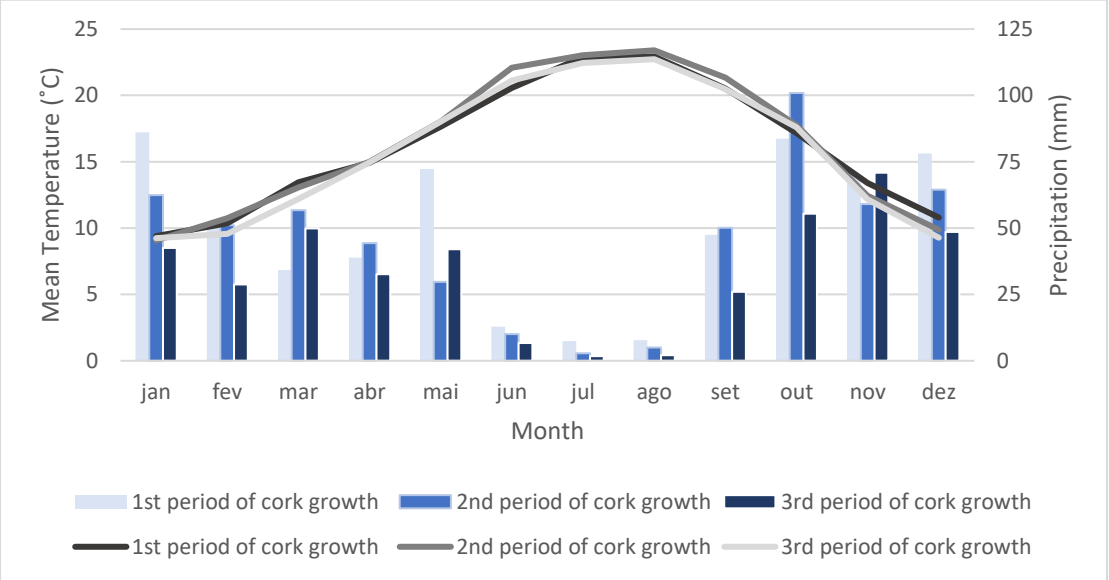

Fig. 3 Climate diagram showing temperature (lines) and precipitation (bars) at study site A for the three periods of cork growth. Climate data: IPMA – Instituto Português do Mar e da Atmosfera (Portuguese Institute for the Sea and the Atmosphere)

A number of measurements (Ribeiro, 2006), such as tree total height (m), tree base of crown height (m), crown rays (m), stem circumference at 130 cm height (at breast height, before and after debark, cm), total debarking height (m), stem height of debark (m), branches debark height and circumference before and after debark (m) and cork weight (Kg) have been collected over years, as well as:

basal area ($m^2\ h^{-1}$) = $(\frac{\pi}{4})*d^2$, which represent a cross-sectional area for trees at 130 cm height, have been analysed over years;

Cork generating surface (m^2) = circunference at breast hieght after debarking + ∑(branches circunference after debarking ∗ branches debark height)

$$\text{Intensity of debark} = \frac{\text{cork generating surface}}{\text{basal area after debarking}} \text{ and}$$

$$\text{Coefficient of debark} = \frac{\text{total debarking height}}{\text{circunference at 130 cm}}.$$

Tree mortality and decline symptoms are, likewise, observed on the study sites.

1.4.2 Avis - Irricork

Irricork is part of an on-going study (GO-Regacork) related with fertirrigation of cork oaks. The main goals of the project are to determining scientific methods of fertirrigation in cork oaks, in order to use marginal irrigation perimeters for cork oak stands, increasing the production of cork and forest areas; to evaluate the effect of fertirrigation on the formation, production and quality of cork from adult and young cork oak stands; to anticipate cork harvests in new areas with rentability and, also, the transference of knowledge.

The study site (Fig. 4 and Fig. 5) is located in Avis, Portugal (39º2'49.77''N, 7º57'32.08''W, UTM coordinates), distant 35 km from the site described in 1.4.1. It was installed in 2003, and is now 18 years old, coupled to an intensive olive plantation. The plantation has 7 x 3.5 m compass and was irrigated since the beginning. 13 centenary cork oaks are scattered on the stand, which cork, harvested in 2017, was analyzed in this thesis.

Since 2003, cork oaks have been irrigated during the summer, usually over four months, as the same water regime as the olive grove nearby, determined by the producer since the beginning and until 2017. Average watering since the last cork formation was 1928.6 $m^3 ha^{-1}$ (2011-2017). Irrigation is drip-surface type with one tube per plantation line and 2.1 L drip emitters spaced 0.75 m apart. Following the cork harvest of 2017, the water regime was determined by the University of Évora, with separate regimes for individual plantation lines in accordance with the different treatments. The plot is located on a Low Satured Gleyic Luvisol, which is defined by the presence of lime at certain depth, responsible for water accumulation. Specific information regarding trees characteristics, such as total height (m), stem circumference at

breast height (cm), basal area, total debarking height (m), and about water regime details are provided in the next chapters.

Fig. 4 Study site - Irricork

Fig. 5 Study site Irricork. Adult trees (amadia cork) are signalised in green.

The study site is characterised by a Mediterranean climate, with hot dry summers and mild winters (Fig. 6). Mean temperature was 16.2°C for the period of cork growth (2011-2017) and mean monthly rainfall was 52.19 mm. The maximum temperatures was recorded in August

(24.2 ˚C) and the minimum in January (7.9 ˚C).

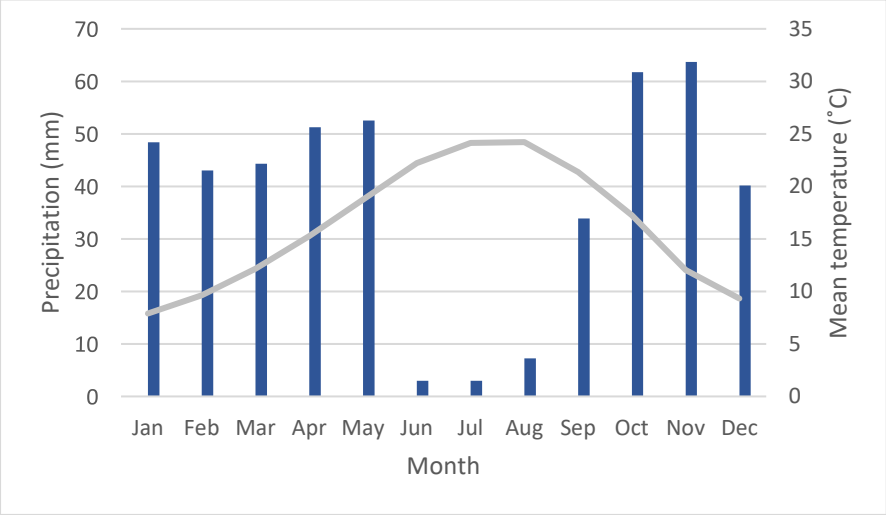

Fig. 6 Climate diagram showing temperature (line) and precipitation (bar) at study site for the period of cork growth – 2011-2017. Climate data: IPMA – Instituto Português do Mar e da Atmosfera (Portuguese Institute for the Sea and the Atmosphere)

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Chapter 2 – Cork influenced by a specific water regime – macro and microstructure characterization – the first approach

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Ana Patrícia Poeiras[1], Maria Emília Silva, Björn Günther, Cordula Vogel, Peter Surový, Nuno de Almeida Ribeiro

Affiliations

University of Évora, Mediterranean Institute for Agriculture, Environment and Development (MED), Pólo da Mitra, Apartado 94, 7006-554 Évora, Portugal

A. P. Poeiras, ORCID: 0000-0002-6049-807X, apcp@uevora.pt
[1]corresponding author

University of Trás-os-Montes e Alto Douro, Department of Forest Sciences and Landscape Architecture, Quinta de Prados, 5001-801 Vila Real, Portugal
M. E. Silva, ORCID: 0000-0002-0624-4247

Chair of Soil Resources and Land Use, Institute of Soil Science and Site Ecology, Technische Universität Dresden, Pienner Str. 19, 01737 Tharandt, Germany
B. Günther, ORCID: 0000-0001-6422-2858
C. Vogel, ORCID: 0000-0002-6525-2634

Czech University of Life Science Prague, Faculty of Forestry and Wood Science, Department of Forest Management, Kamýcká 129, 16500 Praha 6, Suchdol, Czech Republic
P. Surový, ORCID: 0000-0001-6637-8661

University of Évora, Institute of Earth Science (ICT) & Department of Plant Science, School of Sciences and Technology, Pólo da Mitra, Apartado 94, 7006-554 Évora, Portugal
N. A. Ribeiro, ORCID: 0000-0002-6884-2808, mncar@uevora.pt

2.1 Abstract

Cork is the most valuable non-wood product of the cork oak (*Quercus suber* L.). However, the cork oak sector may be at risk due to climatic and economic pressures on cork oak forests, affecting both the quantity and technological quality of products. At some sites, irrigation may present a solution for stimulating cork growth and thereby increasing production. This study presents an initial approach to characterizing cork grown in a forest stand associated with a specific water regime, by comparing cork growth on two plots – irrigated and a traditional rainfed – over an initial five-year period. Samples of cork tissue were analysed and several parameters set: cell area, diameter, cell-wall thickness, number of cells, porosity, growth, and density. Irrigation plot samples showed, on average: 25.83±3.74 mm thickness, 5.17±1.49 mm cork-ring width, 0.149±0.041 $g.cm^{-3}$ density, 13±3.4 % porosity coefficient in the tangential plane, 407.58±268.22 μm^2 cell area in the tangential plane and 887.80±449.14 μm^2 in the transverse plane, a total number of cells of 1232 ±147 per mm^2, and 1.03±0.30 µm cell-wall thickness; whereas traditional rainfed plot samples presented: 21.33±5.48 mm thickness, 3.08±1.44 mm cork-ring width, 0.167±0.068 $g.cm^{-3}$ density, 10±3.5 % porosity coefficient in the tangential plane, 304.31±205.83 μm^2 cell area in the tangential plane and 752.45±398.94 μm^2 in the transverse plane, a total number of cells of 1481±153 per mm^2, and 1.204±0.327 µm cell-wall thickness. As regards irrigation, two parameters: ring width and porosity coefficient, proved to be statistically significant, in contrast to density.

2.2 Introduction

Portugal is regarded as the world's largest cork producer and exporter, accounting for 49.6% of annual global production and a revenue of around 815.6 M euros (APCOR 2019). Despite this, according to the Inventário Florestal Nacional – National Forest Inventory, 11300 ha of cork oak (including 3800 ha accounted for by regeneration of the species) were lost between 2005 and 2015 (ICNF 2020). Forest decline, jeopardizing cork production and affecting both quality and quantity, is due to changes in system management, mostly as a result of agropastoral intensification and/or mechanization (for example, soil disking), as well as pests and diseases and increasingly severe climatic conditions (Ribeiro et al. 2004, 2006, 2010; Pinheiro et al. 2008; Camilo Alves et al. 2013; 2017; Oliveira et al. 2016; Pinto Correia et al. 2018). Climate events, especially the annual distribution of precipitation, influence cork-ring growth, which is

less marked during periods of drought (Schmidt et al. 2009; Vaz et al. 2011; Oliveira et al. 2016; Leite et al. 2019). Several authors have indicated a similar relationship between precipitation and cork-ring width (Oliveira et al. 1996a; Oliveira et al. 1996b; Caritat et al. 1996, Costa et al. 2002, Surový et al. 2009) and solar activity and cork-ring width (Surový et al. 2008), and presented evidence of further factors affecting cork quality: management practices, competition, debark pressure, and soil organic matter content (Montero et al. 1991, 1994, 1998; Montoya et al. 1984; Montoya 1985; Tinoco et al. 2009; Ribeiro et al. 2010; Ribeiro and Surový 2011).

The cork oak grows most intensely during two periods (Oliveira et al. 1996a; Vaz et al. 2011): (1) spring, when growth is greater due to the optimal conditions; and (2) autumn, when growth varies from low to moderate depending on temperature and rainfall. Oliveira et al. (1996a, b) mentioned the high dependency of annual growth dynamics on significant rainfall variation during spring, summer and autumn. If the tree receives sufficient water, nutrients from twiglets migrate to evergreen leaves, initiating both new leaf production and elongation growth, followed by vascular cambium and phellogen activation – factors determining cork growth.

In the present study we seek to determine the contribution of irrigation to cork production and the possible enhancement of production in terms of quantity and quality – the study´s hypothesis. However, management practices may exert an influence on cork characteristics (implemented silviculture model), thus there is a need for providing an understanding of how these may modify cork's cellular structure and, consequently, its quality.

Cork tissue or phellem is defined as the group of cells belonging to the periderm – which derives from traumatic phellogen, as widely described in the literature (Natividade 1950; Graça and Pereira 2004; Pereira 2007, 2015). Its characteristics, as well as cork formation, influence the properties of final products, including three important quality parameters: growth, porosity and density. Cork formation depends on genetic and environmental factors as well as the interaction between them. Pereira et al. (1987) described the structure of cork as homogeneous tissue with no intercellular spaces and thin-walled cells shaped like hexagonal prisms stacked transversely in columns. The different planes observed in cork structures are explained by Pereira (2015), who mentions a similar cell-wall thickness in three planes.

Porosity is a quality parameter defined by the volume occupied by lenticular channels that grow from pith to bark in both a radial and a transverse direction. It is affected by both genetic factors

(lenticular channels and wood inclusions) and external factors (site-specific edaphic-climatic characteristics) (Pinto Correia et al. 2013). This may be observed in radial, transverse and tangential sections: in the first two, pores have an elongated shape, perpendicular to cork-rings, while in tangential sections pores are elliptically shaped (Fortes et al. 2004). Porosity is expressed by the porosity coefficient, whose values establish limits for the cork-stopper industry.

Density, one of cork's main structural characteristics (Fonseca et al. 1994), determines its suitability for different uses (Pereira et al. 1996) and may be influenced by a number of factors: geometry and cell dimension, autumn and spring cell dimension, the presence of lenticular channels, inclusions, discontinuities, cell-wall wrinkling, and the extent of porosity (Fortes at al. 2004; Pereira 2015; Anjos et al. 2008).

In industrial cork processing, the raw material is boiled, which causes radial expansion of about 15%, and 6% transversely and tangentially, producing an increase in thickness. Porosity drops to 50% of gross values and density also undergoes a small change of around of 20% (Fortes et al. 2004). Although the samples used in this study were analysed raw, as the aim was to study the effect of the irrigation of cork oaks on the physiology of cork formation and its characteristics, references to boiled cork have been included throughout this paper due to their importance for the industrial process and the lack of raw cork references. However, this comparison may be made since the boiling process improves the characteristics of cork (leading to an increase in thickness and a decrease in porosity and density), and therefore, when dealing with raw cork, we have a good idea of what its characteristics will be following boiling.

The aim of the study

The aim of this study is to characterize the structure of cork from irrigated cork oaks from a forestry product perspective. Samples were compared with those from a traditional rainfed plot, both groups displaying a marked degree of variability. Cork growth was characterized in accordance with the same growth parameter: initial cork rings. The following parameters were set: cork density, thickness, ring width, porosity coefficient, the number of pores and pore areas, and cellular structure (area, diameter, cell-wall thickness, number of cells). The present study is part of an ongoing project and will provide the basis for the further characterization of cork from cork oaks under different water regimes.

2.3. Material and methods

2.3.1 Cork samples and study sites

Reproduction cork (*amadia*) raw samples from 24 trees at 130 cm height, collected during the 2017 harvest on two research plots maintained by University of Évora were taken as the subject of this study. Each plot has been subjected to a different treatment as regards water availability: at Site A irrigation was used, while Site B corresponds to a traditional rainfed system (Fig. 7). A total of 12 cork samples were collected at each site.

Samples were analysed in their raw state - before any industrial processing – in order to ascertain the real effect of irrigation on cork structure and physiological formation. Samples from site A were harvested following special authorization granted by the Instituto da Conservação da Natureza e das Florestas - Portuguese Institute for Nature Conservation and Forests.

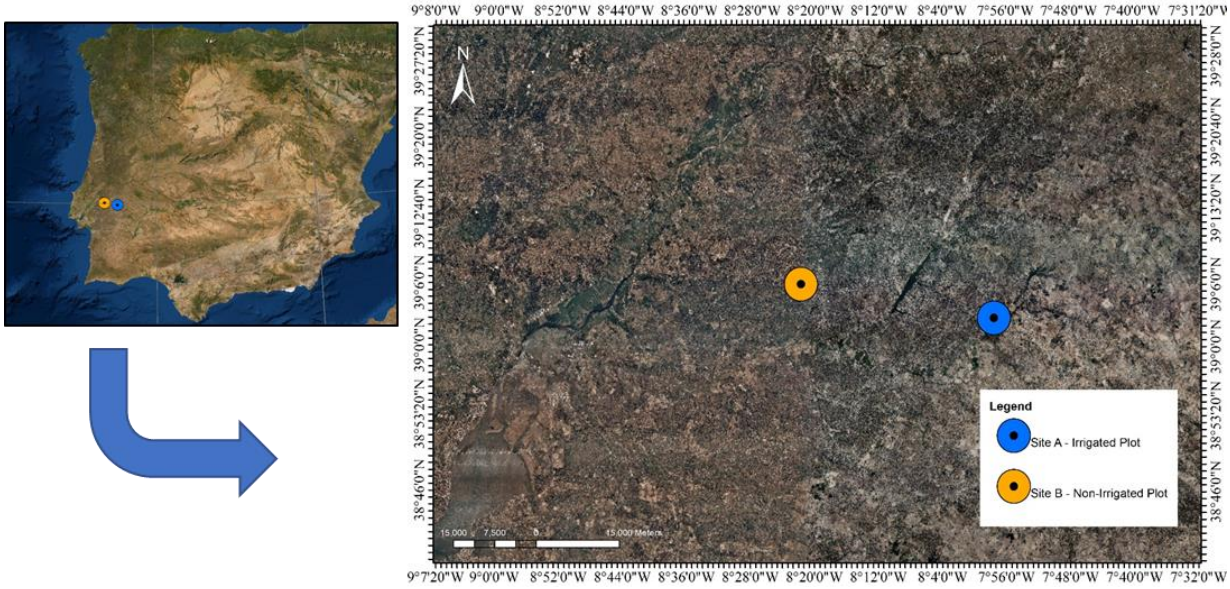

Fig. 7 Research sites: Site A – Irrigated plot; Site B – Non-irrigated plot; Coordinate system: ETRS_1989_Portugal_TM06; Image: Direção geral do território, Portugal, 2018

Samples from Site A presented five complete rings over a six-year growth period. Though located on an 18-year-old plantation, with watering campaigns conducted by the producer from the beginning until 2017, following the same regime as the nearby olive grove, samples were

harvested in 2017 from centenary trees (Table *3*). Irrigation occurred once a week, from June to October, averaging 1928 m^3. ha^{-1} per year (Table 4). At the end of each round of irrigation, Inofert Plus 14:11:6 + 8 B (3,5 Kg. ha^{-1}. $year^{-1}$) was supplied. Irrigation was drip-surface with one tube per plantation line and 2.1 L drip emitters spaced 0.75 m apart. The site presented soil characterized as a Low Satured Gleyic Luvisol. Cork samples from Site B (the control site) were also extracted from centenary adult trees (Table *3*) located on a traditional rainfed plot (not forming part of a plantation) on Non-humic Litolic soil. Samples from Site B presented eight complete rings over a nine-year growth period, harvested in accordance with Portuguese law governing debarking. Trees at both sites had access to underground water.

Table 3 Mean ± Std. deviation for tree characteristics: total height, stem diameter at breast height and stem height of harvesting

	Trees	
Characteristics (average of 12 trees)	**Site A – Irrigated plot**	**Site B – Traditional rainfed plot**
Total height (m)	11.94 ± 1.40	10.88 ± 2.01
Stem diameter at breast height (cm)	198.55 ± 20.84	143.2 ± 34.19
Stem height of harvesting (m)	2.02 ± 0.53	1.68 ± 0.40

Both sites presented a Mediterranean climate, with hot dry summers and mild winters (Fig. 8): Mean annual rainfall and mean temperature: Site A 452.2 mm (Table 4) and 16.2˚C, respectively; Site B 400.9 mm (Table 4) and 15.8˚C, respectively.

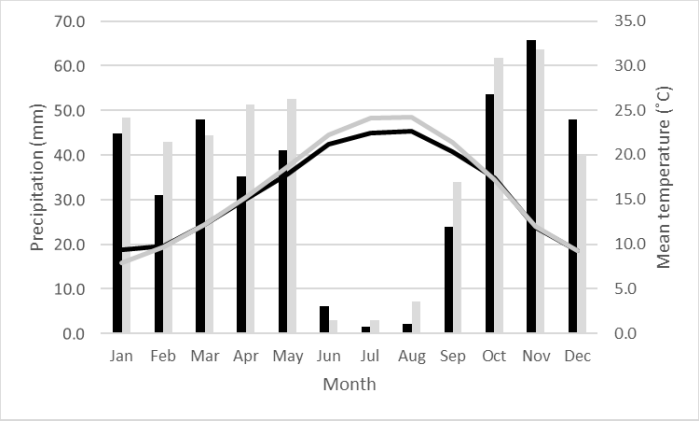

Fig. 8 Climate diagram showing temperature (lines) and precipitation (bars) at the study sites. Site A (irrigated plot) in grey and site B (traditional rainfed plot) in black, for the entire period of cork growth (2011-2017 and 2008-2017, respectively). Climatic data: IPMA – Instituto Português do Mar e da Atmosfera (Portuguese Institute for Sea and Atmosphere)

Table 4 Annual variation of precipitation and irrigation distribution at Site A

Annual precipitation (mm)			Irrigation distribution – Site A (m^3. ha^{-1})
Complete year of cork growth	**Site A – Irrigated plot**	**Site B – Traditional rainfed plot**	
2008*		344.4	
2009*		307	
2010*		310.5	
2011	506.3	502.2	1400
2012	371.5	290.1	2500
2013	453.8	416.3	1800
2014	579.9	579.4	1350
2015	313.3	283	2600
2016	613.2	638	1250
2017	327.3	338.4	2600

*Years that are not part of cork growth for site A

2.3.2 Macrostructure analysis

2.3.2.1 Density and growth

For each sample, a cross-section with 3 cm maximum length and 1.5 cm average thickness was taken using an elementary slicer machine (ABO with 220 mm incorporate blade and sharpener, Oggiona VA, Italy) and conserved under typical environmental conditions. The density and growth of polished samples (using P240 Rhynowood Indasa sandpaper) were analysed by means of X-ray technology using a QTRS-01X Tree Ring Analyser (Quintek Measurement Systems Inc., Knoxville, TN, USA). Measurements were performed automatically using QTRS-01X software (Quintek Measurement Systems Knoxville, Knoxville, TS, USA) (for image output, see Fig. 9). Calculations were made automatically using the following equation, relating X-ray attenuation and density: $\frac{\mathbf{I}}{\mathbf{I_o}} = \boldsymbol{e}^{-\boldsymbol{\mu l}^{\boldsymbol{t}}}$, where **I** is radiation beam intensity after passing through the sample, **Io** is radiation beam intensity not passing through the sample (from bark to pith), $\boldsymbol{\mu l}$ is the sample linear attenuation coefficient, and **t** is sample thickness.
Fixed parameters used included a 3.80 mass absorption coefficient, 200 threshold, and 50 dead band. Bark and belly half-rings were not accounted for in data analysis.

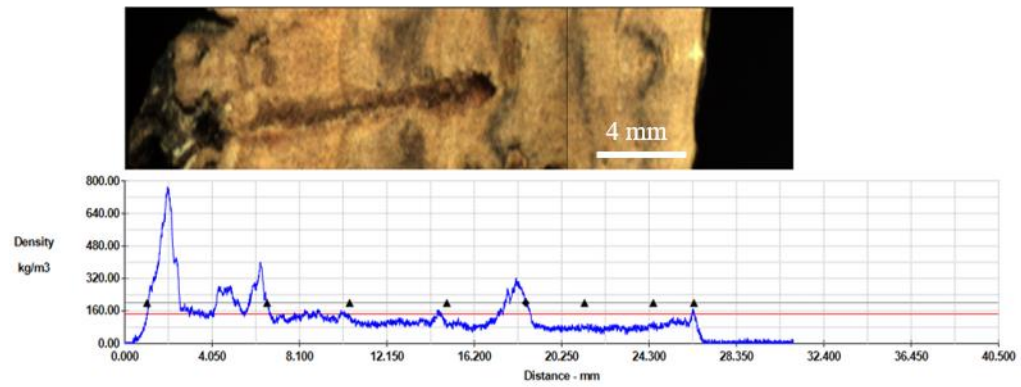

Fig. 9 Image output for one testing samples, using QTMR 01X software: density (kg.m^{-3}) expressed over the ring width (mm) along scan line from bark to pith

2.3.2.2 Porosity

Regarding porosity, samples were cut along the tangential and transverse planes (Fig. 10a, b) and polished prior to analysis. Digital images were obtained by means of a camera (AVT Marlin F-145C2, Stadtroda, Germany). Areas and numbers of pores along the two planes were measured using Image Pro-Plus 6.2 software (Media Cybernetics, Bethesda, USA), and porosity coefficients were calculated. Two regions of interest (ROI) were measured for each sample, with a total of 24 per plot.

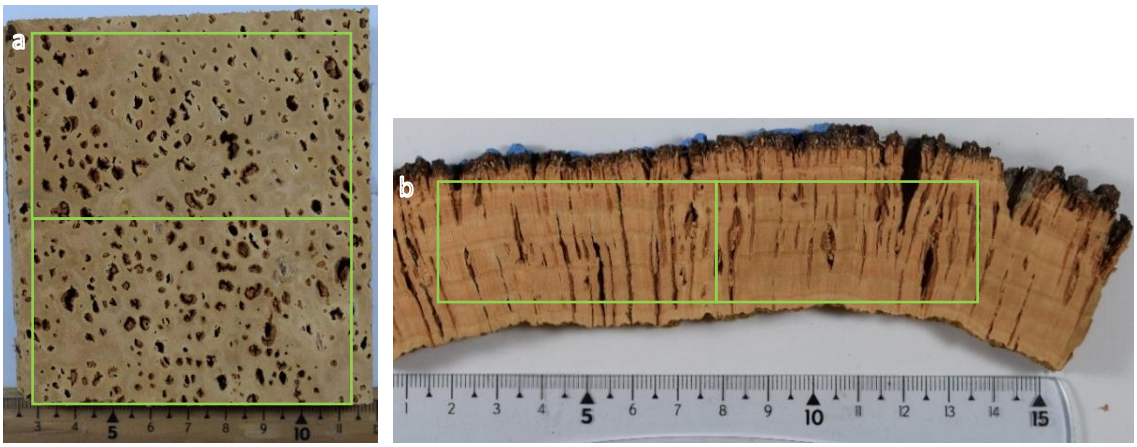

Fig. 10 Cork samples from the irrigated plot prepared for porosity analysis: a - tangential plane; b - transverse plane. Areas within the lines represent the regions of interest (ROI´s)

2.3.3 Microstructure analysis

2.3.3.1 Biometric analysis

For biometric analysis, tangential and transverse samples were prepared (Fig. 11a, b). Tangential samples were taken using a movable blade microtome (Reichert with Jung blades), each 20 µm thick. For each sample, three images were obtained using a binocular magnifying glass (Nikon SMZ-10, Japan) with 40x magnification and analysed using Image Pro-Plus 6.2 software (Media Cybernetics, Bethesda, USA). Four ROIs, randomly distributed per image, were measured (diameter, area and cell count).

Transverse samples were cut 1 mm thick. Using a polarized light microscope (Olympus BX50, Tokyo, Japan), nine images per sample were obtained. Images were distributed along the cork-ring width. Overall, 24 cells per image were analysed using Image Pro-Plus – 6.2 software (diameter and area).

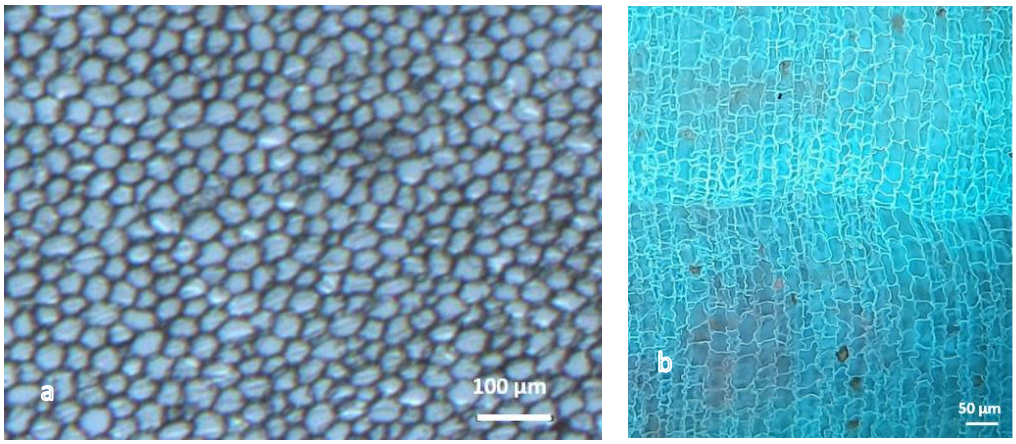

Fig. 11 Microphotographs of cork sample histological sections: a – tangential section (magnifying glass); b – transverse section (polarized light microscope).

2.3.3.2 Cell-wall analysis by means of SEM

For scanning electron microscopic analysis of cork cell features, the transverse samples described in the section *Biometric analysis* were fixed on aluminium specimen holders using conductive double-sided adhesive carbon tabs coated with approximately 40 mm carbon, using an EMITECH K905 Carbon Coater (Emitech Ltd, Ashford, Kent, UK). To minimize charging

effects, thus allowing for higher resolution, they were kept under high vacuum conditions in accordance with the description given by Crouvisier-Urion et al. (2019). For each sample (a total of 24), two images were obtained using MAPS software (MAPS version 2.1.38.1199, Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 10kV beam energy and 2.5 spot size under high vacuum conditions with a scanning electron microscope (Quanta FEG 650, Thermo Fisher Scientific, Waltham, Massachusetts, USA). For each sample and image, 168 to 224 subframes were taken with high magnification and stitched together using MAPS software. The stitched SEM images (Fig. 12) were used for image analysis.

Cell-wall thickness was measured using the ImageJ 1.52a program (Wayne Rasband, National Institutes of Health, USA) and the general structure and features were observed by means of SEM images. Two images per sample were obtained and 200 measurements per image were taken, with a total of 400 cell-wall thickness measurements per cork sample.

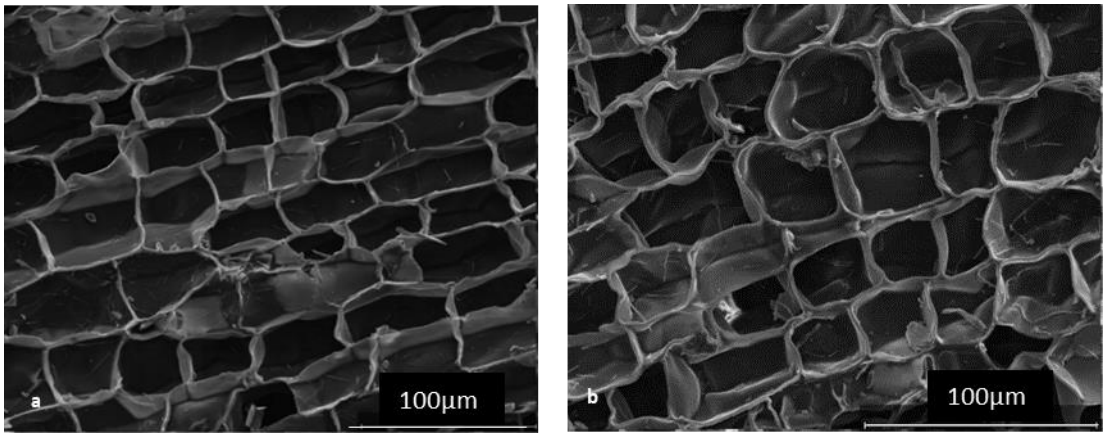

Fig. 12 Cork samples cellular structure from a transverse section obtained with scanning electron microscope: a - sample from the irrigated plot; b – sample from the traditional rainfed plot

2.3.4 Statistical analysis

Statistical analysis was conducted using JMP4.0.2 and SPSS v.25 software package (IBM Corp., Armonk, NY, USA). Analysis of variance (ANOVA) was performed to compare samples from the irrigated plot and the traditional rainfed plot, as well as comparing sample repetitions in each group due to the variability between trees.

Regions of interest for the different samples were compared and variability within trees analysed. A Gauss normal distribution fitted model (General Linear Models) was used to compare the main effects and evaluate the significant differences between means.

In the text the following statistical explanation was used:

n.s. - not significant ($p > 0.05$); * - significant ($p < 0.05$); ** - very significant ($p < 0.01$); **** - highly significant ($p < 0.001$).

2.4 Results and Discussion

2.4.1 Macrostructure analysis

2.4.1.1 Growth and density

Cork thickness for irrigated and non-irrigated plots was 25.83 ± 3.74 mm (5 complete years of growth) and 21.33 ± 5.48 mm (8 complete years of growth) respectively, confirming the contribution of irrigation to cork growth (Table 5). The equivalent cork growth rings at the two sites (the first 5 rings) were compared. Samples from irrigated cork oaks revealed on average greater growth per ring (5.17 ± 1.49 mm) than those of non-irrigated cork oaks (3.08 ± 1.44 mm) (Table 5). Data from non-irrigated trees were in accordance with cork-ring width reported in the literature: Pereira (2007) recorded 3.50 mm cork-ring average growth (sampling across Portugal) and Chorana et al. (2019) an average of 2.43 mm cork annual growth in North-West Algeria. Both articles make reference to boiled cork, and, as this paper deals with raw cork, it is expected that the average cork ring-width would be greater after boiling. Several studies cited environmental effects as the main factors affecting cork growth, particularly cork-ring growth decrease in response to episodes of drought (Costa et al. 2016; Oliveira et al. 1996a, 1996b, 2016). Therefore, it can be stated that the irrigation silvicultural model implemented contributes to cork-ring width increase.

While irrigation resulted in an increase in cork growth, cork producers also find the growth-density relationship to be important. Cork density from irrigated cork oaks averaged 0.149 ± 0.028 $g.cm^{-3}$ while cork density from non-irrigated cork averaged 0.167 ± 0.068 $g.cm^{-3}$ (Table 5), which may be associated with the thinner cell walls of the cork from the irrigated plot. These

characteristics affect the mechanical behaviour of cork (Knapic et al. 2016) and are responsible for the performance of cork products. For example, Oliveira et al. (2014) found a greater resistance to compression in samples with a high density and small ring width.

According to the literature, cork presents a high degree of variation as regards density values, depending on certain features, such as cell-wall thickness and lumen size. The findings of the present study (Table 5) were in accordance with those of Pereira (2007) in Knapic et al. (2016), who presented a density variation ranging from 0.120 to 0.170 $g.cm^{-3}$. Natividade (1934) found a density variation interval of 0.120-0.200 $g.cm^{-3}$ for raw cork, similar to the results of the present study. Additionally, authors such as Silva et al. (2005) and Anjos et al. (2008) presented results (after boiling) of between 0.120 and 0.240 $g.cm^{-3}$. This wide range of values demonstrates the high degree of variability of cork. As already mentioned, the density of samples was determined in the present study for raw cork, which would be expected to produce slightly higher values than those reported in the literature (mostly regarding boiled cork) since the boiling procedure involves great decompression of cell walls and thus a decrease in density.

Table 5 Mean ± Standard deviation for macroscopic characterization of cork thickness, ring width, total density and cork sample ring density by type of treatment (irrigated/non-irrigated). Cork thickness and total density considered the complete years of growth for samples from both plots. Values of cork-ring width and ring density were addressed to the same 5 initial rings of cork formation: Irrigated plot – 2012 to 2016; Non-irrigated plot – 2009 to 2013)

Treatment	Cork thickness (mm)	Cork-ring width (mm)	Total density ($g.cm^{-3}$)	Ring density ($g.cm^{-3}$)
Irrigated	25.83 ± 3.74	5.17 ± 1.49	0.149 ± 0.028	0.149 ± 0.041
Non-Irrigated	21.33 ± 5.48	3.08± 1.44	0.167 ± 0.068	0.167 ± 0.097

Analysis of variance (Table 6) showed a significant degree of influence by *Treatment* and *Ring* on ring width (*p* Value <0.0001). Type of treatment was the main source of ring-width variation, accounting for 46.59 % of variation. In contrast, the source of *Ring* variation, which was highly significant, was only found to be 6.02 %.

Table 6 Analysis of variance in accordance with treatment (T) of different trees (Tr) and cork rings (R), for density and ring width.

		Ring width			Density		
Source	DF	F	*p* Value	Var (%)	F	*p* Value	Var (%)
Treatment (T)	1	39.23 ***	<.0001	46.59	0.56 ns	0.0534	0
Tree/Treatment (Tr/T)	22	2.38 **	0.0023	8.47	6.88 ***	<.0001	51.40
Ring (R)	4	5.70 ***	0.0004	6.02	2.91 ***	0.0258	3.49
R x T	4	4.18 **	0.0038	8.14	1.38 ns	0.2469	1.39
Residual (R x Tr/T)	88		<.0001	30.77		<.0001	43.72

In contrast to growth, there was no significant difference in density associated with irrigation treatment. Variance, associated with the source *Trees within each treatment (Tr/T)*, presented a *p* value of <0.0001 (Table *6*), accounting for 51.40 % of variation. In studies carried out by Silva (1996) and Marrafa (2016), similar results were found, as variation accounted for by the trees proved to be the main source of cork density variation, with a high degree of variability in terms of the genetic dependency of this characteristic. However, Marrafa (2016), Ribeiro et al. (2006) and Ribeiro and Surový (2011) stated that cork-ring width and density are affected by intraspecific competition (intense competition leading to smaller cork-ring width and higher cork density). Thus, these cork characteristics may be controlled using the irrigation silvicultural model, with a view to achieving forest-stand growth stock optimization over time and also individual tree annual cork-ring width and cork-density objectives. In addition, Fonseca et al. (1994) reported a significantly negative relationship between ring-width growth and density.

2.4.1.2 Porosity

Regarding the tangential coefficient of porosity, values of 13 ± 3.4 % were recorded for the irrigated plot and 10 ± 3.5 % for the non-irrigated plot (Table 7). As regards the transverse coefficient of porosity, the findings of the present study are in keeping with the coefficient reported by Pereira et al. (1996) ranging from below 2% to over 15%, while higher values were obtained from irrigated plot samples (14 ± 4 %) than from non-irrigated plot samples (9 ± 3 %). However, these values are expected to decrease after boiling, as indicated by Fortes et al. (2004).

Table 7 Means ± Standard deviation of macroscopic characterization as regards the coefficient of porosity, number of pores and individual pore area for cork samples, according to type of treatment

Treatment	Coefficient of porosity (%)		Number of pores		Individual pore area (mm^2)	
	Tangential	Transverse	Tangential	Transverse	Tangential	Transverse
Irrigated	13 ± 3.4	14 ± 4	385.50 ± 144.73	43.58 ± 9.18	1.448 ± 0.036	6.635± 0.311
Non-Irrigated	10 ± 3.5	9 ± 3	421.67 ± 110.85	30.71 ± 12.95	0.785 ± 0.033	4.284 ± 0.311

A statistically significant relationship between the coefficient of porosity and type of treatment was found using a general linear model. Analysis of variance (Table 8) demonstrated that *Treatment* was the principal source of variation of the transverse coefficient of porosity (49.86%), but this was not the case with the tangential plane, where *Trees within each treatment* was the main source of variation (57.58 %). While porosity in the transverse plane corresponded to different years of cork growth, in the tangential plane it is accounted for by a single year of cork growth. This may be why in the tangential plane *Treatment* was not a significant source of variation as compared with the transverse plane. It would appear that porosity in the tangential plane is determined by genetic variability factors (seeing that the differences between individual trees were more important), whereas in the transverse plane environmental factors, such as irrigation, proved to be determining factors. Marrafa (2016) found that *Trees* was a highly significant source of variation as regards porosity in the tangential plane, while other factors associated with the environment (Year and Plot) were not, which is in accordance with the findings of the present study.

Table 8 Analysis of variance for treatment and trees, as regards the coefficient of porosity in the tangential and transverse planes

		Coefficient of porosity					
		Tangential			Transverse		
Source	DF	F	*p* Value	Var (%)	F	*p* Value	Var (%)
Treatment (T)	1	5.32 *	<.0001	24.04	20.28 ***	<.0001	49.86
Tree/Treatment (Tr/T)	22	7.25 ***	<.0001	57.58	1.63 ns	0.1241	11.93
Residual	24		<.0001	18.38		0.005	38.21

According to Silva (1996), greater soil water availability leads to larger pores. Additionally, Fortes et al. (2004) reported larger pores as a defect associated with fast-growing corks and

recorded a pore length of 14 mm in the cross section and a diameter in the range 4-7 mm in the tangential section (these are higher values than those found in the present study). The same author also observed pore areas of less than 1 mm^2 for most pores in the tangential plane and this is more in keeping with the results of the present study (Table 7): a lower number of pores in the tangential plane for the irrigated plot (385.50 ± 144.73) and a higher coefficient of porosity (13 ± 3.4) and pore area (1.448 ± 0.036 mm^2) than the non-irrigated plot for which the recorded values were: 421.67 ± 110.85 pores, coefficient of porosity 10 ± 3.5 and pore area 0.785 ± 0.033 mm^2.

Regarding number of pores, analysis of variance (Table 9) demonstrated that *Treatment* was the main source of variation in the transverse plane (37.26 %) but not in the tangential plane (0%), while *Trees within each treatment* was found to be the main source of variation in the tangential plane (86.46 %).

The source of variation referred to as *Residual* (which represents repetitions for each sample) accounted for a significant variation in the number of pores in the transverse plane (42.94 %) but only 13.54 % in the tangential plane, thus constituting a source of great variability within the stem.

Table 9 Analysis of variance for treatment for trees, as regards number of pores in the tangential and transverse planes

		Number of pores					
		Tangential			Transverse		
Source	DF	F	*p* Value	Var (%)	F	*p* Value	Var (%)
Treatment (T)	1	0.49 ns	0.016	0.00	11.83 ***	<.0001	37.26
Tree/Treatment (Tr/T)	22	13.78 ***	<.0001	86.46	1.92 ns	0.0607	19.80
Residual	24		<.0001	13.54		0.0071	42.94

The area of individual pores of irrigated cork oaks presented values of 1.448 ± 0.036 mm^2 (Mean ± Std. deviation) in the tangential plane and 6.635 ± 0.311 mm^2 in the transverse plane (Table 6), while cork from the non-irrigated plot presented lower values: 0.785 ± 0.033 mm^2 and 4.284 ± 0.311 mm^2 in the tangential and transverse planes, respectively (Table 7). Thus, cork from non-irrigated cork oaks presented smaller pores in both planes. It should be noted that in the tangential plane when irrigated cork oaks are compared to non-irrigated cork oaks a higher number of pores was found, which were smaller (Table 7).

Variation in individual pore area was mainly accounted for by repetitions (*Residual*) of each sample (p <0.0001) by more than 96.7% and 99.6% in the tangential and transverse planes, respectively (Table 10). Despite the high level of significance in both planes (p <0.0001), the sources of variation designated as *Treatment* and *Tree within each treatment* provided only a small contribution to variation in this case, below 2 %.

Table 10 Analysis of variance by treatment of tree samples, with regard to individual pore area in the tangential and transverse planes

	Individual pore area (mm^2)							
	Tangential				Transverse			
Source	DF	F	*P* Value	Var(%)	DF	F	*P* Value	Var(%)
Treatment (T)	1	13.135 ***	<.0001	1.73	1	8.340 ***	<.0001	0.19
Tree/Treatment (Tr/T)	22	16.921 ***	<.0001	1.61	22	2.823 **	<.0001	0.21
Region/Tree/Treatment	24	0.846 ns	0.6789	0.000	24	0.911 ns	0.587	0.000
Residual	19324		<.0001	96.65	1735		<.0001	99.60

2.4.2 Microstructure analysis

2.4.2.1 Biometric analysis

The cell area of cork samples from irrigated cork oaks was larger in both planes (tangential plane: 407.58 ± 268.22 μm^2; transverse plane: 887.80 ± 449.14 μm^2) than that of samples from the non-irrigated site (tangential plane: 304.31 ± 205.83 μm^2; transverse plane: 752.45 ± 398.94 μm^2) (Tables 11 and 12). With regard to the number of cells, the opposite was found: a lower number of cells were present in samples from the irrigated plot than those from the traditional rainfed plot, the same pattern being found with regard to porosity in the tangential plane (a lower number of pores and a larger area) (Table 7).

Table 11 Mean ± Standard deviation of biometric characterization of the tangential plane, measured in an area of 159913 μm^2, by type of treatment (irrigated/non-irrigated)

Treatment	Number of cells per mm^2	Cell area (μm^2)	Max diameter (µm)	Min diameter (µm)
Irrigated	1232 ± 147	407.58 ± 268.22	27.19 ± 10.81	14.84 ± 6.24
Non-Irrigated	1481 ± 153	304.31 ± 205.83	22.92 ± 9.00	13.04 ± 5.46

Table 12 Mean ± Standard deviation of biometric characterization in the transverse plane, measured in 24 cells, by type of treatment (irrigated/non-irrigated)

Treatment	Cell area (μm^2)	Max diameter (μm)	Min diameter (μm)
Irrigated	887.80 ± 449.14	44.06 ± 12.83	22.21 ± 6.76
Non-Irrigated	752.45 ± 398.94	38.66 ± 11.78	21.36 ± 6.66

Pereira (2007) reported cell diameter values in the range of 10-20 μm, while mean values recorded in the present study were in the upper range of values (Tables 11 and 12). Fortes et al. (2004) reported average cell areas ranging from 400 to 600 μm^2 for boiled cork. Tangential analysis findings for raw samples proved to be similar to the lower values recorded in the present study (Table 11). An increase in diameter and cell area is expected following boiling. However, in the present study, the findings were similar to those of Natividade (1934) as regards raw cork. An average cell height of 37.3 μm was recorded in the radial plane, in keeping with the 38.66±11.78 μm found for maximum diameter on the non-irrigated plot in the transverse plane (Table 12). Natividade reported a 55-25 μm range in cork cell height.

A statistically significant relationship between cell area and type of treatment was established by means of a general linear model (Table 13). Following analysis of variance, it was concluded that in both planes each variation source was statistically significant (p <0.0001), which means that *Treatment, Trees*, S*amples* and the *Regions of interest within each sample* all have an influence on cell area. However, *Treatment* presented a lower variation with regard to the transverse plane (0.245%), which means that irrigation may have a less marked influence on transverse cell area. In the tangential plane, *Sample* (45.54%) and *Region* (42.79%) sources – which represent the variation within tree – were those which accounted for most of the variation. *Region* was also an important source with regard to the transverse plane, accounting for a 78.6% variation in cell area. The transverse plane indicated cork growth for the total number of years and this variability may be accounted for by differences in random cells measured in spring and autumn. It may also be accounted for by cell wall wrinkling, once the analysis was made in raw cork (Fig. 13).

Table 13 Analysis of variance for treatment of tree, sample per tree, and region for each sample, as regards cell area in the tangential and transverse planes

	Cell Area							
	Tangential				Transverse			
Source	DF	F	*p* Value	Var(%)	DF	F	*p* Value	Var(%)
Treatment	1	44.282 ***	<.0001	2.765	1	5.868 *	<.0001	0.245
Tree/Treatment	22	2.750 **	<.0001	0.488	22	5.288 ***	<.0001	0.489
Sample/Tree/Treatment	48	5.258 ***	<.0001	45.543	48	1.171 ns	<.0001	3.356
Region/Sample/Tree/Treatment	216	5.081 ***	<.0001	42.785	144	4.541 ***	<.0001	78.600
Residual	62191		<.0001	8.420	4968		<.0001	17.311

2.4.2.2 Cellular structure and cell walls

Cell walls provide the basis for cork properties. Through SEM it was possible to measure the cell-wall thickness and gain an enhanced view of the cork structure. Under both water regimes it was possible to observe a number of columns composed of cells with a rectangular prism shape (Figure 13), in accordance with the literature (Graça and Pereira 2004; Pereira 2007, 2015). Regarding cork from the irrigated site, some cell walls were wrinkled (Fig. 13a), which may be accounted for by cork which was not boiled, as cells thus did not have the opportunity to expand. In several samples from the two sites, cell lumens and walls showed some solid deposits with different shapes and sizes and soiled surfaces (Fig. 13b), similar to the deposits found by Xiaozhou et al. (2017) in *Quercus variabilis.*

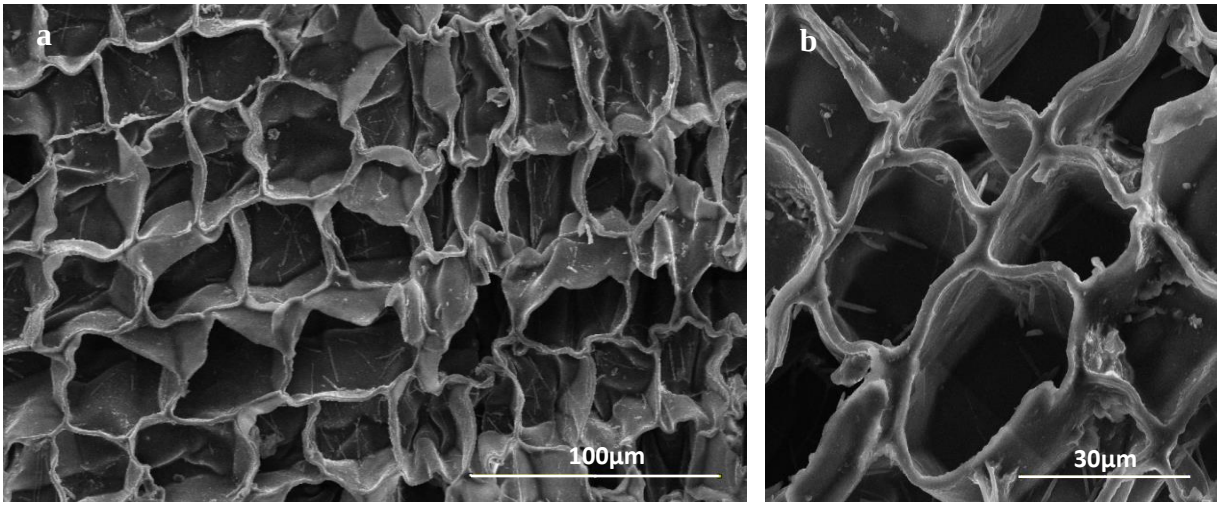

Fig. 13 Cellular structure of a cork sample from the irrigated plot obtained using SEM: a – observation of wrinkled and non-wrinkled cells; b – deposits on cell walls and artefacts in cell lumens

Cork cell-wall thickness random measurements of irrigated cork oaks ranged from a minimum of 0.207 to a maximum of 2.834 µm, with a mean and standard deviation of 1.031 ± 0.300 µm (Table 14), whereas values for non-irrigated cork oaks ranged from a minimum of 0.362 µm to a maximum of 3.463 µm, with a mean and standard deviation of 1.204 ± 0.327 µm. These values provided confirmation of the lower cork density of sample from irrigated cork oaks, although the treatment effect on density is not statistically significant (Table 15).

Table 14 Mean ± Standard deviation of cork cell-wall thickness, by type of treatment

Treatment	Cell-wall thickness (µm)
Irrigated	1.031 ± 0.300
Non-Irrigated	1.204 ± 0.327

Natividade (1934) stated that values ranging from 1 to 2.25 µm are most frequently found in raw cork tissue, depending on the growth season, and the findings of the present study fell within this interval. In slow-growing cork oaks, such as those in Algeria and Morocco, thickness is usually greater (Natividade 1934), which causes slight elasticity.

All tested variation sources were highly significant ($p < 0.0001$, Table 15), demonstrating the important influence of irrigation on cork cell walls. The two regions of interest in each sample are a highly significant source affecting wall-thickness variation, which reflects the great degree of variability within the tree ($p < 0.0001$) (Table 15). Individual cell variation (*Residue*) accounted for most wall-thickness variation (62.6%), in accordance with the results presented by Silva (1996) and Chorana et al. (2019).

Table 15 Analysis of variance by treatment for samples per treatment and ROI in each sample, as regards cell wall thickness

	Cell-wall thickness			
Source	**DF**	**F**	***P* Value**	**VE (%)**
Treatment	1	1007.423 ***	<.0001	11.6
Sample/Treatment	22	118.367 ***	<.0001	11.1
ROI/Sample/Treatment	24	47.858 ***	<.0001	14.7
Residual	9552			62.6

2.5 Conclusion

The comparison of cork samples from two different sites with distinct implemented silvicultural models was conducted. Site A was subject to a specific irrigation regime while at Site B a traditional rainfed system was implemented.

Characteristics such as growth, density and porosity were analysed and tissues were examined (cell area, diameter, and cell-wall thickness).

The findings of this study demonstrate how greatly irrigation impacted most characteristics analysed. Meanwhile, the results of this study (mostly in accordance with published findings) are regarded as falling within the normal range for cork-stopper production, the most important use of cork.

Cork from the irrigated plot presented a greater thickness over a shorter formation period than that from the traditional rainfed plot over a regular formation period. The watering campaign, applied between June and October, contributed to cork-ring width increase, in accordance with the results of studies on the stimulation of greater growth (per ring) due to significant rainfall events (in spring, summer and autumn), carried out by Oliveira et al. (1996a,b), Caritat et al. (1996), Costa et al. (2002) and Surový et al. (2009).

In contrast to growth, no significant impact was found on density due to type treatment. However, some characteristics such as ring width, porosity and cell-wall thickness may have contributed to observed density. Cork from the irrigated plot showed lower density values in keeping with biometric study results. In fact, cork from Site A presented larger cells with greater transverse and tangential dimensions as well as thinner cell walls than cork from the traditional rainfed plot. Additionally, cork from the irrigated plot presented fewer cells per mm^2, which may affect certain properties of cork, such as its insulation capability, by decreasing the number of cells per mm^2, while the conductivity of heat and sound by cork is facilitated by a smaller number of cells. Cork from the irrigated plot showed higher porosity values in both planes than cork from the traditional rainfed plot.

In conclusion, irrigation proved to be a positive factor for cork growth (providing a contribution to growth monitoring), leading to increased cork production at a specific site and under specific conditions. Additionally, the silvicultural model implemented presented changes in cork features. Thus, the findings of this study indicate an opportunity for developing new irrigated cork oak silvicultural models aiming at the higher productivity of cork oak stands (under the

same water regime conditions) and good cork technological quality, both factors being essential for achieving economic sustainability.

This study represents an initial approach to the characterization of cork in terms of a specific water regime, and is part of ongoing research: the next steps will address different water regimes in order to ascertain optimal conditions for cork growth and thus achieve improved cork quality. Suggestions for future research are that the number of trees should be increased and a count of cell rows in cork rings should be conducted.

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Declarations

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Chapter 3 - A cork cell wall approach to swelling and boiling

Ana Patrícia Poeiras [1], Cordula Vogel [2], Björn Günther [2], Maria Emília Silva [3], Peter Surový [4], Constança Camilo-Alves[1] and Nuno de Almeida Ribeiro [5]

[1]University of Évora, Mediterranean Institute for Agriculture, Environment and Development (MED), School of Sciences and Technology, Pólo da Mitra, Ap. 94, 7006-554 Évora, Portugal. [2]Technische Universität Dresden, Chair of Soil Resources and Land Use, Institute of Soil Science and Site Ecology, Pienner Str. 8, 01737 Tharandt, Germany. [3]University of Trás-os-Montes e Alto Douro, Department of Forest Sciences and Landscape Architecture, Quinta de Prados, 5001-801 Vila Real, Portugal. [4]Czech University of Life Sciences Prague, Faculty of Forestry and Wood Science, Department of Forest Management, Kamýcká 129, 16500 Praha 6, Suchdol, Czech Republic. [5]University of Évora, Institute of Earth Science (ICT) & Department of Plant Science, School of Sciences and Technology, Pólo da Mitra, Ap. 94, 7006-554 Évora, Portugal

3.1 Abstract:

The bark of the cork oak (*Quercus suber L).* is mostly used for cork stopper production, the bark 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 scanning electron microscopy under three regimes: under 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.

3.2 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 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 may be analysed using an environmental and scanning electron microscopic approach. In this study, the cell-wall thickness of cork from two study sites was analysed under three sets of conditions: under raw conditions; after immersion in water; and after boiling. The use of environmental scanning electron microscopy (ESEM) enables 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, 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 characteristics both under raw conditions and after boiling, as well as the swelling behaviour of cork from trees grown with and without fertirrigation. 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], therefore 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. Scanning electron microscopy (SEM) under high vacuum conditions was used for raw conditions and after boiling, while environmental SEM (ESEM) was used on samples after swelling following 24h hydration.

3.3 Results

Cork from the fertirrigated plot 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 plot 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. 14). While the cell walls of cork from the fertirrigated plot are thinner than those of cork from the rainfed plot under raw conditions[16], during hydration the former expanded to a greater degree (Fig. 14). 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.

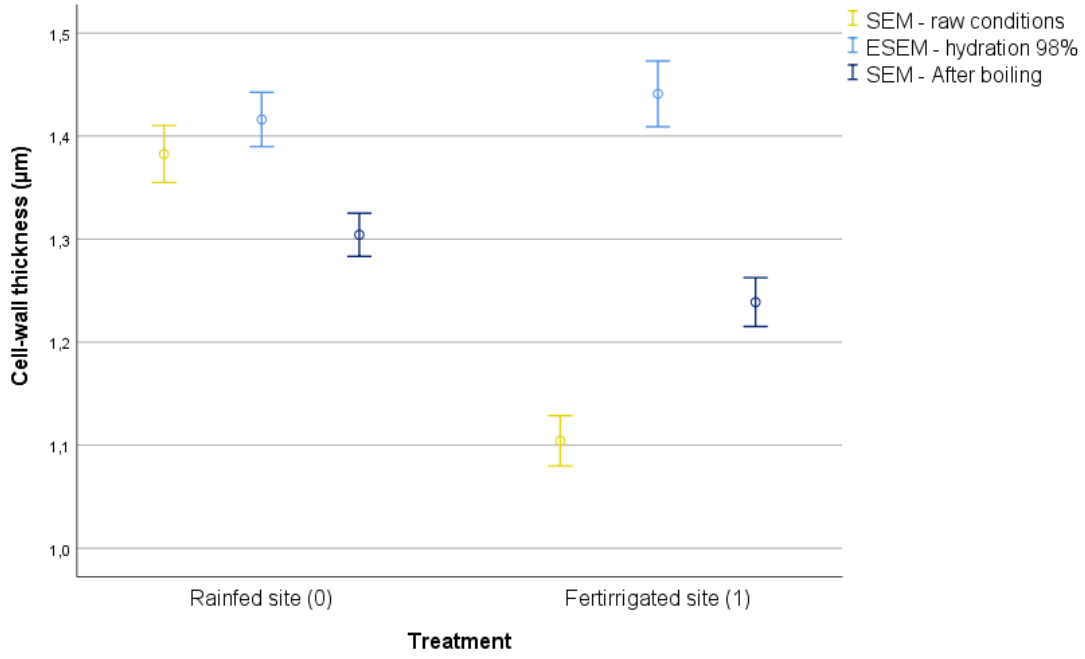

Fig. 14 Mean ± Standard deviation of cell-wall thickness with regard to raw conditions, hydrated conditions and after the boiling stage, for cork samples from the fertirrigated site (1) and the rainfed site (0).

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 16 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 16). 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 16). *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 16). 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.

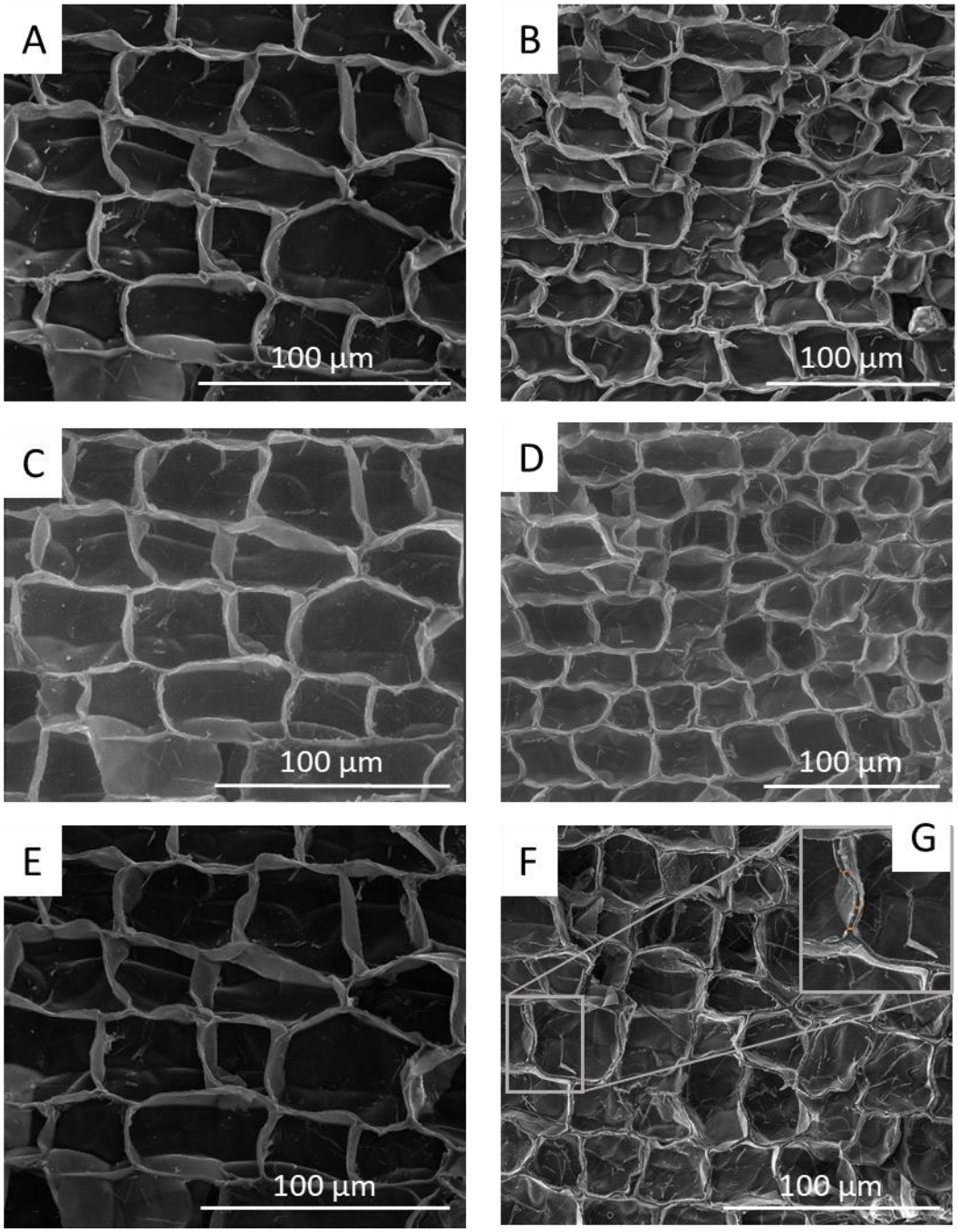

Fig. 15 Microphotographs of the cork cell structure of a transverse section of a cork sample from the irrigated plot (A, C, E) and a cork sample from the rainfed plot (B, D, F): A and B were obtained using SEM on raw cork; C and D were obtained using ESEM after 24h immersion in cold water; E and F were obtained with SEM, after boiling and two days of drying. G: Yellow marks represent an example of a cell wall thickness measurement.

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.

3.4 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.14, 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.

3.5 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.

3.6 Material and Methods

3.6.1 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.

3.6.2 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.

3.6.3 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.15A and B). 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.

3.6.4 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. 15C 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).

3.6.5 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. 15E 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.

3.6.6 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 along the radial length were tagged with a permanent marker pen (Fig. 16) and measured using ImageJ. Samples were boiled at 100º C for one hour, simulating the industrial procedure. After boiling, samples were scanned and radial swelling was measured at the same spots using ImageJ.

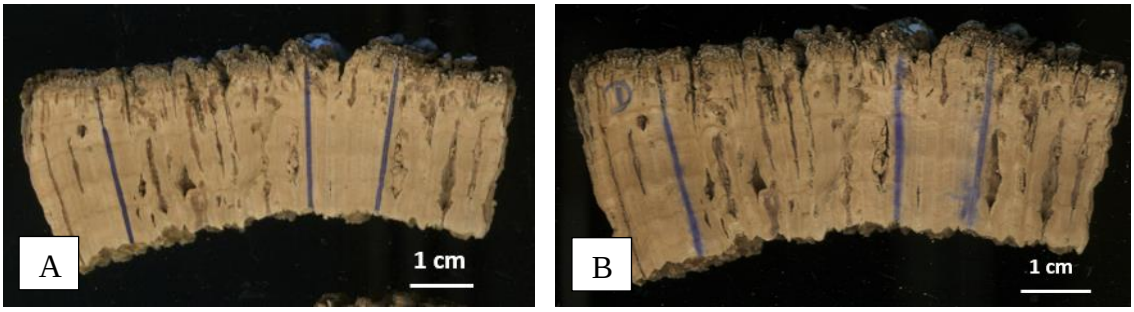

Fig. 16 Example of a macro sample from the irrigated plot: A – before the boiling procedure; B – after the boiling procedure

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Chapter 4 – Influence of water supply on cork increment and quality in *Quercus suber* L.

Ana Patrícia Poeiras [(1)], Teresa Oliveira [(2)], Joana Reis [(3)], Peter Surový [(4)], Maria Emília Silva [(5)] and Nuno de Almeida Ribeiro [(1)]

[(1)] University of Évora, Mediterranean Institute for Agriculture, Environment and Development (MED) & Department of Plant Science, School of Sciences and Technology, Pólo da Mitra, Ap. 94, 7006-554 Évora, Portugal
apcp@uevora.pt
*corresponding author

[(2)] University of Évora, Mediterranean Institute for Agriculture, Environment and Development (MED) & Veterinary Department, Pólo da Mitra, Apart. 94, 7002-554 Évora, Portugal

[(3)] Superior School of Agriculture, Polytechnic Institute of Viana do Castelo, Rua D. Mendo Afonso, 147, Refóios do Lima, 4990-706 Ponte de Lima, Portugal

[(4)] Czech University of Life Sciences Prague, Faculty of Forestry and Wood Science, Department of Forest Management, Kamýcká 129, 16500 Praha 6, Suchdol, Czech Republic

[(5)] University of Trás-os-Montes e Alto Douro, Department of Forestry Sciences and Landscape Architecture, Quinta de Prados, 5001-801 Vila Real, Portugal

[(6)] University of Évora, Institute of Earth Sciences (ICT) & Department of Plant Science, School of Sciences and Technology, Pólo da Mitra, Ap. 94, 7006-554 Évora, Portugal

4.1 Abstract

Cork oak (*Quercus suber* L.) grows in the Mediterranean basis including Portugal and is the main species producing cork which is used prevailingly in stopper industry. In our paper, cork from *Quercus suber* L. over three consecutive harvests from a traditional rainfed plot, between 1999 and 2017, and cork from an irrigated plot, harvested in 2017, were studied. We applied two X-ray image analysis technologies - X-ray micro-computed tomography and X-ray

microdensitometry. Cork development, related with intern porosity, growth and density was studied with the objective of understanding the cork characteristics evolution over the years and with a different water regime. The outcomes of this study suggested an increase in density and porosity over harvests and a slight decrease of the cork growth. Cork samples from the irrigated plot, compared with cork from the same year of extraction in the rainfed plot, showed higher growth rate and higher porosity. The results demonstrated the contribution of climatic factor of precipitation as well as the silviculture model in cork characteristics, showing the relevance of the present work for the definition of the management practices. These may be determinant for enhancing cork quality and quantity production through silviculture measures. Our findings can be particularly useful for stakeholders especially under the conditions of Portugal in terms of increasing the value of the industrial chain of cork.

4.2 Keywords

cork oak; cork characteristics; image analysis; X-ray micro-computed tomography; X-ray microdensitometry

4.3 Introduction

Cork oak (*Quercus suber* L.) is the main species responsible for cork production with sustainable and profitable exploration, growing in the Mediterranean basis. Cork extracted from *Quercus suber* L. is mostly used to the cork stoppers industry (41.6 % for natural cork stopper and 28.9 % for other types of stoppers (APCOR 2019). Porosity is one of the main characteristics responsible for the type of stoppers developed and is in the basis of industrial classification, also associated to cork growth in the radial direction and cork density. Porosity is defined as the fraction occupied by lenticular channels and other structural defects (Fortes et al. 2004), which represent macro porosity across the cork profile. The lenticels' grow from the phellogen to the external surface of the periderm (Pereira 2007) and their borders are composed by thicker cell walls (Pereira 2015), which show higher density. The porosity is affected by genetic and external factors as organisms or striping procedures and could be filled with a movable cellular material. Additionally, it can be affected by management factors, as well, as

the distance between trees (Marrafa 2016; Silva et al. 2017). Density affects positively or negatively the mechanical behaviour of cork, is responsible for the material flexibility and is related with the cork cells structure and the thickness of cork cells walls (Anjos et al. 2008).

The use of X-Ray microdensitometer is commonly employed in wood science to analyse wood cores. It is the reference method in dendrochronology, used to measure growth rings, earlywood and latewood densities and wood density' profiles (Jacquin et al. 2017; Dias et al. 2018). However, already in the mid-nineties, Fonseca et al. (1994) has explored the use of microdensitometry to determining cork quality: namely in the study of the density variation on the suberised tissues around the pores.

In the last decade, Oliveira et al. (2013, 2015, 2016) analysed the intern structure of cork stoppers by X-ray micro-computed tomography (µCT) and authors as Brunetti et al. (2002), Lagorce-Tachon et al. (2015) and Le Barbenchon et al. (2019) studied, using the same technique, the intern structure of cork and granulated cork stoppers. Moreover, as part of a study of imaging of cork over four hundred years, Crouvisier-Urion et al. (2019) also used the X-ray technology to assess the inner porosity in cork stoppers. This method allows the reconstruction of cross-sections of a cork sample into a 3-D object based in a computer algorithm and shows internal defects by a non-destructive method. The µCT method was strongly described by authors as Stock (2009) and Rajczakowska et al. (2015) and in cork stoppers industry has proven to be an added value. Thus, for the reason that it helped and completed the manual chosen procedure. The µCT has been extensively used in wood science in areas such as: anatomical research, rings analysis and wood physical mechanical behaviour as shrinkage (; Bulcke et al. 2009; Belini et al. 2011; Taylor et al. 2013; Van den Bulcke et al. 2014).

The cork oak forest ecosystem is responsible for a healthier and profitable environment, employment in rural regions and is responsible for the economic growth that relies on cork production both in quantity and quality being the market price of cork a major economic drive of system management (Pinheiro et al. 2008; Ribeiro et al. 2010). However, climate changes are affecting all the species, including cork oak, as mentioned in numerous global and specific literature (Gouveia et al. 2017; Lionello et al. 2017; Seidl et al. 2017; Morin et al. 2018;). Due to natural irregularity of the climatic growth factors of precipitation and temperature, cork shows differences in its characteristics along harvests. Events of drought or intensive rainfall have relevant impact in the cork growth as it was described in several studies demonstrating

the influence of annual precipitation on cork rings growth (Caritat et al. 1996; Costa et al. 2002; Leite et al. 2019). Cork characteristics such as growth and density are equally dependent on the implemented silviculture model and specific conditions (Ribeiro et al. 2021) and may be controlled by stand density and structure regulation over time being possible to enhance final cork quality through management (Ribeiro et al. 2011; 2012). Modifications on the management systems in the last decades, mainly by mechanization (soil tillage) and/or intensification of agro-pastoral components are responsible for forest decline and jeopardize the cork quality production (Ribeiro et al. 2004, 2006, 2010; Camilo-Alves et al. 2013, 2017; Godinho et al. 2016; Pinto-Correia et al. 2018). Once the cork production could be at risk, with the reduction of raw material (Camilo-Alves et al. 2020) the application of irrigation systems in some places with available water, and with an efficient use of water, could contribute to increase the cork production.

The aim of this work was to study the principal features responsible for cork quality of those raw natural samples from specific regions in Portugal with two X-ray technologies (µCT and X-ray microdensitometry) trying to understand if both techniques can complement each other in this analysis. The µCT was used for the analysis of the intern porosity, enabling the cross-sections reconstruction along the sample (pith to bark), to understand how the porosity was developed along the cork growth direction and how it fluctuates over time (over harvests). Density and radial growth were analysed with X-ray microdensitometry technology, as in Poeiras et al, (2021) about the cork characteristics from a specific water regime. These two techniques were approached and their potentialities and limitations were evaluated in this study, allowing to understand better the cork features.

4.4 Material and Methods

4.4.1 Sampling and study sites

Raw cork samples from two different sites, all from reproduction cork at 130 cm height, were analysed. The research sites are maintained by the University of Évora and have a different silviculture treatment. Treatments are named Site 1 and Site 2 in the consecutive text.

Site 1: eighteen cork samples from three consecutive harvests (1999, 2008 and 2017) at the same trees (6 trees x 3 years of harvest). Samples had 8 complete years of growth, harvested in

9 and 9 years, and the site was under its natural environmental conditions, rainfed only (Table 1). Precipitation decreased from the first harvest until the third one. Soil varying between Cambisols and Arenosols: Cambisols presented sandy loam of finer subsurface horizons with fast permeability, which come from the mother rock intensively changed. They are commonly used for agriculture and forestry (PROF, ICNF 2019). On the other hand, Arenosols are sandy-textured with any significant soil profile development, with excessive permeability.

Site 2: six cork samples harvested in 2017 with 5 complete years of growth, with the same age that 2017' harvest in the Site 1. Although the natural environmental conditions, related with the average annual precipitation (Table 17), the site was subjected to a different water regime: 2500 $m^3.ha^{-1}$ per irrigation campaign, added in spring/summer periods. Soil was a Gleyic luvisol low saturated, which presented hydromorphic properties within 50 cm of the surface (FAO 2020).

Table 17 Annual precipitation (mm $year^{-1}$) of the study sites, distributed by periods of annual rings cork growth

Average annual precipitation (mm $year^{-1}$)				
Period of cork growth	1991 - 1999	2000 - 2008	2009 - 2017	2012 - 2017
Site 1	589.88	530.56	408.22	
Site 2				528

We decided to study of the volume fraction of lenticels along harvests in the Site 1 and between the last harvest of the Site 1 and Site 2, using micro-computed tomography (SkyScan, Bruker, Belgium), and the density and growth at the same samples, with QTRS-01X Tree Ring Analyser (Quintek Measurement Systems Inc., Knoxville, TN, USA) (Fig. 17). The same technique of X-ray microdensitometry to study growth and density was used in Poeiras et al.2021, but now we intend also to understand if both techniques can complement each other.

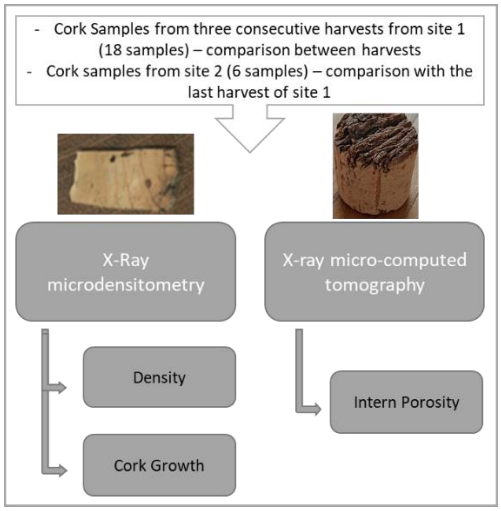

Fig. 17 General scheme about samples, used x-ray techniques and measured parameters measured in each one of the techniques

In the sub-chapters related with the comparison between the 3rd harvest of the Site 1 and Site 2 (both in 2017), is called Treatment 0 and Treatment 1, respectively, due to the water regime applied in the Site 2.

4.4.2 X-ray microdensitometry analysis

Density and growth analysis were obtained by QTRS-01X Tree Ring Analyser (Quintek Measurement Systems Inc., Knoxville, TN, USA) on radial slices with 1.5 cm thickness (Fig. 18), cut with a slicer machine (ABO with 220 mm incorporate blade and sharpener, Oggiona VA, Italy). One measurement per sample were performed (24 in total). The first and last rings (half rings) corresponding to pith and to bark of cork samples were not considered for the analysis. Measurements were achieved automatically with QTRS-01X software, which were based on the principle of absorption of radiation from a collimated beam of X-rays. The density calculation at each measurement point was done from the ratio of the measured attenuation and the beam intensity. The mass attenuation coefficient depends on the energy of the incident radiation and the material composition, which water is the integral element in the measurement accuracy. The microdensitometry analysis provides the capability to recognize the differences in autumn and spring rings cork density and rings growth.

Calibration were done with a mass absorption coefficient of 3.80, a threshold of 200 and a dead band of 50. The radiation beam passed through the sample (bark to pith direction), which length

was changed according to each sample. The target density on the calibration parameters was defined as 0.250 g cm^{-2}, as the maximum in cork density, according Fortes et al. (2004) and Anjos et al. (2008).

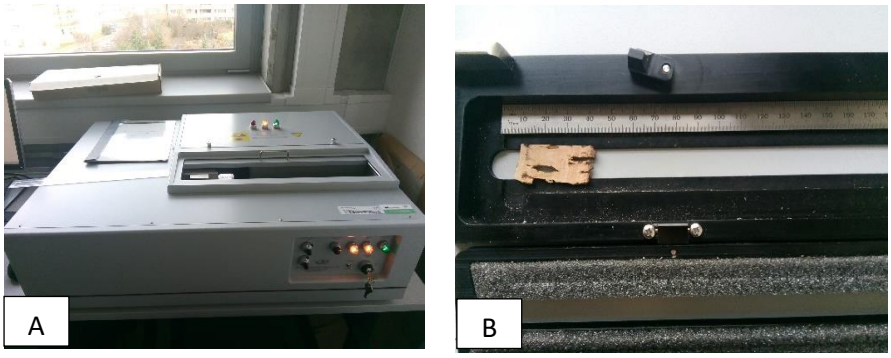

Fig. 18 A - QTRS-01X Data Analyser and Scanner (Quintek Measurement Systems Inc., Knoxville, TN, USA); B – Cassette where the sample is positioned

4.4.3 X-ray micro-computed tomography (µCT) analysis

Samples (18 + 6) were turned into cylinders with a diameter of 25 mm (one cylinder per sample) (see Fig. 19A). A turnstile press machine, from pith to bark, was used in order to fit the tube base of the µCT equipment (SkyScan, Bruker, Belgium). Samples were maintained at regular environmental conditions.

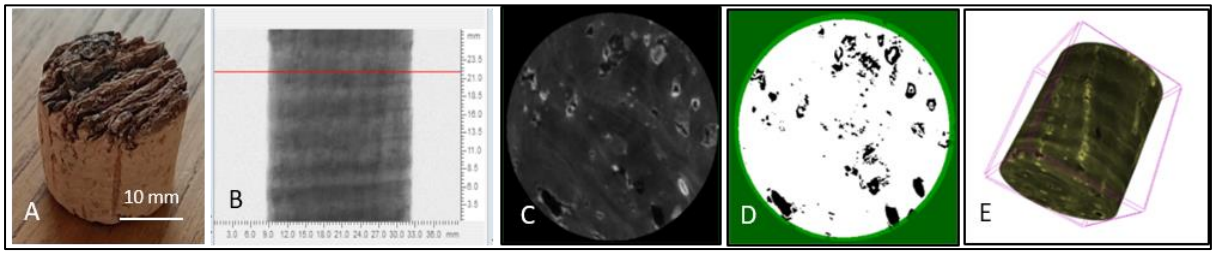

Fig. 19 Sequence of image processing: A – cylindrical cork sample prepared for microCT analysis; B – 1st projection image of a cork sample acquired with CTan version 1.14.4.1+ (64-bit), Bruker microCT; C – reconstructed cross-section of the cork sample (raw image); D – reconstructed cross-section of the cork sample under binarization; E – 3D reconstruction with CTVox (CTVox version 3.0.0r1114 (64-bit)

The general principles and applications of X-ray micro computed tomography have been described in some literature (Elliott et al. 1990; Rajczakowska et al. 2015) – an attenuated X-ray beam passes through the sample and a reconstruction of the sample, through a set of cross-

sections, is performed automatically using mathematic algorithms. It is a non-destructive method, without cell damage during the process of sample's preparation.

The X-ray tube was tuned down to 50 Kv and 800 µA and the projection images were captured with an exposure time of 2200 ms over a 180˚ rotation with a 31 µm pixel size. The process resulting in a total acquisition time of 1h per sample. NRecon (SkyScan1174v2, Bruker microCT, Belgium, version 1.1) was used to make the reconstruction images via the filtered back projection algorithm. The parameters used in the reconstruction of all the databases were: smoothing – 8; ring artefact correction – 13; sharpening – 40 %; beam hardening correction – 28 %.

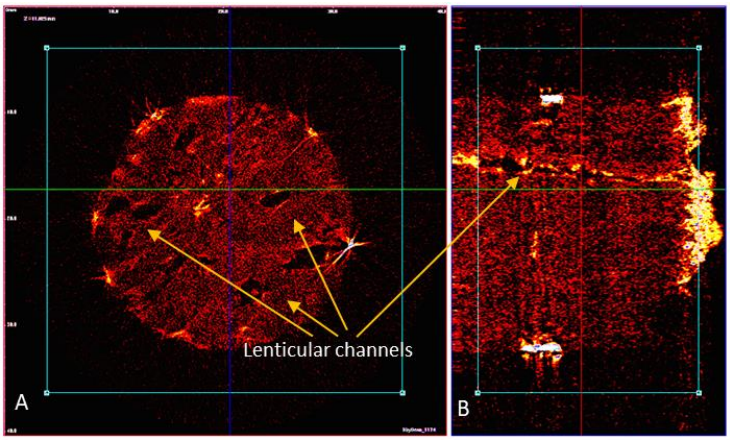

Fig. 20 Tangential section (A) and radial section (B) from a cork sample, obtained with DataViewer (Version 1.5.1.2 64-bit, SkyScan, Bruker microCT): A) shows lenticular channels with circular shapes (in yellow) and B shows one elongated lenticular channel (in yellow).

Data Analyser (CTAn SkyScan, Bruker micro CT, Belgium, version 1.14.4.1) was used to analyse the intern structure and the volume fraction of lenticels. After the image acquisition, a Volume of Interest (VOI) was chosen for each sample and images were binarized for the analysis (Fig. 19B and C). In the binarization the lenticular channels (empty spaces) appeared in black and the solid material in white (Fig. 19D), which histogram was 24-74 for every sample. The lenticular channels present different shapes when visualized from radial and tangential sections, as lengthened and circular shapes, respectively, observed in DataViewer (Version 1.5.1.2 64-bit, SkyScan, Bruker microCT) (Fig.20A and B).

Then, total, close and open porosity were automatically calculated for each sample, in 3D analysis. The porosity along samples was measured in specific cross sections (corresponding to 6 to 18 mm for each volume of interest), in 2D automatically analysis. The pores' area was manually measured and the pores counted, from the binarized images, at the same cross sections. The CTVox (SkyScan, Bruker micro CT, Belgium, 3.0.0r1114, 64-bit) was used to

do the realistic 3D visualization related with the X-ray absorption in the different slices of the object (Fig. 19E and Fig.21).

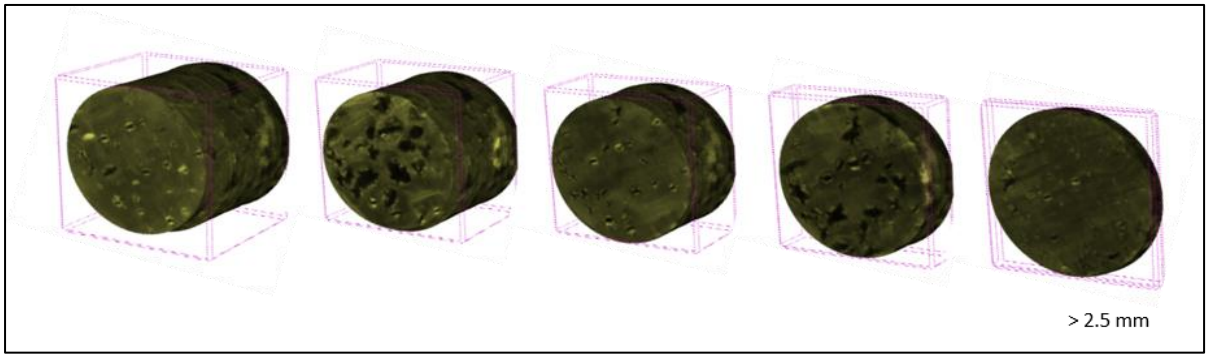

Fig. 21 3-D reconstruction using CTVox version 3.0.0r1114 (64-bit). Image on the left represents a complete sample and image on the right represents a volume of interest (VOI)

4.4.4 Statistical analysis

Statistical analysis was made using SPSS v.25 software package (IBM Corp., Armonk, NY, USA). Analysis of Variance (ANOVA) was done to compare samples from different years of harvest and different treatments. General Linear Models (GLM) of Gauss normal distribution of fitted model were applied to compare the main effects and evaluate the significant differences between the group of means from each harvest and treatment.

In the consecutive text, $p > 0.05$ is used as not significant; $p < 0.05$ is used as significant; $p < 0.01$ is used as very significant and $p < 0.001$ as highly significant, in terms of the statistical explanation.

4.5 Results

4.5.1 Cork density and growth analysis with X-ray microdensitometry technology

4.5.1.1 Harvests in the Site 1

The X-ray microdensitometry technology was used to analyse the total density and growth per sample and per individual cork ring. Samples revealed an increasing on average total density

over extractions (from 1999 to 2017) and a decreasing on total growth. The same happened on the density and growth checked on the cork rings (Table 18).

Table 18 Means ± Std. deviation of total density, average ring density, cork thickness and average cork-ring width according the year of harvest, from the Site 1

Year of harvest	Total density (g cm^{-3})	Ring density (g cm^{-3})	Cork thickness (mm)	Cork-ring width (mm)
1999	0.130 ± 0.019	0.130 ± 0.030	28.22 ± 7.64	3.52 ± 1.48
2008	0.153 ± 0.028	0.159 ± 0.056	26.47 ± 4.22	3.30 ± 1.32
2017	0.157 ± 0.020	0.161 ± 0.036	23.61 ± 6.85	2.94 ± 1.43

Table 19 shows the results from the analysis of variance for the year of harvest on the different cork-ring thickness regarding density and ring-width.

Table 19 Analysis of variance for the year of harvest on the different rings of cork growth, regarding density (g cm^{-3}) and cork-ring width (mm)

	DF	Density		Ring width	
Source of variation		**F**	**P value**	**F**	**P value**
Year of harvest	2	9.055	<0.0001	2.284	0.106
Ring	7	3.004	0.006	4.166	<0.0001
Year of harvest * Ring	14	1.199	0.285	0.602	0.859
Residual	120				

The year of cork harvest represented a highly significant influence ($p < 0.0001$) in cork density. Nevertheless, only the year of 1999 showed a different statistical density (Fig. 22). However, it did not show significance due to cork growth ($p = 0.106$) (Fig. 23). The rings of cork formation (Fig. 24 and Fig. 25) showed very significant variation in regard to density and highly significance regarding ring width. The interaction between each year of cork harvest and ring did not represent a significant effect on density and ring-width ($p = 0.285$ and $p = 0.859$, respectively; Table 19).

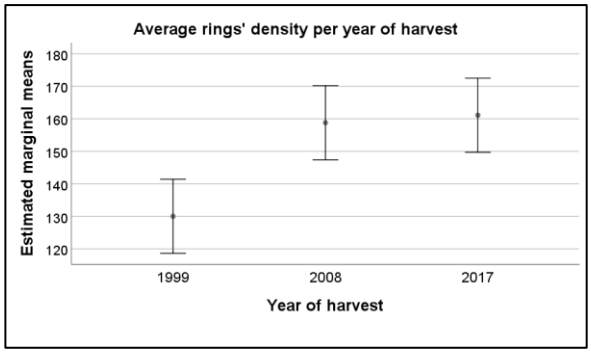

Fig. 22 Result of density (in g cm^{-3}) analysis in three consecutive harvests (average per cork ring). Error bars indicate 95% CI.

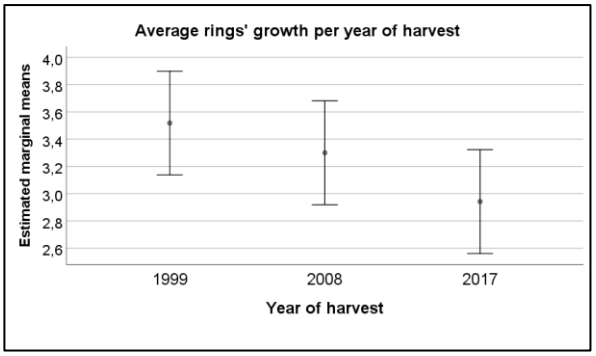

Fig. 23 Result of the average cork-ring width (in mm) analysis in three consecutive harvests. Error bars indicate 95% CI.

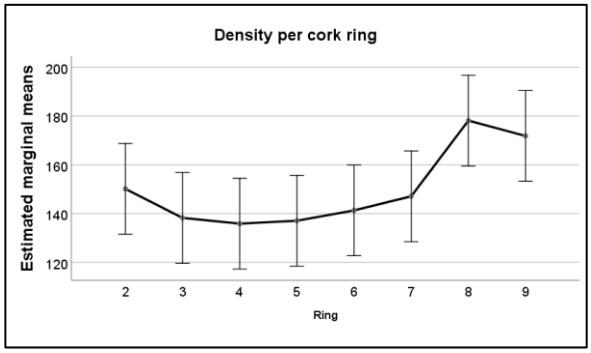

Fig. 24 Result of density (in g cm^{-3}) analysis over cork thickness. Error bars indicate 95% CI.

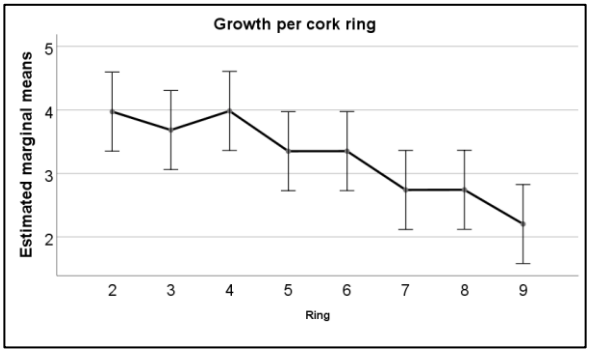

Fig. 25 Result of cork-ring width (in mm) analysis over cork thickness. Error bars indicate 95% CI.

3.5.1.2 Comparison between the 3rd cork harvest of the Site 1 and Site 2

When compared the 3rd cork harvest of the Site 1 (non-irrigated) and the Site 2 (subjected to irrigation), the results demonstrated higher total growth and higher growth per ring in cork from Site 2. On the other hand, the density was lower in the total sample and per ring (Table 20). The comparisons per ring were performed on the same five years of growth in both treatments.

Table 20 Mean ± Std. deviation of total density, ring density, cork thickness and cork-ring width according treatment (0 – non-irrigated; 1 – irrigated)

Treatment	Total density (g cm^{-3})	Ring density (g cm^{-3})	Cork thickness (mm)	Cork-ring width (mm)
0	0.157 ± 0.020	0.154 ± 0.026	23.61 ± 6.85	3.23 ± 1.62
1	0.144 ± 0.021	0.148 ± 0.041	25.50 ± 1.86	5.09 ± 1.36

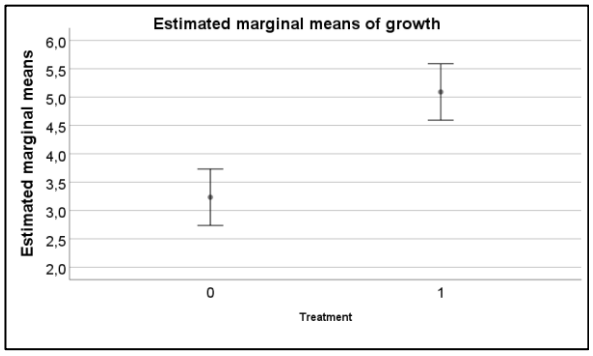

Fig. 26 Result of growth analysis in treatments 0 and 1 (average per ring of cork growth). Error bars indicate 95% CI.

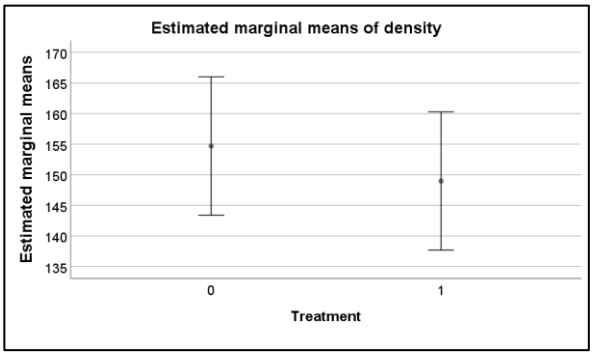

Fig. 27 Result of density analysis in treatments 0 and 1 (average per ring of cork growth). Error bars indicate 95% CI.

The results obtained from ANOVA (Table 21) demonstrated a highly significant influence of the different water regime (Treatment) in the cork growth ($p < 0.0001$) (Fig. 26) Even with the differences of the means related with density, ANOVA did not show significance ($p = 0.475$) (Fig. 27). Rings demonstrated to be a significant origin of variation for density but did not show significance for cork-ring width. The interaction between treatment and rings showed significance for density and ring width (0.049 and 0.022, respectively).

Table 21 Analysis of variance for treatment on the different samples and rings, regarding density (g cm^{-3}) and cork-ring width

	DF	**Density**		**Ring width**	
Source of variation		**F**	**P value**	**F**	**P value**
Treatment	1	0.518	0.475	28.116	<0.0001
Ring	4	3.223	0.020	1.915	0.123
Treatment * Ring	4	2.577	0.049	3.141	0.022
Residual	50				

4.5.2 Inter porosity with X-ray µCT technology

4.5.2.1 Cork harvests in the site 1

Total, open and close porosity, in 3D analysis, were automatically measured in CT Analyser (SkyScan, Bruker micro CT, Belgium, version 1.14.4.1) for each group of samples (harvests of 1999, 2008 and 2017). The analysis demonstrated an increasing of total and open porosity

over years, and a slight regression of close porosity from the first harvest to the second one (Table 22).

Table 22 Mean ± Standard deviation of total, open and close porosity (%) regarding harvests

Year	Total porosity (%)	Open porosity (%)	Close porosity (%)
1999	17.02 ± 6.70	14.96 ± 7.01	2.41 ± 0.73
2008	17.58 ± 7.15	15.90 ± 7.27	2.00 ± 0.48
2017	18.47 ± 6.89	16.34 ± 7.04	2.54 ± 1.02

The results obtained from the ANOVA (Table 23) did not verified a significant influence of the year of cork harvest in total porosity. Only the source of variation samples within each year of harvest showed a highly significance. These analyses demonstrated high variability in the different samples. A high variability within each cross section was markedly presented and the interaction cross-section and year of harvest did not exist.

Table 23 Analysis of variance for the year of harvest on the different samples and cross-sections, regarding total porosity (%)

		Total porosity	
Source of variation	**DF**	**F**	**P value**
Year of harvest	2	0.789	0.456
Sample/Year of harvest	15	5.823	<0.0001
Cross-section	12	0.953	0.496
Cross-section x Year of harvest	24	0.950	0.534
Residual	180		

The findings from the analysis of variance are shown in the Fig. 28 and Fig. 29.

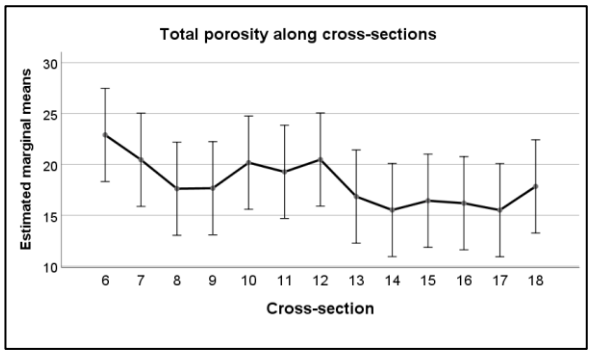

Fig. 28 Total porosity (in per cent) along cross-sections (6-18 mm). Error bars indicate 95% CI.

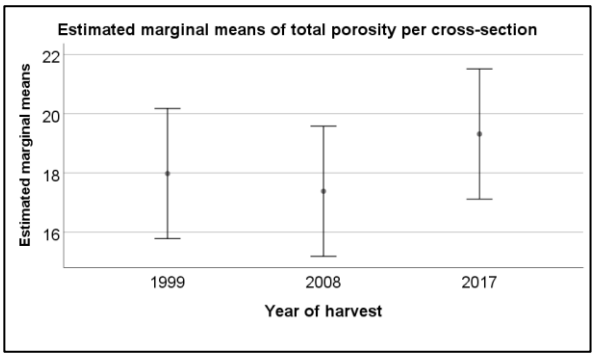

Fig. 29 Result of total porosity (in per cent) per cross-section on each year of harvest. Error bars indicate 95% CI.

At the same time, the evolution of total porosity along cork samples (from pith to bark) in 2D cross-sections between 6 and 18 mm were automatically analysed for each sample. For this intention one millimeter correspond to one cross-section. Higher pores' area was measured in the first harvest. However, the higher number of pores belong to the last harvest.

The ANOVA with regard to the number of pores (Table 24) showed a highly significance for all of the sources of variation. The interaction between cross-section and the year of harvest was the only source of variation that demonstrated to be not significant (Fig. 30 and Fig. 31).

Table 24 Analysis of variance for the year of harvest on the different samples and cross-section, regarding the number of pores and pores area (mm^2)

		Number of pores		**Pores' area (mm^2)**	
Source of variation	**DF**	**F**	**P value**	**F**	**P value**
Year of harvest	2	7.718	<0.0001	12.586	<0.0001
Sample/Year of harvest	15	35.481	<0.0001	10.146	<0.0001
Cross-section	12	6.186	<0.0001	1.835	<0.038
Cross-section x Year of harvest	24	4.074	<0.0001	0.595	0.940
Sample x (cross-section/Year of harvest)	180	2.306	<0.0001	1.462	<0.0001
Residual	4560				

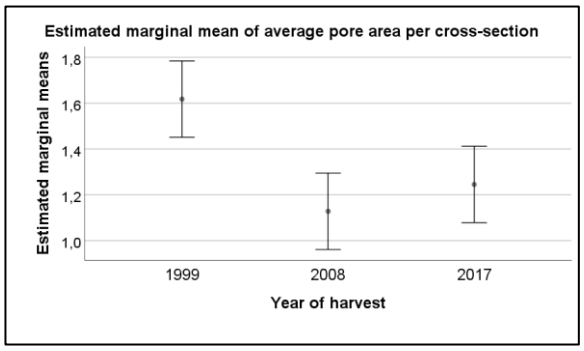

Fig. 30 Result of pores' area (in mm^2) per cross-section on each year of harvest. Error bars indicate 95% CI.

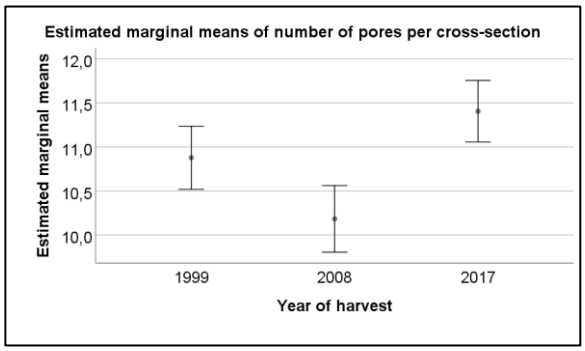

Fig. 31 Result of number of pores per cross-section on each year of harvest. Error bars indicate 95% CI.

4.5.2.2 Comparison between the 3rd cork harvest of the Site 1 and Site 2

Total, open and close porosity, in a 3D analysis in CT Analyser, regarding the complete sample, demonstrated to be higher in the Treatment 1, subjected to a different water regime, when contrasted with theTreatment 0 (the last harvest of site 1) (Table 25).

Table 25 Mean ± Std. deviation of total, open and close porosity according treatment

Treatment	Total porosity (%)	Open porosity (%)	Close porosity (%)
0	18.47 ± 6.89	16.34 ± 7.04	2.54 ± 1.02
1	27.87 ± 9.98	25.77 ± 10.41	2.80 ± 0.96

Along cross-sections, between 6 and 18 mm, the 2D analysis also revealed an evident difference between treatments, with higher values in the Treatment 1 (Fig. 32).

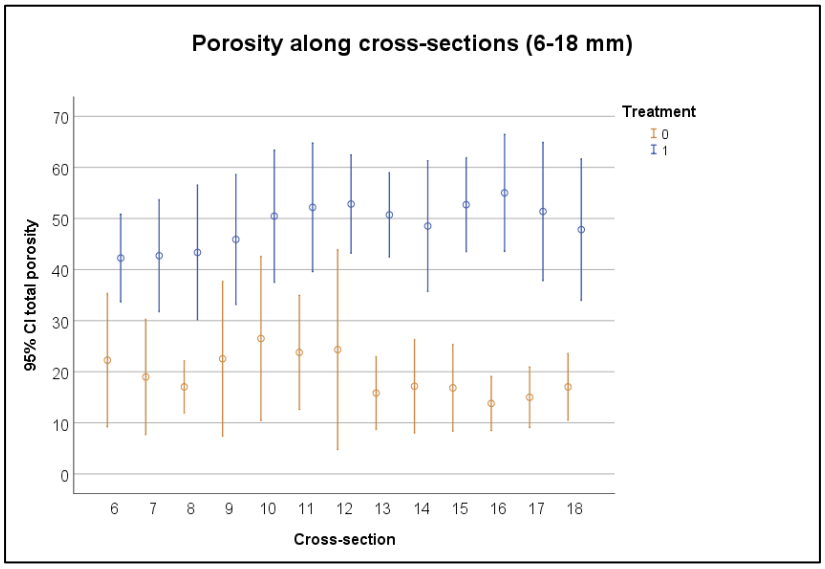

Fig. 32 Total porosity along cross-sections in treatments 0 and 1

The pores' area was bigger in samples from treatment 1 (Fig. 33) while the number of pores counted was smaller.

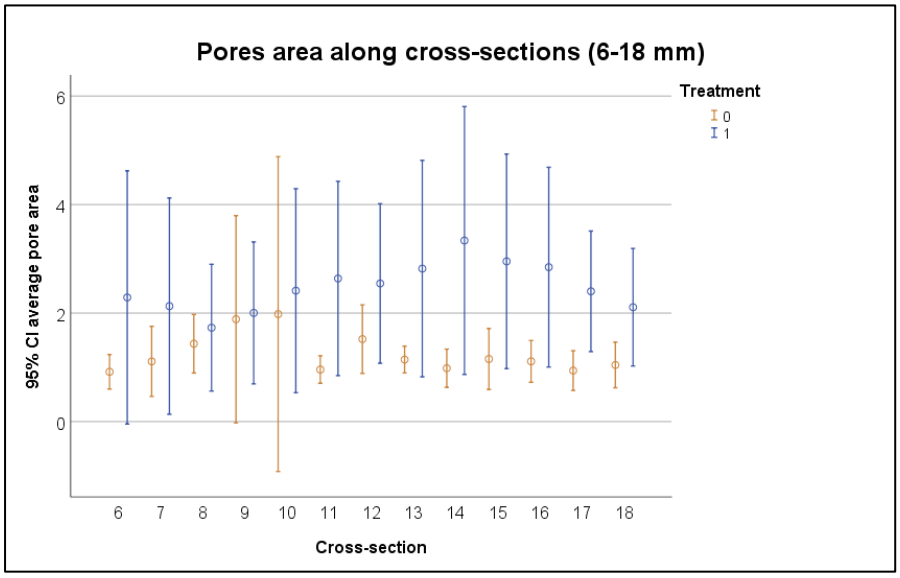

Fig. 33 Pores' area (mm^2), per treatment, along cross-sections (6-18 mm)

The statistical analysis (ANOVA) revealed a highly significant influence of treatment and samples within each treatment for total porosity and pores' area ($p < 0.0001$) (Table 26 and Table 27). The irrigation demonstrated a significant impact on the porosity and pores' area analysed, but was not statistically significant in regard the number of pores. Cross-sections did not demonstrate significance on the total porosity and in the pores area, but revealed a highly significance in the number of pores.

Table 26 Analysis of variance for the total porosity (%) according treatment, samples and cross-section

		Total porosity	
Source of variation	**DF**	**F**	**P value**
Treatment	1	493.922	<0.0001
Sample/Treatment	10	10.331	<0.0001
Cross-section	12	1.302	0.226
Cross-section x Treatment	12	1.674	0.081
Residual	120		

Table 27 Analysis of variance for treatment on the different samples and cross-section, regarding the number of pores and pores' area (mm^2)

		Number of pores		**Pores' area (mm^2)**	
Source of variation	**DF**	**F**	**P value**	**F**	**P value**
Treatment	1	1.722	0.190	94.851	<0.0001
Sample/Treatment	10	24.935	0.0001	13.852	<0.0001
Cross-section	12	6.827	0.0001	1.221	0.261
Cross-section x Treatment	12	4.201	0.0001	1.851	0.036
Sample x (Cross-section/Treatment)	120	2.526	0.0001	1.494	0.001
Residual	3213				

Figures 34, 35 and 36 confirmed the results from the analysis of variance.

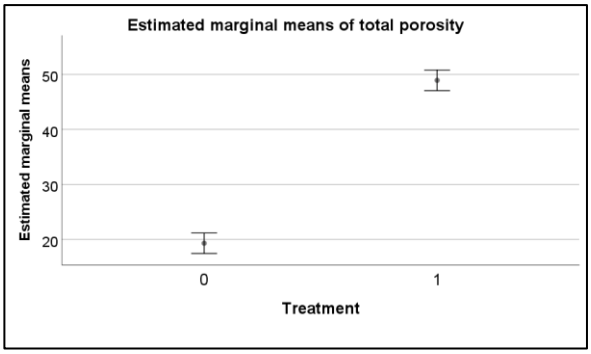

Fig. 34 Result of total porosity (in %) per cross-section on each treatment. Error bars indicate 95% CI.

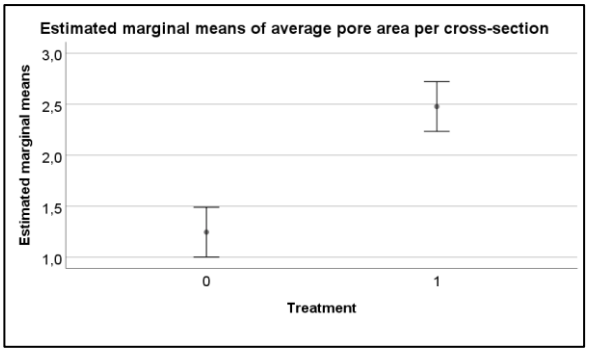

Fig. 35 Result of pores' area (in mm^2) on each treatment. Error bas indicate 95% CI.

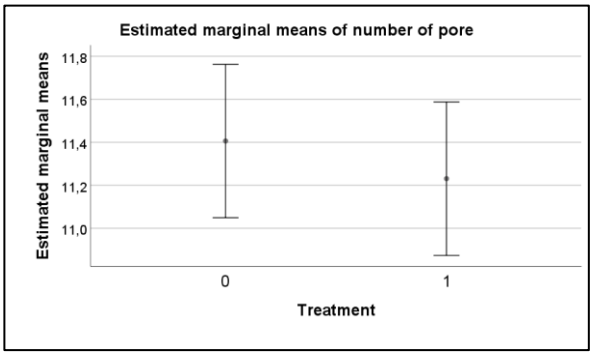

Fig. 36 Result of number of pores on each treatment (measured per cross-section). Error bars indicate 95% CI.

4.6 Discussion

A reduction on cork growth over harvests for the same trees was found with the X-ray microdensitometer, at the same time that an increasing on cork density was revealed. The reduction of growth can be explained by the changes on precipitation - the drops over time cause reductions on cork growth, which is according to literature (Costa el at. 2016; Leite et al. 2019).

For each cork sample, the growth curve (Fig. 25) showed a decreasing cork growth trend, explained by Natividade (1934), Fortes el al (2004) and Ghalem et al. (2016) which is related with the pressure caused by the oldest rings and external enclosure. However, the density curve (Fig. 24) showed an increasing on cork density trend, which can be explained by the smaller growths in the newest rings and consequent lower number of spring cells with thinner cell walls

(Natividade 1934). Once the density analysis was performed in raw cork, the densification of the external rings may be due to the cellular tensions occurred in the radial cork growth.

When compared cork samples from the two different site managements, cork from the Treatment 1 (irrigated) showed higher growth rates. According literature drought episodes are the responsible for low cork growth (Costa et al. 2016) so the higher growth rates can be explained by the conditions that trees have – more water availability. The higher growth of cork from the plot subjected to irrigation contributes to the increasing on cork production.

Despite the lower density presented in cork from the irrigated plot, the statistical analysis did not reveal any significance related with the management, in contrast to cork growth. As Natividade (1934) demonstrated, higher growths showed bigger cells, which correspond to bigger spring cells. Because of that, density is related with cork growth and not directly with the site management. Fonseca et al (1994) found a negative correlation between the average width of growth and the cork density, which was statistically significant, but only 8.6%.

The findings related with the values of density for samples from both plots (0.157 ± 0.020 g.cm^{-3} in site 1 – non-irrigated – and 0.144 ± 0.021 g.cm^{-3} in site 2 – irrigated) were in line with bibliography and suitable for the industry. Knapic et al. (2016) and Pereira (2007) mentioned valued of 0.120-0.170 g.cm^{-3}.

Regarding the µCT analysis, the µCT images resolution depends on the scanner used, which was lower in the Bruker Skyscanner used for this study. Moreover, cork tissue has a lower density compared with other materials, as bone, concrete or wood, largely analysed with µCT technology. As a result of that, some issues related with the cross-sections threshold occurred in the visualization of the lenticular channels in 3D and 2D analysis. However, it was possible to perform the purpose of this analysis. In the 3D analysis, used for the general porosity acquisition, the porosity is smaller than in the 2D analysis (used for a specific volume of interest along cork samples – porosity on cross-sections). These happened due to the close porosity (pores are more probably surrounded by solid material in the 2D sections).

Open porosity was higher than close porosity in every year of cork harvest and plots, which is related with the inner structure of cork. Lenticular channels cross the cork plank radially, from the phellogen to the external surface, allowing gas exchanges (Oliveira & Pereira 2020).

The year of cork harvest and samples in each year showed to be relevant sources of variation in regard to pores area and number of pores. In every analysis samples in each year demonstrated to be a highly significant origin of variation, which shows the genetic variability

presented in cork and the importance of the genetic factors in those characteristics (identical findings were presented by Silva (1996)).

Our results suggested that the formation of lenticels could not only be controlled by genetic factors, as referred by Pereira (2007). The increasing on density over cork harvests for the same plot (which accompanying the increase of porosity on the same samples) could be related by the thicker cell walls – sclereid cell walls – surrounding the lenticular channels (Fonseca et al. 1994; Pereira 2015). Total porosity and the pores area were not influenced by the cross-sections, as showed by the statistical analysis. However, the number of pores was influenced by the position in samples, which suggest their emergence or disappearance along the cork profile or can be related with the discontinuities presented in cork (Fortes et al 2004).

When samples from the different management plots were compared the results demonstrated a notorious difference on them. The source Treatment highly influenced the porosity, pores area and number of pores, which demonstrated that the management practices revealed an impact on porosity. Treatment 1 (irrigated) brought an increasing on porosity due to pores' area and not due to the number of pores. According Natividade (1934) the favourable growth conditions presented in a plot originate a faster cork growth, which contributes to the deformation of lenticular channels. The bigger pores area found in the Treatment 1 could be explained by that.

Variability is markedly present on both sites, represented by the source of variation samples within each treatment, which shows that the genetic factors continuous to be one of the most important factors in the variability of cork quality (Pereira 2007; Silva et al. 2017).

4.7 Conclusions

The comparison of cork porosity between consecutive cork harvests and between the 3rd cork harvest of the Site 1 and Site 2 were quantified in those intern structure with µCT technology, and characteristics as density and total growth were measured with microdensitometry technology.

Both techniques demonstrated to be valuable for the present analysis. The use of µCT could be an opportunity in further studies to compare the inner porosity of cork from *Quercus suber* L. with the surface coefficient porosity of the same samples, in different site managements. µCT proved to be an important technique to analyse open and close porosity, quantify pores and measure pores' area along cork samples. At the same time, the X-ray microdensitometry was

confirmed as a relevant technique that may be used in the same samples as µCT (after sectioning in slices), to study the density and growth of cork profiles, in a 2D analysis. Regarding density and growth, the microdensitometry has the advantage of measure distinctly each cork ring, which was not correctly visualized in µCT analysis. However, to use the microdensitometer is required to convert samples in thinner slices, which is more time-consuming. The techniques used can complement each other and contribute to the main cork features analysis.

Our experiment clearly demonstrated that irrigation can increase cork production, thus it is possible to control the cork growth through the silviculture model. Additionally, management practices shown to have a contribution to changes in cork features.

In future studies the number of samples could be duplicated with the goal of cover a larger number of trees from different sites with different implemented silviculture models.

Acknowledgements

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Chapter 5 – The influence of site quality and intraspecific competition in cork from consecutive debarkings for a specific Portuguese site - density and growth analysis

5.1 Abstract

The cork characteristics response to the silviculture model of a pure cork oak stand for cork production is analysed in this study. The influence of site quality, climate and intraspecific competition indices was analysed on cork thickness and density. These cork characteristics have been approached with X-Ray microdensitometer technology, which samples were extracted from the same trees of permanent plots, from a specific site, during consecutive debarkings. Cork is the most important non-wood forest product in the Mediterranean system and its density and thickness belong to an important quality parameters group, with important applicability on industrial products. Soil limitations to cork oak growth demonstrated no statistical significance to cork thickness or density when associated to climate, which showed a strongly effect on that characteristics. Several climate indices revealed great significance on cork characteristics. However, the Ombrotermic was the one that better explain variability in the full model. Thickness showed a decreasing over debarkings, especially in "poor" site quality zones, with a negative correlation with regards to density. Competition indices showed great relevance on cork characteristics: Hh3 competition index, related to the canopy overlapping and light cone method, proved a high significance on cork density, which means a gain in cork density with the maintenance of a high crown cover (and high basal area); and Hd2 competition index proved a great effect on cork thickness. These results predict a great relevance to the cork oak stands management, with applicability in high density stands. Thus, it is proved that cork characteristics may be controlled by management actions, especially in the early stages, such as initiation and juvenile stages.

5.2 Keywords

Site quality; competition; X-ray microdensitometry, *Quercus suber* L., density, thickness

5.3 Introduction

Cork oak lands are one of the main forest occupations in Portugal (719.900 hectares), according to the last National Forest Inventory (ICNFa, 2020). However, severe cork oak mortality events have repeatedly occurred since the 1980s, disrupting the system in all its aspects (Macara, 1975; Cabral et al, 1992; Brasier et al, 1993; Moreira et al, 2005; Sousa et al, 2007). Cork oak lands are a multifunctional system which represents an important social-economical, environmental and heritage value for the country. In an economic perspective, cork oak lands and cork production contribute to the employment rate and development of inland areas, contributing to the economic growths. As what is happening in other type of forests, their importance goes through environmental goods and ecosystem services, such as carbon sink, soil and water resources protection and biodiversity protection. Nonetheless, climate changes associated to some management practices may contribute to forest damage (Ribeiro, 2006). Soil disking, mechanization and agropastoral intensification are part of forest decline causes (Ribeiro, 2008; Camilo-Alves et al, 2020). Soil disking can destroy about 40% of the cork oak roots (Dinis, 2014), including the roots responsible for water absorption from the groundwater and its redistribution to the tree and superficial roots (Besson et al, 2006; Dinis, 2014). Soil disking also decreases soil organic matter and nutrients, essential to trees vitality. In regards to climate changes, some projections predict not only a decrease in precipitation, but also an extension of the dry season with a possible reduction of water availability (ICNFb, 2019), which could jeopardize cork oaks vitality. Cork production occurs most intensely by the end of Spring and beginning of Autumn (Oliveira et al., 1996; Vaz et al., 2011) but low water availability and high temperatures in these periods may reduce cork productivity. Several studies carried out by Markus et al. (2009), Oliveira et al. (2016) and Leite et al. (2019) verify that lower annual distribution of precipitation have a negative influence on cork-ring growth. Tree competition for water, nutrients and sun light could also affect stands structure. According to Ribeiro (2006) competition studies should go through two levels – the effects of competition on the resource availability; and on the plants response to resource availability by means of reproduction, growth and survival rates. Competition indices may have spatial or non-spatial information, which means the dependency or independency of distance between trees. Spatial competition indices with better performances are those overlapping influence areas and

growing space. Non-spatial competition indices with better performance are those relating tree dimension and average plot dimension, due to the effect of tree dimension on cork production. The effect of spatial competition indices on cork characteristics such as thickness and density were tested on this study. Site quality was calculated and climate and soil factors were also tested on those cork characteristics and site quality analysed. Density is one of cork's main structural characteristic (Fonseca et al, 1994) which determines cork quality for stoppers production – associated with other characteristics such as porosity and thickness. Cork density values may range between 120-240 $Kg.m^{-3}$ (Silva *et al.*, 2005) and some factors, such as cell dimension, cell-wall wrinkling, lenticular channels or wood inclusions have an influence on it (Fortes at al., 2004; Anjos et al, 2008). Spring and Autumn cork rings have different densities, with spring cells showing greater dimensions and thinner walls. In general, cork rings are larger in the first years after cork harvest and a decreasing on cork growth trend is present due to the pressure caused by the oldest rings, located on the outside (Natividade, 1934; Fortes et al, 2004; Ghalem et al, 2016). An increasing on cork density trend is usually verified, in contrast to the growth trend, due to the smaller cork rings-width in the newest rings.

In this study, both characteristics were analysed with x-ray microdensitometry technology, as in Poeiras et al. (2021). This technology is usually used in wood science to measure wood density profiles, reconstruct past climatic variations and analyse wood quality (Jacquin et al., 2017). The applied technology also enables the analysis of radial variation of density at the annual and intra-annual growth ring level (earlywood and latewood densities) with a collimated X-ray source that generates parallel X-rays.

5.4 Material and Methods

5.4.1 Study site

Situs in Tagus River basin (39º07'07.80''N, 8º21'33.78''W) the study site is in a sub Mediterranean ecological zone in a flat terrain with soils varying between Cambisols, Arenosols, Regosols and Podzols. Several management actions have been applied in the study site. The site was managed in a base of forestry techniques until 1999 (which includes the growth period of the 1st cork harvest analysed); from the beginning of 2000 until nowadays the site has been subjected to several different management actions, such as soil disking.

5.4.2 Climatic parameters and site quality

Phytoclimatic classification for each extraction group was based on Termicidity, Ombrothermic and Continentality Indices, following correspondences at Fig. 37.

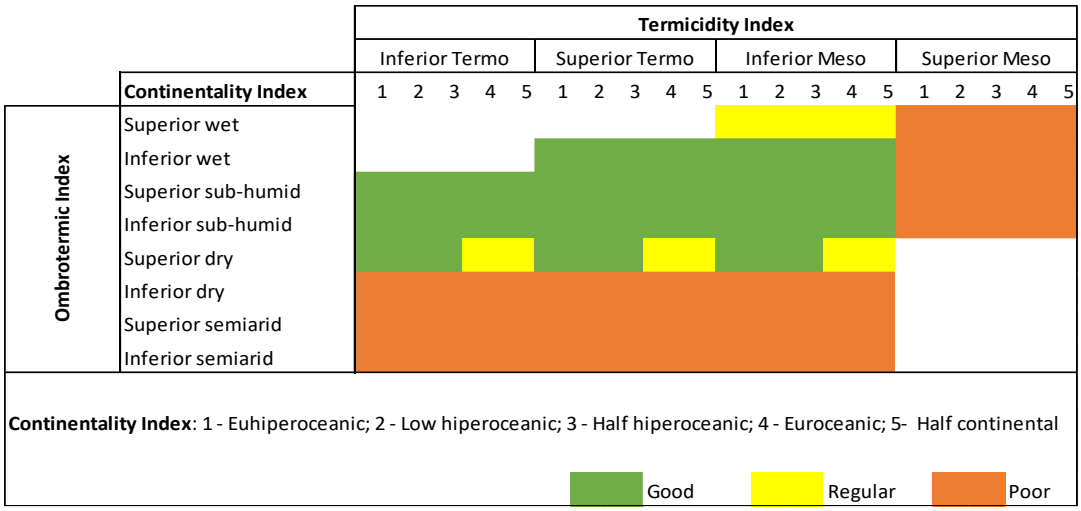

Fig. 37 Site quality chart based on climatic indices (Ombrothermic, Continentality and Termicidity) to *Quercus suber* L. used for phytoclimatic quality determination (PROF, ICNF, 2019)

Termicidity Index (It) was calculated with the formula It = (T+m+M)x10, where T is the average annual temperature, m is the monthly average minimum temperature and M the monthly average maximum temperature of the coldest month. This index weighs the winter cold intensity. Ombrothermic Index (Io) was calculated with the formula Io = Pp/Tp, where Pp is the sum of average precipitation and Tp the sum of monthly average positive temperatures. This index ponders the influence of precipitation. Continentality Index (CI) was calculated with the formula CI = Tmax-Tmin, where Tmax means the average temperature of the hottest month and Tmin the average temperature of the coldest month, which ponders the influence of a landmass, in opposition to oceanity. Due to the reduction on overall precipitation, Ombrothermic Index changed from superior dry to inferior dry on the 3rd harvest (Table 29), which represents a lower potential in the phytoclimatic potential to *Quercus suber* L. according PROF (2019).

Table 28 Climate parameters of the study site distributed by debarking periods

Climatic Parameter (unit)	**Mean ± Standard Deviation**		
	1st debarking	2nd debarking	3rd debarking
Total Annual precipitation (mm.year $^{-1}$)	636.81 ± 194.50	589.62 ± 199.38	421.04 ± 141.86

Average annual temperature (˚C)	16.21 ± 0.64	16.38 ± 0.58	15.87 ± 0.50
Monthly average maximum temperature (˚C)	22.74 ± 0.60	23.41 ± 0.43	23.57 ± 1.11
Monthly average minimum temperature (˚C)	9.68 ± 0.79	9.52 ± 0.86	8.62 ± 0.89

Table 29 Average Climatic Indices for cork rings-growth period on the three consecutives debarkings (Termicidity, Ombrothecmic and Continentality Indices)

Cork growth period	**Termicidity Index (It)**	**Ombrothermic Index (Io)**	**Continentality Index (Ic)**	**Climatic site quality**
1st debarking	336.47 - Inferior mesomediterranic	3.3 - Superior dry	14.6 - Euoceanic	2
2nd debarking	344.73 - Inferior mesomediterranic	3.0 - Superior dry	14.9 - Euoceanic	2
3rd debarking	328.68 - Inferior mesomediterranic	2.2 - Inferior dry	15.2 - Euoceanic	1

However, on the 2nd harvest, the years of cork harvest 2007 and 2008 showed a lower site quality than the previous year, which changed from "regular" to "poor".

Soil quality was calculated in regards to their limitation for trees growth. Following the PROF (2019) soils were classified as: *Good (3)* without any limitation to roots growth, *Regular (2)* soils with low capacity for water retention and *Poor (1)* hydrophilic soils.

Site quality based on both soil type (Fig. 38, according PROF, ICNF, 2019) and climatic indices was calculated based on the minimum' law (Table 29).

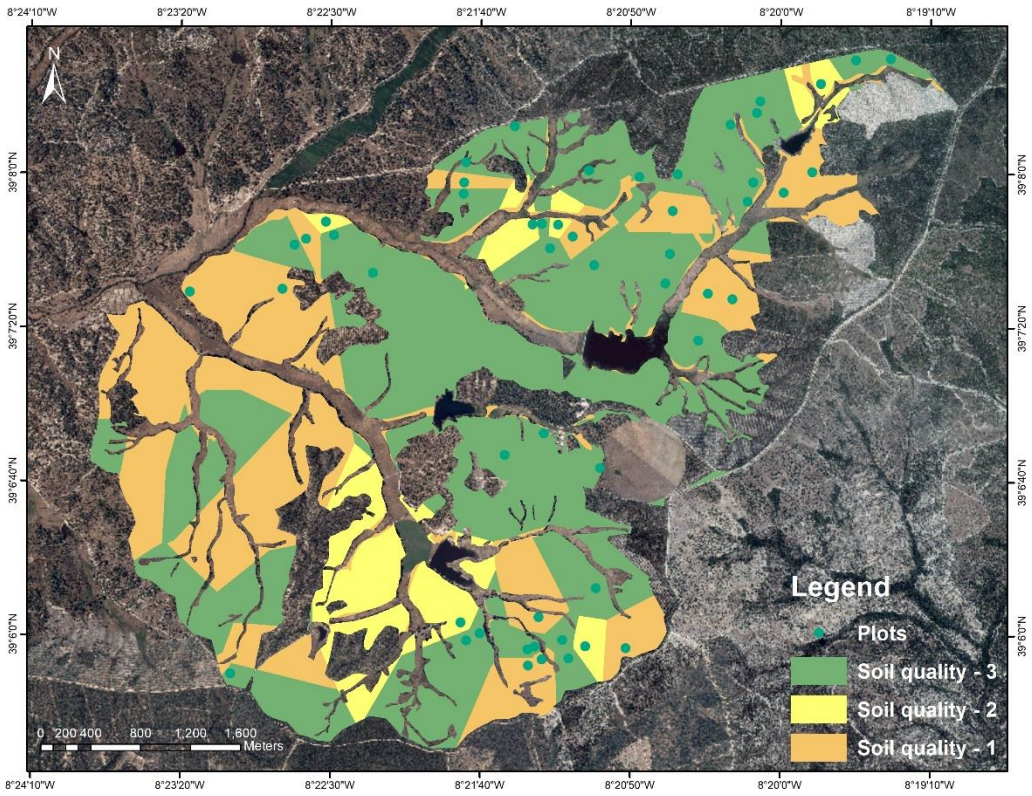

Fig. 38 Soil quality for the study site

Fig. 39, Fig. 40 and Fig. 41 represent the differences on the study site, according site quality, for each period of cork harvest. It is observed a decreasing of site quality over time. Table 30 shows the results of site quality based on climate and soil quality for the study site, according periods of cork growth.

Table 30 Site Quality for cork rings-growth period, based on climatic indices and soil quality

Cork growth period	Site quality based on climatic indices	Site quality based on soil type	Final site quality
1st debarking	2	3	2
		2	2
		1	1
2nd debarking	2	3	2
		2	2
		1	1
	1	3	1
		2	1
		1	1

3rd debarking	1	3	1
		2	1
		1	1

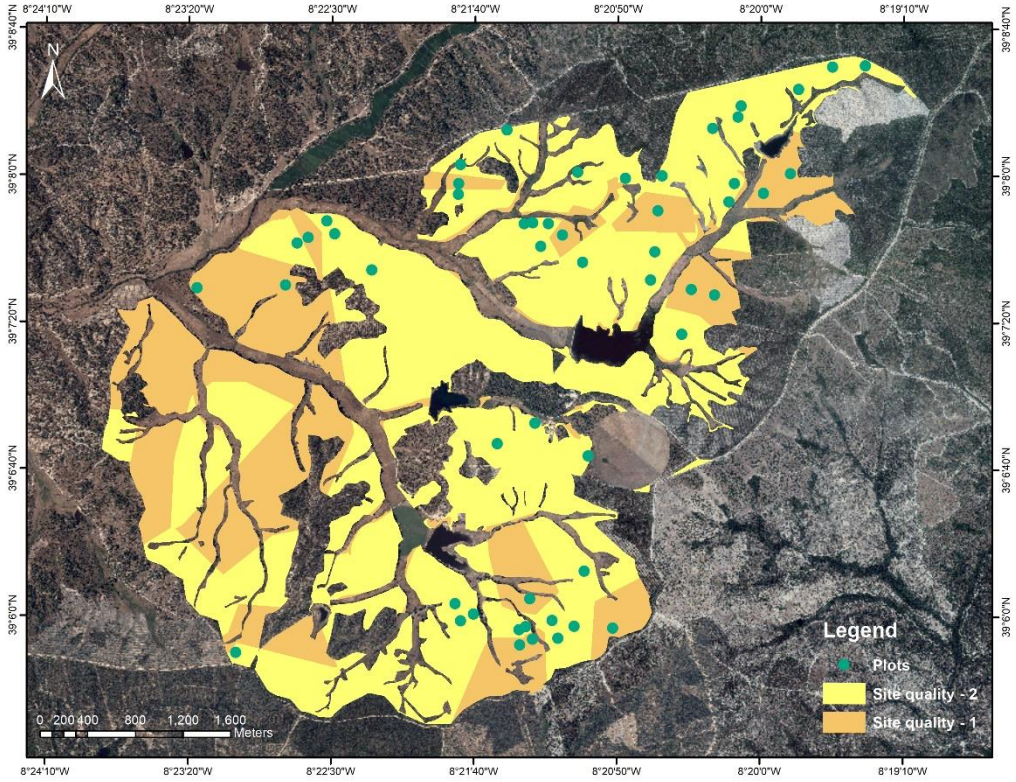

Fig. 39 Site quality for the study site on the 1st debarking (1996, 1997, 1998 and 1999)

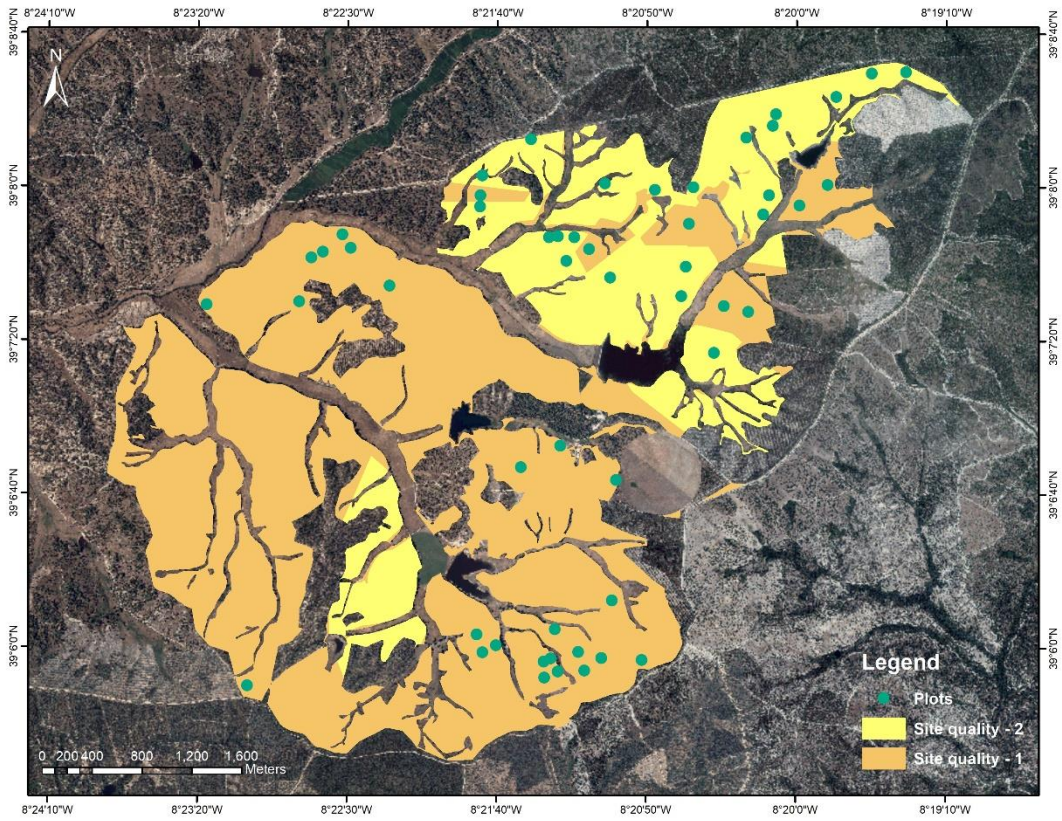

Fig. 40 Site quality for the study site on the 2nd debarking (2005, 2006, 2007 and 2008)

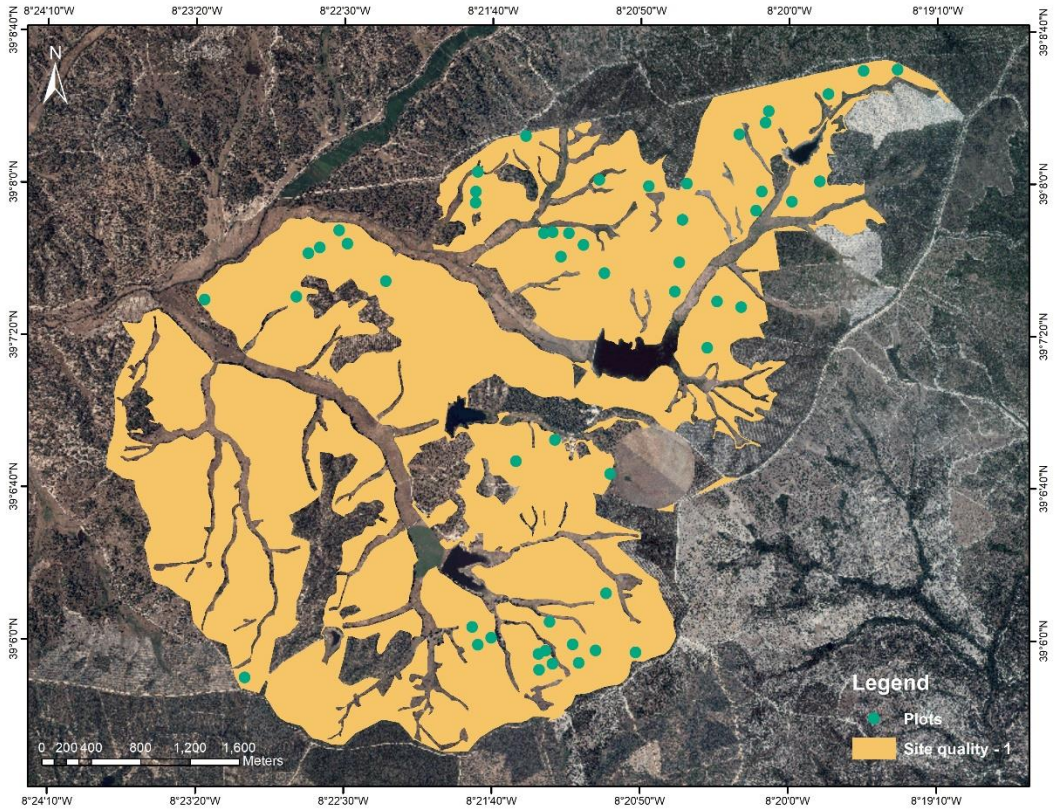

Fig. 41 Site quality for the study site on the 3rd debarking (2014, 2015, 2016 and 2017)

Emberger Coefficient, also calculated, use the precipitation (P, mm) and temperatures (M – average maximum temperatures of the hottest month and m – average minimum temperatures of the coldest month, ºC) in order to quantify the water available to plants (Oliveira, 1998): $Q = \frac{100 \times P}{(M+m) * (M-m)}$. The coefficient enables to classify the Mediterranean climate in Semi-Arid, Sub-Humid, Humid and Super Humid. The average coefficient obtained to the 1º and 2º decades of cork production was Sub-Humid, in contrast to the last period, which corresponded to the Semi-arid classification.

The **Giacobbe Index** was also calculated, determining the dryness type according: $100 \text{ x} \frac{P_{\text{total i}}}{M_i \times (M_i - m_i)}$, where P is the sum of precipitation for the considered period, M is the average maximum temperatures on those period and m represents the average minimum temperatures for the same period (Oliveira, 1998). Every decades of cork production demonstrated an *Arid* type.

5.4.3 Intraspecific Competition Indices

Spatial competition indices based on Ribeiro (2006), mentioned on Chapter 2, were tested in this study. Spatial indices calculate the significance of each stand' tree as a competitor of a certain tree. The competitors' search algorithms provide information about which tree are considered as competitors, based on the maximum radius of influence around the target tree. The spatial dependent size-ratio index based on $\sum_{j=1}^{n} \frac{d_j}{d_i} * \frac{1}{dist_{ij}}$ (Hegyi, 1974), and the algorithms used as a search competitors D2: $Dist_{ij} < 0.33 * d_j$ (based on the basal area method) (Fig. 42) and H3: $Dist_{ij} < \frac{HT_j - HCB_j}{1.19}$ (based on the canopy overlapping method, using the light cone) demonstrated a positive signal in those cork characteristics. In the mathematical formulas: d_j – trunk diameter at 130 cm height of the competitor, d_i - trunk diameter at 130 cm height of the target tree, $dist_{ij}$ – distance between competitor and target tree, HT_j – competitor total height, HCB_j – canopy basis height.

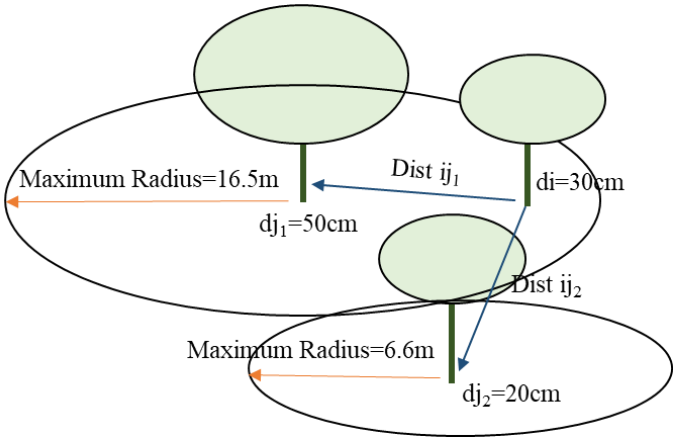

Fig. 42 Example of the D2 search algorithm method

Hegyi determined the competitive relationship between trees, in the physical growing space, based on the sum of all competitors within a fixed radius, in relation to the target tree, according to their diameters (Fabrika and Pretzsch, 2013). The canopy intersection method enables the competitor' identification if the actual crowns, potential crowns or growth areas overlap the central tree. The method is related to tree' diameter or height, which express pressure relative to the tree's spatial location. Daniels (1976) improved the method using the research angle.

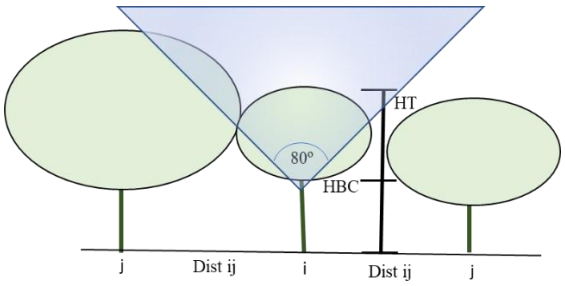

Fig. 43 Example of tree competition based on the light cone method (adapted from Fabrika and Pretzsch, 2013)

5.4.4 Cork samples – collection, processing and analysis

One raw cork sample from reproduction cork oak tree was collected at 130 cm height, in a total of 2430 trees, over three consecutive cork harvests – 653 on the 1st cork harvest, 950 on the 2nd cork harvest and 824 on the 3rd cork harvest.

A cross-section with 3 cm maximum length and 1.5 cm average thickness was taken from each sample (Fig. 44), using an elementary slicer machine (ABO with 220 mm incorporate blade

and sharpener, Oggiona VA, Italy), conserved under typical environmental conditions and polished using P240 Rhynowood Indasa sandpaper.

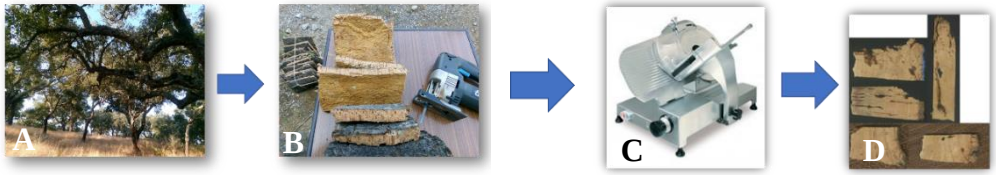

Fig. 44 Steps of cork samples preparation: A – example of a plot; B – samples collected; C – Slicer machine; D – cross-sections

Cork-sections were studied by means of QTRS-01X Tree Ring Analyser (Quintek Measurement Systems Inc., Knoxville, TN, USA). Bark and belly half-rings were not accounted for the analysis (as in Fig. 45). Measurements were performed automatically using QTRS-01X software (Quintek Measurement Systems Knoxville, Knoxville, TS, USA). It provides a sample RGB image, working with an X-Ray system in a radial direction with a collimated X-ray source that generates parallel X-rays. The X-ray technology works on the physical principle from the ratio of the measured attenuation and beam intensity: $\frac{\mathbf{I}}{\mathbf{I_o}} = \boldsymbol{e}^{-\boldsymbol{\mu l}^{\boldsymbol{t}}}$, where **I** is radiation beam intensity after passing through the sample, **Io** is radiation beam intensity not passing through the sample (from bark to pith), **μ*l*** is the sample linear attenuation coefficient, and **t** is sample thickness. Table 31 shows the fixed parameters used, as in Poeiras et al. (2021).

Table 31 QTRS-01X Tree Ring Analyser fixed parameters

Microdensitometer Parameters	
Target Density	250kg/m2
Mass Absorption Coefficient	3.7987
Threshold	200
Dead band	50
Target Length	Changed at each sample

5.4.4 Statistical analysis

Statistical analyses were made using SPSS v.25 software package (IBM Corp., Armonk, NY, USA). Before statistical modeling, all variables were graphically explored regarding distribution patterns and outliers. Generalized Linear Mixed Models (G.L.M.M) were applied to analyze how cork density (dependent variable 1) and cork thickness (dependent variable 2) from the cork samples obtained on the first two consecutive extraction periods (9 years growth between extractions) was related to the independent variables (1) soil quality, (2) climate quality, (3) tree diameter, (4) cork harvest index and (5) several intra-specific competition indices, one by one.

The covariance type selected for the random variables (between subjects and between extraction periods) was the unstructured type.

During the text the subsequent statistical explanation was used:

n.s. - not significant ($p > 0.05$); * - significant ($p < 0.05$); ** - very significant ($p < 0.01$); **** - highly significant ($p < 0.001$).

5.5 Results

Site Quality (SQ) showed three possible sequences (Table 32) to the three periods of cork growth (three consecutive cork harvests), due to climatic factors.

Table 32 Mean ± Standard Error of cork density and cork thickness on each cork harvest, according the site quality (SQ) combinations

Site quality			**Cork density (Kg.m^{-3})**			**Cork thickness (mm)**		
SQ1	SQ2	SQ3	1st debarking	2nd debarking	3rd debarking	1st debarking	2nd debarking	3rd debarking
1	1	1	157.86 ± 3.30	166.08 ± 3.94	175.00 ± 3.65	24.20 ± 0.66	21.43 ± 0.66	21.12 ± 0.55
2	1	1	155.30 ± 2.42	161.64 ± 2.87	166.50 ± 2.94	25.04 ±0.48	23.01 ±0.48	22.03 ±0.46
2	2	1	154.92 ±2.48	156.09 ±2.55	166.61 ± 2.77	23.50 ± 0.46	22.98 ±0.45	23.80 ± 0.46

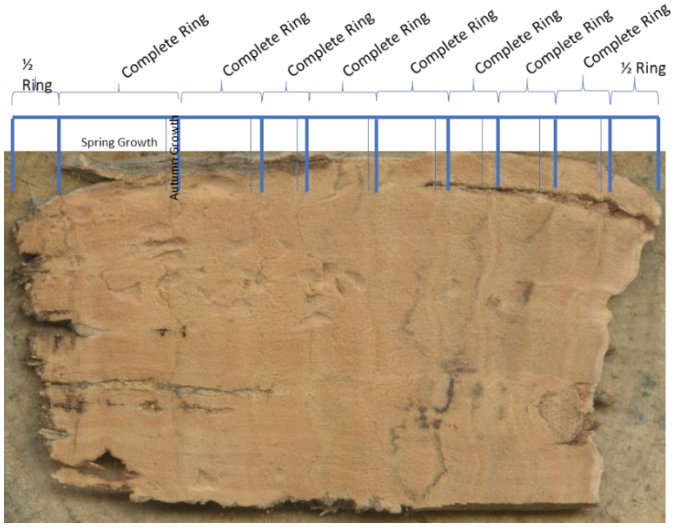

Fig. 45 Cork sample. Half rings were not considered in the analysis.

Cork density increased over debarkings in every SQ combination. On the other hand, cork thickness showed a negative correlation, decreasing in every combination except on the last one – 2:2:1.

Statistical analysis of G.L.M.M. were performed with regards to the 1st and 2nd consecutive debarkings due to several disturbances on the system after the 2000 decade. Stand functional relationships were analysed on those debarkings. Several management actions should be quantified before their introduction on the analysis, such as root' loss by means of soil disking, which may be applied in further studies.

Table 33 Synchronic modelling on the effect on each independent parameter on cork density, by decade of cork production

Parameter		Cork production (decade)	Coef.	Std. Error	95% Confidence Interval		t	*p* value
					Lower	Upper		
Intensity of debark		**1990**	1.423	0.214	1.002	1.843	6.647	< **0.001**
		2000	1.882	0.324	1.245	2.519	5.805	< **0.001**
Site quality	**1**	**1990**	-0.451	2.984	-6.310	5.407	-0.151	0.880
	2		0[a]					
	1	**2000**	-5.395	3.922	-13.104	2.315	-1.375	0.170
	2		0[a]					
Soil quality	**1**	**1990**	-0.748	3.087	-6.810	5.315	-0.242	0.809
	2		-1.478	3.920	-9.177	6.220	-0.377	0.706
	3		0[a]					
	1	**2000**	-3.992	4.885	-13.592	5.609	-0.817	0.414
	2		0.869	5.114	-9.182	10.921	0.170	0.865
	3		0[a]					

Intraspecific competition indices	**Hd2**	**1990**	4.001	3.425	-2.725	10.726	1.168	0.243
	Hd2	**2000**	11.215	5.252	0.893	21.537	2.135	**0.033**
	Hh3	**1990**	12.993	5.286	2.613	23.373	2.458	**0.014**
	Hh3	**2000**	22.092	9.206	3.998	40.185	2.400	**0.017**
	MEh3	**1990**	7.978	3.306	1.486	14.470	2.413	**0.016**
	MEh3	**2000**	12.966	5.530	2.098	23.835	2.345	**0.019**
Tree size	**capi**	**1990**	0.105	0.039	0.029	0.180	2.721	**0.007**
	capi	**2000**	0.078	0.060	-0.040	0.196	1.305	0.192

Table 34 Synchronic modelling on the effect of each independent parameter on cork thickness, by decade of cork production

Parameter		**Cork production (decade)**	**Coef.**	**Std. Error**	**95% Confidence Interval**		**t**	***p* value**
					Lower	**Upper**		
Intensity of debark		**1990**	-0.282	0.042	-0.363	-0.200	-6.766	< **0.001**
		2000	-0.279	0.052	-0.380	-0.178	-5.415	< **0.001**
Site quality	**1**	**1990**	0.356	0.581	-0.785	1.496	0.612	0.541
	2		0[b]	0.000				
	1	**2000**	0.922	0.620	-0.296	2.141	1.487	0.138
	2		0[b]	0.000				
Soil quality	**1**	**1990**	0.432	0.601	-0.748	1.613	0.719	0.472
	2		0.382	0.763	-1.116	1.881	0.501	0.617
	3		0[b]	0.000				
	1	**2000**	0.660	0.772	-0.858	2.177	0.855	0.393
	2		0.656	0.808	-0.933	2.244	0.811	0.418
	3		0[b]	0.000				
Intraspecific competition indices	**Hd2**	**1990**	-5.911	0.626	-7.141	-4.681	-9.437	< **0.001**
	Hd2	**2000**	-5.915	0.786	-7.459	-4.371	-7.529	< **0.001**
	Hh3	**1990**	-6.530	1.002	-8.498	-4.562	-6.515	< **0.001**
	Hh3	**2000**	-7.463	1.421	-10.256	-4.669	-5.251	< **0.001**
	MEh3	**1990**	-3.798	0.629	-5.034	-2.562	-6.034	< **0.001**
	MEh3	**2000**	-4.591	0.852	-6.265	-2.916	-5.387	< **0.001**
Tree size	**capi**	**1990**	0.052	0.007	0.038	0.066	7.185	< **0.001**
	capi	**2000**	0.039	0.009	0.021	0.057	4.171	< **0.001**

The one by one applied G.L.M.M. (Tables 33 and 34) revealed that the intensity of debark shows great significance on these cork characteristics. Additionally, all the intraspecific competition indices tested show great significance on cork thickness ($p < 0.001$). Regarding cork density, the Hh3 index revealed the greatest significance (p=0.014). However, all the other competition indices tested showed significance on this cork characteristic ($p<0.05$).

In regards to the soil and site quality there was a trend related to cork characteristics. However, the margin of error was high and no statistical significance was found (Table 33 and 34).
Despite precipitation and temperature are significantly associated with both cork thickness and density, in regards to density (Table 35), nether annual or precipitation on the dry years revealed to be significant when independently analysed, in contrast to thickness. However, the Continentality and Termicidity indices showed significance on that cork characteristic. Considering the indices combining parameters of temperature and precipitation, the Ombrotermic Index was the one that better explain variability in the full model (Table 37 and 38). In regards to thickness (Table 36), all climatic parameters and indices analysed revealed great significance.

Table 35 Diachronic modelling on the effect of each climatic index on cork density

Parameter	Coef.	Std. Error	95% Confidence Interval		t	*p*-value
			Lower	Upper		
Continentality index	14.116	4.857	4.575	23.658	2.906	**0.004**
Termicity index	0.418	0.128	0.166	0.670	3.257	**0.001**
Av_annual_precipitation	-0.053	0.039	-0.129	0.023	-1.366	0.172
Precipitation dry months	0.016	0.020	-0.024	0.056	0.777	0.437
Ombrothermic index	-15.075	6.471	-27.774	-2.376	-2.330	**0.020**
Giacobbe index	-2.530	3.517	-9.436	4.376	-0.719	0.472
Qemberger index	-0.401	0.293	-0.976	0.174	-1.368	0.172

Table 36 Diachronic modelling on the effect of each climatic index on cork thickness

Parameter	coef.	Std. Error	95% Confidence Interval		t	*p*-value
			Lower	Upper		
Continentality index	-4.431	0.610	-5.630	-3.233	-7.265	< **0.001**
Termicity index	-0.108	0.016	-0.140	-0.077	-6.722	< **0.001**
Av_annual_precipitation	0.030	0.006	0.019	0.041	5.232	< **0.001**
Precipitation dry months	0.012	0.003	0.007	0.018	4.179	< **0.001**
Ombrothermic index	5.904	0.902	4.133	7.674	6.547	< **0.001**
Giacobbe index	3.381	0.469	2.460	4.302	7.209	< **0.001**
Qemberger index	0.231	0.040	0.152	0.310	5.727	< **0.001**

Table 37 Diachronic modelling on the effect of the Tree intensity of debark, Tree size, Intraspecific Competition Index Hh3 and Climatic Ombrothermic index climatic index on cork density

Parameter	Coef.	Std. Error	95% Confidence Interval		t	p value
			Lower	Upper		
Intensity of debark	1.438	0.198	1.05	1.827	7.27	<0.001
Tree size	0.025	0.293	-0.032	0.083	0.858	0.391
Hh3	8.163	4.707	-1.078	17.405	1.735	0.083
Ombrothermic index	-22.211	5.273	-32.558	-11.863	-4.212	<0.001

Table 38 Diachronic modelling on the effect of the Tree intensity of debark, Tree size, Intraspecific Competition Index Hd2 and Climatic Ombrothermic Index climatic index on cork thickness

Parameter	coef.	Std. Error	95% Confidence Interval		t	p value
			Lower	Upper		
Intensity of debark	-0.258	0.037	-0.331	-0.185	-6.972	<0.001
Tree size	-0.006	0.004	-0.014	0.003	-1.351	0.177
Hd2	-5.057	0.568	3.88	6.687	7.39	<0.001
Ombrothermic Index	5.284	0.715	3.88	6.687	7.39	<0.001

The residual effects between 2:2 combination showed a higher variability between trees, which represent a high variability between trees of the 2nd debarking.

In regards to the plots basal area, Mean ± SD was 9.65 ± 3.29 $m^2 ha^{-1}$ for the first decade of production analysed. Total Thickness was 30.42 ± 0.45 mm. According to the literature (Fortes et al, 2004; Silva, 1996), cork planks expand around of 15% transversely and 6% tangentially after the industrial boiling procedure, which means an increase in cork thickness. Thus, the cork analysed showed a good thickness to the natural cork stoppers production. Additionally, Fig. 46 demonstrates that cork thickness does not decrease with greater basal area. Therefore, it is possible to increase cork production maintaining a great basal area (Fig. 46 and 48), such as >12 $m^2 ha^{-1}$, as stated by Ribeiro (2015). In regards to cork density, it is possible, as well, to manage cork stands with >12 m^2 ha of basal area, with no density variation.

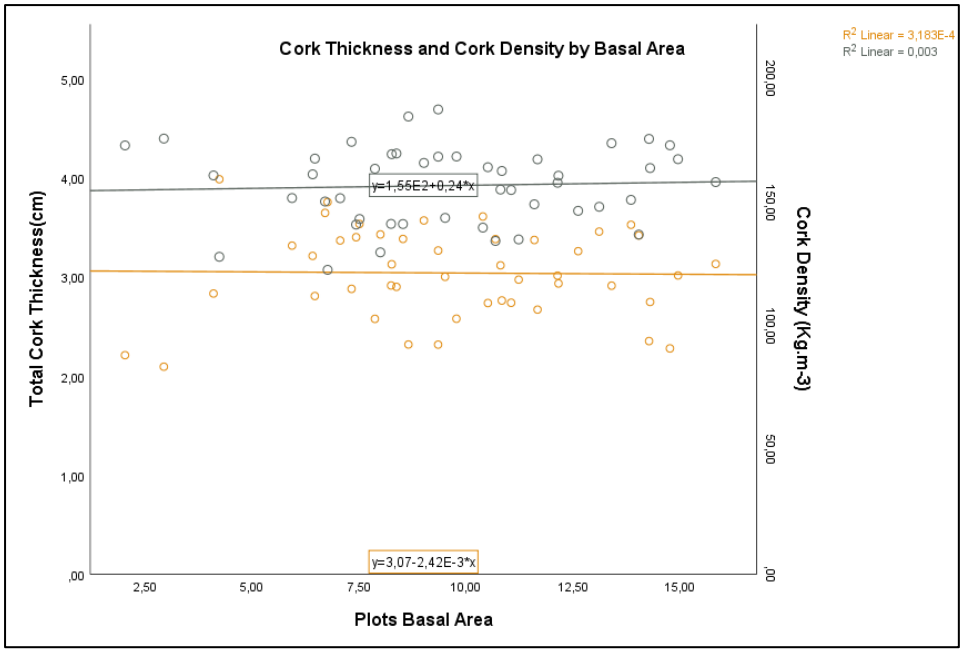

Fig. 46 Relation of cork thickness and cork density by basal area (m2 ha^{-1}). In orange related to thickness and in grey related to density.

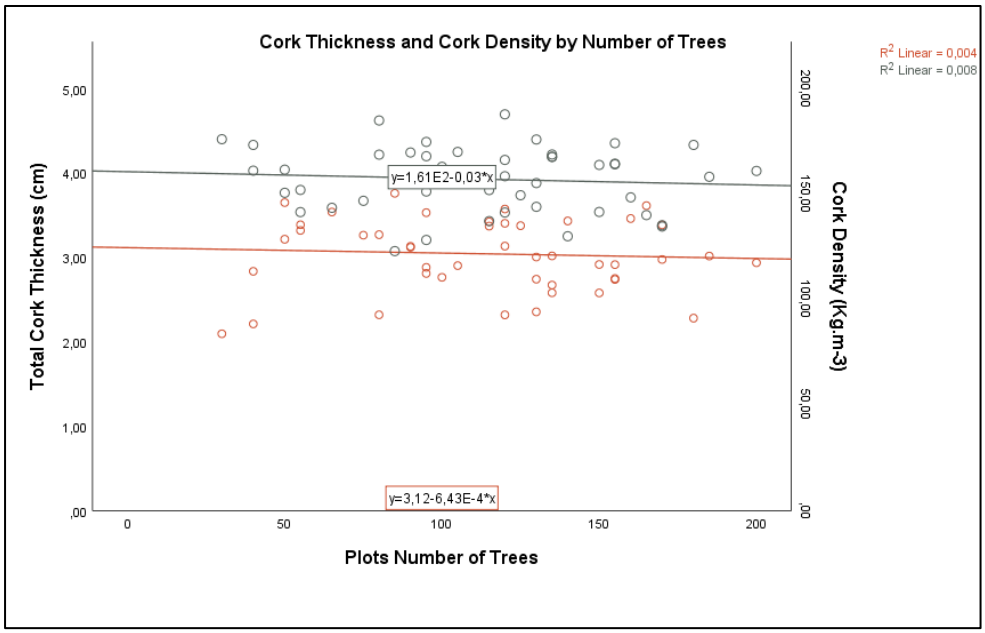

Fig. 47 Relation of cork thickness and cork density by number of trees. In orange related to thickness and in grey related to density.

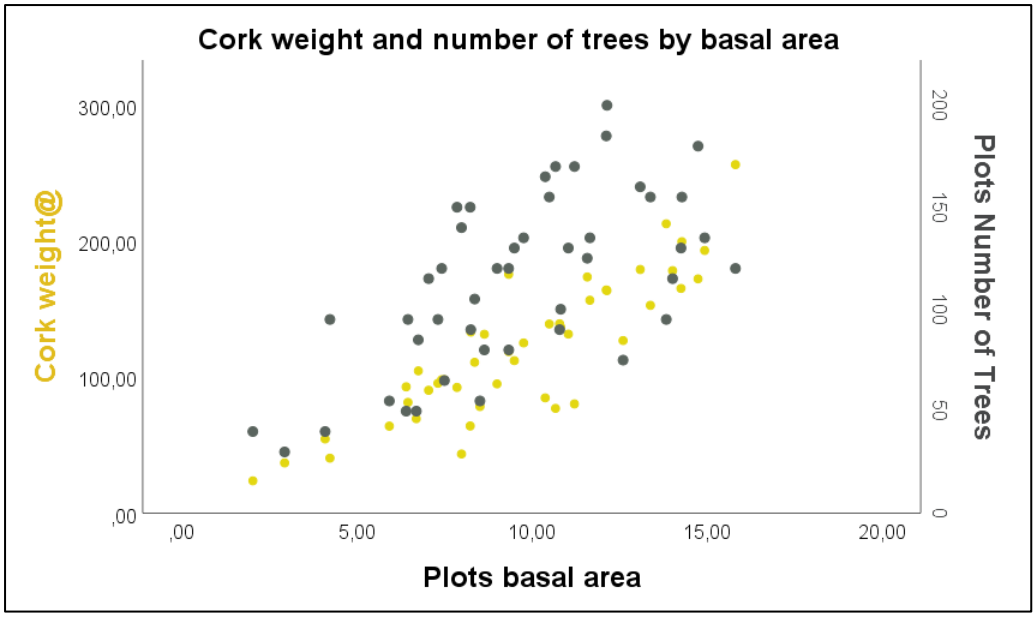

Fig. 48 Relation between cork weight (@), number of trees and basal area (m^2/ha)

5.6 Discussion

Cork characteristics, such as *thickness* and *density*, play an important role on cork products, especially on cork stoppers industry. Mean ± St. Error of cork density analysed on the consecutive debarkings (Table 32) are in line with cork thickness reported in the literature (Chorana et al, 2019; Pereira, 2007). It should be noted that cork in this study was analysed in the raw state to the maintenance of its real characteristics, before any industrial procedure. In the literature cork analysis is mostly performed after cooking. It is expected a thickness expansion after the boiling procedure. Cork density found in this analysis (Table 32), were, likewise, in line with the literature. Natividade (1934) reported a great interval for raw cork density (120-200 $Kg.m^{-3}$), which represent the cork great variability. Following the boiling procedure cork density is predict to decrease due to the release of tangential tensions (Fortes et al, 2004). To the cork stoppers industry, stoppers are usually taken from the most internal side of cork planks (Fig.49), meaning that the oldest cork rings are discarded on the industrial procedures. Cork density, according to the cork density trend (Natividade, 1934) is higher on those cork rings. As an example, cork from the 1st debarking, with 8 complete years of growth showed high density when the most internal 6 years were accounted (163.01±1.38 $kg.m^{-3}$), which is important to consider in sites with low cork densities, such as the fertirrigated site studied in Poeiras et al. (2021).

Fig. 49 Example of a cork plank to cork stoppers production. Cork stoppers are usually extracted from the most internal side of the cork plank.

The management parameter *intensity of debark* demonstrated a high significance on both cork characteristics, enabling control those characteristics through silviculture actions. For the initial stand, as analysed previously, the search index h3, used on competition indices Hh3 and MEh3 demonstrated significance on cork density, representing trees competition for light (Fig. 43).
This result revealed the important influence of high stand density to the increase of cork density, especially in those stands with higher growth rates where density is lower and an undergoes around of 20% (Fortes et al. 2004) is expected on the industrial boiling procedure. Thus, in stands with high basal area, the cork density optimization to the cork stoppers industry is possible to achieve, with gains in productivity, as well.
In stands with high growth rates due to water availability, the possibility of managing cork thickness and maintain cork production rates, which was proved by the significance of the intensity of debark on cork characteristics, are a good insight over silviculture models of pure cork oak stands for cork production. Likewise, Ribeiro et al (2003) and Fig. 48 demonstrated that a basal area loss leads to a decreasing in stands production. Surový at al. (2015) predicted a method for the estimation of cork production using remotely sensed aerial imagery, which may be used as a complement of silviculture techniques.
The competition and search index Hd2, related to the competition through the distance between trees in relation with their basal area revealed a high impact on cork thickness for the two periods analysed. These results showed that competition by soil resources have an influence on cork thickness. This may be related to roots system dynamics, which area of projection is around 2.5 to 3x of the crown projection area (Dinis, 2014; Metro & Sauvage, 1957). Daniels (1976) stated, as well, that competition occurs far beyond the limits of crown projection area.
The findings of this study enable to conclude that cork characteristics, such as density and thickness, may be controlled by management actions, contributing, as well, to stand productivity, and, consequently to the increase of forest areas. Tinoco et al (2009) found, as well, better cork quality in cork oak stands managing with sowing about forty years old. This

stand showed better cork quality than the stand from natural regeneration, than a mixed stand and than a stand with intensive pasture.

Between the debarking periods, climate had changed towards less average precipitation and larger temperature range, with higher maximum temperatures and lower minimum temperatures. Overall reduction in precipitation had more impact than reduction in precipitation on the dry months. Cork oaks are known to rely on groundwater during summer drought (Otieno et al.2006; David et al. 2007; Besson et al. 2014) to maintain their photosynthetic activity. Accordingly, cork growth during spring is usually associated with precipitation in the previous season (Costa, 2002). This may explain why a reduction in overall precipitation may have a stronger effect on cork thickness and density than reduced precipitation during dry months. In relation to temperature, the stronger effect of the Continentality and Termicidity indices suggested that maximum temperatures, as well as minimum ones, are having a limiting effect on growth, in accordance with previous studies (Costa, 2002). The index that better comprised the conjoin effect of precipitation and temperature of cork growth was the Ombrotermic index, in accordance to Leite et al. (2019). Those authors revealed a higher decrease of cork growth with events of drought. According some projections of climate changes that predict a decrease of precipitation and an increase on the dry season (Lionello et al., 2017), cork oaks may show lower growths (Costa *et al.*, 2016), due to the dominant signal of climate on cork growth. Nevertheless, the silviculture model of cork oak pure stands with higher trees' density and, as well, the silviculture model of irrigation in some areas with available water, mentioned in the previous chapters, may lead to an increase of cork production, and to the possibility of cork characteristics improvement in regards to the specific products intended to obtain.

5.7 Conclusions

This study intended to analyse the influence of intraspecific competition and climate factors on cork characteristics, such as density and thickness, which have been analysed by means of X-Ray microdensitometry technology for consecutive debarkings. Despite the high variability presented between trees, the Hd2 and Hh3 competition indices showed a high significance on cork thickness and density, respectively. In the first analysed decade, competition demonstrated

a great impact on cork density, which proved to be related with the tree's proximity on the stand.

The *Ombrotermic* climate index showed great impact on cork characteristics, as well, proved by the impact of precipitation and temperature on cork growth, as mentioned on the literature. Therefore, it was proved that cork characteristics may be controlled through management actions, such as the intensity of debark and stand density, in high density stands, with high basal area, enabling to reach specific cork parameters (density and thickness), important to the cork industry, with the maintenance of stand productivity.

5.8 References

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Chapter 6 – Conclusions and Further Perspectives

This study focused on the analysis of cork characteristics from different applied silviculture models, one of them a pure cork oak stand for cork production and the other a silviculture model of irrigation. Several image analysis and X-ray technologies were applied to understand the response of cork formation, cork characteristics and cork quality to those silviculture models. X-ray techniques, such as microdensitometry and micro-computed tomography, and techniques of scanning electron microscopy (SEM), environmental scanning electron microscopy (ESEM) and a set of image analysis programs were used to analyse cork tissue from macroscopic characteristics to the cellular level.

In the traditional pure cork oak stand for cork production some consecutive debarkings were studied, and a reduction on cork thickness and an increasing on density over debarkings were observed. Climate, especially in regards to the reduction in total precipitation, showed more impact than the reduction in precipitation on the dry months. Larger temperature range, with lower minimum and higher maximum temperatures were found for the study area. Thus, the Ombrothermic Index showed great influence on cork characteristics.

At the dynamics stand level, Hd2 and Hh3 competition and search indices showed high significance on cork thickness and density, respectively. Therefore, it was proved that cork density and thickness may be controlled through management actions. Intraspecific competition is related to the light cone and distance between trees. It was also demonstrated a possibility of cork density and thickness control through the intensity of debark.

As a response to the silviculture model of irrigation, cork demonstrated greater growths than cork from the rainfed plot used for that analysis, lower density (with no statistical significance), higher coefficient of porosity on the tangential and transverse planes, lower number of pores with greater areas in the tangential plane and greater internal porosity. However, the results about competition, on chapter 4, showed that cork characteristics, such as density and thickness, may be controlled through management actions. Those actions, such as high density stands and high intensity of debark, enabling control the specific cork parameters to the specific cork goods produced.

At the cell level, cells from the irrigated plot presented thinner cell-walls, which showed greater expansion when hydrated. After the boiling procedure there was a decrease in the thickness of the cell walls of cork from the rainfed plot between the raw stage and the boiling stage, in contrast to cork from the irrigated plot, where an increase in cell-wall thickness was observed.

The objectives of the present study were achieved, with, at least, some contributions to Knowledge' advances, which is the objective of a PhD.

Several new questions have emerged from this study, which field experiments are still on-going, as well as a several number of studies about the trees and cork tissue in an integrated approach of the research team. Research about the phellogen and phelloderm of cork samples are in progress. The chemical composition of cork from the fertirrigated silviculture model, questions about the mechanical behaviour, the application of the specific competition indices in other sites with different climatic and edaphic characteristics are such examples of further and in progress studies.