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# The impact of drought events on the xylogenesis and phloem anatomy of *Pinus pinaster* Aiton saplings

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## Resumo

As alterações climáticas são reconhecidas como uma ameaça significativa à biodiversidade e aos ecossistemas. Prevê-se um aumento da temperatura, um regime de precipitação irregular e de seca intensa, condicionando o crescimento das árvores. A região do Mediterrâneo não é exceção, onde estas previsões se aplicam. A taxa de mortalidade nas árvores é elevada nas primeiras fases de desenvolvimento, por isso é crucial perceber como estas condições afetam árvores jovens e/ou plântulas. Para tal, foi desenhada uma experiência de manipulação de água. Utilizaram-se plântulas de pinheiro-bravo (*Pinus pinaster*), uma espécie importante na floresta portuguesa e mediterrânica, para entender o impacto da seca ao nível de crescimento, xilogénese e desenvolvimento do floema. Foi realizada uma experiência com plântulas de dois anos de idade sujeitas a diferentes regimes hídricos, um grupo foi submetido a uma seca mais intensa, seguida de um período de irrigação extra (após a seca) e o outro grupo serviu como controlo. A irrigação após a seca foi aplicada para perceber se as plântulas conseguem recuperar após um período de falta de água. Assim, foram estabelecidos quatro tratamentos: CC (controlo + controlo); CI (controlo + irrigação extra); EC (exclusão de água + controlo); EI (exclusão de água + irrigação extra).

Em geral, as plântulas submetidas à exclusão de água apresentaram alturas e diâmetros reduzidos. Em relação à xilogénese, não houve diferenças significativas entre os tratamentos em relação ao número de células do câmbio, células em fase de expansão (E), lenhificação (L) e maduras (M). No entanto, os tratamentos com irrigação extra apresentaram uma elevada produção de células maduras (M) e de floema, com o tratamento EI a apresentar uma recuperação mais rápida.

O facto de as plântulas terem resistido à seca é muito importante para a restauração e reflorestação de florestas em condições desfavoráveis e de *stress*, uma vez que as primeiras fases da vida das árvores são importantes para o estabelecimento inicial de uma floresta.

**Palavras-chave:** Anatomia; floema; irrigação extra; *Pinus pinaster*; seca; xilogénese;



## Abstract

Climate change is widely recognized as a significant threat to biodiversity and ecosystem services which will have an impact on forests. Climate change forecasts a temperature increase, irregular precipitation and intense drought, conditioning tree growth. The Mediterranean region is no exception, with a prediction of more intense droughts and reduced precipitation. In the first stages of development, tree mortality is high and thus it is increasingly important to understand the impact of drought on saplings. *Pinus pinaster* is an important species in the Portuguese and Mediterranean forest. To understand the impact of water availability on growth parameters, xylogenesis and phloem development of *P. pinaster* saplings, a water manipulation experiment was performed using 2-year-old maritime pines. The saplings were submitted to a drought period but also to an extra irrigation period (after the drought) to verify if the saplings could recover from an intense drought period. Four treatments were established: CC (control + control); CI (control + extra irrigation); EC (water exclusion + control) and EI (water exclusion + extra irrigation).

In general, the saplings that were submitted to a water exclusion period showed a lower diameter and height. Concerning the xylogenesis, no differences were observed among the treatments when comparing the number of cambial cells, cells in enlargement (E), in lignification (L) and mature (M). However, the treatments that had the extra irrigation after the drought period, showed a higher rate in the production of mature cells (M and ELM cells) and of phloem, with the EI treatment showing the fastest recovery. Furthermore, the extra irrigation treatments also produced more tracheids during that period.

Overall, the fact that saplings did resist to a drought period is very important for restoration and reforestation programs under stressful and unfavorable conditions because the early life stages of trees are important for the initial establishment of a forest.

**Keywords:** Anatomy; drought; extra irrigation; phloem; *Pinus pinaster*; xylogenesis





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## INTRODUCTION

### Climate change impact on forest ecosystems

Climate change is widely recognized as a significant threat to biodiversity and ecosystem services. The global climate changes are predicted to be an increase in temperature, the alteration of the precipitation regime and an increase in the frequency of extreme events, such as floods and droughts (IPCC, 2007). Drought is defined as “a deficit of rainfall in respect to the long-term mean, affecting a large area for one or several seasons or years, drastically reducing primary production in natural ecosystems and rainfed agriculture” (WMO, 1975). Climate change will have a detrimental impact on forests since tree growth is climate-dependent. Forest ecosystems are being rapidly transformed due to climate change, and increases in the duration, frequency and severity of drought are changing the composition, structure and biogeography of many forest regions. These changes can also have indirect consequences for increased tree mortality, such as insect outbreaks, diseases and increased frequency and severity of wildfires (Allen et al., 2010; Cailleret et al., 2017).

The Mediterranean climate is characterized by hot and dry summers, while winters tend to be cool and moist, with most of the precipitation occurring during these months. All five Mediterranean-climate ecoregions are highly distinctive, collectively harboring 10% of the Earth’s plant species (Cowling et al., 1996). These ecoregions are located at the Mediterranean Basin, Australia, Chile, California and South Africa (Cowling et al., 1996). The Mediterranean region is already suffering the impact of climate change. For example, in the Mediterranean Basin, the Palmer Drought Severity Index (PDSI), a standardized index that uses readily available data of temperature and precipitation to estimate relative dryness, shows a declining trend, meaning that soil water availability for vegetation is decreasing. PDSI values over the last few decades, especially in the western part of the Mediterranean Basin, are linked to increasing temperatures (Nicault et al., 2008). If this warming trend continues as forecasted (Nicault et al., 2008; IPCC 2001; Scott et al. 2003), it is likely to result in prolonged and extreme droughts over the entire Mediterranean Basin. This trend towards increasing periods of drought, coupled with human impact on soil conditions in the

Mediterranean Basin, will have important agricultural and socio-economic consequences and thus, needs to be considered in land management policies.

Predicting how Mediterranean forest species will respond to future climate changes hinges on a better understanding of their response and recovery to drought events. Recent studies have reported climate change induced mortality episodes around the globe (Cailleret et al., 2017). Considering that the climate-change scenarios suggest an increase in aridity in many areas (Petit et al. 1999), research on plant response and recovery to water stress is becoming increasingly important to improve reforestation programs. Mediterranean forests are thus excellent natural laboratories to study the effects of drought on wood formation, growth and tree survival (Vieira et al., 2013).

#### Climate change and its impact on trees

Trees present a long-life span that does not allow for a quick adaptation to environmental changes (Lindner et al., 2010). Increasing temperature will induce higher rates of evapotranspiration, leading to a reduction in stomatal conductance, with a consequent reduction in net primary productivity of forest ecosystems. Intense and prolonged droughts can, in extreme cases, lead to carbon starvation and tree mortality. This can potentially lead to losses and/or changes in biodiversity of woodland communities and in a significant increase of regional-scale mortality of dominant tree species (Breshears et al., 2005; IPCC, 2007; Choat et al., 2012). Although the vulnerability of each individual species will depend on the level of exposure to climate change (its sensitivity to that change and ability to adapt), plants exhibit a wide range of morphological and ecophysiological responses to drought both between and within species (Chaves et al. 2003, Bréda et al. 2006). Climate change will impact forests in a way that will require adaptation measurements that are difficult to design, plan and implement.

Apart from changing the ecophysiological response of a tree, climate change also leaves its mark on tree anatomy. The changes observed inter-annually in tree-ring properties are the result of climate. In water limited environments, such as the Mediterranean region, in years with higher levels of precipitation wider tree-rings are formed. On the opposite, if

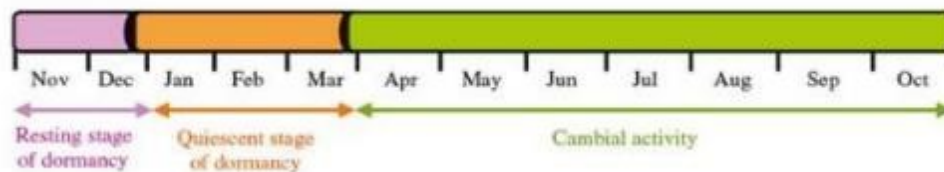
precipitation is lower and temperatures are higher (mainly in summer), cell expansion is lower and so are the tree rings (Vieira et al., 2009). Studies investigating the climatic signal of tree-rings in the Mediterranean region have demonstrated a positive response to spring precipitation and a negative one with summer temperature (Zalloni et al., 2016). Thus, tree rings provide information about the climatic conditions during the growing season. Xylogenesis, the study of tree-ring formation, can improve dendrochronological studies by improving the time resolution of these studies and by analyzing the process of wood formation directly and not retrospectively.

### Cambium and xylem formation

In the Mediterranean area, wood formation occurs seasonally from the division of the vascular cambium and through lignification process (Rowe and Speck, 2005; Rathgeber et al., 2016). The vascular cambium is a secondary meristematic tissue responsible for the increase in diameter of trees and shrubs with secondary growth (Dickison, 2000). However, these growth responses differ among species and environments. The cambium is located between the phloem and the xylem, differentiating xylem cells inwards and phloem cells outward, towards the bark (Rathgeber et al., 2016). The vascular cambium presents two types of cells: fusiform stem cells, highly elongated cells that will differentiate into cells of secondary xylem and phloem; and ray stem cells, which form the radial system that connects xylem and phloem (Taiz & Zeiger, 2002).

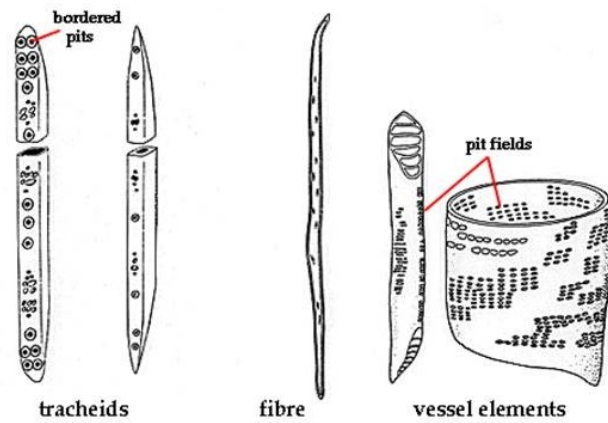
The vascular cambium presents a seasonal activity, being active during the spring and dormant during winter. The dormant phase can be divided in a resting and in a quiescent stage (Figure 1). The resting phase is characterized by the inability of the cambium to produce new cells, even if the right environmental conditions are present; the quiescent phase corresponds to the time window when cell production is dependent on external factors, such as temperature (Begum et al., 2012). When the above-mentioned conditions are met, cambial reactivation occurs, and new cambial cells are formed, leading to the formation of new xylem and phloem cells (Dickison, 2000). To engage and regulate cambial activity, internal and external (environmental) factors are crucial. Hormones are the most important internal factor, especially auxin (indole-3-acetic acid – IAA). This hormone is

involved in cell growth and expansion. It is produced on the top of the plant, near the leaves, and transported basipetally from the leaves to the roots, stimulating growth through the entire plant or tree. Appropriate levels of auxin, together with certain external environmental conditions (temperature and water availability), lead to the reactivation of the cambium and to the production of new cells.



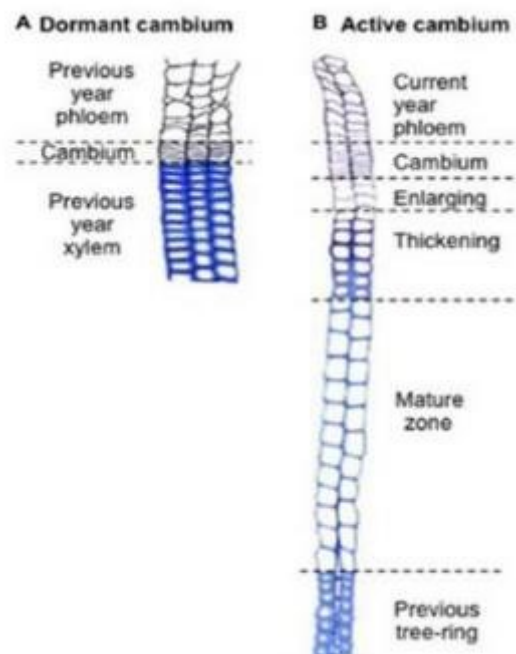
**Figure 1.** Schematic representation of the seasonal cambial activity. In purple: resting stage of dormancy; in orange: Quiescent stage of dormancy; and in green: Cambial activity (Source: Begum et al., 2012).

The xylem tissue is mainly constituted by two types of tracheary elements involved in the transport of water: the tracheids that occur on both Gymnosperms and Angiosperms, although the last group presents fewer tracheids; and vessels, that only occur in Angiosperms (Figure 2). There are several differences between Gymnosperms and Angiosperms wood. The first ones have simple and homogenous wood (softwood), non-porous, with no vessels and small amounts of axial parenchyma. This wood is composed by only one type of cells, the tracheids. On the other hand, angiosperms have a more complex, porous and heterogenous wood (hardwood). This wood is formed by different types of cells: vessels, tracheids, fibers and a more distributed axial parenchyma (Wilson & White, 1986). Tracheids are elongated cells that present numerous pits in the cell walls that interconnect the network of tracheids for water flow. Vessel elements are shorter and wider and present perforation plates on the top and end of each cell. Vessels are disposed on top of each other forming a larger conduit. The vessels are more efficient in the transport of water, presenting a lower resistance pathway than tracheids (Taiz & Zeiger, 2002). Due to its homogenous wood gymnosperms are often selected for xylogenesis studies.



**Figure 2.** Illustration of the main xylem cells: tracheids, fibers and vessel elements. Source: [http://tarahhiemes.com/FWE/modules/module01/media/topic3\\_lecture-notes.html](http://tarahhiemes.com/FWE/modules/module01/media/topic3_lecture-notes.html)

Xylem cells are the result of a process that starts with cambial cell divisions, growth and maturation of a pool of cells that undergo several phases of differentiation before reaching its complete maturation, a process known as xylogenesis (Wodzicki, 1971; Rossi et al., 2006b; Rossi et al., 2012). During development, the cambial cells start to change morphologically and physiologically, originating new xylem cells. Five steps are described for xylem development, from the cambial mother cell to a functional tracheid: (1) cell enlargement (2), cell wall thickening, (3) lignification, (4) and finally cell maturation and programmed cell death (5) (Rossi et al., 2012; Rathgeber et al., 2016, Figure 3). Programmed cell death of tracheary elements is crucial for water transport, since the hollow cells provide a low-resistance pathway and reduced pressure gradients for water transport from the roots to the leaves, with a high efficiency (Taiz & Zeiger, 2002)



**Figure 3.** Wood anatomical representation of the seasonal cambial activity. During dormancy the number of cambial cells is reduced; when the cambium is active there is an increase in the number of cambial cells and several stages of maturation of the tracheary elements occur (enlarging, thickening and fully mature). (Source: Rathgeber et al., 2016).

## Climate control on cambial activity and wood formation

Climate has an enormous influence on xylem development, structure and functionality. The tracheary elements produced in the spring (earlywood), when water availability is higher, have larger lumens, while tracheary elements produced in summer (latewood) have smaller lumens and thicker cell walls (Dickison, 2000). Depending on the prevalent climatic conditions, that vary year-to-year, xylem cells are produced with different structural characteristics (Dickison, 2000). The period of wood formation is linked to environmental conditions that act directly on the cells, forming the tree rings (Vieira et al., 2013). For example, in water limited environments, the number and anatomical properties of the tracheids formed are dependent on water availability. Water availability has a major impact on the process of cell division and enlargement as the presence or absence of water affects turgor pressure. Turgor pressure is responsible for the enlargement process since it is the pressure exerted by water over in the primary cell wall that expands it (Oribe et al., 2003). Water availability affects the cell turgor pressure and connects the quantity of water present with tracheid size (Eilmann et al., 2011; Arzac et al., 2018). If less water is available, fewer tracheids with smaller lumen area will be formed (Vieira et al., 2017).

The mechanisms of xylem formation and development have a great ecological importance (Martin-Benito et al., 2012). To understand the impact of climatic conditions (temperature and/or water availability) on xylogenesis, several manipulative experiments have been performed. Balducci et al. (2015), using four-year-old *Picea mariana* saplings in greenhouse conditions showed that this species, after being subjected to water stress, was able to resume cambium activity and radial growth. However, when subjected to a period of severe drought, the saplings died under warm conditions, due to the loss of hydraulic conductivity.

The impact of climate can be largely different in saplings and in adult trees. Bachofen et al. (2017) refers that tree saplings have been considered less drought tolerant than adult trees because of their small root system and small carbon reserves. To test this, the authors submitted 2/3-year-old *Pinus sylvestris* and *Pinus nigra* saplings to four months of very intense drought, during two consecutive years, and despite the long duration of the drought conditions, mortality rates of the saplings were below 10% by the end of the 2<sup>nd</sup> year. Thus,



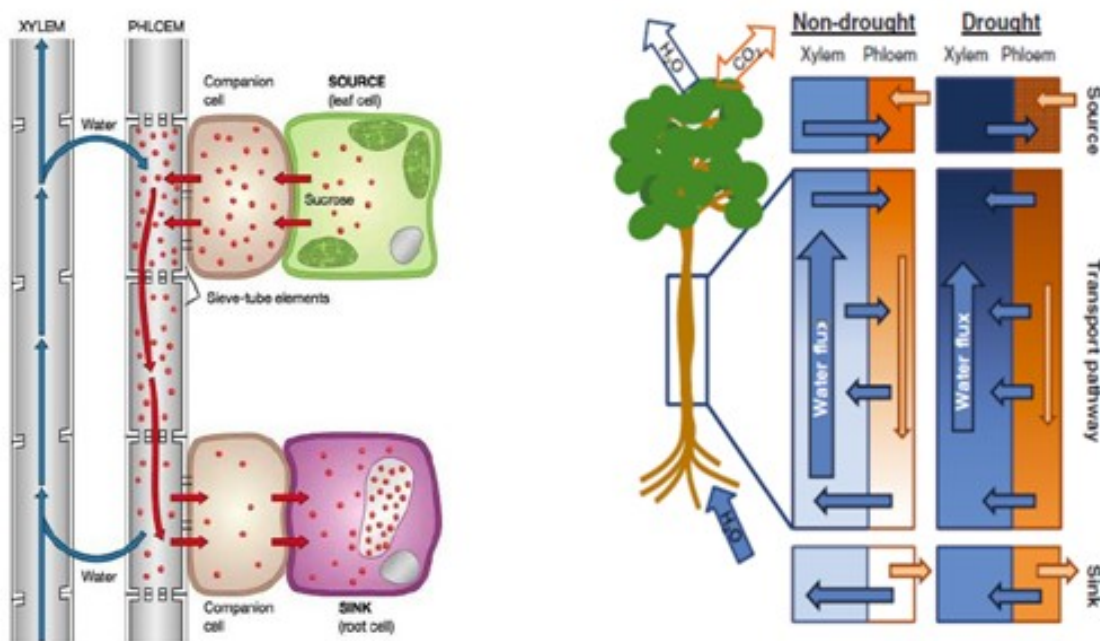
saplings could tolerate and acclimatize to repeated summer droughts (Bachofen et al., 2017). If saplings can be drought tolerant, its establishment will be important for the success of plantations and natural regeneration of forest tree species in long term (López et al., 2009).

### Phloem formation

Phloem is also produced by cell divisions of the vascular cambium, although its differentiation has fewer steps compared with the differentiation of xylem tissue. The phloem consists of conducting sieve elements (SE), companion cells (CC), non-conducting parenchyma cells and fibers and it is located between the cambium and the bark of the tree, presenting living cells with no nucleus (Dickison, 2000). Phloem differentiation is stimulated by low levels of auxin, and its development occurs before the xylem's (where this one occurs with high levels of auxin) (Dickison, 2000). Initially, primary phloem can be designated as protophloem and metaphloem where the first one is short-lived and the first to develop. When the protophloem ceases function the metaphloem is formed. In the stem, this differentiation occurs along the longitudinal plane in an acropetal direction (from the base towards the apex) (Dickison, 2000). The older phloem is pushed outside by the new phloem cells, being exposed to tangential and radial stresses, due to the increase in stem diameter, making the older cells non-functional, deformed or displaced.

Phloem is the tissue responsible for the transport of photosynthates from source to sink. Its main function is the long-distance transport of sugars and carbohydrates from mature leaves (sources) to the roots and meristems (sinks) or reserves/storage tissues located near the roots (De Schepper et al., 2013; Dickison, 2000; Turgeon & Wolf, 2009; Savage et al., 2016). Besides the transport function, phloem is also responsible for long-distance signaling of plant hormones. It also plays a role in plant defense against predators or in case of wounding. When a sieve tube is wounded, it surges due to pressure release and the cellular debris covering the sieve pores seals them (De Schepper et al., 2013). Phloem transport is influenced by xylem water potential, where the flow rate depends on the water potential gradient and the hydraulic conductivity between tissues (Hölttä et al. 2009; Hölttä et al. 2006; Sala et al. 2010; Sevanto et al. 2011) (Figure 4).

Phloem transport is influenced by differentiating pressure between the source and the sink caused by osmotic gradients (Savage et al., 2016), transporting the soluble organic compounds made during photosynthesis. The phloem extracts water from the xylem to use for carbohydrate transport (Savage et al., 2016). However, during a drought period, phloem uses an increasing concentration of solutes to prevent losing water to the xylem, allowing the phloem to keep turgor pressure (Figure 4, Savage et al., 2016). Thus, phloem can also store water and redistribute it when necessary.



**Figure 4.** On the left, a schematic representation of phloem transport (Source: <https://pmgbiology.com/tag/transport/>). On the right, water transport pathway on a drought and non-drought situation (Source: Savage et al., 2016).

## Objectives and hypothesis

Climate in the Mediterranean region is changing with an increased frequency of drought events and reduction of precipitation. Maritime pine forests (*Pinus pinaster* Aiton) are quite important in Portugal representing approximately 23% of the forest area. Maritime pine is adapted to summer drought conditions. However, it is important to understand the impact of increasing dry conditions, in terms of severity and duration, at the anatomical and morphological level, especially of saplings. Mortality occurs mostly in the first life stages of tree development, so it is important to determine how saplings respond to drought to plan for reforestation programs.

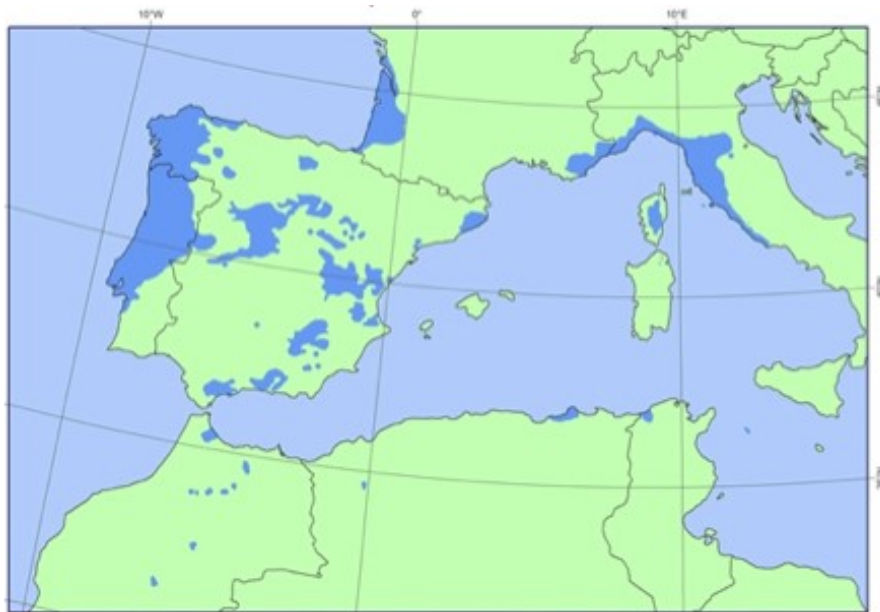
A water manipulation experiment was performed to test the response of maritime pine saplings to different water regimes. The central aim of this study was to understand the impact of a drought period, followed by a recovery period (rehydration) on the xylogenesis and phloem production of maritime pine saplings. The experiment was performed on 2-year-old saplings, under greenhouse conditions.

Our main hypothesis are: (i) saplings subjected to drought periods will present a reduction in the production of xylem and phloem, with fewer cells produced by the vascular cambium; and (ii) saplings subjected to drought followed by rehydration will produce new xylem and phloem cells after the recovery period, indicating a capacity of maritime pine to recover growth after a drought period.

## MATERIALS AND METHODS

### Study species - *Pinus pinaster* Aiton

Maritime pine (*Pinus pinaster* Aiton) is a fast-growing tree of the Pinaceae family and of the genus *Pinus*, that can live up to 300 years and reach up to 40 meters (Farjon, A., 2010). It was described by William Aiton in the 18th century (Brummitt & Powell, 1992), and is characterized by its pyramidal shaped top, straight or curved dark reddish trunk, long and rigid branches, spiny needles and cones, and a deep taproot with well-developed secondary roots (Farjon, A., 2010). This tree species requires total solar exposure and cannot tolerate shade, being described as a heliophytic species (Farjon, A., 2013). Flowering occurs around its sixth year, between late winter and mid-spring. Its distribution covers the west Mediterranean-Atlantic region, being present in Portugal, Spain, coastal area of France and Italy and with small populations in the north of Africa (Figure 5). It is present from sea level to over 1800 meters of altitude (Alfa et al., 1996). Maritime pine can be found in different soil types such as coastal sands, sandstones, dolomites, peridotites, granites, slates and metamorphic rocks as well as coniferous forests and mixed forests.



**Figure 5.** Geographical distribution of maritime pine (*Pinus pinaster* Aiton) in Western Europe (in blue). Source: [www.euforgen.org/species/pinus-pinaster](http://www.euforgen.org/species/pinus-pinaster).

In Portugal, it is the most abundant conifer species and the most important pine species in terms of planted area and economic value (Figueiredo et al., 2014). It occupies over 714.000 ha, mainly distributed in mountain regions of North, Northeast and Center of Portugal, representing over 23% of the national forest area (ICNF, 2013). It is the third most frequent forest species in the country preceded only by *Quercus suber* L. (cork oak) with 737.000 ha and *Eucalyptus globulus* Labill. with 812.000 ha (Aguiar et al., 2007). Its current territorial extension is undeniably caused by human hand and the various plantations of this pine tree across the country for economic reasons, being an important timber tree, used for paper production, wood exportation and extraction of resin (Viñas et al., 2016; Mendes, 2007).

Maritime pine presents a great ecological plasticity and a varied climate spectrum. It is adapted to areas with sub Mediterranean-type climatic conditions, especially those with higher precipitation rates. However, it can support low to high quantity of rainfall throughout the year as well as cold and strict winters and coastal temperate climate. Although it is considered a species that prefers soils with relatively low, acid pH, between 4 and 9 (Alía et al., 1996), it can stand all kinds of substrates, showing a preference for loose, sandy soils, low in nutrients. Some authors considered that due to its high stomatal sensitivity to soil water conditions (Nguyen & Lannat, 2003; Granier & Loustau, 1994), presents an isohydric strategy (“drought-avoiding”) that consists in closing the stomata in response to water stress, to prevent hydraulic failure. By adopting this behavior, carbon uptake, photosynthesis, growth and carbohydrate availability are affected, which can ultimately result in tree mortality (Martinez-Vilalta & Garcia-Forner, 2017).

For this study, maritime pine saplings were selected. Saplings are considered a vulnerable life stage where most of the mortality occurs. Also, saplings are easier to work and to manipulate due to their smaller size and are easier to utilize in hydraulic manipulative experiments comparing with adult trees. Understanding drought tolerance and resistance in saplings is of crucial importance for forest management and reforestation programs. If the saplings are drought tolerant, its establishment will be important for the success of plantations and natural regeneration of maritime pine forests in the long term (López et al., 2009). To reinforce, it is also known that pine species, including *P. pinaster*, are the most frequent species used in reforestation programs in the Mediterranean basin because of their

ability to survive harsh environmental conditions and their rapid growth when established (López et al., 2009).

### Experimental Design

The experiment was conducted in maritime pine saplings (*Pinus pinaster* Ait.), obtained in a forestry nursery. A total of 672 saplings with 2 years-old were used in this experiment. These were obtained in October 2016, transferred from cuvettes to 6 L pots, and put in a greenhouse where they stayed throughout the experiment. The greenhouse is in inProPlant facilities in Coimbra, in an area of 144 m<sup>2</sup>.



**Figure 6.** Distribution of maritime saplings in the greenhouse. Each line of saplings corresponds to a randomly assigned treatment.

Four treatments were defined: control + control (CC; n = 128), control + irrigation (CI; n = 128), exclusion + control (EC; n = 128), and exclusion + irrigation (EI; n = 128). The amount of water provided to both control treatments (CC and CI) were based on meteorological values of the average precipitation level of the region of Coimbra for each month of the year. This data was downloaded from the Royal Netherlands Meteorological Institute (KNMI - <http://www.climex.knmi.nl/>). Based on those values, the amount of water reduced in the exclusion treatments (EC and EI) and increased in the irrigation treatments (CI and EI) was calculated. The exclusion treatment consisted in reducing the amount of water

by 50% in April and May and by 75% in June, July and August (Table 1). The irrigation treatment consisted in providing an extra 100% of water in September, to the CI and EI treatment. To do so, a drip irrigation system was installed in the greenhouse to water the saplings. The saplings were arranged two-by-two in 16 lines of 42 saplings. The lines were then randomly assigned to each treatment (Figure 6).

**Table 1.** Volume of water given to each treatment per month based on the meteorological values for the Coimbra region (data downloaded from the Royal Netherlands Meteorological Institute – KMNI). In gray, the water exclusion period; in blue, the irrigation period.

Months of the year	Volume of water (mL/pot/month)			
	CC	CI	EC	EI
January	3746,87	3746,87	3746,87	3746,87
February	2479,38	2479,38	2479,38	2479,38
March	2040,55	2040,55	2040,55	2040,55
April	2298,17	2298,17	1149,09	1149,09
May	1800,14	1800,14	450,04	450,04
June	717,82	717,82	179,46	179,46
July	255,07	255,07	85,02	85,02
August	340,09	340,09	63,77	63,77
September	1438,49	2143,11	1438,49	2143,11
October	2990,46	2990,46	2990,46	2990,46
November	2905,34	2905,34	2905,34	2905,34
December	2732,46	2732,46	2732,46	2732,46

#### Monitoring of soil water content and plant growth

At the beginning of the experiment soil water content (SWC) was measured in all pots to ensure that there were no significant differences between treatments before the start of the experiment. SWC was also measured to monitor the water availability in the different treatments throughout the experiment to prevent reaching extremely low values (below 10 v/v) that could induce the mortality of the saplings. These values were measured using a HH2 Moisture Device from Delta T Devices and monitored every 12-18 days until the end of 2017.




Besides soil water content, growth parameters for each sapling were also monitored, namely the diameter of the main stem, total height, and height of green leaves. Diameter was measured with a digital calliper (Perel tools) in two directions, to account for irregularities in the stem. The total height was measured using a measuring tape from a

marked point above the soil to the apical meristem, whereas the height of green leaves (in this case, needles) was measured from the first green needle (from the base) to the apical meristem. When the saplings were transferred to the 6L pots, the stems were marked at a given distance from the soil to avoid variation due to the amount of soil in the pots, ensuring that all measurements were performed at the same level. These components were measured on every sampling date performed during the experiment (from February 2017 to February 2018).





Phenology

The phenological development of the apical meristem was recorded along the experimental period. The phenological observations were classified according to a scale from 1 to 6, corresponding to the evolution of the meristem from dormant to the presence of new needles (Table 3). Phenology was monitored every 12-18 days for all treatments until the end of August 2017, the end of the simulated drought period.

**Table 2.** The seven defined phenological phases with their respective description and pictures.

Phase	Description	Picture
1	Dormant apical meristem (undeveloped). Beginning of the bracts opening and bud elongation;	
2	“Swelling” of the bud;	
3	Bracts are fully open, elongation of the meristem but no needles are visible;	



<p><b>4</b> Bracts fully open, where the new needles can be seen of the top of the meristem;</p>	
<p><b>5.1</b> Bracts fully open where the new needles can be seen in the entire meristem. Each bract with one developed needle;</p>	
<p><b>5.2</b> Bracts fully open where the new needles can be seen in the entire meristem. Each bract with two developed needles;</p>	
<p><b>6</b> Apical meristem fully developed;</p>	

### Xylem and phloem anatomical analysis

During the growing season, different phases of xylem and phloem cell development are found within a few microns, going from living bark to mature xylem (Rossi et al., 2006), so it is important to verify its evolution, growth dynamics and the main factors affecting xylogenesis (Caffey, 2002). The anatomy study was conducted between February 2017 and March 2018 using 672 two-year-old maritime pine (*Pinus pinaster* Ait.) saplings. Destructive samplings occurred once every month to extract samples for xylogenesis and phloem anatomy. In each sampling, 32 saplings, 8 for each treatment, were randomly selected to account for variability due to the placement in the greenhouse. A stem disc was collected 5 cm above soil level in a transversal cut.

After collection, the samples were put in histo-cassettes and immersed in a FAA (formaldehyde-acetic-acid-ethanol solution) solution for 7 days, a fixing agent, with the objective to avoid sample degradation. Then the samples were transferred to a 50% ethanol

solution for 5 days. The dehydrations and paraffin embedding proceedings continued following the protocol by Rossi et al. (2006), Table 3. After embedding in paraffin, the samples were cut at 7 microns using a Microm HM 340E Electronic Rotary microtome. The strips produced were immersed in a water bath at 30 °C to stretch the sections and then transferred to a microscope slide previously treated with glycerol albumin for greater adhesion of the sections. Next, the slides were kept in the oven at 30 °C for about 12-24 hours before the staining process.

**Table 3.** Dehydration and paraffin inclusion. Protocol by Rossi et al. (2006).

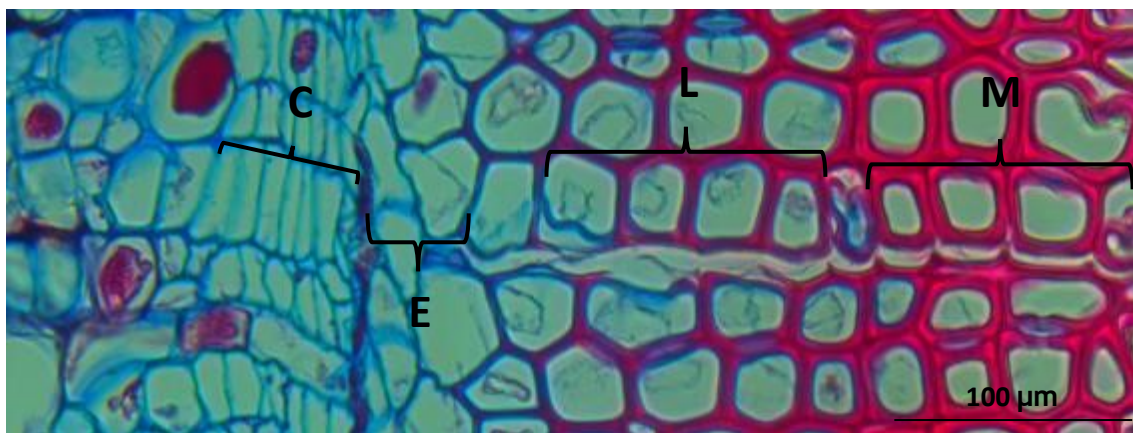
Reagent	Time
FAA	7 days
Ethanol 50%	5 days
Ethanol 70% (2x)	120 minutes
Ethanol 90% (2x)	90 minutes
Ethanol 95%	90 minutes
Ethanol 100% (2x)	90 minutes
Bio-Clear 50%	90 minutes
Bio-Clear 100% (2x)	90 minutes
Paraffin (60°C) (2x)	120 minutes

Once the sections were dry, they were stained and permanently fixed using Eukitt, a mounting medium glue containing 45% acrylic resin and 55% xylenes, for microscopy. The stain consisted of a mixture of Astra Blue (0.15%) and Safranin (0.04%) water solution, and were used with the objective of increasing the contrast between lignified and non-lignified tissues (Von Arx et al., 2016). Astra Blue stains the non-lignified structures blue and safranin the lignified structures red.

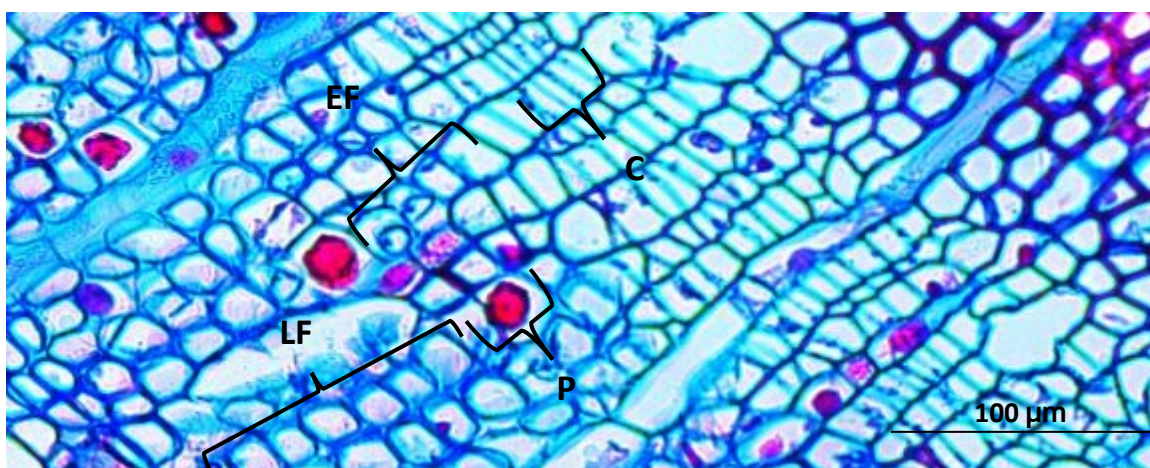
Microscopic analysis were performed using a microscope LEIKA DM4000B under visible and polarized light with a magnification of 5x and 20x. The transverse cuts were used to visualize the progress of xylem and phloem formation. Cambial, enlarging, cell wall thickening and mature cells were counted on three radial files in every sample, for each sampling date. Cambial cells are defined as thin cell walls with small radial diameters while

enlarging cells have a diameter at least twice of a cambial cell. Lignifying cells shine under polarized light and present a blue coloration, changing to red as the lignification process ends. Mature tracheids present a red pigmentation (Gričar et al., 2006; Vieira et al., 2013).

For the phloem, the number of early phloem, parenchyma and late phloem cells were counted on three radial files in all treatments. Parenchyma cells are very visible due to its dark stained contents. Parenchyma cells are used to separate early phloem from late phloem cells in a growing season (Gričar & Čufar, 2008). A magnification of 5x and 20x were also used for the phloem monitoring.



**Figure 7.** Cross section of a tree ring of maritime pine tree sapling (*Pinus pinaster*), in a magnification of 20x. C – Cells of the cambium; E – Cells in enlargement; L – Cells in lignification/thickening; M – Mature xylem cells;



**Figure 8.** Cross section of the phloem region of a maritime pine sapling (*Pinus pinaster*) in a magnification of 20 x. C – Cells of the cambium; EF – Early phloem cells; P – Phloem parenchyma cells; LF – Late phloem cells;

## Statistical Analysis

Soil water content (SWC) was compared between days of the year (DOY) and treatments (CC, CI, EC and EI) using a two-way analysis of variance (ANOVA). This test is applied when there is one measurement variable (the values of SWC, in this case) and two factors (DOY and treatment) in all possible combinations (McDonald, 2014). Differences in diameter, total height and green height were also analyzed using a two-way ANOVA. This analysis was made using SigmaStat 4.0 (Systat Software, Inc., San Jose, CA, USA).

The variation of the number of differentiating xylem cells, enlarging (E), lignifying (L) and mature cells (M), ELM (Figure 15), through time showed a bimodal distribution, thus two Gompertz functions were applied, one for each sigmoid curve. The dynamics of cell production in each treatment (CC, CI, EC and EI) were fitted using a Gompertz function, in JMP (SAS Insititue, Cary, NC, USA). This function is defined as:

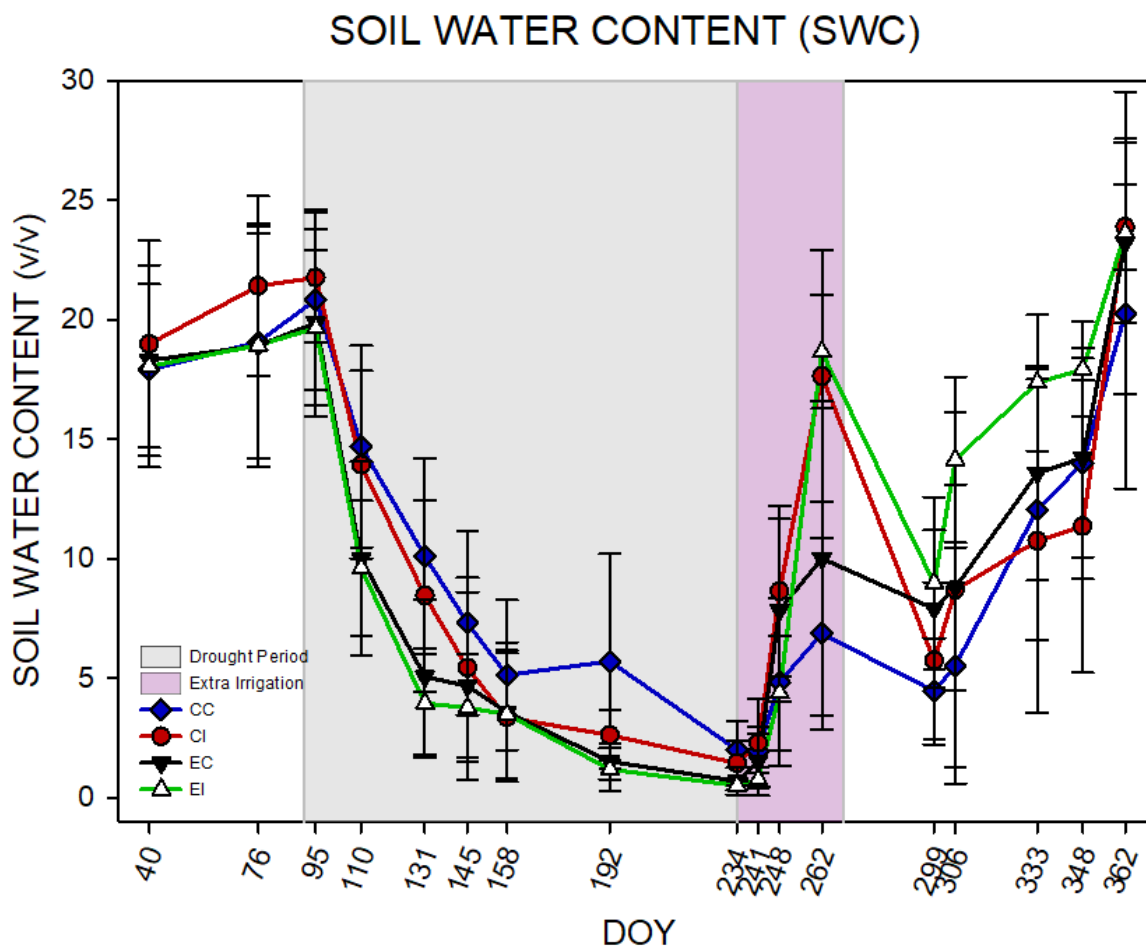
$$y = A \exp(-e^{\beta - k\tau})$$

where  $y$  is the number of cells,  $\tau$  is time computed as DOY (days of the year),  $A$  is the upper asymptote,  $\beta$  is the inflection point and  $k$  is the rate of change parameter (Rossi et al., 2003; Balducci et al., 2013).

## RESULTS

### Soil water content (SWC)

Soil water content presented a seasonal pattern and differences between treatments (Figure 9). A significant rise of SWC was observed from March until April (DOY 95) but then, from the start of the water exclusion period at the beginning of April (DOY 91-95) those values dropped. The water exclusion experiment lasted until the end of August (DOY 252). The exclusion treatments (EC and EI) registered the lowest values, having SWC values below 10 (v/v). After the drought period, in September (DOY 252), a recovery on the soil water content was verified, in all treatments. The irrigation treatments, EI and CI, showed the highest recovery after drought. Soil water content increased more rapidly in EI than in CI. In the end of September SWC decrease in EI and CI, reaching the same value in all treatments. In October (DOY 270-299), SWC increased, reaching the pre-experiment values in all treatments.



**Figure 9.** Soil water content values (v/v) for all four treatments (DOY, days of year). The gray shaded area is the drought period (EC and EI treatments) and the light purple shaded area is the period of extra irrigation (CI and EI treatments).

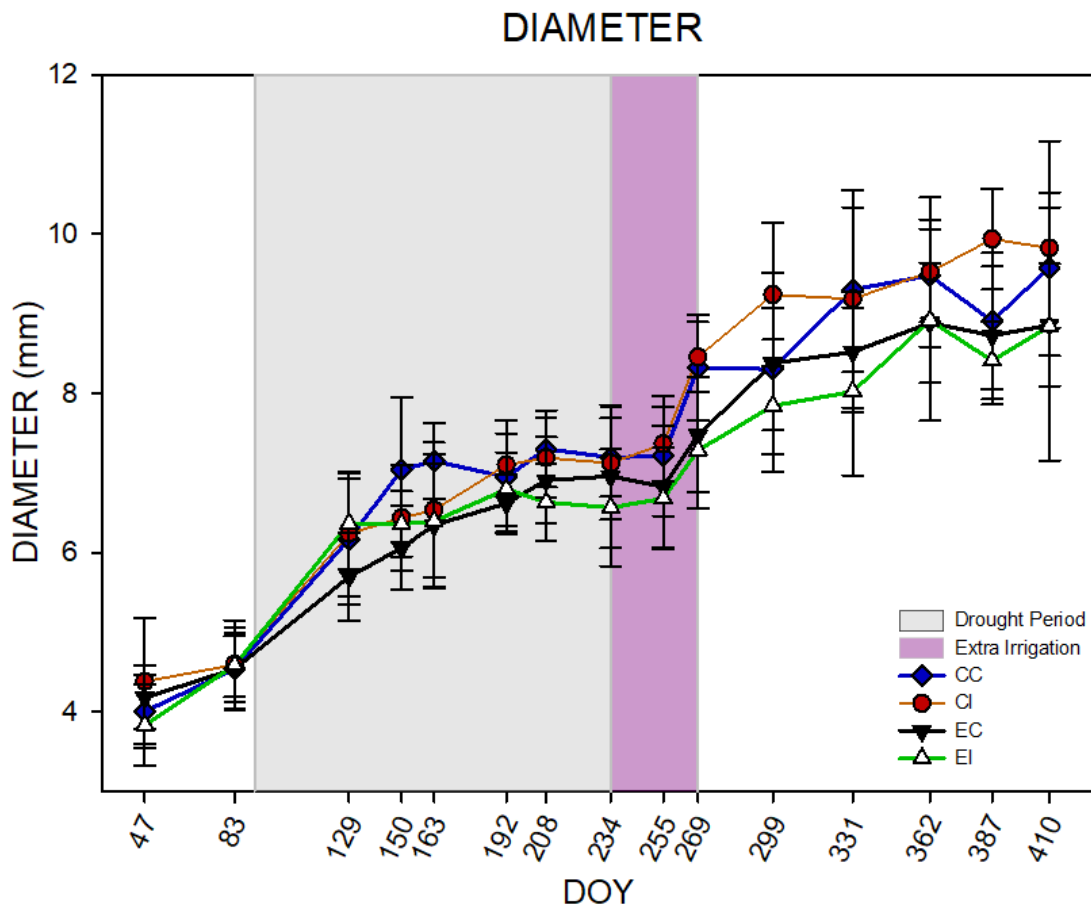
A two-way ANOVA was performed to compare SWC between treatments throughout the experiment. The results showed significant differences in DOY ( $p < 0.001$ ), between treatments ( $p = 0.013$ ) and in the interaction between DOY and treatment ( $p < 0.001$ ).

**Table 4.** Two-way ANOVA analysis of the soil water content (SWC) of the maritime pine saplings using day of year (DOY) and Treatment as factors.

Parameters	Factors	F	p
SWC	DOY	143.185	<0.001
	Treatment	3.627	0.013
	Treatment x DOY	2,945	<0.001

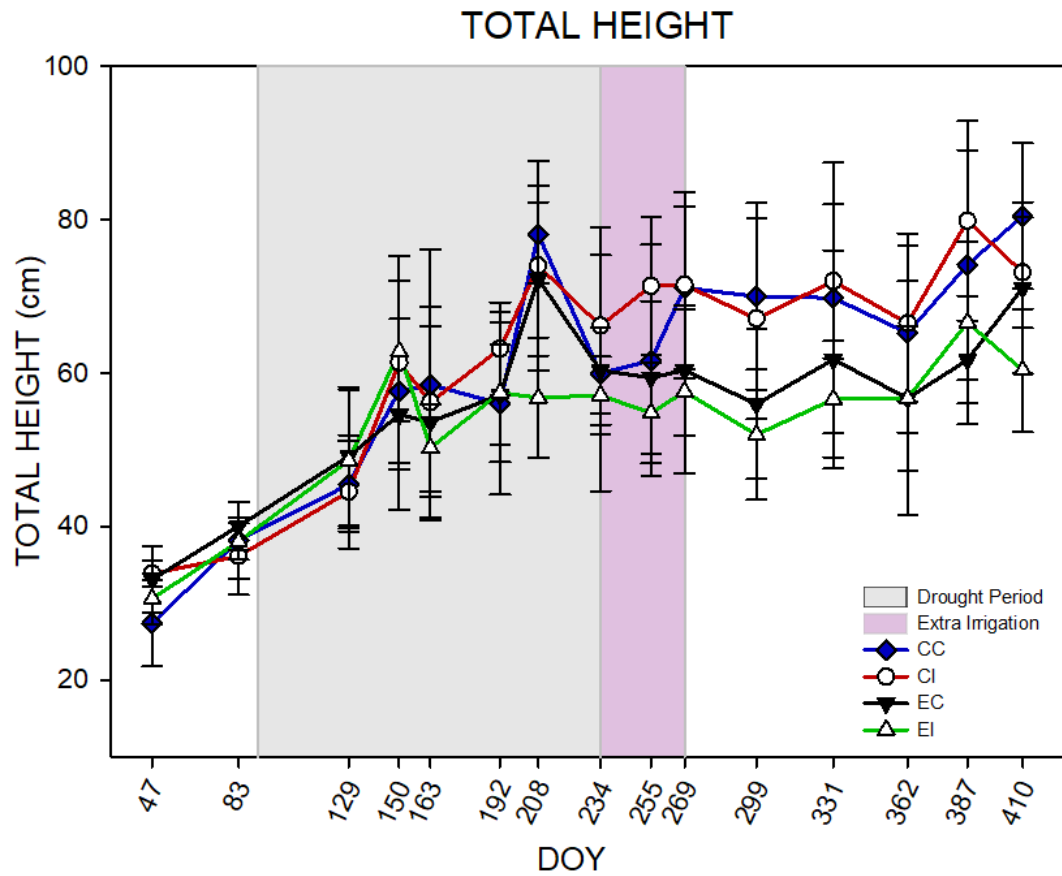
Plant growth parameters: stem diameter, total height and green height

A gradual increase of stem diameter was observed in all treatments until the end of May (DOY 150, Figure 10). During the drought period (DOY 91 to 234), no stem diameter increment was observed for the EI and EC treatments and a very slow growth was observed in the CC and CI treatments. In DOY 255 (September), corresponding to the end of the drought experiment period, the stem diameter increased rapidly (Figure 10). In general, the CI treatment showed higher values of stem diameter, while the EI treatment showed the lowest, despite both receiving an extra irrigation in September.

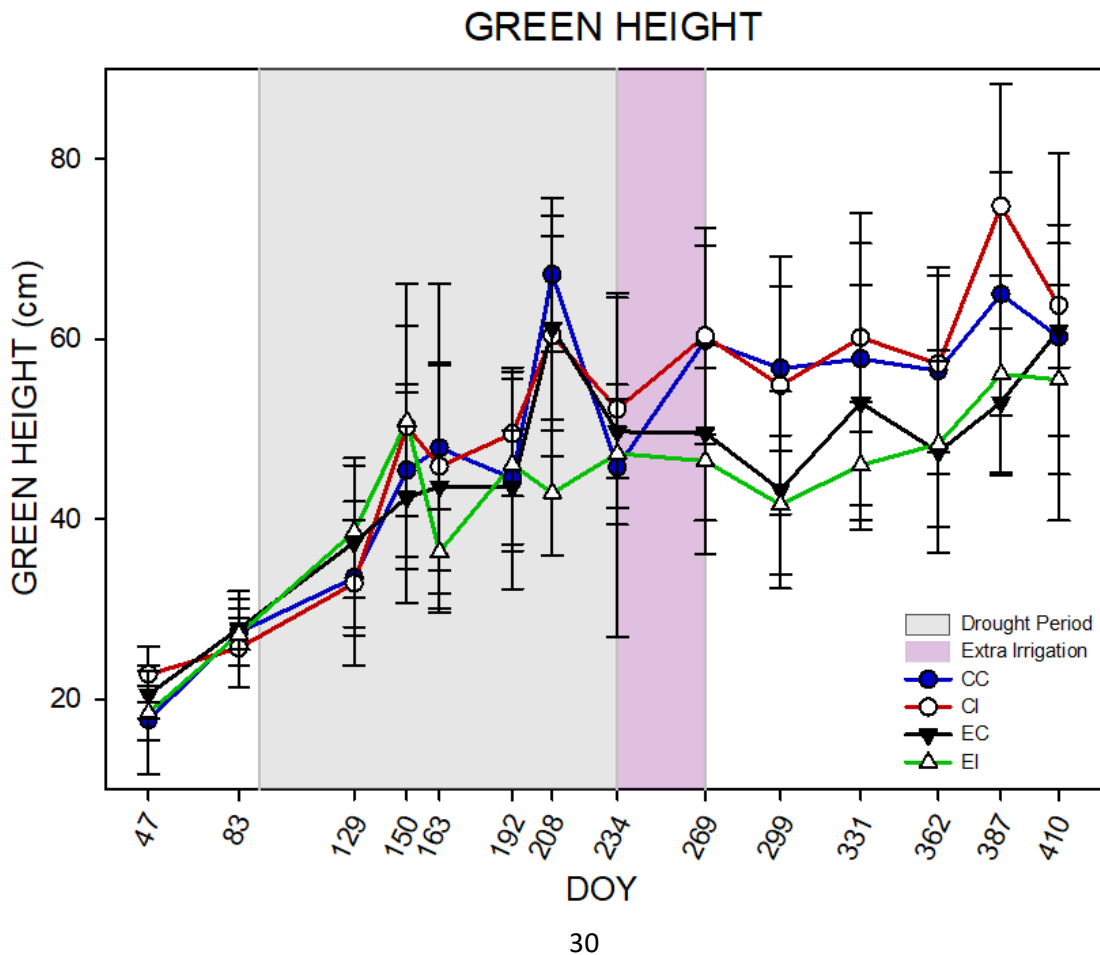


**Figure 10.** Diameter values (mm) for each of the treatments during the 2017 growing season. The gray shaded area is the drought period (EC and EI treatments) and the light purple shaded area is the period of extra irrigation (CI and EI treatments).

Both total and green height showed a gradual increase until the end of May (DOY 150), starting a slowing down process during June (DOY 163, Figures 8 and 9). Although at a slower rate, total and green height continued to increase during the drought period, except for the EI treatment where both heights were stable. From September to December (DOY 255 to 362), total and green height were stable. Overall, the EI and EC treatments showed lower values of total and green height, compared with the CC and CI treatments that were always on a higher level (Figures 11 and 12).



**Figure 11.** Total height of the pine saplings (in cm) for all treatments during the 2017 growing season. The gray shaded area is the drought period (EC and EI treatments) and the light purple shaded area is the period of extra irrigation (CI and EI treatments).





**Figure 12.** Green height of the pine saplings (in cm) for all treatments during the 2017 growing season. The gray shaded area is the drought period (EC and EI treatments) and the light purple shaded area is the period of extra irrigation (CI and EI treatments).

A two-way ANOVA was performed comparing the growth parameters (diameter, total and green height) taking into account the days of the year (DOY) and the treatments (CC, CI, EC and EI). The results showed differences in DOY and treatment for the diameter, total and green height with a  $p < 0.001$ , but no interaction between DOY and Treatment (Table 5).

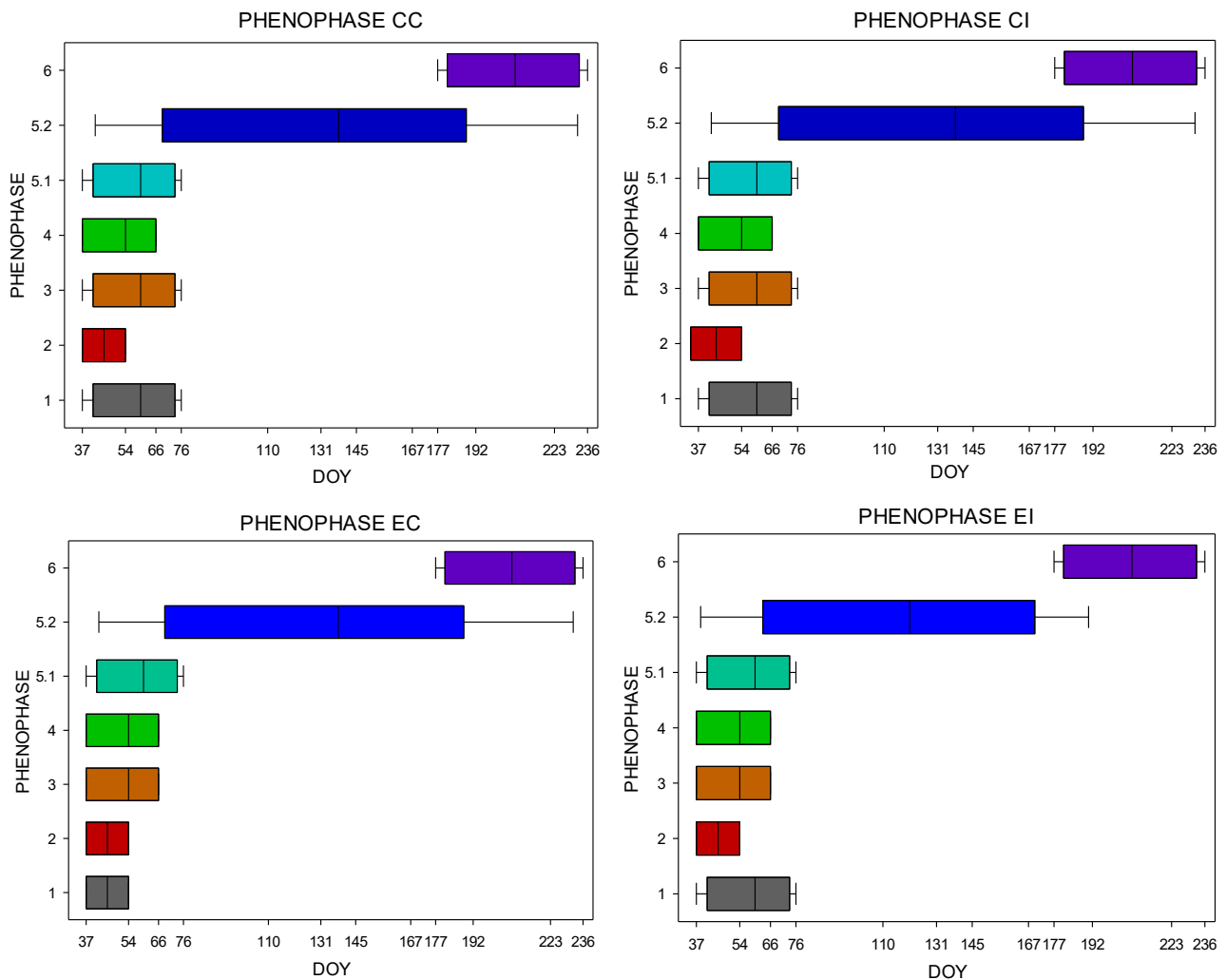
**Table 5.** Two-way ANOVA analysis of growth parameters of maritime pine saplings using day of year (DOY) and treatment as factors.

Parameters	Factors	F	<i>p</i>
<b>Diameter</b>	DOY	11.511	<0.001
	Treatment	100.888	<0.001
	Treatment x DOY	1.029	0.426
<b>Total Height</b>	DOY	8.899	<0.001
	Treatment	25.257	<0.001
	Treatment x DOY	1.105	0.312
<b>Green Height</b>	DOY	10.013	<0.001
	Treatment	28.451	<0.001
	Treatment x DOY	1.217	0.174

### Phenology

In the beginning of the experiment, in the first phenological observation (DOY 37), the saplings were already in different phenological phases (for phenological phase description, see Table 2). There were saplings in almost every phenological stage except for Stage 6. Throughout the course of the experiment (DOY 76-192) the saplings developed from stage 1 to 5.2 until the month of July (DOY 192). In the last month that phenology was monitored, which was August (DOY 223-236), the saplings were in the last defined stage of

phenophase (stage 6), meaning that the meristems were fully developed. All four treatments presented a similar phenological development. The phenophase that lasted longer was phase 5.2 and the one presenting the shorter duration was phase 2 for all treatments.

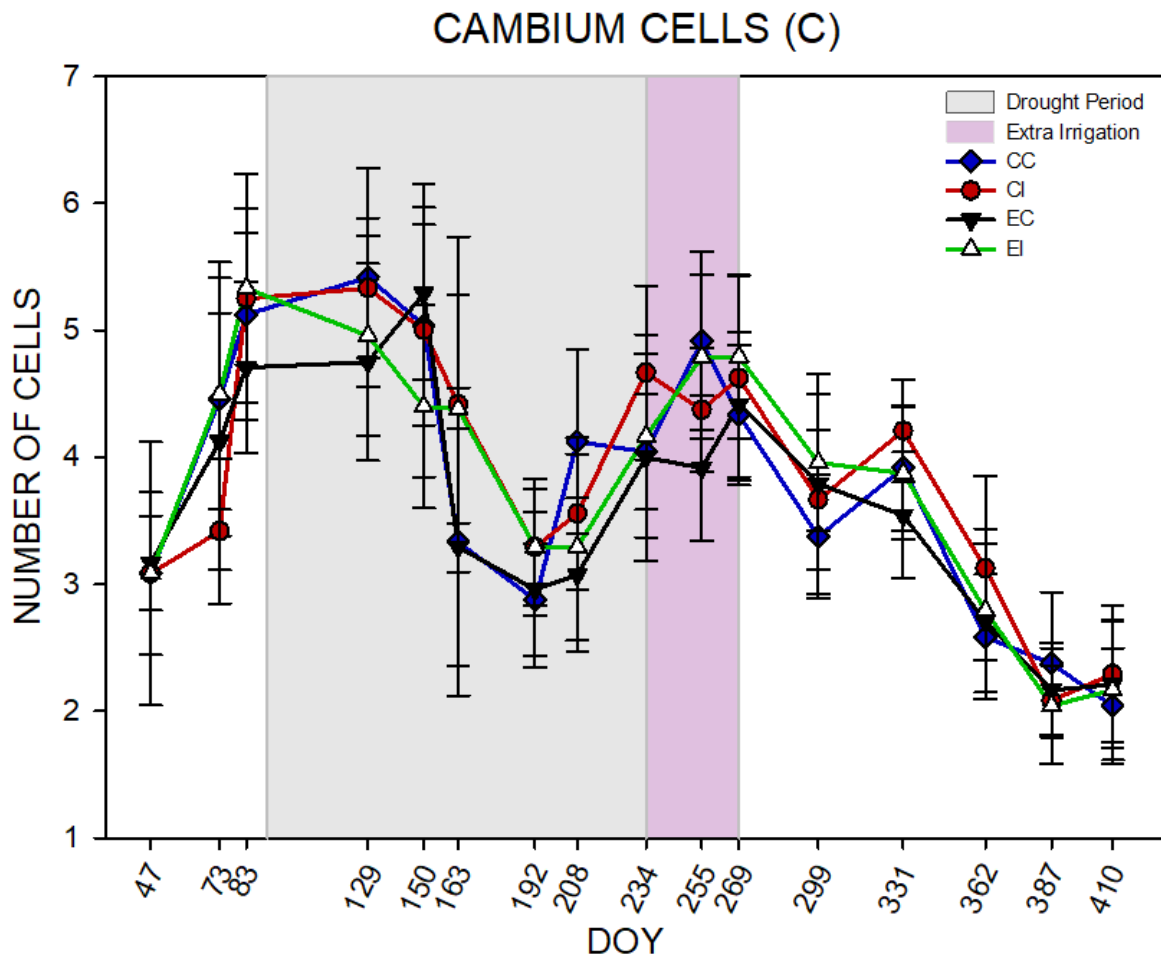


**Figure 13.** Phenological development of maritime saplings in all four treatments (CC, CI, EC and EI) in days of the year (DOY). For phenological phase description see Table 2.

### Xylogenesis

In the beginning of the experiment (DOY 47), the cambium cells were still dormant with an average of three cells in all treatments (Figure 14). With the start of the growing season, in March, the number of cambial cells started to increase, followed by the appearance of cells in enlargement and lignification (DOY 73). In that same period, it was observed new xylem mature cells, an average of ten cells, in all four treatments. The number of cambial cells changed from an average of 3 to 6, between February and May (DOY 47 to

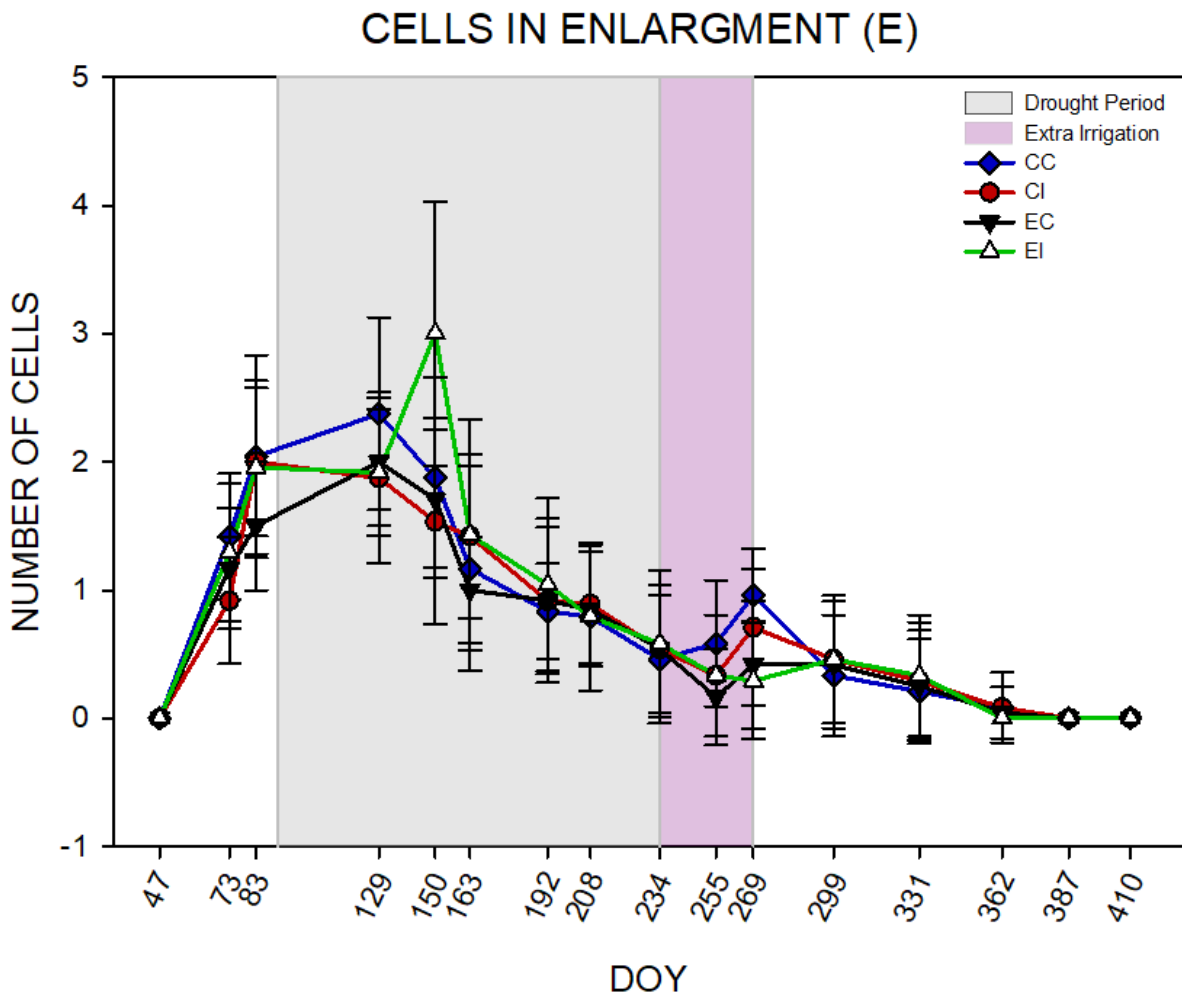
150, Figure 14). During the drought period (from June to July, DOY 163-192) the number of cambial cells reduced to 3, followed by a slight increase to up to 5 cells from the end of July until the middle of September (DOY 208-269). From September onwards, the number of cambial cells was reduced to 2 cells. The four treatments followed a similar pattern of the seasonal variation in the number of cambial cells, and all treatments ended with the same number of cambial cells in the end of the experiment (DOY 410, Figure 14).



**Figure 14.** Number of cambial cells (C) produced by the maritime pine saplings of all four treatments during the course of the experiment (DOY). The gray shaded area is the drought period (EC and EI treatments) and the light purple shaded area is the period of extra irrigation (CI and EI treatments).

The number of cells in the enlargement phase showed a smooth but steady increase until the beginning of May (DOY 129). Afterwards, the number of enlarging cells started to decrease until September (DOY 255). In September, corresponding to the irrigation period,

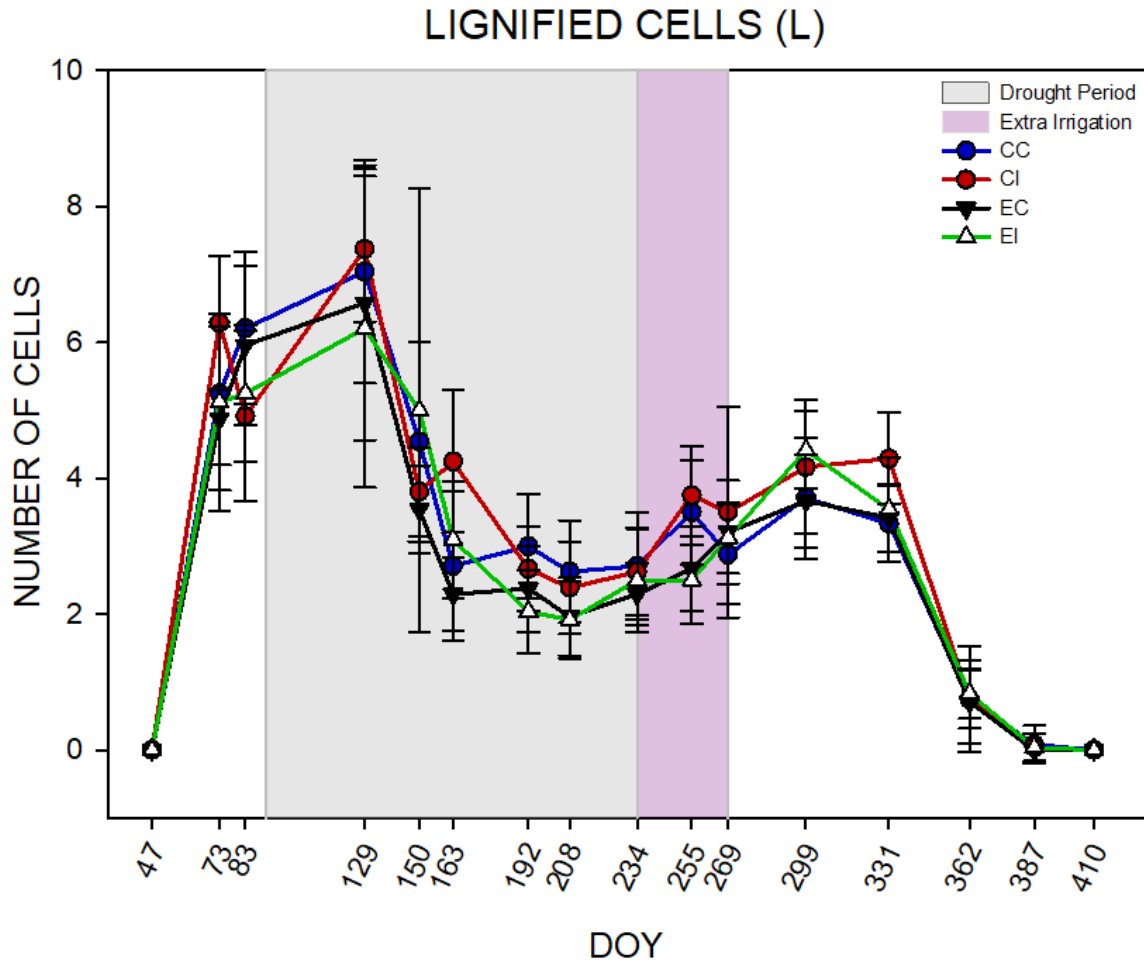
the number of enlarging cells presents an increase. In December (DOY 362) there were no longer cells in enlargement (Figure 15). The EI treatment showed a higher number of cells in enlargement in the end of May, followed by a decrease until the middle of July (Figure 15). From here on, the number of enlarging cells in EI treatment stabilized and presented the same variation as the rest of the treatments.



**Figure 15.** Number of enlarged xylem cells (E) produced by the maritime pine saplings during the course of the experiment for all the four treatments, in DOY. The gray shaded area is the drought period (EC and EI treatments) and the light purple shaded area is the period of extra irrigation (CI and EI treatments).

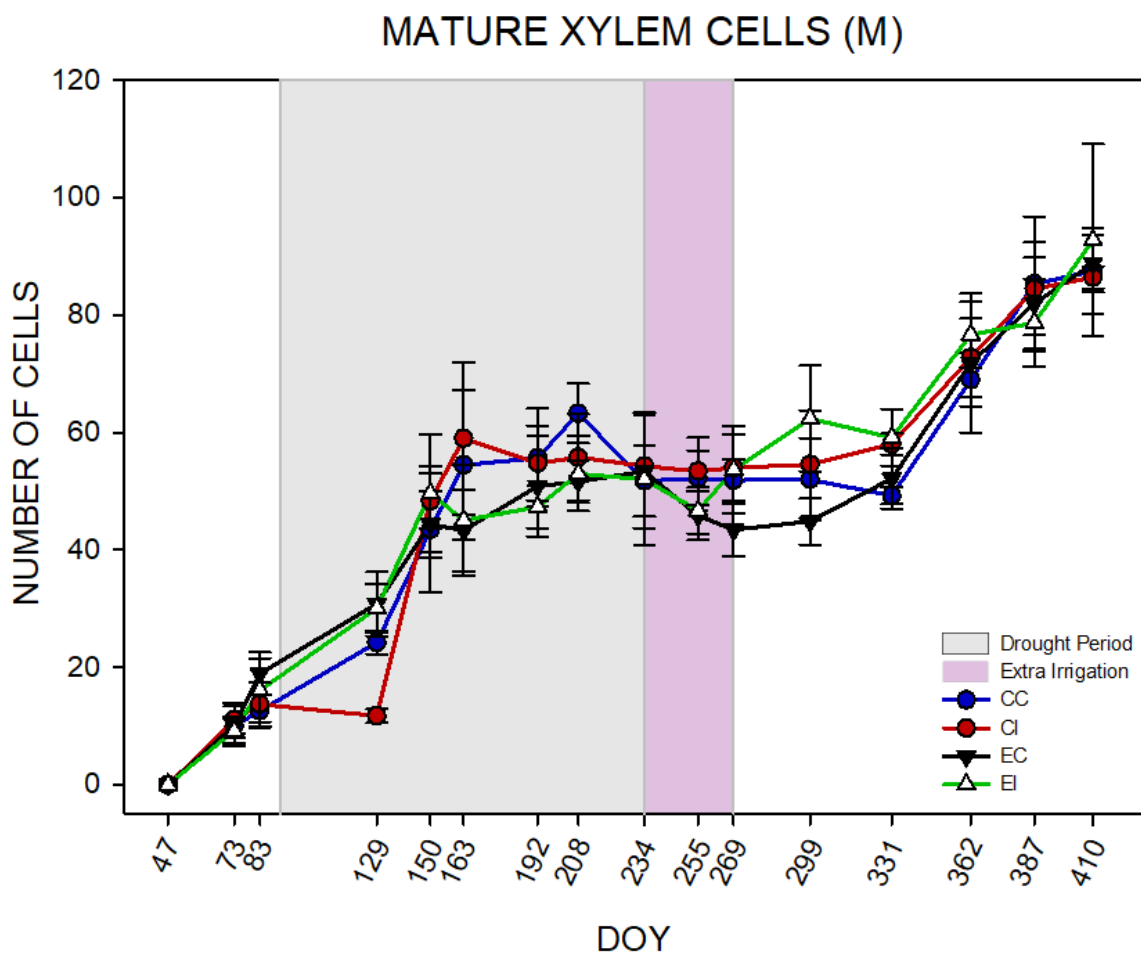
For the cells in cell wall lignification, these showed a steep increase from February to March (DOY 47-73) followed by a smoother increasing trend until the beginning of May (DOY 129, Figure 16). Afterwards there was a declining trend until June (DOY 163) followed by a smaller increase until the end of November (DOY 331). From there on, the number of

lignifying cells drastically decreased until there were no cells in lignification (DOY 387). No significant differences were observed in the number of lignifying cells between treatments.



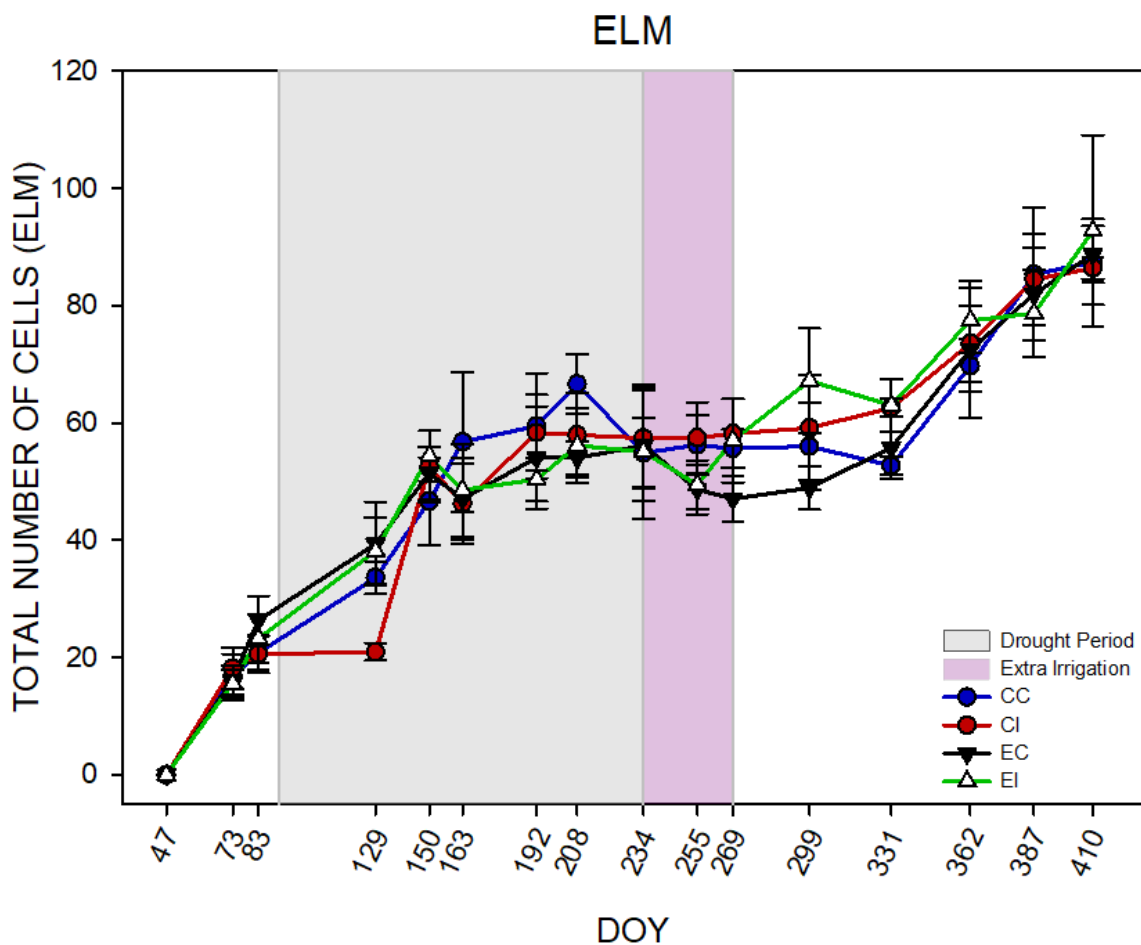
**Figure 16.** Number of xylem cells in lignification process (L) produced by the maritime pine saplings during the course of the experience, for all the treatments, in DOY. The gray shaded area is the drought period (EC and EI treatments) and the light purple shaded area is the period of extra irrigation (CI and EI treatments).

The number of mature cells showed a steady increase until the end of July (DOY 208), followed by a steady state throughout the rest of the summer, until the end of September (DOY 269). A new increase was observed especially from the end of October until the end of the experiment, in February of 2018 (DOY 299 – 410). All treatments showed a similar seasonal pattern. From September (DOY 255) to October (DOY 299), the EC treatment showed a lower number of mature cells, compared with the EI treatment, but by the end of the growing season both treatments showed a similar number of mature cells (Figure 17).



**Figure 17.** Number of mature xylem cells (M) produced by the maritime pine saplings throughout the course of the experiment, in all four treatments, in DOY. The gray shaded area is the drought period (EC and EI treatments) and the light purple shaded area is the period of extra irrigation (CI and EI treatments).

To verify the growth pattern of the xylem cells in a more detailed perspective of the number of formed xylem cells, a graph was made with the sum of the enlarging (E), lignifying (L) and mature cells (M), Figure 18. Here, it can be observed that the pattern is similar to the one exhibited by mature xylem cells (Figure 17), with an increase until the end of July (DOY 208) followed by a stabilization through the rest of the summer months. Next, another increase is verified from October until the end of the experiment (DOY 299-410). A bimodal pattern is verified on this graphical representation, with two major peaks being observed. One from the beginning of the experiment until the drought period (DOY 47-150) and the other after the drought period (DOY 331-410).



**Figure 18.** Graphical representation of the sum of enlarged (E), lignified (L) and mature xylem cells (M) in days of the year (DOY). In gray is the drought period simulated to the saplings. In light purple is the period of extra irrigation for the CI and EI treatments.

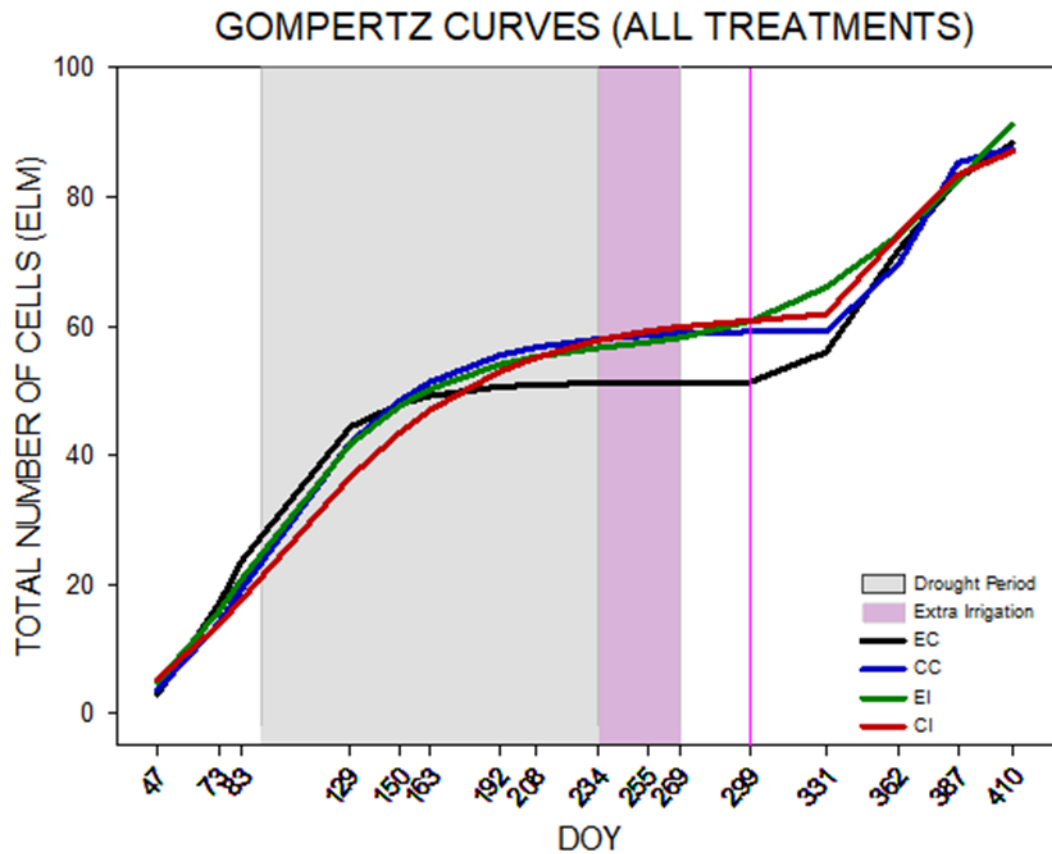
Due to the presence of a bimodal pattern, two Gompertz functions were fitted to the data. Function 1 is from the beginning of the experiment until October 2017 (DOY 47-299) and after that (DOY 299-410). The parameters that define the Gompertz equation were separated,  $A_1$ ,  $\beta_1$  and  $k_1$  correspond to the first curve and  $A_2$ ,  $\beta_2$  and  $k_2$  to the second curve (Table 6).

**Table 6.** Values of the Gompertz functions parameters.  $A$  represents the upper asymptote of the total number of ELM cells,  $\beta$  the inflection point and  $k$  the growth rate parameter. One (1) and two (2), represent the two Gompertz functions.

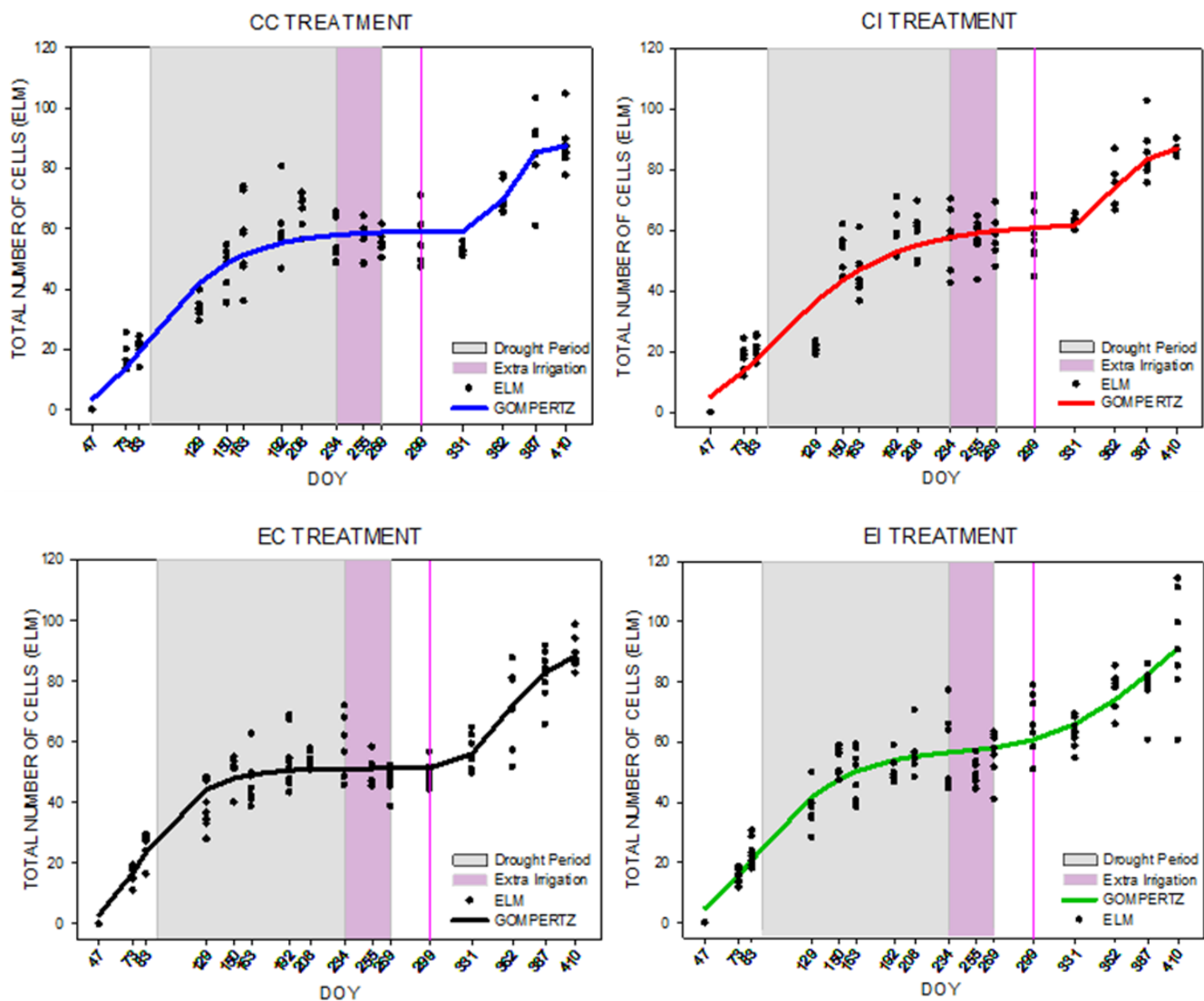
Treatment	A1	A2	k1	k2	$\beta_1$	$\beta_2$
CC	54.42	83.03	0.03	0.09	87.50	361.89
CI	62.20	90.29	0.02	0.05	95.04	355.85
EC	51.35	93.11	0.04	0.03	75.89	352.44
EI	57.87	102.99	0.02	0.01	83.65	421.04

The Gompertz functions fitted the data adequately, reaching an asymptote in the end. Through the observation of the statistical results, in the first Gompertz curve, which goes until the end of October 2017 (DOY 299), the highest number of cells (62.20) and inflection point (95.04) were present in the CI treatment, as the CC treatment presented the lowest number of cells (54.42). The exclusion treatments presented fewer number of cells especially the EC treatment (EC, 51.35; EI, 57.87 – Table 6). Still on the first curve, in general, the control treatments presented lower number of cells than the treatments who received extra irrigation, which was before DOY 299 (Table 6, Figure 19 and 20). In the end of the growing season, the EI treatment stood out as the treatment with the highest number of cells (102.99) and with the highest inflection point (421.04) comparing with the other treatments, where the lowest number of cells was present again on the CC treatment (83.03), despite this treatment having the highest growth rate (0.09), (Table 6, Figure 19 and 20).





**Figure 19.** Graphical representation of the Gompertz functions for ELM cells (enlarged + lignified + mature cells) of all treatments. The lines represent the Gompertz curves for each treatment. The pink vertical line represents the separation of the two Gompertz curves (DOY 299). The gray shaded area is the drought period (EC and EI treatments) and the light purple shaded area is the period of extra irrigation (CI and EI treatments).

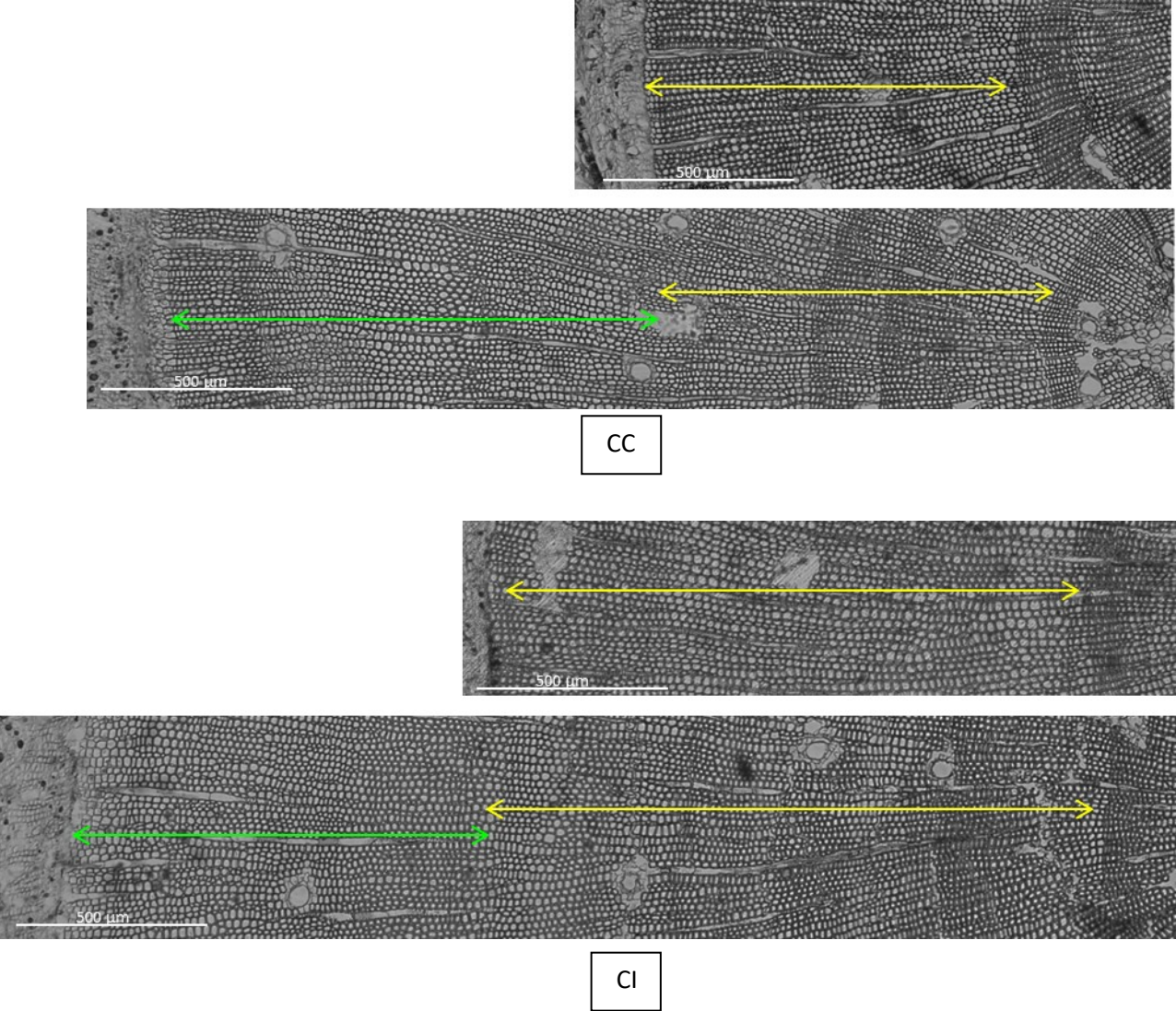


**Figure 20.** Graphical representation of the Gompertz functions for ELM cells (enlarged + lignified + mature cells) for each treatment. The dots represent the number of ELM cells and the line represents the Gompertz curve of the respective treatment. The pink vertical line represents the separation of the two Gompertz curves (DOY 299). The gray shaded area is the drought period (EC and EI treatments) and the light purple shaded area is the period of extra irrigation (CI and EI treatments).

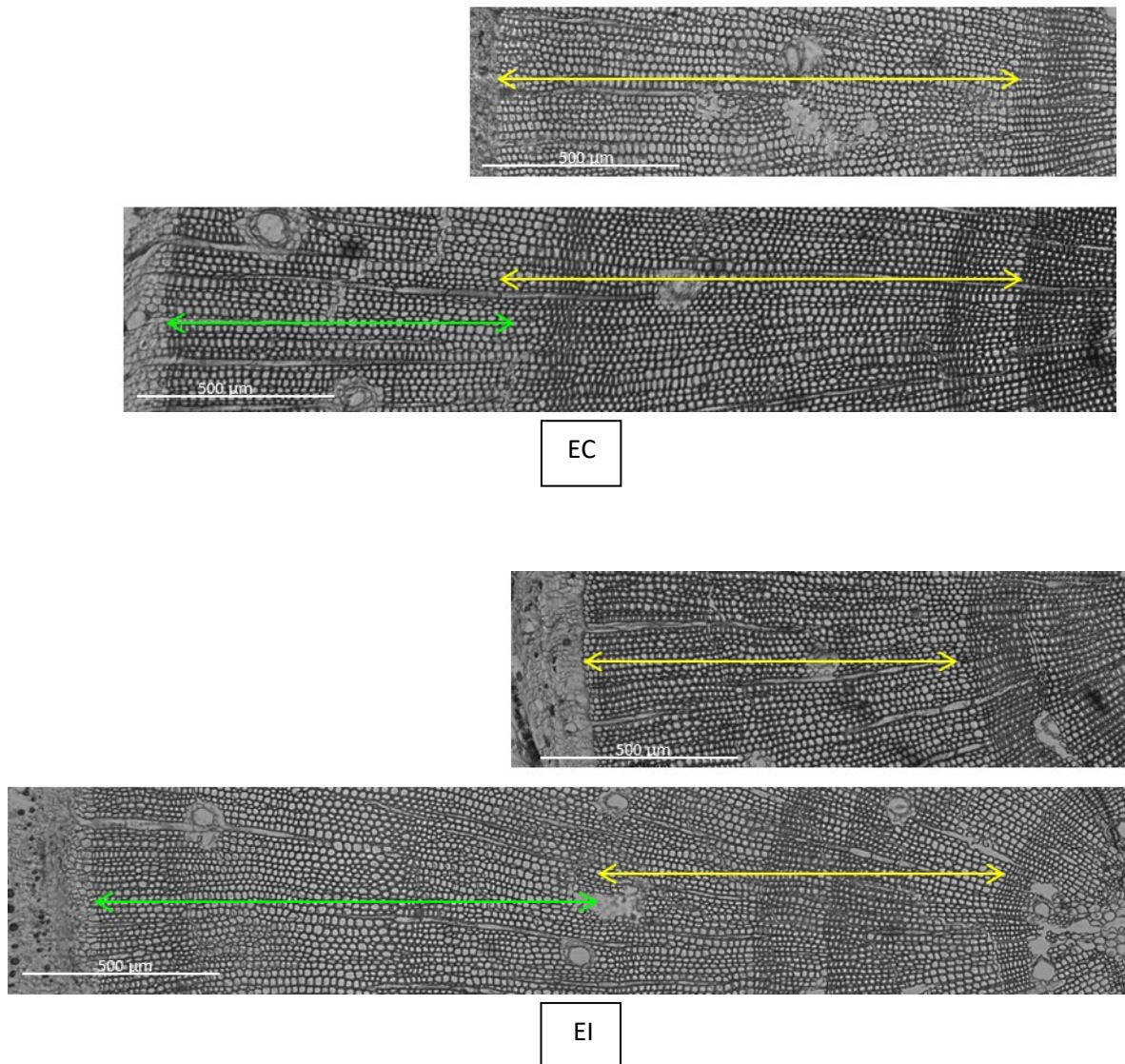
### Characterization of the tree-rings produced by the saplings

A qualitative comparison of the tree-ring formed on each treatment, in August (DOY 234, Figures 21 and 22, yellow arrow), after drought and before the extra irrigation and at the end of the experiment (DOY 410, figures 21 and 22, green arrow), showed that in the extra irrigated treatments (CI and EI) presented a bigger number and larger tracheids produced after the drought period (Figure 21 and 22, green arrow), when compared with

the respective control treatments (CC and EC, Figure 21 and 22). It was also observed that the tree-rings of all treatments had intra-annual density fluctuations (IADFs).



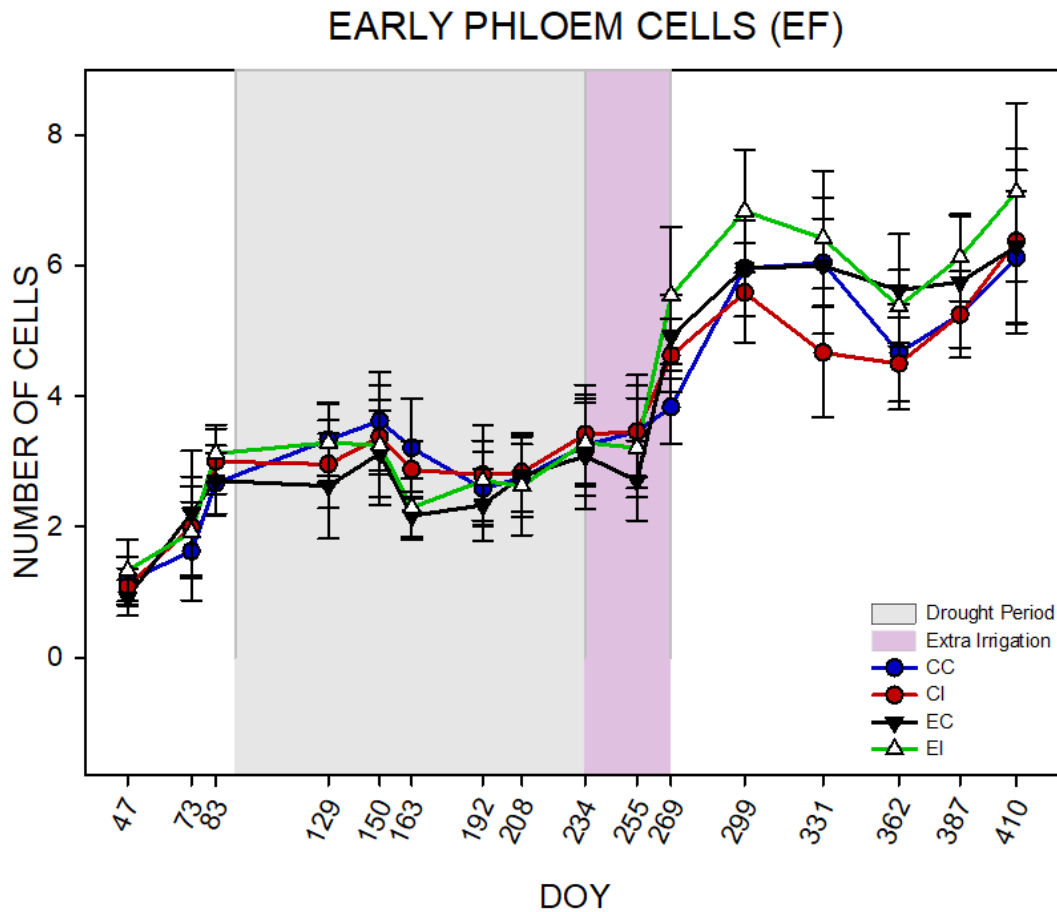
**Figure 21.** Microscopical images of the tree rings of CC and CI treatments. Top picture was taken in August, DOY 234, and bottom picture at the end of the growing season (DOY 410). The yellow arrow represents the growth before the irrigation treatment and the green arrow the growth after irrigation.



**Figure 22.** Microscopical images of the tree rings of EC and EI treatments. Top picture was taken in August, DOY 234, and bottom picture at the end of the growing season (DOY 410). The yellow arrow represents the growth before the irrigation treatment and the green arrow the growth after irrigation.

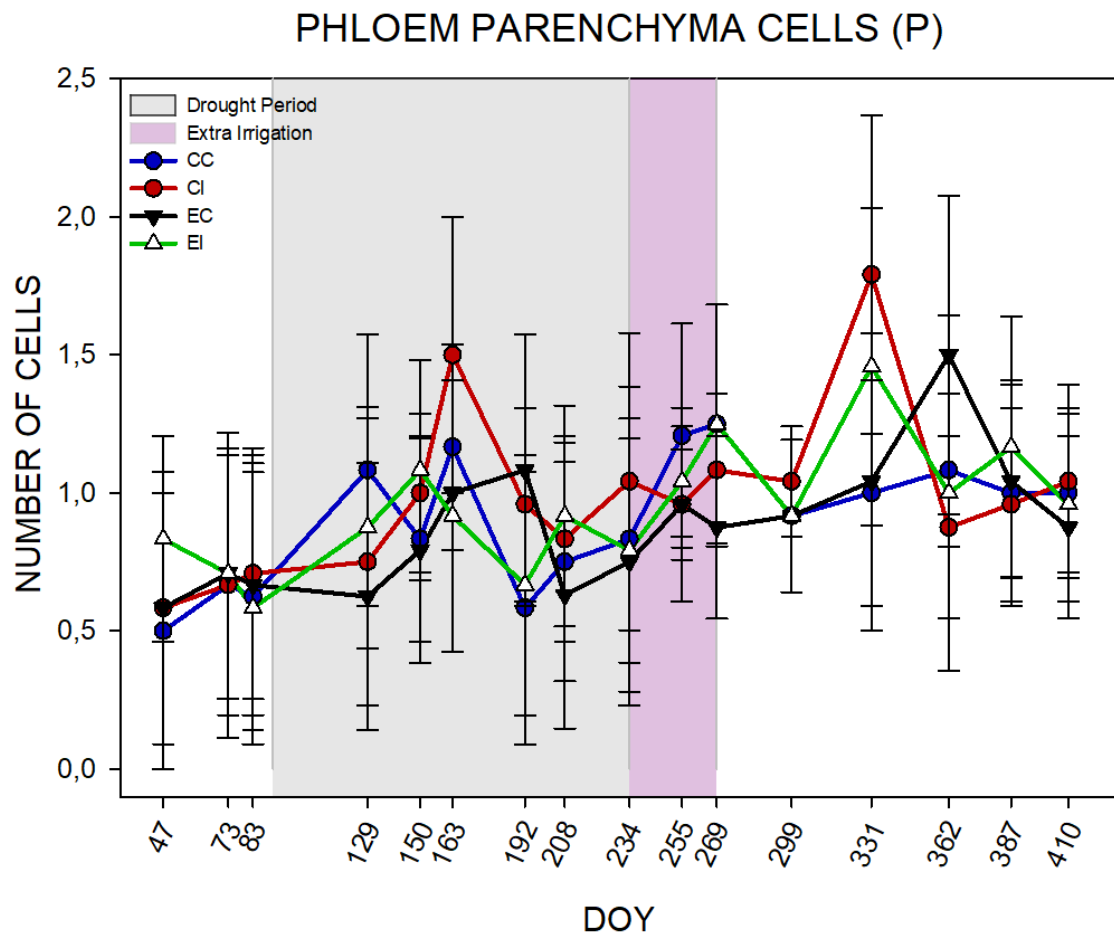
### Phloem phenology

From the beginning of the experiment until the end of May (DOY 150), there was a slight increase in the number of early phloem cells (EF), afterwards, in the experimental drought period, that number stabilized. In September (DOY 255-269), the number of early phloem cells increased, this increase was higher on the EI treatment. Until the end of the experience (DOY 410), the number of cells stabilized between 5 and 7 (Figure 23).



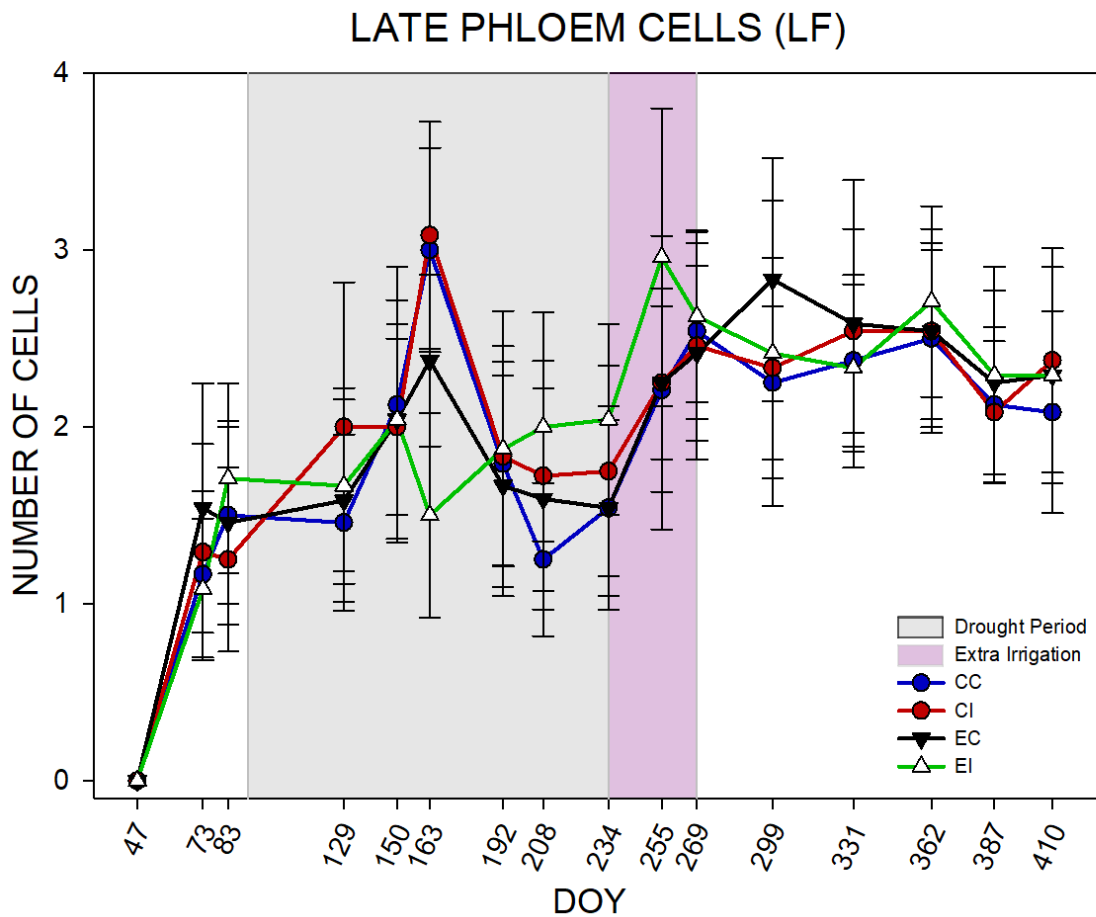
**Figure 23.** Number of early phloem cells (EF) produced by the maritime pine saplings throughout the course of the experiment, for all treatments. The gray shaded area is the drought period (EC and EI treatments) and the light purple shaded area is the period of extra irrigation (CI and EI treatments).

For the parenchyma cells of the phloem, its number was between 1 or 2 cells, with small variations during the course of the experiment (Figure 23). The CI treatment, however had a slight peak in June (DOY 163) and in November (DOY 331) (Figure 24).



**Figure 24.** Number of parenchyma cells of the phloem (P), in all four treatments, produced by the maritime pine saplings during the course of the experiment. The gray shaded area is the drought period (EC and EI treatments) and the light purple shaded area is the period of extra irrigation (CI and EI treatments).

The number of late phloem cells (LP) increased until June (DOY 150), with the exception of the EI treatment where the number of late phloem cells decreased on that month. After that, through the summer months and experimental drought period, the number of LP cells stabilized in all treatments and proceeded to have a new increase, although smaller through September and October (DOY 255-299). In the rest of the months and until the end of the experiment (DOY 331-410) the number of cells stabilized once again between 2 and 3 late phloem cells (Figure 25).



**Figure 25.** Number of late phloem cells (L) produced by all four treatments of maritime pine saplings through the course of the experiment. The gray shaded area is the drought period (EC and EI treatments) and the light purple shaded area is the period of extra irrigation (CI and EI treatments).

## DISCUSSION

This experiment reports the results of a one-year water manipulation experiment in 2-year-old maritime pine saplings. The saplings were divided in four treatments, control-control (CC), control-irrigation (CI), exclusion-control (EC) and exclusion-irrigation (EI). The exclusion treatment consisted in a reduction of water from April to August and the irrigation treatment in providing extra irrigation in September. The main differences observed between treatments were on the growth parameters (height and diameter), with EC and EI showing lower values of height and diameter by the end of the experiment. The dynamics of cambial activity, xylem and phloem differentiation did not present significant differences between treatments, although the tracheids formed after the drought period appeared to present differences between treatments.

At the start of the experiment, diameter, total height and green height of the saplings were similar between treatments. After the drought period, the saplings from the exclusion treatments (EC and EI) presented lower diameters and heights than those in the control treatments (CC and CI), showing that the water exclusion experiment had an impact on these growth parameters. In all treatments, diameter increment stabilized during the drought period but the increment in total and green height continued, mainly in July and August (DOY 192 to 234). Under drought conditions, maritime pine adopts an isohydric behavior, closing its stomata to reduce transpiration and avoid hydraulic failure (McDowell et al., 2008; Vieira et al., 2013). This strategy reduces carbon uptake, carbohydrate availability and photosynthesis, thus reducing growth (Oribe et al., 2003; Vieira et al., 2013). Radial increment was affected by the drought period, indicating that the saplings allocated their resources towards primary growth. Bergeron et al. (2004), using *Picea mariana* seedlings showed that reducing the quantity of irrigated water did not significantly affected height growth or the physiology of the seedlings, as the xylem conductive tissues have the capacity to store water. In this case, seedling growth is not compromised with lower quantity of water available. Gruber et al. (2010) affirms that radial growth is strongly limited by drought, especially during spring, and that cambial activity and cell differentiation are not.



Phenology was not influenced by water availability as all treatments presented a similar phenological development. Kramer et al. (2000), using *Pinus pinaster* described that in Mediterranean coniferous forests, water availability affects more leaf area and needle elongation (as this depends on the quantity of water present in the soil) than the timing of the phenological events. A 3-year rainfall exclusion experiment using *Pinus sylvestris*, showed that water exclusion had no effect on phenology, suggesting that it has more impact in cambial activity rather than leaf phenology (De Uña et al., 2017). Probably a higher intensity of drought is required to affect and/or change leaf phenology.

Regarding xylogenesis, a bimodal growth pattern was observed in all treatments. The bimodal growth pattern consists of two distinctive growth periods (one in spring and another in autumn), separated by a quiescent period in summer, very common in the Mediterranean vegetation (Cherubini et al., 2003; Vieira et al., 2014; Pacheco et al., 2017; Vieira et al., 2017). The first growth period was observed from the beginning of the experiment until the drought period, and the second one after the drought period/extra irrigation and until the end of the experiment. The effect of the water exclusion is not immediately perceptible on the xylogenesis, as the main differences between treatments only occurred after the irrigation. Comparing the total number of cells (ELM, Figure 18) between treatments it is possible to see that EC and EI treatments present the most differences. Saplings from the EI treatment responded to the irrigation treatment by recuperating faster and presenting a higher cell production after irrigation than saplings in the EC treatment. Water availability is crucial for radial growth and plays a fundamental role in the second growth period, after the drought period (De Luis et al., 2011a; De Luis et al., 2011b; Vieira et al., 2014). Cell enlargement is an important process for the xylem's tracheary elements (size and growth) and constitutes the first stage of plant cell differentiation (Rathgeber et al., 2016). Water availability, hormone regulation and turgor pressure are the principal factors regulating this process. If there is water stress, it affects the enlarging process (the increase of cell volume and growth) and the following processes of cell division (Rathgeber et al., 2016). In the end of the experiment, the number of tracheids formed was similar between treatments, indicating that maritime pine saplings can recuperate from a drought period, even if there is not a post-drought irrigation. There were differences on the diameter but not on the number of cells. Gruber et al. (2010), refers that

radial growth is highly limited by drought during spring but, despite the dynamics of cell differentiation and its duration is influenced by water availability, the onset of cambial activity and cell production is controlled by temperature. The saplings started cambial activity early due to favorable conditions prevailed at the beginning of the growing season.

In the first sampling, on February 2017 (DOY 47), there were no differentiating xylem cells (enlarged, lignified and mature), as the cambium was still dormant. However, in the following sampling, in March (DOY 73), there were already cells in enlargement, lignification and even some mature xylem cells. This shows that within a short time period (26 days), cell differentiation was complete. A xylogenesis study made by Vieira et al. (2014) using adult *Pinus pinaster* trees, points out that the first mature xylem cells were only observed in June, with cambial activity also starting in March. The results here present show that cell production and differentiation is much quicker in saplings compared with adult trees. In another study comparing xylogenesis between saplings/pole and adult trees growing in the same area, Vieira et al. (2018) showed that the rate of tracheid production was much higher in saplings/pole trees, compared with adult trees, with younger trees producing more cells during the growing season. This agrees with the results here present.

Although in the end of the growing season there were no differences between treatments in the number of tracheids, the impact of the extra irrigation given to the EI and CI treatment was visible in the number and anatomy of the tracheids produced immediately after the irrigation. In the second period of growth, saplings in the EI and CI treatments presented a slightly higher number of tracheids (Figure 21). This suggests that the saplings responded to the extra irrigation by producing larger tracheids after the drought period. The control treatments (CC and EC), despite also having produced new tracheids after the drought period, formed fewer tracheids due to a less amount of available water. Vieira et al. (2017), in a rain exclusion experiment, refers that the trees with less water availability, produced less tracheids with a narrower lumen area, which agrees with our results. In natural conditions, if the weather conditions after drought are favorable, namely high precipitation and mild temperatures, it induces a second growth period, either in saplings and trees' growing in the Mediterranean region (Camarero et al., 2010; Vieira et al., 2017).

It was also observed that all treatments showed intra-annual density fluctuations (IADFs). IADFs are characterized by the presence of latewood-like cells within the earlywood

and/or earlywood-like cells within latewood (Campelo et al., 2007), and are anatomical indicators of the climatic conditions within the growing season. The IADF associated with a second growth period, with earlywood like cells within latewood, could be more evident in the CI and EI treatments, and could be associated to the extra irrigation. If indeed there are differences between irrigation and control treatments in terms of the anatomy, size and number of tracheids after irrigation, it could be an important topic to explore, since it would reflect the impact of climatic variations on tree-rings. The IADFs associated with the drought period, that would induce latewood-like cells within earlywood, are not so clearly distinguished among treatments, as the CC treatment itself shows IADFs. Thus, further studies are necessary to understand the patterns and climatic signal of the IADFs found. A tracheidogram would allow a more quantitative and comparable analysis between the several treatments, by allowing to analyze and compare lumen diameter and cell wall thickness between treatments.

Regarding the phloem cells, the saplings in the exclusion treatments (EC and EI) produced more early-phloem (EF) after the drought period comparing with the rest of the treatments. The formation of the phloem as well as of the xylem is progressive. This means that, if the plant is forming late phloem cells, it will not increase the number of cells in early phloem. But, since after the drought, occurred a second growth peak, and if the cambium was active, the production of early-phloem cells could have occurred. A similar pattern was observed for the phloem parenchyma cells, where after drought and extra irrigation, a peak occurs for CI and EI and later for the treatments that did not received extra irrigation (CC and EC). A study made by Gričar et al. (2015), in a boreal forest, refers that in general, the number of early phloem cells are positively affected by spring precipitation, and late phloem cells by autumn precipitation. Gričar et al. (2009) explains that phloem cells are important for the long-term survival of trees due to their role in the conduction of assimilates in the sieve cells, and this ability depends on their number and size. However, the production of phloem cells seems to be less dependent on environmental factors than xylem cells (Gričar & Čufar, 2008). Phloem cells are still difficult to study because they are more fragile structurally so further studies and new protocols are needed to comprehend more deeply the phloem's development and role under stressful conditions.

Regardless of being subjected to an intense drought episode, the two-year-old saplings resisted to the low amounts of water supplied, with no mortalities registered, and no differences in the xylogenesis and phloem cell production, despite the significant differences on the growth parameters (diameter and height). A recent study made by Bachofen et al. (2017), where they simulated four months of drought during two consecutive years, concluded that 2 and 3-year-old *Pinus sylvestris* and *Pinus nigra* can tolerate and potentially acclimatize to repeated summer droughts. The follow-up of the *P. pinaster* saplings with a second year of drought stress will allow understanding if this species is also able to adjust to repeated drought.

## **FINAL REMARKS**

The final number of tracheids produced did not present differences between treatments, suggesting that the water exclusion treatment was not intensive enough to trigger a response in the number of cells. However, there were significant differences between the height and diameter of trees in the exclusion and control treatments suggesting that the anatomical properties of the tracheids could be responsible for the differences in radial diameter. Additional studies on the anatomical properties (lumen area and cell wall thickness) are necessary to confirm this hypothesis. Regarding the irrigation experiment, the availability of water after drought is crucial for the saplings to resume cambial activity and wood formation, producing wider and more tracheids, that will probably allow an increase in its hydraulic conductivity.

As a future research aim, it would be very interesting to study the response of saplings to recurrent droughts and to intense drought conditions. This could result in larger differences between treatments in the number of xylem and phloem cells and in its anatomical properties due to a memory effect, for example. Overall, the fact that saplings did resist to a drought period is very important for forests restoration purposes because the early life stages of trees are important for the initial establishment of a forest. The results from this experiment show that *Pinus pinaster* saplings can resist to a pronounced and prolonged drought and are thus a suitable forest species to use in reforestations in the Mediterranean area.

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