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DROUGHT RECOVERY OF MARITIME PINE SEEDLINGS (*PINUS PINASTER* AIT.): SHORT AND LONG-TERM EFFECTS

Dissertação no âmbito do Mestrado em Ecologia orientada pela Professora Doutora Maria Cristina Amaral Penas Nabais dos Santos e pela Doutora Núria Garcia-Forner e apresentada departamento Ciências da Vida da Faculdade de Ciências e Tecnologias da Universidade de Coimbra

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Drought recovery of Maritime pine saplings (*Pinus pinaster* Ait.): Short and Long-term effects

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia, realizada sob a orientação científica da Doutora Núria Garcia-Forner (Faculdade de Ciências e Tecnologia da Universidade de Coimbra) e do Professora Doutora Maria Cristina Amaral Penas Nabais dos Santos (Faculdade de Ciências e Tecnologia da Universidade de Coimbra).

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Ana Margarida Fonseca

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Resumo

O principal objetivo deste trabalho foi avaliar os diferentes constituintes da resiliência relativamente à seca em plântulas de *Pinus pinaster* Aiton para tentar compreender resposta desta espécie ao stress hídrico, a curto e a longo prazo. Mediram-se vários parâmetros em plântulas de *P. pinaster* sujeitas a stress hídrico, seguido de um período de hidratação: fotossíntese, condutância estomática, transpiração, conteúdo relativo de água nas folhas, potencial hídrico (início da manhã e meio do dia), eficiência no uso da água, e parâmetros de crescimento (altura e diâmetro do caule). Calcularam-se índices de resistência, recuperação e resiliência para estimar a capacidade de recuperação após a seca. Não houve diferenças significativas entre tratamentos, mas apenas diferenças sazonais. Durante o período de stress hídrico, todos os parâmetros apresentaram valores menores comparando com o período antes da ocorrência de stress hídrico. Após rega, as variáveis hidráulicas melhoraram rapidamente, e, somente após 27 dias, as plântulas atingiram os níveis mais altos de fotossíntese, transpiração e condutância estomática. Estes resultados indicam que a limitação hidráulica foi o processo que regulou a recuperação das trocas gasosas. Todos os parâmetros conseguiram recuperar para valores superiores após a irrigação, comparando com o período de seca. No entanto, em geral a resiliência foi baixa, ou seja, no tempo de duração da experiência os parâmetros não recuperaram para os níveis antes do stress hídrico.

Abstract

The main goal of this research was to evaluate the different components of drought resilience in *Pinus pinaster* Aiton saplings to understand this species' ability to cope with stress, in short and long-term periods. Photosynthesis, stomatal conductance, transpiration, relative water content, leaf predawn and midday water potential, water use efficiency and growth parameters (height and diameter) were measured in *Pinus pinaster* saplings under water stress, and after rehydration. During water stress, all variables showed lower values, compared with the pre-drought period. After re-watering, hydraulic variables increased soon after, only after 27 days did saplings reach the highest levels of photosynthesis, transpiration, and stomatal conductance. These results indicate that hydraulic limitation was the process governing gas-exchange recovery from drought. After rewatering all the parameters recovered to higher values compared with the drought period. However, in general, the resilience was low within the time of the experience, with most of the parameters not attaining similar values to the ones before the drought.

Chapter 1 – Introduction

1.1 Climate change

The Earth's climate has been changing throughout the ages. In the last 650,000 years, cycles of glacial have advanced and withdrew. The last ice age came to an end about 7,000 years ago marking the beginning of the modern climate era — and human civilization.

According to the Intergovernmental Panel on Climate Change (IPCC, 2007), climate change refers as “a difference in the state of the climate that can be identified by modifications in the mean and/or the variability of its properties and that persists for an extended period, typically decades or longer”. This phenomenon occurs due to natural internal forcings (processes that are primarily due to interactions within the atmosphere as well as those that involve coupling of the atmosphere with various components of the climate system) or external events, such as modulations of the solar cycles, volcanic eruptions and persistent anthropogenic changes in the composition of the atmosphere or in land use. Climate change can be explained by a gradual increase in global surface temperature, mostly caused by anthropogenic emissions such as carbon dioxide (CO_2) and other greenhouse gases. This is commonly referred to as Global Warming.

Undeniably, there is compelling evidence of changing in precipitation or melting snow and ice, thus altering hydrological systems, affecting water resources quantitatively and qualitatively (IPCC, 2014). Over the last decade, scientists have extensively studied the effects of these changes on natural and human systems. Many species (whether terrestrial, freshwater or marine) have altered their geographic ranges, seasonal activities, migration patterns, abundance and interactions due to ongoing climate change (IPCC, 2014). Many studies covering a wide range of regions, crops and forests show numerous negative impacts of climate change in plant life (e.g.:Walther *et al.*, 2002; Patz *et al.*, 2005; Stern, 2008; Mooney *et al.*, 2009) and raise awareness of the impact on human lives and health, ecosystems, economies, societies, cultures, services and infrastructure.

1.2 Drought

The World Meteorological Organization defines drought as “A deficit of rainfall in respect to the long-term mean, affecting a large area for one or several seasons or years, that drastically reduces primary production in natural ecosystems and rainfed agriculture”. The global average temperature has increased about 0.6°C during the last 100 years and is predicted to continue rising at a rapid rate even under a conservative scenario (Root *et al.*, 2003). The direct and indirect effects of increased temperature, changing rainfall patterns and drought are expected to significantly increase extinction rates although the vulnerability of each individual species depends on the level of exposure to climate change, its sensitivity to that change and ability to adapt.

Global warming will likely reduce water availability, thus increasing frequency and severity of drought stress (Chaves *et al.*, 2002). Drought stress is one of the prime determinants of the distribution of different vegetation types (Woodward, 1987). Thus, it becomes crucial to analyse humidity shortage frequency, severity, and duration for a given historical period to calculate the occurrence probabilities of future drought episodes of varying intensity, duration, and spatial coverage (Wilhite & Glantz, 1985).

The rapid pace of climate change may outstrip the capacity of populations to adapt in regions where droughts are predicted to increase in both duration and severity. In a long-term schedule, widespread forest declines could lead to reductions in net primary productivity of forest ecosystems, loss in biodiversity and changes in its composition and woodland communities (Choat *et al.*, 2012). Mortality of plants because of longer droughts and higher temperatures events has become a major focus of attention recently, with various reports highlighting severe mortality episodes around the globe, thus research on plant response to water stress is becoming increasingly important (Petit *et al.*, 1999). Even though there is a rich literature on plant responses to water stress, our current understanding of the causes of tree mortality is still limited (Allen *et al.*, 2010, McDowell *et al.*, 2008). Predicting how forests will respond to future climate changes hinges on a better understanding in the reaction to drought, at a species level, through physiological mechanisms (Choat *et al.*, 2012).

Several ecosystems may already be responding to climate change (Menzel *et al.*, 2006; Allen *et al.*, 2010). Among others, recovery capacity after disturbance depends on the impact and frequency of past drought episodes (Lloret *et al.* 2011). Under current global warming projections, terrestrial ecosystems could take longer to recover after drought, which could increase the vulnerability of these systems to drought. Thus, identifying the underlying mechanisms that control plants' resilience over time is essential to better understand ecosystems capacity to recover.

1.3 Plant responses to drought

Life evolved in the medium of water. The amount of water available to plants is vital, since it justifies 80-90% of the fresh weight of most herbaceous plant structures and over 50% of the fresh weight of woody plants (Kramer & Boyer, 1995). When plants are not able to withdraw water from the soil, under extreme conditions they can reach the so called wilting point, at which plants can no longer obtain the water necessary to meet their needs, thus, wilting and eventually dying from moisture starvation.

Almost every plant process is affected directly or indirectly by water supply, eventually, compromising their survival. Water shortage affects the soil-to-leaf hydraulic conductance, osmotic potential (Chaves & Pereira, 1992; Tognetti *et al*., 1997; Yordanov *et al*., 2000), photosynthesis, growth and the root-shoot ratio at the whole plant level (Chaves, 1991).

Both low soil water contents and high vapour pressure deficits combined can cause xylem embolism, which is defined as the “blockage of xylem conduits by air emboli due to xylem cavitation” (Tyree & Sperry,1989). The vulnerability of xylem to cavitation is considered as a key factor determining the response of plants to water shortage.

Most land plants are exposed to short or long-term water deficits during their life cycle and tend to develop mechanisms to acclimate and/or adapt to shifting environmental conditions (Akıncı & Lösel, 2012). Under hydric stress, plants have established some strategies to avoid tissue dehydration, maintaining tissue water potential as high as possible, or by tolerating low tissue water potential. Responses to water scarcity are highly complex and involve adaptive changes at diverse levels and time scales. These drought-responses change according to species, provenances and individuals within provenance and with drought severity (Akıncı & Lösel, 2012).

Stomatal closure and leaf growth inhibition are among the earliest responses to drought, protecting the plants from extensive water loss. Significantly decrease of stomatal conductance may occur even before significant decrease in leaf water potential can be detected (Flowers *et al*., 1989; Chaves *et al*., 2002; Liu *et al.*, 2004). The stomata also close in response to decline in leaf turgor and/or water potential, or to a high evaporative demand (Bhattacharjee & Saha, 2014). This is a common response to water deficits that develop quickly or slowly and may result from shoot or root dehydration (Chaves, 1991). Decreases in stomatal conductance constrain internal CO_2 concentration and reduce photosynthetic rates that eventually may cease as water deficits increase. Despite Miyashita *et al.* (2005) showed that photosynthetic rate, transpiration rate and stomatal conductance of kidney bean decreased hand-in-hand with the decrease of soil water content during water stress, Liu *et al.* (2004) showed a delay between photosynthetic and stomatal response to soil drying in potato plants.

Among species, there can be significant differences in response to drought even when their physiological performance under no water stress is similar. Faria *et al*. (1998) compared two evergreen oak species, growing side by side near Évora (Portugal) and found that both species displayed similar net carbon assimilation rates under none or moderate water stress. However, by the end of the dry season, *Quercus ilex* spp. *rotundifolia*, the one with a deeper root system, sustained higher photosynthetic capacity than *Quercus suber*. Sustaining carbon (C) uptake even at expenses or water loss may be critical to survive during drought (Garcia-Forner *et al.* 2016).

In nature, plants can either be subjected to slowly developing water shortage (within days to weeks or months) or face fast short-term water deficits (hours to days) thus, time is also a factor to consider. In fact, plant responses depend on the intensity/duration of the induced stress. Desiccation speed can have totally different results in terms of physiological response or adaptation (McDonald & Davies, 1996). Mitchell *et al*. (2014) divided the drought period into (i) a first phase in which photosynthesis was maximal and growth continued at high rates supported by high hydraulic conductance, (ii) a second phase in which growth ceased while photosynthesis continued only at reduced rates, followed by hydraulic conductance; and (iii) a third phase in which carbon assimilation was absent and hydraulic conductance was completely impaired.

Thus, short-term responses are primarily linked to stomatal regulation, with reduction in water loss by transpiration and maximizing CO_2 intake, whereas medium-term responses include steady-state behaviours across the entire plant system, not only with a decrease in plant growth, but also in CO_2 assimilation. Long-term stress will lead plant to either escape dehydration by shortening their life cycle or optimize their resource gain through acclimation, whether by variation in gene responses, anatomical modifications of specific organs and acquisition of physiological adaptively strategies with the purpose of reducing the overall growth and, therefore, to balance resource utilization (Chaves, M *et al*., 2003) (Figure 1).

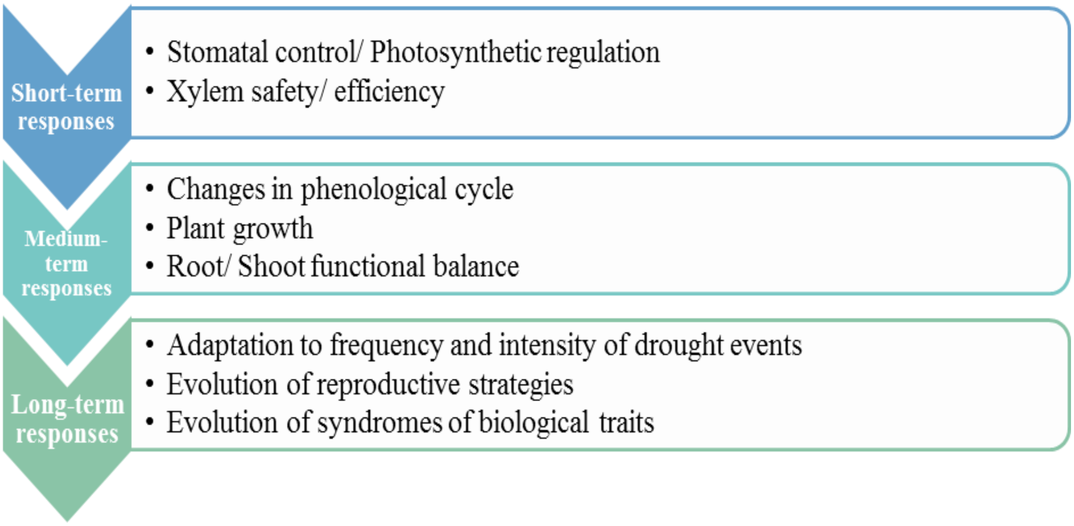


Figure 1- Plant responses to drought in a temporal scale. Based on Mencuccini (2014)

1.4 Plant Recovery, Resistance and Resilience

In nature, sporadic precipitation is essential for maintaining ecosystem structural stability, especially in arid and semi-arid areas. In central North American grasslands, plant richness and growth increased most in wet years followed by dry years (Adler & Levine, 2007). In fact, a small rainfall pulse can induce a rapid response in a desert ecosystem, which quickly triggers plant growth (Reynolds *et al.*, 2004). After re-hydration, drought-stressed individuals recover plant growth, photosynthesis, transpiration, and stomatal conductance (Gallé *et al.*, 2007a). Still, the extent and magnitude of the recovery from re-hydration may depend on drought impact and species resistance as severe stress may irreversibly injury tissues.

At the species level, both drought susceptibility and recovery capacity will depend on the specific trait. Different physiological traits may exhibit major differences in sensitivity – extent and response time – to a pulse of water after drought, however, plant performance results from the complex network of all physiological process. Therefore, to fully understand plants ability to recover from a drought stress it is necessary to consider both carbon and water economies after rewatering, either for a short or long period of time.

Plant resilience broadly defined as the capacity to recover after disturbance, has several components. This involves quantitative estimations of the indicator variable previous, during and after drought. For instance, resistance is the ability to avoid displacement during an environmental disturbance or stress, in other words, the ability of a system to return to its former state following stress. Recovery is defined as a period of time following a stress (for example, rehydration following drought) until a new homeostasis is attained. Thus, it can also be described as the ability of an individual to recover relative to the damage experienced during disturbance. Resilience, often estimated by analysing the impact of disturbance on ecological properties, is the extent to which a parameter is able to return to their equilibrium levels following a disturbance (Pimm 1984).

Recovery of plant function is a gradual process, yet vital for individuals, because if a new drought arrives before full recovery, plants survival could be compromised. The present understanding of drought recovery has generally focused on precipitation that ends a drought by alleviating water deficit as opposed to restoring function in plants. However, ecosystems or individuals' recovery once drought is ameliorated cannot be assumed. Different components and variables need to be evaluated and recovery time is also a critical metric to be considered.

Hydraulic constraints mainly delimit the recruitment niche for any species, and hence defines its recovery from drought (Brodribb & Cochard 2009). However, plants' survival during water stress or their post-drought performance involves an interaction between the hydraulic system and carbon source-sink dynamics during and after the stress. Some studies have also described the recovery of hydraulic conductance following rewatering after drought (Lo Gullo *et al.* 2003; Trifilo *et al.* 2003).

In this study, it was also considered the presence of mycorrhizal fungi and their function in drought recovery. It has been suggested that mycorrhizal hyphae may directly enhance root water uptake, providing adequate water to preserve physiological activity in plants, particularly under severe drought conditions (e.g., Allen, 1982; Faber *et al*., 1991; Read, 1992). For instance, mycorrhizal fungi sometimes increase root density or alter root system morphology, enabling infected plants to explore more soil volume and extract more water than uninfected plants during drought (Davies *et al*., 1996; Kothari *et al*., 1990). Therefore, mycorrhizal plants' may maintain greater cell turgor during water deficits than uninfected plants, and thus, better tolerate and recover from drought.

Considering that projected future climates will display more frequent and intense drought events, a cumulative effect on plant species is expected, especially in conifers in which the hydraulic conductance recovery steers the recovery of fundamental physiological processes such as gas exchange (Brodribb & Cochard 2009). Therefore, apart from the obvious physiological importance, the implications for understanding drought survival and recovery of plants are crucial for the environment.

1.5 Objectives and Hypothesis

The main goal of this research was to evaluate the different components of drought resilience in *Pinus pinaster* Aiton saplings to understand this species' ability to cope with future drought episodes. Plants' behaviour was mainly assessed through the measurement of CO_2 assimilation, stomatal conductance, plant growth and hydraulic performance of *Pinus pinaster* Aiton saplings during drought and after re-watering. The specific aims were: 1) to identify whether recovery and resilience are conditioned by the level of drought induced stress; 2) to evaluate whether resilience and recovery differ at a short and long-term period; 3) to assess if, after rehydration, the amount of water received has an impact on resilience and recovery speed in each measured variable.

I expect lower recovery rates and maybe even non-resilience of plants subjected to a higher drought stress. More specifically, I anticipate lower recovery rates in water related traits but only under higher drought intensity. Under moderate drought stress and non-irreversible damages to the photosynthetic system and/or xylem – cavitation and embolism of tracheids – I hypothesize a parallel recovery pattern of gas-exchange and hydraulics until full recovery.

I expect that increased water availability during rehydration will result in faster recovery from drought under milder stress, but this will not be enough to fulfil water needs at short term when drought stress was more intensive. Only in the long term, all assessed variables are expected to stabilize.

Chapter 2 - Materials and Methods

2.1 Species: *Pinus pinaster* Aiton

The Mediterranean climate is characterized by a hot, dry period in summer and a cool, wet period in winter, as well as by high inter-annual variability. Therefore, natural vegetation of the Mediterranean area has developed an array of adaptations to water stress, resulting in a high diversity of growth forms. In fact, mechanisms of response to environmental stresses include morphological and physiological adaptations. The most common morphological traits are small leaf size (especially in sclerophyllous species), deep root system, thick bark and high sprouting ability. Physiological adaptations include tolerance to tissue dehydration, early spring development of photosynthesis and ability of a complete recovery after a long summer stress period. In the typical Mediterranean zone, forests are composed of broad-leaves, particularly oaks, both evergreen and deciduous, such as *Q. ilex, Q. suber, Q. coccifera, Q. pubescens, Q. cerris, Q. pyrenaica, Q. toza, Q. calliprinos, Q. ithaburensis* and others, or conifers such as *Juniperus* sp, *Pinus halepensis, Pinus brutia, Pinus pinea and Pinus pinaster*.

The distribution of *Pinus pinaster* Aiton, known as maritime pine, goes from Portugal to the mouth of the River Garoone in France. Current geographic distribution is the result of a long history of plantation and cultivation throughout these regions and being uncertain its natural distribution. *P. pinaster* populations are mainly located in coastal areas, although scarcer in the entire coastal region of Cantabria in Spain (Costa *et al*., 2007).

The maritime pine is naturally distributed in the Iberian Peninsula, especially in Portugal, where it is the most abundant conifer and the most important pine species in terms of area planted and economic value (Figueiredo *et al*., 2014). It covers around 1000000 ha that represents 35 % of forested land in Portugal (ICNF, 2013). *P. pinaster* exploitation contributes over the years to the rural economy of the country and its people (Radich & Alves, 2000).

Originally, this species grew in warm and humid regions but, as a result of many years of cultivation and adaptation outside its natural range, now tolerates a broad range of environmental conditions (EUFORGEN, 2009). *P. pinaster* can survive in areas with only 400 mm annual precipitation but grows better in areas with 850 mm mean annual precipitation and at least 75 mm of rainfall between May and September. In its native range it only withstands a maximum of 100 consecutive days without rainfall, but plantations in some areas can tolerate a maximum of 150 days of drought. Desirable temperatures for its growth range between 0 and 12ºC in the winter and between 15 and 26ºC, during summer (CABI, 2014).

2.2 Experimental design

This experiment is part of a long-term greenhouse experience to study the links between wood formation and plant physiological responses performed at the facilities of the University of Coimbra.

The study was conducted in a greenhouse located in *Polo II* of the University of Coimbra (40°10'58.7"N 8°24'52.8"W), Coimbra, Portugal. 1500 *P. pinaster* saplings (~20 cm tall and 2 years-old) from the same population were acquired from Veiga & Silva nursery and transplanted to 8 L pots in October 2017. The substrate was a mixture of blonde peat (0-40 mm), pine bark (0-15 mm) and up to nine months release-fertilizer (annex -technical document-). Plants were well watered for acclimation and to allow root expansion into the pots until March 2017. The experience lasted from March to December 2017.

After measuring saplings basal diameter and height, plants were assigned to one of the four treatments based on a combination of spring-summer and autumn watering regime. The watering regime used was based on the monthly rainfall pattern of Mata Florestal da Tocha (Latitude: 40.375, Longitude: -8.625) where forest plantations of *P. pinaster* trees have been grown during the last centuries. From April to August, half of the saplings were irrigated following the reference rainfall pattern – control – while the other half – exclusion – were subjected to an irrigation reduction of 50% in April and 75% from May to August (Table 1). In September we separated each treatment into control and extra-irrigated saplings (Table 1). The first group followed the same reference irrigation pattern and the extra-irrigated – irrigation – saplings received extra water to raise soil water content to spring levels in 10 days. These combinations resulted in four treatments: control-control (CC), control-irrigation (CI), exclusion-control (EC) and exclusion-irrigation (EI).

A subset of plants from this major experiment were transferred to a greenhouse located on the Botanical Garden of Coimbra (40°12'20.2"N 8°25'14.7"W) from June to October.

32 *Pinus pinaster* saplings, eight plants per combination of spring-summer and autumn treatments, from the main experiment were randomly selected. These groups match the same two treatment combinations that were described above: control + control (CC); control + irrigation (CI), with an extra irrigation from September to October; exclusion + control (EC), with a significant reduction of water irrigation and no extra

irrigation from September onwards; (4) exclusion + irrigation (EI), both treatments considered (Figure 2).

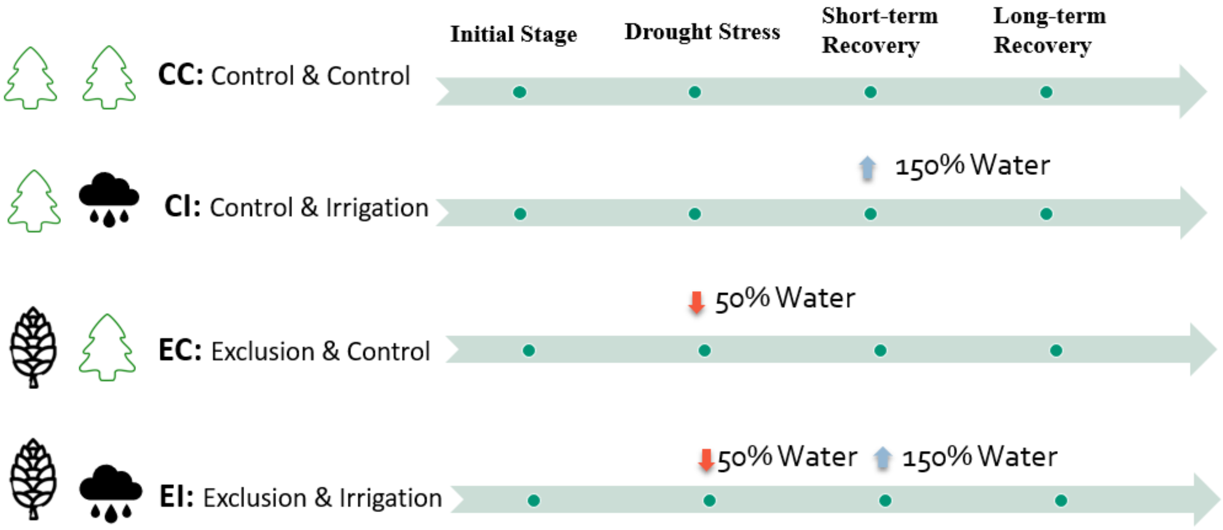


Figure 2 – Experimental design

Table 1. Monthly water applied in spring-summer and autumn treatments (L/m^2)

	CC	CI	EC	EI	Exclusion water
January	132.1	132.1	132.1	132.1	=control
February	87.4	87.4	87.4	87.4	=control
March	71.9	71.9	71.9	71.9	=control
April	81	81	40.5	40.5	-50%
May	63.4	63.4	15.9	15.9	-75%
June	25.3	25.3	6.3	6.3	-75%
July	9	9	2.2	2.2	-75%
August	12	12	3	3	-75%
September	22.2	*	22.2	*	*
October	13.2	43	13.2	43	+31%

**We supplied enough water to achieve soil water content and water potential levels close to those in April 2017, one week before the third campaign. For three days, every sapling was watered hourly during day-time and the soil was allowed to rehydrate from the bottom of the pots making it impossible to quantify the volume of water applied.*

2.3 Soil and plant parameters measured

Soil Water Content (SWC) indicates how much water is present in the soil. Therefore, SWC was measured using 5 cm probes, SM300 Moisture Meter (Delta-T devices Burwell Cambridge, UK.) Each pot was drilled with two holes at ~18 cm from the top soil (11 cm above the base of the pot), to allow SWC horizontal measurements to this depth. Before measuring the SWC, to ensure a good contact between the soil and the moisture probe, the soil from each pot was gently compacted.

Phenology was evaluated on the apical bud and on the number of secondary buds from the top of the plant, every 12 to 18 days. In order to identify the apical bud and its phenophase,a numerical classification scheme was used (Figure 3): (1) bud is dormant and unchanged in size;(2) bud appears to be swelling or growth is observed;(3) needle scales are open due to the swelling of individual leaf bud;(4) there is needle appearance although heterogeneously distributed on the apex; (5.1) Emergence of only 1 per opening and elongation of the apex.; (5.2) Emergence of 2 needles per opening and elongation of the apex; (6.) the apical bud is fully developed and growth has stopped.

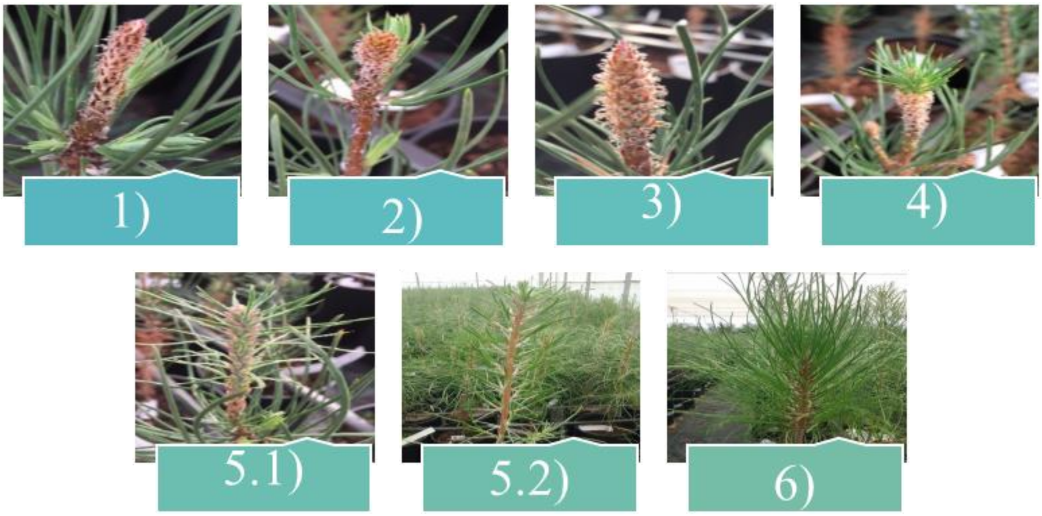


Figure 3 – Phenological stages. See the text for a detailed description of each stage.

For the physiological measurements, data was collected on four campaigns, in total. Two campaigns during drought in: 1) June 28th, and 2) August 18th, milder and intensive drought respectively. All 32 plants were rewatered according to their respective autumn treatment and two more campaigns were carried out: 3) short-term recovery in September 19th and 4) long-term recovery in October 17th. In each campaign, eight individuals per treatment combination were measured. The four physiological campaigns lasted 5 consecutive days during which SWC, plant growth, water potential, and gas-exchange were measured for all trees.

On average plants were 37.90 ± 1.20 cm tall and 4,5 ± 0.03 mm diameter at 2 cm from the topsoil. For a more comprehensive understanding of the morphological progression of pine saplings during the experiment, height and diameter were measured and their relative growth rate (RGR) was calculated at the end of the experiment for all plants and treatments. RGR is the increase in height or diameter relative to the size of the plant present at the start of a given time interval. It also indicates plant strategy concerning its productivity upon disturbance regimes. If two consecutive harvests at times t_1 and t_2 are considered and plant height or diameter are M_1 and M_2 respectively, then RGR is calculated as seen in formula 1:

$$RGR = {(\ln M2 - \ln M1)}/{(t2 - t1)} \tag{1}$$

Water potential (ψ) is regularly used to measure plant water status, an important indicator of physiological water stress (Hsiao, 1973). Water potential is a measure of the resistance pathway for water movement, and is a function of soil water availability, evaporative demand and soil conductivity. It goes from close to zero (negative) in well-watered leaves and fleshy tissue to lower values as tissues dehydrate. Two needles per tree were collected at two times on the same day: before sunrise to measure predawn water potential (ψ_{Pd}) and between 11:30 and 13 h (solar time) to measure midday water potential (ψ_{Md}). Needles were immediately placed in plastic bags and stored in a refrigerator until they were measured (within 1-2 hours) using a Scholander pressure chamber (Manofrigido, Lisboa, Portugal).

Another commonly used indicator of plant water status is relative water content (RWC), although it is more informative under severe water deficits. RWC is the water content (on a percentage basis) relative to the water content of the same tissue at full hydration. To calculate this variable, one needle per seedling was cut upon the harvest of the needles used for the water potential measurements. Afterwards, they were immediately placed in humid plastic bags and kept inside a cooler until processed in the lab within 1 hour. Once in the laboratory, fresh weight (FW) was measured with an electronic balance. Afterwards, all needles were then transferred to test tubes with distilled water and sealed in order to rehydrate tissues for 24 hours (Figure 4). As soon as the weight of the fully hydrated samples (saturated weight, SW) was measured, needles were dried for 48 h at 65°C to get their dry weight (DW). To calculate RWC, the formula 2 was used:

$$\text{RWC}= \frac{((FW-DW)}{(SW-DW)} \text{ x } 100 \tag{2}$$

Figure 4 –Needles placed in test tubes with distilled water for SW measurements

To determine leaf stomatal conductance (g_s), transpiration rates (Tr) and net assimilation rate (A_n), we used an infrared gas-exchange analyser system and the 6400-02B LED Light Source chamber (Li-Cor LI-6400XT Lincoln, Nebraska, USA). Needle measurements were carried out at mid-morning, when highest stomatal conductance could be expected, under the following conditions: 400 ppm of CO_2, 1000 µmol m^{-2} s^{-1} light-saturating photosynthetic photon flux density, block temperature fixed to 20 or 25 ºC depending on the air temperature (to reduce the temperature gradient between inside and outside the leaf chamber), and relative humidity (RH) on full bypass to allow RH fluctuations. In all cases, four needles were placed inside the leaf chamber and measurements were taken once steady state gas exchange had been maintained for at least 2 min. After gas-exchange measurements, measured needles were cut and we measured the real area that was inside the chamber to correct the data.

Water-use efficiency (WUE) refers to the amount of water lost per unite of fixed carbon during photosynthesis. At the leaf level, WUE is calculated as the ratio between A_n and g_s.

For each campaign, mortality was monitored. Although the main goal was to avoid mortality, by the third campaign, 6 saplings succumbed to drought stress. As such, for the third and fourth campaign, the total number of pine saplings was 26.

At the end of the experiment, a destructive sampling was performed. Roots and needles were collected, and the stem was cut into pieces. All plant components were placed in paper bags, properly categorized and were dried for 48 h at 65°C to get their dry weight or biomass.

The ratio between above- and below-ground biomass was calculated.

The roots were analysed for the presence of mycorrhizal fungi to evaluate their potential role on drought recovery. After an abundant rinsing in tap water, the roots were cut in small fractions and were collected in Petri dish. Each section was examined at 35× magnification using a Leica MZ6 Stereozoom Microscope (Leica Microsystems, Heerbrugg,Switzerland) and a gridline millimetre paper to enable the counting of mycorrhizal fungi. The number of mychorrizae was expressed in percentage of root colonization.

2.4. Indices

To evaluate the resilience capacity of *P. pinaster* saplings subjected to different drought stress three indices were calculated for each measured parameter: relative resistance, recovery, and resilience per se.

Resistance is estimated as the ratio between the performance during and before the disturbance. It corresponds to the ratio between the values during the drought and the values during the respective pre-drought period, meaning that the closer the ratio reaches to 1, the greater the resistance to drought stress.

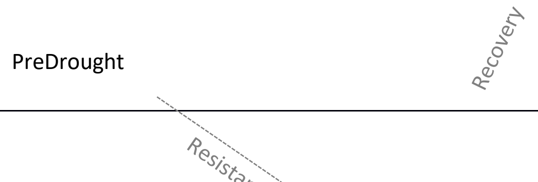
$$Resistance = {}^{Drought}\!/_{PreDrought}$$

Recovery corresponds to the ratio between the post-drought values and the values of the several parameters during the respective drought period.

$$Recovery = {}^{PostDrought}\!/_{Drought}$$

Resilience is calculated as the ratio between post-drought growth and pre-drought growth, providing insight about capacity to reach pre-disturbance performance levels.

$$Resilience = {}^{PostDrought}\!/_{PreDrought}$$



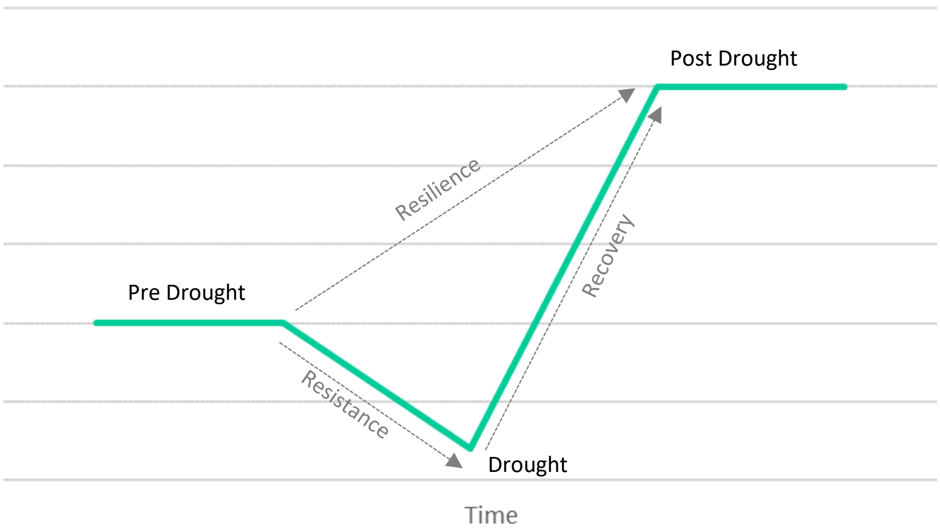


Figure 5– Hypothetical case: Plant stages before stress, during it and after drought relief (in black) and resilience indexes (in grey and respective arrows). The plant is suffering from water stress but has post-drought recovery resulting in high resilience. Based on Lloret (2011).

Since the set of plants used in this experiment is part of a major experiment in which drought started in April 2017, in June — when we conducted the first physiological campaign — plants were under drought stress. Thus, the pre-drought values used to calculate the resilience indices, were those of the main experiment. Pre-drought physiological performance was evaluated in March 2017 on 8 plants per treatment combination and despite there was no treatment yet, there were significant differences between treatments in certain variables and thus, the average initial pre-values per treatment and variable were used as reference for the pre-drought stage

In order to understand the impact of drought and re-watering on recovery and resilience components at short- and long-term, these indices were calculated for campaign 3 or campaign 4, considered to be the short- and long-term recovery respectively.

To calculate the indices using the water potential, due to the fact that they are presented as negative values, some adjustments in the calculation of the indices were made. For this variable we used the inverse of both numerator and denominator. For instance, to calculate water potential recovery, we have used the following equation:

$$Recovery = \left(\frac{1}{\psi PostDrought}\right) \Big/ \left(\frac{1}{\psi Drought}\right)$$

On the third campaign, there were some saplings that presented photosynthetic negative values. Therefore, in order to calculate A_n Recovery, we added a constant to be able to interpret the values in the same way as in the other variables:

$$Recovery = (PostDrought + PostDrought) \Big/ (PostDrought + Drought)$$

None of the resilience components was calculated for the WUE variable. Since it is calculated as the ratio between A_n/g_s, whenever A_n is negative, WUE will be as well. Therefore, this parameter would display both positive and negative values. Furthermore, WUE drought response curve is the contrary to the rest of the studied variables and presents no significant temporal variation, therefore this variable was excluded from these calculations.

2.5. Statistics

Linear mixed models were used to study the time series of all ecophysiological variables: ψ_{Pd}, ψ_{Md}, A_n, g_s, RWC, WUE, Diameter and Height. Saplings were included as a random factor in all statistical models, whereas campaign, treatment (CC, CI, EC, and EI) and their interaction were included as fixed factors. As for indices, resilience and recovery were analysed with the same model but considering only campaign 3 and 4, short and long-term recovery respectively as time factor. On the other hand, RGR, biomass, mycorrhizal colonization and relative resistance were analysed with simple linear models with treatment as a fixed factor.

Prior to all analyses, data were log or square root transformed to achieve normality whenever required. A different model was fitted for each variable in all statistical tests. Selection procedure always started from the saturated model (treatment, campaign and the interaction between the two variables are considered when both considered) and progressively removed the variables with the lowest explanatory power until the minimal adequate model with the lowest Akaike Information Criterion (AIC) was obtained. Models within two AIC units of the best fitting model were considered equivalent in terms of fit and the simplest one was selected.

All analyses were carried out with the R statistical Software v.3.5.0 (R Development Core Team 2018), using the functions lm and lme from the nlme package for linear models and mixed linear models. Significance for all statistical analyses was accepted at $P < 0.05$.

Chapter 3 – Results

3.1 Before drought

The morphological and physiological parameters measured in March were used as reference values before the application of the treatments.

Variable	Mean	SE
An (µmol CO_2 m^{-2} s^{-1})	13.91	0.51
gs (mol H_20 m^{-2} s^{-1})	0.32	0.02
Tr (mol m^{-2} s^{-1})	4.75	0.16
WUE (µmol CO_2 /mol H_2O)	47.41	2.68
ψPd (MPa)	0.50	0.02
ψMd (MPa)	0.71	0.03
Height (cm)	26.70	0.60
Diameter (mm)	4.57	0.09

Table 2- Morphological and physiological variables, measured in March 2017. ψ_{Pd}, pre-dawn water potential; ψ_{Md}, midday water potential; A_n, CO_2 assimilation; g_s, stomatal conductance; Tr, transpiration; WUE: Water Use Efficiency. Means and SE are shown (N=32).

3.2 Seasonal response to drought and re-watering

In general, there were no significant differences between treatments.

Although we tried to avoid plant mortality, during campaign 3, 6 plants had already succumbed to drought. It is noteworthy that all are part of exclusion treatments, two EC and four EI.

3.2.1 Hydraulic relations

There are no significant differences between treatments for ψ_{Pd} and ψ_{Md}. However, both showed significant differences over time (ψ_{Pd} , P= <0.0001; ψ_{Md} , P= 0.006).

ψ_{Pd} and ψ_{Md} became more negative as the soil dried (see annex Table 3 and Figure 17 for soil water content reference) throughout campaigns, with average values in August of −1.77 ± 0.15 and −2.15 ± 0.17 MPa., respectively. Despite watering reduction on EC and EI plants, it did not result in lower predawn or midday water potentials than in the other two treatments. After rewatering, predawn water potential was on average −1.04 ± 0.10 MPa with no significant differences between normal rewatering vs. extra irrigation (Figure 6). In general, predawn water potential decreased to the minimum values achieved during the most stressful campaign (DOY=230), except for EC treatment which reached its lowest values in campaign 3 (DOY=262). At that time, midday water potential tended to increase, reaching higher values during long-term recovery (DOY=289). Nonetheless, there were no significant differences between short and long-term recovery (Figure 6).

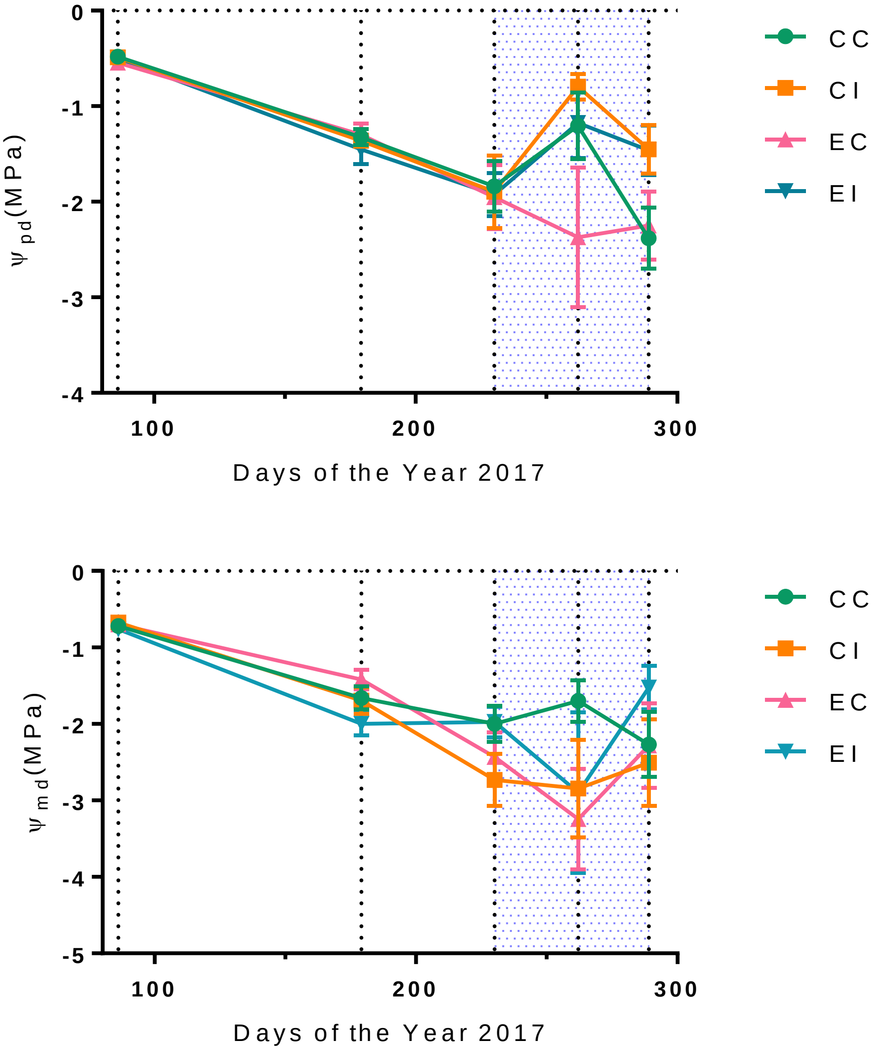


Figure 6– Average of $\Psi_{predawn}$ and Ψ_{midday} throughout time, per treatments. Water potentials were measured on needles. Means and SE are shown (N=32 during campaign 1 and 2; N=26 during campaign 3 and 4). The light blue area represents the autumn campaign period.

Although there was a declining trend from the first to the third campaign, RWC showed no statistical differences throughout time (Figure 7, P= 0.0761).

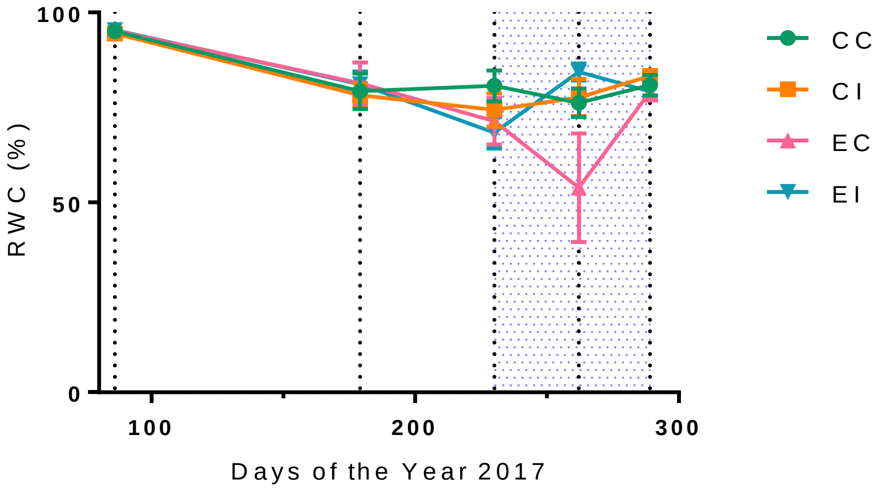


Figure 7 - Average of RWC throughout time, per treatments. Means and SE are shown (N=32 during campaign 1 and 2; N=26 during campaign 3 and 4). The light blue area represents the autumn campaign period.

3.2.2 Gas Exchange

There was a seasonal variation of A_n (Figure 8, P<.0001), but no significant differences were found between treatments (Figure 8, i). During the first campaign the average value of A_n was 8.75 ± 1.46 µmol CO_2 m^{-2} s^{-1}, compared with 13.91 ± 0.51 µmol CO_2 m^{-2} s^{-1} obtained in March, in the beginning of the growing season (Table 2). Regardless of the amount of water applied during re-watering, it is only on the fourth campaign that A_n increased to 15.13 ± 1.65 µmol CO_2 m^{-2} s^{-1} (Figure 8, i). Stomatal conductance (g_s) and Transpiration followed a seasonal pattern similar to A_n (Figure 8, ii and iii). Water Use Efficiency (WUE) showed no significant differences between campaigns (P= 0.1223), nor treatments (P= 0.1460).

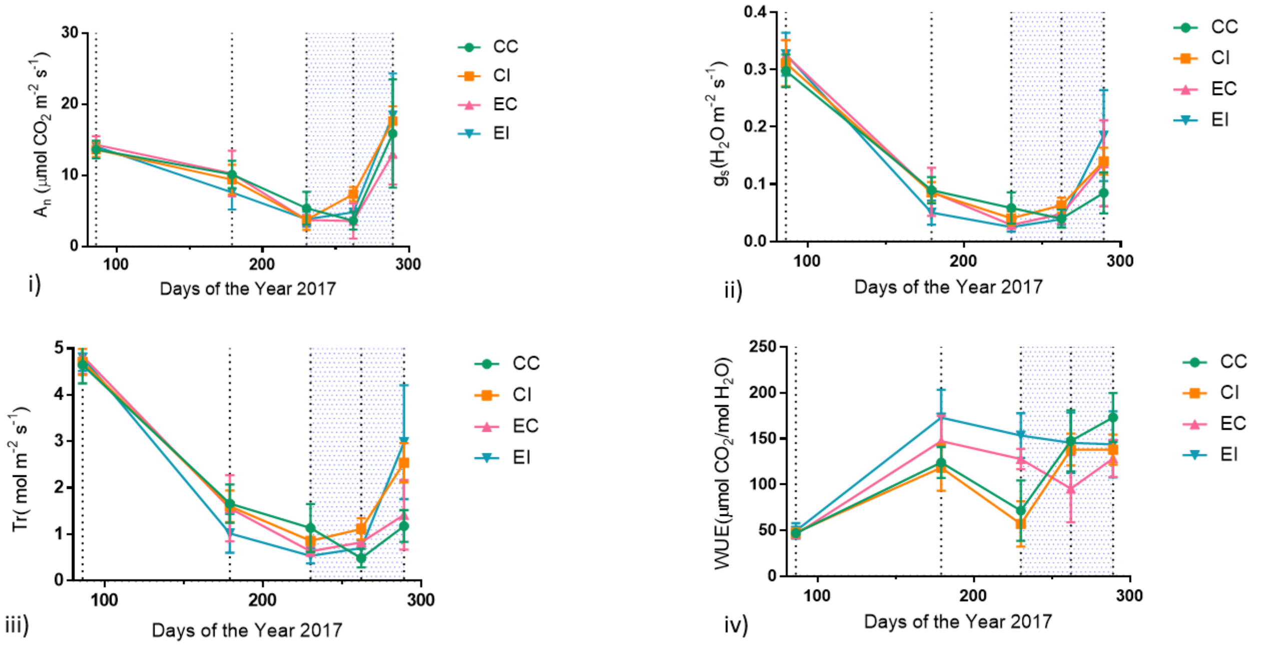


Figure 8 – Average values of gas-exchange throughout time, per treatments: i) CO_2 Assimilation rate; ii) Stomatal conductance; iii) Transpiration rate; iv) Water Use Efficiency. Means and SE are shown (N=32 during campaign 1 and 2; N=26 during campaign 3 and 4). The light blue area represents the autumn campaign period.

3.2.3 Growth and Biomass

P. pinaster saplings grew on average 11.27 ± 1.28 cm from February till the beginning of June, and the height remained stable until the end of the experiment, with an average value of 37.69 ± 0.16 cm (Figure 9). There were no significant differences among the different treatments (P= 0.4329) nor campaigns (P=0.7976).

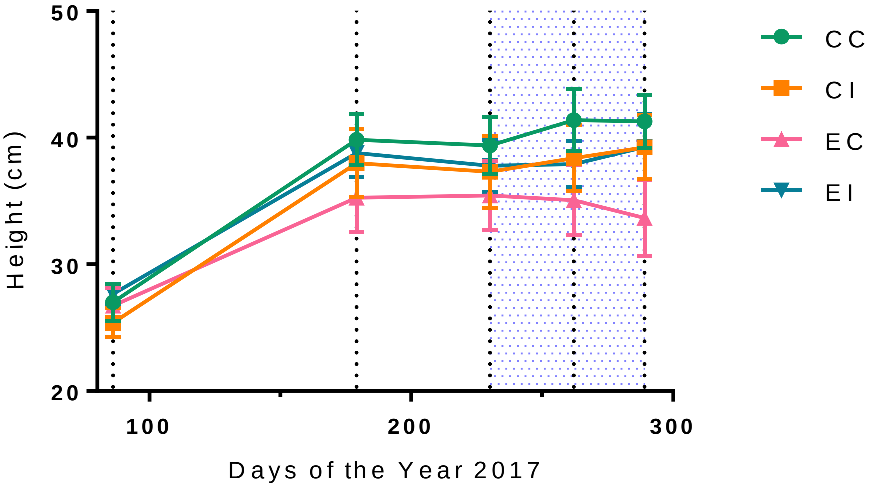


Figure 9 - Average height of *Pinus pinaster* throughout time, per treatments. Means and SE are shown (N=32 during campaign 1 and 2; N=26 during campaign 3 and 4). The light blue area represents the autumn campaign period.

Plant diameter showed significant differences along the campaigns (P <.0001) and among treatments (P=0.004) (Figure 10). The general seasonal pattern was an increasing trend of the diameter until followed by stabilization, although there was a decline in the third campaign. EC plants showed the lowest diameter compared with the other treatments.

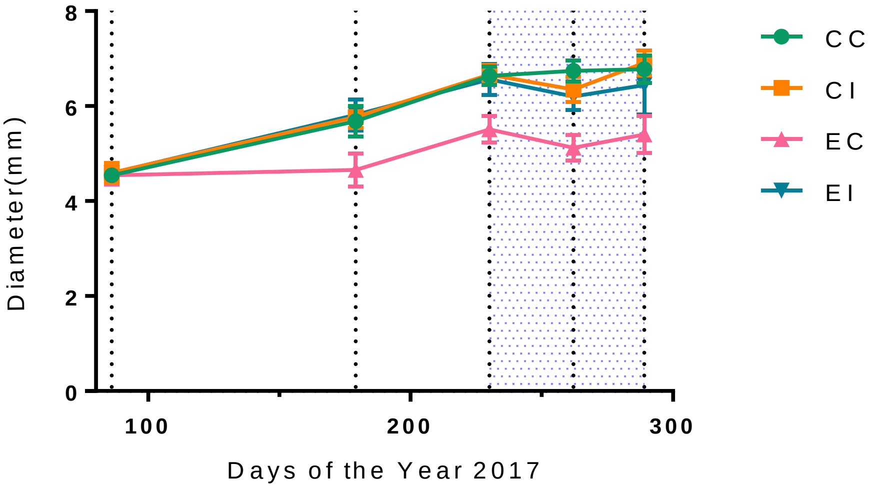


Figure 10 - Figure 10 - Average diameter of *Pinus pinaster* by treatment throughout time. Means and SE are shown (N=32 during campaign 1 and 2; N=26 during campaign 3 and 4). The light blue area represents the autumn campaign period.

Diameter was significantly different between treatments at the end of the study, with EC treatment showing the lowest values.

At the end of the experiment the ratio above/belowground biomass was on average 2.36 g ± 0.12 with no differences between treatments (Figure 11, P=0.8570).

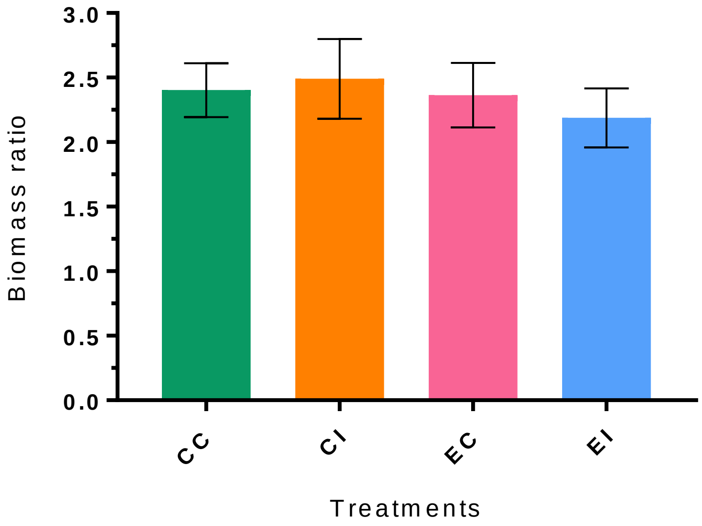


Figure 11- Average ratio above/belowground biomass at the end of the study (October 2017) for each treatment. Above-ground biomass includes stem, branches and needles of *P. pinaster* saplings. Means and SE are shown (N=26).

3.2.4 Mycorrhizal colonization

At the end of the experiment, the average percentage of mycorrhizal colonization was 11.04% ± 2.18, with no significant differences between treatments (Figure 12, P= 0.1598).

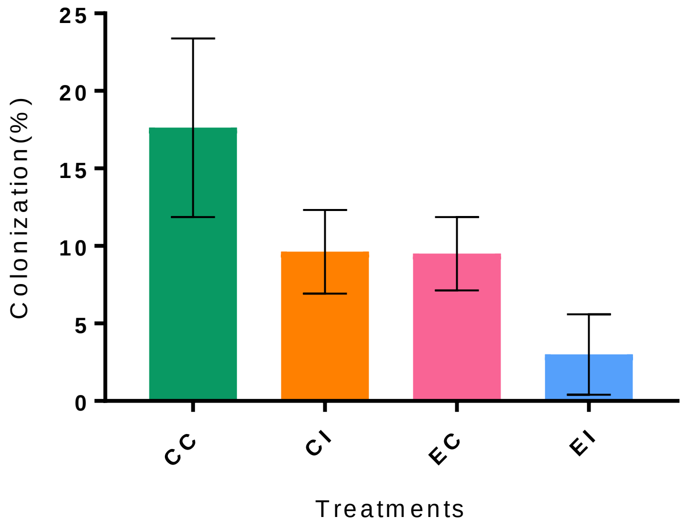


Figure 12- Average mycorrhizal root colonization in percentage at the end of the study (October 2017) for each treatment. Means and SE are shown (N=26).

3.3 Resilience Components

In general, for all the indices calculated (resistance, recovery and resilience) no significant differences were found between treatments.

3.3.1 Resistance

No variables presented significant differences between treatments. Resistance in ψ_{Pd} (P= 0.8677) and ψ_{Md} (P=0.07509) was relatively similar, as well as photosynthetic resistance (P= 0.7563) throughout the four treatments. On the other hand, g_s (P= 0.705) and transpiration (P= 0.6311) present low values, meaning that, the impact of the disturbance acutely distressed both traits. RWC resistance was consistent between treatments (P= 0.2905) (Figure 13).

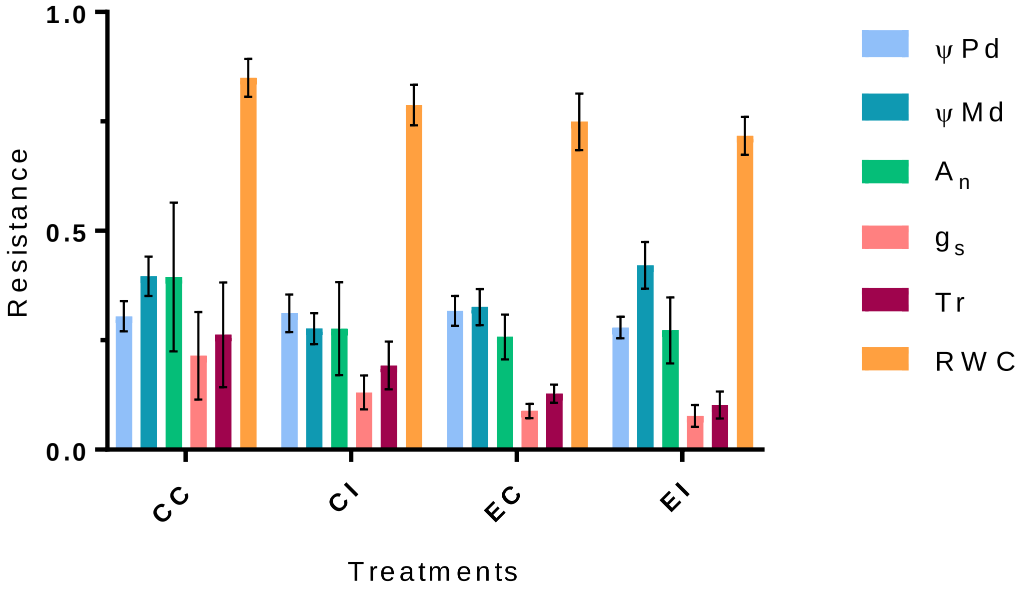


Figure 13 – Resistance of all physiological variables per treatment. Means and SE are shown. ψ_{Pd}, pre-dawn water potential; ψ_{Md}, midday water potential; A_n, CO_2 assimilation; g_s, stomatal conductance; Tr, transpiration; RWC, relative water content.

3.3.2 Recovery

If recovery values are superior to 1, it means that the measured variable present higher values after drought compared with the drought period. All the other traits showed values equal or superior to 1.

Concerning the time factor (short and long-term), most of the traits increased the values with time and presented significant differences between campaigns (A_n:P< 0.0001; Tr: P <.0001; g_s: P <.0001). The exceptions to this trend were ψ_{Pd} , ψ_{Md} and RWC. The first variable recovered quickly after rehydration, followed by a decrease in long-term recovery values even though it still presented significant differences between campaigns (P= 0.0005). The other two variables remained stable in both short and long-term recovery, with no significant differences between this period. (P= 0.713 and P= 0.2867, respectively) (Figure 14).

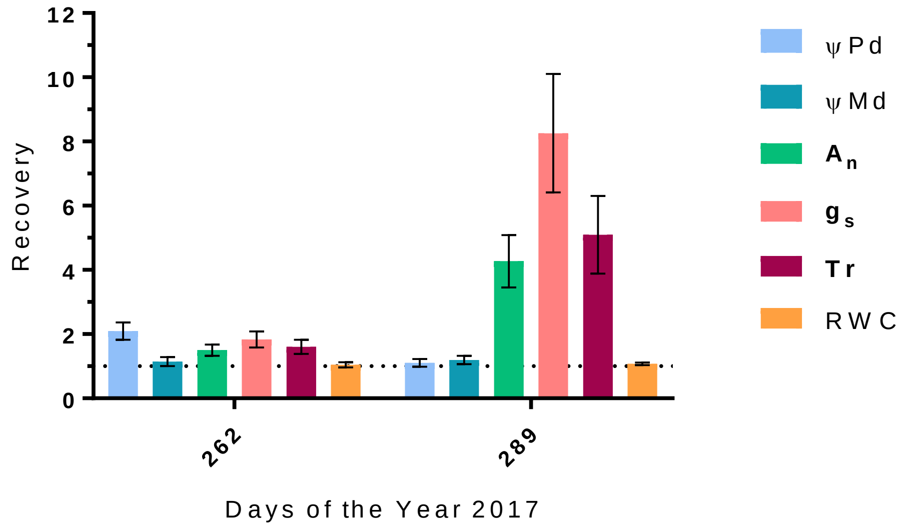


Figure 14 – Short and Long-term recovery of all physiological variables. Means and SE are shown. The variables highlighted in bold have shown significant differences throughout short and long-term recovery. ψ_{Pd}, pre-dawn water potential; ψ_{Md}, midday water potential; A_n, CO_2 assimilation; g_s, stomatal conductance; Tr, transpiration; RWC, relative water conductance.

3.3.3 *Resilience*

Overall, resilience values were below 1, meaning that none of the studied parameters recovered before drought performance (reference values of March 2017), either for short or long-term recovery. A_n is the exception as, at long-term recover, and independently of treatment, *P. pinaster* photosynthetic rates exceed March rates with average resilience of 1.19 ± 0.11 µmol CO_2 m^{-2} s^{-1}.

Concerning the time factor, every parameter except ψ_{Pd} increased slightly overtime. In fact, ψ_{Pd} presents higher short-term resilience values compared with long-term.

ψ_{Pd}, A_n, g*s* and Tr have all shown significant differences between short and long-term (P= 0.0004; P <.0001; P= 0.0002 and P=0.0004, respectively), while the other variables did not (ψ_{Md}: P= 0.4957; RWC: P= 0.0702). Though, ψ_{Pd} decreased from short to long-term resilience, whereas A_n did the opposite, meaning that from the third to the fourth campaign, photosynthetic values increased pointedly.

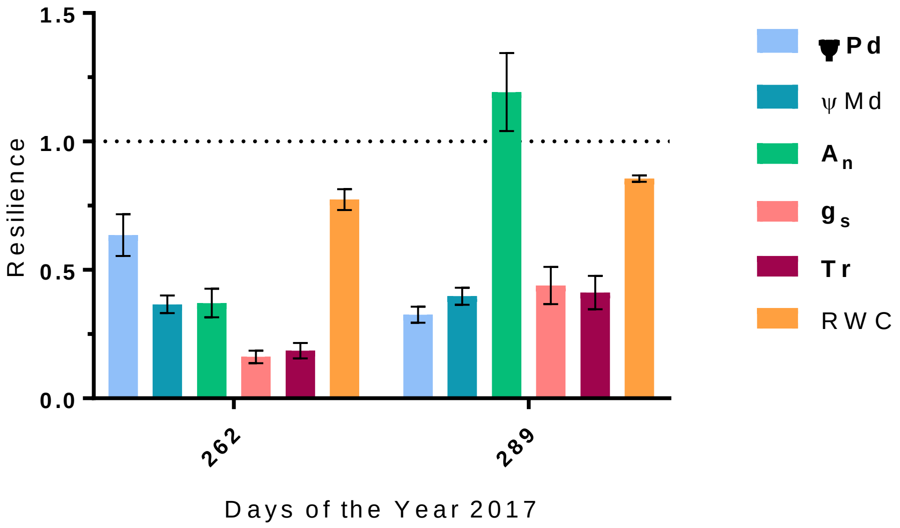


Figure 15 - Short and Long-term resilience of all physiological variables. Means and SE are shown. Variables in bold presented significant differences between short and long-term recovery. ψ_{Pd}, predawn water potential; ψ_{Md}, midday water potential; A_n, CO_2 assimilation; g_s, stomatal conductance; Tr, transpiration; RWC, relative water content.

Chapter 4 – Discussion

We have studied the seasonal variations of leaf gas exchange parameters (net CO_2 assimilation rate, stomatal conductance, transpiration rate), hydraulic relations (predawn and midday water potentials, water-use efficiency and relative water content), growth (diameter and height) of *Pinus pinaster* saplings subjected to drought-stress and following rewatering. In general, there were no differences between treatments. Thus, the group of saplings that were in water exclusion, and the group of saplings that afterwards had extra irrigation from September to October, behaved in a similar way, concerning the traits measured.

Leaf Ψ_{Pd} along the 4 campaigns showed more variability compared with leaf Ψ_{Md}. Leaf Ψ_{Pd} is theoretically in equilibrium with substrate water potential and is an indication of the soil water availability (Medrano *et al*. 2002). The stability of leaf Ψ_{Md} indicates that whatever the available water along the season the potential was kept at similar values, meaning that the plant was able to regulate the stomatal opening to maintain midday leaf Ψ more or less stable under fluctuations of the environmental conditions (Martínez-Vilalta *et al*., 2014). In fact, stomatal conductance showed a significant decreasing trend along the season, following the drying pattern, and increasing after the irrigation period. The same pattern was observed for CO_2 assimilation and transpiration, parameters highly correlated with stomatal conductance. These results point at the regulative function of the stomata in minimizing water loss under limited water supply, increasing water-use efficiency (Galle & Feller, 2007b).

The short and long-term responses to rewatering were different in hydraulic and gas exchange variables. In fact, whereas after rehydration, predawn water potential seemed to increase instantly, only after 27 days did saplings reach the highest levels of photosynthesis, transpiration, and stomatal conductance. RWC, however, was not significantly different. Therefore, after immediate irrigation, we hypothesize that water moves to the tissues for rehydration. As soon as those values are reestablished, there is a major recovery on gas exchange variables. This can indicate that hydraulic limitation was the process governing gas-exchange recovery from drought, since there is a delay between hydraulic and gas exchange recovery (Bogeat-Triboulot *et al*., 2006; Brodribb & Cochard, 2009).

For most variables, there was no resilience, meaning that they did not return to the initial conditions, before drought stress. The only exception to this trend was A_n. As reported, full resilience of net photosynthetic rate has been observed as drought stress ends and when followed by rewatering (Xu *et al*, 2009). For example, after 15 days of rewatering, *Populus nigra L*. A_n completely recovered to the pre-stress level (Fortunati A *et al*, 2008).

It was interesting to observe that g_s did not fully recover. Studies have suggested that full resilience always co-occurred in both photosynthetic function and stomatal aperture after an episode of drought followed by rewatering (Inzaloo *et al*, 2008). However, Xu and Zhou (2008) reported in *Leymus chinensis* leaves, that g_s only had a partial recovery after rewatering, not reaching the level of the well-watered treatment.

All in all, the relative contributions from different limitation components during drought and after rewatering may need to be elucidated in future experiments with a stronger degree and duration of water stress.

Chapter 5 – Conclusion

Our results confirm that *Pinus pinaster* adjust their stomatal opening, meaning that they have a tight regulation of the stomatal conductance (which have direct influence in traits such as CO_2 assimilation and transpiration) reflected in that fact that the midday water potential was kept at similar values, independently of the water availability.

The recovery index showed that hydraulic traits recover faster than gas-exchange parameters. Thus, we hypothesize that the recovery of gas-exchange parameters after a drought period is dependent on the recovery of hydraulic parameters.

Most of the measured traits showed low resilience, except CO_2 assimilation that increased resilience at a long-term scale. Consequently, for most of the traits the values did not recover to pre-drought conditions, which may constrain plants response to future drought events. However, we have to take into account that saplings were growing in pots and, by the end of the experiment, the spatial limitation can affect resilience.

This paper presented time variations of leaf gas exchanges. The fact that there were no significant differences between the treatments can indicate that the drought stress and extra-irrigation was not quantitatively sufficient to trigger a different response of *Pinus pinaster* saplings. Future experiments should consider inducing a stronger water stress (amount and/or duration).

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Annexes

Table 3 – SWC values on 20 different occasions per treatments.

date	DOY	CC	CI	EC	EI	Total Result
2017-02-09	40	17.9	19.0	18.3	18.1	18.3
2017-02-23	54	12.5	11.4	9.8	12.9	11.6
2017-03-07	66	12.3	12.1	11.6	11.8	11.9
2017-03-17	76	19.1	21.4	18.9	18.9	19.6
2017-04-05	95	20.8	21.8	19.9	19.7	20.6
2017-04-20	110	14.7	13.9	10.0	9.6	12.2
2017-05-11	131	10.1	8.5	5.1	4.0	7.0
2017-05-25	145	7.3	5.4	4.7	3.8	5.4
2017-06-07	158	5.1	3.4	3.6	3.5	3.9
2017-07-11	192	5.7	2.6	1.5	1.2	2.8
2017-08-22	234	2.0	1.5	0.7	0.5	1.2
2017-08-29	241	2.0	2.3	1.5	0.8	1.7
2017-09-05	248	4.8	8.7	7.9	4.4	6.4
2017-09-19	262	6.9	17.7	10.0	18.7	13.3
2017-10-26	299	4.5	5.7	7.9	9.0	6.8
2017-11-02	306	5.5	8.7	8.8	14.2	9.4
2017-11-27	331	10.6	12.5	10.8	16.3	12.6
2017-11-29	333	12.1	10.8	13.6	17.4	13.4
2017-12-14	348	14.0	11.4	14.2	17.9	14.3
2017-12-28	362	20.3	23.9	23.2	23.6	22.7
Total Result		14.0	14.7	13.9	14.5	14.3

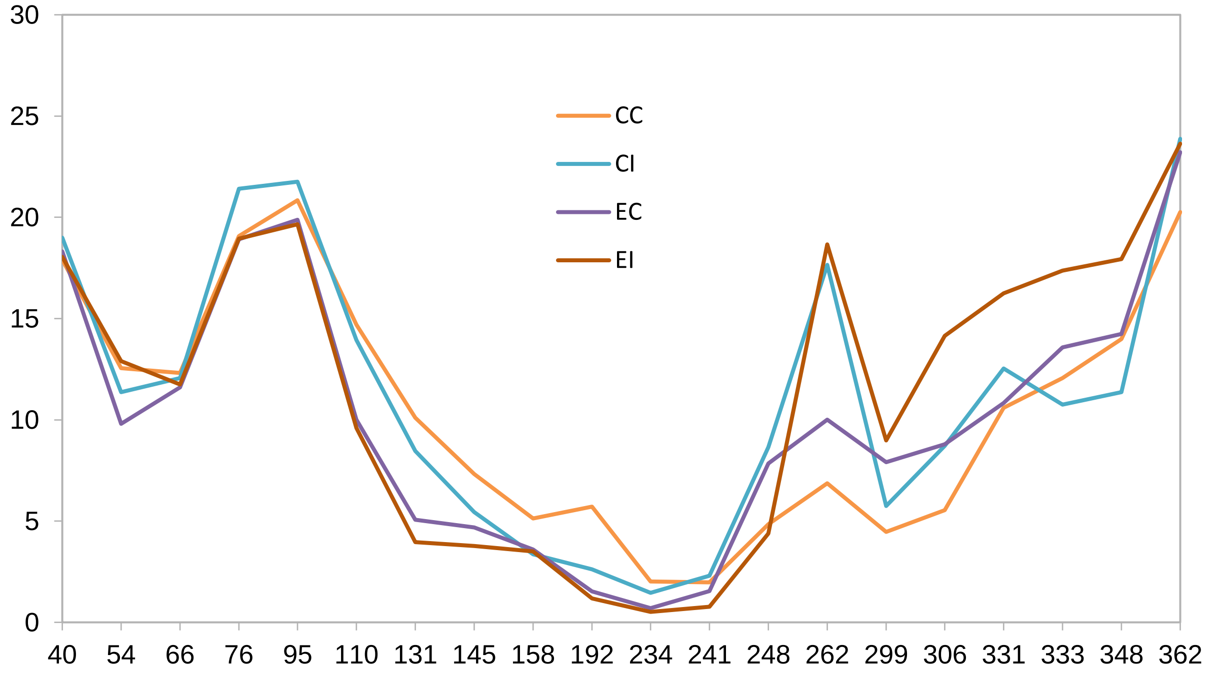


Figure 17 –SWC values throughout time per treatment.

SIRO

FICHA TÉCNICA

FT-11-SV-115
Data:07-04-2017
Rev: 01

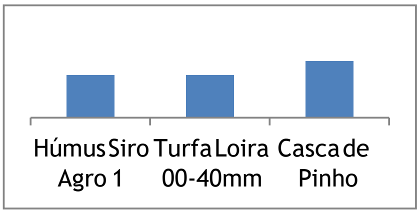
Siro® Ácido

Descrição

Substrato especial para o envasamento e transplante de uma ampla gama de plantas acidófilas tais como: camélias, azáleas, rododendros, gardénias, éricas, hortênsias, entre outras.

Tem uma textura média com pH baixo, enriquecida com adubos de libertação controlada, que permitem às plantas sensíveis ao calcário, o seu desenvolvimento saudável e floração abundante e duradoura.

Composição: Húmus Siro Agro 1 *(Húmus à base de resíduos florestais, com predominância de Casca de Pinho marítimo),* turfa loira selecionada 0-40mm, casca de pinho 0-15 tratada termicamente segundo a legislação em vigor e 1.5 Kg de adubo de libertação controlada até 9 meses de longevidade.



Características Químicas

Fertilização:

NPK 20-05-11	0.5 kg/m³	Longevidade 2-3 meses
NPK 17-9-8 + 4Mg	0.5 kg/m³	Longevidade 8-9 meses
NPK 16-11-10 + 2MgO	0.5 kg/m³	Longevidade 8-9 meses

Condutividade: 60 – 100 µS/cm

pH em $CaCl_2$: 4.0-4.5

MO > 70%

Aplicação: Vaso/solo

Apresentação:

Referência	Designação	Unidade de venda	Palete
SR150900110	Siro Ácido 20L	Saco 20 L	144 sacos
SR150900115	Siro Ácido 45L	Saco 45 L	45 sacos
SR150900118	Siro Ácido 70L	Saco 70 L	39 sacos
SR150900124	Siro Ácido – big bag	bb 2.3m³	1 unidade
SR150900498	Siro Ácido Big Bale	m³	Big Bale ≥ 3 m³
SR150900499	Siro Ácido – granel	m³	35 m³

Notas:

1) Cálculo do Volume – “*de acordo com NP 12580”*
2) *O volume do Big Bale é calculado individualmente e pode variar de acordo com o seu enchimento e densidade do produto.”*