What makes plant succumb under drought conditions: A tissue-level approach to understand and identify tree mortality

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What makes plant succumb under drought conditions:

A tissue-level approach to understand and identify tree mortality

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“The drought was the very worst …

When the flowers that we’d grown together died of thirst”

- Taylor Swift

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Résumé : Les modèles climatiques globaux prévoient une augmentation de la fréquence et de l'intensité des sécheresses affectant la survie des arbres. Afin d'améliorer la précision des modèles mécanistiques prédisant la mort des arbres, il apparait crucial de comprendre ces processus en condition de sécheresse extrême et d'identifier les seuils physiologiques clés au-delà desquels les arbres meurent. Ainsi, l'objectif principal de cette étude était d'identifier les traits physiologiques clés, à l'échelle tissulaire, pouvant servir de seuil de mortalité chez les arbres. Pour cela, plusieurs individus de deux espèces différentes ont été exposé à un événement de sécheresse et leur capacité à récupérer a été suivie après leur réhydratation. Les résultats montrent que les pourcentages de perte de conductance hydraulique communément acceptés comme des seuils de mortalité (P_{50} pour les conifères et P_{88} pour les angiospermes) ne seraient qu'une vague approximation de la capacité des arbres à survivre. Cependant, aucun des traits physiologiques suivi n'a pu déterminer un seuil identifiant la mort des arbres. La présence de cellules vivantes au niveau de l'écorce et du phloème permet de postuler que les arbres pourraient survivre à un événement de sécheresse si la continuité hydraulique entre les racines et les cellules vivantes était maintenue.

Mots clés : *Changement climatique, Sécheresse extrême, Mortalité des arbres, Perte de conductance hydraulique, Capacité à récupérer*

Abstract : Global climatic models predict an increment in the frequency and intensity of drought events which have already shown to have important consequences on tree survival and forest dieback. Therefore, it is crucial to identify and understand not only the mechanisms leading to tree mortality under extreme drought conditions but also the physiological thresholds beyond which trees die. This information is important for improving the precision of mechanistic models predicting tree mortality. Thus, the main aim of this study was to identify key physiological traits that could work as proxies for tree mortality. For this, we exposed individuals from two different species to drought and monitored their ability to recover after rehydration. Results showed that the percentage loss of conductance commonly accepted as threshold for tree mortality (i.e. 50% for conifers and 88% for angiosperms) were only a vague approximation of tree ability to survive extreme drought event. However, none of the traits monitored evinced a clear threshold for identifying tree mortality. Nevertheless, the presence of living cells in the bark and in the phloem made us hypothesize that a minimal hydraulic connection between the soil and such living cells is required for allowing the recovery of the trees from drought.

Key words: *Climate change, Drought, Tree mortality, Hydraulic failure, Recovery capacity*

Abbreviations list

ATR: Able To Recover

BRW: Before Re-Watering

C1: Initial water conductivity

C2: Maximal water conductivity

C-T: Cohesion-Tension

EL: Percentage of electrolyte leakage or Lysis percentage

ET: Evapotranspiration

FDA: Fluorescein diacetate

Fm: Maximal fluorescence

Fv: Variable fluorescence

Fv/Fm: Chlorophyll a fluorescence

Fv'/Fm': Chlorophyll a fluorescence under light conditions

GHGs: Greenhouse Gases

INRA: National Institute for Agricultural Researches

IPCC: Intergovernmental Panel on Climate Change

K: Conductivity

Ki: Initial conductivity

Kmax: Maximal conductivity

L-RWC: Leaf Relative Water Content

LVDT: Linear Variable Differential Transformer

Micro-CT: X ray microtomograph

MPa: Megapascal

mV: millivolts

NATR: Not Able To Recover

NMR: Nuclear Magnetic Resistance

P_{50}: Xylem Water Potential at which 50% loss of hydraulic conductance occurs.

P_{88}: Xylem Water Potential at which 88% loss of hydraulic conductance occurs.

PLC: Percentage Loss of hydraulic Conductance

PRW: Post Re-Watering

PS: Photosystem

RCP: Representative Concentration Pathways

ROS: Reactive Oxygen Species

RWC: Relative Water Content

S-RWC: Stem Relative Water Content

VC: Vulnerability Curve

VPD: Vapor Pressure Deficit

Ψ: Water Potential

Ψ_L: Leaf Water Potential

Ψ_S: Stem Water Potential

Summary

Introduction

Forests, including woodlands and savannas, cover 30% of the world land's surface (FAO 2006) and provide societies with ecosystem services such as biodiversity (Ayres and Lombardero 2000), timber production, watershed protection (Allen *et al.* 2010) but also with carbon storage and its associated atmospheric feedbacks (Reichstein *et al.* 2013). In addition, forests also contribute to aesthetic and spiritual benefits which contribute greatly to the well-being of societies (Allen *et al.* 2010).

Forests are composed by trees that live at the interface between the atmosphere and soil. Even if their physical organization allows them to widely explore the environment they evolve in, i.e. branches and leaves assimilate carbon dioxide from the atmosphere while underground roots absorb water and mineral from the soil, trees are motionless and are therefore highly influenced by the weather where they grow. Because water requirements for a single tree could reach up to 50L per day (for conifers) and up to 500L per day (for angiosperms) (Sperry *et al.* 2008), drought is one of the main limiting factors for forests survival and composition (Anderegg *et al.* 2012b). In fact, numerous studies have been carried out focused on the tolerance of trees to drought, showing some variability both between species (Torres-Ruiz *et al.* 2017b) and within species (Stojnić *et al.* 2018). During the last decades, human-induced climate change has increased the frequency of heat waves and drought events which has induced important tree mortality events worldwide (Anderegg *et al.* 2012b). More recently, the Intergovernmental Panel on Climate Change (IPCC) has reported that the increasing concentration of atmospheric carbon dioxide (CO_2), mainly caused by humans activities, could lead to an increase in the mean global temperature by about 2.6 to 4.8°C according to the representative concentration pathways (RCP) 8.5 scenario (Intergovernmental Panel on Climate Change 2014). Although higher concentrations of CO_2 could lead to a better efficiency of photosynthesis (Ainsworth and Rogers 2007), the rising temperatures associated with the expected changes in the precipitation patterns would increase the high evaporative demand and the plant transpiration rate which would provoke an increase in xylem sap tension and, consequently, exacerbate the risk of hydraulic failure by cavitation. As the percentage of cavitated vessels increases, the hydraulic conductance of the xylem decreases until the flow of water stops and provokes the desiccation of the plant tissues, the cell death and, finally, the death of the tree (McDowell *et al.* 2008). These changing conditions would exacerbate the occurrence of drought-induced tree mortality events (Keenan *et al.* 2013, Duan *et al.* 2014) and consequently forests

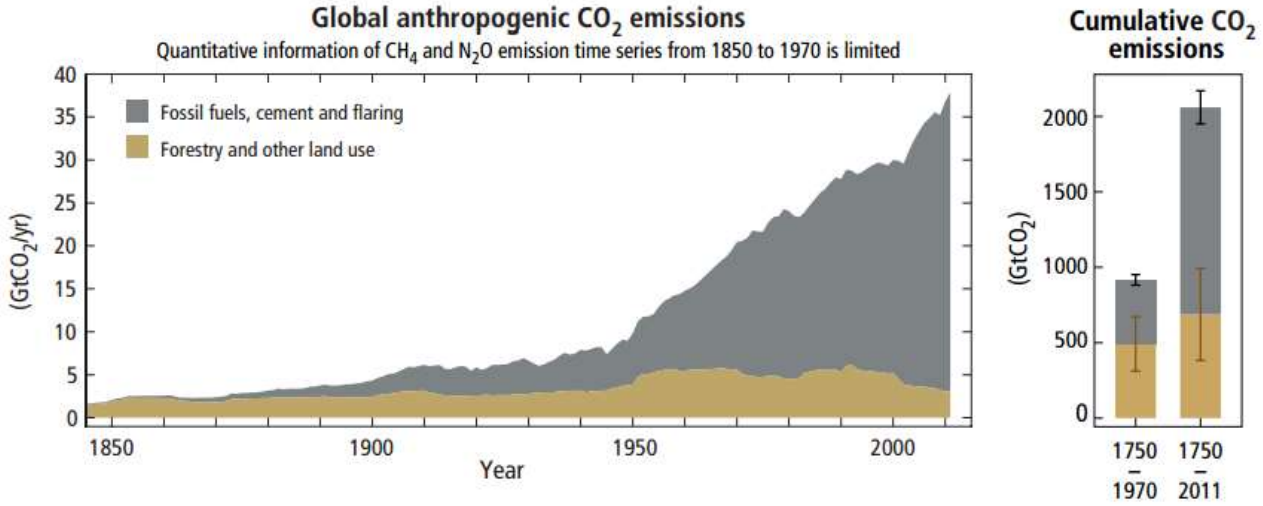


Figure 1: Annual global anthropogenic carbon dioxide emissions.
Half of the cumulative anthropogenic CO_2 emissions between 1750-2011 were emitted in the last 40 years mainly due to fossil fuel combustion linked with economic development.
(Intergovernmental Panel on Climate Change, 2014)

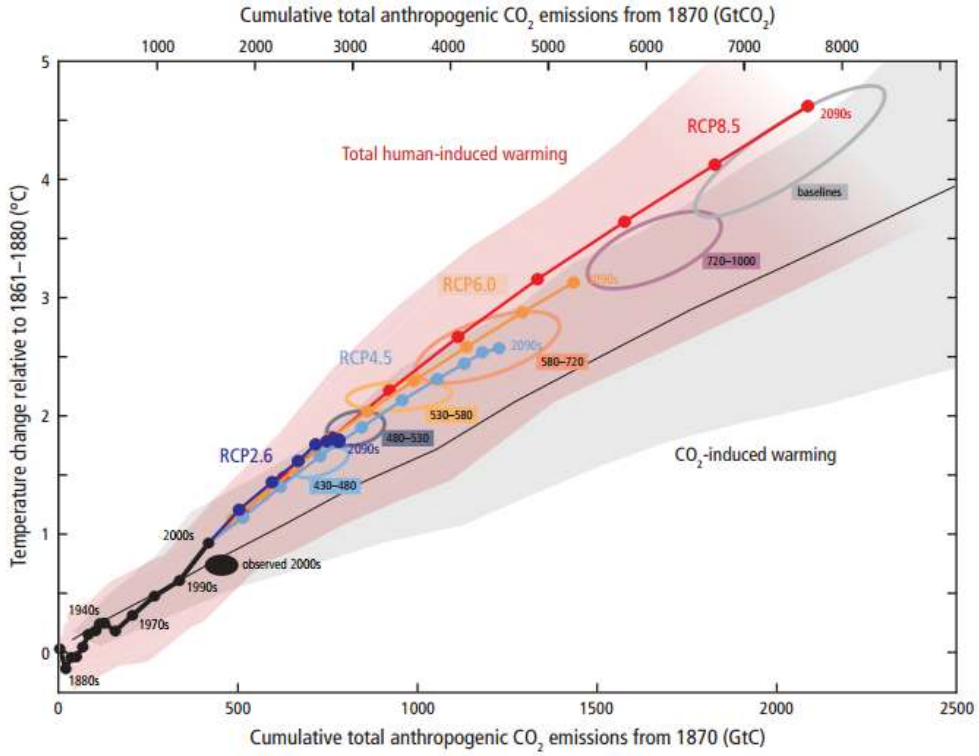


Figure 2: Temperature changes regarding cumulative total anthropogenic CO_2 emissions from 1870.
(Intergovernmental Panel on Climate Change, 2014)

dieback (Hosking and Hutcheson 1988, Lwanga 2003, Landmann and Dreyer 2006).

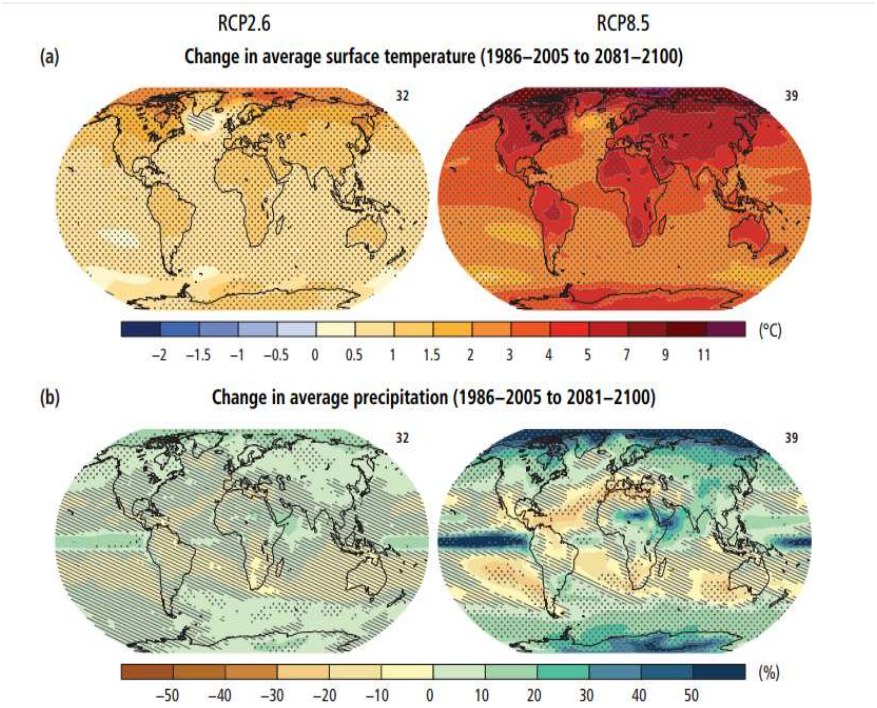
Despite the fact that xylem hydraulic failure is considered to be the main cause of tree mortality under severe drought conditions, it is still unclear when we could consider that a tree is dead, and therefore, is not able to recover and resprout anymore.

Addressing this question is especially relevant to implement models predicting the effect of the expected increase in the frequency and intensity of drought events on trees (Trenberth *et al.* 2014) since, so far, it is not possible to predict accurately when a tree would die from drought. Therefore, to make a significant step forward in our predictions about the variations in the composition of the forests due to climate change, it is crucial to identify those physiological traits able to define the threshold between a living and a dead tree.

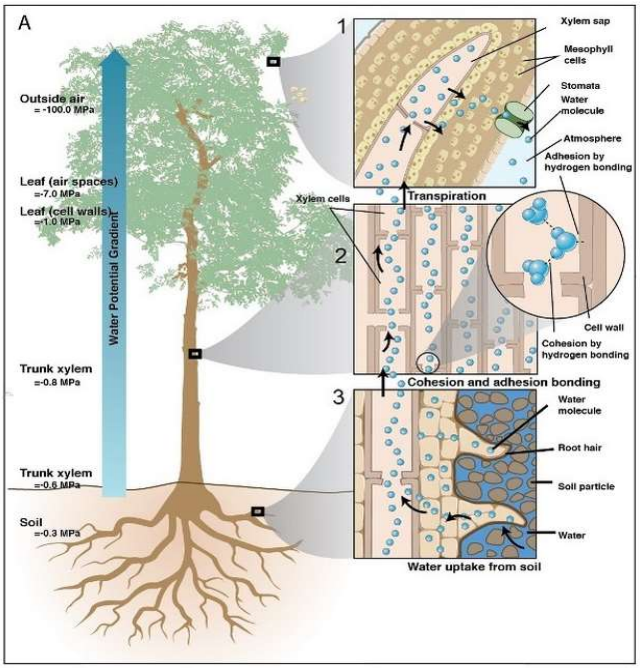
Bibliography Synthesis

1. Climate change, global warming and its consequences

According to the United Nations Framework Convention on Climate Change, climate change is defined as "a change of climate which is attributed directly or indirectly to human activity that alters the composition of the global atmosphere and which is in addition to natural climate variability observed over comparable time periods." In general, climate change is assimilated to global warming which is primarily caused by the accumulation of greenhouse gases (GHGs) in the atmosphere. Indeed, the atmospheric GHGs concentration in 2011 got to levels that were never reached in the last 800,000 years (Intergovernmental Panel on Climate Change, 2014) [Figure 1]. Even if there are natural sinks for carbon, like forests, the IPCC report showed that 40% of the CO_2 emissions remained in the atmosphere since 1750 (Intergovernmental Panel on Climate Change 2014). This accumulation of CO_2 in the atmosphere is directly related with the temperature changes [Figure 2] as the global mean surface temperature increases in a range of 0.8°c to 2.5°c per trillion tons of carbon emitted as CO_2 (Intergovernmental Panel on Climate Change 2014). Therefore, according to the RCP 8.5 scenario (worst-case scenario), the temperature could increase in between +1.5°c in southern Australia and +11°c in the North Pole by 2100 [Figure 3(a)] (Intergovernmental Panel on Climate Change 2014). Also, changes in the water cycle are expected to occur induced by the global warming. Thus, the IPCC reports that changes in average precipitation will not be uniform worldwide (Intergovernmental Panel on Climate Change 2014), with wet regions getting wetter and dry regions getting dryer (Trenberth *et al.* 2014) [Figure 3 (b)]. It has also been reported



<u>Figure 3:</u> Change in average surface temperature (a) and change in average precipitation (b).
The worst-case scenario (RCP8.5) evinced that the temperature elevation could reach between +1.5°c and +11°c in 2100. For precipitations, RCP8.5 scenario show that mid-latitude and subtropical regions will experiment lower means precipitations than actual while in wet regions precipitations will increase.
(Intergovernmental Panel on Climate Change, 2014)



<u>Figure 4:</u> Water circulation in plants : Soil-Plant-Atmosphere continuum.
Xylem sap flow following the cohesion-tension theory.
(McElrone *et al.* 2013)

that changes in the precipitation patterns will not only increase the frequency of the drought events, but also their intensity and duration (Trenberth *et al.* 2014). Even though it exists different definitions for drought, meteorological drought seems to be the more common one (Wilhite and Glantz 1987) and is defined, as mentioned by the IPCC in its fourth assessment report, as "in general terms, drought is a 'prolonged absence or marked deficiency of precipitation', a 'deficiency of precipitation that results in water shortage for some activity or for some group' or a 'period of abnormally dry weather sufficiently prolonged for the lack of precipitation to cause a serious hydrological imbalance'." (IPCC 2007).

2. Drought impact on plant physiology and forests ecosystems

2.1. Drought impact in plant water transport capacity

In plants, water moves from the roots to the leaves through the xylem that is a specialized tissue for water transport (Kirkham 2005, McElrone *et al.* 2013). Once water is absorbed by the plant through the roots, it can move over long distances within the plant due to the cohesion-tension (C-T) theory developed by Dixon and Joly in 1894. The C-T theory is based on the cohesion between water molecules (Milburn 1979, Kirkham 2005) and the pull of the continuous water column in the xylem induced by the plant transpiration, that is what finally causes the ascent of the sap (Meinzer *et al.* 2001, Brown 2013). Therefore, transpiration creates a tension gradient within the plant that allows water to circulate throughout the xylem under tension [Figure 4].

During a drought event, the combination of lower precipitations and high temperatures increases the evaporative demand from the atmosphere which induces higher evapotranspiration (ET) rates in plants (Duan *et al.* 2014) and, therefore, increases the tension on the xylem sap (Choat *et al.* 2018).

2.2. Drought events and forest die-off

Numerous episodes of drought-induced forest mortality have been observed in the last decades (Allen *et al.* 2010). For example, important widespread drought-induced tree mortality events were reported in Africa such as in the tropical moist forest of Uganda (Lwanga 2003), in New Zealand with the mortality of *Nothofagus* forests (Hosking and Hutcheson 1988) and in France during the heat wave and drought during the summer 2003 (Landmann and Dreyer 2006). As those events are likely to get more frequent in a near future (Trenberth *et al.* 2014), it is important to highlight the role of drought in determining the composition and the structure of

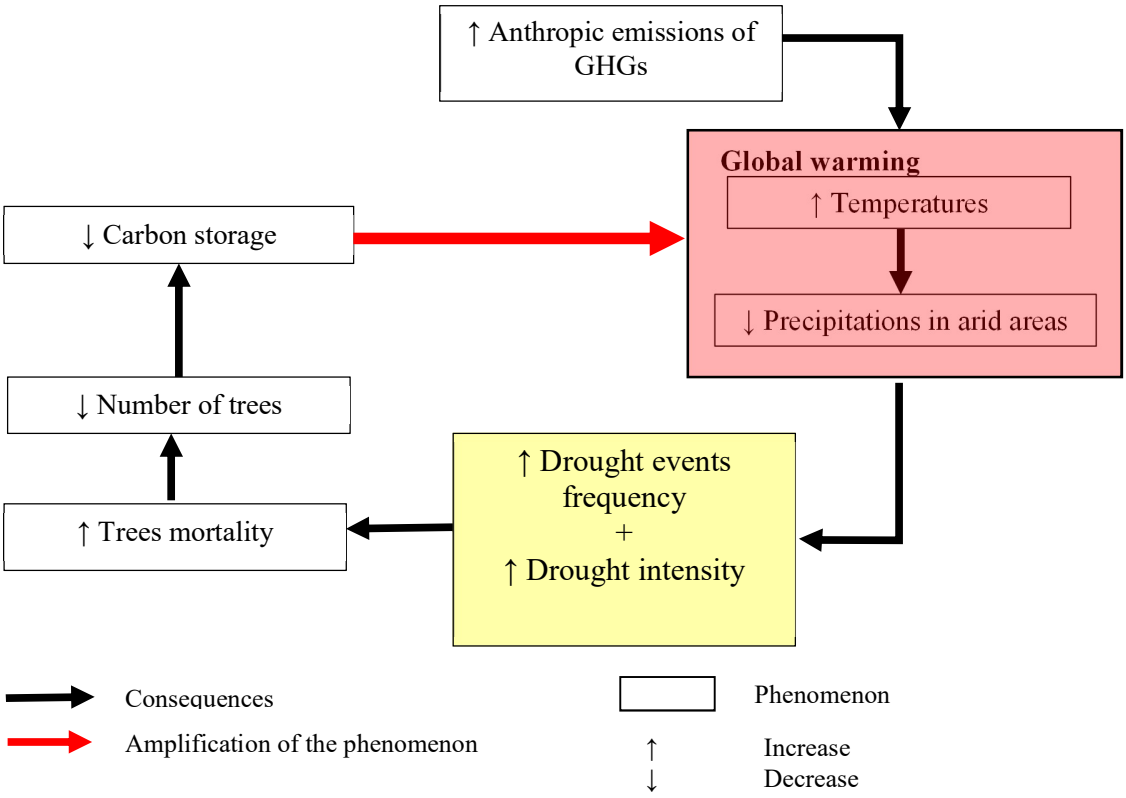


Figure 5: Consequences of drought on forests and climate change.
Anthropic emissions of GHGs are the main reason to global warming causing higher temperatures that will disrupt the water cycle and cause lower precipitations in arid areas. The combination of those two elements is likely to cause an increase in drought frequency, intensity and duration.
Drought stress induces higher tree mortality which reduces the number of trees on Earth. Because trees take a long time to regrow and therefore cannot compensate the humans' emissions of CO_2, a lower number of trees reduces the potential carbon storage, and, as a result, more carbon dioxide will remain in the atmosphere and thus enhance global warming.
(©Marylou MANTOVA)

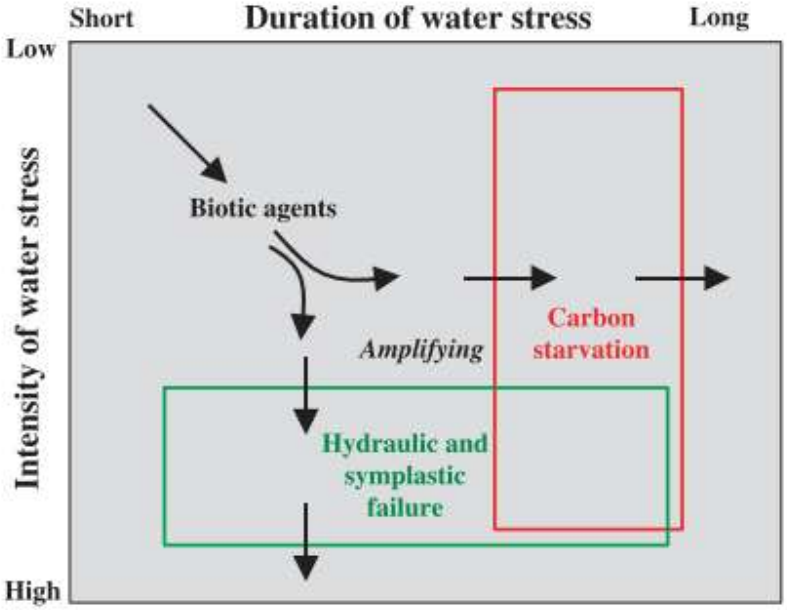


Figure 6: Theoretical relationship between the temporal length of the drought (duration), the relative decrease in water availability (intensity) and the mechanisms underlying mortality. (McDowell *et al.* 2008)

forests globally (Allen *et al.* 2010). Indeed, an important amount of forests worldwide is located in areas where the risk for drought is expected to increase in the next decades. Thus, e.g., the progressive water loss during the California drought (2012-2015) led to the loss of 102 million trees (Asner *et al.* 2016). Contrary to California, which can be considered as a water-limited region, some non-water limited areas experienced similar consequences induced by drought. This is the case for the tropical northern area of Australia, where 6% of the mangrove vegetation died from a drought event combining high temperatures and low precipitations back in late 2015 and early 2016 (Duke *et al.* 2017). Therefore, because forests seem to be sensitive to climate change and play an important role in carbon balance that, at the same time, helps regulating the climate [Figure 5] (Reichstein *et al.* 2013), it is crucial to prevent and predict the occurrence of drought-induced tree mortality events. **For this, it is necessary to understand not only the mechanisms driving tree mortality but also to determine a physiological trait that could be used as a proxy for determining when a tree is dead and not able to recover or resprout anymore.**

3. Drought-induced tree mortality

3.1. Causes of plants death under drought conditions

Vegetation mortality could be induced by multiple factors such as recurrence of climate stress, insects pests and diseases (Franklin *et al.* 1987, Miao *et al.* 2009). Studies in plant mortality frequently agree in that water limitation is one of the main causes of plants death. In fact, it has been shown that when plants undergo recurrent exposure to drought, their growth decreases significantly, and their risk to die increases (Pederson 1998, Suarez and Ghermandi 2004). Also, it seems that plants are likely to die when exposed to a prolonged drought periods with high air temperatures and vapor pressure deficit (VPD) conditions (Swetnam and Betancourt 1998, Breashears *et al.* 2005, Bigler *et al.* 2006). Drought-induced mortality is related to drought intensity and duration (McDowell *et al.* 2008) [Figure 6]. Thus, there are two hypotheses explaining tree mortality depending on the intensity and duration of the drought event. On one hand, the first hypothesis relates tree mortality with the **xylem hydraulic failure** that occurs when plants are exposed to intense drought conditions. On the other hand, tree death seems to be induced by a depleting in carbon reserves, i.e. **carbon starvation** (McDowell *et al.* 2008), induced by the partial closure of the stomata that occurs under prolonged mild drought conditions (McDowell *et al.* 2008, McDowell and Sevanto 2010). However, even if hydraulic failure and carbon starvation are not

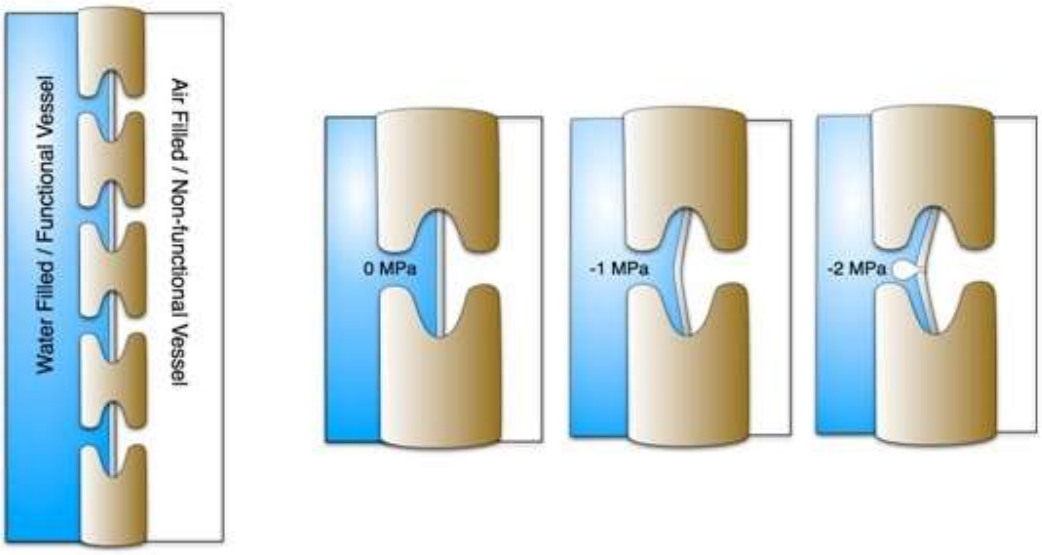


Figure 7: Cavitation mechanism.
As the tension increases at the pit-membrane level, a critical tension is reached and water pass from liquid state to vapor state. Because of xylem tension, the void created extends and generates cavitation. (© 2013 Nature Education Adapted from Tyree & Zimmermann 2002, McElrone *et al.* 2013)

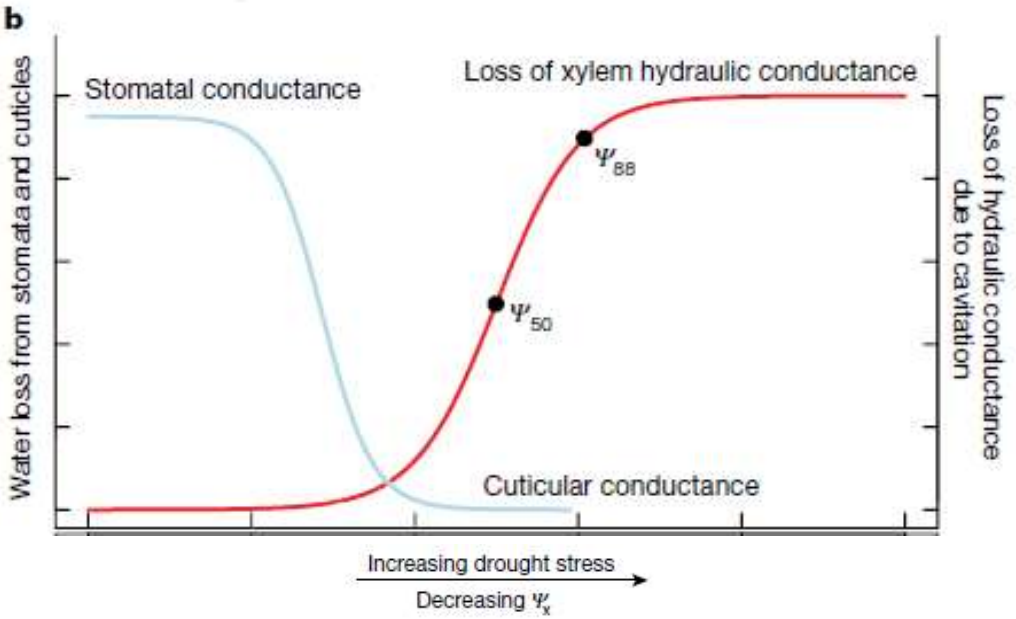


Figure 8: Determining P_{50} and P_{88} by using vulnerability curve.
Vulnerability curve (in red) vs Stomatal conductance (in blue).
During a drought event, when the xylem water potential (Ψ_x) decreases, plants close their stomata. Once the stomata are closed, plant continues losing water due to cuticular conductance. Under water-stress conditions, cavitation events occur reducing the xylem hydraulic conductance. The CAVITRON technique along with the optical method are used to generate vulnerability curves (in red) on which it is possible to determine the P_{50} and the P_{88} which correspond respectively to the xylem water potential at which 50% or 88% of hydraulic conductance are lost.
(Choat *et al.* 2018 modified)

mutually exclusive mechanisms (McDowell 2011b), many studies agrees on that hydraulic failure is one of the main causes of tree mortality under severe drought (Urli *et al.* 2013) or that "one primary cause of productivity loss and plant mortality during drought is hydraulic failure" (Choat *et al.* 2012). Therefore, because this study will be centered on the effect of intense drought-event on trees, a deeper review of the physiological processes and mechanisms related with intense drought induced tree mortality will be established.

3.2. Intense drought: hydraulic failure

3.2.1. Hydraulic failure definition and mechanism

It is well known now that most of the mortality events occurring under severe drought conditions are induced by the **hydraulic failure of the xylem tissue due to the occurrence of cavitation events**. (McDowell *et al.* 2008) [Figure 6]. Thus, during a severe drought event, soil water availability decreases while the evaporative demand and cuticle conductance increase resulting in an increment of the xylem tension that induces the occurrence of cavitation events in the xylem.

Cavitation is the change from liquid water to water vapor [Figure 7] (Dixon and Joly 1894, Tyree and Sperry 1989) that results in the formation of gas bubbles (emboli) in the xylem conduits and provokes the hydraulic dysfunction of the xylem reducing the plant water transport capacity. Thus, during intense drought, as VPD and xylem tension increase, the cavitation can spread throughout the entire xylem (Choat *et al.* 2016). As the percentage of cavitated vessels increases, the hydraulic conductance of the xylem decreases until the flow of water stops and provokes the desiccation of the plant tissues, the cells death and, as a last resort, the death of the tree. Despite the efforts of plants to reduce water loss by closing stomata before the onset of hydraulic failure [Figure 8], plants still suffer from water-stress as the water potential (Ψ) continue decreasing due to the water losses through the cuticle (Kerstiens 1996) and stomatal leakiness (Oren and Pataki 2001).

3.2.2. Vulnerability to cavitation

Vulnerability to cavitation is extremely variable across species and biomes (Delzon *et al.* 2010, Choat *et al.* 2012). In fact, different strategies have been developed throughout evolution to preserve the integrity of the plant vascular system, being all them mostly defined by two different constraints: the ability to maintain relatively high water potential by limiting water losses under

drought conditions (Blackman *et al.* 2016, Martin-StPaul *et al.* 2017); and the physical limits of the xylem vessels to avoid the entry of air into the xylem conduits, i.e., the vulnerability to cavitation. Xylem vulnerability to cavitation is usually evaluated by constructing vulnerability curves (VC). These curves represent how the percentage loss of hydraulic conductance (PLC) induced by cavitation varies with the xylem tension, i.e. the xylem water potential [Figure 8]. Valuable information could be extract from such curves as, e.g., the xylem tension inducing 50% loss of hydraulic conductance (P_{50} value), that is commonly used when comparing the vulnerability to cavitation between species. In conifers, this P_{50} value also represents a threshold value for xylem tension above which plants cannot recover anymore from drought (Brodribb and Cochard 2009). In angiosperms however, the xylem tension above which plants cannot recover anymore is that representing 88% loss in hydraulic conductance and is called the P_{88} (Urli *et al.* 2013).

4. Tree mortality, current methods to evaluate it and ongoing questions

4.1. Death in plant physiology

In plant physiology, one study clearly defined tree death: "Death is defined as thermodynamic equilibrium between the organism and the environment, in which plants no longer have energy gradients to drive metabolism or regenerate." (McDowell 2011a). It was pointed out lately that tree death from drought is still poorly define and that the definition given by McDowell (2011a) remained limited in utility as it does not provide with proxies that can help to identify clearly if a tree is dead or not (Anderegg *et al.* 2012a). Therefore, Anderegg *et al* (2012a), proposed that tree death can be considered as "a complete system failure due to lack of water resources".

Despite these definitions of tree death, in practice, it is difficult to determine if a tree is dead or not. In fact, even if Anderegg *et al.* (2012a), settled a definition for tree death, they pointed out the fact that their definition was based on the **ability of the tree to recover or not from drought events**. They also highlighted the fact that studies needed to clearly define what tree mortality is and provide with criteria that could permit the irrevocable identification of dead trees.

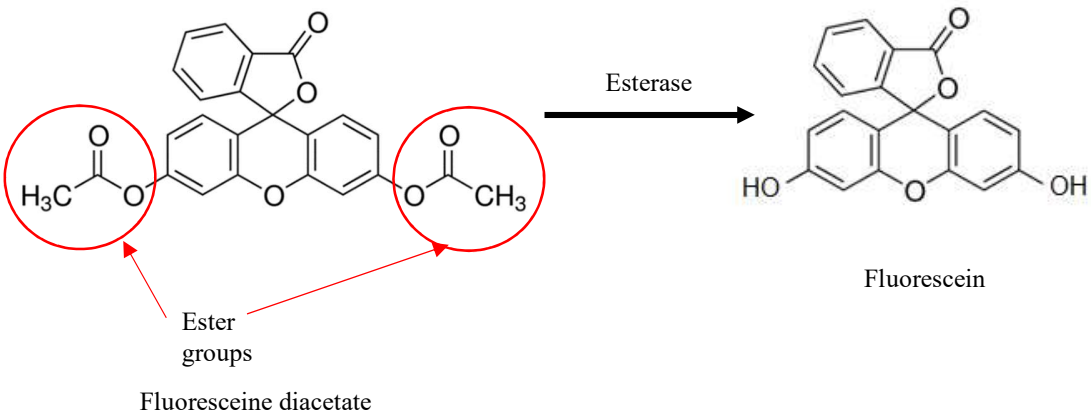
Therefore, with the lack of accuracy in the tree death definition, it seems difficult to determine accurately when a tree is in total failure or at which point its gradients are so low that they cannot maintain the metabolism of the plant anymore. **For this reason, most of the studies on tree mortality are based on methods evaluating the ability of the tree to recover or on the identification of living tissues by direct observation.**

4.2. Evaluating tree mortality

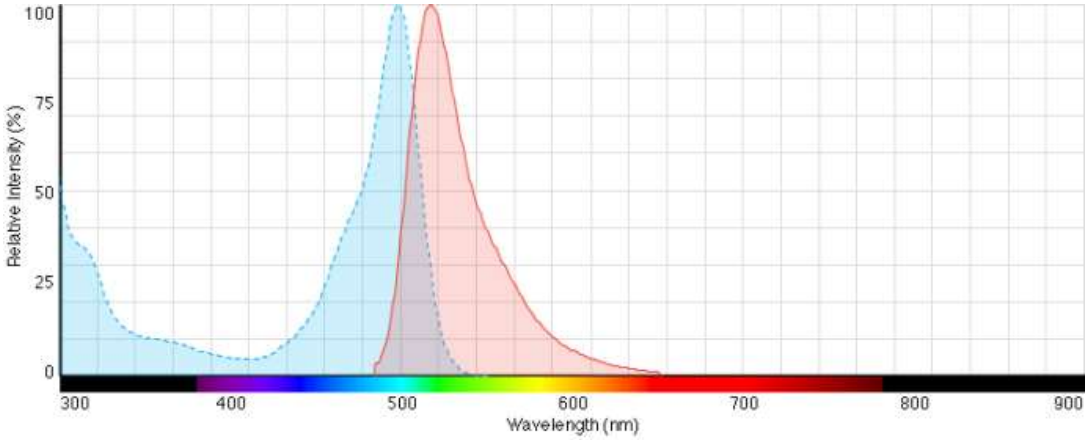
In many studies, trees are considered as dead when they cannot recover from drought-induced cavitation and are unable to grow the next vegetative season (Lloret *et al.* 2004, Rice *et al.* 2004, Brodribb and Cochard 2009, Anderegg *et al.* 2012b, Barigah *et al.* 2013b). Thus, for conifers, it has been shown that when the percentage of loss in conductance (PLC) is higher than 50%, trees cannot recover anymore. In angiosperms however, the percentage of embolism leading to a significant reduction of the recovery capacity of the trees is around 88% (Urli *et al.* 2013). Because of this major difference between angiosperms and gymnosperms and even if the P_{50} and P_{88} are commonly accepted as indicator to identify tree death and as measurable indexes for tree mortality (Sperry and Love 2015), it is not clear what are the changes occurring at tissue level that make plants being not able to recover anymore. **Thus, based on those observations, trees are currently considered as dead from hydraulic failure when they are not able to recover from embolism and/or resprout the next year.** Even if this method of tree death identification based on the tree ability to recover is generally accepted by the scientific community, **it does not allow to identify the main drivers for tree death**. Also, it is necessary to wait until the next growing season to verify if plants are able to recover from drought and this could be an important limiting factor in many studies.

Another common method to determine if a tree is dead from drought can be by direct observation of the color of the tissues beneath the bark. Thus, O'Brien *et al.* (2014) considered a tree as dead "when no green tissue was observable under the bark on the stem" and confirmed their observations by "re-watering some seedlings" and evaluating which plants were able to recover the year after. This direct observation method is generally accepted but it does not provide us with enough physiological information for establishing a clear threshold between living and dead tree.

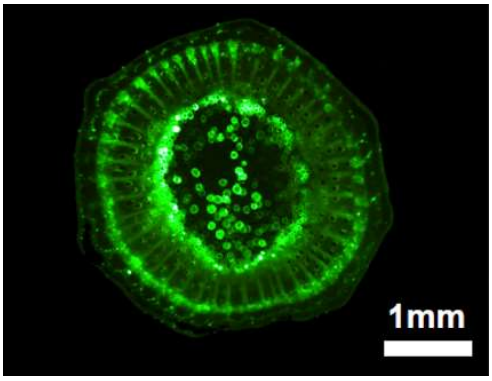
Therefore, it is crucial to identify new proxies based on physiological traits that would allow us to determine when exactly a tree dies, what is essential to predict accurately the time of death of the trees by using mechanistic models (Martin-StPaul *et al.* 2017)**.**



<u>Figure 9:</u> Chemical reaction transforming fluorescein diacetate in fluorescein.
(https://www.thermofisher.com/order/catalog/product/F1303 modified)



<u>Figure 10:</u> Fluorescein diacetate absorption spectrum (in blue) and restitute fluorescence (in red).
FDA absorbs blue wavelength (<500nm) and restitute a green fluorescence with a peak at 520nm.
(https://www.thermofisher.com/order/catalog/product/F1303)



<u>Figure 11:</u> Cross section of the upper stem part of a grapevine plant stained with FDA.
(Charrier *et al.* 2016)

5. Developing new methods to identify and predict tree mortality

5.1. Detecting living cells: a staining process

In order to determine which living tissues are affected by drought, it is necessary to identify where living cells are and how the amount changes during the dehydration of the tissues. For this, Fluorescein Diacetate (FDA), a cell-permanent esterase substrate able to stain the cytoplasm of living cells (Truernit and Haseloff 2008) could be used. In fact, FDA was first used to stain animal cells (Rotman and Papermaster 1966) and then used in plants cells (Widholm 1972). Briefly, FDA is a non-polar molecule that enters the living cells where the esterase (active only in living cells) cleave the acetate residues and form fluorescein molecule, a non-lipophilic molecule that cannot cross the cell membranes and, therefore, accumulates in the living cells (Widholm 1972) [Figure 9]. Once excited by wavelength around 490-500nm, the fluorescein molecule restitutes a yellow/green fluorescence [Figure 10], allowing the identification of the living cells [Figure 11].

Thus, one of the aims of the study is to develop a staining protocol using FDA that can be used for monitoring the changes in the number of living cells in the different plant tissues during drought. Although qualitative, the goal is to evaluate whether the decrease in the amount of living cells in given plant tissues can be used as a proxy for setting a threshold for plant death (i.e. lack of recovery).

5.2. Predicting tree mortality by screening membrane failure

When soil water availability decreases, plant mineral uptake decreases as well and reduces the concentration of key minerals for the cell membrane stability, as potassium or sodium (Wang *et al.* 2013). Such reductions decline the cell membrane stability while the accumulation of reactive oxygen species (ROS) increases. Indeed, normally ROS are destroyed by the antioxidant capacity of the plant. However, under water-stress conditions, the antioxidant capacity of the plant is depleted while the production of ROS by the photosystems (PS) I and II increases (Guadagno *et al.* 2017). Thus, the accumulation of ROS in the cell can certainly leads to the disruption of membranes (Petrov *et al.* 2015). Therefore, due to the disruption of membranes, under water-stress conditions, the cells' electrolyte leakage should increase, and this trait could be followed using the electrolytes leakage protocol developed by Zhang and Willison (1987) and Sutinen *et al.* (1992). In addition, Guadagno *et al.* (2017) also defined plant mortality as a "threshold in membrane disruption for which plants cannot recover" what could suggest that electrolyte leakage could work

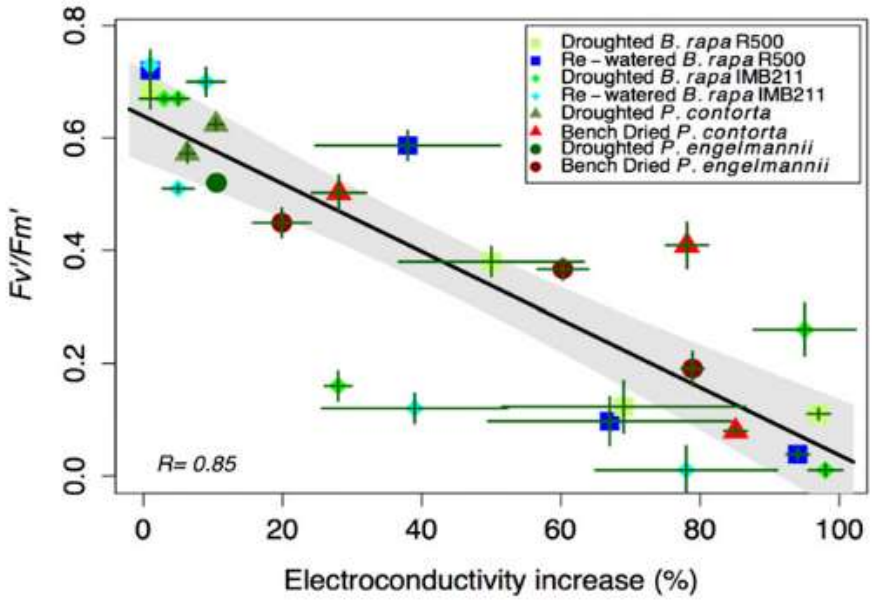


Figure 12: Link between electrolyte leakage and chlorophyll a fluorescence.
Electrolyte leakage is measured as electroconductivity increase (%). Fv'/Fm' represents the maximum efficiency of photosystem II under light conditions. We notice a decrease in Fv'/Fm' as the percentage of electroconductivity increases. Therefore, because electroconductivity is linked with membrane stability, which decreases under water-stress condition, it is possible to conclude that under drought conditions, cells damages increase along with the loss of efficiency of the photosystem II.
(Guadagno *et al.* 2017)

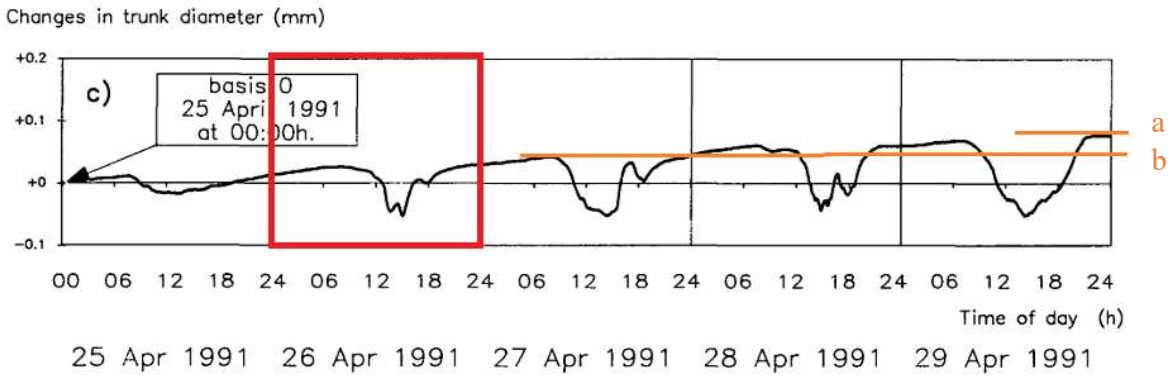


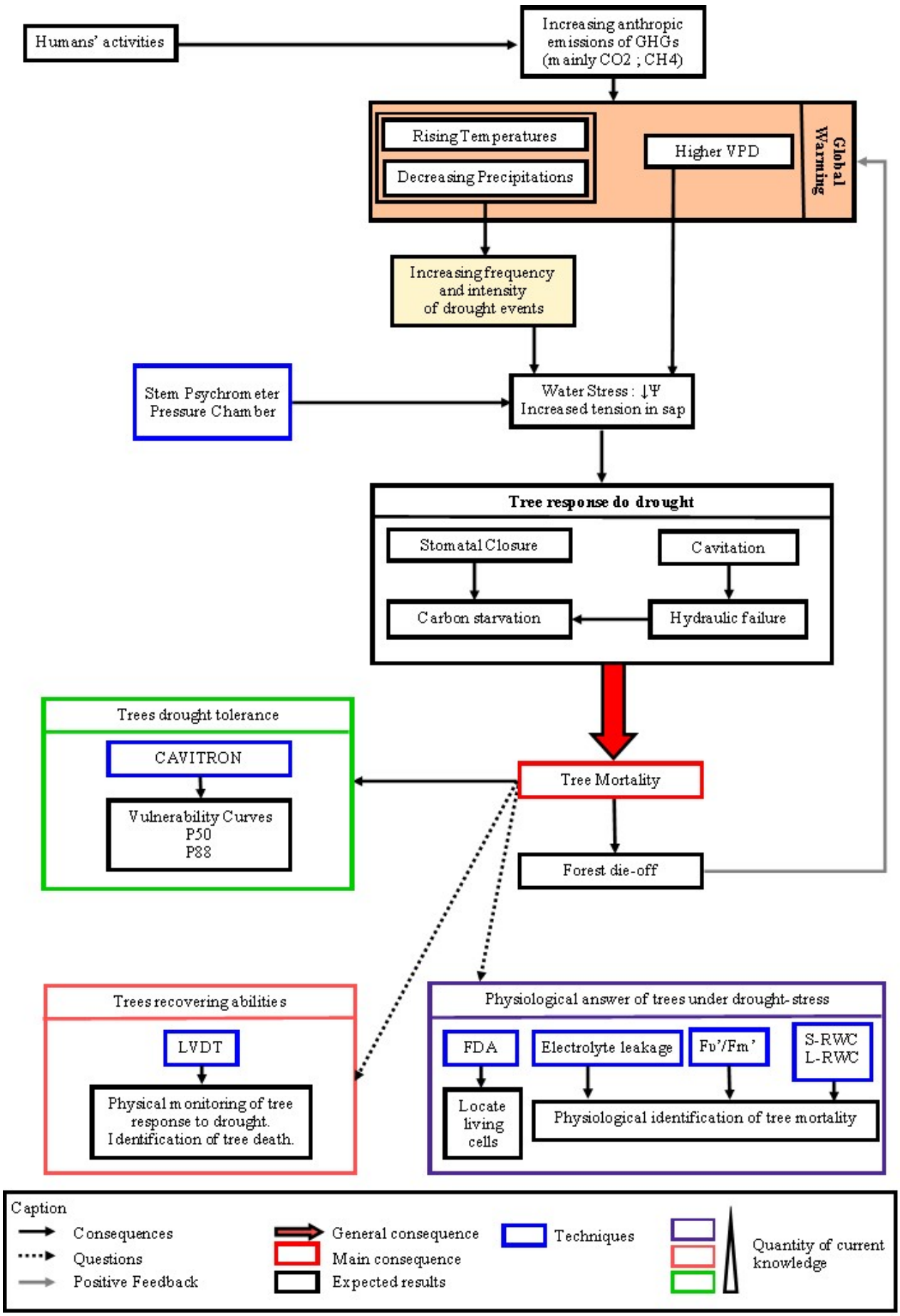
Figure 13: Changes in trunk diameter (mm) recorded with LVDT sensors on young peach trees (*Prunus persica* L.).
Surrounded in red is the daily variation of the trunk diameter of 5-year-old peach tree. It is possible to notice a shrink in diameter in light conditions (from 09:00 to 17:00).
The growth rate of the peach tree can be determined by looking at the differences between a and b levels in regard to the time variation.
(Simonneau *et al.* 1993)

as a proxy for point of death. Therefore, **the aim is to set a threshold in percentage of electrolyte leakage allowing the identification of tree's death.**

It is also known that the variable fluorescence (Fv) is linked with the presence of intact photosystems (Franck *et al.* 2002). Thus, under drought stress conditions, the accumulation of ROS can lead to membrane failure and the photosystems located in the membrane would suffer from the membrane disruption. Hence, Fv should be impacted resulting in a variation of the chlorophyll a fluorescence (Fv/Fm) with water-stress. Therefore, the chlorophyll a fluorescence in light conditions (Fv'/Fm'), which set the maximum efficiency of the PS II, should also be impacted. Considering this, it has been recently reported a link between electrolyte leakage and Fv'/Fm'(Guadagno *et al.* 2017). Thus, the maximum efficiency of the PS II in light conditions, represented by the Fv'/Fm' ratio, decreased along with the increasing percentage of electroconductivity (that is to say, electrolyte leakage) [Figure 12]. Unlike electrolyte leakage that is a destructive technique, the variable fluorescence technique uses a fluorometer which can be used on intact plants and is therefore more appropriate to monitor drought impact on plants. **By combining those two techniques, monitoring electrolyte leakage and looking at loss of variable fluorescence, it could be expected to predict at which physiological threshold a tree dies from drought.**

5.3. Determining tree capacity to recover

The Linear Variable Differential Transformer (LVDT) technology converts a rectilinear motion into an electric signal (Hunter 2007) and is one of the most common methods for monitoring the variation in stem or trunk diameter to evaluate tree growth along the day (Ameglio *et al.* 2010, Adam *et al.* 2013). Indeed, under non-stressing conditions, stem diameter increases each day due to the secondary growth of the tree (Simonneau *et al.* 1993) [Figure 13]. On a daily basis, trunk and stems tends to shrink during the day due to the transpiration of the plant and to expand during the night once the stomata are closed and the transpiration rate is almost zero (Daudet *et al.* 2005). **Under drought conditions, the stems or trunk will shrink and these variations in stem diameter along the day will significantly decrease until showing any change.** The hypothesis is that depending on the duration of the water stress and once rehydrated, plant would show again some variation in stem diameter if they are able to recover from stress. If no changes in diameter are observed after re-watering the plants, the plant can be then considered as dead from drought.



<u>Figure 14:</u> Summary diagram of the state of the art, ongoing questions and purpose of the master project. (©Marylou MANTOVA)

6. Purpose of the master project

In general, there is a lack of studies focusing on the physiological mechanisms behind tree mortality *per se* and the response of plant to water stress (McDowell 2011a ; Guadagno *et al.* 2017). Considering this, **the aim of this master project is to evaluate and develop new techniques that, combined with classical methods used in plant physiology [Figure 14], will enable us to**:

(i) Identify key physiological traits that could work as proxies for tree mortality.

(ii) Determine the main changes occurring at the plant tissue level explaining the lack of recovery after drought.

(iii) Establish a physiological threshold for plant death based on physiological measurements and not only in the capacity of recovery of plant the following growing season.

Materials and methods

Plant material, growth conditions and experimental design

The experiments were conducted from *January 22, 2019* to *May 31, 2019* at the INRA research station of Clermont Ferrand, France (45877'N, 3814'E; altitude of 300m). Two evergreen species were selected to carry out this experiment during wintertime: one angiosperm, *Prunus lusitanica*, and one conifer, *Pseudotsuga menziesii*.

Both *Prunus lusitanica* and *Pseudotsuga menziesii* young trees (8 individuals per species [Table 1]) were grown under non-limiting water conditions in 5L and 9.2L pots respectively, at the Lycée Agricole Louis Pasteur nursery in Lempdes (France) and at the INRA research station of Clermont-Ferrand, respectively. *Pseudotsuga* individuals were 4 years old at the time of the experiments while *Prunus* ones were 2 years old. Fifteen days prior the measurements, all trees were moved to a controlled-environment glasshouse cell and kept under natural light and at a temperature oscillating between 1°C and 23°C. During this period, plants were kept well-irrigated (field capacity) by a drip irrigation system controlled by an electronic timer.

To expose the plants to a severe event of water stress, irrigation was withheld in set of four to six individuals that were removed from the pots, their roots washed with water and the whole individuals were air dried to facilitate dehydration. Plants were let to dehydrate until reaching severe levels of water stress that correspond with high levels of PLC, and then rehydrated at field

Prunus lusitanica		*Pseudotsuga menziesii*	
Individual	**Drought event**	**Individual**	**Drought Event**
Prunus 1	February 14, 2019 to February 22, 2019	Douglas 1	April 05, 2019 to April 18, 2019
Prunus 2	February 14, 2019 to February 22, 2019	Douglas 2	April 05, 2019 to April 23, 2019
Prunus 3	February 14, 2019 to February 22, 2019	Douglas 3	April 05, 2019 to April 24, 2019
Prunus 4	February 14, 2019 to February 22, 2019	Douglas 4	April 12, 2019 to May 07, 2019
Prunus 5	March 22, 2019 to April 02, 2019	Douglas 5	April 16, 2019 to May 13, 2019
Prunus 6	March 12, 2019 to March 20, 2019	Douglas 6	April 19, 2019 to May 13, 2019
Prunus 7	March 12, 2019 to April 02, 2019	Douglas 7	May 03, 2019 to May 21, 2019
Prunus 8	March 22, 2019 to April 01, 2019	Douglas 8	April 30, 2019 to May 21, 2019

Table 1: Summary table of the individuals and their associated period of drought event.

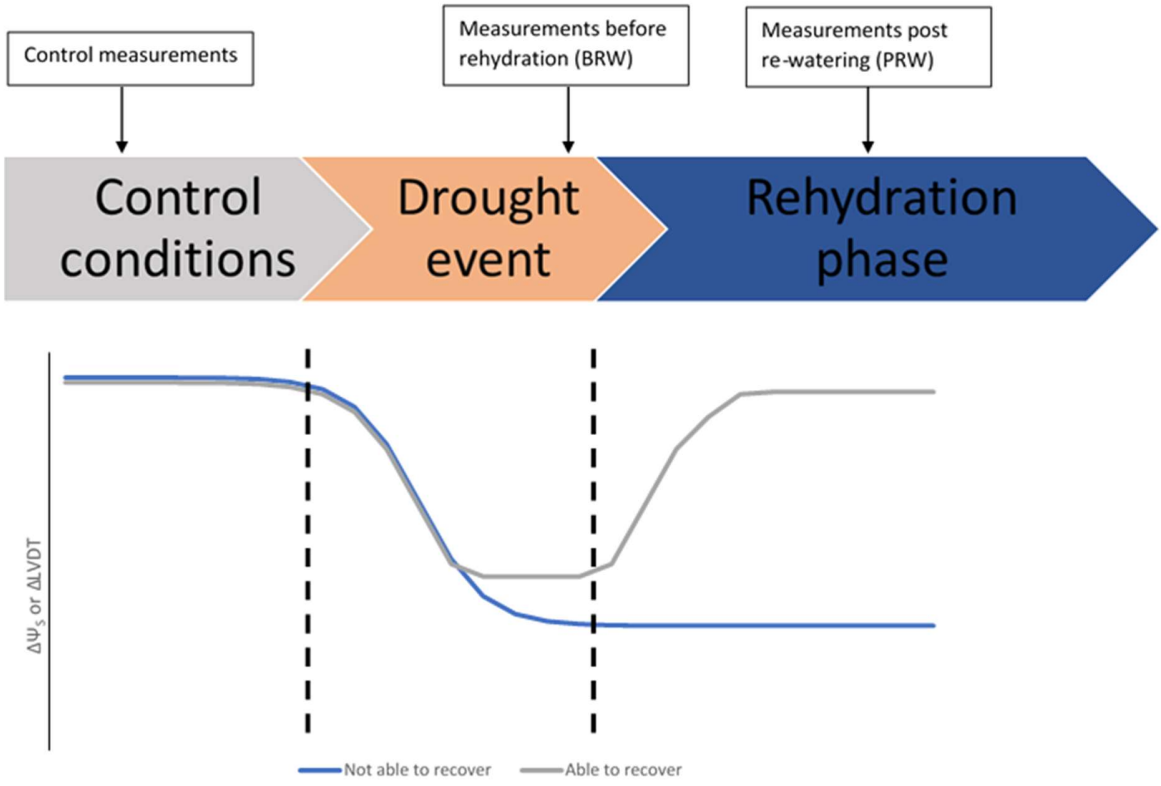


Figure 15: Timeline showing the different phases of the experiment and the time of punctual measurements of the different followed traits associated with the expected variations in stem water potential (Ψ_S) and stem diameter (ΔLVDT).

capacity for evaluating their capacity of recovery and survival. Thus, and according to the vulnerability curves obtained for both species (see more detail below), *Prunus lusitanica* plants were rehydrated once they reached water potential values of ca. -10.0 MPa whereas for *Pseudotsuga menziesii*, the irrigation for rehydrating the plants was applied once they showed water potential of ca. -7.0 to -9.0 MPa. Both during the dehydration and rehydration phases of the experiments, water potential and variation in stem diameter using LVDT were monitored continuously while PSII efficiency, leaf and stem relative water content, and electrolyte leakage were monitored punctually in 8 plants per species [Figure 15].

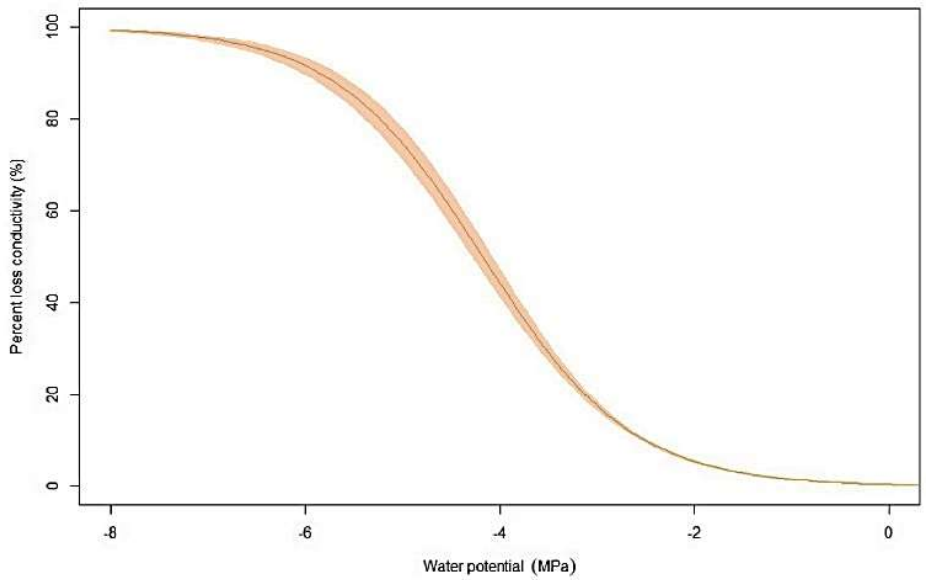
Vulnerability to cavitation

To ensure the accuracy of the results and avoid artifacts due to anatomical characteristics of the two species evaluated (Cochard *et al.* 2013, Torres-Ruiz *et al.* 2017a), two different techniques were used for evaluating the vulnerability to cavitation.

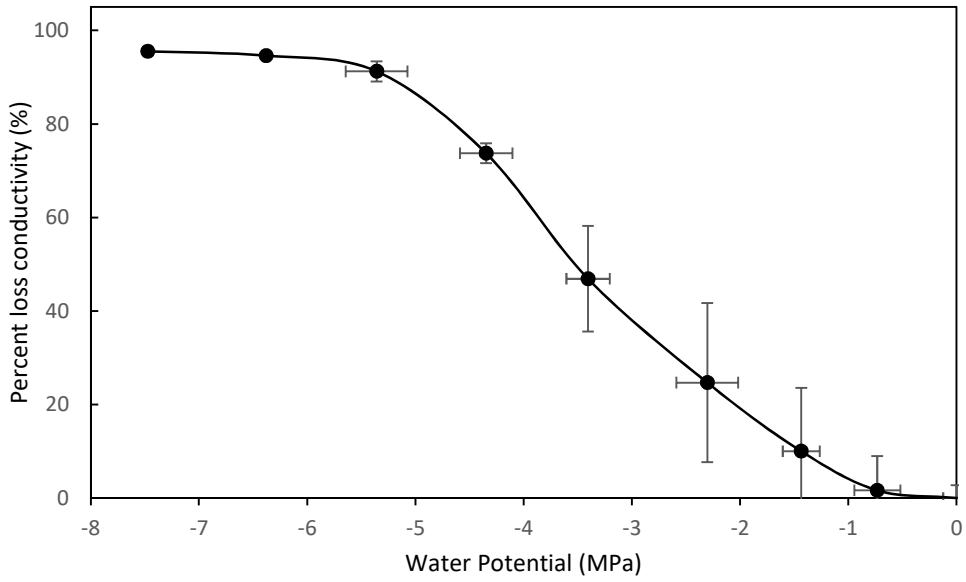
For *Prunus lusitanica*, xylem vulnerability to cavitation [Figure 16 (a)] was determined by using the **optical method** (Brodribb *et al.* 2017). Therefore, a clamp composed of a camera was set on an exposed part of *Prunus* stem xylem and captured images every 5 minutes in four individuals let dehydrating at open air. Based on the principle that light interacts differently with xylem that is water filled or air filled (Overview : The Optical Method - OpenSourceOV, 2019) it was possible to compare the images quantitative differences in brightness caused by cavitation events and to generate vulnerability curves regarding the decreasing water potential recorded by one ICT psychrometer (ICT international, Australia) set near the exposed stem xylem.

For *Pseudotsuga menziesii,* xylem vulnerability to cavitation [Figure 16 (b)] was assessed with the **Cavitron technique** (Cochard 2002) which uses centrifugal force to increase the water tension in a xylem segment while measuring the decrease in its hydraulic conductance. Thus, five 0.45m-long stem samples from five different trees (i.e. one samples per tree) were collected prior exposing the plants to water stress. All branches were debarked to prevent resin contamination and recut under water with a razor blade to a standard length of 0.27m. For calculating the vulnerability curves, the maximum sample conductivity (K_{max}) was measured at low speed and relatively high xylem pressure (−0.75 MPa). The xylem pressure was then decreased stepwise by increasing the rotational velocity, and the conductivity (K) measured at each pressure step. Each pressure was applied on the sample for 2 minutes. Sample loss of conductivity (PLC, %) was computed as follow: $PLC = 100 * (1 - \frac{K}{Kmax})$.

(a)



(b)



<u>Figure 16:</u> Vulnerability curves to cavitation for *Prunus lusitanica* stems (a) and *Pseudotsuga menziesii* stems (b)

a) Vulnerability curve for *Prunus lusitanica* stems obtained on four different samples using the optical method (Brodribb *et al.* 2017). The P_{50} is evaluated at -4.3MPa.

b) Vulnerability curve for *Pseudotsuga menziesii* stems obtained on five different samples using the CAVITRON technique developed by Cochard in 2002. The P_{50} is evaluated at -3.4MPa.

According to these vulnerability curves, *Prunus* individuals seems to be more resistant to cavitation dans *Pseudotsuga* individuals making them more resistant to drought.

The relationship between PLC and the water xylem pressure induced by centrifugation can be described by the following sigmoidal equation (Pammenter and Willigen Vander 1998):

$PLC = \frac{100}{(1+e^{a/25(P-P50)})}$ where *a* is the slope of the curve at the inflection point, and P50 represents the pressure at which 50% loss of conductivity occurred.

Xylem water potential measurements

During the time course of the experiment and for a continuous monitoring of the water potential, 4 psychrometers (ICT international, Australia) were installed in 4 plants (i.e. one psychrometer per plant) at the stem level and covered with aluminum foil to prevent their direct exposure to the sunlight and minimize the effect of external temperature variations (Vandegehuchte *et al.* 2014) [Figure 17]. Psychrometers continuously recorded the stem water potential every 30mn. Regular measurements were also made using a Scholander-type pressure chamber (PMS, Corvallis, Oregon, USA) to monitor the stem xylem water potential (Ψ_S), the leaf water potential (Ψ_L), and assess plant water status (McCutchan and Shackel 1992). These additional measurements confirmed the accuracy of the values collected by the psychrometers (for the stem xylem water potential) and also enabled us to monitor a larger number of plants since the amount of ICT's available was limited. Stem water potential was always measured in two fully developed and healthy leaves previously bagged for at least forty minutes prior the measurements to prevent transpiration and promote equilibrium with the plant axis. The stem xylem water potential was then assumed to be the same as the stem bearing the covered leaves (McCutchan and Shackel 1992, Barigah *et al.* 2013b). When the stem water potential reached values below -8MPa, the terminal part of the stem was directly used to evaluate the stem water potential since, probably due to most hydraulic disconnection of the leaves from the stem vessels, it was not possible to carry out the measurements in the leaves anymore. Ψ_L was measured daily on three healthy leaves directly cut from the plant until their water potential could not be measured anymore using the Scholander-type pressure chamber. Both leaves and stem samples were cut from the plant and immediately stored in a vial containing a piece of wet paper inside to saturate the air and prevent dehydration until carrying out the measurements.

Stem diameter variations

Stem diameter variations were measured continuously by Linear Variable Differential Transformer (LVDT) sensors set up on each plant before exposing them to water stress.

<u>Figure 17:</u> ICT psychrometer covered in aluminum foil to protect it from direct sunlight exposure.
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<u>Figure 18:</u> LVDT sensors installed on plants during the time-course of the experiment.
a) LVDT sensor installed on *Pseudotsuga menziesii* trunk;
b) LVDT sensor set up on *Prunus lusitanica* stem.
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The needle which correspond to the moving part of the measurement sensor was applied on stem with glue [Figure 18]. Sensors were connected to a data logger (Model CR1000, Campbell Scientific LTD, Logan, Utah, USA) and recorded an average of stem diameter variation (in mV) every 10 minutes.

Relative Water Content

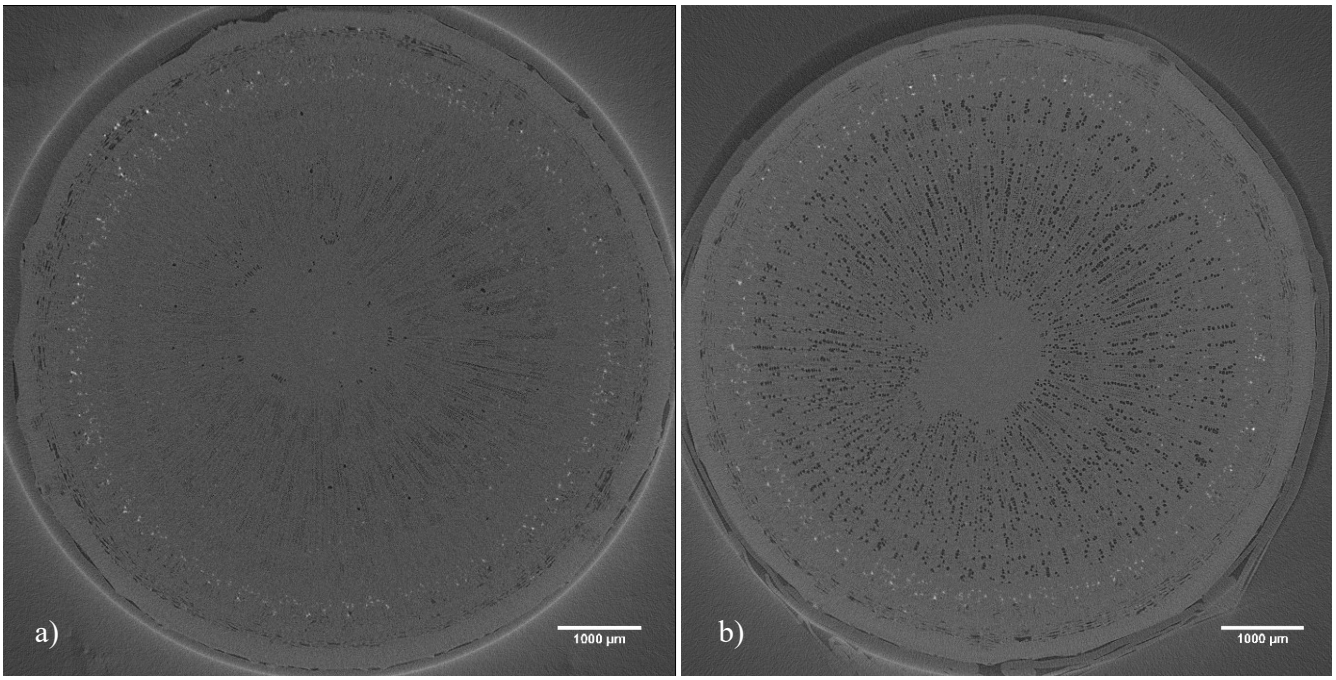
Stem relative water content (S-RWC) and leaf relative water content (L-RWC) were measured every 1 to 3 days according Barrs and Weatherley (1962): $RWC = \frac{(FW-\quad)}{(TW-DW)}$ where FW is the fresh weight measured immediately after sampling; TW is the turgid weight measured after immersing the stem in distilled water for 24 hours (for S-RWC) or after soaking the leaf petiole for 24 hours (or until total weight gain) in distilled water (for L-RWC); and DW is the dry weight of the samples after 24 hours of drying in an oven at 70°c (or until measuring a stable dry weight). All measurements were obtained with a precision scale (METTLER AE 260, DeltaRange ®) and were performed on three healthy leaves or three small stem sections.

Electrolyte leakage

Cell damages induced by drought were determined on three stem samples per plant using the electrolyte leakage test (Zhang and Willison 1987, Sutinen *et al.* 1992). Briefly, stem samples were cut in 10 slices of 2mm of thickness and put in test tubes containing 15mL of pure water. Test tubes were shaken at 60 shakes/min during 24hours at 5°c to stop enzyme activity. Water conductivity of the effusate (C1) was measured at room temperature using a conductimeter (3310 SET1, Tetracon® 325). Then, all the cells contained in the test tubes were killed by autoclaving the samples at 121°c for 30minutes (King and Ludford 1983), let cool at room temperature for 60 minutes and the maximal conductivity (C2) measured. The lysis percentage (EL) was then determined using the following equation: $EL = \frac{C1}{C2} * 100$.

Chlorophyll a fluorescence

Chlorophyll A fluorescence measurements in light conditions (Fv'/Fm') were performed on five healthy leaves per plant using a fluorometer (PAM-210 Chlorophyll Fluorometer). Measures were done on the adaxial side of one sun-exposed leaf and consisted in applying one saturating pulse of light, emitted with a Red LED (emission peak at 665nm, max intensity 3500µmol.m^{-2}.s^{-1} PAR, duration 3µs), and performed approximately in the middle of the leaf while avoiding the mid rib.



<u>Figure 19:</u> X-ray microtomograph (Micro-CT) scans showing the state of embolism of *Prunus lusitanica* individuals in a) native scan and b) after the cut and injection with air. The black holes correspond to embolized vessels of xylem. The percentage loss of conductance is determined by computing the amount of cavitated vessels before and after the cut and is here equal to 4.65%.

Quantification of xylem embolism

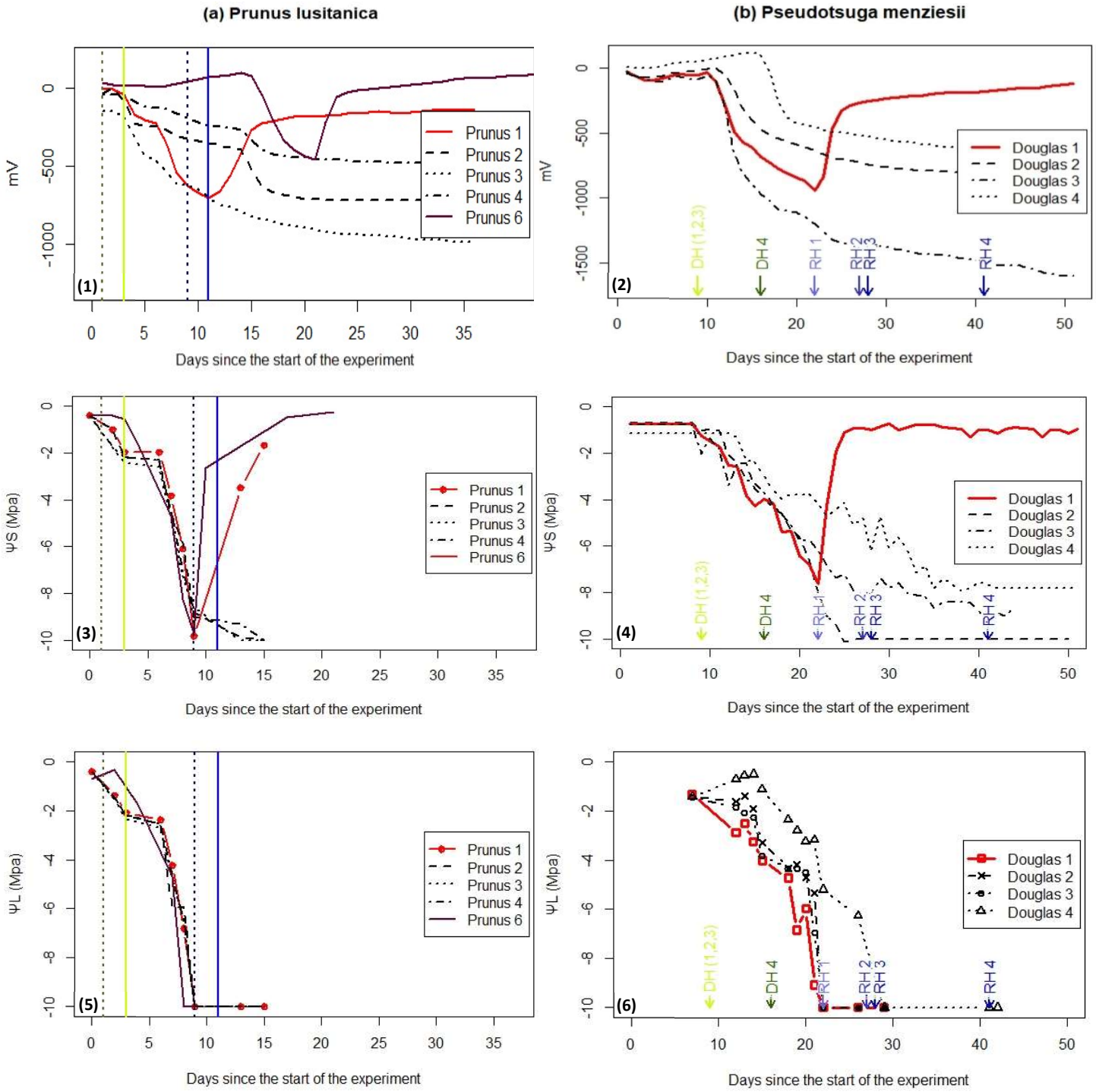
The amount of embolism in the stem was evaluated along all the experiment by determining the loss of hydraulic conductance (PLC) using a xylem embolism meter (XYL'EM, Bronkhorst, Montigny-Les-Corneilles, France) and also by direct observation using X-Ray microtomography (Nanotom 180 XS; GE, Wunstorf, Germany) (Micro-CT) at the PIAF laboratory (INRA,Clermont-Ferrand, France)(Cochard *et al.* 2015). For both techniques, samples were cut underwater to prevent artifactual increases in the amount of embolism in the samples (Torres-Ruiz *et al.* 2015).

For XYL'EM, in both species, the PLC was evaluated using three stem samples from each individual. The sample length was at least of 30mm. For both species, the initial conductance (K_i) was measured at low pressure. In order to determine the maximal conductance (K_{max}), *Prunus* samples were flushed with water at high pressure (2bars) for 20 minutes to remove the embolism. *Pseudotsuga* samples, which are composed of tracheids and not vessels, could not be flushed at high pressure. Therefore, in order to remove embolism, *Pseudotsuga* samples were immersed in water and put under the action of a vacuum pump to create void overnight.

For Micro-CT, for both species, one or two samples (depending on the quantity of plant material) were immersed in liquid paraffin wax to prevent their dehydration during the 21 minutes scan. At the end of the experiment, samples were cut 3mm above the scanned cross section, injected with air (0.1MPa) and re-scanned to visualize all the emptied vessels [Figure 19]. The amount of PLC was computed by determining the amount of cavitated vessels in the samples before and after cutting the sample. This then permitted to determine the PLC for each sample.

Cytology: detecting living cells

Fluorescein diacetate (FDA) (F7378-10G, SIGMA-ALDRICH, Co, St Louis, MO, USA), was used to stain the cytoplasm of living cells and therefore identify qualitatively the amount of living cells and their location while plants were exposed to water stress conditions and after re-watering them. For this, 60µm thick cross sections were obtained with a microtome (Leica RM2165) and stained for 20minutes in a 1% solution of FDA made from a stock solution of 5mg/mL of FDA diluted in acetone (Widholm 1972). Cross sections were mounted in a slide and observed using an inverted fluorescence microscope (Axio Observer Z1, ZEISS; YFP filter) within the following hour. Photos of the entire cross section were obtained by assembling automatically tiles (which correspond of different photos of the cross section) and were obtained with Zen 2 software. The number of tiles per image variated between 16 tiles (for small cross sections) to 110



<u>Figure 20:</u> Dynamic of the stem diameter recorded by LVDT (in mV) (1,2), the stem water potential (Ψ_S) (3,4), the leaf water potential (Ψ_L)(5,6) in *Prunus lusitanica* (a) and *Pseudotsuga menziesii* (b).

(a) For *Prunus* individuals, the dehydration started on the third day of the experiment (green line) for Prunus 1,2,3 and 4 and ended 8 days later with the beginning of the rehydration phase (blue line). The dehydration of Prunus 6 started on the first day (dashed green line) and ended 8 days later (dashed grey line). Ψ_S and Ψ_L consisted of punctual measurements made with the Scholander Pressure Chamber.

(b) For *Pseudotsuga* individuals, the dehydration (DH) started between the 9 and the 16 day of the experiment and ended with the rehydration (RH) between the day 22 and the 41 of the experiment. Ψ_S was continuously recorded with ICTs psychrometers while Ψ_L was measured punctually with the Scholander Pressure Chamber.

For both species, Ψ_L measurements were measured until reaching the limit of the pressure chamber (-10MPa).

tiles for (big cross sections) for both species. Photo processing was done using Zen 2, Image J and Photoshop CS5 softwires.

Statistical analyses

Statistical analyses consisted of *paired t-test* (after testing for normality and homogeneity of variances) and *Wilcoxon test* (for non-normal distribution) and were performed using R program to compare the set before and after the drought event and before re-watering and after re-watering. A *one way analysis of variance (ANOVA)* (for normal distribution and homogeneity of variance) or a *F Test of Welch* (without supposing homogeneity of variance) were used to compare individuals within each set for each trait before (Control), after the drought treatment (BRW) and during the rehydration phase (PRW). When the differences were significant*, a multiple comparison of means (post hoc Tukey honest significant difference test or Tukey contrast)* was carried out. All tests were performed using a level of significance α=0.05.

Results

1. Water potential and trunk diameter dynamics

Linear Variable Differential Transformer (LVDT) sensors showed a noticeable shrinkage in the stems of *Prunus* and *Pseudotsuga* during the time-course of the drought event and a subsequent increase in the stem diameter following rewatering for those individuals that were able to recover from drought. Thus, Prunus 1 and Prunus 6 which reached Ψ_S values of -10MPa [Figure 20 (3)] showed an increase in stem diameter immediately after being re-hydrated [Figure 20 (1)]. However, Prunus 2, 3, 4 [Figure 20 (1)], and the others plants (Prunus 5,7,8 [Annex 1]) which also reached a Ψ_S of -10MPa did not show any increase in stem diameter after the rehydration, being unable to recover from drought and therefore considered as dead plants. For *Pseudotsuga*, only Douglas 1 was able to show a recovery in trunk diameter [Figure 20 (2)] after rehydration at Ψ_S=-7.48MPa [Figure 20 (4)]. For both species, those individuals that were able to recover after drought did not reach the same values for stem diameter than the ones they showed before dehydration despite showing similar Ψ_S values before being dehydrated and after the rehydration. Leaf water potential (Ψ_L) [Figure 20 (5,6)] and stem water potential (Ψ_S) [Figure 20 (3,4)] decreased progressively along the dehydration phase of the study for both species However, those individuals that recovered from drought only show a recovery in water status at the stem level (i.e. only Ψ_S recovered and reached similar values than before dehydration). The Ψ_L did not recover even in

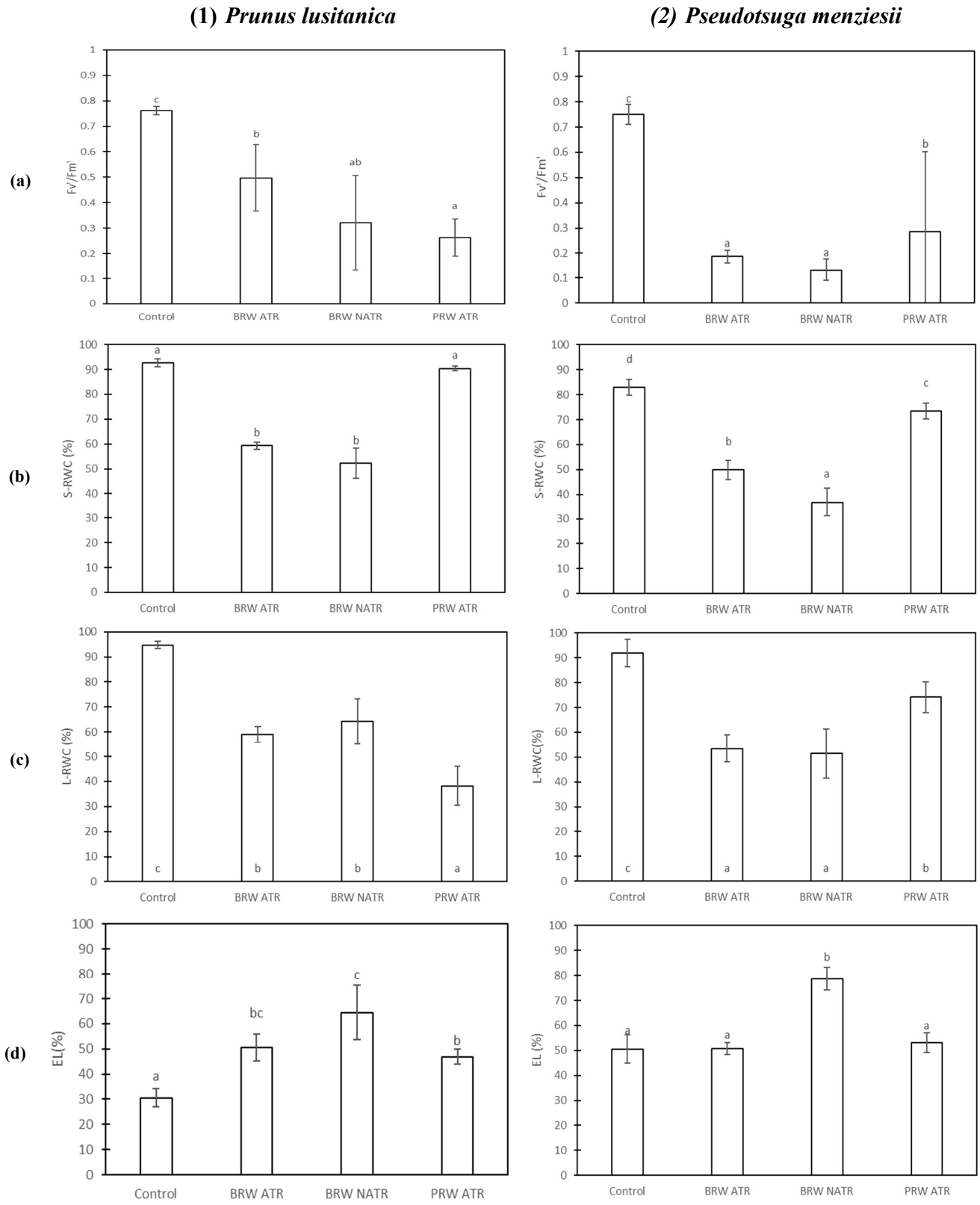


Figure 21: Variation of photosystem II efficiency (Fv'/Fm') (a), stem relative water content (S-RWC) (b), leaf relative water content (L-RWC) (c) and stem electrolyte leakage (EL) (d) in (1) *Prunus lusitanica* and (2) *Pseudotsuga menziesii*. Measurements were performed on all individuals in control conditions (Control) and after the drought event (e.g before re-watering the plants: BRW). The post re-watering (PRW) data correspond to the measurements performed on recovering individuals 27 to 76 days after rehydrating the trees. ATR stands for the individuals able to recover from drought while NATR refers to the individuals not able to recover from drought. Different letters indicate statistically significant differences (α=0.05) within individuals for each species.

recovering plants for both species, remaining at ca. <-10MPa for *Prunus* and <-8MPa for *Pseudotsuga*. Those plants that did not show any increment in stem diameter after the recovery irrigation did not show any recovery in plant water status either in Ψ_S nor Ψ_L.

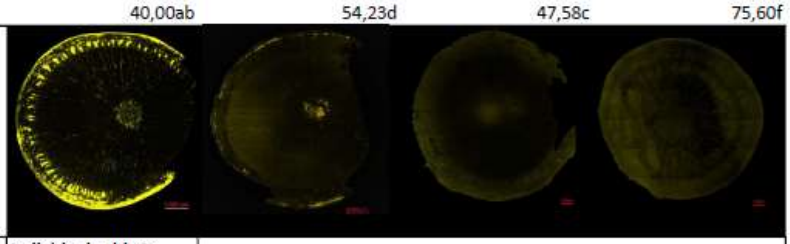
2. Dynamic of photosystem II efficiency, electrolyte leakage and relative water content

During the dehydration of the plants, a significant decrease in photosystem II efficiency (Fv'/Fm') and both in leaf (L-RWC) and stem relative water content (S-RWC) were observed for both species [Figure 21]. Thus, Fv'/Fm' mean values for *Prunus* decreased from 0.763 in control conditions to 0.498 at the end of the dehydration phase for those individuals that were able to recover (as indicated by changes in stem diameter) after irrigation (i.e. Prunus 1 and Prunus 6). However, those individuals that did not show any recovery after being rehydrated showed mean Fv'/Fm' values of 0.322 [Figure 21 (a)]. Similar results were observed for *Pseudotsuga* [Figure 21 (b)], which showed a PS II efficiency for individuals under control conditions of 0.750 and of 0.190 for those individuals able to recover from drought. Those individuals that were not able to recover, however, showed mean Fv'/Fm' values of 0.130. For both species, differences in Fv'/Fm' were not significant between individuals that were able to recover from drought and those that were not able to recover.

The **S-RWC** [Figure 21 (b)] followed the same dynamic as the Fv'/Fm' as its percentage significantly dropped from 92.6% in control conditions to 59.3% in *Prunus* individuals able to recover after the rehydration and 52.3% *Prunus* individuals that did not recover following rehydration. The differences in S-RWC were not significant when comparing individuals able to recover and those not able to recover. Similar results were observed for *Pseudotsuga*, showing S-RWC values that decreased from 83.0% for individuals under control conditions to 49.8% for those able to recover after dehydration (e.g. Douglas 1) and 36.9% for those that were not able to recover. Unlike *Prunus* individuals, the differences between *Pseudotsuga* individuals able to recover and those not able to recover were statistically significant, what suggests that this trait could work as a proxy for this species for determining when a plant is able to recover from drought.

Similarly to S-RWC and the Fv'/Fm', **L-RWC** was significantly impacted in both species by the drought event as it decreased from 94.71% in control conditions to 58.90% and 64.20% in *Prunus* individuals able to recover and those unable to recover respectively, while it goes from 91.80% (control conditions) to 53.50% (able to recover) or 51.50% (not able to recover) for

(a)

	Individuals able to recover		Individuals not able to recover	
	Prunus lusitanica 1	*Prunus lusitanica 6*	*Prunus lusitanica 4*	*Prunus lusitanica 7*
Drought event	February 14, 2019 to February 22, 2019	February 27, 2019 to March 20, 2019	February 14, 2019 to February 22, 2019	February 27, 2019 to April 04, 2019
Ψx (MPa)	<-10	<-10	<-10	<-10
PLC (%) :				
Xyl'em	98,60a	82,00b	95,8a	93,67a
Micro-CT	100,00a	89,27b	97,87a	87.03b
Fv'/Fm' (%)	0,46bc	0,54c	0,77d	0,006a
S-RWC (%)	57,08a	59,98d	67,92e	54,55bc
L-RWC (%)	52,81e	60,93a	45,6c	70,45ab
EL (%)	40,00ab	54,23d	47,58c	75,60f
FDA				

(b)

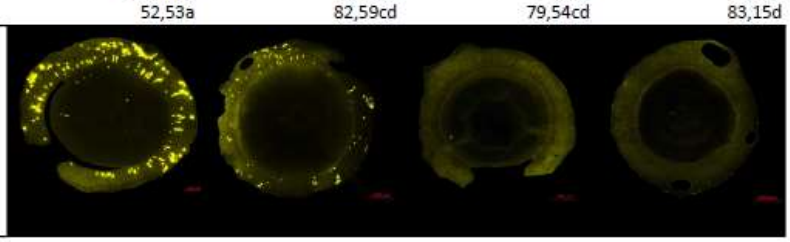
	Individuals able to recover	Individuals not able to recover		
	Douglas 1	*Douglas 4*	*Douglas 2*	*Douglas 3*
Drought event	April 05, 2019 to April 18, 2019	April 12, 2019 to May 07, 2019	April 05, 2019 to April 23, 2019	April 05, 2019 to April 24, 2019
Ψx (MPa)	-7,48	<-10	<-10	-7,68
PLC (%) :				
Xyl'em	96,80a	82,29b	72,40c	93,55a
Micro-CT	67,92a	73,58c	86,66b	68,76a
Fv'/Fm'	0,09a	0,08a	0,21b	0,13a
S-RWC (%)	49,82b	34,94a	34,77a	43,78b
L-RWC (%)	53,47bc	45,79ab	72,26c	31,67a
EL (%)	52,53a	82,59cd	79,54cd	83,15d
FDA				

Table 2: Summary of the *Prunus lusitanica* (a) and *Pseudotsuga menziesii* (b) drought experiment.

Each individual was dehydrated until its water potential surpass the P_{88} for Prunus and the P_{50} for Douglas, respectively. The PLC was measured on three stem samples of each plant using one hydraulic technique (Xyl'EM) and one imaging technique (Micro-CT). PSII efficiency (FV'/FM'), stem and leaf relative water content (S-RWC; L-RWC) and electrolyte leakage (EL) were then determined for each individual. Finally, the staining process using Fluorescein diacetate (FDA) (60µm thick cross section – 1% solution) was applied and microphotographs were taken using an inverted fluorescence microscope.

Different letters indicate statistically significant differences (α=0.05) within individuals for each species.

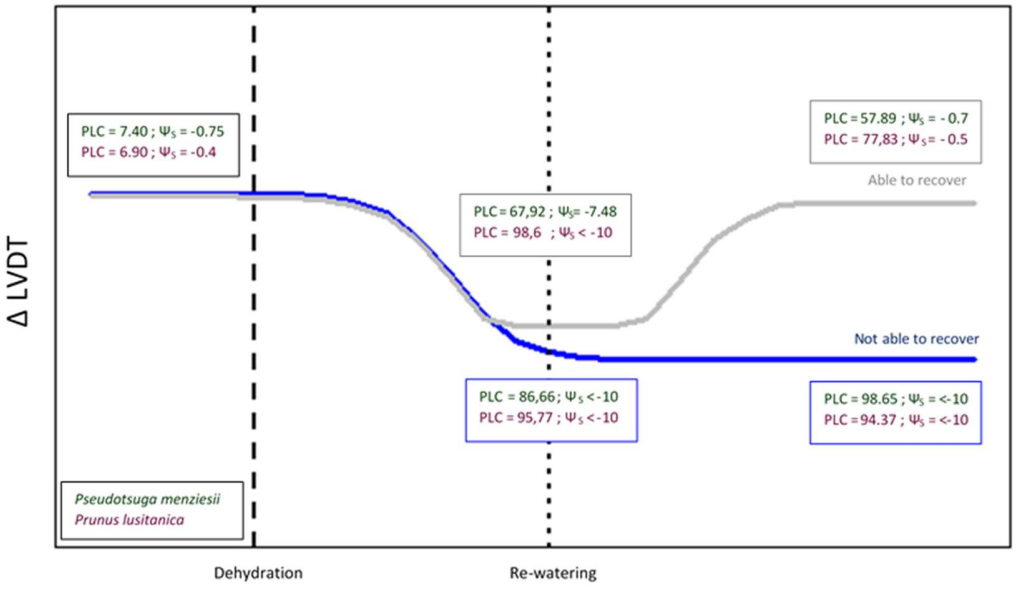


Figure 22: Theoretical curve showing variation in stem diameter in trees able to recover (grey) and trees not able to recover from drought (blue).

The PLC (loss of conductance) reached at the end of the dehydration phase for each individual was above the common threshold for mortality for both species: P_{50} for *Pseudotsuga* and P_{88} for *Prunus*.

Pseudotsuga. None of the two species evaluated showed significant differences between recovering and non-recovering individuals before applying the recovery irrigation [Figure 21(c)]. Contrary to the three previous traits, the **electrolyte leakage** showed a significant increase during the progressive dehydration of the trees for *Prunus* (control conditions: 30.6%; able to recover : 50.7% ; non able to recover 64.4%) and for *Pseudotsuga* (control conditions: 50.5%; not able to recover : 78.8%) [Figure 21 (d)]. However, the *Pseudotsuga* individuals able to recover do not show a higher electrolyte leakage after the drought event (i.e. control: 50.5%; Douglas 1 able to recover: 50.8%) while contrary, Prunus 1 and Prunus 6 that were able to recover showed a higher percentage of electrolyte leakage after the drought event (i.e. BRW: 50.7%%) than in control conditions (i.e. Control: 30.6%).

During the **post-rewatering phase (PRW)**, measurements of Fv'/Fm' and the L-RWC were performed between 27 and 77 days after the rehydration of the plants, with significant differences between the study species [Figure 21(a)]. First of all, in *Prunus*, the PS II efficiency decreased until reaching 0.263 in *Prunus* trees that were able to recover from drought according to results from LVDT and water potential. However, for *Pseudotsuga* a significant increase in the PSII efficiency was observed after the re-hydration of the plants (BRW: 0.186; PRW: 0.284). The **L-RWC** [Figure 21 (c)] showed the same pattern as the Fv'/Fm', e.g. it continues to decrease in *Prunus* individuals (BRW: 58.9%; PRW: 38.2%) while it recovered in *Pseudotsuga* (BRW: 53.5%; PRW: 74.1%). The electrolyte leakage [Figure 21 (d)] remained stable after re-watering the plants for both species (i.e. for *Prunus*: BRW: 50.7%, PRW: 46.84%; for *Pseudotsuga*: BRW 50.8%, PRW: 53.1%). The **S-RWC** [Figure 21(b)] in individuals able to recover increased significantly for both species but only reached its control values for *Prunus* individuals. Indeed, it increased from 59.3% (BRW) to 90.4% (PRW) for *Prunus* and from 49.8% (BRW) to 73.5% (PRW) for *Pseudotsuga*.

3. Hydraulic failure

The stem PLC values for *Prunus* and *Pseudotsuga* when measured via the Xyl'EM technique prior to the drought treatment (under well-watered conditions) was of 6.90% and 18.88%, respectively. PLC prior to the drought, according to the Micro-CT values, was 0.95% and 7.40% for *Prunus* and *Pseudotsuga* respectively. The stem PLC increased during the progressive dehydration of the trees, reaching values between 82.00% to 99,90% according to the Xyl'EM values for *Prunus* (e.g. Prunus 1: 98.60%, Prunus 7: 93.74%)[Table 2 (a)], i.e. above the threshold

Individual	Trait	BRW	PRW
Prunus 1	PLC	98.60	77.83**
	FV'/FM'	0.333	0*
	S-RWC	57.08	90.59**
	L-RWC	52.81	34.20*
	EL	40.00	49.89*
Prunus 6	PLC	89.3	54.83**
	FV'/FM'	0.194	0*
	S-RWC	59.98	90.13**
	L-RWC	60.93	42.28
	EL	54.23	43.80
Douglas 1	PLC	67.92	57.89*
	FV'/FM'	0.088	0.284
	S-RWC	49.81	73.49**
	L-RWC	53.47	74.11*
	EL	50.81	53.01

* significant level α = 0.01

** significant level α = 0.05

Table 3: Table summarizing the evolution of the different traits followed during the time-course of the experiment in plants able to recover from drought.

BRW represents the measurements performed on the individuals the day of the beginning of the re-watering phase while PRW represents measurements performed on the individuals 77 days after re-watering for *Prunus* 1, 62 days after re-watering for Prunus 6 and 27 days after the beginning of the re-watering phase for Douglas 1.

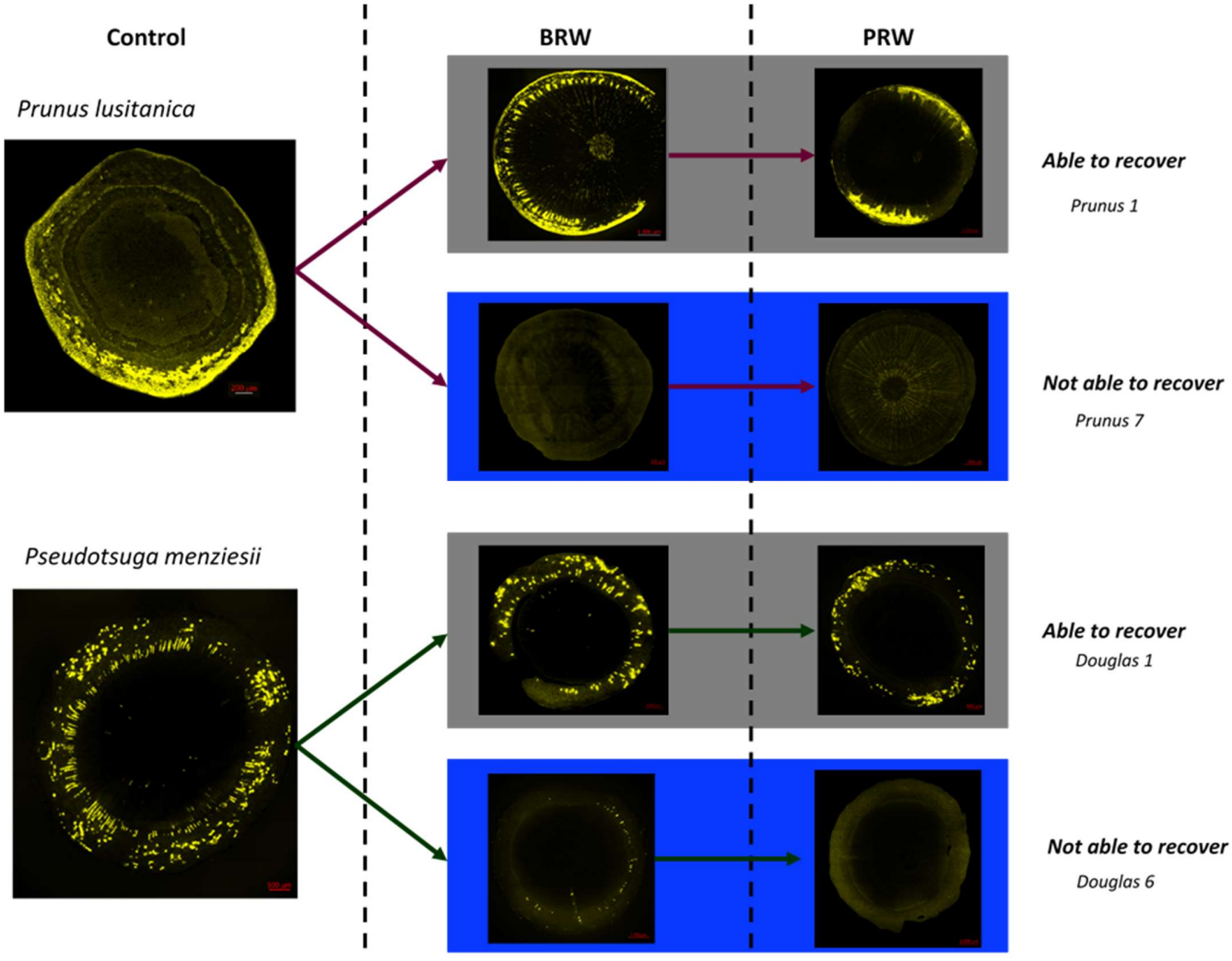


Figure 23: Evolution of the amount of living tissues between control conditions, before re-hydration (BRW), and 27 to 77 days after re-hydration (PRW) in cross section of *Prunus* and *Pseudotsuga* stained with fluorescein diacetate (FDA) and observed with a fluorescence microscope.

for recovery and point of death for angiosperms (i.e. P_{88}). For *Pseudotsuga* [Table 2(b)], and according to the Micro-CT measurements, the PLC values at the end of the dehydration phase of the study variated between 67.90% to 93.85% (e.g. Douglas 1: 67.90%), being therefore much higher than the threshold for recovery and point of death for conifers (i.e. P_{50}). Therefore, for the two species evaluated, some of the trees that reached PLC levels higher than the common threshold for mortality (P_{50} for conifers and P_{88} for angiosperms) were able to keep some living cells and to recover from drought once rehydrated [Figure 22]. Also, the levels of PLC tended to diminish when measured 10 and 4 weeks after rehydration for *Prunus* and *Pseudotsuga* respectively reaching 77.83% for Prunus 1 (Xyl'EM value) and 57.89% for Douglas 1 (Micro-CT value) [Table 3].

4. Effect of drought and recovery on living tissues

The use of FDA allowed to establish if living cells were present or not before and after rehydration [Figure 23]. In control conditions, all of the plants were all showing living cells in the bark and in the phloem proving that the technique was working to detect living tissues. However, no living cells could have been detected in the cambium in both species. The application of FDA BRW showed that the amount of living cells in both species decreased and was then affected by the drought. For *Prunus*, Prunus 4 and Prunus 7 [Table 2(a)] did not show any living cells, all the others showed living cells but the amount was less than in control conditions. However, out of all the plants showing living cells, only Prunus 1 and Prunus 6 [Table 2(a)] were able to recover from drought and to maintain the amount of living cells days after the rehydration [Figure 23]. All the other plants that showed living cells before rehydration (Prunus 2, Prunus 3, Prunus 5, Prunus 8) lost the fluorescence signal during the rehydration phase.

The same results were obtained for *Pseudotsuga*. Douglas 1, Douglas 4 [Table 2 (b)] and Douglas 6 [Figure 23] all showed living cells in the bark and phloem BRW. However, only Douglas 1 was able to recover from drought. All the other individuals did not emit fluorescence before rehydration (e.g. Douglas 2 and Douglas 3) [Table 2(b)]. Douglas 1 was able to maintain its amount of living cells during the rehydration phase while all the other ultimately loose the fluorescence signal after re-watering the plants [Figure 23].

<u>Figure 24:</u> ***Prunus lusitanica*** **n°1 flushing new leaves after experiencing a 8-days-long drought event and reaching a stem water potential of -10MPa.** Before the re-hydration phase of the experiment, measurements showed a loss of conductance at stem level of 98.6% which is higher than the common proxy for angiosperm mortality (88%).

Discussion

1. Is hydraulic failure the main driver for tree mortality?

Results showed that even when trees for both species reached critical levels of embolism (>90% for *Prunus* and >50% for *Pseudotsuga*), they still show evidence of living cells and were able to recover from drought when re-watered. These are very relevant and novel results since, until now, the threshold for recovery and point of death for trees were the water potential value inducing 88% and 50% (i.e P_{88} an P_{50}) of loss in stem conductance for angiosperms and conifers, respectively (Brodribb and Cochard 2009, Barigah *et al.* 2013b, Urli *et al.* 2013). *Prunus* individuals that reached PLC levels of 98.6%, i.e. well above than 88%, were able to recover and even flushed new leaves [Figure 24] after re-watering them to field capacity. Similarly, *Pseudotsuga* individuals showing PLC levels of 67.92% were also able to recover from drought once re-watered. However, our study was conducted in potted plants that were re-watered at field capacity after the dehydration phase. Under natural conditions, such increments in the amount of water available for the trees rarely occur, what would enhance the survival probability of the plants. Similar results have been recently reported for loblolly pine (*Pinus taeda*) by Hammond *et al.* (2019) which reported a lethal PLC threshold of 80%, i.e. much higher than 50% commonly reported for conifers. However, our study showed that recovery did not occur in *Pseudotsuga* individuals when PLC reached values above 70%. Therefore, this raises questions on how lethal PLC thresholds vary not only among tree species, but also within species. Our results, therefore, highlight the importance of revising the actual recovery and point of death thresholds suggested for angiosperms and conifers, and of considering the link between the amount of living cells together with the remaining plant hydraulic functioning when evaluating its recovery capacity from drought. This is especially important considering that these threshold values for mortality are crucial when using mechanistic models aimed to estimate the time to death of plants under drought conditions, as the SUREAU model (Martin-StPaul *et al.* 2017) and T-Crit model (Blackman *et al.* 2016). Our results, therefore, show how **the level of stem embolism should not be considered as a proxy for tree death solely** due to the similar high PLC values reported for both trees that were able to recover and for those that were not.

However because trees able to recover and re-draw water from soil showed similar high PLC values than the ones not able to, this highlights the importance of carrying out accurate PLC measurements both by using the Xyl'EM or the Micro-CT technique. Indeed, the Micro-CT

technique could potentially lead to artefactual PLC values higher than 100% in plants able to recover. The reason is that, by using Micro-CT, PLC is determined by comparing the area of the embolized vessels at a given water potential with the total xylem area of the sample, i.e. the total conductive xylem area. To facilitate the determination of the total xylem area, samples are injected with air and re-scanned (Cochard *et al.* 2015). The second scan can be not exactly at the same position than the first scan so, when the amount of embolized vessels is important, any change in the total amount of vessels just below or above the first scanning point can lead to important over- or underestimations of the PLC. In addition, after the cut, it is necessary to immerse the sample in paraffin wax that can go into some vessels and be considered erroneously as fully functional vessels, underestimating therefore the number of embolized vessels. In addition, the estimation of the PLC using the Xyl'EM is based on the ratio between the initial and the maximal conductance of a sample. However, as discussed by Cochard *et al.* (2013) the presence of already formed but non-functional vessels can lead to an overestimation of the maximal conductance of the sample. In addition, for conifers, pit membranes can remain permanently aspirated against the cell walls of the embolized tracheids leading to an underestimation of the maximal conductance and consequently to unrealistic PLC values (Cochard *et al.* 2013). Thus, the precision of the values estimated by the Xyl'EM can be discussed and the levels of PLC measured with this apparatus should be interpreted carefully.

2. Is there a link between water potential and tree mortality?

As established in the bibliography, a clear link between Ψ_S and mortality should be made. Water potential is the main driver for PLC and it has been traditionally used to set mortality thresholds, i.e. P_{50} and P_{88} (Brodribb and Cochard 2009, Barigah *et al.* 2013b, Urli *et al.* 2013). However, values of ca. 100% of PLC were reached at water potentials below -10.0 MPa, as shown by the fitted vulnerability curves to embolism. This, therefore, did not allow us to determine a threshold water potential value for tree mortality since trees with such high PLC values that were able to recover from drought showed water potential values below -10.0, i.e. below the minimum value that the available techniques are able to measure. Indeed, while both the psychrometers and the Scholander provide accurate water potential values for most of the species at mild water stress conditions, any of these two techniques can be used for such high xylem tensions mostly due to technical limitations (psychrometers) or to safety reasons (Scholander chamber). We tried to resolve this technical limitation by trying to measure those low water potentials using a water

activity meters (WP4-T, Decagon Devices, Pullman, Washington, USA) that was originally designed to follow soil water potentials up to -100MPa. However, when we compared the water potential values reported by this device and the ´gold-standard´ method, i.e. the Scholander pressure chamber, for both stem and leaf samples in between -0.5MPa and -10MPa, we observed how water potential values were highly underestimated by the WP4-T device. This agrees in fact with previous observations by Martínez *et al.* (*2013*) and invalidated therefore the use of the WP4-T as an alternative to measure the very low Ψ_L and Ψ_S values that trees, as in our study, normally reach when evaluating drought-induced tree mortality. Therefore, in order to be able to read accurate water potentials below -10MPa, current techniques need to be improved or new techniques are need to be developed. In fact, after our observations during this study, some suggestions has been made to the manufacturer of the psychrometers (ICT International, Australia) in order to help with the design of new psychrometers able to measure accurately water potential values below -10MPa.

3. Why do plants die?

3.1. Disruption of the continuum between available water for tissues and soil water

For the two species evaluated in this study, and despite the amount of living cells decreasing noticeably during dehydration, trees that were able to recover showed some living cells mostly located in the bark and in the phloem before rehydration. No living cells could be detected in the cambium, in either the control conditions or in recovering plants. This could suggest that either the FDA was not capable of entering the cambial cells probably due to differences in membrane composition or that the staining process was not long enough to allow the transformation from FDA to fluorescein. However, the presence of living cells at the stem level was not always related with the recovery of the plants after rewatering. This was the case for *Pseudotsuga* for which we did not observe any recovery for plants showing similar amount of living cells and at similar locations than those that were able to recover from drought after rewatering. Also, the *Pseudotsuga* individual showing living cells before rehydration (e.g. Douglas 6) and that was not able to recover had lost the FDA fluorescence signal 14 days after the beginning of the re-watering phase of the experiment. The explanation could be that under drought conditions, plants can rely on their own water reserves (Epila *et al.* 2017) which could maintain the metabolism of the cell a bit longer.

However, once the water reserves are drained, the tissues would ultimately dry and cells would dehydrate and die. Therefore, and according to these results, **we hypothesize that not only the presence of living cells is required for allowing the plant to recover from drought, but also a hydraulic connection between them and the root system that would allow the irrigation of the tissues and, therefore, would trigger the recovery of the plants.** Under severe drought conditions, plants would indeed rely on their own water reserves (Epila *et al.* 2017). Nonetheless, in order to survive a drought event those water reserves need to be refilled, re-establishing a hydraulic continuum from the roots able to transport the water from soil to the living tissues. This hypothesis suggests that even when PLC show value near to 100% for angiosperms or above 50% for conifers, a minimal hydraulic functioning between the soil and the living tissues could be enough to survive from drought if plants have access to water.

In addition, if plants are thus able to survive according to this hypothesis, it would be important to quantify the amount of living cells before the rehydration to answer unresolved questions as, e.g., is a small amount of living cell able to trigger the plant recovery from drought and keep it alive? Also, it is well known that, due to their totipotency capacity, plant cells are able to dedifferentiate and generate a whole organ from any cell/explant (Malamy and Benfey 1997, Laux 2004). Thus, under a drought-stress, is any of the remaining living cells able to trigger this dedifferentiation process or are specific cells the ones necessary for regenerating the plant tissues and recovering from drought? Therefore, long-term drought survival could rely on the ability to be able to grow new xylem tissues as suggested by Hammond *et al.* (2019).

3.2. Disruption of the continuum between cells and plant water reserves

It has been described that under severe drought conditions the water demand of the plant is almost zero as stomata are already closed in order to reduce the evaporative demand of the plant (Hochberg *et al.* 2017). Despite this, the different plants tissues remain irrigated until water potential reaches values low enough for inducing the xylem hydraulic failure and, therefore, the tree death because of the failing of the system to provide enough water to the crucial cells for survival. It was demonstrated that, under severe drought conditions, certain plants rely on their own water reserves and are therefore not reliant on water supply (Epila *et al.* 2017). Thus, a focus on cell hydration in living tissues could be the main key for plant survival (Martinez-Vilalta *et al.* 2019). However, our results in stem relative water content (S-RWC) did not show a clear pathway to understand tree mortality as no significant differences were noticed between plants able to

recover and those unable to do so. Therefore, rather than just focusing on the plant water status, a deeper study of water relocation in plants as suggested by Körner (2019) could be the key to understand tree mortality. Indeed, pressing question is to know whether the relocation of water from plant reserves would be enough for the key tissues to survive a drought event.

3.3. Fine root failure hypothesis

Contrary to our study which focused on stem hydraulic failure, a recent study on trees drought-driven mortality in Texas suggested that the fine root failure was probably more related to mortality than stem hydraulic failure as the trees which suffers the most important mortality rates (i.e. *Juniperus*) have lower (more negative) stem P_{88} values (i.e. more resistance to embolism) than those showing lower mortality rates (i.e. *Quercus*) (Johnson *et al.* 2018). So according to their results, a more detailed study considering the loss in hydraulic functioning in other plants organs as fine roots during drought would be required to evaluate relative role of hydraulic failure at root and stem level on drought-induced tree mortality.

4. When do plants die? Which trait can be used as proxy for mortality?

Our results show that most of the plants were still showing living cells prior to rehydration however not all of them were able to recover from drought. Therefore, when does a plant cross the point of no return and cannot recover anymore? This question has been widely debated in recent publications (McDowell *et al.* 2008, Anderegg *et al.* 2012a), where mortality has been defined as "a complex system failure due to lack of water resources". However, this definition still does not define when a plant crosses from life to death which is a current priority to understand the mechanism causing tree mortality (McDowell *et al.* 2011a, Martinez-Vilalta *et al.* 2019).

Unfortunately, our study failed to give a quantitative physiological threshold for drought-induced tree mortality. Indeed, our results contrast with those reported by Guadagno *et al.* (2017) showing how the loss of variable fluorescence could work as an operational proxy for plant mortality in *Brassica rapa*. In our study, no significant differences in PS II efficiency were observed between the trees able to recover and those showing no recovery from drought. This difference between Guadagno's and our study could be related with possible differences in main mechanisms related with the drought-mortality in herbaceous and woody species, although more research on this question should be carried out to confirm this hypothesis. However, our results agree with Guadagno *et al.* (2017) in that membrane failure at the cellular scale could be a possible proxy for mortality in conifers since it clearly seems to be the most proximate cause of death.

However the link between the membrane and the hydraulic failures is still unresolved.

As suggested by Martinez-Vilalta *et al.* (2019) a focus on plant water status is necessary to understand drought-induced mortality in plants. Therefore, the RWC for both leaf and stem was monitored during this study. However, S-RWC differed between plants that recovered from drought and those that did not in the case of *Pseudotsuga*. At the leaf level, despite Kursar *et al.* (2009) showed that tolerance to low leaf water status is correlated with species drought performance (Kursar *et al.* 2009)(Kursar *et al.* 2009)(Kursar *et al.* 2009), differences in leaf relative water content before rehydration of the trees in our study were not significant between recovering and non-recovering trees, invalidating this trait as a possible proxy identifying trees able to recover from drought. This discrepancy between Kursar *et al.* (2009) and our study is probably due to the fact that L-RWC was measured in our study when plants showed levels of water stress much higher than in Kursar *et al* (2009). So, probably, leaves were already hydraulically disconnected from the stems when measuring L-RWC, i.e. just before applying the recovery irrigation, for both species. This would have favored a faster dehydration of the leaves in comparison with the stems. It is also important to consider that this study was conducted on young trees in pots and that, to confirm if the results obtained can be extrapolated to mature trees growing in the field, a more extended and detailed experiment including both young and mature trees of different species and exposed to different levels of drought would be required in order to improve our knowledge about the key physiological thresholds for drought-induced tree mortality.

Conclusions and perspectives

By combining a living-cell staining process with LVDT sensors and PLC measurements, this study showed that the common thresholds for recovery and point of death considered until now, i.e P_{50} for conifers and P_{88} for angiosperms, seem to be not accurate enough for evaluating tree mortality. Our results showed that plants with PLC levels of 98.6% for *Prunus* (angiosperm) and 67.92% for *Pseudotsuga* (conifer) were still able to draw water from soil and recover from drought when they were rewatered. As our results were obtained on young trees, i.e. between 2 and 4 years, and for an angiosperm and a conifer species only, a more exhaustive and wider study including several species for each plant group and including individuals within a wider range of ages, i.e. from young to mature trees, would be required in order to confirm if our observations represent a general pattern for trees. So, if P_{50} and P_{88} cannot stand for threshold of mortality

identification, what physiological trait can be used to this purpose? This question is still unresolved as none of the followed traits during the time-course of this experiment demonstrated a clear threshold for distinguishing, at severe drought conditions, those trees that have succumbed to drought from those that would still be able to recover from it and therefore survive. Therefore, none of them would work as a proxy for tree mortality on their own.

The fact that plants that were exposed to severe drought conditions and still show some living tissues at the stem level as well as a minimal hydraulic functioning, however, makes us to hypothesize that, as far as the remaining living tissues of the stem are hydraulically connected to the roots, the recovery of the tree from drought is possible. Still, after the hydraulic disconnection between the stem and the root, some living tissue could be detected at the stem for a given amount of time, what could generate some confusion at the time of determining if a plant is alive, dying or already dead base on these observations only.

Results from our study have generated new interesting questions about the behavior of the living cells during dehydration. Thus, whether isolated living cells are capable of dedifferentiate and renewal the plant tissues during the recovery from drought is still unclear. Similarly, dynamic of cellular death, i.e. what are the first and last tissues to die when trees reach high levels of water stress, is still unclear. After this study, however, we have identify different techniques that could be very helpful for addressing these unsolved questions. Thus, the FDA staining process developed in this study; correlated with the recovering ability of the tree shown by LVDT sensors, would be helpful to identify key tissues for tree survival. Also, if plants rely on their own water reserves to keep the irrigation of the different tissues during drought, it would be interesting to look at the water dynamic in the different plant tissues during a drought event. For this purpose, Nuclear Magnetic Resistance (NMR) imaging could be used like it was done by Barigah *et al.* (2013a) to identify the water distribution in trees. The use of tracers such as stable isotopes (e.g. deuterium) as suggested by Körner (2019) could also help for identifying if the irrigation of those key tissues or survival are kept during drought until the very end (that is to say, the mortality point).

In addition, if a clear dynamic of water relocation is detected, carrying out a comparison between a wide range of species, with different distribution ranges and different strategies to cope with drought, could be interesting in order to check for possible common evolutionary patterns for the preservation of certain tissues during a drought event.

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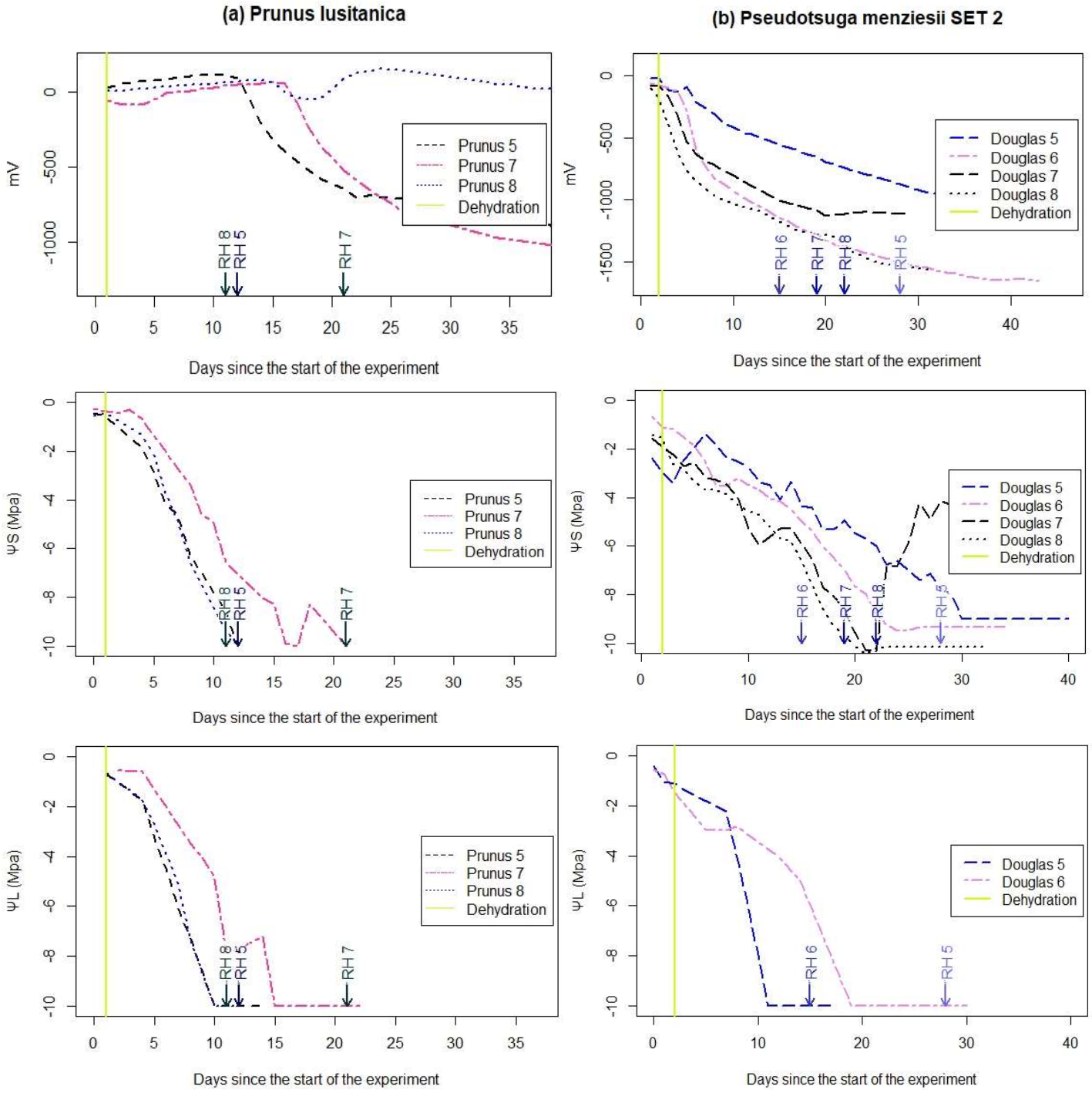
Supplementary data



Annex 1 : Dynamic of the stem diameter recorded by LVDT (in mV) (1), the stem water potential (Ψ_S) (2), the leaf water potential (Ψ_L) (3) in Prunus lusitanica (a) and Pseudotsuga menziesii (b).

a) For Prunus individuals, the dehydration started on the third day of the experiment and ended 8 days later with the beginning of the rehydration phase. Ψ_S and Ψ_L consisted of punctual measurements made with the Scholander Pressure Chamber until -10MPa for Ψ_S and -8MPa for Ψ_L.

b) For *Pseudotsuga* individuals, the dehydration (DH) started the second day of the experiment and ended with the rehydration (RH) between the day 15 and the day 28 of the experiment. Ψ_S was continuously recorded with ICTs psychrometers while Ψ_L was measured punctually with the Scholander Pressure Chamber. Once reaching -5MPa, no measurements could be conducted in leaves with the pressure chamber.

Skills

During this internship, I had the opportunity to develop many skills.

First of all, working in an international team helped me to greatly improve my English speaking and writing skills. I indeed had the opportunity to work with Spanish, Brazilians and Australians people who were all speaking different languages, but the English helped us to communicate. I can now certainly say that my communication skills are enhanced at the end of this internship and that I am now able to write and to speak almost fluently in English. Working in an international team made me encountered new person from different countries with various culture what also allowed me to open my mind.

During this internship, I was able to develop my time management capabilities by scheduling experiments on the 3 to 6 months term. I was also capable to work in team as I was using many different devices that belonged to different teams. This led to better communication skills along with enhanced planning skills. Working with other people can surely bring some disagreement and therefore it helped me to manage problems and to plan decisions that would certainly avoid the problems.

During this internship, I learned how to respect deadlines and therefore I learn how to work under pressure which led to a better stress management capacity.

On overall, this internship also greatly contributed to expand my plant physiology knowledge with everyday learning of new concepts about plant hydraulic or plant anatomy. It also allowed me to work on my critical thinking and to have a perspective on my results. In addition, working in a unit composed of different teams also helped to arouse even more my curiosity by showing interest in the other teams' research projects what expended my knowledge about plant physics.

To finish, my social skills were also greatly improved as I took part of number of activities at, and outside, the research station with my co-workers.