Respuesta a la sequía de *Pinus radiata* D. Don y su implicación en los procesos de tolerancia.

Drought - response of *Pinus radiata* D. Don and its implication in the tolerance processes.

NURIA DE DIEGO SÁNCHEZ

Vitoria - Gasteiz 2012

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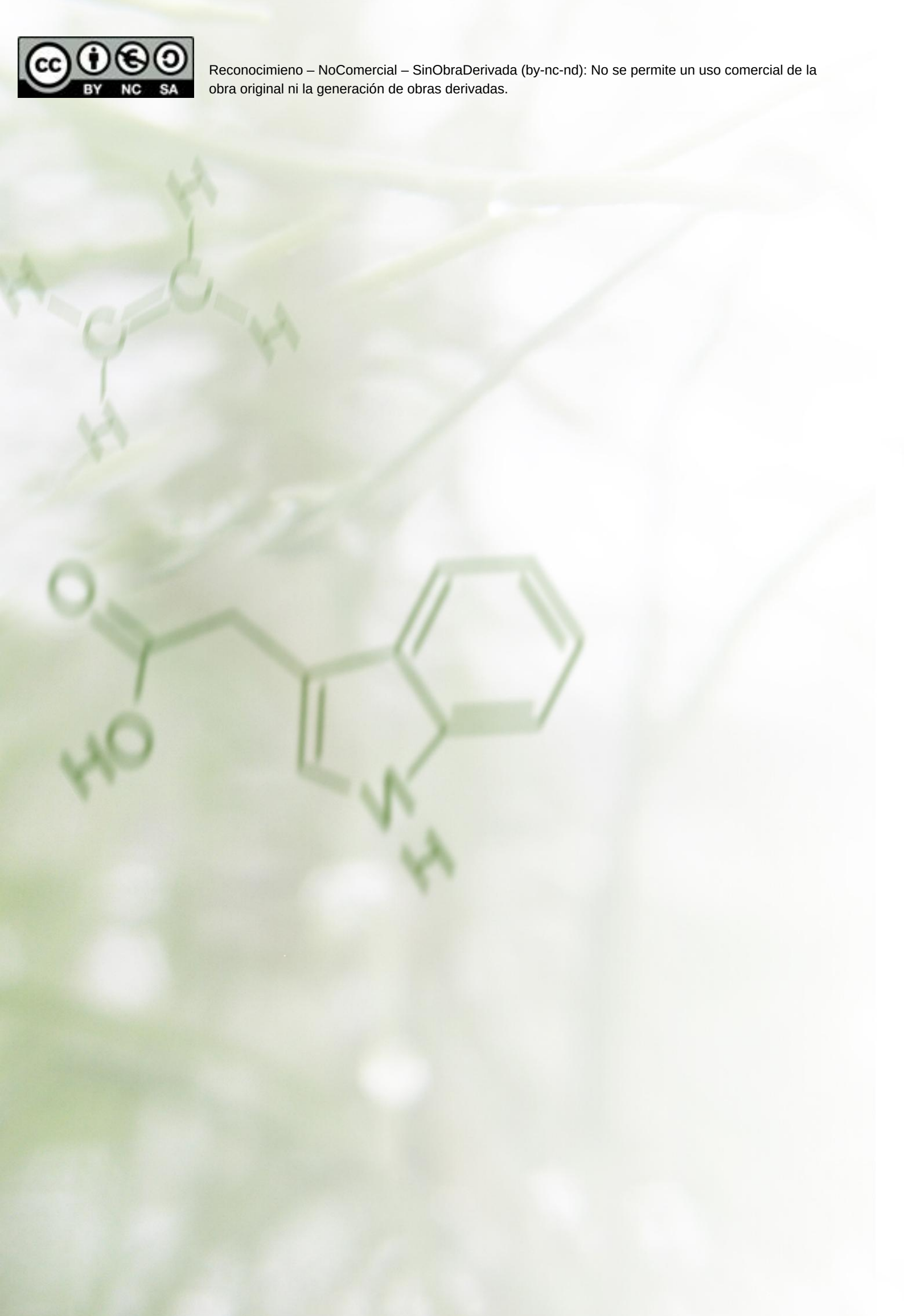

"Prenez intérêt, je vous en conjure, à ces demeures sacrées que l'on désigne du nom expressif de laboratoires. Demandez qu'on les multiplie et qu'on les orne: ce sont les temples de l'avenir; de la richesse, du bien-être. C'est là que l'humanité grandit, se fortifie et devient meilleure. Elle y apprend à lire dans les œuvres de progrès et d'harmonie universelle tandis que ses œuvres à elles sont trop souvent celles de la barbarie, du fanatisme, de la destruction."

-Louis Pasteur-

A mis padres,

Antonio y Mili

Agradecimientos

Hace tiempo que comencé este largo viaje sin fin que es la carrera científica. Con esta tesis concluye esta primera etapa que espero de lugar a muchas otras. La verdad es que este trabajo nunca hubiese comenzado sin el apoyo, impulso y enseñanzas de mis dos codirectoras, la Dra. Paloma Moncaleán y la Dra. Maite Lacuesta. Agradezco a Paloma que me concediese esta gran oportunidad y que me animase a conocer nuevos lugares, laboratorios y gente que forma este pequeño mundo. Gracias por todos estos años de formación y por todos sus consejos. Y a Maite, por acogerme como una más, por su cariño, y por todos esos ratos de conversación entre múltiples papeles, datos, gráficas y relaciones.

Me gustaría también agradecer al Gobierno Vasco por financiarme (VEC2007012, 72.0093.0) y concederme la beca predoctoral para la formación de investigadores, y al Ministerio de Educación y Ciencia por permitirme realizar este trabajo (MEC- AGL2006-13912-C02-02, MEC-INIA-SUM2006-00007-CO2 Y RTA2010-00041-CO2-O2).

A Neiker, y toda la gente que me ha ayudado a lo largo de este proceso. Al departamento de Producción Vegetal, de Producción Animal, a todo el personal de mantenimiento, la gente de campo, y en especial al departamento de Biotecnología y a todos aquellos que lo forman o que formaron parte de él. A su directora, la Dra. Sonia Castañón, a Ana Herrán, Pablo G. Goikoetxea, Sonia Suarez, Pablo Alonso, Isibene, Leire, Enrique, Zior, Judith, Ainara, Javi, Gemma, Marina, Ana Aragonés, Mónica, Isi y en especial a Iratxe, Iñigo, e Inés… ¡¡¡Buf!! Qué decir de esas tardes eternas colgados de los percheros… el "Txoko Txorra"… y Katty casi echándonos a escobazos…

A mis compañeros de caseta, Sergio, Mikel, Patxi, Ander y Vanesa, por hacer los largos días de escritura más amenos.

A las chicas de Administración, Yoli, Cristina, Sonia, Iratxe, y en especial a mi Anita, por los malos y buenos momentos, por saber que siempre estás ahí… Y por ser como eres…¡¡¡no cambies!!!

Al laboratorio de "cultivo *in vitro*", Amaia, Elena Fernández, Ernesto, Montse, Bego, Carlos Herrán y sobre todo a Itzi, con la que he compartido más que una campana, pinzas y bisturí.

Al departamento de Biología Vegetal y Ecología, y todos los que le constituyen, al. Dr. Txema Becerril, Dr. Alberto Muñoz, Nacho, Antonio, Usue, Amaia, Ibón, Esti, María, Bea, Jon, vamos, a todos… y sobre todo a Iñigo y sus eternas discusiones de ciencia.

A todas las personas que he conocido en las diferentes estaciones del mundo por las que he pasado a lo largo de estos años. Al Dr. Mirolav Strand, y su fiel Petra por acogerme en esa ciudad tan bonita como es Olomouc, e introducirme en el maravilloso mundo de las fitohormonas. A la Dra. Krystyna Klimaszewska, por esos largos paseos por los bosques de Quebec, y por convertir la ciencia a la vez que sencilla, en algo profundamente interesante e inquietante. Al Dr. Francisco Pérez-Alfocea, por su acogida, apoyo y consejos, y ese deje Murciano que tanto me agrada. A la Dra. Gloria Pinto, y a todo el departamento de Fisiología Vegetal de la Universidad de Oviedo, al Dr. Ricardo Ordás, a las Dras. Belén Fernández y Ana Rodríguez, a la Dra. Mª Jesús Cañal, al Dr. Roberto Rodríguez, y a todos los técnicos y becarios, especialmente a Millán, Jose, Candela, Norma, Elisa y Chus. Por último, a los servicios generales (Sgiker-Álava) de la Universidad del País Vasco y al departamento de Química Analítica, desde sus capitanes, el Dr. Ramón Barrio y la Dra. Arantza Goicolea, a los Dres. Alberto, Ana y Nora, a Alicia y a todas las becario/as, sobre todo a mis dos chicas predilectas, Itxaso y María, sois las dos muy grandes, ¡¡¡GUAPISIMAS!!! Por último, A mi genial Mamen, que ha conseguido que me entusiasmen todos esos aparatejos, y sobre todo desmontarlos y arreglarlos…;)

Y por supuesto, a todos esos compañeros incondicionales que me han apoyado tanto en los buenos como en los malos momentos. A mis chicas de futbito; Sara, Leire, Ane, Irati, Tami, Lutxi, Lekue, Miren, Txibi, Aitziber, Enara, a nuestro míster, Javi, las Mamaos, Ane, Neri, Yoana, la Zaio, la Jaio, y en especial a mis chicas favoritas, Ana y Dei, que las pobres me han aguantado carros y carretas…

A Ricardo, Aser y Mentzi, porque sin vosotros estos años no hubiesen sido igual… entre charlas, vinitos, y alguna que otra cañita… Por ser lo que sois, y espero que sigáis siendo.

A toda mi gente de Medina, a la Mari, al Jose, al Moreno, a Ana, al Chus, a Fernando, a la Pau, y a mis niños, Mario y Marina. A los más borrachuzos, Pepino y Magdalena, y en especial a Curro, por toda su ayuda, y por no descuartizarme cuando de verdad le entraron ganas…

A mis hermano/as; Luis, Almu, y Esti, sin la cual la portada y presentación no sería lo mismo. A mis cuñado/as Jesus, Santi y Bego, y sobre todo estas pequeñas que me traen loca, María, Alejandra, Lucía, y Gadea, y el recien llegado Bruno.

Y aquí concluye la primera etapa de este largo e intenso viaje, que espero continuar y compartir con todos vosotros y en especial contigo "ricillos" (y nuestros peludos compañeros de piso…), porque mucha parte de esto es tan tuya como mía "Y LO SABES"... ¡¡¡MIL GRACIAS, GUAPO!!!

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Abbreviations

AA	amino acid
ABA	abscisic acid
ACC	1-aminicyclopropane-1-carboxylic acid
Ala	*L*- alanine
$\boldsymbol{A_N}$	instantaneous net photosynthesis
ANCOVA	analysis of covariance
ANOVA	analysis of variance
Arg	*L*- arginine
Asn	*L*- asparagine
Asp	*L*- aspartic acid
ATP	adenosine triphosphate
C	cortex
C1	first drought cycle
C2	second drought cycle
Cad	cadaverine
Chl	chlorophyll
Ck(s)	cytokinin(s)
D	water stressed plant
DW	dry weight
E	instant leaf transpiration
EA	ethylamine
E.L.	electrolyte leakage
End	endodermis
Epi	epidermis
Exo	exodermis
Fv/Fm	the maximum quantum yield of *PSII* (photochemistry)
FW	fresh weight
GABA	γ- aminobutyric acid
Gln	*L*- glutamine
Glu	*L*- glutamic acid
Gly	*L*- glycine
$\boldsymbol{g_s}$	stomatal conductance

HA	histamine
His	*L*- histidine
HPLC	High Performance Liquid Chromatography
HSD	honestly significant difference
Hys	hypodermis
IAA	indole-3-acetic acid
Id	index of injury
Ile	*L*- isoleucine
JA	jasmonic acid
K_{leaf}	leaf hydraulic conductance
Leu	*L*- leucine
LWC_{100}	leaf water content at full turgor
LWC_{Ti}	leaf water content at time Ti
Lys	*L*- lysine
M	mean
MA	methylamine
MANOVA	multivariate analysis of variance
MC	mesophyll cell
Met	*L*- methyonine
O	ecotype
OA	osmotic adjustment
***OH*- Pro**	*L*- hydroxiproline
p	probability
PA	polyamines
PBS	phosphate buffered saline
PC	principal component
PEA	*β*- phenylethylamine
Phe	*L*- phenylalanine
Pro	*L*- proline
PSII	photosystem *II*
Put	putrescine
***q*CN**	complete non-photochemical quenching of chlorophyll fluorescence
R^2	Pearson´s correlation number
R3	three days under rewatering condition after a drought cycle

R7	a week under rewatering condition after a drought cycle
R/A	relation between root and aerial dry weight
RD	resin duct
RdGR	relative growth ratio of root collar diameter
RDW	relative dry weight
RhGR	relative growth ratio of plant height
RWC	relative water content
SA	salicylic acid
S.E.	standard error
Ser	*L*- serine
S*i*	50% of stressed plants from *i* ecotype with external symptoms
Spd	spermidine
Spm	spermine
Sto	stomata
T	treatment
T0	the beginning of the drought cycle
T1	a week under water stress conditions
T2	two weeks under water stress conditions
T3	three weeks under water stress conditions
T4	four weeks under water stress conditions
TA	tiramine
Thr	*L*- threonine
Ti	time
TLP	turgor loss point
Trp	*L*- tryptophan
Tryp	tryptamine
TW	weight at full turgor
Tyr	*L*- tyrosine
Val	*L*- valine
VT	vascular tissue
W	well watered plant
Z	zeatin
ZR	zeatin riboside
$\boldsymbol{\varepsilon}$	cell wall elastic modulus

Φ_{PSII}	the effective quantum yield of photochemical energy conversion in *PSII*
Ψ	water potential
Ψ_{g}	gravity potential
Ψ_{leaf}	midday water potential
Ψ_{pd}	predawn water potential
Ψ_{π}	osmotic potential
Ψ_{π}^{100}	osmotic potential at full turgor
$\Psi_{\pi,osm}$	osmotic contribution of osmolytes

Resumen

En este estudio se evaluaron cinco ecotipos de *Pinus radiata* D. Don y un híbrido de especie (O4, *Pinus radiata* x *Pinus attenuata*) con el fin de caracterizar fisiológicamente su respuesta al estrés hídrico y determinar su tolerancia, capacidad de recuperación y endurecimiento. La plantas de O4 fueron incluidas como modelo de tolerancia debido a la alta resistencia a la sequía descrita en *P. attenuata* (Begley, 2001). En cada ecotipo se estudiaron los cambios fisiológicos producidos durante ciclos de estrés hídrico a corto y a largo plazo, incluyendo las posibles señales hormonales así como sus interconexiones. Para el análisis, se emplearon diferentes herramientas estadísticas como regresiones, ANCOVAs, MANOVAs, y análisis de componentes principales que facilitaron el entendimiento de la compleja respuesta de las plantas al estrés hídrico.

La falta de agua provocó una alteración de los parámetros hídricos y de intercambio gaseoso, una inducción del ajuste osmótico, y un aumento del valor del modulo de elasticidad de las paredes celulares. El crecimiento de las plantas también se vio afectado.

Las fitohormonas jugaron un papel fundamental en la respuesta al estrés hídrico, su capacidad de recuperación y endurecimiento. Así, todas las plantas descendieron sus niveles de citoquininas como primera señal de estrés. El contenido de zeatinas y los niveles de conductancia hidráulica en hoja disminuyeron de forma paralela, y cuando la reducción alcanzó valores de 65%, las plantas estresadas empezaron a acumular otras hormonas como ácido índole-3-acético (IAA) y ácido abscísico (ABA). IAA fue la fitohormona más representativa en plantas de *P. radiata* sometidas a consecutivos ciclos de déficit hídrico. La presencia de IAA en las acículas estuvo relacionada con la aparición de síntomas externos como curvatura apical y epinastia acicular.

La sequía también incrementó los niveles de ácido jasmónico y disminuyó los de ácido 1-aminociclopropano-1-carboxílico (ACC), precursor del etileno. Estos procesos se observaron principalmente en los ecotipos que mostraron mayor porcentaje de plantas con síntomas externos. Por el contrario, el ácido salicílico se acumuló en aquellas plantas que presentaron bajas pérdidas electrolíticas y menores síntomas externos.

Las plantas procedentes de O4 y O5 fueron las más tolerantes, y su tolerancia se basó en un eficiente control de la conductancia hidráulica y del cierre estomático, y un alto ajuste osmótico activo inducido por la síntesis *de novo* de osmolitos, que contribuyó al mantenimiento de la turgencia celular. Entre los osmolitos analizados, los carbohidratos solubles fueron los solutos que contribuyeron en mayor medida al ajuste osmótico, mientras que los aminoácidos y las poliaminas libres mostraron una modesta contribución. Sin embargo, las plantas estresadas incrementaron en gran medida ciertos aminoácidos y poliaminas libres de forma diferencial debido a su implicación en la respuesta a la falta de agua. A este respecto, las plantas incrementaron en gran medida sus niveles de ácido γ-aminobutírico, prolina (Pro) y ácido glutámico (Glu) durante la sequía, y especialmente Glu y Pro tras el endurecimiento. Únicamente los ecotipos más tolerantes, O4 y O5, aumentaron sus niveles de Pro como consecuencia de la aclimatación a condiciones de déficit hídrico, por lo tanto este aminoácido fue el mejor indicador de la capacidad de endurecimiento. Además, las plantas de O5 mostraron los mayores niveles de putrescina, espermidina y espermina.

La mayor tolerancia a la desecación y la capacidad de endurecimiento también estuvo asociada a características estructurales tales como menor cavidad subestomática y mayor tamaño de las células xilemáticas, además de un aumento del tamaño de los canales resiníferos bajo condiciones de estrés.

Nuestro estudio demostró que la tolerancia a la sequía en *Pinus radiata* varía a nivel intraespecífico *y* está modulada por cambios morfológicos, fisiológicos y señales hormonales entre las que el IAA juega un papel principal.

Summary

In this study, five *Pinus radiata* ecotypes and a species hybrid (O4, *Pinus radiata* x *Pinus attenuata*) were analyzed to evaluate their drought response and to determine their tolerance, recovery capacity and hardening. O4 plants were included as tolerance models due to the high drought resistance of *P. attenuata* (Begley, 2001). In each ecotype the physiological changes produced along short and long stress cycles were studied, including the possible hormonal signals and their interconnections. To the analysis, different statistic tools such as regressions, ANCOVAs, MANOVAs and principal component analysis were used for an easier understanding of this complex plant response to water stress.

The water deficit provoked an alteration of hydric status and gas exchange parameters, an osmotic adjustment and an increase of cell wall elastic modulus. Plant growth was also affected.

Phytohormones played a main role in the plant water stress response, recovery capacity and hardening. Thus, stressed plants decreased their cytokinin levels, being the first water stress hormonal signal. The needle zeatin levels and hydraulic conductance decreased together, and when this reduction reached values of 65%, stressed plants started to accumulate indole-3-acetic acid (IAA) and abscisic acid (ABA). IAA was the most representative phytohormone in *P. radiata* plants subjected to sequential drought cycles. The needle IAA presence in stressed plants was mainly related to the external symptoms such as needle epinasty and apical curvature.

Drought also induced jasmonic acid accumulation and diminution of 1-aminocyclopropane-1-carboxilic acid (ACC), the ethylene precursor. These traits were mainly observed in ecotypes with high percentage of external symptoms. On the contrary, salicylic acid was accumulated in plants that presented low electrolyte leakage and less external symptoms.

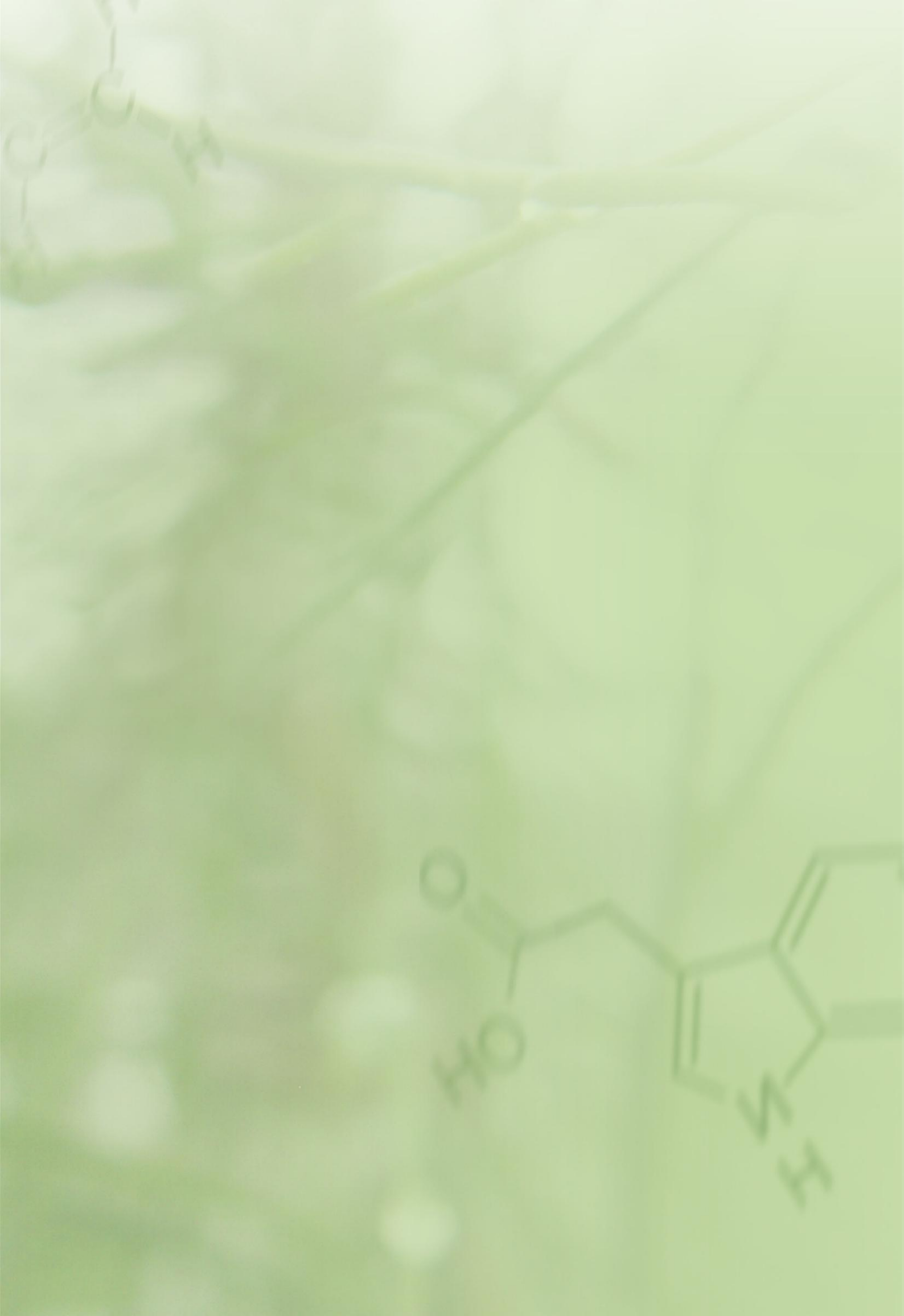
Plants from O4 and O5 were the most drought tolerant ones, and their tolerance was due to an efficient control of hydraulic conductance and stomatal closure, and a high active osmotic adjustment (OA) (70%) induced by *de novo* osmolyte synthesis, that allowed the turgor maintenance. Among analyzed osmolytes, soluble carbohydrates strongly contributed to

OA, whereas free amino acids and free polyamines showed a modest contribution. However, stressed plants highly increased some specific amino acids and polyamines, suggesting an implication in plant water stress response. At this respect, γ- aminobutyric acid, proline (Pro) and glutamic acid (Glu) were accumulated in plants under water stress, and especially Glu and Pro after drought conditioning. The most tolerant ecotypes, O4 and O5, also increased their Pro content during hardening, being a good indicator of acclimation capacity. In addition, O5 showed the highest values of putrescine, spermidine and spermine.

High desiccation tolerance and hardening capacity were also associated to structural characteristics such as less substomatal chamber and higher xylem cell area, and a resin duct size increase under drought conditions.

Our study showed that drought tolerance in *Pinus radiata* varies at intraspecific level and is modulated by physiological and morphological changes and hormonal signals that are interconnected each other, playing IAA the principal role.

I.-Introducción general



I. 1.- Estrés en las plantas.

Debido a las fluctuaciones medioambientales, las plantas raramente se desarrollan bajo óptimas condiciones, teniendo que soportar periodos bajo situaciones desfavorables a lo largo de su ciclo vital. Es por ello que podemos llamar **condiciones estresantes** a aquellas situaciones que reducen la tasa de algún proceso fisiológico por debajo de la máxima que la planta mantendría en otras condiciones más adecuadas (Reigosa & Pedrol, 2003). Así, Larcher (1987) definió el **estrés** de las plantas como "*un estado en el cual las crecientes demandas a las que es sometida la planta conducen a una desestabilización inicial de las funciones, seguidas de un estado de normalización y una mejora de la resistencia*", además, "*si se exceden los límites de la tolerancia y se sobrepasa la capacidad de* ***aclimatación****, el resultado puede ser un daño permanente o incluso la muerte*". Más tarde, Lichtenthaler (1996) extendió el concepto de estrés en las plantas incluyendo la regeneración, cuando ya ha cesado el agente estresante.

De acuerdo con esta definición, se puede concluir que la actual definición de estrés en las plantas lleva implícito un proceso dinámico que describimos a continuación (Fig. I.1) (Lichtenthaler, 1998):

1.- ***Fase de respuesta*****:** reacción de alarma (comienzo del estrés).

- ✓ Desviación de la norma funcional, reducción o aumento anormal de la actividad fisiológica
- ✓ Desestabilización estructural (proteínas, membranas)
- ✓ Disminución de la vitalidad
- ✓ Los procesos catabólicos exceden a los anabólicos

2.- ***Fase de restitución*****:** estado de resistencia (el estrés continúa).

- ✓ Procesos de aclimatación
- ✓ Procesos de reparación
- ✓ Endurecimiento (reactivación y ajuste → estabilidad)

3.- ***Fase final*****:** estado de agotamiento (estrés de larga duración).

- ✓ Intensidad de estrés demasiado alta
- ✓ Inicio del proceso de senescencia
- ✓ Daño crónico, muerte celular

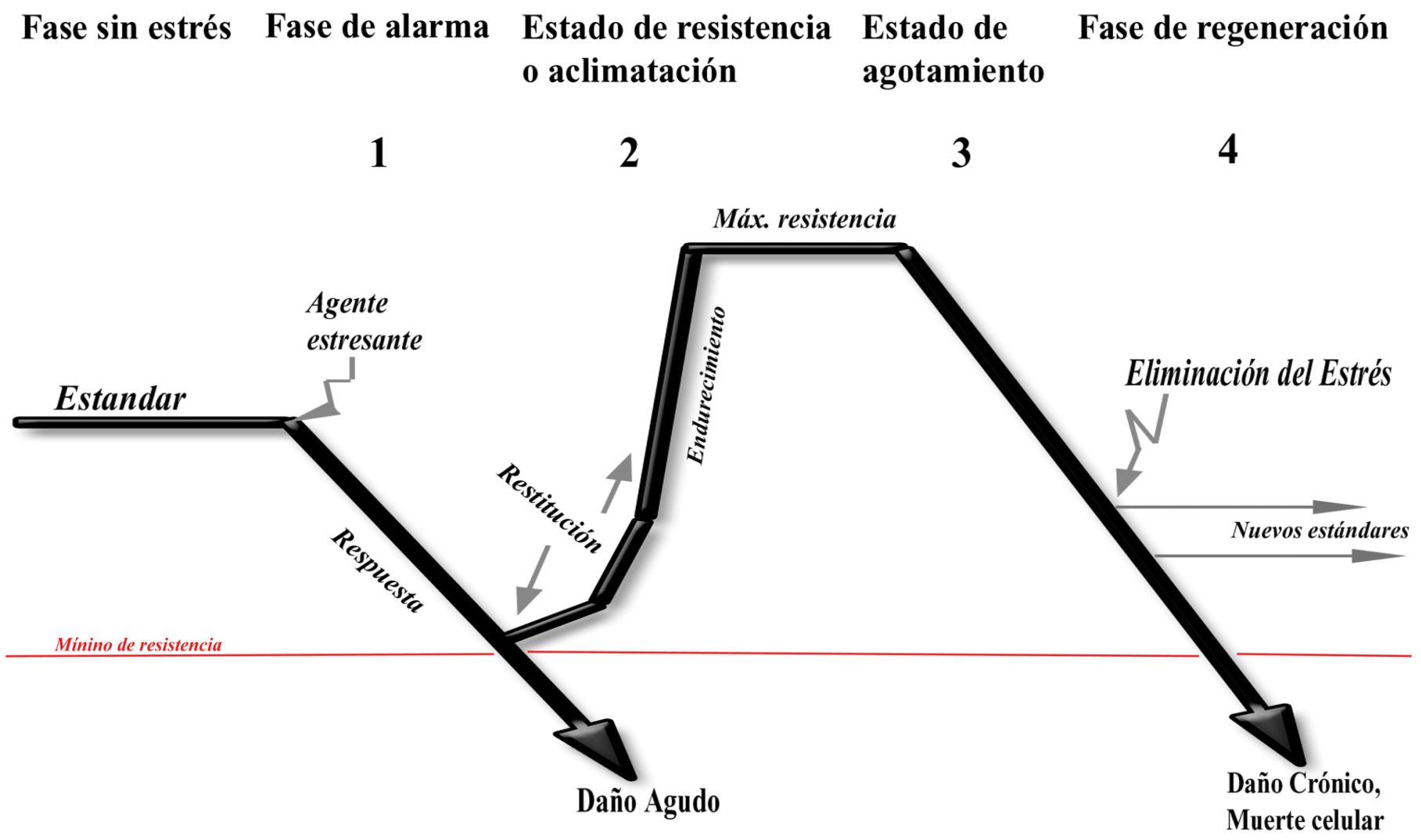


Figura I.1. Secuencia de fases y respuestas inducidas en las plantas por la exposición a una agente estresante (Lichtenthaler, 1998).

4.- ***Fase de regeneración*****:** regeneración parcial o completa del funcionamiento fisiológico, cuando el agente estresante es eliminado y el daño no ha sido muy intenso.

Una larga exposición de las plantas a condiciones de estrés produce, en ocasiones, daños celulares y finalmente la muerte. Pero si el agente estresante cesa su actividad antes del momento en el cual la respuesta de la planta pasa de ser reversible a irreversible y la senescencia no se convierte en el proceso dominante, está se puede mover a un nuevo estándar fisiológico.

Los diversos estreses abióticos y sus efectos, tanto en escenarios naturales como en plantaciones, son un tema de interés científico debido a la necesidad global de mantener e incrementar la productividad agroforestal (Verslues *et al.* 2006). Para ello, es determinante la elección de especies que garanticen su supervivencia y producción, a pesar de la exposición a periodos de estrés (Araus *et al.*, 2002).

La "Ley del mínimo" fue uno de los primeros principios ecofisiólogicos relacionados con el rendimiento de los cultivos. En él se atribuyó a un único recurso con suministro limitado o inexistente, la disminución del funcionamiento de los organismos (Chapin *et al.*, 1987). En principio, el crecimiento de las plantas está limitada por la disponibilidad de un gran número de recursos limitantes (Canham *et al.*, 1996), como son por ejemplo la luz (Balaguer *et al.*, 2001), los nutrientes (Valladares *et al.*, 2000), y/o la disponibilidad de agua en el suelo (Baquedano *et al.*, 2008).

I. 2.- Estrés hídrico en el género *Pinus*

El déficit hídrico es el principal limitante de la producción de las plantas a nivel mundial, y la sequía un importante conductor de la adaptación ecofisiológica de la planta (Flexas *et al.*, 2005; McDowell *et al.*, 2010). De acuerdo con las predicciones sobre cambio climático, en los próximos años se espera un aumento en las temperaturas medias y un incremento en la frecuencia e intensidad de las sequías (Sánchez *et al.*, 2004; Seager *et al.*, 2007). Por este motivo, el destino de muchos ecosistemas dependerá, por un lado, del ritmo en el que se produzcan estos cambios climáticos, y por otro, de la velocidad de adaptación que muestren las plantas que los constituyen (Klein *et al.*, 2011). A este respecto, ciertas evidencias apuntan a que si el proceso de adaptación se produce de forma más lenta que el

cambio medioambiental, podría llegar a poner en riesgo la supervivencia de las plantas (Allen *et al.*, 2010).

En el cultivo de Pináceas, la falta de agua es considerada como uno de los factores más importantes que condicionan su productividad. Así, en *Pinus halepensis* (Prieto *et al.*, 2009), *Pinus nigra* (Martín-Benito *et al.*, 2008), *Pinus pinaster* (Sánchez-Gómez *et al.*, 2010) y *Pinus sylvestris* (Duursma *et al.*, 2008) se ha observado un descenso de la productividad en condiciones de déficit hídrico, en ocasiones ligado al lugar de procedencia del material de partida (Cregg & Zhang, 2001; López *et al.*, 2009b). La distribución de la biomasa de la planta entre parte radicular y aérea también se ve afectada por el déficit hídrico (Callaway & DeLucia, 1994; Canham *et al.*, 1996; Green *et al.*, 1994), no solo entre poblaciones de la misma especie (Aranda *et al.*, 2010), sino que también entre genotipos (Barnes, 2002; Cregg, 1994). Por este motivo, y debido a la amplia respuesta observada tanto a nivel de especie como entre diferentes ecotipos (Tognetti *et al.*, 1997), se hace necesario el uso de material procedente de diferentes áreas geográficas y climatológicas que faciliten el entendimiento de los procesos fisiológicos que, bajo condiciones de estrés, confieren a la planta distintos grados de tolerancia, y/o están involucrados en diferentes mecanismos de adaptación o aclimatación (Corcuera *et al.*, 2011; Zhang *et al.*, 1997).

La especie *Pinus radiata* D. Don, debido a su gran importancia en la industria forestal en muchos países del mundo, ha sido motivo de programas de mejora para seleccionar plantas elites de alta calidad maderera (Codesido & Fernández-López, 2009; Ivkovic *et al.*, 2006). Asimismo, en las últimas décadas, se han realizado estudios dirigidos a un mayor conocimiento de los mecanismos de tolerancia a ciertos estreses bióticos, como son el ataque de hongos e insectos asociados a su cultivo (Grace *et al.*, 2005; Lottmann *et al.*, 2010). Por el contrario, existen pocos trabajos que evalúen la tolerancia del *P. radiata* al déficit hídrico (Conroy *et al.*, 1988a). A este respecto, se ha analizado la competencia por la toma de agua del suelo de las plantas en presencia de malas hierbas (Sands & Nambiar, 1984; Woods *et al.*, 1992), la dinámica de su follaje en relación a la disponibilidad de agua a lo largo de los cambios estacionales (Raison *et al.*, 1992), o incluso la eficiencia de plantas micorrizadas en la absorción del agua (Ortega *et al.*, 2004; Sands & Theodorou, 1978). También ha sido motivo de análisis su supervivencia tras el trasplante, que apunta al déficit hídrico como uno de los principales causantes de un establecimiento exitoso del cultivo (Mena-Petite *et al.*, 2004; Nambiar *et al.*, 1979).

El endurecimiento por estrés hídrico se ha comprobado que mejora la supervivencia de los cultivos tras el trasplante y frente a futuros periodos de déficit hídrico (Domec *et al.*, 2009; Villar-Salvador *et al.*, 1999). Este proceso de endurecimiento se basa en someter a las plantas a déficit hídrico no letal que promueva mecanismos de resistencia (Vilagrosa *et al.*, 2006), como el incremento del ajuste osmótico, la estabilidad de membranas celulares (López *et al.*, 2009a; Tinus *et al.*, 2000), e incluso una reducción en la tasa de transpiración (Cinnirella *et al.*, 2002), aunque estos cambios no siempre ocurren (Villar-Salvador *et al.*, 1999). Con respecto a *P. radiata*, pocos estudios han evaluado su capacidad de aclimatación a condiciones de déficit hídrico (Conroy *et al.*, 1988b), por lo que se desconoce su capacidad de endurecimiento y cuáles son los mecanismos fisiológicos que lo modulan.

I. 3.- *Pinus radiata* D. Don

El género *Pinus* está clasificado en el reino *Plantaea* como división *Spermatophyta*, subdivisión *Gymnospermae*, orden *Coniferae*, y familia *Pinaceae*. Theophrastus estableció la identidad del género que incluye más de cien especies (Richardson, 1998), entre las que se encuentra el *Pinus radiata* D. Don. El *Pinus radiata* D. Don, también denominado Pino radiata, Pino insignis o Pino de Monterrey, evolucionó hace aproximadamente 100 millones de años y es un pino serótino de la costa Pacífica de Norteamérica. Tras el estudio de las poblaciones naturales de la especie realizado por Libby (1997), y posteriormente por Brown (1999), se diferenciaron tres variedades con cinco procedencias originarias (Fig. I.2):

- ✓ ***Pinus radiata* var. *radiata*:** Año Nuevo, Monterrey y Cambria (California-EEUU).

- ✓ ***Pinus radiata* var. *binata*:** Isla Guadalupe (Baja California-México).

- ✓ ***Pinus radiata* var. *cedrosensis*:** Isla de Cedros (Baja California-México)**.**

Figura I.2. Representación geográfica de las procedencias de *Pinus radiata* D. Don.

La historia del cultivo del pino radiata en el País Vasco data de 1897, año en que se realizó la primera plantación importante. Desde entonces hasta ahora, su cultivo ha ido aumentando hasta convertirse, en la actualidad, en la especie que ocupa mayor extensión y proporciona mayor productividad forestal, con cerca del 90% de las cortas anuales (Inventario Forestal CAE 2005). Así, el *P. radiata* cubre unas 137.466 ha que suponen el 35% de la superficie forestal arbolada total. Además, la elevada productividad forestal que reúne la vertiente cantábrica, con clima y suelo aptos para su cultivo, se traduce en importantes crecimientos, con una producción media de madera de 11 m^3 $ha^{-1}$$año^{-1}$. Estas producciones se han visto incrementadas hasta 25-30 m^3 ha^{-1} $año^{-1}$ en algunos países como Australia, Nueva Zelanda y Chile, gracias a la selección realizada en los programas de mejora genética (Li & Wu, 2005; Zamudio *et al.*, 2002). Asimismo, junto a su alta producción, el pino radiata permite turnos de corta de 30 años y proporciona una madera homogénea, características que le hacen ser muy apreciado en la industria maderera.

I. 4.- Indicadores fisiológicos

En las últimas décadas, multitud de parámetros ecofisiólogicos han sido utilizados para determinar el nivel de estrés y la tolerancia de las plantas a diferentes ciclos de déficit hídrico

(Monson & Grant, 1989). Tradicionalmente los más evaluados son los parámetros relacionados con el balance de agua (Dichio *et al.*, 2009), el intercambio gaseoso y la fotosíntesis (Mena-Petite *et al.*, 2003).

En el género *Pinus*, las variaciones de los parámetros hídricos han sido motivo de un exhaustivo estudio en condiciones de déficit hídrico (Blackman *et al.*, 2009; Maherali *et al.*, 2002). Entre ellos, tanto el potencial hídrico (Ψ_{leaf}) (Hacke *et al.*, 2000), como sus componentes, potencial osmótico (Ψ_{π}) y potencial de turgencia (Ψ_{t}), se han considerado buenos indicadores del estado hídrico de la planta (Fan *et al.*, 2006; López *et al.*, 2009a).

El turgor es crítico para la vida de las plantas ya que su pérdida inhibe el crecimiento (Fan *et al.*, 2006). La pérdida de turgor desencadena ajustes fisiológicos y bioquímicos que son importantes para su mantenimiento, destacando la importancia de un eficiente ajuste osmótico (OA) que impida mayores pérdidas de agua a bajo Ψ_{leaf} (Dichio *et al.*, 2009; Chen & Jiang, 2010). En *Pinus* spp., el ajuste osmótico de plantas se considera una de los mejores estrategias fisiológicas para evitar la deshidratación. En relación a esto, se pueden diferenciar dos procesos: el "ajuste osmótico activo", que se basa en la síntesis de *novo* de osmolitos, y el "ajuste osmótico pasivo", donde el aumento de la concentración de solutos se produce por la pérdida de volumen celular como consecuencia del descenso de agua en el simplasto (Dichio *et al.*, 2006; Loustau *et al.*, 1995). Generalmente, los solutos acumulados u osmolitos son productos metabólicos de bajo peso molecular entre los que destacan carbohidratos, ácidos orgánicos, aminoácidos (AAs) y poliaminas (PAs) libres (Sánchez *et al.*, 2011; Warren *et al.*, 2011).

Entre estos solutos, la prolina (Pro) es tradicionalmente el metabolito más vinculado con la respuesta de la planta al estrés (Silveira *et al.*, 2003; Sofo *et al.*, 2004). La Pro es un aminoácido proteinogénico esencial para el metabolismo primario de las plantas con una rigidez conformacional excepcional (Szabados & Savouré, 2010). Desde la primera evidencia de su acumulación en *Lollium perenne* L. en fase de marchitamiento (Kemble & MacPherson, 1954), numerosos estudios han observado que el contenido de Pro aumenta en la mayoría de las plantas bajo diferentes estreses medioambientales, incluido en condiciones de falta de agua (Xiong *et al.*, 2011; Yamada *et al.*, 2005). Este alto contenido de Pro se ha asociado principalmente a funciones de osmoprotección (Ashraf & Foolad, 2007; Kumar & Yadav, 2009), por lo que su acumulación está considerado un importante mecanismo de tolerancia

(Verbruggen & Hermans, 2008). Sin embargo, la correlación entre la acumulación de Pro y la tolerancia al estrés no es siempre clara (Widodo *et al.*, 2009; Xin & Browse, 1998). Por ello, sus variaciones a lo largo de diferentes ciclos de estrés y posterior rehidratación podrían determinar su rol en los procesos de tolerancia de plantas de *P. radiata*.

Junto al ajuste osmótico, las propiedades elásticas de los tejidos son características fisiológicas que también pueden condicionar el mantenimiento del turgor y el volumen celular a bajos Ψ_{leaf} (Hessini *et al.*, 2009). Así, bajos valores del módulo de elasticidad de las paredes celulares (ε) contribuyen al mantenimiento del turgor en condiciones de déficit hídrico, aunque si las células acumulan un alto número de solutos, corren el riesgo de una ruptura o daño del tejido tras la rehidratación (Clifford *et al.*, 1998). A este respecto, estudios realizados en especies arbóreas tales como *Quercus* spp. (Saito & Terashima, 2004) y *Pseudotsuga menziesii* (Joly & Zaerr, 1987) observaron una disminución de los valores de ε como respuesta de las plantas a la falta de agua disponible. Por el contrario, en *Acer pseudoplatanus* (Ayoub *et al.*, 1992) y *Fagus* spp. (Uemura *et al.*, 2000) un aumento de ε confirió la ventaja de poder disminuir su Ψ_{leaf}, y extraer mayor cantidad de agua del suelo sin presentar un gran descenso de su contenido hídrico foliar (Kramer & Boyer, 1995). Por esta variada respuesta, actualmente existe un fuerte debate sobre cuál de las dos estrategias confieren mayor tolerancia a las plantas bajo condiciones estresantes y posterior recuperación.

En relación a los parámetros de intercambio gaseoso, su estudio en el género *Pinus* se remonta a los años 80; en *P. taeda* (Teskey *et al.*, 1987) y en *P. ponderosa* (DeLucia & Heckathorn, 1989). Bajo condiciones de estrés hídrico, las plantas disminuyen tanto su conductancia estomática (g_s) como su actividad fotosintética (Inclán *et al.*, 2005; Letts *et al.*, 2009), llegándose a producir una total fotoinhibición como consecuencia de un estrés severo y, en algunas ocasiones, daños en los fotosistemas y/o en sus componentes (Baquedano & Castillo, 2007; Martínez-Ferri *et al.*, 2000). Además, la regulación estomática se encuentra estrechamente relacionada con el estado hídrico de las plantas, agrupándose en dos categorías denominadas regulaciones isohídricas y anisohídricas (Tardieu & Simonneau, 1998). Las plantas isohídricas reducen su conductancia estomática cuando el potencial hídrico del suelo decrece y las condiciones atmosféricas presentan baja humedad relativa, manteniendo un Ψ_{leaf} relativamente constante a pesar de la limitada disponibilidad de agua (McDowell *et al.*, 2008). Por el contrario, las especies anisohídricas permiten una disminución del Ψ_{leaf} a medida que disminuye el contenido de agua del suelo. Entre las *Pinaceae*, el *P. edulis* es un buen ejemplo

de especie isohídrica, ya que consigue mantener Ψ_{leaf} en valores de -2 MPa mediante un eficiente control estomático bajo condiciones severas de déficit hídrico (McDowell *et al.*, 2008; West *et al.*, 2008).

Igualmente, se ha observado una estrecha relación entre las variaciones de Ψ_{leaf} e intercambio gaseoso, y la conductancia hidráulica (Hubbard *et al.*, 2001; Sperry, 2000). Así, los primeras investigaciones realizadas por Sperry & Tyree (1990) indicaron que la pérdida de conductancia hidráulica que daba lugar a la embolización del tejido xilemático variaba entre especies (Brodribb *et al.*, 2005; Cochard *et al.*, 2002), como también sucede en el género *Pinus* (Charra-Vaskou & Mayr, 2011; Domec *et al.*, 2009; West *et al.*, 2007). Además, las hojas son la parte que más contribuyen a la resistencia global de la planta (Sack & Holbrook, 2006). Sin embargo, en coníferas, la relación del estado hídrico [Ψ_{leaf} y RWC (%)] y parámetros de intercambio gaseoso de las plantas con respecto a su conductancia hidráulica son poco conocidas (Brodribb *et al.*, 2010; Quero *et al.*, 2011), y menos aún en *Pinus radiata* y/o a nivel intraespecífico (Tognetti *et al.*, 1997).

El estrés hídrico no solo causa cambios fisiológicos en las plantas sino que además afecta a gran cantidad de rutas metabólicas (Pospisilova *et al.*, 2005). Por eso, no es de extrañar que los cambios del contenido de fitohormonas sean la base de las respuestas al estrés hídrico, ya que estos compuestos están considerados como la principal señal entre la parte radicular y aérea de la planta (Dodd, 2005; Pérez-Alfocea *et al.*, 2010). En el género *Pinus*, el ácido abscísico (ABA) ha sido ampliamente estudiado como fitohormona de respuesta al estrés hídrico (Kume *et al.*, 2006; Rajasekaran & Blake, 1999), ya que se considera el principal responsable del cierre estomático (Schachtman & Goodger, 2008; Wilkinson & Davies, 2002). En este sentido, recientes trabajos sugieren que el funcionamiento estomático está también regulado por otras fitohormonas como las auxinas, citoquininas (Cks), etileno, ácido jasmónico (JA) y ácido salicílico (SA) (Acharya & Assmann, 2009; Santner & Estelle, 2009). Con el fin de esclarecer la implicación de todas ellas en condiciones de estrés, se hace necesario un mayor análisis de las mismas así como sus posibles interacciones, sobre todo en coníferas.

Respecto a la síntesis de Cks existen controversias sobre su implicación en los mecanismos de tolerancia a sequia (Chernyad'ev & Monakhova, 2003; Tanaka *et al.*, 2006). En coníferas, prácticamente no existen estudios que determinen el papel de las Cks en condiciones de estrés hídrico, aunque si se han observado variaciones de sus niveles bajo otros estreses

abióticos como contaminación del suelo (Kraigher & Hanke, 1996), o presencia de altos niveles de aluminio (Cizkova, 1995).

Entre las auxinas, el ácido índole-3-acético (IAA) es el más estudiado en condiciones de estrés (Albacete *et al.*, 2008; Arbona & Gómez-Cadenas, 2008; Mahouachi *et al.*, 2007), aunque existe relativa poca información sobre la variación de su concentración en condiciones de estrés hídrico (Acharya & Assmann, 2009).

I. 5.- Objetivo

El **objetivo general** de este estudio fue la caracterización fisiológica de plantas de *Pinus radiata* D. Don en respuesta al estrés hídrico, con el fin de determinar indicadores de tolerancia, capacidad de recuperación y endurecimiento. Para ello, se seleccionaron cinco ecotipos y un híbrido de especie (*Pinus radiata* x *Pinus attenuata*) de diferentes zonas geográficas y climatológicas. Para lograr este objetivo general, nos propusimos los siguientes objetivos parciales:

- ✓ **Objetivo 1.-** Evaluar los diferentes parámetros fisiológicos y sus interconexiones a lo largo de un ciclo de sequía y posterior rehidratación (Chapter 1).

- ✓ **Objetivo 2.-** Estudiar la implicación de las principales fitohormonas en la respuesta de las plantas a lo largo de un ciclo de sequía y posterior rehidratación (Chapter 2).

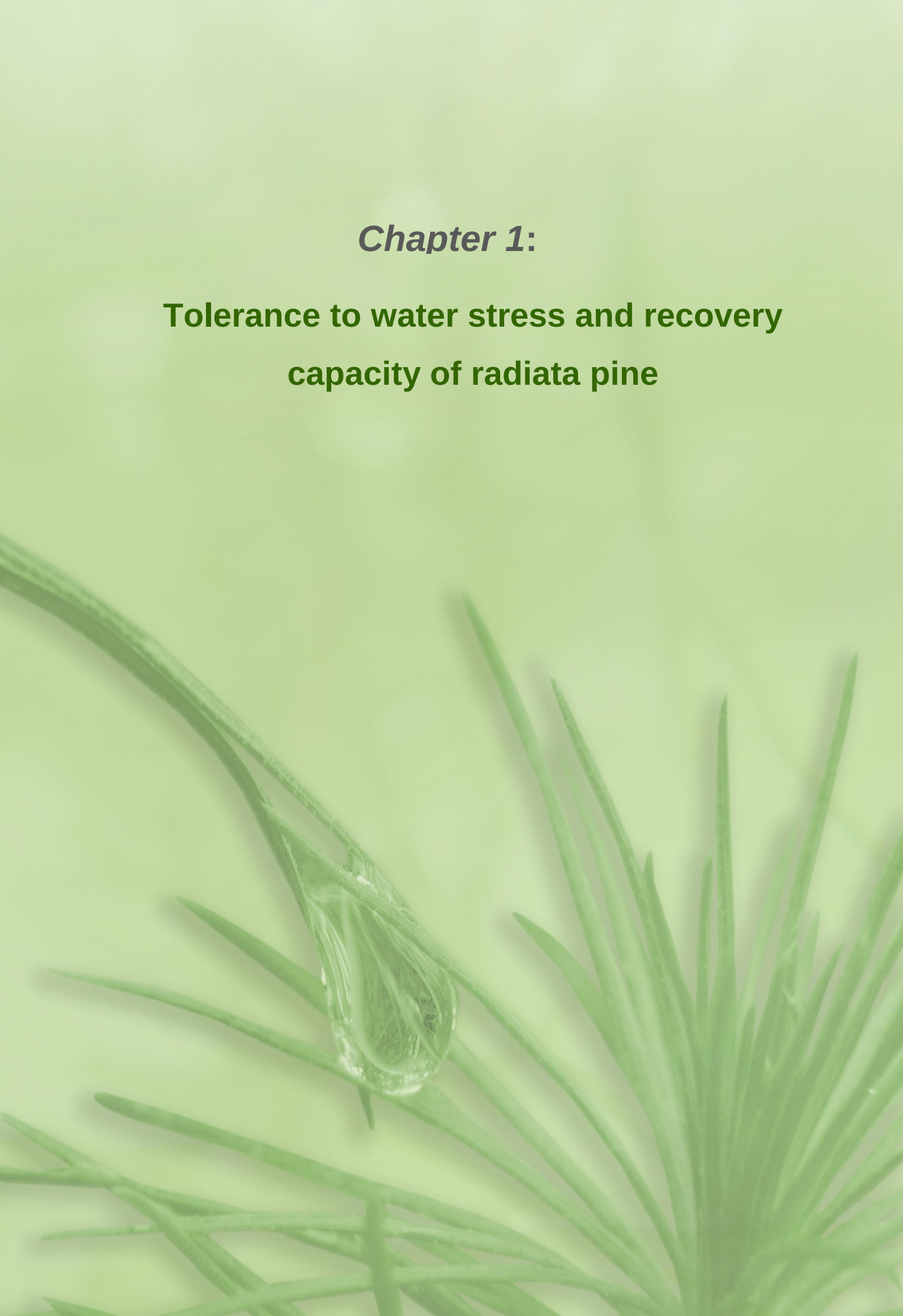
- ✓ **Objetivo 3.-** Analizar la capacidad de ajuste osmótico de las plantas durante un ciclo de estrés y posterior rehidratación, y evaluar en qué grado contribuyen a la tolerancia a la sequía (Chapter 3).

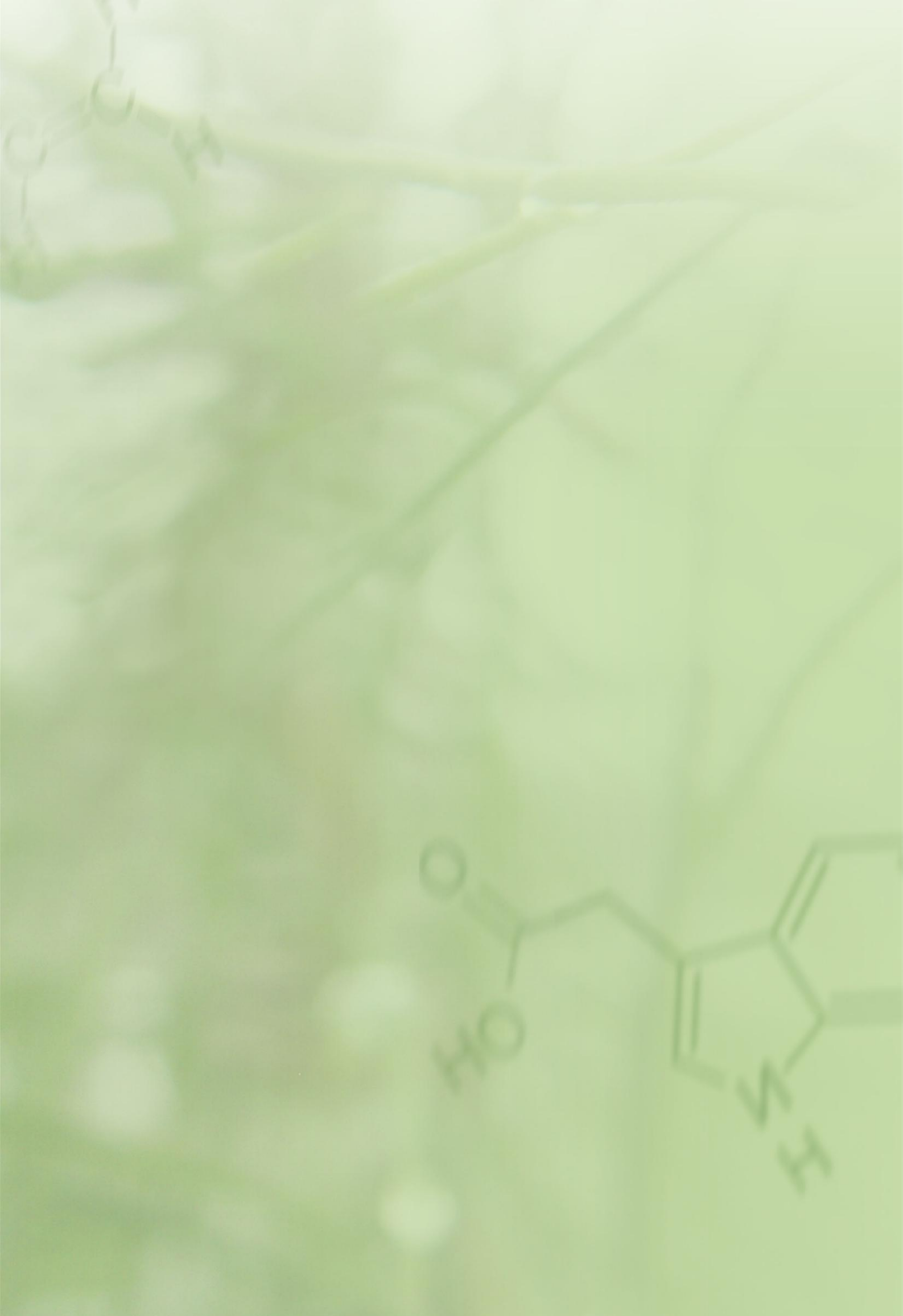
- ✓ **Objetivo 4.-** Determinar si las características morfológicas están asociadas con la tolerancia al estrés hídrico (Chapter 4).

- ✓ **Objetivo 5.-** Comparar la capacidad de endurecimiento de las plantas e identificar los parámetros fisiológicos implicados (Chapter 4).

Chapter 1:

Tolerance to water stress and recovery capacity of radiata pine





1.1.- Introduction

Drought reduces the capacity of plants to take up water from the soil (Medlyn *et al.*, 2011). Nowadays, low water availability is the main environmental factor limiting plant growth and yield worldwide (Flexas *et al.*, 2006). In this regard, the fate of many forest ecosystem will depend on the climate change process ratio in relation to the adaptation grade to such changes (Klein *et al.*, 2011). For this reason, plant drought tolerance will be critical for plantation success and natural forestry regeneration (López *et al.*, 2009b). The improvement in drought resistance of forest species have been one of the main objectives of some breeding programs for a long time, and intensive studies are being carried out to identify factors that can be used as criteria for selection of genotypes with different drought tolerance levels (Cregg, 1994).

Water balance, gas exchange and morphological parameters have been traditionally analyzed to determinate plant drought tolerance (Brodribb & McAdam, 2011; Domisch *et al.*, 2001; Mena-Petite *et al.*, 2003). Furthermore, the photosystem II (*PSII*) efficiency, photosynthetic pigment composition and cell membrane status have been proved to play important roles in water stress and drought tolerance mechanisms (Vilagrosa *et al.*, 2010). In regard to cell membranes, they are considered to be one of the first targets of many stresses, and the maintenance of their integrity and stability under water stress situations is one of the principal conditions of drought tolerance (Lauriano *et al.*, 2000). In addition, some works have showed a strong relationship between gas exchange parameters and leaf water potential with plant hydraulic conductance (Cochard *et al.*, 2002; Hubbard *et al.*, 2001; Sperry, 2000). In this respect, the xylem vulnerability of hydraulic conductance to stress in conifers is recently motive of a further thorough study (Brodribb & Cochard, 2009; Willson & Jackson, 2006), in some *Pinus* species included [*P. edulis* (West *et al.*, 2007), *P. halepensis* (Klein *et al.*, 2011), *P. mugo* (Charra-Vaskou & Mayr, 2011), *P. palustris* (Addigton *et al.*, 2006), *P. ponderosa* (Hubbard *et al.*, 2001), *P. taeda* (Domec *et al.*, 2009) and *P. virginiana* (Johnson *et al.*, 2011)]. However, this study has not been carried out in *P. radiata*, and there is little information at intra-species level (Tognetti *et al.*, 1997).

Pinus radiata D. Don is one of the most cultivated species in the North of Spain, owing to its fast growth and high quality wood production (Codesido & Fernández-López, 2009; Ivkovic *et al.*, 2006). For this reason, the learning of its physiological mechanisms is necessary to guarantee both the survival and productivity under the future climate change conditions, as it

has been previously described in *Pinus canariensis* (López *et al.*, 2009b), *Pinus pinaster* (Sánchez-Gómez *et al.*, 2010) and *Pinus sylvestris* (Cregg & Zhang, 2001).

Due to the complex relationships among all of these physiological parameters through a drought cycle and subsequent recovery, there are few studies that have evaluated their interconnection (Brodribb & Holbrook, 2003; Liu *et al.*, 2003; Niu *et al.*, 2003). The use of statistic tools such as multivariate analysis of variance (MANOVA) and principal component (PC) analysis make easier the understanding of the relationship among the different physiological parameters (Jacobsen *et al.*, 2008; Maherali *et al.*, 2002; Ober *et al.*, 2005).

The physiological traits related to water stress and recovery capacity in *P. radiata* should be studied due to the fact that this knowledge would be highly interesting in genetic improvement programs. For this reason, the aim of this work was to determine the effect of water stress on the early physiological response assessed in plants climatologically different. The evaluation of several physiology parameters and their interconnections along a drought cycle and subsequent rewatering could permit us to identify initial signals of water stress in *P. radiata*.

1.2.- Material and Methods

1.2.1.- Plant material and growth conditions

1.2.1.1.- Plant material

Seeds from different geographical and climatological ecotypes were obtained from the following origins and companies (Table 1.1):

- ✓ **O1- *Pinus radiata* var. *radiata* x *Pinus radiata* var. *binata*:** Provided by Proseed and collected from a seed orchard located in Amberley, New Zealand.

- ✓ **O2- *Pinus radiata* var. *radiata*:** Provided by Servicio de Material Genético of Ministerio de Medio Ambiente and collected from open-pollinated trees grown in the Basque coastline (Spain).

- ✓ **O3- *Pinus radiata* var. *radiata*:** Provided by Australian Tree Seed Centre (CSIRO Forestry and Forest Products) and collected from a seed orchard located in Billapaloola, Australia.

- ✓ **O4- *Pinus radiata* var. *radiata* x *Pinus attenuate*:** Provided by Proseed and collected from a seed orchard located in Amberley, New Zealand.

- ✓ **O5- *Pinus radiata* var. *radiata* x *Pinus radiata* var. *cedrosensis*:** Provided by Proseed and collected from a seed orchard located in Amberley, New Zealand.

- ✓ **O6- *Pinus radiata* var. *radiata* (GF 17):** Provided by Proseed and collected from a control-pollinated trees located in Kaingaroa, New Zealand.

Table 1.1. Location and climate details of the six *Pinus radiata* ecotypes (O1-O6). Data are derived from the Bureau of Meteorology from the Government of each country. (T= Temperature).

Localization	Country	Latitude	Longitude	Altitude (m)	Annual rainfall (mm)	Monthly rainfall (mm)	T.mean (ºC)	T.maximum (ºC)	T.minimum (º C)
Amberley (O1, O4, O5)	New Zealand	27º 62´ S	152º 71´ E	24	845.3	72.3	19.9	26.8	13.1
Kaingaroa (O6)	New Zealand	28º 24´ S	176º 34´ E	544	1545.6	128.9	10.9	16.0	5.9
Oihanberri (O2)	Spain	43º 17´ N	2º 54´ O	42	1434	119.5	14.3	19.1	9.4
Billapaloola (O3)	Australia	35º 18´S	148º 40´E	700	1250	104.1	15	21	9

1.2.1.2.- Growth conditions

Seeds were subjected to cold stratification prior to sowing. They were stored at 4^{o}C under dark conditions for three weeks. Immediately after, they were introduced for two days in sterilized water to induce germination at the same conditions. Finally, seeds were sown in pots of 17 cm ∅ with peat:perlite (7/3, v/v), and maintained in a greenhouse for two years under controlled conditions (T^{a}= 23ºC and RH=70%).

1.2.2.- Experimental design

In order to study *P. radiata* plant response to drought and their recovery capacity after rewatering, two-year old plants were analyzed during summer time. Ten plants per ecotype were

used; half of them were randomly selected for water stress treatment by withholding water (Water stressed plants- D), and the remaining plants were cultured with water supply (Control plants- W). Drought treatment was maintained for four weeks (from T0 to T4), and immediately after water stressed plants were rewatered for a week (Fig. 1.1). Recovery was evaluated after three (R3) and/or seven (R7) days after rewatering. Water status parameters were analyzed at R3 and R7. Gas exchange, fluorescence parameters and electrolyte leakage were measured at R7. Finally, at T4, relative water content and photosynthetic pigments were evaluated.

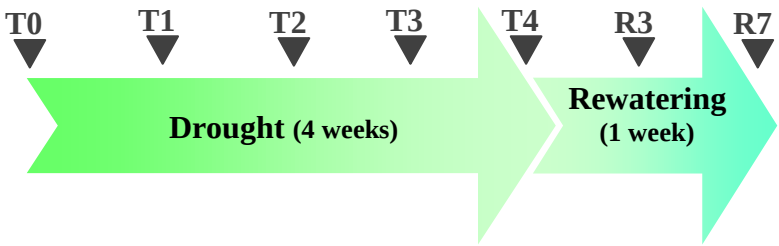


Figure 1.1. Scheme of the experimental design for six *Pinus radiata* ecotypes (O1-O6) subjected to a drought period of four weeks (from T0 to T4), and subsequent rewatering for one week. R3 and R7 indicate three and seven days after rewatering, respectively.

1.2.3.- Water status determination

1.2.3.1.- Relative Water Content

Relative water content [RWC (%)] was determined in two needles collected from apical area of each plant at the end of the drought period (T4), following the method described by Boyer (1969). RWC was calculated as the following equation:

$$RWC\ (\%) = (FW-DW)/(TW-DW) \times 100 \qquad \text{(Eqn. 1.1)}$$

Where,

FW is the fresh weight at the harvesting time; TW is the weight at full turgor estimated after 24 hours of needle imbibition in water. DW is the dry weight after 48 hours at 60 ºC.

1.2.3.2.- Water potential.

Water potential (Ψ) (MPa) of all ecotypes were measured at predawn (from 5:00 to 6:00 a.m.) (Ψ_{pd}) and at midday (Ψ_{leaf}, leaf water potential) along the drought period (from T0 to T4),

and at three (R3) and seven (R7) days after rewatering, using Scholander chamber (Skye SKPM 1400) according to the pressure-equilibration technique (Scholander *et al.*, 1965).

1.2.3.3.- Turgor pressure

Plant turgor pressure (Ψ_t) (MPa) was estimated at the same time as Ψ_{pd} and Ψ_{leaf} and was calculated using the following mathematical equation:

$$\Psi_{leaf} = \Psi_{\pi} + \Psi_t + \Psi_g \qquad \text{(Eqn. 1.2)}$$

where,

Ψ_{π} is osmotic potential calculated by van´t Hoff equation (Ψ_{π}= -R×T^a×c_s); R the gas constant, T^a the absolute temperature (25ºC), and c_s the solute concentration determined by cryoscopic osmometer (Osmomat O30). Ψ_g is the gravity potential estimated as Ψ_g= ρ_w×g×h; ρ_w is the density of water, g the gravity, and h the height of each plant.

1.2.4.- Gas exchange parameters

Instant leaf transpiration (E, mmol H_2O m^{-2} s^{-1}), stomatal conductance (g_s, mmol H_2O m^{-2} s^{-1}), and instantaneous net photosynthesis (A_N, µmol CO_2 m^{-2} s^{-1}) were measured at midday along a drought cycle (from T0 to T4) and after a week of rewatering (R7). An infra-red gas analyzer system (IRGA, ®CIRAS-2 PPSystem) equipped with the universal photosynthesis chamber [PLC(U)] was used.

1.2.5.- Needle hydraulic conductance

Needle specific hydraulic conductance (K_{leaf}) (mmol H_2O m^{-2} s^{-1} MPa^{-1}) is derived from Darcy´s law described by Ewers *et al.* (2000):

$$E = K_{leaf} \times (\Psi_s - \Psi_{leaf} - \Psi_g) \qquad \text{(Eqn. 1.3)}$$

where,

E was instant leaf transpiration, Ψ_s (soil water potential), estimated from Ψ_{pd} according to Hubbard *et al.* (2001); Ψ_g was gravity potential (Eqn. 1.2).

1.2.6.- Electrolyte leakage

In order to determine electrolyte leakage [*E.L.* (%)] two needles per plant and treatment were collected, washed and introduced in a test tube with 5 mL of deionized water. Electrolytic conductivity (EC) was measured using a portable conductivity meter (Cole Parmer-Model 19101-10) at the collection date (EC_i) and after 24 h (EC_f). Then, samples were autoclaved during 10 min at 121ºC, and cooled at room temperature to measure the total electrical conductivity (EC_t). Electrolyte leakage was measured through the drought period (from T0 to T4) and after a week of rewatering (R7), and calculated according to the following equation:

$$E.L.\ (\%) = [(ECf-ECi)/(ECt-ECi)] \times 100 \qquad \text{(Eqn. 1.4)}$$

Index of injury (Id) was determined at T4 as described Flint *et al.* (1967):

$$Id\ (\%) = [(Rs-Rc)/(1-Rc)] \times 100 \qquad \text{(Eqn. 1.5)}$$

where,

Rc and *Rs* are (ECf-ECi)/ (ECt- ECi) for control and stressed plants, respectively.

1.2.7.- Fluorescence and photosynthetic pigment content

Chlorophyll fluorescence was measured in apical needles at room temperature along the drought period (from T0 to T4) and after a week of rewatering (R7) using a portable fluorometer (®Hansatech FMS2). The maximum quantum yield of *PSII* photochemistry (*Fv/Fm*) and the effective quantum yield of photochemical energy conversion in *PSII* (Φ_{PSII}) were estimated as described by Rohácek (2002):

$$Fv/Fm = (Fm-Fo)/Fm \qquad \text{(Eqn. 1.6)}$$

$$\Phi_{PSII} = (Fm´-Fs)/Fm´ \qquad \text{(Eqn. 1.7)}$$

where,

Fo and *Fm* is the minimum and maximum chlorophyll fluorescence yield in the dark-adapted state, respectively; *Fm´* the maximum chlorophyll fluorescence yield in the light-adapted state, and *Fs* the steady-state chlorophyll fluorescence yield in the light-adapted state.

At the end of the drought period (T4), complete non-photochemical quenching of chlorophyll fluorescence (*q*CN) was calculated by the mathematical procedure (Rohácek, 2002):

$$qCN = (Fm-Fm')/Fm \quad \text{(Eqn. 1.8)}$$

Photosynthetic pigment content was determinated in apical needle of each plant and treatment after four weeks under water stress conditions (T4) using dimethylsulfoxide (DMSO) as extraction solvent (Barnes *et al.*, 1992). Chlorophyll as well as carotenoid concentrations (μg mL^{-1}) were calculated as follow:

$$\text{Chlorophyll } a \text{ (Chl } a) = [(12.47 \times A_{665}) - (3.62 \times A_{649})] \quad \text{(Eqn. 1.9)}$$

$$\text{Chlorophyll } b \text{ (Chl } b) = [(25.06 \times A_{649}) - (6.5 \times A_{665})] \quad \text{(Eqn. 1.10)}$$

$$\text{Carotenoid} = (1000 \times A_{480}) - [(1.29 \times \text{Chl } a) - (53.78 \times \text{Chl } b)/220] \quad \text{(Eqn. 1.11)}$$

where,

A_λ is the absorbance at λ wavelength.

1.2.8.- Quantification of parameter decrease

The decrease (%) of plant gas exchange parameters and K_{leaf} was estimated as:

$$\text{Decrease (\%)} = [(Xm-Xi) / Xm] \times 100 \quad \text{(Eqn. 1.12)}$$

where,

Xi was the parameter value measured at time *i* (from T0 to T4) and *Xm* at T0.

1.2.9.- Statistical analysis

Multivariate analysis of variance (MANOVA) was carried out by *proc glm* in the S.A.S® software package to evaluate the relationship among measured parameters and to estimate the possible differences in the response of six *P. radiata* ecotypes along a drought period (from T0 to T4) and a subsequent rewatering for a week (R7). For the analysis, five plants per treatment and ecotype were used according to the following mathematical model:

$$y_{ijkr} = \mu + O_i + T_j + Ti_k + OT_{ij} + OTi_{ik} + TTi_{jk} + OTTi_{ijk} + e_{ijkr} \quad \text{(Eqn. 1.13)}$$

where,

y_{ijkr} was the response variable result of the r^{th} plant of the i^{th} ecotype subjected to j^{th} treatment at k^{th} time; μ was the experimental mean, O_i was the effect of the ith ecotype, T_j the effect of the j^{th} treatment (well watered (W) or water stressed plants (D)), Ti_k the effect of the k^{th} time (T0 to T4 and R7); OT_{ij} was the interaction between the ith ecotypes and j^{th} treatment, OTi_{ik} between the ith ecotype and k^{th} time, TTi_{jk} between the j^{th} treatment and k^{th} time, $OTTi_{ijk}$ among the i^{th} ecotype, the j^{th} treatment and the k^{th} time and e_{ijkr} was the random error component.

Multiple comparisons were calculated by Tukey´s HSD test at $p< 0.05$ to determine the different signification levels among the factors and their possible interactions. Analysis of covariance (ANCOVA) was used to determine the relationship among the parameters highly correlated after MANOVA using *proc glm* in the S.A.S® software.

At the end of the drought (T4), the most relevant physiological parameters were evaluated in stressed plants from six ecotypes (O1-O6) using principal component (PC) analysis to check the possible connection between their levels and the origin climatology. The analysis was performed using The Unscrambler® Version.-9.1 software.

1.3.- Results

1.3.1.- Water status

Water status of each ecotype was analyzed by the determination of predawn (Ψ_{pd}) and midday (Ψ_{leaf}) water potential, and turgor pressure (Ψ_t) in needles along the drought period and after rewatering (R3 and R7).

Concerning $\Psi_{pd,}$ there was not significance among time (Ti), treatment (T) and ecotype (O) according to MANOVA (Table 1.2). Stressed plants from all ecotypes presented variations along the drought period due to the interaction effect of Ti and T ($p<0.001$). Ψ_{pd} was the first physiological parameter which decreased its values from the beginning of the drought, but stressed plants did not showed statistical differences until T2, reaching values below -0.75 MPa (Fig. 1.2). At T4, all stressed plants presented Ψ_{pd} values of -2 MPa, but all of them recovered the initial levels at R3 (Fig 1.2).

Table 1.2. Multivariate analysis of variance (MANOVA) of several physiological variables in six *Pinus radiata* ecotypes (O) under irrigation and no irrigation conditions (T) for four weeks and subsequent rewatering for a week (Ti).

VARIABLE	FACTORS	DF	Sum Sq	Mean Sq	F value	Pr(>F)
Ψ_{pd}	Ti	4	11.46	2.87	93.62	***
	O	5	0.90	0.18	5.24	***
(MPa)	T	1	9.88	9.88	288.13	***
	Ti × O	20	2.01	0.10	2.93	***
	Ti × T	**4**	**9.19**	**2.30**	**67.00**	*******
	O × T	5	0.82	0.16	4.77	***
	Ti × O × T	20	0.79	0.04	1.15	ns
Ψ_{leaf}	Ti	4	9.63	2.41	64.75	***
	O	5	0.66	0.13	3.57	**
(MPa)	T	1	8.85	8.85	238.14	***
	Ti × O	20	1.19	0.06	1.60	ns
	Ti × T	4	9.47	2.37	63.67	***
	O × T	5	0.83	0.17	4.46	***
	Ti × O × T	**20**	**1.53**	**0.08**	**2.06**	******
Ψ_{t}	Ti	4	22.75	5.69	34.07	***
	O	5	2.85	0.57	3.41	**
(MPa)	T	1	11.60	11.60	69.52	***
	Ti × O	20	5.40	0.27	1.62	*
	Ti × T	4	20.05	5.01	30.03	***
	O × T	5	2.88	0.58	3.45	**
	Ti × O × *T	**20**	**5.93**	**0.30**	**1.78**	*****
E	Ti	4	7.122	1.780	97.01	***
	O	5	1.888	0.378	20.57	***
(mmol H_2O $m^{-2}s^{-1}$)	T	1	7.118	7.118	387.85	***
	Ti × O	20	1.104	0.055	3.01	***
	Ti × T	4	1.414	0.354	19.26	***
	O × T	5	0.169	0.034	1.84	ns
	Ti × O × T	**20**	**0.849**	**0.042**	**2.31**	******
g_s	Ti	4	46545.5	11636.38	109.2	***
	O	5	101117.9	2023.6	18.99	***
(mmol H_2O $m^{-2}s^{-1}$)	T	1	42222.9	42222.9	396.25	***
	Ti × O	20	5422.7	271.1	2.54	***
	Ti × T	4	8315.1	2078.8	19.51	***
	O × T	5	1453.7	290.7	2.73	*
	Ti × O × T	**20**	**3756.6**	**187.8**	**1.76**	*****
A_N	Ti	4	32.88	8.22	59.73	***
	O	5	9.50	1.90	13.81	***
(µmol $CO_2m^{-2}s^{-1}$)	T	1	14.88	14.88	108.12	***
	Ti × O	20	11.18	0.56	4.06	***
	Ti × T	**4**	**13.30**	**3.32**	**24.16**	*******
	O × T	5	0.67	0.13	0.97	ns
	Ti × O × T	20	4.38	0.22	1.59	ns
Fv/Fm	Ti	6	0.159	0.032	20.94	***
	O	5	0.063	0.013	8.11	***
	T	1	0.114	0.114	73.69	***
	Ti × O	30	0.082	0.002	1.77	**
	Ti × T	6	0.093	0.015	10.04	***
	O × T	5	0.023	0.004	2.93	*
	Ti × O × T	**30**	**0.076**	**0.002**	**1.64**	*****
Φ_{PSII}	Ti	4	0.119	0.03	9.47	***
	O	**5**	**0.082**	**0.016**	**5.23**	*******
	T	1	0.124	0.124	39.43	***
	Ti × O	20	0.088	0.004	1.39	ns
	Ti × T	**4**	**0.142**	**0.035**	**11.26**	*******
	O × T	5	0.027	0.005	1.72	ns
	Ti × O × T	20	0.081	0.004	1.28	ns
E.L.	Ti	4	4.46	1.11	14.61	***
	O	**5**	**3.12**	**0.62**	**8.17**	*******
(%)	T	1	0.95	0.95	12.40	***
	Ti × O	20	2.06	0.10	1.35	ns
	Ti × T	**4**	**1.19**	**0.30**	**3.90**	******
	O × T	5	0.34	0.07	0.89	ns
	Ti × O × T	20	2.21	0.11	1.39	ns

Note: * < 0.05; ** <0.01 and *** <0.001; ns-non-significant.

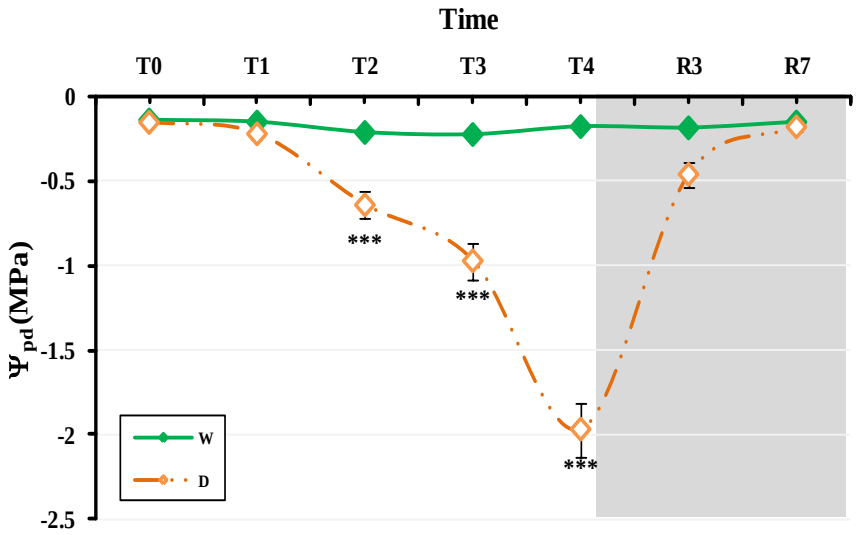


Figure 1.2. Predawn water potential (Ψ_{pd}, MPa) in *Pinus radiata* plants exposed to irrigation (W- Well watered plants) or no irrigation conditions (D- Stressed plants) for four weeks (from T0 to T4) and a subsequent rewatering (Shade area) for three (R3) and seven (R7) days. *M* ± *S.E.* Significant differences with regard to each control are represented by asterisks according to Tukey´s HSD test after MANOVA. * < 0.05; ** < 0.01; *** < 0.001.

Changes of Ψ_{leaf} and Ψ_t were due to the triple effect of Ti, O and T ($p< 0.01$ and $p<0.05$, respectively) according to MANOVA (Table 1.2). Ψ_{leaf} showed statistically significant differences at T3 (Fig. 1.3). Thus, stressed plants from O1, O2, O3 and O5 presented Ψ_{leaf} below -1.5 MPa, whereas O4 and O6 maintained their values near -1 MPa. At T4, stressed plants from all ecotypes were statistically different with respect to each control and showed Ψ_{leaf} lower than -2 MPa, except O4 and O5. At R3, all stressed plants increased their Ψ_{leaf}, reaching their control values at R7 (Fig. 1.3).

Drought caused lower effect in turgor pressure (Ψ_t) than in Ψ_{leaf} (Fig. 1.3). Only stressed plants from O1 presented major differences for Ψ_t at T3, and decreased their values below the turgor loss point (*TLP*) at T4. At this moment, stressed plants from O2, O3 and O6 significantly decreased their Ψ_t as well. Only O4 and O5 maintained their turgor. At R3, all ecotypes recovered their Ψ_t control values, except O1 that slowed down the recovery until R7 (Fig. 1.3).

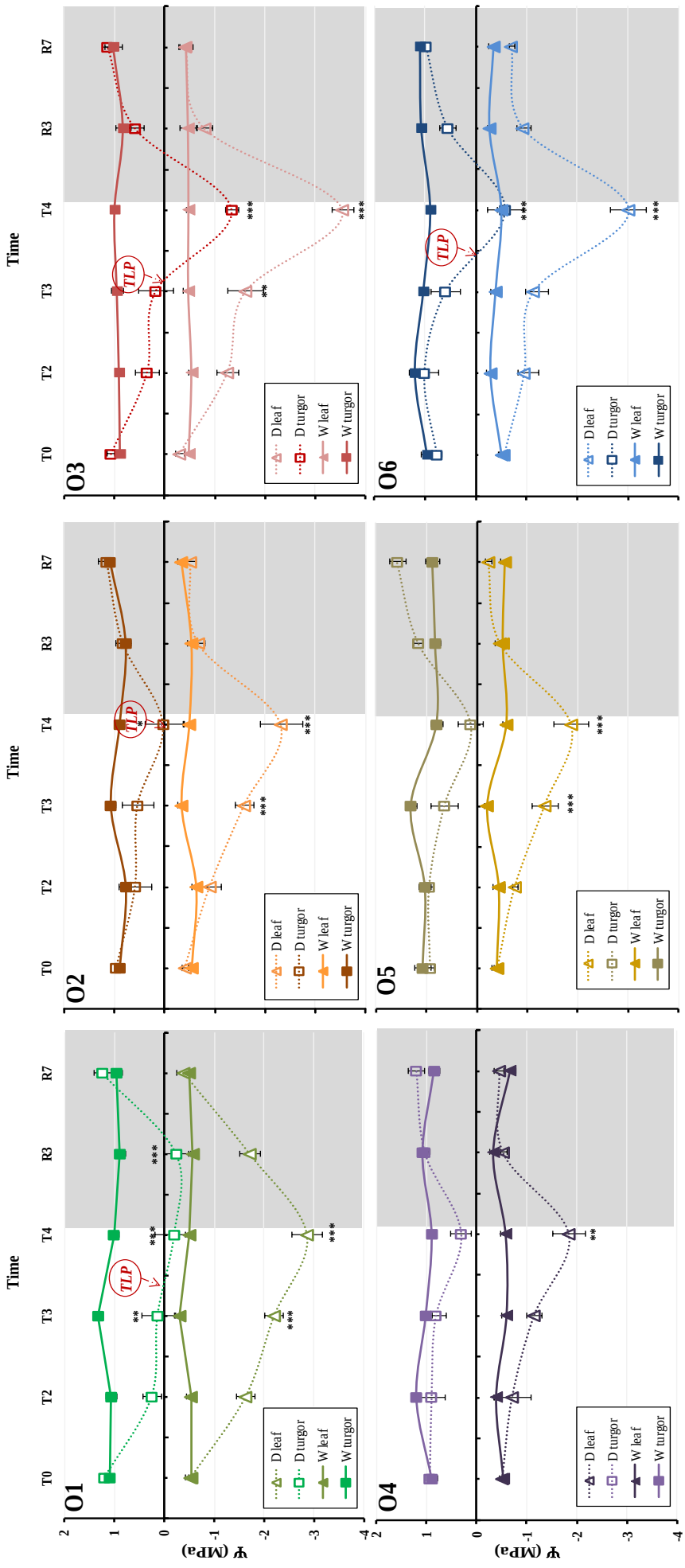


Figure 1.3. Midday water potential (W- and D-leaf) (Ψ_{leaf}, MPa) and turgor pressure (W- and D-turgor) (Ψ_t, MPa) in six *Pinus radiata* ecotypes (O1-O6) exposed to irrigation (W-Well watered, closed symbols) or no irrigation conditions (D- Stressed plants, open symbols) for four weeks (from T0 to T4) and a subsequent recovery after rewatering (Shade area) for three (R3) and seven (R7) days. *M* ± *S.E.* ***TLP***- turgor loss point Significant differences with regard to each control are represented by asterisks according to Tukey´s HSD test after MANOVA. * < 0.05; ** < 0.01; *** < 0.001.

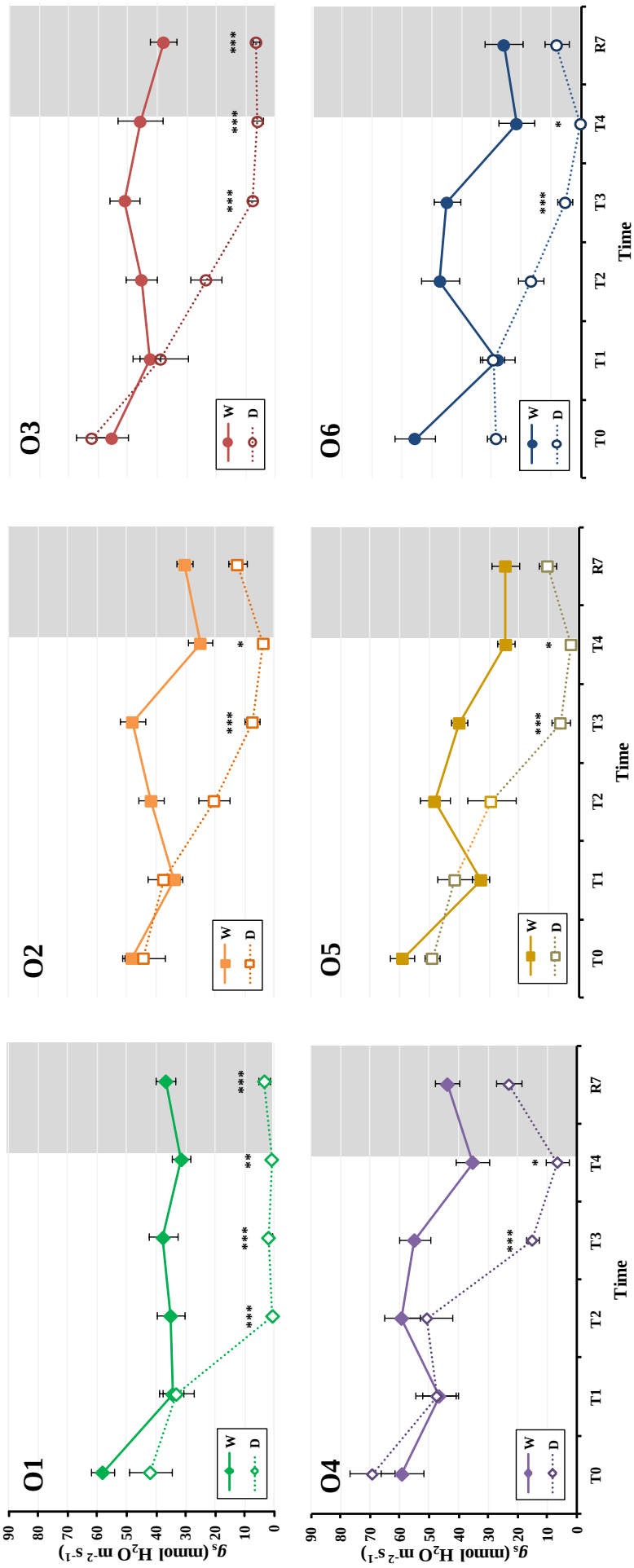


Figure 1.4. Stomatal conductance (g_s, mmol H_2O $m^{-2}s^{-1}$) in six *Pinus radiata* ecotypes (O1-O6) exposed to irrigation (W- Well watered, closed symbols) or no irrigations conditions (D- Stressed plants, open symbols) for four weeks (from T0 to T4) and a subsequent rewatering for a week (R7-Shade area). *M* ± *S.E.* Significant differences are represented by asterisks according to Tukey´s HSD test after MANOVA. * < 0.05; ** < 0.01; *** < 0.001.

1.3.2.- Gas exchange parameters

Stomatal conductance (g_s) and instant transpiration *(E)* presented significative differences in plants subjected to drought due to the interaction effect among Ti, T and O, according to MANOVA (p<0.01 and p<0.05, respectively) (Table 1.2). All stressed plants progressively decreased their g_s levels through the drought period (Fig. 1.4), being statistically significant in O1 at T2, and at T3 in the rest of ecotypes. At T4, g_s was practically valueless in all ecotypes. At R7, all ecotypes maintained their g_s values lower than controls, but only O1 and O3 plants were statistically different (Fig. 1.4).

The triple interaction was not significant for instantaneous net photosynthesis *(A_N)*, and its variations were produced by the reciprocal effect between T and Ti according to MANOVA (p<0.001) (Table 1.2). Drought decreased A_N values from the beginning of the drought period. At T2, all stressed plants showed major differences with respect to their controls (p<0.05) (Fig. 1.5). At T4, A_N was practically negligible in all stressed plants. At R7, plants recovered their control values (Fig. 1.5).

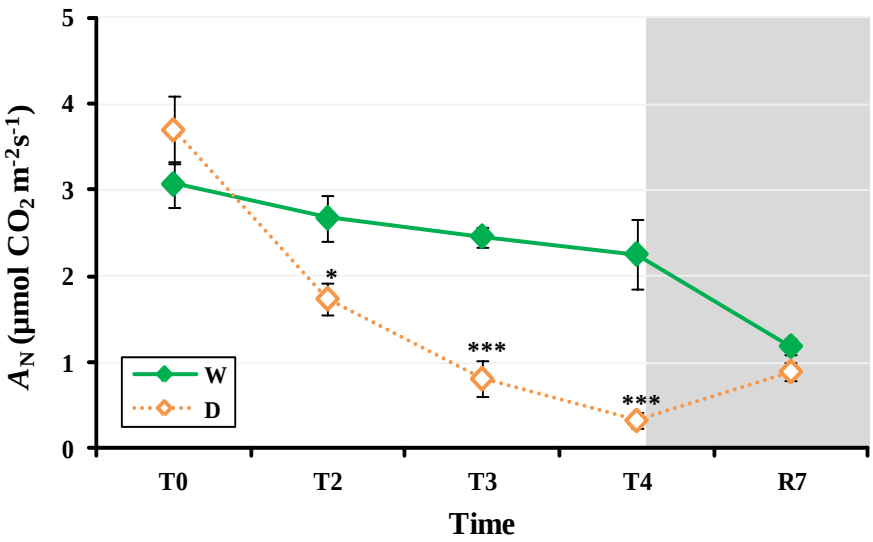


Figure 1.5. Instantaneous net photosynthesis (A_N, µmol CO_2 m^{-2} s^{-1}) in *Pinus radiata* plants exposed to irrigation (W- Well watered plants) or no irrigation conditions (D- Stressed plants) for four weeks (from T0 to T4) and a subsequent rewatering for a week (R7-Shade area). *M* ± *S.E.* Significant differences with regard to each control are represented by asterisks according to Tukey´s HSD test after MANOVA. * < 0.05; ** < 0.01; *** < 0.001.

1.3.3.- Fluorescence, electrolyte leakage and photosynthetic pigments

Maximum quantum yield of *PSII* (*Fv/Fm*) photochemistry showed statistically significant differences for the triple interaction among Ti, T and O according to MANOVA ($p< 0.05$) (Table 1.2). In this matter, stressed plants from O1 and O6 significantly decreased their *Fv/Fm* at T3 (Fig. 1.6). At T4, all stressed plants except those concerning O4 and O5 showed significative differences in their *Fv/Fm* values in relation to their controls. O1 plants presented the highest *Fv/Fm* decreases, reaching values of 0.62. After a week of rewatering, all stressed plants except O1 recovered their *Fv/Fm* (Fig 1.6).

The effective quantum yield of photochemical energy conversion in *PSII* (Φ_{PSII}) did not show significance concerning the triple interaction, and the changes were due to the reciprocal effect between Ti and T according to MANOVA ($p<0.001$) (Table 1.2). Control plants presented values of 0.83 (Fig. 1.7A). Stressed plants significantly decreased to 0.76 at T3. At the end of the drought period, plants decreased their Φ_{PSII} values to 0.71, inducing an increase of the *q*CN values (Data not shown). All stressed plants recovered their initial levels after rewatering.

Electrolyte leakage [*E.L.* (%)] was affected by a double interaction between Ti and T, according to the MANOVA ($p<0.01$) (Table 1.2), and showed an opposite trend as observed in Φ_{PSII} along the drought cycle (Fig. 1.7A and 1.7B).

Whereas the Φ_{PSII} reduction was significant at T3, their *E.L.* (%) of stressed plants did not present significance until T4. At this time, stressed plant reached *E.L.* values of 20.4%. It was remarkable that *E.L.* (%) showed capacity of reversion for all stressed plants which recovered their control values after a week of rewatering (R7) (Fig. 1.7B).

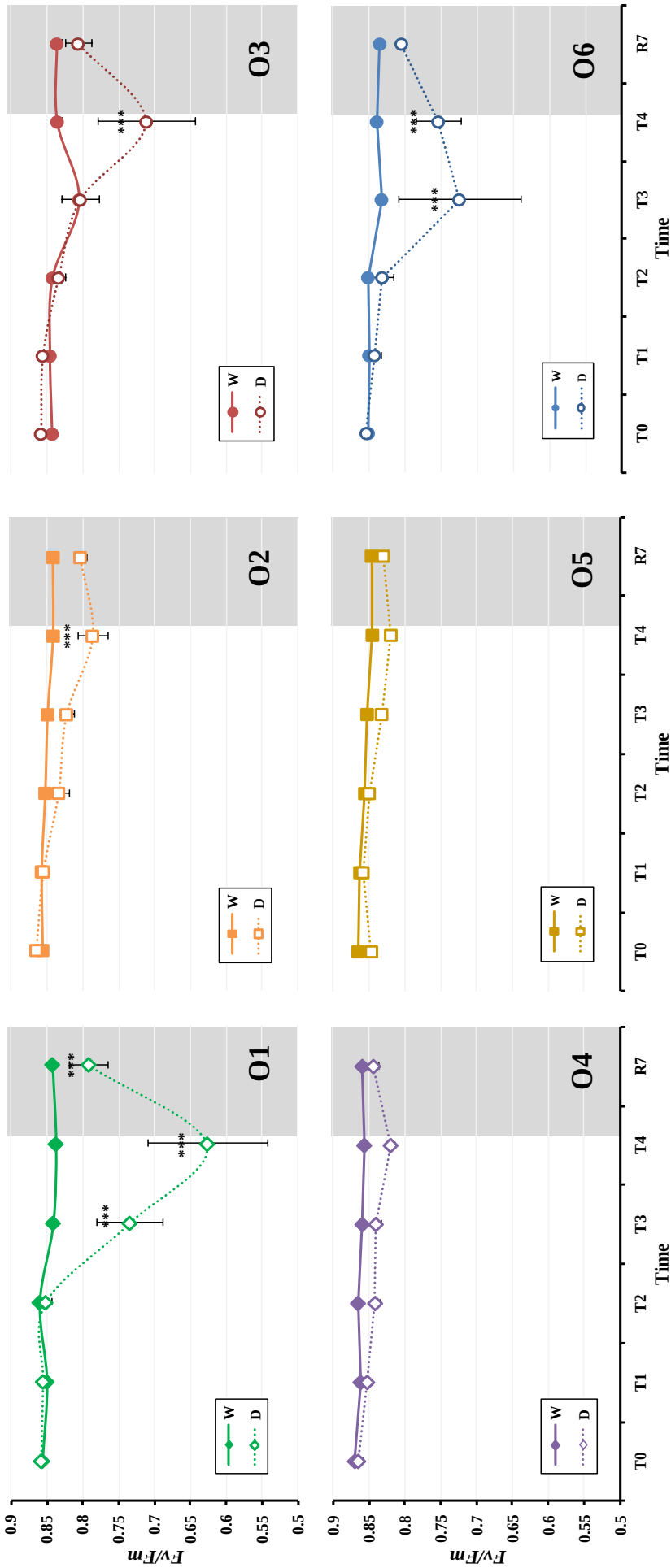


Figure 1.6. Maximum quantum yield of *PSII* (*Fv/Fm*) in six *Pinus radiata* ecotypes (O1-O6) exposed to irrigation (W- Well watered, closed symbols) or no irrigation conditions (D- Stressed plants, open symbols) for four weeks (from T0 to T4) and a subsequent rewatering for a week (R7-Shade area). *M* ± *S.E.* Significant differences with regard to each control are represented by asterisks according to Tukey´s HSD test after MANOVA. * < 0.05; ** < 0.01; *** < 0.001.

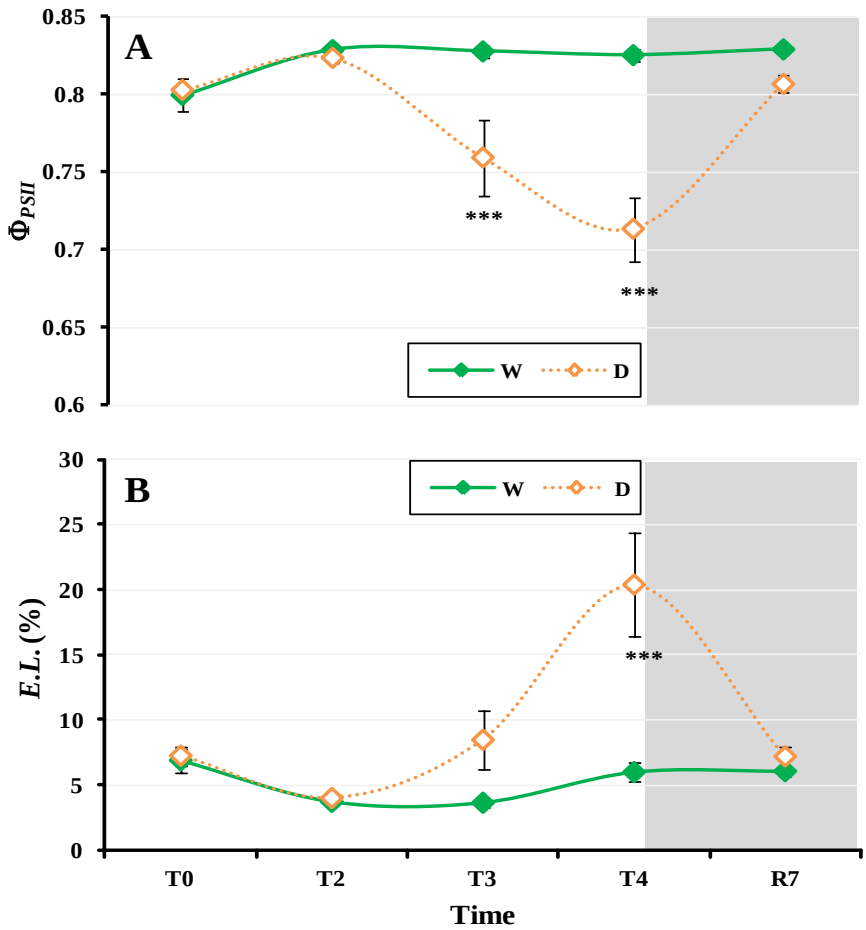


Figure 1.7. The effective quantum yield of photochemical energy conversion in *PSII* (Φ_{PSII}) (A) and electrolyte leakage [*E.L.* (%)] (B) in *Pinus radiata* plants exposed to irrigation (W- Well watered plants) or no irrigation conditions (D- Stressed plants) for four weeks (from T0 to T4) and a subsequent rewatering for a week (R7-Shade area). *M* ± *S.E.* Significant differences with regard to each control are represented by asterisks according to Tukey´s HSD test after MANOVA. * < 0.05; ** < 0.01; *** < 0.001.

At T4, stressed plants not only presented the efficiency of *PSII* yield decrease but also in photosynthetic pigment content (Fig. 1.8). Stressed plants lowered their chlorophyll (*a*+*b*) and carotenoid content with respect to the well watered plants (Fig. 1.8- Inset figures). Regarding chlorophyll (*a*+*b*), the highest decrements were observed in O1. Besides, O1 together with O6 also showed the highest decreases of carotenoids. O4 and O5 presented the lowest changes in photosynthetic pigment content, especially of chlorophyll (*a*+*b*) in O4 and caroteinod content in both ecotypes (Fig. 1.8).

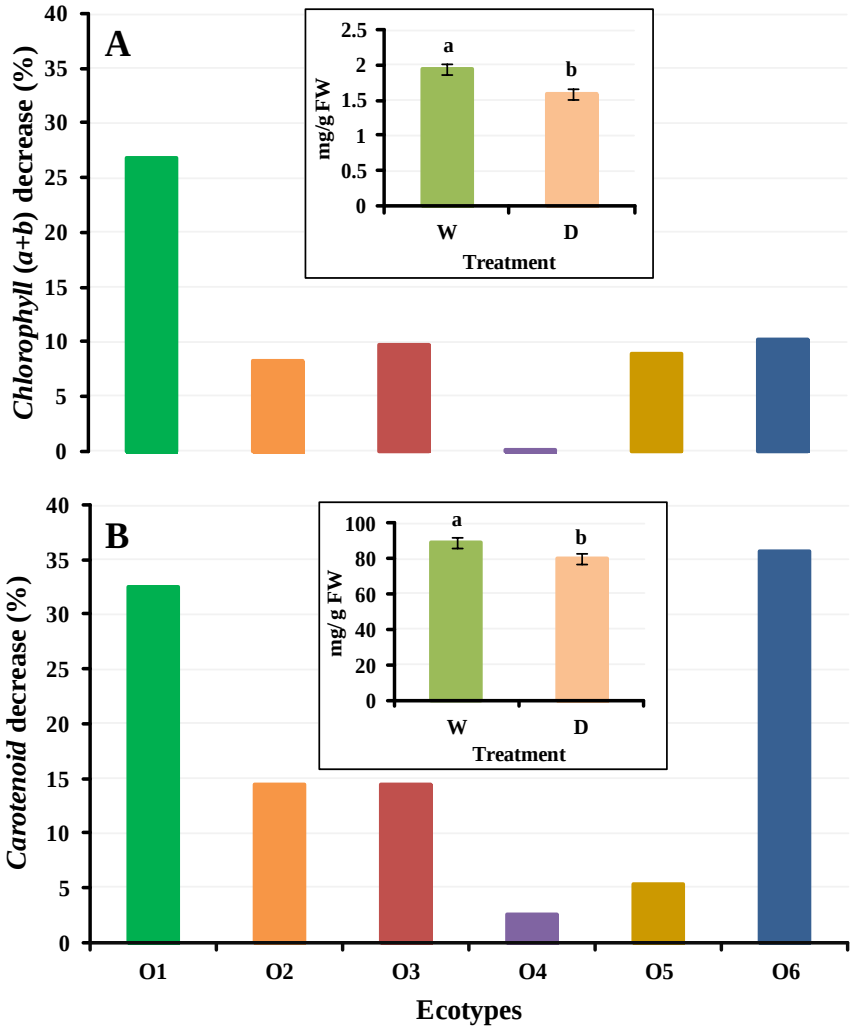


Figure 1.8. Decrease (%) of *Chlorophyll (a+b)* (A) and *carotenoid* (B) ratio content (D-water stressed / W-well watered plants) in six *Pinus radiata* ecotypes (O1-O6) after four weeks under drought conditions (T4). Inset, total pigment content (mg g^{-1} FW). *M* ± *S.E.* Different letter means significant differences according to Tukey´s HSD test after ANOVA.

1.3.4.- Correlation analysis

The correlation matrix was calculated according to MANOVA to determine the strongest relationships among the evaluated parameters (Table 1.3). The most significant correlations were observed between Ψ_{leaf} and Ψ_t, and between *E.L.* (%) and Φ_{PSII}. The high significant relation between *E* and g_s was due to their simultaneous calculation using the same algorithm incorporated into CIRAS-2 PPsystem.

Table 1.3. Lineal correlation matrix among water balance parameters [predawn water potential (Ψ_{pd}), midday water potential (Ψ_{leaf}), and turgor pressure (Ψ_t)], gas exchange [instantaneous net photosynthesis (A_N), instant transpiration (E), and stomatal conductance (g_s)], electrolyte leakage [*E.L.* (%)] and the effective quantum yield of photochemical energy conversion in *PSII* (Φ_{PSII}) according to MANOVA. Numerical values are Pearson´s correlation numbers with the significance**.**

	Ψ_{pd}	Ψ_{leaf}	Ψ_t	A_N	E	g_s	*E.L.* (%)	Φ_{PSII}
Ψ_{pd}	1							
Ψ_{leaf}	0.30***	1						
Ψ_t	0.16*	**0.78*** **	1					
A_N	0.18**	0.10^{ns}	0.04^{ns}	1				
E	0.11^{ns}	0.09^{ns}	0.06^{ns}	0.14*	1			
g_s	0.11^{ns}	0.09^{ns}	0.06^{ns}	0.16*	0.95***	1		
E.L. (%)	-0.00^{ns}	-0.01^{ns}	0.01^{ns}	-0.04^{ns}	0.11^{ns}	0.11^{ns}	1	
Φ_{PSII}	-0.06^{ns}	-0.04^{ns}	-0.07^{ns}	0.14^{*}	0.04^{ns}	0.05^{ns}	**-0.48*** **	1

Note: * < 0.05; ** <0.01 and *** <0.001; ns- non-significant.

Changes of Ψ_t were correlated to the Ψ_{leaf} status, but not influenced by the ecotype according to ANCOVA (Table 1.4), pointing out a global species response. In this matter, stressed plants decreased their Ψ_t and Ψ_{leaf} at the same time (Fig. 1.9). When plants reached Ψ_{leaf} below -2 MPa they lost their turgor (*TLP*) (Fig. 1.9).

Table 1.4. Analysis of covariance (ANCOVA) of turgor pressure (Ψ_t) *vs.* midday water potential (Ψ_{leaf}), and the effective quantum yield of photochemical energy conversion in *PSII* (Φ_{PSII}) *vs.* Electrolyte leakage [(*E.L.* (%)] in *Pinus radiata* plants from *six* different ecotypes (O).

Variable	Factors	P-value
Ψ_t	Ψ_{leaf}	***
	Ecotype (O)	ns
	Ψ_{leaf} * O	ns
E.L. (%)	Φ_{PSII}	**
	Ecotype (O)	**
	Φ_{PSII} * O	***

Note**:** * < 0.05; ** <0.01 and *** <0.001; ns- non-significant.

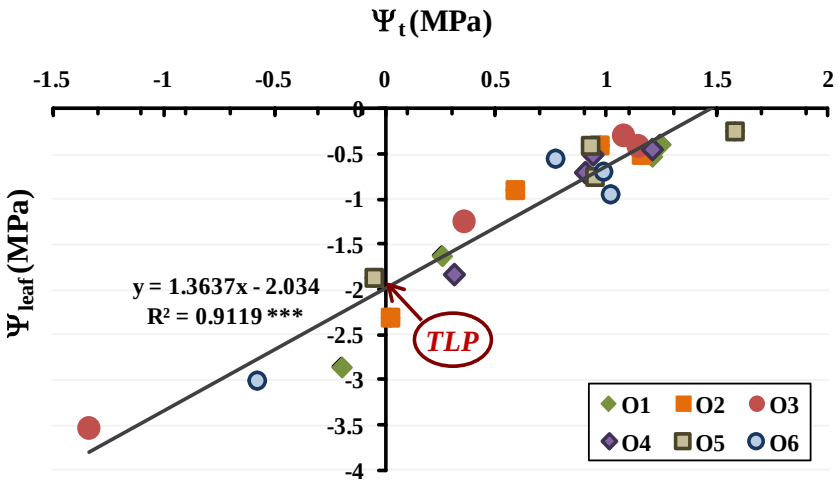


Figure 1.9. Correlation between midday water potential (Ψ_{leaf}) *vs.* turgor pressure (Ψ_t) in six *Pinus radiata* ecotypes (O1-O6) exposed to drought for four weeks (from T0 to T4). ***TLP***- Turgor loss point. R^2- Pearson´s correlation number with the significance according to the ANCOVA. * < 0.05; ** <0.01 and *** <0.001.

There was a significant lineal correlation between *E.L.* (%) and Φ_{PSII}. According to ANCOVA, the effect was due to the interaction between Φ_{PSII} and O ($p< 0.001$). The individual effect of O had also influence on the *E.L.* (%) variations ($p< 0.01$) (Table 1.4). Stressed plants from O1, O3 and O6 presented the highest Φ_{PSII} decreases at T4. These three ecotypes together with O2 increased their *E.L.* over 15% (Fig. 1.10).

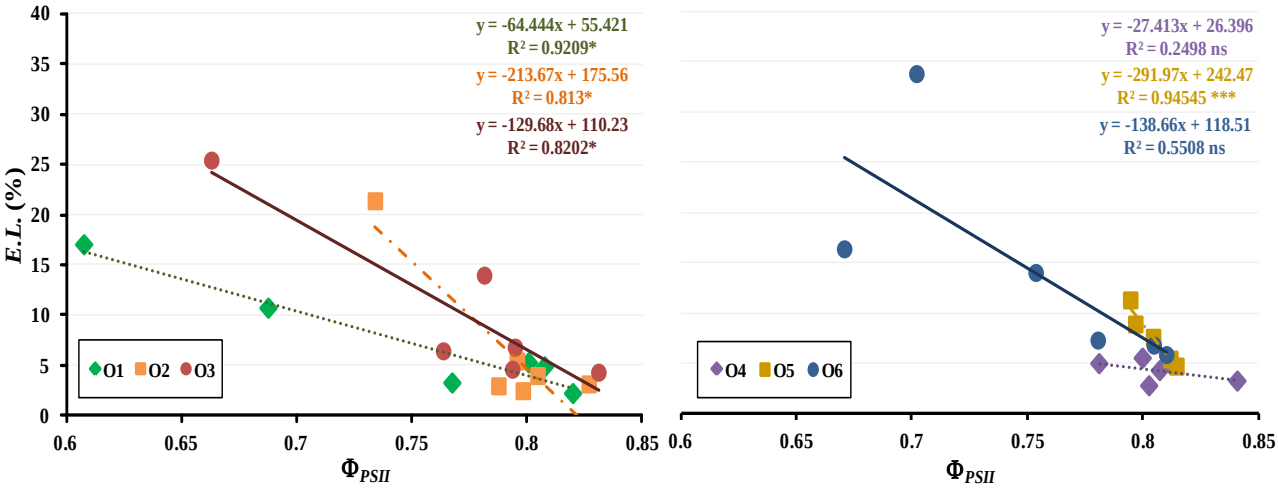


Figure 1.10. Correlation between electrolyte leakage [*E.L.* (%)] *vs.* the effective quantum yield of photochemical energy conversion in *PSII* (Φ_{PSII}) in six *Pinus radiata* ecotypes (O1-O6) exposed to drought for four weeks (from T0 to T4). R^2- Pearson´s correlation number with the significance according to ANCOVA. * < 0.05; ** <0.01 and *** <0.001; ns-non-significant.

When A_N, E and K_{leaf} decrease was evaluated compared to Ψ_{leaf} variation, different drought responses were appreciated among ecotypes (Fig. 1.11). K_{leaf} was the most affected parameter under water stress conditions. A slight decrease of Ψ_{leaf} (-0.7 MPa) was generally accompanied by a K_{leaf} decrement higher than 50%. When K_{leaf} decreased below 90 %, stressed plants reached their *TLP*, except in the case of O4 and O5 which decreased their K_{leaf} near 100% without reaching the *TLP*. Besides, E and A_N decreased in line with K_{leaf} in all ecotypes, except in O1 which dropped its photosynthetic activity later. Drought provoked a similar behaviour between stressed plants from O4 and O5 (Fig. 1.11).

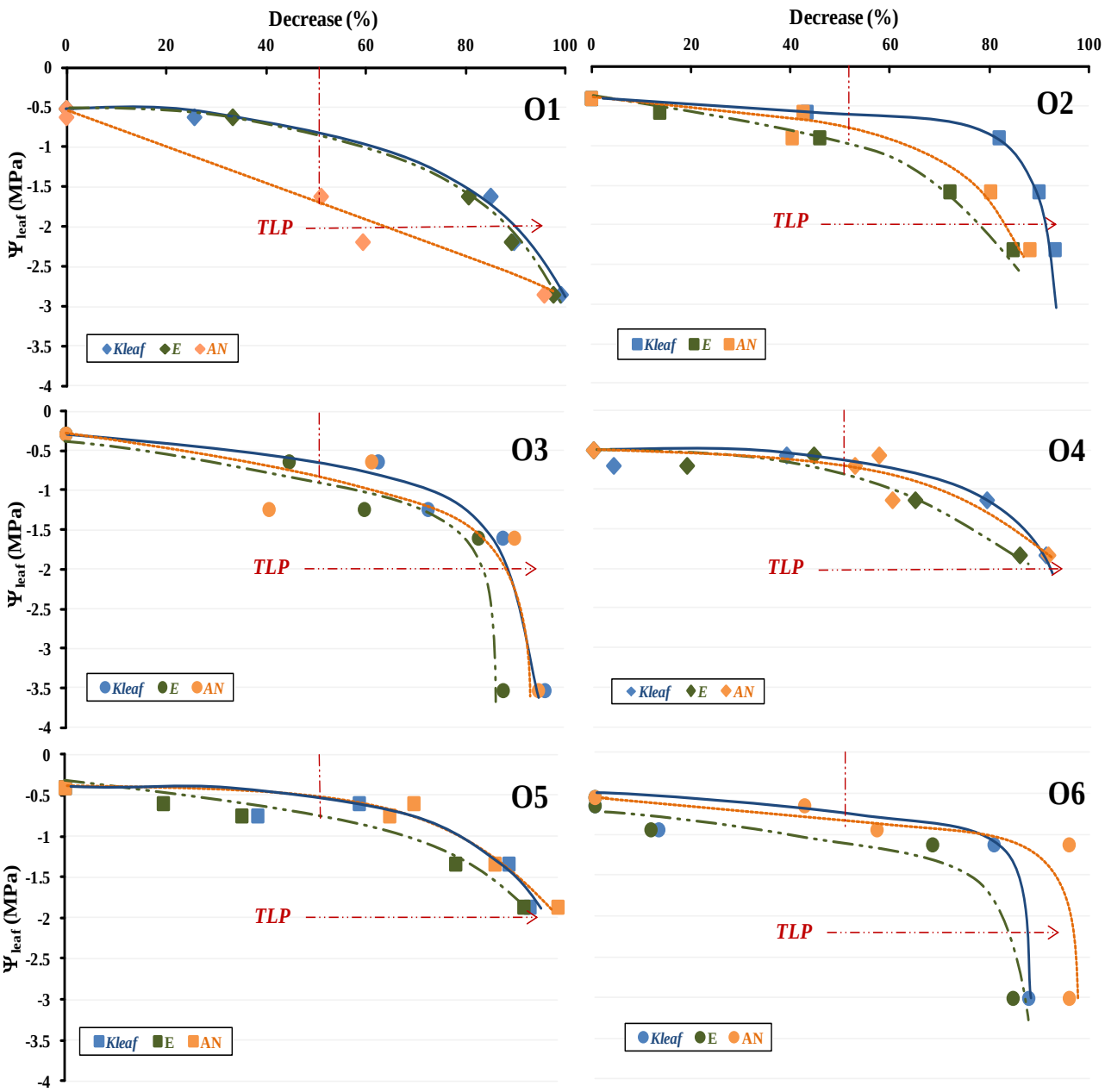


Figure 1.11. Relation among midday water potential (Ψ_{leaf}) *vs.* needle hydraulic conductance (K_{leaf}), transpiration (E) and photosynthesis (AN) decreases (%) in six *Pinus radiata* ecotypes (O1-O6) exposed to drought conditions for four weeks (from T0 to T4). Discontinuous red line represented decreases of 50%. Discontinuous red arrows indicate the turgor loss point- ***TLP***.

The relationship between physiological parameters and drought tolerance in different ecotypes was evaluated at T4 by Principal Component (PC) analysis (Fig. 1.12). The study was well represented by the PC-1 and PC-2 to the fact that they explained a total of 80% of the experiment variance (54% and 26%, respectively).

Negative correlation was observed between O6, and both O4 and O5. Stressed plants from O4 and O5 were closely correlated with the highest values of extreme (minimum and maximum) and mean temperature, Ψ_{leaf}, Ψ_t, RWC (%), and g_s. These two ecotypes showed an inverse correlation with annual-rainfall, monthly-rainfall, Id (%) and *E.L.* (%). On the contrary, PC-2 showed that O1 plants had a direct correspondence with parameters such as K_{leaf} decreases (%) and *q*CN, and an inverse relationship with Ψ_{leaf}, A_N, and *Fv/Fm* (Fig. 1.12).

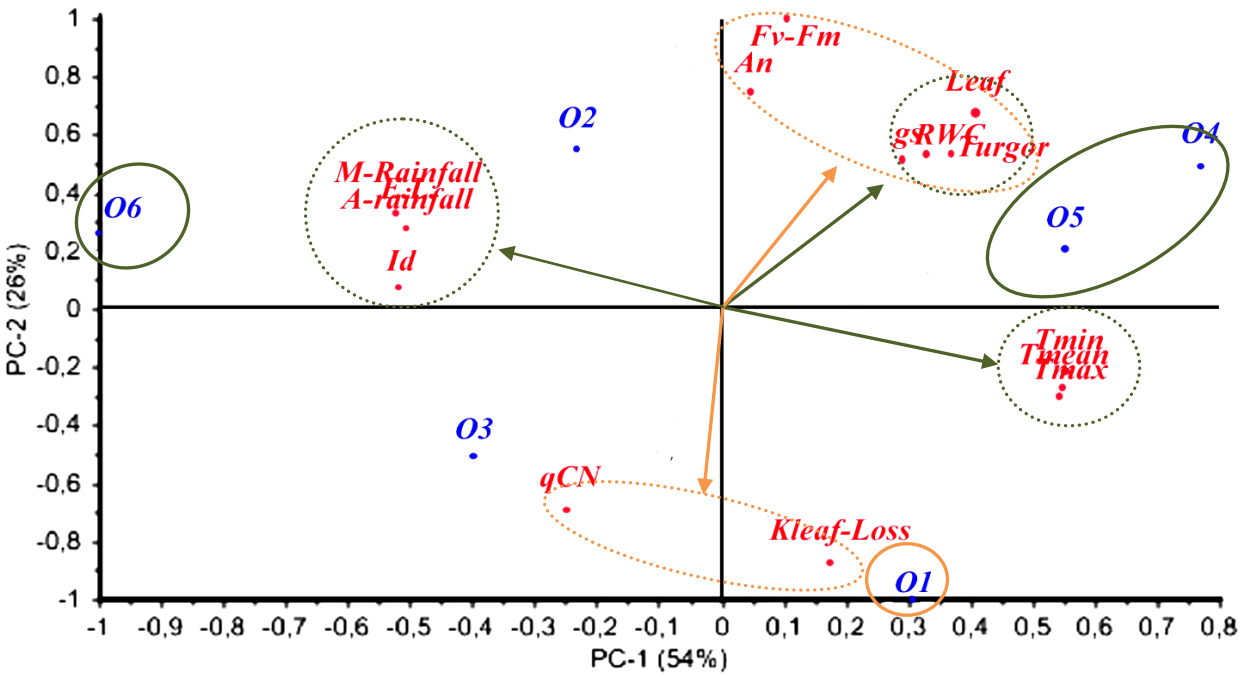


Figure 1.12. Principal component (PC) analysis of physiological and climatological parameters in six *Pinus radiata* ecotypes (*O1-O6*) after four weeks of drought conditions (T4). Annual rainfall- *A-Rainfall*, Monthly rainfall- *M-Rainfall*, temperature mean- *Tmean*, maximum- *Tmax* and minimum- *Tmin*, leaf water potential- *Leaf*, turgor pressure- *Turgor*, Relative water content (%)- *RWC*, leaf hydraulic conductance decrease - *Kleaf-loss*, gas exchange parameters (instantaneous net photosynthesis- *An*, instant transpiration- *E* and stomatal conductance- *gs*), photosystem *II* yield (maximum quantum yield of *PSII* photochemistry-*Fv-Fm*, and non-photochemical quenching of chlorophyll fluorescence –*qCN*), electrolyte leakage (%) - *E.L.*, and the index of injury- *Id*. Results of PC-1 were represented by green colour and PC-2 by orange colour.

1.3.5.- Recovery capacity after drought

When recovery capacity was analyzed, 100% stressed plants survived at R7, except O6 plants (80%) (Fig. 1.13A). All plants practically reached their RWC control levels with recoveries over 95% (Fig. 1.13B). The K_{leaf} recovery was also analyzed and only stressed plants from O4 and O5 increased their values to control ones, with percentages of 84 and 107 %, respectively. O2, O3 and O6 plants only recovered 20% of their K_{leaf} control values, whereas O1 showed a recovery of 40% (Fig. 1.13B).

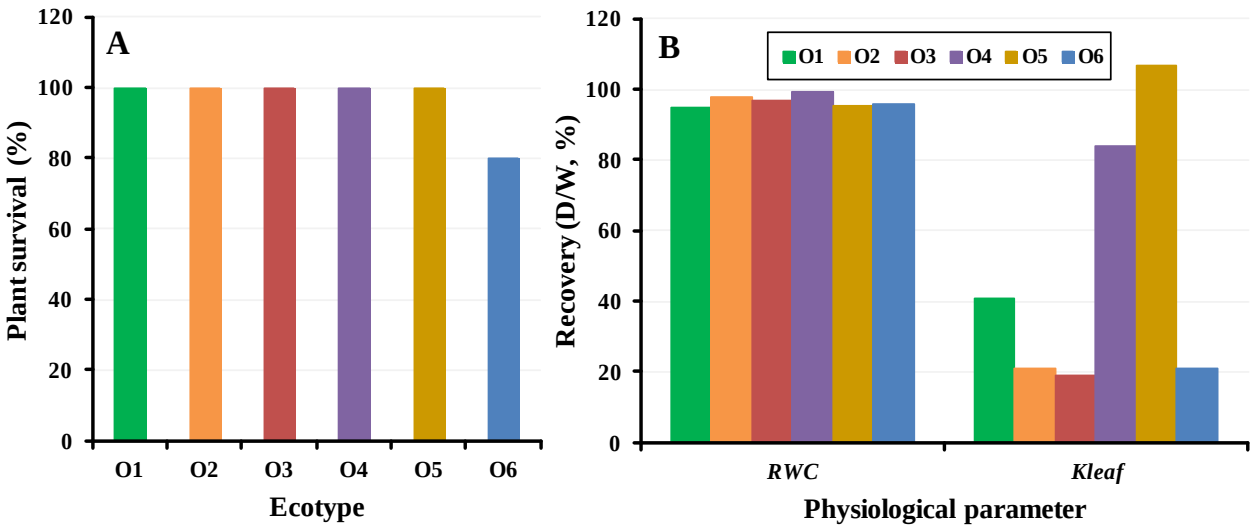


Figure 1.13. Plant survival (%) (A) and recovery capacity of RWC and hydraulic conductance (K_{leaf}) (%) (B) in six *Pinus radiata* ecotypes (O1-O6) rewatered for a week (R7) after a drought period of four weeks (from T0 to T4).

1.4.- Discussion

1.4.1.- Dynamic of *P. radiata* plant response to drought

Plants have different drought response and, while some plants exhibit capacity for acclimatation under adverse conditions, others only show a modest tolerant response to external changes (Valladares *et al.*, 2007). In our study, *Pinus radiata* D. Don plants from six climatologically different ecotypes showed similar values for all evaluated parameters at T0. However, different responses among ecotypes were observed along the drought period and subsequent recovery after rewatering. Regarding Ψ_{pd}, stressed plants from all ecotypes significantly decreased their values at T2 (Fig. 1.2). In this regard, Hubbart *et al.* (2001)

indicated that Ψ_{pd} was a good indicator of soil water status, pointing out that all plants were subjected to the same water stress.

In our study, the event sequence through water stress period underpinned K_{leaf}, together with g_s and E as the first processes affected in radiata pine plants subjected to drought (Fig. 1.4 and 1.9). These results were in accordance with earlier works carried out in another conifers where a strong relationship between K_{leaf} and Ψ_{leaf} (Brodribb & Cochard, 2009; Ewers *et al.*, 2000), and gas exchange parameters (Johnson *et al.*, 2009) were observed. At T4, all ecotypes showed K_{leaf}, g_s, E and A_N decrements higher than 90%. Previous studies have demonstrated that plants limit transpiration by stomatal closure before a serious decrease of hydraulic conductance occurs to prevent embolization (K_{leaf} = 0) (Delzon *et al.*, 2004; Johnson *et al.*, 2011). This fact has also been recognized in conifers, and in *Pinus* species in particular, which exhibit an hydric control through K_{leaf} regulation (Brodribb *et al.*, 2005; Cochard *et al.*, 2004). According to this assumption, previous works realized in *P. ponderosa* (Pinol & Sala, 2000) and *P. sylvestris* (Irvine *et al.*, 1998) showed that the plant transpiration diminution by stomatal control prevented the embolization of xylem elements.

A_N fall was in part to g_s and Ψ_{leaf} reduction. Bauerle *et al.* (2003) suggested that a reduction in A_N could also be attributed to a collapse of the mesophyll cells due to loss of turgor (Chaves *et al.*, 2009). In our work, all stressed plants decreased A_N levels (Fig. 1.5), but the O4 and O5 did not showed significant losses of turgor pressure (Fig. 1.3). In addition, there is not a strong correlation between g_s and A_N (Table 1.3). These results pointed out that the A_N decrease might be caused by other non-stomatal limitations (Escalona *et al.*, 1999) as changes in the functioning of the photosynthetic apparatus (Calatayud *et al.*, 2000), and/or a possible reduction of Rubisco activity (Vu *et al.*, 1999). *Fv/Fm* and Φ_{PSII} provide information about *PSII* status, so they are traditionally considered to be the primary target of environmental stress that lead to photoinactivation (Barber & Andersson, 1992). Except in O4 and O5, all stressed plants of *P. radiata* diminished their *Fv/Fm* at the end of the drought period probably due to a degradation of photosynthetic pigments after a significant A_N decrease (Fig. 1.5, 1.6 and 1.8). Furthermore, all stressed plants increased their non-photochemical quenching (*q*CN), especially in O3 and O6 (Fig. 1.13). On the other hand, *PSII* yield was highly correlated to *E.L.* (%). Regarding *E.L.* (%), thylakoid membranes are considered the primary sites that presented modifications under stress (Calucci *et al.*, 2001; Huseynova *et al.*, 2007) by changes in lipid composition and lipid-protein ratio (Quartacci *et al.*, 1995), membrane fluidity (Quartacci *et al.*, 2000), and superoxide radical

production (Sgherri *et al.*, 1993). Moreover, these changes may produce a decrease of dissipation pigments (carotenoid) and chlorophylls, both thylakoid membrane components (Campos *et al.*, 2003; Ramalho *et al.*, 2002). In our study, a reduction of these components was observed at the end of the drought period, especially in those plants that firstly decreased their *Fv/Fm* (O1 and O6) corroborating the aforesaid assumptions. On these accounts, we think that the cell membrane alteration status [*E.L.* (%) increase] could explain the decrease of *Fv/Fm* and Φ_{PSII}, the increase of *q*CN and partially the reduction of A_N activity.

When the ecotype characteristics were analyzed, we found strong evidences that the high tolerance of O4 and O5 to drought was related with their origin; they were from areas with less annual rainfall and higher mean, maximum (26ºC), and minimum temperature (Table 1.1) (Fig. 1.13). Besides, O6 was from areas with two-fold the annual rainfall of O4 and O5 ones and with milder temperatures. This ecotype reached the *TLP* (-2 MPa) with lower K_{leaf} decreases (80%), being the most sensitive ecotype to water stress. On the other hand, *P. radiata* plants from O4 and O5 showed the highest drought tolerance due to a high control of K_{leaf} decrease along the drought cycle and a more efficient stomatal conductance regulation. This drought-response allowed O4 and O5 plants to preserve higher RWC (%), preventing the reduction of Ψ_{leaf} and Ψ_t even at K_{leaf} decrements near 100%. This behaviour could indicate a hydrostable nature typical for drought avoiding species (Rouhi *et al.*, 2007; Siam *et al.*, 2009), also associated to the isohydric concept (McDowell *et al.*, 2008; Quero *et al.*, 2011). Furthermore, the different physiological traits among ecotypes subjected to water stress lead to the conclusion that the K_{leaf} dynamic not only varied at inter-species (Pockman *et al.*, 1995), but also at intra-species level (Tognetti *et al.*, 2011). These results are in accordance with some studies about stress tolerance concerning other *Pinus* species where phenotype drought tolerance was linked to plant geographical origin as occurred in *P. halepensis* (Calamassi *et al.*, 2001), *P. sylvestris* (Cregg & Zhang, 2001) and *P. canariensis* (López *et al.*, 2009b). It is of interest to highlight that O4 is a species hybrid of *Pinus radiata* var. *radiata* x *Pinus attenuate* which has been referred to be water stress resistant (Begley, 2001; Wright, 1968) and it can serve as reference of tolerance traits.

1.4.2.- Recovery capacity after drought

The ecotypes showed different response to drought stress, they responded to the rewatering, except O6 where 20% plants did not survive. Stressed plants reached their initial

levels in term of Ψ_{pd}, Ψ_{leaf} and Ψ_t , A_N , Φ_{PSII}, *E.L.* (%) and RWC (%) after rewatering. Some conifers showed a strong association between K_{leaf} and plant death (Blackman *et al.*, 2009; Brodribb *et al.*, 2010), and reached a lethal water stress when plants presented Ψ_{leaf} decreases over 95% (Brodribb & Cochard, 2009). Fatal water stress can be operationally defined as the transition point where Ψ_{leaf} passes from a recoverable to nonrecoverable water stress upon soil rewatering (Brodribb *et al.*, 2010). In our result, *P. radiata* plants subjected to drought for a period of four weeks reached K_{leaf} decreases near 95% but, after rewatering, they recovered some aforesaid physiological traits, including Ψ_{leaf}. On the contrary, only O4 and O5 recovered their K_{leaf} levels related to controls after rewatering despite K_{leaf} losses near 100%. The recovery capacity observed in O4 and O5 has been previously reported in *Pinus virginiana* (Johnson *et al.*, 2011). Hydraulically limited recovery of the rest of ecotypes from sub-lethal water stress suggested that xylem cavitation in these plants was either very slow to repair, or the damaged xylem tissue might be replaced by new one (Brodribb *et al.*, 2010; Brodribb & Holbrook, 2005). Moreover, previous studies have also showed that the transpiration ratio and also its recovery after a stress situation were strongly mediated by the hydraulic conductance (Brodribb & Cochard, 2009; Klein *et al.*, 2011). Blackman *et al.* (2009) found a shift in the relationship between E and Ψ_{leaf}, following rewatering, focusing damages of leaf photosynthesis apparatus as a possible candidate that does not allow the gas exchange recovery and/or ABA accumulation in leaves due to its stomatal regulation as other possible candidate. In this study, we demonstrated that the recovery of *P. radiata* plants was attributable to a hydraulic limitation and not due to photosynthetic apparatus damages as was observed by Brodribb & Cochard (2009). The possible hormone implication will be analyzed in following studies.

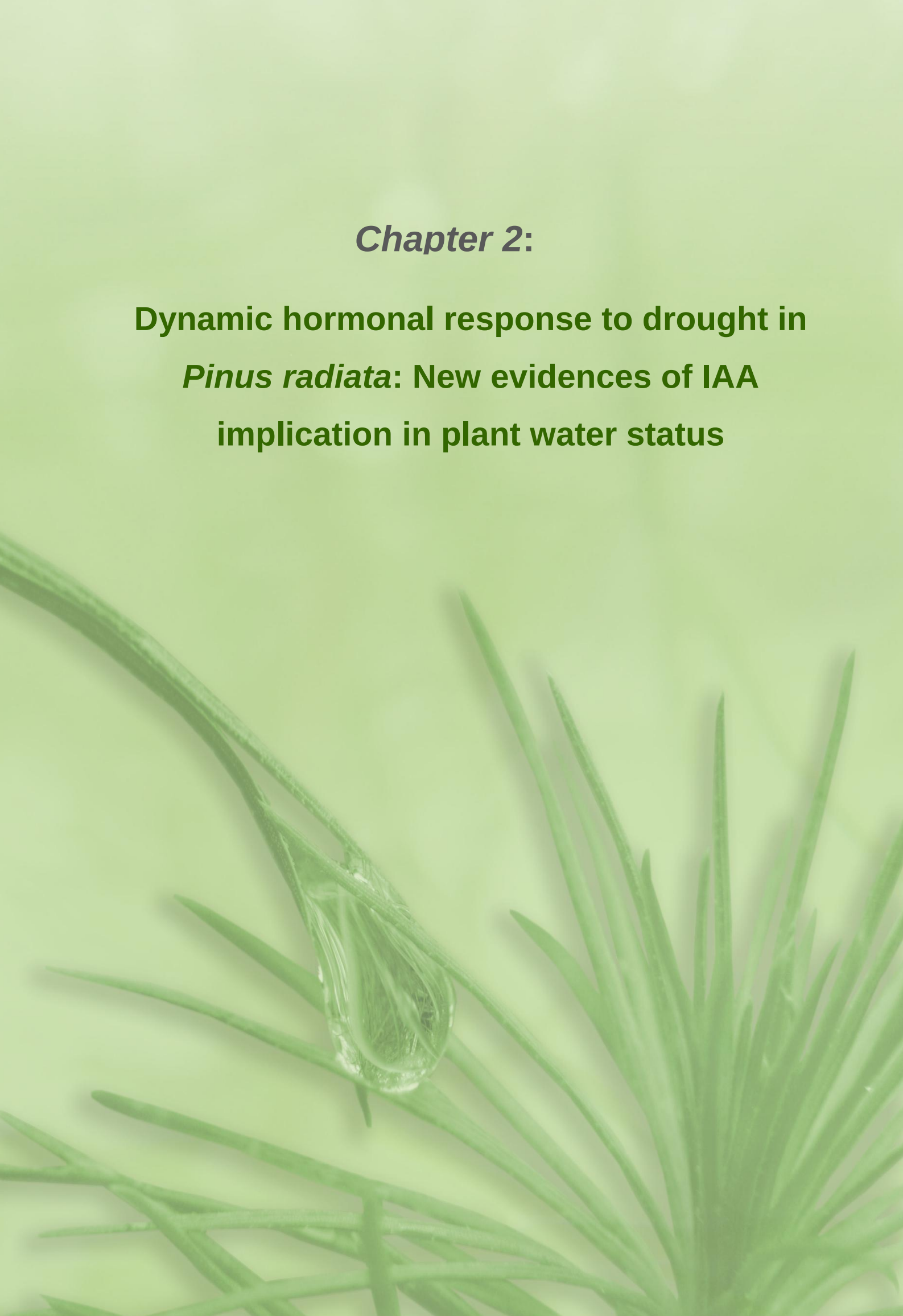
Finally, *E.L.* (%) has been conventionally considered as membrane injury indicator and an irreversible parameter (Bajji *et al.*, 2002), but, at this respect, there are some controversies. Thus, some authors have reported that the membrane leakage can be reversed (Campos *et al.*, 2003), and the reversibility capacity is associated with the stability loss of the lipid layer by peroxidation under stress conditions (Bajji *et al.*, 2002; Navari-Izzo *et al.*, 2000). In accordance with this explanation, in our studies all plants reached their initial *E.L.* (%) levels at R7 (Fig. 1.7B) and corroborated its reversibility.

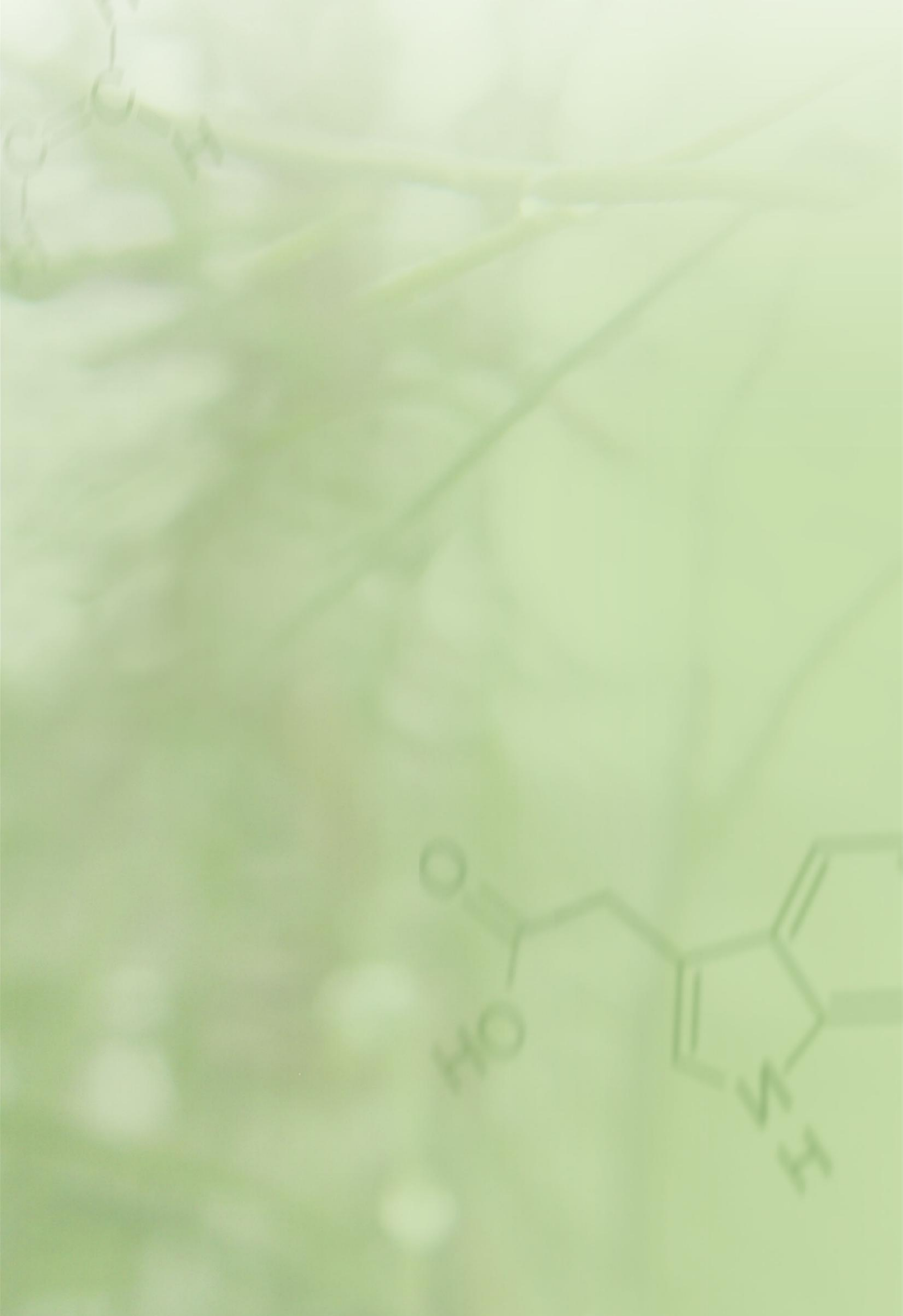
To summarize, this study showed that drought response and tolerance of several *Pinus radiata* plants varied at intra-species level (ecotypes). In addition, these results provide valuable information about the main role of leaf hydraulic properties in the dynamic response of plants

during water stress period and subsequent rewatering. As the same time as the onset of K_{leaf} dysfunction, plants close stomata to prevent embolization and turgor loss. O4 and O5 showed a high drought tolerance produced by the combination of an earlier stomatal closure at less negative Ψ_{leaf} and Ψ_t that slowed down the cell water loss. Stress was reversible in most of ecotypes regarding to water potential, photosynthetic parameters and *E.L.* (%) after rewatering, but plants did not recover their K_{leaf} and g_s levels. Only O4 and O5 got similar values of K_{leaf} and g_s compared with their controls after rewatering, being the most drought-tolerant ecotypes. Our results point out K_{leaf} together with g_s**,** as the best water status markers of *Pinus radiata* D. Don plants. The tolerance of *P. radiata* ecotypes to desiccation was characterized by decrease of K_{leaf} and g_s, less variations of Ψ_{leaf} and turgor maintenance. Finally, *E.L.* (%) increment indicates a "severe water stress", whereas no variations in *Fv/Fm* point out stress tolerance.

Chapter 2:

Dynamic hormonal response to drought in *Pinus radiata*: New evidences of IAA implication in plant water status





2.1.- Introduction

Pinus radiata is one of the most abundant species in the North of Spain, and the knowledge of its drought response mechanism is essential to guarantee the plantation survival under the water reduction conditions predicted in a future scenario of climatic change (Brunet *et al.*, 2009; Sánchez *et al.*, 2004). In order to evaluate the drought response of *Pinus radiata* plants and the recovery capacity after rewatering, preliminary physiological studies were carried out using six ecotypes (O1-O6) from different geographic and climatologic areas (Chapter 1). Different behaviours were observed among ecotypes, varying the intensity of response to stress and the recovery capacity.

Phytohormones are involved in different processes throughout plant growth and development (Ross *et al.*, 2011; Skirycz & Inzé, 2010), and are essential for the ability of plant acclimation to abiotic stresses by mediating a wide range of adaptive response (Santner & Estelle, 2009). The complexity of plant response includes hormone synthesis, transport and signalling pathways as well as by the diversity of interactions among them (Santner & Estelle, 2009). For this reason, growth regulators are being investigated to understand their role in stress situations (Peleg & Blumwald, 2011; Wang *et al.*, 2010). Abscisic acid (ABA) is considered to be one of the main plant signals in drought stress mediating several acclimation responses (Duan *et al.*, 2007; Jiang & Hartung, 2008). Some studies have showed that ABA is transported to the leaves as a root-to-shoot chemical signal (Dodd, 2005; Pospisilova, 2003) to induce stomatal closure (Schachtman & Goodger, 2008; Wilkinson & Davies, 2002) and, recently, Ghanem *et al.* (2008) have suggested that ABA has a long-lasting effect on plant hydraulic properties by stimulating leaf growth recovery after rewatering (Parent *et al.*, 2009). However, other hormones are involved in the plant response to stress. In this matter, it has been suggested that stomatal function is also regulated by other hormones as auxins, cytokinins (Cks), ethylene, jasmonates (JA) and salicylic acid (SA) (Acharya & Assmann, 2009; Santner & Estelle, 2009).

In addition, the synthesis of Cks has been related to osmotic adjustment under stress conditions (Pospisilova, 2003). Some evidences suggest that root-synthesized Cks can ameliorate shoot growth inhibition caused by environmental stress (Ghanem *et al.*, 2008). Indole-3-acetic acid (IAA) is the most studied auxin (Albacete *et al.*, 2008; Arbona & Gómez-Cadenas, 2008; Mahouachi *et al.*, 2007), but relatively little information is available on the changes in auxin content induced by water stress (Acharya & Assmann, 2009).

Ethylene is considered as primary signal in the regulation of the plant´s immune response (Pieterse *et al.*, 2009). Ethylene is synthesized through 1-aminicyclopropane-1-carboxylic acid (ACC) by the action of ACC-oxidase (Alexander & Grierson, 2002) and its increase play a critical signalling role in plant response to stress, promoting senescence (Albacete *et al.*, 2009; Lim *et al.*, 2007)

JA and SA have a role in plant response to stress (Delaney, 2007; Howe, 2007). Concerning angiosperms, it is well established that JA is implicated in response to biotic stresses such as herbivorous attack and wounding (Liechti & Farmer, 2002), pathogenesis defense (Wasternack, 2007) and abiotic stresses such as UV irradiation (Demkura *et al.*, 2010), ozone exposure (Rao *et al.*, 2000), flooding (Arbona & Gómez-Cadenas, 2008) or drought (Shan & Liang, 2010). On the contrary, there is little information about jasmonates and their relation to the defense mechanisms in gymnosperms, particularly in the case of *Pinus* spp. (Pedranzani *et al.*, 2007). SA has also been associated with plant resistance (Delaney *et al.*, 1994; Durner *et al.*, 1997), but the mechanisms of influence are poorly understood, especially in conifers (Rajasekaran & Blake, 1999).

Due to the different responses observed among the *Pinus radiata* ecotypes (Chapter 1), we hypothesized that the different responses observed could be modulated by phytohormone signals. For this reason, we studied the dynamic of the main phytohormones, their roles in the response of *P. radiata* plants to drought and recovery, and their possible relationships with water balance and gas exchange parameters. The general analysis of all these traits could permit us a better understanding of the different plant defense strategies and finally to identify possible markers of drought tolerance in radiata pine plants.

2.2.- Material and Methods

2.2.1.- Plant material, growth conditions and experimental design

Seed characteristics, growth conditions and experimental design were performed as described in Chapter 1 (1.2.1 and 1.2.2). Phytohormones were quantified at T0, T2, T4, and at R7 (Fig. 2.1).

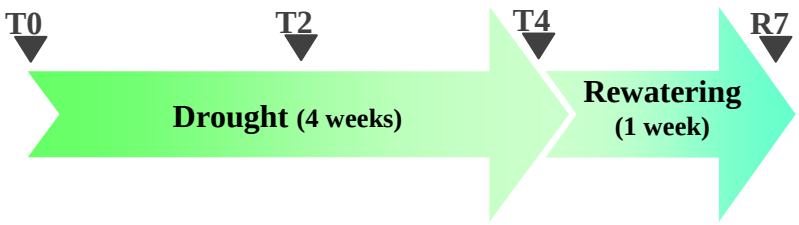


Figure 2.1. Scheme of the experimental design for six *Pinus radiata* ecotypes (O1-O6) subjected to a drought period of four weeks (from T0 to T4), and subsequent rewatering for a week (R7).

2.2.2.- Hormone quantification

2.2.2.1.- Hormone extraction

Hormones were analyzed on two apical needles per plant. Needles were immediately frozen in liquid nitrogen. Samples were pooled for further analysis and then, maintained at -80ºC until extraction. ABA, Cks [zeatin (Z), and zeatin riboside (ZR)], IAA, JA, SA, and ACC were extracted and purified according to the method described by Dobrev & Kaminek (2002), and analyzed mostly as described previously by Albacete *et al.* (2008). In summary, plant material (0.5 g FW) was homogenized in liquid nitrogen and dropped in 2.5 mL of cold (-20ºC) extraction solution of methanol/water (80/20, v/v). The extracts were centrifuged at 20,000 *g* for 15 min at 4ºC and the pellets were re-extracted for 30 min in additional 2.5 mL of the same extraction solution. Supernatants were collected and filtered through Sep-Pak Plus †C_{18} (®Waters, USA) to remove interfering lipids and plant pigments, and evaporated at 40ºC under vacuum. The residues were dissolved in 1 mL methanol/water (20/80, v/v) solution using an ultrasonic bath. The dissolved samples were filtered through 13 mm diameter nylon membrane Millex filters (Ø 0.22 µm) (®Millipore, Bedford, MA, USA) and placed into tubes adjusting the volume to 1.5 mL with the extraction solution.

2.2.2.2.- Hormone analysis

Analyses were carried out with an HPLC/MS system consisting of an Agilent 1100 Series HPLC (©Agilent Technologies, Santa Clara, CA) equipped with an autosampler connected to an Agilent Ion Trap XCT Plus mass spectrometer (Agilent Technologies) using an electrospray interface. Previous to injection, 100 µL of each fraction was again filtered through Millex filters (∅ 0.22 µm). 8 µL of each sample, previously dissolved in mobile phase A, was

injected onto a Zorbax SB-C18 HPLC column (5 μm, 150 × 0.5 mm, Agilent Technologies) at 40°C and eluted at a flow rate of 10 μL min^{-1}. Mobile phase A [water/acetonitrile/formic acid (94.9/5/0.1, v/v/v)] and mobile phase B [water/acetonitrile/formic acid (10/89.9/0.1, v/v/v)] were used for the chromatographic separation. The elution consisted of maintaining 100% A for a period of 5 min, and then a 10 min linear gradient from 0 to 6% B, followed by another 5 min linear gradient from 6 to 100% B, and finally 100% B kept for another 5 min. The column was equilibrated with the starting composition of the mobile phase for 30 min before each analysis. The UV chromatograms were recorded at 280 nm with the diode array detector module (Agilent Technologies®). Different control samples with known concentrations of each component (0.05, 0.075, 0.1, 0.2, and 0.5 mg L^{-1}) were also analyzed in the same conditions. The mass spectrometer was operated in the positive mode with a capillary spray voltage of 3500 V and a scan speed of 22,000 (m/z)/s from 50 to 500 m/z. The nebulizer gas (He) pressure was set to 30 psi, while the drying gas was set to a flow of 6 L min^{-1} at a temperature of 350°C. Mass spectra were obtained using the DataAnalysis program for LC/MSD Trap Version 3.2 (Bruker Daltonik, GmbH, Germany). For quantification of ABA and JA, calibration curves were constructed for each component analyzed using internal standards: [$^2H_6$]*cis,trans*-abscisic acid and [$^2H_5$](±)-jasmonic acid (Olchemin Ltd., Olomouc) (0.05, 0.075, 0.1, 0.2, and 0.5 mg L^{-1}) and corrected for 0.1 mg L^{-1}. ACC and SA were quantified by the external standard method, using the same concentration of the product (©Sigma-Aldrich Inc., St. Louis, MO). Recoveries ranged between 92 and 95%. Three biological replicas were quantified per sample.

2.2.3.- Statistical analysis

Multivariate analysis of variance (MANOVA) was carried out by *proc glm* in the S.A.S ® software package. For the analysis of phytohormone and ACC, three pools per ecotype and treatment were measured through the drought period (T0, T2, and T4) and subsequent rewatering (R7) in order to evaluate the relation between non-irrigated and irrigated plants (D/W). The analysis was realized according to the following mathematical model:

$$y_{ijr} = \mu + O_i + Ti_j + OTi_{ij} + e_{ijr} \qquad \text{(Eqn. 2.1)}$$

where,

y_{ijr} was the response variable result of the r^{th} plant of the i^{th} ecotype subjected at j^{th} time; μ was the experimental mean, O_i was the effect of the i^{th} ecotype, Ti_j the effect of the j^{th} time; OTi_{ij} between the i^{th} ecotype and j^{th} time, and e_{ijr} was the random error component.

Multiple comparisons were calculated using the post hoc Tukey´s HSD test to determinate the different signification levels. To analyze possible correlations among phytohormones and physiological parameters *proc reg* was used, and analysis of covariance (ANCOVA) was carried out by *glm proc* in the S.A.S.® software package.

Finally, the ecotype recovery capacity was evaluated as the relation between controls (W) and water stressed plants (D) at R7, using principal component (PC) analysis in The Unscrambler® Version.-X.1 software.

2.3.- Results

2.3.1.- Hormonal dynamic under water stress

The most evident external symptoms of *P. radiata* plants subjected to water stress were needle epinasty and apical curvature (Fig. 2.2). These symptoms showed high variability among the six evaluated ecotypes. At T2, 40% plants from O1, O3 and O6 and only 20% plants from O2 showed external symptoms (Table 2.1). In contrast, O4 and O5 plants presented a normal appearance. At T4, all plants from O6 showed epinastic needles and apical curvature at the end of the drought period while O4 and O5 only showed a 40% and a 20% stressed plants, respectively. At R7, all plants recovered a normal appearance, except 20% plants from O6 (Table 2.1).

Figure 2.2. Apical part of two-year old plants of *Pinus radiata*. Well-watered plants (A). Plants with needle epinasty and apical curvature after four weeks under drought conditions (from T0 to T4) (B).

Table 2.1. External symptoms (%) in *six Pinus radiata* ecotypes (O1-O6) after two (T2) and four (T4) weeks under drought conditions and subsequent rewatering (7R).

Ecotype	External symptoms (%)		
	Drought		Rewatering
	T2	T4	R7
O1	40	80	0
O2	20	80	0
O3	40	80	0
O4	0	40	0
O5	0	20	0
O6	40	100	20

IAA was the least abundant hormone in *Pinus radiata* needles, with levels around 15-20 ng g^{-1} FW at the beginning of the drought period, whereas the rest of phytohormones showed values 15 or 30-fold higher [from 250 to 600 ng g^{-1} FW (Data not shown)]. However, IAA explained most of the model variance (66%) (Table 2.3). In all ecotypes, the highest IAA increment was observed at T4 related to T0 (Fig. 2.3B), except in O4 that did not show statistically significant differences along the experiment. The highest increments were observed in O1 whose levels increased 27-fold their control values (from 16.8 to 456.0 ng g^{-1} FW), whereas in O5 were only double (from 5.1 to 13.6 ng g^{-1} FW). At R7, O5 recovered their initial values, while IAA was still accumulating in O1, O2 and O3 (Fig. 2.3B).

Table 2.2. Multivariate analysis of variance (MANOVA) of ABA, IAA, Z+ZR, ACC, JA and SA ratio content (D- water stressed /W- well watered plants) in *six Pinus radiata* ecotypes (O) under irrigation and no irrigation conditions (T) for four weeks and subsequent rewatering for a week (Ti).

VARIABLE	FACTORS	DF	Sum Sq	Mean Sq	Fvalue	Pr(>F)
ABA	Ti	3	7.3703	2.4568	906.93	***
	O	5	3.7768	0.7554	278.84	***
	Ti × O	**15**	**15.3582**	**1.0239**	**377.97**	*******
IAA	Ti	3	1209.35	403.12	9454.16	***
	O	5	1449.8857	289.98	6800.73	***
	Ti × O	**15**	**1654.82**	**110.32**	**2587.33**	*******
Z+ZR	Ti	3	3.1438	1.0479	727.34	***
	O	5	6.1948	1.2390	859.92	***
	Ti × O	**15**	**7.8806**	**0.5253**	**364.65**	*******
ACC	Ti	3	0.1578	0.0526	46.78	***
	O	5	0.0301	0.0060	5.35	***
	Ti × O	**15**	**0.4612**	**0.0307**	**27.34**	*******
JA	Ti	3	9.7822	3.2607	1233.70	***
	O	5	6.8941	1.3788	521.68	***
	Ti × O	**15**	**7.0291**	**0.4686**	**177.30**	*******
SA	Ti	3	1.7025	0.5675	553.92	***
	O	5	1.9844	0.3969	387.39	***
	Ti × O	**15**	**4.2663**	**0.2844**	**177.61**	*******

Note: * < 0.05; ** <0.01 and *** <0.001.

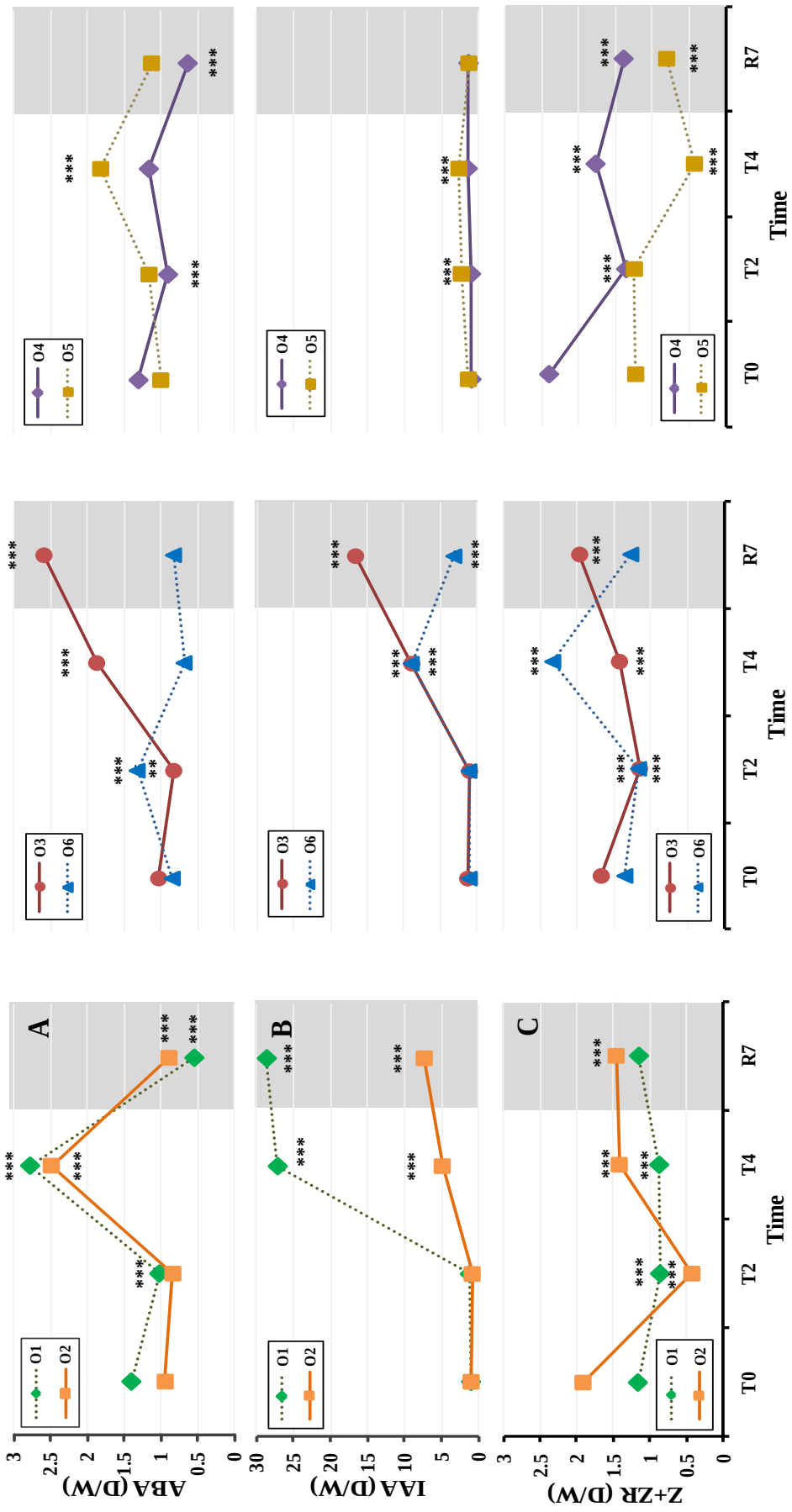


Figure 2.3. ABA (A), IAA (B), and Z+ZR (C) ratio content (D-water stressed / W-well watered plants) in six *Pinus radiata* ecotypes (O1-O6) during a drought period (from T0 to T4) and subsequent rewatering for a week (R7- Shady area). *M* ± *S.E.* Significant differences with respect to each ratio at T0 were represented by asterisks according to Tukey´s HSD test after MANOVA. * < 0.05; ** < 0.01; *** < 0.001

Table 2.3. Characteristic vector of matrix associated to MANOVA for ABA, IAA, Z+ZR, ACC, JA and SA ratio content (D-water stressed / W-well watered plants) variables analyzed in six *Pinus radiata* ecotypes subjected to drought for 4 weeks (from T0 to T4) and subsequent rewatering for a week (R7).

Characteristic root	Percentage	ABA	IAA	Z+ZR	ACC	JA	SA
821.44	**66.01**	-0.127	**0.703**	0.144	-0.292	-0.221	-0.058
207.06	**16.64**	-2.055	0.015	1.328	0.0804	-1.330	**3.863**
129.94	**10.44**	0.648	-0.069	**2.995**	-0.096	1.616	-0.276
56.53	4.54	1.813	-0.026	-0.036	1.533	-1.415	**3.151**
23.38	1.88	-0.054	0.050	**-2.030**	-0.7778	**1.953**	1.220
6.03	0.48	-0.241	0.009	-0.040	**4.017**	0.486	-0.749

In general, all plants contained between 100 and 1000-fold higher values (ng g^{-1} FW) of zeatin (Z) than zeatin riboside (ZR) (Data not shown). For these reason, the Z and ZR were evaluated together. According to characteristic vectors, Z+ZR explained a model variance of 10.44%, being the third most representative hormone of the experimental model (Table 2.3). Z+ZR changes were evident since the beginning of the drought period, so all ecotypes significantly decreased their D/W at T2, except O5 that maintained their levels (Fig. 2.3C). At T4, stressed plants from all ecotypes maintained their Z+ZR levels below the initial amounts. Only O6 increased them. At R7, O1 and O6 recovered their initial Z+ZR relation (Fig. 2.3C).

Stressed plants from O5 did not vary the ACC content along the experiment (Fig. 2.4A). At T4, only stressed plants from O1 and O6 significantly dropped their ACC levels. At R7, O1 recovered its ACC initial levels whereas O3 increased them (Fig. 2.4 A).

Drought also induced changes in endogenous JA levels (Fig. 2.4B). O5 did not change its JA content along the water stress period. At T2, O2 and O6 decreased significantly their JA levels and O3 maintained their values. At T4, these three ecotypes strongly increased their ratios. Stressed plants from O1 and O4 significantly accumulated JA throughout the drought period, showing the highest JA ratios at T4. (Fig. 2.4B).

Finally, SA was the second most representative phytohormone, explaining a model variance of 16.64% (Table 2.3). At T2, all stressed plants, except in O4 and O5, significantly decreased their SA levels compared to T0 (Fig. 2.4C). At T4, O4, O5 and O6 significantly increased their SA content. After rewatering, only stressed plants from O3 and O4 recovered the SA values, whereas O1 and O6 presented higher ratios than those observed in T0. O2 and O5 significantly decreased them (Fig. 2.4C).

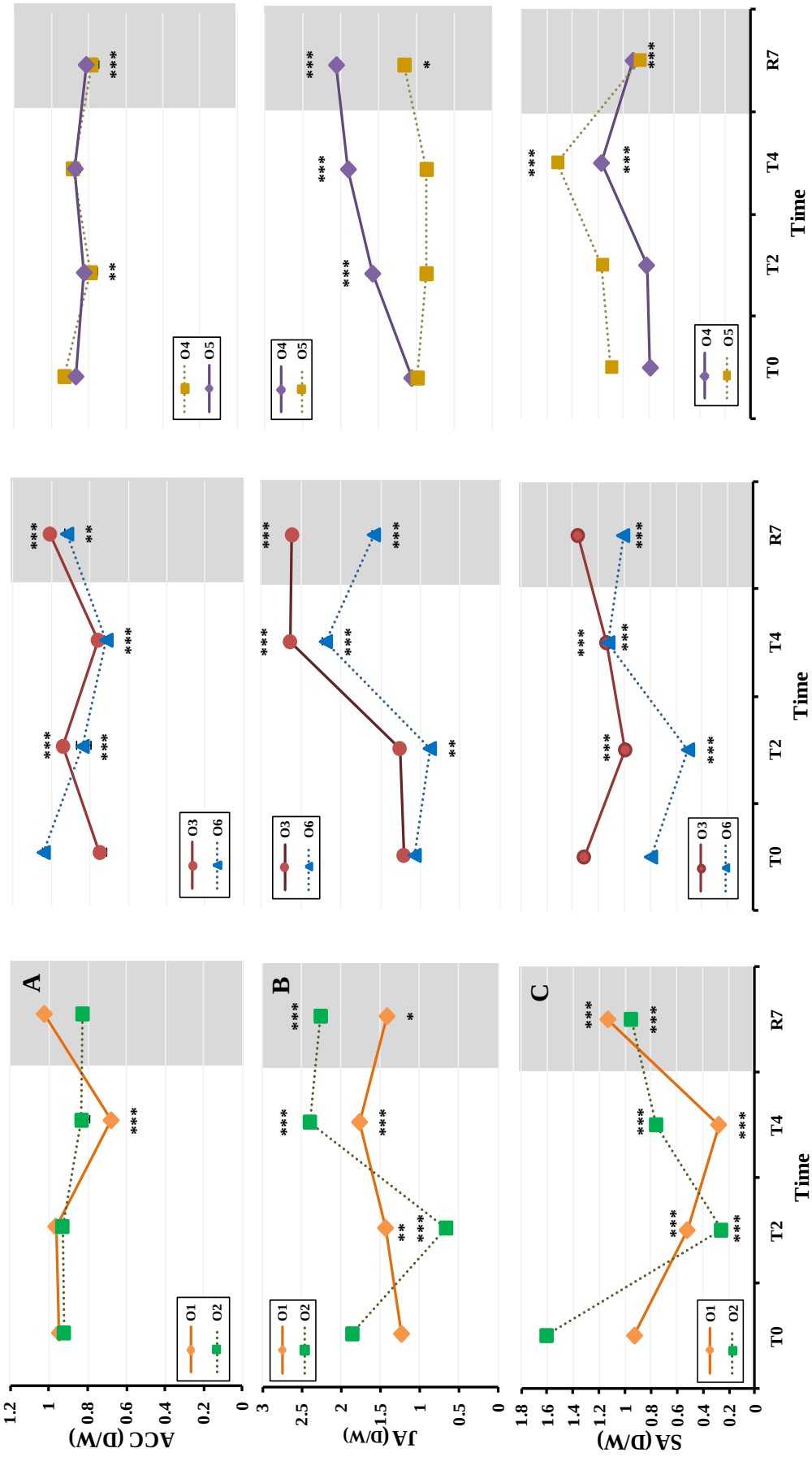


Figure 2.4. ACC (A), SA (B), and JA (C) ratio content (D-water stressed / W-well watered plants) in six *Pinus radiata* ecotypes (O1-O6) during a drought period (from T0 to T4) and subsequent rewatering for a week (R7- Shady area). *M* ± *S.E.* Significant differences with respect to each ratio compared to T0 by asterisks according to Tukey´s HSD test after MANOVA. * < 0.05; ** < 0.01; *** < 0.001.

2.3.2.- Correlation analysis

During the two first weeks of drought, a strong correlation was detected among Z+ZR variations and Ψ_{leaf}, K_{leaf} and g_s (Fig. 2.5). A 50% decrease of Z+ZR content (D/W = 1.20) was observed when plants dropped their Ψ_{leaf} below –0.7 MPa (Fig. 2.5A), and this decrease was related to 50% and 35% reduction of K_{leaf} and g_s, respectively (Fig. 2.5B and 2.5C). When stressed plants reached Ψ_{leaf} of -1.4 MPa, both Cks content and K_{leaf} were reduced by 80% and g_s presented a decrement of 55%.

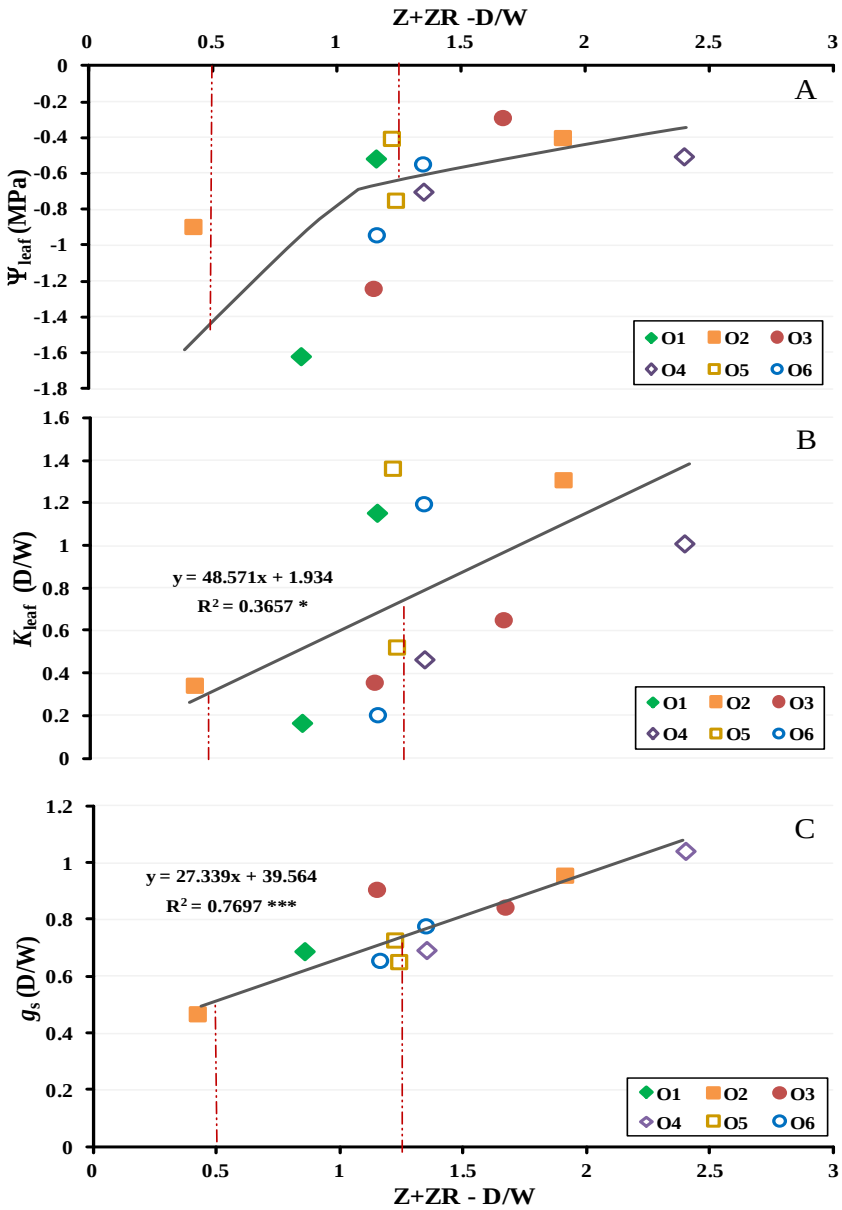


Figure 2.5. Correlation among leaf water potential (Ψ_{leaf}) (A), leaf hydraulic conductance (K_{leaf}) (B) and stomatal conductance (g_s) (C) *vs.* cytokinin (Z+ZR) ratio content (D-water stressed / W-well watered plants) in six *Pinus radiata* ecotypes (O1-O6) from T0 to T2. Red dashed lines represented 50% and 80% of Z+ZR decrease. R^2 is Pearson´s correlation number with the significance. * < 0.05; ** <0.01 and *** <0.001

A highly significant correlation was observed among IAA accumulation and leaf water potential and turgor pressure (Fig. 2.6). Stressed plants started to accumulate IAA in the needles when plants decreased their Ψ_{leaf} to –1 MPa and their Ψ_t to 0.66 MPa (Z+ZR diminution of 65%). When stressed plants reached the *TLP*, they increased their IAA levels to 4-fold the initial ones (Fig. 2.6).

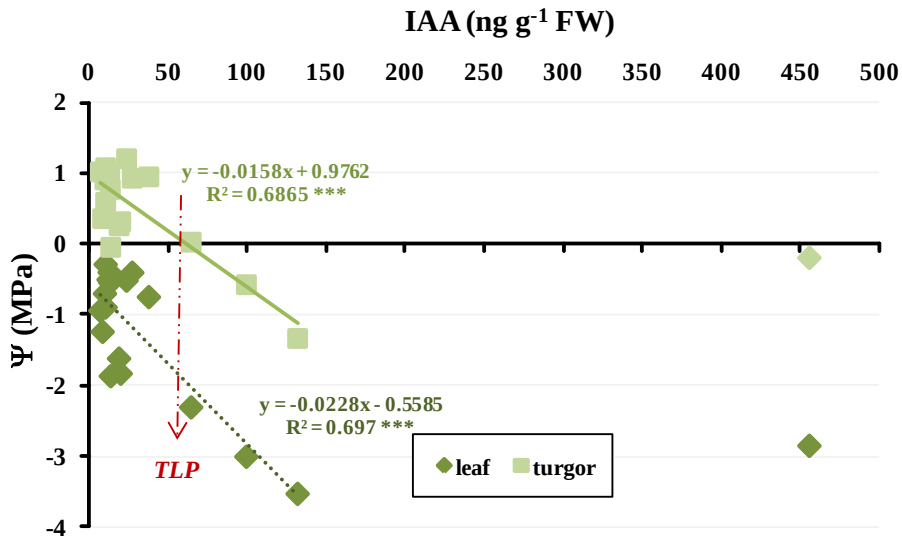


Figure 2.6. Correlation between IAA (ng g^{-1} FW) *vs.* leaf water potential (Ψ_{leaf}-leaf), and turgor pressure (Ψ_t-turgor) (MPa) in six *Pinus radiata* ecotypes along a drought cycle of four weeks (from T0 to T4). R^2 is Pearson´s correlation number with the significance. * < 0.05; ** <0.01 and *** <0.001

Hydraulic conductance, gas exchange, fluorescence and electrolyte leakage showed similar relationship with ABA and IAA content (Fig. 2.7). Stressed plants started to accumulate IAA and ABA when the K_{leaf} and A_N values decreased around 65% and 45% with regard to their control levels, respectively (Fig. 2.7A, B, C and D). *Fv/Fm* and *E.L.* (%) changes were as well analyzed with respect to IAA and ABA levels (Fig. 2.7E, F, G and H), and significant lineal regressions were obtained for both IAA and ABA accumulation (p< 0.001). IAA showed a stronger relationship with *Fv/Fm* variation than ABA according to Pearson´s correlation number (R^2= 0.90 and 0.47) (Fig. 2.7E and F), whereas ABA had higher values related to *E.L.* (%) (R^2= 0.79 and 0.91) (Fig. 2.7G and H).

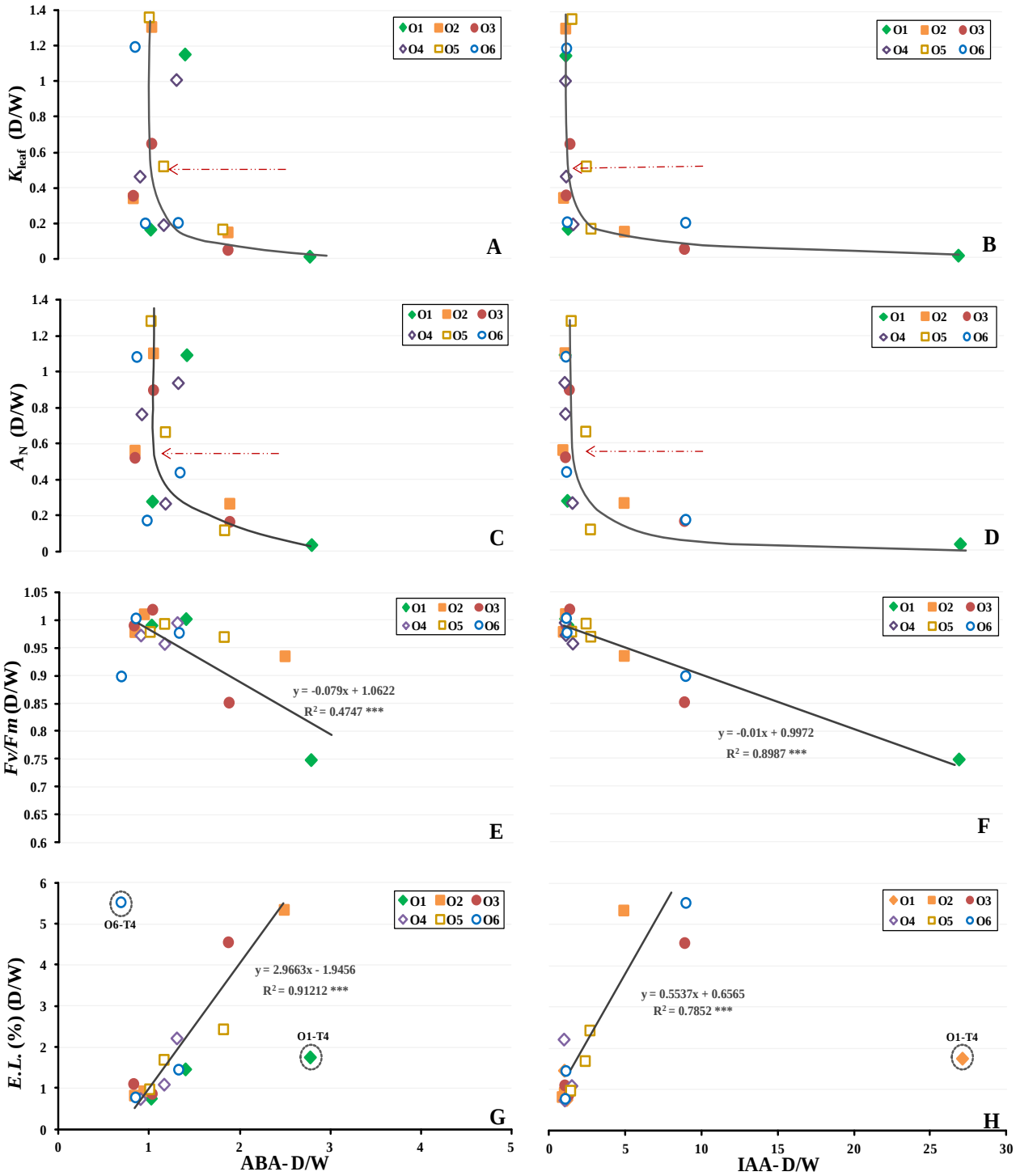


Figure 2.7. Correlations among leaf hydraulic conductance (K_{leaf}) (A, B), instantaneous net photosynthesis (A_N) (C, D), the maximum quantum yield of *PSII* (*Fv/Fm*) (E, F), and electrolyte leakage [*E.L.* (%)] (G, H) *vs.* IAA and ABA ratio content (D-water stressed / W-well watered) in six *Pinus radiata* ecotypes (O1-O6) along a drought period of four weeks (from T0 to T4). Discontinuous circle indicates point out the regression. R^2 is Pearson´s correlation number with the significance. * < 0.05; ** <0.01 and *** <0.001

The relationship between phytohormones and relative water content (RWC %) was evaluated, and a high correlation was observed among ABA and IAA levels with RWC (%) (Fig. 2.8). RWC (%) induced IAA accumulation in all ecotypes, increasing 12-fold the initial

values at T4, whereas only tripled their ABA levels (Fig. 2.8A and B), corroborating a higher influence of IAA than of ABA in *Pinus radiata* plants during water stress.

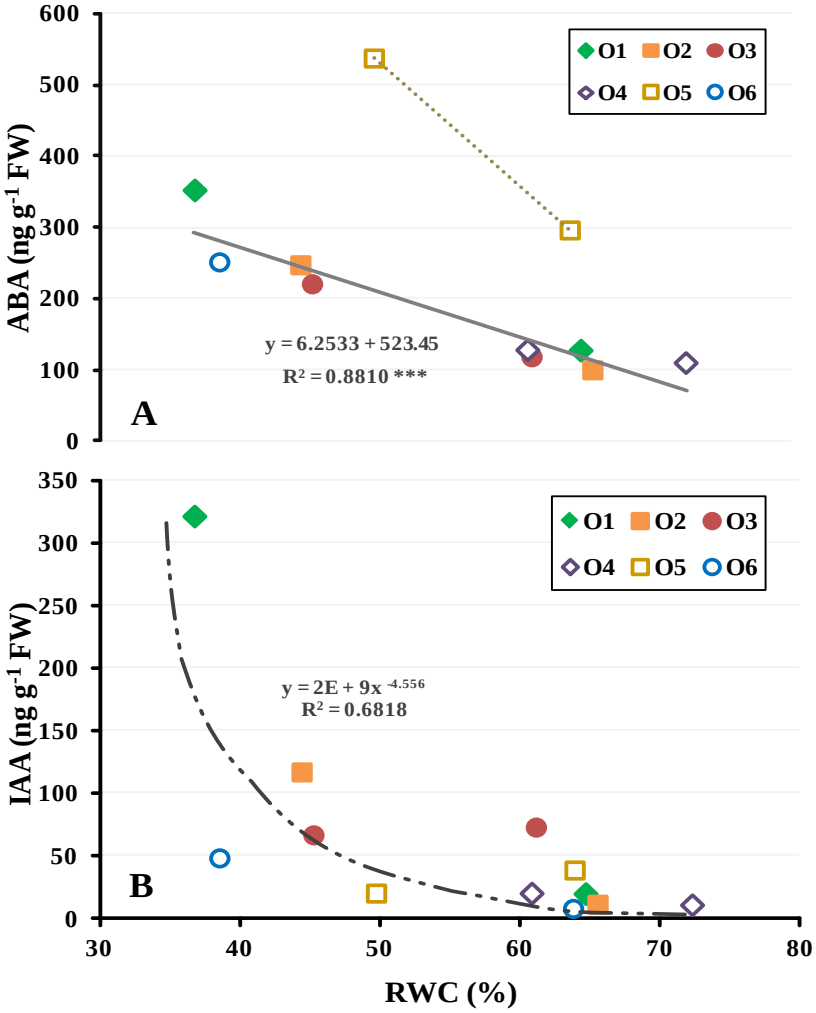


Figure 2.8. Correlations among ABA (A) and IAA (ng g^{-1} FW) (B) *vs.* relative water content [RWC (%)] in six *Pinus radiata* ecotypes (O1-O6) after four weeks under drought conditions (T4). R^2 is Pearson´s correlation number with the significance. * < 0.05; ** <0.01 and *** <0.001

External symptoms were highly interrelated to IAA, JA, and SA levels (Fig. 2.9A and B). When ecotypes presented 50% plants with needle epinasty and apical curvature, their IAA levels were double (Fig. 2.9A). Besides, an IAA accumulation of 30-fold the control levels was reached when all plants showed external symptoms. JA accumulation was related to high percentages of plants with epinasty and apical curvature, whereas a SA increase was associated with the lowest percentages of these symptoms (Fig. 2.9B). Moreover, although a strong correlation was observed between JA and external symptoms (p< 0.01), the relation JA/ACC significantly showed the strongest one (p<0.001) (Fig. 2.9B and C).

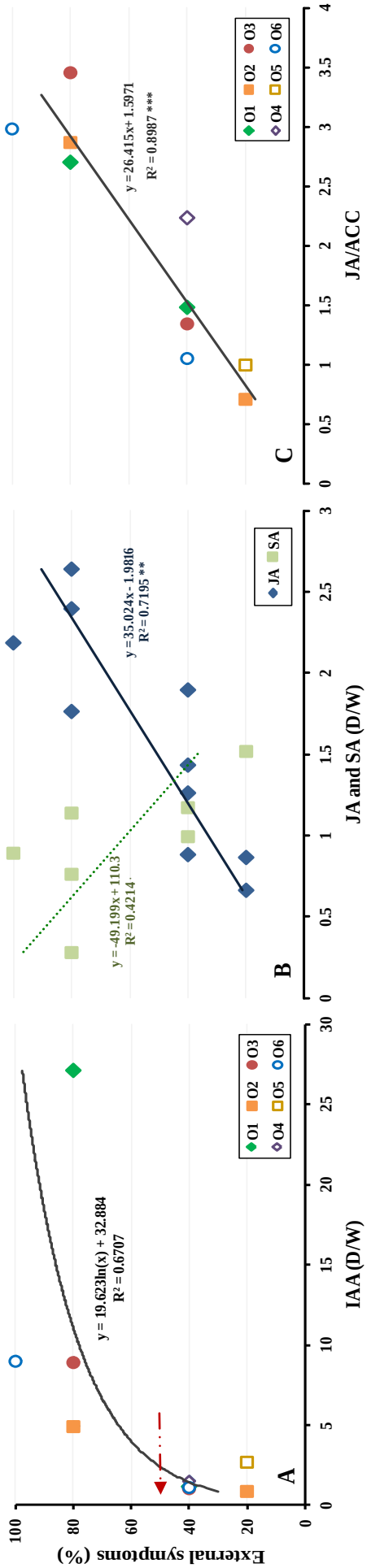


Figure 2.9. Correlations among external symptoms (%) *vs.* IAA (A), JA and SA (B), and JA/ACC (C) ratio content (D-water stressed / W-well watered plants) in six *Pinus radiata* ecotypes (O1-O6) during a drought period of four weeks (from T0 to T4). Red narrow indicates a RWC of 50%. R^2 is Pearson´s correlation number with the significance. * < 0.05; ** <0.01 and *** <0.001

The relationship among several hormones was analyzed along the drought period. According to this, stressed plants did practically not vary their IAA/ABA ratio at T2, except O1, O4 and O5 which increased their relations at T0 (Fig. 2.10A). At T4, some ecotypes strongly augmented their IAA/ABA relation, especially O1, O3 and O6. On the other hand, O4 and O5 maintained similar IAA/ABA values observed at T2. Furthermore, stressed plants from these two ecotypes (O4 and O5) also increased their IAA/ACC levels at T2, and maintained similar values at T4 (Fig. 2.10B). The remaining ecotypes did not show changes at T2 regarding T0, but a strong increase was evident at T4, especially in O1 and O6.

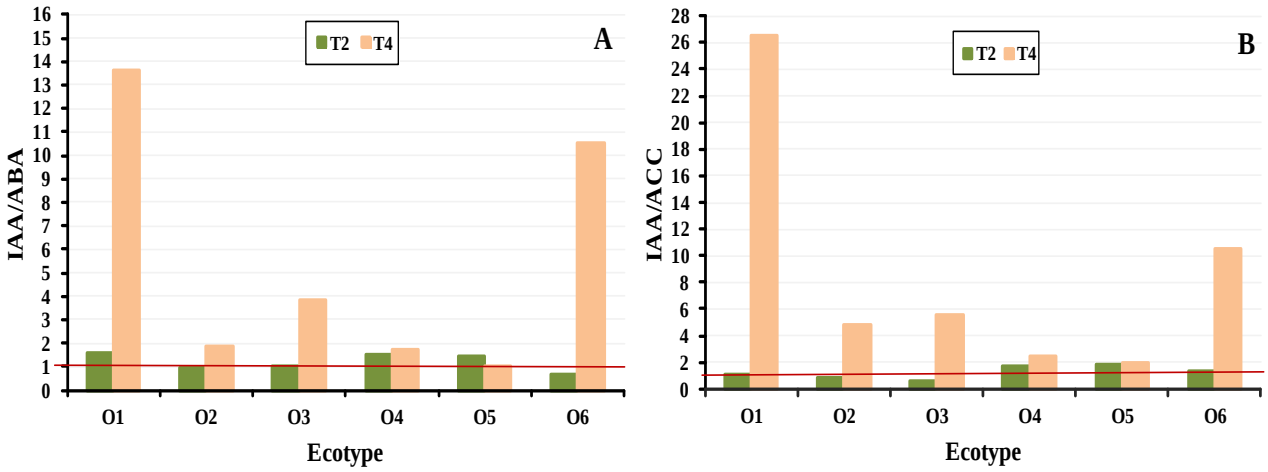


Figure 2.10. IAA/ABA (A) and IAA/ACC (B) ratio content (D-water stressed / W-well watered plants) in six *Pinus radiata* ecotypes (O1-O6) after two (T2) and four (T4) weeks under drought conditions and related to the beginning of the experiment (T0) (Red line)

Only O5 did not vary JA/SA at T2 with respect to T0 (Fig. 2.11A). At T4, stressed plants from O5 and O4 decreased their JA/SA ratio with respect to T2, whereas in O1 JA/SA relation was still rising. Besides, according to matrix after MANOVA, a significant lineal correlation was observed between JA and SA (p<0.01) (Table 2.4). In this matter, O1 and O5 ecotypes showed the same significant tendency (p<0.001) with regard to the relationship between JA and SA (Fig. 2.11B). Whereas O1 increased their JA levels, stressed plants from O5 accumulated SA along the drought cycle (Fig. 2.11B).

Table 2.4. Lineal correlation matrix among ACC, JA; SA; ABA, IAA, and Z+ZR according to MANOVA. Numerical values are Pearson´s correlation numbers with the significance.

	ACC	JA	SA	ABA	IAA	Z+ZR
ACC	1					
JA	0.14^{ns}	1				
SA	0.09^{ns}	**0.45****	1			
ABA	0.04^{ns}	0.04^{ns}	0.12^{ns}	1		
IAA	0.07^{ns}	0.08^{ns}	0.03^{ns}	0.12^{ns}	1	
Z+ZR	0.10^{ns}	0.02^{ns}	0.04^{ns}	0.07^{ns}	0.0^{7ns}	1

Note: * < 0.05; ** < 0.01; *** < 0.001; ns- non-significant.

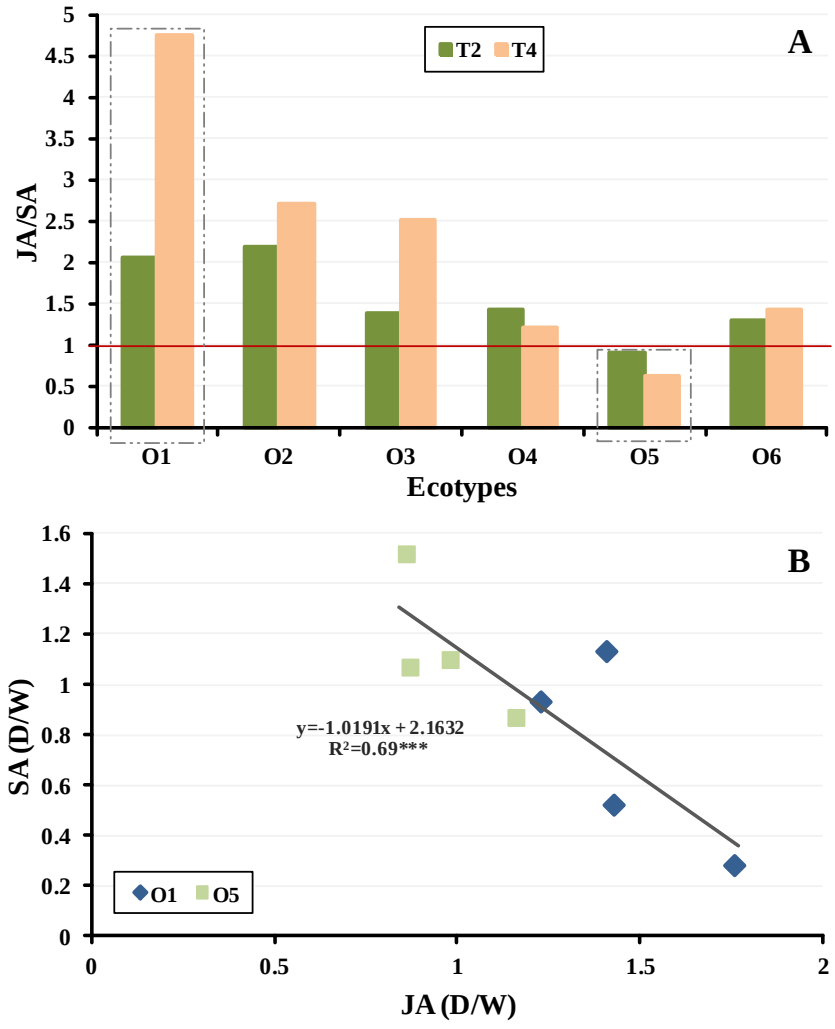


Figure 2.11. JA/SA (A) (D-water stressed / W-well watered plants) in six *Pinus radiata* ecotypes (O1-O6) after two (T2) and four (T4) weeks under drought conditions and related to the beginning of the experiment (T0) (Red line). Relationship between SA *vs.* JA ratio content (D/W) (B) in O1 and O5 ecotypes along the experiment (from T0 to T4 and at R7). R^2 is Pearson´s correlation number with the significance. * < 0.05; ** <0.01 and *** <0.001

2.3.3.- Recovery capacity

When the water supply was re-established, all ecotypes showed recovery capacity after rewatering, but the intensity varied among them. All aforementioned parameters were evaluated by principal component (PC) analysis, explaining PC-1 a total of 44 % of model variance (Fig. 2.12). In this matter, a strong positive correlation among O4 and O5 was noticed. Moreover, stressed plants from O4 and O5 showed a direct correlation with high values of A_N, E, fluorescence parameters (*Fv/Fm* and Φ_{PSII}), K_{leaf}, and high values of Z+ZR/IAA and ACC/IAA ratio. They also presented negative relationship with the IAA, SA and ACC levels, suggesting a strong implication of these hormones in the plant water status. This trend was inverted in O3 (Fig. 2.12).

According to PC-2 (24% of model variance), the recovery of O1 plants was inversely correlated to Z+ZR content, and jasmonate levels and the phytohormones such as JA/ACC and JA/SA (Fig. 2.12).

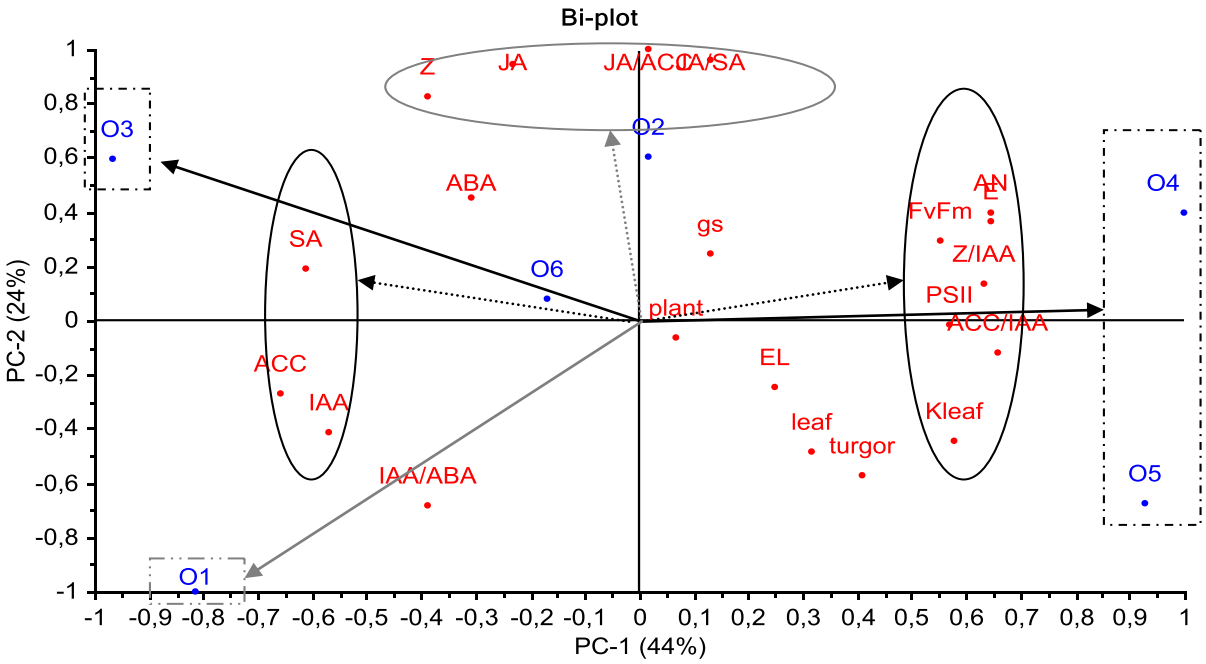


Figure 2.12. Principal component (PC) analysis of recovery capacity after rewatering (R7) between irrigated (W) and non-irrigated (D) plants from six *Pinus radiata* ecotypes (O1-O6) subjected to drought for four weeks (T4). Plant- the percentage of plants with external symptoms, leaf - leaf water potential, turgor - turgor pressure, Kleaf- leaf hydraulic conductance ratio (D/W), AN-net photosynthesis, E- instantaneous transpiration, gs- stomatal conductance, FvFm- the maximum quantum yield of PSII photochemistry, EL- electrolyte leakage, ABA- abscisic acid, IAA- indole-3-acetic acid, Z- zeatin and zeatin riboside, ACC- 1-aminocyclopropane-1-carboxylic acid, JA- jasmonic acid and SA- salicylic acid.

2.4.- Discussion

2.4.1.- Drought and physiological changes

This study provided experimental evidences of the connection between hormonal levels and changes on parameters such as water balance, gas exchange and fluorescence in *Pinus radiata* D. Don plants along a water deficit period and subsequent recovery. Different responses were observed among ecotypes, being O4 and O5 plants those that showed more tolerance.

In *P. radiata* needles a strong decrease of Z+ZR content was observed at T2, and this decrement was closely bound to Ψ_{leaf}, K_{leaf} and g_s variations (Fig. 2.5), corroborating the important role of Cks in the regulation of early plant response to stress (Granda *et al.*, 2011). To this respect, as Goodger *et al.* (2005) suggested that chemical signals are produced before hydraulic signals, and they represented an "early warning" of soil water status. At this respect, Cks play a regulative role in plant response to water deficit (Chernyad'ev & Monakhova, 2003; Shao *et al.*, 2010). Thus, it was observed that minimal changes in Ψ_{leaf} produces significant changes in all plant tissues´ Ck concentration, although the effect is stronger at leaf level as it was previously described in *Medicago sativa* (Goicoechea *et al.*, 1995) and *Vitis vinifera* (Stoll *et al.*, 2000). Furthermore, Cks are considered an ABA antagonist in procedures such as stomatal aperture control during the early response (Haisel *et al.*, 2008; Peleg & Blumwald, 2011), and the low Z+ZR content and subsequent high ABA accumulation could be the responsibles of the negligible g_s noticed at T4.

ABA is known to play an important role in the environment plant acclimation (Sánchez-Díaz *et al.*, 2008). ABA role as a stress signal has been deeply discussed in several studies (Dodd, 2005; Hartung *et al.*, 2002; Pospisilova, 2003), specifically related to the stomatal closure regulation (Acharya & Assmann, 2009; Bauerle *et al.*, 2006; Li *et al.*, 2000). Under drought conditions, all ecotypes presented needle ABA accumulation although the highest levels were detected after four weeks under water stress conditions (Fig. 2.3). The initial low ABA accumulation observed at T2 may be due to the fact that ABA present in leaves might be loaded in the phloem to its recycling (Dodd, 2005) and/or to be transported to other plant areas (Jeschke *et al.*, 1997; Sauter *et al.*, 2001). Other authors pointed out that the changes of needle ABA levels was controlled by a dynamic equilibrium between ABA biosynthesis and catabolism (Ren *et al.*, 2007). Moreover, ABA accumulation requires an activated and accelerated production of

ABA precursors (Ren *et al.*, 2007), processes that could delay the ABA increment in the leaves. The highest ABA values were observed in stressed plants from O5 (537 ng g^{-1} FW), which maintained the turgor, practically losing its K_{leaf} and gas exchange capacity at T4 (Chapter 1). Furthermore, stressed plants from O5 showed the highest recovery capacity of K_{leaf} after rewatering (Chapter 1). In this regard, ABA accumulation is assumed to be a water defense mechanism through the increment of some antioxidant enzyme activity to prevent cell damage (Jiang & Zhang, 2002), and it could be a explanation for the high stress-tolerance observed in O5 and also the lowest damage percentage (Table 2.1). Moreover, O5 presented the highest ABA values with respect to the RWC (%) (Fig. 2.8A), being this fact a possible cause of their faster stomata closure when water potential was less negative.

P. radiata plants significantly accumulated JA after four weeks without water supply, except in O5 (Fig. 2.4B). Stressed plants from O5 did not have significant JA variations related to T0 along the drought cycle, and did not lose their turgor, presenting the lowest number of plants with external symptoms (20%) (Table 2.1). These results corroborated that JA levels were rapidly and transiently increased by plant cell changes under biotic and abiotic stresses (Creelman & Mullet, 1997; Wasternack, 2007), including cell turgor reduction (Schaller & Stintzi, 2009). This behaviour may be due to the fact that jasmonate synthesis is normally stimulated by physical damages as consequence of stress effect (Gould *et al.*, 2009). Thus, stressed plants that showed high stress signs [low Fv/Fm and high *E.L.* (%)] (O1, O3 and O6), increased their JA and their JA/ACC levels (Fig 2.9). According to these results, JA would be a possible indicator of damage response.

SA is also considered a signal molecule that modulates plant responses to stress (Delaney, 2007; Senaratna *et al.*, 2000). Various physiological and biochemical effects of SA in plants including ion uptake, membrane permeability, mitochondrial respiration and photosynthesis have been well documented (Barkosky & Einhellig, 1993; Delaney *et al.*, 1994; Wang *et al.*, 2010). Besides, SA regulates plant growth, triggers local resistance and active Systemic Acquired Resistance response (Delaney, 2007). O5 stressed plants showed the highest SA accumulation, maintained their *Fv/Fm* values and practically did not show apparent external damages (Table 2.1) (Fig. 2.4C and 2.11). The remaining ecotypes presented a high JA accumulation and low SA changes that induced an increment in JA/SA relation. The antagonist behaviour between JA and SA has been well-studied (Felton & Korth, 2000; Howe, 2007; Kunkel & Brooks, 2002). This form of negative cross-talk appears to provide plants with the

plasticity to mount a defense responses (Turner *et al.*, 2002). This possible defense mechanism is specific of the stress situation and minimizes the expression of inappropriate defense genes (Howe, 2007; Kunkel & Brooks, 2002).

2.4.2.- The role of IAA during water stress

Most of plants subjected to abiotic stresses have shown changes in their IAA levels (Albacete *et al.*, 2008; Kong *et al.*, 2008). In our study, IAA was the most influent phytohormone under drought conditions (Table 2.3), showing the highest increments with respect to the control content in plants subjected to water deficit conditions (Fig. 2.2B). Besides, IAA accumulation showed strong correlations with other physiological processes previously studied such as leaf water potential, hydraulic conductance, and instantaneous net photosynthesis, among others (Fig. 2.6 and 2.7). The most tolerance ecotypes (O4 and O5), did not present an IAA accumulation more than 2-fold their control values (Fig. 2.3B). When the relationship between A_N, *Fv/Fm* and *E.L.* (%) and IAA and ABA accumulation was evaluated, similar tendencies were observed but stressed plants showed higher IAA than ABA increments, with a minimum of 10-fold the control values for IAA and 3-fold for ABA (Fig. 2.7). Possible effects of IAA changes were frequently due to crosstalk with other hormones (Chandler, 2009), regulating processes of stomata closure (Pospisilova, 2003), Reactive Oxygen Species activation (Tognetti *et al.*, 2011) and/or ethylene synthesis (Hansen & Grossmann, 2000; Merritt *et al.*, 2001). For example, it is well-known that auxins interact with Cks in the control of many central developmental processes (Tanaka *et al.*, 2006; Zhao, 2008). The crosstalk among them is being still studied due to the difficulty of resolving which induces the cause and/or the effect of their changes (Nordström *et al.*, 2004). In our study, an evident reduction of Z+ZR (65%) was firstly noticed in needles under drought earlier than the increment of ABA and IAA levels. Concerning IAA, its accumulation was well correlated to the decrease of RWC (%) (Fig. 2.8) and the induction of epinasty symptoms (Fig. 2.9A), as it was previously observed in some angiosperms (Kawano *et al.*, 2003; Keller & Van Volkenburgh, 1997) and even in conifers (Blake *et al.*, 1980). In this matter, some studies have demonstrated that leaf curvature is a defense mechanism that slow down the damages under stress conditions (Abreu & Munné-Bosch, 2008). In *Vitis vinifera* it has been reported that shoots with downward orientation accumulated IAA in the apex areas and induced the reduction of hydraulic conductance, this fact did not occur in upward oriented shoots (Lovisolo *et al.*, 2002). According to this assumption, in *P. radiata* plants the apical curvature could be induced by IAA accumulation in the apical needles (Fig.

2.9A), but this accumulation might be a consequence of K_{leaf} reduction (started with K_{leaf} decrease of 65%), and not its possible cause.

In addition, IAA increase also presented a strong negative relationship with *Fv/Fm* and a positive correlation with *E.L.* (%) and the presence of epinasty, which pointed out a high IAA accumulation as indicator of severe plant water deficit and, in agreement with Abreu & Munné-Bosch (2008) as a defense mechanism signal to reduce higher damages in the *PSII* (Fig. 7C and 7D). Finally, some studies have come up with the result that IAA can influence ethylene biosynthesis and *vice versa* (Santner & Estelle, 2009; Swarup *et al.*, 2002). On this account, Tsuchisaka & Theologis (2004) observed that 1-aminocyclopropane-1-carboxylate synthase have been proved to be regulated by auxin presence. Thus, stressed plants from O1 and O6 also showed the highest decrements of ACC levels due to a possible conversion to ethylene at the end of the drought period (Fig. 2.4A), and also a high of IAA/ACC ratio (Fig. 2.10B). Thus, Else *et al.* (1995) observed petiole epinastic on plants under stress stimulated by ACC conversion to ethylene across ACC oxidase (Dodd, 2005; Ghanem *et al.*, 2008). According to this, the needles epinasty observed in *P. radiata* plants under water stress conditions could be due to the additive effect of IAA and ethylene accumulation.

2.4.3.- Recovery capacity after drought

The recovery capacity was also analyzed (Fig. 2.12). Plants from all ecotypes recovered their Ψ_{leaf} and Ψ_{turgor} after a week with water supply (Chapter 1) (Cregg & Zhang, 2001; Medrano *et al.*, 2003). Only stressed plants from O4 and O5 regained their K_{leaf} levels (Table 2). In this matter, Blackman *et al.* (2009) observed that possible candidates to the inhibition of gas exchange recovery were the damages of leaf photosynthesis apparatus or/and ABA accumulation. On this account, O3 showed the lowest recovery percentages of gas exchange parameters and leaf hydraulic conductance, and was still accumulating ABA after rewatering, contrarily to the rest of ecotypes (Fig. 2.3A). Furthermore, ABA accumulation and *Fv/Fm* decrease or *E.L.* (%) increase presented a high correspondence (Fig. 2.7E and G), and these strong relationships could also corroborate the aforesaid evidence (Blackman *et al.*, 2009). This same pattern was appreciated in the case of IAA (Fig. 2.7F and H).

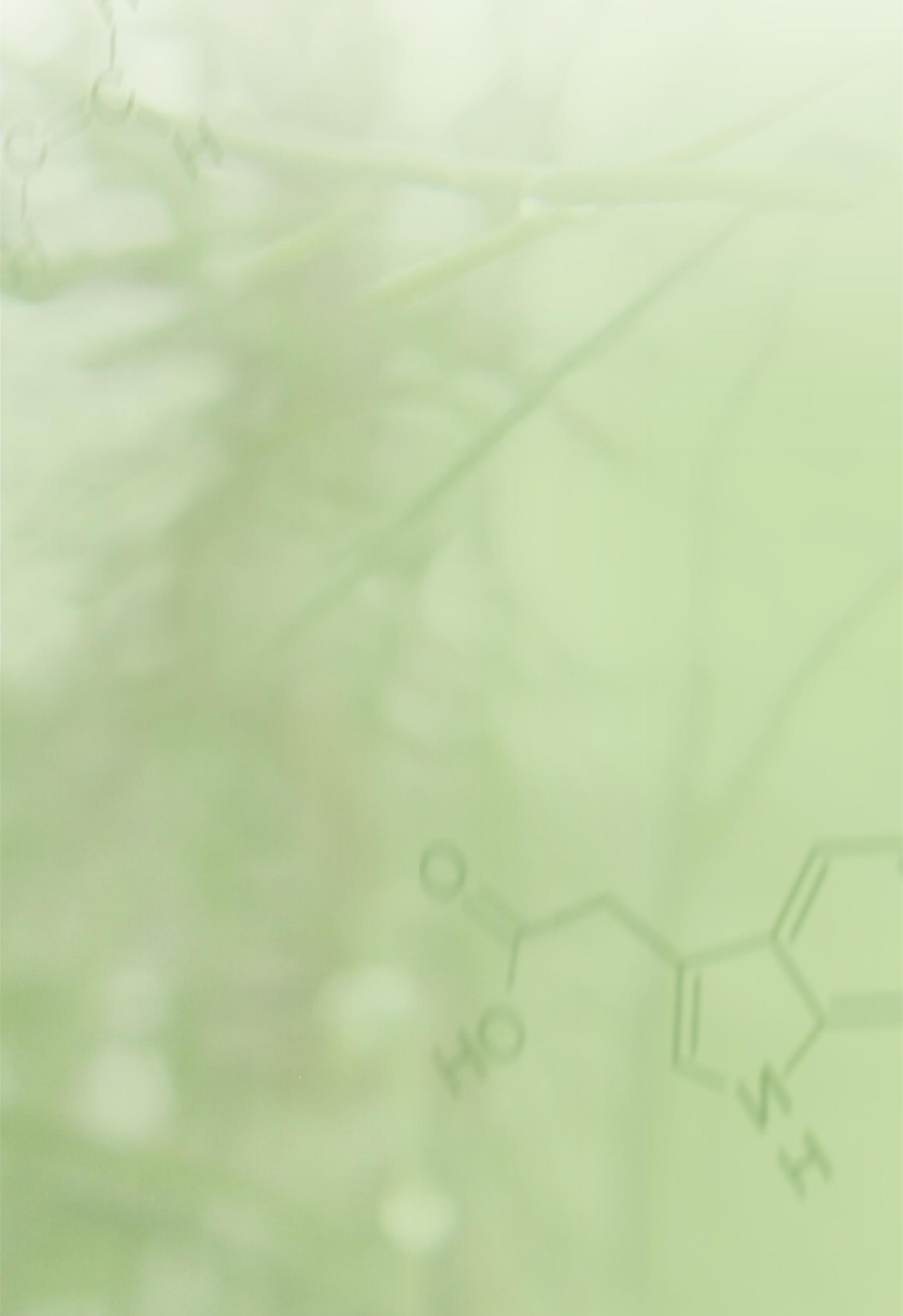
According to PC, recovery capacity of the highest drought tolerance ecotypes (O4 and O5) was correlated to the lowest IAA values, so only these plants recovered their K_{leaf} initial

levels (Fig. 2.12). In this sense, although Salleo *et al.* (1996) reported that high IAA concentrations were necessary to the recovery of hydraulic conductivity, they could also induce a great number of small xylem vessels that delayed the K_{leaf} recovery (Lovisolo *et al.*, 2002) This assumption could be one reason of the low percentage of K_{leaf} observed in stressed plants from O1, O2, O3 and O6 after a week of rewatering, so they did not recover the IAA levels (Fig. 2.3B). Besides, O4 and O5 were also positively correlated to Z+ZR/IAA and ACC/IAA relations that can induce the stomatal aperture due to the Ck increase and a decrease of IAA levels (Acharya & Assmann, 2009; Pospisilova, 2003).

To summarize, our study provides new insights about the role of major phytohormones (ABA, Z, IAA, JA, SA), and ACC in needles of *Pinus radiata* D. Don plants during drought periods and their relationships with some physiological parameters commonly associated to stress (Ψ_{leaf}, K_{leaf}, g_s). Although ABA has been traditionally considered the principal water stress indicator, according to our results, IAA was the most important phytohormone of the model (66% model variance) and the most representative "water deficit signal". The main drought indicator was K_{leaf} which dropped in line to Z+ZR content that acted as a first drought signal. When Z+ZR content decreased over 65%, plants started to accumulate ABA and IAA in the needles. Both, ABA and IAA variations presented a great correlation with the changes in K_{leaf}, A_N, *Fv/Fm* and *E.L.* (%) due to water stress situation. JA accumulation revealed that plants have been subjected to a severe stress, being considered as a "warning alarm". Finally, SA accumulation was shown as a mechanism of "water stress tolerance".

Chapter 3:

Cell wall elastic modulus and osmolyte implication in drought response and recovery in radiata pine



3.1.- Introduction

In nature, many plants are adversely influenced by several environmental factors that have a negative effect on survival and development (Chen & Jiang, 2010). Drought has been associated with regional-scale forest mortality worldwide, and climate change is expected to exacerbate regional mortality events (McDowell *et al.*, 2010). Forest ecosystem productivity is severely constrained by water availability, highlighting the need to know the key processes that allow trees to overcome such severe water shortages (Bréda *et al.*, 2006).

Drought tolerance mechanism have been summarized as (Clifford *et al.*, 1998): (i) avoidance of damaging plant water deficits; (ii) stress tolerance-adaptations that enable plants to continue functioning despite of plant water deficit and (iii) efficiency mechanism that enable the plants to optimize the utilization of resources, especially water. In most cases, when water stress is detected, plant´s first response is to avoid low water potential by decreasing stomatal conductance and, in long term, by root growth changes in order to maximize water uptake (Kramer & Boyer, 1995). With the extension of water deficit these responses no longer confer protection against low water potential (Verslues *et al.*, 2006).

Considering additional tolerance mechanisms, plants must avoid cell dehydration by preventing water loss via cell wall hardening or promoting water influx as a result of accumulation of active solute (osmotic adjustment- OA) that decreases the osmotic potential (Chen & Jiang, 2010; López *et al.*, 2009a; Nguyen-Queyrens & Bouchet-Lannat, 2003). The synthesis of osmolytes is considered as an "active osmotic adjustment", whereas the concentration of solute due to a reduction of cell volume by a net loss of symplastic water is defined as "passive osmotic adjustment" (Dichio *et al.*, 2006). Osmolytes are low molecular weight and highly soluble compounds which protect plants from stress not only through the contribution to osmotic contribution, but also the detoxification of reactive oxygen species, the protection of membrane integrity and the stabilization of enzymes and proteins (Ashraf & Foolad, 2007; Chen & Jiang, 2010). Inorganic and organic solutes such as ions, soluble sugar and free amino acids (AAs) and free polyamines (PAs) contribute like osmolytes to the lowering of osmotic potential (Pérez-López *et al.*, 2010; Serraj & Sinclair, 2002; Silva *et al.*, 2010).

Plant species greatly differ with respect to the type of solutes accumulated and their relative contribution to the reduction of osmotic potential (Hummel *et al.*, 2010; Levy *et al.*,

2006; Martinelli *et al.*, 2007). Substantial differences have been reported between species and even cultivars (Bajji *et al.*, 2001; Levy *et al.*, 2006). In many woody species, the principal osmolytes involved in the OA seem to be organic solutes (Patakas *et al.*, 2002). Among organic solutes, sugar accumulation plays the main role in *Prunus persica* (Escobar-Gutierrez *et al.*, 1998) and *Eucalyptus* spp. (Adams *et al.*, 2005), whereas AAs seems to be the most important in *Morus alba* (Ramanjulu & Sudhakar, 2001). Soluble carbohydrates and amino acids, specifically proline, are especially important in *Ziziphus mauritiana* (Clifford *et al.*, 1998). Proline is traditionally considered the most important osmolyte accumulated under stress conditions due to its osmotic implication (Hare & Cress, 1997; Pérez-Pérez *et al.*, 2009; Verbruggen & Hermans, 2008) and as protector against oxidative damage (Girija *et al.*, 2002). In the last years, some controversies about the positive role of proline on drought stress have been reported, pointing out that the proline implication is species dependent (Silva *et al.*, 2010; Souza *et al.*, 2004).

In addition, some studies suggest that cell wall elasticity properties together with OA are another important defense mechanisms against water stress (Hessini *et al.*, 2009; Saito & Terashima, 2004). The wall cell elastic modulus (ε) of leaf tissue expressed as the change in cell turgor for a unit change in the cell relative water content, has a critical role in water relations (Saito & Terashima, 2004). Indeed, more elastic walls can shrink under osmotic stress to maintain high turgor pressure (Pérez-López *et al.*, 2010; Saito & Terashima, 2004), while less elastic walls permit decreases in leaf water potential and extract water from dry soil with small water losses (Kramer & Boyer, 1995; Navarro *et al.*, 2007). As regards woody plants, this assumption has been a polemical subject for decades. Thus, the increase and decrease of ε have been explained as adaptive changes to water stress conditions (Hessini *et al.*, 2009; Martínez *et al.*, 2007).

Preliminary studies carried out in *Pinus radiata* D. Don ecotypes showed a different drought response and recovery capacity among them. This variable response affected water status, photosynthetic processes, stomata behaviour or membrane functions (Chapter 1). In addition, the changes in these physiological traits were induced by hormonal signals, being IAA the most representative one in the course of the study (Chapter 2).

Due to the different ecotype responses to drought and rewatering, we hypothesize that the variable tolerance to drought may be mediated by osmotic adjustment (OA) and/or wall cell

elastic modulus (ε). In the present study, we focus the analysis on these traits during drought and rewatering, and evaluate in which extent they contributed to drought tolerance in the different *Pinus radiata* ecotypes. Moreover, we will discuss if these parameters could be used as physiological indicators of drought tolerance, facilitating the selection of interesting genotype.

3.2.- Material and Methods

3.2.1.- Plant material, growth conditions and experimental design

Seed characteristics, growth conditions and experimental design were performed as described in Chapter 1 (1.2.1 and 1.2.2). Osmotic potential and osmolyte content were quantified along the drought period (from T0 to T4) and at R7. Active and passive osmotic adjustment was measured at T4, and cell wall elastic modulus was analyzed at T4 and R7.

3.2.2.- Water relations analysis

3.2.2.1.- Water potential

Leaf water potential (Ψ_{leaf}) and turgor pressure (Ψ_t) (MPa) were measured as defined in Chapter 1 (1.2.3.1).

3.2.2.2.- Osmotic potential

Osmotic potential (Ψ_π) (MPa) was determinated as described by Pérez-López *et al.* (2009) with minor modifications. Two needles of each plant were instantaneously frozen in liquid nitrogen, and stored at -80ºC until the analysis. Samples were thawed, placed in vials and centrifugated at 15.000 *g* for 20 min to extract the sap. Extracts were equilibrated at 25 ºC for 15 min. Osmolarity was determinated by freezing point osmometry using an Osmomat 030 osmometer (Gonotec GMBH, Berlin, Germany). The Ψ_π was calculated by van´t Hoff equation:

$$\Psi_\pi = -R \times T^a \times c_s \qquad \text{(Eqn. 3.1)}$$

where,

R is the gas constant, T^a the sample temperature (ºK), and c_s the solute concentration (mol Kg^{-1}).

Osmotic potential at full turgor (Ψ_π^{100}) (MPa) was calculated as described by Dichio *et al.* (2006):

$$\Psi_\pi^{100} \times LWC_{100Ti} = \Psi_\pi \times LWC_{Ti} \quad \text{(Eqn. 3.2)}$$

where,

LWC_{100} is the leaf water content at full turgor and LWC_{Ti} is the leaf water content of the plants at a determinate time (Ti).

Values of LWC were determinated as:

$$LWC = [(FW - DW) / FW] \times 100 \quad \text{(Eqn. 3.3)}$$

3.2.2.3.- Osmotic adjustment and cell wall elastic modulus

Osmotic Adjustment (OA) (MPa) was calculated as the difference in osmotic potential between irrigated and non-irrigated plants ($\Delta\Psi_\pi$).

The wall cell elastic modulus (ε) (MPa) was estimated assuming a linear relationship between turgor pressure and RWC using the method of Rivelli *et al.* (2002) described by Pérez-Lopez *et al.* (2010):

$$\varepsilon = \Delta\Psi_t / (\Delta V/V) \quad \text{(Eqn. 3.4)}$$

where,

$\Delta\Psi_t$ is the difference of turgor pressure, and $\Delta V/V$ the RWC difference between fresh and full hydrated tissue.

3.2.2.4.- Passive osmotic adjustment

The loss contribution of symplastic water to the decline of osmotic potential ($P\Delta\Psi_\pi$) was calculated for each ecotype and treatment at T4 and R7, and determinated as the following mathematical procedure (Dichio *et al.* 2006):

$$P\Delta\Psi_{\pi} = \Delta\Psi_{\pi} - A\Delta\Psi_{\pi} \quad \text{(Eqn. 3.5)}$$

where,

$A\Delta\Psi_{\pi}$ is the difference between estimated osmotic potential at full turgor (Ψ_{π}^{100}) measured in control plants (W) and in water stressed plant (D).

3.2.2.5.- Osmotic contribution of osmolyte

The estimated osmotic contribution of osmolytes to needle ($\Psi_{\pi,osm}$) (MPa) was obtained using the van´t Hoff equation (Pérez-López *et al.*, 2010):

$$\Psi_{\pi,osm} = -0.002479 \times RDW \times c_s \quad \text{(Eqn. 3.6)}$$

where,

$\Psi_{\pi,osm}$ indicates the contribution of individual osmolytes to Ψ_{π}^{100}, c_s is the molar concentration of the solute (mol Kg^{-1}), 0.002479 m^3 MPa mol^{-1} is the RT^a value at 25ºC; osmolytes are assumed to have ideal behaviour (Alarcón *et al.*, 1993). RDW is the relative DW at saturation, determinated using the following equation:

$$RDW = DW/(TW-DW) \quad \text{(Eqn. 3.7)}$$

where,

TW is weight at full turgor.

3.2.3.- Osmolyte quantification

3.2.3.1.- Free amino acid and free polyamine extraction

Free AAs and free PAs were analyzed on two apical needles per plant collected from T0 to T4 and at R7. Needles were immediately frozen in liquid nitrogen. Samples were maintained at -80ºC until extraction. Free AAs; *L*- isoleucine (Ile), *L*- leucine (Leu), *L*- lysine (Lys), *L*-methyonine (Met), L-phenylalanine (Phe), *L*-threonine (Thr), *L*-tryptophan (Trp), *L*-valine (Val), and *L*- histidine (His), *L*- aspartic acid (Asp), *L*- glutamic acid (Glu), *L*- asparagine (Asn), *L*- serine (Ser), *L*- glutamine (Gln), *L*- glycine (Gly), *L*- arginine (Arg), *L*- alanine (Ala), *γ*-aminobutyric acid (GABA), *L*- tyrosine (Tyr), *L*- proline (Pro), and *L*- hydroxiproline (*OH*-Pro), and free PAs; Histamine (HA), ethylamine (EA), methylamine (MA), tryptamine (Tryp), *β*- phenylethylamine (PEA), putrescine (Put), cadaverine (Cad), spermidine (Spd), tiramine (TA), spermine (Spm) were extracted according to the method described by Calanni *et al.* (1999). Each treatment sample was pooled and homogenized in liquid nitrogen. 0.10 g of each

sample (FW) was placed in 2 mL vial, and dropped in 1 mL of extraction mixture of ethanol/water (80/20, v/v). Extracts were centrifuged at 2,000 *g* for 10 min at 4ºC. Pellets were re-extracted for 10 min in additional 1 mL of the same extraction solution. Supernatants were collected and evaporated to dryness by a stream of compressed air. The pellet was dissolved in 1 mL mobile phase at initial conditions. Samples were filtered through 13 mm diameter nylon membrane Millex filters (Ø 0.22 µm) (©Millipore, Bedford, MA, USA), and placed into new tubes.

3.2.3.2.- Free amino acid and free polyamine quantification

Analyses were carried out with a HPLC Model 1100 Agilent (Palo Alto, USA) connected to a fluorescence detector. AAs and PAs derivatization was executed into the loop, mixing 1 µL of borate buffer (pH 10), 2.5 µL of each standard or sample previously filtered, 0.5 µL of o-phthaldehyde-2-mercaptoethanol (OPA), 0.5 µL of 9-fluorenylmethyl chloroformate (FMOC) and 32 µL of filtered Milli-Q® water. 8 µL of each mixture was injected onto a GEMINI (NX)- C18 column (5 µm, 150 × 0.5 mm, ©Phenomenex, Inc.) with a guard column ZORBAX Eclipse AAA-Pack (Analytical guard Column 5 µm, 4.6 × 12.5 mm, Agilent technologies, Inc.) installed in an oven Gecko 2000 (Essex, UK) at 40 °C and eluted at a flow rate of 1.5 mL min^{-1}. Mobile phase A [ammonium formate (20 mM, pH 7.8)] and mobile phase B [acetonitrile/methanol/water formic acid (45:45:10, v/v/v)] were used for the chromatographic separation. The elution consisted of a 42 min linear gradient from 10 to 57% B, followed by another 8 min linear gradient from 57 to 90% B, and finally a 5 min linear gradient from 90 to 100% B. The flow was continuous at a rate of 1.5 mL for 52 min, other continuous flow rate of 0.8 mL min^{-1} for 0.5 min and a last continuous flow rate of 1.5 mL min^{-1} for 2.5 min. Column was equilibrated with the starting composition of the mobile phase for almost 15 min before each analysis. The fluorescent detector operated at excitation wavelength of 220 nm, and emission wavelengths of 350 nm and 440 nm. Pro and *OH*-Pro was detected at 350 nm, and the remainder free AAs and free PAs at 440 nm (Fig. 3.1). Standards with known concentrations of each component (AAs: 10, 25, 50, 100 and 200 mg L^{-1}, and PAs: 5, 12,5, 25, and 50 mg L^{-1}) were also examined under the same conditions. The spectra were obtained using the DataAnalysis program for HPLC-FD. Recoveries were determined using internal standards on each emission wavelength (*L*- sarcosine at 350 nm and α-aminoadipic acid at 440 nm). Recoveries ranged between 85 and 90%. Three biological replicas were quantified per sample.

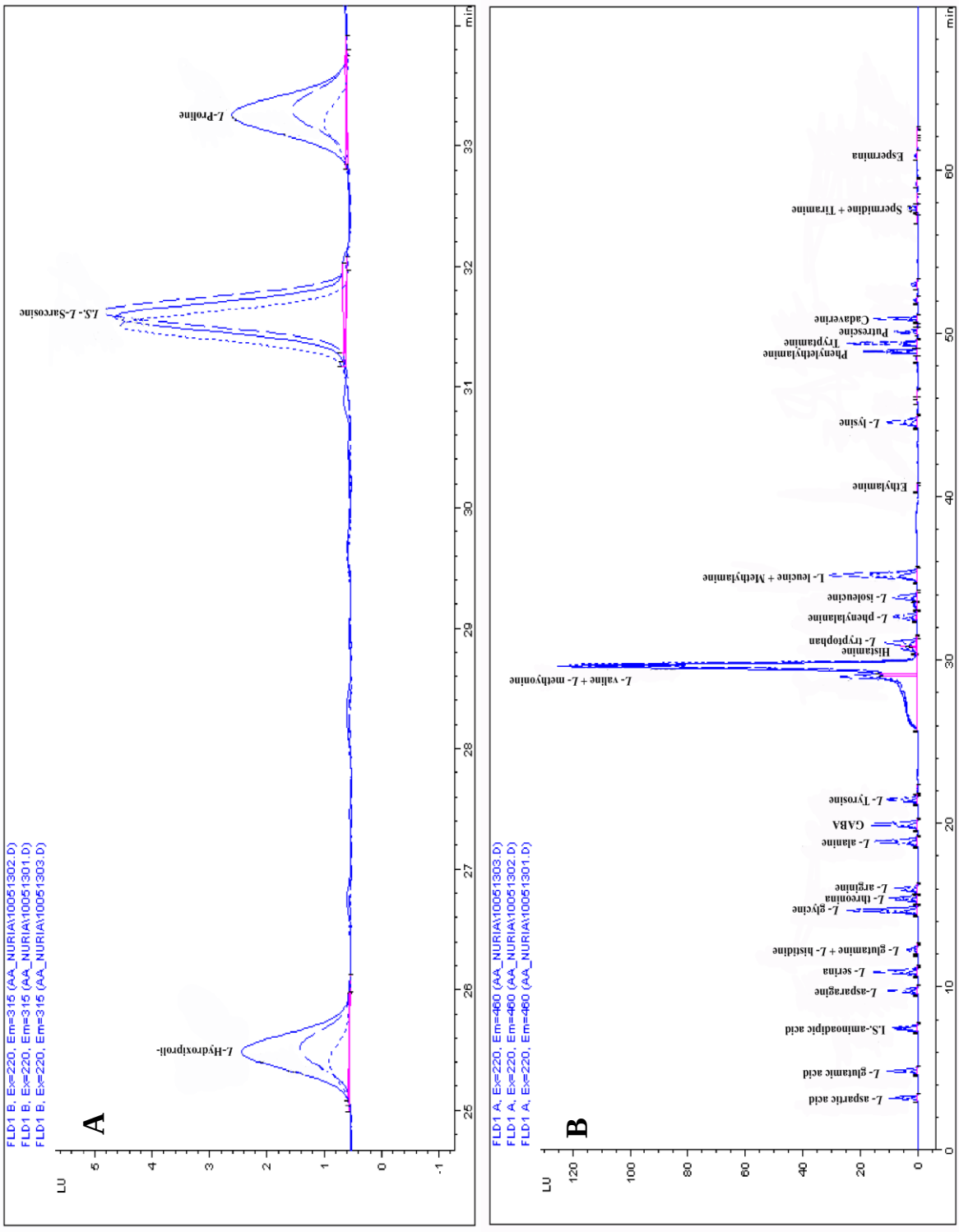


Figure 3.1. Chromatogram of some free amino acid and free polyamine quantification at 350 nm (A) and at 440 nm (B) using two internal standars: *L*- sarcosine at 350 nm and α-aminoadipic acid at 440 nm.

3.2.3.3.- Soluble carbohydrate quantification

Carbohydrate analysis was performed on the same extract as described for the quantification of free PAs and AAs. Sucrose, *D*-glucose and *D*-fructose content was analyzed using the enzymatic Kit extraction ©Boehringer Mannhein/ R-Biopharm (Cat. Nr. 10 716 260 035).

3.2.4.- Statistical analysis

Analysis of variance (ANOVA) was carried out by *proc glm* in the S.A.S ® software package. The Ψ_π was analyzed in five plants per ecotype and treatment in different times (Ti); two-weekly during the drought period (from T0 to T4) and a week after rewatering (R7), according to the following mathematical model:

$$y_{ijkr} = \mu + O_i + T_j + Ti_k + OT_{ij} + OTi_{ik} + TTi_{jk} + OTTi_{ijk} + e_{ijkr} \quad \text{(Eqn. 3.8)}$$

where,

y_{ijkr} was the response variable result of the r^{th} plant of the i^{th} ecotype subjected to j^{th} treatment at k^{th} time; μ was the experimental mean, O_i was the effect of the *i*th ecotype, T_j the effect of the j^{th} treatment (well watered (W) or water stressed plants (D), Ti_k the effect of the k^{th} time (T0 to T4 and R7); OT_{ij} was the interaction between the i^{th} ecotypes and j^{th} treatment, OTi_{ik} between the i^{th} ecotype and k^{th} time, TTi_{jk} between the j^{th} treatment and k^{th} time, $OTTi_{ijk}$ among the i^{th} ecotype, the j^{th} treatment and the k^{th} time and e_{ijkr} was the random error component.

For the analysis of OA and ε, the changes per ecotype were measured through the drought period (from T0 to T4) and subsequent rewatering (R7) (Ti), and were analyzed according to the following mathematical model:

$$y_{ijr} = \mu + O_i + Ti_j + OTi_{ij} + e_{ijr} \quad \text{(Eqn. 3.9)}$$

where,

y_{ijr} was the response variable result of the r^{th} plant of the i^{th} ecotype subjected at j^{th} time; μ was the experimental mean, O_i was the effect of the *i*th ecotype, Ti_j the effect of the *k*th time; OTi_{ij} between the *i*th ecotype and k^{th} time, and e_{ijr} was the random error component.

Multiple comparisons were calculated using the post hoc Tukey´s HSD test for balance data and Tukey-Kramer for unbalance data. To analyze possible correlations among physiological parameters *proc reg* was used, and analysis of covariance (ANCOVA) was carried out by *glm proc* in the S.A.S ® software.

3.3.- Results

3.3.1.- Osmotic adjustment and the bulk elastic modulus

Changes of Ψ_{leaf} showed a positive correlation with Ψ_{π} variations (R^2 = 0.62, p<0.001) (Fig. 3.2). According to ANCOVA, Ψ_{π} variations were due to a direct effect of Ψ_{leaf} (p< 0.001) and were not influenced by the ecotypes. As it was observed in Chapter 1, stressed plants with Ψ_{leaf} of -2 MPa reached their turgor loss point (*TLP*), and also dropped their Ψ_{π} values to -2 MPa (Fig. 3.2).

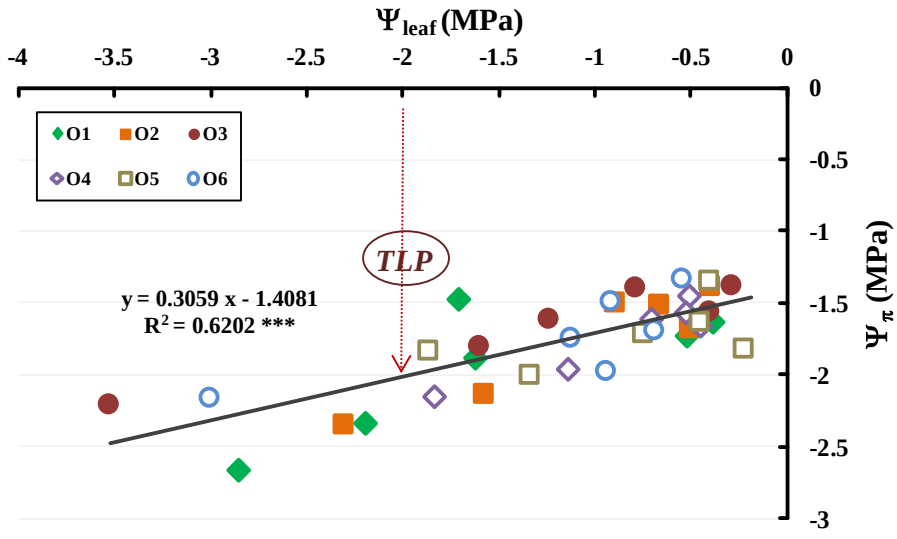


Figure 3.2. Correlation between osmotic potential (Ψ_{π}) *vs.* leaf water potential (Ψ_{leaf}) (MPa) in six *Pinus radiata* ecotypes (O1-O6) along a drought period of four weeks (from T0 to T4). Discontinuous narrow indicates ***TLP***- Turgor loss point. R^2- Pearson`s correlation numbers with the significance according to ANCOVA. * < 0.05; ** <0.01 and *** <0.001.

Drought induced a reduction of Ψ_{π} in all stressed plants (Fig. 3.3), and the effect was due to the triple interaction among Ti, T and O according to ANOVA (Table 3.1). Ψ_{π} variations in stressed plants of all ecotypes were not statistically significant with regard to each control until T4, except in O6 which showed statistical differences at T2 (Fig. 3.3). At T4, stressed plants from O6 presented similar Ψ_{π} levels than obtained at T2, and statistically equal than O2, O3, O4 and O5 (around -2.2 MPa). O1 showed the highest Ψ_{π} decrement with values below -2.6 MPa, and statistically different to the rest of stressed plants (Data not shown). At R7, stressed plants from all ecotypes recovered their Ψ_{π} control values (Fig. 3.3).

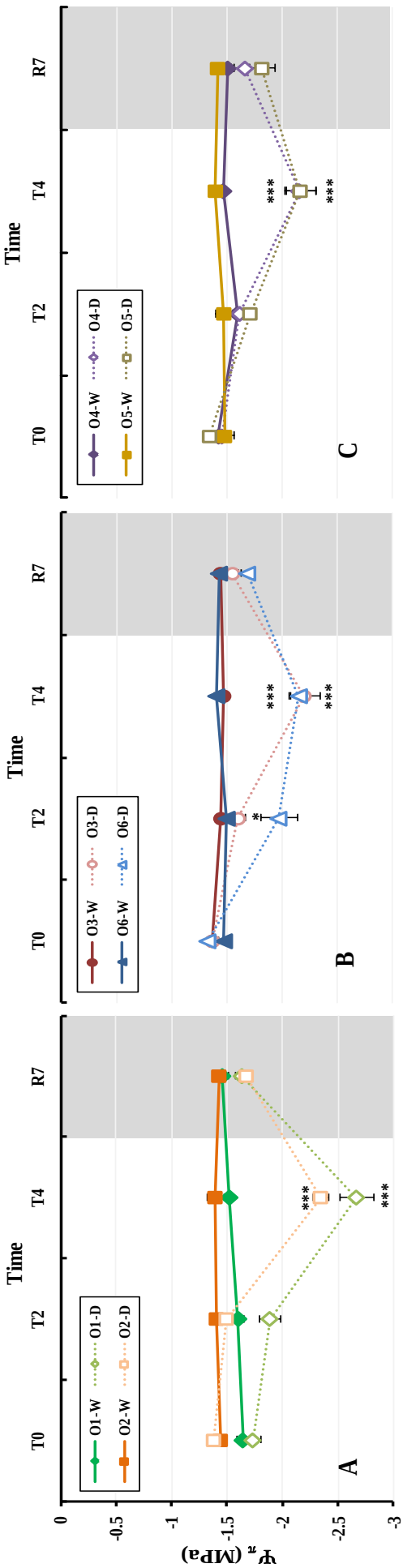


Figure 3.3. Osmotic potential (Ψ_π, MPa) in six *Pinus radiata* ecotypes [O1-O2 (A), O3-O6 (B), O4-O5 (C)] exposed to irrigation (W- Well watered, closed symbols) or no irrigation conditions (D- Stressed plants, open symbols) along a drought period of four weeks (from T0 to T4) and subsequent recovery after rewatering for a week (R7- Shady area). *M* ± *S.E.* Significant differences with regard to each control are represented by asterisks according to Tukey´s HSD test after ANOVA. * < 0.05; ** < 0.01; *** < 0.001.

Table 3.1. Analysis of variance (ANOVA) of osmotic potential (Ψ_π), osmotic adjustment (OA) and cell wall elastic modulus (ε) in six *Pinus radiata* ecotypes (O) under irrigation and no irrigation conditions (T) for four weeks and subsequent rewatering (Ti).

VARIABLE	FACTORS	DF	Sum Sq	Mean Sq	Fvalue	Pr(>F)
Ψ_π (MPa)	Ti	3	5.3910	1.7970	65.65	***
	O	5	1.1421	0.2284	8.34	***
	T	1	5.6438	5.6438	206.18	***
	Ti ×O	15	0.9794	0.0653	2.39	**
	Ti × T	3	6.2601	2.0867	76.23	***
	O × T	5	0.2404	0.0481	1.76	ns
	Ti × O × Ti	**15**	**0.6985**	**0.0466**	**1.70**	*
OA (MPa)	**Ti**	**2**	**7.0566**	**3.5283**	**61.45**	***
	O	5	0.4918	0.0984	1.71	ns
	Ti × O	10	0.9548	0.0955	1.66	ns
ε (MPa)	**Ti**	**2**	**15.4202**	**3.0840**	**6.10**	***
	O	**5**	**7.3243**	**3.6622**	**7.24**	**
	Ti × O	10	6.1034	0.6103	1.21	ns

Note: * < 0.05; ** <0.01 and *** <0.001; ns-non-significant.

When the osmotic adjustment (OA) was analyzed, significant differences were only observed with respect to Ti according to ANOVA (Table 3.1). At T2, stressed plants approximately presented an OA of 0.2 MPa, and increased 4-fold the values at T4 (Fig. 3.4A). At R7, all plants recovered the OA levels observed at T2. Regarding the OA mechanisms to concentrate osmolytes into plant cell (active or passive), different behaviours were noticed among ecotypes at T4 (Fig. 3.4B). Whereas O1, O2, O3 and O6 did not show differences between active and passive OA, in stressed plants from O4 and O5 the synthesis of osmolytes (active OA) was the main contribution with a 78% (Fig. 3.4B).

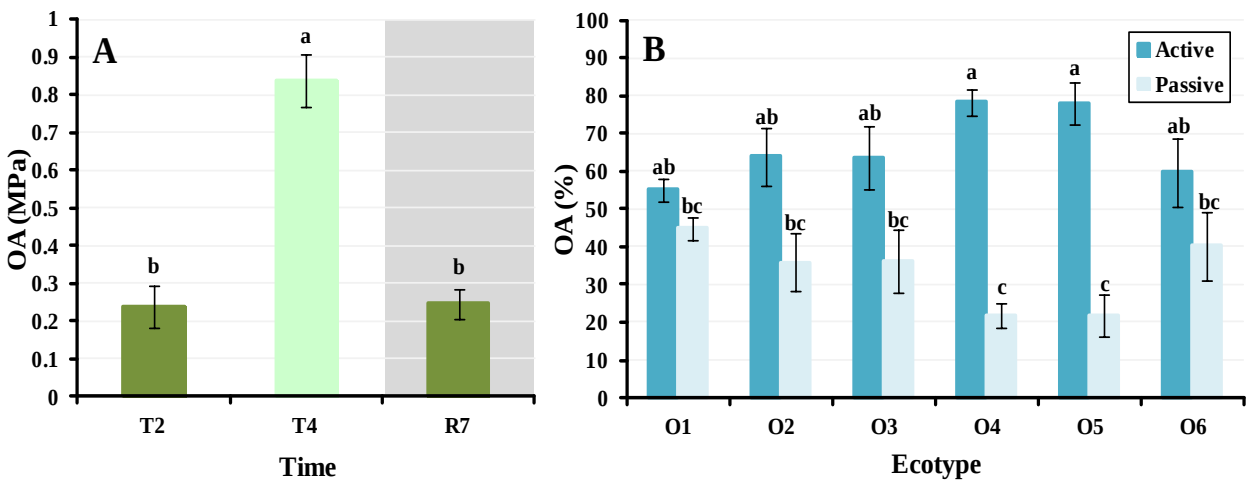


Figure 3.4. Osmotic adjustment (OA, MPa) in *Pinus radiata* plants after two (T2) and four (T4) weeks under drought conditions and subsequent recovery after rewatering for a week (R7- Shady area) (A). Active and passive contribution to osmotic adjustment (OA; %) in each ecotype (O1-O6) at T4 (B). *M* ± *S.E.* Different letters mean significant differences according to Tukey´s HSD test after ANOVA.

Drought also induced variations in the cell wall elastic modulus (ε) and its changes depended on Ti and O according to ANOVA, but not on their interaction (Table 3.1). All stressed plants significantly increased their ε over 1 MPa with respect to each control at T4, recovering their control levels after rewatering (Fig 3.5A). O6 showed the highest ε values, with no significant differences with respect to O1, O2 and O3 (Fig. 3.5B). On the contrary, O5 had the lowest ε together with O4, with values 4-fold lower than observed in O6.

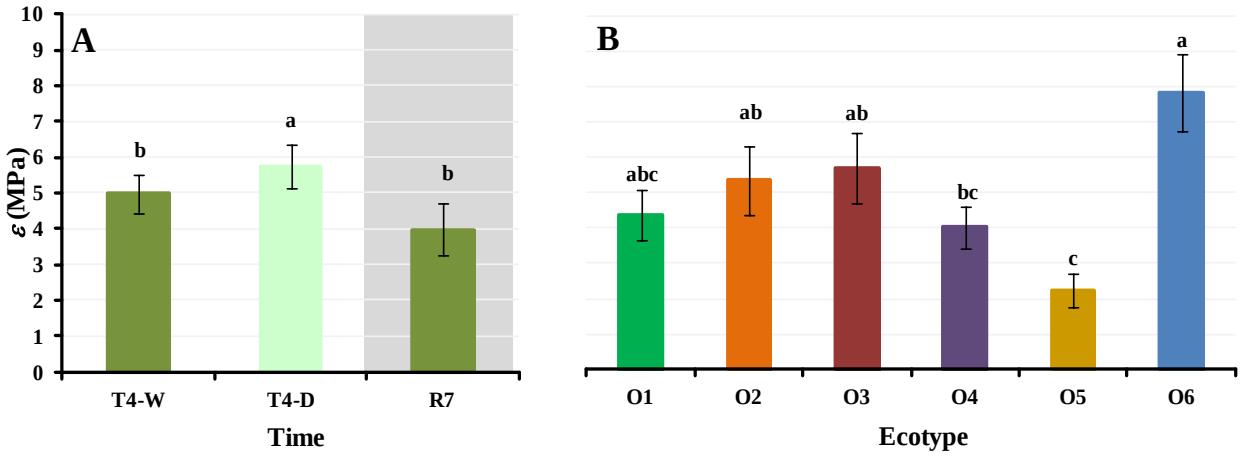


Figure 3.5. Cell wall elastic modulus (ε) (MPa) in irrigated (W) and non-irrigated plants (D) of six *Pinus radiata* ecotypes (O1-O6) after four weeks under drought conditions (T4) and after rewatering for a week (R7- Shady area) (A). ε values of each ecotype at T4 (B). *M* ± *S.E.* Different letters mean significant differences according to Tukey´s HSD test after ANOVA.

3.3.2.- Osmolyte contribution

As it was observed in Fig. 3.4B, active OA varied among O and represented at least a 50% total OA. To determine the relative contribution of organic solutes to OA, some soluble carbohydrates, free AAs, and free PAs were analyzed (Table 3.2 and 3.3). The amount of soluble carbohydrates was higher than free AA and free PAs during drought. However, free AAs increased in a higher extend than soluble sugars or PAs. Soluble carbohydrates were the principal osmolytes which contributed to OA, varying among ecotypes from 13.8% (O5) to 33% (O4), whereas free AAs and PAs modestly contribute to OA with percentages below 8% and 1%, respectively (Table 3.3). It is remarkable that one of the less affected ecotypes (O4) showed the highest soluble carbohydrate values in well watered plants (14.83 mg g^{-1} FW) and decreased after four weeks under drought conditions (9.46 mg g^{-1} FW) (Fig. 3.2). Contrariwise, the rest of ecotypes increased their levels at T4, especially O2 that increased their values from 2.9 to 6.4 mg g^{-1} FW.

Sucrose was the most abundant soluble sugar in all ecotypes and its levels increased during drought, mainly in stressed plants from O1 (Table 3.2 and 3.3). *D*-glucose and *D*-fructose presented the highest increases in all stressed plants, especially *D*-glucose in O2 and O3 with 5.18 and 4.87-fold their control values. On the other hand, stressed plants from O6 showed increases of 7.15-fold their *D*-fructose content compared to their controls, with an OA contribution of 7.46% (Table 3.3).

Regarding free AAs, O1 and O6 showed the highest increases with 7.22 and 6.30-fold with regard to the control values, and an osmotic contribution of 4.11 and 7.72%, respectively (Table 3.3). Stressed plants showed the highest increases in Arg, Asn, Gln, Glu, Gly, Ser, Trp, and especially in Pro and GABA content at T4 (Table 3.2 and 3.3). O1 presented the highest GABA accumulation, with increments of 468.8-fold the values of irrigated plants. O3 showed the strongest Pro increase with values of 868.9-fold the controls (Table 3.3).

Stressed plants from all ecotypes practically did not vary their total free PAs, except O5 that increased 6-fold their levels at T4 (Table 3.2 and 3.3). O5 based their PA increment principally in a strong accumulation of Spd and Spm, showing values of 69.68 $\mu g\ g^{-1}$ FW and 47.41 $\mu g\ g^{-1}$ FW, respectively. Stressed plants from O5 also increased Put in a lesser extent, doubling their contents from 6.5 to 14.7 $\mu g\ g^{-1}$ FW related to the irrigated ones (Table 3.2).

3.3.3.- Correlation analysis

The reduction of RWC (%) observed in *P. radiata* needles was highly correlated to the type of OA (%); active or passive, and the cell wall elastic modulus (ε) (Fig. 3.6). At T4, stressed plants that maintained higher RWC (%) showed higher active than passive OA. Thus, plants with 60% RWC presented 80% active and 20% passive OA. On the contrary, plants which decreased their RWC below 50% had percentage near 50% both, active and passive OA (Fig. 3.6A). Variation in ε also was related to the active and passive OA but the tendency was opposite to the observed in the case of the RWC changes (Fig. 3.6B). In this matter, stressed plants that had high ε, showed a equilibrate OA (50%) between active and passive type and the lowest RWC (35%).

Table 3.2. Soluble carbohydrate (mg g^{-1} FW), free AAs and free PAs (µg g^{-1} FW) in six *Pinus radiata* ecotypes (O1-O6) under irrigated and non-irrigated conditions for four weeks (T4)

Osmolytes	Ecotypes											
	O1		O2		O3		O4		O5		O6	
	W	D	W	D	W	D	W	D	W	D	W	D
Carbohydrates (mg g^{-1} FW)	**3.30 ± 0.08**	**5.30 ± 0.02**	**2.88 ± 0.07**	**6.44 ± 0.28**	**3.98 ± 0.39**	**4.96 ± 0.75**	**14.83 ± 0.36**	**9.46 ± 1.30**	**2.09 ± 0.08**	**3.48 ± 0.44**	**2.78 ± 0.16**	**4.44 ± 0.17**
Sucrose	1.25 ± 0.09	2.86 ± 0.21	2.17 ± 0.02	2.76 ± 0.64	3.36 ± 0.73	3.68 ± 0.87	8.37 ± 1.11	5.92 ± 1.24	1.12 ± 0.08	1.28 ± 0.13	1.92 ± 0.01	0.84 ± 0.18
D-glucose	1.01 ± 0.06	1.18 ± 0.02	**0.51 ± 0.15**	**2.69 ± 0.90**	**0.15 ± 0.00**	**0.72 ± 0.05**	5.09 ± 0.57	1.99 ± 0.05	0.57 ± 0.02	1.35 ± 0.08	**0.58 ± 0.10**	**1.84 ± 0.06**
D-fructose	1.04 ± 0.05	1.26 ± 0.17	**0.20 ± 0.06**	**1.00 ± 0.03**	**0.14 ± 0.08**	**0.57 ± 0.07**	3.38 ± 0.47	1.55 ± 0.11	0.40 ± 0.02	0.85 ± 0.23	**0.28 ± 0.06**	**1.76 ± 0.07**
Free AAs (µg g^{-1} FW)	**198.4 ± 22.0**	**1430.0 ± 55.2**	**314.1 ± 14.5**	**1101.4 ± 5.63**	**182.3 ± 7.19**	**950.8 ± 36.1**	**335.1 ± 15.3**	**662.4 ± 12.9**	**232.9 ± 17.3**	**679.2 ± 27.1**	**289.7 ± 67.3**	**1826.2 ± 69.5**
Asp	8.25 ± 1.04	4.80 ± 0.18	23.40 ± 0.52	8.24 ± 0.02	6.01 ± 0.22	6.70 ± 0.15	7.02 ± 0.08	20.15 ± 0.80	9.60 ± 0.03	< 0.01	10.20 ± 0.49	11.73 ± 1.08
Glu	**48.35 ± 0.84**	**138.2 ± 0.08**	**96.39 ± 1.27**	**218.8 ± 0.69**	**36.46 ± 1.75**	**142.8 ± 1.25**	**86.08 ± 1.38**	**95.65 ± 0.15**	**55.89 ± 1.11**	**75.26 ± 0.62**	**68.10 ± 0.71**	**198.5 ± 0.88**
Asn	< 0.01	8.61 ± 0.07	< 0.01	17.46 ± 0.01	< 0.01	12.86 ± 0.12	< 0.01	8.55 ± 0.04	< 0.01	< 0.01	< 0.01	36.88 ± 0.99
Ser	2.18 ± 0.11	33.86 ± 0.60	8.39 ± 0.09	35.66 ± 0.01	1.80 ± 0.66	31.56 ± 0.24	14.38 ± 0.76	21.51 ± 1.37	6.17 ± 0.55	13.51 ± 0.10	6.92 ± 0.21	48.90 ± 0.08
Gln+His	22.12 ± 1.82	205.9 ± 4.95	59.20 ± 0.32	245.48 ± 0.52	31.06 ± 2.19	153.4 ± 3.74	72.43 ± 3.46	138.4 ± 2.67	37.68 ± 1.97	330.6 ± 2.75	69.09 ± 3.25	592.5 ± 39.92
Gly	< 0.01	12.52 ± 0.38	< 0.01	4.67 ± 0.09	< 0.01	2.53 ± 0.14	6.49 ± 0.67	< 0.01	< 0.01	0.14 ± 0.09	< 0.01	4.13 ± 0.20
Thr	< 0.01	6.32 ± 0.14	< 0.01	5.71 ± 0.01	< 0.01	3.18 ± 0.08	< 0.01	1.92 ± 0.12	< 0.01	< 0.01	< 0.01	< 0.01
Arg	0.15 ± 0.10	84.94 ± 0.52	6.62 ± 0.17	123.7 ± 0.10	8.46 ± 0.66	76.28 ± 1.40	19.31 ± 0.79	50.75 ± 0.59	3.28 ± 0.41	78.27 ± 0.32	8.35 ± 4.04	122.7 ± 3.23
Ala	0.16 ± 0.10	182.6 ± 8.22	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	29.00 ± 6.48	76.25 ± 37.10	14.38 ± 14.38
GABA	**0.17 ± 0.16**	**78.19 ± 1.15**	**< 0.01**	**133.51 ± 0.95**	**< 0.01**	**70.98 ± 3.32**	**< 0.01**	**6.06 ± 1.18**	**< 0.01**	**18.57 ± 0.98**	**< 0.01**	**130.9 ± 1.24**
Tyr	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	16.55 ± 0.35	< 0.01	0.76 ± 0.06	< 0.01	< 0.01	< 0.01	48.49 ± 0.37
OH-Pro	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Val+Met	5.64 ± 0.74	0.62 ± 0.06	20.51 ± 1.66	6.31 ± 0.02	8.80 ± 0.76	11.15 ± 0.47	30.55 ± 0.93	6.45 ± 0.57	8.01 ± 0.69	8.01 ± 0.28	8.87 ± 0.04	29.00 ± 0.69
Trp	< 0.01	60.56 ± 1.79	0.80 ± 0.42	42.69 ± 0.15	< 0.01	38.61 ± 1.24	< 0.01	17.86 ± 0.87	3.43 ± 0.67	38.61 ± 8.84	8.85 ± 0.31	44.16 ± 0.68
Phe	19.26 ± 0.00	19.26 ± 0.01	19.26 ± 0.01	19.26 ± 0.01	19.26 ± 0.01	17.94 ± 1.32	19.26 ± 0.01	19.26 ± 0.01	19.26 ± 0.01	24.19 ± 0.00	19.26 ± 0.00	9.36 ± 0.00
Pro	**11.35 ± 3.37**	**538.3 ± 2.11**	**6.54 ± 5.14**	**174.9 ± 0.34**	**0.44 ± 0.44**	**299.6 ± 21.10**	**4.98 ± 0.37**	**205.3 ± 3.62**	**5.37 ± 1.97**	**34.36 ± 2.23**	**2.61 ± 2.61**	**442.3 ± 3.43**
Ile	< 0.01	0.02 ± 0.00	< 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.03 ± 0.00	0.02 ± 0.01	< 0.01	< 0.01	< 0.01
MA+ Leu	12.63 ± 6.62	4.33 ± 4.33	8.04 ± 4.81	4.43 ± 2.21	< 0.01	0.12 ± 0.12	6.48 ± 6.48	< 0.01	12.51 ± 9.14	24.31 ± 4.35	11.21 ± 8.20	10.37 ± 1.30
Lys	68.15 ± 1.02	51.06 ± 0.63	64.97 ± 0.04	60.57 ± 0.50	70.00 ± 0.50	66.54 ± 1.21	68.09 ± 0.34	69.81 ± 0.83	71.70 ± 0.72	4.34 ± 0.12	< 0.01	81.86 ± 1.05
Free PAs (µg g^{-1} FW)	**65.26 ± 0.28**	**65.16 ± 1.95**	**38.81 ± 1.01**	**43.35 ± 0.83**	**41.47 ± 3.58**	**38.37 ± 0.48**	**56.37 ± 2.57**	**54.23 ± 0.50**	**36.48 ± 1.26**	**217.6 ± 1.73**	**122.4 ± 2.20**	**124.1 ± 8.15**
EA	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	51.34 ± 1.24	75.47 ± 1.75	3.97 ± 0.05
Tryp	19.31 ± 0.00	19.31 ± 0.01	18.40 ± 0.52	19.31 ± 0.01	19.31 ± 0.01	19.31 ± 0.01	19.31 ± 0.01	19.31 ± 0.02	19.31 ± 0.02	5.18 ± 0.00	19.31 ± 0.02	5.18 ± 0.00
PEA	22.84 ± 0.19	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	20.68 ± 0.40	19.81 ± 0.26	< 0.01	17.92 ± 0.01	< 0.01	36.07 ± 0.58
Put	**5.74 ± 0.08**	**2.92 ± 0.01**	**4.24 ± 0.21**	**3.35 ± 0.09**	**8.98 ± 0.05**	**7.09 ± 0.03**	**5.54 ± 0.10**	**6.35 ± 0.24**	**6.47 ± 0.30**	**14.70 ± 0.10**	**6.21 ± 0.04**	**14.02 ± 0.08**
Cad	8.77 ± 0.00	8.77 ± 0.01	8.77 ± 0.01	8.51 ± 0.25	8.77 ± 0.01	8.77 ± 0.01	8.77 ± 0.01	8.77 ± 0.02	8.77 ± 0.02	11.41 ± 0.00	8.77 ± 0.02	11.41 ± 0.00
Spd+TA	< 0.01	< 0.01	< 0.01	0.08 ± 0.00	2.25 ± 2.25	< 0.01	2.08 ± 1.00	< 0.01	< 0.01	**69.68 ± 0.33**	6.43 ± 0.03	33.45 ±3.26
HA	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	1.16 ± 0.07
Spm	**8.60 ± 0.00**	**34.16 ± 7.41**	**7.41 ± 0.28**	**12.10 ± 0.40**	**2.16 ± 1.27**	**3.20 ± 0.46**	**< 0.01**	**< 0.01**	**1.93 ± 0.96**	**47.41 ± 0.06**	**6.25 ± 0.39**	**11.40 ± 3.89**

Table 3.3. Osmolyte content (D-water stressed /W-well watered plants) and each contribution to Ψ_{π}^{100} (%) in six *Pinus radiata* ecotypes (O1-O6) subjected to drought conditions for four weeks (T4).

Osmolyte	**Ecotype**											
	O1		**O2**		**O3**		**O4**		**O5**		**O6**	
	D/W	**%**	**D/W**	**%**	**D/W**	**%**	**D/W**	**%**	**D/W**	**%**	**D/W**	**%**
Carbohydrates (mg g^{-1} FW)	1.61	15..23	2.24	19.14	1.36	16.00	0.65	33.02	1.67	13.80	1.60	18.87
Sucrose	2.34	8.21	1.26	8.19	1.08	11.86	0.88	20.69	1.14	5.07	0.44	3.54
***D*-fructose**	1.20	3.62	6.69	2.97	2.50	1.82	0.47	5.41	2.20	3.38	7.15	7.46
***D*-glucose**	1.18	3.40	5.18	7.98	4.87	2.31	0.39	6.94	2.36	5.34	3.40	7.78
Free AAs (µg g^{-1} FW)	7.22	4.11	3.50	3.27	5.21	3.07	1.98	2.31	2.91	2.69	6.30	7.72
Arg	73.86	0.24	18.69	0.37	9.02	0.25	2.63	0.18	23.86	0.31	14.70	0.52
Asn	8.61	0.02	17.45	0.05	12.86	0.04	2.09	0.00	1.00	0.00	36.87	8.58
Glu	2.86	0.40	2.27	0.65	3.94	0.40	1.12	0.33	1.35	0.30	2.92	0.84
Gln+His	9.31	0.21	4.14	0.30	4.94	0.22	0.52	0.01	8.80	1.31	8.58	1.96
Gly	12.50	0.04	4.67	0.01	2.53	0.01	6.49	0.02	1.14	0.00	4.12	0.02
Ser	15.55	0.10	4.25	0.11	17.52	0.10	0.66	0.50	2.18	0.50	7.06	0.21
GABA	468.40	0.22	133.50	0.40	71.00	0.23	6.10	0.02	18.60	0.07	130.90	0.55
Pro	47.50	1.55	26.70	0.52	868.90	0.97	41.40	0.72	6.40	0.14	169.20	1.87
Free PAs (µg g^{-1} FW)	1.00	0.19	1.12	0.13	0.93	0.12	0.96	0.19	5.97	0.86	1.01	0.52

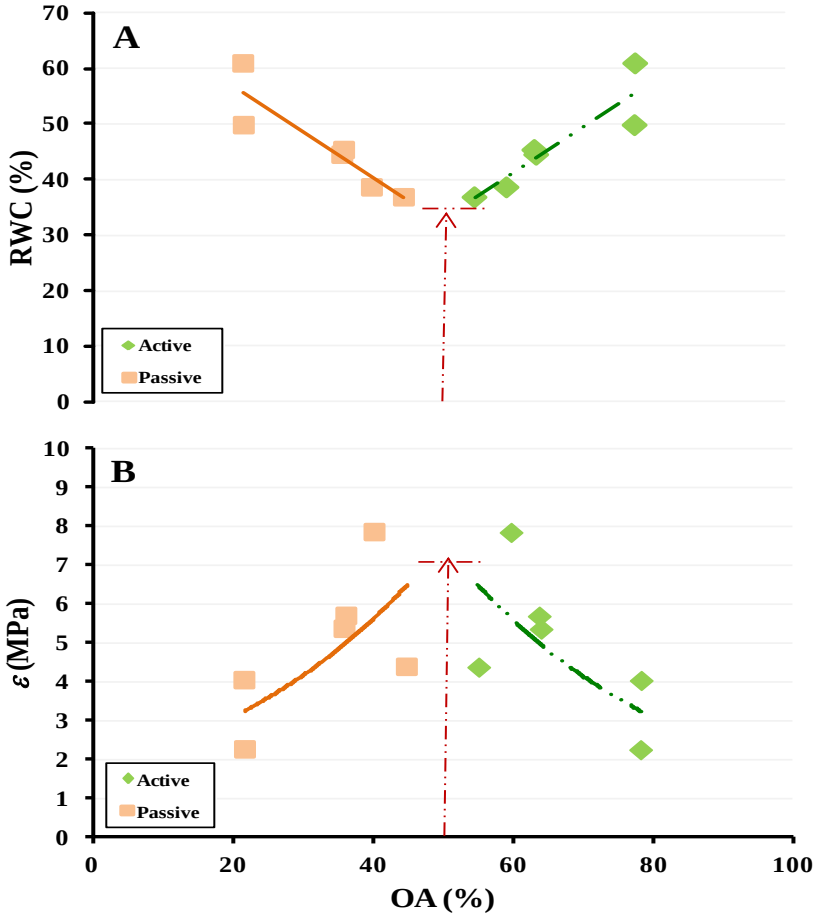


Figure 3.6. Correlation among relative water content (RWC, %) (A) and cell wall elastic modulus (ε, MPa) (B) *vs.* active and passive OA (%) in plants from six *Pinus radiata* ecotypes after four weeks under drought conditions (T4). Red narrows indicate passive or active OA of 50%.

Higher values of ε were also positively correlated to the presence of external symptoms (%) in plants (R^2= 0.81, p<0.001). Thus, when 100% plants showed symptoms ε reached values of 6.8 MPa (Fig 3.7A). Besides, high ε levels were related to strong losses of electrolytes expressed as *E.L.* (R^2=0.65, p<0.05), reaching values of 35% (Fig. 3.7B).

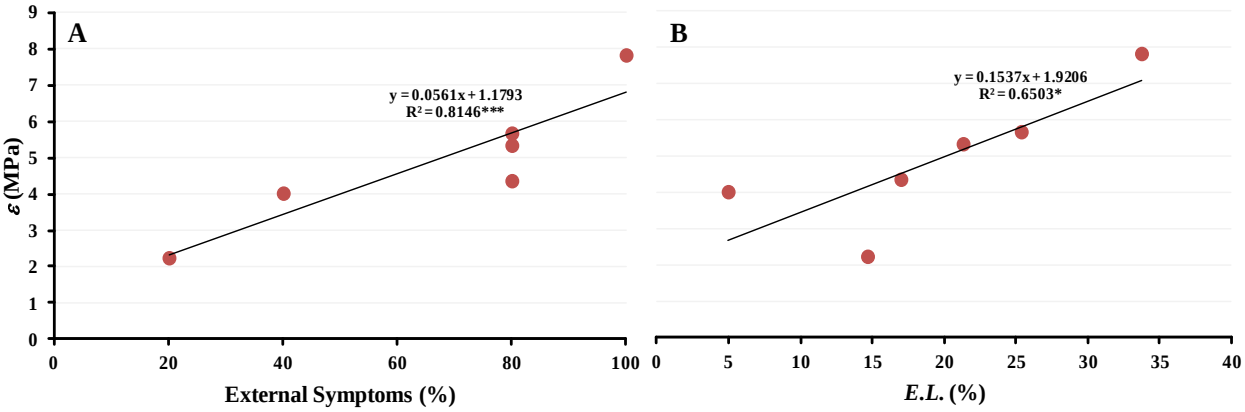


Figure 3.7. Correlation among cell wall elastic modulus (ε) (MPa) *vs.* external symptoms (%) (A) and *E.L.* (%) (B) in plants from six *Pinus radiata* ecotypes after four weeks under drought conditions (T4). R^2- Pearson´s correlation number with the significance- * < 0.05; ** <0.01 and *** <0.001.

Pro accumulation showed a lineal correlation with leaf water potential, turgor pressure, osmotic potential and RWC (%) (Fig. 3.8A and 3.8B). GABA had a strong logarithmic curve, showing high variation related to small reductions in potentials (Fig. 3.8C) and water content (Fig 3.8D). When stressed plants reached their turgor loss point (*TLP*), they showed a Pro and GABA accumulation of 210 and 20 μg g^{-1} FW respectively, (Fig. 3.8) and a RWC of nearly 50% (Fig 3.8B and 3.8D).

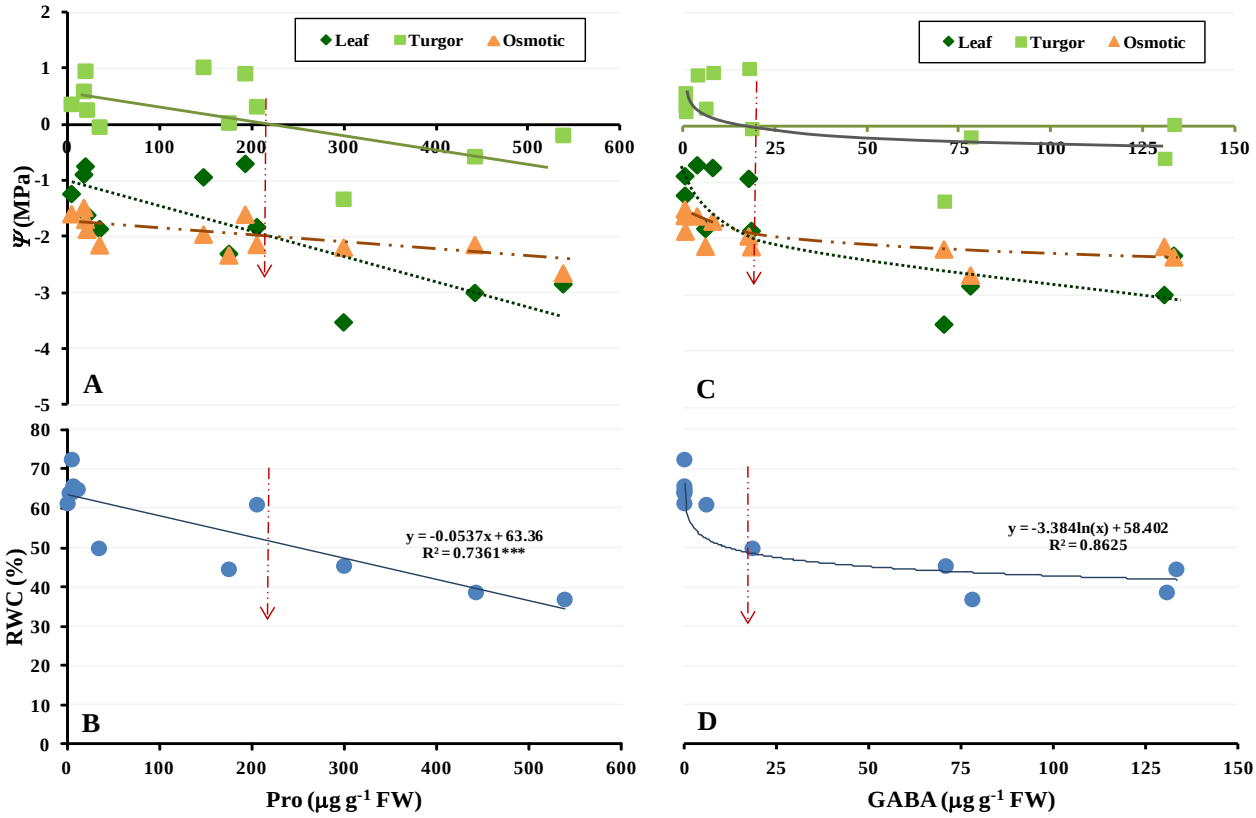


Figure 3.8. Correlation among leaf water potential, osmotic potential, turgor pressure (MPa) (A) and RWC (%) (B) *vs*. *L*-proline (Pro) (μg g^{-1} FW) in plants from six *Pinus radiata* ecotypes along a drought period of four weeks (from T0 to T4). Leaf water and osmotic potential, turgor pressure (MPa) (C) and RWC (%) (D) *vs.* γ-aminobutyric acid (GABA) (μg g^{-1} FW). Discontinuous red narrows indicate turgor loss point. R^2- Pearson`s correlation numbers with the significance. * < 0.05; ** <0.01 and *** <0.001.

The most remarkable phytohormone involved in *P. radiata* drought response, indole-3-acetic acid (IAA) showed a strong relation with some AAs accumulation (Fig 3.9). γ-aminobutyric acid (GABA) was positively correlated to IAA accumulation (Fig. 3.9A), showing a higher relationship than Pro (R^2 = 0.95 and 0.65, respectively). Thus, stressed plants that accumulated IAA levels five times as much as the control references, they showed 100-fold over the GABA values than those observed in well watered plants. Trp accumulation was also observed in plants subjected to drought (Fig. 3.9B). At this regard, stressed plants did not start to increase the IAA content until accumulated Trp levels of 40 μg g^{-1} FW.

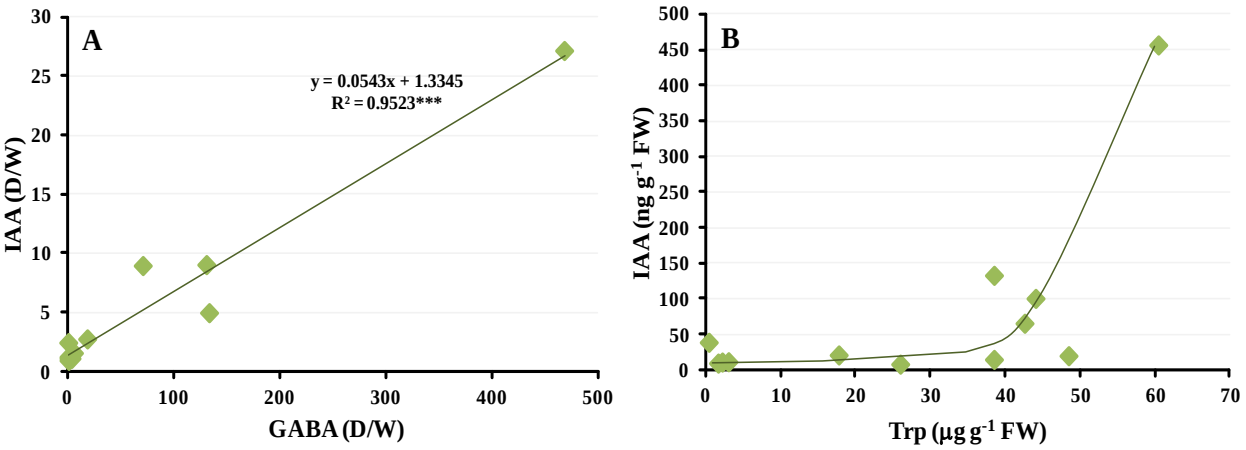


Figure 3.9. Correlation between IAA *vs.* γ-aminobutyric acid (GABA) ratio content (D-water stressed /W-well watered plants) in plants from six *Pinus radiata* ecotypes along a drought period of four weeks (from T0 to T4) (A). IAA (ng g^{-1} FW) *vs. L*-tryptophan (Trp) (µg g^{-1} FW) (B).

3.3.4.- Osmolyte content and recovery

At R7, stressed plants recovered the control Ψ_π values (Fig. 3.1) but did not the solute levels (Fig. 3.10). Regarding soluble carbohydrate content, stressed plants from all ecotypes maintained their values over the controls, particularly O3 that had 7.5-fold higher values than irrigated plants. With regard to free PAs, all stressed plants presented higher levels than well watered plants, showing O4 the highest accumulation. The free AAs content was still over the control levels at R7. Plants from O1 had the highest total AA accumulation, increasing 6-fold the well watered values from 265.4 to 1536.7 µg g^{-1} FW (Fig. 3.10).

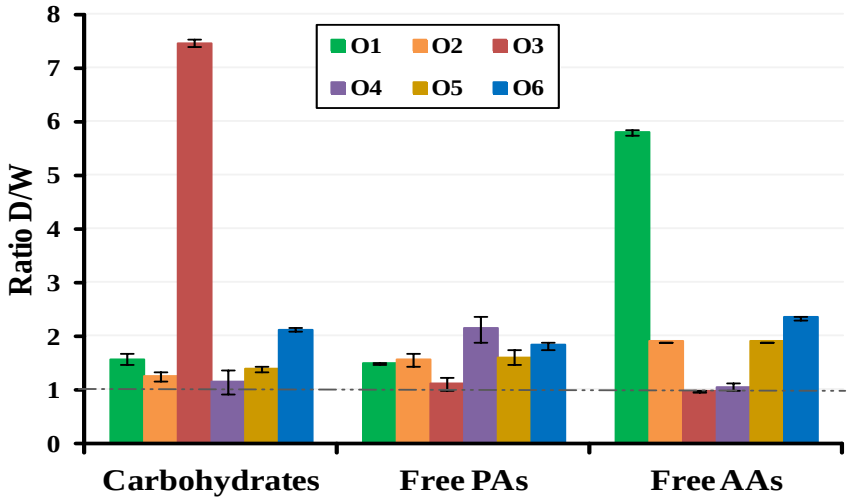


Figure 3.10. Soluble carbohydrates, free polyamine (PAs) and free amino acid (AAs) ratio content (D-water stressed / W-well watered plants) in six *Pinus radiata* ecotypes (O1-O6) subjected to a drought cycle of four weeks (T4) and subsequent rewatering for a week (R7). *M* ± *S.E.* Discontinuous grey line indicates R/W ratio =1.

At R7, only stressed plants from O5 recovered their control *D*-glucose and *D*-fructose values (Fig. 3.11A). The remaining ecotypes maintained their levels over the well watered plants, especially O3 which had 8-fold and 12-fold more levels of *D*-glucose and *D*-fructose respectively than controls. Among AAs, Pro values of all stressed plants were also higher than those levels observed in well watered ones (Fig. 3.11B). O1 showed the highest Pro values (329.3 μg g^{-1} FW), increasing 35-fold their control levels. Regarding GABA accumulation, stressed plants from O2 and O4 recovered their control values. O6 plants showed the highest GABA levels with 110-fold the values of well watered ones. O5 increased its GABA levels with respect to the values observed at T4, doubling the content from 18.6 to 44.1 μg g^{-1} FW (Fig. 3.11 B). Gly and Ser ratios were recovered in all ecotypes, except in O1 and O6 (Fig. 3.11C). Stressed plants from O1 presented the highest Gly and Ser accumulations, being 6 and 20-fold the levels observed in their controls, respectively (Fig. 3.11C).

3.4.- Discussion

Under drought conditions, all *Pinus radiata* D. Don plants showed OA capacity (Fig. 3.2 and 3.3). Previous studies have already observed the capacity of osmotic adjustment (OA) in this species under water stress (Yunusa *et al.*, 2005; Zou *et al.*, 2000) and set the control Ψ_π at similar values (around -1.2 MPa) to those observed in the well watered plants. All stressed plants lowered their Ψ_π values with regard to the well watered ones, but the decreases were not significant until T4, except O6 that presented a significant decrement at T2 (Fig. 3.3). This trait was opposite to the behaviour observed in *Pinus pinaster* Ait. plants under drought that began their osmotic adjustment early in the stress period (Nguyen-Queyrens & Bouchet-Lannat, 2003). Furthermore, all ecotypes presented similar Ψ_π values at T4, except O1 that showed the more negative ones (Ψ_π= -2.6 MPa). The recognized metabolic benefits of solute accumulation may be linked to either active augmentation of them within cell (active OA) or concentration of solute due to a loss of water from plant tissues (passive OA) (Dichio *et al.*, 2009). Joly & Zaerr (1987) suggested that the systems for generating higher intracellular solutes in response to external stress are less developed in woody shrub and trees than in herbaceous plants. However, an active OA has been observed in numerous woody plants (Dichio *et al.*, 2006; Liu *et al.*, 2011) and also in conifers [in *Picea mariana* (Major & Johnson, 2001) and in *Pinus pinaster* (Nguyen-Queyrens & Bouchet-Lannat, 2003)].

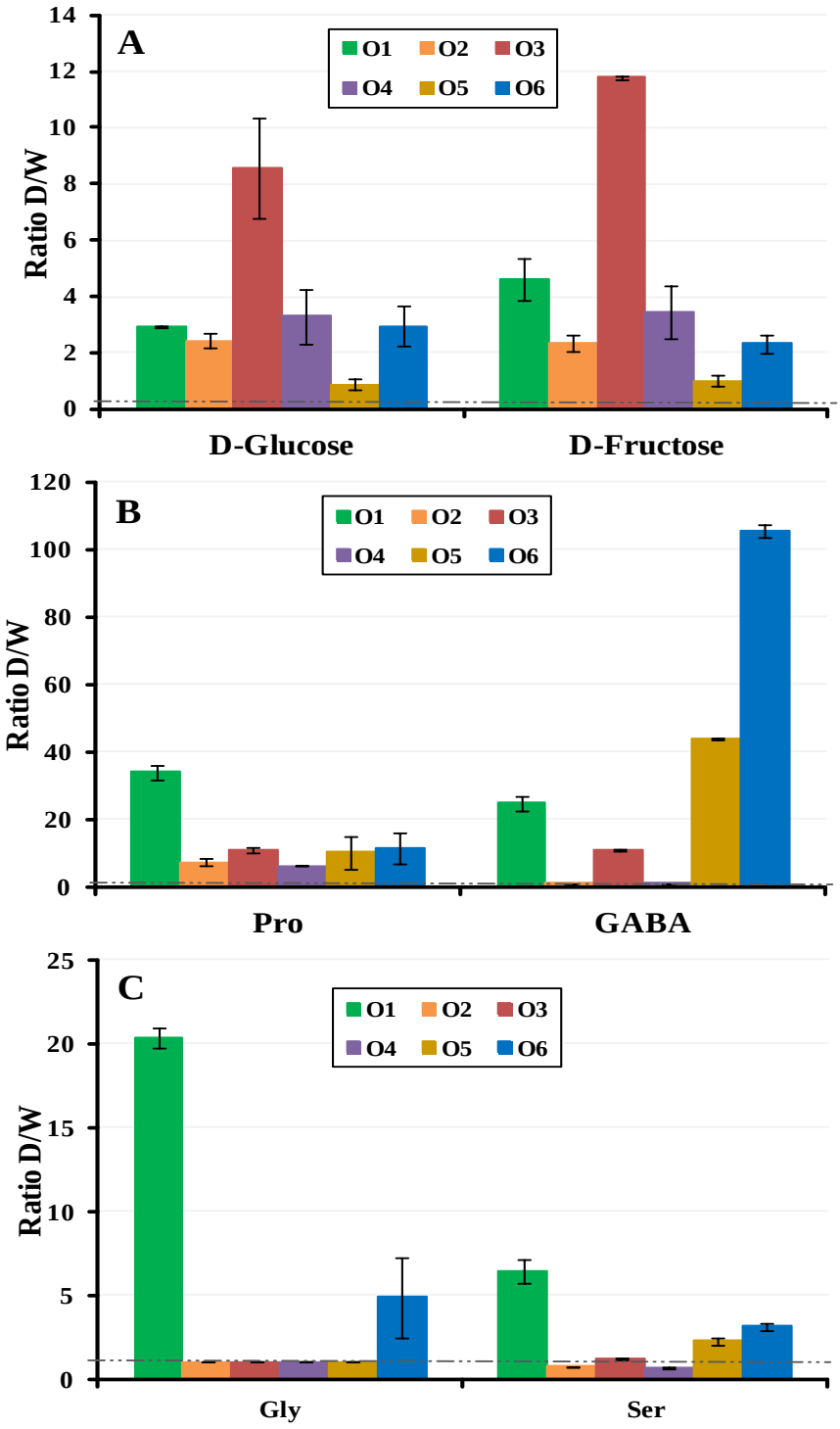


Figure 3.11. *D*-glucose and *D*-fructose (A), *L*-proline (Pro) and *γ*-aminobutyric acid (GABA) (B), and *L*-glycine (Gly) and *L*-serine (Ser) (C) ratio content (D-water stressed / W-well watered plants) in six *Pinus radiata* ecotypes (O1-O6) subjected to a drought cycle of four weeks (T4) and subsequent rewatering for a week (R7). *M* ± *S.E.* Discontinuous grey line indicates R/W ratio =1.

In our study, all plants proportionally increased their OA due to the drought effect, thus, ecotype effect did not show influence (Table 3.1) (Fig. 3.4A). In this respect, statistical differences in the percentage of active and passive OA have been observed among ecotypes (Fig. 3.4B). The most tolerant plants (O4 and O5) based their OA in the *de novo* solute synthesis (active OA, 78%), whereas the other ecotypes showed a percentage balanced between active and passive OA. In addition, opposite to the passive OA, the active OA was directly correlated to RWC (%) and inversely to the ε increases (Fig 3.6A and 3.6B). Stressed plants from O4 and O5 presented the highest RWC (%) among all ecotypes, they did not reach the threshold of *TLP* and had the lowest ε at T4 (Fig. 3.5A). It has been observed that a small ε in the drought environment contributes to the turgor maintenance of the leaf cells under low leaf water content conditions (Saito & Terashima, 2004). Although inelastic cell wall (high ε) precludes turgor maintenance to low water content, some recent studies have observed several potential advantages over elastic cell walls (Patakas *et al.*, 2002; Serrano & Peñuelas, 2005). In gymnosperms, the different ε behaviours have been related to the drought tolerance. Thus, in *Picea glauca* and *Picea mariana* a large increase in cell wall elastic modulus conferred stress tolerance (Major & Johnson, 2001; Marshall & Dumbroft, 1999). On the contrary, in *Thuja plicata* low ε was associated to tolerant plants, whereas in *Pinus halepensis* (Tognetti *et al.*, 1997) and *Tsuga heterophylla* (Kandico *et al.*, 1980) no variations in ε levels were related to stress resistance. In our study, the ecotypes showed different ε values, and all plants increased 1 MPa their values under drought conditions (Fig 3.5A). At this respect, stressed plants with ε levels over 4 MPa dropped their water content to 50% (Fig. 3.6), losing their turgor. In addition, ε increases were positively correlated to *E.L.* (%) and the percentage of plants with external symptoms such as needle epinasty and apical curvature (Fig. 3.7). In accordance to these results, an increase of cell rigidity conferred high drought tolerance in *P. radiata* plants, but ε levels over 4 MPa induced high water loss due to membrane damages.

3.4.1.- Increased osmolyte concentrations during drought

Drought induced soluble carbohydrate accumulation in needles of *P. radiata* plants as the principal osmolytes involved in active OA, especially in O6 (Table 3.2 and 3.3), being remarkable the contribution of *D*-glucose and *D*-fructose. These hexose sugars might result from starch hydrolysis to be used like raw material to the synthesis of wall cell (Clifford *et al.*, 1998; Meinzer *et al.*, 2002) that can be damaged by drought. At this respect, we observed that those

ecotypes with highest ε values (O2, O3 and O6) presented high sugar accumulation, the highest percentages of plants with external symptoms, strongly accumulated jasmonic acid and decreased ACC in the needles. Besides, some studies also observed that the repression of genes involved in the synthesis of Calvin Cycle enzymes under drought conditions has been associated with an increase in sugar concentration in the leaf (Aranjuelo *et al.*, 2011; Stitt *et al.*, 2007), having an influence in the reduction of the A_N previously observed.

Free PAs and free AAs practically did not contribute to OA (Table 3.3) but drought induced strong increases in some of them (Table 3.2 and 3.3). On this account, Hasegawa *et al.* (2000) suggested that the purely osmotic contribution of the metabolites to stress tolerance may not describe their function completely and the pathway leading to a particular osmolyte may be more important than accumulation *per se*. According to this assumption, Pro and GABA highly increased in *P. radiata* plants subjected to drought (Table 3.2 and 3.3), and presented strong negative relationship with the Ψ_{leaf}, Ψ_t and Ψ_π variation, and the RWC (%) (Fig. 3.8). Pro accumulation under stress has been correlated to stress-tolerance in many plant species (Ashraf & Foolad, 2007; Xiong *et al.*, 2011), and it has been associated to the scavenging of free radicals and thereby protecting cellular structures against oxidative damage and denaturation (Pérez-Pérez *et al.*, 2009). At this regard, those ecotypes with the lowest RWC (%) (O1 and O6) accumulated the highest values of Pro. Thus, in our study a Pro accumulation may have been due to the stress-induced response than to drought adaptation.

With regard to GABA, rapid accumulation occurs in response to a variety of stress situations (Shelp *et al.*, 1999; Zushi & Matsuzoe, 2007) as we could appreciate in *P. radiata* at T4 (Table 3.2 and 3.3). In fact, high GABA concentrations induced the stabilization and protection of thylakoids against freezing damage better than Pro (Shelp *et al.*, 1999). Besides, in *P. radiata* plants, a significant correlation was also observed among Pro and GABA and IAA, showing a higher Pearson´s correlation number in the case of GABA (Fig. 3.9). At this respect, the strong increase of Pro and GABA levels suggest that IAA may be a signal pathway leading to the accumulation of these two amino acids under stress conditions. IAA accumulation was also related to great Trp increase in needles of *P. radiata* plants along drought cycle. According to this, it is well-known that Trp is a precursor of IAA synthesis (Strader & Bartel, 2008; Tao *et al.*, 2008) being the principal responsible for this phytohormone accumulation.

An increase of free AAs such as Asn, Arg, Glu, Gln, Gly and Ser was also appreciated in *P. radiata* plants subjected to drought conditions (Table 3.2 and 3.3). This accumulation could be due to the fact that drought induced certain leaf senescence (most of the plants showed external symptoms) that provokes the degradation of compounds such as proteins, lipids and nucleic acids and the release of nitrogen that can be used in biological processes as respiration (Araújo *et al.*, 2011). On this account, the glycine-serine interconversion is an important reaction of primary metabolism in all organisms including plants (Bauwe & Kolukisaoglu, 2003). In addition to the role in metabolism, it is an integral part of the photorespiratory metabolic pathway in which Gly is produced and converted into Ser within the mitochondria (Igarashi *et al.*, 2006; Novistkaya *et al.*, 2002). Besides, the possible increase of photorespiration (high Gly and Ser levels) could be explain the decrease of Φ_{PSII} and *Fv/Fm* observed in stressed plants at the end of the drought period (Chapter 1). Glu and Gln are as well involved in the respiratory processes and new evidences point out that these two amino acid together with Arg are implicated in Pro metabolism (Díaz *et al.*, 2010; Kalamaki *et al.*, 2009). On this account, plants from ecotypes that accumulated more Pro increased their Glu and Gln (Table 3.2). Finally, Asn accumulation have also been reported in other species such as in *Lupinus albus* (Pinheiro *et al.*, 2004), in *Lycopersicum esculentum* (Chaffei *et al.*, 2004) as well as in *Pinus* genus (Gezelius & Näsholm, 1993) under adverse conditions. Asn, being composed of two nitrogen and four carbon atoms, is an "economical" way of storing nitrogen and, under stress conditions, its augmentations could be due to nitrogen accumulation and protein degradation (Martinelli *et al.*, 2007).

Concerning to free PA accumulation, great increases of Put, Spd, and Spm were observed in O5 (Table 3.2 and 3.3). Some studies have demonstrated that Spm plays versatile roles in stress response (Takahashi & Kakehi, 2010). High Spm levels can confer plant stress tolerance controlling ion channel and receptor activities in membranes (Liu *et al.*, 2000; Shabala *et al.*, 2007) and protecting DNA from free radical attack (Ha *et al.*, 1998). In addition, Farooq *et al.* (2009) reported that exogenous application of Spm in *Oryza sativa* L. improved drought resistance, and maintained less negative water potential and membrane properties. According to this, stress plants from O5 not only accumulated Spm, but also maintained less negative water potential and their turgor and practically did not increase their *E.L* (%). Putrescine is also involved in plant tolerance to abiotic stress. In addition, recent studies have related high levels of Put to transpiration control (Alcázar *et al.*, 2010), being other possible cause of the low gas exchange at less negative water potential observed in O5 at T4 (Chapter 1). Finally, the Spd

augmentation could favour the growth under adverse conditions (Imai *et al.*, 2004). Moreover, it has been reported that the high levels of Spd together with Spm induced drought tolerance in angiosperms (Alcázar *et al.*, 2011; Liu *et al.*, 2004). Concerning this assumption, our results pointed out that an increase in the content of these aforesaid PAs could also provide conifers with a higher resistance to drought.

3.4.2.- Osmolyte recovery after rewatering

Stressed plants from all ecotypes recovered their Ψ_π and ε after a week of rewatering (Fig. 3.3 and 3.5A). The reversibility of ε has been previous observed in *Quercus* spp. (Saito & Terashima, 2004) and *Pseudotsuga menziesii* (Joly & Zaerr, 1987) when the water supply was restored. Regarding osmolyte content, soluble carbohydrate accumulation was a little high in water stressed plants than controls, except in O3 that strongly increased their values 7.5-fold than well watered ones (Fig. 3.10). In addition, all ecotypes maintained high levels of free PAs and AAs related to their controls, except O3. The low involvement of these compounds in OA and the total recovery of Ψ_π may point out the high AAs, and especially, the high Pro values such as a carbon and nitrogen reserve that permit plants to activate their growth after stress (Silveira *et al.*, 2003). Regarding PAs, it was remarkable that O5 maintained high levels of Spm, and Put at R7. This trait could be due to a response mechanism that could confer adaptation in future drought cycles (Alcázar *et al.*, 2011). Furthermore, the maintenance of Pro and GABA high levels in all plants subjected to a week with water supply after a drought period, could be a possible conditioning mechanism to subsequent drought cycles that have to be evaluated in future studies. Finally, the great Gly and Ser accumulation observed in stressed plants from O1 (Fig. 3.11C) might be due to their implication in photorespiratory process (Novistkaya *et al.*, 2002), because in previous studies it was observed that this ecotype did not recover the *Fv/Fm* control levels at R7 (Chapter 1).

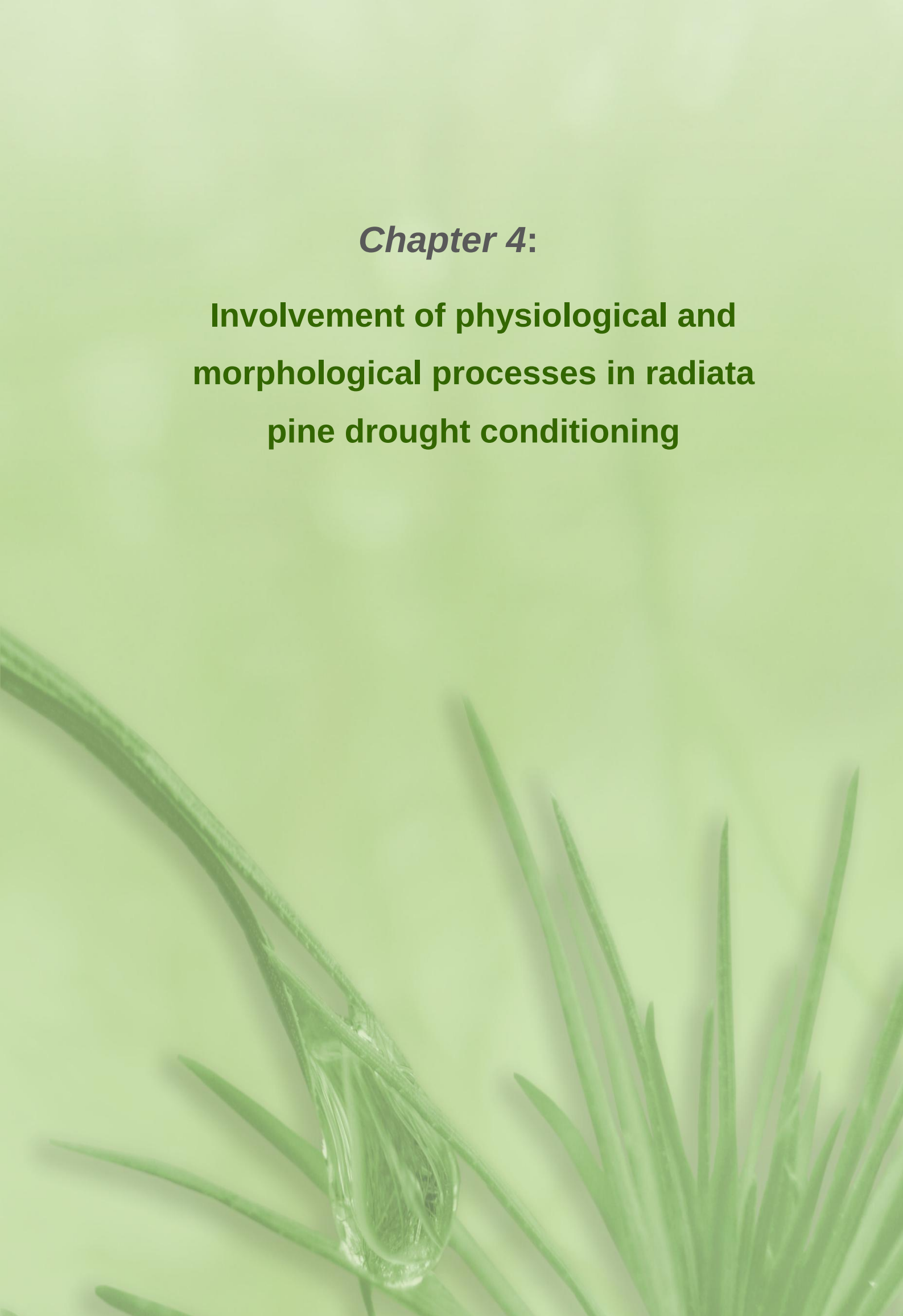
To summarize, drought reversibly induced OA and changes of cell wall elastic modulus (ε) in *Pinus radiata* plants subjected to drought. Tolerance to water stress differed among ecotypes and was related to the maintenance of RWC (%) due to a higher "active" OA and to a low initial ε value as was especially observed in O4 and O5. In this respect, although all ecotypes increased their ε under drought, those plants that reached values over 4 MPa lost their turgor. Among organic solute, soluble carbohydrates showed the highest contribution to OA,

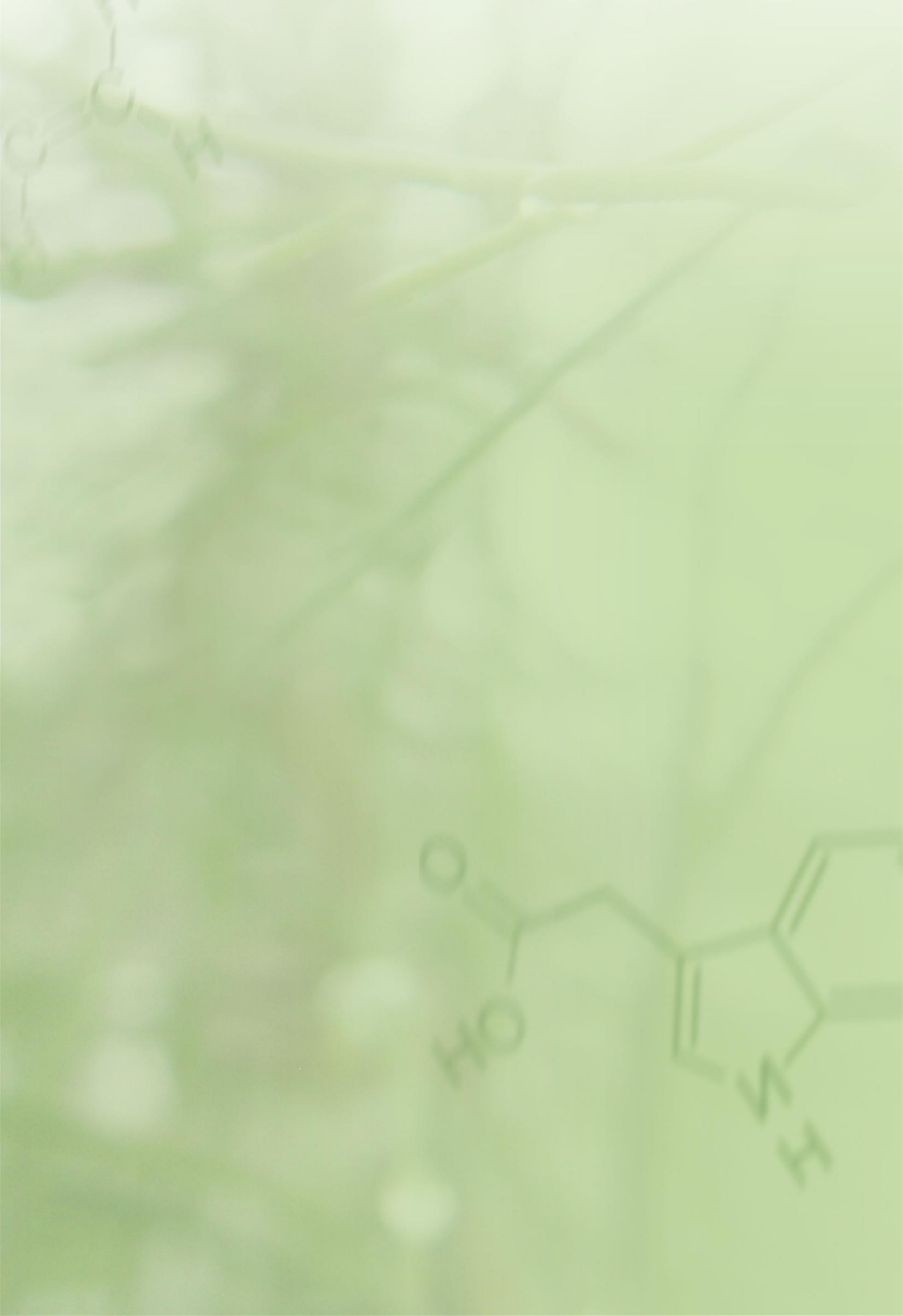
highlighting the increase in *D*-fructose and *D*-glucose in the most stressed ecotypes (O3 and O6).

Free PAs and free AAs slightly contributed to OA, but some of them were significantly accumulated under drought conditions, especially Pro and GABA. The increase of these two amino acids was strongly correlated to a decrease of RWC (%) and water potential. The most tolerant ecotype (O5) not only accumulated low levels of Pro and GABA as drought response but also showed low increases of Glu, Gln, Gly and Ser, AAs involved in respiration and degradation procedures (Aranjuelo *et al.*, 2011; Nunes-Nesi *et al.*, 2008; Pedrol *et al.*, 2000). On the contrary, O5 presented a strong accumulation of PAs related to stress tolerance Put Spd and Spm, and the lowest *D*-fructose and *D*-glucose. Besides, the capacity of recovery observed in these plants (O5) could be due to the recovery of *D*-glucose and *D*-fructose levels, and the maintenance of high Pro, Put and Spm and the increase of GABA content. Finally, the increases of the Put, Spd and Spm contributed to the *Pinus radiata* tolerance under drought conditions.

Chapter 4:

Involvement of physiological and morphological processes in radiata pine drought conditioning





4.1.- Introduction

Plant survival and distribution of plants strongly depend on their adjustment ability to environmental variation (Beikircher & Mayr, 2008). Among environmental fluctuations, drought is perhaps the major factor limiting plant production and survival (Hessini *et al.*, 2009). One procedure for improving forest seedling survival is to condition nursery-grown plants by the exposure to water stress before transplanting to the forest, process traditionally called plant conditioning or hardening (Edwards & Dixon, 1995; Villar-Salvador *et al.*, 2004). The tolerance mechanisms involved in hardening may arise from physiological responses to morphological and structural variations (Henderson & Davies, 1990; Li *et al.*, 2004; Mencuccini, 2003), and it could vary at species level (Augé *et al.*, 1987; Khalil & Grace, 1992; Maury *et al.*, 2000).

Water relations have been widely used to characterize drought resistance (Iannucci *et al.*, 2000; Khalil & Grace, 1992). Leaf water relations, gas exchange, osmotic adjustment and cell wall elastic modulus have been shown as important traits to tolerate periods without water supply (Chen & Jiang, 2010; Fan *et al.*, 2006). These physiological processes also have been involved in the response of *Pinus radiata* to water stress (Chapter 1 and 3). In this respect, it has been reported that drought-hardening could reduce osmotic potential at saturation and at turgor loss point but enhanced cell membrane stability (Villar-Salvador *et al.*, 2004).

Under severe water limitations, leaf anatomy characteristics and their modifications conditioned plant adaptation and survival (Abrams & Kubiske, 1990; Curtis *et al.*, 1996). These modifications comprise a reduction of the leaf size, stomatal density and an increase of epidermis thickness, leaf rolling and mesophyll compactness (Bosabalidis & Kofidis, 2002; Ennajeh *et al.*, 2010). Furthermore, under progressive drought cycles, structural changes could alleviate the direct effects of water stress on stomatal conductance and photosynthesis (Cinnirella *et al.*, 2002). In addition, plants predominantly tend to allocate biomass to roots at the cost of reducing the allocation to leaves (Weih *et al.*, 2011), decreasing photosynthesis dependent processes and plant growth (White *et al.*, 2000).

Other studies have reported that phytohormonal signals activated by adverse environmental conditions also regulated different physiological procedures from stomatal aperture, xylogenesis and senescence to growth (Achard *et al.*, 2006; Ghanem *et al.*, 2008; Granda *et al.*, 2011). Regarding drought-conditioning, there is little information about the

hormone involvement, being ABA the most studied (Tuteja, 2007). ABA has been associated to the regulation of stomatal closure, plant growth and gene expression involved in stress response (Grene *et al.*, 2011; Wu *et al.*, 2006). In our study, IAA was the most representative hormone that determinated *Pinus radiata* plant water status under drought conditions (Chapter 2). According to this result, we hypothesized that this phytohormone could be also implicated in drought-conditioning.

Due to the variety of drought and recovery response previously observed among the different ecotypes during the first drought cycle and rewatering, not only at physiological but also at phytohormonal levels, plants were exposed to a second drought cycle in order to evaluate the ecotype conditioning capacity, and to identify the processes involved in water stress hardening. On this account, the objectives of this work were to study (i) the morphological differences among ecotypes and its changes during water stress, (ii) the water relations, osmotic adjustment, cell wall elastic modulus variation and (iii) phytohormone content during a second drought period to clarify if these modifications differ among ecotypes and in their hardening capacity.

4.2.- Material and Methods

4.2.1.- Plant material and growth conditions

Seed characteristics and growth conditions were performed as described in Chapter 1.

4.2.2.- Experimental design

In order to analyze the drought-conditioning capacity of *P. radiata* plants, the different ecotypes were analyzed at structural and morphological level at the beginning of the experiment and under two consecutive drought cycles (C1 and C2) (Fig 4.1). C1 was from T0 to T4 and C2 started after a week of rewatering (R7) and ended when 50% plants from each ecotype showed external symptoms as needle epinasty and apical curvature (Chapter 2) [S_i; where *i* represented the ecotype (from O1 to O6)]. At this moment, whole plants were collected for biomass determination, and two needles of each plant were conserved to analyze the content of the major phytohormones (ABA, IAA, Z+ZR, ACC, JA and SA). Water balance, gas exchange

parameters, osmotic adjustment, organic solutes (soluble carbohydrates, free AAs and PAs) and cell wall elastic modulus were evaluated along C2.

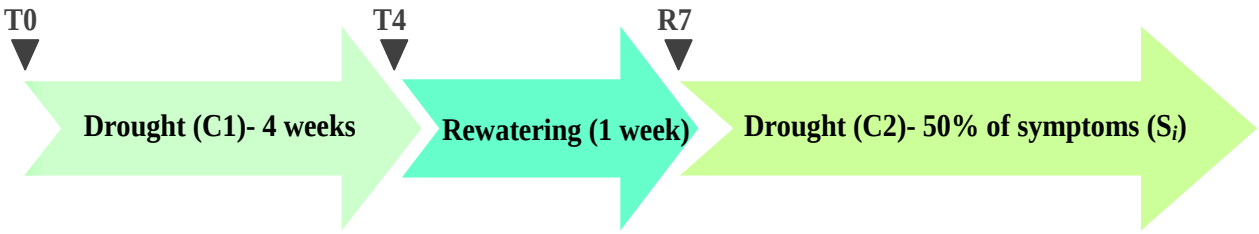


Figure 4.1. Scheme of the experimental design for six *Pinus radiata* ecotypes (O1-O6) exposed to a first drought period of four weeks [C1 (from T0 to T4)] and a second drought period (C2) after a week of rewatering until 50% plants from each ecotype showed external symptoms (S_i). *i* represented the ecotype (from O1 to O6) with external symptoms.

4.2.3.- Structural analysis

Structural analysis was carried out in needles from each ecotype at T0. Tissue was fixed for 24 h with 3% (w/v) paraformaldehyde containing 0,1% (v/v) ®Triton X-100 at 4ºC (Schraut *et al.*, 2004). Samples were washed three time each for 10 min in PBS (phosphate buffered saline: 137 mM NaCl, 2.7 mM KCl, 7.9 mM Na_2HPO_4 and 1,5 mM KH_2PO_4, at pH = 7.3) to remove the fixative. Samples were stored in PBS containing 0.1% (w/v) paraformaldehyde at 4ºC.

Samples were introduced in cryostat medium (Tissue-Tek, Killik®) and were frozen at -23ºC. Transversal needle sections of 50 µm were cut with a sliding cryotome CM1510S (©2002 Leica Microsystems, Wetzlar, Germany). Sections were immersed in ascendant and descendant 25%, 50%, 75% and 100% ethanol solution, washed for 30 min in PBS containing 0.1% (v/v) Tween® 20, and finally, for 10 min in MilliQ water. Sections were assembled on the slides with Mowiol (©Sigma-Aldrich Co.). Samples were viewed with a confocal laser scanning microscope TCS-SP2-AOBS (©2002 Leica Microsystems, Wetzlar, Germany). Structural analysis was carried out by LCS Lite-Leica confocal software V. 2.61. Build 1538 (©Leica Microsystem Heidelberg GmbH).

4.2.4.- Biometric and growth parameters

Each plant height (cm) and root collar diameter (mm) were measured at T0, T4 and S_i. Relative height growth ratio (RhGR) (cm day^{-1}) and relative diameter growth ratio (RdGR) (mm day^{-1}) were estimated as described by Sánchez- Gómez *et al.* (2010). The RhGR and RdGR were calculated at T0, T4 and S_i according to the following mathematical equation:

$$RGR = (\ln G_i - \ln G_j)/(t_i - t_j) \quad \text{(Eqn. 4.1)}$$

where,

G represents the height and root collar diameter in time i and j (t_i and t_j respectively, with $i>j$).

Biomass production for each ecotype was determinated at S_i. Dry mass (g) of root (Root DW), aerial part (aerial DW) and total biomass (Total DW) was measured. The relation between root DW and aerial DW (R/A) was calculated.

4.2.5.- Water status determination and gas exchange parameters

Water relations, including leaf hydraulic conductance and gas exchange parameters, were determinated in all plants as explained in Chapter 1.

4.2.6.- Osmotic adjustment and cell wall elastic modulus

Osmotic adjustment (OA), osmotic contribution of osmolyte and cell wall elastic modulus (ε) were determinated as explained in Chapter 3.

4.2.7.- Soluble osmolyte quantification

Soluble carbohydrates, free AAs and free PAs were quantified as described in Chapter 3.

4.2.8.- Hormone quantification

ABA, IAA, Z+ZR, ACC, JA and SA content was quantified as described in Chapter 2.

4.2.9.- Inmunolocalization of ABA and IAA

4.2.9.1.- Tissue preparation

Tissue preparation procedure was performed according to the method described by Schraut *et al.* (2004) with some modifications. To immobilize ABA and IAA by covalent binding to proteins, all tissues were fixed for 24 h with 3% (w/v) paraformaldehyde in 4% (w/v) 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (©Sigma-Aldrich Co.) containing 0,1% (v/v) Triton® X-100 at 4ºC. Samples were washed three consecutive times for 10 min each in PBS to remove the fix solution, and were stored in PBS containing 0.1% (w/v) paraformaldehyde at 4ºC.

Samples were introduced in cryostat medium (Tissue-Tek, Killik®) and were frozen at -23ºC. Sections of 50 μm were cut with a sliding cryotome CM1510S (©2002 Leica Microsystems, Wetzlar, Germany). Sections were collected on slides and conserved at 4ºC until the immunocytochemistry was done.

4.2.9.2.- Inmunocytochemistry

Sections were immersed in ascendant and descendent 25%, 50%, 75% and 100% ethanol series, washed for 30 min in PBS containing 0.1% (v/v) Tween® 20, and finally, for 5 min in PBS. Before incubating overnight with the primary antibody ABA or IAA (polyclonal -BSA conjugates, ©Agrisera AB, Sweden), samples were pre-treated with 5% (w/v) BSA (Bovine albumin serum) in PBS for 30 min to reduce unspecific bindings. After washing twice with 0.1% (v/v) Tween® 20 in PBS for 10 min, sections were incubated with Alexa 488 (©Molecular Probes, Göttingen, Germany) as a secondary antibody for 1 h in darkness. Samples were washed twice for 10 min with 0.1% (v/v) Tween® 20 in PBS and, immediately after, incubated with toluidine blue to quench the autofluorescence of the lignified cell wall for at least 5 h. Sections were washed in MilliQ® water, and assembled on the slides with Mowiol (©Sigma-Aldrich Co.). Covered slides were sealed with nail varnish. Sections were viewed with a confocal laser scanning microscope TCS-SP2-AOBS (©2002 Leica Microsystems, Wetzlar, Germany).

4.2.10.- Statistical analysis

Multivariate analysis of variance (MANOVA) and one, two and three-way univariate analysis of variance (ANOVA) were carried out by *proc glm* using the S.A.S® software. Multiple comparisons were calculated using the post hoc Tukey´s HSD test for balance data and Tukey-Kramer for unbalance data to determinate the different signification. To analyze possible correlations *proc reg* was used and analysis of covariance (ANCOVA) was carried out by *glm proc* in the S.A.S® software.

4.3.- Results

After seven weeks under drought conditions, O2 and O6 showed 50% plants with external symptoms ($S_{O2,\ O6}$). O3 presented symptoms after nine weeks (S_{O3}), whereas O1, O4 and O5 did not have 50% plants with external symptoms until eleven weeks under drought conditions ($S_{O1,\ O4,\ O5}$) (Fig. 4.2).

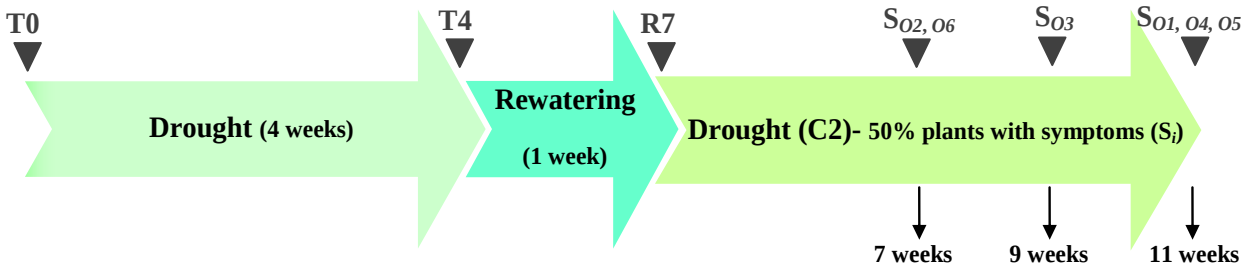


Figure 4.2. Scheme of the experimental design for six *Pinus radiata* ecotypes (O1-O6) grown under a first drought period of four weeks (C1), rewatered for a weeks (R7), and a second drought period (C2) until 50% plants from each ecotype showed external symptoms (S_i). *i* represented the ecotype (from O1 to O6) with external symptoms.

4.3.1.- Structural analysis

When the structural analysis was performed at T0, needle differences were observed according to ANOVA (Table 4.1). The highest divergences were noticed in the xylem cells and substomatal chamber size (Fig. 4.3). O1 presented the highest xylem vessel area, but only showed statistical differences with O6 (Fig. 4.3A). O6 had the biggest substomatal chamber (Fig. 4.3B). The smallest substomatal cavity was appreciated in O4 with 542.5 μm^2, an area 3-fold lower than observed in O6 (1658.6 μm^2). Only O5 plants did not show statistically significant differences with O4 with respect to the substomatal chamber size (Fig. 4.3B).

Table 4.1. Analysis of variance (ANOVA) of xylem cell, substomatal chamber, plant height, root collar diameter, relative growth ratio of height (RhGR) and root collar diameter (RdGR), Aerial dry weight (DW), Root DW, Total DW and the ratio between root and aerial DW (R/A) in six *Pinus radiata* ecotypes (O) under irrigation and no irrigation conditions (T) after a drought cycle of four weeks, and a second drought cycle after rewatering for a week (Ti).

VARIABLE	FACTOR	DF	Sum Sq	Mean Sq	Fvalue	Pr(>F)
Xylem cell (μm^2)	**O**	**5**	**2778.93**	**555.79**	**3.09**	*****
Substomatal chamber (μm^2)	**O**	**5**	**2568429.7**	**5136.94**	**8.88**	*******
Height	**O**	**5**	**7361.47**	**1526.30**	**18.92**	*******
(cm)	Ti	2	12209.44	6104.72	75.68	***
	T	1	345.45	345.45	4.28	*
	O*Ti	10	333.52	33.35	0.41	ns
	O*T	5	743.52	148.78	1.84	ns
	Ti*T	**2**	**1210.72**	**605.36**	**7.50**	*******
	O*Ti*T	10	121.48	12.15	0.15	ns
Diameter	**O**	**5**	**46.06**	**9.21**	**13.76**	*******
(mm)	Ti	2	198.05	99.02	147.93	***
	T	1	44.01	44.01	65.75	***
	O*Ti	10	9.81	0.98	1.46	ns
	O*T	5	3.53	0.71	1.05	ns
	Ti*T	**2**	**27.75**	**13.88**	**20.73**	*******
	O*Ti*T	10	6.43	0.64	0.96	ns
RhGR	O	5	0.00014	0.00003	8.52	***
($cm\ day^{-1}$)	Ti	1	0.00042	0.00042	131.55	***
	T	**1**	**0.00010**	**0.00010**	**34.56**	*******
	O*Ti	**5**	**0.00005**	**0.00001**	**2.99**	*****
	O*T	5	0.00001	0.00000	0.62	ns
	Ti*T	1	0.00000	0.00000	1.01	ns
	O*Ti*T	5	0.00002	0.00000	1.39	ns
RdGR	**O**	5	0.00012	0.00002	4.44	**
($mm\ day^{-1}$)	Ti	1	0.00094	0.00094	172.62	***
	T	1	0.00016	0.00016	29.98	***
	O*Ti	5	0.00014	0.00003	5.30	***
	O*T	5	0.00010	0.00002	3.47	**
	Ti*T	1	0.00000	0.00000	0.17	ns
	O*Ti*T	**5**	**0.00008**	**0.00002**	**2.74**	*****
Aerial DW	**O**	**5**	**1716.05**	**343.21**	**7.56**	*******
(g)	**T**	**1**	**1945.97**	**1945.97**	**42.84**	*******
	O*T	5	246.38	49.28	1.08	ns
Root DW	O	5	1395.69	279.14	8.20	***
(g)	T	1	2398.86	2398.86	70.48	***
	O*T	**5**	**645.86**	**129.17**	**3.80**	******
Total DW	**O**	**5**	**7609.25**	**1521.85**	**6.04**	*******
(g)	**T**	**1**	**11174.26**	**11174.26**	**44.25**	*******
	O*T	5	1525.66	305.13	1.21	ns
R/A	O	5	0.106	0.211	0.36	0.871
	T	**1**	**0.592**	**0.592**	**10.16**	******
	O*T	5	0.379	0.076	1.30	0.279

Note: * < 0.05; ** <0.01 and *** <0.001; ns-non-significant.

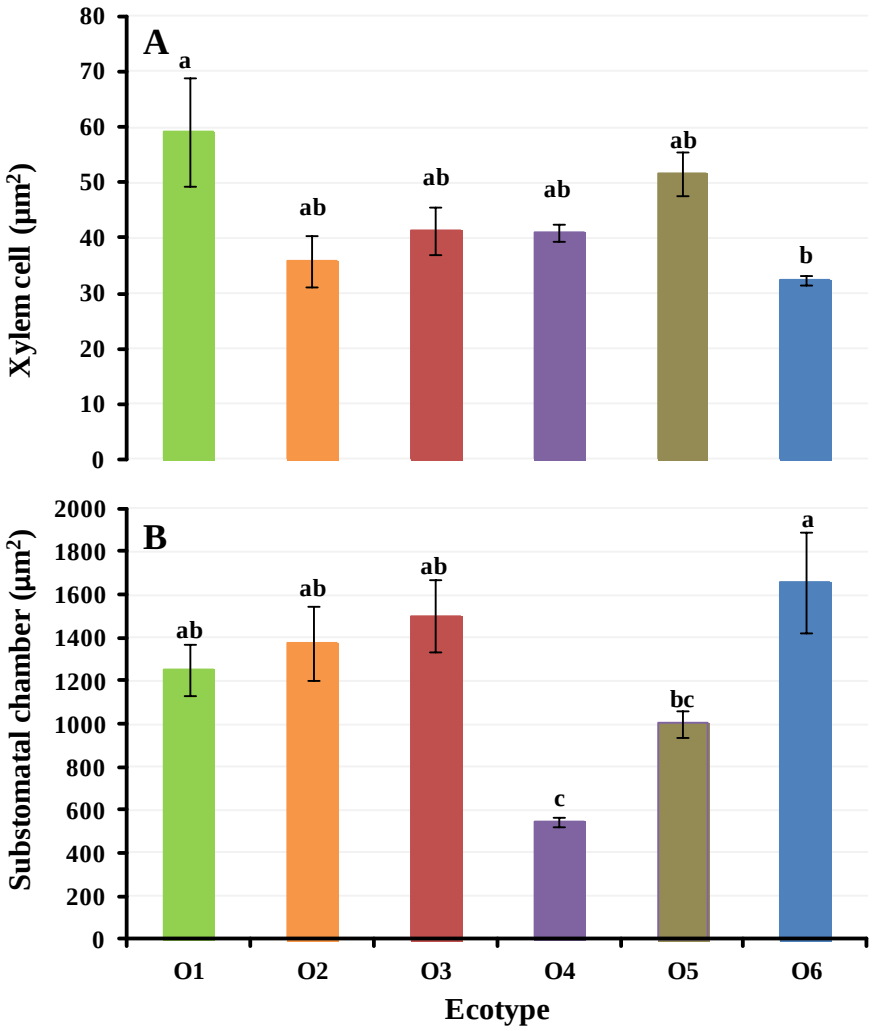


Figure 4.3. Area (µm^2) of xylem cells (A) and substomatal chamber (B) in six *Pinus radiata* ecotypes (O1-O6). *M* ± *S.E.* Different letters mean significant differences according to Tukey´s HSD test after ANOVA.

4.3.2.- Biometric and morphologic parameters

To evaluate morphological parameters, the absolute and relative growth was statistically analyzed in plants of each ecotype between the two drought periods (C1 and C2) (Table 4.1). According to the ANOVA, the changes of height and diameter were due to the interaction effect between T and Ti (p<0.001). All controls equally increased their height and root collar diameter since T0, and presented a significant growth in C1 and C2 (Fig. 4.4). Water stressed plants from all ecotypes showed a significant growth in height during C1, but did not increase it through C2 (Fig. 4.4B). Regarding root collar diameter, all stressed plants significantly decreased their growth during the two drought cycles compared to the well watered ones (4.4B).

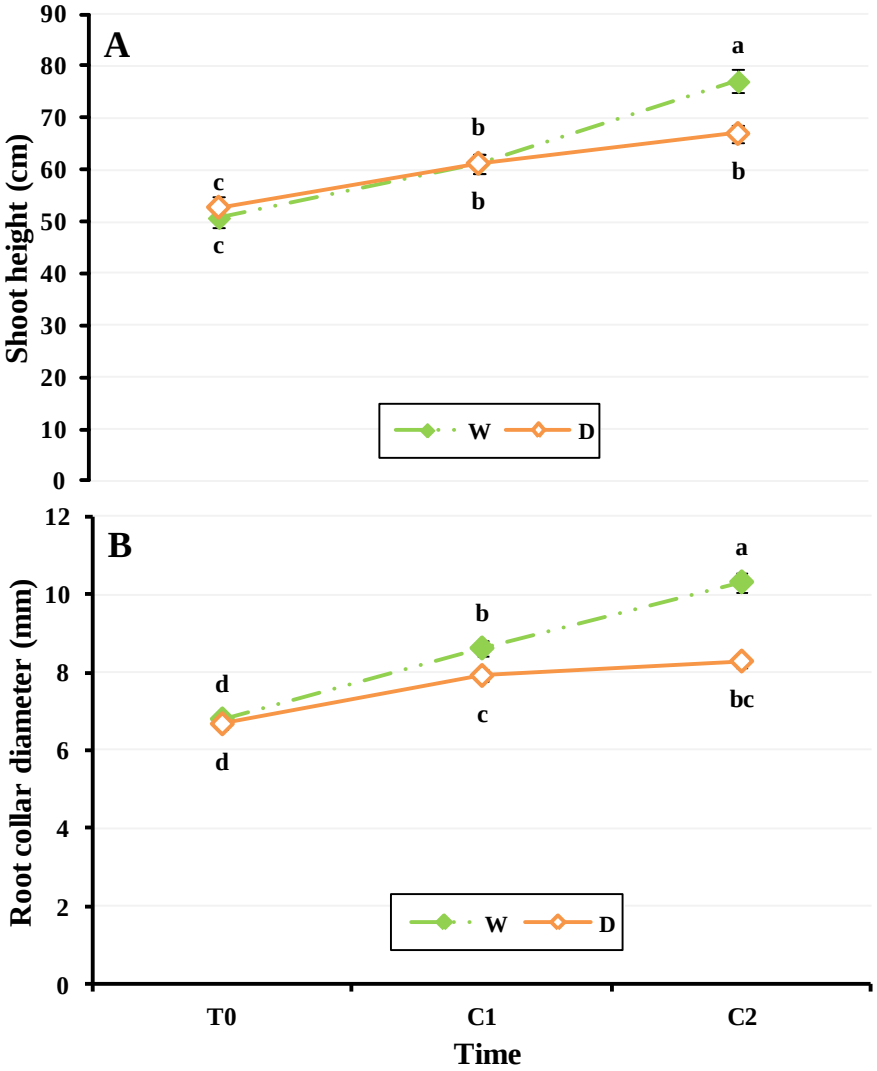


Figure 4.4. Height (cm) (A) and collar root diameter (mm) (B) of *Pinus radiata* plants since the beginning of the study (T0), along a drought cycle of four weeks (C1) and a second drought cycle (C2) after a week of rewatering until S_i. W and D mean irrigated and non-irrigated plants, respectively. *M* ± *S.E.* Different letters mean significant differences according to Tukey´s HSD test after ANOVA.

The increase in RhGR was due to the interaction effect of O and Ti, according to ANOVA (Table 4.1). All stressed plants significantly decreased their RhGR with respect to their controls. The highest RhGR was observed in O4 during C1, being statistically different with regard to the remaining ecotypes (Fig 4.5A). Along C2, all ecotypes did not show statistical differences among them, but significantly decreased their RhGR levels compared to the relative growth observed through C1 (Fig 4.5A).

The RdGR also varied along the experiment (Fig. 4.5B). In this case, the variations were due to the interaction effect among O, Ti and T according to ANOVA (Table 4.1). Only stressed plants from O1 decreased their RdGR with respect to each control during C1, but not at

the end of C2 (Fig. 4.5B). The remaining ecotypes did not show significant differences between irrigated and non-irrigated plants during the two drought cycles.

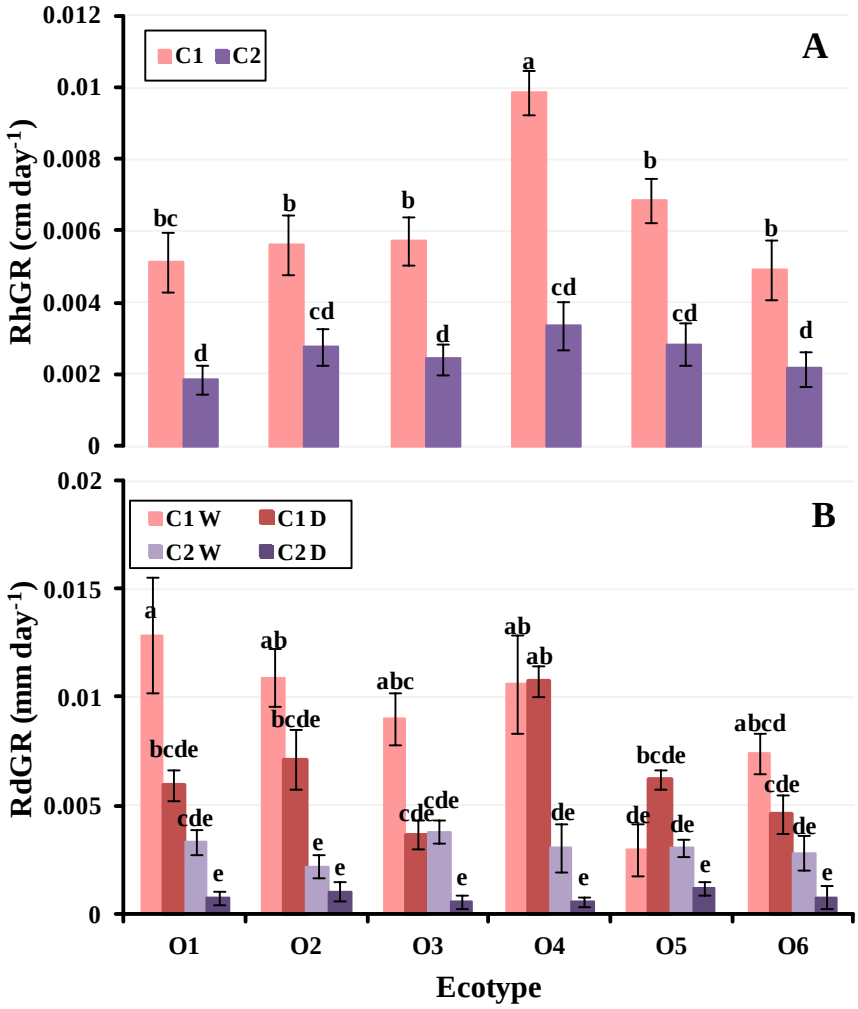


Figure 4.5. Relative growth ratio of height (RhGR, cm day^{-1}) (A) and root collar diameter (RdGR, mm day^{-1}) (B) in six *Pinus radiata* ecotypes (O1-O6) along a first drought cycle of four weeks (C1) and a second drought cycle (C2) after a week of rewatering until S_i. W and D mean irrigated and no irrigated plants, respectively. *M* ± *S.E.* Different letters mean significant differences according to Tukey´s HSD test after ANOVA.

Regarding Aerial DW and Total DW (g), drought induced significant changes, and the variations were due to the individual effect of the O (p<0.001) and T (p<0.001) according to ANOVA (Table 4.1). All stressed plants reduced their biomass production with respect to the well watered plants. O1 showed the highest Total DW, practically duplicating the O1 biomass production (Fig 4.6A). O5 and O6 did not present statistical differences with O1 plants (Fig. 4.6A). The same significant groups were observed in the Aerial DW analysis (Data not shown).

Drought produced variations in Root DW by the interaction between O and T according to ANOVA (Table 4.1). Stressed plants from all ecotypes significantly decreased their Root DW production with regard to each control, except in the case of O2 and O4 (Fig 4.6B). Water stress treatment also induced changes in the R/A ratio due to the treatment ($p<0.01$) (Table 4.1), decreasing the relation in stressed plants compared to the watered plants values [from 0.8 to 0.6 (Data not shown)].

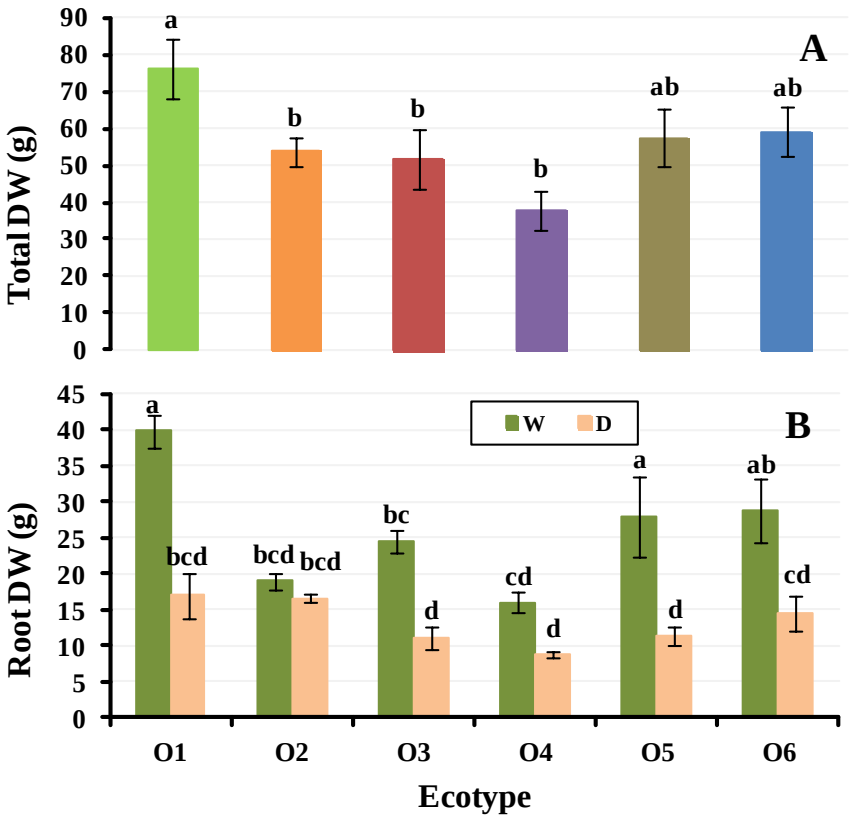


Figure 4.6. Dry weight (g) of total plant (total DW) (A) and root part (root DW) (B) in six *Pinus radiata* ecotypes (O1-O6) at S_i. W and D mean irrigated and no irrigated plants, respectively. *M* ± *S.E.* Different letters mean significant differences according to Tukey´s HSD test after ANOVA.

4.3.3.- Water balance, osmotic adjustment and cell wall elastic modulus

During C2, stressed plants showed a linear loss of needle RWC (%) with respect to the decreases of Ψ_{leaf} values ($p< 0.001$) (Fig. 4.7A). The linear correlation between these two parameters was not statistically different to those observed along the first drought cycle (C1) (Table 4.2). According to ANCOVA, the RWC changes were only due to the Ψ_{leaf} variations ($p< 0.001$) and were not influenced by O (non-significant).

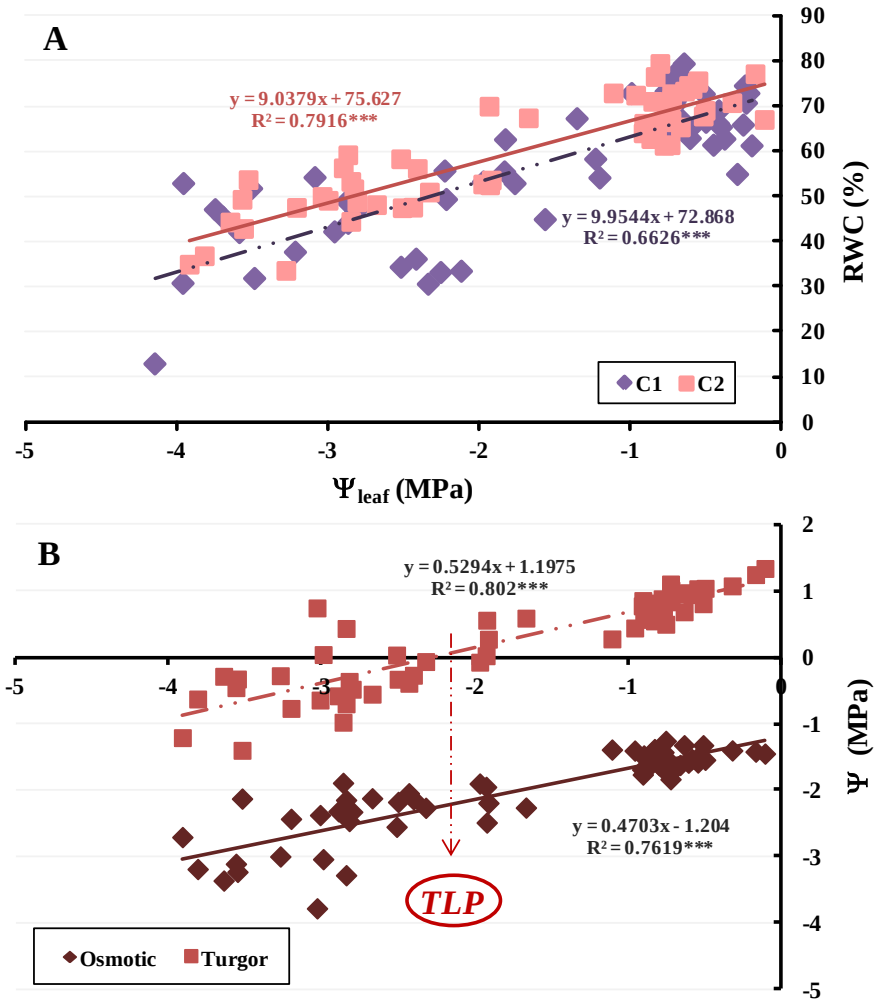


Figure 4.7. Correlation between needle RWC (%) *vs.* leaf water potential (Ψ_{leaf}, MPa) in plants from six *Pinus radiata* ecotypes (O1-O6) exposed to two consecutive drought cycles (C1 and C2) (A). Osmotic potential (Osmotic) and turgor pressure (Turgor) *vs.* Ψ_{leaf} (MPa) (B) during the second drought cycle until S_i. Discontinuous red narrows indicated ***TLP***- Turgor loss point. R^2- Pearson´s correlation number with the significance according to ANCOVA. * < 0.05; ** <0.01 and *** <0.001.

In the same way as observed in RWC (%), Ψ_{leaf} showed significant positive correlation with Ψ_t and Ψ_π (p<0.001) and the O effect was again non-significant according to ANCOVA (Fig. 4.7B). When plants dropped their Ψ_{leaf} and Ψ_π to around -2.2 MPa reached their turgor loss point (*TLP*) (Fig 4.7B).

Table 4.2. Analysis of variance (ANOVA) of the equations obtained from the correlation between RWC (%) (*Y*) *vs.* midday water potential *(X)* in plants from six *Pinus radiata* ecotypes exposed to two different drought cycles (C1 and C2) according to Kruskal-Wallis.

Equation	Variable	Pr(>F)
Y= aX+b	Slope (*a*)	0.15
	Intercept (*b*)	0.70

At $S_{O2,O6}$ (seven weeks), stressed plants from all ecotypes presented valueless gas exchange and K_{leaf} (Data not shown). All ecotypes showed statistical differences in their Ψ_{leaf} and Ψ_t due to the reciprocal effect between O and T according to ANOVA (Table 4.3). Stressed plants decreased significantly their Ψ_{leaf} compared to each control (Table 4.4). O4 showed the less negative Ψ_{leaf}, reaching values of -1.91 MPa against -3.3 MPa of O3. The Ψ_{leaf} values of O1 and O5 were not statistical different regarding O4. Only these three ecotypes did not show differences in RWC (%) changes with respect to each control. O4 plants maintained their turgor pressure under drought conditions, and did not present statistical differences with O5 (Table 4.4.).

Table 4.3. Analysis of variance (ANOVA) of Ψ_{leaf}, Ψ_t, RWC, Ψ_π, OA and ε in six *Pinus radiata* ecotypes (O) under irrigation and no irrigation conditions (T) after seven weeks under a second drought cycle ($S_{O2,O6}$). Type means the mechanism of osmotic adjustment as active or passive OA.

VARIABLE	FACTORS	DF	Sum Sq	Mean Sq	Fvalue	Pr(>F)
Ψ_{leaf}	O	5	2.559	0.512	2.62	*
(MPa)	T	1	61.816	61.516	316.57	***
	O*T	**5**	**4.128**	**0.826**	**4.23**	****
Ψ_t	O	5	1.457	0.291	1.99	ns
(MPa)	T	1	16.791	16.791	114.78	***
	O*T	**5**	**2.207**	**0.441**	**3.02**	*
RWC	O	5	728.94	145.79	3.82	**
(%)	T	1	4708.11	4708.11	109.13	***
	O*T	**5**	**635.93**	**127.19**	**3.34**	*****
Ψ_π	O	5	1.123	0.224	1.73	ns
(MPa)	**T**	**1**	**14.143**	**14.143**	**109.13**	*****
	O*T	5	1.017	0.203	1.57	ns
OA	O	5	0.00	0.00	0.0	ns
(%)	Type	1	17722.39	172239	281.09	***
	O*Type	**5**	**1192.35**	**238.47**	**3.78**	*****
ε (MPa)	**O**	**5**	**3.34**	**0.668**	**2.95**	*****

Note: * < 0.05; ** <0.01 and *** <0.001; ns-non-significant.

The Ψ_π changes induced by drought were only produced by T effect according to ANOVA (p< 0.001) (Table 4.3). Stressed plants from each ecotype presented osmotic adjustment (OA). They significantly decreased their Ψ_π -1 MPa with respect to their controls after seven weeks of drought (Fig.4.8A). When the type of OA was evaluated all stressed plants showed the same OA mechanism without significative differences, being active adjustment the main one, with values in the range of 70% (Fig. 4.8B).

Table 4.4. Leaf water potential (Ψ_{leaf}), turgor pressure (Ψ_t) (MPa) and RWC (%) in six *Pinus radiata* ecotypes (O1-O6) after seven weeks under a second drought cycle ($S_{O2,O6}$). W and D mean irrigated and non-irrigated plants, respectively. *M* ± *S.E.* Different letters mean significant differences according to Tukey´s HSD test after ANOVA.

Ecotype	Treatment	Ψ_{leaf} (MPa)	Ψ_t (MPa)	RWC (%)
O1	**W**	-0.80 ± 0.04 a	0.81 ± 0.02 a	62.50 ± 2.44 abc
	D	-2.61 ± 0.28 bc	-0.41 ± 0.28 c	51.84 ± 1.30 cd
O2	**W**	-0.63 ± 0.12 a	0.83 ± 0.12 a	72.53 ± 1.32 ab
	D	-3.15 ± 0.26 c	-0.31 ± 0.28 c	44.91 ± 2.68 d
O3	**W**	-0.57 ± 0.13 a	1.01 ± 0.09 a	67.90 ± 1.32 ab
	D	-3.33 ± 0.17 c	-0.47 ± 0.10 c	42.32 ± 3.33 d
O4	**W**	-0.72 ± 0.11 a	0.79 ± 0.14 a	71.94 ± 2.35 a
	D	-1.91 ± 0.37 b	0.43 ± 0.21 ab	59.15 ± 4.67 abc
O5	**W**	-0.79 ± 0.05 a	0.59 ± 0.03 a	69.54 ± 2.23 ab
	D	-2.52 ± 0.23 bc	-0.25 ± 0.25 bc	56.61 ± 4.11 bcd
O6	**W**	-0.65 ± 0.11 a	0.86 ± 0.12 a	68.69 ± 1.16 ab
	D	-2.95 ± 0.24 c	-0.52 ± 0.16 c	49.77 ± 2.23 cd

At $S_{O2,O6}$, the main osmolytes which contributed to OA were soluble carbohydrates (Table 4.5). In general, drought increased the soluble sugar levels in all ecotypes except in O2 and O6 plants regarding the controls. The high soluble sugar accumulation was observed in stressed plants from O1 with contents of 23.98 mg g^{-1} FW and an OA contribution of 89.9 %. They strongly increased the three carbohydrates: sucrose, *D*-fructose and *D*-glucose content, with 7.33, 6.65 and 10 mg g^{-1} FW, respectively (Table 4.5). Stressed plants from O1, O3 and O5 also presented higher sugar levels at C2 than observed at C1 (Table 4.5).

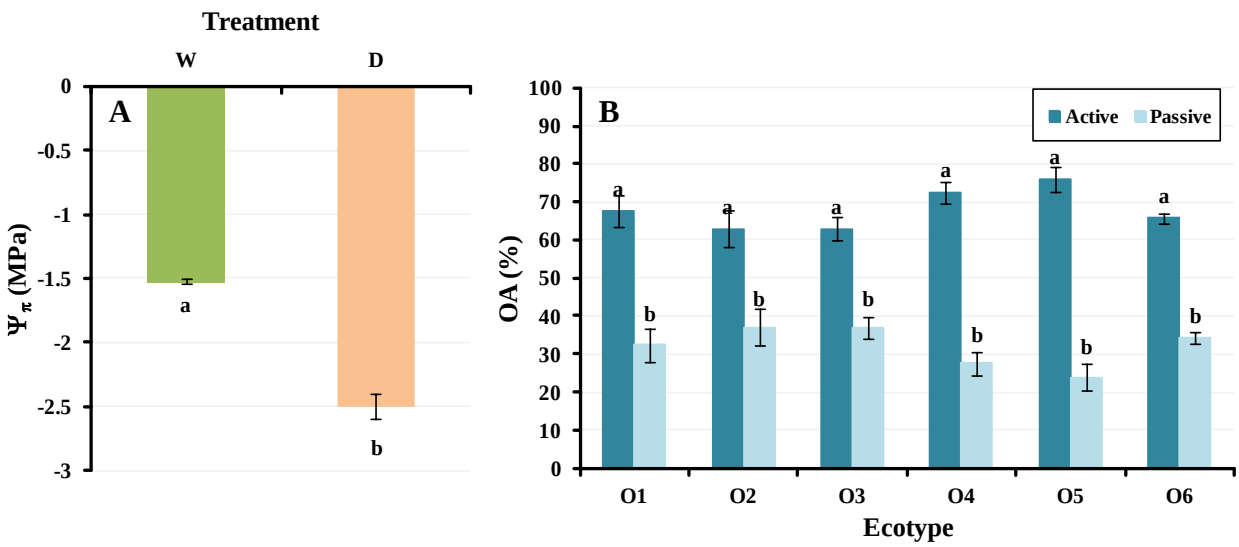


Figure 4.8. Osmotic potential (MPa) in six *Pinus radiata* ecotypes subjected to irrigation (W) and no irrigation conditions (W) for a second drought cycle of seven weeks ($S_{O2,O6}$) (A). Active and passive contribution to osmotic adjustment (OA, %) (B) in each ecotype. *M* ± *S.E.* Different letters mean significant differences according to Tukey´s HSD test after ANOVA.

Table 4.5. Osmolyte content and ratio content (D- water stressed /W-well watered plants) , relation (C1/C2) and each contribution to Ψ_{π}^{100} (%) in six *Pinus radiata* ecotypes (O1-O6) subjected to a drought conditions for four weeks (C1) and a second drought cycle (C2) for seven weeks after a week of rewatering.

Osmolyte	Ecotype																							
	O1				O2				O3				O4				O5				O6			
	Content	D/W	C2/C1	%	Content	D/W	C2/C1	%	Content	D/W	C2/C1	%	Content	D/W	C2/C1	%	Content	D/W	C2/C1	%	Content	D/W	C2/C1	%
Soluble carbohydrates (mg g^{-1} FW	**23.98**	**4.34**	**4.52**	**89.83**	**4.13**	**1.08**	**0.64**	**12.32**	**7.83**	**1.81**	**1.58**	**20.62**	**9.54**	**1.20**	**1.02**	**31.89**	**5.02**	**1.40**	**1.44**	**20.71**	**4.03**	**0.98**	**0.91**	**12.00**
Sucrose	7.33	4.10	2.57	27.47	1.43	0.58	0.51	4.26	2.30	4.38	0.65	6.04	1.33	9.96	0.22	4.44	1.92	1.27	1.51	7.92	1.13	0.92	1.35	3.37
***D*-fructose**	6.65	3.74	5.62	24.91	1.36	2.34	0.50	4.04	2.69	1.29	3.76	7.09	4.36	1.03	2.20	14.64	2.03	2.02	1.51	8.38	1.36	0.85	0.75	4.07
***D*-glucose**	10.00	4.34	7.93	37.45	1.35	1.07	1.35	4.02	2.85	1.81	5.05	7.50	3.83	1.02	2.48	12.81	1.07	1.40	1.26	4.41	1.53	0.88	0.87	4.56
Free AAs (µg g^{-1} FW)	**1600.30**	**6.09**	**1.11**	**5.99**	**2074.84**	**4.65**	**1.88**	**6.19**	**1277.70**	**3.10**	**1.34**	**3.36**	**1032.45**	**1.58**	**2.81**	**4.45**	**1802.49**	**5.80**	**2.74**	**7.44**	**2015.18**	**2.92**	**1.25**	**6.01**
Arg	36.16	36.19	4.20	0.14	60.79	60.79	3.48	0.18	28.54	28.54	2.22	0.08	32.38	2.67	32.38	0.11	51.33	17.79	51.33	0.21	70.97	70.97	1.97	0.21
Asn	233.70	233.70	2.75	0.86	380.51	5.03	3.07	1.13	153.94	1.47	2.01	0.40	522.59	1.10	10.30	1.75	304.41	156.46	3.89	1.26	275.58	0.97	2.24	0.82
Glu	902.90	5.19	6.53	3.38	977.20	3.90	4.47	2.91	636.09	2.54	4.45	1.67	803.50	1.58	8.40	2.69	931.30	4.54	12.37	3.84	960.11	3.50	4.83	2.86
Gln+His	67.84	10.44	0.33	0.25	53.91	4.46	0.22	0.16	31.52	1.65	0.21	0.08	60.66	1.17	0.44	0.20	64.17	1.74	0.20	0.26	60.84	3.20	0.13	0.18
GABA	47.82	47.82	0.31	0.18	161.46	161.46	1.23	0.48	130.87	130.87	1.84	0.34	65.39	3.50	10.78	0.22	122.32	122.32	6.58	0.50	116.08	116.08	0.88	0.35
Pro	56.14	56.14	0.10	0.21	157.42	16.68	0.90	0.47	131.13	32.64	0.44	0.34	214.51	26.43	1.05	0.72	122.87	122.87	3.58	0.51	326.53	20.41	0.74	0.97
Free PAs (µg g^{-1} FW)	**99.75**	**1.02**	**1.51**	**0.37**	**144.94**	**1.25**	**3.34**	**0.43**	**62.61**	**0.91**	**1.64**	**0.16**	**68.06**	**1.13**	**1.18**	**0.23**	**55.96**	**0.80**	**0.25**	**0.20**	**62.21**	**1.28**	**0.31**	**0.19**
Put	1.42	0.31	0.14	0.05	7.08	1.32	2.11	0.02	7.66	1.75	1.08	0.02	7.86	2.48	1.24	0.03	9.25	1.44	0.63	0.04	7.61	2.13	0.54	0.02
Spd	<0.01	1.00	1.00	0.00	<0.01	1.00	1.00	0.00	0.68	1.67	1.68	0.00	<0.01	1.00	1.00	0.00	6.61	1.48	0.10	0.03	<0.01	1.00	1.00	0.00
Spm	44.85	1.26	1.31	0.17	109.83	1.34	9.09	0.33	25.91	0.92	8.09	0.07	5.07	5.07	1.5	0.02	8.57	2.96	0.18	0.04	0.19	0.20	0.00	0.01

Total free AAs contribute to OA with percentages below 7.5% (Table 4.5). All ecotypes except O4 strongly increased their AAs content between 3 and 6-fold their controls, being higher in C2 than in C1. Glu was the most accumulated AA in stressed plants, reaching values over 800 μg g^{-1} FW in all ecotypes, except in O3 that presented contents of 636.09 μg g^{-1} FW. Plants also accumulated Arg, Asn, GABA, Glu, Gln+ His and Pro. With respect to GABA and Pro, only O4 and O5 increased their contents compared to the levels at the end of C1 (Table 4.5).

Put, Spd and Spm were free PAs which presented the highest accumulations after seven weeks under a second drought cycle (Table 4.5). Again, stressed plants from O5 showed the highest values of Put and Spd with 9.25 and 6.61 μg g^{-1} FW, respectively. O5 together with O4 also presented the strongest Spm accumulation with 2.96 and 5.07-fold more than their control levels (Table 4.5)

Statistically different values of cell wall elastic modulus (ε) were observed among ecotypes according to ANOVA (Table 4.3). The ε changes showed a negative correlation with Ψ_t variations (p<0.001) (Fig. 4.9A). Thus, when stressed plants increased ε to 4.4 MPa, they reached the *TLP* as observed during C1. After seven weeks of drought conditions, only O4 and O5 showed ε values below *TLP*, with 3 and 4.3 MPa respectively (Fig. 4.9B).

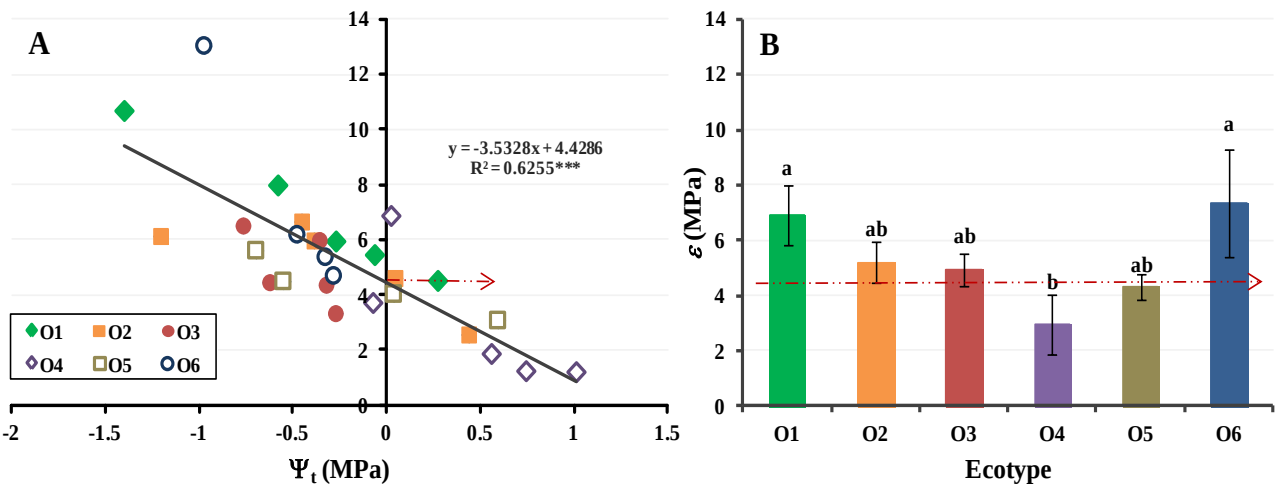


Figure 4.9. Correlation between wall cell elastic modulus (ε) *vs*. turgor pressure (Ψ_t) (MPa) (A) in six *Pinus radiata* ecotypes (O1-O6) exposed to a second drought cycles. ε values of each ecotype (B). Discontinuous narrows indicate the turgor loss point. *M* ± *S.E.* R^2- Pearson´s correlation numbers with the significance. Different letters mean significant differences according to Tukey´s HSD test after ANOVA. * < 0.05; ** <0.01 and *** <0.001.

4.3.4.- Hormonal analysis

At S_i, all stressed plants showed variations of hormonal content. These variations were highly significant and produced by the interaction effect between O and T according to MANOVA (Table 4.6). All ecotypes except O1 and O2 decreased their Z+ZR levels with respect to each control (Fig. 4.10A). The highest decreases were observed in O4, reducing more than 2-fold the control levels.

Table 4.6. Analysis of variance (ANOVA) of Z+ZR, ABA, IAA, ACC, JA and SA content (ng g^{-1} FW) in six *Pinus radiata* ecotypes (O) under irrigation and no irrigation conditions (T) along a second drought cycle until 50% plant from each ecotype showed external symptoms (S_i).

VARIABLE	FACTORS	DF	Sum Sq	Mean Sq	Fvalue	Pr(>F)
Z+ZR	O	5	736764.79	147352.96	2294.11	***
(ng g^{-1} FW)	T	1	92242.07	92242.07	1436.10	***
	O*T	**5**	**962557.63**	**192511.53**	**2997.17**	*******
ABA	O	5	431684.84	86336.97	810.54	***
(ng g^{-1} FW)	T	1	267550.31	267550.31	2511.78	***
	O*T	**5**	**346029.87**	**69205.97**	**649.71**	*******
IAA		5	86939.15	17387.83	5737.92	***
(ng g^{-1} FW)	T	1	176424.71	176427.71	58219.5	***
	O*T	**5**	**91526.89**	**18305.38**	**6040.71**	*******
ACC	O	5	12968.12	2593.63	49.44	***
(ng g^{-1} FW)	T	1	68511.43	68511.43	1306.10	***
	O*T	**5**	**29297.75**	**5859.55**	**111.71**	*******
JA	O	5	139451.74	27890.35	962.18	***
(ng g^{-1} FW)	T	1	23811.68	23811.68	821.47	***
	O*T	**5**	**26462.41**	**5292.48**	**182.58**	*******
SA	O	5	12968.12	2593.63	49.44	***
(ng g^{-1} FW)	T	1	68511.43	68511.43	1306.10	***
	O*T	**5**	**29297.75**	**5859.55**	**111.71**	*******

Note:;* < 0.05; ** <0.01 and *** <0.001; ns-non-significant.

Regarding ABA content, stressed plants from all ecotypes significantly increased its accumulation compared to each control, except O2 which did not vary their levels (Fig. 4.10B).

IAA was the most remarkable phytohormone in C2, explaining a total of 99% of model variance (Table 4.7). IAA was strongly accumulated in all stressed plants with respect to each control (Fig. 4.10C). O4 showed the highest IAA increases.

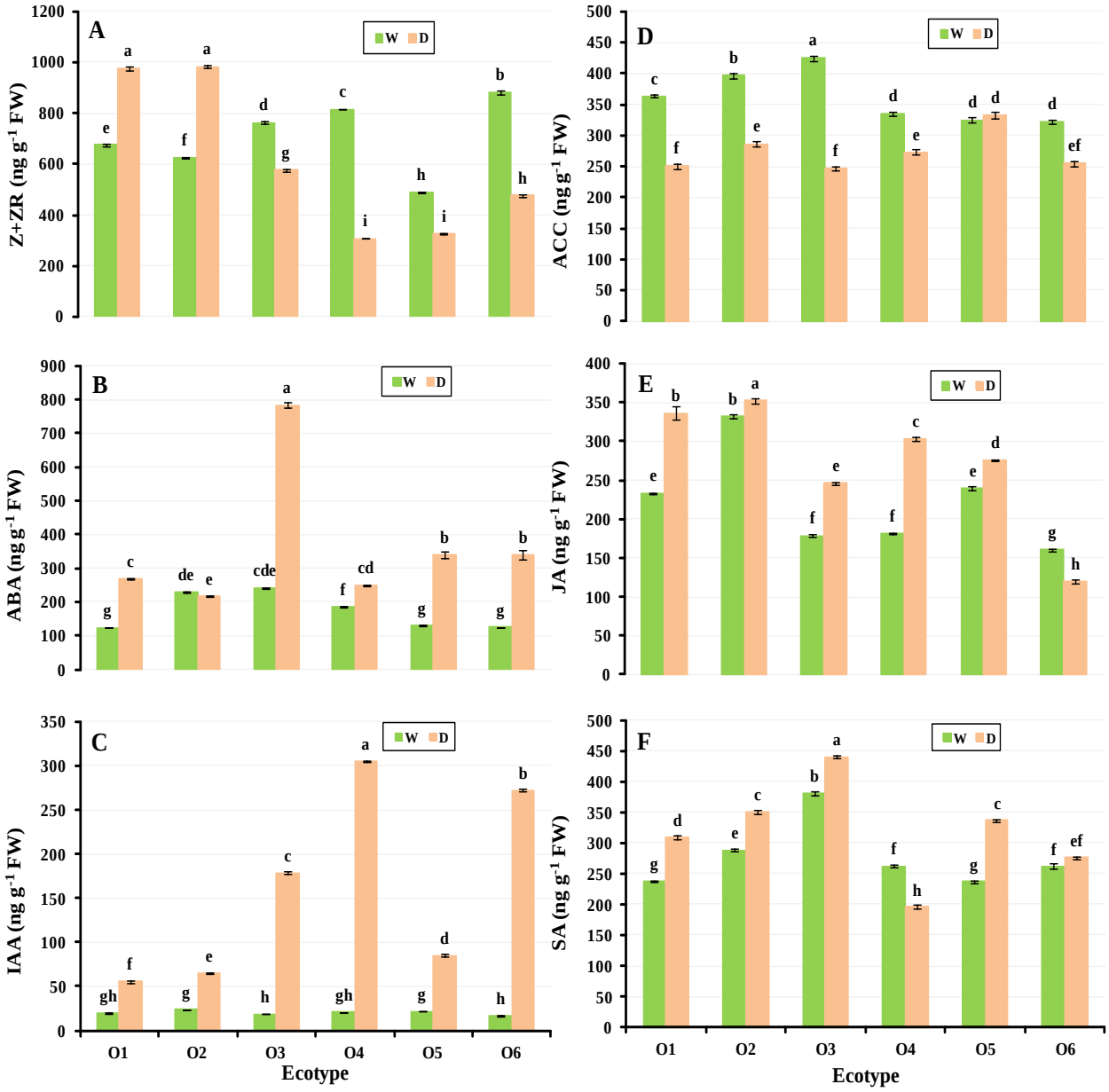


Figure 4.10. Z+ZR (A), ABA (B), IAA (C), ACC (D), JA (E) and SA (F) content (ng g^{-1} FW) in six *Pinus radiata* ecotypes (O1-O6) subjected to a second drought cycle until 50% plant from each ecotype showed external symptoms (S*i*). W and D mean irrigated and non-irrigated plant, respectively. *M* ± *S.E.* Different letters mean significant differences according to Tukey´s HSD test after MANOVA.

Drought induced a reduction of the ACC content in stressed plant from all ecotypes except in O5 (Fig. 4.10D).

Some stressed plants accumulated JA and/or SA when they were subjected to drought conditions (Chapter 2). At S_i, all stressed plants increased their JA, except O6 (Fig. 4.10E). With respect to SA, all plants increased their values, except in O6 and O4 (Fig. 4.10F). Stressed plants from O6 maintained their SA levels whereas O4 decreased them.

Table 4.7. Characteristic vector of matrix associated to MANOVA for ABA, IAA, Z+ZR, ACC, JA and SA ratio content (D-water stressed / W-well watered plants) variables analyzed in six *Pinus radiata* ecotypes subjected to a second drought cycle until 50% plant from each ecotype showed external symptoms.

Characteristic root	Percentage	ABA	IAA	Z+ZR	ACC	JA	SA
3512.70	**92.20**	0.045	**0.1232**	0.0208	0.0098	0.0013	0.0230
186.38	**4.89**	0.0184	**0.0310**	0.0101	0.0072	0.0018	0.0044
72.76	**1.91**	0.0058	**0.0423**	0.0147	0.0148	0.0073	0.0083
37.21	0.98	0.0018	0.0017	0.0037	0.0217	**0.0368**	0.0025

If the two drought periods were compared (C1 and C2), a strong increase in IAA content was observed in O4 and O5 at $S_{O1,\ O4,\ O5}$, showing 15 and 6-fold the levels observed at the end of C1, respectively (Fig. 4.11). Regarding JA, only stressed plant from O4 and O5 presented higher values at C2 than at C1, being over 4-fold higher the JA accumulation in the case of O5 at the end of C1 (Fig. 4.11).

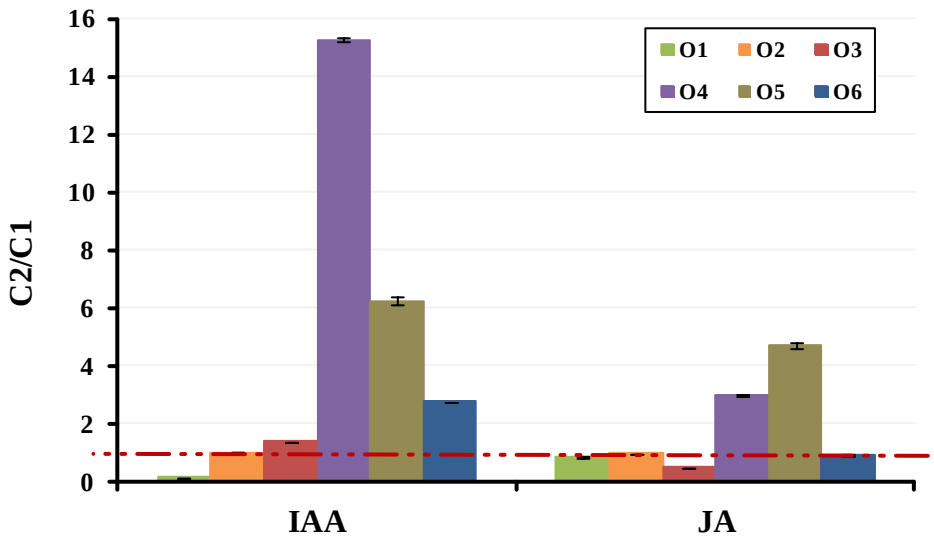
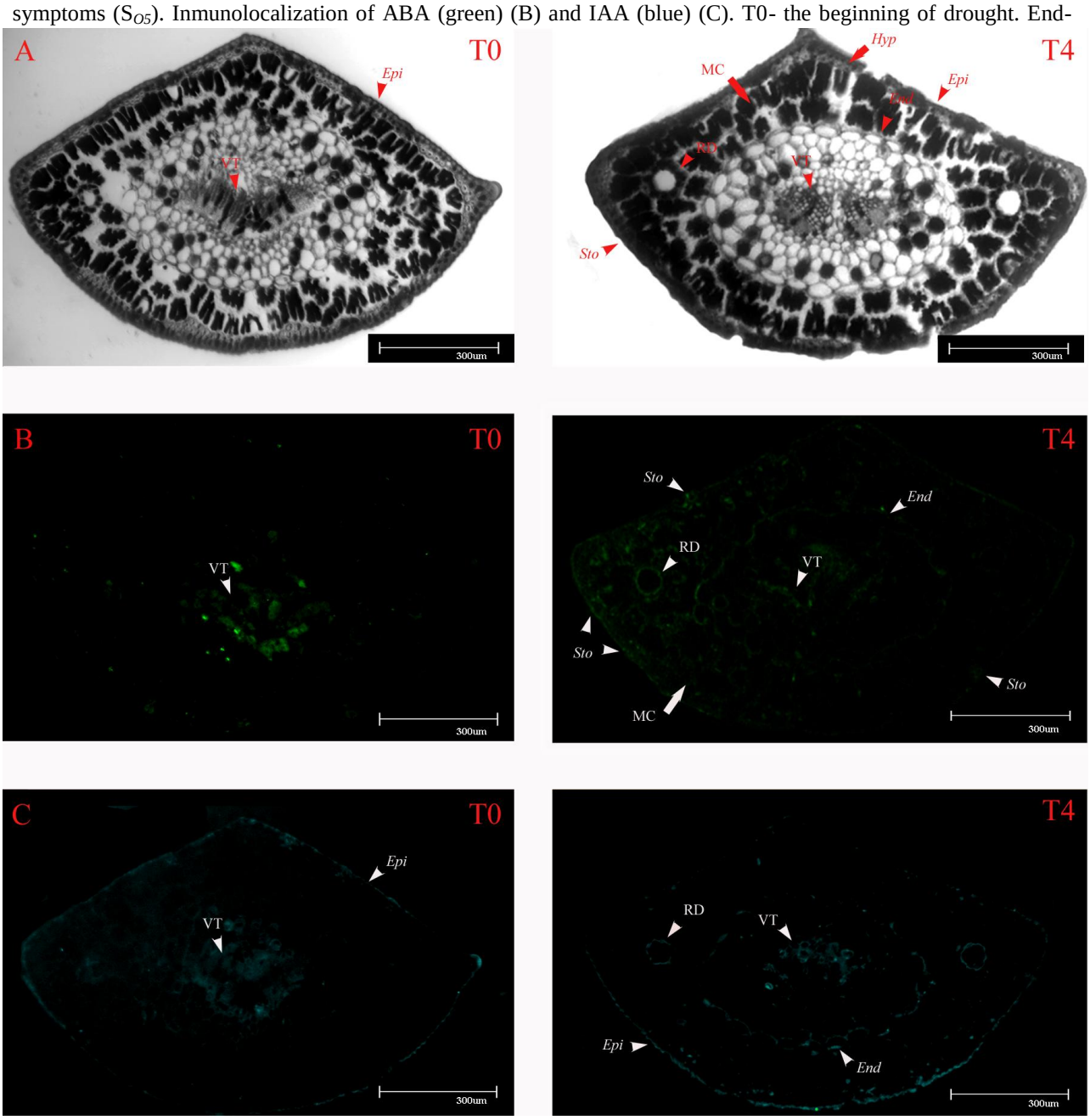
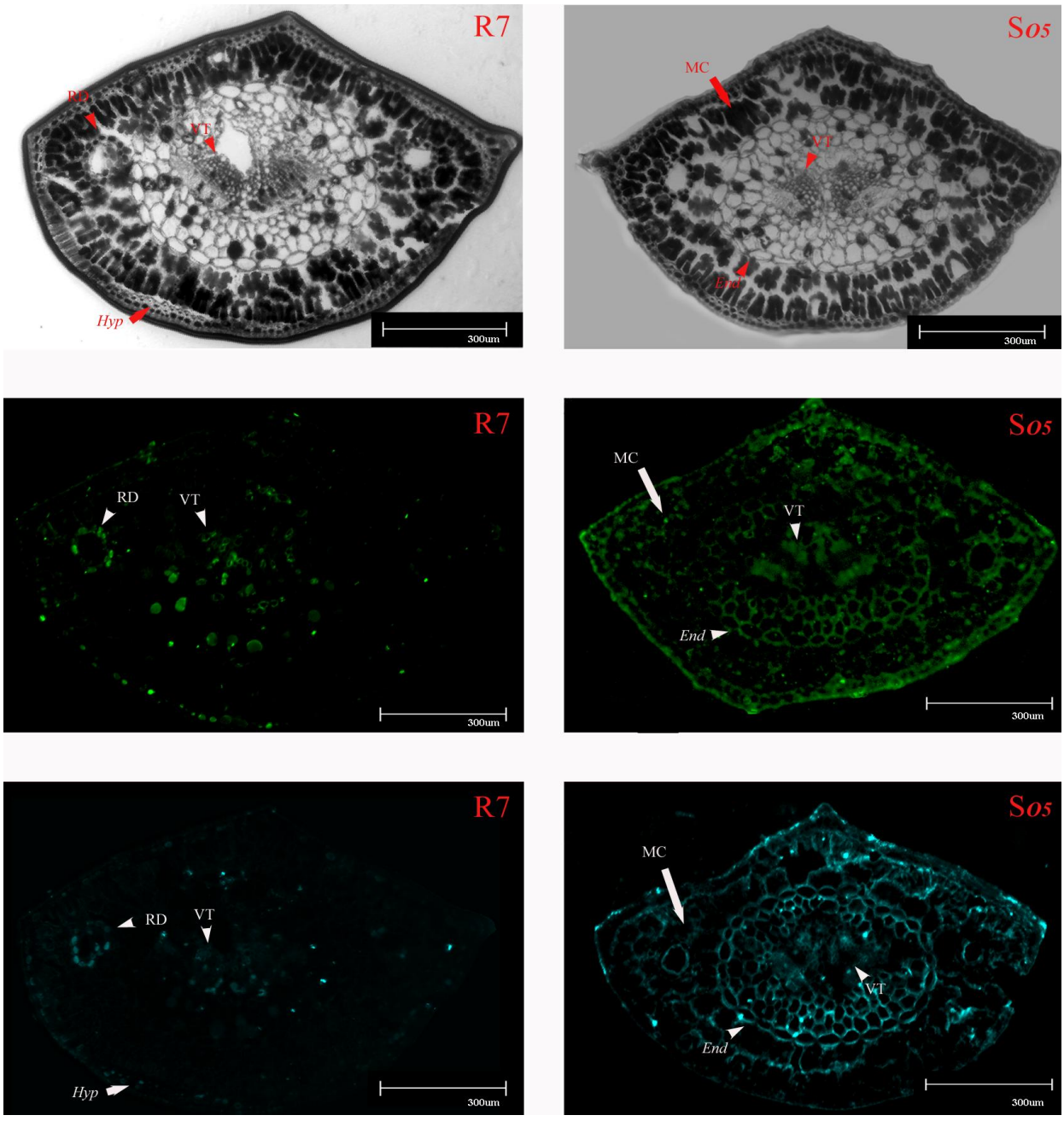


Figure 4.11. Relation of IAA and JA content (ng g^{-1} FW) in six *Pinus radiata* ecotypes (O1-O6) exposed to two consecutive drought cycles (C1/C2). Discontinuous red line indicates C2/C1 ratio = 1.

Figure 4.12. Transmitted light image (A) of *Pinus radiata* needles from O5 subjected to a first drought cycle of four weeks (T4) and a second drought cycle after a week of rewatering (R7) until 50% plants presented external symptoms (S_{O5}). Inmunolocalization of ABA (green) (B) and IAA (blue) (C). T0- the beginning of drought. End-



endodermis, Epi-epidermis, Hyp-Hypodermis, MC- Mesophyll cells, RD- Resin ducts, Sto- Stomata, and VT- Vascular tissue



4.3.4.1.- Inmunolocalization of ABA and IAA

The high drought tolerance, together with the low stress symptoms observed at T4, made O5 interesting samples to evaluate the principal localization areas where IAA and ABA were accumulated.

Plants showed ABA and IAA accumulation in the Vascular Tissue (VT) in all evaluated times. Besides, IAA was located into epidermis (*Epi*) at T0 (Fig. 4.12B and 4.12C). At T4, high accumulations of IAA and ABA were appreciated into mesophyll cells (MC), *Epi* and endodermis (*End*). ABA content was also evident into the guard cells (Fig. 4.12B). At this time, needles showed structural changes (Fig. 4.13). The most evident changes were a strong MC compression, a significant increase of the resin duct (RD) area, and a decrease of the substomatal chamber size (Fig. 4.13) (Table 4.8).

At R7, IAA and ABA were located into RD cells and IAA also into hypodermis (*Hyp*). Finally, at S_{O5}, the structural changes observed in needles at T4 were maintained, and IAA and ABA were accumulated in all needle areas, especially into MC (Fig 4.12 and 4.14).

In apical roots, controls showed IAA and ABA accumulation into the cortex (*C*), and xylem (X) (Fig. 4.14B and 4.14C). IAA was located in the endodermis (*End*), whereas ABA was situated in the apoplast. At T4, roots decreased ABA content and increased IAA, principally in apoplast. At R7, the principal IAA and ABA accumulation was observed into exodermis (*Exo*), especially for ABA. Finally, at S_{O5}, roots showed a compression of *C* cells (Fig. 4.14A) and an evident IAA and ABA accumulation (Fig. 4.14B and 4.14C).

Table 4.8. Analysis of variance (ANOVA) of xylem cell, substomatal chamber and resin duct (RD) area in O5 after a drought cycle of four weeks (from T0 to T4- C1), and a second drought cycle after a week of rewatering until S_{O5} (Ti). Substomatal chamber means substomatal chamber.

VARIABLE	FACTORS	DF	Sum Sq	Mean Sq	Fvalue	Pr(>F)
Xylem cell (μm^2)	Ti	2	142.696	71.348	0.55	ns
Substomatal chamber (μm^2)	**Ti**	**2**	**277905.12**	**138952.56**	**6.57**	****
RD (μm^2)	**Ti**	**2**	**1163903.86**	**581951.93**	**11.11**	****

Note: ns-non-significant;* < 0.05; ** <0.01 and *** <0.001

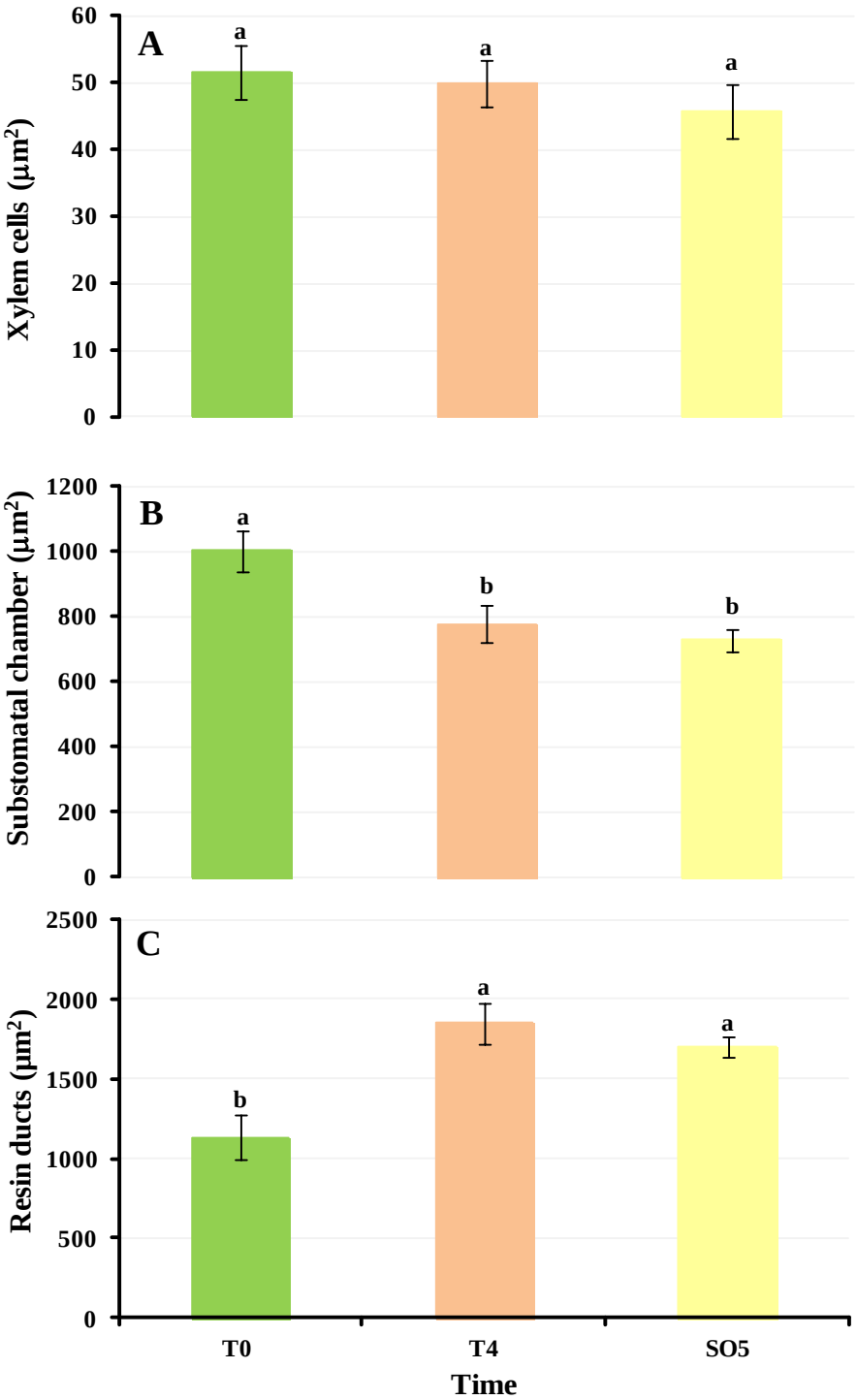
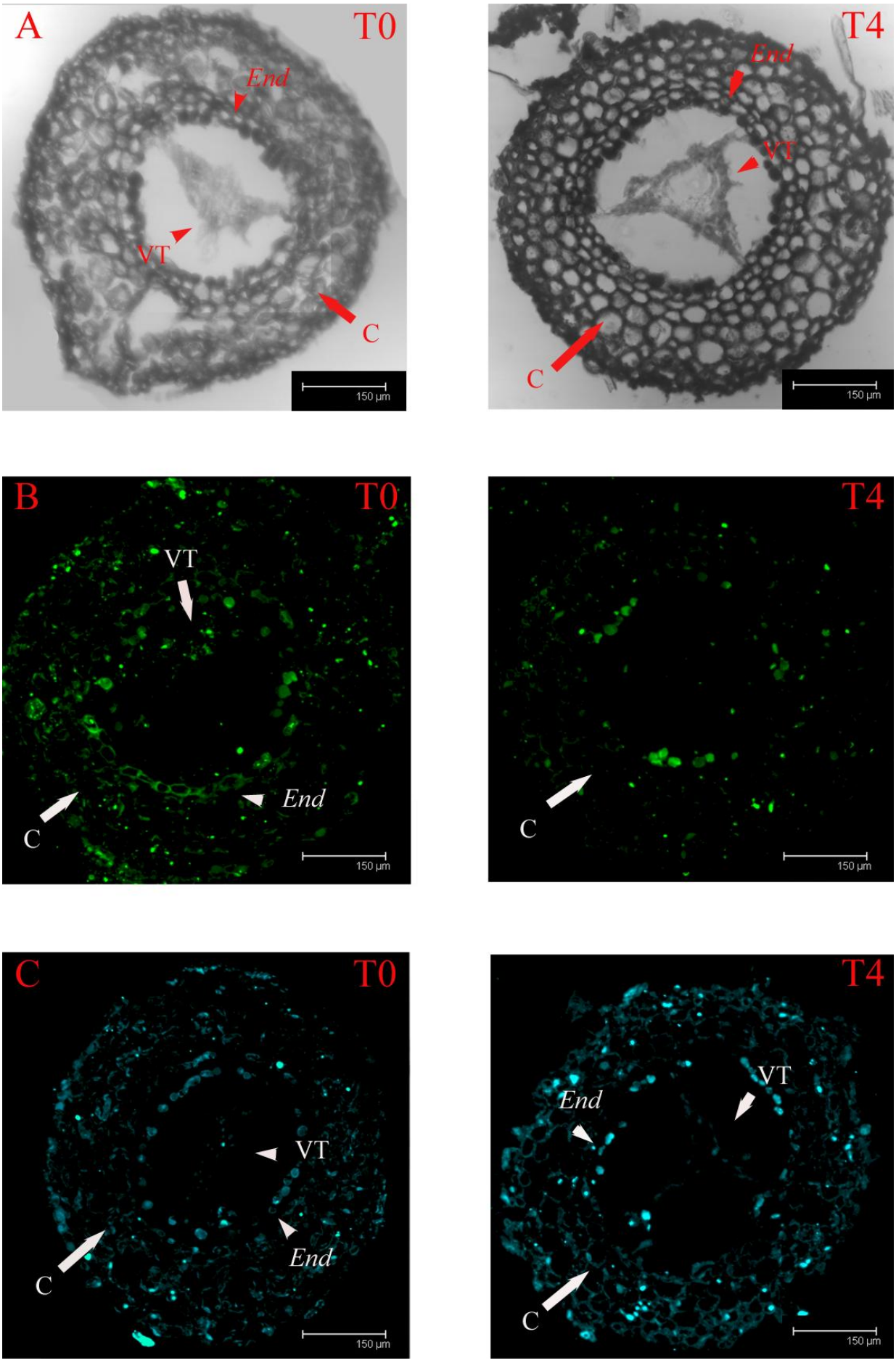
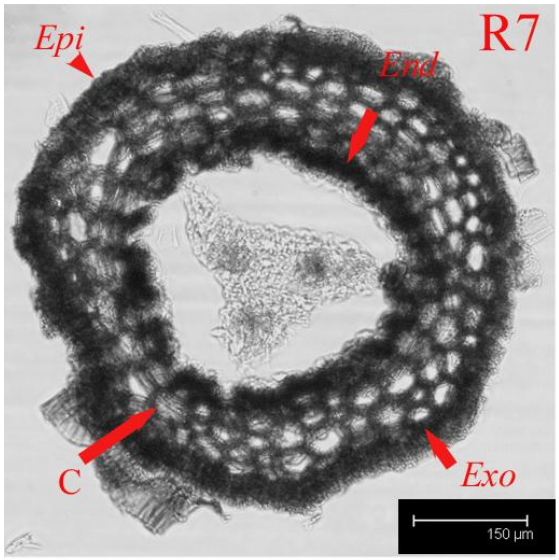


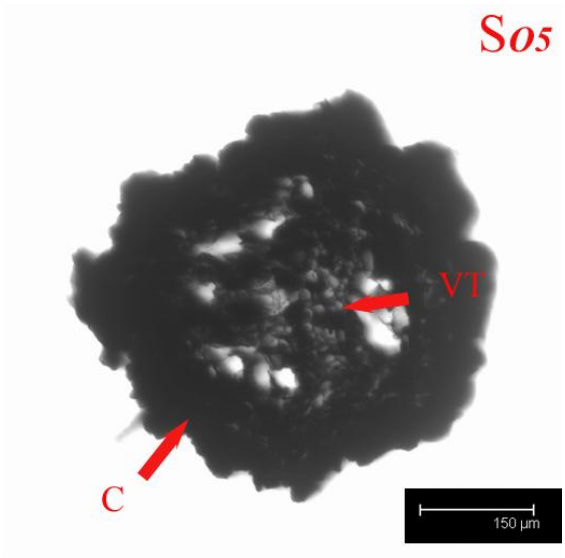
Figure 4.13. Xylem cell (A), substomatal chamber (B), and resin duct areas (C) (µm^2) in *Pinus radiata* plants from O5 at the experiment beginning (T0), at the end of the one drought cycle (T4) and a second drought period after a week of rewatering until 50% plants presented external symptoms (S_{O5}). *M* ± *S.E.* Different letters mean significant differences according to Tukey´s HSD test after ANOVA.

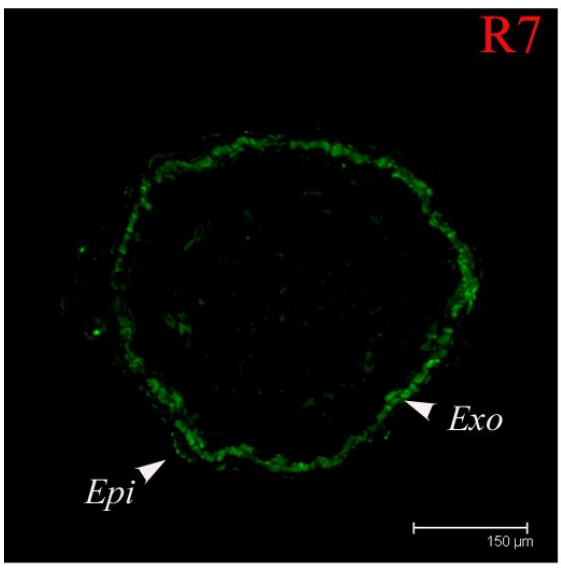
Figure 4.14 Transmitted light image (A) of *Pinus radiata* needles from O5 subjected to a first drought cycle of four weeks (T4) and a second drought cycle after a week of rewatering (R7) until 50% plants presented external

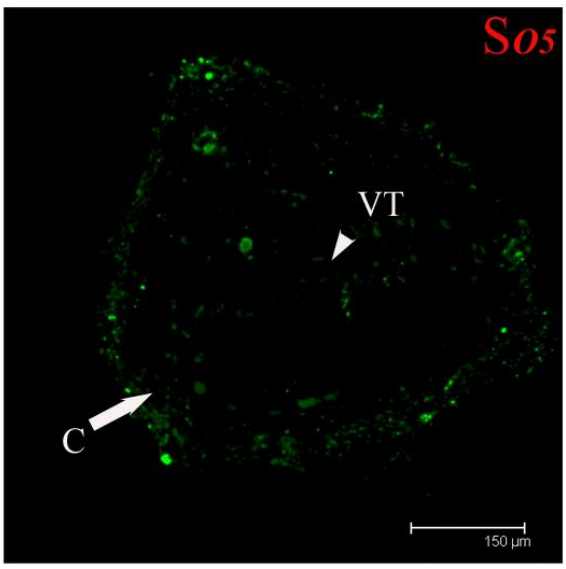


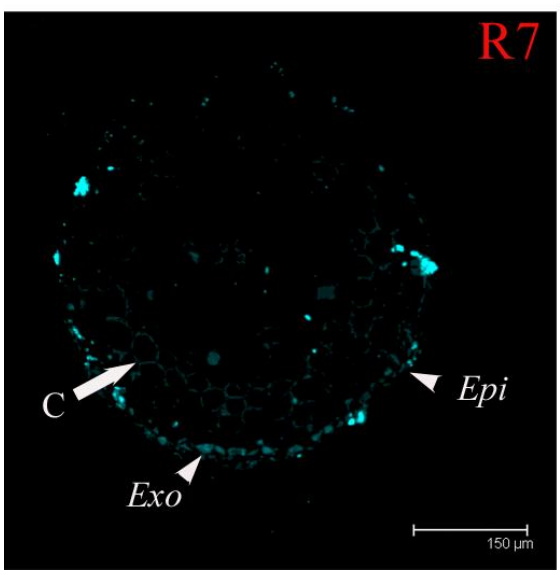
symptoms (S_{O5}). Inmunolocalization of ABA (green) (B) and IAA (blue) (C).T0- the beginning of drought. C-cortex, End- endodermis, Exo-exodermis, Epi-epidermis, and VT- Vascular tissue.

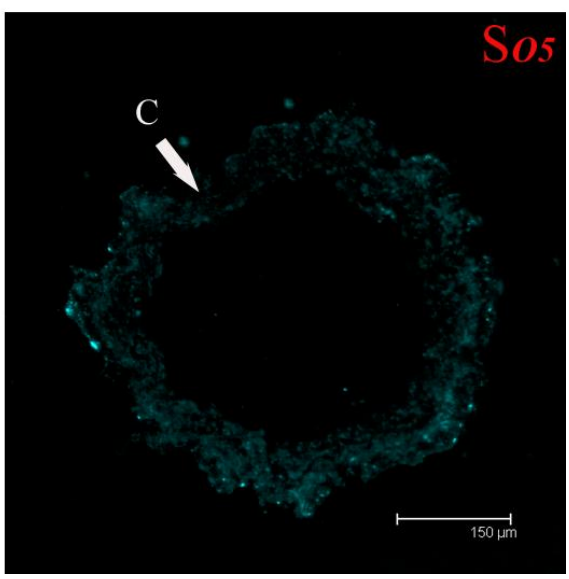












4.4.- Discussion

Along C2, all ecotypes showed hardening capacity. The ecotypes did not show external symptoms until seven weeks under stress conditions, whereas some of them presented symptoms after two weeks under a first drought period (C1) (Chapter 2). O1, O4 and O5 showed a higher drought acclimatation so they did not present external symptoms until eleven weeks under a second drought period (Fig 4.2).

Plant growth was also affected by drought, so radial collar diameter, height and total biomass production of plants was reduced during both first and second drought cycle (Fig 4.4). Root collar diameter was a non destructive biometric parameter more sensitive to drought that plant height and was significant reduced compared to control plants either at C1 and C2. In addition, there were differences in diameter among ecotypes (Fig. 4.5). The decrease of radial growth in forest species has been related to less drought tolerance plant (Ogaya *et al.*, 2003), and only stressed plants from O5 did not change their RdGR along the two drought cycles.

Regarding height, stressed plants from all ecotypes were affected at the same level by the water stress, as it was observed in different population of *Pinus pinaster* under water stress (Sánchez-Gómez *et al.*, 2010).

Biomass production was also affected by drought (Fig. 4.6). Although different sizes were appreciated among ecotypes, the plant biomass diminution was as a result of drought effect, including the R/A ratio. Plants traditionally allocated biomass from shoots to roots under stress conditions (Canham *et al.*, 1996; Prieto *et al.*, 2009). However, almost all *Pinus radiata* ecotypes decreased the root biomass at the end of the second drought cycle (Fig. 4.5B). In this matter, some studies suggested that a diminution of root biomass production was a good signal of hardening (Villar-Salvador *et al.*, 1999; Villar-Salvador *et al.*, 2004). The decrease of root biomass could be due to the induction of secondary roots that could favour a great absorption with a low energy cost (Fujii & Kasuya, 2008). In addition, the IAA content increased in O5 apical roots (Fig. 4.14), and this fact could stimulate lateral root formation to improve water uptake (Seo *et al.*, 2009).

Along C2, IAA was the most representative hormone, increasing the percentage of model variance explanation to 99% with respect to the percentage (66%) obtained during the

first water stress period (Table 2.3 and 4.7). All stressed plants strongly increased their IAA content, especially O4 and O5. The strong IAA accumulation observed in O4 and O5 could be a consequence of a longer and more severe drought (eleven weeks) that induced higher plant response (Fig. 4.10C and 4.11) (Blake *et al.*, 1980; Kawano *et al.*, 2003). Furthermore, ABA accumulation was observed in stressed plants from all ecotypes (Fig. 4.10B). Besides, ABA accumulation was located in guard cells to regulate stomatal closure at T4 and at S_{O5} (Fig. 4.12). A dominant ABA role in stressed plants was the stomata conductance control by the signalling from root to shoot (Schachtman & Goodger, 2008). This ABA translocation to stomata could be a cause of the ABA decrease in roots at T4 (Fig 4.14). On the contrary, at this time IAA content increased in roots and, as it was previously mentioned, IAA accumulation could stimulate the lateral root formation to improve water uptake (Seo *et al.*, 2009). ABA has been predicted to affect lateral root initiation (De Smet *et al.*, 2006; Xiong *et al.*, 2006), modulating IAA transport and/or signalling (Shkolnik-Inbar & Bar-Zvi, 2010). The well-known negative crosstalk between these phytohormones could explain the low ABA and high IAA presence in roots of O5 at T4. In addition, a reduction of intercellular space size and a strong increase of the resin duct area were also observed under stress conditions (Fig. 4.12 and 4.13). A possible explanation of the diminution of intercellular spaces could be due to the increase of ε, so water held by cell wall is more tightly bound, losing mainly the water from the intercellular spaces (Islam *et al.*, 2003). As regards resin ducts, studies carried out in *Pinus ponderosa* have showed that those ecotypes with higher size presented higher drought tolerance (Kane & Kolb, 2010). In this matter, resin duct permits the terpene rich-oleoresin translocation that plays an important role against stress, and some evidences point out the JA and mechanical wounding as up-regulators of these compound productions (Abbot *et al.*, 2010; Phillips *et al.*, 2010).

After rewatering, IAA and ABA were accumulated in apical root exodermis (*Exo*) of O5. *Exo* could serve as a barrier to retard ABA transport to vascular tissue (VT), especially when the redistribution is through the symplast (Hartung *et al.*, 2002; Hose *et al.*, 2001). It was remarkable that the IAA and ABA presence in *Exo* had not previously been described in any gymnosperms, and the *Exo* implication of recovery capacity could be related to the regulation of IAA and ABA transport to other plant areas. At the end of the second drought cycle (S_{O5}), ABA and IAA accumulation was also appreciated in O5 needles (Fig. 4.12) where ABA played a role in stomatal control and IAA was implicated in epinasty processes.

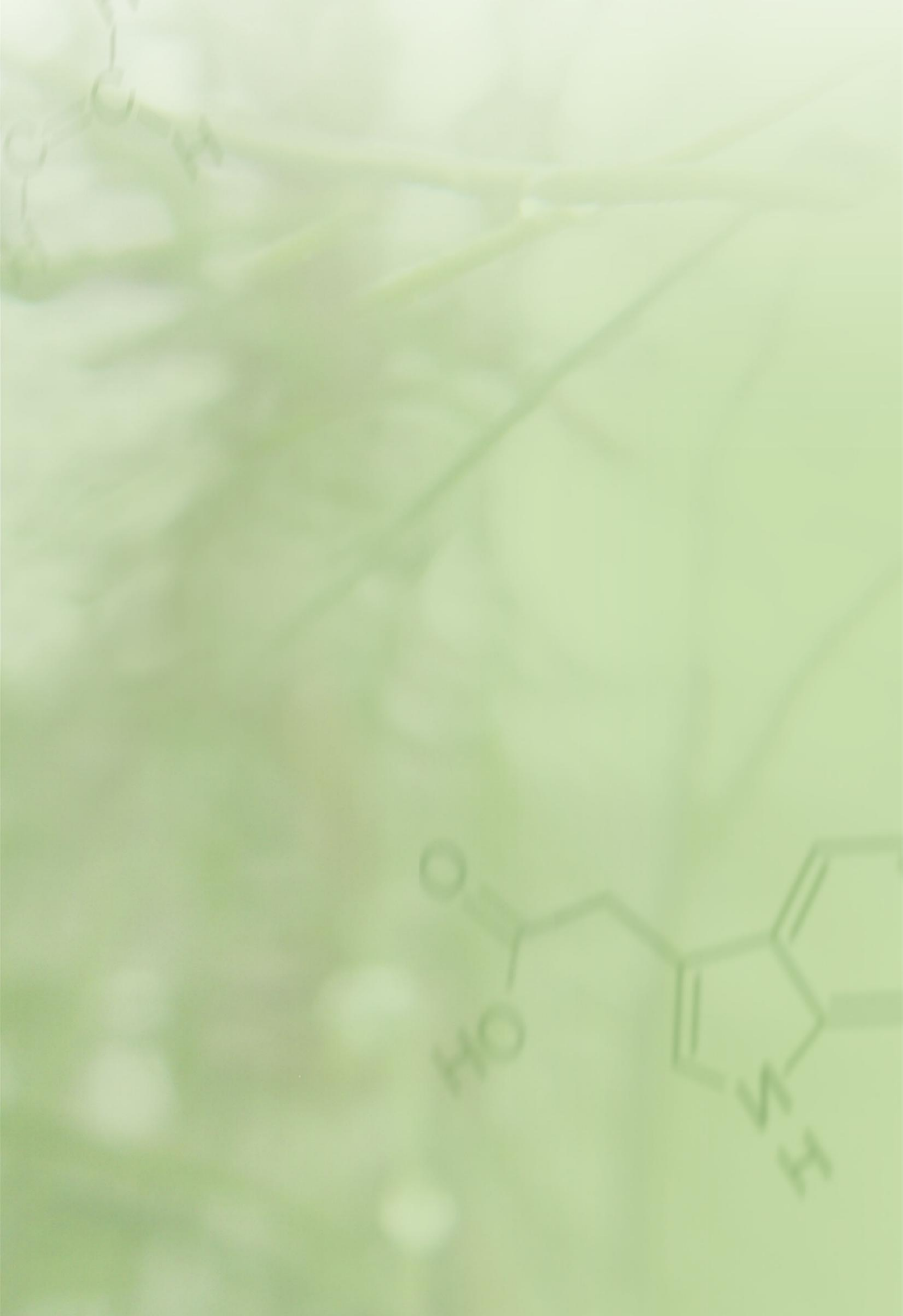
When all ecotypes presented 50% plants with external symptoms (S_i) increased their JA levels in needles (Fig. 4.10E). In this regard, jasmonates has been traditionally related to the damage response and, recently, with an increase of enzymatic antioxidants and the modulation of the membrane lipid peroxidation under drought conditions (Anjum *et al.*, 2011). According to these assumptions, the JA accumulation in *P. radiata* plants subjected to drought could be an initial signal to activate plant drought response when a severe drought condition has provoked cell damages. Although O5 accumulated JA in needles, only this ecotype showed higher values of SA than JA. The positive SA effects could give O5 more tolerance under different water stress cycles, including under severe drought conditions (Delaney, 2007).

All drought-conditioning plants decreased their osmotic potential (Ψ_π) and increased their percentages of active osmotic adjustment (OA) to a range of 70% (Fig. 4.8). The extension of OA has been associated to plant hardening, as it was observed in *Thuja occidentalis* L (Edwards & Dixon, 1995). Soluble carbohydrates presented the highest percentages of OA contribution and an evident increment was observed in O1 and O5 (Table 4.5). The increase in the soluble carbohydrate accumulation has been showed in abiotic stress-conditioning of plants (Gilmour *et al.*, 2000; Tinus *et al.*, 2000) and of course in drought hardening (Arndt *et al.*, 2001; Villar-Salvador *et al.*, 2004). Under stress conditions, it has been reported that the sugar accumulation in plants may be due to an increased partitioning of fixed carbon to soluble sugars (Chaves, 1991; Pinheiro *et al.*, 2001). Moreover, all plants accumulated free AAs after a second drought period of seven weeks (Table 4.5). Glu was the most accumulated AA. The increase of Glu together with the augmentation of Asr, Gln and Arg could be due to the fact that these compounds sequestrate and store ammonia when stress reduces carbon fixation and nitrogen availability by the interaction of nitrogen assimilation pathway, photorespiratory nitrogen cycle and nitrogen translocation (Aranjuelo *et al.*, 2011; Gaufichon *et al.*, 2011). Regarding Pro has been traditionally considered the most relevant AA under stress conditions as osmotical active compound and/or protector against oxidative damages (Szabados & Savouré, 2010; Verbruggen & Hermans, 2008). In our work, only O4 and O5 increased their Pro content with respect to the first drought cycle. According to this, Pedrol *et al.* (2000) reported that hardened plants accumulated high Pro levels when they are subjected to a severe water stress, underpinning a high drought-conditioning capacity in these two ecotypes. Finally, regarding PA accumulation, O5 plant presented the highest putrescine (Put), spermidine (Spd) and spermine (Spm) levels after seven weeks of a second drought cycle. According to this, they are the most common free

PAs that can be observed in plants, due to their implication of plant stress response and growth control (Wimalasekera *et al.*, 2011).

To summarize, in this work we observed new evidences of physiological response, osmotic adjustment, phytohormone regulation, morphological and structural characteristics involved in *P. radiata* drought hardening. In this respect, along the second drought cycle IAA was the most representative phytohormone (Table 4.7). IAA accumulation pointed out the regulation of lateral root formation and the needle epinasty. Structural traits such as small substomatal chamber, and high resin duct areas were related to a better drought acclimation. The increase of active osmotic adjustment was the principal physiological trait that determinated drought-conditioning. Soluble carbohydrates were the most important osmolytes implicated in OA. All stressed plants significantly increased their AA content, especially their Glu levels due to its involvement in the photorespiratory cycle. Furthermore, an increase in Pro accumulation gave *Pinus radiata* plants high capacity of drought conditioning. In addition, an increase of cell wall rigidity (ε) could be an effective mechanism to prevent water loss in cell, while its values did not reach more than 4.4 MPa (*TLP*).

II.-Discusión general



II.- Discusión general

En este estudio se evaluaron cinco ecotipos de *Pinus radiata* D. Don y un híbrido de especie (O4, *Pinus radiata* x *Pinus attenuata*) bajo condiciones de déficit hídrico con el fin de determinar su tolerancia, capacidad de recuperación y acondicionamiento. La plantas de O4 fueron incluidas como modelo de tolerancia debido a la alta resistencia a la sequía descrita en *P. attenuata* (Begley, 2001). En cada ecotipo se evaluaron los cambios fisiológicos que se produjeron a lo largo de ciclos de estrés hídrico a corto y largo plazo, incluyendo las posibles señales hormonales y sus interconexiones. Para el análisis, se emplearon diferentes herramientas estadísticas tales como regresiones, ANCOVAs, MANOVAs, y análisis de componentes principales que facilitaron el entendimiento de esta compleja respuesta de las plantas al estrés hídrico.

Al inicio del experimento (T0) los ecotipos no mostraron diferencias entre los parámetros fisiológicos evaluados. Sin embargo, bajo condiciones de déficit hídrico, presentaron variaciones en los niveles de Ψ_{leaf}, Ψ_t, RWC (%), ajuste osmótico, acumulación de osmolitos solubles y contenido de fitohormonas. La tolerancia a la sequía estuvo más ligada al ecotipo que al área de procedencia, destacando la alta resistencia observada en el híbrido varietal O5, que mostró un comportamiento muy similar al de las plantas de O4. Por el contrario, el híbrido O1 presentó una mayor sensibilidad al estrés a pesar de proceder de la misma zona geográfica y climatológica. Esta variación ecotípica entre plantas procedente de áreas similares también ha sido descrita en *Pinus halepensis* (Tognetti *et al.*, 1997) y en *Pinus pinaster* (Aranda *et al.*, 2010).

De forma general, la sequía indujo una pérdida del RWC (%), y esta disminución estuvo estrechamente relacionada con el descenso del Ψ_{leaf} (Curva de presión-volumen). Así, cuando el RWC de las plantas descendía un 50%, y el Ψ_{leaf} alcanzaba valores de -2 MPa, las plantas perdían su turgencia. Esta curva no se vio modificada tras el proceso de endurecimiento. Los descensos de Ψ_{leaf} también estuvieron estrechamente relacionados con disminuciones de K_{leaf} y de g_s, E y A_N. Además, las pérdidas de K_{leaf} se produjeron paralelamente a una disminución del contenido de Z+ZR en las hojas, actuando como primera señal hormonal de estrés (Granda *et al.* 2011). Tras una disminución del 65% de Z+ZR y K_{leaf}, la plantas comenzaron a acumular ABA e IAA, incrementando sus niveles a medida que el estrés se hacía más severo. IAA fue la fitohormona que explicó el mayor porcentaje de varianza del modelo experimental, con un 66%

durante el primer ciclo de sequía, e incrementando este valor al 99% tras el proceso de endurecimiento. En las plantas de *P. radiata,* la presencia de IAA se observó tanto en las acículas como en las raíces bajo condiciones de estrés hídrico. En las acículas, el incremento de IAA estuvo correlacionado con el descenso de valores de *Fv/Fm*, y una mayor pérdida electrolítica y porcentaje de plantas con síntomas externos, posiblemente debido a la implicación de esta hormona en los procesos de epinastia (Blake *et al.*, 1980; Kawano *et al.*, 2003). Por otra parte, en los ápices radiculares la presencia de IAA en respuesta a la sequía podría inducir la formación de raíces laterales para facilitar la toma de agua.

Las plantas estresadas también acumularon SA y JA, este último como respuesta a la presencia de daños físicos en las plantas, como ha sido previamente descrito en angiospermas (Schaller & Stintzi, 2009; Wasternack, 2007). Sin embargo, el aumento de los niveles de SA no mostró correlación con la presencia de síntomas, y se asoció a mecanismos de defensa como es la protección frente a daños en las membranas de las células [menor *E. L.* (%)], y del *PSII* (mayores valores de *Fv/Fm*). A este respecto, se ha descrito que el SA regula la activación de múltiples mecanismos de respuesta tal y como describen Delaney (2007) y Wang *et al*. (2010), destacando entre ellos la activación del sistema Reactive Oxigen Species de la planta e incluso la inducción del cierre estomático (Khokon *et al.*, 2011; Senaratna *et al.*, 2000). O5 mostró los mayores niveles de SA en los diferentes ciclos de estrés.

Como consecuencia de una menor disponibilidad de agua, todos los ecotipos mostraron ajuste osmótico (OA), favoreciendo la síntesis de nuevos osmolitos (OA activo) como una eficiente respuesta de tolerancia, especialmente tras el endurecimiento. Los carbohidratos solubles fueron los solutos que más contribuyeron al OA, destacando principalmente el incremento del contenido de *D*-glucosa y *D*-fructosa. El aumento de estas dos hexosas puede servir como materia prima en la reparación de las paredes celulares para favorecer una rápida recuperación tras la rehidratación, como ha sido descrito en angiospermas (Clifford *et al.*, 1998; Cuellarar-Ortiz *et al.*, 2008) y también en coníferas (Meinzer *et al*., 2002). Yu (1999) comprobó que ciertos azúcares, además de la expresión de genes relacionados con la fotosíntesis, también regulaban gran número de genes relacionados con respiración, metabolismo de nitrógeno y de ciertas rutas del metabolismo secundario de la planta. En este sentido, la síntesis de nuevos metabolitos no solo puede contribuir al OA sino que puede mediar la respuesta al déficit hídrico (Chen & Jiang, 2010; Patakas *et al.*, 2002).

Los AAs libres, y en menor medida las PAs libres, contribuyeron ligeramente como osmolitos en el OA, aunque en condiciones de déficit hídrico las plantas mostraron un gran aumento de su contenido, especialmente de ciertos AAs. Las plantas estresadas incrementaron los niveles de Pro y GABA, como respuesta a la deshidratación. La acumulación de ambos AAs bajo condiciones de estrés ha sido relacionada con diferentes mecanismos de defensa como protección de membranas, estabilización de proteínas, así como reguladores de la síntesis de enzimas antioxidantes (Bouché & Fromm, 2004; Kumar & Yadav, 2009). Durante la aclimatación, las plantas incrementaron su acumulación de Pro (Pedrol *et al.*, 2000), como se observó en los ecotipos más tolerantes, O4 y O5. Por el contrario, las plantas procedentes de ecotipos menos resistentes (O2 y O6) incrementaron significativamente su contenido de ácido glutámico (Glu), y en menor medida los niveles de arginina (Arg), asparagina (Asn) y glutamina (Gln) durante los sucesivos ciclos de estrés, probablemente como mecanismo de detoxificación del amonio interno liberado por la degradación de proteínas (James *et al.*, 1993; Pinheiro *et al.*, 2001). El fuerte incremento del contenido de Glu también ha sido previamente atribuido en *Pinus radiata* a la alta demanda energética que realiza la planta bajo condiciones de estrés hídrico (Mena-Petite *et al.*, 2006). Así, la planta puede obtener energía del carbono que constituye el Glu en el ciclo del ácido tricarboxílico a partir de la acción de la glutamato deshidrogenasa (GDH), ya que otras fuentes de energía como la síntesis de ATP se ven reducidas en condiciones de estrés (Flexas *et al.*, 2005; Lawlor, 2002).

Los mayores incrementos se produjeron en O5 bajo condiciones de sequía, acumulando principalmente putrescina (Put), espermidina (Spd) y espermina (Spm), PAs tradicionalmente relacionadas con la tolerancia de las plantas frente a condiciones de estrés (Takahashi & Kakehi, 2010; Wimalasekera *et al.*, 2011). Además, las plantas de O5 no variaron sus niveles ACC. En este sentido, tanto Put, Spd como Spm han mostrado efectos anti-senescentes a partir de la retención de clorofilas, así como la inhibición de la síntesis de etileno (Wimalasekera *et al.*, 2011). Los altos niveles de PAs observados en O5, unido al mantenimiento de sus niveles de ACC, de *Fv/Fm* y las bajas pérdidas en el contenido de los pigmentos fotosintéticos, sugieren un papel protector de las PAs mediante la inhibición de la síntesis de etileno, como se ha observado previamente en *Pinus sylvestris* (Jokela *et al.*, 2011).

En conclusión, las plantas más tolerantes mantuvieron su contenido de agua por encima de *TLP* mediante un fuerte descenso de la conductancia hidráulica y posterior inhibición del intercambio gaseoso, un eficiente ajuste osmótico basado en la síntesis *de novo* de solutos, y un

aumento de la rigidez de la pared celular (incremento de ε) (Fig. II.1). Estos procesos han sido tradicionalmente asociados a especies evasoras del estrés o también denominadas isohídricas (McDowell *et al.*, 2008; Quero *et al.*, 2011). Por el contrario, las plantas menos tolerantes mostraron un ajuste osmótico similar pero descendieron sus valores de Ψ_{leaf} por debajo del *TLP*. Además, la acumulación de solutos estuvo más relacionada con procesos de degradación y detoxificación (Glu, Gln, Arg o Asn) que con mecanismos de defensa.

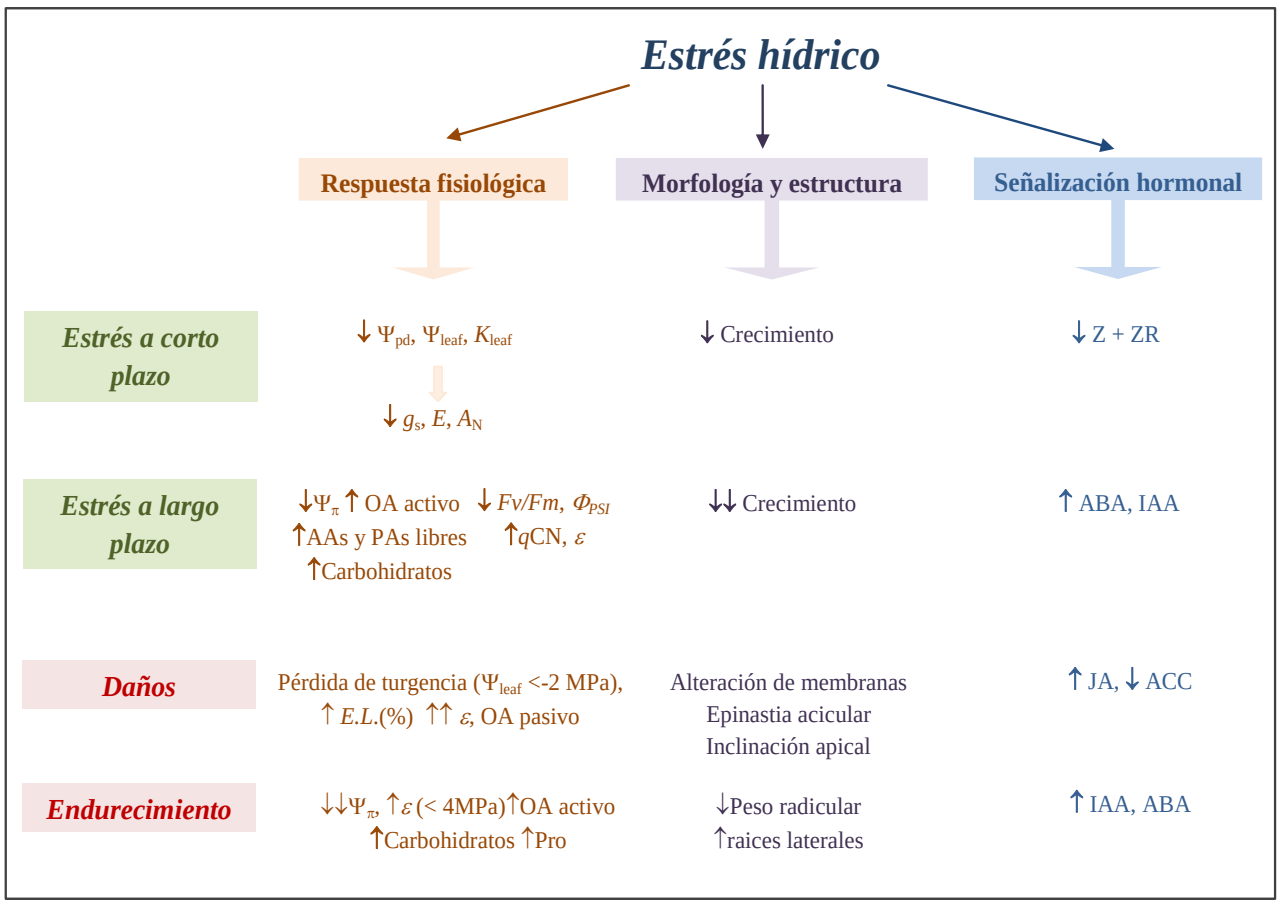


Figura II.1. Mecanismo propuesto de la respuesta al estrés hídrico de plantas de *Pinus radiata* D. Don.

Como respuesta a un estrés hídrico severo, las plantas más tolerantes (O4 y O5) incrementaron sus niveles de SA, pero prácticamente no variaron su contenido de JA y ACC hasta la observación de síntomas externos. En cambio, los ecotipos más sensibles a la sequía incrementaron rápidamente sus niveles de JA, y disminuyeron su contenido de ACC.

Por último, los ecotipos presentaron características estructurales diferentes. A este respecto, las plantas más tolerantes mostraron menor tamaño de las cavidades subestomáticas, y mayor tamaño de las células xilemáticas. Una menor cavidad subestomática reduce las pérdidas

por transpiración y minimiza la pérdida de agua (Gibson, 1996; Pickard, 1982). Por otro lado, células xilemáticas de menor tamaño son muy susceptibles a la cavitación, sufriendo más fácilmente daños que retrasan la recuperación del sistema de transporte de agua tras la rehidratación (Lovisolo *et al.*, 2002). Esto lo corrobora el hecho de que solo los ecotipos O4 y O5 recuperaron los valores de conductividad hidráulica tras el riego.

Las plantas de O5 aunaron una mayor tolerancia al estrés hídrico, alta capacidad de recuperación, y mantuvieron a su vez una buena producción de biomasa con respecto al resto de los ecotipos. Este ecotipo presentó un comportamiento de tipo isohídrico, con un eficiente control de la conductividad hidráulica y del cierre estomático. Además, mostró un alto ajuste osmótico activo, incrementó su ε sin alcanzar valores por encima de 4 MPa, y acumuló SA como mecanismo de defensa frente al estrés (Fig. II.2). La disminución de la cavidad subestomática y aumento de conductos resiníferos fueron modificaciones estructurales que incrementaron su resistencia frente a la deshidratación.

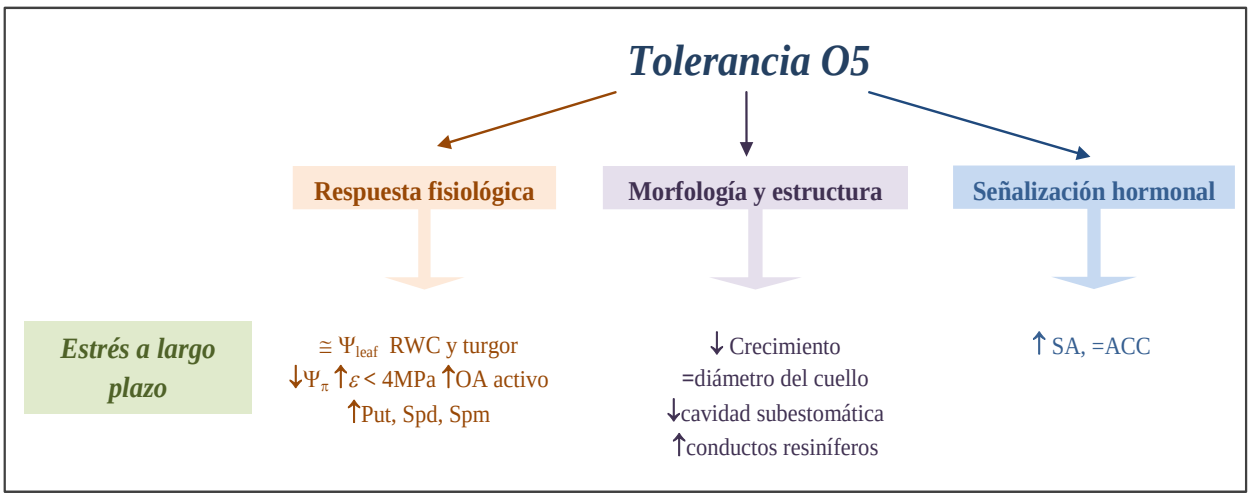
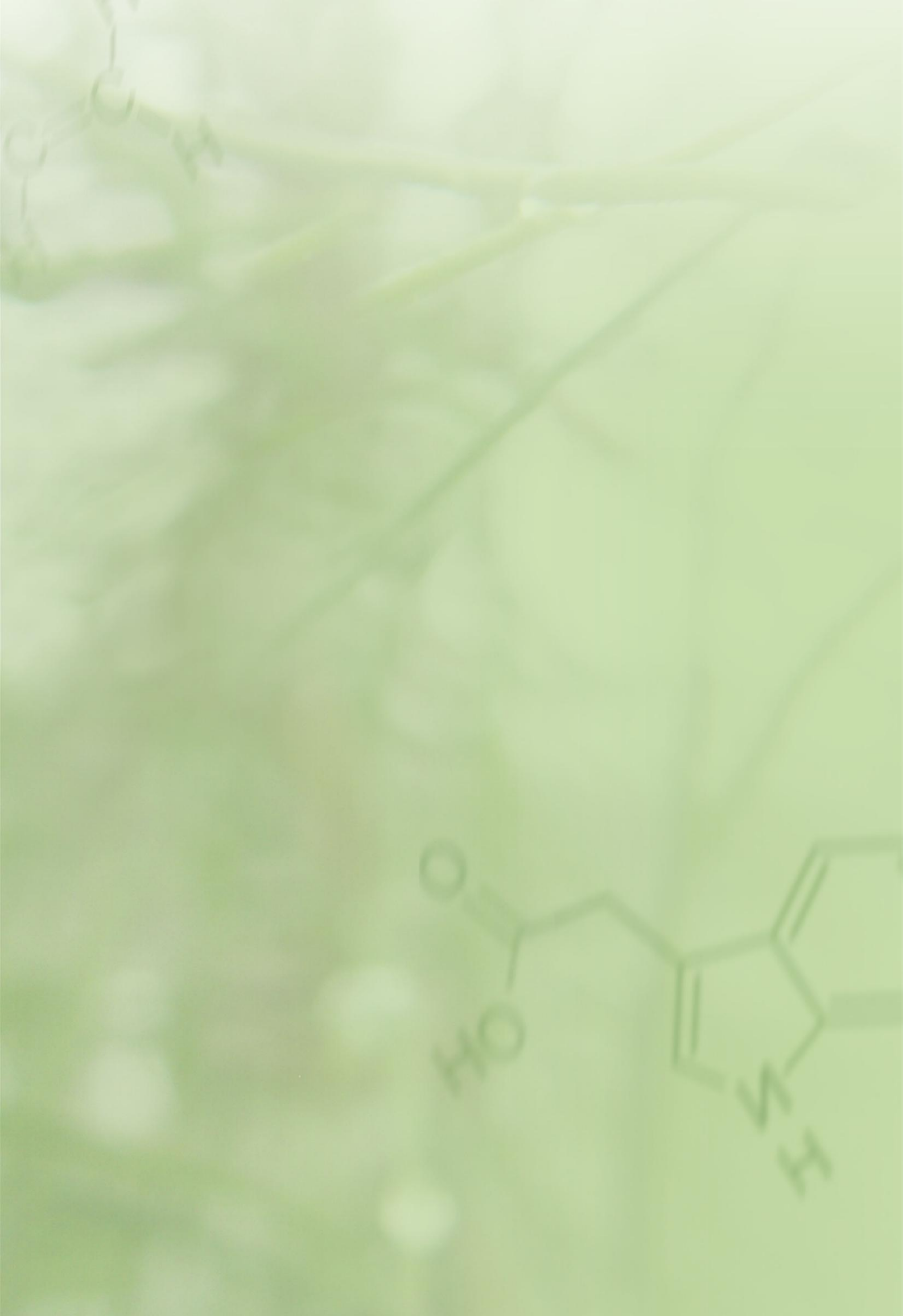


Figura II.2. Mecanismo de tolerancia propuesto en base al comportamiento del ecotipo O5 de *Pinus radiata* D. Don condiciones de estrés hídrico.

III.-Conclusiones/Conclusions



III.1-Conclusiones

1. La respuesta de *Pinus radiata* al estrés hídrico varió a nivel intraespecífico, de manera que cada ecotipo mostró diferente grado de tolerancia, recuperación y aclimatación, siendo O4 y O5 los ecotipos más tolerantes.

2. Los ecotipos O4 y O5 mostraron un comportamiento isohídrico ya que mantuvieron sus niveles de potencial hídrico en hoja por encima del punto de turgor (Ψ_{leaf} = -2 MPa), y presentaron una regulación de la conductividad hidráulica y un control estomático más eficiente, lo que retrasó la disminución de su contenido hídrico relativo.

3. Las citoquininas actuaron como primera señal hormonal de estrés y su descenso estuvo relacionado con una pérdida de conductividad hidráulica y cierre estomático. Cuando el contenido de citoquininas disminuyó un 65%, las plantas comenzaron a acumular IAA y ABA.

4. El IAA explicó un 66% de la varianza durante el primer ciclo de sequía y un 99% durante el endurecimiento, siendo así la fitohormona más significativa del modelo experimental, y un buen indicador del estado hídrico de la planta.

5. Existió una respuesta inversa entre los niveles de SA y JA, de manera que altos niveles de SA confirieron mayor tolerancia frente al estrés hídrico.

6. El incremento del módulo de elasticidad de las paredes celulares contribuyó a la tolerancia de las plantas frente al déficit hídrico, aunque valores superiores a 4 MPa se asociaron a alteraciones de las propiedades de las membranas y pérdida de turgencia.

7. El ajuste osmótico "activo" fue uno de los principales mecanismos de tolerancia y capacidad de endurecimiento de las plantas, siendo los carbohidratos solubles los osmolitos con mayor contribución al mismo.

8. Las plantas incrementaron sus niveles de Glu y GABA, además de su contenido en Pro como respuesta al estrés hídrico. Sin embargo, tras el endurecimiento, la Pro fue mejor indicador de la capacidad de aclimatación a la sequía.

9. Las características estructurales de las hojas como una menor cavidad subestomática, un mayor tamaño de células xilemáticas, así como un aumento de los canales resiníferos confirieron a la planta mayor tolerancia frente al estrés.

III.2-Conclusions

1. The *Pinus radiata* responses varied at intraspecific level, and each ecotype showed different levels of tolerance, recovery and conditioning against water stress, being O4 and O5 the most tolerant ecotypes.

2. O4 and O5 ecotypes showed an isohydric behaviour because they maintained their leaf water potential over the *TLP* (Ψ_{leaf} = -2 MPa), presented more efficient regulation of hydraulic conductance and stomatal control that delayed the RWC decrease.

3. Cks acted as first hormonal signal of stress and their decreases were related to a loss of hydraulic conductance and stomatal closure. When the cytokinin content decreased to 65%, plants started to accumulate IAA and ABA.

4. IAA explained a 66% variance along the first drought cycle and 99% after hardening, being the most significant phytohormone of the experimental model and good indicator of the plants water status.

5. There was an inverse response between SA and JA so high SA levels conferred high protection against water stress.

6. The cell wall elastic modulus increases contributed to plant tolerance against water deficit, although values over 4 MPa were associated with membrane alteration properties and turgor loss.

7. The active osmotic adjustment was one of the main tolerance mechanisms and plant hardening capacity, being the soluble carbohydrates the solutes with the highest contribution.

8. Plants increased their Glu and GABA levels, as well as Pro as water stress response. However, after hardening Pro was better indicator of drought conditioning.

9. The structural characteristics such as a lower substomatal chamber, a high xylem cell area, and an increase of resin duct size gave plants a higher stress tolerance.

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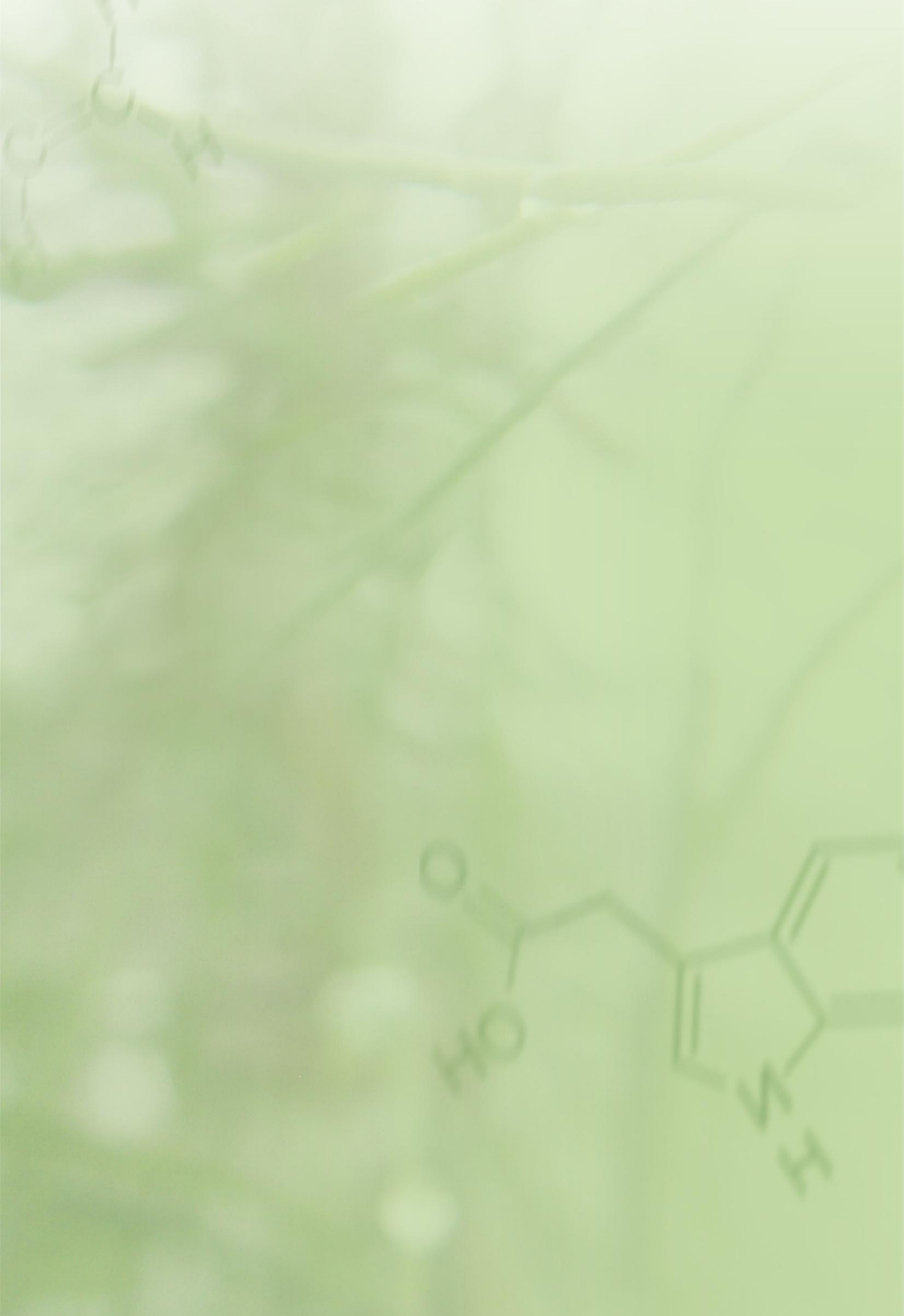

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