FACULTAT DE BIOLOGIA

DEPARTAMENT DE BIOLOGIA VEGETAL

Flavan-3-ol and ascorbate accumulation and oxidation in plants, and its physiological significance

---

Acumulación y oxidación de flavan-3-oles y ascorbato en plantas, y su significado fisiológico

Iker Hernández

Barcelona, 2007

Flavan-3-ol and ascorbate accumulation and oxidation in plants, and its physiological significance

---

Acumulación y oxidación de flavan-3-oles y ascorbato en plantas, y su significado fisiológico

Memoria presentada por Iker Hernández para optar al título de Doctor en el programa de doctorado "La fisiologia de les plantes i l'ambient", bienio 2002-2004, del Departament de Biologia Vegetal de la Facultat de Biologia de la Universitat de Barcelona. Este trabajo ha sido realizado en dicho Departamento y en el Instituto de Investigación sobre el Medio Ambiente y la Sostenibilidad de Newcastle Upon Tyne (IRES Newcastle) del Reino Unido bajo la dirección del Dr Sergi Munné y la Dra Leonor Alegre.

Codirector	Codirectora	Autor
Dr Sergi Munné	Dra Leonor Alegre	Iker Hernández

“If you march your Winter Journeys, you will have your reward, so long as all you want is a penguin’s egg”

Cherry Apsley-Garrard

Discovery expedition to South Pole (1901-1904)

ACKNOWLEDGEMENTS

First and most important, I am indebted to the supervisors of this work, Dr Sergi Munné and Dr Leonor Alegre. They have always represented two sides of the scientific work. On one hand, Dr Munné has led me through the most active and pioneering paths of plant science. And on the other, Dr Alegre has always represented the critical view of plant science as a whole concept. Together, they have supervised the present work by providing me with the best proportion of independence and guidance. Thanks so much Sergi and Henar!

I am also very grateful to Dr Jeremy D Barnes and Dr Anne M Borland, who supervised my stage at the IRES Newcastle. They provided me with nearly unlimited independence to develop my research interests and always appeared open to new ideas. Thanks so much!

There are three lab mates whose influence is reflected in the present work. First, I am very grateful to Tana Jubany, THE lab mate who I have had the most transcendental philosophical conversations with. Dr Tahar Taybi has been key in all experiments performed in tobacco, and he has taught me everything I know about molecular biology. Last, but not least, Holly Smith helped me with all social issues during my stage in Newcastle, including those seminars that lasted until 4 am…

I am also very grateful to the staff of the Services of the Experimental Fields of the UB, especially to Josep Matas, who helped me grow all kinds of plants, tuning devices, etc.

The Chromatography section of the ”Serveis Científic-Tècnics” of the UB, where I performed all HPLC and HPLC-MS experiments, deserves a special mention, especially Dr Olga Jáuregui, Dr Maria Reixach, Teresa Barajas and Dr Isidre Casals for their expertise and advice. Most of the experimental part of this PhD thesis would not have been possible without their help.

I would also like to state my gratitude to the lab mates Beth, Cristina C, Eva C, Fagua, Gemma, Guillem, Iker, Laura, Llorenç, Luisinho, Marta L, Marta P, Pere, Ricard, Sam, Santi, and every person at the Plant Biology Department of the Faculty of Biology at the UB; and Alberto, David, Eduardo T, Elena, Eva F, Igor, Karen, Marta Lopes, Mercè, Mireia, Montse T, Nuria, Rubén, Sergi V and Toni: former members of the mentioned Department.

I keep an especially sweet memory of my stage in Newcastle thanks to the great people I met at the EMPP and the Gatehouse labs: Mirela, Adelina, Chris C, Chris M, Chrysta, Collin, Cristina P, Dodmani, Edna, Eleni, Eva, Evan, Maria, Nacho, Nikos, Petra, Raúl, Sajjad, Simon, Sue, Tahar and Vicky.

There is life beyond the laboratory, and I am very grateful to Cristina, my partner, for sharing with me the best and the worst moments during this time. Although I have made my home wherever I have been working, I would say that the hardest work during this time has been to be away from home. Thus, I am very grateful to my parents, brothers and friends –especially Ángel and Edu- for helping me from Gasteiz.

I am also indebted to the Department of Education, Research and Universities of the Basque Government for the financial support of the PhD grant I was awarded, and the EU Marie Curie Training Network HPMT-DT-2001-00219.

These studies were also supported by the Spanish Government (projects no. BOS2003-01032 and BFU2006-01127).

Thanks all!

TABLE OF CONTENTS

RESUMEN (abstract in Spanish) 1

ABSTRACT 25

INTRODUCTION 33
1.- Flavonoids 35
1.1- Biochemical features of flavonoids 35
1.2.- Flavonoids in plants 38
1.3.- Subcellular compartmentation of flavonoids 38
1.4.- Functions of flavonoids 39
1.5.- Flavonoids and photoprotection 40
1.6.- Antioxidant flavonoids 42
1.7.- Flavan-3-ols 46
1.8.- Other phenolics 47
2.- Ascorbate 49
2.1- Biochemical features of ascorbate 49
2.2.- Subcellular compartmentation of ascorbate 50
2.3.- Functions of ascorbate 51
2.4.- Ascorbate as an antioxidant 51
3.- Plant responses to stress 53
3.1.- Stress concept in plants 53
3.2.- Water deficit 53
3.3.- High light 54
3.4.- Oxidative stress in plants 55
3.4.1.- Oxidative stress in the apoplast 57
3.4.2.- Oxidative stress in the symplast 58
3.4.3.- Oxidative stress signaling 59
3.5.- Plant responses to stress as affected by age 61

4.- Choosing the adequate experimental model 64

OBJECTIVES 65

MATERIALS AND METHODS 69
1.- Plant material 71
1.1.- Mediterranean plants 71
1.2.- Tea plant [*Camellia sinensis* (L.) Kuntze] 74
1.3.- Tobacco (*Nicotiana tabacum* L.) 76
2.- Brief description of the core experiments 78
2.1.- Comparative study of total phenolic levels in *M. officinalis*, *S. officinalis* and *C. clusii* as affected by drought 78
2.2.- Drought-induced changes in flavonoids and ascorbate in field-grown *C. clusii* 78
2.3.- Accumulation and oxidation of flavan-3-ols and ascorbate in *C. clusii* as affected by plant age 78
2.4.- Effects of water deficit on flavan-3-ol accumulation and oxidation in tea plants 79
2.5.- Apoplastic oxidation state as a signal 79
3.- Stress indicators 80
3.1.- Monitoring environmental conditions 80
3.2.- Water relations 80
3.3.- Photosynthesis 80
3.4.- Chlorophyll and total carotenoid content 81
3.5.- Chlorophyll fluorescence 81
3.6.- Lipid peroxidation 82

4.- Studies on flavonoids 83
4.1.- Determination of total phenolics 83
4.2.- Determination of total anthocyanidins 83
4.3.- Analyses of flavonols and flavan-3-ols 84
4.4.- Determination of flavan-3-ols and their quinones 90
4.5.- Total proanthocyanidins 93
4.6.- Determination of flavan-3-ols in chloroplasts 93
4.6.1.- Chloroplast isolation 93
4.6.2.- Chloroplast purity 94
4.6.3.- Chloroplast integrity 94
5.- Studies on ascorbate 95
5.1.- Determination of ascorbate in whole leaves 95
5.2.- Determination of ascorbate in the apoplast and symplast of tobacco leaves 95
5.3.- Ascorbate oxidase 96
5.4.- Analyses of nucleic acids 97
5.4.1.- Total RNA and mRNA isolation 97
5.4.2.- Subtractive hybridisation library construction 98
5.4.3.- Gene expression analyses 99
5.4.4.- DNA and RNA gels 100
6.- Statistical analyses 102
7.- Timing of the experiments 103

RESULTS 105
1.- Comparative study of total phenolic levels in *M. officinalis*, *S. officinalis* and *C. clusii* as affected by drought 107
1.1.- Characterization of the stress imposed 107
1.2.- Changes in total phenolics and anthocyanidins 110

2.- Drought-induced changes in flavonoids and ascorbate in *C. clusii* 113

2.1.- Analysis of oxidative stress indicators 113

2.2.- Identification of antioxidant flavonoids in *C. clusii* leaves and their changes under water deficit 114

2.3.- Drought-induced changes in ascorbate 116

3.- Accumulation and oxidation of flavan-3-ols and ascorbate in field-grown *C. clusii* as affected by plant age 118

3.1- Oxidative stress markers as affected by plant age, high light and water deficit 120

3.2- Flavan-3-ol accumulation and oxidation in response to high light 122

3.3- Flavan-3-ol accumulation and oxidation as affected by plant age 126

3.4.- Determination of flavan-3-ols in chloroplasts 126

3.5- Antioxidant function of ascorbate in seasonal and diurnal timescales 127

3.6- Ascorbate oxidation in response to high light and water deficit as affected by plant age 130

4.- Effects of water deficit on flavan-3-ol oxidation in tea plants 131

4.1.- Characterization of the stress imposed 131

4.2.- Changes in flavan-3-ols and their quinones along the water stress treatment 134

4.3.- Accumulation of proanthocyanidins along the water stress treatment 137

5.- Effects of apoplast ascorbate oxidation state on apoplast-symplast signaling 139

5.1.- Effects on physiological parameters 139

5.2.- Effects on nuclear gene expression 144

DISCUSSION 151

1.- Flavonoids 153

1.1.- Changes in total phenolic levels in *M. officinalis*, *S. officinalis* and *C. clusii* as affected by drought 153

1.2.- Identification of flavonoids in *C. clusii* 156

1.3.- Effects of water deficit and high light on flavan-3-ol accumulation 159

1.4.- Effects of water deficit and high light on flavan-3-ol oxidation 165

1.5.- Effects of plant age on *C. clusii* responses to water deficit and high light stress 166

1.6.- Effects of plant age on flavan-3-ol accumulation and oxidation 167

2.- Ascorbate 169

2.1.- Effects of water deficit and high light on ascorbate accumulation and oxidation 169

2.2.- Effects of plant age on ascorbate accumulation and oxidation 170

2.3.- Apoplastic ascorbate oxidation state as a signal 170

CONCLUSIONS 177

REFERENCES 181

RESUMEN

A lo largo de la historia, la investigación sobre los flavonoides ha ido ligada a algunos de los avances científicos más importantes, como los primeros trabajos de la herencia de los caracteres, el descubrimiento de los transposones y las propiedades ácido-base de los pigmentos florales. Los flavonoides son un grupo de metabolitos secundarios muy heterogéneo. Derivan de las vías del shikimato y del malonato. La estructura básica de cualquier flavonoide consiste en dos anillos de benceno unidos por una cadena de tres carbonos, es decir, una estructura C_6-C_3-C_6. Se pueden encontrar flavonoides prácticamente en cualquier órgano vegetal, e incluso en secreciones como las ceras, exudados radiculares y nódulos de leguminosas. La síntesis de flavonoides está ligada a la cara citosólica de la membrana del retículo endoplasmático, aunque recientemente también se han encontrado enzimas de biosíntesis de flavonoides en el núcleo de *Arabidopsis thaliana*. Se considera que los enzimas de biosíntesis de flavonoides se disponen en complejos multienzimáticos anclados a la membrana del retículo endoplasmático, dando lugar a un fenómeno conocido como canalización de metabolitos, que consiste en la competencia de los enzimas de las diferentes ramas de una vía, por los precursores comunes. Esta canalización de metabolitos es la causa de que el patrón de flavonoides de cada especie en un momento de desarrollo y estado fisiológico concreto sea único (o por lo menos característico). La mayoría de las funciones de los flavonoides descritas hasta ahora están relacionadas con las interacciones planta-patógeno, ya sea con bacterias, hongos, insectos o herbívoros. Algunos flavonoides están implicados en procesos de reconocimiento de simbiontes y en fenómenos de alelopatía. Además, los flavonoides presentan espectros de absorción UV-visible con máximos de absorbancia entre el rango visible y el UV-B, lo que les confiere propiedades fotoprotectoras. En algunos casos, estas propiedades ópticas confieren coloración a los

flavonoides, como en el caso de las antocianidinas, que son responsables de las coloraciones malva-azuladas de muchas flores y la coloración rojiza de muchas hojas en senescencia. También se ha postulado que los fenil propanoides, entre ellos los flavonoides, pueden servir de sumidero de carbono reducido y equivalentes de reducción alternativo al ciclo de Calvin. Los flavonoides han sido objeto de estudio intensivo en las últimas décadas por sus propiedades beneficiosas para la salud humana. Estas propiedades se atribuyen principalmente a la elevada capacidad antioxidante *in vitro* de los flavonoides, sobre todo de los flavan-3-oles, que son los principales responsables de las propiedades beneficiosas del te, el vino, el zumo de arándanos, el chocolate, etc. Se conoce la función *in planta* de este grupo de compuestos cuando se presentan en forma de polímeros, también llamados proantocianidinas o taninos condensados. Las proantocianidinas están implicadas en los mecanismos de defensa de las plantas frente a estreses bióticos y en el endurecimiento de los tegumentos de las semillas. Sin embargo, el proceso de polimerización no se conoce en detalle, ya que la estereoisomería y la composición de las moléculas de proantocianidinas son muy complejas.

El ácido ascórbico es un carbohidrato que puede llegar al 10 % del contenido total de carbohidratos de una célula. No obstante, la vía de síntesis de este compuesto todavía es objeto de estudio y debate, dado que existen varias rutas dependiendo de la especie, órgano, tejido, estado de desarrollo o estado fisiológico. El ascorbato tiene una gran tendencia a donar electrones, obteniéndose como resultado un producto de oxidación muy estable, el dehidroascorbato, lo que le confiere la capacidad de actuar como antioxidante. El ascorbato es el antioxidante más abundante en las plantas, y se encuentra en todos los compartimentos celulares, tejidos y órganos, salvo

en las semillas en dormición. Además, los productos de oxidación del ascorbato se pueden reciclar con la intervención de enzimas y otras fuentes de poder reductor, como la ferredoxina, el NADPH o el citocromo *b*. La acción del ascorbato sirve para detoxificar especies reactivas del oxígeno (ROS), principalmente peróxido de hidrógeno (H_2O_2) y anión superóxido, en cualquier compartimento celular. Sin embargo, en el caso del apoplasto, el reciclaje del ascorbato requiere el transporte de dehidroascorbato a través de la membrana plasmática, que posteriormente es reducido a ascorbato en el apoplasto. También se conoce la implicación del ascorbato en otros procesos como la progresión a lo largo del ciclo celular, la señalización celular y la regulación de la actividad enzimática, sin que su capacidad antioxidante esté directamente relacionada.

Generalmente se considera que las plantas se encuantran en condiciones de estrés cuando uno o más factores se desvía significativamente del óptimo para el crecimiento y desarrollo vegetal. Los factores de estrés se pueden clasificar, según su origen, como factores bióticos o abióticos. Entre los primeros se encuentran por ejemplo la invasión por hongos patógenos, las plagas de insectos o el herbivorismo. Entre los segundos, las bajas temperaturas, la falta o el exceso de agua, la salinidad y los contaminantes. La mayoría de los factores de estrés ejercen su influencia negativa en la fisiología de las plantas causando estrés oxidativo entre otros efectos.

Cualquier metabolismo aerobio, incluyendo la fotosíntesis y la respiración, presentan una formación intrínseca de ROS, que son extremadamente reactivas y tienden a oxidar otros compuestos celulares como ácidos nucleicos, proteínas, carbohidratos o lípidos, alterando su función. A concentraciones bajas, las ROS actuan como señales celulares en procesos de senescencia, señalización hormonal y defensa ante patógenos y ante estreses abióticos. Sin embargo, cuando la formación de ROS supera la

capacidad de los sistemas antioxidantes, pueden causar daño oxidativo oxidando diferentes componentes celulares.

En el simplasto, los cloroplastos son orgánulos muy sensibles al estrés oxidativo, dadas las elevadas tensiones de O_2 (por la fotólisis del agua en el fotosistema II) y por los fenómenos de transferencia de energía y electrones en las membranas de los tilacoides. La formación de ROS en los cloroplastos ocurre principalmente en los fotosistemas y las pantallas colectoras de luz. Los peroxisomas son la mayor fuente de ROS, especialmente de H_2O_2, en las plantas, que se forman como subproducto de determinadas reacciones. En las mitocondrias también se forman ROS, cuando el O_2 actúa como aceptor final en la cadena transportadora de electrones. Las ROS pueden estar también presentes en otros orgánulos bien porque se formen en ellos, bien porque difundan desde otros.

El apoplasto es otra fuente importante de ROS en plantas. En el apoplasto, la formación de ROS puede ser inducida por la actividad de NADPH oxidasas y peroxidasas como mecanismo necesario para la lignificación, formación de aerénquima o la respuesta hipersensitiva. La formación de ROS en el apoplasto también puede ser debida a la acción de agentes oxidantes externos, como los contaminantes atmosféricos dióxido de azufre (SO_2) y ozono (O_3).

Como se menciona anteriormente, el estrés oxidativo puede ser un factor subyacente a diferentes factores de estrés. Por ejemplo, cuando las plantas cierran los estomas para evitar pérdidas de agua por transpiración cuando la disponibilidad hídrica es baja, la limitación estomática de la fotosíntesis puede incrementar la formación de ROS debido a la saturación de la cadena transportadora de electrones fotosintética como consecuencia de la reducción en la velocidad del ciclo de Calvin y la consecuente falta de equivalentes de reducción oxidados. El exceso de luz también induce estrés oxidativo, bien

solo, bien en combinación con otros factores de estrés, como el déficit hídrico, las bajas temperaturas o la salinidad. En general, la mayoría de estreses abióticos hacen menos eficientes los mecanismos de captación y transformación de la energía solar, haciendo que la radiación incidente sea excesiva.

Recientemente se han considerado las ROS y otros radicales libres no sólo como subproductos con efectos negativos para el metabolismo celular, sino también como señales. Para ello, la señal tiene que ser percibida y transducida y la respuesta ejecutada. Actualmente se conocen elementos que ejercen estas funciones en respuesta a las especies reactivas del oxígeno, aunque las rutas completas de percepción y transducción de señales todavía no están del todo definidas.

El envejecimiento se define como la acumulación de cambios en el desarrollo, responsables de la alteración lenta, progresiva y secuencial de las funciones vitales que ocurre en las plantas con el paso del tiempo. Es importante diferenciar envejecimiento de senescencia. La senescencia se considera el proceso biológico que conduce a la muerte de la célula, órgano o planta entera. Por tanto, la senescencia es la última fase del proceso de envejecimiento. Existen dos posibles tipos de factores causantes del envejecimiento: intrínsecos y extrínsecos. Entre los primeros se encuentran los cambios en los meristemas por las repetidas dividiones celulares, el acortamiento de los telómeros, etc. y entre los segundos, el déficit hídrico, deficiencias nutricionales, etc. Teniendo todo esto en cuenta, las causas subyacentes al proceso del envejecimiento en plantas son complejas, y actualmente son objeto de debate.

Las plantas mediterráneas están adaptadas a condiciones de alta radiación solar y déficit hídrico que coinciden cíclicamente en los meses de verano. En estas condiciones, muchas de estas plantas viven durante periodos de tiempo

muy largos. Estas plantas, además, presentan elevados niveles de compuestos cuya función es favorecer la resistencia a diversos estreses, como por ejemplo el ascorbato. Estas características hacen que las plantas mediterráneas sean un buen modelo para los estudios que se describen en esta tesis. Los estudios en plantas mediterráneas (*C. clusii*, *S. officinalis* y *M. officinalis*) combinados con otros en plantas modelo (tabaco) y plantas de te (que se caracterizan por el elevado contenido en flavan-3-oles, sensibilidad al déficit hídrico e importancia agronómica), constituyen un conjunto adecuado para el estudio de la acumulación y oxidación de flavan-3-oles y ascorbato, y sus significado fisiológico en plantas.

El objetivo final del presente trabajo es entender el significado fisiológico de la acumulación y oxidación de flavan-3-oles y ascorbato en plantas. Para abordar este objetivo, el presente trabajo se centró en:

1.- Esclarecer los efectos de los estreses ambientales en la acumulación y oxidación de flavonoides

1.1.- Identificar los flavonoides que se acumulan en *C. clusii* y plantas de te ante un tratamiento de déficit hídrico en condiciones de campo

1.2.- Determinar cuál de los dos siguientes factores, la elevada radiación solar o el déficit hídrico, induce la acumulación de flavonoides en condiciones de campo

1.3.- Identificar productos de oxidación de flavan-3-oles *in vivo*

1.4.- Determinar si los flavan-3-oles efectivamente se oxidan *in vivo* a dichos productos de oxidación, ante el estrés hídrico o lumínico

2.- Estudiar el significado fisiológico de los flavan-3-oles y el ascorbato en las respuestas de las plantas al estrés ambiental, y la influencia del envejecimiento en estos procesos

2.1.- Determinar si el envejecimiento influye en la magnitud del estrés oxidativo experimentado por las plantas en condiciones de estrés hídrico y/o estrés lumínico

2.2.- Estudiar las diferencias en la acumulación de ascorbato y flavan-3-oles en plantas de diferentes edades en condiciones óptimas, estrés hídrico y/o estrés lumínico

2.3.- Estudiar los efectos del envejecimiento en la oxidación del ascorbato y los flavan-3-oles en plantas de *C. clusii* sometidas a elevada radiación y déficit hídrico

3.- Evaluar la posible función del estado de oxidación del ascorbato como señal celular

3.1.- Determinar los efectos de la alteración del estado de oxidación del ascorbato en el apoplasto, sobre los principales enzimas de reciclaje del ascorbato en el citosol, a nivel de expresión génica

3.2.- Indentificar genes cuya expresión se ve afectada como consecuencia del cambio de estado de oxidación del ascorbato del apoplasto

3.3.- Identificar componentes de la vía de transducción de la señal del estado de oxidación del ascorbato en el apoplasto

Para abordar estos objetivos, se diseñaron 5 experimentos principales. En primer lugar, se analizaron los niveles de fenoles totales y los cambios que estos experimentaron durante un tratamiento de 50 días de estrés hídrico en condiciones de campo, en plantas de *Cistus clusii*, *Salvia officinalis* y *Melissa officinalis*. Como se describe posteriormente en detalle, *C. clusii* mostró los mayores niveles, y el mayor incremento de éstos tras el tratamiento de estrés, de las tres especies estudiadas. Por ello, se eligió esta especie para identificar flavonoides que se acumularan en hojas durante el

tratamiento de déficit hídrico mencionado, y comparar los niveles de estos flavonoides con los de ascorbato (segundo experimento).

Para el tercer experimento, se mantuvieron plantas juveniles (1 año de edad, no reproductivas) y maduras (6 años de edad) en condiciones de campo durante la primavera (elevada radicación), verano (elevada radiación y déficit hídrico) y otoño, para determinar el/los factor(es) que determina(n) la acumulación y oxidación de flavan-3-oles (monoméricos y proantocianidinas). Posteriormente se determinó si los flavan-3-oles de las hojas de *C. clusii* se acumulan en los cloroplastos.

Las plantas de te (*Camellia sinensis*), que se caracterizan por su elevado contenido en flavan-3-oles y que tienen un gran interés económico y social, son muy sensibles a la falta de agua y exceso de luz. Para el cuarto experimento, estas plantas fueron expuestas a las condiciones de campo del clima mediterráneo en verano (incluyendo déficit hídrico y elevada radiación) para estudiar la oxidación *in vivo* de los flavan-3-oles en condiciones de estrés.

Finalmente (quinto experimento), para determinar si el estado de oxidación del ascorbato actúa como señal celular, se estudiaron plantas transgénicas de tabaco con la expresión del gen que codifica para la ascorbato oxidasa sobreexpresado o reprimido. Se llevaron a cabo análisis de expresión de genes seleccionados *a priori* y se construyó una librería de hibridación substractiva para identificar genes cuya expresión estuviera alterada en estos transgénicos.

Durante los experimentos descritos, las condiciones climáticas se registraron paralelamente a los indicadores de estrés (peso específico foliar, hidratación foliar, contenido hídrico relativo foliar y eficiencia máxima del fotosistema II). Los análisis de clorofilas, carotenoides, fenoles totales, ascorbato y dehidroascorbato, proantocianidinas, peroxidación lipídica y actividad

ascorbato oxidasa se llevaron a cabo mediante técnicas espectrofotométricas. La pureza de los cloroplastos aislados se determinó por ensayos espectrofotométricos de la actividad de enzimas marcadores de los diferentes orgánulos, y su integridad se determinó por el método de reducción del ferricianuro. La identificación de flavonoides en hojas de *C. clusii* se llevó a cabo mediante cromatografía líquida de alta resolución. La determinación simultánea de flavan-3-oles y sus quinonas se realizó por cromatografía líquida de alta resolución acoplada a espectrometría de masas con ionización por electroespray. Los análisis de expresión génica se llevaron a cabo mediante técnicas basadas en la reacción en cadena de la polimerasa. Por un lado, los análisis de expresión de genes elegidos *a priori* se realizó mediante retrotranscripción seguida de reacción en cadena de la polimerasa, todo en una sola reacción (*one step* SQ-RT-PCR). Por otro lado, se construyó una librería de hibridación sustractiva mediante métodos también basados en la reacción en cadena de la polimerasa. Esta hibridación sustractiva consiste básicamente en hibridar un extracto problema de ARN total, con uno de referencia (control), de manera que los ARNs en exceso se amplifican y se secuencian.

El primer experimento demostró que en óptimo estado hídrico, el contenido en fenoles totales de *C. clusii* es mayor que el de *S. officinalis* y el de *M. officinalis* (89.84 μEq ácido tánico dm^{-2} en *C. clusii* frente a 22.95 y 17.12 μEq dm^{-2} en *S. officinalis* y *M. officinalis*, respectivamente). Además, el contenido en fenoles totales en hojas de *C. clusii* aumentó de *ca.* 90 a 238 μEq dm^{-2} a lo largo de un periodo de 50 días de déficit hídrico en condiciones de campo. *S. officinalis* mostró niveles menores de fenoles totales que sólo aumentaron ligeramente (de 23 a 42 μEq dm^{-2}) durante el tratamiento. *M. officinalis* mostró los niveles más bajos de fenoles totales

(17-24 μmol dm^{-2}), que además no variaron con el tratamiento de estrés. Dado que, entre estas tres especies, *C. clusii* mostró los mayores niveles de fenoles totales y que estos aumentaban con un tratamiento de déficit hídrico, se seleccionó esta especie para identificar flavonoides con capacidad antioxidante y analizar sus posibles variaciones durante un tratamiento de déficiti hídrico en condiciones de campo.

De entre los flavonoides analizados, el galato de (-)-epigalocatequina (EGCG) mostró las mayores concentraciones (hasta 5 μmol dm^{-2}). Otros flavan-3-oles como la (-)-epicatequina (EC) y el galato de (-)-epicatequina (ECG) mostraron concentraciones por debajo de 0.25 y 0.03 μmol dm^{-2}, respectivamente. Ninguno de los flavonoles analizados, quercetina y canferol, fue hallado como aglicona, es decir, sin ningún azúcar esterificado. Los niveles de EGCG, ECG y EC aumentaron progresivamente durante el tratamiento de déficit hídrico en condiciones de campo, alcanzando valores máximos (5, 0.03 y 0.25 μmol dm^{-2}, respectivamente) tras 30 días de estrés. La concentración de ascorbato se duplicó tras 15 días de estrés, y los valores máximos de ascorbato se alcanzaron tras 50 días de sequía (*ca.* 95 μmol dm^{-2}). La eficiencia máxima del fotosistema II y los niveles de peroxidación lipídica se mantuvieron constantes durante el periodo experimental, mientras que el estado de oxidación del ascorbato disminuyó (de *ca.* 0.45 a 0.18) debido al incremento de la forma reducida y la disminución de la forma oxidada, indicando, por tanto, que las plantas no sufrieron daño oxidativo.

En el tercer experimento, las plantas juveniles y maduras de *C. clusii* se sometieron a condiciones de campo en clima mediterráneo durante la primavera (elevada radiación), el verano (elevada radiación y déficit hídrico) y el otoño. Las plantas sufrieron una pérdida de agua moderada, alcanzando valores alrededor de 60 % de contenido hídrico relativo foliar, durante los meses de verano. Los marcadores de estrés oxidativo (eficiencia máxima del

fotosistema II y peroxidación lipídica) revelaron que las épocas más estresantes para la planta fueron el verano y la primavera. Aunque la magnitud del estrés no fue muy pronunciada, los indicadores de estrés oxidativo mostraron que éste fue más marcado en las plantas maduras que en las juveniles. Los niveles del principal flavan-3-ol en hojas de *C. clusii*, EGCG, aumentaron de 1.7 a 2.6 μmol dm^{-2} desde el principio del experimento (17 de mayo) a finales de junio. Posteriormente, los niveles de EGCG disminuyeron a niveles alrededor de 0.1 μmol dm^{-2} desde septiembre hasta el final del experimento (11 de noviembre). Las proantocianidinas incrementaron su concentración en hojas de *C. clusii* desde el principio del estudio hasta el momento de sequía más pronunciada (agosto) de 0.24 a 0.51 y de 0.34 a 0.53 μEq proantocianidina B2 dm^{-2} en plantas juveniles y maduras, respectivamente. Desde entonces, los niveles de proantocianidinas recuperaron progresivamente los valores iniciales. Ni los flavan-3-oles monoméricos ni sus quinonas ni las proantocianidinas mostraron ningún tipo de fluctuación diurna. Los niveles de EGCG mostraron una fuerte correlación positiva con los niveles de radiación máxima diaria (r^2=0.89 y 0.97 para plantas juveniles y maduras, respectivamente), mientras que esta correlación fue negativa y más baja con el contenido hídrico relativo foliar (r^2=0.33 y 0.70 para plantas juveniles y maduras, respectivamente). No hubo diferencias significativas estadísticamente en el contenido en EGCG entre plantas juveniles y maduras durante el experimento, aunque las plantas maduras tuvieron niveles ligeramente superiores a las juveniles durante el verano. Los niveles de quinona de EGCG en plantas maduras fueron mayores que en plantas juveniles durante todo el experimento, en general. Los niveles de proantocianidinas fueron mayores en las plantas maduras que en las juveniles, durante la primavera y el verano. Estos flavan-3-oles no fueron detectados en cantidades cuantificables en cloroplastos de *C. clusii*.

Los niveles de ascorbato aumentaron de *ca.* 230 a. 590 μmol dm^{-2} de la primavera al verano, recuperando los valores iniciales en otoño. Los valores de dehidroascorbato aumentaron rápidamente de 100 a 400 μmol dm^{-2} de principios de mayo a principios de junio. Posteriormente, los niveles de dehidroascorbato recobraron progresivamente los valores iniciales hasta septiembre, que volvieron a aumentar alcanzando valores similares a los mostrados a principios de junio. El estado de oxidación del ascorbato varió con los valores de ascorbato y dehidroascorbato. Sin embargo, independientemente de los niveles de estos compuestos y del estado de oxidación del ascorbato una hora antes del amanecer, el ascorbato al mediodía siempre estaba más oxidado, mostrando un estado de oxidación muy constante, alrededor del 40 %, tanto en plantas juveniles como en maduras. Los niveles de ascorbato y dehidroascorbato en plantas juveniles fueron ligeramente inferiores a los de plantas maduras. El estado de oxidación del ascorbato también fue ligeramente más oxidado en plantas maduras que en juveniles, en los meses de verano (julio y agosto).

En el cuarto experimento se expusieron plantas de te a las condiciones de campo de clima mediterráneo, para determinar si la oxidación de flavan-3-oles con elevada capacidad antioxidante es posible *in vivo*. Tras el experimento se determinaron los niveles de flavan-3-oles (monoméricos y quinonas) y proantocianidinas. La quinona de EC y la de EGCG incrementaron 10 y 30 veces su concentración, respectivamente, tras 19 días de tratamiento. La oxidación de EC y EGCG a sus respectivas quinonas precedió temporalmente a la acumulación de proantocianidinas, que aumentaron de 35 a 53 mg g peso $seco^{-1}$ después de 26 días de tratamiento. La formación de quinonas de flavan-3-oles (o lo que es lo mismo, el estado de oxidación de los flavan-3-oles) se correlacionó estrechamente

(correlación negativa, r^2=0.80 y 0.98, para el EGCG y la EC, respectivamente) con los niveles de peroxidación lipídica.

En el quinto experimento, las plantas de tabaco con el gen de la ascorbato oxidasa sobre expresado (*sense*) o reprimido (*antisense*) no mostraron diferencias significativas entre sí ni con el genotipo salvaje, en lo que respecta a producción de biomasa, tras 6 semanas de crecimiento. El contenido de ascorbato total en el simplasto también fue igual en los tres genotipos, alrededor de 2500 nmol g peso fresco^{-1}, de los cuales, entre el 10 y el 20 % era dehidroascorbato. El contenido de ascorbato en el apoplasto de las plantas *antisense* era mayor (*ca.* 100 nmol g peso fresco^{-1}) que en las salvajes y que en las *sense* (*ca.* 40 nmol g peso fresco^{-1}). La línea *sense* presentó un estado de oxidación del ascorbato apoplástico mayor que la salvaje y la *antisense* (80, 50 i 30 %), respectivamente. Asimismo, la actividad ascorbato oxidasa en la línea *sense* era mayor que en la *antisens*e y que en la salvaje, respectivamente (13, 1.2 i 0.5 nkat g peso fresco^{-1}, respectivamente). En las tres líneas de plantas de tabaco, la mayoría de los genes involucrados en el ciclo del ascorbato-glutation en el citosol mostraron niveles de expresión similares. Sin embargo, un componente de un canal de Ca^{2+} (que funciona como heterodímero) dependiente de H_2O_2 estaba fuertemente silenciado en las dos líneas transgénicas respecto a la salvaje. La librería de hibridación sustractiva mostró 17 ESTs (del inglés *expressed sequence tags*) expresados diferencialmente en las plantas *sense* respecto al salvaje, y 14 en las *antisense* respecto al salvaje. Entre estos ESTs se encontraron genes involucrados en procesos de regulación, estrés, fotorrespiración, asimilación de N, transporte de electrones y otros con funciones desconocidas.

Aunque los compuestos fenólicos se consideran metabolitos secundarios, algunos fenoles cumplen funciones claramente dentro del metabolismo primario, como por ejemplo las plastoquinonas. Además, la segunda molécula más abundante en plantas, sobre todo en plantas leñosas perennes, es la lignina, que es un polifenol. Por lo tanto, no sorprende el hecho de que gran parte del peso de las hojas sea de origen fenólico, como se observa en *C. clusii*. Independientemente del papel que pueda desempeñar individualmente cada fenol, se ha postulado que los fenil propanoides pueden actuar como sumidero de carbono reducido y equivalentes de reducción, alternativo al ciclo de Calvin.

El contenido de fenoles totales de las hojas de *C. clusii* mostró una correlación mejor con el contenido hídrico relativo foliar cuando se expresaba por unidad de área foliar, que cuando se expresaba por unidad de peso, lo que sugiere un efecto de la luz en la acumulación de fenoles. Sin embargo, no se pueden descartar otras funciones de los fenoles como la formación de cutículas, resistencia a estrés biótico o endurecimiento de paredes celulares, que son importantes en condiciones de estrés.

Muchos compuestos fenólicos presentan actividades antioxidantes *in vitro* muy elevadas, de manera que el incremento de su concentración podría proteger las plantas de *C. clusii* ante el estrés oxidativo. Debido a su resistencia a la sequía y al aumento que experimentan los niveles de fenoles totales ante esta situación, se eligió *C. clusii* como material vegetal para estudiar la acumulación y oxidación de flavonoides con capacidad antioxidante en condiciones de déficit hídrico en condiciones de campo.

El tratamiento de 50 días de déficit hídrico en condiciones de campo no afectó negativamente las funciones bioquímicas de las hojas de *C. clusii*, como muestran los niveles de oxidación lipídica y la eficiencia máxima del fotosistema II. Estudios anteriores han demostrado la presencia de flavan-3-

oles en especies del género *Cistus*. En el presente estudio se demuestra la presencia de los flavan-3-oles EGCG, EC y ECG en hojas de *C. clusii* en concentraciones de hasta 5, 0.25 y 0.03 μmol dm^{-2}, respectivamente. En concordancia con estudios anteriores, no se encontraron ni quercetina ni canferol en forma de aglicona. Aunque la localización subcelular de estos compuestos está por determinar, son relativamente lipófilos, de manera que podrían estar asociados a membranas. Además, estos flavan-3-oles pueden polimerizar y acumularse como proantocianidinas en las vacuolas. Los niveles de EC, ECG y EGCG aumentaron significativamente en plantas de *C. clusii* sometidas a déficit hídrico en condiciones de campo. Hasta ahora, sólo se habían descrito incrementos así en plantas de te y dos especies del género *Crataegus*.

Durante los primeros 15 días de tratamiento de estrés, el incremento en los niveles de flavan-3-oles fue debido, por lo menos parcialmente, a los cambios morfológicos que sufrieron las hojas de *C. clusii,* como el incremento del peso específico foliar. En cambio, después de estos primeros 15 días, el peso específico foliar permaneció constante, mientras que los niveles de flavan-3-oles continuaron incrementándose.

Se ha sugerido que los flavan-3-oles actuan como antioxidantes en plantas. Los flavan-3-oles EC, ECG y EGCG interrumpen eficientemente reacciones de oxidación en cadena, como la oxidación lipídica, y quelan metales de transición que pudieran favorecer las reacciones de Fenton y Haber-Weiss. El hecho de que los flavan-3-oles sean sólo un pequeño porcentaje de los fenoles totales indica que en las situaciones estudiadas, la activación de la ruta de los fenil propanoides tiene lugar a nivel de las primeras reacciones de la vía (e.g. fenilalanina amonio liasa y calcona sintasa).

Las funciones que los flavan-3-oles pueden tener en las plantas no son mutuamente excluyentes, de manera que aunque, por ejemplo, las

proantocianidinas puedan servir como sumidero alternativo de carbono fotosintético, también pueden ejercer su función en la resistencia a estreses bióticos.

Como se discute más adelante, el ascorbato también aumentó paralelamente a los fenoles totales. Esto puede ser debido a una posible interacción entre ambos, dado que los radicales fenoxil (primeros productos de oxidación de los fenoles) pueden ser reciclados *in vitro* por el ascorbato.

Durante el experimento con plantas juveniles y maduras de *C. clusii*, las plantas estuvieron sometidas a condiciones de campo desde mayo hasta noviembre, de manera que sufrieron la típica sequía del clima mediterráneo en los meses de verano. El estrés oxidativo causado por las condiciones ambientales adversas fue más pronunciado en el verano (elevada radiación y déficit hídrico), seguido por la primavera (elevada radiación) y el otoño, respectivamente. Los niveles de EGCG, el principal flavan-3-ol en hojas de *C. clusii*, alcanzaron valores máximos alrededor del solsticio de verano, cuando la radición solar al mediodía es mayor y el fotoperiodo más largo que en cualquier otro momento del año. Además, este incremento de EGCG se correlacionó fuertemente con la máxima radiación solar diaria.

Considerando conjuntamente los datos de este experimento con los del experimento anterior (tratamiento de déficit hídrico a plantas de *C. clusii* en condiciones de campo), normalizados para cada caso experimental (año de muestreo y edad de la planta), se aprecia claramente que la acumulación de EGCG responde a la luz incidente (cantidad o fotoperiodo), más que al contenido hídrico foliar. Esta acumulación se desencadena cuando la radiación máxima diaria supera los 1800 μmol m^{-2} s^{-1} o las 13 horas de luz. El hecho de que en el clima mediterráneo el periodo de máxima radiación coincida con el déficit hídrico, hace que los efectos que causan estos factores

puedan confundirse, pudiendo desencadenar respuestas independientes y/o comunes.

Existen muchos trabajos que demuestran que la acumulación de flavonoides está inducida por radiaciones UV. Sin embargo, se requieren experimentos específicos para demostrar que los flavan-3-oles protegen las plantas de estas radiaciones, dado que estos compuestos absorben las radiaciones UV-C (máximos de 275 nm), y que estas radiaciones difícilmente alcanzan la superfície terrestre. Por otro lado, aunque el control fotoperiódico de la síntesis de flavan-3-oles no se ha estudiado, se sabe que otros flavonoides, las antocianinas, presentan este tipo de regulación.

Las plantas de te en condiciones de campo en el clima mediterráneo sufrieron una fuerte pérdida de agua y una fotoinhibición crónica. En estas condiciones, los niveles de EGCG y EC permanecieron constantes. Estos resultados, comparados con otros de la bibliografía, sugieren que dependiendo de la magnitud, duración e intensidad del estrés impuesto, los niveles de flavan-3-oles pueden aumentar o permanecer constantes.

En este estudio se demuestra por primera vez cómo los flavan-3-oles, EC y EGCG, son oxidados a sus respectivas quinonas en condiciones de estrés (alta radiación combinada con déficit hídrico, en este caso). Los niveles de las quinonas de EC y EGCG mostraron una correlación negativa muy alta con los niveles de peroxidación lipídica, lo que sugiere que la oxidación de los flavan-3-oles monoméricos protege las membranas de la oxidación. Esto concuerda con el hecho de que estos flavan-3-oles se puedan integrar profundamente (tanto o más que el α-tocoferol) en membranas modelo. La formación de estas quinonas puede ocurrir por la oxidación no enzimática de los flavan-3-oles reducidos, o mediante enzimas como peroxidasas (que requieren H_2O_2) u oxigenasas (que requieren O_2). Por lo tanto, la oxidación de EC y EGCG puede ser considerada como un simple mecanismo dentro

del metabolismo oxidativo, por ejemplo para formar proantocianidinas, que adicionalmente puede tener una actividad antioxidante relevante en endomembranas.

En el presente estudio se describe cómo en plantas de te, la oxidación de EC y EGCG a sus respectivas quinonas precede la acumulación de proantocianidinas. Aunque no se conoce en detalle el proceso de polimerización de flavan-3-oles para formar proantocianidinas, está ámpliamente reconocido que este proceso requiere un metabolismo oxidativo, por lo menos en las semillas.

Aparentemente, la oxidación de EC y EGCG a sus respectivas quinonas no depende únicamente del estrés oxidativo, dado que esta oxidación sólo ocurre en plantas de te, y no en plantas de *C. clusii* sometidas a diferentes condiciones e intensidades de estrés, lo que hace pensar que dicha oxidación requiere algún factor adicional que se da en plantas de te, y no en *C. clusii*.

En cambio, aunque se requieren estudios en profundidad para confirmarlo, las plantas maduras presentaron niveles de quinonas de EGCG y EC mayores que las juveniles, lo que sugiere que estos compuestos pueden actuar como antioxidantes en *C. clusii* en determinadas condiciones.

En el presente estudio se demuestra por primera vez que los flavan-3-oles, concretamente la quinona de EGCG y las proantocianidinas, se acumulan en mayor medida en hojas de plantas maduras que de plantas juveniles, lo que podría servir a la planta para desviar carbono fotosintético que en condiciones limitantes para el crecimiento se podría acumular en forma de sacarosa y hexosas.

Además, otros estudios han mostrado cómo los niveles de sacarosa y la relación C/N aumentaban con la edad de la planta en condiciones de sequía en esta misma especie. Aunque las plantas de diferentes edades estuvieron expuestas a las mismas condiciones ambientales, y sufrieron la misma

pérdida de agua, los indicadores de estrés oxidativo indican que las plantas de más edad sufren más estrés oxidativo cuando las condiciones son desfavorables.

Las plantas de *C. clusii* respondieron al tratamiento de déficit hídrico incrementando los niveles de ascorbato y su reciclaje, lo que se manifestó en una disminución del dehidroascorbato. Como consecuencia, el estado de oxidación del ascorbato disminuyó como mecanismo de aclimatación.

Los niveles de ascorbato en plantas de *C. clusii* en condiciones de campo se incrementaron durante el periodo de sequía estival, recuperando los valores iniciales en otoño. Las variaciones estacionales del estado de oxidación del ascorbato confirman que, como en otras muchas especies, esta molécula actua como antioxidante. Además, el ascorbato está siempre más oxidado al mediodía que antes del amanecer, indicando que también actúa como antioxidante a escala diurna.

Los niveles de ascorbato en plantas juveniles fueron ligeramente menores que en las plantas maduras, y los niveles de dehidroascorbato siguieron la misma tendencia. Aunque la interacción entre la edad de las plantas y la fecha de muestreo no fue estadísticamente significativa (P=0.061) en cuanto al estado de oxidación del ascorbato, se puede apreciar que el ascorbato estaba ligeramente más oxidado en plantas maduras durante los meses más estresantes, julio y agosto.

Se sabe que el ascorbato, además de antioxidante, tiene funciones importantes en otros procesos, como en la regulación de la progresión del ciclo celular o en la señalización celular. El ascorbato del apoplasto tiene la capacidad de ejercer una gran influencia en el metabolismo del simplasto. En el apoplasto, el ascorbato es el principal componente redox, seguido de los fenoles y las poliaminas, respectivamente. Las plantas de tabaco con el estado de oxidación del ascorbato del apoplasto manipulado genéticamente

(modulando la actividad de la ascorbato oxidasa), no mostraron diferencias en términos de producción de biomasa en condiciones óptimas de crecimiento, respecto al genotipo salvaje. Además, los genes responsables del ciclo del ascorbato-glutation en el citosol tampoco mostraron diferencias, a nivel de expresión génica, con el salvaje. El hecho de que tampoco hubiera diferencias en la expresión del gen que codifica la fenilalanina amonio liasa sugiere que estas plantas no compensan el estado redox del apoplasto con el segundo componente redox de ese compartimento: los fenoles. Estos resultados corroboran los obtenidos por otros autores, que describieron cómo a medida que el ascorbato del apoplasto está más oxidado, la planta es más sensible al O_3.

La expresión del gen que codifica para la proteína NtPC1B, uno de los dos componentes de un canal de Ca^{2+} dependiente de H_2O_2 (que funciona como heterodímero, NtPC1A/B), resultó reprimida tanto en las plantas *sense* como en las *antisense*, respecto al salvaje. Éste es el principal canal de Ca^{2+} implicado en el denominado *oxidative burst*, que depende de la NADPH oxidasa de pared celular, que es un componente común en la señalización de diferentes tipos de estrés que requieren comunicación apoplasto-simplasto. Reduciendo la expresión de este gen, la planta evita una activación constitutiva del proceso. Esto hace que estas plantas sean más sensibles a patógenos avirulentos, dado que son incapaces de desencadenar una respuesta hipersensitiva adecuada.

La librería de sustracción mostró que procesos tan dispares como la fotorrespiración, el transporte de electrones, la asimilación de S y N, las respuestas ante diferentes estreses y la regulación del metabolismo, están afectados, a nivel de expresión génica, por el estado de oxidación del ascorbato en el apoplasto. En particular, los procesos de homeostasis de H_2O_2 (canales de Ca^{2+} dependientes de H_2O_2, enzimas de fotorrespiración

que generan H_2O_2, catalasas, etc.) están fuertemente afectados por este factor.

Del trabajo desarrollado en esta tesis se extraen las siguientes conclusiones:

1.-Se demuestra la presencia de los flavan-3-oles (-)-epicatequina, galato de (-)-epicatequina y galato de (-)-epigalocatequina, en hojas de *C. clusii*. Los niveles de estos compuestos aumentan con un tratamiento de déficit hídrico combinado con elevada radiación en condiciones de campo

2.- El principal factor de estrés que condiciona la acumulación de flavan-3-oles en hojas de *C. clusii* es la radiación máxima recibida (o el fotoperiodo). Aunque los estudios iniciales sugirieron un efecto del déficit hídrico, dicho efecto es consecuencia de que ambos factores de estrés (elevada radiación y déficit hídrico) coinciden temporalmente en el clima meditarráneo

3.- Se demuestra la oxidación *in vivo* de (-)-epicatequina y galato de (-)-epigalocatequina a sus respectivas quinonas en plantas, concretamente en plantas de te sometidas a sequía

4.- El ascorbato reducido se acumula y el dehidroascorbato disminuye en las fases iniciales de un periodo de estrés como mecanismo de la aclimatación. Además, como se ha descrito en otras especies, el ascorbato actúa como antioxidante a escala estacional y diurna

5.- El envejecimiento aumenta el estrés oxidativo en plantas de *C. clusii*, especialmente durante los periodos de estrés abiótico

6.- La acumulación de flavan-3-oles es ligeramente mayor en plantas maduras que juveniles, especialmente durante los periodos de estrés abiótico –elevada radiación y déficit hídrico. Aunque las quinonas de flavan-3-oles no se acumulan en plantas de *C. clusii* en condiciones de déficit hídrico y/o exceso de luz, sus niveles son constitutivamente

mayores en plantas de mayor edad. Esta mayor acumulación podría servir como mecanismo para desviar carbono fotosintético a sumideros alternativos para evitar una inhibición de la fotosíntesis por sus productos

7.- El estado de oxidación del ascorbato en el apoplasto ejerce una profunda influencia en la expresión génica en el núcleo, especialmente en lo que respecta a la homeostasis del H_2O_2.

ABSTRACT

Flavonoids and ascorbate have received much attention because of their health promoting properties, generally exerted through their antioxidant capacity. The main sources of ascorbate and flavonoids in plants include leaves, fruits, seeds and bark. Ascorbate has been extensively studied and it is well established its antioxidant activity *in vivo*. Moreover, evidences accumulate indicating that ascorbate also exerts other functions that are not necessarily linked to its antioxidant capacity, including cell signaling, enzyme activity regulation and cell cycle progress.

The functions of flavonoids are very diverse. These functions normally overlap, so an individual flavonoid may play several roles at the same time, and several individual flavonoids may play similar roles. It has been proposed that phenyl-propanoids, including flavonoids, may act as alternative sinks for reduced carbon and reducing equivalents, helping to prevent overreduction of electron transporters and the accumulation of photosynthetic products such as sugars. Although flavonoids show higher *in vitro* antioxidant activity than ascorbate, their function as antioxidants *in planta* is controversial.

The final objective of the present work is to understand the physiological significance of flavonoids and ascorbate accumulation and oxidation in plants. To achieve this major goal, the present work is aimed particularly at (i) elucidating the effects of environmental stress on the accumulation and oxidation of flavonoids, (ii) studying the physiological significance of flavonoids and ascorbate in plant responses to environmental stress as affected by plant age and (iii) evaluating the possible role of ascorbate oxidation state as a cell signal.

First, the phenolic content of three plant species adapted to Mediterranean climate, that is *Cistus clusii* Dunal, *Salvia officinalis* L. and *Melissa officinalis* L., was screened, and the levels of total phenolics determined

during a water deficit treatment in field conditions. This study revealed that *C. clusii* showed higher total phenolic levels (100 μEq dm^{-2}) than *S. officinalis* and *M. officinalis* (23 and 20 μEq dm^{-2}, respectively). Moreover, *C. clusii* showed enhanced accumulation of total phenolics under stress, attaining the highest total phenolic levels of 238 μEq dm^{-2} after 50 days of stress (2.7 times higher than at the beginning of water deficit treatment).

Thus, *C. clusii* was chosen among the three species studied for the identification of individual flavonoids. The flavan-3-ols (-)-epicatechin, (-)-epicatechin gallate and (-)-epigallocatechin gallate were identified for the first time in *C. clusii* leaves. All these flavan-3-ols reached maximum concentrations after 30 days of drought, attaining concentrations of *ca.* 0.25, 0.025 and 5 μmol dm^{-2}, respectively. Ascorbate accumulated progressively in leaves during drought, reaching maximum concentrations of *ca.* 100 μmol dm^{-2} at the end of the experiment.

C. clusii is a woody perennial, with a lifespan over 10 years, typically exposed, as it occurs to other native Mediterranean plants, to seasonal cycles of water deficit and excess light. To evaluate the effect of these environmental constrains on flavan-3-ol and ascorbate accumulation and oxidation in *C. clusii* leaves, seasonal variations in the levels of reduced and oxidized flavan-3-ols and ascorbate were monitored along with stress markers in plants growing under Mediterranean field conditions. Juvenile (1-year-old) and mature (6-years-old) *C. clusii* plants were used for the experiment, which allowed us to determine the effects of plant age on flavan-3-ol and ascorbate accumulation and oxidation in leaves. Mature plants showed slightly higher oxidative stress, especially under the more stressful conditions of spring and summer, than the juvenile ones. Monomeric flavan-3-ols accumulated in drought, reaching their highest levels at the beginning of July (*ca.* 2.6 μmol dm^{-2} for (-)-epigallocatechin

gallate). During the summer, flavan-3-ol levels were higher in mature than in juvenile plants. Proanthocyanidins also accumulated in drought, the oldest plants showing the highest levels during the more stressful periods of spring and summer, reaching values of *ca.* 0.53 μEq dm^{-2}. Flavan-3-ol quinones, which are flavan-3-ol oxidation products, did not accumulate in drought. However, mature plants showed slightly higher flavan-3-ol quinone levels than juvenile ones. Flavan-3-ol accumulation strongly correlated with maximum solar irradiances, and showed a weak correlation with the relative leaf water content. During this experiment, ascorbate accumulated in leaves of drought-stressed plants, attaining maximum levels of *ca.* 500 μmol dm^{-2}. Mature plants showed a higher oxidation state of ascorbate than the juvenile ones during the summer.

As mentioned above, flavan-3-ol quinones did not accumulate in *C. clusii* plants exposed to drought. To elucidate whether or not flavan-3-ols can be oxidized to their respective quinones *in vivo*, a plant with agronomic interest, with high flavan-3-ol content and sensitive to water deficit and high light was chosen for further studies. Tea (*Camellia sinensis* L. Kuntze) plants were exposed to water deficit under Mediterranean field conditions and levels of reduced and oxidized flavan-3-ols were measured along with stress markers. (-)-Epicatechin quinone and (-)-epigallocatechin gallate quinone levels increased from *ca.* 0.1 to 19, and from undetectable levels to ca. 2.5 μmol g DW^{-1} in drought. Proanthocyanidins also accumulated (up to *ca.* 98 μEq g DW^{-1}) at the end of the drought treatment, while reduced flavan-3-ols remained constant. Accumulation of flavan-3-ol quinones showed a strong negative correlation with the extent of lipid peroxidation in leaves.

Ascorbate is well known to act as an antioxidant in plant cells. However, it exerts a role in many other physiological processes including, among others, cell cycle regulation, and modulation of the production of hormones, such as

gibberellins, and photoprotective compounds, such as zeaxanthin, by acting as an enzyme cofactor. To explore the possible function of ascorbate as a signal in plants, the effects of ascorbate oxidation in the apoplast on nuclear gene expression were evaluated. For this experiment, tobacco transgenic plants over- and under- expressing ascorbate oxidase (and therefore with an enhanced or reduced ascorbate oxidation state in the apoplast) were used. Interestingly, these plants showed altered gene expression patterns in the symplast. Some genes with altered expression include those involved in photorespiration, signal transduction, electron transport and stress metabolism, among other processes. Moreover, Ca^{2+}-dependent cell signaling resulted strongly altered.

In conclusion, it is reported here for the first time that levels of the flavan-3-ols, (-)-epicatechin, (-)-epicatechin gallate and (-)-epigallocatechin gallate increase significantly after a water deficit treatment in leaves of *C. clusii* plants in field conditions. Furthermore, it is shown that plant aging increases oxidative stress in *C. clusii* leaves during the more stressful periods of spring and summer and that flavan-3-ols accumulate to a slightly higher extent under stress in mature plants compared to juvenile ones.

Also, it is shown here that (-)-epigallocatechin gallate accumulation responds to high light exposure rather than to water deficit in field-grown *C. clusii* plants, thus it is suggested that accumulation of (-)-epigallocatechin gallate and proanthocyanidins might protect *C. clusii* leaves from excess light. It is provided evidence for the first time for the *in vivo* oxidation of (-)-epicatechin and (-)-epigallocatechin gallate to their respective quinones in plants. This oxidation occurred in tea plants exposed to water deficit, while in *C. clusii* plants was apparent only as plants aged. It is also shown that accumulation of monomeric flavan-3-ols precedes accumulation of

proanthocyanidins in water-stressed tea and *C. clusii* plants, suggesting that they may be involved in the biosynthesis of proanthocyanidins in drought.

Finally, the present study demonstrates that the ascorbate oxidation state in the apoplast provokes a profound influence on nuclear gene expression. The metabolic pathways affected by the ascorbate oxidation state in the apoplast are very diverse, and include, among others, photorespiration, signal transduction pathways, electron transport processes, stress metabolism and diverse regulatory systems.

INTRODUCTION

1.- Flavonoids

In the 17th century, Robert Boyle described the acid-base properties of plant pigments, unaware that anthocyanins, a particular group of flavonoids, were the pigments responsible for color changes after pH shifts. In the 19th century, Mendel addressed flavonoids while studying seed pigmentation in pea plants. The color pattern he took as inherited character was due to mutations in genes encoding enzymes involved in flavonoid biosynthesis. Barbara McClintock's discovery of transposons also derived from research on seed pigmentation. In this case, maize kernel pigmentation was altered by the insertion of transposable elements into flavonoid biosynthetic genes (Shirley 2001). As one of the most widespread groups of phytochemicals, flavonoids have received much attention in the past and they are still a main focus of research in plant biology.

1.1- Biochemical features of flavonoids

Flavonoids are phenolic compounds that belong to secondary metabolism. They show a basic C_6-C_3-C_6 skeleton, i.e. two benzene rings linked by a three-carbon chain (figure 1). Over 6000 flavonoids have been identified and characterized to date in nature, mainly in plants. Although their classification has been difficult, 6 major classes have been established: anthocyaninidins, biflavonoids (or flavandiols), chalcones, flavanols (including proanthocyanidins, PAs), flavanones and flavonols. Other minor flavonoid groups include isoflavonoids, aurones and stilbenes. Although these compounds are widespread, they are not ubiquitous in plants. The formation of major flavonoids results from the condensation of three malonyl-CoA molecules with a coumaroyl-CoA molecule. When this reaction is catalyzed by the stilbene synthase, this condensation gives rise to stilbenes. In contrast, when the reaction is catalyzed by chalcone synthase, chalcone is formed.

Further biochemical reactions produce the rest of flavonoids found in plants, including aurones, anthocyanidins, flavanols (flavan-3-ols and flavan-4-ols), flavones, flavanones, dihydroflavonols, leucoanthocyanidins, flavonols and isoflavonoids (figure 1). The biosynthesis of flavonoids in plants has been extensively studied and reviewed recently (Dixon and Steele 1999; Koes et al 2005, Shirley 2006).

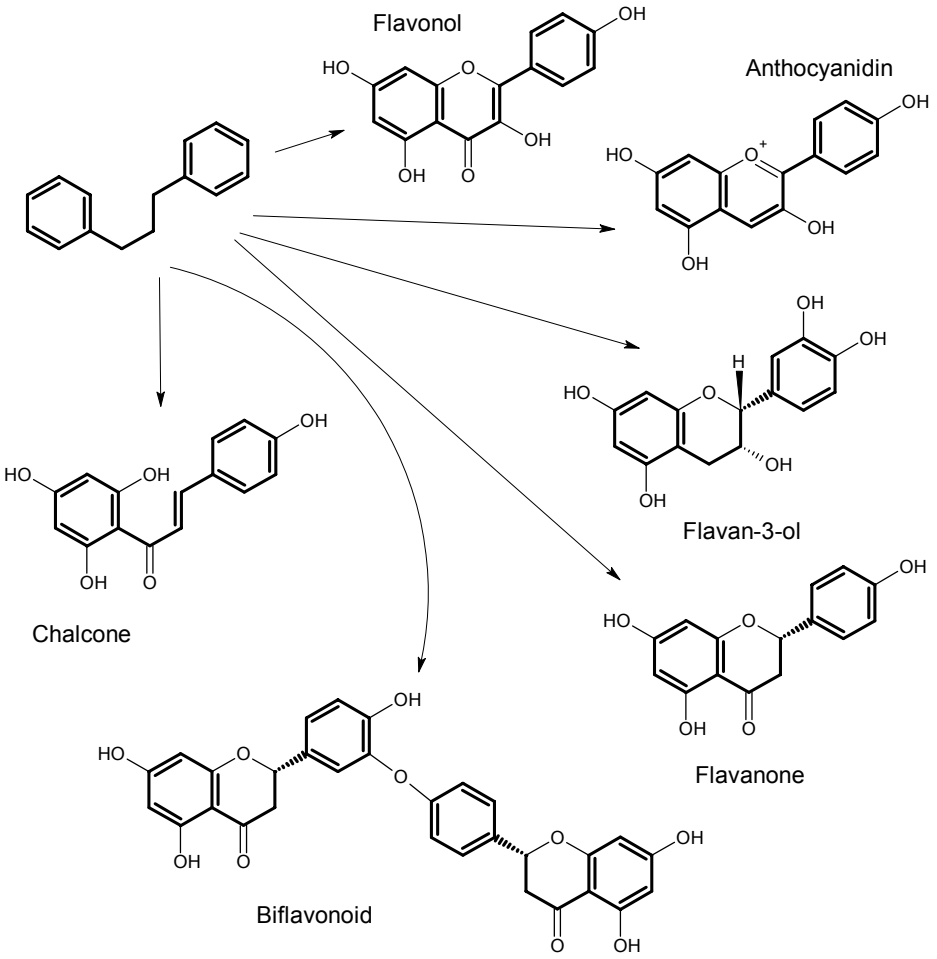

Figure 1: Chemical structures of the 6 major flavonoid types

1.2.- Flavonoids in plants

Flavonoids are universally distributed throughout the Plant Kingdom, including mosses and ferns (Economides and Adam, 1998; Wollenweber et al 1998, Brinkmeier et al 1999). Flavonoid-based chemotaxonomic surveys have been performed in several plant species (Svehlikova et al 2002, Valant-Vetschera et al 2003, Wollenweber et al 2003, Yusuf-Umi et al 2003) and flavonoids have also been used as criteria in studies on the geographic distribution of plant species (Al Wakeel et al 1987, Wells and Bohm 1988, Vieira et al 2003). Peer et al (2001) reported these compounds in the roots, cauline and rosette leaves, inflorescence stems, floral primordia, maturing siliques, stigmata and pollen of Arabidopsis. The main site of flavonoid accumulation in plants is the epidermis and subepidermis of photosynthetic tissues. In species where cuticular waxes are abundant, flavonoids may be major components of these waxes and glandular trichomes, especially those flavonoids strongly hydrophobic (e.g. methylated flavanols) (Vogt et al 1987, Tattini et al 2000). Furthermore, several flavonoids, including glycosylated forms, have been localized in the mesophyll (Tattini et al 2004). High levels of flavonoids have also been detected in nodules of leguminous plants, and more specifically in dividing cortical cells of nodules (Mathesius et al 1998).

1.3.- Subcellular compartmentation of flavonoids

The subcellular localization of flavonoids is highly compartmentalized. Flavonoid biosynthesis occurs in the cytosolic face of the endoplasmic reticulum, where enzymes of the flavonoid biosynthetic pathway form multi-enzymatic complexes. Flavonoid biosynthetic enzymes in these multienzymatic complexes compete for substrates, so assembling of these complexes is key for the prevalence of one or several groups of flavonoids

(Shirley 2001). This phenomenon has been termed metabolite channeling (Stafford 1974) and is responsible for the flavonoid profile exclusive (or at least, characteristic) of each species. Flavonoids are stored in the vacuole of epidermal, subepidermal and stomatal cells (Moskowitz and Hrazdina, 1981; Schnabel et al 1986). Others are located in the cell wall, either loosely associated or covalently linked to cell wall components, mainly to proteins (Strack et al 1988, Schnitzler et al 1996). Flavonoids are also found in the external surface of a number of plant organs and in waxes (Schmutz et al 1994). Recent studies have also demonstrated the presence of flavonoids and some enzymes involved in flavonoid biosynthesis in nuclei (Saslowsky et al 2005). Flavonoids form complexes with water-soluble molecules like glucose, glutathione or glucuronate to make water-soluble compounds that are then removed from the cytosol by membrane-located transporters (Martinoia et al 1993, Marrs et al 1995). Furthermore, several of these compounds are associated with the nuclei and endomembranes of plant cells (Hutzler et al 1998). Green tea catechins (flavan-3-ols), especially galloylated catechins (i.e. gallic acid esters of flavan-3-ols), integrate deep in the lipid bilayer of model membranes and liposomes (Kumazawa et al 2004).

1.4.- Functions of flavonoids

The functions of flavonoids are as heterogeneous as the groups they form. These compounds are involved in a vast array of processes, including light harvesting and screening, plant-microbe and plant-plant interactions, plant responses to both biotic and abiotic stress, cell signaling, defense against herbivores, plant growth and development, and plant reproduction (reviewed by Shirley 1996, Grotewold 2006). In plant-pathogen interactions, many flavonoids act against bacterial, viral and fungal pathogens and insect pests. These flavonoids, regardless of the flavonoid class they belong to, are

usually called phytoalexins. Flavonoids also defend plants against herbivores such as insects and mammalians by providing the plant with an astringent flavor. Others, such as the naturally occurring uncommon epimer (-)-catechin, are powerful allelochemicals that are secreted by roots (Dixon et al 2005). Furthermore, quercetin and kaempferol glycosides have been shown to induce the *vir* (virulence) genes of *Agrobacterium tumefaciens* and several flavonoids in leguminous plants induce the *nod* (nodulation) genes of the symbiotic bacteria of the *Rhizobiaceae* family. Although not as extensively studied, abiotic stress resistance also involves flavonoids. The major roles that these compounds play in plant response to abiotic stress are related to their photoprotective and antioxidant properties, which will be discussed here in detail.

1.5.- Flavonoids and photoprotection

Compelling evidence now indicates that flavonoids may also have an additional photoprotective role. These compounds are highly efficient in screening solar radiation. The light-absorbing properties of flavonoids, especially anthocyanidins, which show maximum absorption at the visible range, have received considerable attention in biochemistry and have been the focus of several studies on light harvesting and screening. Most flavonoids have an absorption spectrum with two bands. Band II is between 240 and 280 nm and is due to the A ring (benzoyl structure) of the flavonoid molecule, while band I, between 300 and 400 nm, is caused by the B ring (cinnamoyl structure) (Markham and Mabry 1975) (figure 2). The composition of these two rings defines the absorption maxima of these two bands. Therefore, modifications like glycosylations, pH shifts, metal chelation, acylation, methylation, oxygenation or desaturation strongly

modify the absorption spectra of flavonoids and can shift the band I maximum to the visible range.

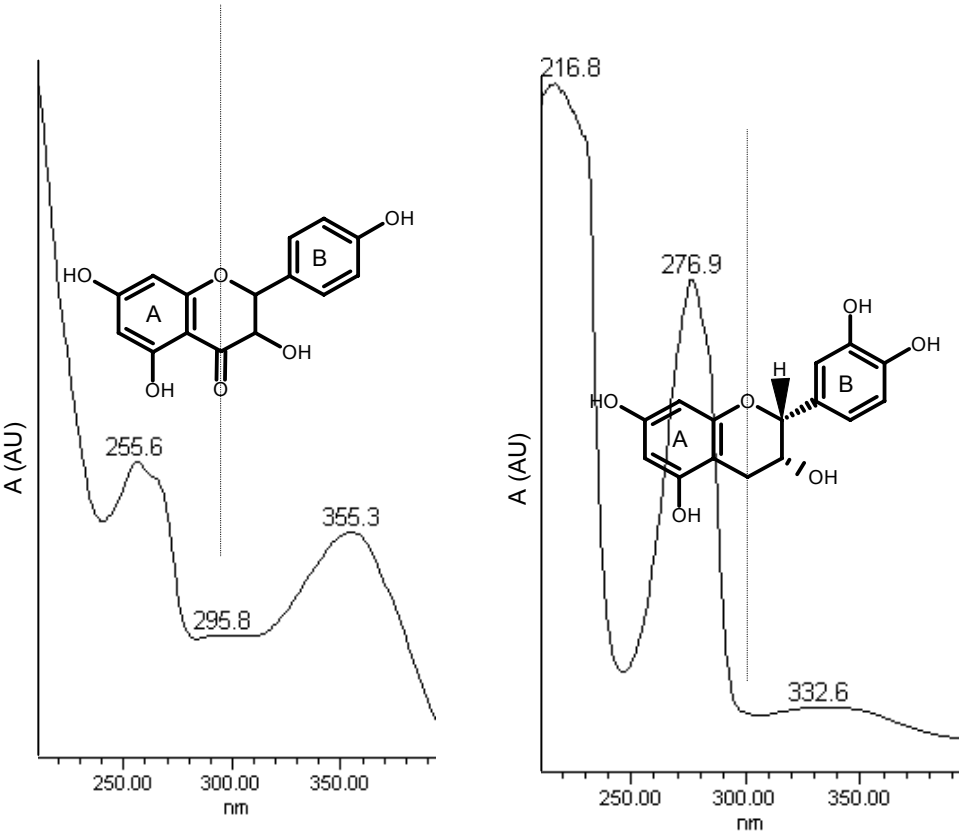

Figure 2: UV-absorption spectra of a flavonol (kaempferol, left) and a flavan-3-ol ((-)-epicatechin, right). UV-absorption peaks between 240 and 285 nm are due to the benzoyl moiety (left side of the dotted line) and those between 300 and 400 nm, to the cinnamoyl system (right side of the dotted line). A stands for absorbance in arbitrary units (AU)

Light-screening flavonoids, like anthocyanins, are often located in the vacuole of epidermal, subepidermal or mesophyll cells so that, although a leaf with a high anthocyanin content absorbs more quanta than an acyanic (anthocyanin-free) leaf, fewer quanta are transferred to the photosynthetic

apparatus. For example, anthocyanins in red-osier dogwood (*Cornus stolonifera*) reduce the frequency and severity of photoinhibition (Feild et al 2001). In contrast, if light is limiting photosynthesis, plants with high flavonoid content are slightly less efficient because of the absorption of these compounds within the photosynthetically-active radiation spectrum (Gould 2004).

In agreement with their absorption spectra, some flavonoids protect plants against harmful UV-B radiation by acting as screens. They are often found in sensitive tissues like epidermal layers of pollen, apical meristems and photosynthetic tissues. The function of flavonoids as UV screens has been supported by many studies exploring either flavonoid accumulation or flavonoid biosynthetic gene expression upon UV-light exposure (Li et al 1993, Bieza and Lois 2001, Brosché et al 1999, Ryan et al 2002). Flavonoids also protect nucleic acids in flavonoid-enriched irradiated cell cultures (Takahashi et al 1991, Stapleton and Walbot 1994).

In addition, Grace and Logan (2000) suggested that phenyl-propanoids, including flavonoids, may serve as an alternative photosynthetic carbon sink. This way, under photoinhibitory conditions, the phenylpropanoid pathway would consume reduced carbon and reducing equivalents, preventing carbohydrate accumulation and subsequent inhibition of photosynthesis.

1.6.- Antioxidant flavonoids

Many flavonoids are anti-inflammatory, anti-allergic, anti-platelet, anti-viral, anti-bacterial and anti-tumoral agents, and it is generally assumed that all these health promoting properties are due to their antioxidant capacity (Dixon and Steele 1999). Antioxidant flavonoids are thought to be responsible for most of the health promoting properties of tea infusions and wines, two of the most commercially relevant drinks in the world. Several

structural features of flavonoids confer high antioxidant potential. The hydroxyl groups are moieties that can donate electrons and protons, and of special relevance in flavonoids is the presence of the *ortho* 3',4'-dihydroxyl moiety in the B ring (*e.g.* catechin, and quercetin) and the *meta* 5,7-dihydroxy moiety in the A ring (*e.g.* kaempferol and apigenin). The double bond between carbons 2 and 3 in combination with a 4-keto group and the 3-OH group (*e.g.* quercetin), in the C ring, provide an effective structure to de-localize electrons. High glycosylation profiles usually reduce antioxidant potential by esterifying flavonoids on hydroxyl groups. In contrast, oligomeric and polymeric flavonoids show great antioxidant activity. In the case of PAs, after a flavan-3-ol is oxidized, the resulting product dimerizes/oligomerizes with other oxidized flavan-3-ols, yielding a series of molecules, PAs, which are stable and show great electron de-localization capacity (reviewed by Rice-Evans et al 1997).

Flavonoids detoxify a wide range of reactive oxygen species (ROS), including singlet oxygen ($^1O_2$), superoxide anions ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$) as well as lipid peroxyl radicals *in vitro* (Bors et al 1990, Terao et al 1994, Rice-Evans 1997, Nagai et al 2005). Flavonoids also interact with other antioxidants, establishing recycling pathways, as demonstrated for ascorbate, which recycles flavonoid phenoxyl radicals back to their reduced forms (Yamasaki et al 1997). Flavonoids also have the capacity to chelate transition metals in several moieties (3',4'-dihydroxy and 4-keto,3(or 5)-hydroxy groups) of their structure, thereby preventing the formation of hydroxyl radicals in the Fenton reaction (Rice-Evans 1997).

Although several flavonoids, like quercetin, catechin gallate esters and theaflavins (flavan-3-ol derivatives), show up to 6 times more antioxidant activity than ascorbic acid or α-tocopherol *in vitro* (Rice-Evans et al 1995

and 1996), it is difficult to demonstrate their antioxidant function *in vivo* (table 1). However, the enhanced biosynthesis of flavonoids with high hydroxylation patterns, which confers high antioxidant potential, suggests that these compounds exert an antioxidant function *in vivo* (Ryan et al 2002). The most likely fate of oxidized flavonoids is the formation of phenoxyl radicals, which are extremely unstable and show strong pro-oxidant activities (Rietjens et al 2002, Labuda et al 2003). These radicals are therefore quickly disproportionated to semi-quinones and these to flavonoid quinones, which are more stable and show lower pro-oxidant activity compared with phenoxyl radicals. Because of the capacity of flavonoids to de-localize unpaired electrons, the rearrangement and stabilization of the oxidation products is extremely fast. Consequently, it is difficult to detect the direct products of flavonoid oxidation, such as phenoxyl radicals, semiquinones and quinones. However, much research effort has been devoted to this topic in recent years. The identification of new flavonoid oxidation products has contributed to determine the antioxidant mechanism of these compounds (Sawai and Sakata 1998, Valcic et al 1999 and 2000, Kondo et al 1998, Bors et al 2000, Sawai and Moon 2000, López-Serrano and Barceló 2002, Mizooku et al 2003, Krishnamachari 2004). Vivas de Gaulejac et al (2001) quantified several flavan-3-ol quinones by derivatization with benzenesulfinate and reported an increase in these quinones upon UV irradiation in wine. During the fermentation of tea (*Camellia sinensis*) leaves to produce black tea, flavanols are also oxidized to quinones, which dimerize to produce theaflavins and thearubigins (Subramanian et al 1999). Using the extract of forty-six plants, Tanaka et al (2002) demonstrated that a mixture of epicatechin and epigallocatechin oxidize and transform into theaflavin derivatives, even when these plants did not contain these catechins. Furthermore, galloylated catechins can integrate

deeply into membranes, which explains, along with their antioxidant properties, their strong inhibitory capacity over lipid peroxidation (Caturla et al 2003).

Compound		*In vitro* antioxidant activity[a] (mM)
Flavan-3-ols	EC	2.4 ± 0.02
	ECG	4.9 ± 0.02
	EGCG	4.8 ± 0.06
Flavonols	Quercetin	4.7 ± 0.10
	Kaemferol	1.3 ± 0.08
Others	Ascorbate	1.0 ± 0.03
	α-Tocopherol	1.0 ± 0.02
	Lutein	1.5 ± 0.10
	β-Carotene	1.9 ± 0.10

Table 1: *In vitro* antioxidant activity, measured by the trolox equivalent antioxidant activity test of several flavonoids (from Rice-Evans et al 1997 and Miller et al 1996). EC, (-)-epicatechin; ECG, (-)-epicatechin gallate; EGCG, (-)-epigallocatechin gallate.

In this scenario, in which flavonoids play a vast array of functions in plants, it has not been unequivocally demonstrated yet that flavonoids act as antioxidants under oxidative stress *in vivo*. Studying the evolution of antioxidant flavonoids and their oxidation products under abiotic stress conditions may help us elucidate the putative antioxidant function of flavonoids.

1.7.- Flavan-3-ols

Flavan-3-ols are a particular group of flavonoids (figure 1). Studies on flavan-3-ols have focused mainly on the biosynthesis, accumulation and properties of PAs (*syn.* condensed tannins). These compounds were named PAs because after acid hydrolysis they yield coloured anthocyanidins, which settles the basis for the main PA determination method. The main PA initiation and elongation building blocks are (+)-catechin and (-)-epicatechin derivatives. It has been clasically thought that they are synthesized by the sequential action of flavanone 3-hydroxylase, dihydroflavonol reductase and leucoanthocyanidin reductase. However this pathway only yields 2,3-*trans* chiral intermediates (e.g. (+)-catechin), either with 2R,3S or 2S,3R conformation. The existence of an alternative pathway involving anthocyanidin synthase and anthocyanidin reductase, that yields 2,3-*cis* intermediates (e.g. (-)-epicatechin) either with 2S,3S or 2R,3R conformation, solved this paradox (Xie et al 2003). Thus, there are two possible ways for the synthesis of flavan-3-ols, depending on their stereochemistry, as depicted in figure 3. Moreover, flavan-3-ols may show different substitutions at the C3, such as the esterification of gallic acid, yielding galloylated flavan-3-ols. The best-described function of flavan-3-ols is to provide protection against microbial pathogens, insect pests, herbivores and fungal pathogens. It is thought that this protection is exerted by divalent metal chelation, such as iron and copper, at *o*-diphenyl moieties. This characteristic of metal complexation of flavan-3-ols (and other flavonoids) also provide plants with tolerance to certain metal toxicities, e.g. aluminium. Moreover, the unusual epimer (-)-catechin acts as a powerful allelochemical in spotted knapweed (*Centaurea maculosa*). Flavan-3-ols, like all phenyl-propanoids, have been suggested to act as alternative sink for photosynthetic carbon and reduction equivalents (for review, see Dixon et al 2005).

1.8.- Other phenolics

The term phenolic includes any molecule with a benzoyl moiety (i.e. a hydroxyl moiety in a benzene ring). All phenolics are synthetized by the shikimate pathway. There is also an enormous variability of phenolic molecules that play a massive array of functions, from photosynthetic electron transport (quinones, FAD, etc.) to structural function (e.g. the aminoacids phenylalanine and tyrosine, lignin, suberin, etc.) and defense against pathogens (phytoalexins, tannins, etc.). It is difficut to talk about phenolics as a group since they exert very different functions, in different organs, tissues, cells and organelles. However, aside from the common biosynthetic pathway, most phenolics show a high antioxidant capacity due to the ability of donating electrons (at the hydroxyl moiety) and the ability to de-localize the donated electron by the resonant bounds of the benzene ring. Another common feature of phenolics is that they show light absorption maxima at the UV range (Rice-Evans et al 1997).

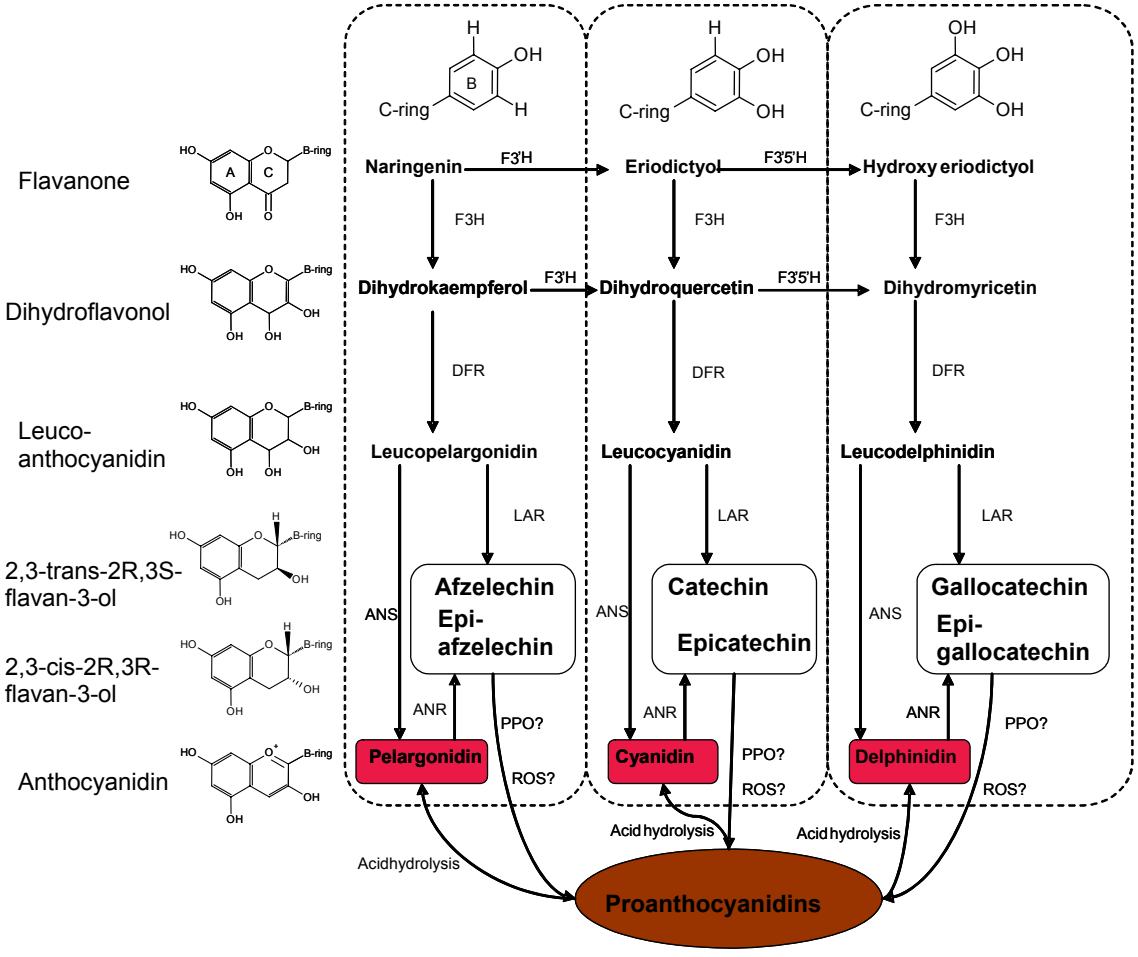

Figure 3: Biosynthesis of stereo-specific flavan-3-ols and proanthocyanidins. F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; F3H, flavanone 3 hydroxylase, LAR, leucoanthocyanidin reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase; PPO, polyphenol oxidases; ROS, reactive oxygen species.

2.- Ascorbate

L-ascorbic acid (also called vitamin C) is the most abundant low-molecular weight antioxidant in plant cells. It accounts for up to 10 % of total soluble carbohydrates (Smirnoff and Pallanca 1996). Despite its importance, ascorbic acid biosynthesis is still a matter of debate today. Several different pathways have been described leading to ascorbic acid formation from hexoses (Wheeler et al 1998, Conklin 1998). Ascorbic acid is present in all higher plants and in all plant organs except in dormant seeds (Loewus 1980 and 1988, DeGara et al 1997). It tends to be more concentrated in aerial parts than in roots, and among different tissues, it is particularly found at high levels in meristems (Franceschi and Tarlyn 2002, Hancock et al 2003).

2.1- Biochemical features of ascorbate

Ascorbic acid is a carbohydrate that shows a C_2-C_3 double bond providing a structure that de-localizes electrons. The hydroxyl moiety of the C3 appears dissociated at physiological pH, so ascorbic acid appears as ascorbate in plant cells (figure 4). Ascorbate may appear alone or bound to other molecules such as glucuronic acid, sulphate or glucose (Davey et al 2002). The most important biochemical feature of ascorbate is its ability to donate electrons yielding monodehydroascorbate (MDHA) radical, which is re-cycled back to ascorbate by MDHA reductases (MDHARs). Two MDHA molecules can also spontaneously yield ascorbate and dehydroascorbate (DHA). The latter may be degraded or re-cycled back to ascorbate by the DHA reductases (DHARs) (figures 4 and 5). The importance of these re-cycling reactions will be discussed later.

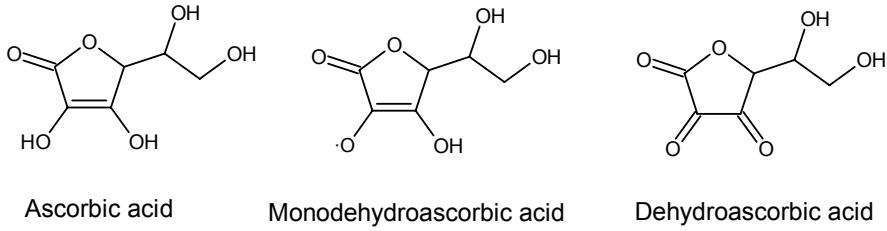

Figure 4: Chemical structures of L-ascorbic acid and its derivatives, monodehydroascorbic acid and dehydroascorbic acid.

2.2.- Subcellular compartmentation of ascorbate

Ascorbate occurs in all subcellular compartments of plant cells, including the apoplast. Some organelles, however, show low levels of ascorbate, like vacuoles (Anderson et al 1983, Rautenkranz et al 1994). The last steps in ascorbate biosynthesis are located in mitochondria, linked to inner mitochondrial membranes (Siendones et al 1999). However, it still remains unclear whether ascorbate is released to the mitochondrial matrix or to the intermembrane space, where it could diffuse freely into the cytosol. Ascorbate does not diffuse through membranes, so it needs to be transported to other organelles. Ascorbate transport to apoplast deserves a special mention. Enzymatic machinery necessary for ascorbate re-cycling has not been found in the apoplast. However, as described below, ascorbate is oxidized in this cellular compartment. Ascorbate transport to apoplast is thought to occur by transporting DHA and then reducing it in the apoplast. The reduction of MDHA and DHA to ascorbate in the apoplast occurs via plasma membrane-integrated cyt *b* proteins (Asard et al 1992 and 1995, Horemans et al 1994, Conklin and Barth 2004).

2.3.- Functions of ascorbate

Ascorbate is involved in a number of processes including cell wall expansion (and also strengthen), hormone metabolism, stress resistance and cell division. In many of these processes ascorbate acts as cofactor of a number of enzymes, typically mono- and di-oxygenases. It is also involved in oxalate and tartrate biosynthesis (Smirnoff 1996). However, the most important functions of ascorbate in plants are due to its antioxidant capacity, which is required in many of the physiological processes described before. Functions of ascorbate have been extensively reviewed (Loewus 1988, Foyer et al 1991 Foyer 1993, Foyer et al 1994, Arrigoni 1994, Smirnoff and Pallanca 1996, Smirnoff 1996, Noctor and Foyer 1998 and Smirnoff 2006).

2.4.- Ascorbate as an antioxidant

Ascorbate interacts with ROS, especially with H_2O_2, both enzymatically (by the means of ascorbate peroxidases, APXs), and non enzymatically. In contrast to other antioxidants, it is able to terminate chain reactions yielding a stable oxidation product, DHA (Davey et al 2002), which is re-cycled back to ascorbate (like MDHA), by other sources of reducing equivalents, typically glutathione, NAD(P)H, Fd or cyt *b* (for review, see Smirnoff 2006). The best-known ascorbate oxidation and re-cycling pathway is the so-called ascorbate-glutathione cycle (figure 5), where DHA and MDHA produced by H_2O_2 scavenging are re-cycled to ascorbate by the co-ordinated action of MDHARs, DHARs and glutathione reductases (GRs). This pathway has been described in different organelles, including chloroplasts, mitochondria, cytosol and peroxisomes.

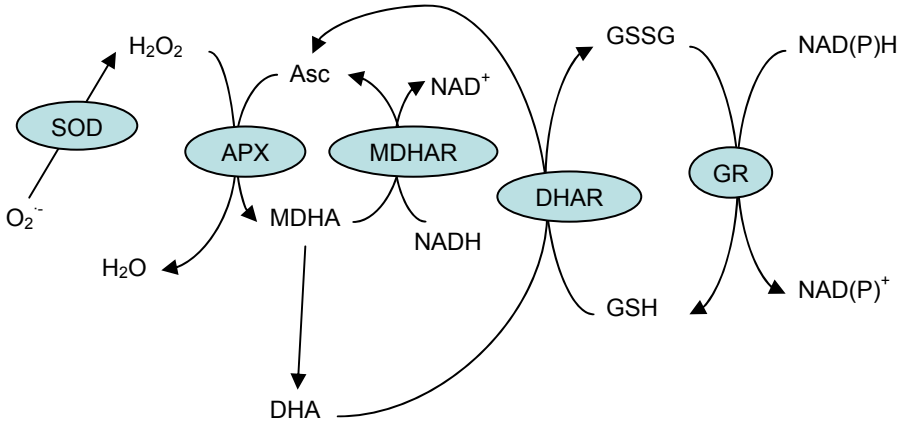

Figure 5: Ascorbate-glutathione cycle. Encircled components stand for enzymes that may show different isoforms depending on the subcellular compartment. SOD, superoxide dismutases; APX, ascorbate (Asc) peroxidases; MDHAR, monodehydroascorbate (MDHA) reductases; DHAR, dehydroascorbate (DHA) reductases; GR, glutathione reductases. $O_2^{\cdot-}$, superoxide anion; H_2O_2, hydrogen peroxide; GSH, reduced glutathione; GSSG, oxidized (dimeric) glutathione.

However, the ascorbate-glutathione cycle in chloroplasts deserves a special mention. If the Mehler reaction occurs, the O_2 released at PSII level may be reduced to water at PSI level by the coordinated action of SODs and APXs, establishing the so-called water-water cycle, which has a role in dissipating excess excitation energy in chloroplasts (Asada 1999):

$2H_2O \rightarrow 4e^- + 4H^+ + O_2$	(water photolysis)
$2O_2 + 2e^- \rightarrow 2O_2^{\cdot-}$	(Mehler reaction)
$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$	(SODs)
$H_2O_2 + 2Asc \rightarrow 2H_2O + 2MDHA$	(APXs)
$2MDHA + 2e^- + 2H^+ \rightarrow 2Asc$	(MDHARs)
$2H_2O + O_2^* \rightarrow 2H_2O^* + O_2$	

3.- Plant responses to stress

3.1.- Stress concept in plants

Levitt, in 1972, defined stress as "any environmental factor potentially unfavorable to living organisms". Factors responsible for stress situations in plants may have abiotic or biotic origin. Common biotic stress factors are pathogen attack, plant parasites, herbivores, etc. Among the abiotic stress factors, water deficit, excess light, chilling, freezing, nutrient deficiencies and air pollutants are major ones (Lichtenthaler 1996, Larcher 2003).

The persistence of a stress factor over time may eventually lead to cell/tissue/plant death if the plant does not find the way to overcome the adverse condition. Some plants escape stressful situations by completing their life cycle during favorable periods. Other plants tolerate stress situations by acquiring thermodynamic equilibrium with the environment (Levitt, 1972). Both acclimation and adaptation are mechanisms leading to stress tolerance. Adaptation refers to the inheritance of features that favor the survival of individuals of a certain species. On the other hand, acclimation refers to non-heritable changes that lead to stress tolerance (Lichtenthaler, 1996). Some of the major environmental constrains in our region, the Mediterranean basin, are water deficit and high light that coincide during the summer.

3.2.- Water deficit

Due to the high vapor pressure deficit (VPD) resulting from the low air relative humidity (often combined with high temperatures), plants tend to increase transpiration. If water availability in roots is also low, plants suffer a net water loss and thus, water deficit. Under this situation, plants generally respond by closing stomata to minimize water loss by transpiration. Stomatal closure occurring in water-stressed plants avoids water loss by transpiration,

but it also prevents CO_2 diffusion into the substomatal cavity. This stomatal limitation of photosynthesis slows Calvin cycle down due to the lack of CO_2 for ribulose 1,5-bisphosphate carboxylation, catalyzed by the ribulose 1,5-bisphosphate carboxylase/oxygenase, the first enzyme involved in the Calvin cycle. If decreased CO_2 availability slows Calvin cycle down, the utilization of ATP and reducing equivalents in other reactions of the cycle also decreases (Schulze and Cadwell 1995). However, under water stress, plants generally continue receiving solar radiation. The absence of oxidized ('free') electron acceptors –mainly $NADP^+$- and substrates for oxidative phosphorylation (ADP) can result in the accumulation of reduced electron transporters and excited chlorophylls. This situation prompts the formation of ROS, as described below, by energy or electron transfer to O_2. The low water availability also causes other effects such as the growth arrest due to the lack of turgor, morphological changes, synthesis of protective compounds (e.g. osmolytes, abscisic acid), and changes in gene expression, among other processes (Chaves et al 2002).

3.3.- High light

High light is one of the most common abiotic stress factors for plants. It can appear alone or more commonly along with other stress factors such as nutrient deficiencies, salinity stress or water deficit. In the Mediterranean basin, for example, water deficit limits photosynthesis by inducing stomatal closure as described before. If at the same time, solar radiation is high, excess light is greater than in well-watered plants, and can overwhelm energy dissipation mechanisms. Another situation where an abiotic stress factor enhances excess light is chilling temperatures. Chilling temperatures slow down all biochemical processes. This makes photosynthesis slower, and thus, solar radiation surpasses light energy processing capacity. In a

similar manner, water stress, chilling, salinity, nutrient deficiencies, metal toxicities and most abiotic stress factors make light to be in excess or enhance this phenomenon (for review, see Møller et al 2007).

3.4.- Oxidative stress in plants

Oxygen in biological systems is generally present in different oxidation states ranging from O_2 to H_2O. Mechanisms to obtain energy implicate oxidative processes in all aerobic organisms. Such oxidative metabolism makes energy obtention processes highly efficient, but it also has disadvantages. Molecular oxygen (O_2) has a very electropositive redox potential (E_0'=0.82 mV for the reaction $O_2+4e^-+4H^+ \rightarrow 2H_2O$), which makes it highly reactive. The O_2 tends to reduce (or to harvest energy) from other compounds, generating partially reduced oxygen intermediates: ROS. In plants, the transference of the energy from triplet chlorophylls (excited chlorophylls) to the O_2, generates $^1O_2$ (singlet oxygen). If instead of energy, electrons are transferred to O_2, $O_2^{\cdot-}$ (superoxide anion) and subsequently H_2O_2 (hydrogen peroxide) are formed. When $O_2^{\cdot-}$ and H_2O_2 accumulate in the presence of a transition metal, they may undergo the so-called Haber-Weiss reaction (where the metal acts as a catalyst), to yield $OH^{\cdot-}$ (hydroxyl radical, Halliwell and Gutteridge 1999, Apel and Hirt 2004). $OH^{\cdot-}$ can also generate when H_2O_2 accumulate in the presence of a transition metal, by the so-called Fenton reaction. In this reaction, the transition metal does not act as a catalyst, it is instead reduced (Halliwell and Gutteridge 1999). The $^1O_2$, $O_2^{\cdot-}$, H_2O_2 and $OH^{\cdot-}$ are the most common ROS in plants (figure 6).

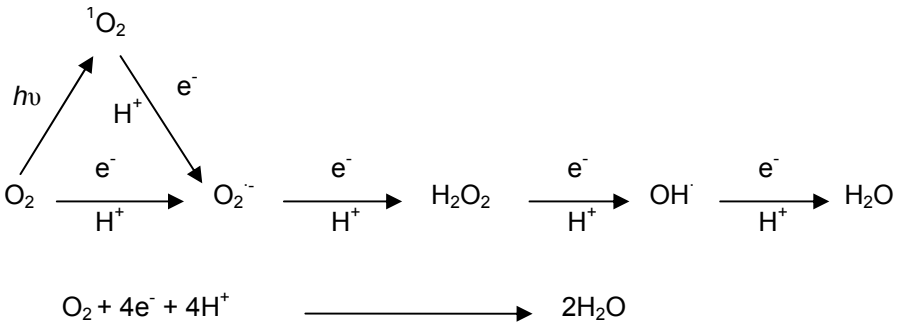

Figure 6: Scheme of the reactions leading to the most common reactive oxygen species in plants after sequential addition of electrons to O_2. O_2, triplet oxygen (basal state); $O_2^{\cdot-}$, superoxide anion; H_2O_2, hydrogen peroxide; $^1O_2$, singlet oxygen; $OH^{\cdot}$, hydroxyl radical.

These ROS are extremely unstable, with an average lifetime ranging from milliseconds (H_2O_2) to nanoseconds ($OH^{\cdot}$) (Perl-Treves and Perl 2002, Møller et al 2007), and they tend to oxidize other molecules in order to stabilize unpaired electrons in their atomic structures. As stated before, under optimal conditions there is a basal, low ROS production intrinsic to every aerobic metabolism. ROS are implicated in cell signaling in different cellular processes, including leaf senescence, defense against pathogen attack and responses against environmental constrains (Doke 1997, Dat et al 2000, Vranová et al 2002, Mahlingam and Fedoroff 2003, Overmyer et al 2003). However, when ROS concentration becomes high enough to overwhelm antioxidant systems, plants suffer oxidative stress as a consequence of the unbalanced cellular redox status. Oxidative stress may cause oxidation of target compounds, including proteins, lipids, sugars and nucleic acids, that may lead to oxidative damage if plant defense mechanisms do not avoid it. Thus, ROS metabolism requires a tight regulation to provide the plant with the adequate ROS levels that allow

signaling processes without any oxidative damage (Mittler 2002, Neill et al 2002, Overmyer et al 2003).

Poly-unsaturated fatty acids are especially susceptible ROS targets. Under oxidative stress conditions, ROS react with poly-unsaturated fatty acids to yield lipid peroxyl radicals. These radicals react with neighboring poly-unsaturated fatty acids yielding new lipid peroxyl radicals, thus establishing a chain reaction that leads to membrane damage (Møller et al 2007). The oxidation of target molecules by ROS alters their functionality due to changes in their redox state, structure and integrity (Asada and Takahashi 1993, Møller et al 2007).

3.4.1.- Oxidative stress in the apoplast

The apoplast is an important source of ROS. In this compartment, NAD(P)H oxidases producing $O_2^{\cdot-}$ (and H_2O_2 afterwards), and peroxidases producing H_2O_2 are abundant (Vianello and Macri 1991). The production of H_2O_2 in the apoplast is key for lignin formation, because by the action of cell wall peroxidases that use H_2O_2 as a substrate, lignin precursors are cross-linked (Gross 1980). Moreover, ROS formation in the apoplast is key for developing the hypersensitive response which essentially consists of the first barrier against avirulent pathogens (Degousee et al 1994, Lamb and Dixon 1997). Apart from the 'self-induced' ROS formation in the apoplast, this cell compartment is the first contact point of plant cell with oxidizing air pollutants such as O_3 and SO_2 (figure 7). The presence of these compounds also leads to the oxidation of cellular (apoplastic, in this case) compounds thus generating oxidative stress. The main molecule with antioxidant capacity in the apoplasts is ascorbate. However, the neccesary enzymatic machinery required for ascorbate re-cycling is not located in the apoplast, so intermediates must be transported through the plasma membrane. Although

candidates for such transporters have been described, the full identification of them still remains elusive (Conklin and Barth 2004).

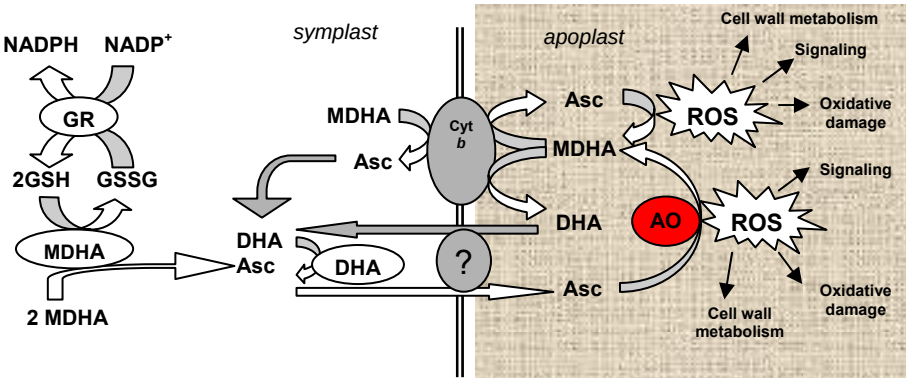

Figure 7: Ascorbate (Asc) metabolism in the apoplast and its relationship with Asc in the symplast. ROS, reactive oxygen species, or other oxidizing agent such as O_3 and SO_2; MDHA, monodehydroascorbate; DHA, dehydroascorbate; Cyt *b* stands for cytochrome *b* proteins that provide electrons through plasmalema to re-cycle MDHA; DHAR, dehydroascorbate reductase; MDHAR, monodehydroascorbate reductase; GSH, glutathione; GSSG, oxidized (dimeric) GSH; GR, glutathione reductase. ? stands for a possible transporter of Asc or DHA.

3.4.2.- Oxidative stress in the symplast

Chloroplasts are very susceptible to suffering oxidative stress essentially because they have high levels of O_2 due to the photolysis of water and because they harvest light energy and transfer it to an electron transport chain that is based on a series of redox reactions. When photosynthetic reactions and energy transfer mechanisms fail, energy or electrons may be transfered to O_2, generating the different ROS (Asada 1999, see figure 6 for further detail). Another important source of ROS in plant cells are peroxisomes (Foyer and Noctor 2003). Part of the carbon lost as a consequence of the oxygenase activity of RuBisCO during photorespiration is re-cycled back by the glycolate cycle (C2 cycle). In this cycle, glycolate is

oxidized to glyoxilate by the glycolate oxidase, which produces H_2O_2 as a byproduct in peroxisomes. The ribulose 1,5-bisphosphate used for glycolate formation in chloroplasts is re-cycled back by the photorespiratory cycle (C_2 cycle). Moreover, $O_2^{\cdot-}$ is also produced in peroxisomes and glyoxisomes by NAD(P)H oxidases, urate oxidases, xanthine oxidases and Acyl-CoA carboxylase (Huang et al 1983, Elstner 1991, Lindqvist et al 1991, McKrise and Leshem 1994, Del Río et al 2002). Mitochondria are also important organelles in ROS production. Like chloroplasts, they have an electron transport chain that involves a series of redox reactions. Leaks in this electron transport chain lead to the transference of electrons directly to O_2, generating $O_2^{\cdot-}$. Between 1 and 2 % of the electrons that flow through the mitochondrial electron chain in optimum conditions leak, yielding $O_2^{\cdot-}$ (Rich and Bonner 1978, Elstner 1991). The occurrence of oxidative stress has been described in many other cell compartments such as the endoplasmic reticulum, vacuoles, nuclei and cytosol. Formation of ROS in these organelles is thought to be quantitatively less important than in the above mentioned ones. However, diffusion of ROS, especially H_2O_2, to nuclei and cytosol, may be of great importance given that it is relatively stable and diffuses across membranes (Pert-Tarves and Perl 2002, Møller et al 2007).

3.4.3.- Oxidative stress signaling

As stated before, ROS formation is intrinsic to every aerobic metabolism, and at low levels, ROS serve a signaling function. Enhanced formation of ROS under stress conditions can occur through diverse mechanisms, many of which are described before. Additionally, ROS can also be actively synthesized by plant cell enzymes such as NADPH oxidases or cell wall peroxidases. In some cases, these enzymatically-formed ROS serve determinant roles in stress resistance, such as lignin formation or localized

cell death in the hypersentitive and systemic acquired responses (Chen et al 1993, Levine et al 1994). Furthermore, a number of processes, including gene expression, are tightly regulated by levels of ROS that are not toxic for plant metabolism, and certain ROS-dependent responses are ROS-specific. For example, $O_2^{\cdot -}$, and no other ROS, is responsible for the induction of phytoalexin accumulation after pathogen attack to tobacco cells (Perrone et al 2003).

To act as signaling molecules, ROS levels have to be perceived somehow. Redox-sensing proteins that serve this role have been described. The best described ROS sensing mechanism to date is the two-component histidine kinase system in yeasts. H_2O_2 induces changes in the sensor part of the system triggering kinase activity of the effector part (Singh 2000, Buck et al 2001). Other sensors include protein phosphatases, thiol peroxidases and thiol-containing proteins (e.g. thioredoxins) and compounds (e.g. glutathione) (for review, see Buchanan and Balmer 2005, Desikan et al 2006). Once ROS signal has been perceived, the signal is transduced by different pathways that can involve Ca^{2+}, K^+ and reversible phosphorylation of proteins in a manner equivalent to other signal transduction pathways with both shared and exclusive components (figure 8). Common responses to ROS signaling include changes in gene expression, enzyme activity shifts and protein stability alterations (for review, see Mittler et al 2004, Buchanan and Balmer 2005, Desikan et al 2006).

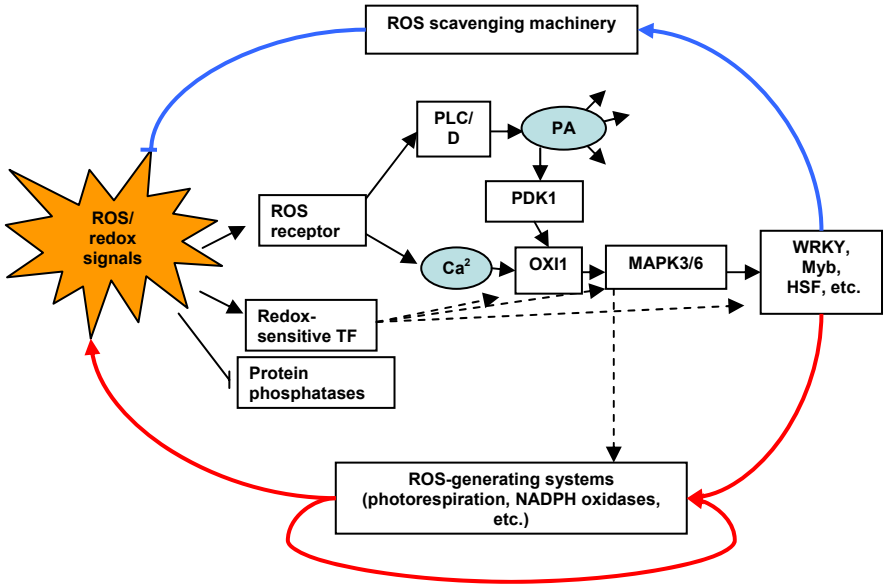

Figure 8: Reactive oxygen species (ROS) and redox signal transduction pathways. Red line indicates a positive feed back loop that enhances ROS production after ROS sensing, while negative feed back regulation (blue line) is aimed to scavenge ROS. PA, phosphatidic acid; PLC/D, phospholipase C or D; PDK, phosphoinositide-dependent kinase; OXI, ROS-induced protein kinase; MAPK3/6, mitogen-activated protein kinase cascade; TF, transcription factor; WRKY, Myb and HSF stand for common transcription factors. Adapted from Mittler et al 2004.

3.5.- Plant responses to stress as affected by age

Aging has been classically defined as the accumulation of changes in plant development responsible for slow, progressive, and sequential alterations that accompany the plants as they age (Noodén and Guiamet 1996, Dangl et al 2000, Munné-Bosch 2007). Plant aging is characterized by a decrease in relative growth rates accompanied by decreases in photosynthesis in recently emerged leaves. It is important to separate aging from senescence. Senescence is considered as the physiological process that leads to plant/organ/cell death. Senescence, then, is the very last stage of aging. In

perennial plants, localized senescence occurs contiuously in certain tissues and organs, e.g old leaves. Hence, non-senescent tissues (e.g. recently emerged leaves) of perennial plants are an adequate model to study the effects of plant age without the interference of the senescence process.

There are two main groups of factors that can determine the aging process in plants: intrinsic and extrinsic factors. Intrinsic factors include changes occurring within the plants as they age, such as increases in plant size and changes in gene expression in the meristems. Somatic mutations, polyploidy and telomere shortening, due to repeated divisions of meristematic cells can occur during aging in perennials and could be fixed to the shoots and leaves originated from these meristems (Gill et al 1995, Salomonson 1996, Walbot 1996, Blackburn et al 2000, Riha et al 2001). Extrinsic factors limiting photosynthesis and growth rates as plants age include all those factors external to the plant, such as water and nutrient avalaibility, or pathogen attack (Munné-Bosch and Lalueza 2007).

The “hydraulic limitation hypothesis” suggests that as plants increase in size and height, the resistance offered by the different elements involved in water transport increases and the water supply to tissues and organs at the top of the canopy may be reduced (Ryan and Yolder 1997, Ryan et al 2006). This may result in a turgor loss and thus, in the reduction of growth. To minimize this effect, the response of plants is to increase abscisic acid levels that help controling water loss by inducing stomatal closure, which also contributes to reduce relative growth rates of recently emerged leaves as plants age (Munné-Bosch and Lalueza 2007).

The free radical theory of aging in animals postulates that aging results from the accumulation of disfunctions caused by free radicals (such as ROS), or the unbalance between free radicals and antioxidants. According to this theory, as animals age they show increased sensitivity to oxidative

stress-induced diseases such as cardiovascular disease, cancer and chronic inflammation (Harman 1981 and 1991). In plants, the role of free radicals and oxidative stress is known in stress responses (Inzé and van Montagu 2002, Smirnoff 2006) and leaf senescence (Smart 1994, Noodén and Guiamet 1996, Buchanan-Wollaston 1997, Dangl et al 2000, Quirino et al 2000), but little is known about the effects of plant age on plant responses to environmental stress. Although studies on perennials have been limited in part by the challenges of working with long-lived organisms, such studies are essential if we are to understand the effects of plant age on plant responses to stress.

4.- Choosing the adequate experimental model

Plants adapted to Mediterranean field conditions have high amounts of secondary metabolites and low molecular weight antioxidants. Thus, they are an adequate model to study the accumulation and oxidation of flavonoids and ascorbate in plants under abiotic stress conditions, taking water deficit and high light as environmental stresses of particular interest. Moreover, perennials adapted to this climate are a useful model to study the involvement of flavonoids and ascorbate in plant responses to these abiotic stresses and the influence of plant age in these processes, since they are very resistant to drought stress and have moderate life spans (at least shorter than those of trees).

Additionally, tea plants have an outstanding agronomic and nutraceutical value, mainly due to their high flavonoid content, especially flavan-3-ols, which give them a number of health promoting properties. Tea plants are very sensitive to water deficit and high light, so they are an adequate model not only to study how flavan-3-ol accumulation is affected by these environmental stresses, but also to unravel to what extent flavan-3-ols can be oxidized in plants.

To study the role of ascorbate in the apoplast as a cell signal, a transgenic approach with tobacco plants over- and under-expressing ascorbate oxidase, was employed. The utilization of transgenic tobacco as plant material allowed us to apply conventional molecular biology techniques based on existing methodology. The transcript profile of these transgenic lines was analyzed by top-up an bottom-up approaches.

OBJECTIVES

The final objective of the present work was to understand the physiological significance of flavonoid and ascorbate accumulation and oxidation in plants. To achieve this major goal, the present work was aimed particularly at:

1.- Elucidating the effects of environmental stress on the accumulation and oxidation of flavonoids

1.1.- Indentifying the flavonoids that accumulate in *C. clusii* and tea plants after a drought period that include high light and water deficit stress

1.2.- Determining which of the two factors, high light or water deficit, induce flavonoid accumulation

1.3.- Identifying *in vivo* flavan-3-ol oxidation products

1.4.- Determining whether flavan-3-ols are readily oxidized *in vivo* to their oxidation products after water deficit and high light stress

2.- Studying the physiological significance of flavonoids and ascorbate in plant responses to environmental stress as affected by plant age

2.1.- Determining whether or not plant age affects the extent of oxidative stress under optimum conditions, water deficit and high light stress

2.2.- Studying differences in ascorbate and flavan-3-ol accumulation in plants of different ages under optimum conditions, water deficit and high light stress

2.3.-Studying the oxidation of ascorbate and flavan-3-ols in *C. clusii* plants exposed to high light and water deficit as affected by plant age

3.- Evaluating the possible role of ascorbate oxidation state as a cell signal

3.1.- Determining the effects of a shifted ascorbate oxidation state in the apoplast, on the major ascorbate re-cycling enzymes in the symplast, at gene expression level

3.2.- Identifying metabolic pathways affected at gene expression level after apoplastic ascorbate oxidation state alteration

3.3.- Identifying components of the apoplastic ascorbate oxidation state signal transduction pathway to the nucleus

MATERIALS AND METHODS

1.- Plant material

1.1.- Mediterranean plants

Cistus clusii (Dunal) is a perennial shrub of the *Cistaceae* family. It can grow up to 1 m high. *C. clusii* shows erect shoots with sessile leaves, which are dark green above and white tomentose beneath, and that sprout in spring and fall. *C. clusii* leaf margins curl downwards. It shows white flowers up to 2.5 cm diameter, blooming in spring (figure 9). It is very resistant to drought and it grows in sandy and perturbed areas of the Mediterranean basin and the bush woods 'maquias' and 'garrigas' (Vogt et al 1987, Pugnaire and Lozano 1997).

Figure 9: *C. clusii* shrub (left) and a detail of the flower and leaves (right)

Melissa officinalis (L.) belongs to the *Lamiaceae* family. It is a perennial plant up to 90 cm high with a characteristic lemon-like flavor. It shows oval-shaped, up to 7.5 cm long, light green and slightly pubescent leaves strongly serrated in the edges. *M. officinalis* leaves appear in verticils of two opposited leaves inserted in square-section shoots, sprouting every spring and fall. *M. officinialis* blooms during summer, showing pale yellow flowers, about 1.3 cm diameter, that appear irregularly disposed in the upper nods of the shoots (figure 10). It is naturally spread in southern Europe and north Africa. It is grown as a medicinal plant because of its essential oils, and as a consequence of its domestication it grows subespontaneously in some areas in the United Kingdom and northern Europe. *M. officinalis* also grows in perturbed areas and road margins, and it needs well drained soils. It can withstand soils with scarce nutrients and high solar radiations (Bolós and Vigo 1984-2001).

Figure 10: *M. officinalis* plants (left) and a detail of the inflorescence (right)

Salvia officinalis (L.) is an aromatic perennial that belongs to the *Lamiaceae* family. It can grow up to 70 cm high and shows erect and pubescent shoots. *S. officinalis* leaves are petiolated, oval-shaped –rarely lanceolated- with a conspicuous central nerve. *S. officinalis* flowers are from white to purplish, disposed in ears, with an up to 3 cm corolla. *S. officinalis* blooms in late spring, and new leaves sprout in early spring and fall. This plant species grows in rocky places of the Mediterranean Europe. It is also grown as a medicinal plant.

Figure 11: *S. officinalis* plants (left) and a detail of the inflorescence (right)

For the experiments, *M. officinalis, S. officinalis* and *C. clusii* seeds were germinated and grown in 0.5 l pots containing a mixture of soil:peat:perlite (1:1:1, v/v/v). Plants were maintained in a greenhouse with a controlled temperature (24 ºC/18 ºC, day/night) and they were watered twice a week, once with tap water and once with Hoagland's solution (Hoagland and Arnon 1950). After one year of growth, plants were transplanted during the spring of 1999 to the experimental fields of the Faculty of Biology

(University of Barcelona, Spain). Before the plants were transferred, the soil -calcic Luvisol (FAO)- was ploughed and treated with N:P:K (1:1:1, v/v/v) fertilizer at the rate of 100 kg ha^{-1}. For the water deficit treatment experiments (described in chapters 1 and 2 of results), plants were watered with tap water before the experiment began. During the experiment, plants were covered with a clear PVC sheet when rainfall was expected, so they did not receive any external water input. Thus, physiological and biochemical parameters were followed during the progression of water deficit under Mediterranean field conditions.

For the aging experiments (described in chapter 3 of results), plants of different ages were used. A group of plants (mature plants) corresponded to those described before, while juvenile plants were obtained as follows. After 5 years of growth, seeds of mature *C. clusii* plants were harvested, germinated as described above and transplanted during the spring of 2004 to the same experimental fields. *C. clusii* flowers by the first time during the second year of growth, so plants younger than 2 years are juvenile, so herein, 'juvenile' refers to 1-year-old plants, and 'mature' to 6 years-old plants. *C. clusii* plants of different ages but with the same genetic background were therefore grown under the same field conditions in two plots that were separated by 2 m.

1.2.- Tea plant [*Camellia sinensis* (L.) Kuntze]

Tea plants [*Camellia sinensis* (L.) Kuntze] are perennial woody plants (figure 11). In this case they are trees given that they can grow up to 9 m high. *C. sinensis* belongs to the *Theaceae* family. It shows lanceolated or elliptic leaves from 5 to 6 cm long and about 2.5 cm wide, blooming in spring. Young leaves, which sprout in spring, are pubescent and they become hairless when mature. Whitish flowers appear alone or in couples

hanging from short petioles from leaf axils. *C. sinensis* is native to mainland south and southeast Asia, but it is grown as a crop in many regions all over the world, especially in south and southeast Asia and India. Compared to *M. officinalis*, *S. officinalis* and *C. clusii*, *C. sinensis* is very sensitive to drought, and it needs shade and a well drained, neutral to slightly acidic soil rich in organic matter for optimal growth (Squire and Callander 1981). Tea plants have an outstanding economical importance because their dried and/or fermented leaves after infusion in water constitute one of the most popular beverages all over the world: tea. *C. sinensis* leaves are well known to have high flavonoid amounts, especially flavan-3-ols. For the experiments, 2-years-old *Camellia sinensis* ssp. *sinensis* (L.) Kuntze cv. Small Leaf Tea were obtained from cuttings in a nursery (Camforest, NC, USA). Plants in 1 l pots were watered twice a week with Hoagland's solution (Hoagland and Arnon 1950) in a glasshouse at the Experimental Fields of the Faculty of Biology from January to May 2005. Plants were transferred to the soil of the Experimental Fields on May 25, 2005, where they were watered twice a week with tap water until the experiment started on June 15, 2005. After this date, plants grew under Mediterranean field conditions without any additional watering, during the following 26 days.

Figure 11: *C. sinensis* tree (left) and a detail of the flower and leaves (right).

1.3.- Tobacco (*Nicotiana tabacum* L.)

Tobacco *(Nicotiana tabacum* L.) is an annual plant which can reach up to 3 m high (figure 12). Leaves of tobacco plants are oval-shaped up to 50 cm long and their flowers show white to pink petals welded in a tube-like corolla. Tobacco is native from America, but it is now sub-spontaneous in many temperate climate areas. It grows in road margins and crops, blooming in summer and fall. Tobacco is used as a model plant because it has a short life cycle (a few months), produces a great amount of seeds, is easily transformed, and yields a high amount of plant material in a short time. Moreover, tobacco genome sequencing is under way. For the experiments, 6 week-old F_2 of 2 tobacco (*Nicotiana tabacum* L. cv. Petit Havana) transformant lines over- and under-expressing ascorbate oxidase (AO), respectively, were employed as well as the wild type. The first line (P3-7-2) consisted of tobacco plants over-expressing pumpkin AO in the sense orientation under the control of a constitutive promoter. The second line (T1-6-1) consisted of plants over-expressing the tobacco AO in the antisense orientation under the control of the same promoter (for further detail, see Pignocchi et al 2003). Seeds of the P3-7-2 (denoted as 'sense' herein), T1-6-1 (denoted as 'antisense' herein) and wild type, were germinated in agar plates -containing kanamycin in the case of the transformants- in growth chambers under constant temperature of 25 °C and 16 h photoperiod of 250 μmol m^{-2} s^{-1}, in the growth chambers placed in the Ridley Building of the Newcastle University (Newcastle Upon Tyne, UK). After germination, seedlings were transferred to 1 l pots with John Innes nº 2 compost (Norwich Research Park, Colney; Norwich, UK) and covered with a Saran Wrap film. Seedlings grew under the conditions described above in the same growth

chambers. When plants reached about 5 cm height, the film was removed. After potting, plants were watered with tap water by subirrigation.

Figure 12: *N. tabacum* plants (left) and a detail of the inflorescence (right).

2.- Brief description of the core experiments

2.1.- Comparative study of total phenolic levels in *M. officinalis*, *S. officinalis* and *C. clusii* as affected by drought

During spring 2001, *M. officinalis*, *S. officinalis* and *C. clusii* plants of the same age were exposed to water deficit by withholding water under Mediterranean field conditions, and were covered with a clear PVC sheet to avoid any external water input. Leaf samples were collected approximately every two weeks at predawn, and different biochemical and physiological parameters were measured, with an emphasis on the accumulation of phenolics in leaves. Total phenolics were measured along with stress indicators, to determine the extent of accumulation of phenolics under water deficit. Given that *C. clusii* showed higher phenolic content and greater increment after the water deficit treatment (see results), *C. clusii* was chosen for further studies on flavonoids.

2.2.- Drought-induced changes in flavonoids and ascorbate in *C. clusii*

C. clusii leaf samples from the experiment described above were further analyzed to identify major antioxidant flavonoids –anthocyanidins, flavonols and flavan-3-ols- responding to the drought treatment. Aside from flavonoids, ascorbate was also determined to compare its response to that of flavonoids.

2.3.- Accumulation and oxidation of flavan-3-ols and ascorbate in field-grown *C. clusii* as affected by plant age

For this experiment, juvenile and mature *C. clusii* plants with the same genetic background growing under Mediterranean field conditions were studied during the experimental period (May-November, 2004). To determine the effects of environmental constrains (water deficit and high

light) on flavan-3-ol and ascorbate accumulation and oxidation, and the influence of plant age on these processes, flavan-3-ol content -including flavan-3-ol oxidation products and flavan-3-ol polymers- and levels of reduced and oxidized ascorbate were measured throughout the experimental period. Environmental parameters and physiological and biochemical indicators of stress were also monitored during the experiment.

To determine whether flavan-3-ols are present in *C. clusii* chloroplasts, these organelles were isolated and flavan-3-ol levels determined in chloroplast extracts.

2.4.- Effects of water deficit on flavan-3-ol accumulation and oxidation in tea plants

Water deficit and excess light are well known to induce oxidative stress. Tea plants are very sensitive to these factors and show great amounts of flavan-3-ols. To determine whether flavan-3-ols are readily oxidized *in vivo* within plant cells under extreme oxidative stress, a water deficit treatment in combination with high light was aplied to tea plants in field conditions. Flavan-3-ols, including reduced and oxidized forms, were analyzed along with stress markers.

2.5.- Apoplastic oxidation state as a signal

To determine whether ascorbate oxidation state in the apoplast affects symplast metabolism, we studied existing tobacco transformants over- and under-expressing AO. These plants allowed us to study the effects of a shift in the apoplastic oxidation state of the ascorbate, on symplast metabolism, without any additional treatment aside from the transformation. In this study we focused on gene expression and some physiological parameters, such as growth, biomass production and photosynthesis.

3.- Stress indicators

3.1.- Monitoring environmental conditions

Environmental conditions were monitored throughout the study with a weather station situated at 300 m from the experimental plot, 30 m above ground level. The weather station, which data are available at www.infomet.am.ub.es/arxiu/estacio_ub/meteodata/, consisted of a pyranometer sensor CM11 (KIPP & ZONEN, Delft, The Netherlands), which records solar radiations between 305 (UV-B) and 2800 nm (infra-red) wavelengths and a HMP35AC thermohygrometer (Väisala, Finland). Vapor pressure deficit (VPD) was calculated from air temperature and relative humidity data according to Nobel (1991).

3.2.- Water relations

Plant water status was estimated by measuring the RWC as 100x(FW-DW)/(TW-DW), where TW is the turgid weight after re-hydrating the leaves at 4 ºC for 24 h in darkness, FW is the fresh weight, and DW is the dry weight after oven-drying the leaves at 80 ºC for 24 h. Leaf area was measured by using a flatbed scanner (Epson, Nagano, Japan), and the leaf mass per area ratio (LMA) was calculated as DW per leaf area. Leaf hydration (H) was measured as (FW-DW)/DW.

3.3.- Photosynthesis

Curves of photosynthesis rates respective to the intracellular CO_2 (A/C_i curves) were obtained in tobacco plants by using a CIRAS 1 portable photosynthesis system (PP Systems, Hitchin; Hertfordshire, UK) as described elsewhere (Sanmartín et al 2003). Measurements were performed inside the growth chambers, and from the curves, the maximum photosynthetic rates and maximum carboxylation speed were obtained.

Maximum photosynthetic rates were calculated as the photosynthesis rates that do not respond to further increment of intracellular CO_2, and maximum carboxylation speeds were calculated as the increment rate of photosynthesis after the increment of intracellular CO_2 levels, within the lineal phase of this response.

3.4.- Chlorophyll and total carotenoid content

Photosynthetic pigments (chlorophylls and total carotenoids) were analyzed spectrophotometrically as described (Lichtenthaler and Wellburn 1983). In short, frozen leaf samples were ground in liquid nitrogen with pre-chilled mortar and pestle. The resulting fine powder was repeatedly extracted (four times) with 80 % acetone by ultrasonication (Vibra-Cell Ultrasonic Processor). Pigment concentrations were calculated as described by Lichtenthaler and Wellburn (1983) by recording A_{663}, A_{646} and A_{470} from the pooled supernatants. Among the different solvents tested by Lichtenthaler and Wellburn (1983), 80 % aqueous acetone is the most suitable for simultaneous extraction of chlorophylls and total carotenoids.

3.5.- Chlorophyll fluorescence

The maximum efficiency of PSII photochemistry (F_v/F_m ratio) was estimated as $(F_m–F_o)/F_m$, where F_m and F_o are the maximum and minimum fluorescence yields obtained from attached leaves with a portable fluorimeter mini-PAM (Walz, Effeltrich, Germany) according to Bilger et al. (1995). Decreases in the F_v/F_m ratio are indicative of chronic photoinhibition, thus reflecting damage to the PSII (Werner et al 2002).

3.6.- Lipid peroxidation

The extent of lipid peroxidation was estimated spectrophotometrically by measuring the amount of malondialdehyde (MDA) in leaves by the method described by Hodges et al (1999), which takes into account the presence of interfering compounds in the thiobarbituric acid (TBA)-reactive substances assay. In short, leaf samples, ground with pre-chilled mortar and pestle in liquid nitrogen, were extracted repeatedly (four times) with 80:20 (v/v) ethanol:water using ultrasonication (Vibra-Cell Ultrasonic Processor). Supernatants were pooled and an aliquot of appropriately diluted sample was added to a test tube with an equal volume of either (i) –TBA solution comprised of 20 % (w/v) trichloroacetic acid and 0.01 % (w/v) butylated hydroxytoluene (BHT), or (ii) +TBA solution containing the above plus 0.65 % (w/v) TBA. Samples were heated at 95 ^{o}C for 25 min, and after cooling, absorbance was read at 440, 532 and 600 nm. MDA equivalents (nmol ml^{-1}) were calculated as 10^{6}x[(A-B)/157,000], where:

$$A=[({}^{+TBA}A_{532})-({}^{+TBA}A_{600})]-[({}^{-TBA}A_{532})-({}^{-TBA}A_{600})]$$

$$B=[({}^{+TBA}A_{440})-({}^{+TBA}A_{600})]x0.0571.$$

4.- Studies on flavonoids

4.1.- Determination of total phenolics

Total phenolics were estimated spectrophotometrically according to Lee et al (1987). In short, leaf samples, ground with pre-chilled mortar and pestle in liquid nitrogen, were extracted four times with 50 % (v/v) methanol by ultrasonication (Vibra-Cell Ultrasonic Processor). After centrifugation, supernatants were pooled, and 2 volumes of 2 N Folin and Ciocalteu's phenolic reagent (Fluka) and 4 volumes of 17 % (w/v) sodium carbonate were added to 1 volume of leaf extract and the A_{765} was measured. Tannic acid (TA, Sigma) was used as a standard for calibration (Schützendübel et al 2001), so all measurements are given as equivalents of tannic acid, since phenolics detected by this method may have very different molecular weights.

4.2.- Determination of total anthocyanidins

Anthocyanidin content was estimated spectrophotometrically essentially as described by Feild et al (2001). In short, 100 mg plant material was ground in liquid nitrogen with a pre-chilled mortar and pestle. Anthocyanidins were repeatedly extracted (four times) from the resulting powder with acidified (0.1 N HCl) n-n-dimethylformamide (Sigma) by ultrasonication (Vibra-Cell Ultrasonic Processor). 30 % (v/v) H_2O_2 (Sigma) was added to an aliquot of the pooled supernatants to eliminate interferences with soluble tannins (Lee et al 1987). A_{525} was read to quantify anthocyanidins. The molar extinction coefficient applied for calculations was that of cyanidin 3-glycoside, 3.8×10^4 l g^{-1} cm^{-1}, with a correction for the interference of phaeophytin: $0.55 \times A_{554}$ (Feild et al 2001).

4.3.- Analyses of flavonols and flavan-3-ols

Antioxidant flavonoids in *C. clusii* leaves (flavan-3-ols and flavonols) were determined by HPLC with a modification of the method described by Revilla and Ryan (2000). Plant material was ground in liquid nitrogen with a pre-chilled mortar and pestle, and the resulting powder was extracted four times with methanol by ultrasonication (Vibra-Cell Ultrasonic Processor). After centrifugation, the resulting supernatants were pooled, evaporated to dryness, and re-suspended in 2 ml of methanol. Flavonoids were separated on a Hypersyl ODS-5 μm column (250x4.6 mm, Teknokroma, Sant Cugat del Vallès, Spain) at a flow rate of 1 ml min^{-1}. The solvents consisted of (A) methanol:water (2:98; v/v; the water being adjusted to pH 3 with phosphoric acid) and (B) acetonitrile. The gradient started with 100 % solvent A, 0–5 min decreasing to 90 % A, 5–13 min decreasing to 82 % A, 13–17 min decreasing to 80 % A, 17–22 min decreasing to 70 % A, 22–26 min decreasing to 62 % A, 26–30 min decreasing to 58 % A, 30–34 min decreasing to 50 % A, 34–39 min decreasing to 30 % A, 39–44 min decreasing to 0 % A, and then the column was re-equilibrated to the initial conditions before the next sample was injected. Detection was carried out at 275 nm (for flavan-3-ols) and 355 nm (for flavonols) with a diode array detector 2996 (Waters, Milford, MA). Compounds were identified by their characteristic spectra and by co-elution with standards obtained from Fluka and Sigma (table 2, figure 13).

The presence of (-)-epicatechin (EC), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG) in samples was confirmed further by liquid chromatography coupled to electrospray ionization mass spectrometry (HPLC-ESI-MS) (PerkinElmer LC Series 200, PerkinElmer Inc., Shelton, CT, USA, coupled to an API 3000, PerkinElmer Sciex, Concord, Ont., Canada) by applying a declustering potential of -60 V. The gradient used

was the same as described previously, except that the flow rate was 0.8 ml min^{-1} and formic acid replaced phosphoric acid, in solvent A. Individual flavonoids were identified by comparing their mass spectra (figures 14 to 16) with those of pure standards, which were obtained from Sigma and Fluka.

		RT HPLC		RT MS	
	m/z	**Standard**	**Sample**	**Standard**	**Sample**
Epigallocatechin	305	10,8	-	6,28	-
Catechin	289	11,7	-	8,59	-
Epicatechin	289	14	12,9	13,87	13,73
Epigallocatechin gallate	457	14,5	14,4	13,43	14,74
Epicatechin gallate	441	18,7	18,4	19,95	18,85
Quercetin	301	27	-	-	-
Kaempferol	285	30	-	-	-

Table 2: Data of the chromatograms recorded at 275 nm and 355 nm of *C. clusii* leaf extracts. RT HPLC stands for the retention times in HPLC analyses, and RT MS stands for the retention times of the MS chromatograms of each mass to charge ratios (m/*z*)

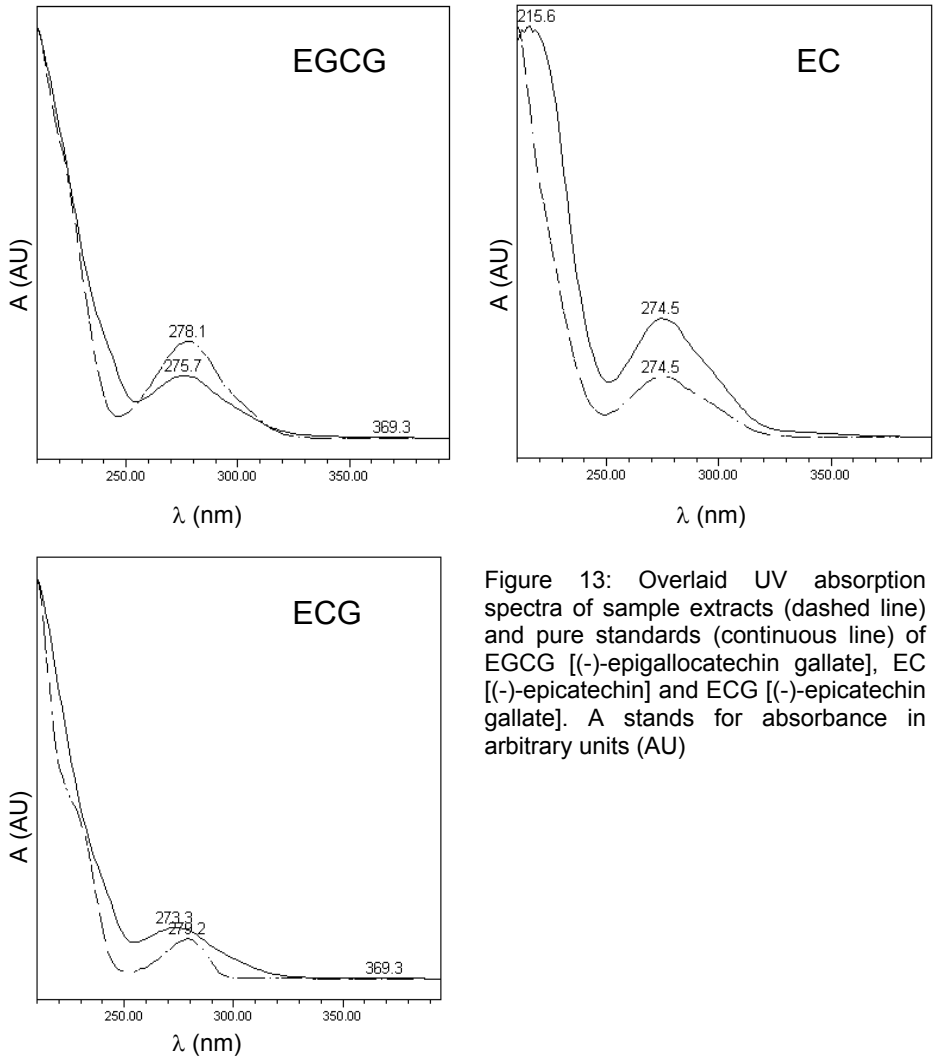

Figure 13: Overlaid UV absorption spectra of sample extracts (dashed line) and pure standards (continuous line) of EGCG [(-)-epigallocatechin gallate], EC [(-)-epicatechin] and ECG [(-)-epicatechin gallate]. A stands for absorbance in arbitrary units (AU)

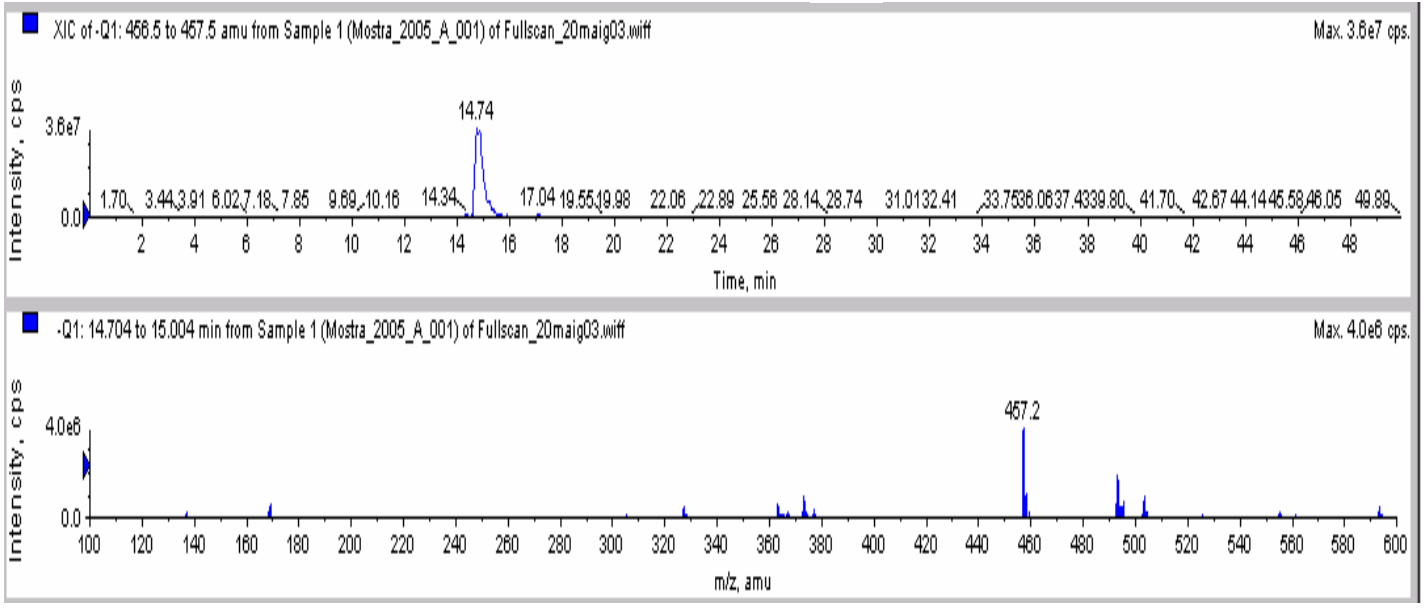

Figure 14: Identification of (-)-epigallocatechin gallate in *C. clusii* leaves. Top; chromatogram of the m/*z* ranging from 456.5 to 457.5 [(-)-epigallocatechin gallate pseudo-molecular weight]. Bottom; mass spectrum of the peak between 14.70 and 15.00 min showing a pseudo-molecular peak at m/*z* 457.2 [(-)-epigallocatechin gallate pseudo-molecular weight]. The term m/*z* stands for the mass to charge ratio, and the intensity of the signal is expressed in counts per second (cps).

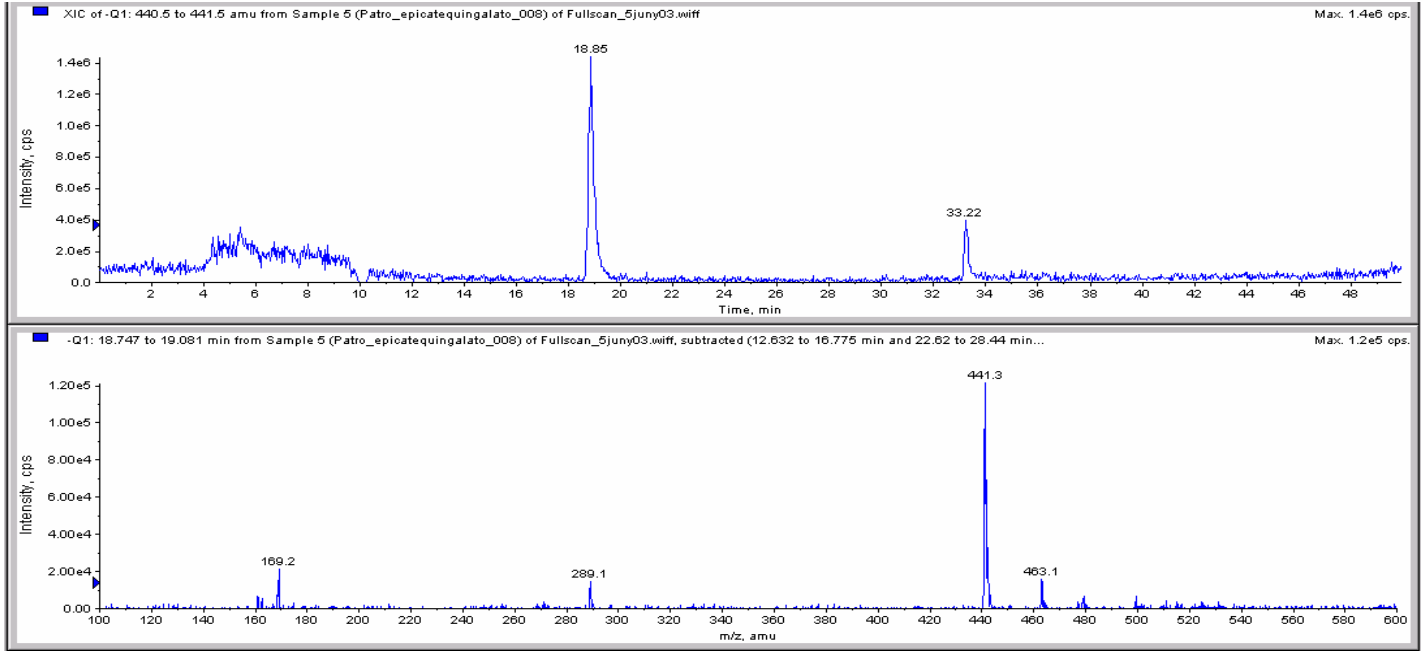

Figure 15: Identification of (-)-epicatechin gallate in *C. clusii* leaves. Top; chromatogram of the m/*z* ranging from 440.5 to 441.5 [(-)-epicatechin gallate pseudo-molecular weight]. Bottom; mass spectrum of the peak between 18.75 and 19.08 min showing a pseudo-molecular peak at m/*z* 441.3 [(-)-epicatechin gallate pseudo-molecular weight] and two main fragments (m/*z* 169.2, gallate; and m/*z* 289.1, (-)-epicatechin]. The term m/*z* stands for the mass to charge ratio, and the intensity of the signal is expressed in counts per second (cps).

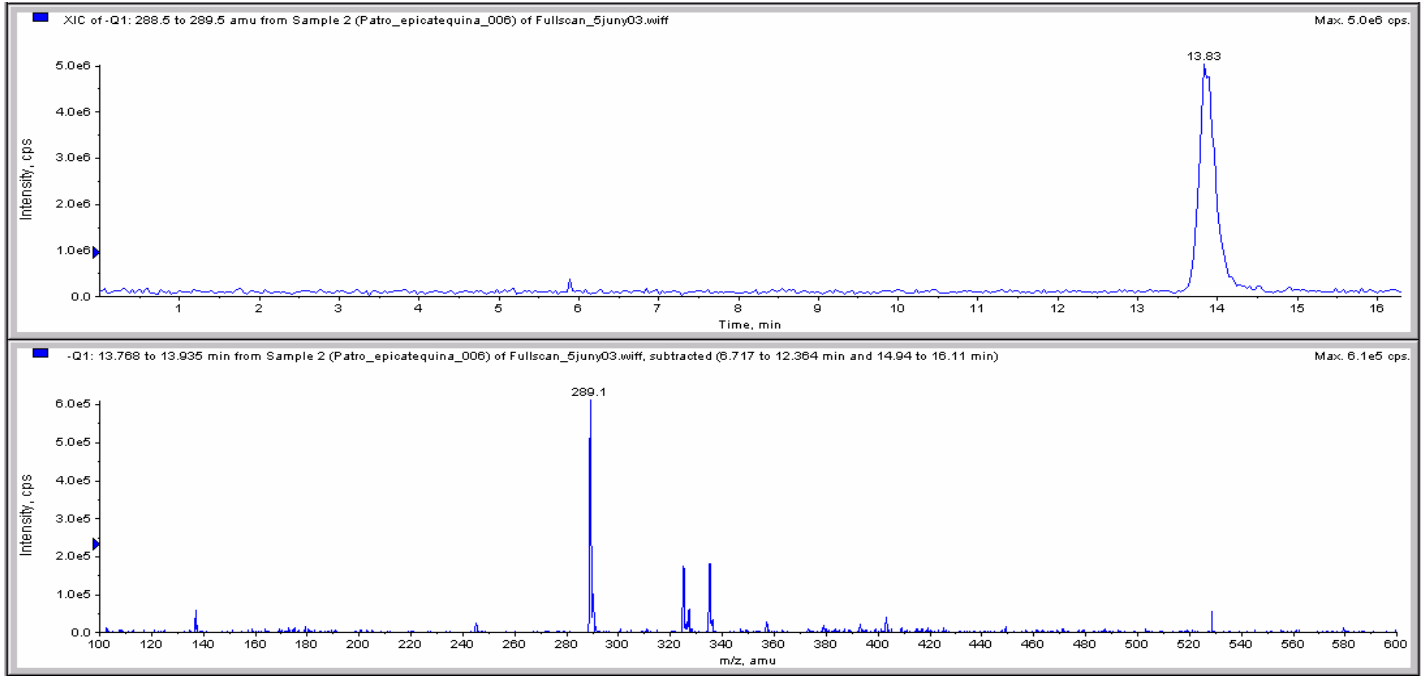

Figure 16: Identification of (-)-epicatechin in *C. clusii* leaves. Top; chromatogram of the m/*z* ranging from 288.5 to 289.5 [(-)-epicatechin pseudo-molecular weight]. Bottom,; mass spectrum of the peak between 13.77 and 13.94 min showing a pseudo-molecular peak at m/*z* 289.1 [(-)-epicatechin pseudo-molecular weight]. The term m/*z* stands for the mass to charge ratio, and the intensity of the signal is expressed in counts per second (cps).

4.4.- Determination of flavan-3-ols and their quinones

For the determination of flavan-3-ols and flavan-3-ol quinones, plant material was ground in liquid nitrogen with pre-chilled mortar and pestle. The monomeric flavan-3-ols, EC and EGCG, and their quinones, ECQ and EGCGQ, respectively, were extracted with ethyl acetate (by using ultrasonication) and derivatized with sodium benzenesulfinate by a modification of the method described by Vivas de Gaulejac et al (2001). Monomeric flavan-3-ols and their quinones were analyzed by HPLC-ESI-MS. The HPLC separation was carried out with a Waters 2996 (Waters, Milford, MA) separation module and a Sunfire™ C_{18} column (3.5 μm particle size; 2.1x50 mm) with a pre-column of the same material (2.1x10 mm) (Waters). The gradient was applied at a 0.6 ml min^{-1} flow rate, as follows: initial conditions, 70 % A and 30 % B; min 1, 20 % A and 80 % B; min 4, 100 % B; min 5, 100 % B; min 6, 100 % C; min 8, 100 % C; min 10, 70 % A and 30 % B; and 3 min of column re-conditioning. Solvent A consisted of acidified water (0.05 % formic acid); solvent B, 1:4 (v/v) acetonitrile:solvent A; solvent C, acetonitrile. The ESI-MS conditions were essentially the following. At the source: capillary, 3 KV; HV lens, 0 KV; Cone, -50 KV. At the MS: ion energy, 3.0 V; ion energy ramp, 6.0 V; LM and HM resolution 12.5; multiplier, 700 V. EC and EGCG were quantified by using the corresponding standards from Sigma. ECQ and EGCGQ were quantified by using these standards once they were oxidized and derivatized as described (Vivas de Gaulejac et al 2001, see figure 17 for further details). The identity of the peaks corresponding to EC, EGCG, ECQ and EGCGQ was confirmed further by their tandem mass spectra (product ion scan of m/z 289, 457, 429 and 597, respectively; figure 18) by using an HPLC (HP 1100 Series, Palo Alto, CA) coupled with an electrospray ionization tandem mass spectrometry system (ESI-MS/MS) (PerkinElmer Sciex).

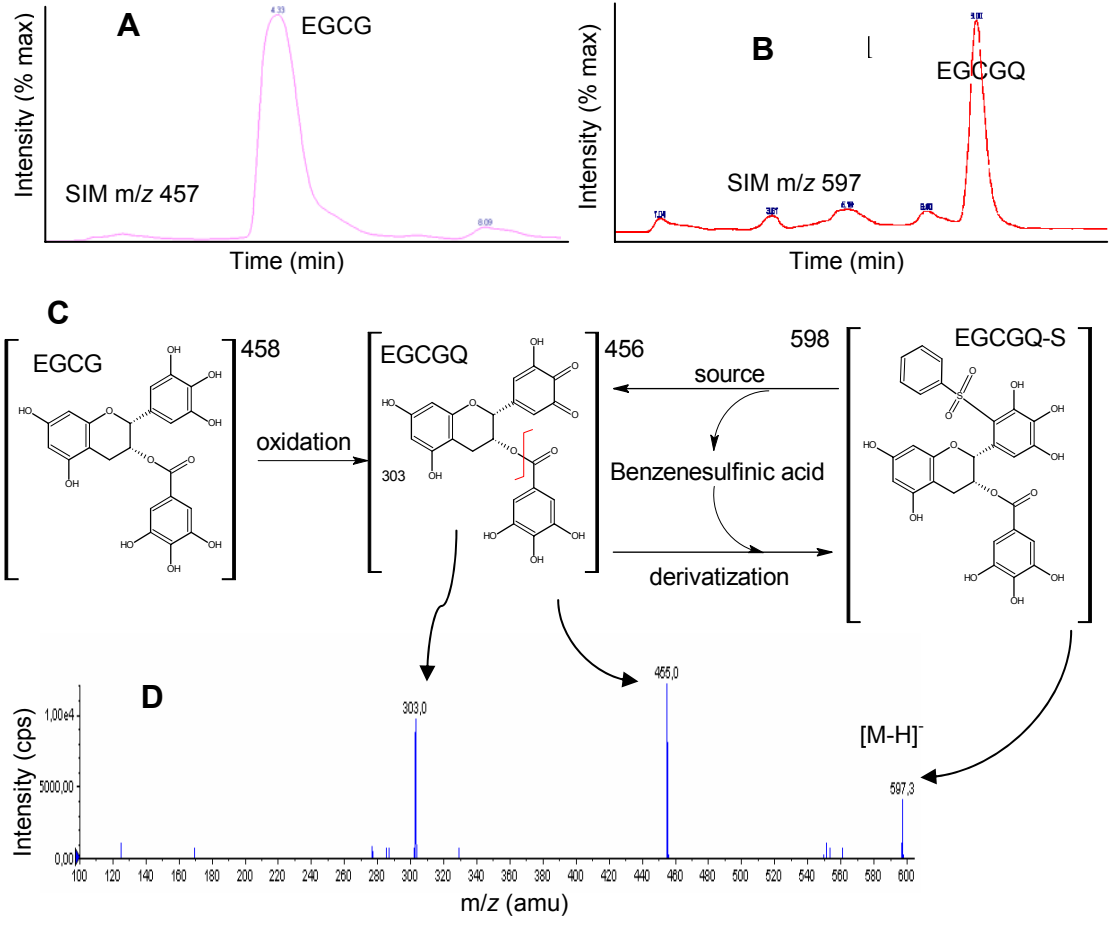

Figure 17: (A) Single ion mode (SIM) chromatogram of (-)-epigallocatechin gallate (EGCG) (m/*z*=457); (B) SIM chromatogram of the sulfonated (-)-epigallocatechin gallate quinone (EGCGQ-S); (C) molecular formula of EGCG, EGCG quinone (EGCGQ), including molecular masses and fragmentation products; Mass spectrum of the EGCGQ-S. m/*z* stands for mass-to-charge ratios; and $[M-H]^-$ stands for pseudo-molecular peak, and the peak intensity is expressed as a percentage of the maximum intensity, or as counts per second (cps)

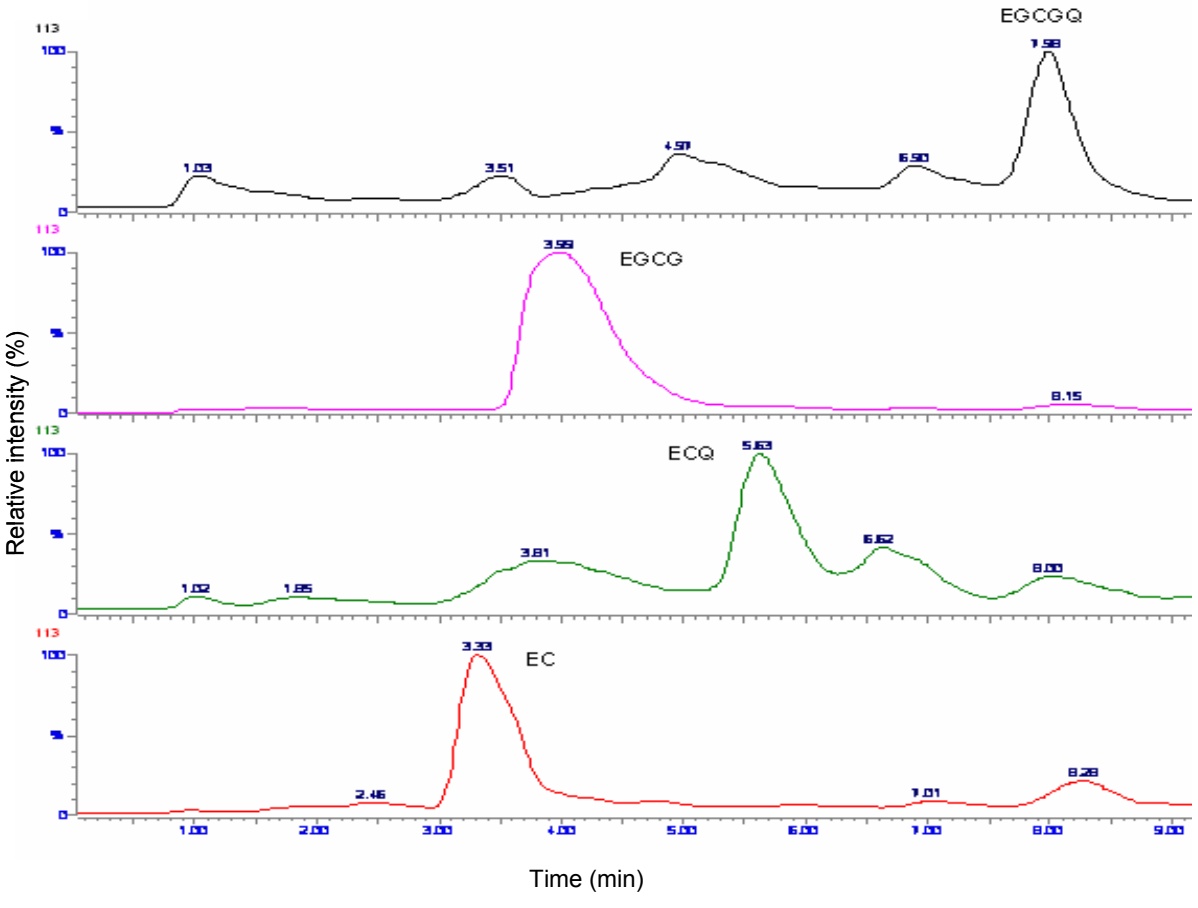

Figure 18: Single ion mode chromatograms of a *C. clusii* leaf extract. From top, (-)-epigallocatechin gallate quinone (EGCGQ) chromatogram (m/*z* 597), (-)-epigallocatechin gallate (EGCG) chromatogram (m/*z* 457), (-)-epicatechin quinone (ECQ) chromatogram (m/*z* 429), and (-)-epicatechin (EC) chromatogram (m/*z* 289).

4.5.- Total proanthocyanidins

Levels of PAs were estimated spectrophotometrically by the butanol:HCl assay as described by Dalzell and Kerven (1998). Essentially, leaves samples were repeatedly extracted (four times) with 70 % (v/v) aqueous acetone containing 5.26 mM sodium metabisulfite using ultrasonication (Vibra-Cell Ultrasonic Processor). One ml of the pooled supernatants was mixed with 5 ml butanol:HCl (95:5, v/v) in a screw-cap tube and incubated at 95 ºC for 1 hour in a waterbath. After cooling at room temperature and darkness, A_{550} was read. The standard calibration curve was built by using pure proanthocyanidin B2 (Fluka). As the stereochemistry of PAs varies with PA chain length, all PA measurements were expressed as proanthocyanidin B2 equivalents.

4.6.- Determination of flavan-3-ols in chloroplasts

Determination of flavan-3-ols in chloroplasts was carried out as described above by using chloroplast-enriched fractions isolated as follows.

4.6.1.- Chloroplast isolation

Chloroplast isolation was carried out essentially as described by Munné-Bosch and Alegre (2001). In short, fresh leaf samples were homogenised in isolation buffer (pH 7.8) containing 0.5 M sorbitol, 50 mM tricine, 1 mM dithiothreitol (DTT), 1 mM magnesium chloride, 1 mM BHT, 0.1 % (v/v) bovine serum albumin. The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 2500 g for 4 min at 4 ºC. The resulting pellet was resuspended in isolation buffer and centrifuged at 200 g for 1 min at 4 ºC. The supernatant was further centrifuged at 2500 g for 4 min at 4 ºC. The resulting pellet was resuspended in an appropriate volume of isolation buffer, layered onto 10 ml of 25 % percoll (Sigma) in isolation buffer and

centrifuged at 15800 g for 20 min at 4 ºC. The resulting chloroplast pellet was resuspended in isolation buffer (without bovine serum albumin) and centrifuged again at 2500 g for 4 min at 4 ºC to remove the remaining percoll. Isolated chloroplasts were used immediately for further analyses.

Protein content in soluble and particulate fractions was determined spectrophotometrically using a commercially available kit (Sigma) based on modifications of the Lowry method.

4.6.2.- Chloroplast purity

The purity of isolated chloroplasts was determined by analyzing the activity of enzymes exclusive of different organelles. NADPH-cyt c reductase (endoplasmic reticulum), cyt c oxidase (mitochondria) and vanadate-sensitive ATP-ase (plasmalemma) activities were measured spectrophotometrically as described by Munné-Bosch and Alegre (2001).

4.6.3.- Chloroplast integrity

The integrity of isolated chloroplasts was determined by the ferricyanide reducing assay (Lilley et al 1975), monitoring O_2 release by PSII with a Clark-type oxygen electrode. The reaction solution consisted essentially of intact or lysed chloroplasts (osmotic shock) in the presence of ferricyanide, an artificial electron aceptor at PSII level that does not cross chloroplast envelopes. Chloroplast integrity was calculated as $100x(b\text{-}a)/b$, where a is the oxygen release by intact chloroplasts and b is that of ruptured chloroplasts.

5.- Studies on ascorbate

5.1.- Determination of ascorbate in whole leaves

Ascorbate and DHA were determined spectrophotometrically by an adaptation of the method described by Kampfenkel et al (1995). This method is based on the reduction of Fe^{3+} to Fe^{2+} by the ascorbate and the reduction of DHA to ascorbate by dithiotreitol. In short, frozen leaf samples were ground in liquid nitrogen and the resulting powder was repeatedly extracted (four times) with 6 % (w/v) trichloroacetic acid using ultrasonication (Vibra-Cell Ultrasonic Processor). An aliquot of the pooled supernatants was assayed as described (Kampfenkel et al 1995). The oxidation state of the ascorbate pool was estimated as $100x(DHA/Asc_t)$, where Asc_t is reduced plus oxidized (DHA) ascorbate.

5.2.- Determination of ascorbate in the apoplast and symplast of tobacco leaves

For the experiments with tobacco, ascorbate and DHA both in the symplast and apoplast were determined spectrophotometrically as described by Sanmartín et al (2003). For the apoplast ascorbate pool size and oxidation state determination, leaf tissue harvested from young fully expanded leaves was infiltrated with a buffer containing 66 mM potassium phosphate (pH 3.0), 0.2 mM diethylenetriamine-pentaacetic acid (DTPA) and 1 mM sodium azide; carefully rolled and placed in a 5 ml pipette tip. Then, the samples were centrifuged at 3000 g for 5 min at 4 ºC. The apoplast washing fluid (AWF) was recovered in 1.5 ml microcentrifuge tubes containing 100 µl of a solution with 6 % (w/v) *m*-phosphoric acid and 0.2 mM DTPA. The residual leaf material after the AWF extraction was used to determine the ascorbate and DHA in the symplast. Residual plant material (*ca.* 0.1 g) was homogenized in liquid nitrogen and extracted in 1 ml 6 % (w/v) *m*-

phosphoric acid containing 0.2 mM DTPA. Afterwards, extracts were centrifuged at 10000 g for 4 min at 4 ºC, and the supernatant was recovered.

Ascorbate and DHA contents in tobacco symplast and apoplast extracts were determined as described by Takahama and Oniki (1992) by following the decrease of A_{265} in the sample after addition of AO. For the ascorbate determination, the reaction mixture consisted of 50 µl of sample extract (for the symplast) or AWF (for the apoplast) in 60 mM potassium phosphate buffer (pH 6.3) and 1 U ml^{-1} AO. For the DHA determination, 2 mM DTT was added, instead of AO, to a different aliquot, and A_{265} was measured after complete reduction of the sample (5 min approx.). Readings were taken before and after adding AO or DTT to the reaction mixture, and the A_{265} of the AO or DTT solution was taken into account in the calculations. The oxidation state of the ascorbate pool was estimated as above.

5.3.- Ascorbate oxidase

The AWF was obtained as described elsewhere (Sanmartín et al 2003) with minor modifications. Essentially, *ca.* 0.5 g leaf tissue -excluding the central nerve- were harvested from young fully developed tobacco leaves. Samples were vacuum infiltrated applying 3 pulses of 10 sec at –70 KPa with a pH 7.4 phosphate buffer containing 66 mM potassium posphate, 2.2 mM DTPA and 40 mM calcium chloride. The tissue was then blotted-dry, carefully rolled and placed in a 5 ml pippete tip. Samples were then spun at 3000 g for 5 min at 4 ºC and the AWF was collected in 1.5 ml microcentrifugue tubes. Infiltration and AWF recovery was repeated 2 more times on the residual leaf material.

After the extraction of the AWF, the residual leaf material was extracted for the proteins in soluble and particulate fraction, essentially as described by Sanmartín et al (2003). For the soluble fraction, the residual leaf material

(0.5 g aprox.) was homogenised in liquid nitrogen and extracted with 2 vol of buffer (pH 7.4; 50 mM potassium phosphate buffer, 1 mM phenylmethylsulfonyl fluoride, 1.5 % polyvinyl polypyrrolidone and 10 % glycerol). The extract was allowed to stand on ice for 20 min vortexing occasionally. Afterwards, the homogenate was centrifugated for 20 min at 15000 g and 4 ºC, and the supernatat was collected. This supernatant along with the AWF constituted the soluble fraction.

The remaining pellet (particulate fraction, i.e. ionically bound fraction) was resuspended in the extraction buffer containing 1 M sodium chloride and 1 M calcium chloride, vortexed occasionally during 10 min at 4 ºC and re-spun under the same conditions as above. The supernatant was recovered and the particulate fraction extraction was repeated in the remaining pellet. The pooled supernatants constituted the particulate fraction.

The AO activity was monitored as described by Moser and Kanellis (1994) following spectrophotometrically the oxidation of ascorbate at 265 nm. The reaction mixture contained 66 mM potassium phosphate (pH 5.3), 0.15 mM ascorbate, and sample solution, and the extinction coefficient applied was 14 mM^{-1} cm^{-1} at 265 nm. One AO unit is defined as the oxidation of 1 μmol ascorbate in 1 min at 25 ºC.

Protein content in soluble and particulate fractions was determined spectrophotometrically using a commercially available kit (Sigma) based on modifications of the Lowry method.

5.4.- Analyses of nucleic acids

5.4.1.- Total RNA and mRNA isolation

Total RNA was extracted by the Tri reagent (Helena Biosciences, Sunderland, Tyne & Wear, UK) method. In short, 100 mg of plant material were ground in liquid nitrogen with pre-chilled mortar and pestle, and

poured into 1 ml Tri reagent. Chloroform was added to separate lipids. After spinning, the RNA fraction (upper supernatant) was collected, and the RNA was precipitated with isopropanol. The precipitate was further cleaned with ethanol, and finally re-suspended in diethyl pyrocarbonate-treated nanopure water. Only RNA with an $A_{260}/A_{280}>1.8$ (indicative of the low contamination with proteins) was used for the experiments. Moreover, isolated RNA was run in de-naturing agarose gels to determine the integrity of the RNA molecules.

mRNA was isolated from total RNA using the PolyAtract® kit (Promega Corp., Woods Hollow Road, Madison WI, USA) following the manufacturer's instructions.

5.4.2.- Subtractive hybridisation library construction

The mRNA was concentrated by ethanol precipitation in order to achieve the manufacturer's suggested concentration. The subtractive hybridisation was carried out using the PCR-Select cDNA subtraction kit (BD Biosciences Clontech, Mountain View, CA, USA) following the manufacturer's instructions. Forward and reversed subtractions were carried out by using the wild type as tester and each transformant line as driver and *vice versa*, in order to obtain up- and down-regulated cDNAs. The amplified differentially expressed cDNAs were cloned unespecifically using a TOPO TA cloning system (Invitrogen, Inchinnan Business Park, Paisley, UK) following the manufacturer's instructions. Plasmid DNA bearing any insert was extracted using a commercially available kit (Qiagen, Crawley, West Sussex; UK) and sequenced using M13 forward and reversed promoters (Macrogen Inc. Seoul, Korea).

5.4.3.- Gene expression analyses

Gene expression analyses were performed by one-step semi-quantitative reverse transcription polymerase chain reaction (SQ-RT-PCR). In short, total RNA was digested with DNase and denatured at 65 °C. The PCR reaction mixture consisted on 100 ng total RNA, dNTPs (deoxy-nucleotide triphosphate), forward and reverse complementary primers (see below), RNase Out –an RNase inhibitor-, Superscript II reverse transcriptase, Taq polymerase, DTT, magnesium chloride and PCR buffer, all purchased from Invitrogen. Samples were placed in a thermal cycler and the following gradient was applied: 50 °C for 30 min, 94 °C for 2 min, 95 °C for 1 min; 20-30 times (94 °C 30 sec, primer specific annealing temperature (see below) for 30 sec, 72°C 45 sec); and 72 °C for 5 min (Peltier Thermal Cycler 200, MJ research Inc., Waltham, MA). Primers were designed according to the following criteria: 18-20 base pairs (bbpp) long; 40-50 % of the bases being G or C; no T in the 3' end; no more than 3 C or T repeated towards the 3' end; no palindromic sequences; no primer-dimer sequences; amplified fragment between 500 and 700 bbpp, including the primers; reversed complementary primer within the 3' untranslated region (UTR) (alternatively, the forward primer within the 5'UTR); theorethical annealing temperature (calculated as 4x[G+C]+2x[A+T], where G, C, A and T indicate the number of G, C, A and T of the primer's sequence) between 50 and 60 ºC. The primer specific annealing temperature was set up for every primer couple employed, and the number of cycles was optimized to keep the amplification within the linear range (figure 19). The specific forward and reverse complementary primers consisted on the following sequences. MDHAR partial EST (expressed sequence tag, acc. nº BQ842867): NTMDHARF: 5'GACAGAAACTTCAAATAGCCG3'; NTMDHARR: 5'GAACATGTTGATCATTCTCGC3'; DHAR (encoding for

dehydroascorbate reductase; acc. nº AY074787): NTDHARF: 5'ATCTGTGTCAAGGCTGCTG3'; NTDHARR: 5'ACTTCCTGCGAAACAACGG3'; cAPX gene (encoding for a cytosolic APX; acc. nº U15933): NTFCAPX: 5'CTGGAGGACCTGATGTTC3'; NTRCAPX: 5'CGTCTAATAACAGCTGCC3'; NtEIG-C08 gene (encoding for GPX; acc. nº AB041518): NTFGPX: 5'CCAATCTAGCAAGCCTCAA3'; NTRGPX: 5'ATGCAGACAAATCCAGAGC3'; PAL gene (encoding for phenylalanine ammonia lyase; acc. nº X78269): NTFPALA: 5'GCGATAGACTTGAGGCATT'; NTRPALA: 5'GATCCTGTTGTTTGGAGAACC3'; NtTPC1B gene (encoding for a two-pore calcium channel; acc. nº AB124647): NTTPC1BF: 5'CCAACGGAGAATGGATTCG3'; NTTPC1BR: 5'CAGCATGGAGAAAGGAGCA3'; Tub A3 gene (encoing for α-tubulin 3; acc. nº AJ421413): NTFTUBA3: 5'TCCTCATATGCTCCtGTC3'; NTRTUBA3: 5'AGCAGACAAGCATTCTAC3'; 18S rRNA (acc. nº NTF18SRNA): 5'GACGAACAACTGCGAAAG'; NTR18SRNA: 5'CATCTAAGGGCATCACAG3'

5.4.4.- DNA and RNA gels

RNA gels were performed on 1.5 % agarose gels containing 0.5 μg ml^{-1} ethidium bromide and 17 % formaldehyde as a denaturing agent, in MOPS (morpholinepropanesulfonic acid) buffer. Total RNA samples were loaded in a commercial loading buffer (Invitrogen) containing formamide, formaldehyde, ethidium bromide, 10X MOPS buffer and loading dye containing glycerol, EDTA (ethylenediaminetetraacetic acid), bromophenol blue and xylene cyanol. Samples were run for *ca*. 15 min at 90 V.

DNA gels were also run in 1.5 % agarose containing 0.5 μg ml^{-1} ethidium bromide, but in TBE (tris/borate/EDTA) buffer. DNA samples were loaded in a commercial DNA loading dye (Invitrogen) containing glycerol, EDTA, bromophenol blue and xylene cyanol.

The band intensities were quantified with the Eagle Eye II (Stratagene, Garden Groove, CA).

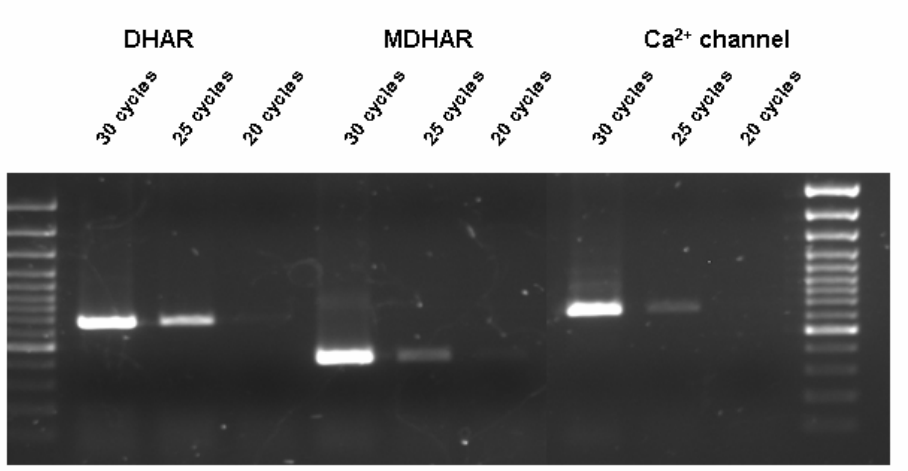

Figure 19: Agarose gel of the PCR products of the dehydroascorbate reductase (DHAR, acc. nº AY074787), monodehydroascorbate reductase (MDHAR, acc. nº BQ842867) and a plasmalemma Ca^{2+} channel (NtPC1B, acc. nº AB124646), showing the number of amplification cycles.

6.- Statistical analyses

In order to determine the statistical significance of the differences observed in the results, ANOVA or *t*-tests were performed at $P<0.05$ with the SPSS software (SPSS Inc. Chicago, IL). All data sets were checked for normal distribution ($P<0.05$) prior to any further analysis. When a variable tested in ANOVA tests showed statistically significant differences after one or several factors, the Tukey post-hoc test was applied to establish homogeneous groups, that are denoted with letters in the graphics presented.

7.- Timing of the experiments

The first water deficit treatment in field conditions to *C. clusii*, *S. officinalis* and *M. officinalis* was applied in spring 2001. Between the rest of 2001 and 2002, total phenolics and anthocyanidins were measured.

In 2003, flavonoids, ascorbate and biochemical stress indicators (e.g. lipid peroxidation) were determined in *C. clusii* leaf samples from the previous experiments. In 2003, experiments with tobacco plants were performed, including the determination of ascorbate, AO activity, photosynthesis measurements and gene expression analyses. In 2004, the experiment with juvenile and mature *C. clusii* plants was run. The analyses of the samples collected in this experiment, including flavan-3-ols, flavan-3-ol quinones, PAs, ascorbate and stress indicators, were carried out between the sampling days and 2006.

In the meantime, in 2005, the experiments with tea plants, which included measurement of flavan-3-ols, flavan-3-ol quinones, PAs and stress indicators, were performed. Moreover, in the same year, 2005, *C. clusii* chloroplasts were isolated and analyzed for the presence of flavan-3-ols and flavan-3-ol quinones. See table 3 for a summary of the timing of the experiments.

Plant material	Year	Total phenolics	Anthocyanins	Flavan-3-ols	Flavan-3-ol quinones	PAs	Ascorbate	Stress indicators	Photosynthesis	AO	Gene expression
C. clusii *S. officinalis* *M. officinalis*	2001-2002	√	√								
C. clusii	2003			√			√	√			
N. tabacum	2003						√		√	√	√
C. clusii (juv and mat)	2004-2006			√	√	√	√	√			
C. clusii chloroplasts	2005			√	√						
C. sinensis	2005			√	√	√		√			

Table 3: Summary of the measurements performed in the different species employed for the experiments (juv and mat stand for juvenile and mature plants). Ant, anthocyanins; Quinones stand for flavan-3-ol quinones; PAs, proanthocyanidins; AO, ascorbate oxidase activity; DHA, dehydroascorbate. Stress indicators stands for relative leaf water content, leaf hydration, leaf mass per area ratio, environmental conditions, lipid peroxidation, chlorophyll and carotenoid contents and maximum quantum yield, together.

RESULTS

1.- Comparative study of total phenolic levels in *M. officinalis*, *S. officinalis* and *C. clusii* as affected by drought

1.1.- Characterization of the stress imposed

Before the experiment began, *C. clusii*, *S. officinalis* and *M. officinalis* were well watered. Thereafter, plants grew under water deficit by preventing water input from rainfall by covering them with a clear PVC sheet when rain was expected, for 50 days. The daily maximum PFD ranged between 1832 and 1910 μmol m^{-2} s^{-1} during these 50 days. The maximum diurnal temperature in the experimental plot ranged between 19.7 and 25.2 ºC and the VPD ranged between 0.84 and 2.27 KPa (table 4). These environmental data indicate that during the experimental period the incident light and the evaporative demand were high, while maximum air temperatures remained mild.

Days of drought	PFD ($\mu molm^{-2}s^{-1}$)	T_{air} (ºC)	VPD (KPa)
0	1837	25.2	2.27
15	1832	19.7	0.84
30	1889	23.0	1.27
50	1910	21.8	1.37

Table 4: Environmental parameters during sampling days of a drought treatment in field conditions. PFD, photon flux density; T_{air}, maximum air temperature; VPD, vapor pressure deficit.

From the three plant species, *M. officinalis* showed the highest RWC at the beginning of the experiment. The RWC in this species decreased from 86 % to 40 % after the 50 days of the experiment. *S. officinalis* showed lower RWC values at the beginning of the experiment (77 %), which decreased to

59 % after 50 days of drought. *C. clusii* showed a RWC of 82% at the beginning of the experiment and decreased to 64 % at the end of it. With similar RWC values at the beginning of the experiment in all three plant species, *M. officinalis* showed the strongest RWC decrease, while *C. clusii* kept the highest RWC among the three species studied. *S. officinalis* showed an intermediate water loss (figure 20).

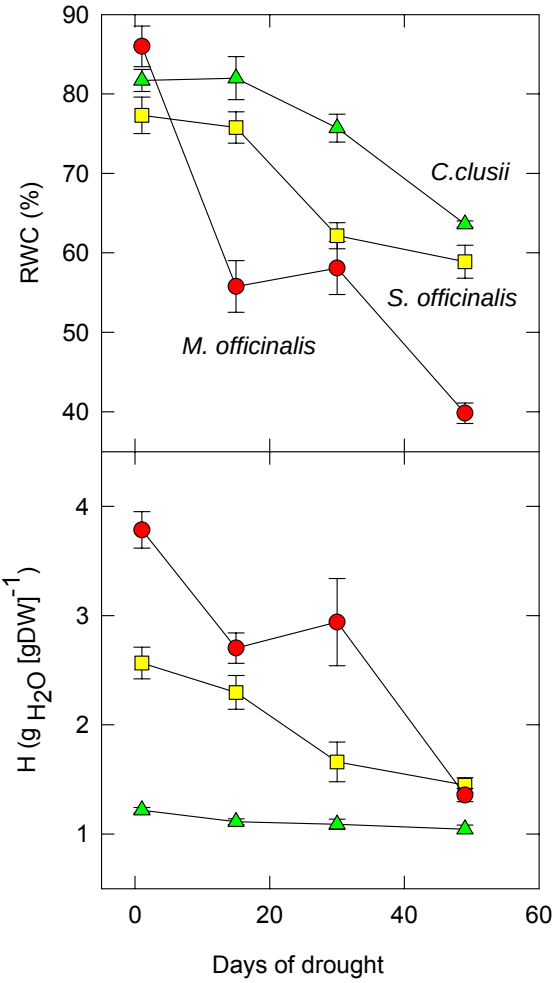

Figure 20: Changes in the relative leaf water content (RWC) and leaf hydration (H) during a drought period in the three species studied

M. officinalis also showed the highest H at the beginning of the experiment (3.8 g H_2O g DW^{-1}). In this species, H decreased to 1.4 g H_2O g DW^{-1} after 50 days of water stress treatment. *S. officinalis* showed initial H values of 2.6 g H_2O g DW^{-1}, decreasing to 1.4 g H_2O g DW^{-1} at the end of the experiment. In *C. clusii* leaves, initial H values were lower (1.5 g H_2O g DW^{-1}), and showed only a slight decrease at the end of the experiment (see figure 20). There were clear differences in the initial H between all three plant species, so the lower the initial H, the smaller the decrease in H after the experiment.

The LMAs at the beginning of the experiment indicates that *C. clusii* leaves (1.72 g dm^{-2}) are more sclerophyllous than those of *S. officinalis* (0.90 g dm^{-2}) and *M. officinalis* (0.35 g dm^{-2}), respectively. There were no significant changes in the LMA neither in *S. officinalis* nor in *M. officinalis* during the experiment ($P<0.05$; figure 21), keeping LMA values between 0.90 and 1.16; and 0.35 and 0.58 g dm^{-2}, respectively. In contrast, *C. clusii* increased its LMA from 1.72 to 3.18 g dm^{-2} after 30 days of treatment, showing the strongest increment during the first 15 days. After these initial 15 days, the LMA in *C. clusii* leaves increased only slightly (figure 21). *C. clusii* nearly doubled its LMA during the treatment, while *M. officinalis* and *S. officinalis* kept their LMA constant.

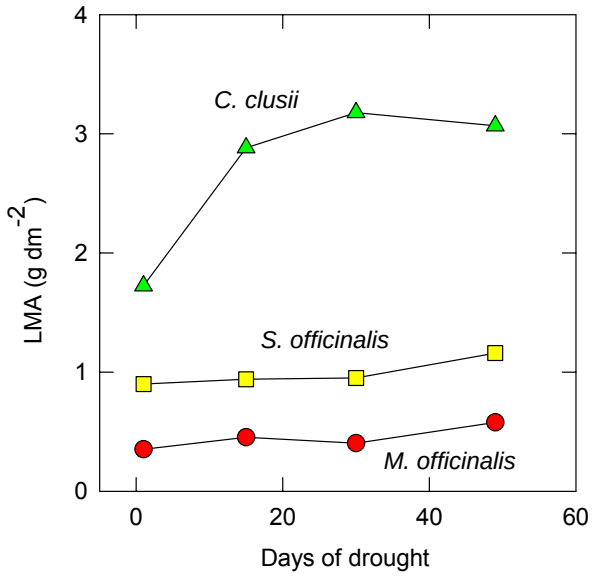

Figure 21: Changes in the leaf mass per area ratio (LMA) during a drought period in all three species studied.

1.2.- Changes in total phenolics and anthocyanidins

Total phenolic content in *M. officinalis*, *S. officinalis* and *C. clusii* at the beginning of the experiment, when expressed on a dry weight basis indicate that the latter species show the highest phenolic content –48.91, 30.59 and 52.23 μEq g DW^{-1}, in *M. officinalis*, *S. officinalis* and *C. clusii*, respectively. When expressed on a leaf area basis, *C. clusii* also showed higher phenolic content than *S. officinalis* and *M. officinalis* (89.84 μEq dm^{-2} in *C. clusii vs.* 22.95 and 17.12 μEq dm^{-2} in *S. officinalis* and *M. officinalis*, respectively) ($P<0.05$) (see figure 22).

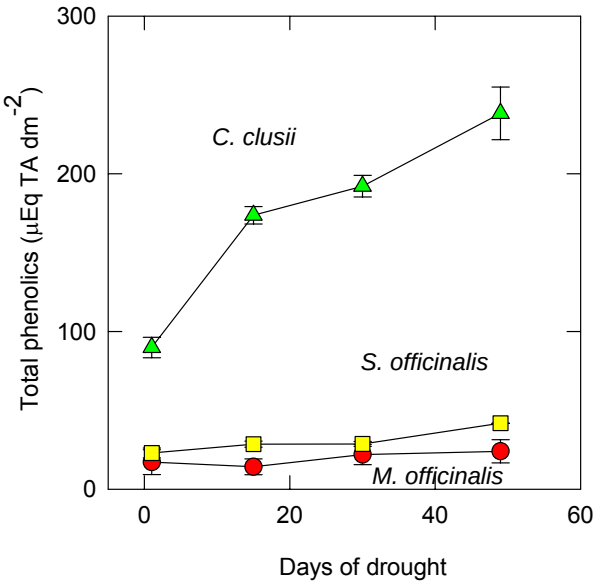

Figure 22: Changes in total phenolics during a drought period in all three species studied (TA, tannic acid).

Levels of total phenolics in *M. officinalis* were constant during the experiment, either on a dry weight basis or on a leaf area basis, ranging around 20 µEq dm^{-2}. In *S. officinalis*, total phenolics increased from 22.95 to 41.82 µEq dm^{-2} (that is an 80% increase) during the treatment. *C. clusii* showed however the strongest increment with a 2,7-fold increase, reaching values of 238 µEq TA dm^{-2} after 50 days of water stress (which corresponds to 77.62 µEq g DW^{-1} or 132 mg g DW^{-1}). This means that approximately a 13 % of the dry matter of *C. clusii* leaves is made of phenolic compounds. In *C. clusii* leaves, the phenolic content showed a strong negative correlation (r^2=0.67) with the RWC. In contrast, the correlation between the phenolic content and the daily incident radiation integral was unclear (figure 23).

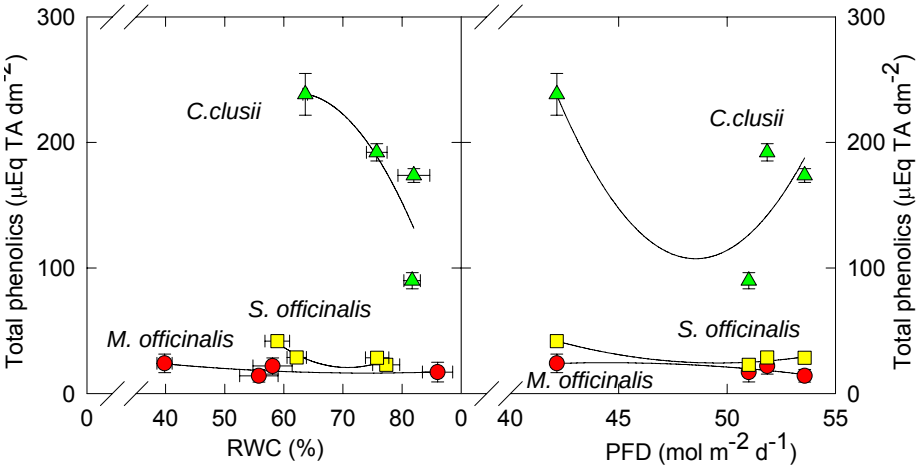

Figure 23: Correlation between total phenolics (TA, tannic acid) and the relative leaf water content (RWC) and the photon flux density (PFD) daily integral.

The amount of anthocyanidins present in the three species studied was always lower than 0.05 nEq cyanidin-3-glucoside dm^{-2}, thus indicationg that all the studied species have acyanic leaves under water stress.

Due to the higher concentration and responsiveness to water deficit of phenolic content in *C. clusii*, compared to *S. officinalis* and *M. officinalis*, *C. clusii* was chosen for further studies on flavonoids.

2.- Drought-induced changes in flavonoids and ascorbate in *C. clusii*

2.1.- Analysis of oxidative stress indicators

No significant changes in chlorophyll levels, the chlorophyll a to b ratio, or the extent of lipid peroxidation in leaves, estimated as MDA concentrations, were observed in *C. clusii* leaves during the drought period. Moreover, the F_v/F_m, which is indicative of photoinhibitory damage to the photosynthetic apparatus, remained constant around 0.8 throughout the study (figure 24). These data together indicate that although plants suffered a moderate water loss, the stress suffered by these plants was mild.

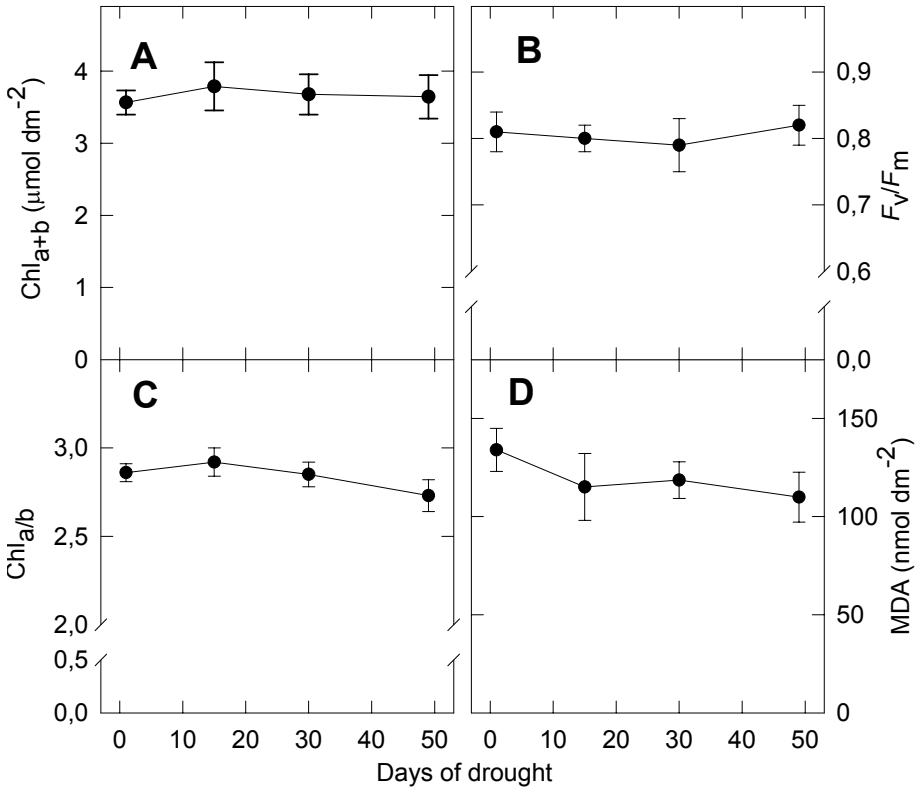

Figure 24: Changes in chlorophyll a plus b (Chl_{a+b}) levels (A), maximum efficiency of PSII photochemistry (F_v/F_m) (B), chlorophyll a to b ratio (Chl a/b) (C), and malondialdehyde (MDA) levels (D), an indicator of lipid peroxidation, in *C. clusii* leaves during a 50-days drought treatment.

2.2.- Identification of antioxidant flavonoids in *C. clusii* leaves and their changes under water deficit

Among the flavonoids analyzed, the flavan-3-ols (-)-epicatechin (EC), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG) were found in *C. clusii* leaves, whereas the flavonols quercetin and kaempferol were not detected (with a detection limit of 0.01 μmol dm^{-2}). The concentrations of EC and ECG were below 0.25 and 0.03 μmol dm^{-2} (below 0.08 and below 0.01 μmol g DW^{-1}, respectively). Among the flavan-3-ols, EGCG was present at the highest concentrations, ranging from 1.97 to 4.99 μmol dm^{-2} (1.14 to 1.57 μmol g DW^{-1}) (figure 25). The concentration of EGCG, which began to increase at the onset of the water stress, reached a maximum after 30 days, when the RWC was about 75 %. As the experiment progressed, the EGCG concentration remained constant, even though RWC continued decreasing up to about 63 %. The EGCG accounted for between 1.8 and 2.6 % of total phenolics and both parameters showed a close positive correlation (r^2=0.92) during the experiment. Concentrations of EC and ECG were 20- and 100-fold lower, respectively, than those of EGCG, although they all changed in parallel during the treatment (figure 25).

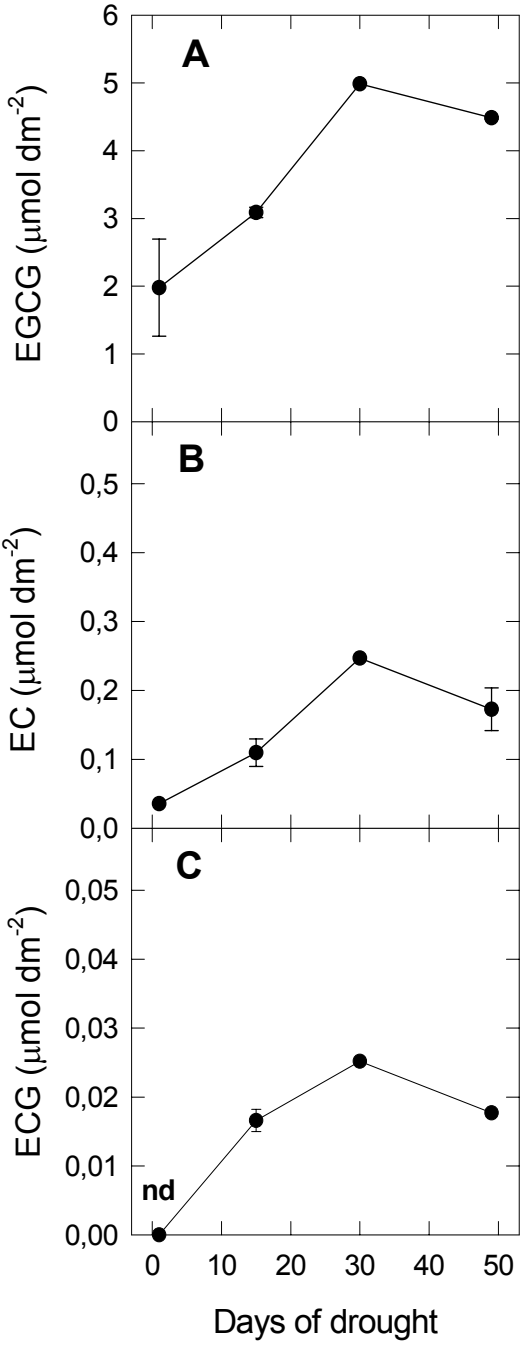

Figure 25: Changes in (-)-epigallocatechin gallate (EGCG) (A), (-)-epicatechin (EC) B) and (-)-epicatechin gallate (ECG) (C) in *C. clusii* leaves during a 50-days drought treatment. nd, not detected.

2.3.- Drought-induced changes in ascorbate

Ascorbate concentrations increased 2.1-fold, from *ca.* 37 to 80 μmol dm^{-2}, after 15 days of water stress treatment and they reached maximum levels of about 97 μmol dm^{-2} after 50 days of drought (figure 26). Ascorbate concentrations positively correlated with EGCG levels during the treatment (r^2=0.75). In contrast, DHA decreased significantly after 15 days, and then remained unchanged for the rest of the experimental period. As a result, the oxidation state of ascorbate shifted toward its reduced form after 15 days of treatment and remained constant at about 0.18 for the rest of the experiment (figure 26). Ascorbate levels were *ca.* 20 times higher than those of EGCG in *C. clusii* leaves.

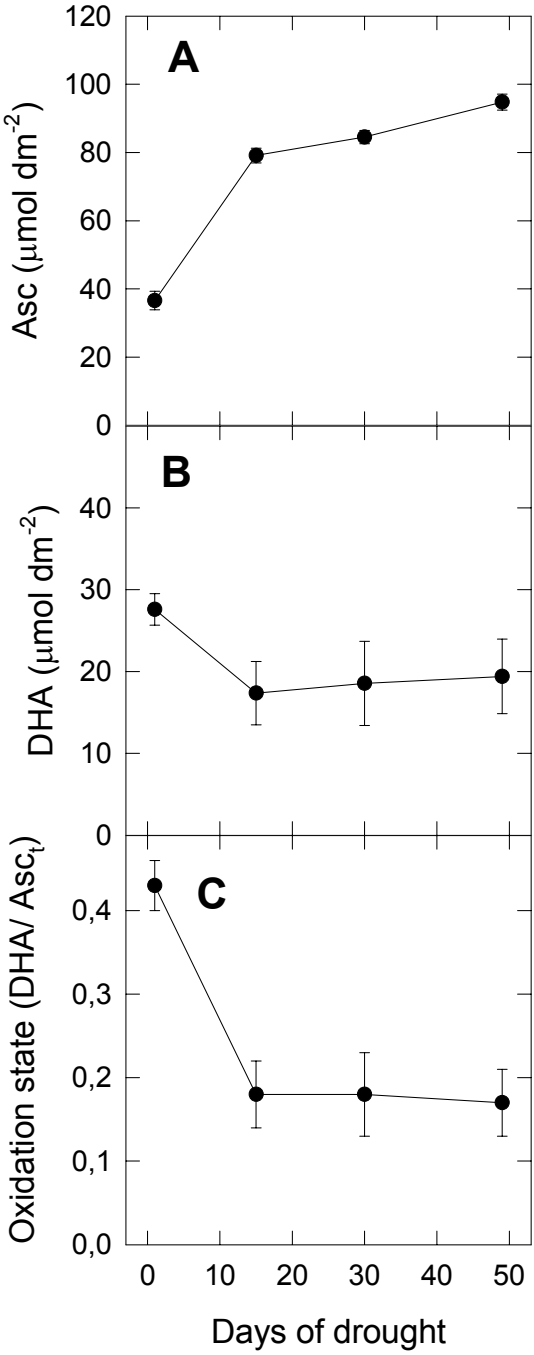

Figure 26: Changes in ascorbate (Asc) (A), dehydroascorbate (DHA) (B) and the oxidation state of the ascorbate pool (Asc_t, Asc plus DHA) (C) in *C. clusii* leaves during a 50-days drought treatment.

3.- Accumulation and oxidation of flavan-3-ols and ascorbate in field-grown *C. clusii* as affected by plant age

The accumulation and oxidation of ascorbate and EGCG, the major flavan-3-ol in *C. clusii* leaves, were studied in juvenile and mature plants in field conditions. These conditions were characterized by a dry summer, with very low precipitation, PFDs around 2000 μmol $m^{-2}s^{-1}$ and maximum diurnal air temperatures ranging between 25-30 ºC. During the experiment, PFD values increased from 1968 μmol $m^{-2}s^{-1}$ on May 17, to reach *ca.* 2100 μmol $m^{-2}s^{-1}$ in dates around the summer solstice (when irradiance and day length are maximum), and decreasing thereafter to *ca.* 1550 μmol $m^{-2}s^{-1}$ on September 24 (figure 27). During the experimental period, RWC decreased from values around 85 % on May 17 to values around 60 % on July 9. Thereafter, RWC values recovered slightly during summer (between July 19 and August 25), reaching values of *ca.* 75 % at the end of it, and fully recovered after typical fall rainfalls. Juvenile plants tended to show a slightly lower RWC ($P<0.05$) (figure 27).

The LMA increased progressively during the drought period, from *ca.* 1.3 g dm^{-2} on May 17 to *ca.* 2.4 g dm^{-2} at the end of summer (August 25). The LMA recovered partially in new leaves that appeared in fall, showing values of *ca.* 1.8 g dm^{-2} on September 24. Thereafter, LMA slightly increased again in mature plants, reaching values of *ca.* 2.2 g dm^{-2} on November 11. Juvenile plants tended to show slightly lower LMA ($P<0.05$) throughout the experiment (figure 27).

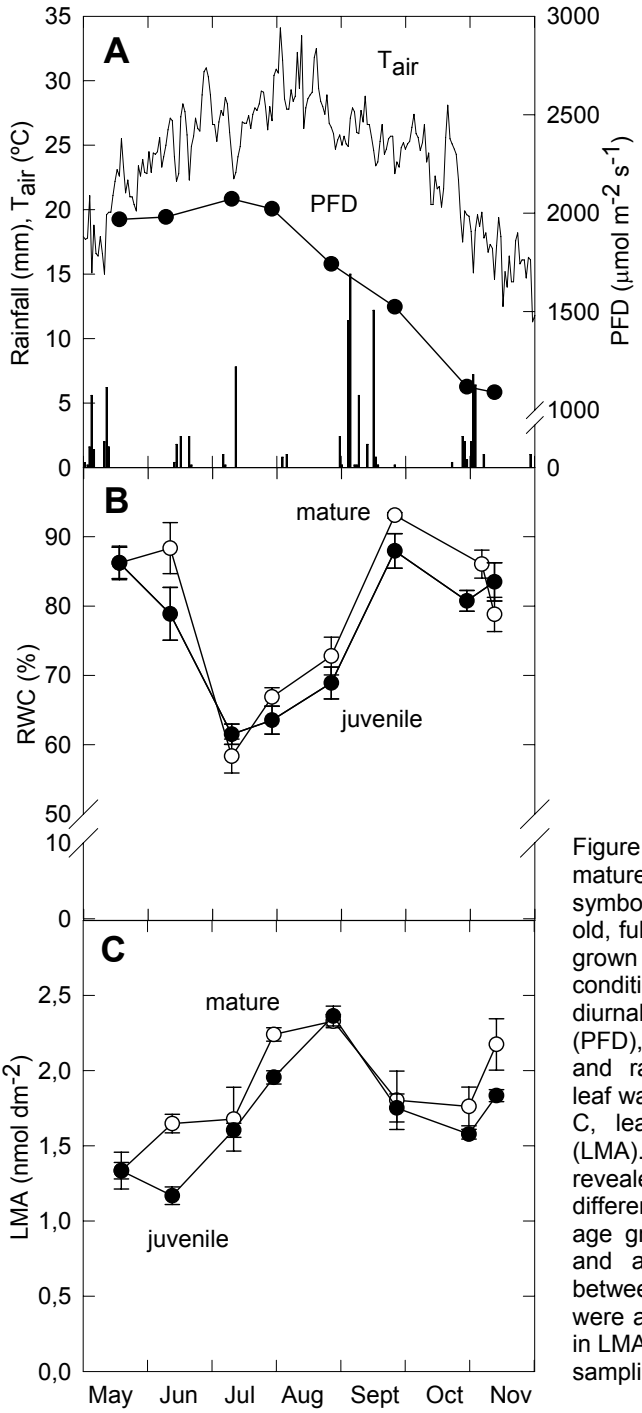

Figure 27: Stress indicators in mature (6-years-old, open symbols) and juvenile (1-year-old, full symbols) *C. clusii* plants grown under Mediterranean field conditions. Panel A, maximum diurnal photon flux density (PFD), air temperature (T_{air}), and rainfall. Panel B, relative leaf water content (RWC). Panel C, leaf mass per area ratio (LMA). ANOVA test (P<0.05) revealed statistically significant differences in RWC between age groups and sampling day, and a significative interaction between these factors. There were also significant differences in LMA between age groups and sampling day (ANOVA P<0.05).

3.1.- Oxidative stress markers as affected by plant age, high light and water deficit

The F_v/F_m ratio was kept above 0.8 during the whole experiment in all cases, indicating the absence of a chronic photoinhibition. However, F_v/F_m values were slightly lower in mature plants compared to that of juvenile ones during the whole experiment ($P<0.05$) (figure 28). Levels of MDA reached their maximum in spring (from May 17 to July 9), with high incident light, attaining values of *ca.* 75 and 100 nmol dm^{-2} in juvenile and mature plants, respectively. During the summer, MDA levels decreased slightly, attaining values of *ca.* 60 and 70 nmol dm^{-2} in juvenile and mature plants, respectively, at the end of this season (August 23). Finally, MDA levels were the lowest at the beginning of fall, showing values of *ca.* 30 nmol dm^{-2} for both plant groups, increasing slightly thereafter until the end of the experiment (figure 28). There were no statistically significant differences in MDA levels between juvenile and mature plants ($P=0.061$), but the interaction between the sampling date and plant age was statistically significant ($P<0.05$), which indicates that older plants showed higher MDA levels than younger ones, but only on sampling days of spring and summer (see figure 28).

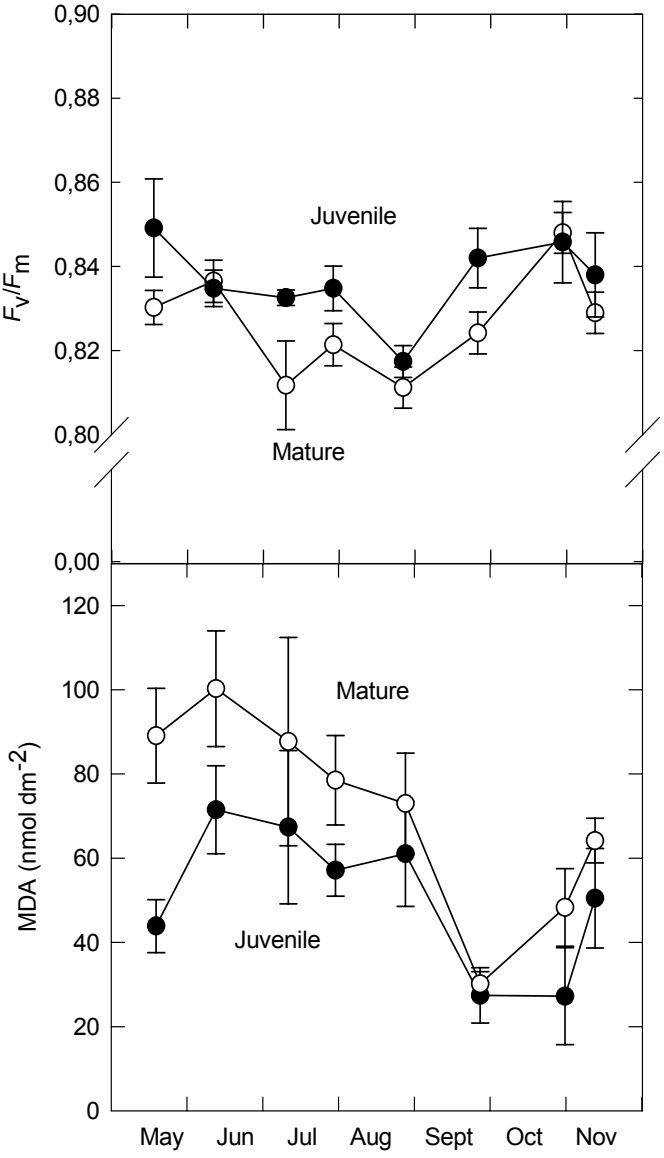

Figure 28: Maximum quantum yield (F_v/F_m) and malondialdehyde (MDA) levels, an indicator of lipid peroxidation, in leaves of mature (6-years-old, open symbols) and juvenile (1-year-old, full symbols) *C. clusii* plants grown under Mediterranean field conditions. ANOVA test (P<0.05) revealed statistically significant differences in F_v/F_m between age groups and sampling day, and a significant interaction between these factors. ANOVA test also showed differences in MDA levels between sampling days and the significance of the interaction between the sampling day and plant age.

3.2.- Flavan-3-ol accumulation and oxidation in response to high light

Both in juvenile and mature plants, levels of EGCG increased from *ca*. 1.7 to 2.6 µmol dm^{-2} from May 17 to June 10 (figure 29). Afterwards, EGCG levels decreased progressively to reach the lowest levels of 0.4 µmol dm^{-2} on September 24. The amount of EGCGQ decreased from spring (May 17 and June 10) to summer (July 9 to August 25) –from *ca.* 0.25 to 0.1 µmol dm^{-2}- and fully recovered after fall rainfalls, in new leaves (figure 29). The amount of PAs increased progressively from spring until the end of summer (from *ca.* 0.24 to 0.51 and from 0.34 to 0.53 µEq B2 dm^{-2} in juvenile and mature plants, respectively), and recovered after fall rainfalls (figure 29).

The analysis of diurnal variations in EGCG, EGCGQ and PAs during spring (May 17), summer (July 28) and fall (November 11) revealed that diurnal fluctuations did not have any short term effect (within hours) on the accumulation of these flavan-3-ol derivatives (figure 31). The levels of EGCG showed a strong positive correlation with maximum diurnal PFD (r^2=0.89 and 0.97 in juvenile and mature plants, respectively) (figure 30). However, correlation between RWC and EGCG levels was lower (r^2=0.33 and r^2=0.70, in juvenile and mature plants, respectively), and negative (figure 30).

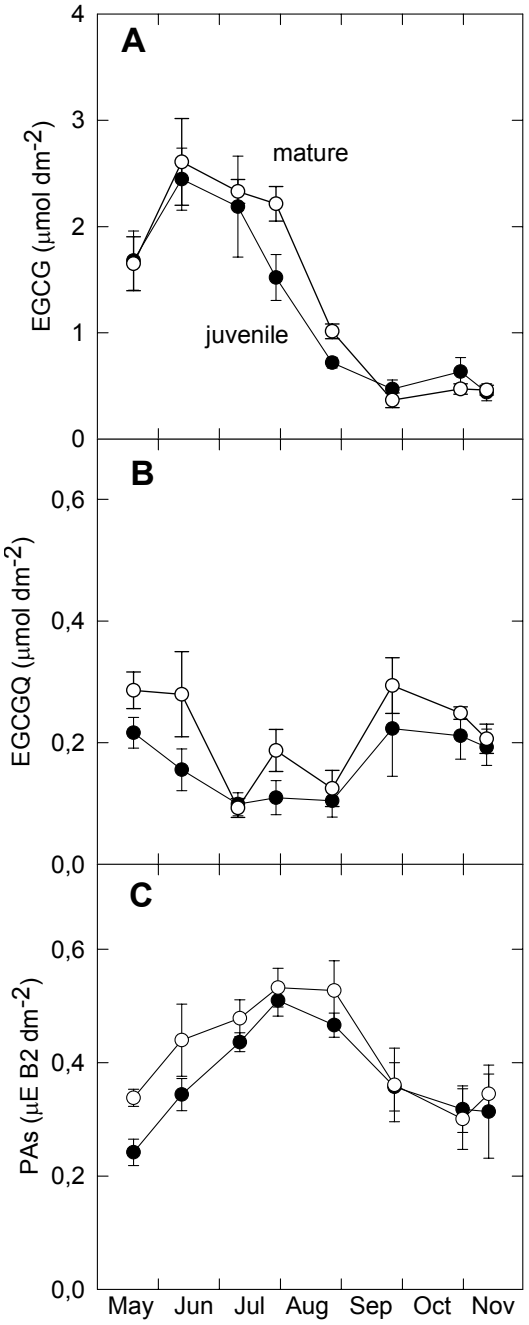

Figure 29: Accumulation of flavan-3-ols in mature (6-years-old, open symbols) and juvenile (1-year-old, full symbols) *C. clusii* plants grown under Mediterranean field conditions. Panel A, (-)-epigallocatechin gallate (EGCG); Panel B, EGCG quinone (EGCGQ); Panel C, proanthocyanidins (PAs). ANOVA test (P<0.05) revealed statistically significant differences in EGCG and EGCGQ between sampling days. ANOVA test (P<0.05) also revealed statistically significant differences in PAs between age groups and sampling day.

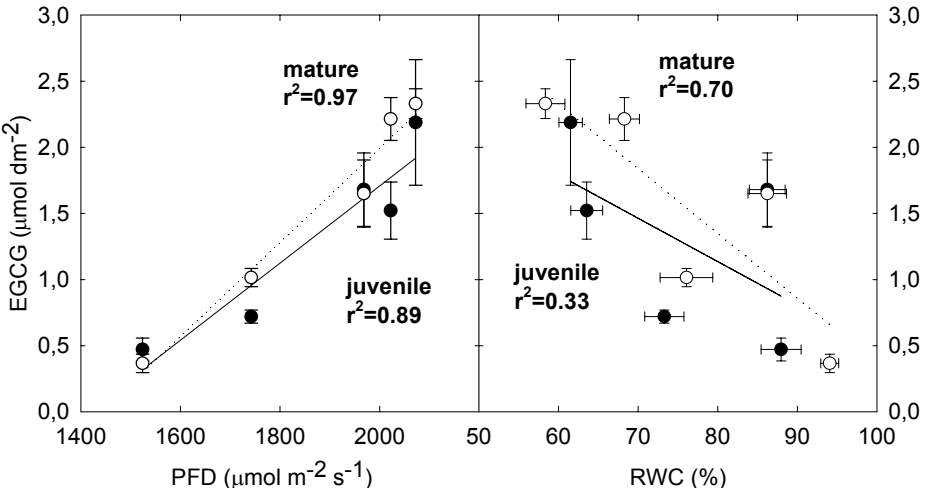

Figure 30: Correlation of flavan-3-ol content in mature (6-years-old, open symbols) and juvenile (1-year-old, full symbols) *C. clusii* plants grown under Mediterranean field conditions, maximum diurnal photon flux density (PFD) and relative leaf water content (RWC).

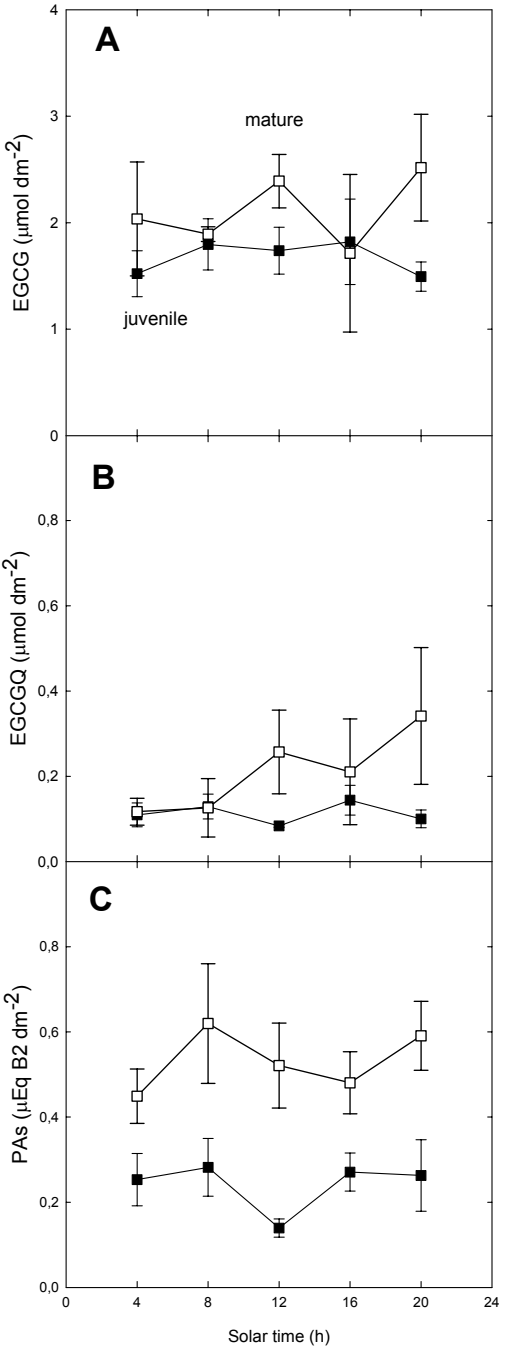

Figure 31: Diurnal changes in flavan-3-ol levels in juvenile and mature *C. clusii* plants grown under Mediterranean field conditions. Panel A, (-)-epigallocatechin gallate (EGCG); Panel B, EGCG quinone (EGCGQ); Panel C, proanthocyanidins (PAs). Data correspond to July 28, 2004.

3.3.- Flavan-3-ol accumulation as affected by plant age

Mature plants showed slightly higher PA content than juvenile ones during spring and summer ($P<0.05$), and these differences disappeared after fall rainfalls (figure 29). Mature plants also showed slightly higher EGCGQ contents ($P=0.069$) than juvenile ones during the whole experiment. Although there were no differences in EGCG content between juvenile and mature plants during the experiment, mature plants showed slightly higher (not statistically significant) EGCG content than juvenile ones during the summer (figure 29).

3.4.- Localization of flavan-3-ols in chloroplasts

Isolated *C. clusii* chloroplasts showed low contamination with other subcellular compartments. Activities of vanadate-sensitive ATP-ase and NADPH-cyt c reductase, which are characteristic of the plasmalemma and endoplasmic reticulum, respectively, were hardly detectable ($1.31x10^{-4}$ μmol P_i min^{-1} mg $prot^{-1}$ and 1.78 nmol min^{-1} mg $prot^{-1}$, respectively) in *C. clusii* chloroplast-enriched fractions. Cyt *c* oxidase activity, which is characterisitic of mitochondria, was not detectable at all. The integrity of the chloroplasts isolated was about 85 %.

Levels of the flavan-3-ols ECG and EC were below the detection limit (0.5 μmol mol Chl^{-1}) and EGCG was detectable but not quantifiable. The detected traces of EGCG were most likely due to small contaminations with other subcellular fractions, given that EGCG levels per chlorophyll unit in whole leaf extracts were at least 1000 times higher than in chloroplast-enriched fractions.

3.5.- Antioxidant function of ascorbate in seasonal and diurnal timescales

Ascorbate levels increased from *ca*. 230 μmol dm^{-2} in spring to *ca*. 590 μmol dm^{-2} in summer, decreasing again in leaves that appeared in fall (figure 32). When expressed on a dry weight basis, ascorbate content remained constant along the experiment around 200 μmol gDW^{-1}. Levels of DHA strongly varied during the experimental period. Initial DHA levels (May 17) were around 100 μmol dm^{-2}. By June 10, DHA levels increased sharply to attain values of *ca.* 400 μmol dm^{-2}. Thereafter, DHA levels decreased progressively during the summer, recovering initial values. After September, DHA levels raised again to values similar to those in June (400 μmol dm^{-2} aprox.). The oxidation state of the ascorbate pool changed in parallel with ascorbate and DHA levels. Moreover, the ascorbate pool was always oxidized from predawn to midday, showing a very constant oxidation state at midday around 40 % in both juvenile and mature plants during the whole experiment (figure 33).

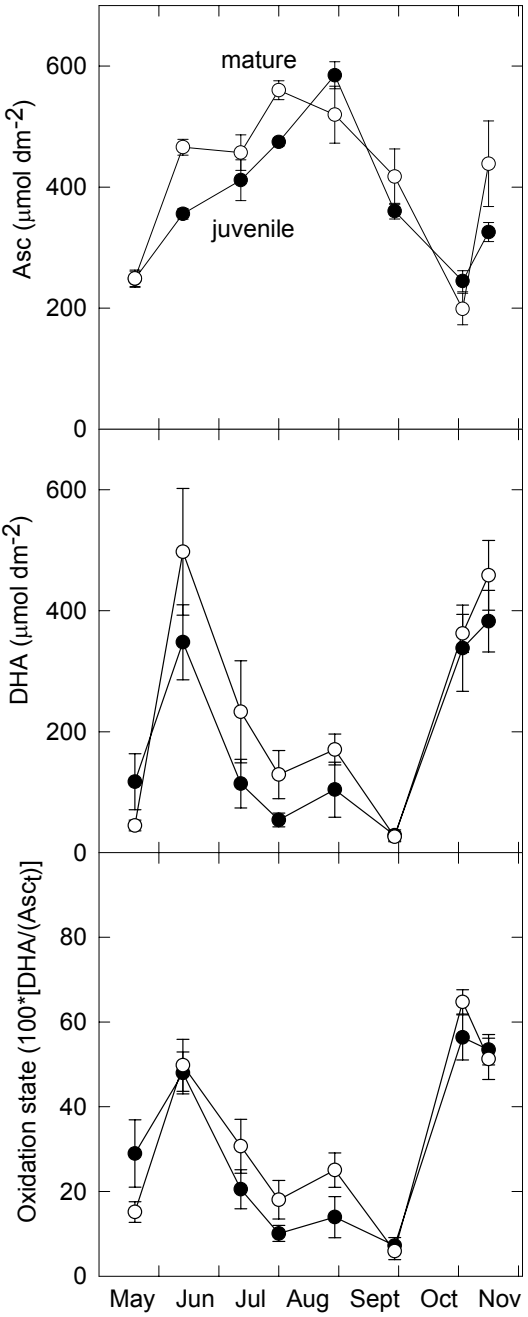

Figure 32: Ascorbate pool indicators at predawn in *Cistus clusii* plants grown under Mediterranean field conditions. Asc, amount of reduced ascorbate (A). DHA, amount of dehydroascorbate (B). Oxidation state of the ascorbate pool, estimated as $100x(DHA/Asc_t)$, where Asc_t is Asc plus DHA (C). Full symbols stand for juvenile (1-year-old) and open symbols stand for mature (6-years-old) plants. ANOVA test ($P<0.05$) revealed statistically significant differences in Asc and DHA between age groups and sampling day, and the significance of the interaction between these two factors. ANOVA test also revealed statistically significant differences in the oxidation state of Asc between sampling days.

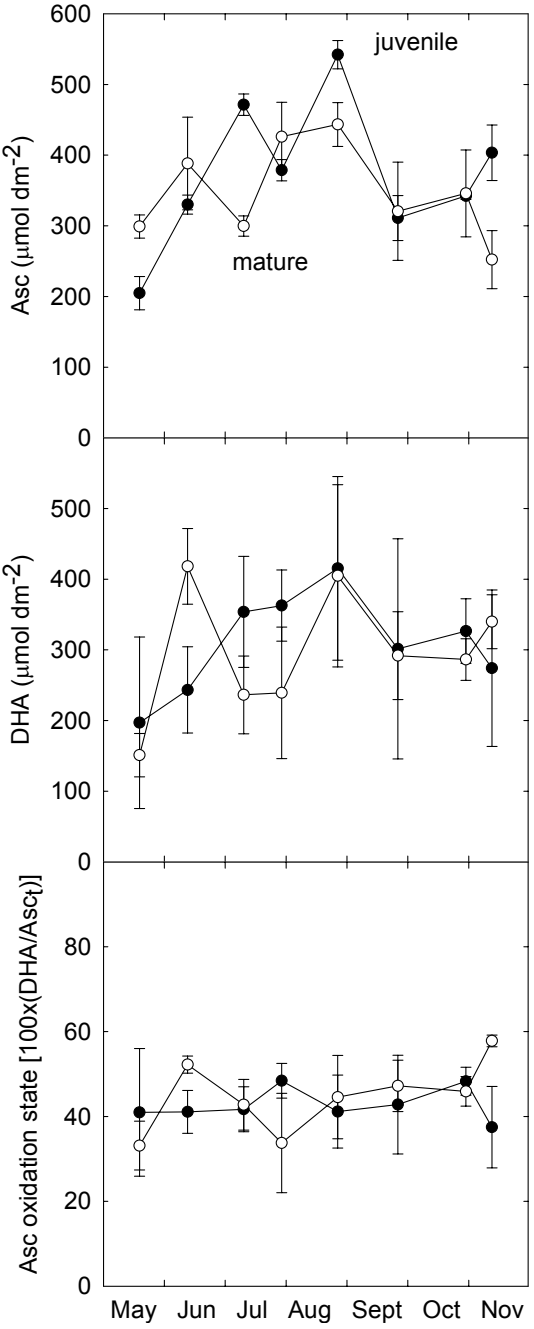

Figure 33: Ascorbate pool indicators at midday in *Cistus clusii* plants grown under Mediterranean field conditions. Asc, amount of reduced ascorbate (A). DHA, amount of dehydroascorbate (B). Oxidation state of the ascorbate pool, estimated as $100x(DHA/Asc_t)$, where Asc_t is Asc plus DHA (C). Full symbols stand for juvenile (1-year-old) and open symbols stand for mature (6-years-old) plants. ANOVA test ($P<0.05$) revealed statistically significant differences in Asc, DHA and the Asc oxidation state between sampling days, and in the interaction between Asc content and the sampling day.

3.6.- Ascorbate oxidation in response to high light and water deficit as affected by plant age

Predawn ascorbate levels in juvenile plants were slightly lower than in mature ones ($P<0.05$), and the same trend was observed for DHA levels (figure 32). The oxidation state of the ascorbate pool did not show any significant difference between both plant ages. Although the interaction between plant age and the sampling date was not statistically significant ($P=0.061$), it can be appreciated that ascorbate pool was slightly more oxidized in the oldest plants during the most stressful months of summer (July and August) (figure 32).

4.- Effects of water deficit on flavan-3-ol oxidation in tea plants

4.1.- Characterization of the stress imposed

Tea plants are water deficit- and high light-sensitive plants with agronomical and nutraceutical interest due to their high flavan-3-ol content. For the present experiment, water stress was imposed by withholding water from plants growing under Mediterranean field conditions. During the 26 days of treatment, maximum diurnal VPD ranged around 2 KPa and midday PFD around 1800 μmol m^{-2} s^{-1} (figure 34). During these 26 days, maximum and minimum air temperatures ranged around 25 and 20 ºC, respectively. Due to these environmental conditions along with the water stress treatment, the RWC in tea plants dropped from *ca.* 85 % to 50 % after the first 19 days of water deficit and then remained constant until the end of the experiment. Between days 19 and 26 there were small rainfalls (*ca.* 1.5 mm), which may explain why RWC did not decrease further after day 19 (figure 34). The F_v/F_m changed in parallel with the RWC, decreasing significantly from *ca.* 0.8 to 0.4 by day 19, and remaining constant until day 26 (figure 34), which indicates a strong chronic photoinhibition by day 19 and therein.

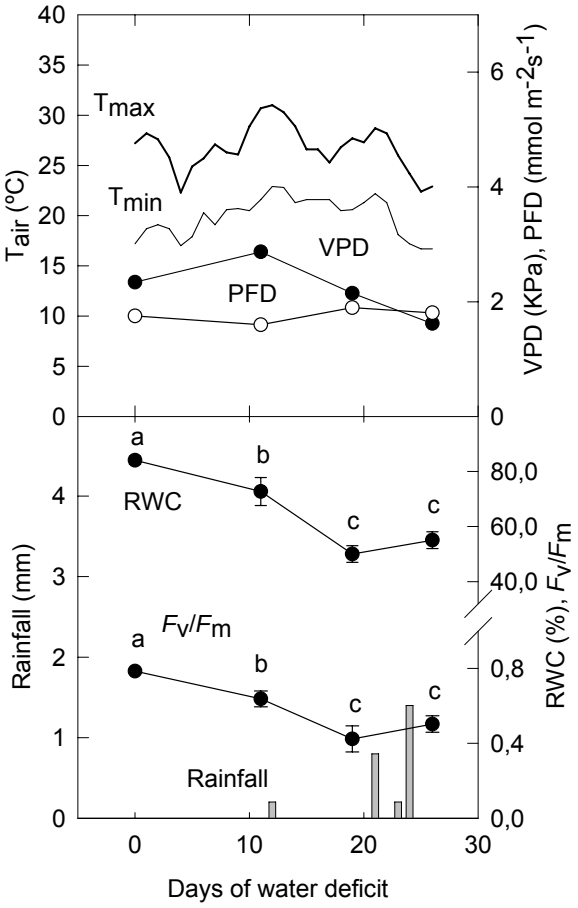

Figure 34: (Top panel) climatologic conditions (maximum and minimum daily air temperatures [T_{max} and T_{min}, respectively], maximum diurnal vapor pressure deficit [VPD] and maximum diurnal photon flux density [PFD]); and (bottom panel) rainfall, relative leaf water content (RWC) and maximum efficiency of photosystem II photochemistry (F_v/F_m) during the experiment. Letters indicate significant statistical difference (P<0.05, ANOVA).

The LMA showed a slight increase (from 0.96 to 1.31 g dm^{-2}) after the first 11 days of water deficit treatment. Thereafter, LMA values recovered to the initial ones. Although the mentioned differences were statistically significant (P<0.05) (figure 35), they are not as notable as they are in *C. clusii*. Thus, the concentrations of different compounds in tea leaves are expressed herein on a DW basis instead of a leaf area basis.

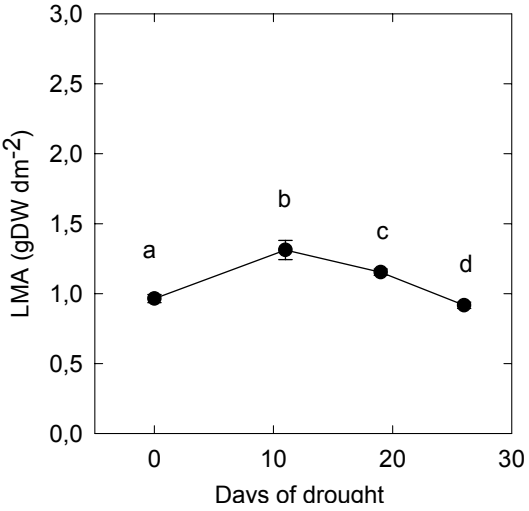

Figure 35: Changes in leaf mass per area ratio (LMA) in drought-stressed *C. sinensis* leaves. Letters indicate a significant statistical difference (P<0.05, ANOVA).

Chlorophyll and total carotenoid contents remained constant throughout the experiment between 3.60 and 3.87 mmol g DW^{-1}, and 0.062 and 0.085 mmol g DW^{-1}, respectively (figure 36).

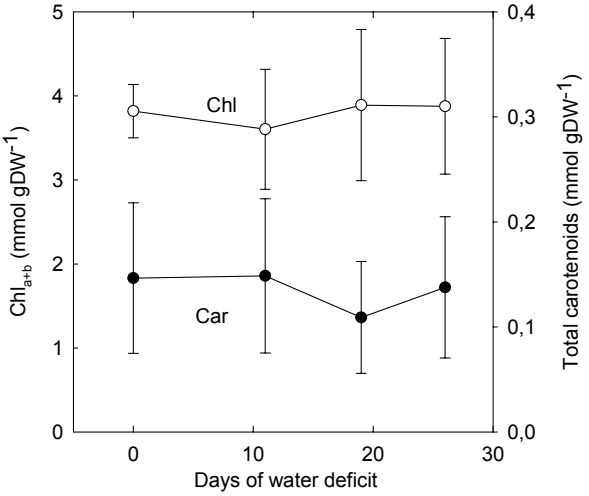

Figure 36: Chlorophyll (Chl) a plus b content (Chl_{a+b}) and total carotenoids (Car); during 26 days of water deficit in tea plants. ANOVA tests (P<0.05) revealed no differences in chlorophyll or carotenoid contents during the water deficit treatment.

The extent of lipid peroxidation, estimated as MDA formation, decreased significantly from *ca*. 75 to 40 nmol g DW^{-1} after 19 days of water deficit, and then remained constant until the end of the experiment (figure 37).

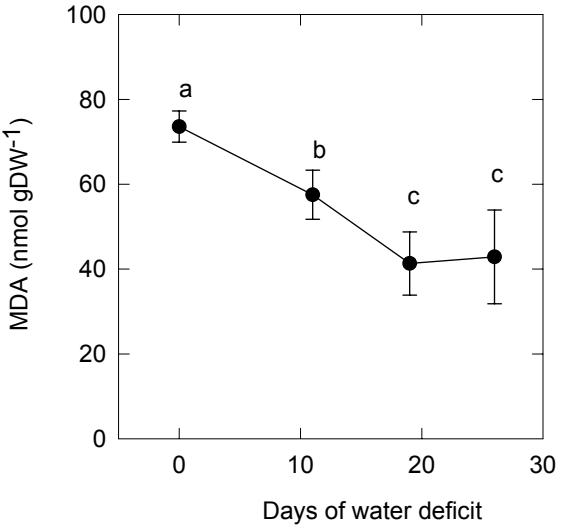

Figure 37: Changes in malondialdehyde (MDA) levels, an indicator of lipid peroxidation, in drought-stressed *C. sinensis* leaves. Letters indicate a significant statistical difference (P<0.05, ANOVA).

4.2.- Changes in flavan-3-ols and their quinones along the water stress treatment

The levels of reduced flavan-3-ols, EC and EGCG, remained unaltered throughout the experiment. Levels of EC were around 1 μmol g DW^{-1}, while those of EGCG were *ca.* 5-fold higher throughout the experiment (figure 38).

The levels of oxidized flavan-3-ols, ECQ and EGCGQ increased sharply under water deficit (figure 38). The strongest increases were observed after 19 days of stress, when levels of ECQ and EGCGQ rose from *ca.* 0.1 to 20 μmol g DW^{-1} and from undetectable levels to 2.5 μmol g DW^{-1}, respectively. ECQ and EGCGQ decreased slightly at the end of the experiment, concomitantly with a small, but non-significant, increase of RWC due to

small rainfalls. The oxidation state of EC and EGCG increased from 0.23 to 0.98 and from undetectable to 0.36 after 19 days of water deficit, respectively, when RWC values were at their lowest.

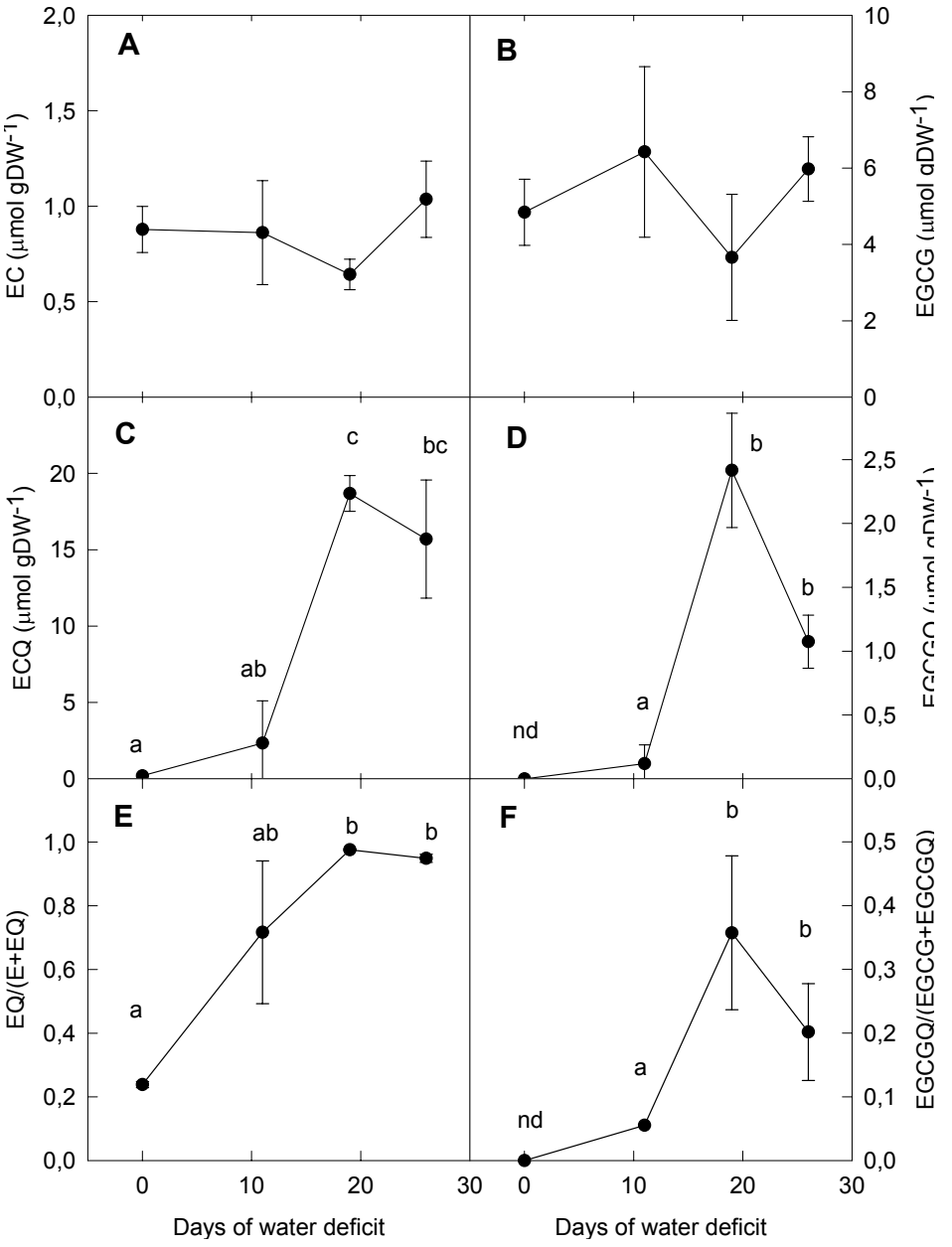

Figure 38: Changes in (-)-epicatechin (EC) (A), (-)-epigallocatechin gallate (EGCG) (B), (-)-epicatechin quinone (ECQ) (C), (-)-epigallocatechin gallate quinone (EGCGQ) (D), and the redox state of EC (E) and EGCG (F), estimated as ECQ/(ECQ+EC) and EGCGQ/(EGCGQ+EGCG), respectively, in *C. sinensis* leaves during the 26 days of water deficit. Letters indicate significant statistical differences (P<0.05, ANOVA).

The accumulation of ECQ and EGCGQ negatively correlated with MDA levels in leaves (r^2=0.975 and 0.798, respectively).

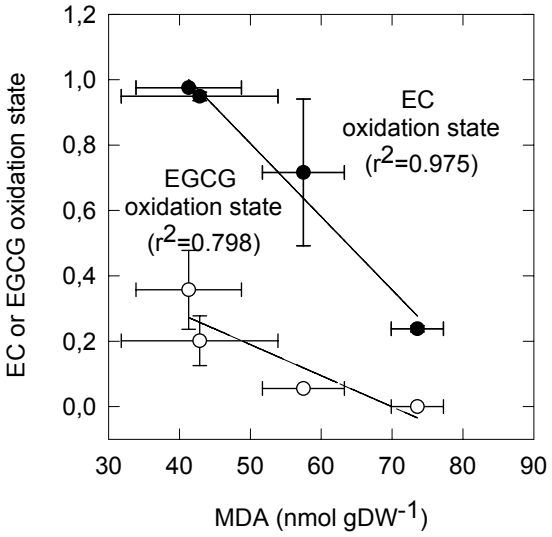

Figure 39: Correlation between malondialdehyde (MDA) –an indicator of lipid peroxidation- and the oxidation states of (-)-epicatechin (EC, full symbols) and (-)-epigallocatechin gallate (EGCG, open symbols).

4.3.- Accumulation of proanthocyanidins along the water stress treatment

An enhanced accumulation of PAs was observed in our study after the water stress treatment, their levels increasing from 61.19 to 92.46 mEq B2 g DW^{-1} (35 to 53 mg g DW^{-1}) (figure 40). ECQ and EGCGQ appearance and increment preceded PAs accumulation after 26 days of water deficit (see figures 38 and 40).

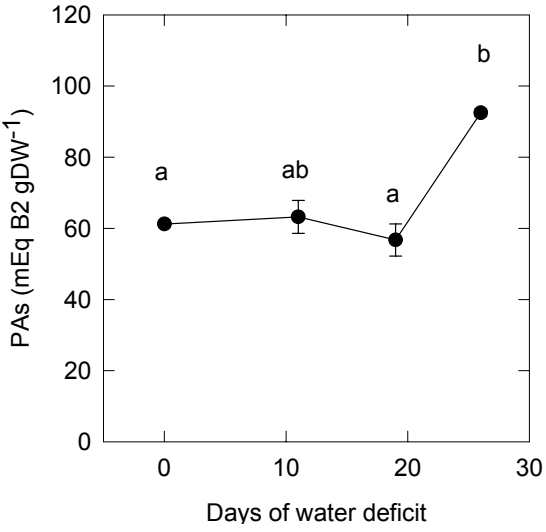

Figure 40: Levels of proanthocyanidins (PAs) during a 26-days drought treatment to tea plants in Mediterranean field conditions. Letters indicate a significant statistical difference (*P*<0.05, ANOVA).

5.- Effects of apoplast ascorbate oxidation state on apoplast-symplast signaling

5.1.- Effects on physiological parameters

Six week-old tobacco plants reached around 150 mm height, and although a slight trend to a reduced growth was observed when AO was less expressed, no statistical differences in growth parameters were observed between the antisense, wild type and sense lines (figure 41). There was no statistical difference in leaf area, around 1500 cm^2, between lines after 6 weeks, although a slight decrease was observed in the sense line. Regarding to the weight distribution, there were no statistical differences in the aerial parts, roots or leaves of any genotype, being the mean values around 5, 2.5 and 3 g FW $plant^{-1}$, respectively. In general, variability was high and statistical analyses revealed no differences in any of the growth parameters analyzed (figure 41).

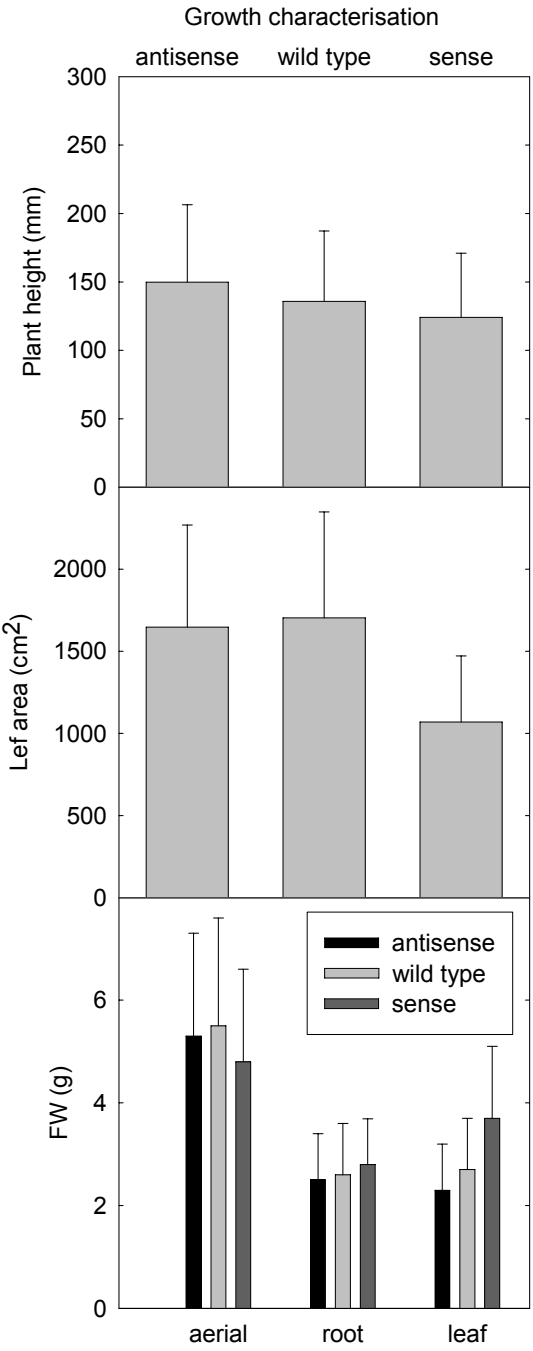

Figure 41: Plant growth indicators in tobacco plants over- (P3-7-2, sense) and under- (T1-6-1, antisense) expressing ascorbate oxidase.

Ascorbate pool size in the symplast -the residual leaf material after extracting the AWF- was about 2500 nmol g FW^{-1}, and between 10 and 20 % in the oxidized form, DHA (figure 42, B and D). There were no differences in the ascorbate pool size or oxidation state in the symplast between the different lines tested (figure 42, B and D). In contrast, the ascorbate pool in the apoplast was around 40 nmol g FW^{-1} in the wild type and the sense line, while that of the antisense line was around 100 nmol g FW^{-1}. The ascorbate content in the apoplast varied from *ca.* 5 nmol g FW^{-1} in the sense line to *ca.* 15 nmol g FW^{-1} in the wild type and to *ca.* 80 nmol g FW^{-1} in the antisense (figure 42, A). The DHA content also varied with the genotype, being highest in the sense line (*ca.* 40 nmol g FW^{-1}) and lowest in the wild type (*ca.* 20 nmol g FW^{-1}). The ascorbate oxidation state in the apoplast strongly depended on the genotype. The antisense line showed the lowest oxidation state in the apoplast (*ca.* 30 %), followed by the wild type (*ca*. 50 %), while the sense showed an oxidation status of *ca.* 80 % (figure 42).

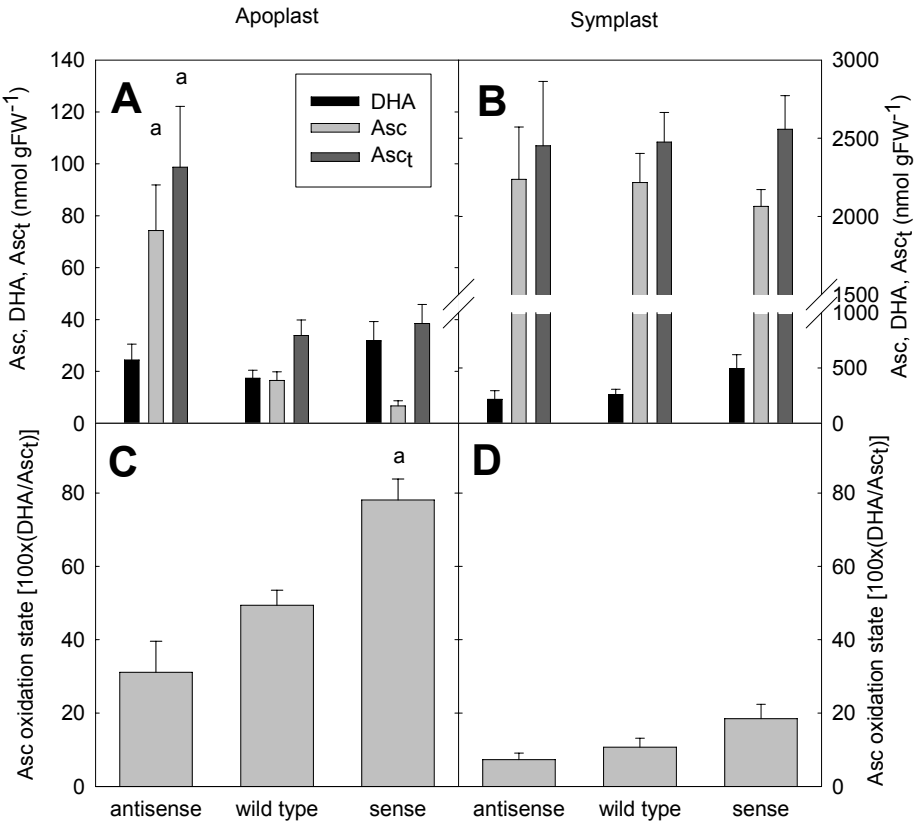

Figure 42: Ascorbate (Asc) pool status in tobacco plants over- (P3-7-2, sense) and under- (T1-6-1, antisense) -expressing ascorbate oxidase, compared to the wild type. Panel A shows Asc, dehydroascorbate (DHA) and total Asc (Asc_t, which corresponds to Asc+DHA) in the apoplast. Panel B shows Asc, DHA and Asc_t in the symplast. Panels C and D show Asc pool oxidation state, calculated as $100x(DHA/Asc_t)$ in the apoplast and symplast, respectively. Letters indicate significant statistical differences between genotypes ($P<0.05$, ANOVA).

The AO activity in the AWF was lowest in the antisense line (*ca.* 0.5 nkat g FW^{-1}), followed by the wild type (*ca.* 1.2 nkat gFW^{-1}). The sense line showed the highest AO activity (*ca.* 13 nkat gFW^{-1}) in the AWF among the tested lines (figure 43).

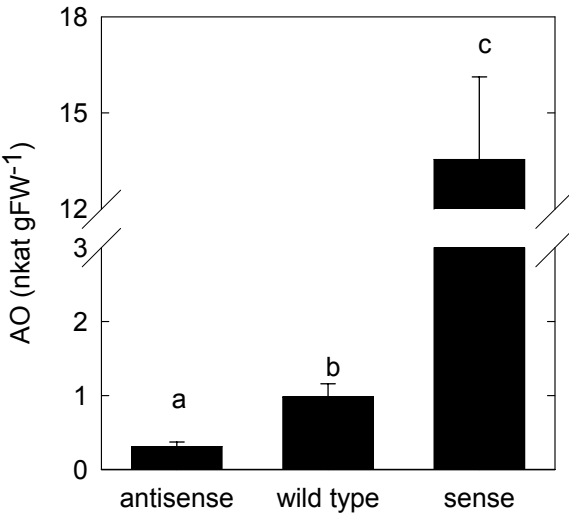

Figure 43: Ascorbate oxidase (AO) activity in the apoplast washing fluid in tobacco plants over- (P3-7-2) and under- (T1-6-1) expressing ascorbate oxidase. Letters indicate significant statistical difference (p<0.05, ANOVA).

In summary, the ascorbate pool size and oxidation state in the symplast remained unaltered in transgenic lines when compared to the wild type. In constrast, in the apoplast, the ascorbate pool size remained constant in the sense line, but it was increased in the antisense line, relative to the wild type, and the oxidation state of the ascorbate pool was lower in the antisense, and higher in the sense relative to the wild type.

The maximum carboxylation speed remained constant (*ca.* 58 μmol CO_2 m^{-2} s^{-1}) in all three lines, and so did the maximum photosynthetic rates (*ca.* 24 μmol CO_2 m^{-2} s^{-1}) (table 5).

	antisense	wt	sense
Vc_{max} (µmol CO_2 m^{-2} s^{-1})	52 ±5	62 ±5	60 ±2
A_{max} (µmol CO_2 m^{-2} s^{-1})	26 ±2	22 ±1	25 ±0

Table 5: Photosynthesis *vs*. increasing intracellular CO_2 *(A/C_i)* curves from tobacco plants over- (P3-7-2, sense) and under- (T1-6-1, antisense) expressing ascorbate oxidase, and wild type (wt). Vc_{max}, maximum carboxyllation speed; A_{max}, maximum photosynthetic capacity. No statistical differences were observed between lines (ANOVA, P<0.05)

5.2.- Effects on nuclear gene expression

The expression analyses of genes involved in the ascorbate re-cycling in the cytosol showed that very slight or null changes were observed in the expression of the genes encoding for MDHAR, DHAR, cAPX, and GPX. The NtTPC1B, a component of a two-pore Ca^{2+} channel, resulted down-regulated in the sense and strongly down-regulated in the antisense line, relative to the wild type (figure 44).

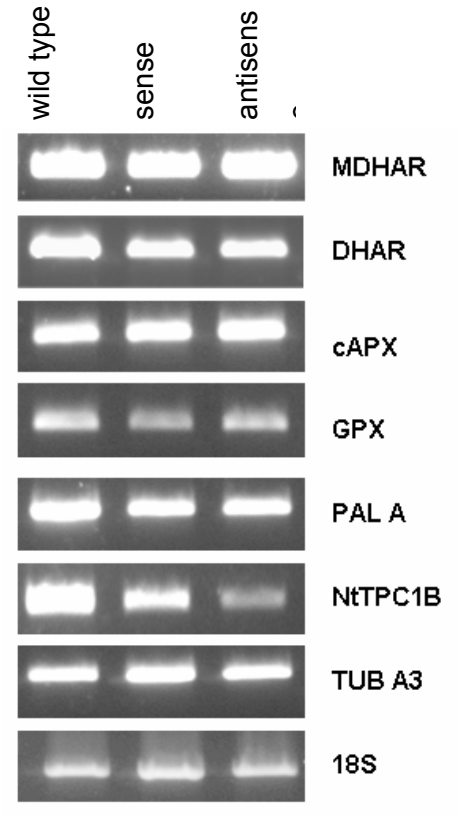

Figure 44: Semi-quantitative PCR gene expression analyses of several mRNAs from tobacco plants over- (P3-7-2, sense) and under- (T1-6-1, sense) expressing ascorbate oxidase. MDAR, monodehydroascorbate reductase (putative) (acc. nº BQ842867); DHAR, dehydroascorbate reductase (acc. nº AY074787); cAPX, cytosolic ascorbate peroxidase (acc. nº U15933); GPX, glutathione peroxidase (acc. nº AB041518); PAL A, phenylalanine ammonia lyase A (acc. nº X78269), NtTPCB1B, a two-pore calcium channel (acc. nº AB124647); TUB A3, α-tubulin 3 (acc. nº AJ421413); 18S, rRNA 18S (acc. nº AJ236016) (equal loading control).

The cDNA subtractive hybridisation library consisted of 31 ESTs (expressed sequance tags) with scores higher than 50. From those, 17 ESTs were differentially expressed in the sense line, while 14 ESTs were differentially expressed in the antisense, respect to the wild type. From the 17 ESTs differentially expressed in the sense line, the following 9 ESTs were underexpressed: protein kinase Ck2 regulatory subunit, chloroplast thiazole biosynthetic protein, glycine-rich protein 2, mitochondrial Rieske Fe-S protein, Fd-NADP reductase, chalcone synthase, protein phosphatase 2A and 2 cDNA-AFLP (amplification fragment length polymorfism) fragments (table 6). The 8 overexpressed ESTs in the sense line respect to the wild

type, were the following: fasciclin-like arabinogalactan protein, chloroplast protein 12, a wound-inductive mRNA, glycine descarboxylase, cysteine synthase, a poly A binding protein and 2 cDNA-AFLP fragments (table 6, figure 45).

In addition, from the 14 ESTs differentially expressed in the antisense, compared to the wild type, the following 5 ESTs were underexpressed: a ripening regulation protein, a RING finger family transcription factor, an UV-B repressible protein, glycine descarboxylase and a cDNA-AFLP fragment (table 6). The remaining 9 ESTs were over-expressed: NT3 mRNA, thiazole biosynthetic protein, Fd-thiorredoxin oxidorreductase catalitic subunit, a homeodomain leu-zipper transcription factor, a cyclophyllin-like protein, nitrate reductase, a chloroplast channel, Voucher ET4-8 protein and a cDNA-AFLP fragment (table 6, figure 45).

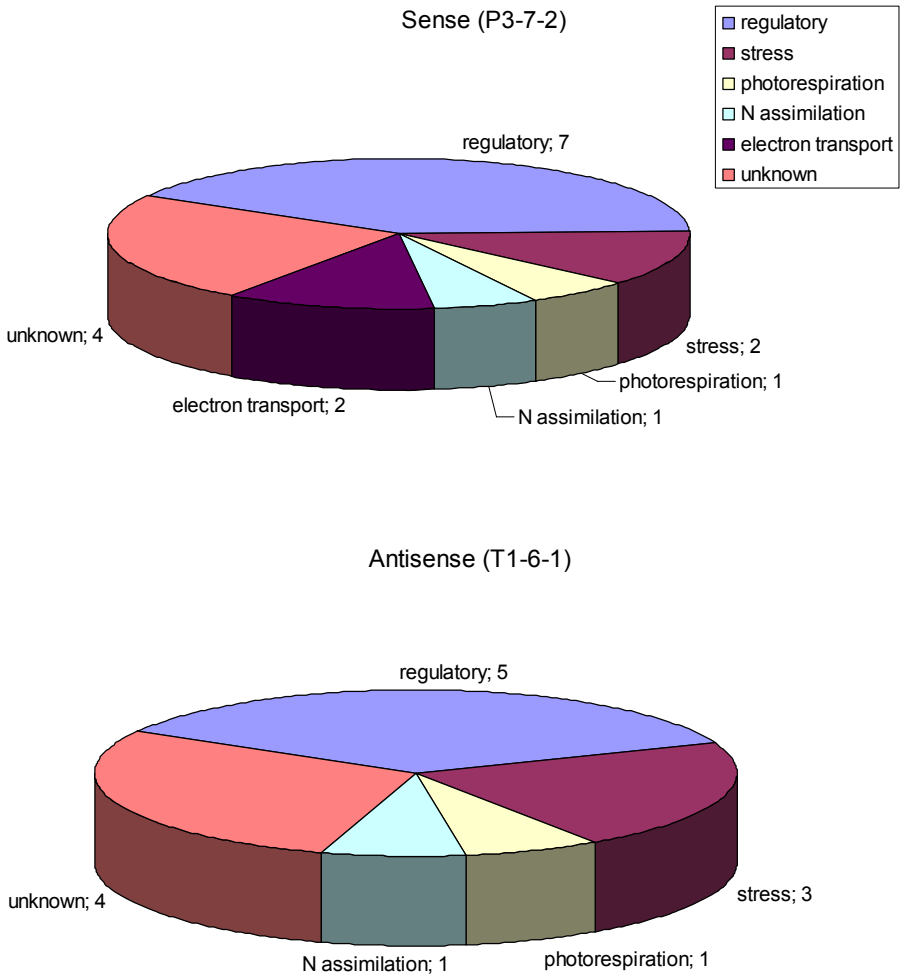

Figure 45: Distribution by categories of the differentially expressed ESTs in tobacco plants over- (P3-7-2, sense) and under- (T1-6-1, antisense) expressing ascorbate oxidase, compared to the wild type.

Alterations in sense line:

Under-expressed genes in sense line:

- ✓ Protein kinase Ck2 regulatory subunit (65Kda) (acc. nº NTA488194): recruited by signalosome to form protein-ubiquitin. Interacts with cdk5/p35. Very ubiquitous
- ✓ Chloroplast thiazole biosynthetic protein (acc. nº AY220080): involved in thiamin pyrophosphate (vitamin B1) biosynthesis
- ✓ Glycine-rich protein RNA interaction domain (acc. nº X60007): interacts with RNA to stabilize it in cold acclimation
- ✓ Mitochondrial Rieske Fe-S protein (acc. nº M77225): electron transporter involved in mitochontrial electron transport chain
- ✓ Fd-NADP reductase (acc. nº Y1432): involved in photosynthetic electron transport
- ✓ Chalcone synthase (acc. nº X14599): involved in flavonoid biosynthesis
- ✓ Protein phosphatase 2A regulatory subunit (acc. nº X97913): involved in many signal transduction pathways and regulatory processes
- ✓ 2 cDNA-AFLP fragments from catalase deficient mutants (acc. nº AJ538743 and AJ538724): unknown function

Over-expressed genes in sense line:

- ✓ Fasciclin-like arabinogalactan protein (acc. nº BT013997): possibly involved in cell adhesion
- ✓ Chloroplast protein 12 (acc. nº AF359459): depending on its redox state, recruits glyceraldehyde-3-phosphate dehydrogenase and phosphoribulokinase
- ✓ Wound inductive mRNA (acc. nº A009882): unknown function
- ✓ Glycine descarboxylase (acc. nº Z25854): involved in photorespiration
- ✓ Cysteine synthase (acc. nº AB029512): involved in cysteine formation and S assimilation
- ✓ Poly A binding protein (acc. nº AF190656): involved in mRNA stabilization
- ✓ 2 cDNA-AFLP fragments from catalase deficient mutants (acc. nº AJ539047 and AJ538450): unknown function

Alterations in antisense line:

Under-expressed genes in anti-sense line:

- ✓ Ripening regulation protein DDTFR6/A (acc. nº AY496106): its function is unknown, but its was cloned by cold-stress induction
- ✓ RING finger family transcription factor (acc. nº AK117718): unknown function
- ✓ UV-B repressible protein (acc. nº AY551823): repressed in UVB-irradiated cotton plants
- ✓ Glycine descarboxylase (acc. nº Z25854): involved in photorespiration
- ✓ cDNA-AFLP fragment from catalase deficient mutants (acc. nº AJ538557): unknown function

Over-expressed genes in anti-sense line:

- ✓ NT3 mRNA (acc. nº AF124369): involved in salinity tolerance
- ✓ Thiazole biosynthetic protein (acc. nº AY220080): involved in thiamin pyrophosphate (vitamin B1) biosynthesis
- ✓ Fd-thioredoxin oxido-reductase catalytic subunit (acc. nº AJ317080): involved in different regulation processes (e.g. Calvin cycle enzymes) by the photosynthetic electron transport chain load
- ✓ Homeodomain leucine-zipper transcription factor (acc. nº AJ439449): regulation of developmental responses to environmental cues (e.g. abscisic acid-dependent effects)
- ✓ Cyclophyllin-like protein (acc. nº AY368274): interacts with ethylene receptors in abiotic stress
- ✓ Nitrate reductase (acc. nº X66147): N assimilation
- ✓ Chloroplast channel (acc. nº NM_187508): component of chloroplast membrane pores
- ✓ Voucher ET4-8 protein (acc. nº: AY491537): ethephon-regulated mRNA
- ✓ cDNA-AFLP fragment from catalase deficient mutants (acc. nº AJ538407): unknown function

Table 6: Identities and accession numbers (acc. nº) of the differentially expressed (under- and over-expressed) ESTs (expressed sequence tags) in tobacco plants over- (P3-7-2, sense) and under- (T1-6-1, antisense) -expressing ascorbate oxidase, compared to the wild type.

As shown in table 6, genes involved in the metabolism of different organelles were differentially expressed in transgenic lines with a shifted apoplastic ascorbate oxidation state.

DISCUSSION

1.- Flavonoids

1.1.- Changes in total phenolic levels in *M. officinalis*, *S. officinalis* and *C. clusii* as affected by drought

C. clusi leaves showed the highest phenolic content under optimum conditions among the three studied species, followed by *S. officinalis* and *M. officinalis*, respectively (figure 22). Moreover, phenolic content of *C. clusii* leaves strongly increased along the water stress treatment, while that of *S. officinalis* increased slightly, and that of *M. officinalis* did not increase at all (figure 22). The term 'phenolic' includes every molecule bearing a hydroxyl moiety on an aromatic ring. Although phenolics are considered secondary metabolites, they include molecules clearly involved in primary metabolism, such as plastoquinones. Moreover, the second most abundant molecule after cellulose in higher plants, especially in woody perennials, is lignin: a phenolic. Apart from the particular functions the different phenolics may play, the biosynthesis of phenolic compounds may serve as a sink for reduction equivalents and reduced carbon compounds. Under stressful conditions, this may be particularly relevant since the Calvin cycle works at low rates and the biosynthesis of phenolic compounds may therefore prevent the photosynthetic electron transport chain from collapsing by over-reduction of electron transporters (Grace and Logan 2000; see figure 46).

The phenolic content in *C. clusii* leaves show better correlation with the RWC when expressed on an area basis than when expressed on a dry weight basis, suggesting that phenolics, at least some of them, may serve functions related to incident light, *e.g.* light screening. However, other functions of phenolics such as cuticle formation, biotic stress resistance and cell wall strengthening, which may be important under stress conditions, cannot be discarded.

Aside from their function as floral pigments, anthocyanins have been shown to be involved in light screening, especially blue light, under stressful conditions, and they also act as light screening pigments in red-senescing woody plants (Feild et al 2001). However, none of the three studied plants showed detectable amounts of anthocyanidins, indicating that they do not accumulate anthocyanins under stress conditions.

Many phenolic compounds show strong antioxidant activity *in vitro* (table 1), so the increase of their concentrations under drought stress in *C. clusii* leaves may enhance antioxidant defenses under oxidative stress. Due to its resistance to drought stress and the fact that the levels of of total phenolics increased under water deficit in *C. clusii*, this species was chosen for further studies on flavonoid accumulation and oxidation in plants.

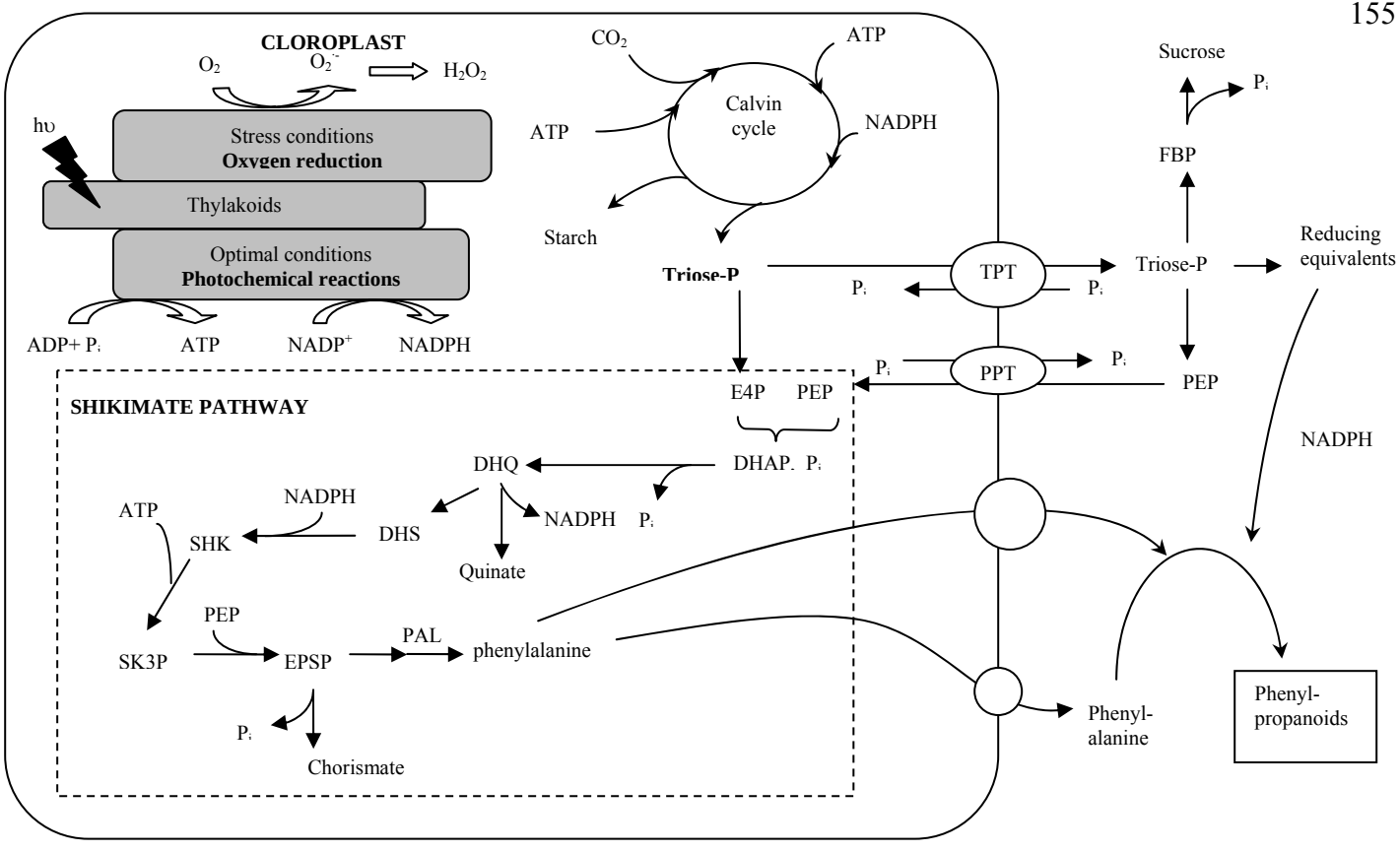

Figure 45: Relationship between the phenyl-propanoid pathway, carbohydrate metabolism and photosynthesis light reactions. E4P, Eritrose 4-phosphate; PEP, phosphoenol pyruvate; DHAP, 3-deoxi-D-arabino-heptulosenate-7-phosphate; DHQ, 3-dehydroquinate; DHS, 3-dehidroshikimate; SHK, shikimate; SK3P, shikimate 3-fosfate; EPSP, 5-enoilpiruvilshikimate 3-phosphate; FBP, fructose 1,6-bisphosphate; triose-P, triose phosphate; TPT, triose phosphate transporter; PPT, phosphoenol pyruvate transporter, PAL, phe ammonia lyase. Adapted from Grace and Logan (2000).

1.2.- Identification of flavonoids in *C. clusii*

HPLC and HPLC coupled to MS was used for the identification of flavonoids in leaves of *C. clusii* plants. As shown in table 2, the retention times of the peaks of flavan-3-ols, obtained by injecting samples in the HPLC might be slightly different from that of the pure standards. This phenomenom is common when a very complex sample, like a raw methanol extract of *C. clusii* leaves, is run in the HPLC. In these situations, the matrix of the extract can slightly shift the retention times of the separated compounds. To further check for the identity of the peaks, the UV-visible absorption spectrum of each peak in samples and standards were obtained with the diode array detector. The absorption peak of EC, at 275 nm, was the same for the standard and the sample, confirming that although the retention times differed by about 1 min, the identification of the compound was correct. The different relative height of the absorption peak at 275 nm between EC in samples and pure standards (see figure 13) may be simply due to differences of concentration, which increase the intensity of the peak without changing the wavelength of the absorption peak. In contrast, EGCG and ECG showed slightly different UV-visible absortion peaks in samples and in pure standards, although the retention times coincided. This may be due to other compounds of the matrix of the *C. clusii* leaf extracts that may slightly absorb at those wavelengths at the same retention time. In that case, further identification by MS is needed, and an HPLC method coupled to MS was used for it. In figure 14 it is shown that at 14.74 min there is a peak with a pseudo-molecular mass of 457.2 amu: EGCG in its de-protonated form. In the mass spectrum of the peak at 14.74 min, the pseudo-molecular peak of EGCG is the most intense peak, with minor peaks that correspond to EGCG fragments (like the m/z 169 amu, that corresponds to gallate anion). Figure 15 shows that there are two peaks at 18.85 and 33.22 min, respectively, in

the chromatogram of 440.5-441.5 amu: ECG in its de-protonated form. The second peak (33.22 min) shows an absolutely different retention time compared to the standards, so it can not be ECG. It is likely to be a dimer of ECG, a proanthocyanidin. In contrast, the mass spectrum of the peak at 18.85 min, shows, at a similar retention time to that of the pure standard, a pseudo-molecular peak (441.3 amu) with maximum intensity, and peaks corresponding to fragments of ECG (289.1 amu, EC; and 169.2 amu, gallate; both in their anionic forms). In figure 16, a peak of m/z 288.5-289.5 amu at 13.83 min: EC in its de-protonated form, is shown. In the mass spectrum of the peak at 13.83 min, the pseudo-molecular peak of EC is the most intense peak. By using the retention times of the peaks in chromatograms recorded at the adient wavelengths, co-elution with internal standards, the UV-visible absorption spectra, the chromatograms of the pseudomolecular peaks, and the mass spectra of the peaks, EC, ECG and EGCG were therefore succesfully identified in *C. clusii* leaf extracts.

Previous studies have demonstrated the presence of flavan-3-ols in plant species belonging to the genus *Cistus* (Poestsch and Reznik 1972, Vogt et al 1987, Demetzos and Perdetzoglou 1999), and we now report on the occurrence of the flavan-3-ols EC, ECG and EGCG in *C. clusii* leaves. Among the flavan-3-ols, EGCG was present in greatest concentration (about 5 μmol dm^{-2}), whereas EC and ECG were found at concentrations below 0.25 and 0.03 μmol dm^{-2}, respectively, which is within the range of concentrations shown in other species (Jeyaramraja et al 2003, Kirakosyan et al 2003). We did not detect the flavonols quercetin and kaempherol in their aglycone forms, in *C. clusii* leaves, which is consistent with previous reports (Vogt et al 1987).

As described previously, flavan-3-ols, like those identified in *C. clusii* leaf extracts, show high antioxidant capacity *in vitro*, which suggests that they

may form oxidation products in oxidizing conditions. Although the oxidation products of flavan-3-ols strongly depend on the conditions of the oxidation process, some authors have suggested that the most likely *in vivo* oxidation products of flavan-3-ols are flavan-3-ol quinones (Mizooku et al 2003, Dixon et al 2005). Furthermore, Vivas de Gaulejac et al (2001) reported on the accumulation of flavan-3-ol quinones in UV-irradiated wines. In the present study we report for the first time on the presence of flavan-3-ol quinones in plants. The methodology employed by Vivas de Gaulejac et al (2001) required deep modifications for its application to leaf extracts. In the present experiments, a more compact column (3.5 μm particle size) was used in order to reduce the chromatogram length. The stabilization of flavan-3-ol quinones by derivatization with benzenesulfinic acid lays on the valence change of the S atom in the benzenesulfinate molecule when it binds the flavan-3-ol quinone. The S of the benzenesulfinate shows a valence of +4. When this molecule gets in contact with the reactive B ring of the flavan-3-ol *o*-quinone, the S of the benzenesulfinate is oxidized (to valence +6), introducing electrons to the B ring of the flavonoid, restoring the resonant bounds of the aromatic ring and reducing the quinones to quinols. However, when the derivatized quinone enters the MS source, the ion energy ramp and the cone voltage applied (see materials and methods) break down the molecule and the derivatization reaction reverts, so the flavan-3-ol quinone can be seen as a fragment of the derivatized quinone (figure 17).

By applying this method, ECQ and EGCGQ were identified in *C. clusii* leaf extracts. For the quantification of these compounds simultaneously with their reduced precursors (EC and EGCG, respectively), MS chromatograms in single ion mode of the pseudo-molecular peaks of EC, EGCG, ECQ and EGCGQ, were used (figure 18). This quantification method minimizes

contaminations because only molecules with the same retention time and molecular weight are quantified.

C. clusii is therefore an adequate plant material for evaluating the relevance of antioxidant flavonoids in abiotic stress resistance, because it contains considerable amounts of reduced and oxidized flavonoids in leaves and is well adapted to withstand environmental constrains in Mediterranean field conditions.

1.3.- Effects of water deficit and high light on flavan-3-ol accumulation

In the present study, the levels of EC, ECG and EGCG increased significantly in *C. clusii* plants after a water deficit treatment in the field. To our knowledge, drought-induced changes in these flavan-3-ols have previously been reported only in tea plants (Jeyaramraja et al 2003) and in two species of the genus *Crataegus* (Kirakosyan et al 2003). In both studies, EC concentrations increased in water-stressed plants, in agreement with our results. However, ECG and EGCG decreased in water-stressed tea plants, which contrasts with what occurs in *C. clusii*. This difference may be due to (i) the magnitude of the stress imposed on the plants: mild water deficit was imposed on field-grown *C. clusii* plants, whereas severe stress was imposed to potted greenhouse-grown tea plants, and/or (ii) other factors that affect flavan-3-ol accumulation in plants such as the incident light, which will be discussed later.

The causes of the accumulation of flavan-3-ols in water-stressed *C. clusii* plants should be evaluated in detail. In some cases, differences can be simply attributed to changes in leaf morphology. For instance, during the first 15 days of the first drought experiment (see sections 1 and 2 of results), the RWC remained unaltered, while flavan-3-ols per leaf area increased significantly. However, EGCG decreased slightly (by 7 %) when expressed

on a dry mass basis, indicating that changes in EGCG responded to morphological alterations of leaves (increment of LMA). In contrast, between days 15 and 30, EGCG per leaf area increased by about 67 %, whereas LMA remained unaltered and RWC decreased to about 75 %, indicating that these changes in EGCG were caused by enhanced synthesis of flavan-3-ols, rather than alterations in leaf morphology. As RWC decreased further reaching about 63 % by day 50, the EGCG concentration remained unaltered, suggesting that rates of EGCG degradation and synthesis were similar. As it will be discussed later many other stress factors in the field, such as the high irradiances happening in the Mediterranean summer, may also have a strong impact on flavan-3-ol accumulation in leaves, even higher than water deficit.

In the third experiment, EGCG, the major flavan-3-ol in leaves of mature and juvenile *C. clusii* plants grown in Mediterranean field conditions, increased from *ca*. 1.7 to 2.5 μmol dm^{-2} from spring to the beginning of summer, when maximum diurnal irradiation was highest. In this longer experiment, EGCG levels showed a strong positive correlation with maximum diurnal PFDs and RWC (figure 30). Which of these two stress factors, water deficit or high light, is however causing changes in EGCG levels in *C. clusii* leaves? Compiled data from the the two experiments (sections 2 and 3 of results), given relative to the maximum levels of each case (sampling year and age), clearly revealed that light levels, rather than water deficit, influence EGCG accumulation in *C. clusii* leaves (figure 47). EGCG levels strongly positively correlated with the maximum diurnal PFD and day length, while correlation of EGCG levels with RWC was much lower (figure 47). Thus, integrating data from these two experiments, it can be deduced that flavan-3-ols respond to maximum irradiances (or photoperiod) rather than water deficit. *C. clusii* plants showed the highest

EGCG levels by the end of June, when maximum PFD values and day length are at their highest during the year.

When EGCG levels are given respect to PFD values or photoperiod, a sharp increase of EGCG levels occurs when PFD values and day length surpass 1800 μmol m^{-2} s^{-1} and 13 h, respectively (figure 47). It is therefore clear that the effects of high light levels during spring and summer on EGCG accumulation may be masked by the coincidence with water deficit in Mediterranean summer. Although in this plant species photosynthesis is saturated at PARs higher than *ca.* 1000 μmol m^{-2} s^{-1} (Munné-Bosch and Alegre 2002), EGCG accumulation does not occur until 1800 μmol m^{-2} s^{-1} aprox. This gap between the energy input that saturates photosynthesis and the energy input that triggers EGCG accumulation can be attributed to the main energy dissipation mechanisms in this plant species, like thermal energy dissipation, ROS formation and scavenging by antioxidant systems, etc. (e.g. α-tocopherol and xanthophyll cycle) (Munné-Bosch and Alegre 2002).

EGCG, like all monomeric flavan-3-ols, shows a light absorption maximum around 275 nm (figure 13), which is within the UV-C band, and very close to the UV-B. Although photons within this wavelength band are very energetic, they hardly reach Earth surface, therefore suggesting that the photoprotective role of EGCG if any, may not be directly linked to UV light absorption. Although photoperiodic control of flavan-3-ol biosynthesis has not been demonstrated thus far, it is possible that the link between light levels and EGCG accumulation in leaves simply responds to a photoperiodic control of flavan-3-ol biosynthesis. Indeed, the biosynthesis of other flavonoids -anthocyanins- has been shown to be under photoperiodic control by photoreceptors (Oelmüller and Mohr 1985, Grace and Logan 2000).

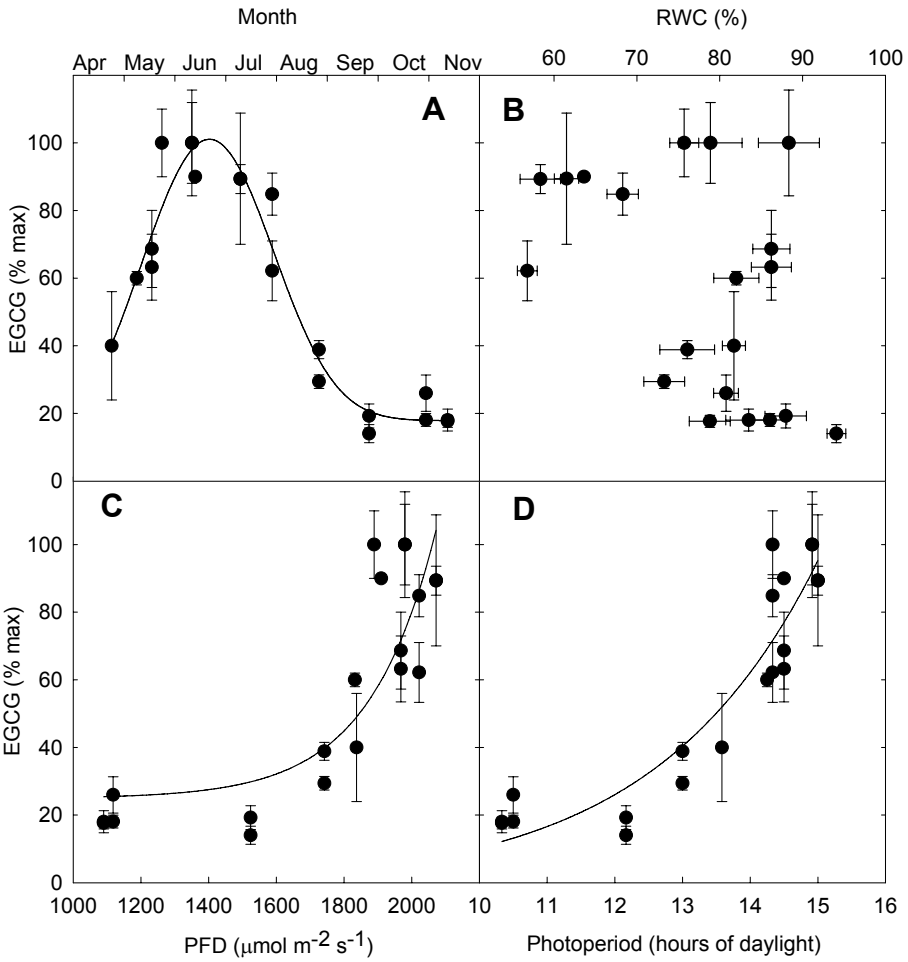

Figure 47: Relationship between (-)-epigallocatechin gallate (EGCG) contents and the sampling date (panel A), the relative leaf water content (RWC, panel B), the maximum diurnal photon flux density (PFD, panel C) or the photoperiod (panel D) in *Cistus clusii* plants grown under Mediterranean field conditions. Data from all experiments performed in *C. clusii* are given relative to the maximum contents obtained for each study.

In addition, it has been suggested that flavan-3-ols act as antioxidants in plants (Miller et al 1996, Shirley 1996, Rice-Evans et al 1997). The flavan-3-ols EC, ECG and EGCG are efficient chain-breaking antioxidants and transition metal chelators, thus they may help inhibiting lipid peroxidation in

membranes (Caturla et al 2003, Potapovich and Kostyuk 2003). Other functions have also been suggested for flavan-3-ols (for review, see Shirley 1996; Marles et al 2003, Dixon et al 2005). When photosynthetic electron transport is limited, metabolic alterations may lead to drought-induced increases in flavan-3-ols as a result of the activation of alternative metabolic routes mediated by phenylalanine ammonia lyase, chalcone synthase and other enzymes. This may lead to accumulation of flavan-3-ols and flavan-3-ol derivatives, such as PAs, which may accumulate in vacuoles and have an anti-herbivore role (Dixon and Paiva 1995). These various roles are not mutually exclusive, and it is likely that flavan-3-ols, like the other antioxidants, serve several functions in plants.

As discussed later, ascorbate also increased in parallel with total phenolics, showing a strong positive correlation. This may be associated with a putative interaction between both groups of compounds, because phenoxyl radicals, i.e., the immediate oxidation products of phenolics, may be recycled back to their reduced forms by ascorbate. Among phenolics, it is likely that flavan-3-ols cooperate with ascorbate in the scavenging of ROS. Ascorbate is found in all subcellular fractions in plant cells, showing low concentrations in the vacuoles. Although the subcellular distribution of the monomeric flavan-3-ols EC, ECG and EGCG in plant cells is unknown, the last steps in the synthesis of flavan-3-ols occur in the cytoplasm, where ascorbate levels are high (Smirnoff and Wheeler 2000, Shirley 2001, Marles et al 2003), suggesting that a physical interaction between both antioxidants at membrane interfaces is possible. One can therefore speculate that flavan-3-ols, similar to α-tocopherol and other antioxidants, but probably with a different subcellular location, function as antioxidants in drought-stressed *C. clusii* plants, and that the resulting phenoxyl radicals are recycled back to

their reduced forms by ascorbate, which in turn, is recycled back to its reduced form, thereby maintaining its oxidation state as drought progresses.

Although flavonoids have been generally considered to be exclusively synthesized in the cytoplasm of plant cells, linked to the cytosolic face of the endoplasmic reticulum, it has been recently shown that some key enzymes of the pathway such as chalcone synthase, co-localize in the nuclei of several *Arabidopsis* cell types (Saslowsky et al 2005). EC is synthesized from cyanidin by the action of the enzyme anthocyanidin reductase, which is localized in the cytoplasmic face of the endoplasmic reticulum (reviewed by Marles et al 2003). However, further research is needed to clarify the subcellular localization of monomeric flavan-3-ols in plants. Analyses of flavan-3-ols in chloroplast-enriched fractions from *C. clusii* leaves, revealed only traces of EGCG in the HPLC-MS analyses. EGCG, ECG, EC, or their respective quinones do not therefore accumulate in chloroplasts of *C. clusii* leaves. It is, however, possible that these flavan-3-ols exert an antioxidant role in other cellular compartments.

Tea plants grown in Mediterranean summer field conditions suffered a strong water loss and chronic photoinhibition. In these conditions, the levels of the reduced flavan-3-ols, EC and EGCG, remained unaltered throughout the experiment. Endogenous concentrations of EC and its gallate ester, EGCG, have been shown to increase (Jeyaramraja et al 2003, Kirakosyan et al 2003) but also to remain unchanged (Arts et al 2000) in previous studies on tea and other plant species exposed to water deficit. Together, these findings suggest that the effect of water deficit on EC and EGCG accumulation strongly depends on the species and conditions of the study.

1.4.- Effects of water deficit and high light on flavan-3-ol oxidation

The oxidation of EC and EGCG to their respective quinones in plants *in vivo* is shown here for the first time in the studies with tea plants. The levels of the oxidized flavan-3-ols ECQ and EGCGQ increased sharply under water deficit (figure 38). The strongest increases were observed when stress was maximum. Levels of ECQ and EGCGQ decreased slightly at the end of the experiment, concomitantly with a small increase of RWC due to small rainfalls. The oxidation state of EC and EGCG increased sharply after the onset of the water deficit treatment. Since levels of EC and EGCG remained unaltered throughout the experiment, this sharp increase suggests that the flavan-3-ols synthesized *de novo* in water-stressed tea plants are rapidly oxidized to their respective quinones.

The extent of lipid peroxidation in tea plants decreased significantly after 19 days of drought treatment, and then remained constant until the end of the experiment (figure 39). The MDA levels suggest that although plants were subject to severe water deficit and chronic photoinhibition, they did not show enhanced lipid peroxidation in leaves. The accumulation of ECQ and EGCGQ strongly correlated with the decrease in MDA levels in leaves (r^2=0.975 and 0.798, respectively), which suggests that the oxidation of EC and its gallate ester, EGCG, to their respective quinones may prevent lipids from oxidation. This is consistent with studies showing that these flavan-3-ols, especially EGCG, can integrate deeply into lipid bilayers, acting as membrane antioxidants (Kumazawa et al 2004, Saffari and Sadrazeh 2004).

Quinonic forms of flavonoids may be formed by non-enzymatic oxidation, or as a result of the activity of peroxidases (in the presence of H_2O_2) and other polyphenol oxidases such as tyrosinase and laccase (in the presence of oxygen) (Mayer and Harel 1979, Guyot et al 1996). The accumulation of ECQ and EGCGQ in water-stressed tea plants may therefore happen as

intermediate products of PA biosynthesis, but also as a result of H_2O_2 scavenging by peroxidases. An enhanced accumulation of PAs was observed in our study after the water stress treatment (figure 40). ECQ and EGCGQ appearance and increases preceded PAs accumulation after 26 days of water deficit. Flavan-3-ols such as EC and EGCG may finally polymerize and accumulate in vacuoles as PAs. The PA polymerization mechanism and the origin of the initiation and elongation building blocks remain to be fully understood (reviewed by Dixon et al 2005), but there is general agreement that an oxidative metabolism may be required for PAs biosynthesis, at least during seed browning (Pourcel et al 2007). Though still to be proven, it is likely that the oxidation of EC and EGCG to their respective quinones observed in water-stressed tea leaves may therefore be a part of an oxidative process linked to the biosynthesis of PAs.

1.5.- Effects of plant age on *C. clusii* responses to water deficit and high light stress

During the experimental period in 2004, mature and juvenile *C. clusii* plants were exposed to typical Mediterranean climatic conditions, experiencing a hot, dry summer, with very low precipitation, PFDs of *ca.* 2000 μmol m^{-2} s^{-1} and maximum diurnal air temperatures ranging 25-30 ºC (figure 27). Under such PFDs, photosynthesis in *C. clusii* leaves is by far saturated (Munné-Bosch and Alegre 2002), so plants were exposed to excess energy during the experiment. Lipid peroxidation in leaves was indeed higher and the F_v/F_m ratio lower during spring and summer, suggesting that the excess incoming energy was dissipated, at least partially, by the formation of ROS.

Although environmental conditions were the same for juvenile and mature plants and both plant groups showed a similar water loss, oxidative stress markers revealed age-related changes in oxidative metabolism.

During spring and summer, the seasons with highest maximum irradiances, lipid peroxidation was higher in older plants. The high F_v/F_m ratios throughout the experiment in both plant age groups indicate the absence of chronic photoinhibition in both plant groups. However, F_v/F_m values were slightly lower in mature compared to juvenile plants, indicating that the electron transport machinery works more efficiently in younger plants (figure 28). These results confirm previous studies showing that plant aging increases oxidative stress in leaves, which appears to be associated with reductions in stomatal conductance, especially during periods of drought. Such lower stomatal conductance, combined with the high PFDs typical of Mediterranean spring and summers, leads to enhanced formation of ROS as an energy dissipation mechanism as plants age (Munné-Bosch and Alegre 2002, Munné-Bosch and Lalueza 2007). In the latter study, based on slightly higher C/N ratios and sucrose levels per unit of chlorophyll found in 7-years-old compared to 2-years-old *C. clusii* plants, it was suggested that age-induced reductions in photosynthesis may not only be caused by stomatal closure, as suggested by the enhanced ABA contents in older plants, but also by a feedback inhibition of photosynthesis by sugars.

1.6.- Effects of plant age on flavan-3-ol accumulation and oxidation

Monomeric and polymeric flavan-3-ol contents increased in leaves as *C. clusii* plants aged, especially during the more stressful periods of spring and summer. We proved therefore for the first time in the present study that flavonoids, and more particularly, EGCGQ and PAs accumulate to a greater extent in leaves of older plants, which suggests that the accumulation of these secondary metabolites may serve the aging plant, at least to some extent, to divert carbon that would otherwise accumulate as sucrose and/or hexoses and inhibit photosynthesis, especially during periods of abiotic

stress. Together, data from present and previous studies (Munné-Bosch and Alegre 2002, Munné-Bosch and Lalueza 2007) suggest that changes in primary and secondary metabolism may serve the aging plant to protect leaves from abiotic stress.

2.- Ascorbate

2.1.- Effects of water deficit and hight light on ascorbate accumulation and oxidation

C. clusii plants responded to a water deficit treatment in the field by quickly increasing their ascorbate content in leaves. Thereafter, the ascorbate content was kept high during the treatment, which indicates that the stress applied was not severe for these plants. Levels of DHA followed exactly the inverse trend. These data suggest *C. clusii* activates its ascorbate reducing and re-cycling machinery as soon as it senses drought, as an acclimation mechanism. The oxidation state of the ascorbate pool, thus, shifted toward its reduced form at the onset of the treatment and then remained unaltered, indicating that ascorbate is efficiently re-cycled once oxidized, in water-stressed plants (Noctor and Foyer 1998, Asada 1999).

Levels of ascorbate in field-grown *C. clusii* leaves increased from spring to summer and recovered back in new leaves that appeared in fall. Although this increase was mostly due to increased LMA, the oxidation state of the ascorbate pool also increased from spring to summer and recovered in fall. Regardless of the oxidation state of the ascorbate pool at predawn, its oxidation state at midday was remarkably constant around 40 %, and always higher than at predawn, indicating that ascorbate oxidation and re-cycling follows diurnal fluctuations. This function of ascorbate as a low molecular weight antioxidant has been extensively studied and reported for all subcellular compartments and plant organs with the exception of dormant seeds (Loewus 1988, Foyer 1993, Foyer et al 1991 and 1994, Arrigoni 1994, Smirnoff and Pallanca 1996, Smirnoff 1996, Noctor and Foyer 1998). Ascorbate is well known to be involved in excess energy dissipation by the water-water cycle in chloroplasts and the xanthophyl cycle (Demmig-Adams and Adams 1996, Asada 1999). Our results confirm that the antioxidant

function of ascorbate reported for many plant species, also occurs in *C. clusii*, and show how this antioxidant function is exerted at both seasonal and diurnal timescales. Moreover, the results presented in the present study suggest that ascorbate oxidation state may be tightly regulated. What is the reason why ascorbate oxidation state is so constant at midday regardless of the environmental and developmental conditions? One can hypothesize that ascorbate oxidation state may act as a sensor for cell redox state signaling, since it is a redox pair (ascorbate/DHA) present in all cell compartments, from the nucleus to the apoplast.

2.2.- Effects of plant age on ascorbate accumulation and oxidation

The oxidation state of ascorbate increased during the summer, but not during spring or fall, as plants aged. The present and previous studies (Munné-Bosch and Alegre 2002) show that plant aging increases oxidative stress in leaves, which appears to be associated with reductions in stomatal conductance during periods of drought. Ascorbate is is one of the major ROS scavenging systems in chloroplasts, where ROS formation as defense mechanism mainly occurs under the above mentioned conditions. Thus, ascorbate is probably being oxidized by ROS formed in chloroplasts as a mechanism for excess energy dissipation, as described for many other plant species (e.g. Asada 1999), further supporting the hypothesis that plant aging enhances oxidative stress under stressful conditions (for review, see Munné-Bosch 2007). As it is shown later, ascorbate may play other additional functions aside its antioxidant role.

2.3.- Apoplastic ascorbate oxidation state as a signal

The oxidation state of the ascorbate pool in the apoplast has the potential to exert a profound influence on symplast metabolism (Pignocchi and Foyer

2003, Foyer and Noctor 2005). Ascorbate, along with phenolics and polyamines, is the major antioxidant in the apoplast, but the later ones show low redox buffering capacity, so changes in the ascorbate oxidation state in the apoplast can be interpreted also as redox shifts in this cell compartment. In the present study, the effects of a shifted apoplastic ascorbate oxidation state on nuclear gene expression were explored. The plant material (tobacco plants) employed for these experiments showed enhanced or decreased ascorbate oxidation state by over- and under-expression of AO. However, the antisense line, under-expressing AO, also showed an increased size of the ascorbate pool in the apoplast, which could interfere with the results attributed to a reduced oxidation state of the apoplastic ascorbate. Plants over- and under-expressing AO did not show any growth or developmental alteration, or any differences in biomass production in all 3 genotypes (wild type, sense and antisense). Both apoplastic and symplastic ascorbate are well known to be involved in oxidative stress resistance (Asada 1999, Pignocchi and Foyer 2003). Many stress factors such as virulent pathogen attack, and oxidant air pollutants (e.g. O_3 and SO_2) alter apoplast ascorbate oxidation state (Degousee et al 1994, Lamb and Dixon 1997). Ascorbate or its major oxidation product, DHA, are thought to be transported across plasma membrane, so plant cells can control an oxidative gradient across it (Horemans et al 2000, Pignocchi and Foyer 2003). In the present study it is shown that the genes encoding for the main enzymes involved in ascorbate recycling in the cytosol (MDHAR and DHAR) and the genes encoding for the main enzymes that catalyze ascorbate and glutathione oxidation (cAPX and GPX) in the same subcellular compartment did not show any expression shift after AO under- or over-expression. Moreover, the expression of the *palA* gene, encoding for the phenylalanine ammonia lyase A, also remained unaltered, suggesting that plant cells do not compensate for the ascorbate

oxidation state with the second redox buffer in the apoplast: phenolics; at least at gene expression level. These findings agree with those reported by other authors (Sanmartín et al 2003, Pignocchi et al 2003) who reported on the enhanced sensitivity of AO over-expressing plants to O_3 and on the enhanced resistance of the tobacco under-expressing AO to the same stress factor.

NtTPC1B, a component of the voltage-dependent Ca^{2+} channel NtTPC1A/B, resulted under-expressed in both transgenic lines, especially in the sense line. This Ca^{2+} channel is the major one responding to H_2O_2 and is implicated in the NADPH oxidase-mediated oxidative burst. This oxidative burst is a common feature on signaling pathways of many different stress kinds, e.g. fungal pathogen attack, air pollutants and insect pests, involving apoplast-symplast communication (Torres and Dangl 2005). With the present data, AO over-expression seems to mimic NADPH oxidase activity so the plant under-expresses the Ca^{2+} channel to prevent a constitutive activation of the enzyme. Moreover, although to a lesser extent, NtTPC1B gene expression is also down-regulated in the antisense line. As stated above, many apoplast-symplast communication pathways are mediated by Ca^{2+} channels and, in this case, the plant may be preventing the constitutive activation of the singnaling pathway that would be operating to detect a less oxidized ascorbate pool or an increased ascorbate pool size, given that the AO antisense plants showed increased ascorbate pool size. These suggestions are in agreement with the finding that AO over-expressing plants show enhanced susceptibility to a virulent strain of *P. Syringae*: in these plants, the formation of the oxidative burst involved in the hypersensitive response is limited by the low abundance of the Ca^{2+} channel involved in the process (Pignocchi et al 2006).

The subtractive hybridisation library showed that different pathways within the symplast were regulated at gene expression level by the ascorbate oxidation state in the apoplast. Among the cloned ESTs, there can be found genes involved in many levels of the apoplast-symplast signal transduction pathway as well as genes altered as a response to different stimuli. The output of the subtractive hybridisation library included genes involved in photorespiration. This process is one of the major sources of H_2O_2 in photosynthetic plant cells. Glycolate oxydase is the enzyme responsible for H_2O_2 formation by catalyzing the conversion of glycolate into glyoxylate (figure 48). This reaction occurs in peroxisomes, where the great amounts of H_2O_2 generated are scavenged by catalases. Pignocchi et al (2006) showed that diurnal cycles of the three catalase isoforms and the amplitude of the diurnal cycles of glycolate oxidase gene expression are altered in these plants. Together, these findings suggest that H_2O_2 homeostasis in the symplast is deeply altered when apoplastic ascorbate oxidation state is manipulated. In the present study it is reported that the expression of the genes encoding for DHAR and APX were not altered in the transgenic lines. However, Pignocchi et al (2006) reported that the activity of many enzymes involved in ascorbate redox metabolism, including DHAR and APX, were altered, especially in the AO overexpressing plants, indicating the presence of regulation mechanisms at post-transcriptional level.

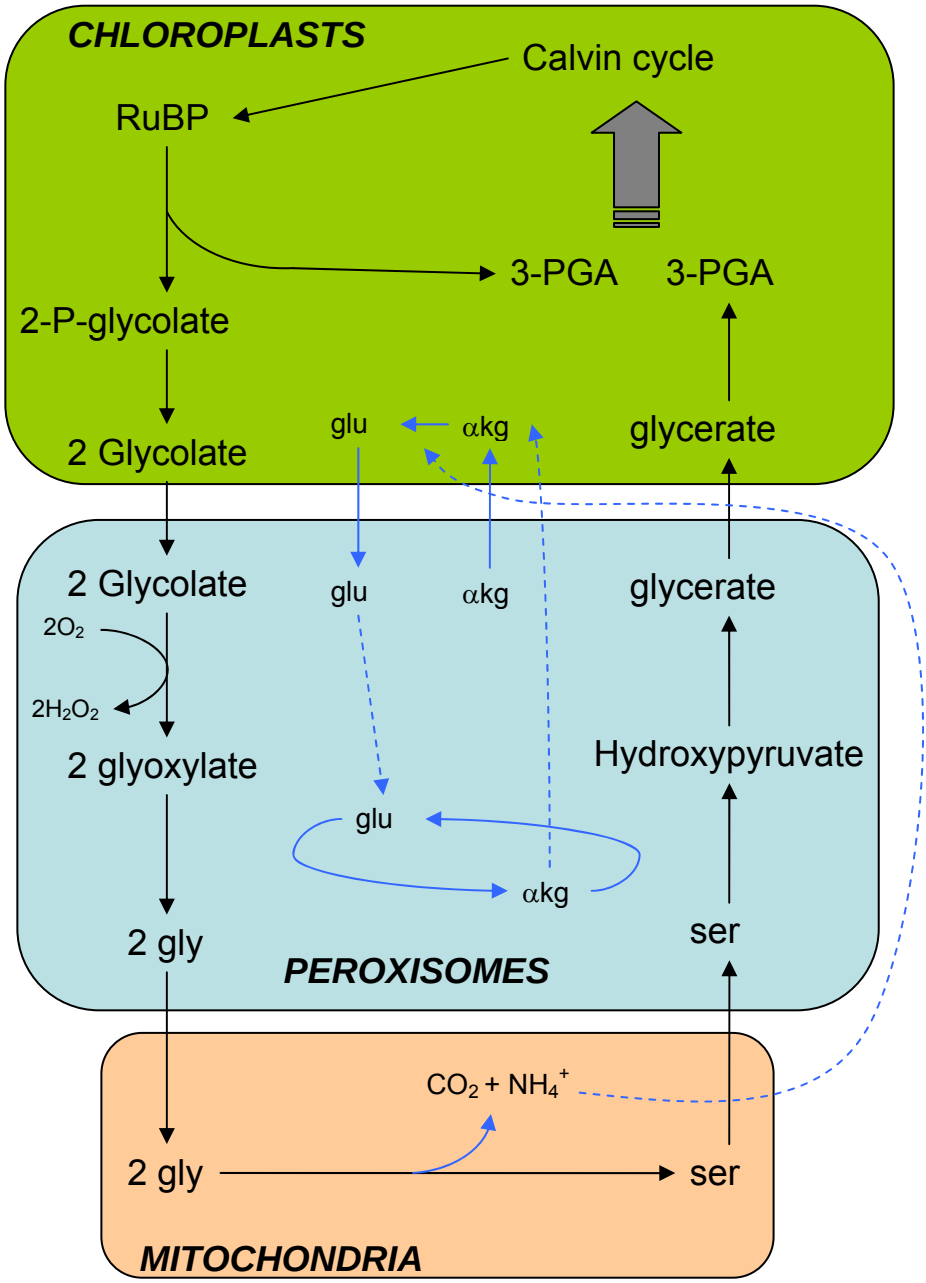

Figure 48: Photorespiratory (C_2) cycle. RuBP, ribulose-1,5-bisphosphate; αkg, α-ketoglutarate; 3-PGA, 3-P-glyceric acid; Blue arrows stand for reactions involving nitrogen re-cycling reactions.

Further research is needed to fully understand the involvlement of the different metabolic pathways in apoplast-symplast signaling, but the implication of apoplastic ascorbate oxidation state in nuclear metabolism is key in apoplast-symplast communication. It is difficult to draw a picture of the physiological meaning of the subtractive hybridisation library output. However, as shown in figure 45 and table 6, it is clear that many different metabolic processes are affected by apoplastic ascorbate oxidation state through nuclear gene expression.

CONCLUSIONS

1.- It is demonstrated the presence of the monomeric flavan-3-ols (-)-epigallocatechin gallate, (-)-epicatechin and (-)-epicatechin gallate, and the absence of quercetin and kampferol free aglycones in *C. clusii* leaves. The levels of these compounds increase under a combination of water deficit and high light in the field

2.- The major stress factor affecting flavan-3-ol accumulation in *C. clusii* leaves is either the maximum diurnal irradiance or the photoperiod. Although initial studies suggested an effect of plant water status on flavan-3-ol accumulation, this effect seems to be a consequence of the coincidence of water deficit with high light in Mediterranean field conditions

3.- Evidence is provided evidence of the *in vivo* oxidation of (-)-epicatechin and (-)-epigallocatechin gallate to their respective quinones in plants, particularly in tea plants under drought stress.

4.- Reduced ascorbate accumulates and DHA decreases at initial stages of a water stress treatment in *C. clusii* as an acclimation mechanism to a subsequent oxidative stress. Moreover, ascorbate is oxidized following diurnal cycles, showing highest oxidation state always at midday.

5.- Plant aging increases oxidative stress in *C. clusii* leaves, particularly during periods of abiotic stress

6.- The accumulation of flavan-3-ols is slighly higher in mature plants than in juvenile ones, especially during periods of abiotic stress –high light and water deficit. Although flavan-3-ol quinones do not accumulate in water- and/or high light-stressed *C. clusii* plants, their levels are constitutively higher in older plants. Those responses may serve as a mechanism to divert photosynthetic carbon to alternative sinks in order to avoid the inhibition of photosynthesis by photosynthetic products.

7.- Ascorbate oxidation state in the apoplast provokes a profound influence on nuclear gene expression, especially in H_2O_2 homeostasis mechanisms, such as photorespiration and the oxidative burst. NtTPC1A/B, a voltage-dependent Ca^{2+} channel, is involved in apoplastic ascorbate redox state signal transduction, at gene expression level

REFERENCES

Al Wakeel SAM, El-Negoumy SI, El-Hadidi MN and Saleh NAM (1987). Flavonoid patterns in *Fagonia mollis*-complex. Biochem. Syst. Ecol. 15: 459-460

Anderson JW, Foyer CH and Walker DA (1983). Light-dependent reduction of dehydroascorbate and uptake of exogenous ascorbate by spinach chloroplasts. Planta 158: 442-450

Apel K and Hirt H (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant. Biol. 55: 373-399

Arrigoni O (1994). Ascorbate system in plant development. J. Bioenerg. Biomembr. 26: 407-419

Arts ICW, van de Putte B, Hollman PCH (2000). Catechin contents of foods commonly consumed in The Netherlands. 2. Tea, wine, fruit juices, and chocolate milk. J. Agric. Food Chem. 48: 1752-1757

Aruoma OI, Halliwell B, Laughton MJ, Quilan GJ and Gutteridge JM (1989). The mechanism of initiation of lipid peroxidation evidence against a requirement for an iron-II-iron-III complex. Biochem. J. 258: 617-620

Asada K (1999). The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50: 601-639

Asada K and Takahashi M (1993). Production and scavenging of active oxygen in photosynthesis. **In:** Kyle DJ, Osmond CB, Arntzen CJ (eds.). Photoinhibition. Elsevier, Amsterdam, pp 227-287

Asard H, Horemans N and Caubergs RJ (1995). Involvement of ascorbic acid and a *b*-type cytochrome in plant plasma membrane redox reactions. Protoplasma 184: 36-41

Asard H, Horemans N and Caubergs RJ (1992). Transmembrane electron transport in ascorbate-loaded plasma membrane vesicles from higher plants involves a *b*-type cytochrome. FEBS Lett. 306: 143-146

Bieza K and Lois R (2001). An Arabidopsis mutant tolerant to lethal ultraviolet-B levels shows constitutively elevated accumulation of flavonoids and other phenolics. Plant Physiol. 126: 1105-1115

Bilger W, Schreiber U and Bock M (1995). Determination of quantum efficiency of photosystem II and non-photochemical quenching of chlorophyll fluorescence in the field. Oecologia 102: 425-432

Blackburn EH, Chan S, Chang J, Fulton TB, Krauskopf A, McEachern M, Prescott J, Roy J, Smith C and Wang H (2000). Molecular manifestations and molecular determinations of telomere capping. Cold Spring Harbor Symp. Quant. Biol. 65: 253-263

Bolós O and Vigo J (eds.) (1984-2001). Flora dels Països Catalans. Barcino, Barcelona

Bors W, Michel C and Stettmaier K (2000). Electron paramagnetic resonance studies of radical species of proanthocynidins and gallate esters. Arch. Biochem. Biophys. 15: 347-355

Bors W, Heller W, Michel C and Saran M (1990). Flavonoids as antioxidants: determination of radical scavenging efficiencies. Method. Enzymol. 186: 343-355

Brinkmeier E, Hahn H, Seeger T, Geiger H and Zinsmeisteret HD (1999). Seasonal variation in flavonoid concentrations of mosses. Biochem. Syst. Ecol. 27: 427-435

Brosché M, Fant C, Bergkvist SW, Strid H, Svensk A, Olsson O and Strid A (1999). Molecular markers for UV-B stress in plants: alteration of the expression of four classes of genes in *Pisum sativum* and the formation

of high molecular mass RNA adducts. Biochim. Biophys. Acta 1447: 185-198

Buchanan BB and Balmer Y (2005). Redox regulation: a broadening horizon. Annu. Rev. Plant Biol. 56: 187-220

Buchanan-Wollaston V (1997). The molecular analysis of leaf senescence. J. Exp. Bot. 48: 181-199

Buck V, Quinn J and Pino TS (2001). Peroxidase sensors for the fission yeast stress-activated mitogen-activated protein kinase pathway. Mol. Biol. Cell 12: 407-419

Cao H, Glazebrook J, Clarke JD, Volko S and Dong XN (1997). The Arabidopsis NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell 88: 57-63

Caturla N, Vera-Samper E, Villalain J, Mateo CR and Micol V (2003). The relationship between the antioxidant and the antibacterial properties of galloylated catechins and the structure of phospholipid membranes. Free Radic. Biol. Med. 34: 648-662

Chaves MM, Pereira JS, Maroco J, Rodrigues ML, Ricardo CPP, Osório ML, Carvalho I, Faria T and Pinheiro C (2002). How plants cope with water stress in the field? Photosynthesis and Growth. Ann. Bot. 89: 907-916

Chen Z, Silva H and Klessig DF (1993). Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. Science 262: 1883-1886

Conklin PL and Barth C (2004). Ascorbic acid, a familiar small molecule intertwined in the response of plants to ozone, pathogens, and the onset of senescence. Plant Cell Environ. 27: 959-970

Conklin PL (1998). Vitamin C: a new pathway for an old antioxidant. Trends Plant Sci. 3: 329-330

Dalzell SA and G Kerven (1998). A rapid method for the measurement of *Leucaena* spp. proanthocyanidins by the proanthocyanidin (butanol/HCl) assay. J. Sci. Food Agric. 78: 405-416

Dangl JL, Dietrich RA and Thomas H (2000). Senescence and programmed cell death. **In:** Buchanan BB, Gruissem W and Jones RL (eds.). Biochemistry and Molecular Biology of Plants. American Society of Plant Physiologists, Rockville, pp 1044-1100

Dat J, Vandenabeele E, Vranova E, Van Montagu M, Inzé D and Van Breuseguem F (2000). Dual action of the active oxygen species during plant stress responses. Cell Mol. Life Sci. 57: 779-795

Davey MW, Van Montagu M and Inzé D (2002). Ascorbate metabolism and stress. **In:** Inzé D and Van Montagu M (eds.) Oxidative stress in plants. Taylor and Francis, London and New York, pp 271-295

De Gara L. De Pinto MC. Arrigoni O (1997). Ascorbate synthesis and ascorbate peroxidase activity during the early stage of wheat germination. Physiol. Plant. 100: 894-900 1997

Degousee N, Triantaphylides C and Montillet JL (1994). Involvement of oxidative processes in the signaling mechanisms leading to the activation of glyceollin synthesis in soybean (*Glycine max*). Plant Physiol. 104: 845-952

Del Río LA, Corpas FJ, Sandalio LM, Palma JM, Gómez M and Barroso JB (2002). Reactive oxygen species, antioxidant systems and nitric oxide in peroxisomes. J. Exp. Bot. 53: 1255-1272

Demetzos C and Perdetzoglou DK (1999). Chemotaxonomic survey of wild and cultivated *Cistus* L. species. Plant Biosyst. 133: 251-254

Demmig-Adams B and Adams III WW (1996). The role of xanthophyll cycle carotenoids in the protection of photosynthesis. Trends Plant Sci. 1: 21–26

Desikan R, Hankock J and Neill S (2006). Reactive oxygen species as signaling molecules. **In:** Smirnoff N (ed.). Antioxidants and reactive oxygen species in plants. Blackwell Publishing, Oxford, pp 169-196

Dixon RA, Xie D and Sharma SB (2005). Proanthocyanidins – a final frontier in flavonoid research? New Phytol. 165: 9-28

Dixon RA and Steele CL (1999). Flavonoids and isoflavonoids - a gold mine for metabolic engineering. Trends Plant Sci. 4: 394-400

Dixon RA and Paiva NL (1995). Stress-induced phenylpropanoid metabolism. Plant Cell 7: 1085-1097

Doke N (1997). The oxidative burst: role in signal transduction and plant stress. **In:** Scandalios SG (ed.). Oxidative stress and the molecular biology of antioxidant defences. Cold Spring Harbor Laboratory Press, New York, pp 785-813

Economides C and Adam KP (1998). Lipophilic flavonoids from the fern *Woodsia scopulina*. Phytochemistry 49: 859-862

Elstner EF (1991). Mechanisms of oxygen activation in different compartments of plant cells. **In:** Pell EJ and Steffen KL (eds.). Active oxygen/oxidative stress and plant metabolism. American Society of Plant Physiologists, Rockville, pp 13-25

Feild TS, Lee DW and Holbrook NM. (2001). Why leaves turn red in autumn. The role of anthocyanins in senescing leaves of red-osier dogwood. Plant Physiol. 127: 566-574

Foyer CH and Noctor G (2005). Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. Plant Cell 17: 1866-1875

Foyer CH and Noctor G (2003). Redox sensing and signaling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. Physiol. Plant. 119: 395-404

Foyer CH, Lelandais M, Edwards EA and Kunert KJ (1994). Photooxidative stress in plants. Physiol. Plant. 100: 241-254

Foyer CH (1993). Ascorbic acid. **In:** Alscher RG and Hess JL (eds.). Antioxidants in higher plants. CRC Press, Boca Raton, pp. 31-58

Foyer CH, Lelandais M, Edwards EA and Mullineaux PM (1991). The role of ascorbic acid in plants, interactions with photosynthesis, and regulatory significance. **In:** Pell E and Steffen K (eds.). Active oxygen/oxidative stress and plant metabolism. Proceedings 6th Annual Penn State Symposium on Plant Physiology, American Society of Plant Physiologists, Rockville, pp. 131-134

Franceschi VR and Tarlyn MR (2002). L-ascorbic acid is accumulated in source leaf phloem and transported to sink tissues in plants. Plant Physiol. 130: 649-656

Gill DE, Chao L, Perkins SL and Wolf JB (1995). Genetic mosaicism in plants and clonal animals. Annu. Rev. Ecol. Evol. Syst. 26: 423-444

Gould KS (2004). Nature's Swiss army knife: the diverse protective roles of anthocyanins in leaves. J. Biomed. Biotechnol. 314-320

Grace S and Logan BA (2000). Energy dissipation and radical scavenging by the plant phenyl propanoid pathway. Philos. Trans. R. Soc. B Biol. Sci. 335: 1499-1510

Gross GG (1980). The biochemistry of lignification. Adv. Bot. Res. 8: 25-63

Grotewold E (ed.) (2006). The science of flavonoids. Springer Science and Business Media Inc, New York

Guyot S, Pellerin P, Brillouet JM, Cheynier V (1996). Inhibition of beta-glucosidase (*Amygdalae dulces*) by (+)-catechin oxidation products and procyanidin dimers. Biosci. Biotechnol. Biochem. 60: 1131-1135

Halliwell B and Gutteridge JMC (eds.) (1999). Free Radicals Biology and Medicine. Oxford University Press, NY

Hancock RD, McRae D, Haupt S and Viola R (2003). Synthesis of L-ascorbic acid in the phloem. BMC Plant Biol. 3: 7

Harman D (1991). The aging process: major risk factors for disease and death. Proc. Natl. Acad. Sci. USA 88: 5360-5363

Harman D (1981). The aging process. Proc. Natl. Acad. Sci. USA 78: 7123-7128

Hoagland DL and Arnon DI (1950). The water-cultured method for growing plants without soil. Calif. Agric. Exp. Sta. Circ. 347

Hodges DM, DeLong JM, Forney CF and Prange K (1999). Improving the thiobarbituric acid-reactive substances assay for estimating lipid peroxidation in plant tissues containing anthocyanidin and other interfering compounds. Planta 207: 604-611

Horemans N, Foyer CH and Asard H (2000). Transport and action of ascorbate at the plant plasma membrane. Trends Plant Sci. 5: 263-267

Horemans N, Asard H and Caubergs RJ (1994). The role of ascorbate free radical as an electron acceptor to cytochrome *b*-mediated trans-plasma membrane electron transport in higher plants. Plant Physiol. 104: 1455-1458

Huang AHC, Trelease RN and Moore Jr. TS (eds.) (1983). Plant peroxisomes. Academic Press, London

Hutzler P, Fischbach R, Heller W, Jungblut T, Reuber S, Schmitz R, Veit M, Weissenbock G and Schnitzler J (1998). Tissue localization of phenolic compounds in plants by confocal laser scanning microscopy. J. Exp. Bot. 49: 953-965

Inzé D and Van Montagu M (eds.) (2002). Oxidative stress in plants. Taylor and Francis, London and New York

Jeyaramraja PR, Pius PK, Kumar RR and Jayakumar D (2003). Soil moisture stress-induced alterations in bioconstituents determining tea quality. J. Sci. Food Agric. 83: 1187-1191

Kampfenkel K, Van Montagu M and Inzé D (1995). Extraction and determination of ascorbate and dehydroascorbate from plant tissue. Anal. Biochem. 225: 165-167

Kirakosyan A, Seymour E, Kaufman PB, Warber S, Bolling S and Chang SC (2003). Antioxidant capacity of polyphenolic extracts from leaves of *Crataegus laevigata* and *Crataegus monogyna* (Hawthorn) subjected to drought and cold stress. J. Agric. Food Chem. 51: 3973-3976

Koes R, Verweij W and Quattrochio F (2005). Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. Trends Plant Sci. 10: 236-242

Kondo K, Kurihara M, Miyata N, Suzuki T and Toyoda M (1998). Mechanistic studies of catechins as antioxidants against radical oxidation. Arch. Biochem. Biophys. 362: 79-86

Krishnamachari V, Levine L, Zhou CH and Paré PW (2004). *In vitro* flavon-3-ol oxidation mediated by a B ring hydroxylation pattern. Chem. Res. Toxicol. 17: 795-804

Kumazawa S, Kajiya K, Naito A, Saitô H, Tuzi S, Tanio M, Suzuki M, Nanjo F, Suzuki E and Nakayama T (2004). Direct evidence of interaction of a green tea polyphenol, epigallocatechin gallate, with lipid bilayers by solid-state nuclear magnetic resonance. Biosci. Biotechnol. Biochem. 68: 1743-1747

Labuda J, Buckova M, Heilerova L, Šilhár S and Štepánek I (2003). Evaluation of the redox properties and anti/pro-oxidant effects of selected flavonoids by means of a DNA-based electrochemical biosensor. Anal. Bioanal. Chem. 376: 168-173

Lamb C and Dixon RA (1997). The oxidative burst in plant disease resistance. Annu. Rev. Plant Physol. Plant Mol. Biol. 48: 251-275

Larcher W (ed.) (2003). Plants under stress. **In:** Physiological plant ecology, Springer, Heidelberg, pp 345-450

Lee DW, Brammeier S and Smithe AP (1987). The selective advantages of anthocyanins in developing leaves of mango and cacao. Biotropica 19: 40-49

Levine A, Tenhaken R and Dixon R (1994). H_2O_2 from the oxidtive burst orchestrates the plant hypersensitive disease resistance response. Cell 79: 583-593

Levitt J (1972). Stress and strain terminology. **In:** Levitt J (ed.). Responses of plants to environmental stresses. Academic Press, London, pp 1-8

Li J, Ou-Lee T, Raba R, Amudson RG and Last RL (1993). Arabidopsis flavonoid mutants are hypersensitive to UV-B irradiation. Plant Cell 5: 171-179

Lichtenthaler HK (1996). Vegetation stress: an introduction to the stress concept in plants. J. Plant Physiol. 148: 4-14

Lichtenthaler HK and Wellburn AR (1983). Determination of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Biochem. Soc. Trans. 11: 591-592

Lilley R, Fitzgerald MP, Rienits KG and Walker DA (1975). Criteria for intactness and the photosynthetic activity of spinach chloroplast preparations. New Phytol. 75: 1–10

Lindqvist I, Baden CI, Mathews FE and Lederer F (1991). Spinach gycolate oxidase and yeast flavocytochrome b2 are struturally homologous and evolutionarily related enzymes with distinctly different function and flavin mononucleotide binding. J. Biol. Chem. 266: 3198-3207

Loewus FA (1988). Ascorbic acid and its metabolic products. **In:** Preiss J (ed.). The biochemistry of plants: a comprehensive treatise, vol. 14. Academic Press, Inc. San Diego, CA, USA; London, England, UK

Loewus FA (1980). L-ascorbic acid: metabolism, biosynthesis, function. **In:** Preiss J (ed.). The biochemistry of plants, vol. 13. Academic Press, Inc. San Diego, CA, USA; London, England, UK, pp 77-79

López-Serrano M and Barceló RS (2002). Comparative study of the products of the preoxidase-catalyzed and the polyphenol oxidase-catalyzed (+)-catechin oxidation. Their possible implications in strawberry (*Fragaria x ananassa*) browning reactions. J. Agric. Food Chem. 50: 1218-1224

Mahalingam R and Fedoroff N (2003). Stress response, cell death signaling: the many faces of reactive oxygen species. Physiol. Plant. 119: 56-68

Markham KR and Marbry TJ (1975). Ultraviolet-visible and proton magnetic resonance spectroscopy of flavonoids. **In:** Harbone JB, Marbry TJ and Marbry H (eds.) The Flavonoids. Chapman and Hall Ltd., London

Marles MAS, Ray H and Gruber MY (2003). New perspectives on proanthocyanidin biochemistry and molecular regulation. Phytochemistry 64: 367-383

Marrs KA, Alfenito MR, Lloyd AM and Walbot V (1995) A glutathione S-transferase involved in vacuolar transfer encoded by the maize gene *Bronze-2*. Nature 375: 397-400

Martinoia E, Gril E, Tommasini R, Kreuz K and Amrhein N (1993). ATP-dependent glutathione *S*-conjugate 'export' pump in the vacuolar membrane of plants. Nature 364: 247-249

Mathesius U, Bayliss C, Weinmann JJ, Schlaman HRM, Spaink HP, Rolfe BG, McCully ME and Djordjevic MA (1998). Flavonoids synthesised in

cortical cells during nodule initiation are early developmental markers in white clover. Mol. Plant-Microbe Interact. 11: 1223-1232

Mayer AM and Harel E (1979). Polyphenol oxidases in plants. Phytochemistry 18: 193-215

Miller JN, Sampson J, Candeias LP, Bramley PM and Rice-Evans CA (1996). Antioxidant activities of carotenes and xantophylls. FEBS Lett. 384: 240-242

Mittler R (2002). Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7: 405-410

Mittler R, Vanderawera S, Gollery M and Van Breusegem F (2004). The reactive oxygen gene network of plants. Trends Plant Sci. 9: 490-498

Mizooku Y, Yoshikawa M, Tsuneyoshi T and Arakawa R (2003). Analysis of oxidized epigallocatechin gallate by liquid chromatography/mass spectrometry. Rapid Commun. Mass Spectrom. 17: 1915-1918

Møller IM, Jensen PE and Hansson A (2007). Oxidative modifications to cellular components in plants. Annu. Rev. Plant Biol. 58: 459-481

Moser O and Kanellis AK (1994). Ascorbate oxidase of *Cucumis melo* L. var. reticulatus: purification, characterization an antibody production. J. Exp. Bot. 45: 717-724

Moskowitz AH and Hrazdina G (1981). Vacuolar contents of fruit subepidermal cells from *Vitis* sp. Plant Physiol. 68: 686-692

Munné-Bosch S (2007). Aging in perennials. Crit Rev. Plant Sci. 26: 123-138

Munné-Bosch S and Lalueza P (2007). Age-related changes in oxidative stress markers and abscisic acid levels in a drought-tolerant shrub, *Cistus clusii* grown under Mediterranean field conditions. Planta 225: 1039-1049

Munné-Bosch S and Alegre L (2002). Plant aging increases oxidative stress in chloroplasts. Planta 214: 608-615

Munné-Bosch S and Alegre L (2001). Subcellular compartmentation of the diterpene carnosic acid and its derivatives in the leaves of rosemary. Plant Physiol. 125: 1094-1102

Nagai S, Phara K and Mukai K (2005). Kinetic study of the quenching reaction of singlet oxygen by flavonoids in ethanol solution. J. Phys. Chem. *B* 109: 4234-4240

Neill S, Desikan R and Hancock J (2002). Hydrogen peroxide signaling. Curr. Opin. Plant Biol. 5: 388-395

Nobel PS (1991). Physicochemical and Environmental Plant Physiology. Academic Press. San Diego

Noctor G and Foyer CH (1998). Ascorbate and glutathione: keeping active oxygen under control. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49: 249-279

Noodén LD and Guiamét JJ (1996). Genetic control of senescence and aging in plants. **In:** El Schneider and JW Rowe (eds.) Handbook of the biology of aging. Academic Press, San Diego, pp 94-118

Oelmüller R and Mohr H (1985). Mode of coaction between blue/UV light and light absorbed by phytochrome in light-mediated anthocyanin formation in milo (*Shorgum vulgare* Pers) seedling. Proc. Natl. Acad. Sci. USA. 82: 6124-6128

Overmyer K, Brosche M and Kangasjarvi J (2003). Reactive oxygen species and hormonal control of cell death. Trends Plant Sci. 8: 335-342

Peer WA, Brown DE, Tague BW, Muday GK, Taiz L and Murphy AS (2001). Flavonoid accumulation patterns of transparent testa mutants of Arabidopsis. Plant Physiol. 126: 536-548

Perl-Treves R and Perl A (2002). Oxidative stress, an introduction. **In:** Inzé and Van Montagu (eds.). Oxidative stress in plants. Taylor and Francis, London, pp: 1-32

Perrone ST, McDonald KL, Sutherland MW and Gues DI (2003). Superoxide release is necessary for phytoalexin accumulation in *Nicotiana tabacum* cells during the expansion of cultivar-race and non-host resistance towards *Phytophtora* spp. Physiol. Mol. Plant Pathol. 62: 127-135

Pignocchi C, Kiddle G, Hernández I, Foster S, Asensi A, Taybi T, Barnes JD and Foyer CH (2006). Ascorbate oxidase-dependent changes in the redox state of the apoplast modulate gene transcript accumulation leading to modified hormone signaling and orchestration of defense processes in tobacco. Plant Physiol. 141: 423-435

Pignocchi C and Foyer CH (2003). Apoplastic ascorbate metabolism and its role in the regulation of cell signaling. Curr. Opin. Plant Biol. 6: 379-389

Pignocchi C, Fletcher JM Wilkinson JE, Barnes JD and Foyer CH (2003). The function of ascorbate oxidase in tobacco. Plant Physiol. 132: 1631-1641

Poetsch I and Reznik H (1972). The flavonoids of the genus *Cistus*. I. The aglycones. Ber. Deutsch. Bot. Ges. 85: 209-218

Potapovich AI and Kostyuk VA (2003). Comparative study of antioxidant properties and cytoprotective activity of flavonoids. Biochemistry 68: 514-519

Pourcel L, Routaboul JM, Cheynier V, Lepiniec L and Debeaujon I (2007). Flavonoid oxidation in plants: from biochemical properties to physiological functions. Trends Plant Sci. 12: 29-36

Pugnaire FI and Lozano FJ (1997). Effect of soil disturbance, fire and litter accumulation on the establishment of *Cistus clusii* seedlings. Plant Ecol. 131: 207-213

Quirino BF, Noh Y, Himelblau E and Amasino RM (2000). Molecular aspects of leaf senescence. Trends Plant Sci. 5: 278-282

Rautenkranz AAF, Li L, Mächler F, Märtinoia E and Oertli JJ (1994). Transport of ascorbic and dehydroascorbic acids across protoplast and vacuole membranes isolated from barley (*Horeum vulgare* L. cv Gerbel) leaves. Plant Physiol. 106: 187-193

Revilla E and Ryan JM (2000). Analysis of several phenolic compounds with potential antioxidant properties in grape extracts and wines by high-performance liquid chromatography-photodiode array detection without sample preparation. J. Chromatogr. A 881: 461-489

Rice-Evans CA, Miller NJ and Paganga G (1997). Antioxidant properties of phenolic compounds. Trends Plant Sci. 2: 152-159

Rice-Evans C, Miller NJ, Bollwell PG, Bramley PM, Pridham JB (1995). The relative antioxidant activities of plant-derived polyphenolic flavonoids. Free Radic. Res. 22: 375-383

Rich PR and Bonner WD (1978). The sites of superoxide anion generation in higher plant mitochondria. Arch. Biochem. Biophys. 188: 206-213

Rietjens IMCM, Boersma MG, de Haan L, Spenkelink B, Awad HM, Cnubben NHP, van Zanden JJ, van der Woude H, Alink GM and Koeman JH (2002). The pro-oxidant chemistry of the natural antioxidants vitamin C, vitamin E, carotenoids and flavonoids. Environ. Toxicol. Pharmacol. 11: 321-333

Riha K, McKnight TD, Griffing LR and Shippen DE (2001). Living with genome instability: plant responses to telomere dysfunction. Science 291: 1797-1800

Ryan MG, Phillips N and Bond MJ (2006). The hydraulic limitation hypothesis revisited. Plant Cell Environ. 29: 367-381

Ryan KG, Swinny EE, Markham KR and Winefield C (2002). Flavonoid gene expression and UV photoprotection in transgenic and mutant *Petunia* leaves. Phytochemistry 59, 23-32

Ryan MG and Yoder BJ (1997). Hydraulic limits to tree height and tree growth. BioScience 47: 235-242

Saffari Y and Sadrazeh SMH (2004). Green tea metabolite EGCG protects membranes against oxidative damage *in vitro*. Life Sci. 74: 1513-1518

Salomonson A (1996). Interactions between somatic mutations and plant development. Vegetatio 127: 71-75

Sanmartín M, Drogoudi PD, Lyons T, Barnes JD, Kanellis AK (2003). Over-expression of ascorbate oxidase in the apoplast of transgenic tobacco results in altered ascorbate and glutathione redox states and increased sensitivity to ozone. Planta 216: 918-928

Saslowsky DE, Warek U and Winkel BSJ (2005). Nuclear localization of flavonoid enzymes in *Arabidopsis*. J. Biol. Chem. 280: 23735-23740

Sawai Y and Moon J (2000). NMR analytical approach to clarify the molecular mechanisms of the antioxidative and radical-scavenging activities of antioxidants in tea using 1,1-diphenyl-2-picrylhydrazyl. J. Agric. Food Chem. 48: 6247-6253

Sawai Y and Sakata K (1998). NMR analytical approach to clarify the antioxidative molecular mechanism of catechins using 1,1-diphenyl-2-picrylhydrazyl. J. Agric. Food Chem. 46: 111-114

Schmutz A, Buchala A, Jenny T, Ryser U (1994). The phenols in the wax and in the suberin of green cotton fibres and their function. Acta Hortic. 381: 269-75

Schnabel H, Weissenböck G, Scharf H (1986). *In vivo* microspectrophotometric characterisation of flavonoid glycosides in *Vicia faba* guard and epidermal cells. J. Exp. Bot. 37: 61-72

Schnitzler JP, Jungblut TP, Heller W, Hutzler P, Heinzmann U, Schmelzer E, Ernst D, Langebartels C and Sandermann H (1996). Tissue localization of UV-B screening pigments and chalcone synthase mRNA in Scots pine (*Pinus sylvestris* L.) needles. New Phytol. 132: 247-258

Schulze ED and Cadwell MM (1995). **In:** Ecophysiology of photosynthesis, Springer, Heidelberg

Schützendübel A, Schwanz P, Teichmann T, Gross K, Langenfeld-Heyser R, Godbold DL and Polle A (2001). Cadmium-induced changes in antioxidative systems, hydrogen peroxide content, and differentiation in scots pine roots. Plant Physiol. 127: 887-898

Shirley BSJ (2006). The biosynthesis of flavonoids. **In:** Grotewold E (ed.). The science of flavonoids. Springer Science and Business Media Inc, New York

Shirley BW (2001). Flavonoid biosynthesis. A colourful model for genetics, biochemistry, cell biology and biotechnology. Plant Physiol. 126: 485-493

Shirley BW (1996). Flavonoid biosynthesis: 'new' functions for an 'old' pathway. Trends Plant Sci. 1: 377-382

Siendones E, González-Reyes JA, Santos-Ocaña C, Navas P and Córdoba P (1999). Biosynthesis of ascorbic acid in kidney bean. L-galacto-γ-lactone dehydrogenase is an intrinsic protein located at the mitochondrial inner membrane. Plant Physiol. 120: 907-912

Singh KK (2000). The *Saccharomyces cerevisiae* SLN1P-SSK1P two-component system mediated response to oxidative stress and in an oxidant-specific fashion. Free Radic. Biol. Med. 29: 1043-1050

Smart CM (1994). Gene expression during leaf senescence. New Phytol. 125: 27-58

Smirnoff N (ed.) (2006). Antioxidants and reactive oxygen species in plants. Blackwell Publising, Oxford

Smirnoff N and Wheeler GL (2000). Ascorbic acid in plants: biosynthesis and function. Crit. Rev. Plant Sci. 19: 267-290

Smirnoff N (1996). The function and metabolism of ascorbic acid in plants. Ann. Bot. 78: 661-669

Smirnoff N and Pallanca JE (1996). Ascorbate metabolism in relation to oxidative stress. Biochem. Soc. Trans. 24: 472-478

Squire GR and Callander B (1981). Tea plantations. **In:** Kozlowski (ed.). Water deficits and plant growth vol. VI. Academic Pres, London, pp 471-510

Stafford HA (1974). Possible multi-enzyme complexes regulating the formation of C_6-C_3 phenolic compounds and lignins in higher plants. Rec. Adv. Phytochem. 8: 53-79

Stapleton AE and Walbot V (1994). Flavonoids can protect maize DNA from the induction of ultraviolet radiation damage. Plant Physiol. 105: 881-889

Strack D, Heiemann J and Klinkott JS (1988). Cell wall-bound phenolics from spruce needles. Z. Naturforsch. 43c: 37-41

Subramanian N, Venkatesh P, Ganguli S and Sinkar VP (1999). Role of polyphenol oxidase and peroxidase in the generation of black tea theaflavins. J. Agric. Food Chem. 47: 2571–2578

Svehlikova V, Mráz P, Piacente S and Marhold K (2002). Chemotaxonomic significance of flavonoids and phenolic acids in the *Hieracium rohacsense* group (*Hieracium* sect. *Alpina*; *Lactuceae*, *Compositae*). Biochem. Syst. Ecol. 30: 1037-1049

Takahama U and Oniki T (1992). Regulation of peroxidase-dependent oxidation of phenolics in the apoplast of spinach leaves by ascorbate. Plant Cell Physiol. 33: 379-387

Takahashi A, Takeda K and Ohnishi T (1991). Light-induced anthocyanin reduces the extent of damage to DNA in UV-irradiated *Centaurea cyanus* cells in culture. Plant Cell Physiol. 32: 541-548

Tanaka T, Mine C, Inoue K, Matsuda M and Kouno I (2002). Synthesis of theaflavin from epicatechin and epigallocatechin by plant homogenates and role of epicatechin quinone in the synthesis and degradation of theaflavin. J. Agric. Food Chem. 50: 2142-2148

Tattini M, Galardi C, Pinelli P, Massai R, Remorini D and Agati G (2004). Differential accumulation of flavonoids and hydroxycinnamates in leaves of *Ligustrum vulgare* under excess light and drought stress. New Phytol. 163: 547-561

Tattini M, Gravano E, Pinelli P, Mulinacci N and Romani A (2000). Flavonoids accumulate in leaves and glandular trichomes in *Phillyrea latifolia* exposed to solar irradiation. New Phytol. 148: 69-77

Terao J, Piskuli M and Yao Q (1994). Protective effect of epicatechin, epicatechin gallate and quercetin on lipid peroxidation in phospholipid bilayers. Arch. Biochem. Biophys. 308: 278-284

Torres MA and Dangl JL (2005). Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. Curr. Opin. Plant Biol. 8: 1-7

Valant-Vetschera KM, Roitman JN and Wollenweber E (2003). Chemodiversity of exudate flavonoids in some members of the Lamiaceae. Biochem. Syst. Ecol. 31: 1247-1255

Valcic S, Burr JA, Timmermann BN and Liebler DC (2000). Antioxidant chemistry of green tea catechins. New oxidation products of (-)-

epigallocatechin galate and (-)-epigallocatechin from their reactions with peroxyl radicals. Chem. Res. Toxicol. 13: 801-810

Valcic A, Muders A, Jacobsen NE, Liebler DC and Timmermann BN (1999). Antioxidant chemistry of green tea catechins. Identification of products of the reaction of (-)-epigallocatechin gallate with peroxyl groups. Chem. Res. Toxicol. 12: 382-386

Vianello A and Macri F (1991). Generation of superoxide anion and hydrogen peroxide at the surfce of plant cells. J. Bioenerg. Biomembr. 23: 409-423

Vieira RF, Grayer RJ and Paton AJ (2003). Chemical profiling of *Ocimum americanum* using external flavonoids. Phytochemistry 63: 555-567

Vivas de Gaulejac N, Vivas N, Nonier M, Absalon C and Bourgeois G (2001). Study and quantification of monomeric flavan-3-ol and dimeric procyanidin quinonic forms by HPLC/ESI-MS. Application to red wine oxidation. J. Sci. Food Agric. 81: 1172-1179

Vogt T, Proksch P and Gülz P (1987). Epicuticular flavonoid aglycones in the genus *Cistus*, *Cistaceae*. J. Plant Physiol. 131: 25-36

Vranová E, Inzé D and Van Breusegem F (2002). Signal tranduction during oxidative stress. J. Exp. Bot. 53: 1227-1236

Walbot V (1996). Sources and consequences of phenotypic and genotypic plasticity in flowering plants. Trends Plant Sci. 1: 27-32

Wells TC and Bohm BA (1988). Flavonoids of *Luetkea pectinata* (*Rosaceae*: *Spiraeoideae*). Biochem. Syst. Ecol. 16: 479-484

Werner C, Correia O and Beyschlag W (2002). Characteristic patterns of chronic and dynamic photoinhibition of different functional groups in a Mediterranean ecosystem Funct. Plant Biol. 29: 999-1011

Wheeler GJ, Jones MA and Smirnoff N (1998). The biosynthetic pathway of vitamin C in higher plants. Nature 393: 365-369

Wollenweber E, Stevens JF, Dörr M, and Rozefelds AC (2003).Taxonomic significance of flavonoid variation in temperate species of *Nothofagus***.** Phytochemistry 62: 1125-1131

Wollenweber E, Stevens JF, Ivanic M, Deinzer ML (1998). Acylphloroglucinols and flavonoid aglycones produced by external glands on the leaves of two *Dryopteris* ferns and *Currania robertiana*. Phytochemistry 48: 931-939

Xie DY, Sharma SB, Paiva NL, Ferreira D and Dixon RA (2003). Role of anthocyanidin reductase, encoded by *BANYULS* in plant flavonoid biosynthesis. Science 299: 396-399

Yamasaki H, Sakihama Y and Ikehara N (1997). Flavonoid-peroxidase reaction as a detoxification mechanism of plant cells against H_2O_2. Plant Physiol. 115: 1405-1412

Yusuf-Umi K, Abdullah N, Bakar B, Itam K, Abdullah F and Sukari MA (2003). Flavonoid glycosides in the leaves of *Mimosa* species. Biochem. Syst. Ecol. 31: 443-445