CREAF

Universitat Autònoma de Barcelona

Departament de Biologia Animal,
de Biologia Vegetal i d'Ecologia
Unitat d'Ecologia

Changes in terpene production and emission in response to climate change and eutrophication

Canvis en la producció i emissió de terpens en resposta al canvi climàtic i a l'eutrofització

Ph.D. Thesis
Tesi Doctoral

Josep-Salvador Blanch i Roure

Bellaterra, juny de 2010

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Memòria presentada per:

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per optar al grau de Doctor

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A tots els que heu estat al meu costat quan calia

Lost in thought and lost in time
While the seeds of life and the seeds of change were planted
Outside the rain fell dark and slow
While I pondered on this dangerous but irresistible pastime
I took a heavenly ride through our silence
I knew the moment had arrived
For killing the past and coming back to life

(David Gilmour, Coming back to life)

Croyez ceux qui cherchent la vérité, doutez de ceux qui la trouvent

(André Gide , Journal 1889-1939)

Agraïments

Caminante no hay camino, se hace camino al andar
(Antonio Machado)

Aquesta tesi és el resultat d'un llarg camí que vaig començar ara fa uns anys, i mentre l'he anat recorrent, m' he trobat amb molta gent que ha influït d'una manera més o menys directa sobre el resultat final, que està plasmat en aquestes fulles. Vull agrair doncs a totes les persones que m'heu ajudat durant aquest temps tant laboral com personalment.

En primer lloc, agraeixo al meu director de tesi, en Josep Peñuelas, per haver confiat en mi, i per tot l'ajut prestat al llarg de la tesi. També agraeixo al meu codirector, en Joan Llusià, qui m'ha ensenyat tot el que sé de laboratori, per la seva paciència –que és molta- i amistat. Com no, mai estaré prou agraït amb la Sue Owen, treballar amb ella va ser una experiència summament enriquidora, i a més, em va obrir les portes de casa seva durant la meva estada a Edinburgh, fent que no em sentís tan lluny dels meus. També dono les gràcies a en Ulo Niinemets i a en Steffen Noe, per la seva ajuda i atencions durant la meva estada a Estònia, i a en Luis Sampedro i a en Xoaquin Moreira pel mateix motiu durant l'estada a Pontevedra. Vull agrair també a en Jordi Sardans i a en Sergi Munné el seu ajut prestat per fer anàlisis diverses de laboratori. Finalment, a tot el grup Peñuelas en general, perquè sempre estan disposats a ajudar: he aprés molt treballant al seu costat.

Durant aquest temps, la meva segona casa ha estat el CREAF. Agraeixo als companys del despatx, des de la Maria, Edu i Marc fins als membres "frikydespatx": Nacho, Jara, Núria i membres itinerants de la cinquena taula (Míriam-chocho *et al*) per tots aquests anys cohabitant a la masmorra? Sense vosaltres no hauria estat el mateix. Això es fa extensible a "los vecinos de al lado", Roger, Maiky..., i com no a la Loles, miembro anexo del despacho(*). No m'oblido tampoc dels que ja no hi son, Rosie, Imma, Laia, Patri, Giorgio..., per les giraldillas, sopars, cafès, i més.

(*) La Loles es mereix un apartat especial, per tot el que m'ha ajudat a tots els nivells, començant perquè també va fer VOCs, i gràcies a això, hem pogut compartir patrons al cromatògraf, integrar pics, resultats ... A més, i entre d'altres moltíssimes coses, vam tenir un grup de rock? amb en Nacho (Lo vuestro!), hem anat de congressos junt amb en Roger (qui sempre aconsegueix el pressupost mínim!) i ens hem fet un tip de mirar pelis de dubtós valor cinèfil junt amb la Jaripeig (mis niñas!)...

El camí que he fet durant aquesta tesi, com a camí que és, té un inici i té un final, i tampoc cal oblidar-los. Així doncs, de la part del començament vull agrair a tota la gent de Lleida, amb una menció especial a en Joan Ramon, i com no, a la "*Secta Leridae*" (o sigui: Z, O, X, G, B, J, L), i de la part del final a tota la gent del Servei de Gestió Forestal, que m'han acollit molt bé en aquesta nova etapa (tot i les sinceritats extremes d'en Joaquim). A tots vosaltres, gràcies pels ànims i el suport en el dia a dia.

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How I wish, how I wish you were here.
We're just two lost souls swimming in a fish bowl, year after year
(David Gilmour)

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Chapter 3.2

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Chapter 4.2

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Our research on volatile compound emissions has been supported by the funding from the European Science Foundation (Programme VOCBAS), the European Commission Marie Curie research training network ISONET (MC-RTN-CT-2003 504720), the Spanish Government (grants CGL2006-04025/BOS and Consolider-Ingenio Montes CSD2008-0040), and the Catalan Government (grant SGR2009/458).

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Chapter 1. General introduction

Introducció general

1.1. Volatile Organic Compounds

Compostos Orgànics Volàtils

The air that we breathe is a mixture of gases sustained by the gravity which envelops the Earth creating the atmosphere. This air is composed by a combination of nitrogen (78%), oxygen (21%), water vapor (variable between 0-7%) and other substances (1%) such as ozone, carbon dioxide, hydrogen and other noble gases like krypton and argon. The atmosphere is a dynamic layer that continuously interacts with the human beings: all the living elements presents in the surface of the Earth, including plants and animals, interchange gases with the atmosphere by processes like respiration (in animals and plants) and photosynthesis (in plants) originating the "Carbon cycle" (*Fig.1*).

Volatile Organic Compounds (VOCs) belong to the 1% of other substances which constitute the atmosphere, and they come from gaseous interchanges of animals and plants. Their principal characteristic is their volatility: once they are released in the atmosphere, they have a short life from minutes to hours or days depending on the compound and on the atmospheric composition (*Table 1*).

L'aire que respirem és una barreja de gasos que estan sostinguts per la gravetat, i que envolten la Terra formant l'atmosfera. Aquest aire està compost per una combinació de nitrogen (78%), oxigen (21%), vapor d'aigua (variable entre 0 i 7%) i altres substàncies (1%) com per exemple ozó, diòxid de carboni, hidrogen i altres gasos nobles com el criptó i l'argó. L'atmosfera és una capa dinàmica que interactua contínuament amb els elements vius: tots els elements vius presents a la superfície de la Terra, incloent-hi animals i plantes, intercanvien gasos amb l'atmosfera per processos com ara la respiració (en animals i plantes) i la fotosíntesi (en plantes) originant el cicle del Carboni *(Fig.1)*.

Els Compostos Orgànics Volàtils (COVs) pertanyen a aquest 1% d'altres substàncies que formen l'atmosfera, i que provenen d'intercanvis de gasos entre animals i plantes. La seva principal característica és la seva volatilitat: un cop són alliberats a l'atmosfera, tenen un període de vida curt que va des de minuts fins a hores o dies en depenent del compost i de la composició atmosfèrica (*Taula 1*).

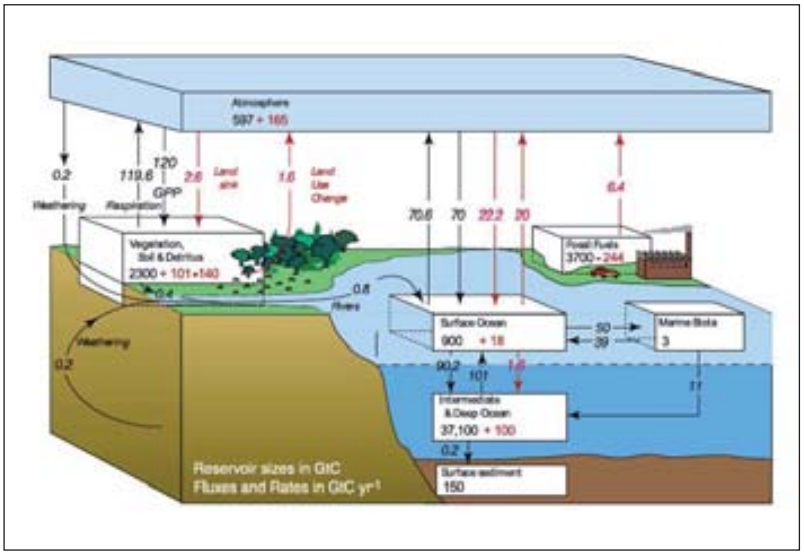


Fig.1 - The Global Carbon cycle
El Cicle Global del Carboni
(Font: IPCC 2007)

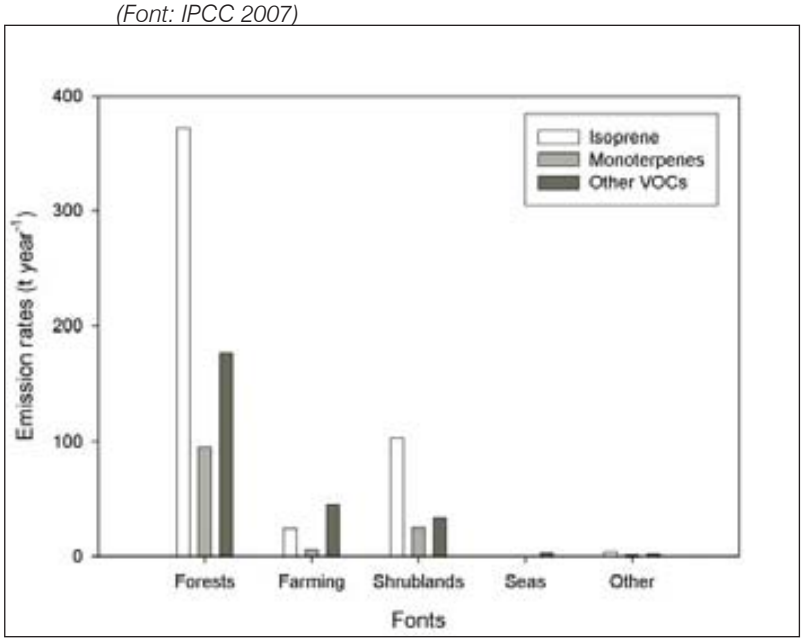


Fig.2 - Estimation of BVOC global emission per font and type of compound
Estimació de l'emissió global de COVBs per font i tipus de compost
(Font: Guenther et al 1995)

Table 1 - Mean estimated life for the main monoterpenes present in the troposphere due to their reaction with different radicals
Taula 1 - Vida mitjana estimada pels principals monoterpens presents a la troposfera degut a la seva reacció amb diferents radicals

(*Font: Seinfeld and Pandis 1998*)

Compound	OH^-	O_3	NO_3^-
Isoprene	1.7 h	1.3 d	0.8 h
α-pinene	3.4 h	4.6 h	2 h
β-pinene	2.3 h	1.1 d	4.9 h
Camphene	3.5 h	18 d	1.5 d
2-carene	2.3 h	1.7 h	36 min
3-carene	2.1 h	10 h	1.1 h
Limonene	1.1 h	1.9 h	53 min
Terpinolene	49 min	17 min	7 min

VOC may have either antropic or biotic origin. Antropic VOC sources come mainly from industrial activities such as painting, shoe or iron and steel industries, evaporation of organic solvents, cars or even smoke from cigarettes. These sources have historically been the ones that have worried us, and have been the center of most studies, leaving apart other possible natural sources. However, there are big amounts coming from natural sources, such as vegetation, oceans and superficial continental water, soils, sediments, microbial decomposition of organic matter, geological pools of hydrocarbons and volcanoes (Fehsenfeld *et al* 1992). In 1960, Went suggested that the "blue hazes" which appear over forests in summer may be the result of photochemical reactions from biogenic VOCs. Moreover, he made the first estimation of biogenic VOCs (BVOCs), which was of 175 Tg year^{-1}. Later on, Guenther *et al* (1995) made an estimation of BVOC in a global level of 1150 Tg year^{-1} (*Fig.2*). Forests contribute with more than 70% of the emission from all those sources.

BVOCs include different families of compounds: isoprenoids, alkanes, alkenes, alcohols, esters, carbonils and acids (Kesselmeier and Staudt 1999). Isoprenoids constitute a heterogenic group of products, which have very different structures and functions. Some plants, especially the ones belonging to the families Coniferae, Laminaceae, Labiatae, Compositae and Rutaceae are capable of synthesizing big amounts of isoprenoids. Chapman and Hall (1996) described more than 29,000 compounds with isoprenoid character.

All the isoprenoids are formed by a basic structural unit, isoprene (C_5H_8, *Table 1*), which repeats itself. Thus, is possible to classify isoprenoids depending on the number of Carbons that they have: hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}), tetraterpenes (C_{40}), and politerpenes ($>C_{40}$).

Isoprenoids can also be classified into primary and secondary metabolites: primary metabolites are the ones indispensables for plant growing and developing while secondary metabolites are not indispensables for the plant's live, but they are also important for the plant's response to the environment (Newman

Els COVs poden tenir tan origen antròpic com biòtic. Les fonts d'origen antròpic dels COVs provenen principalment d'activitats industrials, com l'industria de la pintura, del calçat o la del ferro i acer; d'evaporació de dissolvents orgànics, dels cotxes o fins i tot del fum de les cigarretes. Aquestes fonts han estat les que històricament més ens han preocupat, i han estat el centre d'atenció de la majoria dels estudis, deixant de banda altres possibles fonts, com les d'origen natural. No obstant, hi ha una gran quantitat de compostos provinents de fonts naturals, com la vegetació, oceans i aigües superficials continentals, sòl, sediments, descomposició microbiana de la matèria orgànica, contenidors geològics d'hidrocarburs i volcans (Fehsenfeld *et al* 1992). A l'any 1960 Went va suggerir que les "boirines blaves" que apareixen sobre els boscos a l'estiu són el resultat de diverses reaccions fotoquímiques dels COVs biogènics. A més, va fer la primera estimació de COVs biogènics (COVBs), que va ser de 175 Tg any^{-1}. Més tard, Guenther *et al* (1995) van fer una estimació de COVBs a nivell global de 1,150 Tg any^{-1} (*Fig.2*). Els boscos contribueixen amb més d'un 70% de l'emissió d'entre totes les fonts.

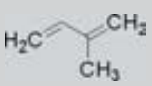
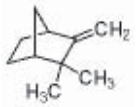
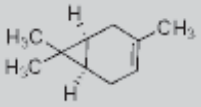
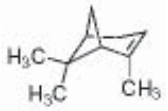
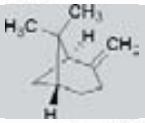
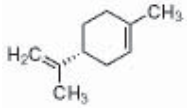
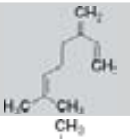
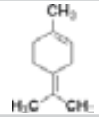
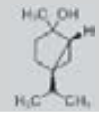
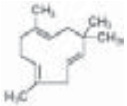
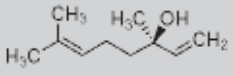
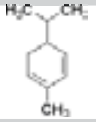
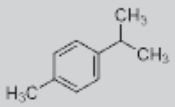
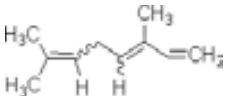
Els COVBs inclouen diferents famílies de compostos: isoprenoides, alcans, alquens, alcohols, èsters, carbonils i àcids (Kesselmeier i Staudt 1999). Els isoprenoides constitueixen un grup molt heterogeni de productes, que tenen estructures i funcions molt diferents entre ells. Algunes plantes, especialment les pertanyents a les famílies coníferes, laminàcies, labiades, compostes i rutàcies tenen la capacitat de sintetitzar grans quantitats d'isoprenoides. Chapman i Hall (1996) van descriure més de 29,000 compostos amb caràcter isoprenoide.

Tots els isoprenoides estan formats per una unitat estructural bàsica, l'isoprè (C_5H_8, *Taula 1*), que es va repetint. Per consegüent, és possible classificar els isoprenoides en funció del número de Carbons que tinguin. D'aquesta manera tenim: hemiterpens (C_5), monoterpens (C_{10}), sesquiterpens (C_{15}), diterpens (C_{20}), triterpens (C_{30}), tetraterpens (C_{40}) i politerpens ($>C_{40}$).

Els isoprenoides també es poden classificar en metabòlits primaris i secundaris: els metabòlits primaris són aquells indispensables per al creixement i desenvolupament de la planta, mentre que els metabòlits secundaris no són

Table 2 - Non-methane organic compounds emitted by vegetation, along with their molecular weight, Bowling point and Chemicals structure. (From Asensio 2007)

Taula 2 - Compostos orgànics no-metànics emesos per la vegetació, junt amb el seu pes molecular, punt d'ebullició i estructura química (de Asensio 2007)

Compound name	Chemical formula	Molecular weight (g mol^{-1})	Boiling point (K)	Chemical structure
Isoprene	C_5H_8	68.12	307	
Camphene	$C_{10}H_{16}$	136.24	320	
Δ^3-Carene	$C_{10}H_{16}$	136.24	441	
α-Pinene	$C_{10}H_{16}$	136.24	428	
β-Pinene	$C_{10}H_{16}$	136.24	436	
Limonene	$C_{10}H_{16}$	136.24	448	
Myrcene	$C_{10}H_{16}$	136.24	440	
Terpineole	$C_{10}H_{16}$	136.24	459	
Sabinene	$C_{10}H_{16}$	136.24	437	
α-Humulene	$C_{15}H_{24}$	204.35	396	
Linalool	$C_{10}H_{16}O$	154.25	469	
α–Phellandrene	$C_{10}H_{16}$	136.24	446	
p-Cymene	$C_{10}H_{14}$	134.22	450	
Ocimene	$C_{10}H_{16}$	136.24	373	

and Chappel 1999). Primary metabolites include sterols, carotenoids and diverse hormones, and secondary metabolites include monoterpenes, sesquiterpenes and diterpenes. A list of the main principal terpenes along with their main physical and structural characteristic is shown on *Table 2*.

indispensables per la vida de la planta tot i que són importants per la resposta de la planta al medi (Newman i Chappel 1999). Els metabòlits primaris inclouen esterols, carotenoids i diverses hormones, i els metabòlits secundaris inclouen monoterpens, sesquiterpens i diterpens. A la *Taula 2* es mostra un llistat dels principals terpens junt amb les seves principals característiques físiques i estructurals.

The present PhD dissertation is focused in the study of mono and sesquiterpenes, commonly called "volatile terpenes", including their formation inside the plant and their emission to the air.

Aquesta tesi doctoral està centrada en l'estudi dels mono i sesquiterpens, correntment anomenats "terpens volàtils", incloent des de la seva formació a l'interior de la planta fins a la seva emissió a l'aire.

VOCs environmental aspects

VOCs take part in the atmospheric chemistry and in different processes related to climatology, which are summarized following (Sabillon 2001):

- The existence of VOCs in a Nitrogen oxides (NO_x) high-concentrated atmosphere facilitates the formation of tropospheric ozone.
- VOCs control the concentration of hydroxyl ions (OH^-) in the atmosphere. The terpene photooxidation could be the main source of tropospheric CO which influences the OH^- concentration in the atmosphere.
- VOCs are related to the formation of organic nitrates. They can react with NO_x and transport them to long distances.
- The oxidation of some biogenic hydrocarbons can originate organic aerosols which are the responsible of the formation of smog.
- Monoterpenes can react with water vapor present in the air and create soft organic acids, which can be deposited with the rain and affect the soil chemistry.

Aspectes mediambientals dels COVs

Els COVs prenen part en la química de l'atmosfera i en diferents processos relacionats amb la climatologia, com són: (Sabillon 2001):

- L'existència de COVs en una atmosfera amb alta concentració d'òxids de nitrogen (NO_X) facilita la formació d'ozó troposfèric.
- Els COVs controlen la concentració d'ions hidroxil (OH^-) a l'atmosfera. La fotooxidació dels terpens podria ser la font principal de CO troposfèric que influència la concentració de OH^- a l'atmosfera.
- Els COVs estan lligats a la formació de nitrats orgànics. Poden reaccionar amb els NO_x i transportar-los a llargues distàncies.
- L'oxidació d'alguns hidrocarburs biogènics pot originar aerosols orgànics que són els responsables de la formació de l'smog fotoquímic.
- Els monoterpens poden reaccionar amb el vapor d'aigua present a l'aire i crear àcids orgànics dèbils, que es poden dipositar amb la pluja i afectar a la química del sòl.

VOCs biological aspects

The reason why plants produce and emit VOCs is still uncertain. Many hypotheses have been proposed, such as:

- Plant physiology: the production of isoprenoids is supposed to provide a protection of possible damages on the cell membranes. Those damages could be caused by extreme conditions such as drought stress, high temperatures, oxidative stress or high irradiation (Sharkey and Singsaas 1995, Loreto and Velikova 2001, Peñuelas and Llusià 2003).
- Ecological interactions: different studies have proposed hypotheses about the function of those compounds in the interaction between the different agents of the trophic chain.
- Attract pollinators and predators of its herbivores (Croteau 1987, Pichersky and Gershenzon 2002, Peñuelas and Llusià 2004, Moreira *et al* 2008)
- Communication between plants (Peñuelas *et al* 1995, Shulaev 1997)
- Disturbance of the flowering period in neighbour plants (Terry *et al* 1995)
- Importance on forest fires. Some VOCs (including monoterpenes) have been detected durinig wood combustion (Ciccoli *et al* 2001, Alessio *et al* 2004).

Aspectes biològics dels COVs

La raó per la qual les plantes produeixen i emeten COVs no està del tot clara. Tot i això, s'han proposat diverses hipòtesis, que són:

- Fisiologia de la planta: la producció d'isoprenoides suposadament aporta protecció en front a possibles danys a les membranes cel·lulars. Aquests danys podrien ser causats per condicions extremes com ara estrès hídric, altes temperatures, estrès oxidatiu o alta irradiació (Sharkey i Singsaas 1995, Loreto i Velikova 2001, Peñuelas i Llusià 2003).
- Interaccions ecològiques: diversos estudis han proposat hipòtesis sobre la funció d'aquests compostos en la interacció entre diferents agents biològics de la cadena tròfica.
- Atracció de pol·linitzadors i predadors dels seus herbívors (Croteau 1987, Pichersky i Gershenzon 2002, Peñuelas i Llusià 2004, Moreira *et al* 2008)
- Comunicació entre plantes (Peñuelas *et al* 1995, Shulaev 1997)
- Alteració del període de floració de plantes veïnes (Terry *et al* 1995)
- Importància dins dels incendis forestals. S'han detectat alguns COVs (incloent-hi els monoterpens) durant la combustió de la fusta (Ciccoli *et al* 2001, Alessio *et al* 2004).

1.2. Factors which determine terpene synthesis and emission

Factors que determinen la síntesi i emissió de terpens

Terpene biosynthesis cycle

The biosynthesis of terpenes is divided in 3 steps (*Fig.3*): first step consists of biosynthesis of isopentenil biphosphate (IPP), which is the basic structure of the terpene composition. IPP has two different formation pathways: classic pathway, also called mevalonic acid pathway (MVA), located in the citosol and in the endoplasmatic reticle, and the alternative Rohmer pathway, also called methileritritophosphate pathway (MEP) or 1-deoxi-D-xylusole pathway (DOX), located in the chloroplast (Kreuzwieser et al 1999, Owen and Peñuelas 2005).

Second step consists of the biosynthesis of prenilphosphates of different lengths. They will be the starting point of different ramifications that will conduct to the synthesis of isoprenoids. The 3 prenilphosphates that are synthesized are: geranildiphosphate

El cicle de la biosíntesi dels terpens

La biosíntesi dels terpens es divideix en 3 etapes (*Fig.3*): la primera etapa consisteix en la formació de isopentenil bifosfat (IPP), què és l'estructura bàsica de la composició dels terpens. L'IPP té dos vies de formació: la via clàssica, o via del àcid mevalònic (MVA), localitzada al citosol i al reticle endoplasmàtic, i la via alternativa de Rohmer, també anomenada via del metileritritofosfat (MEP) o via de 1-deoxi-D-xilulosa (DOX) què està localitzada al cloroplast (Kreuzwieser et al 1999, Owen i Peñuelas 2005).

La segona etapa consisteix en la biosíntesi de prenilfosfats de diferents llargades. Aquests, seran el punt d'origen de diferents ramificacions que portaran a la síntesi d'isoprenoides. Els tres prenilfosfats que es sintetitzen són: geranildifosfat (GPP, C_{10}), farnersildifosfat

(GPP, C_{10}), farnesildiphosphate (FPP, C_{15}) and geranilgeranildiphosphate (GGPP, C_{20}).

Third step consists of the synthesis of diverse compounds: monoterpenes are synthesized from GPP; sesquiterpenes, esterols, brasinoesteroids, ubiquinone, dolicol and prenilated proteins are synthesized from FPP; and carotenes, chlorophylls, plastoquinone,

(GPP, C_{15}) i geranilgeranildifosfat (GGPP, C_{20}).

La tercera etapa consisteix en la síntesi de diversos compostos: a partir del GPP es sintetitzen els monoterpens, a partir del FPP es sintetitzen els sesquiterpens, esterols, brasinoesteroides, ubiquinona, dolicol i les proteïnes prenilades, i a partir del GGPP es sintetitzen els carotens, clorofil·les,

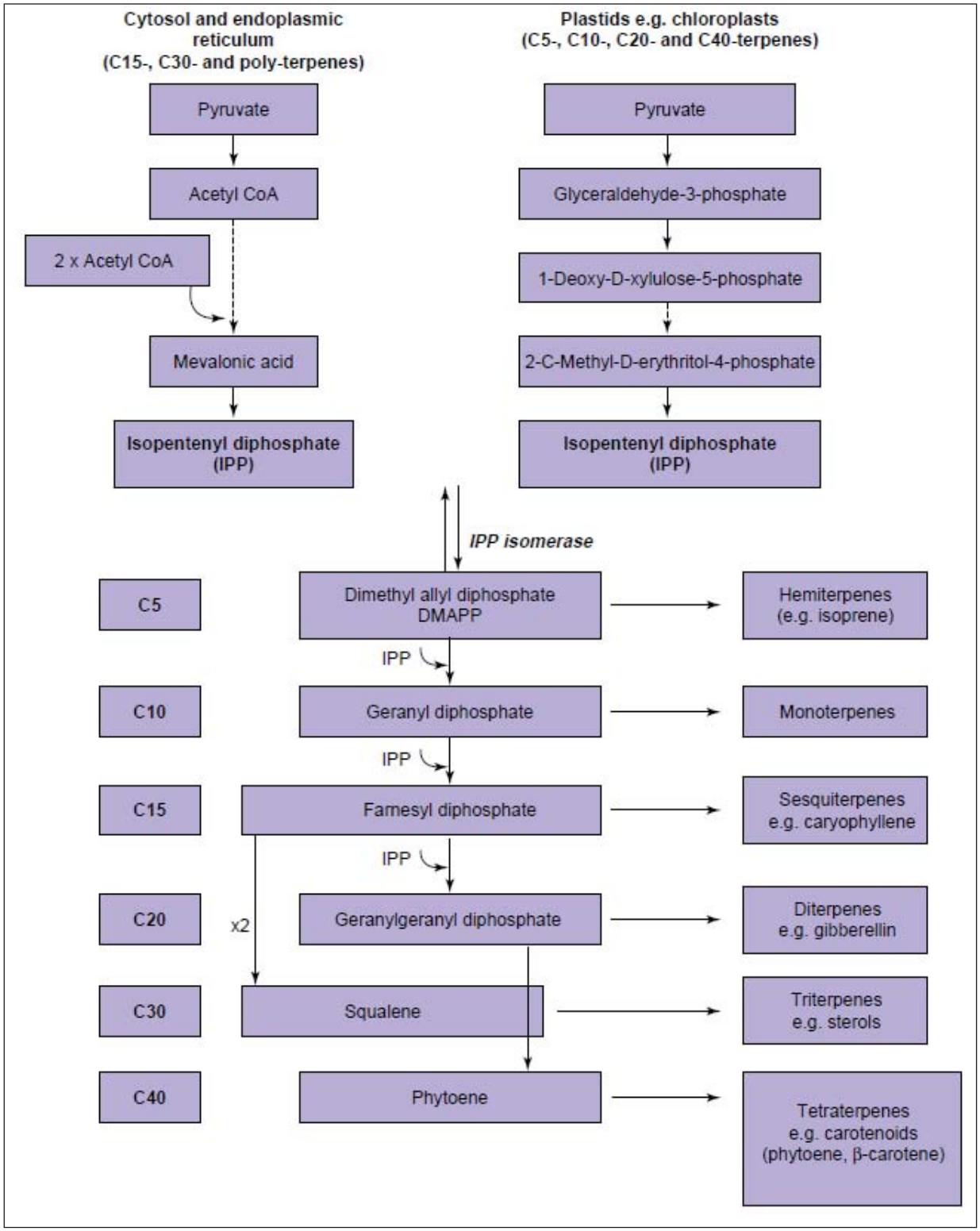


Fig.3 - BVOC biosynthesis
Biosíntesi dels BVOCs
(Font: Owen and Peñuelas 2005)

giberelins and other prenilated proteins are synthesised from GGPP (Lichtenthaler *et al* 1997, Sharkey *et al* 2001).

Terpene storage in plants: storing species vs non-storing species

Storing species present specialized organs located inside or outside the leaves, where they can accumulate the terpenes once they have been produced. Some examples are the resin ducts of the pines, the resin exudates of firs, the glandules in the trichomes of the mints or the storing cavities of the eucalypts (Gershenzon and Croteau 1991). Some examples of plants with storing organs are the conifers, *Cistus albidus* L. or *Bupleurum fruticosum* L. (Loreto *et al* 1996, Llusià and Peñuelas 2000).

plastoquinona, giberelines i d'altres proteïnes prenilades (Lichtenthaler *et al* 1997, Sharkey *et al* 2001).

Emmagatzematge de terpens a les plantes: espècies acumuladores vs no acumuladores

Les espècies acumuladores tenen òrgans especialitzats localitzats dins o fora de les seves fulles, on hi poden emmagatzemar els terpens un cop produïts. Alguns exemples d'organs d'emmagatzematge són els conductes resinífers dels pins, els exsudats de resina dels avets, les glàndules als tricomes de les mentes, o les cavitats de magatzem dels eucaliptus (Gershenzon i Croteau 1991). Alguns exemples de plantes acumuladores són les coníferes, *Cistus albidus* L. o bé *Bupleurum fruticosum* L. (Loreto *et al* 1996, Llusià i Peñuelas 2000)

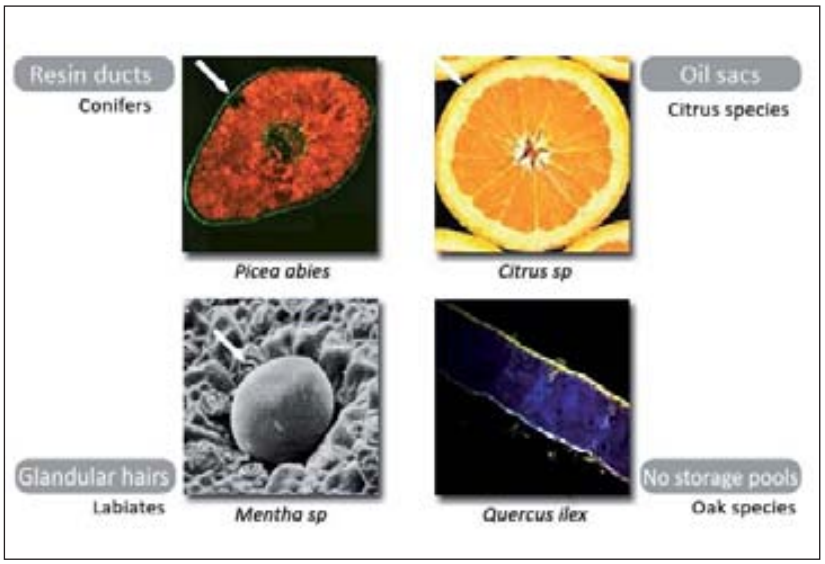


Fig.4 - Storing organs on different species
Òrgans d'emmagatzematge algunes espècies
(Font: Lange and Croteau 1999)

On the contrary, other species do not have that kind of storing organs; those plants emit the terpenes once they have produced them. Some examples of non-storing species are *Quercus ilex* L., *Quercus coccifera* L., *Arbutus unedo* L. and *Erica arborea* L (Llusià and Peñuelas 2000).

Per contra, hi ha espècies que no tenen aquests òrgans d'emmagatzematge; aquestes plantes emeten els terpens un cop els han produït. Alguns exemples de plantes no acumuladores són *Quercus ilex* L., *Quercus coccifera* L., *Arbutus unedo* L. i *Erica arborea* L (Llusià i Peñuelas 2000).

Mechanisms of terpene emission

Plant terpene emissions depend on: the source inside the plant, the diffusion pathway, the volatility of the compounds and the environmental conditions (Tingey *et al* 1991). The emission rate will be more or less dependant on these factors depending on the existence of storing organs on the plant: non-storing species will be more dependant than storing species as species with storing organs have an extra conductance factor than non-storing species (*Fig.5*).

Monoterpenes that have been produced inside the leaf can be released to the open air

Mecanismes d'emissió de terpens

L'emissió de terpens per part de les plantes depèn de: la font a l'interior de la planta, la via de difusió, la volatilitat del compost i les condicions ambientals (Tingey *et al* 1991). La tassa d'emissió serà més o menys depenent d'aquests factors segons si la planta té òrgans d'emmagatzematge o no: les espècies no acumuladores seran més depenents que les espècies acumuladores ja que les espècies amb òrgans d'emmagatzematge tenen un factor de conductància afegit respecte a les espècies no acumuladores (*Fig.5*).

Els monoterpens produïts a l'interior de la fulla poden ser alliberats a l'aire lliure per tres

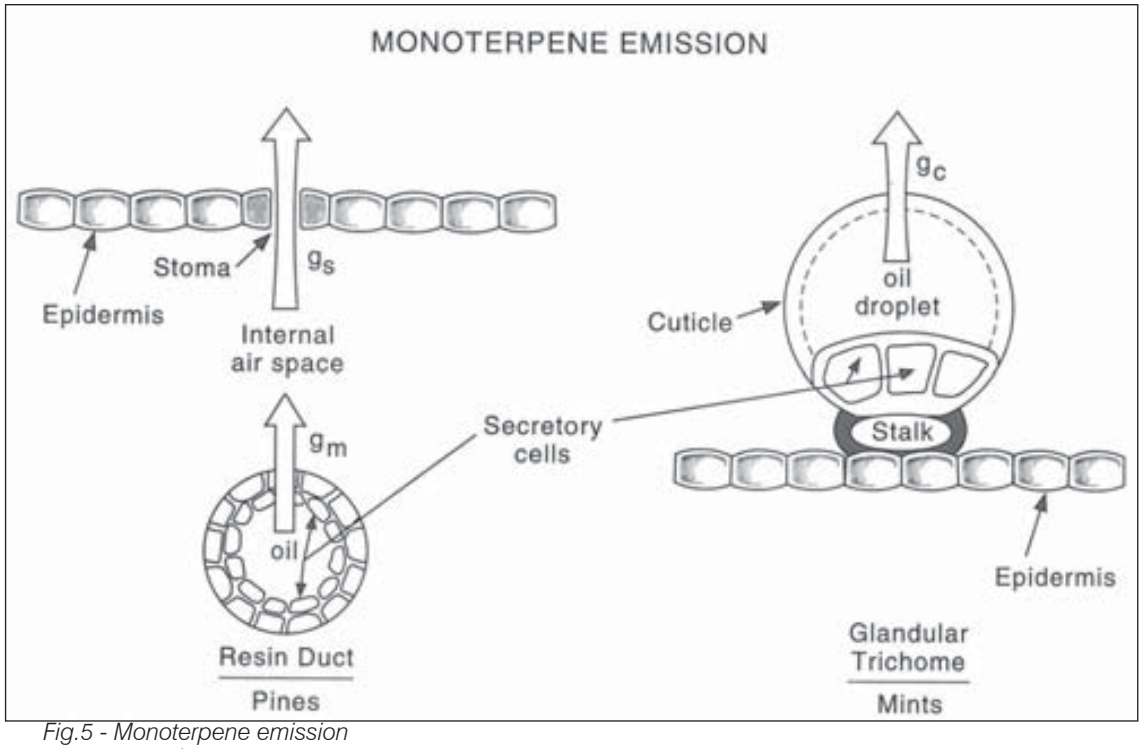


Fig.5 - Monoterpene emission
Emissió de monoterpens
(Font: Fall 1999)

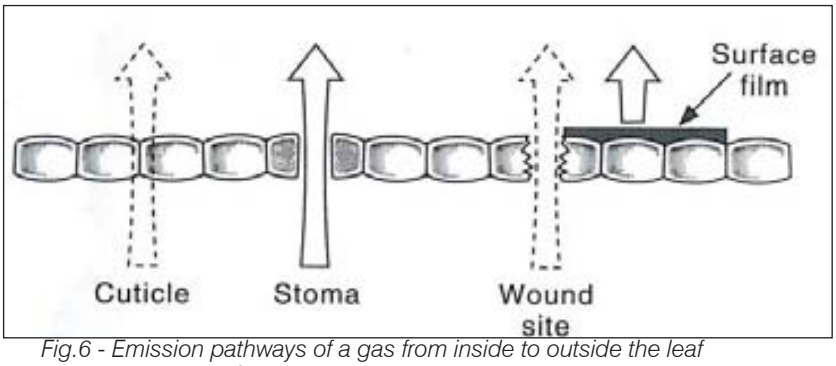


Fig.6 - Emission pathways of a gas from inside to outside the leaf
Vies d'emissió d'un gas des de l'interior d'una fulla cap a l'exterior
(Font: Fall 1999)

through three different ways: stomata, cuticle and through wound sites (*Fig.6*).

It is not always clear whether emission rates and stomatal conductance (g_s) are related, as sometimes they are not. Fall and Monson (1992) postulated that isoprene emission rates are independent of the aperture/ occlusion of the stomata. However, Peñuelas and Llusià (1999) showed that emission rates from the non-storing species *Quercus ilex* significantly correlated with the stomatal conductance. Consequently, emission rates of non-storing species depend more on stomatal conductance than storing species due to the fact that they have to immediately emit the compounds after their production.

Emission through cuticle diffusion is originated from a pressure gradient that is created because of the different concentrations of the compounds. This kind of emissions would be more important in storing species which present higher concentrations than non-storing species due to their storing organs.

Emission rates will depend on the size of those storing organs (if any) and on the relative humidity of the air (RH): the higher the RH the greater emission rates, because of the increase of the cuticle permeability regarding the rest of the compounds.

Plants can emit terpenes to the environment through wounds directly or indirectly by evaporation of volatile compounds coming from the material that the plant uses to sane the wound. This type of emission has to be taken into account especially when we perform terpene sampling of storing species: if a leaf is damaged when placing the cuvette or when manipulating the plant, a big amount of compounds should be released through the wound and that emission could contaminate the sampling. For this reason, we have to take extra-care when sampling storing species.

vies diferents: estomes, cutícula i a través de ferides (*Fig.6*).

No està clar del tot que la tassa d'emissió i la conductància estomàtica (g_s) estiguin sempre relacionades, ja que de vegades no ho estan. Fall i Monson (1992) van postular que la tassa d'emissió de l'isoprè és independent de l'obertura/oclusió dels estomes. Per contra, Peñuelas i Llusià (1999) van mostrar que la tassa d'emissió de l'espècie no acumuladora *Quercus ilex* està significativament correlacionada amb la conductància estomàtica. Així doncs, la tassa d'emissió en espècies no acumuladores depèn més de la conductància estomàtica que no en espècies acumuladores donat que les primeres tenen que emetre immediatament els compostos just després de produir-los.

L'emissió mitjançant difusió cuticular s'origina per un gradient de pressions creat per les diferents concentracions dels compostos. Aquest tipus d'emissions seran més importants en espècies acumuladores què tenen concentracions més grans que les no acumuladores degut als òrgans d'emmagatzematge.

La tassa d'emissió dependrà de la mida dels òrgans d'emmagatzematge (si és que n'hi ha) i de la humitat relativa de l'aire (HR): com més HR, més gran serà la tassa d'emissió, degut a l'augment de la permeabilitat de la cutícula respecte a la resta dels compostos.

Les plantes poden emetre terpens al medi directament a través de ferides, o indirectament per evaporació dels compostos volàtils provinents del material que la planta utilitza per curar la ferida. Aquest tipus d'emissió s'ha considerar sobre tot quan fem mesures de terpens en espècies acumuladores: si es danya una fulla a l'hora de situar la pinça de mostreig o durant la manipulació de la planta, aquesta alliberarà una gran quantitat de compostos a través de la ferida, i aquesta emissió ens podria contaminar la mostra. Per aquesta raó, tenim que ser molt precisos durant els mostrejos en espècies acumuladores.

1.3. Influence of environmental variables in the production and emission of VOCs

Influència de les variables ambientals sobre la producció i emissió de COVs

VOCs production and emission can be altered by the environmental conditions where the plant grows. The main environmental variables that can influence are temperature and light.

In both storing and non-storing species, VOC emission rates are directly related to temperature following an exponential curve (*Fig.7*).

Light specially influences on non-storing species. Light stimulates photosynthesis through the activation of the enzyme Rubisco, and consequently, the production of VOCs increases. If the plant has storing organs, emission rates would be less related to the production, and more related to the size of the storing pools (*Fig.8*).

The dependance of tempertaure on terpene emission rates in storing species is modelled

La producció i emissió de COVs pot variar en funció de les condicions ambientals on creix la planta. Les principals variables ambientals que poden influir són la temperatura i llum.

Tant en espècies acumuladores com en no acumuladores, la tassa d'emissió de COVs està directament relacionada amb la temperatura, seguint una corba exponencial (*Fig.7*).

La llum influeix especialment a les espècies no acumuladores. Aquesta, estimula la fotosíntesi mitjançant l'activació de l'enzim Rubisco, i d'aquesta manera incrementa la producció de COVs. Si la planta té òrgans d'emmagatzematge, la tassa d'emissió estarà menys relacionada amb la producció, i més relacionada amb la mida dels òrgans d'emmagatzematge (*Fig.8*).

La dependència de la tassa d'emissió amb la temperatura en espècies acumuladores es modelitza mitjançant la equació 1 (Tingey 1980,

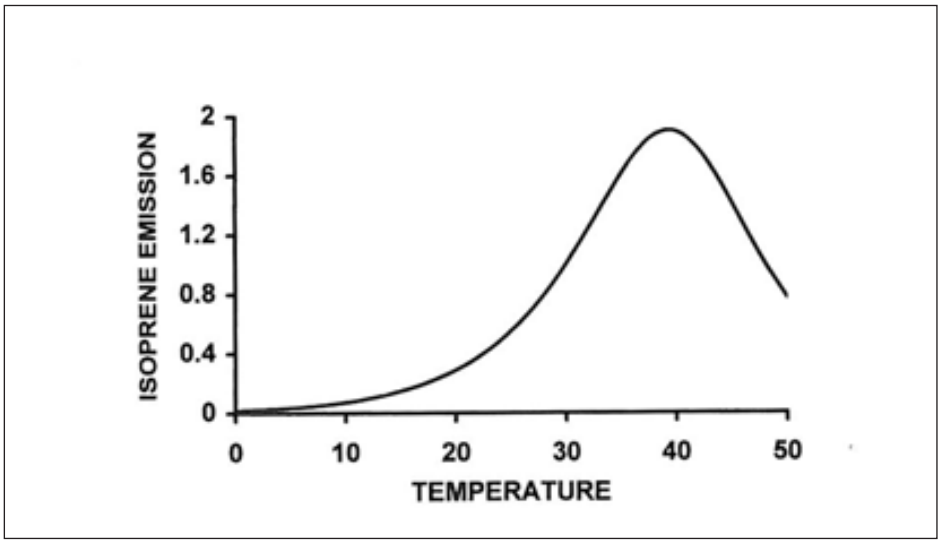


Fig.7a - Isoprene emission rate dependence temperatura curves
Corbes de dependència de la tassa d'emissió de l'isoprè amb la temperatura
(Font: Fall 1999)

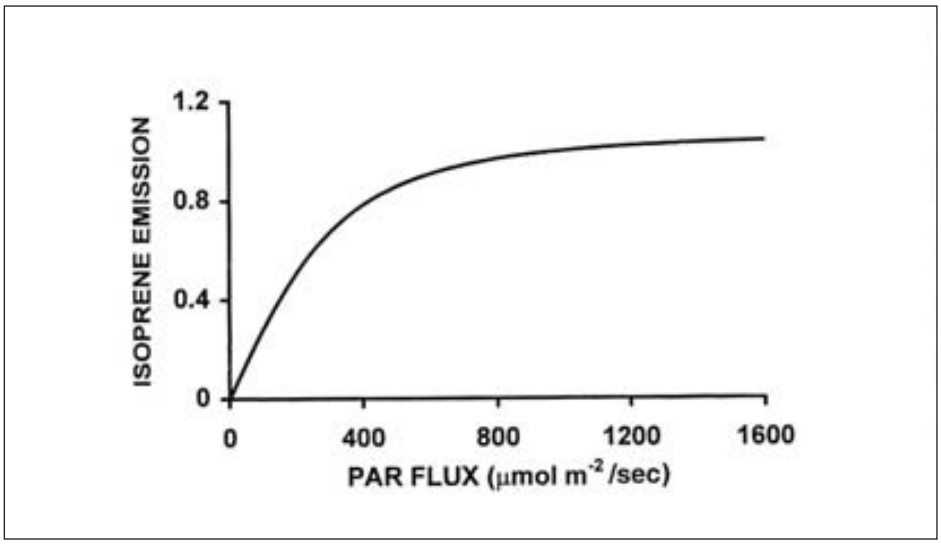


Fig.7b - Isoprene emission rate dependence with light curve
Corba de dependència de la tassa d'emissió de l'isoprè amb la llum
(Font: Fall 1999)

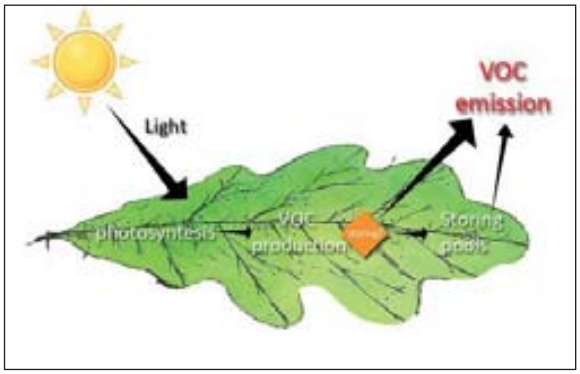


Fig.8 - Light effect on VOC production and emission
Influència de la llum en la producció i emissió de COVs

by equation 1 (Tingey 1980, Guenther *et al* 1993), where E_0 is the basal emission for each species, β is an empirical coefficient whose value is 0.09 based on a review of measurements (Guenther *et a*l 1993), and T_S is the leaf temperature at standard conditions (30 ºC).

For non-storing species, we use the model described by Guenther *et al* (1995) for isoprene emissions, which consider both light and temperature (equations 2, 3, 4), where C_L is the light correction factor and C_T the temperature correction factor, α=0.0027 and k=1.066 are the empirical coefficients describing the light-dependence and f=95,000 J mol^{-1}, d=230,000 J mol^{-1} are the empirical coefficients describing the temperature dependence, T_m is the temperature optimum of monoterpene emission (314 K), and R is the gas constant (8.314 J K^{-1} mol^{-1}).

Guenther *et al* 1993), on E_0 és l'emissió base per cada espècie, β és un coeficient empíric amb valor 0.09, basat en una recopilació bibliogràfica de valors mesurats (Guenther *et al* 1993), i T_S és la temperatura de la fulla en condicions estàndard (30 ºC).

Per a les espècies no acumuladores, s'utilitza el model descrit per Guenther *et al* (1995) per a les emissions d'isoprè, que considera tan la llum com la temperatura (equacions 2, 3, 4), on C_L és el factor de correcció de la llum, i C_T és el factor de correcció de la temperatura, α=0.0027 i k =1.066 són coeficients empírics que descriuen la dependència de la llum i f=95,000 J mol^{-1} i d=230,000 J mol^{-1} són coeficients empírics que descriuen la dependència de la temperatura, T_m és la temperatura òptima per a l'emissió de monoterpens (314 K) i R és la constant dels gasos (8.314 J K^{-1} mol^{-1}).

$$E = E_0 \cdot e^{\beta(T-T_s)} \quad (1)$$

$$E = E_0 \cdot C_L \cdot C_T \quad (2)$$

$$C_L = \frac{\alpha \cdot k \cdot Q}{\sqrt{1+\alpha^2 Q^2}} \quad (3)$$

$$C_T = \frac{e^{\frac{f(T-T_S)}{R \cdot T_S \cdot T}}}{1+e^{\frac{d(T-T_m)}{R \cdot T_S \cdot T}}} \quad (4)$$

Those models are suitable for the particular scenarios in which were formulated, but they have to be adapted to different scenarios by parameterisation. Nevertheless, there could be other environmental variables that influence VOCs such as CO_2 concentration (Constable *et al* 1999) or ozone concentration (Li *et al* 2009).

Aquests models són vàlids pels escenaris particulars pels que es van formular, però es poden adaptar a altres escenaris diferents mitjançant una parametrització del model. Tot i això, podrien haver-hi altres variables ambientals que influenciéssin als COVs com ara la concentració de CO_2 (Constable *et al* 1999) o la concentració d'ozó (Li *et al* 2009).

1.4. Influence of global change on the production and emission of VOCs

Influència del canvi global sobre la producció i emissió de COVs

Global change

The term global change is referred to the changes that have been produced in different basic functioning-processes of the Earth due to human activity. Man has been living on the Earth surface during millions of years, but during the last century and especially due to industrial revolution, their activities have increased their effects on the environment.

According to the last intergovernmental panel of climate change (IPCC 2007) temperatures have increased and precipitations have decreased in the Mediterranean region, and an increase of 0.4 ºC is expected for the next two decades (*Fig.9*). As a consequence of that increase, plant phenology has also been altered: some plants have advanced their growing season (Peñuelas and Filella 2001).

Canvi global

El terme canvi global es refereix als canvis que s'han produït en diferents processos bàsics de funcionament de la Terra degut a l'activitat humana. L'home ha viscut a la superfície de la Terra durant milions d'anys; tot i això, durant l'últim segle, i especialment a partir de la revolució industrial, les seves activitats han incrementat el seu efecte sobre el medi ambient.

Segons el panel intergovernamental del canvi climàtic (IPCC 2007) les temperatures han augmentat i les precipitacions han disminuït a la regió mediterrània, i s'espera un augment de 0.4 ºC per a les pròximes dues dècades (*Fig.9*). Com a conseqüència d'aquest increment, la fenologia de les plantes també s'ha vist alterada: algunes plantes han avançat el seu període de creixement (Peñuelas i Filella 2001).

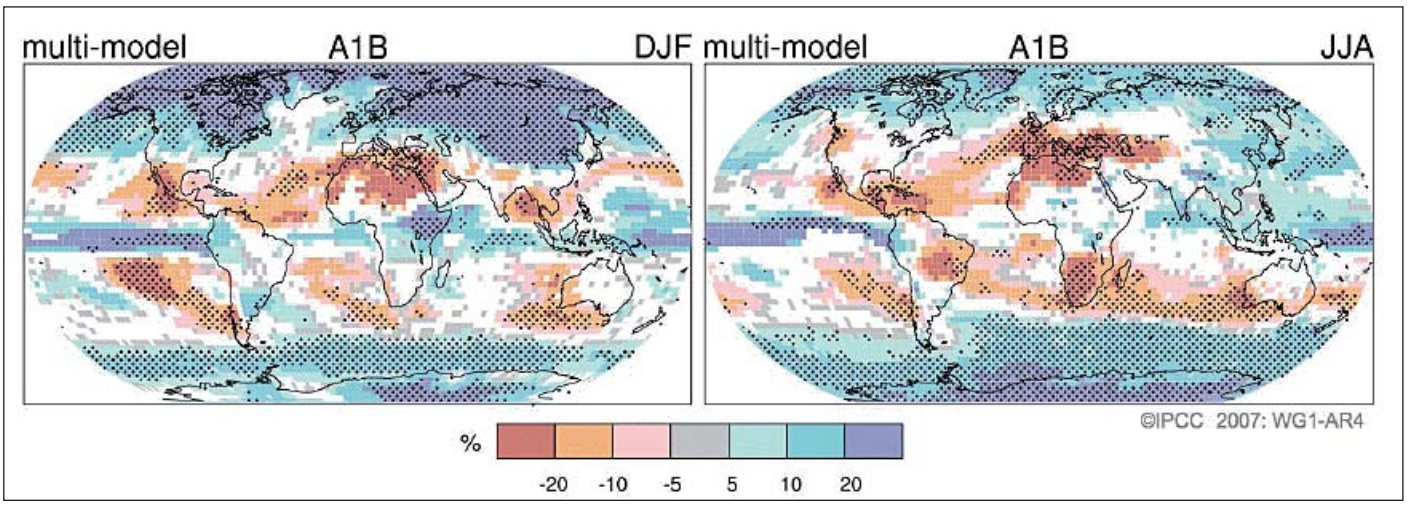


Fig.9a - Precipitation projections
Projeccions de precipitació
(Font: IPCC 2007)

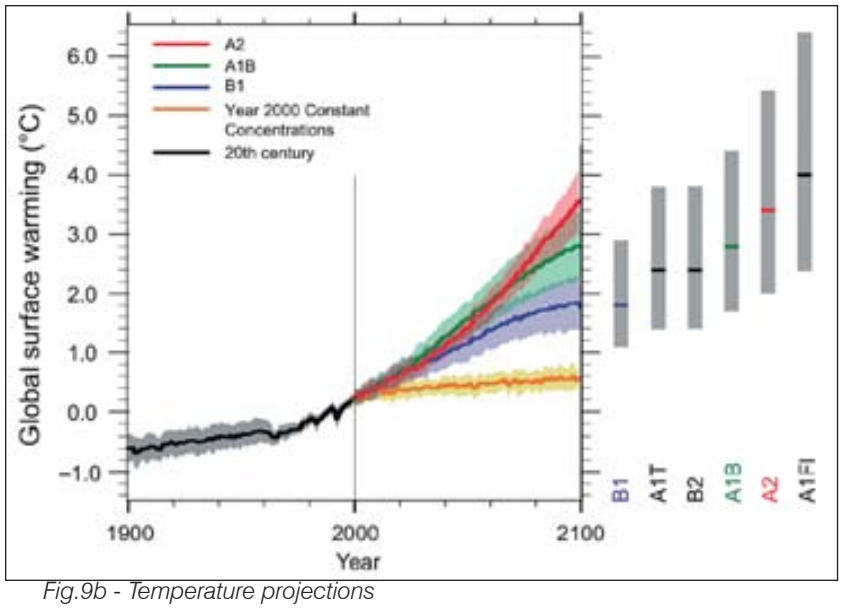


Fig.9b - Temperature projections
Projeccions de temperatura
(Font: IPCC 2007)

Despite the fact that climate change is the most known effect of global change, there are other effects important as well, such as increase of CO_2 and eutrophication of ecosystems. Those effects can affect the plants, and consequently the production and emission of BVOCS (*Fig.10*).

Drought and warming

Plant responses to drought and warming are variable and depend on each plant: the most plastic plants will be capable to adapt their physiology to stress, while the more exigent plants will end up disappearing overridden by other plants in a lower position in the succession line. Some studies show how drought limits photosynthesis through stomatal conductance: some plants close

Tot i que el canvi climàtic és l'efecte més conegut del canvi global, hi ha d'altres efectes que també s'han de tenir en compte, com ara l'augment de CO_2 i l'eutrofització dels ecosistemes. Aquests efectes poden afectar les plantes, i per consegüent, la producció i emissió de COVBs (*Fig.10*).

Sequera i escalfament

Les respostes de les plantes a la sequera i a l'escalfament són diverses i depenen de cada planta: les plantes més plàstiques tindran la capacitat d'adaptar la seva fisiologia a l'estrès, mentre que les plantes més exigents acabaran desapareixent i essent suplantades per altres plantes que es troben en una posició inferior a la línia de successió. Alguns estudis mostren que la sequera pot limitar la fotosíntesi a través de la conductància estomàtica: algunes plantes

their stomata under drought conditions (Sharkey 1990, Chaves 1991, Ort *et al* 1994, Cornic and Massacci 1996).

Among the abiotic factors affecting plant terpene emission rates, temperature is outstanding (Tingey *et al* 1980, Guenther *et al* 1993, Staudt and Seufert 1995, Loreto *et al* 1996a, b, Peñuelas and Llusià 1999, Llusià and Peñuelas 2000). Warming increases the production and emission rates of most terpenes exponentially up to maximum by enhancing the synthesis enzymatic activities, raising the terpene vapour pressure, and decreasing the resistance of emission pathway (Tingey *et al* 1991, Loreto *et al* 1996a). A further 2–3 ºC rise in the mean global temperature, which is predicted to occur early this century (IPCC 2007), could increase BVOC global emissions by an additional 30–45% (Peñuelas and Llusià 2003, Peñuelas and Staudt 2010).

Mediterranean ecosystems are water-limited (Sardans and Peñuelas 2004). Water-stress usually increases terpene concentrations in many storing and non-storing species (Kainulainen *et al* 1992, Llusià and Peñuelas 1998, Loreto *et al* 2001), and usually increases terpene emission rates (Loreto *et al* 1998, Peñuelas and Llusià 1999).

tanquen els seus estomes en condicions de sequera (Sharkey 1990, Chaves 1991, Ort *et al* 1994, Cornic i Massacci 1996).

D'entre els factors abiòtics que afecten a la tassa d'emissió de les plantes, el més important és la temperatura (Tingey *et al* 1980, Guenther *et al* 1993, Staudt i Seufert 1995, Loreto *et al* 1996a, b, Peñuelas i Llusià 1999, Llusià i Peñuelas 2000). L'escalfament incrementa la producció i la tassa d'emissions de la majoria dels terpens de forma exponencial fins a un màxim, ja que potencia l'activitat de síntesi dels enzims, incrementant la pressió de vapor dels terpens i fent disminuir la resistència de la via d'emissió (Tingey *et al* 1991, Loreto *et al* 1996a). Un futur increment de 2-3 ºC en la mitjana global de temperatures, que és el que s'ha previst que passi durant aquest segle (IPCC 2007), podria incrementar les emissions globals de COVBs fins un 30-45% (Peñuelas i Llusià 2003, Peñuelas i Staudt 2010).

Els ecosistemes mediterranis estan limitats per l'aigua (Sardans i Peñuelas 2004). L'estrès hídric normalment incrementa les concentracions de terpens, tant en espècies acumuladores com en no acumuladores (Kainulainen *et al* 1992, Llusià i Peñuelas 1998, Loreto *et al* 2001), i normalment incrementa la tassa d'emissió de terpens (Loreto *et al* 1998, Peñuelas i Llusià 1999).

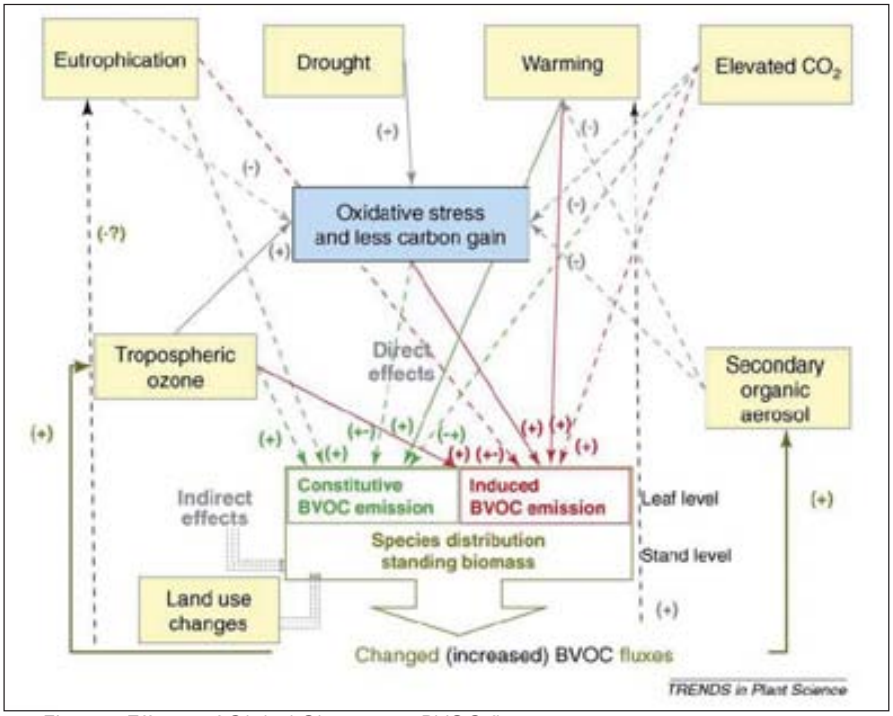


Fig.10 - Effects of Global Change on BVOC fluxes
Efectes del canvi global en els fluxos de COVBs)
(Font: Peñuelas and Staudt 2010)

Eutrophication of ecosystems

In general, Mediterranean ecosystems are considered to be poor in nutrients (Mooney and Dunn 1970, Ellis and Kummerow 1989): mediterranean soils often suffer from nutrient deficiencies (Specht 1973, Kruger 1979, Terradas 2001, Sardans *et al* 2006). However, there has been an increase of nitrogen and phosphorus availability during the last decades, and their cycles have been altered (Peñuelas and Filella 2001b, Rodà *et al* 2002, Sardans and Peñuelas 2004, Sardans *et al* 2006). Total atmospheric deposition was estimated at 15-22 kg N ha^{-1} $year^{-1}$, most of it being retained within the studied broadleaved evergreen forests. Ecosystem N availability is thus likely to be increasing in these forests (Rodà *et al* 2002). N availability has increased last decades due to anthropogenic sources such as fertilizers, combustion of fossil fuels and cattle residuals (Vitousek *et al* 1997). P concentrations have increased mostly due to agricultural practices (Rubaek *et al* 2000).

Our ecosystems are more eutrophicated than some years ago: the inputs of nutrients (and especially of nitrates) have increased progressively in the last years.

Eutrofització dels ecosistemes

Els ecosistemes mediterranis normalment estan considerats com ecosistemes pobres en nutrients (Mooney i Dunn 1970, Ellis i Kummerow 1989): els sòls mediterranis normalment sofreixen deficiències de nutrients (Specht 1973, Kruger 1979, Terradas 2001, Sardans *et al* 2006). No obstant, en les darreres dècades ha un incrementat la disponibilitat de nitrogen i fòsfor, i els seus cicles respectius s'han vist alterats (Peñuelas i Filella 2001b, Rodà *et al* 2002, Sardans i Peñuelas 2004, Sardans *et al* 2006). La deposició atmosfèrica total s'ha estimat en 15-22 kg N ha^{-1} any^{-1}, la majoria de la qual resta retinguda als boscos de planifolis (Rodà *et al* 2002). La disponibilitat de nitrogen ha augmentat en les darreres dècades a causa de les fonts antropogèniques com són els fertilitzants, la combustió de combustibles sòlids i els residus ramaders (Vitousek *et al* 1997). Les concentracions de fòsfor han incrementat principalment degut a les pràctiques agrícoles (Rubaek *et al* 2000).

Els nostres ecosistemes estan més eutrofitzats que fa uns anys: les entrades de nutrients (i especialment les de nitrats) han crescut progressivament els darrers anys. Una gran

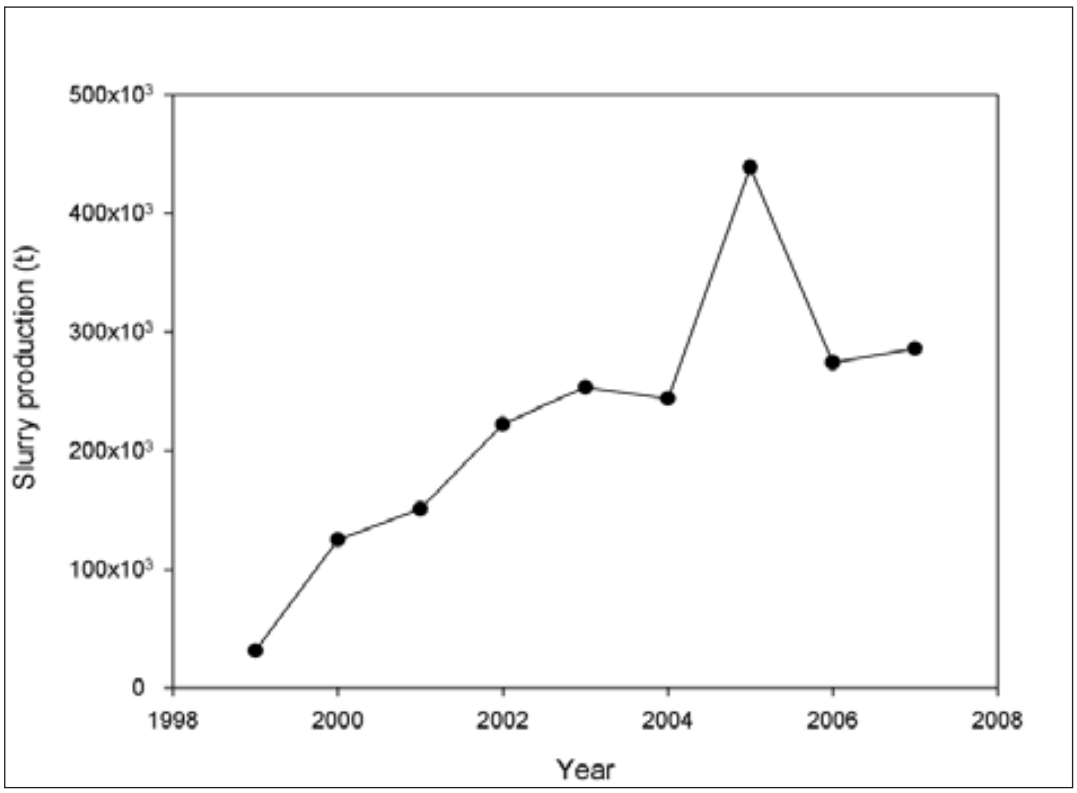


Fig.11 - Temporal evolution of the slurry producction in Catalonia, from 1999 to 2007
Evolució temporal de la producció de purins a Catalunya des de 1999 fins a 2007
(Font: Agència Catalana de Residus website)

A big contribution to this increase is due to the increase of slurries in agriculture in an indiscriminate way (*Fig.11*).

Apart from slurries, other factors which are consequence of climate change such as temperature increase or drought also influence this phenomenon: heating increases the mineralization (nitrates) and drought makes nutrients unavailable for plants and facilitates system loses when there are rains.

The global nitrogen cycle has now reached the point in which more nitrogen is fixed annually by human-driven processes (fertilizers, combustion of fossil fuels, and waste from stock raising) than by natural processes (Vitousek *et al* 1997, Rodà *et al* 2002). Along with nitrogen, phosphorus is also a frequent limiting factor in Mediterranean ecosystems (Sardans *et al* 2006). Similarly to nitrogen, phosphorus input to ecosystems, especially aquatic ones, has increased in last decades (Rubaek *et al* 2000). Nutrient supplies have often been shown to be an important factor in growth, structure and distribution of mediterranean communities (Kruger 1979, Henkin *et al* 1998). And as a result of these increases in nitrogen and phosphorus, nitrogen and phosphorus foliar concentrations have increased in some Mediterranean species in the last few decades (Peñuelas and Filella 2001).

Some theories have stated that these eutrophication of the ecosystem could alter the secondary metabolite production of the plants: The carbon/nutrient balance hypothesis (CNBH) (Bryant *et al* 1983) predicts that when a resource, such as nitrogen, is abundant, a plant will allocate proportionately less carbon toward carbon based secondary compounds (reserve and defence) and more toward growth (Lerdau *et al* 1995, Peñuelas and Estiarte 1998). Similar hypotheses can be developed for the availability of other resources such as CO_2, phosphorus or water (Peñuelas and Estiarte 1998). The growth differentiation balance hypothesis (Lorio 1986) recognizes that all secondary metabolites have an ontogenetically determined phenology and that their synthesis is emphasized during periods of plant differentiation. Growth dominates during favorable conditions, and differentiation is at a maximum only when

part d'aquest creixement es deu a l'augment indiscriminat de l'ús de purins en agricultura (*Fig.11*).

A banda dels purins, també influencien en aquest fenomen altres factors que són conseqüència del canvi climàtic com l'augment de la temperatura o la sequera: l'escalfament fa créixer la mineralització (nitrats) i la sequera fa que els nutrients no estiguin disponibles per les plantes i facilita pèrdues del sistema quan hi ha pluges.

El cicle global del nitrogen ha arribat a un punt en què cada any es fixa més nitrogen per processos humans (fertilitzants, combustió de combustibles fòssils, i residus per l'augment d'estocs) que no per processos naturals (Vitousek *et al* 1997, Rodà *et al* 2002). Junt amb el nitrogen, el fòsfor és sovint un factor limitant en els ecosistemes mediterranis (Sardans *et al* 2006). Anàlogament al nitrogen, l'entrada de fòsfor als ecosistemes, especialment en els aquàtics, ha crescut en les darreres dècades (Rubaek *et al* 2000). Les aportacions de nutrients sovint s'han descrit com a factors importants en el creixement, estructura i distribució de les comunitats mediterrànies (Kruger 1979, Henkin *et al* 1998). Com a resultat d'aquests increments de nitrogen i fòsfor, les concentracions foliars d'aquests dos elements també han crescut en algunes espècies de caire mediterrani en les darreres dècades (Peñuelas i Filella 2001).

Algunes teories han postulat que aquesta eutrofització del ecosistema podria alterar la producció dels metabòlits secundaris de les plantes: la hipòtesi del balanç de carboni / nutrients (CNBH) (Bryant *et al* 1983) postula que quan un recurs, com ara el nitrogen, és abundant, la planta assignarà proporcionalment menys carboni a la producció de compostos carbònics secundaris (reserva / defensa) i més al creixement (Lerdau *et al* 1995, Peñuelas i Estiarte 1998). D'aquesta manera, es poden formular hipòtesis similars per la disponibilitat d'altres recursos com el CO_2, el fòsfor o l'aigua (Peñuelas i Estiarte 1998). La hipòtesi del balanç de diferenciació del creixement (Lorio 1986) reconeix que els metabòlits secundaris tenen una fenologia ontològicament predeterminada i que la seva síntesi s'emfatitza durant els períodes de diferenciació de la planta. El creixement domina quan hi ha condicions favorables, i la diferenciació és màxima només

conditions are suboptimal for growth. This could be more evident in tree species with predeterminated growth such as pine trees. The optimal allocation model (Tuomi *et al* 1991) predicts decreasing investment in defence with increasing resource availability, because reduced costs of tissue production could compensate higher risks of herbivore predation. The plant stress hypothesis (Mattson and Haack 1987) states that environmental stresses on plants decrease plant resistance to insect herbivory by altering whole-plant source-sink resource allocation schedules and foliar chemistry, thus changing food palatability. Since phosphorylated compounds such as isopentenyl diphosphate and dimethylallyl diphosphate are immediate precursors of isoprene it is likely that also P availability influence isoprenoid emission rates.

quan les condicions són subòptimes per al creixement. Això podria ser més evident en espècies arbòries amb un creixement predeterminat com podrien ser els pins. El model d'assignació òptima (Tuomi *et al* 1991) prediu que s'inverteix menys en defensa quan més recurs disponible hi ha, perquè els baixos costos de producció de teixits compensarien el risc de predació per part d'herbívors. La hipòtesi de l'estrès de la planta (Mattson i Haack 1987) postula que els estressos ambientals disminueixen la resistència de la planta a l'atac d'insectes herbívors mitjançant una alteració del patró de distribució de les fonts de recursos globals de la planta i de la química foliar, canviant així la palatabilitat del menjar. Donat que alguns compostos fosforilats com el isopentenil difosfat i el dimetilalil difosfat són precursors immediats de l'isoprè és probable que la disponibilitat de fòsfor influeixi en la tassa d'emissió d'isoprenoides.

1.5. Description of the main studied species: Quercus ilex, Pinus halepensis, Pinus pinaster and *Arabidopsis thaliana*

Descripció de les principals espècies estudiades: Quercus ilex, Pinus halepensis, Pinus pinaster, i Arabidopsis thaliana

Quercus ilex L.

Quercus ilex is a tree species which can reach 25 m high. The leaf shape is variable, the adult leaves are entire, 4–8 cm long and 1–3 cm broad, while those on the lower branches of young trees are often larger (to 10 cm long), and are toothed or somewhat spiny. The flowers are catkins, produced in the spring; the fruit is an acorn, which matures in about six months (*Fig.12*).

Mediterranean Holm oak is considered one of the species with higher terpene emission rates

Quercus ilex L.

Quercus ilex és una espècie arbòria que pot arribar fins als 25 m d'alçada. La forma de la fulla és variable, les fulles adultes són enteres, de llargada 4-8 cm i 1-3 cm d'amplada, mentre que les que estan a branques baixes d'arbres joves sovint són més llargues (fins a 10 cm), són dentades i una mica espinoses. Les flors apareixen en aments a la primavera; el fruit és la gla, que madura al voltant d'uns sis mesos (*Fig.12*).

L'alzina mediterrània està considerada com

Fig.12 - 2-year potted Quercus ilex seedlings, in the experimental fields of the Universitat Autònoma de Barcelona (April 2004)
Plançons de Quecus ilex de dos anys en contenidors, als camps experimentals de la Universitat Autònoma de Barcelona (abril 2004)

Fig.13a - Distribution of Quercus ilex in Spain
Distribució de Quercus ilex a Espanya
(Font: www.anthos.es)

Fig.13b - Distribution of Quercus ilex in Catalonia
Distribució de Quecus ilex a Catalunya
(Font: Mapa de cobertes del Sòl de Catalunya, versió 3)

(Kesselmeier and Staudt 1999, Llusià and Peñuelas 2000). The main emitted terpenes are monoterpenes, being the most common α-pinene, β-pinene, sabinene and myrcene (Llusià and Peñuelas 2000). The most emitted compound is usually α-pinene, which can reach until 40% of the total emission rates (Loreto *et al* 1996a).

Pinus halepensis Mill.

Pinus halepensis (*Fig.14*) is a tree which can reach 20-25 m high. Its leaves, flexibles and light green colored, of 6-12 (15) cm length,

una de les espècies amb una major tassa d'emissió de terpens (Kesselmeier i Staudt 1999, Llusià i Peñuelas 2000). Els principals terpens que emet són monoterpens, i els més comuns són: α-piné, β-piné, sabiné i mircé (Llusià i Peñuelas 2000). El principal compost emès és l'α-piné, el qual pot arribar fins a un 40% de la tassa d'emissió (Loreto *et al* 1996a).

Pinus halepensis Mill.

Pinus halepensis (*Fig.14*) és un arbre que pot créixer fins a 20-25 m d'alçada. Les seves fulles, flexibles i de color verd clar, de 6-12 (15) cm de llargada, estan normalment disposades

are usually disposed in groups of 2 leaves, rarely from 3 to 5 leaves. The cones are narrow conic, 5-12 cm long and 2-3 cm broad at the base when closed, green at first, ripening glossy red-brown when 24 months old. The seeds are 5-6 mm long, with a 20 mm wing, and are wind-dispersed.

P. halepensis is a tree that can grow indifferently of the soil type, which appears in xerophytic forests, brushes and machias in the Mediterranean area, ranging from sea level to 1000 m. It is drought resistant and can grow in areas with precipitations from 250 to 800 mm (Gil *et al* 1996). In the Iberian Peninsula appears mainly in the eastern half and in the Balearic Islands. In Catalonia is

en grups de 2, rarament de 3 a 5. Les seves pinyes tenen forma cònica, de 5-12 cm de llarg i 2-3 cm d'ample a la base quan estan tancades, verdes al començament, i viren cap a un roig-marronenc quan tenen 24 mesos. Les llavors fan 5-6 mm de llarg, amb un ala de 20 mm, i són dispersades pel vent.

P. halepensis és un arbre que pot créixer amb indiferència del tipus de sòl, i normalment apareix en boscos xerofítics, matollars i màquies a l'àrea mediterrània, des del nivell del mar fins als 1000 m d'alçada. És resistent a la sequera i pot créixer en àrees amb precipitacions entre 200 i 800 mm (Gil *et al* 1996). A la Península Ibèrica apareix majoritàriament a la meitat est i a les illes Balears. A Catalunya, és present en

Fig.14 - Two-year potted Pinus halepensis seedlings, in the experimental fields of the Universitat Autònoma de Barcelona (april 2004)
Plançons de 2 anys de Pinus halepensis en contenidor, als camps experimentals de la Universitat Autònoma de Barcelona (abril 2004)

Fig.15a - Distribution of Pinus halepensis in Spain
Distribució de P. halepensis a Espanya
(Font: www.anthos.es)

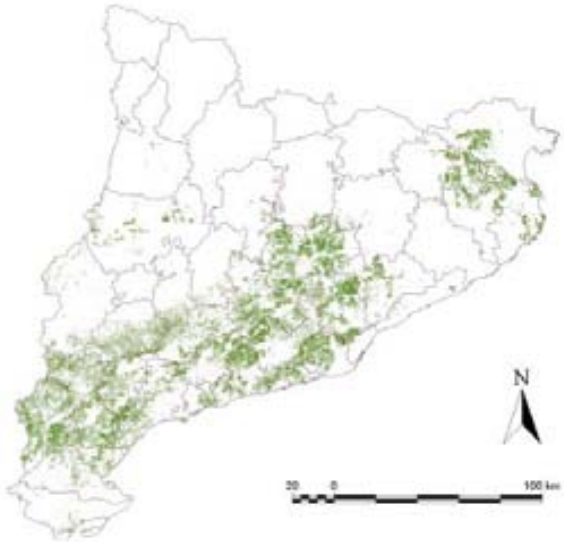


Fig.15b - Distribution of Pinus halepensis in Catalonia
Distribució de P. halepensis a Catalunya
(Font: Mapa de cobertes del Sòl de Catalunya, versió 3)

present in 9.82% of the surface (IFN 2000). Its distribution in Spain and Catalonia is shown in *Figs.15 a, b*.

Regarding terpene emission rates, despite the fact that *P. halepensis* has not been considered a high-emitter species (Owen *et al* 2002) there are several studies which show emission rates are dominated by α-pinene and myrcene (Llusià and Peñuelas 2000, Owen *et al* 2002), which represent an important percentage of the total emission amount.

Pinus pinaster Ait.

Pinus pinaster (*Fig.16*) is a medium-size tree, reaching 20-35 m tall. The bark is orange-red, thick and deeply fissured at the base of the trunk, somewhat thinner in the upper crown. The leaves ('needles') are in pairs, very stout (2 mm broad), 12-22 cm long, and bluish-green to distinctly yellowish-green. The cones are conic, 10-20 cm long and 4-6 cm broad at the base when closed, green at first, ripening glossy red-brown when 24 months old. They open slowly over the next few years, or after being heated by a forest fire, to release the seeds, opening to 8-12 cm broad. The seeds are 8-10 mm long, with a 20-25 mm wing, and are wind-dispersed.

P. pinaster prefers non-carbonated soils, and usually lives in mediterranean lands with certain influence of maritime climate, ranging from sea level to 1000 m. Maritime pine is a heliofitic species, which usually appears in brushes and in forests with low density of trees, sometimes can form secondary pine forests. This species is characteristic of the occidental half of the Mediterranean region and from Atlantic zones of the south of France, Spain and Portugal (*Fig.17a, b, c*).

Maritime pine has been widely chosen as forestation species in Galicia (NW Spain) since the XVIIIth century. Despite being partly replaced in the last decades by species with higher productions like *Pinus radiata* and *Eucalyptus globulus*, *P. pinaster* is still the most important forest tree species in Galicia (DGCN 2000).

Regarding terpene emission rates, *P. pinaster* is considered a low-emitting species (Kesselmeier and Staudt 1999). Terpene

un 9.82% de la superfície (IFN 2000). La seva distribució a Espanya i Catalunya es mostra a les *Figs.15 a,b*.

Pel que fa a l'emissió de terpens, tot i que *P. halepensis* no està considerat com un gran emissor de terpens (Owen *et al* 2002), hi ha diversos estudis en els que es mostra que la seva tassa d'emissió està dominada per α-piné i mircé (Llusià i Peñuelas 2000, Owen *et al* 2002), els quals representen un percentatge important de l'emissió total.

Pinus pinaster Ait.

Pinus pinaster (*Fig.16*) és un arbre de mida mitjana que pot arribar fins als 20-35 m d'alçada. L'escorça és vermellosa-ataronjada, gruixuda i molt fisurada a la base del tronc, i menys a la part de la copa. Les fulles (acícules) estan disposades en parelles, gruixudes (2 mm d'ample), de 12-22 cm de llargada, i blau-grogues a groc-verdes. Les pinyes són còniques, 10-20 cm de llarg i 4-6 cm d'ample a la base quan estan tancades, verdes a l'inici, i viren cap a un roig-marronenc quan tenen 24 mesos. Aquestes s'obriran gradualment durant els pròxims anys o bé si hi ha un incendi forestal, alliberant les seves llavors. Les llavors fan 8-10 mm de llargada, amb una ala de 20-25 mm, i es dispersen pel vent.

P. pinaster prefereix sòls no carbonatats, i normalment viu a zones mediterrànies amb certa influència de clima marítim, des del nivell del mar fins a 1000 m d'alçada. El pi marítim és una espècie heliòfila, i normalment apareix en matollars i en boscos amb baixa densitat d'arbres, de vegades també forma pinedes secundàries. Aquesta espècie és característica de la meitat occidental de la regió mediterrània i de zones atlàntiques del sud de França, Espanya i Portugal (*Fig17 a,b, c*).

El pi marítim s'ha triat molt sovint com a espècie per a reforestació a Galicia (NW Espanya) des del segle XVIII. Tot i que ha estat parcialment substituït a les darreres dècades per espècies més productores com el *Pinus radiata* o l'*Eucalyptus globulus*, *P. pinaster* encara és una de les espècies forestals més importants a Galicia (DGCN 2000).

Pel que fa la tassa d'emissions, *P. pinaster* està considerat com una espècie amb baixes

Fig.16 - Pinus pinaster in pots in the experimental station of Lourizan (Pontevedra), july 2006
Pinus pinaster en contenidors a l'estació experimental de Lourizan (Pontevedra), juliol de 2006.

Fig.17a - Distribution of Pinus pinaster in Spain
Distribució de Pinus pinaster a Espanya
(Font: www.anthos.es)

Fig.17b - Distribution of Pinus pinaster distribution in Catalonia
Distribució de Pinus pinaster a Catalunya
(Font: Mapa de cobertes del Sòl de Catalunya, versió 3)

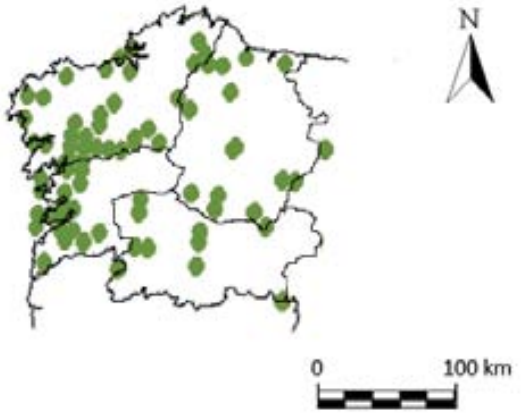


Fig.17c - Distribution of Pinus pinaster distribution in Galicia
Distribució de Pinus pinaster a Galicia
(Font: www.anthos.es)

emission rates are dominated by α-pinene and β−pinene, which represent an important percentage of the total emission amount (Simon *et al* 2005).

Arabidopsis thaliana

Arabidopsis thaliana (*Fig.18*) is a small flowering plant native to Europe, Asia, and northwestern Africa (*Figs9 a, b*). *A. thaliana* is a spring annual with a relatively short life cycle, and is popular as a model organism in

emissions (Kesselmeier and Staudt 1999). La tassa d'emissions està dominada per α-piné i β-piné, els quals representen un percentatge important de la tassa d'emissió total (Simon *et al* 2005).

Arabidopsis thaliana

Arabidopsis thaliana (*Fig.18*) és una planta petita nativa d'Europa, Àsia i del nord-oest d'Àfrica (*Figs.9 a, b*). *A. thaliana* és una planta anual amb un cicle de vida relativament curt, i és popular perquè s'ha utilitzat sovint com a

Fig.18 - Arabidopsis thaliana at the final stage of its vegetative period. Universitat Autònoma de Barcelona, July 2008
Arabidopsis thaliana a la fase final del seu període vegetatiu. Universitat Autònoma de Barcelona, juliol 2008.

Fig.19a - Distriburion of Arabidopsis thaliana in Spain
Distribució de Arabidopsis thaliana a Espanya
(Font: www.anthos.es)

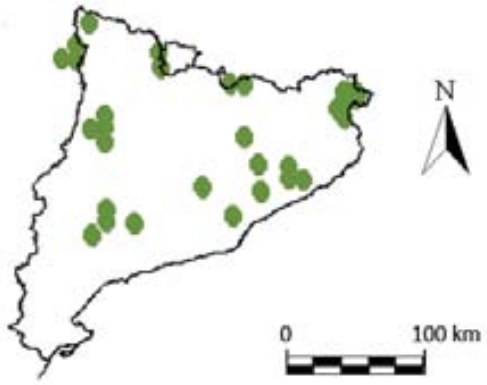


Fig.19b - Distribution of Arabidopsis thaliana in Catalonia
Distribució de Arabidopsis thaliana a Catalunya
(Font: www.anthos.es)

plant biology and genetics. Its genome is one of the smallest plant genomes and was the first plant genome to be sequenced.

Regarding emission rates, it is known that its flowers are terpene-emitters, and traces of terpene emissions have also been found on its leaves (Chen *et al* 2003, Aharoni *et al* 2006).

organisme model en biologia d'espècies i en genètica. El seu genoma és un dels genomes més petits de les plantes, i va ser el primer genoma vegetal que es va seqüenciar.

Pel que fa a la tassa d'emissions, es sap que les flors emeten terpens, i s'han trobat indicis d'emissió de terpens a les seves fulles (Chen *et al* 2003, Aharoni *et al* 2006).

1.6. Objectives

Objectius

General objectives

The general objectives of this PhD thesis were to study the effect of some of the most prominent global change components, **climate change** (mostly drought as major factor in our region) and **eutrophication** (increase of nutrient availability in the environment) on terpene **production (chapter 2)** and **emission (chapters 3.1 and 3.2)**. We aimed to study such effects in both storing and non-storing species. Moreover, we aimed to study if there is a **genotypic effect (chapters 4.1 and 4.2)** on the terpene production and emission (*Fig. 20*). These general objectives are common to all chapters. In addition, the specific aims of each chapter are listed below. To accomplish these aims we have conducted three field-lab experiments in increasingly controlled

Objectius generals

Els objectius generals d'aquesta tesi doctoral van ser estudiar l'efecte d'alguns dels components més prominents del canvi global, el **canvi climàtic** (majoritàriament sequera, què és el factor més important a la nostra regió) i **eutrofització** (augment de la disponibilitat de nutrients al medi) en la **producció (capítol 2)** i **emissió (capítols 3.1 i 3.2)**. El nostre objectiu va ser estudiar aquests efectes tant en espècies acumuladores com en no acumuladores. A més, també vàrem voler estudiar si hi ha un **efecte del genotip (capítols 4.1 i 4.2)** a la producció i emissió de terpens (*Fig.20*). Aquests objectius generals són comuns a tots els capítols. Així mateix, a continuació es citen els objectius específics per a cada capítol. Per complir aquests objectius hem realitzat tres experiments en camp i laboratori, en

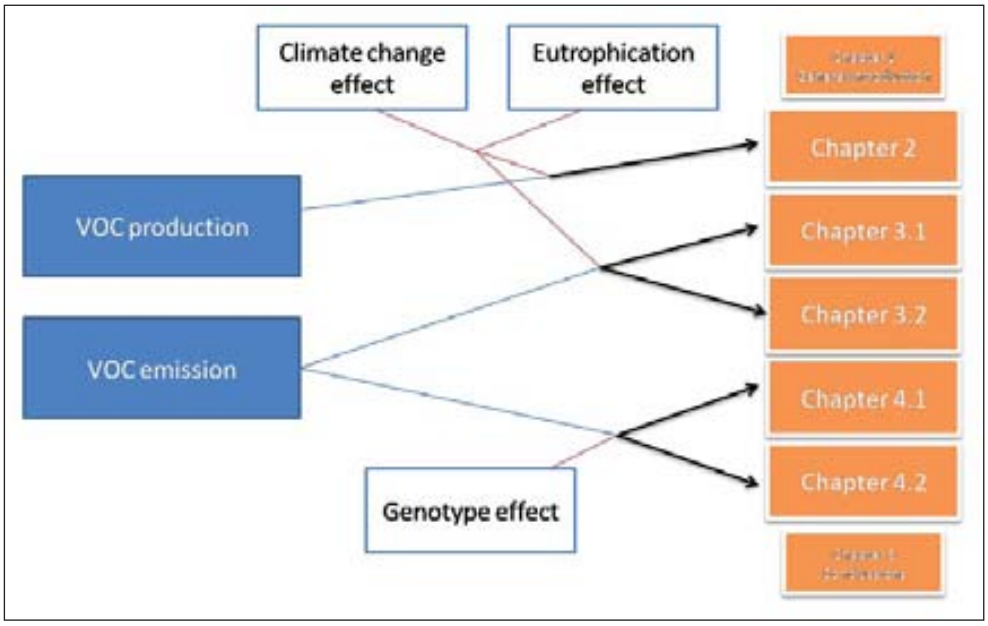


Fig.20 - PhD chapter scheme
Esquema dels diferents capítols de la tesi doctoral.

conditions in order to reduce the variability produced by the environment.

condicions controlades creixents per a reduir la variabilitat produïda per l'ambient.

Specific objectives

Chapter 2 - Drought, warming and soil fertilization effects on leaf volatile terpene concentrations in *Pinus halepensis* and *Quercus ilex*

The aim of the present work was to conduct a controlled study of the interaction between temperature, water stress and nutrient doses on monoterpene content of foliage. The objective was to contribute to a better understanding of increasing drought, eutrophication and temperature on terpene concentrations in a terpene-storing (*P. halepensis* Mill.) and in a non-storing (*Q. ilex* L.) Mediterranean species. We aimed to answer the following research questions:

1. What is the effect of drought, warming and fertilization on leaf terpene production?
2. Are there differences in the leaf terpene production pattern and quantities in storing and non-storing species?

Objectius específics

Capítol 2 - Efectes de la sequera, escalfament i fertilització del sòl sobre les concentracions de terpens volàtils a les fulles de *Pinus halepensis* i *Quercus ilex*

L'objectiu d'aquest treball va ser realitzar un estudi controlat de la interacció entre la temperatura, estrès hídric i diferents dosis de nutrients als continguts de monoterpens de les fulles. Aquest objectiu va contribuir a entendre millor efecte d'una sequera creixent, eutrofització i temperatura sobre les concentracions de terpens en una espècie acumuladora (*P. halepensis* Mill) i en una no acumuladora (*Q. ilex* L.). Ens vàrem fer les següents qüestions de recerca:

1. Quin és l'efecte de la sequera, escalfament i fertilització en la producció de terpens de la fulla?
2. Hi ha diferències al patró de producció de terpens i en quantitats en espècies acumuladores i no acumuladores?

Chapter 3.1 - Different sensitivity of terpene emissions to drought and fertilization in terpene storing *Pinus halepensis* and in non storing *Quercus ilex*

In this work we studied and compared the terpene emission rates of these two dominant species of the Mediterranean ecosystems: *P. halepensis* (a terpene-storing species) and *Q. ilex* (a terpene nonstoring species) in response to increasing water stress and fertilization (nitrogen and phosphorus addition) along a spring-summer growth period. Our aim was to estimate the changes in terpene emissions that can be expected in the next decades if projected climate change and increased ecosystems fertilization occur. Hence, we aimed to answer the following research questions:

1. What are the effects of drought and fertilization on leaf terpene emission rates?
2. Are there differences in the leaf terpene emission pattern in storing and non-storing species?

Capítol 3.1 - Diferent sensibilitat de les emissions de terpens a la sequera i fertilització en l'espècie acumuladora *Pinus halepensis* i en la no acumuladora *Quercus ilex*

En aquest treball vàrem estudiar i comparar les tasses d'emissió de terpens de dues espècies dominants en ecosistemes mediterranis: *P. halepensis* (una espècie acumuladora de terpens) i *Q. ilex* (una espècie no acumuladora de terpens) en resposta a un estrès hídric creixent i a fertilització (addició de nitrogen i fòsfor) al llarg del període de creixement primavera-estiu. El nostre objectiu va ser estimar els canvis en la tassa d'emissió de terpens que es poden esperar a les pròximes dècades si les previsions de canvi climàtic i augment de fertilització dels ecosistemes es fan realitat. Així doncs, ens vàrem fer les següents qüestions de recerca:

1. Quins són els efectes de la sequera i la fertilització a la tassa d'emissió de terpens de la fulla?
2. Hi ha diferències al patró d'emissió de terpens de la fulla en espècies acumuladores i no acumuladores?

Chapter 3.2 - Instantaneous and historical temperature effects on α-pinene emissions in *Pinus halepensis* and *Quercus ilex*

In the present study, we focused on the most emitted terpene for the previous studied species, α-pinene, in order to reduce the variability to the maximum. We aimed to evaluate the relative importance of instantaneous temperature and temperature history in determining α-pinene emissions in *P. halepensis* and in *Q. ilex* L. Emissions were monitored during the entire season simultaneously with leaf environmental conditions and the correlations of emission rates (*E*) and photosynthetic electron transport (J_{CO2+O2}) with instantaneous temperatures and with average temperature over different number of days prior to measurements were determined to assess the strength of instantaneous and historical temperature signals. We aimed to answer the following research questions:

Capítol 3.2 - Efectes de la temperatura instantània i històrica en les emissions d'α-piné en *Pinus halepensis* i *Quercus ilex*

En aquest estudi ens vàrem centrar en el terpé més emès als estudis previs, què és l'α-piné, per a reduir la variabilitat al màxim. Vàrem voler estudiar d'importància relativa de la temperatura instantània i de l'històric de temperatures a les emissions d'α-piné en *P. halepensis* i *Q. ilex*. Les emissions es van mesurar durant una estació sencera junt amb les condicions ambientals de la fulla, i es van determinar les correlacions de la tassa d'emissió (*E*) i del transport fotosintètic d'electrons (J_{CO2+O2}) amb les temperatures instantànies i amb la mitjana de temperatura dels dies anteriors al mostreig per veure la força de la temperatura instantània en comparació amb la temperatura històrica. Ens vàrem fer les següents qüestions de recerca:

1. Are there differences in the temperature dependence of α-pinene of emission rates and photosynthetic electron transport in storing and non-storing species?
2. a) Does the historic temperature (mean temperature of previous days) affect emission rates and photosynthetic electron transport?

   b) Are there any differences between storing and non-storing species?

1. Hi ha diferències en la dependència a la temperatura de la tassa d'emissió del α-piné i del transport fotosintètic d'electrons en espècies acumuladores i no acumuladores?
2. a) Hi ha un efecte de la temperatura històrica (mitjana de la temperatura dels dies anteriors) sobre la tassa d'emissió i el transport fotosintètic d'electrons?

   b) Hi ha diferències entre espècies acumuladores i no acumuladores?

Chapter 4.1 - Effects of phosphorus availability and genetic variation of leaf terpene contents and emission rates in *Pinus pinaster* seedlings susceptible and resistant to the pine weevil *Hylobius abietis*

The aim of this study was to analyze the effect of phosphorus fertilization on total terpene emission rates and on terpene concentrations in half-sib families of *P. pinaster* seedlings cultivated under controlled conditions, previously found to be resistant or susceptible to the large pine weevil in field conditions. We aimed to answer the following research questions:

Capítol 4.1 - Efectes de la disponibilitat de fòsfor i de la variació genètica sobre la producció i la tassa d'emissió de terpens foliars de plançons de *Pinus pinaster* susceptibles i resistents al corc del pi *Hylobius abietis*

L'objectiu d'aquest estudi va ser analitzar l'efecte de la fertilització amb fòsfor sobre la tassa d'emissió de terpens i sobre les concentracions de terpens en famílies de mitjos-germans de *P. pinaster* cultivades sota condicions controlades, i que prèviament s'ha vist que eren resistents o susceptibles a l'atac del corc del pi en condicions de camp. Ens vàrem fer les següents qüestions de recerca:

1. What is the effect of phosphorus deficiency on plant physiology?
2. Are there differences between families on plant physiology?
3. What is the pattern of leaf terpene production and emission in *P. pinaster*?
4. What is the effect of phosphorus deficiency on terpene production?
5. What is the effect of phosphorus deficiency on terpene emission rates?
6. Are there differences between families regarding the terpene production?
7. Are there differences between families regarding the terpene emission rates?

1. Quin és l'efecte de la deficiència de fòsfor sobre la fisiologia de la planta?
2. Hi ha diferències entre famílies pel que fa a la fisiologia de la planta?
3. Quin és el patró de producció i emissió de terpens foliars en *P. pinaster*?
4. Quin és l'efecte de la deficiència de fòsfor en la producció de terpens?
5. Quin és l'efecte de la deficiència de fòsfor en la tassa d'emissió de terpens?
6. Hi ha diferències entre famílies pel que fa a la producció de terpens?
7. Hi ha diferències entre famílies pel que fa a la tassa d'emissió de terpens?

Chapter 4.2 - Investigating the photosynthesis and terpene-content strategies of two different genotypes of *Arabidopsis thaliana* (wild-type and *CoxIV-FANES I* transgenic)

We aimed to test the hypothesis that the *A. thaliana* genotype which is modified to emit nerolidol from mitochondrial synthesis will show other differences in terpene production, and physiology in leaves and roots. We aimed to answer the following research questions:

1. Are there differences between wyld-type and transgenic plants regarding the plant physiology?
2. What is the pattern of leaf terpene production in *A. thaliana*?
3. Are there differences between genotypes of *A. thaliana* regarding leaf terpene production?
4. What is the pattern of root terpene production in *A. thaliana*?
5. Are there differences between genotypes of *A. thaliana* regarding root terpene production?

Capítol 4.2 - Investigació de les estratègies fotosintètiques i de producció de terpens de dos genotips diferents de *Arabidopsis thaliana* (genotip salvatge, i genotip transgènic *CoxIV-FANES1*)

Vàrem voler testar la hipòtesi que el genotip modificat per emetre nerolidol a partir de la síntesi mitocondrial d'*A. thaliana* mostraria diferències en la producció de terpens i en la fisiologia de les fulles i arrels. Ens vàrem fer les següents qüestions de recerca:

1. Hi ha diferències entre el genotip salvatge i el transgènic pel que fa a la fisiologia de la planta?
2. Quin és el patró de producció de terpens foliars a *A. thaliana*?
3. Hi ha diferències entre genotips de *A. thaliana* pel que fa a la producció de terpens foliars?
4. Quin és el patró de producció de terpens a les arrels a *A. thaliana*?
5. Hi ha diferències entre genotips de *A. thaliana* pel que fa a la producció de terpens a les arrels?

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Chapter 2. Drought, warming and soil fertilization effects on leaf volatile terpene concentrations in *Pinus halepensis* and *Quercus ilex*

Efectes de la sequera, escalfament i fertilització del sòl sobre les concentracions de terpens volàtils a les fulles de *Pinus halepensis* i *Quercus ilex*

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2.1. Abstract

The changes in foliar concentrations of volatile terpenes in response to water stress, fertilization and temperature were analyzed in *Pinus halepensis* and *Quercus ilex*. The most abundant terpenes found in both species were α-pinene and Δ^3-carene. β-Pinene and myrcene were also abundant in both species. *P. halepensis* concentrations were much greater than those of *Q. ilex* in agreement with the lack of storage in the latter species (15,205.60 ± 1,140.04 vs 0.54 ± 0.08 μg g^{-1} [d.m.]). The drought treatment (reduction to 1/3 of full watering) significantly increased the total terpene concentrations in both species (54% in *P. halepensis* and 119% in *Q. ilex*). The fertilization treatment (addition of either 250 kg N ha^{-1} or 250 kg P ha^{-1} or both) had no significant effects on terpene foliar concentrations. Terpene concentrations increased from 0.25 μg g^{-1} [d.m.] at 30 ºC to 0.70 μg g^{-1} [d.m.] at 40 ºC. in *Q. ilex* (the non storing species) and from 2,240 μg g^{-1} [d.m.] at 30 ºC to 15,621 μg g^{-1} [d.m.] at 40 ºC in *P. halepensis* (the storing species). Both species presented negative relationship between terpene concentrations and Relative water contents (RWC). The results of this study show that higher foliar terpene concentrations can be expected in the warmer and drier conditions predicted for the next decades in the Mediterranean region.

Key words: fertilization, *Pinus halepensis*, *Quercus ilex*, temperature, Terpene concentration, water stress.

Resum

Es van analitzar els canvis en les concentracions de terpens volàtils en resposta a l'estrès, fertilització i temperatura en *Pinus halepensis* i *Quercus ilex*. Els terpens més abundants que es van trobar en ambdues espècies van ser α-piné i Δ^3-caré. També s'hi van trobar β-piné i mircé en abundància. Les concentracions de *P. halepensis* van ser molt més grans que les de *Q. ilex*, tal i com era d'esperar ja que aquesta segona és una espècie no acumuladora (15,205.60 ± 1,140.04 vs 0.54 ± 0.08 μg g^{-1} [p.s.]). El tractament de sequera (reducció de la dosi complerta de reg a 1/3) va incrementar significativament les concentracions de terpens totals en ambdues espècies (54% a *P. halepensis* i 119% a *Q. ilex*). El tractament de fertilització (adició de 250 kg N ha^{-1} o 250 kg P ha^{-1} o ambdues) no va tenir efectes significatius sobre les concentracions foliars de terpens. Les concentracions de terpens van augmentar des de 0.25 μg g^{-1} [p.s.] a 30 ºC fins a 0.70 μg g^{-1} [p.s.] a 40 ºC. a *Q. ilex* (l'espècie no acumuladora) i des de 2,240 μg g^{-1} [p.s.] a 30 ºC fins a 15,621 μg g^{-1} [p.s.] a 40 ºC a *P. halepensis* (l'espècie acumuladora). Les dos espècies van presentar una correlació negativa entre les concentracions de terpens i el contingut relatiu d'aigua (CRA). Els resultats d'aquest estudi mostren que es poden esperar concentracions més grans de terpens a les fulles sota les prediccions de major escalfor i sequera que s'han fet per a la regió Mediterrània.

Paraules clau: fertilització, *Pinus halepensis*, *Quercus ilex*, temperatura, Concentració de terpens, estrès hídric.

2.2. Introduction

Plants produce Biogenic Volatile Organic Compounds (BVOCs) that include many groups of compounds: isoprene, terpenes, alkanes, alkenes, alcohols, esters, carbonyls and acids (Kreuzwieser *et al* 1999, Peñuelas and Llusià 2001). The most abundant BVOCs are the volatile isoprenoids. Terpenes can be stored in specialized structures (storing plants) or not (non-storing plants). Storing plants have specialized storage organs such as resin ducts (in pines), resin blisters (in firs), glandular trichomes (in mints), or leaf storage cavities (in eucalypts) (Gershenzon and Croteau 1991). Plant terpene concentrations usually are approximately 1-2% of the dry weight, but in some cases they may reach up to 15-20% of the dry weight of a plant (Ross and Sombrero 1991, Langenheim 1994). However, non-storing plants do not have these storing structures and they emit the terpenes immediately after their production (Loreto *et al* 2001). In storing plants the main functions of terpenes appears to be defence against pathogens and herbivores, and wound healing. In non-storing plants the production of isoprenoids could be linked to thermotolerance helping plants to conduct photosynthesis at high temperatures by avoiding cellular membranes damages and alleviating oxidative stress (Sharkey *et al* 2001, Peñuelas and Llusià 2002). Moreover, some recent studies report that BVOCs attract pollinators and herbivore predators, and mediate communication between the plant and other organisms (Peñuelas *et al* 1995, Peñuelas and Llusià 2003, 2004).

The production of terpenes can be affected by diverse factors, both biotic and abiotic (Peñuelas and Llusià 2003).The most important ones are abiotic factors: temperature (Tingey *et al* 1980), light (Banthorpe and Njar 1984), CO_2 concentrations (Peñuelas and Llusià 1997), soil nutrient availability (Schonwitz *et al* 1991), and water availability (Kainulainen *et al* 1992). Water availability, soil nutrient and temperature are three factors directly linked with global change that are very important in Mediterranean ecosystems (Peñuelas and Llusià 2002, 2005, Sardans *et al* 2005, Sardans and Peñuelas 2007).

Global circulation and ecophysiological models project further warming and further aridification for the next decades in the Mediterranean region due to the warming, and the consequent increased evapotranspirations without increases in precipitations (Piñol *et al* 1998, Peñuelas and Llusià 2002, 2005, IPCC 2007). Warming increases the production and emission rates of most terpenes exponentially up to maximum by enhancing the synthesis enzymatic activities, raising the terpene vapour pressure, and decreasing the resistance of emission pathway (Tingey *et al* 1991, Loreto *et*

al 1996, Peñuelas and Llusià 2001). Terpene concentrations have been generally found to increase in drought conditions (Hodges and Lorio 1975, Kainulainen *et al* 1992, Llusià and Peñuelas 1998, Turtola *et al* 2003). However, terpene concentrations may be reduced when the water stress is severe (Bertin and Staudt 1996, Llusià and Peñuelas 1998).

Regarding nutrients, there has been an increase of Nitrogen and Phosphorus availability during the last decades, and their cycles have been altered (Peñuelas and Filella 2001, Rodà *et al* 2002, Sardans and Peñuelas 2004, Sardans *et al* 2006). N availability has increased last decades due to anthropogenic sources such as fertilizers, combustion of fossil fuels and cattle residuals (Vitousek *et al* 1997). P concentrations have increased mostly due to agricultural practices (Rubaek *et al* 2000). Both trends are expected to continue in the next decades with the increasing population and the increasing use of resources. The carbon/nutrient balance hypothesis (CNBH) (Bryant *et al* 1983) and the growth differentiation balance hypothesis (GDBH) (Loomis and Croteau 1973, Lorio 1986) both address how the relative availabilities of resources affect their allocation to the production of new tissue and to the defence of existing tissues. The CNBH predicts that when a resource, such as Nitrogen, is abundant, a plant will allocate proportionately less carbon toward carbon based secondary compounds (reserve and defence) and more toward growth (Lerdau *et al* 1995, Peñuelas and Estiarte 1998). Similar hypotheses can be developed for the availability of other resources such as CO_2, Phosphorus or water (Peñuelas and Estiarte 1998). However, in a study of *Eucalyptus* spp, King *et al* (2004) found that neither water stress, measured using carbon isotope ratios as an indicator, nor nutrient stress, measured as foliar nitrogen and phosphorus content, accounted for observed variation in terpene content.

There are several reports in the literature studying the effects of temperature, drought and nutrients on monoterpene contents in plant foliage (McKinnon *et al* 1998, Turtola *et al* 2003, King *et al* 2004, Rennenberg *et al* 2006), and Rennenberg *et al* (2006) reported a significant interaction between drought and high temperature on VOC formation and nutrient uptake, but there are very few studies investigating the effect of the interaction between these three factors.

The aim of the present work was to present, for the first time, a controlled study of the interaction between temperature, water stress and nutrient doses on monoterpene content of foliage. The results contribute to a better understanding of increasing drought, eutrophication and temperature on terpene concentrations in a terpene-storing (*Pinus halepensis* Mill.) and in a non-storing (*Quercus ilex* L.) Mediterranean species.

2.3. Material and Methods

Study site

This experiment was carried out in a greenhouse (plastic tunnel 28 m long and 6 m wide) located in the experimental fields at the Universitat Autònoma de Barcelona (Bellaterra, Barcelona, NE Spain). This greenhouse is located 147 m above the sea level at the UTM coordinates 31T x = 0425064 y = 459446. It prevents water incomings from rain, and maintains homogeneous Mediterranean-like environmental conditions.

Experimental design

24 two-year-old plants of *Pinus halepensis* purchased in Apromi breeding ground (Juneda, Lleida, Spain) and 24 two-year-old plants of *Quercus ilex* purchased in Forestal Catalana (Breda, Girona, Spain) were grown in 2 l pots containing a mixture of peat (Sphagnum neutral peat, H-Terraplant-1, Compo) and perlite (2:1) from June to August 2004. The peat contained approximately 160 mg l^{-1} of N and 150 mg l^{-1} of P_2O_5. The treatments were distributed randomly in the greenhouse. Previously, they were well watered and maintained in Mediterranean-like environmental conditions until the beginning of the experiment.

Two dose treatments were applied: drought and fertilization. The drought treatment had two doses: Control (C), l liter water per week and plant, and Drought (D), 0.33 liters water per week and plant, half of the pines and oaks were subjected to drought treatment and the other half were control plants. The fertilization treatment had four doses: control (O) without fertilization, 250 kg N ha^{-1} Nitrogen (N), 250 kg P ha^{-1} Phosphorus (P) and both 250 kg N ha^{-1} + 250 kg P ha^{-1} (NP). Chemicals used were NH_4NO_3 for Nitrogen fertilization and $Ca_3(PO_4)_2$ for Phosphorous fertilization (both from Fluka, Buchs, Switzerland). The fertilization dose was distributed homogeneously during the two and a half months of the experiment from June to August. Six plant replicates were conducted for each treatment level.

Sampling and Relative Water Contents analysis

In total, 6 sampling campaigns were conducted, one every 2 weeks from June to mid August. One shoot per *P. halepensis* and one leaf per *Q. ilex* and were cut and immediately put into liquid Nitrogen. These samples were kept in a freezer (-30 ^{o}C) until their analysis. Sampled shoots and leaves were always those submitted to direct radiation (sunlit parts of the plant). Sampled leaves were one-year old well developed leaves.

Relative Water Content (RWC hereafter) was measured in each plant on each of the six sampling dates. The sampling procedure followed the steps described by Barrs and Weatherley (1962): fresh weight (FW) was obtained for 3-4 *P. halepensis* needles and one *Q. ilex* leaf by the difference between [tube+water+leaf] and [tube+water]. After at least 12 hours of moisturizing, the saturated weight (SW) was obtained. Dry weight (DW) was obtained after 72 hours at 60^{o}C until weight constancy. The following formula was applied to obtain the RWC:

$$RWC = \frac{(FW - DW)}{(SW - DW)}$$

Leaf temperature was also calculated using an ADC-LCA4 (ADC Inc. Hoddesdon, Hertfordshire, England) connected to a cuvette model PLCA4 (ADC Inc. Hoddesdon, Hertfordshire, England).

Laboratory analyses: terpene contens

Terpene extraction method from the frozen samples was different in *P. halepensis* than in *Q. ilex* (Llusià and Peñuelas 1998). Three-four needles of *P. halepensis* were introduced in a Teflon tube with liquid Nitrogen and they were mechanically crushed with a Teflon embolus in order to extract terpenes. 1 ml of pentane was added together with a non-terpenoid internal standard (0.1 μl of dodecane). This sample was centrifuged 5 minutes at 5000 rpm and 5-10^{o}C in order to separate the liquid and solid phases. 3 μl of the extract were directly injected into a GC-MS (model Hewlett Packard HP59822B, Palo Alto, California). Each *Q. ilex* leaf was introduced in a Teflon tube filled with liquid Nitrogen and then placed into a glass of boiling water. When liquid Nitrogen disappeared, volatile terpenes were liberated from the leaf, carried by a stream of Nitrogen and trapped in a multibed cartridge (Carbotrap C (300 mg), Carbotrap B (200 mg) and Carbosieve S-III (125 mg) from Supelco (Belmonte, Pa)). VOCs adsorbed in these cartridges were analyzed by GC-MS being previously desorpted in a Thermal Desorbtion Unit (model 890/891, Supelco, INC, Bellefonte, Pennsylvania) during 4 minutes to 250 ^{o}C using Helium as a carrier gas and injected into a 30 m x 0.25 mm x 0.25 μm film thickness capillary column (Supelco HP-5, Crosslinked 5% pH Me Silicone). Full scan method was used to perform the chromatography. After sample injection at 40 ^{o}C, temperature was increased at 30 ^{o}C min^{-1} up to 70 ^{o}C, and thereafter at 10 ^{o}C min^{-1} up to 150 ^{o}C, where temperature was maintained for 5 min, and thereafter at 70 ^{o}C min^{-1} up to 250 ^{o}C, which was maintained for another 5 min. Helium flow was 1 ml min^{-1}. For both species, 2 blank analyses per day were also conducted.

The identification of terpenes was conducted by GC-MS and comparison with standards from Fluka (Buchs, Switzerland), literature spectra and GCD Chemstation G1074A HP with the Wiley275 library. Terpene calibration curves (n=4 different terpene concentrations) were always significant (R^2>0.99) in the relationship between signal and terpene concentration. The most abundant terpenes had very similar sensitivity (differences were less than 5%). Total terpene concentrations were calculated as the sum of these main terpenes.

Leaf dry mass weight was determined after drying the residual vegetal material at 60 ^{o}C for 48 hours.

P and N leaf concentrations

P and N leaf concentrations were measured once at the end of the sampling period. P concentrations were analyzed by atomic emission spectroscopy with Inductive Coupled Plasma (ICP-AES). Needles and leaves were crushed and dried at 60 ^{o}C during 48 hours. The ICP-AES analyses were conducted after acid digestion (HNO_3:$HClO_4$, 2:1, v/v) in a microwave Moulinex Optiquick Duo Y92 using open fluorinated ethylene propylene flasks (Nalge Company, Rochester, UK). The concentrations were determined in a Polyscan Thermo Jarrel ASH Model 61 E spectrophotometer (Waltham, MA, USA).

N concentrations were analyzed by combustion followed by gas chromatography (GC) using a NA2100 C.E. Instrument (Thermo Electron, Milano, Italy). The sample was prepared weighting between 1 and 2 mg of previously ground leaves in a tin small capsule and adding 2 mg of Vanadium Pentoxid as an oxidant additive.

Statistical analyses

Repeated measures analyses of variance (RM-ANOVA) were conducted for total terpene concentrations as dependent variable and the two treatments (drought and fertilization) as independent variables. Differences between control and drought for the drought treatment and between control and N, P, NP for the fertilization treatment were compared with Fisher post-hoc tests. Correlation analyses were conducted among the studied variables: leaf RWC, leaf concentrations of Nitrogen and Phosphorus and leaf concentrations of terpenes. All these analyses were conducted with the software package STATISTICA 6.0 (StatSoft Inc, Tulsa, USA).

2.4. Results

RWC was reduced by drought treatment from 0.92 ± 0.01 to 0.89 ± 0.01 and from 0.88 ± 0.01 to 0.86 ± 0.01 for *P. halepensis* and *Q. ilex* respectively (data not shown). The drought treatment decreased the growth of the stem diameter of *P. halepensis* ($P<0.01$) but not of *Q. ilex* (Fig. 1). However, despite the fact that *Q. ilex* drought plants were not significantly different from the well watered ones for the overall period of measures (all data), there were significant differences ($P<0.01$) at the end of the experiment (6th measure, julian day 224). There was also a significant effect ($P<0.001$) for the time course and for the interaction drought per time, since in both species the drought effect became more significant with time course (*Fig.1*). There were no significant differences in plant height between control and drought plants for both species. Nevertheless there was a significant effect ($P<0.001$) on plant height for the time course in both species, *P. halepensis* and *Q. ilex*, and there was a significant interaction effect between drought and time ($P<0.001$) for *P. halepensis*. No significant effect of fertilization treatments was found neither on stem diameter nor on plant diameter (data not shown).

No significant responses of leaf concentrations of Nitrogen and Phosphorus to fertilization treatment were found neither in *P. halepensis* nor in *Q. ilex* (*Fig.2*). However, drought treatment significatively increased ($P<0.001$) N concentrations in *P. halepensis* and tended to do it also in *Q. ilex* (*Fig.2*). However, the drought treatment had no significant effect on *Q. ilex* N concentrations when NP and N treatments were applied.

The main terpenes detected in both species were: α-pinene, β-pinene, β-myrcene, Δ^3-carene, 2-carene, camphene, sabinene, sabinene, β-phellandrene, limonene and β-ocymene (*Table 1*). There was a huge difference for total terpene concentrations corresponding to the species' capacity or incapacity of storing terpenes in specialized organs between *P. halepensis* and *Q. ilex* (*Fig.3*): The average terpene concentration was 14,866 ± 1,678 $\mu g\ g^{-1}$ [d.m.] for *P. halepensis* needles and 0.39± 0.06 $\mu g\ g^{-1}$ [d.m.] for *Q. ilex* leaves.

Drought significatively increased total terpene concentrations from 13,425.57 ± 330.43 μg g-1 [d.m.] to 21,127.45 ± 3,656.47 μg g^{-1} [d.m.] in non-fertilized plants of *P. halepensis* ($P<0.05$, mean of the overall period of measures, n=72, all data) (*Fig.3*) and from 0.324 ± 0.0395 μg g^{-1} [d.m.] to 0.749 ± 0.141 μg g^{-1} [d.m.] in non-fertilized plants of *Q. ilex* ($P<0.001$, mean of the overall period of measures, n=72, all data) (*Fig.3*). The drought effect of increasing terpene concentrations in the control *P. halepensis* plants is evident in P ($P<0.05$) and NP (not significant, but a trend, $P<0.1$) fertilized plants, but N fertilization alone canceled this effect (*Fig.3*). This was not observed in *Q. ilex*.

Fertilization treatments did not affect significantly terpene concentrations in any of the two studied species (*Fig.3*). Moreover, only *P. halepensis* showed significant differences for drought treatment in control (O, $P<0.1$) and phosphorus (P, $P<0.05$) fertilization treatment (*Fig.3*).

The most abundant terpenes in *P. halepensis* and *Q. ilex* were α-pinene, β-pinene, β-myrcene and Δ^3-carene (*Fig.4*). For *P. halepensis* plants, drought significantly increased α-pinene, β-myrcene and Δ^3-carene concentrations except when there was a nitrogen addition (*Fig.4*). These greater concentrations of terpenes in droughted plants were especially significant in *P. halepensis* plants with Phosphorus addition, especially for the most abundant terpenes: α-pinene and Δ^3-carene. On the other hand, no differences were found between the fertilization treatments (*Fig.4*). Drought treatment significatively increased β-pinene and Δ^3-carene total concentrations in *Q. ilex* plants ($P<0.01$ and $P<0.05$ respectively) (*Fig.4*). No differences were found between different fertilization treatments.

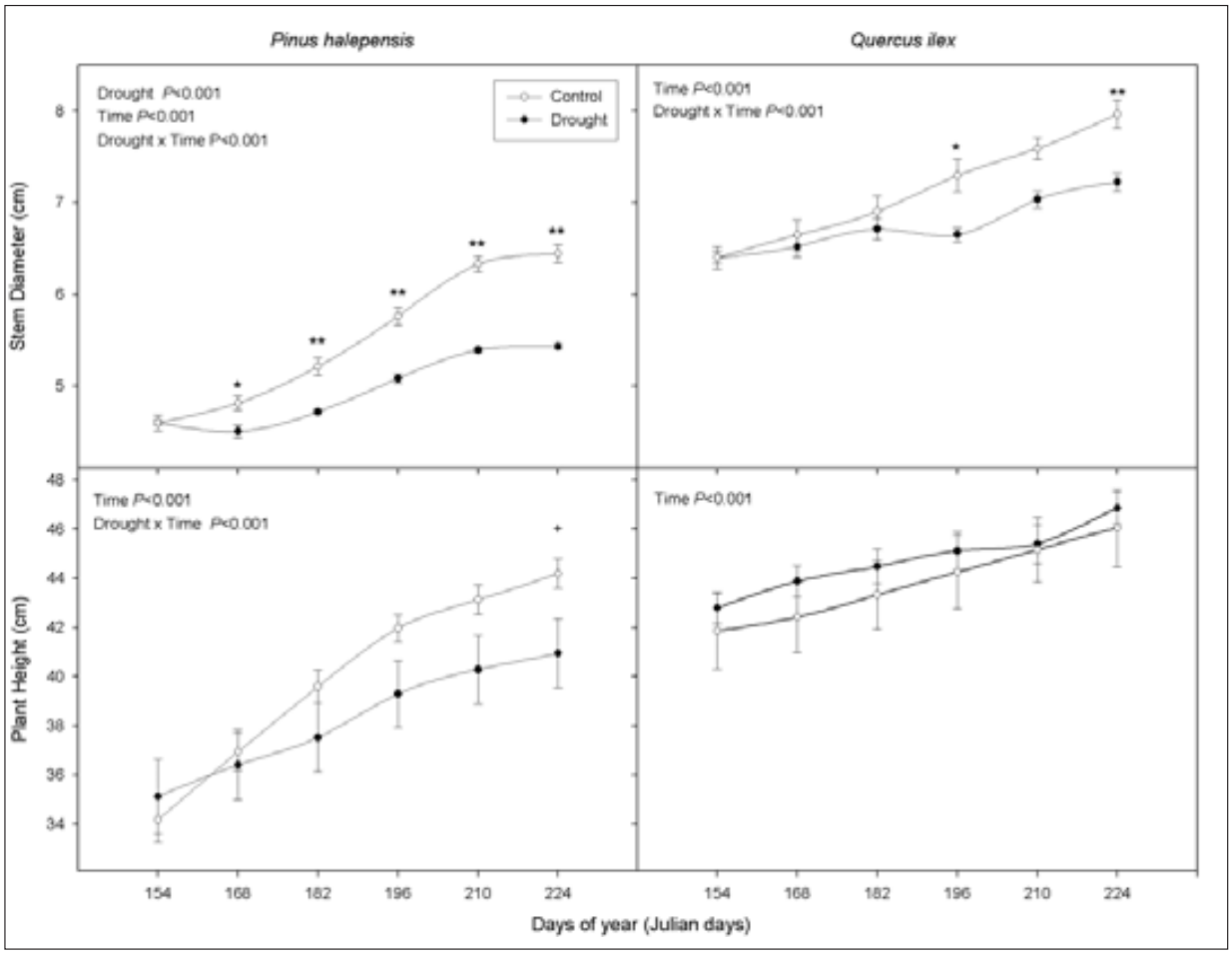

*Fig.1 - Stem diameter and plant height (cm) along the experiment (julian days) for P. halepensis and Q.ilex under control and drought treatments. Vertical bars indicate standard errors of the mean (n=12). Statistical significance for the overall effect of drought on stem diameter and plant height (repeated measurements ANOVA) is indicated inside the panels. ** P<0.01, * P<0.05 , + P<0.1*

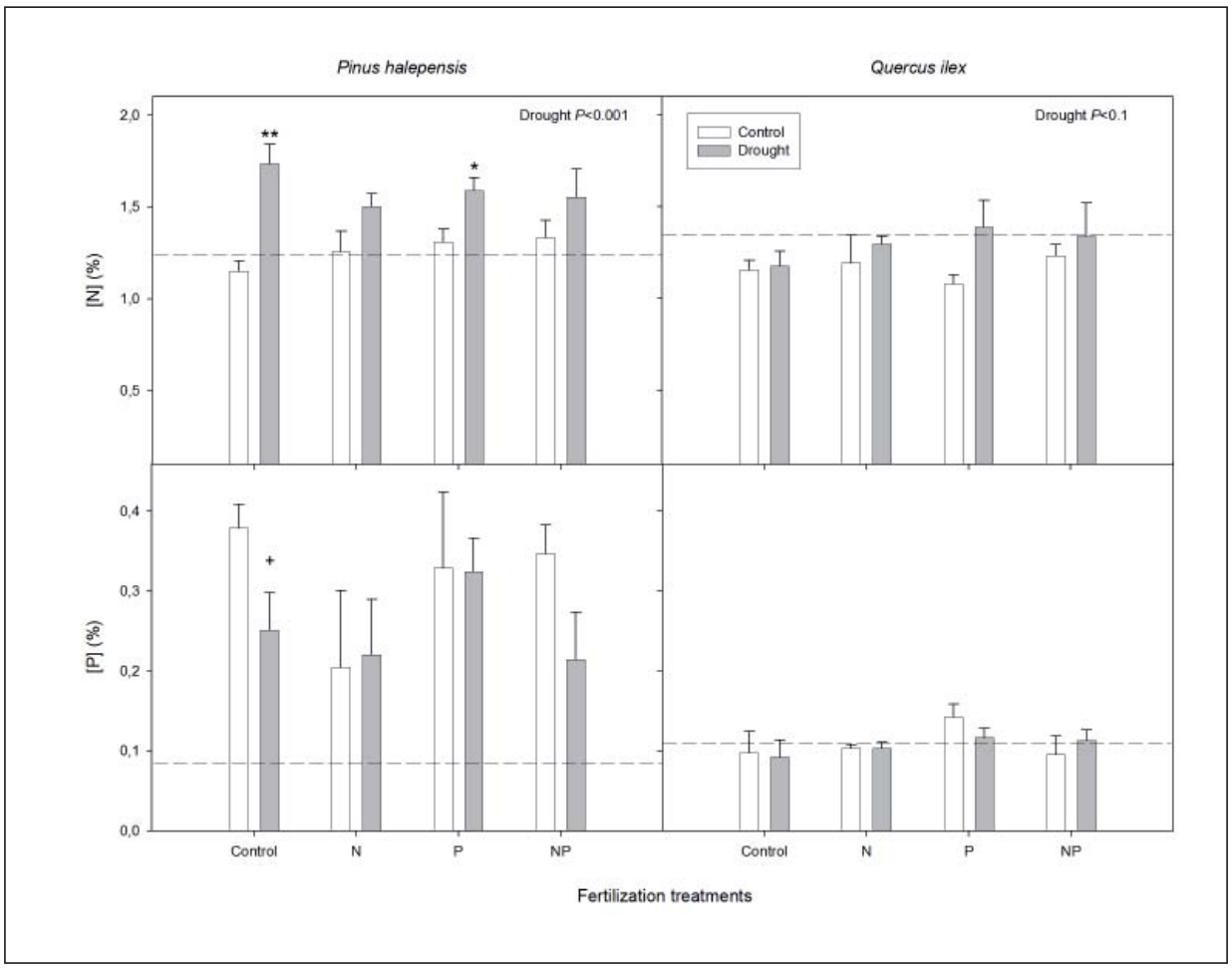

*Fig.2 - Leaf concentrations of Nitrogen (N) and Phosphorus (P) for the fertilization treatments (Control, N = Nitrogen, P = Phosphorus, NP = Nitrogen + Phosphorus), for the two studied species, P. halepensis and Q. ilex. Vertical bars indicate standard errors of the mean (n=3) Statistical significance for the overall effect of drought on Leaf concentrations of Nitrogen (N) and Phosphorus (P) (ANOVA) is indicated inside the panels. ** P<0.01 ,* P<0.05 , + P<0.1. Slashed line indicates the average values in Spain according to EC-UN/ECE-FBVA (1997)*

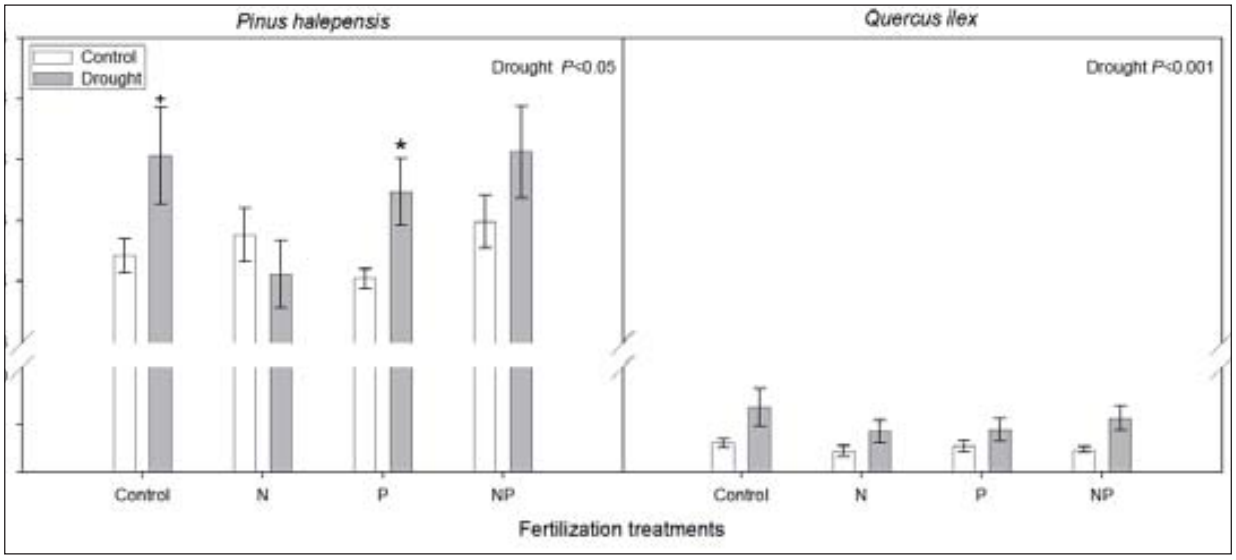

*Fig.3 - Total terpene concentrations, for drought and fertilization treatments (Control, N = Nitrogen, P = Phosphorus, NP = Nitrogen + Phosphorus), and for P. halepensis and Q. ilex plants, expressed in μg g^{-1} [d.m.]. Vertical bars indicate standard errors of the mean (n=4 sampling dates means of 3 plant replicates each for P. halepensis and 3 sampling dates means of 3 plant replicates each for Q. ilex). Statistical significance for the overall effect of drought on monoterpene concentrations (repeated measurements ANOVA) is indicated inside the panels. * P<0.05 , + P<0.1*

Table 1 - Total terpene leaf concentrations (μg g^{-1}[d.m.]) (mean ± S.E., n=12) for P. halepensis and Q. ilex plants for drought and control treatments.

Pinus halepensis

Treatment	α-Pinene	β-Pinene	β-Myrcene	Δ^3-Carene	2-Carene	Camphene	Sabinene	Limonene	β-Ocimene	β-Phellandrene	Caryophyllene	α-Caryophyllene
June 16 2004												
Control	4,837.5±128.1a	534.1±65.4a	605.6±141.5a	2,135.2±62.6a	**123.6±10.1a**	91.4±32.5a	123.6±18.1a	0.0±0.0a	0.0±0.0a	46.7±25.0a	76.7±16.5a	29.7±6.6a
Drought	4,729.3±1,435.5a	589.2±218.2a	519.2±160.5a	2,000.3±666.8a	**55.6±21.8b**	64.0±27.7a	78.3±27.7a	0.0±0.0a	13.4±10.1a	19.0±5.3a	49.2±4.1a	20.4±2.0a
June 30 2004												
Control	11,295.2±2689.8a	1,196.9±214.5a	1,402.4±104.8a	4,564.7±521.5a	264.8±48.2a	95.7±25.9a	162.8±59.2a	0.0±0.0a	**0.9±0.9a**	52.9±10.3a	245.2±72.5a	117.1±40.0a
Drought	15,300.7±3599.3a	1,212.0±228.7a	1,863.1±660.2a	5,382.9±1,546.7a	368.1±115.0a	134.7±29.6a	259.2±108.6a	0.0±0.0a	**59.6±20.6b**	64.4±18.2a	328.0±128.7a	157.7±66.7a
July 28 2004												
Control	5,059.5±475.4a	374.5±36.7a	723.9±124.6a	1,955.3±124.6a	86.9±12.7a	54.0±8.7a	53.9±7.7a	0.0±0.0a	11.1±7.8a	**16.1±1.7a**	71.8±12.5a	135.0±53.3a
Drought	8,833.4±3,896.6a	809.6±380.1a	1,336.0±368.8a	5,179.7±2,698.3a	308.0±170.9a	122.4±55.1a	130.0±79.5a	0.6±0.6a	12.5±3.7a	**70.3±29.6b**	216.9±99.4a	132.5±42.4a
August 11 2004												
Control	7,873.2±764.6a	650.2±57.2a	1,034.6±224.3a	3,206.4±157.7a	156.0±15.1a	75.0±8.5a	**74.1±13.5a**	2.0±2.0a	**4.5±0.9a**	45.6±13.0a	153.0±20.0a	155.0±5.2a
Drought	9,033.3±2,036.0a	776.1±226.6a	1,668.9±665.0a	4,651.2±1,316.9a	241.1±67.8a	94.3±30.6a	**141.0±28.7b**	0.0±0.0a	**17.7±5.7b**	63.1±17.6a	251.5±74.8a	209.5±54.6a

Quercus ilex

Treatment	α-Pinene	β-Pinene	β-Myrcene	Δ^3-Carene	Limonene	β-Ocimene	α-Phellandrene	Caryophyllene	α-Caryophyllene
June 16 2004									
Control	0.059 ± 0.020a	0.012 ± 0.007a	0.004 ± 0.004a	0.009 ± 0.004a	0.001 ± 0.001a	0.0±0.0a	0.0±0.0a	0.017 ± 0.006a	0.013 ± 0.007a
Drought	0.039 ± 0.015a	0.003 ± 0.002a	0.006 ± 0.006a	0.008 ± 0.004a	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.013 ± 0.007a	0.008 ± 0.008a
July 14 2004									
Control	0.131 ± 0.012a	**0.029 ± 0.001a**	0.051 ± 0.023a	0.069 ± 0.019a	0.0±0.0a	0.0±0.0a	0.004 ± 0.004a	0.045 ± 0.020a	0.037 ± 0.012a
Drought	0.175 ± 0.040a	**0.109 ± 0.025b**	0.080 ± 0.023a	0.104 ± 0.046a	0.02 ± 0.02a	0.0±0.0a	0.005 ± 0.005a	0.042 ± 0.017a	0.068 ± 0.033a
August 11 2004									
Control	**0.116 ± 0.023a**	**0.050 ± 0.006a**	0.050 ± 0.015a	**0.036 ± 0.011a**	0.004 ± 0.004a	**0.002 ± 0.002a**	0.0±0.0a	0.017 ± 0.004a	0.008 ± 0.002a
Drought	**0.212 ± 0.023b**	**0.133 ± 0.033b**	0.067 ± 0.024a	**0.156 ± 0.026b**	0.188 ± 0.124a	**0.051 ± 0.015b**	0.0±0.0a	0.048 ± 0.020a	0.035 ± 0.020a

Different letters indicate significant differences between drought and control treatment (t-student, P<0.05). They are highlighted in bold type.

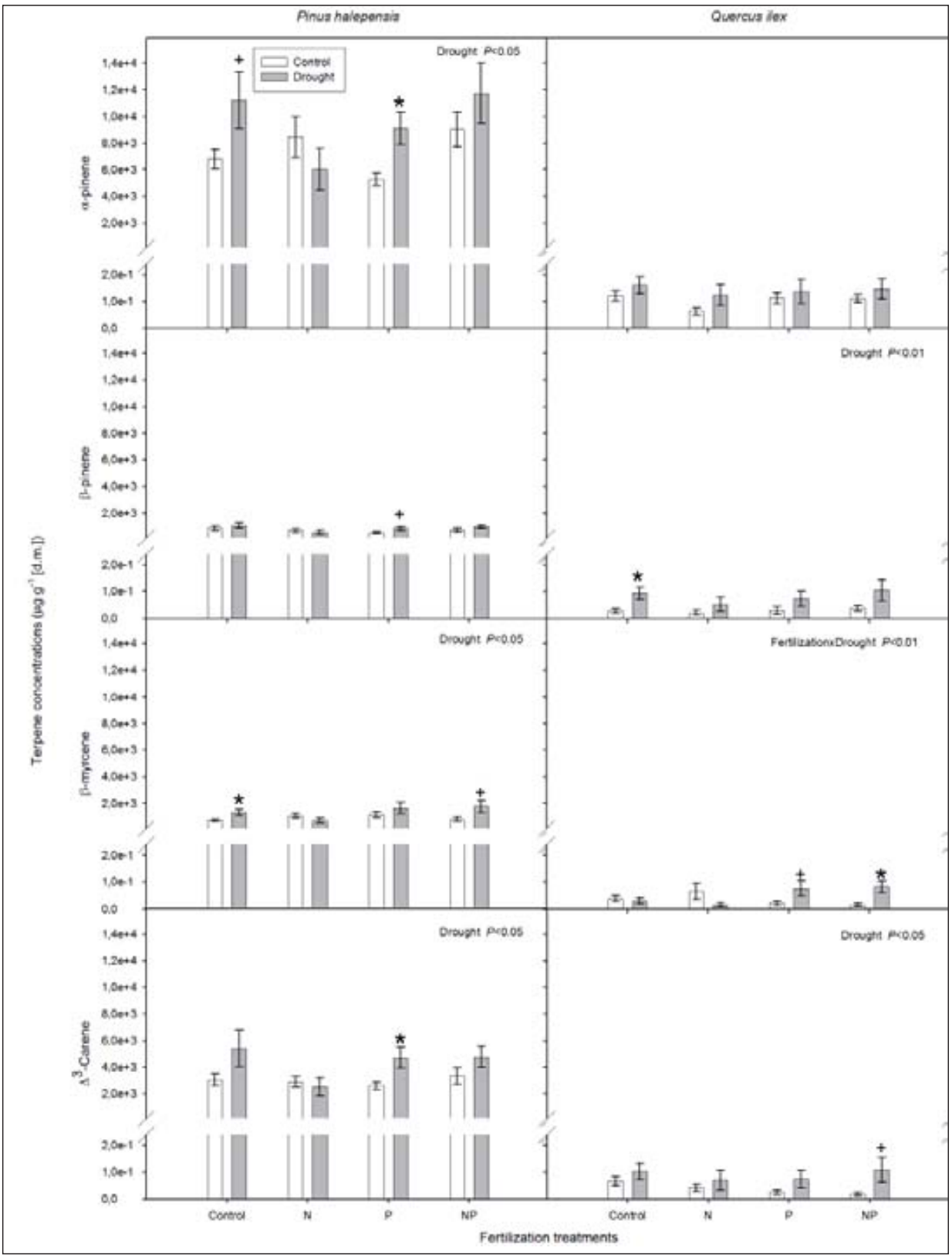

*Fig.4 - Concentrations (µg g^{-1}[d.m.]) of the most abundant terpenes for drought and fertilization treatments (Control, N = Nitrogen, P = Phosphorus, NP = Nitrogen + Phosphorus), in P. halepensis and Q. ilex plants. Vertical bars indicate standard errors of the mean (n=4 sampling dates means of 3 plant replicates each for P. halepensis and 3 sampling dates means of 3 plant replicates each for Q. ilex). Statistical significance for the overall effect of drought on monoterpene concentrations (repeated measurements ANOVA) is indicated inside the panels. * P<0.05, + P<0.1*

Drought treatment tended to increase total terpene concentrations in both species during the experiment especially in *Q. ilex* which presented a significant interaction between drought and time ($P< 0.001$, ANOVA) (*Fig.5*). This increase was independent from the fertilization treatment.

A negative correlation was found between total terpene concentrations and RWC for both species *P. halepensis* ($R^2=0.37$, $P<0.01$) and Q. ilex ($R^2=0.37$, $P<0.01$) (*Fig.6*). There were no significant relations between total terpene concentrations and N (*Fig.6*) and P (data not shown) leaf concentrations for *P. halepensis*, but in *Q. ilex* plants total terpene concentrations increased with leaf Nitrogen concentration (*Fig.6*). *Q. ilex* terpene concentrations of α-pinene, β-pinene, β-myrcene and Δ^3-carene also showed a tendency to increase with leaf Nitrogen concentration ($R^2=0.15$, 0.15 and 0.13 respectively, $P<0.1$, data not shown).

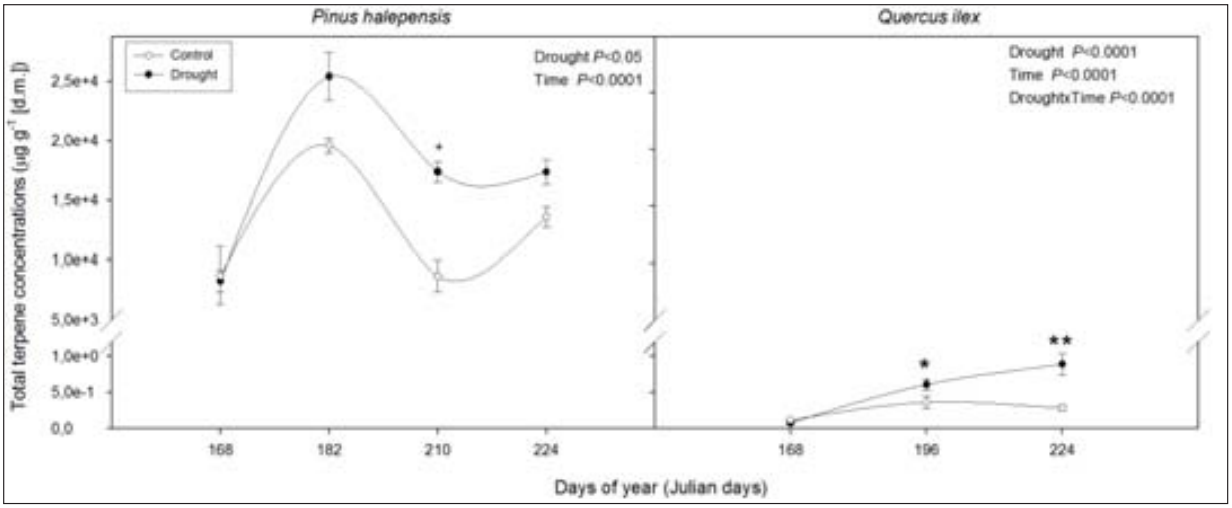

Fig.5 - Total terpene concentrations expressed in µg g-1 [d.m.] along the experiment, for control and drought treatments and for P. halepensis and Q. ilex. Vertical bars indicate standard errors of the mean (n=3). Statistical significance for the overall effect of drought on monoterpene concentrations (repeated measurements ANOVA) is indicated inside the panels . P<0.05, ** P<0.01, + P<0.1. Treatment started in 148 julian day*

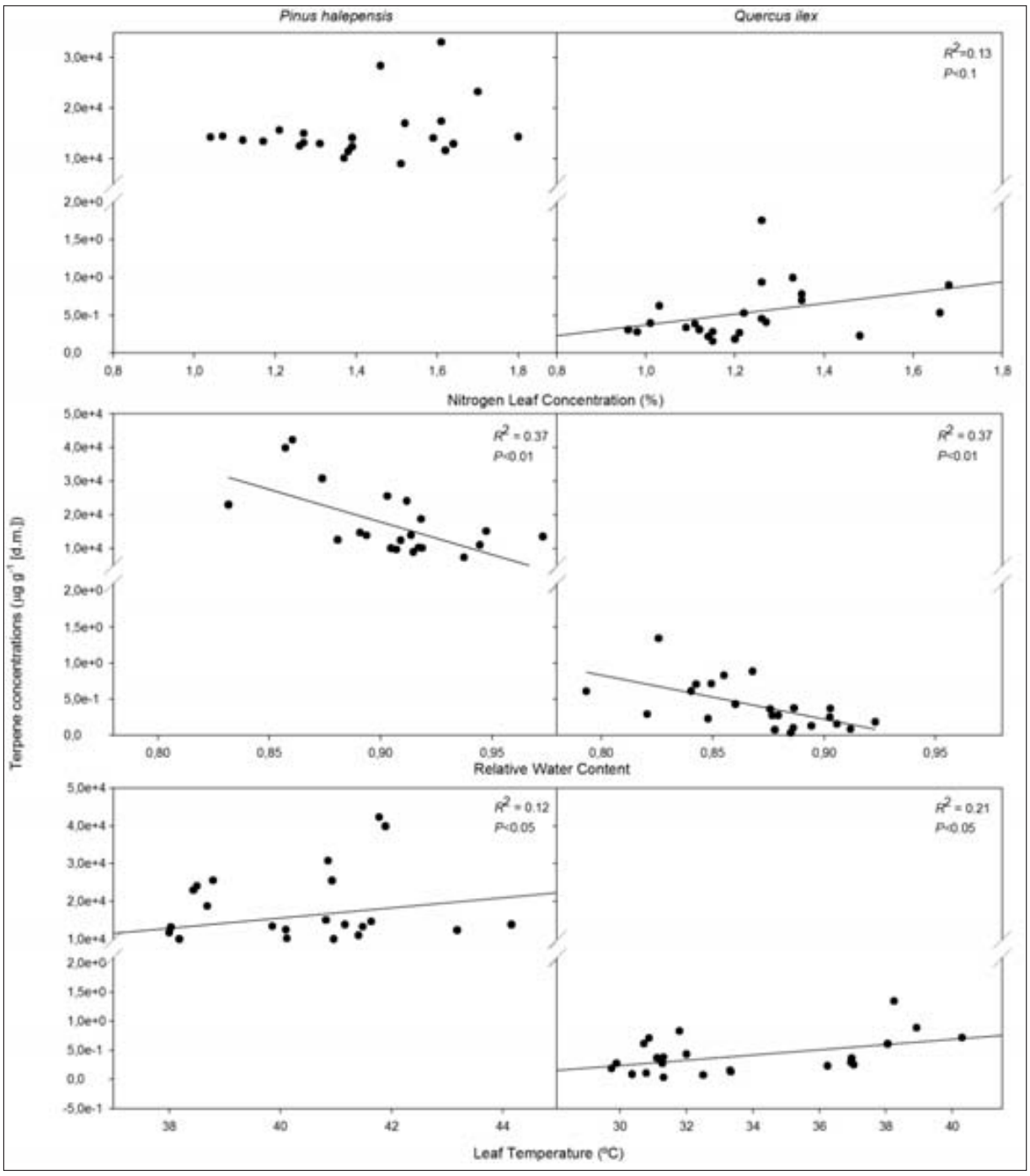

Fig.6 - Relationships of total terpene concentrations with leaf relative water contents, temperature and foliar nitrogen concentrations, for P. halepensis and Q. ilex plants (n=24)

2.5. Discussion

There was a large difference between terpene concentrations in *P. halepensis* and *Q. ilex* species, corresponding to a storing and a non storing species, respectively. Previous authors have also reported the existence of concentrations of these compounds in *P. halepensis* needles (Llusià and Peñuelas 2000, Llusià *et al* 2006), and in *Q. ilex* leaves (Loreto *et al* 2001, Peñuelas and Llusià 2002, Llusià *et al* 2005). However, other studies have found that the most abundant terpenes in *P. halepensis* are different ones, for example limonene (Llusià and Peñuelas 2000). These differences show once more the variable terpene content of different ecotypes and even individuals (Staudt *et al* 2001). The existance of VOCs on leaves can be interpreted as physiological effects (protection against oxidative and high temperature stress), ecological effects (VOCs as signals: pollination attractors, herbivore deterrents) (Peñuelas and Llusià 2003) and environmental effects (VOCs contribution to wild-fire, i.e., Alessio *et al* (2008) show correlation of VOC content and flammability in *P. halepensis* and *Q. ilex*).

Total terpene concentrations increased significantly under the experimental drought conditions in the two studied species *P. halepensis* and *Q. ilex* ($P<0.05$ and $P<0.001$, respectively). There was no effect of drought in the N fertilized *P. halepensis* plants (*Fig.3*), perhaps because of enhanced synthesis of amino acids such as proline, which increases resistance to drought (Vendruscolo *et al* 2007). However, not all terpene compounds increased in drought, and these different responses may be linked either to different effects of drought on particular terpene synthase enzymes, or to possible different protective roles for different terpene compounds in the face of drought.

The effect of water-stress increasing monoterpene concentrations has been previously reported by several authors in many storing and non-storing species (Hodges and Lorio 1975, Gershenzon *et al* 1978, Kainulainen *et al* 1992, Llusià and Peñuelas 1998, Loreto *et al* 2001, Delfine *et al* 2005). Drought-induced monoterpene concentration increases was also verified by the significant negative correlations between terpene concentrations and relative water content (*Fig.6*). Moreover, drought plants grew less than control plants in terms of plant height and stem diameter (*Fig.1*) allowing for a larger fraction of carbon being allocated to monoterpenes formation instead of allocating it to growth (Bradford and Hsiao 1982, Llusià and Peñuelas 1998, Peñuelas and Estiarte 1998). It seems that in typical drought-stress conditions of hot Mediterranean summer days with decreased photosynthetic rates and stomatal conductances at midday, an important part

of photosynthetic C fixation is still used for terpene production and emission (Yani *et al* 1993, Peñuelas and Llusià 1999, Vallat *et al* 2005). Accumulation of monoterpenes in water-stressed leaves may have ecological functions such as defence or storage (Peñuelas and Estiarte 1998). Drought induces oxidative stress in plants. Plants can reduce the damage caused by oxidative stress with monoterpenes. Thus, plants with more compounds can fight better against oxidative stress. For this reason, storage makes plants more resistant in front of oxidate stress in drought conditions. For the same reason, the bigger the pool size, the higher capability of the plant to respond to oxidative stress. Under stress conditions the build-up of secondary compounds like terpenes could replace photorespiration in protection from photodamage (Peñuelas and Llusià 2002). However, in the non-storing species *Q. ilex*, a decrease of monoterpene production could be expected with drought, considering that *Q. ilex* monoterpene biosynthesis like isoprene is directly dependent on the photosynthetic activity (Niinemets *et al* 2002). Net photosynthetic rates and stomatal conductance decreased with drought (data not shown, (Blanch *et al* 2007)) Heat and drought have a negative effect on photosynthesis by deactivating 1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Rennenberg *et al* 2006). However, *Q. ilex* monoterpene concentrations increased with heat and drought. The response observed could be explained by the fact that monoterpene production is less sensitive than photosynthetic activity to water stress (Bertin and Staudt 1996), partly because the lack of terpene storage structures may be compensated by an increase in the internal BVOCs concentrations in both lipid and aqueous phases of leaves (Niinemets *et al* 2004). These results suggest that under heat and drought stress there may be alternative unknown sources for monoterpene production as suggested in previous studies (Plaza *et al* 2005, Ormeño *et al* 2007, Brilli *et al* 2007).

Terpene concentrations increased progressively during summer in *Q. ilex* following increasing temperatures, and differences between control and drought plants became significant especially in August, when temperature was higher, thus supporting the existence of an interaction between drought and temperature (*Fig.5*). This fact indicates that concentrations of individual terpenes are more sensitive to environmental changes in the non-storing species *Q. ilex* than in the storing species *P. halepensis*, as expected given the much lower concentrations.

There was not effect of fertilization on growth. The effect of fertilization on terpene concentrations was not wholly clear (trends but not significant). Several previous studies have found no relation between terpene concentrations and nutrient addition (Muzika *et al* 1989, Manninen *et al* 1998). In the present experiment, P fertilization had no significant effect on its own, but when there was P fertilization and drought effect which was highly significant in *P. halepensis* plants for the total terpene contents, and for the principal individual terpenes: α-pinene and Δ^3-carene. This effect may be due to the fact that increasing drought decreases P availability (Sardans and Peñuelas 2004). On the contrary, P fertilization had no significant effect on *Q. ilex* leaf terpene concentrations.

Q. ilex plants increased terpene concentrations with the increasing N leaf concentration (*Fig.6*) resulting from the drought treatment (*Fig.2*). Higher nitrogen concentration in leaves might indicate higher enzyme activity resulting in more terpene production in a species like *Q. ilex* which is dependent on short-term production (Litvak *et al* 1996). No significant relationship was found between foliar terpene concentrations and increasing nitrogen leaf concentrations in the terpene-storing species *P. halepensis*. The basal nutrient levels in the soil (not measured) and in the leaves were probably high enough to make the response of growth and terpene concentrations not limited by N and P. Plants generally control their N and P uptake and will not take up much more than their requirements (Barceló *et al* 1992). So adding nutrients to a growth medium (peat and perlite) which already contains sufficient nutrients, is not likely to change N, P or terpene content. However, the drought situation is likely to very much change the plants' metabolism and requirements for nutrients and terpenes. N and P leaf concentrations were in the range or slightly above the range of values given by the European Commission-United Nations/Economic Commission for Europe (1997) for *P. halepensis* and *Q. ilex* in Spain (*Fig.2*).

In summary, monoterpene concentrations were 54% higher for drought than for control plants in *P. halepensis* and 119% higher for drought than for control plants in *Q. ilex*. The fertilization treatments conducted in this study had no significant effects on terpene foliar concentrations, but the increased N foliar concentrations generated by the drought treatment were accompanied by increased terpene concentrations in *Q. ilex*. Terpene concentrations in *P. halepensis* and *Q. ilex* increased with higher temperatures of summer. All together these results show that higher terpene concentrations can be expected in the warmer and drier conditions projected for the next decades in the Mediterranean region by climatic and ecophysiological models (Peñuelas *et al* 2005, IPCC 2007).

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Chapter 3.1. Different sensitivity of terpene emissions to drought and fertilization in terpene storing *Pinus halepensis* and in non storing *Quercus ilex*

Diferent sensibilitat de les emissions de terpens a la sequera i fertilització en l'espècie acumuladora *Pinus halepensis* i en la no acumuladora *Quercus ilex*

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3.1.1. Abstract

We studied the effects of water stress, fertilization and time course on foliar volatile terpene emission rates by *Quercus ilex* and *Pinus halepensis* in a garden experiment. The terpenes mostly emitted by both species were α-pinene, β-pinene, β-myrcene and Δ^3-carene. *P. halepensis* emission rates (average 31.45 μg g^{-1} [d.m.] h^{-1}) were similar to those of *Q. ilex* (average 31.71 μg g^{-1} [d.m.] h^{-1}). The effects of drought (reduction to 1/3 of full watering) and fertilization (250 kg N ha^{-1}, 250 kg P ha^{-1}, or both) were different depending on the species: the drought treatment significantly increased the terpene emissions from *Q. ilex* by 33% and the fertilization treatments reduced the terpene emissions from *P. halepensis* by 38%. Terpene emission rates increased with time course in parallel to raising summer temperatures in *P. halepensis* and *Q. ilex*, whose emission rates were temperature related ($r = 0.42$ and $r = 0.68$ respectively) and light related ($r = 0.32$ and $r = 0.57$ respectively). There was a positive relationship for *P. halepensis*, and a negative relationship for *Q. ilex*, between emission rates and relative water contents. No relationship was found between emission rates and N or P foliar concentrations. The results of this study show complex species-specific responses with stronger and faster short term responses in terpene non-storing than in storing species and indicate that terpene emissions may significantly change in the warmer, drier and more fertilized conditions predicted for the next decades in the Mediterranean region.

Key words: VOC, monoterpene emission rates, *Pinus halepensis*, *Quercus ilex*, fertilization, drought stress.

Resum

Vàrem estudiar els efectes de l'estrès hídric, fertilització i el pas del temps en la tassa d'emissions de terpens volàtils de *Quercus ilex* i *Pinus halepensis*, en un experiment en hivernacle. Els terpens que més van emetre ambdues espècies van ser α-piné, β-piné, β-mircé i Δ^3-caré. La tassa d'emissions de *P. halepensis* (de mitjana 31.45 μg g^{-1} [p.s.] h^{-1}) va ser similar a la de *Q. ilex* (de mitjana 31.71 μg g^{-1} [p.s.] h^{-1}). Els efectes de la sequera (reducció fins a 1/3 de la dosi de reg) i la fertilització (250 kg N ha^{-1}, 250 kg P ha^{-1} o ambdues) van ser diferents depenent de l'espècie: el tractament de sequera va incrementar significativament les emissions de terpens de *Q. ilex* en un 33% i la fertilització va disminuir les emissions de *P. halepensis* en un 38%. La tassa d'emissió de terpens va augmentar amb el pas del temps conjuntament amb l'increment de temperatures

de l'estiu tant en *P. halepensis* com en *Q. ile*x, les emissions dels quals van estar correlacionades tant amb la temperatura (r = 0.42 i r = 0.68 respectivament) com amb la llum (r = 0.32 i r = 0.57 respectivament). Hi va haver una correlació positiva per a *P. halepensis* i negativa per a *Q. ile*x de la tassa d'emissió de terpens amb el contingut relatiu d'aigua. No es va trobar relació entre la tassa d'emissió de terpens i les concentracions foliars de N i P. Els resultats d'aquest ens mostren respostes complexes espècie-específiques i respostes ràpides a curt termini a les espècies no acumuladores de terpens en comparació amb les espècies acumuladores, i indican que les emissions de terpens probablement canviaran si les prediccions de més escalfor i sequera per a l'àrea mediterrània en les pròximes décades es fan realitat.

Paraules clau: COV, tassa d'emissió de terpens, *Pinus halepensis*, *Quercus ile*x, fertilització, estrès hídric

3.1.2. Introduction

Volatile Organic Compunds (VOCs) have an important role in atmospheric chemistry (Singh and Zimmerman 1992, Lerdau and Peñuelas 1993) and particularly in the development of aerosols and ozone (Andreae and Crutzen 1997). Went (1960) already recognized that foliar emissions of VOCs could have a significant impact on tropospheric chemistry by influencing the processes that control the formation of atmospheric haze.

For a number of years, much research effort has been invested in studying the importance of VOCs emitted by natural sources and their role in photochemical formation of ozone (Chameides *et al* 1988, Atkinson 2000). Plants produce and emit a wide range of VOCs (Fehsenfeld *et al* 1992). Biogenic emissions have been estimated to globally exceed anthropogenic emissions (Guenther *et al* 1995, Simpson *et al* 1995).

In Mediterranean forests *Pinus halepensis* Mill. and *Quercus ilex* L. are dominant tree species, and they both emit terpenes (Llusià and Peñuelas 1998, 2000). However, *P. halepensis* stores terpenes and *Q. ilex* does not (Llusià and Peñuelas 1998). The pattern of terpene emission from plants that do not store terpenes in specialized structures may be different from that of plants having specialized structures for their storage (Lerdau 1991, Seufert *et al* 1995, Loreto *et al* 1996a, Lerdau *et al* 1997, Llusià and Peñuelas 1999). In terpene-storing species, pool size in resin ducts and internal or external glands affects the emission rates, and it can be expected that the short-term response of terpene emission rates to photosynthetic photon flux density (PPFD) and photosynthetic rates could be stronger and faster in nonstoring species than in storing species (Staudt and Seufert 1995).

Aleppo pine *Pinus halepensis* Mill. is considered a low emitting species (Owen *et al* 2002). *P. halepensis* emission rates are maximal in the middle of the day and become negligible during the night (Simon *et al* 2005). VOC emissions from *P. halepensis* are dominated by α-pinene and myrcene which may represent 70% of the total emission (Llusià and Peñuelas 2000, Owen *et al* 2002, Ormeño *et al* 2007). The remaining 30% of the terpene emissions is often constituted by β-pinene, caryophyllene and α-caryophyllene (Llusià and Peñuelas 2000). Only limited data are available concerning the influence of the environment, particularly light and temperature, on terpene emission rates (Peñuelas and Llusià 1999a).

Mediterranean evergreen oak *Quercus ilex* L. is among the heaviest terpene emitters (BEMA 1997, Llusià and Peñuelas 2000). Leaves of *Q. ilex* emit large amounts of terpenes that can affect the regional air quality and climate (Kesselmeier and Staudt 1999) and may contribute to changes in the atmospheric composition (BEMA 1997). The principal terpenes emitted by *Q. ilex* are α-pinene, β-pinene, sabinene and myrcene (Kesselmeier *et al* 1996, BEMA 1997, Llusià and Peñuelas 2000). α-Pinene emissions in *Q. ilex* may represent about 40% of the total emission in this species (Loreto *et al* 1996b). *Q. ilex* emission is light dependent (Staudt and Seufert 1995, Loreto *et al* 1996b, Peñuelas and Llusià 1999b) and CO_2 dependent (Loreto *et al* 1996a, b, Peñuelas and Llusià 1999a). The dependence on these factors suggests that α-pinene biosynthesis may be related to photosynthesis and that the emission is controlled by the availability of photosynthesis intermediates (Loreto *et al* 1996a, b).

Mediterranean ecosystems are water-limited (Sardans and Peñuelas 2004). Among the abiotic factors affecting plant terpene emission rates, temperature is outstanding (Tingey *et al* 1980, Guenther *et al* 1993, Staudt and Seufert 1995, Loreto *et al* 1996a, b, Peñuelas and Llusià 1999a, b, Llusià and Peñuelas 2000). However, water availability plays a significant role too (Bertin and Staud 1996, Staud and Bertin 1998, Peñuelas and Llusià 1999b). Water availability in the Mediterranean regions is likely to be reduced in the near future by the predicted increases of temperatures, and the consequent increases of evapotranspiration rates (Piñol *et al* 1998, Peñuelas *et al* 2002, 2005). As a consequence of this water reduction, terpene emissions are expected to increase except when the drought is severe; in that case emissions are drastically reduced (Llusià and Peñuelas 1998, 1999, Peñuelas and Llusià 1999a, b).

In general, Mediterranean ecosystems are considered to be poor in nutrients (Mooney and Dunn 1970, Ellis and Kummerow 1989): mediterranean soils often suffer from nutrient deficiencies (Specht 1973, Kruger 1979, Terradas 2001, Sardans *et al* 2006). However, the global Nitrogen cycle has now reached the point in which more N is fixed annually by human-driven processes (fertilizers, combustion of fossil fuels, and waste from stock raising) than by natural processes (Vitousek *et al* 1997, Rodà *et al* 2002). Along with nitrogen, phosphorus is also a frequent limiting factor in Mediterranean ecosystems (Zinke 1973, Sardans 1997, Henkin *et al* 1998, Hanley and Fenner 2001, Sardans *et al* 2006). Similarly to N, P input to ecosystems, especially aquatic ones, as increased in last decades (European Environment Agency 1998, Rubaek *et al* 2000). Nutrient supplies have often been shown to be an important factor in growth, structure and distribution of Mediterranean communities (Kruger 1979, Carreira *et al* 1992, Sardans 1997, Henkin *et al* 1998). And as a result of these increases in N and P, N and P foliar concentrations have increased in some Mediterranean species in the last few decades (Peñuelas and Filella 2001). The carbon-nutrient balance theory predicts that any lack in nutrients will affect the production of secondary metabolites (Gershenzon and Croteau 1991, Peñuelas and Estiarte 1998). When nutrient availability is limited, growth rate is reduced, but photosynthesis remains constant due to carbon availability. Concentrations of nitrogen-based compounds will decline, but accumulation of carbohydrate will lead to the synthesis of terpenoids (Gershenzon 1994, Peñuelas and Estiarte 1998). Past studies indicate that basal emission rates are influenced apart from temperature and PPFD by growth, CO_2, water and nitrogen supply (Guenther *et al* 1993, Llusià and Peñuelas 1999). Since phosphorylated compounds such as isopentenyl diphosphate and dimethylallyl diphosphate are immediate precursors of isoprene it is likely that also P availability influence isoprenoid emission rates.

In this work we studied and compared the terpene emission rates of these two dominant species of the Mediterranean ecosystems: *P. halepensis* (a terpene-storing species) and *Q. ilex* (a terpene nonstoring species) in response to increasing water stress and fertilization (Nitrogen and Phosphorus addition) along a spring-summer growth period. Our aim was to estimate the changes in terpene emissions that can be expected in the next decades if predicted climate change and increased ecosystems fertilization occur.

3.1.3. Material and Methods

Study site

This experiment was carried out in a greenhouse (plastic tunnel 28 m long and 6 m wide) located in the experimental fields at the Universitat Autònoma de Barcelona (Bellaterra, Barcelona, NE Spain). This greenhouse is located 147 m above the sea level at the UTM coordinates 31T x = 0425064 y = 459446. It prevents water incomings from rain, and maintains homogeneous Mediterranean-like environmental conditions. Mediterranean conditions include a marked seasonality, with a long summer, where the lowest precipitation rate and the highest annual irradiance coincide.

Experimental design

24 two-year-old plants of *Pinus halepensis* purchased in Apromi breeding ground (Juneda, Lleida, Spain) and 24 two-year-old plants of *Quercus ilex* purchased in Forestal Catalana (Breda, Girona, Spain) were grown in 2 l pots containing a mixture of peat (Sphagnum neutral peat, H-Terraplant-1, Compo) and perlite (2:1) from June to mid August 2004. Previously, they were well watered and maintained in Mediterranean-like environmental conditions until the beginning of the experiment.

Two treatments were applied simultaneously: drought and fertilization. The drought treatment had two doses: Control (C) l liter water per week and plant and Drought (D) 0.33 liters water per week and plant; so half of the pines and oaks were subjected to drought treatment and the other half were control plants. The fertilization treatment had four doses: control (O) without fertilization, 250 kg N ha^{-1} Nitrogen (N), 250 kg P ha^{-1} Phosphorus (P) and both 250 kg N ha^{-1} + 250 kg P ha^{-1} (NP). Chemicals used were NH_4NO_3 for Nitrogen fertilization and $Ca_3(PO_4)_2$ for Phosphorous fertilization (both from Fluka, Buchs, Switzerland). The fertilization dose was distributed homogeneously during the two and a half months of the experiment from June to August. Six plant replicates per treatment levels were monitored.

Field measurements of growth, relative water content, photosynthetic rates, soil moisture, stomatal conductance and sampling of emitted VOC

Diameter and height were measured in each each one of the six sampling dates.

Relative Water Content (RWC) was measured in each plant in each one of the six sampling dates. For 3-4 needles per *P. halepensis* and one leaf per *Q. ilex*, fresh weight (FW) was obtained by difference between tube+water+leaf less tube+water. After at least 12 hours of moisturizing the saturated weight (SW) was obtained. Drought weight (DW) was obtained after drying for 72 hours at 60 ^{o}C. The following formula was applied to obtain the RWC:

$$RWC = \frac{(FW - DW)}{(SW - DW)}$$

Soil moisture was measured in each plant pot along the experiment by using Time Domain Reflectometry (TDR) (Tektronix 1502C, Beaverton, Oregon, US).

Measurements of net photosyntetic rates, stomatal conductance and VOCs emissions were conducted every 15 days from June to mid-August, i.e. six sample time points. Measurements were conducted on sunny cloudless days. The average PPFD of the measurements was 1,270 μmol m^{-2} h^{-1}.

CO_2 exchange was measured using a non-dispersive infra-red gas analyzer (IRGA), model ADC-LCA4 (ADC Inc. Hoddesdon, Hertfordshire, England) connected to a cuvette model PLC2P (ADC Inc. Hoddesdon, Hertfordshire, England). CO_2 uptake (A) and stomatal conductance (g_s) were measured in sunlit shoots on *P. halepensis* and in sunlit leaves of *Q. ilex*. Both shoots and leaves were from the previous year. A and g_s values were expressed on a projected leaf area basis measured with Li-Cor 3100 Area Meter (Li-Cor Inc., Nebraska, USA).

In order to sample VOCs, a T-system was installed outside the cuvette of the IRGA-porometer. Part of the air passed through cartridges filled with three different phases separated by plugs of quartz wool: Carbotrap C (300 mg), Carbotrap B (200 mg) and Carbosieve S-III (125 mg) from Supelco (Belmonte, Pa) by using a pump at constant flow. The multibed glass cartridges were 11.5 cm long x 4 mm interior diameter, and were previously preheated 5 minutes to 300 ^{o}C in order to activate the Carbon properties for terpene absorbance. The hydrophobic properties of the tubes were supposed to minimize sample displacements by water. Inside the cartridges, terpenes did not suffer chemical transformations as checked with standards (α-pinene, β-pinene and limonene). Sampling time was 5 minutes and the pump flow was 500 ml min^{-1}. One blank every three samples was taken to substract the effect of "sticky-mononoterpenes" inside the cuvette. The glass tubes (with trapped VOC) were stored in a portable refrigerator at 4 ^{o}C, and taken to the adjacent laboratory. At the laboratory, the glass tubes were stored at -30 ^{o}C before analysis, for no longer than 15 days.

Laboratory analyses: VOCs and P and N foliar concentrations

The identification and quantification of the VOCs trapped in the cartridges was done in a Gas Chromatography-Mass Spectrometry (GC-MS, model Hewlett Packard HP59822B, Palo Alto, California) linked to a thermal desorption unit (model 890/891, Supelco, INC, Bellefonte, Pennsylvania).

VOCs were extracted from the cartridges in the thermal desorption unit by heating at 250^{o}C during 2 minutes, and using Helium as a carrier gas (flow was 1ml min^{-1}) and then introduced into the GC-MS. We used a 30 m x 0.25 mm x 0.25 μm film thickness capillary column (Supelco HP-5, Crosslinked 5% pH Me Silicone). Full scan method was used to perform the chromatography, taking 22 minutes time. After sample injection at 40 ^{o}C, temperature was increased at 30 ^{o}C min^{-1} up to 70 ^{o}C, and thereafter at 10 ^{o}C min^{-1} up to 150 ^{o}C, where temperature was maintained for 5

min, and thereafter at 70 ^{o}C min^{-1} up to 250 ^{o}C, which was maintained for another 5 min. Helium flow was 1 ml min^{-1}.

The identification of terpenes was conducted by GC-MS and comparison with standards from Fluka (Buchs, Switzerland), literature spectra and GCD Chemstation G1074A HP with the Wiley275.L library. To focus on terpenes, only compounds that had, between others, mass 93 were selected.

Terpene calibration curves (n=4 different terpene concentrations) were always significant (R^2>0.99) in the relationship between signal and terpene concentration. The most abundant terpenes had very similar sensitivity (differences were less than 5%).

P concentrations were analyzed by atomic emission spectroscopy with Inductive Coupled Plasma (ICP-AES). Needles and leaves were crushed and dried at 60^{o}C during 48 hours. The ICP-AES analyses were conducted after acid digestion (HNO_3:$HClO_4$, 2:1, v/v) in a microwave Moulinex Optiquick Duo Y92 using open fluorinated ethylene propylene flasks (Nalge Company, Rochester, UK). The concentrations were determined in a Polyscan Thermo Jarrel ASH Model 61 E spectrophotometer (Waltham, MA, USA).

N concentrations were analyzed by combustion followed by gas chromatography (GC) using a NA2100 C.E. Instrument (Thermo Electron, Milano, Italy). The sample was prepared weighting between 1 and 2 mg of previously ground leaves in a tin small capsule and adding 2 mg of Vanadium pentoxide as an oxidant additive.

Statistical analyses

Repeated measures analyses of variance (ANOVA) were conducted for total VOC emissions as dependent variable and the two treatments (drought and fertilization) as independent variables. Differences between particular levels of the treatments were assessed with post-hoc Fisher tests. Correlation analyses were conducted among all the measured variables. All statistical analyses were conducted with the software package STATISTICA 6.0 (StatSoft Inc, Tulsa, USA).

3.1.4. Results

Temperature ranged from 26 ^{o}C at the beginning of the experiment (spring) to 35 ^{o}C at the end (summer) and Photon Flux Density averaged about 1,300 μmol m^{-2} s^{-1} during the spring-summer period of study (*Fig.1*).

The drought treatment reduced soil moisture in both species pots (*Table 1*), in *P. halepensis* from 0.19 to 0.06 and in *Q. ilex* from 0.20 to 0.04. Drought decreased height and diameter in both species: *P. halepensis* control plants increased 30% their height and 41% their diameter, while droughted plants only increased them 17% and 19% respectively. *Q. ilex* control plants increased 11% their height and 25% their diameter, while droughted plants only increased them 10% and 14% respectively.

No significant responses of leaf concentrations of nitrogen and phosphorus to fertilization treatment were found neither in *P. halepensis* nor in *Q. ilex*. However, drought treatment increased N concentrations in *P. halepensis* and tended to do it also in *Q. ilex* (*Table 1*).

Net photosynthetic rates did not present differences among the different levels of the fertilization treatment but were lower in the drought treatment (Fig. 2), especially in *Q. ilex* ($P<0.001$, ANOVA). For both species, *P. halepensis* and *Q. ilex*, the effect of time and its interaction with drought treatment were significant ($P<0.001$, ANOVA). Net photosynthetic rates were higher in *Q. ilex* (data not shown) than in *P. halepensis* during the studied period of summer (*Fig.2*). Drought decreased stomatal conductance 42% in *P. halepensis* and 28% in *Q. ilex* (*Fig.2*). Time and interaction between drought and time significantly ($P<0.001$, ANOVA) affected stomatal conductance in both species *P. halepensis* and *Q. ilex*. Stomatal conductance values for *Q. ilex* were also higher than those of *P. halepensis* (*Fig.2*).

Total terpene emission rates were similar in both species, *P. halepensis* (31 μg g^{-1} [d.m.] h^{-1}) and *Q. ilex* (32 μg g^{-1} [d.m.] h^{-1}) (*Fig.3*). *P. halepensis* total terpene emission rates decreased by 38% in fertilization treatments while drought treatment increased them by 33% in *Q. ilex* (*Fig.3*). Post-hoc tests for *P. halepensis* showed that there were no differences between any of the different fertilization levels in fertilized plants (*Fig.3*). The effect of phosphorus fertilization decreasing total terpene emission rates in *P. halepensis* plants was significant on 30 June ($P<0.1$), 14 July ($P<0.1$) and 28 July ($P<0.05$) (*Fig.4*).

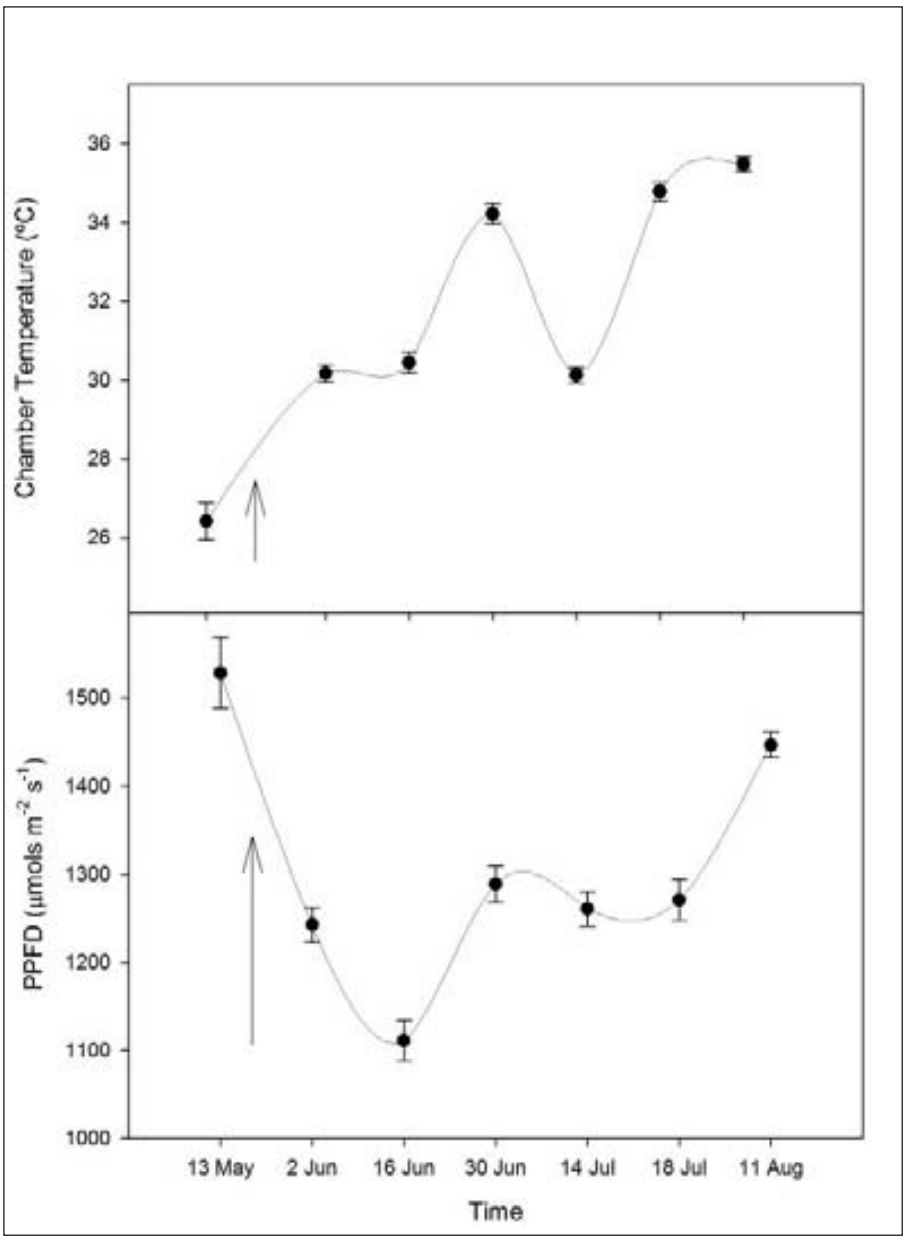

Fig.1 - Ambient variables measured when sampling along the experiment: Temperature (ºC), and Photosynthetic Photon Flux Density (µmol m^{-2} s^{-1}). Arrows indicate start of drought and fertilization treatments

Table 1 - Diameter and height increase, Relative Water Content (RWC) and soil moisture (SM) (mean ± SE) for P. halepensis and Q. ilex plants for drought and control treatments (n = 24) (0 initial, f final). Different letters indicate significant differences between treatments (P<0.05, t-Student)

	ΔDiameter (cm)	ΔHeight (cm)	RWC_0 (% v/v)	RWC_f (% v/v)	SM_0 (% v/v)	SM_f (% v/v)
Pinus halepensis						
Control	0.42 ± 0.03a	0.30 ± 0.03a	0.91 ± 0.02a	0.98 ± 0.01a	0.215 ± 0.011a	0.134 ± 0.011a
Drought	0.19 ± 0.02b	0.17 ± 0.08b	0.88 ± 0.04a	0.93 ± 0.01b	0.08 ± 0.008b	0.043 ± 0.003b
Quercus ilex						
Control	0.25 ± 0.13a	0.11 ± 0.07a	0.81 ± 0.03a	0.96 ± 0.01a	0.202 ± 0.01a	0.164 ± 0.012a
Drought	0.14 ± 0.10b	0.09 ± 0.08b	0.87 ± 0.03a	0.92 ± 0.02a	0.05 ± 0.005b	0.027 ± 0.002b

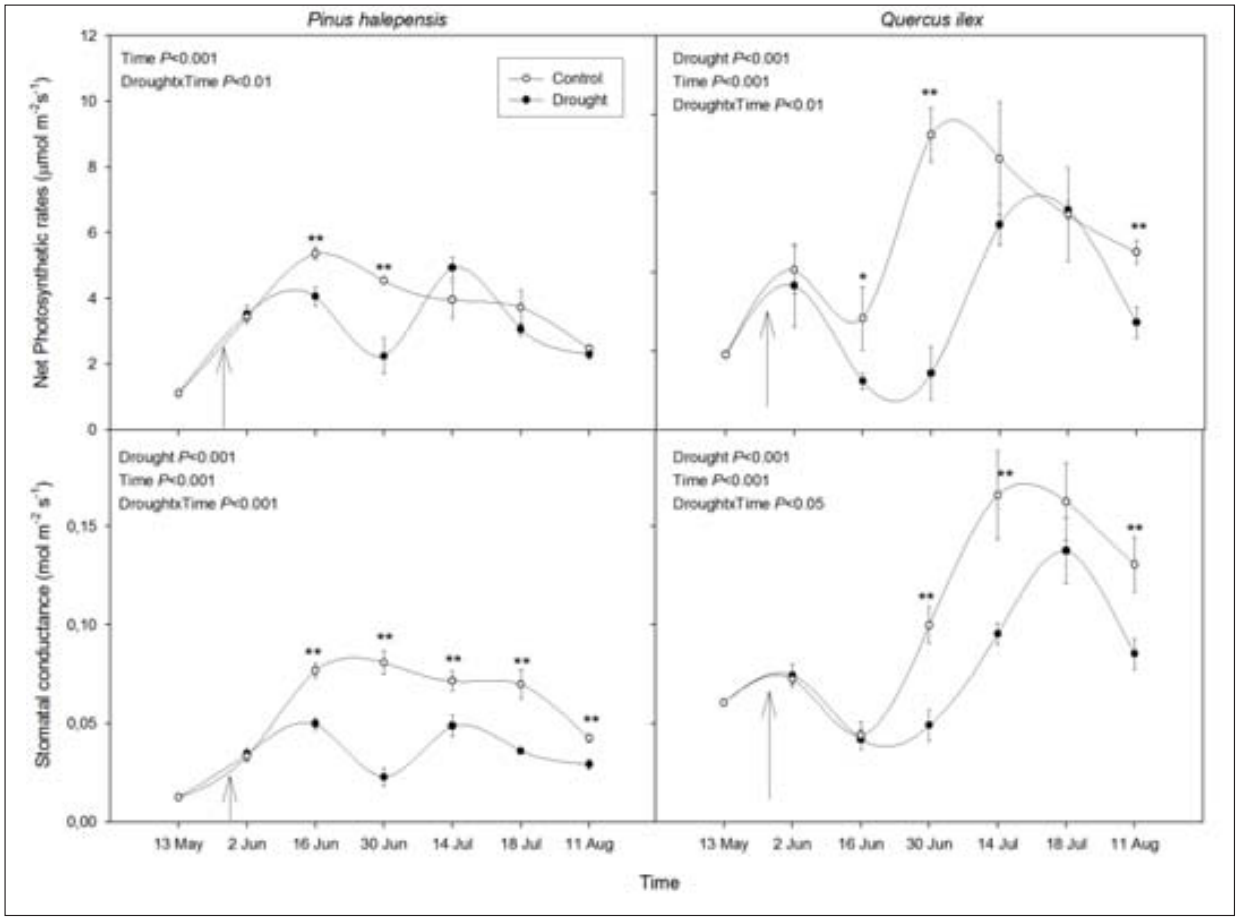

Fig.2 - Net photosynthetic rates (μmol $m^{-1}s^{-1}$) and stomatal conductances (mol m^{-2} s^{-1}) along the experiment, for P. halepensis and Q. ilex plants and for control and drought conditions. Arrows indicate starting of drought and fertilization treatments. Repeated measures ANOVA significant variables are shown in each panel. Asterisks indicate significant differences among the two watering levels (P<0.05 ** P<0.01, post-hoc Fisher test, repeated measures ANOVA)*

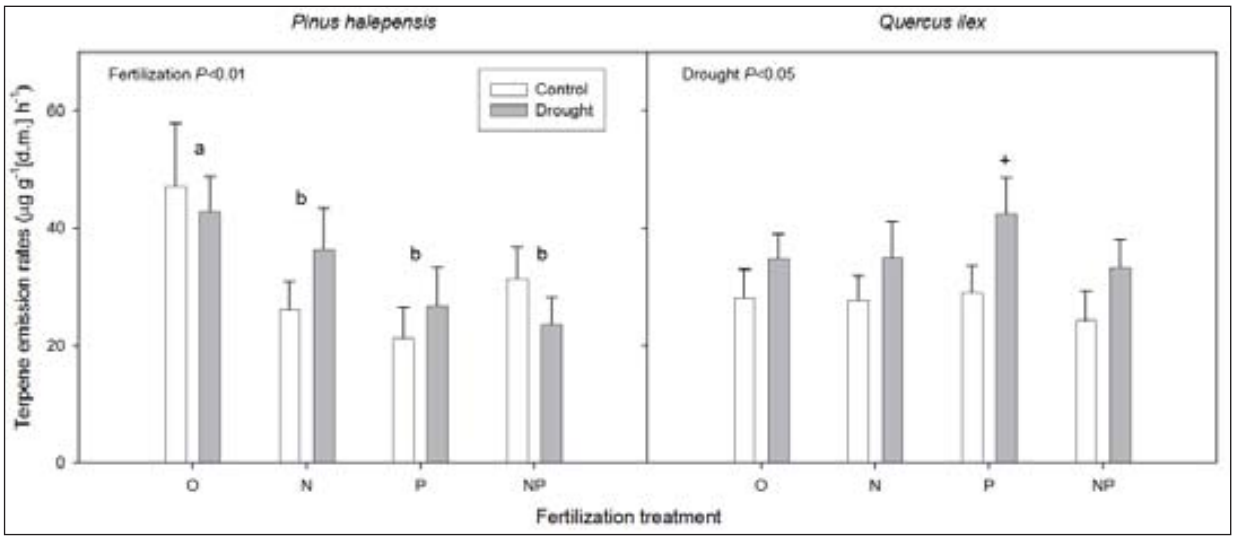

Fig.3 - Total terpene Emission Rates (μg g^{-1}[d.m.] h^{-1}) for fertilization and drought treatments. Vertical bars indicate standard error of the mean (n=3). Reapeated measures ANOVA significant variables are shown in each panel. Different letters indicate significant statistical differences among fertilization levels (P<0.05, post-hoc Fisher test). Asterisks indicate significant differences among watering doses (+ P<0.1, post-hoc Fisher test, repeated measures ANOVA)

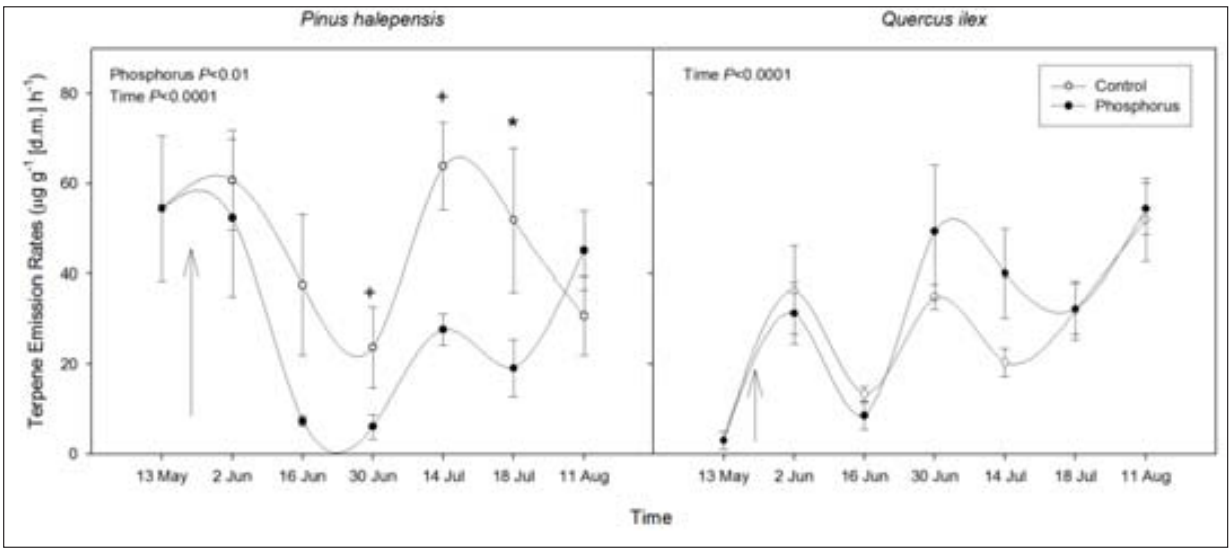

Fig.4 - Total Emission Rates (µg g^{-1} [d.m.] h^{-1}) for Control and Phosphorus fertilized plants. Vertical bars indicate standard error of the mean (n=3). Arrows indicate starting of treatments. Repeated measures ANOVA significant variables are shown in each panel. Asterisks indicate significant differences among watering doses (P<0.05, + P<0.1, post-hoc Fisher test, repeated measures ANOVA)*

No differences between drought and control plants were observed in *P. halepensis*. In *Q. ilex* there were higher emission rates in two sampling dates (*Fig.5*): 16 June ($P<0.05$, ANOVA) and 28 July ($P<0.05$, ANOVA).

The most emitted terpenes in both species *P. halepensis* and *Q. ilex* were the monoterpenes α-pinene, β-pinene, β-myrcene, Δ^3-carene, 2-carene, camphene, α-phellandrene, limonene and β-ocymene and the sesquiterpenes sabinene, caryophyllene and α-caryophyllene. The emission rates of those compounds are shown in *Tables 2* and *3* and in *Fig.6*. Comparing both species, α-pinene, β-myrcene and Δ^3-carene emission rates were higher in *P. halepensis* plants whereas β-pinene was emitted at greater emission rates by *Q. ilex* (*Tables 2, 3, Fig. 6*). The fertilization treatment had significant ($P<0.05$) effects on the emission rates of α-pinene, β-pinene and

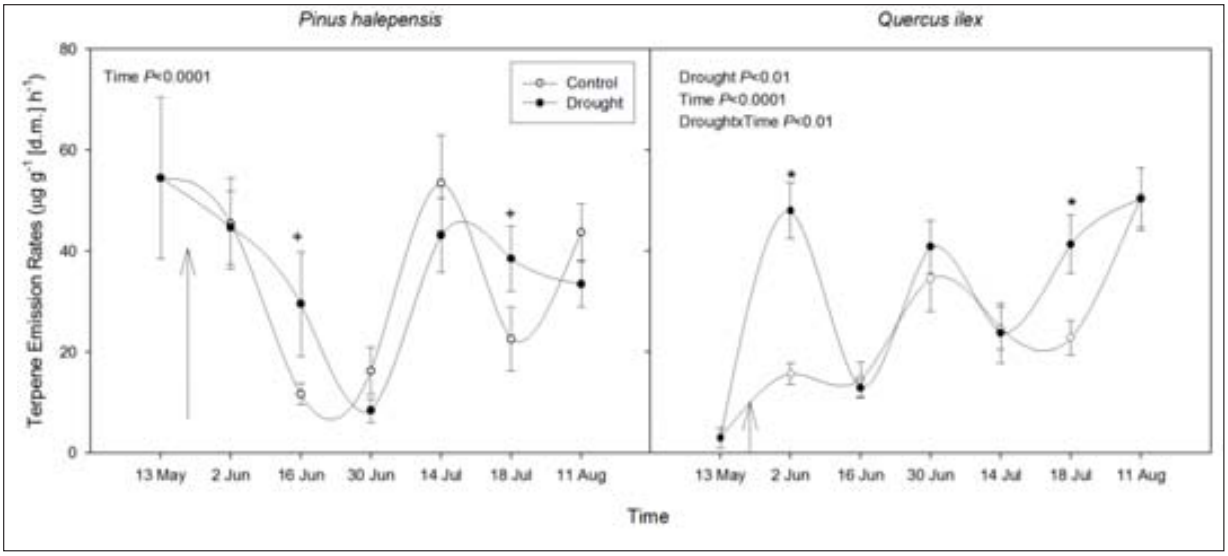

Fig.5 - Total Emission Rates (µg g^{-1} [d.m.] h^{-1}) for control and water stressed plants. Vertical bars indicate standard error of the mean (n=3). Repeated measures ANOVA significant variables are shown in each panel. Arrows indicate starting of treatments. Asterisks indicate significant differences among watering doses (P<0.05,+ P<0.1 post-hoc Fisher test)*

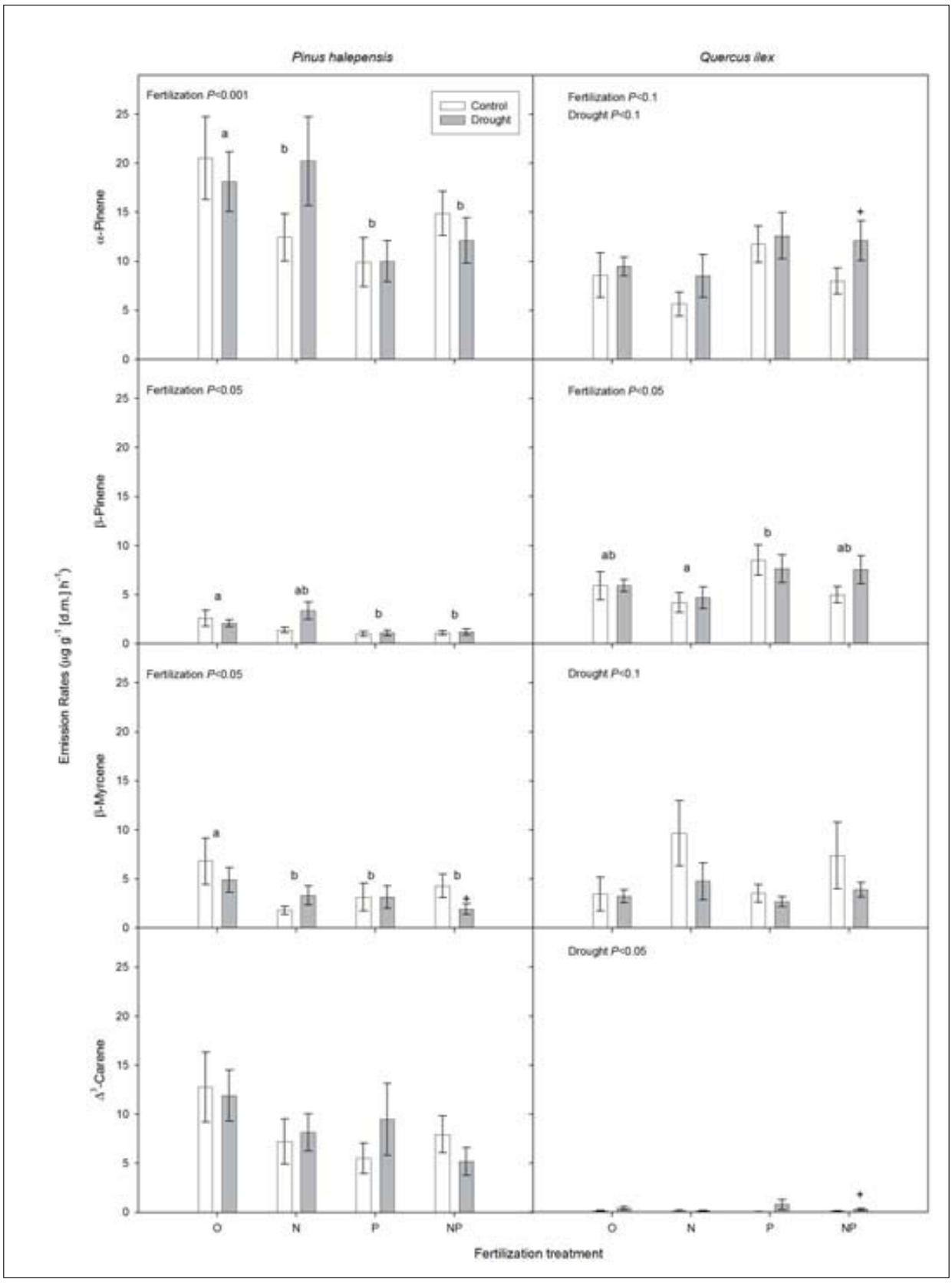

Fig.6 - Emission Rates (μg g^{-1} [d.m.] h^{-1}) of the main terpenes, for drought and fertilization treatments. Vertical bars indicate standard error of the mean (n=6 sampling date means of 3 plants per treatment). Repeated measures ANOVA significant variables are shown in each panel. Different letters indicate significant statistical differences among fertilization levels (P<0.05, post-hoc Fisher test). Asterisks indicate significant differences among watering doses (+ P<0.1, post-hoc Fisher test, repeated measures ANOVA)

Table 2 - Total terpene emission rates (μg g^{-1} [d.m.] h^{-1}) (mean ± standard error, n=12) for P. halepensis plants for drought and control treatments. Different letters indicate significant (t-student, P<0.05) differences between drought and control treatment. They are highlighted in bold type

P. halepensis												
Treatment	α-Pinene	β-Pinene	β-Myrcene	Δ^3-Carene	2-Carene	Camphene	Sabinene	Limonene	β-Ocimene	β-Phellandrene	Caryophyllene	α-Caryophyllene
June 2 2004												
Control	23.9±5.8a	1.9±0.6a	9.9±0.8a	17.5±4.5a	0.8±0.4a	0.5±0.2a	0.5±0.2a	3.3±0.5a	0.8±0.1a	0.4±0.1a	0.2±0.0a	0.5±0.2a
Drought	17.9±1.3a	3.3±0.5a	5.8±1.1a	14.2±4.0a	0.3±0.1a	1.1±0.1a	0.1±0.0a	0.7±0.4a	0.5±0.2a	0.2±0.0a	0.1±0.0a	0.2±0.1a
June 16 2004												
Control	5.6±0.7a	0.7±0.1a	1.5±0.5a	2.7±1.1a	0.1±0.0a	0.4±0.2a	0.1±0.0a	0.8±0.2a	0.1±0.1a	**0.0±0.0a**	0.0±0.0a	0.0±0.0a
Drought	16.4±5.3a	7.2±3.8a	4.7±2.1a	12.1±5.3a	0.4±0.2a	1.0±0.3a	0.1±0.1a	2.6±1.3a	0.4±0.1a	**0.1±0.1b**	0.0±0.0a	0.0±0.0a
June 30 2004												
Control	**8.8±3.2a**	**1.0±0.3a**	2.2±1.0a	**4.5±2.6a**	**0.3±0.1a**	**0.0±0.0a**	0.2±0.1a	0.3±0.3a	0.9±0.3a	0.1±0.1a	**0.2±0.1a**	0.0±0.0a
Drought	**4.0±1.3b**	**0.3±0.1b**	0.5±0.1a	**1.2±0.2b**	**0.1±0.0b**	**1.1±0.6b**	0.1±0.0a	0.3±0.2a	0.6±0.1a	0.2±0.1a	**0.0±0.0b**	0.0±0.0a
July 14 2004												
Control	30.7±4.5a	4.5±1.6a	9.6±2.6a	**25.8±7.3a**	**1.3±0.4a**	0.2±0.0a	**0.9±0.1a**	0.9±0.3a	0.3±0.1a	0.3±0.1a	0.5±0.1a	0.2±0.0a
Drought	20.6±4.8a	2.8±1.2a	4.5±1.6a	**11.2±3.2b**	**0.5±0.2b**	0.2±0.1a	**0.4±0.1b**	0.6±0.2a	0.4±0.1a	0.1±0.1a	0.4±0.1a	0.1±0.0a
July 28 2004												
Control	**10.9±4.5a**	1.30±0.7a	2.2±1.1a	**5.8±3.0a**	0.3±0.2a	0.1±0.0a	0.2±0.1a	**0.3±0.1a**	2.3±1.3a	0.1±0.0a	0.3±0.2a	0.1±0.1a
Drought	**21.2±5.8b**	1.6±0.3a	3.6±1.5a	**11.8±4.2b**	0.6±0.2a	0.2±0.0a	0.2±0.1a	**0.6±0.2b**	3.5±0.8a	0.2±0.1a	0.4±0.1a	0.1±0.1a
August 18 2004												
Control	34.2±7.5a	5.0±2.1a	12.6±6.5a	14.3±3.1a	0.8±0.3a	**0.3±0.1a**	0.3±0.1a	1.0±0.3a	1.9±0.3a	0.3±0.1a	0.7±0.3a	0.2±0.1a
Drought	21.4±4.8a	1.1±0.2a	1.7±0.3a	5.7±1.1a	0.3±0.1a	**0.1±0.0b**	0.2±0.1a	0.3±0.1a	1.5±0.3a	0.2±0.1a	0.1±0.0a	0.1±0.0a

Table 3 - Total terpene emission rates (μg g^{-1} [d.m.] h^{-1}) (mean ± standard error, n=3) for Q. ilex plants for drought and control treatments. Different letters indicate significant (t-student, P<0.05) differences between drought and control treatment. They are highlighted in bold type

Q. ilex												
Treatment	α-Pinene	β-Pinene	β-Myrcene	Δ^3-Carene	2-Carene	Camphene	β-Phellandrene	Limonene	β-Ocimene	α-Phellandrene	Caryophyllene	α-Caryophyllene
June 2 2004												
Control	**4.8±1.8a**	3.6±0.7a	3.0±1.2a	**0.0±0.0a**	0.0±0.0a	0.1±0.0a	0.3±0.3a	**3.6±1.9a**	0.0±0.0a	**0.2±0.1a**	**0.0±0.0a**	0.1±0.1a
Drought	**11.3±2.9b**	7.0±2.4a	5.2±2.7a	**0.3±0.1b**	0.2±0.1a	1.0±1.0a	4.5±2.1a	**16.2±8.0b**	0.1±0.1a	**0.4±0.2b**	**0.1±0.0b**	0.3±0.1a
June 16 2004												
Control	5.0±0.9a	3.7±0.7a	1.2±0.2a	0.1±0.0a	0.0±0.0a	**0.1±0.0a**	1.4±0.4a	1.7±0.7a	0.0±0.0a	0.2±0.0a	0.0±0.0a	0.1±0.1a
Drought	4.7±1.1a	2.5±1.0a	1.0±0.3a	0.0±0.0a	0.0±0.0a	**0.0±0.0b**	1.1±0.4a	2.2±0.9a	0.0±0.0a	0.2±0.1a	0.0±0.0a	0.1±0.1a
June 30 2004												
Control	10.1±3.8a	6.9±3.4a	3.5±1.9a	0.4±0.2a	0.1±0.0a	0.1±0.0a	3.0±1.4a	9.6±6.3a	0.2±0.2a	**0.2±0.1a**	0.0±0.0a	0.1±0.1a
Drought	14.5±4.0a	8.3±1.6a	2.7±0.8a	1.3±0.7a	0.2±0.0a	0.4±0.2a	3.8±1.9a	7.9±3.5a	0.1±0.0a	**0.8±0.3b**	0.1±0.1a	0.6±0.4a
July 14 2004												
Control	8.2±1.9a	5.0±1.3a	6.2±5.4a	0.0±0.0a	0.0±0.0a	0.1±0.0a	1.6±0.6a	3.2±1.9a	0.2±0.2a	0.1±0.1a	0.0±0.0a	0.1±0.1a
Drought	6.4±1.9a	4.3±1.3a	3.0±2.0a	0.1±0.0a	0.0±0.0a	0.1±0.0a	1.2±0.7a	3.5±1.8a	0.0±0.0a	0.1±0.1a	0.1±0.1a	5.0±4.8a
July 28 2004												
Control	6.3±1.8a	4.6±1.9a	7.0±5.2a	0.0±0.0a	0.0±0.0a	0.3±0.1a	1.7±0.5a	**2.7±1.2a**	0.1±0.1a	0.0±0.0a	0.0±0.0a	0.0±0.0a
Drought	13.8±5.1a	10.0±3.8a	6.1±1.3a	0.4±0.2a	0.1±0.1a	0.4±0.2a	2.8±1.5a	**12.9±4.6b**	0.1±0.0a	0.4±0.2a	0.1±0.1a	0.3±0.2a
August 18 2004												
Control	15.5±4.2a	11.0±2.9a	15.0±7.7a	**0.1±0.0a**	0.1±0.0a	0.2±0.1a	3.6±1.6a	**4.4±2.7a**	0.0±0.0a	0.5±0.2a	0.0±0.0a	0.2±0.1a
Drought	15.8±5.3a	8.6±3.2a	4.3±1.2a	**0.4±0.2b**	0.2±0.1a	0.6±0.3a	5.3±2.3a	**15.1±5.1b**	0.3±0.2a	0.6±0.3a	0.0±0.0a	3.3±1.8a

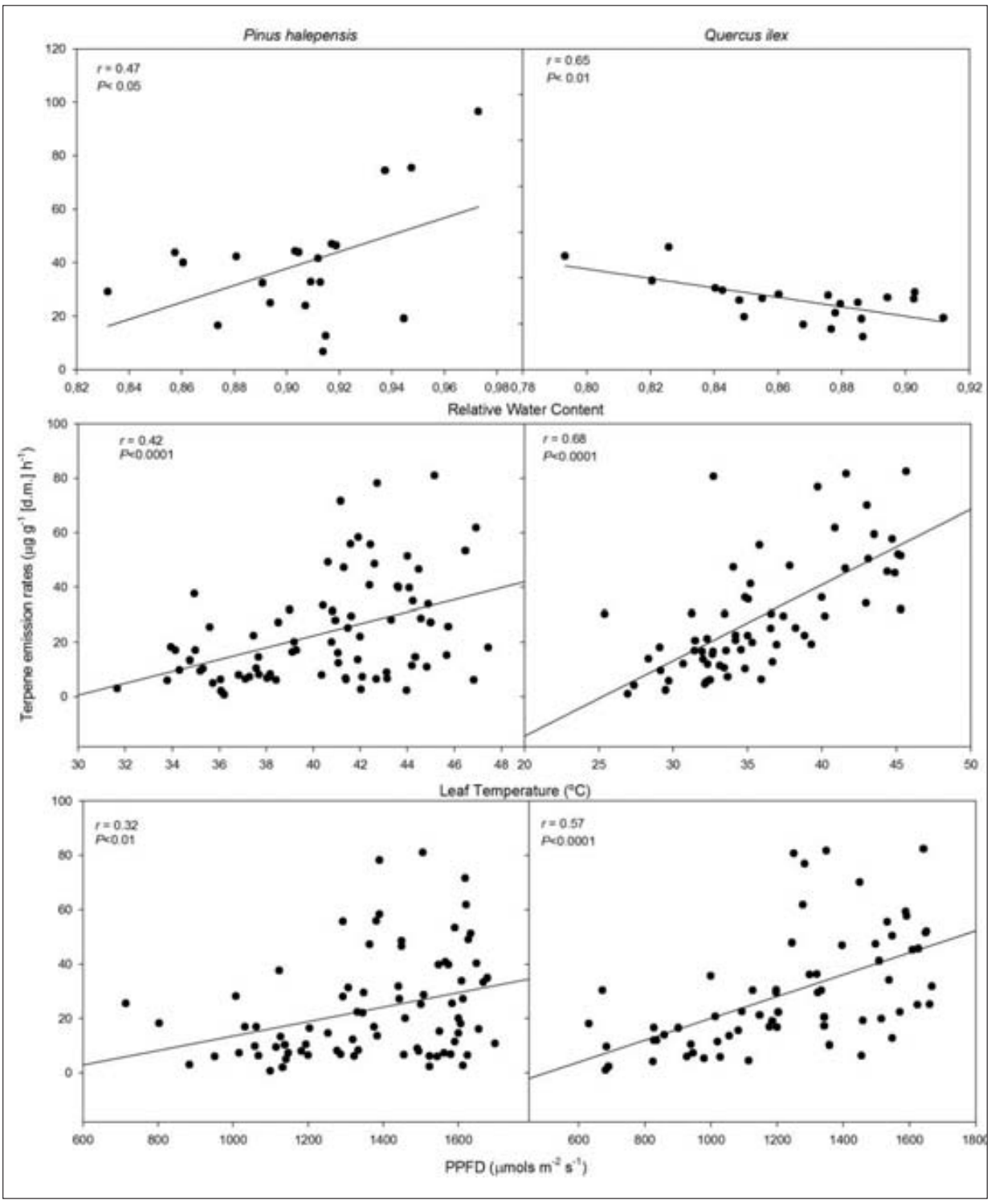

Fig.7 - Relations of total terpene emission rates (µg g^{-1} [d.m.] h^{-1}) with leaf Relative Water Content, chamber temperature and incident PPFD (Photosynthetic Photon Flux Density) for P. halepensis and Q. ilex

β-myrcene in *P. halepensis,* and on Δ^3-carene in *Q. ilex* (*Fig.6*). *P. halepensis* plants fertilized with Phosphorus addition (P and N+P) emitted lower amounts of α-pinene and β-pinene than the control plants (22% and 28% respectively). Plants fertilized with both Nitrogen and Phosphorus (N, P and N+P) emitted lower amounts of β-myrcene (48%) than control plants (*Fig.6*). For individual terpenes, the irrigation increased α-pinene β-myrcene and Δ^3-carene emission in *Q. ilex* plants (*Fig.6*).

A positive correlation was found between total terpene emission rates and RWC for *P. halepensis* (r=0.47, P<0.05) and a negative correlation was found in *Q. ilex* (r=0.65, P<0.01) (*Fig.7*). *Figs.4* and *5* show a significant effect of the time course on the emission rates. With increasing temperatures in the summer there were increasing emissions especially in *Q. ilex* and also, although less evident, in *P. halepensis*. The emission rates of *P. halepensis* and especially of *Q. ilex* showed thus a significant relation to temperature (r=0.42, P<0.0001 and r=0.68, P<0.0001 respectively) (*Fig.7*). They were also significantly related to PPFD again specially in *Q. ilex* (r=0.32, P<0.01 and r=0.57, P<0.0001 respectively) (*Fig.7*). No significant relationship was found for terpene emission and N and P foliar concentrations (*Fig.8*), at least in the range of values of this study.

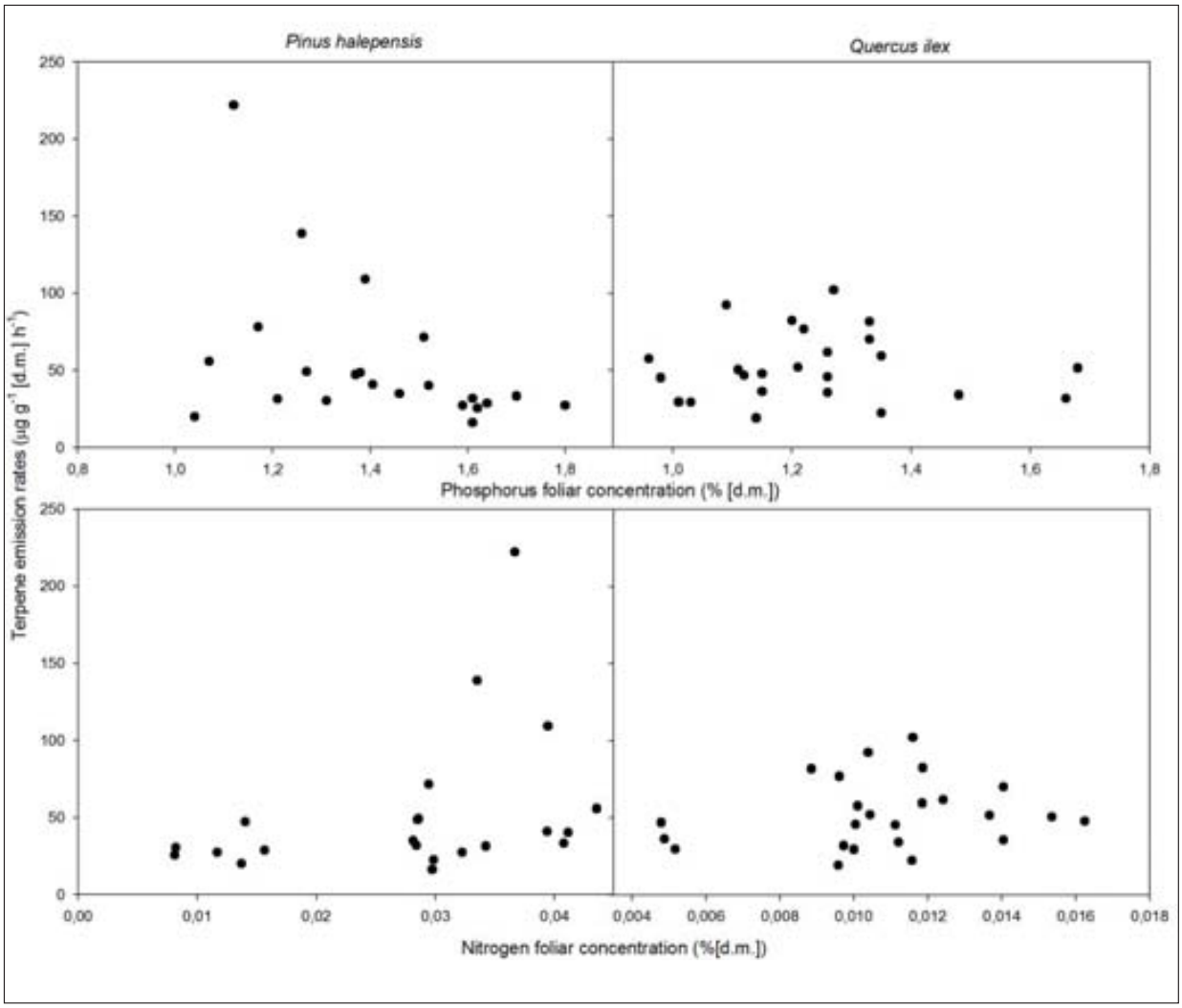

Fig.8 - Relationships between total terpene emission rates μg g^{-1} [d.m.] h^{-1}) and N and P foliar concentrations

3.1.5. Discussion

P. halepensis normalized values with Guenther algorithm (Guenther *et al* 1993, 1995) at 30 ^{o}C temperature and 1,000 μmol m^{-2} s^{-1} PPFD were on avarage 43.90 ± 7.41 μg g^{-1} [d.m.] h^{-1} for non-fertilized and well irrigated plants, more than reported in most studies for *P. halepensis*. Kesselmeyer and Staud (1999) found emissions below 1.0 μg g^{-1} [d.m.] h^{-1}, Owen *et al* (1997) found emissions between 5 and 10 μg g^{-1} [d.m.] h^{-1}, Simon *et al* (2005) found mean emission rates of 14.76 μg g^{-1} [d.m.] h^{-1} and Peñuelas and Llusià (1999b) found emissions of 20 μg g^{-1} [d.m.] h^{-1}. Despite the latter emissions were measured in an aerea geographically close to our emissions, ours were higher. These could be due to the different environmental conditions since emissions depend on temperature, light and physiological state of the plants among many other factors (Street *et al* 1997, Peñuelas and Llusià 2001). Needle damage could have been involved in *P. halepensis* measurements, which are extremely delicate, however we took precautions to avoid it as much as possible.

Q. ilex normalized values with Guenther algorithm (Guenther *et al* 1993, 1995) at 30 ^{o}C and 1,000 μmol m^{-2} s^{-1} were on average 12.82 ± 2.37 μg g^{-1} [d.m.] h^{-1} for non-fertilized and well irrigated plants. These rates are within the range of emission factors previously reported for *Q. ilex*. Kesselmeyer and Staud (1999) found emissions between 6 and 58 μg g^{-1} [d.m.] h^{-1}, Owen *et al* (1997) reported emissions >10 μg g^{-1} [d.m.] h^{-1} and Llusià and Peñuelas (2000) found emissions of 11 μg g^{-1} [d.m.] h^{-1}. The closest results are those of Peñuelas and Llusià (1999a), probably because of the geographical proximity of the experiment sites also in Catalonia.

The results showed larger emission rates of most volatile terpenes such as α-pinene and β-pinene because measurements were done in summer. This agrees with Llusià and Peñuelas (2000). The most volatile terpenes are more responsive to temperature than the least volatile terpenes that are more responsive to PPFD and photosyntetic rates (Peñuelas and Llusià 1999a).

The main terpenes emitted by *P. halepensis* were α-pinene, β-pinene, β-myrcene and Δ^3-carene. These terpenes emissions have also been reported in previous studies (Llusià and Peñuelas 1999, 2000). However, other studies of *P. halepensis* emissions have found high percentages of other compounds such as limonene (Llusià and Peñuelas 2000), β–trans-ocimene and linalool (Simon *et al* 2005). The terpenes found in this study have also been reported in emissions of other Mediterranean pines like *Pinus pinea* L. (Kesselmeier *et al* 1997, Owen *et al* 1997, Sabillon and Cremades 2001) and *Pinus sylvestris* L. (Rinne *et al* 1999, Komenda *et al* 2003).

The main terpenes emitted by *Q. ilex* were α-pinene, β-pinene, β-myrcene and Δ^3-carene. These terpenes emissions have also been reported in previous studies such as Kesselmeier *et al* (1996), Loreto *et al*(1996 a, b), BEMA (1997) and Llusià and Peñuelas (1998, 1999, 2000). However, other studies have found other compounds in *Q. ilex* emissions such as limonene, cineole, ocimene and linalool (Street *et al* 1997).

It has been reported that under severe drought conditions terpene-storing species decrease their terpene emissions (Llusià and Peñuelas 2000). However, the drought treatment had no significant global effect on *P. halepensis* emission rates (*Fig.3*), probably because the drought treatment was not severe enough for this species well adapted to summer drought. The drought treatment resulted nevertheless in a range of relative water contents that presented a positive relationship with emission rates (*Fig.7*). On the contrary, the drought treatment increased *Q. ilex* terpene emission rates. Terpene emission rates were negatively correlated with relative water contents in *Q. ilex* (*Fig.7*). This result coincides with former studies (Loreto *et al* 1998, Peñuelas and Llusià 1999b). This different behaviour of the two species may be linked to *P. halepensis* being a storing species whose emissions are facilitated by higher humidity (Llusià and Peñuelas 1999) and being *Q. ilex* a non-storing species that increases production and concentration with drought (Llusià and Peñuelas 1998). The production of terpenes has been linked to an increased water stress tolerance in some species (Peñuelas and Llusià 2001). Stress protection of terpenes may be attributed to their capacity to increase membrane fluidity and stability due to their lipophility and to their antioxidant capacity similarly to what occurs with other isoprenoids (Peñuelas and Llusià 2002).

Terpene precursors contain high-energy phosphate bonds and require ATP and NADPH for their synthesis. Hence, P can be a limiting factor in terpenoid biosynthesis. Increased application of fertilizers has been shown to affect oil yield and composition in different plant species (Tiwari and Banafar 1995, Dethier *et al* 1997). However, our fertilization treatments reduced emissions in *P. halepensis* (*Fig.3*), which fits better with the carbon/nutrient balance hypotheses stating that there can be a decrease of carbon-based secondary compounds such as terpenoids as a consequence of greater allocation of carbon to plant growth induced by higher nutrient availability (Peñuelas and Estiarte 1998). According with the carbon/nutrient balance hypotheses (CNBH) (Bryant *et al* 1983) and the growth differentiation balance hypothesis (GDBH) (Loomis and Croteau 1973, Lorio 1986) when a resource, such as Nitrogen or Phosphorus, is abundant, plants will allocate less carbon toward carbon based secondary compounds and more toward growth. On the contrary, when such a resource is scarce, a plant will allocate proportionately more of an abundant resource in carbon based secondary compounds (Lerdau *et al* 1995, Peñuelas and Estiarte 1998). Moreover, because fertilization generally increases tree growth, resin duct density is lower in the N-fertilized trees (Kyto *et al* 1999). For that reason, non-fertilized plants (control plants) would use more resources to produce and emit carbon-based secondary metabolites, which fit well the results of this study with lower emissions in fertilized plants in *P. halepensis*. This result is in accordance with Holopainen *et al* (1995), who reported that young pine seedlings growing at high availability of Nitrogen have longer needles and reduced chemical defenses. This result also agrees with Waring *et al* (1985) and with Margolis and Waring (1986) who found that nutrient deficient environments produced a greater amount of structural carbohydrates, which are directly linked with terpene production (Charlwood and Banthorpe 1978, Croteau 1984). However, the CNB and the GDB hypotheses are not fully in accordance with the similar growth of fertilized and non fertilized plants in our study. Since all the effects of the fertilization treatment were quite weak, future research is clearly needed where wider ranges of fertilization and nutrient contents are tested to disentangle fertilization effects on terpene emissions.

Emission rates also increased in both species with the increasing temperatures of summer: time was a significant variable (*Figs.4, 5*) and there was a significant relationship with leaf temperature (*Fig.7*). It is well known that terpene emissions depend on temperature (Guenther *et al* 1995, Guenther 1997, Kesselmeier and Staudt 1999, Peñuelas and Llusià 2001, Llusià *et al* 2005). However, the relationship was stronger in *Q. ilex* than in *P. halepensis*, probably because *P. halepensis* data had more variation than *Q. ilex* data. Although strong precaution taken in measurements seems to preclude this possibility, needle damage might have been involved in the variability of the high

emission rates found for *P. halepensis*. But in any case, emissions from the non-storing species *Q.ilex* were more influenced by changes in RWC, temperature and light than those from the storing species *P. halepensis*.

The results of this study show that terpene emission rates may significantly change with important biological and environmental consequences (Peñuelas and Llusià 2003) if climate warming and drought occur as predicted by IPCC and ecophysiological models such as GOTILWA (IPCC 2007, Sabaté *et al* 2002, Peñuelas *et al* 2005), and if global fertilization (Vitosuek *et al* 1997) continues as expected in the next decades. However, the species-specific terpene storing characteristics and the complex responses found here, warrant much more research on this issue to arrive to reliable predictions.

3.1.6. References

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Chapter 3.2. Instantaneous and historical temperature effects on α-pinene emissions in *Pinus halepensis* and *Quercus ilex*

Efectes de la temperatura instantània i històrica en les emissions d'α-piné en *Pinus halepensis* i *Quercus ilex*

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3.2.1. Abstract

We compared the role of instantaneous temperature and temperature history in the determination of α-pinene emissions in Mediterranean conifer *Pinus halepensis* that stores monoterpenes in resin ducts, and in Mediterranean broad-leaved evergreen *Quercus ilex* that lacks such specialized storage structures. In both species, α-pinene emission rates (*E*) exhibited a significant exponential correlation with leaf temperature, and the rates of photosynthetic electron transport (J_{CO2+O2}) started to decrease after an optimum at approximately 35 ºC. However, there was a dependence of *E* on mean temperature of previous days than on mean temperature of current day for *P. halepensis* but not for *Q. ilex*. J_{CO2+O2} showed a maximum relationship to mean temperature of previous 3 and 5 days for *P. halepensis* and *Q. ilex* respectively, compared with the current day mean temperature. We conclude that although the best correlation of emission rates were found for instantaneous foliar temperatures, the effect of accumulated previous temperature conditions should also be considered in models of monoterpene emission, especially for terpene storing species.

Key Words: acclimation, emission model, *Quercus ilex*, *Pinus halepensis*, previous climate effects.

Resum

Vàrem comparar els rols de la temperatura instantània i de la temperatura històrica en la determinació de les emissions d' α-piné a la conífera mediterrània *Pinus halepensis* que emmagatzema monoterpens als conductes resinífers, i a la caducifòlia *Quercus ilex*, que no té aquestes estructures d'emmagatzematge especialitzades. En ambdues espècies, la tassa d'emissió d'α-piné (*E*) va mostrar una correlació exponencial significativa amb la temperatura de la fulla, i la tassa del transport fotosintètic d'electrons (J_{CO2+O2}) decreixia després d'un òptim aproximadament a 35 ºC. No obstant, *E* va mostrar una dependència amb la temperatura mitjana dels dies previs comparat amb la del mateix dia per a *P. halepensis*, però no per *Q. ilex*. J_{CO2+O2} també va mostrar una correlació amb la temperatura mitjana dels 3 i 5 dies previs per a *P. halepensis* i *Q. ilex* respectivament més alta que no amb la mitjana del dia de mostreig. Concloem que tot i que les millors correlacions de la tassa d'emissió de terpens es van trobar per a les temperatures foliars instantànies, l'efecte de la temperatura acumulada en els dies previs també s'hauria de considerar als models d'emissions de monoterpens, especialment per a les espècies acumuladores de terpens.

Paraules clau: aclimatació, model d'emissions, *Quercus ilex*, *Pinus halepensis*, efectes del clima previ.

3.2.2. Introduction

Biogenic Volatile Organic Compounds (BVOCs) are produced and emitted by many plant species and have a series of relevant physiological and ecological functions (Peñuelas and Llusià, 2001, 2004). Emission of these compounds has also major consequences for ambient air quality. In particular, these plant-generated compounds can react rapidly with anthropogenic and biogenic trace components of atmosphere (e.g. OH radical, ozone and NO3 radical) and contribute to tropospheric ozone and photochemical smog formation, thereby significantly curbing the quality of ambient air (Fehsenfeld *et al* 1992, Chameides *et al* 1988, Atkinson 2000). In addition, BVOCs might play an important role in altering the climate at regional and global scales (Peñuelas and Llusià 2003, Kulmala *et al* 2004, Tunved *et al* 2006). Because of potentially important role of BVOC in tropospheric air quality and climate, there is continuous interest in developing BVOC emission models to quantify plant-generated volatile flux over large areas (Guenther *et al* 1993, 1995, 2006, Martin *et al* 2000, Niinemets *et al* 2002, Arneth *et al* 2007).

Some monoterpene-emitting species like needle-leaved conifers all across the world and many odorous species in Mediterranean macchia have specialized tissues such as resin ducts or glandular trichomes for storage of produced volatile isoprenoids. On the contrary, some other strong monoterpene-emitting species like Mediterranean evergreen oaks such as *Quercus ilex* L. do not have specific storage tissues for monoterpenes (Loreto *et al* 1996a, Llusià and Peñuelas 2000). These anatomical differences are important as the terpenoid emission from specialized storage is expected to depend only on the diffusion from the storage pools, while in the species lacking the storage pools, the emission is mainly driven by the immediate rate of synthesis (Fall 1999, Kesselmeier and Staudt 1999, Niinemets *et al* 2004).

The rate of terpene emission (E) strongly depends on environmental conditions, in particular, on instantaneous leaf temperature (Tingey *et al* 1980, Kesselmeier and Staudt 1999, Atkinson 2000, Peñuelas and Llusià, 2001, 2003). Typically, the emission rates depend exponentially on temperature, and such an exponential dependence of emissions on instantaneous temperature has been implemented in all terpene emission models. Currently, plant terpene emissions are mostly predicted using Guenther et al. algorithm (G93 model (1993)). For species with specialized storage structures, this model simulates the emission rates using a species-specific basal emission rate (E_0) and scaling the values of E_0 to different temperatures according to an exponential relationship (the temperature correction factor). For monoterpene-emitting species lacking storage structures,

isoprene emission algorithm (Guenther *et al* 1993) that uses additionally light as an emission driver has been implemented (Bertin *et al* 1997, Ciccioli *et al* 1997).

Recently, complementary modeling approaches have been developed that use plant physiological properties to predict emissions (Niinemets *et al* 1999, Martin *et al* 2000, Zimmer *et al* 2000). For instance, the rate of monoterpene emission in species lacking specialized storage structures has been predicted on the basis of photosynthetic electron transport rate (J_{CO2+O2}) and monoterpene synthase activity (Niinemets *et al* 2002). Monoterpene synthase activity provided an estimate of basal emissions analogous to E0 (fraction of electrons in monoterpene synthesis on the model), while J_{CO2+O2} that depends on instantaneous environmental variables, temperature and photosynthetic photon flux density, was used to scale the basal emissions to different temperature and light conditions (Niinemets *et al* 2002). In other physiological models, the emission were also linked to photosynthetic carbon metabolism in various ways (Niinemets *et al* 1999, Martin *et al* 2000, Zimmer *et al* 2000, Bäck *et al* 2005). It has been stated that such modeling approaches allow consideration of stress effects on volatile isoprenoid emissions (Grote and Niinemets 2008). For example, stress-dependent reductions in J_{CO2+O2} are suggested to explain the rapid decline in monoterpene emissions in stressed plants (Niinemets *et al* 2002).

E_0 values were initially supposed to be constant and represent the inherent plant capacity for production of a particular volatile compound (Winer *et al* 1992, Seufert *et al* 1995, Karlik and Winer 2001). However, it has become increasingly apparent that the basal emission rates can change over time (Goldstein *et al* 1998, Llusià and Peñuelas 2000, Gray *et al* 2003, 2006, Kuhn *et al* 2004), but the factors controlling such temporal modifications are still poorly understood. Furthermore, the available emission algorithms have mainly focused on the influence of instantaneous leaf temperature on the emission rates and J_{CO2+O2}. However, many plant physiological processes are known to strongly acclimate to previous leaf temperature environment (Yamori *et al* 2005, Hüve *et al* 2006, Bauerle *et al* 2007), and temperature history likely alters terpene emission rates as well. Already Schurmann (1993) suggested that in some plants, the monoterpene emission may involve distinct long-term kinetic mechanisms. It has been further suggested that isoprene basal emission rate is altered by leaf thermal history (Sharkey *et al* 1999, Fuentes and Wang 1999, Geron *et al* 2000, Lehning *et al* 2001, Petron *et al* 2001), but the way plant emissions adjust to leaf temperature environment is not fully understood. While it has been suggested that leaves respond to average temperature of previous days (Sharkey *et al* 1999, Fuentes and Wang 1999, Geron *et al* 2000, Lehning *et al* 2001, Petron *et al* 2001), it is also not clear over what time period ambient leaf temperatures alter leaf emissions. Given that the internal pool sizes are much larger in species with specialized storage structures, it is expected that the emissions respond to longer historical temperature signal in species with specialized storage structures than in species lacking such storage structures in the foliage.

The aim of the present study was to evaluate the relative importance of instantaneous temperature and temperature history in determining α-pinene emissions in monoterpene-storing Mediterranean evergreen conifer *Pinus halepensis* Mill. and in Mediterranean evergreen broad-leaved species *Quercus ilex* L. that lacks specialized monoterpene storage tissues. Emissions were monitored during the entire season simultaneously with leaf environmental conditions and the correlations of E and J_{CO2+O2} with instantaneous temperatures and with average temperature over different number of days prior to measurements were determined to assess the strength of instantaneous and historical temperature signals.

3.2.3. Material and Methods

Plant material

Full experimental details and the protocol for a-pinene emission measurements are provided in Blanch *et al* (2007). In short: the experiment was conducted in the campus of the Universitat Autònoma of Barcelona, Catalonia, Spain (41°29' N, 2°6' E, elevation 147 m) throughout the summer of 2004. Two-year-old potted (2 l pots) seedlings of *Pinus halepensis* (seedling source: Apromi breeding ground, Juneda, Lleida, Spain) and *Quercus ilex* (seedling source: Forestal Catalana, Breda, Girona, Spain) were used for the experiments. The plants were grown outside under typical Mediterranean conditions in an open plastic tunnel. The plants were watered every two days up to soil field capacity, giving an equivalent of 1 l of water per week and pot.

α-Pinene emission rates in *Pinus halepensis* and *Quercus ilex* were measured every 6 days over the growing season. Instantaneous leaf temperatures and incident quantum flux densities were measured during the emission measurements, while mean daily temperatures were obtained from a climatic station in the locality of the study. The a-pinene emission measurements were conducted at the leaf-level: one leaf of *Q. ilex* and one shoot of *P. halepensis* were clamped in the cuvette, the emission measurements were conducted using an ADC gas exchange system: we divided the output flow tube using a T-system: a part of the flow went into a new tube, in which we placed a three-bed carbon trap tube, and a pump at the end, so, the output air from the cuvette was forced to pass through the carbon tube at a controlled flow (with the pump). In order to substract the outgoing monoterpenes from the ingoing air stream and in order to consider the carry-over effect of the cuvette we made one blank sample every 3 samples: we sampled one cartridge with the cuvette closed without clamping any shoot or leaf. Moreover, we waited 10 minutes between each sample with the cuvette opened in order to get the system ventilated.

Estimation of photosynthetic electron transport rate (J_{CO2+O2})

The photosynthetic electron transport rate (J_{CO2+O2}, μmol m^{-2} s^{-1}) needed to achieve a rate of net carbon assimilation A (μmol m^{-2} s^{-1}) was calculated as Brooks and Farquhar (1985, equation 1), where R_d is rate of mitochondrial respiration continuing in the light (μmol m^{-2} s^{-1}), Γ^* (μmol mol-

[1]) is the hypothetical CO^2 compensation point in the absence of R_d (Laisk 1977), and C_i is the intercellular CO_2 concentration (μmol mol^{-1}).

$R_d$is estimated from the proportionality between A and R_d observed at 25 ^oC (R_d=0.15 A). Γ^* at

$$J_{CO_2+O_2} = \frac{(A+R_d)\cdot(4\cdot C_i + 8\Gamma^*)}{C_i - \Gamma^*} \quad (1)$$

$$\Gamma^* = \Gamma^*_{25} + 0.0188\cdot(T-25) + 0.0036\cdot(T-25)^2 \quad (2)$$

$$T_n = \frac{\sum_{d=1}^{d=n} T_d}{n} \quad (3)$$

different leaf temperatures was estimated according to Lambers et al (1998), using equation 2, where Γ^*_{25} is constant (3.7 Pa).

Measurement of average temperature of previous days

Average temperature of days preceding the measurements Tn (n = 1-15) was calculated using equation 3, where n is the number of days preceding the measurements, and T_d is the average daily air temperature corresponding to day d.

Statistical analyses

In both species, correlations between E and J_{CO2+O2} and averages of temperature were calculated with different number of days prior to measurements, starting with the mean temperature of the day of sampling (T_1) and ending with the mean temperature of the 15 days preceding the measurements (T_{15}).

The effects of leaf temperature and mean temperature of previous days (T_n, *Eq.4*) on the instantaneous a-pinene emission rates (*E*) were analyzed by exponential regressions. The effects of leaf temperature and mean temperature of previous days (T_n, *Eq.4*) on the rate of photosynthetic electron transport (J_{CO2+O2}) were analyzed by quadratic regressions (adjust to a 2nd grade polynomial).

All the statistical analyses were performed with R 2.7.2 for Windows (R Foundation for Statistical Computing, Vienna, Austria).

3.2.4. Results and Discussion

Emission rates and relationship

The emission rates of α-pinene in *P. halepensis* range from 1.3 to 19.8 μg g^{-1} h^{-1} and those of *Q. ilex* between 0.11 and 14.7 μg g^{-1} h^{-1}. *E* exhibited a significant positive exponential correlation with leaf temperature during the measurements in both species, *P. halepensis* (r=0.64, P<0.001) and *Q. ilex* (r=0.57, P<0.001) (*Fig.1*). J_{CO2+O2} showed a significant quadratic correlation with leaf temperature in *P. halepensis* (r=0.59, P<0.01) but not in *Q. ilex* (Fig. 2).

The storing species *P. halepensis* showed an increase of the correlation coefficient of α-pinene emission rates with mean temperature of previous days when considering increasing number of days, reaching the highest correlation coefficient with the mean temperature of the previous 13 days (T_{13}, *Fig.3*).

The non-storing species *Q. ilex* did not show any improvement of the correlation coefficient with previous days for *E* (*Fig.3*): on the contrary, the best correlation was found with the mean temperature of the day of sampling (T_1, *Fig.3*, r=0.27, P<0.1).

The correlation coefficient of J_{CO2+O2} with mean temperature of previous days increased from the first to the following previous days reaching a maximum at the mean temperature of the three previous days for *P. halepensis* (r=0.47, P<0.05) and at the mean temperature of the five previous days for *Q. ilex* (*Fig.4*, r=0.39, P<0.1).

Previous studies have generally reported lower total monoterpene emission rates for *P. halepensis*: Alessio *et al* (2004) found emissions of a-pinene of 0.4 μg g^{-1} [d.m.] h^{-1}, Ormeño *et al* (2007) found emissions between 0.5 and 1.2 μg g^{-1} h^{-1}, and Peñuelas and Llusià (1999) found emissions of 1.5 μg g^{-1} [d.m.] h^{-1}. However, our data was collected in a Mediterranean ecosystem during the high temperatures of the Mediterranean summer in a range between 30 and 44 ^{o}C. High precaution was taken while conducting the measurements to avoid clamping damage of the needles.

Regarding the a-pinene emission rates of *Q. ilex*, our data (0.11 to 14.7 μg g^{-1} h^{-1}) agrees with previous studies. Alessio *et al* (2004) found emissions of 1.7 μg g^{-1} [d.m.] h^{-1}, Street *et a*l (1997)

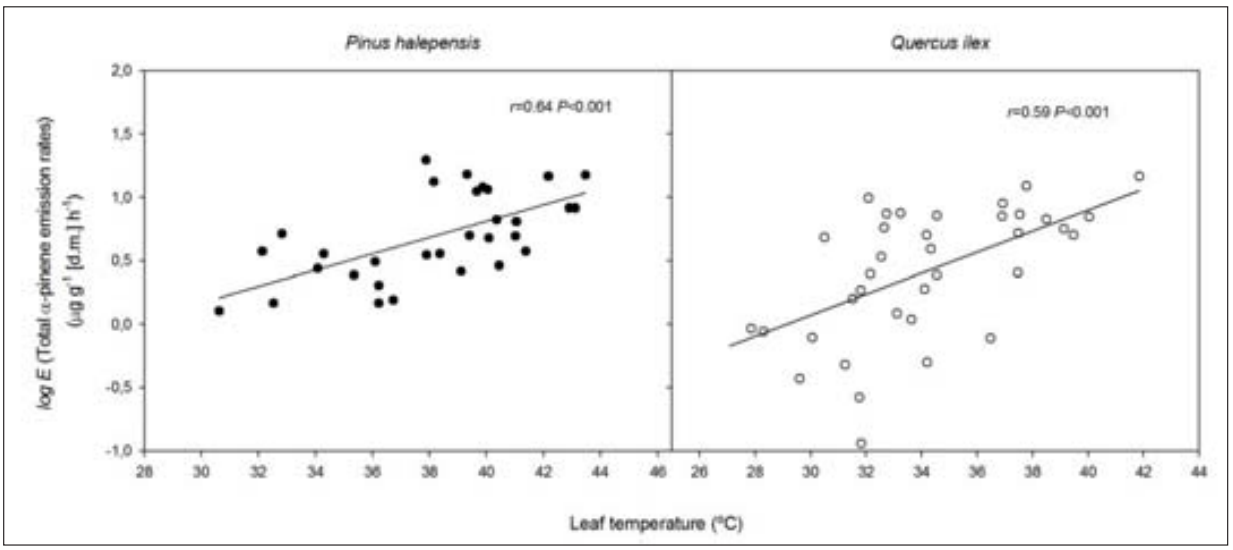

Fig.1 - Relationships between α-pinene emission rates (E) and leaf temperature in evergreen conifer P. halepensis and evergreen broad-leaved tree Q. ilex. Each datapoint corresponds to a separate leaf. Data were fitted by exponential regressions

found emissions between 2.5 and 3 μg g^{-1} [d.m.] h^{-1}, Owen *et al* (1997) reported α-pinene emissions rates between 0.5 and 20 μg g^{-1} [d.m.] h^{-1} and Llusià and Peñuelas (2000) found maximum α-pinene emissions of 5 μg g^{-1} [d.m.] h^{-1}.

Both species, *P. halepensis* and *Q. ilex* showed high correlation coefficients (*r*) of α-pinene emission rates (*E*) with instant leaf temperature (0.64 and 0.59 respectively, *Fig.1*), as it was expected. It has been widely reported that monoterpene emission rates depend exponentially on instantaneous temperatures (Tingey *et al* 1980, Kesselmeier and Staudt 1999, Atkinson 2000, Peñuelas and Llusià 2003).

The Arrenhius-type curve describing the dependence of J_{CO2+O2} on instant temperatures in both species (*Fig.2*) indicates that the measurements were done around the maximum J_{CO2+O2} of the plant, which supports the high values of α-pinene emissions. Because of that, there were also some measurements that were conducted above the maximum, and therefore those plants could have suffered photoinhibition due to the high temperatures.

Terpene emissions in a terpene-storing species such as *P. halepensis* are expected to rely mainly on the extensive storage pools, and are thus, believed to be less sensitive to rapid modifications in the rate of terpene synthesis, for instance, after changes in light (Tingey *et al* 1991, Guenther *et al* 1993). The increase of the correlation coefficients of *E* from T_1 to T_{13} (*Fig.3*) indicates that the pools of monoterpenes depend more on the historical temperature of previous days than of the current day.

The emissions of a-pinene in non-storing species such as *Q. ilex* are on the contrary directly dependent on the rate of terpene synthesis (Fall 1999), that can be altered by temperature, light and water availability (Staudt and Seufert 1995, Kesselmeier *et al* 1996, Loreto *et al* 1996b). Consequently, the emissions of non-storing species are more dependent on the temperature and light conditions in the day of sampling than on the mean temperature of previous days (*Fig.3*). These weaker correlation of *E* with historical temperature may reflect the importance of the initial rapid change in the emission potentials as outlined by Hanson and Sharkey (2001).

In both species *P. halepensis* and *Q. ilex* there was evidence of previous days adjustment in J_{CO2+O2} that was completed after three and five days respectively (*Fig.3*). Given that J_{CO2+O2} may partly control terpene emission rate through NADPH and ATP availability for terpene synthesis (Niinemets *et al* 2002), such long-term changes may reflect coupled adjustment in J_{CO2+O2}, for

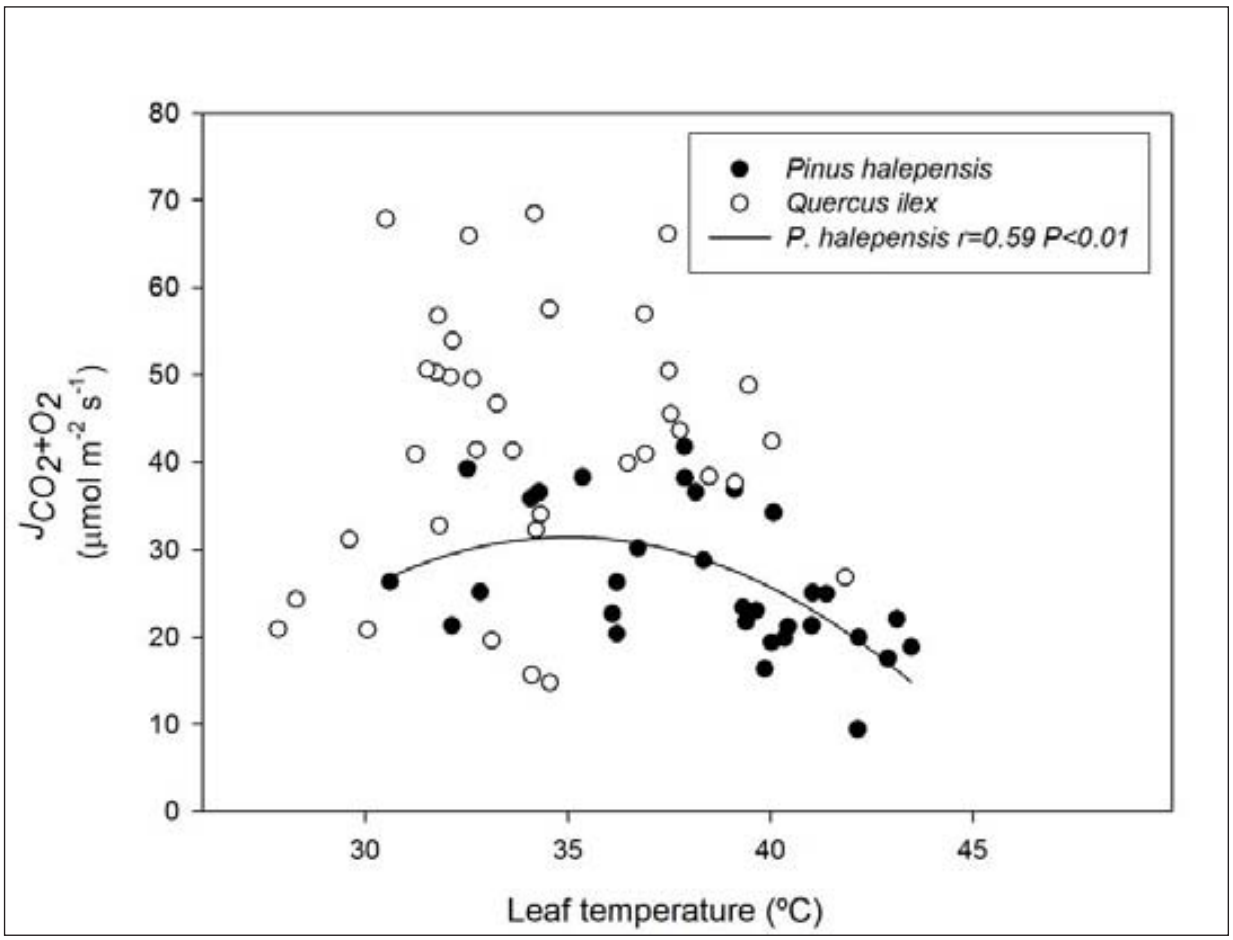

Fig.2 - Relationships between the rate of photosynthetic electron transport (J_{CO2+O2}) and leaf temperature in evergreen conifer P. halepensis and evergreen broad-leaved tree Q. ilex. Each datapoint corresponds to a separate leaf. Data were fitted by quadratic regressions. J_{CO2+O2} values are expressed per unit projected leaf area in both species

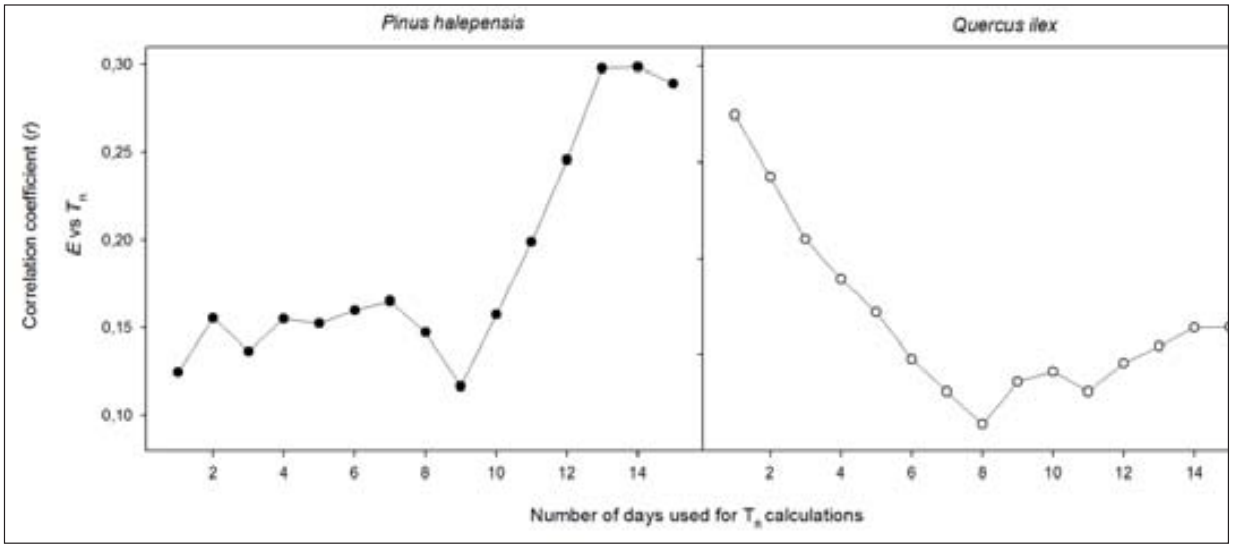

Fig.3 - Correlation coefficient (r) of α-pinene emission rate (E) with the historical average temperature (T_n) while varying the number of days for calculation in P. halepensis and Q. ilex. The number of days ranged from 1 (mean temperature of the day of sampling) until 15 (mean temperature of the preceding 15 days). The maximum values correspond to T_{13} (r=0.29, P<0.1) for P. halepensis and T_1 (r=0.27, P<0.1) for Q. ilex.

instance through anatomical adjustments such as modifications in chloroplast to total leaf surface area ratio (Oguchi *et al* 2003, 2005) or modifications in nitrogen investment in the components of photosynthetic machinery and in enzymes controlling terpene synthesis (e.g.Hikosaka *et al* 1999). It has been observed that acclimation in the heat-stability of photosynthetic electron transport takes between 5-7 days in deciduous trees (Hüve *et al* 2006).

The best correlations of emission rates were found for instantaneous foliar temperatures, partly explaining the success of simple empirical models based on temperature response such as the Guenther model (Guenther *et al* 1993), but overall, these data also underscore the importance of previous leaf temperature environment in determining monoterpene emission rate, in particular in species with extensive foliar monoterpene reservoirs. There have been attempts to include such adaptation responses in the volatile isoprenoid emission models (Guenther *et al* 2000), but species-specific variation in the previous environmental signal and environmental signals of various time length have, to our knowledge, not been considered. The effect of accumulated previous day conditions should thus be considered and implemented in modeling of volatile isoprenoid emissions.

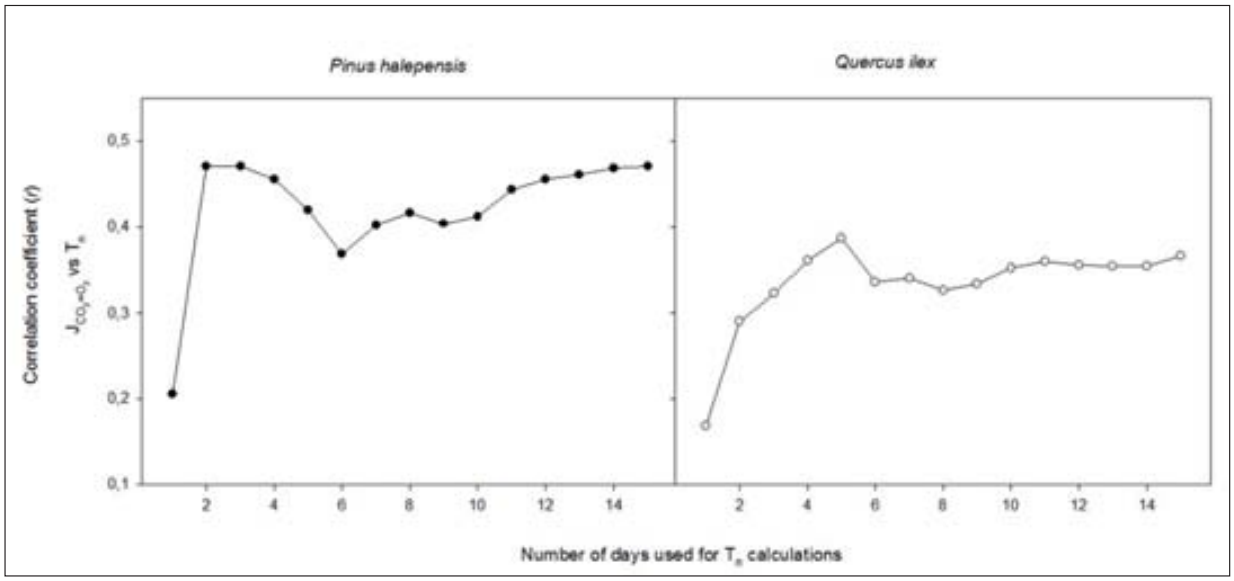

Fig.4 - Correlation coefficient (r) of the rate of photosynthetic electron transport (J_{CO2+O2}) with the historical average temperature (T_n) while varying the number of days for calculation in P. halepensis and Q. ilex. The number of days ranged from 1 (mean temperature of the day of sampling) until 15 (mean temperature of the preceding 15 days). The maximum values correspond to T_3 (r=0.47, P<0.05) for P. halepensis and T_5 (r=0.39, P<0.1) for Q. ilex

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Chapter 4.1. Effects of phosphorus availability and genetic variation of leaf terpene contents and emission rates in *Pinus pinaster* seedlings susceptible and resistant to the pine weevil *Hylobius abietis*

Efectes de la disponibilitat de fòsfor i de la variació genètica sobre la producció i la tassa d'emissió de terpens foliars de plançons de *Pinus pinaster* susceptibles i resistents al corc del pi *Hylobius abietis*

4.1.1. Abstract

We studied the effects of phosphorus fertilization on foliar terpene concentrations and on foliar volatile terpene emission rates in six half-sib families of *Pinus pinaster* Ait. seedlings. Half of them appeared to be resistant to the attack of the pine weevil *Hylobius abietis* L., a generalist phloem feeder, and the other half appeared to be susceptible to this insect. We hypothesized that P stress could modify the terpene concentration in the needles and thus derive to altered terpene emission patterns relevant to plant-insect signaling. The total concentrations and emission rates ranged between 5,732 and 13,995 μg g^{-1} [d.m.] and between 2 and 22 μg g^{-1} [d.m.] h^{-1} respectively. The storage and emission were dominated by the isomers α and β-pinene (77.2 % and 84.2 % of the total terpene amount respectively). P stress caused in both resistant and susceptible families an increase of 31% of the foliar terpene concentrations with an associated 5-fold decrease of the terpene emission rates in sensible seedling families. Those higher contents would indicate an allocation of the "excess of carbon" generated due to growth being limited because of P scarcity, to terpene emissions. The higher increase of terpene emission rates in fertilized plants of sensible families could be related to plant-animal communication and could explain the pattern of weevil damage observed in the field.

Keywords: Maritime pine, nutrient stress, plant resistance to insects, herbivory, plant-insect interactions, large pine weevil, Galicia.

Resum

Vàrem estudiar els efectes de la fertilització amb fòsfor sobre les concentracions de terpens en fulla i sobre la tassa d'emissions de volàtils en fulla en sis famílies de plançons de *Pinus pinaster* Ait. mitjos-germans. La meitat d'elles eren resistents a l'atac del corc del pi *Hylobius abietis* L., un generalista consumidor de floema, i l'altra meitat eren susceptibles a aquest insecte. Vàrem hipotetitzar que l'estrès de P podria modificar a la concentració de terpens de les fulles i per tant, derivar en una alteració del patró d'emissions que influeix en la comunicació planta-insecte. Les concentracions totals i la tassa d'emissions oscil·laren entre 5,732 i 13,995 μg g^{-1} [d.m.] i entre 2 i 22 μg g^{-1} [d.m.] h^{-1} respectivament. L'estrès de P va provocar un increment d'un 31% de les concentracions de terpens foliars en ambdues famílies, amb un decrement associat de 5 cops en la tassa d'emissió de terpens. Aquests majors continguts indicarien una reassignació del "excés de carboni" generat a causa de la limitació del creixement que causa la falta de P, cap a les emissions de terpens. Les famílies sensibles van mostrar un increment més gran de la tassa d'emissions, fet que podria estar relacionat amb la comunicació planta-animal i això podria explicar el patró del dany observat a camp.

Paraules clau: Pi marítim, estrès de nutrients, resistència de la planta a insectes, herbivoria, interaccions planta-insecte, corc del pi, Galicia.

4.1.2. Introduction

Phosphorus has many roles in plant growth and metabolism. One of the principal functions of P is energy transfer through the action of adenosine triphosphate (ATP). ATP and its derivatives, ADP and AMP, are involved in different aspects of energy transfer in all plant growing tissues. Apart from this global function, P is also necessary for assembling nucleic acids (DNA and RNA), proteins, enzymes and carbohydrates. It plays an essential role in photosynthesis and is involved in the formation of sugars and starch. The various roles of P denote its relevance in many vital processes such as the formation of seeds or the development of roots. It also speeds plant maturity and helps the plant resist stresses (Urbano 1999).

Fertilization of young pine seedlings and the subsequent boosting of primary growth rates could, however, could lead to increased susceptibility to pests and diseases due to altered allocation patterns of energy to growth and defence, improved tissue quality for the insects, or both. In this sense, in a field study, Zas *et al* (2006a, 2008) found that traditional silvicultural practices such as P fertilization could lead to greater susceptibility to the pine weevil *Hylobius abietis* L. in seedlings of *Pinus pinaster* Ait. and *Pinus radiata* D. Don, which may be at least partially explained by a reduction in resistance (Moreira *et al* 2008). The pine weevil *H. abietis* is a generalist phloem-feeder that constitute a major pest in conifer plantations all around Europe, where causes important regeneration problems through feeding on the bark of the young pine seedlings (Leather *et al* 1999, Conord *et al* 2006). The susceptibility of *P. pinaster* to this insect has been found to be under strong genetic control, with some families were consistently more damaged than others (Zas *et al* 2005).

Greater nutrient availability could directly increase the nutritional value of the plant tissues and thus increase the preference by the insects (Ayres and Lombardero 2000; Moreira *et al* 2009). Phosphorus fertilization on P stressed pine seedlings may also diminish the allocation of energy to constitutive and induced defences by favouring the growth rates. Several models of plant defence suggest altered patterns of allocation to chemical defences in environments with increased nutrient availability. The Carbon nutrient balance (Bryant *et al* 1983) stated that when growth is limited by nutrients, plants allocate the “excess carbon” to the production of secondary metabolites. The Growth differentiation balance (Lorio 1986) recognizes that all secondary metabolites have an ontogenetically determined phenology and their synthesis is emphasized during periods of plant differentiation. Growth dominates during favourable conditions, and differentiation is at a

maximum only when conditions are suboptimal for growth. The Optimal allocation model (Tuomi *et al* 1991) predicts decreasing investment in defence with increasing resource availability, because the reduced costs of tissue production could compensate the higher risks of herbivore predation. Greater P availability could also lead to a higher appearance of the fertilized plants to the insect. Changes in leaf-contained organic compounds due to fertilization can be translated in changes in the emitted carbon based secondary volatile compounds, as stated by "excess carbon" hypotheses (Peñuelas and Estiarte 1998), thus altering plant-animal interactions,.

Maritime pine (*P. pinaster*) has been widely chosen for forestation in Galicia (NW Spain) since the XVIIIth century. Despite being partly replaced in the last decades by species with higher productions like *P. radiata* and *Eucalyptus globulus*, *P. pinaster* is still the most important forest tree species in Galicia. According to the last forest survey (DGCN 2000), Galicia contains more than 500,000 ha of pure and mixed *P. pinaster* stands, which represents around 44% of the total Galician wooded area. The intensive silviculture applied to *P. pinaster* stands in Galicia entails short rotations (15 to 45 years), in which there is an important extraction of nutrients from the system (Merino *et al* 2003).

Maritime pine plantations in Galicia commonly suffer important nutrient deficiencies (Martins *et al* 2009). These plantations are usually located on acid and sandy soils with low amounts of available nutrients, especially P. Moreover, the loss of nutrients through harvesting can lead to decreased reserves of soil available nutrients (Dambrine *et al* 2000, Merino *et al* 2003). Under these conditions, P appears as one of the most limiting factors for growth in *P. pinaster* stands in NW Spain (Martins *et al* 2009).

The main objective of the present study is to determine the effect of P availability on the content and emission rate of leaf volatile terpenes. We hypothesized that P availability could modify the terpene concentration in the needles and the photosynthetic activity of *P. pinaster* thus leading to altered terpene emission patterns relevant to plant-insect signalling. To this end and with the additional aims of studying the effect of genetic variation and the relationships with the resistance to pests, we analyzed the effect of P fertilization on terpene concentrations and on terpene emission rates in half-sib families of *P. pinaster* seedlings cultivated under controlled conditions, previously found to be resistant or susceptible to the large pine weevil in field conditions in Galicia forests.

4.1.3. Material and Methods

Experimental design and plant material

We performed a two factorial experiment with different pine genetic entries and P fertilization treatments under controlled conditions. The experimental layout was a randomized split-plot design replicated in three blocks, with four phosphorus fertilization treatments acting as the whole factor and six genetic entries as the split factor. In total, we sampled 72 pine seedlings, corresponding to 3 blocks ($\times$ 4 phosphorus fertilization treatments $\times$ 6 genetic entries nested into two susceptibility groups, 'susceptible' and 'resistant' families.

P. pinaster families belonged to six half-sibs families (open-pollinated, known mother trees), all native from the coastal region of Galicia (NW Spain).Three families were previously recognized to be susceptible to the attack by the pine weevil (H. abietis) in an extensive field study, while the other three families appeared to be more resistant to this plague (Zas *et al* 2005). Damage (debarked area by the pine weevil) to the susceptible families in that field study was more than two-fold greater than that suffered by the resistant families (Zas *et al* 2005).

Plant material, greenhouse conditions and experimental fertilization

On 7 February 2006, *P. pinaster* seeds were individually sown in 2 l pots containing perlite in a glass greenhouse (36.5 m long and 15 m wide) with controlled temperature (10-22 ^{o}C at night and day, respectively) and daily water irrigation.

On 15 March 2006, we started to apply the fertilization treatments by sub-irrigation (every two days) with four different fertilization treatments. The complete balanced fertilizer ("*P20*") was prepared according optimum requirements for maritime pine tree growth, containing 100 ppm of N, 20 ppm of P, 40 ppm of K, 10 ppm of Ca, 20 ppm of Mg, and the necessary amounts of micronutrients and trace elements. The other fertilizer solutions ("*P10*", "*P5*" and "*P2*") differed only in the concentration of P, which was reduced to 10, 5 and 2 ppm, respectively, in order to promote growth restrictions through increasing P limitations. The pH values were adjusted to 6.5 in all the solutions. Fertilizer solutions were replaced every two weeks. The experiment was carried out in the facilities of CIF Lourizán (Pontevedra, NW Spain, UTM coordinates 29T 42^{o}24'33'' N 8^{o}39'47''W).

Photosynthetic activity and terpene emission collection

On 24-27 July 2006, measurements of net photosynthetic rates, stomatal conductance and terpene emissions were conducted. These measurements were done at controlled standard conditions (30^{o}C and 1000 μmol m^{-2} h^{-1} PAR). CO_2 exchange was measured using a non-dispersive infrared gas analyzer (IRGA), model ADC-LCPro+ (ADC Inc. Hoddesdon, Hertfordshire, England) connected to a conifer leaf chamber (ADC Inc. Hoddesdon, Hertfordshire, England). CO_2 uptake (*A*) and stomatal conductance (g_s) were measured in lateral shoots on *P. pinaster*. A and gs values were expressed on a projected leaf area basis measured with Li-Cor 3100 Area Meter (Li-Cor Inc., Nebraska, USA).

In order to sample terpene emissions, a T-system was installed outside the cuvette of the IRGAporometer. We used a calibrated air sampling pump at constant flow (Qmax, Supelco, Bellefonte, Pennsylvania) to trap isoprenoids passing part of the air through cartridges (8 cm long and 0.3 cm internal diameter) filled with terpene adsorbents Carbopack B, Carboxen 1003, and Carbopack Y (Supelco, Bellefonte, Pennsylvania) separated by plugs of quartz wool. The sampling time was 5 min, and the flow varied between 470 and 500 ml min^{-1} depending on the tubes' adsorbent and quartz wool packing. The hydrophobic properties of the tubes were supposed to minimize sample displacement by water. In these tubes, terpenes did not suffer chemical transformations as checked with standards (α-pinene, β-pinene, camphene, myrcene, *p*-cymene, limonene, sabinene, camphor, and dodecane). Prior to use, these tubes were conditioned for 10 min at 350 ^{o}C with a stream of purified helium. The trapping and desorption efficiency of liquid and volatilized standards such as α-pinene, β-pinene or limonene was practically 100%. In order to eliminate the problem of memory effect of previous samples, blanks of 5-min air sampling without plants were carried out immediately before and after each measurement. The glass tubes were stored in a portable fridge at 4 ^{o}C and taken to the laboratory where they were stored at -28 ^{o}C until analysis (within 24-48 h). There were no observable changes in terpene concentrations after storage of the tubes as checked by analyzing replicate samples immediately and after 48-h storage. Emission rate calculations were made on mass balance basis and by subtracting the control values (without plants) from the values of samples with plants.

Seedling harvesting and nutrient analyses

On 1 August 2006, we measured height and basal diameter (mean of two measures). A composite sample of primary needles from different parts of each tree was collected, deep frozen and preserved at -80 ^{o}C into close-tight glass vials for the analysis of foliar terpene content. Then pine were destructively sampled, and roots, stems and mature and young needles from each seedling were carefully separated, dried during 72 h at 65 ^{o}C and weighed to the nearest 0.001 g. The needle samples were finely grounded, labelled and preserved for nutrient analysis.

For the analysis of N and P content, 0.3 g of needles were digested in a mixture of selenous sulphuric acid and hydrogen peroxide (Walinga *et al* 1995). Nitrogen was colorimetrically analyzed in diluted aliquots of this digestion using a BioRad 680 microplate reader (California, USA) at λ = 650 nm according the method proposed by Sims *et al* (1995). Phosphorus was analysed in the same diluted aliquots by inductively coupled plasma optical emission spectroscopy (ICP-OES) using a Perkin-Elmer Optima 4300DV (Massachusetts, USA) in the central laboratory facilities at Universidade de Vigo – CACTI (www.uvigo.es/webs/cactiweb/). Nitrogen and P concentration were expressed in mg g^{-1} dried weight of tissue.

Terpene analysis

Tubes with trapped emitted monoterpenes were inserted in an OPTIC3 injector (ATAS GL International BV 5500 AA Veldhoven, The Netherlands) connected to a Hewlett Packard H*P5*9822B GC-MS (Palo Alto, CA, USA), where they were desorbed at 250 ^{o}C during 3 min. Terpenes were separated

using a TRB-5 Fused Silica Capillary column, 30m x 0.25mm x 0.25μm film thickness (Teknokroma, Barcelona, Spain). After sample injection, the initial temperature (40 °C) was increased at 30 ^{o}C min^{-1} up to 60 ^{o}C, and thereafter at 10 ^{o}C min^{-1} up to 150 ^{o}C maintained for 3 min, and thereafter at 70 ^{o}C min^{-1} up to 250 ^{o}C, which was maintained for another 5 min. Helium flow was 1 ml min^{-1}. The identification of terpenes was conducted by GC-MS and comparison with standards from Fluka (Buchs, Switzerland), literature spectra and GCD Chemstation G1074A HP with the Wiley275 library. Terpene calibration curves (for 4 different terpene concentrations) were always significant (R^2>0.99). The most abundant terpenes had very similar sensitivity (differences were less than 5%). Terpene concentration was referred to needle dried weight [d.w.].

For extraction of resin terpenoids in the needles, three-four needles were grounded under liquid nitrogen in Teflon tubes with a Teflon embolus. Then, we added 1 ml of pentane as extractant and 0.1 μl of dodecane, a non-terpenoid internal standard. Teflon tubes with pentane samples were centrifuged in an ultrasonic bath for 5 minutes at 5000 rpm and 5-10 ^{o}C to separate the liquid and solid phases. Pentane extracts were immediately recovered and transferred to chromatography glass vials. After recovering the pentane extract, the mass of the needle pellet was determined by oven-drying at 65 ^{o}C for 4 days. Terpenes in the extract were analyzed using a GC-MS (Palo Alto, CA, USA) with a robotic sample processor (FOCUS) (ATAS GL International BV 5500 AA Veldhoven, The Netherlands). Separation, quantification and identification were performed as described above.

Statistical analyses

All traits were analyzed by the following model:

$$Y_{ijkl} = \mu + B + P + R + G(R) + P*G(R) + P*R + B*R + B*P + \varepsilon_{ijk}$$

, where Yijkl is the variable of the trait, μ is the overall mean, B, P, R and G are the main fixed effects of block, P fertilization, resistance group and genotype, and ε_{ijk} is the experimental error. Genotype was nested within resistance types G(R). The B*P interaction was considered a random factor for properly analyze the split plot design (Littell *et al* 2006). The MIXED procedure of SAS was used. When main effects were significant, differences among treatment means were tested for significance using the LSMEAN statement. The PROC GLM procedure of SAS was used for the MANOVA analyses; Wilk's Lambda statistics were used.

4.1.4. Results

Plant growth and needle nutrient concentrations

Fertilizer treatments strongly affected plant growth (F=20.82, P<0.001) and phosphorus concentration in plant tissues (F=141.39, P<0.001) (*Table 1*). Plants with complete fertilization (*P20*) produced 2.5-fold greater biomass than plants with lower P fertilization (*Fig.1*). P concentration in needles was strongly influenced by fertilization, showing increasing values accordingly to the P fertilizers. Plants under balanced fertilization exhibited P concentrations 3-fold greater than P stressed plants (*Fig.1*). The only treatment that drove P concentration in needles under critical levels was *P2*; this treatment was therefore the one that generated the clearest P deficiency.

Nitrogen concentration in needles was only slightly greater, but significant (F=5.97, P<0.05) in complete fertilization than in P stressed plants (*Table 1*, *Fig.1*).

Those families with a resistant behaviour at field showed slightly higher concentrations of P (F=7.79, P<0.01) in leaf tissues than susceptible families, but no differences in terms of N concentrations (F=3.16, P > 0.05) and total biomass (F= 2.73,P > 0.05) were detected (*Table 1*, *Fig.1*).

Photosynthesis (*A*), stomatal conductance (g_s) and transpiration rates (*E*)

Fertilizer treatments decreased photosynthesis (F=4.48, P<0.05) and transpiration (F=6.12, P<0.05) (*Table 1, Fig.2*): complete fertilization (*P20*) produced lower *A* and *E* than the lowest fertilizer treatment *P2* (*Fig.2*). However, these effects were different in resistant families than in sensible families as revealed by the strong interaction P*R (*Table 1*) for *A*, g_s and *E* .Sensible families showed the lowest values of *A* and *E* at *P10* treatment, and resistant families showed the lowest values of *A* and *E* at *P20* treatment.

Significant differences among families in photosynthesis (F=2.72, P<0.05) and stomatal conductance (F=10.23, P<0.001) were found (*Table 1*).

Table 1 - Summary of the split-plot model for P and N concentration in needles, total biomass, net photosynthetic rates, stomatal conductance, transpiration rates, Total Terpene Contents and Total Terpene Emission Rates of P. pinaster. B P R and G are the main effects of block, fertilization, resistance and genotype. Genotype was nested in resistance G(R)

			P needles		N needles		Total biomass		Net photosynthetic rate		Stomatal conductance		Transpiration Rate		Total terpene concentration		Terpene emission rate	
	DF num	DF den	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
B	3	9	1.19	0.3661	0.89	0.4841	1.81	0.2151	0.01	0.9981	0.07	0.9730	0.17	0.9151	0.48	0.7038	1.61	0.2544
P	3	9	141.39	**<.0001**	5.97	**0.0160**	20.82	**0.0002**	4.48	**0.0347**	2.58	0.1179	6.12	**0.0148**	4.25	**0.0396**	9.76	**0.0034**
R	1	33	7.79	**0.0087**	3.16	0.0846	2.73	0.1081	1.30	0.2623	0.28	0.5977	0.19	0.6685	0.22	0.6456	19.48	**0.0001**
G(R)	4	33	1.15	0.3526	0.78	0.5483	2.95	**0.0345**	2.72	**0.0460**	10.23	**<.0001**	1.76	0.1603	4.16	**0.0078**	16.78	**<.0001**
P*G(R)	12	33	1.85	0.0793	0.89	0.5632	1.94	0.0659	2.03	0.0539	2.70	**0.0118**	1.49	0.1788	1.99	0.0584	3.56	**0.0028**
P*R	3	33	3.75	**0.0201**	1.45	0.2462	4.53	**0.0091**	7.35	**0.0007**	6.17	**0.0019**	5.08	**0.0053**	0.94	0.4329	5.32	**0.0046**
B*R	3	33	0.21	0.8917	0.68	0.5697	1.52	0.2271	0.79	0.5106	0.88	0.4595	0.38	0.7658	1.05	0.3823	2.47	0.0813

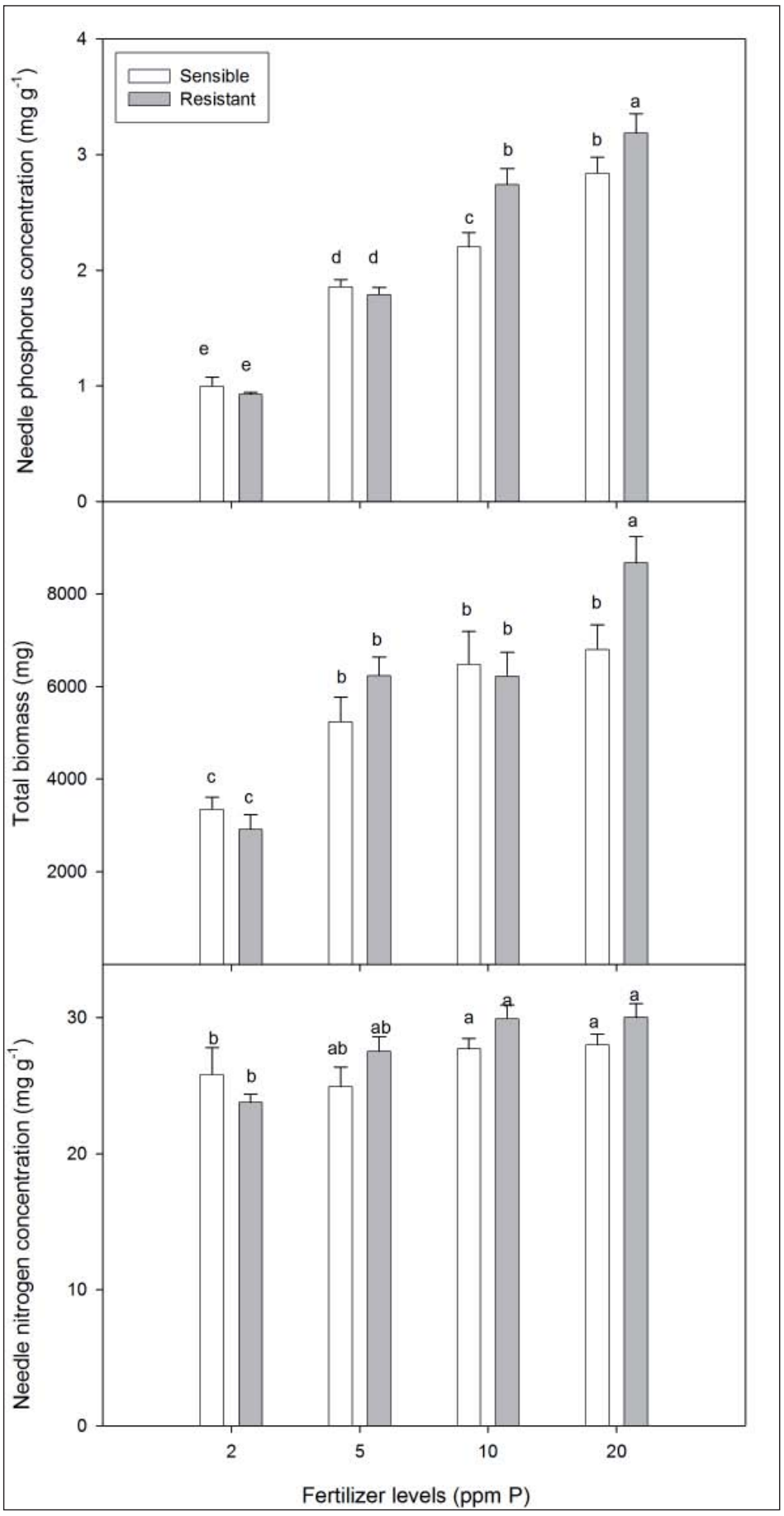

Fig.1 - Nitrogen and Phosphours concentrations in needles and total biomass, for different P fertilizer treatments and resistance family groups. Vertical bars indicate standard error of the mean (n=9). Different letters indicate significant statistical differences among fertilizer levels

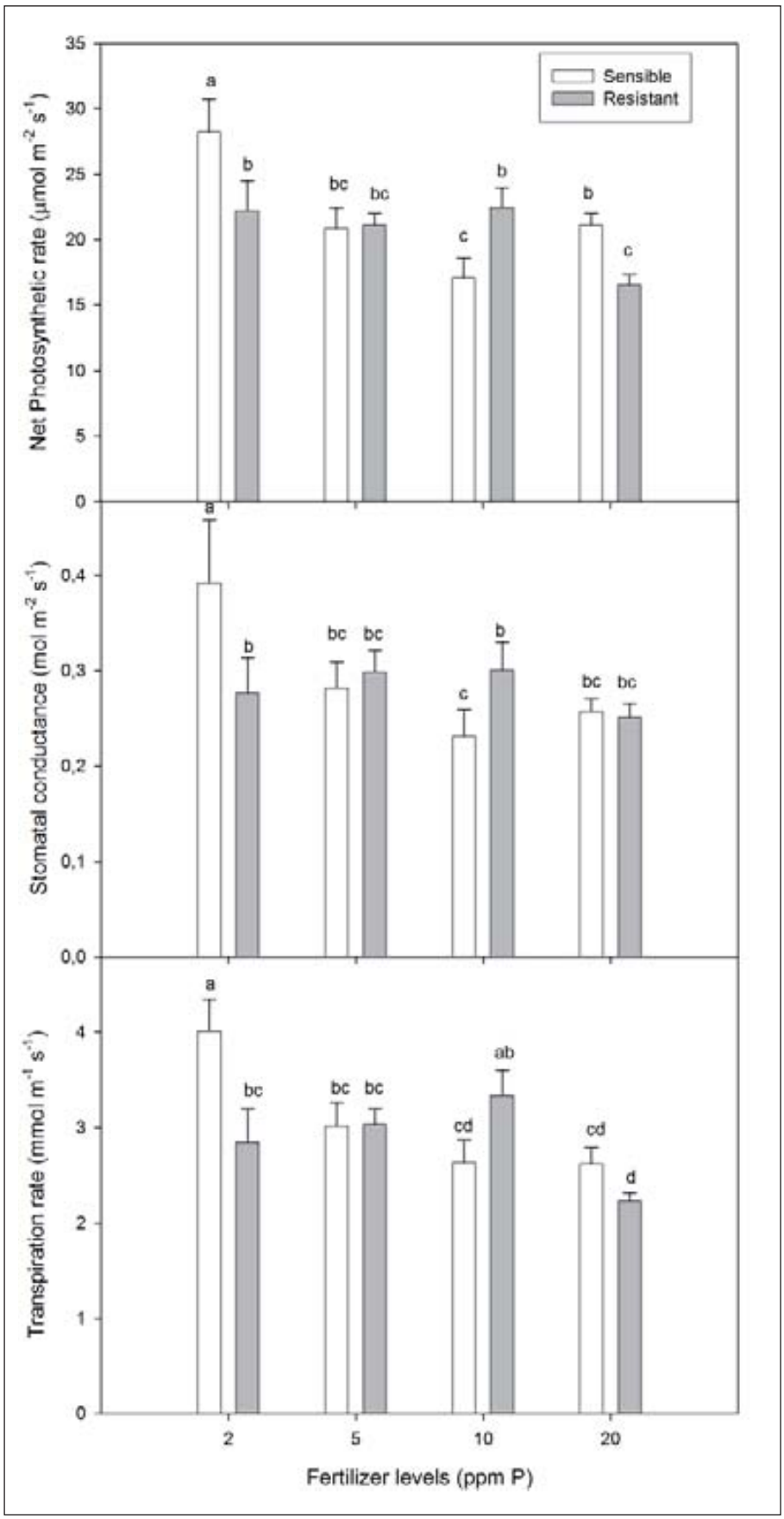

Fig.2 - Net photosynthetic rates, stomatal conductance and transpiration rates for different P fertilizer treatments and resistance family groups. Vertical bars indicate standard error of the mean (n=9). Different letters indicate significant statistical differences among fertilizer levels

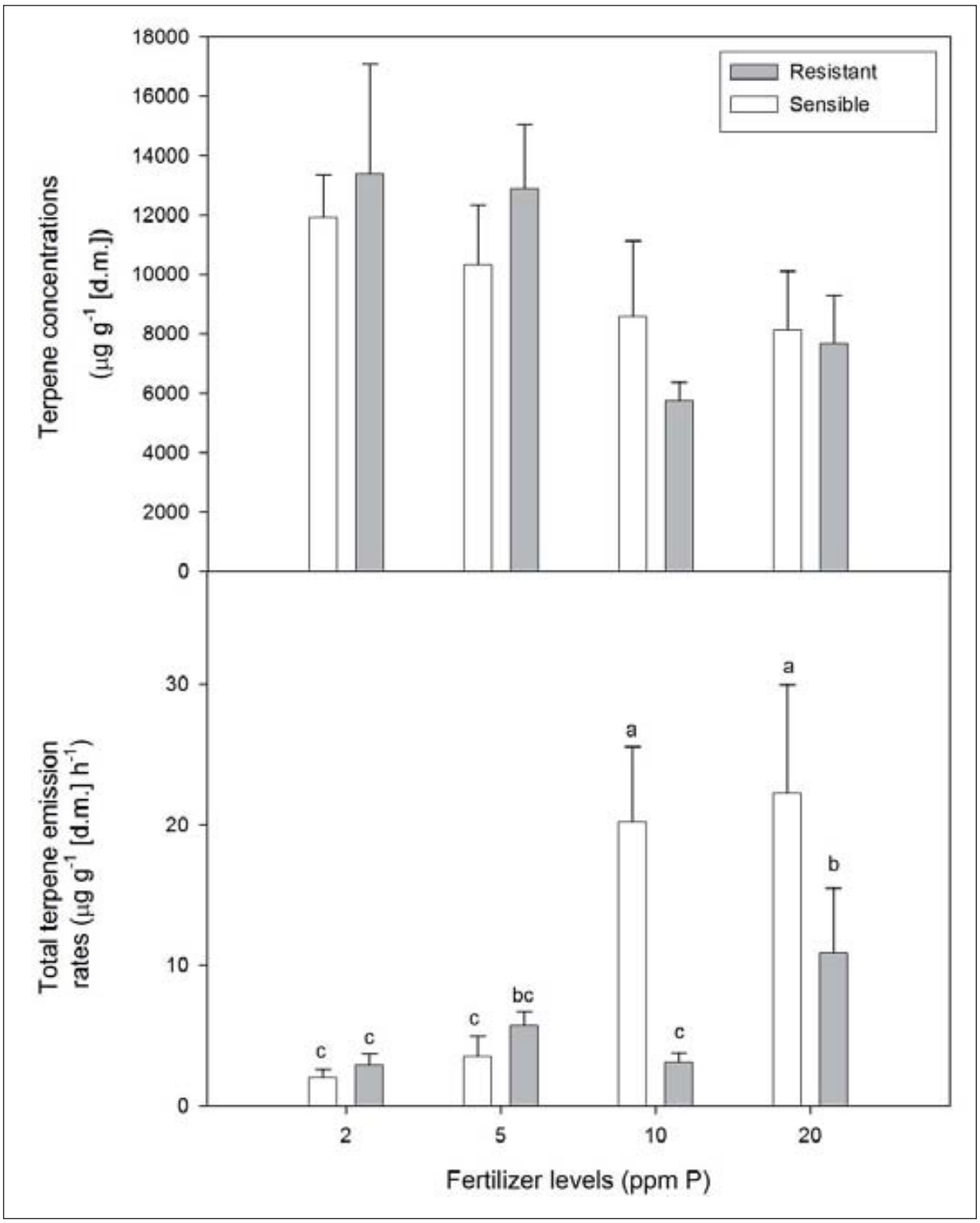

Fig 3. - Total Terpene Contents and Total Terpene Emission Rates for different fertilizer treatments and resistance family groups. Vertical bars indicate standard error of the mean (n=9). Different letters indicate significant statistical differences among fertilizer levels

Table 2 - Individual and total terpene concentrations (n=68) and emission rates (n=70) for all families and all treatments.

	Terpene concentration		Terpene emission	
	$\mu g\ g^{-1}$	%	$\mu g\ g^{-1}h^{-1}$	%
cis-ocimene	14.66	0.16		
α-pinene	4,203.48	46.65	4.33	46.80
camphene	63.36	0.70	0.34	3.70
β-pinene	2,757.90	30.60	3.46	37.45
myrcene	133.80	1.48	0.05	0.57
Δ^3-carene	1,288.85	14.30	0.47	5.05
sabinene	296.44	3.29	0.06	0.67
β-phellandrene			0.25	2.69
terpinolene	30.39	0.34	0.04	0.39
α-fenchene	27.68	0.31		
trans-caryophyllene	65.16	0.72		
α-humulene	29.49	0.33		
germacrene	50.92	0.57		
limonene+β-phellandrene			0.17	1.80
other compounds	47.59	0.53	0.08	0.89

Table 3 - Summary of the Multivariance Analysis for Total Terpene Contents for P. pinaster. B P R and G are the main effects of block, fertilization, resistance and genotype. Genotype was nested in resistance G(R).

Manova hypothesis	Wilk's Lambda	*P*-value
Non-general P effects	0.15194675	0.0036
Non-general R effects	0.44274740	0.0060
Non-general G(R) effects	0.03232137	<.0001
Multivariance analysis	0.28185726	0.3472

Volatile terpenes

Several mono- and sesquiterpenes were found in both leaf concentrations and in terpene emissions. The relative percentages of the different compounds in the total amount are shown in *Table 2*. The isomers α and β-pinene dominate the accumulation (77.2 %) and emission (84.2 %) of the total terpene amount. Δ^3-carene is also present at high proportions accounting for 14.3% of the concentrations, and 5% of the emission rates. The rest of the compounds appeared in smaller percentages (*Table 2*).

The mean terpene concentration values ranged from an average of 7.9 μg g^{-1} in *P20* to an average of 12.6 μg g^{-1} in *P2* (*Fig.3*). The mean emission rates values were however fairly high, ranging from 2.5 μg g^{-1} h^{-1} (*P2*) to 16 μg g^{-1} h^{-1} (*P20*) (Fig.3)

MANOVA analysis for the contents of the individual compounds showed significant differences among P treatments (λ=0.15, P<0.01), resistance types (λ=0.44, P<0.01) and genotypes (λ=0.03, P<0.0001), but there was not significant effect of P*R.Thus the different P treatments influenced not only the individual compound concentrations but also the whole terpene profile of our samples (*Table 3*).

Total terpene concentration significantly increased with P deficiency (F=4.25, P<0.05) (*Table 1, Fig.3*). On the contrary, total terpene emission rates significantly decreased with P fertilization (F=9.76, P<0.01) (*Table 1*, *Fig.3*). This effect was much higher in sensible than in resistant families (P*R interaction F=5.32; P=0.0046; *Fig.3*).

There was a strong effect of family within resistance (F=26.78, P<0.0001) and of the interaction P*G(R) (F=3.56, P=0.0028) for terpene emission rates evidencing that different families showed different behaviours. Not all sensible families increased significantly their emission under high P availability.

4.1.5. Discussion

Terpene contents and emissions

The observed mean terpene concentrations in the P. pinaster leaves were lower than reported in other studies for the same species (Arrabal *et al* 2005) or in other pine species (e.g. Blanch *et al* 2009). The mean terpene emission rates were, however, higher than the values reported in the available literature (e.g. 0.2 $\mu g\ g^{-1}\ h^{-1}$ reported by Simon *et al* 1994). Those differences of our data compared with literature values could be explained by the differences in the environmental conditions during the measures, which were relatively warmer in our experiment (standard conditions 30 ^{o}C and 1000 $\mu mol\ m^{-2}\ h^{-1}$ PAR) (Kesselemeier and Staudt 1999). The low concentration values in leaves and the relatively high emission rates observed in the present study suggest that *P. pinaster* tends to emit the monoterpenes instead of keeping them in the terpene pools.

Our results, where α- and β-pinene were the 77.2% and 84.2% of the total emission rates and concentrations respectively (*Table2*) agree with previous studies that have shown that α- and β-pinene are the principal terpenes emitted (Simon *et al* 1994) and accumulated (Arrabal *et al* 2005, Ormeño *et al* 2009) by *P. pinaster*. Apart from being the terpenes with higher concentrations, α- and β-pinene have vapour pressures two to three times higher that the rest of the emitted terpenes. As the reaction rate constants of those compounds, α- and β-pinene, with O_3, OH^- and NO_3^- are lower (Atkinson 1990), and as their mean estimated life is below 5 h (Seinfeld and Pandis 1998), they have a key role in the environmental chemistry.

Phosphorus and genetic effects on photosynthesis and terpene content and emission

P concentration in leaves was above the P deficiency levels proposed for field studies (Bonneau 1995) in plants with *P5*, *P10* and *P20* fertilizer levels. That is, our fertilization ranged from high levels to low levels, but always within the regular physiological margin. The fertilization treatment

was significantly effective: the higher the fertilization dose, the higher the concentration of P in the plant, as previously reported (Keay *et al* 1968). Moreover, P fertilization also increased the biomass of fertilized plants (*Fig.1*) accordingly to the growth response to P fertilization observed at field on P impoverished soils (Martins *et al* 2009). Curiously, photosynthesis rates, stomatal conductance and transpiration showed a slight tendency to decrease with greater P doses the days of measurement (*Fig.3*). Despite the fact that P plays an essential role in photosynthesis and is involved in the formation of sugars and starch (Urbano 1999), previous authors have also reported negative correlations between P fertilization and A (Loustau *et al* 1999, Cheaib *et al* 2005). Warren and Adams (2002) suggested that the lack of photosynthetic response to P supply was the result of a deficiency of N induced by high P supply. That deficiency of N in plants would decrease the activity of the enzyme Rubisco, and consequently the photosynthetic parameters (A, g_s) would decrease. Since the biomass clearly increased with the fertilization, the higher photosynthetic rates of *P2* had to be limited in time.

Resistant and non-resistant families showed contrary responses to initial P deficiency. The significant interaction fertilization × genotype (*Table1*) suggests some genetic variation in the nutrient use efficiency among the studied genotypes. These differences may arise by different nutrient use efficiency between them. This agrees with genetic differences in nutrient use efficiency in response to fertilization reported in many tree species (i.e. Baligar *et al* 2001, Zas *et al* 2006b, 2008).

The most P-stressed conditions (doses *P2* and *P5*) led to higher leaf terpene concentrations (*Fig.3*) accompanying lower biomass accumulations. These higher terpene contents can be explained by many of the theories based on the "excess carbon" hypothesis (Peñuelas and Estiarte 1998) such as the Carbon-Nutrient Balance theory (Bryant *et al* 1983) and the Growth Differentiation theory (Lorio 1986). These theories state that plants use resources to produce carbon based secondary metabolites when they do not use those resources for growth (Peñuelas and Estiarte 1998).

Higher amounts of terpenes were emitted in the less stressed conditions (doses *P10* and *P20*) in comparison with the most stressed conditions especially in sensible species (*Fig.3*)

The fact that sensible families emitted higher amounts of terpenes strengthens the theory that there is a genetic component in the terpene production and emission patterns, similarly to the above discussed genetic differences in nutrient use efficiency in response to fertilization. Moreover, the fact that sensible families emitted higher amounts of terpenes could be related to plant-animal communication: an increase of P could increase the attack of *H. abietis* at field through the increase in the amount of α-pinene emitted since it attracts *H. abietis* (Moreira *et al* 2008). In fact, the amount of debarked area in young seedlings at field has been found to increase with higher P availability (Zas *et al* 2006b). The preference of pine weevil for sensible families could be explained by the higher emission rates of those families under the most fertilized conditions (*P10* and *P20*), compared to resistant families (*Fig.3*).

In conclusion, higher phosphorus availability altered the plant physiology (higher biomass, higher nutrient concentrations) decreased the accumulation of leaf terpenes and increased the emission rates of terpenes in *Pinus pinaster*, a terpene storing species. There was a genetic effect, and different responses in physiology and in terpene production and emission of pine families depending on their susceptibility to weevil damage at field. The higher terpene emission rates of susceptible families under high nutrient availability could explain the pattern of weevil damage observed at field.

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Chapter 4.2. Investigating the photosynthesis and terpene-content strategies of two different genotypes of *Arabidopsis thaliana* (wild-type and *CoxIV-FaNES1* transgenic)

Investigació de les estratègies fotosintètiques i de producció de terpens de dos genotips diferents de *Arabidopsis thaliana* (genotip salvatge, i genotip transgènic *CoxIV-FaNES1*)

4.2.1. Abstract

We investigated the hypotheses that an Arabidopsis genetically modified to emit nerolidol will show other differences in terpene production, and physiology. To this end, photosynthetic rate, electron transport rate, fluorescence, and volatile terpene concentrations were analyzed in leaves, and volatile terpene concentrations in roots, of two genotypes of *Arabidopsis thaliana* (a wild-type "WT", and a transgenic line (*CoxIV-FaNES1*) with linalool/nerolidol synthase "TR"). For both genotypes, we found low concentrations of α-pinene+β-ocimene, limonene and humulene in leaves; and higher concentrations of α-pinene+β-ocimene, sabinene+β-pinene, β-myrcene, limonene and humulene in roots. TR plants tended to have lower pools of terpene compounds in their leaves, with lower photosynthesis rates, electron transport rates and stomatal conductance, compared with WT plants. The maximal photochemical efficiency *Fv/Fm* was also significantly lower in TR plants, indicating that these genotypes were more stressed than WT plants. However, TR plants had higher root terpene concentrations. Thus TR plants appear to deflect the resources towards root production of volatile terpenes in detriment to leaf production. We conclude that the TR plants which are genetically modified to emit nerolidol showed significant differences in pools of other terpenoids, and also showed significant differences in stress status and physiology.

Keywords: *Arabidopsis thaliana*, *FaNES I*, leaf terpene concentrations, root terpene concentrations, genetic effect.

Resum

Vàrem investigar la hipòtesi que una Arabidopsis genèticament modificada per a emetre nerolidol mostraria diferències en la producció de terpens i en la fisiologia. Per a tal fi, vàrem analitzar la tassa fotosintètica, el transport d'electrons, la fluorescència i les concentracions de terpens volàtils a les fulles, i les concentracions de terpens volàtils a les arrels de dos genotips diferents d'*Arabidopsis thaliana* (un genotip salvatge "WT", i una línia transgènica (*CoxIV-FaNES1*) amb la sintasa linalool/nerolidol "TR"). Als dos genotips vàrem trobar concentracions baixes de α-piné+β-ocimené, limoné i humulé a les fulles, i concentracions altes de α-piné+β-ocimené, sabiné+β-piné, β-mircé, limoné i humulé a les arrels. Les plantes TR van tendir a tenir menors acumulacions de terpens a les seves fulles, on la tassa fotosintètica, el transport d'electrons i la conductància estomàtica van ser baixes en comparació amb les plantes WT. La màxima eficiència

del fotosistema II *Fv/Fm* també va ser significativament més baixa a les plantes TR, indicant que aquest genotip patia més estrès que no les plantes WT. No obstant, les plantes TR van presentar concentracions més grans de terpens a les arrels. D'aquesta manera sembla ser que els recursos es desvien cap a la producció de terpens a les arrels en detriment de la producció a les fulles. Concloem que les plantes TR, que estan genèticament modificades per emetre nerolidol, varen mostrar diferències significatives en les acumulacions de terpens, i també varen mostrar diferències significatives en l'estat d'estrès i en la fisiologia.

Paraules clau: *Arabidopsis thaliana*, *FaNES I*, concentracions de terpens a les fulles, concentracions de terpens a les arrels, efecte genètic.

4.2.2. Introduction

Plants produce a variety of VOCs of which the most representative and abundant group is isoprenoids (Kesselmeier and Staudt 1999). Mono and sesquiterpenes are C_{10} and C_{15} isoprenoid compounds that can be produced in the chloroplasts (MEP pathway) and in the cytosol (MVA pathway) (Kreuzwieser *et al* 1999, Owen and Peñuelas 2005). The physiological function of these isoprenoids is to avoid damages in cellular membranes when the plants are under physiological stresses, for example, water stress, high temperatures, oxidative stress and high irraditation (Sharkey and Singsaas 1995, Loreto and Velikova 2001, Peñuelas and Llusià 2003, Peñuelas and Munné-Bosch 2005). Mono and sesquiterpnes also have ecological functions; they are defensive compounds in case of pathogens attack or herbivory (Croteau 1987, Pichersky and Gershenzon 2002) and they can act as pollinator attractants (Kesselmeier and Staudt 1999), and may also play a role in allellopathy (Fischer *et al* 1994, Peñuelas *et al* 1996). Terpenoids can have impact on regional air quality reacting with anthropogenic and biogenic nitrogen oxides, contributing to tropospheric ozone and photochemical smog formation (Chameides *et al* 1988).

Volatile isoprenoids are mostly produced and emitted by the aerial parts of the plant (leaves and flowers). However, Janson *et al* (1993) suggested roots as a possible source of monoterpenes in soil and recent studies also show that there is terpenoid production and emission in roots (Asensio *et al* 2007). This has been supported by measurements of monoterpene emissions in laboratory experiments from pine roots with qualitative and quantitative evidence of the existence of monoterpenes in soils under pine trees (Lin *et al* 2007, Asensio *et al* 2008).

The commonly used model plant *Arabidopsis thaliana* does not emit isoprene (Loivamaeki *et al* 2007). However, *A. thaliana* is thought to have over 30 putative genes belonging to the terpene synthases (TPSs), a multigene family (Aubourg *et al* 2002, Chen *et al* 2003). Most of these are almost exclusively expressed in flowers (Chen *et al* 2003, Tholl *et al* 2005, Aharoni *et al* 2006), but low terpene emissions from leaves (Chen *et al* 2003) and even from roots (namely, 1,8-cineole) have been detected (Chen *et al* 2004).

Previous studies referring to *A. thaliana* terpene production focused in the emission of terpenoids by its flowers (Chen *et al* 2003, Aharoni *et al* 2003). Although Chen et al (2003) pointed that there are trace amounts of the monoterpenes limonene and β-myrcene within its leaves (Chen *et al* 2003), there is generally a lack of information regarding leaf or root production of terpenes in

this species. However, Kappers (2005) developed a transgenic line (*CoxIV-FaNES1*) with linalool/nerolidol synthase, targeted specifically to the mitochondria. By studying this transformed genotype (TR) with a wild-type (WT) Arabidopsis, we aimed to the hypothese that the Arabidopsis genotype which is modified to emit nerolidol from mitochondrial synthesis will show other differences in terpene production, and physiology in leaves and roots.

4.2.3. Material and Methods

Plant material and plant growth

We used *Arabidopsis thaliana* genotype *Landsberg erecta* (*Ler-0*) (WT) and the transgenic *FaNES1* line (TR) from Iris Kappers (Wageningen University), which expresses a linalool/nerolidol synthase gene. *A. thaliana* seedlings were placed for 4 days at 4 ^{o}C in Petri dishes, and were cultivated in 475 cm^3 plastic pots filled with peat and perlite (2:1, v/v) in a controlled environment chamber (14 h photoperiod, 130–150 μmol quanta m^{-2} s^{-1}, 21 ^{o}C air temperature).

The growth medium used was based on that optimized by Gibeaut *et al* (1997). The final concentrations were 1.5 mM $Ca(NO_3)_2$, 1.25 mM KNO_3, 0.75 mM $MgSO_4$, 0.5 mM KH_2PO_4, 70 μM Fe-diethylenetriamine pentaacetate, 50 μM KCl, 50 μM H_3BO_3, 10 μM $MnSO_4$, 2 μM $ZnSO_4$, 1.5 μM $CuSO_4$, and 0.075 μM ammonium molybdate (chemicals were from Fluka, Buchs, Switzerland).

Plant measurements: basal rosette diameter, CO_2 exchange and chlorophyll flourescence

The diameter of the basal rosette was measured in each plant throughout the experiment. CO_2 exchange was measured at the end of the growing cycle using a portable non-dispersive infra-red gas analyzer (IRGA), model ADC-LCi (ADC Inc. Hoddesdon, Hertfordshire, England) connected to an Arabidopsis leaf chamber (ADC Inc. Hoddesdon, Hertfordshire, England). CO_2 uptake (A) and stomatal conductance (g_s) were measured in leaves of the basal rosette. A and g_s values were expressed on a projected leaf area basis measured with Li-Cor 3100 Area Meter (Li-Cor Inc., Nebraska, USA).

The maximum photochemical efficiency of PSII (*Fv/Fm*) and the apparent photosynthetic electron transport rate (*ETR*) were measured at the end of the growing cycle with a PAM-2000 fluorometer (Walz, Effeltrich, Germany). ETR was estimated as:

$$ETR = \frac{\Delta F}{F'_m} \cdot PPFD \cdot 0.84 \cdot 0.5$$

, where $\Delta F/F'_m$ (actual photochemical efficiency of PSII) was calculated within the software, according to Genty *et al* (1989), 0.84 is the coefficient of absorption of the leaves, and 0.5 is the fraction of electron involved in the photoexcitation produced by one quanta, since two photosystems are involved. Chlorophyll fluorescence was measured twice a day: after turning the lights on and after 7 hours of lighting. The maximum PSII photochemical efficiencies (*Fv/Fm*) were measured after keeping leaves in the dark for at least 25 min.

Laboratory analyses: leaf and root terpene contents

For measurement of monoterpenes, leaves and roots were ground in liquid nitrogen and repeatedly extracted (three times) with pentane, with a non-terpenoid internal standard (0.1 μl of dodecane). Before chromatographical analysis, we centrifuged extracted leaves and roots with pentane at 10,000 rpm for 10 min. Extracts were then concentrated with a stream of nitrogen, because low concentrations were expected.

Monoterpene separation and analyses were conducted in a GC-MS system (Hewlett Packard HP59822B, Palo Alto, CA, USA). Extracts (3 μl) were injected in to the GC-MS system and passed into a 30 m x 0.25 mm x 0.25 μm film thickness capillary column (Supelco HP-5, Crosslinked 5% pH Me Silicone). Full scan method was used to perform the chromatography. The GC oven was programmed to start at 40 ^{o}C, then the temperature was increased at 30 ^{o}C min^{-1} up to 70 ^{o}C, and thereafter at 10 ^{o}C min^{-1} up to 150 ^{o}C, when the temperature was maintained for 5 minutes, and thereafter at 70 ^{o}C min^{-1} up to 250 ^{o}C, which was maintained for another 5 min. Helium flow was 1 ml min^{-1}. For both species, 2 blank analyses per day were also conducted.

The identification of terpenes was conducted by GC-MS and comparison with standards from Fluka (Buchs, Switzerland), and GCD Chemstation G1074A HP with the Wiley275 library. An internal standard dodecane was used to determine extraction efficiency. Dodecane did not co-elute with any terpene. Calibrations was performed with the common terpenes α-pinene, Δ^3-carene, β-pinene, β-myrcene, *p*–cymene, limonene and sabinene standards once every five analyses. The major ions of each compound were used for quantification. Terpene calibration curves (n=4 different terpene concentrations) were always significant ($R^2>0.99$) in the relationship between signal and terpene concentration. The most abundant terpenes had very similar sensitivity (differences were less than 5%). Total terpene concentrations were calculated as the sum of these main terpenes.

Leaf dry mass weight was determined after drying the residual vegetal material at 60 ^{o}C until constant weight.

Statistical analyses

Analysis of variance (ANOVA) with Fisher post hoc tests for all the studied dependent variables, and Student's t-tests were used to test the significance of differences in response between transformed and wild type plants in each fertilization and drought treatment, using R 2.7.2 software for Windows (R Foundation for Statistical Computing, Vienna, Austria). Differences were considered significant at a probability level of $P<0.05$.

4.2.4. Results

Growth: mean diameter of the basal rosette

The growing pattern was different in WT than in TR: at the end of the experiment TR plants reached 55.5 % bigger basal rossetes than WT plants. WT plants reached their maximum diameter half way through the experiment, with very low increase during the two last weeks. During this time plants increased from 4.1 to 4.5 cm. TR plants had bigger basal rosettes diameters that increased continuously during the 4 weeks of the experiment. During the two last weeks of the experiments, plants grew from 4.3 to 7.0 cm.

Net photosynthetic rates, stomatal conductance and fluorescence measurements

Net photosynthetic rates (*A*) were 78.6% lower in TR plants than in WT plants ($P<0.001$; *Fig.1*).

Stomatal conductance (g_s) tended to be lower in TR plants compared to WT plants (not significant $P=0.12$, *Fig.1*). The apparent photosynthetic electron transport rate (ETR) was 30.8% lower ($P<0.001$) in TR plants than in WT plants (*Fig.1*). The maximum photochemical efficiency of PSII (*Fv/Fm*) was 25.5% lower ($P<0.001$) in TR plants than in WT plants (*Fig.1*).

Leaf VOC contents

There was no significant difference in leaf terpene concentration between the two genotypes, but there was a tendency for higher terpene concentrations in WT plants (*Fig. 2*). The main terpenes produced in both genotypes were the monoterpenes α-pinene+β-ocimene and limonene and the sesquiterpene humulene (*Fig.3*). Other terpene-like compounds were found: "terpene-like compound 1" (possibly myrtenal), "terpene-like compound 2" (possibly β-ionone) and "terpene-

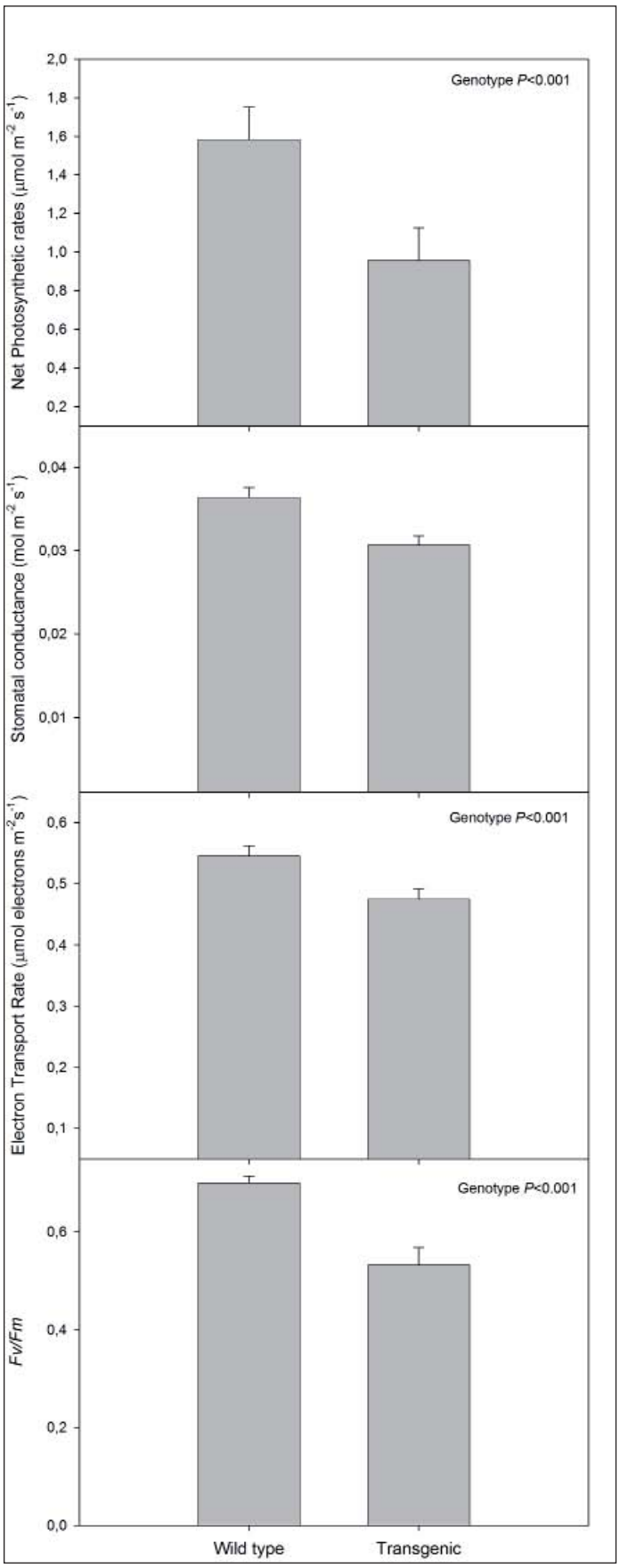

Fig.1 - Net photosynthetic rates (μmol m^{-2} s^{-1}), stomatal conductance (mol m^{-2} s^{-1}), apparent photosynthetic electron transport rate (μmol m^{-2} s^{-1}) and Photochemical efficiency (Fv/Fm) for Wild type WT and Trangenic TR A. thaliana plants. Statistical significance for the overall effect of genotype is indicated inside the panels. Vertical bars indicate standard errors of the mean (WT n=50; TR n=42)

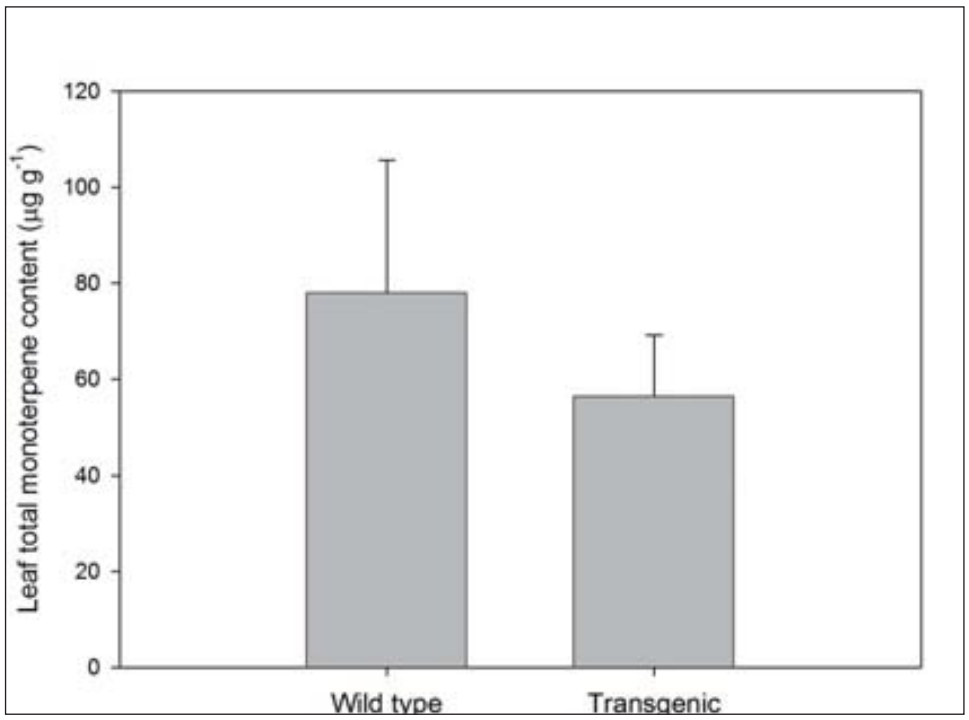

Fig.2 - Total leaf terpene concentrations (µg g-1 [d.m.]) for wild type WT and Trangenic TR A. thaliana plants. "Total" only includes the identified terpenes α-pinene+β-ocymene, limonene and humulene. Vertical bars indicate standard errors of the mean (WT n=50; TR n=42)

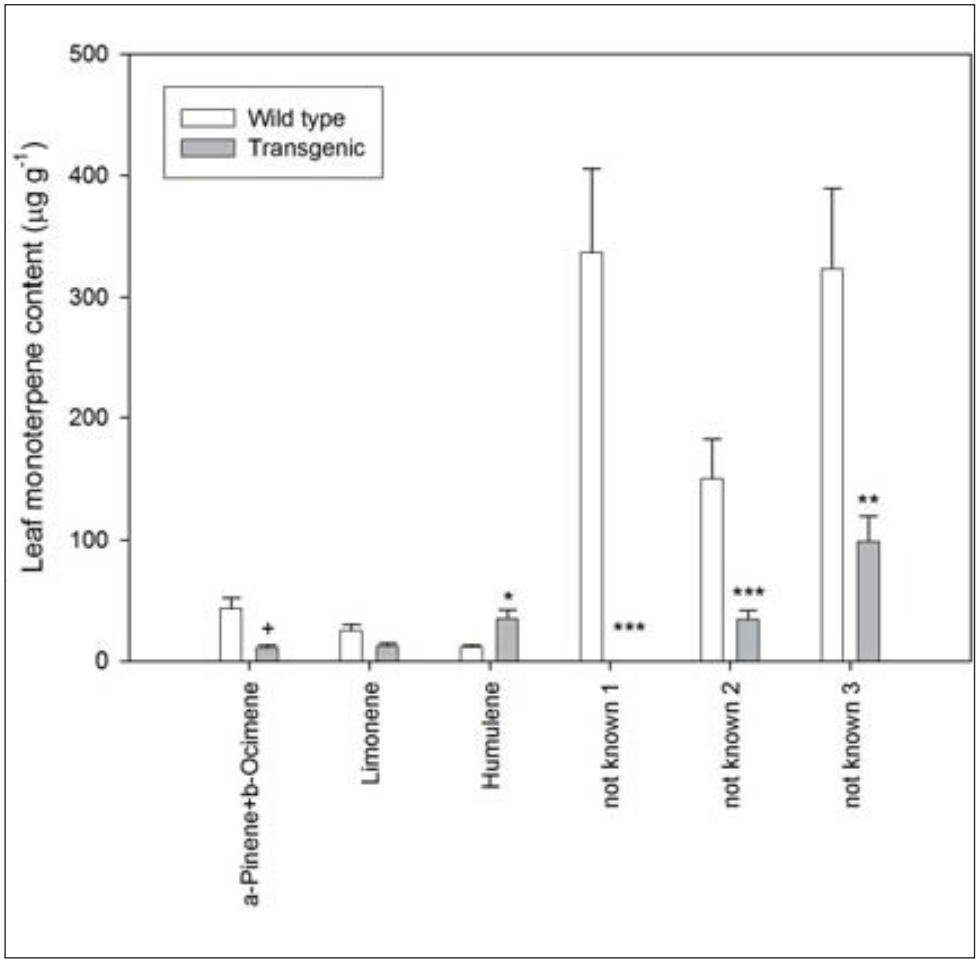

*Fig.3 - Individual leaf terpene and unknown componds'concentrations (µg g-1 [d.m.]) for wild type WT and Trangenic TR A. thaliana plants. Vertical bars indicate standard errors of the mean (WT n=50; TR n=42). Asterisks indicate significant differences among the drought treatment. (+ P<0.1, * P<0.05, ** P<0.01, *** P<0.001)*

like compound 3" (*Fig.3*). TR plants tended to produce lower amounts of terpenes than WT plants (*Fig.3*). The "unknown 2" compound was not produced in TR plants (*Fig.3*).

Root VOC contents

TR plants produced significantly ($P<0.001$) higher (239%) concentrations of terpenes than WT plants (*Fig.4*). The main terpenes produced in both genotypes were the monoterpenes α-pinene+β-ocimene, sabinene+β-pinene, β-myrcene and limonene and the sesquiterpene humulene (*Fig.5*). Other terpenes were found: "unknown 3" and "unknown 4" (*Fig.5*).

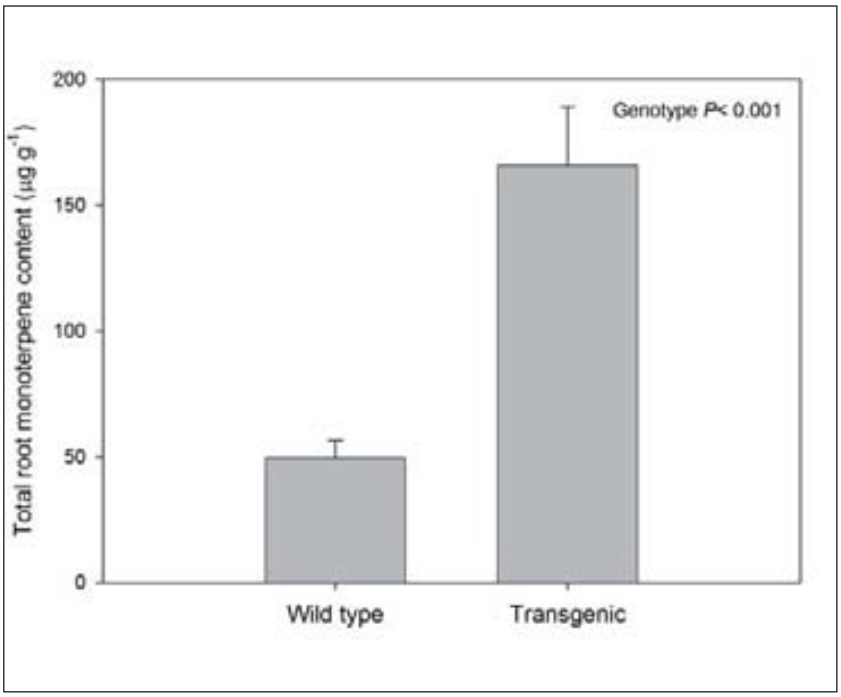

Fig.4 - Total root terpene concentrations (µg g^{-1} [d.m.]) for wild type WT and Trangenic TR A. thaliana plants. "Total" includes α-pinene+β-ocimene, sabinene+β-pinene, β-myrcene, limonene and humulene. Vertical bars indicate standard errors of the mean (WT n=50; TR n=42). Statistical significance for the overall effect of genotype is indicated inside the panels

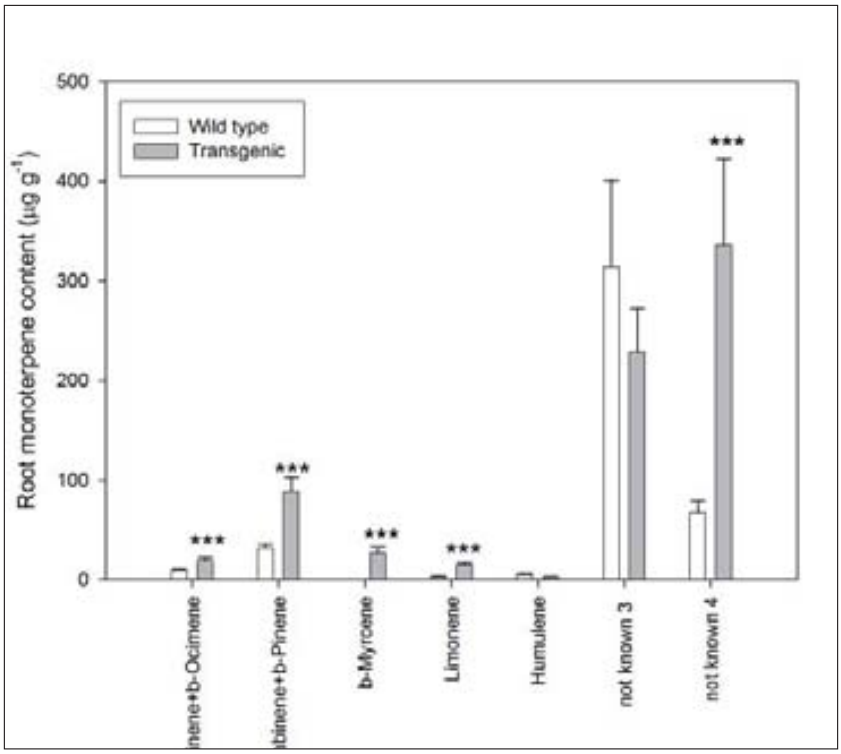

*Fig.5 - Individual root terpene and unknown compounds' concentrations (µg g^{-1} [d.m.]) for wild type WT and Trangenic TR A. thaliana plants. Vertical bars indicate standard errors of the mean (WT n=50; TR n=42). Asterisks indicate significant differences among the drought treatment (*** P<0.001)*

4.2.5. Discussion

Compounds detected in leaf and root extracts

Our results agree with and expand the previous results of Chen *et al* (2003) who found traces of monoterpenes in leaves of *Arabidopsis thaliana* plants, such as β-caryophillene and thujopsene (higher concentrations, but lower than 0.6 ng h^{-1} $plant^{-1}$) or β-farnesene and β-chamigrene (lower concentrations). There is clear evidence of terpene production in leaves and roots of both WT and TR *A. thaliana*. For both genotypes, we found low concentrations of α-pinene+β-ocimene (WT 42.67 ± 20.64 μg g^{-1}, TR 10.55 ± 2.45 μg g^{-1}), limonene (WT 24.64 ± 6.73 μg g^{-1}, TR 11.58 ± 2.81 μg g^{-1}) and humulene (WT 10.64 ± 5.76 μg g^{-1}, TR 34.31 ± 7.65 μg g^{-1}) in leaves; and higher concentrations of α-pinene+β-ocimene (WT 9.5 ± 1.32 μg g^{-1}, TR 21.58 ± 3.61 μg g^{-1}), sabinene+β-pinene (WT 32.16 ± 3.66 μg g^{-1}, TR 100.91 ± 15.34 μg g^{-1}), β-myrcene (WT 0 ± 0 μg g^{-1}, TR 23.44 ± 5.95 μg g^{-1}), limonene (WT 2.35 ± 1.25 μg g^{-1}, TR 16.75 ± 2.56 μg g^{-1})and humulene (WT 5.44 ± 1.45 μg g^{-1}, TR 2.97 ± 1.34 μg g^{-1}) in roots.

Aharoni *et al* (2003) found small amounts of linalool (from 0.02 to to 13.3 μg day^{-1} $plant^{-1}$ depending on the transgenic line) in the headspace of transformed Arabidopsis plants, with the *FaNES1* gene expressed in the plastids, while Kappers *et al* (2005) expressed the *FaNES1* gene in the mitochondria and also observed nerolidol emissions from the transformed plants' foliage. We did not find linalool or nerolidol in the foliage and root extracts. We did not investigate floral emissions because we removed the flowers to retard the senescence processes in the leaves (Meir *et al* 1994). It is possible that linalool might have been produced in leaves but released immediately after production (similar to isoprene). Our extraction technique would not have captured such compounds. It is also possible that no linalool or nerolidol was produced, as Kappers *et al* (2005) detected no linalool emissions from any of their plants' foliage, and no nerolidol in 25% of the transformed plants.

Effect of genotype

WT plants reached their maximum diameter before the TR plants. The TR plants' diameters increased gradually and consistently from the germination until the mature state. Kappers *et al* (2005) also found that first- and second-generation TR plants showed some growth retardation of the basal rosette, but both WT and TR plants flowered at approximately the same time.

The two genotypes used here had different morphology of the basal rosette. The WT had smaller and higher number of leaves which were shed and replaced when they reached a certain size, while the TR species had fewer leaves whose length increased constantly along the vegetative cycle. Despite the fact that the two genotypes (WT and TR) are morphologically different, the experimental plants were comparable in terms of health and phenology to satisfy the aims of the experiment, providing two different genotypes of the same species, one of which was genetically transformed.

TR plants showed lower photosynthetic activity and production in leaves than the WT plants. Comparing our transformed and WT plants, we found that stomatal conductance (*Fig.1*) and calculated electron transport rates (*ETR*) were lower in transformed plants than in WT, and this appeared to result in lower photosynthesis rates (*Fig.1*). The mean ratio *Fv/Fm* was significantly lower in transformed plants compared with WT, indicating that transformed plants were generally more stressed (Butler and Kitajima 1975, Oxborough and Baker 1997). However, there was a tendency for transformed plants to have lower foliar VOC concentrations than the WT plants, though this difference was not significant. While root VOC concentrations are much higher in transformed plants than in WT plants, it is not known if this is related in any way to the foliar biochemistry. However, Basyuni *et al* (2009) found that leaf isoprenoid concentration generally declined while root concentrations increased in salt-stressed mango plants. In our study, the same relationship of lower leaf terpene content and higher root terpene content in the TR plants also reflects the higher stress status in the TR plants, as indicated by the lower *Fv/Fm* vlaues.

However, it is clear that the genetic modification has effects throughout the plants, other than the targeted effects.

Concluding remarks

We have shown that the Arabidopsis genotype which is modified to emit nerolidol from mitochondrial synthesis shows other differences in terpene production, and physiology in leaves and roots. These plants deflect the resources towards root production in detriment of the leaf production.

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Chapter 5. Conclussions

Conclusions

5.1. Specific conclussions

Conclusions específiques

The specific conclusions of each chapter-study are the following ones:

Les conclusions específiques de cada capítol-estudi són les següents:

Chapter 2 - Drought, warming and soil fertilization effects on leaf volatile terpene concentrations in *Pinus halepensis* and *Quercus ilex*

1. The seedlings responded to drought and warming (* results showed in chapter 2.1):

   a) Decreasing their growth in response to drought. Both, *P. halepensis* and *Q. ilex*, had significantly smaller increase in stem diameter and height than well-watered plants.

   b) Showing lower relative water content (RWC) in response to drought.

   c) (*) Decreasing net photosynthetic rates and stomatal conductance in both species in response to drought.

   d) (*) Warming increased net photosynthetic rates and stomatal conductance in both species.

   The fertilization treatment did not show any significant pattern in the physiology of fertilized plants.

Capítol 2 - Efectes de la sequera, escalfament i fertilització del sòl sobre les concentracions de terpens volàtils a les fulles de *Pinus halepensis* i *Quercus ilex*

1. Els plançons van respondre a la sequera i a l'escalfament (* resultats provinents del capítol 2.1):

   a) Disminuint el seu creixement com a resposta de la sequera. Tant *P. halepensis* com *Q. ilex* van tenir diàmetres al coll de l'arrel i alçades significativament menors que les plantes amb condicions hídriques normals.

   b) Mostrant un menor contingut d'aigua relatiu (RWC) en resposta a la sequera

   c) (*) Disminuint la tassa fotosintètica neta i conductància estomàtica, en ambdues espècies, com a resposta a la sequera

   d) (*) L'escalfament va incrementar la tassa fotosintètica neta i la conductància estomàtica en ambdues

2. The main terpenes found in *P. halepensis* (storing species) were the same than in *Q. ilex* (non-storing species): α-pinene, β-pinene, β-myrcene and Δ^3-carene. However, evidently the concentrations in storing species were extraordinarily greater than in non-storing species.

3. Drought treatment tended to increase total terpene concentrations in both species during the experiment especially in *Q. ilex* which presented a significant interaction between drought and warming. Fertilization treatments did not affect significantly terpene concentrations.

espècies

El tractament de fertilització no va mostrar cap patró significatiu en la fisiologia de les plantes en que se'ls va aplicar fertilització.

2. Els principals terpens que es van trobar a *P. halepensis* (espècie acumuladora) van ser els mateixos que a *Q. ilex* (espècie no acumuladora): α-piné, β-piné, β-mircè, i Δ^3-caré. Òbviament, les concentracions en les espècies acumuladores van ser extraordinàriament més grans que les de les espècies no acumuladores.

3. El tractament de sequera va tendir a incrementar les concentracions totals de terpens en ambdues espècies al llarg de l'experiment, especialment a *Q. ilex*, que va presentar una interacció significativa entre la sequera i l'escalfament. Els tractaments de fertilització no van afectar significativament a les concentracions de terpens.

Chapter 3.1 - Different sensitivity of terpene emissions to drought and fertilization in terpene storing *Pinus halepensis* and in non storing *Quercus ilex*

1. The main emitted compounds were also the same in *P. halepensis* (storing species) than in *Q. ilex* (non-storing species): a-pinene, b-pinene, b-myrcene and D3-carene. Comparing both species, a-pinene, b-myrcene and D3-carene emission rates were higher in *P. halepensis* plants whereas b-pinene was emitted at greater emission rates by *Q. ilex*.
2. The emitted amounts were similar in both species.
3. a) Drought increased emissions of the non-storing species *Q. ilex*, but there was no effect on the storing species.

   b) Fertilization decreased the emission rates of the storing species *P. halepensis*, but there was no effect on the non-storing species.

Capítol 3.1 - Diferent sensibilitat de les emissions de terpens a la sequera i fertilització en l'espècie acumuladora *Pinus halepensis* i en la no acumuladora *Quercus ilex*

1. Els principals terpens que es van trobar a *P. halepensis* (espècie acumuladora) van ser els mateixos que a *Q. ilex* (espècie no acumuladora): α-piné, β-piné, β-mircè i Δ^3-caré. Comparant les dues espècies, les emissions de α-piné, β-mircè i Δ^3-caré van ser més grans en *P. halepensis* mentre que les emissions de β-piné van ser-ho a *Q. ilex*.
2. Les quantitats de terpens emeses van ser similars en ambdues espècies.
3. a) La sequera va incrementar les emissions a l'espècie no acumuladora *Q. ilex*, però no hi va haver cap efecte sobre l'espècie acumuladora.

   b) La fertilització va fer disminuir la tassa d'emissions de l'espècie acumuladora *P. halepensis* però no va tenir cap efecte sobre l'espècie no acumuladora.

Chapter 3.2 Instantaneous and historical temperature effects on α-pinene emissions in *Pinus halepensis* and *Quercus ilex*

1. a) Emission rates (*E*) showed a lineal dependence to instant leaf temperatures for both storing and non-storing species.

   b) Photosynthetic electron transport (J_{CO2+O2}) exhibited a exponential correlation with instant leaf temperature in storing species, showing a maximum at 35 ºC; but no trends were found for non-storing species.

2. Although the best correlation of emission rates were found for instantaneous foliar temperatures, the effect of accumulated previous temperature conditions should also be considered:

   a) There was a higher dependence of *E* on mean temperature of previous days than on mean temperature of current day for storing species but not for non-storing species.

   b) J_{CO2+Q2} showed a maximum relationship to mean temperature of previous 3 and 5 days for storing and non-storing species respectively.

Capítol 3.2 - Efectes de la temperatura instantània i històrica en les emissions d'α-piné en *Pinus halepensis* i *Quercus ilex*

1. a) La tassa d'emissions (*E*) va mostrar una dependència lineal amb la temperatura foliar instantània tant a l'espècie acumuladora com a la no acumuladora.

   b) El transport fotosintètic d'electrons (J_{CO2+O2}) va mostrar una correlació exponencial amb la temperatura instantània de la fulla en l'espècie acumuladora, mostrant un màxim a 35 ºC, però no es van trobar tendències a l'espècie no acumuladora.

2. Tot i que les correlacions van ser millors amb la temperatura foliar instantània, l'efecte acumulat de la temperatura dels dies previs també s'hauria de considerar:

   a) Hi ha una més dependència d'*E* amb la temperatura mitjana dels dies previs que no de la mitjana diària de temperatura, a l'espècie acumuladora, però no a la no acumuladora.

   b) J_{CO2+O2} va mostrar una correlació màxima amb la temperatura dels 3 i 5 dies previs, per espècies acumuladores i no acumuladores respectivament.

Chapter 4.1 Effects of phosphorus availability and genetic variation of leaf terpene contents and emission rates in *Pinus pinaster* seedlings susceptible and resistant to the pine weevil *Hylobius abietis*

1. a) Plants with complete fertilization produced 2.5-fold greater biomass than plants with lower fertilization

   b) Plants under balanced fertilization exhibited P concentrations 3-fold greater than P stressed plants

   c) Nitrogen concentration in needles was greater in complete fertilization than in P stressed plants

   d) Plants under complete fertilization showed lower photosynthesis and transpiration rates than the lowest

Capítol 4.1 Efectes de la disponibilitat de fòsfor i de la variació genètica sobre la producció i la tassa d'emissió de terpens foliars de plançons de *Pinus pinaster* susceptibles i resistents al corc del pi *Hylobius abietis*

1. a) Les plantes amb fertilització complerta van produir 2,5 cops més biomassa que les plantes amb menor fertilització.

   b) Les plantes amb fertilització complerta van mostrar 3 cops més concentracions de P que les plantes amb menor fertilització.

   c) La concentració de nitrogen a les fulles va ser més gran a les plantes amb fertilització complerta que no a les plantes amb dèficit de nutrients.

   d) Les plantes amb fertilització complerta van mostrar menors tasses fotosintètiques i respiratòries que les

fertilization treatment.

2. a) Families with a resistant behaviour at field showed slightly higher concentrations of P in foliar tissues than susceptible families, but no differences in terms of N concentrations and total biomass were detected.

   b) Different families had significant differences in photosynthesis and stomatal conductance: sensible families showed the lowest values of *A* and *E* at low (10 ppm) fertilization, and resistant families showed the lowest values of *A* and *E* at high (20 ppm) fertilization

3. The isomers α and β-pinene dominated the production and emission of the total terpene amount. Δ^3-carene was also present with high percentage.
4. Terpene concentrations increased with phosphorus deficiency.
5. Total terpene emission rates decreased with phosphorus fertilization.
6. Different families showed different behaviours, but there was not a pattern distinguishing between resistant and sensible families.
7. Different families showed different behaviours: the increase of terpene emission rates with P deficiency was much higher in sensible families than in resistant families.

plantes amb dèficit de nutrients.

2. a) Les famílies amb un comportament resistent a camp van mostrar concentracions de P als teixits foliars lleugerament majors que les famílies susceptibles, però no hi van haver diferències pel que fa a les concentracions de N i a la biomassa total.

   b)Hi van haver diferències significatives entre famílies pel que fa a la tassa fotosintètica i a la conductància estomàtica: les famílies sensibles van mostrar els valors més baixos de *A* i *E* a dosis baixes de fertilització (10 ppm), i les famílies resistents van mostrar els valors més baixos de *A* i *E* a dosis altes de fertilització (20 ppm).

3. Els isòmers α i β-piné van dominar la producció i la emissió del total de terpenes. Δ^3-caré també va ser-hi present en un alt percentatge.
4. Les concentracions de terpens van augmentar quan més deficiència de fòsfor tenia la planta.
5. La tassa total d'emissions va disminuir amb la fertilització de fòsfor.
6. Famílies diferents van presentar comportaments diferents, però no hi va haver un patró clar.
7. Famílies diferents van presentar comportaments diferents: l'augment de la tassa d'emissió de terpens amb la deficiència de fòsfor va ser molt més gran a les famílies sensibles que a les resistents.

Chapter 4.2 Investigating the photosynthesis and terpene-content strategies of two different genotypes of *Arabidopsis thaliana* (wild-type and *CoxIV-FaNES1* transgenic)

Capítol 4.2 Investigació de les estratègies fotosintètiques i de producció de terpens de dos genotips diferents de *Arabidopsis thaliana* (genotip salvatge, i genotip transgènic *CoxIV-FaNES1*)

1. a) The growing pattern was different in WT than in TR: at the end of the experiment TR plants reached bigger basal rossetes than WT plants. However, WT plants reached their maximum diameter earlier than TR plants.

   b) Net photosynthetic rates (*A*), the apparent photosynthetic electron transport rate (*ETR*) and the maximum photochemical efficiency of PS II (*Fv/Fm*) were lower in TR plants than in

1. a)El patró de creixement va ser diferent a les famílies amb genotip salvatge (WT) que a les famílies amb genotip transgènic (TR): al final de l'experiment, les plantes TR van formar rosetes basals més grans que les de les plantes WT. No obstant, les plantes WT van arribar al seu diàmetre màxim de roseta basal abans que les plantes TR.

   b)La tassa neta de fotosíntesi (*A*), la tassa de transport aparent d'electrons (*ETR*) i la eficiència màxima del

WT plants. Stomatal conductance (g_s) tended to be lower in TR plants compared to WT plants.

2. Leaves produce terpenes: The main terpenes produced in both genotypes were the monoterpenes α-pinene+β-ocimene and limonene and the sesquiterpene humulene.

3. There was no significant difference in leaf terpene concentration between the two genotypes, but there was a tendency for higher terpene concentrations in WT plants.

4. Roots produced terpenes as well: The main terpenes produced in both genotypes were the monoterpenes α-pinene+β-ocimene, sabinene+β-pinene, β-myrcene and limonene and the sesquiterpene humulene

5. TR plants produced higher root terpene concentrations than WT plants.

PSII (*Fv/Fm*) van ser inferiors a les plantes TR que a les plantes WT. La conductància estomàtica (g_s) va tendir a ser inferior a les plantes TR que a les plantes WT.

2. Les fulles produeixen terpens: els principals terpens produïts en ambdós genotips van ser els monoterpens α-piné+β-ocimé i limoné, i el sesquiterpé humulé.

3. No hi van haver diferències significatives a les concentracions foliars de terpens entre els dos genotips, però hi va haver una tendència a que les plantes TR produïssin majors concentracions de terpens.

4. Les arrels també produeixen terpens: els principals terpens produïts en ambdós genotips van ser els monoterpens α-piné+β-ocimé, sabiné+β-piné, β-mircé i limoné, i el sesquiterpé humulé.

5. Les plantes TR van produir majors concentracions de terpens a les arrels que les plantes WT.

5.2. General conclussions

Conclusions generals

Mild drought produced an increase of the production (content) of terpene and an increase of the terpene emission rates in non storing species. In storing species, drought also increased the production of terpenes but no the emission rates, as the compounds remained in the storing organs instead of being emitted.

The fertilization effect was not as clear as the drought effect. We only found nutrient effect on terpenes in storing species, where the higher the nutrient availability (fertilization), the lower the terpene emission rates. Leaf terpene concentrations increased, but only in the most controlled conditions experiments: in experiments with more variability there were tendencies to decrease (when fertilizing) but they were not statistically significant.

Plant genotype plays an important role in the terpene production and emission pattern: we have found very significant differences among families and transgenic clones of the same species.

The results indicate that there is an effect of drought and fertilization on terpene production and emission rates and therefore if climate

La sequera moderada produeix un augment de la producció (contingut) de terpens i un augment de la tassa d'emissió de terpens a les espècies no acumuladores. A les espècies acumuladores, la sequera també incrementa la producció de terpens però no la tassa d'emissió, ja que els compostos queda retinguts als òrgans d'emmagatzematge en lloc de ser emesos.

L'efecte de la fertilització no va ser tan clar com l'efecte de la sequera. Només vam trobar efecte d'aquesta sobre els terpens de les espècies acumuladores, on una major disponibilitat de nutrients (fertilització) produïa menors tasses d'emissió de terpens. Les concentracions foliars de terpens van augmentar, però només als experiments realitzats sota condicions controlades: als experiments amb més variabilitat hi van haver tendències a disminuir (amb la fertilització) però no van ser significatives estadísticament.

El genotip de la planta juga un paper important en els patrons de producció i emissió de terpens: vàrem trobar diferències significatives importants tant entre famílies com entre clons transgènics de la mateixa espècie.

Aquests resultats indiquen que hi ha un

warming and drought occur as predicted by IPCC (IPCC, 2007) and GOTILWA models (Sabaté et al. 2002), and if eutrophication continues as expected (Vitosuek 1997), terpene emissions may significantly change in the next decades with important biological and environmental consequences (Peñuelas and Llusià 2003, Peñuelas and Staudt 2010). However, the species-specific complex responses found here, warrant much more research on this issue to arrive to reliable predictions.

efecte de la sequera i de la fertilització sobre les tasses de producció i emissió de terpens, i que si es fan realitat prediccions d'escalfament i sequera com les previstes pel IPCC (IPCC 2007) o pels models GOTILWA (Sabaté et al 2002), i que si la eutrofització continua com es previst (Vitosuek 1997), les emissions de terpens canviaran significativament durant la pròxima dècada, portant conseqüències biològiques i ambientals importants (Peñuelas i Llusià 2003, Peñuelas i Staudt 2010). No obstant, la complexitat de respostes espècie-específiques trobada aquí garanteix que fa falta molta més recerca en aquest tema per poder fer prediccions més certes.