UNIVERSIDADE TÉCNICA DE LISBOA
Instituto Superior de Agronomia

Diferenças na resposta a défices hídricos e a baixas temperaturas em dois clones de *Eucalyptus globulus* Labill. com contrastante sensibilidade à secura

Filipe Miguel de Carvalho Costa e Silva

ORIENTADOR: Doutor João Santos Pereira
CO-ORIENTADOR: Doutora Maria Helena Almeida

JÚRI:

Presidente: Reitor da Universidade Técnica de Lisboa

Vogais: Doutor João Manuel Dias dos Santos Pereira, professor catedrático do Instituto Superior de Agronomia da Universidade Técnica de Lisboa;
Doutor Ângelo Manuel Melo de Carvalho Oliveira, professor catedrático do Instituto Superior de Agronomia da Universidade Técnica de Lisboa;
Doutora Maria Manuela Coelho Cabral Ferreira Chaves, professora catedrática do Instituto Superior de Agronomia da Universidade Técnica de Lisboa;
Doutora Otília da Conceição Alves Correia Vale de Gato, professora associada da Faculdade de Ciências da Universidade de Lisboa;
Doutor António Manuel Dorotêa Fabião, professor associado do Instituto Superior de Agronomia da Universidade Técnica de Lisboa;
Doutor Júlio Armando Perestrelo da Cunha Osório, professor auxiliar da Faculdade de Engenharia de Recursos Naturais da Universidade do Algarve;
Doutor Nuno Manuel Gonçalves Borralho, na qualidade de especialista.

Doutoramento em Engenharia Florestal

Lisboa
2008

Resumo

Com o objectivo de identificar marcadores morfológicos, fisiológicos e bioquímicos de mecanismos de resistência ao stress estudaram-se as respostas a défices hídricos e a baixas temperaturas em dois clones de *Eucalyptus globulus* Labill. com sensibilidades à secura putativamente contrastantes. O clone CN5 (resistente à secura) em resposta ao défice hídrico e às baixas temperaturas, mostrou maior desenvolvimento do sistema radicular e maior capacidade de aumentar a proporção de biomassa distribuída para as raízes do que o clone ST51 (sensível à secura). Também, em ambas as condições de stress, o clone CN5 manteve um estado hídrico foliar mais favorável e mostrou maiores reduções do potencial osmótico do que o clone ST51. A maior resistência à secura do clone CN5, baseou-se principalmente na optimização da relação entre a área de transpiração e a área de absorção e na manutenção da condutância hidráulica em condições de secura. Em resposta ao frio, o clone CN5 mostrou ainda uma mais rápida capacidade de aclimatação do que o clone ST51. Prevê-se uma melhor adaptabilidade do clone CN5, do que do clone ST51, a condições naturais de limitação hídrica ou sujeitas à ocorrência de geadas ocasionais, alargando-se, assim, os seus limites de plantação.

Palavras-chave: aclimatação, crescimento das raízes, frio, genótipos, propriedades hidráulicas, stress hídrico.

Abstract

We evaluated responses to water deficits and low temperatures in two E*ucalyptus globulus* Labill. clones with contrasting drought sensitivity. Our aim was to identify morphological, physiological and biochemical markers of stress resistance mechanisms. In response to water deficit and chilling, CN5 clone (drought-resistant) sustained a higher root growth and displayed greater carbon allocation to the root system than ST51 clone (drought-sensitive). In addition, under drought and low temperature conditions, CN5 ramets maintained higher leaf water potential (better water status) and decreased leaf osmotic potential significantly more than the drought-sensitive ST51 ramets. Differences in the response to drought in root biomass, coupled with changes in hydraulic properties, accounted for the clonal differences in drought tolerance, allowing CN5 ramets to balance transpiration and water absorption during drought and thereby prolong the period of active carbon assimilation. Moreover, in response to low temperatures, CN5 clone exhibited a higher capacity to acclimate in a shorter period than ST51. We conclude that Clone CN5 has greater plasticity in terms of adaptive traits than ST51, allowing its plantation range to increase to sites subject to seasonal droughts or sudden frosts.

Keywords: acclimation, root growth, cold, genotypes, hydraulic properties, water stress.

Agradecimentos

Já se sabe que na investigação científica raramente pertence ao autor o inteiro mérito do trabalho realizado. Esta dissertação e os estudos nela contidos não são excepção e, particularmente, tenho noção da importância que tiveram todos quanto, de uma maneira ou de outra, ao longo dos últimos 4 anos, contribuíram para que chegasse a bom termo. Ainda que não possa nomear e agradecer a todos, devo e quero deixar aqui alguns principais agradecimentos:

Ao Professor João Santos Pereira, meu orientador (e por vezes desorientador!), devo quase tudo o que aprendi e quanto evoluí no desafiante ofício de fazer ciência. À Professora Helena Almeida, para além da amizade e de uma co-orientação essencial, devo um enorme optimismo científico e uma disponibilidade sem reservas. À Professora Manuela Chaves devo agradecer as importantes e rigorosas correcções científicas sempre feitas com simpatia. À Drª Alla Shvaleva devo um grande 'spasiba' pela partilha do trabalho mais difícil e desanimador de investigador, mas também pela partilha de muitas horas alegres a construir e destruir experimentos científicos. Por fim e em geral, quero agradecer a todos os co-autores dos artigos científicos publicados e a todos os amigos que me acompanharam e acompanham, lado a lado, na longa batalha por uma dissertação.

Índice

Prefácio

Ao longo do tempo, e mesmo antes de dar início aos trabalhos desta tese, fui-me deparando com a oposição latente e generalizada à espécie que é objecto deste estudo – o eucalipto. Esta oposição, de que damos conta muitas vezes, desde as conversas à mesa de café até aos meios mais informados e científicos, parece ter-se enraizado como um preconceito na mentalidade geral e tudo indica que dificilmente será ultrapassada, independentemente do conhecimento efectivo que já existe sobre a espécie, fruto da larga investigação das últimas décadas (e.g., Alves et al. 2007). Mais cedo ou mais tarde, inevitavelmente, também a mim me surgiu a necessidade de tomar uma posição e de, portanto, saber o que escrevia a ciência e o que alvitravam os investigadores, formados à beira desses eucaliptais que cresciam tão bem como os seus opositores. Mergulhei então nas polémicas dos impactes ambientais do eucalipto sobre os diferentes recursos – água, solo, biodiversidade e paisagem – desde os autores que escrevem "cobras e lagartos" dessas plantações que acusam de não os ter (Caldas 1990), até aos outros mais apologistas do uso desta espécie (Soares et al. 2007).

Do ponto de vista científico, e em poucas palavras porque não cabe aqui alongar-me sobre o assunto, o que encontrei tranquilizou-me e vejo-me tentado a resumi-lo da seguinte forma: Utilizando as técnicas de silvicultura adequadas às específicas condições de cada meio é possível reduzir os impactes ambientais a níveis negligenciáveis. Por outro lado, não deixa de ser preciso reconhecer que ao nível da paisagem e numa escala regional, se encontram por vezes verdadeiros atentados, quer devido ao incumprimento das boas práticas, quer em consequência da ocupação desregrada de grandes extensões contínuas com plantações desta espécie (Silva et al. 2007).

No entanto, para além dos possíveis efeitos deletérios numa hipotética e indefinida qualidade ambiental, a expansão do eucalipto é significativa pelas importantes e concomitantes transformações do mundo rural que lhe estão na origem e que lhe estão associadas. E é por essa perspectiva que se compreende que a atribuída degradação estética da paisagem, se deve não tanto ao aumento das áreas de eucaliptal mas à "desordenação" do território, resultante, em grande parte, do abandono dos campos pela agricultura e à perda dessa malha estruturante. O tão apregoado drama da

eucaliptização do país foi, afinal, muito mais o drama do fim de uma ordem tradicional rural onde as gentes tinham o seu lugar definido, num equilíbrio com o meio e numa harmonia que atravessava os séculos. Aliás, como bem o viu e expressou Oliveira Baptista (2007) num ajustado trecho: "Esta resistência (ao eucalipto) correspondeu ao confronto com uma mudança profunda, à constatação inevitável e visual que o espaço deixara de ser os campos que se trabalhavam e que se percorriam. Os eucaliptais apareciam com o consagrar da ruptura das populações com o espaço que as rodeava, e que estas agora viam com distância e exterioridade. A recusa dos eucaliptos era, assim, a descoberta da paisagem e, simultaneamente, a recusa do símbolo que as populações associavam às transformações que viviam". Esta exterioridade forçada, que correspondeu na prática ao fim de um modo de vida secular, imposta pelas altas leis das economias e do mercado comum, foi o verdadeiro drama de um mundo rural que agonizava, órfão das preocupações governamentais e abandonado à sua sorte. E com o fim desse mundo, o camponês, esse "homem eterno" que atravessava imutavelmente o tempo, deixou aí de ter lugar e de integrar o espaço, completando-o. Assim, a natural revolta dos camponeses contra o eucalipto transcende em muito a árvore que lhe invadiu os campos ainda antigos.

Sem dúvida, que mesmo para quem não tem laços profundos com o mundo rural, há algo de chocante quando, passeando por esse país fora, a única ordem que se encontra em muitas paisagens nos é dada pelas fileiras alinhadas das plantações de eucaliptos. Decerto, essa floresta não satisfaz a nossa necessidade, muitas vezes subconsciente, do elemento natural. Todavia, não faz sentido exigir a esta silvicultura de produção intensiva que cumpra as funções que se esperam das florestas semi-naturais. Não é, pois, o eucalipto que está a mais nesse território entregue a si próprio, mas um ordenamento e uma responsabilidade de intervenção sobre a paisagem que se encontram em falta. Como já muitas vezes se repetiu, o eucalipto é uma árvore "decente" e cumpre a sua função, isto é, a de criar um espaço muito humano algures a meio caminho entre uma seara e uma floresta. E como qualquer árvore, para além de ser um elemento vertical da paisagem, evoca também as colunas ascendentes dos templos sagrados, simbolizando pontes vivas entre a terra e o céu.

Referências bibliográficas

Alves, A.M., J.S. Pereira e J.M.N. Silva 2007. O Eucaliptal em Portugal. Impactes Ambientais e Investigação Científica. Eds. A.M. Alves, J.S. Pereira e J.M.N. Silva. ISAPress, Lisboa, 398 p.

Caldas, E.C. 1990. O drama da campanha do Eucalipto. *In* Colóquio: Eucalipto. Economia e Território. Sociedade Portuguesa de Estudos Rurais, Lisboa, pp. 99-108.

Oliveira Baptista, F. 2007. Prefácio. *In* O Eucaliptal em Portugal. Impactes Ambientais e Investigação Científica. Eds. A.M. Alves, J.S. Pereira e J.M.N. Silva. ISAPress, Lisboa, pp. 6-10.

Silva, J.S., E. Sequeira, F. Catry e C. Aguiar 2007. Os contras. *In* Pinhais e eucaliptais – A floresta cultivada. Ed. J.S. Silva. Jornal Público/ Fundação Luso – Americana para o Desenvolvimento/ Liga para a Protecção da Natureza., Lisboa, pp. 221-259.

Soares, J., L. Leal, P. Canaveira, F. Goes e A. Fialho 2007. Porquê cultivar o eucalipto? *In* Pinhais e eucaliptais - A floresta cultivada. Ed. J.S. Silva. Jornal Público/ Fundação Luso-Americana para o Desenvolvimento/ Liga para a Protecção da Natureza, Lisboa, pp. 185-219.

CAPÍTULO 1

Introdução

1. Introdução

1.1. Enquadramento do estudo

1.1.1. O eucalipto em Portugal

O eucalipto (*Eucalyptus globulus* Labill.), depois de uma rápida expansão ao longo das últimas cinco décadas, ocupa hoje um dos lugares centrais na floresta portuguesa. A sua actual relevância advém, não apenas da importância da área ocupada – cerca de 21% da área florestal nacional – mas também da positiva contribuição para a actividade económica do país, de que é exemplo o peso significativo da indústria de pasta para papel na balança comercial externa com 40% do valor das exportações florestais e 6% do valor total de exportações nacionais (Borges e Borges 2007). No entanto, apesar da sua expressão actual, a área de cultivo do eucalipto cresceu lentamente desde a sua introdução em meados do século XIX até aos anos 50 do século XX, ocupando então uma área de cerca de 50 000 ha.

É a partir desta época que se conjugam as influências das políticas públicas industriais com os interesses dos proprietários privados e a pressão das indústrias em expansão, necessitadas de matérias-primas, resultando na grande expansão da área de eucaliptal até se chegar ao presente valor de 647 000 ha (Alves et al. 2007). Para o sucesso desta expansão concorreram as características da *E. globulus*, nomeadamente, uma elevada qualidade do material lenhoso como matéria-prima para pasta para papel e uma elevada produtividade da espécie associada às favoráveis condições climáticas e de solos em muitas regiões do país. Por outro lado, a aplicação de novas técnicas de silvicultura e o contínuo desenvolvimento dos programas de melhoramento genético possibilitaram a intensificação da cultura e o aumento da sua produtividade.

Foi por iniciativa das empresas de celulose que se iniciaram e se têm desenvolvido em Portugal, há mais de 40 anos, as actividades de melhoramento genético da *E. globulus*. No entanto, a utilização extensiva de sementes melhoradas ou plantas clonais apenas teve início nos finais da década de 90. Actualmente, a florestação por parte das empresas de celulose assenta quase exclusivamente em material seleccionado ou testado no âmbito dos seus programas de melhoramento (Almeida 2004). O objectivo geral destes programas de melhoramento genético é

disponibilizar populações mais produtivas, quer por um acréscimo do crescimento, quer pelo desenvolvimento de genótipos qualitativamente superiores pelas suas características tecnológicas. A possibilidade de intensificação em plantações de alta produtividade permite também reduzir o esforço produtivo noutras regiões, contribuindo para resolver conflitos de competição (e.g., agricultura, expansão urbana, áreas de lazer) pelas áreas disponíveis.

Para além de procurar seleccionar e propagar genótipos mais produtivos, o melhoramento genético pode trazer outras vantagens. Para conseguir produtividades elevadas as plantas têm que se manter saudáveis. As doenças e os insectos herbívoros que se alimentam de folhas são, com frequência, causas do decréscimo na Produção Primária Bruta. Até ao início dos anos 80 do século XX o eucalipto beneficiou, como espécie exótica, da quase total ausência dos seus inimigos naturais. Actualmente, no entanto, o número e dimensão dos focos de pragas e doenças aumentaram quase exponencialmente (e.g., *Phoracantha* sp., *Gonipterus scutellatus*, *Mycosphaerella* sp.), tornando-se num dos principais problemas do eucalipto em Portugal (Branco 2007). Também, neste contexto, é provável que o melhoramento genético possa contribuir para a produção de genótipos mais resistentes ou tolerantes a pragas e doenças.

O impacto do melhoramento genético na cultura do eucalipto está dependente do valor genético das plantas utilizadas, da proporção destas no total das plantações realizadas e do seu comportamento nas condições de campo (Almeida et al. 2005). No contexto da Silvicultura e das actividades de melhoramento, é a floresta clonal de eucalipto que apresenta os maiores desafios tecnológicos, desde a produção de plantas até ao planeamento da floresta e condução dos povoamentos. Para uma silvicultura clonal se desenvolver plenamente, Libby e Ahuja (1993) consideraram três aspectos essenciais: (1) as operações culturais (e.g., fertilização, alocação) devem ser específicas para cada clone seleccionado, (2) a diversidade genética das plantações clonais deve ser rigorosamente controlada e mantida e (3) as estratégias de melhoramento devem ser desenvolvidas continuamente dando resposta aos desafios colocados pela silvicultura clonal. Escusado será dizer que, necessariamente, todas estas condições implicam e se baseiam na detenção de um conhecimento sólido e aprofundado dos clones da população de produção.

Na última década a proporção de plantas melhoradas nas plantações de eucalipto têm crescido significativamente. Do total da área plantada anualmente com

eucalipto cerca de 36% correspondem a arborizações com plantas melhoradas sendo de realçar o peso da utilização de plantas clonais que representam 70% dessas áreas melhoradas (Almeida et al. 2005). De facto, e apesar de a técnica de clonagem apresentar dificuldades específicas de aplicação à *E. globulus* (e.g., Borralho e Wilson 1994), a floresta clonal tem vindo a aumentar regularmente e a ganhar importância entre nós produzindo-se anualmente e em média 2,5 milhões de plantas clonais nos viveiros da Aliança Florestal. Deste modo, passados dez anos das primeiras plantações clonais em larga escala, a floresta clonal no país deve rondar presentemente os 25 000 ha, com taxas anuais de florestação clonal da ordem dos 2000 ha. No entanto, se a área florestal com material melhorado – clonal e seminal – corresponde a cerca de 50 000 ha, representa ainda menos de 10% da floresta de eucalipto em Portugal (Almeida et al. 2005). Existe, portanto, uma clara oportunidade de alargar os benefícios para a economia do sector por um maior investimento na utilização de material melhorado que se estima poder levar a um aumento da produtividade entre 25 e 50% (Borralho et al. 2007).

1.1.2. Condicionantes da Produtividade: a água e a temperatura

A produtividade, isto é, a produção de biomassa por unidade de área e por unidade de tempo, depende da capacidade das árvores em obterem recursos do ambiente (radiação, água e nutrientes minerais) e da eficiência de utilização desses recursos na fixação de CO_2 atmosférico em biomassa. Deste modo, a maior produção de lenho de um determinado genótipo pode dever-se a uma maior capacidade de capturar os recursos disponíveis, a uma melhor eficiência no uso desses recursos ou a uma maior partição de biomassa para a formação de lenho (Binkley et al. 2004). Por outro lado, a produtividade encontra-se limitada pelas condições do meio que influenciam a quantidade de recursos disponíveis. Essencialmente, são as características do clima como a precipitação e a temperatura que limitam a produtividade, embora as características do solo (nutrientes e capacidade de armazenamento de água) possam também ser um factor limitante (Whitehead e Beadle 2004). Considerando as diversas condições edafo-climáticas das plantações de eucalipto em Portugal e os principais factores limitantes da sobrevivência e crescimento, interessam-nos em particular, no

âmbito do presente estudo, os stresses abióticos decorrentes de uma baixa disponibilidade hídrica e de baixas temperaturas.

O desenvolvimento da *E. globulus* é muito sensível aos défices hídricos (Osório et al. 1998; Pereira et al. 1994), sendo a sua produtividade essencialmente afectada através da redução da área foliar e das taxas de fotossíntese. Por exemplo, comparando as produtividades de povoamentos de eucalipto em diferentes regiões do país de acordo com as suas disponibilidades hídricas, observou-se um aumento de 0.9 Mg ha^{-1} ano^{-1} na biomassa aérea para cada 100 mm de aumento na precipitação anual (Soares et al. 2007). Igualmente, para uma região do Nordeste do Brasil e com clones de *E. grandis x urophylla*, esta dependência foi observada ao longo de um gradiente geográfico com um aumento de biomassa aérea de 2.3 Mg ha^{-1} ano^{-1} por cada aumento de 100 mm na precipitação anual (Stape et al. 2004). Nas últimas décadas fizeram-se largos progressos na compreensão das respostas das plantas ao défice hídrico cobrindo os seus mais variados aspectos – morfológicos, fisiológicos e bioquímicos – desde o nível molecular ao da planta inteira (ver, por exemplo, Chaves et al. 2003; Chaves e Oliveira 2004; Flexas et al. 2006). O facto de se terem já encontrado diferenças significativas entre genótipos de eucalipto ao nível, por exemplo, da eficiência do uso da água (Castro 2004; Le Roux et al. 1996) e da partição de biomassa pelas componentes da planta (Osório et al. 1998), deixa pressupor a existência de uma variabilidade intra-específica nas estratégias de resposta ao défice hídrico. Assim, estes resultados apoiam o interesse de seguir esta linha de investigação e permitem prever a possibilidade de utilizar estes conhecimentos tanto ao nível da silvicultura clonal como do melhoramento genético.

As plantações de eucalipto feitas no fim do Inverno e princípio da Primavera permitem fazer coincidir o crescimento inicial das plantas com o período de mais alta disponibilidade de água no solo. Desta forma o sistema radicular tem a possibilidade de se desenvolver e colonizar o solo antes de se iniciar o défice hídrico nos meses mais secos. No entanto, o crescimento das plantas, e portanto a sua produtividade, são influenciados negativamente pelas baixas temperaturas na estação fria. Por outro lado, a ocorrência ocasional de temperaturas negativas é importante, mesmo em regiões de clima Mediterrânico, limitando as áreas de plantação da *E. globulus*. Sendo as plantas jovens de eucalipto mais sensíveis do que as adultas ao frio, o grau de tolerância ao frio pode determinar o sucesso das plantações e, assim, limitar as distribuições da espécie e genótipos por certas áreas ou micro-estações.

Em geral, uma diminuição da temperatura para valores inferiores à temperatura óptima tende a reduzir o crescimento (Gavito et al. 2001; Peng e Dang 2003) a eficiência fotossintética (Allan e Ort 2001; Close et al. 2000) e a capacidade de absorção e transporte de água pelas raízes (Fennell e Markhart 1998; Markhart et al. 1979; Wan et al. 2001). Comparando as respostas de *E. nitens* com as de *E. globulus* em povoamentos sujeitos a baixas temperaturas (temperatura média anual de 8-10 ºC), Battaglia et al. (1998) reportaram uma menor redução do índice de área foliar (área foliar por unidade de área de terreno, L) para a *E. nitens* com uma intercepção da radiação 10 a 15% superior à da *E. globulus*. Assim, para além das diferenças entre espécies, estes autores mostraram que uma temperatura de crescimento abaixo do óptimo (13 a 14 ºC), resulta numa redução de L e num consequente e substancial decréscimo da produtividade. Também entre genótipos contrastantes de uma espécie são de esperar diferenças nas respostas às baixas temperaturas, ao longo do processo de aclimatação ao frio que decorre numa escala de tempo de dias a semanas em resultado de uma combinação de mudanças fisiológicas e metabólicas. Por exemplo, Leborgne et al. (1995) mostraram haver diferenças significativas na tolerância ao frio entre genótipos de *E. gunnii* relacionadas com diferentes metabolismos do carbono e atribuídas a uma acumulação de açúcares solúveis e ao seu efeito de crio-protecção.

A maior parte dos cenários para as alterações climáticas na Península Ibérica sugerem um agravamento da aridez e um aumento da frequência de eventos extremos num futuro próximo (IPCC 2001). Neste contexto, reforça-se o interesse do estudo das respostas morfológicas, fisiológicas e bioquímicas da *E. globulus* às baixas disponibilidades hídricas.

1.1.3. O potencial genético

Para além das condições do meio de crescimento outro factor limitante e responsável pela produtividade de um clone é o seu potencial genético. Porém, a maior ou menor expressão deste potencial genético em produtividade está dependente da existência de interacções genótipo x ambiente (GxA) que, quando importantes, implicam uma troca de posições no desempenho entre clones (e.g., crescimento em altura) em ambientes diferentes. Não só as condições edafo-climáticas das estações,

mas também diferentes anos ou operações culturais, podem representar diferenças ambientais passíveis de causar interacções GxA. O desconhecimento destas interacções em plantações clonais pode levar, em casos extremos, à morte das plantas ou a uma redução do crescimento e da qualidade, sendo muitas vezes difícil reconhecer as verdadeiras causas da perda de produtividade dessas plantações (Zobel e Talbert 1984). De qualquer modo, a oportunidade de explorar estas interacções é uma vantagem desde há muito tempo reconhecida da silvicultura clonal, ajustando os clones com os locais de plantação e com as operações culturais. Esta vantagem existe com os clones de eucalipto, que mostram geralmente uma significativa interacção GxA (Borralho et al. 2007; Zobel 1993), quando as condições ambientais variam consideravelmente. Por exemplo, algumas empresas no Brasil, Colômbia e Venezuela, para além de uma distribuição específica dos clones de eucalipto pelos locais de plantação, aplicam esquemas de fertilização ajustados a cada clone (Zobel 1993).

Por outro lado, a quantidade de testes clonais e os detalhes de caracterização das áreas de plantação necessários para uma elevada especificidade de distribuição podem ser tecnicamente e economicamente inviáveis (Kleinschmit et al. 1993). Geralmente, os testes clonais incluem somente um número limitado de ensaios, não permitindo, por isso, conhecer os limites das plasticidades fenotípicas dos clones (*i.e.* a amplitude de uma característica de um genótipo avaliada ao longo de um gradiente ambiental (Eriksson et al. 2006)). Assim, a dificuldade de distribuir clones muito específicos, isto é, com uma alta plasticidade fenotípica, pelas suas melhores estações, pode justificar uma estratégia de selecção por clones com maiores capacidades de adaptação. A plantação destes clones, mais estáveis nas suas respostas ao ambiente, é vantajosa em áreas de plantação espacialmente heterogéneas onde os clones mais específicos não poderão alcançar as suas maiores produtividades. De todas as formas, um correcto programa operacional de uma silvicultura clonal deve assegurar que o inerente potencial dos clones de produção não é comprometido por uma distribuição por locais de plantação desajustados. Em Portugal, o grau de adaptabilidade (e.g., resistência a agentes bióticos e abióticos) tem ainda uma importância secundária nos programas de melhoramento das empresas, sendo as variáveis-chave de selecção o rendimento em pasta, a densidade da madeira e o volume por hectare (Borralho et al. 2007).

Os testes clonais de campo, devido à multiplicidade de factores ambientais que intervêm na formação do fenótipo observado, não permitem identificar nem quantificar

com rigor as variáveis que contribuem para a plasticidade fenotípica dos clones. De facto, o efeito das condições ambientais pode considerar-se aleatório, na medida em que não sendo controlado, representa uma amostra do total de áreas de plantação possíveis (Matheson e Cotterill 1990). No entanto, os experimentos em condições controladas, estudando efeitos fixos e separando os efeitos de cada factor (e.g., temperatura, luz, disponibilidade de água), são uma ferramenta que poderá permitir uma avaliação mais precisa do valor dos genótipos e complementar a informação de campo. Assim, e dadas as grandes variabilidades edafo-climáticas das áreas de plantação, a exploração das diferenças entre os potenciais genéticos dos clones de *E. globulus* é uma possibilidade de aumentar a produtividade da floresta clonal.

1.2. Escolha dos dois clones em estudo

O material vegetal utilizado no presente estudo consistiu em rametos de dois clones de *E. globulus* Labill., designados CN5 e ST51, pertencentes à população de produção do programa de melhoramento desenvolvido pelo RAIZ (Instituto de Investigação da Floresta e Papel). Os ortetos destes clones foram árvores de uma sub-população de uma mata comercial, seleccionadas por apresentarem elevado crescimento, bom estado fitossanitário e fuste rectilíneo. O orteto do clone CN5 foi seleccionado em 1987 na Caniceira (Abrantes) e o orteto do clone ST51 em 1986 na zona de Santo Tirso. Estas árvores seleccionadas foram abatidas, os seus rebentos de touça recolhidos e postos a enraizar por macro-estacaria. Posteriormente, os rametos destes clones foram plantados em ensaios clonais distribuídos pela área de cultivo do eucalipto.

A escolha destes clones para este estudo deveu-se aos seus desempenhos contrastantes nos ensaios clonais em distintas condições edafo-climáticas. Assim, observou-se que em ensaios de campo sujeitos a elevados défices hídricos estivais, o clone CN5 apresentava uma maior taxa de sobrevivência (17%) e de crescimento (14%) quando comparado com o clone ST51. Por outro lado, em ensaios instalados em zonas de elevada produtividade, onde a mortalidade é praticamente nula, o clone ST51 apresentava maiores valores de produtividade (13%) do que o clone CN5. Com base nestas observações, assumiu-se que o clone CN5 teria características de resistência à

secura ao contrário do clone ST51 que seria sensível à deficiência hídrica. Desta forma, o fundamento desta escolha é também a possibilidade do estudo dos processos biológicos envolvidos na resposta a diferentes stresses, em clones que à partida apresentarão características morfológicas e fisiológicas contrastantes.

1.3. Objectivo geral e interesse do estudo

O objectivo deste estudo é avaliar os mecanismos de resistência a diferentes stresses em clones de *Eucalyptus globulus* e identificar marcadores morfológicos, fisiológicos e bioquímicos associados às principais diferenças nos processos biológicos envolvidos. Como hipótese geral considera-se que para sobreviver e crescer num ambiente com défices hídricos sazonais, como são as regiões de clima Mediterrânico, as plantas perenifólias ou reduzem a transpiração, a assimilação de carbono e o crescimento, economizando água durante o período de défice hídrico, ou utilizam maior percentagem da água disponível, com raízes profundas. Em ambos os casos as plantas têm a maior parte da sua assimilação de carbono e de crescimento no período chuvoso, *i.e.*, no Inverno Mediterrânico e sujeitas ao efeito das baixas temperaturas.

Clones com diferentes capacidades produtivas face às disponibilidades em água devem ter caracteres biológicos distintos. Assim, estudaram-se em particular as respostas morfológicas, fisiológicas e bioquímicas dos clones a défices hídricos, a baixas temperaturas e a temperaturas negativas. A compreensão dos processos biológicos e a identificação das principais diferenças entre clones nas respostas aos diferentes stresses têm interesse pela aplicação desse conhecimento na selecção da população de melhoramento, na distribuição dos genótipos da população de produção e na implementação da silvicultura clonal:

a) Conhecimento biológico dos clones. Para além da compreensão dos processos biológicos os experimentos em condições controladas permitem identificar e caracterizar as plasticidades fenotípicas dos clones. Esta informação tem uma importância fulcral, sobretudo como complemento aos resultados dos ensaios clonais, permitindo uma melhor interpretação e consolidação desses resultados.

b) Programa de melhoramento genético. Este conhecimento permite fundamentar as decisões e apoiar os critérios de selecção, quer na população de melhoramento, quer na população de produção. Por outro lado, este conhecimento é imprescindível para a identificação dos genes responsáveis pelas diferenças genéticas e aplicação da genética molecular aos programas de melhoramento.
c) Eficiência da silvicultura clonal. Uma identificação e quantificação dos efeitos ambientais no crescimento, em condições controladas, permite prever as respostas dos clones em condições naturais e apoiar as decisões de distribuição dos clones pelas diferentes áreas edafo-climáticas, aumentando a produtividade dos povoamentos.

1.4 Estrutura da dissertação

A presente dissertação baseia-se essencialmente nos artigos científicos que foram publicados ao longo do tempo e à medida que os trabalhos de investigação se desenvolveram. Naturalmente, os resultados que se foram obtendo, a sua análise e discussão, suscitaram novas questões e, por vezes, aconselharam novas metodologias ou apontaram novas abordagens de estudo. Assim, e para maior consistência da dissertação, procurou-se, através da presente introdução (Capítulo 1), fazer o enquadramento do estudo, expor o seu principal objectivo e interesse. Depois, do Capítulo 2 ao Capítulo 6, apresentam-se em sequência cronológica – e porventura desprovida de lógica funcional – os artigos publicados ou submetidos em revistas internacionais e que são os seguintes:

Capítulo 2 – Costa e Silva, F.; Shvaleva, A.; Maroco. J.P.; Almeida, M. H.; Chaves, M.M.; Pereira, J.S. (2004). Responses to water stress in two *Eucalyptus globulus* clones differing in drought tolerance. *Tree Physiology* 24:1165-1172.

Capítulo 3 – Shvaleva, A.; Costa e Silva, F.; Breia, E.; Jouve, L.; Hausman, J.F.; Almeida, M. H.; Maroco. J.P.; Rodrigues, M.L.; Pereira, J.S.; Chaves, M.M. (2006).

Metabolic responses to water deficit in two *Eucalyptus globulus* clones with contrasting drought sensitivity. *Tree Physiology* 26:239-248.

Capítulo 4 – Costa e Silva, F.; Shvaleva, A.; Almeida, M. H.; Chaves, M.M.; Pereira, J.S. (2007). Responses to chilling of two *Eucalyptus globulus* clones with contrasting drought resistance. *Functional Plant Biology* 34:793-802.

Capítulo 5 – Shvaleva, A.; Costa e Silva, F.; Scotti, P.; Oufir, M.; Hausman, J.F.; Guignard, C.; Ramos, P.; Almeida, M.H.; Rodrigues, M.L.; Pereira, J.S.; Chaves, M.M. (2008). Physiological and biochemical responses to low non-freezing temperature of two *Eucalyptus globulus* clones differing in drought resistance. *Annals of Forest Science*, 65, 204. DOI: 10.1051/forest:2007087.

Capítulo 6 – Costa e Silva, F.; Shvaleva, A.; Broetto, F.; Ortuño, M. F.; Almeida, M. H.; Rodrigues M.L.; Chaves, M.M.; Pereira, J.S. (2008). Responses to chilling and freezing in two *Eucalyptus globulus* clones with contrasting drought resistance. *Tree Physiology* (Submetido).

Assim, os artigos publicados, respeitantes aos capítulos 2 a 5, foram revistos por investigadores reconhecidos internacionalmente, especialistas nas matérias em questão, sendo uma medida da sua relevância o Factor de Impacto (IF) da respectiva revista. Deste modo, para o ano de 2006, o IF da revista “Tree Physiology” foi de 2.297, para a “Functional Plant Biology” foi de 2.272 e para a “Annals of Forest Science” foi de 1.29.

Finalmente, no Capítulo 7, faz-se um resumo dos resultados obtidos e das suas implicações, tecendo-se umas breves considerações finais.

Referências bibliográficas

Allan, D.J. e D.R. Ort 2001. Impacts of chilling temperatures on photosynthesis in warm-climate plants. Trends in Plant Science. 6:36-42.

Almeida, M.H. 2004. O melhoramento genético do Eucalyptus globulus em Portugal. *In* Comunicando nº 2. DVPF, DGRF, pp. 6-13.

Almeida, M.H., C. Araújo, J.A. Araújo, F. Costa e Silva, I. Neves, V. Paiva, A. Santiago e D. Ribeiro 2005. Melhoramento genético do eucalipto: que impacto na realidade? *In* A Floresta e as Gentes. 5º Congresso Florestal Nacional. Eds. R. Silva e F. Páscoa. Sociedade Portuguesa de Ciências Florestais, Viseu, pp. 145-146.

Alves, A.M., J.S. Pereira e J.M.N. Silva 2007. A introdução e a expansão do eucalipto em Portugal. *In* O Eucaliptal em Portugal. Impactes Ambientais e Investigação Científica. Eds. A.M. Alves, J.S. Pereira e J.M.N. Silva. ISAPress, Lisboa, pp. 13-24.

Battaglia, M., M.L. Cherry, C.L. Beadle, P.J. Sands e A. Hingston 1998. Prediction of leaf area index in eucalypt plantations: effects of water stress and temperature. Tree Physiology. 18:521–528.

Binkley, D., J.L. Stape e M.G. Ryan 2004. Thinking about efficiency of resource use in forests. Forest Ecology and Management. 193:5-16.

Borges, J.G. e G.C. Borges 2007. Impactes socioeconómicos da expansão do eucaliptal. *In* O Eucaliptal em Portugal. Impactes Ambientais e Investigação Científica. Eds. A.M. Alves, J.S. Pereira e J.M.N. Silva. ISAPress, Lisboa, pp. 315-326.

Borralho, N.M.G., M.H. Almeida e B.M. Potts 2007. O melhoramento do eucalipto em Portugal. *In* O Eucaliptal em Portugal. Impactes Ambientais e Investigação Científica. Eds. A.M. Alves, J.S. Pereira e J.M.N. Silva. ISAPress, Lisboa, pp. 61-110.

Borralho, N.M.G. e P.J. Wilson 1994. Inheritance of initial survival and rooting of *Eucalyptus globulus* Labill. stem cuttings. Silvae Genetica. 43:238-242.

Branco, M. 2007. Agentes bióticos do eucalipto em Portugal. *In* O Eucaliptal em Portugal. Impactes Ambientais e Investigação Científica. Eds. A.M. Alves, J.S. Pereira e J.M.N. Silva. ISAPress, Lisboa, pp. 255-282.

Chaves, M.M., J.P. Maroco e J.S. Pereira 2003. Understanding plant responses to drought - from genes to the whole plant. Functional Plant Biology. 30:239-264.

Chaves, M.M. e M.M. Oliveira 2004. Mechanisms underlying plant resilience to water deficits: prospects for water-saving agriculture. Journal of Experimental Botany. 55:2365-2384.

Close, D.C., C.L. Beadle, P.H. Brown e G.K. Holz 2000. Cold-induced photoinhibition affects establishment of Eucalyptus nitens(Deane and Maiden) Maiden and Eucalyptus globulus Labill. Trees. 15:32–41.

Eriksson, G., I. Ekberg e D. Clapham 2006. An introduction to Forest Genetics. Genetic center, Department of Plant Biology and Forest Genetics, SLU, Uppsala. 185 p.

Fennell, A. e A.H. Markhart 1998. Rapid acclimation of root hydraulic conductivity to low temperature. Journal of Experimental Botany. 49:879-884.

Flexas, J., J. Bota, J. Galmés, H. Medrano e M. Ribas-Carbó 2006. Keeping a positive carbon balance under adverse conditions: responses of photosynthesis and respiration to water stress. Physiologia Plantarum. 127:343–352.

Gavito, M.E., P.S. Curtis, T.N. Mikkelsen e I. Jakobsen 2001. Interactive effects of soil temperature, atmospheric carbon dioxid and soil N on root development, biomass and nutrient uptake of winter wheat during vegetative growth. Journal of Experimental Botany. 52:1913-1923.

IPCC 2001. Climate change 2001: the scientific basis. Contribution of working group I to the third assessment report of the Intergovernmental Panel on Climate Change (IPCC).

Eds. J.T. Houghton, Y. Ding, D.J. Griggs, M. Noguer, P.J. van der Linden e D. Xiaosu. Cambridge University Press, Cambridge.

Kleinschmit, J., D.K. Khurana, H.D. Gerhold e W.J. Libby 1993. Past, Present and Anticipated Applications of Clonal Forestry. *In* Clonal Forestry II. Conservation and Application. Eds. M.R. Ahuja e W.J. Libby. Springer-Verlag, Berlin Heidelberg, pp. 9-41.

Le Roux, D., W.D. Stock, W.J. Bond e D. Maphanga 1996. Dry mass allocation, water use efficiency and $\delta^{13}C$ in clones of *Eucalyptus grandis*, *E. grandis* x *camaldulensis* and *E. grandis* x *nitens* grown under two irrigation regimes. Tree Physiology. 16:497-502.

Leborgne, N., C. Teulieres, B. Cauvin, S. Travert e A.M. Boudet 1995. Carbohydrate content of Eucalyptus gunnii leaves along an annual cycle in the field and during induced frost-hardening in controlled conditions. Trees. 10:86-93.

Libby, W.J. e M.R. Ahuja 1993. Clonal Forestry. *In* Clonal Forestry II. Conservation and Application. Eds. M.R. Ahuja e W.J. Libby. Springer-Verlag, Berlin Heidelberg, pp. 1-8.

Markhart, A.H., E.L. Fiscus, A.W. Naylor e P.J. Kramer 1979. Effect of temperature on water ion transport in soybean and broccoli systems. Plant Physiology. 64:83-87.

Matheson, A.C. e P.P. Cotterill 1990. Utility of Genotype x Environment Interactions. Forest Ecology and Management. 30:159-174.

Osório, J., M.L. Osório, M.M. Chaves e J.S. Pereira 1998. Water deficits are more important in delaying growth than in changing patterns of carbon allocation in *Eucalyptus globulus*. Tree Physiology. 18:363-373.

Peng, Y.Y. e Q. Dang 2003. Effects of soil temperature on biomass production and allocation in seedlings of four boreal tree species. Forest Ecology and Management. 180:1-9.

Pereira, J.S., M.V. Madeira, S. Linder, T. Ericsson, M. Tomé e M.C. Araújo 1994. Biomass production with optimised nutrition in *Eucalyptus globulus* plantations. *In* Eucalyptus for Biomass Production Eds. J.S. Pereira e H. Pereira. Instituto Superior de Agronomia, Lisboa, pp. 13-30.

Soares, P., M. Tomé e J.S. Pereira 2007. A produtividade do eucaliptal. *In* O Eucaliptal em Portugal. Impactes Ambientais e Investigação Científica. Eds. A.M. Alves, J.S. Pereira e J.M.N. Silva. ISAPress, Lisboa, pp. 27-59.

Stape, J.L., D. Binkley e M.G. Ryan 2004. Eucalyptus production and the supply, use and efficiency of use of water, light and nitrogen across a geographic gradient in Brazil. Forest Ecology and Management. 193:17-31.

Wan, X., J.J. Zwiazek, V.J. Lieffers e S.M. Landhaüsser 2001. Hydraulic conductance in aspen (*Populus tremuloides*) seedlings exposed to low root temperatures. Tree Physiology. 21:691-696.

Whitehead, D. e C.L. Beadle 2004. Physiologycal regulation of productivity and water use in *Eucalyptus*: a review. Forest Ecology and Management. 193:113-140.

Zobel, B.J. 1993. Clonal Forestry in the Eucalypts. *In* Clonal Forestry II. Conservation and Application. Eds. M.R. Ahuja e W.J. Libby. Springer-Verlag, Berlin Heidelberg, pp. 139-148.

Zobel, B.J. e J. Talbert 1984. Applied Forest Tree Improvement. John Wiley & Sons. Inc., New York. 505 p.

CAPÍTULO 2

Responses to water stress in two *Eucalyptus globulus* clones differing in drought tolerance

2. Responses to water stress in two *Eucalyptus globulus* clones differing in drought tolerance

Summary

We evaluated drought resistance mechanisms in a drought-tolerant clone (CN5) and a drought-sensitive clone (ST51) of *Eucalyptus globulus* Labill. based on the responses to drought of some physiological, biophysical and morphological characteristics of container-grown plants, with particular emphasis on root growth and hydraulic properties. Water loss in excess of that supplied to the containers led to a general decrease in growth and significant reductions in leaf area ratio, specific leaf area and leaf-to-root area ratio. Root hydraulic conductance and leaf-specific hydraulic conductance decreased as water stress became more severe. During the experiment, the drought-resistant CN5 clone maintained higher leaf water status (higher predawn and midday leaf water potentials), sustained a higher growth rate (new leaf area expansion and root growth) and displayed greater carbon allocation to the root system and lower leaf-to-root area ratio than the drought sensitive ST51 clone. Clone CN5 possessed higher stomatal conductances at moderate stress as well as higher hydraulic conductances than Clone ST51. Differences in the response to drought in root biomass, coupled with changes in hydraulic properties, accounted for the clonal differences in drought tolerance, allowing Clone CN5 to balance transpiration and water absorption during drought treatment and thereby prolong the period of active carbon assimilation.

Keywords: acclimation, allocation, hydraulic properties, root growth, water stress.

Introduction

Economically, *Eucalyptus globulus* Labill. is one of the most important members of its genus because of its high growth rate and superior pulp properties. More than 700,000 ha have been planted with *E. globulus* in Portugal. As a result of a combination of breeding programs and improved techniques for the rooting of cuttings, Portuguese plantations have recently been established with clones selected for their high growth rates, high pulp yield and environmental adaptability.

Portugal has a Mediterranean climate with a severe summer drought, even though winter rain may be abundant. To develop improved breeding programs, it is important to gain a better understanding of the physiological responses to drought of clones selected for drought tolerance. Plant responses to water stress involve morphological and biochemical changes that lead first to acclimation and later, as water stress becomes more severe, to functional damage and the loss of plant parts (Chaves et al. 2003). During the acclimation phase, water stress typically results in slower growth rates because of inhibition of cell expansion and reduced carbon assimilation (Osório et al. 1998*a*, 1998*b*). Aboveground plant growth can be further decreased by changes in carbon partitioning that favor root system development (Sharp and Davies 1979), mainly because root growth is less affected by drought than shoot growth (Sharp 1990, Hsiao and Xu 2000). A change in the balance between leaf surface (highly sensitive to drought) and root surface (less sensitive to drought) has obvious advantages for survival, because it permits water savings in relation to water uptake potential. Improved water balance also depends on the capacity to transport water through the plant from roots to leaves. As water stress increases in severity, plant survival depends on the maintenance of xylem integrity as a hydraulic conducting system (Sperry et al. 2002). Root and leaf-specific conductances are generally lower in drought-adapted species than in more water-demanding species (Nardini et al. 1999). There are also differences in xylem cavitation vulnerability to drought, which is lower (occurs at more negative water potentials) in drought-tolerant plants than in more mesophytic plants (Tyree and Ewers 1991, Tyree 1999).

Plant responses to drought depend heavily on the root-to-shoot balance; however, shoots and leaves have been studied in greater detail than roots. For the plant to acclimate to water stress and survive drought, roots have to maintain a viable water flow path along the xylem, and root cells have to withstand some water stress and grow into new unexploited soil to absorb water. Because the roles of root and leaf responses to drought in *E. globulus* have not been fully elucidated, we evaluated the relationship between water supply and demand and the hydraulic properties of two *E. globulus* clones differing in drought sensitivity. Specifically, we studied the drought responses of some physiological, biophysical and morphological plant variables, with particular emphasis on hydraulic properties and root growth.

Materials and methods

Plant materials and treatments

We selected a drought-tolerant clone (CN5) and a drought sensitive clone (ST51) of *E. globulus*. Based on observations in field plantations subjected to summer drought, Clone CN5 has 29% higher survival and 41% higher growth rates (volume ha^{-1}) than Clone ST51. Rooted cuttings of both clones were grown in plastic containers filled with peat (60%) and Styrofoam beads (40%), and transplanted after 11 months to 10-l pots filled with a fine sandy soil. One month after transplanting, 32 cuttings per clone were transferred from the nursery to a controlled-environment greenhouse that provided a day/night temperature of 22/16 °C and relative humidity of about 60%. The mean reduction in solar irradiance in relation to outdoor conditions on sunny days was about 25% (Faria et al. 1996). Sixteen cuttings per clone were assigned to either a well-watered regime (WW; water supplied to equal transpirational losses) or a water-stress regime (WS; water supplied equal to 50% of transpirational losses). Each pot was enclosed in a dark plastic bag tied to the stem to prevent soil evaporation. The experiment lasted 7 weeks (September 9 to October 29, 2002). All plants were watered to runoff on the first day and then twice per week (Mondays and Fridays). To avoid effects caused by

microenvironmental differences (light gradient), the plants were sorted by treatment and moved to the neighboring position every watering day.

Measurements

Transpiration rate in every plant per clone and treatment (n = 16) was determined by measuring differences in pot weight between successive waterings. Stomatal conductance (g_s) was measured in fully expanded leaves at midday (solar time) with a steady-state porometer (Li-1600, Li-Cor, Lincoln, NE). The leaf-to-air vapor pressure deficit during g_s measurements varied between 1.61 and 2.84 kPa. Leaf xylem water potential (predawn, Ψ_{pd}, and midday, Ψ_{md}) was measured with a Scholander-type pressure chamber (PMS Instruments, Corvallis, OR). Measurements of g_s, Ψ_{pd} and Ψ_{md} were made on six plants per treatment (n = 6) four times during the experiment (Weeks 1, 3, 5 and 7). At Weeks 1, 5 and 7, stem xylem water potential (Ψ_x) was measured at midday in attached leaves (n = 6) that were sealed in aluminum bags at dawn (Jones 1992). Hydraulic conductances of the plant–soil system (K_{sp}) and leaf (K_l) were calculated on a leaf area basis, assuming Ψ_{pd} is an estimate of the soil water potential (Jones 1992, Saito et al. 2003):

$$K_{sp} = \frac{E}{\Psi_{pd} - \Psi_{md}} \text{ and } K_l = \frac{E}{\Psi_x - \Psi_{md}}$$

where E is transpiration rate ($m^3\ m^{-2}\ s^{-1}$) through the system measured between predawn and midday.

Plant biomass was evaluated at Weeks 1, 5 and 7 by destructively sampling five plants per treatment (n = 5). These plants were used to determine morphological parameters (height, diameter, number of branches, biomass partition, leaf area and root length). Specific leaf area (SLA) was calculated as the ratio between leaf area and leaf dry mass (DM), and leaf area ratio (LAR) was calculated as the ratio between total leaf area and total plant DM. All dry mass values were obtained after 48 h at 80 °C. Leaves and roots were scanned and leaf area and root parameters (length, diameter, area) were

calculated with Delta T scan software (Delta-T Devices, Hoddeson, U.K.). Roots were assumed to be cylindrical and root surface area was calculated by multiplying the projected area by π.

Nondestructive measurements of leaf expansion on selected leaf blades (from the second leaf pair) were recorded every 3 days on six plants per treatment (n = 6), from Day 12 to Day 47 (one stem leaf per plant). When the selected leaves reached full expansion (during Week 5), the measurements began again with the youngest expanding leaves.

Root water flow

Root water flow was evaluated at Weeks 1, 5 and 7 in the same root systems as the biomass study (n = 5). Steady-state water flow rates in whole root systems (Q_v; mm^3 s^{-1} $plant^{-1}$) were measured by the hydrostatic pressure method (Wan et al. 1999, Wan and Zwiazek 1999) with some modifications. A rigid plastic cylinder was inserted into a pressure chamber and filled with distilled water. The plant stem was cut 20 mm above the cutting end and the root system immediately immersed in distilled water in the pressure chamber. Samples were pressurized at 0.3, 0.4, 0.5, 0.6 and 0.7 MPa. Flow was measured by collecting the exudate for 5 min at each pressure in a pre-weighed capillary vial containing cotton wool that was placed over the cut stem protruding through the stopper in the pressure chamber. Volume flow density (J_v; m^3 m^{-2} s^{-1}) was determined as a steady-state flow rate per unit of root surface area. Root hydraulic conductance (K; mm^3 s^{-1} MPa^{-1}) was calculated as the slope of pressure versus flow rate where the relationship was linear. Because this method measures hydraulic conductance on branched systems with distal components present in parallel, hydraulic conductivity cannot be accurately calculated (Kolb et al. 1996). Measurements were standardized for the size of the root system by dividing K by the total leaf area of the plant, thereby obtaining the leaf-specific hydraulic conductance (LSC; m s^{-1} MPa^{-1}).

Data analysis

Data were subjected to two-way analysis of variance (ANOVA) to test for the effects and interactions of watering treatment and clone, using the STATISTICA (Version 6, 2001, StatSoft, Tulsa, OK) data analysis software system. All variables were tested for normality and homogeneity of variances. Differences were considered statistically significant at $P \leq 0.05$.

Results

Transpiration

Under well-watered conditions, *E* per plant increased throughout the experiment (Figure 1A). Well-watered ST51 plants, with their larger leaf area, exhibited a higher *E*

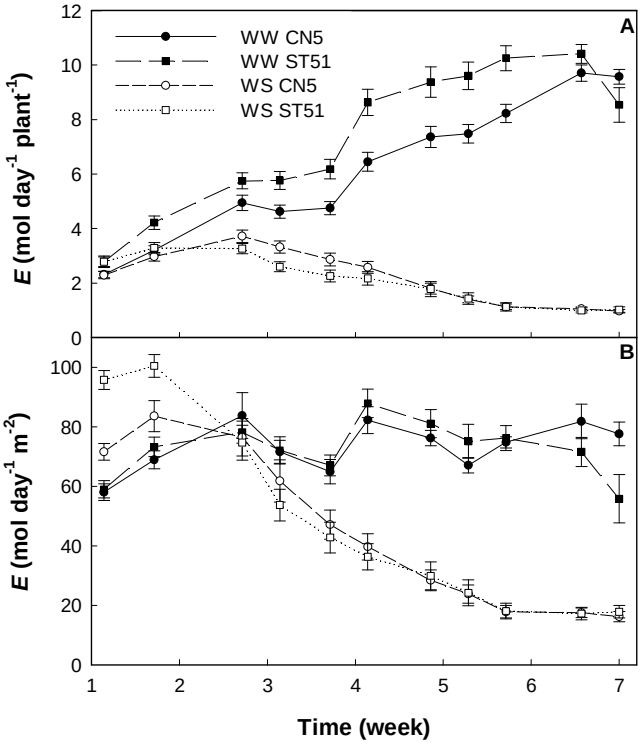

Figure 1. Leaf transpiration rate (*E*) expressed on a per plant basis (A) and leaf area basis (B) in well-watered (WW) and water-stressed (WS) plants belonging to a drought-tolerant clone (CN5) and a drought-sensitive clone (ST51) of *Eucalyptus globulus.* Data are means ± SE (n = 11–16).

per plant than the CN5 plants. However, on a leaf area basis, the clones had similar *E* values (Figure 1B). In water-stressed plants of both clones, *E* increased until Day 14, and then decreased until the end of the experiment (Figures 1A and 1B).

Plant water status and stomatal conductance

Plant water status was assessed by measuring Ψ_{pd} and Ψ_{md}. Well-watered plants of both clones maintained Ψ_{pd} at about –0.30 MPa throughout the experiment, whereas Ψ_{pd} of water-stressed plants declined throughout the experiment and, in Week 7, it fell to –1.71 ± 0.06 and –2.43 ± 0.27 MPa in CN5 and ST51, respectively. In both clones, the leaf water potential curves of the water-stressed plants can be divided into two phases (Figures 2A and 2B). In the first phase, moderate water stress developed slowly from the beginning of the experiment until Week 5. During the second phase, from Week 5 to Week 7, water stress became increasingly more severe. Midday leaf water potential did not vary significantly in well-watered plants, whereas by the end of the experiment, it declined to –2.46 ± 0.05 and –3.26 ± 0.26 MPa in water-stressed plants of CN5 and ST51, respectively (Figure 2B). Not only were the differences between watering regimes statistically significant ($P < 0.001$), but there were also significant differences between clones. Clone CN5 had higher Ψ_{pd} and Ψ_{md} than ST51 during the experiment ($P < 0.05$). Water-stressed ST51 plants exhibited a greater difference between Ψ_{pd} and Ψ_{md} than water-stressed CN5 plants until Week 5, indicating that they experienced more severe stress during the day.

The fall in Ψ_{pd} and Ψ_{md} in water-stressed plants of both clones was concomitant with a decline in g_s from 167 mmol m^{-2} s^{-1} in Week 1 to about 12 mmol m^{-2} s^{-1} in Week 7 (Figure 2C). Water-stressed ST51 plants displayed significantly lower g_s than water-stressed CN5 plants in Week 3 ($P < 0.05$). Well-watered plants of both clones had similar g_s that increased to about 342 mmol m^{-2} s^{-1} at Week 7. The increase in the g_s of well-watered plants from Week 3 to Week 5 was probably associated with changing light conditions because it closely followed the PPF curve (Figure 2D).

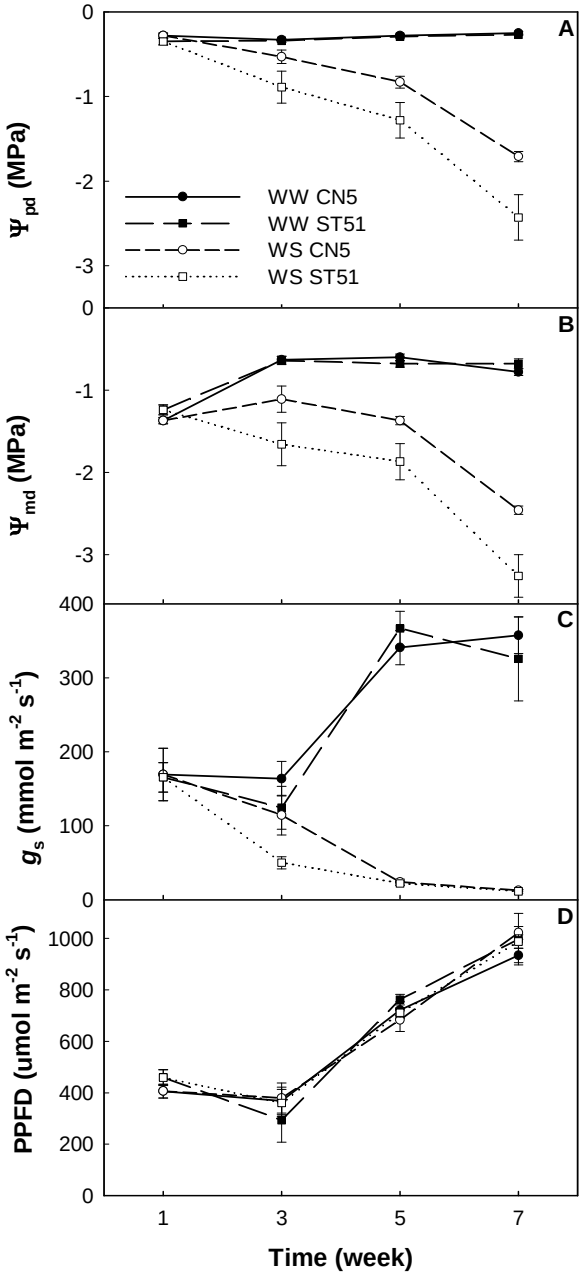

Figure 2. Predawn leaf water potential (Ψ_{pd}; A), midday leaf water potential (Ψ_{md}; B), midday stomatal conductance (g_s; C) and photosynthetic photon flux (PPF; D) in well-watered (WW) and water-stressed (WS) plants belonging to a drought-tolerant clone (CN5) and a drought-sensitive clone (ST51) of *Eucalyptus globulus*. Data are means ± SE (n = 6).

Growth response

Water stress led to a general decrease in growth that was reflected in reductions in total biomass, leaf area, number of branches and total root length (Table 1).

Under well-watered conditions, Clone ST51 had a greater leaf area than Clone CN5 (Figure 3A), which explains its higher growth rate. Despite having similar leaf areas

Table 1. Total biomass, leaf area, number of branches, total root length, dry mass partitioning (percent of total biomass) and leaf growth analysis in well-watered (WW) and water-stressed (WS) plants belonging to a drought-tolerant clone (CN5) and a drought-sensitive clone (ST51) of *Eucalyptus globulus* evaluated at the end of experiment (Week 7). Data are means ± SE (n = 5). Symbols: *, **, *** represent statistical significance at P = 0.05, 0.01 and 0.001, respectively; and ns = nonsignificant at P = 0.05.

Morphological characteristics	WW CN5	WW ST51	WS CN5	WS ST51	Significance of 2-way ANOVA		
					Clone (C)	Watering regime (W)	C x W
Total biomass (g)	15.4±1.1	18.7±1.6	9.2±0.6	9.3±0.4	ns	***	ns
Leaf area (m^2)	0.12±0.007	0.16±0.008	0.05±0.005	0.05±0.003	**	***	**
Number of branches	10.8±0.6	10.0±0.3	6.2±0.7	5.6±0.5	ns	***	ns
Total root length (m)	111±10	121±21	98±13	59±4	ns	*	ns
Dry-mass partitioning							
Stem (%)	27.8±1.5	23.8±1.2	31.7±1.3	35.4±1.3	ns	***	*
Branches (%)	5.3±0.22	6.4±0.24	1.7±0.63	2.8±0.52	*	***	ns
Leaves (%)	51.4±1.6	56.0±1.1	46.8±2.3	48.4±1.3	ns	**	ns
Root (%)	15.5±0.8	13.7±1.2	19.8±1.7	13.5±0.8	**	ns	ns
Leaf growth analysis							
Leaf area ratio ($m^2\ kg^{-1}$)	8.1±0.3	8.8±0.5	5.8±0.3	5.7±0.3	ns	***	ns
Specific leaf area ($m^2\ kg^{-1}$)	15.6±0.3	15.7±0.6	12.4±0.3	11.8±0.4	ns	***	ns
Leaf area / root area	0.98±0.10	1.12±0.13	0.55±0.12	0.76±0.08	ns	**	ns

at the beginning of the experiment (Figure 3A), Clone CN5 had a greater total root length than Clone ST51 ($P < 0.05$). At Week 5, both clones displayed greater increases in root length in the water-stress regime than in the well-watered regime (59 and 16% in CN5 and ST51, respectively) ($P < 0.05$). Thereafter, water-stressed CN5 plants showed continual increases in root growth (32 and 66% at Weeks 5 and 7, respectively), whereas root growth ceased completely after Week 5 in water-stressed ST51 plants (Figure 3B).

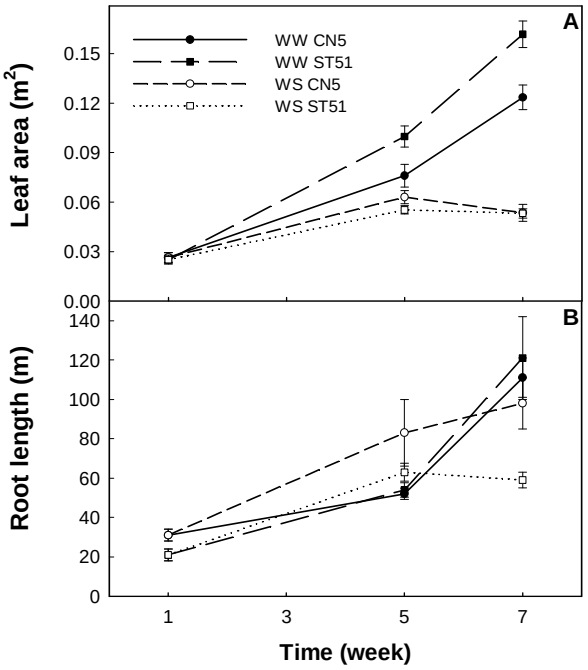

Figure 3. Total leaf area (A) and total root length (B) in well-watered (WW) and water-stressed (WS) plants belonging to a drought-tolerant clone (CN5) and a drought-sensitive clone (ST51) of *Eucalyptus globulus.* Data are means ± SE ($n = 5$).

At the end of the 7-week experiment, water-stressed plants of both clones had significantly decreased the proportion of biomass allocated to branches and leaves (Table 1). On the other hand, the water stress treatment caused an increase in the ratio of stem axis biomass to total biomass, particularly in ST51 (11.6%). In addition to differences in responses to water availability, the clones differed in biomass partitioning. The CN5 plants invested a larger proportion of total dry mass in roots than the ST51 plants (particularly water-stressed plants), whereas ST51 plants invested a larger proportion of total dry mass in leaves and branches, especially under well-watered conditions.

At Week 7, decreases in leaf area ratio and specific leaf area were observed in water-stressed plants of both clones, with no statistically significant clonal differences (Table 1). As a result of restrained leaf area growth and sustained root growth in response to drought (Figure 3), the leaf area/root area ratio decreased to 0.55 in CN5 and to 0.76 for ST51.

Leaf growth was strongly correlated with water supply (Figure 4). The effect of water shortage on leaf area expansion was detectable in the first phase of the experiment (up to Week 4), with a decrease of 24 and 44% in clones CN5 and ST51, respectively ($P < 0.001$). In this phase of moderate stress, leaf area expansion was 25% greater for water-stressed CN5 plants than for water-stressed ST51 plants. In the second phase (from Week 5 to Week 7), when severe water stress developed, leaf growth decreased by 44 and 53% in water-stressed plants of CN5 and ST51, respectively ($P < 0.001$).

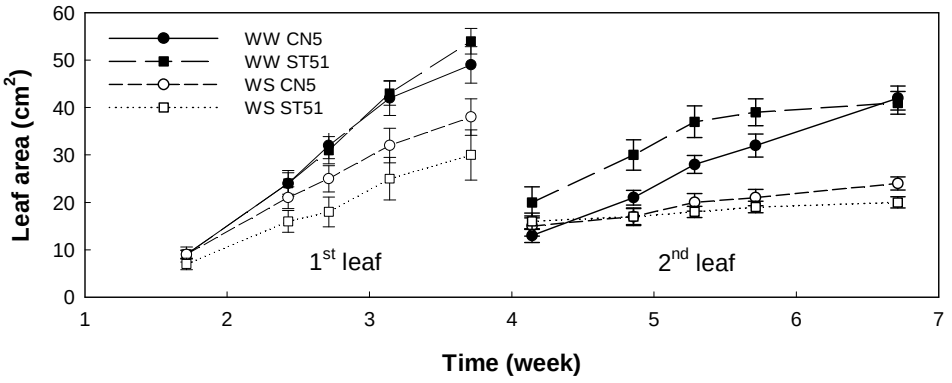

Figure 4. Leaf area expansion measured in the first and second leaves that appeared after the beginning of the experiment in well-watered (WW) and water-stressed (WS) plants belonging to a drought-tolerant clone (CN5) and a drought-sensitive clone (ST51) of *Eucalyptus globulus.* Data are means ± SE ($n = 6$).

Hydraulic properties

Root water flux (J_v) decreased from the beginning of the experiment, particularly in plants in the water stress treatment (Figure 5A), and was strongly correlated with root growth. The watering regime had a significant effect on J_v, with higher values for well-watered plants ($P < 0.001$).

Root hydraulic conductance (*K*) was reduced by soil water deficits in Weeks 5 and 7 ($P < 0.01$ and $P < 0.001$, respectively) (Figure 5B). Although there was no significant clone effect on *K*, there was a significant interaction between clone and treatment effects ($P < 0.05$), so that, by Week 7, ST51 plants exhibited the highest (+36%) and the lowest (–45%) *K* in well-watered and water-stressed conditions, respectively. At Week 7, both CN5 and ST51 water-stressed plants displayed a decrease in *K* (–27 and –35%, respectively) compared with values at Week 5.

Throughout the experiment, LSC decreased in plants in all treatments (Figure 5C).

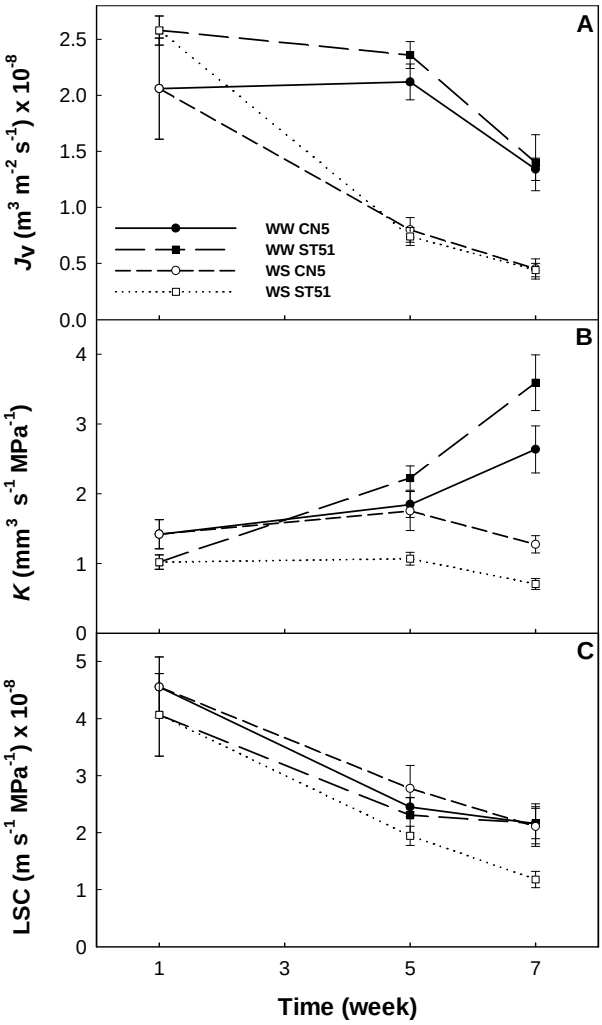

Figure 5. Root water flux (J_v; A), root hydraulic conductance (*K*; B) and leaf specific conductance (LSC; C) in well-watered (WW) and water-stressed (WS) plants belonging to a drought-tolerant clone (CN5) and a drought-sensitive clone (ST51) of *Eucalyptus globulus.* Data are means ± SE ($n = 5$).

However, LSC declined in well-watered plants because of a large increase in leaf area, whereas it declined in water-stressed plants because of reduced *K*. During drought treatment, Clone CN5 maintained higher LSC than Clone ST51 (+43% at Week 5 and +79% at Week 7).

In well-watered plants, hydraulic conductance of the soil–plant system (K_{sp}) remained stable throughout the experiment, although Clone ST51 clone had higher mean values than Clone CN5 (0.54×10^{-7} versus 0.69×10^{-7} m s^{-1} MPa^{-1} in CN5 and ST51, respectively) (Figure 6A). In both clones, K_{sp} decreased with increasing soil water stress at Weeks 5 and 7 ($P < 0.001$); however, water-stressed CN5 plants displayed a smaller decrease in K_{sp} in the first 5 weeks and maintained higher values until Week 7 than water-stressed ST51 plants (on average, +26%).

The development of drought stress led to comparable trends in leaf conductance (K_l) in both clones (Figure 6B). There were significant treatment differences in K_l at Weeks 5 and 7 ($P < 0.001$ and $P < 0.01$, respectively). Nevertheless, water-stress had a greater effect on K_l of ST51 plants compared with CN5 plants at both Weeks 5 and 7 (–54 and –58%, respectively).

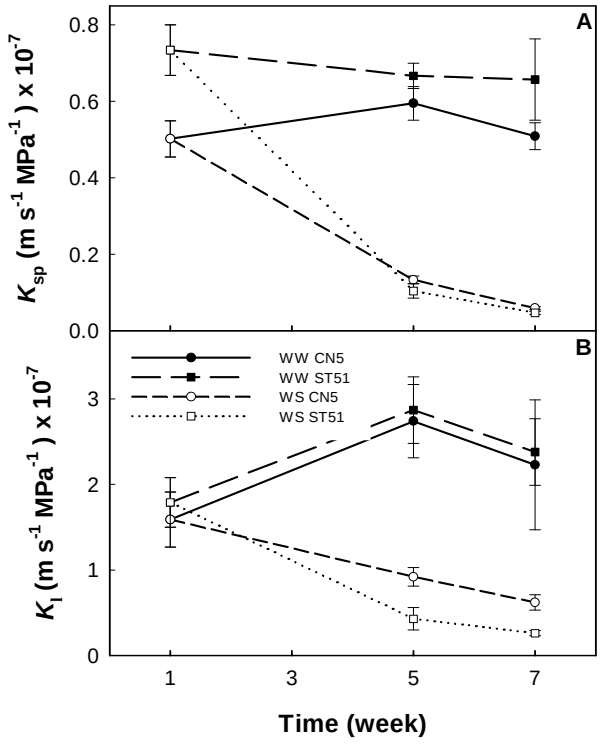

Figure 6. Hydraulic conductance of the soil–plant system (K_{sp};A) and leaf (K_l; B) in well-watered(WW) and water-stressed (WS) plants belonging to a drought-tolerant clone (CN5) and a drought-sensitive clone (ST51) of *Eucalyptus globulus.* Data are means ± SE ($n = 6$).

Discussion

We observed a reduction of about 46% in mean total biomass growth in young container-grown *Eucalyptus* plants when water supplied to the pots was only 50% of plant water use. This treatment resulted in a 63% decrease in total leaf area and a 45% decrease in the ratio of leaf area to total biomass relative to values for well-watered control plants. In addition to the decrease in leaf area, stomata of water-stressed plants closed for longer each day, contributing to decreased growth as a result of reduced carbon assimilation (Figures 1 and 2C).

Acclimation to slowly declining soil water availability occurs before the onset of tissue dehydration and results in physiological and morphological adjustments that improve plant water balance (Pereira and Chaves 1993). We distinguished an initial period of slowly developing water stress during the first 5 weeks of treatment when the tested clones responded differently. Stomatal conductance in ST51 plants fell steeply in response to 3 weeks of moderate water stress, following which the plants entered a period of severe water stress. As a consequence, the plants had only limited time for drought acclimation. A plant's ability to prolong moderate stress or postpone severe stress and thereby maintain a more favorable leaf water status during the first phase of a drought may enable the plant to avoid damage by severe water stress later on. We observed that, in response to water stress, the drought-tolerant CN5 clone had a significantly smaller difference between Ψ_{pd} and Ψ_{md} than the drought-sensitive ST51 clone, leading to a more favorable leaf water status as a result of a higher water supply for a given stomatal conductance (Figure 1). In addition, under drought conditions, CN5 plants had noticeably greater root length (Figure 3B) and rate of new leaf expansion (Figure 4) than ST51 plants. The maintenance of a continued higher growth rate in young leaves of CN5 plants under drought conditions compared with ST51 plants may have contributed to recovery of carbon assimilation after rehydration (data not shown), because the photosynthetic capacity of *E. globulus* is robust during periods of drought (Quick et al. 1992) and younger leaves are generally less affected by drought than older leaves (Pereira and Chaves 1993).

Until Week 5, increases in root length were greater in water-stressed plants than in well-watered plants of both clones (Figure 3B), indicating that water stress had less effect on root growth than on leaf growth (Sharp 1990, Hsiao and Xu 2000). Similar results were reported by Blum et al. (1983) and by Mc-Donald and Davies (1996). Enhanced biomass partitioning to roots may result from a drought-induced reduction in the sink strength of the aboveground plant tissues, making more assimilates available for root growth.

Compared with the drought-sensitive ST51 clone, the drought-tolerant CN5 clone had a higher investment in root system development before drought was imposed (assessed on Day 1 of the experiment), suggesting that this characteristic partially accounts for the enhanced drought tolerance exhibited by this genotype. In addition, water-stressed CN5 plants showed continually greater root growth until Week 7, whereas root growth of water-stressed ST51 plants ceased completely after Week 5 (Figure 3B). Thus, we conclude that the initially larger root system of Clone CN5, coupled with its ability to rapidly increase the proportion of biomass allocated to the root, resulting in optimization of the relationship between transpiration area and absorption area under drought conditions, explains its superior drought resistance compared with plants of Clone ST51. Moreover, we predict that, in field conditions where soil volume is unrestricted, the benefits of a larger investment in root extension under drought conditions will be enhanced because Clone CN5 will be able to access as yet unexplored volumes of soil, resulting in increased water uptake.

Water transport in trees is regulated by the hydraulic conductance of the soil–root–shoot–leaf pathway. Because stomatal conductance and photosynthesis depend on the transport of water from soil to leaf to atmosphere, changes in whole-tree hydraulic conductance may affect gas exchange (Tyree and Ewers 1991, Hubbard et al. 1999). It is possible that, with the intensification of water stress after Week 5, a hydraulic limitation developed – mainly in Clone ST51 – that considerably reduced young leaf expansion and root growth. During drought, ST51 plants displayed a greater restriction in water supply to leaves, with both lower K and LSC values (Figures 5B and 5C) and lower K_{sp} and K_l values (Figure 6) than CN5 plants.

Several studies have shown that changes in whole-plant hydraulic conductance affect g_s and photosynthesis (Bond and Kavanagh 1999, Wan et al. 1999, Brodribb and Field 2000, Hubbard et al. 2001). In our study, although midday g_s was low after Week 5 in water-stressed plants of both clones (Figure 2C), plants with a more limited water supply closed their stomata earlier in the day than plants with a greater water supply. When measured in the afternoon in Week 5, g_s of water-stressed ST51 plants was 45% lower than in water-stressed CN5 plants (data not shown), indicating a difference between the clones in hydraulic systems. In well-watered conditions, despite similar total root system length, Clone ST51 exhibited higher K and K_{sp} in both Weeks 5 and 7 compared with Clone CN5. This matches the general findings of lower root and shoot hydraulic conductances in drought-adapted species (Nardini et al. 1999). However, we cannot disregard the possibility that genotypic differences in root architecture influenced the hydraulic systems. We can assume that water-stressed plants were subjected to a certain loss in conductance as a result of embolism or cavitations, or both, given both the low Ψ_x values that were attained and the higher K values exhibited by well-watered plants throughout the experiment. At Week 5, for similar root system dimensions between clones (Figure 3B), water-stressed Clone ST51 displayed a significantly lower K (–52%), presumably as a result of cavitation. Compared with water-stressed CN5 plants, water-stressed ST51 plants displayed lower stem xylem pressures (–33% at Week 5) and lower K values. We speculate that, compared with Clone CN5, Clone ST51 suffered from a greater cavitation-induced loss in conductance, which took place before Week 5. Differences in vulnerability to cavitation have been associated with drought tolerance both between species (e.g., Tyree and Ewers 1991, Cochard 1992) and between genotypes of the same species (e.g., Tognetti et al. 1997, Vander Willigen and Pammenter 1998).

In summary, our data show that successful drought acclimation in *E. globulus* clones may be the result of different processes, including changes in root biomass coupled with changes in hydraulic properties of the root systems. A greater allocation of biomass to roots and higher hydraulic conductances made it possible to prolong the water-stress-free period for active carbon assimilation in the clone that was least susceptible to drought. These developmental changes, which maintained the balance

between transpiration and absorption areas when soil water availability declined, seemed to be the key determinant of performance under drought conditions.

Acknowledgments

This research received financial support from the Commission of the European Communities (Contract No. QLK5-CT-2000-01377), Directorate-General Research – Quality of Life and Management of Living Resources Programme. This paper does not necessarily reflect the Commission's views and in no way anticipates its future policy in this area. A. Shvaleva was supported by FCT, Lisbon, Grant SFRH/BPD/5667/2001.

References

Blum, A., J. Mayer and G. Gozlan. 1983. Association between plant production and some physiological components of drought resistance in wheat. Plant Cell Environ. 6:219–225.

Bond, B.J. and K.L. Kavanagh. 1999. Stomatal behaviour of four woody species in relation to leaf-specific hydraulic conductance and threshold water potential. Tree Physiol. 19:503–510.

Brodribb, T.J. and T.S. Field. 2000. Stem hydraulic supply is linked to leaf photosynthetic capacity: evidence from New Caledonian and Tasmanian rainforests. Plant Cell Environ. 23:1381–1388.

Chaves, M.M., J.P. Maroco and J.S. Pereira. 2003. Understanding plant response to drought—from genes to the whole plant. Funct. Plant Biol. 30:1–26.

Cochard, H. 1992. Vulnerability of several conifers to air embolism. Tree Physiol. 11:73–83.

Faria, T., D. Wilkins, B. Besford, M. Vaz, J.S. Pereira and M.M. Chaves. 1996. Growth at elevated CO2 leads to down-regulation of photosynthesis and altered response to high temperature in *Quercus suber* L. seedlings. J. Exp. Bot. 47:1755–1761.

Hsiao, T.C. and L.K. Xu. 2000. Sensitivity of growth of roots versus leaves to water stress: biophysical analysis and relation to water transport. J. Exp. Bot. 51:1595–1616.

Hubbard, R.M., B.J. Bond and M.G. Ryan. 1999. Evidence that hydraulic conductance limits photosynthesis in old *Pinus ponderosa* trees. Tree Physiol. 19:165–172.

Hubbard, R.M., M.G. Ryan, V. Stiller and J.S. Sperry. 2001. Stomatal conductance and photosynthesis vary linearly with plant hydraulic conductance in ponderosa pine. Plant Cell Environ. 24:113–121.

Jones, H.G. 1992. Plants and microclimate. 2nd Edn. Cambridge University Press, Cambridge, pp 95–98.

Kolb, K.J., J.S. Sperry and B.B. Lamont. 1996. A method for measuring xylem hydraulic conductance and embolism in entire root and shoot systems. J. Exp. Bot. 47:1805–1810.

McDonald, A.J.S. and W.J. Davies. 1996. Keeping in touch: responses of the whole plant to deficits in water and nitrogen supply. Adv. Bot. Res. 22:229–300.

Nardini, A., M.A. Lo Gullo and S. Salleo. 1999. Competitive strategies for water availability in two Mediterranean *Quercus* species. Plant Cell Environ. 22:109–116.

Osório, J., M.L. Osório, M.M. Chaves and J.S. Pereira. 1998*a*. Effects of water deficits on ^{13}C discrimination and transpiration efficiency of *Eucalyptus globulus*. Aust. J. Plant Physiol. 25:645–653.

Osório, J., M.L. Osório, M.M. Chaves and J.S. Pereira. 1998*b*. Water deficits are more important in delaying growth than in changing patterns of carbon allocation in *Eucalyptus globulus*. Tree Physiol. 18:363–373.

Pereira, J.S. and M.M. Chaves. 1993. Plant water deficits in Mediterranean ecosystems. *In* Water Deficits: Plant Responses from Cell to Community. Eds. J.A.C. Smith and H. Griffiths. Bios Scientific Publishers, Oxford, pp 235–251.

Quick,W.P., M.M. Chaves, R.Wendler et al. 1992. The effect of water stress on photosynthetic carbon metabolism in four species grown under field conditions. Plant Cell Environ. 15:25–35.

Saito, T., T. Tanaka, H. Tanabe, Y. Matsumoto and Y. Morikawa. 2003. Variations in transpiration rate and leaf cell turgor maintenance in saplings of deciduous broad-leaved tree species common in cool temperate forests in Japan. Tree Physiol. 23:59–66.

Sharp, R.E. 1990. Comparative sensitivity of root and shoot growth and physiology to low water potentials. *In* Importance of Root-to-Shoot Communication in the Response to Environmental Stress. Eds. W.J. Davies and B. Jeffcoat. British Society for Plant Growth Regulation, Bristol, U.K., pp 29–44.

Sharp, R.E. and W.J. Davies. 1979. Solute regulation and growth by roots and shoots of water-stressed maize plants. Planta 147:43–49.

Sperry, J.S., U.G. Hacke, R. Oren and J.P. Comstock. 2002. Water deficits and hydraulic limits to leaf water supply. Plant Cell Environ. 25:251–263.

Tognetti, R., M. Michelozzi and A. Giovannelli. 1997. Geographical variation in water relations, hydraulic architecture and terpene composition of Aleppo pine seedlings from Italian provenances. Tree Physiol. 17:241–250.

Tyree, M.T. 1999. Water relations and hydraulic architecture. *In* Handbook of Functional Plant Ecology. Ed. F. Valladares. Marcel Dekker, New York, pp 221–268.

Tyree, M.T. and F.W. Ewers. 1991. The hydraulic architecture of trees and other woody plants. New Phytol. 119:345–360.

Vander Willigen, C. and N.W. Pammenter. 1998. Relationship between growth and xylem hydraulic characteristics of clones of *Eucalyptus* spp. at contrastings sites. Tree Physiol. 18:595–600.

Wan, X. and J.J. Zwiazek. 1999. Mercuric chloride effects on root water transport in aspen seedlings. Plant Physiol. 121:939–946.

Wan, X., S.M. Landhaüsser, J.J. Zwiazek and V.L. Lieffers. 1999. Root water flow and growth of aspen (*Populus tremuloides*) at low root temperatures. Tree Physiol. 19:879–884.

CAPÍTULO 3

Metabolic responses to water deficit in two *Eucalyptus globulus* clones with contrasting drought sensitivity

3. Metabolic responses to water deficit in two *Eucalyptus globulus* clones with contrasting drought sensitivity

Summary

We compared the metabolic responses of leaves and roots of two *Eucalyptus globulus* Labill. clones differing in drought sensitivity to a slowly imposed water deficit. Responses measured included changes in concentrations of soluble and insoluble sugars, prolin, total protein and several antioxidant enzymes. In addition to the general decrease in growth caused by water deficit, we observed a decrease in osmotic potential when drought stress became severe. In both clones, the decrease was greater in roots than in leaves, consistent with the observed increases in concentrations of soluble sugars and proline in these organs. In roots of both clones, glutathione reductase activity increased significantly in response to water deficit, suggesting that this enzyme plays a protective role in roots during drought stress by catalyzing the catabolism of reactive oxygen species. Clone CN5 has stress avoidance mechanisms that account for its lower sensitivity to drought compared with Clone ST51.

Keywords: *antioxidant enzymes, osmotic potential, proline, sugars, water stress.*

Introduction

Soil and atmospheric water deficits are among the most important factors limiting plant growth and photosynthesis. Both kinds occur during the Mediterranean summers, together with high temperatures and high irradiances. *Eucalyptus globulus* Labill., an economically valuable species in Portugal, is an evergreen tree that can survive all but extreme Mediterranean summer conditions. It grows best along the Atlantic coast where the Mediterranean climate is tempered by oceanic influence (Pereira and Chaves 1993).

To cope with periods of drought, plants rely on various drought-avoidance and drought-tolerance mechanisms that vary with genotype (Chaves et al. 2002). Adaptive mechanisms enabling plants to withstand abiotic environmental stress include changes in morphological, physiological and biochemical characteristics such as: (1) root system deph (Volaire et al. 1998); (2) control over leaf transpiration rates (Maroco et al. 1997) or transpirational surface, either through leaf abscission or growth inhibition (Chaves et al. 2003, Munné-Bosch and Alegre 2004); (3) osmoprotectant pool sizes (Delauney and Verma 1993); and (4) tissue dehydration tolerance (Volaire et al. 1998). At the cellular level, drought can affect the production of reactive oxygen species (ROS) (Smirnoff 1998), that may play a role in intracellular signaling (Finkel 1998) beside causing oxidative stress, which can be diagnosed by the accumulation of lipid peroxides, oxidized proteins and modified DNA bases (Rubio et al. 2002). Detoxification of ROS is dependent on a system of antioxidant enzymes and metabolites (Polle and Rennenberg 1992). Enzymes such as glutathione reductase (GR), ascorbate peroxidase (APX), superoxide dismutase (SOD) and catalase (CAT) play a key role in the scavenging of ROS such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl ($OH^\cdot$) and singlet oxygen ($^1O_2$), which are the initiators of a reaction chain leading to the degradation of cellular components (Sgherri et al. 2000). Under optimal growth conditions, antioxidant enzymes and metabolites from leaves detoxify ROS, thus minimizing oxidative damage (Smirnoff 1998). During periods of environmental stress, e.g., periods of drought or high irradiance, additional protective processes involving β-carotene, zeaxanthin, and anteraxanthin synthesis participate in the deactivation of ROS (Garcia-Plazaola et al. 1997, Medrano et al. 2002).

Although the antioxidant defenses of trees have been studied in relation to environmental stresses, such as high altitude (Polle and Rennenberg 1992), pollution (Wingsle and Hallgren 1993) and low temperature (Nakagara and Sagisaka 1984), few studies have focused on the activity of such systems in response to water deficits, and rarely in water-stressed eucalyptus plants (Osawa and Namiki 1985, Osawa et al. 1992). Because some regions in which *E. globulus* is grown commercially, e.g., southern Portugal, experience hot, dry summers, knowledge of the role of antioxidants as a protective system may be useful in tree breeding programs.

During dehydration, osmolytes (mainly proline, glycine betaine and sugars) can help preserve protein and membrane structure and function (Smirnoff 1998). Although changes in soluble and insoluble sugars concentrations in leaves of water-stressed *E. globulus* have been studied (Quick et al. 1992), little is known about the changes in osmotically active compounds in roots of water-stressed *E. globulus*.

Fast growing *Eucalyptus* species are likely to be severely affected by drought. Costa e Silva et al (2004) studied two *E. globulus* clones differing in drought sensitivity in the field (Clone ST51 is more drought sensitive than Clone CN5) and found clonal differences in the type and magnitude of response to water stress. The better performance of Clone CN5 under drought conditions was associated with faster root growth and higher stem hydraulic conductance compared with Clone ST51.

In recent years, new *E. globulus* plantations have used clones selected for high pulp yield. In Portugal, it is desirable that selected clones are well adapted to the Mediterranean summer drought. The present study was undertaken to determine if the leaves and roots of *E. globulus* Clones ST51 and CN5 exhibit metabolic differences when subjected to a gradually imposed water deficit. Metabolic responses to water deficit were investigated by measuring leaf pigment composition as well as osmotic potential, osmotically active compounds (proline, sugars) and enzymes with antioxidant activity in leaves and roots.

Materials and Methods

Plant material

Rooted cuttings of *E. globulus* Clones ST51 and CN5, grown in plastic containers filled with a 3:2 (v/v) peat:styrofoam mix, were obtained from Aliança Florestal, Portugal, and transplanted after 11 months of growth in a nursery to 10-l plastic pots filled with a fine sandy soil. One month after transplanting, each pot was enclosed in a dark plastic bag tied to the stem to prevent soil evaporation. The potted cuttings were placed in a greenhouse in a day/night temperature of 22/16 ºC and a relative humidity of about 60%. The mean reduction in solar irradiance in relation to outdoor conditions on a sunny day was about 25% (Faria et al. 1996). On September 9, 2002, 16 cuttings per clone were assigned to a well-watered regime with watering equal to transpiration loss. The remaining 16 cuttings per clone were assigned to a water-stressed regime with watering equal to 50% of transpiration loss. The amount of water supplied was calculated from the difference in pot weight between successive watering. All plants were watered to the point of runoff on the first day and then watered twice per week (Monday and Friday) according to treatment regime. The treatments continued for 7 weeks (September 9 to October 29, 2002).

Plant water status

Predawn (Ψ_{pd}) and midday (Ψ_{md}) leaf water potential were measured daily at 0500 and 1300 h, respectively, with a Scholander-type pressure chamber (PMS Instruments, Corvallis, OR) in six plants per treatment (n = 6).

Measurements of osmotic potential (Ψ_{π}) were made on previously frozen 6-mm diameter leaf discs and root segments by thermocouple psychrometry, using C-52 sample chambers connected to a Wescor HR-33T dew-point microvoltmeter (Wescor, Logan, UT). The chambers were calibrated with standard NaCl solutions. After thawing, and following a 2-h equilibration period, osmotic potential of the samples were measured by the dew-point method. Room temperature during the measurements was 20 ± 1 °C.

Sampling

At the end of the 7-week treatment, 6-mm diameter leaf discs were collected from fully expanded leaves (0.5 g fresh mass) at predawn and root segments were excised from the central part of the root system (0.5 g fresh mass and diameter < 2 mm). Samples were collected from five plants per treatment, frozen immediately in liquid nitrogen and stored at –80 ºC until analyzed.

Growth analysis

At the end of the 7-week treatment, the plants were harvested and shoots were separated into stem, lateral branches and stem leaves. Roots were gently washed and carefully separated from soil and other debris. Plant components were dried for at least 48 h at 80 ºC in the oven and cooled in desiccators for dry mass determination. Leaves and roots were scanned before drying and leaf area and root parameters (length, diameter, area) of each seedling (five plants per treatment) were calculated with *Delta-T scan* software (Delta-T Devices, Cambridge, U.K.). Roots were assumed to be cylindrical and root surface area was calculated by multiplying the projected area by π.

Soluble and insoluble sugars

Soluble and insoluble sugars in leaves and roots were assayed by the anthrone method (Robyt and White 1987). Frozen leaf discs (0.02 g) and root segments (0.05 g) were ground with a cold mortar and pestle in liquid N_2 with 1 ml of 70% (v/v) ethanol. The homogenate was thermomixed twice at 60 ºC for 30 min, centrifuged at 14,000 g for 5 min and the supernatant used for determination of soluble sugars. To extract insoluble sugars from the pellet, 1 ml of acetone was added, the mixture centrifuged at 14,000 g for 5 min and the supernatant discarded. One ml of HCl (1.1%) was added to the dry pellet, which was thermomixed twice at 60 ºC for 30 min and centrifuged at 14,000 g for 5 min. Absorbance of the insoluble sugars in the supernatant was determined at 620 nm with a spectrophotometer (U-2001; Hitachi, Japan). A calibration curve was prepared with standard glucose solutions.

The contribution of sugars and proline to osmotic potential was calculated, according to the Van't Hoff equation:

$$\Psi_{\pi} = -cRT$$

where *c* is molal concentration of soluble sugars or proline, *R* is the gas constant and *T* is absolute temperature. Estimates of the contribution of these solutes to osmotic potential were based on the water content of the samples.

Proline and leaf pigments

About 100 mg of fresh plant material was homogenized in 1.5 ml of 3% thiobarbituric acid, shaken vigorously for 1 min and centrifuged at 15,000 g for 10 min at 4 ºC. The supernatant was assayed for proline, as described by Bates et al. (1973), by incubating 0.5 ml of extract with 1 ml ninhydrin acid and 1 ml glacial acetic acid for 1 h at 100 ºC. The reaction mixture was rapidly cooled in ice and 1 ml of toluene added and mixed vigorously. Absorbance of the toluene phase was measured at 520 nm. Proline concentration was determined against a standard curve (0 to 0.5 μmol ml^{-1}) with L-proline (Sigma-Aldrich CHEMIE GmbH, Steinheim, Germany).

Pigments were extracted from frozen leaf discs by adding 2 ml acetone:water (9:1, v:v) and grinding with a pestle and mortar. The extract was centrifuged at 10,000 g for 10 min at 4 ºC and the supernatant was filtered through a 0.2-μm filter. Pigments were analyzed by high performance liquid chromatography (HPLC) as described by Wright et al. (1991).

Antioxidant enzymes

For SOD, GR and CAT, frozen leaves (0.5 g fresh mass) and roots (0.5 g fresh mass and diameter < 2 mm) were ground with 2% polyvinylpolypyrrolidone (PVPP) (Sigma Chemical Co., St. Louis, USA) and sea sand and then homogenized with 5 ml of 100 mM phosphate buffer, pH 7.8, containing 2% Triton X-100 (Solon Ind. Pkwy. Solon, Ohio) (Gogorcena et al.1995). The same extraction medium supplemented with

2% ascorbic acid (10 mM) was used for APX (Nakano and Asada 1981). The homogenates were centrifuged at 15,000 g for 20 min and the supernatants assayed for enzyme activity. All steps were performed at 4 °C.

Catalase (EC 1.11.1.6) activity was determined by H_2O_2 consumption measured as the decrease in absorbance at 240 nm, according to the method of Aebi (1983). The assay medium contained 50 mM KH_2PO_4/K_2HPO_4 (pH 7.0), 40 mM H_2O_2 and 100 μm extract. Catalase activity was calculated based on an extinction coefficient of 3.94 mM^{-1} cm^{-1}. Controls lacking either extract or H_2O_2 showed no changes in absorbance.

Glutathione reductase (EC 1.6.4.2) was measured by following the oxidation of NADPH at 340 nm by a modification of the method of Foyer and Halliwell (1976). The assay medium contained 500 mM HEPES (Sigma Chemical) (pH 8.0), 0.25 mM EDTA (Sigma Chemical), 2 mM NADPH (Sigma Chemical), 20 mM oxidized glutathion (GSSG) and 100 μl extract. Control rates were obtained in the absence of GSSG or NADPH. Glutathione reductase activity was calculated based on an extinction coefficient of 6.22 mM^{-1} cm^{-1}.

Ascorbate peroxidase (EC 1.11.1.11) was measured by a modification of the method of Nakano and Asada (1981). The assay medium contained 50 mM KH_2PO_4/K_2HPO_4 (pH 7.0), 20 mM H_2O_2, 8 mM ascorbate, and 100 μl extract. Control rates were obtained in the absence of extract, ascorbate, or H_2O_2. Ascorbate peroxidase activity was calculated base on an extinction coefficient of 2.8 mM^{-1} cm^{-1} for ascorbate at 290 nm.

Total superoxide dismutase (EC 1.15.1.1) activity was determined by the inhibition of the formation of epinephrine at pH 10.4 and 30 ºC (Kroniger et al. 1995). The assay medium contained 62.5 mM Na_2CO_3 (pH 10.4), 0.125 mM EDTA, 20 mM KH_2PO_4/K_2HPO_4 (pH 7.8), 20 mM epinephrine and 100 μl extract. Control rates were obtained in the absence of extract. One unit of superoxide dismutase activity was defined as the amount of enzyme that inhibited epinephrine formation by 50%.

Standard enzymatic assays were performed in a total volume of 1 ml at 25 °C. A commercial Bio-Rad protein assay (Bio-Rad Laboratories GmbH, Munich, Germany) was used to measure soluble protein concentration by the Bradford method (Bradford 1976).

Statistical analysis

Data were subjected to two-way analysis of variance (ANOVA) to assess the effects and interactions of treatment and clones, using the STATISTICA data analysis software (Version 6, StatSoft, Tulsa, OK). Values presented are means ± SE. All statistically significant differences were tested at the $P \leq 0.05$ level.

Results

Leaf water potential and growth response

The Ψ_{pd} of well-watered plants of both clones was maintained at about −0.30 MPa throughout the experiment (Table 1), whereas Ψ_{pd} of water-stressed ST51 and CN5 plants declined to −2.43 ± 0.27 and −1.71 ± 0.06 MPa, respectively (cf. Costa e Silva et al. 2004). Midday leaf water potential did not vary significantly in well-watered plants, whereas it declined to −3.26 ± 0.26 and −2.46 ± 0.05 MPa in water-stressed ST51 and CN5, respectively by the end of the experiment. There were significant differences not only between watering regimes ($P < 0.001$), but also between clones ($P < 0.05$) (Table 2), with Clone CN5 maintaining a higher leaf water status than ST51 in the water-stress treatment.

Table 1. Predawn and midday leaf water potential (MPa) in *Eucalyptus globulus* Clones ST51 and CN5 subjected to water deficit. Measurements were made throughout the 7-week experiment in well-watered (WW) and water-stressed (WS) plants. Values are means ± SE ($n = 5$).

Leaf water potential	Week	ST51 WW	ST51 WS	CN5 WW	CN5 WS
Predawn	1	−0.35 ± 0.04	−0.35 ± 0.04	−0.28 ± 0.03	−0.28 ± 0.03
	3	−0.34 ± 0.02	−0.89 ± 0.19	−0.33 ± 0.01	−0.53 ± 0.08
	5	−0.29 ± 0.02	−1.28 ± 0.21	−0.28 ± 0.02	−0.83 ± 0.07
	7	−0.27 ± 0.01	−2.43 ± 0.27	−0.25 ± 0.02	−1.71 ± 0.06
Midday	1	−1.24 ± 0.06	−1.24 ± 0.06	−1.37 ± 0.04	−1.37 ± 0.04
	3	−0.64 ± 0.05	−1.66 ± 0.26	−0.63 ± 0.04	−1.11 ± 0.05
	5	−0.68 ± 0.04	−1.87 ± 0.22	−0.60 ± 0.04	−1.37 ± 0.05
	7	−0.68 ± 0.06	−3.26 ± 0.26	−0.78 ± 0.04	−2.46 ± 0.05

The water-stress treatment significantly reduced growth in terms of total biomass ($P < 0.001$), leaf area ($P < 0.001$), root length ($P < 0.05$) and leaf to root area ratio ($P < 0.01$) (Figure 1 and Table 2). In addition to differences in responses to water availability, the clones differed in leaf area, with Clone ST51 having higher values under well-watered conditions than Clone CN5 ($P < 0.01$). Relative to control values, the water-stress treatment caused a greater decrease in growth (particularly root length) in ST51 plants than in CN5 plants (Figure 1).

Table 2. Statistical significance of the effects of watering regime (W), clone (C) and their interaction as determined by 2-way analysis of variance of leaf variables: leaf water potential (predawn, Ψ_{pd} and midday, Ψ_{md}), leaf area, leaf area/root area, soluble and insoluble sugar concentration, protein, proline, osmotic potential at full turgor, violaxanthin + antheraxanthin + zeaxanthin (VAZ), lutein, total chlorophyll, glutathione reductase, ascorbate peroxidase and catalase in two *Eucalyptus globulus* clones. Symbols: *, ** and *** represent statistical significance at $P < 0.05$, 0.01 and 0.001, respectively; and ns = not significant at $P = 0.05$.

Leaf parameters	Watering regime	Clone	W x C
Ψ_{pd}	***	*	*
Ψ_{md}	***	*	**
Total biomass	***	ns	ns
Leaf area	***	**	**
Leaf area/root area	**	ns	ns
Soluble sugars	ns	ns	ns
Insoluble sugars	ns	ns	ns
Protein	ns	ns	ns
Proline	***	ns	**
Osmotic potential	***	ns	ns
VAZ	*	ns	ns
Lutein	*	ns	ns
β-carotene	***	**	*
Total chlorophyll	ns	ns	ns
Glutathione reductase	***	ns	*
Ascorbate peroxidase	**	ns	***
Catalase	ns	ns	ns

Osmotic potential

Well-watered plants of ST51 and CN5 had leaf Ψ_π of −1.14 ± 0.03 and −1.19 ±

0.05 MPa, respectively (Table 3). By the end of the experiment, Ψ_π had declined by 43% in water-stressed ST51 leaves and 75% in water-stressed CN5 leaves with significant differences between watering treatments ($P < 0.001$). In roots, drought stress caused a significant ($P < 0.001$) and larger reduction in Ψ_π than in leaves. By the end of the experiment, Ψ_π had declined by 92% in water-stressed ST51 roots and 87% in water-stressed CN5 roots with no significant differences between clones.

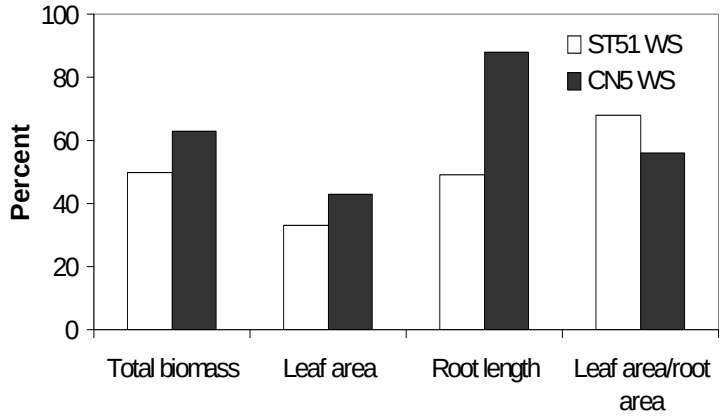

Figure 1. Morphological characteristics of plants of *Eucalyptus globulus* clones ST51 and CN5 subjected to water deficit: total biomass (g), leaf area (cm ^{2}), root length (m) and leaf-to-root area ratio (cm^2 leaf area/cm^2 root area). Measurements were made at the end of the 7-week experiment and are presented as a percentage of the value of well-watered plants. Abbreviation: WS = water-stressed

Table 3. Osmotic potential (MPa) in leaves and roots of *Eucalyptus globulus* clones ST51 and CN5 subjected to water deficit. Measurements were made at the end of the 7-week experiment in well-watered (WW) and water-stressed (WS) plants. Values are means ± SE (n = 5).

Treatment	Osmotic potential	
	Leaves	Roots
ST51 WW	–1.14 ± 0.03	–0.36 ± 0.04
ST51 WS	–1.63 ± 0.41	–0.69 ± 0.10
CN5 WW	–1.19 ± 0.05	–0.37 ± 0.04
CN5 WS	–2.08 ± 0.12	–0.69 ± 0.11

Carbohydrates and soluble protein concentration

There were no statistically significant differences between clones or watering regimes in leaf soluble sugars concentration (Table 2 and 4). By contrast, there was a significant ($P < 0.05$) increase in soluble sugars concentration in roots of water-stressed plants and the increase was higher in ST51 plants than in CN5 plants (55 versus 21%).

There were no significant differences in leaf insoluble sugars concentrations between watering regimes or between clones (Table 2 and 4). However, water stress led to a significant ($P < 0.05$) increase in insoluble sugars concentration in roots, with ST51 plants showing a slightly higher increase than CN5 plants (49 versus 39%) (Table 4 and 5).

In leaves of well-watered plants, soluble sugars accounted for 40% of the osmotic potential value in ST51 and 37% in CN5, and the corresponding values in water-stressed plants were 51 and 28. In roots of well-watered plants, soluble sugars accounted for 25% of the osmotic potential in ST51 and 24% in CN5, whereas in roots of water-stressed plants the corresponding values were 41 and 33%.

There were no significant differences in leaf or root soluble protein concentrations between treatments or clones (Table 2 and 5).

Proline concentration

The water-stress treatment caused a significant increase ($P < 0.001$) in leaf proline concentration in ST51 plants but not in CN5 (Figure 2A). In roots, water stress led to a significant increase ($P < 0.001$) in proline concentration in both clones (Figure 2B), and the increase was greater in CN5 roots than in ST51 roots (253 versus 194%).

In leaves of well-watered plants of both clones, the contribution of proline to the osmotic potential was 0.97% (on average) compared with 1.16% (on average) in leaves of water-stressed plants. In roots of both clones, the contribution of proline to osmotic potential was 0.12 and 0.43% (on average) in well-watered and water-stressed plants, respectively.

Table 4. Soluble and insoluble sugars concentrations in leaves and roots of *Eucalyptus globulus* clones ST51 and CN5 subjected to water deficits. Measurements were made at the end of the 7-week experiment in well-watered (WW) and water-stressed (WS) plants. Values are means ± SE (n = 5).

Treatment	Leaf sugars (µmol g^{-1} dry mass)		Root Sugars (µmol g^{-1} dry mass)	
	Soluble	Insoluble	Soluble	Insoluble
ST51 WW	460 ± 50	200 ± 30	310 ± 60	200 ± 30
ST51 WS	520 ± 90	190 ± 30	480 ± 40	290 ± 20
CN5 WW	400 ± 70	280 ± 70	270 ± 40	180 ± 30
CN5 WS	450 ± 60	140 ± 40	320 ± 40	250 ± 40

Table 5. Statistical significance of the effects of watering regime (W), clone (C) and their interaction as determined by 2-way analysis of variance of root variables: root length, soluble and insoluble sugar concentration, protein, proline,, osmotic potential and activities of glutathione reductase, ascorbate peroxidase, catalase and superoxide dismutase in two *Eucalyptus globulus* clones. Symbols: *, ** and *** represent statistical significance at P < 0.05, 0.01 and 0.001, respectively; and ns = not significant at P = 0.05.

Root parameters	Watering regime	Clone	W x C
Total root lenght	*	ns	ns
Soluble sugars	*	ns (0.07)	ns
Insoluble sugars	*	ns	ns
Protein	ns	ns	ns
Proline	***	ns	ns
Osmotic potential	***	ns	ns
Glutathione reductase	***	***	ns
Ascorbate peroxidase	***	ns	ns
Catalase	ns	ns	ns
Superoxide dismutase	*	*	*

Leaf pigments

In both clones, violaxanthin + antheraxanthin + zeaxanthin (VAZ) and lutein concentrations were significantly higher (P < 0.05) in water-stressed plants than in well-watered plants (Figure 3). Water-stressed caused opposite effects on the β-carotene concentrations of the clones (Figure 3C). The significant clone x treatment interaction (P < 0.05) resulted from a 226% increase in β-carotene concentration in ST51 plants and a 56% decrease in CN5 plants. There were no significant treatment or clonal differences in total chlorophyll concentration (Figure 3D).

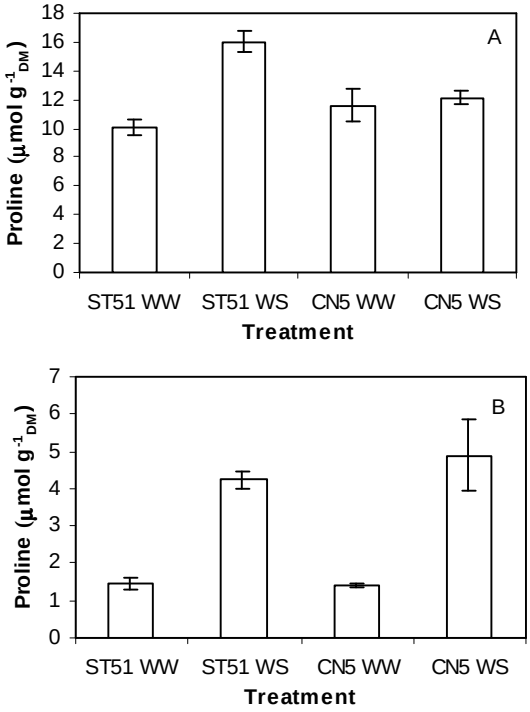

Figure 2. Proline concentrations in leaves (A) and roots (B) of *Eucalyptus globulus* clones ST51 and CN5 subjected to water deficit. Measurements were made at the end of the 7-week experiment in well-watered (WW) and water-stressed (WS) plants. Values are means ± SE (n = 5). Abbreviation: DM = dry mass.

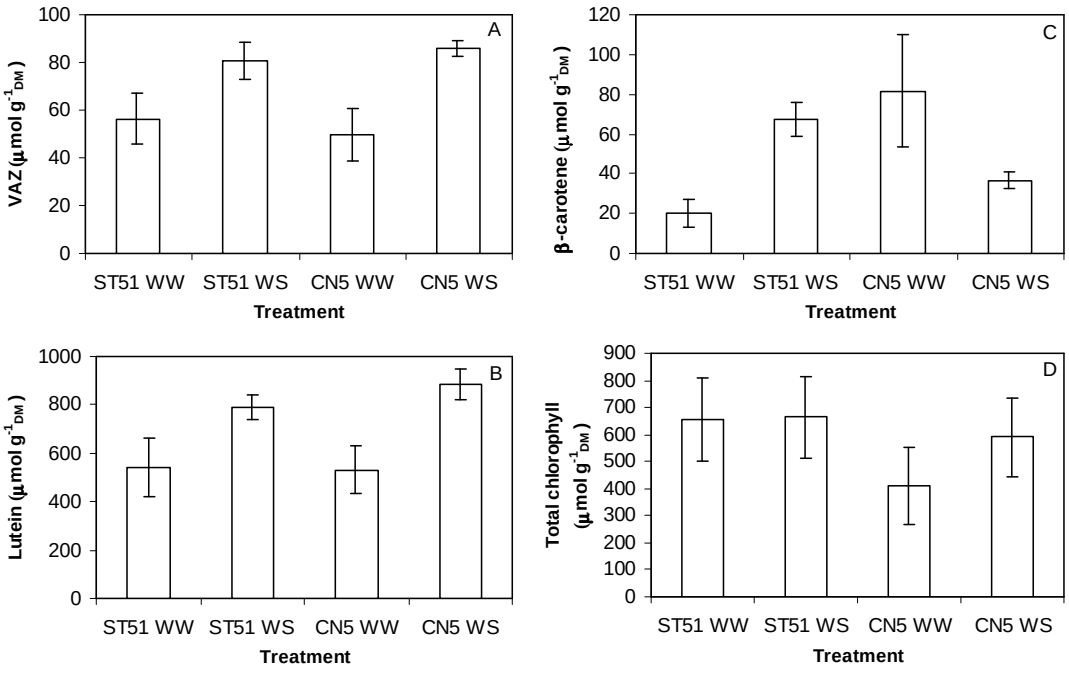

Figure 3. Pigment concentrations in leaves of *Eucalyptus globulus* clones ST51 and CN5 subjected to water deficit. Measurements were made at the end of the 7-week experiment in well-watered (WW) and water-stressed (WS) plants. (A) VAZ = violaxanthin + antheraxanthin + zeaxanthin, (B) lutein, (C) β-carotene and (D) total chlorophyll. Values are means ± SE (n = 5). Abbreviation: DM = dry mass.

Antioxidant enzymes

The effects of water stress on antioxidant enzymatic activities in leaves of the clones were variable (Figure 4). Leaf GR activity in both clones was significantly ($P < 0.001$) decreased by water stress, but particularly in Clone CN5, resulting in a significant clone x treatment interaction. The water stress treatment also decreased APX activity, but only in CN5 plants (Figure 4A and 4B), again leading to a significant clone x treatment interaction (Table 2). There were no significant differences in leaf CAT activity between treatments or clones (Figure 4C).

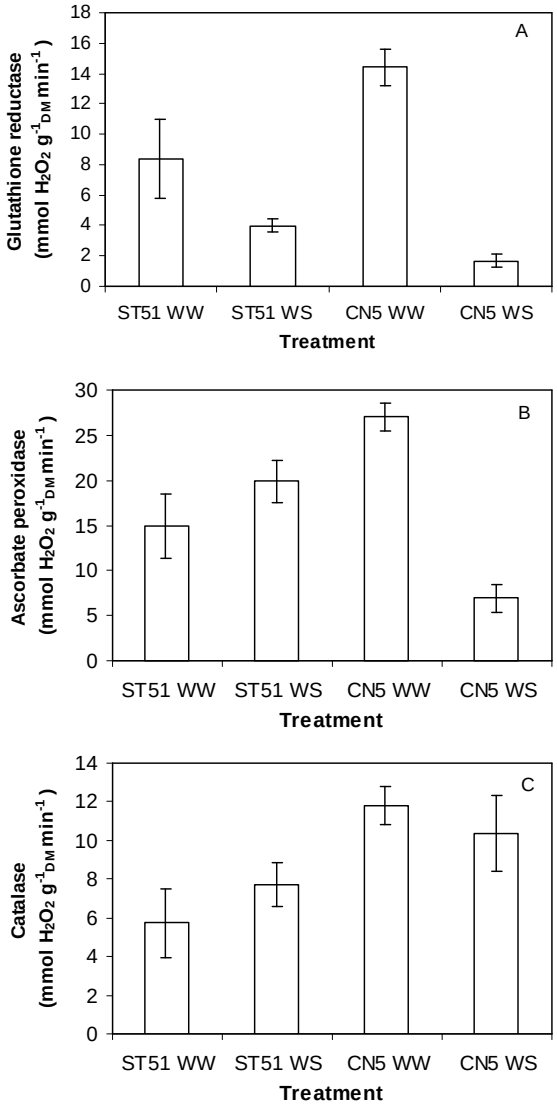

Figure 4. Glutathione reductase (A), ascorbate peroxidase (B) and catalase (C) activities in leaves of *Eucalyptus globulus* clones ST51 and CN5 subjected to water deficit. Measurements were made at the end of the 7-week experiment in well-watered (WW) and water-stressed (WS) plants. Values are means ± SE ($n = 5$). Abbreviation: DM = dry mass.

In roots, the effects of water stress on antioxidant enzymatic activities were more marked than in leaves (Figure 5). In both clones, GR activity was observed only in water-stressed plants and it was significantly ($P < 0.001$) higher in ST51 plants than in CN5 plants (Figure 5A). The activity of APX in roots increased significantly ($P < 0.001$) in both clones in response to water stress (332% and 613%, respectively), but there were no statistically significant differences between clones (Figure 5B). There was no significant effect of water stress ($P = 0.09$) or clone on root CAT activity (Figure 5C). The activity of SOD in roots remained stable in CN5 plants and declined in ST51 plants under water stress. There was a significant interaction ($P < 0.05$) between treatment and clone.

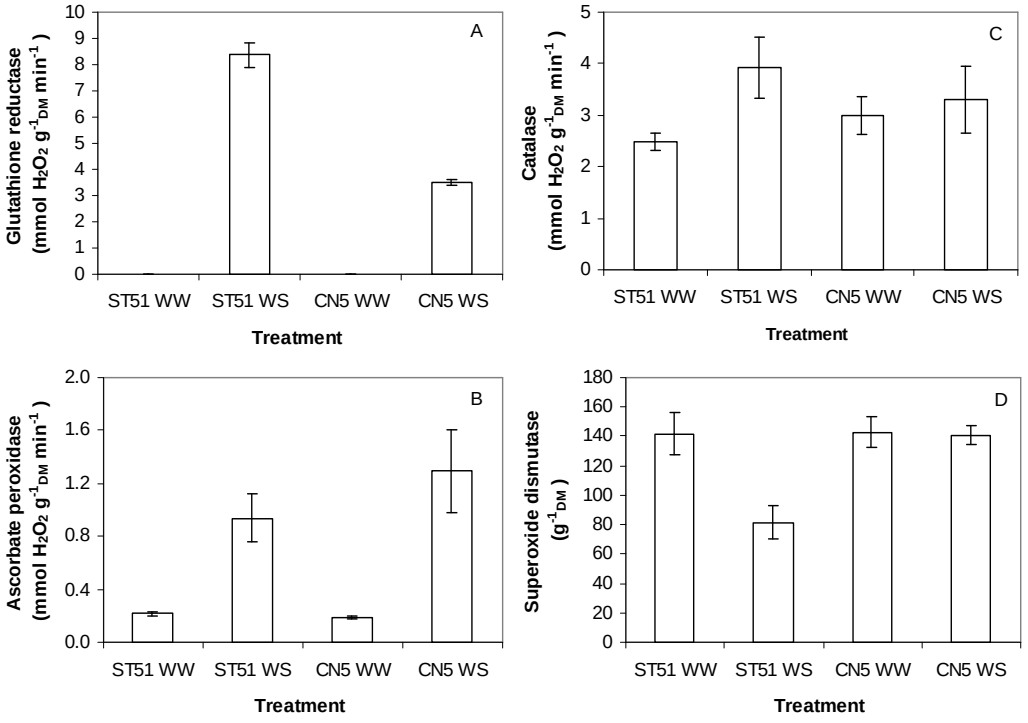

Figure 5. Glutathione reductase (A), ascorbate peroxidase (B), catalase (C) and superoxide dismutase (D) activities in roots of *Eucalyptus globulus* clones ST51 and CN5 subjected to water deficit. Measurements were made at the end of the 7-week experiment in well-watered (WW) and water-stressed (WS) plants. Values are means ± SE ($n = 5$). Abbreviation: DM = dry mass.

Discussion

We examined several biochemical and physiological responses to water deficits in leaves and roots of *E. globulus* clones, reported to differ in drought sensitivity (Costa e Silva et al. 2004). We confirmed that Clone CN5 maintained higher leaf water status (higher Ψ_{pd} and Ψ_{md}) and sustained a higher growth rate than Clone ST51 as a result of drought avoidance mechanisms (Costa e Silva et al. 2004). A slowly imposed water deficit resulted in a reduction in total biomass in both *E. globulus* clones, and the general decline in growth was accompanied by a reduction in the leaf area/root area ratio. Although the drought-induced reductions in total biomass and leaf area did not differ between clones, total root length of Clone CN5 was significantly higher than that of Clone ST51 in the water stress treatment. A reduction in shoot/root ratio, attributable mainly to a reduction in shoot growth, has been associated with tolerance to limited water availability (Pereira and Chaves 1993).

Both clones responded to water stress by altering osmotic potential, osmoprotectants (sugars and proline), antioxidant activity and pigment composition. In general, osmolyte accumulation in plant cells results in a decrease in cell osmotic potential and thus improves water absorption and cell turgor pressure, which might help sustain physiological processes, such as stomatal opening, photosynthesis and expansion growth under drought conditions (Blum 1996). Furthermore, the accumulation of sugars and proline, mostly in the cytoplasm, can protect cell membranes and proteins and enhance dehydration tolerance (Rathinasabapathi 2000).

In response to the water stress treatment, there was a small accumulation of soluble sugars in leaves of both clones and a significantly higher accumulation in roots, especially in ST51 plants. The increased accumulation of soluble sugars may reflect osmolyte accumulation as a consequence of water deficits causing a decrease in growth and hence reduced consumption of organic solutes, rather than a physiological mechanism involved in an adaptive plant response (Munns 1988). The difference in growth rates between clones supports this hypothesis: roots of ST51 had lower growth rates and higher soluble sugar concentrations than roots of CN5, whereas leaves of both clones ceased growth and showed similar increases in soluble sugar concentrations in response to water stress.

Water stress led to a decline in Ψ_π in both clones. The contribution of proline to the change in osmotic potential was only about 1%, however, indicating that the drought-induced increase in proline concentration did not significantly contribute to osmotic adjustment in the water-stressed plants. As indicated in other studies, the role of proline in drought-stressed plants may be associated with preservation of enzyme structure and activity (Delauney and Verma 1993, Hare et al. 1999), and with the protection of membranes from damage by ROS produced in response to drought (Hamilton and Heckathorn 2001). However, the greater increase in leaf proline concentration in the drought-sensitive ST51 clone than in the less sensitive CN5 clone in response to the water-stress treatment cannot be explained on the basis of either a protective or osmotic role. In both treatments, soluble sugars in leaves and roots contributed less to the Ψ_π in Clone CN5 than in the drought-sensitive Clone ST51 (30 versus 50%), indicating that there is no link between the better performance of CN5 plants and an osmotic role of soluble sugars.

The absence of significant differences in protein concentrations both in leaves and in roots indicates that short-term water stress had little or no effect on the synthesis and hydrolysis of soluble proteins, contrary to previous reports of inhibition of protein synthesis in response to drought (Chaves 1991). On the other hand, the increase in insoluble sugar concentrations in roots of both clones under severe water stress suggests a storage function of these organs in drying soil. In summary, the absence of clonal differences in leaf and root Ψ_π and osmolytes in the water-stress treatment indicates that differences in drought sensitivity between the clones are not associated with osmotic adjustment in leaves and roots.

Under well-watered growth conditions, the production and destruction of ROS is well regulated in plant cells. However, under environmental stress, the balance between the production of ROS and the quenching activity of the antioxidant system may be upset (Polle 2001). The capacity of the antioxidative defense system determines whether a cell under stress continues to function or suffers oxidative stress.

Activity of GR in leaves of both clones decreased in response to water stress, particularly in CN5 plants (Figure 4A). Thus, under our experimental conditions, we found no evidence that GR plays a protective role in leaves of water-stressed plants. In contrast, the induction of a high GR activity in roots of water-stressed ST51 and

CN5 plants (Figure 5A) suggests that GR has a protection function against drought in roots of both clones, perhaps by preventing oxidative stress or slowing the progression of root senescence, or both (Munné-Bosch and Alegre 2004). The large increase in root GR activity in response to drought closely resembled the results obtained by Porcel et al. (2003) for soybean (*Glycine max* (L) Merr.) plants.

The significant treatment x clone interaction on leaf APX activity suggests that APX has no protective effect against oxidative stress in leaves of the ST51 and CN5 clones. In contrast, water stress resulted in an increase in root APX activity in both clones, particularly in CN5 plants. Because both GR and APX are key enzymes of the ascorbate-glutathione cycle, we speculate that this cycle provides a mechanism for drought adaptation in *Eucalyptus* roots. Increases in GR and APX activity have also been observed in roots of salt-stressed barley (*Hordeum vulgare* L.) (Liang et al. 2003), conforming to a commonly observed response to water and salt stress (Munns 2002). Thus, although leaves are highly prone to ROS generation, oxidative stress protection also appears to be critical in plant roots during soil drying.

The lack of statistically significant effects of treatments or clones on CAT activity suggests that this enzyme does not provide protection against drought-induced ROS in leaves or roots of *E. globulus* plants. The only alteration in SOD activity occurred in ST51 roots, where drought led to a decrease in activity. Similarly, decreasing SOD activity has been reported in sunflower in response to salt stress, in cucumber in response to chilling stress and in wheat seedlings in response to water deficit (Lee and Lee 2000, Sgherri et al. 2000, Santos et al. 2001). According to Lee and Lee (2000), the metabolism of ROS is dependent on several functionally interrelated antioxidant enzymes. Chilling stress induced a significant increase in SOD activity in leaves of cucumber only during the first hours of stress imposition and thereafter, SOD activity decreased to control values. A similar transient increase in SOD activity may have occurred in roots of water-stressed CN5 plants. Alternatively, it is possible that the higher basal root SOD activity in CN5 plants than in ST51 plants was sufficient to cope with the drought-induced increase in superoxide production and thereby contribute to the higher root growth rate of CN5 plants.

Overall, our results reflect the importance of GR, APX and SOD as a system for scavenging ROS in *E. globulus* roots. These enzymes appear to be more important than CAT in ROS detoxification in *E. globulus*.

Leaf concentrations of VAZ and lutein increased in both clones in response to severe water stress, suggesting that carotenoids may be involved in stress protection of leaves, even in plants subjected to moderate irradiance, as was the case in our study. Based on the finding that water stresss caused a 226% increase in β-carotene in ST51 plants and a 56% decrease in CN5 plants, we hypothesize that leaf β-carotene was more rapidly metabolized in CN5 plants than in ST51 plants, indicating that it served as a protective mechanism in deactivation of ROS (cf. Peñuelas and Munné-Bosch 2005). The concentration of carotenoids did not differ significantly between the clones under severe water stress, suggesting that clonal differences in drought sensitivity are related more to drought avoidance mechanisms than to tolerance of water deficits. In previous work, a comparison of the leaf-level mechanisms for coping with summer stress in central Portugal in four evergreen tree species indicated that *E. globulus* was able to utilize more light in PSII photochemistry and therefore down-regulation of photosynthesis was less severe, than in the evergreen oaks and olive trees (Faria et al. 1998).

In studies of plant responses to drought, it has often been found that one specific mechanism does not confer resistance on its own, but that the interplay of several mechanisms simultaneously is essential (Chaves et al. 2003). We found that both *E. globulus* clones had the ability to respond to water deficits at the cellular level by altering both osmotic components and the activity of the antioxidant protection system. Differences in metabolic responses between clones may reflect different degrees of stress experienced during the drought treatment, because the drought avoiding CN5 plants never reached the same degree of dehydration as the ST51 plants. An important finding of this study was the metabolic response of roots to drought. Although GR activity was not detected in roots of well-watered plants, it increased dramatically in response to water stress, suggesting that GR plays an important role in root responses to drought in *E. globulus*, possibly preventing fine-root death caused by dehydration.

Acknowledgements

This research was carried out with financial support from the Commission of the European Communities (contract No. QLK5-CT-2000-01377), Directorate-General Research-Quality of Life and Management of Living Resources Programme. It does not necessarily reflect the Commission`s views and in no way anticipates its future policy in this area. AL Shvaleva was supported by Fundação para a Ciência e a Tecnologia (FCT, Lisboa) (grant No. SFRH/BPD/5667/2001). The authors thank Viveiros Aliança for providing the plant material.

References

Aebi, H. 1983. Catalase. *In*: Methods of Enzymatic Analysis. Ed. H. Bergmeyer. Verlag Chemie, Weinheim 3:273-277.

Bates, L.S., R.P. Waldren and D. Teare. 1973. Rapid determination of free proline for water tress studies. Plant and Soil 39:205-207.

Blum, A. 1996. Crop responses to drought and the interpretation of adaptation. Plant Growth Reg. 20:135-148.

Bradford, M. N. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Chaves, M.M. 1991. Effects of water deficits on carbon assimilation. J. Exp. Bot. 42:1-16.

Chaves, M.M., J.S. Pereira, J.P. Maroco, M.L. Rodrigues, C.P.P. Ricardo, M.L. Osorio, I. Carvalho, T. Faria and C. Pinheiro. 2002. How plants cope with water stress in the field. Photosynthesis and growth. Ann. Botany 89:907-916.

Chaves, M.M., J. P. Maroco and J. S. Pereira. 2003. Understanding plant responses to drought – from genes to the whole plant. Functional Plant Biol. 30:239-264.

Costa e Silva, F., A.L. Shvaleva, J.P. Maroco, M.H. Almeida, M.M. Chaves and J.S. Pereira. 2004. Responses to water deficits in two clones of *Eucalyptus globulus* Labill of contrasting drought tolerance. Tree Physiol. 24:1165-1172.

Delauney, A.J. and D.P.S. Verma. 1993. Proline biosynthesis and osmoregulation in plants. Plant J. 4:215-223.

Faria, T., D. Wilkins, B. Besford, M. Vaz, J.S. Pereira and M.M. Chaves. 1996. Growth at elevated CO_2 leads to down-regulation of photosynthesis and altered response to high temperature in *Quercus suber* L. seedlings. J. Exp. Bot. 47:1755-1761.

Faria, T., D. Silveri, E. Breia, A. Abadia, J. Abadia, J.S. Pereira and M.M. Chaves. 1998. Differences in the response of carbon assimilation to summer stress (water deficits, high light and temperature) in four Mediterranean tree species. Physiol. Plant. 102:419-428.

Finkel, T. 1998. Oxygen radicals and signalling. Curr. Opin. Biol. 10: 248-253.

Foyer, C. and B. Halliwell. 1976. The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta 133:21-25.

Garcia-Plazaola, J. I., T. Faria, J. Abadia, A. Abadia, M.M. Chaves and J.S. Pereira. 1997. Seasonal changes in xanthophyll composition and photosynthesis of cork oak (*Quercus suber* L.) leaves under Mediterranean climate. J. Exp. Bot. 48:1667-1674.

Gogorsena, Y., I. Iturbe-Ormaetxe, P.R. Escuredo and M. Becana. 1995. Antioxidant defenses against activated oxygen in pea nodules subjected to water stress. Plant Physiol. 108:753-759.

Hamilton, E.W. and S.A. Heckathorn. 2001. Mitochondrial adaptations to NaCl. Complex I is protected by anti-oxidants and small heat shock proteins, whereas complex II is protected by proline and betaine. Plant Physiol. 26:1266-1274.

Hare, P., W. Cress and J. van Staden. 1999. Proline synthesis and degradation: a model system for elucidating stress-related signal transduction. J. Exp. Bot. 50:413-434.

Kroniger, W., H. Rennenberg, H. Tadros and A. Polle. 1995. Purification and properties of manganese superoxide dismutase from spruce (*Picea abies*, L. Karst). Plant Cell Physiol. 36:191-196.

Lee, D.H. and C.B. Lee. 2000. Chilling stress-induced changes of antioxidante enzymes in the leaves of cucumber: in gel enzyme activity assays. Plant Sci. 159:78-85.

Liang, Y., Q. Chen, Q. Liu, W. Zhang and R. Ding. 2003. Exogenous silicon (Si) increases antioxidant enzyme activity and reduced lipid peroxidation in roots of salt-stressed barley (*Hordeum vulgare* L.). J. Plant Physiol. 159:112-121.

Maroco, J.P., J.S. Pereira and M.M. Chaves. 1997. Stomatal responses of leaf-to-air vapour pressure deficit in sahelian species. Aust. J. Plant Physiol. 24:381-387.

Medrano, H., J.M. Escalona, J. Boto, J. Gulias and J. Flexas. 2002. Regulation of photosynthesis of C3 plants in response to progressive drought: Stomatal conductance as a reference parameter. Ann. Bot. (Lond.) 89:895-905.

Munné-Bosch, S. and L. Alegre. 2004. Die and let live: leaf senescence contributes to plant survival under drought stress. Funct. Plant Biol. 31:203-216.

Munns, R. 1988. Why measure osmotic adjustment? Aust. J. Plant Physiol. 83:1052-1059.

Munns, R. 2002. Comparative physiology of salt and water stress. Plant Cell Environ. 25:239-250.

Nakagara, S. and S. Sagisaka. 1984. Increases in enzyme activities related to ascorbate metabolism during cold acclimation in poplar twigs. Plant Cell Physiol. 25:822-824.

Nakano, Y. and K. Asada. 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22:867-880.

Osawa, T. and M. Namiki. 1985. Natural antioxidants isolated from Eucalyptus leaf waxes. J. Agric. Food Chem. 33:777-780.

Osawa, T., N. Ramarathnam, S. Kawakishi and M. Namiki. 1992. Antioxidative defense systems generated by phenolic plant constituents. *In* Phenolic Compounds in Food and their Effects on Health II. Antioxidants and Cancer Prevention. Eds. M.T. Huang, C.T. Ho and C.Y. Lee. ACS Symp. Ser, 507:122-134.

Peñuelas, J. and S. Munné-Bosch. 2005. Isoprenoids: an evolutionary pool for photoprotection. Trends Plant Sci. 10:166-169.

Pereira, J.S. and M.M. Chaves. 1993. Plant water deficits in Mediterranean ecosystems. *In* Water Deficits: Plant responses from cell to community. Eds J.A.C. Smith and H. Driffiths. BIOS Scientific Publishers, Oxford, pp 235-251.

Polle, A. 2001. Dessecting the superoxide dismutase-ascorbate peroxidase-glutathione pathway in chloroplasts by metabolic modeling. Computer simulations as a step towards flux analysis. Plant Physiol. 126:445-462.

Polle, A. and H. Rennenberg. 1992. Field studies on Norway spruce trees at high altitudes. II. Defense systems against oxidative stress in needles. New Phytol. 121:635-640.

Porcel, R., J.M. Barea and J.M. Ruiz-Lozano. 2003. Antioxidant activities in mycorrhizal soybean plants under drought stress and their relationship to the process of nodule senescence. New Phytol. 157:135-143.

Quick, W.P, M.M. Chaves, R. Wendler, M. David, M.L. Rodrigues, J.A. Passaharinho, J.S. Pereira, M.D. Adcock, R.C. Leegood and M. Stitt. 1992. The effect of water stress on photosynthetic carbon metabolism in four species grown under field conditions. Plant Cell Environ. 15:25-35.

Rathinasabapathi, B. 2000. Metabolic engineering for stress tolerance: installing osmoprotectant synthesis pathways. Ann. Bot. (Lond.) 86:709-716.

Robyt, J. and B.J. White. 1987. Biochemical Techniques: Theory and Practice. Brooks/Cole Pub., Monterey, CA, USA, 407 p.

Rubio, M.C., E.M. Gonzalez, F.R. Minchin, K.J. Webb, C. Arrese-Igor, J. Ramos and M. Becana. 2002. Effects of water stress on antioxidant enzymes of leaves and nodules of transgenic alfalfa overexpressing superoxide dismutases. Physiol. Plant. 115:531-540.

Santos, V.C.L., A. Campos, H. Azevedo and G. Caldeira. 2000. In situ and in vivo senescence induced by KCL stress: nutritional imbalance, lipid peroxidation and antioxidant metabolism. J. Exp. Bot. 52:351-360.

Sgherri, C.L.M., M. Maffei and F. Navari-Izzo. 2000. Antioxidative enzymes in wheat subjected to increasing water deficit and rewatering. J. Plant Physiol. 157:273-279.

Smirnoff, N. 1998. Plant resistance to environmental stress. Current Opinion in Biotechnol. 9:214-219.

Volaire, F., H. Thomas and F. Lelievre. 1998. Survival and recovery of perennial forage grasses under prolonged Mediterranean drought. I. Growth, death, water relations and solute content in herbage and stubble. New Phytol. 140:439-449.

Wingsle, G. and J.E. Hallgren. 1993. Influence of SO_2 and NO_2 exposure on glutathione, superoxide dismutase and glutathione reductase activities in Scots pine needles. J. Exp. Bot. 259:463-470.

Wright, S. W., S. W. Jeffrey, R.F.C. Mantoura, C. A. Lewellyn, T. Bjornlund, D. Repeta and N. Welchmeyer. 1991. Improved HPLC method for analysis of chlorophyll and carotenoids in marine phytoplankton. Mar. Ecol. Prog. Ser. 77:193-194.

CAPÍTULO 4

Responses to chilling of two *Eucalyptus globulus* clones with contrasting drought resistance

4. Responses to chilling of two *Eucalyptus globulus* clones with contrasting drought resistance

Abstract

The effect of chilling on growth and plant hydraulic properties in a drought-resistant clone (CN5) and a drought-sensitive clone (ST51) of *Eucalyptus globulus* Labill. was evaluated. Chilling (10/5 ºC, day/night) led to a general decrease in growth of both clones and significant reductions in root hydraulic conductivity, rate of photosynthesis and stomatal conductance in comparison to plants grown at control temperature (24/16 ºC). The drought-resistant CN5 clone maintained higher root growth and lower leaf-to-root-area ratio than the drought-sensitive ST51 clone, in both temperature treatments. Conversely, ST51 exhibited greater carbon allocation to the foliage and higher hydraulic conductance than clone CN5 at both temperatures. Plants of both clones, when acclimated to chilling, maintained a higher hydraulic conductivity than control plants exposed to chilling temperatures without acclimation. Under chilling, the main differences between clones were a higher water status and anthocyanin concentration in CN5 plants, and a stronger inhibition of root growth in ST51 plants. Except for roots, the hypothesis of a lower depression of growth rate in the drought-resistant clone under chilling was not verified. However, higher root growth under low temperatures, as observed in CN5, can be an advantageous trait in Mediterranean-type environments, protecting trees against summer water-stress.

Additional keywords: acclimation, allocation, hydraulic properties, root growth.

Introduction

Due to its fast growth and fibre properties, in particular high pulp quality, *Eucalyptus globulus* Labill. is one of the most commonly planted hardwood trees in the world. Although widely distributed, its expansion is mostly restricted by its drought and cold sensitivity. To overcome these limitations, development of breeding programs require detailed physiological information of the stress-response of the clones selected for nursery production. In addition, such information is necessary for further selection and to support decisions to allocate clones to different climatic regions.

In Mediterranean-type climates, water is available during the cool winter, whereas hot and dry conditions prevail in the summer. Therefore, a successful evergreen tree must be capable of acquiring carbon under lower rather than higher temperatures. Plants exposed to chilling tend to decrease their growth rate (Wan *et al*. 1999; Gavito *et al*. 2001; Peng and Dang 2003), photosynthetic efficiency (Close *et al*. 2000; Allen and Ort 2001) and their water uptake capacity (Markhart *et al*. 1979; Fennell and Markhart 1998; Wan *et al*. 2001). However, it is expected that contrasting genotypes respond differently to low temperatures in the process of cold acclimation that takes place on the time scale of days or weeks as a result of a combination of physiological and metabolic changes. Moreover, several studies confirmed that plant responses to low temperatures show many similarities with responses to water deficits, suggesting that cold resistance and drought resistance mechanisms often share the same pathways (Sung *et al*. 2003; Atkin *et al*. 2005; Blödner *et al*. 2005; Suzuki and Mittler 2006).

For these reasons we hypothesised that, under a Mediterranean-type climate, *E. globulus* genotypes more resistant to dry environments might exhibit lower depression of growth rates under chilling than drought-sensitive plants. If this is true, it will allow a clone less susceptible to drought to prolong carbon assimilation and active growth throughout the water-stress-free period, thus allowing those plants to enter spring with a larger leaf surface area or more reserves than more drought sensitive plants. Likewise, root growth might also benefit from more carbon available (Reich *et al*. 1998). In stands with a mixture of clones, plants with larger surface leaf areas and root growth at the start of spring may become dominant because they acquire a larger share of resources. In

previous work (Costa e Silva *et al.* 2004; Shvaleva *et al*. 2006), the two clones under study were shown to differ in their sensitivity to water deficits (CN5 was drought resistant and ST51 was drought sensitive). Under drought conditions, the better performance of clone CN5 was associated with faster root growth, maintenance of hydraulic conductance and lower values in the leaf-to-root-area ratio compared with clone ST51. The aims of the present work were to: (1) evaluate the effect of chilling in growth and plant hydraulic properties of two clones of *E. globulus* with contrasting responses to drought, and (2) test whether the drought-resistant clone is less affected by chilling than the drought-sensitive clone.

Materials and methods

Plant material and treatments

Ramets produced by rooted cuttings of two *Eucalyptus globulus* Labill. clones (drought-resistant CN5 and drought-sensitive ST51) were grown in plastic containers containing peat (60%) and styrofoam (40%), and were transplanted at 4 months to pots (5.3 L) filled with a fine sandy soil. Two months after transplanting, 32 cuttings per clone were transferred from the nursery to a growth chamber with controlled conditions (24/16 ºC, day/night). Another 32 cuttings per clone were placed in a growth chamber subjected to an acclimation period of 10 days with a gradual temperature decrease (1ºC per day) from 24/16 ºC to 10/5 ºC (day/night). Both growth chambers had similar lighting systems (c.a. 220 µmol m^{-2} s^{-1} at the canopy level), a photoperiod of 12/12 h (day/night) and relative humidity of ~60%. To avoid effects caused by microenvironmental differences (light and temperature gradients), the plants were sorted by treatment and moved to the neighbouring position every watering day. The experiment lasted for 42 days (counted after the 10 days of acclimation period) from 18 January to 1 March 2005. All plants were watered to the point of runoff on day 1 and then watered twice per week (Mondays and Fridays) according to evapotranspiration values.

Gas exchange, hydraulics and plant biomass

Transpiration rates were determined in five plants by measuring differences in pot weight between successive watering operations. In addition, three pots without plants were used to monitor evaporative water loss from the soil. Gas exchange was measured with an LI-6400 portable photosynthesis system (Li-Cor, Lincoln, NE) in full-expanded leaves at midday (solar time) and under the light and temperature conditions of the controlled environment chambers. Leaf xylem water potential (predawn Ψ_{pd} and midday Ψ_{md}) was measured with a Scholander-type pressure chamber (PMS Instruments, Corvallis, OR). These measurements were carried out on a sample of five plants per treatment three times during the experiment (days 1, 8 and 42).

Hydraulic properties analysed through the relation between E and $\Delta\Psi$ gave a relative but integrated measure of the hydraulic conductance of the soil-plant system. Thus, hydraulic conductance was calculated on a plant basis (K_{sp}) and on a leaf area basis (leaf specific conductance (LSC)), assuming Ψ_{pd} is an estimate of the soil water potential in the rooting zone (Jones 1992):

$$K_{sp} = \frac{E}{\Psi_{pd} - \Psi_{md}} \quad \text{and} \quad \text{LSC} = \frac{E_l}{\Psi_{pd} - \Psi_{md}}$$

where E is the transpiration rate on a plant basis (kg s^{-1}) and E_l is the transpiration rate on a leaf area basis (kg m^{-2} s^{-1}) through the system measured between predawn and midday.

Plant biomass was evaluated three times during the experiment (days 1, 8 and 42) through the destructive sampling of five plants per treatment. These plants were used to determine morphological parameters (height, diameter, number of branches, biomass partition, leaf area and root length). Specific leaf area (SLA) was calculated as the ratio between leaf area and leaf dry mass (DM), and leaf area ratio (LAR) was calculated as the ratio between total leaf area and total plant DM. All dry mass values were obtained after 48 h at 80 ºC. Leaves and roots were scanned and leaf area and root parameters (length, diameter, area, forks and tips) were calculated using the WinRhizo software (Regent Instruments Inc., Quebec, Canada). Specific root length (m g^{-1}) was calculated as

the ratio between root length and root DM, and specific root surface area ($cm^2\ g^{-1}$) was calculated as the ratio between root surface area and root DM. We estimated specific root volume ($cm^3\ g^{-1}$) as the ratio between root volume and root DM.

Height, diameter and the number of new leaves were determined every week on five plants per treatment. These same plants were used for non-destructive measurements every 3 days of leaf expansion on selected leaf blades (from the second leaf pair), from day 1 to 42 (one stem leaf per plant). When the selected leaves reached full expansion (day 21) the measurements began again with the youngest expanding leaves.

Anthocyanins

Total anthocyanins were analysed by bisulfite bleaching method (Jordão *et al.* 1998) on day 42 in the same five plants used for biomass determination. Samples were collected from the third leaf pair, frozen immediately in liquid nitrogen and kept at –80 ^{o}C until further assay. The anthocyanin concentration was expressed in mg m^{-2}.

Root water flow

Root water flow was evaluated three times during the experiment (days 1, 8 and 42) in the root systems harvested for biomass estimates. Steady-state water flow rates in whole root systems (Q_v) were measured using the hydrostatic pressure method (Nardini *et al.* 1998; Wan and Zwiazek 1999), with some modifications. A rigid plastic cylinder was inserted in a pressure chamber and filled with distilled water. The plant stem was cut 20 mm above the root collar and the root system immediately immersed in distilled water in the pressure chamber.

The pressure in the chamber was increased continually at a rate of ~0.07 MPa min^{-1} up to 0.7 MPa min^{-1} and then was maintained constant during 30 min. Flow was measured every 5 min by collecting the exudate for 1 min, using a pre-weighed capillary vial containing cotton wool, placed over the cut stem protruding through the stopper in the pressure chamber. At constant pressure the flow was approximately stable (s.d. of the measured flows ranged between 1 and 10%) so that measurements were quasi steady-

state. The pressure was then decreased in steps of 0.15 MPa each with a rate of 0.07 MPa min^{-1} and the same procedure was used to measure the flow at each pressure level tested (0.7, 0.55, 0.4 and 0.25 MPa). Plants from treatment under 10/5 ºC (day/night) were measured in a temperature-controlled pressure chamber at a temperature of 10 ± 1 ºC and control plants grown under 24/16 ºC (day/night) were measured at 24 ± 1 ºC.

Linear root flow rates were obtained and Q_v values were expressed on a per plant basis (kg s^{-1}). The volume flow density (J_v) was determined as a steady-state flow rate per unit of root surface area (kg m^{-2} s^{-1}). Root hydraulic conductance (K) was calculated as the slope of pressure *v.* flow rate where the relationship was linear ($R^2 \geq 0.98$), and is expressed in kg s^{-1} MPa^{-1}. In order to normalise data from plants having different root system dimensions K was referred to the unit root surface area, thus obtaining root hydraulic conductivity (L_p) expressed in kg m^{-2} s^{-1} MPa^{-1}.

Acclimation versus non-acclimation: hydraulic responses

At the end of the experiment, the response and recovery of non-acclimated plants after a change in growth temperature were examined. Plants grown under 24/16 ºC (day/night) were transferred to 10/5 ºC (day/night) for 24 h and then transferred again for control conditions for 5 days. Root water flow was measured at 10 ± 1 ºC, before transference, after 24 h under chilling and after 5 days of recovery in control conditions.

Data analysis

Data were subjected to two-way ANOVA to test for the effects and interactions of temperature treatment and clone, using the STATISTICA (Version 6, StatSoft, Tulsa, OK) data analysis software system. All variables were tested for normality and homogeneity of variances. Differences were considered statistically significant at $P \leq 0.05$.

Results

Transpiration

E values per plant in controls increased in both clones throughout the experiment (Figure 1A) due to leaf area development. However, on a per unit leaf area basis, transpiration rates slightly decreased with time in both clones (Figure 1B). Under chilling, the plants maintained lower transpiration rates, with statistically significant ($P < 0.01$) higher *E* per plant in clone ST51 than CN5 due to a larger leaf area (Figure 1A). However, differences in *E* on a per unit leaf area basis between clones under chilling were only marginally higher in ST51 than CN5 on day 0 ($P < 0.05$) and days 14, 21 and 42 ($P < 0.1$).

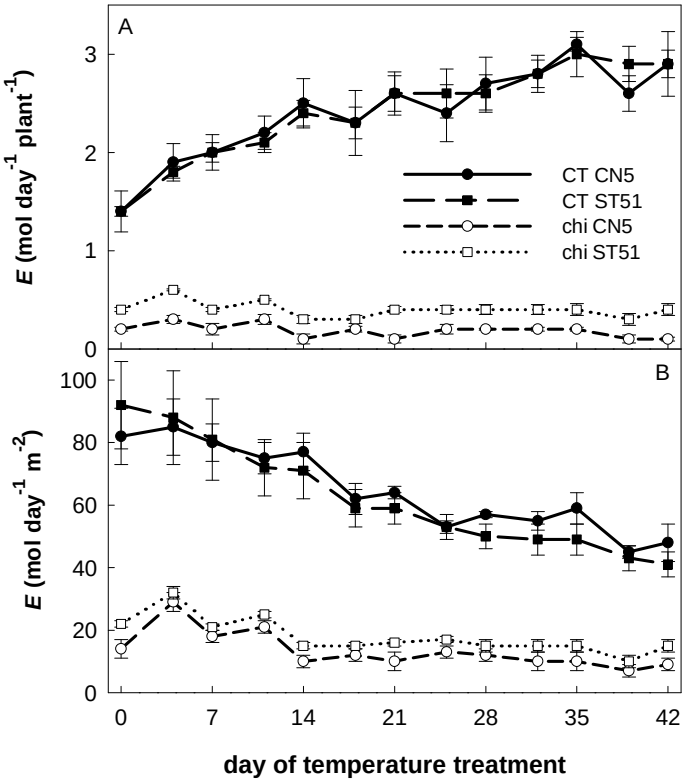

Figure 1. Leaf transpiration rate (*E*) expressed on a (A) per plant basis and (B) leaf area basis of control (CT) and chilled (chi) plants of a drought-resistant clone (CN5) and a drought-sensitive clone (ST51) of *Eucalyptus globulus*. Data are means ± s.e. (*n*=5).

Plant water status and gas exchange

Plants of the two treatments maintained stable Ψ_{pd} throughout the experiment, varying between -0.36 and -0.53 MPa. Clone ST51 had lower Ψ_{pd} than CN5 ($P < 0.05$) during the experiment in particular under chilling, which coincides with the higher *E* of this treatment (Figure 2A). Midday leaf water potential also did not change significantly throughout the experiment, although there were statistically significant differences between the temperature regimes until day 8, with chilling inducing lower Ψ_{md} ($P < 0.05$) in ST51 plants but not in CN5 plants (Figure 2B).

At day 1, g_s decreased by 28% in chilling treated plants of both clones as compared to controls ($P < 0.05$), as a result of acclimation to low temperatures (Figure 2C). After days 8 and 42, g_s difference to control plants increased ($P < 0.001$) to 59 and 51%, respectively.

Similar to g_s, there was a significant effect ($P < 0.001$) of low temperature in *A* of both clones starting on day 1 after acclimation, and although there were only significant differences between the clones on day 42 ($P < 0.05$), clone ST51 showed slightly higher *A* throughout the experiment in both regime temperatures (Figure 2D).

Growth response

Growth of new leaf was strongly dependent on temperature (Figure 3). The strong effect ($P < 0.01$) of temperature in the number of new leaves formed was detectable from day 1 to 42 after acclimation with a final reduction of 65 and 76% in clones CN5 and ST51, respectively, as compared to control plants. Under control conditions, ST51 plants showed a noticeable 171% higher new leaf growth than CN5 plants. Expansion of new leaves was also highly influenced by temperature (Figure 4). At day 21, leaf growth had significantly decreased ($P < 0.001$) by 73 and 44% in plants of CN5 and ST51, respectively. After day 21, although the temperature effect was maintained ($P < 0.001$), there was a decrease in leaf area expansion in both control and treated leaves. In the first 21 days of the experiment, there was also a significant interaction between clone and treatment effects ($P < 0.05$), so that by day 21, CN5 plants exhibited the highest and the

lowest leaf areas, in control and low temperature conditions, respectively. Therefore, the effect of chilling was more marked in CN5.

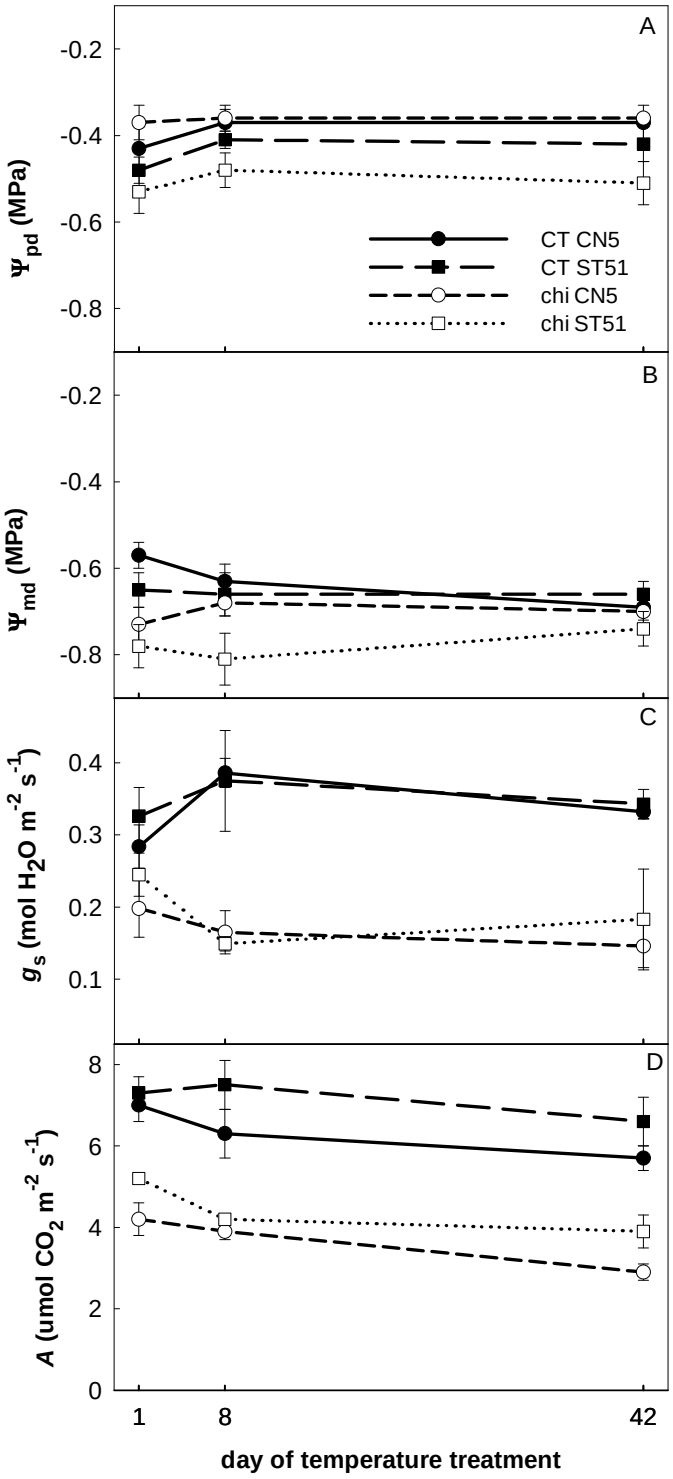

Figure 2. (A) Predawn leaf water potential (Ψ_{pd}), (B) midday leaf water potential (Ψ_{md}), (C) midday stomatal conductance (g_s) and (D) net photosynthesis (A) of control (CT) and chilled (chi) plants of a drought-resistant clone (CN5) and a drought-sensitive clone (ST51) of *Eucalyptus globulus*. Data are means ± s.e. (n=5).

After 42 days, chilling led to a general decrease in growth in both clones with reductions in total biomass, leaf area, number of branches and total root length (Table 1). Plants of CN5 had lower number of branches ($P < 0.05$) and invested a significantly larger proportion of total dry mass in roots than the ST51 plants ($P < 0.01$) in both temperature regimes. In contrast, ST51 plants invested a significantly larger proportion of total dry mass in leaves ($P < 0.05$). When grown at chilling temperatures, the proportion of biomass allocated to branches and leaves decreased significantly in the two clones, and the ratio of stem axis to total biomass and of root to total biomass increased. Chilling affected more the root growth of ST51 than CN5 ($P < 0.01$) with decreases in root biomass of 21 and 9%, respectively, in chilled compared to control plants.

At the end of the experiment, significant decreases in LAR and SLA were observed in plants under low temperature ($P < 0.001$); ST51 plants showed significantly higher LAR values ($P<0.01$) than CN5 plants (Table 1). As a result of the alteration in biomass partitioning from leaves to roots under low temperatures, the leaf area to root area ratio decreased on average 50% in both clones ($P < 0.01$). Moreover, CN5 plants exhibited significant lower values of this ratio ($P < 0.05$) and greater total root length than clone ST51 ($P < 0.05$).

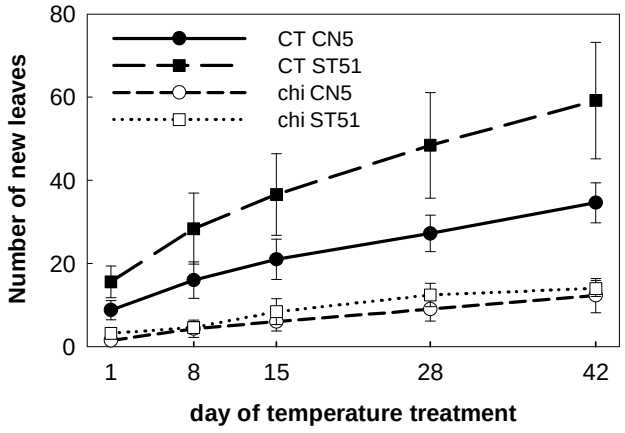

Figure 3. Total number of new leaves of control (CT) and chilled (chi) plants of a drought-resistant clone (CN5) and a drought-sensitive clone (ST51) of *Eucalyptus globulus*. Data are means ± s.e. (n=5).

Table 1. Total biomass, leaf area, number of branches, total root length, dry mass partitioning (percent of total biomass) and leaf growth analysis in control (CT) and chilled (chi) plants of a drought-resistant clone (CN5) and a drought-sensitive clone (ST51) of *Eucalyptus globulus* (day 42). Data are means ± s.e. (n=5). *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant at P > 0.05.

Morphological characteristics	CT CN5	CT ST51	chi CN5	chi ST51	Significance of 2–way ANOVA		
					Clone (C)	Temperature regime (T)	C x T
Total biomass (g)	9.4±0.2	8.7±0.9	6.1±0.9	5.5±0.2	ns	***	ns
Leaf area (cm^2)	621±67	734±105	238±48	289±26	ns	***	ns
Number of branches	8.8±0.7	12.8±2.7	5.6±1.7	8.8±1.0	*	*	ns
Total root length (m)	102±9	75±0.8	71±12	50±9	*	**	ns
Dry mass partitioning							
Stem (%)	29±1.7	26±1.6	35±3.3	35±2.4	ns	**	ns
Branches (%)	2.3±0.5	4.1±1.3	0.8±0.2	1.7±0.3	ns	*	ns
Leaves (%)	52±2.7	57±1.2	40±4.2	49±1.7	*	**	ns
Root (%)	17±1.7	12±1.6	24±3.5	15±1.0	**	*	ns
Leaf growth analysis							
Leaf area ratio ($cm^2\ g^{-1}$)	65.8±6.3	82.8±4.1	38.2±3.0	52.1±3.6	**	***	ns
Specific leaf area ($cm^2\ g^{-1}$)	139±4	158±3	120±3	121±7	*	***	ns
Leaf area / root area	0.59±0.08	0.95±0.17	0.28±0.03	0.48±0.05	*	**	ns

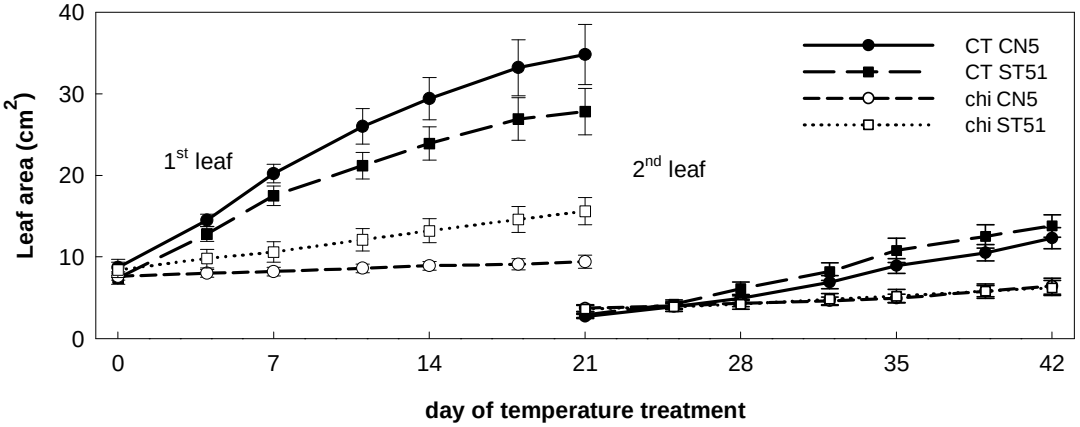

Figure 4. Leaf area expansion measured of emerged first and second leaves of control (CT) and chilled (chi) plants of a drought-resistant clone (CN5) and a drought-sensitive clone (ST51) of *Eucalyptus globulus*. Data are means ± s.e. (*n*=5).

New root growth

When a sample of new roots formed during the course of the experiment was analysed on day 42, there were significant differences between plants due to the temperature regime (Table 2). Plants of both clones under chilling developed new roots with lower specific length and area, as well as lower number of tips and forks ($P < 0.001$). New roots formed under low temperatures showed a significant higher average diameter ($P < 0.001$) than new roots formed under control conditions. In addition, there were also differences in new root morphology between the two clones. New roots in ST51 plants exhibited a marginally significant higher specific length ($P < 0.09$) and area ($P < 0.06$) than CN5 plants. Furthermore, roots of ST51 showed a marginally significant higher number of tips ($P < 0.06$) and a higher specific volume ($P < 0.05$) than the roots of CN5.

Table 2. Specific root length, specific root surface area, average diameter, number of tips, number of forks and specific root volume of a new root sample of control (CT) and chilled (chi) plants of a drought-resistant clone (CN5) and a drought-sensitive clone (ST51) of *Eucalyptus globulus* (day 42). Data are means ± s.e. (n=5). *P < 0.05; ***P < 0.001; ns, not significant at P > 0.05

New root analysis	CT CN5	CT ST51	chi CN5	chi ST51	Significance of two-way ANOVA		
					Clone (C)	Temperature regime (T)	C x T
Specific length (m g DM^{-1})	105±8	125±15	32±4	51±11	0.09	***	ns
Specific surface area (cm^2 g DM^{-1})	1086±37	1216±119	538±22	753±95	0.06	***	ns
Average diameter (mm)	0.33±0.01	0.31±0.01	0.55±0.07	0.50±0.05	ns	***	ns
Number of tips (mg DM^{-1})	35±4	50±8	7±1	12±3	0.06	***	ns
Number of forks (mg DM^{-1})	48±8	63±12	10±2	21±6	ns	***	ns
Specific volume (cm^3 g DM^{-1})[A]	5.7±0.4	6.9±0.6	6.2±0.5	8.1±0.6	*	ns	ns

[A] in total root system

Anthocyanins

At the end of the experiment, the leaves of the plants subjected to low temperature displayed a distinctive reddish colour. There was a significant increase in anthocyanin concentrations ($P < 0.05$) in the leaves under the chilling treatment (Figure 5), with CN5 showing a significantly larger accumulation of anthocyanins than ST51 plants ($P < 0.05$).

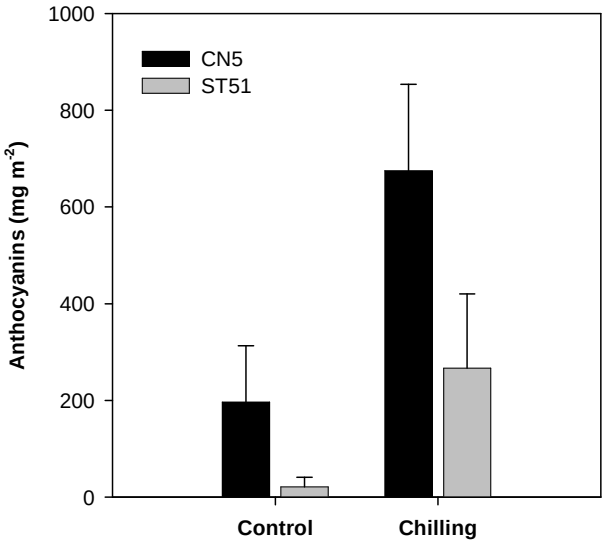

Figure 5. Anthocyanins content measured on day 42 of control and chilled plants of a drought-resistant clone (CN5) and a drought-sensitive clone (ST51) of *Eucalyptus globulus*. Data are means ± s.e. (n=5).

Hydraulic properties

Root hydraulic conductance (K) was strongly related with root growth and increased throughout the experiment in both chill and control treatments (Figure 6A). The temperature regime had a significant effect on K, with lower values in chill-treated plants ($P < 0.001$). Similar to K, root hydraulic conductivity (L_p) was also related to root growth, and decreased under low temperatures ($P < 0.001$). However, there were significant differences in L_p between the clones on day 42 ($P < 0.01$), with higher values in ST51 plants (Figure 6B).

Hydraulic conductance of the soil-plant system (K_{sp}) by the end of the experiment (day 42) showed a significant difference both between temperature regimes and clones

(Figure 7A). Thus, low temperatures led to a decrease in K_{sp}, and clone ST51 had higher K_{sp} values than clone CN5 ($P < 0.001$) in both temperature regimes. There were significant differences between the temperature regimes ($P < 0.001$) on LSC on days 1 and 8. However, there were no differences in LSC values between temperature regimes at the end of the experiment (Figure 7B). The decrease in LSC in control plants, especially from day 1 to 8 was related to a reduction of E on a leaf area basis, and with the initial great development in foliage. In contrast, plants in the low temperature treatment increased their LSC due to limited leaf growth and to increased E values on a leaf area basis. Furthermore, at day 42, there were significant differences in LSC between the two clones ($P < 0.05$), with ST51 plants showing higher capacity (1.9-times, on average) to supply foliage with water than CN5 plants.

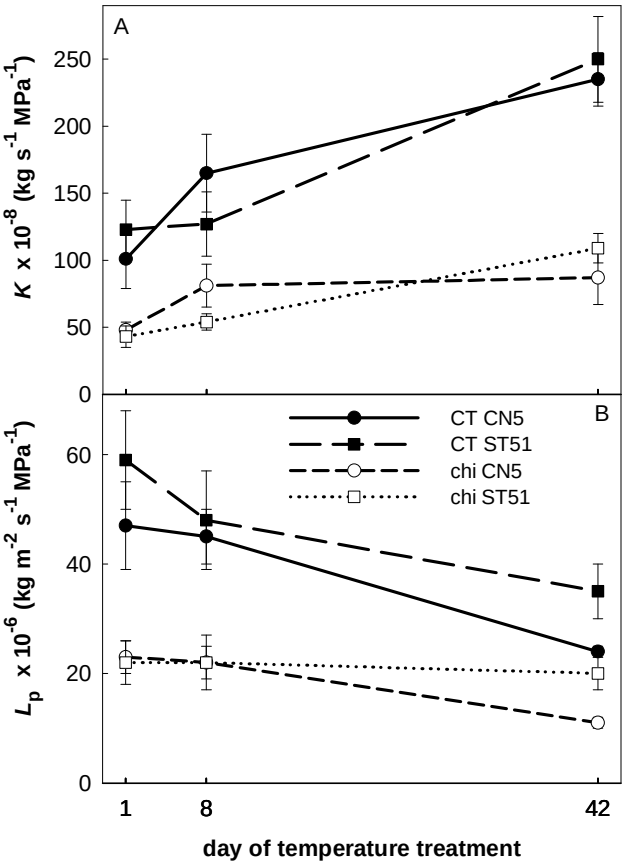

Figure 6. (A) Root hydraulic conductance (K) and (B) root hydraulic conductivity (Lp) of control (CT) and chilled (chi) plants of a drought-resistant clone (CN5) and a drought-sensitive clone (ST51) of *Eucalyptus globulus*. Data are means ± s.e. (n=5).

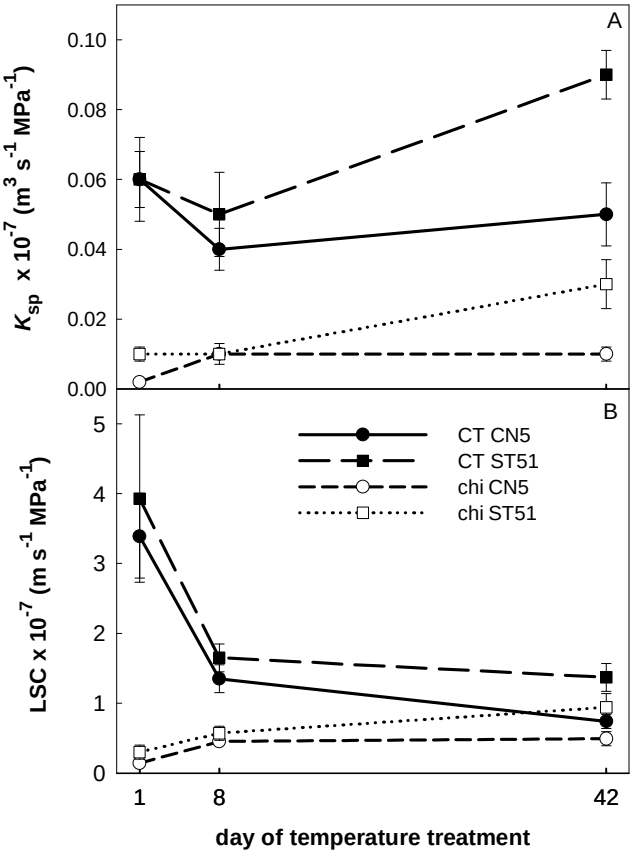

Figure 7. (A) Hydraulic conductance of the soil-plant system (K_{sp}) and (B) leaf specific conductance (LSC) of control (CT) and chilled (chi) plants of a drought-resistant clone (CN5) and a drought-sensitive clone (ST51) of *Eucalyptus globulus*. Data are means ± s.e. (n=5).

Acclimation versus non-acclimation: hydraulic responses

When L_p was measured in control plants at 10 ºC, there was a significant average decrease of 65% ($P < 0.001$) compared to L_p measured at 24 ºC, corresponding with a Q_{10} of 1.9 and 2.4 in CN5 and ST51 plants, respectively (Figure 8). Moreover, after 24 h, under low temperatures, there was an additional decrease in L_p by 45% ($P < 0.01$). The consequences of long-term acclimation to chilling temperatures in L_p can be observed comparing control plants growing 24 h at 10 ºC with plants grown for 42 days at 10 ºC. Acclimation led to a significant increase in L_p in both clones ($P < 0.001$) of ~2-times and 4-times in CN5 (Q_{10} =1.74) and ST51 (Q_{10} =1.49), respectively. Finally, after 5 days in control conditions, there was a marginally significant recovery in L_p ($P < 0.09$) of ~1.3-times.

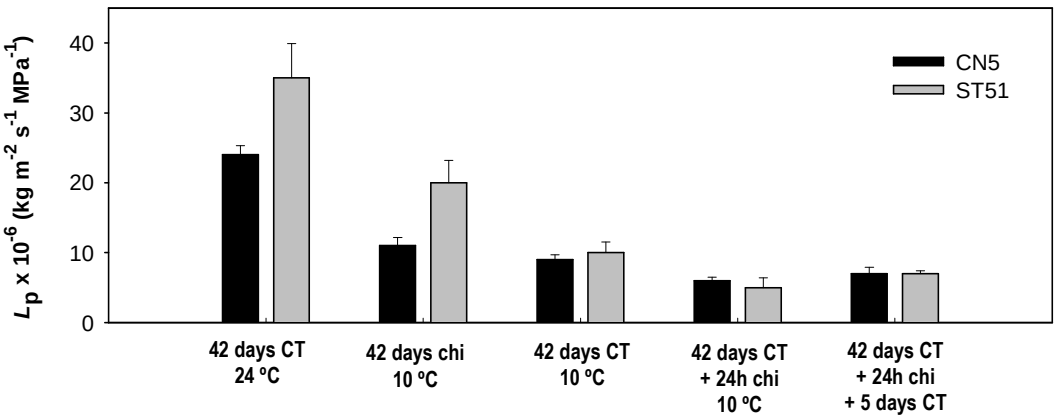

Figure 8. Root hydraulic conductivity (L_p) of control (CT; measured at 24 ± 1 ºC) and chilled (chi; measured at 10 ± 1 ºC) plants at 42 days. Control plants grown at 24/16 ºC (day/night) were also measured at 10 ± 1 ºC, before and after 24 h at 10/5 ºC (day/night), and after 5 days of recovery in control conditions. Plants belonged to a drought-resistant clone (CN5) and a drought-sensitive clone (ST51) of *Eucalyptus globulus*. Data are means ± s.e. (*n*=4).

Discussion

When ramets of the two *E. globulus* clones were grown at chilling temperatures of 10/5 ºC (day/night), a reduction of ~34% in mean total biomass as compared to growth at 24/16 ºC was observed. This cold treatment also resulted in a 61% decrease in total leaf area and a 39% decrease in the ratio of leaf area to total biomass relative to control plants. In addition to the decrease in leaf area under low temperatures, there was a decrease in stomatal conductance, which contributed to reduced carbon assimilation (Figure 2C, D). This reduction in growth due to chilling was similar to an imposition of water stress with the same clones in a previous study (Costa e Silva *et al.* 2004). Furthermore, reductions in the number of branches and alterations in biomass partitioning, resulting in a higher investment in the root system, were also coincident under chilling and drought stress. Temperatures below the plant's optimum for growth usually result in increased relative investment of biomass in roots (Clarkson *et al.* 1988). This may be a consequence of a

reduction in the sink strength of the aboveground plant tissues, making more assimilates available for root growth. In addition, low soil temperatures are known to reduce overall growth and tend to increase carbon allocation to roots (Stoneman and Dell 1993; Gavito *et al*. 2001) due to reductions in nutrient and water uptake (e.g. Markhart *et al*. 1979; Running and Reid 1980; Grossnickle 1988; Wan *et al*. 1999). Moreover, since both shoots and roots were subjected to chilling, the hypothesis of possible feedback mechanisms affecting hydraulic properties has to be accepted (Matzner and Comstock 2001).

The two clones tested responded differently to chilling. Under low temperatures, ST51 clone maintained higher transpiration rates than CN5 clone throughout the experiment and this was accompanied by more negative leaf water potentials. Although there were no differences in g_s or A between the two clones until day 8, ST51 showed a higher rate of carbon assimilation at both temperature regimes on day 42 (Figure 2D). Furthermore, it is interesting to confirm the previous results under drought conditions of different growth characteristics between the two clones. Compared with the drought-sensitive ST51 clone, the drought-resistant CN5 clone had a higher percentage of biomass investment in root system, in control and low temperature conditions (Table 1). In contrast, with CN5 clone, ST51 showed a greater investment in leaf area and higher ratios of leaf area to total biomass, in control or chilling temperatures. Although the effect of chilling in shoot growth was similar in both clones, root growth was more affected in ST51 than in CN5 with higher decreases in root biomass in comparison to control plants.

In many *E. globulus* clones, chilling under high light induces the accumulation of anthocyanins in the leaves, which become reddish. The observed accumulation of anthocyanins induced by low temperatures (Figure 5) followed an accumulation of soluble sugars, occurred under low values of chlorophyll content (Shvaleva *et al*. 2007) and was more intense in CN5, the drought-resistant clone. As proposed by Steyn *et al*. (2002), anthocyanins may prevent possible photoinhibition damages, when low temperatures limit carbon assimilation.

Although the initial hypothesis of a lower depression in growth rate in the drought-resistant clone under chilling cannot be supported, greater allocation of carbon to root growth in clone CN5 under low temperatures is an advantageous trait in the

Mediterranean environment. In fact, it can be predicted that reduction in the transpiration area and increase in root absorption area under low temperatures can also favour drought resistance in the summer. Drought-resistant genotypes will explore more soil volume during the water-stress-free period, enabling increased water uptake during the drought season.

By studying xylem hydraulic properties of the two clones, responses to chilling temperatures may be related with their growth characteristics. It was hypothesised that a higher susceptibility to cavitation/embolism in the drought-sensitive clone result from a steeper decline in hydraulic conductance under drought conditions (Costa e Silva *et al.* 2004). Clonal differences in hydraulic architecture have also been reported by previous studies (Vander Willigen and Pammenter 1998; Sangsing *et al*. 2004).

In this experiment, a decline in L_p was observed, with ST51 clone showing significantly higher water transport efficiency, either in control or chilling treatments. After 42 days, ST51 clone showed a higher capacity to deliver water through the roots (L_p) to leaves (LSC) or the whole plant (K_{sp}) compared with CN5. Limited water transport due to reductions in hydraulic conductance may enhance a conservative water use (Hubbard *et al*. 2001) and is a trait presented by drought-adapted species (Nardini *et al*. 1999) and slow growing species (Tyree *et al*. 1998). Thus, the higher water transport efficiency in clone ST51 matches its higher transpiration rates and is in accordance with its higher growth rate; these characteristics are consistent with its sensitivity to drought conditions.

Root water flow measured in control plants at 10 ºC yielded an average decrease of 65% in L_p compared to control plants measured at 24 ºC. A further decrease of 45% occurred after 24 h under chilling. This decrease in L_p was greater than what should be expected from changes due to water viscosity and density (Q10 =1.25; Matzner and Comstock 2001). Likewise, decreases in L_p unrelated to changes in viscosity were reported in several studies (Fennell and Markhart 1998; Vernieri *et al*. 2001; Wan *et al*. 2001; Melkonian *et al*. 2004) and have been attributed to low-temperature-induced alteration of membrane properties that lowers the hydraulic conductance of radial root water flux (Markhart *et al*. 1979). In this experiment, clone ST51 showed higher decrease of L_p than CN5 (Q_{10} of 2.4 and 1.9, respectively), denoting a stronger effect of low

temperatures on one or more components of the pathway for water movement within the root system. These components may either involve different clone alterations in apoplastic barriers, such as exo- and endodermal Casparian bands (Steudle and Peterson 1998), or differences in water channel activities (Javot and Maurel 2002; Aroca *et al*. 2005). Recently, Lee *et al*. (2005) found a strong channel closure and gating of aquaporins activity as result of low temperature in a chilling-sensitive species showing a high Q_{10} value.

With time under low temperature, some plants can exhibit partial recovery of L_p (Fennell and Markhart 1998; Vernieri *et al*. 2001; Melkonian *et al*. 2004). When control plants growing 24 h and plants growing 42 days under chilling were compared, a long-term acclimation was observed, which led to a significant increase in L_p in both clones (Figure 8). One factor that may be responsible for L_p recovery with time is root growth under low temperatures (Sanders and Markhart 2001). Root growth analysis on day 42 showed that plants of both clones under low temperature developed new roots with lower specific length and area, as well as lower number of tips and forks. In contrast, these new roots exhibited higher average diameters. A decrease in specific root length and increase in diameter with decreasing temperature was also found by Stoneman and Dell (1993) and Gavito *et al*. (2001), respectively. These alterations in root morphology can be driven by higher water availability in the soil under low temperatures and be related with a lower requirement of soil exploration for water acquisition. Thus, the plant under low temperatures develops a root system that can favour hydraulic conductivity through less surface area and higher root conduit diameters (Linton *et al*. 1998). However, a higher root diameter with a larger cortex section can also have a negative effect in L_p (Rieger and Litvin 1999). The possible influence of the root system size in partly explaining the difference in L_p between control and acclimated plants cannot be disregarded. An increase in size of the root system was often associated with a decrease in L_p (Rüdinger *et al*. 1994; Steudle and Meshcheryakov 1996).

Differences in new root morphology between the two clones can also be related to their different hydraulic capacities. The higher specific root length, number of tips and specific root volume in ST51 plants under both treatments can be associated with better soil exploitation capacities favouring uptake and hydraulic conductance, particularly

under optimal conditions for growth. Thus, in comparison to CN5 clone, higher specific root length of ST51 means that for the same root length, it has a lower biomass partition cost. A greater specific root length was suggested to support higher hydraulic conductances (Eissenstat 1991), a plant growth characteristic of fast-growing species (Comas *et al*. 2002).

In summary, this data indicate that chilling (10/5 ºC, day/night) led to a general decrease in growth of both clones and significant reductions in root hydraulic conductivity, rate of photosynthesis and stomatal conductance in comparison to plants grown at control temperature (24/16 ºC). The main differences in the responses to chilling in *E. globulus* clones include a lower depression of root growth in the drought-resistant clone, as well as a better water status and a higher anthocyanin concentration as compared to ST51 plants. Except for roots, the results did not support the initial hypothesis of a lower inhibition of growth rate in the drought-resistant clone under chilling. However, the higher root growth under low temperatures, as observed in CN5 in comparison to ST51 clone, can be an advantageous trait of drought-resistant genotypes in a Mediterranean-type environment. A growth pattern leading to a reduction of the transpiration area and the increase of root absorption area under low temperatures will permit these genotypes to explore more soil volume during the water-stress-free period, which will enable an increased water uptake under the drought season.

Acknowledgements

This research was carried out with financial support from the POCI 2010 and FSE. F. Costa e Silva and A. Shvaleva were supported by FCT, Lisbon, grant SFRH/BD/13211/2003 and grant SFRH/BPD/5667/2001, respectively. We also acknowledge the expert technical assistance of Elsa Breia in this research.

References

Allen DJ, Ort DR (2001) Impacts of chilling temperatures on photosynthesis in warm-climate plants. *Trends in Plant Science* **6**, 36–42. doi: 10.1016/S1360-1385(00)01808-2

Aroca R, Amodeo G, Fernández-Ilescas S, Herman EM, Chaumont F, Chrispeels MJ (2005) The role of aquaporins and membrane damage in chilling and hydrogen peroxide induced changes in the hydraulic conductance of maize roots. *Plant Physiology* **137**, 341–353. doi: 10.1104/pp.104.051045

Atkin OK, Bruhn D, Hurry VM, Tjoelker MG (2005) The hot and the cold: unravelling the variable response of plant respiration to temperature. *Functional Plant Biology* **32**, 87–105. doi: 10.1071/FP03176

Blödner C, Skroppa T, Johnsen Ø, Polle A (2005) Freezing tolerance in two Norway spruce (*Picea abies* [L.] Karst.) progenies is physiologically correlated with drought tolerance. *Journal of Plant Physiology* **162**, 549–558. doi: 10.1016/j.jplph.2004.09.005

Clarkson DT, Earnshaw MJ, White PJ, Cooper HD (1988) Temperature dependent factors influencing water uptake: an analysis of responses at different levels of organization. In 'Plants and temperature'. (Eds SP Long, FI Woodward) pp. 281–309. (The Company of Biologists Ltd.: Cambridge)

Close DC, Beadle CL, Brown PH, Holz GK (2000) Cold-induced photoinhibition affects establishment of *Eucalyptus nitens* (Deane and Maiden) Maiden and *Eucalyptus globulus* Labill. *Trees (Berlin)* **15**, 32–41. doi: 10.1007/s004680000070

Comas LH, Bouma TJ, Eissenstat DM (2002) Linking root traits to potential growth rate in six temperate tree species. *Oecologia* **132**, 34–43. doi: 10.1007/s00442-002-0922-8

Costa e Silva F, Shvaleva A, Maroco JP, Almeida MH, Chaves MM, Pereira JS (2004) Responses to water stress in two *Eucalyptus globulus* clones differing in drought tolerance. *Tree Physiology* **24**, 1165–1172.

Eissenstat DM (1991) On the relationship between specific root length and rate of root proliferation: a field study using citrus rootstocks. *The New Phytologist* **118**, 63–68. doi: 10.1111/j.1469-8137.1991.tb00565.x

Fennell A, Markhart AH (1998) Rapid acclimation of root hydraulic conductivity to low temperature. *Journal of Experimental Botany* **49**, 879–884. doi: 10.1093/jexbot/49.322.879

Gavito ME, Curtis PS, Mikkelsen TN, Jakobsen I (2001) Interactive effects of soil temperature, atmospheric carbon dioxid and soil N on root development, biomass and nutrient uptake of winter wheat during vegetative growth. *Journal of Experimental Botany* **52**, 1913–1923. doi: 10.1093/jexbot/52.362.1913

Grossnickle SC (1988) Planting stress in newly planted jack pine and white spruce. 1. Factors influencing water uptake. *Tree Physiology* **4**, 71–83.

Hubbard RM, Ryan MG, Stiller V, Sperry JS (2001) Stomatal conductance and photosynthesis vary linearly with plant hydraulic conductance in ponderosa pine. *Plant, Cell & Environment* **24**, 113–121. doi: 10.1046/j.1365-3040.2001.00660.x

Javot H, Maurel C (2002) The role of aquaporins in root water uptake. *Annals of Botany* **90**, 301–313. doi: 10.1093/aob/mcf199

Jones HG (1992) 'Plants and microclimate.' (Cambridge University Press: Cambridge)

Jordão AM, Ricardo-da-Silva JM, Laureano O (1998) Evolution of Anthocyanins during grape maturation of two varieties (*Vitis vinifera*): Castelão Francês and Touriga Francesa. *Vitis* **37**, 93–94.

Lee SH, Chung GC, Steudle E (2005) Gating of aquaporins by low temperature in roots of chilling-sensitive cucumber and chilling-tolerant figleaf gourd. *Journal of Experimental Botany* **56**, 985–995. doi: 10.1093/jxb/eri092

Linton MJ, Sperry JS, Williams DJ (1998) Limits to water transport in *Juniperus osteosperma* and *Pinus edulis*: implications for drought tolerance and regulation of transpiration. *Functional Ecology* **12**, 906–911. doi: 10.1046/j.1365-2435.1998.00275.x

Markhart AH, Fiscus EL, Naylor AW, Kramer PJ (1979) Effect of temperature on water ion transport in soybean and broccoli systems. *Plant Physiology* **64**, 83–87.

Matzner S, Comstock JP (2001) The temperature dependence of shoot hydraulic resistance: implications for stomatal behaviour and hydraulic limitation. *Plant, Cell & Environment* **24**, 1299–1307. doi: 10.1046/j.0016-8025.2001.00785.x

Melkonian J, Yu L, Setter L (2004) Chilling responses of mayze (*Zea mays* L.) seedlings: root hydraulic conductance, abscisic acid and stomatal conductance. *Journal of Experimental Botany* **55**, 1751–1760. doi: 10.1093/jxb/erh215

Nardini A, Lo Gullo MA, Salleo S (1999) Competitive strategies for water availability in two Mediterranean Quercus species. *Plant, Cell & Environment* **22**, 109–116. doi: 10.1046/j.1365-3040.1999.00382.x

Nardini A, Salleo S, Lo Gullo MA (1998) Root hydraulic conductance of six forest trees: possible adaptative significance of seasonal changes. *Plant Biosystems* **132**, 97–104.

Peng YY, Dang Q (2003) Effects of soil temperature on biomass production and allocation in seedlings of four boreal tree species. *Forest Ecology and Management* **180**, 1–9. doi: 10.1016/S0378-1127(02)00486-3

Reich P, Walters M, Tjoelker M, Vanderklein D, Buschena C (1998) Photosynthesis and respiration rates depend on leaf and root morphology and nitrogen concentration in nine boreal tree species differing in relative growth rate. *Functional Ecology* **12**, 395–405. doi: 10.1046/j.1365-2435.1998.00209.x

Rieger M, Litvin P (1999) Root system hydraulic conductivity in species with contrasting root anatomy. *Journal of Experimental Botany* **50**, 201–209. doi: 10.1093/jexbot/50.331.201

Rüdinger M, Hallgren SW, Steudle E, Schulze E (1994) Hydraulic and osmotic properties of spruce roots. *Journal of Experimental Botany* **45**, 1413–1425. doi: 10.1093/jxb/45.10.1413

Running SW, Reid CP (1980) Soil temperature influences on root resistance of *Pinus contorta* seedlings. *Plant Physiology* **65**, 635–640.

Sanders PL, Markhart AH (2001) Root system functions during chilling temperatures: injury and acclimation. In 'Crop responses and adaptations to temperature stress'. (Ed. S Basra) pp. 77–108. (The Haworth Press Inc.: New York)

Sangsing K, Kasemsap P, Thanisawanyangkura S, Sangkhasila K, Gohet E, Thaler P, Cochard H (2004) Xylem embolism and stomatal regulation in two rubber clones (*Hevea brasiliensis* Muell. Arg.). *Trees (Berlin)* **18**, 109–114.

Shvaleva A, Costa e Silva F, Breia E, Jouve J, Hausman JF, Almeida MH, Maroco JP, Rodrigues ML, Pereira JS, Chaves MM (2006) Metabolic responses to water deficit in two *Eucalyptus globulus* clones with contrasting drought sensitivity. *Tree Physiology* **26**, 239–248.

Shvaleva A, Costa e Silva F, Scotti P, Oufir M, Hausman JF, *et al.* (2007) Physiological and biochemical responses to low non-freezing temperature of two *Eucalyptus globulus* clones differing in drought resistance. *Annals of Forest Science*, in press.

Steudle E, Meshcheryakov AB (1996) Hydraulic and osmotic properties of oak roots. *Journal of Experimental Botany* **47**, 387–401. doi: 10.1093/jxb/47.3.387

Steudle E, Peterson CA (1998) How does water get through roots? *Journal of Experimental Botany* **49**, 775–788. doi: 10.1093/jexbot/49.322.775

Steyn W, Wand S, Holcroft D, Jacobs G (2002) Anthocyanins in vegetative tissues: a proposed unified function in photoprotection. *New Phytologist* **155**, 349–361. doi: 10.1046/j.1469-8137.2002.00482.x

Stoneman GL, Dell B (1993) Growth of *Eucalyptus marginata* (Jarrah) seedlings in a greenhouse in response to shade and soil temperature. *Tree Physiology* **13**, 239–252.

Sung D, Kaplan F, Lee K, Guy CL (2003) Acquired tolerance to temperature extremes. *Trends in Plant Science* **8**, 179–187. doi: 10.1016/S1360-1385(03)00047-5

Suzuki N, Mittler R (2006) Reactive oxygen species and temperature stresses: a delicate balance between signaling and destruction. *Physiologia Plantarum* **126**, 45–51. doi: 10.1111/j.0031-9317.2005.00582.x

Tyree MT, Velez V, Dalling JW (1998) Growth dynamics of root and shoot hydraulic conductance in seedlings of five neotropical tree species: scaling to show possible adaptation to differing light regimes. *Oecologia* **114**, 293–298. doi: 10.1007/s004420050450

Vander Willigen C, Pammenter NW (1998) Relationship between growth and xylem hydraulic characteristics of clones of *Eucalyptus* spp. at contrastings sites. *Tree Physiology* **18**, 595–600.

Vernieri P, Lenzi A, Figaro M, Tognoni F, Pardossi A (2001) How the roots contribute to the ability of *Phaseolus vulgaris* L. to cope with chilling-induced water stress. *Journal of Experimental Botany* **52**, 2199–2206.

Wan X, Landhäusser SM, Zwiazek JJ, Lieffers VL (1999) Root water flow and growth of aspen (*Populus tremuloides*) at low root temperatures. *Tree Physiology* **19**, 879–884.

Wan X, Zwiazek JJ (1999) Mercuric chloride effects on root water transport in aspen seedlings. *Plant Physiology* **121**, 939–946. doi: 10.1104/pp.121.3.939

Wan X, Zwiazek JJ, Lieffers VJ, Landha¨usser SM (2001) Hydraulic conductance in aspen (*Populus tremuloides*) seedlings exposed to low root temperatures. *Tree Physiology* **21**, 691–696.

CAPÍTULO 5

Physiological and biochemical responses to low non-freezing temperature of two *Eucalyptus globulus* clones differing in drought resistance

5. Physiological and biochemical responses to low non-freezing temperature of two *Eucalyptus globulus* clones differing in drought resistance

Abstract

We have compared the metabolic responses of leaves and roots of two *Eucalyptus globulus* L. clones CN5 and ST51 that differ in their sensitivity to water deficits (ST51 is more drought sensitive), with regard to the effect of chilling (10/5 ºC, day/night). We studied changes in growth, osmotic potential and osmotically active compounds, soluble proteins, leaf pigments, and membrane lipid composition. Our data showed that both clones have the ability to acclimatize to chilling temperatures. As a result of 10 days of acclimation, an increase of soluble sugars in leaves of treated plants of both clones was observed that disappeared later on. Differences between clones were observed in the photosynthetic pigments and soluble protein content which were more stable in CN5 under chilling. It also was apparent that CN5 presented a less negative predawn water potential (Ψ_{pd}) and a higher leaf turgor than ST51 throughout the chilling treatment. In the case of the CN5, increased total lipids (TFA) and concomitant increase of linolenic acid (C18:3) in leaves after acclimatization may be related to a better clone performance under chilling temperatures. Moreover, a higher constitutive investment in roots in the case of CN5 as compared to ST51 may benefit new root regeneration under low temperatures favoring growth after cold Mediterranean winter.

Keywords: carbohydrates / chilling / *Eucalyptus globulus* L. / lipids / membranes

Introduction

Eucalyptus globulus Labill. is an evergreen tree that grows in many regions of winter-rain climates of the Mediterranean-type with a dry and hot summer. In such conditions, a more efficient clone should not only use more water (through deep rooting) but also take advantage of the water availability of the cold season through greater chilling tolerance. Previous studies of two highly productive *E. globulus* clones, CN5 and ST51, with different sensitivity to drought, indicated that these clones exhibited different strategies to cope with water deficit [6]. The investment in root system development before drought, a continuous greater root growth and higher xylem hydraulic conductance under water stress explained superior drought resistance of CN5 clone compared with ST51 clone. Under gradual subjection to water stress both clones CN5 and ST51 had the ability to respond to water deficit at the cellular level by altering their osmotic components and the activity of the antioxidant protection system [24].

Eucalyptus globulus is susceptible to cold and does not tolerate below-freezing temperatures [2]. Moreover, growth is limited by chilling temperatures, e.g., from (0 ºC) 4 ºC to 15 ºC, which may be too low for normal growth. The plants exposed to chilling temperatures undergo a process of acclimation associated with several physiological and biochemical alterations in the plants [2, 12, 25]. The best-characterized changes under chilling, as well as under different types of stresses, include alterations in gene expression, changes in hormone level, accumulation of osmolytes (compatible solutes) and protective proteins as well as modification of cell membranes [4].

According to previous studies [13, 16], the thermotropic phase transition of membrane lipids might play an initiative role in the chilling sensitivity of plants. In chill-sensitive plants, the lipid bilayer has a high percentage of saturated fatty acids chains, and this type of membrane tends to solidify into a semicrystalline state at a temperature well above 0 ºC [27]. As the membranes become less fluid, permeability is affected. During acclimation of plants to low temperature the fatty acids in their membrane lipids become more unsaturated, resulting in enhanced membrane stability [22, 26].

In Mediterranean ecosystems, plant performance during winter is poorly studied, maybe because summer-drought constrains are much more conspicuous. However, “cold”

is a relative term and even the “mild” temperatures of Mediterranean winters may be too low for plant species which have to cope with wide thermal amplitude over the year. However, climate change scenarios for the western Mediterranean (including Portugal) suggest lengthening of the dry season [15], which may turn plants even more dependent from a relatively cool but shorter rainy season. There is also the possibility that global warming will enhance the frequency of extreme weather events including cold spells [7]. The comparison of the dynamics of physiological and biochemical changes between non-acclimated and acclimated plants, is of the utmost importance to understand stress coping mechanism in trees. Considering that resistance of plants to drought and low temperatures share common mechanisms [25], the aim of the present work was to investigate whether the two clones with contrasting response to drought (CN5 and ST51) also exhibit differences (growth and metabolic) in response to low nonfreezing temperature. We analysed the effect of gradual temperature decrease and the effect of chilling on morphological parameters, membrane lipid composition and compatible solutes in leaves and roots of both clones, as well as osmotic potential, soluble proteins and pigments in leaves.

Material and methods

Plant material

Rooted cuttings of the two clones ST51 and CN5 were grown in plastic containers filled with 60% peat and 40% styrofoam beads. ST51 is considered more drought sensitive than CN5. After four months the rooted cuttings of both clones were transplanted to 5.3 L plastic pots. At six months old, 32 plants per clone were transferred from nursery and placed in a growth chamber subjected to a gradual temperature decrease (1.4 ºC per day) from 24/16 ºC to 10/5 ºC (day/night), which took 10 days (acclimation). Measurements were started at Day 1 after plants had reached 10/5 ºC, the beginning of the chilling treatment. Another 32 plants per clone remained in control conditions (24/16 ºC). Air and pot soil temperatures in the growth chamber were monitored through a data

logger (DL2e, delta-t Device, UK) to follow temperature changes during the day. It must be taken into account that under natural conditions soil temperature does not vary as rapidly as air temperature. Other growth conditions were: photoperiod: 12/12 h (day/night), relative humidity of approximately 60%, photosynthetic photon flux density: 220 µmol m^{-2} s^{-1}. The experiment lasted for 52 days (7th January 2005 to 1st March 2005). All plants were watered to runoff on the first day and then twice per week.

Growth analysis and sampling dates

Plants were harvested 42 days after the beginning of the chilling treatment. Shoots were separated into stem, lateral branches and stem leaves. Roots were gently washed and carefully separated from soil and other debris. Plant components were then dried for at least 48 h at 80 ºC in the oven and cooled in desiccators for dry mass determination. Leaves and roots were scanned before drying and then leaf and root area of each seedling (five plants per treatment) were calculated with WinRhizo software (Regent Instrument Inc., Canada). Samples for carbohydrates, lipids, proline, soluble protein analyses and osmotic potential were collected on Days 1, 8 and 42 after the beginning of chilling at predawn on full-expanded leaves (0.5 g fresh mass) and at midday on root segments (0.5 g fresh mass and diameter < 2 mm) excised from the central part of the root system, using five plants per treatment. Samples were removed, frozen immediately in liquid nitrogen and kept at –80 ºC until further analysis.

Plant water relations

Predawn water potential (Ψ_{pd}) was measured with a Scholander-type pressure chamber (PMS Instruments, Corvallis, OR) on five plants per treatment. From the same plants, leaf discs (6 mm diameter) were taken at predawn for osmotic potential (Ψ_{π}) determination, frozen in liquid nitrogen and stored at –80 ºC until analysis. The measurements of Ψ_{π} were made after thawing the samples at room temperature, using C-52 sample chambers connected to a Wescor HR-33T dew-point microvoltmeter (Wescor,

INC Logan, UTAH, USA). Leaf turgor (Ψ_p) was calculated according to the equation: $\Psi_p = \Psi_{pd} - \Psi_\pi$. Osmotic potential at full turgor (Ψ_π^{100}) was calculated from Ψ_π corrected by relative water content values (RWC), measured in samples of 10 leaf discs of 0.7 cm diameter. RWC was calculated as RWC (%) = (FW-DW)/(TW-DW) × 100, where FW, TW and DW are the fresh, turgid (after floating the samples for 3 h on distilled water at room temperature) and dry mass (after oven-drying at 80 ºC), respectively.

Leaf pigments

Pigments were extracted from frozen leaf discs as described in Shvaleva et al. [24] and then analysed by HPLC according to Wright et al. [32] and quantified by custom-made external standard solutions (DHI Water and Environment; Denmark and Carotenature, Switzerland). Twenty-five microliter samples were injected in Zorbax (Agilent Tech., USA) Bonus-RP C18 column and eluted with a quaternary gradient composed of water, acetonitrile, ethyl acetate and 0.5 M ammonium acetate in methanol (20:80, v/v) at flow-rate of 1.0 mL min^{-1}. Pigments content were measured after 42 days of chilling.

Lipid analysis

For lipid analysis, the general procedure of Pham Thi et al. [20] was used with modification according to Scotti Campos et al. [22]. Lipids were extracted in chloroform/methanol/water (1/1/1, v/v/v) according to Allen et al. [1]. After saponification, fatty acids were methylated with BF_3 (Merck) according to Mercalfe et al. [14] using heptadecanoic acid (C17:0) as an internal standard. Subsequently they were analysed by gas-liquid chromatography as described in Mercalfe et al. [22].

Soluble proteins

Soluble proteins were extracted and measured as detailed in Bradford [3].

Carbohydrates and polyols extraction and analysis

Carbohydrates and polyols were extracted from leaves and roots (100 mg FW), according to Van Huylenbroeck and Debergh [30] and then analysed using High Performance Anion Exchange Chromatography coupled with Pulsed Amperometric Detection HPAEC-PAD (Dionex ED 40, Dionex Corp., USA) according to Wilson et al. [31]. The analytical column for carbohydrates was a Dionex Carbopac PA-20 (3 mm × 150 mm) kept at 30 ºC and eluted by on on-line generated KOH at 0.5 ml min^{-1}, whereas polyols were analysed on Dionex Carbopac MA-1 (4 mm × 250 mm) stored at 48 ºC and eluted by a gradient of NaOH (500 mM) at 0.3 ml min^{-1}. Carbohydrates and polyols were quantified using calibration curves with standard solutions [10].

Proline and proline analogues extraction and analysis

Approximately 100 mg of fresh plant material was extracted according to Naidu [17] and then analysed using High Performance Ligand-Exchange Chromatography coupled with Mass Spectrometry HPLEC-MS. N-acetyl DL-proline (Sigma-Aldrich Chemical Company) was used as internal standard [18].

Data analysis

Data were subjected to two-way analysis of variance (ANOVA) to test for the effects and interactions of temperature treatment and between clones, using the STATISTICA (Version 6, 2001, StatSoft, Tulsa, OK) data analysis software system. Data are shown as the mean ± SE in tables and figures. All statistically significant differences between treatments were tested at the $P \leq 0.05$ level.

Results

Growth response

Forty-two days of chilling had a negative effect on growth of both clones with reductions of total biomass, leaf area ratio and total root length (ca. 35%, 40% and 30%, respectively), in relation to control values (Figure 1). At the end of the experiment, ST51 plants showed significantly higher ($P < 0.01$) values of leaf area ratio than CN5 plants, whereas the CN5 clone exhibited greater total root length in both treated and control plants.

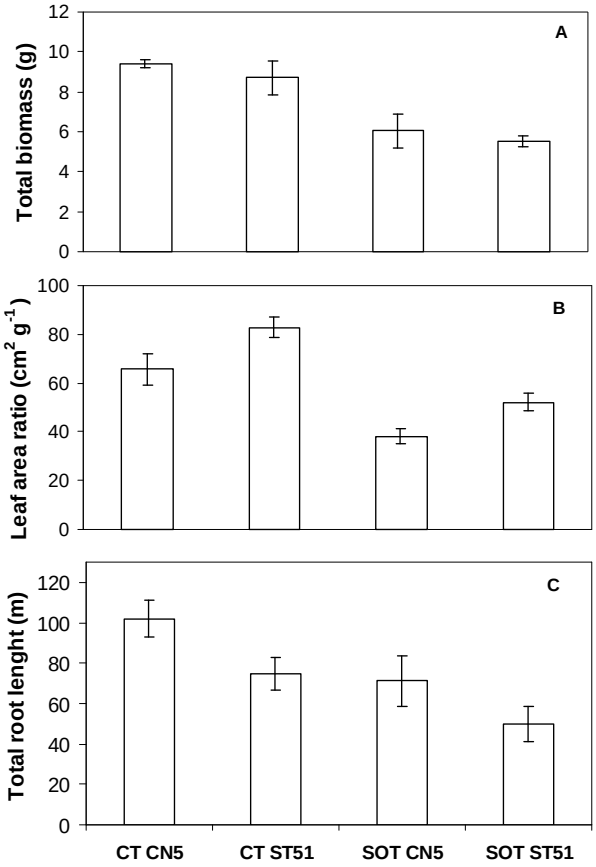

Figure 1. Some morphological characteristics of two *Eucalyptus globulus* Clones CN5 and ST51 subjected to low temperature treatment: total biomass (A), leaf area ratio (B) and total root lenght (C) evaluated at the end of the experiment (42 d after acclimation).

Plant water relation

Predawn water potentials (Ψ_{pd}) were maintained stable throughout the experiment, varying between –0.36 and –0.53 MPa (Figure 2A). Under low temperatures clone ST51

had more negative Ψ_{pd} than clone CN5 ($P < 0.05$). Control plants maintained leaf osmotic potentials around –0.58 MPa, whereas under low temperature Ψ_{π} declined significantly ($P < 0.01$) in both clones to –0.85 and –0.77 MPa in CN5 and ST51 plants, respectively. So, leaf turgor (Ψ_p) in both clones increased significantly ($P < 0.01$) during chilling (Figure 2B). The decrease of Ψ_{π} was a consequence of leaf osmotic adjustment, which mean degree ($\Delta\Psi_{\pi}^{100} = \Psi_{\pi}^{100}$ control – Ψ_{π}^{100} low temperature) throughout the experiment was of 0.18 and 0.13 MPa for CN5 and ST51 clones, respectively (Figure 2C).

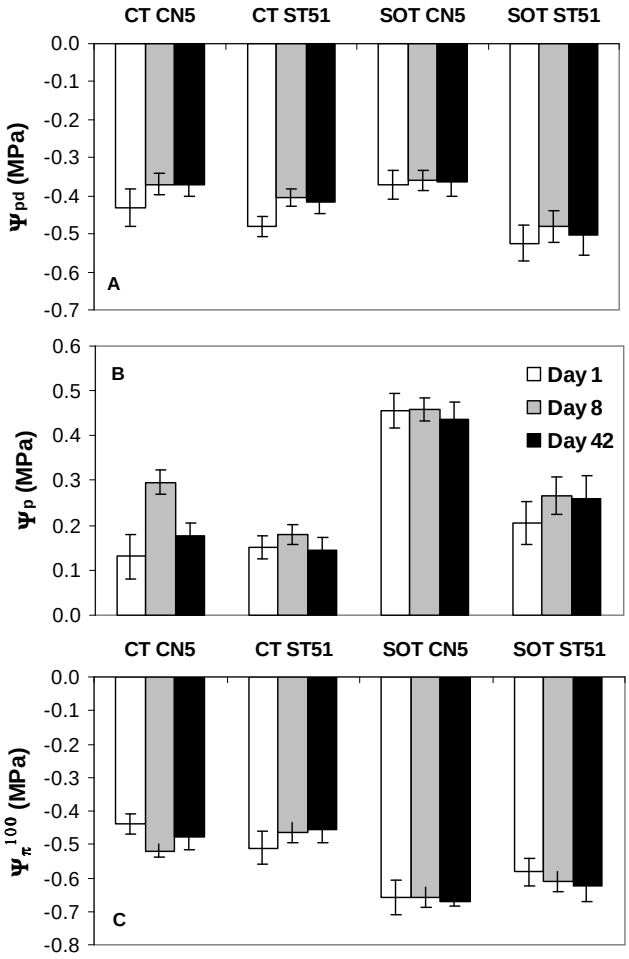

Figure 2. Predawn water potential (Ψ_{pd}, A), leaf turgor (Ψ_p, B) and osmotic potential at full turgor (Ψ_{π}^{100}, C) in leaves of *Eucalyptus globulus* Clones CN5 and ST51 at Day 1, 8 and 42 after suboptimal temperature. CT – control (24/16 ºC), SOT – suboptimal temperature (10/5 ºC). Values are mean ± SE ($n = 5$).

Carbohydrates in leaves

Acclimation led to a clear increase in the content of glucose (Glu), sucrose (Suc) and fructose (Fru) in leaves of both clones (Table I). There were significant differences between the clones ($P < 0.01$) with higher values of these carbohydrates in CN5 plants.

Table I. Galactose, glucose, sucrose, fructose, arabinose and inositol content (μmol g^{-1} dry mass) in leaves of *E. globulus* Clones CN5 and ST51 after cold acclimation (Day 1), after 8 and 42 days of suboptimal temperature. CT – control (24/16 ºC), SOT – suboptimal temperature (10/5 ºC). **P* < 0.05; ***P* < 0.01; ****P* < 0.001; ns, not significant at *P* > 0.05.

Days after acclimation	CT CN5	CT ST51	SOT CN5	SOT ST51	Significance of 2–way ANOVA		
					Clone (C)	Temp. (T)	C x T
Day 1							
Galactose	1.5±0.2	1.0±0.1	1.6±0.2	1.5±0.2	ns	ns	ns
Glucose	6.3±0.6	4.2±0.4	11±1.5	6.8±0.9	**	**	ns
Sucrose	1.9±0.4	0.7±0.2	6.1±1.2	3.0±0.5	**	***	ns
Fructose	6.8±0.6	5.9±1,2	12±0.7	7.6±1.0	**	**	ns
Arabinose	0.1±0.02	0.09±0.02	0.15±0.02	0.15±0.03	ns	ns	ns
Inositol	16±3.1	21±2.1	16±2.9	10.5±1.9	ns	0.06	0.06
Day 8							
Galactose	1.2±0.05	1.2±0.1	1.6±0.2	1.5±0.1	ns	*	ns
Glucose	7.8±0.5	7.3±1.6	16.8±4.3	14.9±2.4	ns	**	ns
Sucrose	2.2±0.5	1.9±0.7	9.1±2.6	7.1±0.6	ns	**	ns
Fructose	6.3±0.4	5.3±0.9	12.7±0.8	10.5±0.9	0.06	***	ns
Arabinose	0.07±0.01	0.11±0.03	0.14±0.01	0.11±0.01	ns	ns	ns
Inositol	20.0±1.1	20.7±1.8	14.2±1.0	12.2±0.5	ns	***	ns
Day 42							
Galactose	1.4±0.1	2.3±0.3	1.6±0.1	1.5±0.2	ns	ns	*
Glucose	5.5±0.5	8.1±0.8	12.5±4.0	9.6±1.9	ns	ns	ns
Sucrose	1.8±0.4	2.5±0.5	9.2±3.6	6.0±1.2	ns	*	ns
Fructose	5.2±0.8	6.7±0.9	11.9±0.9	10.2±0.7	ns	***	ns
Arabinose	0.11±0.05	0.15±0.02	0.16±0.02	0.15±0.02	ns	ns	ns
Inositol	10.6±1.3	22.8±0.5	18.8±1.6	19.7±2.9	**	ns	**

Table II. Galactose, glucose, sucrose, fructose and inositol content (μmol g^{-1} fresh mass) in roots of *E. globulus* Clones CN5 and ST51 after cold acclimation (Day 1), after 8 and 42 days of sub-optimal temperature. CT – Control (24/16 ºC), SOT – suboptimal temperature (10/5 ºC). **P* < 0.05; ***P* < 0.01; ****P* < 0.001; ns, not significant at *P* > 0.05.

Days after acclimation	CT CN5	CT ST51	SOT CN5	SOT ST51	Significance of 2–way ANOVA		
					Clone (C)	Temp. (T)	C x T
Day 1							
Galactose	0.8±0.3	0.41±0.2	0.49±0.11	0.91±0.16	ns	ns	ns
Glucose	2.0±0.7	0.26±0.12	1.17±0.45	2.82±0.46	ns	*	**
Sucrose	1.1±0.4	0.69±0.38	1.55±0.49	2.29±0.4	ns	*	ns
Fructose	0.5±0.1	0.21±0.13	0.44±0.06	0.71±0.18	ns	ns	*
Inositol	0.2±0.04	0.12±0.06	0.13±0.02	0.23±0.05	ns	ns	*
Day 8							
Galactose	0.19±0.03	0.33±0.1	1.45±0.5	0.38±0.08	ns	*	*
Glucose	0.27±0.05	0.43±0.13	2.67±1.31	1.42±0.67	ns	*	ns
Sucrose	0.19±0.05	0.28±0.08	2.17±1.18	1.46±0.57	ns	*	ns
Fructose	0.18±0.03	0.33±0.05	0.83±0.42	0.65±0.15	ns	*	ns
Inositol	0.09±0.02	0.05±0.01	0.36±0.13	0.24±0.06	ns	**	ns
Day 42							
Galactose	0.23±0.08	0.21±0.05	1.3±0.4	0.85±0.24	ns	**	ns
Glucose	0.63±0.2	0.5±0.16	2.94±0.72	2.98±0.98	ns	***	ns
Sucrose	0.27±0.05	0.41±0.17	3.26±0.48	2.82±0.64	ns	***	ns
Fructose	0.51±0.08	0.74±0.05	1.29±0.24	1.02±0.15	ns	***	ns
Inositol	0.21±0.03	0.19±0.04	0.92±0.28	0.7±0.28	ns	**	ns

After 8 days of chilling, in addition to the accumulation of Glu, Suc and Fru, galactose was also significantly higher in both clones as compared to controls. On the contrary, the content of inositol significantly decreased ($P < 0.001$). After 42 days of chilling, the accumulation of Suc and Fru persisted in both clones, although with no significant differences between clones. Among the accumulated carbohydrates Suc showed the highest increases throughout the experiment, whereas arabinose displayed very low contents and without significant changes with the chilling treatment.

Carbohydrates in roots

Acclimation led to a significant ($P < 0.05$) increase of root Glu (10-fold) and Suc (3-fold) content in ST51 plants (Table II). After 8 days, chilling led to a significant ($P < 0.05$) increase of all carbohydrates in both clones but more evident in CN5. After 42 days of chilling the increase of carbohydrates ($P < 0.01$) was also observed in both clones. Among the accumulated carbohydrates Suc showed the highest increases at Day 42: 13-fold and 7-fold in CN5 and ST51, respectively as compared to controls.

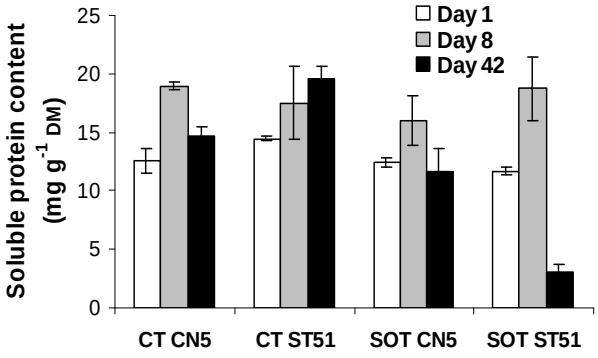

Figure 3. Soluble proteins content in leaves of *Eucalyptus globulus* Clones CN5 and ST51 at Day 1, 8 and 42 after suboptimal temperature. CT – control (24/16 ºC), SOT – suboptimal temperature (10/5 ºC). Values are mean ± SE ($n = 5$).

Soluble proteins in leaves

There were no significant changes in soluble proteins in leaves until Day 42 (Figure 3). After 42 days of chilling, soluble protein content decreased ($P < 0.001$) in both ST51 and CN5 clones (ca. 84% and 27%, respectively) as compared to controls.

Leaf pigments

Pigment content showed a statistically significant ($P < 0.001$) temperature effect after 42 days, which led to a reduction on chlorophyll contents. Clone ST51 showed higher reductions of total chlorophyll content than CN5 under low temperatures, ca. 48% and 22% in relation to control plants, respectively (Figure 4A). Fucoxanthine (Figure 4B), lutein (Figure 4C) and β-carotene (Figure 4D) content in control ST51 plants were significantly higher when compared with control CN5 plants (110%, 138% and 127%, respectively). Forty-two days of chilling led to significant reductions of fucoxanthine, lutein and β-carotene in ST51 plants (57%, 49% and 57%, respectively), whereas in CN5 plants no significant changes were observed.

Proline and proline analogues in leaves

Proline content was higher than betaine and trigonelline in leaves of both clones for control and treated plants throughout the experiment (Table III). After 8 days under 10/5 ºC there was a significant ($P < 0.01$) proline reduction, more evident in ST51 leaves than in CN5 leaves. At Day 42 of chilling trigonelline content showed a significant ($P < 0.05$) reduction in leaves of CN5 and ST51 clones (63% and 43%, respectively). There were significant differences between clones ($P < 0.05$) in trigonelline content at Day 1 (CN5 had higher content than ST51) and in proline content at Day 42 (ST51 had higher content then CN5).

Proline and proline analogues in roots

Acclimation led to a significant decrease ($P < 0.01$) in betaine root content in CN5 plants but not in ST51 (Table IV). After 8 days, proline was higher ($P < 0.06$) in plants under low temperature of both clones and trigonelline was significantly higher ($P < 0.01$) in ST51 clone as compared to CN5 plants. Forty-two days of chilling led to decrease (ca. 50%) in trigonelline in ST51 ($P < 0.05$) without changes in CN5.

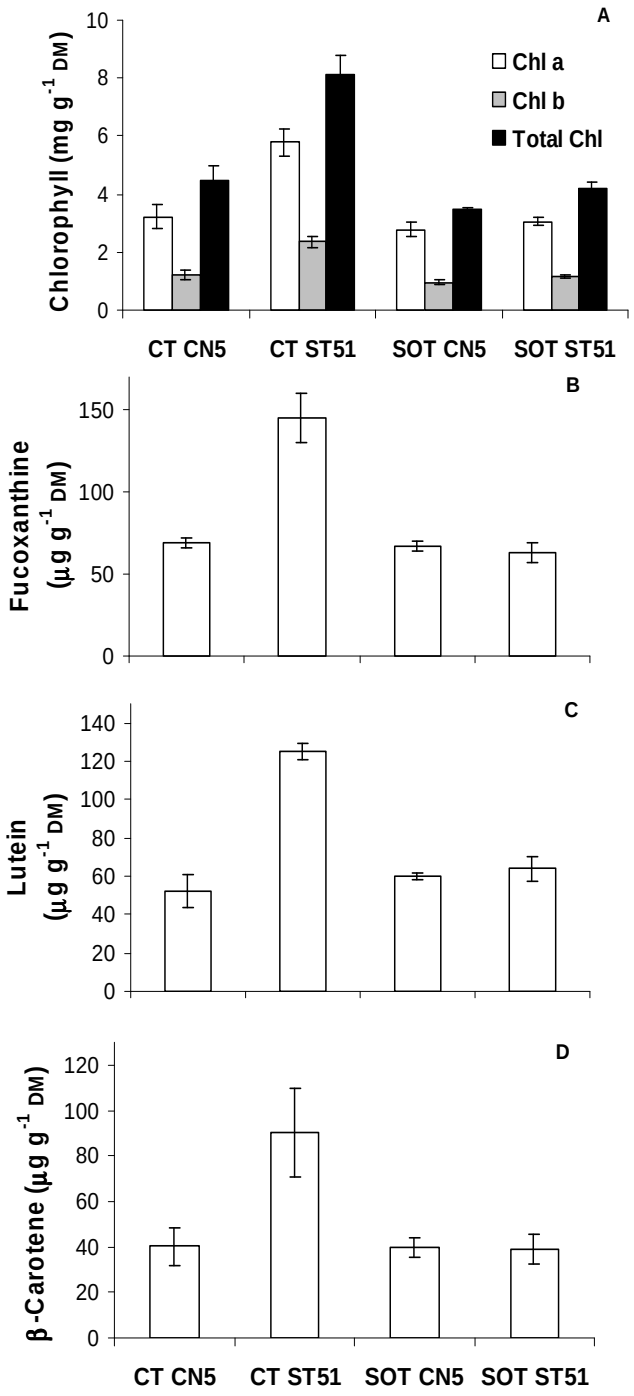

Figure 4. Chlorophyll a, b, total chlorophyll (A), fucoxanthine (B), lutein (C) and β-carotene (D) content in leaves of *Eucalyptus globulus* Clones CN5 and ST51 evaluated after 42 days of chilling. CT – control (24/16 ºC), SOT – suboptimal temperature (10/5 ºC). Values are mean ± SE (n = 5).

Table III. Proline (nmol g^{-1} dry mass) and proline analogues concentration in leaves of *E. globulus* Clones CN5 and ST51 after cold acclimation (Day 1), after 8 and 42 days of suboptimal temperature. CT – control (24/16°C), SOT – suboptimal temperature (10/5°C). **P* < 0.05; ***P* < 0.01; ****P* < 0.001; ns, not significant at *P* > 0.05.

Days after acclimation	CT CN5	CT ST51	SOT CN5	SOT ST51	Significance of 2–way ANOVA		
					Clone (C)	Temp. (T)	C x T
Day 1							
Proline	213±45	145±40	145±5	78±12	ns	ns	ns
Betaine	28±1	27±2	34±4	27±3	ns	ns	ns
Trigonelline	94±15	65±8	77±6	50±7	*	ns	ns
Day 8							
Proline	186±21	155±27	122±36	54±2	ns	**	ns
Betaine	4.6±1.1	5.0±1.1	6.4±1.3	3.4±0.7	ns	ns	ns
Trigonelline	74±5	62±9	65±5	62±9	ns	ns	ns
Day 42							
Proline	110±6	157±17	90±9	141±28	*	ns	ns
Betaine	6.7±0.9	8.9±1.8	6.0±1.9	3.2±1.2	ns	ns	ns
Trigonelline	70±11	105±21	26±2	60±17	ns	*	ns

Table IV. Proline (nmol g^{-1} fresh mass) and proline analogues concentration in roots of *E. globulus* Clones CN5 and ST51 after cold acclimation (Day 1), after 8 and 42 days of sub-optimal temperature. CT – control (24/16 ºC), SOT – suboptimal temperature (10/5 ºC). **P* < 0.05; ***P* < 0.01; ****P* < 0.001; ns, not significant at *P* > 0.05.

Days after acclimation	CT CN5	CT ST51	SOT CN5	SOT ST51	Significance of 2–way ANOVA		
					Clone (C)	Temp. (T)	C x T
Day 1							
Proline	25.1±9	8.5±1.5	5.6±3,0	9.4±0.7	ns	0.07	0.054
Betaine	12.9±2	5.5±1.4	5.6±0.6	5.5±0.7	**	**	**
Trigonelline	5.9±2.	6.4±1.1	6.1±0.7	5.5±0.6	ns	ns	ns
Day 8							
Proline	16.8±2	15.2±3.5	23.7±7	49.3±18.5	ns	0.06	ns
Betaine	17.1±2	11.7±1.6	11.1±2.1	11.4±0.09	ns	ns	ns
Trigonelline	19.8±3	10.8±1.3	20.7±2.1	15±0.4	**	ns	ns
Day 42							
Proline	13.1±1.6	23.9±7.0	10.8±2.6	24.8±8.2	0.055	ns	ns
Betaine	15.3±1.8	19.3±4.9	13.4±2.3	9.6±0.8	ns	ns	ns
Trigonelline	10.2±1.6	10.3±1.1	7.7±2.2	5.2±0.8	ns	*	ns

Lipids in leaves

Total fatty acid (TFA) content in leaves of control plants of both clones was similar (Table V). As a result of acclimation, TFA increased significantly in CN5 (40%), but not in ST51. After 8 days and 42 days of chilling, TFA content remained stable in both clones. As for the individual fatty acids, at Day 1 clone ST51 presented an increase of 14% in C18:2 and clone CN5 an increase of 14% in C18:3 as compared to their respective controls (Table V).

After 8 days, chilling led to a significant increase ($P < 0.05$) in C16:0 (20%) in ST51 leaves and in C18:2 in ST51 and CN5 leaves (36% and 45%, respectively). However, C18:3 was reduced ($P < 0.001$) in both clones (ca. 14%).

Such a tendency was also observed after forty-two days of chilling. In leaves of ST51, C16:0 significantly increased ($P < 0.01$) 32% in comparison with control plants. C18:2 increased by 31% in leaves of both clones. As for C18:3, it was reduced 15% in chilling treated ST51 plants and only 6% in CN5 plants ($P < 0.01$).

Throughout the duration of the experiment no significant changes were observed in C16:1*t* in leaves of both clones.

Lipids in roots

No changes of TFA content were observed at Day 1 in roots of both clones (Table VI). However, after 8 days and 42 days under low temperatures TFA increased ($P < 0.01$) in CN5 plants (95% and 69%, respectively).

In what concerns fatty acids, at Day 1 there was a significant increase ($P < 0.01$) of C18:3 in both clones (29% and 23% in ST51 and CN5, respectively). After forty-two days of chilling, C18:2 content increased 12% in CN5 in roots in relation to control values, while no significant changes occurred in ST51. As for C18:3, a larger increase was observed in roots of ST51 than in roots of CN5 plants (22% and 8%, respectively).

Table V. TFA (mg g^{-1} dry mass) and main fatty acids (mol %) content in leaves of *E. globulus* Clones CN5 and ST51 after cold acclimation (Day 1), after 8 and 42 days of suboptimal temperature. CT – control (24/16 ºC), SOT – suboptimal temperature (10/5 ºC). **P* < 0.05; ***P* < 0.01; ****P* < 0.001; ns, not significant at *P* > 0.05.

Days after acclimation	CT CN5	CT ST51	SOT CN5	SOT ST51	Significance of 2–way ANOVA		
					Clone (C)	Temp. (T)	C x T
Day 1							
TFA	18.1±1.8	22.3±2.5	25.3±1.7	20.8±1.7	ns	ns	*
C16:0	28.2±0.9	22.8±1.2	25.4±1.0	24.2±1.9	*	ns	ns
C16:1*t*	3.5±0.2	3.0±0.4	3.1±0.2	2.7±0.4	ns	ns	ns
C18:2	14.6±0.5	14.9±0.5	13.9±0.6	16.9±0.6	*	ns	*
C18:3	44.8±1.5	54.0±1.3	51.1±1.4	50.7±2.4	*	ns	*
Day 8							
TFA	22.7±1.8	20.6±2.1	18.8±0.9	24.5±2.4	ns	ns	ns
C16:0	23.0±0.2	20.9±0.8	23.9±0.9	25.0±1.5	ns	*	ns
C16:1*t*	3.4±0.3	3.5±0.1	4.0±0.2	3.1±0.4	ns	ns	ns
C18:2	10.7±0.4	12.9±0.2	15.5±1.2	17.6±1.3	*	***	ns
C18:3	56.3±0.6	57.3±0.8	49.1±1.5	49.1±2.0	ns	***	ns
Day 42							
TFA	14.7±0.6	18.9±1.6	17.3±0.4	17.3±2.0	ns	ns	ns
C16:0	21.3±0.6	19.2±0.8	22.4±0.5	25.4±1.4	ns	**	*
C16:1*t*	3.01±0.2	2.8±0.3	3.0±0.1	2.5±0.3	ns	ns	ns
C18:2	12.2±0.2	11.8±0.9	16.0±0.7	15.4±0.4	ns	***	ns
C18:3	57.1±0.8	61.8±1.9	53.9±0.8	52.7±2.2	ns	**	ns

Table VI. TFA (mg g^{-1} fresh mass) and main fatty acids (mol %) content in roots of *E. globulus* Clones CN5 and ST51 after cold acclimation (Day 1), after 8 and 42 days of sub-optimal temperature. CT – control (24/16 ºC), SOT – suboptimal temperature (10/5 ºC). **P* < 0.05; ***P* < 0.01; ****P* < 0.001; ns, not significant at *P* > 0.05.

Days after acclimation	CT CN5	CT ST51	SOT CN5	SOT ST51	Significance of 2–way ANOVA		
					Clone (C)	Temp. (T)	C x T
Day 1							
TFA	1.40±0.13	1.10±0.11	1.20±0.08	1.32±0.14	ns	ns	ns
C16:0	32.1±1.1	29.5±2.0	28.1±0.7	27.8±0.4	ns	*	ns
C18:2	51.3±2.3	54.5±1.6	55.8±0.9	55.4±0.3	ns	ns	ns
C18:3	7.3±0.6	7.8±0.3	9.0±0.5	10.0±0.6	ns	**	ns
Day 8							
TFA	0.75±0.08	1.09±0.11	1.47±0.05	1.20±0.17	ns	**	*
C16:0	30.2±1.6	28.3±0.7	29.6±0.7	31.3±0.6	ns	ns	ns
C18:2	49.3±2.1	52.4±1.4	51.9±0.7	50.2±1	ns	ns	ns
C18:3	11.5±2.1	11.0±1.9	12.6±0.9	12.5±1.0	ns	ns	ns
Day 42							
TFA	1.08±0.10	1.42±0.08	1.82±0.11	1.51±0.05	ns	***	**
C16:0	33.6±1.4	31.4±0.5	30.8±0.8	30.7±0.9	ns	ns	ns
C18:2	46.4±2.2	49.9±0.4	52.0±0.9	50.4±1.1	ns	*	ns
C18:3	10.9±0.8	10.6±0.3	11.8±0.4	13.0±0.7	ns	*	ns

Discussion

Our results showed that although both clones reduced growth in response to chilling, total root length of CN5 was significantly higher in comparison with ST51 in both control and treated plants. This was accompanied by a less negative predawn water potential and a higher leaf turgor in CN5 clone throughout the chilling treatment. This characteristic of CN5 plants will offer an advantage over the drought sensitive ST51 clone, not only under water-stress conditions, due to the possibility to explore more volume of soil [6], but also under cold temperatures through the benefits of higher new root regeneration.

The slowdown of growth during chilling was concomitant with an increase of carbohydrates in leaves and roots of both clones. Interestingly, it was observed at Day 1, as a result of acclimation, an increase of content of glucose, sucrose and fructose in leaves of treated plants of both clones that disappeared later on. The increase in carbohydrates in leaves may reflect the reduction in the sink strength of the aboveground plant tissues. On the other hand, this will lead to more assimilates available for root growth.

Plant water status was not affected in chilled plants, as also observed in other species [8, 33]. In fact, predawn water potentials were unaltered by chilling. Moreover, leaf turgor remained high in chilled plants of both clones, due to the decrease of osmotic potential as also observed in other *Eucalytus* species [29]. The degree of leaf osmotic adjustment given by $\Delta\Psi_{\pi}^{100}$, was initially higher in CN5 comparatively to ST51 plants, in parallel with the higher sugars accumulation observed, but similar afterwards. In fact, a higher content of carbohydrates in leaves of CN5 chilled plants compared to ST51 was only observed at Day 1.

Acclimation of the photosynthetic apparatus to chilling and to high light is well documented but the mechanisms are not completely understood. Karpinska et al. [11] showed in Scots pine that chlorophyll synthesis is temperature sensitive, and under low non-freezing temperature it decreases, due to arrest of chloroplast biogenesis. According to our pigment analysis the same phenomena happened in both clones of *E. globulus* with

higher reduction in ST51 plants; the same occurred as far as fucoxanthine, lutein and β-carotene is concerned. We can hypothesize that ST51 plants suffered more under suboptimal temperatures.

Our data also showed that the amount of proteins in clone ST51 after 42 days of chilling was reduced drastically, which may have a negative impact in the long term response of this clone to suboptimal temperatures. This was not the case for clone CN5.

The role of osmoprotectants along with carbohydrates has also been frequently assigned to proline [9], although, some authors have considered proline accumulation as a symptom of damage rather than an adaptive response [19]. On the other hand, proline contribution to osmoregulation is small in most cultivated species under stress conditions [28]. This was also the case with the *E. globulus* clones under water stress, in which the contribution of this amino acid to the osmotic potential was around 1% [24]. In the present study the lower values of Ψ_π in leaves under chilling temperatures observed in both clones were not accompanied by proline accumulation either in leaves and roots. This suggests that proline did not act as a relevant active osmolite in *Eucalyptus* tissues under suboptimal temperatures. However proline and its analogues may be associated with other roles under stress conditions: protection of cytosolic enzymes and membrane structure, stabilisation of proteins, antioxidant and storage functions [23, 25] and these roles are not ruled out. In this respect the higher values of proline observed in CN5 leaves may play a positive role in growth under low temperatures. The unsaturation of membrane lipids is considered a critical parameter for the functioning of plant membranes. Membrane lipids may suffer changes with growth temperature, particularly in what concerns linolenic acid (C18:3) [5]. An increase of unsaturation may compensate the decrease in the fluidity of membrane that is brought about by the downward shift in temperature [21], and therefore sustained activity of membrane-bound enzymes at lower temperature.

At the beginning of chilling treatment (Day 1), an increased lipid amount (TFA) and a concomitant increase of C18:3 percentage were observed in CN5 leaves, probably due to an activation of lipid synthesis resulting in more unsaturated lipid molecular species. A higher degree of unsaturation could increase membrane fluidity and constitute

an advantage for leaves of CN5 clone under cold conditions. The fact that TFA amounts were not reduced by cold stress suggests that molecular adaptation of lipids occurs apparently without lipid loss, resulting in a decrease of membrane unsaturation.

In roots a higher degree of unsaturation (as inferred from the increase in C18:3 percentage) was observed in treated plants of both clones, which may depend on compositional changes resulting from lipid turnover [22]. An enhanced lipid synthesis in CN5 roots was observed but not in ST51, suggesting a better preservation of root metabolism in CN5 under low temperature.

In summary, our data showed that both CN5 and ST51 *E. globulus* clones have the ability to acclimate to chilling temperatures. Changes, observed in carbohydrates and membrane lipid content following acclimation may play a role in the resistance of *E. globulus* to chilling. Differences between clones were observed in soluble protein and pigment content which were more stable in CN5 than ST51 under chilling temperatures. It also was apparent that CN5 presented a less negative predawn water potential (Ψ_{pd}) and a higher leaf turgor than ST51 throughout the chilling treatment. As a result of acclimation, an increase of total lipids (TFA) and concomitant increase of C18:3 in leaves of CN5 clone may confer to this clone a better performance under chilling temperatures. Although clone CN5 did not present a higher growth under chilling relative to ST51, it showed a lower inhibition of root growth and a greater carbon allocation to roots.

Acknowledgements

Shvaleva A. was supported by FCT, Lisbon (Grant SFRH/BPD/5667/2001) and Costa e Silva F. by FCT, Lisbon (Grant SFRH/BD/13211/2003). The authors thank to Dr. Colin Mc Vey for English revision of the manuscript and for Viveiros Aliança for providing the plant material.

References

[1] Allen C.F., Good P., Davis H.S., Chisum P., Fowler S.D., Methodology for the separation of plant lipids and application to spinach leaf and chloroplast lamellae, J. Am. Oil Chem. Soc. 43 (1966) 223–230.

[2] Almeida M.H., Chaves M.M., Silva J.C., Cold acclimation in eucalypt hybrids, Tree Physiol. 14 (1994) 921–932.

[3] Bradford M.N., A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding, Anal. Biochem. 72 (1976) 248–254.

[4] Chaves M.M., Pereira J.S., Maroco J.P., Understanding plant response to drought – from gene to the whole plant, Funct. Plant Biol. 30 (2003) 1–26.

[5] Cossins A.R., Homeoviscous adaptation of biological membranes and its functional significance, in: Cossins A.R. (Ed.), Temperature Adaptation of Biological Membranes, Portland Press, London, 1994, pp. 63–76.

[6] Costa e Silva F., Shvaleva A, Maroco J.P., Almeida M.H., Chaves M.M., Pereira J.S., Response to water stress in two *Eucalyptus globulus* clones differing in drought tolerance, Tree Physiol. 24 (2004) 1165–1172.

[7] Easterling D.R., Meehl G.A, Parmesan C., Changnon S.A., Karl T.R., Mearns L.O., Climate extremes: observations, modeling, and impacts, Science 289 (2000) 2068–2074.

[8] Equiza M.A., Mirave J.P., Tognetti J.A., Morphological, anatomical and physiological responses related to differential shoot vs. root growth inhibition at low temperature in spring and winter wheat, Ann. Bot. 87 (2001) 67–76.

[9] Gibon, Y., Ronan S., Larher F., Proline accumulation in canola leaf discs subjected to osmotic stress in related to the loss of chlorophylls and to the decrease of mitochondrial activity, Physiol. Plant. 110 (2000) 469–476.

[10] Guignard C., Jouve L., Bogeat-Triboulot M.B., Dreyer E., Hausman J.-F., Hoffmann L., Analysis of carbohydrates in plants by high performance anion-exchange chromatography coupled with electrospray mass spectrometry, J. Chromatogr. 1085 (2005) 137–42.

[11] Karpinska B., Karpinski S., Krol M., Hällgren J.F., Developmental regulation of light independent transcription of nuclear and plastid encoded chloroplast proteins in Scots pine, in: Mathis P. (Ed.), Photosynthesis: from light to biosphere, Kluwer Acad. Publishers, Dordrecht, 1995, pp. 571–574.

[12] Levitt J., Responses of plants to environmental stresses: chilling, freezing and high temperature stresses, 2nd ed., Academic Press, New York, 1980.

[13] Lyons J.M., Chilling injury in plants, Ann. Rev. Plant Physiol. 24 (1973) 445–466.

[14] Metcalfe L.D., Schemitz A.A., Pelka J.R., Rapid preparation of fatty-acid esters from lipids for gas chromatographic analysis, Anal. Chem. 38 (1966) 514–515.

[15] Miranda P.M.A., Valente M.A., Tomé A.R., Trigo R., Coelho M.F., Aguiar A., Azevedo E.B., O clima de Portugal nos séculos XX e XXI, in: Santos F.D., Miranda P. (Eds), Alterações Climáticas em Portugal. Cenários, Impactes e Medidas de Adaptação, Gradiva, Lisboa, 2006, pp. 45–113.

[16] Murata N., Ishizaki-Nishizawa O., Higashi S., Hayashi H., Tasaka Y., Nishida I., Genetically engineered alteration in the chilling sensitivity of plants, Nature 356 (1992) 710–713.

[17] Naidu B.P., Separation of sugars, polyols, proline analogues, and betaines in stressed plant extracts by high performance liquid chromatography and quantification by ultraviolet detection, Aust. J. Plant Physiol. 25 (1998) 793-800.

[18] Oufir M., Schulz N., Sha Valli Khan P.S., Wilhelm E., Burg k., Hausman J.F., Hoffmann L., Guignard C., Analysis of proline, glycine betaine analogues and quaternary ammonium compounds in plants by high-performance ligand-exchange chromatography coupled with electrospray mass spectrometry for environmental stress studies, submitted to J. Chrom. A (2008).

[19] Pérez-Alfocea F., Santa-Cruz A., Guerrier G., Bolarin M.C., NaCl stress-induced organic solute changes on leaves and calli of *Lycopersicon esculentum* L. pennelli and their interspecific hydrofit, J. Plant Physiol. 143 (1994) 106–111.

[20] Pham Thi A.T., Borrel-Flood C., Vieira da Silva J., Justin A.M., Mazliak P., Effects of water stress on lipid metabolism in cotton leaves, Phytochem. 24 (1985) 723–727.

[21] Raison J.K., The influence of temperature-induced phase changes on kinetics of respiratory and other membrane-associated enzymes, J. Bioenerg. 4 (1973) 258–309.

[22] Scotti Campos P., Quartin V., Ramalho J.C., Nunes M.A., Electrolyte leakage and lipid degradation account for cold sensitivity in leaves of *Coffea* sp. plants, J. Plant Physiol. 160 (2003) 283–292.

[23] Shomer-Ilan A, Jones G.P., Paleg L.G., In vitro thermal and salt stability of pyruvate kinase are increased by proline analogs and trigonelline, Aust. J. Plant Physiol. 18 (1991) 279–286.

[24] Shvaleva A., Costa e Silva F., Breia E., Jouve L., Hausman J-F., Almeida M.H., Maroco J.P., Rodrigues M.L., Chaves M.M., Pereira J.S., Metabolic response to water deficit in two *Eucalyptus globulus* clones with contrasting drought sensitivity, Tree Physiol. 26 (2006) 239–248.

[25] Smirnoff N., Environment and plant metabolism: flexibility and acclimation, Univ. Lancaster, UK, 1996.

[26] Somerville C., Direct tests of the role of membrane lipid composition in low-temperature-induced photoinhibition and chilling sensitivity in plants and cyanobacteria, Proc. Natl. Acad. Sci. 92 (1995) 6215–6218. Physiol. 25 (1998) 793–800.

[27] Steponkus P.L., Role of the plasma-membrane in freezing-injury and cold-acclimation, Ann. Rev. Plant Physiol. Plant Mol. Biol. 35 (1984) 543–584.

[28] Taylor C.B., Proline and water deficit. Ups, downs, ins and outs, Plant Cell, 8 (1996) 1221–1224.

[29] Valentini R., Scarascia Mugnozza G., Giordano E., Kuzminsky E., Influence of cold hardening on water relations of three *Eucalyptus* species, Tree Physiol. 6 (1990) 1–10.

[30] Van Huylenbroeck J.M., Debergh P.C., Physiological aspects in acclimatization of micropopagated plants, Plant Tissue Culture Biotechnol. 2 (1996) 136–141.

[31] Wilson L., Amy C., Gordon H., Johnson B., Miller J., Water quality changes during soil aquifer treatment of tertiary e.uent, Water Environ. Res. 67 (1995) 371–376.

[32] Wright S.W., Jeffrey S.W., Mantoura R.F.C., Llewellyn C.A., Bornland T., Repta D., Welschmeyer N., Improved HPLC method for the analysis of chlorophylls and carotenoids from marine phytoplankton, Marine Ecol. Progress Series, 77 (1991) 183–196.

[33] Woodward F.I., Friend A.D., Controlled environment studies on the temperature responses of leaf extension in species of *Pea* with diverse altitudinal ranges, J. Exp. Bot. 39 (1988) 411-420.

CAPÍTULO 6

Responses to chilling and freezing in two *Eucalyptus globulus* clones with contrasting drought resistance

6. Responses to chilling and freezing in two *Eucalyptus globulus* clones with contrasting drought resistance

Summary

We tested the hypothesis that *E. globulus* genotypes more resistant to dry environments might also exhibit higher cold tolerances than drought-sensitive plants. The effect of chilling and freezing was evaluated in acclimated and unacclimated ramets of a drought-resistant clone (CN5) and a drought-sensitive clone (ST51) of *Eucalyptus globulus* Labill. Responses measured included changes in concentrations of soluble sugars, several antioxidant enzymes, anthocyanins, leaf water and osmotic potentials, stomatal conductance, rate of photosynthesis and leaf electrolyte leakage. Progressively lowering air temperatures (from 24/16° C to 10/-2 ºC, day/night) led to acclimation of both *E. globulus* clones. Acclimated ramets exhibited higher photosynthetic rates and stomatal conductances and lower membrane relative injuries when compared to unacclimated ramets. Moreover, low temperatures led to significant increases of soluble sugars and antioxidant enzymes activity (GR, APX and SOD) of both clones in comparison to plants grown at control temperature (24/16 ºC). On the other hand, none of the clones, either acclimated or not exhibited signs of photoinhibition under low temperatures and moderate light. The main differences in the responses to low temperatures between the two clones resulted mainly from differences in carbon metabolism, including a higher accumulation of soluble sugars in the drought-resistant CN5 as well as a higher capacity for osmotic regulation, as compared to the drought-sensitive clone ST51. Although membrane injury suggested that both clones had the same inherent freezing tolerance before and after cold acclimation, the results support the hypothesis that the drought-resistant clone had a greater cold tolerance at intermediate levels of acclimation than the drought-sensitive clone. A higher capacity to acclimate in a shorter period can allow a clone to maintain an undamaged leaf surface area along sudden frost events increasing

growth capacity. Moreover it can enhance survival chances in frost-prone sites expanding the plantations range of more adaptive clones.

Keywords: acclimation, cold tolerance, low temperatures

Introduction

Eucalyptus globulus plantations continue to increase annually and worldwide due to high growth rate and pulping properties (Carbonnier 2004). However, this has resulted in a tendency to include sites for planting with more demanding climatic conditions, such as those with more frequent frost conditions. Even in Mediterranean areas episodic occurrences of below-zero temperatures are important, limiting the expansion of *E. globulus* plantations. Moreover, because young *Eucalyptus* plants are less tolerant to extreme environmental conditions than adults, the degree of frost stress tolerance can determine successful establishment and thereby limit species/genotypes distributions to certain regions or microsites. In addition, with the predicted increase in weather variability induced by global climate change (IPCC 2001), it is expectable that plants will be subjected to sudden frost events with variable hardening possibilities.

Plants face three major problems when exposed to low temperature: an alteration in the spatial organization of the cell membranes, a slowing down of their chemical and biochemical reactions and, under freezing conditions, changes in water status and availability (Sakai and Larcher 1987). Alterations induced by low temperatures comprise changes in the concentrations of a wide range of metabolites, including sugars, protective proteins, as well as modification of cell membranes, changes in hormone levels and alterations in gene expression (Zhu et al. 2007). Moreover, exposure to low temperatures may cause mild oxidative stress, which generates and accumulates reactive oxygen species (ROS) capable of causing oxidative damage to proteins, DNA, and lipids (Apel and Hirt 2004). Generally, cold acclimation ensures protection to plants through enzymatic ROS-scavenging mechanisms (Wise 1995). However, when plants are rapidly subjected to low temperature without acclimation, damages to the enzymatic ROS-

scavengers might be too high and excess ROS can initiate cell death. Furthermore, because relatively mild below-zero temperatures can be lethal even for the more hardy species in the unacclimated state, timing of acclimation is crucial for plant survival in a given area, sometimes independent of the tolerance level to be acquired.

A large amount of research on cold stress and tolerance mechanisms accumulated in the last decades (Levitt 1980; Sakai and Larcher 1987) although there are not many published data on the frost tolerance of *E. globulus* (Almeida et al. 1994; Tibbits et al. 2006; Volker et al. 1994). Recently, it was shown that winter-frost tolerance is a trait with considerable variation within E. *globulus* with most tolerant families tolerating late-winter temperatures 1.4 ºC colder than the overall families average (Tibbits et al. 2006). Thus, it is expected that contrasting genotypes respond differently to low temperatures in the process of cold acclimation that takes place on the time scale of days or weeks as a result of a combination of physiological and metabolic changes under decreasing temperatures. Moreover, plant responses to low temperatures show many similarities with responses to water deficits, suggesting that cold resistance and drought resistance mechanisms often share the same pathways (Atkin et al. 2005; Beck et al. 2007; Sung et al. 2003).

For these reasons we hypothesised that, under a Mediterranean-type climate, *E. globulus* genotypes more resistant to dry environments might also exhibit higher frost tolerances than drought-sensitive plants. If this is true, it will allow a clone less susceptible to drought to maintain an undamaged leaf surface area along the frost periods, thus allowing those plants to enter spring with a higher capacity for growth than more drought sensitive plants. In addition, detailed physiological information of the stress-response of clones is necessary for development of breeding programs and is essential to support decisions to allocate clones to different climatic regions. In previous work (Costa e Silva et al. 2004; Shvaleva et al. 2006), the two clones under study were shown to differ in their sensitivity to water deficits (CN5 was drought resistant and ST51 was drought sensitive) and in their capacity of long-term acclimation to chilling (Costa e Silva et al. 2007; Shvaleva et al. 2008). Under chilling conditions, the better performance of clone CN5 was associated with maintenance of root growth, higher water status and anthocyanin concentration compared with clone ST51. The aims of the present work were

to: (1) evaluate the effect of rapid acclimation to chilling and freezing in physiological and biochemical properties of two clones of *E. globulus* with contrasting responses to drought, (2) compare the responses to chilling and freezing in clones without acclimation and (3) test whether the drought-resistant clone is less affected by freezing than the drought-sensitive clone.

Material and Methods

Plant material and treatments

We studied two *Eucalyptus globulus* Labill. clones (CN5-drought resistant and ST51-drought sensitive). Ramets produced by rooted cuttings of both clones were grown in plastic containers containing peat (60%) and styrofoam (40%), and were transplanted at four months to pots (1.5 l) filled with peat and vermiculite (2/1 v/v). One month after transplanting, 30 cuttings per clone were transferred from the nursery to a growth chamber with controlled conditions (24/16 °C, day/night) (control plants). Another 18 cuttings per clone were placed in a growth chamber subjected to an acclimation period of 14 days with a gradual temperature decrease (1° C per day) from 24/16° C to 10/6 °C (day/night) (acclimation treatment). After acclimation, plants were subjected to a further decline in night temperature during 9 days and measurements were done at days 1, 5 and 9 with temperatures of 10/6, 10/2 and 10/-2 ºC (day/night), respectively. In addition, another group of plants (unnaclimated) were measured in the same days, after transfer 24 h earlier from the control to the low temperature chamber (direct chilling/freezing treatment). Both growth chambers had similar lighting systems (c.a. 220 µmol m^{-2} s^{-1} at the canopy level), a photoperiod of 12/12 hours (day/night) and relative humidity of approximately 60%. To avoid effects caused by microenvironmental differences (light and temperature gradients), the plants were sorted by treatment and moved to the neighbouring position every other day. The experiment was carried during January 2007. All plants were watered to the point of runoff in the first day and then watered twice per week (Mondays and Fridays) according to evapotranspiration values.

Water relations

Leaf xylem water potential was measured at predawn (Ψ_{pd}) with a Scholander-type pressure chamber (PMS Instruments, Corvallis, OR) on one leaf from four plants per treatment. Soon after measuring ψ_{pd}, leaf discs (7 mm diameter) were taken of each leaf, frozen in liquid nitrogen and stored at – 80ºC for later determination of osmotic potential (Ψ_{π}). After thawing the samples at room temperature, Ψ_{π} was measured using C-52 chambers (2 h for equilibration) connected to a Wescor HR-33T dew-point microvoltmeter (Wescor, INC Logan, UTAH, USA) operating in the dew-point mode. The chambers were calibrated with standard NaCl solutions. The prevailing room temperature during the measurements was 20 ± 1 °C.

Gas exchange and chlorophyll fluorescence

Gas exchanges were measured with a LI-6400 portable photosynthesis system (Li-Cor, Lincoln, NE) in one full-expanded leaf from four plants per treatment at midday (solar time). Measurements took place under the light conditions of the controlled environment chambers and temperature fixed in 15 ºC in the low temperature treatments. Pre-dawn maximal photochemical efficiency, *F*v/*F*m, was assessed using a Mini-PAM fluorometer (Walz GmbH, Effeltrich, Germany) under chamber conditions. The same leaves used in gas exchange were measured, taking care to avoid the midrib.

Artificial freezing and membrane injury

Three leaf discs per plant (10 mm in diameter) were punched from full-expanded leaves of six plants per treatment (control and acclimated) and placed in test tubes. Racks of test tubes were placed inside a controlled freezer (Aralab, Lisbon, Portugal) in baths containing an aqueous ethylene glycol solution at 2 ºC. A controlled freezing program followed a constant cooling and thawing rate of 4 ºC h^{-1} and a 2 h exposure to five different target freezing temperatures (-2.6, -3.4, -4.6, -6.2 and -8 ºC). When the

temperature of the bath was at –2 ºC, approximately 0.5 g of finely crushed ice (from deionised water) was added to each tube to make contact with the leaf disk. Membrane injury was determined by measuring cell conductivity after artificial freezing. Electrolyte conductivity of 15 ml deionised water containing leaf discs was measured after 24 h at 25 ºC (T_1) with a K220 conductivity meter (Consort, Turnhout, Belgium). The samples were then boiled in an autoclave at 120 °C for 10 min, held at 25 °C for 2 h and total electrolyte conductivity was measured (T_2). Relative injury (RI) was expressed as a ratio of electrolyte conductivity measured after freezing treatment relative to maximum electrolyte conductivity, $RI = (T_1/T_2) \times 100$.

Pigments analysis

For chlorophyll extraction two leaf discs (10 mm diameter) were incubated in 1.0 mL of dimethyl sulfoxid (DMSO) at 25 ºC during 24 h. After incubation the extracts were transferred to glass cuvettes and measured in a spectrophotometer (DU-79, Beckman, Germany) on DMSO solutions. Total chlorophyll concentration was determined according to (Richardson et al. 2002). Anthocyanins were extracted in 1 mL of methanol-HCl (0.1% HCl, v/v) at -16 ºC and kept at 4 ºC in the dark during 24 h. After 24 h the extracts were transferred to glass cuvettes and the absorbance was read on a methanol-HCl solution. Anthocyanins concentration was calculated according to (Murray and Hackett 1991) with correction of the effect of chlorophylls.

Soluble sugars

Soluble sugars in leaves were assayed by the anthrone method (Robyt and White 1987) as described in (Shvaleva et al. 2006). Frozen leaf discs (0.02 g) were ground with a cold mortar and pestle in liquid N_2 with 1 mM of 70% (v/v) ethanol. The homogenate was thermomixed twice at 60° C for 30 min, centrifuged at 14,000 g for 5 min and the supernatant used for determination with a spectrophotometer (U-2001; Hitachi, Japan).

Antioxidant enzymes

Sample leaves were excised and immediately immersed in liquid nitrogen and stored at -80 °C. The extract for enzymatic analyses was obtained by the suspension of the plant material (300 mg) in 5.0 mL of potassium phosphate buffer (0.1 M, pH 6.8). After centrifugation for 10 minutes at 20,000 g, the supernatant was collected and stored at -80° C. The concentration of soluble protein in the extracts was determined according to Bradford (1976) with bovine serum albumin (BSA) as protein standard. For determination of glutathione reductase (EC 1.6.4.2) and ascorbate peroxidase (EC 1.11.1.11) activity in leaves (0.5 g fresh mass) the general procedure of Foyer and Halliwell (1976) and Nakano and Asada (1981), respectively, were used with some modifications (Shvaleva et al. 2006). For GR the assay medium contained 500 mM HEPES (Sigma Chemical) (pH 8.0), 0.25 mM EDTA (Sigma Chemical), 2 mM NADPH (Sigma Chemical), 20 mM oxidized glutathione (GSSG) and 100 µl extract. Control rates were obtained in the absence of GSSG or NADPH. For APX the assay medium contained 50mM KH_2 PO_4 /K_2 HPO_4 (pH 7.0), 20 mM H_2O_2, 8 mM ascorbate and 100 µl extract. Control rates were obtained in the absence of extract, ascorbate, or H_2O_2.

The determination of the activity of superoxide dismutases (SOD, EC1.15.1.1) considered the capacity of the enzyme to inhibit the photoreduction of nitroblue tetrazolium chloride (NBT). The enzyme activity was determined according to Giannopolitis and Ries (1977) and Del Longo et al. (1993) by mixing 50 µL of crude extract to a solution containing 13 mM metionine, 75 µM p-nitro blue tetrazolium chloride, 100 nM EDTA and 2 µM riboflavin in a 50 mM sodium phosphate buffer (pH 7.8). It was expressed as U mg^{-1} protein, considering that one SOD unit (U) was defined as the amount of enzyme required to inhibit 50% of the NBT photoreduction.

Statistical analysis

Data were subjected to two-way analysis of variance (ANOVA) to test for the effects and interactions of temperature treatment and clone, using the STATISTICA (version 6, StatSoft, Inc. 2001) data analysis software system. Whenever means

difference was significant Student-Newman-Keuls test was used to identify differences between treatments. All variables were tested for normality and homogeneity of variances. Differences were considered statistically significant at $P \leq 0.05$.

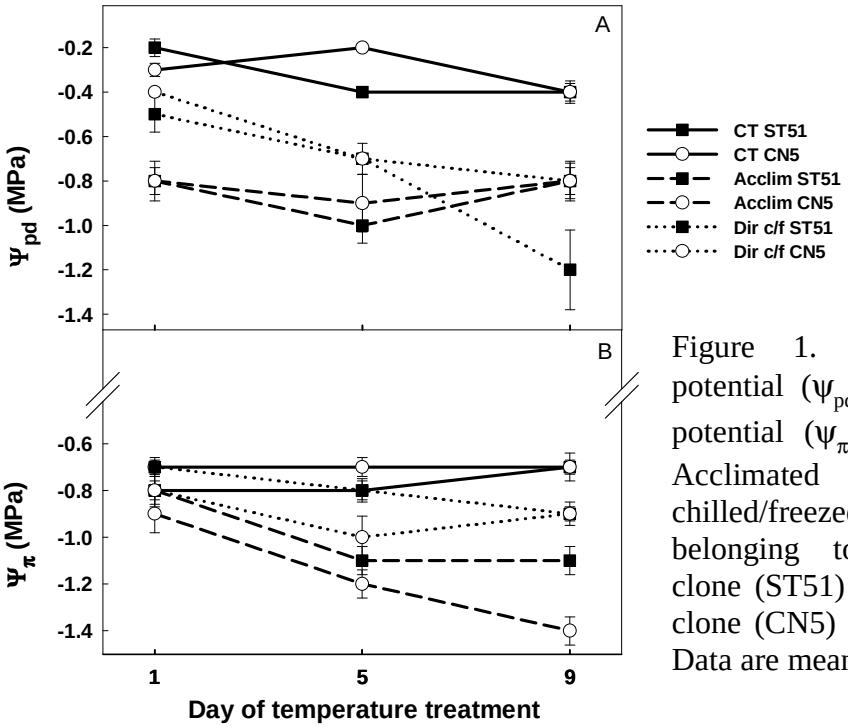

Figure 1. Predawn leaf water potential (ψ_{pd}; A) and leaf osmotic potential (ψ_{π}; B) in Control (CT), Acclimated (Acclim) and Direct chilled/freezed (Dir c/f) plants belonging to a drought-sensitive clone (ST51) and a drought-resistant clone (CN5) of *Eucalyptus globulus*. Data are means ± SE (n=4).

Results

Water relations

Low temperatures led to a significant ($P < 0.001$) decrease in Ψ_{pd} in all the treatments as compared to control plants (Figure 1A). Acclimated plants maintained stable Ψ_{pd} values throughout the experiment (ranging from -0.75 to -0.99 MPa) but much lower than those of control plants (varying between -0.24 and -0.41 MPa). However, a decrease in Ψ_{pd} was associated with the decrease in temperature along the experiment in the direct chilling/freezing treatment. From 10/6 ºC (day 1) to 10/2 ºC (day 5) Ψ_{pd}

declined on average from -0.47 to -0.71 MPa in both clones subjected to low temperatures without acclimation. With lower temperatures, *i.e.* at 10/-2 ºC (day 9), a further decline to -1.16 MPa was observed in ST51 clone, whereas in CN5 clone there was only a slight decline to – 0.83 MPa, a value similar to that presented by the acclimated plants.

Control plants of both clones presented similar and constant Ψ_π values throughout the experiment. Conversely, acclimated plants of both clones showed a decrease in Ψ_π at 10/2 and 10/-2 ºC in comparison to control ($P < 0.001$), although more marked ($P < 0.05$) in CN5 than in ST51 plants (Figure 1B). In addition, CN5 subjected to direct chilling/freezing also exhibited a decrease in Ψ_π from 10/6 to 10/-2 ºC, whereas ST51 only decreased Ψ_π at 10/-2 ºC.

Gas exchange and chlorophyll fluorescence

Stomatal conductance declined significantly ($P < 0.05$) in both clones and all the treatments when temperatures attained 10/2 ºC (day 5). At 10/-2 ºC (day 9) there was a further decrease ($P < 0.001$), with acclimated and unaclimated plants presenting g_s values corresponding to 18% and 7% from those of control plants, respectively (Figure 2A). In day 1 and 5, clone ST51 exhibited higher values of g_s ($P < 0.001$) than CN5 clone in all treatments.

Similarly to g_s, there was a significant effect ($P < 0.001$) of low temperature in *A* of both clones at 10/2 ºC (day 5) causing an average decrease of 23% in comparison to control either in acclimated or unacclimated plants (Figure 2B). Moreover, at 10/-2 ºC (day 9) there was a further decline in *A*, although with a clear effect of acclimation ($P < 0.001$). Acclimated plants showed a 47% decrease in A as compared to control plants, whereas a higher reduction (79%) was observed in unnaclimated plants. Throughout the experiment, Clone ST51 showed higher *A* than CN5 clone ($P < 0.01$) either in control or acclimation treatments. In response to direct chilling/freezing, CN5 plants showed higher *A* than ST51 plants at 10/6 ºC ($P < 0.05$), whereas at 10/2 ºC it was Clone ST51 that showed higher *A* ($P < 0.01$).

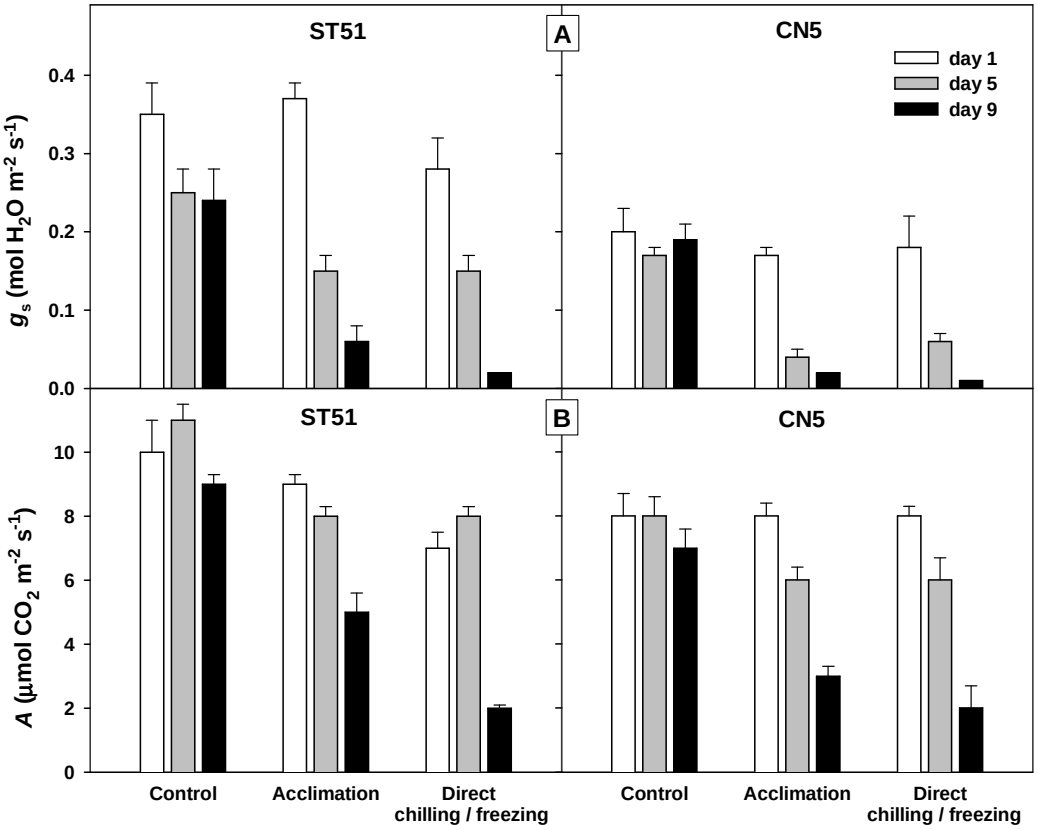

Figure 2. Midday stomatal conductance (g_s; A) and net photosynthesis (A; B) in Control, Acclimated and Direct chilled/freezed plants belonging to a drought-sensitive clone (ST51) and a drought-resistant clone (CN5) of *Eucalyptus globulus*. Control treatment was measured at 24/16 ºC and Acclimation and Direct chilling/freezing treatments were measured at 10/6, 10/2 and 10/-2 ºC in Day 1, 5 and 9, respectively. Data are means ± SE (n=4).

Low temperatures led to a decrease of Fv/Fm (P < 0.001) in both acclimated and unacclimated plants although within relatively constant and high values (Fv/Fm > 0.75) throughout the experiment indicating that no photoinhibition occurred (Figure 3). There were not significant differences between clones although ST51 showed a stronger decrease in Fv/Fm than CN5 in the direct chilling/freezing treatment.

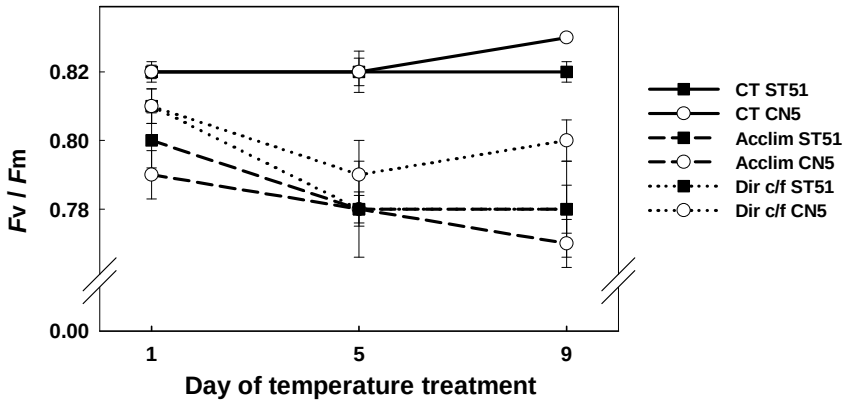

Figure 3. Pre-dawn maximal photochemical efficiency (*F*v/*F*m) in Control (CT), Acclimated (Acclim) and Direct chilled/freezed (Dir c/f) plants belonging to a drought-sensitive clone (ST51) and a drought-resistant clone (CN5) of *Eucalyptus globulus*. Data are means ± SE (*n*=4).

Membrane injury

Both clones showed similar membrane relative injury when subjected to negative temperatures ranging from –2.6 to –8 ºC (Figure 4). Leaf discs of control plants grown at 24/16 ºC and successively subjected to lower negative temperatures showed a gradual increase in membrane damages attaining in both clones an average relative injury of 50% at the temperature –3.8 ± 0.1 ºC. On the other hand, acclimation led to a significant ($P < 0.001$) decrease in membrane damage in relation to control plants, with acclimated plants maintaining low relative injury up to –8 ºC (<25%).

Leaf pigments

Progressively lower temperatures of 10/6, 10/2 and 10/-2 ºC led to an average decrease in anthocyanin concentration of 29% in acclimated plants of ST51 in comparison to control plants. On the contrary, anthocyanin concentration significantly increased ($P < 0.05$) in acclimated CN5 plants at 10/6 and 10/2 ºC (18% and 40%, respectively) and remained stable at 10/-2 ºC (Table 1). Direct chilling/freezing treatment

of 10/6 and 10/2 ºC led to significant ($P < 0.01$ and $P < 0.05$, respectively) reductions of anthocyanin concentration that were similar in both clones. However, when directly subjected to 10/-2 ºC, only ST51 plants showed a decrease in anthocyanins of 28% in comparison to control plants. In addition, control plants of Clone ST51 showed higher anthocyanin concentration than CN5 plants, leading to a clone x treatment interaction effect at day 1 and 5 ($P < 0.05$ and $P < 0.01$, respectively).

Increasingly lower temperatures from 10/6, to 10/2 and to 10/-2 ºC led to a decrease in total chlorophyll concentration of 28%, 28% and 44%, respectively, in acclimated plants of ST51 in comparison to control plants. Moreover, a significant interaction occurred between clone and treatment effects all along the experiment ($P < 0.05$), so that, total chlorophyll increased in acclimated CN5 plants at 10/6 and 10/2 ºC (17% and 44%, respectively) and remained stable at 10/-2 ºC (Table 2), indicating a protective role of acclimation in this clone. Direct chilling/freezing treatment of 10/6 and 10/2 ºC led to small reductions in total chlorophyll ($P < 0.05$) in both clones. However, at 10/-2 ºC only ST51 plants showed a decrease in total chlorophyll of 30% in comparison to control plants.

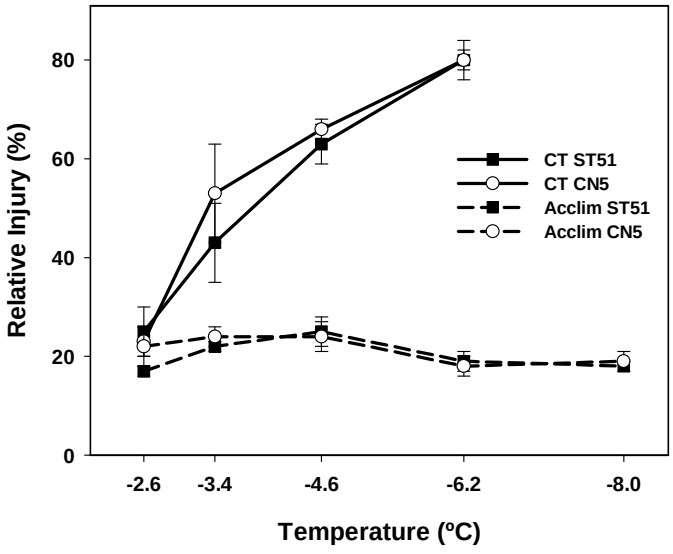

Figure 4. Membrane relative injury in Control (CT) and Acclimated (Acclim) plants belonging to a drought-sensitive clone (ST51) and a drought-resistant clone (CN5) of *Eucalyptus globulus*. Data are means ± SE (n=6).

Table 1. Anthocyanin concentration in Control, Acclimation and Direct chilling/freezing treatments with plants belonging to a drought-sensitive clone (ST51) and a drought-resistant clone (CN5) of *Eucalyptus globulus* evaluated throughout the experiment (Day 1, 5 and 9). Data are means ± SE (*n*=4). Symbols: *, **, *** represent statistical significance at *P* = 0.05, 0.01 and 0.001, respectively; ns = nonsignificant at *P* = 0.05.

Temperature treatment	Anthocyanin concentration (μg m^{-2})		
	day 1 (10/6 ºC)	day 5 (10/2 ºC)	day 9 (10/-2 ºC)
Clone ST51			
Control (24/16 ºC)	173 ± 6	183 ± 5	173 ± 7
Acclimation	130 ± 3	143 ± 5	105 ± 19
Direct freezing/chilling	123 ± 18	150 ± 11	125 ± 12
Clone CN5			
Control (24/16 ºC)	130 ± 12	118 ± 15	115 ± 13
Acclimation	153 ± 9	165 ± 6	120 ± 17
Direct freezing/chilling	97 ± 8	103 ± 19	128 ± 22
Significance of 2-way ANOVA			
Clone (C)	ns	**	ns
Temperature regime (T)	**	ns (0.06)	ns
C x T	*	**	ns

Soluble sugars

Acclimated plants showed an increase in soluble sugars concentration at 10/2 and 10/-2 ºC ($P < 0.001$) in both clones (Table 3). However, contrary to ST51, CN5 showed an earlier increase in soluble sugars at 10/6 ºC ($P < 0.05$) and, moreover, significant higher concentrations at 10/2 ($P < 0.001$) and 10/-2 ºC ($P < 0.05$). In response to direct chilling/freezing, differences between clones were clearer, with CN5 showing increases in soluble sugars of 45%, 69% and 34% at 10/6, 10/2 and 10/-2 ºC, respectively, whereas ST51 clone only slightly increased sugars (23%) at 10/-2 ºC in comparison to control plants.

Table 2. Total chlorophyll concentration in Control, Acclimation and Direct chilling/freezing treatments with plants belonging to a drought-sensitive clone (ST51) and a drought-resistant clone (CN5) of *Eucalyptus globulus* evaluated throughout the experiment (Day 1, 5 and 9). Data are means ± SE (*n*=4). Symbols: *, **, *** represent statistical significance at *P* = 0.05, 0.01 and 0.001, respectively; ns = nonsignificant at *P* = 0.05.

Temperature treatment	Total chlorophyll concentration (mg m^{-2})		
	day 1 (10/6 ºC)	day 5 (10/2 ºC)	day 9 (10/-2 ºC)
Clone ST51			
Control (24/16 ºC)	310 ± 16	351 ± 16	321 ± 19
Acclimation	225 ± 5	253 ± 11	180 ± 31
Direct freezing/chilling	254 ± 39	268 ± 18	224 ± 22
Clone CN5			
Control (24/16 ºC)	228 ± 22	205 ± 25	205 ± 23
Acclimation	267 ± 18	294 ± 15	213 ± 33
Direct freezing/chilling	159 ± 18	188 ± 32	237 ± 47
Significance of 2-way ANOVA			
Clone (C)	ns	*	ns
Temperature regime (T)	ns	ns	*
C x T	**	***	*

Antioxidant enzymes

Acclimation treatment to progressively lower temperatures of 10/6, 10/2 and 10/-2 ºC led to similar responses of both clones with significant increases (at least $P < 0.01$) in all antioxidant enzymes activity in comparison to control plants (Figure 5). From all enzymes, ascorbate peroxidase activity showed the larger increases in relation to control values, particularly at 10/6 and 10/2 ºC (100%, on average). The only significant difference between clones occurred in GR with ST51 plants showing higher activity than CN5 plants all along the experiment ($P < 0.001$ at 10/2 and 10/-2 ºC) (Figure 5A).

Clone responses to direct chilling/freezing were not clear, with only slight increases of antioxidant enzymes activity. Thus, both clones subjected to low temperatures without acclimation only increased GR activity at 10/2 ºC by 26%, on

average, in comparison to control plants ($P < 0.001$). As well, SOD activity in both clones only significantly increased ($P < 0.01$) at 10/-2 ºC by 35%, on average, as compared to control plants (Figure 5C). There were no significant differences between clones under direct chilling/freezing treatment although CN5 showed a clear increase in APX activity of 77% and 69% at 10/6 and 10/2 ºC, respectively, in opposition to ST51 (Figure 5B).

Table 3. Soluble sugars concentration in Control, Acclimation and Direct chilling/freezing treatments with plants belonging to a drought-sensitive clone (ST51) and a drought-resistant clone (CN5) of *Eucalyptus globulus* evaluated throughout the experiment (Day 1, 5 and 9). Data are means ± SE (n=4). Symbols: *, **, *** represent statistical significance at P = 0.05, 0.01 and 0.001, respectively; ns = nonsignificant at P = 0.05.

Temperature treatment	Soluble sugars concentration (mmol m^{-2})		
	day 1 (10/6 ºC)	day 5 (10/2 ºC)	day 9 (10/-2 ºC)
Clone ST51			
Control (24/16 ºC)	31 ± 3.2	21 ± 1.9	30 ± 2.8
Acclimation	26 ± 2.1	38 ± 2.9	46 ± 4.2
Direct freezing/chilling	23 ± 2.0	25 ± 4.8	36 ± 2.5
Clone CN5			
Control (24/16 ºC)	23 ± 2.5	26 ± 3.8	32 ± 3.7
Acclimation	33 ± 0.4	50 ± 4.7	55 ± 2.7
Direct freezing/chilling	33 ± 5.5	44 ± 3.5	43 ± 2.3
Significance of 2-way ANOVA			
Clone (C)	ns	***	*
Temperature regime (T)	ns	***	***
C x T	*	ns	ns

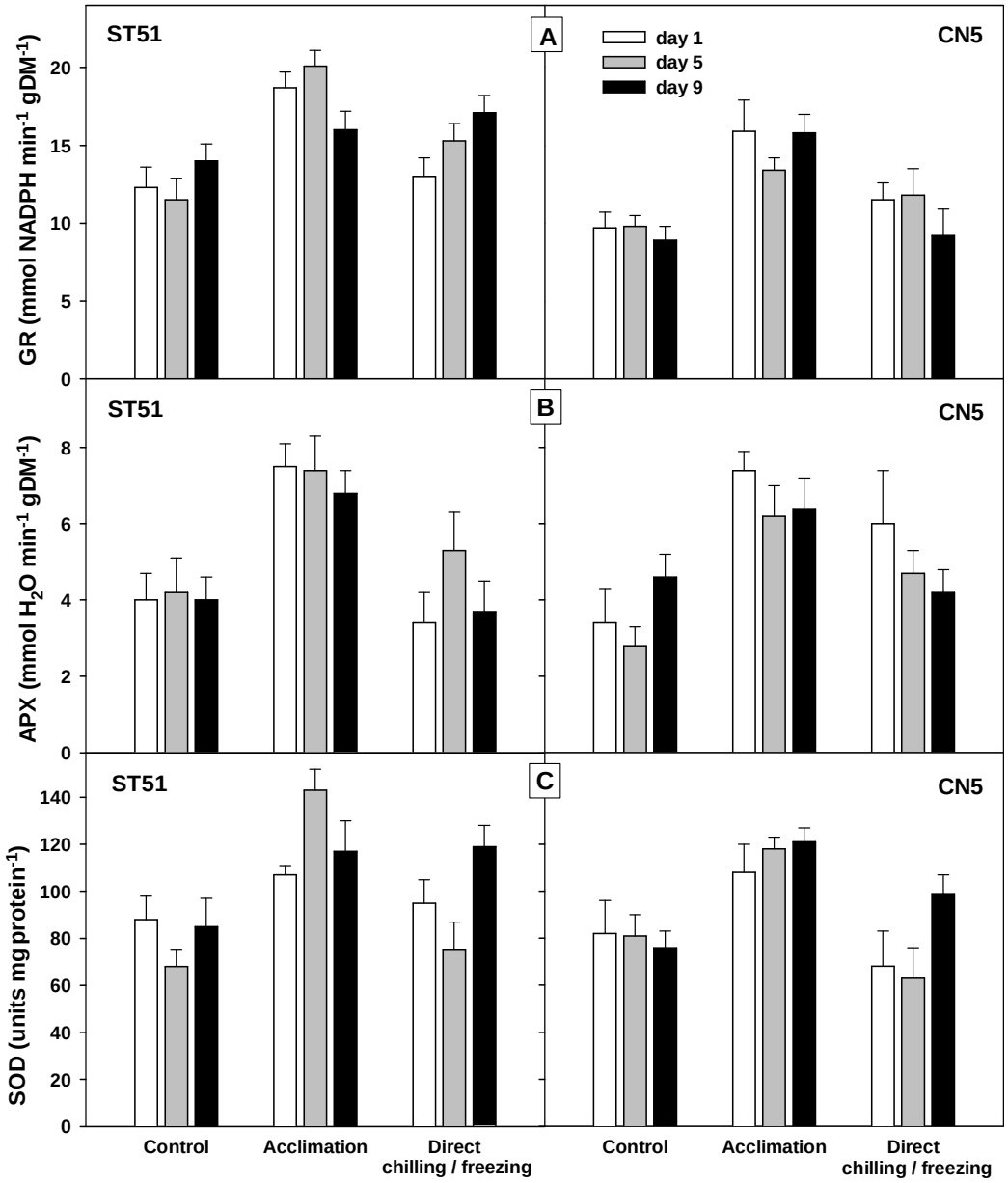

Figure 5. Glutathione reductase (GR; A), ascorbate peroxidase (APX; B) and superoxide dismutase (SOD; C) in Control, Acclimated and Direct chilled/freezed plants belonging to a drought-sensitive clone (ST51) and a drought-resistant clone (CN5) of *Eucalyptus globulus*. Control treatment was measured at 24/16 ºC and Acclimation and Direct chilling/freezing treatments were measured at 10/6, 10/2 and 10/-2 ºC in Day 1, 5 and 9, respectively. Data are means ± SE (*n*=4).

Discussion

In this experiment, given that both shoots and roots were subjected to low temperatures, we can expect some low root temperature influence on leaf metabolism as generally observed – e.g., on stomatal conductance (Almeida et al. 1994). However, an unrealistic drought stress during the day can be dismissed since our low day temperatures prevented high evaporative demands. On the other hand a 10 ºC gradient between soil and air temperatures is a likely event in clear winter days of the Mediterranean climate due to slow soil warming.

The water status of a plant influences its frost resistance via the cell sap concentrations and the degree of hydration of the protoplasm (Sakai and Larcher 1987). In our experiment, relative water content was not altered by any treatment (data not shown). However, predawn leaf water potential exhibited significant changes. Clone CN5 when subjected to freezing temperatures (10/-2 ºC, day 9) without acclimation was able to maintain Ψ_{pd} whereas ST51 did not (Figure 1A). In addition, clone CN5 had a higher capability for osmotic regulation either in acclimation or direct chilling/freezing treatments along the progressively lower temperatures (Figure 1B). A decrease in Ψ_{π}, lowering the freezing point of tissues, can decrease the amount of ice formed, and therefore improve the avoidance of freeze-induced dehydration (Sakai and Larcher 1987). Good correlations between Ψ_{π} and frost resistances were found for *Eucalyptus* sp. (Valentini et al. 1990) although not always associated to a significant decrease in the temperature of ice formation but to an increased ability to endure extracelular ice formation (Almeida et al. 1994). In parallel with the decrease in Ψ_{π} there was a significant increase in leaf soluble sugars concentration more noticeable in CN5 plants. Particularly, CN5 showed a rapid (24 h) increase of soluble sugars in unacclimated plants as compared to ST51 (Table 3).

A strong relationship between leaf soluble carbohydrate accumulation and cold tolerance have been reported for conifers (Greer et al. 2000; Ögren 1997; Repo et al. 2004; Tinus et al. 2000) and for *Eucalyptus* sp. (Almeida et al. 1994; Leborgne et al. 1995b; Leborgne et al. 1995a). Furthermore differences in cold tolerance between

genotypes attributed to different carbohydrate metabolisms and related to the effects of soluble sugars accumulation in cryoprotection have also been reported (Bourion et al. 2003; Leborgne et al. 1995b). The amount of soluble sugars present corresponds to a balance between the rate of photosynthesis, consumption by respiration and export to parts of the plant that are growing. A higher accumulation of soluble sugars in the drought-resistant CN5 clone in spite of its lower photosynthetic rates suggests a more efficient reprogramming of carbon metabolism under low temperatures in CN5 than in ST51. In addition, the significantly lower rates of respiration of CN5 clone under chilling temperatures (data not shown) can as well have contributed to his higher acclimation capacity as it was proposed by Ögren (1997) for several conifers, where sugar consumption led to significant decreases in freezing tolerance.

Both clones showed similar membrane relative injury when subjected to freezing temperatures ranging from –2.6 to –8 ºC (Figure 4). These results indicate that acclimation resulted in fully acclimated plants since both clones maintained very low values of electrolyte leakage until –8 ºC. In addition we can conclude that both clones have the same inherent freezing tolerance before cold acclimation. The observed values of membrane injury of acclimated and unacclimated plants are in accordance with the reported in the literature for *E. globulus* (Almeida et al. 1994; Tibbits et al. 2006). Furthermore, given the observed differences in the time course of sugar accumulation between the two clones and its well correlation with the development of freezing tolerance, we can speculate that CN5 clone can acclimate more rapidly and has higher tolerances for intermediate levels of acclimation than ST51 clone.

Low temperatures are known to inhibit rates of photosynthesis through limiting enzymatic rates of the Calvin Cycle. In addition, a light-dependent decrease and slowly reversible retardation in photosynthetic efficiency or rate may occur following low temperature events, a process termed cold-induced photoinhibition. It has been shown that cold-induced photoinhibition and photodamage under high levels of irradiance affects *E. globulus* development after transplanting (Close et al. 2000). Moreover, when the environmental conditions do not promote carbon fixation, even moderate light may lead to high levels of photoinhibition (Close and Beadle 2005; Govindachary et al. 2004).

In our experiment, there was a significant acclimation effect with acclimated plants maintaining higher net photosynthesis at 10/-2 ºC than unacclimated plants. The reduction in photosynthetic rates caused by low temperatures is strongly influenced by the degree of acclimation of plant material (Greer et al. 2000; Weger et al. 1993). Results similar to ours were found in *E. globulus* by Davidson et al. (2004). However, in spite of the great decrease in photosynthesis (Figure 2B) throughout the experiment none of the clones, either acclimated or unacclimated, exhibited signs of photoinhibition assessed by photochemical efficiency evolution (Figure 3). Thus, *E. globulus* seem to not suffer from cold-induced photoinhibition under moderate levels of light as it has been observed in other species (Govindachary et al. 2004) or in *Eucalyptus sp*. under high irradiances (Close et al. 2000; Close et al. 2001; Egerton et al. 2000). Furthermore, mild frost temperatures alone seem not to be sufficient to cause photoinhibition in *E. globulus* and we can assume that the observed decrease of photosynthetic rates were solely due to the effects of low temperature either by stomatal or non-stomatal limitations. In addition, considering that a lower light utilisation capacity due to cold-induced decrease in photosynthesis requires the dissipation of greater levels of excess light energy, we can conclude that in our conditions, nonphotochemical, heat-dissipation mechanisms were sufficient to deal with excess excitation. Nevertheless, it has been suggested that *E. globulus* can have a lower inherent capacity for the dissipation of excess energy than more cold tolerant *Eucalyptus sp.* (Close et al. 2000).

Many plants accumulate anthocyanin under chilling temperatures. Several functions for anthocyanin have been proposed, including as an anti-oxidant, a UV protectant and as providing protection from visible light through light attenuation (Close and Beadle 2003; Steyn et al. 2002). In opposition to our previous results under long-term (42 days) chilling (Costa e Silva et al. 2007) there was no significant accumulation of anthocyanins in this short-term experiment (9 days) exposure to low temperatures. Nevertheless, under chilling temperatures (day 1 and 5), acclimated CN5 plants showed a trend for anthocyanin accumulation in opposition to ST51 plants. This trend of anthocyanin accumulation as well as the maintenance of total chlorophyll in CN5 and chlorophyll degradation in ST51 plants is in accordance with a lower predisposition for photoinhibition in CN5 as was observed in *E. nitens* and *E. globulus* (Close et al. 2000).

Under optimal environmental conditions, light reaction and electron transport in photosynthesis leads to minimal production of ROS, which otherwise, can cause some photooxidative damage to chloroplasts, carotenoids, and proteins. To cope with stress, plants developed an enzymatic antioxidant defence system which enhancement is often correlated with the acquiring of cold tolerance (Tao et al. 1998; Verhoeven et al. 2005; Wise 1995). In the present study we examined whether antioxidant enzymes capacity are involved in cold tolerance and differ between drought resistant and drought sensitive clones of *Eucalyptus globulus*.

Acclimation to low temperatures led to similar responses of both clones with significant increases in GR, APX and SOD activity in comparison to control plants. Thus, the combined action of these three enzymes seems to have a protective role against chilling induced active oxygen species. In contrast, this clear enhancement in antioxidant capacity of both clones under low temperatures was not observed under drought stress (Shvaleva et al. 2006) where leaf enzymes activity was not significantly altered. Nevertheless, the absence of clone differences in leaf antioxidant enzymes activity after full acclimation suggests that differences in cold tolerance between the clones are not associated with antioxidant capacity. On the other hand, we cannot disregard possible differences between the two clones in antioxidant capacities at intermediate levels of acclimation. In fact, the results of direct chilling/freezing treatment after 24 h of cold exposure showed a significant increase in APX activity only in CN5 clone which can consequently result in different antioxidant capacities between the two clones, or at least, suggest different resistance pathways in each clone when unacclimated.

When we compare the responses of both clones to low temperatures with responses to drought stress from a previous experiment (Costa e Silva et al. 2004; Shvaleva et al. 2006) some common trends arise. In response to low temperatures and to water deficit, the drought-resistant CN5 clone maintained higher leaf water status (higher predawn and midday leaf water potentials) and decreased Ψ_π significantly more than the drought-sensitive ST51 clone (Costa e Silva et al. 2004; Shvaleva et al. 2006). In addition, under drought and chilling conditions, CN5 ramets exhibited a lesser inhibition of root growth than ST51 (Costa e Silva et al. 2007; Costa e Silva et al. 2004). The higher capacity to

deliver water to the leaves given by a more extensive root system is an advantageous trait under water deficits conditions. It is worth mentioning that the higher growth rate of ST51 ramets in optimal conditions (Costa e Silva et al. 2004) can also be related to his higher cold sensitivity. In fact, strong tradeoffs often exist between growth and cold hardiness, even if these negative genetic correlations are weaker and more variable within than among populations (Howe et al. 2003).

In summary, our data indicate that progressively lowering air temperatures (to 10/-2 ºC, day/night) led to acclimation of both *E. globulus* clones. Acclimated ramets exhibited higher photosynthetic rates and stomatal conductances and lower membrane relative injuries when compared to unacclimated ramets. Moreover, low temperatures led to significant increases of soluble sugars and antioxidant enzymes activity (GR, APX and SOD) of both clones in comparison to plants grown at control temperature (24/16 ºC). On the other hand, none of the clones, either acclimated or not exhibited signs of photoinhibition under low temperatures and moderate light. The main differences in the responses to low temperatures between the two clones resulted mainly from differences in carbon metabolism, including a higher accumulation of soluble sugars in the drought-resistant CN5 as well as a higher capacity for osmotic regulation, as compared to the drought-sensitive clone ST51. Although membrane injury suggested that both clones had the same inherent freezing tolerance before and after cold acclimation, the results support the hypothesis that the drought-resistant clone had a greater cold tolerance at intermediate levels of acclimation than the drought-sensitive clone. A higher capacity to acclimate in a shorter period can allow a clone to maintain an undamaged leaf surface area along sudden frost events increasing growth capacity. Moreover it can enhance survival chances in frost-prone sites expanding the plantations range of more adaptive clones.

Acknowledgements

This research was carried out with financial support from the POCI 2010 and FSE. F. Costa e Silva and A. Shvaleva were supported by FCT, Lisbon, grant SFRH/BD/13211/2003 and grant SFRH/BPD/5667/2001, respectively. M. F. Ortuño was granted a Postdoctoral research fellowship from the Spanish Ministry of Education.

References

Almeida, M.H., M.M. Chaves and J.C. Silva 1994. Cold acclimation in eucalypt hybrids. Tree Physiology. 14:921-932.

Apel, K. and H. Hirt 2004. Reactive oxygen species: metabolism, oxidative stress and signal transduction. Annual Review of Plant Biology. 55:373–399.

Atkin, O.K., D. Bruhn, V.M. Hurry and M.G. Tjoelker 2005. The hot and the cold: unravelling the variable response of plant respiration to temperature. Functional Plant Biology. 32:87-105.

Beck, E.H., S. Fettig, C. Knake, K. Hartig and T. Bhattarai 2007. Specific and unspecific responses of plants to cold and drought stress. Journal of Biosciences. 32:501-510.

Bourion, V., I. Lejeune-Hénaut, N. Munier-Jolain and C. Salon 2003. Cold acclimation of winter and spring peas: carbon partitioning as affected by light intensity. European Journal of Agronomy. 19:535-548.

Bradford, M. N. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Carbonnier, L. 2004. The future of Eucalyptus plantations. *In* Eucalyptus in a Changing World Eds. N.M.G. Borralho, J.S. Pereira, C. Marques, J. Coutinho, M. Madeira and M. Tomé. RAIZ - Instituto de Investigação da Floresta e Papel, Aveiro, Portugal, p. 29.

Close, D.C. and C.L. Beadle 2003. The Ecophysiology of Foliar Anthocyanin. The Botanical Review. 69:149–161.

Close, D.C. and C.L. Beadle 2005. Xanthophyll-cycle dynamics and rapid induction of anthocyanin synthesis in Eucalyptus nitens seedlings transferred to photoinhibitory conditions. Journal of Plant Physiology. 162:37-46.

Close, D.C., C.L. Beadle, P.H. Brown and G.K. Holz 2000. Cold-induced photoinhibition affects establishment of Eucalyptus nitens(Deane and Maiden) Maiden and Eucalyptus globulus Labill. Trees. 15:32–41.

Close, D.C., C.L. Beadle and M.J. Hovenden 2001. Cold-induced photoinhibition and foliar pigment dynamics of Eucalyptus nitens seedlings during establishment. Australian Journal of Plant Physiology. 28:1133–1141.

Costa e Silva, F., A. Shvaleva, M.H. Almeida, M.M. Chaves and J.S. Pereira 2007. Responses to chilling of two Eucalyptus globulus clones with contrasting drought resistance. Functional Plant Biology. 34:793-802.

Costa e Silva, F., A. Shvaleva, J.P. Maroco, M.H. Almeida, M.M. Chaves and J.S. Pereira 2004. Responses to water stress in two *Eucalyptus globulus* clones differing in drought tolerance. Tree Physiology. 24:1165-1172.

Davidson, N.J., M. Battaglia and D.C. Close 2004. Photosynthetic responses to overnight frost in Eucalyptus nitens and E. globulus. Trees. 18:245–252.

Del Longo, O.T., C.A. González, G.M. Pastori and V.S. Trippi 1993. Antioxidant defenses under hyperoxygenic and hyperosmotic conditions in leaves of two lines of maize with differential sensitivity to drought. Plant and Cell Physiology. 34:1023-1028.

Egerton, J.J.G., J.C.G. Banks, A. Gibson, R.B. Cunningham and M.C. Ball 2000. Facilitation of Seedling Establishment: Reduction in Irradiance Enhances Winter Growth of Eucalyptus pauciflora. Ecology. 81:1437-1449.

Foyer, C. and B. Halliwell 1976. The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta. 133:21-25.

Giannopolitis, C.N. and S.K. Ries 1977. Superoxide Dismutases: I. Occurrence in Higher Plants. Plant Physiology. 59:309-314.

Govindachary, S., N.G. Bukhov, D. Joly and R. Carpentier 2004. Photosystem II inhibition by moderate light under low temperature in intact leaves of chilling-sensitive and -tolerant plants. Physiologia Plantarum. 121:322-333.

Greer, D.H., L.A. Robinson, A.J. Hall, K. Klages and H. Donnison 2000. Frost hardening of Pinus radiata seedlings: effects of temperature on relative growth rate, carbon balance and carbohydrate concentration. Tree Physiology. 20:107-114.

Howe, G.T., S.N. Aitken, D.B. Neale, K.D. Jermstad, N.C. Wheeler and T.H.H. Chen 2003. From genotype to phenotype: unraveling the complexities of cold adaptation in forest trees. Canadian Journal of Botany. 81:1247–1266.

IPCC 2001. Climate change 2001: the scientific basis. Contribution of working group I to the third assessment report of the Intergovernmental Panel on Climate Change (IPCC). Eds. J.T. Houghton, Y. Ding, D.J. Griggs, M. Noguer, P.J. van der Linden and D. Xiaosu. Cambridge University Press, Cambridge.

Leborgne, N., C. Teulieres, B. Cauvin, S. Travert and A.M. Boudet 1995b. Carbohydrate content of Eucalyptus gunnii leaves along an annual cycle in the field and during induced frost-hardening in controlled conditions. Trees. 10:86-93.

Leborgne, N., C. Teulieres, S. Travert, M.P. Rols, J. Teissie and A.M. Boudet 1995a. Introduction of specific carbohydrates into Eucalyptus gunnii cells increases their freezing tolerance. European Journal of Biochemistry. 229:710-717.

Levitt, J. 1980. Responses of plants to environmental stresses. Vol. I Chilling, freezing, and high temperature stress. Academic Press, London. 497 p.

Murray, J.R. and W.P. Hackett 1991. Difydroflavonol reductase activity in relation to differential anthocyanin accumulation in juvenile and mature phase Hedera helix L. Plant Physiology. 97:343-351.

Nakano, Y. and K. Asada 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant and Cell Physiology. 22:867-880.

Ögren, E. 1997. Relationship between temperature, respiratory loss of sugar and premature dehardening in dormant Scots pine seedlings. Tree Physiology. 17:47-51.

Repo, T., I. Leinonen, A. Ryyppö and L. Finér 2004. The effect of soil temperature on the bud phenology, chlorophyll fluorescence, carbohydrate content and cold hardiness of Norway spruce seedlings. Physiologia Plantarum. 121:93-100.

Richardson, A.D., S.P. Duigan and G.P. Berlyn 2002. An evaluation of noninvasive methods to estimate foliar chlorophyll content. New Phytologist. 153:185-194.

Robyt, J. and B.J. White 1987. Biochemical Techniques. Theory and Practice. Eds. L. McCloud and J. Marsh. Wadsworth, Inc., Belmont, California. 302 p.

Sakai, A. and W. Larcher 1987. Frost survival of plants. Responses and adaptation to freezing stress. *In* Ecological Studies 62. Springer-Verlag, Berlin. 321 p.

Shvaleva, A., F. Costa e Silva, E. Breia, L. Jouve, J.F. Hausman, M.H. Almeida, J.P. Maroco, M.L. Rodrigues, J.S. Pereira and M.M. Chaves 2006. Metabolic responses to water deficit in two *Eucalyptus globulus* clones with contrasting drought sensitivity. Tree Physiology. 26:239-248.

Shvaleva, A., F. Costa e Silva, P. Scotti, M. Oufir, J.F. Hausman, C. Guignard, P. Ramos, M.H. Almeida, M.L. Rodrigues, J.S. Pereira and M.M. Chaves 2008. Physiological and biochemical responses to low non-freezing temperature of two Eucalyptus globulus clones differing in drought resistance. Annals of Forest Science. 65:DOI: 10.1051/forest:2007087.

Steyn, W., S. Wand, D. Holcroft and G. Jacobs 2002. Anthocyanins in vegetative tissues: a proposed unified function in photoprotection. New Phytologist. 155:349-361.

Sung, D., F. Kaplan, K. Lee and C.L. Guy 2003. Acquired tolerance to temperature extremes. Trends in Plant Science. 8:179-187.

Tao, D., G. Öquist and G. Wingsle 1998. Active Oxygen Scavengers during Cold Acclimation of Scots Pine Seedlings in Relation to Freezing Tolerance. Cryobiology. 37:38–45.

Tibbits, W.N., T.L. W., R.H. Gary and N.M.G. Borralho 2006. Genetic variation in frost resistance of Eucalyptus globulus ssp. globulus assessed by artificial freezing in winter. Australian Journal of Botany. 54:521–529.

Tinus, R.W., K.E. Burr, N. Atzmon and J. Riov 2000. Relationship between carbohydrate concentration and root growth potential in coniferous seedlings from three climates during cold hardening and dehardening. Tree Physiology. 20:1097-1104.

Valentini, R., G. Scarascia Mugnozza, E. Giordano and E. Kuzminsky 1990. Influence of cold hardening on water relations of three Eucalyptus species. Tree Physiology. 6:1–10.

Verhoeven, A.S., A. Swanberg, M. Thao and J. Whiteman 2005. Seasonal changes in leaf antioxidant systems and xanthophyll cycle characteristics in Taxus x media growing in sun and shade environments. Physiologia Plantarum. 123:428-434.

Volker, P.W., J.V. Owen and N.M.G. Borralho 1994. Genetic variances and covariances for frost tolerance in Eucalyptus globulus and E. nitens. Silvae Genetica. 43:366–372.

Weger, H.G., S.N. Silim and R.D. Guy 1993. Photosynthetic acclimation to low temperature by western red cedar seedlings. Plant, Cell and Environment. 16:711-717.

Wise, R.R. 1995. Chilling-enhanced photooxidation: The production, action and study of reactive oxygen species produced during chilling in the light. Photosynthesis Research. 45:79-97.

Zhu, J., C.-H. Dong and J.-K. Zhu 2007. Interplay between cold-responsive gene regulation, metabolism and RNA processing during plant cold acclimation. Current Opinion in Plant Biology. 10:290-295.

CAPÍTULO 7

Conclusões gerais

7. Conclusões gerais

7.1. Principais diferenças entre os clones

Os vários estudos realizados e expostos nos capítulos anteriores permitem-nos distinguir os dois clones, de contrastante sensibilidade à seca, com base nas suas características morfológicas e fisiológicas responsáveis pelos diferentes mecanismos de resposta aos stresses impostos. Claramente, a diferença mais importante entre os dois clones encontra-se ao nível do metabolismo do carbono e na partição de biomassa pelas componentes da planta. O clone CN5, quer em resposta ao défice hídrico (Capítulo 2), quer em resposta às baixas temperaturas (Capítulo 4), mostrou um maior desenvolvimento do sistema radicular e uma maior capacidade de rapidamente aumentar a proporção de biomassa distribuída para as raízes do que o clone ST51. Ao contrário, o clone ST51 mostrou distribuir mais biomassa para as folhas, em detrimento das raízes, em condições de secura ou de baixas temperaturas. Em resultado desta diferente distribuição de biomassa, o clone CN5 exibiu sempre menores valores da razão entre a área foliar e a área radicular do que o clone ST51.

Para além de um diferente investimento de biomassa nas raízes, os clones distinguiram-se também pelas características dos seus sistemas radiculares. O clone ST51, em condições bem regadas, apresentou uma maior condutância e condutividade hidráulica das raízes (K, L_p) e uma maior condutância foliar específica (LSC) do que o clone CN5. No entanto, o clone CN5 durante a imposição do stress hídrico mostrou uma menor limitação no fornecimento de água às folhas, com valores mais altos de K e LSC, do que o clone ST51 (Capítulo 2). As maiores perdas de condutância hidráulica das plantas ST51 em stress hídrico, em comparação com as plantas CN5, sugerem ainda uma maior susceptibilidade deste clone a embolismos e cavitações. Por outro lado, diferenças entre os dois clones na morfologia das raízes finas podem também contribuir para as suas diferentes capacidades hidráulicas. Em particular, é de referir que o clone ST51 mostrou ter raízes finas com maior comprimento especifico – *i.e.* com maior comprimento por unidade de biomassa – do que o clone CN5 (Capítulo 4), sendo esta uma característica de espécies com crescimento rápido e estando associada a maiores valores de condutância hidráulica.

Também ao nível das relações hídricas da folha se encontraram diferenças entre os clones. Quer em condições de défice hídrico ou de baixas temperaturas, o clone CN5 manteve um estado hídrico foliar mais favorável (maiores valores de Ψ_{pd} e de Ψ_{md}) e mostrou maiores reduções do potencial osmótico do que o clone ST51 o que lhe permitirá uma maior capacidade de osmoregulação. Por outro lado, ao nível celular e das respostas metabólicas ao défice hídrico e no processo de aclimatação a baixas temperaturas, as diferenças entre os clones não foram tão evidentes. Por exemplo, em resposta ao stress hídrico, os clones mostraram semelhantes alterações nos componentes osmóticos e na actividade do sistema de protecção antioxidante e, em resposta a baixas temperaturas, uma semelhante capacidade de aclimatação (Capítulo 3 e 5). No entanto, apesar de os resultados sugerirem uma igual sensibilidade dos dois clones às temperaturas negativas, antes e depois de aclimatados, os resultados suportam também a hipótese de o clone CN5 ter uma maior tolerância ao frio do que o ST51 para graus intermédios de aclimatação (Capítulo 6).

As plantas respondem fortemente às mudanças do meio de crescimento, no entanto, quando testadas em semelhantes condições, diferentes genótipos mostram distintas capacidades de crescimento. Os genótipos que crescem mais perante uma eventual escassez de recursos fazem-no porque obtêm mais recursos ou porque são mais eficientes. Aparentemente, a maior parte dos processos metabólicos (e.g., fotossíntese, respiração) apesar de explicarem bem as diferenças de crescimento para contrastantes condições ambientais, são conservativos entre genótipos. No presente estudo, por exemplo, os valores de eficiência do uso da água (EUA, também designada eficiência da transpiração, *i.e.* biomassa produzida por unidade de massa de água transpirada num dado intervalo), apesar de aumentarem em resposta ao défice hídrico e às baixas temperaturas, mantiveram-se sem diferenças entre os dois clones (dados não apresentados). Por outro lado, os nossos resultados apoiam fortemente a hipótese de que as diferenças genéticas entre clones nos mecanismos de resistência à secura estão principalmente relacionadas com uma maior capacidade de captação dos recursos do solo e não tanto com um aumento da eficiência no uso desses recursos.

7.2. Adaptabilidade dos clones

Os diferentes mecanismos de resposta aos stresses e as diferentes características morfológicas e fisiológicas dos dois clones, implicam também diferentes adaptabilidades dos clones aos meios naturais de crescimento. A maior resistência à secura do clone CN5, quando comparado com o clone ST51, baseou-se principalmente na optimização da relação entre a área de transpiração e a área de absorção e na manutenção da condutância hidráulica em condições de secura, permitindo assim a este clone prolongar o período de assimilação activa de carbono. Podemos prever também que em condições de campo, onde o volume de solo é praticamente ilimitado, o maior investimento do clone CN5 na extensão do sistema radicular, em condições de secura, levará ainda a maiores benefícios uma vez que permitirá aceder a inexplorados volumes de solo, aumentando a absorção de água. Por outro lado, uma menor capacidade de transporte de água devido a uma menor condutância hidráulica, do clone CN5 em relação ao clone ST51, pode favorecer um uso conservativo da água, sendo uma característica de espécies bem adaptadas à secura. Ao contrário, a maior eficiência de transporte de água do clone ST51 é uma característica favorável em condições de elevada disponibilidade de água, permitindo suportar uma maior área de transpiração e mais elevadas taxas de crescimento.

Em resposta às baixas temperaturas o clone CN5 mostrou uma menor inibição do crescimento das raízes do que o clone ST51, característica que pode ser vantajosa em ambiente de clima Mediterrânico. Um aumento da razão entre a biomassa das raízes e a da parte aérea em condições de baixas temperaturas, levará a uma maior exploração do solo durante o período de maior disponibilidade de água, *i.e.*, no Inverno, permitindo uma maior capacidade de obtenção de água durante a subsequente estação seca. A capacidade de aclimatação a baixas temperaturas, ou seja, o ajustamento do metabolismo das plantas de maneira a melhorar o seu desempenho nas novas temperaturas de crescimento, é também importante para a maior adaptabilidade de um genótipo. O clone CN5 mostrou uma maior capacidade de aclimatação – num período de tempo mais curto – do que o clone ST51. Esta maior tolerância ao frio para graus intermédios de aclimatação pode permitir a um genótipo manter uma área foliar sem danos durante a ocorrência de geadas ocasionais, aumentando assim o seu potencial de crescimento após o período de frio. Desta forma,

o clone CN5 terá maior probabilidade de sobrevivência e/ou crescimento do que o clone ST51 em locais sujeitos a geadas súbitas, sendo maiores os seus limites de plantação.

As implicações na produtividade de uma maior distribuição de biomassa para as raízes dependem dos ganhos marginais em termos de assimilação de carbono das folhas. Se um maior crescimento das raízes se fizer em detrimento da área foliar, os benefícios desse crescimento dependerão do grau de limitação na produtividade que exercem os diferentes recursos do meio: água, nutrientes e luz. Deste modo, é relevante também analisar as diferentes capacidades de captação de recursos dos dois clones. Enquanto, o clone CN5 tem uma maior capacidade de obtenção de água (maior investimento nas raízes), o clone ST51 tem uma maior capacidade de intercepção de luz (maior investimento nas folhas). Assim, é de prever uma melhor adaptabilidade do clone CN5, do que do clone ST51, a condições de limitação hídrica e de nutrientes ou de competição por esses recursos. Inversamente, o clone ST51 apresentará maiores produtividades sempre que os recursos do solo não sejam limitantes.

7.3. Considerações finais

Em última análise, o valor da silvicultura clonal depende do valor intrínseco dos clones e do seu ajustamento ao ambiente em que são plantados. Por sua vez, o valor dos clones e a previsão do seu comportamento dependem em grande parte dos critérios de selecção usados e da eficácia dos testes clonais. Portanto, o valor da silvicultura clonal está essencialmente assente sobre a qualidade e o nível de conhecimento que se dispõe sobre o material vegetal empregue. E é precisamente neste ponto que os estudos fisiológicos em condições controladas e a compreensão dos mecanismos biológicos em geral podem contribuir, completando o conhecimento quase sempre empírico dos testes clonais. Assim, é importante uma interacção concertada entre fisiólogos e melhoradores genéticos para que o conhecimento científico possa ser útil, por exemplo, aplicado no aumento de eficiência dos processos de selecção das populações de melhoramento e de produção e na distribuição dos clones nas plantações.

Por outro lado, o valor do conhecimento adquirido sobre os clones depende também da sua manutenção e aproveitamento ao longo do tempo. O sucesso da introdução de novos clones na população de produção pressupõe que estes novos clones não só são superiores aos já existentes, como é viável a sua propagação em larga escala. De facto, um novo clone deve ser suficientemente superior de maneira a compensar o valor do conhecimento empírico e científico que já se detém com os clones actuais (e.g., técnicas de propagação, produção, mecanismos biológicos). Deste modo, uma demasiado rápida substituição dos clones seleccionados, devido a uma agressiva estratégia de melhoramento genético, pode impedir que se adquira um completo e específico conhecimento técnico dos clones e, assim, obstar ao pleno desenvolvimento de uma silvicultura clonal.

O presente conjunto de estudos mostram que os experimentos em condições controladas permitem identificar e caracterizar as plasticidades fenotípicas dos clones. Este conhecimento permite-nos prever as respostas dos clones em condições naturais e apoiar as decisões da sua distribuição pelas diferentes áreas edafo-climáticas. Concluímos, assim, que esta linha de investigação, possibilitando a compreensão e a exploração das diferenças entre os potenciais genéticos dos clones de *E. globulus*, é uma importante ferramenta de suporte do melhoramento genético e pode contribuir para aumentar a produtividade da floresta clonal.

Outros estudos, mais específicos e com objectivos práticos mais imediatos, podem também ser propostos. Nomeadamente, experimentos de caracterização dos clones da população de produção para determinação rigorosa dos limites de tolerância aos stresses mais relevantes. Em particular, estudos de respostas dos clones a vários graus de secura e vários graus de aclimatação ao frio, com vista a uma hierarquização dos clones em função das suas tolerâncias.