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EFEITOS DE CARBOIDRATOS E QUALIDADE DE LUZ NA RIZOGÊNESE ADVENTÍCIA DE *Eucalyptus grandis* Hill ex Maiden e *Eucalyptus globulus* Labill

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RESUMO

O Brasil é um dos maiores produtores de polpa de eucalipto e suas plantações são dependentes do enraizamento adventício de genótipos selecionados. Neste trabalho foram analisados os efeitos de diferentes fontes de carboidratos e de qualidade de luz no enraizamento adventício *in vitro* de duas espécies de eucalipto economicamente importantes, *Eucalyptus grandis*, de fácil enraizamento e *Eucalyptus globulus*, recalcitrante ao enraizamento. As fontes de carboidratos testadas em meio de cultura líquido foram sacarose, glicose e frutose. Microestacas de ambas as espécies e plantas-matrizes de *Eucalyptus globulus* foram expostas a comprimentos de onda enriquecidos para luz branca, azul, vermelha e vermelho-extrema e seus efeitos foram testados em relação ao enraizamento. O enraizamento adventício aumentou em ambas as espécies quando foi fornecida sacarose durante a fase de indução e frutose na fase de formação. Fazendo uma analogia entre o enraizamento adventício e a tuberização de batata, este resultado pode ser atribuído a atividade de invertases na fase de indução e fructoquinase na fase de formação, porém mais estudos devem ser conduzidos para confirmar esta hipótese. Não houve efeito de qualidade de luz no enraizamento adventício quando os tratamentos de luz foram aplicados nas microestacas. A exposição de plantas-matrizes crescidas em meio de cultura sem sacarose à ambiente enriquecido com comprimento de onda vermelho-extremo proporcionou um aumento de 255% na porcentagem de enraizamento de suas microestacas, mesmo na ausência de auxina exógena no meio de enraizamento, quando comparado com plantas-matrizes expostas à luz branca nas mesmas condições de cultura. Este resultado está aparentemente relacionado com o balanço

endógeno de açúcares solúveis e amido na parte aérea e raízes em desenvolvimento, com maior conteúdo de ambos na região das raízes.

ABSTRACT

Brazil is one of the largest producers of eucalypt pulp and its plantations are dependent of adventitious rooting of selected genotypes. In this work we analyzed the effects of different carbohydrate sources and light qualities on *in vitro* adventitious rooting of two economically important eucalypt species, the easy-to-root *Eucalyptus grandis* and the recalcitrant *Eucalyptus globulus*. The carbohydrate sources tested in static liquid medium were sucrose, glucose and fructose. The effect of white, blue, red and far-red light exposure on microcuttings of both species and on donor-plants of *E. globulus* was evaluated in relation to rooting. Rooting was improved in both species by supplying sucrose in the induction phase and fructose in the formation phase. By a putative analogy of adventitious rooting with tuberization in potato stems, this result was attributed to activities of invertases in the induction phase and fructokinase in the formation phase, but more studies will be needed to confirm this hypothesis. There was no effect of light quality on adventitious rooting when light treatments were applied on microcuttings. Compared to the white light-treated control donor-plants grown on medium without sucrose, donor-plants grown under a far-red light enriched environment on medium devoid of sucrose yielded 255% in the rooting percentage of microcuttings derived therefrom, even in the absence of exogenous auxin in rooting medium. This result was apparently related to the balance between endogenous hexoses and starch content in shoots and developing roots, with a higher content of both in the rooting zone.

INTRODUÇÃO

O eucalipto é a árvore mais plantada no mundo, com mais de 17,8 milhões de hectares (FAO, 2000), sendo o Brasil o sétimo país em área plantada, com cerca de três milhões de hectares (ABRAF, 2007). Não existe setor do florestamento mundial que esteja expandindo mais que o uso industrial de eucalipto, sendo que a maioria das plantações está sendo estabelecida para fornecer polpa para papel (TURNBULL, 1999). No ranking mundial de produção de celulose o Brasil ocupa a sétima posição, porém como fabricante de celulose de fibra curta, o Brasil é o principal produtor mundial, ocupando a primeira posição (ABRAF, 2007). Enquanto na Índia, outro grande produtor de eucalipto, o plantio é extensivo e de baixa produtividade, no Brasil a eucaliptocultura é intensiva e baseada principalmente em floretas clonais formadas com materiais-elite e de elevada produtividade média, chegando a atingir valores 45-60 m^3/ha/ano (MORA & GARCIA 2000). Este sucesso deve-se ao estabelecimento da propagação vegetativa ou clonagem, primeiramente por estaquia tradicional (macroestaquia) e posteriormente por mini e microestaquia, e a programas eficientes de melhoramento genético, com utilização de híbridos.

O enraizamento adventício é uma etapa essencial na propagação vegetativa (DE KLERK *et al*., 1999) de espécies lenhosas economicamente importantes (FETT-NETO *et al*., 2001). Utiliza os princípios da regeneração, fenômeno em que células somáticas diferenciadas de plantas ou animais podem reiniciar desenvolvimento e produzir um novo órgão ou restituir partes somáticas que foram perdidas por injúria ou autotomia (DE KLERK, 2000). Em plantas, o termo regeneração, como regra, é restrito à formação de uma nova parte aérea, raiz ou embrião a partir de tecidos sem um meristema pré-existente

(DE KLERK, 2002). O enraizamento adventício pode ser dividido em duas fases principais: (1) indução, correspondendo a eventos moleculares e bioquímicos, sem mudança morfológica visível, e (2) formação, que compreende divisões celulares envolvidas na organização do meristema da raiz e estabelecimento do primórdio radicular, seguido do alongamento e emergência da raiz (FETT-NETO *et al*., 2001). Em plântulas de *Arabidopsis*, raízes consideradas adventícias e laterais se originam no periciclo do hipocótilo ou de raízes, respectivamente, com o primórdio radicular crescendo através da endoderme e tecidos corticais (TAKAHASHI *et al*., 2003).

Muitos fatores ambientais e endógenos podem regular o desenvolvimento de raízes adventícias (CORRÊA & FETT-NETO, 2004), incluindo luz, temperatura, hormônios e açúcares (TAKAHASHI *et al*., 2003). Os açúcares constituem um dos fatores mais importantes, visto que não são utilizados somente como fonte energética, mas também na construção de componentes estruturais das células e parede celular (JARVIS, 1986; TAKAHASHI *et al*., 2003). Como produto da fotossíntese, a sacarose é descarregada de tecidos-fonte, nas folhas, para tecidos-dreno, nas raízes, como a principal forma de açúcar transportada (GIFFORD & EVANS, 1981). Nos tecidos-dreno, a sacarose é convertida em glicose e frutose ou UDP-glicose e frutose, pela ação de invertases ou sacarose sintase, respectivamente, as quais podem ser fosforiladas pela fructoquinase e hexoquinase (PEGO & SMEEKENS, 2000; WILLIAMS *et al*., 2000).

As plantas contêm múltiplas formas de invertases, purificadas de uma gama de diferentes espécies e tecidos (GODT & ROITSCH, 2006). São caracterizadas pela sua localização subcelular, pH ótimo e pontos isoelétricos (ROITSCH & GONZÁLEZ, 2004; TYNOWSKA-LALANNE & KREIS, 1998). As invertases vacuolar e de parede celular compartilham algumas propriedades bioquímicas, como, por exemplo, clivam sacarose de

maneira mais eficiente no pH entre 4,5 e 5,0 e atacam o dissacarídeo pelo resíduo de frutose (STURM, 1999). A invertase vacuolar apresenta ponto isoelétrico baixo e é responsável pelo nível de sacarose estocada neste compartimento (LEIGH *et al*., 1979; LINGLE & DUNLAP, 1987). Já a invertase de parede celular, também chamada extracelular ou apoplástica (ROITSCH & GONZÁLEZ, 2004), caracteriza-se por um ponto isoelétrico alto e é a enzima chave para suprir de carboidratos os tecidos-dreno através de uma via apoplástica (ROITSCH *et al*., 2003). Há ainda um terceiro tipo de invertase, conhecida como alcalina ou citoplasmática devido ao seu pH ótimo estar entre 6.8 e 8,0 e sua localização subcelular (ROITSCH & GONZÁLEZ, 2004), porém é pouco caracterizada e suas funções são pouco conhecidas. MURAYAMA & HANDA (2007) demonstraram a presença de uma invertase alcalina em mitocôndrias e cloroplastos de arroz, porém sua função não foi definida.

A sacarose sintase é localizada no citoplasma, mas dependendo do seu status de fosforilação, pode estar solúvel ou fortemente aderida ao complexo de celulose sintase da membrana plasmática ou ao citoesqueleto de actina (AMOR *et al*., 1995; WINTER *et al*., 1997; WINTER *et al*., 1998). Esta enzima é particularmente importante na síntese de componentes de reserva (CHOUREY & NELSON, 1976; WEBER *et al*., 1996) e na determinação da potência do dreno em associação com descarregamento simplástico do floema via plasmodesmos (KOCH & NOLTE, 1995; SUNG *et al*., 1989).

Hexoquinases de plantas têm como principal substrato a glicose, porém fosforilam outras hexoses incluindo frutose, manose e galactose, diferenciando-se de fructoquinase, manoquinase e galactoquinase por sua inespecificidade (CÁRDENAS *et al*., 1998). Além de sua função catalítica, apresenta importante papel na sinalização celular, como sensor de hexose, mediando respostas na expressão de genes, germinação, crescimento,

desenvolvimento vegetativo e reprodutivo, estresse e senescência (ROLLAND *et al*., 2006).

Fructoquinases são enzimas que apresentam alta afinidade específica à frutose, sendo sua atividade de fosforilação dependente de ATP e Mg^{2+} (PEGO & SMEEKENS, 2000). Sua atividade, coordenada com a de sacarose sintase, está relacionada com síntese de amido em tubérculo de batata (APPELDOORN *et al*., 2002) e em fruto de tomate (SCHAFFER & PETREIKOV, 1997).

Observações feitas em raízes, tubérculos, frutos e sementes demonstram que a degradação de sacarose via invertase vacuolar ou de parede celular predomina na iniciação e expansão do tecido-dreno (CLAYESSEN & RIVOAL, 2007). Neste estágio, alta atividade de invertase e a resultante alta taxa hexose:sacarose é geralmente associada à altas taxas de divisão celular (WEBER *et al*., 1997; KOCH 2004; VIOLA *et al*., 2001; LI & ZHANG, 2003; APPELDOORN *et al*., 2002). Já o aumento de atividade de sacarose sintase coincide com uma mudança de divisão celular para diferenciação e alongamento celular, possivelmente devido a um decréscimo na taxa hexose:sacarose (WEBER *et al*., 1997; KOCH 2004; VIOLA *et al*., 2001; LI & ZHANG, 2003; APPELDOORN *et al*., 2002). Da mesma forma, alta atividade de hexoquinase está relacionada às regiões de alta divisão celular, durante desenvolvimento do estolão em batata, enquanto que fructoquinase tem maior atividade no tubérculo já formado (APPELDOORN *et al*., 2002).

Em cultura de tecidos, a sacarose é usualmente utilizada como fonte de carboidrato, justamente por ser o açúcar mais abundante no floema. Porém, invertases que são liberadas pelo explante para o meio de cultura, quebram a sacarose em glicose e frutose (GEORGE, 1993); desse modo, os explantes ficam expostos a uma mistura de sacarose, glicose e frutose (CALAMAR & DE KLERK, 2002). Os efeitos dessas fontes de

carboidratos no processo de rizogênese adventícia têm sido estudados em diversas espécies. Em maçã e *Arabidopsis*, a presença de açúcar no meio de cultivo foi determinante para o desenvolvimento de raízes adventícias (PAWLICKI & WELANDER, 1995; TAKAHASHI *et al*., 2003). Sacarose, glicose e frutose na concentração de 1% foram capazes de induzir raízes adventícias em *Arabidopsis*, porém no caso da frutose concentrações mais baixas foram mais efetivas (TAKAHASHI *et al*., 2003). Em maçã, com baixas concentrações de sacarose ou altas concentrações da combinação glicose:frutose obteve-se os melhores resultados (PAWLICKI & WELANDER, 1995). Outro estudo com maçã demonstrou que a presença de sacarose nas primeiras quarenta e oito horas da indução de raízes foi essencial para a formação destas, sendo que sua ausência após esse período não teve importância significativa no processo (CALAMAR & DE KLERK, 2002). Neste mesmo estudo, uma interação entre sacarose e auxina foi evidenciada através de uma curva de dose-resposta, sendo que sacarose na concentração de 7% permite que as células do meristema de raiz fiquem mais sensíveis ou mais competentes para responder à auxina, tendo como resultado final um aumento no número de raízes por microestaca.

Em *Quercus suber*, sorbitol e frutose autoclavada foram incapazes de induzir raízes adventícias, enquanto que sacarose e glicose autoclavadas e frutose filtrada tiveram resultados positivos (ROMANO *et al*., 1995). O resultado negativo da frutose autoclavada foi atribuído a diferenças de sensibilidade entre as espécies aos produtos decorrentes da autoclavagem da frutose, como furfural e hidroximetilfurfural (HSIAO & BORNMAN, 1989). Este mesmo resultado deletério da frutose quanto ao enraizamento adventício foi observado em maçã - *Malus* Jork 9 (MONCOUSIN *et al*., 1992).

A importância de sacarose e glicose nas diferentes fases do enraizamento adventício *in vitro* de *Eucalyptus saligna* e *Eucalyptus globulus* foi evidenciada por CORRÊA *et al*. (2005), sendo que a presença de glicose na fase de indução e sacarose na de formação possibilitou melhores resultados, principalmente em *E. globulus*. Este trabalho utilizou meio de cultivo sólido, e, em decorrência disso, não pôde ser avaliado o papel da frutose neste processo, devido à sua presença e de seus produtos de degradação impedirem a solidificação adequada do ágar, sendo necessários estudos em meio líquido para determinar sua importância no enraizamento de *Eucalyptus*. A determinação de fontes de carboidratos mais efetivas no enraizamento adventício *in vitro* de uma espécie arbórea economicamente importante como o *Eucalyptus*, pode ter utilidade prática direta, visto que a micropropagação é utilizada por empresas produtoras de papel e celulose para estabelecer novos clones .

Dentre os fatores ambientais que atuam na rizogênese adventícia, a luz possui posição de destaque, pois não atua somente como a fonte primária de energia através da fotossíntese, mas também fornece à planta em desenvolvimento um modo de monitorar o ambiente (SULLIVAN & DENG, 2003). A radiação fotossinteticamente ativa (PAR) compreende a faixa de 400 a 700 nm do espectro de qualidade luminosa (TAIZ & ZEIGER, 2004) e apresenta papel fundamental no crescimento e desenvolvimento das plantas na fotossíntese, fotomorfogênese e fototropismo (ANTONOPOULOU *et al*., 2004). As plantas, como organismos sésseis, necessitam ser especialmente "plásticas" em seu desenvolvimento (WHITELAND & DEVLIN, 1998), pois, ao contrário dos animais, são incapazes de fugir de estímulos ambientais desfavoráveis (SULLIVAN & DENG, 2003). Para isso, desenvolveram métodos complexos de perceber a qualidade, quantidade e direção da luz e interpretar esses sinais para produzir respostas fisiológicas e de

desenvolvimento apropriadas (MÖLLER *et al*., 2002; MONTGOMERY & LAGARIAS, 2002). Para monitorar o ambiente luminoso, as plantas desenvolveram uma série de fotorreceptores, caracterizados pelo comprimento de onda que percebem (SULLIVAN & DENG, 2003). Luz vermelho/vermelho-extremo (600-750 nm) é percebida pelos fitocromos e luz azul/UVA (320-500 nm) pelos criptocromos e fototropinas (SULLIVAN & DENG, 2003).

Os fitocromos foram os primeiros fotorreceptores a serem isolados, e devido a isso são os mais estudados até o momento. Caracterizam-se por serem proteínas fotocromáticas, codificadas nas plantas por uma pequena família de genes de 3 a 5 membros (phyA-phyC em arroz e phyA-phyE em *Arabidopsis*). Todos os fitocromos das plantas consistem de uma apoproteína de aproximadamente 124 kDa, covalentemente ligada a um cromóforo tetrapirrólico linear (QUAIL, 1997). Eles existem nas formas vermelho e vermelho-extremo, Pr e Pfr, com a luz vermelha sendo absorvida por Pr levando à conversão para Pfr e, a absorção de luz vermelho-extrema convertendo Pfr de volta a Pr (ROCKWELL *et al*., 2006). Pfr é geralmente considerado a forma biologicamente ativa do fitocromo (QUAIL, 1997). A forma inativa (Pr) acumula em plantas estioladas, sendo que na presença de luz vermelha ocorre a conversão para a forma ativa (Pfr), causando de-estiolamento. A ação de Pfr envolve sua entrada no núcleo para ativação de genes. Isto ocorre via degradação pelo proteassoma de um complexo repressor formado pelo fator de transcrição PIF3 ligado a promotores de genes envolvidos na fotomorfogênese (MARTINEZ-GARCÍA *et al*., 2000). A fotoconversão entre Pr e Pfr envolve isomerização reversível no cromóforo, que induz mudanças conformacionais na proteína (WHITELAND & DEVLIN, 1998). O espectro de absorção de Pr e Pfr envolve uma considerável sobreposição, fazendo com que na maioria das situações ocorra

equilíbrio entre as duas formas (WHITELAND & DEVLIN, 1998). Os fitocromos podem ser classificados em dois grupos baseados na sua estabilidade. O tipo I (lábil) degrada rapidamente quando exposto à luz vermelha ou branca, enquanto que no tipo II (estável) isso não ocorre (CLOUGH & VIERSTRA, 1997). O fitocromo A (phyA) é do tipo I, enquanto que os fitocromos B-E (phyB-phyE) são do tipo II (QUAIL, 1997).

Os criptocromos são flavoproteínas com dois cromóforos, muito similares em estrutura às fotoliases (SANCAR, 2004), enzimas que catalisam o reparo de DNA dependente de luz azul/UVA em bactérias (SANCAR, 2000; LIN, 2002). Todas as fotoliases contêm um cromóforo flavina adenina dinucleotídeo (FAD) e também deazaflavina ou pterina, como cromóforo que absorve luz (SANCAR, 1994), sendo a presença de FAD e pterina demonstradas nos criptocromos de plantas (LIN *et al*., 1995; Malhotra *et al*., 1995). Os criptocromos também apresentam uma extensão C-terminal, não encontrada nas fotoliases (SULLIVAN & DENG, 2003). Existem dois genes similares codificando criptocromos em *Arabidopsis*, CRY1 e CRY2, cujas proteínas apresentam funções sobrepostas (KANG *et al*., 2008). Entretanto, sob luz intensa contínua, a proteína cry2 é mais instável e rapidamente degradada, sendo cry1 o fotorreceptor dominante sob condições de alta irradiância (AHAMAD *et al*., 1998, LIN *et al*., 1998). CRY1 e CRY2 são responsáveis pela inibição por luz azul do alongamento do hipocótilo, embora CRY1 desempenhe um papel mais importante nesta resposta (AHAMAD & CASHMORE, 1993; GUO *et al*., 1998; LIN *et al*., 1998). Assim como os fitocromos, os criptocromos também são capazes de penetrar o núcleo das células, atuando diretamente na percepção e transdução. No escuro, cry formaria um complexo com o repressor de fotomorfogênese COP1 e o fator de transcrição HY5, o qual é degradado no proteassoma sob estas

condições. Na presença de luz azul, ocorre uma reação de fosforilação em cry, separando COP1 do complexo e liberando HY5 para ativação de genes (LISCUM *et al*., 2003).

As fototropinas são os mais recentes fotorreceptores caracterizados (BRIGGS & CHIRSTIE, 2002). Essa classe de fotorreceptores é definida por uma seqüência amino-terminal com 100 aminoácidos repetidos que contêm significante homologia com os domínios Per-Arnt-Sim (PAS), encontrados em moléculas de sinalização (KANG *et al*., 2008). A homologia é particularmente forte entre proteínas envolvidas com sinalização redox, denominadas domínios LOV (BRIGGS *et al*., 2001). Existem dois genes similares para fototropinas em *Arabidopsis*, PHOT1 e PHOT2, codificando proteínas com domínios idênticos e com funções sobrepostas (KANG *et al*., 2008). Phot1 é envolvida em repostas de baixa fluência e é mais sensível à luz, enquanto que Phot2 é mais ativa em condições de alta fluência de fótons, sob contínua irradiância (KANG *et al*., 2008). Fototropinas catalisam uma reação de autofosforilação dependente de luz azul e estão envolvidas com fototropismo, rearranjo de cloroplastos dependente de luz azul e abertura estomática (BRIGGS, 2006; KIMURA & KAGAWA, 2006; WHIPPO & HANGARTER 2006; CHIRSTIE, 2007).

A ação do espectro de radiação luminosa e seus diferentes fotorreceptores no desenvolvimento das plantas vêm sendo estudados. Apesar de resultados prévios confirmarem efeitos morfológicos e fisiológicos da qualidade de luz na rizogênese, as respostas variam de acordo com a espécie vegetal (ANTONOPOULOU *et al*., 2004).

CHÉE & POOL (1989) aumentaram o enraizamento de explantes de *Vitis* em radiação vermelha e BARALDI *et al*. (1998) obtiveram enraizamento de pessegueiro (híbrido GF 655/2) sem adição de auxina em luz vermelha. Já as radiações verde, amarela e azul têm inibido a formação de raízes adventícias, o que tem sido atribuído à destruição

de auxina (FUERNKRANZ *et al*., 1990; GEORGE, 1996). A exposição de *Prunus insititia* (GF655-2) à luz vermelha foi tão eficiente em induzir enraizamento quanto o tratamento com a auxina ANA, sendo que sob luz vermelha as microestacas enraizaram de modo mais rápido (ROSSI *et al*., 1993). Porém, ANTONOPOULOU *et al*. (2004) descrevem diminuição do enraizamento adventício em pessegueiro (híbrido GF 677) com luz vermelha, e aumento sob luz branca, por esta apresentar todos os comprimentos de onda necessários para fotossíntese e outros processos fisiológicos. Curtos períodos (2 ou 4 dias) de exposição ao escuro, o que protegeria a auxina da fotodegradação (BASSUK & MAYNARD, 1987) ou sob luz amarela ou azul, seguida de luz branca, também aumentou o desenvolvimento de raízes adventícias em pessegueiro (híbrido GF 677) (ANTONOPOULOU *et al*., 2004).

O status fisiológico da planta-matriz no momento da excisão da estaca é de extrema importância no processo de enraizamento (MOE & ANDERSEN, 1988), sendo essa condição resultante da interação entre genótipo (espécies e cultivares) e fatores ambientais (luz, temperatura, água, CO_2 e nutrição) (MOE & ANDERSEN, 1988). Tratamentos de qualidade de luz em plantas-matrizes de *Betula pendula* tiveram um maior efeito no enraizamento do que tratamentos aplicados durante o próprio período de rizogênese (SAEBO *et al*., 1995). Neste caso, o pré-tratamento com luz azul fez com que as estacas enraizassem mais rápido e tivessem maior número de raízes, quando comparado com luz vermelha. Tratamentos de intensidade de luz no período de enraizamento de *Pinus sylvestris* também tiveram menor efetividade do que na planta-matriz (HANSEN *et al*., 1978). Baixa irradiância na planta-matriz acarretou em menor conteúdo de carboidratos e maior enraizamento das estacas. Tratamentos de baixa irradiância juntamente com baixa concentração de nutrientes na planta-matriz resultaram em estacas

maiores e com maior capacidade de enraizamento em *Albizia guachapele* (MESÉN *et al.*, 2001). Em *Eucalyptus globulus* e *Eucalyptus saligna* a intensidade de luz (30 ou 60 µmol m^{-2} s^{-1}) fornecida à planta-matriz teve influência limitada em ambas as espécies; porém, um período no escuro foi prejudicial, especialmente para *E. globulus* (CORRÊA *et al*., 2005). O enraizamento de *E. grandis ex vitro* foi estimulado por exposição das plantas-matrizes a baixas taxas de irradiância vermelho:vermelho-extremo, sendo o sucesso no enraizamento associado com baixas concentrações de amido e açúcares solúveis e um aumento no conteúdo total de carboidratos solúveis por estaca (HOAD & LEAKEY, 1996). Em *Triplochiton scleroxylon* baixa irradiância e baixas taxas de vermelho/vermelho-extremo parecem pré-condicionar fisiologicamente e morfologicamente a parte aérea no sentido de aumentar a habilidade de enraizamento das estacas (LEAKEY & STORETON-WEST, 1992). Já para *Terminalia spinosa*, a exposição das plantas-matrizes a altas taxas de vermelho/vermelho-extremo, acarretou em maior atividade fotossintética, maior condutância estomática e mais eficiência no uso de água, sendo mais eficiente na promoção do enraizamento das estacas quando comparado com baixas taxas (NEWTON *et al*., 1996).

De acordo com MOE & ANDERSEN (1998), a importância da irradiância aplicada à planta-matriz é largamente reconhecida; porém, seus efeitos no subseqüente enraizamento são controversos, variando conforme a espécie. Dessa forma, a luz também constitui fator importante no enraizamento adventício, sendo que a quantidade e qualidade de luz podem ser moduladas na planta-mãe e/ou durante o processo de enraizamento adventício, de modo a se obter bom desempenho na formação de raízes.

Auxina tem um papel central na determinação da capacidade de enraizamento, e as condições de luz afetam o metabolismo de auxina e a sensibilidade dos tecidos (REID *et*

al., 1991). O transporte polar de auxina do ápice da parte aérea até a raiz é requerido para acumulação local de auxina observada em diferentes contextos de desenvolvimento (VIETEN *et al*., 2007), incluindo a manutenção do meristema de raiz (SABATINI *et al*., 1999). Foi demonstrado que o transporte polar basípeto de auxina é afetado de modo luz-dependente (JENSEN *et al*., 1998), e um crescente conjunto de evidências sugere uma interação íntima entre fitocromos e sinalização de auxina (MORELLI & RUBERTI 2002). Em *Arabidopsis* a inibição do alongamento do hipocótilo na presença de luz é dependente do transporte polar basípeto de auxina, com envolvimento dos fitocromos A e B e do criptocromo 1 (JENSEN *et al*., 1998). Na presença de luz são necessárias doses mais altas do inibidor de transporte de auxina 2,3,5-ácido triiodo benzóico (TIBA) para bloquear o enraizamento adventício em tomate, relacionando aumento no transporte de auxina em condições de luz (TYBURSKI & TRETYN, 2004). SORIN *et al*. (2005) sugerem que o fator de transcrição responsivo a auxina ARF17 poderia regular o enraizamento adventício de *Arabidopsis*, reprimindo a expressão de genes regulados por luz do tipo GH3 (codificadores de enzimas conjugadoras de auxinas), dessa forma modulando a homeostase de auxina de modo luz dependente.

A condução de experimentos de enraizamento *in vitro* tem algumas vantagens: as condições de cultura de tecido facilitam a administração de auxina e outros compostos, evita degradação microbiana de compostos aplicados, permite a adição de nutrientes inorgânicos e carboidratos e permite o experimento com explantes menores, como segmentos do caule (VAN DER KRIEKEN *et al*., 1993). Dessa forma, estudos que envolvem a caracterização da importância de carboidratos na rizogênese adventícia têm no cultivo *in vitro* uma ferramenta importante.

A utilização comparativa de duas espécies de eucalipto, com diferentes capacidades de enraizamento, pode ser útil em estudos que visam avaliar parâmetros fisiológicos envolvidos no controle desse processo do desenvolvimento. Dentre as mais de 100 espécies de eucalipto introduzidas no Brasil, a mais comum é *Eucalyptus grandis*, seguida por *Eucalyptus saligna* e *Eucalyptus urophylla* (CANETTIERI *et al*., 2007). *Eucalyptus globulus*, mais comumente plantada no Chile (WAGNER *et al*., 2006), têm despertado interesse da indústria de papel e celulose do sul do Brasil.

Eucalyptus grandis possui madeira clara, relativamente macia e menos densa que outras espécies de eucalipto, ideal para diferentes utilidades, incluindo papel e celulose, carvão, postes de iluminação e madeira para construção (ELDRIDGE *et al.*, 1993). É amplamente utilizado para produção de híbridos na indústria de papel e celulose, tendo como características importantes, boa adaptabilidade e boa qualidade de fibra (ALFENAS *et al*., 2004). Pode ser considerada uma espécie de propensão média ao enraizamento, sendo sua propagação clonal dependente de enraizamento adventício.

Eucalyptus globulus apresenta características que interessam particularmente à indústria de papel e celulose do sul do Brasil, como a resistência à geada e o baixo teor de lignina. A geada é uma característica do inverno do sul do Brasil, e a lignina interfere negativamente na extração da celulose (CHIANG 2002; BISON *et al.*, 2007) Esta espécie tem baixa capacidade de enraizamento – recalcitrante (LE ROUX & VAN STADEN, 1991; SERRANO *et al*., 1996) e suas mudas são consideradas mais difíceis de propagar do que *E. grandis*.

Dessa forma, selecionaram-se *E. grandis* e *E. globulus* como o alvo dos estudos de caracterização da importância de diferentes fontes de carboidratos e qualidade de luz no enraizamento adventício relatados neste trabalho.

OBJETIVO GERAL

Avaliar o papel de qualidade de luz e diferentes fontes de carboidratos no controle do processo de enraizamento adventício de *Eucalyptus grandis* e *E. globulus*.

OBJETIVOS ESPECÍFICOS

Caracterizar o enraizamento *in vitro* de *E. grandis* e *E. globulus*, modulando o uso de diferentes fontes de carboidratos (sacarose, glicose e frutose) nas fases de indução e formação de raízes adventícias;

Caracterizar o efeito de qualidade de luz durante o período de enraizamento *in vitro* de *E. grandis* e *E. globulus*, através da exposição das microestacas à luz branca, azul, vermelha ou vermelho-extrema;

Caracterizar os efeitos interativos da qualidade de luz e da disponibilidade de sacarose no enraizamento *in vitro* de *E. globulus*, por meio da exposição da planta-matriz à luz branca, luz azul, luz vermelha e vermelho-extrema, na presença ou ausência de sacarose, no subseqüente enraizamento das microestacas;

Caracterizar o conteúdo de carboidratos em plantas-matrizes submetidas a tratamentos de qualidade de luz na ausência de sacarose.

Pre- and post-severance roles of carbohydrates and light quality on adventitious root development in microcuttings of *Eucalyptus grandis* and *Eucalyptus globulus*

Artigo a ser submetido ao periódico Tree Physiology

Pre- and post-severance roles of carbohydrates and light quality on adventitious root development in microcuttings of *Eucalyptus grandis* and *Eucalyptus globulus*

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Abstract

Brazil is one of the largest producers of eucalypt pulp and its plantations are dependent of adventitious rooting of selected genotypes. In this work we analyzed the effects of different carbohydrate sources and light qualities on *in vitro* adventitious rooting of two economically important eucalypt species, the easy-to-root *Eucalyptus grandis* and the recalcitrant *Eucalyptus globulus*. The carbohydrate sources tested in static liquid medium were sucrose, glucose and fructose. The effect of white, blue, red and far-red light exposure on microcuttings of both species and on donor-plants of *E. globulus* was evaluated in relation to rooting. Rooting was improved in both species by supplying sucrose in the induction phase and fructose in the formation phase. By a putative analogy of adventitious rooting with tuberization in potato stems, this result was attributed to activities of invertases in the induction phase and fructokinase in the formation phase, but more studies will be needed to confirm this hypothesis. There was no effect of light quality on adventitious rooting when light treatments were applied on microcuttings. Compared to the white light-treated control donor-plants grown on medium without sucrose, donor-plants grown under a far-red light enriched environment on medium devoid of sucrose yielded 255% in the rooting percentage of microcuttings derived therefrom, even in the absence of exogenous auxin in rooting medium. This result was apparently related to the balance between endogenous hexoses and starch content in shoots and developing roots, with a higher content of both in the rooting zone.

Keywords: adventitious rooting, carbohydrates, *Eucalyptus*, light quality

Introduction

There is no sector of world forestry that is expanding as rapidly as the industrial use of eucalypts. Most plantations are being established to provide pulp for paper (Turnbull, 1999). Brazil is one of the largest producers of eucalypt pulp (ABRAF 2007) and its plantations are based on the vegetative propagation of selected genotypes. Adventitious rooting is an essential step in the vegetative propagation of trees and may be divided in two main phases: (1) induction, corresponding to the molecular and biochemical events prior to any visible morphological changes, and (2) formation, comprising cell divisions involved in root meristem organization and radical primordial establishment, followed by root elongation and emergence (Fett-Neto et al. 2001). This complex developmental process can be affected by internal and external factors including light, temperature, hormones and sugars (Takahashi et al. 2003).

The effect of sugars on the development of adventitious rooting has been studied in several species. Sucrose, the most transportable sugar in plants and more often employed in tissue culture and micropropagation of woody species (Romano et al. 1995), is converted to glucose and fructose or UDP-glucose and fructose by invertase or sucrose synthase, respectively, and then phosphorylated by fructokinase and hexokinase (Pego and Smeekens 2000; Williams et al. 2000). The presence of sugar in the culture medium was determinant for rooting development in apple and *Arabidopsis* (Pawlicki and Welander 1995, Takahashi et al. 2003, Calamar and De Klerk 2002), especially in the first 48 h for apple, when a dose-response curve with auxin, the main phytohormone involved in root induction, showed an interaction between these factors (Calamar and De Klerk 2002). Despite the widespread use of sucrose, supported by numerous culture success cases obtained, reducing sugars such as glucose and fructose can be readily taken up and

metabolized, potentially providing a better carbon source (Welander et al. 1989). Sucrose, glucose and fructose were capable to induce adventitious rooting in *Arabidopsis* and apple in different concentrations and/or combinations (Takahashi et al. 2003, Pawlicki and Welander 1995) but, in some cases, the effect of fructose was inhibitory (Moncousin et al. 1992, Romano et al. 1995). Corrêa et al. (2005) demonstrated an important role for sucrose and glucose in the two phases of *in vitro* adventitious rooting in *Eucalyptus saligna* and *Eucalyptus globulus*. A positive effect of glucose on cutting rhizogenesis was found if this hexose was supplied during the root induction phase, followed by sucrose in the root formation step, especially for *E. globulus* in agar containing medium (Corrêa et al. 2005). Additional studies with *Eucalyptus* using fructose and liquid medium (fructose partly impairs agar solidification) are needed to obtain a more detailed characterization of the influence of sugars in adventitious rooting of this genus.

Light is not only the energy source for photosynthesis, but also a fundamental regulatory factor in development (Corrêa et al. 2005). Plants have evolved complex methods of sensing quality, quantity, direction and duration of light and interpreting these signals to produce the appropriate physiological and developmental response (Möller et al. 2002; Montgomery and Lagarias 2002). Although previous results have confirmed morphological and physiological effects of radiation on rooting, responses vary according to plant species (Antonopoulou et al. 2004). Chée and Pool (1989) enhanced rooting of *Vitis* explants under red radiation and Baraldi et al. (1988) obtained rooting of peach without auxin under the same light condition, whereas blue light inhibited adventitious rooting (Fuernkranz et al. 1990). In contrast, Antonopoulou et al. (2004) described an inhibitory effect of red light on peach rooting and a positive response to white and blue light.

The physiological status of the donor-plants when the cuttings are excised is of considerable importance. This condition is the result of the interaction between genotype (species and cultivars) and environmental factors (light, temperature, water, CO_2 and nutrition) (Moe and Andersen 1988). Treatments differing in light quality on donor-plants yielded better results in subsequent adventitious rooting of *Betula pendula* cuttings than when the same conditions were applied during the rooting period (Saebo et al. 1995). Similarly, light intensity modification during the rooting of *Pinus sylvestris* was less effective to improve adventitious rooting when compared with equivalent treatments applied to donor-plants, in which low irradiance diminished the carbohydrate content and enhanced rooting (Hansen et al. 1978). Light intensity supplied to donor-plants (30 or 60 µmol m^{-2} s^{-1}) had limited influence on the rooting responses of *E. saligna* and *E. globulus*, whereas dark periods were detrimental, particulary for *E. globulus* (Corrêa et al. 2005). Rooting of *Eucalyptus grandis ex vitro* has been shown to be stimulated by low red : far-red irradiance ratios in donor plants and rooting success was associated with low pre-severance starch and water-soluble sugars concentrations in shoots, and a greater total water-soluble carbohydrate content per cutting (Hoad and Leakey 1996).

Light quantity and quality could be modulated in donor-plants and/or during the rooting period, affecting the content of carbohydrates, auxins and their interactions, providing a potential means of improving adventitious rooting of the cuttings. Auxin has a central role in determination of rooting capacity, and light conditions affect auxin metabolism and tissue sensitivity (Reid et al. 1991). It has been shown that polar auxin transport is affected in a light-dependent manner (Jensen et al. 1998). Under light, high concentrations of 2,3,5-triiodobenzoic acid (TIBA), an inhibitor of auxin polar transport, are needed to block root formation in tomato cuttings (Tyburski and Tretyn 2004).

In the present investigation, the effects of different sources of carbohydrates and light qualities during adventitious rooting of two commercially relevant *Eucalyptus* species with contrasting rooting capacity were analyzed. *E. grandis*, easy-to-root, is the most common eucalypt planted in Brazil and the difficult-to-root *E. globulus* has characteristics of interest for the pulp industry in southern Brazil, such as frost resistance and low lignin content, making cellulose easily extracted.

The effect of sucrose, glucose and fructose combinations in static liquid medium during the two phases of adventitious rooting on the rhizogenic response were examined, and, in a second set of experiments, treatments of different light qualities applied during the rooting of microcuttings or on donor-plants were carried out to evaluate the subsequent rooting of cuttings derived therefrom. Interactions between light quality and sucrose supplied to donor-plants and the internal content of carbohydrates during the whole process were also analyzed.

Material and methods

Plant material

Seeds of *E. grandis* and *E. globulus* (kindly provided by Aracruz Celulose, Unidade Guaíba, RS, Brazil) were surface sterilized in 70% (v/v) ethanol (1 min) and 1,5% (v/v) NaOCl (15 min) with constant stirring, followed by four washes in sterile distilled water. About fifteen seeds were planted on 300 ml glass jars (capped with a double layer of aluminum foil or plastic film in light experiments) containing 60 ml of germination medium (Table 1) and kept at 25 ± 2 ^{o}C and 16 h photoperiod (30 μmol m^{-2} s^{-1} of photosynthetic active radiation). After 3.5 and 4 months for *E. globulus* and *E. grandis*, respectively, microcuttings (about 3-cm-long tip cuttings, containing the meristematic

apex) were excised from seedlings and used for *in vitro* rooting experiments. In donor-plant assays, pre-treatments were applied during the last month of seedling growth.

Culture conditions

Rooting experiments were carried out according to a two-step basal sequencial medium protocol (Fett-Neto et al. 2001). Microcuttings were placed in an induction medium (Table 1) for 4 days and then transferred to formation medium (Table 1) for 20 days. Culture flasks were 20 ml vials with 6 ml medium covered with a double layer of aluminum foil or plastic film (in the light quality experiments) with 2 microcuttings per vial. All reagents were analytical grade and media were prepared with distilled water. Media were sterilized by autoclaving at 121 ^{o}C and 0.15 MPa for 20 min.

Table 1. Composition of culture medium in each step of the experiments

Germination	Root Induction	Root Formation
6 g/l agar	6 g/l agar (or 0 g/l)	6 g/l agar (or 0g/l)
58.67 mM sucrose (or 0 mM)	88 mM sucrose (or 88 mM glucose, 88 mM fructose)	88 mM sucrose (or 88 mM glucose, 88 mM fructose)
MS (Murashige and Skoog , 1962) salts 0.5X	MS salts 0.3X	MS salts 0.3X
	100 mg/l myo-inositol	100 mg/l myo-inositol
	0.4 mg/l thiamine. HCl	0.4 mg/l thiamine. HCl
	10 mg/l IBA (or 0 mg/l)	1 g/l activated charcoal (or 0 g/l)

Experiments with different carbohydrate sources

The carbohydrate sources used were sucrose, glucose and fructose and their effects were evaluated in both phases of adventitious rooting (induction/formation) in equimolar concentrations (88 mM) according to the following combinations: sucrose/sucrose (control), glucose/glucose, fructose/fructose, sucrose/glucose, sucrose/fructose, glucose/sucrose, glucose/fructose, fructose/sucrose and fructose/glucose. The microcuttings used were excised from seedlings growing on germination medium supplied with sucrose and kept in a growth room at 25 ± 2 ^{o}C and 16h photoperiod (30 μmol m^{-2} s^{-1} of photosynthetic active radiation) during the experiment. The media used were liquid, with no activated charcoal in the root formation medium. The experiments of *E. globulus* and *E. grandis* were conducted separately. Each experiment had 30 explants per treatment and was repeated at least twice.

Experiments with different light qualities on microcuttings (post-severance)

Microcuttings excised from seedlings growing in germination medium with sucrose were exposed to white (control), blue, red or far-red light during the rooting period. Light enrichments were provided by filtering the output of the white fluorescent tubes through double cellophane sheets (Lin and Yang 1999, Héraut-Bron et al. 2001). The absorbance spectra of the filters were measured and recorded in a Cintra 5 spectrophotometer (GBC, Victoria, Australia**;** Figure 1). Microcuttings were kept in a growth room at 27 ± 2 ^{o}C and a 16 h photoperiod (40 μmol m^{-2} s^{-1}). Light intensity was normalized for all light treatments. Experiments of *E. globulus* and *E. grandis* were conducted separately. Each experiment had 20 replicate explants per treatment and was independently repeated at least twice with similar results.

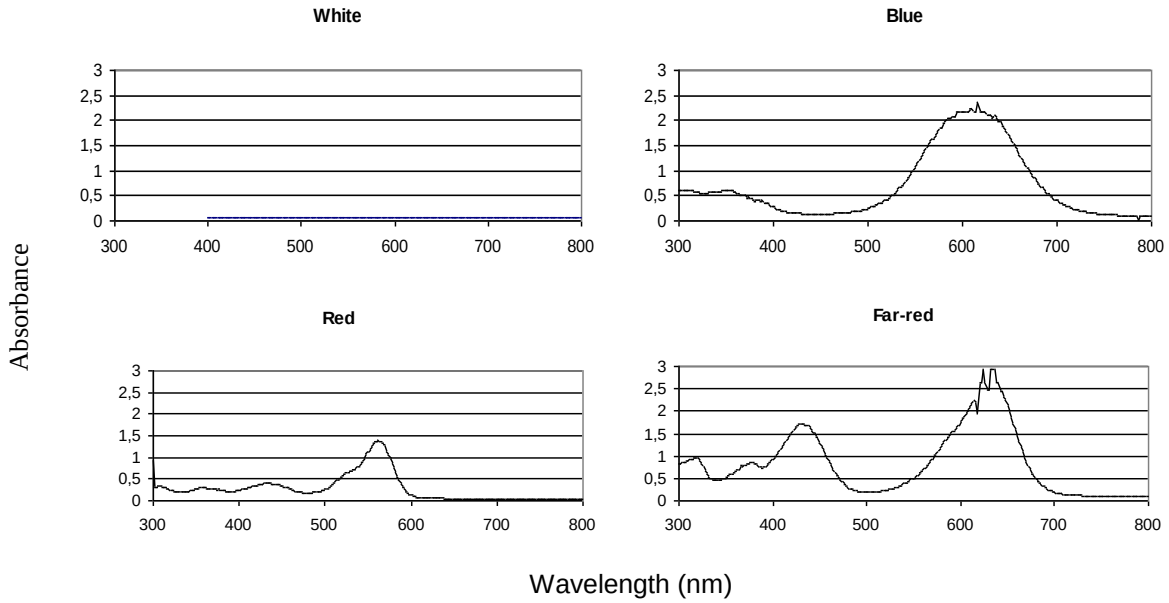

Figure 1. Absorbance spectra of cellophane filters measured in a spectrophotometer.

Experiments with different light qualities on donor-plants (pre-severance)

Seedlings of *E. globulus* growing on germination medium with or without sucrose under white light (30 μmol m^{-2} s^{-1} of photosynthetic active radiation) for 2.5 months were exposed to white (control), blue, red or far-red light for 1 additional month. Light sources were provided by the same cellophane filters and culture conditions described above. Filters were replaced every five days to avoid changes in spectral quality of the transmitted light due to possible color fading. Light intensity was normalized for all light treatments. Microcuttings obtained were used in rooting experiments with or without auxin in the induction medium under the same culture conditions described for the carbohydrate source experiments.

Samples of about 10 mg (FW) of shoots from donor-plants before and after light treatments, and shoots and roots of microcuttings obtained at the end of the experiment were frozen and analyzed for the contents of total soluble hexoses and starch.

Total soluble sugars content

The extraction and quantification of soluble sugars was done according to Dubois et al. (1956) modified by Ghosh (2003). Frozen samples were homogenized in liquid nitrogen, extracted with 1 ml 80% (v/v) ethanol and incubated in a water bath at 75 ^{o}C for 15 min. The extracts were centrifuged at 10,000 *g* for 15 min and the supernatant was recovered. The pellets were re-extracted with 500 µl 80% (v/v) ethanol. For the quantification, 500 µl of the sample, diluted in the same proportion of 80% ethanol, were mixed with 5 ml of concentrated sulphuric acid and 1 ml 5% (w/v) phenol. After agitation, solutions were maintained for 20 min at room temperature (25 ± 3ºC). The absorbance at 490 nm was measured in a Spectramax automated spectrophotometer (Molecular Devices, USA). The standard curve was established with D-glucose.

Starch content

The pellet obtained from the hexose extraction was used for starch extraction as described by McReady et al. (1950) with some modifications. The pellets were homogenized with 250 µl of distilled water and 320 µl of 52% (v/v) perchloric acid, submitted to sonication in a water bath for 15 min and centrifuged at 10,000 *g* for 15 min. The supernatant was collected and the pellet was re-extracted. For the quantification, 500 µl of the extract reacted with 500 µl of freshly prepared anthrone reagent (10 mg anthrone + 5 ml 95% (v/v) sulphuric acid); the resulting solution was mixed and kept in a boiling water bath for 10 min. After cooling, the absorbance at 630 nm was determined in a Spectramax. The standard curve was established with D-glucose in perchloric acid. The glucose content found was multiplied by 0.90 to estimate starch amount.

Plant measurements and statistical analyses

The analyzed morphological parameters were percent rooting, mean number of roots per rooted microcutting (root number), mean length of longest root (root length) and mean rooting time (as described by Fett-Neto et al. 2001). Data were measured 20 days after transferring to formation medium, except by the mean rooting time raw data, that was taken every 2 days after transferring to formation medium. Analyses of variance (ANOVA) followed by Duncan test when appropriate ($P \leq 0.05$) was performed for morphological parameters.

Results

Effects of different carbohydrate sources on microcuttings

The percent rooting was not significantly affected by carbohydrate source in *E. grandis* and *E. globulus* (Figure 2).

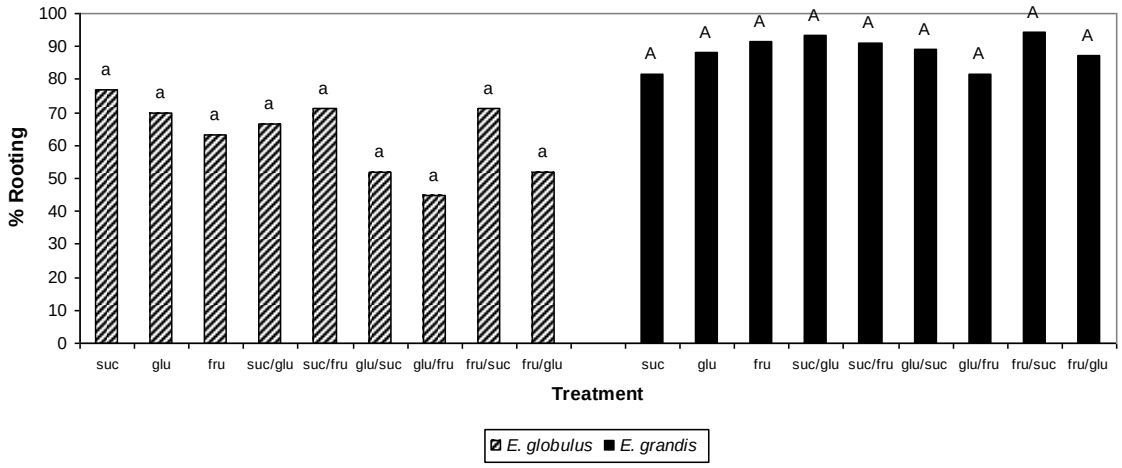

Figure 2. Percent rooting of microcuttings of *E. globulus* and *E. grandis* submitted to treatments with different sources of carbohydrates in equimolar concentrations (88 mM), after 20 days in formation medium. The treatments were not significantly different by ANOVA at ($P \leq 0.05$).

Considering the overall of analyzed parameters, for both species the treatment with sucrose in the induction phase and fructose in the formation phase showed the best results. Mean rooting time for *E. globulus* was significantly reduced in microcuttings submitted to sucrose/fructose (3.68 days) when compared with the control sucrose (5.82 days) (Figure 3). With this treatment microcuttings rooted 42% faster than the control average of *E. globulus* and 25% faster than *E. grandis* control average, apparently reducing recalcitrance to rooting of the former species.

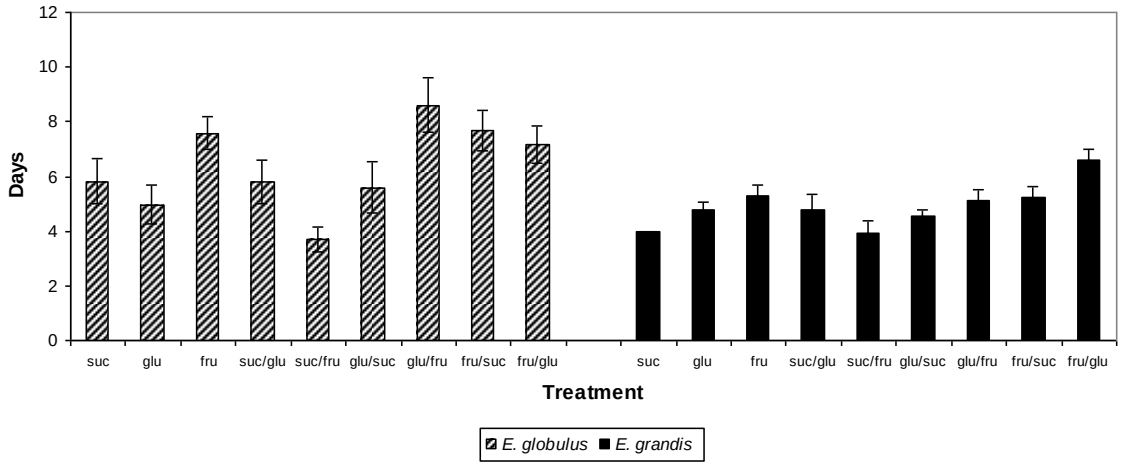

Figure 3. Mean rooting time of microcuttings of *E. globulus* and *E. grandis* submitted to treatments with different sources of carbohydrates in equimolar concentrations (88 mM), after 20 days in formation medium. Lines on top of bars are the trust intervals ($P \leq 0.05$).

For the number of roots in *E. globulus* this advantage was less significant, but sucrose/fructose (7.52 roots) showed a trend toward inducing more roots when compared with the sucrose control (5.65 roots)**:** sucrose/fructose grown cuttings had 25% more roots than sucrose grown ones (Figure 4). In the same species, glucose in both phases (6.76 roots) also showed a trend toward a greater number of roots (Figure 4). *E. grandis* had consistently more roots than *E. globulus* and showed less differences between treatments,

but sucrose/fructose treatment (12.89 roots) was equivalent to the sucrose control (13.81 roots; Figure 4).

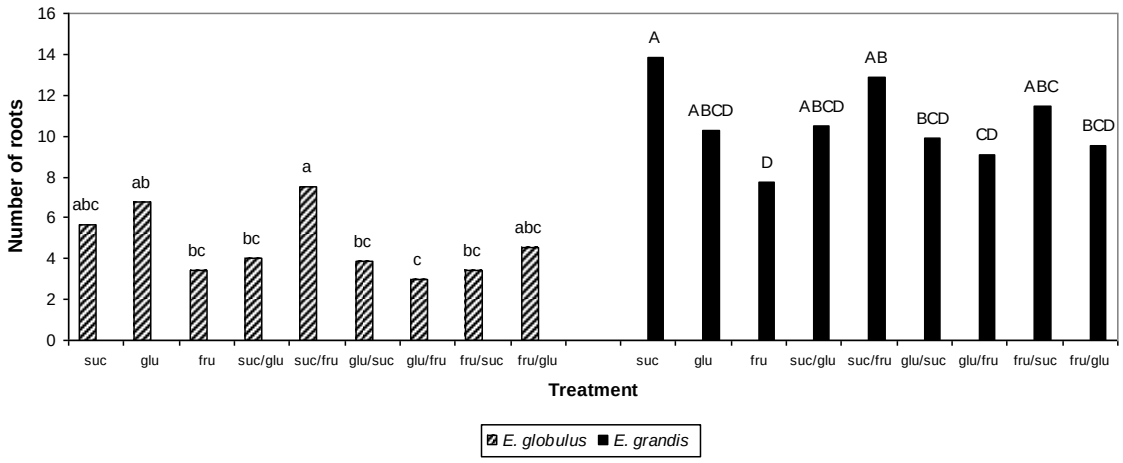

Figure 4. Mean number of roots per rooted microcutting of *E. globulus* and *E. grandis* submitted to treatments with different sources of carbohydrates in equimolar concentrations (88 mM), after 20 days in formation medium. Bars with different letters within each species are significantly different according to a Duncan test ($P \leq 0.05$).

Root length of *E. globulus* had no significant difference among treatments, but microcuttings exposed to fructose showed a tendency of producing shorter roots, except for sucrose/fructose (Figure 5). In *E. grandis*, microcuttings of sucrose/fructose treatment (3.61 cm) significantly produced longer roots when compared to the sucrose control (2.68 cm; Figura 5).

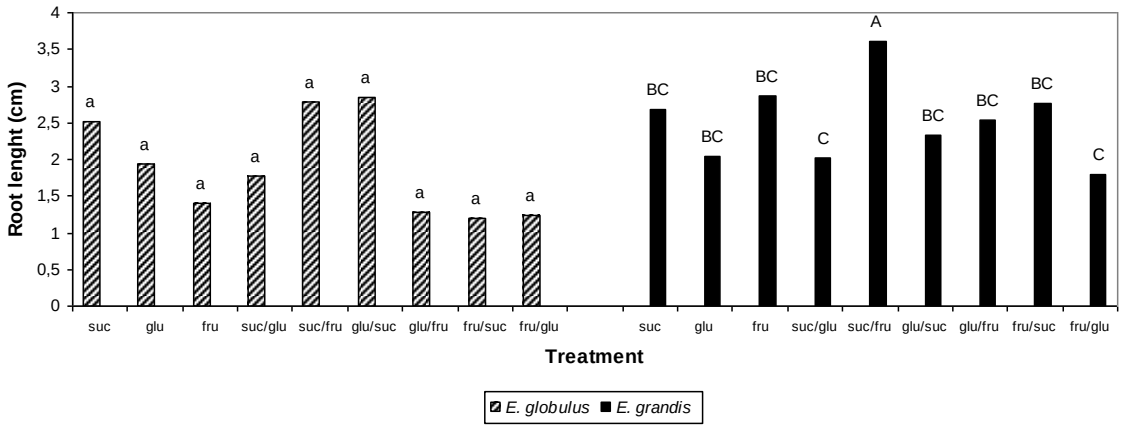

Figure 5. Mean length of longest root in microcuttings of *E. globulus* and *E. grandis* submitted to treatments with different sources of carbohydrates in equimolar concentrations (88 mM), after 20 days in formation medium. Bars with different letters are significantly different according to a Duncan test ($P \leq 0.05$).

E. grandis had a higher rooting percentage, rooted faster and developed higher number of roots and longer roots when compared with *E. globulus* (Figures 2, 3, 4 and 5), confirming the recalcitrance of the latter. Taking all treatments together, the overall average percent rooting of *E. globulus* was 63%, whereas for *E. grandis* it was 88.72% (Figure 2). Roots in *E. grandis* took an average of 4.91 days to develop, about 28% faster than in *E. globulus* (6.32 days) (Figure 3). These differences between species became more obvious when the number of roots was compared. *E. grandis* had 10.56 roots per rooted cutting, whereas *E. globulus* had 4.67 roots, more than a 50% reduction (Figure 4). Again, roots of *E. grandis* (2.52 cm) were on average 25% longer than roots of *E. globulus* (1.88 cm).

Effects of different light qualities on microcuttings (post-severance)

The different light treatments during the rooting period had no significant effect on adventitious rooting of microcuttings in both species (data not show). The only differences

detected in *E. globulus* were observed due to the presence or absence of auxin in the induction control, when microcuttings exposed to IBA had a higher percentage of rooting (white - control: 25% ± 38.48 auxin: 92.86% ± 26.06; blue - control: 32.5% ± 47.43 auxin: 95% ± 22.07; red – control: 17.5% ± 37.72 auxin: 84.61% ± 38.48; far-red – control: 32.5% ± 38.09 auxin: 80% ± 40.5) and more roots (white – control: 1.28 ± 0.53 auxin: 5.95 ± 3.19; blue – control: 1.31 ± 0.48 auxin: 5.26 ± 3.43; red – control: 1.71 ± 1.08; auxin: 5.97 ± 3.57; far-red – control: 2 ± 1.08 auxin: 5.5 ± 3.57) than the control without auxin. This response was not observed in *E. grandis*, which had a high rooting percentage even in the absence of auxin in the induction medium (white – control: 55% ± 35.89 auxin: 65% ± 32.74; blue – control: 60% ± 20.08 auxin: 75% ± 34.35; red – control: 45% ± 32.33 auxin: 70% ± 49.72; far-red – control: 55% ± 42.77 auxin: 70% ± 33.96).

Experiments with different light qualities on donor-plants (pre-severance)

Far-red light and absence of sucrose in donor-plants had a strong positive effect on percent rooting of *E. globulus* microcuttings; microcuttings grown without auxin in the induction medium derived from donor plants grown without sucrose under FR enriched environment (FR cont.) yielded 51% of rooting (Figure 6). The combination of these two factors increased more than two fold the ability of microcuttings to develop roots when compared with microcuttings derived from control donor plants in white light both without and with sucrose in the medium (W cont. and W suc. cont.), which resulted in about 20% rooting (Figure 6). For all of the other morphological parameters evaluated, light treatment or exogenous sucrose availability had no significant effect. Moreover, blue and red light treatments on donor-plants had no effect on rooting of microcuttings when compared with the control in white light (data not show).

As previously reported (Fett-Neto et al., 2001), rooting percentage, root number and root length in the presence of IBA was higher for all light treatments, representing an auxin effect (data not show).

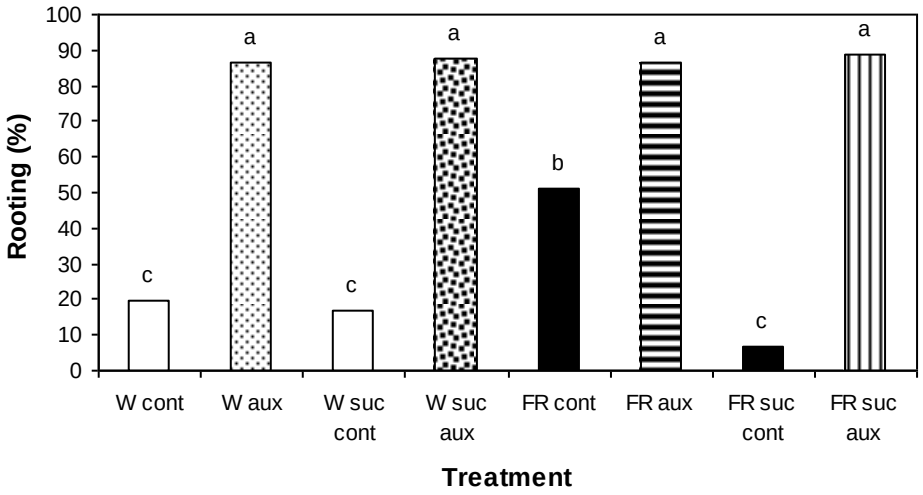

Figure 6. Percent rooting of microcuttings of *E. globulus* excised from donor-plants submitted to treatments with different light qualities in the presence or absence of sucrose (58.67 mM) in the germination medium. Data was taken after 20 days on the formation medium. White and dark columns represent the control without IBA in the induction medium. Values with different letters are significantly different according to a Duncan test ($P \leq 0.05$).

Donor-plants and microcuttings had about five to ten fold more soluble sugars than starch for all light treatments (Figures 7a and b, 8a and b). The content of total soluble hexoses and starch in donor-plants was higher in red light treatment (Figure 7a and b). Far-red light had similar content of hexoses and starch of seedlings before light treatments (pre-treat), blue treatment and the white control (Figure 7a and b).

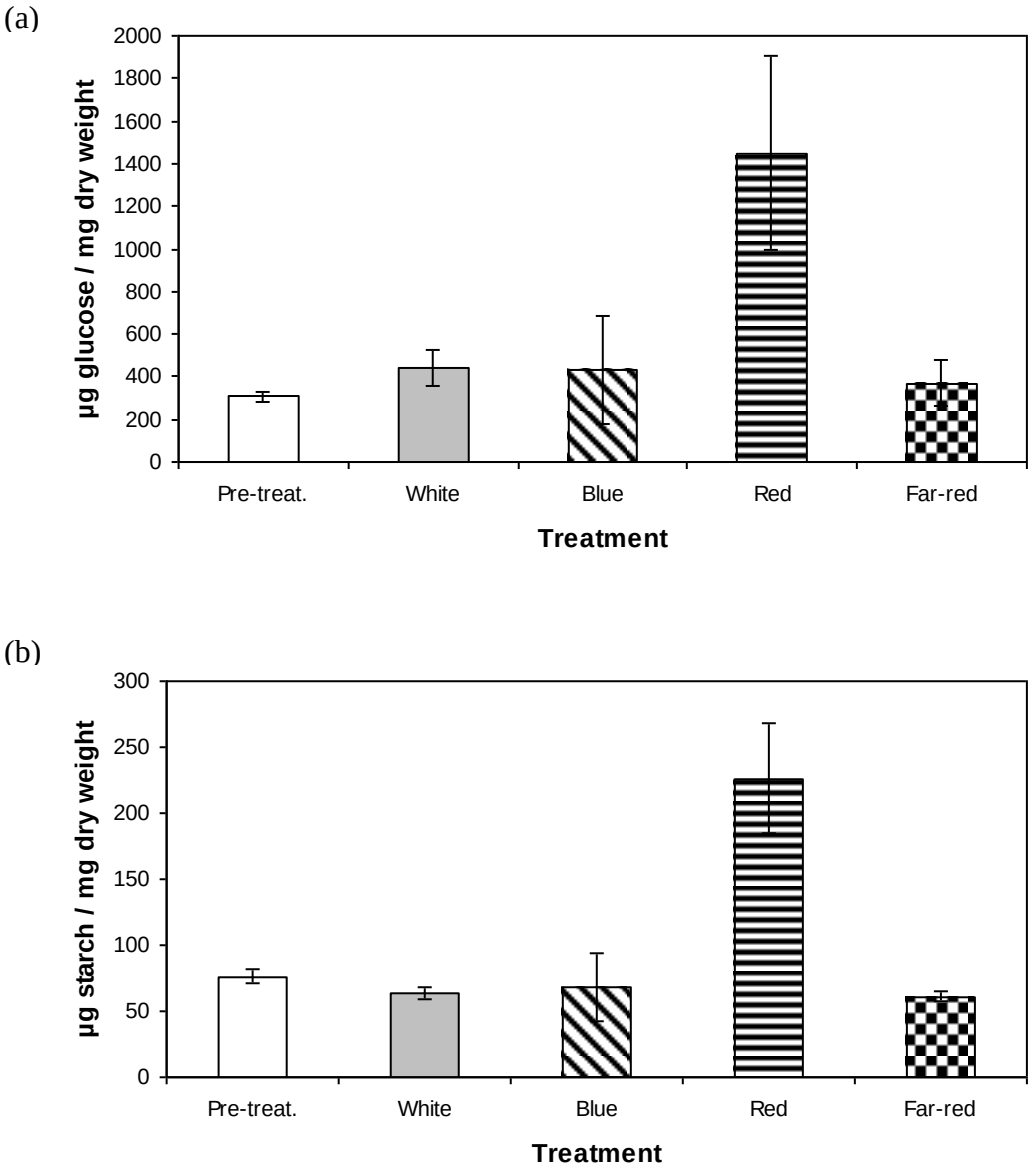

Figure 7. Quantification of total soluble sugars (a) and starch (b) in donor-plants of *E. globulus* exposed to different light treatments during 1 month. Pre-treat. refers to seedlings before light treatment. Top vertical lines indicate standard error of the means.

In microcuttings derived from donor-plants exposed to far-red, roots had a higher content of soluble sugars and starch when compared with the shoots (Figure 8a and b). There was no difference between shoot and root content in white light for both carbohydrates evaluated (Figure 8a and b) and in blue light for starch (Figure 8b), whereas the shoots had more soluble sugars than the root zone in this latter light treatment

(Figure 8a). Microcuttings derived from donor plants grown under red light had more soluble sugars and starch in the shoot (Figure 8a and b).

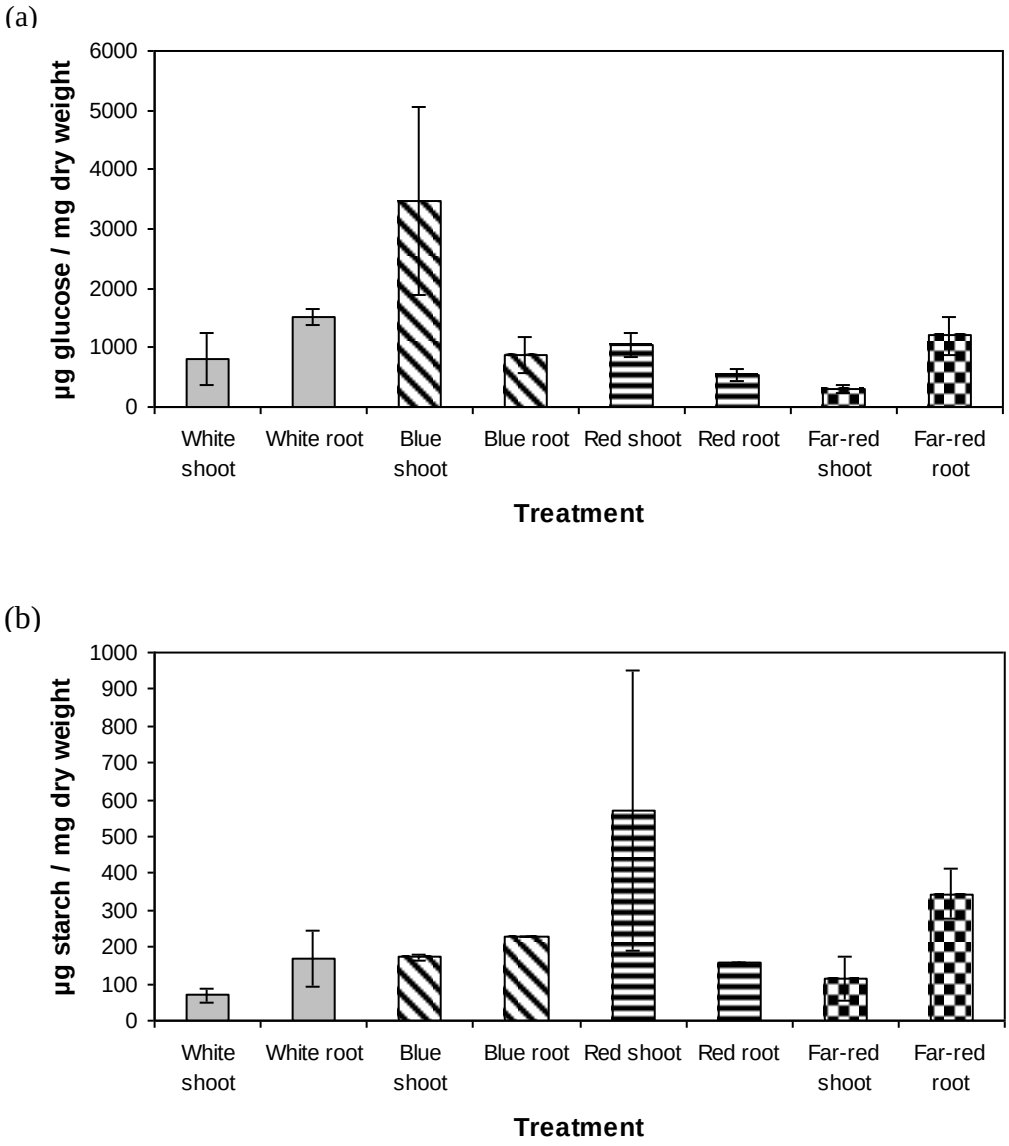

Figure 8. Quantification of total soluble sugars (a) and starch (b) in shoots and root area of microcuttings of *E. globulus* excised from donor-plants exposed to different light treatments during 1 month, after 20 days in the formation medium and without auxin in the induction medium. Top vertical lines indicate standard error of the means.

Discussion

The adventitious rooting of *E. grandis* and *E. globulus* could be improved by supplying different sources of carbohydrate during its two phases, suggesting that the events occurring in induction and formation require different types of sugars. When all the rooting parameters evaluated are considered, the presence of sucrose in the induction phase and fructose in the formation phase yielded better results for both species (Figures 3, 2 and 5). This response could be related to the metabolism of these sugars and the activity of the enzymes involved in this process. In potato, the development of tubers is associated with different enzyme activities, showing a higher activity of invertases and hexokinases in the developing stolon and of sucrose synthase and fructokinase during tuber formation (Appeldoorn et al. 2002). The fructokinase activity is often associated with sucrose synthase and both enzymes are related to sink storage functions and starch production (Ross et al. 1994, Kanayama et al. 1998, Appeldoorn et al. 2002). By analogy with the tuberisation process, during adventitious rooting, the presence of sucrose in the induction medium could activate invertase activity and possibly genes involved with the initial development of the sink organ (Appeldoorn et al. 2002, Viola et al. 2001). The supply of fructose during the formation phase could activate fructokinase activity and genes related with storage functions, leading to starch accumulation (Appeldoorn et al. 2002). Adventitious root formation in *Pinus radiata* is associated with starch accumulation (Li and Leung 2000) and in the present study higher starch content in roots was associated with higher percent rooting (Figure 8b). Besides, reducing sugars can be readily taken up and metabolized, potentially providing a better carbon source (Welander et al. 1989), which could have also contributed to the good performance of microcuttings exposed to

fructose in the formation phase. To confirm this hypothesis further studies will be carried out monitoring the activities of the proposed enzymes during adventitious rooting.

This result does not agree with previous studies on *E. globulus* rooting using agar solidified media, which indicated that better rhizogenesis was observed when glucose was used in the induction and sucrose in the formation phase (Corrêa et al. 2005). This discrepancy could be explained by the different culture conditions used (agar solidified versus static liquid media), which could cause changes in uptake rates of medium components, aeration and turgor status of tissues, ethylene production and accumulation, release and diffusion of wound-related compounds and growth inhibitors, among other factors that can influence rooting (De Klerk et al. 1999).

The strong inhibitory effect of autoclaved fructose described for *Malus* Jork 9 and *Quercus suber* (Moncousin et al. 1992, Romano et al. 1995) were not observed in the present study, since the treatments with carbohydrate sources showed no difference in rooting percentage (Figure 2). However, for both *Eucalyptus* species, a slightly negative effect on root number and length of some treatments with fructose were observed when sucrose was not included in the induction medium (Figures 4 and 5).

The results of light quality experiments indicated that adventitious rooting of both eucalypt species was more influenced by exposing the donor-plants to light treatments than during the rooting process *per se* (Figure 6 and data not show). These data support the important role of the physiological status of donor-plants at the time that cuttings are excised and emphasizes the importance of controlling the growth conditions of donor-plants. Others studies with light quality and quantity also reported that light treatments could be more effective when applied to donor-plants (Hansen et al. 1978, Saebo et al. 1995).

Far-red light and absence of sucrose in donor-plants increased by more than two fold the percentage of rooting of *E. globulus* microcuttings without auxin in the induction medium (Figure 6). This result could be correlated with the higher content of total soluble sugars and starch in the rooting zone when compared with the shoots in microcuttings derived from far-red treated donor plants (Figure 8b). None of the other light treatments displayed this balance, yielding much lower rooting percentage (Figure 8b). Also the starch content in microcutting shoots was higher than in donor-plant shoots in far-red light (Figure 7b and 8b). Similar to our results, starch accumulation has been associated with rooting success in *Pinus radiata* (Li and Leung 2000). These results support the view that relationships between rooting ability and the availability of stored and/or current supplies of carbohydrates are important for clonal propagation, as already suggested by Hoad and Leakey (1996). The competition for assimilates between shoot growth and root formation also influence rooting ability (Eliasson 1971) and an adequate balance could result in root improvement, as observed in donor plants exposed to far-red treatment. Hoad and Leakey (1996) also described a positive effect on adventitious root formation when donor-plants of *E. grandis* were exposed to low red : far-red ratios. In this study, under such light conditions, better rooting response was associated with pre-severance lower starch and hexose content in shoots (no measurement was done for the rooting zone of cuttings). This correlation was not apparent in the present study with *E. globulus*; content of hexoses and starch in shoots of far red treated donor plants was not significantly different from those of white light grown control plants (Figure 7a and b). This difference could be related to species-specific responses or to experimental conditons. The analysis of the same parameters for *E. grandis* under our experimental conditions, currently under way, may help clarify this point. The rooting ability of *Triplochiton scleroxylon* was also

improved by exposure to low red : far-red ratios (Leakey and Storeton-West 1992, Newton et al. 1996). On the other hand, high red : far-red ratios and blue light yielded better rooting responses for *Terminalia spinosa* and *Betula pendula* (Saebo et al. 1995, Newton et al. 1996). These results confirm that the effects of light quality vary considerably between species (Moe and Andersen 1988, Antonopoulou et al. 2004).

Auxin has a central role in determining rooting capacity, and light conditions affect auxin metabolism and tissue sensitivity (Reid et al. 1991). Far-red light could also be affecting auxin metabolism and/or polar transport. Polar auxin transport is affected by light (Jensen et al. 1998) and an increasing body of evidence suggests intimate interactions between phytochrome and light signaling (Morelli and Ruberti 2002). The study of several *aux/aia* mutants has established a strong link between auxin signaling and photomorphogenesis (Kim et al. 1998, Reed et al. 1998, Nagpal et al. 2000). Sorin et al. (2005) suggested that ARF17 and GH3, auxin responsive genes encoding an auxin transcription factor and an auxin conjugating enzyme, respectively, could be involved in the control of adventitious rooting, modulating auxin homeostasis in a light-dependent manner. Tyburski and Tretyn (2004) established a positive relation between light, auxin transport and adventitious rooting in tomato, but without evidence for the action of photoreceptors.

The presence of auxin in the induction medium caused higher rooting percentage in all light treatments; however, most of the pulp and paper companies do not use this phytohormone in large scale to root cuttings of eucalypt hybrids in clonal gardens. Far-red enrichment treatments in donor-plants, as described herein, could possibly be used in commercial scale at relatively low cost to improve the adventitious rooting of “elite” *E. globulus* hybrids that display rooting limitations.

To sum up, carbohydrate and light quality could be modulated in a manner to improve the adventitious rooting of *Eucalyptus* species. The positive results with sucrose in the induction phase and fructose in the formation phase may be attributed to the sequential action of different enzymes related with sugar metabolism, but further studies will be needed to confirm this hypothesis. The improvement of adventitious rooting capacity of microcuttings derived from donor-plants exposed to far-red light in an autotrophic system (without sucrose in the culture medium) was probably associated with an adequate balance between the content of endogenous sugars in shoots and developing roots, with a higher content in the rooting zone. Future studies with auxin transport inhibitors should examine the possible contribution of this light treatment on the modulation of polar auxin transport to the rooting zone.

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CONCLUSÕES E PERSPECTIVAS

Carboidratos e qualidade de luz regulam a rizogênese adventícia de eucalipto, de modo que a modulação desses fatores durante o período de enraizamento e nas plantas-matrizes pode melhorar o enraizamento de micro e miniestacas. Este fato tem desdobramentos importantes para a indústria de papel e celulose, a qual depende do eficiente enraizamento de estacas de híbridos-elite para a produção industrial dessa espécie.

Os bons resultados alcançados quando os meios de cultivo das microestacas contêm sacarose durante a fase de indução e frutose no período formação de raízes, podem ser diretamente empregados na micropropagação de eucalipto, utilizada no estabelecimento de novos clones. Além disso, foram geradas hipóteses que, ao serem testadas, poderão contribuir para maior entendimento do metabolismo de carboidratos durante o enraizamento, caracterizando a importância da ação de enzimas envolvidas na quebra de sacarose e frutose durante esse processo de desenvolvimento.

A exposição das plantas-matrizes a tratamentos de qualidade de luz foram mais efetivos aos explantes do que durante o enraizamento dos próprios, reforçando a importância do status fisiológico da planta-matriz no subseqüente enraizamento e, por conseqüência, a necessidade de controlar de modo eficiente o ambiente em que se encontram as plantas-matrizes.

A exposição das plantas-matrizes de *E. globulus* à luz vermelho-extrema sob condições fotomixotróficas gerou resultados bastante promissores, aumentando mais de duas vezes a capacidade das microestacas geradas em produzir raízes. A aplicação desse resultado pode ser de grande valia para a indústria de papel e celulose no enraizamento em

larga escala de híbridos de *E. globulus* que apresentam baixa porcentagem de enraizamento. Além disso, o balanço de açúcares solúveis totais e amido, encontrado nestas microestacas, pode vir a ser utilizado como um marcador bioquímico para seleção de clones mais aptos a formar raízes e/ou para determinação do momento mais propício de coleta de miniestacas em jardins clonais.

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