DENYS MATHEUS SANTANA COSTA SOUZA

INFLUÊNCIA DA QUALIDADE DE LUZ NO CULTIVO *IN VITRO* DE *Eucalyptus grandis* Hill ex Maiden × *Eucalyptus urophylla* S.T. Blake

LAVRAS - MG
2022

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Engenharia Florestal, área de concentração em Silvicultura e Genética Florestal para obter o título de Doutor.

Prof. Dr. Gilvano Ebling Brondani

Orientador

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Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Souza, Denys Matheus Santana Costa.
Influência da qualidade de luz no cultivo *in vitro* de *Eucalyptus grandis* Hill ex Maiden × *Eucalyptus urophylla* S.T. Blake / Denys Matheus Santana Costa Souza. - 2022\'.
104 p. : il.

Orientador(a): Gilvano Ebling Brondani.

Tese (doutorado) - Universidade Federal de Lavras, 2022\'.
Bibliografia.

1. Micropropagação. 2. Clonagem. 3. Fotomixotrofismo. I. Brondani, Gilvano Ebling. II. Título.

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APROVADA em 8 de julho de 2022.

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LAVRAS – MG

2022

Ao meu pai Espedito, pelo amor, carinho e apoio constante durante essa árdua trajetória.

Dedico

AGRADECIMENTOS

À Deus por tudo que tenho e sou, por guiar sempre os meus passos, iluminar meu caminho e por todas as oportunidades que me fizeram chegar até aqui.

Agradeço aos meus pais que eu tanto amo e são a minha base, pela educação que me deram, apoio incondicional em todos os momentos, carinho e amor.

À minha esposa, pelo apoio, companheirismo, incentivo e paciência, me mostrando o lado positivo em todas as situações, obrigado meu amor.

Á minha filha maravilhosa, que me trouxe muita luz, serenidade e força para continuar as provas e expiações do dia a dia.

Aos meus irmãos, pelo carinho e companheirismo de sempre.

Ao meu grande amigo Sérgio, pelo companheirismo e apoio durante essa jornada.

Aos colegas e amigos de Laboratório e UFLA, Anatoly, Adênio, Júlio, Douglas, Maria, Letícia e Andressa, vocês fizeram essa jornada ser bem mais leve.

Ao meu orientador, Prof. Dr. Gilvano Ebling Brondani, pela orientação, paciência, disponibilidade e ensinamentos, sou muito grato por ter tido a oportunidade de trabalhar sob sua orientação.

Ao Prof. Dr. e amigo Eduardo Oliveira Silva por todo companheirismo e ensinamentos.

À Prof. Dra. Dulcinéia de Carvalho e Prof Dr. Vinícius Politi Duarte por todos os ensinamentos e dedicação.

À Universidade Federal de Lavras e ao Departamento de Ciências Florestais pela formação profissional proporcionada.

Aos professores e técnicos do DCF, pela disponibilidade e prontidão em ajudar.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), pelo suporte financeiro. Obrigado.

"Basta acreditar que um novo dia vai raiar, sua hora vai chegar"

Alexandre Assis/Carlos Rodrigues/ Gilson Bernini

RESUMO

A expansão de plantios e a busca de melhor adequação na produção de mudas têm ampliado a utilização de espécies e combinações híbridas. Dessa forma, a hibridação de *Eucalyptus grandis* com *Eucalyptus urophylla* tem proporcionado excelentes resultados em qualidade da madeira, desenvolvimento, adaptação edafoclimática e maior facilidade de propagação vegetativa. Contudo, alternativas para o rejuvenescimento/revigoramento em tecidos de clones híbridos do gênero *Eucalyptus*, visando maior multiplicação e melhoria do enraizamento adventício, tem - se recomendada a técnica de micropropagação, a qual permite avanços consideráveis na produção de mudas clonais. Diante do exposto, o presente estudo teve como objetivo avaliar o efeito do fotomixotrofismo e uso de biorreatores de imersão temporária (BIT), no processo de fotomorfogênese e desenvolvimento de *E. grandis* × *E. urophylla*, sendo dividido em quatro capítulo. O objetivo do primeiro capítulo foi avaliar o efeito da qualidade de luz nas fases de estabelecimento, multiplicação, alongamento e enraizamento *in vitro*. Diante disso, observou –se que a utilização da qualidade de luz LEDs vermelho/azul e lâmpada fluorescente proporcionaram os melhores resultados para todas as fases da micropropagação. Para o segundo capítulo, o objetivo do estudo foi analisar a influência da intensidade de luz na introdução e multiplicação *in vitro*. Quanto aos resultados, a utilização da qualidade e intensidade de luz lâmpada fluorescente 40 µmol m^{-2} s^{-1} e LEDs vermelho/azul 80 µmol m^{-2} s^{-1} são os mais adequados para serem utilizadas na introdução e multiplicação *in vitro*. No terceiro capítulo, o trabalho teve como objetivo avaliar o efeito do fotomixotrofismo (intensidade de luz e formas de vedação) no alongamento e enraizamento *in vitro*. Com base nos resultados obtidos, os tratamentos lâmpada fluorescente 40 µmol m^{-2} s^{-1} e o uso de uma membrana porosa (1M) são os mais adequados para serem utilizadas no alongamento e enraizamento *in vitro*. Em relação ao quarto capítulo, teve como objetivo avaliar o efeito da qualidade espectral na fase de multiplicação *in vitro* em biorreator de imersão temporária. Foi possível concluir que a qualidade espectral amarela e azul, proporcionaram os melhores resultados para a fase de multiplicação *in vitro*.

Palavras-chave: Micropropagação. Clonagem. Fotomixotrofismo. LEDs. Biorreator.

ABSTRACT

The expansion of plantations and the search for better adaptation in the production of clonal plants have increased the use of species and hybrid combinations. Thus, the hybridization of *Eucalyptus grandis* with *Eucalyptus urophylla* has provided excellent results in wood quality, development, edaphoclimatic adaptation and greater ease of vegetative propagation. However, alternatives for the rejuvenation/reinvigoration in tissues of hybrid clones of the *Eucalyptus* genus, aiming at greater multiplication and improvement of adventitious rooting, the micropropagation technique has been recommended, which allows considerable advances in the production of clonal plants. In view of the above, the present study aimed to evaluate the effect of photomyxotrophism and the use of temporary immersion bioreactors (TIB) in the process of photomorphogenesis and development of *E. grandis* × *E. urophylla*, being divided into four articles. The aim of the first article was to evaluate the effect of light quality in the establishment, multiplication, elongation and rooting phases *in vitro*. Therefore, it was observed that the use of red/blue LED and fluorescent lamp light quality provided the best results for all phases of micropropagation. For the second article, the aim of the study was to analyze the influence of light intensity on *in vitro* introduction and multiplication. As for the results, the use of light quality and intensity fluorescent lamp 40 μmol m^{-2} s^{-1} and red/blue LEDs 80 μmol m^{-2} s^{-1} are the most suitable for use on *in vitro* introduction and multiplication. In the third article, the study aimed to evaluate the effect of photomyxotrophism (light intensity and sealing forms) on elongation and rooting *in vitro*. Based on the results obtained, the treatments fluorescent lamp 40 μmol m^{-2} s^{-1} and the use of a porous membrane (1/M) are the most suitable for use in *in vitro* elongation and rooting. Regarding the fourth article, it aimed to evaluate the effect of spectral quality in the *in vitro* multiplication phase in a temporary immersion bioreactor. It was possible to conclude that the yellow and blue spectral quality provided the best results for the *in vitro* multiplication phase.

Keywords: Micropropagation. Cloning. Photomyxotrophism. LEDs. Bioreactor.

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1. INTRODUÇÃO

No Brasil, as áreas de florestas plantadas de eucalipto crescem a cada ano e são responsáveis pela maior parte da produção de madeira para fins industriais (CARRILLO *et al*., 2018; BRITO *et al*., 2021). A contribuição é elevada para o setor florestal brasileiro que, por sua vez apresenta expressiva representatividade na economia, justificando dessa forma, a busca para a otimização do cultivo, por meio de pesquisa em melhoramento genético e silvicultura clonal, no qual vêm proporcionando ganhos significativos em produtividade e qualidade da madeira de florestas plantadas (ASSIS, 2014; CASTRO *et al*., 2021; OLIVEIRA; HIGA; SILVA, 2021).

A expansão de plantios para regiões não tradicionais e a busca de melhor adequação na produção de mudas têm ampliado a utilização de espécies e combinações híbridas. Dessa forma, nos últimos anos, a hibridação de *Eucalyptus urophylla* S.T. Blake com *Eucalyptus grandis* Hill ex Maiden e outras espécies do gênero *Eucalyptus* tem proporcionado excelentes resultados, tanto em qualidade da madeira, quanto em crescimento, além de uma melhor adaptação ambiental e maior facilidade de propagação vegetativa (TRUEMAN; HUNG; WENDLING, 2018; CASTRO *et al*., 2021; OLIVEIRA; HIGA; SILVA, 2021). Contudo, alternativas para o rejuvenescimento/revigoramento de clones híbridos selecionados do gênero *Eucalyptus*, visando a maior multiplicação e melhoria do enraizamento adventício no processo de produção de mudas clonais, a micropropagação via proliferação de gemas axilares tem sido recomendada, permitindo avanços consideráveis na propagação clonal (ABIRI *et al*., 2020; SOUZA *et al*., 2020a; SOUZA *et al*., 2022a).

Nos últimos anos, as pesquisas sobre as espécies de *Eucalyptus urophylla*, *Eucalyptus grandis* e seus híbridos, envolvendo a micropropagação, aumentaram considerando desde o processo de introdução (MOURA *et al*., 2017, KERET; NAKHOODA; HILLS, 2018; SOUZA *et al*., 2020a), multiplicação (TRUEMAN; HUNG; WENDLING, 2018; CARVALHO *et al*., 2019; SOUZA *et al*., 2020b; SOUZA *et al*., 2022a), alongamento (GALLO *et al*., 2017; MIRANDA *et al*., 2020; MOLINARI *et al*., 2021), enraizamento e aclimatização (NAKHOODA; WATT, 2017; BRONDANI, *et al*., 2018; MIRANDA *et al*., 2020; SOUZA *et al*., 2020a).

Para solucionar os fatores limitantes de cada processo, fica evidente a necessidade de estudos básicos para maximização da produção de microcepas *in vitro*. Diversas tecnologias vêm sendo propostas com o intuito de automatizar o processo da micropropagação, entre elas:

adequação de protocolos quanto ao controle ambiental mais eficiente em sala de crescimento, como diferentes qualidades e intensidade de luz por meio de lâmpadas LEDs (BATISTA *et al.*, 2018); a adoção de sistemas com mais trocas gasosas, como a utilização de membranas porosas (SOUZA *et al.*, 2019); além da automatização das operações dos sistemas de cultura e de procedimentos rotineiros, por meio do uso de biorreatores de imersão temporária (BIT) (BATISTA *et al.*, 2018; CARVALHO *et al.*, 2019; SOUZA *et al.*, 2020c).

Estudos recentes sobre o uso do fotomixotrofismo, tais como a qualidade e intensidade de luz nas salas de crescimento com lâmpadas LEDs e o emprego de sistemas de ventilação dos recipientes (BATISTA *et al.*, 2018; SOUZA *et al.*, 2019; SOUZA *et al.*, 2020b; OLIVEIRA; HIGA; SILVA, 2021; ZEPS *et al.*, 2022) têm chamado atenção, possibilitando criar um ambiente mais favorável para o desenvolvimento das plantas *in vitro*. Além disso, proporciona maior produção de fotoassimilados e estímulos percebido por fotorreceptores que desencadeiam a fotomorfogênese em plantas (GUPTA; KARMAKAR, 2017; OLIVEIRA; HIGA; SILVA, 2021; NERY *et al.*, 2021), e consequentemente maior produtividade das microcepas *in vitro*.

Uma resposta positiva à micropropagação, dentre diversos fatores, deve-se também ao uso de biorreatores por imersão temporária (BIT), sendo uma importante ferramenta para aumentar a proliferação de gemas e multiplicação de brotos em laboratórios e biofábricas (SOUZA *et al.*, 2020c; GAGO *et al.*, 2021; SOUZA *et al.*, 2022b). Esse tipo de sistema pode otimizar o cultivo *in vitro* pela automatização dos processos (LOYOLA-GONZÁLEZ *et al.*, 2019; SOTA *et al.*, 2021), permitindo a obtenção de ganhos em biomassa e a redução do tempo necessário para a propagação (HWANG *et al.*, 2022; ZHANG *et al.*, 2022), além de aumentar a produção de microcepas por unidade de área (SOTA *et al.*, 2021; SOUZA *et al.*, 2022b).

Considerando a importância que o gênero *Eucalyptus* e seus híbridos representam atualmente para o setor florestal, o presente estudo teve como objetivo avaliar o efeito do fotomixotrofismo e o uso de biorreatores de imersão temporária (BIT), no processo de fotomorfogênese e desenvolvimento de *Eucalyptus grandis* Hill ex Maiden × *Eucalytpus urophylla* S.T. Blake.

2. REFERENCIAL TEÓRICO

2.1. *Eucalyptus grandis* × *Eucalyptus urophylla*

O setor florestal brasileiro ocupa, atualmente, uma posição de destaque no cenário mundial, graças às condições ambientais favoráveis ao crescimento das florestas, principalmente do gênero *Eucalyptus*, bem como aos programas de pesquisa em melhoramento genético e silvicultura clonal que vêm proporcionando ganhos significativos em produtividade e qualidade da madeira de florestas plantadas (ASSIS, 2014; BRITO *et al*., 2021; CASTRO *et al*., 2021; OLIVEIRA; HIGA; SILVA, 2021). Diante disso, no Brasil os 9,55 milhões de hectares de florestas plantadas em 2020, cerca de 7,47 milhões são de eucalipto. Isso representa um montante de 68,69 % do total, principalmente distribuídos nos estados de Minas Gerais, São Paulo, Mato Grosso do Sul e Bahia (IBÁ, 2021).

Entre as principais razões para o cultivo intenso deste gênero inclui, o seu rápido crescimento, forma do fuste, valiosas propriedades da madeira e ampla adaptabilidade edafoclimática, tendo recursos essenciais para a produção de celulose, papel, carvão, biomateriais, bioenergia, entre outros, sendo amplamente difundidos em cerca de cem países e seis continentes (CARRILLO *et al*., 2018; CASTRO *et al*., 2021; OLIVEIRA; HIGA; SILVA, 2021). Como resultado de muitos anos de estudo, grandes avanços foram feitos no melhoramento genético e silvicultura clonal, principalmente na utilização de híbridos interespecíficos, no entanto, ainda há espaço para melhorias (ASSIS, 2014; ABIRI *et al*., 2020; OLIVEIRA; HIGA; SILVA, 2021).

A silvicultura clonal com *Eucalyptus* constitui uma das mais evoluídas e bem estabelecidas, sendo uma realidade na maioria das empresas florestais brasileiras. Ela permite, dentre outras vantagens, a uniformização dos plantios, maximização dos ganhos em produtividade e qualidade da madeira, melhor adaptação dos clones à área a ser plantada e aproveitamento de combinações híbridas específicas, aliado a racionalização das atividades operacionais e custos competitivos (XAVIER, 2013; BRITO *et al*., 2021).

Por meio da hibridação de *Eucalyptus urophylla* com *Eucalyptus grandis* e outras espécies do gênero *Eucalyptus* tem proporcionado excelentes resultados, tanto em qualidade da madeira, quanto em crescimento, além de uma melhor adaptação ambiental e maior facilidade de propagação vegetativa (XAVIER, 2013; TRUEMAN; HUNG; WENDLING, 2018; ABIRI *et al*., 2020). Desta

forma, a opção por esses híbridos tende a prevalecer nas regiões tropicais e subtropicais (REIS *et al.*, 2014; ASSIS, 2014).

No Brasil, o *Eucalyptus grandis* caracteriza-se pela alta produtividade, em materiais selecionados (ASSIS *et al.*, 2017), enquanto o *Eucalyptus urophylla* tem sido uma espécie importante para plantios em regiões tropicais úmidas devido ao excelente vigor, resistência ao cancro e capacidade de rebrota (NICOLLE *et al.*, 2015; MANASA *et al.*, 2022). A hibridação entre essas espécies tem demonstrado grande potencial e superioridade aos demais híbridos devido: diâmetro altura do peito (DAP) e altura (HENRIQUES *et al.*, 2018), viabilidade de produção de sementes (PUPIN, 2018), resistência ao cancro, homogeneidade na qualidade da madeira e para plantios clonais em larga escala estabelecidos por propagação vegetativa (SILVA *et al.*, 2019; REZENDE *et al.*, 2021). A busca de plantas produtivas com qualidades específicas faz híbridos, como o *Eucalyptus urophylla* × *Eucalyptus grandis* adequados para o reflorestamento no Brasil (ASSIS, 2014; REZENDE *et al.*, 2021).

Desta forma, o uso de híbridos, aliada às vantagens de rejuvenescimento/revigoramento de materiais genéticos selecionados por meio da técnica de micropropagação em espécies florestais, tem sido uma importante ferramenta a ser utilizada no melhoramento genético, podendo contribuir para a multiplicação rápida dos genótipos selecionados precocemente *in vitro* (SOUZA *et al.*, 2020a; NAZIRAH *et al.*, 2021). Além disso, a utilização dessas metodologias pode favorecer a redução do tempo em programas de melhoramento genético e o aumento da produtividade (YANG *et al.*, 2018; REZENDE *et al.*, 2021; ZIEGLER *et al.*, 2022).

2.2. Micropropagação

As técnicas de propagação vegetativa são a base da silvicultura clonal, sobretudo pela sua efetividade em capturar os ganhos genéticos obtidos dos programas de melhoramento, constituindo atualmente um dos principais processos de produção de mudas do gênero *Eucalyptus* (TRUEMAN; HUNG; WENDLING, 2018; ABIRI *et al.*, 2020). A propagação clonal representa uma alternativa em situações em que a espécie apresenta limitações na propagação sexuada, sendo amplamente utilizada para as espécies florestais, por possibilitar a multiplicação de genótipos selecionados, conservação de germoplasma e pesquisas em geral, com as técnicas de enxertia, estaquia e micropropagação (TRUEMAN; HUNG; WENDLING, 2018; LIMA *et al.*, 2022).

A micropropagação é uma das técnicas de propagação vegetativa mais utilizadas dentre a cultura de tecidos, em que apresentam importante impacto na multiplicação clonal em várias espécies, incluindo arbóreas (SOUZA *et al*., 2018; SOUZA *et al*., 2020a; NAZIRAH *et al*., 2021). Podem ser utilizados como fonte de explantes meristemas, embriões zigóticos ou somáticos, folhas, segmentos caulinares e raízes (RATHORE *et al*., 2014; ZURAIDA *et al*., 2017; ABIRI *et al*., 2020). Estes explantes quando inoculados em condições assépticas, em meio de cultura e com condições físicas e biológicas controladas, podem solucionar os fatores limitantes de cada processo da micropropagação, expressando a capacidade da organogênese, através da neoformação de órgãos, como brotos e raízes, maximizando a produção de mudas. (BIANCHETTI *et al*., 2017; BATISTA *et al*., 2018; ABIRI *et al*., 2020).

Como ferramenta de produção de mudas clonais, a micropropagação tem inúmeras vantagens em que se destacam: a possibilidade de propagação massal de clones em curto espaço de tempo; o maior controle nutricional, ambiental e fitossanitário; possibilitar a formação e manutenção de microjardins clonais; transporte do material clonal para grandes distâncias; o armazenamento por longos períodos; a retenção do vigor híbrido; e acelera os programas de propagação clonal, possibilitando a clonagem de híbridos com alta heterose (KERET; NAKHOODA; HILLS, 2018; TRUEMAN; HUNG; WENDLING, 2018).

Na busca por alternativas para o rejuvenescimento/revigoramento em clones selecionados de *Eucalyptus*, visando a melhoria do enraizamento adventício no processo de produção de mudas clonais a micropropagação via proliferação de gemas axilares tem sido recomendada como técnica para alcançar esse objetivo. Dentre as etapas, em virtude da metodologia adotada para a proliferação das gemas axilares, tem as fases de introdução, multiplicação e alongamento *in vitro*, que é necessária para a obtenção de brotações com tamanho adequado para a fase de enraizamento destas, a qual tem sido realizada *ex vitro* (BRONDANI, *et al*., 2018; ZORZ *et al*., 2020) ou *in vitro* (OBSERSCHELP *et al*., 2018; MIRANDA *et al*., 2020; SOUZA *et al*., 2019; SOUZA *et al*., 2020a).

Nos últimos anos, as pesquisas sobre as espécies de *Eucalyptus urophylla*, *Eucalyptus grandis* e seus híbridos, envolvendo a micropropagação, aumentaram considerando desde o processo de introdução (MOURA *et al*., 2017, KERET; NAKHOODA; HILLS, 2018; SOUZA *et al*., 2020), multiplicação (TRUEMAN; HUNG; WENDLING, 2018; CARVALHO *et al*., 2019; SOUZA *et al*., 2020a; SOUZA *et al*., 2022a), alongamento (GALLO *et al*., 2017; MIRANDA *et*

al., 2020; SOUZA *et al*., 2020b; MOLINARI *et al*., 2021), enraizamento e aclimatização (NAKHOODA; WATT, 2017; BRONDANI, *et al*., 2017; MIRANDA *et al*., 2020; SOUZA *et al*., 2020a).

Diante do exposto, para solucionar alguns fatores que podem ser limitantes na micropropagação, fica evidente a necessidade de estudos para maximização da produção de microestacas *in vitro*, entre eles: o conhecimento e adequação de protocolos, bem como o tipo de explante a ser utilizado; o desenvolvimento de tecnologias de produção em recipientes alternativos; a adoção de sistemas com mais trocas gasosas, como membranas porosas; o conhecimento do ambiente mais eficiente para o sistema e entre outros (BATISTA *et al*., 2018; TRUEMAN; HUNG; WENDLING, 2018; ABIRI et al., 2020).

2.3. Fotomixotrofismo

Quando é passível de maximizar a utilização de uma espécie e, ou, híbrido tem - se o objetivo de intensificar e aprimorar o domínio de técnicas, principalmente aquelas que servem de base para as demais, e isso reflete sobre a propagação vegetativa. Com isso, fica evidente a necessidade de pesquisas para maximização da produção de microestacas *in vitro*, entre eles: o uso de membranas porosas com ventilação natural ou forçada; e o conhecimento do ambiente físico mais eficiente para o sistema, como diferentes qualidades e intensidades de luz através de lâmpadas LEDs (SALDANHA *et al*., 2012; BATISTA *et al*., 2018; MARTINS *et al*., 2020; SOUZA *et al*., 2022a).

Na micropropagação fotomixotrófica sob a qualidade e intensidade de luz emitida e por aumento da troca gasosa por meio de membranas porosas, o desenvolvimento e crescimento das culturas ou acumulação de carboidratos depende em grande parte da fotossíntese e absorção de nutrientes inorgânicos (HERINGER *et al*., 2017; SOUZA *et al*., 2019; MARTINS *et al*., 2020). No cultivo *in vitro*, as plantas perdem parcialmente o autotrofismo e, consequentemente, necessitam de uma fonte exógena de carboidratos, sendo a sacarose a mais utilizada na cultura de tecidos vegetais, contudo, a concentração empregada pode ser ajustada de acordo com a troca gasosa e a fonte de luz à qual as plantas estão expostas (BIANCHETTI *et al*., 2017; SOUZA *et al*., 2019; GAGO *et al*., 2022).

Entre os fatores externos, a intensidade, qualidade de luz e o uso de membranas porosas é de fundamental importância para otimizar o cultivo *in vitro*, pois atua na produção de fotoassimilados, e também como estímulo percebido por fotorreceptores que desencadeiam a fotomorfogênese nos vegetais (GUPTA *et al*., 2017; OLIVEIRA *et al*., 2021; NERY *et al*., 2021). Contudo, as plantas são organismos sésseis, não apenas a morfologia e anatomia do vegetal estão relacionadas ao fotomixotrofismo, mas interações físicas, químicas e bioquímicas também estão sob sua influência (BATISTA *et al*., 2018; SOUZA *et al*., 2022a).

Além disso, fontes de luz LEDs com diferentes comprimentos de onda (< 400 ultravioleta; 400 a 450 violeta; 450 a 500 azul; 500-570 verde; 570-590 amarelo; 590-610 laranja; 610 a 760 vermelho; e > 760 nm de cor infravermelha), pode ser usado sozinho ou combinados para otimizar os processos morfofisiológicos (BATISTA *et al*., 2018; SOUZA *et al*., 2020; ZEPS *et al*., 2022). Devido a capacidade dos diodos emissores de luz (LEDs) tem de modificar diversos metabolismos no vegetal, outras vantagens também podem – se denotar, como fornecer condições ambientais ideais, principalmente luz e temperatura, onde a substituição da iluminação fluorescente padrão com LEDs economizaria custos de energia e seria mais ecológico (BUGBEE, 2016; MILER *et al*., 2019).

Contudo, diferentes estratégias vêm sendo utilizadas, tais como a qualidade e intensidade de luz nas salas de crescimento e o emprego de sistemas de ventilação dos recipientes (PAWLOWSKA *et al*., 2018; BATISTA *et al*., 2018; SOUZA *et al*., 2019; SOUZA *et al*., 2020b; SOUZA *et al*., 2022a), visando criar um ambiente mais favorável para o desenvolvimento das plantas *in vitro*. Assim, várias pesquisas são encontradas com bons resultados e pode desempenhar diversos efeitos na propagação vegetativa.

Para maior multiplicação, alongamento e vigor de brotos foi observado utilizando a LEDs vermelho/azul em *Populus euroamericana* e *Corymbia torelliana* × *Corymbia citriodora*, respectivamente (ARENCIBIA *et al*., 2017; SOUZA *et al*., 2020b); maior concentração de clorofila e enraizamento com LEDs azul em *Vaccinium corymbosum* (HUNG *et al*., 2016); aumento da proliferação de brotos em *Eucalyptus grandis* × *E. urophylla* (SOUZA *et al*., 2022a) e produtividade de microestacas em *Eucalyptus urophylla* com a LEDs vermelho/azul e intensidade luminosa de 80 µmol m^{-2} s^{-1} (MIRANDA *et al*., 2020); para o cultivo *in vitro* e aclimatação *ex vitro* de *Hevea brasiliensis* e *Eucalyptus dunnii*, melhores resultados para comprimento e número de

raiz, sobrevivência e enraizamento, foram obtidos em frascos com membranas porosas (TISARUM *et al*., 2017; SOUZA *et al*., 2019).

2.4. Biorreator de imersão temporária (BIT)

O uso do biorreator foi descrito pela primeira vez por Steward em 1952, mas sua o uso massivo começou anos depois, após os estudos de Alvard e Teisson. Os sistemas de biorreatores por imersão temporária (BIT) permitem contato temporário entre as plantas e o meio líquido, evitando assim a imersão contínua, e consequentemente maior adequação no fornecimento de oxigênio para as culturas (BELLO-BELLO *et al*., 2021; DE CARLO *et al*., 2021; HWANG *et al*., 2022).

A tecnologia dos biorreatores consiste em um sistema automatizado usado na cultura de tecidos de plantas e pode ser aplicado para otimizar todas as fases da micropropagação em laboratórios e biofábricas, bem como: estabelecimento e multiplicação (SOUZA *et al*., 2020c; GAGO *et al*., 2021; SOUZA *et al*., 2022b); alongamento (VIDAL *et al*., 2019; CARVALHO *et al*., 2019; ALVES *et al*., 2021) enraizamento e aclimatização (ALVES *et al*., 2021; BELLO – BELLO *et al*., 2021; CHÁVEZ – ORTIZ *et al*., 2021)

Esse tipo de sistema pode maximizar os protocolos da micropropagação, permitindo a obtenção de ganhos em biomassa e a redução do tempo necessário para a propagação, como observado em plantas medicinais (DE CARLO *et al*., 2021); aumentar a produção de plantas por unidade de área em *Mimosa calodendron* (SOUZA *et al*., 2022b); maior proliferação de brotos em *Castanea dentata* (LIU *et al*., 2022), *Eucalyptus grandis* × *E. urophylla* (SOUZA *et al*., 2020a) e *Verbena officinalis* (KOKOTKIEWICZ *et al*., 2021); maior porcentagem e comprimento de raízes em *Hylocereus undatus* (BELLO – BELLO *et al*., 2021) e *Agave guiengola* (CHÁVEZ – ORTIZ *et al*., 2021).

Nesse contexto, o objetivo da utilização do biorreator é fornecer condições ótimas de crescimento, regulando parâmetros químicos ou físicos, a fim de alcançar rendimento máximo e alta qualidade dos explantes, ou para reduzir os custos de produção por meio da automatização dos processos de cultivo (CARVALHO *et al*., 2019; VIDAL *et al*., 2019) Os biorreatores por imersão temporária são capazes de melhorar o fornecimento de nutrientes e a transferência de gases, e os

distúrbios fisiológicos podem ser minimizados, o que resulta em maior desenvolvimento das culturas micropropagadas (CARVALHO *et al*., 2019; ZHANG *et al*., 2022).

Além disso, o aumento do metabolismo nos tecidos vegetais (respiração aeróbica e fotossíntese), permitem maior aclimatização, enraizamento e/ou qualidade da raiz para as condições *ex vitro* (CARVALHO *et al*., 2019; HWANG *et al*., 2022), No entanto, há necessidade do maior controle da hiperidricidade para proporcionar maior proliferação e qualidade das plantas obtidas (VIDAL *et al*., 2019; SOUZA *et al*., 2020c; SOUZA *et al*., 2022b).

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Artigo publicado na Revista Scientia Forestalis

https://www.ipef.br/publicacoes/scientia/nr127/2318-1222-scifor-48-127-e3329.pdf

https://doi.org/10.18671/scifor.v48n127.03

SCIENTIA FORESTALIS

ISSN 2318-1222 (online)

ORIGINAL ARTICLE

Light quality in micropropagation of *Eucalyptus grandis* × *Eucalyptus urophylla*

Qualidade de luz na micropropagação de Eucalytpus grandis × Eucalyptus urophylla

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How to cite: Souza, D. M. S. C., Fernandes, S. B., Avelar, M. L. M., Frade, S. R. P., Molinari, L. V., Gonçalves, D. S., Pinto, J. E. B. P., Brondani, G. E. (2020). Light quality in micropropagation of *Eucalyptus grandis* × *Eucalyptus urophylla*. *Scientia Forestalis*, 48(127), e3329. https://doi.org/10.18671/scifor.v48n127.03

Abstract

The importance of eucalypts species and their interspecific hybrids has been evidenced in forestry programmes due to their wood quality and adaptation to diverse environmental conditions. To solve the limiting factors in the *in vitro* cultivation of the A211 clone of *Eucalyptus grandis* Hill ex Maiden × *Eucalyptus urophylla* S. T. Blake, the aim of the study was to evaluate the light quality on *in vitro* establishment, multiplication, elongation and rooting stages. The tissues used to obtain explants were nodal segments derived from ministumps of the A211 clone. The effects of light quality on *in vitro* establishment, multiplication, elongation and rooting were evaluated using five sources (fluorescent lamp, white LEDs, red LEDs, blue LEDs and red/blue LEDs). At 30 days, the percentage of contamination and non-responsive explants, length and number of shoots per explant, oxidation, shoot vigor, pigment content, leaf anatomy, root length and number of roots per explant were evaluated. Fluorescent lamps and red/blue LEDs are more suitable for use in the *in vitro* establishment, multiplication, elongation and rooting of the A211 clone, without hindering the development of shoots for the production of micropropagated clonal plants.

Keywords: *In vitro* propagation; Wave-length; LEDs, Cloning.

Resumo

A importância das espécies de eucalipto e de seus híbridos interespecíficos tem sido evidenciada nos programas de silvicultura, devido à qualidade da madeira e adaptação a diversas condições ambientais. Para solucionar os fatores limitantes ao cultivo *in vitro* do clone A211 de *Eucalyptus grandis* Hill ex Maiden × *Eucalyptus urophylla* S. T. Blake, o presente trabalho teve como objetivo avaliar a influência da qualidade de luz nas fases de introdução, multiplicação, alongamento e enraizamento *in vitro*. Os tecidos utilizados para a obtenção dos explantes foram segmentos nodais, provenientes de minicepas do clone A211 de *E. grandis* × *E. urophylla*. Os efeitos no cultivo *in vitro* foram avaliados por meio de cinco fontes de qualidade de luz (Lâmpada fluorescente, LEDs branco, LEDs vermelho, LEDs azul e LEDs vermelho/azul). Dados de porcentagem de contaminação e explantes não responsivos, comprimento e número de brotos por explante, oxidação, vigor das brotações, conteúdo de pigmentos, anatomia foliar, comprimento e número de raízes por explante foram coletados aos 30 dias de cultivo *in vitro*. Com base nos resultados obtidos, pôde-se inferir que as lâmpadas fluorescentes e LEDs vermelho/azul são as mais adequadas para serem utilizadas na introdução, multiplicação, alongamento e enraizamento *in vitro* do clone A211 de *E. grandis* × *E. urophylla*, não ocasionando prejuízos no desenvolvimento de brotações destinadas à produção de plantas clonais micropropagadas.

Palavras-chave: Propagação *in vitro*; Comprimento de onda; LEDs, Clonagem.

Financial support: We thank the National Counsel of Technological and Scientific Development (CNPq), Coordination for Improvement of Higher Education Personnel (CAPES) and Research Support Foundation of the State of Minas Gerais (FAPEMIG) for the financial support and scholarship.
Conflict of interest: nothing to declare.
Correspondig author: gilvano.brondani@ufla.br
Received: 05 abril 2019.
Accepted: 02 agosto 2019.
Editor: Paulo Henrique Müller Silva.

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INTRODUCTION

The expansion of forest plantations to non-traditional regions and the search for improved plant production have increased the use of species and hybrid combinations. The hybridization of *Eucalyptus grandis* with *Eucalyptus urophylla* has provided excellent results in wood quality and growth, in addition to providing better environmental adaptation and easier vegetative propagation (Assis et al., 2018; Trueman et al., 2018). In recent years, research on the micropropagation of *E. grandis*, *E. urophylla* and their hybrids has increased and has included the *in vitro* establishment (Keret et al., 2018), multiplication (Trueman et al., 2018), elongation (Gallo et al., 2017), rooting and acclimatization stages (Brondani et al., 2018).

To understand the limiting factors of each stage of *in vitro* cultivation, it is necessary to conduct basic studies to maximize the production of microstumps. Several technologies have been proposed to automate the micropropagation process, including innovations in the culture environment such as alternative containers that allow gas exchange, use of bioreactors, new LED light sources, and automation of culture systems and routine procedures, such as medium preparation, transplant and acclimatization (Brondani et al., 2018; Batista et al., 2018; Souza et al., 2019).

Recent studies report how different wavelengths influence plant metabolism. According to Batista et al. (2018), blue light (450 – 495 nm), red light (620 – 750 nm), far-red light (750 – 850 nm) and green light (495 – 570 nm) influence the *in vitro* morphogenesis of the plant. In addition to its role in photosynthetic processes, light can act as an external regulation factor in several morphogenic and physiological processes that alter the architecture and phytochemical levels of the plant (Ouzounis et al., 2015). Studies on the effect of different wavelengths of LED lamps on morphogenesis and growth are still scarce for eucalypts species.

Given the need to adapt micropropagation for *E. grandis* × *E. urophylla* A211 clone, this study aimed to evaluate the effect of light quality on *in vitro* establishment, multiplication, elongation and rooting stages.

MATERIAL AND METHODS

Study site and experimental material

The experiments were conducted at the Tissue Culture Laboratory of the Department of Agriculture (DAG), Federal University of Lavras - UFLA, Lavras, Minas Gerais (MG), Brazil. The material used to obtain the explants originated from ministumps of the A211 clone of *Eucalyptus grandis* Hill ex Maiden × *Eucalyptus urophylla* S. T. Blake. They were provided by the 'Viveiro Esteio Produção de Mudas' company, located in the municipality of São João Del Rei - MG, Brazil.

The ministumps were established in UFLA's Department of Forestry Sciences Forest Nursery in a gutter for the clonal mini-garden system containing sand substrate, under a semi-hydroponic system with nutrient solution dripping four times a day at a total flow rate of 4 L m^{-2} day.

Collection of shoots and preparation of explants

Nodal segments (3 – 4 cm) sectioned from the third and fourth terminal nodes of the shoots were collected 20 days after pruning the ministump apex. Subsequently, the explants were immersed in deionized and autoclaved water and transported to the tissue culture laboratory for the *in vitro* inoculation step.

In vitro establishment

The nodal segments were washed in running water and immersed in a fungal solution containing 2.4 g L^{-1} of Orthocide 500® (50% Captan as active principle) for 15 minutes. Next, the explants were washed five times in autoclaved deionized water and immersed in 70%

alcohol solution (v/v) for 30 seconds with constant agitation inside a horizontal laminar flow cabinet. Then, they were immersed in 1% NaClO (v/v) Clarix® solution for 15 minutes. Finally, the nodal segments were washed in autoclaved deionized water five times, and the explants were prepared and inoculated vertically under aseptic conditions in test tubes (25 × 150 mm) containing 10 mL of the culture medium.

The basic culture medium used in the experiments was the saline MS formulation (Murashige & Skoog, 1962) supplemented with 30 g L^{-1} of sucrose (Synth Ltda) and 6 g L^{-1} of agar (Merck SA). The pH was adjusted to 5.8 ± 0.05. The culture media was autoclaved at 127°C and pressure of 1.5 kgf cm^{-2} for 20 minutes. The explants were kept in a growth room at a temperature of 24 ± 1 °C, a photoperiod of 16 hours of light and irradiance of 40 µmol m^{-2} s^{-1} (quantified by radiometer, LI-COR®, LI-250A Light Metre) using different light sources.

The data on mean contamination percentage (fungal and/or bacterial), non-responsive explants, oxidation and vigor were evaluated according to the scoring scale proposed by Oliveira et al. (2016) (Figure 1A-F), and the length of shoots larger than 0.5 cm and mean number of shoots per explant greater than 0.5 cm were evaluated 30 days after inoculation.

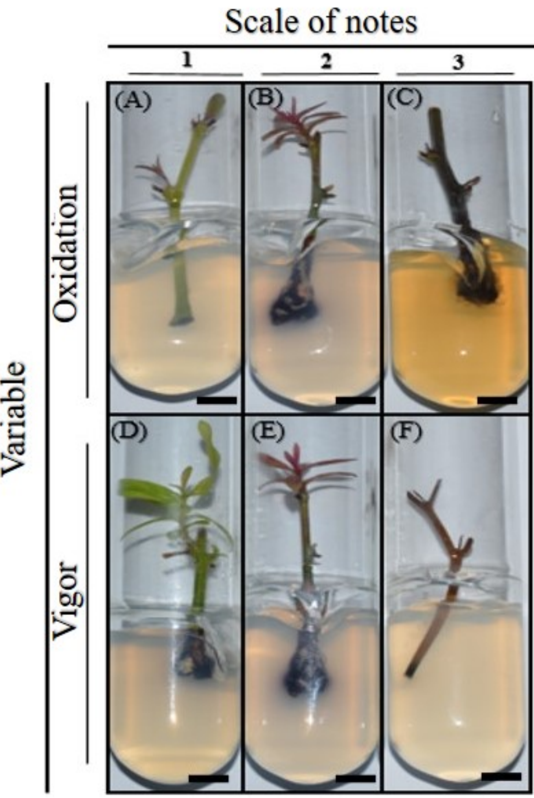

Figure 1. Oxidation and vigor assessments according to a scale of notes. **(A)** OXI 1 = Null: no oxidation; **(B)** OXI 2 = Average: reduced oxidation at the base of the explants medium with grayish tonality; **(C)** OXI 3 = High: complete oxidation of shoots); **(D)** VIG 1 = Optimum: induction of shoots with active growth, without apparent nutritional deficiency; **(E)** VIG 2 = Good: induction of shoots, but with leaves of reduced size; **(F)** VIG 3 = Low: no induction of shoots and, senescence and death.

In vitro multiplication

Shoots (± 0.5 cm) produced during the *in vitro* establishment stage were subcultured into test tubes (25 mm × 150 mm) containing 10 mL of MS culture medium supplemented with 2.22 µM of 6-benzylaminopurine - Sigma® (BAP), 0.05 µM of naphthaleneacetic acid - Sigma® (NAA) and 6 g L^{-1} of agar.

Data on oxidation, vigor, shoot length (> 0.5 cm), mean number of shoots per explant (> 0.5 cm) and photosynthetic pigments were collected at 30 days of culture.

In vitro elongation and rooting

The shoots produced in the *in vitro* multiplication stage were prepared by isolating four standard shoots of 0.5 cm and inoculated under aseptic conditions in glass flasks (250 mL capacity). Shoots were grown for 30 days in flasks containing 50 mL of MS culture medium supplemented with 0.22 μM of BAP and 2.46 μM of indole-3-butyric acid - Sigma® (IBA), 30 g L^{-1} of sucrose, and 6 g L^{-1} of agar.

Data on mean oxidation and vigor according to a scoring scale, shoot length (> 0.5 cm), mean number of shoots per explant (> 0.5 cm), number of roots and length of the longest root (cm) were collected at 30 days of culture.

Light quality

A spectrophotometer (Ocean Optics Spectra-Suite) was used for the analysis of the variations of absolute irradiance (μW cm^{-2} nm^{-1}) and the wavelength (nm) of light emitted by the different types of lamps. That data was then used to plot the different light spectra in graphs (Figure 2A-E).

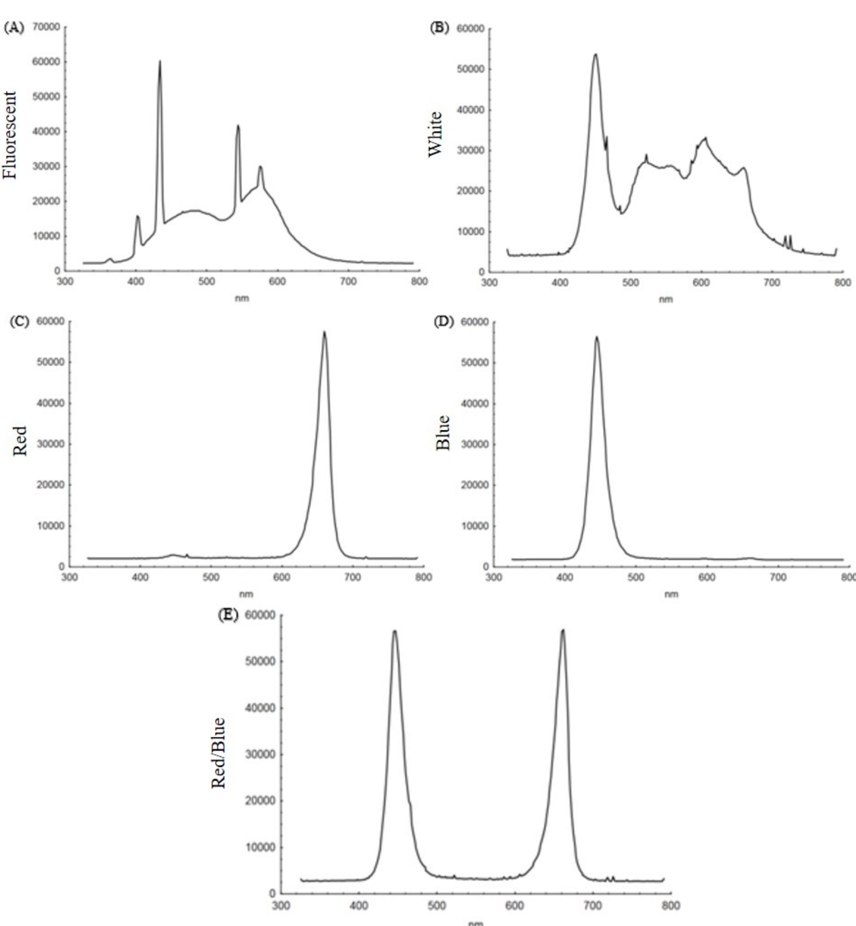

Figure 2. Variations of absolute irradiance (μW cm^{-2} nm^{-1}) and wavelength (nm) of light emitted by: **(A)** Fluorescent lamp; **(B)** White LEDs; **(C)** Red LEDs; **(D)** Blue LEDs; **(E)** 1:1 Red/Blue LEDs used in the *in vitro* condition of the hybrid clone A211 of *E. grandis* × *E. urophylla*.

Photosynthetic pigment analysis

Leaf discs (i.e., 25 mg of leaf fresh matter) were sampled from shoots of the *in vitro* multiplication assay after 30 days under the different light sources and inoculated in 5 mL of DMSO solution (Sigma Aldrich) for 48 hours in the dark (Lichtenthaler, 1987). The absorbance of the samples was determined in triplicate in a quartz cuvette with a 10 mm pathlength in a Genesys 10UV spectrophotometer (ThermoScientific, USA).

Leaf anatomy

Representative samples of leaves from each treatment were collected and kept for 48 hours in 70% formaldehyde acetic acid solution, followed by a transfer to 70% ethanol and a graded ethanol series (80, 90 and 100%) and kept for 30 minutes in each solution (Johansen, 1940). Then, the samples were transferred to a 100% alcohol solution and historesin (Leica®) in a 1:1 ratio in a hot oven (overnight). The embedding was performed with pure hydroxyethyl methacrylate resin, and 7 mm cross sections were obtained with a manual rotary microtome and a knife. The tissues were stained with toluidine blue, mounted on histological slides, and photomicrographed with a coupled digital camera (AxionCam ERc5s) on a micrometre scale and 20x and 40x objectives.

Experimental design and data analysis

In vitro establishment, multiplication, elongation and rooting stages were arranged in a completely randomized design with five different light sources: fluorescent lamp - L/F (HO Sylvania T12, 110 W, São Paulo, Brazil), white LED lamp - L/W (SMD 100, 18 W, Vilux®, Vitória, ES, Brazil), red LED lamp - L/R (LabPARLL-HR/DB-480, 11.6 W, LabLumens®, Carapicuíba, SP, Brazil), Blue LED lamp - L/B (LabPARLL-HR/DB-480, 11.6 W, LabLumens®, Carapicuíba, SP, Brazil) and 1:1 red/blue LEDs lamp - R/B (LabPARLL-HR/DB- 480, 11.6 W, LabLumens®, Carapicuíba, SP, Brazil). Thirty samples/replicates of one explant each were used for the establishment and multiplication. Twelve samples/replicates, consisting of four explants each, were used for *in vitro* elongation and rooting.

The analyses were processed with the R Core Team software, 2018, using the ExpDes package, version 1.1.2 (Ferreira et al., 2013). The data were subjected to analysis of variance (ANOVA) with Tukey's test at 5% significance level.

RESULTS AND DISCUSSION

Effect of light quality on *in vitro* establishment

The appearance of the *E. grandis* × *E. urophylla* A211 clone explants, according to the studied characteristics, can be observed in Figure 3A-D.

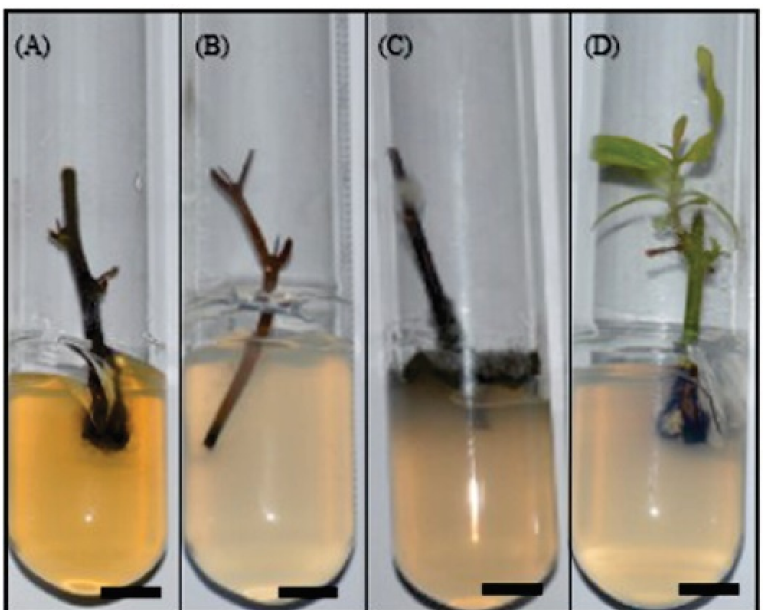

Figure 3. Explants of *E. grandis* × *E. urophylla* clone A211, at 30 days after inoculation on *in vitro* condition: **(A)** Oxidized explant; **(B)** Non responsive explant; **(C)** Explant contaminated; **(D)** Reactive explant. Bar = 0.5 cm.

The different light sources used in the *in vitro* culture of A211 clone explants influenced the percentage of contamination at 30 days after inoculation (Figure 4A). The lowest mean contamination occurred with the use of the R/B source (6.6%) but did not differ from the use of separate L/R and L/B lights ($p < 0.05$). Most likely, this result may be related to the metabolic pathway of microorganisms given that the fungi are receptors of the blue and red wavelengths, absorbing photons into the cells, regulating the fungal photoresponses by differential genetic expression in the carotenoid biosynthesis and hyphae aggregation (Postemsky & Curvetto, 2016).

Low percentages of explants without shoots were observed mainly with light source L/F (mean of 3.3%) (Figure 4B). In an *E. urophylla* × *E. globulus* hybrid, it was possible to obtain 95% of explants with shoots using the fluorescent lamp (Borges et al., 2011). Oliveira et al. (2015) found 51.2% of explants with shoots in *Eucalyptus cloeziana*. Thus, the establishment of tissues during the *in vitro* establishment stage may depend on the plant material (genotype) and on the culture conditions used, such as the light quality in the growth room.

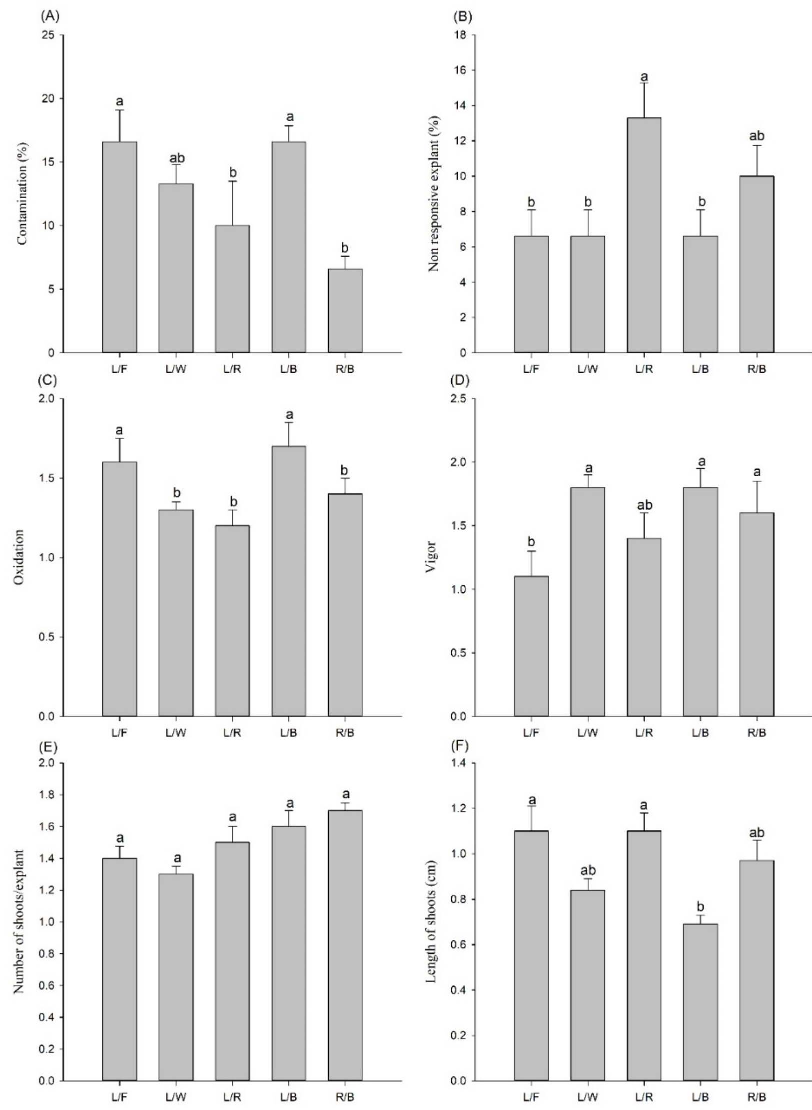

Figure 4. Characteristics observed on *in vitro* introduction of *E. grandis* × *E. urophylla* clone A211 under qualities of light [Fluorescent lamp (L/F), White LEDs (L/W), Red LEDs (L/R), Blue LEDs (L/B) and Red/Blue LEDs (R/B)]. **(A)** Percentage of contamination; **(B)** Percentage non responsive explant; **C-D**: Scale of notes (**Figure 1): (C)** Oxidation; **(D)** Vigor; **(E)** Number of shoots per explant; **(F)** Length of shoots (cm). * Means followed by the same letter do not differ from each other, by the Tukey test at 5% of significance. Bars represent standard deviation.

The oxidation and vigor, according to the scoring scale, showed the lowest means in the light sources L/F, L/W, L/R and R/B, with a significant difference ($p < 0.05$) for the L/B (Figure 4C and 4D). These results are close to those found by Souza et al. (2018), who observed low phenolic oxidation and good vigor of the explants in the *in vitro* establishment of *Corymbia citriodora* × *C. torelliana.*

Phenolic oxidation has been a problem associated with the micropropagation of woody species, especially when considering the *in vitro* establishment stage, which may influence culture growth. These results may be related to culture factors, such as flask size (with smaller flasks leading to low carbon dioxide concentrations and high ethylene concentrations), light intensity, air temperature and relative humidity (Xiao et al., 2011).

The number of shoots, explants under the light source R/B had a higher mean (1.7 shoots) but showed no significant difference for the other treatments ($p > 0.05$) (Figure 4E). The combination of red and blue lights using LED lamps resulted in a higher number of shoots per explant in *Acer saccharum* (Singh et al., 2017), indicating the importance of this light combination for *in vitro* culture.

Regarding the shoot length, it was found that the light quality also affected the development of the explants with significant changes in shoot growth. The L/F and L/R sources promoted higher mean length (1.1 cm) and differed from the L/B light source (Figure 4F). Light-emitting diodes (LEDs) are alternative light sources due to their wavelength specificity, low amount of heat produced, low degradation and high durability, which benefits *in vitro* culture and reduces costs when compared to fluorescent lamps (Bugbee, 2016). However, the fluorescent light also favored the development of A211 clone explants under *in vitro* conditions.

Effect of light quality on *in vitro* multiplication

The different light sources influenced oxidation of the tissues, with a lower mean score (1.1) for the L/W source, differing ($p < 0.05$) only from the L/B source (Figure 5A). The lowest mean shoot vigor score (1.2) was observed with the L/F light source (Figure 5B), resulting in tissue growth and the absence of nutritional deficiency, according to the scoring scale. Using a fluorescent lamp in the *in vitro* multiplication stage of *E. cloeziana*, by Oliveira et al. (2015), found similar results when considering the scoring scale for the classification of shoot vigor.

For the number of shoots per explant, equal mean values (9.3 shoots) were observed using the light sources R/B and L/F; however, they differ significantly ($p < 0.05$) from the L/B treatment (Figure 5C). Likewise, the R/B and L/F sources promoted similar mean shoot lengths (~ 1.7 cm) and differed ($p < 0.05$) from the L/W, L/R and L/B sources (Figure 5D). The data in the literature corroborate those found in the present study because the combination of red/blue LEDs in *Populus euramericana* induced a greater number and length of shoots (Kwon et al., 2015). Batista et al. (2018) confirmed the superiority of LED lamps in the *in vitro* development of plants when compared to fluorescent lamps.

The amounts of chlorophyll a, b and carotenoids were influenced by the different light qualities (Figure 5E). The results indicate that the light sources L/F, R/B and L/W generated higher amounts of the aforementioned photosynthetic pigments. The biosynthesis of chlorophyll a, b and carotenoids is influenced by the quality and intensity of emitted light, with greater efficiency of the photosynthetic process predominantly in the combined blue and red wavelengths (Dutta Gupta & Karmakar, 2017).

Higher levels of chlorophyll a and b were observed in the *in vitro* multiplication of *Gerbera jamesonii* under red/blue LED light compared to separate blue and red LED lights (Pawłowska et al., 2018). In *Stevia rebaudiana,* the amounts of carotenoids in shoots exposed to red/blue LED light were higher compared to blue LED light (Ramírez-Mosqueda et al., 2017).

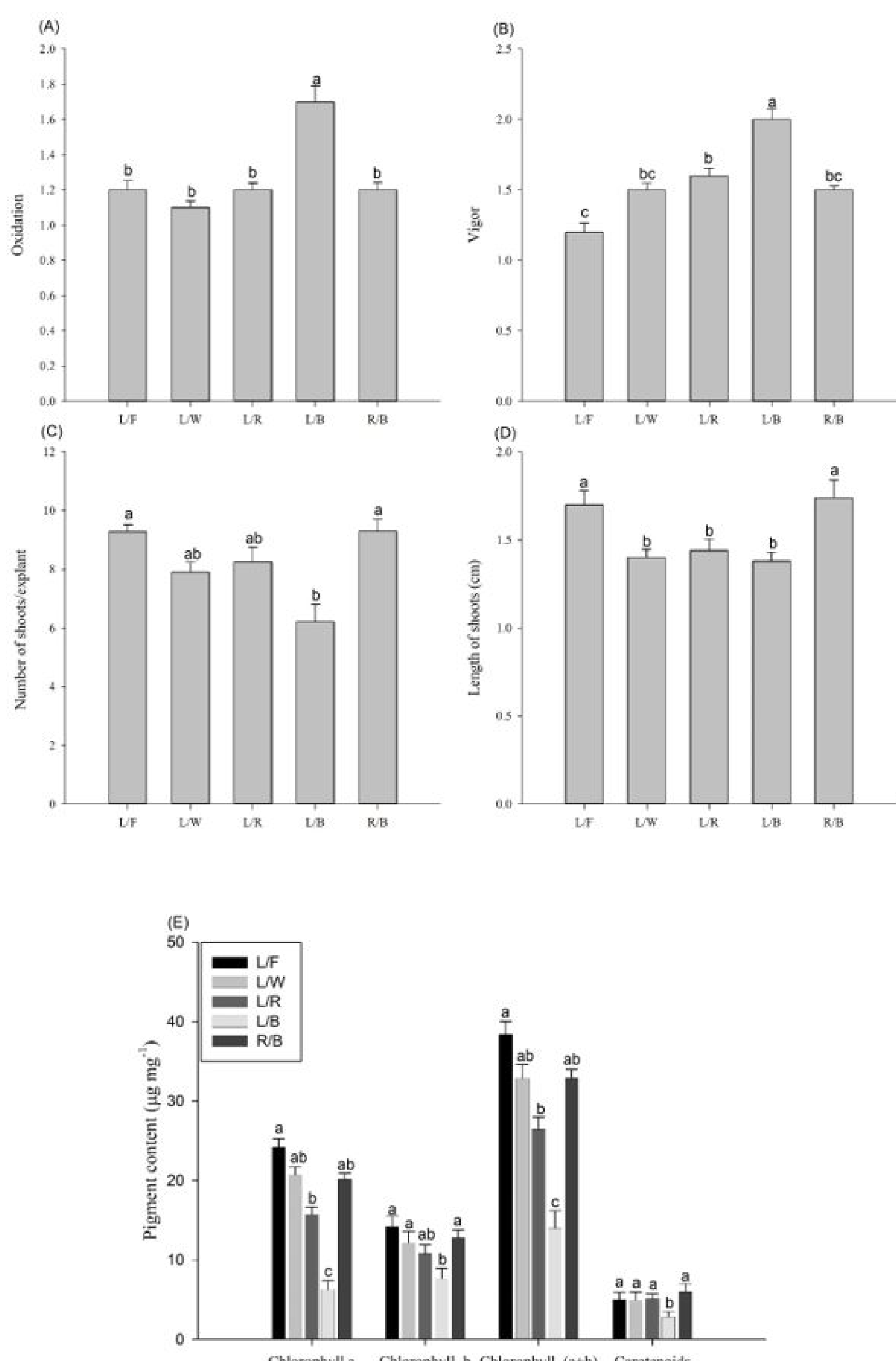

Figure 5. Characteristics observed on *in vitro* multiplication of *E. grandis* × *E. urophylla* clone A211 under qualities of light [Fluorescent lamp (L/F), White LEDs (L/W), Red LEDs (L/R), Blue LEDs (L/B) and Red/Blue LEDs (R/B)]. **A-B:** Scale of notes: **(A)** Oxidation; **(B)** Vigor; **(C)** Number of shoots per explant; **(D)** Length of shoots (cm); **(E)** Pigment content. *Means followed by the same letter do not differ from each other by the Tukey test at 5% of significance. Bars represent standard deviation.

Effect of light quality on *in vitro* elongation and rooting

Regarding the *in vitro* elongation of shoots, the L/F light sources produced the best results for mean length (7.2 cm) and vigor (1.2) of shoots (Figure 6B and 6D), differing significantly ($p < 0.05$) for light sources L/R and L/B. Similar results using a fluorescent lamp source were observed for *Abies borisii-regis* explants (Smirnakou et al., 2016). The adverse effect of the light spectrum (450 nm to 500 nm) may inhibit stem elongation (Taiz & Zeiger, 2013).

Regarding the mean oxidation score (1.1), R/B showed the lowest mean values but did not differ statistically ($p > 0.05$) from the other treatments (Figure 6A). For the mean number of shoots, R/B had the best morphogenetic responses under *in vitro* cultivation (Figure 6C), differing significantly ($p < 0.05$) for the L/W light source.

The control of LED light quality during plant culture is important because it can optimize the photosynthetic rate and increase shoot production (Silva et al., 2017). However, light can also influence the concentrations of plant growth regulators, acting as a morphogenic signal for the proliferation of shoots, whereas the early stages in the light-signalling pathway involve the activation of cytokinins (Roman et al., 2016).

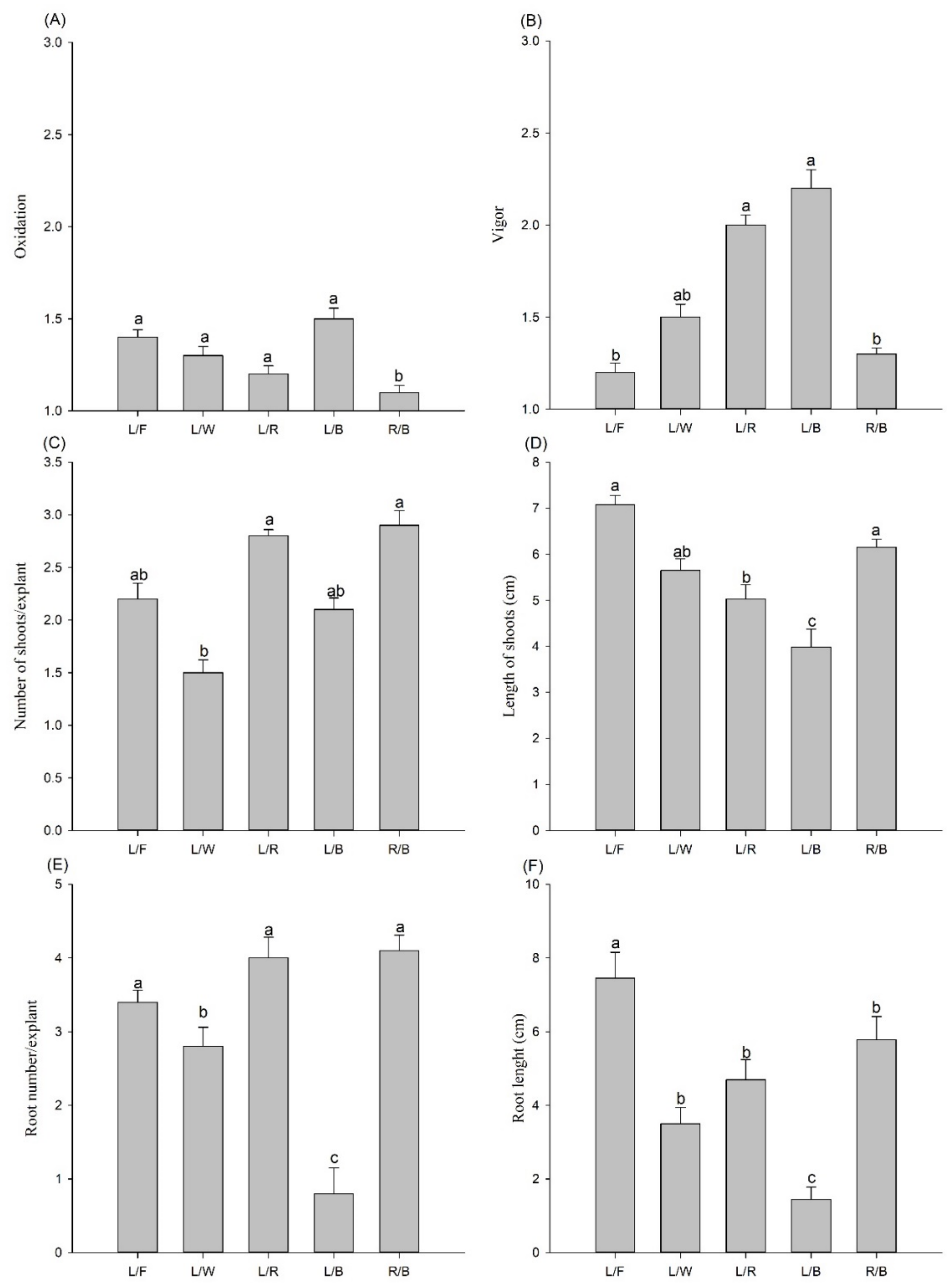

Figure 6. Characteristics observed on *in vitro* elongation of *E. grandis* × *E. urophylla* clone A211 under qualities of light [Fluorescent lamp (L/F), White LEDs (L/W), Red LEDs (L/R), Blue LEDs (L/B) and Red/Blue LEDs (R/B)]. **A-B**: Scale of notes (**Figure 1): (A)** Oxidation; **(B)** Vigor; **(C)** Number of shoots per explant; **(D)** Length of shoots (cm); **(E)** Root number per explant; **(F)** Root length (cm) * Means followed by the same letter do not differ from each other, by the Tukey test at 5% of significance. Bars represent standard deviation.

Regarding the root system of microcuttings, the use of R/B provided a higher mean number of roots (4.1), with a significant difference for L/W and L/B (Figure 6E). Similar results were found in *Microlaelia lundii* microcuttings, where the main advantages were the increase in rooting percentage, number of roots and survival percentage (Favetta et al., 2017). Regarding the mean length of the longest root per microcutting, the use of L/F (7.6 cm) was superior to the other treatments (Figure 6F). Similar results were observed in *Populus americana* microcuttings (Kwon et al., 2015).

Understanding the relationship between wavelengths and plant growth regulators under *in vitro* culture enables micropropagation protocols to be better defined for the species studied here (Figure 7).

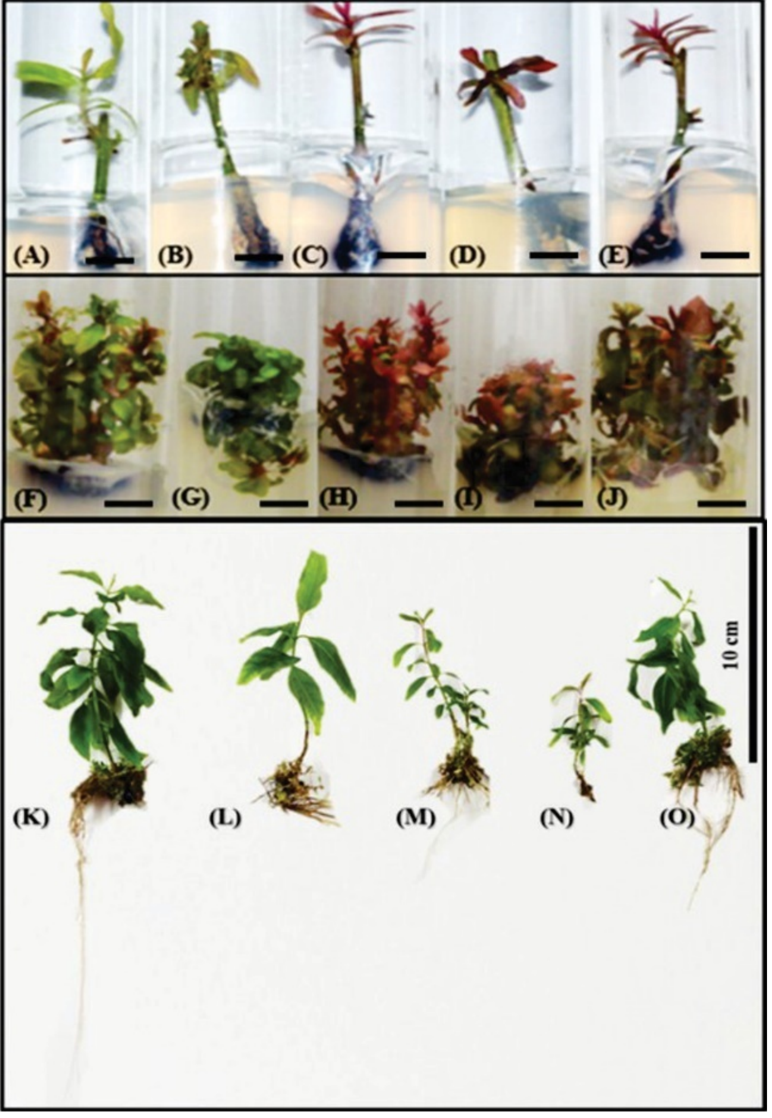

Figure 7. Sprouts of *E. grandis* × *E. urophylla* clone A211 *in vitro* cultivated under different light sources. **(A-E)**: 30 days after inoculation; **(F-J)**: *In vitro* multiplication; **(K-O)**: *In vitro* elongation and rooting; **(A, F** and **K)**: Fluorescent lamp; **(B, G** and **L)** White LEDs; **(C, H** and **M)** Red LEDs; **(D, I** and **N)** Blue LEDs; **(E, J** and **O)** Red/Blue LEDs. Bar = 0.5 cm.

Modifications in the structural organization of leaf cells can be observed at the anatomical level when plants are exposed to different light spectra (Figures 8A-C). Greater mesophyll cell development and organization (Figures 8A and 8C) is observed under L/F and R/B lights (Figures 8A and 8C), characterized by a reduction in the volume of intercellular spaces. The opposite can be observed in leaves under L/B light (Figure 8B).

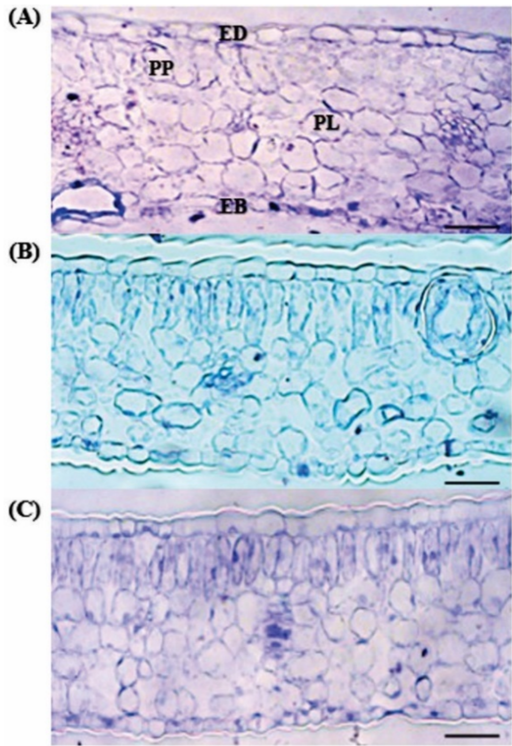

Figure 8. Cross sections of the leaf limb of *E. grandis* × *E. urophylla* clone A211 in different light qualities: **(A)** Fluorescent lamp; **(B)** Blue LEDs; **(C)** Red / blue LEDs. * **ED =** Adaxial epidermis; **PP =** Palisade parenchyma; **PL =** Lacunar parenchyma; **EB =** Abaxial epidermis. Bar = 200 μm.

The red spectrum has a strong influence on the development and expansion of mesophyll cells, while the opposite is true for blue light in ornamental plants. In addition, each spectrum is capable of altering expression levels of specific genes involved in the structural organization of plant cells as well as of photosynthetic genes (Fan et al., 2013).

CONCLUSIONS

The fluorescent and red/blue LED lamps showed the best results in the *in vitro* establishment, multiplication, elongation and rooting of *E. grandis* × *E. urophylla* A211 clone. These two light sources led to lower tissue oxidation, and higher vigor, shoot length, mean number of shoots per explant, photosynthetic pigment content, number of roots and length of the longest root.

ACKNOWLEDGMENT

We thank the National Council of Technological and Scientific Development (CNPq), Coordination for Improvement of Higher Education Personnel (CAPES) and Research Support Foundation of the State of Minas Gerais (FAPEMIG) for the financial support and scholarship.

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Author contributions: DMSCS: conceptualization, data curation, formal analysis, methodology, writing – original draft, writing – review & editing; SBF: conceptualization, data curation, formal analysis, methodology, writing – original draft; MLMA: conceptualization, data curation, formal analysis, methodology, writing – original draft, writing – review & editing; SRPF: formal analysis, methodology, writing – original draft; LVM: formal analysis, methodology, writing – original draft; DSG: formal analysis, methodology, writing – original draft; JEBPP: supervision, validation, visualization, writing – review & editing; GEB: supervision, validation, visualization, writing – review & editing.

ARTIGO 2

Effect of light intensity on *in vitro* introduction and multiplication of *Eucalyptus grandis* × *Eucalyptus urophylla*

Artigo publicado na Revista In Vitro Cellular & Developmental Biology - Plant

https://link.springer.com/article/10.1007/s11627-021-10237-6

https://doi.org/10.1007/s11627-021-10237-6

In Vitro Cellular & Developmental Biology - Plant
https://doi.org/10.1007/s11627-021-10237-6

MICROPROPAGATION

Effect of light intensity on in vitro introduction and multiplication of *Eucalyptus grandis* × *Eucalyptus urophylla*

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Received: 12 August 2021 / Accepted: 2 October 2021 / Editor: Yong Eui Choi
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Abstract

Micropropagation technique has been recommended for plant tissue rejuvenation/reinvigoration and the improvement of clonal plants production commercially. Concerning the limiting factors on in vitro cultivation of *Eucalyptus grandis* × *E. urophylla* hybrid clone, the effects of light intensity on in vitro introduction and multiplication phases were assessed. The experimental tissue was collected from ministumps cultivated in a semi-hydroponic system. The results of light intensity on in vitro introduction and multiplication were evaluated with four treatments (fluorescent lamp/40 μmol m^{-2} s^{-1}, red/blue LEDs 20 μmol m^{-2} s^{-1}, red/blue LEDs 40 μmol m^{-2} s^{-1}, and red/blue LEDs 80 μmol m^{-2} s^{-1}). The explants' morphological, anatomical, and genetic characteristics were evaluated at 30 d in the in vitro introduction, and 12 subcultures for in vitro multiplication. Based on the results, the treatments F/L 40 μmol m^{-2} s^{-1} and R/B 80 μmol m^{-2} s^{-1} are the most suitable during in vitro introduction and multiplication of *Eucalyptus grandis* × *E. urophylla*. It provides a higher percentage of responsive explants, shoots per explant, vigor, photosynthetic pigment content, adaxial epidermis thickness, abaxial epidermis, mesophyll, palisade parenchyma, spongy parenchyma, and vascular tissues. Furthermore, it was found that the micropropagated plants are identical clones of the *Eucalyptus grandis* × *E. urophylla* selected tree; that is, there was no somaclonal variation during the twelve subcultures on in vitro multiplication.

Keywords Vegetative propagation · In vitro cultivation · Spectral quality · Tissue rejuvenation · Genetic fidelity

Introduction

Eucalyptus and *Corymbia* are the most cultivated in the Brazilian forest sector (6.97 million of hectares) (IBÁ 2020), due to diversity, adaptation to different edaphoclimatic conditions, fast growth, and raw material source for industrial segments (Carrillo *et al*. 2018). These characteristics have expanded the species usage and hybrid combinations of *Eucalyptus grandis* W. Hill ex Maiden with *Eucalyptus urophylla* S. T. Blake (known as urograndis eucalypt). However, one of the significant challenges of forestry is developing efficient methods for clonal propagation in industrial scale (Trueman *et al*. 2018).

Micropropagation technique is advantageous for the production of eucalypt clones since it makes possible mass propagation in short periods; greater nutritional, environmental, and phytosanitary control; straightforward transportation of clonal material over great distances; long-term storage; hybrid vigor retention; and tissue rejuvenation/reinvigoration of selected trees (Aggarwal *et al*. 2012; Ahad *et al*. 2018; Abiri *et al*. 2020). Therefore, micropropagation allows the formation of extensive and homogeneous commercial plantations.

However, in the clonal production of eucalypts, there are technical and economic limitations on in vitro cultivation for

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Published online: 23 November 2021

commercial-scale applications. The high cost, the need for protocol adjustment, the small investment in innovation on in vitro propagation process, and the limited survivability in ex vitro conditions are some factors that hinder its usage on a large scale (Shukla *et al*. 2017; Nunes *et al*. 2018). For cost reduction and maximization of the plant production process, micropropagation technologies with different sources of lighting, tissue rejuvenation/reinvigoration, and assessment of genetic fidelity have been studied (Batista *et al*. 2018; Carvalho *et al*. 2019; Solano *et al*. 2019; Miranda *et al*. 2020).

Light-emitting diode (LED) lamps are often used in place of fluorescent lamps, as they are more efficient and economically viable for in vitro plant growth and development. It emerges as an alternative for maximizing in vitro plant multiplication (Miler *et al*. 2019; Jung *et al*. 2021; Oliveira *et al*. 2021). Light intensity and quality are factors that influence photosynthesis, morphogenesis, flowering, and secondary metabolism (Batista *et al*. 2018; Kepenek 2019; Miranda *et al*. 2020; Zarei *et al*. 2021). In environments with low light intensity, photosynthesis can be inefficient, influencing growth and development, while excessive light can alter physiological processes that are critical to plant survival and photorespiration induction and consequently damage photosynthetic structures (Silva *et al*. 2017; Oliveira *et al*. 2021); thus, the adequate spectral qualities and luminous intensity in the growth pattern of the plants, allows greater knowledge about the process of in vitro production of clonal plants.

A positive factor concerning in vitro multiplication is the number of subcultures that target tissue rejuvenation/reinvigoration. It is an attribute of great relevance for cloning and improvement of plants (Tambarussi *et al*. 2018), and the production of plants on a large scale and with the same genetic characteristics as the parent plant is achievable (Shanthi *et al*. 2015; Brondani *et al*. 2018). In vitro multiplication by successive subcultures has provided tissue rejuvenation/reinvigoration (Oliveira *et al*. 2016; Souza *et al*. 2020c;Wang and Yao 2020). However, despite the economic importance of the *Eucalyptus* and its hybrids, studies on the effect of different light intensities in successive subcultures in the morphogenesis and growth process are still lacking.

The present study aimed to evaluate the effect of light intensity on in vitro introduction and multiplication phases considering hybrid clone, tissue rejuvenation and/or reinvigoration. It meets the principle that there is actual need for production maximization of micropropagated plants of *Eucalyptus grandis* ×*E. urophylla*.

Material and methods

Experimental material The genetic material used for making the explants (i.e., a nodal segment of 1 cm in length and an axillary bud without leaves), previously in vitro established and multiplied, were obtained from ministumps of the A211 hybrid clone (M1, selected tree) of *Eucalyptus grandis* W. Hill ex Maiden × *Eucalyptus urophylla* S. T. Blake, known as urograndis eucalypt, from "*Viveiro Esteio Produção de Mudas*" located in São João Del-Rei, MG, Brazil.

The ministumps were established in a clonal minigarden, under a semi-hydroponic system of "*canaletão*" type in a medium sand bed. The plants received a drip of nutrient solution four times a day, at a total daily flow of 4 L m^{-2}. The nutrient solution was composed of calcium nitrate (57.18 mg L^{-1}), potassium nitrate (206.85 mg L^{-1}), monoammonium phosphate (44.57 mg L^{-1}), magnesium sulfate (121.67 mg L^{-1}), calcium sulfate (87.18 mg L^{-1}), ammonium nitrate (140.50 mg L^{-1}), iron sulfate (9.95 mg L^{-1}), sodium-EDTA (13.31 mg L^{-1}), boric acid (6.20 mg L^{-1}), zinc sulfate (8.60 mg L^{-1}), manganese sulfate (4.92 mg L^{-1}), copper sulfate (0.39 mg L^{-1}), and sodium molybdate (0.05 mg L^{-1}). The electrical conductivity of the nutrient solution was maintained at around 2.0 mS m^{-2}, and the pH was adjusted to 6.0 (± 0.2) at a temperature of 25 °C with hydrochloric acid (HCl) or sodium hydroxide (NaOH), both at 1 M, according to Molinari *et al*. (2020).

Collection of shoots and preparation of explants Twenty days after pruning the ministump apex, shoots were collected from each nodal segment 3–4 cm long, without leaves, from the third and fourth terminal nodes. Subsequently, the nodal segments were immersed in autoclaved deionized water and transported to the laboratory for the in vitro introduction.

In vitro introduction Nodal segments (i.e., explants) were washed five times in running water and immersed in a fungicide solution containing 2.4 g L^{-1} of Orthocide 500®, São Paulo, Brazil (Captan 50% as the active ingredient) for 15 min. Subsequently, they were washed five times in autoclaved deionized water and immersed in a 70% (v v^{-1}) hydroalcoholic solution for 30 s with constant agitation inside a horizontal laminar flow chamber.

Then, explants were immersed in NaOCl solution Clarix® (2.0–2.5% of active chlorine) for 15 min. Finally, the explants were washed in autoclaved deionized water five times, and were inoculated vertically, under aseptic conditions in test tubes (25 × 150 mm), containing 10 mL of MS culture medium (Murashige and Skoog 1962) added with 30 g L^{-1} sucrose (Synth Ltda, Diadema, Brazil) and 6 g L^{-1} agar (Merck SA, Rio de Janeiro, Brazil).

In vitro multiplication Shoots produced in the in vitro introduction phase were isolated and standardized to 0.5 cm and inoculated under aseptic conditions in test tubes

(25 × 150 mm), containing 10 mL of MS culture medium, supplemented with 30 g L^{-1} of sucrose (Vetec®, Ceará, Brazil), 0.5 mg L^{-1} BAP (6-benzylaminopurine, Sigma®), 0.01 mg L^{-1} NAA (α-naphthaleneacetic acid, Sigma®, Barueri, Brazil), and 6 g L^{-1} of agar (Merck®).

For 360 d, 12 subcultures were evaluated (S1, S2, …, S12), with the culture medium being renewed every 30 d. The culture medium used for all experiments was prepared with deionized water, and the pH was adjusted to 5.8 (± 0.05). Autoclaving was carried out at the temperature of 127 ºC and a pressure of 1.5 kgf cm^{-2} for 20 min.

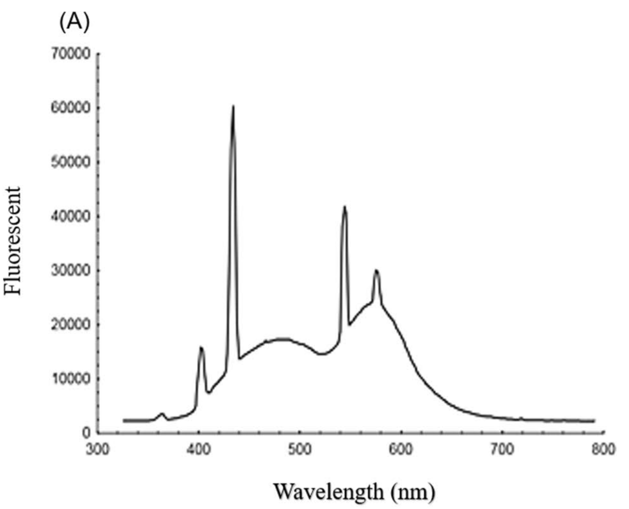
Light intensity The in vitro introduction phase was evaluated by four luminosity intensities (fluorescent lamp) (F/L 40 µmol m^{-2} s^{-1}) (HO Sylvania T12, 20 W, São Paulo, Brazil), red/blue LEDs 1:1 (R/B 20 µmol m^{-2} s^{-1}) (LabPARLL-HR/DB-480, 20 W, LabLumens®, Carapicuíba, SP, Brazil), 1:1 red/blue LEDs (R/B 40 µmol m^{-2} s^{-1}), and 1:1 red/blue LEDs (R/B 80 µmol m^{-2} s^{-1}). For the in vitro multiplication phase, the experiment was conducted in a 4 × 12 factorial arrangement, with four light intensities being tested [fluorescent lamp (F/L 40 µmol m^{-2} s^{-1}), red/blue LEDs 1:1 (R/B 20 µmol m^{-2} s^{-1}), red/blue LEDs 1:1 (R/B 40 µmol m^{-2} s^{-1}), and red/blue LEDs 1:1 (R/B 80 µmol m^{-2} s^{-1}) and twelve subcultures]. The experiments were arranged in randomized design, with thirty replications, consisting of one explant each.

For variations analysis in absolute irradiance (µW cm^{-2} nm^{-1}) and wavelength (nm) of the light emitted by the different spectral qualities, a portable SPECTRA PEN Z850 spectroradiometer (Qubit Systems-Kingston, Ontario, CA) was used. The spectral distributions of each treatment from the experiment are shown in Fig. 1.

The experiments were carried out in a growth room at 24 °C (± 1 °C) under a 16-h photoperiod. The irradiance of each fluorescent lamp or red/blue LEDs corresponded to 20 µmol m^{-2} s^{-1}, which was measured by the photoradiometer model QSO-S Procheck + Sensor-PAR Photon Flux (Decagon Devices, Pullman, Washington). Therefore, tow lamps were used for treatments with a light intensity of 40 µmol m^{-2} s^{-1} and four lamps for the treatment 80 µmol m^{-2} s^{-1}.

At 30 d on in vitro introduction phase, the percentage of total contamination (i.e., fungal and/or bacterial manifestation), unresponsive explants, tissue oxidation, and vigor were evaluated according to the scale of notes adopted by Oliveira *et al*. (2016) and Souza *et al*. (2020b) (Fig. 2), the number of shoots per explant (> 0.5 cm), and the length of shoots (> 0.5 cm) per explant. For in vitro multiplication, morphological characteristics of tissue oxidation and vigor were evaluated according to the scale of notes (Fig. 2), the number of buds per explant, the length of the shoots (> 0.5 cm), the content of photosynthetic pigments (µg mg^{-1}); anatomical: adaxial epidermis thickness (µm), abaxial epidermis thickness (µm), mesophyll thickness (µm), palisade parenchyma thickness (µm), spongy parenchyma thickness (µm), and vascular tissue thickness (µm^2); and genetic fidelity.

Content analysis of photosynthetic pigments Leaf discs (i.e., 25 mg of fresh leaf matter) were sampled after 360 d (12 subcultures) under different light intensities and were inoculated into a 5-mL DMSO solution (Sigma-Aldrich®)



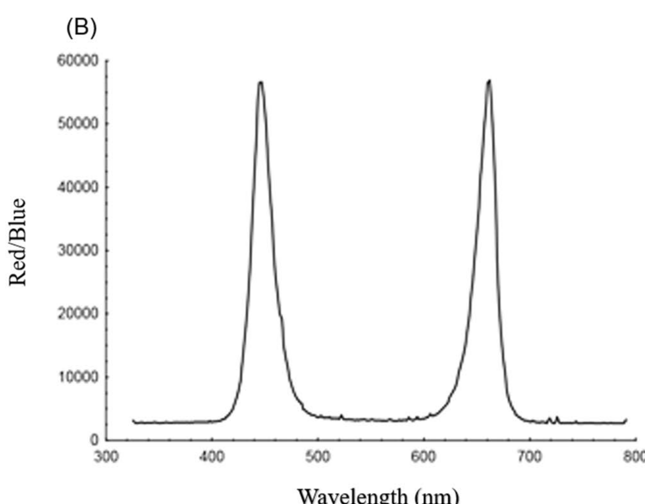


Figure. 1 Variations in absolute irradiance (µW cm^{-2} nm^{-1}) and wavelength (nm) of light emitted on in vitro culture of *Eucalyptus grandis* × *E. urophylla*. (**A**) Fluorescent lamp (F/L); (**B**) red/blue LEDs (R/B)

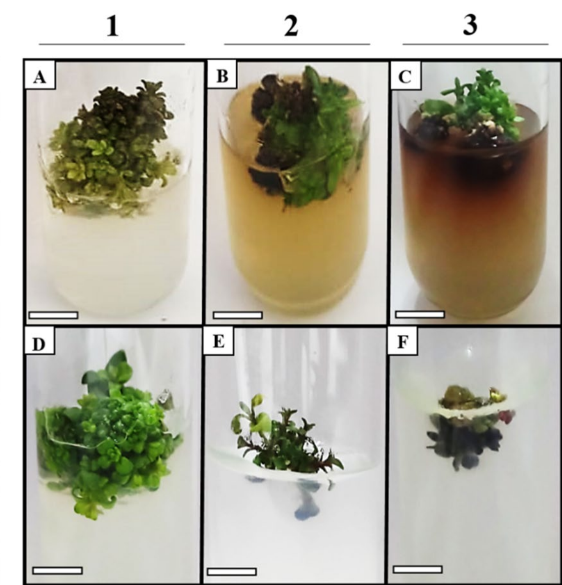


Figure. 2 Scale of notes for the assessment of tissue oxidation and vigor of *Eucalyptus grandis* × *E. urophylla*. (**A**) Note 1: no oxidation; (**B**) note 2: reduced oxidation at the base of the explants (grayish culture medium); (**C**) note 3: complete bud oxidation (blackened culture medium); (**D**) note 1: induction of shoots with growth active, with no apparent nutritional deficiency; (**E**) note 2: shoot induction, but with reduced size leaves; (**F**) note 3: low shoot induction and/or senescence and death. *Bar* = 0.5 cm

Table 1 ISSR primers used in the analysis of genetic fidelity of *Eucalyptus grandis* × *E. urophylla* clone

Primer	Sequence
Becky	(CA) 7-YC
John	(AG) 7-YC
UBC807	(AG) 8-T
UBC809	(AG) 8-G
UBC827	(AC) 8-G
UBC835	(AG) 8-YC
UBC840	(GA) 8-YT
UBC841	(GA) 8-YC
UBC842	(GA) 8-YG

R purine (A or G); *Y* pyrimidine (C or T).

stored for 48 h in the dark, according to the methodology developed by Lichtenthaler (1987).

The absorbance of the samples was determined in triplicate in a 10-mm optical path quartz cuvette in a Genesys 10UV spectrophotometer (Thermo Scientific, São Paulo, Brazil). The wavelengths (665, 649, and 480 nm) and the equations for calculating the concentrations of chlorophylls *a*, *b*, and *a* + *b*, and total carotenoids were based on the method described by Wellburn (1994).

Leaf anatomy Representative samples of leaves from each treatment were collected after 360 d (12 subcultures) and kept for 48 h in FAA solution (formaldehyde, acetic acid, 70% ethanol, 1:1:18), followed by transfer to ethanol 70%, and dehydrated in an alcoholic-ethyl series in increasing concentrations (80%, 90%, and 100%) for 30 min in each solution (Johansen 1940), and subsequently soaked in historresin (Biosystems, Nussloch, Germany) in the proportion 1:1 in a hot oven (overnight). The blockage was processed with pure hydroxyethyl methacrylate resin, and the cross-sections were obtained with a manual rotating microtome and a knife thickness of 7 µm. Tissues were contrasted with toluidine blue (Vetec Química Fina Ltda, Rio de Janeiro, Brazil), mounted on Entellan histological slides (Merck KGaA, Darmstadt, Germany), and photomicrographed with an attached digital camera (AxionCam ERc5s) on micrometric and objective lens 20 × and 40 ×.

For thickness determination of the adaxial epidermis, the thickness of the abaxial epidermis, mesophyll, palisade parenchyma, spongy parenchyma, and the vascular tissues (xylem and phloem) were assessed. Three fields of the organ view were randomly photographed with the leaf area corresponding to 0.04 mm^2. They consisted of fifteen replications (5 anatomical sections × 3 fields of view of the organ), with one leaf each.

Genetic fidelity Young leaves were collected and in vitro multiplied over the 12 subcultures (i.e., S1, S6, and S12) and from the parent plant (M1) from ministumps of the *Eucalyptus grandis* × *E. urophylla* clone in a clonal minigarden for genetic fidelity evaluation. DNA extractions were performed according to the protocol adapted from Ferreira and Grattapaglia (1998). For genetic fidelity assessment, 9 primers were used—specifications are in Table 1. The ISSR reactions were prepared in microplates (PCR-96-Axygen Scientific, Goiânia, Brazil), and in each well were placed 3 µL of DNA (standardized at 20 ng μL^{-1} for all samples) and 10 µL of reaction mix [1.5 mM Phoneutria® PCR Buffer, 1.5 mM dNTP, 1 U Phoneutria® Taq polymerase (5 U μL^{-1}), Belo Horizonte, Brazil Taq, diluent (based on BSA and Tris HCl) and 0.2 mM of each primer, making up the final volume with ultrapure water (4.2 μL^{-1})]. The reactions were amplified in a Gene Amp PCR System 9700 thermocycler, following the steps: 2 min at 94 °C for initial denaturation; 37 cycles of 30 s at 94 °C, 30 s at 42 °C, and 1 min at 72 °C; and final extension for 7 min at 72 °C. The amplification products were separated on a 1.5% agarose gel and stained with Gel Red TM (Uniscience, Osasco, Brazil). Amplicon analyses were performed visually.

Data analysis The analyses were processed with R software, version 3.0.3 (R Core Team 2014), with the aid of the ExpDes package, version 1.1.2 (Ferreira *et al*. 2013). The treatment means were used to perform the statistical analysis and adjustments to the regression equations. Variables that did not show normal distribution before the Shapiro–Wilk test at 5% significance were transformed into arcsen. For significant variables, the Tukey's test at 5% significance was performed.

Results

In vitro introduction A difference in morphological characteristics was observed between the light intensities' responses for in vitro introduction of *Eucalyptus grandis* × *E. urophylla* explants in 30 d of culture. The lowest values for the percentage of contamination were for the R/B 20 μmol m^{-2} s^{-1} (average: 6.6%), R/B 40 μmol m^{-2} s^{-1} (average: 6.6%), and R/B 80 μmol m^{-2} s^{-1} (average: 10.0%), differing statistically from the F/L 40 μmol m^{-2} s^{-1} (average: 18.8%) (Fig. 3*A*). The low percentage of contamination for R/B 20, R/B 40, and R/B 80 μmol m^{-2} s^{-1} denotes the potential of using LED lamps on in vitro introduction phase (Fig. 3*A*).

Regarding the response process in shoot induction, low percentages of explants without shoots were observed, with the F/L, being 40 μmol m^{-2} s^{-1} with the highest mean (96.7% responsive explant), differing for the R/B 20 μmol m^{-2} s^{-1} (Fig. 3*B*). Therefore, it is possible to proceed with the in vitro multiplication phase, denoting the importance of the high proliferation of shoots.

An undesirable aspect of the in vitro introduction process is the phenolic oxidation of the explants. The results showed no significant difference between treatments, but the use of R/B 20 μmol m^{-2} s^{-1} provided the lowest means (1.1) according to the scale of notes (Fig. 3*C*). On the other hand, for explant vigor, the use of R/B 20 μmol m^{-2} s^{-1} showed the worst results (on average: 2.0), differing from the others treatments (Fig. 3*D*).

As for the number of shoots per explant, the use of LEDs R/B 80 μmol m^{-2} s^{-1} provided the best results (on average: 2.1 shoots), differing statistically only for the treatment R/B 20 μmol m^{-2} s^{-1} (average: 1.1 shoots) (Fig.3*E*). However, for the length of the shoots, the means were very close between the F/L 40 μmol m^{-2} s^{-1} (1.5 cm length), R/B 20 μmol m^{-2} s^{-1} (1.4 cm length), R/B 40 μmol m^{-2} s^{-1} (1.1 cm length), and R/B 80 μmol m^{-2} s^{-1} (1.3 cm length) (Fig. 3*F*).

In vitro multiplication The experimental conditions presented a difference in the response of light intensities and the subcultures evaluated on in vitro multiplication phase of the *Eucalyptus grandis* × *E. urophylla* clone. For all characteristics assessed, its factors (i.e., light intensity and subculture) showed no significant interaction. In addition, the regression equations showed second-degree polynomial behavior.

According to the scale of notes (Fig. 2*A–C*), the lowest mean of phenolic oxidation of the explants (1.03) was observed for the treatment R/B 20 μmol m^{-2} s^{-1} (Fig. 4*A*). However, the lowest values were found in the fourth and fifth subcultures, with a gradual increase in all treatments (Fig. 4*A*). Concerning explant vigor (Fig. 2*D–F*), the best results were observed in R/B 80 μmol m^{-2} s^{-1} (average: 1.1) (Fig. 4*B*). In addition, the critical point varied between the seventh and ninth subcultures (Fig. 4*B*), contrary to what was observed in the regression equation, with the best results for the phenolic oxidation of the explants (Fig. 4*A*). One of the main limiting factors for the in vitro multiplication phase is phenolic oxidation; therefore, strategies to overcome or reduce it must maintain vigorous explants.

For the number of buds per explant, the highest multiplication percentage was observed with the use of LEDs R/B 80 μmol m^{-2} s^{-1} (on average: 8.73 buds), with a significant difference for the R/B 20 μmol m^{-2} s^{-1} (average: 6.16 buds) (Fig. 4*C*). Furthermore, there was a gradual increase in the number of buds per explant for all treatments from the first to the ninth subculture (Fig. 4*C*), following the same trend observed for the best vigor results (Fig. 4*B*). The number of buds have fundamental importance in the efficiency of in vitro multiplication phase in successive subculture assessment.

As for the length of the shoots, the highest average was the R/B 20 μmol m^{-2} s^{-1} (1.25 cm) (Fig. 4*D*), probably due to the low intensity of light emitted and, consequently, etiolation of the shoots. However, considering all light sources and intensities evaluated, the critical points for the length of the shoots were found in the third subculture (Fig. 4*D*), contrasting the regression curves for the number of buds per explant (Fig. 4*C*). Regarding the contents of photosynthetic pigments, the evaluations were carried out in the last subculture (twelfth), and the results showed differences regarding the light intensities. The highest means for chlorophyll *a* (25.55 μg mg^{-1}), chlorophyll *b* (15.60 μg mg^{-1}), chlorophyll *a* + *b* (41.15 μg mg^{-1}), and carotenoids (5.12 μg mg^{-1}) were observed in the treatment F/L 40 μmol m^{-2} s^{-1}, differing statistically of R/B 20 μmol m^{-2} s^{-1} (Fig. 4*E*).

Given the above, the efficiency performed by the low light intensity (20 μmol m^{-2} s^{-1}) for *Eucalyptus grandis* × *E. urophylla* proved to be inadequate for the in vitro multiplication step in large-scale production systems. The explants' appearance of *Eucalyptus grandis* × *E. urophylla* clone, given the characteristics studied in introduction and in vitro multiplication, can be seen in Fig. 5*A–H*.

Figure. 3 Morphological characteristics observed on in vitro introduction phase of *Eucalyptus grandis* × *E. urophylla* and different light intensities (F/L 40, R/B 20, R/B 40, and R/B 80 µmol m^{-2} s^{-1}). (**A**) Percentage of contamination; (**B**) percentage of unresponsive explants; (**C**) tissue oxidation (scale of notes according to Fig. 2*A–C*; (**D**) vigor (scale of notes according to Fig. 2*D–F*; (**E**) number of shoots per explant; (**F**) length of shoots (cm). *Means followed by equal *letters* do not differ, by Tukey's test at 5% probability of error. *Bars* represent the standard deviation

Leaf anatomy Morphological responses were observed between the leaf anatomy characteristics and the luminosity intensities used on in vitro multiplication phase (Fig. 6*A–F*). The highest means observed were the thickness of the adaxial epidermis (11.84 µm) (Fig. 6*A*), the abaxial epidermis (6.21 µm) (Fig. 6*B*), spongy parenchyma (39.67 µm) (Fig. 6*D*), and mesophyll (66.88 µm) (Fig. 6*E*) in the treatment with the quality and intensity of light R/B 80 µmol m^{-2} s^{-1}. As for the best results for the palisade parenchyma thickness (average: 30.93 µm) (Fig. 6C) and the area of vascular tissues (average: 11,226.13 µm^{2}) (Fig. 6F) (xylem and phloem), the highest means were found with the treatment F/L 40 µmol m^{-2} s^{-1}.

In contrast to the above results, the low efficiency of treatment with light intensity (20 µmol m^{-2} s^{-1}) provided the most inferior means (Fig. 6*A–F*), corroborating the previous experiment (Fig.4*A–E*), which evaluated the effect of light intensity in relation to the number of subcultures on in vitro multiplication phase.

Furthermore, changes in the structural organization of leaf cells were observed at the anatomical level when plants are exposed to different environments, including other light qualities and intensities (Fig. 7*A–H*). Under higher light intensity F/L 40 µmol m^{-2} s^{-1}, R/B 40 µmol m^{-2} s^{-1}, and R/B 80 µmol m^{-2} s^{-1}, larger dimensions were observed in the adaxial epidermis, parenchyma palisade, spongy parenchyma, and vascular tissue (Fig. 7*A*, *B*, *E*, *F*, *G*, and *H*), and the opposite can be observed in the R/B 20 µmol m^{-2} s^{-1} (Fig. 7*C*, *D*).

Genetic fidelity Samples of micropropagated plants of subcultures 1, 6, and 12 (i.e., S1, S6, and S12) were found to be identical clones of the parent plants (M1) of *Eucalyptus grandis* × *E. urophylla* (Fig. 8). Nine tested primers amplified produced stable amplicons and were genetically the same.

Furthermore, it was found that none of the micropropagated individuals presented polymorphism; that is, the amplicons exhibited the same profile for all individuals. The presence of monomorphism confirms that there was no somaclonal variation during the twelve subcultures on in vitro multiplication of *Eucalyptus grandis* × *E. urophylla*.

Discussion

In vitro introduction The improvement of protocols for in vitro establishment that influences micropropagation development was studied to establish cultivation conditions that can maximize the production of clonal plants on a commercial scale. The morphological characteristics of the *Eucalyptus grandis* × *E. urophylla* clone provided the optimization of the in vitro introduction phase through the specificity of light qualities and intensities.

The urograndis eucalypt hybrid clone used in the study indicated different responses concerning the light intensities and quality. The use of R/B LEDs provided the lowest percentages of contamination when compared to L/F. The metabolic pathway of microorganisms may be subject to regulation by the intensity and quality of light, as many living beings, as well as fungal, are blue and red wavelength receptors, absorbing and regulating photoresponses through gene expression, carotenoid biosynthesis, and aggregation of hyphae (Postemsky and Curvetto 2016). In studies on the in vitro introduction phase of *Corymbia citriodora* × *C. torelliana* (Souza *et al*. 2018), *Eucalyptus grandis* × *E. urophylla* (Souza *et al*. 2020b), and *E. urophylla* (Miranda *et al*. 2020), a lower percentage of explant contamination with red/blue LEDs was observed. However, answers may vary according to plant species and genotypes.

For the characteristics of non-responsive explant percentage, vigor, and the number of shoots per explant, the best results were observed in F/L 40, R/B 40, and R/B 80 µmol m^{-2} s^{-1}. Different studies suggest better in vitro development of plants grown under LED lighting conditions and light intensities above 20 µmol m^{-2} s^{-1} (Batista *et al*. 2016; Ferreira *et al*. 2017). These results are close to those found by Molinari *et al*. (2020) and Souza *et al*. (2020b) in *Eucalyptus grandis* × *E. urophylla*, who observed adequate vigor, shoot development, and percentage of responsive explants above 75%. However, Oliveira *et al*. (2015) found an average of 51.2% of the explants with shoots in *Eucalyptus cloeziana*. Thus, different results are observed on in vitro introduction phase, and the responses vary depending on the genotype and the cultivation conditions tested (i.e., quality and light intensity).

In the culture of *Mouriri elliptica*, supplementation of the culture medium with sucrose became unnecessary when using 50 to 150 µmol m^{-2} s^{-1} of light intensity (Assis *et al*. 2019). In *Castanea sativa* Mill., the increase in light intensity provided a more significant response of the explants to the number of shoots, vigor, and photosynthetic capacity compared to the traditional in vitro culture (F/L 40 µmol m^{-2} s^{-1}) (Sáez *et al*. 2012). Light-emitting diodes (LEDs) are an alternative light source, with potential for in vitro cultivation, due to their wavelength specificity, narrow bandwidth, small amount of thermal emissions, low degradation, and long life, providing better cost–benefit (Bugbee 2016; Abiri *et al*. 2020; Jung *et al*. 2021). However, fluorescent light allowed the *Eucalyptus grandis* × *E. urophylla* clone explants to be in vitro developed.

Regarding the phenolic oxidation of the explants and the length of the shoots, the means were very close, with no difference between the treatments. Oxidation has been a problem associated with the micropropagation of woody species, which can influence crop development. These results may be linked to internal environmental factors that affect the vigor and growth of explants, in which smaller bottles tend to have reduced concentrations of carbon dioxide and high concentrations of ethylene, as well as being affected by the intensity and quality of light, air temperature, and relative humidity (Xiao *et al*. 2011; Miranda *et al*. 2020; Souza *et al*. 2020b). Thus, the methodology used provided the reduction of tissue oxidation and better explant growth and development, which are essential strategies for the propagation system.

In vitro multiplication The effect of exposure to different light intensities and subcultures acted significantly on the morphological characteristics studied on in vitro multiplication phase of *Eucalyptus grandis* × *E. urophylla*. From subcultures three to five, the lowest averages of oxidation of the explants according to the scale of notes and the highest

(A)

Oxidation

F/L 40 $Y= 1.1980 - 0.0412x + 0.0042x^2$ $R^2= 0.84$
R/B 20 $Y= 1.2091 - 0.0586x + 0.0053x^2$ $R^2= 0.96$
R/B 40 $Y= 1.3077 - 0.0376x + 0.0029x^2$ $R^2= 0.85$
R/B 80 $Y= 1.2602 - 0.0612x + 0.0050x^2$ $R^2= 0.84$

(B)

Vigor

F/L 40 $Y= 1.5898 - 0.0901x + 0.0060x^2$ $R^2= 0.96$
R/B 20 $Y= 2.3275 - 0.1849x + 0.0103x^2$ $R^2 =0.98$
R/B 40 $Y= 1.8895 - 0.1032x + 0.0052x^2$ $R^2= 0.98$
R/B 80 $Y= 1.6414 -0.1251x + 0.0079x^2$ $R^2=0.94$

(C)

Number of buds per explant

F/L 40 $Y= 3.4409 + 0.5861x - 0.0188x^2$ $R^2= 0.99$
R/B 20 $Y= 2.4118 + 0.6963x - 0.0337x^2$ $R^2= 0.98$
R/B 40 $Y= 2.6091 + 1.0475x - 0.0499x^2$ $R^2= 0.96$
R/B 80 $Y= 4.0848 + 0.5963x - 0.0162x^2$ $R^2= 0.95$

Subculture

(D)

Shoots length (cm)

F/L 40 $Y= 1.2652 - 0.0738x + 0.0008x^2$ $R^2= 0.96$
R/B 20 $Y= 1.4339 - 0.0731x + 0.0001x^2$ $R^2= 0.94$
R/B 40 $Y= 1.3136 - 0.0980x + 0.0027x^2$ $R^2= 0.97$
R/B 80 $Y= 1.2739 - 0.0517x - 0.0011x^2$ $R^2=0.97$

Subculture

(E)

Pigment content (μg mg^{-1})

F/L 40
R/B 20
R/B 40
R/B 80

Chlorophyll *a* Chlorophyll *b* Chlorophyll *a* + *b* Carotenoids

◂**Figure. 4** Morphological characteristics observed on in vitro multiplication phase of *Eucalyptus grandis* × *E. urophylla* and different light intensities (F/L 40, R/B 20, R/B 40, and R/B 80 µmol m^{-2} s^{-1}). (**A**) Tissue oxidation (scale of notes according to Fig. 2*A–C*); (**B**) vigor (scale of notes according to Fig. 2D–F); (**C**) number of buds per explant; (**D**) length of shoots (cm); (**E**) photosynthetic pigment content (µg mg^{-1}). *Averages followed by equal *letters* do not differ, by Tukey's test at 5% probability of error. *Bars* represent the standard deviation

for the length of shoots were observed, resulting from the treatment with light intensity R/B 20 µmol m^{-2} s^{-1}.

Presumably, the absence or reduced light intensity on in vitro cultivation of woody species is reported in several studies to reduce the phenolic oxidation of tissues in *E. cloeziana* (Oliveira *et al*. 2015); *E. benthamii* (Baccarin *et al*. 2015); *Corymbia citriodora* × *C. torelliana*; and *Corymbia torelliana* × *C. citriodora* (Souza *et al*. 2018). Light intensity and quality are essential for photosynthesis, morphogenesis, flowering, and secondary metabolism (Batista *et al*. 2018; Kepenek 2019). Excessive light can damage photosynthetic structures, while low light intensifies photosynthesis. It can be inefficient, cause nutritional deficiency and etiolation, and delay plant development (Silva *et al*. 2017; Raffo *et al*. 2020), as observed in shoots under treatment R/B 20 µmol m^{-2} s^{-1}.

However, from the seventh (S7) to the ninth (S9) subcultures, the treatment with higher light intensity (i.e., R/B 80 µmol m^{-2} s^{-1}) provided the best results for shoots vigor, the number of buds per explant, and consequently higher multiplication percentage. In general, a minimum of 8 to 12 in vitro subcultures is recommended for the tissue rejuvenation in eucalypts and its hybrids (Wendling *et al*. 2014; Trueman *et al*. 2018). The regeneration of tissues under in vitro conditions through successive subcultures improves the multiplication percentage of the propagules and enables the acceleration of eucalypts genetic improvement programs through the mass propagation genotypes (Tormen *et al*. 2018; Zorz *et al*. 2020). In this context, similar results were found by Oliveira *et al*. (2016) that demonstrated variations on in vitro multiplication across subcultures for *Eucalyptus urophylla* × *E. globulus* clones, making it difficult to distinguish a specific point of stabilization.

Furthermore, light's different qualities and intensities influence the growth and development of plant cells, tissues, and organs, triggering different morphological responses (Abiri *et al*. 2020; Miranda *et al*. 2020; Souza *et al*. 2020a). Previous reports validate this study since the use of R/B LEDs and light intensities above 40 µmol m^{-2} s^{-} in *Microlaelia lundii* (Favetta *et al*. 2017), *Corymbia torelliana* × *C. citriodora* (Souza *et al*. 2018), *Urtica dioica* (Coelho *et al*. 2021), and *Mentha arvensis* (Oliveira *et al*. 2021) induced

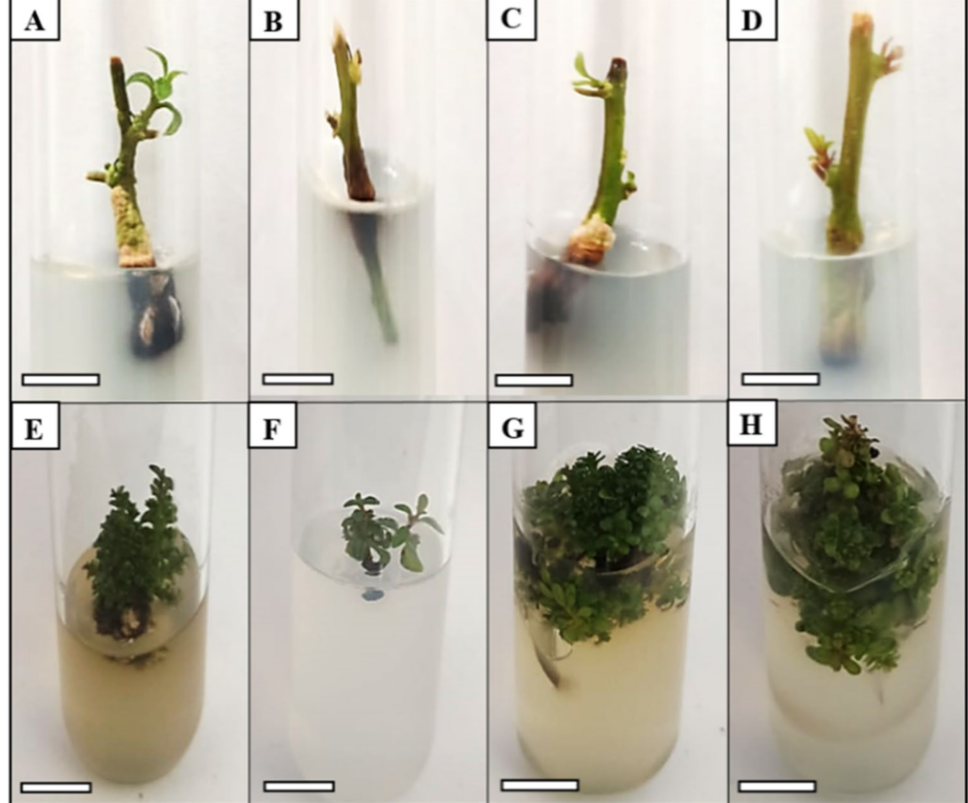


Figure. 5 Visual characteristics of *Eucalyptus grandis* × *E. urophylla* clone at different light intensities. (*A–D*) At 30 d after in vitro introduction phase. (*E–H*) Twelfth subculture on in vitro multiplication phase; (*A*, *E*) F/L 40 µmol m^{-2} s^{-1}; (*B*, *F*) R/B 20 µmol m^{-2} s^{-1}; (*C*, *G*) R/B 40 µmol m^{-2} s^{-1}; (*D*, *H*) R/B 80 µmol m^{-2} s^{-1}. *Bar* = 0.5 cm

Figure. 6 Anatomical characteristics observed on in vitro multiplication phase of *Eucalyptus grandis* × *E. urophylla* and different light intensities (F/L 40, R/B 20, R/B 40, and R/B 80 μmol m^{-2} s^{-1}). (**A**) Adaxial epidermis; (**B**) abaxial epidermis; (**C**) palisade parenchyma; (**D**) spongy parenchyma; (**E**) mesophyll; (**F**) vascular tissues. *Means followed by equal letters do not differ from each other, by Tukey's test at 5% probability of error. *Bars* represent the standard deviation

higher bud numbers and explant vigor. On the other hand, for *Acacia melanoxylon*, there was a more significant proliferation of buds with the use of fluorescent lamps when compared to red/blue LEDs (Li *et al*. 2019). The number of buds per explant and vigor are characteristics that stand out as a tool to assess the efficiency of the multiplication phase in successive subcultures (Silva *et al*. 2016), proving to be factors that have a significant influence on in vitro cultivation.

As for the content of photosynthetic pigments (chlorophyll *a*, *b*, chlorophyll *a* + *b*, and carotenoids), the highest values were found for F/L 40, R/B 40, and R/B 80 μmol m^{-2} s^{-1}, when compared to R/ B 20 μmol m^{-2} s^{-1}. In *Urtica dioica* explants, treatment with white LEDs and a light intensity of 51 μmol m^{-2} s^{-1} provided higher averages for chlorophyll and carotenoids, contrasting with the use of 26 μmol m^{-2} s^{-1} (Coelho et al. 2021). The biosynthesis of chlorophyll *a*, *b*, chlorophyll *a* + *b*, and carotenoids is influenced by the quality and intensity of the light emitted, showing greater efficiency in the photosynthetic process,

predominantly at wavelengths with a broad light spectrum (Gupta and Karmakar 2017; Tian *et al*. 2019; Lei *et al*. 2021).

The absorption of a broad spectrum of light and specific light intensities promotes a higher energy state (Lanoue *et al*. 2018; Klein *et al*. 2018). The excitatory energy of the chlorophyll molecule can be used in the photochemical step, providing greater efficiency to the photosynthetic apparatus (Taiz and Zeiger 2013; Raffo *et al*. 2020). In explants of *Withania somnifera*, a higher content of chlorophyll *a*, *b* and carotenoids were found with a light intensity of 60 µmol m^{-2} s^{-1} (Lee *et al*. 2007). Due to their wavelength specificity, LEDs influence the photomorphogenic responses of explants grown in controlled environments, emerging as a valuable technology to increase productivity (Batista *et al*. 2018; Faria *et al*. 2019). The broad light spectrum through the treatment R/B 80 µmol m^{-2} s^{-1} proved viable and valuable for the in vitro multiplication phase of *Eucalyptus grandis* × *E. urophylla*.

Leaf anatomy The use of different luminosity qualities and intensities had a direct implication on leaf anatomy in *Eucalyptus grandis* × *E. urophylla* explants, with the largest dimensions for the adaxial epidermis, abaxial epidermis, spongy parenchyma, and mesophyll in the R/B 80 µmol m^{-2} s^{-1} treatment and the palisade parenchyma and vascular tissues with F/L 40 µmol m^{-2} s^{-1}. These results are close to those found by Souza *et al*. (2020a) in *Eucalyptus urophylla* × *E. grandis* explants under broad light spectrum (450–700 nm) and light intensity of 40 µmol m^{-2} s^{-1}. However, with low light intensities, tissues tend to develop less

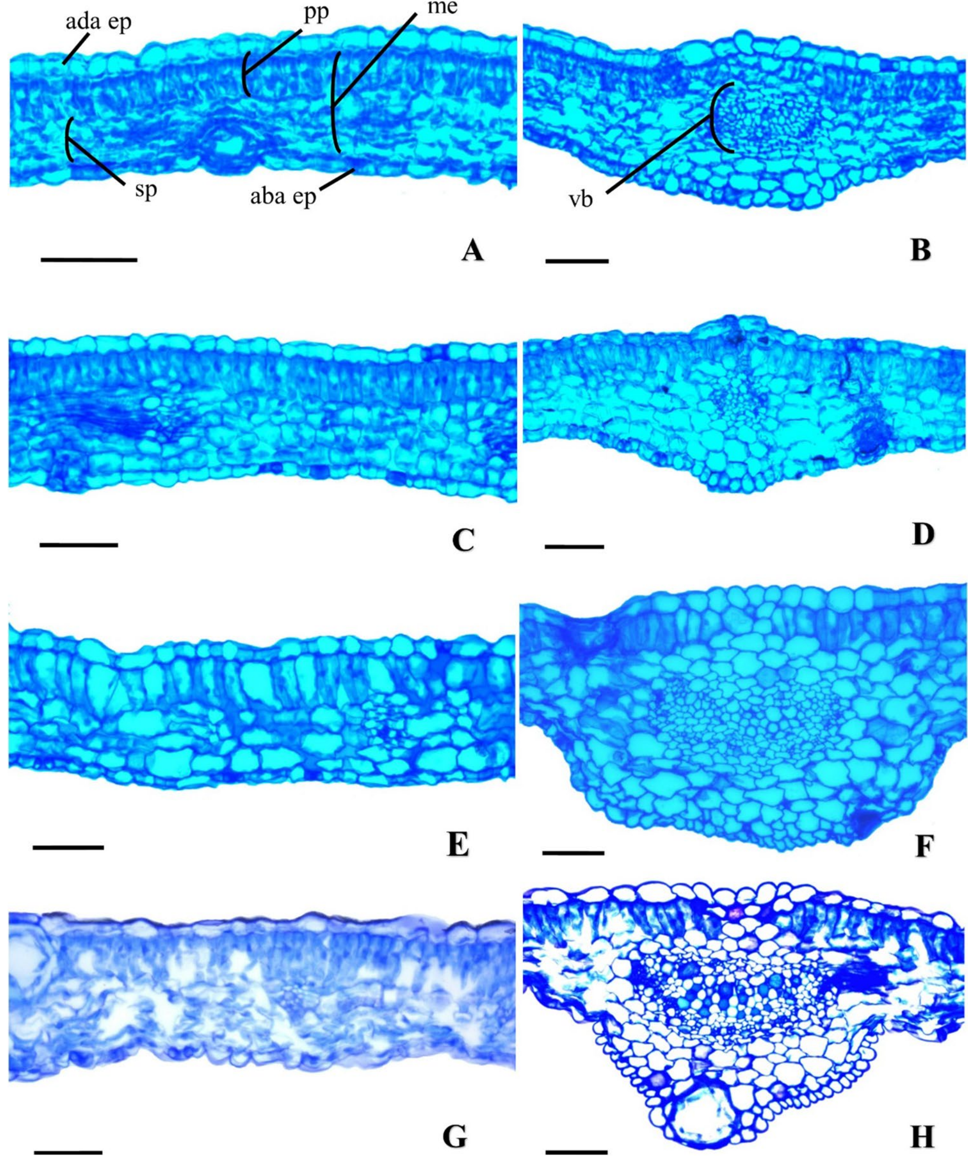


Figure. 7 Cross-sections of the leaf blade (*A*, *C*, *E*, *G*) and midrib (*B*, *D*, *F*, *H*) observed on in vitro multiplication phase of *Eucalyptus grandis* × *E. urophylla* at different light intensities. (**A**) F/L 40 µmol m^{-2} s^{-1}; (**B**) F/L 40 µmol m^{-2} s^{-1}; (**C**) R/B 20 µmol m^{-2} s^{-1}; (**D**) R/B 20 µmol m^{-2} s^{-1}; (**E**) R/B 40 µmol m^{-2} s^{-1}; (**F**) R/B 40 µmol m^{-2} s^{-1}; **G** R/B 80 µmol m^{-2} s^{-1}; (**H**) R/B 80 µmol m^{-2} s^{-1}; *ada ep = adaxial epidermis; aba ep = abaxial epidermis; pp = palisade parenchyma; sp = spongy parenchyma; me = mesophyll; vb = vascular tissue. *Bar* = 200 µm

due to lower production of photoassimilates (Taiz and Zeiger 2013), as observed in this study.

Kwon *et al*. (2015) observed a more significant amount of parenchymal cells dividing when subjected to red/blue light quality, providing greater shoots development and leaves in *Populus euramericana*. According with Macedo *et al*. (2011), the use of the blue wavelength (450 nm) provided an increase in the thickness of epidermis and mesophyll cells on in vitro culture of *Alternanthera brasiliana*. Furthermore, when subjected to higher light intensities, Castro *et al*. (2007) verified better development of conduction tissues and photosynthetic apparatus in *Mikania glomerata*. Li *et al*. (2018) observed more advantageous using a broad light spectrum than green LEDs in the morphogenesis and anatomy of *Solanum tuberosum*. The different light qualities and intensities influence the growth and development of plant cells, tissues, and organs, triggering different morphological and anatomical responses (Hogewoning *et al*. 2012; Jung *et al*. 2021; Lei *et al*. 2021). Each spectrum can alter expression levels of specific genes involved in the structural organization of plant cells and photosynthetic pathway genes (Fan *et al*. 2013).

Although light sources and intensities are critical factors on in vitro culture, few studies still involve their effect on the morphology and anatomy of eucalypts species and hybrids. The knowledge of the relationship between light intensities and the growth pattern of *Eucalyptus grandis* × *E. urophylla* explants allowed greater efficiency in the in vitro establishment, providing a difference in the responses to micropropagation.

Genetic fidelity The micropropagated plants are clones of the donor selected plant (M1), considering that there was no genetic variation over the twelve subcultures (360 d), considering the primes used.

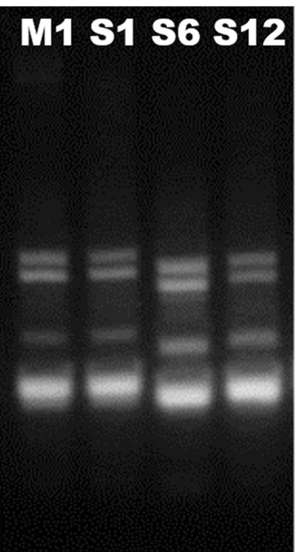


Figure. 8 Gel from the ISSR Becky primer for *Eucalyptus grandis* × *E. urophylla* in S1, S6, and S12 in vitro subculture

Even though somaclonal variation was not found in the present study, with an increase in subcultures, this phenomenon may occur due to the influence of several factors, as well as the addition of high concentrations of plant growth regulators to the culture conditions (Sandhu *et al*. 2018; Solano *et al*. 2019). Those can cause cell cycle disorders (Nasri *et al*. 2019). According with Solano *et al*. (2019), verifying the genetic fidelity of early micropropagated individuals can contribute to the definition of reliable protocols, avoiding future problems with plants in the field after planting.

Research has been carried out with eucalypt species to verify the genetic fidelity of clones compared to selected tree, through the use of molecular markers, as in *Eucalyptus globulus* (Oliveira *et al*. 2017) and *Eucalyptus camaldulensis* (Shanthi *et al*. 2015). No genetic variation of explants was observed in this study, even after 1 yr of in vitro culture with plant growth regulators. Therefore, it is possible to suggest that the protocol was efficient for in vitro cloning.

Conclusions

Our study provided evidence that wavelength specificity influences the morphology and anatomy of *Eucalyptus grandis* × *E. urophylla* cultivated under a controlled environment, emerging as a valuable technology to optimize the production of large-scale clonal plants.

The quality and intensity of light F/L 40 µmol m^{-2} s^{-1} and R/B 80 µmol m^{-2} s^{-1} are the most suitable for in vitro introduction and multiplication phases of *Eucalyptus grandis* × *E. urophylla*, providing a higher percentage of responsive explants, number of shoots per explant, vigor, photosynthetic pigment content, adaxial epidermis thickness, abaxial epidermis, mesophyll, palisade parenchyma, spongy parenchyma, and vascular tissues.

It was observed that micropropagated plants are clones of *Eucalyptus grandis* × *E. urophylla* selected tree; amplicons exhibited the same profile for all individuals, without somaclonal variation during the twelve subcultures on in vitro multiplication phase.

Acknowledgements We thank the National Council for Scientific and Technological Development, Brazil ("*Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq*"); Coordination for Improvement of Higher Education Personnel, Brazil ("*Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES – Código de Financiamento 001*"); and Foundation for Research of the State of Minas Gerais, Brazil ("*Fundação de Amparo a Pesquisa do Estado de Minas Gerais – FAPEMIG*").

Author contribution Souza D. M. S. C.: conduction of experiment, analyses, review, discussion of theme. Fernandes S. B.: conduction of

experiment, discussion of theme. Silva E. O., Duarte V. P., Gonçalves D. S.: discussion of theme. Carvalho D., Teixeira G. L.: discussion of theme, review. Brondani G. E.: supervisor, analyses, discussion of theme, review. All authors contributed to the writing of the manuscript.

Data availability All data and materials support published claims and comply with field standards.

Code availability Not applicable.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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ARTIGO 3

Effect of photomixotrophism on the *in vitro* elongation and rooting of *Eucalyptus grandis* × *Eucalyptus urophylla*

Artigo formatado conforme as normas da Universidade Federal de Lavras - UFLA

Effect of photomixotrophism on the *in vitro* elongation and rooting of *Eucalyptus grandis* × *Eucalyptus urophylla*

ABSTRACT

The rejuvenation/reinvigoration of tissues through micropropagation has become an important tool for clonal propagation in *Eucalyptus* and *Corymbia* species. This study evaluated the effect of photomixotrophism (light intensity and seal type) on *in vitro* elongation and rooting to identify the limiting factors in the *in vitro* culture of the *E. grandis* × *E. urophylla* hybrid clone. Nodal segments (i.e., explants) were collected from ministumps grown in a semi-hydroponic system. The effects of light intensity and seal type on the elongation and rooting stages *in vitro* were evaluated from a 40 μmol m^{-2} s^{-1} fluorescent lamp and 20 μmol m^{-2} s^{-1}, 40 μmol m^{-2} s^{-1}, and 80 μmol m^{-2} s^{-1} red/blue L.E.D.S. with or without gas exchange. Based on the results at 35 days, the 40 μmol m^{-2} s^{-1} fluorescent lamp and gas exchange combination was the most suitable for *in vitro* elongation and rooting stages of *E. grandis* × *E. urophylla*. It increased the vigor, shoot length, photosynthetic pigment content, xylem, phloem, stomatal number and density, root length, diameter, number of roots per explant, and rooting percentages of plants.

Keywords: clonal propagation, micropropagation, light intensity, LEDs, porous membranes.

INTRODUCTION

The expansion of forest plantations and the improved suitability for plant production have increased the use of *Eucalyptus* and *Corymbia* species and their hybrid combinations. In recent years, the hybridization of *Eucalyptus grandis* × *Eucalyptus urophylla* (the hybrid is known as urograndis eucalypts) has provided excellent results in terms of wood quality and growth, in addition to better edaphoclimatic adaptation and easier vegetative propagation (CARRILLO *et al*. 2018). In this context, different ways to improve the production of eucalyptus plants using the micropropagation technique via proliferation of axillary buds have been recommended (TRUEMAN; HUNG; WENDLING, 2018; ABIRI *et al*. 2020; SOUZA *et al*. 2020a).

Tissue rejuvenation through *in vitro* cultivation has become an important tool for the clonal micropropagation of *Eucalyptus* and *Corymbia* species, since they usually have problematic

adventitious rooting when the cutting and mini-cutting techniques are applied (LOPES *et al*. 2019; KUPPUSAMY *et al*. 2019). Among the micropropagation stages, *in vitro* elongation is essential for obtaining shoots for rooting and for acclimatization of microcuttings (SOUZA *et al*. 2020b; ZORZ *et al*. 2020), performed both in the ex-vitro (BRONDANI *et al*. 2018; LI *et al*. 2019) and *in vitro* conditions (MIRANDA *et al*. 2020; MOLINARI *et al*. 2021).

Given the above, to optimize micropropagation protocols for *in vitro* elongation and rooting stages of different eucalyptus genotypes, the need for basic research to improve the production and quality of microstumps is clear. Biotechnology is commonly applied to enhance the micropropagation process, including the control of spectral quality (BATISTA *et al*. 2018; SOUZA *et al*. 2020a;), light intensity (MIRANDA *et al*. 2020; COELHO *et al*. 2021), and gas exchange (SOUZA *et al*. 2019; MARTINS *et al*. 2020; MOLINARI *et al*. 2021) in plant cultivation. Thus, the use of systems that increase the supply of light and CO_2 to some *in vitro* species may promote more significant development of their photosynthetic apparatus, resulting in high multiplication rates, growth, rooting, and the subsequent acclimatization of plants to *ex vitro* conditions (SALDANHA *et al*. 2012; LOUBACK *et al*. 2021).

Plants require a broad light spectrum and efficient gas exchange to optimize the photosynthetic processes, reduce relative humidity, increase aeration, produce hardier plants, and, consequently, favour survival and rooting (LAZZARINI *et al*. 2017; NGUYEN; XIAO; KOZAI, 2020). Conversely, photosynthesis may be inefficient at low light intensity and gas exchange -the results are slow growth and development. Also, excessive light and high CO_2 concentrations can alter physiological processes that are important to plant survival, inducing photorespiration and damaging photosynthetic structures (SILVA *et al*. 2017; OLIVEIRA *et al*. 2021).

Despite the economic importance of eucalyptus species and their hybrids to the forestry sector, studies on the effect of different light intensities and gas exchange scenarios on morphogenesis and growth for plant production are still scarce. This study evaluated the effect of photomixotrophism (light intensity and gas exchange) on the *in vitro* elongation and rooting of *Eucalyptus grandis* Hill ex Maiden × *Eucalyptus urophylla* S. T. Blake microcuttings.

MATERIALS AND METHODS

Experimental material

The genetic material used for acquiring the explants (i.e., 1-cm-long nodal segment and an axillary bud without leaves), previously established and multiplied *in vitro*, was acquired from ministumps of the A211 hybrid clone of *Eucalyptus grandis* Hill ex Maiden × *Eucalyptus urophylla* S. T. Blake, from Esteio Seedling Production Nursery, located in São João Del Rei, Minas Gerais, Brazil.

The ministumps were established in a clonal mini hedge under a semi-hydroponic system of the large channel type in a bed of medium-coarseness sand. The plants received nutrient solution (Table 1) by dripping, four times a day, at a total daily flow rate of 4 L m^{-2}. The electrical conductivity of the nutrient solution was kept at approximately 2.0 mS cm^{-2}.

Table 1. Composition of the nutritive solution for fertigation of *E. grandis* × *E. urophylla* plants.

Nutrient	Nutritive solution[1] (mg L^{-1})	Molecular weight
N-NO_3^-	60.00	14.00
N-NH_4^+	30.00	14.00
P	12.00	30.97
Ca	30.00	40.08
K	80.00	39.10
S	18.92	32.06
Mg	12.00	24.31
Cu	0.10	63.54
Fe	2.00	55.85
Mo	0.02	95.94
Mn	1.60	54.94
Zn	1.96	65.37
B	1.08	10.81
Macro and micronutrient source	QF / MW	(mg L^{-1})
Potassium nitrate (Nuclear®)	KNO_3 / 101.10	206.8500
Monoammonium phosphate (Mallinckrodt®)	$NH_4H_2PO_4$ / 115.03	44.5700
Ammonium nitrate (Reagex®)	NH_4NO_3 / 80.4	140.5000
Calcium sulfate (Vetec®)	$CaSO_4.2H_2O$ / 172.17	87.1817
Calcium nitrate (Labsynth®)	$Ca(NO_3)2.4H_2O$ / 236.15	57.1800
Magnesium sulfate (Mallinckrodt®)	$MgSO_4.7H_2O$ / 246.48	121.6680
Manganese sulfate (Ecibra®)	$MnSO_4.H_2O$ / 169.01	4.9223
Copper sulfate (Mallinckrodt®)	$CuSO_4.5H_2O$ / 249.68	0.3929
Iron sulfate (Synth®)	$FeSO_4.7H_2O$ / 278.02	9.9520
Sodium - EDTA (Nuclear®)	Na_2-EDTA.$2H_2O$ / 372.24	13.3110
Sodium molybdate (Merck®)	$Na_2MoO_4.2H_2O$ / 241.95	0.0504
Zinc sulfate (Mallinckrodt®)	$ZnSO_4.7H_2O$ / 287.54	8.6000
Boric acid (Ecibra®)	H_3BO_3 / 61.83	6.2000

[1] pH was adjusted to 6.0 (±0.2) at 25°C with HCl and NaOH, both at 1 M. Q.F. = chemical formula, M.W. = molecular weight.

In vitro elongation and rooting

In the *in vitro* elongation and rooting stages, the shoots produced in the *in vitro* multiplication stage were prepared by isolating four standardized 0.5 cm shoots with adequate vegetative vigor. Inoculation was performed under aseptic conditions in 250 mL glass flasks. Culture lasted for 35 days in 50 mL of M.S. culture medium (MURASHIGE; SKOOG, 1962). Shoots were supplemented with 30 g L^{-1} sucrose (Vetec®), 6 g L^{-1} agar, 0.05 mg L^{-1} 6-benzyladenine purine (B.A.P.) (Sigma Co.), and 0.5 mg L^{-1} indole-3-butyric acid (I.B.A.) (Sigma®).

The culture medium for the experiment was prepared with deionized water, and the pH was adjusted to 5.8 (± 0.05) at 25 °C. Autoclaving was performed at 121 °C and 1.0 kgf cm^{-2} for 20 min.

Light intensity and seal types

The experiments were conducted in a 4 × 2 factorial arrangement. Four light intensities were tested: fluorescent lamp (F/L 40 µmol m^{-2} s^{-1}) and 1:1 red/blue LEDs (R/B) at 20, 40, and 80 µmol m^{-2} s^{-1}. Two seal types were tested: rigid polypropylene caps without a membrane (0/M) and polypropylene caps with a hole (1.0 cm in diameter) covered with a 1.0-cm^2 membrane (1/M). The experiments were arranged in a randomized design, with 40 replicates, consisting of one explant per replicate. The natural ventilation systems were obtained by the presence of porous membranes manufactured in the lids of the culture recipients, following Saldanha *et al*. (2012) guidelines.

For the description of variations in the absolute irradiance (µW cm^{-2} nm^{-1}) and wavelength (nm) of light emitted by the different spectral qualities analyses, a SPECTRA PEN Z850 a portable spectroradiometer (Qubit Systems, Kingston, Ontario, Canada) was used (Figure 1).

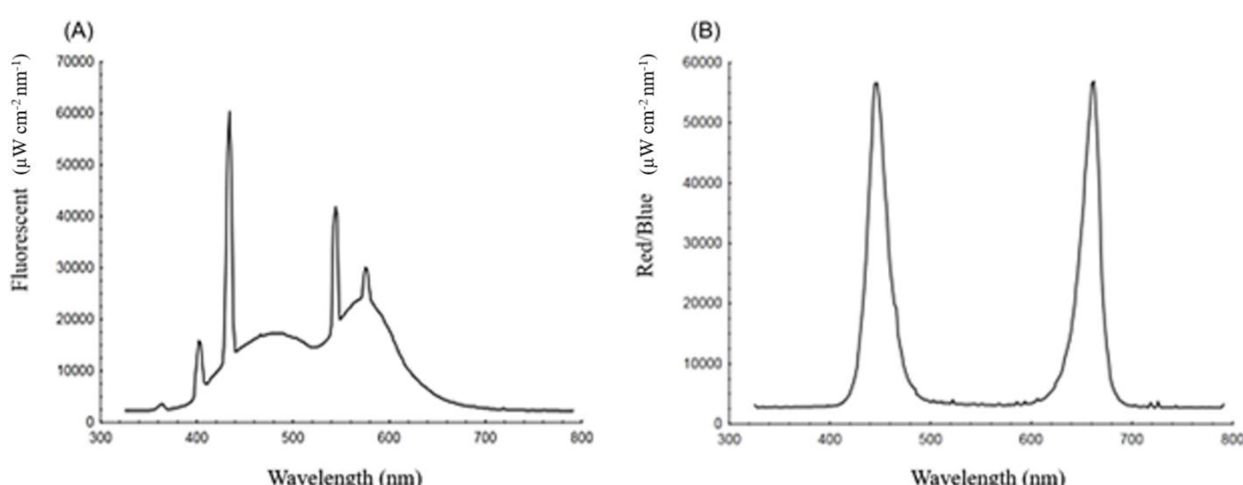


Fig. 1 Variations in absolute irradiance (μW cm^{-2} nm^{-1}) and wavelength (nm) of light emitted in the *in vitro* culture of *Eucalyptus grandis* × *E. urophylla*. (A) Fluorescent lamp (F/L); (B) Red/Blue LEDs (R/B).

The experiment was conducted in a growth room at 24 °C (± 1 °C) under a 16-hour photoperiod. The irradiance of each fluorescent lamp (HO Sylvania T12, 20 W, São Paulo, Brazil) or red/blue LEDs (LabPARLL- HR/DB- 480, 20W) corresponded to 20 μmol m^{-2} s^{-1} and was measured with a QSO-S Procheck + Sensor-PAR Photon Flux photoradiometer (Decagon Devices, Pullman, Washington, U.S.A.). Therefore, in treatments with a light intensity of 40 μmol m^{-2} s^{-1}, two lamps were used, and for 80 μmol m^{-2} s^{-1}, four lamps were used.

In the *in vitro* elongation stage, the following morphological characteristics were evaluated: tissue oxidation and vigour according to a scoring scale (OLIVEIRA *et al*. 2016; SOUZA *et al*. 2020a) (Figure 2), number of shoots per explant (> 0.5 cm), length of shoots (> 0.5 cm), and photosynthetic pigment content (μg mg^{-1}). For *in vitro* rooting, the following characteristics were evaluated: length of the largest root (cm), the diameter of the largest root (mm), number of roots per explant, and rooting percentage.

Fig. 2 Scale of notes for the assessment of tissue oxidation and vigor of *Eucalyptus grandis* × *E. urophylla*. (A) Note 1: no oxidation; (B) Note 2: reduced oxidation at the base of the explants (grayish culture medium); (C) Note 3: complete bud oxidation (blackened culture medium); (D) Note 1: induction of shoots with active growth, with no apparent nutritional deficiency; (E) Note 2: shoot induction, but with reduced size leaves; (F) Note 3: low shoot induction and/or senescence and death. Bar = 1.0 cm.

Analysis of photosynthetic pigments

Leaf discs (i.e., 25 mg of leaf fresh matter) were sampled after 35 days under different light intensities and seal types and were inoculated in 5 mL of dimethyl sulfoxide solution (Sigma Aldrich) and stored for 48 hours in the dark, according to the method developed by Lichtenthaler (1987). The absorbance of the samples was determined in triplicate in a quartz cuvette with a 10-mm optical path in a Genesys 10UV spectrophotometer (Thermo Scientific, U.S.A.). The wavelengths (665, 649, and 480 nm) and the equations for calculating the concentrations of chlorophyll a, b, and $a + b$ and total carotenoids were based on the method described by Wellburn (1994).

Leaf anatomy

Representative samples of the leaves of each treatment were collected after 35 days of *in vitro* culture. They were kept for 48 hours in F.A.A. solution (formaldehyde, acetic acid, 70% ethanol, 1:1:18), then transferred to 70% ethanol, dehydrated in an increasing ethyl alcohol series (80%, 90% and 100%) for 30 min in each solution (JOHANSEN, 1940), and embedded overnight in historesin (Biosystems, Nussloch, Germany) at a 1:1 ratio in a hot oven.

The blockage was processed with pure hydroxyethyl methacrylate resin, and the cross-sections were obtained with a manual rotating microtome and a 7 μm razor. The tissues were contrasted with toluidine blue (Vetec Química Fina Ltda, Rio de Janeiro, Brazil), mounted on Entellan histological slides (Merck KGaA, Darmstadt, Germany), and photomicrographed with a coupled digital camera (AxioCam ERc5s) on a micrometric scale with the 20× and 40× objective lenses.

To determine the anatomical characteristics in cross-section and paradermal sections, three fields of the organ view were randomly photographed - the leaf area corresponded to 0.04 mm².

There were fifteen repetitions (5 anatomical sections × 3 fields of view of the organ), with one leaf each.

The following anatomical characteristics were measured: adaxial epidermis thickness (µm), abaxial epidermis thickness (µm), mesophyll thickness (µm), palisade parenchyma thickness (µm), spongy parenchyma thickness (µm), vascular tissue area (xylem and phloem) (μm^2), number of stomata, stomatal density (μm^2), polar diameter (µm), and equatorial diameter (µm).

Data analysis

The analyses were processed with R software, version 3.0.3 (R CORE TEAM, 2014), with the aid of the Exp- Des package, version 1.1.2 (FERREIRA; CAVALCANTI; NOGUEIRA, 2013). The treatment means were used to perform the statistical analysis. Variables that did not show normal distribution before the Shapiro–Wilk test at 5% significance were transformed into arcsen. For significant variables, the Tukey's test at 5% significance was performed.

RESULTS

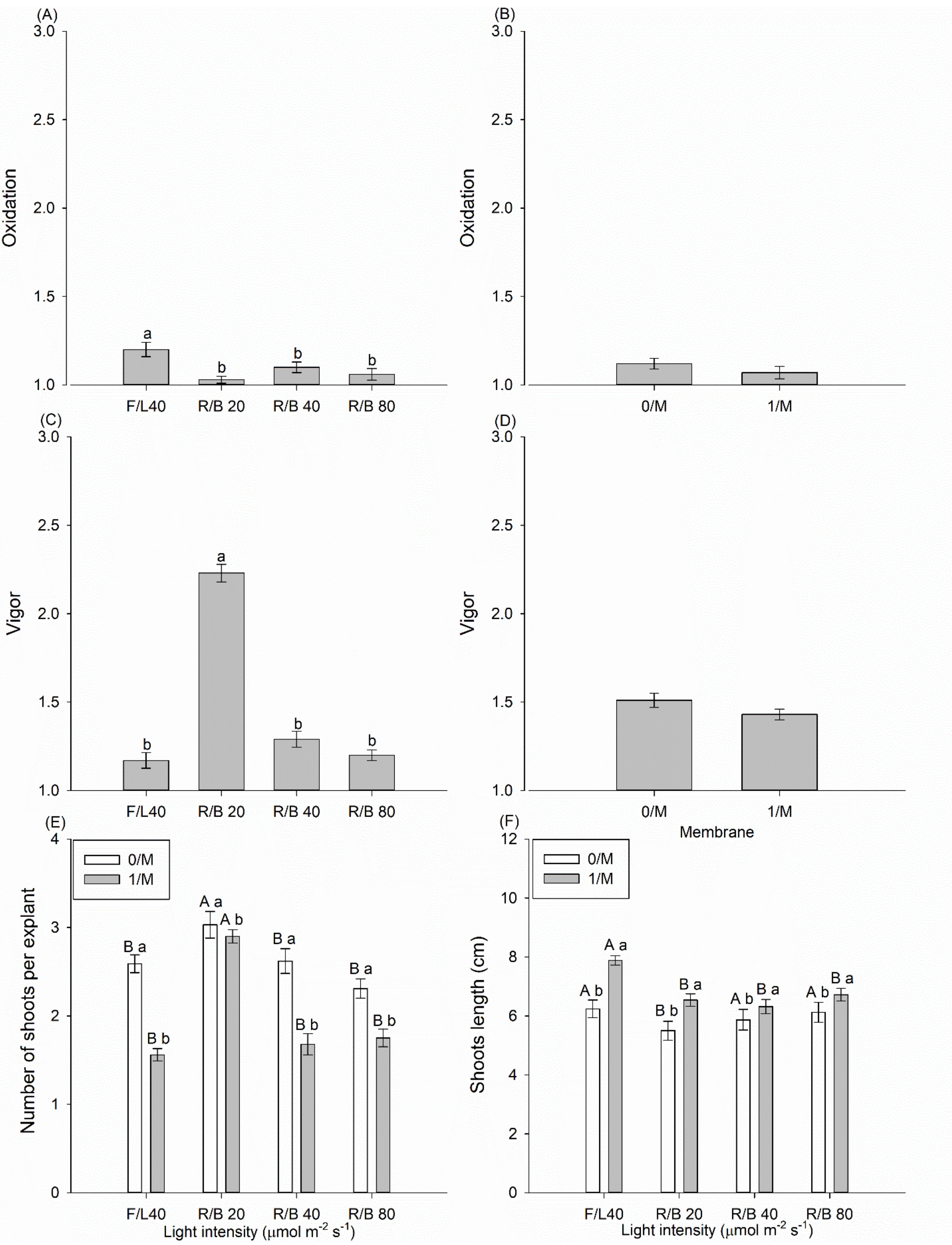
In vitro elongation

The different light intensities and seal types used in the *in vitro* elongation of the *E. grandis* × *E. urophylla* clone influenced the characteristics evaluated at 35 days after inoculation (Figure 3). There was a significant interaction between the factors only for the number of shoots per explant and shoot length.

An undesirable aspect of the *in vitro* elongation process is the phenolic oxidation of explants. The results showed different oxidation responses at different light intensities. According to the scoring scale, all the R/B treatments resulted in lower tissue oxidation than the F/L 40 µmol m^{-2} s^{-1} treatment (score 1.2) (Figure 3A). Evaluating the different seal types, the results were statistically similar between treatments 0/M (score 1.12) and 1/M (score 1.07) (Figure 3B).

In contrast to the previous results, explant vigor was increased by the F/L 40 µmol m^{-2} s^{-1} treatment (score 1.17), significantly differing from the R/B 20 µmol m^{-2} s^{-1} value (2.23) (Figure 3C). The same behaviour was observed in the face of sealing forms, with no significant difference being detected between treatments (Figure 3D).

The greatest number of shoots per explant was observed in the R/B 20 µmol m^{-2} s^{-1} and 0/M (3.03 shoots) (Figure 3E). The 0/M seal type provided the best results for the number of shoots per explant between all treatments evaluated (Figure 3E). However, for shoot length, an inverse pattern was observed. The highest values were F/L 40 µmol m^{-2} s^{-1} (6.24 cm) and with 1/M in all treatments (Figure 3F).



(A)
Oxidation
a
b
b
b
F/L40
R/B 20
R/B 40
R/B 80
(B)
Oxidation
0/M
1/M
(C)
Vigor
a
b
b
b
F/L40
R/B 20
R/B 40
R/B 80
(D)
Vigor
0/M
1/M
Membrane
(E)
0/M
1/M
Number of shoots per explant
B a
B b
A a
A b
B a
B b
B a
B b
F/L40
R/B 20
R/B 40
R/B 80
Light intensity (μmol m-2 s-1)
(F)
0/M
1/M
Shoots length (cm)
A b
A a
B b
B a
A b
B a
A b
B a
F/L40
R/B 20
R/B 40
R/B 80
Light intensity (μmol m-2 s-1)

Fig. 3 Characteristics observed in the *in vitro* elongation of *Eucalyptus grandis* × *E. urophylla* under different spectral intensities (F/L 40, R/B 20, R/B 40, and R/B 80 μmol m^{-2} s^{-1}) and seal types (0/M and 1/M). (A) Oxidation (scoring scale according to Figures 2A-2C). (B) Oxidation (scoring scale according to Figures 2A-2C). (C) Vigour (scoring scale according to the Figures 2D-2F). (D) Vigour (scoring scale according to Figures 2D-2F). (E) Number of shoots per explant. (F) Shoot length (cm). *For (E and F) capital letters represent statistical differences comparing the different spectral intensities in the same treatment (seal types). Lowercase letters represent statistical differences comparing the seal types in the same treatment (spectral intensities). *Averages followed by equal letters do not differ, by Tukey's test at 5% probability of error. Bars represent the standard deviation from the mean value.

The contents of photosynthetic pigments differed by light intensity and seal type, but there was no interaction between the factors. The highest means for chlorophyll *a* (36.30 μg mg^{-1}), chlorophyll *b* (18.70 μg mg^{-1}), chlorophyll *a* + *b* (55.00 μg mg^{-1}), and carotenoids (6.30 μg mg^{-1}) were observed in the F/L 40 μmol m^{-2} s^{-1} treatment, which differed significantly from the other treatments (Figure 4A). Comparing the seal types, the highest values for chlorophyll *a* (30.07 μg mg^{-1}), chlorophyll *b* (16.50 μg mg^{-1}), chlorophyll *a* + *b* (46.57 μg mg^{-1}), and carotenoids (5.72 μg mg^{-1}) were found when using 1/M, with a significant difference from the 0/M treatment (Figure 4B).

According to these results, the efficiency of the F/L 40 μmol m^{-2} s^{-1} treatment and the use of a membrane for *E. grandis* × *E. urophylla* was adequate for the *in vitro* elongation stage in large-scale production systems.

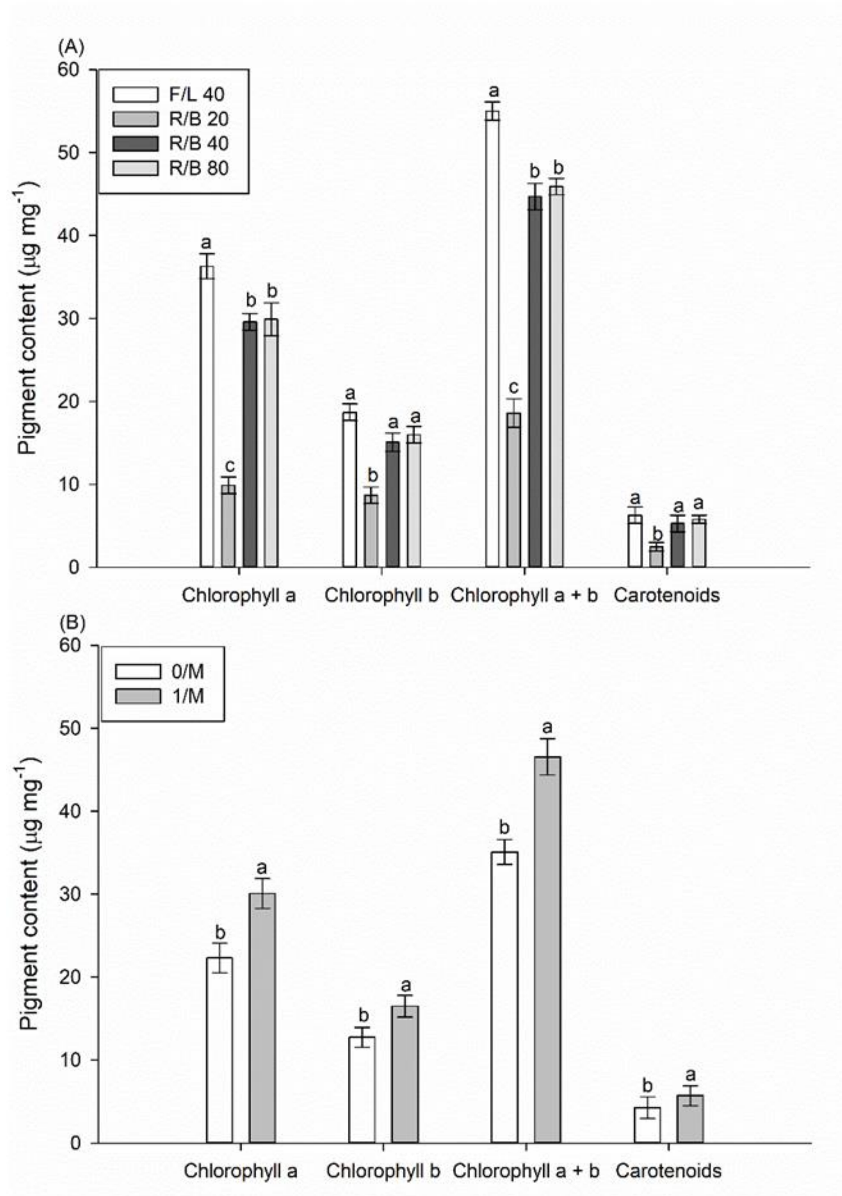


Fig. 4 Contents of photosynthetic pigments (chlorophyll *a*, *b*, and *a* + *b*, and carotenoids) observed in the *in vitro* elongation of *Eucalyptus grandis* × *E. urophylla*. **(A)** Spectral intensity (FL 40, R/B 20, R/B 40, and R/B 80 µmol m^{-2} s^{-1}). **(B)** Type of seal (0/M and 1/M). *Averages followed by equal letters do not differ, by Tukey's test at 5% probability of error. Bars represent the standard deviation from the mean value.

Leaf anatomy

The use of different light intensities and seal types influenced the leaf anatomy characteristics evaluated in cross-sections. There was a significant interaction between the factors for the thickness

of the adaxial epidermis, abaxial epidermis, palisade parenchyma, spongy parenchyma, and mesophyll (Figure 5). There was no interaction between the factors for the thickness of the xylem, phloem, or total vascular tissues (xylem + phloem) (Figure 7).

The highest mean thickness of the adaxial epidermis (15.62 µm and 14.11 µm) (Figure 5A), abaxial epidermis (11.12 µm and 10.95 µm) (Figure 5B), spongy parenchyma (75.13 µm and 54.58) (Figure 5C), and mesophyll (110.64 µm and 131.56 µm) (Figure 5D) of the *E. grandis* × *E urophylla* clone were observed with the 1/M seal type under R/B 80 µmol m^{-2} s^{-1} and R/B 40 µmol m^{-2} s^{-1}, respectively. In contrast to the previous results, there was a low efficiency of the treatment with a light intensity of 20 µmol m^{-2} s^{-1} and without the use of a porous membrane (0/M), resulting in smaller sizes of the leaf tissues evaluated (Figures 5A-F).

In addition, changes were observed in the structural organization of the leaf cells at the anatomical level when the plants were exposed to different spectral qualities or seal types (Figures 6A-H). The appearance of the cross-sections of the leaf blade from microstumps in the *E. grandis* × *E. urophylla* clone, according to the characteristics studied in *in vitro* elongation, are in Figures 6A-H.

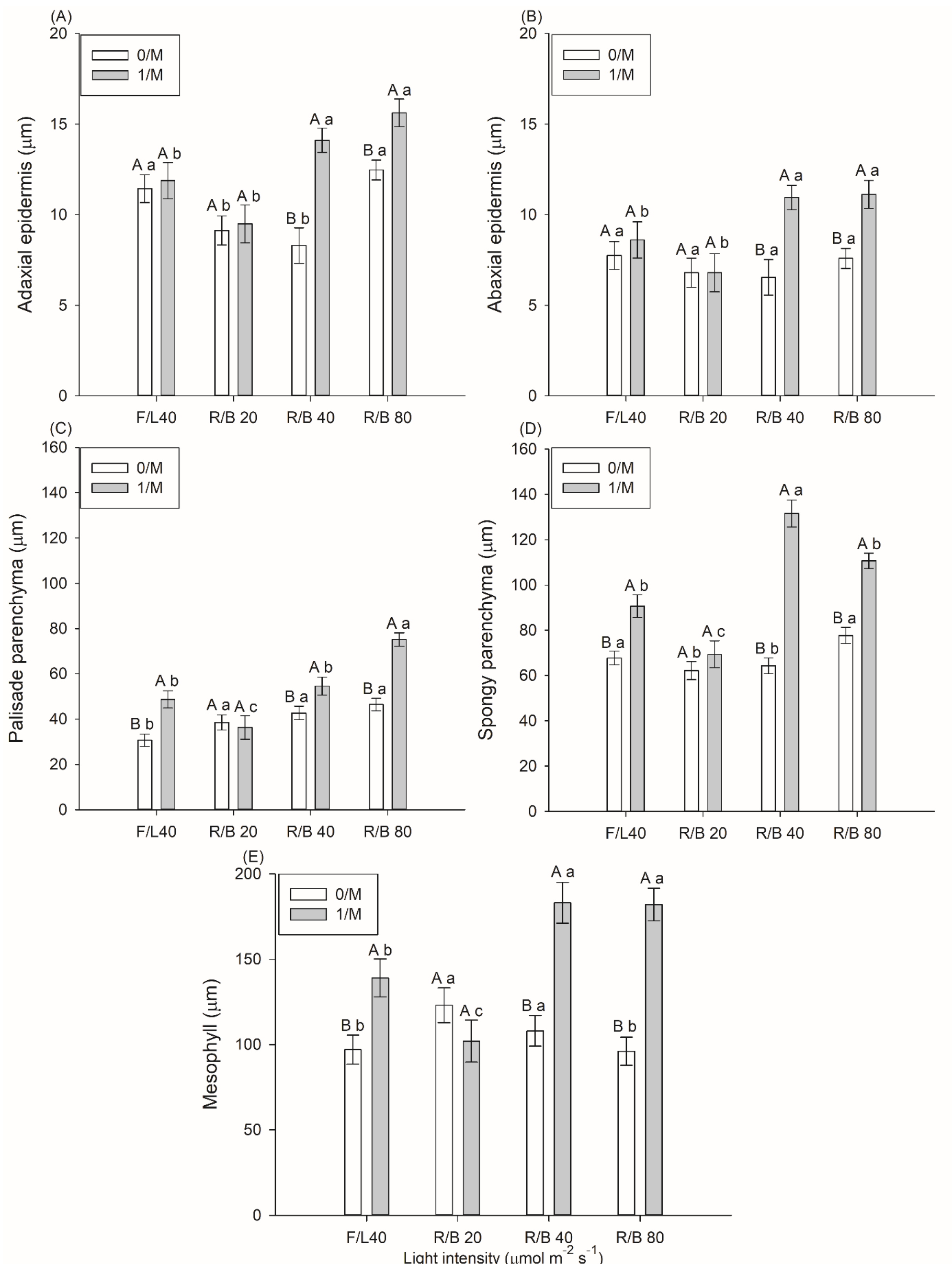


Fig. 5 Anatomical characteristics observed in *in vitro* elongation according to spectral intensity (FL 40, R/B 20, R/B 40, and R/B 80 µmol m^{-2} s^{-1}) and seal type (0/M and 1/M). (A) Adaxial

epidermis. (B) Abaxial epidermis. (C) Palisade parenchyma. (D) Spongy parenchyma. (E) Mesophyll. * Lowercase letters represent statistical differences between the different spectral intensities in the same treatment (seal types) and capital letters that compare the seal types in the same treatment (spectral intensities). *Averages followed by equal letters do not differ, by Tukey's test at 5% probability of error. Bars represent the standard deviation from the mean value.

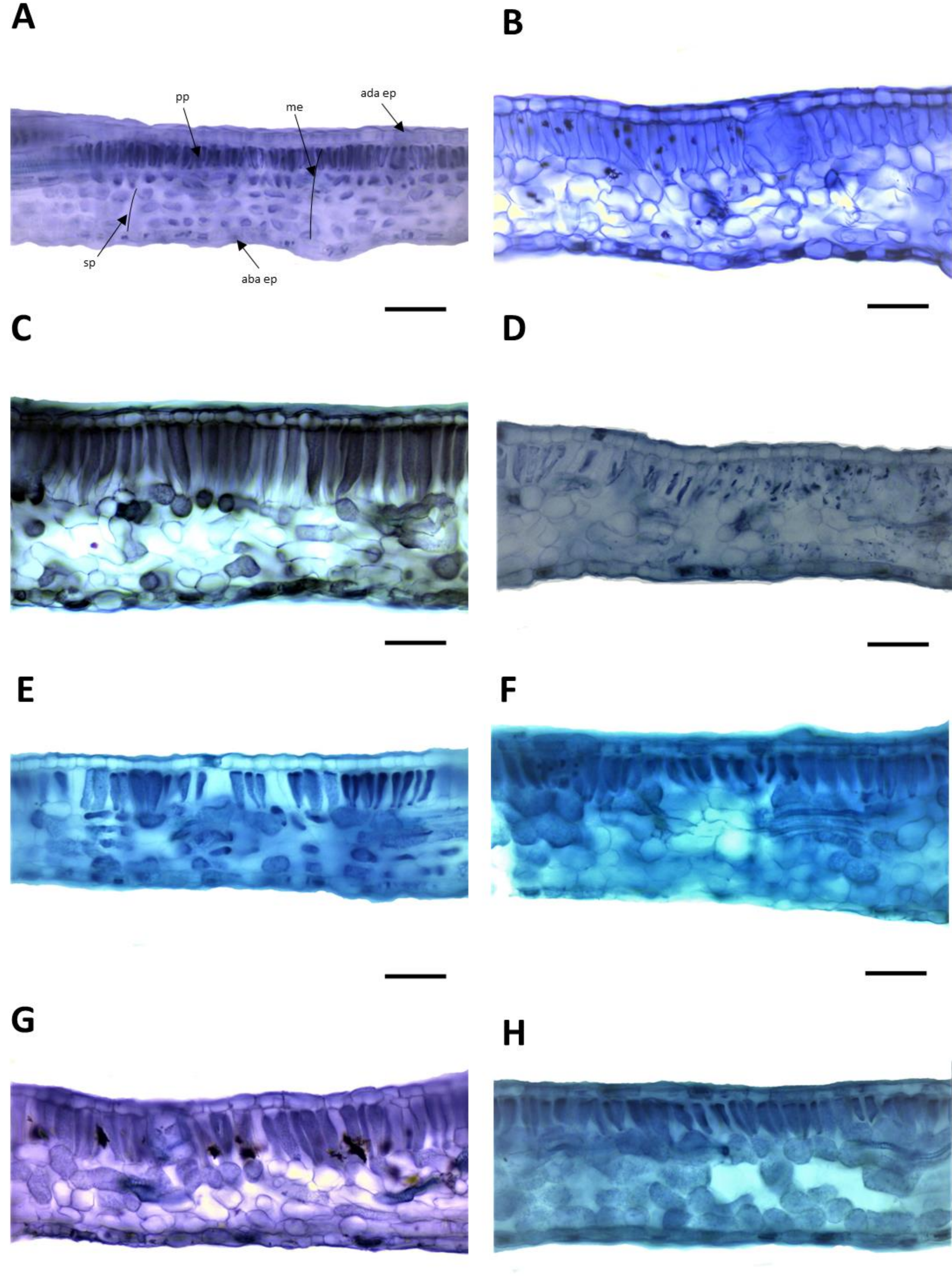

A
B
pp
me
ada ep
sp
aba ep
C
D
E
F
G
H

Fig. 6 Cross-sections of the leaf blade observed in the *in vitro* elongation of *Eucalyptus grandis* × *E. urophylla*. (A) FL 40 µmol m^{-2} s^{-1} and 0/M. (B) FL 40 µmol m^{-2} s^{-1} and 1/M. (C) R/B 20 µmol m^{-2} s^{-1} and 0/M. (D) R/B 20 µmol m^{-2} s^{-1} and 1/M. (E) R/B 40 µmol m^{-2} s^{-1} and 0/M. (F) R/B 40 µmol m^{-2} s^{-1} and 1/M. (G) R/B 80 µmol m^{-2} s^{-1} and 0/M. (H) R/B 80 µmol m^{-2} s^{-1} 1/M. *ada ep = adaxial epidermis; aba ep = abaxial epidermis; pp = palisade parenchyma; sp = spongy parenchyma; me = mesophyll. Bar = 50 µm.

The best results concerning the area of the xylem (mean: 16,128 µm) (Figure 7A), phloem (mean: 15,574 µm) (Figure 7C), and total vascular tissues (mean: 31,703 µm) (Figure 7E) were found for the FL 40 µmol m^{-2} s^{-1} treatment, which differed significantly from the other treatments.

Between the different seal types used (0/M and 1/M), the means of xylem area (9040 µm and 8925 µm) (Figure 7B), phloem area (9631 µm and 8613 µm) (Figure 7D), and total vascular tissue area (18672 µm and 17539 µm) (Figure 7F) did not differ.

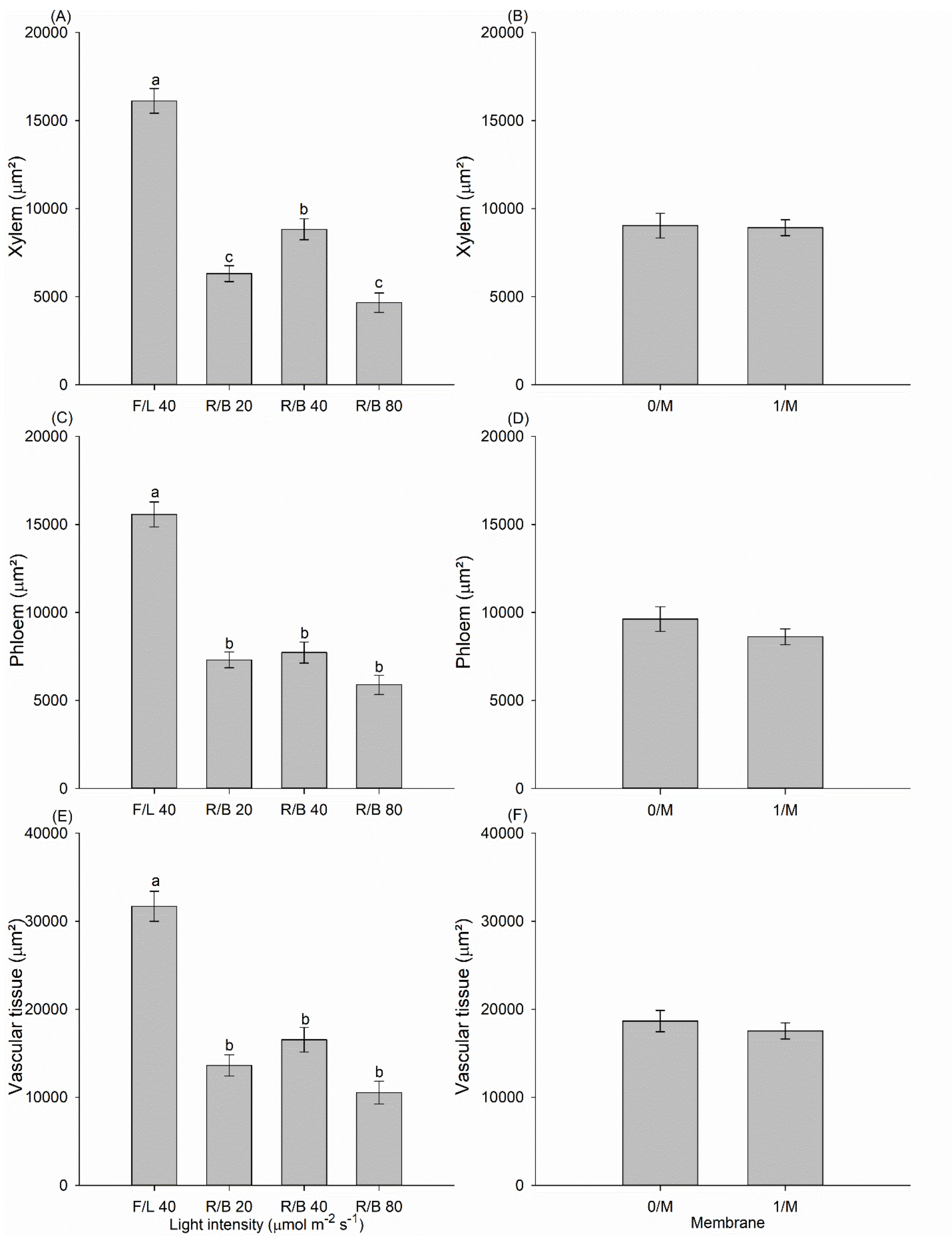


Fig. 7 Anatomical characteristics observed in *in vitro* elongation according to spectral intensity (FL 40, R/B 20, R/B 40, and R/B 80 μmol m^{-2} s^{-1}) and seal type (0/M and 1/M). (A) Xylem. (B)

Xylem. (C) Phloem. (D) Phloem. (E) Vascular tissues. (F) Vascular tissues. *Averages followed by equal letters do not differ, by Tukey's test at 5% probability of error. Bars represent the standard deviation from the mean value.

As for the leaf anatomy characteristics studied in paradermal sections, a significant interaction was observed between the factors (light intensity and seal type) for stomatal number and density. The polar and equatorial diameters did not differ significantly.

The highest mean stomatal number and density were found in the FL 40 µmol m^{-2} s^{-1} plus 1/M treatment (357 stomata and 861.56 $µm^{-2}$) (Figure 8A, 8B and 9B), which differed significantly from the other treatment' values. This same pattern was observed for the vascular system (xylem + phloem) and morphological characteristics evaluated (Figures 3, 4, and 7).

Regarding the polar and equatorial diameter, the largest stomata were observed in the R/B 20 µmol m^{-2} s^{-1} (mean: 20.21 µm and 12.96 µm) (Figure 8C and 8D) and 0/M (mean: 19.07 µm and 12.72 µm) treatments (Figure 8E and 8F), but with no significant difference from the other treatments. The appearance of the stomata in paradermal sections of the *E. grandis* × *E. urophylla* clone are shown in Figures 9A-H.

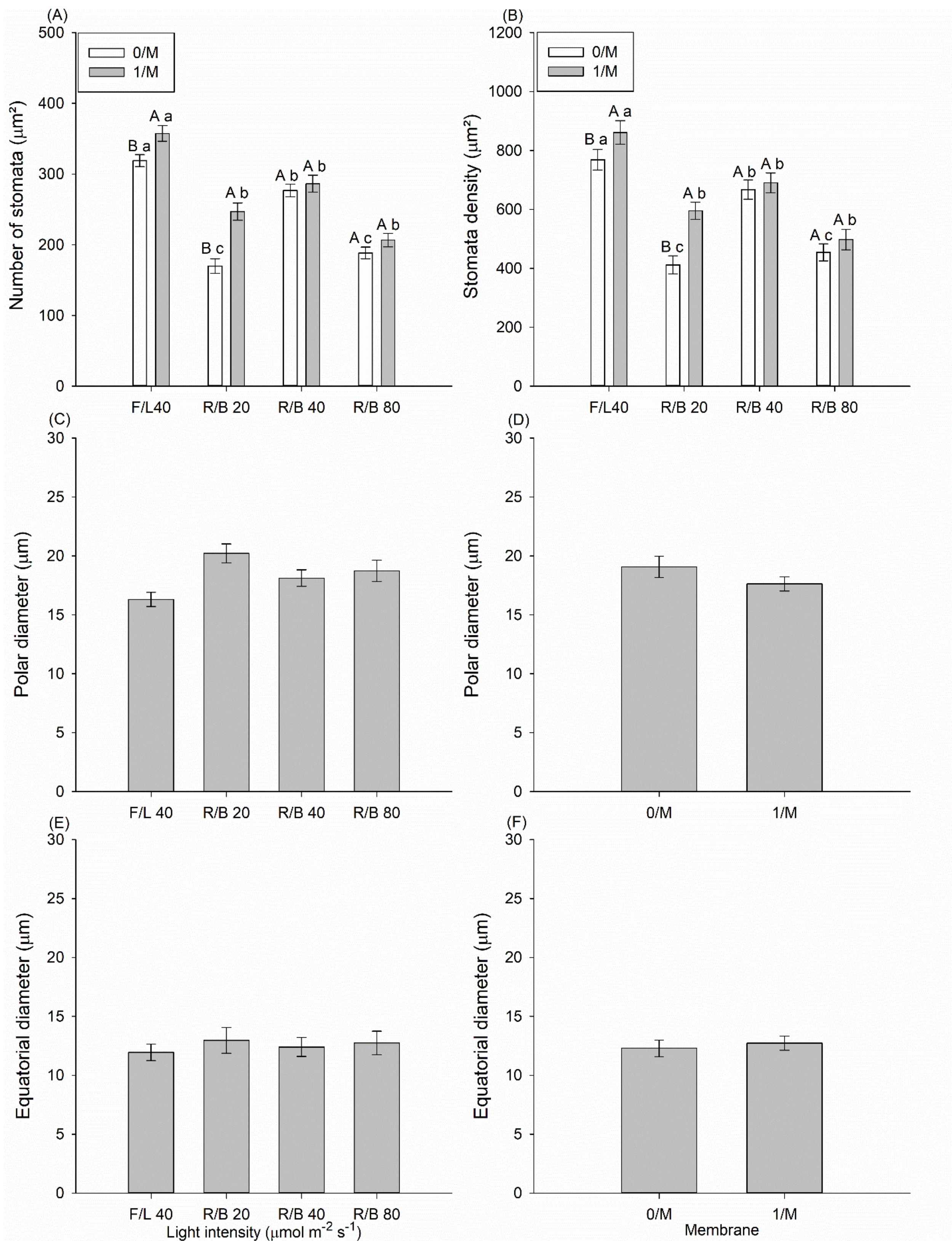


Fig. 8 Anatomical characteristics observed in *in vitro* elongation according to spectral intensity (FL 40, R/B 20, R/B 40, and R/B 80 μmol m^{-2} s^{-1}) and seal type (0/M and 1/M). (A) Number of

stomata. (B) Stomatal density. (C) Polar diameter. (D) Polar diameter. (E) Equatorial diameter. (F) Equatorial diameter. *For (A and B) lowercase letters represent statistical differences comparing the different spectral intensities in the same treatment (seal types) and capital letters represent statistical differences comparing the seal types in the same treatment (spectral intensities). *Averages followed by equal letters do not differ, by Tukey's test at 5% probability of error. Bars represent the standard deviation from the mean value.

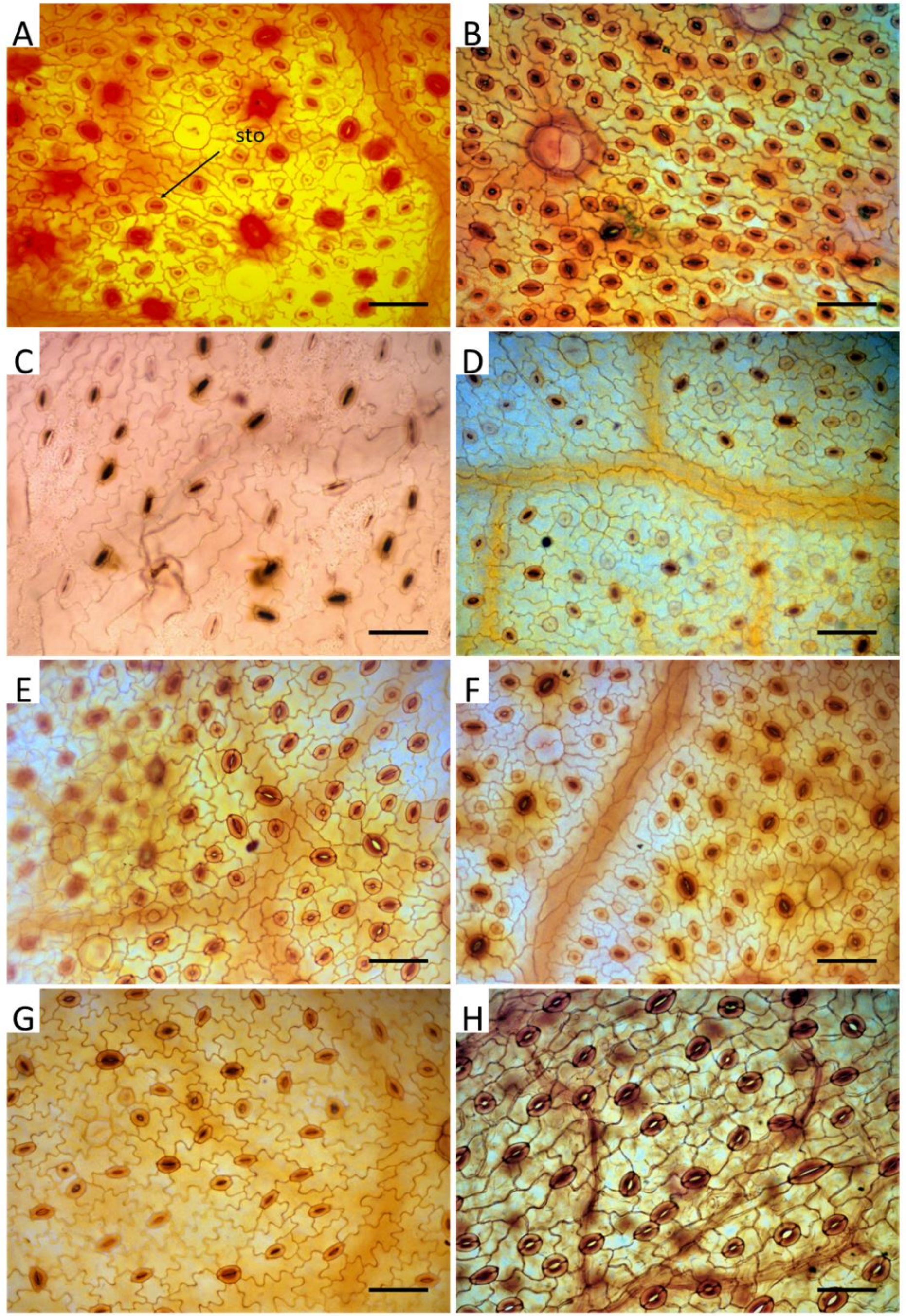


Fig. 9 Paradermal sections of the leaf blade from the *in vitro* elongation of *Eucalyptus grandis* × *E. urophylla*. (A) FL 40 µmol m^{-2} s^{-1} and 0/M. (B) FL 40 µmol m^{-2} s^{-1} and 1/M. (C) R/B 20 µmol m^{-2} s^{-1} and 0/M. (D) R/B 20 µmol m^{-2} s^{-1} and 1/M. (E) R/B 40 µmol m^{-2} s^{-1} and 0/M. (F) R/B 40

μmol m^{-2} s^{-1} and 1/M. (G) R/B 80 μmol m^{-2} s^{-1} and 0/M. (H) R/B 80 μmol m^{-2} s^{-1} 1/M. *STO = stomata. Bar = 50 μm.

In vitro rooting

The characteristics of *in vitro* rooting differed by light intensity and seal type at 35 days of cultivation. There was no interaction between the factors (light intensity and seal type) for root length, root diameter, or rooting percentage, while for the number of roots per explant, the factors were dependent.

Root length and diameter were maximum under FL 40 μmol m^{-2} s^{-1} (6.45 cm and 0.87 mm, respectively), which differed significantly from the R/B 20 μmol m^{-2} s^{-1}, R/B 40 μmol m^{-2} s^{-1}, and R/B 80 μmol m^{-2} s^{-1} treatments (Figure 10A and 10C). The use of a membrane (1/M) also led to a higher mean root length (5.73 cm) and diameter (0.71 mm) than 0/M (4.91 cm in length and 0.61 mm in diameter) (Figure 10B and 10D).

Following the same trend as the aforementioned characteristics, the number of roots per explant was maximum under FL 40 μmol m^{-2} s^{-1} and 1/M (4.09 cm) (Figure 10E). Considering all characteristics evaluated under different spectral intensities and seal types, the lowest performance was observed in the treatment with R/B 20 μmol m^{-2} s^{-1} and 0/M (Figure 10A-E). Thus, a low light intensity (20 μmol m^{-2} s^{-1}) was inadequate for *in vitro* rooting of *E. grandis* × *E. urophylla*.

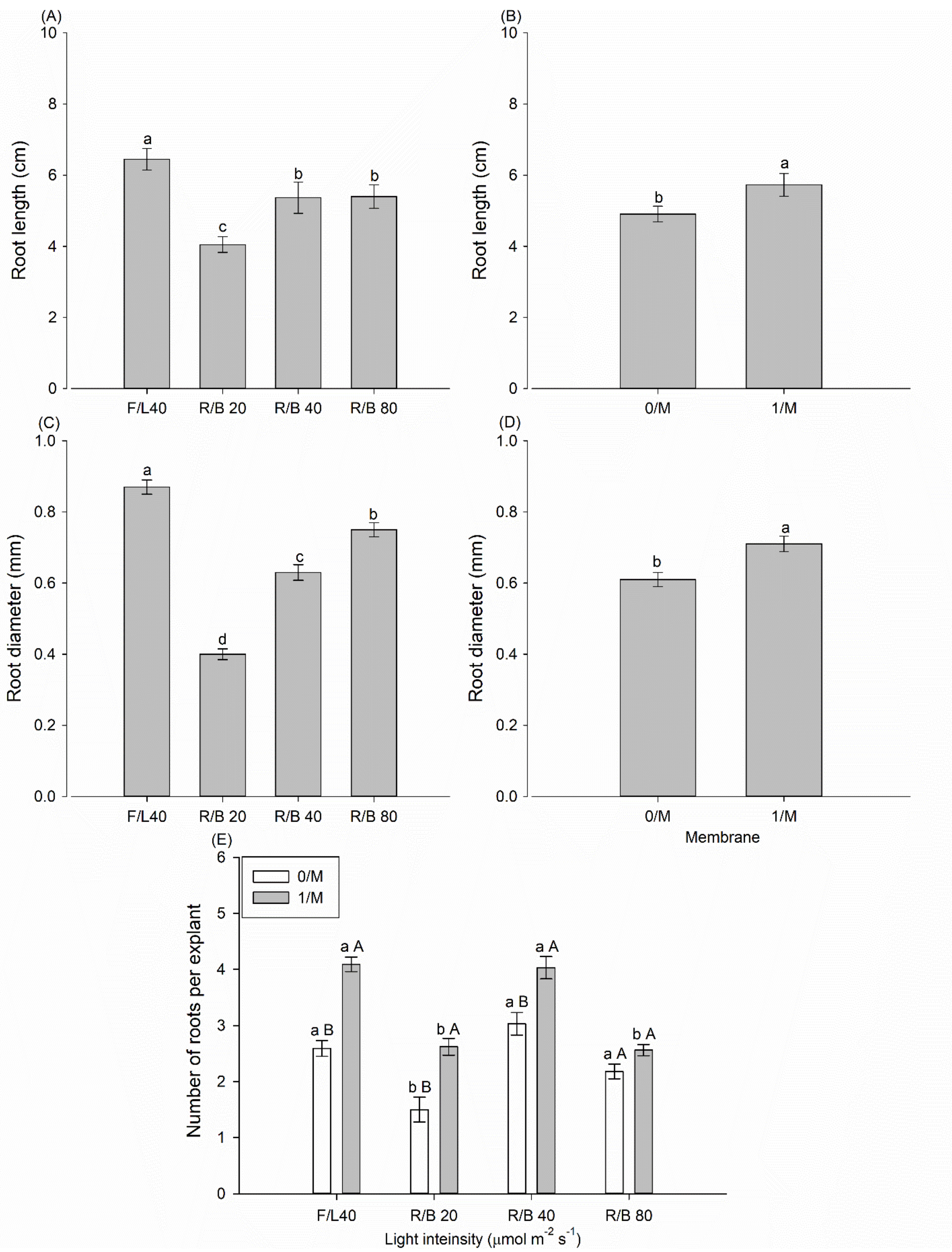


Fig. 10 Morphological characteristics observed in *in vitro* rooting of E. grandis × E. urophylla according to different spectral intensities (FL 40, R/B 20, R/B 40, and R/B 80 μmol m^{-2} s^{-1}) and

seal types (0/M and 1/M). (A) Root length (cm). (B) Root length (cm). (C) Root diameter (mm). (D) Root diameter (mm) (E) Number of roots per explant. *For (E), lowercase letters represent significant differences when comparing the different light sources within the same seal type, and uppercase letters represent significant differences when comparing the seal types within the same light treatment. *Averages followed by equal letters do not differ, by Tukey's test at 5% probability of error. Bars represent the standard deviation from the mean value.

The rooting of the microstumps was influenced by the light intensity and seal type, resulting in different responses of the *E. grandis* × *E. urophylla* clone. The FL 40 µmol m^{-2} s^{-1} treatment provided the highest rooting percentages (96.8%), but it differed significantly only from the R/B 20 µmol m^{-2} s^{-1} treatment (82.8%) (Figure 11A). Comparing the seal types, 1/M yielded a significantly higher rooting percentage (95.3%) than 0/M (87.5%) (Figure 11B).

The results suggest that it is possible to produce seedlings with high root proliferation and rooting percentage, as observed in the FL 40 µmol m^{-2} s^{-1}, R/B 40 µmol m^{-2} s^{-1}, and R/B 80 µmol m^{-2} s^{-1} light treatments. The high rooting percentages indicate the potential to use specific light intensities and seal types. The appearance of the *E. grandis* × *E. urophylla* clone microstumps in terms of the characteristics studied in *in vitro* elongation and rooting are shown in Figures 12A-E.

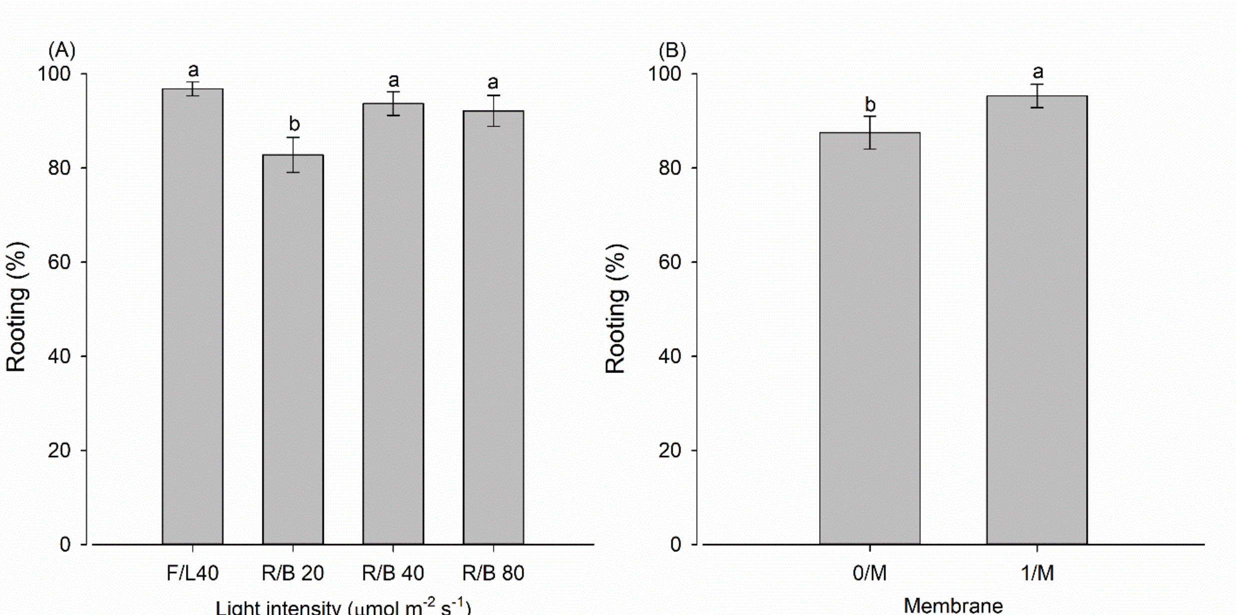


Fig. 11 *In vitro* rooting percentage of *E. grandis* × *E. urophylla*. (A) Spectral intensity (FL 40, R/B 20, R/B 40, and R/B 80 µmol m^{-2} s^{-1}). (B) Seal type (0/M and 1/M). *Averages followed by equal

letters do not differ, by Tukey's test at 5% probability of error. Bars represent the standard deviation from the mean value.

Fig. 12 Microplants of the *Eucalyptus grandis* × *E. urophylla* clone elongated and rooted *in vitro*. (A, C, E, and G) 0/M. (B, D, F, and H) 1/M. (A and B) FL 40 µmol m^{-2} s^{-1}. (C and D) R/B 20 µmol m^{-2} s^{-1}. (E and F) R/B 40 µmol m^{-2} s^{-1}. (G and H) R/B 80 µmol m^{-2} s^{-1}. Bar = 1.0 cm.

DISCUSSION

In vitro elongation

In vitro elongation and rooting responses were studied under specific light intensities and seal types to establish more efficient protocols for producing clonal seedlings on a commercial scale. The different light intensities and seal types used in the *in vitro* elongation of the microstumps influenced tissue oxidation, vigor, number of buds per explant, and shoot development in the *E. grandis* × *E. urophylla* clone.

The lowest phenolic oxidation in the tissues of *E. grandis* × *E. urophylla* was observed in the treatment with the lowest light intensity (20 µmol m^{-2} s^{-1}) and 1/M. Low light or darkness during *in vitro* cultivation of woody species reduces the phenolic oxidation of explants of *Eucalyptus cloeziana* (OLIVEIRA *et al*. 2015), *Eucalyptus benthamii* (BACCARIN *et al*. 2015), *Corymbia citriodora* × *Corymbia torelliana*, and *C. torelliana* × *C. citriodora* (SOUZA *et al*. 2018). *In vitro* conditions that are stressful for plant growth include low CO_2 concentrations and high ethylene concentrations which cause tissue oxidation, senescence, and reduced vigour (TISARUM *et al*.

2017; NGUYEN; XIAO; KOZAI, 2020), as we observed under the condition with lower gas exchange in the treatment without membrane (0/M).

The use of 20 µmol m^{-2} s^{-1} resulted in the worst vigor rates for the microstumps, with apparent etiolation, reduced leaf size, senescence, and/or death (Figure 12C and 12D). Previous research corroborates with the data that was found in this study, as the use of light intensities above 40 µmol m^{-2} s^{-1} in the orchid *Microlaelia lundii* (FAVETTA *et al*. 2017), C*orymbia torelliana* × *C. citriodora* (SOUZA *et al*. 2018), *Urtica dioica* (COELHO *et al*. 2021), and *Mentha arvensis* L. (OLIVEIRA *et al*. 2021) induced greater explant development and vigor. Good quality effects of membranes are also described, as they may be associated with increased photosynthesis caused by high CO_2 availability. In the *in vitro* culture of *Plectranthus amboinicus*, greater vigor of the explants was observed with the use of three membranes in the flasks (SILVA *et al*. 2017).

The number of shoots per explant was highest under the R/B combination of the R/B 20 µmol m^{-2} s^{-1} treatment and 0/M. These findings contrast the results regarding the growth and development of the microstumps, which showed the highest means in the FL 40 µmol m^{-2} s^{-1} plus 1/M treatment. Many studies have shown the superiority of a light source with a broad light spectrum in *in vitro* plant development (BATISTA *et al*. 2018; SANTOS *et al*. 2020; MIRANDA *et al*. 2020), though a response pattern has not been established for all species.

The control of spectral quality, light intensity, and gas exchange during plant cultivation is essential because it can optimize the photosynthetic rate and shoot development (SANTOS *et al*. 2020; SILVEIRA *et al*. 2020). Therefore, *in vitro* propagation with porous membranes that allow greater gas exchange between the external and internal atmospheres of the flasks results in a higher plant growth rate as long as the plant has enough CO_2 (NGUYEN; XIAO; KOZAI, 2020; NÚÑEZ-RAMOS *et al*. 2020).

Regarding the content of photosynthetic pigments (chlorophyll *a*, *b*, and *a*+*b*, and carotenoids), the highest values were observed for the FL 40 µmol m^{-2} s^{-1} treatment. In *U. dioica* explants, treatment with white L.E.D.s and a light intensity of 51 µmol m^{-2} s^{-1} resulted in the highest mean chlorophyll *a*, chlorophyll *b*, and carotenoid contents, unlike 26 µmol m^{-2} s^{-1} (COELHO *et al*. 2021).

The biosynthesis of chlorophyll *a* and *b* and carotenoids is influenced by the quality and intensity of the light emitted and by the seal types used. Greater photosynthetic efficiency and explant development are seen at wavelengths with a broad light spectrum and flasks that allow

greater gas exchange (GUPTA; KARMAKAR 2017; TIAN *et al*. 2019; LEI *et al*. 2021), as observed in the present study.

In a study of *Vaccinium ashei*, the system using membranes led to greater shoot length, number of shoots, leaf area, and chlorophyll content (HUNG *et al*., 2016). However, in the *in vitro* elongation of *Corymbia torelliana* × *C. citriodora*, higher pigment content and microstump development have been observed with the R/B 40 µmol m^{-2} s^{-1} treatment without a porous membrane (SOUZA *et al*. 2020b). Thus, different microstump responses were observed in the *in vitro* elongation stage according to the light intensity and seal type used. Therefore, knowledge of the relationship between light intensities, seal types, and plant growth patterns in micropropagation will lead to greater knowledge of the hybrid, improving the production of clonal seedlings.

Leaf anatomy

The anatomical analyses showed differences between the treatments used in *E. grandis* × *E. urophylla* microstumps, with the largest adaxial epidermis, abaxial epidermis, palisade parenchyma, spongy parenchyma, and mesophyll thicknesses found in the R/B 40 and R/B 80 µmol m^{-2} s^{-1} treatments combined with 1/M. These results are similar to those from Souza *et al*. (2020a) in *E. urophylla* × *E. grandis* microstumps under red/blue L.E.D.s and a light intensity of 40 µmol m^{-2} s^{-1}. In *Cattleya walkeriana* (SILVA *et al*. 2014), *Populus euramericana* (KWON *et al*. 2015), and *Eucalyptus dunnii* microstumps (SOUZA *et al*. 2019), the use of a porous membrane increased the thickness of the leaf epidermis, parenchyma, and mesophyll, corroborating our results.

Light intensity and seal type influence the growth of plant cells, tissues, and organs, triggering different morphological and anatomical responses (MARTINS *et al*. 2020; NGUYEN; XIAO; KOZAI, 2020). Many studies show the superiority of LEDs lamps in *in vitro* plant development over fluorescent lamps (BATISTA *et al*. 2018), but the response to photomixotrophism depends on the genotype (ZIENKIEWICZ *et al*. 2015; NGUYEN; XIAO; KOZAI, 2020).

Vascular tissues, stomatal number, and density were higher under the FL 40 µmol m^{-2} s^{-1} light. When light intensities are low and there is little supply of CO_2, tissues tend to be less developed due to the lower production of photoassimilates (TAIZ; ZEIGER, 2013), as was observed in the R/B 20 µmol m^{-2} s^{-1} with 0/M treatment. Seedlings grown *in vitro* with low light intensity, low CO_2 concentration, and exogenous carbohydrates as the only energy source usually

show anomalies, such as low photosynthetic capacity, stomatal malfunction, absence of leaf cuticle, and abnormal leaf parenchyma (NÚÑEZ-RAMOS *et al*. 2020).

These results corroborate the previous results (Figures 3 and 4) on the effect of light intensity on morphological characteristics in the *in vitro* elongation stage. We infer that a larger vascular system may lead to better shoot development, as observed in the microstumps of the *E. grandis* × *E. urophylla* clone. Castro *et al*. (2007) found the best vascular tissue development in *Mikania glomerata* when it was subjected to a broad light spectrum (450-700 nm) and higher light intensities (40 µmol m^{-2} s^{-1} and 80 µmol m^{-2} s^{-1}).

In Mohamed and Alsadon (2010) study, the use of the membrane sealing system resulted in thicker leaves and more developed vascular tissues (xylem and phloem) in *Solanum tuberosum* than the conventional system. The control of light and CO_2 in plant cultivation is of paramount importance. At low intensity, photosynthesis can be inefficient and reduce development, while excessive photosynthesis can damage photosynthetic structures (SILVA *et al*. 2017).

Another relevant effect caused by increased CO_2 availability and adequate light intensity is the increased stomatal number and density, which we observed mainly with FL 40 µmol m^{-2} s^{-1} and 1/M. In the *in vitro* culture of *Plectranthus amboinicus*, greater stomatal density and better plant development were observed using a membrane system in the flasks (SILVA *et al*. 2017), similar to our results. Regarding *Withania somnifera* explants, higher photosynthetic pigment production and efficiency, stomatal number, and stomatal conductance were observed a light intensity of 60 µmol m^{-2} s^{-1} (LEE *et al*. 2007). In *E. urophylla* microstumps, different light intensities (60, 85, 100, and 140 µmol m^{-2} s^{-1}) had no influence on leaf anatomy (MIRANDA *et al*. 2020), in contrast to what was found in our study.

Photomixotrophism through light intensity and gas exchange, is an important factor in *in vitro* cultivation, besides, there are still few studies of its effects on woody species. A better understanding of the relationship between spectral quality and the use of porous membranes in the growth and development of microstumps would provide a basis for making protocols more efficient, thus allowing for planning and large-scale propagation.

***In vitro* rooting**

In the adventitious rooting process, several factors underlie root formation in microstumps. Among these are plant hormones and the cultivation environment (light, temperature, and gas

exchange), which play an important role in rhizogenesis (DE ALMEIDA *et al*. 2017; DÍAZ-SALA 2020). The difficulty of propagation through *in vitro* adventitious rooting of microstumps is one of the main problems in producing clonal seedlings of many *Eucalyptus* species (TRUEMAN; HUNG; WENDLING, 2018; ABIRI *et al*. 2020). In the present study, root length and diameter, number of roots per explant, and rooting percentage showed the best results in the FL 40 µmol m^{-2} s^{-1} with 1/M treatment.

The use of white or fluorescent spectral quality and specific light intensities in *in vitro* cultures may lead to greater root ad shoot development by allowing light to penetrate the leaves better, maximizing the photosynthetic rate, when compared to blue and red monochromatic lights (TAIZ; ZEIGER, 2013), as observed for *Lactuca sativa* (LIN *et al*. 2013). Different studies also indicate better development of plants grown *in vitro* under light intensities more significant than 20 µmol m^{-2} s^{-1} (BATISTA *et al*. 2016, FERREIRA *et al*. 2017).

These studies show that plants require a broad light spectrum and efficient gas exchange to optimize photosynthetic processes, reduce relative humidity, increase their aeration, become hardier, favour greater survival and rooting (SALDANHA *et al*. 2012; LOUBACK *et al*. 2021). In *Hevea brasiliensis*, *C. walkeriana*, and *E. dunnii*, the best results for length and number of roots, survival, and rooting were obtained with flasks that allowed improved natural ventilation (TISARUM *et al*. 2017; SOUZA *et al*. 2019).

Systems that increase the supply of CO_2 and light to some species *in vitro* usually promote greater development of the photosynthetic apparatus, resulting in high rates of multiplication, growth, rooting, and subsequent acclimation of the seedlings to *ex vitro* conditions (SALDANHA *et al*. 2012; LOUBACK *et al*. 2021). Sometimes, a gradual adaptation to *ex vitro* conditions may be required for plants grown in *in vitro* photomixotrophic systems to undergo autotrophic growth in a greenhouse (PÉREZ *et al*. 2015; SILVEIRA *et al*. 2020).

Thus, the microstumps treated with the 40 µmol m^{-2} s^{-1} and 80 µmol m^{-2} s^{-1} light intensities and 1/M responded uniformly, with high rooting percentages (92.1% to 96.8%) and a high probability of acclimatization, compared to those obtained with the use of 20 µmol m^{-2} s^{-1} and 0/M, being indicative of optimization of the *in vitro* rooting protocol. The treatments with light intensity equal to or greater than 40 µmol m^{-2} s^{-1} and 1/M were more suitable for *in vitro* rooting of the microstumps.

CONCLUSIONS

- Light intensity specificity and the use of a porous membrane influence the morphology and anatomy of *E. grandis* × *E. urophylla* grown under a controlled environment. Therefore, it is a valuable technology to optimize the large-scale production of clonal seedlings.

- The quality and intensity of the FL 40 µmol m^{-2} s^{-1} light combined with 1/M are the most suitable for use in the *in vitro* elongation and rooting of *E. grandis* × *E. urophylla*, providing greater vigor, shoot length, photosynthetic pigment content, xylem, phloem, stomatal number and density, root length and diameter, number of roots per explant, and rooting percentage.

- The light intensity of 20 µmol m^{-2} s^{-1} and the lack of a membrane were inadequate for the *in vitro* elongation and rooting of *E. grandis* × *E. urophylla*.

ACKNOWLEDGMENTS

This work was carried out with the support of the Coordination for the Improvement of Higher Education Personnel - Brazil (CAPES), the National Council for Scientific and Technological Development (CNPq) and the Research Support Foundation of the State of Minas (FAPEMIG).

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ARTIGO 4

Spectral quality and temporary immersion bioreactor for *in vitro* multiplication of *Eucalyptus grandis* × *Eucalyptus urophylla*

Artigo publicado na Revista 3 Biotech
https://link.springer.com/article/10.1007/s13205-020-02447-3
https://doi.org/10.1007/s13205-020-02447-3

3 Biotech (2020) 10:457
https://doi.org/10.1007/s13205-020-02447-3

ORIGINAL ARTICLE

Spectral quality and temporary immersion bioreactor for in vitro multiplication of *Eucalytpus grandis* × *Eucalyptus urophylla*

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Received: 12 July 2020 / Accepted: 21 September 2020
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Abstract
Spectral quality is an important factor for in vitro development of explants in a bioreactor system. Based on the need to optimize micropropagation for *E. grandis* × *E. urophylla* clones, the aim of the study was to assess the spectral quality of in vitro multiplication in temporary immersion bioreactor (TIB). The tissue used to generate the explants (i.e., the nodal segment with 1 cm of length and two axillary bud without leaves) was previously in vitro established and multiplied, it derived from ministumps of *E. grandis* × *E. urophylla* clone grown in a semi-hydroponic system. The spectral quality of in vitro multiplication was assessed through five light sources (i.e., fluorescent lamp, red, green, blue, and yellow cellophane). Morphological and anatomical features of tissues grown in TIB were evaluated at 90 days. Based on the results, yellow and blue spectral qualities were the most suitable to be adopted for in vitro multiplication of *E. grandis* × *E. urophylla*, since they enabled lesser hyperhydricity, favors high number of shoots per explant and shoot length, as well as thicker mesophyll and spongy parenchyma; arise as an alternative for large-scale production of eucalypts clonal plants.

Keywords Cloning · In vitro cultivation · Vegetative propagation · Wavelength

Introduction

The expansion of forest plantations to non-traditional regions and the search for better adaptation in plant propagation has increased the use of species and hybrid combinations. The hybridization of *Eucalyptus grandis* W. Hill ex Maiden with *Eucalyptus urophylla* ST Blake has provided excellent results, both in terms of wood quality and growth, in addition to better environmental adaptation and easier vegetative propagation (Trueman et al. 2018). However, alternatives for the tissue rejuvenation/reinvigoration of selected hybrids of the *Eucalyptus* genus, aiming at greater multiplication of plants, and micropropagation technique has been recommended, allowing considerable advances in clonal propagation (Wendling et al. 2014; Zhang et al. 2020).

Studies focused on investigating the micropropagation of *Eucalyptus grandis*, *Eucalyptus urophylla*, and of their hybrids, have been important for the industrial forestry sector. These studies comprise in vitro establishment (Moura et al. 2017; Keret et al. 2018), bud multiplication (Businge et al. 2017; Máximo et al. 2018; Trueman et al. 2018; Carvalho et al. 2019), shoot elongation (Gallo et al. 2017; Miranda et al. 2020), as well as adventitious rooting and acclimatization phases (Nakhooda and Watt 2017; Brondani et al. 2018).

Basic studies focused on improving microcutting production processes to solve the limiting factors in each micropropagation phases are fundamental. Several technologies have been suggested to optimize micropropagation processes. Innovations in plant growth environments such as the use of

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Published online: 30 September 2020

new lighting sources based on their spectral quality, as well as the automation of culture system operations and routine procedures through the use of temporary immersion bioreactors (TIB) stand out among these technologies (Batista et al. 2018; Carvalho et al. 2019).

The TIB consists of an automated system used to grow plant tissues. It can be applied to improve plant multiplication in laboratory and biofactory environments (Máximo et al. 2018). This type of system optimizes plant cultivation through automating processes (Mendonça et al. 2016), enables biomass gain and shortens propagation time (Máximo et al. 2018), as well as increases plant production per area (Regueira et al. 2017). Accordingly, TIBs are capable of improving nutrient supply and gas transfer processes, as well as of minimizing physiological disturbances in plants, thus improving the development of micropropagated cultures (Carvalho et al. 2019).

Recent studies have reported the influence of wavelength types such as 450–495 nm (blue light), 620–750 nm (red), 750–850 nm (extreme red), and 495–570 nm (green) (Batista et al. 2018) on plants morphophysiological processes since light is used as an energy source for photosynthesis (Heringer et al. 2017). However, despite the economic importance of eucalypts (e.g., *Eucalyptus* and *Corymbia*), and of its hybrids, studies focused on investigating the effect of different spectral qualities on plant growth and development, based on the use of TIB in the in vitro multiplication, remain incipient.

Our aim was to evaluate the in vitro multiplication phase of *E. grandis* × *E. urophylla*, based on spectral quality use and on cultivation in TIB system.

Material and methods

Study site and tissues source

The experiment was conducted in the Laboratory of In Vitro Culture of Forest Species at the Department of Forestry Sciences of the Federal University of Lavras (UFLA), Lavras, Minas Gerais, Brazil. The tissues used to generate the explants (i.e., nodal segment with 1 cm of length and axillary bud without leaves) was previously in vitro established and multiplied; it derived from ministumps of the A211 hybrid clone of *Eucalyptus grandis* Hill ex Maiden × *Eucalyptus urophylla* ST Blake grown at "Viveiro Esteio Produção de Mudas", São João Del Rei, Minas Gerais, Brazil. Ministumps were established in a clonal mini-garden under semi-hydroponic system of the sand-bed channel type. Plants received dripping nutrient solution, which was applied four times a day, at total daily flow of 4 L m^{-2}.

Shoot multiplication in temporary immersion bioreactor (TIB)

Shoots produced at the in vitro establishment stage were isolated, standardized in length (0.5 cm and two buds), and inoculated in glass flasks (250 mL) under aseptic conditions. To the glass flasks were added 150 mL of WPM liquid culture medium (Lloyd and McCown, 1980), which was supplemented with 20 g L^{-1} of sucrose (Vetec®), 0.5 mg L^{-1} of 6-benzylaminopurine (BAP—Sigma®), and 0.01 mg L^{-1} of naphthaleneacetic acid (NAA—Sigma®). Plant tissues were immersed in TIB for 30 s, at 3 h intervals between immersion cycles, throughout 90 culture days. Subcultures were carried out every 30 days for culture medium renewal purposes. The liquid culture medium was prepared with deionized water and its pH was adjusted to 5.8 ± 0.05, before autoclaving. The culture medium was autoclaved at a temperature of 127 ºC and pressure of 1.5 kgf cm^{-2}, for 20 min.

Spectral quality

The experiment has followed a completely randomized design based on five spectral qualities [fluorescent lamp (F/L), red (R/E), green (G/R), blue (B/L), and yellow (Y/E) cellophane], with twenty replications comprising one explant, each. Treatments with red, green, blue, and yellow spectral quality were applied by filtering the light output of fluorescent lamps through double cellophane sheets, which were used to wrap the TIB flasks. SPECTRA PEN Z850 portable spectroradiometer (Qubit Systems-Kingston, Ontario-USA) was used to analyze variations in absolute irradiance (μW cm^{-2} nm^{-1}) and the wavelength (nm) of light emitted by different spectral qualities. Spectral of each treatment used in this experiment are shown in Fig. 1.

The experiment was carried out in growth room at 24 ± 1 °C, under 16 h of photoperiod. Two cold white fluorescent tubes (Philips T10, 0.60 m long, 20 W and color 6400–6500 k) were used on each shelf. Photoradiometer model QSO-S Procheck + Sensor-PAR Photon Flux (Decagon Devices, Pullman, Washington-USA) was used to measure the irradiance of the two lamps (40 μmol m^{-2} s^{-1}).

Plant vigor (VIG) and hyperhydricity (HYP) were evaluated at 90 days of cultivation, based on the scale of notes by Oliveira et al. (2016), as shown in Fig. 2. Number of shoots per explant (> 0.5 cm) (NSE) and shoot length (> 0.5 cm) per explant (SL), as well as photosynthetic pigment content (PPC) were evaluated.

Fig. 1 Variations in absolute irradiance (μW cm^{-2} nm^{-1}) and wavelength (nm) of light emitted for in vitro cultivation of *E. grandis* × *E. urophylla*. **a** Fluorescent lamp; **b** red cellophane; **c** green cellophane; **d** blue cellophane; **e** yellow cellophane

Photosynthetic pigment analysis

Leaf discs (i.e., 25 mg of fresh matter) were sampled after 90 days of exposure to different spectral qualities. Samples were inoculated in 5 mL of DMSO solution (Sigma aldrich) and stored in the dark for 48 h, based on the methodology by Lichtenthaler (1987). Sample absorbance was determined in triplicate with the aid of a quartz cuvette with 10 mm of optical path, in Genesys 10UV spectrophotometer (ThermoScientific, USA). The wavelengths (665, 649 and, 480 nm) and equations used to calculate chlorophylls *a*, *b*, *a* + *b* and total carotenoid concentration were based on the method by Wellburn (1994).

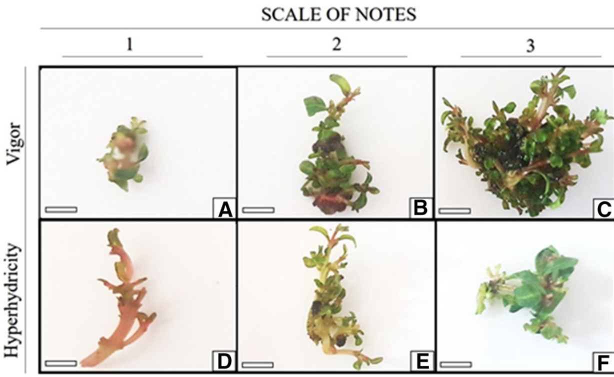

Fig. 2 Scale of notes used to assess vigor and hyperhydricity in *E. grandis* × *E. urophylla* tissues. **a** Scale 1-VIG: low shoot induction, senescence and death; **b** scale 2-VIG: shoots induction, although with reduced leaves; **c** scale 3-VIG: shoot induction and active growth, without apparent nutritional deficiency; **d** scale 1-HYP: hypertrophy in leaf tissues and explant internodes; **e** scale 2-HYP: reduced leaf tissue hypertrophy; **f** scale 3-HYP: no hyperhydricity. Bar = 0.5 cm

Leaf anatomy

Representative leaf samples from each treatment were collected and kept in FAA solution (formaldehyde, acetic acid, 70% of ethanol solution, 1:1:18) for 48 h. Next, they were transferred to 70% ethanol solution and dehydrated at increasing ethanol series concentrations (80, 90, and 100%) in each solution for 30 min (Johansen 1940). Subsequently, they were soaked in historesin (Biosystems, Nussloch, Germany) at the ratio of 1:1, in a hot oven (overnight). Blocking was processed with pure hydroxyethyl methacrylate resin and cross-sections (7 µm of thick) were obtained with the aid of manual rotating microtome and razor. Tissues were stained with toluidine blue (Vetec Química Fina Ltda, Rio de Janeiro, Brazil), mounted on Entellan histological slides (Merck KGaA, Darmstadt, Germany) and photomicrographed with a digital camera (AxionCam ERc5s) at micrometric and objective scale: 20× and 40×, respectively. Adaxial (ADE) and abaxial epidermis (ABE), mesophyll (ME), palisade parenchyma (PP), and spongy parenchyma (SP) thicknesses were measured in three fields of organ view, with leaf area corresponding to 0.04 mm^2; measurements comprised fifteen replications (5 anatomical sections × 3 fields of organ view) with one leaf, each.

Data analysis

Analyses were processed in the R software, version 3.0.3 (R Development Core Team 2018), using the ExpDes package, version 1.1.2 (Ferreira et al. 2013). Variables that did not show the normal distribution in the Shapiro–Wilk test, at 5% of significance level, were transformed by arcsen. Treatment means were subjected to analysis of variance (ANOVA) in Tukey's test at 5% of probability.

Results and discussion

The visual appearance of *E. grandis* × *E. urophylla* clone explants can be seen in Fig. 3a–e and morphological features in Fig. 4a–e. Different spectral qualities used in explant of *E. grandis* × *E. urophylla* (A211 clone) under in vitro culture have influenced the plant vigor, depending on the scale of notes, at 90 days after inoculation (Fig. 4a). The highest mean recorded for VIG was associated with the use of Y/E (2.75), the closest being the scale of notes 3 represented in Fig. 2c, which was significantly different from that recorded for F/L (Fig. 4a) ($p < 0.05$). These results are close to the ones reported by Souza et al. (2018) in *Corymbia citriodora* (Hook.) KD Hill & LAS Johnson × *C. torelliana* (F. Muell.) and by Miranda et al. (2020) in *Eucalyptus urophylla*, who observed good vigor in explants subjected to spectral qualities at peak wavelengths of 450 and 600 nm. On the other hand, Smirnakou et al. (2016) recorded the best vigor results for *Abies borisii-regis* explants subjected to fluorescent lamp light source.

HYP is an undesirable aspect of in vitro multiplication, and have shown different HYP responses according to spectral qualities. Based on the scale of notes 3 represented in Fig. 2f, R/E and G/R recorded the best HYP results (2.75, on average), which were significantly different ($p < 0.05$) from that recorded for B/L, as shown in Fig. 4b. Silva et al. (2017) conducted a study with *Plectranthus amboinicus* (Lour.) Spreng explants grown under red light; results have shown good vegetative vigor and lack of hyperhydricity. Studies have evidenced the influence of red and green light on low stomatal conductance, which is an ecological adaptation to avoid excessive leaf water loss (Aasamaa and Aphalo 2016; Batista et al. 2018).

HYP has been associated with the micropropagation of woody species, which can influence crop development. This outcome may be linked to cultivation factors such as the use of a liquid medium in bioreactor systems, which tend to increase water and mineral salts' absorption by plants. In addition, plants can be affected by light irradiation, air temperature and relative humidity (Xiao et al. 2011). Thus, methodologies aimed at overcoming or mitigating HYP in tissues are important strategies to be adopted in propagation systems.

Explants subjected to B/L presented the best SL results (2.26 cm, on average), which were significantly different from that recorded for R/E and G/R light spectra ($p < 0.05$) (Fig. 4c). The use of blue LED lamps enabled the shoots length per explant in *Acer saccharum* Marsh (Singh et al.

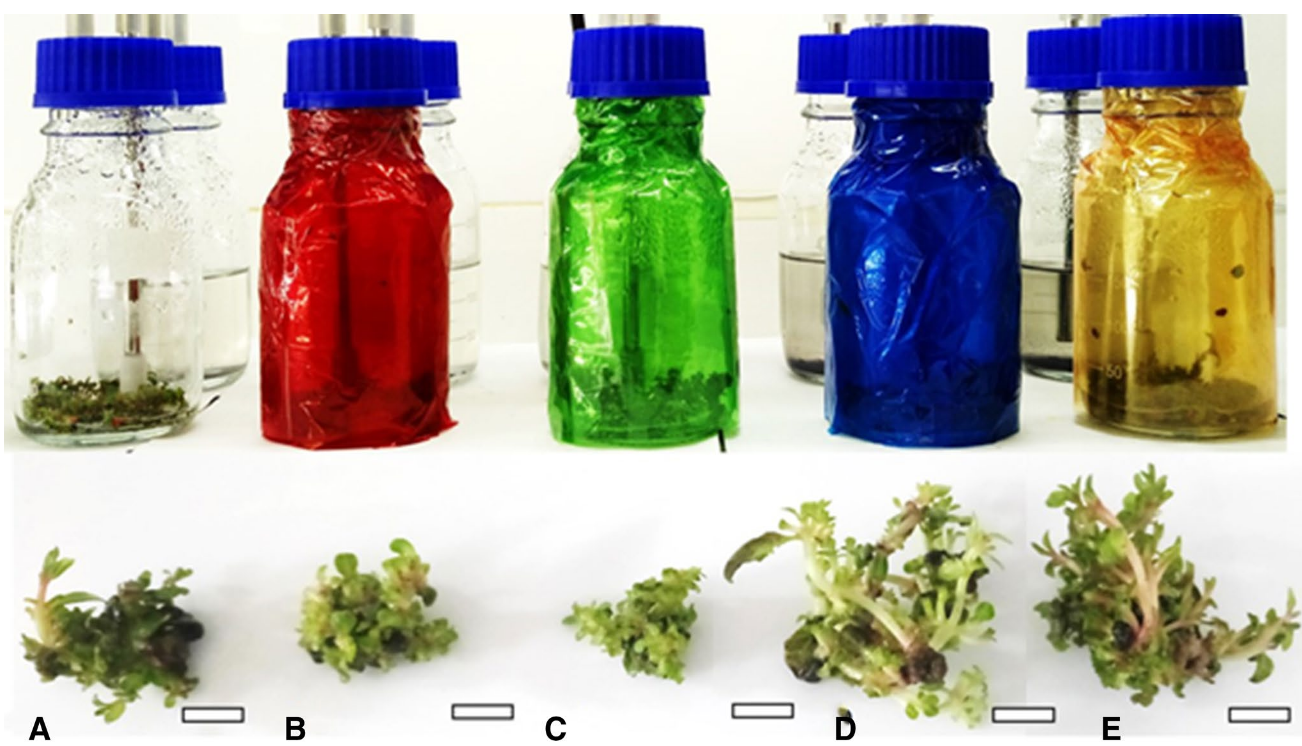

Fig. 3 Details of *E. grandis* × *E. urophylla* shoots in TIB under different spectral qualities on in vitro multiplication phase. **a** Fluorescent lamp (F/L); **b** red (R/E); **c** green (G/R); **d** blue (B/L); **e** yellow (Y/E). Bar = 0.5 cm

2017) and *Bambusa oldhamii* Munro (Silveira et al. 2020); this outcome highlights the important role played by wavelength (400–500 nm) in photomorphological responses of propagules grown in controlled environments—it appears to be a useful technology capable of optimizing shoot development and yield.

There is variation in plant morphophysiological responses to blue light (400–500 nm), such as changes in plant growth and development to allow adaptations to changes in environmental conditions (Lazzarini et al. 2017). However, plant species, such as *Rehmania glutinosa* (Gaertn) Steud. (Manivannan et al. 2015), *Plectranthus amboinicus* (Lour.) Spreng (Noguchi and Amaki 2016), *Solanum lycopersicum* L., *Cucumis sativus* L. and *Capsicum annum* L. (Snowden et al. 2016) grown under wavelengths of 400–500 nm recorded reduced growth.

With respect to NSE, there was a significant difference in shoot proliferation, depending on the adopted spectral quality. Y/E enabled higher NSE (7.70 shoots, on average) than R/E, G/R, and F/L (Fig. 4d). Data described in the literature corroborate the one found in the current study since greater peaks in the spectrum of wavelengths 450 and 600 nm induced a larger number of shoots in *Platycodon grandiflorum* (Jacq.) A. DC. (Liu et al. 2014), *Populus euramericana* Moench. (Kwon et al. 2015) and *Phalaenopsis amabilis alba* Blume (Massaro et al. 2018).

The application of white or yellow LED light to crops can help increasing plant growth and yield, since it enables deeper light penetration in leaves and maximizes photosynthetic rates, in comparison to monochromatic blue and red lights, as observed in *Lactuca sativa* hydroponics (Lin et al. 2013). These studies have shown that plants need a broad light spectrum to optimize photosynthetic processes and that this needs changes from species (Lazzarini et al. 2017). Thus, the application of yellow spectral quality to *E. grandis* × *E. urophylla* has proved to be suitable for shoot multiplication processes in large scale production systems based on TIB.

The best PPC (chlorophyll *a*, *b* and carotenoids) results were compared to explants subjected to Y/E spectral quality (Fig. 4e). Chlorophyll *a*, *b* and carotenoid biosynthesis is influenced by the quality and intensity of the emitted light;

Fig. 4 Morphological features observed in *E. grandis* × *E. urophylla* on in vitro multiplication under different spectral qualities [Fluorescent lamp (F/L), Red (R/E), Green (G/R), Blue (B/L) and Yellow (Y/E) cellophane]. **a** Scale of notes—Vigor (VIG); **b** scale of notes—Hyperhydricity (HYP); **c** shoot length (SL); **d** number of shoots per explant (NSE); **e** Photosynthetic PIGMENT contents (PPC). *Means followed by equal letters did not differ from each other, according to the Tukey's test at 5% of probability. Bars represent the standard deviation from the mean value

the best efficiency in photosynthetic processes prevails at wavelengths ranging from 450 to 600 nm (Gupta and Karmakar 2017).

In vitro multiplication of *Gerbera jamesonii* Bollus ex Hook. F. presented higher chlorophyll *a* and *b* contents under wavelength peaks at 450 and 600 nm (red/blue LEDs) than under monochromatic light sources of 450 nm (blue LEDs) and 600 nm (red LEDs) (Pawlowska et al. 2018). Carotenoid concentrations observed in *Stevia rebaudiana* (Bertoni.) shoots exposed to wavelengths of 450 nm and 600 nm (red/blue LEDs) were higher than that of shoots exposed to 450 nm (blue LEDs) (Ramírez-Mosqueda et al. 2017).

Broad light spectrum absorption promotes greater energy state (Silveira et al. 2020). The excitation energy in the chlorophyll molecule can be used in the photochemical step, be lost in the form of heat or even cause damage to the photosynthetic apparatus such as blue free radical formation (Neto et al. 2020). Thus, knowledge about the association between wavelengths and plant growth patterns on in vitro cultivation can contribute to the understanding of the micropropagation protocols to be applied to the species investigated in the present study.

The leaf anatomy (Fig. 5a–e) evidenced different responses between spectral qualities adopted on in vitro multiplication based in TIB system (Fig. 6a–e). *E. grandis* × *E. urophylla* clone subjected to treatment based on Y/E spectral quality recorded the highest ADE mean (14.64 µm), which was significantly different ($p < 0.05$) from that recorded for the R/E treatment (Fig. 5a). Schuerger et al. (1997) have found thicker leaves and a larger number of chloroplasts per cell in *Capsicum annum* plants subjected to blue LEDs than in plants treated with red LEDs in association with infrared (735 nm) and fluorescent lamp.

On the other hand, the highest ABE values were observed for plants grown under R/E spectral quality (9.94 µm, on average); they were significantly different ($p < 0.05$) from values recorded for the G/R treatment (Fig. 5b). According to Li et al. (2018), yellow and blue LEDs were more advantageous for the morphogenesis of *Solanum tuberosum* plants than green LEDs. Different spectral qualities have influenced the growth and development of plant cells, tissues, and organs, as well as triggered different morphological and anatomical responses (Macedo et al. 2011; Hogewoning et al. 2012).

Results recorded for ME and SP presented the same trend, the highest means were recorded for plants grown under spectral qualities Y/C (150.16 µm and 109.63 µm) and B/C (121.93 µm and 90.04 µm), which differed from the other treatments ($p < 0.05$) (Fig. 5c, e). Kwon et al. (2015) reported a great number of parenchymal cells undergoing cellular division process in *Populus euramericana* plants subjected to treatment with red/blue light, and it enabled greater shoot and leaf development. In addition, Macedo et al. (2011) reported that the 450 nm wavelength in the blue light treatment led to the thicker epidermis and mesophyll cells in *Alternanthera brasiliana* (L.) Kuntze on in vitro cultivation.

Changes in the structural organization of leaf cells can be observed at the anatomical level in plants exposed to different environments, including different light spectra (Fig. 6a–e). Plants subjected to broad light spectrum (450–600 nm) presented larger adaxial epidermis, palisade parenchyma, and mesophyll (Fig. 6e) dimensions, the opposite outcome was observed in plants subjected to fluorescent (Fig. 6a), red (Fig. 6b) and green (Fig. 6c) light. These results are similar to the ones reported by Miranda et al. (2020) for *Eucalyptus urophylla* explants grown under a broad light spectrum. Each spectrum is capable of changing the expression levels of specific genes involved in the structural organization of plant cells, as well as of photosynthetic pathway genes (Fan et al. 2013).

Based on the results, Y/E spectral quality was efficient in enabling the organized development of adaxial and mesophyll epidermis cells, including substantial chlorophyll concentrations in the spongy parenchyma (Fig. 6e). This anatomical development is causally linked to the more SL and NSE recorded in the current study. *E. grandis* × *E. urophylla* explants subjected to G/R treatment recorded the highest PP means (32.55 µm), although there was not significant difference between treatments ($p > 0.05$) (Fig. 5d).

Morphological and anatomical results have implications in the optimization of TIB systems used to produce clonal plants of eucalypts species, based on the micropropagation technique. Broad yellow light spectrum has proved to be viable and important for explant in vitro multiplication in controlled environments.

Conclusions

- The current study provided evidences that wavelength specificity influences the morphology and anatomy of *E. grandis* × *E. urophylla* plants grown in controlled environment; thus, it emerges as a useful technology to optimize clonal plant production at large scale.
- The shoots can be obtained in explants in a temporary immersion bioreactor (TIB) system. Yellow and blue spectral quality can be used on in vitro cultivation, without harming the development of the shoots.
- Yellow and blue spectral qualities are the most suitable to be used in *E. grandis* × *E. urophylla* on in vitro multipli-

Fig. 5 Leaf anatomy features observed in *E. grandis* × *E. urophylla* on in vitro multiplication under different spectral qualities [Fluorescent lamp (F/L), Red (R/E), Green (G/R), Blue (B/L) and Yellow (Y/E) cellophane]. **a** Adaxial Epidermis (ADE); **b** abaxial epidermis (ABE); **c** mesophyll (ME); **d** palisade parenchyma (PP); and **e** spongy parenchyma (SP) thickness. *Means followed by equal letters did not differ from each other, according to the Tukey's test at 5% of probability. Bars represent the standard deviation from the mean value

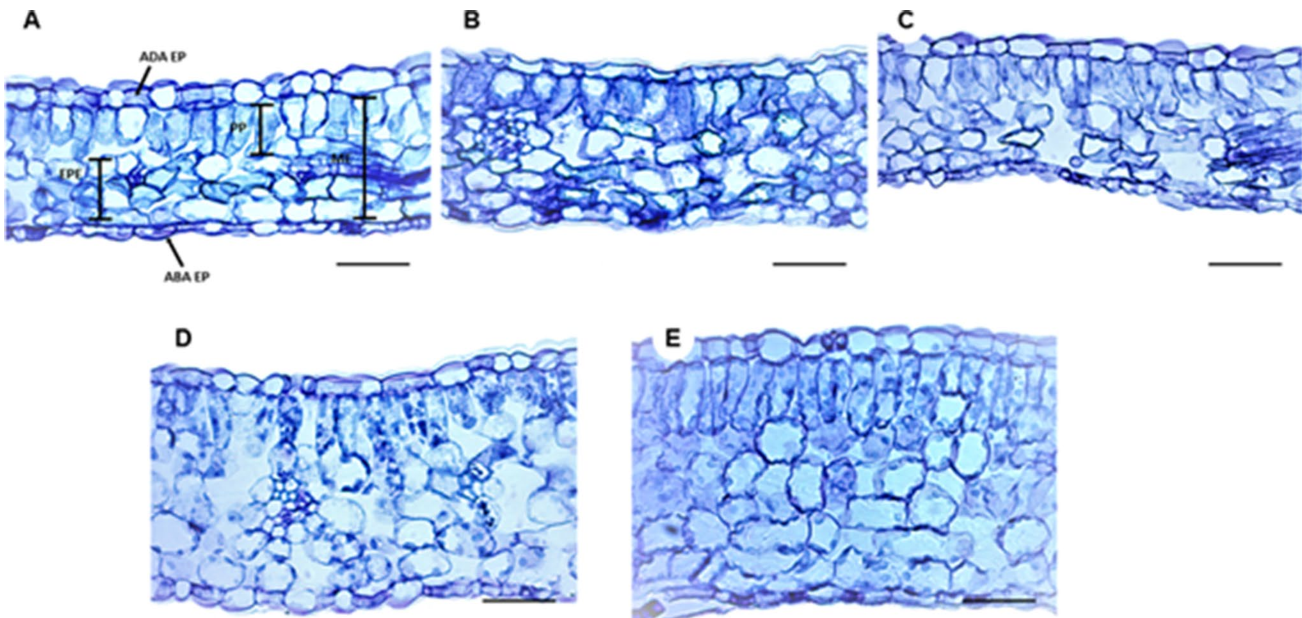

Fig. 6 Cross-sections of leaf blade observed in *E. grandis* × *E. urophylla* on in vitro multiplication under different spectral qualities. **a** Fluorescent lamp (F/L); **b** red (R/E); **c** green (G/R); **d** blue (B/L); **e** yellow (Y/E). * *ADE* adaxial epidermis; ABE abaxial epidermis; *ME* mesophyll; *PP* palisade parenchyma; *SP* spongy parenchyma. Bar = 200 µm

cation, since they enabled lesser hyperhydricity, greater number of shoots per explant, length of shoots, as well as thicker mesophyll and spongy parenchyma.

Acknowledgements We thank the National Counsel of Technological and Scientific Development (CNPq), Coordination for Improvement of Higher Education Personnel (CAPES) and Research Support Foundation of the State of Minas Gerais (FAPEMIG) for the financial support and scholarship.

Author contributions Study group: M.Sc. DMSCS—conduction of experiment, analyses, review, discussion of the theme; M.Sc. SBF—conduction of experiment, analyses, review, discussion of the theme; M.Sc. MLMA—conduction of experiment, analyses, review, discussion of the theme; M.Sc. LVM—analyses, discussion of the theme; Dr. ESVPD conduction of experiment, review, discussion of the theme; Dr. GEB—Supervisor, analyses, review, discussion of the theme.

Funding This work was carried out with the support of the Coordination for the Improvement of Higher Education Personnel—Brazil (CAPES), the National Council for Scientific and Technological Development (CNPq) and the Research Support Foundation of the State of Minas (FAPEMIG).

Availability of data and materials All data presented in this study are available for consultation.

Code availability Not applicable' for that section.

Compliance with ethical standards

Conflicts of interest/Competing interests Not applicable' for that section.

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4. CONSIDERAÇÕES FINAIS

Diante das dificuldades encontradas atualmente na propagação clonal de clones selecionados de algumas espécies e híbridos de *Eucalyptus*, o presente estudo possibilitou a otimização da produção de mudas por meio da micropropagação (clonagem), proporcionando protocolos eficientes e possíveis de serem replicados para o estabelecimento, multiplicação, alongamento e enraizamento *in vitro* e aclimatização.

Os resultados deste estudo indicam novas possibilidades para o rejuvenescimento/revigoramento de propágulos quando submetidos ao cultivo *in vitro*, com a utilização de lâmpadas LEDs vermelho/azul (qualidade de luz), intensidade luminosa 40 e 80 µmol m^{-2} s^{-1}, membranas porosas (1/M) e a tecnologia do biorreator por imersão temporária (BIT). O maior controle dos fatores ambientais, como o fotomixotrofismo (qualidade de luz, intensidade de luz e trocas gasosas) foram primordiais para obtenção de melhores resultados, quanto ao crescimento e desenvolvimento dos explantes *in vitro* de *Eucalyptus grandis* × *Eucalyptus urophylla*.

Diante desse contexto, diodos emissores de luz (LEDs) são potenciais para serem utilizados em larga escala no cultivo *in vitro*, devido à sua especificidade de comprimento de onda, largura de banda estreita, baixa quantidade de emissões térmicas, baixa degradação e longa duração, quando comparado ao sistema convencional (lâmpada fluorescente). Concomitantemente, a especificidade do comprimento de onda por meio da adequada intensidade de luz, possibilitou maximizar os resultados para todas as fases da micropropagação. De acordo as informações supracitadas, é possível inferir e recomendar essas metodologias para serem utilizadas no cultivo *in vitro* de *Eucalyptus grandis* × *Eucalyptus urophylla* para produção de mudas clonais.

Visando proporcionar um ambiente mais favorável para o desenvolvimento das plantas *in vitro* de *Eucalyptus grandis* × *Eucalyptus urophylla*, recomenda – se também a utilização de uma membrana porosa, proporcionando os melhores resultados no presente estudo, quando comparado ao sistema convencional (sem membrana). O uso da ventilação forçada proporciona redução da umidade relativa do ar no interior dos frascos de cultivo e aumento significativo da troca gasosa com a atmosfera exterior, otimizando a transpiração, absorção de água e nutrientes pela planta, podendo possibilitar uma melhor aclimatização *ex vitro.*

Além disso, observou – se que a tecnologia de biorreator por imersão temporária (BIT) possibilitou a obtenção de ganhos em biomassa na fase multiplicação *in vitro*, fornecendo condições ótimas de crescimento e alta qualidade dos explantes, resultando em maior desenvolvimento do material vegetal micropropagado. Portanto, esse sistema de cultivo também foi considerado promissor, podendo ser utilizado em biofábricas para produção de mudas em larga escala.