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ASPECTOS MORFOANATÔMICOS, ULTRAESTRUTURAIS E BIOQUÍMICOS DO PROCESSO DE ESTIOLAMENTO *IN VITRO* DE *GUADUA CHACOENSIS* (ROJAS) LONDOÑO & P.M. PETERSON

Dissertação submetido(a) ao Programa de Pós-Graduação em Recursos Genéticos Vegetais da Universidade Federal de Santa Catarina para a obtenção do Grau de mestre em Ciências, área de concentração em Recursos Genéticos Vegetais
Orientadora: Prof. Dra. Rosete Pescador
Coorientador: Prof. Dr. Hugo Pacheco de Freitas Fraga

Florianópolis
2018

Ficha de identificação da obra elaborada pelo autor
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da UFSC.

Polesi , Luiza Giacomolli
Aspectos morfoanatômicos, ultraestruturais e
bioquímicos do processo de estiolamento in vitro de
Guadua chacoensis (Rojas) Londoño & P.M. Peterson
/ Luiza Giacomolli Polesi ; orientadora, Rosete
Pescador , coorientador, Hugo Pacheco de Freitas
Fraga, 2018.
103 p.

Dissertação (mestrado) - Universidade Federal de
Santa Catarina, Centro de Ciências Agrárias,
Programa de Pós-Graduação em Recursos Genéticos
Vegetais, Florianópolis, 2018.

Inclui referências.

1. Recursos Genéticos Vegetais. 2.
Micropropagação bambu. 3. Guadua chacoensis. 4.
Estiolamento in vitro. 5. Respostas fisiológicas. I.
Pescador , Rosete . II. Pacheco de Freitas Fraga,
Hugo . III. Universidade Federal de Santa Catarina.
Programa de Pós-Graduação em Recursos Genéticos
Vegetais. IV. Título.

Aspectos morfoanatômicos, ultraestruturais e bioquímicos do processo de estiolamento *in vitro* de *Guadua chacoensis* (Rojas) Londoño & P.M. Peterson

por

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Dissertação julgada e aprovada em 20/02/2018, em sua forma final, pelo Orientador e membros da Banca Examinadora, para obtenção do título de Mestre em Ciências. Área de Concentração Recursos Genéticos Vegetais, no Programa de Pós-Graduação em Recursos Genéticos Vegetais, CCA/UFSC.

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Florianópolis, fevereiro de 2018

A minha família pelo apoio incondicional ao longo desta caminhada. Dedico.

AGRADECIMENTOS

Á Universidade Federal de Santa Catarina (UFSC) e ao programa de Pós-Graduação em Recursos Genéticos Vegetais (PPG-RGV) pela oportunidade de realização do mestrado.

À minha orientadora professora Rosete Pescador pela oportunidade de realização deste mestrado, por todos os ensinamentos transmitidos e pela confiança.

Ao meu coorientador professor Hugo Pacheco de Freitas Fraga, por todo o apoio, incentivo e ajuda na execução deste projeto, bem como por todos as respostas imediatas as minhas inúmeras perguntas e pelos conhecimentos transmitidos.

Aos membros da banca, professora Dr. Karin Esemann de Quadros, professor Dr. Paulo Tamaso Mioto e professor Dr. Miguel Pedro Guerra pela disponibilidade, leitura e contribuições no trabalho.

Aos meus amados pais João Batista e Rejane pelo apoio incondicional, todo o amor, carinho, incentivo e compreensão ao longo desta caminhada.

Aos meus queridos irmãos Carolina e Carlos Augusto por todo o amor, incentivo, companheirismo e carinho envolvidos.

Ao Marlon, por todo o incentivo, carinho, paciência, compreensão e por estar sempre ao meu lado mesmo nos momentos mais estressantes ao longo deste mestrado.

Aos amigos e colegas de trabalho Leila do Nascimento Vieira e Angelo Schuabb Heringer por todo o auxílio prestado na execução do projetos, pelas correções de textos e pelas ideias sempre muito boas.

À todos os professores do RGV que fizeram parte da minha formação pelos conhecimentos transmitidos.

Aos amigos e colegas dos laboratórios "Fitotron" e LFDGV por todos os momentos de descontração, pelas colaborações e convivência.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão da bolsa de estudos.

Ao CNPq pelo apoio financeiro para o desenvolvimento das atividades de pesquisa.

A todos os amigos, que de perto ou de longe sempre estiveram ao meu lado transmitindo boas vibrações, meu muito obrigada!

À todos o meu muito obrigada!

É preciso se expor sem medo de dar vexame. É preciso colocar o trabalho na rua. É preciso saber ouvir um não e, depois de secar as lágrimas, seguir batalhando. Arriscar é o nome do jogo. Muitos perdem, poucos ganham. Mas quem não tenta, não tem ao menos o direito de reclamar.
(Martha Medeiros)

RESUMO

Guadua chacoensis é um espécie de bambu com colmos lignificados, da família *Poaceae*, nativo do bioma Mata Atlântica, que apresenta restrições quanto a sua propagação vegetativa, sendo a propagação *in vitro* uma alternativa promissora para a obtenção de mudas desta espécie. Este processo pode ser influenciado por vários fatores, tais como a luz, e ausência dela - estiolamento, uma vez que a luz esta diretamente relacionada com a morfogênese das plantas. Diante do exposto, o objetivo deste trabalho foi avaliar a influência do estiolamento *in vitro* em plantas micropropagadas de *G.chacoensis*, por meio de análises morfoanatômicas, ultraestruturais e bioquímicas. Estas análises foram realizadas em materiais vegetais coletados aos 0, 10, 20 e 30 dias de cultivo tanto de plantas mantidas no escuro quanto de plantas mantidas sob luz do tipo LED branca (controle). Os resultados obtidos na caracterização morfológica de colmos de *G. chacoensis* demonstraram uma promoção no número de brotos gerados por explante induzida pela luz, e ainda, que a altura dos colmos não diferiu estatisticamente entre os dois tratamentos, provavelmente em função do aparecimento de características de material estiolado nos colmos verdes somente aos 20 dias de cultivo no escuro. O período de indução ao estiolamento avaliado afetou o conteúdo de poliaminas livres em colmos de *G. chacoensis*, visto que foram observadas variações nos conteúdos endógenos de poliaminas totais, putrescina, espermidina e espermina. Os conteúdos endógenos dos hormônios ácido abscíssico (ABA), giberelina (GA) e ácido jasmônico (JA) reduziram em colmos estiolados, indicando que a luz pode estar envolvida no mecanismo de síntese destes fitormônio. Por sua vez, a zeatina (Z) manteve seus conteúdos similares na luz e no escuro. Pela análise histológica, por microscopia óptica não foram observadas alterações drásticas entre os colmos mantidos na luz e escuro. Já na análise de microscopia eletrônica de transmissão (MET) foi notado um acúmulo de amido em diferentes tempos de desenvolvimento na luz e no escuro e ainda, a presença de etioplastos não foi observada. Com base nos resultados obtidos, pode-se concluir que o crescimento e desenvolvimento de plantas, bem como a biossíntese de poliaminas e hormônios, foram afetados pelo estiolamento, com reduções em suas concentrações, demonstrando a importância da luz na promoção de respostas fisiológicas *in vitro* em colmos de *G. chacoensis.*

Palavras chaves: bambu, micropropagação, poliaminas, hormônios vegetais, morfoanatomia

RESUMO EXPANDIDO

Introdução

Os bambus são pertencentes a família *Poaceae*, subfamília *Bambusoideae* e compreendem em torno de 1600 espécies distribuídas globalmente. No Brasil são encontradas cerca de 250 espécies de bambus, sendo que entre elas está o *Guadua chacoensis,* uma espécie de bambu do tipo lignificado e nativo da Mata Atlântica. Esta espécie apresenta problemas quanto a sua propagação, sendo necessário o uso de micropropagação para a obtenção de mudas. A micropropagação pode ser afetada por diversos fatores, incluindo a luz, objeto do presente estudo, que encontra-se diretamente relacionada com a morfogênese das plantas. Nas plantas a luz é percebida por fotorreceptores tais como os fitocromos. Uma vez que estes estão em sua forma ativa as diversas respostas fotomorfogênicas são geradas. Entretanto, uma vez que as plantas estão em condição de crescimento na ausência da luz, processo este conhecido como estiolamento, diversas implicações podem ocorrer em alguns processos, tendo como exemplos a diferenciação dos cloroplastos, a biossíntese de poliaminas e os teores endógenos de hormônios vegetais. Os cloroplastos fazem parte do grupo dos plastídios que são organelas relacionados com diversos processos essenciais para as plantas tais como fotossíntese, armazenamento de amido, entre outros. Todos os plastídios são gerados a partir do seu precursor proplastídios, apresentando interconversão entre os diferentes plastídios. Sabe-se que a diferenciação dos cloroplastos é um processo dependente de luz, assim, uma vez que a luz não esteja presente pode ocorre a diferenciação em etioplasto. O etioplasto é o plastídio típico do escuro, que apresenta uma estrutura única de corpo pró-lamelar conectado aos pró-tilacóides e ainda ausência de pigmentos fotossintéticos ativos, possuindo apenas precursores de clorofila e carotenóides. Como consequência, plantas estiolados apresentam caules alongados e coloração amarela a esbranquiçada. Outro processo que podem ser afetado pelo uso de estiolamento *in vitro* é a biossíntese de poliaminas. As poliaminas tais como putrescina, espermidina e espermina estão relacionadas a diferentes processos fisiológicos, tais como divisão celular, organogênese, embriogênese somática, crescimento, florescimento e senescência. Desta forma, a quantificação dos teores de poliaminas pode auxiliar no entendimento do processo de estiolamento *in vitro* de plantas micropropagadas de *G. chacoensis*. Além das poliaminas, a quantificação dos teores endógenos hormonais podem auxiliar na compreensão deste processo e possíveis implicações. Isto

porque os hormônios agem como moléculas sinalizadoras que fazem a percepção dos sinais externos e modulam a expressão gênica.

Objetivos

Este trabalho teve como objetivo geral elucidar e investigar as alterações morfoanatômicas, ultraestuturais e bioquímicas do processo de estiolamento *in vitro* em plantas pré-estabelecidas *in vitro* de *Guadua chacoensis* durante 30 dias de cultivo. Morfologicamente teve-se por objetivo avaliar a altura de colmos e o número de brotos formados em plantas submetidas ao processo de estiolamento *in vitro* e comparar com as plantas mantidas sob luz branca. Anatomicamente e ultraestruturalmente buscou-se caracterizar o processo de estiolamento *in vitro* em colmos de *G. chacoensis* por meio de microscopia óptica e microscopia eletrônica de transmissão e traçar um comparativo com plantas mantidas em iluminação branca. Ainda teve-se como objetivo quantificar e avaliar os teores de poliaminas livres e hormônios vegetais em diferentes tempos durante o estiolamento *in vitro* e comparar com o material mantido sob luz branca.

Metodologia

Colmos de *Guadua chacoensis* pré-estabelecidos *in vitro* foram individualizados e inoculados em frascos contendo 15 ml de meio de cultura MS (Murashige e Skoog) suplementado com sacarose, vitaminas de Morel, 6-benzil-amino-purina (BAP) e geleificado com Phytagel®. Cada frasco continha 5 colmos e foram utilizados dois tratamentos: indução ao estiolamento (escuro) e iluminação do tipo LED branca (controle). Para a indução ao estiolamento utilizou-se papel alumínio para simular o escuro. Estes frascos foram mantidos em sala de crescimento a temperatura de 25 ±2°C e fotoperíodo de 16 horas. Foram realizadas coletas aos 0, 10, 20 e 30 dias de cultivo para a caracterização ultraestrutural e bioquímica do processo de estiolamento *in vitro*. Os materiais para as análises bioquímicas foram coletados e imediatamente congelados em nitrogênio líquido e em seguida mantidos em ultra-freezer até a realização das quantificações de conteúdos endógenos de poliaminas e hormônios vegetais, enquanto que o material para a microscopia eletrônica de transmissão e óptica foram imediatamente colocados em tampão de fixação. Para a caracterização morfológica foram escolhidos aleatoriamente 5 frascos de cada tratamento, sendo que estes foram identificados e utilizados ao longo do tempo para as medições de altura dos colmos e contagem do número de brotos gerados. Para a extração das poliaminas livres foram utilizadas 3

repetições de 300 mg de massa fresca de cada um dos tempos para os tratamentos estiolado e luz e a quantificação foi realizada utilizando HPLC (cromatografia líquida de alta eficiência) com detector UV acoplado. Para a extração dos hormônios vegetais utilizou-se 3 repetições de 100 mg de massa fresca de cada um dos tempos nos dois tratamentos e a quantificação foi efetuada em UHPLC (cromatografia líquida de ultra eficiência) com sistema acoplado de espectrometria de massa sequencial (MS/MS). Para os dados obtidos para altura dos colmos, número de brotos, quantificação de conteúdo endógeno de poliaminas livres e quantificação dos níveis hormonais vegetais fez-se o teste da homogeneidade e uma vez homogêneos, estes dados foram submetidos a análise de variância e posterior teste de separação de médias SNK - Student-Newman-Keuls ($p \leq 0.05$).

Resultados e Discussão

Os resultados encontrados neste trabalho evidenciaram que o estiolamento *in vitro* pode provocar alterações morfológicas, anatômicos, ultraestruturais e bioquímicos em colmos de *G.chacoensis* durante 30 dias de cultivo. Analisando a caracterização morfológica observou-se que parâmetro de altura de colmos não foi influenciado pela indução ao estiolamento no período analisado neste estudo, uma vez que colmos mantidos na luz e no escuro apresentavam em média 3,8 cm aos 30 dias de cultivo. A ausência de diferenças significativas entre os tratamentos está de acordo com a ausência de plantas totalmente estiolados ao 30 dias. O número de brotos gerados foi afetado pelo uso do estiolamento, uma vez que materiais mantidos na luz apresentavam em média 4 brotos aos 30 dias, enquanto que os materiais vegetais estiolados apresentavam em média 2 brotos. Através da caracterização histológica por meio de microscopia óptica observou-se que o estiolamento não afetou a configuração interna dos colmos durante 30 dias de cultivo. Uma única diferença encontrada foi a presença de células mais lignificadas na luz do que em materiais induzidos ao estiolamento. Por sua vez, através das análises de ultraestruturalmente foram observados presença de grãos de amido na luz aos 10 dias e decréscimo destes aos 20 e 30 dias, estando isto possivelmente relacionado com o consumo destes como energia para o crescimento e desenvolvimento dos colmos. Observou-se ainda a presença do retículo periferial do cloroplasto aos 30 dias da luz, sendo que a função exata desta estrutura ainda não é conhecida. Ela pode estar relacionado com o transporte de metabólitos ou ainda com o comportamento fotossintético da espécie. Já nos materiais vegetais induzidos ao estiolamento, embora

fosse esperando a presença do etioplasto, ele não foi observado, sendo visualizados somente cloroplastos com estrutura desorganizada aos 20 e 30 dias de cultivo. Visualizou-se ainda a presença de cloroplastos em estágio de senescência aos 10 dias no escuro. Cloroplastos em senescência foram observados também na luz aos 20 dias de cultivo. Aos 30 dias de cultivo em condições de estiolamento foi observado a formação de amiloplastos - plastídios armazenadores de amido, evidenciando a ocorrência de acúmulo de amido ao longo do tempo. Este acúmulo pode estar ocorrendo como consequência da não utilização do amido como fonte de energia por materiais mantidos no escuro, sendo este resultado contrário ao que ocorre na luz. Em relação as análises bioquímicas foram observadas mudanças nos conteúdos endógenos de poliaminas e hormônios em colmo de *G. chacoensis* submetidos ao estiolamento *in vitro* quando comparados a luz branca. A quantificação de poliaminas (PAs) livres totais na luz evidenciou que o conteúdo endógeno destas PAs apresentaram um aumento na sua concentração até o dia 20 em colmos mantidos na luz, seguida de um decréscimo aos 30 dias. Para as poliaminas livres dos tipos putrescina, espermidina e espermina observou-se que, de maneira geral, os materiais mantidos na luz apresentavam maiores conteúdos endógenos. Assim, supõe-se que estes resultados podem estar relacionados a atividade dos fitocromos no processo de morfogênese *in vitro* e que a produção de poliaminas livres seria influenciada pela morfogênese *in vitro*. Isto porque em materiais mantidos na luz a atividade dos fitocromos favorece o processo de crescimento e desenvolvimento das plantas e a biossíntese de poliaminas também aumenta como resultado da morfogênese. No processo de estiolamento, a atividade dos fitocromos e as consequentes respostas morfogenéticas podem ser inibidas, ocorrendo também um decréscimo nos conteúdos endógenos de poliaminas. A quantificação hormonal revelou que o processo de estiolamento *in vitro* em colmos de *G.chacoensis* pode afetar a biossíntese de fitormônios tanto positivamente como negativamente. Para a zeatina (Z) não foram observados diferença estatística nos conteúdos endógenos de colmos mantidos na luz ou no escuro, e, ainda, a zeatina foi o hormônio mais predominante nos colmos de *G.chacoensis*. Elevados teores de zeatina podem estar correlacionados com o envolvimento deste fitormônio nos processos de maturação e desenvolvimento dos cloroplastos e ainda com a adição de citocinina ao meio de cultura. Na quantificação de ácido abscíssico (ABA) foi observado decréscimo significativo nos teores endógenos dos colmos mantidos no escuro quando comparado com os mantidos na luz. Isto foi

correlacionado com a diminuição dos conteúdos de carotenóides obtidos em matérias vegetais mantidos no escuro, uma vez que estes são os precursores da rota de biossíntese de ABA. Na quantificação de giberelina (GA) os teores obtidos para os colmos mantidos sob condição de estiolamento permaneceram inalterados ao longo do tempo, enquanto que na luz os valores foram aumentando ao longo do tempo. Sabe-se que a biossíntese de giberelina está relacionada com a resposta morfogenética, sendo portanto a o decréscimo na luz relacionada com as alterações na morfogênese no escuro. Quanto aos teores de ácido jasmônico (JA) foi demonstrado um acúmulo em colmos de *G.chacoensis* mantido na luz e um decréscimo quando mantidos no escuro aos 30 dias de cultivo, evidenciando que o estiolamento *in vitro* afeta a biossíntese deste fitormônio.

Considerações finais

O estiolamento *in vitro* afetou os parâmetros morfológicos, histológicos, ultraestruturais e bioquímicos de plantas pré-estabelecidas de *G. chacoensis.* Morfologicamente foi possível observar que plantas mantidas na luz apresentaram um metabolismo mais acelerado, resultado em maior número de brotos do que plantas mantidas no escuro, sendo este valor duas vezes maior. Histologicamente não foram observadas alterações drásticas provocadas pelo estiolamento, com exceção da presença de células mais lignificadas na luz do que em materias estiolados. A estrutura dos cloroplastos foi modificada como consequência do uso do estiolamento e não foi observada a presença de etioplasto nas análises ultraestruturais. Observou-se uma correlação positiva entre a luz, respostas morfogênicas e biossíntese de poliaminas. Ainda notou-se que a biossíntese de Z correlacionou-se positivamente com a maturação dos plastídios enquanto que a biossíntese de ABA correlacionou-se negativamente com os teores endógenos de carotenóides no escuro. A ausência de luz provocou diminuição nos teores de GA e JA. Os resultados encontrados neste trabalho trouxeram um maior entendimento a respeito dos efeitos do processo de estiolamento *in vitro* em plantas de *G. chacoensis* e evidenciaram a importância da luz na promoção de respostas fisiológicas. No entanto, em trabalhos futuros será importante deixar o material vegetal mais tempo no escuro.

Palavras-chave: *Guadua chacoensis*, micropropagação, morfogênese, microscopia, poliaminas, hormônios vegetais.

ABSTRACT

Guadua chacoensis is woody bamboo species, from the family Poaceae, native from Atlantic forest biome, which presented restrictions in it vegetative propagation, being the *in vitro* propagation a valuable tool to obtain propagative material. This process can be influenced by a variety of factors, including light or the absence of them - etiolation, as light is direct correlated with plant morphogenesis. In this sense, the objective of this study was to evaluate the influence of *in vitro* etiolation in micropropagated plants of *G. chacoensis* through morphoanatomical, ultrastrutural and biochemistry analysis. Plant material were collected at 0, 10, 20 e 30 days of culture in light and darkness treatments, and the control used was white LED light. The results obtained in morphological characterization of *G. chacoensis* culms showed an improved number of shoots generated per explant in light, and the culms height did not differs statistically between the two treatments, probably as a result of the later starting of conversion of green culms in etiolated culms, which started only at 20 days in darkness. The content of free polyamines in *G. chacoensis* culms was affected by *in vitro* etiolation during the time of evaluation, as the endogenous contents of total free polyamines, putrescine, spermidine and spermine indicated variation. Reduction in the endogenous levels of the phytohormones abscisic acid (ABA), gibberellin (GA), jasmonic acid (JA) was observed in plants kept in darkness, showing that light is involved in its biosynthesis pathways. Differently, zeatin (Z) showed similar contents in light and darkness conditions. Histological analysis through light microscopy did not shows significant alteration in culms under light and darkness conditions. Transmission electron microscopy analysis showed starch accumulation in different stages of development in light and darkness conditions, and, the presence of etioplast was not observed. Our results showed that etiolation affects plant growth and development, as well as polyamines and hormone biosynthesis, with reduction in its contents during etiolation, showing the importance of light in the promotion of *in vitro* physiological response in culms of *G. chacoensis.*

Key words: bamboo, micropropagation, polyamines, hormones, morphoanatomy

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LISTA DE ABREVIATURAS E SIGLAS

% - porcentagem
µg - microgramas
µl- microlitros
µM - micromolar
cm - centímetros
mg - miligramas
mL - mililitro
ABA - ácido absíssico
ADC - arginina descarboxilase
ANOVA - análise de variância
BAP - 6-benzilaminopurina
GA - giberelina
HPLC - cromatografia líquida de alta eficiência
JA - ácido jasmônico
LED - do inglês light emitting diodes, diodo emissor de luz
MS - meio de cultura (Murashige e Skoog, 1962)
ODC - ornitina descarboxilase
PA - poliaminas
phyA - fitocromo A
Put - putrescina
SAM - S-adenisolmetionina
SAMDC - S-adenosilmetionina descarboxilase
SNK - Student-Newman-Keuls
Spd - espermidina
Spm - espermina
Z - zeatina

SUMÁRIO

1 INTRODUÇÃO E JUSTIFICATIVA

Membros de um dos maiores grupos da família das Poaceae - gramíneas, pertencentes a subfamília Bambusoideae, os bambus apresentam em torno de 1600 espécies e 120 gêneros catalogados no mundo (Soreng *et al.,* 2015). Eles são considerados plantas de múltiplos usos em função do seu potencial econômico, social e ambiental (Akinlabi; Anane-Fenin; Akwada, 2017).

A subfamília Bambusoideae, é subdivida em três tribos: Bambuseae, Arundinarieae e Olyreae, onde as duas primeiras tribos abrangem bambus dos tipos lignificados tropicais, subtropicais e temperados, enquanto a última inclui bambus do tipo herbáceo (Soreng *et al*, 2015).

Eles podem ser encontrados em uma diversidade de habitats e possuem distribuição global, tendo como exceção somente a Antártica (Kelchner e BPG, 2013; Akinlabi; Anane-Fenin; Akwada, 2017). A Ásia é conhecida por ser o maior centro de diversidade de espécies de bambus, sendo a América do Sul o segundo maior, com um endemismo de mais de 40% das espécies de bambus catalogadas no mundo (Kelchner e BPG, 2013).

No Brasil, atualmente, são catalogadas 256 espécies nativas de bambus, das quais 163 espécies são do tipo lignificados, e 93 são herbáceos (Greco *et al.,* 2015). Eles estão localizados principalmente nas regiões remanescentes do bioma Mata Atlântica, onde são encontradas aproximadamente 60% das espécies nativas do país (Filgueiras e Gonçalves, 2004). Dentre estas espécies encontra-se o *Guadua chacoensis*, um bambu do tipo lenhoso que apresenta potencial de uso na construção civil e no artesanato (Akinlabi; Anane-Fenin; Akwada, 2017). Sabe-se, porém, que embora a Mata Atlântica abranja a maior biodiversidade do pais, as perdas sofridas nos últimos anos em função dos desmatamentos demonstram a necessidade de se buscar formas de conservação das diversas espécies que nela habitam, incluindo os bambus.

Métodos de propagação convencional de bambus apresentam uma diversidade de problemas que os tornam inviáveis para a obtenção de mudas em grandes quantidades. Eles apresentam ciclos de produção de sementes bastante variados, sendo de aproximadamente 27 anos para *G. chacoensis*, por exemplo, e ainda estas sementes são de baixa viabilidade (Sandhu; Wani; Jiménez, 2017). Além disso, métodos de propagação vegetativa são trabalhosos, de baixo rendimento e podem

levar à degradação total ou parcial das touceiras (Azzini e Salgado 1993).

Frente a isso, técnicas biotecnológicas, tais como a micropropagação, vem sendo utilizada na propagação de bambus. A organogênese corresponde à rota mais utilizada na micropropagação de bambus, com diversos protocolos já estabelecidos, para diferentes espécies (Singh *et al*., 2013). A micropropagação pode ser afetada por diversos fatores, sendo a luz um dos principais, visto que esta atua diretamente no processo de morfogênese das plantas, afetando assim seu crescimento e desenvolvimento. A técnica de estiolamento *in vitro*, ou crescimento no escuro, tem auxiliado na micropropagação de outras monocotiledôneas. Entretanto, pouco se sabe a respeito do comportamento dos bambus e os potenciais efeitos causados pelo uso de estiolamento *in vitro*, evidenciando a importância de estudos na área.

No Brasil, os bambus vêm ganhando seu espaço em diversos setores devido ao imenso potencial e devido ao aumento dos estudos relacionados a este grupo de plantas. O projeto intitulado "Tecnologias para o desenvolvimento sustentável da cadeia produtiva de Bambu no sul do Brasil" (Chamada MCTI/Ação Transversal/CNPq N.º 66/2013), que está sendo desenvolvido junto a Universidade Federal de Santa Catarina no Laboratório de Fisiologia do Desenvolvimento e Genética Vegetal sob coordenação do Prof. Miguel Pedro Guerra, tem por objetivos gerais identificar e superar os gargalos científicos e tecnológicos na cadeia produtiva de bambu. Dentre os 10 subprojetos propostos neste grande projeto, esta dissertação encontra-se inserida no subprojeto 2 denominado "Macro e micropropagação e conservação *in vitro* de germoplasma de bambu", que tem por objetivo realizar estudos relacionados a micropropagação de espécies pré-estabelecidas, incluindo *Guadua chacoensis*.

Desta forma, esta dissertação teve como objetivo a realização de estudos relacionados à técnica de estiolamento de plantas pré-estabelecidas *in vitro* de *G.chacoensis* e comparar com plantas mantidas sob iluminação do tipo LED branca por meio de análises morfoanatômicas, ultraestruturais e bioquímicas, que correspondem as técnicas de microscopia ótica e eletrônica de transmissão, quantificação de poliaminas livres e de hormônios vegetais. Esta dissertação esta subdivida em três seções, as quais correspondem respectivamente a uma revisão bibliográfica seguida de dois capítulos, sendo que o primeiro dele se refere à análise morfológica, a quantificação de poliaminas e microscopia ótica e o segundo à quantificação hormonal e microscopia eletrônica de transmissão.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Investigar e elucidar as alterações morfoanatômicas, ultraestruturais e bioquímicas de plantas pré-estabelecidas *in vitro* de *Guadua chacoensis* durante o processo de estiolamento.

2.2 OBJETIVOS ESPECÍFICOS

- Avaliar os parâmetros morfológicos de altura da parte aérea e o número de brotos formados em plantas *in vitro* de *G. chacoensis* durante o processo de estiolamento e comparar com plantas mantidas sob iluminação branca.
- Quantificar e analisar os conteúdos de poliaminas livres e hormônios vegetais em diferentes tempos durante o estiolamento *in vitro* de *G. chacoensis* e traçar um comparativo com plantas mantidas sob iluminação branca.
- Analisar anatomicamente e ultraestruturalmente o desenvolvimento de plantas *in vitro* de *G. chacoensis* submetidas ao estiolamento e traçar um comparativo com plantas mantidas sob iluminação branca.

3 REVISÃO BIBLIOGRÁFICA

3.1 OS BAMBUS, SUAS CARACTERÍSTICAS E DISTRIBUIÇÃO.

Conhecidos como plantas de múltiplos usos, os bambus têm chamado a atenção devido ao seu potencial de uso econômico, ambiental e social (Akinlabi; Anane-Fenin; Akwada, 2017). Os bambus vêm sendo apontados como uma alternativa sustentável de substituição da madeira, pois estes apresentam colmos lignificados e crescimento rápido (Clark; Londoño; Ruiz-Sanchez; 2015). Ainda, economicamente, eles podem ser utilizados como matéria prima para a produção de carvão, para a indústria do papel, para a construção civil, como fonte de fibras, na produção de alimentos, para ornamentação, entre outros (Singh *et al*., 2013). Além disso, os bambus apresentam benefícios ecológicos ou ambientais, visto que podem ser utilizados na contenção de erosões, na fixação de carbono, na conservação do solo e da água e na reabilitação de áreas degradadas (Song *et al*., 2011).

Os bambus pertencem a um dos 12 grupos de gramíneas, sendo um dos maiores da família Poaceae, apresentando 1641 espécies e 120 gêneros catalogados no mundo (Soreng *et al*, 2015). São angiospermas, monocotiledôneas, de crescimento rápido (Akinlabi; Anane-Fenin; Akwada; 2017) e ciclo de vida longo (Singh *et al*., 2012).

Membros da subfamília Bambusoideae, os bambus podem ser divididos com base em suas características moleculares e morfológicas em três tribos: Arundinarieae, Bambuseae e Olyrea (Sungkaew *et al.*, 2009; Kelchner e BPG, 2013; Soreng *et al*., 2015). A tribo Bambuseae, dos bambus lenhosos tropicais e subtropicais, possui cerca de 893 espécies em 68 gêneros catalogados (Soreng *et al*., 2015), incluindo as subtribos *Guaduinae* e *Chusqueinae* (Kelchner e BPG, 2013). Já a tribo Arundinarieae, dos bambus lignificados temperados, possui cerca de 621 espécies distribuídas em 31 gêneros, enquanto na tribo Olyreae, dos bambus herbáceos, são incluídas 127 espécies distribuídas em 21 gêneros (Soreng *et al.,* 2015).

Bambus do tipos lignificados e herbáceos apresentam diferenças quanto à sua altura, número de ramificações, tipo de colmos, comportamento de florescimento e tolerância ao sol. Os bambus do tipo lignificados medem entre 1,0 e 35,0 metros, enquanto os herbáceos medem menos de 2,0 metros, e em geral, os primeiros apresentam ramificações complexas, enquanto os segundos apresentam ramificações simples (Filgueira e Gonçalves, 2004). Ainda, bambus do tipo lignificados apresentam colmos lignificados e florescimento sazonal ou

monocárpico, que ocorrem em geral entre 7 e 120 anos e que apesar de gerarem um número grande de sementes, podem levar a morte da planta parental (Kelchner e BPG, 2013). Por outro lado, bambus do tipo herbáceos apresentam colmos pouco lignificados e florescimento anual, que pode se estender por meses, sendo classificados como policárpico (Clark; Londoño; Ruiz-Sanchez; 2015).

Outra maneira de classificação dos bambus é quanto à forma de crescimento de seus rizomas como entouceirantes ou alastrantes. Nos entouceirantes, ou de rizoma do tipo paquimorfo ou ainda simpodial, novos rizomas são produzidos horizontalmente na base de colmos pré existentes e formam novos brotos, que crescem de maneira compacta e formam touceira (Banik, 2015). Bambus com este tipo de rizomas se desenvolvem bem em regiões tropicais, como por exemplo, as espécies *Dendrocalamus asper* e *Bambusa vulgaris* (Tombolato *et al*., 2012). Já nos alastrantes, de rizomas do tipo leptomorfo ou monopodial, os rizomas são alongados e finos, e novos colmos originam-se de brotações laterais advindas de um colmo principal (Banik, 2015). Eles se desenvolvem melhor nas regiões de clima temperado, como por exemplo as espécies *Phyllostachys edulis* e *P. aurea* (Tombolato *et al*., 2012).

Os bambus encontram-se distribuídos globalmente nos continentes Americano, Africano e Asiático, e ainda, recentemente algumas espécies foram introduzidas no Europeu (Akinlabi; Anane-Fenin; Akwada, 2017), sendo assim a única exceção o continente Antártico (Fig. 1) (Sungkaew *et al.,* 2009; Kelchner e BPG, 2013). Eles estão presentes desde o nível do mar até acima de 4.000 metros de altitude (Sungkaew *et al.,* 2009), em solos variados - com exceção dos solos alcalinos, de deserto e pântano (Akinlabi; Anane-Fenin; Akwada, 2017) e sua distribuição está entre 51°N latitudinal no Japão até 47°S latitudinal no sul da Argentina (Yeasmin *et al.,* 2015).

Eles encontram-se em uma variedade de hábitats, incluindo florestas de planícies tropicais, temperadas deciduais, montanhosas, úmidas e de coníferas, desde as áreas de clima temperado até de clima tropical (Akinlabi; Anane-Fenin; Akwada, 2017). Em geral, os bambus apresentam-se como dominantes ou em grande número no meio da floresta, sendo muitas vezes conhecidos como gramíneas de floresta (BPG, 2012; Clark; Londoño; Ruiz-Sanchez; 2015).

Embora os bambus sejam cosmopolitas, a distribuição das espécies não ocorre de maneira igual. O continente Asiático apresentam-se como o maior detentor de riqueza de espécies (somente na China são encontradas cerca de 626 espécies, enquanto a América do Sul, é a

segunda no ranking de maior detentora de riqueza de espécies, sendo Brasil, Venezuela e Colômbia os países que apresentam os maiores números de espécies em ordem decrescente (Bystriakova; Kapos; Lysenko; 2004). Ainda, de acordo com Kelchner e BPG (2013), 40% das espécies de bambus conhecidas são endêmicas das Américas.

Figura 1 - Distribuição mundial dos bambus representados na figura pela coloração lilás - adaptado de Kelchner e BPG (2013).

No Brasil já foram descritas cerca de 256 espécies nativas de bambus, das quais 163 espécies são da tribo Bambuseae - bambus lignificados, e 93 são da tribo Olyreae - bambus herbáceos (Greco *et al.,* 2015) (Fig. 2). Estas espécies encontram-se distribuídas em 34 gêneros, sendo 17 gêneros de cada uma das tribos e ainda, de todas as espécies brasileiras, 176 são consideradas endêmicas, sendo a grande maioria pertencentes à tribo *Bambuseae* (76% do total). Ainda, cerca de 60% destas espécies estão no bioma Mata Atlântica, seguido da Amazônia e do Cerrado (Filgueira e Gonçalves, 2004).

A maior diversidade de espécies de bambus no Brasil encontra-se no Sudeste - abrangendo cerca de 46,9% do total ou 120 espécies, seguida de Norte, Nordeste, Sul e Centro- Oeste. Já em relação a distribuição destas espécies em gêneros, observa-se que o maior número de gêneros está na região Nordeste, seguida de Norte, Sudeste, Sul e Centro-Oeste (Greco *et al.,* 2015).

O sul do Brasil possui cerca de 63 espécies, sendo 8 da tribo Olyreae e 54 da tribo Bambuseae e estas abrangem aproximadamente 38,2% do total de gêneros de bambus encontrados no país, comprimindo 13 gêneros. Destas 63 espécies, 45 são consideradas como endêmicas (44 da tribo Bambuseae e uma da tribo Olyreae) o que corresponde a

25,5% do total. Em Santa Catarina foram catalogadas 44 espécies, das quais 40 pertencem à tribo Bambuseae, sendo 36 destas endêmicas, e 4 da tribo Olyreae, sendo uma endêmica (Greco *et al.,* 2015).

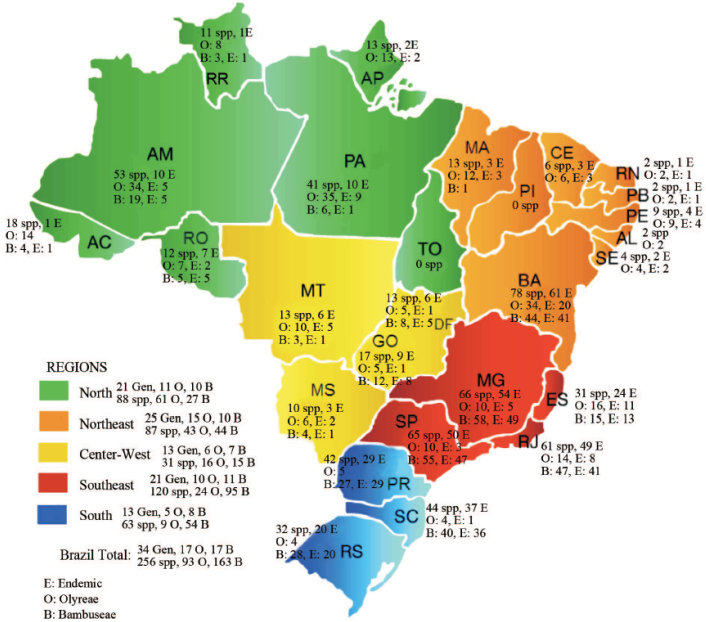

Figura 2 - Distribuição dos bambus nos estados e regiões brasileiras. Fonte: Adaptado de Greco *et al.* (2015).

3.1.1 *Guadua chacoensis*

O gênero *Guadua* compreende aproximadamente 38 espécies de bambus lignificados que se desenvolvem bem em regiões tropicais, tais como América do Sul e Central e abrangem os maiores bambus da América tropical (Akinlabi; Anane-Fenin; Akwada, 2017), sendo também o gênero de maior importância econômica direta das Américas (Clark; Londoño; Ruiz-Sanches, 2015). Os maiores centros de diversidade deste gênero estão no México e América Central, Amazônia, Planalto Central Brasileiro e costa da Bahia (Judziewich e Clark, 2007).

Este gênero corresponde ainda a um dos cinco de maior abrangência de espécies do Brasil. Segundo Filgueiras e Gonçalves (2004), 62,9% das espécies de bambus catalogadas no país são

pertencentes aos gêneros *Merostachys* (23%), *Chusquea* (17%), *Olyra* (7,8%), *Pariana* (7,8%) e *Guadua* (7%).

Espécies pertencentes a este gênero podem atingir 30,0 m de altura e 20,0 cm de diâmetro e são encontradas principalmente na América do Sul e Central (Akinlabi; Anane-Fenin; Akwada, 2017), desde o nível do mar a 2200,0 m de altitude (Judziewich e Clark, 2007). Por apresentarem colmos grandes, bambus deste gênero são comumente utilizados na construção civil e na indústria, mas também no artesanato (Chaowana, 2013).

Dentro do gênero Guadua, destaca-se a espécie *Guadua chacoensis* (Rojas) Londoño & P.M. Peterson (Fig. 3), popularmente conhecido pelo nome de taquaruçu (Bencke *et al.,* 2008) corresponde a um bambu lignificado, nativo da mata Atlântica, que se distribui pelo Brasil, Argentina, Paraguai e Uruguai (Guerreiro, 2014). No Brasil, encontra-se distribuído nos 3 estados do da Região Sul, na Região Sudeste, no estado de São Paulo e na Região Centro Oeste, no Mato Grosso do Sul (Jardim Botânico do Rio de Janeiro, 2017).

G. chacoensis apresenta colmos robustos que chegam a atingir mais de 20,0 m de altura e 18,0 cm de diâmetro. Seus colmos apresentam espinhos (Fig. 3c), suas folhas possuem formato triangular e faixas esbranquiçadas ocorrem no colmo na região dos nós (Fig. 3a). Em geral, estes bambus crescem em florestas tropicais, muitas vezes próximos de rios, e o ciclo de floração é estimado entre 27-28 anos (Vega e Hernandez, 2008). Seus principais usos incluem artesanato e construção civil (Akinlabi; Anane-Fenin; Akwada, 2017).

Figura 3 - *Guadua chacoensis*. a) vista geral da planta; b) destaque para a altura dos colmos; c) detalhe dos espinhos nos colmos, indicados pela seta.
Fonte: Autora.

3.2 PROPAGAÇÃO DE BAMBUS

Métodos tradicionais de propagação de bambus, tais como via sementes ou vegetativa, têm se mostrado bastante ineficientes e insuficientes na obtenção de mudas de bambus (Singh *et al.,* 2013).

A propagação via sementes ou sexuada apresenta como limitações: a) floração esporádica e com grandes intervalos entre os ciclos de produção de sementes (Bystriakova *et al*., 2003), visto que em bambus lignificados, por exemplo, esta produção pode ocorrer entre 7 e 120 anos (Kelchner e BPG, 2013); b) recalcitrância e viabilidade baixa, variando entre 3 e 6 meses (Sandhu; Wani; Jiménez, 2017); c) consumo destas sementes por animais selvagens, tais como roedores e pássaros (Saxena, 1990; Sandhu; Wani; Jiménez, 2017), d) comportamento monocárpico de algumas espécies de bambus, nas quais o florescimento

é seguido pela morte da planta (Singh *et al.,* 2013) e ainda e) uniformidade genética incerta (Sandhu; Wani; Jiménez; 2017).

A propagação vegetativa, por meio de subdivisão de touceiras, plantio de colmos, corte de ramos e macropropagação (Singh *et al.,* 2012) apresentam como limitações do método: a) baixo rendimento; b) alta exigência de mão-de-obra; c) destruição total ou parcial da planta matriz (Azzini e Salgado, 1993); d) dificuldade de transporte, visto que em sua maioria são volumosos e grandes (Saxena, 1990); e) coleta do material dependente do estágio e época do ano, que são em geral restritos (Sandhu; Wani; Jiménez, 2017).

Frente a isto, a cultura de tecidos, ou micropropagação, vem sendo vista como uma alternativa para a propagação de bambus, que pode contornar as limitações apresentadas pelos métodos tradicionais (Singh *et al.,* 2013) e permitir que seja atendida a demanda de produção de mudas de bambus (Goyal e Sen, 2016). Através desta técnica obtêm-se uma rápida multiplicação, visto que é gerados um grande número de plantas em um tempo reduzido, e a conservação de germoplasma, bem como a disponibilidade de material são assegurados (Mudoi *et al.,* 2013).

Embora os primeiros relatos de micropropagação de bambus datam em 1968, quando Alexander e Rao obtiveram a primeira germinação *in vitro* de sementes de *Dendrocalamus strictus*, a atenção dos pesquisadores tem se voltado para estudos nestas espécies somente nos últimos anos (Mudoi *et al.,* 2013).

Duas rotas morfogenéticas distintas podem ser utilizadas na micropropagação de bambus: embriogênese somática e a organogênese. A embriogênese somática (ES) é o processo no qual uma ou várias células somáticas se diferenciam em embriões somáticos, sendo que estes embriões apresentam estrutura análoga aos embriões zigóticos, com eixos bipolares que não exibem conexões vasculares com o tecido parental (Von Arnold *et al*., 2002). Este processo pode ocorrer de maneira direta ou indireta, sendo que na embriogênese direta, o embrião somático é originado diretamente do explante, e na indireta estes se desenvolvem após uma fase intermediária de calos (Lin; Huang; Fang, 2012).

Por sua vez, a organogênese, corresponde à rota de propagação em que os explantes são induzidos a originarem uma estrutura unipolar, sendo gerados brotos ou raízes, de acordo com os estímulos dados. O uso de brotos maduros, juvenis e axilares, como explantes para a organogênese de bambus, tem se mostrado bastante eficientes, sendo atualmente gerados protocolos para cerca de 54 espécies (Singh *et al.,*

2013). Tendo em vista algumas vantagens apresentadas por materiais micropropagados a partir de brotos, tais como maior estabilidade genética e consequente menor variação somaclonal, a organogênese tem se mostrado a via mais promissora para a micropropagação de bambus (Singh *et al.,* 2013).

3.3 O ESTIOLAMENTO E POSSÍVEIS IMPLICAÇÕES

O sucesso da micropropagação é dependente de uma variedade de fatores que incluem seleção de explante, composição do meio de cultura - fitorregulares, fonte de carbono, pH, tempo de subcultivos e condições de crescimento e desenvolvimento oferecidos, incluindo temperatura e qualidade e intensidade luminosa (Singh *et al.,* 2013; Goyal e Sen, 2016).

Para os bambus, a formulação salina MS - Murashige e Skoog (Murashige and Skoog 1962), suplementada com fonte de carbono, tem sido amplamente utilizada e vem apresentando resultados satisfatórios (Singh *et al.,* 2013). Além disso, sabe-se que os fitorregulares mais utilizados na micropropagação de bambus são as citocininas, tais como 6-benzilaminopurina e benzil adenina, e as auxinas, incluindo ácido indolacético, ácido naftaleno acético e 2,4 ácido diclorofenoxiacético (Goyal e Sen, 2016). Contudo, em relação às condições de cultivo, sobretudo relacionadas à luz, diversas são as áreas a serem exploradas.

A luz está diretamente relacionada com o desenvolvimento e crescimento de plantas, de forma que a morfogênese *in vitro* é dependente do comprimento de onda (qualidade), fotoperíodo e intensidade luminosa. Durante o crescimento das plantas, tanto o uso de diferentes espectros de luz, como a ausência de luz - processo este conhecido como estiolamento (George; Hall; De Klerk, 2008), podem influenciar na organogênese e a proliferação de brotos e raízes em cultivos *in vitro* (Moshe e Dalia, 2007).

A percepção da luz pelas plantas ocorre por meio da ação de diversos fotorreceptores, tais como criptocromos, fototropinas e fitocromos (Briggs e Olney, 2001). Os criptocromos são responsáveis por mediar as respostas à luz azul e radiação UVA, enquanto que as fototropinas atuam em respostas da luz azul, radiação UVA e verde (Casal, 2000). Já os fitocromos medeiam as respostas a luz vermelha e vermelha distante, e possuem duas formas fotoreversíveis: Pfr (vermelho-distante) e Pf (vermelho), sendo a primeira delas a forma fisiologicamente ativa. A família dos fitocromos é composta por 5 genes: phy A- E, sendo que o phy A é o que apresenta maior relação

com estiolamento, uma vez que este fitocromo é o mais abundante no crescimento do escuro de sementes (Li *et al.,* 2011).

A ação destes fotorreceptores desencadeia as diversas respostas fotomorfogênicas, por meio da regulação da expressão de genes envolvidos neste processo (Quail *et al.,* 2002). Por outro lado, quando as plantas estão em condições de ausência de luz, os fitocromos são convertidos em sua forma biologicamente inativa e sob estas condições pode ocorrer a ativação de repressores, que bloqueiam as respostas fotomorfogênicas (Xu *et al.,* 2015).

Plantas que crescem no escuro em geral apresentam características típicas de plantas estioladas, tais como a coloração amarela a esbranquiçada, devido à presença de aparatos fotossintéticos não funcionais, e o alongamento da parte aérea (George; Hall; De Klerk, 2008). Em plantas estioladas, a diferenciação de proplastídios em cloroplastos pode ser comprometida, visto que este processo é considerado dependente de luz, podendo assim ocorrer a diferenciação em etioplastos ao invés de cloroplastos (Staehelin, 2005). Uma vez expostas à luz, plantas estioladas passam rapidamente a responder aos estímulos gerados por ela, e os etioplastos são convertidos a cloroplastos (Wise, 2007), permitindo assim que estas plantas voltem a se desenvolver normalmente.

A diferenciação dos cloroplasto, a ação de fitocromos nas respostas fotomorfogênicas e a biossíntese de poliaminas e hormônios vegetais podem ser influenciados pelo uso do estiolamento *in vitro*. Esta técnica pode ser utilizada como objeto de estudo da influência direta ou indireta da luz, nestes processos e serão analisadas em maiores detalhes.

3.3.1 Diferenciação dos cloroplastos

Os plastídios correspondem a um grupo de organelas envolvidas em processos fundamentais do metabolismo das plantas tais como fotossíntese, síntese de lipídeos e amido e assimilação de nutrientes (Wise, 2007). As formas mais conhecidas de plastídios incluem proplastídios, cloroplastos, etioplasto, gerantoplasto, cromoplasto e leucoplastos - incluindo amiloplastos e estes são conhecidos pela sua capacidade de interconversão entre estas diversas formas (Solymosi e Keresztes 2012; Lindquist; Solymosi; Aronsson, 2016).

Os proplastídios correspondem a forma não diferenciada da qual os demais plastídios são gerados e estão presentes em tecidos meristemáticos, reprodutivos e desdiferenciados (Lindquist; Solymosi; Aronsson, 2016). A diferenciação de proplastídios nas demais formas

acontece de acordo com os estímulos recebidos. Por exemplo, proplastídios se diferenciam em cloroplastos quando na presença da luz, entretanto, se a luz estiver ausente, estes podem se diferenciar em etioplastos (Staehelin, 2005).

Os cloroplastos são responsáveis pelas reações da fotossíntese e correspondem ao tipo de plastídio do qual se tem maior conhecimento de suas características, tanto bioquímicas como fisiológicas (Solymosi e Aronsson, 2013). Estas organelas possuem aproximadamente de 5-10 μm de diâmetro e são delimitadas por uma dupla membrana (Lindquist; Solymosi; Aronsson; 2016), que age no transporte de metabólitos, proteínas e lipídeos para o exterior dos cloroplastos (Staehelin, 2005). No interior do cloroplastos está o estroma, uma matriz aquosa contendo ribossomos, grãos de amido, plastoglobulos e tilacóides (Staehelin, 2005). Os tilacóides são as membranas nas quais ocorrem as reações fotossintéticas e em geral são encontradas em pilhas, denominadas *grana* que estão interconectadas entre si (Solymosi e Keresztes, 2012). Os plastoglóbulos correspondem a corpos lipídicos que podem ser encontrados próximos ou conectados às membranas dos tilacóides (Bréhelin; Kessler; Wijk, 2007), sendo que a quantidade presente nos cloroplastos varia de acordo com o estágio de desenvolvimento (Austin *et al.,* 2006).

Os etioplastos correspondem aos plastídios que podem ser formados durante o desenvolvimento no escuro (Wise, 2007). Eles apresentam características únicas, como a presença de um corpo pró-lamelar (PLB), que encontra-se conectado às membranas pro-tilacóides, onde estão localizados os precursores de clorofilas e carotenóides (Solymosi e Keresztes, 2012). Etioplastos possuem aproximadamente 1-5 μm de diâmetro e podem conter plastoglóbulos e grãos de amido em seu interior (Solymosi e Aronsson, 2013).

A diferenciação dos cloroplastos, por ser um processo totalmente dependente de luz, pode servir de ferramenta para os estudos das respostas de morfogênese de plantas induzidas a condição de estiolamento, bem como revelar as respostas resultantes deste processo de estiolamento na interconversão dos diferentes tipos de plastídios.

3.3.2 Biossíntese de poliaminas

As poliaminas (PA) correspondem a pequenas moléculas alifáticas de amina, tais como putrescina (Put), espermidina (Spd) e espermina (Spm) (Bouchereau *et al.,* 1999). Elas são equivalentes respectivamente a diamina, triamina e tetraamina, sendo estas as formas

de poliaminas mais encontradas em plantas (Tiburcio *et al.,* 2014). Elas estão presentes tanto na forma livre como na conjugada, sendo esta última especialmente com compostos fenólicos (Evans e Malmberg, 1989).

Estas moléculas estão frequentemente associadas com diversos processos fisiológicos que afetam o crescimento e desenvolvimento de plantas, incluindo divisão celular, embriogênese, organogênese, enraizamento, crescimento do tubo polínico, florescimento, senescência, bem como nas respostas a estresses biótico e abiótico (Evans e Malmberg, 1989; Tiburcio *et al.,* 2014).

A biossíntese das poliaminas em plantas pode ocorrer por meio de duas rotas diferentes: ODC - ornitina descarboxilase e ADC - arginina descarboxilase (Alcázar *et al.,* 2006) (Fig.4). A rota ODC é conhecida como rota direta, visto que a produção de putrescina ocorre diretamente ela ação da enzima ornitina descarboxilase, enquanto que a ADC é conhecida como indireta, já que a transformação da arginina em putrescina é dependente da ação de arginina descarboxilase e mais duas enzimas (Alcázar *et al*., 2006; Baron e Stasolla, 2008).

A biossíntese de espermidina e de espermina se dá a partir da formação da putrescina (Fig.4). A adição de um grupo aminopropil à putrescina por meio da enzima espermidina sintase resultará na produção de espermidina. Já a adição subsequente de um grupo aminopropil pela espermina sintase à uma molécula de espermidina previamente formada, resultará na produção de uma espermina. Estes grupos aminopropil são gerados a partir da S-adenosilmetionina (SAM) pela ação da enzima S-adenosilmetionina descarboxilase (SAMDC). Além da SAM participar da biossíntese das poliaminas, gerando novos grupos aminopropil, ela participa da rota de biossíntese de etileno (Alcazar *et al.,* 2006), podendo assim a biossíntese de um afetar na biossíntese do outro.

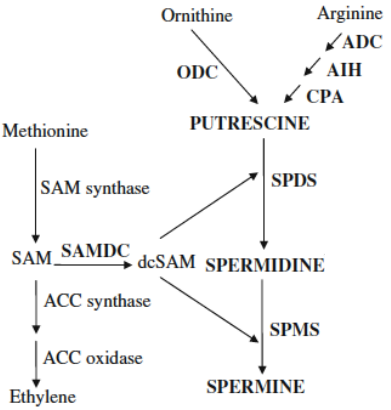

Figura 4 - Esquema simplificado das rota ADC e ODC de biossíntese de poliaminas. Fonte: Adaptado de Alcázar *et al.* (2006).

Devido ao seu envolvimento em diversos eventos de desenvolvimento das plantas, algumas vezes as poliaminas são classificadas como hormônios vegetais. Entretanto, estas não podem ser classificadas desta forma, pois a quantidade de poliaminas necessárias para efeitos biológicos é bem maior que a quantidade de hormônio necessária (Evans e Malmberg, 1989; Kerbauy, 2004). Enquanto as poliaminas são encontradas nas plantas em concentrações na ordem de milimolar, os hormônios são em níveis de micromolar (Evans e Malmberg, 1989).

Poliaminas estão ainda diretamente relacionadas com respostas aos estresses bióticos e abióticos, conforme demonstrado em diversos estudos (Hussain *et al.,* 2011; Tiburcio *et al.,* 2014; Minocha; Majumdar; Minocha; 2014). Entretanto, nenhum destes trabalhos relata o uso do estiolamento, evidenciado a necessidade de um maior entendimento deste processo.

Diante do envolvimento das poliaminas em processos de crescimento e desenvolvimento de plantas e resposta ao estresse, a quantificação de poliaminas de plantas micropropagadas de *G. chacoensis* submetidas ao estiolamento pode evidenciar uma influência das poliaminas também neste processo.

3.3.3 Teores endógenos de hormônios vegetais

As plantas, por serem organismos sésseis, precisam se adaptar rapidamente, tanto em niveis fisiológicos como moleculares, às várias alterações impostas pelo ambiente para se manterem vivas (Pandey *et al.,* 2017). Os hormônios vegetais têm sido considerados chave no processo de adaptação das plantas às diversas flutuações ambientais, visto que estes fazem a integração dos sinais externos com os sinais internos das plantas (Stamm e Kumar, 2010) e assim, coordenam seu desenvolvimento (Wolters e Jürgens, 2009).

Os hormônios vegetais ou fitohormônios são biomoléculas de baixo peso molecular, que agem em baixas concentrações nas plantas, funcionando como moléculas sinalizadoras (Wolters e Jürgens 2009; Erland *et al.,* 2017). Entre os principais hormônios vegetais incluem-se as auxinas, citocininas, giberelinas, brassinosteróides, ácido abcísico, ácido jasmônico e etileno (Wolters e Jürgens, 2009; Stamm e Kumar, 2010).

Por serem moléculas sinalizadoras, a atuação dos hormônios ocorre por meio da percepção dos sinais externos, seguida da modulação da expressão gênica (Kohli *et al.,* 2013). Eles atuam nos processos de crescimento, desenvolvimento e adaptação, bem como nos processos fisiológicos e em resposta a estresse (Pandey *et al.,* 2017; Erland *et al.,* 2017).

Alterações nos teores hormonais podem ser percebidos como resposta ao estresse em algumas plantas, mas, esta resposta é variável de acordo com o condição de estresse e o genótipo (Kohli *et al.,* 2013). Desta forma, a quantificação hormonal de plantas micropropagadas de *G. chacoensis* submetidas ao estiolamento podem ser uma informação importante sobre este processo.

3.4 REFERENCIAS

AKINLABI, Esther Titilayo; ANANE-FENIN, Kwame; AKWADA, Damenortey Richard. Bamboo taxonomy and distribution across the globe. In: **Bamboo, The multipurpose plant.** Springer, Cham, p.1-37, 2017.

ALCÁZAR, Rubén; MARCO, Francisco; CUEVAS, Juan C; PATRON, Macarena; FERRANDO, Alejandro; CARRASCO, Pedro; TIBURCIO, Antonio F.; ALTABELLA, Teresa. Involvement of polyamines in plant response to abiotic stress. **Biotechnology letters**, v. 28, n. 23, p. 1867-1876, 2006.

AUSTIN, Jotham R; FROST, Elizabeth; VIDI, Pierre-Alexandre; KESSLER, Feliz; STAEHELIN, Andrew. Plastoglobules are lipoprotein subcompartments of the chloroplast that are permanently coupled to thylakoid membranes and contain biosynthetic enzymes. **The Plant Cell**, v. 18, n. 7, p. 1693-1703, 2006.

AZZINI, Anísio; SALGADO, Antonio Luiz de Barros. Enraizamento de propágulos de bambu em diferentes substratos. **Bragantia, Campinas**, v. 52, n. 2, p. 113-118, 1993.

BAMBOO PHYLOGENY GROUP. An updated tribal and subtribal classification of the bamboos (Poaceae: Bambusoideae). **The Journal of the American Bamboo Society**, p. 1, 2012.

BANIK, Ratan Lal. Morphology and growth. In: **Bamboo, The Plant and its Uses**. Springer, Suíça, 2015. p. 43-89.

BARON, Kevin; STASOLLA, Claudio. The role of polyamines during in vivo and in vitro development. **In Vitro Cellular and Developmental Biology-Plant**, v. 44, n. 5, p. 384-395, 2008.

BENCKE, Glayson Ariel; DIAS, Rafael Antunes; FONTANA, Carla Suertegaray. Observações ornitológicas relevantes incluindo o primeiro registro de *Campylorhynchus turdinus* para o Paraná. **Atualidades Ornitológicas**, n.145, 2008.

BOUCHEREAU, A; AZIZ, A; LARHER, F; MARTIN-TANGUY, J. Polyamines and environmental challenges: recent development. **Plant Science**, v. 140, n. 2, p. 103-125, 1999.

BRÉHÉLIN, Claire; KESSLER, Felix; VAN WIJK, Klaas J. Plastoglobules: versatile lipoprotein particles in plastids. **Trends in plant science**, v. 12, n. 6, p. 260-266, 2007.

BRIGGS, Winslow R; OLNEY, Margaret A. Photorecpetors in plant photomophogenesis to date. Five phytochromes, two cryptochromes, one phototropin, and one superchrome. **Plant Physiology**, v.125, n.1, p.85-88, 2001.

BYSTRIAKOVA, Nadia; KAPOS, Valerie; LYSENKO, Igor. Distribution and conservation status of forest bamboo biodiversity in the Asia-Pacific Region. **Biodiversity & Conservation**, v. 12, n. 9, p. 1833-1841, 2003

BYSTRIAKOVA, Nadia; KAPOS, Valerie; LYSENKO, Igor. **Bamboo biodiversity: Africa, Madagascar and the Americas**. UNEP/Earthprint, 2004.

CASAL, Jorge J. Phytochromes, Cryptochromes, Phototropin: Photorecepetor Interactions in Plant. **Photochemistry and Photobiology**, v.71, n.1, p.1-11, 2000.

CHAOWANA, Pannipa. Bamboo: an alternative raw material for wood and wood-based composites. **Journal of Materials Science Research**, v. 2, n. 2, p. 90, 2013.

CLARK, Lynn G.; LONDOÑO, Ximena; RUIZ-SANCHEZ, Eduardo. Bamboo taxonomy and habitat. In: **Bamboo, The Plant and its Uses**. Springer, Suíça, p.1-30, 2015.

ERLAND, Lauren AE; SHUKLA, Mukund R.; GLOVER, W.Broe; SAXENA, Praveen K. A simple and efficient method for analysis of plant growth regulators: a new tool in the chest to combat recalcitrance in plant tissue culture. **Plant Cell, Tissue and Organ Culture (PCTOC)**, v. 131, n. 3, p. 459-470, 2017.

EVANS, Phillip T.; MALMBERG, Russell L. Do polyamines have roles in plant development? **Annual review of plant biology**, v. 40, n. 1, p. 235-269, 1989.

FILGUEIRAS, Tarciso S; GONÇALVES, Ana Paula Santos. A checklist of the basal grasses and bamboos in Brazil (Poaceae). **the journal of the American Bamboo Society**, v. 18, n. 1, p. 7-18, 2004.

GEORGE, Edwin F.; HALL, Michael A.; DE KLERK, Geert-Jan. Effects of the physical environment. In: **Plant propagation by tissue culture**. Springer Netherlands, 2008. p. 423-464.

GOYAL, Arvind Kumar; SEN, Arnab. In vitro regeneration of bamboos, the "Green Gold": An overview. 2016.

GRECO, Thiago Machado; PINTO, Moisés Medeiros; TOMBOLATO, Antonio Fernando Caetano. Diversity of bamboo in Brazil. **J Trop Subtrop Botany**, v. 23, p. 1-16, 2015.

GUERREIRO, Carolina. Flowering cycles of woody bamboos native to southern South America. **Journal of plant research**, v. 127, n. 2, p. 307-313, 2014.

Guadua in **Flora do Brasil 2020 under construction.** Jardim Botânico do Rio de Janeiro.Available at: <http://floradobrasil.jbrj.gov.br/reflora/floradobrasil/FB13250>. Acesso em 15 de dezembro de 2017.

HUSSAIN, Syed Sarfraz; ALI, Muhammad; AHMAD, Maqbool; SIDDIQUE, Kadambot H.M. Polyamines: natural and engineered abiotic and biotic stress tolerance in plants. **Biotechnology advances**, v. 29, n. 3, p. 300-311, 2011.

JUDZIEWICZ, Emmet J.; CLARK, Lynn G. Classification and biogeography of new world grasses: Anomochlooideae, Pharoideae, Ehrhartoideae, and Bambusoideae. **Aliso: A Journal of Systematic and Evolutionary Botany**, v. 23, n. 1, p. 303-314, 2007.

KELCHNER, Scot A.; BAMBOO PHYLOGENY GROUP. Higher level phylogenetic relationships within the bamboos (Poaceae:

Bambusoideae) based on five plastid markers. **Molecular phylogenetics and evolution**, v. 67, n. 2, p. 404-413, 2013.

KERBAUY, Gilberto Barbante. **Fisiologia vegetal**. Rio de Janeiro: Guanabara Koogan, 2004.

KOHLI, Ajay et al. The phytohormone crosstalk paradigm takes center stage in understanding how plants respond to abiotic stresses. **Plant cell reports**, v. 32, n. 7, p. 945-957, 2013.

LI, Jigang; LI, Gang; WANG, Haiyang; XING WANG, Deng. Phytochrome Signaling Mechanisms. **The Arabidopsis Book.** The American Society of Plant Biologists, 2011.

LIN, Xinchun; HUANG, Lichun; FANG, Wei. **Bamboo Regeneration via Embryogenesis and Organogenesis**. INTECH Open Access Publisher, 2012.

LINDQUIST, Emelie; SOLYMOSI, Katalin; ARONSSON, Henrik. Vesicles are persistent features of different plastids. **Traffic**, v. 17, n. 10, p. 1125-1138, 2016.

MINOCHA, Rakesh; MAJUMDAR, Rajtilak; MINOCHA, Subhash C. Polyamines and abiotic stress in plants: a complex relationship. **Frontiers in plant science**, v. 5, 2014.

MOSHE, R; DALIA. E. On the effect of light on shoot regeneration in petunia. **Plant cell, tissue and organ culture**, v. 89, n. 1, p. 49-54, 2007.

MUDOI, Kalpataru Dutta et al. Micropropagation of important bamboos: a review. **African Journal of Biotechnology**, v. 12, n. 20, 2013.

MURASHIGE, Toshio; SKOOG, Folke. A revised medium for rapid growth and bio assays with tobacco tissue cultures. **Physiologia plantarum**, v. 15, n. 3, p. 473-497, 1962.

PANDEY, Neha et al. Phytohormones and Drought Stress: Plant Responses to Transcriptional Regulation. **Mechanism of Plant Hormone Signaling under Stress**, p. 477-504, 2017.

QUAIL, Peter H. Phytochrome photosensory signalling networks. **Nature Reviews Molecular Cell Biology**, v. 3, n. 2, p. 85-93, 2002.

SANDHU, Manpreet; WANI, Shabir H.; JIMÉNEZ, Víctor M. In vitro propagation of bamboo species through axillary shoot proliferation: a review. **Plant Cell, Tissue and Organ Culture (PCTOC)**, p. 1-27, 2017.

SAXENA, Sanjay. In vitro propagation of the bamboo (Bambusa tulda Roxb.) through shoot proliferation. **Plant cell reports**, v. 9, n. 8, p. 431-434, 1990.

SINGH, Sharbati R. *et al*. Limitations, progress and prospects of application of biotechnological tools in improvement of bamboo—a plant with extraordinary qualities. **Physiology and Molecular Biology of Plants**, v. 19, n. 1, p. 21-41, 2013.

SINGH, Sharbati R. et al. Micropropagation of Dendrocalamus asper {Schult. & Schult. F.} Backer ex K. Heyne): an exotic edible bamboo. **Journal of plant biochemistry and biotechnology**, v. 21, n. 2, p. 220-228, 2012.

SOLYMOSI, Katalin; ARONSSON, Henrik. Etioplasts and their significance in chloroplast biogenesis. In: **Plastid Development in Leaves during Growth and Senescence**. Springer Netherlands, 2013. p. 39-71.

SOLYMOSI, Katalin; KERESZTES, Áron. Plastid structure, diversification and interconversions II. Land plants. **Current chemical biology**, v. 6, n. 3, p. 187-204, 2012.

SONG, Xinzhang et al. Carbon sequestration by Chinese bamboo forests and their ecological benefits: assessment of potential, problems, and future challenges. **Environmental Reviews**, v. 19, n. NA, p. 418-428, 2011.

SORENG, Robert J. et al. A worldwide phylogenetic classification of the Poaceae (Gramineae). **Journal of Systematics and Evolution**, v. 53, n. 2, p. 117-137, 2015.

STAEHELIN, L. Andrew. Chloroplast structure: from chlorophyll granules to supra-molecular architecture of thylakoid membranes. **Photosynthesis research**, v. 76, n. 1-3, p. 185-196, 2003.

STAMM, Petra; KUMAR, Prakash P. The phytohormone signal network regulating elongation growth during shade avoidance. **Journal of experimental botany**, v. 61, n. 11, p. 2889-2903, 2010.

SUNGKAEW, Sarawood et al. Non-monophyly of the woody bamboos (Bambuseae; Poaceae): a multi-gene region phylogenetic analysis of Bambusoideae ss. **Journal of plant research**, v. 122, n. 1, p. 95-108, 2009

TIBURCIO, Antonio F. et al. The roles of polyamines during the lifespan of plants: from development to stress. **Planta**, v. 240, n. 1, p. 1-18, 2014.

TOMBOLATO, AFC; GRECO, T. M.; PINTO, M. M. Dez espécies de bambus exóticos mais comuns no paisagismo no Brasil. **Revista Brasileira de Horticultura Ornamental**, v. 18, n. 2, p. 105-114, 2012

VEGA, Andrea S.; HERNÁNDEZ, J. Cámara. La floración de Guadua chacoensis (Poaceae, Bambusoideae, Bambuseae). **Revista de la Facultad de Agronomía**, v. 28, p. 107-110, 2008.

VON ARNOLD, Sara et al. Developmental pathways of somatic embryogenesis. **Plant Cell, Tissue and Organ Culture**, v. 69, n. 3, p. 233-249, 2002.

WISE, Robert R. The diversity of plastid form and function. In: **The structure and function of plastids**. Springer Netherlands, 2007. p. 3-26.

WOLTERS, Hanno; JÜRGENS, Gerd. Survival of the flexible: hormonal growth control and adaptation in plant development. **Nature Reviews Genetics**, v. 10, n. 5, p. 305-317, 2009.

XU, Xiaosa et al. Illuminating progress in phytochrome-mediated light signaling pathways. **Trends in plant science**, v. 20, n. 10, p. 641-650, 2015.

YEASMIN, Lucina et al. Bamboo: an overview on its genetic diversity and characterization. **3 Biotech**, v. 5, n. 1, p. 1-11, 2015.

4 - CAPÍTULO 1 - Morphohistological features and free polyamine contents of *Guadua chacoensis* (Rojas) Londoño & P.M. Peterson culms subjected to *in vitro* etiolation process

Abstract

Native from the Atlantic forest biome, *Guadua chacoensis* is a woody bamboo that presents limitation in its conventional methods of propagation. In this context, micropropagation though organogenesis arises as a valuable tool for bamboo *in vitro* propagation. Light is an important factor that affects micropropagation and can be used to elucidate modifications in plant morphogenetic responses. In this study, we evaluated the effects of *in vitro* etiolation process in morphological and histological features, as well as free polyamine levels characterization of *G. chacoensis* culms. Our results regarding morphological features showed that green plantlets started to etiolate after 20 days in darkness, with only the neoformed culms generally presenting etiolated features. Furthermore, culms heights did not show considerable difference between light and darkness conditions. On the other hand, the shoot number generated per culm was twice higher in light than in darkness condition. Endogenous contents of free polyamines were influenced by the light regime. Additionally, a correlation between polyamine content, phytochrome action and photomorphogenesis response was hypothesized. Histological characterization of culms of *G. chacoensis* under light and darkness conditions showed difference in vascular bundle size, which appeared smaller in darkness conditions along the evaluation time.

Keywords: bamboo, micropropagation; polyamine levels; photomorphogenesis response

4.1 Introduction

Bamboos correspond to one of the biggest group of grass family (Poaceae), subfamily Bambusoideae and comprise 1641 species and 120 genera around the world (Soreng et al. 2015). They are distributed in all continents, except Europe and Antarctica, from sea levels to high altitudes, and Americas comprise about 40% of endemic bamboo species (Kelchner and BPG 2013). In Brazil, it is already characterized 256 native species, divided in 34 genera, and most of them belong to Bambuseae tribe (Greco et al. 2015).

Guadua genus belongs to the group of woody bamboos (tribe Bambuseae) and comprises the biggest bamboos of tropical America (Akinlabi, Anane-Fenin and Akwada 2017), including *Guadua chacoensis* (Rojas) Londoño & P.M. Peterson (Londoño and Peterson 1992), a native bamboo from Atlantic forest (Guerreiro 2014).

Micropropagation is a valuable tool for bamboo propagation, which provides large-scale multiplication (Singh et al. 2013) in short time and allows germplasm conservation (Mudoi et al. 2013). Bamboo micropropagation can be achieved using axillary shoots and nodal segments as explants, where the explants are stimulated to develop new shoots (Sandhu et al. 2017).

In vitro propagation can be affected by many factors; however, among them, light is one that have directly influence in plant development and growth. Plant response to light, depends on the photoperiod, wavelength and intensity (Moshe and Dalia 2007). The perception of light by plants depends on the action of photoreceptors, such as phytochromes (Shikata et al. 2015). Phytochromes are presented in two different forms: biologically inactive (Pr - red absorbing) and biologically active (Pfr - far-red absorbing) which are reversible.

The inactive form is synthesized in the cytosol (Baba-Kasai et al. 2014) and can be converted to Pfr by the absorption of red light (Franklin and Quail 2009). As a consequence of the Pfr activation forms, a signal transduction pathway is activated in nucleus and gene expression related to photomorphogenesis is altered (Quail 2002). In consequence, responses such as cell differentiation, plastid development, germination, hypocotyl elongation (Mathews et al. 1995) and seedling de-etiolation (Quail 2002) can occurs.

Among the phytochrome family, phytochromes A (phyA) and B (phyB) are considered the most important ones (Franklin and Quail 2009). The phyA family is the most predominant form during darkness

growth, while phyB is the most predominant in light-growth plants (Li et al. 2011). Additionally the Pr form, or the inactive form, is present during dark growth (Jumtee et al. 2008). Previous studies in *Arabidopsis thaliana* have showed the interaction between phytochromes and light in different process, such as polyamine biosynthesis (Jumtee et al. 2008), regulation of primary metabolism with focus in starch production (Han et al. 2017) and in the photomorphogenesis response (Sheerin et al. 2015).

Darkness, as well as light, affects *in vitro* plant morphogenesis (Moshe and Dalia 2007). The process of plant development in the absence of light is known as etiolation. Etiolated plants spend energy in rapid elongation of hypocotyl and present yellow to white color, and nonfunctional photosynthetic apparatus (George et al. 20008). However, as light is supplied, the nonfunctional photosynthetic apparatus is rapidly reverted to functional apparatus (Solymosi and Kereszles 2012). Etiolation can also be seen as an abiotic stress in plant development, thereby can influence polyamines endogenous levels and culm anatomy.

Polyamines (PAs) correspond to small aliphatic molecules of amines, positively charged, such as diamine putrescine (Put), triamine spermidine (Spd) and tetraamine spermine (Spm), found in almost all plant cells (Bouchereau et al. 1999; Minocha et al. 2014; Tiburcio et al. 2014). Putrescine is the precursor of the other two forms, and in plants, its synthesis can occur thought two pathways: decarboxylation of ornithine or arginine by ornithine decarboxylases (ODC) or arginine decarboxylases (ADC), respectively (Alcazar et al. 2006; Hussain et al. 2011).

With Put biosynthesis, aminopropyl groups formed by decarboxylation of S-adenosyl-methionine (SAM), are added to Put and generate Spd, and the successive addition of aminopropyl groups to Spd generate Spm (Pang et al. 2007). The involvement of PAs in response to biotic and abiotic stress has been related in previous studies (Alcázar et al. 2006; Alcázar et al. 2010; Hussain et al. 2011; Tiburcio et al. 2014; Minocha et al. 2014) However, stress response is variable according to plant species and time of exposure to stress (Liu et al. 2008) and studies demonstrating the PAs endogenous levels in *in vitro* culture of bamboos under stress conditions were not found.

The morphoanatomical characterization of bamboo culms can helps in the determination of bamboo usability (Grosser and Liese 1971; Londoño et al. 2002). Most of the studies found in bamboo culms characterization are carried with culms in field conditions (Metcalfe 1960; Grosser and Liese 1971; Liese 1985; Liese 1987; Londoño et al.

2002; Agrasar and Rodríguez 2003), demonstrating the necessity of studies in micropropagated plantlets. The following tissues compose bamboo culms: epidermis, hypodermis and cortical parenchyma; parenchyma; vascular bundles, composed by xylem - metaxylem, protoxylem, phloem - sieve tubes and sclerenchyma sheet; and fibers (Metcalfe 1960; Liese 1987; Londoño et al. 2002). Parenchyma cells are the most predominant tissues found in bamboo culms, representing 51% of culm composition in *Guadua angustifolia*, followed by 40% fibers and 9% conducting cells (Londoño et al. 2002).

In this sense, the present study investigated the morphological features, free polyamine levels and histological characteristics of *in vitro* culms of *G. chacoensis* subjected to *in vitro* etiolation process.

4.2 Material and Methods

4.2.1 Plant material

Nodal segments of *G. chacoensis* were *in vitro* introduced according to Ornellas et al. (2017), with some modifications. The multiplication culture medium consisted of MS basal salts (Murashige and Skoog 1962) supplemented with Morel vitamins (Morel and Wetmore 1951), 30 g L^{-1} of sucrose, 13 μM of 6-benzylaminopurine (BAP), and gelled with 2 g L^{-1} of Phytagel®. The pH was adjusted to 5.8, prior to autoclaving for 20 minutes at 121°C, 1.5 atm. The cultures were kept in shelves with white LED light (Green Power TLED W; PhilipsTM; 77 par) at 25 ±2°C and 16 h photoperiod. Subculturing was performed every 30 days to a fresh culture medium.

4.2.2 Etiolation induction experiment

For the *in vitro* etiolation induction, we used thoroughly individualized culms, measuring between 2,0 and 3,0 cm (Fig. 1a) in flasks containing 30 ml of MS-multiplication medium.

For this experiment, culms were subjected to two growth conditions: darkness and white LED light (Green Power TLED W; PhilipsTM; 77 par).

The experiment was conducted in a completely randomized design, with 65 flasks per treatment, each one containing five individualized culms. The flasks used for induction of etiolation (darkness condition) were wrapped with aluminum foil in order to maintain the darkness condition. All flasks were kept in the same growth room at 25 ±2°C, 16

h photoperiod. Culms samples were collected at 0, 10, 20 and 30 days after inoculation, being fixed for light microscopy analysis or stored in -80 °C ultrafreezer for polyamine analysis.

4.2.3 Morphological characterization of culms

Morphological characterization of culms were performed by measuring the culm length (cm) and counting the neoformed shoots per culm explant at 0, 10, 20 and 30 days of light or darkness treatment.

For this characterization, five flasks from each treatment were randomly selected.

After the verification of the variance homogeneity of the data sets, data quantified was subject to analysis of variance (ANOVA). Student-Newman-Keuls (SNK) post-hoc test ($p \leq 0.05$) was used and the statistical analysis were performed using the software Statistica®.

4.2.4 Polyamines quantification

Polyamines quantification was performed according to Silveira et al. (2004). Three samples (300 mg fresh mass) for each treatment and each collection time were used for polyamine extraction. Briefly, samples were ground in liquid nitrogen, 1.6 ml of 5% perchloric acid (v/v) were added, and the extracts were incubated in room temperature for 1 h. Then, the microtubes were centrifuged for 20 minutes at 4°C and 20.000 g, and the supernatant were collected. The extracted material was dansylated according to the method described by Silveira et al. (2004).

PAs quantification was performed in HPLC with reverse-phase column C18 (Shimadzu Shim-pack CLC ODS). Mobile phase consisted of 10% acetonitrile in milli-Q water (pH 3.5 adjusted with HCl 1N) and absolute acetonitrile HPLC grade. The flow rate was 1,0 mL min^{-1} a 40°C. A fluorescent detector at 381 nm (excitation) and 510 nm (emission) was used to determine PAs content.

After the verification of the variance homogeneity of the data sets, data quantified was subject to analysis of variance (ANOVA). Student-Newman-Keuls (SNK) post-hoc test ($p<0.05$) was used and the statistical analysis were performed using the software Statistica®.

4.2.5 Light microscopy

Three samples of each treatment were collected in the middle region of culms and fixed in 1.25 % paraformaldehyde in 0.1M sodium

phosphate buffer (pH 7.2). The samples were washed twice in sodium phosphate buffer diluted in distillated water (1:1, v:v) and dehydrated in increasing series of ethanol until ethanol absolute. Then, the material was pre-infiltrated in gradual series of solutions composed by hydroxyethylmethacrylate (Leica® Historesin, Heidelberg, Germany) and ethanol and then infiltrated in pure hydroxyethylmethacrylate (Leica® Historesin, Heidelberg, Germany).

Sections of 10 μm were obtained by using Slee Technik® microtome (Slee Cut 4055, Mainz, Germany), stained with toluidine blue (O'Brien et al 1965) and subjected to histochemical analysis to characterize lignified regions, and periodic acid - Schiff reagent (PAS) (McManus 1948) to characterize starch and neutral polysaccharides. Images were acquired in inverted microscopic Olympus IX81, coupled with digital camera DP71 and software Image Q Capture Pro 5.1 (QImaging Corporation, Austin, TX, USA).

4.3 Results

4.3.1 Morphological features of culms

The culms showed some differences on the morphological features between light and darkness conditions. Under light, culms development occurred normally, with the same color in the original explant and neoformed culms (Fig.1b-d). In darkness condition, the original culms continued with green color, and the majority of neoformed culms showed etiolated characteristics, with white-yellow color (Fig.1e-g).

Morphological characterization of *G. chacoensis* culms under light and darkness treatments among 30 days of culture showed similar responses when the culm height were analyzed, and distinct responses when the number of shoot per explant where evaluated (Fig. 2). Additionally, only few differences were observed between culms growth among light and darkness treatments during the evaluation time.

Culms height at day 30 presented a progressive elongation, with similar features between light and darkness conditions, reaching approximately 3.8 cm (Fig. 2a). On the other hand, the number of shoots per explant considerable differed among treatments; reaching almost 4 and 2 shoots per explant in light and darkness conditions, respectively (Fig. 2b).

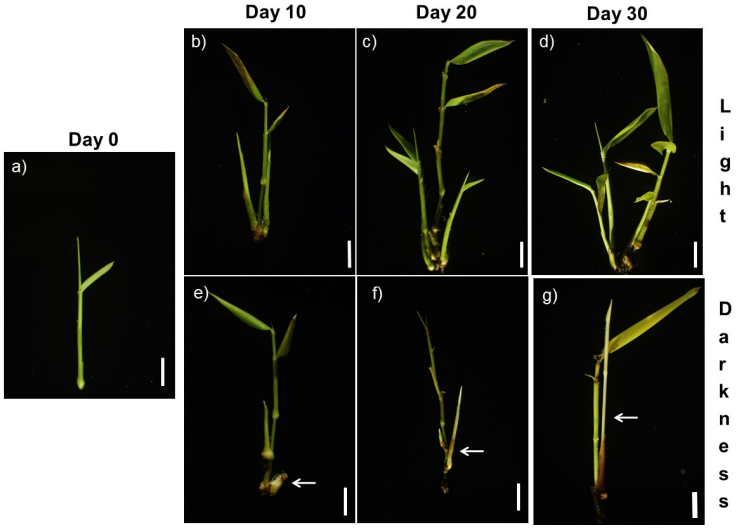

Fig. 1 Culms morphological features of *Guadua chacoensis* culms during 30 days culture under light and darkness conditions. a) Day 0; Light: b) Day10; c) Day 20; d) Day 30; Darkness: e) Day 10; f) Day 20; g) Day 30. Arrows indicate culms with etiolated characteristics.

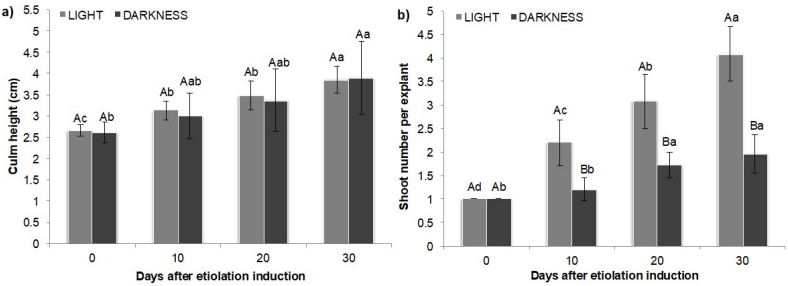

Fig. 2 Height (a) and number (b) of *Guadua chacoensis* culms obtained after 30 days in light and darkness conditions. Mean values ± standard deviation. Different uppercase letters indicates significant difference between treatments, and different lowercase letters indicates significant differences along the time, according to SNK test ($p<0,05$). Coefficient of Variance: Culm height - Light: 7.55%; Darkness: 17.81%. Shoot number per explant - Light: 13.77%; Darkness: 14.23%.

4.3.2 Free polyamines quantification

Contents of total free polyamines of *G.chacoensis* culms under light and darkness condition showed a similar pattern until day 20 of culture, with continuously increasing values (Fig. 3a). A tendency of increase in total free PAs levels was observed in light condition between day 10 and 20, reaching its highest levels in day 20. At day 30, the levels of light and darkness conditions were similar.

Free putrescine (Put) contents showed a continuous increasing until day 20 with these levels remaining unchangeable until day 30 (Fig. 3b) under light and darkness treatment. Additionally, contents found in days 20 and 30 of light condition were significantly different compared to day 0.

Free spermidine (Spd) contents of culms under light conditions presented a constant increasing during the time of evaluation, reaching the highest values at day 20 and a considerable reduction in day 30 (Fig. 3c). An evident difference among treatments was observed in day 20, when light treatment presented higher contents of free Spd than darkness condition.

For spermine (Spm) (Fig. 3d) the results showed that in darkness condition, culms presented continuous increasing content among the 30 days of evaluation. Differently, in light conditions, a continuous increase occurred until day 20, followed by a slight decrease at day 30. Additionally, in light treatment, the most considerable change occurred between day 10 and 20, with the highest Spm content in day 20.

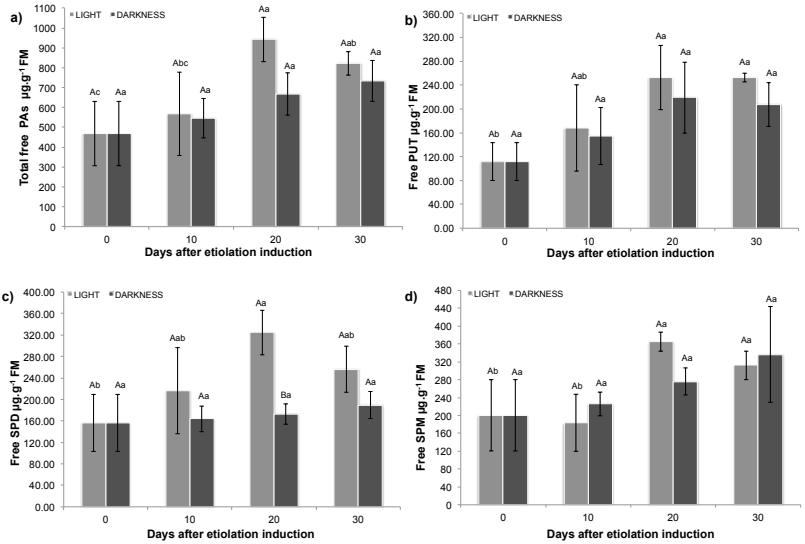

Fig. 3 Free polyamine contents of *Guadua chacoensis* culms obtained under light and darkness treatments among 30 days culture. a) Total Free polyamine contents; b) Free putrescine contents; c) Free spermidine contents; d) Free spermine contents. PUT: putrescine; SPD: spermidine; SPM: spermine. Mean values ± standard deviation. Different uppercase letters indicates significant difference between treatments, and different lowercase letters indicates significant differences along the evaluation time, according to SNK test ($p<0,05$). CV: Total Free PAS - Light: 23%; Darkness: 21%. Put - Light: 24%; Darkness: 26%. Spd - Light: 25%; Darkness: 18%. Spm - Light: 23%; Darkness: 24%.

4.3.3 Light microscopy analysis

Light microscopy analysis of *G. chacoensis* culms under light and darkness conditions did not show dramatic differences in culm morphoanatomy (Fig. 4 and 5).

The culms presented a typical anatomy of bamboo culms, composed by epidermis, hypoderm and cortical parenchyma, parenchyma and vascular bundles (Fig. 4, 5). Small thick cells formed epidermis, and in hypoderms the cells size increased according to the proximity of central region of culms, also presenting thick cell walls (Fig. 4a). Adjacent to the epidermis and hypodermis is the cortical parenchyma that corresponds to some cell layers in the periphery of the culm (Fig. 4a and 5a).

Parenchyma cells presented large cells in the periphery and smaller ones in the middle, in culms at day 0 of culture (Fig. 4a). Along the evaluation time in both conditions, parenchyma cells appeared to increase their size and its walls become thinner, probably as consequence of dense cytoplasm (Fig. 4c-g).

Vascular bundles were formed by xylem, which was composed by two huge metaxylem vessels and one small protoxylem cell surrounded by a sclerenchyma sheet; phloem and fibers. The vascular bundles were usually presented in great quantity in the periphery and in small concentration in the middle of culm, in light and darkness treatments, along the evaluation time (Fig. 4d, g).

Additionally, different sizes of vascular bundle were observed. Apparently, the size of vascular bundle in darkness treatment was smaller than in light along all the evaluation time, with also a diverse shape in darkness condition (Fig. 4 and 5).

Our results of histochemical analysis with toluidine blue showed that culms maintained in light conditions were more lignified than the ones found in darkness treatment, as showed at day 30 of culture (Fig. 4d, g). Lignified cortex could be seen along the evaluation time in light treatment (Fig. 4b-d); however, in darkness treatment it was not observed (Fig. 4e-g).

In contrast, histochemical analysis with PAS did not show differential starch and neutral polysaccharides accumulation in culms under light and darkness conditions (Fig. 5).

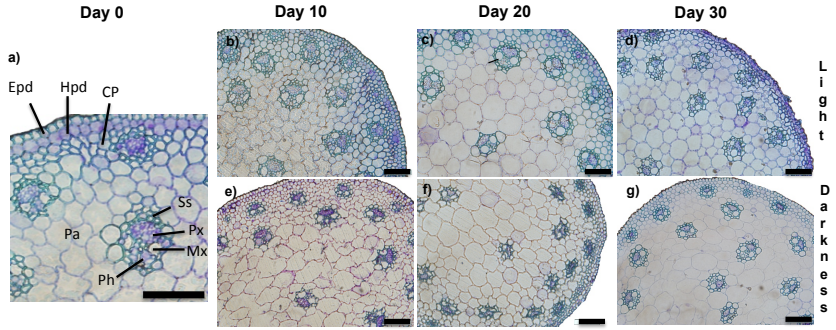

Fig. 4 Histochemical analysis of *Guadua chacoensis* culms stained with toluidine blue along 30 days in light and darkness conditions with the aim to characterized lignified regions. a) Day 0; Light: b) Day10; c) Day 20; d) Day 30; Darkness: e) Day 10; f) Day 20; g) Day 30. Epd: epidermis; Hpd: hypoderm; CP: cortical parenchyma; P: parenchyma; MX: metaxylem; PX: protoxylem; Ph: phloem; Ss: sclerenchyma sheet.

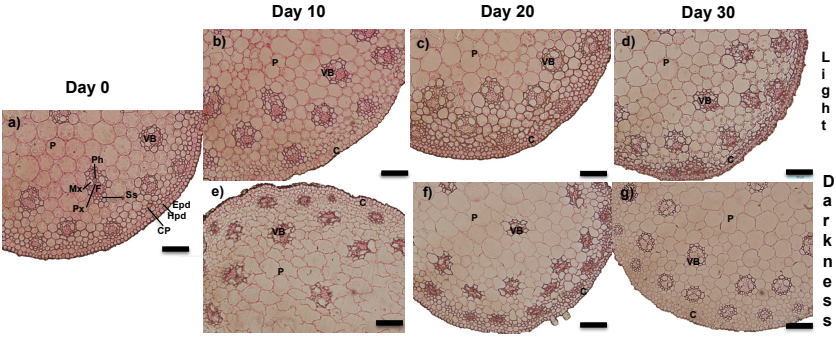

Fig. 5 Histochemical analysis of *Guadua chacoensis* culms of stained with PAS along 30 days in light and darkness conditions with the aim to characterized starch and neutral polysaccharides. a) Day 0; Light: b) Day10; c) Day 20; d) Day 30; Darkness: e) Day 10; f) Day 20; g) Day 30. Epd: epidermis; Hpd: hypoderm; CP: cortical parenchyma; P: parenchyma; MX: metaxylem; PX: protoxylem; Ph: phloem; Ss: sclerenchyma sheet.

4.4 Discussion

4.4.1 Morphological characterization of culms

Analysis of culm height along the 30 days of evaluation under light and darkness conditions revealed a similar pattern between the treatments. Darkness growth was expected to generate plants with etiolated features, such as shoot elongation and white-yellow color (George et al. 2008); however, these responses where only observed in the neoformed culms generated under darkness. Our results suggest that culms started to acquire etiolation features after 20 days in darkness, when etiolated culms were formed (Fig. 1f). Additionally, neoformed shoots developed under darkness presented typical etiolation color, white-yellow, without changes in culm length (Fig. 1e-g).

Suzuki et al. (2004) showed that *in vitro* plantlets of *Catasetum fimbriatum* when transferred from light to darkness conditions only formed etiolated plants after approximately 60 days of incubation. Additionally, previous studies have demonstrated that 60 days of incubation in darkness is necessary to improvement of *in vitro* multiplication of different pineapple genotypes (Barboza and Caldas 2001; De Carvalho et al. 2009). Thus, we can hypothesize for our results that absence the etiolated plants in 30 days of darkness was possibly associated with the short period of incubation under this condition.

Morphological characterization of culms also demonstrated the importance of light in the enhancement of shoot induction in *G. chacoensis* culms. We observed that the number of shoots generated in light were two times higher than the number obtained in darkness at 30 days (Fig.2b). This result could be related to photomorphogenesis response in light and darkness conditions.

In light, normal photomorphogenesis response occurs by the action of phytochromes that perceive the light and activate a myriad of responses, including cell differentiation, plastid development and hypocotyl elongation (Mathews et al. 1995). In contrast, in darkness, the phytochromes are, in general, found in the biologically inactive form (Xu et al. 2015). In this condition, the photomorphogenesis response is blocked by the action of repressors, such as COP/DET/FUS (Constitutively Photomorphogenenic/Deetiolated/ Fusca) and PIFs (Phytochrome Interacting Genes) (Xu et al. 2015; de Wit et al. 2016).

4.4.2 Polyamines quantification

Changes in PAs endogenous contents are normally associated with plant growth and development, although the exact function of PAs in these processes is not fully elucidated. Polyamines accumulation can be either a necessary factor in plant growth process or accumulation of polyamines can be a consequence of it (Desai and Mehta 1985). Additionally, among the three most common PAs, Put and Spd are considered essential in plant morphogenesis, and Spm appears to be involved in other processes, such as production of nitric oxide (NO) and response to stress (Kusano et al. 2007).

In our results, an expressive increase in contents of total free PAs, free Put, Spd and Spm were observed in *G. chacoensis* culms under light conditions at day 20 (Fig. 3 a-d). We suggest that an intense activity of plant photomorphogenesis was induced in this period of culture, and, as a consequence, PAs contents also increased.

Aragão et al. (2015) observed that increased contents of total free PAs in day 30 of *in vitro* plantlets of *Cedrela fissilis* were related with intense metabolic cell elongation, differentiation and division. Zhu and Chen (2005) showed that the enhancement in adventitious morphogenesis in cotyledons of *in vitro* plants of cucumber was probably associated with increased in PAs endogenous contents. Desai and Mehta (1985) also demonstrated that the increase in Put endogenous levels were associated with shoot induction and that this polyamines may have a role in organogenesis of *Passiflora*.

Furthermore, we believe that the increase in plant morphogenesis can be also associated with the availability of nutrients presented in the culture medium in this stage of *in vitro* culture. Long period in a same culture medium without sub culturing can result in nutrient depletion, pH changes and accumulation of toxic compounds that can also results in necrosis of material (Sandhu et al. 2017). However, as the culture is in the beginning of the cycle of multiplication, macro and micronutrients, as well as carbohydrates, are presented in major availability between the first 20 days, favoring plant development.

In the present study we highlighted that the contents of Spd observed at day 20 in light treatment were higher than the contents observed in darkness condition (Fig. 3c). Furthermore, a significant increase in shoot number generated was also in this time (Fig. 2b). Based on these findings, we suggests that an increase in free Spd contents in light treatment at day 20 combined to this shoot formation improvement can

be related to photomorphogenesis response and phytochrome action (Dai and Galston 1980).

Phytochromes are an important group responsible by plant perception of light for morphogenesis response (Shikata et al. 2015). Among photoreceptors, phytochromes and cryptochromes are known to control plant morphogenesis response (Folta and Childers 2008), however phytochromes are the most studied one (Baba-Kasai et al. 2014). They are continuous reversibility between the inactive form, Pr and active form, Pfr (Franklin and Quail 2009), being the inactive form the most predominant in darkness (Jumtee et al. 2008). When the phytochromes are in their biological active form, Pfr, a signal transduction pathway is activated and the transduction of genes related to photomorphogenesis starts (Quail 2002).

A correlation between polyamines contents and phytochromes has been found in previous studies (Dai and Galston 1980; Yoshida and Hirasawa 1998; Jumtee et al. 2008) with emphasis in ADC expression levels, which corresponds to the gene precursor of the indirect pathway of PAs biosynthesis (Alcázar et al. 2006). In an interesting study, Jumtee et al. (2008) showed a direct relationship between phyA and light in the PAs contents. The authors observed a decreased in contents of Put in etiolated wild-type *Arabidopsis thaliana* plantlets compared with *phyA* mutants, which were both subjected to white and far-red light, demonstrating this response only under far-red light.

These authors also investigated this behavior under qRT-PCR analysis with the aim to determine which gene was responsible by the down-regulation of Put in etiolated plantlets. It was found that the *ADC2* gene (arginine decarboxylases 2) were transcript in lower levels in etiolated WT plantlets than in *phyA* mutants in both conditions and concluded that a possible reduction in Put contents could be associated with reduction in ADC2 levels. Furthermore, Yoshida and Hirasawa (1998) demonstrated that ADC activity could be induced by light, and that ADC pathway is probably the preferred pathway of polyamines biosynthesis in functional photosynthetic tissues (Yoshida and Hirasawa, 1998). ADC synthesis was suggested to be localized inside the chloroplast associated with thylakoids membranes (Borrell et al. 1995).

A significant correlation between ADC and light conditions has been demonstrated by Dai and Galston (1980) in etiolated *Pisum sativum* seedling. These authors verified that that buds of etiolated pea seedling presented higher ADC activity under irradiation with red light in comparison with dark. The authors also observed that increase in

ADC levels were accomplished by enhancement in bud growth. So, they suggested that phytochromes action under light conditions promoted ADC activity and growth and that polyamines regulation should be also related with phytochromes function in pea buds.

By this ways, changes in PAs endogenous contents in light in comparison with darkness as showed in Spd levels (Fig. 3c) could be strongly interconnected with changes in ADC expression levels and phytochrome action. Moreover, it seems reasonable to suppose that there is a positive correlation between light and polyamines biosynthesis, as a consequence of phytochrome action and its influence in plant growth, development and gene expression. However, molecular studies with *in vitro* etiolated culms of *G. chacoensis* will be important to confirm our findings.

Plant response to environmental fluctuations, such as stress conditions, is accomplished by changes in physiological, morphological and biochemical composition (Alcázar et al. 2006). Endogenous PAs contents suffer constant changes in response to stress conditions, however it is not known yet if these changes are generated in plants as a result or a way of protection against stress condition (Alcázar et al. 2010; Hussain et al. 2011). Moreover, stress response is variable according to plant species and time of exposure to stress (Liu et al. 2008).

The absence of significant differences between light and darkness regarding te content of for total free PAs (Fig.3a), Put (Fig.3b) and Spm (Fig.3c) found in our work may be associated with PAs oxidation or involvement in other biochemical pathways (Tiburcio et al 2014), as well as PAs conjugation (Bouchereau et al 1999).

Our results suggest that etiolation process affects polyamines endogenous contents. The increased in the contents of free PAs, Put, Spd and Spm from day 10 to 20 in light treatment showed that a possible accumulation of these polyamines is maybe due to photomorphogenesis response. Also we highlighted that Put, Spd and Spm were probably produced in consequence of growth and development of *G. chacoensis* culms, since increase in their contents are accomplished by increase in shoot number per explant and in plant height. Additionally, the existence of a positive correlation between phytochromes function and polyamines biosynthesis is proposed based on the increased in the levels of Spd in light in contrast to darkness conditions.

4.4.3 Light microscopy

Similar morphoanatomical features were found in bamboos culms under light and darkness conditions (Fig. 4, 5). No significant morphological changes were observed in material (Fig. 1), probably as a result of insufficient time under darkness conditions, as earlier discussed.

As we could see in our results, *G. chacoensis* culms are formed by epidermis, hypodermis, parenchyma and vascular bundles (Fig. 4, 5). These characteristics of the culms demonstrated in our study were in agreement with features described to bamboo in previous studies (Metcalfe 1960; Grosser and Liese 1971; Liese 1987; Liese 1985; Londoño 1992; Agrasar and Rodríguez 2003).

Vascular bundles are localized embedded in parenchyma tissues of bamboo culms (Londoño et al. 2002). In our study we could observe that vascular bundles distribution among the culm do not followed an organized order. This is a typical characteristic of monocotyledons (Apezzato-da-Gloria and Carmello-Guerreiro 2006). Additionally, the number and concentration of bundle sheets were variable along the culms, showing more concentration in the periphery than in central area (Fig.4 and Fig.5). Grosser and Liese (1971) in a study realized with 52 species of Asian bamboos proposed that vascular bundles are presented in higher concentration and smaller size in peripheral region and in smaller concentration and higher size in central region. This behavior was also observed latter in other bamboo studies (Liese, 1987; Londoño 2002), suggesting that this is a typical characteristic of bamboo culms.

The presence of more lignified tissues in culms under light than in darkness conditions were also observed (Fig. 4). The process of lignification of bamboo culms started to happen since the beginning of plant development (Liese 1987) and it is associated with culm ageing. A possible reason for the presence of more lignified culms in light than in darkness is that the plant morphogenesis response continues to happen in light and in darkness these response could be blocked, as a consequence of dark condition (Xu et al. 2015; de Wit et al. 2016).

4.5 Concluding Remarks

Our results suggested a positive influence of light in morphological features of *G. chacoensis*. The enhancement in the number of shoots generated per explant was almost twice in light than in darkness, reaching approximately four and two culms, respectively, indicating that

light promotes shoot proliferation in *G. chacoensis in vitro* culture. Moreover, we could see that green plantlets started to present etiolate characteristics after day 20 in darkness and that the neoformed culms generally presented etiolated features, such as white to yellow color. Probably the presence of partially etiolated plants for this specie could be associated with the insufficient time of incubation in darkness in our study.

Different responses in endogenous polyamine contents observed in our study revealed an influence of etiolation process in polyamines contents. It was seen that changes in polyamines contents could be a result of a complex interaction between phytochromes, polyamines biosynthesis and photomorphogenesis response.

Furthermore, the histological characterization of culms of *G. chacoensis* under light and darkness conditions shows similar results, with exception of more lignified culms in light than in darkness, and changes observed in vascular bundle organization, suggesting one more time influence of light in morphogenesis response.

Taken together, ours results indicated that light is essential to *in vitro* propagation of *G. chacoensis* and that its presence allows plant growth and development. Additionally, our studies suggested that futures studies with focus in molecular characterization of ADC genes for the *in vitro* etiolated culms of *G. chacoensis* could be important to confirm our hypothesis of a correlation between light, phytochromes and polyamines endogenous contest.

4.6 References

Akinlabi ET, Anane-Fenin K, Akwada DR (2017) Bamboo Taxonomy and Distribution Across the Globe. In *Bamboo* (pp. 1-37). Springer, Cham.

Alcázar R, Altabella T, Marco F, Bortolotti C, Reymond M, Koncz C, Tiburcio AF (2010) Polyamines: molecules with regulatory functions in plant abiotic stress tolerance. Planta 231(6): 1237-1249. https://doi.org/ 10.1007/s00425-010-1130-0.

Alcázar R, Marco F, Cuevas JC, Patron M, Ferrando A, Carrasco P, Altabella T (2006) Involvement of polyamines in plant response to abiotic stress. Biotechnology letters 28(23): 1867-1876. https://doi.org/ 10.1007/s10529-006-9179-3

Agrasar ZER, Rodríguez MF (2003) Culm anatomy of native woody bamboos in Argentina and neighboring areas: cross section. The journal of the American Bamboo Society 17(1): 28-43.

Appezzato-da-Glória B, Guerreiro C (1992) Anatomia vegetal. Universidade de São Paulo. ESALQ.

Aragão VPM, de Souza Ribeiro YR, Reis RS, Macedo AF, Floh EIS, Silveira V, Santa-Catarina C (2015) In vitro organogenesis of *Cedrela fissilis* Vell.(Meliaceae): the involvement of endogenous polyamines and carbohydrates on shoot development. Plant Cell, Tissue and Organ Culture 124(3): 611-620. https://doi.org/ 10.1007/s11240-015-0919-8

Baba-Kasai AKIKO, Hara N, Takano M (2014) Tissue-specific and light-dependent regulation of phytochrome gene expression in rice. Plant, cell & environment 37(12): 2654-2666. https://doi.org/ 10.1111/pce.12354

Barboza SBSC, Caldas LS (2001) Estiolamento e regeneração na multiplicação in vitro do abacaxizeiro híbrido PE x SC-52. Pesquisa agropecuária brasileira, 36(3), 417-423.

Borrell A, Culianez-Macia FA, Altabella T, Besford RT, Flores D, Tiburcio A (1995). Arginine decarboxylase is localized in chloroplasts. *Plant Physiology*, *109*(3), 771-776.

Bouchereau A, Aziz A, Larher F, Tanguy J-Martin (1999) Polyamines and environmental challenges: recent development. Plant Science 140(2): 103-125.

Dai YR, Galston AW (1981) Simultaneous phytochrome-controlled promotion and inhibition of arginine decarboxylase activity in buds and epicotyls of etiolated peas. Plant physiology, 67(2): 266-269. https://doi.org/10.1104/pp.67.2.266

De Carvalho ACP, Pinheiro MVM, DIAS G, Morais JPS (2009) Multiplicação in vitro de abacaxi ornamental por estiolamento e regeneração de brotações. *Embrapa Agroindústria Tropical-Artigo em periódico indexado (ALICE)*.

De Wit M, Galvão VC, Fankhauser C (2016) Light-mediated hormonal regulation of plant growth and development. Annual review of plant biology 67:513-537. https://doi.org/10.1146/annurev-arplant-043015-112252p

Desai HV, Mehta AR (1985) Changes in polyamine levels during shoot formation, root formation, and callus induction in cultured *Passiflora* leaf discs. Journal of plant physiology 119(1): 45-53. https://doi.org/10.1016/S0176-1617(85)80214-5

Folta KM, Childers KS (2008) Light as a growth regulator: controlling plant biology with narrow-bandwidth solid-state lighting systems. *HortScience*, *43*(7), 1957-1964.

Franklin KA, Quail PH (2009) Phytochrome functions in *Arabidopsis* development. Journal of experimental botany 61(1): 11-24. https://doi.org/10.1093/jxb/erp304

George, EF, Hall MA, De Klerk GJ (2008) Effects of the physical environment. In: Plant propagation by tissue culture, Springer, Netherlands, pp. 423-464 https://doi.org/10.1007/978-1-4020-5005-3_12

Greco TM, Pinto MM, Tombolato AFC, Xia N (2015) Diversity of bamboo in Brazil. *J Trop Subtrop Botany*, *23*, 1-16.

Grosser D, Liese W (1971) On the anatomy of Asian bamboos, with special reference to their vascular bundles. Wood Science and technology 5(4): 290-312. https://doi.org/10.1007/BF00365061

Guerreiro C (2014) Flowering cycles of woody bamboos native to southern South America. *Journal of plant research*, *127*(2), 307-313.

Han X, Tohge T, Lalor P, Dockery P, Devaney N, Esteves-Ferreira A, Fernie AR, Sulpice R (2017) Phytochrome A and B regulate primary metabolism in *Arabidopsis* leaves in response to light. Frontiers in Plant Science 8 1394. https://doi.org/10.3389/fpls.2017.01394

Hussain SS, Ali M, Ahmad M, Siddique KH (2011) Polyamines: natural and engineered abiotic and biotic stress tolerance in plants. Biotechnology advances 29(3): 300-311.https://doi.org/10/1016/j.biotechadv.2011.01.003

Jumtee K, Bamba T, Okazawa A, Fukusaki E, Kobayashi A (2008) Integrated metabolite and gene expression profiling revealing phytochrome A regulation of polyamine biosynthesis of *Arabidopsis*

thaliana. Journal of experimental botany 59(6): 1187-1200. https://doi.org/10.1093/jxb/ern026

Kelchner SA, Bamboo Phylogeny Group (2013) Higher level phylogenetic relationships within the bamboos (Poaceae: Bambusoideae) based on five plastid markers. Mol Phylogenet Evol 67: 404-413. https://doi.org/10.1016/j.ympev.2013.02.005

Kusano T, Yamaguchi K, Berberich T, Takahashi Y (2007) Advances in polyamine research in 2007. Journal of plant research 120(3): 345-350. https://doi.org/10.1007/s10265-007-0074-3

Li J, Li G, Wang H, Wang Deng X (2011) Phytochrome signaling mechanisms. The *Arabidopsis* Book, e0148. https://doi.org/10.1199/tab.0148

Liese W (1985) Anatomy and properties of bamboo. In Proceedings of the International Bamboo Workshop. pp. 196-208

Liese W (1987) Research on bamboo. Wood Science and Technology 21(3): 189-209. https://doi.org/10.1007/BF00351391

Liu JH, Inoue H, Moriguchi T (2008) Salt stress-mediated changes in free polyamine titers and expression of genes responsible for polyamine biosynthesis of apple in vitro shoots. Environmental and Experimental Botany 62(1): 28-35. https://doi.org/10.1016/j.envexpbot.2007.07.002

Londoño X, Peterson PM (1992) *Guadua chacoensis* (Poaceae: Bambuseae), its taxonomic identity, morphology, and affinities. Novon (St Louis) 2:41-47.

Londoño X, Camayo GC, Riaño NM, López Y (2002) Characterization of the anatomy of *Guadua angustifolia* (Poaceae: Bambusoideae) culms. Bamboo Science and Culture: The Journal of the American Bamboo Society 16(1): 18-31.

Mathews S, Lavin M, Sharrock RA (1995) Evolution of the phytochrome gene family and its utility for phylogenetic analyses of angiosperms. Annals of the Missouri Botanical Garden, 296-32. https://doi.org/10.2307/2399882.

Metcalfe CR (1960) Anatomy of the monocotyledons. 1. Gramineae. *Anatomy of the monocotyledons. 1. Gramineae.*

Minocha R, Majumdar R, Minocha SC (2014) Polyamines and abiotic stress in plants: a complex relationship. Frontiers in plant science 5. https://doi.org/10.3389/fpls.2014.0017

Morel G, Wetmore RH (1951) Tissue culture of monocotyledons. Am J Bot 38: 138-140.

Moshe R, Dalia E (2007) On the effect of light on shoot regeneration in petunia. Plant cell, tissue and organ culture 89(1): 49-54. https://doi.org/10.1007/s11240-007-9215-6

Mudoi KD, Saikia SP, Goswami A, Gogoi A, Bora D, Borthakur M (2013) Micropropagation of important bamboos: a review. African Journal of Biotechnology, 12(20): 2770-2785. https://doi.org/10.5897/AJB12.2122

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15: 473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Ornellas TS, Werner D, Holderbaum DF, Scherer RF, Guerra MP (2017) Effects of Vitrofural, BAP and meta-Topolin in the *in vitro* culture of *Dendrocalamus asper*. Acta Hortic 1155: 285-292. https://doi.org/ 10.17660/ActaHortic.2017.1155.41

Pang XM, Zhang ZY, Wen XP, Ban Y, Moriguchi T (2007) Polyamines, all-purpose players in response to environment stresses in plants. Plant Stress 1(2): 173-188.

Quail PH (2002) Phytochrome photosensory signalling networks. Nature Reviews Molecular Cell Biology 3(2): 85-93. https://doi.org/10.1038/nrm728

Sandhu M, Wani SH, Jiménez VM (2017) *In vitro* propagation of bamboo species through axillary shoot proliferation: a review. Plant Cell Tissue Organ Cult 132: 27-53. https://doi.org/10.1007/s11240-017-1325-1

Sheerin DJ, Menon C, zur Oven-Krockhaus S, Enderle B, Zhu L, Johnen P, Hiltbrunner A (2015) Light-activated phytochrome A and B interact with members of the SPA family to promote photomorphogenesis in *Arabidopsis* by reorganizing the COP1/SPA complex. The Plant cell 27(1): 189-201. https://doi.org/10.1105/tpc.114.134775

Shikata H, Hanada K, Ushijima T, Nakashima M, Suzuki Y, Matsushita T (2014) Phytochrome controls alternative splicing to mediate light responses in *Arabidopsis*. Proceedings of the National Academy of Sciences 111(52): 18781-18786. https://doi.org/10.1073/pnas.1407147112

Silveira V, Floh EIS, Handro W, Guerra MP (2004) Effect of plant growth regulators on the cellular growth and levels of intracellular protein, starch and polyamines in embryogenic suspension cultures of Pinus taeda. Plant Cell, Tissue and Organ Culture, *76*(1), 53-60. https://doi.org/10.1023/A:1025847515435

Singh SR, Singh R, Kalia S, Dalal S, Dhawan AK, Kalia RK (2013) Limitations, progress and prospects of application of biotechnological tools in improvement of bamboo - a plant with extraordinary

qualities. Physiol Mol Biol Plants 19: 21-41. https://doi.org/10.1007/s12298-012-0147-1

Solymosi K, Keresztes Á (2012) Plastid structure, diversification and interconversions II. Land plants. Curr Chem Biol 6:187-204.

Soreng RJ, Peterson PM, Romaschenko K, Davidse G, Zuloaga FO, Judziewicz EJ, Morrone O (2015) A worldwide phylogenetic classification of the Poaceae (Gramineae). J Syst Evol 53: 117-137. https://doi.org/10.1111/jse.12150

Suzuki RM, Kerbauy GB, Zaffari GR (2004) Endogenous hormonal levels and growth of dark-incubated shoots of Catasetum fimbriatum. *Journal of plant physiology*, *161*(8), 929-935. https://doi.org/10.1016/j.jplph.2003.11.001

Tiburcio AF, Altabella T, Bitrián M, Alcázar R (2014) The roles of polyamines during the lifespan of plants: from development to stress. Planta 240(1): 1-18. https://doi.org/10.1007/s00425-014-2055-9

Xu X, Paik I, Zhu L, Huq E (2015) Illuminating progress in phytochrome-mediated light signaling pathways. Trends in plant science 20(10): 641-650. https://dx.doi.org/10.1016/j.tplants.2015.06.010

Yoshida I, Hirasawa E (1998) Photoinduction of arginine decarboxylase activity in leaves of *Pharbitis nil*. Phytochemistry, 49(8): 2255-2259. https://doi.org/10.1016/S0031-9422(98)00338-0

Zhu C, Chen Z (2005) Role of polyamines in adventitious shoot morphogenesis from cotyledons of cucumber in vitro. Plant Cell, Tissue and Organ Culture, 81(1): 45-53. https://doi.org/10.1007/s11240-004-2773-y.

5. CAPÍTULO 2 - Etiolation process affects chloroplast ultrastructure and hormone endogenous levels during *Guadua chacoensis* (Rojas) Londoño & P.M. Peterson *in vitro* culture

Abstract

Guadua chacoensis is a woody native bamboo from the Atlantic forest biome. This species produces scarce and low viability seeds, and its vegetative propagation is also season dependent, with low propagules availably. Biotechnology tools, based on micropropagation, comprise efficient tools for its propagation. In addition, these techniques can provide an excellent system to investigate ultrastructure and physiological processes. In the present study we evaluated the influence of etiolation during *in vitro* culture of *G. chacoensis*, with emphasis on the chloroplast biogenesis and the endogenous levels of zeatin (Z), gibberellin (GA), abscisic acid (ABA) and jasmonic acid (JA). Our results indicated, in light treatment, a decreased number of starch grains during *in vitro* culture, probably as consequence of the use of starch as energy providing for plant initiation and posterior development. Differently, in darkness conditions, improved amyloplast formation was observed. The etioplast formation, as would be expected in darkness conditions, was not observed and could be associated with the high endogenous levels of Z observed in culms of *G. chacoensis* as supplementation of cytokinin in the culture medium generated similar results. ABA, GA and JA biosynthesis decreased in darkness conditions, suggesting a strong relationship between light and these phytohormone levels. Additionally, Z and ABA showed to be closely related with plastids formation, by a positive and negative way, respectively. The first one influenced chloroplast maturation, and carotenoid levels apparently influenced the second.

Keywords: bamboos; micropropagation; transmission electron microscopy; plant physiology; phytohormone

5.1 Introduction

Bamboos belong to the grass family Poaceae, subfamily Bambusoideae, comprising 1641 species and 120 genera (Soreng et al. 2015). They are spread in almost all continents, except Europe and Antarctic, from the lowlands to more than 4000 meters of altitude (Kelchner and BPG 2013). South America is the second continent in richness of bamboo species (Bystriakova et al. 2004).

Bambuseae is the largest tribe among the three tribes of Bambusoideae, along with Arundinareae and Olyreae. Bambuseae covers the tropical and subtropical woody bamboos, with about 893 species and 68 genera (Soreng et al. 2015). The genus *Guadua* corresponds to one of five biggest genera of bamboos in Brazil (Filgueiras and Gonçalves 2004) and also the most economically important genus in tropical America (Londoño 1998). *Guadua chacoensis* (Rojas) Londoño & P.M. Peterson (Londoño and Peterson 1992) is a woody native bamboo from the Atlantic forest, with natural occurrence in southern Brazil, Paraguay, Uruguay and northeastern Argentina (Guerreiro 2014).

The flowering intervals of *Guadua chacoensis* is estimated in 28 years (Vega and Hernández 2008), producing scarce and low viability seeds. Vegetative propagation through offset and culm cutting are also seasonal dependent, with low availably of propagules, being conventional propagation methods insufficient and inefficient for bamboos mass propagation (Singh et al. 2013). Thus, the use of biotechnology tools, such as micropropagation, is essential for large-scale bamboo propagation (Singh et al. 2013).

Organogenesis is the morphogenetic route where the explant is induced to generate shoots or roots (Singh et al. 2013). The physiological and genetic conditions of the explant are essential factors in the achievement of *in vitro* propagation. The use of axillary shoot in bamboo micropropagation has been applied to promote high *in vitro* multiplication rates (Jiménez et al. 2006; Jiménez and Guevara 2007; Singh et al. 2013; Sandhu et al. 2017). Another factor that affects micropropagation is light condition, which is directly related with *in vitro* growth and morphogenesis as consequence of wavelength, time of exposure and photoperiod (George et al. 2008). Furthermore, its influence in plastid differentiation, photosynthesis and chloroplast maturation has been demonstrated (Solymosi and Aronsson 2013).

Chloroplast belongs to the diverse group of organelles known as plastids, which includes proplastids, etioplasts, gerantoplasts,

chromoplasts and leucoplasts - for instance amyloplasts (Lindquist et al. 2016). These plastids diverge in their function, chemical compounds and inner structure (Solymosi and Aronsson 2013) and also reveal remarkable interconvertion plasticity, according to the given conditions (Lindquist et al. 2016; Solymosi and Keresztes 2012). Chloroplast differentiation has been designated as a light dependent process, so as light is reduced or either not supplied, the proplastid can differentiate into etioplast (Staehelin 2003). Etioplasts are usually seen as a temporary stage of plastids, which are formed during the dark-growth, although they are rapidly converted to functional chloroplast with light supplying (Wise 2007). Thus, the investigation of the plastid interconversion dynamics can help in the better understanding plants *in vitro* morphogenesis.

Phytohormones are also essential in plant development, acting as signaling molecules in plant response during development, growth, adaptation, stress and physiological response (Erland et al. 2017). They are also involved in many plastid differentiation process and function. Cytokinins (CK) are strongly related with chloroplast biogenesis, especially with its maturation, which has been related in many studies (Stetler and Laetsch 1965; Polanská et al. 2006; Cortleven and Schmülling 2015). They are also involved in the promotion of thylakoid formation; disintegration of prolamellar bodies in de-etiolation process (Cortleven and Schmülling 2015), participates in photomorphogenesis stimulus in dark growth of *Arabidopsis* (Lochmanová et al. 2008) and in promotion of chloroplast related gene expression (Parthier 1979). On the other hand, abscisic acid (ABA) is generally associated with the repression of chloroplast related genes (Yamburenko et al. 2013). ABA biosynthesis pathway is also related with plastids, as the production of carotenoids, its precursor occurs in plastids (Seo and Koshiba 2002).

Previous reports have been demonstrated the effect of gibberellic acid (GA) in plant morphogenesis responses (Fleet and Sun 2005; Alabadí et al. 2008; Li et al. 2015). GA is known to repress the photomorphogenesis in darkness as a consequence of the negative regulation of DELLA proteins. This regulation results in repression of PIFs (phytochrome interacting factors) that are also responsable by repress photomorphogenesis and consequently etiolation responses do not occurs (Li et al. 2015). Another phytohormone that is related with different response in interplay with light is jasmonic acid (JA), where both JA co-receptors and components of light signaling can influence each other response (Kazan and Manners 2011).

In the present work we evaluated the influence of etiolation process during *in vitro* culture of *G. chacoensis*, with emphasis on the chloroplast biogenesis through transmission electron microscopy. We also analyzed the endogenous levels of the phytohormones zeatin (Z), a type of cytokinins, GA, ABA and JA, through reverse-phase ultra-performance liquid chromatography.

5.2 Material and methods

5.2.1 Plant material

Nodal segments of *G. chacoensis* were *in vitro* introduced according to Ornellas et al. (2017), with some modifications. The multiplication culture medium consisted of MS basal salts (Murashige and Skoog 1962) supplemented with Morel vitamins (Morel and Wetmore 1951), 30 g L^{-1} of sucrose, 13 μM of 6-benzylaminopurine (BAP), and gelled with 2 g L^{-1} of Phytagel®. The pH was adjusted to 5.8, prior to autoclaving for 20 minutes at 121°C, 1.5 atm. The cultures were kept in shelves with white LED light (Green Power TLED W; PhilipsTM; 77 par) at 25 ±2°C and 16 h photoperiod. Subculturing was performed every 30 days to a fresh culture medium.

5.2.2 Induction of etiolation

For the *in vitro* etiolation induction, we used thoroughly individualized culms (Fig. 1a) measuring 2-3 cm in flasks (67.2 mm × 129.3 mm/350 ml) containing 30 ml of MS multiplication culture medium.

The experiment had two conditions (darkness and light) and was held in a completely randomized design, with 30 flasks per treatment, each one containing five individualized culms. The flasks used for etiolation were wrapped with aluminum foil in order to maintain the darkness condition. All flasks were kept in the same growth room at 25 ±2°C, with white LED light (Green Power TLED W; PhilipsTM; 77 par) 16 h photoperiod. Samples of culms were collected at 0, 10, 20 and 30 days after inoculation, and stored at -80°C for hormone quantification analysis, or fixed for transmission electron microscopy (TEM) characterization.

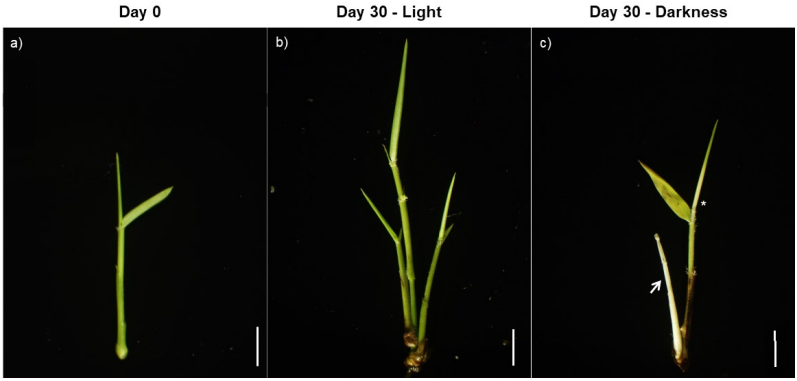

Fig. 1 *Guadua chacoensis* culms. a) Day 0; b) Day 30 at light condition; c) Day 30 at darkness. Bars represent 5 mm. Arrow indicates etiolated culms. *Shows partially etiolated culms.

5.2.3 Transmission electron microscopy

TEM analysis was used to evaluate the chloroplast ultrastructure under light and darkness conditions. Three samples from each treatment were collected from the middle region of culms and fixed with 2.5 % glutaraldehyde, 0.1 M sodium cacodylate buffer (pH 7.2) and 18 mM sucrose per 48 h. Samples were washed with sodium cacodylate buffer and post-fixed with1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 4 h. Then, the samples were dehydrated in acetone series, and embedded in Spurr's resin (EMS Diasum, Hatfield, PA), as the manufacturer instructions. Ultrathin sections were treated with uranyl acetate followed by Reynolds' lead citrate (1963). TEM representative images were obtained with TEM JEM 1011 (JEOL Ltd., Tokyo, Japan) operated at an accelerating voltage of 80 kV.

5.2.4 Hormone quantification

Hormone quantification (zeatin, GA_4, ABA and JA) was performed according to Fraga et al. (2016), with modifications.

Samples of 100 mg of fresh material were ground in liquid nitrogen and transferred to a 2 mL microtube containing 1 mL of extraction buffer (methanol, water and formic acid, 75:20:5, v.v.v with 2 mM of citric acid). The tubes were mixed using a vortex mixer, incubated at -20°C for 3 h, followed by ultrasonic bath (40 kHz frequency) at 4°C for 25 minutes and a centrifugation at 9800 g at 4°C for 10 min, and the

supernatant was collected. The pellets were re-extracted twice, with 750 µL of extraction buffer with time of incubation of 6 h and 12 h and sonicated and centrifuged as described above. Finally, the three supernatants were combined and dried in a vacuum concentrator at 40°C for 22500 g and resuspended in 1 mL of mili-Q water.

The resuspended extract was purified through Oasis MCX column (150 mg Sorbent, Waters Technologies, USA), according to the manufacturer instructions. The eluted was collected, concentrated and dried in vacuum concentrator at 40°C for 22500 g, resuspended in 100 µL of methanol and filtered through 0.22 µm PTFE filter.

Samples quantification was performed by LC-MS/MS consisting of an Acquity UPLC™ System (Waters, USA) quaternary pump equipped with an autosampler. The column used was Acquity UPLC BEH C18 (2.1 x 50 mm, 1.7µm) (Waters, USA) and the mobile phase in the chromatographic separation consisted of eluent A (0.1% formic acid in water) and eluent B (0.1% formic acetic acid in acetonitrile). Gradient used was 1% B until 1 mim, followed by a linear increased up to 6 min reaching 38% B, followed by 100% B until 8,5 min, as a cleaning step, and finally changing to initial 1% B condition up to 9.5 min. The flow rate was 0.45 ml min^{-1} and column temperature of 40°C. A Waters Xevo™ triple quadrupole mass spectrometer system (MS/MS) with an ESI interface was used in tandem MS analyses with the following conditions: capillary voltage, 2.7 kV; source temperature, 150°C; desolvation temperature, 400°C, desolvation gas flow, 800 L h^{-1}; cone gas flow, 50 L h^{-1}.

The parameters of MS/MS detection were optimized to each hormone and multiple reaction-monitoring (MRM) mode was applied in this analysis. Concentrations of 5, 10, 50, 100, 500, 1000 and 1500 ng mL^{-1} was prepared in three independents dilutions in methanol to obtain standard curve, and the analysis/quantification was performed in LC-MS/MS in triplicate. The quantification was achieved by the use of TargetLynz™ software (Waters, USA), with limit of detection (LOD) greater than 3, and the limit of quantification (LOQ) greater than 10. The recovery efficiency and matrix effect was determined according to Trufelli et al. (2010), with standard spikes (40 ng mL^{-1} of all hormones) during the extraction and detection steps. All variations in recovery and matrix effect were considered in the final concentration of each hormone.

After the verification of the variance homogeneity of the data sets, data quantified was subject to one-way analysis of variance (ANOVA). Student-Newman-Keuls (SNK) post-hoc test ($p<0.05$) was used for the

separation of means and the statistical analysis were performed using the software Statistica®.

5.2.5 Chlorophyll and carotenoid quantification

At day 30 of etiolation induction, three types of culms were selected for quantification of chlorophyll a, b, total and carotenoids: green culms, partially etiolated culms (presenting some green and white regions) and totally etiolated culms (presenting yellow to white color) (Fig.1b and Fig.1c). The extraction was realized according to Hiscox and Israelstam (1979) with modifications, where 20 mg of fresh culms were added in microtube with 2 mL of DMSO (Dimethyl sulfoxide). The microtubes were kept in bath water at 65°C for 2 h and then the extracted material were filtered and pippeted in a micro plate. The quantification was performed spectrophotometrically, at 665 nm for Chlorophyll a (Chl-a), 649 nm for Chlorophyll b (Chl-b) and 480 nm for carotenoids (C x+c) (Wellburn 1994), using microplate reader SpectraMax® Paradigm® Multi-Mode. The final concentration of chlorophyll a, b, total and carotenoids were calculated according to Wellburn (1994).

The analysis was performed in biological and technical triplicate, and the data were subject to Student's t-test to make pairwise comparisons ($p \leq 0.05$).

5.3Results

5.3.1 Chloroplast ultrastructure under light and darkness conditions

When observed under light, the chloroplasts at day 0 appeared to be huge organelles, occupying a large space of plant cell (Fig. 2a) and showing features of a mature chloroplast, i.e. the presence of double membrane envelope (the inner and outer membranes), plastoglobuli, starch grains and organized thylakoids stacked forming grana (Fig. 2b).

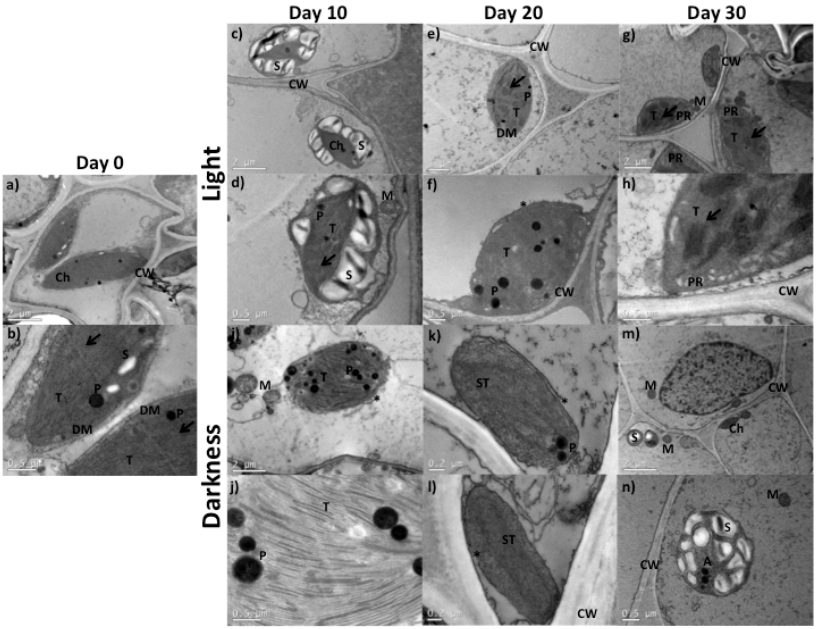

Fig. 2 Plastids ultrastructure under light and darkness conditions at days 0, 10, 20 and 30 in culture of *Guadua chacoensis in vitro* plantlets. a) Day 0 with the presence of two mature chloroplasts; b) Day 0, detail of mature chloroplast with double membrane envelope, thylakoids stacked in *grana* (black arrows), and presence of plastoglobuli; c) Incorporation of starch grain in chloroplast at day 10 in light conditions; d) Detail of a mature chloroplast, with thylakoids stacked in *grana* (black arrow), plastoglobuli and incorporation of starch grains; e) Mature chloroplast with double membrane envelope, thylakoids stacked in *grana* (black arrows), and presence of plastoglobuli, at day 20 in light conditions; f) Chloroplast in degradation with disorganized thylakoids, increased plastoglobuli number and rupture of the double membrane (*); g) Mature chloroplast with peripheral reticulum (PR) formed at day 30 in light conditions; h) Detail of PR formation; i) Chloroplast in degradation with disorganized thylakoids, increased plastoglobuli number and rupture of the double membrane (*), at day 10 in darkness conditions; j) Detail of thylakoids derangement; k-l) Stroma thylakoids formed instead of stacked thylakoids, at day 20 in darkness conditions; m) Chloroplast transition to amyloplast with starch granules incorporated in chloroplast and normal chloroplast and mitochondria, at day 30 in darkness conditions; n) Amyloplast and mitochondria. Ch: chloroplast; CW: cell wall; DM: double membrane envelope; T: thylakoids; P: plastoglobuli; S: starch; M: mitochondria; PR: peripheral reticulum; ST: stroma thylakoids; A: amyloplast; black arrows: grana formation; asterisk: absence of double membrane.

Under light development, the first modification observed at day 10 of culture was the presence of starch grains incorporated in chloroplasts (Fig. 2c). These chloroplasts presented normal features such as thylakoids stacked in *grana*, presence of plastoglobuli, starch grains in large amount and small mitochondria with spherical shape (Fig. 2d).

Mature chloroplasts were observed at day 20 in light conditions, without the presence of starch grains (Fig. 2e) and also some chloroplasts probably in senescence stage, with the absence of double envelope membrane, increased plastoglobuli number and disorganized thylakoids (Fig. 2f). At day 30 in light conditions, peripheral reticulum was observed attached to a mature chloroplast, also without the presence of starch grains (Fig. 2g-h).

The chloroplast development was influenced by the darkness condition. We observed chloroplasts with unorganized double membrane envelope and thylakoids, and increased plastoglobuli number after day 10 in darkness condition (Fig. 2i-j). The presence of mitochondria was also observed (Fig. 2i). At day 20 of darkness, the presences of stroma thylakoids, disposed in lines in chloroplast stroma and in minor number, were observed (Fig. 2k and Fig. 2l). Electron-dense plastoglobuli were also observed (Fig. 2k-l) as well as mitochondria with spherical shape (Fig. 2k). Amyloplast formation, with the incorporation of starch grain in chloroplast, was observed at day 30 in darkness (Fig. 2m). Amyloplast was also observed with the presence of spherical mitochondria in the same cell (Fig. 2n).

5.3.2 Hormone Quantification

Quantification of zeatin (Z) (Fig. 3) demonstrated that the levels of this hormone in light and darkness conditions during the time of evaluation followed a similar behavior. Zeatin levels at day 10 indicated a decrease, followed by an increase at days 20 and 30. In addition, the culms showed the highest Z endogenous level at day 30 of darkness, while the lowest Z endogenous level were observed at day 10 of light condition.

Abscisic acid (ABA) endogenous levels indicated two distinct responses under light and darkness (Fig. 4). Under light condition, ABA levels showed a decrease at day 10 and a considerable increase at day 20 that was maintained at day 30. Otherwise, ABA levels under darkness condition did not present considerable changes and, in overall, it was kept in lowest levels than in light conditions.

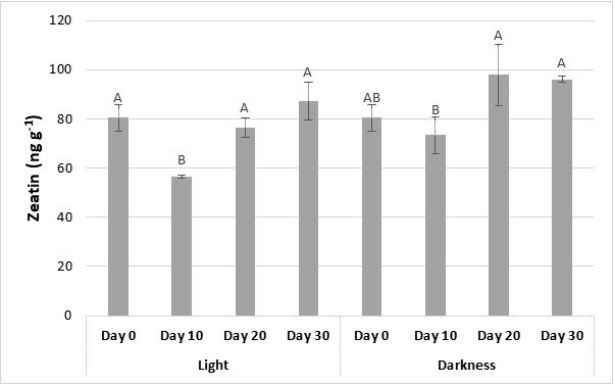

Fig 3 Zeatin (Z) endogenous levels in *Guadua chacoensis in vitro* culms after 0, 10, 20 and 30 days cultured in light and darkness conditions. Mean values ± standard deviation. Different uppercase letters indicate differences in same treatment along the evaluation time, according to SNK test ($p<0.05$). Coefficient of variation (CV) - Light: 6.74%; 0.93%; 5.05%; 8.92; Darkness: 6.74%; 10.23%; 12.75%; 1.23%.

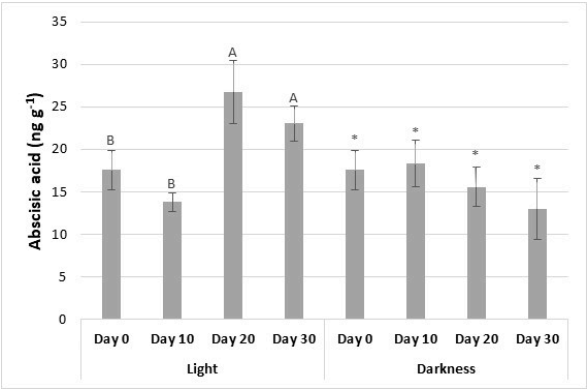

Fig. 4 Abscisic acid (ABA) endogenous levels in *Guadua chacoensis in vitro* culms after 0, 10, 20 and 30 days cultured in light and darkness conditions. Mean values ± standard deviation. Different uppercase letters indicate differences in same treatment along the evaluation time, according to SNK test ($p<0.05$) and * indicate no significant difference. CV - Light: 13.01%; 8.26%; 13.96; 8.79%. Darkness: 13.01%; 14.99%; 14.86%; 27.63%.

Gibberellic acid (GA_4) endogenous levels along light and darkness treatments (Fig. 5) revealed different responses. In light conditions, GA_4 levels showed crescent levels, with the highest level at day 30, a high accumulation of GA_4. In darkness, the level of this hormone was kept unchanged along the evaluation time.

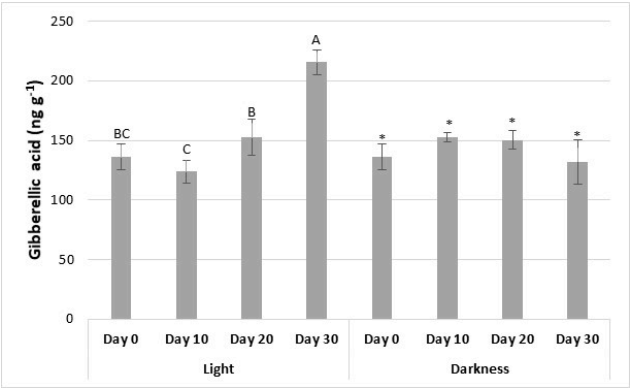

Fig. 5 Gibberellic acid (GA_4) endogenous levels in *Guadua chacoensis in vitro* culms after 0, 10, 20 and 30 days cultured in light and darkness conditions. Mean values ± standard deviation. Different uppercase letters indicate differences in same treatment along the evaluation time, according to SNK test ($p<0.05$) and and * indicate no significant difference. CV - Light: 7.79%; 7.78%; 9.93%; 4.89%. Darkness: 7.79%; 2.51%; 5.18%; 14.14%.

Jasmonic acid (JA) presented a similar response in their levels in light and dark conditions, although in light the values are higher. A considerable increase in its levels was observed at day 10, followed by a decrease in days 20 and 30 (Fig. 6). Although the JA level increased at day 10 in darkness, at days 20 and 30 these values returned to the same levels as day 0. Differently, in light treatment, JA levels were higher at days 20 and 30 than day 0, demonstrating an accumulation of JA in light conditions.

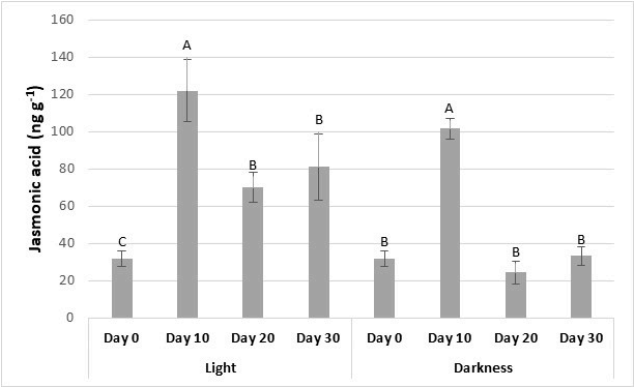

Fig. 6 Jasmonic acid (JA) endogenous levels in *Guadua chacoensis in vitro* culms after 0, 10, 20 and 30 days culltured in light and darkness conditions. Mean values ± standard deviation. Different uppercase letters indicate differences in same treatment along the evaluation time, according to SNK test ($p<0.05$). CV - Light: 13.65%; 13.55%; 11.27%; 22.14%. Darkness: 13.65%; 5.62%; 25.07; 14.87%.

5.3.3 Chlorophyll and carotenoids quantification

Chlorophyll and carotenoids quantification demonstrated that partially etiolated and etiolated culm presents, in general, lower content of these pigments than green culms, as expected (Table 1).

Table 1. Levels of chlorophyll a, b and total, and carotenoids of green, partially etiolated and etiolated culms of *Guadua chacoensis* at day 30 in culture.

	Chl a (µg mg^{-1} FM)	**Chl b (µg mg^{-1}FM)**	**Chl total (µg mg^{-1} FM)**	**C $_{x+c}$ (µg mg^{-1} FM)**
Green	0.133 a	0.040 a	0.173 a	0.0273 a
Partially Etiolated	0.042 b	0.007 a	0.049 b	0.0126 b
Etiolated	*	*	*	*

Different lowercase letters in column indicate significant differences according to the Student's t-test ($p < 0.05$). *Values below the detection limit. FM: fresh material. C $_{x+c}$: carotenoids

5.4 Discussion

5.4.1 Chloroplast ultrastructure under light and darkness conditions

Chloroplast ultrastructure analyses during 30 days of *in vitro* culture of *G. chacoensis* culms provided some evidences that light is a key factor in normal chloroplast development. In this condition, chloroplasts usually presented a mature structure, with all the typical characteristics, including double membrane envelope, thylakoids organized in *grana* and plastoglobuli. In contrast, in darkness condition, we observed some atypical characteristics, such as the reduction of thylakoids membranes.

In vitro cultures usually require carbohydrate supplementation as a source of carbon and osmotic agents, as they are in a photomixotrophic environment with low rates of gas exchange, low CO_2 concentration and poor quality of light conditions (Sandhu et al. 2017). As a consequence of the environment conditions, *in vitro* cultures are not able to produce its own carbohydrate, so sucrose is usually supplemented to the culture medium.

Sucrose can be catabolized and stored as starch, the major form of carbohydrate storage in plants, with its synthesis occurring inside the plastids (Geigenberger 2011). In our study, we observed the accumulation of starch granules in the early stages of chloroplast development under light at day 10 (Fig.2c-d), and it is subsequent absence in days 20 and 30 (Fig.2e-g). Previous studies (Mangat et al. 1990; Sulpice et al. 2009; Lee and Huang 2013) have demonstrated the relationship between the starch granules formation as a consequence of sucrose supplementation to different species *in vitro* cultured, and in both organogenesis and somatic embryogenesis morphogenetic routes.

Mangat et al. (1990) demonstrated that during *Begonia rex* organogenesis, with addition of 3% sucrose in the culture medium, starch accumulation was observed in the first days of culture, with a maximum in about 4-6 days. These starch granules decreased as the shoots were under development, concluding that starch was probably an energy source used in plant initiation and posterior development. Lee and Huang (2013), studying callus of two rice cultivars, observed that in the most responsive cultivar, the glucose, fructose and starch levels were higher during callus induction and were gradually reduced when transferred to regeneration culture medium. Sulpice et al. (2009) also emphasizes that starch accumulation can be considered the driver of

growth and development of plants because they control carbon availability for plants.

In the present study, we propose that starch accumulation in chloroplasts at day 10 in light condition could be associated with the sucrose supplementation in culture medium, which was stored as starch. Furthermore, the absence of these starch granules in chloroplasts during latter stages (days 20 and 30) could be a consequence of the use of these carbohydrates as energy source to sustain plant development process.

During chloroplast development of some species, a structure named chloroplast peripheral reticulum (PR) can appears connected to the inner envelope membrane. The presence of PR could be detected in our study, at day 30 of light condition (Fig.2 g-h). These PRs observed could be classified as PR Type II, which are characterized as a system of densely and packed vesicles in the plastid periphery and are usually found in C3 and C4 species (Wise and Harris 1984). Many authors suggested that PR is an adaptive structure that facilitates the metabolites transport into and out the chloroplast, as the presence of PRs increase the area of the inner envelope membrane (Laetch 1968; Heldt and Sauer 1971; Wise 2007; Szczenpanik and Sowiński 2014).

Chloroplast PR sometimes is associated with low respiration rates, where the photorespiration is probably dependents of the PR presence, as proposed by Hilliard and West (1971). PR presence is also related with high light intensity according to Szczenpanik and Sowiński (2014) that have observed PR system better developed under high light intensity than in low light intensity in four different grasses species. The same authors additionally investigated if PR is a phylogenetic partner or a photosynthetic partner in grasses, and concluded that PR is usually associated with photosynthetic behavior, and that in C4 plants this may be a fundamental adaptation for metabolic transport during photosynthesis reaction. In our results, the presence of PR in chloroplasts at day 30, in light condition (Fig. 2h), corroborates with the possible function as facilitator of metabolic transport. At this step of culture, the quantity of metabolites available for plant growth are probably scarce, and PR can provide the migration of some metabolites from the outside to the inside of the chloroplast, allowing chloroplast to keep their functionality and ensure plant development.

During the life cycle of organelles, senescence is a natural occurring process that corresponds to the last development step before organelles death. Chloroplast is the first organelle to show ultrastructural indicators of senescence, such as thylakoids disorder, increased number of plastoglobuli and collapse of double membrane envelope (Blswal and

Biswal 1988). In the present study, these same signals were observed in chloroplast at day 10 of darkness condition (Fig.2i-j), as well as in chloroplast at day 20 of light condition (Fig.2 e-f).

Senescence can be seen as a natural process along plastids development or can be induced by some stress condition. Plastoglobuli number can increase in response to abiotic conditions that promote stress in photosynthetic apparatus (Austin et al. 2006), such as drought and high saline concentration. Bréhélin et al. (2007) also reported the involvement of plastoglobuli in stress response, and suggested that plastoglobuli should be seen as a cellular organelle involved with secondary metabolism compounds and stress response, rather than as a simples storage organelle. In our study, senescence chloroplasts in day 20 of light could be associated with natural degradation along plastid living. In contrast, the senescing chloroplast observed during the darkness growth, at day 10, is probably consequence of the dark treatment.

Although etioplast formation was expected to occur during dark growth, in our study it was not observed. The most important characteristic of etioplasts is the unique paracrystalline membrane system known as prolamellar bodies (PLB) connected to prothylakoids membranes (Solymosi and Keresztes 2012). In our study, at day 20 of darkness (Fig. 2k), it was observed the presence of stroma thylakoids organized in lines, without the presence of PLB, indicating that these plastids can not be classified as etioplast. We believe that etioplast formation was inhibited as a consequence of the cytokinins supplementation in the culture medium, as well as the high endogenous level of Z observed (Fig. 3). Moreover, the conversion of chloroplast to etioplast could be repressed because the initial chloroplasts were fully developed.

Cytokinins are usually related with chloroplast maturation (Stetler and Laetsch 1965; Polanská et al. 2006; Cortleven and Schmülling 2015). Stetler and Laetsch (1965) demonstrated that chloroplast maturation of tobacco culture tissues were dependent on the kinetin supplementation to develop a complete mature chloroplast. Also that cultures growth in dark conditions presented few thylakoids and was not able to develop *grana* and PLB, not converting to etioplast. Even though plastids are known to be easily reversible between the different types, the only exception of total reversibility is between fully development chloroplast to etioplast (Lindquist et al. 2016).

5.4.2 Hormone Quantification

The signaling function of phytohormone in many processes of plant development and in physiological and stress response (Erland et al. 2017) indicates that quantification of endogenous levels of hormones can provide important insights about the behavior of *G. chacoensis* culms under etiolation condition.

In our study, Z endogenous levels showed a similar response in light and darkness conditions, with a considerable decrease at day 10, follow by and increase at day 20 and 30 (Fig. 3) Cytokinins appear to be involved in sucrose transport and metabolism (Gibson 2004), and the sucrose supplementation in culture medium could lead to alterations in its endogenous levels.

A previous report has shown that the glucose supplementation in darkness growth can promote a decrease followed by an increase in cytokinins endogenous levels (Stirk et al. 2014). This behavior was considered a consequence of the plant metabolism adjustment to initiate CK biosynthesis, using the energy supply provided by the glucose supplementation (Stirk et al. 2014). The same authors additionally proposed that energy supply is most important to cytokinins biosynthesis than light supply.

Suzuki et al. (2010) also found similar results in dark-induced treatment of *Catasetum fimbriatum* (Orchidaceae), and proposed that an increase in CK endogenous level could be understood as a signal of nutrient translocation to guarantee etiolation. In our study, we observed a higher Z endogenous content in comparison with the other hormones analyzed. We believe that the accumulation of this hormone in *G. chacoensis* culms could be a result of the BAP supplementation in the culture medium.

Additionally, CK are strongly related with plastids, such as chloroplast maturation (Stetler and Laetsch 1965; Polanská et al. 2006; Cortleven and Schmülling 2015), thylakoids formation (Cortleven and Schmülling 2015) expression of some chloroplast related genes (Parthier 1979), and inhibition of etioplast formation as we proposed. So, the higher endogenous levels observed compared to the other analyzed phytohormones can be expected in our results.

ABA corresponds to another class of phytohormone directly interconnected with chloroplast. ABA has been associated with the repression of gene expression of some important chloroplast-related genes (Kusnetsov et al. 1994; Yamburenko et al. 2013). In the present work, the quantification of ABA endogenous level in darkness did not

present considerable changes in their levels along the 30 days of evaluation (Fig.4). This behavior suggests a possible reduction in the ABA biosynthesis in darkness, which can be related with the reduction of carotenoids (ABA precursors) endogenous content (Seo and Koshiba 2002).

In our study, we also observed lower carotenoids endogenous content, as well as lower levels of chlorophyll a, b and total in etiolated and partially etiolated material at day 30 (Table 1). Thus, we proposed that ABA biosynthesis in darkness condition is inhibited as a consequence of the reduction in carotenoids content. Grybz et al. (2017) also reported reduction on ABA endogenous levels in etiolated explants as compared with light-cultured explants, and also suggested that it could be a consequence of the reduction in carotenoids levels.

Additionally, the reduction of ABA endogenous levels during darkness condition can be associated with reduction in thylakoids number during darkness, as most of carotenoids are found in thylakoids membranes (Sun et al. 2017). This could be perceived especially at day 30 in darkness, where a drastically reduction in the presence of thylakoids membranes was observed when compared with day 30 of light treatment.

GA influence in plant morphogenesis has already been shown in previous studies (Fleet and Sun 2005; Alabadí et al. 2008; Li et al. 2015). GA quantification in *G. chacoensis* culms showed that GA_4 endogenous levels in light are higher than in darkness conditions, and likewise, the levels on darkness growth are almost unchangeable (Fig. 5). The reduction in the production of GA_4 in darkness treatment is probably correlated with the repression of photomorphogenesis driven by PIFs. Photomorphogenesis response can also be repressed indirectly by the action of DELLA proteins that provoke repression of PIFs (Li et al. 2015). Additionally, DELLA proteins are already characterized as negative regulator of GA response (Sun 2008).

Li et al. (2015) proposed that the gene *DET1* is responsable by repression of photomorphogenesis in darkness. Its action is drived in parts by the negative regulation of DELLA proteins, which is a promoter of photomorphogenesis. These negative regulation estimulated the repression of PIFs, consequently photomorphogenesis response is altered. The same authors also demonstrated that although gene expression quantification of DET1 shows difference, the content of bioactive GA does not show considerable difference between the samples evaluated.

Jasmonic acid response to biotic stress, such as pathogens attack, is further best characterized than its response to abiotic stress. However, it is well known that plant response to biotic and abiotic stress is accomplished by changes in endogenous levels of hormones (Du et al. 2013). Accumulation of JA endogenous levels in *G. chacoensis* culms subjected to light condition and its reduction in darkness proposes a negative response in JA biosynthesis under stress caused by etiolation treatment (Fig. 6).

A significant relationship between JA biosynthesis and light has been demonstrated. A variety of JA co-receptors can influence light response and light-signaling compounds such as phytochrome can influence JA gene expression and response (Kazan and Manners 2011). So, the higher JA endogenous levels in light treatment are probably associated with the active JA biosynthesis when culms are exposed to light. As etiolation is sensed by the plant as an abiotic stress, the increased drastically decrease at day 30 in darkness conditions demonstrated that darkness evokes a reduction in JA endogenous levels.

Our results regarding JA decrease in darkness are in agreement with those reported by Du et al. (2013). These authors observed that the heat stress in rice reduced JA endogenous levels and also the gene expression of some JA biosynthesis genes. Despite of this, it is important to emphasize that knowledge about changes in JA levels in plants in response to abiotic stress is still reduced, and different responses can be observed to different abiotic stress. Du et al. (2013) also observed that cold and drought treatments cause opposite responses, with increased JA levels as well as up-regulation in gene expression. Many questions about JA response to abiotic stress still open; however, our study provides new insights about plant response under darkness growth.

5.5 Concluding remarks

Taken together, our results indicated different starch accumulation pattern in chloroplasts in light or darkness conditions. In light treatment, a decreased number of starch grains was observed during *in vitro* culture, probably associated to providing energy for plant initiation and posterior development. Differently, in darkness conditions, improved amyloplast formation was observed. Complete etioplast formation was not observed in darkness conditions, as expected. We hypothesize that this process could be inhibited by cytokinin supplementation in the culture medium.

Variable responses in endogenous levels of phytohormones, as a consequence of light or darkness conditions, could be observed in *G. chacoensis* culms. ABA, GA and JA biosynthesis decreased in darkness conditions, suggesting a strong relationship between light and these phytohormone levels. Additionally, Z and ABA showed to be closely related with plastids formation, by a positive and negative way, respectively. The first one influences chloroplast maturation, and the second were apparently influenced by carotenoid levels.

References

Alabadí D, Gallego-Bartolomé J, Orlando L, García-Cárcel L, Rubio V, Martínez C, Blázquez MA (2008) Gibberellins modulate light signaling pathways to prevent *Arabidopsis* seedling de-etiolation in darkness. Plant J 53:324-335. https://doi.org/10.1111/j.1365-313X.2007.03346.x

Austin JR, Frost E, Vidi PA, Kessler F, Staehelin L A (2006) Plastoglobules are lipoprotein subcompartments of the chloroplast that are permanently coupled to thylakoid membranes and contain biosynthetic enzymes. Plant Cell 18:1693-1703. https://doi.org/10.1105/tpc.105.039859

Blswal UC, Biswal B (1988) Ultrastructural modifications and biochemical changes during senescence of chloroplasts. Int Rev Cytol 113:271-321. https://doi.org/10.1016/S0074-7696(08)60851-7

Bystriakova N, Kapos V, Lysenko I (2004) Bamboo biodiversity: Africa, Madagascar and the Americas. UNEP-WCMC Biodiversity

Bréhélin C, Kessler F, van Wijk KJ (2007) Plastoglobules: versatile lipoprotein particles in plastids. Trends Plant Sci 12:260-266. https://doi.org/10.1016/j.tplants.2007.04.003

Cortleven A, Schmülling T (2015) Regulation of chloroplast development and function by cytokinin. J Exp Bot 66: 4999-5013. https://doi.org/10.1093/jxb/erv132

Du H, Liu H, Xiong L (2013) Endogenous auxin and jasmonic acid levels are differentially modulated by abiotic stresses in rice. Front Plant Sci 4:1-10 https://doi.org/10.3389/fpls.2013.00397

Erland LAE, Shukla MR, Glover WB, Saxena PK (2017) A simple and efficient method for analysis of plant growth regulators: a new tool in the chest to combat recalcitrance in plant tissue culture. Plant Cell Tissue Organ Cult 131:459-470. https://doi.org/10.1007/s11240-017-1297-1

Filgueiras TS, Santos-Gonçalves AP (2004) A checklist of the basal grasses and bamboos in Brazil (Poaceae). J Amer Bamboo Soc 18:7-18.

Fleet CM, Sun TP (2005) A DELLAcate balance: the role of gibberellin in plant morphogenesis. Curr Opin Plant Biol 8:77-85. https://doi.org/10.1016/j.pbi.2004.11.015

Fraga HPF, do Nascimento Vieira L, Puttkammer CC, dos Santos HP, de Andrade Garighan J, Guerra MP (2016) Glutathione and abscisic acid supplementation influences somatic embryo maturation and hormone endogenous levels during somatic embryogenesis in

Podocarpus lambertii Klotzsch *ex* Endl. Plant Sci 253:98-106. https://doi.org/10.1016/j.plantsci.2016.09.012

Geigenberger P (2011) Regulation of starch biosynthesis in response to a fluctuating environment. Plant Physiol 155: 1566-1577. https://doi.org/10.1104/pp.110.170399

George, EF, Hall MA, De Klerk GJ (2008) Effects of the physical environment. In: Plant propagation by tissue culture, Springer, Netherlands, pp. 423-464. https://doi.org/10.1007/978-1-4020-5005-3_12

Gibson SI (2004) Sugar and phytohormone response pathways: navigating a signalling network. J Exp Bot 55:253-264. https://doi.org/10.1093/jxb/erh048

Grzyb M, Kalandyk A, Waligórski P, Mikuła A (2017) The content of endogenous hormones and sugars in the process of early somatic embryogenesis in the tree fern *Cyathea delgadii* Sternb. Plant Cell Tissue Organ Cult 129:387-397. https://doi.org/10.1007/s11240-017-1185-8

Guerreiro C (2014) Flowering cycles of woody bamboos native to southern South America. J Plant Res 127: 307-313.https://doi.org/10.1007/s10265-013-0593-z

Heldt HW, Sauer F (1971) The inner membrane of the chloroplast envelope as the site of specific metabolite transport. Biochim Biophys Acta 234: 83-91. https://doi.org/10.1016/0005-2728(71)90133-2

Hilliard JH, West SH (1971) The association of chloroplast peripheral reticulum with low photorespiration rates in a photorespiring plant species. Planta 99:352-356. https://doi.org/10.1007/BF00385827

Hiscox JT, Israelstam GF (1979) A method for the extraction of chlorophyll from leaf tissue without maceration. Can J Bot 57:1332-1334. https://doi.org/10.1139/b79-163

Jiménez VM, Guevara E (2007) Micropropagation of bamboo species through axillary shoot proliferation. In: Protocols for micropropagation of woody trees and fruits, Springer Netherlands, pp. 465-476. https://doi.org/10.1007/978-1-4020-6352-7_43

Jiménez VM, Castillo J, Tavares E, Guevara E, Montiel M (2006) *In vitro* propagation of the neotropical giant bamboo, *Guadua angustifolia* Kunth, through axillary shoot proliferation. Plant Cell Tissue Organ Cult 86:389-395. https://doi.org/10.1007/s11240-006-9120-4

Kazan K, Manners JM (2011) The interplay between light and jasmonate signalling during defence and development. J Exp Bot 62:4087-4100. https://doi.org/10.1093/jxb/err142

Kelchner SA, Bamboo Phylogeny Group (2013) Higher level phylogenetic relationships within the bamboos (Poaceae: Bambusoideae) based on five plastid markers. Mol Phylogenet Evol 67: 404-413. https://doi.org/10.1016/j.ympev.2013.02.005

Kusnetsov VV, Oelmüller R, Sarwat MI, Porfirova SA, Cherepneva GN, Herrmann RG, Kulaeva ON (1994) Cytokinins, abscisic acid and light affect accumulation of chloroplast proteins in *Lupinus luteus* cotyledons without notable effect on steady-state mRNA levels. Planta 194: 318-327. https://doi.org/10.1007/BF00197531

Laetsch WM (1968) Chloroplast specialization in dicotyledons possessing the C4-dicarboxylic acid pathway of photosynthetic CO_2 fixation. Am J Bot 55:875-883.

Lee ST, Huang WL (2013) Cytokinin, auxin, and abscisic acid affects sucrose metabolism conduce to *de novo* shoot organogenesis in rice (*Oryza sativa* L.) callus. Bot Stud 54: 5. https://doi.org/10.1186/1999-3110-54-5

Li K, Gao Z, He H, Terzaghi W, Fan LM, Deng XW, Chen H (2015) *Arabidopsis* DET1 represses photomorphogenesis in part by negatively regulating DELLA protein abundance in darkness. Mol Plant 8:622-630. https://doi.org/10.1016/j.molp.2014.12.017

Lindquist E, Solymosi K, Aronsson H (2016) Vesicles are persistent features of different plastids. Traffic 17: 1125-1138. https://doi.org/10.1111/tra.12427

Lochmanová G, Zdráhal Z, Konečná H, Koukalová Š, Malbeck J, Souček P, Brzobohatý B (2008) Cytokinin-induced photomorphogenesis in dark-grown *Arabidopsis*: a proteomic analysis. J Exp Bot 59:3705-3719. https://doi.org/10.1093/jxb/ern220

Londoño X (1998) A decade of observations of a *Guadua angustifolia* plantation in Colombia. J Amer Bamboo Soc 12:37-42.

Londoño X, Peterson PM (1992) *Guadua chacoensis* (Poaceae: Bambuseae), its taxonomic identity, morphology, and affinities. Novon (St Louis) 2:41-47.

Mangat BS, Pelekis MK, Cassells AC (1990) Changes in the starch content during organogenesis in *in vitro* cultured *Begonia rex* stem explants. Physiol Plant 79: 267-274. https://doi.org/10.1111/j.1399-3054.1990.tb06741.x

Morel G, Wetmore RH (1951) Tissue culture of monocotyledons. Am J Bot 38:138-140.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Ornellas TS, Werner D, Holderbaum DF, Scherer RF, Guerra MP (2017) Effects of Vitrofural, BAP and meta-Topolin in the *in vitro* culture of *Dendrocalamus asper*. Acta Hortic 1155: 285-292. https://doi.org/ 10.17660/ActaHortic.2017.1155.41

Parthier B (1979) The role of phytohormones (cytokinins) in chloroplast development. Biochem Physiol Pflanz174:173-214. https://doi.org/10.1016/S0015-3796(17)30575-9

Polanská L, Vičánková A, Nováková M, Malbeck J, Dobrev PI, Brzobohatý B, Vaňková R, Macháčková I (2006) Altered cytokinin metabolism affects cytokinin, auxin, and abscisic acid contents in leaves and chloroplasts, and chloroplast ultrastructure in transgenic tobacco. J Exp Bot 58:637-649. https://doi.org/10.1093/jxb/erl235

Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17:208-212.

Sandhu M, Wani SH, Jiménez VM (2017) *In vitro* propagation of bamboo species through axillary shoot proliferation: a review. Plant Cell Tissue Organ Cult 132:27-53. https://doi.org/10.1007/s11240-017-1325-1

Seo M, Koshiba T (2002) Complex regulation of ABA biosynthesis in plants. Trends Plant Sci 7:41-48.

Singh SR, Singh R, Kalia S, Dalal S, Dhawan AK, Kalia RK (2013) Limitations, progress and prospects of application of biotechnological tools in improvement of bamboo - a plant with extraordinary qualities. Physiol Mol Biol Plants 19:21-41. https://doi.org/10.1007/s12298-012-0147-1

Solymosi K, Aronsson H (2013) Etioplasts and their significance in chloroplast biogenesis. In: Plastid development in leaves during growth and senescence, Springer, Netherlands, pp. 39-71. https://doi.org/10.1007/978-94-007-5724-0_3

Solymosi K, Keresztes Á (2012) Plastid structure, diversification and interconversions II. Land plants. Curr Chem Biol 6:187-204.

Soreng RJ, Peterson PM, Romaschenko K, Davidse G, Zuloaga FO, Judziewicz EJ, Morrone O (2015) A worldwide phylogenetic classification of the Poaceae (Gramineae). J Syst Evol 53:117-137. https://doi.org/10.1111/jse.12150

Staehelin LA (2003) Chloroplast structure: from chlorophyll granules to supra-molecular architecture of thylakoid membranes. Photosynth Res 76:185-196. https://doi.org/10.1023/A:1024994525586

Stetler DA, Laetsch WM (1965) Kinetin-induced chloroplast maturation in cultures of tobacco tissue. Science 149:1387-1388. https://doi.org/10.1126/science.149.3690.1387

Stirk WA, Bálint P, Tarkowská D, Novák O, Maróti G, Ljung K, Van Staden J (2014) Effect of light on growth and endogenous hormones in *Chlorella minutissima* (Trebouxiophyceae). Plant Physiol Biochem 79:66-76. https://doi.org/10.1016/j.plaphy.2014.03.005

Sulpice R, Pyl ET, Ishihara H, Trenkamp S, Steinfath M, Witucka-Wall H, Gibon Y, Usadel B, Poree F, Piques MC, Korff MV, Steinhauser MC, Keurentjes JJB, Guenther M, Hoehne M, Selbig J, Fernie AR, Altmann T, Stitt M (2009) Starch as a major integrator in the regulation of plant growth. Proc Nat Acad Sci USA 106:10348-10353. https://doi.org/10.1073/pnas.0903478106

Sun TP (2008) Gibberellin metabolism, perception and signaling pathways in *Arabidopsis*. *Arabidopsis* Book 6:e0103 https://doi.org/10.1199/tab.0103

Sun T, Yuan H, Cao H, Yazdani M, Tadmor Y, Li L (2017) Carotenoid metabolism in plants: the role of plastids. Mol Plant 11:58-74.https://doi.org 10.1016/j.molp.2017.09.010

Suzuki RM, Kerbauy GB, Pescador R, Purgatto E, Ceccantini GC, Ferreira, WDM (2010) Dark-induced hormone changes coincide with the resumption of light-inhibited shoot growth in *Catasetum fimbriatum* (Orchidaceae). J Plant Physiol 167:375-381. https://doi.org/10.1016/j.jplph.2009.10.002

Szczepanik J, Sowiński P (2014) The occurrence of chloroplast peripheral reticulum in grasses: a matter of phylogeny or a matter of function? Acta Physiol Plant 36:1133-1142. https://doi.org/10.1007/s11738-014-1488-x

Trufelli H, Palma P, Famiglini G, Cappiello A (2011) An overview of matrix effects in liquid chromatography–mass spectrometry. Mass Spectrom Rev 30:491-509. https://doi.org/10.1002/mas.20298

Vega AS, Hernández JC (2008) La floración de *Guadua chacoensis* (Poaceae, Bambusoideae, Bambuseae). Agron Ambient 28:107-110.

Wellburn AR (1994) The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. J Plant Physiol, 144: 307-313. https://doi.org/10.1016/S0176-1617(11)81192-2

Wise RR (2007) The diversity of plastid form and function. In: The structure and function of plastids. Springer, Netherlands, pp. 3-26. https://doi.org/10.1007/978-1-4020-4061-0_1

Wise RR, Harris JB (1984) The three-dimensional structure of the *Cyphomandra betacea* chloroplast peripheral reticulum. Protoplasma 119:222-225. https://doi.org/10.1007/BF01288877

Yamburenko MV, Zubo YO, Vanková R, Kusnetsov VV, Kulaeva ON, Börner T (2013) Abscisic acid represses the transcription of chloroplast genes. J Exp Bot 64:4491-4502. https://doi.org/10.1093/jxb/ert258

6 CONCLUSÕES E PERSPECTIVAS

Os resultados apresentados neste trabalho indicam que o estiolamento *in vitro* pode afetar os parâmetros morfológicos, bioquímicos e histológicos de plantas pré-estabelecidas *in vitro* de *G. chacoensis*. Isto porque o uso desta técnica resultou em algumas alterações importantes na morfologia das plantas, na biossíntese de poliaminas e hormônios vegetais, bem como na configuração dos feixes vasculares e dos cloroplastos, organelas importantes para o crescimento e desenvolvimento de plantas.

Plantas mantidas sob condição de luz parecem ter um metabolismo acelerado, apresentando maior proliferação de brotos, apesar de não diferirem altura dos colmos quanto as plantas são mantidas no escuro. Ademais, plantas mantidas na luz parecem apresentar cloroplastos totalmente funcionais, tendo uma coloração esverdeada, enquanto que plantas mantidas no escuro, após 20 dias, começam a apresentar alterações em sua coloração, ficando amareladas a esbranquiçadas, sendo esta uma características típica de plantas estioladas e ainda, que os brotos que se desenvolveram no escuro já apresentavam estas características. Embora as plantas tenham começado a conversão de plantas verdes para plantas estioladas, observou-se que este tempo de mantimento *in vitro* não foi suficiente para obter brotos totalmente estiolados de *G. chacoensis*. Desta forma, estudos sequentes com tempo de manutenção *in vitro* superior a 30 dias, serão importantes para definir o exato comportamento de plantas de *G. chacoensis* no estiolamento.

Os teores de poliaminas obtidos neste trabalho evidenciam a existência de uma correlação positiva entre a luz e a sua biossíntese. Elas estão completamente interligados, visto que alterações nos conteúdos de poliaminas foram mais expressivos em materiais vegetais mantidos na luz e ainda, de forma generalizada, os conteúdos do material vegetal mantido no escuro apresentava-se inferiores ao da luz. Embora estudos tenham sido encontrados comprovando a interação entre fitocromos - receptores da luz nas plantas, biossíntese de poliaminas por análises moleculares da expressão do gene ADC - um dos precursor da rota de biossíntese de PAs e respostas fotomorfogênicas, não foram encontrados estudos com bambus. Diante disso sugere-se que a realização da análise da expressão gênica do gene ADC em plantas de *G. chacoensis* nos mesmos tempos analisados neste estudo seriam de grande valia para a confirmação desta hipótese.

A biossíntese de hormônios vegetais também parece estar ligada a luz de acordo com os resultados obtidos. Respostas variadas nos teores endógenos dos fitormônios Z, ABA, GA e JA para as condições de luz e escuro foram observadas. Os teores de Z praticamente não foram afetados pelo estiolamento, enquanto que nos conteúdos de ABA, GA e JA ocorreram decréscimo, indicando que sua biossíntese é afetada pelo estiolamento. Estas diferentes respostas estão completamente interligadas com a ação destes hormônios vegetais nos diferentes processos relacionados ao crescimento e desenvolvimento das plantas. Por exemplo, a presença de zeatina em quantidades adequadas no escuro esta relacionada com a atuação deste hormônio no processo de maturação e desenvolvimento dos cloroplastos. Concomitantemente, as reduções dos teores de ABA no escuro, estão relacionadas com o decréscimo nos teores de carotenóides - precursores da biossíntese de ABA, que ocorre no escuro.

A ausência da visualização de etioplastos em materiais vegetais mantidos no escuro analisados através da técnica de MET, no presente estudo, se justifica pela ausência de plantas totalmente estioladas e ainda, que este processo pode ter sido inibido pela presença de citocina no meio de cultura, sendo esta considerada inibidora do processo de conversão de plastídios a etioplastos. Novos estudos com objetivo de elucidar pontos de controle da conversão de cloroplastos a etioplastos utilizando meio de cultura com e sem a adição de citocinina serão importantes para a confirmação desta hipótese. Ainda, diferentes comportamentos quando a presença de grãos de amido na luz e no escuro parecem estar relacionados com os processos de crescimento e desenvolvimento dos materiais e a necessidade do uso de amido nestes processos.

A caracterização dos colmos por meio de microscopia óptica evidencia que o tempo de estiolamento utilizado neste estudo parece não influenciar drasticamente a configuração geral dos colmos, e ainda, que os feixes vasculares parecem ser os primeiros tecidos influenciados pelo estiolamento.

Como perspectivas de trabalho no doutorado pretendo tentar responder algumas das perguntas que ainda ficaram abertas nesta dissertação, e ainda desenvolver estudos a respeito da quantificação de carboidratos e de metilação de DNA global de materiais de *G. chacoensis* submetidos ao processo de estiolamento. Acredita-se que a resolução destas perguntas levariam a uma maior entendimento da influência do uso de estiolamento *in vitro* em plantas de *G. chacoensis* e obtenção de um número maior de propágulos obtidos por

micropropagação. Além disso, pretendo estudar o processo de embriogênese somática para esta espécie, visto que os estudos de propagação de bambus por meio desta rota ainda são escasso.