Universidade de São Paulo
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Controle do metabolismo e desenvolvimento da
orquídea epífita *Catasetum fimbriatum* em resposta à
incidência de luz no sistema radicular.

Control of metabolism and development of the epiphyte
orchid *Catasetum fimbriatum* in response to light
incidence on the root system.

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Sobre a ciência

“Nem sempre funciona, mas em teoria funciona sempre“.

Paulo Marcelo Rayner Oliveira

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1. INTRODUÇÃO GERAL

1.1. O surgimento do sistema radicular

Na condição de organismos sésseis, a evolução dos vegetais terrícolas foi orquestrada pelo surgimento de mecanismos adaptativos marcados pela elevada plasticidade morfológica e metabólica em resposta às condições ambientais circundantes, os quais permitiram maior eficiência na aquisição e no uso dos recursos disponíveis a fim de garantir sua sobrevivência e sucesso reprodutivo (Aasamaa e Aphalo, 2016; Degenhardt e Gimmmler, 2000; Muller e Schmidt, 2004; Robert e Friml, 2009; Walter e Schurr, 2005).

Diversas inovações adaptativas contribuíram para a irradiação das plantas por toda a superfície terrestre (Jones e Dolan, 2012), sendo que o surgimento de rizoides - estruturas primitivas do sistema radicular - durante o período Devoniano (416 a 310 milhões de anos atrás) contribuiu massivamente para a conquista do ambiente terrestre pelas plantas. Por exemplo, postula-se que o alcance de regiões mais profundas do solo por estruturas radiculares permitiu uma maior eficiência na infiltração da água e na aeração do substrato ocupado por organismos vegetais, resultando em importantes modificações físico-químicas do solo (Berner, 1997; Gibling e Davies, 2012; Xue et al., 2016). Além disso, a redução dos níveis de dióxido de carbônico (CO_2) na atmosfera foi acelerada pelo surgimento dos sistemas radiculares devido à liberação de ácidos na rizosfera. De acordo com tal proposição, os ácidos liberados pelas raízes das plantas causaram a erosão de silicatos que, em reação com o cálcio e o magnésio, acelerou o sequestro do CO_2 atmosférico (Berner, 1997). De tal modo, o conjunto dessas alterações levou ao estabelecimento de uma interface entre solo e atmosfera, onde a água presente no solo era absorvida pelos rizoides e transportada pelo corpo da planta até a parte caulinar, sendo, então, perdida para o ambiente na forma de vapor e, com isto, promovendo o estabelecimento de um fluxo de água entre solo e atmosfera. Tal ciclo hídrico afetou substancialmente o regime pluviométrico na face da terra e, como consequência, propiciou o surgimento de uma litosfera e atmosfera propícias à colonização pelas plantas terrícolas primitivas (Algeo e Scheckler, 1998; Rellán-Álvarez et al., 2016).

Assim, a partir do surgimento de estruturas radiculares relativamente simples, diversas modificações morfológicas presentes no corpo vegetal, tais como o surgimento de um sistema vascular altamente desenvolvido capaz de se adaptar aos mais diversos tipos de substrato, permitiram o aparecimento de uma diversidade considerável de sistemas radiculares com variadas características adaptativas (Kenrick e Strullu-Derrien, 2014), as quais estavam

voltadas não apenas à fixação do vegetal ao substrato, mas também à aquisição de água e nutrientes essenciais para o desenvolvimento vegetal.

1.2. Diversidade morfo-funcional radicular: destaque para as raízes de plantas epífitas

Dentre os ambientes ocupados pelos organismos vegetais, o ambiente epifítico é considerado um dos mais instáveis devido à disponibilidade irregular de nutrientes e água no dossel, principalmente, durante períodos prolongados de seca (Benzing e Ott, 1981). O sucesso evolutivo de certos grupos de plantas na conquista do nicho epifítico encontra-se intimamente relacionado ao surgimento de especializações morfológicas e metabólicas essenciais à interceptação, absorção e uso eficiente da água e nutrientes minerais (Benzing et al., 1983). Dentre tais especializações, destacam-se, por exemplo, certas modificações ocorridas no sistema radicular de espécies epífitas da família Orchidaceae – uma das famílias de plantas mais diversificadas, representada por cerca de 19.000 espécies pertencentes a 543 gêneros, sendo 69% delas de hábito epifítico. Além disso, vale ressaltar que estima-se que 68% de todas as plantas epífitas pertençam à família das orquídeas (Benzing, 1990; Pridgeon, 1986; Zotz, 2016).

Dentre as especializações morfológicas essenciais à interceptação e absorção de água e nutrientes minerais presentes nas raízes de orquídeas epífitas, destacam-se, sobretudo, o velame e a exoderme (Benzing et al., 1982b; Moreira et al., 2009; Zotz e Winkler, 2013). O primeiro é um tecido especializado derivado da epiderme de vital importância para essas plantas, uma vez que facilita a absorção de água e nutrientes, e protege contra a desidratação e alta incidência luminosa (Benzing et al., 1983; Dycus e Knudson, 1957; Pridgeon, 1987; Zotz e Winkler, 2013). Além disso, raízes clorofiladas estão presentes em muitas orquídeas epífitas, sendo a coloração verde das mesmas derivada da presença de plastídios localizados, principalmente, no parênquima cortical devido à exposição da raiz à incidência luminosa. Nessas raízes, encontra-se todo o aparato fotossintético necessário para fixação de CO₂, indicando, portanto, a potencial capacidade de realizarem atividade fotossintética (Ho et al., 1983; Moreira et al., 2009; Martin et al., 2010). No entanto, com exceção de algumas espécies de orquídeas epífitas desprovidas de sistema caulinar, muito pouco se sabe sobre o impacto das raízes clorofiladas no balanço de carbono em todo o corpo de orquídeas epífitas portadoras de sistema caulinar bem desenvolvido. Essa questão é ainda mais pertinente quando levamos em consideração certas espécies de orquídeas epífitas, tais como as pertencentes ao gênero *Catasetum*, que apresentam folhagem sazonal e um sistema radicular

que representa pelo menos metade da biomassa que compõe o corpo vegetativo, o qual se encontra em grande proporção exposto à luz (Benzing, 1990).

1.3. Sinalização luminosa no controle do desenvolvimento radicular

A luz é o sinal posicional do ambiente mais importante para as plantas, podendo atuar como fonte de energia na fotossíntese (Hohmann-Marriott e Blankenship, 2011; Nelson e Ben-Shem, 2004; Ruban, 2014) e como componente chave na cascata de sinalização fotomorfogênica (de Wit et al., 2016; Terzaghi e Cashmore, 1995; Wu, 2014; Yokawa et al., 2013). Além de regular importantes eventos na parte aérea das plantas, a luz participa de muitos aspectos do crescimento e desenvolvimento radicular em todas espécies vegetais (Lee et al., 2016), sendo sua provável participação no crescimento e desenvolvimento das raízes aéreas de plantas epífitas potencialmente ainda mais acentuada.

Embora os sistemas radiculares da maioria das plantas se desenvolvam sob o solo e, portanto, não permaneçam diretamente expostos à luz, há várias maneiras pelas quais as raízes podem ser expostas à luz, tais como a exposição direta ao sinal luminoso quando são descobertas na superfície do substrato (Molas et al., 2006), pela difusão do sinal luminoso através do substrato (Mandoli et al., 1990; Mo et al., 2015) ou ainda pela transmissão do sinal luminoso para as raízes através de tecidos aéreos (Sun et al., 2003). Dentre os eventos de sinalização modulados pela luz conhecidos para raízes de algumas plantas modelo destacam-se o gravitropismo (Hopkins e Kiss, 2012; Kiss, 2000; Millar et al., 2010), a formação de pelos absorventes (De Simone et al., 2000), o fototropismo negativo (Okada e Shimura, 1992) e o positivo (Kiss, 2003; Ruppel et al., 2001), o ganho de coloração verde pelo sistema radicular (Usami et al., 2004), a produção de metabólitos secundários (Hemm et al., 2004), o alongamento da raiz primária (Correll e Kiss, 2005; Lariguet et al., 2003), o crescimento e orientação de raízes laterais (Bhalerao et al., 2002; Kiss et al., 2002) e tolerância à seca (Galen et al., 2007).

Assim como em todos os outros órgãos da planta, as respostas do desenvolvimento radicular à luz são mediadas por sistemas de fotorreceptores (Smith, 2000), os quais monitoram as mudanças nas condições luminosas do ambiente circundante e as transformam em sinais endógenos capazes de modular repostas fotomorfogênicas apropriadas. Os fitocromos são os fotorreceptores melhor caracterizados até o momento, constituindo uma família de cromo proteínas fotorreversíveis que são responsáveis pela absorção da luz na região do espectro luminoso do vermelho e vermelho extremo (Chen e Chory, 2011;

Demotes-Mainard et al., 2016; Gu et al., 2011; Reed, 1993; Rockwell et al., 2006; Salisbury et al., 2007). Por outro lado, as plantas também são capazes de perceber o espectro da luz azul, neste caso por meio dos fotorreceptores denominados criptocromos e fototropinas (Briggs e Christie, 2002; Chaves et al., 2011; Christie, 2007; Liu et al., 2011, 2016; Quail, 2002).

Poucas moléculas que atuam *downstream* dos fotorreceptores têm sido caracterizadas para as raízes (Molas et al., 2006). No entanto, alguns indícios apontam para atuação de alguns fatores de transcrição em uma possível intersecção das vias de sinalização dos fitocromos e de fitormônios (Cluis et al., 2004; Sibout et al., 2006) para regulação de respostas fotomorfogênicas em raízes (Bauer et al., 2004; Kutschera e Briggs, 2012). Um ponto interessante nessa integração entre o sinal luminoso e o sinal hormonal é que não apenas a luz afeta a resposta hormonal, mas segundo a literatura, a biossíntese ou a resposta hormonal também podem influenciar as respostas da planta à luz. Dessa forma, as pesquisas realizadas até o momento nessa complexa área da fisiologia vegetal apontam para a existência de mecanismos de transdução de sinal altamente flexíveis e inter-relacionados entre a luz e os fitormônios (Nemhauser, 2008).

1.4. Controle hormonal do desenvolvimento radicular

Sabe-se que os fatores ambientais podem influenciar o crescimento e o desenvolvimento radicular por meio de cascatas de sinalização frequentemente mediadas por pequenas moléculas orgânicas coletivamente denominadas fitormônios (Chapman et al., 2012; De Rybel et al., 2015; Deak e Malamy, 2005; Malamy, 2005; Nagel et al., 2015; Spartz e Gray, 2008; Vanstraelen e Benková, 2012). Ao contrário dos animais, nas plantas não existem órgãos especializados para a biossíntese hormonal, embora possam existir diferenças na capacidade biossintética de cada órgão vegetal (Trewavas, 1981). Os efeitos dessas moléculas podem ser altamente complexos, pois uma única célula pode responder, simultaneamente, a vários hormônios, e um único hormônio pode atuar em vários tipos de tecidos (Spartz e Gray, 2008).

No entanto, sabe-se que os fitormônios não atuam apenas como simples transdutores ou mensageiros de sinais ambientais; na realidade, as informações fornecidas por essas moléculas constituem a base para decidir entre alternativas de programas de desenvolvimento, ou a criação de domínios de identidade dentro de um determinado tecido (Alabadí et al., 2009; Vanstraelen e Benková, 2012). Além disso, sabe-se que, praticamente, todos os hormônios

vegetais conhecidos até o momento afetam em algum grau a organização do sistema radicular, sendo que várias evidências indicam o papel chave dessas moléculas na manutenção do crescimento de raízes primárias e na iniciação e desenvolvimento de raízes laterais (Benková e Bielach, 2010; Celenza et al., 1995; De Smet, 2012; Gutierrez et al., 2012; Lavenus et al., 2013; Petricka et al., 2012; Rowe et al., 2016; Vanstraelen e Benková, 2012; Zhao e Hasenstein, 2010). Nesses estudos, as vias de sinalização da auxina, etileno e ácido abscísico adquirem posição particularmente importante na mediação de respostas ambientais no controle do desenvolvimento do sistema radicular (Casimiro et al., 2001; Lavenus et al., 2013; Orman-Ligeza et al., 2013; Potters et al., 2009; Rodrigues et al., 2014; Steffens e Rasmussen, 2016).

1.4.1. Auxinas no desenvolvimento radicular

Sob o ponto de vista do controle do desenvolvimento vegetal, as auxinas ocupam uma posição de destaque. Auxinas têm papel vital no estabelecimento do polo radicular do embrião, bem como no amadurecimento, crescimento e desenvolvimento radicular (Ding e Friml, 2010; Himanen et al., 2002; Jiang e Feldman, 2002; Petricka et al., 2012). Por muito tempo, acreditou-se que a produção de auxina ocorria exclusivamente na parte caulinar, sendo as raízes dependentes do transporte basípeto para o seu suprimento. Entretanto, agora se sabe que a parte radicular também é capaz produzir auxina (Zhao, 2010). Os níveis de ácido indol acético (AIA), principal representante da classe das auxinas, dependem não só da sua taxa de síntese, mas também de sua conjugação, formando centros inativos de auxina que podem ser mobilizados por ação enzimática ou pelo seu transporte (Alabadí et al., 2009; Ljung, 2013; Novák et al., 2012). O transporte de auxina ocorre de forma controlada e direcionada por proteínas denominadas de PINFORMED (PIN). Estas proteínas encontram-se ligadas à porção apical ou basal da membrana celular e transportam a auxina de maneira polar e específica (Benková et al., 2003; Friml, 2010; Kagenishi et al., 2016; Malenica et al., 2007; Viaene et al., 2013). Vários eventos relativos ao desenvolvimento radicular tem a participação crítica da auxina, sendo que essa classe hormonal está diretamente envolvida na regulação do crescimento da raiz primária e na formação de raízes laterais. Vários estudos com plantas mutantes ou transgênicas de *A. thaliana* têm confirmado que essa classe hormonal é essencial na morfogênese do sistema radicular (Benková e Bielach, 2010; Lavenus et al., 2013; Orman-Ligeza et al., 2013).

A interação entre auxina e a luz é crucial em diversos processos no desenvolvimento vegetal (Halliday et al., 2009). Por exemplo, plântulas de milho (*Zea mays*) ao serem expostas ou abrigadas da luz, mostraram que na presença da luz havia maior nível de auxina no ápice radicular do que no escuro (Suzuki et al., 2016). Além disso, plantas de tomateiro e *Arabidopsis* crescidas no escuro, ao serem transferidas para a luz, apresentaram um forte aumento no transporte polar da auxina (Liu et al., 2011). Experimentos com plantas de *Arabidopsis* crescendo no escuro, constatou uma drástica redução na expressão de *PIN1*, em contrapartida, ao serem expostos à luz sua expressão foi induzida (Sassi et al., 2012). Além disso, trabalhos sugerem que *PIN1* seria o principal transportador de auxina no processo de comunicação entre parte caulinar e a radicular (Liu et al., 2011; Sassi et al., 2012), sendo que a polarização das proteínas PIN mediada pela luz não ocorre exclusivamente na parte caulinar. Na realidade, em raízes de *Arabidopsis*, a migração das proteínas *PIN1*, *PIN2*, *PIN3* e *PIN7* para compartimentos do vacúolo foi induzida em resposta à ausência da luz e o tráfego das proteínas PIN entre a membrana celular e compartimentos vacuolares foi resultado da comunicação à longa distância via fator de transcrição *CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1)* (Kleine-Vehn et al., 2008; Laxmi et al., 2008; Sassi et al., 2012; Wan et al., 2012).

1.4.2. Ácido abscísico no desenvolvimento radicular

O ácido abscísico (ABA) também é um hormônio extremamente importante para o desenvolvimento radicular (Harris, 2015a). Inicialmente chamado de (+)-abscisina II, o ácido abscísico recebeu este nome pela sua ação estimulante na abscisão de frutos (Cornforth et al., 1966). Paralelamente, outro nome também surgiu, dormina, pela sua capacidade de induzir a dormência em sementes (Eagles e Wareing, 1963). Apenas em 1968 foi constatado que tanto a abscisina quanto a dormina eram estruturalmente iguais, recebendo o nome de ácido abscísico (Addicott et al., 1968).

O ABA é frequentemente relacionadas a eventos de sinalização em respostas a condições de estresse, tais como baixa disponibilidade de água, baixas ou altas temperaturas, salinidade elevada, entre outros (Finkelstein et al., 2002; Jones, 2016). Sabe-se que, em *Arabidopsis*, a emergência de raízes laterais a partir de primórdios pré-formados de raiz lateral é reprimida sob déficit hídrico com a participação do ABA como mediador (Deak e Malamy, 2005). Em um estudo mais detalhado, Tan e colaboradores (2003) detectaram a presença marcante de uma das isoformas da enzima-chave na biossíntese do ABA, a 9-cis

“*epoxycarotenoid dioxygenase*” (NCED2), nas células do periciclo envolvidas na iniciação do primórdio radicular, bem como nas células do córtex que circundam a nova raiz em formação. Além disso, o alongamento radicular também se mostrou negativamente regulado pelo ABA (Chen et al., 2008; Rowe et al., 2016; Saab et al., 1992). Por outro lado, plantas como *Mendicacum trumculata*, *Medicago sativa* e *Mimosa pudica*, apresentaram aumento na formação de raízes laterais quando tratadas com 10 μ M de ABA, enquanto que em *Arabidopsis thaliana* a concentração de 1 μ M mostrou-se suficiente para inibir a formação de raízes laterais (Liang e Harris, 2005). A manutenção do crescimento radicular também um evento o qual o ABA mostra se importante. Em trabalho realizado com plantas de arroz mutante *srt6* (“*short-root*”) defectivo para a percepção do ABA, houve menor taxa de crescimento radicular devido ao menor alongamento celular (Yao et al., 2003). Neste mesmo cenário, plantas de milho (*Zea mays*) acumularam ABA quando expostas a baixo potencial osmótico, o que manteve o crescimento radicular (Spollen et al., 2000). Similarmente, plantas de *Arabidopsis* e arroz (*Oriza sativa*) submetidas ao tratamento com ABA ou estresse osmótico, apresentaram aumento nos teores endógenos de ácido abscísico, bem como uma intensificação da taxa de transporte polar da auxina (Xu et al., 2013b).

Outro evento importante que possui participação do ABA é a produção de biomassa. Em um estudo feito com plantas de tomateiro (*Solanum lycopersicon*) e ervilha (*Pisum sativum*), foi constatado que as plantas selvagens apresentaram maiores valores de biomassa, tanto da parte caular quanto da parte radicular, quando comparadas com os respectivos mutantes *sitiens* e *wilty*, ambos deficientes na produção de ácido abscísico (McAdam et al., 2016). Observa-se com isto, que as respostas mediadas pelo ABA, bem como sua percepção e regulação da sinalização podem ser dose- e tecido- dependentes e podem diferir entre espécies vegetais (Harris, 2015). Tal qual a auxina, a luz também regula a biossíntese (Thompson et al., 2000) e eventos de sinalização mediados pelo ABA (Chen et al., 2008). Além disso, as vias de sinalização do ABA também interagem com as do etileno por meio do aumento de seus efeitos mutuamente antagônicos durante várias respostas fisiológicas e morfogênicas sob condições de estresse (Beaudoin et al., 2000; Cheng et al., 2009; Wilkinson e Davies, 2010).

1.4.3. Etileno no desenvolvimento radicular

O etileno foi relatado pela primeira vez em 1901 por Neljubov, um pesquisador Russo, em um trabalho feito com crescimento do hipocótilo de ervilhas em resposta à luz (Neljubov, 1901). A biossíntese do etileno, também conhecido como Ciclo de Yang, se dá a partir no

aminoácido metionina que é convertido a S-AdoMet pela enzima S-Adomet-sintase que por sua vez é convertido a ácido-1-aminociclopropano-1-carboxílico (ACC) por meio da enzima ACC-sintase (ACS), para logo em seguida ser oxidado pela ACC-oxidase (ACO), resultando no etileno (Yang e Hoffman, 1984). Tem-se observado que a presença deste hormônio em baixas concentrações está positivamente envolvida na rizogênese. Por exemplo, estacas caulinares de plantas mutantes deficientes para o gene *ETR* (“*Ethylene Receptor*”), responsável pela sensibilidade ao etileno, apresentaram baixo número de raízes adventícias, evidenciando assim a necessidade do etileno na rizogênese adventícia destas plantas (Clark et al., 1999).

Sabe-se que o etileno possui forte interação com a auxina no crescimento e desenvolvimento radicular (Hansen e Grossmann, 2000; Ivanchenko et al., 2008; Rahman et al., 2002; Vandenbussche et al., 2003). Swarup *et al.* (2007) observaram que a presença do etileno está intimamente ligada à taxa biossintética do AIA, sendo que a diminuição nos níveis endógenos de etileno na raiz de *Arabidopsis* levava a um decréscimo na taxa de biossíntese da auxina. Além disso, a emergência de raízes laterais mostra-se intimamente ligada à ação do etileno. Por exemplo, plantas de *Arabidopsis* tratadas com ACC apresentaram aumento no número de raízes laterais formadas, sendo a presença da auxina indispensável para que este processo ocorresse com sucesso (Ivanchenko et al., 2008).

Além disso, estudos realizados por Hansen e Grossmann (2000), mostraram que o aumento nos níveis de auxina resultava no acréscimo da atividade das enzimas envolvidas na biossíntese do etileno, que por sua vez levava à produção do etileno e, de forma bastante interessante, gerava um acúmulo de ABA no tecido radicular. Curiosamente, mutantes de *Arabidopsis eto1* (“*ETHYLENE OVERPRODUCER1*”), apresentam baixa sensibilidade ao ABA mesmo quando ele está presente em concentrações elevadas (Ghassemian et al., 2000). Similarmente, plantas de milho (*Zea mays*) tratadas com fluridona (inibidor da síntese de ABA) mostraram conspícuo aumento na emissão de etileno e, consistentemente, o mutante *vp5* (deficiente na síntese de carotenoides e ABA) também apresenta altos níveis de etileno (Spollen et al., 2000). Aparentemente, o conjunto destes dados sugere uma ação antagônica entre estes dois hormônios. Por fim, interações hormonais também podem envolver mudanças estruturais como, por exemplo, o caso de plantas transgênicas que sobre-expressam os genes relacionados à via biossintética da auxina *YUCCA8* e *YUCCA9*, as quais apresentam maiores níveis de lignificação celular com proeminente participação do etileno (Hentrich et al., 2013).

1.4.4. Interações entre hormônios e carboidratos no controle do desenvolvimento vegetal

Sabe-se que além dos hormônios, outras substâncias também podem regular o crescimento e desenvolvimento vegetal, dentre as quais destacam-se os carboidratos. Por exemplo, as auxinas possuem intrincadas relações com o metabolismo de açúcares. Plantas de *Arabidopsis* crescidas em meio de cultura suplementado com 3% de glicose apresentaram aumento no número de transcritos das proteínas PIN bem como na localização dessa proteína na membrana celular, e, conseqüentemente, no transporte polar de auxina. Além disso, a transcrição do gene *YUCCA2*, que codifica uma enzima chave na biossíntese da auxina, também foi positivamente regulada pela glicose (Mishra et al., 2009). Da mesma forma, a glicose foi capaz de aumentar o número de transcritos dos genes *YUCCA8* e *YUCCA9*, também relativos à biossíntese da auxina, assim como as concentrações de precursores da biossíntese de AIA, de alguns de seus catabólitos e formas conjugadas (Sairanen et al., 2012). Adicionalmente, o mutante *gin2* (“*GLUCOSE INSENSITIVE2*”), defeitivo no sensor de glicose HXK1 (HEXOKINASE1), apresenta insensibilidade à auxina. Curiosamente, os mutantes resistentes à auxina *axr1*, *axr2*, e *tir1* foram capazes de crescer em meio suplementado com 6% de glicose com fenótipo semelhante ao *gin2* (Moore et al., 2003). Tais dados sugerem um sinergismo entre glicose e auxina.

Além da auxina, o etileno também apresenta envolvimento com a sinalização mediada por açúcares. Zhou e colaboradores (1998) mostraram que níveis elevados de glicose podiam inibir o desenvolvimento de cotilédones e hipocótilos de *Arabidopsis*, entretanto, este efeito inibitório podia ser bloqueado na presença de ACC (precursor do etileno). Esses autores também observaram que o mutante *etr1-1* (“*ETHYLENE RECEPTOR1*”), defeitivo na percepção do etileno, apresentava drástica inibição do crescimento ao ser incubado *in vitro* na presença de 4% e 6% de glicose, sugerindo que, de alguma forma, o etileno poderia antagonizar o efeito inibitório da glicose (Zhou et al., 1998). Curiosamente, mutantes para a produção elevada de etileno como *eto1* (“*ETHYLENE OVER PRODUCER1*”) e *ctr1* (“*CONSTITUTIVE TRIPLE RESPONSE1*”) mostraram-se insensíveis à ação inibitória da glicose (Cheng et al., 2002; Zhou et al., 1998).

Assim como observado para auxina e etileno, o ácido abscísico e o metabolismo de açúcares também apresentam estreitas relações. Por exemplo, plantas de arroz (*Oriza sativa*) submetidas ao tratamento de seca moderada apresentaram aumento nos níveis de ABA com significativa acentuação da força do dreno e enchimento dos grãos, sendo que este processo ocorreu via ativação de enzimas relacionadas ao metabolismo de açúcares (Wang et al.,

2015). Cheng e colaboradores (2002) mostraram que genes relacionados à biossíntese do ABA, tais como *ABA2/GIN1*, *ABA1*, *AAO3* e *ABA3*, foram positivamente regulados em plantas de *Arabidopsis* crescidas em meio suplementado com glicose. O mesmo ocorreu com genes relacionados à sinalização do ABA, como *ABI3*, *ABI4* e *ABI5*, onde houve um aumento no número de transcritos em resposta ao tratamento com glicose. Um ponto curioso é que para a ativação dos genes *ABA2/GIN1*, *ABI3* e *ABI4* induzida pela glicose, a presença do ABA é indispensável (Cheng et al., 2002). Interessantemente, os mutantes *gin1* e *gin-6* (*glucose insensitive1* e *6*), bem como *sis4* e *sis5* (*sugar insensitive4* e *5*), além do *sun6* (*sucrose-uncoupled*) são derivados de mutações em genes ligados à biossíntese ou sinalização do ABA (Gazzarrini e McCourt, 2001), indicando, portanto, que as relações entre o ácido abscísico e os açúcares são bastante próximas.

Assim, a interação entre hormônios e outras substâncias sinalizadoras podem se dar em vários níveis em diferentes contextos como visto acima, tornando imperativo o uso de abordagens diversificadas para obter novas informações que ajudem a elucidar estas relações.

1.5. Breve histórico dos estudos com o gênero *Catasetum* e a espécie *Catasetum fimbriatum*

Um dos primeiros relatos científicos sobre o gênero *Catasetum* deu-se na área taxonômica ainda em 1822 por Karl Sigismund Kunth em seu livro intitulado “*Synopsis plantarum*” (Kunth, 1822). Poucos anos depois, mais precisamente em 1827, William Jaskson Hooker publicou em seu livro “*Exotic flora*” informações adicionais, inclusive com ilustrações coloridas, onde descrevia o novo gênero de orquídea (Hooker, 1827). Três anos mais tarde, John Lindley lançou o primeiro livro voltado exclusivamente à taxonomia das orquídeas, intitulado “*The genera and species of Orchideous plants*”, o qual foi dividido em quatro partes: a primeira dedicada à tribo Malaxideae (Lindley, 1830), a segunda à tribo Epidendreae (Lindley, 1831), a terceira à tribo Vandaeae (na qual foi inserido o gênero *Catasetum* Lindley, 1833) e, por fim, a quarta parte dedicada à tribo Ophrydeae (Lindley, 1835).

Na mesma época James Bateman publicou outro livro dedicado também à família Orchidaceae. Neste livro, Bateman também se reporta à ocorrência do gênero *Catasetum* em coletas feitas no México e na Guatemala (Bateman, 1843). Cerca de 30 anos depois, foi lançado o primeiro manual voltado ao cultivo de orquídeas, incluindo plantas do gênero *Catasetum* (Williams, 1877). Neste período, Charles Morren descrevia pela primeira vez a

espécie *Myanthus fimbriatus* (Morren, 1848). Entretanto, dois anos mais tarde, John Lindley propôs mudanças nomenclaturais, dentre as quais a espécie *Myanthus fimbriatus*, seria classificada como pertencente ao gênero *Catasetum*. Dessa forma, surgindo pela primeira vez na literatura o nome *Catasetum fimbriatum* (Lindley e Paxton, 1850).

Os primeiros estudos com plantas do gênero *Catasetum* envolviam prioritariamente a parte reprodutiva desta orquídea. Charles Darwin, quando de suas buscas sobre a ocorrência da fecundação cruzada em plantas, a exemplo do que já sabia à época para o reino animal, encontrou neste gênero de orquídeas os elementos de convicção contrários à autofertilização como veículo de diversidade. Neste caso, não lhe passou despercebido a ocorrência de flores masculinas e flores femininas morfologicamente tão distintas, bem como o fato de possuírem um mecanismo sofisticado de ejeção de suas políneas (Darwin, 1862). Essa admiração foi claramente retratada por Darwin em um de seus livros, onde perpetuou seu arrebatamento para com estas orquídeas “*I have reserved for separate description on sub-family of the Vandaeae, namely, Catasetidae, which may, I think, to be considered as the most remarkable of all Orchids*” (Darwin, 1862). Em 1912, a espécie *Catasetum darwinianum* foi nomeada justamente em homenagem Darwin (Rolfe, 1913). Desde então, o gênero *Catasetum* vem sendo estudado mais intensamente no que diz respeito às estratégias reprodutivas (Janzen, 1981a).

De fato, pesquisas sobre a biologia floral são as mais frequentes para este gênero, sendo a polinização *per se* a mais abordada desde o século XVIII (Baillon, 1855, 1854; Darwin, 1862; Janzen, 1981b; Milet-Pinheiro et al., 2015; Nicholson et al., 2008; Porsch, 1955; von Guttenberg, 1930). Além disso, o dimorfismo floral das plantas do gênero *Catasetum* tem sido outro aspecto bastante abordado (Darwin, 1862; Rolfe, 1890; Romero e Nelson, 1986). Um dos estudos pioneiros sobre a fisiologia do dimorfismo floral estabeleceu uma possível relação entre o efeito de altas intensidades luminosas e a formação de flores femininas (Gregg, 1982, 1975; Zimmerman, 1991). No plano bioquímico, estudou-se os componentes responsáveis fragrância floral (Hills et al., 1972; Lindquist et al., 1985) e a composição do viscidio - uma pequena massa de substância adesiva presente no polinário ejetável (Schlee e Ebel, 1983).

Devido ao fato de serem plantas decíduas, o que, aliás, as diferem da grande maioria das demais Orchidaceae, levou Benzing e colaboradores (1982b) a estudar a fotossíntese tanto nas folhas quanto nos pseudobulbos destas plantas. Os autores concluíram que pseudobulbos desprovidos de folhas não eram realizando trocas gasosas devido à ausência de estômatos e

eram, portanto, em grande parte dependentes das folhas como fonte de fotoassimilados (Benzing et al., 1982a). Também foram abordadas questões relacionadas ao balanço de carbono e eficiência no uso do nitrogênio (Zotz e Winter, 1994), além de estudos farmacológicos (Shimizu et al., 1988) e sobre a velocidade da ejeção do polinário (Nicholson et al., 2008).

Apesar do gênero *Catasetum* ser estudado já há um bom tempo, análises fisiológicas são relativamente recentes. Um dos primeiros trabalhos com esse gênero envolvendo a ação de hormônios vegetais foi realizado com um híbrido de *Catasetum trulla* x *Catasetum Berthrand*. Neste trabalho foi demonstrada pela primeira vez a ocorrência da conversão direta de ápices radiculares em gemas caulinares capazes de originar rapidamente uma nova planta completa (Kerbaudy, 1984). Utilizando raízes isoladas de *C. fimbriatum*, foi observado que auxinas, em geral, possuem um papel inibidor sobre esse evento organogênico, enquanto que citocininas promovem tal processo de conversão com a subsequente formação de estruturas semelhantes a protocormos (Colli e Kerbaudy, 1993; Peres et al., 1999). Adicionalmente, foi visto que a partição de carboidratos também possui papel importante nesse processo de conversão (Vaz et al., 1998). Outros temas fisiológicos também foram abordados, como a nutrição mineral, sobretudo do metabolismo do nitrogênio, quando foi observado que plantas de *C. fimbriatum* apresentavam nítida preferência pela forma amoniacal (NH_4^+) ao invés da forma nítrica (NO_3^-) e por aminoácidos (Majerowicz et al., 2000; Majerowicz e Kerbaudy, 2002). Peres e colaboradores (2001) trabalhando com a relação entre a parte caulinar e a parte radicular em dois genótipos de *C. fimbriatum*, observaram que o genótipo que apresentava a parte caulinar mais desenvolvida possuía maiores níveis de citocininas, enquanto que o genótipo que apresentava sistema radicular mais desenvolvido, possuía maiores níveis endógenos de AIA (Peres et al., 2001). Sendo o ambiente epifítico bastante instável (como descrito acima), Peres e colaboradores (2009) avaliaram a resposta de raízes isoladas em meio de cultura com baixo potencial osmótico, onde verificou-se um efeito promotor do crescimento radicular coincidentemente ao aumento nos níveis endógenos de ABA e de AIA nesses tecidos.

Paralelamente, análises morfo-histológicas revelaram a presença de espessamento de parede celular tanto em folhas quanto em raízes de *C. fimbriatum*, sendo tal característica proposta como possível caráter taxonômico por alguns autores (Oliveira e Sajo, 2001; Pedroso de Moraes et al., 2012; Silva et al., 2015; Stern e Judd, 2001). Curiosamente, estrutura bastante semelhante foi descrita em 1864 em velame de raízes de orquídeas

(Leitgeb, 1864, 1865). Embora muitos tenham reportado a ocorrência desta especialização da parede celular, poucos trabalhos investigaram a modulação fisiológica deste evento em orquídeas. Sabe-se, no entanto, que o espessamento da parede celular em *C. fimbriatum* é fortemente regulado pelo etileno, uma vez que a aplicação de diferentes concentrações desse fitormônio intensificou o espessamento das paredes celulares em diferentes tecidos radiculares e a aplicação de 1-metilciclopropeno – MCP - (inibidor da percepção de etileno) inibiu tal processo de morfogênese celular (Rodrigues et al., 2014).

Trabalhos mostraram ainda que as plantas de *C. fimbriatum* apresentam uma substancial plasticidade morfológica em resposta à presença ou ausência de luz. Por exemplo, tais plantas originam pseudobulbos na presença de luz e, quando incubadas no escuro, originam estolões aclorofilados com desenvolvimento rápido e crescimento indefinido. Quando transferidas da luz para o escuro, dá-se uma diminuição nos níveis de AIA concomitantemente a um incremento nos teores endógenos de citocininas. Sob este novo *status* hormonal ocorre a quebra da dominância apical com a liberação de gemas laterais e a formação de estolões (Suzuki et al., 2004, 2010). Entretanto, a utilização do etileno inibe fortemente o crescimento do estolão na ausência da luz (Suzuki e Kerbauy, 2006).

Como visto acima, o gênero *Catasetum* vem sendo foco de interesse científico há algum tempo. Algumas de suas respostas morfológicas *in situ* são bastante particulares dentre as Orchidaceae. Dentre elas vale destacar as fases do desenvolvimento sazonal de orquídeas desse gênero, que podem ser divididas em: (1) fase de crescimento ativo, onde as plantas utilizam a grande disponibilidade de água e nutrientes da estação chuvosa para desenvolvimento rápido de novos órgãos; e (2) a fase de dormência, que coincide com os períodos de seca, quando ocorre a perda das folhas, restando apenas os pseudobulbos e raízes no corpo vegetativo das plantas (Benzing, 1990; Lacerda 1995). Outro ponto interessante é a flexibilidade morfológica do sistema radicular que plantas de *Catasetum* podem apresentar durante sua fase de crescimento, frente a diferentes condições ambientais. Sabe-se, por exemplo, que as plantas deste gênero se destacam pela capacidade de colonização de troncos em decomposição (Zotz, 2016). Ao crescerem neste tipo de substrato que é úmido e com elevada disponibilidade de matéria orgânica, estas plantas, em geral, não apresentam um grande número de raízes crescendo diretamente expostas ao ambiente. Dessa forma, a formação de raízes aclorofiladas fica direcionada para o interior do substrato. Por outro lado, estas plantas também são capazes de se estabelecer em ambientes menos favoráveis. Uma vez fixadas em substratos secos ou pouco penetráveis, as raízes clorofiladas crescem diretamente

expostas às adversidades ambientais, podendo apresentar tanto crescimento gravitrópico positivo, quanto negativo. O resultado é uma estrutura em forma de cesto que circunda o substrato capaz de acumular água e matéria orgânica (Benzing, 1986, 1990; Da Silva e Da Silva, 1998). Em virtude desta característica, uma parte do sistema radicular cresce com exposição restrita à luz e ao ar atmosférico, seja por crescer em troncos podres ou no interior dessa estrutura de cesto (Benzing, 1986, 1990).

Tendo em vista toda a plasticidade morfogênica apresentada por orquídeas epífitas do gênero *Catasetum*, sobretudo nos diferentes tecidos que compõem o seu sistema radicular (Benzing 1986, 1990; Zimmerman 1990, 1991; Da Silva e Da Silva 1998), o presente trabalho propôs-se a explorar algumas das respostas morfo-fisiológicas moduladas pela presença (ou ausência) de incidência luminosa direta no sistema radicular de *Catasetum fimbriatum*.

1.6. Referências Bibliográficas

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2. HIPÓTESE E OBJETIVOS

Tendo em vista que o fenótipo das raízes aéreas que compõem o sistema radicular da orquídea *Catasetum fimbriatum* é claramente modulado pelo microambiente no qual a planta está se desenvolvendo, como é o caso da presença ou ausência de incidência luminosa direta nas raízes, optou-se por se utilizar desse modelo vegetal para o estudo dos possíveis eventos de sinalização mediados por hormônios durante as respostas de fotomorfogênese radicular. Assim, em consonância com pesquisas realizadas no Laboratório de Fisiologia do Desenvolvimento Vegetal do IBUSP com esta orquídea, o presente estudo teve como objetivo central a investigação das possíveis respostas morfo-fisiológicas moduladas em plantas de *Catasetum fimbriatum* devido à exposição (ou não) do seu sistema radicular à luz direta.

Para tal, traçamos os seguintes objetivos específicos:

- Avaliar as respostas de crescimento, diferenciação celular e teores endógenos de três classes hormonais (auxina, do etileno e do ácido abscísico) em tecidos radiculares submetidos (ou não) à exposição direta à luz por diferentes períodos;
- Investigar a influência da exposição do sistema radicular à incidência luminosa sobre as relações de fonte e dreno ocorridas entre os órgãos do sistema caulinar (pseudobulbo e folhas), avaliando-se o possível papel sinalizador do ácido abscísico e auxinas entre os diferentes órgãos vegetativos.

3. CAPÍTULO I

Light modulates auxin, abscisic acid and 1-aminocyclopropane-1-carboxylic acid contents and the cell wall modifications in aerial roots of the epiphytic orchid *Catasetum fimbriatum*.

3.1 Abstract

The epiphytic habitat is considered one of the most changeable environments occupied by plants, mainly due to its discontinuous availability of water and nutrients. However, some groups of plants, such as in Orchidaceae family, are recognized for their successful colonization of this biotope. An interesting example of epiphytic specialization is represented by the aerial root system of the orchid *Catasetum fimbriatum*, as it can display a notable developmental plasticity when exposed to variable light conditions: its absorptive roots can penetrate through organic substratum nearly cut off from luminosity while the upward-growing roots exposed to light and can photosynthesize. Since indole-3-acetic acid (IAA), abscisic acid (ABA) and ethylene are crucial signals modulating the root architecture in most plant species, and are also key mediators during numerous photomorphogenic responses, this study investigated the potential involvement of these hormones during the light-mediated growth responses in *C. fimbriatum* roots. For this, the root systems of plants at the growing phase were either darkened or exposed to light, while the shoot system was kept fully illuminated. The youngest root of each plant was analyzed throughout the experiments for the growth rate, morpho-histological modifications, and endogenous levels of IAA, ABA, and 1-aminocyclopropane-1-carboxylic acid (ACC; the ethylene precursor). Covered roots presented higher volume and growth rate, but lower dry mass than the exposed ones. The higher accumulation of root biomass in the light-exposed roots were closely correlated with a strong cell wall thickening in the root cortex, which appeared to be specifically induced by blue light. In general, root exposure to light induced increasing levels of ABA and reduction of ACC content. This suggests that light might modulate *C. fimbriatum* root development through a fine-tuned hormonal mediation which depends on coordinated adjustments of IAA, ABA and ACC levels.

Key words: Aerial roots, cell wall differentiation, epiphytic orchid, phytohormones, photomorphogenesis.

3.2. Introduction

Since the beginning of terrestrial environment conquest, the plants had changed drastically. The advent of some structures such as seeds, flowers, leaves and root system was determinant to allow the plant irradiation through the earth surface (Algeo and Scheckler, 1998). Since the appearance of the first root-like structure 419-408 million years ago, this organ has changed dramatically throughout plant evolution (Gensel and Edwards, 2002). Fossil evidences suggest that the increasing level of specialization in the root system, such as the formation of lateral roots, contributed to plant exploration and colonization of earth surface (Meyer-Berthaud et al., 2013). In this context, the root system is highly important for most plant species since it often represents the key organ responsible for water and nutrient uptake, besides allowing the plant fixation on substrate.

Among the different biotopes colonized by plants, the epiphytic niche is one of the most unstable and challenging for plant survival due the frequent nutritional scarcity and the intermittent availability of water caused by long periods of severe drought (Benzing and Ott, 1981). A plant family that massively contributed to the epiphytic diversity is the Orchidaceae (Zotz, 2016), which presents rich diversity and the expressive number of approximately 19,000 species. A number that represents 68% of all epiphyte plants (Zotz, 2016). This family presents some interesting and complex examples of adaptations, such as those found in the shootless epiphytic species in which all metabolic and physiological processes are performed by the roots, including the presence of crassulacean acid metabolism (CAM) as the photosynthetic mode for CO₂ fixation (Benzing and Ott, 1981). Additionally, plants of *Oncidium* “Aloha” shows a remarkable plasticity in response to water deficit within its vegetative organs, where leaves presents the standard C₃ photosynthesis while the pseudobulbs and green roots can express the CAM pathway (Rodrigues et al., 2013).

The structure of root orchids has been explored since 1824 (Link, 1824); according to Benzing et al. (1983) several modifications of these organs provided the necessary conditions to orchid survival on the canopy. This includes the remarkable morphogenetic plasticity found among species included in the *Catasetum* genus, in which the root system holds a significant role for guaranteeing the survival of those plants in a hostile environment (Host, 1999). For example, the root tip of *Catasetum* plants is capable of converting itself into a shoot primordium when exposed to certain stressful conditions, originating a complete new plant (Colli and Kerbauy, 1993; Kerbauy, 1984). Essentially, the architectural organization of the root system of *Catasetum* plants presents some other interesting morphological features, such

as the formation of a nestle-like structure that creates a rhizosphere with roots growing protected from light (inside this structure) and roots growing under total light exposure (surrounding the inner roots), remaining, therefore, more susceptible to environmental factors. This structure formed by the whole root system allows water and organic matter retention (Benzing, 1990).

Concurrently, one of the most conspicuous tissues in the *Catasetum* roots is the velamen, which is formed by several layers of dead epidermal cells and functions like a sponge for water and nutrient retention (Dycus and Knudson, 1957; Zotz and Winkler, 2013). The velamen cells display thickenings on their cell walls, and this remarkable arrangement was firstly described for orchid tissues by Link (1824) and characterized by Leitgeb (1865) as a cellular conformation potentially involved in providing structural support to avoid extreme cellular dehydration. The different thickening patterns found on cell walls have been used as a taxonomic character by some researches (Freudenstein and Rasmussen, 1999; Pridgeon, 1982; Silva et al., 2015; Stern and Judd, 2001). In fact, cell walls in both velamen and cortex of *Catasetum fimbriatum* roots often present this kind of specialization (Pedroso de Moraes et al., 2012) which is partially controlled by modifications in the ethylene production and/or perception under potentially stressful growing conditions (Rodrigues et al., 2014).

Actually, the root development can be modulated by virtually all environmental changes such as drought, cold, salinity (Knight and Knight, 2001; Mahajan et al., 2005; Vinocur and Altman, 2005), nutrient status (Carvalho et al., 2016; López-Bucio et al., 2003; Nibau et al., 2008) and light (De Wit et al., 2016; Tombesi et al., 2015). Light is mandatory for the existence of plant life and it can act as both the energy source in photosynthesis (Hohmann-Marriott and Blankenship, 2011; Matsuda et al., 2011; Ruban, 2014; Tombesi et al., 2015) and the environmental signal that drives photomorphogenic responses (De Wit et al., 2016; Wu, 2014). During the plant evolution, a complex system of light sensors was established, in which three main families of photoreceptors are responsible to orchestrate the light perception, signaling and responses: the phytochromes that respond to red light (Chen and Chory, 2011; Klose et al., 2015; Rockwell et al., 2006; Salisbury et al., 2007), and the cryptochromes and the phototropins, which are both responsive to blue light (Ahmad, 2016; Briggs and Christie, 2002; Chaves et al., 2011; Christie, 2007; Liu et al., 2011, 2016; Sullivan et al., 2016). Several physiological events in plants are directly controlled by light, including the developmental control of the root system in terrestrial plants (Mo et al., 2015) during the following processes: phototropism (Hopkins and Kiss, 2012; Kutschera and Briggs, 2012;

Vitha et al., 2000), gravitropism (Correll and Kiss, 2005; Hopkins and Kiss, 2012; Millar et al., 2010), root growth (Millar et al., 2010; Moni et al., 2015), and lateral root formation (Bellini et al., 2014; Lariguet et al., 2003; Salisbury et al., 2007). In *Catasetum* species light also participates in the control of flowering sex expression (Gregg, 1975; Moraes and Almeida, 2004). Curiously, when plants of *C. fimbriatum* are cultured in dark, etiolated shoots with indeterminate growth are formed in place of the regular pseudobulb development (Suzuki et al., 2004).

Additionally, root development is controlled by hormones from the embryo pole establishment until the post-vegetative, mature phase (Petricka et al., 2012; Smith and De Smet, 2012), being the auxin IAA the most important one (Overvoorde et al., 2010; Tian and Reed, 1999). The interplay between light and auxin is well known (Halliday et al., 2009). IAA is responsible for the establishment of the root pole meristematic during embryo formation (Furuta et al., 2014; Weijers and Wagner, 2016), lateral root initiation, development and emergence (Benková and Bielach, 2010; Lavenus et al., 2013; Péret et al., 2009; Porco et al., 2016), maintenance of meristematic cells (Ding and Friml, 2010; Jiang and Feldman, 2002; Tian et al., 2014), and procambial and vasculature development (De Rybel et al., 2015; Furuta et al., 2014; Lucas et al., 2013), particularly the protoxylem formation on root tip (Bishopp et al., 2011) and xylogenesis (Della Rovere et al., 2015).

Microarrays analyses revealed that transgenic plants of *Brassica napus* overexpressing the gene *CRYPTOCHROME1 BnCRY1-OE* presented modifications in the cell wall extensibility due the downregulation of *XTH18*, a gene codifying for an enzyme related to the control of cell wall extensibility (Sharma et al., 2014). Interestingly, the deposition of lignin on the cell wall seems to be induced by light presence (Syros et al., 2005). Furthermore, the *Arabidopsis* mutant *wat1* presented defects on secondary cell wall deposition when grown under short day conditions. Interestingly, this mutant also present defects on the polar auxin transport (PAT). Nevertheless, submitting this mutant to continuous light, were detected lignified cell walls. However, the PAT was maintained with no alteration independently of light condition, showing that the disturbance on cell wall might compromise the auxin transport (Denancé et al., 2010). Microarray studies revealed that several genes are co-regulated by *WAT1* gene, among them are some key genes involved in the auxin transport and signaling (Manfield et al., 2006), and in the vasculature patterning and secondary cell wall deposition (Ranocha et al., 2013). Complementarily, the mutant *repp3* (regulator of PIN

polarity) presents defects on PIN polarization, cellulose deficiency and abnormalities in the cell wall composition (Feraru et al., 2011).

As important as auxin, ethylene also have strong influence on root development and displays several layers of signaling interaction with auxin itself (Stepanova et al., 2007). For example, an interplay between auxin and ethylene was shown in two lines of *Arabidopsis* overexpressing genes *YUCCA* (*YUC8ox* and *YUC9ox*), which encodes key enzymes in the auxin biosynthesis. These plants displayed increased production of both IAA and ethylene, whereas the combination of these two hormones resulted in the secondary cell wall growth and lignin deposition (Hentrich et al., 2013). Ethylene is also involved with callose deposition (De Cnodder et al., 2005), lateral root formation interacting with auxin (De Smet, 2012; Fukaki and Tasaka, 2009; Ivanchenko et al., 2008), root growth acting on auxin biosynthesis and distribution (Růžicka et al., 2007), root hair formation (Gilroy and Jones, 2000; Rahman et al., 2002; Vissenberg et al., 2001) and vasculature tissue proliferations with auxin interplay (Smet and De Rybel, 2016). Interestingly, plants of *C. fimbriatum* treated with ethylene presented a substantial impairment of pseudobulb development with increased formation of adventitious roots, root hairs (instead of velamen) and intense differentiation of wall thickenings in the cortex cells (Rodrigues et al., 2014). Accordantly, this hormone is also capable to modify the cell wall structure by inducing cell lignification in roots of *Vigna radiate* (Huang et al., 2013).

Another hormone that presents a prominent influence in the root development, mainly in response to abiotic stress (Jones, 2016; Wilkinson and Davies, 2002), is the abscisic acid (ABA). On barley plants, the application of exogenous ABA increased cell hydraulic conductivity in roots by inducing the intensification of aquaporins abundance (Sharipova et al., 2016). The root growth is also influenced by ABA, for instance, maize plants submitted to low potential water treatment presented impaired root growth that was restored by applying exogenous ABA (Sharp, 2004). Xu et al. (2013) observed in *Arabidopsis* plants under moderate water deficit the enhancement of ABA accumulation, auxin polar transport and root growth stimulation. Besides, auxin signaling might be also ABA-mediated via expression induction of *AUXIN RESPONSE FACTOR 2* (*ARF2*) (Wang et al., 2011). ABA is also involved in cell wall differentiation, since the *Arabidopsis* mutant *abi4* (Finkelstein, 1994) is not capable to induce callose deposition (Ton and Mauch-Mani, 2004). Gravitropic response might be also regulated by ABA interacting with IAA and ethylene. *Arabidopsis* mutants defective in root gravitropic responses - such as *eir1-1* (*ETHYLENE INSENSITIVE ROOT 1*),

aux1-7 and *axr4-2* (*AUXIN RESISTENT*) -, respond to the ABA treatments with roots growing against the vector of gravity (Han et al., 2009). Interestingly, roots of *C. fimbriatum* submitted to osmotic stress displayed enhanced levels of both ABA and IAA (Peres et al., 2009).

Therefore, a variety of factors might regulate both growth and development of root systems with the key participation of hormones. However, our understanding about orchid developmental control is still rather superficial. Since the Orchidaceae family represents the most important contributor for the epiphytic diversity, is necessary new integrative approaches for a better understand of this group of plants (Zotz, 2016). Recent studies have raised some questions about the light being a non-natural, stressful condition to the root system of the classical plant models (Xu et al., 2013; Yokawa et al., 2014). In this way, our work aimed to provide new information about the light action on root morphology and how the plant respond to this environmental factor, since that the light presence or absence on root system is a natural condition for *C. fimbriatum* species. Moreover, since the beginning of orchid root morphology exploration by Link (1824), experimental investigations targeting the modulation of cell wall thickening (Rodrigues et al., 2014) or investigating its development (Idris and Collings, 2015) are relatively recent and still scarce. Furthermore, bringing new information that help to a better understand on how light might influence the cell wall modification in root tissues, a remarkable adaptive characteristic of *C. fimbriatum*, is highly important for the comprehension about how this root architecture and organization contributed to evolutionary success of this plant.

3.3. Material and Methods

Plant material and growth conditions

Micropropagated plants of *Catasetum fimbriatum* Lindl (Orchidaceae) were obtained in Vacin and Went solution (1949), according to the procedure described by Suzuki *et al.* (2004), and incubated under 20 $\mu\text{mol m}^{-2}\text{s}^{-1}$ of light intensity emitted LED lamps, temperature 25°C $\pm$ 2, and 12 hours of photoperiod. These plants were kept under controlled conditions for either 6 months or 40 days, depending to experiment.

Experimental design with six-month old plants

After six months under the growth conditions described above, plants with the youngest pseudobulb holding green leaves and only one adventitious root (length between 1.5 and 2.0 centimeters) were selected for experimentation (FIG. 1A). As illustrated in the figure 1A, the plant portion designated for the experimental analysis corresponded to the newly formed organs during their active growth phase. The selected plants were transferred to an assay tube containing 10 mL of the same medium culture used to the plant micropropagation, with the careful inoculation of the initial roots into the culture medium (approximately one-centimeter deep). The root systems were either darkened or exposed to light under controlled *in vitro* conditions, while the shoot system was kept fully illuminated with white light emitted by a LED RGB (Red, Green, Blue) system with continuous light intensity of $90 \mu\text{mol m}^{-2}\text{s}^{-1}$, and temperature $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$. Throughout the subsequent 45 days of treatments, the youngest root of each plant was photographed every 5 days for morphometric analyses. At the end of the light treatments, each monitored root of ten independent plants was measured for the fresh weigh (FW), followed by dehydration in a dry oven to calculate the dry weigh (DW) and the water content. Following the same arrangement, root tissues were also collected from six plants of each light treatment to perform the histological analysis. For the hormonal analyses, the same general experimental design described above was followed, while root samples were harvested from 20 plants at three different time-points: at the beginning of experiment (T0), after fifteen days under the light treatments (T15), and after thirty days under each light treatment (T30). Additionally, hormonal quantification was also performed in tissue samples of the initially formed roots collected from the older set of organs already established during the previous growing period, being separated into three equals parts (named as tip, middle or base) (FIG. 1A).

For hormonal evaluation, all the harvested roots (both sets of first roots and younger roots) were immediately fragmented into small pieces and subsequently weighted, frozen in nitrogen liquid, and stored at -80°C until the hormonal extraction.

Experimental design with forty-day old plants

After forty days under the same growth conditions described above, young plants holding only one initial root (nearly 1.5 cm long) and one unit of pseudobulb were selected for a second set of experimentation (FIG. 1B). These young plants were carefully transferred

to assay tubes containing 40 mL of the same growth medium previously described, and their root systems were either darkened or exposed to light under controlled *in vitro* conditions, while the shoot system were kept fully illuminated with white light LED (white lamps) system with continuous light intensity of $100 \mu\text{mol m}^{-2}\text{s}^{-1}$, temperature $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$. Throughout the subsequent 20 days of light treatments, the initial root of each plant was photographed every 5 days for morphometric analyses. At the end of the light treatments, each monitored root of ten independent plants was measured for the fresh weigh (FW), followed by dehydration in a dry oven to calculate the dry weigh (DW) and the water content. Following the same arrangement, root tissues were also collected from six plants of each light treatment to perform the histological analysis. For the hormonal analyses, root samples were harvested from 20 plants at four different time-points: at the beginning of experiment (T0), after five days under each light treatment (T5), after ten days under each light treatment (T10), and after fifteen days under each light treatment (T15). All the first roots were harvested, immediately fragmented into small pieces and subsequently weighted, frozen in nitrogen liquid, and stored at -80°C until the hormonal extraction.

Figure 1 – Morphological representation of *Catasetum fimbriatum* plants used in the experiments. **A)** A six-month old plant displaying a set of older organs formed during the previous growing cycle (*i.e.* older pseudobulb and initial roots) and the new set of developing organs currently growing (*i.e.* younger pseudobulb and younger root). **B)** A fourth-day old plant during its first growing cycle showing the first set of developing organs (*i.e.*, first formed root and the first pseudobulb). White bars correspond to 1 centimeter.

Growth Analysis

The total growth, absolute rate growth, relative rate growth and root volume were calculated with the data obtained through the photographs analyzed by the software ImageJ with the plug-in SmartRoot (Lobet et al., 2011).

Histological analysis

The roots were fixed in Karnovsky's solution (1965) for 24 hours under vacuum at room temperature. The fixed material was dehydrated with acetone starting with 30% until 70%, following by their hand-cut sectioning and staining with toluidine blue 0,05% of phosphate buffer pH 6.8. Photomicrographs were taken with a digital Leica DFC320 camera on a Leica DM LB microscope.

Hormonal analysis

Endogenous IAA, ABA and ACC levels were simultaneously quantified by GC-MS procedure according to Melo et al., (2016) with some modifications. Approximately 200 mg of frozen roots samples were ground in liquid nitrogen in the presence of 20 mg poly(vinylpolypyrrolidone) (PVPP) and afterward homogenized in 1,25 mL extraction solution composed of 10 mM ascorbic acid, 10 mM ethylenediamine tetraacetic acid (EDTA) and 10 mM dithiothreitol (DTT). The internal standard was included, 0,5 µg [¹³C₆]-IAA (Cambridge Isotopes, Inc.) and 0,5 µg [²H₆]-ABA (OlChemIm Ltd), and 0,5µg [²H₄]-ACC (Sigma-Aldrich) were added to each sample as internal standards. After centrifugation (25,000 g, 20 min, 4°C), 1 ml of the supernatant was collected for analysis of IAA and ACC, the remaining sample was used to ABA quantification. The IAA and ACC samples were purified via solid phase extraction (SPE) column (Supelclean LC-NH₂, Supelco) as described in Chen *et al.*(1988). The eluates were evaporated, resuspended in 100 µL of methanol and transferred to a vial. The methanol was totally dried, and the samples resuspended in 50 µL pyridine followed by a 60 minutes derivatization at 92°C using 50 µL *N*-tert-Butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA).

For ABA, the sample were submitted to liquid extraction. For this procedure, 500 µL of ethyl acetate were added on tube followed by mixing and centrifuged for 5 minutes at 25,000 g. This step was repeated twice. The ethyl acetate was totally dried, followed by resuspension with 100 µL of methanol, and then transferred to a vial with subsequent

evaporation of the methanol. Next step the simple sample was resuspended in 20 μ L of methanol and followed by 30 minutes of derivatization in room temperature using 10 μ L (Trimethylsilyl)diazomethane. For all the compounds, the analysis was performed on a gas chromatograph coupled to a mass spectrometer (model GCMS-QP2010 SE, Shimadzu) in selective ion monitoring mode. The chromatograph was equipped with a fused-silica capillary column (30m, ID 0.25 mm, 0.25 μ m thick internal film) DB-5 MS (Agilent Technologies) stationary phase using helium as the carrier gas at a flow rate of 4.5 mL min⁻¹ in the following program: 2 min at 100°C, followed a ramp by 10°C min⁻¹ to 140°C, 25°C min⁻¹ to 160°C, 35°C min⁻¹ to 250°C, 20°C min⁻¹ to 270°C and 30°C min⁻¹ to 300°C. The injector temperature was 250°C and for the MS settings operating were used: ionization voltage, 70 eV (electron impact ionization); ion source temperature, 230°C; interface temperature, 260°C. Ions with a mass ratio/charge (m/z) of 244, 202 and 130 (corresponding to endogenous IAA), 250, 208 and 136 (corresponding to [¹³C₆]-IAA), 272, 244, 160 (corresponding to endogenous ACC), 276, 248, 164 (corresponding to [²H₄]-ACC), 190, 162 and 130 (corresponding to endogenous ABA), and 194, 166 and 134 (corresponding to [²H₆]-ABA) were monitored. Endogenous concentrations were calculated based on extracted chromatograms at m/z 244 and 250 for IAA, 272 and 276 for ACC and 190 and 194 for ABA.

Statistical Analyzes

Statistical analyses were performed using SAS Jmp statistical software 13.0. Results with $p < 0.05$ were considered significantly different.

3.4. Results

Light-induced responses in the morphology of younger roots from six-month old plants

According to the figure 2A, covered roots presented higher values of both fresh weight (FW) and water content than the correspondent exposed ones. Accordingly, the total dry mass (μ g/mm³) of exposed roots was significantly higher than in the covered roots, a trend that becomes even clearer when comparing the proportion of dry weight in relation to the FW (DW/g FW), where a higher ratio was found in roots exposed to light (8.8%) than those protected from light (5.1%) (FIG. 2A). By analyzing the root length measurements taken at each 5 days, is possible to notice that covered roots displayed higher values since the 5th day

of the experimental period, which resulted in a higher final length of roots growing protected from light (FIG. 2B), whereas such growth difference was mainly established during the time frame comprised between 5 and 15 days of light treatments (FIG. 2C). Accordingly, the representation of the root growth rate in a daily basis (FIG. 2D) corroborated the observation that the strongest light-induced growing responses in *C. fimbriatum* roots occurred during the first fifteen days of treatment.

Figure 2 – Morphometric values relative to the development of young roots (from 6-month-old plants) grown under light and protected light conditions **A**). Fresh Weigh (FW) and dry Weight (DW). **B**) Growth rate percentage. **C**) Total root length in each 5 days **D**) Growth rate in mm per day. For **B**, **C** and **D** continuous line represent root-covered condition, dashed line represent root exposed condition. On letter A different letter represent statistical difference according *t* Student test *p* value <0,05. For letter B, C and D different letter represent statistical difference on the same experimental condition according Tukey test *p* value <0,05. Capital letters represent Root covered and lowercase Root exposed. Asterisk means statistical difference among both experimental conditions at the same time experiment according *t* Student test *p* value < 0,05. One asterisk represents *p* value <0,05, two asterisk represent *p* value <0,001. Bars represent standard error.

Since the exposure of the root system to light triggered expressive morphological variations, we performed histological analyses of the treated roots in order to evaluate the light impacts on the cellular level of this organ. For these comparative analysis, special attention was directed to the light-modulated cellular features in the cortical tissue and velamen (FIG. 3A). The results revealed that under both light conditions studied, the youngest

tissues located at the apical portion of the treated roots did not present any clear difference in the cellular organization of both cortex (FIG. 3B and 3C) and velamen (FIG. 3D and 3E) tissues. In addition to velamen as the prevalent trait in the specialized epidermis of *C. fimbriatum* roots, some root hairs were also found scattering the epidermal surface of all growing roots analyzed (FIG. 3D). On the other hand, analyzing the mature region of the root is clear the light response on cortex cell (FIG. 4). When grown exposed to light the cortex cell present markedly thickening cell wall (FIG. 4A). Surprisingly, the light absence on root system left to not induction of modifications on cell wall (FIG. 4B). Trying to clarify the light response, the roots growing exposed to light were transferred to covered root condition. As a surprise the thickening cell wall was no longer formed (FIG. 4C). This results confirm the remarkable effect of light on root morphology.

Figure 3 - Transversal sections of *C. fimbriatum* roots submitted to the different light treatments. (A and F) Sections from mature segments isolated from the middle portion of light-exposed roots to illustrate the general organization of the main tissues (A) in the roots studied with a detail of root hairs (F). (B - E) Sections from young segments isolated from the apical portion of either light-exposed (B and D) or covered (C and E) roots. Cellular details of cortical tissue (B and C) and velamen (D and E).

Figure 4- Longitudinal sections of mature segments isolated from the middle portion of *C. fimbriatum* roots submitted to the treatments of light exposition (A) or darkness condition (B). (C) Illustration of the transition zone that marks the cellular rearrangement in the root cortex triggered by the transference of roots from the light-exposed condition to the darkness. Arrows indicate the presence of cell wall thickenings induced by light until the border of this transition zone.

Light-induced responses in the hormonal status of roots from six-month old plants

Exception for ACC level, those related to IAA and ABA were significantly higher in illuminated than in covered roots (FIG. 5A, B and C). Interestingly, all of them attained the maximum levels at the 15th day, regardless of light condition used. The strong enhancement of IAA and ABA contents showed by exposed roots until day 15, was followed by significance decrease. However, along of the experiment time, any significant variation of these hormones took place when the roots were grown under protected light condition. As to ACC, contrarily to that observed with IAA and ABA, the significant enhancement amount at the 15th occurred in the protected (FIG. 5C), followed also by a reduction on day 30.

Figure 5 - Hormonal content of young roots (from 6-month-old plants). Comparison between the roots growing exposed or protected to light. **A)** Free Auxin content (IAA), **B)** abscisic acid content and **C)** 1-aminocyclopropane-1-carboxylic acid (ACC). **DW** means Dry Weigh. Different letter represent statistical difference on the same experimental condition (according Tukey test considering p value $<0,05$). Capital letters represent Root covered and lowercase represent Root exposed. Asterisk means statistical difference among both experimental conditions at the same time experiment (according t Student test considering different p value $<0,05$). One asterisk represent p value $<0,05$, two asterisk represent p value $<0,001$ and three asterisk represent p value $<0,0001$. Bars represent standard error.

Aiming aim was compare the light response of an organ actively growing with other in stationary growth phase. We also analyzed the initial roots. For our surprise, protecting the root system to light led to growth resumption, in contrast to exposed roots. In order to evaluate changings that might led the meristematic activity return, we divided the roots in three equal parts: tip, middle and base. This approach permitted us to isolate analyze in detail the region that return to active growth. In figure 6 are presented the results obtained with IAA, ABA and ACC levels in three different parts. Is possible to observe a prominent increase on auxin level found in covered root tips at the 15th day, followed by a marked decrease (FIG

6A), being less intense on the mature regions (FIG. 6D and 6G). The ABA content present the same pattern as those observed on young roots with higher levels, on both parts, on exposed root condition (FIG. 6B, 6E and 6H). In the same way, the ACC content was also really similar to young roots, with higher levels on root covered condition. (FIG. 6C, 6F and 6I).

Figure 6 - Hormone contents of initial roots incubated under light and protected light conditions (from 6-month-old plants). Comparison between the roots growing exposed or protected to light. Tip, middle and base represent the apical root part, Middle represents the second part and base the most distant part from the root apex respectively. **A, D and G** free auxin content (IAA), **B, E and H** abscisic acid content (ABA) **C, F and I** 1-aminocyclopropane-1-carboxylic acid content (ACC). DW Dry Weigh. Different letter represent statistical difference on the same experimental condition (according Tukey test considering p value $<0,05$). Capital letters represent Root covered and lowercase represent Root exposed. Asterisk means statistical difference among both experimental conditions at the same time experiment (according t Student test considering different p value $<0,05$). One asterisk represent p value $<0,001$, two asterisk represent p value $<0,0001$ and three asterisk represent p value $<0,001$. Bars represent standard error.

Light-induced responses in the morphology of initial roots from forty-day old plants

Owning the light promote conspicuous modifications on root morphology, we decided to explore more details. For this, we used plants with 40 days old. This plant phase was chosen for presenting only one root, the initial root, and an active growth. It ensures us the plants will not stop growing due the organ age, and certainly will keep active during all experiment time. We used the same approach of plants well developed, covering or exposing the root system to white light. In covered and exposed roots both growth rates presented virtually the same tendency (Figs 7A & 7B), including also, the significant drop occurred from day 5 to day 10 in root-exposed condition.

In order to evaluate the process that induces the cell wall reinforcement, we submitted the roots to white light, and also to blue monochromatic light. As expected on with light was possible detect the cell wall thickening (FIG 8A and 8B). Surprisingly, exposing the roots to red light, any thickening was observed (FIG 8C and 8D). On the other hand, this process largely stimulated with the use of blue monochromatic light (8E and 8F).

Figure 7 – Growth analyzes of the first root (from 40-day-old plants) incubated under light and light protected conditions. **A)** growth rate in mm per day and **B)** growth rate in percentage both taken at each five days. Different letters represent statistical difference on the same experimental condition (according Tukey test considering p value < 0.05). Capital letters represent Root covered and lowercase represent Root exposed.

Figure 8 - Longitudinal section of equivalent root region of the first root formed (from 40-day-old plants) incubated under different light quality. **A)** Cell wall thickenings in roots exposed to white light. **B)** Same image observed with polarized light. **C)** Absence of thickening in red light-exposed roots, confirmed by means of polarized light use **D.** **E)** Intense cell wall thickening in root n exposed to blue light confirmed by the used of polarized light **F).**

Light-induced responses in the hormonal status of initial roots from forty-day old plants

Despite IAA measurements did not reveal significant differences in illuminated and protected roots (FIG. 9A). The same result was obtained with the analyses of ABA (FIG. 9B). On the other hand, ACC showed significant enhancement in the two experimental light situations, but with significantly larger values in covered roots (Fig. 9C).

Figure 9 - Hormone contents of the first root formed (40 days old plants). A) Free auxin (IAA), B) Absciscic acid (ABA) and C) 1-aminocyclopropane-1-carboxylic acid (ACC). DW means Dry Weigh. Different letters represent statistical difference on the same experimental condition (according Tukey test considering p value $<0,05$). Capital letters represent Root covered and lowercase represent Root exposed. Asterisk means statistical difference among both experimental conditions at the same time experiment (according t Student test considering different p value $<0,05$). One asterisk represents p value $<0,05$, two asterisk represent p value $<0,001$. Bars represent standard error.

3.5. Discussion

Light is an environmental cue deeply involved in the root development of the epiphytic orchid *C. fimbriatum*. When grown protected from light exposure, roots displayed conspicuous growth responses in terms of higher length and volume (FIG. 2A). Taken together, these aspects seem to reflect, primordially, the root cell expansion, which is known as an outstanding plant process favored by light absence (Moni et al., 2015; Xu et al., 2013). The cell expansion is an event dependent on both cell wall extensibility and hydraulic force exerted by turgor pressure (Wolf et al., 2012). According to the revision of Beauzamy et al. (2014), turgor pressure impacts not only cell enlargement but also influences several important developmental processes. Besides, secondary wall deposition might restrict the cell growth (Li et al., 2016). In view of this, presumably, the occurrence of cell wall thickening only in cortical cells of the light-exposed roots (FIG. 4A) might have restricted the root growth.

However, despite of being thinner and shorter than the covered roots, the light-exposed roots exhibited higher biomass values both in individual roots and in its relative distribution throughout the root system (FIG. 2A). At some extent, the light-induced thickening in the cell wall of cortical cells might contribute for such morphological response (FIG. 4B). In accordance, the orchid *Phalaenopsis* cultivated in elevated light intensities had increased biomass in both root and shoot systems, and also higher content of lignin in the roots (Ali et al., 2006). Additionally, seedlings of *Ebenus cretica* growing exposed to light presented higher lignin content than seedlings growing under darkness (Syros et al., 2005). Cell wall thickening was also induced when plants of *Helianthus annuus* were transferred from the dark condition to the continuous light (Kutschera, 1990). Accordingly, tomato plants exposed to high light intensities (300, 400 and 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$) presented higher leaf thickness than the plants exposed to lower light intensities (Fan et al., 2013). Furthermore, the orchid *Doritaenopsis* cultivated under blue light presented higher biomass content in its leaves and roots than the plants cultivated under red light (Shin et al., 2008).

The light condition also influenced conspicuously the hormonal content of *C. fimbriatum* roots. For instance, while IAA and ABA levels were higher in young-illuminated roots, ACC content was predominant in the covered roots (FIG. 5A, B & C). In general, the light-induced hormonal patterns observed in the initial roots of forty-day old plants (FIG. 9) were similar to those observed in the younger roots of six-month old plants (FIG. 5). It is known that following the transfer of intact plants of *C. fimbriatum* from the light to the dark

condition triggered the resumption of the shoot meristem activity (Suzuki et al., 2010). In the present study, the transference of the root system of *C. fimbriatum* to the darkness also triggered the growth reactivation of the organ due to an apparent resumption of the root apical meristem activity. Since the auxin maximum is crucial for the root meristem activity (Ding and Friml, 2010; Overvoorde et al., 2010), the observed resumption of the *C. fimbriatum* root meristem might be a consequence of the increased levels of IAA content in roots maintained under darkness (FIG. 6A).

Furthermore, the auxin distribution in the *Arabidopsis thaliana* mutant *repp3* caused cellulose deficiency and defects in cell wall composition (Feraru et al., 2011). Besides, the expression of genes that codify the central transcription factors involved the secondary cell wall production *VND6* and *VND7* is stimulated by *ASL19* (*ASYMMETRIC LEAVES-LIKE*), which is controlled by *ARF7* (*AUXIN RESPONSE FACTOR7*) (Soyano et al., 2008). Additionally, analyses with the mutants *sitiens* (*Solanum lycopersicum*) and *wilty* (*Pisum sativum*), both defectives in the ABA biosynthesis, showed reduced biomass in comparison to the wild type (McAdam et al., 2016). Taking into account the higher levels of ABA and IAA observed in roots maintained under continuous light, they could be considered strong putative candidates related to the observed thickening in cortical cells (FIG. 4A). On the other hand, we did not observe any positive correlation between cell wall thickening and the endogenous levels of ACC of *C. fimbriatum* roots; on the contrary, the occurrence of cell wall thickening was less pronounced in root cells kept under darkness (FIG. 4B) even when they presented relatively elevated levels of ACC (FIG. 5C).

In view of the conspicuous effects of light and darkness on the developmental pattern of the shoot system of *C. fimbriatum* and their marked effects on endogenous hormone status (Suzuki et al. 2004, 2010), coupled with the above exposed results on the root cortex cell wall thickening, a study was performed to verify the type of light on this event. In plants with 40 days old treated with blue and red light types inducing of cell wall thickening occurred only in the presence of blue light (FIG. 9E & 9F). It is known that in epidermal maize coleoptiles, blue light induced the longitudinal orientation, but the application of auxin overcome the effect of blue light reorienting transversally. On the other hand, red light red light prompt the microtubule transversally orientated on intact plants, but sectioned explants, this modification only occur with auxin presence (Zandomeni and Schopfer, 1993). The cell wall composition also shows to be influenced by light, in microarray analyzes of genes light regulated, was found that eight genes related to cell wall synthesis was positively regulated by light. On the

other hand, 26 genes related to cell wall degradation were downregulated by light presence (Ma et al., 2001). Additionally, *Triticum aestivum* seedlings growing exposed to continuous light presented higher activity levels of cell wall enzymes both on coleoptiles and roots, when compared with plants growing in complete absence of light (Krishnan et al., 1985). The presence of cell wall reinforcements in such a grade in the cortex of an epiphytic roots, naturally exposed to light and others stressing environmental cues (Benzing, 1990), would corroborate to minimize the effects dehydration and collapse of the cells, such as occurs in xylem vessels (Rodrigues et al., 2014; Li et al., 2016). The effects of blue light would be represent an important target to biotechnological research (Loqué et al., 2015), is a clear process light dependent strictly induced by blue light (FIG 9E & 9F). Interestingly, independently of light condition the cell wall thickening of velamen kept invariable indicating that the cortex cell wall thickening and velamen one would be modulated by different control signals. The control of hormonal content and morphology, growth, cell differentiation was clearly modulated by light. Additionally, the blue light seems to be more effective on cell wall modifications than the red light. However, the network behind this mechanism is still unclear, approaches more in-deep is necessary to clarify the control on root development of *Catasetum fimbriatum*.

3.6. References

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4. CAPÍTULO II

Auxin and abscisic acid interplay in coordinating carbon partition between leaves and pseudobulbs of the epiphytic orchid *Catasetum fimbriatum* in response to light exposition of its aerial root system.

4.1. Abstract

Catasetum fimbriatum is a seasonally-deciduous epiphytic orchid with a complex and flexible aerial root system, which can grow unexposed or exposed to light. As for the light-exposed roots, pseudobulbs might also contribute positively to carbon balance by recycling respiratory CO₂. In fact, pseudobulbs are essential storage organs for epiphytic orchids and they can support growth and metabolic demands of aerial roots lacking light exposure. Recent studies with some classical plant models have shown that light can coordinate shoot and root development by reciprocally regulating auxin transport between these organs. Besides, the balance between indole-3-acetic acid (AIA) and abscisic acid (ABA) is an important signal for determining the source-to-sink relationship between vegetative organs. This study investigated the potential involvement of auxin and ABA during the light-induced (re)mobilization of carbohydrates in pseudobulbs and leaves of *C. fimbriatum*. The source-to-sink relationship between these organs was manipulated by either darkening or exposing the roots to light, while the shoot system was kept fully illuminated for 10 days. Concurrently, treatments with 10 µM NPA (N-1-naphthylphthalamic acid), an inhibitor of polar auxin transport, were comparatively studied. The youngest vegetative organs were analyzed for the endogenous content of AIA, ABA, sucrose, glucose, fructose, malate and citrate. Covering the roots increased in pseudobulbs the levels of AIA and all carbon sources studied (specially glucose and fructose), while the covered roots showed slightly higher levels of AIA. The concomitant treatment with NPA and root covering caused a sharp decrease of AIA levels in all organs and an ABA increase in the root system. Interestingly, this last condition induced a conspicuous carbohydrate accumulation in pseudobulbs, with sucrose as the predominant form. A possible interplay between auxin and ABA might contribute for these light-controlled responses that influence the root capability of regulating carbohydrate partition in the shoot system by a long-distance signaling mechanism.

Key words: abscisic acid, aerial root, carbon partitioning, epiphytic orchid, polar auxin transport.

4.2. Introduction

Plants are often exposed to environmental variations and, due to their sessile nature, must have the necessary ability to survival and use the physical changing factors to control their development. Certainly, among them, light is one of the most prominent one, both as energy source (Hohmann-Marriott and Blankenship, 2011; Nelson and Ben-Shem, 2004) and environmental signal controlling the photomorphogenic responses (Rockwell et al., 2006; de Wit et al., 2016; Wu, 2014). The plant development is highly modulated by light in several important processes, such as carbohydrate status (Kim et al., 2013; Tombesi et al., 2015), cell division (Okello et al., 2016), carotenoid accumulation (Zhang et al., 2015), de-etiolation (Melo et al., 2016), root halotropism (Yokawa et al., 2014), and control of hormonal signaling and/or production (de Wit et al., 2016).

Among of the plant hormones with recognized signaling mechanisms controlled by light, the auxin deserves special attention. It is well known the importance of the polar auxin transport (PAT), mediated by PIN proteins - a family of efflux auxin transporters -, which is crucial for controlling plant growth and development, and represents the key pathway behind the phototropic responses (Blakeslee et al., 2004; Friml et al., 2002a). Besides, PAT plays a conspicuous role during virtually all phases of root growth and development (Casimiro et al., 2001; Friml et al., 2002b; Hopkins and Kiss, 2012; Laxmi et al., 2008; Lucas et al., 2011; Malenica et al., 2007; Sassi et al., 2012; Zeng et al., 2010). Root responses to light appear to be mediated by the photoreceptors phototropins, phytochromes, UVR-8 (Yokawa et al., 2014), and cryptochromes (Zeng et al., 2010).

The influence of light is also well establish for controlling the transport of metabolites in different plant species and organs (Giaquinta, 1978; Häusler et al., 2014; Keiller and Smith, 1989; Matsuda et al., 2011, 2014; Sauter and Ambrosius, 1986; Savage et al., 2015). In fact, the sugars produced by photosynthesis, mainly the sucrose, can be transported from the source to the sink organs through the vascular system or be destined to storage within the organ (Ruan, 2014; Griffiths et al., 2016). For instance, when wheat shoots were submitted to low light treatment, the root growth was compromised, which was interpreted as a decrease in carbohydrate production caused by a transport reduction (Nagel et al., 2015). Complementary, the capacity for lateral roots rhizogenesis and the levels of soluble carbohydrates in leaves of petunia cuttings were enhanced when submitted to dark treatment before light exposure (Klopotek et al., 2010). Accordingly, higher levels of soluble sugars and relatively low starch amounts were found in shoots of the epiphytic orchid *Dendrobium* “Second Love” when

plants were incubated in the dark; however, a contrary situation occurred in the presence of light (Ferreira et al., 2011). Besides functioning as carbon sources, the carbohydrates produced by photosynthesis can also act as signaling molecules (Rolland et al., 2006). For example, in the *Arabidopsis* mutant *stp-2* (also called *WOX9*), the cell cycle arresting effect seems to be dependent on the presence of sucrose added to the growth medium (Wu et al., 2005), while the supplementation with glucose and fructose restored the cell cycle by activation of *CYCBI;1* expression (Skylar et al., 2011; Wu et al., 2005).

Furthermore, some close relations are found between plant hormones and sugars. For example, the expression of genes coding for YUCCA2 (an enzyme in the auxin biosynthetic pathway), PIN2 (an auxin efflux transporter), two ARFs (Auxin Response Factors) and ABP1 (a receptor of abscisic acid, ABA) were up-regulated by glucose in *Arabidopsis* (Mishra et al., 2009). Besides, the involvement of ABA with the sugar signaling was revealed by the *Arabidopsis* mutant *glucose-insensitive (gin1)* which shows insensitivity to glucose even when growing in a culture medium supplemented with this sugar (Zhou et al., 1998). However, the glucose sensibility was restored in *gin-1* when this mutant was incubated in a medium supplemented with 4% of glucose and 100 nM of ABA (Cheng et al., 2002). Complementarily, the *aba1-1*, *aba2-1* and *aba3-2* mutants, with deficiency in the ABA production, were insensitive to the glucose presence in the culture medium, suggesting an ABA-dependence for glucose perception (Arenas-Huertero et al., 2000). On the other hand, genes related to ABA signaling, such as *ABI3* and *ABI4*, were not induced by the simple presence of ABA or glucose in the medium, suggesting the necessity of presence of both molecules to induce the expression of these aforementioned genes. In addition, the mutant *aba2-gin1* did not present any expression of neither *ABI3* or *ABI4*, indicating the involvement of a synergistic role of glucose and ABA in the ABA signaling pathway as well (Cheng et al., 2002). Moreover, organic acids, such as malate and citrate, are other putative molecules capable of acting as both carbon source and signaling molecule within plant organs. Malate can participate in photosynthetic reactions as a CO₂ concentrator mechanism and can also be used as a substrate for ATP production through the tricarboxylic acid cycle in the mitochondria (Peckmann et al., 2012). In plastids of sunflower embryos supplied with different carbon sources, malate supported the highest fatty acid production (Pleite, 2005). Furthermore, this organic acid act as an important osmoticum (Lee et al., 2008).

For terrestrial plants, the aerial system is responsible for capturing most light signals from the surrounding environment, whereas the aboveground root system is mainly involved

with the plant fixation and the uptake of water and nutrients from the substrate. However, in most epiphytic species of orchids the entire plant body, including the root system, can be exposed to light incidence. According to Benzing (1990), amongst three epiphyte plants two are orchids, which exemplifies the enormous importance of Orchidaceae as one of the largest and most diverse cosmopolitan families among angiosperms. It is suggested that roots were the most modified organs during the evolutionary passage from the soil to the canopy niche in the tropical-subtropical rainforests, due to the necessity of several adaptations to ensure the plant survival in a highly hostile habitat (Holst, 1999). Among the morphological specializations present in the root system of epiphytic orchids that deserves particular attention is the velamen; a structure formed by the differentiation of a dead multilayer epidermis which is closely related to both water retention and absorption (Benzing, 1990). Besides, most aerial roots of epiphytic orchids can also photosynthesize when exposed to light, a fact best exemplified by the extreme cases of some shootless orchids that completely rely on their green aerial roots for performing (Benzing et al., 1983).

In terms of morphological plasticity, the Neotropical species of epiphytic orchids included in the *Catasetum* genus show notable developmental flexibility in response to a wide array of environmental and endogenous cues mediated (Majerowicz and Kerbaudy, 2002; Rodrigues et al., 2014; Suzuki et al., 2004, 2010). For example, *in situ* conditions this orchid usually form a huge nestle-like structure consisted of fine and short lateral roots, inside of which are accumulated organic debris and humidity (Holst, 1999). Interestingly, while all these lateral roots are typically gravitropic negative, the largest first formed are gravitropic positive, growing inside the suspended basket roots, absorbing nutrients and water. Besides, root tips of *Catasetum fimbriatum* are able to convert into shoots, giving rise to new plants (Kerbaudy, 1984; Colli and Kerbaudy, 1993), while this process was inhibited by the exogenous auxin IBA (indolebutyric acid) with a concomitant decrease in the levels of soluble carbohydrates (Vaz et al., 1998). Other interesting characteristic of this plant is the capacity to maintain an indeterminate growth of the shoot system in absence of light, giving rise to stolon-like structures instead of developing pseudobulbs as the typical store organs present in several orchids (Suzuki et al., 2004). A significant drop of IAA level in the shoots took place when they were transferred from light to dark condition (Suzuki et al., 2010). A possible interaction between the shoot and the root systems was also described for *C. fimbriatum* plants due to differences in dry matter partitioning between these organs in response to the

nitrogen source. Furthermore, the levels of soluble sugars also presented variation according to nitrogen source (Majerowicz and Kerbaudy, 2002).

Therefore, the main goal of this study was to evaluate the impact of the light exposition of *C. fimbriatum* root system in the source-to-sink relationship between leaves and pseudobulbs. Concomitantly, we also investigated the potential involvement of auxin and ABA as mediators of the light-induced responses in both shoot and root systems.

4.3. Material and Methods

Plant growth conditions

Micropropagated plants of *Catasetum fimbriatum* Lindl (Orchidaceae) were obtained in Vacin and Went solution (1949), according to procedure described by Rodrigues *et al.* 2014. After six months of development *in vitro*, the plants were transferred to collective pots using dried moss fiber as substrate and they were kept during twelve months in a green house. Following this period, the plants was transferred to a climatic chamber with $200 \mu\text{mol m}^{-2}\text{s}^{-1}$ of light intensity emitted by fluorescent lamps, temperature $25 \text{ }^{\circ}\text{C} \pm 2$, 70% relative humidity, and 12 h of photoperiod. After 30 days under this conditions, the plants were gently removed and transferred to transparent glass vials (10-cm heigh and 3.6-cm wide), filled with the same inorganic solution previously described (without agar), and with the root system fully exposed to light. Nearly only one centimeter of the roots was plunged in the nutritional solution. During 30 days of incubation the nutritional solution was renewed every three days. Subsequently this experimental condition acclimatization period, the plants were transferred to the same type of vials containing distilled water and incubated for more twenty days after which they were separates in two experimental groups. The course time of the experiments was 10 days. In one of them the plants were remained in 5.8 adjusted pH distilled water with the roots both light exposed or protected conditions, with the respective shoots continuously illuminated. In the second the experimental conditions were exactly the same but, however, the plants treated with $10 \mu\text{M}$ of *N*-(1-Naphthyl)phthalamic acid (NPA) a potent inhibitor of polar auxin transport, added to distilled water with adjustment of pH 5.8. For each experimental condition were used 15 individuals, totalizing 60 plants. The plants remained at $25 \text{ }^{\circ}\text{C} \pm 2$ and $150 \mu\text{mol m}^{-2}\text{s}^{-1}$ of light intensity supplied by LED. After 15 days of incubation the plants were divided in leaves and pseudobulbs for the analysis.

Hormonal analysis

Endogenous IAA and ABA amounts were simultaneously quantified by GC-MS procedure of Melo et al. (2016). Approximately 200 mg of frozen roots samples were ground in liquid nitrogen in the presence of 20 mg poly(vinylpyrrolidone) (PVPP) and subsequently homogenized in 1.25 mL extraction solution composed of 10 mM ascorbic acid, 10 mM ethylenediamine tetraacetic acid (EDTA) and 10 mM dithiothreitol (DTT). The internal standard was included, 1 µg [$^{13}\text{C}_6$]-IAA (Cambridge Isotopes, Inc.) and 2 µg [$^2\text{H}_6$]-ABA (OlChemIm Ltd) were added to each sample as internal standards. After centrifugation (25000 g, 20 min, 4°C), the supernatant was purified via solid phase extraction (SPE) column (Supelclean LC-NH₂, Supelco) as described in Chen et al. (1988). Purified samples were evaporated, resuspended in 200 µL of methanol and divided in two parts, both was again dried and one of them was used to IAA quantification and the other one for ABA quantification. For IAA, the sample was resuspended in 50 µL pyridine followed by a 60-min derivatization at 92 °C using 50 µL *N*-tert-Butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA). For ABA, the sample was resuspended in 20 µL of methanol and followed by 30 min derivatization in room temperature using 10 µL (Trimethylsilyl)diazomethane. For both hormones, the analysis was performed on a gas chromatograph coupled to a mass spectrometer (model GCMS-QP2010 SE, Shimadzu) in selective ion monitoring mode. The chromatograph was equipped with a fused-silica capillary column (30 m, ID 0.25 mm, 0.25 µm thick internal film) DB-5 MS (Agilent Technologies) stationary phase using helium as the carrier gas at a flow rate of 4.5 mL min⁻¹ in the following program: 2 min at 100°C, followed a ramp by 10°C min⁻¹ to 140°C, 25°C min⁻¹ to 160°C, 35°C min⁻¹ to 250°C, 20°C min⁻¹ to 270°C and 30°C min⁻¹ to 300°C. The injector temperature was 250°C and the following MS operating parameters were used: ionization voltage, 70 eV (electron impact ionization); ion source temperature, 230°C; interface temperature, 260°C. Ions with a mass ratio/charge (*m/z*) of 244, 202 and 130 (corresponding to endogenous IAA), 250, 208 and 136 (corresponding to [$^{13}\text{C}_6$]-IAA), 190, 162 and 130 (corresponding to endogenous ABA), and 194, 166 and 134 (corresponding to [$^2\text{H}_6$]-ABA) were monitored. Endogenous concentrations were calculated based on extracted chromatograms at *m/z* 244 and 250 for IAA and 190 and 190 for ABA.

Sugars and organic acids analysis

Soluble sugars and organic acids were performed as described by Roessner et al. (2001), with some modifications. 100 mg of fresh sample were ground in liquid nitrogen and extracted with solution methanol:chloroform:water proportion 12:5:1 (v/v/v). It was added 40 µg of salicylic acid as internal standard for organic acid and 200 µg of phenyl-β-glucopyranoside as soluble sugars internal standard, then the sample was vigorously mixed and extracted at 60°C during 30 min. After were added 500 µL of water and centrifuged at 16000 g for 10 min, next the supernatant was collected. For soluble sugars analyses was dried 25 µL of extract under vacuum at 60°C, then was add 25 µL of pyridine and the derivatization was performed with 25 µL of *N,O*-bis(trimethylsilyl)trifluoroacetamide with 1% of trimethylchlorosilane (BSTFA 1% TMCS) incubated for 60 min at 75°C, in the end was injected. For the organic acids was dried 50 µL of extract under vacuum at 60°C, 25 µL of pyridine was added and the derivatization was performed with 25 µL of *N-tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) incubated for 60 min at 92°C, then it was injected. For both of them, the analyses were performed with the same method configuration on gas chromatograph coupled mass spectrometer (model QP2010 SE Shimadzu) in scan mode. The chromatograph was equipped with a fused-silica capillary column (30 m, ID 0.25 mm, 0.25 µm thick internal film) DB-5 MS (Agilent Technologies) stationary phase using helium as the carrier gas at a total flow rate 24 mL min⁻¹ and column flow 1 mL min⁻¹ in the following program: initial temperature at 100°C, followed a ramp by 6°C min⁻¹ to 300°C, remaining during 10 min. The injector temperature was 290°C and the following MS operating parameters were used: ionization voltage 70 eV (electron impact ionization), ion source temperature 200°C, interface temperature 250°C, scan mode, event time 0.30 seconds, scan speed 3333. We verified if the endogenous amount of salicylic acid would be able to interfere in this analysis, and we found that, in this case, the endogenous content it was not detectable in scan mode.

Statistical Analyzes

Statistical analyses were performed using SAS Jmp statistical software 13.0. Results with $p < 0.05$ were considered significantly different.

4.4. Results

According to showed in table 1 the amounts of endogenous IAA of leaves changed largely depending on the light conditions of the root system. Thus, while in root-exposed condition the IAA leaves content was 3.0-fold higher than those of root-covered situation; however, contrarily, under this protected light condition the IAA levels of pseudobulbs and roots enhanced 1.8 and 1.62-fold, respectively. Being the roots of *Catasetum fimbriatum* typically aerial organs, it is interesting to highlight the positive influence of light on the IAA leaf amount, which was 5.27 higher than roots itself. However, a rather unexpected significant drop on this IAA ratio (1.0 fold) was observed when the roots were maintained under light protection condition. This ratio decrease was due to mainly a diminishing of IAA level in the leaves itself and not to any putative IAA synthesis in the roots, since their amounts did not change both in the presence or in absence of light. Anyway, it is interesting to highlight also a gradient of IAA established from shoot to root direction in root exposed plants, with the intermediated level of the auxin of pseudobulbs. On the other hand, light absence on system root, the highest amount of IAA was in the pseudobulb. Shoot and root presented such similar results. Notice that there is a tendency of inversion of IAA content comparing the same organ of both condition. According to showed in table 1 NPA treatment disturbed significantly IAA status in studied organs by reducing drastically on the one hand the presence of the auxin in leaves and pseudobulbs and enhancing in their amounts in light exposed roots. Curiously, the valor of auxin acquired in this organ (42.31) was nearly the sum of three ones in the absence of NPA.

Table 1 – Auxin content in leaves, roots and pseudobulb of plants with root system growing either exposed or covered to light. Comparison among control group and plants treated with NPA, a polar auxin transport blocker. Different letters means statistical difference (Tukey test p value $< 0,05$), $\pm$ represent the standard deviation. nd means non detected.

Treatment Organ	<i>Root Exposed</i>		<i>Root Covered</i>	
	Control	NPA	Control	NPA
Leaves	27,00 $\pm$ 5,32 b	nd	8,99 $\pm$ 1,79 cd	nd
Pseudobulb	11,44 $\pm$ 3,89 cd	nd	20,71 $\pm$ 3,11 bc	2,32 $\pm$ 1,30 d
Roots	5,12 $\pm$ 1,63 d	42,31 $\pm$ 10,89 a	8,30 $\pm$ 3,41 d	7,10 $\pm$ 2,32 d

As described above, the light absence on root system caused expressive change on IAA amounts in shoot organs such as leaves and pseudobulbs of *Catasetum fimbriatum* plants (Table 1). In view of the long distance putative effect of the dark on the auxin level, we decided to verify some presumable involvement of the root system on the transport of IAA, as

well as ABA, carbohydrates and organic acids too. As a whole, the treatment with N-1-naphthylphthalamic acid (NPA), a polar auxin transport inhibitor, resulted in expressive effects on IAA, ABA, sugars and organic acids endogenous amounts on leaves and pseudobulbs, either illuminates or dark maintained roots. Neither on root-exposed nor root-covered conditions was possible to detect any amount of IAA on leaves. The same was verified on pseudobulb of plants with root exposure to light. On dark root treatment, the NPA caused remarkable reduction on IAA content. In contrast, surprisingly, the amount of IAA content enhanced on light exposed roots and did not modified in those kept under dark condition.

Table 2 - Absciscic acid content in leaves, roots and pseudobulb of plants with root system growing either exposed or covered to light. Comparison among control group and plants treated with NPA, a polar auxin transport blocker. Different letters means statistical significant difference (Tukey test p value $< 0,05$), $\pm$ represent the standard deviation. nd means non detected.

Treatment Organ	<i>Root Exposed</i>		<i>Root Covered</i>	
	Control	NPA	Control	NPA
Leaves	2004,92 $\pm$ 22,34 ab	427,61 $\pm$ 32,43 ef	1090,02 $\pm$ 71,67 cde	682,62 $\pm$ 55,73 def
Pseudobulb	1211,09 $\pm$ 8,01 bcde	1419,10 $\pm$ 103,654 bcd	1171,40 $\pm$ 71,95 cde	1207,22 $\pm$ 13,46 bcde
Roots	170,10 $\pm$ 140,05 f	1663,45 $\pm$ 71,79 bc	208,92 $\pm$ 96,03 f	2777,67 $\pm$ 4,85 a

According displayed in table 2 the treatment with the polar auxin transport blocker, left to a strongly decrease of ABA amounts on root-exposed plants, as well a clear diminishing tendency in root-covered plants. Interestingly, the tendency to ABA dropping in the leaves was not followed by the pseudobulbs (Table 2). However, any significant difference was detected in NPA treatment, regardless of the root light incubation (Table 2). According to displayed in figure 1, the pattern of accumulation of fructose, glucose and sucrose on leaves and pseudobulbs did not vary significantly when the roots were exposed to light. Only a, tendency to storing these monosaccharides was observed in the pseudobulbs of root-light protected plants. Soluble sugars contents were also largely influenced by the presence of NPA (FIG. 1). Plants with root exposed to light presented a substantial enhancement of fructose and glucose amounts in the pseudobulb, but not in the leaves (FIG. 1). On root-covered condition, either on leaves or pseudobulbs showed an increasing tendency on the amounts of fructose and glucose, being although only the pseudobulbs results statistically significant. Differently from the both monosaccharides, sucrose showed a different dynamic of accumulation. Thus, while in plants with light exposed roots NPA treatment enhanced significantly their amount only in the leaves, in those with protected roots

NPA a significant increase in glucose content occurred on either leaves or pseudobulbs (FIG. 1).

The amounts of organic acids in leaves and pseudobulbs did not vary significantly in the root light exposed plants; however, their accumulation in the pseudobulbs of root light protected plants was 7-fold and 3 times higher for citrate and malate than in leaves, respectively (FIG. 2A and B). A tendency of increase was observed comparing the pseudobulb and leaves of light root condition. Besides, NPA treatment changed also the distribution of the studied organic acid in leaves and pseudobulbs, resulting in large differences between them depending on the light root conditions. In comparison to the control, an increasing tendency on the contents of citrate caused by NPA (3.6 fold higher) was observed only in leaves of protected root plants. The same tendency took place also with malate in those plants with protected root system (FIG. 2B). However, a significant increase on malate content was detected on leaves of plants with root exposed to light. In root-protected condition, a clear similar increasing propensity in leaves was observed too.

Figure 1 (A) Fructose, (B) Glucose, (C) Sucrose, content in leaves and pseudobulb (PB). White bars columns represent control plants and black columns plants treated with NPA, a polar auxin transport blocker. The shaded area indicates plants with system root protected to light. Bars represent the standard deviation. Different letter indicate significant difference (Tukey test $p < 0.05$).

Figure 2 - (A) citrate and (B) malate content in leaves and pseudobulb (PB). White columns represent control plants and black columns plants treated with NPA, a polar auxin transport blocker. The shaded area indicates plants with system root protected to light. Bars represent the standard deviation. Different letter indicate significant difference (Tukey test p value $<0,05$).

4.5. Discussion

Plants of *Catasetum fimbriatum*, a Neotropical epiphytic orchid, showed prominent changes in IAA, ABA, sugars and organic acid amounts when the root system was maintained in the dark. According to studies performed with this orchid IAA synthesis can occur both in attached and isolated light exposed roots (Peres et al., 1999; Peres and Kerbauy, 1999), as well in their shoots (Suzuki et al., 2004). A positive light influence on auxin root synthesis was observed also to *Zea mays* (Suzuki et al., 2016). However, an unexpected response on IAA accumulation was observed when the root systems of the plants were maintained under dark condition, resulting in a rather and noteworthy significant drop in the IAA leaves suggesting, at first view, the involvement of a long distance hormonal control process.

In *Arabidopsis* was shown that the PIN location in the roots could be positively modulated by light, and the absence of light drive the PIN protein to vacuolar compartments (Laxmi et al., 2008). Therefore, the decrease observed on plants with protected roots, might

reflect alterations in the PIN protein traffic. In this case, the conspicuous IAA accumulation in the pseudobulbs could indicate an enhancement of polar auxin transport to these organs, but not for the roots. Thus, it is plausible to suggest that in this orchid the leaves auxin content would be a root dependent process; if so, a long-distance modulating process would be involved. In line with these results, NPA-light treated plants caused also a similar response in promoting IAA accumulation in the roots, but not in pseudobulbs, while in NPA-dark root treatment auxin amount was comparatively low. In this orchid, the effect of NPA seems to vary according to organ type.

Furthermore, the light root condition seems to influence auxin status in entire plant. Presumably, this unexpected endogenous change in auxin distribution could be related to the fact the roots of *Catasetum fimbriatum* are aerial photosynthesizing organs (Kerbaudy et al., 2012), and so with some particular physiological attributes did still not describe to terrestrial ones. A singular ability of the *Catasetum* is the apexes meristems of isolated roots to convert directly into buds (Kerbaudy, 1984), an event controlled by endogenous auxin-to-cytokinin ratio (Peres et al., 1999). In the later study, significant enhancements of both IAA (3.5 fold) and IAA conjugates (12.9 fold) were found at the 10 days of incubation under light. Virtually, the ability of *Catasetum fimbriatum* roots to produce auxin (Peres et al., 1999; Peres and Kerbaudy, 1999) is deeply corroborated by the high IAA status displayed in the shootless epiphytic orchid *Campylocentrum burchellii* (Peres et al., 1997). In fact, the apparent importance of light on auxin augment was showed also to roots of terrestrial plants according demonstrated in *Zea mays* exposed to a flash of white light (Suzuki et al., 2016). Altogether, these data could indicate that beyond auxin transport effect light presence could also positively influence their production and/or conjugation in *Catasetum fimbriatum*.

Similarly, to that happened with IAA leaf content, the levels of ABA in root protected plants dropped also significantly in that organ. Interestingly, a positive correlation between these hormones was reported on shoot tissue of *Galium aparine* that, when treated with auxin, presented an increase of ABA content (Hansen and Grossmann, 2000) even with synthetic auxin (Grossmann et al., 1996). To clarify this interface, we used the NPA to verify whether this ABA change occurred as a direct consequence of light absence in the roots or due to the auxin change in the leaves. Surprisingly, even with the root system exposed to light, a noticeable coincidence among abscisic acid and auxin reduction in leaves (Table 2). These data might be an indication of a close relationship between IAA presence and ABA levels.

It is well reported in the literature relationships between hormones and exogenous carbohydrates (León and Sheen, 2003; Rolland et al., 2006). Sugar treatments are capable to increase IAA synthesis through induction of gene expression related to auxin biosynthesis like *YUCCA8*, *YUCCA9*, *CYP79B2* and *CYP79B3* (Sairanen et al., 2012). Furthermore, genes related to auxin response as *IAA17*, *IAA19* and *IAA29*, and transport as *PIN7*, are also positively modulated by sugar (Lilley et al., 2012). Additionally, the sugar content modulated by exogenous hormones application has been also explored. Plants of *Phaseolus vulgaris* when treated with IAA, resulted on increased of total sugar content (Altman and Wareing, 1975). Additionally, *Cucurbita maxima* IAA-treated resulted in different effects on sugar content, on outer tissues of hypocotyl the total soluble carbohydrate increased while on outer tissues the content of these molecules decreased (Wakabayashi et al., 1990). Since that, the studied sugar contents did not vary significantly both in leaves and in pseudobulbs regardless of root light conditions. We explored the response to auxin variation triggered by NPA exposure. Blocking the polar auxin transport, resulted to significant change on sucrose leaves contents, the main form of sugar transport in plants (Kühn and Grof, 2010; Ward et al., 1998), suggesting so some relationship between polar auxin transport and sucrose transport in the leaves. The significant low amount of sucrose in pseudobulbs seems to corroborate this point of view. The significant enhancement of glucose and fructose levels in these organs could reflect their transport from the leaves or to a sucrose molecule breakdown, both influenced by NPA. On root-covered condition, the effect of NPA followed a different pattern. The fructose and glucose presented a tendency to higher level on leaves, and in contrast to root exposed condition, the sucrose amount presented prominent elevation. On pseudobulb, glucose and fructose alterations in response to NPA were similar either in exposed or covered root system. However, the sucrose amount increased deeply. Is possible perceive that the blockage of polar auxin transport increased the content of sugar, but covering the root system, the response to NPA was intensified. Actually, hybrid plants of *Dendrobium* presented elevated sugar content in response to dark grow condition while light exposed plants presented higher amount of starch (Ferreira et al., 2011). Thus, the diminishing IAA amount seems to modify fructose, glucose and sucrose levels. However, a possible additional signal that is responsive to light lack and to auxin decrease, might be enhancing the change the carbohydrate profile mainly when it come sucrose. Apparently, the ABA is not involved in these processes once this hormone did not show any modification in pseudobulbs.

The most conspicuous modification involving citrate occurred in response to light. Notice that there is no difference between leaves pseudobulb in root-lightning condition. However, the root-light absence resulted a difference of eight folds higher on pseudobulb. Furthermore, a tendency of increasing was detected on leaves with root covered using NPA. Apparently, the changes in citrate content are not due to polar auxin transport inhibition because the NPA caused just slight and no significant changing. Conversely, since the light depletion on the system root left a massive increase of citrate content, is possible to infer that this organic acid amount is modulate by light and not by auxin. In contrast, malate content presented clear modulation in response to IAA transport inhibition. Leaves of plants of root-exposed condition treated with NPA, presented a consistent increase on malate amount. Interestingly, wild-type *Arabidopsis* plants when treated with IAA, presented a decrease on shoot malate content and the mutant *slr* (*SOLITARY-ROOT*), that presents low sensitive to auxin, showed higher concentrations of organic acids than the wild type (Anegawa et al., 2015). In summary, citrate is apparently induced by the root darkened condition and the presence of auxin might downregulate the malate quantity on leaves.

Our data suggest a close relation among the hormone content and the source and sink interaction. In view of all modifications on carbon sources in response to light condition on root system. Showing that the root system is capable to change the metabolism on shoot part by the simple presence or absence on roots.

4.6. References

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5. CONCLUSÕES GERAIS E PERSPECTIVAS

O conjunto de dados obtidos neste trabalho nos permite concluir que a presença ou ausência de incidência de luz no sistema radicular de *Catasetum fimbriatum* gerou respostas, em geral, antagônicas. Primeiramente, a morfologia da raiz mostrou ser fortemente modulada pela luz, uma vez que raízes protegidas da luz apresentaram maiores taxas de crescimento. Entretanto, mesmo sendo menos alongadas, as raízes crescidas na luz apresentaram maior conteúdo de biomassa. Este resultado apresenta fortes ligações com as análises histológicas que revelaram a formação de um intenso espessamento da parede celular no tecido cortical apenas das raízes expostas à luz, sendo o comprimento de onda na faixa do azul o principal sinal indutor desse processo. As análises hormonais revelaram que os níveis de AIA e ABA, em geral, são positivamente regulados pela presença da luz, ao passo que teores do ácido 1-aminociclopropano-1-carboxílico foram superiores em raízes protegidas da incidência luminosa. Tais resultados sugerem que a luz module o desenvolvimento radicular de *C. fimbriatum* por meio de uma intrincada cascata de sinalização envolvendo ajustes finos e coordenados nos teores de AIA, ABA e ACC. Outro ponto importante que apresenta estreita relação com a presença ou ausência da incidência luminosa no sistema radicular de *C. fimbriatum* foi revelado pelo grupo de resultados que apontaram para a existência de comunicação entre as partes caulinar e radicular dessa orquídea mediada pelos hormônios AIA e ABA, o qual se mostrou dependente da exposição (ou não) do sistema radicular à incidência luminosa e pareceu capaz de regular a longa distância as relações de fonte e dreno entre as folhas e pseudobulbos.

Assim, algumas abordagens complementares poderiam auxiliar a elucidar os resultados obtidos neste trabalho. Primeiramente, necessita-se de uma investigação mais aprofundada sobre os possíveis mecanismos de comunicação de longa distância entre os órgãos de plantas de *C. fimbriatum* que foram descobertos nesse trabalho por meio da exposição do sistema radicular à luz ou escuridão. Essa é uma questão extremamente inovadora que possui potencial para múltiplas explorações experimentais visando o entendimento da influência do sistema radicular sobre a parte caulinar das plantas. Além disso, uma investigação da dinâmica hormonal em resposta às luzes azul e vermelha poderia ser um primeiro passo rumo ao estabelecimento das relações existentes entre a exposição das raízes à luz e a diferenciação celular das células do córtex. Adicionalmente, a utilização de substâncias reguladoras de crescimento, seja o fornecimento de hormônios *per se* ou de substâncias inibidoras de seu transporte, biossíntese ou percepção poderia ajudar a entender

melhor a participação de cada hormônio neste processo. Outra abordagem interessante seria uma análise da composição da parede celular das células do córtex em resposta às diferentes condições luminosas. Tendo em vista que nossos resultados mostraram um gradiente de diferenciação celular induzido pela luz em sentido oposto ao ápice radicular, seria interessante levar essa questão para se estudar variações de respostas ao longo da raiz como, por exemplo, a o estudo da expressão de genes relacionados à diferenciação da parede celular, bem como analisar a expressão diferenciada destes frente às diferentes condições de exposição luminosa do sistema radicular de *C. fimbriatum*.

7. RESUMO

O ambiente epifítico é considerado um dos habitats mais desafiadores para as plantas, pois a disponibilidade hídrica e nutricional pode ser bastante escassa. Além disso, as plantas que colonizam este ambiente estão mais expostas aos fatores ambientais, dentre eles a luminosidade se mostra bastante importante, uma vez que esta atua tanto como fonte de energia na fotossíntese quanto como sinal ambiental em repostas fotomorgênicas. Sabe-se, por exemplo, que a luz exerce forte influência sobre a morfogênese radicular de plantas em geral, porém impacta ainda mais o desenvolvimento de orquídeas epífitas, dada a frequente exposição de suas raízes aéreas à incidência luminosa. Tendo em vista que a auxina ácido indolil-3-acético (AIA), o ácido abscísico (ABA) e o etileno são moduladores cruciais no controle da arquitetura radicular na maioria das plantas, sendo também mediadores chave em várias respostas fotomorfogênicas, este estudo propôs-se a investigar o possível envolvimento destes hormônios durante diferentes respostas morfo-fisiológicas desencadeadas pela exposição à luz do sistema radicular de plantas de *C. fimbriatum*. A ausência de incidência luminosa sobre as raízes resultou em maiores taxas de crescimento e volume radicular, porém, com menor acúmulo de biomassa em relação às raízes expostas à luz. O incremento na biomassa em raízes expostas à luz esteve correlacionado ao espessamento da parede celular na região cortical, o qual ocorreu em resposta especificamente à luz azul. Em termos gerais, a exposição das raízes à luz induziu o aumento nos níveis de AIA e ABA, enquanto que os teores de ACC foram superiores em raízes protegidas da incidência luminosa. Estes resultados sugerem que a luz pode modular o desenvolvimento radicular de *C. fimbriatum* através de um fino controle hormonal que depende de ajustes coordenados dos níveis de AIA ABA e ACC. Também foi investigado o potencial envolvimento das auxinas e do ABA durante a remobilização de carboidratos entre pseudobulbos e folhas de plantas que tiveram seus sistemas radiculares expostos à (ou protegidos da) luz. Os resultados revelaram que a manutenção das raízes sob condições de escuro levou ao aumento dos teores de AIA e de todas as fontes de carbono estudadas (especialmente de glicose e frutose) nos pseudobulbos, enquanto que as raízes cobertas apresentaram apenas um leve aumento no conteúdo de AIA. O tratamento concomitante das raízes com a condição de escuro e a aplicação de um inibidor do transporte polar de auxina causou uma diminuição abrupta nos teores de AIA em todos os órgãos analisados e a elevação do conteúdo de ABA no sistema radicular. De maneira interessante, essa última condição experimental induziu um conspícuo acúmulo de carboidratos nos pseudobulbos, principalmente de sacarose. Assim, os dados deste trabalho

reforçam a importante participação do AIA e ABA como possíveis mediadores da sinalização desencadeada pela luz incidente no sistema radicular de *C. fimbriatum*, cujas respostas induzidas regulam não somente a morfogênese de tecidos radiculares, mas também influenciam na regulação da partição de carbono no sistema caulinar por meio de um provável mecanismo de sinalização à longa distância.

Palavras chave: Desenvolvimento radicular, luz, morfogênese, hormônios, carboidratos, *Orchidaceae*.

8. ABSTRACT

The epiphytic environment is considered one of the most challenging for plants, due to frequent scarcity of water and nutrients. Furthermore, the plants that colonized this biotope are usually more exposed to the environmental cues. Light is considered one of the most important signals controlling plant development because it can act as both an energy source for photosynthesis and an environmental signal for photomorphogenic responses. Besides, light can influence the root morphology of most plants, with even stronger impacts expected in aerial roots of epiphytic orchids due their frequent exposition to direct light. Since indole-3-acetic acid (IAA), abscisic acid (ABA) and ethylene are crucial hormonal signals modulating the root architecture in most plant species, and key mediators during numerous photomorphogenic responses, this study investigated the potential involvement of these hormones in different morpho-physiological responses regulated by either the darkness treatment or the light exposure of *Catasetum fimbriatum* root system. The absence of light incidence on the roots resulted in higher root volume and growth rate, but lower dry mass accumulation than the light-exposed ones. The higher accumulation of biomass in the light-exposed roots was closed correlated with a more intense cell wall thickening in the root cortex, which appeared to be specifically induced by the blue light. In general, root exposure to light induced increasing levels of ABA and AIA, while the ACC content was higher in roots protected from light. This suggests that light might modulate *C. fimbriatum* root development through a fine-tuned hormonal mediation, which depends on coordinated adjustments of IAA, ABA and ACC levels. This study also investigated the potential involvement of auxin and ABA during the (re)mobilization of carbohydrates in pseudobulbs and leaves of plants that had their root systems either exposed to (or protected from) light. The results revealed that covering the roots increased in pseudobulbs the levels of AIA and all

carbon sources studied (specially glucose and fructose), while the covered roots showed slightly higher levels of AIA. The concomitant treatment with NPA and root covering caused a sharp decrease of AIA levels in all organs and an ABA increase in the root system. Interestingly, this last condition induced a conspicuous carbohydrate accumulation in pseudobulbs, with sucrose as the predominant form. The data obtained in this study reinforce the remarkable participation of IAA and ABA as possible mediators of the signaling cascades triggered by the light incidence on *C. fimbriatum* root system, which was capable of inducing photomorphogenic responses not only in root tissues, but was also able to influence the carbon portioning in the shoot system by a potential long-distance signaling mechanism.

Key words: Root development, light, morphogenesis, hormones, carbohydrates, Orchidaceae.