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EDISON CARDONA MEDINA

Interação parasita-hospedeiro e síntese de azafranina em raízes da hemiparasita de raiz *Escobedia grandiflora*

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Tese submetida ao Programa de Recursos Genéticos Vegetais da Universidade Federal de Santa Catarina para a obtenção do título de Doutor em Ciências
Orientador: Prof. Dr. Rubens Onofre Nodari
Coorientador: Prof. Dr. Fernando Joner

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EDISON CARDONA MEDINA

Interação parasita-hospedeiro e síntese de azafranina em raízes da hemiparasita de raiz *Escobedia grandiflora*

O presente trabalho em nível de doutorado foi avaliado e aprovado por banca examinadora composta pelos seguintes membros:

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Certificamos que esta é a **versão original e final** do trabalho de conclusão que foi julgado adequado para obtenção do título de doutor em Ciências.

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Prof. Dr. Rubens Onofre Nodari
Orientador

Florianópolis, 2021.

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"*Parasitic flowering plants are as beautiful as any orchid, as interesting as any carnivorous plant, and as important to the ecosystems and human life as any other organism.*"

(Henning S. Heide-Jørgensen)

RESUMO

Escobedia grandiflora (L.f) Kuntze é uma planta hemiparasita de raiz que possui raízes de cor laranja, caracterizadas pela presença de azafranina, que são usadas com fins medicinais e como corante alimentar. Apesar da importância desta planta, pouco se conhece sobre o seu hábito parasítico e o pigmento da raiz. O presente trabalho objetivou avaliar as interações de *E. grandiflora* com as hospedeiras, bem como investigar sobre a biossíntese e a função da azafranina nas raízes de *E. grandiflora.* Foram desenvolvidos quatro capítulos que abordaram: (i) a sua influência na composição e diversidade de plantas em quatro comunidades; (ii) a indagação sobre a biossíntese da azafranina e sua função nas raízes de *E. grandiflora*; (iii) a determinação da preferência por hospedeiros em duas fases do desenvolvimento de *E. grandiflora* e (iv) a avaliação da interação entre *E. grandiflora* e duas hospedeiras, *Paspalum glaucescens* e *Eryngium elegans*. Os principais resultados obtidos foram que: (i) na presença de *E. grandiflora* houve variação na composição de espécies em quatro comunidades de plantas do sul do Brasil. Ademais, ela foi associada com maior riqueza de espécies, maior diversidade e uniformidade, bem como houve redução da cobertura das plantas mais dominantes em quatro comunidades de plantas. Os resultados encontrados foram similares nas quatro comunidades avaliadas, que apresentaram fisionomias marcadamente diferentes. (ii) Foi confirmada a presença maioritária da azafranina nas raízes de *E. grandiflora,* assim como foram identificados os genes que codificam as enzimas associadas a biossíntese dos carotenoides e apocarotenoides. Adicionalmente, foi revelado que a azafranina é armazenada nos espaços intercelulares do córtex radicular e na área de interação entre o haustório e a raiz hospedeira. (iii) Plantas adultas de *E. grandiflora* são generalistas conseguindo parasitar uma ampla gama de hospedeiros. Porém, no crescimento inicial de *Escobedia* tem preferência por hospedeiras específicas, que permitem o maior crescimento (iv) Constatou-se que *E. grandiflora* não afetou o desenvolvimento das hospedeiras, nem influenciou na competição entre elas. Por outro lado, quando os dois hospedeiros estavam juntos, *E. elegans* afetou notavelmente o desenvolvimento de *P. glaucescens*, independente da presença do hemiparasita. Entretanto, a presença de *P. glaucescens* afetou negativamente a sobrevivência e crescimento de *E. grandiflora*. Contudo, a presença de *E. elegans* beneficiou a sobrevivência e crescimento de *E. grandiflora*, mesmo na presença de *P. glaucescens*.

Palavras-chave: Apocarotenoides da raiz. Diversidade de plantas. Haustório. Hemiparasita de raiz. Preferência de hospedeiros. Orobanchaceae.

ABSTRACT

Escobedia grandiflora is a root hemiparasitic plant with orange-coloured roots used for medicinal and cooking dye purposes. However, much uncertainty still exists about the root orange pigment and the parasitic habit. In this sense, the objective of this Ph.D. Dissertation was to evaluate the interactions between *E. grandiflora* and their hosts and investigate azafrin biosynthesis and its function in *E. grandiflora* roots. Four chapters were developed that addressed: (i) the influence of *E. grandiflora* on the structure and diversity of four plant communities in which it occurs of southern Brazil; (ii) the azafrin biosynthesis and function in *Escobedia* roots; (iii) the host preference in two development stages of *E. grandiflora* and (iv) the interaction between *E. grandiflora* and two host species, *Paspalum glaucescens* and *Eryngium elegans*. The main results obtained were: (i) species composition differed in *E. grandiflora* presence and their presence was associated with higher species richness, diversity, and evenness. Furthermore, the percentage of dominant species decreased with the presence of *E. grandiflora*. These results were consistent over the four grasslands evaluated in the present study (ii) The major compound of *E. grandiflora* roots was confirmed as azafrin. Also, the genes that encode the carotenoid and apocarotenoid pathways were identified in *E. grandiflora* roots. In addition, azafrin is stored in the intercellular spaces of the root cortex and the interface between the haustorium and the host root. (iii) Mature plants of *E. grandiflora* are generalist, parasitizing a wide range of host species from natural populations, however, during the early growth, *E. grandiflora* exhibited a better development when in contact with specific host species. (iv) *Escobedia grandiflora* did not affect the host development. In contrast, *E. elegans* positively influenced the survival and development of Escobedia and negatively the *P. glaucescens* development. Furthermore, this rosette species also changed the outcome of the interaction between Escobedia and *P. glaucescens*, reducing grass's competitive strength and benefiting the presence of the hemiparasite. Hence, the improvement of Escobedia development by hosts promoted a substantial azafrin level in the root, being major particularly in the presence of both hosts.

Keywords: Apocarotenoids root. Haustorium. Host preference. Plant diversity. Root hemiparasite. Orobanchaceae.

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1 INTRODUÇÃO E JUSTIFICATIVA

As plantas parasitas são especializada em adquirir recursos essenciais de outras plantas, invadindo o tecido vivo por meio de uma estruturas conhecidas como haustório (HEIDE-JØRGENSEN, 2008; TĚŠITEL, 2016). O haustório invade e estabelece conexão vascular com o tecido do hospedeiro, servindo como ponte estrutural e fisiológica para a absorção de água e solutos inorgânicos ou orgânicos do hospedeiro (JOEL, 2013; TEIXEIRA-COSTA, 2021). As plantas parasitas ocorrem em quase todos os ecossistemas terrestres e evoluíram de forma independente por ao menos doze vezes, resultando em 292 gêneros e cerca de 4750 espécies, agrupados em 20 famílias, sendo aproximadamente 60% parasitas de raiz e 40% de caule (HEIDE-JØRGENSEN, 2008; NICKRENT, 2020). Neste vasto grupo, a ordem Santalales contém o maior número de gêneros e espécies, enquanto Orobanchaceae é a maior família (NICKRENT, 2020).

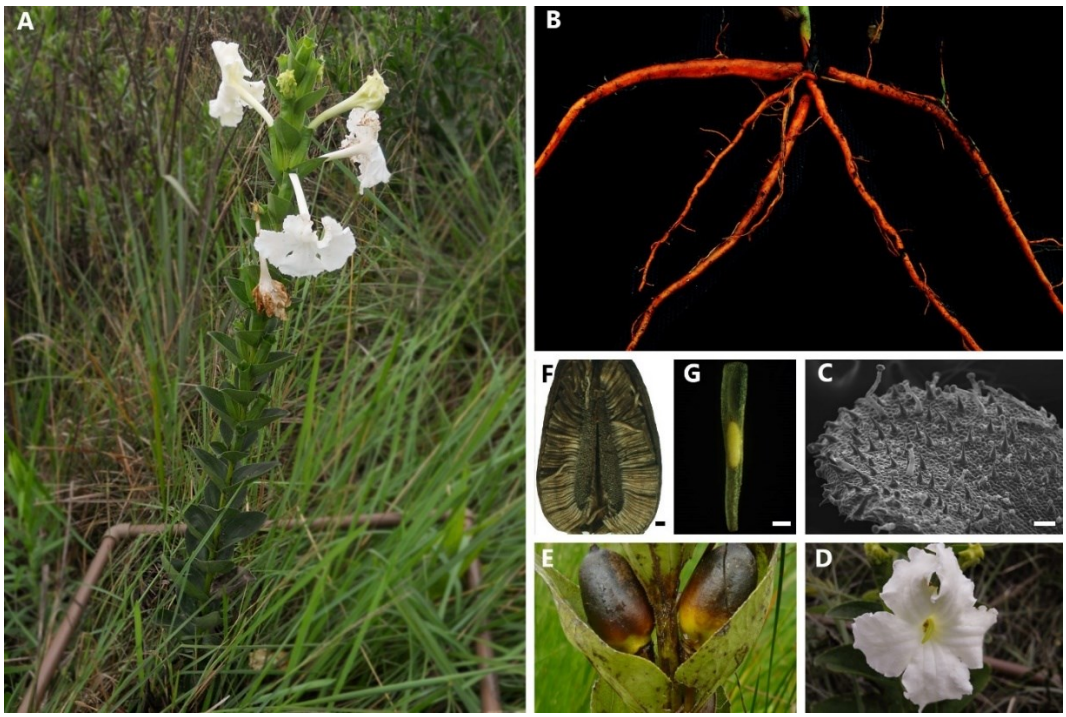
Em virtude da sua diversidade taxonômica, plantas parasitas possuem diversos hábitos de crescimento e diferentes características funcionais (TĚŠITEL, 2016). As plantas hemiparasita fazem fotossínteses e absorvem, da hospedeira, água e solutos inorgânicos, e eventualmente elementos orgânicos (TĚŠITEL et al., 2011). Já as plantas holoparasitas possuem pouca ou nula atividade fotossintética, absorvendo do hospedeiro todos os recursos necessários para sobreviver (MCNEAL et al., 2013). Em casos extremos, alguns holoparasitas são endoparasitas, que desenvolvem todo seu ciclo de vida no tecido hospedeiro, emergindo apenas para formar estruturas reprodutivas (THOROGOOD et al., 2021).

Plantas parasitas são comumente conhecidas por serem infestantes em cultivos de importância econômica como os gêneros *Orobanche*, *Alectra* e *Striga*, da família Orobanchaceae, que afetam negativamente a produtividade de plantas de lavoura como milho, arroz e leguminosas; ou da Santalaceae como *Arceuthobium*, que afeta cultivos florestais (FERNÁNDEZ-APARICIO; FLORES; RUBIALES, 2016; NICKRENT, 2020; TĚŠITEL et al., 2021). No entanto, o número de plantas parasitas prejudiciais em cultivos é relativamente baixo (cerca de 25 gêneros), mas a maioria delas possui funções benéficas e são componentes das comunidades de vegetação natural, influenciando positivamente a biodiversidade ou sendo amplamente aproveitadas pelos humanos para fins alimentares, medicinais e culturais (NICKRENT, 2020; TĚŠITEL et al., 2021; WATSON, 2009a). Por exemplo, *Rhinanthus alectorolophus* (Scop.) Pollich (Orobanchaceae) tem sido usado como restaurador ecológico (TĚŠITEL et al., 2017), *Santalum album* L (Santalaceae) pela valiosa madeira e óleo essencial

(TEIXEIRA DA SILVA et al., 2016), *Cistanche deserticola* Ma. (Orobanchaceae) exibe propriedades neuroprotetoras, hepatoprotetoras e antioxidantes (FU et al., 2018). *Anacolosa frutescens* (Blume) Blume (Olacaceae) e *Melientha suavis* Pierre (Opiliaceae) têm sido aproveitadas para uso de seus frutos (PIGNONE; HAMMER, 2016). As raízes de *Krameria triandra* Ruiz & Pav. (Krameriaceae) e *Centranthera grandiflora* Wall. ex Benth. (Orobanchaceae) possuem efeitos anti-inflamatórios (CARINI et al., 2002; YANG; CHOU; LI, 2018). A maior proporção dos usos das plantas parasitas tem sido associados à medicina tradicional, especialmente na Ásia, Europa e África, sendo que as famílias Orobanchaceae (52 espécies) e Loranthaceae (Santalales) (22 espécies) são as que registram o maior número de espécies utilizadas para esta finalidade (TĚŠITEL et al., 2021). Porém, ainda existe pouca informação sobre a biologia e a potencialidade das plantas parasitas, principalmente na América do Sul (TĚŠITEL et al., 2021).

Este é o caso da planta hemiparasita de raiz *Escobedia grandiflora* (Figura 1A), conhecida localmente como açafrão do campo ou açafrão de raiz, que ocorre de forma natural nas Américas Central e do Sul apresentando grande potencial de uso. *Escobedia grandiflora* é uma espécie herbácea (Figura 1A), possui raízes alaranjadas (Figura 1B), ramos eretos glabros ou pubescentes, cilíndricos, com folhas simples, opostas, ásperas em ambas as faces e com tricomas concentrados nas nervuras (Figura 1C). As flores são solitárias, com pedicelo ereto a semiereto e bractéolas opostas inseridas abaixo do cálice. A corola é de cor branca, com tricomas capitados (Figura 1D). O fruto é uma cápsula elipsoide e deiscente, que contém centenas de sementes com cerca de 5 mm de comprimento (Figure 1E-G) (BURGUER; BARRINGER, 2000; SOUZA; GIULIETTI, 2009; CARDONA; MURIEL, 2015).

Figura 1. Características morfológicas da planta hemiparasita *Escobedia grandiflora*: (A) Vista externa da planta hemiparasita; (B) sistema radicular laranja; (C) electromicrografia em MEV da superfície folhar denotando a presença de tricomas (CARDONA-MEDINA; SANTOS; NODARI, 2019); (D) vista frontal da flor e (E) do fruto elipsoide; (F) vista longitudinal interna do fruto, composto de centos de sementes; (G) semente destacando a exotesta que recobre o embrião. Scale: (C)= 100 µm, (F)=1 mm; (G)=500 µm.



As raízes de *E. grandiflora* foram amplamente utilizadas como corante alimentar desde a época pré-colombiana até meados dos anos 60, principalmente na região da cordilheira dos Andes, sendo considerada um dos corantes alimentícios mais importantes da época (PENNELL, 1931; MURIEL et al., 2015). O uso da raiz como corante foi diminuindo devido ao surgimento dos corantes artificiais (tartrazina) e a redução das populações naturais de *E. grandiflora,* o que dificultou a obtenção da raiz (MURIEL et al., 2015). Por consequência, gerou-se uma perda enorme do conhecimento associado a raiz de *E. grandiflora*. Ainda, o uso que mais prevalece é o medicinal, no qual as raízes têm sido usadas na medicina popular para o tratamento de hepatites e hiperlipidemia (SILVA et al., 2010a; MURIEL et al., 2015). Contudo, *E. grandiflora* não é considerada como domesticada e seu uso continua baseado no extrativismo (PENNELL, 1931; CARDONA; MURIEL, 2015). As propriedades da coloração da raiz são em razão da presença abundante de um pigmento laranja pertencente ao grupo dos apocarotenoides

conhecido como azafranina ($C_{27}H_{38}O_4$) (KUHN, 1935). Em geral, a produção e acumulação de apocarotenoide C_{27} como a azafranina é um evento raro na natureza (FLOSS et al., 2008). A azafranina apresenta atividade antioxidante e tem sido usada na prevenção e tratamento de doenças cardiovasculares (YANG; CHOU; LI, 2018). No entanto, pouco se conhece sobre a função e a biossíntese da azafranina nas raízes de *E. grandiflora*, sendo essa uma informação essencial para promoção do seu uso e cultivo.

Segundo registros de herbários, esta planta ocorre naturalmente em diferentes localidades do Brasil, desde o nordeste (Bahia), passando pelo centro-oeste (Distrito Federal, Goiás, Mato Grosso do sul) até o sul (Paraná, Santa Catarina, Rio Grande do Sul); ocorrendo principalmente em campos e banhados (SOUZA; GIULIETTI, 2009; "speciesLink network", 2021). Porém, muitos dos registros dos herbários são antigos e pouco se conhece sobre o estado atual de conservação das populações de *E. grandiflora* no Brasil. Em alguns casos não se observam populações, mas apenas indivíduos isolados. Não existe um plano de conservação desta espécie e são poucas as populações atualmente conhecidas que ocorrem dentro de áreas protegidas, como as populações encontradas no Parque Municipal da Lagoinha do Leste, em Florianópolis (SC). No estado de São Paulo a espécie foi considerada em perigo de extinção (VELLEDA, 2016). Estes aspectos são ainda mais problemáticos pelo hábito parasita de *E. grandiflora*.

A conservação de plantas parasitas envolve o conhecimento sobre a interação com as espécies hospedeiras, entendendo que a sobrevivência de uma planta hemiparasita é garantida pela absorção de água e nutrientes da hospedeira, por meio de diversos mecanismos de interação (MARVIER; SMITH, 1997; PRESS; PHOENIX, 2005; SAUCET; SHIRASU, 2016). Estudos mostraram que algumas plantas parasitas possuem o potencial de moldar a estrutura da comunidade pela redução no crescimento de alguns hospedeiros, que diminuem a competição com as espécies hospedeiras e não hospedeiras (GIBSON; WATKINSON, 1992; DAVIES et al., 1997; BOROWICZ; WALDER; ARMSTRONG, 2019). Como esperado, existe uma grande variabilidade nas respostas das comunidades às plantas parasitas, dependendo dos mecanismos de interação através dos quais o parasita altera os hospedeiros. Por outro lado, uma vegetação com uma maior riqueza de espécies poderia ser mais benéfica para algumas plantas hemiparasitas, devido à presença e diversidade de potenciais hospedeiros (JOSHI; MATTHIES; SCHMID, 2000; FIBICH et al., 2017).

Apesar da distribuição e importância de *E. grandiflora*, seu hábito parasitário passou muito tempo despercebido pela comunidade científica, limitado apenas a estudos filogenéticos

da família. O primeiro estudo que trata sobre o parasitismo de *E. grandiflora* emergiu em 2015, confirmando que o crescimento e a formação de estruturas reprodutivas de *E. grandiflora* dependiam do desenvolvimento de haustórios e da aderência às raízes hospedeiras (CARDONA; MURIEL, 2015). A germinação das sementes é alta sem a presença do hospedeiro, porém com poucas reservas nutricionais para sustentar as futuras plântulas que possuem uma alta taxa de mortalidade (CARDONA; MURIEL, 2015; CARDONA-MEDINA; SANTOS; NODARI, 2019). Diferente de outras espécies hemiparasitas de raiz que desenvolvem haustório terminais e parasitam o hospedeiro logo depois da germinação, as plântulas de *E. grandiflora* dependem da própria capacidade de absorver nutrientes até desenvolver haustórios laterais e parasitar as raízes de hospedeiros adequados (CARDONA-MEDINA; SANTOS; NODARI, 2019). Porém, o parasitismo nem sempre é atingido, fazendo do desenvolvimento inicial da plântula um estádio crítico (CARDONA; MURIEL, 2015; CARDONA-MEDINA; SANTOS; NODARI, 2019). Como resposta a essa limitada produção científica, ao estado atual de conservação e importância de *E. grandiflora*, informações sobre a interação dessas espécies nas comunidades de plantas são necessárias. Neste contexto, o entendimento do efeito da planta parasita na comunidade, o aprofundamento sobre a azafranina, a preferência por determinados hospedeiros, assim como a interação com eles são essenciais para a conservação e uso sustentável da planta parasita.

Diante do exposto, apresenta-se a primeira tese de doutorado realizada sobre *E. grandiflora*, a qual teve como objetivo (1) avaliar as interações de *E. grandiflora* com os hospedeiros e (2) investigar a biossíntese e função da azafranina nas raízes de *E. grandiflora.* Desta maneira a tese foi estruturada em quatro capítulos que são o resultado do estudo de duas características muito importantes para o melhor entendimento de *E. grandiflora*: a interação parasita-hospedeiro e a caracterização do pigmento laranja das raízes.

No primeiro capítulo foi avaliado a influência de *E. grandiflora* na composição e diversidade de plantas em quatro comunidades. Os resultados mostraram que a composição de espécies diferiu nas parcelas com o hemiparasita e que a presença de *E. grandiflora* foi associada com maior riqueza de espécies, maior diversidade e equabilidade. Dentre os grupos funcionais, os mais influenciados foram os graminóides (gramíneas, juncos e monocotiledôneas de folhas estreitas) e as ervas. Ademais, houve uma diminuição na cobertura das espécies mais dominantes na presença do hemiparasita. Os resultados encontrados foram similares nas quatro comunidades avaliadas, que apresentaram fisionomias marcadamente diferentes.

No segundo capítulo foi investigado a biossíntese da azafranina nas raízes de *E. grandiflora*, assim como sua localização e função nas raízes e no haustório durante a interação com os hospedeiros. Para isso utilizaram-se análises de HPLC, da sequência de RNA, e estudos filogenéticos e estruturais. Os resultados mostraram a presença maioritária da azafranina nas raízes de *E. grandiflora*, assim como foi possível identificar os genes mais importantes que codificam as enzimas da via dos carotenoides, além dos genes candidatos que codificam as enzimas da biossíntese da azafranina nas raízes de *E. grandiflora*. Ademais, destaca-se que a azafranina é armazenada nos espaços intercelulares do córtex radicular e foi observada como deposição na interface entre o haustório e a raiz hospedeira, hipotetizando a possível relação da azafranina com a interação parasita-hospedeira.

No terceiro capítulo determinou-se a gama de hospedeiras para plantas maduras de *E. grandiflora* em quatro comunidades de plantas obtidas através de análises microscópicas das conexões entre os haustórios e as raízes hospedeiras. Além disso, foi avaliada a preferência por diferentes hospedeiros durante a etapa inicial do desenvolvimento de *E. grandiflora*. Os resultados indicaram que plantas maduras de *E. grandiflora* são generalistas. Porém, no início do desenvolvimento *E. grandiflora* têm preferência por dois hospedeiras (*Eryngium elegans* e *Evolvulus glomeratus*), que promovem seu crescimento de forma diferenciada.

No quarto capítulo foi avaliada a interação entre *E. grandiflora* e dois hospedeiros provenientes das comunidades naturais: *Paspalum glaucescens* (Poaceae) e *Eryngium elegans* (Apiaceae). Primeiramente foi avaliado o efeito de *E. grandiflora* no desenvolvimento dos dois hospedeiros e na competição entre eles. Em seguida, foi avaliada a influência de cada hospedeiro e sua combinação no crescimento de *E. grandiflora* e na acumulação de azafranina na raiz. Verificou-se que *E. grandiflora* não afeta significativamente o desenvolvimento dos hospedeiros, nem influencia a competição entre eles. Entretanto, quando os dois hospedeiros estavam juntos, *E. elegans* afetou notavelmente o desenvolvimento de *P. glaucescens,* independente da presença do hemiparasita. Por outro lado, a presença de *P. glaucescens* afetou negativamente a sobrevivência e crescimento de *E. grandiflora*. Contudo, a presença de *E. elegans* beneficiou a sobrevivência e crescimento de *E. grandiflora*, mesmo na presença de *P. glaucescens*.

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2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar as interações de *Escobedia grandiflora* com os hospedeiros, e estudar a biossíntese e a função da azafranina nas raízes de *E. grandiflora*

2.3 OBJETIVOS ESPECÍFICOS

CAPÍTULO 1

1. Avaliar comparativamente a influência da planta hemiparasita *Escobedia grandiflora* na estrutura e diversidade de comunidades de plantas.

CAPÍTULO 2

2. Investigar a biossínteses da azafranina e sua função nas raízes de *Escobedia grandiflora*.

CAPÍTULO 3

3. Determinar a preferência de hospedeiros em duas fases do desenvolvimento de *Escobedia grandiflora.*

CAPÍTULO 4

4. Avaliar a interação entre *E. grandiflora* e dois hospedeiros, *Paspalum glaucescens* e *Eryngium elegans.*

3 HIPOTESES

CAPÍTULO 1

- Ho: A presença de *Escobedia grandiflora* não está associada com mudanças na composição de quatro comunidades de plantas.

  Ha: A presença de *Escobedia grandiflora* está associada com mudanças na composição de quatro comunidades de plantas

- Ho: A presença de *Escobedia grandiflora* não está associada com o aumento dos índices de diversidade e com a redução da cobertura das plantas mais dominantes de quatro comunidades de plantas.

  Ha: A presença de *Escobedia grandiflora* está associada com o aumento dos índices de diversidade e com a redução da cobertura das plantas mais dominantes de quatro comunidades de plantas.

CAPÍTULO 2

- Ho: A presença abundante do pigmento laranja nas raízes de *Escobedia grandiflora* não corresponde ao apocarotenoide azafranina.

  Ha: A presença abundante do pigmento laranja nas raízes de *Escobedia grandiflora* corresponde ao apocaroteonide azafranina.

- Ho*: Escobedia grandiflora* não acumula a azafranina no apoplasto da raiz.

  Ha: Escobedia grandiflora acumula a azafranina no apoplasto da raiz

CAPÍTULO 3

- Ho: Na fase adulta, *Escobedia grandiflora* é uma planta especialista que parasita espécies hospedeiras específicas.

  Ha: Na fase adulta, *Escobedia grandiflora* é uma planta generalista que parasita uma grande variedade de plantas

- Ho: A preferência por hospedeiros na etapa inicial do crescimento de *Escobedia grandiflora* é similar à fase adulta.

  Ha: A preferência por hospedeiros na etapa inicial do crescimento de *Escobedia grandiflora* é diferente à fase adulta.

CAPÍTULO 4

- Ho: *Escobedia grandiflora* afeta notavelmente o desenvolvimento dos hospedeiros.

  Ha: *Escobedia grandiflora* não afeta notavelmente o desenvolvimento dos hospedeiros.

- Ho: A combinação de hospedeiros não influencia diferenciadamente o desenvolvimento de *Escobedia grandiflora*.

  Ha: A combinação de hospedeiros influencia diferenciadamente o desenvolvimento de *Escobedia grandiflora.*

4 CAPÍTULO 1- EFFECTS OF ROOT HEMIPARASITE *Escobedia grandiflora* (Orobanchaceae) ON SOUTHERN BRAZILIAN GRASSLANDS: DIVERSITY, COMPOSITION, AND FUNCTIONAL GROUPS.

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4.1 ABSTRACT

Escobedia grandiflora is a root hemiparasite from Central and South America, and its orange roots are used as a phytomedicine and food coloring. Since root hemiparasites from South America have not been extensively investigated, we asked how *E. grandiflora* would affect the structure and diversity of plant communities in four locations in southern Brazil, all belonging to the Atlantic Forest biome. Specifically, we asked if the presence of the hemiparasite would 1) affect plant species composition, 2) influence the plant diversity, and 3) influence the percentage cover of dominant plant species. We conducted a paired-quadrat, *i.e*., with and without *E. grandiflora*, observational study in four locations in southern Brazil. For each quadrat, species composition and percent cover of each species were visually evaluated. Species were also grouped into functional groups. Species composition differed between quadrats with and without *E. grandiflora*. Quadrats with *E. grandiflora* showed higher species richness, Shannon´s diversity, and Pielou´s evenness. These results varied also among functional groups. These results vary among functional groups; graminoids increased in richness, diversity, and evenness whereas herb´s evenness decreased. Furthermore, the percentage of dominant species decreased with the presence of *E. grandiflora*. There is a clear association between the neotropical root hemiparasite *E. grandiflora* and the grassland plant community structure. Higher plant diversity, dominance reduction, and changes in species composition are associated with the presence of this perennial hemiparasite. These findings were consistent among the four grasslands with markedly different physiognomies and the first study conducted on a Latin American root hemiparasite species. However, future manipulative experiments are necessary to fully disentangle the cause-effect relation between higher plant diversity and the presence of *E. grandiflora*.

Key words: *Escobedia*, grasslands, parasitic plants, plant community, plant diversity, species richness**.**

4.2 INTRODUCTION

Parasitic plants need resources of other plants, known as hosts, to survive, and their activity is mediated by a unique structure called haustorium (HEIDE-JØRGENSEN, 2008; WESTWOOD et al., 2010). Parasitic plants are important elements of natural and semi-natural ecosystems over the planet (PRESS; PHOENIX, 2005; HEIDE-JØRGENSEN, 2008; CAMERON; PHOENIX, 2013). Notably, many life forms of parasitic plants are known to, directly and indirectly, affect the structure of host plants and other organisms in plant communities (PRESS; PHOENIX, 2005; GRAFFIS; KNEITEL, 2015). In general, the impact varies, depending on the type of parasitic plant and the host range species it prefers. Two types are generally recognized: holoparasites and hemiparasites (TĚŠITEL, 2016). Holoparasites do not have photosynthetic activity and absorb sap from the host phloem (HIBBERD et al., 1999). Hemiparasite plants have photosynthetic activity and absorb water and inorganic nutrients from the host xylem (TĚŠITEL et al., 2017). They may strongly compete with the host, decreasing its growth (BOROWICZ; ARMSTRONG, 2012).

Still, root hemiparasites can increase the richness and diversity of plant communities (JOSHI; MATTHIES; SCHMID, 2000; FIBICH et al., 2017). However, to do this, hemiparasite plants need to grow their root system, including the development of haustoria, to attach to one or more suitable host roots (TĚŠITEL et al., 2011; CARDONA-MEDINA; SANTOS; NODARI, 2019). Thus, while suitable hosts will benefit hemiparasites in terms of growth, performance, and reproduction (CAMERON; COATS; SEEL, 2006; REN et al., 2010), these benefits may result in the reduction of the host's biomass and growth (MUDRÁK; LEPŠ, 2010; HELLSTRÖM; BULLOCK; PYWELL, 2011). This reduction may, in turn, affect vegetation structure and diversity (GIBSON; WATKINSON, 1992; MUDRÁK; LEPŠ, 2010; DEMEY et al., 2015; HEER et al., 2018), creating gaps and altering the competitive balance between host and non-host plants (MUDRÁK; LEPŠ, 2010; DEMEY et al., 2015). This means that hemiparasites can suppress and reduce abundant or dominant hosts to promote plant coexistence and even increase the diversity of plant communities (TĚŠITEL et al., 2017; HEER et al., 2018). These effects are stronger when the density of hemiparasites increases (HEER et al., 2018). For example, *Rhinanthus* is used in high densities in restoration programs to increase the diversity in grassland communities owing to the suppression by parasitism of competitive and dominant plants (TĚŠITEL et al., 2017). However, hemiparasites can also suppress non-dominant host

species and, hence, decrease the diversity of plant communities (GIBSON; WATKINSON, 1992).

The studies are focused on controlling and eliminating parasites populations (MARVIER; SMITH, 1997; JOEL; GRESSEL; MUSSELMAN, 2013). Studies addressing the effects of hemiparasites on plant communities are still scarce. These studies are mainly concentrated in temperate regions and were carried out using only a few hemiparasite plant taxa, such as *Rhinanthus* spp., *Castilleja* spp., and *Triphysaria* spp. (MARVIER, 1998; SPASOJEVIC; SUDING, 2011; HEER et al., 2018). Currently, little is known about the impact of other important root hemiparasites in the communities of other regions, especially in South America. Such is the case of the hemiparasite plant *Escobedia grandiflora* (L. f) Kuntze (Orobanchaceae), which occurs naturally in non-forested communities in Central and South America (SOUZA; GIULIETTI, 2009). The orange roots of *E. grandiflora* present high medicinal and food value, and they were one the most important food dyes until the early 20th century in the Andean region of Colombia (MURIEL et al., 2015). Despite the relevance of *E. grandiflora*, it is recently that its parasitic habit has been discovered (CARDONA; MURIEL, 2015) and how it happens in the seedling stage (CARDONA-MEDINA; SANTOS; NODARI, 2019). To the best of our knowledge, no studies have reported the effects of *E. grandiflora* on plant communities. Therefore, we undertook a study of the root hemiparasite *E. grandiflora* to investigate its positive and negative effects on plant communities, especially any differences in plant diversity and composition in the presence of this root hemiparasite.

Here, we conducted a paired-quadrat observational study in four regions in southern Brazil to evaluate the influence of the hemiparasite plant *E. grandiflora* on the structure and diversity of plant communities in which it occurs. We tested three hypotheses: that the presence of *E. grandiflora* (1) affects plant community composition, (2) influences the plant diversity, and (3) the relative percentage cover of dominant plant species. Based on our best knowledge, the present study is the first to report to the effect of *E. grandiflora* on plant communities and the first to examine the effect of root hemiparasite on plant communities of South America.

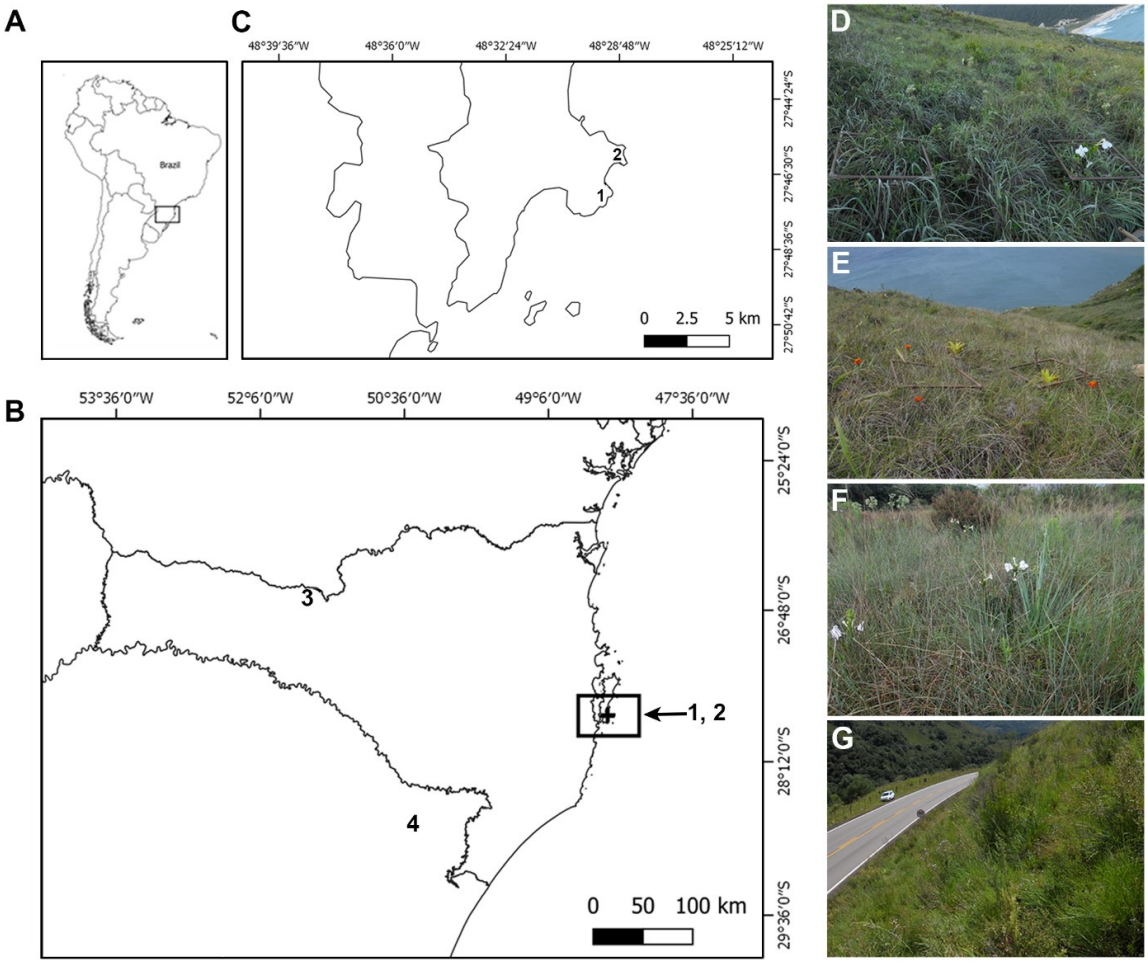
4.3 MATERIAL AND METHODS

4.3.1 Study areas

The present study was carried out in four locations in southern Brazil (Figure 1), all belonging to the Atlantic Forest biome. Exact point coordinates were selected according to the

occurrence of *E. grandiflora* populations through the information of its distribution in the species link project ("speciesLink Network", 2018). The first (27°46'53.0"S, 48°29'16.1"W, 190 m.a.s.l.) and second (27°46'05.9"S, 48°28'46.2"W, 27 m.a.s.l.) sampled areas are located in the mountains of the Lagoinha do Leste Municipal Park (LLMP), municipality of Florianópolis, state of Santa Catarina (SC). The LLMP areas present mean annual precipitation of 1497 mm, a mean monthly maximum temperature of 25.3ºC, and a mean monthly minimum temperature of 17.5ºC. The main vegetation types of the park are coastal vegetation, locally known as "restinga", and mosaics of grasslands with rocky outcrops. The coastal vegetation of LLMP has suffered anthropogenic fires; the last one was recorded in 2016 (Pers. Comm). The most abundant species in location 1 are *Paspalum glaucescens* Hack., *Axonopus siccus* (Nees) *Kuhlm.*, *Bulbostylis capillaris* (L.) Kunth ex C.B. Clarke, and *Calliandra tweediei* Benth., and in location 2, they are *Paspalum glaucescens*, *Tibouchina urvilleana* (DC.) Cogn., *Andropogon selloanus* (Hack.) Hack., and *Paspalum* cf. *ramboi*. The third sampled location (26°36'56.9"S, 51°29'51.0"W, 1235 m.a.s.l.) is located in the municipality of Água Doce, SC, near a roadside. Mean annual precipitation is 1707 mm, mean monthly maximum temperature is 21.2ºC, and mean monthly minimum temperature is 11.5ºC. The vegetation is characterized by mosaics of *Araucaria angustifolia* forests with dry and wet grasslands dominated by tall grasses and shrubs, and exotic *Pinus* monoculture plantations. In this area, *E. grandiflora* occurs both in dry and wet grasslands, although cattle grazing was not observed. The most abundant species are *Anthenantia lanata* (Kunth) Benth., *Baccharis* cf. *myriocephala*, and *Andropogon lateralis* Nees. The fourth sampled location (28°53'06.0"S, 50°27'52.9"W, 927 m.a.s.l.) is private property near a roadside located in a small valley in the municipality of Jaquirana, state of Rio Grande do Sul. Mean annual precipitation is 1725 mm, mean monthly maximum temperature is 21.1ºC, and mean monthly minimum temperature is 11.6ºC. The vegetation includes cattle-grazed grasslands in mosaics with *Araucaria* forest. The most abundant species are *Schizachyrium* sp., *Paspalum glaucescens*, and *Raulinoreitzia crenulate* (Spreng.) R.M. King & H. Rob.

Figure 1. Geographic location of study areas. (A) Map of South America. (B) Map of the southern states of Brazil, Santa Catarina (SC) at the center, Rio Grande do Sul (RS) to the south, and Paraná (PR) in the north. Numbers represent the location of the four sampled areas described in the text. (C) Map of sampled locations 1 and 2 located in Florianópolis, Santa Catarina Island. (D-G) Photos of sampled locations 1 to 4, respectively.

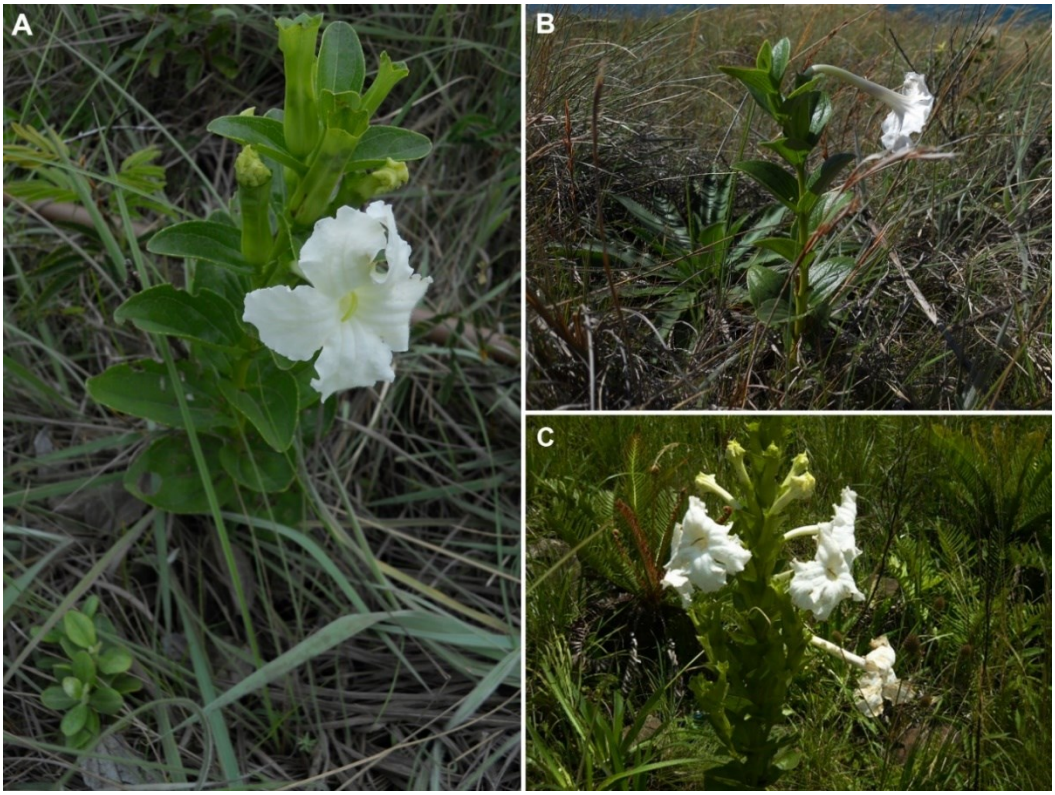


4.3.2 Sample design

Sampling was done in the four areas between December of 2018 and March of 2019, during the flowering period of *E. grandiflora* (Figure 2). In each area, 60 quadrats (or plots) of 50 x 50 cm in a paired design were surveyed. For each pair, one quadrat was positioned with one individual of *E. grandiflora* in the center, and the other was placed one meter apart, in a random direction, in a location without *E. grandiflora* (control quadrat) (GIBSON; WATKINSON, 1992). The 30 pairs were spaced by at least 10 m. In the quadrats, the mean cover of *E. grandiflora* was 9.5% (± 5.6 SD). Considering the four areas, a total of 240 quadrats were established. For each quadrat, the percentage of the vegetative cover of each species was visually estimated using the decimal scale for relevés (LONDO, 1976). Additionally, we

counted the number of different plant species in each quadrat, and the plants were identified (supplementary Table S1) using scientific literature and databases like the Missouri Botanical Garden (www.tropicos.org), speciesLink (www.splink.org.br), and species list of the Brazilian Flora, Jardim Botânico do Rio de Janeiro (http://floradobrasil.jbrj.gov.br/).

Figure 2. Overview of *Escobedia grandiflora* individuals during the flowering period in locations 1 (A) and 2 (B) of Florianópolis municipality and in location 3 (C) of Água Doce municipality.



4.3.3 Functional groups

In parasitic plant studies, functional group divisions are commonly used to facilitate the interpretation of parasitic plant effects on natural communities (FISHER et al., 2013; MATTHIES, 2017; SANDNER; MATTHIES, 2017). Therefore, species of the four locations were classified into five functional groups: graminoids (grasses, sedges, and narrow-leaved monocots from Xyridaceae and Amaryllidaceae), rosettes, herbs (including subshrubs, legumes, and non-graminoids and non-rosette herbs), shrubs, and cryptogams (including pteridophytes, and lichens) (supplementary Table S1). The selection of these functional groups

was based on the morphological and habit patterns of the plants in the four evaluated communities.

4.3.4 Statistical analysis

The number of species (species richness), Shannon's diversity, and Pielou's Evenness were calculated for each quadrat using the vegan package (HEER et al., 2018; OKSANEN, 2020). The hemiparasite *E. grandiflora* was omitted from the species richness, diversity, evenness, and plant composition (BARDGETT et al., 2006; DEMEY et al., 2015; HEER et al., 2018). To test the first hypothesis, *i.e.*, that *E. grandiflora* modifies plant community composition, we performed a generalized linear model for multivariate responses (GLMmv) in which the condition (with and without *E. grandiflora*) was used as the explanatory variable, and the matrix of plant community composition, using plant cover for each species in each quadrat, was chosen as the response variable. We chose negative binomial distribution because it visually fit the residuals better compared to other distributions. To control for the paired sampling design and the four different locations, a permutation matrix was generated where pairs of samples (sub-blocks) and locations (blocks) were fixed but blocks and factor levels within sub-blocks (with and without *E. grandiflora*) were randomized. *P*-values were calculated based on the 9999 matrices via PIT-trap resampling (adjusted for multiple testing) calculated using a stepdown resampling algorithm (WANG et al., 2020). We measured the proportion of variance explained by the model through a pseudo R2 for multivariate GLM, following SLAVICH et al.(2014). To visually check for differences in the community composition of each location with and without *E. grandiflora*, we explored the data with non-metric multidimensional scaling (nMDS) ordinations.

To test the second hypothesis, *i.e.*, that *E. grandiflora* increases plant diversity, we built generalized linear mixed models (GLMM) in which the condition (with and without *E. grandiflora*) was the explanatory variable, and richness, diversity, and evenness were the response variables. We also modeled diversity indices for each functional group and performed the same GLMMs. To evaluate if the presence of *E. grandiflora* is related to a decrease in the percentage cover of the most dominant species in each quadrat, we performed two GLMMs. First, we tested if quadrats containing the parasite presented less percentage cover assigned to the first most dominant species in each quadrat. Then, we performed the same test for the sum of the two most abundant species per quadrat. To control the effect of paired quadrats (sub-blocks) and locations (blocks), these were considered as random variables in the GLMMs. For

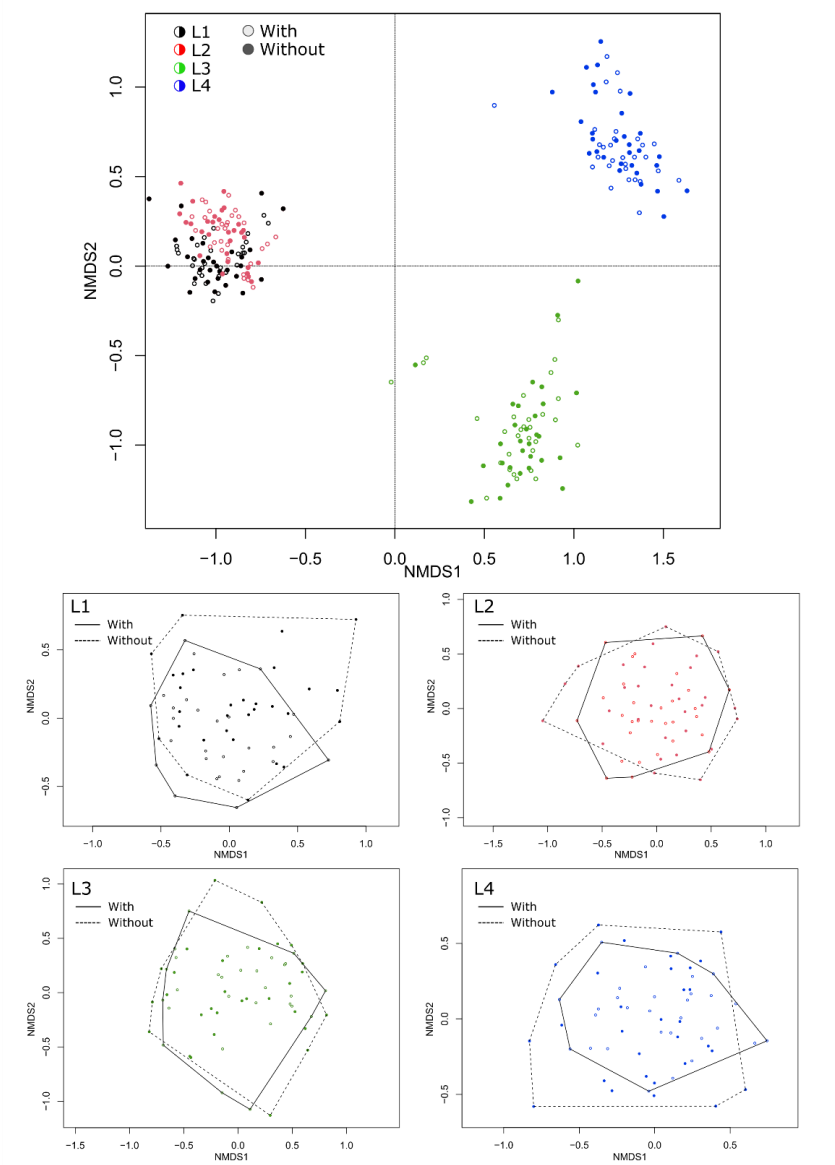
GLMMs, a Gaussian distribution was used. Model validation has based analysis of plotting residuals versus fitted values. Finally, for GLMMs, we measured the proportion of variance explained by fixed factors (R2 marginal) and random and fixed factors (R2 conditional) using the MuMIn package (BARTOŃ, 2020). All data were analyzed in R environment, version 3.5.0 (R Core Team, 2018), using the "vegan" package for producing the ordinations (OKSANEN, 2020), "permute" for permutations (SIMPSON et al., 2019), "mvabund" for GLMmv (WANG et al., 2020) and "glmmTMB" for GLMMs (MAGNUSSON et al., 2019).

4.4 RESULTS

4.4.1 Plant community composition

Our results showed that *E. grandiflora* can change the plant community composition (Likelihood ratio test (LR) = 254, P-value< 0.001, global pseudo $R^2 = 0.1$; Figure 3).

Figure 3. Non-metric multidimensional scaling (nMDS) ordinations of plant community composition in quadrats with (open circles/ solids lines) and without (filled circles/ dashed lines) *Escobedia grandiflora* in four locations (L1, L2, L3, L4) in southern Brazil. These ordinations are for visualization only.



4.4.2 Diversity

Quadrats with *E. grandiflora* exhibit high values of all three diversity indices: species richness, Shannon's diversity, and Pielou's evenness in the four grassland communities compared to control (Table 1). The number of species in quadrats with *E. grandiflora* was higher than that in the control quadrats when considering the four locations of the study (z=7.621, std error=1.237, $p < .001$), as 9.4 (± 3.4 SD) species were found in quadrats with *E. grandiflora*, and 8.7 (± 3.2 SD) species were found in the control. The species number ranged from 3 to 19 in the presence of *E. grandiflora* and ranged from 2 to 17 in control quadrats (Figure 4). Fixed effects explained 1% of species richness (marginal R^2) differences, achieving 80% with both fixed and random effects (conditional R^2). Considering the functional groups (Table 1), the richness of the graminoids increased from 4.1 (± 2 SD) species to 4.9 (± 2.1 SD)

species with *E. grandiflora* presence. For the other functional groups, species richness was not influenced by the presence of *E. grandiflora*.

Table 1. Summary of generalized linear models to test the effect of *Escobedia grandiflora* on species richness, Shannon's diversity, and Pielou's evenness, including the effect on each functional group.

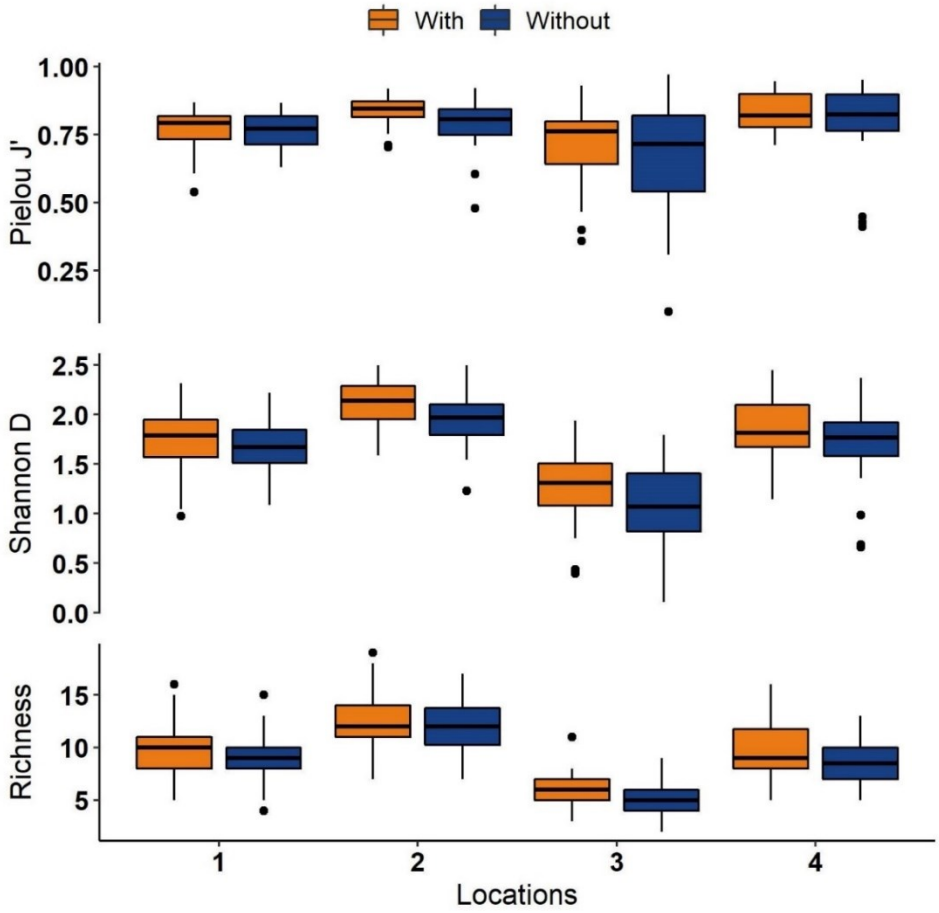
Model	Parasite	Estimate	Std. Error	z-Value	Pr(>|z|)	R^2m	R^2c
Richness							
Total	With	9.433	1.237	7.621	< .001	0.011	0.810
	Without	-0.708	0.189	-3.739	< .001		
Graminoids	With	4.867	0.733	6.640	< .001	0.033	0.795
	Without	-0.767	0.123	-6.239	< .001		
Herbs	With	2.375	0.348	6.824	< .001	0.000	0.518
	Without	0.050	0.150	0.333	0.739		
Shrubs	With	1.200	0.169	7.063	< .001	0.002	0.334
	Without	0.091	0.103	0.892	0.372		
Rosette	With	0.675	0.131	5.159	< .001	0.000	0.281
	Without	0.017	0.080	0.209	0.835		
Cryptogams	With	0.325	0.095	3.406	< .001	0.011	0.414
	Without	-0.116	0.052	-2.204	0.027		
Shannon´s Diversity							
Total	With	1.737	0.159	10.873	< .001	0.023	0.721
	Without	-0.143	0.031	-4.493	< .001		
Graminoids	With	1.123	0.154	7.263	< .001	0.040	0.427
	Without	-0.196	0.047	-4.128	< .001		
Herbs	With	0.557	0.120	4.628	< .001	0.004	0.181
	Without	0.071	0.062	1.146	0.252		
Shrubs	With	0.228	0.056	4.039	< .001	0.001	0.079
	Without	0.030	0.043	0.689	0.491		
Rosette	With	0.077	0.027	2.849	0.004	0.001	0.043
	Without	-0.013	0.025	-0.529	0.60		
Cryptogams	With	0.030	0.017	1.758	0.078	0.002	0.038
	Without	-0.013	0.016	-0.801	0.423		
Pielou's Evenness							
Total	With	0.789	0.026	30.034	< .001	0.020	0.463
	Without	-0.037	0.012	-3.009	0.003		

Graminoids	With	0.730	0.050	14.740	< .001	0.015	0.470
	Without	-0.059	0.023	-2.615	0.009		
Herbs	With	0.472	0.091	5.169	< .001	0.013	0.443
	Without	0.094	0.040	2.382	0.017		
Shrubs	With	0.277	0.069	4.035	< .001	0.002	0.268
	Without	0.039	0.045	0.851	0.395		
Rosette	With	0.091	0.038	2.413	0.016	0.000	0.251
	Without	-0.002	0.028	-0.080	0.937		
Cryptogams	With	0.038	0.021	1.861	0.063	0.005	0.273
	Without	-0.022	0.017	-1.270	0.204		

R^2 denotes variance explained by fixed factors (Marginal R^2 -R^2m) and variance explained by fixed and random factors (Conditional R^2-R^2c).

Shannon's diversity also increased in quadrats when *E. grandiflora* was present (z=10.873, std error= 0.160, p< .001) (Table 1, Figure 4). Fixed effects explained 2% of Shannon's diversity (marginal R^2) differences, achieving 72% with both fixed and random effects (conditional R^2). For functional groups, only graminoids were affected by the presence of the hemiparasite. Graminoid diversity increased with *E. grandiflora* presence (z=7.263, std error=0.154, p< .001).

Figure 4. Community parameters differences among quadrats with and without *Escobedia grandiflora.* Box plots illustrate medians (thick line), quartiles (box), maximum and minimum values observed (whiskers), and outliers (points).



Evenness increased with *E. grandiflora* presence, considering the four locations of the study (z=30.034, std error= 0.026, $p< .001$, Table 1, Figure 4). Fixed effects explained 2% of the difference in evenness (marginal R^2), achieving 46% with both fixed and random effects (conditional R^2). Considering the functional groups, the evenness of graminoids and herbs was affected differently with hemiparasite presence. Graminoid evenness increased, and herb evenness decreased with *E. grandiflora* presence (Table 1). Therefore, the vegetative cover of graminoid species was more homogeneous, and herbs, more heterogeneous, with *E. grandiflora*.

4.4.3 Dominant species percentage cover

Relative percentage cover of dominant species was also different between plots without and with *E. grandiflora* (Table 2). The relative cover of the most dominant species in quadrats

with *E. grandiflora* was lower than that in control quadrats (z=8.620, std error=4.259, *p*=<.001) since the cover of the most dominant species in the presence of *E. grandiflora* was 36.7% (+/- 15 SD) and in control quadrats 44% (+/-17 SD). Fixed effects explained 5% of the differences of one dominant species (marginal R^2), achieving 54 % with both fixed and random effects (conditional R^2). In the same way, the summed cover of the two most dominant species in quadrats (Table 2) decreased in the presence of *E. grandiflora* (z=11.548, std error=4.930, *p*<.001) since the cover was 57% in the presence of *E. grandiflora* (+/-16.5 SD) and in control quadrats 62.3% (+/-17.1 SD).

Table 2. Summary of generalized linear models to test the effect of *Escobedia grandiflora* presence on the relative percentage cover of one and two dominant species of whole plant communities.

		Dominant Species Relative Cover					
Model	**Parasite**	**Estimate**	**Std. Error**	**z-Value**	**Pr(>|z|)**	**R^2m**	**R^2c**
One species	With	36.717	4.259	8.620	< .001	0.048	0.542
	Without	7.191	1.429	5.031	< .001		
Two species	With	56.937	4.930	11.548	< .001	0.026	0.523
	Without	5.424	1.515	3.579	< .001		

R^2 denotes variance explained by fixed factors (Marginal R^2- R^2m) and variance explained by fixed and random factors (Conditional R^2- R^2c).

4.5 DISCUSSION

The results indicate a significant association between the presence of *E. grandiflora* and differences in community composition, increases in diversity indices, and decreases in the relative cover of the dominant species. Furthermore, the presence of *E. grandiflora* was positively associated with the increase of graminoid diversity indices and decrease of herb evenness. This study is the first to report the association of *E. grandiflora* with plant community structure and diversity and the first study on hemiparasites of South America. Our results align with a previous observational study of another perennial root hemiparasite *Pedicularis canadensis* L. (DIGIOVANNI et al., 2017). Moreover, it is important to note that the effects of *E. grandiflora* were consistent over the four grasslands evaluated in the present study. These four grasslands display very evident differences in species compositions and physiognomies.

Generally, the effect of root hemiparasite species on community structure and diversity should be interpreted with caution because results are limited by the type of the study

(observational/experimental) (AMELOOT; VERHEYEN; HERMY, 2005; BULLOCK; PYWELL, 2005). Our study is observational, i.e., evaluate the differences of vegetation in plots with and without *E. grandiflora*. Experimental studies (manipulations/ hemiparasite sown) are adequate to visualize the community effect of hemiparasites (BULLOCK; PYWELL, 2005). Moreover, the interpretation of the results are limited to only a few hemiparasite species and ecosystems with specific life history (PRESS; PHOENIX, 2005; SPASOJEVIC; SUDING, 2011; TĚŠITEL et al., 2017; HEER et al., 2018). In contrast to other observational studies from root hemiparasite species (GIBSON; WATKINSON, 1992; AMELOOT; VERHEYEN; HERMY, 2005), the effects of *E. grandiflora* on community diversity and species composition were mild. This may suggest that not all root hemiparasite plants strongly associated with the diversity and composition of plant communities. Indeed, the strength with which root hemiparasites influence plants at the community level depends on diverse traits of root hemiparasite interactions with host and non-host (CAMERON; PHOENIX, 2013). Besides, determining the positive or negative relationship of hemiparasites and diversity depends on whether dominant or subordinate species are preferred as hosts (PRESS; PHOENIX, 2005). Experimental field studies demonstrated that when dominant species as grasses are parasitized and their abundance is reduced, subdominant species and subordinate species as forbs may thrive, increasing local species diversity (JOSHI; MATTHIES; SCHMID, 2000; PYWELL et al., 2004; WESTBURY; DUNNETT, 2007). In contrast, when subordinate species are negatively affected to the detriment of dominant species, diversity levels will decrease (Gibson & Watkinson, 1989). Positive effects of *Rhinanthus mino*r L. and *Rhinanthus alectorolophus* (Scop.) Pollich were reported to be strong in grassland plant communities. Consequently, these plants can be introduced as a management and restoration tool in pastures (PYWELL et al., 2004; HEER et al., 2018). Otherwise, in an observational study, other perennial species, such as *Castilleja* (SPASOJEVIC; SUDING, 2011), produced no effects in species richness, evenness, and diversity on plant communities. In the cases of *E. grandiflora*, positive effects on species diversity and differences in species composition are important to explain biodiversity patterns and the mechanisms at play among interacting species in these communities. Root hemiparasite species may affect neighbors by other means, such as direct competition with hosts and non-hosts and the positive effects of high-quality litter deposition (FISHER et al., 2013). The expected net effects of parasitism on species diversity could be neutralized by these complex interactions, such as those found in the hemiparasite *Castilleja* (SPASOJEVIC;

SUDING, 2011). Nevertheless, these issues have never been studied in *E. grandiflora*. Furthermore, *E. grandiflora* commonly attached many hosts roots simultaneously (CARDONA; MURIEL, 2015). This feature is beneficial to hemiparasite performance since it allows the absorption of different amounts of nutrients from host plant roots (PENNINGS; CALLAWAY, 2002; ROWNTREE et al., 2014). Therefore, simultaneous parasitism of several hosts could decrease host damage by root hemiparasites (PENNINGS; CALLAWAY, 2002) and dilute the effect on the plant community as a whole. Such effects were also verified in other organisms like herbivore insect-host interactions (FRANZKE et al., 2010).

The strength of the effect on plant communities depends on the life history characteristics of parasitic plants (PRESS; PHOENIX, 2005). Hemiparasite density, dispersion, size, and lifespan (annual or perennial) should also be considered in determining the hemiparasite's impact. Strong effects by hemiparasites on plant communities are commonly found in experimental studies, in species with high density, such as *R. alectorolophus* and *Pedicularis sylvatica* L. (JOSHI; MATTHIES; SCHMID, 2000; DEMEY et al., 2015). A high density of some hemiparasites is related to a high survival rate of hemiparasite seedlings (JOSHI; MATTHIES; SCHMID, 2000; MUDRÁK et al., 2014; DEMEY et al., 2015). On the contrary, findings in *E. grandiflora* show that seedlings have a low survival rate and low growth (CARDONA; MURIEL, 2015; CARDONA-MEDINA; SANTOS; NODARI, 2019). In the four areas studied, *E. grandiflora* populations were composed of sparse individuals, not dense aggregations. Observational study using a hemiparasite density approach revealed that the high density of *R. alectorolophus* strongly influenced plant composition and increased richness and evenness in plant communities (HEER et al., 2018). The possible interference of *E. grandiflora* density on the effect on plant communities cannot be ruled out, and it would be interesting to be evaluated. In addition, root hemiparasites are generally considered to be poor competitors for light (CAMERON et al., 2005; SVENSSON; CARLSSON, 2004a); however, in contrast to other herb root hemiparasite species, mature *E. grandiflora* is a large plant, ranging from 0.5 up to 2 m of vegetative height, and thus might present a strong competitive ability toward plant communities.

Moreover, *E. grandiflora* presents a perennial root system that will last several years and sprouts flowering shoots in the same spot, promoting a long-term interaction with hosts and non-host neighbors. We know by personal observation that the same individual rhizomes planted in pots have been sprouting for seven years. Collected specimens in the field presented comparatively large root systems, indicating old individuals (> 10 years). The effect of

perennial or biennial may be different from annual hemiparasites. The hemiparasite *Bartsia alpina* L presents perennial rhizomes and annual dense clones with branching that constantly benefits the growth of co-occurring plants (QUESTED; PRESS; CALLAGHAN, 2003a; TAYLOR; RUMSEY, 2003). In addition, a long-term experimental study (seven years) demonstrated that the biennial hemiparasite *Pedicularis palustris* L. has a strong negative effect on the biomass of the dominant Cyperaceae species, which lead to a change in species composition, allowing the colonization of non-dominant grasses and herbs (DECLEER; BONTE; VAN DIGGELEN, 2013). Another long-term study (ten years) indicated that the perennial hemiparasite *P. canadensis* also alters the community's composition and productivity; however, these results varied with time and space (BOROWICZ; WALDER; ARMSTRONG, 2019). Therefore, these life history aspects may intensify the long-term effects of *E. grandiflora* on community diversity and structure, although this question, too, remains to be elucidated.

The positive relationships we found between diversity indices and the presence of the hemiparasite could also implicate that *E. grandiflora* presents a greater chance of establishment in more diverse spots over grassland areas. First, richer communities may present better hosts or a combination of better hosts providing root systems for hemiparasite attachment and establishment (JOSHI; MATTHIES; SCHMID, 2000; ROWNTREE et al., 2014; SANDNER; MATTHIES, 2018). This is especially relevant considering that *E. grandiflora* haustoria are attached to host roots of seven plant families (CARDONA; MURIEL, 2017). Second, areas with greater diversity may present lower competition intensity with dominant plants (BOROWICZ; ARMSTRONG, 2012; TĚŠITEL et al., 2013). Using a database of 18,101 vegetative plots of temperate communities from the Czech Republic, one study found that incidences of 11 to 16 root hemiparasites are higher in regions with greater plant diversity (FIBICH et al., 2017). Nevertheless, our study concerns local community patterns, not regional or broad landscape scales. However, in addition to the positive relationship between diversity and the presence of *E. grandiflora*, the present study also verified the association between a lower vegetative cover of dominant plants and *E. grandiflora* presence. In this sense, we are aware that causality between the hemiparasite and vegetation structure could be ambiguous in observational studies. Some studies have addressed the effects of vegetation density on the population of *Rhinanthus* (DE HULLU, 1985), for instance. Therefore, a more comprehensive study is required to further and adequately disentangle the effects of root hemiparasites on ecosystem function, species effects, and habitat suitability. Whether the presence of *E.*

grandiflora affects the structure and diversity of plant communities, or the reverse is yet to be elucidated by well-designed manipulative experiments.

Our results demonstrate that the root hemiparasite *E. grandiflora* is associated with a high number of species, diversity, and evenness in local communities. Furthermore, cover reduction of dominant plants was associated with *E. grandiflora*, pointing to changes in species composition. Functional groups were differentially affected in that graminoids were favored to the detriment of herbs. Our results are consistent across physiognomically different grasslands. The role of this tropical root hemiparasite on ecosystem functioning, nonetheless, requires further experimental studies, especially with removal and sown of hemiparasite. To conserve this hemiparasite is necessary to maintain the diversity of plant communities, especially the diversity of suitable host species (MARVIER; SMITH, 1997). Therefore, research should be undertaken to investigate the quality and host preference of *E. grandiflora* in the evaluated plant communities.

To celebrate its 125th anniversary, the Senior Editorial Board of Science selected the top 25 unresolved scientific questions. Among them was the following: "What Determines Species Diversity?" Seen from this perspective, the present study has provided the first scientific insights into root hemiparasite effects of Orobanchaceae in plant communities of South America, thereby inching closer toward explaining species diversity in a complex ecosystem southern Brazil.

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4.6 SUPPLEMENTARY MATERIAL

Table S1. Functional group (graminoids, herbs, rosettes, shrubs, and cryptogams), scientific name, family, and location of the species with occurrence within the pair plots, in four plant communities of southern Brazil. Species were identified using scientific literature and databases like the Missouri Botanical Garden (www.tropicos.org), speciesLink (www.splink.org.br), and species list of the Brazilian Flora, Jardim Botânico do Rio de Janeiro (http://floradobrasil.jbrj.gov.br/).

Functional Group	Scientific Name	Family	Location
Cryptogams	*Anemia* sp.	Anemiaceae	Jaquirana
Cryptogams	*Blechnum serrulatum* Rich.	Blechnaceae	Florianópolis
Cryptogams	cf. *Cladonia* sp.	Cladoniaceae	Florianópolis
Cryptogams	*Lindsaea botrychioides* A. St.-Hil.	Lindsaeaceae	Florianópolis
Cryptogams	*Lycopodiella cernua* (L.) Pic. Serm.	Lycopodiaceae	Florianópolis
Cryptogams	*Adiantum* sp.	Pteridaceae	Jaquirana
Cryptogams	Undetermined	Pteridaceae	Água Doce
Cryptogams	*Anemia* cf. *tomentosa* (Savigny) Sw.	Schizaeaceae	Florianópolis
Cryptogams	*Selaginella* sp.	Selaginellaceae	Florianópolis
Cryptogams	*Thelypteris* sp.	Thelypteridaceae	Jaquirana
Cryptogams	Undetermined	Thelypteridaceae	Água Doce
Cryptogams	Undetermined	Undetermined	Jaquirana
Graminoids	*Habranthus robustus* Herb.	Amaryllidaceae	Florianópolis
Graminoids	*Nothoscordum* sp.	Amaryllidaceae	Florianópolis
Graminoids	*Bulbostylis capillaris* (L.) Kunth ex C.B. Clarke	Cyperaceae	Florianópolis
Graminoids	*Rhynchospora barrosiana* Guagl.	Cyperaceae	Florianópolis
Graminoids	*Scleria sellowiana* Kunth	Cyperaceae	Florianópolis
Graminoids	*Scleria hirtella* (Sw.)	Cyperaceae	Florianópolis
Graminoids	Undetermined	Cyperaceae	Água Doce

Graminoids	*Bulbostylis* sp.	Cyperaceae	Jaquirana
Graminoids	*Cyperus aggregatus* (Willd.) Endl.	Cyperaceae	Jaquirana
Graminoids	*Cyperus brasiliensis* (Kunth) Bauters	Cyperaceae	Jaquirana
Graminoids	*Cyperus reflexus* Vahl	Cyperaceae	Jaquirana
Graminoids	*Fimbristylis* sp.	Cyperaceae	Jaquirana
Graminoids	*Rhynchospora* sp.	Cyperaceae	Jaquirana
Graminoids	Undetermined	Cyperaceae	Jaquirana
Graminoids	Undetermined	Cyperaceae	Jaquirana
Graminoids	*Hypoxis decumbens* L.	Hypoxidaceae	Florianópolis
Graminoids	*Neomarica* sp.	Iridaceae	Florianópolis
Graminoids	*Sisyrinchium vaginatum* Spreng.	Iridaceae	Água Doce
Graminoids	*Sisyrinchium* sp.	Iridaceae	Jaquirana
Graminoids	*Andropogon selloanus* (Hack.) Hack.	Poaceae	Florianópolis
Graminoids	*Andropogon* sp.1	Poaceae	Florianópolis
Graminoids	*Andropogon* sp.2	Poaceae	Florianópolis
Graminoids	*Axonopus siccus* (Nees) Kuhlm.	Poaceae	Florianópolis
Graminoids	*Aristida* sp.	Poaceae	Florianópolis
Graminoids	*Chascolytrum uniolae* (Nees) L.Essi, Longhi-Wagner & Souza-Chies	Poaceae	Florianópolis
Graminoids	*Dichanthelium sabulorum* (Lam.) Gould & C.A. Clark 1	Poaceae	Florianópolis
Graminoids	*Dichanthelium* sp.	Poaceae	Florianópolis
Graminoids	*Eriochrysis cayennensis* P. Beauv.	Poaceae	Florianópolis
Graminoids	*Ischaemum minus* J. Presl	Poaceae	Florianópolis
Graminoids	*Paspalum glaucescens* Hack. (1)	Poaceae	Florianópolis
Graminoids	*Paspalum polyphyllum* Nees ex Trin.	Poaceae	Florianópolis
Graminoids	*Paspalum* cf. *ramboi* I.L. Barreto	Poaceae	Florianópolis
Graminoids	*Schizachyrium* sp.1	Poaceae	Florianópolis
Graminoids	Undetermined	Poaceae	Florianópolis
Graminoids	Undetermined	Poaceae	Florianópolis
Graminoids	*Andropogon lateralis* Nees	Poaceae	Água Doce
Graminoids	*Anthenantia lanata* (Kunth) Benth.	Poaceae	Água Doce
Graminoids	*Dichanthelium sabulorum* (Lam.) Gould & C.A. Clark 2	Poaceae	Água Doce
Graminoids	*Digitaria* sp.	Poaceae	Água Doce
Graminoids	*Eriochrysis holcoides* (Nees) Kuhlm.	Poaceae	Água Doce
Graminoids	*Festuca ampliflora* Döll	Poaceae	Água Doce
Graminoids	*Panicum* sp.	Poaceae	Água Doce
Graminoids	*Paspalum glaucescens* Hack. 2	Poaceae	Água Doce
Graminoids	*Paspalum polyphyllum* Nees ex Trin.	Poaceae	Água doce
Graminoids	*Schizachyrium* sp.2	Poaceae	Água Doce
Graminoids	*Stipa airoides* Ekman	Poaceae	Água Doce
Graminoids	Undetermined	Poaceae	Água Doce
Graminoids	*Andropogon lateralis* Nees	Poaceae	Jaquirana

Graminoids	*Cortaderia selloana* (Schult. & Schult. f.) Asch. & Graebn.	Poaceae	Jaquirana
Graminoids	*Eragrostis airoides* Nees	Poaceae	Jaquirana
Graminoids	*Eriochrysis cayennensis* P. Beauv.	Poaceae	Jaquirana
Graminoids	*Paspalum glaucescens* Hack. (3)	Poaceae	Jaquirana
Graminoids	*Schizachyrium* sp.3	Poaceae	Jaquirana
Graminoids	Undetermined	Poaceae	Jaquirana
Graminoids	Undetermined	Poaceae	Jaquirana
Graminoids	Undetermined	Poaceae	Jaquirana
Graminoids	Undetermined	Poaceae	Jaquirana
Graminoids	Undetermined	Poaceae	Jaquirana
Graminoids	*Xyris jupicai* Rich.	Xyridaceae	Jaquirana
Herbs	*Alstroemeria isabellana* Herb.	Alstroemeriaceae	Água Doce
Herbs	*Centella asiatica* (L.) Urb.	Apiaceae	Florianópolis
Herbs	*Asclepias mellodora* A. St.-Hil.	Apocynaceae	Florianópolis
Herbs	*Gonioanthela axillaris* (Vell.) Fontella & E.A. Schwarz	Apocynaceae	Florianópolis
Herbs	*Oxypetalum tomentosum* Wight ex Hook. & Arn.	Apocynaceae	Florianópolis
Herbs	Undetermined	Apocynaceae	Florianópolis
Herbs	*Hydrocotyle pusilla* A. Rich.	Araliaceae	Jaquirana
Herbs	*Aristolochia robertii* Ahumada	Aristolochiaceae	Florianópolis
Herbs	*Achyrocline flaccida* (Weinm.) DC.	Asteraceae	Florianópolis
Herbs	*Acmella bellidioides* (Sm.) R.K. Jansen	Asteraceae	Florianópolis
Herbs	*Baccharis trimera* (Less.) DC.	Asteraceae	Florianópolis
Herbs	*Baccharis* sp.1	Asteraceae	Florianópolis
Herbs	*Chromolaena ascendens* (Sch. Bip. ex Baker) R.M. King & H. Rob.	Asteraceae	Florianópolis
Herbs	*Chrysolaena flexuosa* (Sims) H. Rob. 1	Asteraceae	Florianópolis
Herbs	*Chrysolaena flexuosa* (Sims) H. Rob. 2	Asteraceae	Florianópolis
Herbs	*Lepidaploa chamissonis* (Less.) H. Rob.	Asteraceae	Florianópolis
Herbs	*Lucilia acutifolia* (Poir.) Cass.	Asteraceae	Florianópolis
Herbs	*Lucilia linearifolia* Baker	Asteraceae	Florianópolis
Herbs	*Lucilia lycopodioides* (Less.) S.E. Freire	Asteraceae	Florianópolis
Herbs	*Mikania* cf. *involucrata* Hook. & Arn.	Asteraceae	Florianópolis
Herbs	*Phyllanthus subemarginatus* Müll. Arg.	Asteraceae	Florianópolis
Herbs	*Praxelis sanctopaulensis* (B.L. Rob.) R.M. King & H. Rob.	Asteraceae	Florianópolis
Herbs	*Pterocaulon* sp.	Asteraceae	Florianópolis
Herbs	*Achyrocline alata* (Kunth) DC.	Asteraceae	Água Doce
Herbs	*Austroeupatorium inulifolium* (Kunth) R.M. King & H. Rob.	Asteraceae	Água Doce
Herbs	*Austroeupatorium* sp.	Asteraceae	Água Doce
Herbs	*Baccharis* cf. *erioclada* DC.	Asteraceae	Água Doce
Herbs	*Baccharis weirii* Baker	Asteraceae	Água Doce
Herbs	*Chromolaena congesta* (Hook. & Arn.) R.M. King & H. Rob.	Asteraceae	Água Doce

Herbs	*Grazielia intermedia* (DC.) R.M. King & H. Rob.	Asteraceae	Água Doce
Herbs	*Stevia collina* Gardner	Asteraceae	Água Doce
Herbs	*Vernonanthura* sp.	Asteraceae	Água Doce
Herbs	*Achyrocline flaccida* (Weinm.) DC.	Asteraceae	Jaquirana
Herbs	*Acmella bellidioides* (Sm.) R.K. Jansen	Asteraceae	Jaquirana
Herbs	*Aldama anchusifolia* (DC.) E.E. Schill. & Panero	Asteraceae	Jaquirana
Herbs	*Aldama pilosa* (Baker) E.E. Schill. & Panero	Asteraceae	Jaquirana
Herbs	*Aspilia montevidensis* (Spreng.) Kuntze 1	Asteraceae	Jaquirana
Herbs	*Aspilia montevidensis* (Spreng.) Kuntze 2	Asteraceae	Jaquirana
Herbs	*Baccharis* sp.2	Asteraceae	Jaquirana
Herbs	*Chromolaena hirsuta* (Hook. & Arn.) R.M. King & H. Rob.	Asteraceae	Jaquirana
Herbs	*Chrysolaena flexuosa* (Sims) H. Rob.	Asteraceae	Jaquirana
Herbs	*Erechtites hieraciifolius* (L.) Raf. ex DC. 1	Asteraceae	Jaquirana
Herbs	*Erechtites hieraciifolius* (L.) Raf. ex DC. 2	Asteraceae	Jaquirana
Herbs	*Gamochaeta americana* (Mill.) Wedd.	Asteraceae	Jaquirana
Herbs	*Jaegeria hirta* (Lag.) Less.	Asteraceae	Jaquirana
Herbs	*Lucilia linearifolia* Baker	Asteraceae	Jaquirana
Herbs	*Pterocaulon balansae* Chodat	Asteraceae	Jaquirana
Herbs	*Solidago chilensis* Meyen	Asteraceae	Jaquirana
Herbs	*Stevia* sp.	Asteraceae	Jaquirana
Herbs	Undetermined	Asteraceae	Jaquirana
Herbs	*Thaumatocaryon tetraquetrum* (Cham.) I.M. Johnst.	Boraginaceae	Água Doce
Herbs	*Evolvulus glomeratus* Nees & C. Mart.	Convolvulaceae	Florianópolis
Herbs	*Jacquemontia densiflora* (Meisn.) Hallier f.	Convolvulaceae	Florianópolis
Herbs	Undetermined	Convolvulaceae	Florianópolis
Herbs	*Drosera communis* A. St.-Hil.	Droseraceae	Jaquirana
Herbs	*Euphorbia insulana* Müll. Arg.	Euphorbiaceae	Florianópolis
Herbs	*Aeschynomene* sp.1	Fabaceae	Florianópolis
Herbs	*Aeschynomene* sp.2	Fabaceae	Florianópolis
Herbs	*Collaea stenophylla* (Hook. & Arn.) Benth.	Fabaceae	Jaquirana
Herbs	*Desmodium cuneatum* Hook. & Arn.	Fabaceae	Jaquirana
Herbs	*Desmodium uncinatum* (Jacq.) DC.	Fabaceae	Jaquirana
Herbs	*Desmodium* sp.1	Fabaceae	Jaquirana
Herbs	*Desmodium* sp.2	Fabaceae	Jaquirana
Herbs	*Eriosema tacuaremboense* Arechav.	Fabaceae	Jaquirana
Herbs	*Rhynchosia corylifolia* Mart. ex Benth.	Fabaceae	Jaquirana
Herbs	*Rhynchosia diversifolia* Micheli	Fabaceae	Jaquirana
Herbs	Undetermined	Fabaceae	Jaquirana
Herbs	*Zygostigma australe* (Cham. & Schltdl.) Griseb.	Gentianaceae	Água Doce
Herbs	*Hypericum connatum* Lam.	Hypericaceae	Jaquirana
Herbs	*Hoehnea parvula* Epling	Lamiaceae	Água Doce

Herbs	*Hyptis muelleri* Briq.	Lamiaceae	Água Doce
Herbs	*Hyptis radicans* (Pohl) Harley & J.F.B. Pastore	Lamiaceae	Água Doce
Herbs	*Linum selaginoides* Lam.	Linaceae	Florianópolis
Herbs	*Cuphea urbaniana* Koehne	Lythraceae	Água Doce
Herbs	Undetermined	Malvaceae	Florianópolis
Herbs	*Pavonia dusenii* Krapov.	Malvaceae	Água Doce
Herbs	*Myrcia* cf. *selloi* (Spreng.) N. Silveira	Myrtaceae	Florianópolis
Herbs	Undetermined	Myrtaceae	Florianópolis
Herbs	Undetermined	Myrtaceae	Florianópolis
Herbs	*Sauvagesia erecta* L.	Ochnaceae	Florianópolis
Herbs	*Cleistes libonii* (Rchb. f.) Schltr.	Orchidaceae	Florianópolis
Herbs	*Epidendrum fulgens* Brongn.	Orchidaceae	Florianópolis
Herbs	*Habenaria* sp.1	Orchidaceae	Florianópolis
Herbs	*Habenaria* sp.2	Orchidaceae	Água Doce
Herbs	*Habenaria* sp.3	Orchidaceae	Jaquirana
Herbs	*Buchnera longifolia* Kunth	Orobanchaceae	Florianópolis
Herbs	Undetermined	Orobanchaceae	Florianópolis
Herbs	*Buchnera* sp.	Orobanchaceae	Água Doce
Herbs	*Oxalis barrelieri* L.	Oxalidaceae	Florianópolis
Herbs	*Oxalis bipartita* A. St.-Hil.	Oxalidaceae	Água Doce
Herbs	*Passiflora suberosa* L.	Passifloraceae	Florianópolis
Herbs	*Polygala pulchella* A. St.-Hil. & Moq.	Polygalaceae	Jaquirana
Herbs	*Coccocypselum* cf *lanceolatum* (Ruiz & Pav.) Pers.	Rubiaceae	Florianópolis
Herbs	*Galium* cf. *atherodes* Spreng.	Rubiaceae	Água Doce
Herbs	*Galium atherodes* Spreng.	Rubiaceae	Jaquirana
Herbs	*Galium* sp.1	Rubiaceae	Florianópolis
Herbs	*Galium* sp.2	Rubiaceae	Jaquirana
Herbs	*Smilax campestris* Griseb.	Smilacaceae	Florianópolis
Herbs	Undetermined	Undetermined	Florianópolis
Herbs	Undetermined	Undetermined	Florianópolis
Herbs	Undetermined	Undetermined	Água Doce
Herbs	Undetermined	Undetermined	Jaquirana
Herbs	*Stachytarpheta cayennensis* (Rich.) Vahl	Verbenaceae	Florianópolis
Herbs	*Verbena* sp.1	Verbenaceae	Jaquirana
Herbs	*Verbena* sp.2	Verbenaceae	Jaquirana
Herbs	*Glandularia* sp.	Verbenaceae	Jaquirana
Herbs	*Glandularia marrubioides* (Cham.) Tronc.	Verbenaceae	Jaquirana
Herbs	*Verbena* sp.3	Verbenaceae	Jaquirana
Rosettes	*Eryngium elegans* Cham. & Schltdl.	Apiaceae	Florianópolis
Rosettes	*Eryngium megapotamicum* Malme	Apiaceae	Água Doce
Rosettes	*Eryngium* sp.	Apiaceae	Água Doce
Rosettes	*Chaptalia runcinata* Kunth	Asteraceae	Florianópolis
Rosettes	*Orthopappus angustifolius* (Sw.) Gleason	Asteraceae	Florianópolis
Rosettes	Undetermined	Asteraceae	Florianópolis
Rosettes	*Erigeron maximus* (D. Don) Otto ex DC.	Asteraceae	Água Doce

Rosettes	*Lessingianthus cataractarum* (Hieron.) H. Rob.	Asteraceae	Água Doce
Rosettes	*Vernonanthura* sp.	Asteraceae	Água Doce
Rosettes	*Chrysolaena flexuosa* (Sims) H. Rob.	Asteraceae	Jaquirana
Rosettes	*Holocheilus brasiliensis* (L.) Cabrera	Asteraceae	Jaquirana
Rosettes	*Orthopappus angustifolius* (Sw.) Gleason	Asteraceae	Jaquirana
Rosettes	*Pterocaulon polystachyum* DC.	Asteraceae	Jaquirana
Rosettes	*Verbesina sordescens* DC.	Asteraceae	Jaquirana
Rosettes	*Aechmea lindenii* (E. Morren) Baker	Bromeliaceae	Florianópolis
Rosettes	*Dyckia encholirioides* (Gaudich.) Mez	Bromeliaceae	Florianópolis
Rosettes	*Vriesea friburgensis* Mez	Bromeliaceae	Florianópolis
Rosettes	Undetermined	Orchidaceae	Jaquirana
Shrubs	*Ilex dumosa* Reissek	Aquifoliaceae	Florianópolis
Shrubs	*Symphyopappus casarettoi B.L. Rob.*	Asteraceae	Florianópolis
Shrubs	*Baccharis vulneraria* Baker	Asteraceae	Água Doce
Shrubs	*Baccharis* cf. *myriocephala* DC.	Asteraceae	Água Doce
Shrubs	*Campovassouria cruciata* (Vell.) R.M. King & H. Rob.	Asteraceae	Água Doce
Shrubs	*Solidago chilensis* Meyen	Asteraceae	Água Doce
Shrubs	*Baccharis erioclada* DC.	Asteraceae	Jaquirana
Shrubs	*Baccharis pentaptera* DC.	Asteraceae	Jaquirana
Shrubs	*Baccharis subdentata* DC.	Asteraceae	Jaquirana
Shrubs	*Baccharis* sp.2	Asteraceae	Jaquirana
Shrubs	*Campovassouria cruciata* (Vell.) R.M. King & H. Rob.	Asteraceae	Jaquirana
Shrubs	*Chromolaena ivifolia* (L.) R.M. King & H. Rob.	Asteraceae	Jaquirana
Shrubs	*Symphyopappus* sp.	Asteraceae	Jaquirana
Shrubs	*Raulinoreitzia crenulata* (Spreng.) R.M. King & H. Rob.	Asteraceae	Jaquirana
Shrubs	*Agarista chlorantha* (Cham.) G. Don	Ericaceae	Água Doce
Shrubs	*Escallonia* sp.	Escalloniaceae	Jaquirana
Shrubs	*Croton allemii* G.L. Webster	Euphorbiaceae	Florianópolis
Shrubs	*Calliandra tweediei* Benth.	Fabaceae	Florianópolis
Shrubs	*Vitex megapotamica* (Spreng.) Moldenke	Lamiaceae	Florianópolis
Shrubs	*Tibouchina urvilleana* (DC.) Cogn.	Melastomataceae	Florianópolis
Shrubs	*Rhynchanthera brachyrhyncha* Cham.	Melastomataceae	Água Doce
Shrubs	*Tibouchina gracilis* (Bonpl.) Cogn.1	Melastomataceae	Água Doce
Shrubs	*Tibouchina gracilis* (Bonpl.) Cogn.2	Melastomataceae	Jaquirana
Shrubs	*Tibouchina ursina* (Cham.) Cogn.	Melastomataceae	Água Doce
Shrubs	*Myrcia palustris* DC.	Myrtaceae	Florianópolis
Shrubs	*Guapira opposita* (Vell.) Reitz	Nyctaginaceae	Florianópolis
Shrubs	*Ouratea salicifolia* Engl.	Ochnaceae	Florianópolis
Shrubs	*Ludwigia sericea* (Cambess.) H. Hara	Onagraceae	Água Doce
Shrubs	*Ternstroemia brasiliensis* Cambess.	Pentaphylacaceae	Florianópolis
Shrubs	*Myrsine parvifolia* A. DC.	Primulaceae	Florianópolis

Shrubs	*Galianthe* sp.	Rubiaceae	Água Doce
Shrubs	*Dodonaea viscosa* Jacq.	Sapindaceae	Florianópolis
Shrubs	*Lantana camara* L.	Verbenaceae	Florianópolis

5 CAPÍTULO 2-THE HEMIPARASITIC PLANT *Escobedia grandiflora* ACCUMULATES THE APOCAROTENOID PIGMENT AZAFRIN IN THE ROOT APOPLAST AND IN THE HAUSTORIUM-HOST ROOT INTERFACE.

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5.1 ABSTRACT

The herbaceous hemiparasite plant *Escobedia grandiflora* (Orobanchaceae) (hereafter referred to as *Escobedia*) is a plant native to Central and South America used in traditional medicine in Andean region. The roots of this plant accumulate azafrin, an orange apocarotenoid pigment with a significant local relevance as a cooking dye that exhibits antioxidant and cardioprotective properties. Despite these important traditional uses of *Escobedia* species, commercial cultivation has not been implemented in part due to the challenges derived from their parasitic habit. The present work combines chromatography analyses, a *de novo* transcriptome assembly, and phylogenetic analyses with qPCR, confocal microscopy, and cytological studies to investigate azafrin biosynthesis and function in *Escobedia* roots. We confirmed that azafrin is the major compound in *Escobedia* root. Also, we identified *Escobedia* genes encoding carotenoid pathway enzymes for the production of β-carotene and derived xanthophylls and found they were highly expressed in azafrin-accumulating roots. Regarding apocarotenoids, the genes encoding carotenoid cleavage dioxygenase (CCD) enzymes, necessary for abscisic acid biosynthesis, were expressed at much higher levels than those potentially involved in the production of strigolactones. Based on transcript accumulation and phylogeny reconstruction of CCDs, we suggest that azafrin production relies on the activity of the *Escobedia* isoform CCD4b, belonging to a hemiparasite-exclusive clade of CCD4 comprises only by root-specific sequences. We also show that azafrin is not stored in amyloplasts or cytosolic compartments but released into the root apoplast. Furthermore, an exceptionally high accumulation of this apocarotenoid pigment was observed in the area where the *Escobedia* haustorium contacts the host's root, suggesting a role of azafrin in the parasitization process. Altogether, our data provide an unanticipated understanding of the *Escobedia* parasitization system, an indispensable step towards cultivation of this valuable medicinal plant.

Key Words: Azafrin, apocarotenoid, carotenoid cleavage dioxygenase, haustorium, parasitic plant, *de novo* transcriptome assembly.

5.2 INTRODUCTION

Escobedia grandiflora (L. f.) Kuntze (Orobanchaceae) (hereafter referred to as *Escobedia*) is a perennial herbaceous plant native to Central and South America, where it grows in dry and wetland non-forested ecosystems, and it associates with high plant diversity in the communities (BURGUER; BARRINGER, 2000; CARDONA-MEDINA et al., 2021). It is a root hemiparasite that parasitizes other plant roots through a structure known as haustorium (CARDONA; MURIEL, 2015; CARDONA-MEDINA; SANTOS; NODARI, 2019). *Escobedia* is an important medicinal plant for the Andean region. The abundant orange pigment in its root is used as a cooking dye and local medicine for hepatitis, jaundice, hyperlipidaemia, and obesity (MURIEL et al., 2015; SILVA et al., 2010b). This pigment was identified in *Escobedia* roots as the apocarotenoid azafrin ($C_{27}H_{38}O_4$) (KUHN, 1935; ESCHENMOSER; EUGSTER, 1975). Azafrin is also present in other parasitic plants such as in the roots of *Centranthera grandiflora* Wall. ex Benth., in the rhizomes of *Alectra parasitica* Hochst. ex A. Rich. but also in non-parasitic medicinal plants such as *Caralluma umbellata* Haw. (AGRAWAL; LADDHA; TIWARI, 2014; EVANJALINE; VR, 2018; VERMA et al., 2019; ZHANG et al., 2019). This apocarotenoid exhibits antioxidant and cardioprotective properties (YANG; CHOU; LI, 2018).

Apocarotenoids are cleavage products of carotenoids (Fig. 1), presenting different functions, and some have roles as plant growth regulators such as abscisic acid (ABA), strigolactones (SL), and others yet to be fully characterized (FELEMBAN et al., 2019; HOU et al., 2016; MORENO et al., 2021; WALTER; FLOSS; STRACK, 2010a). Biosynthesis of plant carotenoids takes place in plastids, and it begins with the formation of C_{40} 15-*cis*-phytoene through condensation of two molecules of C_{20} geranylgeranyl diphosphate (GGPP) by phytoene synthase (PSY), the first and main rate-determining enzyme of the pathway (RODRIGUEZ-CONCEPCION et al., 2018; MORENO et al., 2021). PSY is usually encoded by small gene families in plants (STAUDER et al., 2018). Phytoene desaturation and isomerization produces lycopene, the red carotenoid responsible for the colour of ripe tomatoes. Formation of β rings in the two ends of the lycopene molecule generates β-carotene, the main pro-vitamin A carotenoid and the proposed precursor of azafrin (RODRIGUEZ-CONCEPCION et al., 2018; ZHANG et al., 2019). Cleavage of the C_{40}-skeleton of carotenoids can either take place non-

enzymatically or be catalysed by carotenoid cleavage dioxygenases (CCDs) to produce apocarotenoids (FELEMBAN et al., 2019; MORENO et al., 2021). In the model plant *Arabidopsis thaliana*, 9-*cis*-epoxycarotenoids dioxygenases (NCEDs) are involved in the biosynthesis of ABA whereas CCD7 and CCD8 participate in the first steps of SL production. Other CCD enzymes such as plastid-localized CCD4 and cytosolic CCD1 are involved in the production of other apocarotenoids, including volatiles and growth regulators (AULDRIDGE; MCCARTY; KLEE, 2006; WALTER; FLOSS; STRACK, 2010a; HOU et al., 2016; FELEMBAN et al., 2019; MORENO et al., 2021). The biosynthetic pathway of azafrin in the hemiparasitic plant *Centranthera* was proposed to begin with the isomerization of β-carotene to 9-*cis*-β-carotene by the enzyme DWARF27 (D27), followed by cleavage by CCD7 to produce 10'-apo-β-carotenal, a common precursor of strigolactones (BRUNO; AL-BABILI, 2016; ZHANG et al., 2019). Then, an unknown aldehyde dehydrogenase would transform aldehyde into a carboxylic acid, and a cytochrome P450 monooxygenase would catalyse the oxidation reactions to produce azafrin (ZHANG et al., 2019) (Fig. 1).

Plant apocarotenoids often modulate the interaction of plants with other organisms, such as herbivores, arbuscular mycorrhizal (AM) fungi, and parasitic plants (MORENO et al., 2021; WANG; LIN; AL-BABILI, 2021). A well-known case is SL, which stimulates the germination of some parasitic seeds from Orobanchaceae, contributes to establishing Arbuscular mycorrhiza (AM) symbiosis, and modulates other rhizospheric communication with symbionts and parasites (Torres-Vera et al., 2016; Mutuku et al., 2021). Other apocarotenoids with roles in rhizospheric interactions such as AM symbiosis include blumenols, mycorradicins, zaxinone and anchorene (MORENO et al., 2021; WANG; LIN; AL-BABILI, 2021). Although the accumulation of the orange apocarotenoid pigment azafrin in the roots of *Escobedia* is one of the main applied interests of this medicinal plant, very little is known about its biosynthesis and function. Here we investigate azafrin biosynthesis and insights on a possible function in parasitic plant-host interactions.

5.3 MATERIALS AND METHODS

5.3.1 Plant material

Roots and dried fruits of *Escobedia grandiflora* were collected from nine mature individuals (i.e. in flowering period) in a natural population located in the Lagoinha do Leste Municipal Park (27°46'53.0"S, 48°29'16.1"W, 190 m.a.s.l.), municipality of Florianópolis,

Brazil. After collection, the roots were frozen at -80ºC, lyophilized, and then pulverized with a TissueLyser (Qiagen) to obtain a fine powder for further chromatography, RNA-Seq, and RT-qPCR analyses.

5.3.2 Root and haustoria development

The seeds from fruits were extracted and imbibed for five days in distilled H2O and sown as described by CARDONA-MEDINA; SANTOS; NODARI (2019). Twenty imbibed seeds were sown in 2L pots without a host in greenhouse conditions with a long day photoperiod. After nine months, roots were collected and processing as described above for UPLC and RT-qPCR analyses. Furthermore, twenty imbibed seeds were sown in 5L pots with a host for nine months to observe different root and haustoria development phases, as reported by CARDONA-MEDINA; SANTOS; NODARI (2019). Haustoria and roots were collected and processed for further microscopy analyses. The selected host plant was *Pennisetum purpureum* Schumach (Poaceae); it has been used because intrusive cells of the haustorium penetrated *P. purpureum* roots xylem (CARDONA; MURIEL, 2015; CARDONA-MEDINA; SANTOS; NODARI, 2019).

5.3.3 Azafrin analyses

Azafrin identification in roots of *Escobedia grandiflora* was based on the chromatographic behaviour, UV-visible spectrum and mass spectroscopic characteristics obtained by HPLC-DAD-MS(ESI+). Data was compared with those of literature (BRITTON, 1991; BRITTON; LIAAEN-JENSEN; PFANDER, 2004). Chromatographic analysis was carried out using a Waters e2695 Alliance chromatograph (Waters Cromatografía, SA, Barcelona, Spain) coupled in series with a Waters 2998 photodiode array detector (DAD) and with an ACQUITY QDa Mass Detector with electrospray ionization (ESI) source running in positive mode. Chromatographic analysis was performed in Instituto de la grassa (Sevilla/ Spain) The entire system was controlled with Masslynx v4.2 MS software (Waters Corporation, Manchester, UK). The chromatographic method was as described in DELGADO-PELAYO; GALLARDO-GUERRERO; HORNERO-MÉNDEZ (2016), with slight modifications. Briefly, carotenoid separation was carried out by a binary-gradient elution using an initial composition of 75 % acetone and 25 % deionized water (both containing 0.1 % formic acid), which was increased linearly to 95 % acetone in 10 min, then hold for 7 min and raised to 100 % in 3 min, and maintained constant for 10 min. Initial conditions were reached in 5 min. The separation

was performed in a reversed-phase C18 (20 mm x 4.6 mm i.d., 3 µm, Mediterranea SEA18; Teknokroma, Barcelona, Spain) fitted with a guard column of the same material. The temperature of column was kept at 25 ºC and the sample compartment was refrigerated at 15 ºC. An injection volume of 10 µL and a flow rate of 1 mL min-1 were used. UV-visible detection was performed at 450 nm and the online spectra were acquired in the 350–700 nm wavelength range. The settings for MS analysis were: nebulizing gas (nitrogen) pressure 95 psi; capillary voltage 0.8 kV; cone voltage 20 v; source temperature 120 ºC; probe temperature 600 ºC; scanning range of m/z 150-1,000.

To quantification, lyophilized roots from *E. grandiflora* grown with hosts (natural populations) and without a host were extracted to quantify azafrin levels. To extraction, 1 mg of fine root powder was mixed with 900 µL of methanol and 100µL of 2 % (w/v) solution of tartrazine in ethanol 70% as internal standard, in cold and absence of light. The solution was mixed by agitation for 1 min. Then, it was separated by centrifugation at 1300 rpm for 5 min at 4°C. The upper phase was collected and filtered through a 0.2 µm microfilter. For each sample, 10 µL was injected onto an Agilent Technologies 1290 Infinity UPLC system in the Centre of Research in Agricultural Genomics (CRAG). The mobile phases consisted of 100% acetonitrile (A) and water with 0.1% formic acid (B). Azafrin was monitored at 417 and 450 nm using the Agilent ChemStation software. Three biological repetitions were performed. Finally, quantification was done by comparison of peaks areas with those of tartrazine standard.

5.3.4 Transcriptome sequencing and assembly

Total RNA extracted from a pool of *Escobedia* roots grown with their hosts in a natural population. Analyses were performed in the Centre of Research in Agricultural Genomics (CRAG). Samples representing different stages of the parasitizing process (i.e. root development) were extracted using the Maxwell® RSC Plant RNA kit (Promega) with the Maxwell® RSC Instrument (Promega), according to the manufacturer´s instructions. RNA samples were pooled in equivalent amounts and library construction was performed with Illumina TruSeq Stranded mRNA kit, according to the manufacturer's instructions. A single library was sequenced by IGATech. Raw paired-end (2x150) reads were processed as follows: 1. Removal of adaptor sequences, sliding-window trimming, length and quality filtering of the raw paired-end reads were performed with *fastp* v0.20.0 (CHEN et al., 2018) using *-w* 16 -5 -3 *-r -W* 4 *-M* 20 *-l* 40. All other settings were left at default. 2. *de novo* transcriptome assembly

of surviving reads was performed with *Trinity* v2.11.0 (HAAS et al., 2013) using default settings.

5.3.5 Transcriptome analysis and functional annotation

Surviving paired-end reads were aligned against the refence assembly using bowtie2 (LANGMEAD; SALZBERG, 2012) using the local read alignment mode (--local). Read summarization was performed using Feature Counts (LIAO; SMYTH; SHI, 2014) with default settings except that both ends must be aligned and chimeric fragments excluded (i.e. -B and -C option enabled). Fragments Per Kilobase of transcript per Million mapped reads (FPKM) were used as expression units. Protein-coding region prediction was first performed with TransDecoder v5.5.0 (http://transdecoder.github.io) with homology searches against the UniProt90 Reference Clusters (https://www.uniprot.org/) using DIAMOND (BUCHFINK; XIE; HUSON, 2015) and Pfam-A database (http://pfam.xfam.org/) using HMMER (MISTRY et al., 2013) to maximize the prediction sensitivity. Additionally, identification of accurate gene sets, removal of assembly redundancies, and protein-coding selection were also performed using Evidential Gene tr2aacds pipeline (DON GILBERT, 2013) with minimum amino acid length of 100 (-MINAA=100). All other settings were default. Transcripts classified as 'okaysets' were retained for further downstream analysis. Functional annotation of the reference transcriptome was performed as follows: 1. Using Mercator4 web application (https://www.plabipd.de/portal/web/guest/mercator4) for assigning BIN categories (SCHWACKE et al., 2019). 2. Retaining the best Blastx search hit against the plant UniProt sprot database (https://www.uniprot.org/) using DIAMOND with the --more-sensitive, --max-hsps 1, --max-target-seqs 1 option enabled. 3. Incorporation of Pfam domain and homolog annotation from TransDecoder's protein-coding prediction pipeline. Gene space completeness was assessed using Benchmarking Universal Single-Copy Orthologs (BUSCO) v3 (WATERHOUSE et al., 2018) with default settings except the lineage database and assessment mode set to -l embryophyta_odb10 and -m genome, respectively. For *Centranthera grandiflora* transcriptome, differential expression between leaf, root, and stem tissues and transcript abundance data (transformed and expressed as FPKM in this study) were obtained from ZHANG et al. (2019). *Escobedia grandiflora* orthologs from *Arabidopsis thaliana* and *Centranthera grandiflora* were inferred using CRB-BLAST with default settings (AUBRY et al., 2014).

5.3.6 Phylogenetic analysis

Phylogenetic reconstructions were performed with MrBayes (HUELSENBECK; RONQUIST, 2001). The resulting topology based on a phylogram was constructed through the standard stepwise pipeline. Firstly, the candidate protein sequences were aligned with MUSCLE (Edgar, 2004; parameters by default) and the multiple sequence alignment outcome was exported in nexus format (MADDISON; SWOFFORD; MADDISON, 1997). The best substitution protein model (with the lowest Bayesian information criterion -BIC- score) was identified using MEGAX (KUMAR et al., 2018). In both our cases, the best model was the Jones-Taylor-Thornton (JTT) with rates among sites following a gamma distribution. During the search of the best substitution model each sequence was considered as 'partially-deleted' in which a candidate is discarded if it presents higher percentage of ambiguous sites than the threshold specified in the Site Coverage Cutoff parameter in MEGAX options (similar to the complete deletion option but with a threshold set to 100%, i.e. absence of ambiguous sites). Once the multiple sequence alignment was performed, the best substitution model was identified and the distribution of rates among sites was selected. The phylogenetic analysis was then conducted by bayesian inference, using the Markov Chain Monte Carlo algorithm (MCMC) available in MrBayes. The different clusters' reliability was calculated with the posterior probabilities (GELMAN et al., 1995), being the values higher than 0.70 acceptable and the values higher than 0.90 highly supported.

5.3.7 Real-time RT-qPCR

RNA extracted from roots of *Escobedia* grown with and without host were used to analyze the expression of four candidate genes potentially involved in azafrin biosynthesis in *Escobedia,* by means of quantitative real-time RT-qPCR. mRNA was retrotranscribed into cDNA using oligo-dT as a primer with NZY First-Strand cDNA Synthesis kit (nzytech). RT-qPCR was performed in a Light Cycler 480 apparatus (Roche), using SYBRGreen I as a fluorescent reporter and Platinum Taq Polymerase. For 10 µl of quantitative PCR, 2 µl of the cDNA was mixed with 5 µl of 2× SYBRGreen (Roche), 0.3 µm of forward and reverse primers, and 2,4 µl of dH2O. RT-qPCR data were normalized with the actin gene (DN8798_c0_g1_i1). The amplification conditions were as follows: an initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 10 s and 60°C for 30 s. Moreover, melting at 95°C for 2 s,

65°C for 15 sec, and a gradual increment at 95. Three technical replicates for each biological repetition and three biological repetitions were performed. Primers are listed in supplementary Table S1.

5.3.8 Structural and anatomical investigation

An anatomical investigation was done to identify and determine the localization of orange pigment inside the roots and in the haustoria attached to the host root. First, fresh samples were incubated in cryoprotectant solution series (sucrose). Subsequently, samples were embedded in a tissue-freezing medium (Fisher, Houston, USA) and frozen up to -19°C. Longitudinal and cross-sections (10-30 μm) were cut in a cryostat Thermo Scientific HM525 NX (Walldorf, Germany) and then analysed in light and with a Leica TCS SP5 Confocal Laser Scanning Microscope (CLSM). Carotenoid detection in CLSM was performed with the method described by D'Andrea et al. (2014), using the 488 nm ray line of an argon laser for excitation and 500-550 nm of emission window. First, the chromoplasts of carrot were detected in fresh hand-cut sections of root to corroborate that the signal belongs to carotenoids. Then, in the same conditions, carotenoid-derived were detected in a cryostat and fresh hand-cut sections of *Escobedia*.

Haustoria samples at different developmental stages were processed to analyse haustorium structure in light microscopy, fluorescence microscopy, and transmission electron microscopy (TEM). Haustoria were fixed in a solution with 2.5 % glutaraldehyde in a 0.1 M sodium phosphate buffer and dehydrated through an ethanolic series (RUZIN, 1999). Fixed and dehydrated samples to analyse the internal structure of the haustorium were processed as described by Cardona-Medina et al. (2019). Sections were stained with toluidine blue (O'BRIEN; FEDER; MCCULLY, 1964). The sections were visualized using a BX-40 microscope (Olympus, Tokyo, Japan), and images were taken with a DP71 digital camera (Olympus, Tokyo, Japan). Fluorescence microscopy was evaluated using an inverted IX81 microscope (Olympus, Tokyo, Japan; emission wavelength: 330-385 nm). Images were taken with a DP71 digital camera (Olympus, Tokyo, Japan).

Analyses of hyaline body ultrastructure were visualized in a TEM. According to PUESCHEL (1979), samples were fixed in 2.5 % glutaraldehyde in 0.1M sodium cacodylate buffer 0.1 M, pH 7.2, and sucrose 0.2 M for 12 h. Furthermore, samples were post-fixed using a solution containing 2% osmium tetroxide and a sodium cacodylate buffer (1:1) for 4 h at room temperature. Finally, samples were washed in a cacodylate buffer, dehydrated in an acetone

series, and embedded in Spurr's resin (Hatfield, USA). Semi-thin 700 nm sections were performed on glass slides to select the region for ultrastructural analysis. Afterward, ultrathin 60 nm sections were contrasted with uranyl acetate 1% and lead citrate 1% according to REYNOLDS (1963), images were taken and recorded with a JEOL JEM1011 (Tokyo, Japan) transmission electron microscope for further analysis.

5.3.9 Statistical analyses

For qRT-PCR analyses, we performed a one-way ANOVA in which the host conditions were the explanatory variable, and azafrin and relative transcript levels of candidate genes were the response variable. Response variables were square-root transformed. ANOVA validation was based on the Shapiro-Wilk test and the analysis of plotting residuals. Significant differences ($p \leq 0.05$) were tested using Tukey´s test. The data were analyzed in R environment version 4.0.3 (R Core Team, 2020).

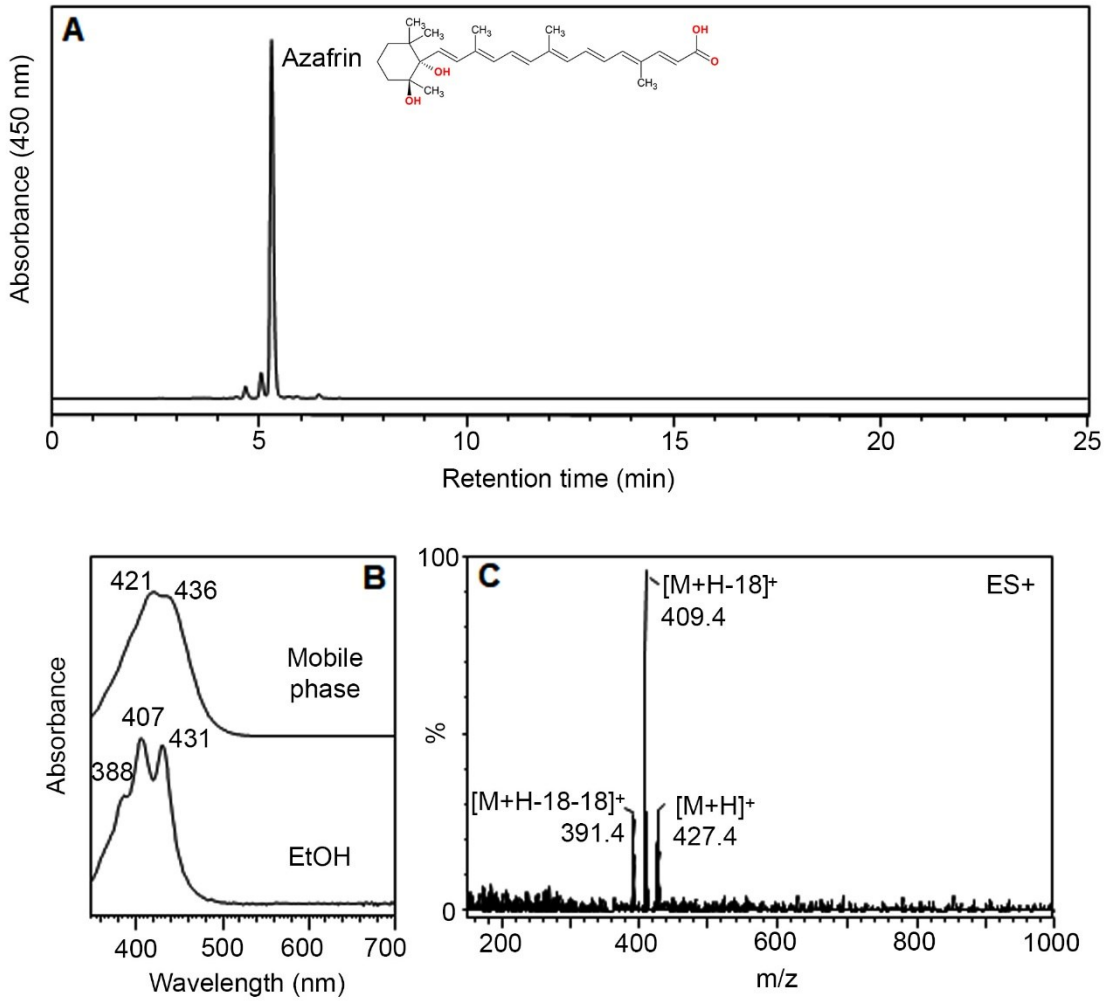
5.4 RESULTS AND DISCUSSION

5.4.1 Azafrin identification in the roots of *Escobedia grandiflora*

As expected from early studies in *Escobedia* species (KUHN, 1935; KARRER; JUCKER, 1948; ESCHENMOSER; EUGSTER, 1975), the HPLC analysis of the carotenoid extract prepared from *Escobedia* roots (Figure 1A) revealed the presence of a major compound (>95%) with an on-line UV-visible spectrum (390, 421, 436 nm). This spectrum agrees with a chromophore structure with seven to eight conjugated double bonds (c.d.b) and is following the structure proposed for azafrin with a seven c.d.b polyene chain and a conjugated carbonyl group. Notably, the UV-visible spectrum obtained during the HPLC analysis (Figure 1B) was affected by the acid mobile phase and as a result presented absorption maxima and fine structure slightly different from those reported in the literature (BRITTON, 1991; BRITTON; LIAAEN-JENSEN; PFANDER, 2004). To check this, the major peak (Rt 5.3 min) was collected and purified from the DAD detector outlet, and the resulting UV-visible spectrum (Figure 1B) in ethanol (388, 407, 431 nm) matched the data reported in previous studies (BRITTON, 1991; BRITTON; LIAAEN-JENSEN; PFANDER, 2004). Probably the observed effect was due to the acidic nature of azafrin as affected by the chromatographic conditions assayed. Eventually, the structure of azafrin was confirmed by mass spectrometry. Thus, the spectrum of the major HPLC peak was consistent with the formula $C_{27}H_{38}O_4$ (Mw=426.2770), with a fragmentation

pattern presenting three characteristic fragments corresponding to the protonated molecule ([M+H]+, 427.4) and the loss of one and two water molecules derived from the hydroxy groups ([M+H-18]+, 409.4; M+H-18-18]+, 391.4) (Figure 1C). Azafrin has been also identified in non-parasitic species, although it has only been found abundantly in the roots (*Escobedia grandiflora* and *Centranthera grandiflora*) and rhizomes (*Alectra parasitica*) of root hemiparasitic plants (AGRAWAL; LADDHA; TIWARI, 2014; ZHANG et al., 2019). It is interesting to note that these species are classified in the same subclade within the Buchnerae clade of Orobanchaceae family (NICKRENT, 2020). Additionally, the other two species from this subclade (*Notochilus* and *Melasma*) were reported to have orange roots, however, the identity of the major compound has not yet been identified (SAFFORD, 1999; "speciesLink network", 2021). It may be the case, therefore, that the abundant production of azafrin would be related to a specific group of root parasitic plants.

Figure 1. HPLC-DAD-MS(ESI+) analysis of the extract prepared from *Escobedia grandiflora* roots. (A) DAD chromatogram 450 nm; (B) UV/Vis spectra of the major peak (azafrin) in the mobile phase and after isolation in ethanol, and (C) mass spectrum.



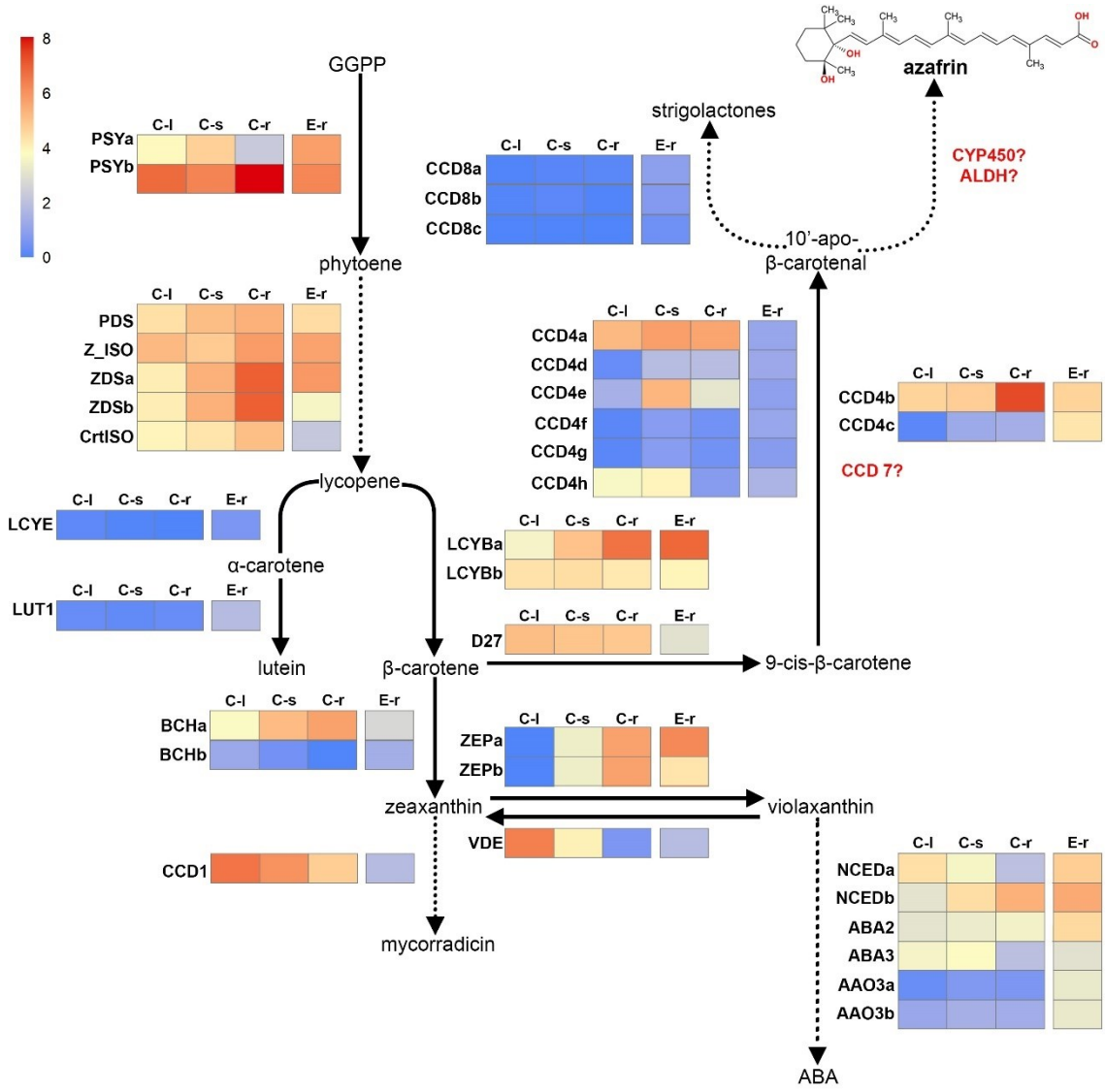
5.4.2 Inspection of the *Escobedia grandiflora* root transcriptome allows the identification of genes belonging to the complete carotenoid pathway.

Escobedia accumulates high levels of azafrin in the roots from natural populations. Therefore, to identify tentative genes involved in the synthesis of this apocarotenoid pigment, we extracted RNA from these roots and performed an RNA-Seq study to identify *Escobedia* genes potentially encoding enzymes of the carotenoid and apocarotenoid biosynthetic pathways. Following adapter removal, trimming, and quality filtering with *fastp*, 76 million 2x150bp paired-end reads (~ 10.8 gigabases of sequence data) were used for *de novo* transcriptome assembly using *Trinity*. Summary statistics of the final assembly include, among others, a total of 115,882 transcripts (65,701 'genes') ranging between 200 – 12,858 nt in length, a mean and N50 sequence length of 1111 and 1798 nt, a GC-content of 0.43, and 25.5% of total transcripts were deemed lowly/not expressed (i.e. FPKM < 0.5) (Supporting Information Figure

S1A, Table S1). Additionally, there were 61,325 and 48,576 transcripts with evidence-supported coding sequences predicted using *TransDecoder* and *EvidentialGene* pipeline, respectively. For example, transcripts containing predicted CDS by *TransDecoder* revealed that many were complete (i.e. full-length and containing both start and stop codons) and predominantly being >1kb in length (Supporting Information Figure S1B). BUSCO assessments with the embryophyta lineage database also indicated very high predicted completeness of the full transcriptome assembly and reduced CDS-only set (i.e. 93.7% - 94.2% of 1,375 BUSCOs evaluated) (Supporting Information Figure S1C). Functional annotation of the reference transcriptome with MapMan BIN v4 categories, matching Pfam domain of predicted peptides, and shared homology with plant Uniprot database sequences revealed that 51302, 51578, and 66335 transcripts (44 – 57% of total transcripts) were successfully annotated to these three criteria, respectively. Notably, we observed a greater representation of transcripts involved in BIN15_RNA biosynthesis, BIN18_Protein modification, BIN50_Enzyme classification, BIN24_Solute transport, among others (Supporting Information Figure S1D). Nonetheless, BIN categories related to the carotenoid (BIN9.1.6.1), xanthophylls (BIN9.1.6.2), apocarotenoid, (BIN9.1.6.3), and related abscisic acid (BIN11.1.1) biosynthetic pathways, among others, were also successfully assigned to transcripts relevant for azafrin biosynthesis (Supporting Information Table S1D). Together, the assembly statistics, gene completeness scores, and a reasonably high number of functional categories and domain assignments to transcripts, suggest a reasonably high assembly quality of the pooled root transcriptomes.

According to our annotation pipelines, we identified thirty-five genes potentially involved (5 partial and 30 complete sequences) in carotenoid and apocarotenoid pathways that were expressed in the roots of *Escobedia* (Figure 2). In the first section of the carotenoid pathway (i.e. leading to the synthesis of lycopene), corresponding to only one gene per each enzyme were found for phytoene desaturase (PDS), ζ-carotene isomerase (Z-ISO) and carotenoid isomerase (CRTISO), whereas two genes encoding PSY and ζ-carotene desaturase (ZDS) were expressed in azafrin-producing roots.

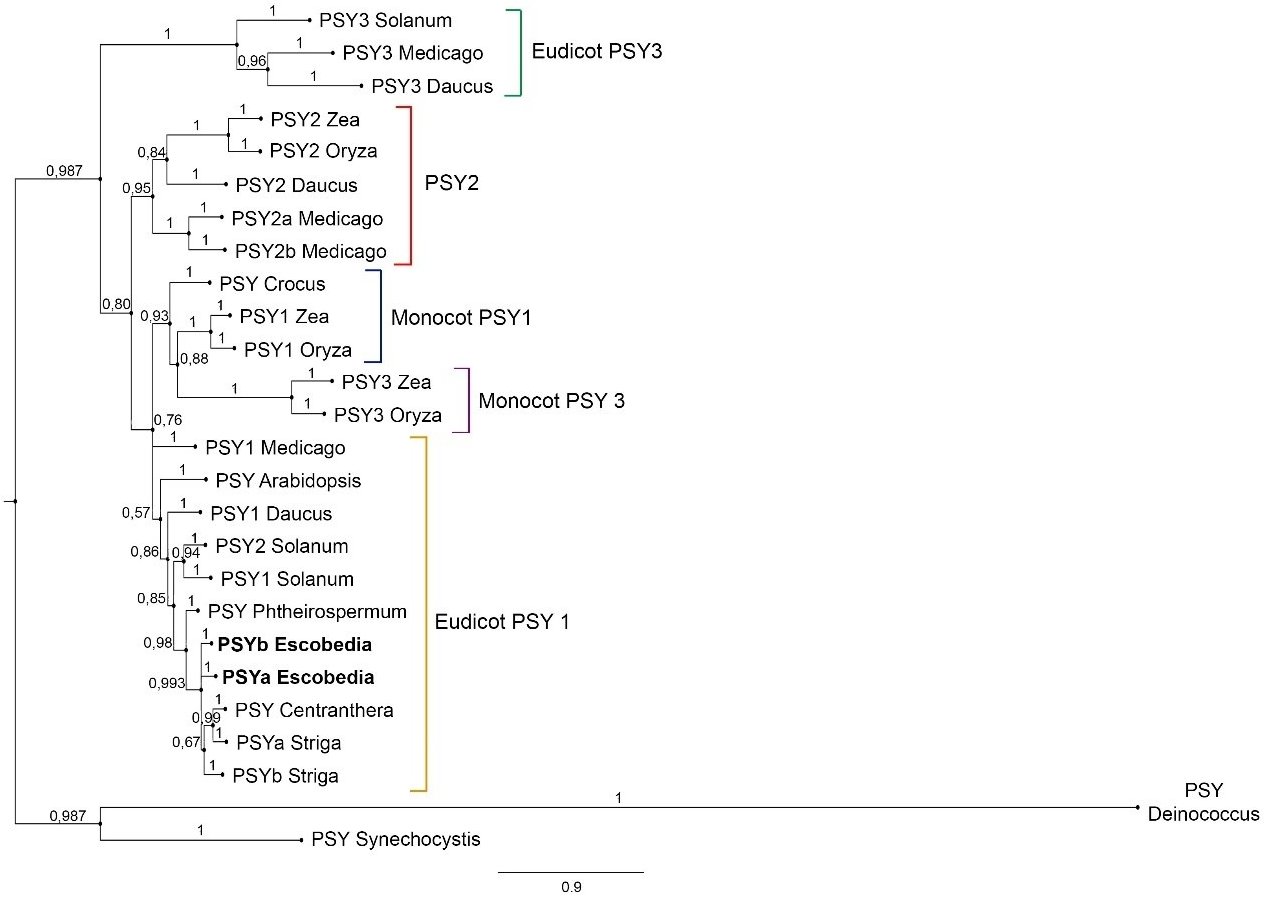
Figure 2. Proposed pathway for carotenoid and azafrin biosynthesis and expression level of enzyme-encoding genes in *Escobedia grandiflora* roots (E-r) in comparison with those of *Centranthera grandiflora* in root (C-r), stem (C-s), and leaf (C-l). Dotted lines implicate multiple steps. Colours represent transcript abundance based on the RNA-Seq analysis (acronyms and FPKM values are listed in Supplementary Table S2). Scale corresponds to $\log_2$ transformed values (FPKM+1). Transcripts for the enzymes indicated in red were not identified in the root *de novo* transcriptome. Data for *Centranthera grandiflora* are retrieved from ZHANG et al. (2019) and reanalysed.



We further inspected the different protein families of the carotenoid pathway to gain insights in their evolution in *Escobedia*. PSY is the main flux-controlling enzyme of the carotenoid pathway (FRASER et al., 2002; RODRIGUEZ-CONCEPCION et al., 2018). Unlike ZDS, PSY is usually encoded by small gene families encoding distinct isoforms associated with

organ- or tissue-specific production of carotenoids. For example, tomato PSY1 is essential for fruit carotenoid production during ripening, while PSY2 is preferentially found in photosynthetic tissues, and PSY3 is a member of a widespread phylogenetic clade in dicots found to participate in Arbuscular mycorrhiza (AM) interactions and distinct from monocot PSY3 isoforms involved in ABA formation (STAUDER et al., 2018). In this work, two PSY isoforms were found to be expressed in *Escobedia* roots, namely PSYa and PSYb (Figure 2). The phylogenetic comparison of the *Escobedia* PSYa and PSYb isoforms with PSY sequences from three other root hemiparasitic species (*Phtheirospermum japonicum* (Thunb.) Kanitz, *Striga asiatica* (L.) Kuntze, and *C. grandiflora*) and several plants with well-characterized PSY families (including tomato, carrot, maize, and rice) or a single PSY gene (*Arabidopsis*) led to their classification in five clades, designated as eudicot PSY1 (EPSY1), monocot PSY1 (MPSY1), PSY2 (including both eudicot and monocot species), eudicot PSY3 (EPSY3) and monocot PSY3 (MPSY3) (Figure 3). *Escobedia* PSYa and PSYb were grouped in the EPSY1 clade, together with *Arabidopsis* PSY, carrot PSY1, and both tomato PSY1 and PSY2 (Figure 3). A sub-clade of only root hemiparasitic PSY sequences (including those from *Escobedia*) was found within this clade with a 98% bootstrap support (Figure 3). It is interesting to note that higher levels of transcripts encoding PSY were found in azafrin-producing *C. grandiflora* roots than to leaves and stems (Figure 2) (ZHANG et al., 2019), suggesting that azafrin production requires an active metabolic flux into the carotenoid pathway.

Figure 3. Phylogenetic analysis of the phytoene synthase (PSY) family in several plants. The tree includes twenty-five angiosperm species and two bacteria as outgroups. The resulting five distinctively separate clades are indicated. Gene accessions are listed in Supplementary Table S3.



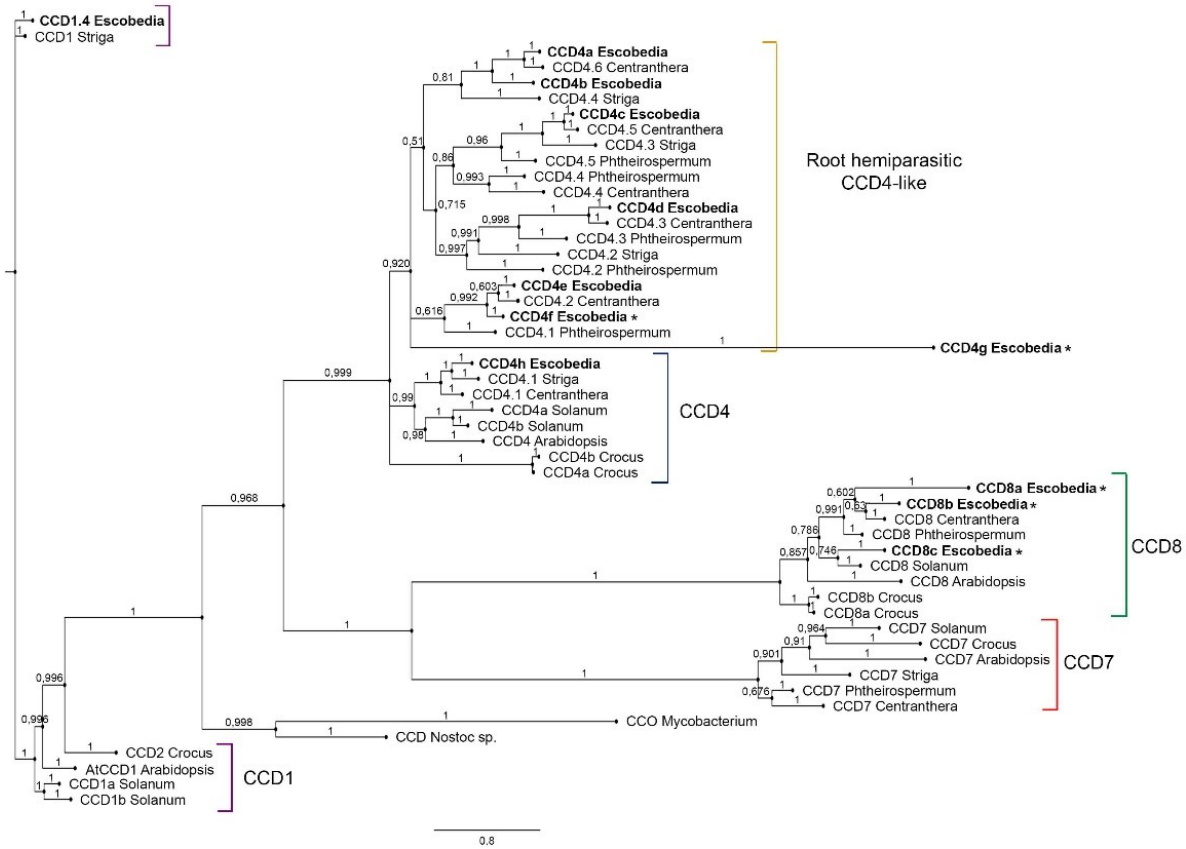
After lycopene, the carotenoid pathway branches out (Figure 2). Cyclization of the two ends of the linear lycopene molecule to produce one β and one ε ring generates α-carotene. These reactions are catalysed by lycopene β and ε cyclases (LCYB and LCYE, respectively). By contrast, β-carotene is synthesized from lycopene when only β rings are formed by LCYB enzymes. Transcripts encoding LCYE were found at much lower levels than those encoding the two LCYB isoforms expressed in *Escobedia* roots, LCYBa and LCYBb (Figure 2), suggesting a higher flux through the β, β branch compared to the β, ε branch. In agreement with this conclusion, transcripts for the LUT1 enzyme, which catalyzes the hydroxylation of ε rings to produce lutein (the most abundant carotenoid in green photosynthetic tissues), are much less abundant than those for β ring hydroxylases (BCHa and BCHb). Based on the higher abundance of transcripts for BCHa, this is likely that the main isoform transforming β-carotene into zeaxanthin (Figure 2). Then, two highly expressed zeaxanthin epoxidase isoforms (ZEPa and

ZEPb) produce violaxanthin. The very low levels of transcripts found for violaxanthin epoxidase (VDE) suggest that the main metabolic flux is from zeaxanthin to violaxanthin in *Escobedia* roots. High levels of transcripts encoding NCED and other ABA biosynthetic enzymes such as ABA2, ABA3, AAO3 (Figure 2), further suggest that violaxanthin is readily converted into ABA in this tissue. A similar scenario was described in azafrin-producing *C. grandiflora* roots, with low levels of transcripts for LUT1 and VDE but higher expression of genes encoding enzymes of the β, β branch and the ABA biosynthesis pathway (Figure 2) (ZHANG et al., 2019).

5.4.3 Azafrin production in *Escobedia grandiflora* roots might rely on CCD4 rather than CCD7 enzymes.

Besides ABA, other apocarotenoids are formed by the activity of CCD enzymes. Among them, SL and azafrin production are proposed to share the first isomerization step from β-carotene (Figure 2). Transcripts encoding D27 were expressed in azafrin-accumulating roots, supporting the conclusion that β-carotene is actively isomerized to 9-*cis*-β-carotene in this tissue, hence supporting the production of strigolactones or/and azafrin (Figure 2). In *Centranthera grandiflora*, it was highly expressed in all tissues evaluated (Figure2) (ZHANG et al., 2019). Both apocarotenoids were also proposed to share the next step of the pathway, i.e. the cleavage of C_{40} 9-*cis*-β-carotene by CCD7 to produce C_{27} 10'-apo-β-carotenal (ZHANG et al., 2019). In-depth phylogenetic analysis of the CCD family from *Escobedia* and several other plants, including hemiparasitic species, showed five clades designated as CCD1 (paraphyletic), CCD4, root hemiparasitic CCD4-like (RHCCD4), CCD7, and CCD8 (Figure 4). The only gene encoding CCD1 was also found to be poorly expressed in roots (Figure 2). The RHCCD4 clade, which comprises only sequences of root hemiparasitic plants and it clusters separately of CCD4 with 92% bootstrap support, included the six CCD4-like sequences of *Escobedia* (CCD4a to f), including the two most expressed *Escobedia* CCD-encoding transcripts (CCD4b and CCD4c) (Figure 2). The *Escobedia* CCD4-like isoforms in the RHCCD4 clade were closely related to those from *C. grandiflora*, which also produces azafrin in the roots (Supporting Information Table. S4).

Figure 4. Phylogenetic analysis of the Carotenoid Cleavage Dioxygenase (CCD) family in several plants. The tree includes 202 sequences from nine angiosperms and two bacteria as outgroups. The analysis identified five distinctively separate clades as indicated. Asterisks indicate incomplete amino acid sequences of *Escobedia grandiflora*. Gene accessions are listed in Supplementary Table S3.



A striking conclusion of our phylogenetic analysis is that no sequences of *Escobedia* were present in the CCD7 clade, which included sequences of the other hemiparasitic species and typical CCD7 enzymes of *Arabidopsis* and tomato, among other species. We speculate that the RHCCD4 clade enzymes most highly expressed in *Escobedia* roots (CCD4b) might catalyse the same C9-C10 cleavage reaction that CCD7 enzymes perform in other plants or tissues. The CCD4 subfamily is probably the worst-characterized CCD enzymes. They are encoded by several genes in many species, resulting in isoforms that often differ in their expression profile and substrate selectivity (Hou et al., 2016). In general, CCD4 enzymes have broad substrate specificity, and many of them appear to have a role in carotenoid catabolism, particularly in carotenoid-sink tissues such as flowers, fruits, seeds, and roots (WALTER; FLOSS; STRACK,

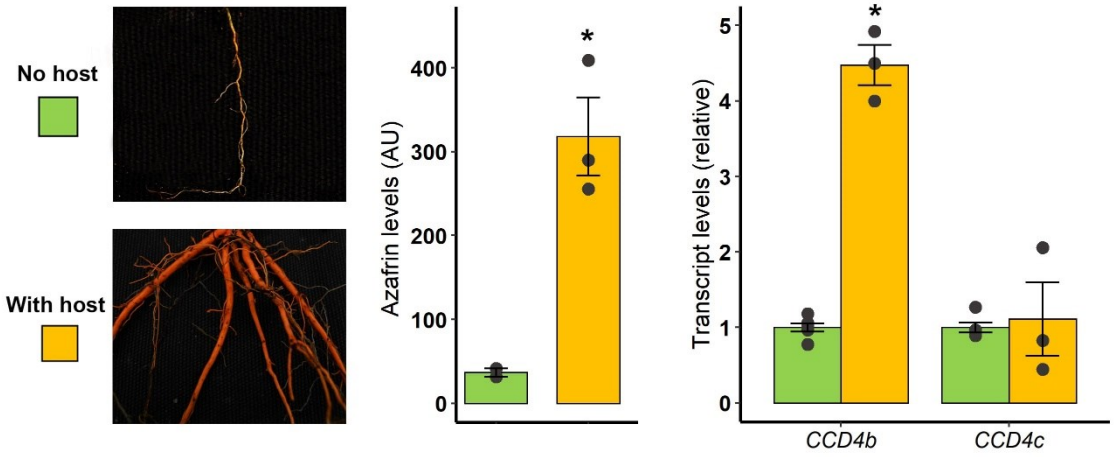
2010; HOU et al., 2016; MORENO et al., 2021). They usually cleave carotenoids (notably β-carotene) at the C9–C10/C9'-C-10' double bond, but the *Arabidopsis* CCD4 enzymes also catalyses the C9–C10 cleavage of β, β xanthophylls such as zeaxanthin while other CCD4 enzymes cleave asymmetrically at the C7–C8/C7'-C-8' double bond (RUBIO et al., 2008; HUANG; MOLNÁR; SCHWAB, 2009; MA et al., 2013; LÄTARI et al., 2015; BRUNO et al., 2016). Interestingly, *Arabidopsis* CCD4 has been shown to catalyse the cleavage of β-carotene to all-*trans*-10'-apo-β-carotenal and, at a much lower efficiency, of 9-*cis*-β-carotene to the SL precursor 9-*cis*-10'-apo-β-carotenal (BRUNO et al., 2016). It can thus be suggested that *Escobedia* CCD4b or/and CCD4c isoforms might also catalyse these reactions to deliver precursors for SL and azafrin biosynthesis, hence making the participation of a CCD7 enzyme unnecessary. Interestingly, transcripts encoding CCD7 were expressed at much lower levels than those encoding D27, and CCD4b was highly expressed than CCD4c in the azafrin-producing roots of *Centranthera* (Figure 2) (ZHANG et al., 2019), suggesting that CCD4 isoforms might also produce the precursors for SL and azafrin in this hemiparasitic plant.

C_{27} 10'-apo-β-carotenal is the substrate of CCD8 enzymes in the next step of the SL biosynthesis pathway (Figure 2). By contrast, *Escobedia* genes encoding homologs for CCD8 (CCD8a to c) were found to be expressed at much lower levels than the one for D27 in roots, being similar in *C. grandiflora* roots (Figure 2). Based on these data, we hypothesized that the pathway for producing strigolactones (via CCD8) is not very active in *Escobedia* roots. This might not be a general trend in parasitic plants. For example, the tubercle of the holoparasitic plant *Phelipanche aegyptiaca* (Pers.) Pomel showed a high expression of *D27*, *CCD7*, and *CCD8* when parasitizing the host roots (EMRAN et al., 2020). However, mycorrhizal plants that produce high levels of apocarotenoids reduce their production and secretion of SL, likely because abundant apocarotenoid production in AM-colonized roots might generate a metabolic sink and successfully compete for SL precursors (WALTER; FLOSS; STRACK, 2010). Similarly, the presumably low flux towards SL in azafrin-producing *Escobedia* and *C. grandiflora* might be related to the requirement of very high levels of common precursors to support the massive production of azafrin in the roots of these hemiparasitic plants. Strikingly, we were unable to detect any carotenoid species in *Escobedia* roots, suggesting that the carotenoid pathway in this organ is mainly directed to provide substrate for apocarotenoid production, and no intermediates are accumulated. Similarly, no detectable amounts of carotenoids were found in AM roots of all plants investigated despite the required high flux through the carotenoid pathway (FESTER et al., 2002).

5.4.4 Root CCD4b expression increases when azafrin production is activated by the parasitization of host plants.

To complement the RNAseq analysis, transcript levels of candidate genes encoding the RHCCD4 isoforms most abundant in *Escobedia* roots (CCD4b and CCD4c) were measured in roots of *Escobedia* plants grown either with or without hosts (Figure 5). Previous studies observed that the roots of *Escobedia* were colourless in the initial developmental stages (i.e., before parasitizing a host), while the orange pigment became most visible after haustoria penetration in the host root (CARDONA-MEDINA; SANTOS; NODARI, 2019). HPLC analyses confirmed that azafrin levels were substantially increased in *Escobedia* roots when growing in the presence of host plants compared to those grown in their absence (Figure 5). *Escobedia* showed poor root development in the absence of the host. Furthermore, the level of transcripts encoding CCD4c were similar regardless of the presence of a host, those encoding CCD4b was present at much higher levels in azafrin-producing roots (Figure 4): these results suggest that CCD4c might participate in the constitutive production of apocarotenoids whereas CCD4b would be up-regulated in conditions requiring an extra production of azafrin.

Figure 5. Azafrin and transcript levels of selected genes in roots of *Escobedia grandiflora* plants grown with (orange) and without a host (green). Arbitrary units (AU). Asterisks represent significant differences by Tukey test ($P<0.05$). Error bars represent standard errors (SE) of the means (n=3).



Confirming the specific biological function of these carotenoid-cleaving enzymes *in vivo* is very challenging, as the carotenoid substrates and physiological context that these

particular isoforms have in *Escobedia* roots (including subplastidial location, protein partners and cofactors) might be very different from those available in test systems such as *Escherichia coli* cells or *Nicotiana benthamiana* leaves (AHRAZEM et al., 2017; BRUNO et al., 2016; ZHENG et al., 2021). Furthermore, assessing the role of CCD4b in azafrin production would require demonstrating the transformation of their products into azafrin, which cannot be done until the specific genes and enzymes involved in this transformation are identified.

5.4.5 Azafrin accumulates in the apoplast of the *Escobedia grandiflora* root cortex.

An anatomical analysis was next performed to investigate where pigment formation and storage takes place in *Escobedia* roots. The internal structure of the root revealed that the abundant orange pigment likely corresponding to azafrin was not stored in plastids (the site where all plant carotenoids are made) or in the cell cytosol (where the synthesis of many apocarotenoids is completed) but accumulated in the intercellular spaces (i.e., apoplast) of the root cortex (Figure 6A). Confocal Laser Scanning Microscopy analyses based on the autofluorescence produced by the system of conjugated double bonds present in the polyene chain of carotenoids and monocyclic apocarotenoids of sufficient length such as C_{27} azafrin (D'ANDREA; AMENÓS; RODRÍGUEZ-CONCEPCIÓN, 2014) confirmed that the orange pigmentation detected by light microscopy was due to the presence of azafrin as both autofluorescence and color signals overlapped (Figure 6B; Figure 7). Light and confocal microscopy of carrot (*Daucus carota*) roots also showed an overlap of color and autofluorescence signals, but in this case, they were both detected inside plastids (i.e., chromoplasts) as they correspond to carotenes (β-carotene and, to a lower extent, α-carotene) instead of their cleavage products (Figure 7A-C). By contrast, azafrin was virtually absent from the large starch-filled plastids (i.e., amyloplasts) present in *Escobedia* roots (Figure 6; Figure 7D-F).

Figure 6. Distribution of azafrin in the root cortex apoplast of *Escobedia grandiflora*. (A) Micrographs under light microscopy show azafrin as an orange pigment in the intercellular spaces of the cortex (arrowheads). (B) Micrographs under confocal microscopy show azafrin as a green autofluorescence (arrowheads). Fluorescence images (right panels) are shown next to the corresponding merged micrographs of fluorescence and bright field images of the same field. Images show representative images of root longitudinal sections (upper panels) and cross-sections (central and lower panels). Abbreviations: SG= Starch grains. Scale bars: (Upper and central panels) = 50 µm, (lower panels) = 20 µm.

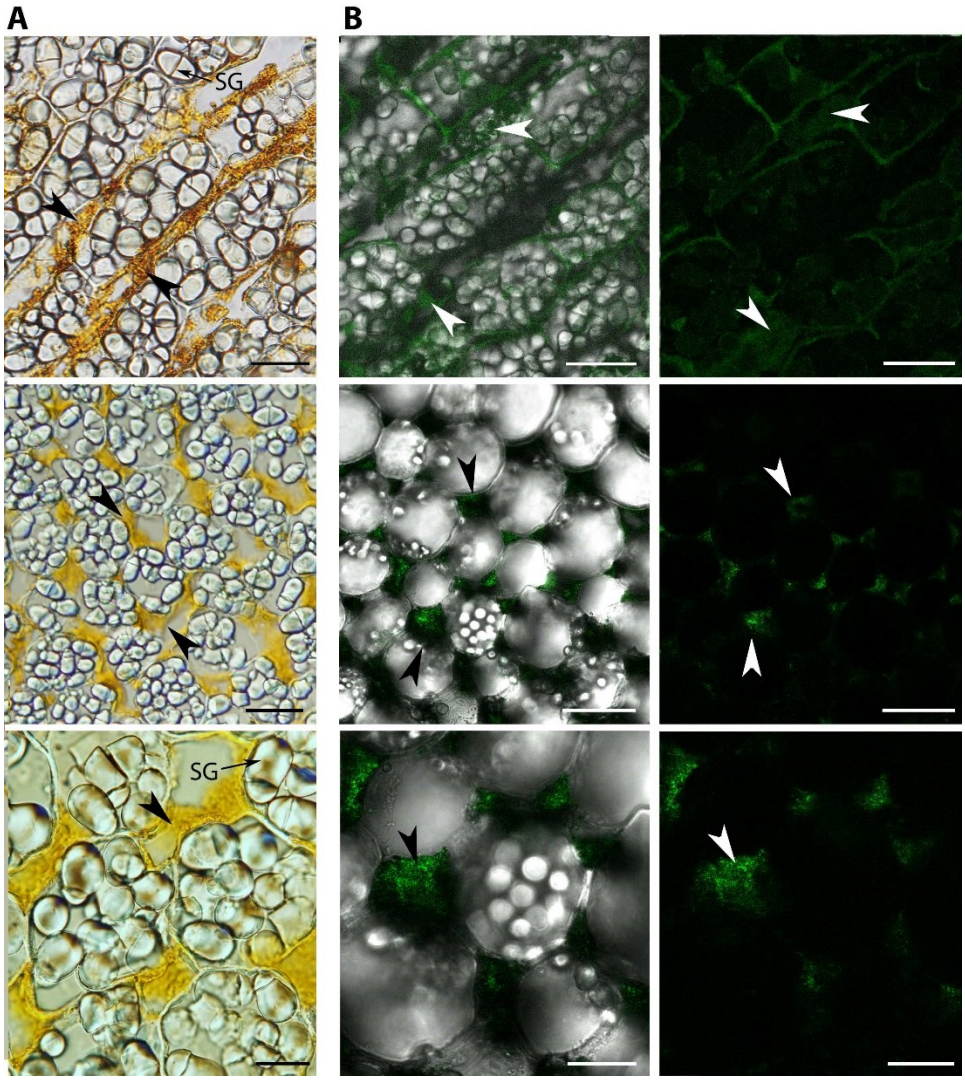
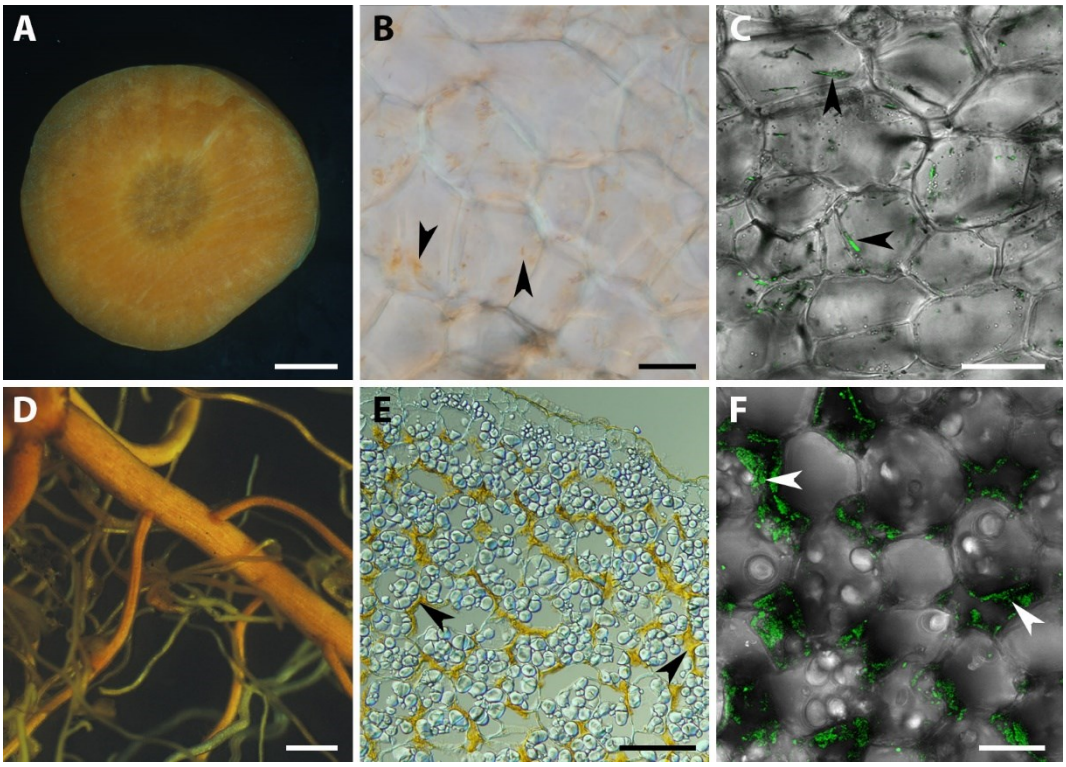


Figure 7. Carotenoid and carotenoid-derived distribution on carrot root (A-C) and *Escobedia grandiflora* root (D-F), respectively. Overview of carrot (A) and *E. grandiflora* roots (D). Micrograph under light microscopy (arrowheads) show a chromoplasts of carrot root cells (B) and an orange pigment in the intercellular spaces of *E. grandiflora* cortex (E). Micrographs emitted at 500-550 nm and excited with the 488 nm of an argon laser under confocal microscopy, show green autofluorescence corresponding to carotenoids in the chromoplasts (C), and carotenoid-derived azafrin in the intercellular spaces of cortex (F) (arrowheads). Scale bar: (A)=5 mm, (B, C, E)=50 µm, (D)= 1 mm, (F)=20 µm.



Carotenoids are synthesized and accumulated in different plastid types, including amyloplasts (HORNER et al., 2007; RODRIGUEZ-CONCEPCION et al., 2018; SUN et al., 2018). Studies of apocarotenoid formation in mycorrhizal roots have led to conclude that the C_{27} products of CCD4 or/and CCD7 activities are exported from the plastid and used in the cytosol as substrates of CCD1 enzymes that convert them into downstream products such as C_{13} (colorless) blumenols and C_{14} (yellow) mycorrhadicins (WALTER; FLOSS; STRACK, 2010b; FIORILLI et al., 2019; MORENO et al., 2021). Similarly, the C_{27} product of CCD4b activity in the amyloplasts of *Escobedia* roots might be exported from the plastids to the cytosol. Accumulation of C_{27} is uncommon in the nature, probably because CCD1 activity (FLOSS et al., 2008; WALTER; FLOSS; STRACK, 2010b). The very low expression level of the only gene encoding CCD1 in *Escobedia* roots (Figure 2) suggests that most of this C_{27} intermediate might remain available for other cytosolic enzymes to transform it into downstream products.

The differences between azafrin and 10'-apo-β-carotenal are one terminal carboxyl group and two hydroxyl groups in the cyclohexane skeleton (ZHANG et al., 2019). The activity of cytosolic aldehyde dehydrogenase and cytochrome P450 monooxygenase enzymes could transform the aldehyde group of 10'-apo-β-carotenal into carboxylic acid and insert oxygen atoms, respectively, eventually improving hydrophilicity (Figure 2). Water-soluble azafrin might then be released from the root cells and accumulate in the apoplast. Based on the putative role reported for the accumulation of colored apocarotenoids in AM-inoculated roots, it is possible that azafrin might participate in the interaction with the rhizosphere, e.g. by providing protection from oxidative damage caused by biotic or abiotic stresses (STRACK; FESTER, 2006). Because the root of *Escobedia* is colourless after seed emergence and the orange coloration caused by azafrin accumulation is most obvious upon parasitization of host plants (CARDONA-MEDINA; SANTOS; NODARI, 2019), it is likely that azafrin also can modulates one or several aspects of the parasitism process.

5.4.6 High levels of azafrin in the interface between *Escobedia grandiflora* haustorium and the host root suggest a role for this apocarotenoid in the parasitization process.

Following germination, *Escobedia* seedlings grow very slowly until their roots find a host to parasitize, which involves the formation of specialized organs (haustoria) to penetrate the host root (CARDONA; MURIEL, 2015; CARDONA-MEDINA; SANTOS; NODARI, 2019). The haustorium is an organ characteristic of parasitic plants that have evolved in multiple independent angiosperms. It contains structures for mechanical attachment to the host root and vascular tissue connections that involve the differentiation of various specialized cell types (TEIXEIRA-COSTA, 2021). A detailed exploration of the *Escobedia* haustorium attached to host roots revealed a complex internal structure, presenting four recognizable regions (Figure 8): haustorial base, vascular tissue, hyaline body, and intrusive cells (endophyte). The haustorial base connects the parasitic root with the haustorium, morphologically similar to root tissue. The vascular tissue comprised by provascular cells and tracheary elements (haustorium xylem) is arranged perpendicularly to the haustorial base and towards the host root xylem. Intrusive cells of the haustorium penetrate and advance inside the host root, constituting the endophyte. Tracheary elements of the haustorium were observed inside the host's metaxylem, indicating parasitism success in the host root (Figure 8). This study noticed that every haustorium attached to host roots contained orange pigment depositions in the region directly contacting the host

root interface (Figure 9A-C). Confocal analyses confirmed that this orange pigment corresponds to carotenoid-derived (Figure 9D) equal to found in the root apoplast (Figure 6).

Figure 8. Overview of the *Escobedia grandiflora* haustorium parasitizing *Pennisetum purpureum* roots. An illustration showing a longitudinal section of the mature haustorium attaching to the host root (scale= 100 μm), Toluidine blue-stained sections are also shown. The haustorium base (depicted in blue) connects with the host root through the xylem bridge of the haustorium (depicted in purple), consisting of provascular tissue and tracheary elements that grow towards the host root. The hyaline body (depicted in red) shows cells with dense cytoplasm and starch grains (black arrows), Golgi vesicles (narrow arrow) (scale= 2 μm), and large nuclei (white arrow). The endophyte is formed by intrusive cells from the haustorium embedded within the host body (depicted in green). Detailed haustorium xylem inside the host's metaxylem (asterisk) indicates parasitism success in the host root with disruption of host tissues. The localization of the azafrin pigment in the apoplast space and in the interface between the haustorium and the host root is depicted in orange. Scale bars=50 μm. Abbreviations: AZ = Azafrin; HB = Hyaline body; HR = Host root; PV = Provascular tissue; PH = Parasite haustorium; RX = Root xylem; TE = Tracheary elements

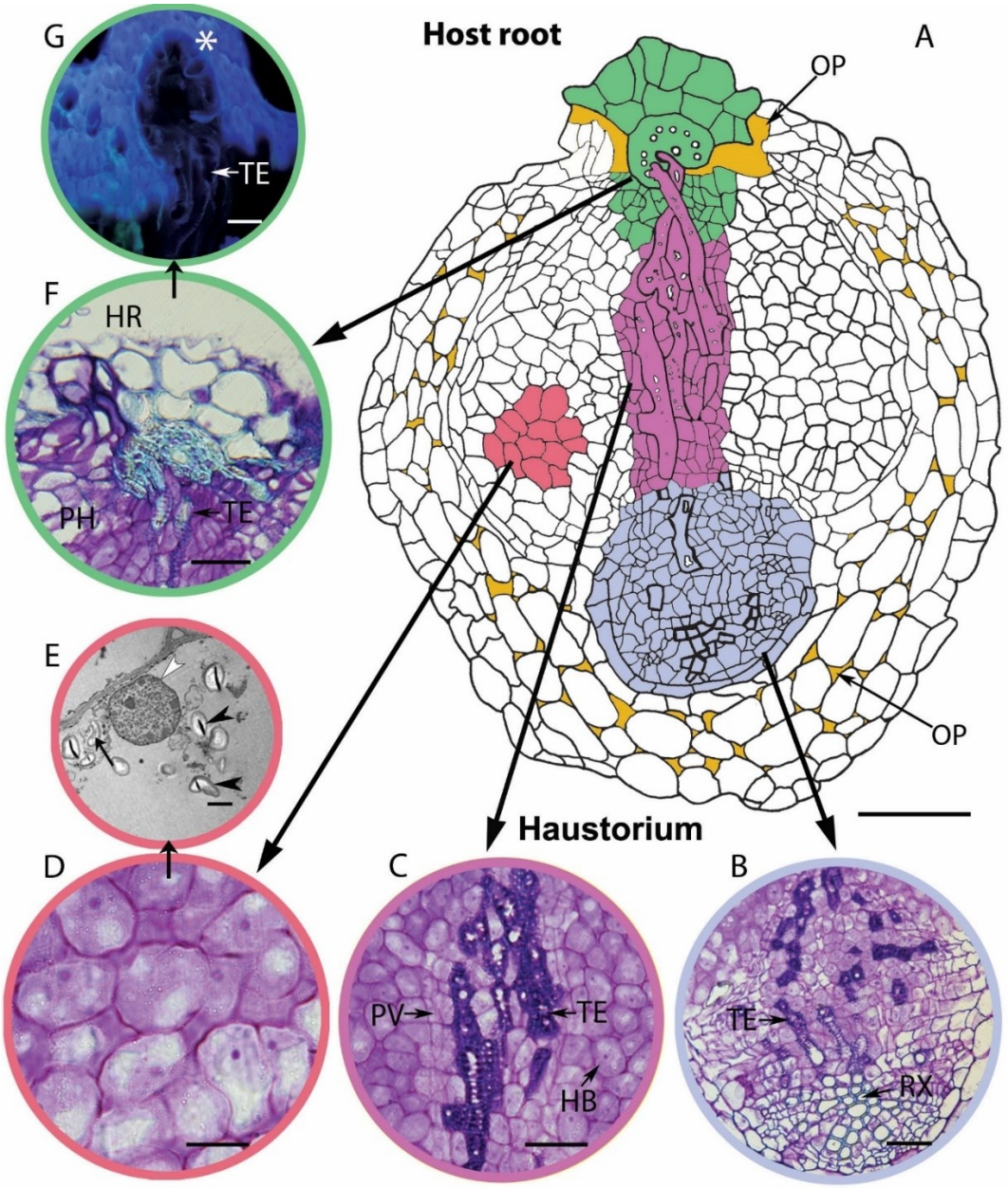
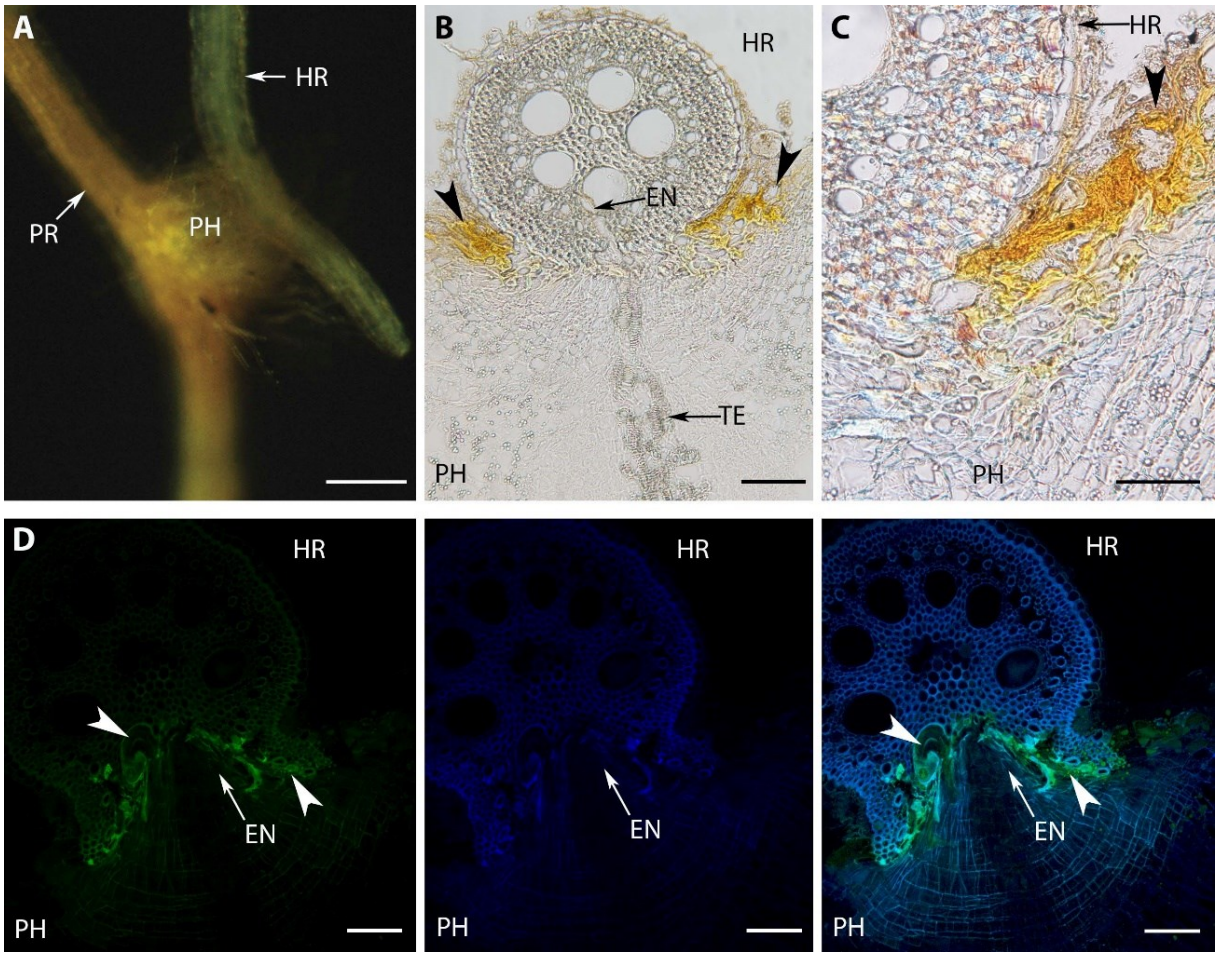

G
TE
F
HR
PH
TE
E
D
Host root
A
OP
OP
Haustorium
C
PV
TE
HB
B
TE
RX

Figure 9. *Escobedia grandiflora* haustorium attached to a host root (*Pennisetum purpureum*). (A) External view of haustorium attached to host root. (B) Representative light microscopy picture of the haustorium-host root interface showing an accumulation of the orange pigment azafrin (arrowhead). (C) Detail of azafrin pigment accumulation in the haustorium-host root interface (arrowhead). (D) Representative confocal microscopy images showing the localization of azafrin fluorescence in the haustorium-host root interface. From left to right: autofluorescence emitted at 500-550 nm and excited with the 488 nm of an argon laser, corresponding to azafrin (arrowhead); blue autofluorescence emitted at 409-482 nm and excited with the 405 nm of an argon laser, corresponding to cell walls; and merged green and blue autofluorescence. Abbreviations: HR= Host root; EN= Endophyte; PH= Haustorium, PR= Parasite root; TE= Tracheary elements. Scale bars: (A) = 200 µm, (B, D) = 100 µm, (C)=50 µm.



The reason why azafrin accumulates in the haustorium-host root interface is still unknown. We propose that azafrin might inhibit the host defence responses during the penetration of the haustorium inside the roots. Haustorium penetration in host roots involves enzymatic secretion and mechanical pressure (by haustorial hairs and cellular division) that

degrade and disrupt the host cells walls (HEIDE-JORGENSEN; KUIJT, 1995; HOOD et al., 1998; LOSNER-GOSHEN et al., 1998). This process causes a wound in the host root that allows the entry of haustorium intrusive cells into the host vascular system. Host roots quickly respond to the wound by activating the production of reactive oxygen species (ROS), which can activate programmed cell death (PCD) and trigger plant defence responses (MINIBAYEVA et al., 2009; TRIPATHY; OELMÜLLER, 2012), including the generation of phenolic compounds and callose deposition, induction of immunity-related genes, and deposition of lignin and suberin to avoid the advance of parasitization (HIRAGA et al., 2001; MINIBAYEVA et al., 2009; SAUCET; SHIRASU, 2016). Evidence of necrosis involving ROS was found in resistant hosts during unsuccessful penetration by the haustorium of *Orobanche cumana* Wallr. (LETOUSEY et al., 2007). Thus, the haustorium might inhibit host PCD and defense responses by eliminating extracellular ROS as a strategy to facilitate parasitization (MOR; MAYER; LEVINE, 2008). Inactivation of defence responses has also been observed in biotrophic pathogens during the parasitism of host plants, due to its need to proliferate in living host cells (SIDDIQUE et al., 2014). Likewise, endophytic fungi can produce antioxidants to circumvent damage by ROS during beneficial interaction with the host plant (HAMILTON et al., 2012). We speculate that azafrin accumulation in the haustorium-host root interface might also play an antioxidant role to counteract the ROS-related host defence responses, hence allowing the parasitism to success.

Biotrophic pathogens can also suppress plant defence responses by inducing the production of ABA, an apocarotenoid hormone with an antagonistic interaction with defence-activating hormones such as salicylic acid (DE TORRES-ZABALA et al., 2007; CAO; YOSHIOKA; DESVEAUX, 2011; MOEDER et al., 2010; SIVAKUMARAN et al., 2016). Seedlings of *Monochasma savatieri* Franch. ex Maxim. (Orobanchaceae) a increment of ABA levels after parasitization of host roots than without host (CHEN et al., 2021). Similarly, high production and exudation of ABA was observed in the seedlings of the hemiparasite *Striga hermonthica* (Delile) Benth., suggesting that ABA could increase the susceptibility of the host to parasitism (FUJIOKA et al., 2019). Interestingly, genes related to ABA biosynthesis were highly expressed in *Escobedia* roots (Figure 2) but also in the roots of the hemiparasite *C. grandiflora* (Figure 2) (ZHANG et al., 2019). It is therefore possible that ABA production in roots of hemiparasitic plants might contribute to inhibit the host response during haustorium penetration, similar to that proposed for azafrin. Further work should experimentally address

this and other unanswered questions about the role of azafrin and other apocarotenoids in the parasitization strategy of *Escobedia*. The information would be vital improving the cultivation and hence exploitation of this important medicinal plant.

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5.5 SUPPLEMENTARY MATERIAL

Table S1. Primers used for RT-qPCR

Name	Sequence
Actin-F	5′TGCAGCTGATAGGTGGAGTG3′
Actin-R	5′GGCTTCATGAGAAGGAATGG3′
CCD4b-F	5′CGGAGGATGAGGGTTATCTG3′
CCD4b-R	5′GATCCGGCATCCATGACTAC3′
CCD4c-F	5′ATGAGGCGTCGGTTGTCTAC3′
CCD4c-R	5′GTGACCATTGATGGGGCTAC3′

Table S2. Code, identification and FPKM values used in the proposed pathway for azafrin biosynthesis in *Escobedia*. Expression values were log_2 transformed (FPKM+1).

Gene code	Gene ID	Log_2 (FPKM+1)
PSYa	DN945_c0_g1_i1	5,758
PSYb	DN945_c0_g1_i4	6,265
PDS	DN2843_c1_g2_i1	4,473
Z_ISO	DN3052_c2_g1_i3	5,677
ZDSa	DN1140_c0_g1_i9	5,885
ZDSb	DN1140_c0_g1_i2	3,588
CrtISO	DN3485_c0_g1_i5	2,214
LCYBa	DN13078_c0_g2_i1	6,850
LCYBb	DN13078_c0_g3_i1	3,968
LCYE	DN15003_c0_g1_i1	0,607
BCHa	DN12727_c0_g1_i3	2,693
BCHb	DN26818_c0_g1_i2	1,410
LUT1	DN8641_c0_g1_i7	1,829
ZEPa	DN3699_c0_g1_i1	6,189
ZEPb	DN3699_c0_g1_i2	4,302
VDE	DN27435_c0_g1_i2	1,839
D27	DN2368_c0_g1_i1	2,993
CCD1	DN12527_c0_g1_i4	1,817
CCD4a	DN11277_c0_g1_i1	1,128
CCD4b	DN5855_c0_g1_i1	4,649
CCD4c	DN2213_c1_g4_i1	4,266
CCD4d	DN24736_c0_g1_i1	1,217
CCD4e	DN32036_c0_g1_i1	0,936
CCD4f	DN9499_c0_g1_i1	1,148
CCD4g	DN9499_c0_g1_i2	0,806
CCD4h	DN11481_c0_g1_i6	1,579
CCD8a	DN57235_c0_g1_i1	0,923
CCD8b	DN2370_c0_g1_i4	0,754

CCD8c	DN61814_c0_g1_i1	0,401
NCEDa	DN22079_c1_g1_i1	4,774
NCEDb	DN71_c1_g1_i2	5,539
ABA2	DN1245_c0_g2_i1	4,554
ABA3	DN3600_c0_g1_i2	3,007
AAO3a	DN2103_c0_g1_i7	3,232
AAO3b	DN7225_c0_g2_i2	3,246

Table S3. Gene code and IDs of amino acid sequences of PSY and CCDs used in phylogenetical analysis. Asterisks symbols indicate incomplete amino acid sequences of *Escobedia*.

Gene type	**Species**	**Gene code**	**Gene ID**
PSY	*Arabidopsis thaliana*	PSY *Arabdopsis*	At5g17230
PSY	*Centranthera grandiflora*	PSY *Centranthera*	Cluster-13824.83791
PSY	*Crocus sativus*	PSY *Crocus*	QCI61139.1_Cs
PSY	*Daucus carota*	PSY1 *Daucus*	DCAR_010057
PSY	*Daucus carota*	PSY2 *Daucus*	DCAR_023043
PSY	*Daucus carota*	PSY3 *Daucus*	DCAR_024333
PSY	*Deinococcus sp.*	PSY *Deinococcus*	WP_162177599.1
PSY	*Escobedia grandiflora*	PSYa *Escobedia*	Eg_DN945_c0_g1_i1
PSY	*Escobedia grandiflora*	PSYb *Escobedia*	Eg_DN945_c0_g1_i4
PSY	*Medicago truncatula*	PSY1 *Medicago*	Mtr5g076620
PSY	*Medicago truncatula*	PSY2a *Medicago*	Mtr3g450510
PSY	*Medicago truncatula*	PSY2b *Medicago*	Mtr5g090780
PSY	*Medicago truncatula*	PSY3 *Medicago*	Mtr3g083630
PSY	*Oryza sativa*	PSY1 *Oryza*	LOC_Os06g51290
PSY	*Oryza sativa*	PSY2 *Oryza*	LOC_Os12g43130
PSY	*Oryza sativa*	PSY3 *Oryza*	LOC_Os09g38320
PSY	*Phtheirospermum japonicum*	PSY *Phtheirospermum*	GFQ03426.1
PSY	*Solanum lycopersicum*	PSY1 *Solanum*	Solyc03g031860.2
PSY	*Solanum lycopersicum*	PSY2 *Solanum*	Solyc02g081330.2
PSY	*Solanum lycopersicum*	SlPSY3 *Solanum*	Solyc01g005940.2
PSY	*Striga asiatica*	PSYa *Striga*	GER47037.1_Striga
PSY	*Striga asiatica*	PSYb *Striga*	GER27235.1_Striga
PSY	*Synechocystis sp.*	PSY *Synechocystis*	WP_193386659.1
PSY	*Zea mays*	PSY1 *Zea*	NP_001108124.2_Zm

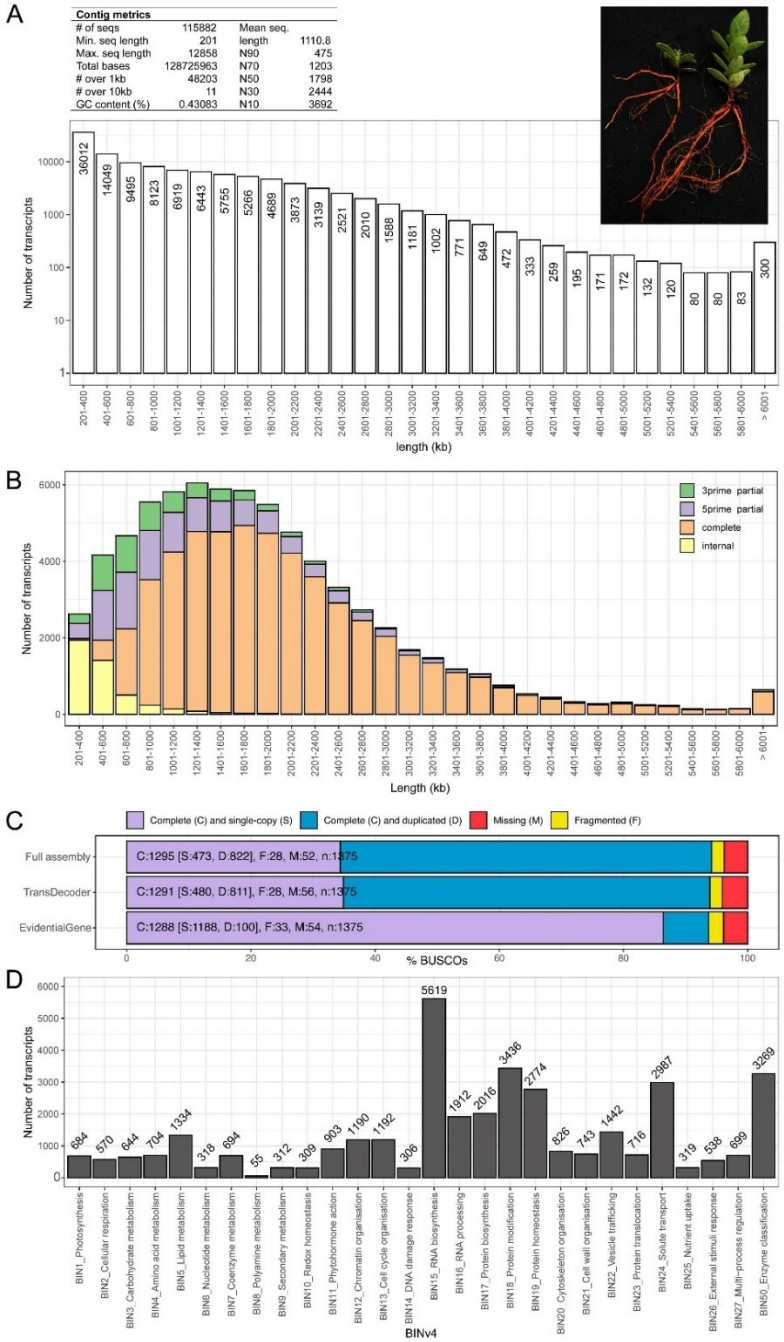
PSY	*Zea mays*	PSY2 *Zea*	NP_001108117.1_Zm
PSY	*Zea mays*	PSY3 *Zea*	ACG30201.1_Zm
CCD	*Arabidopsis thaliana*	CCD1 *Arabidopsis*	AT3G63520
CCD	*Arabidopsis thaliana*	CCD4 *Arabidopsis*	AT4G19170
CCD	*Arabidopsis thaliana*	CCD7 *Arabidopsis*	AT2G44990
CCD	*Arabidopsis thaliana*	CCD8 *Arabidopsis*	AT4G32810
CCD	*Centranthera grandiflora*	CCD4.1 *Centranthera*	Cluster_13824.41915
CCD	*Centranthera grandiflora*	CCD4.2 *Centranthera*	Cluster_13824.6803
CCD	*Centranthera grandiflora*	CCD4.3 *Centranthera*	Cluster_13824.133194
CCD	*Centranthera grandiflora*	CCD4.4 *Centranthera*	Cluster_13824.943
CCD	*Centranthera grandiflora*	CCD4.5 *Centranthera*	Cluster_13824.134694
CCD	*Centranthera grandiflora*	CCD4.6 *Centranthera*	Cluster_13824.63869
CCD	*Centranthera grandiflora*	CCD7 *Centranthera*	Cluster_13824.3681
CCD	*Centranthera grandiflora*	CCD8 *Centranthera*	Cluster-2227.0
CCD	*Crocus sativus*	CCD2 *Crocus*	AIG94929.1
CCD	*Crocus sativus*	CCD4a *Crocus*	ACD62476.1
CCD	*Crocus sativus*	CCD4b *Crocus*	ACD62477.1
CCD	*Crocus sativus*	CCD7 *Crocus*	AIF27228.1
CCD	*Crocus sativus*	CCD8a *Crocus*	AIF27229.1
CCD	*Crocus sativus*	CCD8b *Crocus*	AIF27230.1
CCD	*Escobedia grandiflora*	CCD1 *Escobedia*	Eg_DN12527_c0_g1_i4
CCD	*Escobedia grandiflora*	CCD4a *Escobedia*	Eg_DN11277_c0_g1_i1
CCD	*Escobedia grandiflora*	CCD4b *Escobedia*	Eg_DN5855_c0_g1_i1
CCD	*Escobedia grandiflora*	CCD4c *Escobedia*	Eg_DN2213_c1_g4_i1
CCD	*Escobedia grandiflora*	CCD4d *Escobedia*	Eg_DN24736_c0_g1_i1
CCD	*Escobedia grandiflora*	CCD4e *Escobedia*	Eg_DN32036_c0_g1_i1
CCD	*Escobedia grandiflora*	CCD4f *Escobedia*	Eg_DN9499_c0_g1_i1*
CCD	*Escobedia grandiflora*	CCD4g *Escobedia*	Eg_DN9499_c0_g1_i2*
CCD	*Escobedia grandiflora*	CCD4h *Escobedia*	Eg_DN11481_c0_g1_i6
CCD	*Escobedia grandiflora*	CCD8a *Escobedia*	Eg_DN57235_c0_g1_i1*
CCD	*Escobedia grandiflora*	CCD8b *Escobedia*	Eg_DN2370_c0_g1_i4*
CCD	*Escobedia grandiflora*	CD8c *Escobedia*	Eg_DN61814_c0_g1*
CCD	*Mycobacterium tuberculosis*	CCO *Mycobacterium*	CEJ50676.1
CCD	*Nostoc sp.*	CCD *Nostoc* sp.	RUR87155.1
CCD	*Phtheirospermum japonicum*	CCD4.1 *Phtheirospermum*	GFP80922.1
CCD	*Phtheirospermum japonicum*	CCD4.1 *Phtheirospermum*	GFP81524.1

CCD	*Phtheirospermum japonicum*	CCD4.2 *Phtheirospermum*	GFP79372.1
CCD	*Phtheirospermum japonicum*	CCD4.3 *Phtheirospermum*	GFP79363.1
CCD	*Phtheirospermum japonicum*	CCD4.4 *Phtheirospermum*	GFP79368.1
CCD	*Phtheirospermum japonicum*	CCD4.5 *Phtheirospermum*	GFP90552.1
CCD	*Phtheirospermum japonicum*	CCD8 *Phtheirospermum*	GFQ02826.1
CCD	*Solanum lycopersicum*	CCD1a *Solanum*	Solyc01g087250.2
CCD	*Solanum lycopersicum*	CCD1b *Solanum*	Solyc01g087260.2
CCD	*Solanum lycopersicum*	CCD4a *Solanum*	Solyc08g075480.2
CCD	*Solanum lycopersicum*	CCD4b *Solanum*	Solyc08g075490.2
CCD	*Solanum lycopersicum*	CCD7 *Solanum*	Solyc01g090660.2
CCD	*Solanum lycopersicum*	CDD8 *Solanum*	Solyc08g066650.2
CCD	*Striga asiatica*	CCD1 *Striga*	GER27697.1
CCD	*Striga asiatica*	CCD4.1 *Striga*	GER56223.1
CCD	*Striga asiatica*	CCD4.2 *Striga*	GER26752.1
CCD	*Striga asiatica*	CCD4.3 *Striga*	GER48996.1
CCD	*Striga asiatica*	CCD4.4 *Striga*	GER30315.1
CCD	*Striga asiatica*	CCD7 *Striga*	GER49360.1

Table S4. Percent identity matrix (PIM) of CCD4 and CCD4-like amino acid sequences between *Escobedia* and *Centranthera* (blue). PIM shows that sequences of *Escobedia* CCD4-like are related to those from *Centranthera grandiflora. Asterisks represent* partial sequences.

	Amino acid sequences	**Percent identity matrix**													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	CCD4g_*Escobedia**	100	20,61	20,34	18,11	19,69	28,91	28,35	25,2	21,88	24,81	27,91	20,93	20,31	18,75
2	CCD4.3_*Centranthera*		100	88,38	51,64	50,93	46,01	46,35	51,29	51,26	51,41	49,23	51,99	49,77	51,61
3	CCD4d_*Escobedia*			100	49,63	48,7	45,26	44,5	51,6	53,78	49,72	48,29	48,54	47,64	52,8
4	CCD4.5_*Centranthera*				100	89,66	51,8	51,08	54,57	54,17	51,64	50,34	52,18	51,82	53,96
5	CCD4c_*Escobedia*					100	52,16	51,08	54,33	51,67	50,55	50	52,55	51,82	54,25
6	CCD4.1_*Centranthera*						100	86,68	53,74	56,2	52,83	57,53	52,18	54,09	56,52
7	CCD4h_*Escobedia*							100	53,97	60,33	53,94	56,16	52,92	53,97	58,14
8	CCD4.4_*Centranthera*								100	52,1	55,16	58,38	52,34	52,57	52,63
9	CCD4f_*Escobedia**									100	82,79	-nan	51,24	51,24	51,24
10	CCD4.2_*Centranthera*										100	86,44	52,57	52,47	56,3
11	CCD4e_*Escobedia*											100	56,51	55,14	58,72
12	CCD4b_*Escobedia*												100	72,05	74,93
13	CCD4.6_*Centranthera*													100	87,9
14	CCD4a_*Escobedia*														100

Figure S1. *de novo* transcriptome assembly from roots of *Escobedia grandiflora:* (A) Summary statistics of the final assembly and length distribution frequency of transcripts; (B) length distribution frequency of transcripts containing both start and stop codons; (C) BUSCO assessments with the embryophyte lineage database; (D) Functional annotation of the reference transcriptome with MapMan BIN v4 categories.



6 CAPÍTULO 3-ONTHOGENETIC CHANGES IN HOST PREFERENCE OF ROOT HEMIPARASITE *Escobedia grandiflora*: FROM FAIRLY SPECIALIST TO GENERALIST

6.1 ABSTRACT

Escobedia grandiflora (L.f) Kuntze (hereafter referred to as *Escobedia*) is a perennial root hemiparasite that occurs in dry and wet non-forested ecosystems from central and South America. Their orange-colored roots have medicinal and cooking dye purposes. However, very little is currently known about the identity of parasitized species by the root hemiparasite *Escobedia*. The present study aimed to determine the host range of *Escobedia* mature plants and evaluate the host preference during early growth. First, we examine haustoria attachments of *Escobedia* in four natural populations in southern Brazil and confirm the vascular tissue connection with host root through microscopical analyses. Additionally, we performed a pot experiment to understand whether, during early growth, the development of *Escobedia* is affected by its consortium with nine different host species. We found that *Escobedia* haustoria effectively connected the xylem of 38 host root species with different taxa and root morphologies. Most of the hosts belong to graminoids and herbs. Furthermore, during early growth *Escobedia* displayed high development with *Eryngium elegans* (Apiaceae) and *Evolvulus glomeratus* (Convolvulaceae) host. These results confirm that mature *Escobedia* from natural communities are generalist, parasitizing a broad host range. However, *Escobedia* showed preference with specific hosts during the early growth because their development is only possible with two hosts. The findings reported here shed new light on the host preferences of *Escobedia* and further implications for the *Escobedia* populations in the plant communities.

Key words: haustorium, host-parasite interaction, host range, host specificity, juvenile plants, Orobanchaceae, seedlings.

6.2 INTRODUCTION

Parasitic plants are a fascinating group that is characterized to obtain part (hemiparasite) or all (holoparasite) the resources from adjacent plants (shoot or root) by a typical structure known as haustorium. Holoparasites are non-green and dependent on carbon

from other plants known as hosts, whereas hemiparasites present photosynthetic activity, and are characterized by obtaining only water and inorganic nutrients from hosts (WESTWOOD et al., 2010). The haustorium appears to be the crucial organ that allows parasitism through vascular tissue connection with the host, providing an effective biological bridge to the interchange of material (MASUMOTO et al., 2021). In root hemiparasites from Orobanchaceae family, the haustorium's internal structure comprises five distinctive cell types: parenchyma cells, hyaline body, procambium-like cells, tracheary elements, and intrusive cells (MASUMOTO et al., 2021; see chapter 2). Intrusive cells invade the host tissue, and some haustorium cells are differentiated in tracheary elements from procambium-like cells to connect with the host xylem (MASUMOTO et al., 2021).

Root hemiparasites have a broad host range, attaching to the roots of several species, sometimes simultaneously (GIBSON; WATKINSON, 1989; PRESS; PHOENIX, 2005; CAMERON; PHOENIX, 2013), and obtaining different resources from each host species (GOVIER; NELSON; PATE, 1967). Host range can be limited by the ability of a parasite to recognize a potential host, develop haustoria, and penetrate on their root (PRESS; PHOENIX, 2005; FURUTA et al., 2021). Host recognition requires host-derived signalling molecules that stimulate the germination of parasites and the haustorium-inducing factors (HIF) that triggers the haustoria formation (FURUTA et al., 2021; MUTUKU et al., 2021). The potential host can avoid the haustorium invasion stimulating varied defence mechanisms to activate a hypersensitive response, release cytotoxic compounds and establish physical barriers (lignin and suberin deposition and cells wall thickening) (CAMERON; COATS; SEEL, 2006; CLARKE et al., 2019). These mechanisms may cause fragmentation and damage of haustorium tissue, resulting in an incompatible interaction (CAMERON; COATS; SEEL, 2006; THOROGOOD; HISCOCK, 2010). Hence, the amount of a given hosts is another factor in determining host range in parasites, whereas the existence of abundant hosts increases the probability of contact with parasite haustoria (MARVIER; SMITH, 1997; NORTON; CARPENTER, 1998). Parasites may thus operate as specialists choosing hosts with specific attributes that improve their performance (PRESS; PHOENIX, 2005). Host attributes are associated with shoot, and root architecture, growth rate and nitrogen content (TĚŠITEL et al., 2011; MATTHIES, 2017).

The interaction among both organisms can benefit the development and reproduction of parasites but may damage the host (CAMERON et al., 2005; PRESS; PHOENIX, 2005). These interactions are represented in the root parasites of the Orobanchaceae family, occurring

in a broad range of habitats worldwide (PHOENIX; PRESS, 2005; MUTUKU et al., 2021). Members as *Striga*, *Pheliphanche*, and *Orobanche*, are well-known for their harmful effect on crops (TĚŠITEL et al., 2021). Other species, such as the annual herb *Rhinanthus*, play an essential role in modifying the diversity and structure of plant communities being used as a restoration tool in grasslands (TĚŠITEL et al., 2017; HEER et al., 2018). In addition, several species have been widely used for humans by their medicinal and alimentary properties, such as *Centranthera grandiflora* Wall. ex Benth., *Cistanche deserticola* Ma, and *Escobedia grandiflora* (L. f) Kuntze (MURIEL et al., 2015; ZHANG et al., 2019; TĚŠITEL et al., 2021).

Escobedia grandiflora (Hereafter referred to as *Escobedia* avoiding confusion with host species) is a perennial hemiparasitic herb with orange-colored roots used for medicinal and cooking dye purposes (MURIEL et al., 2015). The main compound in Escobedia roots is the apocarotenoid Azafrin that is accumulated in the intercellular spaces of the root cortex and the haustorium (see chapter 2). *Escobedia* occurs in dry and wet non-forested ecosystems from central and South America, where it was found as a species positively associated with high diversity of plants in the communities, being that the functional group graminoids was the most frequent in the *Escobedia* presence (see chapter 1). However, much uncertainty still exists about the identity of parasitized species by *Escobedia*, regardless of their importance (MURIEL et al., 2015, see chapters 1 and 2) and as being categorized as endangered species (VELLEDA, 2016). Conservation and use of parasitic plants thus involve understanding host range and the main hosts that support parasite growth (MARVIER; SMITH, 1997).

A previous study based on haustoria attachments suggested that *Escobedia* is a generalist hemiparasite, where twenty-two species of potential hosts were recorded for natural populations plants in the Andean region (CARDONA; MURIEL, 2017). However, this approach is not enough to confirm the host range because vascular tissue connections were not corroborated (THOROGOOD; HISCOCK, 2010; SUETSUGU et al., 2012). Therefore, confirmation of the compatible interactions is necessary to determine the host species. Conversely, host preference could be more restricted in the seedling stage of root hemiparasitic plants. After germination, hemiparasites seedlings depend on their resources to develop roots as quickly as possible to recognize and form haustorial attachments to suitable parasitize host roots (TĚŠITEL et al., 2011, 2013; MUTUKU et al., 2021). However, if the host is not suitable, the seedling cannot survive without the host due to inefficient resource uptake and higher sensitivity of seedlings to light competition with the co-occurring species (TĚŠITEL et al.,

2011). In this way, hosts might promote the early growth of hemiparasite seedlings differently. Only *Pennisetum purpureum* Schumach. and *Paspalum notatum* Flüggé are recorded as hosts in seedlings, although these species do not belong to the natural communities where *Escobedia* occurs. The early growth of *Escobedia* begins with the emergence of roots from the seeds until the development of haustoria and true leaves; this stage appears to be the most critical in their life-cycle (CARDONA-MEDINA; SANTOS; NODARI, 2019). The seeds of *Escobedia* have insufficient reserves, but they germinate without chemical host signs and have a high germination percentage, yet the seedlings showed high mortality rates and slow development with *P. purpureum* and *P. notatum* (CARDONA; MURIEL, 2015; CARDONA-MEDINA; SANTOS; NODARI, 2019). In line with this, much remains unclear about the hosts from natural communities that promote the development of *Escobedia* during its early growth.

In this sense, the objectives of this study were to determine the host range for mature *Escobedia* and evaluate the host preference for *Escobedia* during early growth. To achieve the objectives, (1) we examined the haustoria attachments of co-occurring plants from natural communities, (2) performed microscopical analyses to determine the host range in natural populations based on the xylem/xylem connection between the haustoria and the potential host roots and (3) evaluated host preference to understand whether a specific host may influence the development of juvenile plants of *Escobedia*.

6.3 MATERIAL AND METHODS

6.3.1 Examination of haustoria attachments

To determine the host range, we firstly examined haustoria attachments of *Escobedia* in natural populations. Microscopical analyses of haustoria were performed to confirm whether a vascular tissue connection existed between the haustoria and the potential hosts roots. Thus, haustoria attachments were examined on different co-occurring plant species in four non-forested areas localized in southern Brazil, from December 2018 to March 2019, during the flowering period of *Escobedia*. These areas were selected by the presence of flowering plants of *Escobedia* according to the characterized populations in chapter 1: The first (27°46'53.0"S, 48°29'16.1"W, 190 m.a.s.l.) and second populations (27°46'05.9"S, 48°28'46.2"W, 27 m.a.s.l.) were localized in the Lagoinha do Leste Municipal Park (LLMP) Florianópolis, Santa Catarina (SC) state. The third population (26°36'56.9"S, 51°29'51.0"W, 1235 m.a.s.l.) in Água Doce municipality (SC), and the last (28°53'06.0"S, 50°27'52.9"W, 927 m.a.s.l.) in Jaquirana municipality, state of Rio Grande do Sul (RS). In each area, individuals of *Escobedia* were

delimited by 50 x 50 cm quadrats to examine the haustoria attachments (see chapter 1). The soil was removed from the different co-occurring species in each quadrat, and their roots verified for the presence of orange-coloured haustoria (GIBSON; WATKINSON, 1989; CARDONA; MURIEL, 2017). Haustoria attached to potential host roots were collected and fixed in microtubes with 2.5% paraformaldehyde in 0.1 M sodium phosphate buffer and dehydrated by ethanol series (RUZIN, 1999) for further microscopical analyses of haustorium connections. For each potential host, at least three haustoria samples were collected. Potential host species were classified into five functional groups based on the morphology and habit patterns in the four assessed communities (see chapter 1): graminoids (Poaceae and Cyperaceae), rosettes, herbs (including subshrubs, legumes, and non-graminoids and non-rosette herbs), shrubs, and pteridophytes. Finally, potential host species were collected for taxonomic assessment, as described in chapter 1.

6.3.2 Microscopical confirmation

Haustoria attachments were analyzed under light and fluorescence microscopy to confirm the successful xylem/xylem connection with the potential host roots. At least three haustoria attached to each potential host root were evaluated, considering successful connection when at least one haustorium connected their xylem in the host root xylem. To light microscopy, fixed and dehydrated samples were processed using the same method previously reported by (CARDONA-MEDINA; SANTOS; NODARI, 2019). Briefly, they were infiltrated and embedded with hydroxyethyl methacrylate (Leica, Heidelberg, Germany) (GERRITS; SMID, 1983), and 5 μm thicks sections were cut using a RM 2125RT rotary microtome (Leica®, Nussloch, Germany) and stained with toluidine blue (O'BRIEN; FEDER; MCCULLY, 1964). A BX-40 microscope was used to visualize the sections, and a DP71 digital camera to capture images (Olympus, Tokyo, Japan). To fluorescence microscopy, fixed samples were incubated in sucrose solution series (10-40%) and embedded in a tissue-freezing medium (Fisher, Houston, USA), and frozen up to -19°C. Cross-sections of 50 μm in thickness were cut in a cryostat Thermo Scientific HM525 NX (Walldorf, Germany) to visualize xylem connection using an inverted fluorescence microscope (IX81, Olympus, Tokyo, Japan; emission wavelength: 330-385 nm). Images were taken with a DP71 digital camera (Olympus, Tokyo, Japan).

6.3.3 Host preference during early growth

To evaluate the host preference during early growth, we performed a co-cultivation pot experiment to understand whether, during early growth (90 days), the development of *Escobedia* is affected by its consortium with nine different host species: three species of the graminoid group *Bulbostylis capillaris* (L.) /(Poaceae); four species of herbs *Acmella bellidioides* (Sm.) R.K. Jansen (Asteraceae), *Epidendrum fulgens* Brongn. (Orchidaceae), *Evolvulus glomeratus* Nees & C. Mart. (Convolvulaceae), *Lucilia linearifolia* Baker (Asteraceae); one species of pteridophyte *Anemia* cf. *tomentosa* (Anemiaceae) and one species of rosette *Eryngium elegans* Cham. & Schltdl. (Apiaceae). These species were selected based on the results of host range determination for mature plants described above and to represent different functional groups of plants. It is important to note that the haustoria successfully connected in seven of these species, but not in two of them (*I. minus* and *A. tomentosa*). The plant material used to install the experiment was collected in the first and second populations above described. For the hemiparasite and *E. fulgens* we used seeds, obtained from pre-deiscent fruits. For the remaining eight species, rhizomes or other vegetative structures were used. Plants of *E. fulgens* were obtained by *in vitro* asymbiotic seed germination, according to the method of VOGES et al. (2014).

Host rhizomes were planted in 0.5 L pots and cultivated for 30 days in a greenhouse before of the introduction of *Escobedia* seedlings, in order to ensure host establishment and root development (CARDONA-MEDINA; SANTOS; NODARI, 2019). *Escobedia* seeds were imbibed in water for five days, and later, twenty *Escobedia* seeds were sown in each pot (CARDONA-MEDINA; SANTOS; NODARI, 2019), with 5 cm of distance from the host species (MATTHIES, 2017). *Escobedia* was grown in a randomized design with each of the nine host species and individually (no-host) to evaluate the host preference during the early growth. The design consisted of ten treatments with seven repetitions for each treatment. After 90 days, we measured the number of leaves. Afterward, *Escobedia* was removed from the pots and counted of haustorium number connected with the host root. Aboveground and below parts of *Escobedia* were frozen at -80 °C and lyophilized to measure dry weight. The fixed haustoria attachments were stored in microtubes and fixed in 2.5% paraformaldehyde in 0.1 M sodium phosphate buffer. Fixed samples were also cleared with sodium hypochlorite 2.5% to confirm and count the number of xylem/xylem connection in a BX-40 microscope. In addition, fixed and dehydrated samples were also infiltrated and embedded, following the methodology

mentioned above. Finally, *Escobedia* plants were frozen at -80 and lyophilized to determine the dry weight.

6.3.4 Statistical analyses

To evaluate the host preference for *Escobedia* during early growth in different host species, we performed a one-way ANOVA, in which the host species were the explanatory variable, and the growth parameters were the response variables (dry weight, leaf number, haustorium number, and number of xylem/xylem connections). Dry weight and leaf number data were log-transformed, and haustorium number and xylem connection data were square-root transformed. To confirm the ANOVA assumptions, Shapiro-Wilk test and graphical analyses of the residuals were performed. Tukey's test was used to detect the statistically significant differences between treatments ($p \leq 0.05$). In addition, the Pearson correlation was used to estimate the correlation coefficient between the haustorium number and the dry weight, leaf number, and xylem connection of *Escobedia*. All statistical analysis was performed in R environment v4.0.3 (R core team, 2020).

6.4 RESULTS

6.4.1 Host range for mature *Escobedia grandiflora* plants

Examination of haustoria presence in roots of species naturally occurring in four plant communities showed that *Escobedia* haustoria were attached to the roots of 43 potential hosts from twelve botanic families (Table 1). However, it is important to note that this number is possibly underestimated because some haustoria adhered to host roots were delicate and may have been lost during collection.

Table 1. The host range for mature plants of *Escobedia grandiflora* occurring in four plant communities of Southern Brazil. The success of parasitism was determined by examining the haustoria attached to the potential host root and the microscopical confirmation of xylem/xylem connection. Signs corresponds to successful (+) and unsuccessful (-) xylem/xylem connection.

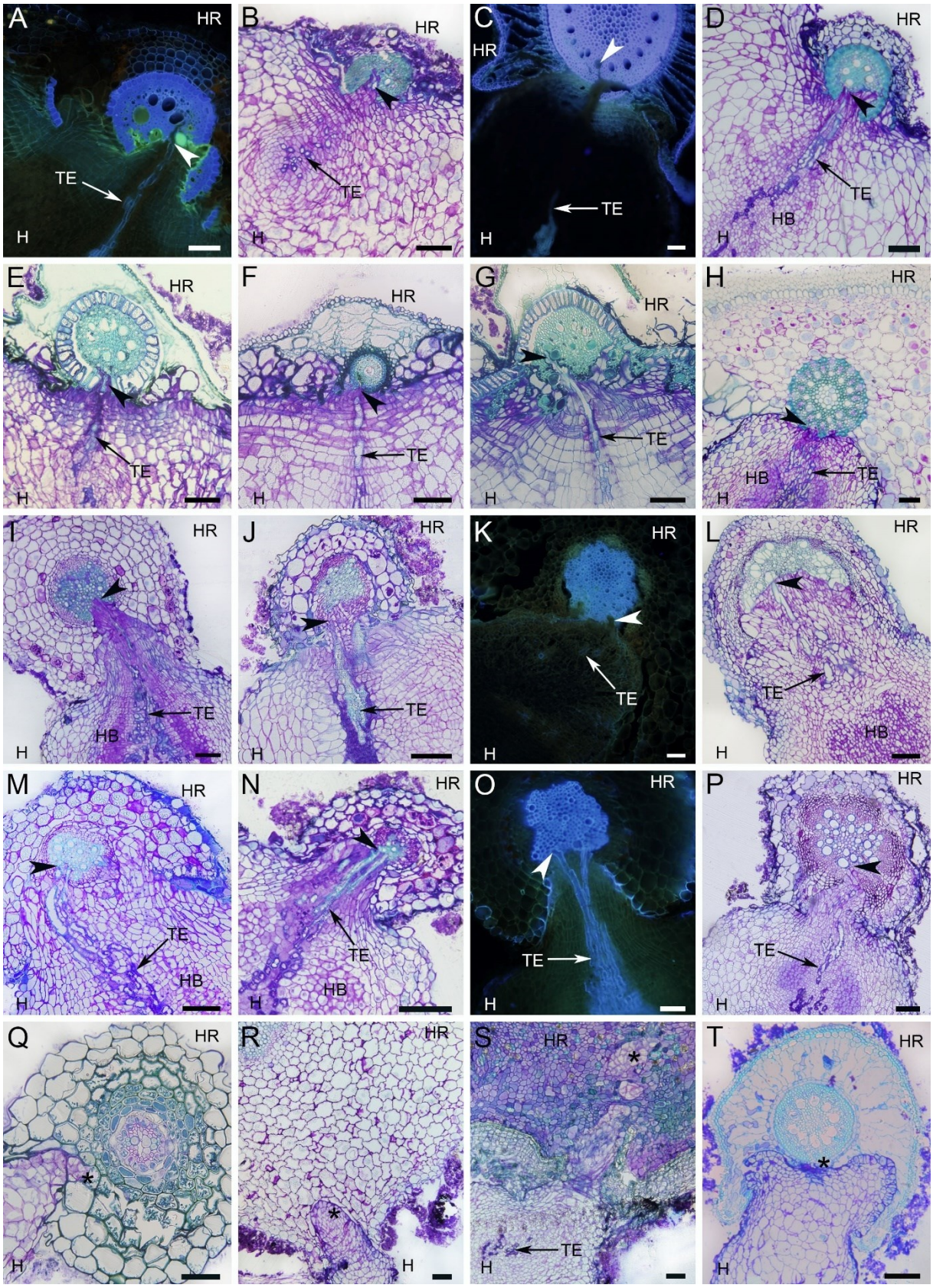
Species	**Family**	**Functional group**	**Xylem/xylem connection**
Bulbostylis capillaris (L.) Kunth ex C.B. Clarke	Cyperaceae	Graminoids	+
Bulbostylis sp.	Cyperaceae	Graminoids	+
Rhynchospora barrosiana Guagl.	Cyperaceae	Graminoids	+
Scleria hirtella (Sw.)	Cyperaceae	Graminoids	+

Scleria sellowiana Kunth	Cyperaceae	Graminoids	+
Undetermined	Cyperaceae	Graminoids	+
Andropogon lateralis Nees	Poaceae	Graminoids	+
Andropogon selloanus (Hack.) Hack.	Poaceae	Graminoids	+
Andropogon sp.	Poaceae	Graminoids	+
Anthaenantia lanata (Kunth) Benth.	Poaceae	Graminoids	+
Aristida sp.	Poaceae	Graminoids	+
Axonopus siccus (Nees) Kuhlm.	Poaceae	Graminoids	+
Cortaderia selloana (Schult. & Schult. f.) Asch. & Graebn.	Poaceae	Graminoids	+
Dichanthelium sabulorum (Lam.) Gould & C.A. Clark	Poaceae	Graminoids	+
Eriochrysis cayennensis P. Beauv.	Poaceae	Graminoids	+
Ischaemum minus J. Presl	Poaceae	Graminoids	-
Paspalum cf. *ramboi I*	Poaceae	Graminoids	+
Paspalum polyphyllum Nees ex Trin.	Poaceae	Graminoids	+
Paspalum glaucescens Hack.	Poaceae	Graminoids	+
Schizachyrium sp.	Poaceae	Graminoids	+
Undetermined	Poaceae	Graminoids	+
Undetermined	Poaceae	Graminoids	+
Undetermined	Poaceae	Graminoids	+
Acmella bellidioides (Sm.) R.K. Jansen	Asteraceae	Herbs	+
Chrysolaena flexuosa (Sims) H. Rob.	Asteraceae	Herbs	+
Lucilia linearifolia Baker	Asteraceae	Herbs	+
Praxelis sanctopaulensis (B.L. Rob.) R.M. King & H. Rob.	Asteraceae	Herbs	+
Evolvulus glomeratus Nees & C. Mart	Convolvulaceae	Herbs	+
Aeschynomene sp.	Fabaceae	Herbs	+
Rhynchosia diversifolia Micheli	Fabaceae	Herbs	+
Undetermined	Myrtaceae	Herbs	+
Epidendrum fulgens brongn.	Orchidaceae	Herbs	+
Blechnum serrulatum Rich.	Blechnaceae	Pteridophytes	+
Anemia cf. tomentosa (Savigny) Sw.	Schizaeaceae	Pteridophytes	-
Eryngium elegans Cham. & Schltdl.	Apiaceae	Rosettes	+
Eryngium sp.	Apiaceae	Rosettes	-
Chaptalia runcinata Kunth	Asteraceae	Rosettes	+
Dyckia encholirioides (Gaudich.) Mez	Bromeliaceae	Rosettes	+
Baccharis cf. *myriocephala* DC.	Asteraceae	Shrubs	+
Baccharis pentaptera DC.	Asteraceae	Shrubs	+
Baccharis subdentata DC.	Asteraceae	Shrubs	-
Calliandra tweediei Benth.	Fabaceae	Shrubs	+
Tibouchina urvilleana (DC.) Cogn.	Melastomataceae	Shrubs	-

Haustoria attachments were abundant in the roots of graminoids such as *Paspalum glaucescens*, *Bulbostylis capillaris*, *Axonopus siccus*, *Andropogon selloanus*, and *Schizachyrium* sp. These species are quite abundant in the evaluated communities. To determine the host range of *Escobedia*, analysis of haustorial connections revealed that 88% of the

haustoria effectively connected to the host root xylem, forming xylem bridges (compatible interaction) (Table 1, Figure 1). Therefore, *Escobedia* haustoria successfully parasitized the roots of 38 species, most of which belong to graminoids (22 species), followed by herbs (nine species), shrubs (three species), rosettes (two species), and pteridophytes (one species) (Table 1). In this way, *Escobedia* is considered a generalist hemiparasitic plant, and it can parasitize not only hosts from different taxa but also different root morphologies (Figure 1). For instance, the root of the terrestrial orchid *Epidendrum fulgens* (Figure 1H), with a multiestratified epidermis (velamen), the wall thickened endodermis of some Poaceae and Cyperaceae species (Figure 1A-G), and host species with secondary root growth (Figure 1I-M, P). In successful connections, no damage or fragmented cells were observed in the haustorium. Although haustoria with successful connections exhibited similar patterns, differences in the hyaline body's development were noticed. The hyaline body showed most development in haustoria attached to herbs, shrubs, and rosettes (Fig 1H-P), while it was poorly developed in haustoria attached to graminoids roots (Figure 1A-G). In unsuccessful connections, host penetration was observed, but the intrusive cells of haustoria only reached the cortex of potential hosts' roots (Figure 1Q-T). In the case of *Ischaemum minus*, the haustorium began to gain entry into their root cortex. Moreover, hyaline bodies were not verified in any of the haustoria with the unsuccessful connection.

Figure 1. Haustorial connections between *Escobedia grandiflora* and different root species. a-p: Successful connections of haustorium xylem with the host root xylem (arrowhead). q-t: Unsuccessful connection in which the haustorium does not reach the host root's vascular tissue (asterisk). (A) *Paspalum glaucescens* (Poaceae), (B) *Andropogon selloanus* (Poaceae), (C) *Schizachyrium* sp. (Poaceae), (D) *Paspalum polyphyllum* (Poaceae), (E) *Bulbostylis capillaris* (Cyperaceae), (F) *Bulbostylis* sp. (Cyperaceae), (G) *Rhynchospora barrosiana* (Cyperaceae), (H) *Epidendrum fulgens* (Orchidaceae), (I) *Praxelis sanctopaulensis* (Asteraceae), (J) *Lucilia linearifolia* (Asteraceae), (K) *Acmella bellidioides* (Asteraceae), (L) *Baccharis* cf. *myriocephala* (Asteraceae), (M) *Evolvulus glomeratus* (Asteraceae), (N) *Chaptalia runcinata* (Asteraceae), (O) *Aeschynomene* sp. (Fabaceae), (P) *Eryngium elegans* (Apiaceae), (Q) *Anemia* cf. *tomentosa* (Anemiaceae), (R) *Baccharis subdentata* (Asteraceae), (S) *Tibouchina urvilleana* (Melastomataceae), (T) *Ischaemum minus* (Poaceae). Scale=100µm. Abbreviations: H= haustorium; HR= Host root; TE= Tracheary elements.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6.4.2 Host preference during the early growth of *Escobedia grandiflora*

Despite the broad host range in mature plants, *Escobedia* seedlings showed preference with restricted hosts (Figure 2). *Escobedia* displayed the most remarkable development in consortium with *E. elegans* and *E. glomeratus* (Figure 2F, I), and much less with *A. tomentosa*, *B. cappillaris*, *I. minus*, *P. glaucescens*, *A. bellidioides*, and without a host (Figure 2A-E, G, H, J). In the case of *Escobedia* grown in consortium with *E. fulgens*, only one individual showed considerable development (Figure 2G). At least one *Escobedia* individual survived in consortium with each one of the nine host species. The most striking visual observation from these results is that root pigmentation was more visible in the roots of developed *Escobedia* individuals in consortium with *B. cappillaris*, *E. elegans*, *E. glomeratus*, and *A. bellidioides* (Figure 2F, I).

Figure 2. Early growth of *Escobedia grandiflora* (arrowhead) in consortium with nine host species for 90 days in pots, showed better development with specific hosts: (A) No host; (B) *Anemia* cf. *tomentosa*; (C) *Bulbostylis capillaris*; (D) *Ischaemum minus*; (E) *Paspalum glaucescens*; (F) *Eryngium elegans*; (G) *Epidendrum fulgens*; (H) *Lucilia linearifolia*; (I) *Evolvulus glomeratus*; (J) *Acmella bellidioides*. Scale=5 mm.

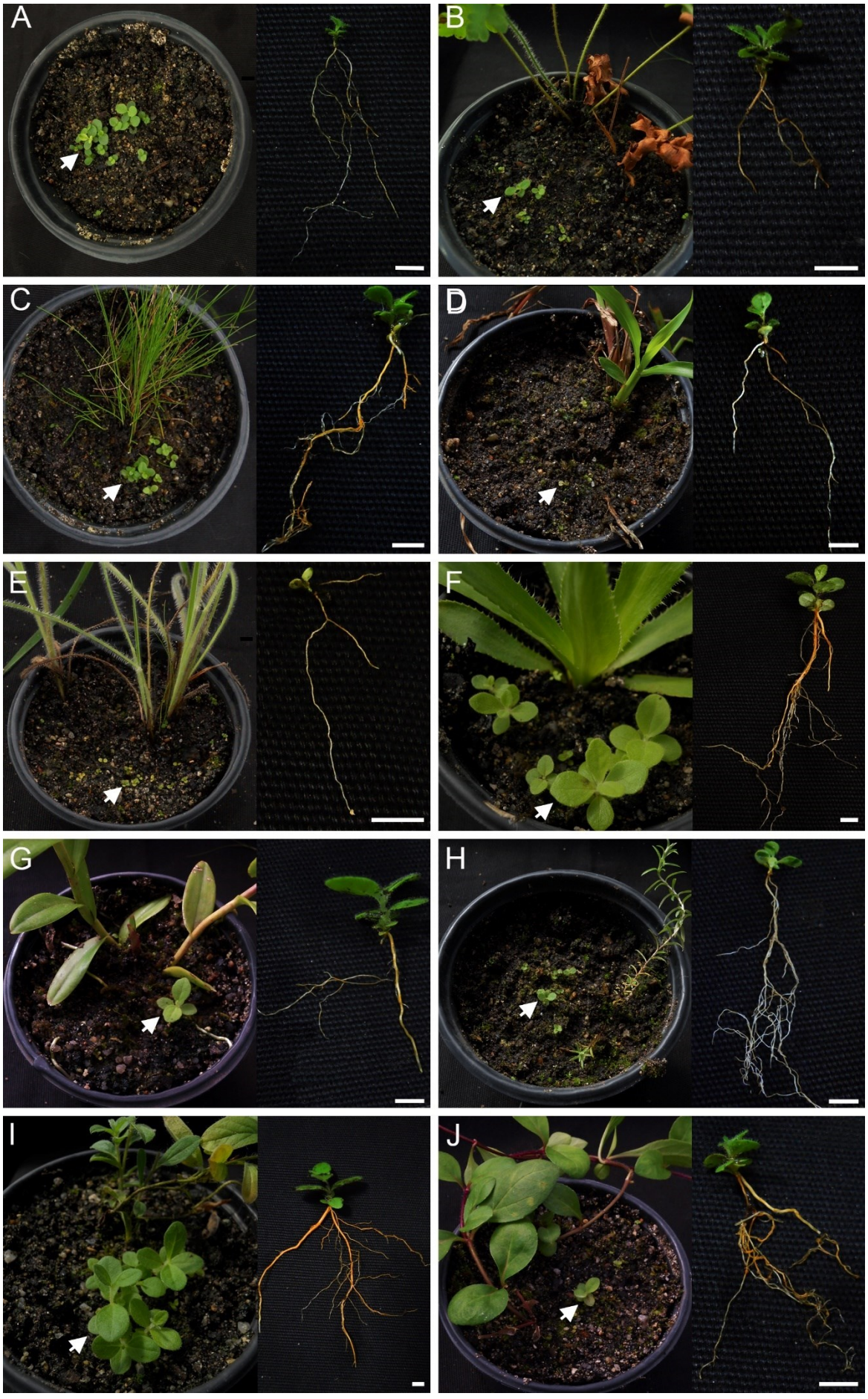
A
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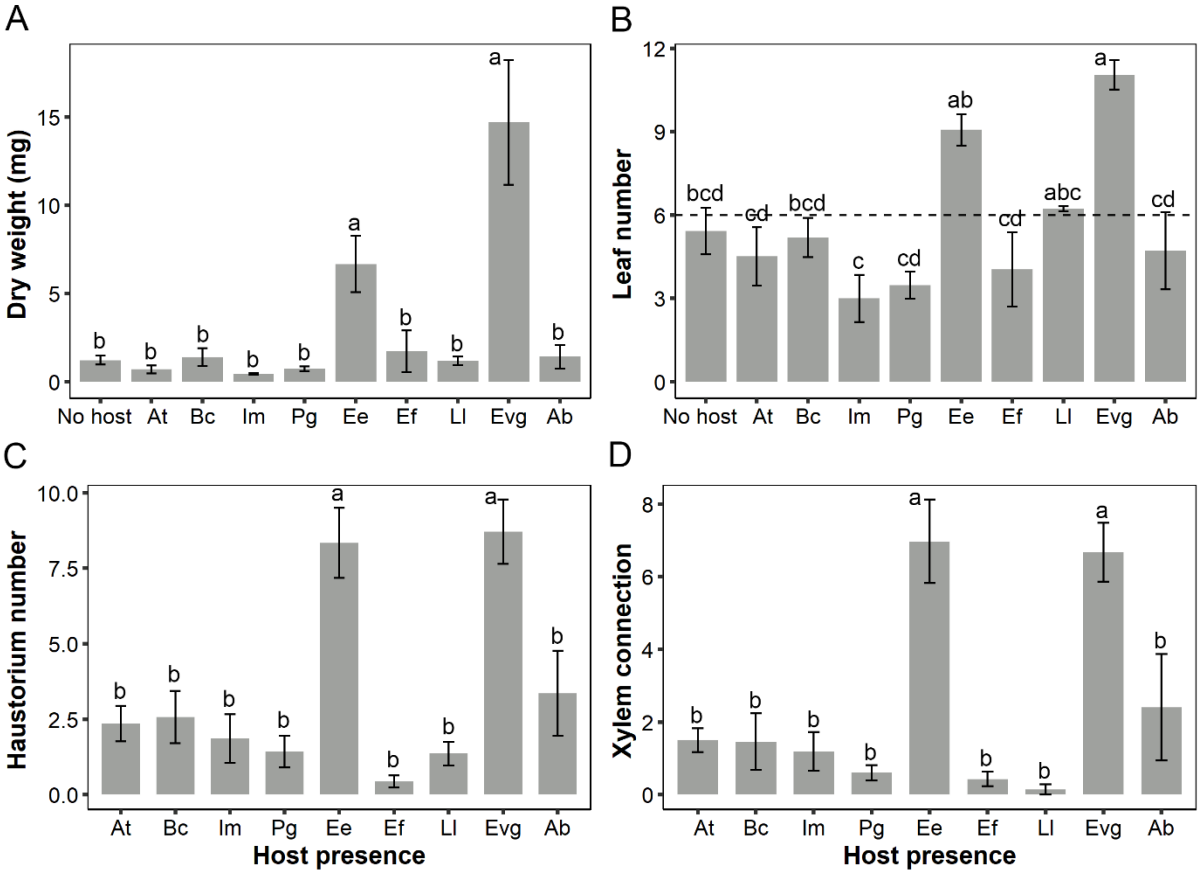
Table 2. Dry weight, leaf number, haustorium number and xylem-xylem connection of *Escobedia grandiflora* during the initial development on nine host species showed better development with specific hosts. Means of seven repetitions followed by standard errors (SE).

Functional group	**Host species**	**Family**	**Dry weight (mg)**	**Leaf number**	**Haustorium number**	**Xylem connection**
-	No host	-	1,2(±0.2)	5,4 (±0.8)	-	-
Graminoids	*Bulbostylis capillaris*	Cyperaceae	1,4(±0.5)	5,2(±0.7)	2,6(±0.9)	1,5(±0.8)
Graminoids	*Ischaemum minus*	Poaceae	0,5(±0.04)	3,0(±0.8)	1,9(±0.8)	1,2(±0.5)
Graminoids	*Paspalum glaucescens*	Poaceae	0,8(±0.1)	3,5(±0.5)	1,4(±0.5)	0,6(±0.2)
Herbs	*Acmella bellidioides*	Asteraceae	1,4(±0.7)	4,7(±1.4)	3,4(±1.4)	2,4(±1.4)
Herbs	*Epidendrum fulgens*	Orchidaceae	1,7(±1.2)	4,0(±1.3)	0,4(±0.2)	0,4(±0.2)
Herbs	*Evolvulus glomeratus*	Convolvulaceae	14,7(±3.5)	11,0(±0.5)	8,7(±1)	6,7(±0.8)
Herbs	*Lucilia linearifolia*	Asteraceae	1,2(±0.2)	6,2(±0.1)	1,4(±0.4)	0,1(±0.1)
Pteridophyte	*Anemia* cf. *tomentosa*	Schizaeaceae	0,7(±0.2)	4,5(±1)	2,4(±0.4)	1,5(±0.3)
Rosettes	*Eryngium elegans*	Apiaceae	6,7(±1.6)	9,1(±0.6)	8,3(±1.2)	7,0(±1.1)

The growth parameters of *Escobedia* increased considerably only in consortium with two hosts (*E. glomeratus* and *E. elegans*) (Table 2, Figure 3, supplementary Figure S1). The host preference was particularly evident in *Escobedia* biomass ($F=35.43$, $p<0.001$) (Table 2). The biomass of *Escobedia* was 13-fold times larger in consortium with *E. glomeratus* and 6-fold in consortium with *E. elegans* than those with the other hosts (Figure 3A). Furthermore, the leaf number also varied with *E. glomeratus* and *E. elegans* ($F=13.39$, $p<0.001$); however, their effect was smaller than the measured through biomass (Table 2, Figure 3B). *Escobedia* only developed eophylls in consortium with *A. tomentosa, I. minus,* and *P. glaucescens.* The haustorium number incremented notably on roots in consortium with *E. glomeratus* and *E. elegans*, being similar among them (Table 2, Figure 3C,). *Escobedia* haustorium developed xylem connection at least in one root of each host evaluated (Table 2, supplementary Figure S2). Hence, the haustoria of juvenile *Escobedia* were connected on the *A. tomentosa* and *I. minus* roots (supplementary Figure S2A), regardless of the unsuccessful connection found in mature plants of *Escobedia* (Figure 1Q, T). However, xylem connections were higher only in the roots of *E. glomeratus* and *E. elegans* (Table 2, Figure 3D); consequently, *Escobedia* greatly parasitized those host roots. Despite the striking similarity in haustorium number and xylem

connection of the two best host species, *E. glomeratus* accumulated two times more biomass than *E. elegans*.

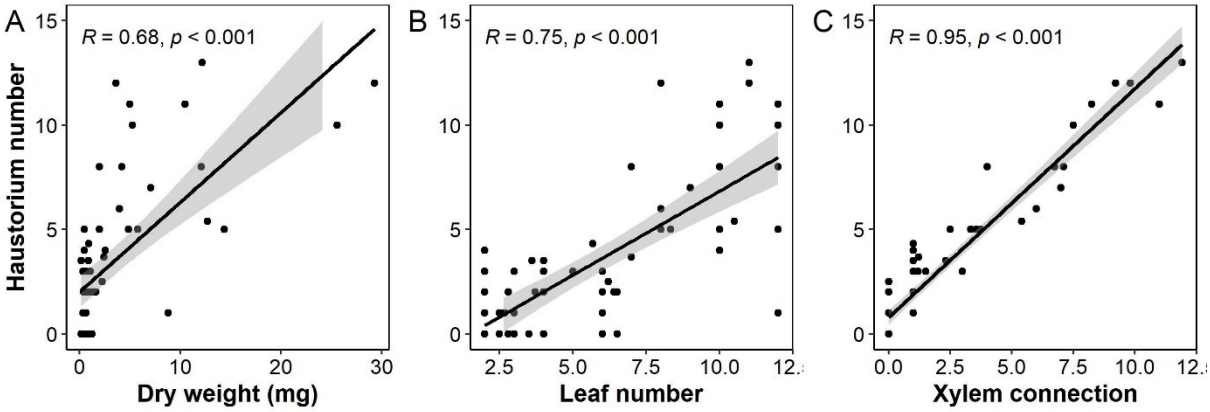
Figure 3. Early growth of *Escobedia grandiflora* associated with nine host species, showed better development with specific hosts (A-D): **At**, Anemia cf. tomentosa; **Bc**, Bulbostylis capillaris; **Im**, Ischaemum minus; **Pg**, Paspalum glaucescens; **Ee**, Eryngium elegans; **Ef**, Epidendrum fulgens; **Ll**, Lucilia linearifolia; **Evg**, Evolvulus glomeratus; **Ab**, Acmella bellidioides. The dashed reference line in "B" indicates the development of true leaves. Haustorium number and leaf number variables included only Escobedia-host data. Different letters above error bars represent significant differences by Tukey test ($P<0.05$). Error bars represent standard errors (SE) of the means (n=7).



Correlation analysis showed that the number of haustoria is associated with the biomass and the leaf number (Figure 4A-B). Therefore, higher haustoria attachments on the host's root promote biomass gain and increase the leaf number of *Escobedia*. Furthermore, the increment in the haustoria number strongly increases the haustorium connection in the host root xylem

(parasitism) (Figure 4C). The *Escobedia* individuals that presented a low number of haustoria showed reduced growth. Only several successful connections in the host xylem allowed the differential growth of *Escobedia*, as occurred in consortium with *E. glomeratus* and *E. elegans*. Therefore, *Escobedia* exhibited preferences with specific hosts during the early growth because just a subset of two out of nine hosts greatly enhanced their development.

Figure 4. Pearson correlation coefficient between haustoria number and (a) dry weight, (b) leaves number and xylem connection of haustorium in host root xylem, showed that only increase of haustorium number allowed high Escobedia development. Only *Escobedia grandiflora*-host data was included. *p*-values are statistically significant at p<0.05.



6.5 DISCUSSION

6.5.1 Host range of *Escobedia grandiflora* in natural populations

This study demonstrated that *Escobedia* parasitizes a wide range of host species from natural populations, and juvenile plants exhibit a significantly better development when in contact with specific host species. We determined that *Escobedia* has a broad host range (i.e., generalist), confirmed by a successful xylem connection between the haustorium and the different host roots. *Escobedia* parasitized the roots of 38 species, belonging to eleven plant families in four different plant communities. Our results confirm our previous findings for *Escobedia* (CARDONA; MURIEL, 2017), where the xylem connection was not evaluated. Our results are also consistent with other root hemiparasites (Orobanchaceae), which showed a broad range of host species, such as *Rhinanthus minor* L. (approx. 50 host species) and *Parentucellia viscosa* (L.) Caruel (23 hosts species) (GIBSON; WATKINSON, 1989; CAMERON; COATS; SEEL, 2006; SUETSUGU et al., 2012; CAMERON; PHOENIX, 2013). Sometimes, host range has been inferred only by examining haustorial attachments (GIBSON;

WATKINSON, 1989; NILSSON; SVENSSON, 1997). However, before defining a co-occurring species as a host, the haustorium connection must be functional, because not all haustoria effectively connect to the host xylem (SUETSUGU et al., 2012; CAMERON; PHOENIX, 2013).

No evidence of defence response, such as haustorium fragmentation, lignin deposition, or cell wall thickening, was observed in unsuccessful connections between *Escobedia* haustorium and potential hosts. Host defence response is one factor that can limit the number of hosts in the roots of hemiparasites, such as in some forbs during the incompatible interaction with *Rhinanthus* (CAMERON; COATS; SEEL, 2006; CAMERON; PHOENIX, 2013). In our case, some haustoria penetrated the potential host roots, but it was not functional because the xylem-to-xylem connection was not achieved. Thus, compatible interactions may require that the parasite disable the host defensive response in order to successfully connect to host vasculature (CLARKE et al., 2019). It seems that this is likely one of the reasons to not detect incompatible interactions between *Escobedia* and hosts. However, the mechanisms by which this occurs are still poorly studied. Recently, it was suggested that the azafrin (C_{27} apocarotenoid), localized in high levels in the haustorium attached to a host root, might be related to the inhibition of host response during the parasitism (see chapter 2). This link remains to be experimentally elucidated.

Our results show that *Escobedia* haustoria can penetrate and connect (i.e., functional haustoria) with the host vasculature from diverse plant taxa with different root morphologies. These results follow the high levels of plant diversity found in the areas with *Escobedia* (see chapter 1). Therefore, the high diversity of plant species may improve the development of parasite populations due to increasing the chance of finding multiple hosts and parasitizes them simultaneously, absorbing different nutritional components from each (GOVIER; NELSON; PATE, 1967; JOSHI; MATTHIES; SCHMID, 2000; ROWNTREE et al., 2014). This is likely because *Escobedia* is a perennial hemiparasite with rhizomes that develop large annual shoots and possess a long-living root system (CARDONA; MURIEL, 2015; see chapter 1). The roots accumulate considerable amounts of starch grains inside the cells, high levels of azafrin in the apoplast, and develop many lateral haustoria (CARDONA-MEDINA; SANTOS; NODARI, 2019; see chapter 2). These root characteristics may drive the long-term parasitization of the co-occurring host species in the communities, supporting the idea that host selection is positively correlated with their abundance (PRESS; PHOENIX, 2005).

6.5.2 Host preference during the early stage of *Escobedia grandiflora*

Interestingly, although *Escobedia* successfully parasitized a broad range of host roots, we detected preference by specific hosts in the early growth of *Escobedia* because their development (dry weight, leaf number and, haustorium number) was notably higher only with two hosts, namely *E. elegans* and *E. glomeratus*. Increment of haustoria attachments promoted successful connections with the host root and higher growth of *Escobedia*. Thus, the success of the growth in seedlings and juvenile parasitic plants is limited by the ability to develop higher haustoria number in a shorter period of time and invading the root of specific hosts (KEITH; CAMERON; SEEL, 2004b; TĚŠITEL et al., 2011). The early growth is the most critical stage of *Escobedia* life-cycle; it begins after the emergence of roots from the seeds, followed by the expansion of the cotyledons and the development of eophylls (seedlings), culminating with the formation of leaves (Juvenile plant) (CARDONA-MEDINA; SANTOS; NODARI, 2019). *Escobedia* growth in consortium with the most suitable hosts after 3 months of development is higher than that found in the perennial root hemiparasite *Monochasma savatieri* Franch. ex Maxim., but it is lower when compared with other perennials species from *Pedicularis* and *Castilleja* genera (MATTHIES, 1997; REN et al., 2010; MATTHIES, 2017; CHEN et al., 2020). Furthermore, the most developed individuals of *Escobedia* displayed great orange pigmentation in their roots. Orange pigment corresponds to azafrin, stored in the root apoplast and more concentrated in *Escobedia* parasitizing host roots (see chapter 2).

In this way, the reasons for selecting a specific host might be related to signaling molecules derived from the host that trigger haustoria formation and indicate the location of a host root mediated by haustorial hairs (CUI et al., 2020; FURUTA et al., 2021; MUTUKU et al., 2021). Fast recognition and attachment to hosts are crucial to the survival of some parasites (PRESS; PHOENIX, 2005). Some of these molecules are known as haustorium-inducing factors (HIF), which include chemical compounds as flavonoids, phenolics, and quinones such as dimethoxy-p-benzoquinone (DMBQ), the most extensive haustorium-inducing molecule among root hemiparasites within Orobanchaceae (ALBRECHT; YODER; PHILLIPS, 1999; FURUTA et al., 2021). HIF is potentially associated with the determination of hosts: low concentration of HIF promotes a fewer haustoria (GURNEY et al., 2003; SAUCET; SHIRASU, 2016). In addition to host-derived molecules, host selection can also be influenced by host attributes such as shoot and root architecture (MATTHIES, 2017; TWYFORD, 2018). Host species with abundant and superficial tiny roots can benefit hemiparasite seedling performance

due to easy penetration in the host root (MARVIER; SMITH, 1997; MATTHIES, 2017), as observed in *E. elegans* and *E. glomeratus* hosts. Graminoids also have abundant roots, although they roots are strongly lignified compared with *E. elegans* and *E. glomeratus* roots. Thickness or lignified roots may be difficult for the penetration of the haustoria, affecting the seedlings´ development and establisment (DAVIES; GRAVES, 2000; MATTHIES, 2017). Moreover, seedlings have inefficient uptake of resources and higher sensitivity to light competition with some host species, being light competition more intense in species with quick shoot growth (MATTHIES, 1995; TĚŠITEL et al., 2011), as verified in the graminoids species and *A. bellidioides*. In contrast, *Eryngium* spp increases light incidence in neighboring plants due to their architecture (i.e., rosette-shaped with rigid leaves), facilitating plants' survival and establishment (FIDELIS et al., 2009). Therefore, to explain the preferences with specific hosts found in this study, we hypothesized that the HIF and other host signals, as ethylene (CUI et al., 2020), might be found notably in *E. elegans* and *E. glomeratus* roots than in the other host, stimulating the haustoria formation of seedlings and juvenile plants of *Escobedia* to penetrate and connect into to the host vasculature. Additionally, the root and shoot architecture of both hosts can also facilitate the establishment and development of *Escobedia* by light and tiny host roots availability.

An *Escobedia* individual plant produces hundreds of viable seeds, which are dispersed by the wind, and it has high rates of germination, but a high seedling mortality (FALCÃO, 1980; CARDONA; MURIEL, 2015; CARDONA-MEDINA; SANTOS; NODARI, 2019). In other root hemiparasites, the seeds are the best propagation strategy, as observed in *Rhinanthus alectorolophus* (Scop.) Pollich (annual hemiparasite) or *Pedicularis palustris* L. (perennial), where seedlings are able to achieve high densities and form large number of individuals in the communities (DECLEER; BONTE; VAN DIGGELEN, 2013; HEER et al., 2018). In contrast, *Escobedia* has scattered mature individuals in natural populations that do not correspond to the hundreds of viable seeds it produces. One explanation for this might be the high levels of host specificity in the early growth of *Escobedia*. Our experimental study showed that only two hosts (*E. elegans* and *E. glomeratus*) promote the growth of *Escobedia* juvenile plants. These hosts are less abundant in the communities where *Escobedia* occurs, whereas the less preferred hosts (*P. glaucescens* and *B. capilaris*) were the most abundant in the communities (see chapter 1). Hemiparasites' presence may depend on the host distribution in the community (WATSON, 2009b). Thus, the presence of particular hosts, such as *E. elegans* (rosette) and *E. glomeratus* (herbs) could determine the occurrence of *Escobedia* seedlings in the communities.

Furthermore, the optimal foraging theory (MACARTHUR; PIANKA, 1966) should be a helpful approach to elucidate how to root hemiparasites choose their hosts in different ontogenetic stages. Consumers would balance the gross energy intake and the energy spent to search or handle the host (CHARNOV, 1976). Assuming that there is a wide variety of potential host species frequently available around and that the seedlings of *Escobedia* must quickly obtain resources to enable its establishment before it dies, the better strategy should be to maximize its effort on hosts that are easy to parasitize, do not compete for light and that provide the necessary amount and diversity of nutrients and water. That is, becoming a specialist. On the other hand, once the perennial root of the hemiparasite is established it would live and grow for many years, attaching to different host roots and absorbing different resources from each, presenting a generalist strategy.

Nevertheless, these results should be interpreted with caution because they were obtained in greenhouse conditions, and the seedlings and juvenile plants of *Escobedia* might be affected by the interactions with other abiotic and biotic factors. Considering the evidence in this study and in the literature, we propose that during the early growth of *Escobedia*, seedlings and juvenile plants have a preference by specific hosts, but as its parasitism increases, it may parasitize a great range of hosts. These findings have implications for conserving this important parasitic plant because the knowledge and management about the host preference are necessary for establishing efficient conservation strategies (MARVIER; SMITH, 1997).

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6.6 SUPPLEMENTARY MATERIAL

Figure S1. Principal component analysis (PCA) plot showing the relationship among the initial growth of *Escobedia grandiflora* and nine host species. Vectors (blue arrows) represent growth parameters. Symbols represent host species. **At**, *Anemia* cf. *tomentosa*; **Bc**, *Bulbostylis capillaris*; **Im**, *Ischaemum minus*; **Pg**, *Paspalum glaucescens*; **Ee**, *Eryngium elegans*; **Ef**,

Epidendrum fulgens; **Ll**, *Lucilia linearifolia*; **Evg**, *Evolvulus glomeratus*; **Ab**, *Acmella bellidioides*.

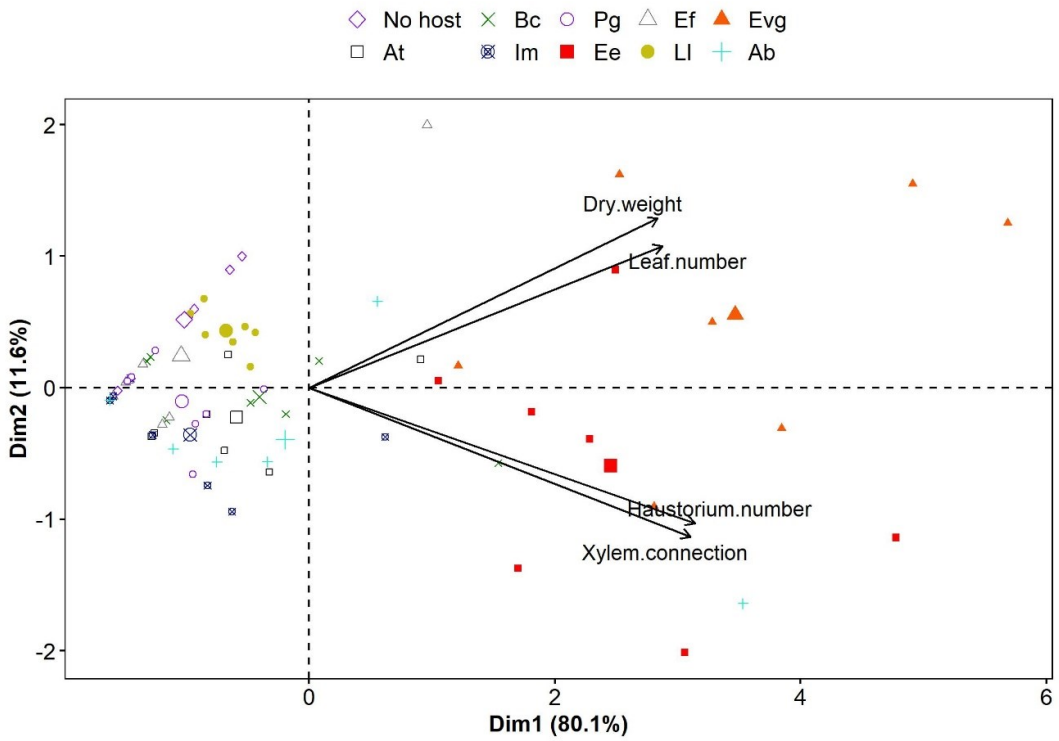
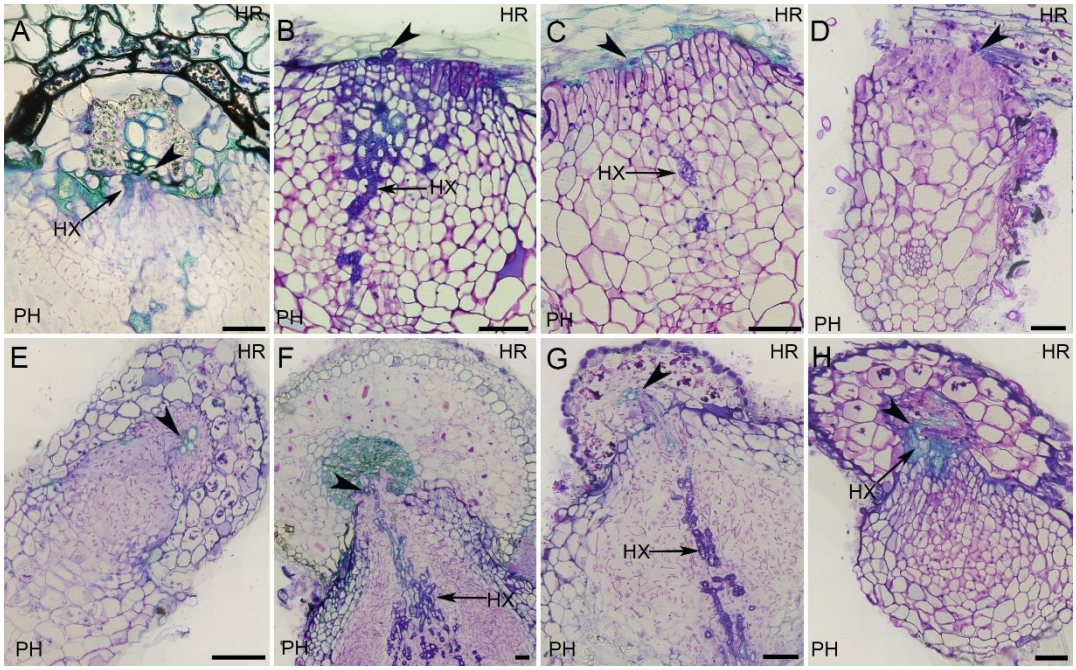


Figure S2. Confirmation of haustorial connection of *Escobedia grandiflora* during their early growth with different host root species. (A) *Anemia* cf. *tomentosa,* (B) *Bulbostylis capillaris*, (C) *Ischaemum minus*, (D) *Paspalum glaucescens*, (E) *Eryngium elegans*, (F) *Epidendrum fulgens*, (G) *Evolvulus glomeratus*, (H) *Acmella bellidioides.* Scale= 50 µm. HR= Host



7 CAPÍTULO 4- THE ROLE OF ROOT HEMIPARASITE *Escobedia grandiflora* on *Paspalum glaucescens* AND *Eryngium elegans*: INTRIGUING PARASITE-GRASS-ROSETTE INTERACTION

7.1 ABSTRACT

Root hemiparasite can affect the host's performance and influence the other interactions among their hosts and other neighbours. However, root hemiparasite interactions are widely unexplored in South America. Thus, this study evaluates the parasite-grass-rosette interactions with the hemiparasite *Escobedia grandiflora* (Orobanchaceae) and their hosts, *Paspalum glaucescens* (Poaceae) and *Eryngium elegans* (Apiaceae). We performed a nine-month pot experiment to assess the effect of the root hemiparasite on the development and competition between the two hosts, and the influence of each host, and the combination of both on the hemiparasite development. We found no significant reduction of hosts growth in the presence of *Escobedia*. Furthermore, *E. elegans* exerted a high competition intensity on *P. glaucescens* indifferent to *Escobedia* presence. Otherwise, *P. glaucescens* prejudiced the survival and development of *Escobedia*, whereas *E. elegans* benefited *Escobedia* even in the presence of *P. glaucescens*. In addition, the presence of both hosts significantly promoted azafrin accumulation in *Escobedia* roots. The empirical results in the present study provide a new understanding of *Escobedia*-grass-rosette interaction, indicating the facilitator effect of *E. elegans* on the survival and development of *Escobedia*.

Key words: Azafrin, Competition, facilitation, haustorium, Orobanchaceae

7.2 INTRODUCTION

Interactions between plant species influence the structure, functioning, and stability of ecosystems. Understanding the mechanisms that determine the outcomes of interactions allows the development of tools for the management and conservation of biodiversity and the restoration of degraded ecosystems (HARVEY et al., 2017). Among the interspecific interactions in herbaceous plant communities, competition is one of the main factors influencing the successional development of these ecosystems, as they determine patterns of species coexistence and the composition of the various successional stages (TILMAN, 1994).

In grasslands and other open ecosystems, highly competitive species tend to become excessively dominant and can reduce biological diversity in the absence of agents to control them (GRIME, 2006). Other biological interactions, such as parasitism, can alter competition outcomes. Some root hemiparasitic plants are considered ecosystem engineers precisely because of their potential to modify the interactions of their hosts with other species (TĚŠITEL et al., 2017; BOROWICZ; WALDER; ARMSTRONG, 2019; TĚŠITEL et al., 2021).

Root hemiparasites are a specific group of parasitic plants with a photosynthetic activity that obtain water and mineral nutrients from a host root through a specialized structure named haustorium (TĚŠITEL, 2016). Competition and facilitation mechanisms can influence the hemiparasite-plant interaction on community (PRESS; PHOENIX, 2005). On one hand, hemiparasites directly extract the nutrients and water directly from the hosts, consequently decreasing host growth and driven in a modification of competitive relations between other plants (PRESS; PHOENIX, 2005; TĚŠITEL, 2016). On the other hand, co-occurring species can considerably affect the performance of hemiparasitic plants (PRESS; PHOENIX, 2005). Above-ground competition of neighboring species can reduce the growth of hemiparasites because they are sensitive to competition for light, especially in the seedling stage (MATTHIES, 1995; TĚŠITEL et al., 2011). However, occurrence of different functional groups can facilitate the survival and performance of hemiparasites because it increases the possibility of finding a diverse range of hosts and establish proper interactions (GOVIER; NELSON; PATE, 1967; JOSHI; MATTHIES; SCHMID, 2000). Besides, high-quality litter from hemiparasite can positively influence the growth of surrounding species by releasing high nutrients (QUESTED; PRESS; CALLAGHAN, 2003a) since leaves of some hemiparasites have great nutrients content from the parasitization and fast decomposition in the soil (SPASOJEVIC; SUDING, 2011; FISHER et al., 2013). Most studies of hemiparasite-host interactions have only focused on a few species as *Rhinanthus*, *Pedicularis,* and *Melampyrum* from temperate grasslands (CAMERON; WHITE; ANTONOVICS, 2009; DECLEER; BONTE; VAN DIGGELEN, 2013; MATTHIES, 2017). In contrast, root hemiparasite interactions are largely unexplored in South America.

Escobedia grandiflora (L.f) Kuntze (referred next as *Escobedia*) is a perennial root hemiparasitic plant used for food and medicinal purposes around the Andean region (MURIEL et al., 2015). Escobedia roots contain an abundant apocarotenoid known as azafrin, related to the vigorous root growth and the success of parasitism in the host root system (see chapters 2 and 3). An observational study demonstrated that areas where *Escobedia* is found, are generally

associated with cover reduction of dominant species and at the same time with a high diversity of species, suggesting that *Escobedia* prefers more diverse areas (see chapter 1). According to empirical evidence from the annual hemiparasite *Rhinanthus*, these effects could relate to growth suppression of dominant species (e.g., grasses) by the parasitism that changes the competitive balance between hosts and allows the growth of subordinate species (e.g., forbs) (MATTHIES, 1996; PRESS; PHOENIX, 2005; MUDRÁK et al., 2016; TĚŠITEL et al., 2017). However, it is still unclear whether *Escobedia* can considerably suppress host species growth by mediating the competition between them. *Escobedia* is a generalist hemiparasite with a broad host range from different functional groups of the natural communities (see chapter 3). Among these, *Escobedia* parasitizes, for example, rosette-shaped species (*Eryngium* spp., *Dyckia encholirioides* (Gaudich.) Mez) and the root of graminoids species (*Paspalum glaucescens* Hack., *Bulbostylis capillaris* (L.) Kunth ex C.B. Clarke, and *Andropogon lateralis* Nees) (see chapter 3).

Paspalum glaucescens is a perennial grass with dense tufts, and it is a frequent component of highland grasslands in southern Brazil (POZZOBON; VALLS, 2000; ANDRADE et al., 2019). It is a dominant grass in plant communities where *Escobedia* occurs in southern Brazil (see chapter 1). Roots of *P. glaucescens* are frequently parasitized by the haustoria of mature *Escobedia* in natural communities (see chapter 3). Surprisingly *P. glaucescens* promotes insignificant development during the early growth of *Escobedia* because the seedlings showed stagnant growth and poor haustoria formation (see chapter 3). In contrast, *Eryngium* spp are rosette-shaped plants with rigid leaves, frequent in grasslands in southern Brazil (FIDELIS et al., 2008; BOMFIM et al., 2021; see chapter 1). It is also a common host for root hemiparasites, where two species of *Eryngium* are hosts for *Escobedia* (see chapters 1and 2). *Eryngium* play an important role as a facilitator in communities since its architecture allows opening in the dense dominant grass tufts, providing gaps with high light incidence that allows the establishment of a wide number of species (FIDELIS et al., 2009). Furthermore, *Eryngium elegans* Cham. & Schltdl. significantly promotes the development of *Escobedia* seedlings and juvenile plants, which presented a high number of haustoria connected to the host vascular system (see chapter 3).

Despite the importance of the host-hemiparasite interactions in natural communities, there is no experimental studies that evaluate, at the same time, the effects of *Escobedia* on host species and the influence of hosts on the hemiparasite development. Therefore, based on the

available evidence about these hemiparasite-host interactions, our study aimed to evaluate the parasite-grass-rosette interactions with the neotropical hemiparasite *Escobedia grandiflora* (Orobanchaceae) and their hosts, *Paspalum glaucescens* (Poaceae) and *Eryngium elegans* (Apiaceae). We evaluated (1) the effect of the root hemiparasite *Escobedia grandiflora* on the development and competition of the two hosts, and (2) the influence of each host and the combination of both on the hemiparasite development.

7.3 MATERIALS AND METHODS

7.3.1 Plant material

Mature dried fruits of *Escobedia grandiflora* and rhizomes of the selected hosts, *Paspalum glaucescens* (Poaceae) and *Eryngium elegans* (Apiaceae), were collected in a natural community located in the Lagoinha do Leste Municipal Park (27°46'53.0"S, 48°29'16.1"W, 190 m.a.s.l.), municipality of Florianópolis, state of Santa Catarina, Brazil.

7.3.2 Experimental design

A complete randomized experiment was designed with all possible combinations between *Escobedia* and two host species, *P. glaucescens* (*Pg*) and *E. elegans* (*Ee*), in a total of seven combinations with 20 replicates each. *Escobedia* was grown individually without the presence of host (*E*) and in consortia with *P. glaucescens* (*E*/*Pg*) or *E. elegans (E/Ee),* as with both *(E/Pg/Ee)*. Hosts were also grown individually, *P. glaucescens* (*Pg*) or *E. elegans* (*Ee*), and together (*Pg/Ee*).

Host rhizomes were grown in 2 L^{-1} pots for one month under greenhouse conditions for root establishment. *Escobedia* seeds were imbibed for five days in distilled water previously to sowing (20 seeds per pot), as described by Cardona-Medina et al. (2019). Imbibed seeds were grown in individual pots or sown to the corresponding host combination (see above).

Experiment was evaluated nine months after *Escobedia* sowing. *Escobedia* plants survival were counted and aboveground characteristics stem length, size leaf, and the number of leaves were measured on *Escobedia* and on its hosts. Aboveground and below parts of *Escobedia* were collected and dried for 72 h at 80 °C, as well for the aboveground parts of the hosts. *Escobedia* roots were frozen at -80 °C and lyophilized. Haustoria were collected and fixed in a 2.5 % glutaraldehyde solution in 0.1 M sodium phosphate buffer and dehydrated through an ethanolic series (RUZIN, 1999) for further microscopy analyses.

7.3.3 Microscopy analyses

The fixed haustoria samples were analyzed by light microscopy. Samples were processed as described by Cardona-Medina et al. (2019) and the images were captured with a DP71 digital camera (Olympus, Tokyo, Japan) using a BX-40 microscope (Olympus, Tokyo, Japan).

7.3.4 UPLC analysis of azafrin

Extraction of azafrin was performed in the dark at 4 °C (cold room). For extraction and quantification of root apocarotenoid, 1 mg of lyophilized *Escobedia* roots was mixed with 900 µL of methanol and 100 µL of 2 % (w/v) solution of tartrazine in ethanol 70%, as an internal standard, and agitated for 1 min. Followed by centrifugation at 1300 rpm for 5 min at 4°C. The upper phase was collected and filtered through a 0.2 µm microfilter. For each sample, 10 µL was injected onto an Agilent Technologies 1290 Infinity UPLC system. Peak areas of apocarotenoid at 419 nm were determined using the Agilent ChemStation software and quantified by comparison with tartrazine standard.

7.3.5 Statistical analyses

A one-way ANOVA was performed in which the treatments (*Escobedia* presence and host species combination) were the explanatory variable, and the morphological characteristics of *P. glaucescens* and *E. elegans* were the response variables (shoot, root, and total biomass, length, and number of leaves). Response variables were square-root transformed. ANOVA validation was based on the Shapiro-Wilk test and the analysis of plotting residuals. Additionally, the Relative Competition Intensity Index (RCI index) was calculated for each host in the presence and absence of *Escobedia*. The RCI index was calculated as the difference of the total biomass of each host alone, minus the biomass of the host grown together with the other host, divided by the biomass of the host grown alone (WEIGELT; JOLLIFFE, 2003). Finally, we performed a two-way permutational analysis of variance to determine the effect of competition between the two hosts (PERMANOVA). The hosts and the hemiparasite (absence or presence) were explanatory variables; the RCI index was the response variable. We used the "aovp" function from the package "lmPerm" to PERMANOVA analysis (WHEELER;

TORCHIANO, 2016). The confidence intervals of the parameters and p-value were calculated based on 5000 iterations.

To test the host combination influence in the development of *Escobedia*, two analyses were performed: for the first analysis, logistic regression was used to estimate the influence of host combination in the *Escobedia* survival rate in which the host combination was the explanatory variable and *Escobedia* survival was the response binary variable. As the response variable is binary (dead=0 and alive=1), a quasibinomial distribution was used. The model fit was based on the receiving operator characteristics (ROC) curve (Roman et al., 2014) using the package "pROC" (ROBIN et al., 2021) and with a Nagelkerke R2, a pseudo-R2 metric (HAMMOND et al., 2019) using the package "DescTools" (SIGNORELL et al., 2021). For the second analysis, a one-way ANOVA was performed in which the host combination was the explanatory variable, and the morphological and physiological characteristics of *Escobedia* were the response variables (shoot, root, and total biomass, stem length, width leaves, length leaves, number of leaves and azafrin levels). Response variables were square-root transformed. ANOVA assumptions were validated by the Shapiro-Wilk test and by analysing the plotted residuals. For all the analyses, significant differences ($p \leq 0.05$) among treatments were measured using Tukey's test. Additionally, correlations were estimated between the total biomass of *P. glaucescens* and *E. elegans* using Pearson's correlation coefficient. All statistical analyses were performed in R environment v4.0.3 (R Core Team, 2020).

7.4 RESULTS

7.4.1 The effect of host combination and *Escobedia grandiflora* presence on host species development

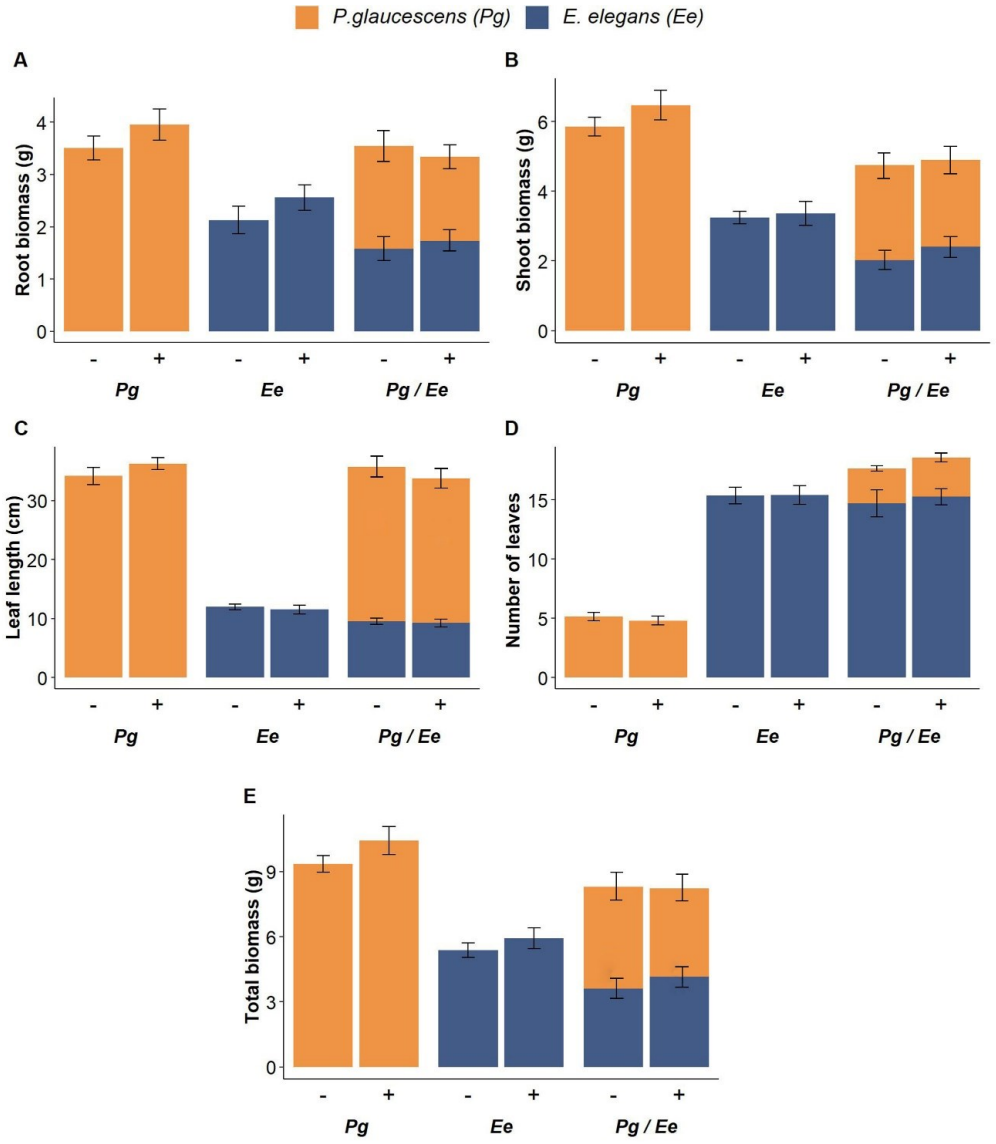
The presence of *Escobedia* did not significantly affect any of the morphological characteristics of the two hosts, grown individually or in intercropping (Table 1, Fig. 1). In contrast, the host species greatly affected each other's development. The presence of *E. elegans* reduced 56 % of *P. glaucescens* total biomass, affecting both shoot and root biomass (leaf length and number decreased 28% and 37%, respectively). Nevertheless, *P. glaucescens* also affected *E. elegans* negatively, with over 30% total biomass loss, reducing both shoot and root biomass but not the total number of leaves. Particularly, a negative correlation of the biomass (r^2=-0.65, $p<0.001$) between *P. glaucescens* and *E. elegans* indicated that when the biomass of

one host increases, the other biomass decreases, sustaining the evidence of competition between these hosts.

Table 1. Effect of the hemiparasite plant *Escobedia grandiflora* and the host species combination on morphological characteristics of *Paspalum glaucescens* (*Pg*) and *Eryngium elegans* (*Ee*). Different letters represent significant differences by Tukey test ($p \leq 0.05$), standard error in parenthesis.

		Mean			
Morphological characteristics	**Host**	**Single host**	**Host+ *Escobedia***	**Two host**	**+ *Escobedia* + two hosts**
Shoot biomass (g)	*Pg*	a 5.86(±0.3)	a 6.47 (±0.4)	b 2.73 (±0.4)	b 2.48 (±0.4)
	Ee	a 3.25 (±0.2)	a 3.36 (±0.3)	b 2.03 (±0.3)	b 2.41 (±0.3)
Root biomass (g)	*Pg*	a 3.50 (±0.2)	a 3.95 (±0.3)	b 1.96 (±0.3)	b 1.61 (±0.2)
	Ee	ab 1.41 (±0.3)	a 1.56 (±0.2)	b 1.20 (±0.2)	ab 1.26 (±0.2)
Total biomass (g)	*Pg*	a 9.36 (±0.4)	a 10.43 (±0.6)	b 4.69 (±0.6)	b 4.09 (±0.6)
	Ee	ab 5.38 (±0.3)	a 5.92 (±0.5)	c 3.61 (±0.5)	bc 4.14 (±0.5)
Length leaves (cm)	*Pg*	a 34.15 (±1.5)	a 36.25 (±1)	b 26.15 (±1.8)	b 24.51 (±1.7)
	Ee	a 3.45 (±0.5)	ab 3.37 (±0.8)	bc 3.07 (±0.5)	c 2.98 (±0.7)
Number of leaves	*Pg*	a 2.25 (±0.3)	a 2.16 (±0.4)	b 1.69 (±0.2)	b 1.77 (±0.4)
	Ee	a 15.35 (± 0.7)	a 15.42 (±0.8)	a 14.70 (±1.1)	a 15.25 (±0.7)

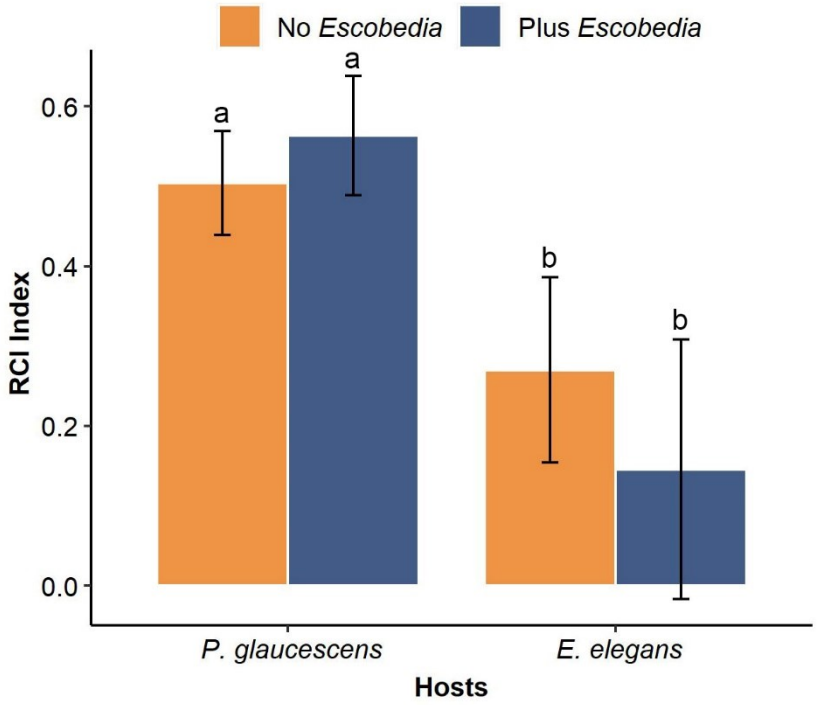
Figure 1. Effect of the hemiparasite plant *Escobedia grandiflora* and the host species combination on morphological characteristics of *Paspalum glaucescens* and *Eryngium elegans*. The signs represent the absence (-) or the presence (+) of the hemiparasite. Host species combination comprises of *P. glaucescens* (*Pg*) or *E. elegans* (*Ee*) and *P. glaucescens* plus *E. elegans* (*Pg/Ee*). Bars represent standard error of the means.



To determine the effect of competition between the two hosts, the index of Relative Competition Intensity (RCI index) was calculated (Fig. 2). A two-way PERMANOVA revealed a significant difference between the RCI index of *P. glaucescens* and *E. elegans* hosts (df=1, Inter=5000, p <0.001), regardless of the presence or absence of *Escobedia* (Fig. 2). This demonstrates that *E. elegans* exerted a high competition intensity on *P. glaucescens* (0.53 ± 0.05), leading to significant reductions of *P. glaucescens* biomass. In contrast, *P. glaucescens*

exerted a low competition intensity on *E. elegans* (0.21 ± 0.1). The presence of *Escobedia* has not significantly influenced the RCI index of the two hosts (df=1, Inter=5000, p=0.764). Therefore, the parasitism of *Escobedia* did not alter the competitive balance of *P. glaucescens* and *E. elegans* hosts.

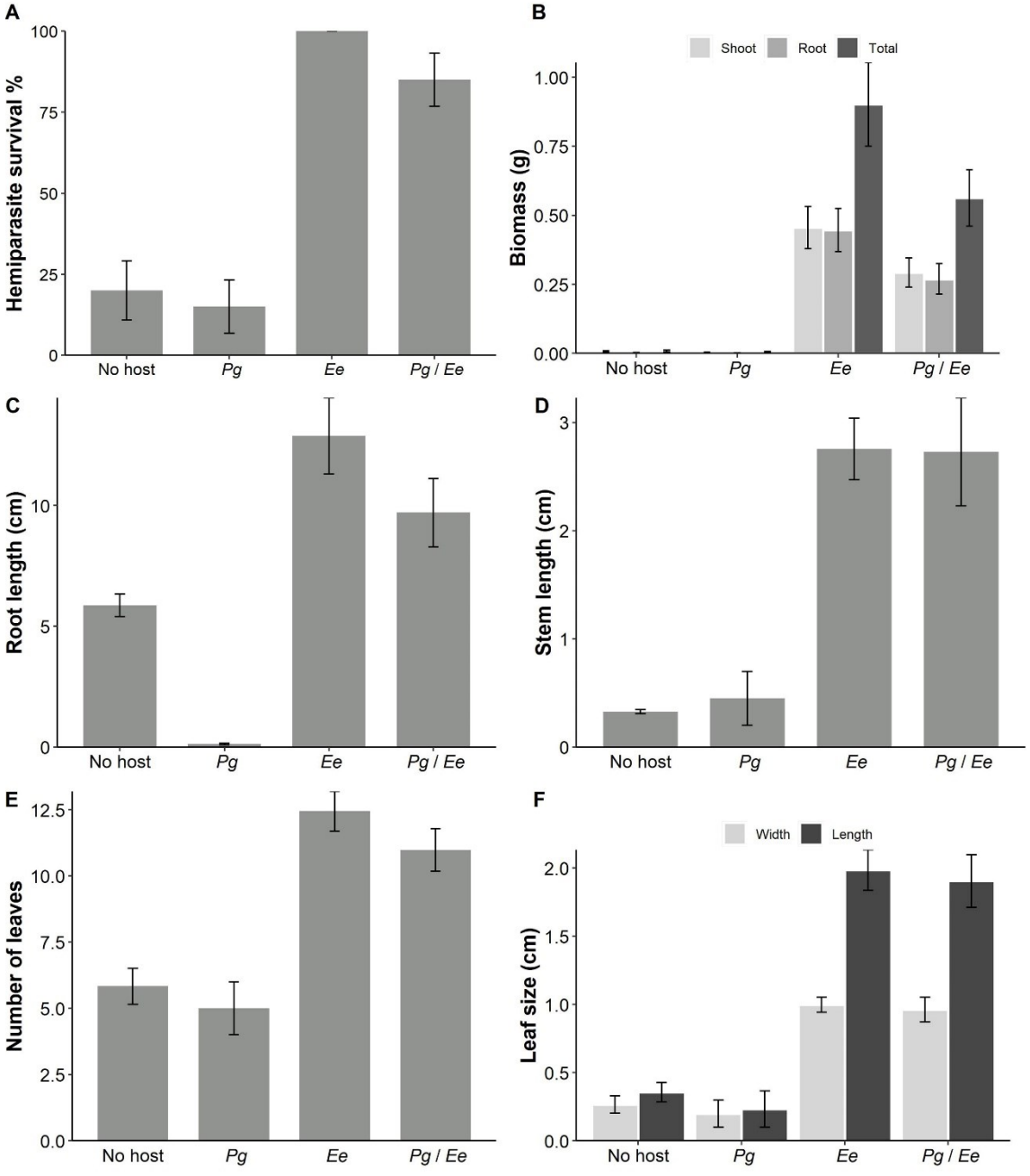
Figure 2. Relative competition intensity index (RCI index) of *Paspalum glaucescens* and *Eryngium elegans* in presence or absence of the hemiparasite *Escobedia grandiflora*, where the host species with the highest value is most affected by competition intensity. Bars represent standard errors of the means. Means with different letters represent significant differences by Tukey test ($p \leq 0.05$).



7.4.2 Influence of hosts on *Escobedia grandiflora* development

The results demonstrated that *Paspalum glaucescens* and *Eryngium elegans* hosts notably influenced the survival and development of the hemiparasite *Escobedia* (Fig. 1). The survival of *Escobedia* after nine months was strongly influenced by hosts (Fig. 1A), being higher with *E. elegans* (estimate= 0.55, p<0.001) and with both hosts (estimate=0.47, p<0.001). The presence of *P. glaucescens* (estimate=-0.04, p=0.614) and host absence (estimate= -2.75, p<0.001) drastically reduced the survival of *Escobedia* (Fig. 1). The post hoc test demonstrated no significant differences between *E. elegans* and both *E. elegans* and *P. glaucescens* (p= 0.619), indicating a similar influence of one and two hosts on *Escobedia* survival.

Figure 3. Influence of host species combination, *Paspalum glaucescens* and *Eryngium* elegans, on plant survival (A) and the morphological characteristics of the hemiparasite *Escobedia grandiflora* (B-F). Species combination comprises *Escobedia* grown individually without the presence of host (no host), and in consortia with *P. glaucescens* (*Pg*) or *E. elegans (Ee),* and with both *(Pg/Ee)*. Bars represent standard errors of the means. Means with different letters represent significant differences by Tukey test ($p \leq 0.05$).



The host combination strongly influenced the morphological characteristics of *Escobedia* (Table 2, Fig. 3). The biomass and the stem length significantly increased when *Escobedia* grew in consortia with *E. elegans* and with both hosts (Table 1, Fig. 4B). Moreover, the consortium with only *P. glaucescens* did not influence the biomass accumulation in

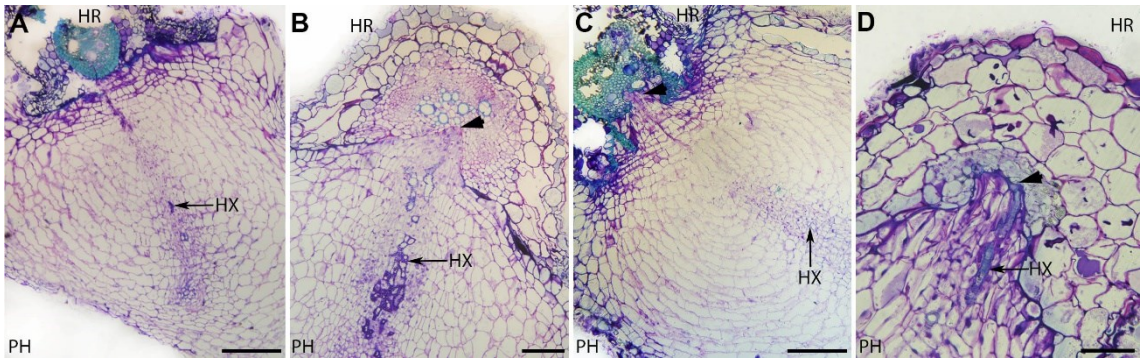
Escobedia, similarly found without a host. The host presence also influenced the width length and number of leaves in *Escobedi*a (Table S2). These values notably increased when *Escobedia* grew with *E. elegans* or with both host species (Table 2, Fig. 3). Overall, after nine months *E. elegans* presence favoured the survival and development of *Escobedia* independent of the *P. glaucescens* presence, hence, individuals did not produce reproductive structures during this experiment.

Table 2. Influence of host species combination, *Paspalum glaucescens* (*Pg*) and *Eryngium elegans* (*Ee*), on development of *Escobedia grandiflora* (*E*). Different letters represent significant differences by Tukey test ($p \leq 0.05$), standard error in parenthesis.

Development parameters	**Mean**			
	***Escobedia* (E)**	***E* + *Pg***	***E* + *Ee***	***E* + *Pg* + *Ee***
Shoot biomass (gr)	b 0.007 (±.002)	b 0.004 (±.001)	a 0.456 (±0.08)	a 0.293 (±0.05)
Root biomass (gr)	b 0.002 (±.001)	b 0.002 (±.001)	a 0.446 (±0.08)	a 0.270 (±0.05)
Total biomass (gr)	b 0.010 (±.003)	b 0.005 (±.001)	a 0.902 (±0.1)	ab 0.563 (±0.1)
Stem length (cm)	c 0.642 (±.01)	bc 0.571 (±0.2)	a 1.610 (±0.3)	ab 1.544 (±0.5)
Number of leaves	b 5.833 (±0.7)	b 5 (±1)	a 12.442 (±0.7)	a10.980 (±0.8)
Leaves width (cm)	b 0.267 (±0.06)	b 0.2 (±0.1)	a 0.998 (±0.05)	a 0.962 (±0.1)
Leaves length (cm)	b 0.356 (±0.07)	b 0.233 (±0.1)	a 1.984 (±0.1)	a 1.905 (±0.2)
Root length (cm)	b 2.418 (±0.5)	ab 0.404 (±0.03)	a 3.479 (±1.5)	a 3.004 (±1.4)
Azafrin level (u.a)	c 36.880 (±5)	c 23.754 (±16)	b 143.395 (±5.2)	a 185.502 (±11.2)

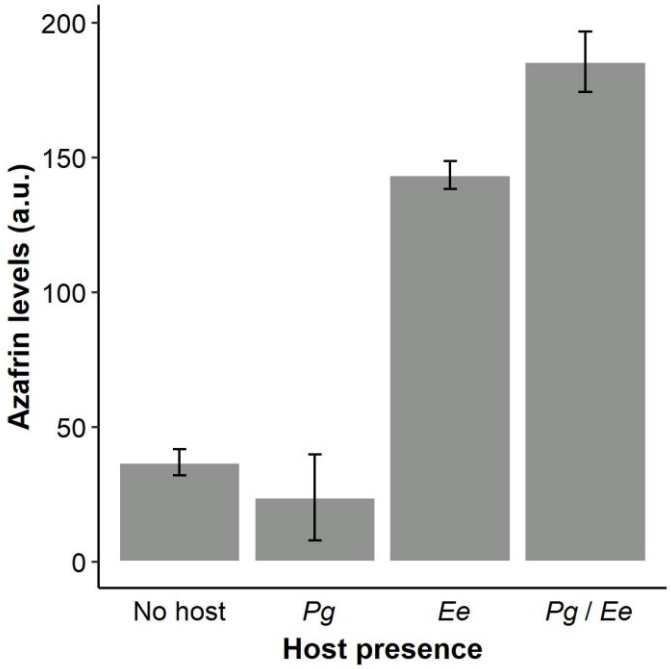
Following the poor survival and development of *Escobedia* without a host, their roots did not develop any haustoria. Furthermore, *Escobedia* developed just one haustorium that attached to *P. glaucescens* host root (Fig. 4A). Structural analysis of this haustorium confirmed that the intrusive cells invaded the root cortex of *P. glaucescens*, but only a few effectively penetrated the central cylinder of the host (Fig. 4A). Therefore, *Escobedia*, in consortium with *P. glaucescens*, did not legitimately parasitize their root, as no evidence of the vascular tissue connection between the haustoria and the host roots xylem was found. In contrast, when *Escobedia* grew with *E. elegans*, more than 80 haustoria was formed, many of them established vascular tissue connections with the host root (e.g. figure 4B). Furthermore, *Escobedia* grown with *E. elegans* and *P. glaucescens* developed around 50 haustoria attached to both host roots, often simultaneously. In both cases, intrusive cells invaded the host's xylem and developed vascular tissue connections (Fig. 4C-D). However, the haustorium xylem was larger when connected to *E. elegans* than to *P. glaucescens* (Fig. 4C-D).

Figure 4. Parasitism evidence (xylem-xylem connection) of *Escobedia grandiflora* grown with two different host species and their combination. Cross-sections of *Escobedia* haustoria and host roots under light microscopy. (A) Haustorium attached to *P. glaucescens* root, note to initial connection of the intrusive cells with host xylem; (B) haustorium attached to *E. elegans* root (C) haustorium attached to *P. glaucescens* root when *E. elegans* was present; (D) haustorium attached *to E. elegans* when *P. glaucescens* was present. Haustoria successfully invaded the host's roots (B, C, D) developing numerous xylem elements connected to the host's xylem (arrowhead). Abbreviations: HR= host root, HX= haustorium xylem, PH= parasite haustorium. Scale bars: (A-B) =200 µm, (C) =100 µm, (D) = 50 µm.



Interestingly, host combination also strongly influenced the azafrin levels of *Escobedia* roots ($F=38.49$, $p<0.001$) (Table 2, Fig. 5). Azafrin levels on *Escobedia* roots grown together with both hosts substantially increased when grown with hosts alone (Table 2, Fig. 5). The increase of azafrin levels also differed considerably in *Escobedia* grown with *E. elegans* than with *P. glaucescens*. In this way, the presence of two hosts species notably influenced the increment in the azafrin levels of *Escobedia* roots.

Figure 5. Azafrin levels in the root of *Escobedia grandiflora*. *Escobedia* was grown individually without the presence of host (no host) and in consortia with *P. glaucescens* (*Pg*) or *E. elegans* (*Ee*) and with both (*Pg/Ee*). Arbitrary units (a.u.). Bars represent standard errors of the means. Means with different letters represent significant differences by Tukey test ($p \leq 0.05$).



7.5 DISCUSSION

The hemiparasite *Escobedia* did not significantly affect host growth, and consequently, did not influence the competition outcome between the two hosts. The effect of parasitic plants on hosts largely depends on the parasitized species identities (PRESS; PHOENIX, 2005). As shown in pot experiments, hemiparasites have a strong depletory effect on its most beneficial host species (MATTHIES, 1996, 1997). Interestingly, this was not observed in the interaction of *Escobedia* and the most fit host here evaluated, *E. elegans*, that did not show significant biomass reduction during nine months of development. A study evaluating 27 hosts interacting with the hemiparasite *Melampyrum arvense* L. (Orobanchaceae) highlighted that a fast-growing host circumvents the detrimental competition for nutrients and water caused by the parasitism (MATTHIES, 2017). Similar results were found for *E. elegans* which thrived faster than *Escobedia*. In this way, negative effects on the host by root hemiparasite can occur when the growth of a good host are slower (MATTHIES, 2017). *Escobedia* was found associated with lower evenness of herbs in natural plant communities (See chapter 1), in this case, the hemiparasite might exert, at some extent, negative effect in the establishment of specific herbaceous hosts. The possible adverse effect of *Escobedia* on slow-growing hosts cannot be ruled out and it would be interesting to be evaluated for the consortia with distinct hosts.

Additionally, adverse effects on the host during parasitism may be diminished by litter effects of perennial hemiparasites (QUESTED; PRESS; CALLAGHAN, 2003b; SPASOJEVIC; SUDING, 2011). Leaves of hemiparasite concentrate two to four times more nutrients with fast decomposition than the host leaves, allowing high nutrient release in the soil (QUESTED; PRESS; CALLAGHAN, 2003; SPASOJEVIC; SUDING, 2011). The concentrated nutrient litter of hemiparasites can positively influence the growth of neighbouring plants (QUESTED et al., 2004; QUESTED; PRESS; CALLAGHAN, 2003; SPASOJEVIC; SUDING, 2011). This effect may be greater in slow-growing host species with long lifespan (PRESS; PHOENIX, 2005). In our study, *Escobedia* produced litter from leaves in the vicinity of *E. elegans*. This characteristic is typical of *Escobedia* development, where the stems die, subsequently, the rhizomes develop new shoots until the stems produce reproductive structures (CARDONA; MURIEL, 2015).

Despite no effect of *Escobedia* on host growth, the present study found a significant difference in the competition between hosts. In *E. elegans* presence, *P. glaucescens* biomass decreased by an average of 56%, the leaf number by 37%, and the leaf length by 28%. The RCI index results indicated that competition intensity caused by *E. elegans* significantly reduced the *P. glaucescens* biomass. The plant-plant competition involves the conflict to seize limited resources (such as access to light, water, and nutrients), which regulates the rate of carbon capture (CALLAWAY, 1997). The competitive abilities of resources acquisition may be affected by specific plant characteristics, such as shoot architecture, root structure, and dispersion in the soil (GUREVITCH; SCHEINER; FOX, 2006). According to FIDELIS et al. (2009), the rosette-shaped architecture of *Eryngium horridum* Malme with rigid and thorny leaves can reduce the competition by dominant grasses, pushing away the tussocks dense grass. Therefore, a possible explanation for the negative effect of *E. elegans* may be related to its morphological characteristics (including the rosette-shaped and abundant root system with small roots) with lateral development that affects the *P. glaucescens* development.

Besides the competitive effect on *P. glaucescens*, *E. elegans* positively influenced the high survival and growth of *Escobedia*. A previous study also displayed a strong preference of *Escobedia* for *E. elegans* over other hosts after three months of development, which formed several haustoria and successfully parasitized the host roots (see chapter 3). These results further support the idea of *Eryngium* species facilitating effect (FIDELIS et al., 2009; BOMFIM et al., 2021). The *E. horridum* architecture also provides gaps that provides high light incidence, benefiting the establishment and survival of forb species (FIDELIS et al., 2009). These

beneficial effects can be extended to other trophic levels, such as spider species, due to physical properties of rosette leaves (BOMFIM et al., 2021). In contrast, *P. glaucescens* negatively affected the survival and growth of *Escobedia*. Survived plants showed poor growth and did not parasitize host roots when grew only with *P. glaucescens*. A previous study also found that *P. glaucescens* was not a fit host for *Escobedia* during its early development; seedlings were undeveloped and formed few haustoria (see chapter 3). The establishment and survival of seedlings is harder in dense grass matrices due to shading of the profuse architecture (FIDELIS et al., 2009). Seedlings of some root hemiparasites are very susceptible to light competition with the host, because they have less efficiency to resources acquisition and subsequent low growth (TĚŠITEL et al., 2011; TĚŠITEL; PLAVCOVÁ; CAMERON, 2010). In *Escobedia*, seedling stage is the most critical in their development, due to limited reserves found in seeds and the strong dependence on host specificity to succeed parasitism (CARDONA-MEDINA; SANTOS; NODARI, 2019; see chapter 3). In this context, an excessive shading by the host can limit parasite photosynthetic activity and parasitism establishment, resulting in low biomass accumulation and possible parasite death (MATTHIES, 1995; PRESS; PHOENIX, 2005; TĚŠITEL et al., 2011), as observed in our study.

Regardless of the harmful effects of *P. glaucescens* on *Escobedia*, a significant survival and growth with both *P. glaucescens* and *E. elegans* was found. Furthermore, the haustoria parasitized the root of both hosts, often simultaneously. The remarkable development of *Escobedia*, even with *P. glaucescens* presence, corroborates the facilitation effect of *E. elegans*. After overcoming the early stage of development, the hemiparasite can be less susceptible to competition for resources, and it can parasitize other hosts in its vicinity (MATTHIES, 1996; KEITH; CAMERON; SEEL, 2004a; SVENSSON; CARLSSON, 2004b; TĚŠITEL et al., 2011). Facilitative plant interactions may directly result from environmental improvement conditions or may indirectly suppress species that could compete by resources with the beneficed species (CALLAWAY, 1995, 1997). Thus, our results indicate that besides the direct benefits of *E. elegans* for *Escobedia* growth, the notable biomass reduction of *P. glaucescens* by *E. elegans* may have indirectly benefited *Escobedia* development, possibly due to the decrease of the harmful effects of *P. glaucescens* on survival and growth of the hemiparasite. Graminoids are the most frequent host of mature plants of *Escobedia* in natural communities (see chapter 3), where *P. glaucescens* was the most abundant species among populations from southern Brazil (see chapter 1). Therefore, these results are consistent with

the suggestion that during early growth (see chapter 3), *Escobedia* was notably favoured by specific hosts and might lead to subsequent parasitism of a wider and more diverse range of hosts.

Following substantial development of *Escobedia* in consortia with *E. elegans* and both hosts, the roots displayed high levels of azafrin. Azafrin is a C27 apocarotenoid (i.e., carotenoid-derived) with antioxidant properties. It is stored in the intercellular root spaces and the haustorium-host root interface (see chapter 2). Due to these characteristics, azafrin has been related to interactions of *Escobedia* root with the rhizosphere and the parasitization process in the hosts' root (see chapter 2). It is interesting to note that azafrin levels were higher in *Escobedia* roots in the presence of both hosts, which together had extensive host root biomass than with only *E. elegans*. In this way, the presence of both host roots might promote azafrin accumulation in *Escobedia* roots due to major interaction with the rhizosphere and parasitism with the host roots. In natural communities of southern Brazil, higher azafrin levels (400 a.u.) also have been found in the roots of mature plants, which are associated with high plant diversity and parasitizing a wide host range (see chapters 1 and 2).

In conclusion, we provide novel empirical evidence about the hemiparasite-grass-rosette interaction. These results suggest that *Escobedia* did not affect hosts development in early stages. In contrast, *E. elegans* positively influenced the survival and development of *Escobedia* and negatively the *P. glaucescens* development. Furthermore, this rosette species also changed the outcome of the interaction between *Escobedia* and *P. glaucescens*, reducing grass's competitive strength and benefiting the presence of the hemiparasite. Hence, the improvement of *Escobedia* development by hosts promoted a substantial azafrin level in the root, being major particularly in the presence of both hosts.

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7.6 SUPPLEMENTARY MATERIAL

Table S1. Summary of ANOVA showing the effect of hemiparasite presence (E) and the host species combination on morphological characteristics of *Paspalum glaucescens* (Pg) and *Eryngium elegans* (Ee). The variable number of leaves for *P. glaucescens* and root biomass leaves length for *E. elegans* were square-root transformed, and significant P-values are in bold (P<0.05).

	P. glaucescens	*E. elegans*

Morphological parameters	df	Ee and E effect			Pg and E effect		
		MSQ	F	P	MSQ	F	P
Total biomass	3	206.86	31.06	**<0.001**	22.892	5.966	**<0.001**
Shoot biomass	3	90.52	19.11	**<0.001**	8.409	5.589	**0.001**
Root biomass	3	26.264	18.6	**<0.001**	3.634	3.634	**0.016**
Leaves length	3	673.1	14.21	**<0.001**	38.25	4.898	**0.004**
Number of leaves	3	1.5383	11.62	**<0.001**	2.135	0.149	0.93
Residuals	76						

Table S2. Summary of ANOVA showing the influence of two host species combination, *Paspalum glaucescens* and *Eryngium elegans,* on morphological characteristics of *Escobedia grandiflora*. Significant P-values are in bold (P<0.05).

Morphological characteristics	df	MSQ	F	P
Shoot biomass	3	0.323	4.236	**0.01**
Root biomass	3	0.322	4.067	**0.013**
Total biomass	3	1.289	4.335	**0.001**
Stem length	3	9.710	7.194	**<0.001**
Number of leaves	3	73.32	7.223	**<0.001**
Leaves width	3	0.941	10.75	**<0.001**
Leaves length	3	4.612	9.646	**<0.001**
Root length	3	97.24	5.269	**0.004**
Residuals	39			

8 CONSIDERAÇÕES FINAIS E PERPECTIVAS FUTURAS

Escobedia grandiflora é um importante recurso genético com potencial medicinal e como corante de alimentos. As análises das raízes das populações naturais confirmaram a presença abundante do apocarotenoide azafranina. A análise do RNA possibilitou identificar os genes que codificam as enzimas responsáveis pela biossíntese de azafranina. A sua biossíntese ocorre dentro da célula, porém é armazenada fora dela nos espaços intercelulares do córtex da raiz. A análise da estrutura interna do haustório mostrou também a presença da azafranina na interface entre o haustório e a raiz hospedeira. Além disso, os resultados da tese revelaram que populações naturais de *E. grandiflora* estão associadas com maiores índices de riqueza, diversidade e equabilidade de plantas em quatro comunidades. Destaca-se que, nessas comunidades, *E. grandiflora* parasita as raízes de uma ampla gama de plantas hospedeiras, em especial graminóides e ervas. No entanto, na etapa inicial do crescimento, *E. grandiflora* se desenvolveu bem apenas com hospedeiros específicos, entre os quais encontrou-se *Eryngium elegans* e *Evolvulus glomeratus*. Além disso, durante a interação de *E. grandiflora* com *Paspalum glaucescens* e *E. elegans* foi observado que o parasitismo de *E. grandiflora* não afetou consideravelmente o desenvolvimento dos dois hospedeiros durante nove meses de interação. Pelo contrário, a presença desses dois hospedeiros influenciou notavelmente a sobrevivência, o crescimento e acumulação da azafranina na raiz de *E. grandiflora*, sendo *E. elegans* a espécie chave nesta interação. Portanto, uma vez que o desenvolvimento inicial de *E. grandiflora* é superado na presença de *E. elegans*, pode parasitar hospedeiros que no início não foram possíveis de serem parasitados, conforme observado com espécies mais abundantes como *P. glaucescens*.

O avanço no conhecimento científico proporcionado pela presente tese está relacionados com aspectos críticos associados à biologia de *E. grandiflora,* que permitirão estabelecer estratégias de conservação e uso. Esta tese apresenta também importantes contribuições na caracterização do transcriptoma de *E. grandiflora,* fornecendo informações relevantes sobre os genes associados aos diferentes processos do metabolismo, os quais serão a base para futuros estudos sobre o desenvolvimento parasítico de *E. grandiflora.* Além disso, os resultados sugerem a realização de novos experimentos para aprofundar a relação da azafranina na raiz de *E. grandiflora* com a rizosfera e a interação com os hospedeiros.

Outro desafio importante é a realização de estudos experimentais sobre o efeito de *E. grandiflora* (adição do parasita) nas comunidades, avaliando, entre outros aspectos, o efeito da

densidade do hemiparasita na composição e diversidade de plantas. Visto que há indícios na literatura que o efeito de hemiparasitas de raiz nas comunidades de plantas são maiores conforme a maior densidade de hemiparasita. Estes estudos deveriam ser efetuados em regiões onde populações de *E. grandiflora* se encontram mais ameaçadas, para assim contribuir com a conservação da espécie.

Para recuperação das populações de *E. grandiflora* ou a realização de estudos experimentais nas comunidades por semeadura é recomendável realizar primeiro o desenvolvimento inicial das plântulas em casa de vegetação, para logo serem levadas para campo com hospedeiros específicos, conforme indicam os resultados dos capítulos 3 e 4 da presente tese. Contudo, estes hospedeiros podem mudar dependendo das espécies vegetais de cada comunidade. Assim, para futuros estudos recomenda-se verificar os hospedeiros específicos para cada comunidade que será avaliada, conforme realizado no capítulo 3.

Uma alternativa ao desenvolvimento de plantas juvenis em casa de vegetação é o cultivo *in vitro* de *E. grandiflora* a partir de sementes. Dentre as vantagens, destaca-se a obtenção de grande número de indivíduos, mantendo condições ideais de crescimento para *E. grandiflora*. Testes *in vitro* já foram realizados, nos quais observou-se melhor crescimento com a presença do hospedeiro (tomate). Contudo, esse hospedeiro não foi o mais adequado. Assim, com base nos resultados obtidos, o crescimento de plântulas de *E. grandiflora in vitro* a partir de sementes poderá ser maior com *E. glomeratus ou E. elegans*.

No entanto, como é uma planta parasita, o estabelecimento de novas plantas de *E. grandiflora* para a conservação e o uso deve ser realizado com a devida precaução. As populações estabelecidas deveriam passar por uma avaliação de risco, para saber o comportamento delas nas comunidades. Além disso, recomenda-se não estabelecer plantas de *E. grandiflora* fora da sua área de ocorrência natural pelo risco de efeitos negativos aos ecossistemas, como observado em outras plantas parasitas. Por enquanto, nossos resultados confirmam a associação positiva de *E. grandiflora* com os índices de diversidade e a baixa afetação que exerce nos hospedeiros.