Development of novel approaches to study *Cuscuta campestris* biology

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Plant Pathology, Physiology and Weed Science

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July 6, 2021

Blacksburg, VA

Key words: Parasitic plant, haustoria, *in vitro* culture, hormones, carotenoids, defense response

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Abstract

Cuscuta campestris is an obligate parasitic plant that lacks expanded leaves and roots and requires a host to complete its lifecycle. Parasite-host connections occur via an haustorium, a unique organ that acts as a bridge for the exchange of water, nutrients, macromolecules like mRNA, microRNA, and proteins, and microorganisms. Studies of *Cuscuta* spp. are challenging due to its dependence on the host and other host influences on the parasite. Recent research has shown intriguing aspects of *Cuscuta* biology like exchange genetic material with its hosts and loss of genes involved in processes such as high photosynthetic rates and defense. We developed new tools and methodologies that allow us to explore *C. campestris* biology in an unprecedent way. Foremost of these is an axenic method to grow *C. campestris* on an Artificial Host System (AHS). The AHS allows *C. campestris* to display its entire life cycle *in vitro,* including seed production. Using the AHS, we studied haustorial function, determining the role of nutrients and phytohormones on parasite haustorium development and growth, and found genes involved in haustorial function. The AHS allowed us to demonstrate the positive effect of light on *C. campestris* growth in the absence of a photosynthetic host and to investigate carotenoid- and ABA- related processes in the haustorial regions. We also wanted to understand how *C. campestris* defenses work independently from a plant host, so we studied the parasite responses to the bacterial epitope flg22 and the bacteria *Peudomonas syringe*. Our findings indicate that *C. campestris* is able to sense flg22, but its response differs from those observed in other non-parasite plants. Transcriptomic analysis revealed up-regulation of genes related to biotic and abiotic stresses, and downregulation of genes related to cuticle development. Our study contributes to understanding the *C. campestris* immune response in the absence of a host plant. Taken together, this research contributes novel methodologies that enable insights into *C. campestris* biology without the interference of a plant host on the parasite.

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General Audience Abstract

Field dodder (*Cuscuta campestris*) is a parasitic plant that lacks leaves and roots and attacks a wide range of plants, such as tomato and beets. Dodders are not able to carry out full photosynthesis and thus are incapable of producing enough food or obtaining water to survive on their own, so they parasitize other plants. Dodders have developed specialized structures called haustoria that allow them to take resources directly from their hosts. Studying dodder is challenging due to the dependence of the parasite on its host, such that effects of one plant on the other are hard to disentangle. We developed new tools and methodologies that allow us to explore the biology of dodder in unprecedent ways. We developed an Artificial Host System (AHS) that allows the growth and study of dodder without involving a living host plant. Thanks to this new tool, we were able to improve understanding of the function of the haustorium, discover nutrients and growth factors that are indispensable for dodder development, and prove that dodder growth benefits from light. Using the AHS, we compared haustorial regions and shoot tips of dodder to identify genes specific to haustorial function. Additionally, we studied the responses of dodder to bacteria to understand how it reacts to microbial colonization. Our studies contribute with the development of novel methodologies that allow unprecedent discoveries into the biology of dodder. We expect that this work will promote the study of parasitic plant biology.

To all my family,

my most precious treasure.

Especially to my parents Amparo and Mariano

Acknowledges

This is a perfect opportunity to acknowledge all the people that have played an impactful role in my life, people who helped me to achieve my goals. Family, mentors and friends that have encouraged me to cultivate my passions and interests, they have supported me and taught me simple and complex things that now dance sparklingly in my mind.

Thanks to …

My parents and brother for their always constant and unconditional support every day of my life, you are my foundation!

My husband whose lovely care made of the last months of my Ph.D. a better experience.

My friends for the stimulating discussions, for their disposition to listen to me and for sharing their advice.

My teachers and mentors who have encouraged my curiosity and provided me with incredible learning opportunities.

Dr. Westwood for his support on pursuing new research ideas, which allowed me to develop autonomy, creativity and leadership.

Dr. Boris Vinatzer for giving me the opportunity to come to Virginia Tech in the first place and allow me to learn and contribute to his research program.

Dr. George Norton for his great disposition and patience to teach me about International Development.

Thanks to all the people in the Translational Plant Science Center and Latham Hall, fellow students, postdocs, technicians and professors, who always had a friendly piece of advice and help to build a supportive community.

Table of Contents

List of figures

Chapter 1

Chapter 2

Table S1. Genes involve in carotenoid-related pathways and their expression values (DESeq2 results) in haustorial regions.

Chapter 4

Apendix 1

Attributions

Chapter 3:

Sukhmanpreet Kaur was a PhD student in the Translational Plant Sciences Program at Virginia Tech rotating at Westwood Laboratory at the moment of the study. She helped with extractions of pigments.

Chapter 4:

Hannah Ambrose was an undergraduate research assistant in Virginia Tech at Westwood Lab at the moment of the study. She helped with bacterial growth curves.

Chapter 1: Literature review

Parasitic weeds cause a negative impact on agriculture but may offer advantages to ecosystems

Parasitic plants are important and intractable agricultural pests all around the world. Witchweeds (*Striga* spp.), broomrapes (*Orobanche* spp. and *Phelipanche* spp.), and dodders (*Cuscuta* spp.) are some of the parasitic plants classified as agricultural weeds. These parasites pose challenges for agriculture due to the limited availability of management strategies for their control. *Striga* spp. are root parasites that cause major losses in cereal production, affecting smallholder farmers in Africa, and generating annual crop yield losses of about $7 billion in sub-Saharan Africa alone (Ejeta, 2007). *Orobanche* spp. and *Phelipanche* spp. are root parasites of legumes, tomato, potato, sunflowers, among others (Costea & Tardif, 2006). They represent an important biological constraint to crops grown in a Mediterranean climate, including the Mediterranean area, California and Chile in the Americas, and Australia (Casadesús & Munné-Bosch, 2021). The stem parasite dodders are distributed globally and affect a wide range of crops belonging to different plant families of eudicots (Albert *et al.*, 2008).

Although most studies of parasitic plants are centered on parasitic weeds due to their negative effects on agriculture and forestry, they also have a role in natural ecosystems. Parasitic plants cause a reduction in host fitness and biomass because their growth depends partially or completely on the host. Models have shown that a parasitized host accumulates less biomass than an un-parasitized host (Hautier *et al.*, 2010). From an ecological point of view, parasitic plants reduce biomass of plant communities, alter the structure of microbial communities associated with plants, and enhance the cycling of nutrients like nitrogen in the soil in both natural and agricultural ecosystems (Quested, 2008; Li *et al.*, 2014; Casadesús & Munné-Bosch, 2021). These effects are not necessarily a drawback. Parasitic plants play an important role in their natural ecosystems. For example, they may attach themselves to multiple plant hosts at the same time, generating physical networks between plant communities (Těšitel *et al.*, 2020). Thanks to these connections, systemic signals travel between different host individuals, some of them warning other plants against

herbivore insects and salt stress as has been shown in interactions between *C. australis* and its host (Hettenhausen *et al.*, 2017; Li *et al.*, 2020). Parasitic plants also promote horizontal gene flow in the plant community, for example *C. campestris* has acquired genes from other plants by horizontal gene transfer that seems to be involve in haustorial biology, defense and amino acid metabolism in the parasite (Yang *et al.*, 2019). Parasitic plants may act as microorganism vectors facilitating their transmission between two related or non-related host plants (Hartung *et al.*, 2010; Jelkmann & Kuehn, 2010). These types of plant parasites could be used as control agents for exotic plant species, for instance infestation with *C. campestris* has been shown to have a negative effect on *Mikania micrantha*, a native plant from South America but exotic in Asia (Shen *et al.*, 2005). However, control strategies for weeds using parasitic plants have to be applied with great caution and only using native species, as otherwise the parasitic plant itself could become a constraint for agriculture and natural ecosystems (Těšitel *et al.*, 2020). These close ecological relationships between parasitic plant and their host may contribute to natural and agricultural ecosystem maintenance and development.

Parasitic plants share important characteristics

Parasitic plants are distributed among 12 plant families and represent about 1% of existing flowering plant species (Westwood *et al.*, 2010; Nickrent, 2020). Despite the independent evolution of these different families of parasitic plants, they all possess specific adaptations that allow them to take resources directly from host plants, exhibiting a complete (obligate) or partial (facultative) heterotrophic host dependence (Westwood *et al.*, 2010). They offer a model for evolution of intra-kingdom parasitism, which involve several stages such as host detection, attachment to host roots or shoots, exploitation of host resources, and suppression of host defenses (Bouwmeester *et al.*, 2021). Interactions may also be characterized as compatible or incompatible, depending on the specific parasite, host and the environment.

The most important organ and exclusive trait shared by all parasitic plants is the haustorium. The haustorium (plural: haustoria) is an example of convergent evolution of parasitism since it shows common shared structures and function across evolutionarily independent linages (Westwood *et al.*, 2010). The haustorium is a specialized multicellular structure involved in the attachment to the host, penetration of host tissues, establishment of a close physical connection between parasite and host vasculatures, and exchange of substances, such as nutrients and water (Kuijt, 1969). A

parasitic plant has one of two known types of haustorium depending on the species: terminal haustorium, observed only in roots of obligate parasites, or lateral haustorium observed in stems or roots of facultative parasites and some obligate parasites (Yoshida *et al.*, 2016). For example, species of the genus *Cuscuta* present lateral haustoria produced from their stem tissues.

Cuscuta species are globally important stem parasitic plants

The stem parasite *Cuscuta* spp. is the only genus from the family Convolvulaceae that has a parasitic lifestyle. Nearly 200 *Cuscuta* species have been described, and they are distributed around the world, with about 75% of *Cuscuta* species found in the Americas (Yuncker, 1932). *Cuscuta* is usually spread by seed, and *C. campestris*, originally from North America, has been spread in an almost cosmopolitan level, mostly as a contaminant in seeds of clover (*Trifolium* spp.) and alfalfa (*Medicago* spp.) (Parker, 2012). Due to its wide distribution, this species may be the most agriculturally damaging *Cuscuta* (Goldwasser *et al.*, 2016).

Currently, *Cuscuta* spp. affect thousands of hectares of agricultural land and cause reduced crop yields. They have been reported attacking a wide range of cover and food crops like alfalfa, clover, beet, faba bean, tomato, cranberry, soybean, and others (Parker, 2012). Herbicides have shown some efficacy for controlling *Cuscuta* (Nadler-Hassar *et al.*, 2009), however, their phytotoxicity to parasites varies and may not be completely effective to kill the parasite. *Cuscuta* seedlings present a different herbicide resistance profile than plants that are already attached to hosts. For instance, the *Cuscuta* response to imazamox (a systemic herbicide from the ALS-inhibitors group) varies, with seedlings being resistant while the growth of the parasite that is already attached to imazamox-resistant hosts is inhibited by the herbicide. *Cuscuta* presents a different response to glufosinate, with seedlings being susceptible while *Cuscuta* attached to a glufosinate-resistant host are slightly affected (Nadler-Hassar *et al.*, 2009). The mechanism of this resistance relies on the exchange of the protein phosphinothricin acetyl transferase (PAT), which is responsible for host glufosinate resistance (Jiang *et al.*, 2013). PAT migrates from a glufosinate-resistant host to the parasite through the haustoria, providing resistance to *Cuscuta* against this herbicide until the protein is degraded. This indicates that the parasite benefits from the host herbicide resistance. In contrast, *C. campestris* is sensitive to the carotenoid biosynthesis inhibitors, such as the

herbicides flurochloridone, sulcotrione, and mesotrione, as all of them cause a bleaching phenotype in the parasite (Weinberg *et al.*, 2003).

Cuscuta species present singular morphological and physiological features

All *Cuscuta* spp. have similar vegetative morphology characterized by narrow yellow stems with reduced leaves and vestigial roots (Parker, 2012). Species of this genus are classified in three subgenera: *Monogyna*, *Cuscuta* and *Grammica*. In general, classification follows sexual characters like flower size, floral cluster density, number of floral segments, and style, stigma and capsule traits (Mcneal *et al.*, 2007). *Cuscuta* species are difficult to identify considering only morphological characters due to their homogeneity. Other information like genome size, chloroplast genome sequence, habitat and host preferences can support species taxonomic assignment (Mcneal *et al.*, 2007, Park, et al 2019).

Phylogenetic studies have shown that despite *Cuscuta* spp. lack of a large photosynthetic surface like leaves, most *Cuscuta* species have conserved photosynthetic genes (Mcneal *et al.*, 2007). Nevertheless, photosynthetic capacity varies widely among the members of this genus. For example, *C. reflexa*, *C. gronovii* and *C. campestris* have thylakoids, low levels of the large subunit of rubisco, and the capacity to produce chlorophyll, while *C. odorata* and *C. grandiflora* do not possess thylakoids and chlorophyll has not been detected in their tissues (Van Der Kooij *et al.*, 2000). It has been reported that *C. reflexa* obtains 99% of its carbon from the host despite having photosynthetic machinery (Jeschke *et al.*, 1994; Jeschke and Hilpert, 1997), and *C. campestris* lost genes responsible for high photosynthesis activity and nutrient uptake from the soil (Vogel *et al.*, 2018). Low photosynthetic rates in *Cuscuta* species are not surprising since these plants are holoparasites, and must have a host to obtain carbohydrates needed to complete their life cycle (Westwood *et al.*, 2010). Although it has been proposed that photosynthesis in *Cuscuta* mainly functions to supply lipids for seed production and recycle carbon from respiration (Mcneal *et al.*, 2007), more research is needed to understand why *Cuscuta* species have conserved some genes that are involved in highly energetic cost and complex photosynthetic processes if the carbon yield is not enough to maintain an independent life style from the host (Van Der Kooij *et al.*, 2000).

Cuscuta life cycle

Germination, host localization and attachment

The *Cuscuta* life cycle depends on its close association with a host. *Cuscuta* is an annual plant and its life cycle begins in the spring, when growing in temperate regions. Members of this genus have impermeable seeds that can remain viable in a dormant state in the soil for years and germinate once the epidermal layer has been scarified (Mcneal *et al.*, 2007). After germination, occurring at temperatures between 15-38°C (Hutchison & Ashton, 1979), the thread-like shoot emerges from the soil. *Cuscuta* spp. can follow volatile chemical signals produced by potential host plants while searching for a nearby host (Runyon *et al.*, 2006). Seedlings must find a compatible host within a couple of weeks after germination and make vascular connections, otherwise they will die from starvation.

As soon the parasite shoot has found a host, the attachment phase starts with the shoot tip coiling around the host stem. Light conditions can affect the ability of the parasite to coil on its host stem, and low ratios of red:far-red light exposure increase *Cuscuta* coiling ability (Johnson *et al.*, 2016). Once the coil has been completed, the prehaustorium formation starts in the internal tissues that will become haustoria. At the same time, a holdfast is developed and its surface cells secrete pectins that help the parasite attach to the host epidermis (Vaughn, 2002). Additionally, the production of xyloglucan endotransglucosylase hydrolase (XTH) starts, and this protein is involved in cell wall modification important for penetration of host tissue (Olsen *et al.*, 2016; Olsen & Krause, 2017). At the same time, a reciprocal recognition between both plants may occur. For instance, the membrane receptor CuRe1, in tomato (*Solanum lycopersycum*), recognizes the *C. reflexa* peptide Crip21, a minimal epitope in the cell-wall glycine-rich protein (CrGRP) (Hegenauer *et al.*, 2016, Hegenauer et al. 2020). Upon recognition, a classical plant immune response is activated including generation of Reactive Oxygen Species (ROS) and ethylene production in order to contain the parasite colonization. Additionally, it is possible that multiple host cytoplasmatic genes are also involved in the resistance to *Cuscuta.* This is the case of the *Cuscuta* receptor for Lignin-based Resistance 1 (CuRLR1), a cytosolic receptor, that together with a couple of transcription factors seem to be involved in the recognition of an unknown parasite signal and the activation of local lignification that prevents further *C. campestris* penetration (Jhu *et al.*, 2021). The case involving the membrane receptor CuRe1, and the case involving the

cytoplasmatic receptor CuRLR1 resemble Pathogen Triggered Immunity (PTI) and Effector Triggered Immunity (ETI) respectively, two branches of plant immunity observed during plant pathogen interactions (Albert *et al.*, 2021)

In order to continue with the invasion, the parasite may use phytohormones that affect host responses. Increasing indole-3-acetic acid (IAA, an auxin) in the haustorial region can cause not only changes in haustorial cells but also in the morphology of the host tissues in contact with the haustoria, as described in the case of *C. reflexa* colonizing tomato tissues in an incompatible interaction (Löffler *et al.*, 1999). On the other hand, the host also may use hormones like salicylic acid (SA) and jasmonic acid (JA) to mediate an effective defense against the parasite, for example *C. pentagona* showed a larger growth on hosts that had deficiencies in SA and JA (Runyon *et al.*).

Cuscuta *obtains resources through the haustorium*

Haustorium formation is the most important part of the *Cuscuta* spp. cycle of life because it is essential for establishing a connection with the host and getting resources. Haustorium formation and invasion of host tissue consist of three general stages: 1) initial stage, when parasite stem wraps around the host and initial swelling of the holdfast occurs; 2) penetration stage, when the haustorium grows from parasite through host tissue; and 2) maturation stage, when the pre-haustorium extends from the parasite stem tissues towards the host, and transitions to mature-functional haustorium. At this point, connection is established to host vascular tissues, and the parasite feeds from its host (Lee, 2007; Olsen & Krause, 2017). In the penetrating stage, cells start to divide and elongate, and searching hyphae form and grow between the host cells (Vaughn, 2003). After searching hyphae cells reach host vascular tissues, the mature stage is reached, terminal hyphae cells adjacent to host's xylem elements become xylem elements; terminal hyphae cells adjacent to the host's sieve tubes become absorbing hyphae that closely attach to host sieve tubes; and parenchyma cells in the haustorium differentiate into phloem or xylem as appropriate (Jeschke *et al.*, 1994; Birschwilks *et al.*, 2006).

Once the connections have been completed, *Cuscuta* assumes the role of a strong sink. It takes not only water and minerals from its host, but increases the host photosynthetic rate and competes for assimilates (Jeschke *et al.*, 1994; Birschwilks *et al.*, 2006). In the interaction between faba beans

and *Cuscuta*, the parasite changes the direction of photo-assimilate transport until it becomes a sink that impedes pod and fruit development (Wolswinkel, 1975).

In compatible interactions, after *Cuscuta* has successfully established, the parasite propagates through shoots as well as seeds. *Cuscuta* produces new shoots that grow in toward other host stems following light clues in order to make new attachments (Johnson *et al.*, 2016). The parasite flowers and produces capsule fruits. Interestingly, *C. australis* has been reported to synchronize its flowering time with the host, relying on host flowering signals (Shen *et al.*, 2020). However, the flowering process in other *Cuscuta* species has not been fully studied and other signals may be involved in flowering control.

Haustoria are not only channels for resources uptake but also highways for genetic information exchange

Molecules exchanged through the parasite-host connections are diverse in nature, among them water, metabolites, herbicides, proteins, viroids and phytoplasmas (Kim & Westwood, 2015). Several studies have shown the movement of molecules from host to parasite tissues, including: sugars (Tsivion, 1978), amino acids (Birschwilks *et al.*, 2006), the reporter protein GFP, the tobacco mosaic virus movement protein and other fluorescent probes (Haupt *et al.*, 2001; Birschwilks *et al.*, 2006). Protein movement is influenced by its size as was demonstrated by host-to-parasite translocation of GFP, but not a GFP-ubiquitin fusion (Birschwilks *et al.*, 2007). Studies also have shown how *Cuscuta* spp. can act as bridges between two different plants (Birschwilks *et al.*, 2006, 2007). Some examples correspond to the movement of GFP from a transgenic host to a wild-type plant, and of a virus from a carrier host to a new one through *Cuscuta*.

Additionally, genetic material, like DNA, mRNA and miRNA, also move in a bi-directional way between parasite and host (Kim & Westwood, 2015). It has been reported that at least three mitochondrial genes have been transferred from *Cuscuta* to *Plantago* (Mower *et al.*, 2010). Although the mechanisms have not been completely clarified yet, this suggests horizontal gene transfer via a strictly DNA-level intermediate (Yang *et al.*, 2019). On the other hand, *Cuscuta* and its host bi-directionally exchange a large amount of mRNA while growing on Arabidopsis or tomato (Kim *et al.*, 2014), and proteins when growing on Arabidopsis and soybean (Liu *et al.*,

2020). Additionally, the production of miRNAs and their translocation from *Cuscuta* to its host has been demonstrated (Shahid *et al.*, 2018). These molecules are involved in the production of some siRNA in the host that can silence genes in the host in order to increase the virulence of the parasite.

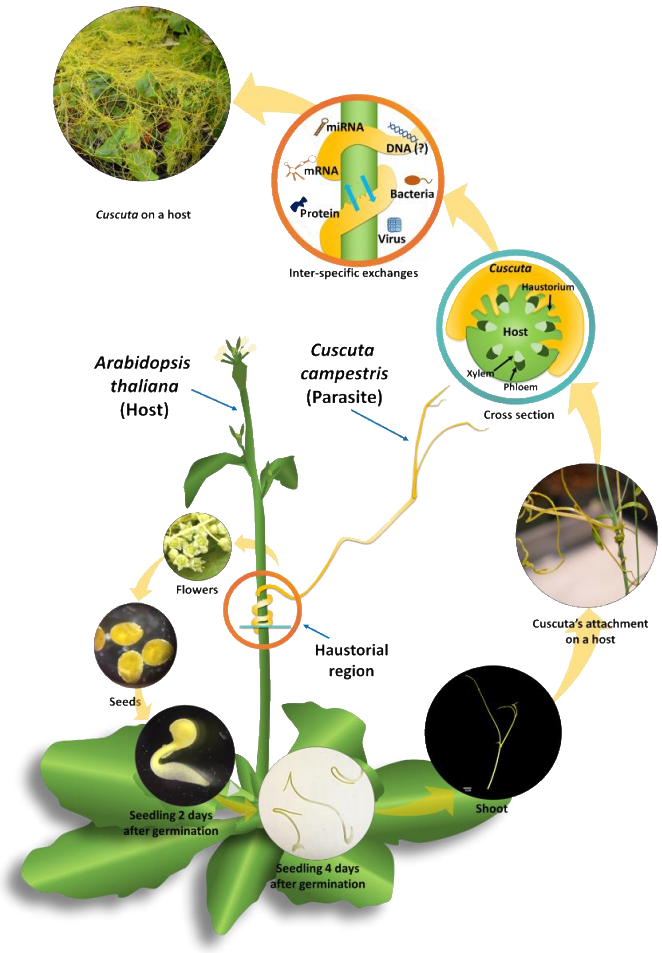

Fig. 1 *Cuscuta campestris* life cycle

Experimental methodologies that enable the study of *Cuscuta* biology and the interaction with its host need to be developed

Cuscuta *growth*

Interest in *Cuscuta* research is increasing, but there is no consensus on how to conduct experiments with this plant. Most of the basic experimental protocols for growing and evaluating *Cuscuta* have

not been standardized, leading to variability in data collected by different researchers. For example, studies differ in the units of inoculation/propagation used. A survey of the literature shows that several starting forms of parasite shoots, seedlings and seeds were used, as well as shoots cut from nursery plants (David-Schwartz *et al.*, 2008; Bleischwitz *et al.*, 2010; Goldwasser *et al.*, 2012; Alakonya *et al.*, 2012; Kim *et al.*, 2014; Li *et al.*, 2014; Connie Tjiurutue *et al.*, 2016; Hegenauer *et al.*, 2016, 2017; Furuhashi *et al.*, 2016). Some studies used pre-germinated *Cuscuta* seedlings while others used seeds that had been germinated *in situ*. Others used different sizes of vegetative shoots coming from a nursery plant, in some cases with a preliminary training by having them coil on sticks before final inoculation on the host plant. Additionally, some studies monitor *Cuscuta* success by measuring parameters like fresh weight, biomass or length without considering the large variation that the plant exhibits while growing on different hosts. Therefore, developing a set of standardized techniques would be beneficial so data produced by different studies could become easily comparable.

Transformation and in vitro culture

The availability of new sequencing technologies has allowed the production of a large amount of transcriptomic and genomic data during the interaction of *Cuscuta* with its hosts (Kim *et al.*, 2014; Shahid *et al.*, 2018). However, progress in understanding the function of specific genes, mostly in the parasite, remains challenging because there is no methodology for stable genetic transformation of *Cuscuta*.

Previous efforts to transform *Cuscuta* species have been partially successful, but have not resulted in regeneration of a transformed plant. In one report, *C. trifolii* callus was obtained after 4-6 months of culture in liquid induction media, and was transformed with *Agrobacterium tumefaciens* (Borsics *et al.*, 2002). Although transgenic callus was observed, no new whole transgenic plants were reported. Later, a transient transformation was accomplished for studying actin filament organization and cytoskeleton formation. This transformation was achieved for *C. europea* using *A. tumefaciens* LBA4404 and GFP as a marker gene, however they report a relatively short period of marker expression in the transgenic tissue (Kaštier *et al.*, 2017). Another study reports the transformation of *C. europea* callus for up to 21 days. However, this transformed tissue failed in its morphogenic and regeneration potential (Švubová & Blehová, 2013). In our lab, we developed

and tested different strategies for *C. campestris* transformation (Fig. 2), and although we have been able to obtain transient transformation, stable transformation is still elusive. *Cuscuta* transformation is challenging due to limiting factors like poor knowledge of the parasite biology while growing *in vitro* conditions, and restricted availability of different types of target tissues; for example, the transformation of other plants starts with leaf tissue, this is not possible in *Cuscuta* due to its lack of leaves.

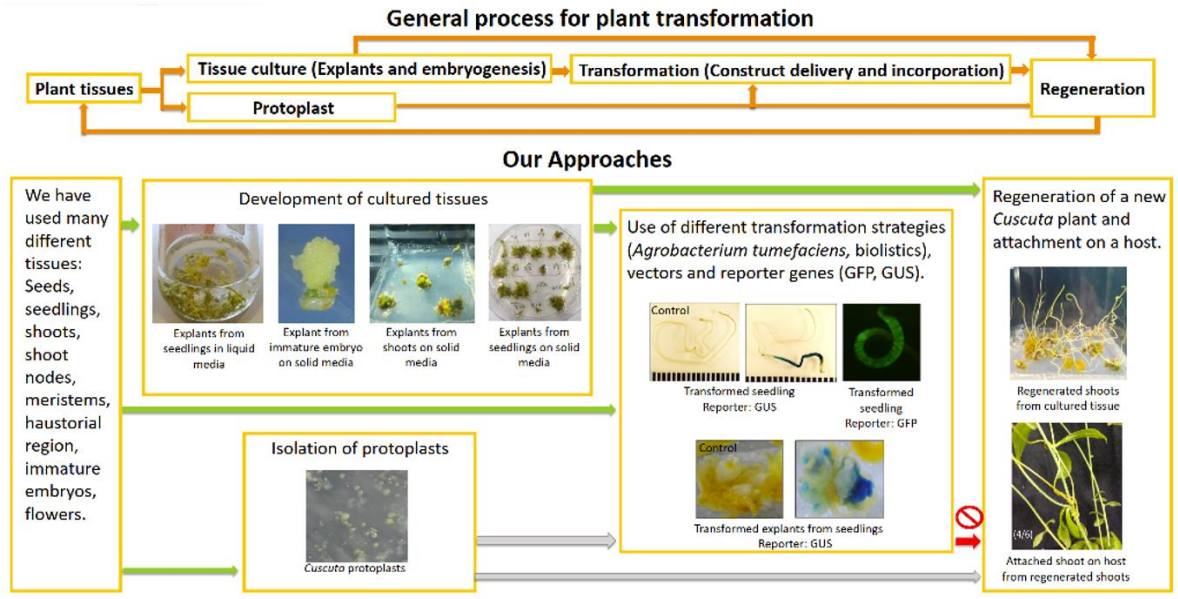

Fig. 2 Different strategies for *C. campestris* transformation tested in the Westwood Lab.

In conclusion, novel methodologies that facilitate the use of new molecular tools, like gene-editing, are needed to explore and understand the biology of *Cuscuta* and the molecular basis of parasitism. *Cuscuta* has drawn the interest of scientists for over 150 years (Engelmann, 1859). However, recently this interest has had an extraordinary resurgence partly due to recent reports of exchange of genetic material between the parasite and the host. Methodologies such as standardized guidelines for growing the parasite and parasite stable transformation may be of great help to improve parasite manipulation and experimentation. These insights have the potential for biotechnological advances, parasitic weed control and research on intra-kingdom parasite evolution.

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Chapter 2: An artificial host system enables the obligate parasitic plant *Cuscuta campestris* to grow and complete its life cycle *in vitro*

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This chapter is publicly available on BioRxiv (doi: 10.1101/2021.06.21.449293) and was submitted to New Phytologist Journal on June 25, 2021

Summary

- *Cuscuta campestris* is an obligate parasitic plant that requires a host to complete its lifecycle. Parasite-host connections occur via an haustorium, a unique organ that acts as a bridge for the uptake of water, nutrients and macromolecules. Research on *Cuscuta* is often complicated by host influences, but comparable systems for growing the parasite in the absence of a host do not exist.
- We developed an axenic method to grow *C. campestris* on an Artificial Host System (AHS). We evaluated the effects of nutrients and phytohormones on parasite haustoria development and growth. Haustorium morphology and gene expression were characterized.
- The AHS consists of an inert, fibrous stick that mimics a host stem, wicking water and nutrients to the parasite. It enables *C. campestris* to exhibit a parasitic habit and develop through all stages of its lifecycle, including production of new shoots and viable seeds. Phytohormones NAA and BA affect haustoria morphology, and increase parasite fresh

weight and biomass. Gene expression in AHS haustoria reflect process similar to those in haustoria on actual host plants.

- The AHS is a methodological improvement for studying *Cuscuta* biology by avoiding specific host effects on parasite and giving researchers full control of the parasite environment.

Key words: field dodder, haustorial gene expression, *in vitro* growth, obligate parasitic plant

Introduction

Dodders are parasitic plants of the genus *Cuscuta* (Convolvulaceae) that can invade a wide range of dicot plants. *Cuscuta* spp. are characterized by their vining growth habit and lack of roots and expanded leaves. They use an organ unique to parasitic plants, the haustorium, to invade host stems to gain access to essential nutrients and water resources. *Cuscuta* spp. exhibit compromised or no photosynthetic activity (Van Der Kooij *et al.*, 2000), so they rely at various levels on their hosts as their only carbon source. Thus their survival depends on parasitic features like the ability to find a host, produce functional haustoria and efficiently take up resources.

Cuscuta spp. are obligate parasites, so must have a host to complete their life cycles (Westwood *et al.*, 2010). The *Cuscuta* spp. life cycle comprises the stages seed, seedling, host colonization, haustoria development, vegetative growth, flower production, and fruit and seed set. *Cuscuta* seeds germinate on the soil without any chemical stimulant (Benvenuti *et al.*, 2005), and the thread-like seedlings must find a suitable plant host to avoid death due to exhausting its limited resources (Shimizu & Aoki, 2019). After the parasite has found a host, its shoot coils around the host stem, and the haustorium starts to develop from within the internal face of the coil. Coiling and haustorium formation respond positively to the exposure to enriched far-red light (700-800 nm) and tactile stimuli (Lane & Kasperbauer, 1965; Tada *et al.*, 1996). These stimuli provoke an increase in internal levels of the phytohormone cytokinin, leading to haustoria induction (Haidar *et al.*, 1998; Furuhashi *et al.*, 2011).

Haustorium development involves tissue dedifferentiation and redifferentiation, and can be divided into three phases: adhesive, intrusive, and conductive (Heide-Jørgensen, 2008). In the adhesive phase, epidermal cells redifferentiate into trichome-like cells that form a holdfast or adhesive disc (Vaughn, 2002). Holdfast cells secrete pectin and other compounds that mediate the adhesion of parasite tissues to host tissues. Meanwhile, meristematic cells in the inner cortex behind the holdfast generate the prehaustorium (Shimizu & Aoki, 2019).

During the intrusive phase, the prehaustorium becomes the haustorium (Heide-Jørgensen, 2008). The haustorium, formed by intrusive cells, elongates through the center of the holdfast and penetrates the host stem epidermis and cortex (Vaughn, 2003). Then terminal digitate cells, or searching hyphae, elongate to encounter host vascular elements. Initial haustoria formation is independent of host factors, however, they may be required for further differentiation of xylem vessels (Kaga *et al.*, 2020). Searching hyphae cells adjacent to host xylem elements become xylem hyphae, while those adjacent to sieve tubes become absorbing hyphae. Subsequently, cortex cells in the haustorium also differentiate into phloem or xylem elements as appropriate (Vaughn, 2006; Shimizu & Aoki, 2018; Shimizu *et al.*, 2018).

The conductive phase starts after searching hyphae differentiate to vascular conductive elements in the mature haustoria, completing the vascular connection between the host and the parasite (Heide-Jørgensen, 2008). The parasite becomes a strong sink competing with the host for resources as exemplified by *C. reflexa*, which obtains 99% of its carbon from the host (Jeschke *et al.*, 1994). Once the connections are established, the *Cuscuta* shoot resumes growth to produce new shoots that reach other host stems, making additional points of connection and ultimately produces flowers, fruits, and seeds.

Recent studies have described interesting features of the host-*Cuscuta* system including the reciprocal exchange of large amounts of macromolecules such as proteins (Liu *et al.*, 2020), mRNAs (Kim *et al.*, 2014), and microRNAs (Shahid *et al.*, 2018). Some of the exchanged microRNAs have been shown to function by affecting gene expression in the host plant (Shahid *et al.*, 2018). There is an urgent need to develop new methods to study *Cuscuta* in ways that can

distinguish parasite processes from host processes in order to define the contributions of each to the interaction.

One approach to studying *Cuscuta* in the absence of a host is through tissue culture. Published methodologies exist for obtaining callus and shoot regeneration for *C. trifolii* (Bakos A. *et al.*, 1995; Borsics *et al.*, 2002), *C. europea* (Švubová & Blehová, 2013), and *C. reflexa* (Srivastava & Dwivedi, 2001), and floral induction for *C. reflexa* (Baldev, 1962). Other studies reported the production of haustoria by applying a mechanical pressure between two surfaces, along with a far-red light stimulus on shoots of *C. japonica* (Tada *et al.*, 1996; Furuhashi *et al.*, 1997), *C. reflexa*, and *C. campestris* shoots (Kaga *et al.*, 2020; Lachner *et al.*, 2020). These systems have certain advantages, but tissue culture does not allow investigations related to haustorial form or function, and none of them accurately reflect normal *Cuscuta* parasitic growth habit with production of new shoots and viable seeds.

Here we report the development of an axenic system to grow *C. campestris* on an artificial host that provides water, nutrients, and phytohormones to the parasite. We demonstrate that *C. campestris* growing in this Artificial Host System (AHS) exhibits all the stages observed during its typical lifecycle, including flower, fruit, and production of viable seeds. Functional characterization, including transcriptomic analysis, of the haustorial region of *C. campestris* growing in the AHS shows growth that is similar to that on a living host.

Materials and methods

Preparation of the artificial host system

The AHS was assembled using a magenta GA-7 plant culture box (Sigma) and a plastic autoclavable lamina (Polypropylene No. 5, 0.4 mm thick) cut to fit the bottom of the box and serve as a support to hold the artificial hosts (Fig.**1**, **S1**). The paper spindle from a cotton swab was used as the "host stem". Each magenta box held four spindles. When *Cuscuta* seedlings were used as inoculum, 0.2ml microcentrifuge tubes were attached to the base of each spindle to hold the seedling. All the system components, magenta boxes, sheets with artificial hosts and aluminum foil strips (used to attach the plant to the artificial host) were autoclaved before assembly.

Media preparation

Modified Murashige and Skoog liquid media (MMS) (Srivastava & Dwivedi, 2001) was used as base media for most of the experiments. It contained 300 mg l^{-1} NH_4NO_3, 800 mg l^{-1} KNO_3, 250 mg l^{-1} $CaCl_2.2H_2O$, 260 mg l^{-1} $MgSO_4.7H_2O$, 120 mg l^{-1} KH_2PO_4, and 3% glucose, pH 5.7. Sterile 1X MS micronutrients (Phytotechlab, USA) were added after autoclaving. This MMS media was supplemented with filter-sterilized phytohormones 1-naphthaleneacetic acid (NAA) (3 mg l^{-1}) or 6-benzylaminopurine (BA) (1 mg l^{-1}) for specific experiments.

Preparation of parasitic plant material

Vegetative *C. campestris* shoot tips were used as inoculum for most of the experiments. Nurseries of the parasite were established on beets (*Beta vulgaris*, Detroit Dark Red) as follows. *C. campestris* seeds were scarified by immersion in sulfuric acid (95%) for 20 min, followed for 3 washes with water for 10 minutes. Seeds were germinated on wet filter paper in Petri dishes for 4 days, then seedlings were attached to petioles of one-month-old beet plants using a small piece of tape. Host and parasite were placed under direct full spectrum light (Mercury-Vapor lamp) and far-red enriched light (Sylvania Spot-Gro bulb, enriched in >750nm wavelength, 35 µmol $m^{-2}s^{-1}$) under 14h light /10h dark photoperiod. Plants were watered as needed and fertilized with All Purpose Plant Food (Miracle-Gro, USA). Once the *C. campestris* plants produced large numbers of shoots (around 1.5 months after inoculation), 3 cm shoot tips were harvested for inoculum. Shoot tips were surface sterilized with 3.5% sodium hypochlorite and 0.04% Tween20 for 10 min, followed by 5 washes with sterile water just before attaching them to the artificial host.

When seedlings were used as inoculum, parasite seeds were scarified as described above and surface sterilized for 5 minutes with 3.5% sodium hypochlorite and 0.1% SDS followed by 5 washes with sterile water. Sterilized seeds were germinated as described above under sterile conditions. For the germination of seeds collected from plants growing in the AHS, mature seeds were scarified and germinated as described above, except the scarification period was 7 min. Seedlings and new shoots were attached to *Arabidopsis thaliana* inflorescences under light conditions described above. Aracones (Arasystems, Belgium) were used to increase relative humidity in the parasite-host microenvironment and to avoid the parasite growing onto neighboring plants in later stages.

Artificial host system assembly

Working under aseptic conditions, surface-sterilized *C. campestris* shoots were attached to a spindle using a sterile strip of aluminum foil that held the parasite shoot securely to the spindle with a gentle twist of the foil (e.g., Fig. **2a,b**). The foil was placed approximately 1 cm from the tip of the *C. campestris* shoot, and each parasite shoot was placed high enough on the spindle to keep its lowest extremity away from any contact with media in the bottom of the box. After attaching the shoots, liquid media was added to the box, avoiding touching the plants. Magenta boxes were sealed with parafilm and placed under a Sylvania Spot-Gro bulb for 2.5 days with a 14h light/10h dark photoperiod at 33°C. Boxes were then transferred to a growth chamber under full spectrum light (61 µmol $m^{-2}s^{-1}$, 25°C). Relative humidity in the boxes with liquid media reached 100%, whereas boxes without media were at 43%. AHS boxes were kept under full spectrum light at 25°C for 33.5 days in the case of growth assays, this time was reduced to 12.5 days for other assays like RNA-seq. When seedlings were used as inoculum, the procedure was the same except a 3-day-old *C. campestris* seedling was placed in a 0.2ml microcentrifuge tube containing 150 µl of sterile water to keep the seedling hydrated and the tube was attached to the base of each spindle.

Measuring parasite growth

For evaluating parasite growth, AHS boxes were opened, plants were detached from artificial hosts, extended on a white surface with a ruler, and photographed. Pictures were analyzed using ImageJ 1.51j8 (National Institutes of Health, USA) to obtain the total length of the plant, including all branches and the initial 3 cm shoot. Shoots were then weighed to obtain fresh weight and dried at 50°C for four days to before reweighing to obtain biomass data (dry weight). Data were collected from three independent experiments with a total of 96, 105 and 76 plants, respectively. Only plants that coiled and produced healthy haustoria were considered for growth analyses. Statistical analysis used JMP Pro 15 (SAS Institute Inc.).

CFDA Assay

C. campestris plants were grown for 15 days in AHS containing MMS media with NAA and BA. The spindle "hosts", along with the connected parasites, were then transferred to a 1.5-ml microcentrifuge tube containing the same media plus 0.3 mg ml^{-1}carboxyfluorescein diacetate

(CFDA, initially dissolved in DMSO) and held inside a closed sterile glass tube. Negative controls consisted of the equivalent concentration of DMSO without CFDA. In this way, the spindle conducted media containing CFDA to the point of the parasite attachment that was the only point of transfer to the parasite. After four days, plants were detached, rinsed thoroughly with water, and examined for CFDA fluorescence using a stereoscope under UV light with an EGFP filter.

Microscopy

C. campestris was grown in the AHS with MMS, NAA and BA. Plants were collected 16 days post inoculation (dpi), hand-sectioned, and stained with Toluidine Blue-O. Cross-sections were evaluated under an inverted microscope (Olympus CKX53) equipped with a camera (Olympus EP50).

RNA-seq and transcriptomic analysis

Vegetative parasite shoot tips were introduced to the AHS containing MMS media with NAA and BA. After 15 dpi, tissue was collected for RNA extraction (Fig. **S4**). Plants with healthy holdfasts and newly developed shoots were detached from the artificial host. For haustorial samples, regions harboring visibly healthy holdfasts (identified by formation of new shoots) were separated from any shoot or lateral meristem. For non-haustorial stem samples, new healthy and turgid shoots were collected after removing 5 mm of the stem connecting to the haustorial region to avoid tissue contamination. All tissues were immediately frozen in liquid nitrogen. Samples from 3-4 plants were pooled for each replicate and four replicates were used for each type of tissue. For RNA extraction, frozen tissue was ground using glass beads in a bead beater and extracted using the RNeasy Plant Mini Kit (Qiagen). RNA was precipitated using 3M NaOAc and Ethanol 100%, and resuspended in RNAse-free water. RNA concentration and purity were evaluated using a Nanodrop OneC (ThermoScientific), and quality was confirmed using an RNA TapeStation System (Agilent). A mRNA library was prepared using Poly-A enrichment, and sequencing was performed by Novogene using Illumina NovaSeq 6000 platform with PE150.

Bioinformatic analysis

RNA-seq raw data quality was initially evaluated using FASTQC v.0.11.9 (Braham Bioinformatics). Then, FASTP v.1.0.0 (Chen *et al.*, 2018) was used to detect and trim adaptors, and filter out sequences shorter than 50nt, with low quality or low complexity. Clean reads were

mapped with STAR 2.5.2b (Dobin *et al.*, 2013) against the genome of *C. campestris* (Vogel *et al.*, 2018). Gene counts obtained using STAR 2.5.2b were processed by DESeq2 1.30.1 (Love *et al.*, 2014) using R (https://www.r-project.org/), with differential expression analysis using the default settings and a threshold of genes with more than 10 counts. To increase the stringency of differentially expressed gene (DEG) detection and filter out biases related to the structured distribution of the samples (haustoria vs stem from the same plant pool), gene counts were also processed by edgeR 3.32.1 (Robinson *et al.*, 2010; McCarthy *et al.*, 2012) using R to perform a second DEG analyses accounting for plant pool as blocking factor using the option of generalized linear model - Likelihood Ratio Test (glmLRT). Genes with low counts were filtered using count-per-million (CPM) and normalization was done using trimmed mean of M-values (TMM) according to default settings. DEGs were determined by the genes in the intersection from the two approaches and considering thresholds at adjusted p-value (padj) < 0.05 for DESeq2 or False Discovery Rate (FDR) < 0.05 for edgeR.

Protein sequences reported for *C. campestris* (Vogel *et al.*, 2018) were used for a functional annotation using eggNOG-mapper v2 (Huerta-Cepas *et al.*, 2017, 2019) to obtain their associated GO terms. Then a Kolmogorov-Smirnov like enrichment test (also known as GSEA) was performed. Enrichment analysis was done independently for down- and up-regulated genes. DEGs, their expression p-value according to DESeq2, and their GO terms were analyzed by topGO R package version 2.42.0 (Alexa & Rahnenfuhrer, 2021) considering the settings weight01 and NodeSize 10. Top enriched GO terms (p-value ≤ 0.001) were plotted using REVIGO (Supek *et al.*, 2011) under default conditions.

Comparison with other *C. campestris* expression datasets

Genes found to be up-regulated in the haustorial region of *C. campestris* growing in AHS (with a padj < 0.01) were compared to other published gene expression datasets (Fig. **S4**). The first selected dataset corresponds to up-regulated genes in micro-dissected haustoria tissue, 87 hours after the forced induction of haustoria while in contact with a leaf of *A. thaliana* (Kaga *et al.*, 2020). The second dataset corresponds to up-regulated genes in fully formed haustorial region of *C. campestris* (formerly published as *C. pentagona*) growing on two hosts, *Solanum lycopersicum* and *Nicotiana tabacum* (Ranjan *et al.*, 2014). A third dataset was taken from Kim, *et al.* (2014), that used functional connections between *C. campestris* (also formerly published as *C. pentagona*)

and *A. thaliana*. In this case, we used clean gene counts of total expressed genes in the interface (corresponding to the haustorial region) and parasite (stem tissue) to perform DEG analyses. Data corresponding to three replicates was processed by to edgeR 3.32.1 1 (Robinson *et al.*, 2010; McCarthy *et al.*, 2012) to perform a DEG analysis accounting for batch effect and using default settings. DEGs were determined by FDR < 0.05.

Genes IDs used by Vogel *et al.* (2018) for *C. campestris* were used as a convention. A BlastX (-evalue 1e-50, -max_target_seqs 1, -max_hsps 1) against the proteins reported by Vogel *et al.* (2018) was used to retrieve the gene IDs that correspond to the data obtained from Ranjan *et al.* (2014) and Kim *et al.* (2014). Gene IDs utilized by Kaga *et al.* (2020) were used directly as they correspond to Vogel *et al.* (2018) gene IDs. We grouped the datasets of Ranjan *et al.* (2014) and Kim, *et al.* (2014) based on their study of functional haustorial regions of *C. campestris* growing on a plant host.

Once the shared upregulated genes in haustorial regions among the selected studies were identified, we used the *C. campestris* protein sequences in Vogel *et al.* (2018) to identify their homologues in *A. thaliana* TAIR10 (Phytozome v12.1.6) using BlastX (-evalue 1e-50, -max_target_seqs 1, -max_hsps 1). Candidate *A. thaliana* homologues were subjected to Single Enrichment Analysis (SEA) using agriGO v2.0 (Tian *et al.*, 2017) using Go slim and Phytozome v11 as reference, with default settings.

Results

An artificial host system for growing *C. campestris* under axenic conditions

We developed a system that provides the essential components for *C. campestris* growth in a way that mimics growth on a plant host. The main elements of the AHS are a solid vertical support with capillary capacity, liquid media with proper nutrients and phytohormones, and a translucent container to hold these together under aseptic conditions (Fig. **1, S1**). We evaluated several materials for use as artificial hosts, and found the best to be the paper spindle from a cotton swab, which has a firm structure, yet sufficient porosity to allow the movement of liquid media by capillary action. This vertical artificial host "stem" provides a tactile stimulus that, when combined with far-red enriched light, induces the parasite to coil and form haustoria (Tada *et al.*, 1996;

Furuhashi *et al.*, 2011). The AHS works with *C. campestris* shoots originating from either vegetative cuttings or seedlings (Fig. **1**). However, shoot tips exhibited a higher rate of coiling and holdfast production (84% and 82%, respectively) than seedlings (74% and 49%, respectively). Therefore, we used shoot tips for further experiments.

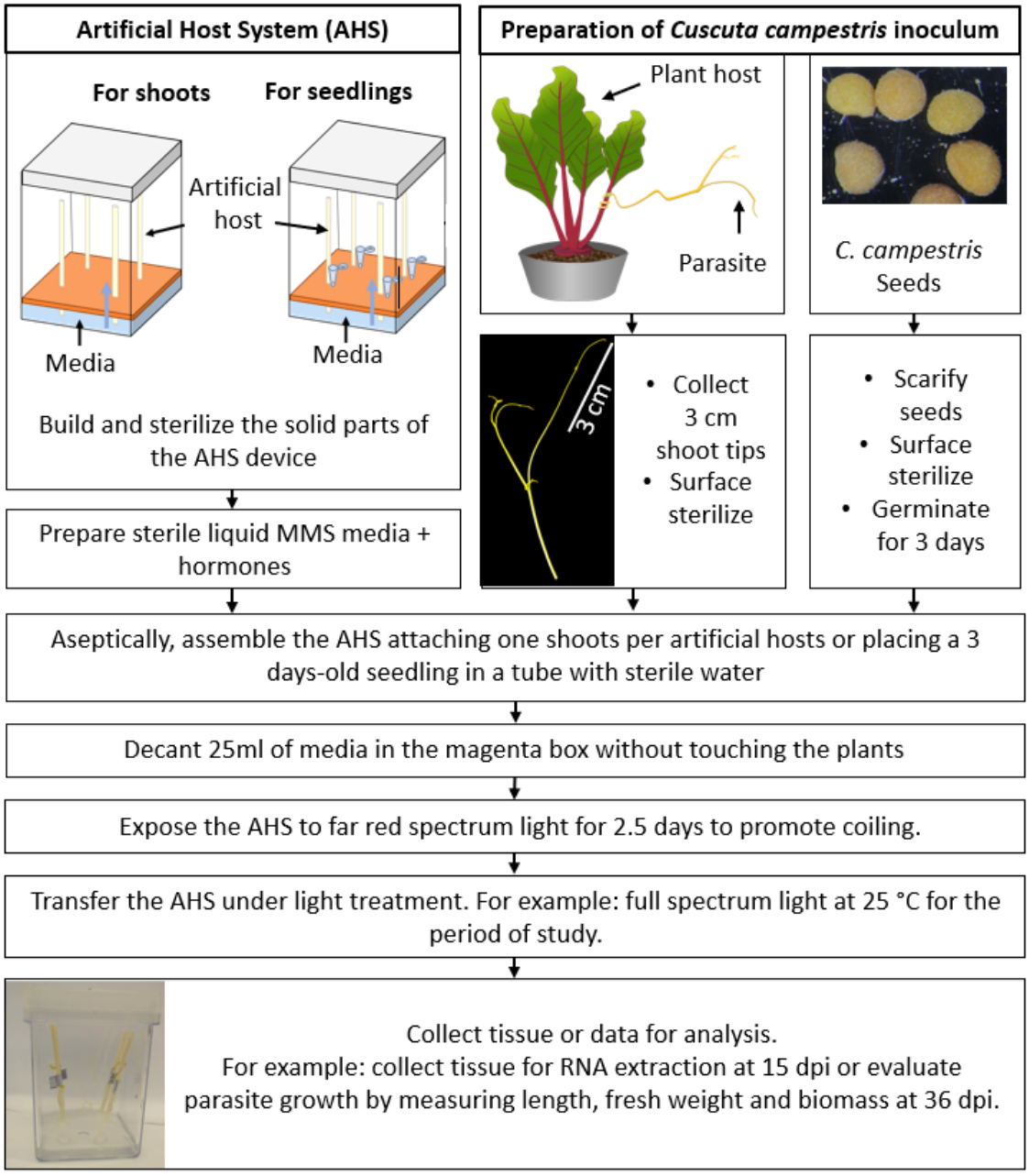


Fig. 1. Schematic depiction of the artificial host system (AHS) and the key steps needed to grow *Cuscuta campestris* on the artificial host. Days post inoculation (dpi).

C. campestris displays all lifecycle stages under the AHS conditions

Cuscuta species are obligate parasites, so normally cannot grow beyond the seedling stage without a plant host (Kaiser *et al.*, 2015), but the AHS adequately substituted for the host, enabling *C. campestris* to complete all life stages. We attached 3-cm shoot tips to artificial hosts and evaluated their development up to 36 dpi. At 2.5 dpi, parasites had coiled and formed holdfast structures on the shoot in contact with the artificial host (Fig. **2a,b**). We consider the presence of a holdfast to be a sign of initiation of haustorial development. At 15 and 36 dpi parasites had conspicuous enlargements in the haustorial region and produced new vegetative shoot growth, flowers, or both (Fig. **2c-e**). After 36 dpi, some plants were still elongating (Fig. **2d, S3a**) whereas others had long shoots that had died back to a small section (< 3 cm) of green turgid tissue at the tip, which we considered to be senescent shoots (Fig. **S3a**). In the cases where artificial hosts were supplied with pure water or no media at all, *C. campestris* was not able to grow (Fig. **2f, S2**). Generally, no healthy holdfasts developed under these treatments, but some formed when just water was applied, resulting in a slight increase in fresh weight. However, addition of nutrient media led to significant gains in length and biomass (Fig. **S2**).

C. campestris colonizes new hosts through either vegetative shoot branches or seedlings coming from germinated seeds. To be useful, the AHS should be able to support growth of *C. campestris* from both sources. We confirmed vegetative propagation using shoot cuttings from AHS-grown plants at 36 dpi by taking active shoots of at least 3 cm and applying them to new plant hosts (Fig. **S3**) as well as new artificial hosts. These shoots were viable, coiled, produced holdfasts, and grew on both types of hosts. For propagation by seed, we scarified the AHS-produced seeds and determined that they are able to germinate (Fig. **2g**), with a germination rate of 28% (n=47). We attached these seedlings to new plant hosts as well as new artificial hosts and found that they are able to grow on plant hosts as well as on artificial hosts (Fig. **2h,i**).

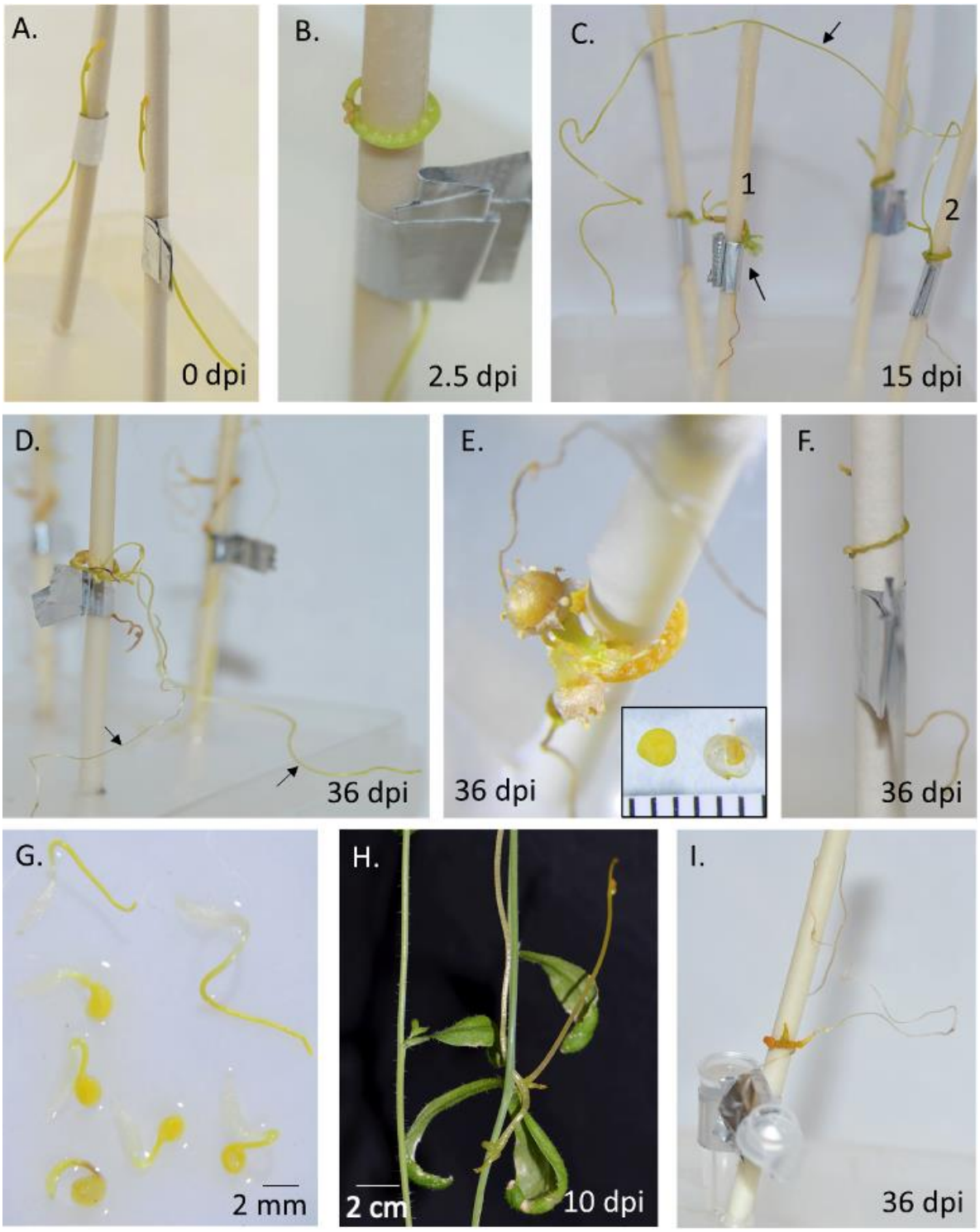


Fig. 2. *Cuscuta campestris* grows without a plant host under the controlled conditions of the artificial host system (AHS). **a-f.** *C. campestris* 3 cm shoot tips were used as inoculum. **a**. 3 cm shoot tips attached to an artificial host at 0 days post inoculation (dpi). **b**. Parasite coiled with holdfasts after 2.5 dpi. **c**. Parasite at 15 dpi, growth is evident as flower development (arrow) in

plant No. 1 and production of a new shoot in plant No. 2 **d**. New shoots elongated, 36 dpi. **e**. Parasite flowered and produced seeds (inset; scale is in millimeters). **f**. *C. campestris* attached to an artificial host without any media or water at 36 dpi. Parasite did not develop swollen holdfast or subsequent growth. **g**. Seedlings after scarification and germination of seeds obtained from *C. campestris* grown in the AHS. **h**. Seedling from seed obtained through AHS colonizing *A. thaliana* 10 dpi. **i**. Seedling growing 36 dpi after being used as inoculum in a second AHS.

The parasite in the AHS feeds through the haustorial region

The haustorium is the signature organ of parasitism in parasitic plants and is essential for uptake of water and nutrients from the hosts (Kuijt, 1977). To characterize the ability of haustoria to take up resources, we added the phloem-mobile dye CFDA (Grignon *et al.*, 1989) to the media and monitored its uptake and translocation into the parasite. CFDA is not fluorescent, but once inside a cell it is cleaved to carboxyfluorescein, which is fluorescent. We observed the assimilation of fluorescent signal in the parasite tissues, both in association with the holdfast/haustorium region and in scale leaves that cover apical and lateral meristems, which are located away from the haustorial region (Fig. **3**). These observations indicate that the haustoria of *C. campestris* growing on the AHS function in uptake of small molecules.

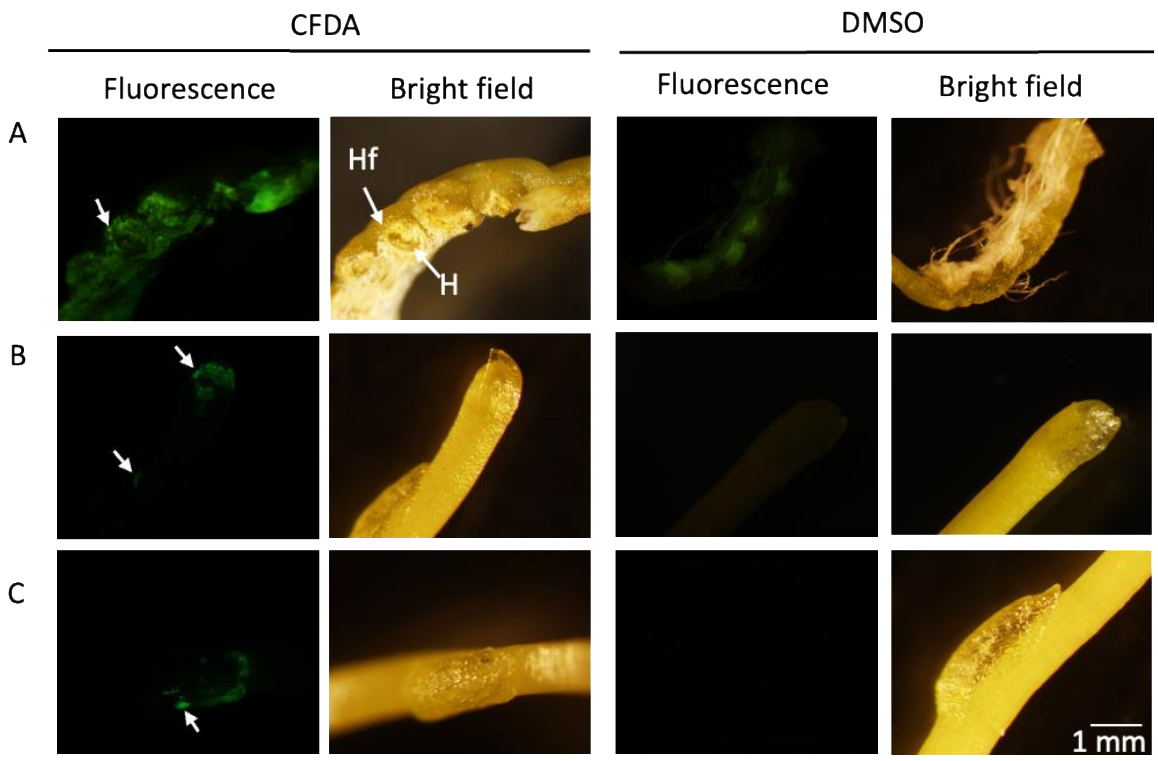


Fig. 3. *Cuscuta campestris* growing in artificial host system (AHS) exposed to the fluorescent dye carboxyfluorescein diacetate (CFDA) or DMSO solvent control from the artificial host. **a.** Haustorial region. Cells of the holdfast show fluorescence. Holdfast (Hf), Haustorium (H). **b** and **c** Scale leaves protecting apical and lateral meristems.

The AHS allows study of *C. campestris* under controlled axenic conditions

Establishment: Parasite establishment is independent of the media composition, although holdfast morphology is sensitive to external phytohormones

To evaluate the impact of media composition on parasite establishment (here defined as stem coiling and formation of the holdfast), we applied *C. campestris* shoot tips to artificial hosts supplied with six different media treatments: nothing (no media at all), pure water, MMS media, MMS with NAA, MMS with the BA, and MMS with NAA plus BA. The coiling phase occurred by 1 dpi and we found no significant differences in the coiling rates among the treatments (Fig. **4a**). The holdfast formation phase occurred by 2 dpi, and the percentage of formation was similar among all treatments except for the dry spindle (Fig. **4g**). The media composition did affect

holdfast morphology. At 8 dpi, holdfasts showed altered phenotypes in response to the different media compositions (Fig. **4b-g**). Parasite holdfasts exposed to MMS media with NAA and or BA exhibited a more complex structure than ones exposed to MMS media or water, and inclusion of BA produced the greatest effect, leading to more developed and thicker holdfasts (Fig. **4c,e**) that more closely resembled those formed on a plant host such as *N. benthamiana* (Fig. **4h**). To further examine the parasitic organ produced on the artificial host, we inspected the anatomy of the haustorial region. The holdfast cells form an adhesion disc on the outer section of the haustoria that adhered firmly to the artificial host and resisted removal as evidenced by spindle fibers remaining attached to the cells (Fig. **5a-c**). Cross-sections of the holdfasts revealed that each haustorial region contains a range of holdfasts at different developmental stages (Fig. **5d-f**). Immature organs, usually located at the extremes of the haustorial region, had holdfasts but no haustorial development (Fig. **5d**). Mature organs with a developed haustorium were often located in the center of the haustorial region, and these showed substantial protrusion toward the artificial host (Fig. **5e,f**). Within the files of elongated cells radiating toward the host, tracheary elements of xylem were identifiable by their ringed secondary walls (Fig. **5e,f**). Although our sectioning did not reveal a continuous file of tracheary elements, we observed them both near the parasite stem and close to the distal part of the haustorium that contacts the host surface (Fig. **5e,f**).

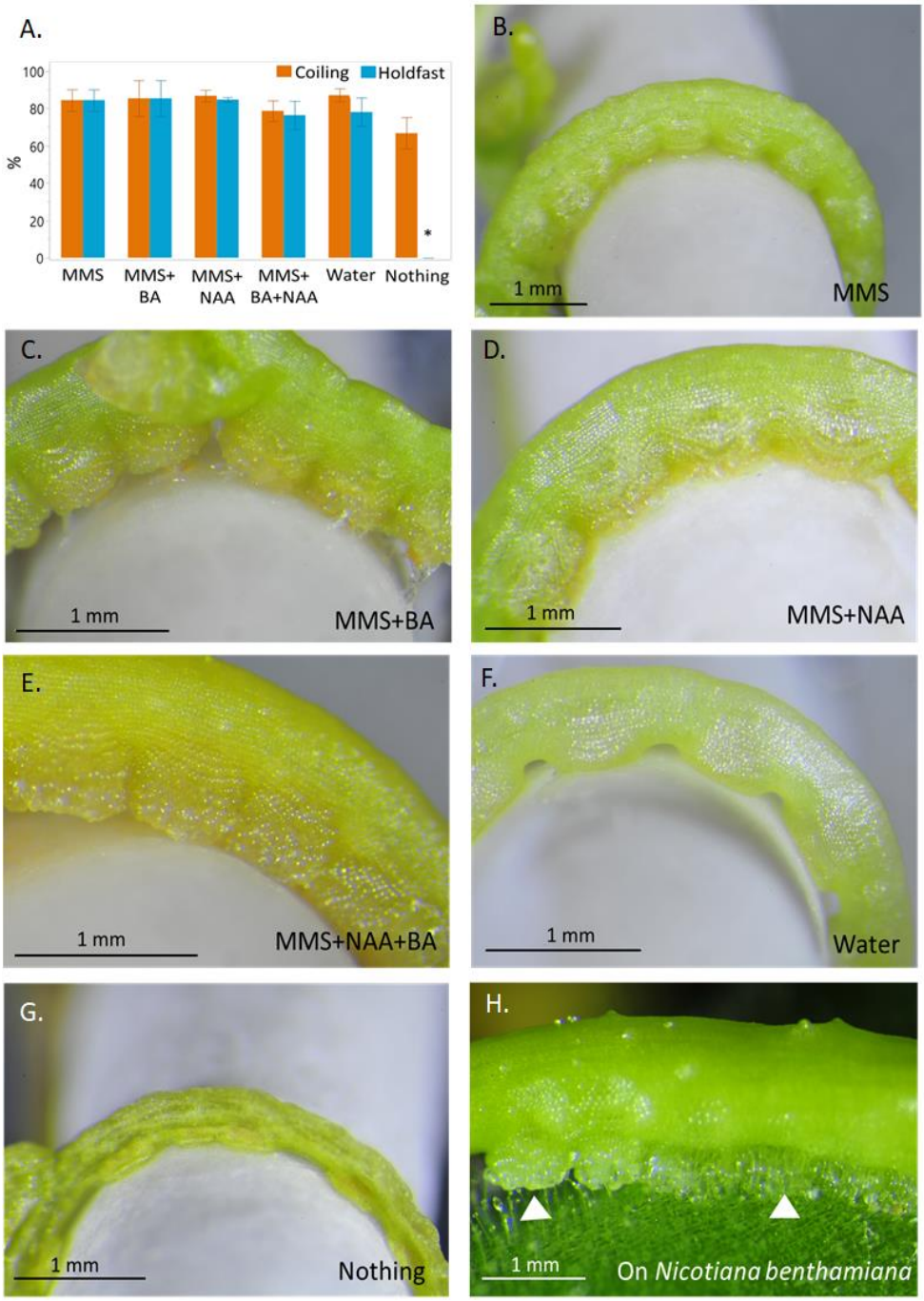


Fig. 4. Characterization of *Cuscuta campestris* coiling and holdfast morphology in the artificial host system (AHS). **a**. Frequencies of coiling and holdfast production evaluated at 36 days post inoculation (dpi). The experiment was repeated three independent times, with 15 replicates in average. Graph presents means between the three experiments and ±SE. **b**-**g**. Holdfast morphology as influenced by phytohormones in the media. Holdfasts of parasites growing with MMS (Modified Murashige and Skoog) media (**b**) **c.** MMS+6-benzylaminopurine (BA) (**c**), MMS+1-

naphthaleneacetic acid (NAA) (**d**), MMS+NAA+BA (**e**) or just water (**f**). **g**. Parasite growing on an artificial host without any media, "Nothing". **h**. Holdfasts (white arrows) of *C. campestris* growing on *Nicotiana benthamiana*. Pictures were taken at 8 dpi.

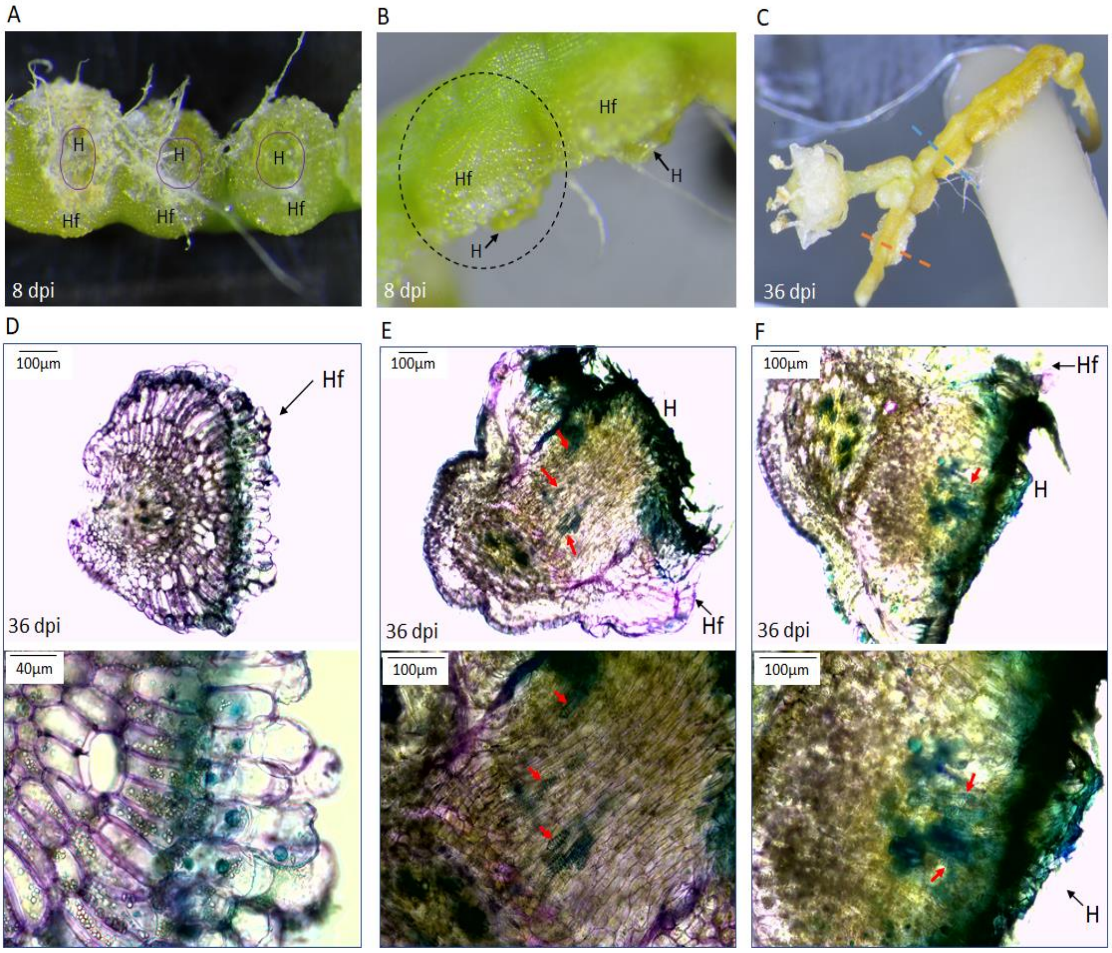


Fig. 5. Anatomy of parasitic organs produced by *Cuscuta campestris* growing on an artificial host with MMS (Modified Murashige and Skoog) media including 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BA). **a**. haustorial region inner face view at 8 days post inoculation (dpi). Haustoria (H) is observed in the center of the parasitic organ surrounded by the holdfast (Hf). Paper fibers from the artificial host are visible. **b**. Lateral view of haustorial region with visible holdfast and protruding haustoria at 8 dpi. Dotted line circle indicates a parasitic organ. **c**. haustorial region with parasitic organs at different developmental stages (36 dpi). **d-f**. Cross sections of parasitic organs of *C. campestris* growing on an artificial host at 36 dpi. Bottom panels

show higher magnification of upper panels. **d**. Parasitic organ in an early developmental stage corresponding to the orange dotted line in Fig. **c**. Holdfast epidermal cells on the face were in contact with the host, so represent the adhesion disk. Higher magnification (bottom panel) shows these cells to be elongated. **e** and **f**. Mature parasitic organs corresponded to the blue dotted line in Fig. **c**. Haustorium development is conspicuous with elongated cells protruding towards the artificial host. Hand-cut cross-sections were stained with Toluidine blue-O to reveal tracheary elements of the xylem (in blue, indicated by red arrows).

Parasite growth and development: Phytohormones affect C. campestris *weight, length, and fitness*

To evaluate the effect of the AHS media composition on parasite growth, we inoculated *C. campestris* shoot tips on artificial hosts and evaluated changes in parasite fresh weight, biomass, and total length at 36 dpi. The parasite exhibited significantly higher fresh weight and biomass when exposed to MMS media containing phytohormones as compared to only MMS media or water (Fig. **6a,b**). The greatest gain in fresh weight and biomass occurred in treatments containing BA compared to MMS alone or containing only NAA. Parasites exposed to only MMS or water treatments showed comparable fresh weights, while MMS allowed significantly higher biomass accumulation than just water. Fewer differences were found when measuring shoot length. Parasites exposed to media with MMS and BA attained the greatest length but did not differ significantly from plants with NAA-containing media. No significant length gain was found in parasites growing on AHS media without phytohormones (Fig. **6c**). Our results show that the fresh weight, biomass, and total length of *C. campestris* are significantly enhanced by externally-provided phytohormones.

C. campestris propagation occurs through seeds and shoots, so measuring these aspects of parasite growth in the AHS provides an indication of *C. campestris* fitness. We assessed the percentage of new active shoots and fruits as indicators of the parasite potential to propagate and colonize new hosts. We consider actively growing shoots to be those with turgid shoot tips of at least 3 cm and able to colonize new hosts (Fig. **S3**). Only plants growing with media containing BA showed a significant increase in the percentage of active shoots (Fig. **7a**). With respect to sexual reproduction, flowers were formed under all MMS media conditions, regardless of phytohormones

(**Fig. 7b**). Parasites with fruits generally produced one fruit with 1 to 2 viable seeds. Only *C. campestris* exposed to media containing phytohormones produced seed. These results suggest that externally supplied phytohormones BA and NAA enhance parasite fitness in the AHS.

In measuring *C. campestris* fitness and growth, we noted a frequent tradeoff in type of growth. Some plants grew long vegetative shoots, while others would thicken and produce flowers. In order to understand parasite growth parameters, we evaluated the relationship between biomass, fresh weight, and total length. Data from each parameter were grouped, regardless of the media, and Pearson's coefficient of correlation was calculated between the pairs of parameters (Fig. **S4**). We found the strongest correlation between biomass and fresh weight (r=0.869). We also analyzed the distribution of plants with flowers and fruits in relation to biomass and total length, and this confirmed that most parasites with flowers or flowers and fruits displayed reduced total length and elevated biomass compared to the parasites without flowers or fruits (Fig. **S5**). We conclude that reliance on any single parameter can be misleading and recommend the use of more than one of these three growth parameters to capture the phenotypic response of *C. campestris*.

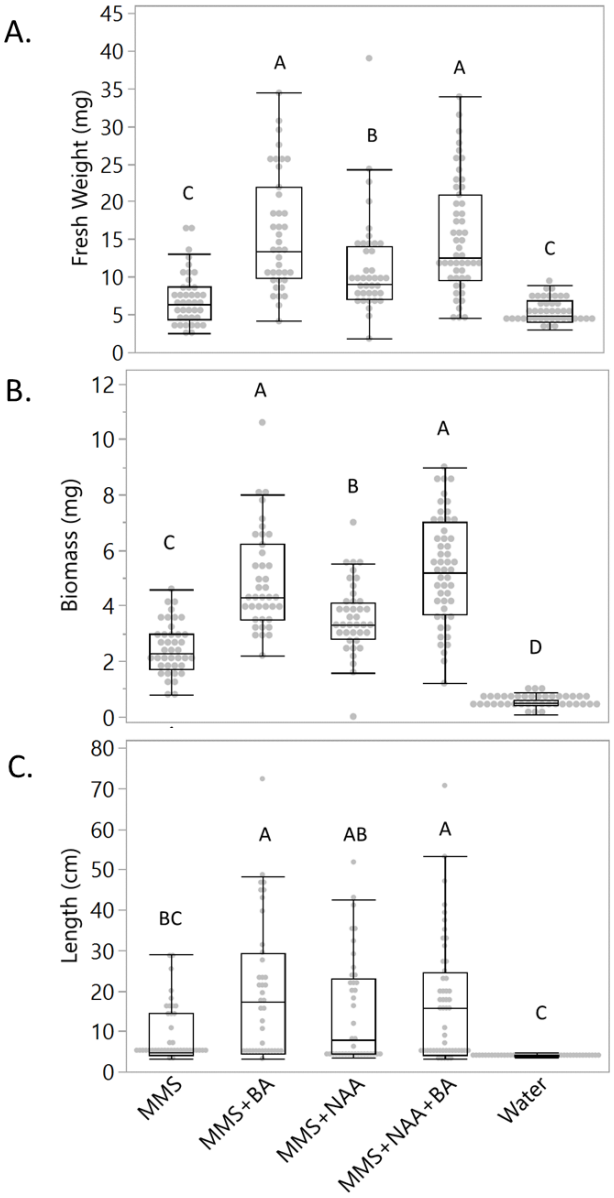


Fig. 6. *Cuscuta campestris* growth in the artificial host system (AHS) supplied with different media. Parasite fresh weight (**a**), parasite biomass (**b**) and parasite total length (**c**) were measured at 36 days post inoculation. Different media were tested: MMS (Modified Murashige and Skoog) media, MMS with 1-naphthaleneacetic acid (NAA), MMS with 6-benzylaminopurine (BA), MMS with NAA and BA, and water alone. Statistical differences were detected by ANOVA with post-hoc Tukey HSD test. Differences were considered statistically significant at $P<0.05$ and indicated with different letters. Data are pooled from three independent experiments. Analysis included only plants that coiled and developed healthy holdfasts. Number of samples ranged from 39 to 51 per treatment.

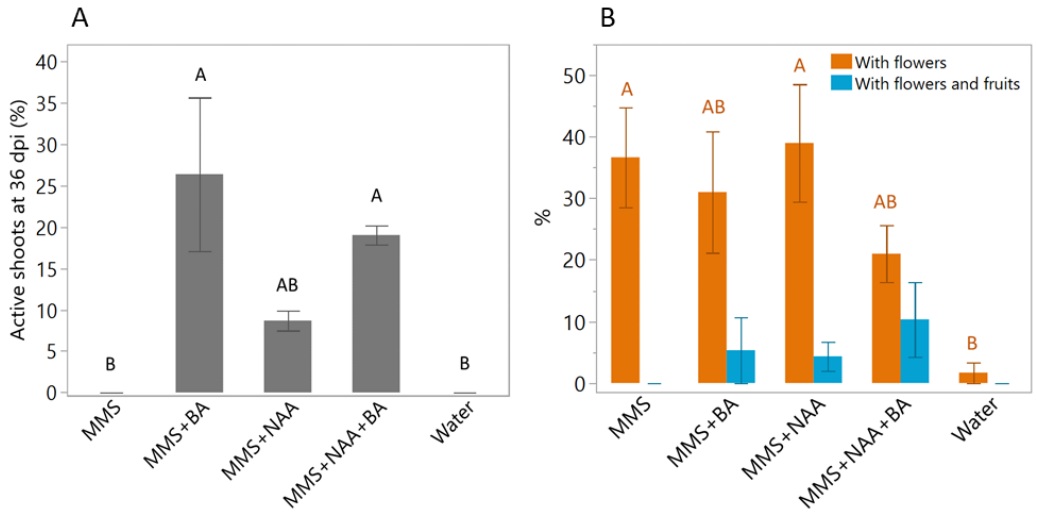


Fig. 7. Effect of artificial host system (AHS) media composition on the ability of *Cuscuta campestris* to generate parasitically competent shoots, flowers, and fruits. **a**. The percentage of plants with active shoots after 36 days post inoculation (dpi). Active shoots were defined as those with tips of at least 3 cm of healthy, turgid shoot, which are characteristic of shoots capable of forming new host connections. **b**. The percentage of plants with flowers only or with fruit production. Analyses included only plants that coiled and developed a healthy holdfast. Graph presents means ±SE between three independent experiments, each experiment with a number of plants ranged from 6 to 19 per treatment. Statistical differences were detected by ANOVA with post-hoc Tukey HSD test. Differences were considered statistically significant at $P<0.05$ and indicated with different letters.

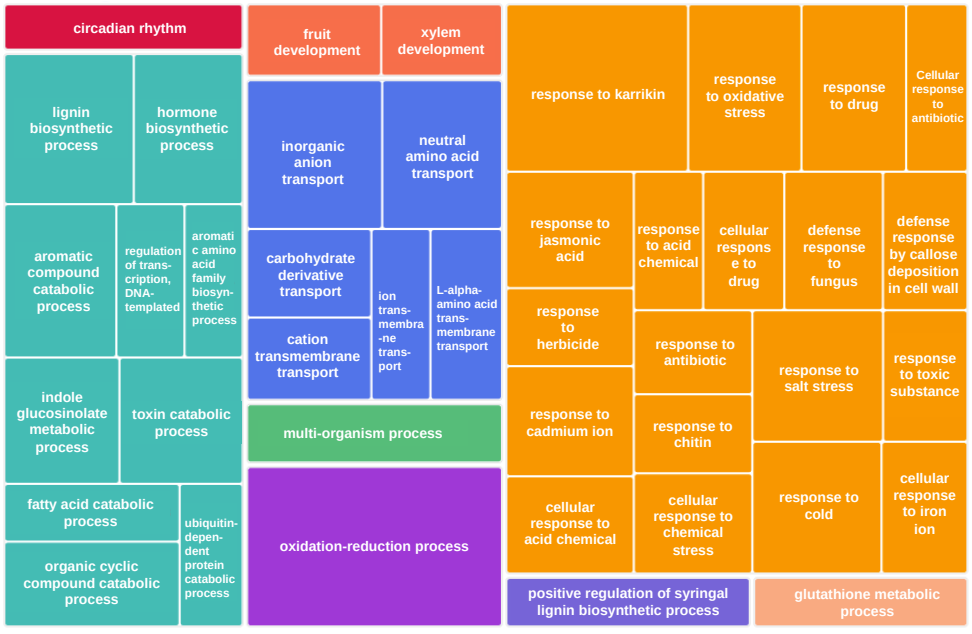
Haustorial function: Characterization of the gene expression profile of a functional haustorial region in the absence of a plant host

In order to explore features associated with a functional haustoria in the absence of a plant host, we analyzed differential gene expression between the haustorial region and the newly developed stem of plants growing in the AHS (Fig. **S6**). We grew *C. campestris* in the AHS (MMS media with NAA and BA at 15 dpi) and sequenced mRNA extracted from the haustorial region and vegetative shoots. On average, 90% of the reads uniquely mapped to the previously sequenced *C.*

campestris genome (Vogel *et al.*, 2018). We found a total of 20,749 DEG, with 9,939 up-regulated and 10,810 down-regulated in the haustorial region compared to stem (Fig. **S6**).

We performed a Gene Ontology (GO) enrichment analysis to determine which biological processes were associated with AHS grown haustorial development and function. Among the overrepresented processes in the haustorial region are lignin biosynthesis, xylem development, and response to hormones, which may be related to haustoria development (Fig. **8a**, Table S1). Other enriched processes include transport (ions, carbohydrates and amino acids), hormones biosynthesis, response to abiotic stress, and defense, which may be associated with haustorium interaction with a host. On the other hand, genes underrepresented in the haustorial region compared to the shoot include those functioning in organ and stomatal morphogenesis, gravitropism, response to hormones, cuticle and cell wall-related biosynthetic processes, auxin efflux, cell differentiation, DNA-related processes, and translation (Fig. **8b**, Table S1).

A.



B.

Fig. 8. Enriched Gene Ontology (GO) terms associated to Biological Process corresponding to differentially expressed genes (DEGs) in haustorial region of a parasite growing in artificial host system (AHS). **a**. 9,939 up regulated genes were included. After the enrichment analysis, the top 55 enriched GO Terms (enrichment p-value < 0.001) were selected to build a TreeMap. **b**. 10,810 down regulated genes were included. After the enrichment analysis, the top 35 enriched GO Terms (enrichment p-value < 0.001) were selected to build a TreeMap. Kolmogorov-Smirnov like enrichment tests (also known as Gene Set Enrichment Analysis - GSEA) was performed for **a** and **b**, considering expression p-value. Each rectangle is a single cluster representative, its size represents the frequency of the GO term in the underlying GO Annotation database. Colors indicate superclusters of loosely related terms.

Genes upregulated in haustorial regions of plants in the AHS are also expressed during parasitism of actual plant hosts

Finally, we compared the DEG found in the haustorial region of a parasite growing in AHS with other published gene expression datasets from haustorial tissues associated with real plant hosts. We used datasets from two categories of haustoria, “functional” or “immature”. Functional haustoria come from studies that report established haustoria that allow parasite growth. Data in

the "functional" category was derived from a union of data from analysis of *C. campestris* growing on *A. thaliana* inflorescences (Kim *et al.*, 2014) and on *S. lycopersicum* and *N. tabacum* stems (Ranjan et al., 2014). Immature haustoria have visible holdfasts, but growth of new shoots or flowers is not reported. Data in the "immature" category are from *C. campestris* haustoria 87 hours after haustorial induction in contact with a leaf of *A. thaliana* (Kaga et al., 2020).

Comparison of induced DEGs from the AHS-grown haustoria to the functional and immature haustoria in contact with a plant host data revealed 220 genes shared by all three datasets regardless of haustorial functional status or contact with actual plant tissues (Fig. **S7**) (Table **S2**). Another 62 genes were uniquely shared by functional haustorial tissues even though AHS-grown plants lacked contact with a real plant host. To assess functions of the 220 and 62 shared genes, we searched for homologues of the *C. campestris* genes in *A. thaliana*, finding 148 and 45 genes, respectively (Fig. **S8**). Genes present in all haustoria were grouped in biological processes categories related to anatomical structure development, multicellular organism development, and response to endogenous stimulus, biotic stimulus, external stimulus, abiotic stimulus, and stress (Fig. **S8a**). Genes shared by just functional haustoria were grouped in biological processes related to anatomical structure development, response to abiotic stimulus and response to stress (Fig. **S8b**).

Discussion

Features of the AHS

One of the primary challenges of studying obligate parasitic plants is the difficulty in separating the biology of the parasite from that of its host. Although *Cuscuta* species have previously been grown under tissue culture conditions (Loo, 1946; Baldev, 1962; Bakos A. *et al.*, 1995; Srivastava & Dwivedi, 2001; Borsics & Lados, 2002; Švubová & Blehová, 2013), the phenotype of the parasite under such conditions does not correspond to the coiling and haustorial feeding habit that characterizes normal growth. To overcome this limitation, we developed an AHS to grow the obligate parasite *C. campestris* without a plant host. The AHS allows growth of *C. campestris* under axenic conditions while exhibiting a parasitic habit including haustorial function. This enables studies of *Cuscuta* that more fully approximate natural mechanisms of nutrient acquisition and growth of the parasite. Additionally, the AHS supports production of viable seeds and new

shoots under *in vitro* conditions (Fig. **2**), which may be useful in the genetic manipulation of *Cuscuta*.

Cuscuta will coil around almost any solid object of the appropriate size, shape, and orientation, which is usually anything resembling a vertical plant stem (Furuhashi *et al.*, 2011). During the optimization of the AHS, we tested different diameters and types of artificial host, including paper straws, rolled filter papers, and solid paper spindles. The best substrates were cylindrical with a relatively small diameter and a firm structure. The material also must deliver water and nutrients to the parasite. We tested liquid and solid media inside straws, but ultimately recognized that substrates with capillary capacity provided sufficient delivery of liquid media to the parasite. It is possible that other materials can be used for artificial hosts, but we found these to be simple and effective. Haustoria structures developed in the AHS appear to function in uptake similar to natural haustoria. In this system, *C. campestris* interacts with the artificial host to take up water and small molecules as demonstrated by the uptake of CFDA and phytohormones (Fig. **3, 4, 6, 7**). The CFDA uptake and translocation provided evidence of a symplastic route from the haustorium structure to the shoot tips of *Cuscuta*. Gene expression in AHS-grown haustorial regions also shows an alignment with its function in nutrient uptake and interaction with hosts, with similarities to genes induced in haustoria of parasites on actual plant hosts (Ranjan *et al.*, 2014). For example, we found that haustorial regions had enhanced expression of genes categorized as associated with increased response to stress, catabolism, and transport (carbohydrates, amino acids and ions), but reduced expression for morphogenesis and cell wall associated genes (Fig. **8**). Haustorial regions also showed activation of genes involved in response to abiotic and biotic stress.

Insights into parasitism

The AHS provides a system in which to explore other aspects of *Cuscuta* biology. For example, what is the nature of host specificity for *C. campestris* when it grows relatively normally on a non-living host? *Cuscuta* species are recognized as having broad host ranges (Mishra, 2009), but the bases for host specificity are uncertain and have been variously attributed to host anatomy (Dawson *et al.*, 1994), host chemical composition (Honaas *et al.*, 2013), or perhaps some type of passive or active defense mechanism (Kaiser *et al.*, 2015). The AHS described herein suggests that a minimum host provides water, sugar, mineral nutrients and at least one cytokinin. It should be recognized that *Cuscuta* in the AHS is not equivalent to one growing on a preferred plant host in

terms of vigor, so the AHS lacks certain aspects of the parasite-host interaction that may contribute to optimal parasite growth. But the ability of the parasite to subsist and produce viable seeds in such a system serves as a strong reminder that this parasite is extremely adaptable and can complete its life cycle with few resources.

A notable aspect of the AHS is how well the haustorial development appears to parallel that of normal haustoria on living host plants. We found evidence for an effect of the hormones NAA and BA on the parasite, with holdfast phenotype (Fig. **4**) and parasite growth (Fig. **6**) responding to hormone treatment applied through the artificial host. Elongation of cells at the holdfast surface agree with reports on the effect of injected auxin in parasite tissues (Löffler *et al.*, 1999). On the other hand, our results contrast with those of another system for artificial haustoria induction where no visible alterations in haustoria structures were observed after 48 hours in contact with a solid media containing phytohormones and without contact with the host tissues (Kaga *et al.*, 2020). We postulate that the shorter time allowed for haustorial development or different media composition could explain the conflicting results.

The addition of the cytokinin BA to the media had a pronounced effect on parasite development and growth. Parasites in the AHS with BA showed more robust holdfast morphology and greater biomass accumulation, as well as a capacity to form shoots capable of making new host connections and setting viable seeds (Fig. **4,6,7**). Gene expression data showed "response to cytokinin" as an enriched process in parasite shoots as compared to haustoria (Fig. **8b**). In a previous study, BA applied to cut ends of *C. reflexa* stem sections induced haustoria in the absence of the host and in darkness, with the maximum number of individual haustoria formed close to the apex (Ramasubramanian *et al.*, 1988). Although we did not monitor the number of individual haustoria (holdfasts in our case), we did not note differences in the capacity to form holdfasts in parasites receiving diverse treatments such as water or media, including media with BA (Fig. **4a**).

Another example of the minimum host requirements for *C. campestris* growth is the formation of vascular tissue in the haustorial structure. The development of the *C. pentagona* xylem has been correlated with differentiation of haustorial searching hyphae that contact host xylem (Vaughn, 2006). In the AHS, tracheary elements formed in the haustorial structure in contact with host-derived auxin (NAA) and cytokinin (BA), sugar (glucose) and other nutrients in the media, suggesting that these are sufficient to induce differentiation in at least some cells (Fig. **5**). This

agrees with the crucial role for auxin and cytokinin in vascular differentiation (Wetmore & Rier, 1963; Aloni, 1987) and vascular reconnection during formation of grafting unions (Melnyk *et al.*, 2015). Additionally, our observation of xylem elements is supported by transcriptional data that show an enrichment in xylem development and lignin biosynthetic process in haustorial regions compared to stems 15 dpi (Fig. **8a**). Kaga et al. (2020) report the expression of genes related to xylem differentiation in *C. campestris* at 57 hours post inoculation without penetrating a plant host, indicating that haustoria acquire potential for xylem differentiation at early stages.

Other topics where the AHS may provide insight include defense and reproduction. We found genes related to defense induced in the haustorial region relative to the stem, which may indicate a preparation of the parasite for the biological interaction. It is clear that a complex molecular interaction occurs between parasite and host (Hegenauer *et al.*, 2016, 2020), leading to outcomes such as host resistance, or parasite success (Hegenauer *et al.*, 2016; Jhu *et al.*, 2019). The AHS may be useful in dissecting aspects of host and parasite defenses. The issue of flowering is another exciting topic, with recent work suggesting that the parasite relies on its host for regulation of flower induction (Shen *et al.*, 2020). Some have contended that *C. campestris* has lost many of its own flowering regulator genes through evolutionary reduction (Vogel *et al.*, 2018), so it must therefore rely on flowering signals from a host plant that serve to synchronize its flowering times with the host. However, we observed parasites flowering and producing seeds in the absence of a flowering – or even a living - plant host. Further study is needed to understand flowering dynamics in *Cuscuta*, and the AHS may be a useful tool.

Historically microbial cultures and especially axenic cultures have allowed a deeper understanding of microorganisms and parasites biology. For example, the growing of human parasites *in vitro* has enabled the study of their biological features and requirements without the influence or contamination of the host. It has also facilitated their identification and the assessment of control methods (Visvesvara & Garcia, 2002) . The AHS presents a valuable new approach to studying *Cuscuta*. In this system, it is possible to manipulate "host" chemical composition such as phytohormones (e.g., Fig. **4,6,7**), or to test hypothesis involving environmental conditions that would otherwise be impractical with a live host (e.g., growth in darkness). The AHS is versatile and may be modified to explore different substrate material or compare specific host requirements of different *Cuscuta* species, although the AHS was optimized for *C. campestris*. We expect this tool to be a valuable addition to the workbench of *Cuscuta* researchers.

Acknowledgements

Hannah Ambrose helped with material preparation for AHS experiments.

Author contributions

VBG and JHW conceived the project. VBG developed the system, conducted the experiments, analyzed the data and drafted the manuscript. JHW edited the manuscript.

Data availability

The data that support the findings of this study are openly available in the NCBI Gene Expression Omnibus (GEO) archive at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE178396, reference number GSE178396.

Funding

This project was supported by the US National Science Foundation (IOS-1645027) and National Institute of Food and Agricultural award 160111 to JHW.

Declarations

- Ethics approval and consent to participate

  Not Applicable

- Consent for publication

  Not Applicable

- Competing interests

  The authors declare that they have no competing interests.

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Supporting information

Fig. S1 Materials for building the artificial host system (AHS).

Fig. S2 *C. campestris* with developed turgid holdfast gained fresh weight, biomass and length compared to parasites with no developed turgid holdfast.

Fig. S3 New parasite shoots produce in the AHS are viable.

Fig. S4 Relationship among biomass, length, and fresh weight in *C. campestris*.

Fig. S5 Relationship among biomass, length, and flowers and fruits presence in *C. campestris.*

Fig. S6 Differential gene expression analysis on haustorial region and stem tissues collected from *C. campestris* growing in the AHS.

Fig. S7 Comparison between up regulated genes present in three categories of haustoria developmental conditions.

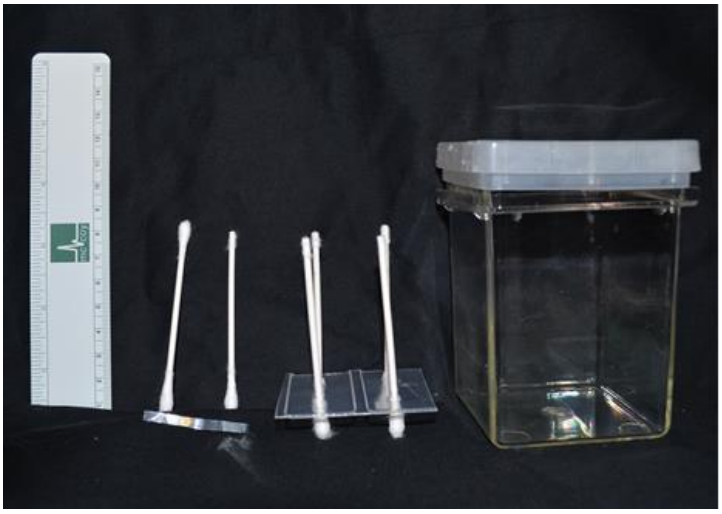
Fig. S8 Singular enrichment analysis (SEA) of *C. campestris* up regulated genes in haustorial regions under different conditions.

Table S1 Enriched GO terms for upregulated and down regulated genes

Table S2 DEGs shared by datasets considering haustoria functional status

Supplementary figures

Fig. S1 Materials for building the artificial host system (AHS). Cotton is removed from one end of a swab to yield a 0.3 cm diameter, 7 cm long paper spindle, the spindle is held vertical by a thin sheet of plastic, with the cotton end directed towards the bottom of the box to be immersed in media. Four “artificial host” spindles are thus held together and placed in a culture box.



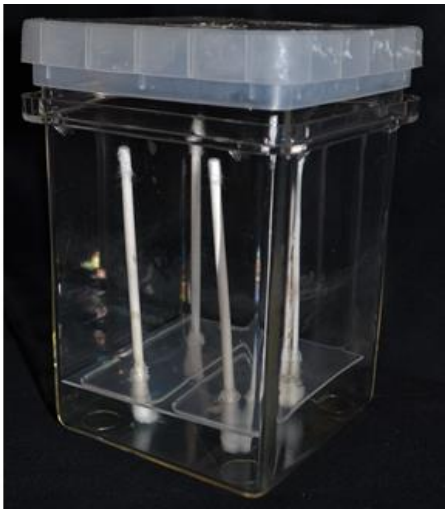


Fig. S2 *C. campestris* with developed turgid holdfast (Hf) growing in the artificial host system (AHS) with MMS (Modified Murashige and Skoog) media including 1-naphthaleneacetic acid (NAA) (3 mg l-1) and 6-benzylaminopurine (BA) (1 mg l-1) gained fresh weight (**a**), biomass (**b**) and length (**c**) compared to parasites with no developed turgid holdfast (No Hf) under the same media, or under water as media or without any media ("nothing"). Parasites growing without any media did not present developed turgid holdfast or growth. Evaluation was made at 36 days post inoculation (dpi). Statistical differences were detected by ANOVA with post-hoc Tukey HSD test. Differences were considered statistically significant at $P<0.05$ and indicated with different letters. Number of samples ranged from 10 to 51 per treatment. Data correspond to three independent experiments.

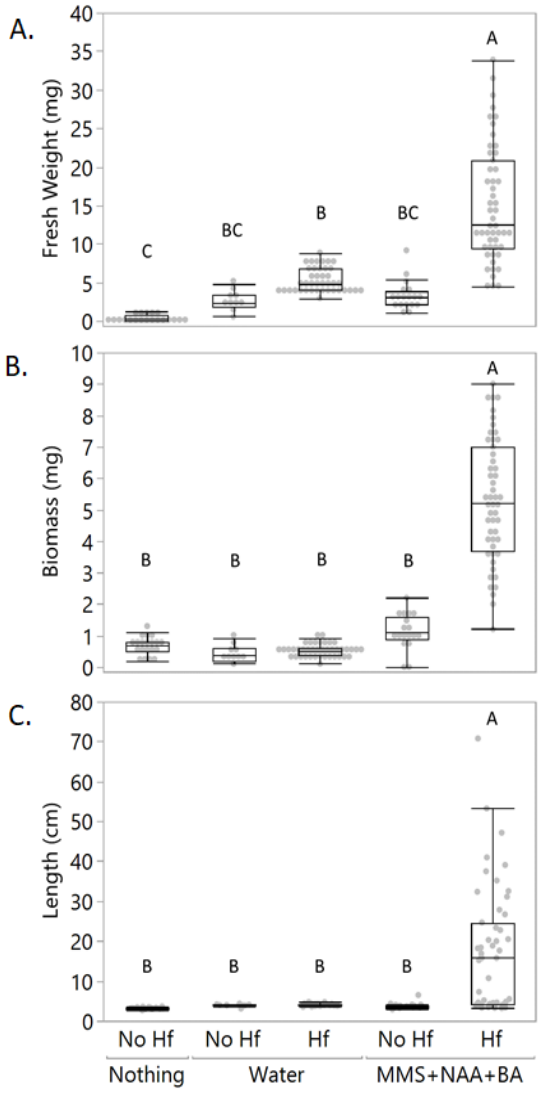


Fig. S3 New parasite shoots produce in the artificial host system (AHS) are viable. **a.** *Cuscuta campestris* new shoots obtained through the AHS using a 3cm shoot tip from nursery as initial inoculum 36 days post inoculation. Orange arrow shows an active shoot. Blue arrow shows a senescent shoot. **b**. Active shoots were able to colonize a new plant host (*Arabidopsis thaliana*).

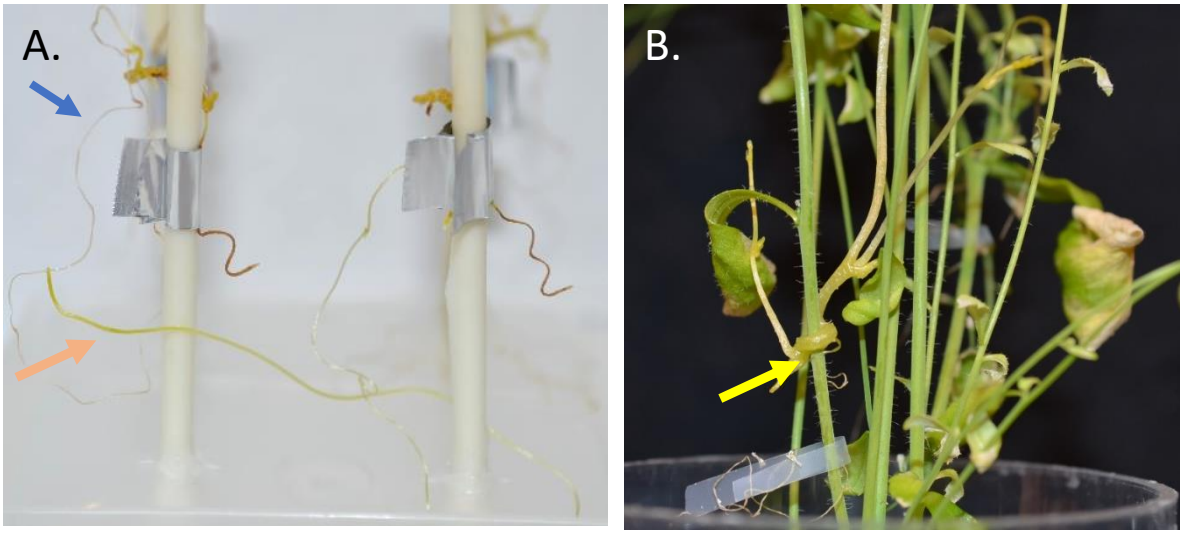


Fig. S4 Relationship among biomass, length, and fresh weight in *Cuscuta campestris*. Graphic collects data from parasites growing under different treatments in the artificial host system (AHS) 36 dpi. Pearson's coefficient (r) was calculated regardless of treatments. Colors correspond to different treatments and are shown only for reference. Ellipses indicate the area where 95% of the data fall. n= 210. Stars indicate the mean point of the ellipse and correspond total average. Data correspond only to parasites that coiled and developed holdfast.

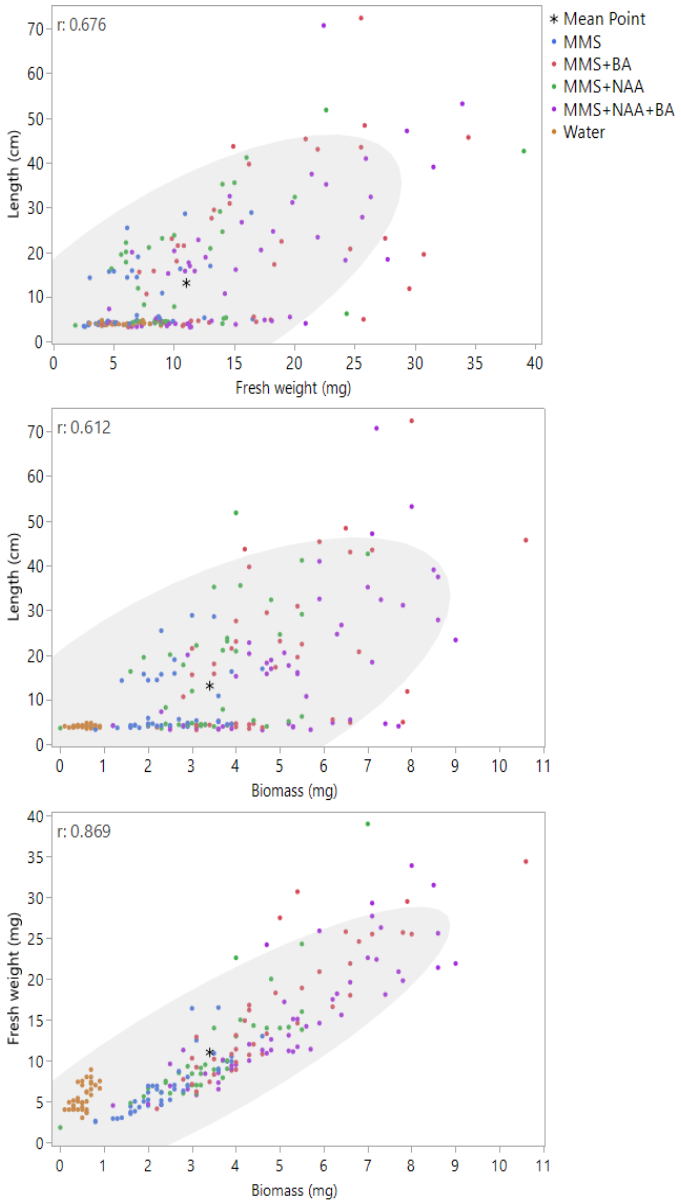


Fig. S5 Relationship among biomass, length, and flowers and fruits presence in *Cuscuta campestris*. Graphic collects data from parasites growing under different treatments in the artificial host system (AHS) 36 days post inoculation (dpi). Ellipses indicate the area where 50% of the data corresponding to a specific condition fall: parasites with no flowers or fruits (grey, n=147), parasites with only flowers (orange, n=50), parasites with flowers and fruits (blue, n=13). Stars represent the average for the values in each ellipse. Data correspond only to parasites that coiled and developed a turgid holdfast.

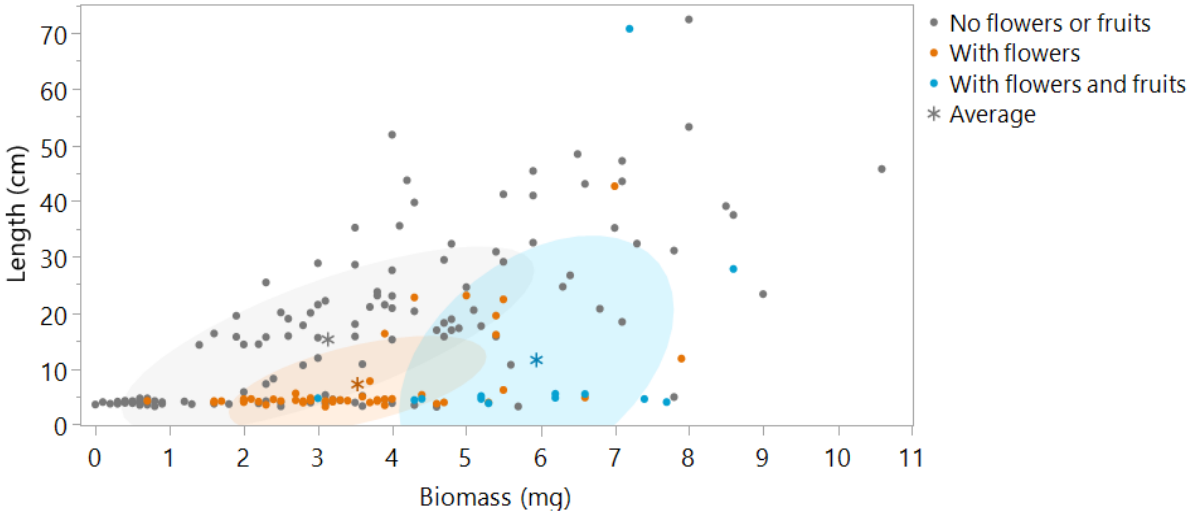
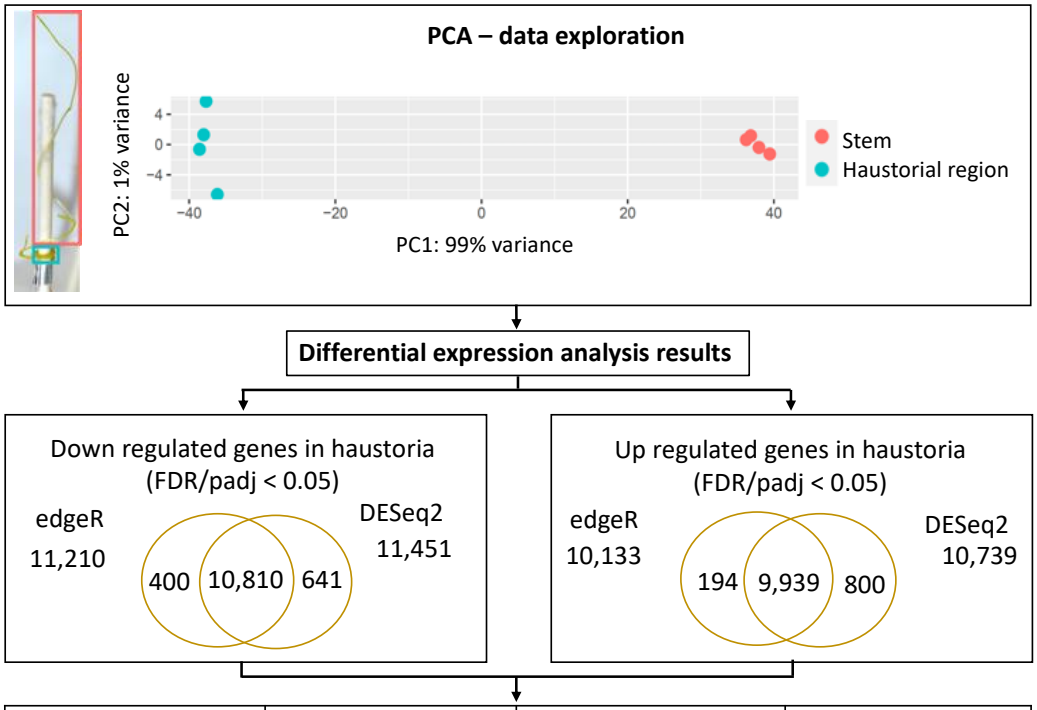


Fig. S6 Differential gene expression analysis on haustorial region and stem tissues collected from *Cuscuta campestris* growing in the artificial host system (AHS). Principal component analysis (PCA) shows a segregation on the samples by type of tissue. Differential expression analysis results show the number of down regulated or up regulated genes in the haustorial region tissues compare to the stem tissues after filters were applied. Four biological replicates of each tissue were sequenced. Illumina RNA-seq yielded an average of 63.4 million total raw reads per sample and, after filtering by length and low quality, 97.8% passed quality control. False discovery rate: FDR. Adjusted p-value: padj.



Filter	Total DEG	Up regulated in Haustoria	Down regulated in Haustoria
padj < 0.05	20,749	9,939	10,810
padj < 0.01	11,479	8,969	2,510

Fig. S7 Comparison between up regulated genes present in three categories of haustoria developmental conditions. First category corresponds to up regulated genes (8.969 genes, padj<0.01) in the functional haustorial regions of parasites growing in artificial host system (AHS). The second category includes independently reported genes expressed in functional haustorial region of *C. campestris* growing on different plant hosts. Out of the 469 genes in this group, 177 correspond to genes reported by Kim et al (2014) and 308 to Ranjan et al (2014), of which 16 were shared by both studies. The third category corresponds to up regulated genes in haustorial tissue, 87 hours after the artificial-induction of haustoria in contact with a leave of a plant host (Kaga *et al*., 2020). 220 genes were shared between all categories, with haustoria formation regardless function or contact with plant host. 62 genes were shared between the groups that harbored functional haustorial regions regardless the presence of a plant host. While 78 genes were shared only between the groups that had a contact with plant host tissues regardless haustorial function. Adjusted p-value: padj.

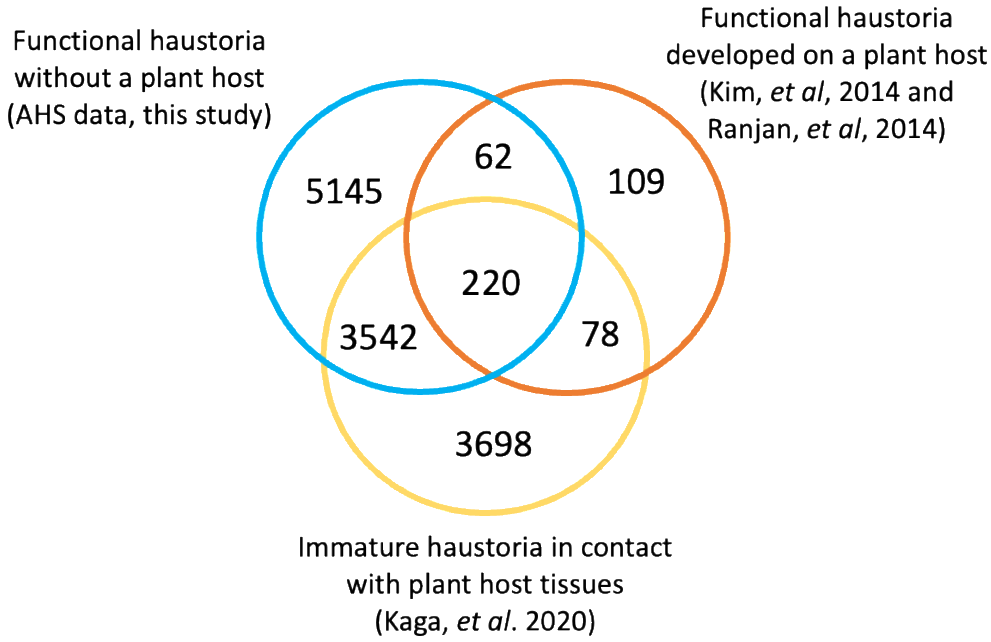
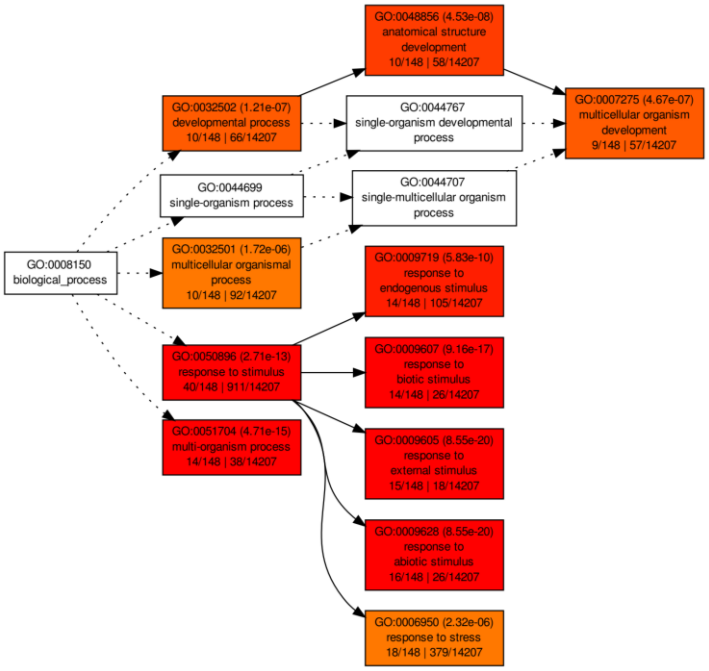
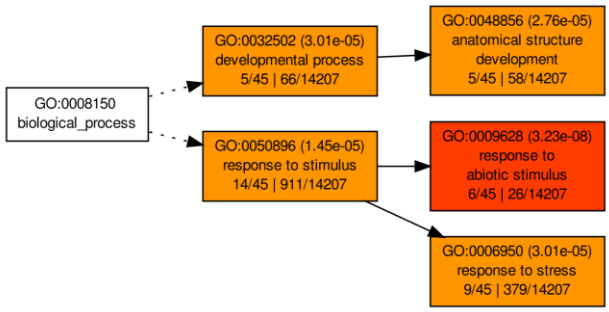


Fig. S8 Singular enrichment analysis (SEA) of *Cuscuta campestris* up regulated genes in haustorial regions under different conditions. Gene ontology (GO) Slim terms are based on homologue candidate genes in *Arabidopsis thaliana* and SEA was made considering *A. thaliana* genome as reference. GO Slim plant terms are organized in a hierarchical way and show **a**. GO terms associated to genes shared between datasets that include functional and immature haustoria regardless contact with a host plant, and **b**. GO terms associated to genes shared between datasets that include only functional haustoria regardless contact with a host plant. A functional haustoria is determined by formation of conspicuous holdfasts, and growth and development of new shoots and flowers after haustoria formation.

A.



B.



Supplementary tables

Table S1. Gene Set Enrichment Analysis - GSEA results for upregulated and downregulated genes in haustorial regions compared to stem.

Gene Set Enrichment Analysis - GSEA results				
Analysis	**No**	**GO.ID**	**Term**	**weight01_KS**
GO Terms Upregulated in haustorial regions compared to shoots	1	GO:0055114	oxidation-reduction process	8.10E-15
	2	GO:0080167	response to karrikin	5.30E-11
	3	GO:0015698	inorganic anion transport	1.30E-07
	4	GO:0009809	lignin biosynthetic process	2.40E-07
	5	GO:0006979	response to oxidative stress	3.90E-07
	6	GO:0015804	neutral amino acid transport	7.80E-07
	7	GO:0042493	response to drug	1.20E-06
	8	GO:0019439	aromatic compound catabolic process	1.40E-06
	9	GO:0009651	response to salt stress	1.60E-06
	10	GO:0009409	response to cold	1.80E-06
	11	GO:0042446	hormone biosynthetic process	2.70E-06
	12	GO:0051704	multi-organism process	4.00E-06
	13	GO:0009407	toxin catabolic process	4.90E-06
	14	GO:0009699	phenylpropanoid biosynthetic process	5.80E-06
	15	GO:0044550	secondary metabolite biosynthetic proces...	8.70E-06
	16	GO:1901361	organic cyclic compound catabolic proces...	8.70E-06
	17	GO:0009753	response to jasmonic acid	9.80E-06
	18	GO:0042343	indole glucosinolate metabolic process	1.10E-05
	19	GO:0009850	auxin metabolic process	1.20E-05
	20	GO:0046686	response to cadmium ion	1.80E-05
	21	GO:0050832	defense response to fungus	2.40E-05
	22	GO:0009737	response to abscisic acid	2.90E-05
	23	GO:1901430	positive regulation of syringal lignin b...	4.40E-05
	24	GO:1902475	L-alpha-amino acid transmembrane transpo...	6.50E-05
	25	GO:0071229	cellular response to acid chemical	6.60E-05
	26	GO:0007623	circadian rhythm	0.0001
	27	GO:0062197	cellular response to chemical stress	0.0001
	28	GO:0052544	defense response by callose deposition i...	0.00011
	29	GO:0009414	response to water deprivation	0.00014
	30	GO:0006749	glutathione metabolic process	0.00015
	31	GO:0071281	cellular response to iron ion	0.00015
	32	GO:0035690	cellular response to drug	0.00016
	33	GO:1901264	carbohydrate derivative transport	0.00017
	34	GO:0009636	response to toxic substance	0.00018
	35	GO:0034614	cellular response to reactive oxygen spe...	0.00021
	36	GO:0042447	hormone catabolic process	0.00025
	37	GO:0006355	regulation of transcription, DNA-templat...	0.00027
	38	GO:0098655	cation transmembrane transport	0.00031
	39	GO:0071236	cellular response to antibiotic	0.00034
	40	GO:0034220	ion transmembrane transport	0.00035
	41	GO:0009062	fatty acid catabolic process	0.00036
	42	GO:0010154	fruit development	0.00037
	43	GO:0000041	transition metal ion transport	0.00038
	44	GO:0098656	anion transmembrane transport	0.00039
	45	GO:0046677	response to antibiotic	0.00044
	46	GO:0009635	response to herbicide	0.00044
	47	GO:0034754	cellular hormone metabolic process	0.00054
	48	GO:0001101	response to acid chemical	0.00057
	49	GO:0010200	response to chitin	0.00067
	50	GO:0009739	response to gibberellin	0.00069

	51	GO:0042542	response to hydrogen peroxide	0.0008
	52	GO:0010089	xylem development	0.0008
	53	GO:0006511	ubiquitin-dependent protein catabolic pr...	0.00086
	54	GO:0009073	aromatic amino acid family biosynthetic ...	0.0009
	55	GO:0071805	potassium ion transmembrane transport	0.00095
GO Terms Downregulated in haustorial regions compared to shoots	1	GO:0006355	regulation of transcription, DNA-templat...	1.50E-09
	2	GO:0006412	translation	1.90E-06
	3	GO:0042335	cuticle development	2.20E-06
	4	GO:0007018	microtubule-based movement	2.50E-06
	5	GO:0009664	plant-type cell wall organization	4.60E-06
	6	GO:0010143	cutin biosynthetic process	5.00E-06
	7	GO:0009630	gravitropism	9.30E-06
	8	GO:0010315	auxin efflux	3.30E-05
	9	GO:0006268	DNA unwinding involved in DNA replicatio...	3.50E-05
	10	GO:0051603	proteolysis involved in cellular protein...	3.80E-05
	11	GO:0030244	cellulose biosynthetic process	4.80E-05
	12	GO:0010025	wax biosynthetic process	4.90E-05
	13	GO:1905421	regulation of plant organ morphogenesis	5.30E-05
	14	GO:0055085	transmembrane transport	5.40E-05
	15	GO:0009739	response to gibberellin	6.30E-05
	16	GO:0009833	plant-type primary cell wall biogenesis	8.80E-05
	17	GO:0010345	suberin biosynthetic process	0.00012
	18	GO:0009735	response to cytokinin	0.00012
	19	GO:0080022	primary root development	0.00013
	20	GO:0010103	stomatal complex morphogenesis	0.00024
	21	GO:0030154	cell differentiation	0.00025
	22	GO:0034220	ion transmembrane transport	0.00027
	23	GO:0009733	response to auxin	0.00035
	24	GO:0009734	auxin-activated signaling pathway	0.00039
	25	GO:0010229	inflorescence development	0.0005
	26	GO:0009926	auxin polar transport	0.00057
	27	GO:0032875	regulation of DNA endoreduplication	0.00058
	28	GO:0009226	nucleotide-sugar biosynthetic process	0.00082
	29	GO:0006631	fatty acid metabolic process	0.00083
	30	GO:0034219	carbohydrate transmembrane transport	0.00084
	31	GO:0007154	cell communication	0.00087
	32	GO:0009965	leaf morphogenesis	0.00089
	33	GO:0010375	stomatal complex patterning	0.0009
	34	GO:0043622	cortical microtubule organization	0.00091
	35	GO:0010187	negative regulation of seed germination	0.001

Table S2.

Group	Gene ID	Annotation according to Vogel, et al 2018
220 Differentially expressed genes (DEGs) in all haustoria datasets	Cc009934	Solute transport.primary active transport.ABC superfamily.ABC2 family.subfamily ABCG transporter
	Cc018521	Membrane vesicle trafficking.regulation of membrane tethering and fusion.RAB-GTPase activities.F-class RAB GTPase
	Cc018673	Cell wall.lignin.monolignol synthesis.hydroxycinnamoyl-CoA:quinate/shikimate O-hydroxycinnamoyltransferase (HCT)
	Cc004308	Phytohormones.gibberellin.synthesis.gibberellin 3-oxidase
	Cc006923	hypothetical protein
	Cc024384	RNA biosynthesis.transcription factor families.AS2/LOB transcription factor
	Cc000492	hypothetical protein
	Cc019959	RNA biosynthesis.transcription factor families.WRKY transcription factor
	Cc031652	Photosynthesis.CAM/C4 photosynthesis.phosphoenolpyruvate (PEP) carboxylase activity.regulation.PEP carboxylase kinase
	Cc003443	hypothetical protein
	Cc026980	hypothetical protein
	Cc008785	hypothetical protein
	Cc025210	hypothetical protein
	Cc046498	RNA biosynthesis.transcription factor families.OFP transcription factor
	Cc046186	hypothetical protein
	Cc002392	Cell wall.cell wall proteins.expansins.alpha-type expansin
	Cc001676	Phytohormones.auxin.conjugation and degradation.indole-3-acetic acid-amido synthetase
	Cc006374	hypothetical protein
	Cc031513	Phytohormones.gibberellin.synthesis.ent-kaurene oxidase
	Cc012682	Solute transport.primary active transport.ABC superfamily.ABC1 family.subfamily ABCB transporter
	Cc015170	RNA biosynthesis.transcription factor families.WRKY transcription factor
	Cc007810	hypothetical protein
	Cc017119	hypothetical protein
	Cc002425	Protein degradation.peptide tagging.Ubiquitin (UBQ)-anchor addition (ubiquitylation).UBQ-ligase E3 activities.RING-domain E3 ligase activities.RING-H2-type E3 ligase
	Cc015809	RNA biosynthesis.RNA polymerase III-dependent transcription.TFIIIf transcription factor complex.small subunit
	Cc021738	hypothetical protein
	Cc006049	hypothetical protein
	Cc046181	hypothetical protein
	Cc023546	hypothetical protein
	Cc046589	hypothetical protein
	Cc020997	Secondary metabolism.terpenoids.terpenoid synthesis.mono-/sesquiterpene-/diterpene synthase
	Cc013390	hypothetical protein
	Cc045095	Secondary metabolism.phenolics.flavonoid synthesis and modification.flavonoid glycosylation.flavonol-3-O-glycoside-rhamnosyltransferase
	Cc014521	hypothetical protein
	Cc014332	hypothetical protein
	Cc023826	hypothetical protein
	Cc033782	hypothetical protein
	Cc022075	hypothetical protein
	Cc008764	RNA biosynthesis.transcription factor families.MYB superfamily.G2-like GARP transcription factor
	Cc000405	Cell wall.cellulose.synthesis.cellulose microfibrils and hemicellulose interaction.COB-type protein
	Cc021850	Solute transport.carrier-mediated transport.MFS superfamily.PHT1 phosphate transporter
	Cc009779	hypothetical protein
	Cc014313	hypothetical protein
	Cc001394	hypothetical protein
	Cc021564	RNA biosynthesis.transcription factor families.bHLH transcription factor

	Cc028535	Solute transport.carrier-mediated transport.APC superfamily.AAAP family.GABA transporter (GAT-type)
	Cc017835	hypothetical protein
	Cc005572	hypothetical protein
	Cc001428	Protein degradation.peptide tagging.Ubiquitin (UBQ)-anchor addition (ubiquitylation).UBQ-ligase E3 activities.RING-domain E3 ligase activities.RING-HCa-type E3 ligase
	Cc046185	hypothetical protein
	Cc047148	Phytohormones.auxin.transport.PILS-type auxin efflux transporter
	Cc027530	hypothetical protein
	Cc021184	RNA biosynthesis.transcription factor families.WRKY transcription factor
	Cc001165	Environmental stimuli response.temperature.Hsp (heat-shock-responsive protein) families.Hsp70 family.DnaK protein
	Cc041495	Protein modification.phosphorylation.TKL kinase superfamily.DUF26 kinase
	Cc025869	RNA biosynthesis.transcription factor families.bZIP superfamily.bZIP transcription factor
	Cc012194	Photosynthesis.CAM/C4 photosynthesis.phosphoenolpyruvate (PEP) carboxylase activity.regulation.PEP carboxylase kinase
	Cc046172	Carbohydrate metabolism.trehalose metabolism.trehalose-6-phosphate phosphatase
	Cc013624	hypothetical protein
	Cc023733	RNA biosynthesis.transcription factor families.C2C2 superfamily.DOF transcription factor
	Cc006024	RNA biosynthesis.transcription factor families.HSF (heat shock) transcription factor
	Cc042068	hypothetical protein
	Cc016007	Solute transport.carrier-mediated transport.MFS superfamily.NRT1/PTR anion transporter
	Cc004966	hypothetical protein
	Cc032608	Protein degradation.peptide tagging.Ubiquitin (UBQ)-anchor addition (ubiquitylation).UBQ-ligase E3 activities.RING-domain E3 ligase activities.RING-HCa-type E3 ligase
	Cc002917	hypothetical protein
	Cc031337	hypothetical protein
	Cc015995	RNA biosynthesis.transcription factor families.MYB superfamily.MYB transcription factor
	Cc033885	Protein degradation.peptide tagging.Ubiquitin (UBQ)-anchor addition (ubiquitylation).UBQ-ligase E3 activities.RING-domain E3 ligase activities.RING-H2-type E3 ligase
	Cc006378	Protein modification.phosphorylation.TKL kinase superfamily.G-Lectin kinase families.SD-1 kinase
	Cc031953	hypothetical protein
	Cc034939	hypothetical protein
	Cc016807	RNA processing.mRNA decay.mRNA deadenylation.CCR4-NOT complex.CAF1 deadenylase component
	Cc021186	Solute transport.channel-mediated transport.channels.SLAC anion channel
	Cc025183	hypothetical protein
	Cc029755	hypothetical protein
	Cc014359	Protein modification.phosphorylation.TKL kinase superfamily.WAK/WAKL kinase
	Cc046301	Carbohydrate metabolism.trehalose metabolism.trehalase
	Cc021807	hypothetical protein
	Cc021392	hypothetical protein
	Cc019087	hypothetical protein
	Cc009646	Protein degradation.peptidase families.serine-type peptidase activities.serine carboxypeptidase
	Cc032310	Protein degradation.peptidase families.serine-type peptidase activities.serine carboxypeptidase
	Cc021462	Solute transport.primary active transport.P-type ATPase superfamily.P3 family.AHA P3A-type proton-translocating ATPase
	Cc036899	Carbohydrate metabolism.raffinose family oligosaccharide biosynthesis.galactinol synthase
	Cc006661	hypothetical protein
	Cc041789	Solute transport.carrier-mediated transport.DMT superfamily.PUP organic cation transporter
	Cc032033	Solute transport.channel-mediated transport.channels.VIC superfamily.voltage-gated potassium cation channel (AKT/SKOR/GORK-type)
	Cc030833	hypothetical protein
	Cc037266	RNA biosynthesis.transcription factor families.PLATZ transcription factor
	Cc029220	Solute transport.channel-mediated transport.channels.OSCA calcium-permeable channel

	Cc025355	Environmental stimuli response.pathogen-associated molecular pattern (PAMP).PTI (pattern-triggered immunity) network.bacterial elicitor response.non-canonical heterotrimeric G-protein complex.XLG atypical G-alpha component
	Cc006867	hypothetical protein
	Cc014343	Cell wall.cell wall proteins.expansins.alpha-type expansin
	Cc001702	Nutrient uptake.nitrogen assimilation.nitrate assimilation.nitrite reductase
	Cc010790	hypothetical protein
	Cc012599	hypothetical protein
	Cc023054	hypothetical protein
	Cc000592	hypothetical protein
	Cc023492	hypothetical protein
	Cc010836	hypothetical protein
	Cc011169	Protein modification.O-linked glycosylation.hydroxyproline O-linked glycosylation.HPAT hydroxyproline-O-arabinosyltransferase
	Cc009894	RNA biosynthesis.transcription factor families.WRKY transcription factor
	Cc041892	hypothetical protein
	Cc034808	hypothetical protein
	Cc011457	Secondary metabolism.terpenoids.mevalonate pathway.3-hydroxy-3-methylglutaryl-CoA reductase
	Cc034895	RNA biosynthesis.transcription factor families.C2H2 zinc finger transcription factor
	Cc030682	Solute transport.primary active transport.ABC superfamily.ABC1 family.subfamily ABCC transporter
	Cc008169	hypothetical protein
	Cc021338	Protein modification.phosphorylation.TKL kinase superfamily.RLCK-VII kinase families.RLCK-VIIa kinase
	Cc038393	Protein modification.phosphorylation.AGC kinase superfamily.AGC-VII/NDR kinase
	Cc011499	Redox homeostasis.glutathione S-transferase activities.class tau
	Cc032750	Phytohormones.gibberellin.perception and signal transduction.GID1-type receptor
	Cc033164	Solute transport.carrier-mediated transport.DMT superfamily.PUP organic cation transporter
	Cc023941	Solute transport.primary active transport.ABC superfamily.ABC2 family.subfamily ABCG transporter
	Cc008877	hypothetical protein
	Cc030825	hypothetical protein
	Cc021719	hypothetical protein
	Cc034615	hypothetical protein
	Cc006848	Solute transport.carrier-mediated transport.MFS superfamily.PHT4 phosphate transporter
	Cc030002	Redox homeostasis.glutathione S-transferase activities.class phi
	Cc022032	hypothetical protein
	Cc046982	Protein modification.phosphorylation.TKL kinase superfamily.RLCK-VII kinase families.RLCK-VIIa kinase
	Cc044103	Cell wall.hemicellulose.heteromannan.modification and degradation.endo-beta-1,4-mannanase
	Cc003195	hypothetical protein
	Cc008856	hypothetical protein
	Cc001410	Carbohydrate metabolism.sorbitol metabolism.sorbitol dehydrogenase
	Cc012485	Solute transport.carrier-mediated transport.MFS superfamily.NRT1/PTR anion transporter
	Cc023287	hypothetical protein
	Cc011501	Redox homeostasis.glutathione S-transferase activities.class tau
	Cc018208	Solute transport.carrier-mediated transport.CDF superfamily.CaCA family.magnesium cation:proton antiporter (MHX-type)
	Cc017781	RNA biosynthesis.transcription factor families.Trihelix transcription factor
	Cc026586	Phytohormones.abscisic acid.transport.NRT-type abscisic acid transporter
	Cc037644	RNA biosynthesis.transcription factor families.C3H zinc finger transcription factor
	Cc020737	hypothetical protein
	Cc007916	hypothetical protein
	Cc001798	hypothetical protein
	Cc014713	hypothetical protein
	Cc045793	hypothetical protein

	Cc020357	hypothetical protein
	Cc029734	hypothetical protein
	Cc044726	Protein degradation.peptide tagging.Ubiquitin (UBQ)-anchor addition (ubiquitylation).UBQ-ligase E3 activities.RING-domain E3 ligase activities.RING-H2-type E3 ligase
	Cc034504	hypothetical protein
	Cc029702	hypothetical protein
	Cc001515	hypothetical protein
	Cc037850	hypothetical protein
	Cc036773	RNA biosynthesis.transcription factor families.Trihelix transcription factor
	Cc012240	Cell wall.hemicellulose.xyloglucan.modification and degradation.xyloglucan endotransglucosylase/hydrolase
	Cc007841	Solute transport.carrier-mediated transport.CDF superfamily.CaCA family.cation antiporter (CAX-type)
	Cc020988	hypothetical protein
	Cc044218	hypothetical protein
	Cc019313	hypothetical protein
	Cc022848	Solute transport.carrier-mediated transport.APC superfamily.AAAP family.amino acid transporter (AAP-type)
	Cc030011	Redox homeostasis.hydrogen peroxide removal.glutaredoxin
	Cc002506	Phytohormones.auxin.conjugation and degradation.indole-3-acetic acid carboxyl methyltransferase
	Cc021431	Solute transport.carrier-mediated transport.DMT superfamily.UmamiT-type solute transporter
	Cc018519	hypothetical protein
	Cc001912	RNA biosynthesis.transcription factor families.WRKY transcription factor
	Cc019134	hypothetical protein
	Cc006392	hypothetical protein
	Cc009585	hypothetical protein
	Cc027379	hypothetical protein
	Cc014618	RNA biosynthesis.transcription factor families.WRKY transcription factor
	Cc047087	Secondary metabolism.terpenoids.mevalonate pathway.3-hydroxy-3-methylglutaryl-CoA reductase
	Cc044805	hypothetical protein
	Cc044853	hypothetical protein
	Cc040876	hypothetical protein
	Cc010305	Environmental stimuli response.temperature.Hsp (heat-shock-responsive protein) families.Hsp70 family.DnaK protein
	Cc000120	Cell wall.hemicellulose.heteromannan.synthesis.galactoglucomannan galactosyltransferase (MUCI10)
	Cc029995	Solute transport.primary active transport.ABC superfamily.ABC1 family.subfamily ABCC transporter
	Cc045128	hypothetical protein
	Cc025211	hypothetical protein
	Cc044191	Cellular respiration.oxidative phosphorylation.alternative NAD(P)H dehydrogenase activities.alternative oxidase
	Cc045381	hypothetical protein
	Cc019474	hypothetical protein
	Cc015200	hypothetical protein
	Cc029299	Phytohormones.strigolactone.synthesis.CCD8 carotenoid cleavage dioxygenase
	Cc004177	hypothetical protein
	Cc033724	hypothetical protein
	Cc012653	Phytohormones.gibberellin.conjugation and degradation.gibberellin 2-oxidase
	Cc015187	RNA biosynthesis.transcription factor families.MYB superfamily.G2-like GARP transcription factor
	Cc006316	hypothetical protein
	Cc020986	hypothetical protein
	Cc047279	hypothetical protein
	Cc004058	hypothetical protein
	Cc011330	Protein modification.phosphorylation.TKL kinase superfamily.LRR-XV kinase

	Cc031487	hypothetical protein
	Cc019824	Protein modification.phosphorylation.AGC kinase superfamily.AGC-VII/NDR kinase
	Cc000126	Amino acid metabolism.biosynthesis.glutamate family.glutamate-derived amino acids.ornithine.N-acetylglutamate synthase
	Cc041374	hypothetical protein
	Cc017704	Cell wall.pectin.rhamnogalacturonan I.modification and degradation.beta-galactosidase
	Cc014726	hypothetical protein
	Cc029651	Solute transport.carrier-mediated transport.MFS superfamily.NRT1/PTR anion transporter
	Cc043224	hypothetical protein
	Cc016683	Protein degradation.peptidase families.aspartic-type peptidase activities.pepsin-type protease
	Cc017699	Protein degradation.peptidase families.cysteine-type peptidase activities.papain-type protease
	Cc009826	Cytoskeleton.microfilament network.actin polymerisation.cofilin-like actin depolymerizing factor
	Cc008824	Solute transport.carrier-mediated transport.TOC superfamily.SWEET sugar efflux transporter
	Cc007654	hypothetical protein
	Cc003877	hypothetical protein
	Cc003916	hypothetical protein
	Cc025698	hypothetical protein
	Cc025442	hypothetical protein
	Cc030734	hypothetical protein
	Cc013479	hypothetical protein
	Cc011814	hypothetical protein
	Cc046171	Cellular respiration.oxidative phosphorylation.cytochrome c oxidase complex.assembly.HCC/SCO1 component
	Cc008031	hypothetical protein
	Cc006175	hypothetical protein
	Cc002541	RNA biosynthesis.transcription factor families.AP2/ERF superfamily.ERF-type transcription factor
	Cc020917	hypothetical protein
	Cc012811	Protein degradation.peptide tagging.Ubiquitin (UBQ)-anchor addition (ubiquitylation).UBQ-ligase E3 activities.RING-domain E3 ligase activities.RING-H2-type E3 ligase
	Cc041478	hypothetical protein
	Cc002111	hypothetical protein
	Cc041869	hypothetical protein
	Cc016575	Cell wall.pectin.rhamnogalacturonan I.modification and degradation.beta-galactosidase
	Cc006921	hypothetical protein
	Cc023446	Secondary metabolism.phenolics.flavonoid synthesis and modification.flavons and flavonols.flavone synthase activities.type-I flavone synthase
	Cc016335	hypothetical protein
	Cc009621	hypothetical protein
62 Differentially expressed genes (DEGs) in funtional haustoria datasets	Cc011507	hypothetical protein
	Cc026247	Nutrient uptake.nitrogen assimilation.nitrate assimilation.nitrate reductase
	Cc027434	hypothetical protein
	Cc019072	Chromatin assembly and remodeling.histone modifications.histone lysine methylation/demethylation.PRC2 histone methylation complex.VRN/FIS/EMF core complexes.FIE component
	Cc032486	hypothetical protein
	Cc027141	Phytohormones.strigolactone.synthesis.MAX1 monooxygenase
	Cc029155	hypothetical protein
	Cc017491	RNA biosynthesis.transcription factor families.bHLH transcription factor
	Cc017713	hypothetical protein
	Cc025227	hypothetical protein
	Cc045085	Polyamine metabolism.spermidine/spermine.synthesis.S-adenosyl methionine decarboxylase
	Cc006170	Protein modification.hydroxylation.prolyl hydroxylase
	Cc005014	hypothetical protein
	Cc017627	hypothetical protein
	Cc023554	hypothetical protein

	Cc002886	hypothetical protein
	Cc007871	Solute transport.carrier-mediated transport.APC superfamily.AAAP family.AAAP-type transporter
	Cc018583	hypothetical protein
	Cc047498	hypothetical protein
	Cc009763	Protein modification.phosphorylation.TKL kinase superfamily.LRR-V kinase
	Cc047633	hypothetical protein
	Cc036033	Phytohormones.strigolactone.synthesis.DWARF27 biosynthesis protein
	Cc004856	Solute transport.carrier-mediated transport.IT superfamily.phosphate transporter (PHO-type)
	Cc031064	Solute transport.carrier-mediated transport.MOP superfamily.MATE family.metabolite transporter (DTX-type)
	Cc004305	RNA biosynthesis.transcription factor families.DBP transcription factor
	Cc009634	RNA biosynthesis.transcription factor families.B3 superfamily.REM transcription factor
	Cc007122	hypothetical protein
	Cc019353	Secondary metabolism.phenolics.p-coumaroyl-CoA synthesis.phenylalanine ammonia lyase
	Cc002268	RNA biosynthesis.transcription factor families.NAC transcription factor
	Cc012179	hypothetical protein
	Cc032013	hypothetical protein
	Cc021178	Solute transport.channel-mediated transport.channels.OSCA calcium-permeable channel
	Cc039229	hypothetical protein
	Cc027611	hypothetical protein
	Cc044688	Redox homeostasis.enzymatic reactive oxygen species scavengers.superoxide dismutase activities.copper/zinc superoxide dismutase
	Cc033156	Amino acid metabolism.biosynthesis.shikimate family.shikimate pathway.3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase
	Cc047628	hypothetical protein
	Cc021927	hypothetical protein
	Cc037370	hypothetical protein
	Cc026439	hypothetical protein
	Cc003540	hypothetical protein
	Cc005437	hypothetical protein
	Cc043978	RNA biosynthesis.transcription factor families.HB (Homeobox) superfamily.HD-ZIP I/II transcription factor
	Cc020523	Phytohormones.cytokinin.synthesis.cytokinin phosphoribohydrolase
	Cc013975	hypothetical protein
	Cc005472	Solute transport.carrier-mediated transport.MFS superfamily.NRT1/PTR anion transporter
	Cc005020	Environmental stimuli response.temperature.Hsp (heat-shock-responsive protein) families.Hsp70 family.DnaK protein
	Cc000225	hypothetical protein
	Cc031699	RNA biosynthesis.transcription factor families.MADS box transcription factor
	Cc017659	Protein degradation.peptidase families.serine-type peptidase activities.subtilisin-type protease families.SBT5 protease
	Cc017739	hypothetical protein
	Cc007278	RNA processing.ribonuclease activities.RNase P ribonuclease activities.RNA-dependent RNase P complex.RPP14/POP5 component
	Cc018614	hypothetical protein
	Cc012157	hypothetical protein
	Cc034787	Cytoskeleton.actin and tubulin folding.CCT chaperonin folding complex.CCT2 beta subunit
	Cc017156	hypothetical protein
	Cc030614	hypothetical protein
	Cc030285	hypothetical protein
	Cc047408	hypothetical protein
	Cc034973	Solute transport.channel-mediated transport.channels.QUAC/ALMT anion channel
	Cc004113	hypothetical protein
	Cc047144	hypothetical protein

Chapter 3: *Cuscuta campestris* grown on an artificial host benefits from light and ability to produce carotenoid pigments

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Abstract

Cuscuta campestris is an obligate parasitic plant that lacks expanded leaves and roots. This plant parasite depends on its host to complete its life cycle, and uses a specialized organ called a haustorium to extract the nutrients and water. The Artificial Host System (AHS) is a unique system that allows research on light effects on *C. campestris* in the absence of a photosynthetic host. Using the AHS, we grew *C. campestris* under diurnal or constant dark conditions and assessed the role of light on parasite growth and carotenoid content. We also investigated the differences associated to carotenoid-related processes between the haustorial regions and stems without haustoria. We examined the transcriptional profile of genes in the pathways leading to carotenoid, abscisic acid (ABA) and strigolactone (SL) biosynthesis, and evaluated the carotenoid content between haustorial regions and stems, and their response to ABA application. Our results showed that *C. campestris* gained biomass under dark conditions, but more biomass accumulated under diurnal conditions, and that exposure to light affected pigment content. At the level of transcriptional regulation, the carotenoid biosynthetic pathway and the ABA and SL core pathways operate differently in haustorial regions compared to stems, but we did not detect differences in total carotenoid content between the two plant organs. Conversely, application of ABA through the artificial host provoked significant differences in carotenoid content between the haustorial region and the stem, without affecting parasite growth. In sum, we demonstrated that light has a positive effect on parasite growth, and influences pigment content, whereas the application of the carotenoid-derived phytohormone ABA through the artificial host influences pigment content but not parasite growth.

Introduction

Cuscuta campestris (subgenus *Grammica*) is a parasitic plant characterized by extremely simple morphology, without roots or extended leaves (Hibberd *et al.*, 1998). This parasite is a yellowish vine, whose anatomy comprises a stem, a parasitic organ called a haustorium, and flowers and fruits once it has reached a reproductive stage. *C. campestris* is able to colonize a wide range of dicots, coiling around their stems and producing haustoria that establish vascular connections to their hosts. These connections are bridges for the exchange of nutrients, water and genetic materials (Birschwilks *et al.*, 2007; Kim *et al.*, 2014; Sun *et al.*, 2018; Liu *et al.*, 2020). *C. campestris* presents a significantly higher content of carotenoids and a lower content of chlorophylls than the autotrophic plant relative, *Ipomoea tricolor* (Van Der Kooij *et al.*, 2000). *C. campestris* is an obligate parasite that relies on a living host (Kuijt, 1969) or an artificial one (Bernal-Galeano & Westwood, 2021) to obtain necessary resources to complete its life cycle. This parasite has shown a significatively lower photosynthetic rate than other *Cuscuta* species and *I. tricolor* (Van Der Kooij *et al.*, 2000) and, despite the presence of most photosynthetic genes in the *Cuscuta* genus (Mcneal *et al.*, 2007), *C. campestris* lacks some of the genes involved in a high-level photosynthetic rate (Vogel *et al.*, 2018). It has been hypothesized that the conservation of some photosynthesis-related components is important for the production of lipids used in seed setting or for recycling of host-derived carbon (Mcneal *et al.*, 2007). However, the specific roles of the photosynthetic-related components or the specific roles of carotenoids and their derivates in *Cuscuta* remain unclear.

Carotenoids are terpenoid compounds produced by plants and some microorganism (Nisar *et al.*, 2015). They are a large and diverse class of molecules that include colorless but also yellow, orange and red compounds. Carotenoids are able to absorb light in the visible spectrum range from 450 to 550 nm, increasing the spectral range for light absorption of plants, and ultimately photosynthetic capacity (Hashimoto *et al.*, 2016).

Carotenoids and their derivates play a crucial role in several plant processes such as photosystem assembly, light-harvesting and photoprotection (Giuliano *et al.*, 2003; Cazzonelli & Pogson, 2010; Águila Ruiz-Sola & Rodríguez-Concepción, 2012; Havaux, 2014), regulation of growth (Tian, 2015), and interaction of plants with their environment (Cazzonelli & Pogson, 2010; Walter & Strack, 2011). Carotenoid precursors are produced by the methylerythritol 4-phosphate (MEP)

pathway (Eisenreich *et al.*, 2001; Rodríguez-Concepción & Boronat, 2002) In this pathway, the rate-determining enzymes 1-deoxyxylulose-5-phosphate synthase (DXS) and (1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) are important for flux regulation (Nisar et al, 2015). In *A. thaliana* seedlings, the overexpression of the genes encoding these two enzymes have shown increased carotenoid production (Estévez *et al.*, 2001; Carretero-Paulet *et al.*, 2006).

Plant carotenoid synthesis starts with the production of phytoene, the first carotenoid produced by the phytoene synthetase enzyme (PSY) (Shewmaker *et al.*, 1999; Bramley, 2002; Sieiro *et al.*, 2003). The PSY is a rate-limiting enzyme for carotenoid synthesis (Shewmaker *et al.*, 1999). *A. thaliana* only has one gene that encodes for PSY, whereas tomato (*Solanum lycopersicum*) and tobacco (*Nicotiana* spp) have three genes with different tissue-specific expression patterns (Bramley, 2002; Giorio *et al.*, 2008; Wang *et al.*, 2021). The expression of the genes PSY1 and PSY2 in *Nicotiana tabacum* increased upon treatment with abscisic acid (ABA), or gibberellic acid (GA)(Wang *et al.*, 2021). Silencing of PSY in orchids showed tissues with reduced carotenoid content, increased expression of genes encoding enzymes involved downstream of the carotenoid biosynthesis pathway due to relaxed product feedback regulation, a reduction in endogenous levels of gibberellic acid and abscisic acid, and semidwarf plants (Diretto *et al.*, 2010; Liu *et al.*, 2014).

The carotenoid biosynthetic pathway continues towards the production of all-trans-lycopene. After a series of reactions catalyzed by the enzymes phytoene desaturase (PDS), (zeta) z-carotene isomerase (Z-ISO), (zeta) z-carotene desaturase (ZDS) (Chen *et al.*, 2010), and carotenoid isomerase (CRTISO), the newly synthesized and colorless phytoene is converted into the red colored lycopene (Isaacson *et al.*, 2004). After the formation of all-trans-lycopene, this is modified by the enzymes lycopene β-cyclase (β-LCY) and lycopene ε-cyclase (ε-LCY) leading to the formation of α-carotene (with one β and one ε ring) and β-carotene (with two β rings) (Cunningham *et al.*, 1996; Pogson *et al.*, 1996). Lutein is an α-carotene–derived xanthophyll, and zeaxanthin, violaxanthin, and neoxanthin are β-carotene–derived xanthophylls (Rissler & Pogson, 2001). Lycopene is the compound responsible for the red color of tomato fruit, and during fruit development transcriptional control is reflected by the increased expression of the genes involved in the first steps of the carotenoid pathway (SlPSY1 and SlPDS), and the decreased expression of genes involved in converting lycopene to other subproducts (SlLCYE and SlLCYB) (Pecker *et al.*, 1992; Alba *et al.*, 2005).

The level of carotenoids in plant tissues depends on the equilibrium between their biosynthesis, storage and degradation (Hannoufa & Hossain, 2012; Li & Yuan, 2013). The enzymatic cleavage and oxidation of carotenoids produce apocarotenoids, which include volatile and flavor compounds, as well as phytohormones like ABA and strigolactones (SL) (Auldridge *et al.*, 2006). Apocarotenoids can be enzymatically produced by members of the carotenoid cleavage dioxygenase (CCD) family, with nine enzymes in *A. thaliana* (Tan *et al.*, 2003). Five of them belong to the nine-cis-epoxycarotenoid cleavage dioxygenases *(NCED2, NCED3, NCED5, NCED6, and NCED9).* In *A. thaliana*, NCEDs are rate-limiting enzymes for ABA biosynthesis (Tan *et al.*, 2003), as they are involved in the production of the ABA precursor, xanthonin (Nambara & Marion-Poll, 2005). A second group is formed by CCD1, CCD4, CCD7 (MAX3), and CCD8 (MAX4). CCD7 and CCD8 are involved in the production of the SL precursor, carlactone (Gomez-Roldan *et al.*, 2008; Seto *et al.*, 2014).

The carotenoid-derivate phytohormone ABA is important for plant stress responses and development. ABA regulates tissue-specific responses to several environmental stresses as well as endogenous signals related to water deficit (Wright & Hiron, 1969; Pierce & Raschke, 1980; Henson, 1982; McAdam & Brodribb, 2016), seed development and dormancy (Zeevaart & Creelman, 1988). When environmental conditions are optimal, plants maintain ABA at basal levels which promotes growth (Chen *et al.*, 2020). After a plant is affected by changing environmental conditions such as drought, the accumulation of ABA increases causing the activation of stress responses like stomal closure and changes in root architecture (Mustilli *et al.*, 2002; De Smet *et al.*, 2003). ABA levels are controlled transcriptionally and pos-transcriptionally, and they are tuned via synthesis, degradation, metabolism, deconjugation and transport (Chen *et al.*, 2020). Recent studies determined that leaves are the primary source for ABA biosynthesis (McAdam *et al.*, 2016), from where it is transported through the phloem from shoots to the roots, either during drought stress or unstressed conditions(Ikegami *et al.*, 2009; McAdam & Brodribb, 2016; Manzi *et al.*, 2016).

Cuscuta spp. are plants adapted to support drought. *Cuscuta* spp. have a reduced transpiration (Fuentes *et al.*, 2014) and a low density of stomata that develop in the parasitic stage (Lyshede, 1985). Some *Cuscuta* spp. have shown the capacity of sensing drought stress and synthesizing ABA (Qin *et al.*, 2008; Li *et al.*, 2015b). However, it has been shown that *C. australis* seedlings

have a very low sensitivity to ABA, since their growth was only affected by large concentrations of ABA (700uM), while tomato seedlings showed arrested growth with just 1 uM ABA (Li *et al.*, 2015b). On the other hand, reports have shown how tomato plants hosting *C. pentagona* increased their ABA content after a second attachment of the parasite, which also presented more ABA but no increased amounts of other hormones like auxin (RUNYON *et al.*, 2010).

It is clear that *Cuscuta* does not possess the typical organs of an autotrophic plant, such as leaves and roots, organs that normally contribute to the production of phytohormones that are distributed across the plant and act as developmental determinants (McAdam *et al.*, 2016). This leads to questions like: how does the organ compartmentalization work in this parasite? Or what are the localization patterns of the phytohormones or sites of production of other substances in *Cuscuta*? We hypothesize that despite the parasite simplicity, there is a distribution of functions between the haustorial tissues and the shoots related to parasite development and functioning, for example during the parasitic phase.

In order to gain a better understanding into the role of carotenoids in the parasite biology when living without a plant host, we investigated the relation among total carotenoid content, carotene-related expression genes in haustorial regions vs stems, parasite growth and the effect of the carotenoid-derivate phytohormone ABA. We used the Artificial Host System (AHS) (Bernal-Galeano & Westwood, 2021), a methodology that allows growth of the parasite without a living host and thus enables experiments that test conditions such as constant darkness, or exposure to substances such as herbicides and hormones that would normally impact host physiology and thereby confound conclusions about parasite response to such treatments.

Materials and methods

Plant material and AHS

C. campestris was grown in the Artificial Host System (AHS) as described by Bernal-Galeano and Westwood (2021). Three-centimeters parasite vegetative shoot tips were taken from a nursery grown on beets. Shoot tips were surface sterilized and attached to artificial hosts inside sterile magenta boxes. Modified Murashige and Skoog liquid media (MMS) (Srivastava & Dwivedi, 2001) was used as base media for most of the experiments containing 3% glucose, pH 5.7, 1X MS micronutrients (Phytotechlab, USA), 1-naphthaleneacetic acid (NAA) (3 mg l^{-1}) and 6-

benzylaminopurine (BA) (1 mg l^{-1}), and we refer to this media as basal media. After parasite attachment, AHSs were placed under enriched far red light for 2.5 days to promote coiling and haustoria formation, followed by 12.5 or 33.5 days under full spectrum light in a diurnal regimen (14 hrs light/10 hrs dark) at 25°C. We will refer to the diurnal regimen as "light". Additionally, we will refer to shoots as "stems", this are shoots mainly from by stems, with meristematic regions but not haustorial regions or flowers, as described by Bernal-Galeano and Westwood (2021).

Darkness, ABA and mesotrione treatments

Experiments under darkness were set as described above. Parasites were attached to artificial hosts and basal media was used. After the parasites were exposed to far-red enriched light for 2.5 days the AHS boxes were transferred to complete and continuous darkness for 12.5 or 33.5 days. Experiments that included abscisic acid (ABA) were set using the AHS and the basal media as described above, but with the addition of 100uM ABA to the media. All parasites were exposed to 2.5 days of enriched far-red light and then moved to full spectrum light for 12.5 days for pigment extraction or 33.5 days for growth evaluation. Experiments with mesotrione were set up using the AHS and the basal media as described above, with the difference that filter sterilized mesotrione (Callisto Herbicide, Syngenta) was added directly to the media after the incubation under far-red light for 2.5 days and before the AHS were moved to the full spectrum light for 33.5 days.

Parasite growth evaluation

Parasite growth was measured as described by Bernal-Galeano and Westwood (2021). After 36 days post inoculation (dpi) boxes were opened and parasites were removed. Only parasites that made good connections to artificial hosts, as indicated by turgid and healthy holdfasts, were considered for further analysis. After the plant material was collected, parasites were extended on a white surface, photographed next to a ruler and their total length, including the initial 3cm of the inoculum, was measured using the software ImageJ 1.51j8 (National Institutes of Health, USA). Then the fresh weight of each plant was measured after drying for 3 days at 50 C. Statistical analysis was done using JMP Pro 15 (SAS Institute Inc.).

Pigment extraction

Plants growing under AHS under light, darkness or with added ABA were collected 15 dpi. Only plants with turgid and healthy holdfast were included for analysis. Plant material was weighed and

80% acetone was used to obtain a ratio of 10 mg of plant fresh weight per ml of solvent. Plant material was ground using beads and bead beater until complete disintegration of the tissue. Then centrifugation was used to remove debris from the extract. Pigment content was evaluated through spectrometry following the protocol in Lichtenthaler (Lichtenthaler, 1987).

Bioinformatic analysis

A list of *A. thaliana* genes related to carotenoid or ABA pathways was built based on reports from the literature. For the identification of homologue candidate genes, protein sequences corresponding to the selected genes of *A. thaliana* were extracted from Phytozome TAIR10 version. Arabidopsis sequences were used in a BlastP against a *C. campestris* protein sequence database (Vogel *et al.*, 2018). All the hits that presented an e-value <1e-50 were collected. Then, a reciprocal BlastP was run using the *C. campestris* sequences from the hits in the first BlastP, against the *A. thaliana* protein database. For the reciprocal Blast, only the best hit was considered (-evalue 1e-50, -max_target_seqs 1, -max_hsps 1). After obtaining the results, the *A. thaliana* gene ID was compared to the initial gene ID of the selected genes. If the gene ID was the same, the homologue candidate was considered for further analysis. The gene ID of *C. campestris* genes were used to extract transcriptomic information from the differential expression analysis made by Bernal-Galeano and Westwood (2021). For simplicity, only the results of the DESq2 analysis were considered. An adjusted p-value (padj) < 0.05 was considered as threshold for differential expression.

Results

Light has a measurable positive impact on* C. *campestris weight and length gain

To experimentally evaluate the role of light on *C. campestris* weight and length without the effect of a living host, we grew parasites using the AHS with MMS media and the phytohormones NAA, and BA. After the parasites were exposed to far-red light to promote coiling and haustorium formation, they were transferred to either a light regime or to permanent darkness. Our results showed that *C. campestris* significantly gained fresh weight and biomass compared to the initial inoculum regardless of light availability (**Fig. 1 a-e**). However, parasites under light conditions gained significantly more biomass than those under permanent darkness. Parasites in light conditions were also longer than the ones in darkness, while parasites in darkness did not differ in length from the shoots at the initial time (**Fig. 1 f**).

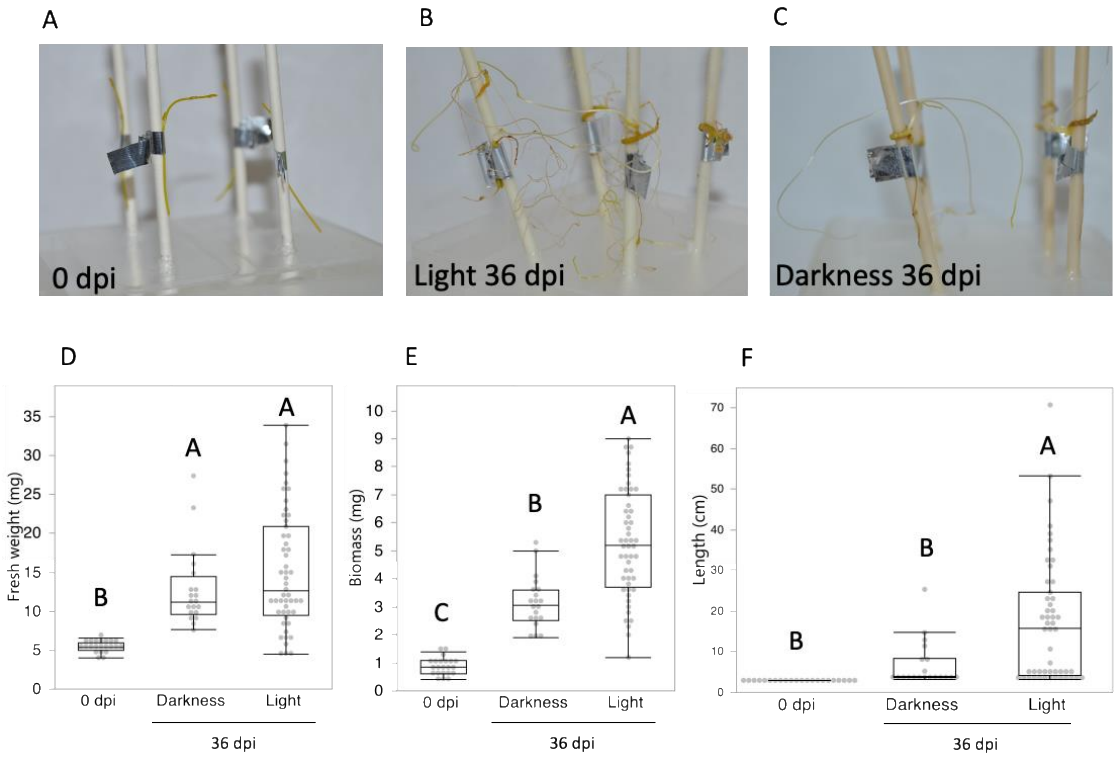


Fig. 1. Effect of light on *Cuscuta campestris* growing in the artificial host system (AHS). **a.** *C. campestris* shoot tips were inoculated in AHS. Parasites were exposed to far-red enriched light for 2.5 days, and then to either light conditions (**b**) or permanent darkness (**c**) for 33.5 days. Data was collected at 36 dpi. **d**) Parasite fresh weight. **e**) Parasite biomass. **f**) Parasite length. **d-f**) Statistical differences were detected by ANOVA with post-hoc Tukey HSD test. Differences were considered statistically significant at $P<0.05$ and indicated with different letters. Experiment was repeated three times. Analyses included only plants that coiled and developed a turgid holdfast. Number of plants ranged from 20 to 51 per treatment.

Light activates the production of pigments in* C. *campestris tissues

Since etiolated non-parasitic plants present a lower content of chlorophylls and carotenoids as well as low biomass (Von Lintig *et al.*, 1997), we wondered whether the differences in biomass accumulation had a relation with pigment content in *C. campestris* growing in the AHS. Our results show differences in the pigmentation between parasites kept in darkness compared to the ones

under light conditions. In order to analyze the pigment content in the parasite tissues under light and dark conditions, we extracted and quantified their pigments. Our results confirmed a change in the pigment content profile of the parasite in response to light (**Fig. 2**). We observed that light caused a significant increase in total carotenoids, total chlorophylls, and chlorophyll a, while chlorophyll b content was not affected. We found that the larger the parasite biomass under light conditions, the higher levels of chlorophyll a, total chlorophylls and total carotenoids. We also observed that the amount of carotenoids (ug) per unit of fresh tissue (mg) were significantly higher than the amount of chlorophylls regardless of light or darkness exposure.

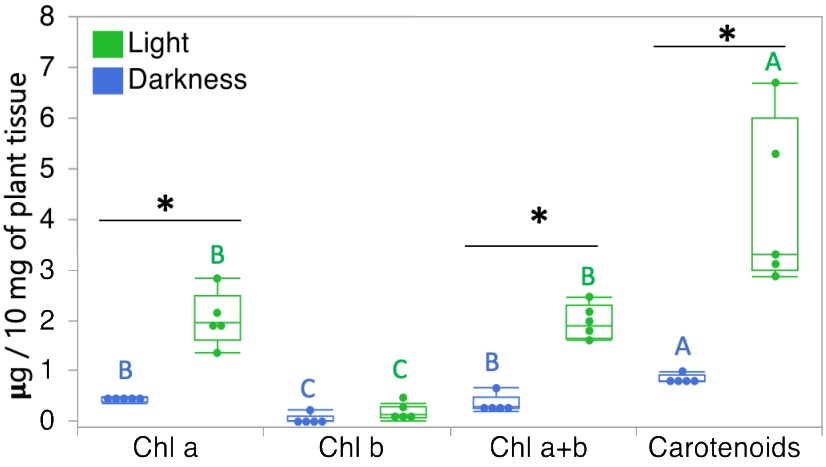


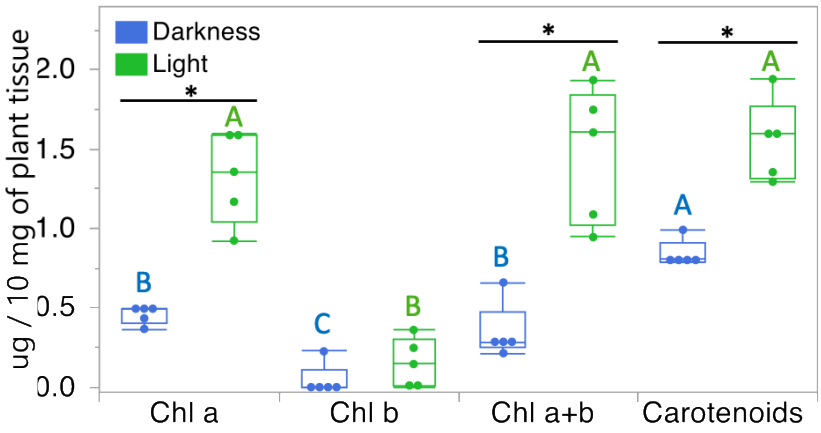
Fig. 2. Pigment content in whole *C. campestris* growing in artificial host system (AHS) under light or darkness conditions at 15 dpi. Statistical differences between light and dark treatments are indicated with Asterix above the horizontal bars that indicate the compared groups. Differences were detected by ANOVA with post-hoc Student-T test and considered statistically significant at $P<0.05$. Comparisons of pigment concentrations within light treatments are indicated by letters above bars. Within each treatment statistical differences in pigment content were detected by ANOVA with post-hoc Tukey HSD test and were considered statistically significant at $p<0.05$.

We noticed that haustorial regions of parasites held in darkness presented a conspicuous accumulation of an orange-colored substance that co-localized with the portion of the holdfast in contact with the artificial host. This substance remained on the paper spindle that makes up the

artificial host after the parasite was peeled off, indicating an exudation of pigments from the parasite (Fig. **3a**). In order to understand the effect of light on pigment content of the haustorial regions, we examined these regions at 15 dpi under diurnal or dark conditions. We observed a shift in levels of different pigments under dark, the concentration of total carotenoids was larger than that of chlorophylls. Under light exposure there were no significant differences in the amount of chlorophyll a, chlorophyll a and b, and total carotenoid in the haustorial regions (Fig. **3b)**. When comparing the specific pigment amount between plants growing under the different light conditions, all pigments except chlorophyll b displayed significantly reduced pigment content under darkness compared to light (Fig. **3b**). The ratio between total carotenoids, and chlorophylls a and b in haustorial regions under darkness was significantly higher than similar regions under light (Fig. **3c**).

A.

B.



C.

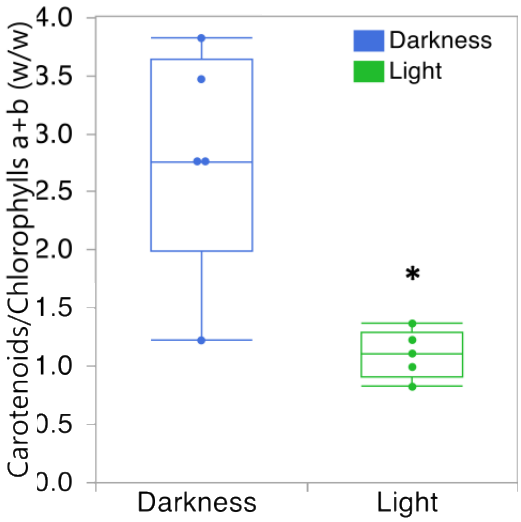


Fig. 3. Differences in pigment content of haustorial regions of parasites growing in diurnal (light) and dark conditions in the artificial host system (AHS). **a**) Haustorial regions of *Cuscuta campestris* 36 days post inoculation (dpi). **b**) Quantification of pigment content in haustorial regions grown under light or dark conditions and measured at 15 dpi. Statistical differences were detected by ANOVA with post-hoc Student-T test. Differences were considered statistically significant at $p<0.05$ and indicated with stars. Five replicates were evaluated per treatment. **c**) Comparison of the Carotenoid/chlorophyll ratio presented by parasite growing in diurnal and dark conditions at 15 dpi. Statistical differences were detected by ANOVA with post-hoc Student-T test. Differences were considered statistically significant at $p<0.05$ and indicated with stars.

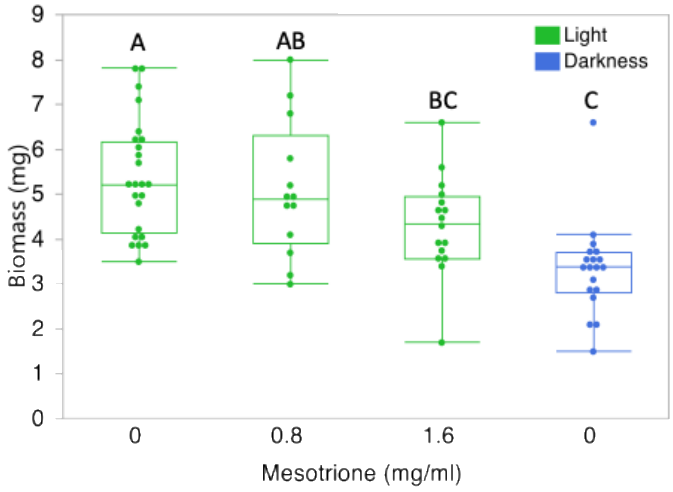
Cuscuta campestris presents decreased biomass and pigmentation after application of a carotenoid biosynthesis pathway inhibitor through the artificial host

The differences in *C. campestris* growth under darkness as compared to light, combined with decreased pigment concentrations in darkness, suggests a causative linkage between these two parameters. To test this idea, we inhibited the carotenoid biosynthetic pathway of *C. campestris* by application of mesotrione, a carotenoid biosynthesis inhibitor that ultimately causes plant tissue bleaching and is used for the control of broadleaf and some grass weeds (Mitchell *et al.*, 2001).

This herbicide led to the bleaching of the tissues and a reduction in biomass of *C. campestris* growing on carrot, however the host was sensitive to the treatment (Weinberg *et al.*, 2003). The mesotrione mechanism of action relies on the competitive inhibition of the enzyme 4-Hydroxyphenylpyruvate dioxygenase (HPPD) involved in the biosynthetic pathway of plastoquinone, a cofactor of phytoene desaturase which is an important enzyme for carotenoid production (Mitchell *et al.*, 2001).

For our experiment, *C. campestris* shoot tips were grown in AHS under diurnal conditions with and without mesotrione, and parasite color and growth were evaluated at 36 dpi. We observed an herbicide concentration-dependent reduction of parasite biomass (Fig. **4a**), in which the parasites exposed to the highest concentration of mesotrione (1.6 mg ml^{-1}) exhibited a significant decrease in biomass compared to the control. No significant differences were observed in the fresh weight or length of the parasites (Fig. S1 a,b). Pigmentation of the haustorial region exhibited clear differences (Fig.4**b**). Parasites growing with media containing mesotrione presented a bleached phenotype 36 dpi. Changes in biomass and pigmentation of the parasite under mesotrione suggest that a light dependent process involving carotenoids contributes to *C. campestris* growth.

A.



B.

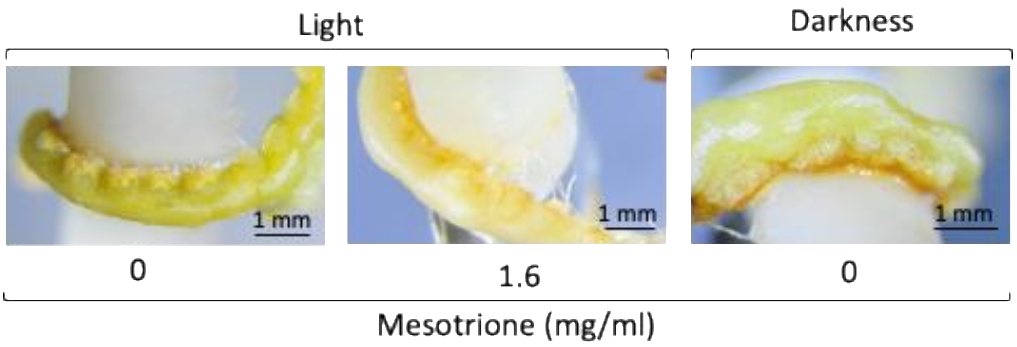


Fig. 4. Effect of the bleaching herbicide mesotrione on biomass accumulation and pigmentation of *Cuscuta campestris* growing on artificial host system (AHS). **a**. Mesotrione causes a decreased biomass accumulation in parasites growing on AHS after 36 dpi. Statistical differences were detected by ANOVA followed by a post-hoc Tukey HSD test. Differences were considered statistically significant at $P<0.05$ and indicated with different letters. Analyses included between 13 and 24 replicates, and only plants that coiled and developed turgid holdfasts. **b**. Mesotrione and darkness provoked a change in the pigmentation of haustorial regions compared to the control. Pictures taken 36 dpi.

Genes involved in carotenoid biosynthesis pathway are mostly downregulated in haustorial regions compared to stems in* C. *campestris growing in AHS

After observing the differences in the pigmentation of haustorial regions under the different treatments and the high concentrations of carotenoids compared to chlorophylls, we wondered whether differences would also be reflected in transcriptional regulation of carotenoid-related genes between the haustorial region and stems. We cataloged genes that have been reported as involved in carotenoids biosynthesis and extracted their corresponding sequences from *A. thaliana*. We discovered that some genes have several copies in *C. campestris* (Table S1). Then, we collected transcriptomic data associated to all the copies of the studied genes from the data generated by Bernal-Galeano & Westwood (2021). This data was the product of a differential expression analysis between haustorial region and stem of parasites growing in AHS condition with MMS media, NAA and BA under a diurnal regimen at 15 dpi. The presence of these genes, either in haustorial regions or stems, confirms the expression of genes involved in the carotenoid biosynthetic pathway in *C. campestris* under the tested conditions. Only genes that presented a

differential expression (padj < 0.05) between haustorial regions and stems were plotted on the schematic view of the carotenoid biosynthetic pathway and other associated pathways (**Fig. 5**). The general profile showed that the expression of many of the genes in the carotenoid biosynthetic pathway are downregulated in haustorial regions compared to stem tissues. For example, the gene *PSY*, an important flux-controlling element of the pathway, is downregulated in haustorial regions as well as the genes *PDS3, ZDS, CRTISO, LUT2, LCYB, LUT1, BCH1, NPQ1, ABA1* and *ABA4.* Only two *C. campestris* genes in this pathway presented the opposite pattern, *ZIC1* and *LUT5* were upregulated in haustorial regions compared to stem. The gene encoding for the enzyme BCH2 did not show a homologue candidate under the selected parameters.

The transcriptional factors PIF1 and HY5 are regulators of the expression of *PSY* in response to light cues. PIF1 represses the expression of *PSY* in *A. thaliana* plants growing under darkness, but after plants are exposed to light *PSY* expression is derepressed and activated by HY5 (Quian-Ulloa & Stange, 2021). Transcriptomic data of *C. campestris* growing in the AHS under diurnal conditions showed that all copies of *PIF1* are downregulated in the haustorial region compared to stem tissues. Our data also show that the only copy of *C. campestris* HY5 is expressed in haustorial regions and stems, although not differentially (Table S1). At a transcriptomic level of regulation, we did not find evidence of a positive association between the expression of the genes encoding PIF1 and HY5 and the expression of *PSY* in *C. campestris.*

Other pathways that show an effect on carotenoid production are the MEP pathway and the plastoquinone biosynthesis pathway. The MEP pathway provides the necessary precursors for the carotenoid synthesis. In this pathway, DXS and DRX are flux-controlling enzymes (Águila Ruiz-Sola & Rodríguez-Concepción, 2012). *C. campestris* transcriptomic data showed that *DXS* and *DXR* are slightly upregulated in haustorial tissues compared to stem. On the other hand, the plastoquinone biosynthesis pathway is important for carotenoid biosynthesis because its product plastoquinone acepts electrons produced by the action of the PDS enzyme (Norris *et al.*, 1995). Additionally, this pathway contains the enzyme HPPD, the target of mesotrione. Transcriptomic data showed that the gene *PDS1* encoding HPPD, is being upregulated in haustorial tissues compared to stems. Other genes in this pathway, *PDS2* and *VTE3,* showed an opposite pattern, being downregulated in the haustorial region.

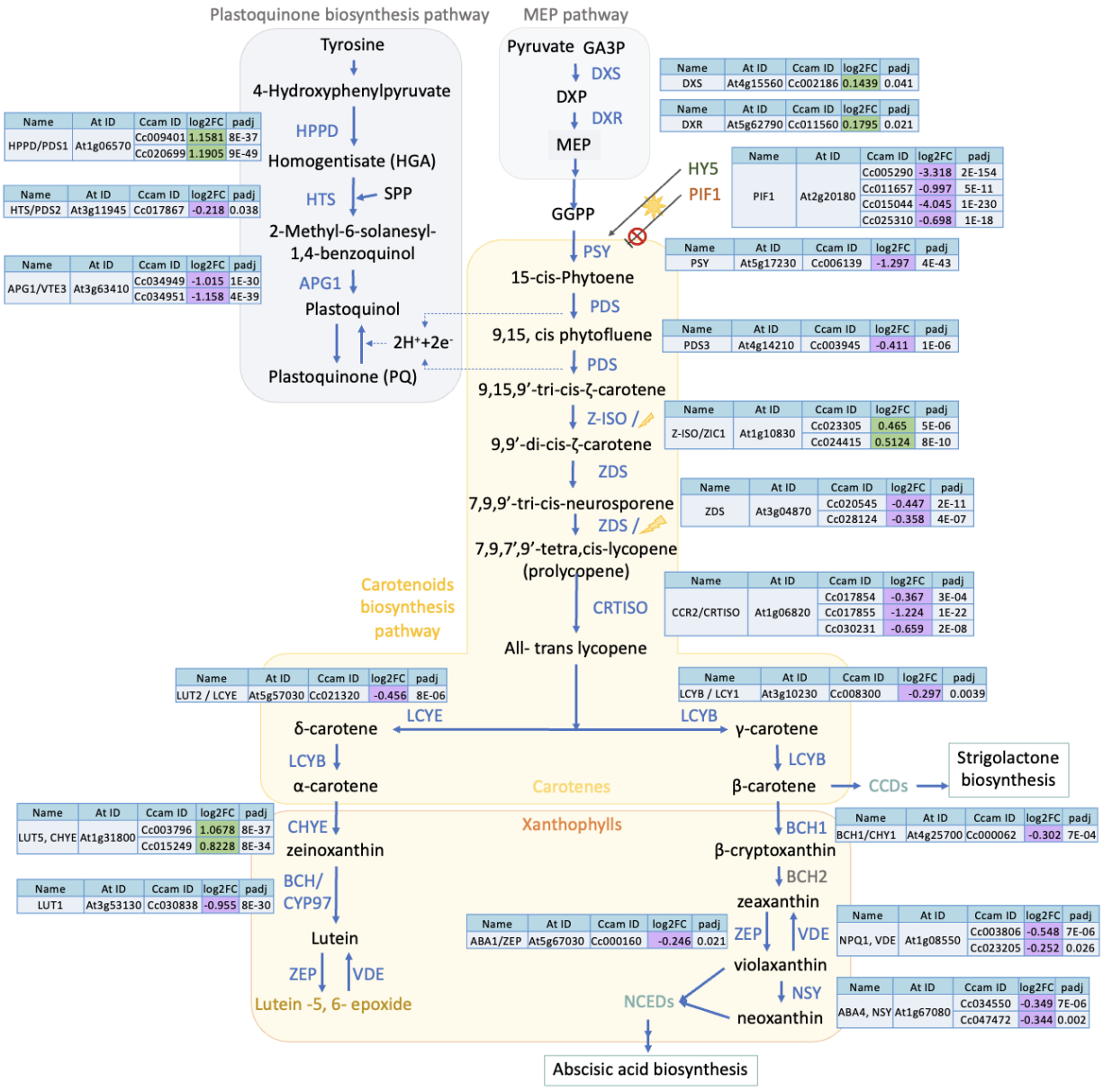


Fig. 5. Gene expression profiles of *Cuscuta campestris* genes previously reported as involved in carotenoid, plastoquinone and MEP biosynthetic pathways. Genes listed in the tables embedded in the figure are homologues of *A. thaliana* genes found through reciprocal BlastP and presenting differential expression between haustorial regions and stems, "Name" corresponds to *C. campestris* gene ID according to Vogel et. Al. (Vogel *et al.*, 2018). In green, fold changes of upregulated genes in haustorial regions. In purple, fold changes of downregulated genes in haustorial regions, hence these genes are upregulated in the same magnitude in stems. padj <0.05. Some of the genes present more copies in *C. campestris* but no differential expression. The gene

BCH2 did not present a homologue candidate. The transcription factor *HY5* presented a homologue candidate but no differential expression. For more information consult Table S1.

Genes related to the core of the abscisic acid and strigolactones biosynthetic pathways are mostly upregulated in the haustorial regions compared to stem tissues

Carotenoids are precursors of several important compounds for plant development and stress adaptation such as the phytohormones abscisic acid (ABA) and strigolactones (SL). We analyzed the differences in the expression profiles of the genes involved in the core biosynthetic pathway of these two phytohormones in the haustorial regions and stems of *C. campestris* growing on artificial hosts using the data reported by Bernal-Galeano and Westwood (2021). The enzymes NCED are key for the production of ABA (Frey *et al.*, 2012). We found candidate homologues in *C. campestris* for three out of the five *A. thaliana* NCED genes (Fig. **6a**). NCED3 has two homologous copies in *C. campestris,* both are being expressed but not differentially between the haustorial regions and the stems. While CcNCDE5 and CcNCDE9 were found to have two homologous copies each, and only one of the two copies is expressed more in haustorial regions compared to stems. In the case of the strigolactone biosynthetic pathway, we found homologues in *C. campestris* for the three genes involved in the core of the biosynthetic pathway (Fig. **6b**). A single copy of D27 is present, three copies of CCD7 and two copies of CDD8. All copies of D27 and CCD8 are upregulated in the haustorial region compared to stem tissues, while only one of the CCD7 copies is differentially expressed, being downregulated in the haustorial region. The overall expression profiles of genes involved in the biosynthesis of ABA and SL, present upregulation in haustorial regions compared to stem tissues.

A. B.

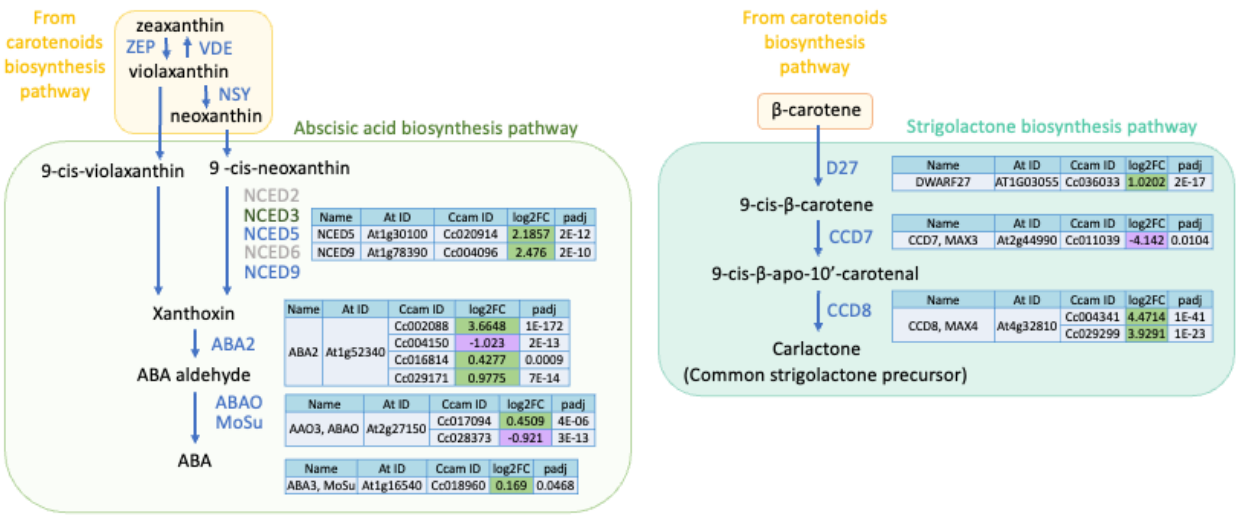


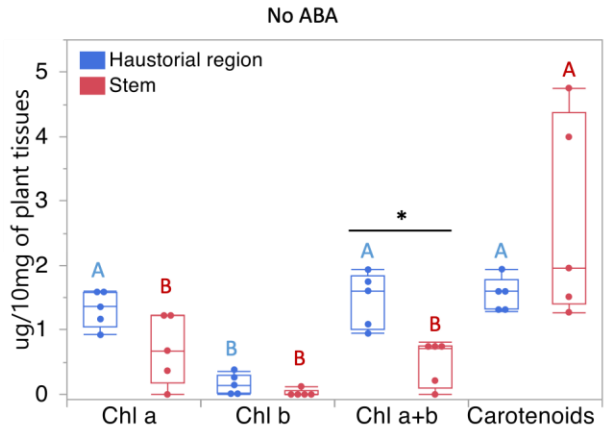
Fig. 6. Expression profile of genes in the ABA and SL core biosynthetic pathways in *Cuscuta campestris* growing in AHS. **a**. Core ABA biosynthetic pathway. **b**. Core SL biosynthetic pathway. Genes reported in the figures are homolog candidates according to reciprocal Blast results considering *Arabidopsis thaliana* (At) as reference. In green, fold changes of upregulated genes in haustorial regions. In purple, fold changes of downregulated genes in haustorial regions, these genes are upregulated in the same magnitude in stems. padj <0.05. Only *C. campestris* differential expressed genes are presented in tables in the figure. Some of the genes present more copies in *C. campestris* but no differential expression. The genes At*NCED2* and At*NCED6* did not present homologue candidates in *C. campestris*. The genes At*NCDE3* and Cr*NCDE1* presented homologue candidates but no differential expression. For more information consult Table **S1**.

Difference between pigment content in haustorial regions and stem increased under exogenous ABA treatment, however plant growth was not affected

The transcriptional evidence for increased expression of the ABA biosynthetic pathway in haustorial regions compared to stems led us to hypothesize that ABA is important for haustorial function. To mimic host-derived ABA on parasite growth, we applied exogenous ABA to the parasite through the AHS and evaluated *C. campestris* growth and pigment content in haustorial regions and stems. Parasite shoots were grown in the AHS and ABA (100uM) was added to the basal media. After 15 dpi, parasites with control media (without ABA) presented significant differences in chlorophyll a and b content between haustorial regions and stems, and only stems

accumulated significantly more carotenoids than chlorophylls (Fig. **7a**). On the other hand, haustorial regions of parasites under ABA treatment showed a significantly greater accumulation of carotenoids than stems, in contrast to controls without ABA (Fig. **7b**). Similar to the control, only stems had significantly more carotenoids than chlorophylls. Interestingly, comparing ABA-induced changes in pigment accumulation in haustorial regions and stems showed changes in patterns of pigment accumulation. The haustorial region pigments generally decreased as result of ABA application, whereas pigments generally increased in the stem, although only the decrease of chlorophyll a in haustorial regions was statistically significant (Fig. **7c**). We did not find that ABA in the media affected *C. campestris* growth (Fig. **7d**). This agrees with a report that *C. australis* seedlings did not present changes in length under comparable ABA concentrations, contrasting to the drastic decrease exhibited by tomato under the same conditions (Li *et al.*, 2015a).

A.



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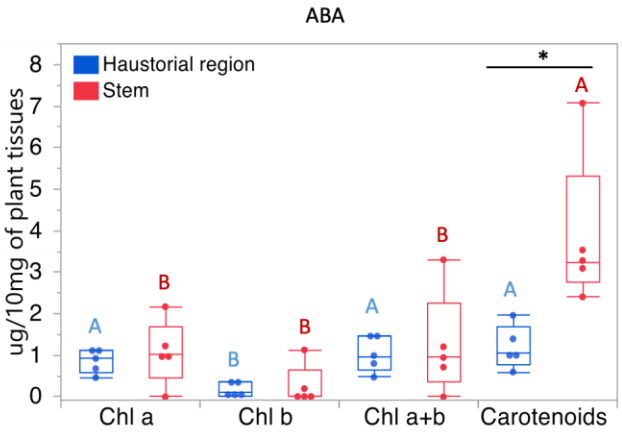
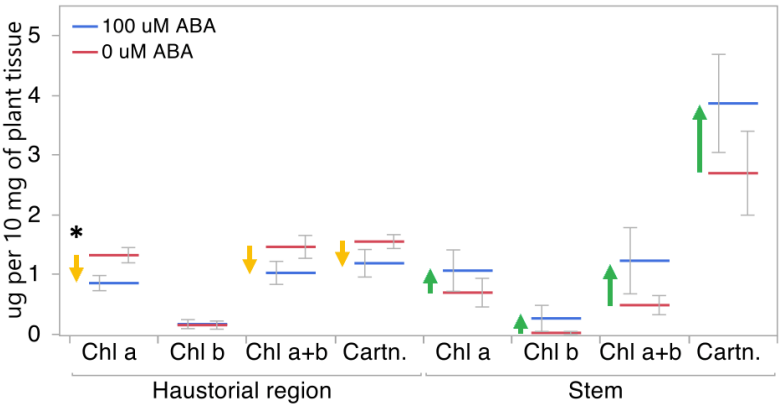


C.



D.

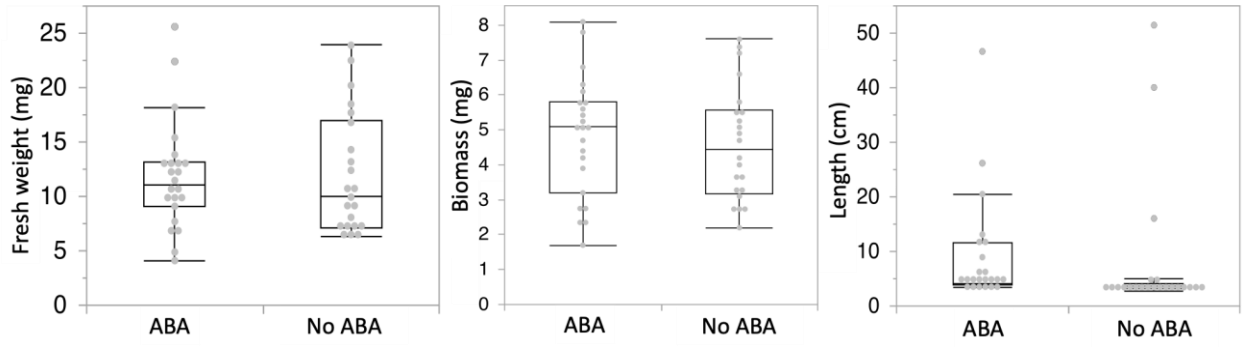


Fig. 7. Effect of exogenous abscisic acid (ABA) applied through haustoria on pigment content and growth of *Cuscuta campestris*. **a**) Pigment content in haustorial regions and stems. **b**) Pigment content in haustorial regions and stems of parasites growing with ABA (100uM). **a** and **b)** Data was collected 15 dpi. Significant differences were evaluated by ANOVA test followed by a post-hoc Student T test when comparisons were made between different tissues for the same pigment, or Tukey HDR when comparisons were made among different pigments for the same tissue. Differences were considered statistically significant at $P<0.05$ and indicated with a star or different letters. Analyses included 5 replicates. **c**) Comparison of pigment contents in haustorial regions and stem tissues between control (red) and treatment conditions with ABA (100uM) (blue). Data was collected 15 dpi. Graphic represents the media with a +/- SE. Arrows show positive (green) and negative (yellow) change trend. Significant differences were evaluated by ANOVA test followed by a post-hoc Student T test. Differences were considered statistically significant at $P<0.05$ and indicated with a star. Analyses included 5 replicates. **d**) Comparison between fresh weight, biomass and length of parasites grown under control or ABA (100 uM) treatment conditions. Data was collected 36 dpi. Significant differences were evaluated by ANOVA test. Analyses included 23 replicates, and only plants that coiled and developed turgid holdfasts.

Discussion

Previously, we demonstrated that *C. campestris* is able to complete its life cycle on an artificial host that provides water and nutrients (Bernal-Galeano and Westwood, 2021). For this study, we took advantage of the versatility of the AHS to evaluate the growth of the parasite under dark

conditions and its pigment content, avoiding constraints related to light requirements imposed by autotroph plant hosts. Specifically, this approach eliminates the variable of "type of plant host", which is important given that different host species and nutrient supply have a strong effect on the content of photosynthetic pigments in *Cuscuta* species (Panda & Choudhury, 1992).

Previous studies of *Cuscuta* have primarily focused on how light cues play a role in host localization, attachment and establishment (Tada *et al.*, 1996; Haidar *et al.*, 1998; Johnson *et al.*, 2016). Far-red light activates phototropic responses in *Cuscuta* (Orr *et al.*, 1996), as well as their coil around host stems and the production of haustoria (Haidar *et al.*, 1998). On the other hand, *Cuscuta* spp. are considered holoparasites, meaning that they depend on their host to complete their life cycle, and in consequence, *Cuscuta* have seemingly little need to produce carbohydrates (Mcneal *et al.*, 2007). Some *Cuscuta* spp. have chlorophyll and are able to perform low levels of photosynthesis (Van Der Kooij *et al.*, 2000). In the case of *C. campestris*, genomic analyzes have shown that this parasite lacks some of the genes responsible for high photosynthetic rates (Vogel *et al.*, 2018). Experimental analysis showed that the photosynthetic rates of *C. campestris* are so low that they never reach a zero-net balance with respect to the respiration rate (DINELLI *et al.*, 1993; Van Der Kooij *et al.*, 2000). This low photosynthetic rate has been presumed to be involved in recycling host-derivate carbon (Hibberd *et al.*, 1998) or in lipid biosynthesis during seed production (Mcneal *et al.*, 2007). These studies suggest that photosynthesis is not a main contributor for *C. campestris* growth.

In the present study, we demonstrate that light has a clear effect on *C. campestris* growth. Considering the reports on low photosynthetic rates of *C. campestris*, as well as its larger abundance of carotenoids compared to chlorophylls (Van Der Kooij *et al.*, 2000), we decided to focus our analysis on the role of carotenoids in the parasite growth. Our results show that *C. campestris* is able to accumulate more biomass under light than under continuous darkness without the input from a living host. We also found that chlorophyll and total carotenoids content increased with light exposure in *C. campestris*, which agrees with observations on autotrophic flowering plants (Hirschberg, 2001), and that parasites growing on AHS presented larger amounts of carotenoids than chlorophylls regardless the light input. Treatment with mesotrione, an inhibitor of the carotenoid biosynthesis, showed a similar outcome with reduced pigment content and reduce biomass. These findings showed a correlation between pigment content and biomass accumulation

in *C. campestris*, suggesting that the parasite endogenous pigments, or their derivates, have an effect on its growth.

Carotenoid biosynthetic pathway provides precursors to the ABA biosynthetic pathway. Studies report that the overexpression of phytoene synthase (PSY) (Cao *et al.*, 2015) or zeaxanthin epoxidase (ZEP) (Frey *et al.*, 1999) belonging to the carotenoid pathway provokes an increase in ABA levels. Additionally, levels of ABA increased at the expense of a severe decline in β,β-carotenoid content in water-stressed citrus roots (Walter *et al.*, 2015). Interestingly, we found that genes in the carotenoid biosynthetic pathway were in general downregulated in the haustorial region compared to the stem, whereas, genes in the subsequent biosynthetic core pathways of ABA and SL were in general upregulated in the haustorial region compared to the stem.

This may indicate that at a transcriptomic level of regulation the production of carotenoids is not as necessary in haustorial regions as it is in stems, while the production of ABA and SL are prioritized in haustorial regions. Based on this, we hypothesize that levels of carotenoids in the haustoria are depleted as result of a low flux into carotenoid biosynthesis combined with a high flux of carotenoid usage, for example to be converted into ABA. Though, the carotenoid content in samples under the same conditions than the transcriptomic data showed no significant differences in carotenoid content between haustorial regions and stems (Fig. **7a**). The influence of other factors such as carotenoid storage, transport of precursors, or posttranslational regulation could be additionally studies. However, we observed that exogenous application of ABA through the haustoria had a significant effect on carotenoid accumulation, being higher in stems compared to haustorial regions. This indicates that carotenoid levels in the parasite are sensitive to the carotenoid-derived phytohormone ABA, when applied through the artificial host.

We found that the presence of pigments in *C. campestris* are correlated to its biomass gain and our pigment and transcriptomic analyses provided clues about the carotenoid-related processes that are happening in the haustorial regions and the stems. Up-regulation of genes involved in the biosynthesis of ABA and changes in pigment content after ABA treatment show a role in the parasite for this hormone. A study showed an enrichment in genes related to processes like response to ABA, cold and salt stress in the haustorial region (Bernal-Westwood, 2021). It is possible that the haustorial region is sensitive to ABA coming from the host, which may be sensed in the haustorial region but affects pigment content in the stem. Previous hypotheses have stated

that ABA may increase the flow of water and nutrients to the parasite (Bock & Fer, 1992; Taylor *et al.*, 1996), our results did not show changes in *C. campestris* fresh weight, biomass or length after ABA treatment. Other hypotheses postulate that ABA function as a signal for the activation or modification of defenses (Bostock, 1999; Adie *et al.*, 2007; Bodenhausen & Reymond, 2007). More evidence is needed to know the function of this hormone in *C. campestris* biology.

In this study, we used the AHS to grow and study the parasite *C. campestris* without the influence of a plant host. We investigated the effect of light on *C. campestris* growth and its influence on carotenoid content, as a result we found that light has a significant positive impact on *C. campestris* growth which seems to be correlated to carotenoid production. We also studied the differences between the haustorial region and the stem associated to carotenoid-related processes. We found that genes involved in carotenoid biosynthesis pathway are mostly downregulated, while genes related to the core of the abscisic acid and strigolactones biosynthetic pathways are mostly upregulated in the haustorial regions compared to stem. Although these differences are not captured by the total carotenoid content, they indicate that ABA plays a differential role in the haustorial region and in the shoot. Then, we assessed the effect of the carotenoid-derived phytohormone ABA on *C. campestris* growing on the AHS and found that although differences between pigment content in haustorial regions and stem increased under host-derived ABA, parasite growth was not affected. *Cuscuta* has been shown to have an unusual carotenoid composition (Bungard *et al.*, 1999) and it is possible that not well studied carotenoid-derived molecules play a role in parasite biology, this is why further investigation on the relation between apocarotenoids and other aspects of *Cuscuta* biology is needed.

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Supplementary information

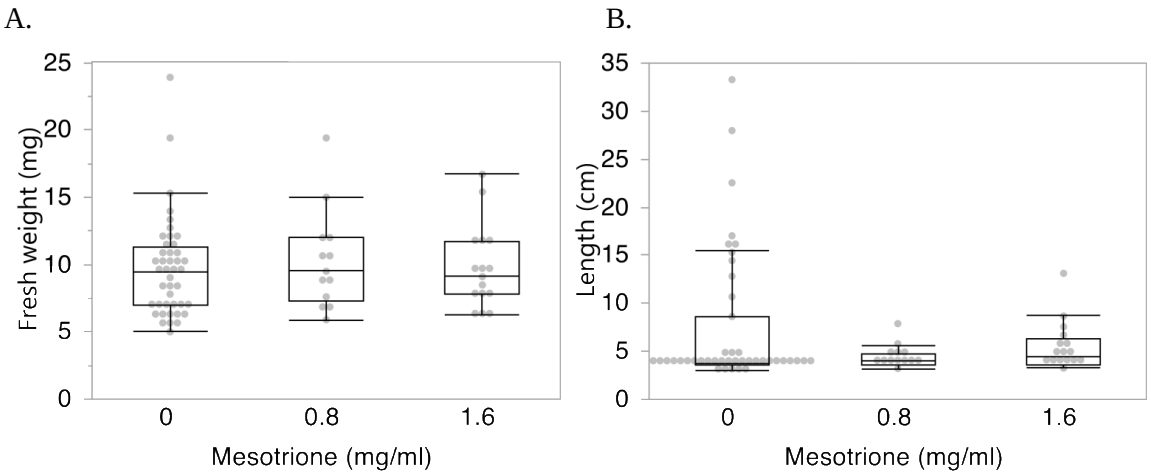

Fig. S1. Effect of the bleaching herbicide mesotrione on fresh weight (A) and length (B) of *C. campestris* growing on AHS. No significant differences were found after evaluation by ANOVA test. Analyses included between 13 and 24 replicates and only plants that coiled and developed a turgid holdfast were considered.

Table S1. Genes involve in carotenoid-related pathways and their expression values (DESeq2 results) in haustorial regions.

Pathway	Main genes involved in selected pathways			DESeq2 results		
	Gene symbol	At ID	Cc ID	log2 FoldChange	pvalue	padj
Carotenoid regulation	HY5	At5g11260	Cc019794	-0.03024	0.81728454	8.6E-01
	PIF1	At2g20180	Cc005290	-3.31794	1.12E-156	2.0E-154
	PIF1	At2g20180	Cc011657	-0.99660	9.59E-12	4.7E-11
	PIF1	At2g20180	Cc015044	-4.04505	2E-233	9.7E-231
	PIF1	At2g20180	Cc025310	-0.69771	1.32E-19	1.1E-18
Plastiquinone	APG1/VTE3	At3g63410	Cc034949	-1.01526	8.15E-32	1.1E-30
	APG1/VTE3	At3g63410	Cc034951	-1.15846	1.92E-40	3.7E-39
	HPPD, PDS1	At1g06570	Cc009401	1.15808	4.43E-38	7.9E-37
	HPPD, PDS1	At1g06570	Cc020699	1.19046	3.55E-50	9.2E-49
	HTS, PDS2	At3g11945	Cc030218	0.18303	0.0393503	6.3E-02

	HTS, PDS2	At3g11945	Cc017867	-0.21823	0.02289402	3.8E-02
MEP	DXS	AT4G15560	Cc002186	0.14386	0.024914879	4.1E-02
	DXS	AT4G15560	Cc019629	0.16168	0.079029308	1.2E-01
	DXR	AT5G62790	Cc011560	0.17946	0.011777096	2.1E-02
Carotenoid core	PSY	At5g17230	Cc006139	-1.29674	2E-44	4.4E-43
	PSY	At5g17230	Cc034337	0.18031	0.09958157	1.4E-01
	PDS3	At4g14210	Cc003945	-0.41089	0.00000031	1.0E-06
	PDS3	At4g14210	Cc027773	-0.08825	0.27434961	3.5E-01
	ZDS	At3g04870	Cc020545	-0.44701	3.29E-12	1.7E-11
	ZDS	At3g04870	Cc028124	-0.35768	0.000000129	4.4E-07
	ZIC1	At1g10830	Cc023305	0.46496	0.00000148	4.6E-06
	ZIC1	At1g10830	Cc024415	0.51241	1.84E-10	8.1E-10
	CCR2/CRTISO	At1g06820	Cc017854	-0.36664	0.00011149	2.7E-04
	CCR2/CRTISO	At1g06820	Cc017855	-1.22350	1.29E-23	1.3E-22
	CCR2/CRTISO	At1g06820	Cc030231	-0.65944	3.9E-09	1.5E-08
	CCR2/CRTISO	At1g06820	Cc030232	-0.06100	0.43758315	5.2E-01
	LCY1	At3g10230	Cc008300	-0.29719	0.00191817	3.9E-03
	LCY1	At3g10230	Cc009784	0.09416	0.29441898	3.7E-01
	LCY1	At3g10230	Cc010704	0.06402	0.86852692	9.0E-01
	LCY1	At3g10230	Cc026751	0.09079	0.79761355	8.4E-01
	LUT2	At5g57030	Cc021320	-0.45612	0.00000258	7.7E-06
	LUT2	At5g57030	Cc045745	-0.32625	0.06402424	9.7E-02
	BCH1/CHY1	At4g25700	Cc000062	-0.30183	0.00031255	7.2E-04
	BCH1/CHY1	At4g25700	Cc029107	-0.05260	0.6170302	6.8E-01
	LUT5	At1g31800	Cc003796	1.06782	4.66E-38	8.3E-37
	LUT5	At1g31800	Cc015249	0.82283	5.18E-35	8.2E-34
	LUT1	At3g53130	Cc030838	-0.95537	5.64E-31	7.6E-30
	ABA1	At5g67030	Cc000160	-0.24605	0.01205493	2.1E-02
	ABA1	At5g67030	Cc022657	0.00609	0.95163733	9.6E-01
	NPQ1	At1g08550	Cc003806	-0.54831	0.00000213	6.5E-06

	NPQ1	At1g08550	Cc023205	-0.25209	0.01475469	2.6E-02
	ABA4	At1g67080	Cc034550	-0.34943	0.00000213	6.5E-06
	ABA4	At1g67080	Cc047472	-0.34428	0.00069603	1.5E-03
ABA biosynthesis	NCED3	At3g14440	Cc001928	0.06168	0.8443426	8.8E-01
	NCED3	At3g14440	Cc022856	0.30244	0.37676186	4.6E-01
	NCED5	At1g30100	Cc006229	NA	NA	NA
	NCED5	At1g30100	Cc020914	2.18574	3.69E-13	2.0E-12
	NCED9	At1g78390	Cc002701	NA	NA	NA
	NCED9	At1g78390	Cc004096	2.47598	3.28E-11	1.5E-10
	AAO3	At2g27150	Cc017094	0.45089	0.00000131	4.1E-06
	AAO3	At2g27150	Cc028373	-0.92111	4.53E-14	2.6E-13
	ABA2	At1g52340	Cc002090	-0.06925	0.66109118	7.2E-01
	ABA2	At1g52340	Cc013931	-0.16332	0.21120699	2.8E-01
	ABA2	At1g52340	Cc002088	3.66476	4.62E-175	1.0E-172
	ABA2	At1g52340	Cc004150	-1.02313	3.08E-14	1.8E-13
	ABA2	At1g52340	Cc016814	0.42766	0.00039036	8.9E-04
	ABA2	At1g52340	Cc029171	0.97755	1.2E-14	7.1E-14
	ABA2	At1g52340	Cc040130	NA	NA	NA
	ABA2	At1g52340	Cc040135	NA	NA	NA
	ABA2	At1g52340	Cc029173	-0.13707	0.87829918	9.1E-01
	ABA3	At1g16540	Cc000521	0.02997	0.72777694	7.8E-01
	ABA3	At1g16540	Cc000522	-0.45502	0.08387872	1.2E-01
	ABA3	At1g16540	Cc018959	-0.11980	0.55607098	6.3E-01
	ABA3	At1g16540	Cc018960	0.16899	0.02848053	4.7E-02
SL biosynthesis	CCD7	At2g44990	Cc004240	NA	NA	NA
	CCD7	At2g44990	Cc011039	-4.14233	0.00548373	1.0E-02
	CCD8	At4g32810	Cc004341	4.47145	4.82E-43	1.0E-41
	CCD8	At4g32810	Cc029299	3.92907	9.89E-25	1.0E-23
	DWARF27	AT1G03055	Cc036033	1.02023	2.28E-18	1.7E-17

Chapter 4: *Cuscuta campestris* is able to recognize the bacterial epitope flg22 and trigger an immune response involving genes related to biotic and abiotic stresses

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Abstract

Cuscuta campestris is a parasitic plant that makes close connections with its hosts. Connections are formed by a parasite organ called a haustorium which is capable of exchanging different substances, ranging from water and nutrients to microorganisms. Recent genome sequencing suggests that *Cuscuta* spp. lack genes involved in defense against microorganisms. We want to understand how the *C. campestris* immune response works, so we challenged parasite tissue with the bacterial epitope flg22. We analyzed diverse aspects of the plant response by evaluating visual changes in stem tissues, ROS production, transcriptomic analysis and *in planta* bacterial growth. Our findings indicate that *C. campestris* is able to sense flg22, but ROS production-related responses differ from those observed in *A. thaliana* and *N. benthamiana*. Transcriptomic analysis revealed changes at a transcriptomic level start 0.5 hours after treatment. At 2 hours after treatment, upregulated genes are predominantly related to biotic and abiotic stresses, while downregulated genes are related to cuticle development. We found that flg22 treatment affects subsequent bacterial colonization in *C. campestris.* Our study contributes to understanding the *C. campestris* immune response in the absence of a host plant.

Introduction

Cuscuta campestris, commonly known as field dodder, is a thread-like parasitic plant whose life cycle depends on obtaining resources from its host. Host colonization starts when shoots from parasite seedings or branches coil around the stems of a potential host. After achieving close contact, *C. campestris* develops a specialized parasitic organ called haustorium. The haustorium grows invasively through the host stem tissues by using cell-wall modifiers, such as xyloglucan

endotransglucosylase/hydrolase (Olsen & Krause, 2017), until its cells reach the host vasculature components, establishing xylem-xylem and phloem-phloem connections between the parasite and host. This intimate connection allows the bidirectional transit of substances like water, nutrients, proteins, and genetic material (e.g., mRNA and microRNA), but also microorganisms (Birschwilks *et al.*, 2006; Kim *et al.*, 2014; Shahid *et al.*, 2018).

C. campestris exhibits promiscuous parasitism that may expose it to a wide range of hosts and their associated microorganisms. *C. campestris* colonizes primarily dicots and is able to parasitize species representing many families, including plants from the *Solanaceae* family like tomato and tobacco, and Brassicaceae members such as the model plant *Arabidopsis thaliana*. *Cuscuta* has also shown the ability to parasitize more than one plant at the same time, and even plants from different species at once, such that *Cuscuta* may link together diverse groups of plants and enable physiological exchange of materials (Liu *et al.*, 2020).

Connections between the parasite and the host also permit the exchange of microorganisms, in some cases pathogens of the invaded host. Among these are viruses, mycoplasma, phytoplasma, fungal endophytes and bacteria (Costa, 1944; Siller *et al.*, 1987; Kamihska & Korbin, 1999; Suryanarayanan *et al.*, 2000; Jelkmann & Kuehn, 2010; Morgan *et al.*, 2012). Some *Cuscuta* species can maintain certain microorganisms and allow them to multiply. For example, *C. campestris* is able to acquire the *grapevine leafroll-associated virus-7* (GLRaV-7), the cucumber mosaic virus (CMV) and the tobacco mosaic virus (TMV) from infected hosts (Costa, 1944; Jelkmann & Kuehn, 2010). Although *C. campestris* facilitates viral replication of GLRaV-7 and CMV, but not of TMV, it allows the transmission of the three types of viruses to a new host. Interestingly, *C. campestris* did not present any symptoms when infected with GLRa7 or TMV, and only showed a subtle growth change under CMV infection.

Cuscuta also transmits and maintains bacterial plant pathogens. *Cuscuta indecora* transmits the unculturable pathogens '*Candidatus Liberibacter asiaticus*' and '*Candidatus Liberibacter americanus*', causal agents of Huanglongbing disease (citrus greening) (Hartung *et al.*, 2010). '*Ca Liberibacter'* spp. multiplies inside *C. indecora* and is distributed unevenly across the infected plants. Although the bacteria travel up to 18 cm away from the source of infection, *C. indecora*

shoots' tips showed a low frequency of colonization. The asymptomatic vectoring of the bacteria suggests that *Cuscuta* spp. have unusual relationships with some plant pathogens. Few pathogens of *Cuscuta* have been identified and studied. Two fungi, *Alternaria destruens* and *Colletotrichum gloeosporioides* f. sp. *cuscuta*, have been reported as *Cuscuta* spp. pathogens and are used as biocontrols for the spread of *Cuscuta* (Sandler, 2010). Only one bacterial species, *Dickeya chrysanthemi*, has been reported as producing recognizable disease symptoms in *Cuscuta* spp. (Schaad & Brenner, 1977).

Plants possess an array of constitutive and defense components that protect them from pathogen colonization. Among these components are physical barriers like the plant cuticle and cell-wall, chemical barriers like accumulation of secondary metabolites, and the recognition of non-self or damaged-self signals that leads to a response like changes in gene expression (Lee *et al.*, 2017). Microbe colonization often occurs through wounds or natural apertures, such as stomata, that facilitate the penetration, for example this enables bacterial pathogens to traverse barriers like the plant cuticle (Melotto *et al.*, 2008). Once pathogens are inside the plant, they can move through the mesophyll or the vascular bundles and colonize other tissues. Plants detect microbial molecules by recognizing Pathogen Associated Molecular Patterns (PAMPs) or Microbe Associated Molecular Patterns (MAMPs). This recognition initiates a well-conserved immune response known as PAMP/MAMP triggered immunity (PTI/MTI) comprised of general responses to inhibit pathogen proliferation (Jones & Dangl, 2006). An example of PTI is the response upon the recognition of flg22, a region of the bacterial flagellin, by the transmembrane pattern recognition receptor (PRR) FLS2 (Zipfel *et al.*, 2004). Recognition of flg22 contributes to a basal resistance even in the cases when the interaction occurs between certain plant species and a nonadopted pathogen (Zipfel *et al.*, 2004; Zhang *et al.*, 2010).

Upon recognition, the flg22-FLS2 interaction triggers a sequence of responses. These include PRR complex formation and protein phosphorylation, Ca+2 influx, extracellular alkalinization, reactive oxygen species (ROS) burst, intracellular mitogen-activated protein kinase (MAPK) activation, ethylene production, expression of defense genes, stomatal closure, callose deposition, salicylic acid (SA)-, ethylene (ET)- and jasmonic acid (JA)- related hormonal responses and growth inhibition (Chinchilla *et al.*, 2007; Gust *et al.*, 2007; Tsuda *et al.*, 2009; Ranf *et al.*, 2015; Guzel

Deger *et al.*, 2015; Yu *et al.*, 2017). The response related to the SA pathway is linked to resistance to biotrophic pathogens, like the bacterial plant pathogen *Pseudomonas syringe* pv *tomato* (Pto). During the immune response, after PAMP detection, SA contributes to PTI. SA accumulation is promoted by genes like Enhanced Disease Susceptibility1 and its co-regulator Phytoalexin Deficient4 (EDS1/PAD4), mutation of these genes causes increased *Pto* growth in *A. thaliana* (Cui *et al.*, 2017).

The conserved mechanisms of plant defense against pathogens are well understood, but little research has been done to understand the responses of *Cuscuta* to microbes. Genomic analyses have shown how some genes involved in the plant defense are lacking in *Cuscuta* spp. genomes. *C. australis* displays a reduction of genes encoding Receptor-like kinases (RLK) and resistance (R) proteins (339 and 15 genes, respectively) in comparison with *A. thaliana* (670 and 170 genes, respectively) or *Ipomoea nil* (992 and 148 genes, respectively), a close relative of *Cuscuta* in the Convolvulaceae family (Sun *et al.*, 2018). In *C. campestris*, EDS1 is also missing along other genes related with defense and other processes (Vogel *et al.*, 2018). Some of the missing genes, like PAD4 or EDS1, are involved in a positive regulation of salicylic acid (SA)-dependent signaling during defense response (Rustérucci *et al.*, 2001; Mateo *et al.*, 2004). *Arabidopsis* mutants for PAD4 exhibit lower levels of SA as well as defects in defense response (Zhou *et al.*, 1998).

Given the wide host range of *Cuscuta* that exposes the parasite to diverse pathogens its capacity to act as a vector of these pathogens, and the evidence of reduced numbers of genes associated with plant defense, we sought to obtain information on how *Cuscuta* responds to microbial colonization. In this study, we explored the response of *C. campestris* to the PAMP flg22, to get a deeper understanding on how the innate immune response in this parasitic plant works. Our results point to the ability of *C. campestris* to recognize and respond to the PAMP/MAPM flg22. However, we observed that some aspects of the response, like ROS production, contrast to responses in other plants. Additionally, our transcriptomic analysis showed enrichment in abiotic responses despite the treatment with a biotic-stress-related elicitor. Our goal is to contribute to understand another aspect of the *C. campestris* biology, its immune response.

Materials and methods

Plant material

Nurseries of *C. campestris* were established on beets (*Beta vulgaris*, Detroit Dark Red) as describe by Bernal-Galeano and Westwood (2021). *C. campestris* seeds were scarified and germinated, then seedlings were attached to petioles of one-month-old beet plants. After one month, parasite shoot tips were collected and attached to one-month-old *Nicotiana benthamiana* plants. Host and parasite were placed under direct full spectrum light (Mercury-Vapor lamp) and far-red enriched light (Sylvania Spot-Gro bulb, enriched in >750nm wavelength, 35 µmol $m^{-2}s^{-1}$) under 14h light /10h dark photoperiod. Plants were watered as needed and fertilized with All Purpose Plant Food (Miracle-Gro, USA). One month later, new *C. campestris* produced shoots were detached from the host and collected for immediate experimentation. Only parasite stem sections with 1.2 to 1.5 mm diameter and localized at least 10 cm away from the point of attachment to the host were used. These sections did not include shoot tips, meristems or nodes. Plant material from leaves, petioles, or inflorescence stems from one-month-old non-inoculated *N. benthamiana* and 1.5-month-old *A. thaliana* grown under the same conditions were used for controls.

Sample preparation for Reactive Oxygen Species assay

Harvested *C. campestris* stems were immediately used after collection. Sections were cut in multiple 3 mm-long units, which were distributed in a 96-wells LUMITRAC 200 clear flat bottom white plate (Greiner Bio-One, Monroe, NC, USA), with two stem sections per well, and kept in sterile water (HyClone, Fisher Scientific) for 20 hours in darkness. A Reactive Oxygen Species (ROS) assay was adapted for *C. campestris* based on Clarke and Vinatzer (2017) and Smith and Heese (Smith & Heese, 2014). The water containing the stem sections was replaced by 100 ul of ROS elicitation media composed of water (HyClone, Fisher Scientific), 0.02 mg ml^{-1} horse radish peroxidase (Sigma) and 0.034 mg ml^{-1} luminol (Sigma). For samples treated with flg22, 1µM flg22 (EZBiolab, Seq: QRLSTGSRINSAKDDAAGLQIA) was added to the ROS elicitation media before use. In the experiments where *Pseudomonas syringe pv tomato* (Pto) DC3000 was used, an overnight liquid culture of the bacteria was centrifuged and resuspended in water, then added to the ROS elicitation media instead of flg22 to obtain 0.5 OD_{600}. The sealed plate was immediately

place in Biotek HD plate reader (Winooski, VT, USA) for data collection, and pictures of the samples were taken 1.5 and 17 hours after treatment.

In planta bacterial growth assay

Pto DC3000 was recovered from a cryopreserved stock. Bacteria were grown on LB plates with 50 mg L^{-1} rifampicin for two days at 28ºC. An isolated colony was used to start a liquid culture which was grown overnight with continuous agitation at 28ºC. To prepare bacterial inoculum, the culture was centrifuged, media was discarded and bacteria was resuspended in sterile water to a concentration of 0.1 OD.

For each replicate, two 3-cm-long by 1-1.2 mm diameter stem sections of *C. campestris* growing on beets were collected and placed in a 2ml centrifuge tube. Pretreatment consisted of 150 µl sterile water or 1 µM flg22 applied to the bottom of the tube in contact with the lower terminal end of the *C. campestris* stems. Soft vacuum was applied for 10 min and then plants were incubated for 22 h at room temperature. Stems were then transferred to a new tube and 100 µL of bacterial inoculum (0.1OD) was applied to the bottom of the tube tubes for 10 minutes at -0.07 MPa, and plants were incubated for 24 h at room temperature. Plant stems were blotted to remove extra water and weighed. Then stems were surface sterilized with a solution of 3% bleach and 0.04% Tween 20 for 5 min, and rinsed 3 times with sterile water to eliminate any bleach residue. Plant stems were ground and serial dilutions were prepared from the plant extract. Serial dilutions were plated on LB plus rifampicin media and incubated for two days at 28C. Resulting colonies were counted and calculations were made to obtain the number of colony former units per mg of tissue. The same procedure was used for *A. thaliana* except that 3 cm sections of inflorescence stems were used rather than *Cuscuta* stems.

Sample preparation and RNA extraction for transcriptomic analysis

Tissues used were as described above for the ROS assay. Harvested *C. campestris* stem sections were cut in multiple 3 mm-long units of around 1.2 mm diameter. The sections were placed in sterile water (HyClone, Fisher Scientific) for 20 h in darkness. Ten sections were transferred to

each 1.5 ml centrifuge tube. Samples corresponding to time zero were collected immediately into liquid nitrogen, and the treatment with flg22 (1µM, EZbiolab) was applied to the other samples. Samples were collected 0.5, 1 and 2 h s after treatment and frozen immediately on liquid nitrogen.

For RNA extraction, frozen tissue was ground to a powder using glass beads in a bead beater and extracted using the RNeasy Plant Mini Kit (Qiagen). RNA was precipitated using 3M NaOAc and Ethanol 100%, and resuspended in RNAse-free water. RNA concentration and purity were evaluated using a Nanodrop OneC (ThermoScientific), and quality was confirmed using an RNA TapeStation System (Agilent). A mRNA library was prepared using Poly-A enrichment, and sequencing was performed by Genewiz using Illumina HiSeq with PE150.

Bioinformatic analysis

RNA-seq raw data quality was initially evaluated using FASTQC v.0.11.9 (Braham Bioinformatics). Then FASTP v.1.0.0 (Chen *et al.*, 2018) was used to detect and trim adaptors, and filter out sequences shorter than 50 nt, with low quality or low complexity. Clean reads were mapped with STAR 2.5.2b (Dobin *et al.*, 2013) against the genome of *C. campestris* (Vogel *et al.*, 2018). A Sperman correlation test was conducted using R. Gene counts obtained using STAR 2.5.2b were processed by DESeq2 1.30.1 (Love *et al.*, 2014), with differential expression analysis using the default settings and a threshold of genes with more than 10 counts. Protein sequences corresponding to DEGs genes were retrieved from *C. campestris* genome (Vogel, 2018). Then a BLASTP search against *A. thaliana* protein database (TAIR 10) was done (-evalue 1e-30, -max_target_seqs 1, -max_hsps 1) in order to identify candidate homologous genes, their anotations and associated Gene Ontology (GO) terms. We then used the *A. thaliana* gene ID to perform a GO term analysis using Panther 16.0.

FLS2 multiple sequence alignments were done using MUSCLE (EMBL-EBI).

Results

Cuscuta campestris *is sensitive to flg22 but exhibits differences in ROS production compared to* A. thaliana *and* N. benthamiana

In order to evaluate whether *C. campestris* is able to detect a purified synthetic flg22, based on the epitope found in *Pseudomonas syringe* pv *tomato* (Pto) DC3000, we confirmed the presence of a FLS2 homolog in *C. campestris* and developed an assay to test PTI elicitation in plant stems. Our methodology is based on a previously developed methodology to test ROS production in plant leaf tissues (Smith & Heese, 2014; Clarke & Vinatzer, 2017) and considers visual changes in plant tissue provoked by the treatments. Our methodology is designed to suit experiments with *Cuscuta* because it uses just plant stems, which is essential given the lack of leaves and roots in this species, and allows evaluation of the parasite response without the host interference. After exposing *C. campestris* stem sections to flg22 (1 µM) and to a *Pto* culture, the terminal ends of the stems exhibited a change in color that increased in intensity over time (Fig. 1). The change of color was evident at the cut ends of the sections and following similar patterns of distribution in all three tested plant species tissues, *C. campestris*, *A. thaliana* and *N. benthamiana.* Treatments with flg22 exhibited a faster and stronger response than the ones with *Pto*, as expected due to the higher availability of the purified peptide compared to that naturally found in the bacterium. Treatment of *C. campestris* with only flg22 without the ROS elicitation media did not present change in color 2 h after elicitation (data not shown), suggesting that the change of color is related to ROS production.

We evaluated the activation of the ROS burst in *C. campestris*, *A. thaliana* and *N. benthamiana* tissues after treatment with flg22 and Pto. Intriguingly, the parasitic plant did not present the same pattern as *A. thaliana* or *N. benthamiana* (Fig.2 a-c). No production of luminescence associated with ROS production was detected in *C. campestris* in spite of the changes in tissue color similar to the other two plants (Fig.1). *A. thaliana* inflorescence stems and *N. benthamiana* petioles showed a clear luminescence peak associated to a high ROS production around 10 min after elicitation with flg22. *A. thaliana* presented a stronger response than *N. benthamiana* to flg22 as evidenced by the amplitude of the peak. In contrast, *N. benthamiana* exhibited a greater response to *Pto*, with a peak around 17 min after elicitation with *Pto* that was absent in the other two plants.

To explore the molecular interaction between flg22 and the receptor FLS2, we aligned the protein sequences of the ectodomain of CcFLS2, AtFLS2, NbFLS2 and the FLS2 of *Solanum lycopersicum* (Fig. 3). The FLS2 ectodomain is the receptor portion that is outside of the cell membrane and interacts with the PAMP/MAMP. Previous studies have identified 18 amino acid residues that are involved in the recognition of flg22 in *A. thaliana* (Sun *et al.*, 2013). Of the 18 important residues for flg22 perception, four of them contain critical changes in *Cc*FLS2 compared to the other three analyzed FLS2. Amino acids in these positions in CcFLS2 correspond to the residues 220, 272, 414 and 417 in AtFLS2, and changes include variation in charge, polarity and size of the side chain of the amino acid.

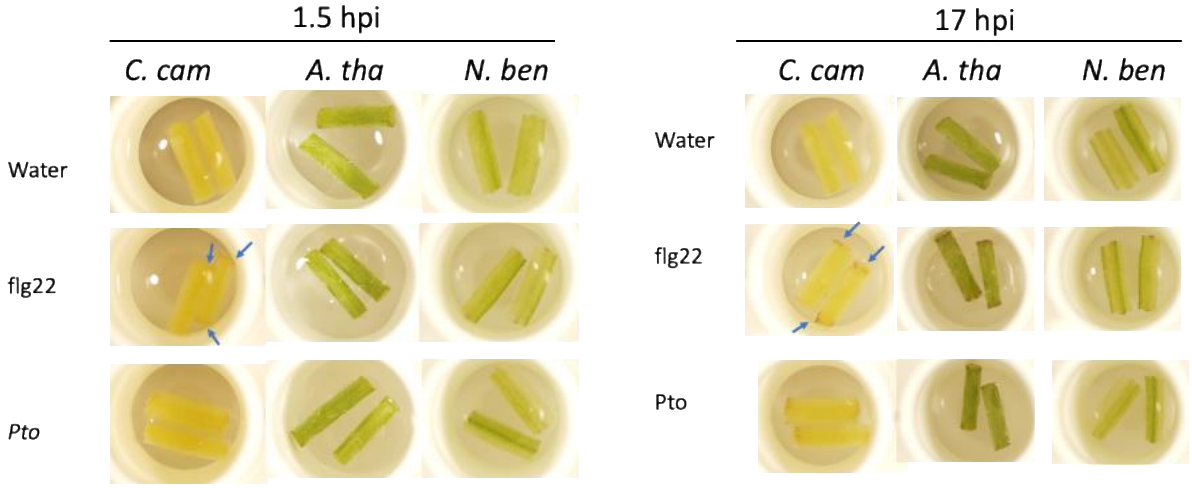

Fig. 1 Response of *C. campestris* (*C. cam*) stems, *A. thaliana* (*A. tha*) inflorescence stems, *and N. benthamiana* (*N. ben*) petiole sections to flg22 and *Pseudomonas syringe* pv. tomato (*Pto*) DC3000 at 1.5 hours post inoculation (hpi) and 17 hpi.

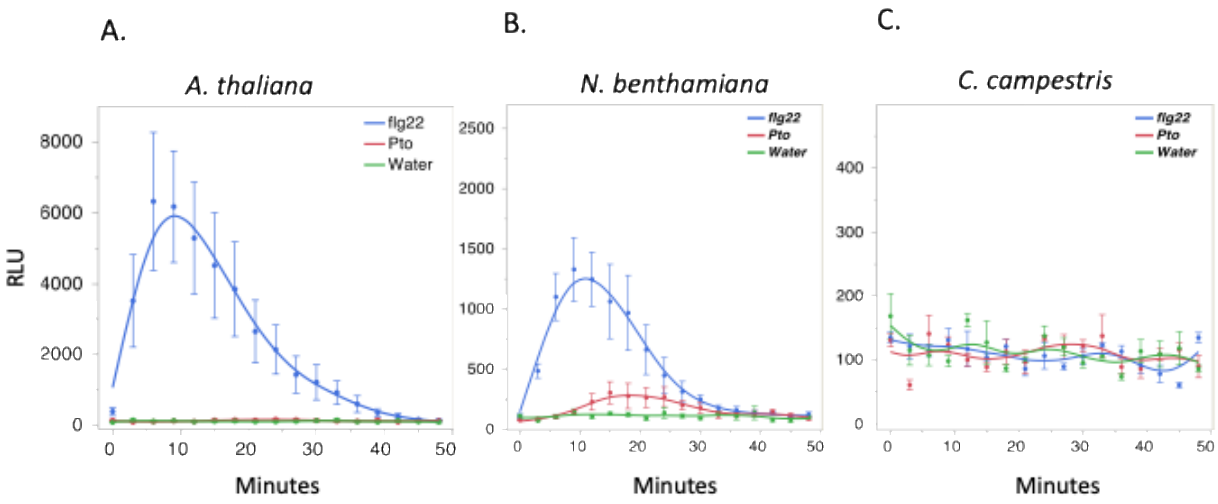

Fig. 2 ROS production in inflorescence stems of *A. thaliana* (A), petioles of *N. benthamiana* (B), and stems of *C. campestris* (C), in response to 1µM flg22, *Pseudomonas syringe* pv. *tomato* (*Pto*) DC3000, and control (water). RLU: relative light units.

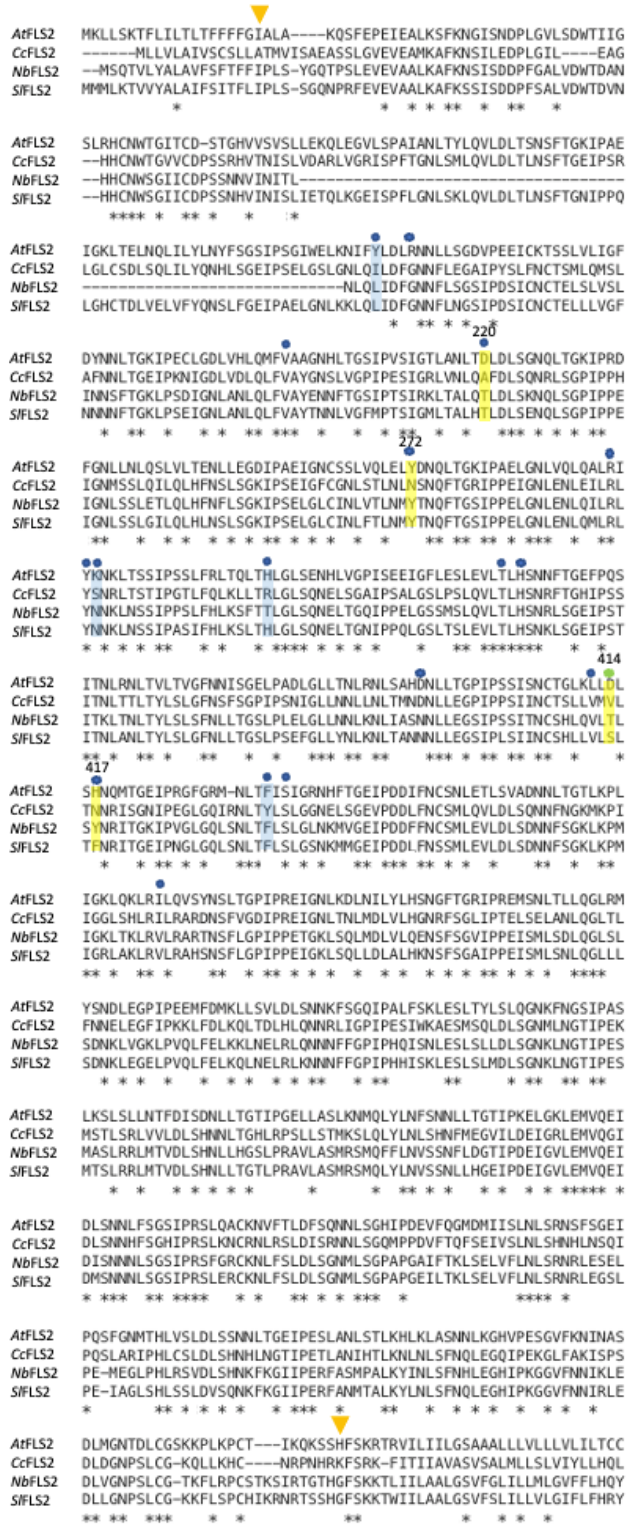

Fig. 3 Multiple protein sequence alignment between *At*FLS2, *Cc*FLS2, *Nb*FLS2, and *Sl*FLS2. Exact coincidences in amino acid residues are marked with asterisks. Yellow arrows indicate the ends of the ectodomain of FLS2 based on the *A. thaliana* sequence. Circles indicate 18 amino acid

residues involved in flg22 recognition (Sun *et al.*, 2013). Yellow bands indicate critical amino acid residues changes in *C. campestris* compared to amino acids in the same position in the other three plants. Blue bands indicate low impact amino acid changes in *C. campestris* compared to amino acids in the same position in the other three plants.

Cuscuta campestris *exhibit reduced bacterial titers after pretreatment with flg22*

In order to assess the effect of the response of *C. campestris* to flg22 on its ability to control bacterial invasion, we perform an *in planta* bacterial growth assay with *Pseudomonas syringe* pv. *tomato* (*Pto*) DC3000 after a pre-treatment of sections of *C. campestris* stems with mock or with flg22 (Fig. 4). We found that after a day of infection, *C. campestris* showed significantly lower bacterial titers after treatment with flg22. Although we observed a decrease in bacterial growth in the *A. thaliana* inflorescence stem tissues pre-treated with flg22 compared to the mock-treated control, the change was not statistically significant.

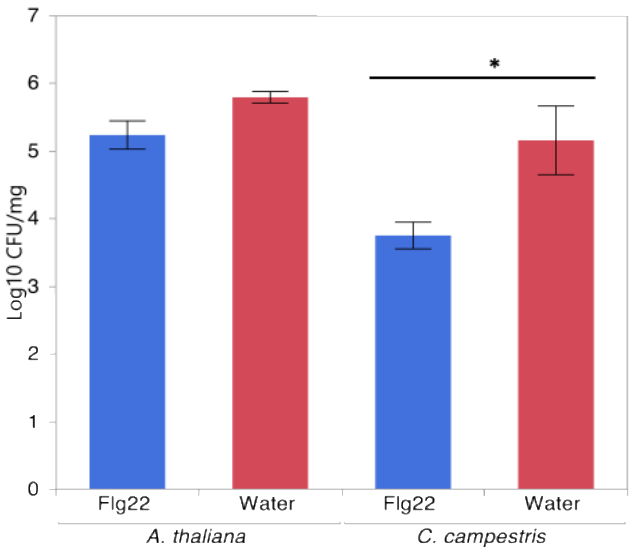

Fig. 4 *in planta* bacterial growth assay of *Pto* in *A. thaliana* and *C. campestris* pre-treated with flg22. Pretreatment with 1µM flg22 was done for 22 h before bacterial inoculation. Growth evaluation was done 1 day after inoculation. Significant differences between the two treatments were evaluated through ANOVA test and Student T test. Differences that are significant at $P<0.05$ are indicated by an asterisk. 3 to 5 samples were used per treatment.

Transcriptomic analysis of* C. campestris *treated with flg22

With the goal of understanding which processes are involved in the activation of the immune response in *C. campestris* after the elicitation with the PAMP/MAMP flg22, we performed a time course transcriptomic analysis. We treated *C. campestris* stem sections with flg22 employing the methodology described above for evaluating the PTI elicitation in plant stems. We collected tissue at 0, 0.5, 1 and 2 hours after elicitation, extracted RNA and perform RNA-seq. Although our analysis started with four replicates per time, we dropped one of the replicates of time 0h because of its low correlation with the other three replicates after applying an initial analysis using Spearman correlation test (Fig. **S1**). Differential gene expression (DGE) analysis was done on the remaining replicates. We compared the times 0.5h, 1h and 2h independently against time 0h. Considering a padj < 0.05, time 0.5h displayed a total of 14 Differentially Expressed Genes (DEGs) (9 up-regulated, and 5 downregulated), time 1h displayed 280 DEGs (231 up-regulated, and 49 downregulated), whereas time 2h displayed a total of 601 DEGs (405 up-regulated, and 196 downregulated) (Fig. **5a-c**).

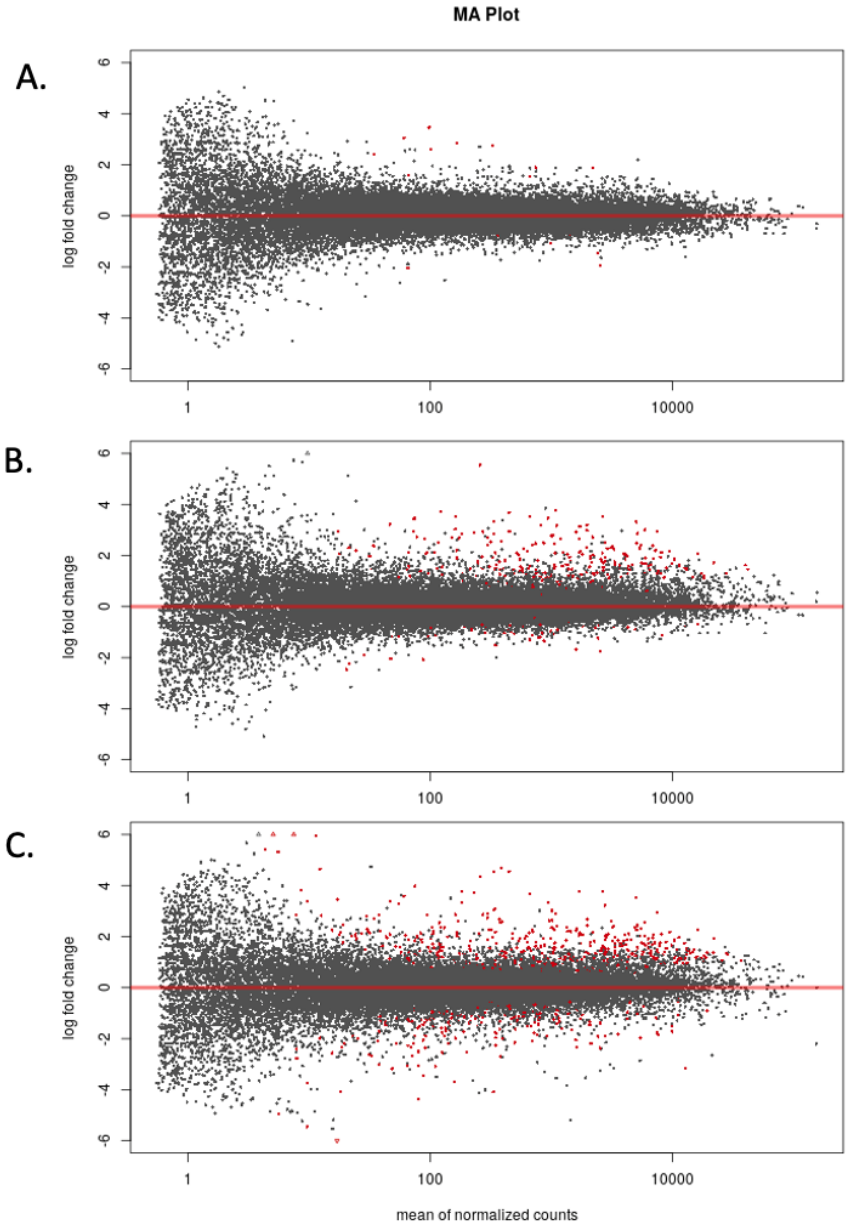

Fig. 5 Differential expressed genes in *C. campestris* at three different times after treatment with the MAMP/PAMP flg22. A) 0.5h compared to 0h. B) 1h compared to 0h. C) 2h compared to 0h. MA plots show in red those genes that are differentially express between the mentioned times time 0 at a padj<0.05. Genes above the red line are up-regulated, and genes below are downregulated.

We conducted a BLAST search for homolog candidates of the DEGs found in *C. campestris* in *A. thaliana* to know more about their associated functions. Out of the 895 DEGs found in *C. campestris* considering all the evaluated times, 731 were unique, and 624 presented at least one

homolog candidate in *A. thaliana*. Using unique *A. thaliana* corresponding gene IDs, we performed a Gene Ontology (GO) enrichment analysis on the DEGs found at the time points 1h (Table **1**) and 2h (Table **2**).

Table 1. GO term enrichment analysis based on homolog candidates in *A. thaliana* of DEGs in *C. campestris* at 1h after treatment with flg22.

GO terms enriched in up-regulated genes 1h after flg22 treatment		
GO biological process	**Fold Enrichment**	**P value**
response to chitin	17.49	8.55E-05
response to organonitrogen compound	10.86	5.69E-04
response to nitrogen compound	8.79	3.16E-03
response to organic substance	4.2	4.21E-06
response to oxygen-containing compound	4.13	7.58E-05
response to endogenous stimulus	3.99	3.16E-03
cellular response to chemical stimulus	3.87	4.42E-02
response to hormone	3.85	1.08E-02
response to chemical	3.23	2.14E-05
protein modification process	3.06	9.03E-04
cellular protein modification process	3.06	9.03E-04
macromolecule modification	2.7	8.55E-03
response to stimulus	2.64	6.04E-09
response to stress	2.6	6.34E-03
cellular protein metabolic process	2.44	2.08E-02
organonitrogen compound metabolic process	2.25	8.29E-03
cellular metabolic process	1.79	4.66E-02
metabolic process	1.76	1.59E-02
cellular process	1.69	2.14E-05
GO molecular function	**Fold Enrichment**	**P value**
catalytic activity, acting on a protein	2.6	4.63E-02
transferase activity	2.44	8.08E-03
catalytic activity	1.94	8.66E-05

*GO cellular components analysis for up-regulated genes, and all GO categories for downregulated DEGs at this time point exhibited unclassified terms.

Table 2. GO term enrichment analysis based on homolog candidates in *A. thaliana* of DEGs in *C. campestris* at 2h after treatment with flg22

GO terms enriched in up-regulated genes 2h after flg22 treatment		
GO biological process	**Fold Enrichment**	**P value**
protein folding	9.43	1.38E-04
response to heat	9.34	2.20E-06
response to fungus	5.35	1.11E-02
response to temperature stimulus	4.71	1.09E-04
cellular response to endogenous stimulus	4.45	6.18E-03
cellular response to hormone stimulus	4.43	1.43E-02
cellular response to organic substance	4.41	2.90E-04
cellular response to chemical stimulus	4	1.71E-06
response to biotic stimulus	3.81	1.02E-05
biol. proc. in interspecies interaction between organisms	3.77	1.23E-05
response to external biotic stimulus	3.67	4.21E-05
response to other organism	3.67	4.21E-05
response to lipid	3.62	5.37E-03
response to inorganic substance	3.52	2.26E-03
response to organic substance	3.44	6.93E-08
defense response	3.41	3.71E-03
response to endogenous stimulus	3.4	5.59E-05
response to external stimulus	3.37	2.99E-06
response to hormone	3.36	1.31E-04
response to oxygen-containing compound	3.35	3.40E-06
response to abiotic stimulus	3.34	8.70E-10
response to chemical	3.05	2.70E-10
signal transduction	3.02	1.75E-03
signaling	2.94	2.96E-03
cellular response to stress	2.9	4.20E-02
response to stress	2.89	1.21E-10
cell communication	2.76	2.17E-03
cellular response to stimulus	2.73	2.05E-06
response to stimulus	2.51	5.55E-16
phosphorus metabolic process	2.5	1.48E-02
phosphate-containing compound metabolic process	2.48	2.62E-02
protein modification process	2.46	5.11E-04
cellular protein modification process	2.46	5.11E-04

macromolecule modification	2.29	1.42E-03
cellular protein metabolic process	2.01	2.18E-02
organonitrogen compound metabolic process	1.81	2.82E-02
cellular metabolic process	1.74	2.43E-05
organic substance metabolic process	1.72	3.36E-05
cellular process	1.71	3.67E-13
metabolic process	1.71	8.41E-06
primary metabolic process	1.64	7.07E-03
GO molecular function	**Fold Enrichment**	**P value**
unfolded protein binding	12.43	1.65E-04
protein threonine phosphatase activity	8.88	3.69E-02
protein serine phosphatase activity	8.88	3.69E-02
protein threonine kinase activity	4.75	7.70E-04
protein serine kinase activity	4.75	7.70E-04
protein serine/threonine kinase activity	3.43	2.34E-02
protein kinase activity	3.03	2.37E-02
phosphotransferase activity, alcohol group as acceptor	3.01	5.53E-03
kinase activity	2.95	4.63E-03
catalytic activity, acting on a protein	2.44	2.47E-04
protein binding	1.93	3.61E-05
catalytic activity	1.75	2.05E-06
binding	1.53	8.71E-04
GO cellular component	**Fold Enrichment**	**P value**
plasma membrane	2.03	1.64E-03
cell periphery	2.01	3.70E-04
membrane	1.73	4.85E-03
GO terms enriched in downregulated genes 2h after flg22 treatment		
GO biological process	**Fold Enrichment**	**P value**
cuticle development	49.53	3.09E-04

* GO molecular function and cellular component for downregulated DEGs at this time point exhibited unclassified terms.

Discussion

Our goal in this study was to explore the molecular bases of the immune response of *C. campestris*. *Cuscuta* spp. are obligate parasites that rely on their host to complete their life cycle. Over the

course of evolution, species in this linage have experienced genomic reductions that include losing genes that are well conserved in other plants (Sun *et al.*, 2018; Vogel *et al.*, 2018). Among the lost genes are some that in autotrophic angiosperms play critical roles in defense responses, like PAD4 and EDS1 (Rustérucci *et al.*, 2001; Mateo *et al.*, 2004). These two genes are highly upregulated during biotrophic pathogen infections and are involved in the activation of the SA pathway (Yang *et al.*, 2015; Baggs *et al.*, 2020). How does *C. campestris* immune response work when it is challenged by a biotrophic microorganism? For our study, we selected the well-characterized epitope flg22, a component of the flagellin protein of *Pseudomonas syringe* pv *tomato*, a biotrophic pathogen that attacks tomato, a common host of *C. campestris*.

We discovered that *C. campestris* recognizes and responds to flg22, but our data suggest differences in downstream responses upon the recognition of the PAMP/MAMP with respect to responses in other plants. The recognition of flg22 by *C. campestris* is supported by visual evidence that suggests the activation of molecular responses after PAMP/MAMP treatment occurs in a similar way to that of *A. thaliana* or *N. benthamiana*. Recognition of the bacteria *Pto* was also observed in *C. campestris*. No changes in *C. campestris* stem color were seen following treatment with flg22 but without luminol or HRP, the two main components of the ROS elicitation media used for the detection of reactive oxygen species. This observation may indicate that the change of color in presence of the ROS elicitation media is related to ROS production.

However, when the ROS production was assayed, luminescence was not detected for *C. campestris* stems treated with flg22 one hour after elicitation. In contrast, stem tissues from *A. thaliana* and *N. benthamiana* showed a clear and early response. Responses associated to ROS production observed in *A. thaliana* stems were activated among the first minutes after elicitation with flg22, this agrees with a typical response of *A. thaliana* leaf tissues (Chinchilla *et al.*, 2007). However, we observed a greater amplitude of the ROS burst based on the luminescence production in *A. thaliana* stems compared with its leaves (data not shown), this comparison is not possible in *C. campestris* given its lacking of leaves.

Our analysis of the amino acid sequences of CcFLS2 found that the receptor has some changes in important residues involved in the interaction of flg22 that may alter its performance compared to

that exhibited by AtFLS2 or NbFLS2. Previous studies involving AtFLS2 showed that disruption of recognition of flg22 caused by mutations in specific amino acid residues may compromise the immune response (Sun *et al.*, 2013). For example, when the amino acid residue D414A was mutated in AtFLS2, the interaction between the receptor and flg22 was partially compromised, as well as the recruitment of other associated receptors (Sun *et al.*, 2013). CcFLS2, presents the amino acid valine in the same position were the mutation in *At*FLS2 was found. Valine (V) shares hydrophobic features with alanine (A), and may suggest that there are changes in the flg22 recognition by *Cc*FLS2 compared to *At*FLS2, which may impact responses downstream of detection.

Nevertheless, our data show that *C. campestris* is able to sense flg22 and trigger a response. We observed DEGs in *C. campestris* after treatment with flg22. A large group of DEGs was detected at 1h and 2h after elicitation compared to a very few DEGs detected at 0.5h after treatment. This indicates that transcriptional responses in *C. campestris* provoked by flg22 occur after 0.5 h. Larger numbers of DEGs observed at 1h and 2h coincide with color changes in the *C. campestris* stem after flg22 treatment.

Enriched GO terms corresponding to upregulated DEGs in *C. campestris* show associations with biological interactions but also with responses to abiotic stresses. Among the enriched biological processes corresponding to upregulated genes at 1h after treatment are response to chitin, which is a PAMP/MAMP found in fungi and responds to oxygen-containing compounds, which may be related to ROS production. These two biological processes agree with early responses upon the PAMP/MAMP elicitation, since recognition is the first steps followed by ROS related processes (Yu *et al.*, 2017). Then, among the enriched biological processes corresponding to upregulated genes at 2h after treatment we found several processes related to biological interactions like response to external biotic stimulus, biological processes involved in interspecies interaction, response to other organisms, defense response and response to fungus. We also found other enriched processes related to abiotic stresses like response to temperature and to abiotic stimulus; as well as related to communication like signal transduction and cell communication. Interestingly, most of the enriched molecular functions in upregulated genes at 2h are related to kinase activity, which commonly have functions that include protein activation and signal transduction (Hohmann

et al., 2017). We also observed that at this point there is an enrichment of cellular components associated to cell membrane and periphery. Intriguingly, the only enriched biological process associated to downregulated genes at 2h after treatment with flg22 was cuticle development. Previous reports have shown that a mutant of *A. thaliana* with compromised cuticle and wax production is more resistant to the biotrophic bacteria *P. syringae* and the necrotrophic fungus *Botritis cinerea.* A further study of the specific DEGs in *C. campe*stris is needed in order to understand differences at gene expression levels compared to the patterns observed in the defense responses of other plants, considering the lack of key defense related genes in *C. campestris* (Sun *et al.*, 2018; Vogel *et al.*, 2018).

Changes in stem color and differential gene expression shows that *C. campestris* is able to sense and react to the PAMP/MAPS flg22. Our observations on the reduced bacterial titers in *C. campestris* stems pre-treated with flg22 suggest that the response exhibited by *C. campestris* is a type of immune response. According to hour observations, the response activated by a PAMP/MMAP may last for at least one day, or activate other responses that may contribute to control of future bacterial invaders for at least hours or days. This response may be SA-independent or related to other ways of SA production, as *C. pentagona* seedlings conserve the ability to produce SA (Runyon *et al.*, 2010).

In this study, we have explored the basal immune response of *C. campestris* triggered by the bacterial epitope flg22. We found that *C. campestris* is able to recognize and respond to the PAMP/MAPM. However, some aspects of the response, like ROS production, differ from responses in other plants. Transcriptomic analysis showed enrichment in abiotic responses despite the treatment with a biotic-stress-related elicitor. Studies have demonstrated an antagonistic relationship between the biotic-stress-related hormone SA and the abiotic-stress-related hormone abscisic acid (ABA) (Lievens *et al.*, 2017). However, other studies have suggested a connection between plant immunity and abiotic stress. Genes related to the SA pathway have been shown to have a role in drought stress response and ABA-antagonism (Szechyńska-Hebda *et al.*, 2016; Baggs *et al.*, 2020). Many aspects of the *C. campestris* biology are still unknown, so more studies analyzing aspects like the relationships between immunity and response to abiotic stress in *C. campestris* are needed.

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Supplementary information

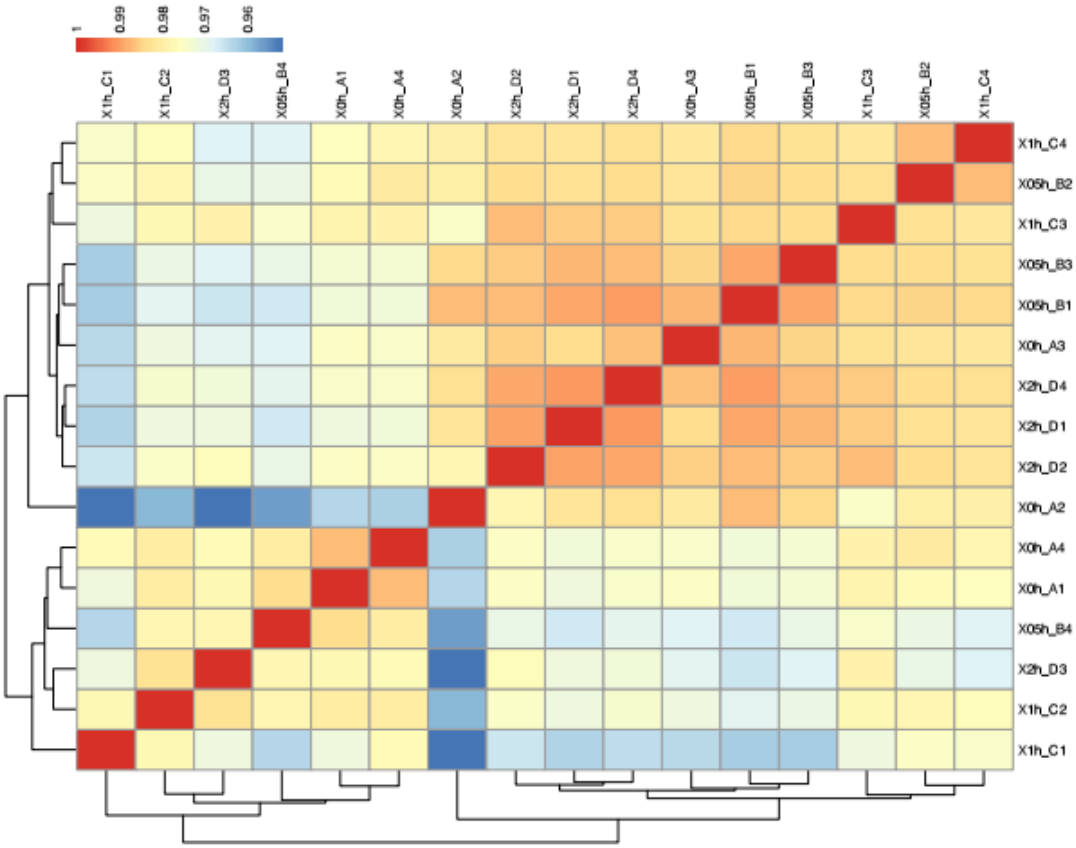

Fig. S1. Spearman correlation test among the data corresponding to RNA-seq of samples treated with flg22 and evaluated at times 0 (A1-4), 0.5 (B1-4), 1 (C1-4), and 2 (D1-4) hours.

Appendix 1: How to grow *C. campestris* and succeed in the first try: a practical guide for growing dodder for laboratory research

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Abstract

Parasitic plants live by taking their nutritional needs from other plants through specialized structures called haustoria. *Cuscuta* species are stems parasites, which are totally dependent on their hosts to complete their life cycle. Interest in *Cuscuta* research is increasing, but no standardized protocols exist for working with this parasite. An example of that is the diversity of experimental approaches that use different starting material like different types of shoots or seedlings. What are the advantages of using one or another? We want to present the alternatives that researchers interested in initiating a *Cuscuta* research program can use to growth and manipulate *C. campestris*. We expect that this guide helps new researchers to start their programs in an easier, quicker and more informed way to meet the objectives of their investigation.

Introduction

Field dodder (*Cuscuta campestris*) is a parasitic plant that lives entirely off the water and nutrients it takes from a host plant. The communicating bridge between *Cuscuta* and its host is the haustorium, a specialized organ that connects parasites with host allowing exchange of molecules including genetic material and proteins (Kim et al 2014, Shahid et al 2018, Liu et al, 2020). Growing dodder for research purposes is not difficult, but it can seem tricky to anyone not familiar with the parasite. Dodder can be sensitive to environmental conditions and choice of host species, so this guide was developed to help new dodder researchers overcome some of the more common problems encountered when trying to grow this parasite.

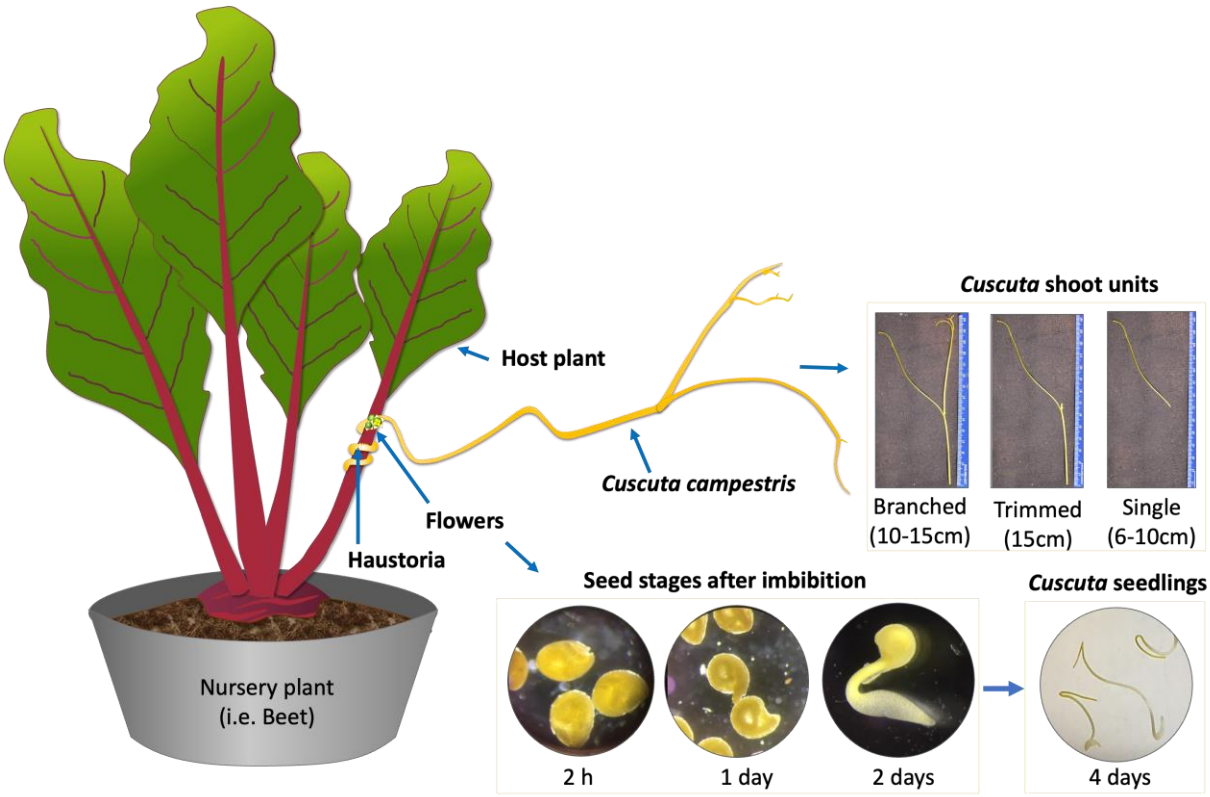

Fig. 1 *Cuscuta* life cycle

Dodder

The dodder plant consists primarily of a vining stem that wraps around a host plant and forms haustoria at points of contact. Generally, the parasite coils around the stem of its host but in rare occasions it also can grow around leaves. As the haustoria connect to the host vascular tissue, it fuels growth of the dodder shoots, which branch at every node and each new branch is able to make connections to the same or neighboring plants. In this way, dodder forms a network of shoots and connections, often resulting in dense mats of dodder.



Fig. **2** *Cuscuta campestris* growing plant hosts, on the petiole of a beet leaf (Left), and on the back of an *A. thaliana* leaf (Center). Infestation of *C. campestris* on beet plants (Right)

Dodder will flower and produce seeds as long as it has sufficient energy from its host. The dodder seeds have a tough seed coat, able to withstand passage through an animal gut. This seed coat must be broken by either physical or chemical treatment, after which the dodder seeding will emerge. Unlike some other well-known parasitic plants (e.g., witchweeds, broomrapes), dodder seeds have no special germination mechanism.

Although most species of dodder have functional photosynthetic systems and may appear greenish at times, their color generally ranges from a creamy yellow to orange or red (depending on the species). We find that stressed dodder turns greener, whereas happy dodder is yellow/orange.

A key concept for parasitic plants is host specificity. All parasites are specialized to grow better on certain host species than others, but the range of acceptable hosts varies greatly among different parasites. Among parasitic plants, the dodders have some of the broadest host ranges, and will parasitize hosts from many different families. In general, dodders grow on broadleaf hosts (grasses alone cannot support them), but there are clearly host species that the dodder prefers over others (more on this topic below), so one of the first steps to growing dodder is finding a good host that you can grow well.

Procedures

1. **Preparing the Host**

Fig. 3 Beets (top) and tomatoes (foreground) to be use as hosts for *C. campestris*

Start growing host plants in advance of planting dodder seeds so that hosts are ready to support the parasite. The precise time of planting will depend on the specific host and the objectives of your experiment, but when you are starting dodder from seeds, it is best to have host plants that are also very young (seedling stage) or with thin stems to be inoculated.

1.1 Prepare pots or flats with potting medium (any soil or medium appropriate for growing the host plants will work).

1.2 Select host species to use. Some hosts are preferred, and this may depend on the species of dodder in question. These recommendations are based on our experience with *C. campestris* (or *C. pentagona*). Some host plants may be parasitized easily by the parasite, while others are non-hosts and are largely resistant to parasite attack (Table **1**). There may also be variation in host varieties, with some being more susceptible to dodder than others. Dodder that has established on a suitable host may have a better invasion success rate on less suitable plants as secondary hosts.

Table 1. Host of *C. campestris*

Great hosts – Highly susceptible species (Best for primary attachments)	
Arabidopsis (inflorescence stem)	
Beet	
Coleus	
Cosmos	
Good hosts – Susceptible species.	
Solanum penelli	Alfalfa (Medicago)
Pepper	Clover
Carrot	Bean
Petunia	*Nicotiana tabacum*
Nicotiana benthamiana	
Host that presents features of resistance	
Tomato *(Solanum lycopersicum)*	

Table 2. Non-host of *C. campestris*

Non-hosts **Dodder may parasitize them if first established on good hosts, but cannot survive solely on these.**
Grasses, such as: Sorghum Millet Wheat Fescue

Although our dodder has been seen growing on tomato fields, it often has a tough time establishing on tomato hosts unless the tomato is a seedling. Tomato is a great secondary host, meaning that dodder already growing on a host plant can readily make new connections to tomato. We recommend using the inflorescence stem of *Arabidopsis* (or similar highly susceptible plant) as a primary host to get the dodder started. Then, the growing dodder shoots can be propagated on a secondary host.

Fig. 4. *C. campestris* shoot tip coiling around tomato stem

2. Starting Dodder from Seeds

Dodder can be started from seeds by two different ways: Sowing the seeds into the soil close to a selected host and waiting for their germination, or attaching previously germinated seedlings to the host stem. Dodder seeds have a tough seed coat that may cause the seed to remain dormant in the soil for years. The best way to stimulate germination is by scarifying the seeds by soaking in acid, that simulates passage through an animal digestive system.

2.1 Scarify dodder seeds for germination

2.1.1 Place seeds in a beaker with concentrated sulfuric acid for 15 – 30 min. Freshly harvested seeds require shorter scarification time.

2.1.2 Carefully decant acid from the seeds (or otherwise remove seeds from the acid).

2.1.3 Rinse dodder seeds in several changes (3-5) of tap water stirring constantly. A black layer will fall off from the seeds surface.

2.1.4 Proceed immediately to the sections 2.2 or 2.3, or let seeds dry out on a filter paper. Dried scarified seed can be stored for months until time for planting.

2.1.5 Germination rates in our experience may vary according to the seed batch, but usually range from 60-80%.

2.2 Sowing dodder directly on soil

2.2.1 Plant the dodder seeds 5-7 days before you want them to attach to hosts. Plant seeds by mixing them into the soil at a depth of < 1 cm around the host plant. Most dodder seedlings will emerge within a week of planting, but may continue to emerge sporadically for two or three more weeks.

2.2.2 After dodder emergence, avoid growing plants under only fluorescent lighting. Lights with a spectrum emphasizing far-red wavelengths will promote coiling, while fluorescent light inhibits coiling. Natural daylight also works well. We use lights shifted toward the far-red spectrum (often alone or in supplement to fluorescent lights) to encourage attachments. Note that many dodder researchers have noticed seasonal effects: Dodder grows better and attaches more readily in spring and summer than in fall or winter.

Fig. 5 A *C. campestris* seed emerging from the soil next to a potential host, in this case a beet stem

2.3 Manually attaching seedlings to hosts

It is possible to germinate seeds in a Petri dish and then place the resulting seedling directly on host stems. This method increases the coiling and attachment rate of dodder on the hosts compared to starting dodder from seeds germinating in the soil and allows the researcher to control the site of attachment. However, since seedlings are fragile, a gentle touch is required to avoid causing damage to them.

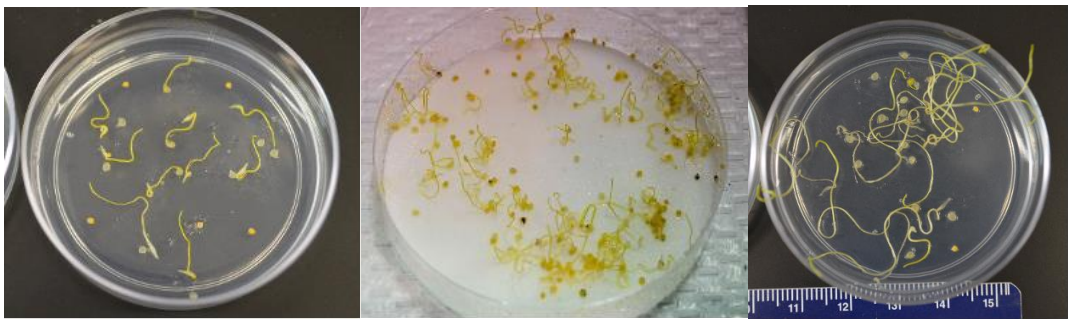

Fig. 6 *C. campestris* seedlings on solid B5 media 3 days after imbibition (left), filter paper with water 3 days after imbibition (center), on solid B5 media 6 days after imbibition

2.3.1 Once dodder seeds have been scarified, place them on a wet filter paper in a Petri dish at a temperature between 20-30°C. They will germinate.

2.3.2 After 3-5 days seedlings will have emerged and can be placed directly on the stems of selected hosts. Carefully orient a seedling in parallel to the host stem. Gently attach it with a narrow piece of tape at one point.

2.3.3 Seedlings are able to coil and make successful attachments after 4-7 days. Seedlings are susceptible to desiccation, so make sure that the plants (host and parasite) are under high relative humidity conditions (around 50%), especially during the first days after inoculation. Additionally, it is important to provide proper light conditions (see 2.2.2).

3. Propagating Vegetative Shoots

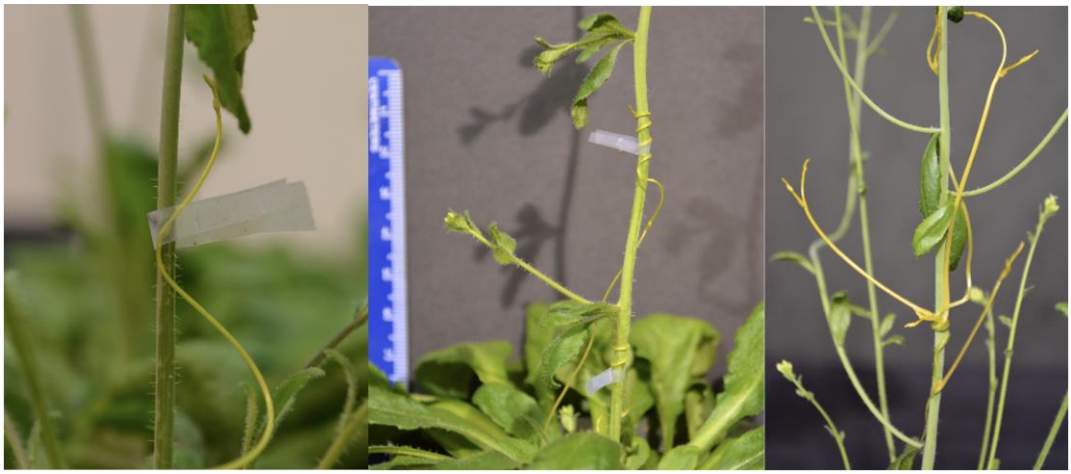

Fig. 7. *C. campestris* growing on *A. thaliana* inflorescence stem. *C. campestris* shoot tip attached manually to the host stem (Left). *C. campestris* shoot tips coiling around host stem after exposition to enriched far-red spectrum light (Center). *C. campestris* growing around host stem (Right).

The easiest method for propagating dodder is through stem cuttings (shoot tips). Once a plant has attached to a host and started to grow, it will branch profusely and make additional attachments from each new tendril. To propagate, cut about a 6-10 cm section of free dodder shoot, and tape it to the stem of another plant. This is best done using a thin piece of scotch tape placed 1.5 cm below the tip of the dodder shoot/tendril. The dodder tip will coil around the host stem over the next 12 hours and then not develop for the next 4-5 days. Once the haustorial attachments are made, the dodder will resume growth.

Fig. 8. Attachment of *C. campestris* stem cutting (shoot tips). Examples of stem cutting to be attached to a host stem (Left). Procedure to attach *Cuscuta* cuttings to a host plant using a tape (Center and Right).

4. Manipulation and evaluation of *C. campestris* growth

After *C. campestris* is established and has made connection with the vascular bundles of the host plant, it grows. We have seen that the larger the initial stem cutting is the more growth variation. This is related to the number of meristems found in a cutting (Fig. 9). So, if the goal of your experiment is to evaluate small differences of the parasite growth, we recommend to use small stem cuttings. For example, 3-cm shoot tips work well.

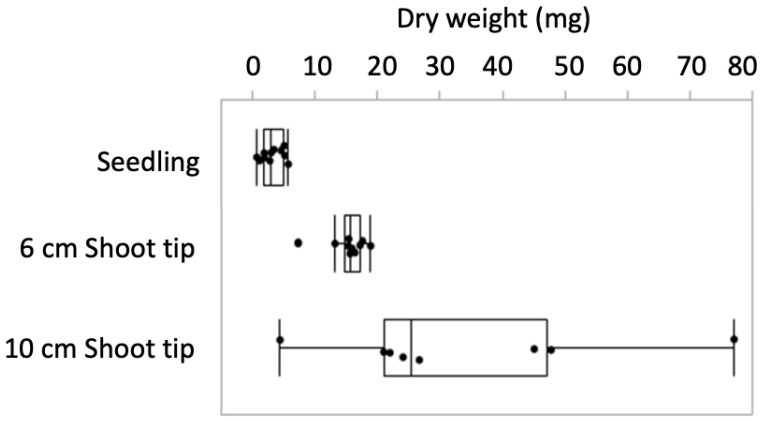

Fig. 9 Comparison of *C. campestris* growth 15 days after attachment to host using different types of initial inoculum.

Some experiments require to growth many samples at the same time. If you have reduced space availability, it could be tricky to keep samples separated, since the new shoots of a *Cuscuta* growing on a host will search for other host stems nearby. We found that Arasystem (translucent sheets held by a plastic base) (Ghent, Belgium) is a good alternative to keep *Cuscuta* contained when growing on *A. thaliana* (Fig. **10**). This technique allows to have many samples growing in a small space. If the host plant is different from Arabidopsis, modification by joining two or more upper parts of the Arasystem without the base can make a tube with a wider diameter. Humidity domes can be used instead of the Arasystem when host plants are bigger than Arabidopsis. Additionally, this technique increases seedling survival after attachment to the host and before

establishment of the connection between the parasite and the host, due to increased relative humidity.

Fig. 10 *Cuscuta* growing on *Arabidopsis* plants using Arasystem to maintain *Cuscuta* contained avoiding it to colonize other plants.

4. Seed Production

Fig. 11 *C. campestris* flowering and producing fruits (capsules) with seeds.

Dodder is a selfing species, so seeds can be produced from plants without need for crossing. Each dodder fruit produces up to four seeds, but actual yield varies. Plants growing on feeble hosts will not produce as much seed as those growing on robust hosts. The low output of each flower is compensated by the large number of flowers that may be produced by vigorous plants.

To collect seeds, let the flowers mature, and fruit and vines senesce and dry on the host. Harvest fruit capsules and crush them over a soft sieve that will catch the larger dried flower remains while letting the seeds pass through. Collect seeds on rough paper and then allow seeds to roll off while small debris stays on the paper. Repeat this winnowing process until seeds are free of debris. Seeds can be stored at room temperature for years.

Note

C. campestris is able to grow on an artificial host. For more information consult: Bernal-Galeano and Westwood, 2021.

References

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