CHEMICALLY-MEDIATED INTERACTIONS BETWEEN THE PARASITIC
PLANT CUSCUTA PENTAGONA, ITS HOSTS PLANTS, AND
INSECT HERBIVORES

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ABSTRACT

Parasitic plants are some of the world’s most destructive agricultural pests and have significant impacts on the dynamics of the communities they inhabit. Yet, the ecology of interactions between parasitic plants and their hosts remain largely unexplored. In this work we investigate the chemical-mediation of interactions between the economically important parasitic plant, *Cuscuta pentagona*, and its host plants. *Cuscuta* spp. (dodders) are yellowish vines that lack roots or expanded leaves, and are dependent on aboveground attachment to other plants for water and nutrients. After germination, seedlings must forage and locate a suitable host within a few days in order to survive. We show that location and discrimination of hosts by *C. pentagona* seedlings is guided by plant volatiles, thus providing a compelling example of volatile communication between plants. We next examined how *C. pentagona* affected host plant (tomato) defenses against insect herbivores. Parasitism greatly reduced host-plant production of the anti-herbivore phytohormone jasmonic acid (JA) and plant volatiles induced by caterpillar feeding, in part via antagonism of the herbivore- and parasite-induced defense signaling pathways. We also examined the induced responses of tomato to attack by *C. pentagona* and show that both JA and salicylic acid (SA) defense pathways are sequentially activated. Moreover, these pathways both regulate effective defenses against *C. pentagona*. Lastly, we review the chemical ecology of seed germination and host location by the major parasitic weeds of crops and discuss the potential for manipulating these mechanisms for sustainable control of these agricultural pests.
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Chapter 1

Overview

Among flowering plants, an estimated 4,500 species are parasitic and obtain at least some of their water and nutrients from other plants (Nickrent, 2007). Parasitism originated independently several times during angiosperm evolution (at least 11; Nickrent et al., 1998), resulting in a diversity of morphology and life histories among lineages (Kuijt, 1969). For example, some species are facultative and can survive in the absence of a host, whereas others are obligate parasites that are unable to establish and develop independently. A number of species possess chlorophyll and are able to produce at least some of their required nutrients through photosynthesis (hemiparasitic) whereas others lack chlorophyll and are completely dependent on the host for nutrients (holoparasitic) (Parker and Riches, 1993; Press and Graves, 1995). Parasitic species can be further divided into those that attach belowground to host-plant roots and those that attach aboveground to host-plant shoots. A common feature of all parasitic plants is the haustorium, the connective organ used to withdraw water and nutrients from the host; however, the structure of haustoria varies with the lifestyle of the parasite (Kuijt, 1969).

Parasitic plants are important components of natural ecosystems and play important roles in determining community structure and dynamics. For instance, the root hemiparasite *Rhinanthus minor* is a major driver of both aboveground and belowground properties in grasslands (Bardgett et al., 2006). Similarly, the obligate holoparasite
Cuscuta salina significantly affects the community structure of the salt marshes in which they occur (Pennings and Callaway, 1996). The removal of resources by the parasite also has direct negative effects on the host and can severely reduce host plant growth and reproduction (Goldwasser et al., 2001; Press and Graves, 1995; Wolswinkel, 1974). A small percentage of parasitic plant species have become agricultural pests, causing severe yield losses and limiting crop production in many parts of the world (Parker and Riches, 1993; Musselman et al., 2001). Moreover, effective and economical means of their control are generally lacking, in part because of their close physiological connection to host plants (Gressel et al., 2004; Rispail et al., 2007). Despite their ecological and economic importance, the interactions between parasitic plants and their hosts remain poorly understood.

This thesis explores the chemical ecology of interactions between the economically important parasitic plant Cuscuta pentagona, and several host plants, in particular, tomato (Solanum lycopersicum). The genus Cuscuta (dodders) represents the only parasitic lineage within the Morning Glory Family, Convolvulaceae (Stefanović and Olmstead, 2004). These obligate holoparasites are yellow-to-orange, epiphytic vines with no roots or expanded leaves that attach to stems and leaves of their hosts. Cuscuta spp. generally lack chlorophyll and have little photosynthetic capability, but still retain the genes necessary for photosynthesis, perhaps to synthesize lipids for allocation to developing seeds (McNeal et al., 2007a, 2007b). Several species of Cuscuta inflict serious damage to many crops worldwide including forage legumes (alfalfa, clover, lespedeza), potato, carrot, sugar beets, chickpea, onion, cranberry, blueberry, and citrus
(Dawson et al., 1994), and *C. pentagona* is a major weed of tomato in California causing yield losses of 50 to 75% (Goldwasser et al., 2001).

The seeds of *Cuscuta* contain limited energy reserves allowing seedling growth of only several centimeters. Chemical cues secreted from host-plant roots facilitate host location in root parasitic plants by inducing germination only in the presence of a host (Bouwmeester et al., 2003), but *Cuscuta* have no specialized germination requirement and seedlings must ‘forage’ to locate hosts (Dawson et al., 1994). Evidence suggests that *Cuscuta* seedlings grow toward neighboring green plants (Fritsché et al., 1958), and are able to “choose” among potential hosts (Kelly, 1992), but the role of host-produced stimulants in above-ground ecology of *Cuscuta* remains largely unexplored.

The presumably significant selection pressure for *Cuscuta* seedlings to employ efficient strategies for host location, together with the established role for plant volatiles as important cues for foraging insects (De Moraes et al., 1998; 2001), led us to hypothesize that host plant volatiles may similarly provide relevant directional cues to foraging parasites. The idea of volatile communication between plants, in particular that communication between damaged plants and their neighbors induces the neighbors to increase their levels of insect resistance, was first proposed by Rhoades (1983). Since then, communication between plants has been the subject of considerable contention (e.g., Fowler and Lawton 1985, Bruin et al. 1995, Karban and Baldwin 1997). Despite mounting evidence for volatile-mediated interactions (e.g., Baldwin and Schultz 1983, Bruin et al. 1992, Karban et al. 2000, 2003), many ecologists remain skeptical, in part because of issues with the experimental design of many studies (e.g. sealing plants in
chambers with unrealistic volatile concentrations) and the availability of alternative explanations (e.g., Dicke et al. 2003; Paschold et al., 2006). More recently researchers have provided evidence that more realistic volatile concentrations likely induce priming of the defenses of receiving plants, rather than the initiation of full scale responses (Engelberth et al., 2004).

In chapter two, we explored the possibility that host-plant volatiles might mediate host-location by seedlings of *C. pentagona*. We placed newly germinated seedling in a vial of water located at the center of a dry filter paper disk and recorded their growth by tracing the seedling’s position on the filter paper. Using this simple experimental design, we were able to test growth toward various plants, several controls representing possible alternative cues, and ultimately extracted host-plant volatiles released independently of the plant itself. We further tested seedlings’ ability to “choose” between hosts of differing quality and assessed growth responses to individual compounds from the complex blend of volatiles released by plants. Our findings demonstrate that plant volatiles play a role in mediating ecologically significant interactions and provide an example of plant-to-plant communication in a system other than the transfer of herbivore-induced warning signals. This work was published in *Science* 313: 1965-1967 (2006).

Chapter 2 illustrates the ability of plants to perceive and respond to their environment. Plants show similar responses to the myriad organisms that attack them. In response to pathogens and herbivores, plants activate defenses including the production of defensive compounds that directly affect the attacker, and plant volatiles that indirectly reduce damage by attracting natural enemies of herbivores (Karban and Baldwin, 1997;
Dangl and Jones, 2001). Plant induced defenses can be highly specific and tailored to the particular species of attacker (De Moraes et al., 1998). The physiological changes that occur in response to herbivores and pathogens are well studied and regulated by complex signaling pathways involving the phytohormones jasmonic acid (JA) and salicylic acid (SA). Defenses induced by pathogens generally result from the SA pathway, whereas JA plays a primary role regulating herbivore-induced defenses (Dangl and Jones, 2001; Schilmiller and Howe, 2005).

The JA and SA pathways can negatively interact (termed cross-talk), so that response to one enemy can interfere with responses to subsequent enemies (Stout et al., 2006). For example, SA can inhibit synthesis and action of JA (Peña-Cortés et al., 1993; Doares et al., 1995) resulting in increased performance of insects that feed on infected plants (Felton et al., 1999; Stout et al., 2006). Although considerable research has examined how plants respond to concurrent attack by herbivores and pathogens, the effects of attack by parasitic plants on defense of other enemies has not been studied. In chapter 3 we examine how parasitism by C. pentagona affected tomato defenses against beet armyworm caterpillars (Spodoptera exigua; BAW). Parasitism diminished tomato production of BAW-induced JA and blocked herbivore-induced volatiles. We report that parasitized plants contained increased amounts of SA, and using SA-deficient transgenic tomato plants provide evidence that C. pentagona-induced SA inhibits JA production against BAW. These results show that, like pathogens, parasitic plants can impact host plant defense against herbivores. This research was published in Plant Physiology 146: 987-995 (2008).
The responses induced in plants by herbivores and pathogens are well characterized, but the defenses induced by parasitic plant attack remain largely unknown. The results presented in chapter 3 provided insight into these responses and suggest a SA-mediated, pathogen-like response by tomato to *C. pentagona*. In the fourth chapter of this thesis, we directly investigated the induced responses of tomato to attack by *C. pentagona* by measuring changes in several phytohormones, including JA and SA, during the first six days of parasitism. The first attachment of parasite seedlings to 10-day-old tomato plants induced few changes in the tomato host. In contrast, a second attachment by *C. pentagona* 10 days later elicited large increases in both JA and SA, and a hypersensitive response. Using various tomato mutants with JA- and SA-altered signaling, we provide evidence that both JA and SA mediate effective defenses against *C. pentagona*. It is thought that cross-talk between the JA and SA pathways may allow plants to fine-tune responses to contain a combination of defenses from both pathways (Reymond and Farmer, 1998). Therefore, it is interesting to note that the timing of JA and SA synthesis by parasitized plants was staggered, which suggests that tomato plants may coordinate defenses against *C. pentagona* by sequentially activating these hormones. These findings are the first to directly measure hormonal defense signaling induced in a host plant by a parasitic plant, and indicate that host plant responses to *C. pentagona* are a mixture of known herbivore and pathogen defenses. This research is to be submitted to *The Plant Journal*.

The fifth chapter of this thesis reviews the chemical ecology of host location and selection by economically important parasitic plants. We focus on the host secondary
metabolites that influence the below-ground interactions of parasitic plants that attach to host roots (e.g. *Striga* and *Orobanche*) and the use of volatile compounds by *Cuscuta* (chapter 2). We discuss the potential to exploit these critical, chemically-mediated early stages of parasitism to provide sustainable control of these parasitic plants in agriculture. This review chapter has been submitted to *Agronomy for Sustainable Development*.

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Chapter 2

Volatile chemical cues guide host location and host selection by parasitic plants

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The importance of plant volatiles in mediating interactions between plant species is much debated. Here we demonstrate that the parasitic plant *Cuscuta pentagona* (dodder) uses volatile cues for host location. *Cuscuta pentagona* seedlings exhibit directed growth toward nearby tomato plants (*Lycopersicon esculentum*) and toward extracted tomato-plant volatiles presented in the absence of other cues. Impatiens (*Impatiens wallerana*) and wheat plants (*Triticum aestivum*) also elicit directed growth. Moreover, seedlings can distinguish tomato and wheat volatiles and preferentially grow toward the former. Several individual compounds from tomato and wheat elicit directed growth by *C. pentagona*, whereas one compound from wheat is repellent. These findings provide compelling evidence that volatiles mediate important ecological interactions among plant species.
Plant volatiles serve as important foraging cues for both insect herbivores and their natural enemies and can convey complex information regarding plant location, identity, and condition (1-5). It has been suggested that volatiles may have similar importance for interactions among plants, but such claims have remained controversial (6-13) and where plant-plant volatile effects have been demonstrated, their ecological importance remains unclear (6-9). Previous work on volatile-mediated interactions among plant species has dealt with the role of volatiles induced by herbivory or other environmental stressors in initiating defensive responses in neighboring plants (7, 14-19). Parasitic plants, which to survive must rapidly locate and attach to other plants, provide an alternative system in which host-plant volatiles might be expected to play an important role.

Parasitic plants are important components of both natural and agricultural ecosystems and have significant influence on the structure and dynamics of the communities they inhabit (20, 21). Yet, little is known about the ecology of interactions between parasitic plants and their hosts. Like insect herbivores, parasitic plants exhibit various “foraging” patterns (22-25) and are capable of “selecting” among potential hosts (22-25), but the mechanisms involved in host location and discrimination are not well understood.

Flowering plants in the genus *Cuscuta* are obligate parasites with little photosynthetic capability; they obtain nutrients by attaching to above-ground shoots of other plants (26) (Fig. 1). *Cuscuta* spp. are important agricultural pests, included on the United States Department of Agriculture’s *Top Ten Weeds List*, and can be difficult to
control without also impacting host plants (27). Seeds of *Cuscuta* spp. contain minimal energy reserves, allowing growth of only several centimeters, and upon germination, the rootless seedlings must locate and attach to a suitable host within a few days (26). In some parasitic plants, contact with chemical cues secreted from host-plant roots is required for germination (28, 29), but *Cuscuta* spp. have no specialized germination requirements and must depend on seedling “foraging” for host-plant location (26) (Fig. 3). After germination *C. pentagona* seedlings exhibit a rotational growth habit (circumnutation) until contacting a host (26). Host secondary metabolites are known to influence the belowground growth of parasitic plants that attach to host roots (28, 29), and host-derived chemicals also induce haustorial development by these parasites (30). However, the role of host-derived compounds in aboveground host location by *Cuscuta* spp. has not previously been determined.

In this study, we explored host finding by seedlings of *C. pentagona*. First, we examined whether *C. pentagona* seedlings exhibit directed growth toward host plants (potted 20-day-old tomato seedlings). The basal end of a *C. pentagona* seedling was inserted into a water vial placed at the center of a dry filter-paper disc. A host plant was placed near the edge of the disc (Fig. 1D) and the seedling was allowed to “forage” for four days. Seedlings’ growth across the disc was recorded by tracing their position on the filter paper (Figs. 1D and 2A). Our initial assay determined whether plants grew into the semicircle (disc half) adjacent to the target plant or into the semicircle opposite the target. This assay yielded statistically significant results (80% grew toward host plant) (Table 1) indicating that directed growth does occur. Visual observation of the recorded
growth patterns further suggested that a large proportion of plants grew more or less directly toward the target plant. To quantify this impression, we divided the disc into four quadrants (Fig. 2A) and used chi-square analysis to compare expected and observed numbers of plants growing into each. More seedlings than expected by chance grew into the quadrant nearest the target, whereas significantly fewer grew into the quadrant directly opposite the target (Table 1).

These results provide strong evidence for directed growth by *C. pentagona* seedlings toward host plants, but do not establish the cues responsible for eliciting this growth. Because we suspected a role for host-plant volatiles, we used the experimental design described above to test seedling growth responses to control targets designed to mimic possible alternative cues. Targets included pots of moist soil without plants, artificial tomato seedlings, and vials of green- or red-colored water. None of these control targets elicited a growth response from *C. pentagona* seedlings (Table 1). However, these controls provided at best a crude representation of the cues available from actual host plants, and the lack of response to these targets does not conclusively eliminate a possible role for shading or other light cues in host location. The moist soil control does indicate that the cues involved in host location, volatile or otherwise, are derived from the host plants themselves (Fig. 2A and Table 1).

To demonstrate more firmly a role for volatile cues in host location, we placed *C. pentagona* seedlings, arranged on filter paper discs as before, in a small open-air enclosure linked to two enclosed ‘target’ chambers by short lengths of black polyvinyl chloride pipe, each with an intervening 90° bend (Fig. 2B). Four potted 20-day-old
tomato seedlings were placed in one of the target chambers and four artificial tomato plants in pots of moist soil in the other. This configuration was designed to permit volatile transmission while blocking most light cues. Previous studies testing plant response to volatiles have been criticized for using airtight chambers that produce elevated volatile concentrations and may influence the physiological status of the plants (6-8, 13). Our open system avoided such problems. Multiple plants were used to increase volatile concentrations because the design of this experiment necessitated placing host plants unrealistically far away from the *C. pentagona* seedlings (i.e., farther than the seedling could grow before exhausting its energy reserves). Here we observed a directed growth response similar to that in our first experiment. Significantly more *C. pentagona* seedlings grew toward the target chamber containing host seedlings than toward the chamber containing artificial plants (77% grew toward host plants) (Table 1). This response was statistically indistinguishable from that to a single tomato plant in a completely open system (Table 2). Dividing the discs into quadrants again revealed more seedlings than would be expected by chance growing more or less directly toward the target, and fewer growing directly away from the target (Table 1).

This result strongly suggests a role for host-plant volatiles in host location by *C. pentagona* seedlings; however, we cannot rule out the possibility that this experimental design still allows the transmission of some alternative cues. To establish conclusively a role for volatile cues, we used the same experimental design to test seedling growth responses to extracted host volatiles experimentally released from rubber septa in the absence of any other plant-derived cues. Volatiles were collected from four twenty-day-
old tomato plants onto SuperQ (Alltech Associates, Deerfield, IL) adsorbent filters. Extracts from these filters were then released from a rubber septum placed in one of the target chambers (Fig. 2B). A septum treated with solvent alone was placed in the other chamber. Gas chromatographic analysis revealed that undamaged tomato seedlings released eight major volatile compounds [α-pinene, β-myrcene, 2-carene, p-cymene, β-phellandrene, limonene, (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, and one unidentified monoterpene] and that rubber septa treated with tomato volatile extracts released the same compounds in approximately the same proportions as intact plants but in lesser amounts (Table 3). We observed a growth response to extracted volatiles similar to that observed in response to whole plants: significantly more C. pentagona seedlings grew toward the target chamber containing the septum with extracted host volatiles than toward the chamber containing the septum with solvent alone (73% grew toward host plant volatiles) (Table 1 and Fig. 2A). Once again, dividing the discs into quadrants revealed an excess of seedlings growing more or less directly toward the target and fewer than would be expected by chance growing directly away from the target (Table 1 and Fig. 2A).

A pairwise comparison using logistic regression showed no significant difference in seedling responses to the three tomato volatile treatments (a single tomato plant, four tomato plants in the experimental enclosure, or extracted volatiles) but did show significant differences between the tomato volatile treatments and all other targets (Table 2), providing further confirmation of a role for host-plant volatiles in foraging by C. pentagona seedlings. These results demonstrate decisively that C. pentagona seedlings
exhibit directed growth toward volatile compounds derived from tomato plants and strongly suggest that this is an adaptive mechanism for host location.

In a subsequent experiment, we found that *C. pentagona* seedlings also exhibited directed growth toward nearby cultivated *Impatiens wallerana* ‘Dazzler’ (disc half: $\chi^2 = 6.53, P = 0.01$, quadrant: $\chi^2 = 10.27, P = 0.01, n = 30$). Wheat plants (*Triticum aestivum* ‘McNeal’), an unsuitable host on which *Cuscuta* do not survive (26), elicited a growth response that was statistically marginal ($\chi^2 = 3.33, P = 0.06, n = 30$); however, a small increase in sample size yielded a significant result (disc half: $\chi^2 = 5.57, P = 0.01$, quadrant: $\chi^2 = 8.09, P = 0.04, n = 34$). These results suggest that *C. pentagona*’s host-location mechanism operates across a wide range of plant species.

Having established the role of volatiles in host plant location by *C. pentagona*, we examined whether *C. pentagona* seedlings were also able to distinguish between potential hosts of differing quality. When *C. pentagona* seedlings were planted between tomato (host) and wheat (non-host) seedlings and equidistant from each, they exhibited a strong and consistent growth bias toward tomato ($\chi^2 = 12.57, P < 0.001, n = 23$). This result cannot be explained by contact cues, because there were no cases in which *C. pentagona* seedlings contacted one host before attaching to the other. To confirm that this host preference was mediated by plant volatiles, we gave seedlings a choice between rubber septa treated with extracted tomato and wheat volatiles (using the set-up described above for extracted tomato volatiles) (Fig. 2B). *Cuscuta pentagona* seedlings exhibited a clear preference for extracted tomato volatiles ($\chi^2 = 6.53, P = 0.011, n = 30$). This result
suggests that, although *C. pentagona* may respond to a variety of plant odors, it is capable of preferentially responding to volatiles produced by its preferred hosts.

To explore the contribution of individual compounds to the attractiveness of host volatiles, we used the same assay previously described for whole plants (Fig. 1D) to examine the growth responses of *C. pentagona* seedlings to synthetic standards released from rubber septa. When we tested seven identified compounds from the tomato blend, a significant positive response was observed to β-phellandrene, β-myrcene, and, after a small increase in sample size, α-pinene (Table 4). Notably, β-myrcene is also released by wheat seedlings. Six other compounds released by 20-day-old wheat seedlings did not show a significant positive growth response (Table 4). Unexpectedly, one wheat compound, (Z)-3-hexenyl acetate, appeared to have a repellant effect—although this result was not significant ($\chi^2 = 3.33$, $P = 0.06$, $n = 30$), a small increase in sample size yielded statistical significance (Table 4). This finding suggests a possible mechanism for the observed preference for the volatile blend produced by the preferred host tomato over that produced by the non-host wheat.

The positive growth response observed to individual compounds suggests that these compounds may be important for host location and discrimination. However, complex qualitative features of the blend may play an important role (31). Until the detailed mechanisms of by which *C. pentagona* perceives and responds to host-plant volatiles are elucidated, it will be difficult to determine exactly how the information content of the signal is encoded in the volatile blend, because cross talk may occur between components of the blend or their effects on the receiver (6). Because of its parasitic life-style and the
concomitant reduction in physiological complexity (e.g., the almost complete absence of photosynthesis and leaves) *C. pentagona* may provide an excellent model system for further investigation of the mechanisms by which plants perceive and respond to volatile signals.

Aboveground plant structures have previously been shown to exhibit directed growth in response to light, gravity, humidity, and physical contact (32). Our results demonstrate that directed growth can also be elicited by airborne chemical cues. In addition, our findings provide insight into the host-location and host-selection mechanisms used by parasitic plants, showing that host-plant volatiles play a role in this system similar to that previously described for foraging insect herbivores (1) and thus revealing unexpected convergence in the host-location strategies used by disparate natural enemies of plants. Finally, our results provide an example of chemical communication between plant species that plays an important role in mediating interspecific ecological interactions. We expect these findings will have broad implications for research in a variety of fields, including chemical ecology, parasite-host interactions, and plant biology. Moreover, these results provide knowledge that may be useful in developing new tactics for controlling parasitic plants that attack agricultural crops.
References


Acknowledgements

We thank J. Tumlinson, J. Schultz, J. Tooker, G.W. De Moraes, T. Turlings, J. Dean, and C. Delphia for helpful comments on the manuscript; D. Weaver and T. Lanini for seeds; J. McNeal for help with Cuscuta germination; J. Zhu for help with statistical analysis; W. Boland for supplying \((E,E)-4,8,12\text{-trimethyl-1,3,7,11-tridecatetraene}\), and the David and Lucile Packard Foundation and the Beckman Foundation for financial support.

Materials and Methods

Foraging assays. Experiments testing C. pentagona seedling preferences between tomato or wheat plants and C. pentagona seedling response to individual compounds were conducted in a greenhouse. All other experiments were conducted inside reach-in growth chambers (25°C, 16L:8D). Tomato plants (Lycopersicon esculentum ‘Halley 3155’) were grown in square plastic pots (9 cm tall x 10 cm wide) in a separate, insect-free growth chamber (25°C, 16L:8D) and used when plants had two expanded leaves (20 days post-planting). Seeds of Cuscuta pentagona were collected from an infested tomato crop in Yolo County, California. The seeds were soaked in concentrated sulphuric acid for 1 h, rinsed with distilled water, and placed in a Petri dish on moist filter paper to germinate.

The basal end of newly emerged C. pentagona seedlings that were approximately 2-3 cm long were placed in 2 ml glass vials filled with distilled water and covered with aluminum foil in which a small hole was created to hold the seedling. A piece of filter paper was inserted into the vial for support so that approximately 1-2 cm of the seedling
apex was visible. The top of each vial was inserted into a hole in the center of a round piece of cardboard on which was placed a dry 90 mm diameter piece of Whatman® No. 1 filter paper. *Cuscuta pentagona* seedlings were allowed to grow for four days and then the positions of the seedlings were traced on the filter paper and the disc half occupied by the apical end of each seedling was recorded. A new filter paper disc was used for each trial. These data were analyzed using chi-square tests (SAS v.8.2).

To determine if *C. pentagona* seedlings grew toward nearby tomato plants, one 20-day-old tomato plant was placed so that the disc was at soil level and the tomato leaves were positioned at the disc edge (45 mm from the *Cuscuta* seedling, Fig. 1D). The filter paper disc was supported on a piece of cardboard and did not contact the soil or tomato leaves. Up to four discs were run simultaneously by placing them as above on four sides of the tomato plant, but only one tomato plant was assayed at a given time in each growth chamber. Tomato plants were 24-days-old at the end of each trial. We repeated this assay using *Impatiens wallerana* ‘Dazzler’ (Burpee Co., Warminster, PA) as the host plant. *Impatiens* spp. are preferred hosts of *Cuscuta* spp. (*S1-S2*). The *Impatiens* plants used were approximately 5 cm tall (typically 40 d after seeding) and had six expanded leaves.

To account for potential light cues we enclosed four 20-day-old tomato plants inside a 17.9 L clear plastic Rubbermaid® container (26.3 cm wide x 27.8 cm tall x 41.6 cm long) with a hole cut in one end to allow insertion of the end of a 7.6cm ID black PVC pipe with a 90° bend. The length of the PVC pipe from bend to opening was 7 cm. Four artificial 20-day-old tomatoes made of green felt on chenille sticks were placed in moist
soil in a second plastic container. *Cuscuta pentagona* seedlings and filter paper discs were placed on a square plastic pot (9 cm tall x 10 cm wide) inside an open-top cardboard box (10 cm wide x 25 cm tall) with holes cut in two sides to allow insertion of the other ends of the PVC pipes (Fig. 2B). The positions of the treatments were switched for each trial.

Previous studies testing plant response to volatiles have been criticized for using airtight chambers that produce elevated volatile concentrations and may influence the physiological status of the plant ([S3-S6]). Our open system was designed to avoid such problems. We used multiple target plants in enclosed chambers (open only to the open-air chamber enclosing the *C. pentagona* seedling) to provide sufficient concentrations of volatiles because the experimental set-up required placing target plants farther away from *C. pentagona* seedlings (~20 cm) than the range over which seedlings would normally forage before exhausting their energy reserves (< 10 cm). Observed responses of *C. pentagona* seedlings were statistically indistinguishable from those to a single tomato plant in a completely open system (Table 1).

The above trials were repeated using rubber septa (Wheaton, Millville, NJ) treated with volatiles collected in 24-h intervals from four 20-day-old tomato plants (see below). A septum with tomato volatiles was placed in one container and a second septum treated with only solvent was placed in the other (Fig. 2C); both septa were replaced every 24 h. The positions of the container with volatiles and the container with solvent were switched between each trial.
To investigate host choice by seedlings of *C. pentagona*, a tomato plant and a wheat plant (‘McNeal’ spring wheat) were seeded 10 cm apart in a plastic pot. When these plants were 20-days-old (tomato had 2 expanded leaves, wheat had 3 leaves), a scarified *C. pentagona* seedling was planted in the center, 5 cm from each plant, and allowed to grow until it attached to a plant. A small number of seedlings that did not attach to either plant were not included in the analysis. Daily observations revealed that no *Cuscuta* seedlings contacted one host prior to attaching to the other. To confirm a role for volatiles in host choice, *Cuscuta* seedlings were given a choice between extracted tomato and wheat volatiles using the experimental enclosure setup described above (Fig. 2B). Volatiles collected in 24-h intervals from four 20-day-old tomato plants or four 20-day-old wheat plants were placed on rubber septa. A septum with tomato volatiles was placed in one container and a septum with wheat volatiles was placed in the other. Both septa were replaced every 24 h, and the positions of the boxes were switched between each trial.

Trials assaying individual tomato volatiles were conducted on open benches in a greenhouse. Synthetic standards of individual volatiles were placed on rubber septa (100 μg / septum). A filter paper disc with *C. pentagona* seedling (prepared as above) was placed near the volatile-treated septum (45 mm from seedling); a septum treated with only solvent was used as a control. Seedlings were allowed to forage for three days and the positions of the seedlings were recorded.
**Volatile collection and analysis.** Plant volatiles were collected in 24-h intervals by pulling 1 l of the air passing over tomato plants or rubber septa (2 l min⁻¹) in a volatile collection chamber (S7) through SuperQ® adsorbent (25 mg) filters; the remainder of the air vented out of the bottom of the system. Traps were rinsed with 150 μl methylene chloride into sample vials or into rubber septa for use in foraging assays; 400 ng of n-octane and nonyl acetate were added to sample vials as internal standards. Septa were stored at -80 C until use. Samples were analyzed by gas chromatography and mass spectrometry, and volatile compounds were identified by comparison of chromatographic retention times and mass spectra with those of commercially available standards. Quantification was based on peak area (GC/FID) relative to that of internal standards.

To ensure that plant volatiles were not absorbed and re-released by the filter paper discs, volatiles were collected from discs exposed to tomato seedlings or to septa containing volatiles for 48 hours. No measurable volatiles were observed in either case.

Supporting References


**Figure 1.** Parasitic plants in the genus *Cuscuta*. (A) *Cuscuta pentagona* seedling attaching to a tomato plant. (B) Vines of *C. pentagona* coiled around the petiole of a tomato leaf. (C) Growth habit of *Cuscuta*. (D) *C. pentagona* seedling growing toward a tomato plant across a filter paper disc.
Figure 2. Foraging by *Cuscuta pentagona* seedlings. (A) Summary of *Cuscuta* seedling growth responses to: a pot containing moist soil, a nearby 20-day-old tomato plant, and tomato volatiles released from rubber septa. The position of the ‘target’ is indicated by a circled “X”. The final position of the apex of each seedling is highlighted with a solid black circle. The numbers of seedlings growing into each disk half and quadrant are summarized in the smaller circles below each disc. (B) Experimental setup for the release of plant volatiles while blocking light cues.
Figure 3. *Cuscuta pentagona* seedling growing toward a 20-day-old tomato seedling.

For this picture, a *C. pentagona* seed was scarified and planted in soil about 10 cm from the tomato seedling. If the *Cuscuta* seedling is unable to locate and attach to the tomato within a few days it will die.
Table 1. Foraging of *Cuscuta pentagona* seedlings on filter paper discs to various targets.

<table>
<thead>
<tr>
<th>Exp</th>
<th>Target</th>
<th>Seedlings choosing disc half with or without targets</th>
<th>Seedlings choosing quadrants (direction relative to target)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. with target (%)</td>
<td>% with target (%)</td>
</tr>
<tr>
<td>1</td>
<td>10-day-old plants</td>
<td>23</td>
<td>77</td>
</tr>
<tr>
<td>2</td>
<td>20-day-old plants</td>
<td>24</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>Red glass</td>
<td>14</td>
<td>47</td>
</tr>
<tr>
<td>4</td>
<td>Green glass</td>
<td>12</td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>Artificial plant</td>
<td>12</td>
<td>40</td>
</tr>
<tr>
<td>6</td>
<td>Moist soil</td>
<td>14</td>
<td>47</td>
</tr>
<tr>
<td>7</td>
<td>20-day-old plants</td>
<td>23</td>
<td>77</td>
</tr>
<tr>
<td>8</td>
<td>Volatile extracts*</td>
<td>22</td>
<td>73</td>
</tr>
</tbody>
</table>

*Target tested in experimental enclosure.

Table 2. Pair-wise test using logistic regression to contrast different target treatments. $\chi^2$ (P value).

<table>
<thead>
<tr>
<th>Exp</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td>0.098 (0.754)*</td>
<td>5.47 (0.019)‡</td>
<td>7.82 (0.005)‡</td>
<td>7.82 (0.005)‡</td>
<td>5.47 (0.019)‡</td>
<td>0.0 (1.0)*</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>6.75 (0.009)‡</td>
<td>9.25 (0.002)‡</td>
<td>9.25 (0.002)‡</td>
<td>6.75 (0.009)‡</td>
<td>0.089 (0.754)*</td>
<td>0.371 (0.543)*</td>
<td>4.31 (0.038)‡</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>0.271 (0.603)†</td>
<td>0.271 (0.603)†</td>
<td>0.0 (1.0)†</td>
<td>0.271 (0.603)†</td>
<td>0.089 (0.754)*</td>
<td>0.371 (0.543)*</td>
<td>6.49 (0.011)‡</td>
</tr>
<tr>
<td>4</td>
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<td>0.271 (0.603)†</td>
<td>0.271 (0.603)†</td>
<td>0.271 (0.603)†</td>
<td>5.47 (0.019)‡</td>
</tr>
<tr>
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<td>0.271 (0.603)†</td>
<td>0.271 (0.603)†</td>
<td>0.271 (0.603)†</td>
<td>5.47 (0.019)‡</td>
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</tr>
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<td></td>
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<td></td>
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<td></td>
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<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

*Group A (Exp. 1, 2, 7, and 8) ‡Group B (Exp. 3, 4, 5, and 6) †Contrast tests between groups A and B
Table 3. Average volatiles released by 20-day-old tomato plants and by rubber septa treated with tomato volatiles.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Volatiles released (ng/24hours ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Four 20-day-old tomato plants</td>
</tr>
<tr>
<td>α-Pinene</td>
<td>83.8 ± 13.9</td>
</tr>
<tr>
<td>β-Myrcene</td>
<td>93.5 ± 6.2</td>
</tr>
<tr>
<td>2-Carene</td>
<td>1131.6 ± 173.4</td>
</tr>
<tr>
<td>p-Cymene</td>
<td>53.6 ± 13.1</td>
</tr>
<tr>
<td>β-Phellandrene</td>
<td>2843.9 ± 395.8</td>
</tr>
<tr>
<td>Limonene</td>
<td>602.7 ± 64.6</td>
</tr>
<tr>
<td>(E,E)-4,8,12-Trimethyl-1,3,7,11-tridecatraene</td>
<td>376.9 ± 141.8</td>
</tr>
<tr>
<td>Unidentified monoterpene</td>
<td>138.9 ± 18.2</td>
</tr>
</tbody>
</table>

Table 4. Foraging of Cuscuta pentagona seedlings on filter paper discs to individual tomato (top) and wheat (bottom) volatiles released from rubber septa.

<table>
<thead>
<tr>
<th>Volatile compound</th>
<th>Seedlings choosing disc half with or without volatile</th>
<th>Seedlings choosing quadrants (direction relative to volatile)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. with volatile</td>
<td>No. without volatile</td>
</tr>
<tr>
<td>α-Pinene</td>
<td>23</td>
<td>11</td>
</tr>
<tr>
<td>β-Myrcene</td>
<td>21</td>
<td>9</td>
</tr>
<tr>
<td>2-Carene</td>
<td>14</td>
<td>20</td>
</tr>
<tr>
<td>p-Cymene</td>
<td>13</td>
<td>17</td>
</tr>
<tr>
<td>β-Phellandrene</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>Limonene</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>TMTT*</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>(Z)-3-Hexen acetate</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>(E)-3-Hexen-1-ol</td>
<td>15</td>
<td>19</td>
</tr>
<tr>
<td>(E)-β-Ocimene</td>
<td>16</td>
<td>14</td>
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<tr>
<td>Linalool</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>Decanal</td>
<td>22</td>
<td>12</td>
</tr>
<tr>
<td>Nonanal</td>
<td>17</td>
<td>17</td>
</tr>
</tbody>
</table>

* (E,E)-4,8,12-Trimethyl-1,3,7,11-tridecatraene
Chapter 3

Parasitism by *Cuscuta pentagona* attenuates host plant defenses against insect herbivores

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Abstract

Considerable research has examined plant responses to concurrent attack by herbivores and pathogens, but the effects of attack by parasitic plants—another important class of plant-feeding organisms—on plant defenses against other enemies has not been explored. We investigated how attack by the parasitic plant *Cuscuta pentagona* impacted tomato (*Solanum lycopersicum*) defenses against the chewing insect *Spodoptera exigua* (beet armyworm, BAW). In response to insect feeding, *C. pentagona*-infested (parasitized) tomato plants produced only one-third of the anti-herbivore phytohormone jasmonic acid (JA) produced by unparasitized plants. Similarly, parasitized tomato, in contrast to unparasitized plants, failed to emit herbivore-induced volatiles after three days of BAW feeding. Although parasitism impaired anti-herbivore defenses, BAW growth was slower on parasitized tomato leaves. Vines of *C. pentagona* did not translocate JA from BAW-infested plants: amounts of JA in parasite vines grown on caterpillar-fed and control plants were similar. Parasitized plants generally contained more salicylic acid (SA), which can inhibit JA in some systems. Parasitized mutant (*NahG*) tomato plants deficient in SA produced more JA in response to insect feeding than parasitized wild type plants, further suggesting cross-talk between the SA and JA defense signaling pathways. However, JA induction by BAW was still reduced in parasitized compared to unparasitized *NahG*, implying that other factors must be involved. We found that parasitized plants were capable of producing induced volatiles when experimentally treated with JA, indicating that resource depletion by the parasite does not fully explain the observed attenuation of volatile response to herbivore feeding. Collectively, these
findings show that parasitic plants can have important consequences for host plant
defense against herbivores.

**Introduction**

Plants have evolved the ability to perceive attack and respond by activating induced
defenses (Karban and Baldwin, 1997; Dangl and Jones, 2001). The defensive strategy
utilized is dependent on the attacker and can be highly specific. For example, plants can
distinguish feeding by closely related herbivore species and tailor induced volatiles to
attract specialist parasitoids (De Moraes et al., 1998). The induced physiological changes
of plants in response to herbivores and pathogens are well studied and result from
complex defense signaling networks regulated by the plant hormones jasmonic acid (JA)
and salicylic acid (SA). In general, the JA pathway is activated in response to herbivores
and regulates production of compounds that impair digestion (Chen et al., 2005, 2007)
and of induced plant volatiles that attract natural enemies (Turlings et al., 1990) and repel
ovipositing moths (De Moraes et al., 2001). The SA pathway is typically activated in
response to pathogens and mediates a hypersensitive response and the production of an
array of antimicrobial phytoalexins and pathogenesis-related (PR) proteins that results in
systemic acquired resistance (SAR) to a broad-spectrum of pathogens (Durrant and
Dong, 2004). However, the categorization of JA as an herbivore-defense signal and SA
as a pathogen-defense signal is imperfect, as JA-mediated defenses are induced by some
pathogens and SA-mediated defenses by some herbivores (Moran and Thompson, 2001;
Glazebrook, 2005).
The defenses that plants deploy against one enemy may or may not be effective against other enemies (Stout et al., 2006). Moreover, the JA and SA signaling pathways can negatively interact, so that resistance to one pest may increase the vulnerability to another. For example, SA-mediated responses to pathogens have been found to negatively affect subsequent JA-mediated defenses against herbivores, resulting in increased performance of insects that feed on infected plants (Preston et al., 1999; Felton et al., 1999; Thaler et al., 1999, 2002; Stout et al., 2006). Although it is well established that SA can inhibit production of JA and the expression of JA-induced defenses (Peña-Cortés et al., 1993; Doares et al., 1995; Thaler et al., 1999; Cipollini et al., 2004), predicting positive or negative effects on subsequent enemies has proved difficult since a strict dichotomy between the defense pathways for pathogen and insect attack does not always exist and the range of organisms affected by each pathway varies (Felton and Korth, 2000; Thaler et al., 2002; Cardoza et al., 2003; Thaler et al., 2004; Stout et al., 2006). Defense signaling cross-talk may allow plants to minimize costly, ineffective defenses and fine-tune responses to specific enemies (Reymond and Farmer, 1998; Kunkel and Brooks, 2002), but the mechanisms underlying JA/SA cross-talk are not understood.

To date, research on induced plant defenses and defense signaling cross-talk has focused almost exclusively on herbivorous arthropods and pathogens. But plants also must defend themselves from attack by other plants. Approximately 4500 species of flowering plants (about 1%) are parasitic (Nickrent, 2007) and attach to other plants to obtain water and nutrients (Kuijt, 1969). Parasitic plants can severely impact host growth
and reproduction (Wolswinkel, 1974; Press and Graves, 1995) and have significant
effects on the structure and productivity of ecosystems in which they occur (Press and
Phoenix, 2005; Bardgett et al., 2006). Parasitic plants also account for some of the
world’s most destructive agricultural pests (Parker and Riches, 1993; Musselman et al.,
2001). Dodders, genus *Cuscuta* (Convolvulaceae), are one of the most ecologically and
economically significant groups of parasitic plants (Kuijt, 1969). *Cuscuta* spp. have
yellow-to-orange vines that lack obvious chlorophyll, roots, and expanded leaves, and
thus are completely dependent on aboveground attachment to other plants for survival
and reproduction (Dawson et al., 1994). We recently demonstrated that *Cuscuta*
*pentagona* seedlings use plant volatiles to locate and choose among hosts (Runyon et al.,
2006). Once a host is located, *C. pentagona* vines twine around the host stem and
produce haustoria, specialized organs that grow into the host to extract nutrients from
both xylem and phloem (Dawson et al., 1994). *Cuscuta* spp. cause extensive damage
each year to numerous agricultural crops (e.g., tomato [*Solanum lycopersicum*], alfalfa
[*Medicago sativa*], potato [*Solanum tuberosum*], soybean [*Glycine max*], onion [*Allium
cepa*], and cranberry [*Vaccinium macrocarpon*]) and, because of their close physiological
connection to hosts, are difficult to control without also impacting the crop plants
(Nadler-Hassar and Rubin, 2003). Despite their economic importance and the profound
effects they have on host plants and community dynamics, relatively little is known about
the defenses induced by parasitic plant attack or how these defenses affect host plant
interactions with other organisms.
Trade-offs in plant defenses against different attackers are likely central to the ecology and evolution of induced defenses. Moreover, understanding such trade-offs is key to avoiding unwanted side-effects if these pathways are to be manipulated to control pests in agriculture. In this study, we examined how parasitism by *C. pentagona* affects tomato plants’ induced defenses against a chewing insect, *Spodoptera exigua* (the beet armyworm, BAW), by comparing production of JA and plant volatiles from parasitized and unparasitized tomato plants. We also determined the growth rate of BAW caterpillars on parasitized and unparasitized plants. Finally, we investigated several mechanisms that might explain the observed impact of *C. pentagona* parasitism on tomato herbivore defenses, including the removal of JA by *Cuscuta*, negative cross-talk between the JA and SA pathways, and the availability of resources needed for induced defenses.

**Results**

**Production of JA and SA by Parasitized and Unparasitized Tomato Plants**

To investigate how *C. pentagona* infestation affected herbivore-induced defenses of the tomato host, we first constructed a time-course tracking concentrations of JA and SA during the first 24 h of BAW feeding (Figs. 1 and 2). Amounts of JA began to increase as soon as 15 minutes after insect feeding began, and the highest JA concentrations occurred after BAW had fed for 24 h (Fig. 2A). The production of JA by parasitized and unparasitized plants was not statistically different during the first 2 h of insect feeding, but after 24 h of feeding *Cuscuta*-infested tomato plants contained only about 30% of the
JA found in unparasitized plants (mean ± SE ng/g JA: 278 ± 77 parasitized, 812 ± 112 unparasitized; Fig. 2A). Parasitized and unparasitized control plants, which received no insect feeding, did not differ in JA content (Fig. 2A). *Cuscuta pentagona*-infested plants generally contained greater amounts of SA than unparasitized plants (Fig. 2B), but this difference was not consistently significant due to the large variability in SA content in parasitized plants (Fig. 2B).

**Production of Herbivore-Induced Volatiles by Parasitized and Unparasitized Tomato Plants**

We next examined the impact of parasitism on host-plant volatile production induced by BAW feeding. Undamaged tomato plants released 13 volatile compounds which included the monoterpenes α-pinene, β-pinene, β-myrcene, 2-carene, p-cymene, β-phellandrene, limonene, (E)-β-ocimene, linalool, and 2 others that are unidentified; the sesquiterpene β-caryophyllene; and the homoterpene (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT). Aside from small, inconsistent amounts of several 6-carbon green leaf volatiles [(Z)-3-hexenal, (Z)-3-hexenol, and (Z)-3-hexenyl acetate], plants that received BAW feeding produced these same 13 volatile compounds, but in greater amounts. Total volatile production induced by BAW feeding did not differ between parasitized and unparasitized plants during the first two days; however, unparasitized plants released significantly more total volatiles than parasitized plants on day three of feeding (Fig. 3). Moreover, BAW feeding induced a significant increase in total volatiles produced by unparasitized plants (*P* = 0.0371), while total volatile production by
parasitized plants damaged by BAW did not differ among the three days ($P = 0.6590$). At no time did *C. pentagona*-parasitized plants fed on by BAW produce more volatiles than parasitized plants without BAW (Fig. 3). Among individual volatile compounds, three days of BAW feeding on unparasitized plants induced significant increases in $\alpha$-pinene, 2-carene, $\beta$-phellandrene, limonene, and one unidentified monoterpene (Fig. 4). None of these volatile compounds were induced by caterpillar feeding on parasitized plants (Fig. 4). Interestingly, *Cuscuta*-infested control plants released greater total volatiles (encompassing the same individual volatiles induced by BAW) during the first two days of the experiment than unparasitized control plants (Fig. 3).

**BAW Feeding and Growth on Parasitized and Unparasitized Tomato**

Because JA content and volatile production can be positively correlated with amounts of damage (Ohnmeiss et al., 1997; Gouinguené et al., 2003; Dean and De Moraes, 2006; Tooker and De Moraes, 2007), we compared the leaf area consumed by BAW on parasitized and unparasitized tomato plants over a 24-h period. Although BAW tended to remove more leaf area from unparasitized than parasitized plants (44 ± 8 and 33 ± 7 cm$^2$ 24h$^{-1}$, respectively), this difference was not significant ($t$-test, $P = 0.304$) nor did the proportion of total leaf area eaten differ (0.041 ± 0.005 unparasitized, 0.048 ± 0.009 parasitized; $t$-test, $P = 0.456$). There were no noticeable differences in the feeding pattern of BAW on leaves of parasitized and unparasitized plants.
BAW caterpillars feeding on leaves of parasitized tomato plants grew much slower than those feeding on unparasitized tomato leaves (Fig. 5; mean ± SE relative growth rate: 0.63 ± 0.05 and 1.76 ± 0.11, respectively; *t*-test, *P* < 0.0001).

Translocation of Herbivore-Induced JA by *Cuscuta*

The haustoria of *Cuscuta* form vascular connections with the host, creating a powerful sink that transports sugars, amino acids, and other nutrients from host to parasite (Dawson et al., 1994; Birschwilks et al., 2007). We investigated the possibility that *C. pentagona* might withdraw JA from BAW-infested tomato plants. After 24 h of BAW feeding, the amounts of JA in *C. pentagona* vines growing on caterpillar-fed plants were not different from those growing on uninfested plants (mean ± SE ng/g JA: 26.5 ± 3.5 and 23.5 ± 2.5, respectively; *t*-test, *P* = 0.624; *n* = 12).

Parasitized *NahG* Tomato Plants Produce More Herbivore-Induced JA than Parasitized Wild Type Plants

Parasitized Wild Type Plants

We used SA-deficient *NahG* tomato plants to explore the possible inhibition of JA by SA induction, comparing JA in parasitized transgenic and wild-type plants after 24 h of BAW feeding. In all treatments, transgenic *NahG* plants produced significantly less SA than wild-type plants (Fig. 6A). However, degradation of SA by the enzyme salicylate hydroxylase was not complete in *NahG* plants which contained about 20 ng g⁻¹ of SA, amounts similar to those reported by Li et al. (2006). Parasitized *NahG* plants produced more JA in response to BAW feeding than parasitized wild type plants (Fig. 
6B). Unparasitized *NahG* plants tended to produce more herbivore-induced JA than unparasitized wild type plants, but this difference was not significant (Fig. 6B; *P* = 0.096). In both *NahG* and wild-type plants, parasitism by *C. pentagona* significantly reduced production of JA in response to BAW feeding (Fig. 6B; *P* = 0.033 and 0.004, respectively).

**Parasitized Tomato Plants Produce Induced Volatiles when Treated with JA**

To determine if *C. pentagona*-infested tomato plants could produce induced volatiles, we treated parasitized and unparasitized wild-type plants with synthetic JA and compared subsequent volatile production. Application of JA induced a significant increase in volatiles after three days in both parasitized and unparasitized plants (Fig. 7). Total JA-induced volatile production by parasitized plants was not different from that of unparasitized plants for any day (Fig. 7).

**Discussion**

*C. pentagona* impacts on herbivore-induced JA and volatiles

Tomato plants parasitized by *C. pentagona* contained only about one-third as much JA as unparasitized plants after 24 h of caterpillar feeding (Fig. 1A). The role of JA in regulating induced plant defenses against chewing insects (e.g. proteinase inhibitors) is well established, and tomato served as a model system for much of this work. For example, loss of function tomato mutants for JA production have been shown to be more
susceptible to insect feeding (Orozco-Cardenas et al., 1993; Howe et al., 1996; Li et al., 2003), whereas gain of function mutants have increased resistance to herbivores (Li et al., 2002; Chen et al., 2005). Furthermore, application of exogenous jasmonate has been shown to promote resistance of tomato plants to BAW in agricultural fields (Thaler, 1999). Although not verified in this study, reduced production of caterpillar-induced JA in parasitized tomato plants should translate into less proteinase inhibitors and other foliar anti-herbivore compounds compared to parasitized plants.

In contrast to unparasitized plants, tomato plants parasitized by \textit{C. pentagona} failed to produce herbivore-induced volatiles three days after insect feeding began (Figs. 3 and 4). Because JA mediates the production of induced plant volatiles in tomato (Ament et al., 2004; Thaler et al., 2005), reduced JA production in parasitized plants may explain the absence of herbivore-induced volatiles. Volatiles induced by insect feeding are known to serve as important cues that can both repel ovipositing herbivores and attract their natural enemies, significantly reducing herbivore pressure in nature (Kessler and Baldwin, 2001; De Moraes et al., 2001). Our results suggest that \textit{C. pentagona}-infested tomato plants would be unable to gain these benefits of volatile induction. When JA was supplied exogenously, parasitized tomato plants produced amounts of volatiles similar to unparasitized plants (Fig. 7), suggesting that the absence of induced volatiles cannot be explained solely by the removal of resources by the parasite.

Despite the attenuation of herbivore-induced JA and volatiles, the growth rate of BAW was greatly reduced on parasitized plants (Fig. 5). Slower growth of BAW may be explained by reduced water and nutrient availability in parasitized plants. \textit{C. pentagona}
acts as a strong sink withdrawing water and nutrients from the host plant, which can reduce sugar and nitrogen content of host plant leaves (Jeschke et al., 1994). Nutritional inadequacy of the host plant may also explain the slower growth rate of *Chilo partellus* (Swinhoe), a lepidopteran stem borer, on maize infested by the parasitic plant *Striga hermonthica* (Del.) Benth. (Mohamed et al., 2007). However, we cannot rule out the possibility that compound(s) produced in the course of defense against *Cuscuta* might negatively affect BAW caterpillars.

**Cuscuta Does Not Translocate Herbivore-Induced JA**

In response to herbivory, plant volatiles are released not only at the site of feeding but also systemically from undamaged leaves (Paré and Tumlinson, 1997). Systemic responses in tomato are mediated by a phloem-mobile signal originating at the site of damage, and recent work indicates that this signal is likely to be JA (Schilmiller and Howe, 2005). We hypothesized that phloem-feeding parasites might withdraw JA from BAW-fed plants, reducing JA levels in leaves and precluding a systemic volatile response. However, we found no evidence that *C. pentagona* removed JA since parasite vines grown on uninfested and BAW-infested tomato contained the same amount of JA.

**Cuscuta-induced SA may inhibit JA**

Studies using tomato have shown that SA, either applied exogenously or induced by pathogens, can inhibit production of herbivore-induced JA (Doares et al., 1995; Stout et al., 1999; Thaler et al., 2002). Several lines of evidence from the current study and
others indicate that plant defenses induced by Cuscuta spp. attack are ‘pathogen-like’ and might be mediated by SA. For example, reported host plant responses to Cuscuta spp. include hypersensitive reactions and phytoalexin production (Bringmann et al., 1999) as well as the expression of pathogenesis-related genes (Borsics and Lados, 2002). In this study, we also observed localized cell death at the point of Cuscuta attachment.

Furthermore, parasitized plants tended to contain more SA than unparasitized plants (Fig. 2B). To examine whether Cuscuta-induced SA might be inhibiting JA, we compared BAW-induced JA production in parasitized wild-type and SA-deficient NahG tomato plants. Parasitized NahG plants produced more JA than parasitized wild type plants in response to BAW feeding (Fig. 6), implying that SA inhibited JA production. However, JA/SA crosstalk alone does not fully explain these results since Cuscuta still reduced BAW-induced JA production in NahG plants (Fig. 6B). Though the effect was not statistically significant, BAW-induced JA appeared to be elevated in uninfested NahG plants (Fig. 6B), a pattern that might result from reduced SA suppression (Spoel et al., 2003).

Some plant pathogens and herbivores are known to manipulate host defenses by interfering with plant defense signaling. For example, Pseudomonas syringae injects the JA mimic coronatine into tomato, eliciting JA responses and suppressing effective SA responses to promote pathogenesis (Zhao et al., 2003). Moreover, silverleaf whitefly feeding activates SA defenses and reduces operative JA defenses in Arabidopsis (Zarate et al., 2007). In broad terms, Cuscuta ‘feeding’ resembles that of whiteflies; both are ‘stealthy’ phloem feeders (i.e. cause little tissue damage) that feed continuously from the
same location over an extended period of time. We cannot rule out the possibility that, like some pathogens and insects, *C. pentagona* co-opts JA/SA crosstalk to manipulate host defenses. We are currently investigating which defense pathways are activated in parasitized tomato plants and the efficacy of JA and SA responses in defense against *C. pentagona*.

In summary, herbivore-induced production of JA and volatiles are compromised when tomato plants are infested by the parasitic plant *C. pentagona*. SA-Mutant (*NahG*) tomato plants deficient in SA production contained significantly more BAW-induced JA when parasitized than wild-type plants, providing some evidence of SA-JA antagonism in host plant defense signaling. Our results further suggest that parasitism by *C. pentagona* induces plant volatiles and may elicit an SA-mediated ‘pathogen-like’ response in tomato. However, a better understanding of host plant perception and physiological responses to attack by parasitic plants is needed to identify the mechanisms underlying *C. pentagona*-mediated effects on host plant defenses against herbivores.

**Materials and Methods**

**Plant/Insect Material and Growth Conditions**

Seeds of *Cuscuta pentagona* collected from an infested tomato field in Yolo County, California were obtained from Dr. Tom Lanini (University of California, Davis). Seeds were soaked in concentrated sulfuric acid for 1 h using a Gooch crucible, rinsed for 1 min with distilled water, and placed in a Petri dish on moist filter paper to germinate. Tomato plants (*Solanum lycopersicum* cv Halley 3155) were grown in an insect-free growth
chamber (25°C, 16-h photoperiod at 250 µmol m⁻² s⁻¹ provided by cool white fluorescent tubes) in 9 cm tall x 10 cm wide square plastic pots filled with a peat-based general-purpose potting soil with fertilizer (Osmocote®/ The Scotts Company, Marysville, OH). Seeds of NahG tomato plants and the corresponding wild type (cv MoneyMaker) were obtained from Dr. Harry Klee (University of Florida) and grown similarly, except that they received low light intensity (75 µmol m⁻² s⁻¹) to prevent development of necrotic leaf spots. Beet armyworm (Spodoptera exigua) eggs were obtained from the USDA/ARS Research Laboratory in Tifton, Georgia and reared on a casein-based artificial diet in a growth chamber (25/22°C day/night, 16-h photoperiod).

*C. pentagona* Attachment and Growth on Tomato

Newly germinated *C. pentagona* seedlings, approximately 4 cm long, were allowed to attach to 10-day-old tomato seedlings (first true leaves just beginning to expand) by leaning the *C. pentagona* seedling against the right side of the tomato meristem (*Cuscuta* are left-handed and coil from right to left). Because far-red light promotes tight coiling of *Cuscuta* spp. (Haidar and Orr, 1999), two incandescent 75W bulbs (75A/CL/DL/RP 120V, Orsam Sylvania Inc., Danvers, MA) per 15 pots in 30 cm x 50 cm flats were placed 1 m above plants and left on for 24 h (off for 8 h of scotophase). Using this setup, *C. pentagona* seedlings coiled tightly around the tomato seedlings within 6 h and haustorial swellings at points of contact with the host were evident within 24 h. Control plants received the same treatment, and plants exposed to incandescent light for this short period showed no noticeable physiological effects. Ten days later, the growing *Cuscuta*
vine was allowed to attach a second time to the petiole of the second expanded true leaf (the youngest expanded leaf) of the same now-20-day-old tomato host (Fig. 1). To control the site of attachment, the parasite vine at approximately 2 cm from apex was placed against the right side of the appropriate tomato petiole. Subsequent brief exposure to incandescent light (as above) usually induced coiling around the petiole at this point. Five days later, the apical leaflet attached to the parasitized petiole of the 25-day-old tomato received insect feeding for phytohormone analysis, volatile collection, and caterpillar growth trials (Fig. 1, arrow).

**Extraction and Quantification of JA and SA**

A time-course of changes in JA and SA in 25-day-old tomato was conducted for the following treatments: (1) Tomato control (no parasitism or BAW feeding), (2) Tomato + parasite control (C. pentagona parasitism only), (3) Tomato + BAW (BAW feeding only), and (4) Tomato + C. pentagona + BAW (Parasitized tomato with BAW feeding). For treatments with insect feeding, one third-instar BAW was confined to the apical leaflet of the parasitized petiole leaf (Fig. 1, arrow) using a round 3 cm diameter clip-cage. The corresponding leaf of plants in insect-free treatments received empty cages. Insects were watched until they began to feed. At 0, 15, 30, 45, 60, 120 min and 24 h after feeding began approximately 100 mg of the leaf (incorporating the feeding site) was removed, immediately snap-frozen in liquid nitrogen in FastPrep® tubes (Q-BIOgene, Carlsbad, CA) with 1 g of Zirmil beads (1.1 mm; Saint-Gobain ZirPro, Mountainside, NJ), weighed, and held at -80º C until processed. We used vapor phase extraction to
extract and measure JA and SA following the method of Schmelz et al. (2003, 2004).

Briefly, plant tissue was homogenized using Zirmil beads in a FastPrep® shaker, and the phytohormones were partitioned into an organic layer (dichloromethane), transferred to a 4 ml glass vial, and derivatized from carboxylic acids to methyl esters using trimethylsilyldiazomethane (Sigma-Aldrich, St. Louis, MO). The solvent was evaporated under an air stream, and the dry vial was heated to 200º C for 2 min to expedite volatilization of analytes which were collected at this time from the headspace using volatile traps containing 30 mg of Super-Q® (Alltech, Deerfield, IL) attached to a vacuum (1 l/min). The phytohormones were eluted from the traps using 150 μl of dichloromethane and analyzed by gas chromatography-mass spectrometry with isobutane chemical ionization with select-ion monitoring (settings described by Schmelz et al., 2004). Amounts of methyl jasmonate and methyl salicylate were quantified using standard curves made with pure standards (Sigma-Aldrich, St. Louis, MO); internal standards were used to confirm derivatization and recovery.

**Collection and Analysis of Plant Volatiles**

Volatiles were collected from the four plant treatments described above from intact, potted 25-day-old tomato plants using a closed push/pull system. A guillotine Teflon® base with a small hole in the center for the plant stem rested on the pot and plants were enclosed in a glass dome (15 cm tall x 16 cm wide at base). Filtered air was pushed into the top of the chamber (2 l/min), passed over the plant, and was pulled out the side (1 l/min) through volatile traps containing a 30 mg bed of the adsorbent Super-Q®.
Volatiles were eluded from traps with 150 μl dichloromethane; 200 ng of \( n \)-octane and 400 ng of \( n \)-nonyl-acetate were added as internal standards. Samples were analyzed with an Agilent 6890 gas chromatograph (injector: splitless mode, 220º C, 1 μl sample volume) equipped with a flame ionization detector. Compounds were separated on a HP-1 (15 m x 0.25 i.d, 0.1 μm film thickness) column held at 35 º C for 1 min after injection, and then programmed at 4º C min\(^{-1}\) to 140º C, then 20º C min\(^{-1}\) to 220º C. Quantifications were made relative to internal standards using ChemStation software (Agilent Technologies, Wilmington, DE). Identifications of compounds were confirmed using mass spectrometry (HP 5973) by comparing retention times and mass spectra to commercial standards (De Moraes and Mescher, 2004). To investigate herbivore-induced volatiles, one third instar BAW was confined, using a round 3 cm diameter clip-cage, to the apical leaflet of the parasitized tomato petiole leaf (Fig. 1) or to the corresponding leaf of unparasitized plants. Empty cages were clipped on parasitized and unparasitized control plants. Volatiles were collected for three days between 1,000 and 2,200 h (light period: 6 AM - 10 PM).

**BAW Feeding and Performance on Parasitized and Unparasitized Tomato**

Third-instar BAW caterpillars were caged individually on the parasitized petiole leaf (Fig. 1) or on the corresponding leaf of unparasitized 25-day-old tomato plants. At the beginning and end of the experiment, caterpillars were starved for 24 h to void gut contents and then weighed. Caterpillars were allowed to feed for 24 h and the relative growth rate \([(\text{final weight-initial weight}) / (\text{initial weight x number of days})]\) (RGR) was
calculated (Waldbauer, 1968). In a separate experiment, we compared the total amount and proportion of total leaf area consumed by BAW on parasitized and unparasitized plants. Caterpillars were allowed to feed as above for 24 h, then all leaves were removed, taped to a white piece of paper, digitally scanned, and leaf area was determined using the imaging analysis software SigmaScan Pro 5 (SPSS Inc., Chicago, IL).

Translocation of JA by *C. pentagona* from BAW-infested tomato

One third-instar BAW was allowed to feed on parasitized and unparasitized tomato plants as described above. After 24 h of feeding, the entire *C. pentagona* plant (approximately 300 mg fresh weight) was removed, immediately frozen in liquid nitrogen, and stored at -80°C until processed. The vines were ground in liquid nitrogen with a mortar and pestle to a fine powder, and an aliquot of approximately 100 mg (fresh weight) was used for JA extraction and measurement (as described above).

JA Production by *NahG* Tomato in Response to BAW Feeding

To examine the possibility that SA inhibits BAW-induced JA production in parasitized plants, the production of JA and SA by *NahG* and wild-type (MoneyMaker) tomato plants in response to BAW feeding was determined. *NahG* plants express a gene encoding a bacterial enzyme, salicylate hydroxylase, that converts SA immediately to inactive catechol, and are thus deficient in accumulation of this plant hormone (Brading et al., 2000). Caterpillars were allowed to feed for 24 h on 25-day-old transgenic and wild-type plants and amounts of JA and SA were measured as described above.
**Induction of Volatiles with Synthetic JA**

The ability of parasitized and unparasitized tomato plants to produce induced volatiles upon treatment with synthetic JA was investigated. JA was synthesized from methyl jasmonate (Farmer et al., 1992) and suspended in 70% ethanol:water. The average fresh weigh of the appropriate apical leaflet was determined and the amount of JA typically found in unparasitized plants after 24 h of caterpillar feeding (about 800 ng g\(^{-1}\); Fig. 2A) was evenly applied with a pipette to the apical leaflet attached to the parasitized petiole of the 25-day-old tomato (Fig. 1). JA was applied on morning of day 1 and volatiles were collected for 3 days.

**Statistical Analyses**

Comparisons were made among treatments for each sampling period in the JA/SA time-courses, and to test for treatment effects on volatile production, using analysis of variance (ANOVA); individual means were compared with Tukey’s honestly significantly different (HSD) means separation test. All statistics were done using SAS (version 8.2; SAS Institute, Cary, NC). Amounts of JA and SA were analyzed on a per-gram-fresh-weight basis and were natural log (ln) transformed to stabilize variance. Volatile data were square-root transformed to meet variance assumptions. Because parasitized tomato plants were typically smaller than unparasitized plants, volatiles were analyzed by leaf area (ng/cm\(^2\)). Leaf area was determined using SigmaScan Pro 5 (as described above). The RGR of BAW on healthy and *Cuscuta*-infested tomato leaves, and JA in *Cuscuta* after 24 h BAW feeding were compared using *t*-tests.
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Figure 1. Schematic showing a 25-day-old tomato plant with attached *C. pentagona* as used in this study. Tomato plants were first parasitized by *C. pentagona* seedlings when 10 days old (attachment point below cotyledons). The parasite vine was allowed to grow for 10 days and to attach again to the petiole of the second expanded leaf of the now-20-day-old tomato. Five days later, the leaf of the parasitized petiole (indicated with arrow) of the 25-day-old plant received caterpillar feeding for volatile collection or phytohormone analysis.
Figure 2. Time-course of changes in jasmonic acid (A) and salicylic acid (B) in unparasitized tomato plants and plants parasitized by *C. pentagona* in response to BAW feeding. Parasitized and unparasitized plants that did not receive insect feeding served as controls. Note breaks in the x-axis (A and B) and the y-axis (A). Data show the mean and SE of untransformed values from six replicates. Different letters indicate significance differences within each timepoint (*P* < 0.05); “n.s.” = no significance.
Figure 3. Total volatile production (mean ± SE) by unparasitized tomato plants and plants parasitized by *C. pentagona* on days 1-3 of BAW feeding. Parasitized and unparasitized plants that did not receive insect feeding served as controls. Data show untransformed values from six replicates. Different letters indicate significance differences within each day (*P* < 0.05).
Figure 4. Amounts (mean ± SE) of α-pinene, 2-carene, β-phellandrene, limonene, and one unidentified monoterpane produced by unparasitized tomato plants and plants parasitized by *C. pentagona* on day 3 of BAW feeding. Parasitized and unparasitized plants that did not receive insect feeding served as controls. These five volatile compounds are induced by BAW feeding on unparasitized plants. Data show untransformed values from six replicates. Different letters indicate significance differences between treatments (*P* < 0.05).
Figure 5. Relative growth rate of BAW on unparasitized tomato and tomato parasitized by *C. pentagona*. Data show mean and SE of untransformed values from 15 replicates. Different letters indicate significance differences between treatments (*P* < 0.05).
Figure 6. Comparison of salicylic acid (A) and jasmonic acid (B) in unparasitized NahG and wild type tomato plants and plants parasitized by C. pentagona after 24 h of BAW feeding. Data represent mean and SE of untransformed values from six replicates. An asterisk (*) denotes significant differences in SA or JA between NahG and wild type plants within treatments ($P < 0.05$).
Figure 7. Total volatile production (mean ± SE) by unparasitized tomato plants and plants parasitized by *C. pentagona* on days 1-3 after treatment with JA. Data show untransformed values from six replicates. Different uppercase letters indicate significance differences among days for unparasitized plants; lowercase letters indicate significance differences among days for parasitized plants (*P* < 0.05); *P*-values indicate differences between unparasitized and parasitized plants within days.
Parasitism by *Cuscuta pentagona* sequentially induces effective
JA and SA defence pathways in tomato

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Summary

Induced plant responses to herbivores and pathogens have been studied extensively but the perception and response of plants to attack by other plants remains largely unknown. Here we profile changes in several phytohormones and C18 fatty acids in tomato during attachment by the parasitic plant *Cuscuta pentagona* (field dodder). Newly germinated *C. pentagona* seedlings largely avoided activation of host defences upon first attachment to 10-day-old tomato seedlings: small increases in cis-jasmonic acid (JA) and auxin were the only observed responses. In contrast, a second attachment by the growing *C. pentagona* vine to the tomato 10 days later elicited a 2.5-fold increase in trans-JA, a 60-fold increase in cis-JA, and a 30-fold increase in salicylic acid (SA), followed by a hypersensitive response. Parasites grew significantly larger on transgenic tomato plants deficient in SA (*NahG*) or mutants insensitive to JA (*jai1*) compared to wild type plants, suggesting that both SA and JA mediate effective defences against *C. pentagona*. Moreover, maximum amounts of JA peaked 12 h before SA, indicating that tomato may regulate defences against *Cuscuta* via the sequential action of these hormones. *Cuscuta*-infestation also induced significant increases in free linolenic- and linoleic acids, and abscisic acid. These findings provide the first direct documentation of hormonal defence signalling induced by a parasitic plant and show that host plant defences against attack by *Cuscuta* display characteristics of both herbivore- and pathogen-induced responses.
Introduction

The defences induced in plants to attack by pathogens and herbivorous arthropods are known to result from complex defence signalling networks regulated by the plant hormones salicylic acid (SA) and jasmonic acid (JA) (see recent reviews by Wasternack et al., 2006; Glazebrook, 2005; Schilmiller and Howe, 2005; Durrant and Dong, 2004). In response to pathogens, SA activates and regulates a hypersensitive response (HR) and the synthesis of an array of antimicrobial phytoalexins and pathogenesis-related (PR) proteins that results in systemic acquired resistance (SAR) (Durrant and Dong, 2004). The JA pathway plays a major role in herbivore-induced responses and mediates the production of metabolites that reduce insect growth (Chen et al., 2005, 2007) and of plant volatiles that attract natural enemies (Turlings et al., 1990; De Moraes et al., 1998) and repel foraging herbivores (De Moraes et al., 2001; Kessler and Baldwin, 2001). Much less is known about the defences induced in host plants by parasitic plant attack. This is surprising since parasitic plants represent some of the world’s most destructive agricultural pests (Parker and Riches, 1993; Musselman et al., 2001) and have significant impacts on the dynamics of the ecosystems in which they occur (Press and Phoenix, 2005; Bardgett et al., 2006).

A common feature of parasitic plants is the haustorium, the organ that penetrates host tissues and fuses with the host vascular system to withdraw water and nutrients. The widespread and recognizable genus Cuscuta L. (Convolvulaceae) is one of the most ecologically and economically significant groups of parasitic plants (Kuijt, 1969).
*Cuscuta* spp. (dodders) are yellow-to-orange vines that lack roots or expanded leaves and require aboveground attachment to other plants to survive and reproduce (Dawson *et al.*, 1994). *Cuscuta* generally lack obvious chlorophyll but have retained the genes necessary for photosynthesis, probably to synthesize lipids for allocation to seeds rather than carbohydrate production (McNeal *et al.*, 2007a, 2007b). Upon germination, *Cuscuta* seedlings are dependent on energy reserves in seeds to grow and forage for hosts. We recently showed that seedlings of *C. pentagona* exploit plant volatiles to locate and select hosts (Runyon *et al.*, 2006). Once a host is located, *Cuscuta* vines twine around the host stem or petiole and development of haustoria begin when *Cuscuta* epidermal cells enlarge and secrete glue-like substances containing primarily de-esterified pectins that adhere the parasite to the host (Vaughn, 2002). Recently it was shown that *Cuscuta reflexa* attachment induces the tomato host to synthesize an arabinogalactan protein which promotes parasite adherence (Albert *et al.*, 2006). Following attachment, haustorial cells elongate and penetrate the host tissue using both enzymes and mechanical pressure (Nagar *et al.*, 1984) and individual cells of the haustoria elongate into searching hyphae which connect with vascular bundles of the host (Birschwilks *et al.*, 2006, 2007). Upon successful formation of vascular connections with the host, *Cuscuta* becomes a powerful sink withdrawing water, sugars, amino acids, and other nutrients from host to parasite (Dawson *et al.*, 1994; Birschwilks *et al.*, 2007).

Reported host plant responses to *Cuscuta* include a HR and phytoalexin production by a non-host tropical liana in response to *C. reflexa* (Bringmann *et al.*, 1999), and the expression of a pathogenesis-related gene by *Cuscuta*-infested alfalfa (Borsics and
Lados, 2002). The responses of resistant tomato varieties to *C. reflexa* represent the best studied host plant defences against *Cuscuta*. The growth of *C. reflexa* haustoria into the host is prevented by a mechanical barrier resulting from elongation of hypodermal host cells, a HR, and accumulation of phenolics and peroxidases at the attachment site (Ihl et al., 1988; Sahm et al., 1995). Experimental removal of this dead cell layer allowed the formation of functional haustoria and parasite growth (Ihl et al., 1988). Recent molecular work has shown that two aquaporin genes (*LeAqp2, TRAMP*) and a cell wall-modifying enzyme (*LeXTH1*) are expressed in tomato during unsuccessful *C. reflexa* attack, but their roles in defence remain uncertain (Werner et al., 2001; Albert et al., 2004). We recently reported a HR at the attachment site, and elevated amounts of SA in leaves of *C. pentagona*-infested tomato plants (Runyon et al., 2008).

Host defences against parasitic plants whose haustoria attach belowground to host roots are also poorly known and existing studies have failed to provide a clear picture. For example, application of BTH, a functional analogue of SA, promoted resistance of several hosts including tobacco to *Orobache* spp. (broomrapes) (Sauerborn et al., 2002; Gonsior et al., 2004; Pérez-de-Luque et al., 2004; Kusumoto et al., 2007). But *O. aegyptiaca* parasitism of tobacco did not induce expression of *PR-1a*, a marker of the SA pathway and SAR (Griffitts et al., 2004). Reported changes in *Arabidopsis thaliana* gene expression in response to *O. ramosa* include several genes regulated by JA, but not SA-dependent genes (Vieira Dos Santos et al., 2003a, 2003b). However, treatment with JA analogues did not affect resistance of red clover to *O. minor* (Kusumoto et al., 2007).
Vieira Dos Santos et al. (2003a) did report that several genes known to be involved in *Arabidopsis* responses to pathogen attack were induced by *O. ramosa*.

These studies provide insights into host plant responses to parasitism, but the specific phytohormone(s) induced in defence of parasitic plants remains unknown. In this study, we investigated the induced defences of a susceptible tomato variety to attack by *C. pentagona* by tracking changes in JA, SA, abscisic acid (ABA), auxin (indole-3-acetic acid; IAA), and free linoleic- and linolenic acids during the first six days of parasitism. This was done for the first attachment of *C. pentagona* seedlings to 10-day-old tomato plants in which no HR results, and for the second attachment 10 days later to the tomato leaf petiole which results in a strong HR. Lastly, to assess the effectiveness JA and SA defences we determined the performance of *C. pentagona* on several tomato signalling mutants.

**Results**

*C. pentagona* seedlings largely avoid activating defences in 10-day-old tomato

We first investigated the parasitism of 10-day-old tomato plants by newly germinated *C. pentagona* seedlings. This first attachment did not affect concentrations of SA, total JA, ABA, free linolenic- or linoleic acids (Figure 1). However, although there was no difference in total JA between parasitized and unparasitized plants, separation of the two
JA isomers revealed that *C. pentagona* seedlings did induce a small increase in *cis*-JA 36 hr (*P* = 0.0070) and 48 hr (*P* = 0.0474) after development of haustoria began (Figure 2). The only other documented parasite-induced change in tomato seedlings was an increase in amounts of auxin at 48 hr (*P* = 0.0032). Parasitism by *C. pentagona* seedlings did not induce a HR in 10-day-old tomato plants. Interestingly, in both time courses, amounts of SA in undamaged tomato plants displayed a cyclical diurnal pattern with SA concentrations generally lower early in the day (time points 0, 24, 48, 120 sampled approximately 9AM) and higher late in the day (points 12, 36, 60 sampled approximately 9PM) (Figures 1 and 3a).

*A second attachment by C. pentagona induces SA, JA, ABA, and free fatty acids*

We next investigated hormonal changes induced in the tomato host when the growing *C. pentagona* vine reattaches 10 days later. In contrast to the first attachment, the second attachment to a leaf petiole of 20-day-old tomato plants elicited significant increases in the defensive phytohormones SA and JA, as well as ABA and free fatty acid content (Figures 3 and 4), followed by a strong HR (Figure 5a). Amounts of SA began to increase 24 hr after the first observed growth of haustoria, reached a maximum at 48 hr, and remained significantly elevated five days after infection began (Figure 3a). In response to parasitism, total JA increased rapidly between 24 and 36 hr after initiation of haustoria growth, and decreased to control levels by 60 hr (Figure 3b). The change in
total JA was primarily the result of an increase in cis-JA which remained significantly higher five days after attachment began (Figure 6; $P = 0.0142$). This maximum induction of cis-JA at 36 hr mirrors that seen in 10-day-old plants in response to C. *pentagona* seedlings (Figure 2). Interestingly, maximum production of JA by parasitized plants occurred 12 hr before maximum production of SA (Figure 3). In addition to SA and JA, amounts of ABA were greater in parasitized plants at 36 hours post-infection and accumulated over time (Figure 3c). Unlike the first attachment, amounts of auxin did not increase in the tomato petiole during the second attachment (Figure 3c). Parasitism by *C. pentagona* also significantly increased free fatty acid content in the host (Figure 4). In general, amounts of free linoleic and linolenic acids in affected tomato petioles began to increase around 36 hr and remained elevated through 120 hr (Figure 4). The first indications of a HR (indicated by collapsed, but not darkened epidermal cells) appeared about three days after haustoria began to enlarge, subsequent to peak increases in JA and SA (Figure 3). Lastly, the petioles of 20-day-old tomato plants with only the first parasite attachment, which were sampled as an additional control, generally did not differ from petioles of unparasitized plants, except in containing less auxin at two time points (Figure 3).

*C. pentagona*-induced JA and SA mediate effective defences

To assess the impact of JA and SA defences on *C. pentagona*, we determined the biomass of parasites grown on several tomato mutants with altered JA and SA signalling,
(1) 10 days after the first attachment to 10-day-old tomato seedlings, and (2) 10 days after the second attachment to the tomato leaf petiole. A similar trend was seen for the biomass of the host plants and *C. pentagona* 10 days after the first attachment to both JA- and SA altered plants (Figure 7). Biomass of *C. pentagona* grown on wild type and SA-deficient *NahG* plants was not different (Figure 7a; \( P = 0.8478 \)), nor did biomass of parasites grown on wild type, JA-insensitive, or JA enhanced plants differ (Figure 7b; \( P = 0.0661 \)). However, mean parasite biomass on JA-insensitive plants, though no significant, did tend to be greater (Figure 7b). Although mean biomass of parasitized tomato plants from all treatments tended to be less than uparasitized plants after only 10 days of parasitism, these differences were not significant (Figure 7; \( P > 0.05 \)).

We next determined *C. pentagona* and host plant biomass 10 days after the second attachment. Here, parasite biomass was significantly greater on both SA-deficient *NahG* and JA-insensitive plants (Figure 8). Parasites grown on SA-deficient plants were about 40% larger than those grown on the wild type (Figure 8a; mean ± SE biomass: 0.115 ± 0.01 and 0.073 ± 0.01, respectively; \( P = 0.0331 \)). Biomass was also different for parasites grown on the JA altered and wild type plants (Figure 8b; \( P < 0.0001 \)). Parasitic plants from JA-insensitive plants (*jai1*) were more than twice the size of those grown on wild type plants with JA defences intact (Figure 8b; mean ± SE biomass: 0.310 ± 0.02 and 0.154 ± 0.02, respectively). Mean parasite biomass was reduced on plants with the JA pathway constitutively activated (35S::prosys) compared to the wild type, but the effect was not statistically significant (Figure 8b; mean ± SE biomass: 0.125 ± 0.02 and 0.154 ± 0.02, respectively; \( P > 0.05 \)). Ten days after the second attachment, *C.*
parasitism had significantly reduced the biomass of all tomato hosts (Figure 8; $P < 0.001$). Moreover, for the JA altered plants, biomass of parasitized wild type plants was larger than that of jai1 or 35S::prosys plants (Figure 8b). However, it should be noted that 35S::prosys plants are known to display a stunted phenotype, likely due to the constitutive production of large amounts of JA-inducible defensive proteins (McGurl et al., 1994).

Notably, in contrast to the wild type, NahG plants did not develop a HR after the second attachment by C. pentagona (Figure 5). Also, although a strong HR was evident in all parasitized JA wild type and 35S::prosys plants, JA-insensitive plants varied in the ability to produce a HR in response to the second attachment. For example, a strong HR was produced only 40% of the time, a partial HR (cell necrosis present only at part of the attachment) 20% of the time, and no HR the other 40% ($N = 10$).

**Discussion**

Despite their ecological and economic significance as plant-feeding organisms, the defences induced by parasitic plant attack remain largely unknown. In this study, we used a metabolomic profiling technique (vapour phase extraction) to measure the changes in phytohormones that occur within tomato plants during parasitism by C. pentagona.

Our results show that parasite seedlings largely avoided eliciting host defences when attaching to 10-day-old tomato seedlings (Figure 1), whereas a second attachment by the
growing parasite vine 10 days later induced large increases in several plant hormones and a strong HR (Figures 3 and 4a). We also assessed the effectiveness of SA- and JA-mediated defences using transgenic and mutant plants. These methods give the first clear picture of the composition and timing of hormonal signalling induced in defence of a parasitic plant.

First attachment by C. pentagona seedlings

The only changes induced by the first parasite attachment to 10-day-old tomato plants were small increases in cis-JA and auxin (Figures 1 and 2). This stands in sharp contrast to the large responses elicited during the second attachment (Figures 3-6), and suggest that C. pentagona seedlings were able to avoid or suppress the activation of host defences. Alternatively, it is possible that young tomato seedlings may simply be unable to fully respond to parasitism. However, cotyledon stage tomato plants (similar to the size used in this study) are able to produce large amounts of SA and a HR in response to fungal pathogens (Hammond-Kosack et al., 1996). Observations show that parasite seedlings do frequently induce a HR upon first attachment to 20-day-old tomato plants (Supplemental Figure S1), suggesting that older plants are better able to respond, or that effectiveness of the purported mechanisms by which C. pentagona subvert defences is dependent on the size of the host plant.

The increased mean biomass of C. pentagona on JA-insensitive plants (Figure 7b), though not significant, suggests that defences mediated by the small increase in cis-JA
may be negatively affecting early growth of the parasite. Increases in auxin have been reported for another *Cuscuta*-tomato interaction (Löffler *et al.*, 1999). In that study, cell elongation at the attachment site in both *C. reflexa* and tomato was accompanied by increased concentrations of auxin.

*Second attachment by C. pentagona to tomato petioles*

The second attachment by *C. pentagona* induced large increases in the defensive phytohormones SA and JA and a subsequent HR (Figures 3a, b and 5a). It is well established that SA responses protect plants against many pathogens, including fungi and bacteria (Durrant and Dong, 2004) and that JA responses provide resistance to chewing and sucking herbivores (Walling, 2000). We found that *C. pentagona* grew larger on JA insensitive or SA deficient tomato hosts (Figure 8) indicating that SA and JA responses are both effective against a parasitic plant. The defensive compounds that accumulate in response to pathogen-induced SA and herbivore-induced JA have been characterized (Walling, 2000; Durrant and Dong, 2004), but the mechanisms by which these could operate to reduce *C. pentagona* growth are unknown. However, SA plays a crucial role in the production of a HR (Alvarez, 2000), which is an effective defence against *C. reflexa* (Ihl *et al.*, 1988). The absence of a HR in parasitized *NahG* plants (Figure 5b) supports an essential role for SA in HR development in this interaction. Interestingly, parasite-induced HR was present in JA wild type and 35S::*prosys* plants, whereas HR
development was highly variable in *jail* plants, suggesting that JA also contributes to this response. JA has been shown to accumulate in pathogen-induced HR lesions in tobacco (Kenton *et al.*, 1999). It is noteworthy that *C. pentagona* induced predominantly cis-JA (Figure 6), which is believed to be the more biologically active of the two naturally occurring forms (Beale and Ward, 1998). *cis*-JA is also synthesized in response to insect feeding and application of microbial or insect elicitors (Blechert *et al.*, 1995; Engelberth *et al.*, 2007). This increase in JA provides a plausible explanation for our recent report of increased volatile production by *C. pentagona*-parasitized tomato plants and the reduced caterpillar growth on parasitized tomato leaves (Runyon *et al.*, 2008).

The SA- and JA-dependent pathways are known to be antagonistic and their simultaneous activation can inhibit defence responses. For example, it is well established that SA can inhibit the synthesis and action of JA (Peña-Cortés *et al.*, 1993; Doares *et al.*, 1995; Spoel *et al.*, 2003). This cross-talk can allow plants to minimize activation of ineffective defences in favour of operative ones (Rayapuram and Baldwin, 2007), or may permit fine-tuning of defences using a combination of both signalling molecules (Reymond and Farmer, 1998). Interestingly, our data show that the production of JA by tomato plants in response to parasitism precedes subsequent accumulation of SA (Figure 3a, b). Because JA and SA appear to independently mediate effective defences (Figure 8), this would suggest that tomato plants actively coordinate the synthesis of these hormones to enact a defensive phenotype containing components of both pathways. This may be similar to the sequential use of hormones by tomato against the bacterium
*Xanthomonas campestris* pv *vesicatoria*, in which jasmonate production must precede that of SA to reduce bacterial growth (O’Donnell *et al.*, 2003).

Amounts of free linolenic- and linoleic acids increased in tomato following parasitism by *C. pentagona* (Figure 4). Free linolenic acid and linoleic acid are known to increase in response to tissue damage by wounding or insect attack, and their oxidation is an early step in the biosynthesis of JA (Conconi *et al.*, 1996; Schilmiller and Howe, 2005). However, free fatty acids amounts continue to increase well beyond peak JA synthesis at 36 hr (Figures 3 and 6), suggesting their increases may some other function(s). For example, increases in free linolenic- and linoleic acids and their subsequent oxidation could play a role in the development of a HR (Rustérucci *et al.*, 1999).

The second attachment by *C. pentagona* also induced increases in ABA (Figure 3c). ABA accumulates in plants under drought stress (Seki *et al.*, 2007), and tomato plants may produce ABA in response to water removal by the parasite. Parasite vines began to grow from the attachment between 36-48 hr, which corresponds with increases in ABA (Figure 3c). However, growth of *C. pentagona* from the first attachment at about the same time did not induce ABA in young tomato seedlings (Figure 1). In general, hormone content of *C. pentagona* vines did not differ greatly from that of undamaged tomato plants (amounts in *C. pentagona* provided in Supplementary Table S1), except that parasite vines growing from the second attachment contained noticeably more ABA (mean ± SE ng g⁻¹ ABA: 1929 ± 809 for vines and 340 ± 17 for tomato). A similar distribution of ABA occurs between the unrelated parasitic *Striga hermonthica* and maize.
(Taylor et al., 1996). The function of increased ABA in the parasite is unknown, but may increase the flow of water and nutrients to the parasite (Taylor et al., 1996). Alternatively, some evidence suggests ABA could function as a signal in the activation or modification of defences (Adie et al., 2007; Bodenhausen and Reymond, 2007).

Lastly, in this compatible C. pentatogona-tomato interaction, most tomato responses occurred after the parasite began to grow from the host (between 36 and 48 hr). It would be interesting to compare the timing and extent of tomato responses to C. pentagona with those to C. reflexa, in which tomato plants successfully prevent parasitism by blocking initial haustorial growth using a HR. Perhaps, like plant interactions with pathogens, the speed of perception and response to parasitic plants can determine resistance or susceptibility.

In summary, seedlings of C. pentagona generally avoided the activation of host defences upon first attachment to tomato seedlings. Tomato plants responded to a second attachment by activating the JA and SA signalling pathways, both of which appear to mediate effective defences that reduce parasite growth. Moreover, our results suggest that by varying the timing of JA and SA synthesis, parasitized plants may achieve a defensive response containing elements of both pathways. Parasitism also induced increases ABA and free fatty acid content of the host, but their roles in defence are uncertain. We conclude that similar to herbivore and pathogen attack, plants are able to perceive invasion by parasitic plant haustoria and respond by activating induced defences.
Experimental procedures

Plant material and growth conditions

Seeds of *Cuscuta pentagona* were collected from an infested tomato field in Yolo County, California. Seeds were soaked in concentrated sulphuric acid for 1 h using a Gooch crucible, rinsed for 1 min with distilled water, and placed in a Petri dish on moist filter paper to germinate. Tomato plants (*Solanum lycopersicum*) ‘Halley 3155’ including JA signalling mutants (*jai1, 35S::prosys*, and wild type ‘Castlemart’) were grown in an insect-free growth chamber (25°C, 16-h photoperiod at 250 μmol m⁻² s⁻¹ provided by cool white fluorescent tubes) in 9 cm tall x 10 cm wide square plastic pots filled with a peat-based general-purpose potting soil with fertilizer (Osmocote; The Scotts Company). The SA-deficient *NahG* and wild type ‘MoneyMaker’ tomato plants were grown similarly, except that they received low light intensity (75 μmol m⁻² s⁻¹) to prevent development of necrotic leaf spots.

*C. pentagona* attachment and growth on tomato

Newly germinated *C. pentagona* seedlings, approximately 4 cm long, were allowed to attach to 10-day-old tomato seedlings (first true leaves just beginning to expand) by leaning the *C. pentagona* seedling against the right side of the tomato meristem (*Cuscuta* are left-handed and coil from right to left). Because far-red light promotes tight coiling
of *Cuscuta* spp. (Haidar and Orr, 1999), two incandescent 75W bulbs (75A/CL/DL/RP 120V, Orsam Sylvania) per 15 pots in 30 cm x 50 cm flats were placed 1 m above plants and left on for 24 h (off for 8 h of scotophase). Using this setup, *C. pentagona* seedlings coiled tightly around the tomato seedlings within 6 h and haustorial swellings at points of contact with the host were evident within 24 h (Supplemental Figure S2). Control plants received the same treatment, and plants exposed to incandescent light for this short period showed no noticeable physiological effects. Ten days later, the growing *Cuscuta* vine was allowed to attach a second time to the petiole of the second expanded true leaf (the youngest expanded leaf) of the same now-20-day-old tomato host. This was done by repeating brief exposure to incandescent light (as above). After 1-2 hr of exposure the apex of the parasite vines would typically coil and the site of attachment was controlled by carefully placing the coiled vine around the tomato petiole.

*Time-course of C. pentagona attachment*

A time course of phytohormone and free fatty acid changes in tomato was conducted for the first attachment of *C. pentagona* seedlings to 10-day-old tomato plants and for the second attachment to the tomato leaf petiole 10 days later. These points were chosen because we observed that the first attachment does not induce a HR whereas the second attachment elicits a strong HR. Moreover, in nature once *Cuscuta* seedlings successfully parasitize and grow from a host, they frequently reattach to this host (Runyon, personal observations). The entire tomato seedling was sampled for the first attachment time course. For the second attachment, approximately 100 mg of the tomato petiole
incorporating the *C. pentagona* attachment sites were sampled and the petiole of a tomato
with only the first parasite attachment was sampled as an additional control. In both
cases, the parasite vine including haustoria were removed from the host and immediately
snap-frozen in liquid nitrogen in FastPrep tubes (Q-BIOgene) with 1 g of Zirmil beads
(1.1 mm; Saint-Gobain ZirPro), weighed, and held at -80º C until processed. The time
courses consisted of nine sampling points as follows: (1) time 0; (2) parasite tightly
wrapped, 5-6 hr after time 0; (3) first signs of haustoria swelling corresponding to the
elongation of *Cuscuta* epidermal cells and adherence of parasite to host, approximately
24 hr after time 0; (4-9) 12, 24, 36, 48, 60, and 120 hr after first signs of haustoria
development.

*Extraction and quantification of phytohormones and fatty acids*

We used vapor phase extraction to extract and measure phytohormones and fatty acids
following the method of Schmelz et al. (2003, 2004). Briefly, plant tissue was
homogenized using Zirmil beads in a FastPrep® shaker or ground to a fine powder in
liquid nitrogen (petioles) prior to using Zirmil beads, and the phytohormones were
partitioned into an organic layer (dichloromethane), transferred to a 4 ml glass vial, and
derivatized from carboxylic acids to methyl esters using trimethylsilyldiazomethane
(Sigma-Aldrich, St. Louis, MO). The solvent was evaporated under an air stream, and
the dry vial was heated to 200º C for 2 min to expedite volatilization of analytes which
were collected at this time from the headspace using volatile traps containing 30 mg of
Super-Q® (Alltech, Deerfield, IL) attached to a vacuum (1 l/min). The phytohormones were eluted from the traps using 150 µl of dichloromethane and analyzed by gas chromatography-mass spectrometry with isobutane chemical ionization with select-ion monitoring (settings described by Schmelz et al., 2004). Amounts of JA, SA, ABA, IAA, and free linoleic- and linolenic acids were quantified using these internal standards (100 ng) added prior to homogenization with beads: $^2\text{H}_6\text{SA}$, dhJA, $^2\text{H}_6\text{ABA}$, $^2\text{H}_5\text{IAA}$, and gamma-linolenic acid. Gamma-linolenic was used to quantify linolenic and linoleic acids. Metabolites were analyzed on a per-gram-fresh-weight basis and were natural log (ln) transformed to meet variance assumptions. Comparisons were made among treatments for each sampling period in the time-courses using one-way analysis of variance (ANOVA); individual means were compared with Tukey’s honestly significantly different (HSD) means separation test. All statistics were done using SAS (version 8.2; SAS Institute).

Performance of C. pentagona on JA and SA signalling mutants

The biomass of C. pentagona on JA and SA tomato mutants was determined 10 days after the first attachment of seedlings to 10-day-old tomatoes, and 10 days after the second attachment to the 20-day-old tomato leaf petiole. At these times, all of the C. pentagona vine and the aboveground tomato shoot was dried in an oven at 55º C for 72 h and weighed. Cuscuta biomass is known to be positively correlated with fitness (Koskela et al., 2001). To determine effectiveness of JA defences, we used jasmonic acid-
insensitive1 (jai1) tomato mutants which have lost the function of the tomato homolog of  
CORONATINE-INSENSITIVE1, fail to express JA-responsive genes, and have severely  
compromised resistance to herbivores (Li et al., 2004). We also used tomatoes  
transformed to overexpress prosystemin (35S::prosys), a positive regulator of the JA  
pathway, which exhibit increased resistance to herbivores (Chen et al., 2005). To assess  
SA defences, we used transgenic NahG plants expressing the enzyme salicylate  
hydroxylase, which converts SA immediately to inactive catechol, and are deficient in  
accumulation SA (Brading et al., 2000). Comparisons of biomass were done using one-  
way ANOVA and individual means were compared with Tukey’s HSD means separation  
test.

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35S::prosys seeds, J. Zhu for help with statistics, and J. Tooker, J. Saunders, and E. Bogus  
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References


Figure 1. Time course of changes in salicylic acid (SA), total jasmonic acid (JA), abscisic acid (ABA), auxin (IAA), free linoleic acid (18:2 FA), and free linolenic acid (18:3 FA) in 10-day-old tomato plants (mean ± SE, n = 6) during first attachment by Cuscuta pentagona seedlings. Compounds are marked by lines and abbreviations, white symbols represent parasitized plants and dark symbols represent unparasitized controls. Significant difference: * $P < 0.05$. 
Figure 2. Time course of changes in the cis- and trans-isomers of jasmonic acid (JA) in 10-day-old tomato plants (mean ± SE, n = 6) during first attachment by Cuscuta pentagona seedlings. Compounds are marked by lines and abbreviations, white symbols represent parasitized plants and dark symbols represent unparasitized controls. Significant differences: * $P < 0.05$. 
Figure 3. Time course of changes in the phytohormones (a) salicylic acid (SA), (b) total jasmonic acid (JA), and (c) abscisic acid (ABA) and auxin (IAA) in 20-day-old tomato petioles (mean ± SE, n = 6) during second attachment by *Cuscuta pentagona* seedlings. ABA and IAA are marked by lines and abbreviations. White circles represent parasitized petioles, dark circles represent unparasitized control petioles, and dark triangles represent unparasitized petioles of plants with the first parasite attachment. Significant differences between treatments: *P < 0.05, **P < 0.001, ***P < 0.0001.
Figure 4. Time course of changes free fatty acids, (a) linoleic (18:2 FA) and (b) linolenic (18:3 FA) in 20-day-old tomato petioles (mean ± SE, n = 6) during second attachment by Cuscuta pentagona seedlings. White circles represent parasitized petioles, dark circles represent unparasitized control petioles, and dark triangles represent unparasitized petioles of plants with the first parasite attachment. Significant differences between treatments: *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$. 
Figure 5. Salicylic acid-deficient tomato plants (NahG) do not develop a HR in response to parasitism by C. pentagona. Images are of wild type (WT) and NahG petioles 12 days after second attachment by the C. pentagona vine.
Figure 6. Time course of changes in the cis- and trans-isomers of jasmonic acid (JA) in 20-day-old tomato petioles (mean ± SE, n = 6) during second attachment by Cuscuta pentagona seedlings. White circles represent parasitized petioles, dark circles represent unparasitized control petioles, and dark triangles represent unparasitized petioles of plants with the first parasite attachment. Significant differences between treatments: *P < 0.05, ***P < 0.0001.
Figure 7. Biomass of *C. pentagona* 10 days after first attachment to tomato plants altered in (a) SA and (b) JA defence signalling (mean ± SE dry weight, n = 10). *NahG* plants are SA-deficient, *jai1* are JA-insensitive, and *35S::prosys* constitutively express the JA pathway. n.s. = no significance between treatments ($P > 0.05$).
Figure 8. Biomass of *C. pentagona* 10 days after second attachment to tomato plants altered in (a) SA and (b) JA defence signalling (mean ± SE dry weight, n = 10). *NahG* plants are SA-deficient, *jai1* are JA-insensitive, and 35S::prosys constitutively express the JA pathway. Different letters indicate significant differences within treatments (*P* < 0.05), n.s. = no significance between treatments.
**Figure S1.** The first attachment by *C. pentagona* seedlings to 20-day-old tomato plants typically elicit a HR.
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**Table S1.** Amounts of SA, JA, ABA, IAA, and free linolenic- and linoleic acids in *C. pentagona* seedlings and *C. pentagona* vines growing from the first attachment to 10-day-old tomato seedlings and from the second attachment to 20-day-old tomato petioles.
Parasitic plants in agriculture: chemical ecology of germination and host-plant location as targets for sustainable control

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Abstract – Parasitic plants are among the most problematic pests of agricultural crops worldwide. Effective means of control are generally lacking, in part because of the close physiological connection between the established parasite and host plant hindering efficient control using traditional methods. Seed germination and host location are critical early growth stages that occur prior to host attachment and provide promising targets for ecologically sound management of parasitic weeds. Knowledge of parasite-host interactions, particularly chemical cues that induce parasite seed germination and mediate host location should facilitate development of novel management approaches. In parasitic plants that attach to host roots (e.g. *Striga* and *Orobanche* spp.) seed germination is known to occur only in the presence of chemical stimulants released from plant roots. The recent finding that these same chemicals promote colonization of beneficial fungi has potentially important implications for control of parasitic plants. Far less is known about the early stages of parasitic plants that attach aboveground to host shoots (e.g. *Cuscuta* spp.). Seeds of these parasites lack germination stimulants, and it was only recently shown that foraging *C. pentagona* seedlings use airborne cues to locate and select among hosts. We review research on seed germination and host location by the major parasitic weeds that attack agricultural crops, and discuss the implications of recent findings for the development of sustainable and effective management strategies.
1. INTRODUCTION

Approximately 4500 species of flowering plants (more than 1%) are parasitic, obtaining some or all of their water and nutrients from other plants (Kuijt, 1969; Nickrent, 2007). A small percentage of these parasitic species infest agricultural crops and cause serious problems for farmers in many parts of the world (Parker and Riches, 1993; Musselman et al., 2001). Few practical and economically sound methods are available for controlling parasitic plant species (Gressel et al., 2004; Rispail et al., 2007), in part because their physiological connection to host plants limits the usefulness of most herbicides. Parasitic weeds can also be difficult to eradicate because they often produce large numbers of long-lived seeds. For example, a single *Orobanche* sp. plant can produce over 200,000 dust-like seeds that remain viable for 8-10 years (Parker and Riches, 1993). In addition, parasitic plants that attack host roots can inflict serious damage to crop plants before the latter emerge from the soil, making it difficult to diagnose infestations before economic losses occur.

Breeding for host-plant resistance offers a potentially economical approach to controlling parasitic plants. However, with a few exceptions (e.g. resistance of cowpea to *Striga*; Lane et al., 1993) breeding programs have not provided effective control and are challenging because plant resistance traits are often poorly characterized, genetically complex, and of low heritability (Rispail et al., 2007). Genetic engineering might help to overcome some of these difficulties (Bouwmeester et al., 2003), but societal concerns
about genetically modified technology may prevent widespread adoption (Humphrey et al., 2006).

The search for improved or alternative approaches to controlling parasitic plants in agriculture will be facilitated by increased understanding of the complex ecological and physiological interactions between parasitic plants and their hosts. Host location is a critical part of the life cycle of the most damaging parasitic weeds, which are obligate parasites that depend on the limited reserves available in seeds to quickly locate suitable hosts. Host location thus seems a promising target for control strategies. In this paper, we review the most important plant parasites of agricultural crops, focusing on the chemical ecology of seed germination and host location, and discuss the potential for manipulating these mechanisms to control these important weeds.

2. THE MAJOR PARASITIC PLANTS IN AGRICULTURE

Parasitism originated independently several times during angiosperm evolution, and the lifestyles of parasitic plants vary greatly across taxa (Kuijt, 1969; Nickrent et al., 1998). Some species are facultative parasites able to survive in the absence of hosts while others are obligately parasitic and cannot develop independently. A distinction can be drawn between hemiparasitic plants which possess chlorophyll and are able to produce some of their required nutrients through photosynthesis and holoparasitic plants which lack chlorophyll and are completely dependent on host resources, but this distinction is
not always clear-cut (Parker and Riches, 1993; Press and Graves, 1995). A more definitive division can be drawn between parasitic plants that make belowground attachments to host-plant roots and those that attach aboveground to host-plant shoots (Fig. 1). This review will focus on the most economically important groups of plant parasites: witchweeds, *Striga* spp. (Scrophulariaceae), and broomrapes, *Orobanche* spp. (Orobanchaceae), which attach to host roots, and dodders, *Cuscuta* spp. (Convolvulaceae), which make above-ground attachments to host shoots (Parker, 1991).

*Striga* spp. are obligate root hemiparasites and infest an estimated two-thirds of the cereals and legumes in sub-Saharan Africa, causing annual crop losses estimated at US$7 billion annually and negatively influencing the lives of more than 300 million people (Berner et al., 1995; Musselman et al., 2001). Several species of *Striga* attack the major cereal crops in Africa (e.g. maize, sorghum, millet, rice), but *S. hermonthica* and *S. asiatica* are the most widely distributed and destructive (Oswald, 2005). *Striga gesnerioides* parasitizes broadleaf plants and is a serious threat to cowpea production in many parts of Africa (Parker and Riches, 1993). In the 1950s, *S. asiatica* was discovered parasitizing maize in the southeastern USA, but its spread there has been halted by an intensive eradication program (Parker, 1991).

*Orobanche* spp. are obligate root holoparasites which constrain production of many crops, primarily in the Mediterranean region, the Middle East, and northern Africa (Parker and Riches, 1993). Among the six *Orobanche* species considered serious pests, *O. ramosa* and *O. aegyptiaca* have the widest host ranges and heavily damage a variety of crops including tomato, potato, eggplant, faba bean, lentil, peanut, chickpea,
cucumber, and sunflower (Parker and Riches, 1993). *Orobanche cumana* has a host range limited to Asteraceae, and it is an important pest of cultivated sunflowers (Parker and Riches, 1993; Press and Graves, 1995). Infestation by *Orobanche* spp. can result in total crop loss (Bernhard et al., 1998).

*Cuscuta* spp. have yellow-to-orange, rootless, leafless vines that attach to shoots of host plants. They are obligate holoparasites, typically exhibiting broad host ranges, and inflict serious damage to many crops, including forage legumes (alfalfa, clover, lespedeza), potato, carrot, sugar beets, chickpea, onion, cranberry, blueberry, and citrus (Dawson et al., 1994). Seeds of *Cuscuta* spp. have been transported worldwide in contaminated shipments of crop plant seeds. *Cuscuta pentagona* is a major weed of tomato in California, causing yield losses of 50 to 75% (Goldwasser et al., 2001). In China, several *Cuscuta* species inflict severe damage on soybeans (Dawson et al., 1994).

### 3. PARASITIC PLANTS USE CHEMICAL CUES TO LOCATE HOSTS

Seeds of most parasitic plants contain few energy reserves that allow limited growth. Consequently, seedlings can survive only a few days after germination before attaching to a host. The imperative of finding hosts quickly presumably imposes strong evolutionary selection pressure favoring the development of efficient host-location mechanisms. Both root and shoot parasitic plants utilize chemical cues released by host plants for this purpose (Fig. 1).
3.1. Root parasitic plants: Germination stimulants

Seeds of *Striga* and *Orobanche* spp. germinate only in the presence of chemical compounds exuded from host roots (Fig. 1; Bouwmeester et al., 2007). Because these germination stimulants, collectively called strigolactones, are unstable and degrade rapidly in the soil, they occur at concentrations sufficient to induce germination only within a few millimeters of host roots (Fate et al., 1990). Concentration gradients of strigolactones may also facilitate directed growth of the parasite radicle toward the host root (Dubé and Olivier, 2001). The sensitivity of parasite seeds to these germination stimulants depends upon a conditioning period under warm and humid conditions and concomitant synthesis of gibberellins in seed tissues (Matusova et al., 2004). To date, several germination stimulants have been isolated and identified from root exudates of both host and non-host plants. In work with *Striga lutea*, the first germination stimulant, strigol, was isolated from root exudates of the non-host cotton (Cook et al., 1966). Strigol has since been found to be released by roots of true hosts including maize and millet (Siame et al., 1993). Additional strigolactone germination stimulants that have been identified include sorgolactone from sorghum, orobanchol and alectrol from red clover, and 5-deoxy-strigol from *Lotus japonicus* (Hauck et al., 1992, Yokota et al., 1998, Akiyama et al., 2005). Recently, strigolactones have been shown to be apocarotenoids produced by plants via the carotenoid pathway, rather than sesquiterpenoids as had previously been assumed (Matusova et al., 2005). The details of germination induction by strigolactones are not understood (Bouwmeester et al., 2007), though possible mechanisms have been proposed (Mangnus and Zwanenburg, 1992). Application of
ethylene can trigger seeds of *Striga* and *Orobanche* spp. to germinate, indicating that strigolactones may act by stimulating ethylene biosynthesis (Logan and Stewart, 1991). The recent discovery that strigolactones serve as important cues for plant-beneficial arbuscular mycorrhizal fungi (AMF; Akiyama et al., 2005; Besserer et al., 2006), suggests that parasitic plants may have co-opted these signals to recognize and locate host roots.

### 3.2. Shoot parasitic plants: Plant volatiles

In contrast to root parasitic plants, germination of *Cuscuta* spp. seeds is not dependent on stimulants derived from a host plant (Dawson et al., 1994). Rather seedlings must forage to locate potential hosts nearby. We recently reported that seedlings of *C. pentagona* use host plant volatiles to guide host location and selection (Fig. 1; Runyon et al., 2006). It had previously been suggested that *Cuscuta* spp. seedlings forage randomly (Dawson et al., 1994) or orient their growth to various light cues associated with the presence of host plants (Benvenuti et al., 2005). While light cues may play a role in host location, we found that *C. pentagona* seedlings exhibited directed growth toward tomato volatiles experimentally released in the absence of any other plant-derived cues. Moreover, seedlings used volatile cues to “choose” tomato, a preferred host, over the non-host wheat. Several individual compounds from the tomato volatile blend were attractive to *C. pentagona* seedlings, including α-pinene, β-myrcene, and β-phellandrene, while one compound from the wheat blend, *(Z)*-3-hexenyl acetate, had a repellent effect. We subsequently confirmed that *C. pentagona* seedlings respond
to volatiles from a range of host plants, including *Impatiens*, wheat (Runyon et al., 2006), and alfalfa (Mescher et al., 2006). These findings provide a plausible mechanism to explain previous reports of selective foraging by *Cuscuta* spp. (Kelly, 1990; 1992; Sanders et al., 1993; Koch et al., 2004). It is tempting to speculate that the remarkably similar but unrelated shoot-parasitic plants in the genus *Cassytha* (Lauraceae), and perhaps climbing vines in general, might also use volatile cues to locate their hosts, but this possibility has yet to be examined empirically.

4. CONTROL STRATEGIES TARGETING GERMINATION/HOST LOCATION

Considerable research has examined the possibility of exploiting germination stimulants for control of *Striga* and *Orobanche*. Control strategies include: (1) inducing “suicidal germination”, (2) inhibiting germination, and (3) reducing the production of germination stimulants by crop plants. In addition, the newly discovered role of strigolactones in the recruitment of symbiotic AMF (Akiyama et al., 2005) has opened new possibilities for modifying the production of germination stimulants by host plants. We are not aware of any studies exploring the possibility of disrupting host location by *Cuscuta* spp., which have no germination stimulants. However, the recently documented role of volatiles in host location by *C. pentagona* and the identification of several attractive and repellant compounds (Runyon et al., 2006), suggests that such strategies might be plausible.
4.1. Suicidal germination

Inducing germination of *Striga* and *Orobanche* spp. seeds in the absence of a suitable host plants results in “suicidal germination” and subsequent reduction in numbers of parasitic-plant seed in soil. Both man-made and natural compounds have been investigated for their ability to induce germination. Analogs of strigol have been synthesized (e.g., GR 24 and Nijmegen 1) and are potent elicitors of germination in both *Striga* and *Orobanche* spp. (Wigchert et al., 1999); however, their instability in soil (Barbiker et al., 1987) and the high cost of producing large quantities of these compounds have so far prohibited their use in agriculture (Humphrey et al., 2006). Ethylene has been a valuable component of the eradication program targeting *Striga asiatica* in the USA, where it induces about 90% germination when injected into the soil (Parker, 1991). However, fumigating soil with ethylene is likely to negatively influence AMF and other non-target soil microorganisms (Lendzemo et al., 2005). It has been proposed that ethylene-producing nonpathogenic bacteria could be used to induce suicidal germination of *Striga* (Berner et al., 1999), but a better understanding of bacteria/ethylene/crop interactions is needed before this method can be used in agriculture. Other natural compounds including fungal toxins (Evidente et al., 2006) and methyl jasmonate (Yoneyama et al., 1998) have been shown to induce germination of *Striga* and *Orobanche* spp. seeds, but their potential uses in agriculture remain largely unexplored.

Planting non-host trap crops that induce suicidal germination is perhaps the most effective strategy currently available for *Striga* control (Oswald, 2005). Recent studies in this area have focused on identifying and assessing the effectiveness of potential trap
crops (Gbêhounou and Adango, 2003; Lins et al., 2006; Fenández-Aparicio et al., 2007; Khan et al., 2007) and the possibility of breeding for increased production of germination stimulants (Botanga et al., 2003). Use of nitrogen-fixing legumes as trap crops has the added benefit of increasing soil fertility which can further assist in Striga control because Striga thrive in poor soils (Parker and Riches, 1993). The efficacy of legume rotations could potentially even be improved by inoculating crops with supplemental nitrogen-fixing rhizobia in combination with ethylene-producing bacteria to simultaneously increase suicidal germination and soil fertility (Ahonsi et al., 2003; Babalola et al., 2007).

Legumes have also proven useful as part of a novel “push-pull” (stimulo-deterrent) pest management approach that illustrates the utility of increased plant diversity, simultaneously reducing Striga and lepidopteran stemborer infestations (Kahn et al., 2000). Intercropping maize or sorghum with the leguminous trap crop Desmodium spp. decreases parasitism by Striga spp. and repels ovipositing stemborers, which subsequently move toward grasses bordering the field. Desmodium suppress Striga not only by producing a germination stimulant, but also by producing chemicals that interfere with development of haustoria (Khan et al., 2002).

4.2. Inhibiting germination of parasitic plants

Sensitivity of Orobanche spp. seeds to germination stimulants is positively correlated with production of gibberellin during seed conditioning; therefore, their germination can be inhibited by gibberellin biosynthesis inhibitors (Joel, 2000). Applying the gibberellin inhibitor uniconazole to soil near sunflowers significantly
decreased broomrape parasitism and increased sunflower performance (Joel, 2000). Sunflower varieties that are resistant to *O. cernua* exude coumarins that inhibit germination and are toxic to newly germinated seedlings (Serghini et al., 2001). More recently, unidentified allelochemicals from oats appeared to inhibit seed germination of *O. crenata* and reduced parasitism when intercropped with legumes (Fenández-Aparicio et al., 2007). Seed germination can also be influenced by some amino acids, which have been shown recently to have profound effects on development of *O. ramosa*. For instance, applying exogenous methionone almost completely inhibited seed germination and reduced the number of developing *Orobanche* spp. tubercles on tomato roots, possibly indicating that soil applications of amino acids or amino acid-producing microbes might be used to manage parasitic weeds (Vurro et al., 2006).

### 4.3. Reducing the production of germination stimulants by crop plants

Decreased production of germination stimulants is the best characterized mechanism of crop resistance to parasitic plants (Rispail et al., 2007). This strategy has been exploited successfully in sorghum breeding to confer resistance of certain sorghum varieties to *Striga* (Haussmann et al., 2000). Resistance is apparently absent in some crop plants including cowpea and maize (Rubiales, 2003), though considerable variation has been reported among genotypes of tomato and *Arabidopsis* (Goldwasser and Yoder, 2001; El-Halmouch et al., 2006). Recent findings suggest that selecting for reduced production of germination stimulants might negatively influence crop interactions with beneficial AMF (Akiyama et al., 2005). Recognition that strigolactones that induce
parasitic plant seeds to germinate also recruit nutrient-supplying AMF suggests that manipulating mycorrhizal colonization could be used to manage parasitic plants (Akiyama et al., 2005). Recent reports show that nutrient deficiency, which can in some cases is mitigated by AMF, can increase strigolactone production by potential host plants (Yoneyama et al., 2007). Moreover, colonization of host plants by AMF can down-regulate production of germination stimulants (Lendzemo et al., 2007; Bouwmeester et al., 2007), suggesting that enhancing AMF colonization of crop seedlings in fields could reduce strigolactone production and possibly reduce the numbers of parasitic plant seeds that germinate.

4.4. Disruption of volatile host location by Cuscuta spp.

The discovery that Cuscuta spp., like root-parasitic plants, use chemical cues to find hosts may lead to control strategies aimed at disrupting host location analogous to those described for root-parasitic plants. Plant volatiles, even more so than strigolactones, are sensitive to environmental variables (De Moraes et al., 1998; 2001; Tooker and De Moraes, 2007; Tooker et al., submitted) and could potentially be manipulated (cf. Turlings and Ton, 2006) to reduce attraction of Cuscuta spp. seedlings. In addition, the production of plant volatiles is a heritable trait (Degen et al., 2004) that could potentially be incorporated into a plant breeding program for Cuscuta resistance. Moreover, because at least one repellent compound ([Z]-3-hexenyl acetate) has been identified, a “push-pull” approach for control of Cuscuta spp. can be envisioned similar to that used for African stemborers. However, little or no work to date has examined the feasibility of such
approaches and further work is needed to elucidate how *Cuscuta* spp. perceive and respond to plant volatiles.

5. CONCLUSIONS

In spite of intensive research, adequate strategies for controlling parasitic plants remain elusive, and these weeds continue to threaten agricultural crops worldwide. Chemically mediated interactions between early-stage parasitic plants and their hosts play a key role in infestation and may be exploited for control. Recent advances in this area suggest a number of potentially fruitful approaches, including the prospect of simultaneously managing beneficial symbionts and parasitic weeds. For example, implementing cultural practices that favor AMF, such as reducing tillage and fungicide application could improve growth and increase drought tolerance in crops (Plenchette et al., 2005), and potentially reduce *Striga* infestations (Lendzemo et al., 2007). Additional research is needed to understand the mechanisms underlying strigolactone perception and responses in both parasitic plants and AMF. Intercropping with non-host plants that induce “suicidal germination” and/or are allelopathic to root parasites (e.g. Khan et al., 2002) is another promising approach that warrants continued efforts to identify potential trap crops and improve their efficacy. Recent work on the role of volatiles in host location by *C. pentagona* suggests that control strategies aimed at disrupting host location might be used against parasites that make above-ground attachments, but more work is needed in this area. It seems unlikely that any single method alone will provide long-term control of parasitic weeds. An integrative approach incorporating one or
several methods targeting the chemistry used in host location by parasitic weeds is more likely to provide sustainable strategies that will minimize crop losses.

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Figure 1. Plant-derived chemical cues are used by both shoot and root parasitic weeds to locate hosts. Upon germination, the growth of *Cuscuta* seedlings is directed toward volatile compounds released from nearby tomato plants (aboveground at left). The entire blend of tomato volatiles (at least seven compounds) is most attractive, but three compounds from this blend individually elicit directed growth of *Cuscuta*: (A.) β-
phellandrene, (B.) β-myrcene, and (C.) α-pinene (Runyon et al., 2006). Seeds of the root parasites *Striga* and *Orobanche* will only germinate in response to specific chemicals released by plant roots (belowground at right). These germination stimulants, called strigolactones, are active only within several millimeters of the host root. *Orobanche* seedlings are shown with haustoria attaching to tomato (inset, lower right). Strigol (D.) was the first germination stimulant identified. Strigol has not been isolated from tomato roots, but similar strigolactones are produced. The chemical ecology of host location by parasitic weeds provides early developmental points that could be exploited and manipulated for sustainable control.
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