Development of Behavior-semiochemical-based IPM Approach for Invasive Kudzu Bug, *Megacopta cribraria* (F.) (Hemiptera: Plataspidae)

by

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Abstract

The kudzu bug, *Megacopta cribraria* (F.), is newly detected invasive pest insect native to Asia. Since the first detection in 2009, it has spread rapidly throughout the southeastern U.S. It has become a key pest of legume crops, particularly soybeans (*Glycine max* L.), and a threat to the international trade of agricultural produces. However, chemical insecticides are currently the only tool to control *M. cribraria*. The searching for alternative IPM approaches to control *M. cribraria* based on manipulating behavior and programs exploring semiochemicals against *M. cribraria* are needed, both for the preservation of the environment and from an economic point of view. In this study, we first investigated the effect of plant growth stage on the attraction of adult *M. cribraria* to soybeans, and whether the attraction was plant constitutive volatiles. Greenhouse assays examined behavioral orientation preference of adults when given choices of four growth stages of whole soybean plants (V2, R1, R3, and R5). Significantly more adults landed on the early reproductive stage R1, followed by R3, compared with V2 and R5 stages. Laboratory olfactometer assays elucidated that plant constitutive volatiles were the cues used by adult *M. cribraria* in locating and selecting the preferred growth stage. Females were significantly more olfactory sensitive than males. Electroantennogram (EAG) results indicated strong antennal responses to constitutive volatiles emitted by the whole soybean plant, though the antennal olfactory responses were not statistically different among volatiles of different growth stages. Then, two greenhouse choice assays were conducted to evaluate the foraging orientation preference of *M. cribraria* adults to six legume species and to plant growth stages (V2, V4, R1 and R5) of each plant species. The R1stage was the most attractive in soybean and lima bean (*Phaseolus lunatus* (L.), whereas the preferred stage varied for the other legumes.

Given their respective attractive growth stages, adults significantly preferred lima bean over soybean and kidney bean (*Phaseolus vulgaris* (L.), but showed little interest in other legume species. Two greenhouse no-choice assays assessed the suitability of the six legumes for adult fecundity and nymph development. Though oviposition occurred to females caged with every legume species and no significant differences in the number of eggs among the species, females showed ovipositional preference to soybean, lima bean and mung bean plants. The hatch rates of eggs laid by adults fed on soybean, lima bean and mung bean were significantly higher than other legumes. Nymphs completed development only on soybean, lima bean, and mung bean, but the survivorships were significantly greater on soybean and mung bean than on lima bean. Lastly, electrophysiological and behavioral responses of adult *M. cribraria* to plant volatile compounds from kudzu (*Pueratia montana* (Loureiro) Merrill var. lobate (Willdenow)) and soybean, were examined to identify plant semiochemicals used in host location and attraction of *M. cribraria*. Analysis of the headspace volatiles of the kudzu and soybean plants by gas chromatography-electroantennographic detection (GC-EAD) with female *M. cribraria* revealed six compounds that elicited antennal response. The host volatile compounds were identified using gas chromatography-mass spectrometry (GC-MS). EAG from female *M. cribraria* were recorded in response to 15 compounds. Among them, 1-octen-3-ol, nonanal, ocimene and benzaldehyde, eliciting the strongest EAG responses were selected to evaluate the dose-dependent EAG responses and behavioral responses to *M. cribraria*. The host plant chemicals, 1-octen-3-ol and benzaldehyde were significantly more attractive to *M. cribraria* at higher doses in EAG and olfactometer bioassays, indicating a dose-dependent effect. Our results provide an insight into *M. cribraria* chemical and behavioral ecology and is of great significance for optimal

timing of field scouting and treatment, and the development of semiochemical-based management of *M. cribraria*.

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CHAPTER 1

General Introduction

Biology, Ecology and Management of Invasive Kudzu Bug, *Megacopta cribraria* (Hemiptera: Plataspidae)

The kudzu bug, *Megacopta cribraria*, is an invasive member of the family Plataspidae originating from Asia (Jenkins et al. 2010). First discovered in Georgia in October of 2009 (Eger et al. 2010), since then its distribution has grown to include nearly every county in Georgia, Alabama, North Carolina and South Carolina, and has been confirmed in at least 13 states (Gardner et al. 2013a), www.kudzubug.org/distribution-map/, 2016). With widespread damage to soybean (*Glycine max* L.), *M. cribraria* has continued to expand its range eastward into the interior of North America, north into Virginia, and south into Florida. In their native region of Asia, *M. cribraria* is considered a occasional pest of leguminous (Fabaceae) plants (Eger et al. 2010). In the U.S., this insect has become not only a residential nuisance pest as initially thought but also a serious economic pest of soybeans and a threat to international trade of agricultural products to Central America (Ruberson et al. 2013).

Biology

Taxonomy

Megacopta cribraria was first described as *Cimex cribraria* in 1798 by Fabricius from individuals collected in India (Fabricius 1798). Since then, it has been described as *Tetyra cribraria* (Fabricius 1803), *Thyreocoris cribarius* [sic] (Burmeister 1835), *Coptosoma cribrarium* (Amyot and Serville 1843), *Coptosoma xanthochlora* Walker (Walker 1867); *Coptosoma cribrarium* (Ahmad and Moizuddin 1975). In 1977, these names were synonymized as *Megacopta cribraria* by Hsiao et al. (Hsiao et al. 1977). There is some disagreement with regard to the taxonomy of the *Megacopta species* in the U.S. Jenkins et al. (2010) found that molecular characters for Georgia specimens were closer to *M. cribraria*. However, further research on the mitochondrial genome of the bacterial symbionts of *M. cribraria* suggested that the 16*S* rRNA gene and the *wsp* gene were closer to the gene of *Candidatus* Ishikawaella capsulate from *Megacopta punctatissima*; the *groEL* chaperone gene was 99% identified from the GenBank sequences of *Candidatus* Ishikawaella capsulate from *M. punctatissima* and *M. cribraria*. According to Hosokawa et al. (2014), phylogeographical analyses based on 8.7 kb mitochondrial DNA sequences of the introduced and East Asian native *Megacopta* populations strongly suggests that the invading *M. cribraria* populations in the U.S. are derived from a *M. punctatissima* population in the Kyushu region in Japan.

Life Cycle and Development

The life cycle of *M. cribraria* consists of egg stage, five nymphal stages and adult stage. *Megacopta cribraria* development from egg to adult ranges from 45-58d. It completes its whole life cycle in 45-290 days depending on the location, temperature, generation, food source and other conditions (Ahmad and Moizuddin 1977, Wu et al. 1992, Del Pozo-Valdivia and Reisig 2013, Shi et al. 2014, Golec et al. 2015). *Megacopta cribraria* are very active during warmer weather; however, when temperatures decrease, adults start to locate overwintering areas, and enter dormancy during the winter. When temperatures warm in spring, adults will again become active. Yet in warmer areas, *M. cribraria* may be active year around (Thippeswamy and Rajagopal 1998). The developmental threshold temperature estimated for egg to adult was 14.25 °C, with a thermal constant of 849.56 °C degree-days (Shi et al. 2014). The pre-oviposition period of *M. cribraria* ranges from 3-54 days, the longer pre-oviposition period may due to its capability to store sperm overwinter (Golec and Hu 2015). Females deposit 1-5 egg masses, and lay approximately 3-46 eggs per egg-mass; the incubation period lasts from 4-12 days depending on the temperature (Ahmad and Moizuddin 1977, Wu et al. 1992, Del Pozo-

Valdivia and Reisig 2013). After incubation period, first-instars nymphs hatch and ingest the bacteria contained in the capsules before dispersing to obtain plant food (Fukatsu and Hosokawa 2002). The total time required for nymphal development ranges from 34 to 96 days, varying with temperature (Shi et al. 2014) and food source (Del Pozo-Valdivia and Reisig 2013, Golec et al. 2015). Adult *M. cribraria* longevity ranges from 20-250 days depending on the temperature and generation (Wu et al. 1992).

During egg deposition, the female simultaneously places small, brown-colored endosymbiont capsules underneath the egg mass. These contain two essential obligate bacterial endosymbionts: the primary γ-proteobacterial, *Candidatus* Ishikawaella capulata and the secondary α-proteobacterium, *Wolbachia* (Hosokawa et al. 2005, Hosokawa et al. 2007, Jenkins et al. 2010), which serves as the essential food for newly hatched first instar, plays an essential role for proper development and reproduction of this insect, and may also support the ecological expansion of *M. cribraria* (Jenkins and Eaton 2011). It has been reported that the development period from first instar to adult was significantly longer, and the adult survival rate, body size and pigmentation significantly reduced after the removal of endosymbiont capsules (Fukatsu and Hosokawa 2002, Hosokawa et al. 2006).

Feeding Damage

Both nymph and adult *M. cribraria* feed on phloem sap of above-ground plant parts with their piercing-sucking stylets, referentially feeding on young and tender growth. Feeding damage can result in necrosis, discolored spotting, defoliation, improper development of pod, wilting and poor seed set (Tayutivutikul and Yano 1990, Xing et al. 2006, Suiter et al. 2010). In the U.S., damage caused by *M. cribraria* can result in soybean yield loss up to 60% if left uncontrolled.

(Seiter et al. 2013b). With the continued expansion of the range of this invasive pest in the U.S., its significant impact on soybeans and other cultivated legumes will continue to increase. This is problematic because soybean ranks as the second most planted field crop in the U.S. with an estimated annual market value of about $43 billion (USDA-NASS 2015). A previous study suggested that soybean planting date might influence soybean field invasion of *M. cribraria* adults, based on field observations that populations were often greater on early-planted than on late-planted soybeans (Del Pozo-Valdivia et al. 2016). However, that hypothesis does not explain the much greater populations of the second generation on early-planted soybeans after the late-planted soybeans become available.

Ecology

Seasonal Distribution and Activity

Megacopta cribraria is a strong and rapid flyer and hitchhiker (Leslie et al. 2014, Medal et al. 2015). Weather fronts and strong air currents were likely involved in long-range dispersal of adults to the east and northeast (Leslie et al. 2014). When arrived at a new location, the availability of host plants and suitable climate contribute to its successful establishment and continual spread. Seasonal movement (short-range dispersal) of *M. cribraria* has also been observed. *Megacopta cribraria* undergoes two generations a year with three adult peaks of activity in the U.S. (Zhang et al. 2012a, Seiter et al. 2013a). The first peak movement is seen in early April after overwintering adults become active in late March; then some first generation adults move from overwintering sites or kudzu to early planted soybean when they become available (Golec et al. 2015); the timing may differ with the phenology of kudzu vines and the planting schedule for soybeans. The second peak occurs in late July to early August; the trigger(s) for this dispersal are still unknown, but high population density and soybean chemical cues may be the factors. The last peak is seen in late September to early October when temperatures drop in the fall and second generation adults seek refuge to overwinter in host fields or near houses in reproductive dormancy (Golec and Hu 2015, Lahiri et al. 2015).

Host Plants of Megacopta cribraria

Megacopta cribraria has been reported to feed on more than 33 plant species worldwide. It is frequently reported as a pest of legumes in Asia, and its primary developmental hosts in the U.S. are known as kudzu (*Pueratia montana* (Lour.) Merr. var. *lobate* (Willd.) Maesen and S. Almeida) and soybean, but other legume plants and various fruit trees can be infested before availability of the primary developmental hosts (Eger et al. 2010, Gardner et al. 2013a). Studies by Zhang et al. (2012) demonstrated that even though large numbers of *M. cribraria* were found on yellow wood (*Cladrastis kentukea* (Dum. Cours.) Rudd) and black locust (*Robinia pseudoacacia* L.), no eggs were deposited on these two plant species. Kudzu and soybean were the only two hosts that *M. cribraria* can complete the development on among 11 tested plant species. Medal et al. (2013b) evaluated the host suitability of 12 plants, including 11 legume species in greenhouse no-choice tests and found that, besides kudzu and soybean, *M. cribraria* also can complete development on pigeon pea, followed by black-eye pea (*Vigna sinensis* L), lima bean (*Phaseolus lunatus* L.) and pinto bean (*Phaseolus vulgaris* L.). Other authors also reported some other plants as the reproduction host of *M. cribraria*. For example, mung bean (*Vigna radiate* L. Wilczek) (Golec et al. 2015), fava bean (*Vicia faba* L.) (Blount et al. 2015), hummingbird tree (*Sesbania grandiflora* Pers.), fire cracker flower (*Crossandra undulaefolia* Salisb), and cotton (*Gossypium hisutum* L.) (Srinivasaperumal et al. 1992). Though *M. cribraria* will infest and cause economic damage to a wide range of hosts, many crop plants may be infected as bridge hosts to avoid overspecialization on preferred host plants, which are only available at certain seasons (Bernays and Chapman 1994, Palumbo et al. 2016).

Phytophagous insects utilize host plants for feeding and developing (Bernays and Chapman 1994). To do so, monophagous or oligophagous insects use specific cues from host plants that are detectable and reliable (Jermy et al. 1988). Among the cues, plant volatiles play a predominant role in mediating orientation of phytophagous insects (Finch and Collier 2000, Bruce et al. 2005, Fujii et al. 2010). Plant volatile emission can be constitutive or can be induced in response to a variety of stresses. Some host plants release unique blends of constitutive volatiles that can attract phytophagous insects, particularly inexperienced insects (Visser 1986, Dickens 2000, Brilli et al. 2009, Dicke and Baldwin 2010). These constitutive volatiles mainly consist of terpene, ester, ketone, aldehyde, and alcohol (Szendrei and Rodriguez-Saona 2010). However, the induced volatiles could make the plants adaptive if their effects cause a behavioral change in the interacting herbivore that result in a fitness benefit for the plant (Bruce et al. 2005). For example, induced volatiles in soybean and pigeon pea plants artificially infested with the neotropical brown stink bug, *Euschistus heros* (F.) can help to attract its egg parasitoid, *Telenomus podisi* Ash. (Moraes et al. 2005). According to the work of Szendrei and Rodriguez-Saona (2010), only 3% of the studies confirmed that the tested plant volatiles had repellent effects, while 76% of all plant-insect interaction studies demonstrated the attraction of insects by plant volatiles. When using attractants in baits for insects that mimic plants, aldehydes were the most effective chemicals. However, whether or not there are any volatiles attractants in host plants that can be used for alternatives IPM program of *M. cribraria* is unknown.

Pest Management

Biology and Cultural Control

Some natural enemies entered in North America immediately after the invasion and establishment of *M. cribraria* (Gardner et al. 2013a). Among the natural enemies of *M. cribraria* identified, two parasitoids, *Paratelenomus saccharalis* (Dodd) (Hymenoptera: Platygastridae) (a specialist to Plataspidae), *Ooencyrtus nezarae* Ishii (Hymenoptera: Encyrtidae), and one entomopathogens, *Beauveria bassiana* (Balsamo) Vuillemin have emerged as important and successful. Field parasitism rates by *P. saccharalis* were recorded as 51% of eggs in China, 43%-100% of eggs in Japan (Wall 1928, Takasu and Hirose 1985), and 44.9%-85.7% of egg masses with a range of 47.5-84.8% of egg parasitism in the southeastern U.S (Gardner et al. 2013, Tillman 2016). High parasitism of 100% for egg masses and 68.6% for eggs by *O. nezarae* were observed in Japan (Hirose et al. 1996). The fungus *B. bassiana* found infecting and killing *M. cribraria*, resulted in up to 70% to 83% mortality in nymphs and adults in laboratory bioassay (Borah and Dutta 2002, Borah and Sarma 2009). Nearly 60% eggs of *M. cribraria* were parasitized by *Alberus* sp. (Hymenoptera: Aphelinidae) in the field (Rajmohan and Narendran 2001). The less dominant egg parasitoids *Trissolcus* sp. (Hymenoptera: Scelionidae), that co-exist with *O. nezarae* in China were found for less than 2% of total parasitoids in the field (Zhang et al. 2003). Many other parasitoids including families Aphelinidae, Encyrtidae, Scelionidae, Tachinidae, and some predators from families Pentatomidae, Geocoridae, Anthocoridae, Formicidae, Coccinellidae, Chrysopidae, and Oxyopidae have been reported (Greenstone et al. 2014), while the efficacy of these natural enemies in the field is unknown. Though high parasitism was observed in some natural enemies, such as *P. saccharalis*, the control efficacy is limited, due to its seasonal occurrence (mainly from May to July) and its short life cycle (5-9 days) (Takasu 2012).

Some cultural control methods for controlling *M. cribraria* have been described. In the fall, removing kudzu patches and other weeds in nearby residential areas can prevent the bugs from overwintering there (Wang et al. 2004). However, removing kudzu by mowing is not practical in the soybean fields, because it must be repeated nearly weekly during the growing season for multiple years (Golec et al. 2014). White color traps such as cross-vane traps (Horn and Hanula 2011) and white polyvinyl chloride adult sticky traps (Dhammi et al. 2016), can only be used to monitor the insect population in the soybean fields. Potential resistant soybean varieties to *M. cribraria* have been evaluated and selected using quantitative trait loci (QTLs) analysis (Xing et al. 2008) and six screening technique (Bray et al. 2016), but the yield protection hasn’t been determined. Insecticides are currently the only widely used and successful control measure for *M. cribraria*.

Thresholds and Chemical Control

The large crop losses caused by *M. cribraria*, coupled with the limitations of cultural and biological control, have driven the wide use of insecticide in management of this pest. Currently, the recommend action threshold for insecticide application of *M. cribraria* is 5 or more adults per plant before R1, and one or more nymph per sweep between R1 to R7 (Reed 2013) (http://www.aces.edu/pubs/docs/A/ANR-0500-B/ANR-0500-B.pdf). The susceptibility of *M. cribraria* to several insecticide classes, including pyrethroid, neonicotinoid, oxadiazine, organophosphate, chitin synthesis inhibitor, and molting hormone agonist has been studied (Wang et al. 2004, Seiter et al. 2013c, Seiter et al. 2015). The suppression and residual efficacy against *M. cribraria* varies among rates and types of insecticides, insect stages, formulas and application timing. Treatments targeting at the nymphal stage of *M. cribraria* had the greatest impact on populations (Wang et al. 2004, Seiter et al. 2015), and the control effects were above 90% with the utility of 5% chlorpyrifos, 2.5% deltamethrin, and 20% fenvalerate. For adult control, broad-spectrum insecticides in the pyrethroid and organophosphate have a high efficacy against *M. cribraria* in China (Wu et al. 1992, Wang et al. 1996). The mixture of organophosphates and pyrethroids offered a good control above 85% on adults, such as the mixture of 50% chlorpyrifos and 5% cypermethrin (Wang et al. 2004). In the U.S., Seiter et al. (2015) evaluated the efficacy of different insecticides labeled for soybean pests in controlling *M. cribraria* and calculated economic benefits in field trials. Several pyrethroid compounds, particularly bifenthrin, cyhalothrin, and cypermethrin were more effective against *M. cribraria* adults and nymphs than other commonly used chemical. The pyrethroid 11.2% bifenthrin showed an immediate control up to 97.5% 2-6 day after treatment. Net marginal benefits were typically greatest for pyrethroids, either alone or tank-mixed with other materials. The carbamate carbaryl also showed higher efficacy in controlling of *M. cribraria* than did organophosphate. Some new insecticides classes, such as neonicotinoids, chitin synthesis inhibitors and molting hormone agonists are not as efficacious as the pyrethroids (Seiter et al. 2016).

Objectives

Early-planted soybeans are at greater risk of infestation than later-planted soybean (Del Pozo-Valdivia et al. 2016), and yet we know very little about the reason and mechanisms of this directional movement. Understanding host finding behavior will aid in understanding the mechanism of *M. cribraria* adult movement, which is a key factor for successful management of this pest. Currently, development of resistant varieties is likely to reduce the population of *M. cribraria* in the field, but the yield protection of soybean production has not been determined.

Though several natural enemies, specialist and generalist, have been discovered, the control efficacy is limited, due to their seasonal occurrence and short life cycle. The white color traps and white polyvinyl chloride adult sticky traps are not effective and eradicating the kudzu host is not practical. Therefore, insecticide applications are currently the only short-term solution to manage this pest in soybeans. However, repeated use of a limited number of insecticides could favor the development of insecticide resistance. Opportunities exist to find alternative IPM approaches to control *M. cribraria* based on manipulating behavior and programs exploring semiochemicals against *M. cribraria* are needed, both for the preservation of the environment and from an economic point of view. Therefore, the long term goal of this project is develop effective alternative IPM approaches for managing invasive *M. cribraria* to enhance soybean productivity and sustainability. The objectives for this project are to:

1) Investigate the effects of plant growth stages of soybean on attractions to adults
2) Determine the foraging orientation, oviposition preference and development of *M. cribraria* on six bean crops
3) Identify plant bioactive compounds in host plants that attract *M. cribraria*

CHAPTER 2

Differential Attraction of *Megacopta cribraria* (Heteroptera: Plataspidae) to Different Growth Stages of Soybean Plant: Comparative Responses to Whole Plant and Plant Volatiles

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Introduction

Phytophagous insects must find host plants for feeding and development (Bernays and Chapman 1994). Host finding is more challenging for herbivores with narrow host ranges, because foraging insects must locate the species of host plant against a background of many irrelevant species within diverse and often varying quantitative and spatial vegetational contexts (Tahvanainen and Root 1972, Bruce and Pickett 2011). To do so, monophagous or oligophagous insects use specific cues from host plants that are detectable and reliable (Jermy et al. 1988). Among these cues, olfaction is the primary sense used by phytophagous insects to detect and locate hosts (Finch and Collier 2000, Bruce et al. 2005, Fujii et al. 2010, Schroder et al. 2015). Plant volatile emissions can be constitutive or they can be induced in response to a variety of stresses. Constitutive volatile profiles are specific to a plant species and mediate the interaction between a plant and its environment, often repelling, poisoning or attracting insects (Whittaker and Feeny 1971), particularly those inexperienced phytophagous individuals and populations (Visser 1986, Dickens 2000, Brilli et al. 2009, Dicke and Baldwin 2010). Constitutive emissions occur both during periods when plants do not experience stress and when they do (Dicke 2000). Volatile profiles differ between plant species, cultivars, as well as over the growing season at different growth stages (Schoonhoven et al. 1998, Bruce et al. 2005, Dicke and Baldwin 2010, Zhang et al. 2015a). Phytophagous insects find their host by perceiving specific olfactory signals (Bruce and Pickett 2011). Therefore, variations in volatile emission, although these differences may be subtle, impact insect behavioral responses to host plants (Bruce and Pickett 2011, Zhang et al. 2015a). Previous studies have documented effects of host plant age and olfactory cue on host-finding behaviors of phytophagous insects (Visser 1979, Van Rensburg et al. 1987). For example, when given a choice between intact young and mature potato plants, *Solanum* *tuberosum* L. Colorado potato beetles, *Leptinotarsa decemlineata* (Say), prefer mature plants (Dickens 2000). However, young potato plants became attractive to this insect after they were damaged with conspecific or heterospecific herbivores, possibly due to an increase of attractive compounds in herbivore-induced volatiles (Bolter et al. 1997). The flowering and early pod-filling stages of soybean plants, *Glycine max* Merrill, are known to attract species of Pentatomidae stink bugs, regardless of soybean planting date or maturity group (McPherson 1996, Smith et al. 2009, Permana et al. 2012).

The kudzu bug, *Megacopta cribraria* (F.), is an invasive member of the family Plataspidae originating from Asia (Jenkins et al. 2010). First discovered in Georgia in October of 2009 (Eger et al. 2010), the kudzu bug has since spread rapidly throughout the mid-southern and southeastern U.S. (www.kudzubug.org, 2016). In their native Asia, the kudzu bug is considered a primary pest of leguminous (Fabaceae) plants (Eger et al. 2010). In the U.S., this insect has elevated its pest status from the initially thought nuisance to homeowners to a serious pest of soybeans and a risk to international trade of agricultural products (Ruberson et al., 2013). The kudzu bug has two generations per year and overwinters as adults in reproductive dormancy, clustered in various sheltered areas, under tree bark and rocks, or other debris in the vicinity of host fields or residences (Golec and Hu 2015, Lahiri et al. 2015). Overwintered adults become active again in April. The first generation of adults emerges in late June and the second generation adults appear in August (Del Pozo-Valdivia and Reisig 2013). Emerged adults disperse from overwintering sites to host plants or from the infested host plants to new habitats (Golec et al. 2015). Soybean and the invasive weed kudzu, *Pueraria montana* (Loureiro) Merrill var. *lobate* (Willdenow), are the two primary reproductive host plants in the U.S. (Zhang et al. 2012b), even though several legume crops have been reported as developmental hosts under greenhouse conditions (Medal et al. 2013a, Blount et al. 2015). Additionally, adults are commonly found on a variety of taxonomically diverse plants during their dispersal periods before they locate preferred host plants or overwintering sites (Gardner et al. 2013a, Golec and Hu 2015). Unlike other stink bugs, kudzu bug rarely feeds on the pod or seed of the plant but reduces yield indirectly by both nymphs and adults removing vascular fluids, typically from the stems and petioles of the plant (Tayutivutikul and Yano 1990, Xing et al. 2006, Suiter et al. 2010). If left uncontrolled, yield loss of up to 60% in a confined environment, such as field cages, was recorded (Li et al. 2001, Seiter et al. 2013b). This is problematic because soybean ranks as the second most planted field crop in the U.S. and produces an estimated annual market value of about $43 billion (USDA-NASS 2015).

Recent studies reported that early-planted soybeans (especially those planted in April and May) are more prone to kudzu bug infestation and harbor greater populations throughout the season than late-planted soybeans (those planted in June and July), likely because of the coincidence of their availability with adults dispersing from overwintering sites, as well as the fact that they can support both generations of this bivoltine pest (Blount et al. 2016, Del Pozo-Valdivia et al. 2016). However, the hypothesized link between environmental conditions and the biology of the kudzu bug does not explain the much greater populations of the second generation on early-planted soybeans after the late-planted soybeans become available. This hypothesis is also unable to explain the cues that attract dispersing adults to soybean plants. Understanding the cues attracting dispersing kudzu bugs to soybean will aid in forecasting population dynamics and in developing more effective management programs against this pest. Currently, insecticide applications are the only short-term solution to manage this pest in soybeans (Seiter et al. 2016).

The objectives of this study were twofold: (i) to determine whether the growth stage of soybean plant plays a role in kudzu bug attraction using greenhouse choice assays, and (ii) to determine whether the attractive cue is olfactory using both olfactometer and electroantennogram assays. This study also examined whether male and female adults have the same olfactory sensitivity and if there were differences in behavioral responses to soybean whole plants and constitutive plant volatiles between kudzu bug generations.

Materials and Methods

Insects

Adult kudzu bugs were collected from a naturally infested wild kudzu patch (32.363301 N 85.300183 W) in Auburn, Lee County, AL. Overwintered adults were collected in mid-April and the first generation adults were collected in July, both during their peak dispersal period. Adults were collected using a sweep net, brought to a greenhouse, and maintained in a plastic framed mesh cages (30 x 30 x 30 cm^3) (BugdormTM, BioQuip Products, Rancho Dominguez, CA) supplied with fresh kudzu shoots, changed daily. Adults were used for assays within 2 days of field collection.

Plants

Soybean plants were grown from seeds (Mycogen 5N501R2, G5 cultivar, Mycogen Seeds Company, Indianapolis, IN) in a greenhouse on the Auburn University campus (32.586691 N, 85.488764 W) under constant conditions (24 ± 3 °C, 50% - 70% RH, and ambient light). Four seeds were planted in each plastic pot (17.5 cm diameter 17.5 cm, 17.5 cm height) with moistened Sunshine Professional Soil Mix (SunGro Horticulture Canada Ltd., Seba Beach, Alberta). Seeds were sowed weekly until termination of the study so enough plants of specific growth stages were available for experiments on the designated assay dates. Plants were watered as needed and extra seedlings were removed, so that each pot for this study contained only three soybean plants.

Soybean growth stages were determined using the system developed by Fehr et al. (1971). Briefly, the vegetative stages (V1 to Vn) are defined by the number of nodes on the main stem, while the reproductive stages (R1 to Rn) are determined according to flowering and pod development at the upper portion of the main stem. This study evaluated the following four distinct growth stages for the adult kudzu bug foraging orientation preference: V2, R1, R3, and R5. These stages were selected to represent vegetative (V2: a fully unrolled leaf at the node above the unifoliolate node), flowering (R1: one flower at any node), pod (R3: pod beginning to develop at one of the four uppermost nodes with a fully unrolled leaf), and seed (R5: seeds beginning to develop in the pod at one of the four uppermost nodes with a fully unrolled leaf) growth stages.

Greenhouse Multiple-Choice Behavioral Orientation Preference Assay

Greenhouse multiple-choice assays were conducted to investigate possible soybean growth stage preference in kudzu bug adults. The assay was carried out under constant conditions (24 ± 3 °C, 50% - 70% RH, and ambient light). Four pots containing plants at a specific growth stage (V2, R1, R3 or R5) were randomly arranged, adjacent to each other, inside a large screen cage (173 x 71 x 91 cm^3, Dura-Tent, Poway, CA). The plants were examined carefully and only intact and healthy plants with no injury or damage were used. Groups of 100 adults (50 females and 50 males) were introduced into Petri dish (10 cm diameter, 1.5 cm height, Thermo Fisher Scientific, Waltham, MA) and were released in the center of the cage at an equal distance from each plant at 1100 hrs. Adults were allowed to freely select the plants at their preference for a given time. The number of insects landing on each plant within the cage was recorded at 1, 24, and 48 post-release h. The recorded number was then transformed to the proportion of adults observed on a particular plant (sum observed on a particular plant divided by the sum of adults observed on all the plants within a cage). This assay used a completely randomized block design with cage as block each containing a pot of each of the four growth stages, two independent sets with four replicates for each of the overwintered and first generation to gain a comprehensive understanding of the possible effects of insect generation and physiological status on behavioral response. A total of 1,600 adults were tested: 100 for each replicate, 400 for each set, and 800 for each generation.

Olfactometer Assay

Y-tube olfactometer assays were designed to determine whether the host-seeking behaviors observed in greenhouse choice assays were plant odor mediated responses. The behavioral responses of adults (males and females) to constitutive volatiles of whole plants at the four selected growth stages were recorded. The plants were examined carefully and only intact and healthy plants with no injury or damage were used to ensure the emitted volatiles were constitutive.

The olfactometer was a Y-shaped tube (diameter: 2.5 cm; common tube length: 25.0 cm; length of each arm: 21.0 cm) with the two arms extended at 90°. The two arms were connected to two cylindrical glass chambers (22.8 cm diameter and 40.6 cm height). The glass chambers were connected through a Teflon-lined glass tube connector to two air delivery system (ARS Inc., Gainesville, FL) (Fig. 2.1). The two glass chambers each had an inlet at the top and an outlet at the bottom: one held potted soybean plants at a specific growth stage and the other being air control (the same pot with soil but no plant). Airflow was charcoal-filtered, and humidified by passing through two glass bubblers (ARS Inc., Gainesville, FL) filled with purified water, then pushed into the two glass chambers, and from there to the test insect. Airflow through the olfactometer arms was maintained at a rate of 200 mL/min at the entrance and the vacuum pump evacuated air at a rate of 380 mL/min (190 mL/min for each arm). The olfactometer apparatus was held vertically by a wooden support, placed in a cardboard box (82 x 82 x 61 cm^3) lined with white paper, and positioned under a fluorescent light source (100 lux) for uniform lighting and visual record of insect movement. Before initiating the experiments, a pre-assay was run and the results showed no behavioral effect of clean air or soil-pot on kudzu bug adults.

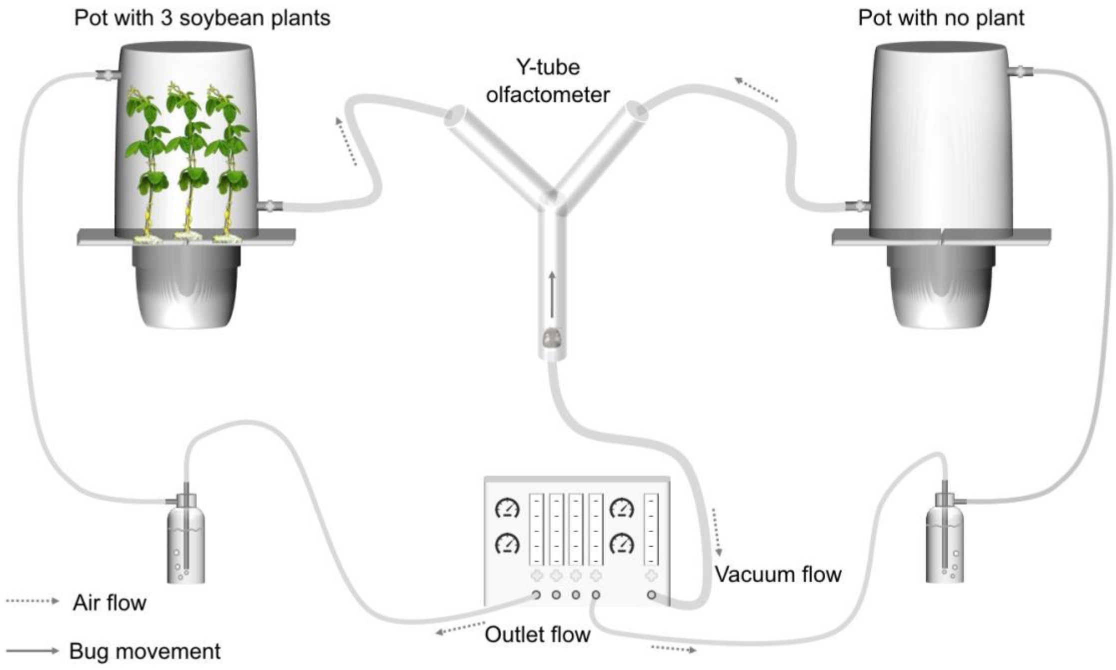

Fig. 2.1. The illustration of Y-tube olfactometer and air deliver system

One insect at a time was introduced at the entrance of common tube and given 10 min to make a choice between the two arms (clean air vs. air carrying plant odor). The insects that passed the 10 cm mark on the arm was recorded as having made a choice (Bertschy et al. 1997). If no choice was made within 10 min, the assay was concluded. The insects were used only once. After each insect was tested, the Y-tube was washed using water, rinsed with 95% ethanol, and air-dried. After two insects were tested, the position of the olfactometer arms was alternated to avoid directional bias. After ten insects were tested, the glass chambers were replaced with new ones and the potted plants and pots were replaced to avoid olfactory bias. For each plant growth stage, 60 insects (30 males and 30 females) were tested. The numbers of female and male insects were recorded separately. The data were generated using 240 different individual adults. The experiments were conducted from 09:00 until 14:00 in the laboratory under 24±3°C and 50-70% RH.

Electroantennography (EAG)

To further understand the degree of response of kudzu bugs to constitutive volatile extracts from four different growth stages of soybean, antennae responses were quantified using EAG.

Volatile collection: We collected the plant constitutive volatiles from the headspace of intact whole plants at a specific growth stage placed in glass chambers (17.5 cm in diameter by 85 cm high) (Ceballos et al. 2015). Each glass chamber contained an inlet at the top and eight outlets at the bottom and was connected by Teflon-lined glass tube connectors to pumps in an air delivery system (ARS Inc., Gainesville, FL). Charcoal-filtered and humidified air was blown at a rate of 1 L/min and was evacuated from the chamber at a rate of 900 mL/min. The flow difference for pushing and vacuum pumps were set to prevent collecting outside air from possible leakages.

Plant volatiles were collected by connecting a HSP-Q material volatile collection trap (http://www.volatilecollectiontrap.com/) at the outlet of the jar and the vacuum pump for 24h. Then, the samples were eluted by 0.2ml hexane and stored at -18°C.

EAG: EAG measurements were made using a commercially available electroantennographic system (Model CS-05; Syntech, Hilversum, Netherlands, consisting of an Ag-AgCl electrode for antenna fixation, a CS-05 stimulus controller, and an IDAC box for data acquisition), using a modified method by Ho and Millar (2002). Each antenna was carefully amputated at the base and the distal tip cut-off with a scalpel. The cut end of the antenna was immediately attached to an electrode with non-drying electrode gel (Spectra 360 Electrode Gel, Parker Laboratories, INC. Fairfield, NJ). The electrode was connected through a high-impedance DC amplifier to send signals to a PC equipped with an IDAC interface box and appropriate software (EAG Pro from Synthech, Hilversum, Netherlands). An eluted extract (10 μL) was applied to a piece of filter paper strip (0.8 x 5cm^2). After solvent evaporation, the paper strips were placed inside a glass Pasteur pipette (Fisher Scientific, Pittsburgh, PA, USA) for EAG test. The tip of the pipette was inserted about 3 mm into a small hole through the wall of a glass tube (0.8 cm diameter, 10 cm long) directed at the antennal preparation. A continuous flow of 0.6 L/min of purified air was passed over the antenna through the pipette. The stimulus was set at 10 s puffs at 90 s intervals. The EAG response to 10 μL of hexane was tested as control. To compensate for antennal fatigue, blank stimulus and standard stimulus (10 μL kudzu volatile extract) were tested before and after soybean volatiles and hexane. EAG responses to volatiles were calculated as corrected absolute EAG values (Kang and Hopkins 2004). Four antennae from four female adults (one from each adult) were carried out for each plant growth stage.

Data Analysis

Greenhouse behavioral data were analyzed for responsiveness (proportion of adults observed on a particular plant relative to the sum of adults observed on all the plants within a cage) using the generalized linear mixed model procedures with a binomial distribution function and the default logit canonical link as implemented in PROC GLIMMIX (SAS Institute 2012). The fixed effects were the soybean growth stage (SGS) and time after release, where that latter was modeled as a continuous covariate nested within soybean growth stage. The random effects were set, cage and cage x SGS. The least squares means (LS-means) and 95% confidence intervals (CI) for each observation (time after release) were calculated using the AT (modifies covariate value in computing LS-means) option. Means and CIs were then expressed on the original scale using the ILINK (inverse link) option for suitably chosen estimable functions. Individuals that did not land on soybean plants were excluded from the statistical analysis.

Results of olfactory assays were analyzed with a χ^2 test to test the null hypothesis of no preference of males and females to odors of a particular soybean growth stage. The 95% confidence intervals were counted using Agresti & Coull interval (Brown et al. 2002). The responses of males and females to the respective choices were analyzed separately. Individuals that did not make a choice were excluded from the statistical analysis.

EAG response values were corrected by subtracting the blank value and were determined to be normally distributed and then analyzed using one-way analysis of variance (ANOVA) following by a Tukey’s HSD (Honest Significance Difference) test ($P = 0.05$).

Results

Greenhouse Behavioral Orientation Preference to Different Growth Stages

Overall, soybean growth stages had a significant effect on attraction of dispersal adults of both the overwintered ($F = 16.73$, df = 3, 42, $P < 0.01$) (Fig. 2.2a) and first generation ($F = 20.14$, df = 3, 42, $P < 0.01$) (Fig. 2.2b) kudzu bugs.

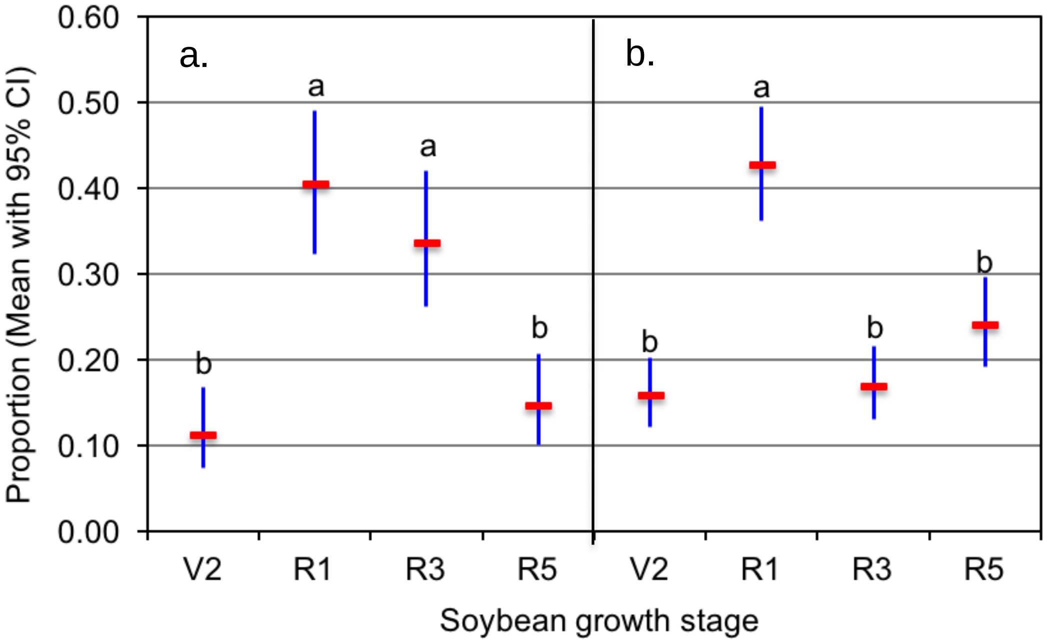

Fig. 2.2. The mean proportions (mean and 95% CI) of overwintered (a) and 1st generation (b) adult kudzu bugs observed on different soybean growth stages over a 3-d observation in greenhouse choice assay. The same letter above CI intervals indicates no significant difference within growth stages at alpha = 0.05 (GLIMMIX)

For the overwintered generation (Fig. 2.3a), a significantly greater proportion of adults preferably located the R1 stage, followed by R3, over the vegetative V2 stage and late reproductive R5 stage during the 3-d period. One hour after experiment initiation, the greatest proportion of adults was observed on R1, and the lowest proportion (≤ 0.13) was recorded on V2 and R5. Then we observed a slight decrease on R1 and a slight increase on R3 as the adults moved around among the choice plants to reach equilibrium, while the number on R1 remained the greatest. Averaging across the 48 hour of observation, the mean proportions of adults, in the order of proportion, were 0.40, 0.34, 0.15, and 0.11 on R1, R3, R5, and V2, respectively. This indicates that overwintered adults orientated preferentially to early reproductive stages over early vegetative and late reproductive stages.

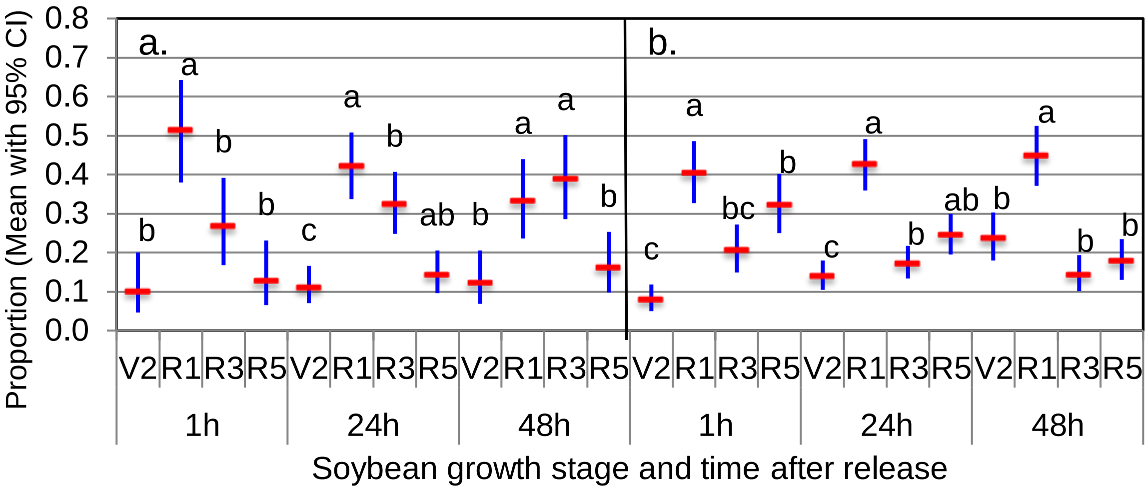

Fig. 2.3. The proportion (mean and 95% CI) of overwintered (a) and 1st generation (b) adult kudzu bugs observed on different soybean growth stages at post-release 1h, 24h and 48h in greenhouse choice assay. The same letter above CI intervals within growth stages indicates no significant difference among observation intervals at alpha = 0.05 (GLIMMIX)

A similar pattern of growth stage attractiveness was observed in the 1st generation (Fig. 2.3b), with R1 plants consistently attracting the greatest proportion (> 0.40) of adults over the 3-d experiment. At 1h observation, the greatest proportion (0.40) was observed on R1, followed by 0.32 on R5, 0.20 on R3 and the lowest proportion (0.08) on V2. Afterward, the proportions (0.42 - 0.45) on R1 remained the greatest, whereas the proportions among the other three stages changed as some adults started shifting among the plants within the cage, showing increasing proportions on V2 and decreasing proportions on R5. Nevertheless, averaging across observation hours, the proportions of adults, in the order of proportion, were 0.43, 0.24, 0.17 and 0.16, and on R1, R5, R3, and V2, respectively. The comparison of distribution patterns (Table 2.1) showed a negligible difference on the most preferred growth stage between overwintered and 1st generation adults, indicating that the most preferred growth stage was not impacted by kudzu bug generation and soybean planting date.

			Confidence Interval		
Soybean Growth Stage	**Kudzu Bug Generation**	**Mean**	**LL**	**UL**	***P* value**
V2	Overwintered adults	0.11	0.07	0.17	0.161
	First Generation adults	0.16	0.12	0.20	
R1	Overwintered adults	0.40	0.32	0.49	0.675
	First Generation adults	0.43	0.36	0.49	
R3	Overwintered adults	0.34	0.26	0.42	0.000
	First Generation adults	0.17	0.13	0.22	
R5	Overwintered adults	0.15	0.10	0.21	0.020
	First Generation adults	0.24	0.19	0.30	

Table 2.1 The proportions of adult kudzu bugs selected soybean plants at V2, R1, R3, or R5 stage in greenhouse choice assay. Data were analyzed by the generalized linear mixed model procedures with a binomial distribution function and the default logit canonical link as implemented in PROC GLIMMIX (SAS Institute 2012).

Olfactory Behavioral Responses to Whole Soybean Plant Volatiles

Plant volatiles from soybean of different growth stages elicited differential olfactory response from adult kudzu bugs. The olfactory responses to constitutive volatiles of the same growth stage differed between male and female adults (Fig. 2.4).

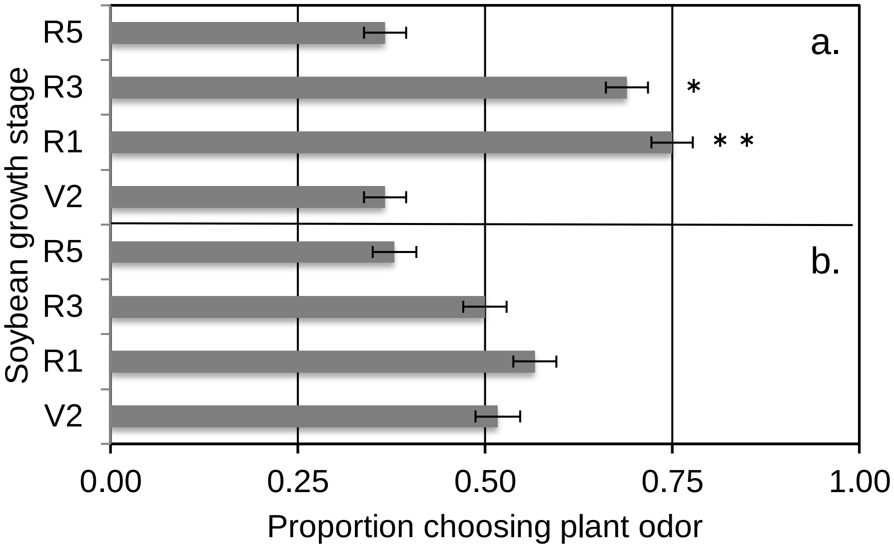

Fig. 2.4. Behavioral orientation choice of adult kudzu bug females (a) and males (b) in laboratory Y-tube assay. Bars represent the percentage of kudzu bugs choosing constitutive odor associated with whole soybean plant at different growth stage. Asterisks (*) indicate a significant difference at $P < 0.05$, asterisks (**) indicate a significant difference at $P < 0.01$ (χ^2 test).

To understand the specific foraging orientation behaviors observed through the greenhouse experiments, female and male responses were analyzed independently. Female responsiveness (number of females that made a choice between a plant odor and the control) to odors of different growth stages was shown in Fig. 2.4a. Significantly more females preferred odors from R1 (χ^2 = 7, df = 1, P = 0.01) and R3 (χ^2 = 4.17, df = 1, P = 0.04), compared with odors from V2 (χ^2= 2.13, df = 1, P = 0.14) or R5 (χ^2= 2.13, df = 1, P = 0.14). The olfactory response of females validated the orientation preference observed in greenhouse assays and indicated that the greenhouse orientation preference was a plant volatile mediated behavior.

Males displayed much less olfactory sensitivity (Fig. 2.4b) than females, and showed no significant preference to plant volatiles (V2: χ^2 = 0.03, df = 1, P = 0.85; R1: χ^2 = 0.53, df = 1, P = 0.47; R3: χ^2 = 0, df = 1, P = 1; and R5: χ^2= 1.69, df = 1, P = 0.19) compared to the control.

Despite the lack of statistical difference, the frequencies of positive responses to soybean plant odors were constantly higher on R1 than R3 and R5.

EAG Response to Whole Soybean Plant Volatiles

The corrected absolute EAG values of EAG responses are presented in Fig. 2.5. The EAG responses to the four soybean volatiles were significantly greater than to the control hexane (F = 3.57, df = 4, P = 0.03). Volatiles of R1 soybean plant elicited the greatest EAGs responses than volatiles of other plant growth stages, but the difference among the four growth stages was not statistically significant (F = 1.76, df = 3, 19, P = 0.21).

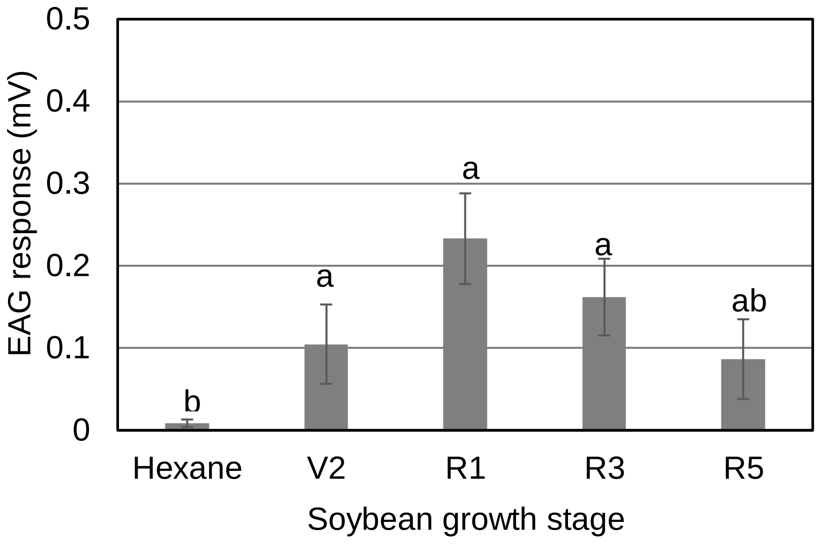

Fig. 2.5. Absolute EAG response (Mean ± SE, n = 4) of female kudzu bug antennae to different constitutive volatiles from soybean whole plant at specific growth stages. Means with different letters indicate significant (ANOVA, Tukey’s HSD test, $P < 0.05$) differences between treatments.

Discussion

The present study provides the first empirical evidence that plant growth stage affects the attraction of adult kudzu bugs to host soybeans, and that the attractive cue is the constitutive volatiles emitted from whole soybean plants. This growth stage effect was not impacted by soybean planting data or kudzu bug generation. It is therefore way likely that, host-finding and colonization are driven by olfactory sensitive females.

When presented with choices of soybean plants at different growth stages, significantly more adults selected plants at flowering stage (R1) than plants at other growth stages. The large preference for R1 plant was not influenced by soybean planting date or the generation of the insect. This result indicates that the physiological stage of the host contributes strongly to host-finding behavior of the kudzu bug. This growth-stage-effect provides a reasonable explanation for the greater populations and infestation of early-planted soybeans than late-planted soybeans (Blount et al. 2016, Del Pozo-Valdivia et al. 2016). In the spring when overwintered adults become active to seek out a food source with a fierce appetite, early-planted soybeans are the only available host plants in agricultural areas where the invasive weed kudzu is rarely present. The dispersing adults readily recognize the host plants, though they are still small at V4 or younger, lay eggs and develop the first generation on them. When the first generation develops into adults in June, the early-planted soybeans are at R1 or older and the late-planted soybeans are small at V3 or younger. Given the choice, the newly emerged adults that are already on early-planted soybeans choose to remain on or re-infest the early reproductive stage plants after pesticide treatments, rather than leaving the most attractive for the less attractive late-planted young plants. This is true in the field even when the late-planted and early-planted fields are close-by (Golec et al. 2015). Additionally, some newly emerged adults may disperse from overcrowded kudzu vines and be attracted to the early reproductive stage plants after pesticide treatments, because of the migratory nature of this insect. Adult kudzu bugs rapidly lay eggs after landing on host plants (Del Pozo-Valdivia et al. 2016). As a result, the early-planted soybeans have greater populations and the late-planted soybeans suffer less infestation. This phenomenon has been proposed as planting-date-effect, meaning the timing of host availability impacts insect colonization (Blount et al., 2016; Del Pozo-Valdivia et al., 2016;). However, the planting-date-effect theory is unable to explain the propensity of adults to retain on early-planted soybeans after late-planted soybeans become available and why adults would re-infest early-planted plants following insecticide treatment than move on to untreated late-planted young plants, considering the tender parts of the host are their preferred food source (Ruberson et al. 2013, Del Pozo-Valdivia et al. 2016). Furthermore, our data have demonstrated that neither planting date nor insect generation influenced preference of adult kudzu bugs to R1 – R3 plants. Another possible explanation for the insect remaining on infested plants is that kudzu bugs, like other Heteropteran species (true bugs) (McBrien et al. 2002, Aldrich et al. 2009), might produce an aggregation pheromone that directly or indirectly attracts subsequent immigrant adults in the field. However, the existence of semiochemical aggregation pheromone in kudzu bug has not been proven.

The effect of plant growth stage on host preference has been reported in members of the Pentatomidae that prefer to feed on developing seeds, such as brown marmorated stink bug, *Halyomorpha halys* (Stål) (Cissel et al. 2015), southern green stink bug, *Nezara viridula* (Linnaeus) (Thomas et al. 1974, Smith et al. 2009), and brown stink bug, *Euschistus serous* (Say) (Smith et al. 2009). However, kudzu bug is neither a seed-feeder nor a pod-feeder. Both nymphs and adults are sap suckers with a preference of tender vegetative parts (Ruberson et al. 2013). Theoretically, the predicted favorable stages should be the vegetative ones, but not the reproductive stages. We do not have a reasonable explanation for vegetative stages being the less-recognizable choice among the four tested growth stages. If the optimality model applies, the host choice of phytophagous insects would maximize fecundity and/or benefit the fitness of offspring (Jaenike 1990). Srinivasaperumal et al. (1992) reported that kudzu bu*g* preferred to feed on plants containing higher concentrations of proteins and reducing sugars and lower concentrations of phenols. Two recent studies proposed that the kudzu bug abundance varied from different plant genotypes (Bray et al. 2016, Fritz et al. 2016). Future studies may investigate nutrition value variations among soybean growth stages and nutrition influences on kudzu bug nymph development and adult fecundity.

The olfactometer and EAG revealed that growth stage preference behavior was modulated by constituent volatile cues emitted by live host plant. Female adults showed higher olfactory responsiveness than males, with an olfactory attraction pattern remarkably similar to behavioral response to live plants observed in greenhouse assays. The difference in growth stage attractiveness could be due to quantitative and qualitative changes of volatile emission of the plants at different growth stages (Boue et al. 2003, Silva et al. 2013). In general, plants release more volatiles at flowering stage (Ceballos et al. 2015). Boue et al. (2003) documented increasing amounts of several volatile compounds, including 3-hexanone, (E)-2-hexenal, 1-hexanol, and 3-octanone, during early reproductive stages but altered concentrations at late growth stages of soybean plants. Further work needs to elucidate specific growth stage volatiles, their compositions, and influence on the interactions between the soybean plant and the insect. The electrophysiological response confirms that adult kudzu bugs are capable of detecting volatile blends at the level of the peripheral nervous system. Soybean plant volatiles elicited significant EAG antennal responses but the difference was not significant between different volatile blends of the four growth stages. This is because that EAG only provides information about activation of receptors but does not provide information about the type of insect behavior the compound elicits; which could be as an attractant, repellent or other behavioral response.

The finding of females being more responsive to soybean odors than males is particularly interesting because Golec and Hu (2015) hypothesized that pre-winter mated females may be the first to exploit hosts and potentially increase the invasion of new habitats without the need to mate again in spring. Their hypothesis was based on observations that female kudzu bugs had a greater overwintering survival rate and a portion of females mated prior to overwintering. Our greenhouse choice assays were not designed to differentiate the 3d proportions between males and females landing on soybean plants. However, our olfactometer data revealed stronger odor-evoked response in females than males, and the R1 and R3 growth stages produced the most behaviorally active constituent odors. Several hypotheses have been proposed to link female-biased host-odor responsiveness to oviposition considerations; such that plant growth stage used for oviposition maximizes offspring fitness (Knolhoff and Heckel 2014). Seiter et al. (2013b) reported that peak numbers of total egg masses deposited by kudzu bug occurred in early August on soybean plants of the R2/R3 stage, but Blount et al. (2016) observed no oviposition preferences dependent on the physiological stage of the host plant. In contrast, males exhibited no significant preference to tested plant odors. Low or no responses were likely due to (a) males were less sensitive to the constitutive odors from whole soybean plants or (b) odors presented in this experiment were below male behavioral threshold levels, additional studies are needed. Similar behaviors have been documented in *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae) (Chinta et al. 1994), *Cylas formicarius* (Fab.) (Korada et al. 2010), and *Delia antiqua*

(Diptera: Anthomyiidae) (Romeis et al. 2003), with females being more sensitive to plant volatiles than males.

Our experimental set-ups were designed to only test the specific olfactory response of kudzu bugs to constitutive volatiles that confer the characteristic soybean smell. The integrity of plants was carefully maintained to ensure no biotic/abiotic injury or damage that would cause the release of induced volatiles that consequently alter the amount and composition of constitutive volatile blends (Pichersky et al. 2006, Copolovici et al. 2011). Numerous studies have demonstrated that herbivore induced plant volatiles are used as a defense against herbivorous insects (Schoonhoven et al. 2005). There are also studies showing that herbivore induced volatiles act as attractants or oviposition stimulants of pest insects (Silva et al. 2013). One typical example is that young potato plants were not constitutively attractive to Colorado potato beetle (Dickens 2000), but became attractive when they were damaged with conspecific or heterospecific herbivores (Bolter et al. 1997). Additionally, our study was designed to test naïve kudzu bugs developed on kudzu plant, inexperienced with soybean plants. Insect behaviors can be plastic and previous feeding experience can modulate behavioral responses of phytophagous insects. McCormick et al. (2016) demonstrated that naïve gypsy moth (*Lymantria dispar* L.) caterpillars prefer the odor of undamaged foliage of black poplar (*Populus nigra* L.), while both naïve and experienced gypsy moth caterpillars are attractive to constitutive and herbivore-induced volatiles.

Overall, our study is expected to contribute to the basic and applied knowledge of kudzu bug biology for developing integrated pest management program. Delaying soybean planting date has been proposed as an alternative method to reduce infestation risk (Blount et al. 2016, Del Pozo-Valdivia et al. 2016). However, delaying planting date is neither an effective nor feasible practice because the adult host-finding behavior is independent of soybean planting date. If late-planted soybeans are the only available choice, kudzu bugs will readily infest regardless they are young or old; similar to what happens with early-planted soybeans in early spring. It is only after choices become available that kudzu bug adults prefer to infest and re-infest plants at early reproductive stages over other growth stages. Besides, planting later will reduce potential soybean yield because of the shorter growing period than early-planted soybeans with a longer growing season (Torrion et al. 2012). Our finding that the early reproductive stage attracts most adults implies it is a critical stage for pest management. This finding provides empirical support for pesticide applications at early reproductive stages providing the best protection of soybean yield (Seiter et al. 2016). Our findings that adults used constitutive volatiles from soybean plant as innate cues in host finding and the volatiles of early reproductive growth stage are the most attractive have particular implications for developing monitoring and trapping techniques and for the potential application of "push-pull" method against this pest. EAG screening process helps reduce the number of volatiles tested to identify promising bioactive components. We are underway in testing the attraction of naïve and experienced kudzu bug to constitutive volatiles of soybean plants and characterizing the compounds that elicited behavioral and antennal activities. Whether young soybean plants after herbivory would become attractive to naïve and experienced (reared on soybean plants) kudzu bugs needs to be investigated.

CHAPTER 3

Differential Attraction, Fecundity and Development of Kudzu Bug, *Megacopta cribraria* (Heteroptera: Plataspidae) to Different Growth Stages and Legume Species

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Introduction

The kudzu bug, *Megacopta cribraria* (Fabricius) (Heteroptera: Plataspidae), native to Asia and Oceania, was first discovered congregating on homes near stands of the invasive weed kudzu, *Pueraria montana* Lour. (Merr.) variety *lobata* (Willd.), in residential areas of northeast Georgia in 2009 (Suiter et al. 2010). This was the first time a member of the family Plataspidae was reported in the western hemisphere (Eger et al. 2010, Jenkins et al. 2010). Since its detection, the kudzu bug has rapidly spread throughout the southeastern United States (Gardner et al. 2013a), www.kudzubug.org/distribution-map/, 2016), where it is not only a residential nuisance pest, but also a serious economic pest of soybean (Fabaceae: *Glycine max* (L.) Merrill) and a threat to international trade of agricultural products to Central America (Ruberson et al. 2013).

In its temperate Oriental distribution, the kudzu bug completes one to three generations per year (Tayutivutikul and Yano 1990, Wu et al. 1992, Li et al. 2001, Chen et al. 2009). In the invaded range of the United States, the kudzu bug completes two generations per year (Zhang et al. 2012b). Its life cycle consists of an egg stage, 5 nymphal stages, and adult stage (Tayutivutikul and Yano 1990, Wu et al. 1992). Eggs are laid typically on plant leaves and new buds, nymphs and adults suck sap from stems, leaf petioles and leaves. Feeding by this insect does not damage seeds directly, but can cause visually apparent necrotic lesions at feeding sites and reduce seeds per pod and individual seed weight (Seiter et al. 2013b). The first generation adults emerge in June. Second generation adults occur in August and migrate to overwinter in protected areas adjacent to host fields or residential houses in late fall (Zhang et al. 2012b, Golec and Hu 2015, Lahiri et al. 2015). Overwintered adults become active again in the spring. Then, adults disperse to seek host plants, mates, and the oviposition peaks occurs in April (Golec et al. 2015). An interesting biological trait is that a female deposits small brown capsules containing symbiotic bacteria before laying egg-masses on them. Newly hatched nymphs consume the obligate bacteria before dispersing to obtain plant food (Hosokawa et al. 2007). The dominate bacterium of the endosymbionts is *Candidatus* Ishikawaella capsulate Mpkobe, which impact the development and host range of this insect (Jenkins and Eaton 2011, Hosokawa et al. 2014).

In its native range, the kudzu bug is considered polyphagous, primarily feeding on legume hosts but also feeding on various non-legume crops and fruit trees (Eger et al. 2010). In China and Japan, it is sometimes a serious pest of soybeans and edible vegetable bean crops (Wu et al. 2006, Kikuchi and Kobayashi 2010). In the southeastern United States, kudzu bug adults have been anecdotally observed on many legume and non-legume species during flight dispersal (Gardner et al. 2013b, Ruberson et al. 2013); but its naturally-occurring completion of full life cycle was only observed on soybean and kudzu (Zhang et al. 2012b). Although the current known host range is limited, the prospect of yet unidentified hosts supporting kudzu bug development in kudzu- and soybean-growing areas is alarming (Dhammi et al. 2016). Several legumes are reported to support development from egg hatch to adults in no-choice greenhouse or laboratory trials (Medal et al. 2013b, Blount et al. 2015, Golec et al. 2015, Huskisson et al. 2015, Medal et al. 2016). However, these studies do not always agree with each other in insect preference and/or plant suitability for oviposition and development, possibly due to the differences in the source/physiological condition of kudzu bugs, plant variety/cultivar/maturity group (Fritz et al. 2016), experimental method, time and condition. Soybean planting date (Blount et al. 2016, Del Pozo-Valdivia et al. 2016) and plant growth stage (Yang et al. unpublished) has been reported having significant impact on soybean susceptibility to infestation by kudzu bugs. However, whether the growth stages of other legume species also affect the attractions of kudzu bug adults are unknown. This information will aid in developing sampling strategies and insecticide application placement for successful management of this pest. This, in combination with contradictory information on the true host range of the kudzu bug led to this study.

We evaluated six legume species which are either economically important to the United States, reported in the literature as host plants, or have been evaluated with contradictory conclusions in the literature. The objectives of this study were four-fold: 1) to assess attraction of kudzu bug adults to different growth stages of each of the selected legume species; 2) to evaluate the comparative attraction of kudzu bug adults to the six legume species at their respective attractive growth stages; 3) to examine the fecundity of kudzu bugs on each of the selected legume species; and 4) to elucidate the suitability of the legumes to serve as a developmental host.

Materials and Methods

Insects and Plants

Kudzu bug adults and egg-masses were collected from a naturally infested wild kudzu patch (32.609111°N, 85.391889 °W) near Town Creek Park in Auburn, Lee County, AL. One day before start of an assay, adults were collected using a sweep net, kept in a mesh-lidded glass container (10 cm diameter, 12 cm height, Uline, Pleasant Prairie, WI) provided with fresh leafed kudzu twigs, and brought to a greenhouse on the Auburn University campus (Auburn, AL). Six legume species (Table 3.1) were selected for this study. The legume species were chosen based on agronomic crops of interest in the southeastern states of the United States. Four seeds were planted in black plastic pots (17.5 cm diameter, 17.5 cm height) containing moistened Sunshine Professional Soil Mix (SunGro Horticulture Canada Ltd., Seba Beach, Alberta, Canada) and thinned to 2 plants/pot for choice assays in 2014 and 1 plant/pot for no-choice assays in 2015. Seeds were planted weekly from February 20th until termination of choice assays in the year of 2014, and from February 23rd until termination of no-choice assays in the year of 2015, to assure availability of the expected growth stages of all the plant species for the assays. Potted plants were maintained in the greenhouse and watered as needed. Plant growth stages were determined using the system developed by Fehr et al. (1971) with slight modifications to generally apply to selected species (Pookpakdi and Chuangphetchinda 1988, Raven et al. 1992)Schwartz and Langham http://beanipm.pbgworks.org/lima-bean). The growth stages were defined based on the characteristics of the legume plants. All assays were carried out in the same greenhouse on Auburn University campus under natural light, 25 ± 2°C (February through April) or 27 ± 2°C (May through June), 50% - 70% RH).

Scientific Name	Common Name	Variety (Cultivar)	Producer
Glycine max (L.) Merrill	Soybean	Mycogen 5N501R2, G5 cultivar	Mycogen seeds company, Indianapolis, IN
Phaseolus lunatus (L.)	Lima bean	Christmas Lima (a.k.a. Giant Lima , Giant Florida Pole)	Alabama Farmers Cooperative, AL
Vigna radiate (L.) R. Wilczek	Mung bean	Crystal (upright type)	http://www.nuts.com, Cranford, NJ
Vigna unguiculata subsp. *unguiculata* (L.) Walp	Black-eyed pea	California blackeye	http://www.nuts.com, Cranford, NJ
Phaseolus vulgaris (L.)	Kidney bean	Red Rover (EX 08520700)	Hirt's Gardens, Wadsworth, OH
Phaseolus vulgaris (L.)	Green bean	Derby (Bush)	Ferry-Morse Seed Company, Fulton, KY

Table 3. 1 Legume species evaluated in greenhouse choice and no-choice assays

Choice Attraction of Adults to Different Growth Stages

Adult choice assay was to study differential attractions of adults to different plant growth stages of each plant species. Four plant growth stages were evaluated and these stages were chosen to represent two vegetative stages (V2: two unfolded trifoliolate leaves and V4: four unfolded trifoliolate leaves) and two reproductive stages (R1: one open flower on the plant and R5: one pod with fully developed seeds). Overwintered adults were collected in April and May 2014 during dispersal peak period before the appearance of egg-mass. Adults were sexed based on external morphology of the terminal abdominal segments (Eger et al. 2010) and were allowed to acclimate to the greenhouse for 24 h before being subjected to assays.

Four potted plants of a single legume species (V2, V4, R1, and R5) were placed at a 40-cm distance to each of the adjacent pots, inside a screen cage (173 x 71 x 91 cm^3, Dura-Tent, Poway, CA). A completely randomized block design with four replicates was used, where cages were considered blocks and potted plants were considered treatment. All replicates were carried out simultaneously for each respective assay. A group of 100 adults (50 females and 50 males) was released at 1100 hrs in the center of the cage from a Petri dish (10 cm diameter, 1.5 cm height, Fisher Scientific, Pittsburgh, PA) which was at an equal distance to each pot. Observations consisted of recording the number of adults landed on each growth stage at 1, 24 and 48 post-release hours as a measure of plant growth stage preference. Preliminary tests showed little change in adult preference after 48 h. Assays were conducted at different dates (20th, 26th of April, and 10th, 16th of May, 2015) for different species due to their different growth rates. In total, the assays used 24 cages (4 cages per plant species), tested 96 potted plants (4 pots per cage) and 2,400 kudzu bug adults (100 adults per cage).

Choice Attraction of Adults to Different Legume Species at Their Respective Attractive Stages

Adult choice assay was carried out to determine if adults would preferably select soybean plants (the preferred reproductive host) over other legume species each at their respective preferred growth stages identified in the previous assay. This information will be useful to determine if any non-soybean species could be the potential trapping crop for managing this pest.

Using the same assay protocol described in the previous adult choice assay, the completely randomized block design had four blocks (cages). Each consisted of six treatments (potted plants) including soybean as positive control. The four replicates were carried out simultaneously. The six treatments were lima bean (R1), soybean (R1), black-eyed pea (R1), kidney bean (R1), mung bean (R5), and green bean (V4). One pot of each legume species were placed inside a custom-made screen cage (2 m diameter, 1.5 m height), arranged in a circle, adjacent to one another in equal distance, with a 10-cm space from the wall of the cage. Newly emerged first generation adults were collected, sexed and allowed to acclimate to greenhouse for 24 h before starting the assay in June of 2014. We have previously reported no difference in adult attraction to host between overwintered and first generation adults (Yang et al. Unpublished data). A group of 100 adults (50 females and 50 males) was released at the center of the cage at 1100 hrs. Observations were conducted as described in the previous section. This assay used 4 cages, 24 potted plants (6 pots per cage), and 400 kudzu bug adults (100 adults per cage).

No-choice Fecundity on the Six Legume Species

Adult no-choice assay was conducted in the same greenhouse to determine the suitability of the six selected legume species for kudzu bug fecundity. Groups of seven caged pots (one legume species per pot plus one empty pot as negative control) were arranged on separate bench tables (2.4 x 0.9 m^2) in a randomized completed block design, and replicated three times. Benches were considered as blocks and cages were considered as treatments. The bench tables were 2-m apart from one another to allow free movement of the observers for observation and data collection. Potted plants were caged individually in front opening collapsible cages (30.3 x 30.3 x 60.6 cm^3, BioQuip 1450M, Rancho Dominguez, CA). All the potted plants were in V3 growth stage (3 unfolded trifoliolate leaves) to be consistent with other studies (Golec et al. 2015; Blount et al. 2016; Medal et al. 2016). Overwintered adults at copulation peak were collected in April 2015 and handled as described in choice assays. Ten pairs of adults were placed into each cage at 1100 hrs, and allowed to feed and lay eggs freely in the cage. The cage and caged plant were inspected daily and the numbers of egg-masses and eggs were counted until five days after no new egg was observed. The hatch rate of eggs within a cage (on plants and on cage) was recorded until all the eggs had hatched.

No-choice Nymphal Development on the Six Legume Species

Nymph no-choice assay was carried out using the same protocol and the same randomized completed block design with 3 replicates, as described in the adults no-choice assay and all the plants were at V3 growth stage when the assay was initiated. The difference was that, instead of adults, twenty 1st instar nymphs were inoculated onto one leaf per caged plant, using a fine pen brush. A caged pot without plant was used as negative control and the 1st instar nymphs were placed on cage screen wall. The 1st instar nymphs were obtained from egg-masses on kudzu plants at the same site of adult collections. Forty-two leaves with egg-masses were cut off, placed in twelve petri dishes lined with moistened filer paper at the bottom, and brought back to the greenhouse in late April 2015. Upon hatching, 1st instar nymphs were allowed 24 h to ingest the endosymbiont capsule contents, after which time they displayed wandering behavior away from the egg-masses. Twenty randomly selected individuals were transferred, using a fine pen brush, to a leaf-node of each caged plant. Plants were inspected every other day until all live nymphs had emerged to adults to obtain data on nymphal development days and survivorships.

Data Analysis

Greenhouse choice assays data were analyzed for responsiveness (proportion of adults observed on a specific plant growth stage (or a specific legume species) relative to the sum of adults observed on all the plant growth stages (or legume species) within a cage using non-parametric Kruskal-Wallis H test at $P < 0.05$ (SPSS version 22, 2013). Individuals that did not land on legume plants were excluded from the statistical analysis. Adults counted at the three post release inspections were analyzed with combined numbers because not all adults had dispersed from Petri dish during the first inspection, and then separately for time-specific numbers because not all plants showed similar time trend of attraction. Data obtained from adult no-choice assay (number of egg-mass as and eggs, and egg hatch rate on each plant species) and nymph no-choice assay (nymphal development days and survivorship on each plant species) were analyzed using separate one-way analysis of variance (ANOVA) and the significant differences among legume species were determined by the Tukey's Honestly Significant Difference (HSD) at $P < 0.05$, with plant species as the fixed effect in the model (SPSS version 22, 2013).

Results

Choice Attraction of Adults to Different Growth Stages

The pooled mean proportions of kudzu bug adults on each growth stage over the 48-h observation are presented in Fig. 3.1. Kudzu bug adults were found on all growth stages with significant differential preferences for different plant growth stages. Furthermore, the most preferred growth stage was not the same for the six plant species. The early reproductive stage (i.e., flowering R1 stage) was clearly the most attractive one or among the most attractive stages in all legumes except for green bean. The young vegetative stage (i.e., V2) proved to be the least attractive, or among the least attractive stages of all legumes except for green bean. R1was the most attractive stage in lima bean ($\chi^2 = 36.718$; df = 3; $P < 0.01$) and soybean ($\chi^2 = 21.830$; df = 3; $P < 0.01$). It also was the most attractive in black-eyed pea ($X^2 = 21.975$; df = 3; $P < 0.01$), though the difference compared with R5 was not significantly different. Kudzu bugs significantly preferred R1 and V4 of kidney bean ($\chi^2 = 33.134$; df = 3; $P < 0.01$) and R1 and R5 of mung bean ($\chi^2 = 32.352$; df = 3; $P < 0.01$). However, adults significantly preferred the V4 stage over the two reproductive stages of green bean (R1 and R5) ($\chi^2 = 20.509$; df = 3; $P < 0.01$).

During the 48-h assay, kudzu bug adults displayed differential orientation penchant to different growth stages of respective legume species (Fig. 3.2). For lima bean (Fig. 3.2a) and soybean (Fig. 3.2b), R1 stage was consistently the most attractive and V2 and R5 the least attractive stages. For kidney bean (Fig. 3.2c), R1 and V4 showed increasing attractiveness while V1 and R5 displayed declining attractiveness. For black-eyed pea (Fig. 3.2d) and mung bean (Fig. 3.2e), adults displayed a slightly decline in orientation penchant to R1 but a slightly increase to R5. For green bean (Fig. 3.2f), adults demonstrated no steady preference pattern to any growth stages.

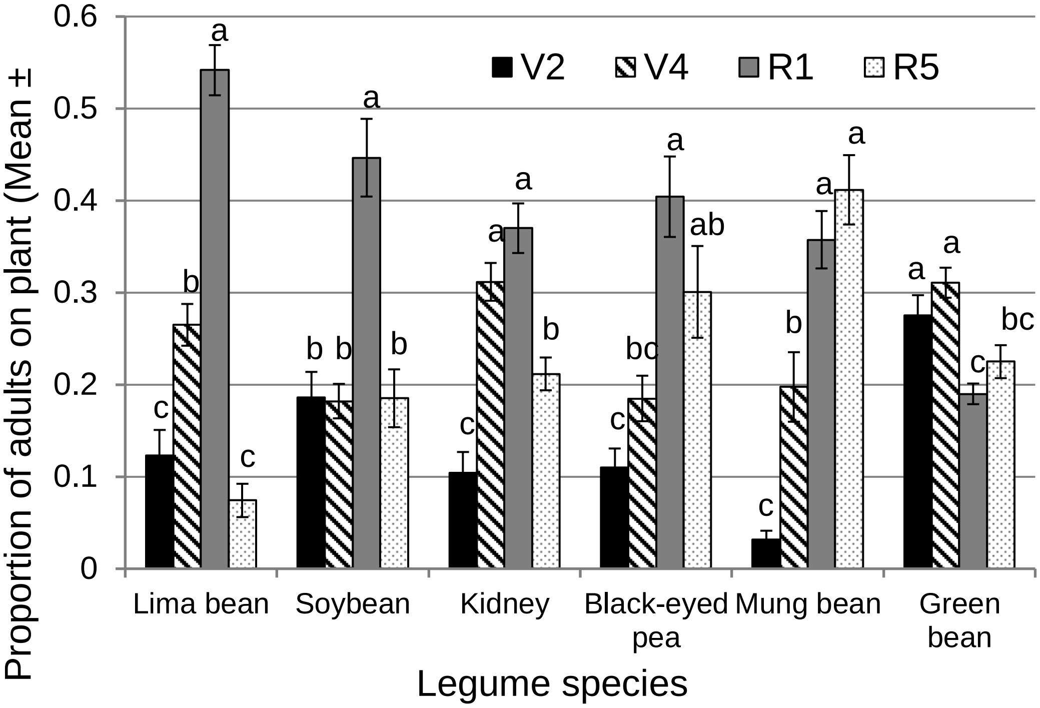

Fig. 3.1 Differential attractions of kudzu bug adults to different plant growth stages in greenhouse choice assay. Pooled 48-h observation proportions (Mean ±SE) of the released adults counted on different growth stages. The same letter above SE bars indicates no significant difference within growth stages (alpha = 0.05).

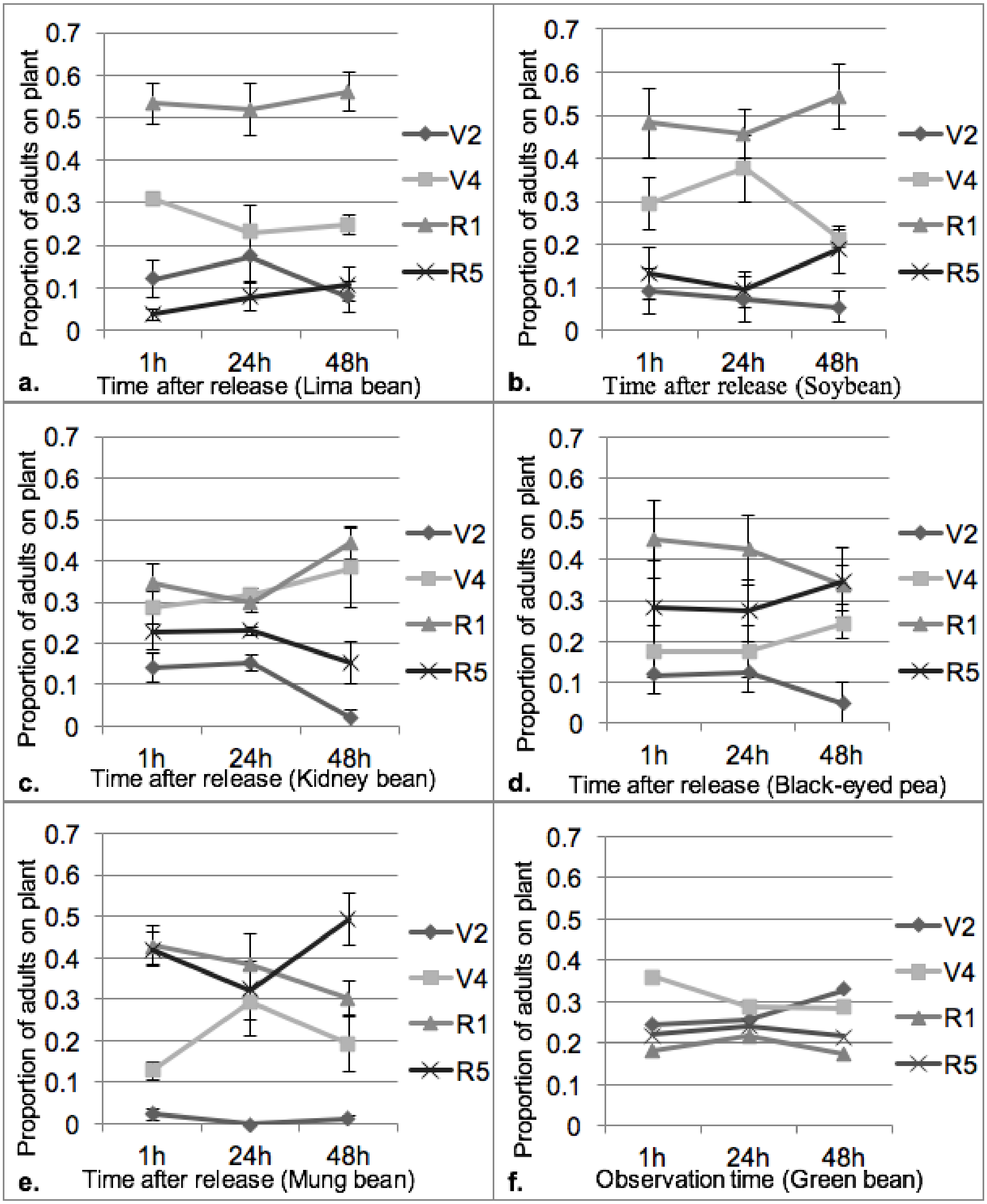

Fig. 3.2 Time-trend of kudzu bug adult attractions to different plant growth stages during the 48-h greenhouse choice assay. The proportions (Mean ±SE) of the released adults counted on different growth stages of each legume species. (a) Lima bean, (b) Soybean, (c) Black-eyed pea, (d) Mung bean, (e) Kidney bean, and (f) Green bean.

Choice Attraction of Adults to Different Legume Species at Their Respective Attractive Growth Stages

When given choice of the most attractive stages, kudzu bug adults exhibited significantly different orientation preference to the respective six legume species ($\chi^2 = 60.079$; df = 5; $P < 0.01$) (Fig. 3.3). Clearly, lima bean was the most preferred, followed by soybean and kidney bean which were not significantly different from one another. The other three legumes were the least preferred. As shown in the growth-stage choice assay, kudzu bug adults moved around seeking a preferred host during the 48-h assay (Fig. 3.4) but the order of preference to the six legume species remained the same.

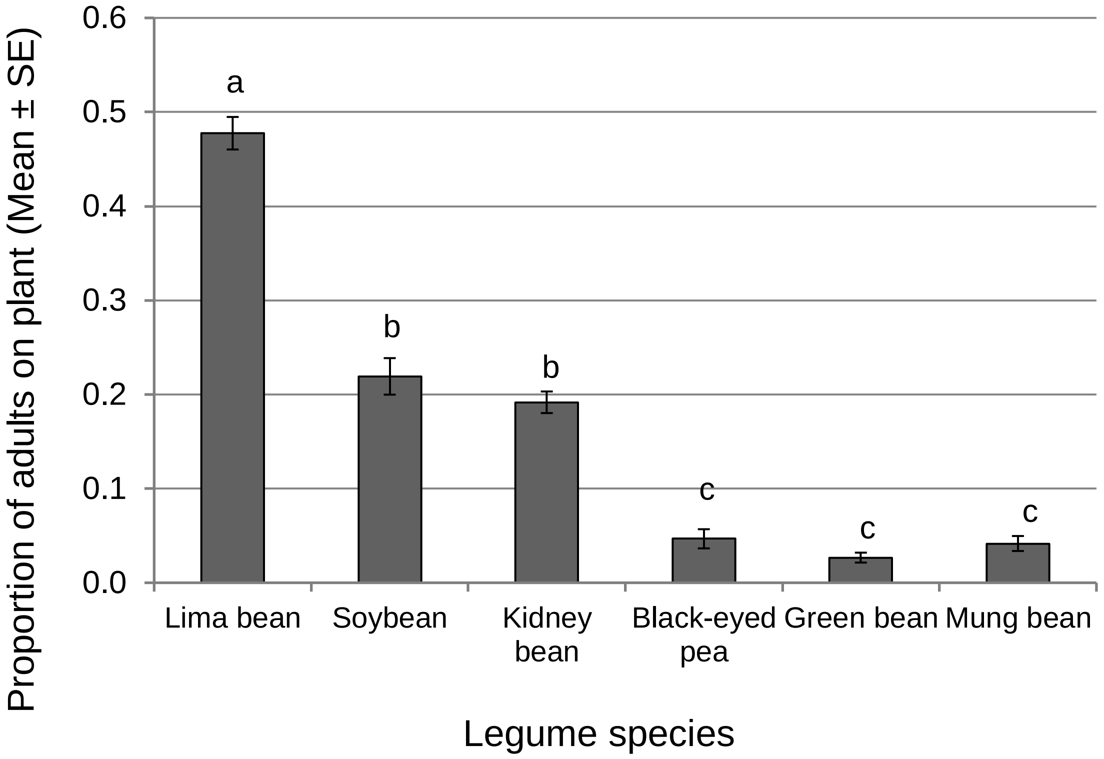

Fig. 3.3 Differential attractions of kudzu bug adults to different legume species at their respective attractive growth stages in greenhouse choice assay. Pooled 48-h observation proportions (Mean ±SE) of the released adults counted on different legume species. The same letter above SE bars indicates no significant difference within growth stages (alpha = 0.05).

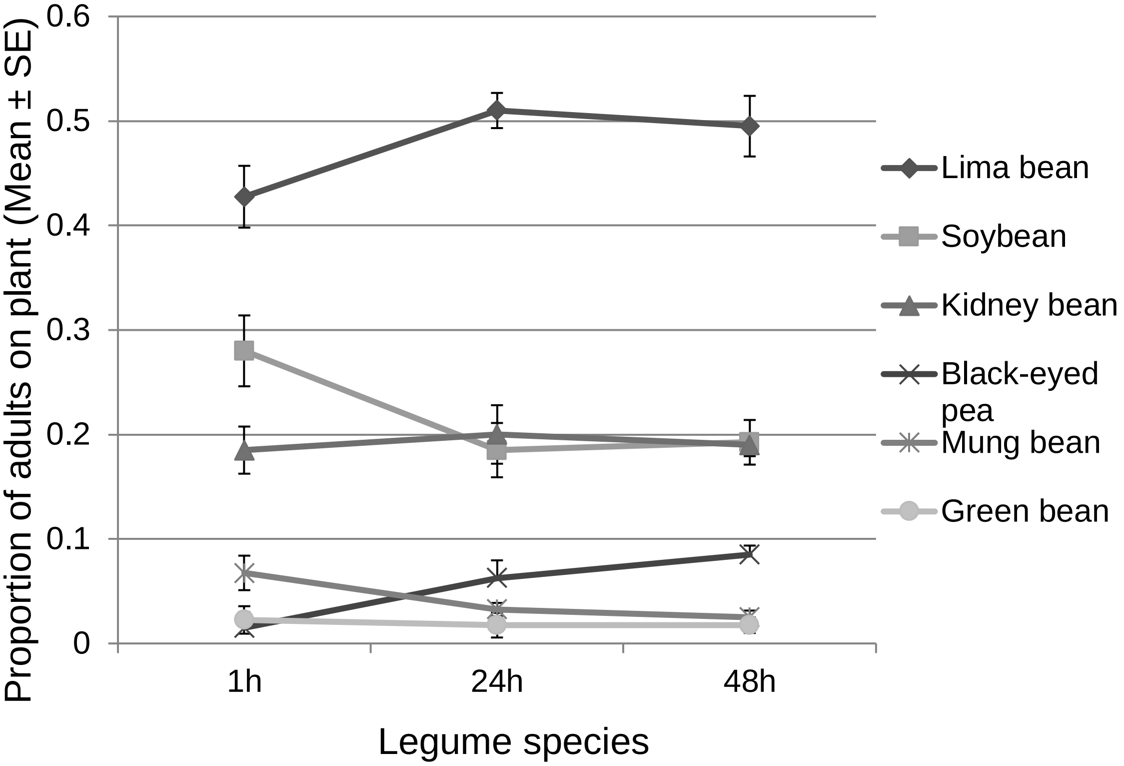

Fig. 3.4 Time-trend of kudzu bug adult attractions to different legume species at their respective attractive growth stages plant growth stages during the 48-h greenhouse choice assay. The proportions (Mean ±SE) of the released adults counted on different growth stages of each legume specie

No-choice Fecundity on the Six Legume Species

Females caged on each legume species laid eggs that hatched at different rates (Table 2). Females of control died without oviposition. However, eggs were only deposited on soybean, lima bean and mung bean. Females caged with black-eyed pea, kidney bean and green bean deposited eggs everywhere but not on plants. Although there was no significant difference in the numbers of egg-masses (F = 1.47, df = 5, 12, P = 0.27) and eggs (F = 0.92, df = 5, 12, P = 0.50), egg hatch rates differed significantly among eggs produced by females feeding on different legume species (*F* = 3.95, df = 5, 12, *P* = 0.02). Eggs produced by females feeding on soybean, lima bean and mung bean had the highest hatch rates, which were higher but did not differ significantly compared with eggs produced by females feeding on black-eyed pea. Eggs deposited on cages hosting kidney bean and green bean hatched at much lower level.

Parameter	Legume common name Soybean* (plant/cage)	Lima bean* (plant/cage)	Mung bean* (plant/cage)	Black-eyed pea**	Kidney bean**	Green bean**	Control	df	*F*-value	*P*-value
Egg-mass	9.0±1.0 (8.7/0.3)	6.7±0.3 (1.7/5)	6.3±2.6 (0.3/6)	5.0±0.6	4.3±1.2	6.0±1.0	0	5,12	1.47	0.27
Egg	68.0±10.5	62.3±5.9	67.3±19.2	67.7±4.9	52.7±8.6	44.7±1.8	0	5,12	0.92	0.50
Egg duration	9.7±1.5	9.0±1.2	9.0±1.2	6.7±0.3	8.5±1.5	9.6±1.5	n/a	5,11	0.88	0.53
Hatch rate (%)	67.9±10.1a	58.1±13.8a	42.6±13.4a	31.3±5.0ab	14.7±7.4b	20.9±9.4b	n/a	5,12	3.95	0.02

Table 3.2 Suitability of legume species for kudzu bug fecundity. Mean (±SE) number of egg-masses, eggs, and egg hatch rates of kudzu bug females caged with a single legume species. Hatch rate is for the total eggs irrespective they were on plant or on cage. Different letters within a row indicate a significant difference using Tukey's HSD (ANOVA, alpha = 0.05). (*Females laid eggs on both plant and cage; **Females laid eggs everywhere but not on plant

No-choice Nymphal Development on the Six Legume Species

Legume species had a significant effect on nymphal survival (*F* = 15.16, df = 2, 6, *P* < 0.01). Nymphs only completed development to adults on soybean, lima bean and mung bean (Table 3). Those on the rest of the legume species died at various nymphal stages. The nymphs had a significantly higher survivorship on soybean and mung bean than on lime bean. Only a few nymphs reached the 5th, 3rd, and 2nd instar stage on kidney bean, green bean, and black-eyed pea, respectively. All the nymphs in the control cage died at the 1st instar stage. Mean developmental days from 1st instar to adult were not significantly different among soybean, lima bean and mung bean (*F* = 1.42, df = 2, 5, *P* = 0.33). Our effort on recording the developmental day and mortality of respective instars produced little meaningful data (data not shown) because this assay was not designed to allow feasibility to accurately track individuals. However, close examination indicated that the greatest mortality across all legume species occurred in the 2nd and 3rd instars. The sex ratio of adults on each plant species was not noted due to an unexpected greenhouse shutdown at the end of this study.

	Legume common name									
Parameter	Soybean	Lima bean	Mung bean	Black-eyed pea	Kidney bean	Green bean	Control	df	*F*-value	*P*-value
Stage of 100% mortality	n/a	n/a	n/a	2nd instar	5th instar	3rd instar	1st instar	2,5	9.22	0.02
Developmental days (1st instar to adult)	47.7±3.4	46.7±0.7	42.0±1.0	-	-	-	-	2,5	1.42	0.33
Final survival (%)	55.0±7.6a	8.3±10.4b	45.0±5.0a	n/a	n/a	n/a	n/a	2,6	15.16	<0.01

Table 3.3 Suitability of legume species for nymphal development. Twenty newly emerged 1st instar nymphs were inoculated on each plant. Mean (±SE) nymphal developmental days and survivorship. Different letters within a row indicate a significant difference using Tukey's HSD (ANOVA, alpha = 0.05).

Discussion

Our greenhouse choice assays provided the first empirical evidence that growth-stage affect the attraction of kudzu bug adults to all legume species but green bean. The results confirm our previous finding that soybean R1 stage was the most attractive (Yang et al. unpublished), supports the recommended soybean treatment during early reproductive stages for kudzu bug control (Reed 2013 http://www.aces.edu/pubs/docs/A/ANR-0500-B/ANR-0500-B.pdf), and helps to explain how soybean growth stages affect control efficacy (Seiter et al. 2016). The finding that the early reproductive stage R1 also was the most attractive, or among the most attractive growth stages, in other legume species (with the exception of green bean, which had vegetative stages more attractive than reproductive stages) is of interest. Our previous study reported that soybean R1 was the most, followed by R3, and soybean R5 and V2 were the least preferred; and the soybean growth-stage effect was attributed to plant volatiles. Would it be possible that these legume species share the same chemical attractants during early reproductive stages? We are currently working on identifying the chemical constituents responsible for the attraction of kudzu bug adults. Our finding offers insight for optimal time of field scouting, developing sampling strategies, and developing kudzu bug management programs. Future study will look into the chemical ecology of kudzu bugs in relations to different legume species.

Legume species choice assay showed a significant orientation penchant of kudzu bug adults for lima bean over soybean and kidney bean, and much less preference for mung bean, black-eyed pea and green bean. This result contradicts the result from Huskission et al. (2015) who used two-choice experiment design and reported that adults preferred kudzu over soybean, and preferred soybean over lima bean. Blount et al. (2015) conducted choice assays of 17 plant species and varieties for adult longevity and reported that edamame-soybean and soybean were the most preferred, though a few adults also were recorded on all the other legumes including mung bean, lima bean, black-eyed pea, fava (*Vicia faba*), pinto-deorho (*Phaseolus vulgaris*) and lablab (*Lablab purpureus*). Their 2012 trial showed a few adults on mung bean, lima bean, and black-eyed pea, but their 2013 trail showed almost no insects on mung bean. The inconsistent results could result from the differences in kudzu bug adults (i.e. physiological conditions), plants (growth stage, variety/cultivar, genotype), and experiment method and conditions. Huskisson et al. (2015) used adults that were collected from various temporary plants in late fall when the adults were preparing to overwinter, tested only 15 adults per replicate, and used soybean and lima bean plants 5 wk from seeds (no information on variety). Small sample sizes are known to lead to incorrect *P* values in animal behavior experiments (Mundry and Fischer 1998). Blount et al. (2015) used adults collected in summer when adults were at various physiological stages (virgin, mated, reproductive stage, or post-oviposition) and plants of 3-leaf stage of all the species. They sowed the plants in 10-cm pot, and conducted the 17-choice assay in small cages of 75 x 75 x 175 cm^3 where the plants not only touched each other, but were also at unequal distances from where the adults were released. Furthermore, in the 19-d trials, they only recorded the number of adults on respective plants (not adult longevity as they stated) and collected no information about female oviposition and fecundity. Previous exposure to different foods may affect insect host selection (Jaenike 1988). Plant growth stage significantly influences the attraction of kudzu bug adults to legume plants, as we have proven in this study. Too many plants in a choice test are known to inevitably affect host location of insects, because many key odor compounds often occur at very low concentrations in complex background of volatiles (Mauchline et al. 2008, Sun et al. 2010, Lu et al. 2012). In our study, we used kudzu bug adults collected from the same kudzu patch during the period they were actively seeking food and mate, utilized specific attractive growth stages of plant species, employed much bigger cages so the plants not only had a spacious distance from one another but also were equal distant to the insect-releasing center.

Adult orientation preference data offered no information about whether the insects would feed or simply rest on the plants, but may be indicative of the likelihood that migrating adults feed on the plants. Our no-choice assays confirmed that confined female oviposited on every legume species that and no egg-laying were laid by the control females that did not have access to a plant that died, and likely died of starvation. This result provides the empirical evidence that all the tested legume plants, including the least preferred green bean, were used for temporary feeding needed for oviposition. Our data on female fecundity indicates that plant food source had a significant impact on female fecundity. Egg hatch rates were significantly higher for females fed on soybean, lima bean and mung bean, compared with the rest of the plants. Previous observations suggested kudzu bug adults during migration feed on a broader range of plants (Gardner et al. 2013a). A recent DNA analysis of gut-content from migrating kudzu bug adults showed presence of kudzu, lettuce, loblolly pine, red oak and walnut, though not knowing if the plant materials were ingested by adult feeding on plants or acquired from pollen-contaminated capsules by 1st instar nymph (Lovejoy and Johnson 2014). Feeding on mixed diets of different host plants (polyphagous feeding behavior) may be an adaptive strategy of insects to avoid overspecialization (dependency) on preferred host plants which are only available at certain seasons (Bernays and Chapman 1994).

While it is clear adult *M. cribraria* can be found on a wide range of plant species, they only have a limited range of host plants for oviposition and development. Our no-choice assay shows that, among the tested legume species, only soybean, lima bean and mung bean were the choice for oviposition, and the only three plants species that supported complete development from 1st instar to adults. In field observations, Blount et al. (2015) also reported adults on soybean, Edamame-soybean (*Glycine max*, Mosley' O.P. prolific), mung bean, lima bean and several other legumes. This may supports the performance-preference hypothesis that females made their oviposition choice to maximize offspring fitness (Cunningham et al. 1999). Previous greenhouse studies and field observations agree that kudzu and soybean are the primary host plants and on which adults lay eggs and nymphs develop, however differences exist regarding other host plants (Zhang et al. 2012b, Medal et al. 2013b, Blount et al. 2015). The disagreements were the black-eyed pea, lima bean and mung beans where results were inconsistent between studies. Our results agree with Zhang et al. (2012) that there is no adult development occurred on black-eyed pea while Medal et al (2013) had opposite results. Our results corroborated Medal et al. (2013) and Golec et al. (2015) that nymphs completed development on lima bean while Blount et al. (2013)

showed all nymphs died within 1 wk. Our results confirmed that nymphs can complete development on mung bean (Golec et al. 2015) while Blount et al. (2015) demonstrated the opposite. Medal et al. (2013) reported pigeon bean (*Cajanus cajan*) an excellent host for laboratory rearing of kudzu bugs. The difference could be due to the differences in plant variety/cultivar or/and the difference of in physiological state and plant growth stage. The composition of the symbiotic gut capsule may have a major influence on host range of the kudzu bug (Hosokawa et al. 2014). Nutritional strength also may influence kudzu bug host preference (Srinivasaperumal et al. 1992).

The no-choice tests clearly show that some economically important agricultural crops are at risk of being infested by this newly introduced plant pest, particularly as there are more legume species from the kudzu bug's native regions that are being grown in the United States. The finding that lima bean at the early reproductive stage was more attractive to kudzu bug adults than other legume species, including soybean, but poorly supported oviposition and progeny development, indicates that this plant species may have potential as a trap crop for this invasive pest. Trap cropping is a traditional tool that manipulates an agroecosystem as part of an integrated pest management strategy (Shelton and Badenes-Perez 2006). Trap cropping encompasses the inherent characteristics of the trap crop plants as well as the strategies associated with their development to serve as a sink for an interested insect pest. We plan to investigate why kudzu bug adults preferred lima bean to soybean yet females had an ovipositional preference for soybean and examine preferences during the time a trap crop should be available to migrate adults. Future studies will extend the preference study under field conditions to evaluate the potential of utilizing lima bean as perimeter trap crop to intercept overwintered kudzu bug adults migrating into early-planted soybean field as a tactic of integrated management, and explore the relationship between host preference and plant semiochemicals and nutrients.

CHAPTER 4

Electrophysiological and Behavioral Responses of the Kudzu Bug, *Megacopta cribraria* (Hemiptera: Plataspidae) to Volatile Compounds Identified from Host Plants, Kudzu (*Pueraria montana* (Loureiro) Merrill var. *lobate* (Willdenow)) and Soybean (*Glycine max* Merrill.)

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Introduction

Successful host location by phytophagous insects is crucial for their development and reproduction (Dicke and van Loon 2000, Bruce et al. 2005). This complex process is mediated by sensory cues including both long-range attraction and short-range cues, such as olfactory, visual, gustatory, and tactile stimuli (Bernays and Chapman 1994). Among them, plant odor cues play an important role in host location. Plant volatile emission can be constitutive, or induced in response to a variety of stresses. The constitutive volatiles provide valuable information for insects that facilitate locations of food (Byers et al. 1985, Blight et al. 1995, Stensmyr et al. 2001), as well as those plants that are suitable oviposition sites (Showler 2001, Broad et al. 2008). Furthermore, the presence of conspecific or heterospecific phytophagous insects on plants alters the blend of volatiles emitted by plants (Dicke and van Loon 2000, Tumlinson and Engelberth 2008), thereby providing additional information for host plant evaluate and the potential suitability of plants for colonization (De Moraes et al. 2001, Meiners et al. 2005). Whereas induced volatile emission of certain plant volatiles can be adaptive if their presence alters the behavior of the interacting herbivore with the resultant effect of a fitness benefit for the plants (Bruce et al. 2005).

According to the meta-analysis from Szendrei and Rodriguez-Saona (2010), only 3% of studies have confirmed that the plant volatiles examined had repellent effects, while 76% of plant-insect interaction studies have demonstrated attractions of insects to plant volatiles. The classes of attractive chemicals in plant volatiles consist primarily of aldehydes, alcohols, esters, ketones. When using attractants in baits for insects that mimic plants, aldehydes were the most effective chemicals. Among aldehydes, phenyl acetaldehyde has been reported to be used in 65% of the examined studies. (Szendrei and Rodriguez-Saona 2010). The green leaf volatiles (i.e., 3-

hexen-1-ol, 3-hexenyl-acetata, and hexanal), which are highly volatile and ubiquitous in plants, are easily detectable by a diverse range of insects including both specialists and generalists (Schoonhoven et al. 1998, Arimura et al. 2005). For example, green leaf volatiles play an important role in the orientation of the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) (Visser 1979). Other chemicals had also been reported, for example, adults and larvae of the coding moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) are highly attracted to the pear ester (ethyl, (E, Z)-2,4-decadienoate) (Light et al. 2001). Once the active chemicals in host plant volatiles are identified, their functions need to be further explored to understand how they contribute to plant-insect interactions, and then they can be exploited for the development of alternative IPM program for pest control.

The kudzu bug, *Megacopta cribraria*, is an important pest of legume plants, and in the U.S. it primarily develops on kudzu (*Pueraria montana* (Loureiro) Merrill var. *lobate* (Willdenow)) and soybean (*Glycine max* (L.) Merrill.) (Zhang et al. 2012a). Damage to soybeans results in necrosis, spot discoloration, defoliation, improperly developed pods, wilt and poor seed set (Tayutivutikul and Yano 1990, Xing et al. 2006, Suiter et al. 2010). Soybean yield losses around 60% can occur if left field are left untreated (Li et al. 2001, Seiter et al. 2013b). Since its earliest detection in Georgia in 2009, its distribution has grown to include nearly every county in Georgia, Alabama, North Carolina and South Carolina with confirmed presence in at least 13 additional states in the U.S. (Gardner et al. 2013b)www.kudzubug.org, 2016). With the continued geographic expansion of this pest in the U.S., its impact on soybeans will continue to increase. Not only is it a pest of soybeans, it is continually reported as a residential nuisance pest during time it seeks overwintering sites, and cause a threat to international trade of agricultural products to Central America (Ruberson et al. 2013). Though several natural enemies (a specialist and several generalists) have been discovered in (Gardner et al. 2013b, Golec et al. 2013, Ruberson et al. 2013); however their efficacy is limited due to their seasonal occurrence and short life cycle (Takasu 2012), and most do not reduce economically damaging populations. White color traps, such as cross-vane trap (Horn and Hanula 2011) and white polyvinyl chloride adult sticky traps (Dhammi et al. 2016), are effective for monitoring the insect population in the soybean fields but is not useful for their control. Physical control, such as removing kudzu by mowing is not practical in the soybean fields, because it must be repeated nearly weekly during the growing season for multiple years (Golec et al. 2014). Insecticide application is currently the only short-term management solution of this pest. Therefore, the development of alternative approaches to control *M. cribraria* based on manipulation of behavior by exploitation of semiochemicals against *M. cribraria* may offer a different mechanism of control, thus contributing to a better IPM approaches to its management.

Megacopta cribraria has been reported to feed on more than 34 plant species worldwide. Several legumes were reported to be potential hosts of *M. cribraria* by greenhouse or laboratory trails. Studies by Zhang et al. (2012) demonstrated that even though large numbers of *M. cribraria* were found on yellow wood (*Cladrastis kentukea* (Dum. Cours.) Rudd) and black locust (*Robinia pseudoacacia* L.), kudzu and soybean were the only two hosts that *M. cribraria* can complete the development on among 11 tested plant species. Medal et al. (2013) evaluated the host suitability of 12 plants, including 11 legume species in greenhouse no-choice tests and found that, besides kudzu and soybean, *M. cribraria* also can complete development on pigeon pea, followed by black-eye pea (*Vigna sinensis* L), lima bean (*Phaseolus lunatus* L.) and pinto bean (*Phaseolus vulgaris* L.). Other authors also reported some other plants as the reproduction host of *M. cribraria*. For example, mung bean (*Vigna radiate* L. Wilczek) (Golec et al. 2015), fava bean (*Vicia faba* L.) (Blount et al. 2015), hummingbird tree (*Sesbania grandiflora* Pers.), fire cracker flower (*Crossandra undulaefolia* Salisb), and cotton (*Gossypium hisutum* L.) (Srinivasaperumal et al. 1992). However, these studies do not always agree with each other in insect preference and/or plant suitability for oviposition and development (Medal et al. 2013b, Blount et al. 2015, Golec et al. 2015, Huskisson et al. 2015, Medal et al. 2016, Yang et al. unpublished data). Although more host plants may exist in the U.S. that will support the full life cycle of *M. cribraria*, only kudzu and soybean support the production of a complete generation in the field (Dhammi et al. 2016).

Our preliminary studies demonstrated that host-finding behavior of *M. cribraria* is an olfactory response to constitutive volatile chemicals associated with whole soybean plants where adults were most attracted to early reproductive stages of soybean (Yang et al. unpublished data). To understand the chemical elicitors involved in the interactions of host plants with kudzu bugs, this study aimed to identify the bioactive volatile compounds in host plants (kudzu and soybean) by using a combined approach consisting of gas chromatography-electroantennographic detection (GC-EAD), gas chromatography-mass spectrometry (GC-MS), electroantennogram (EAG) and olfactometer bioassay.

Materials and methods

Insect

First generation *M. cribraria* adults used for antennal response assays were collected from kudzu in Gainesville, FL (29°35’39.12” N 82°25’36.01” W) and provisioned with fresh kudzu leaves. Second generation adults used for behavioral response assays were collected from a wild kudzu patch (32°36’33.01” N 85°30’01.83” W) in Auburn, Lee County, AL, using a sweep net from

August through September 2016. Adults were brought to a greenhouse and maintained in plastic framed mesh cages (30 x 30 x 30 cm^3) (Bugdorm TM, BioQuip Products, Rancho Dominguez, CA) supplied with fresh kudzu shoots and changed daily.

Plants

Soybean plants were grown from seeds (Mycogen 5N501R2, G5 cultivar, Mycogen Seeds Company, Indianapolis, IN) in a greenhouse on the Auburn University campus (32.586691 N, 85.488764 W), and maintained under 24 ± 3 °C, 50% - 70% RH, and ambient light. Three seeds were planted in each plastic pot (17.5 cm in diameter, 17.5 cm in height) with moistened Sunshine Professional Soil Mix (SunGro Horticulture Canada Ltd., Seba Beach, Alberta). Seeds were planted weekly, begin on April 20th until May 30th to ensure the availability of R1-R2 stage (growth stage commonly preferred by kudzu bugs) soybean plants for GC-EAD and GC-MS assays that were conducted in the United States Department of Agriculture (USDA), in Gainesville, FL. Kudzu plants were grown from field-collected root crowns (32°36’32.80” N, 85°30’02.66” W, Auburn, Lee Co., AL) on March 2nd, 2016.

Chemicals

Synthetic chemicals included 1-octen-3-ol (98%), 3-octanol (99%), decanal (99%); methyl salicylate (99%), D-nerolidol (98%), farnesene (≥85%, with isomer), tridecane (99%), L-linalool (95%), D-limonene (97%), dodecane (99%), 2-ethyl-1-hexanol (99%), ocimene (90%, with isomer), 3-hexen-1-ol (97%), and benzaldehyde (98%) were purchased from Sigma-Aldrich, St. Louis. MO. Nonanal (98%) was purchased from Acros Organic, Fisher Scientific, Pittsburgh, PA.

Plant Volatile Collection

Whole, undamaged soybean plants at R1-R2 stage (growth stage commonly preferred by kudzu bugs) and kudzu, were placed into a large glass volatile collection chamber (17.5 cm in diameter by 85 cm in height) (Analytical Research Systems (ARS), Gainesville, FL) for headspace volatile collection. Prior to use, glass chambers were washed with soap and water, rinsed with distilled water, then acetone, and heated to 40^{o}C for at least 6 hrs. For volatile collection, the port at the top of the glass chamber was used for air inlet and a port at the bottom of the chamber used for an outlet port. A Teflon guillotine system isolated the above-ground plant structures in the chamber with the pot below. Inlet and outlet ports were connected with corrugated Teflon tubing to both an air delivery system and volatile collection system, respectively (ARS Inc., Gainesville, FL). Charcoal-filtered and humidified air was delivered into the collection chambers through the inlet port at 1L/min and air drawn from the collection chambers through the outlet port at 900 mL/min. The flow difference for pushing and vacuum pumps was set to prevent collecting outside air from possible leakages. Volatile collection traps containing 30 mg HaySep-Q (80-100 mesh) (ARS Inc., Gainesville, FL) were placed at the outlet post and collections made for 24 hrs. Immediately after removal of collection traps from the apparatus, volatiles were eluted with 200 µL hexane (Sigma-Aldrich, St. Louis. MO), and stored at -18°C. Collections were conducted in the greenhouse at 27 ± 3°C and 50 - 70% RH in USDA, Gainesville, FL.

Chemical Analysis (GC-MS)

Chemical analysis was conducted using an HP 6890 gas chromatograph with an HP 5973 MS detector in EI mode. Plant extract samples (1 µL) were injected into a splitless injector onto an HP-5MS column (30 m x 250 µm (inner diameter) x 0.25 µm film (Agilent Technologies, Santa

Monica, CA). The GC oven was programmed to 50°C (initial), holding for 10 min, then increasing to 180°C at 2°C/min, held for 1 min, then increasing 10°C/min to 240°C. The carrier gas was helium (1.2 mL/min). Chemicals were identified through comparison with mass spectra libraries and retention times of standards.

GC-EAD

To determine which individual volatile compounds elicited the antennal response from female *M. cribraria*, the GC-EAD analysis was conducted using a gas chromatograph (GC) (6890N, Agilent Technologies) equipped with a flame ionization detector (FID) and an electroantennographic detector (Syntech, Hilversum, Germany). The GC was equipped with a fused silica capillary column (30 cm x 0.25 µm) coated with (5%-phenyl) dimethypolysiloxane (HP-5, 0.25 µm thick, Agilent Technologies). One µL of each sample was injected (splitless mode) with injector and detector temperatures at 250°C and 275°C, respectively. The oven temperature was programmed at 40°C for 1 min, rising to 250°C at 10°C /min, and then held for 4 min. The column effluent was split 50:50 with a Gerstel 4-way cross (Gerstel, Mulheim, Germany) (one outlet blocked) between the FID and EAD with two 100 cm lengths of deactivated fused silica capillaries (0.25 µm ID, Agilent Technologies). Helium was used as the carrier gas at 0.4 m/sec for the FID and EAD. The capillary leading to the EAD ran through a heated transfer line and temperature control unit (Syntech) (250°C) with the capillary end discharging into a glass tube (1 cm diameter) containing charcoal-filtered, humidified air (0.5 m/sec). The antennal preparation was 0.5 cm from the tube opening. Each antenna was amputated at the base and the tip of the antenna was removed. The dissected antenna was immediately attached with electrode gel (Spectra 360® Electrode Gel, Parker Laboratories, Orange, NJ) to an Ag-AgCl electrode with 10-x amplification (Syntech). The signal was processed through a 2 channel serial-bus acquisition controller (IDAC2, Syntech) and analyzed with software (EAD ver. 2.5 Syntech). For each plant volatile extract, electroantennograms were recorded from more than three antennae from three female adults (one from each adult) (Zhang et al. 2008). Antennal responses were matched with FID signals of compounds eluting from the GC.

Comparative EAG Responses to Selected Compounds

To further identify chemicals from plant volatile extracts that may be potentially responsible for the attractions of *M. cribraria*, the antennal receptivity of adult female *M. cribraria* to 15 selected chemicals (10 µg/µL) was determined by EAG. The antennae were removed from the base, and the tip of the antenna was removed. Dissected antenna was immediately fastened with electrode gel (Spectra 360 Electrode Gel) onto an Ag-AgCl electrode.

Each test chemical was diluted in hexane to 10 µg/µL for use in comparative EAG response tests. To prepare treatments for testing, 10 µL of each chemical solution was applied to a piece of filter paper strip (0.8 x 5cm^2, , Fisher Scientific, Pittsburgh, PA). The solvent was allowed to evaporate from the filter paper for 1 min, then the paper strip was placed inside a glass Pasteur pipette (15 cm in length) (Fisher Scientific, Pittsburgh, Pennsylvania, USA). The tip of the pipette was inserted about 3 mm into a small hole through the wall of a glass tube (0.8 cm diameter, 10 cm long) directed at the antennal preparation. A continuous flow rate (0.6 L/min) of charcoal-filtered and humidified air was provided by a stimulus controller (CS-05, Syntech), with a stimulus duration of 10 s. A 90 s interval between successive stimulations was allowed for antennal recovery (Ceballos et al. 2015). EAG responses to 10 µL of hexane was tested as control. Responses to blank (clean air) and standard (a hexane extract of kudzu volatiles) treatments were obtained before and after all the test chemicals so that correction could be made in the event of loss of sensitivity of the preparation during the recording. It was assumed that the decrease in sensitivity was linear with time for the correction. EAG recordings were obtained from five antennae for each solution. Signals were stored and analyzed using EAD ver. 2.5 software (Synthech, Hilversum, The Netherlands). EAG responses to volatiles were calculated as corrected absolute EAG values (Kang and Hopkins 2004).

Dose-dependent EAG Response

Four chemicals that elicited the strongest EAG responses (mV) were selected for evaluation of the effect of concentration on responses of *M. cribraria*. These included 1-octen-3-ol, nonanal, benzaldehyde, and ocimene. The EAG method was the same as aforementioned in comparative EAG test. Five concentrations (10^{-2}, 10^{-1}, 1, 10, and 100 µg/µL) (Michereff et al. 2013) of each chemical were prepared in hexane and tested. Treatments of each chemical were made in order of increasing concentration with 90 sec intervals between stimuli. Hexane was used as a solvent control and 10 µL of kudzu volatile extracts was used as a reference standard. Five replicates (each antenna was treated as one replicate) were obtained for each solution. Data were calculated as corrected absolute EAG values (Kang and Hopkins 2004).

Y-tube Olfactometer Bioassays

Four chemicals were selected for evaluation for attraction responses in bioassays based on both the strongest EAG responses elicited in this study and their roles in attraction or having high EAG responses on other insects (Table 4.1). These included 1-octen-3-ol, nonanal, benzaldehyde, and ocimene. Additionally, two compounds (farnesene and methyl salicylate), previously reported as attractants to natural enemies (Table 4.1), were also tested. The solvent for all synthetic standard solutions of volatile compounds was hexane (EMD Millipore Corporation Billerica, MA).

Chemical	Test insect	Response	Reference
Nonanal	*Culex pipiens quinquefasciatus* (Diptera: Culicidae)	Attract	(Syed and Leal 2009)
	Galerucella placida Baly (Coleoptera: Chrysomelidae)	Attract	(Malik et al. 2016)
	Triatoma infestans (Hemiptera: Reduviidae)	EAG	(Guerenstein and Guerin 2001)
	Aedes aegypti (Diptera: Culicidae)	EAG	(Ghaninia et al. 2008) (Logan et al. 2008)
	Microplitis mediator (Hymenoptera: Braconidae)	Attract	(Li et al. 2014)
	Graphium sarpedon nipponum (Lepidoptera: Papillionidae)	EAG	(Li et al. 2010)
	Callosobruchus maculatus (Coleoptera: Bruchidae)	Attract	(Adhikary et al. 2015)
	Sitotroga cerealella (Lepidoptera: Gelechiidae)	Attract	(Fouad et al. 2013)
1-octen-3-ol	*Galerucella placida* (Coleoptera: Chrysomelidae)	Attract	(Malik et al. 2016)
	Anopheles (Kerteszia) sp. (Diptera: Culicidae)	Attract	(Laporta and Sallum 2011)
	Glossina spp. (Diptera: Glossinidae)	Attract	(Vale and Hall 1985)
	Cis boleti (Coleoptera: Ciidae)	Attract	(Thakeow et al. 2008)
	Lutzomyia (Lutzomyia) longipalpis (Diptera: Psychodidae)	EAG	(Sant'ana et al. 2002)
Methyl salicylate	*Cnaphalocrocis medinalis* (Lepidoptera: Pyralidae)	EAG	(Sun et al. 2014)
	Hypothenemus hampei (Coleoptera: Curculionidae)	Attract	(Cruz-López et al. 2016)
	Prorops nasuta (Hymenoptera: Bethylidae)	Attract	(Cruz-López et al. 2016)
	Phymastichus coffea (Hymenoptera: Eulophidae)	Attract	(Cruz-López et al. 2016)
	Geocoris pallens (Hemiptera: Geocoridae)	Attract	(James 2003)
	Chrysopa nigricornis (Neuroptera: Chrysopidae)	Attract	(James 2003)
	Diaphorina citri (Hemiptera: Psyllidae)	Attract	(Mann et al. 2012)

E,E-α-farnesene	*Microplitis mediator* (Hymenoptera: Braconidae)	Attract	(Li et al. 2014)
	Lobesia botrana (Lepidoptera: Tortricidae)	GC-EAD	(Anfora et al. 2009)
	Anaphes iole (Hymenoptera: Mymaridae)	EAG	(Williams III et al. 2008)
	Lobesia botrana (Lepidoptera: Tortricidae)	EAG	(Tasin et al. 2005)
	Cydia pomonella (Hymenoptera: Mymaridae)	EAG	(Ansebo et al. 2004)
Ocimene	*Prorops nasuta* (Hymenoptera: Bethylidae)	Attract	(Cruz-López et al. 2016)
	Phymastichus coffea (Hymenoptera: Eulophidae)	Attract	(Cruz-López et al. 2016)
	Anthonomus eugenii (Coleoptera: Curculionidae)	Attract	(Muniz-Merino et al. 2014)
	Myllocerinus aurolineatus (Coleoptera: Curculionidae)	Attract	(Sun et al. 2012)
	Cephus cinctus Norton (Hymenoptera: Cephidae)	Attract	(Piesik et al. 2008)

Table 4.1 Previously reported chemicals identified from kudzu and soybean as well as biological responses in other organisms.

Bioassays were conducted using a Y-tube olfactometer (diameter: 2.5 cm; common tube length: 25.0 cm; length of each arm: 21.0 cm) with the two arms extended at 90° to confirm the bioactivities of single compounds on female *M. cribraria* (Horton and Landolt 2007). In addition, behavioral bioassays of male *M. cribraria* to benzaldehyde were also tested, because males showed stronger behavioral responses than females to benzaldehyde in the pre-test. The olfactometer apparatus (Fig. 4.1) consisted of an air pump (Hydrofarm, Inc. Petaluma, CA) producing two air streams, with each going through a glass bottle (100 mL) each containing 25g of activated charcoal, bubbled through 50 mL of purified water in the second glass bottle, an odor-source-glass bottle containing a filter paper strip (2 x 1cm^2, Fisher Scientific) and a flowmeter. An aliquot (10 µL) of each test solution was applied to a filter paper strip (2 x 1cm^2), and the solvent was allowed to evaporate (1 min) before inserting into an odor-source glass bottle connected to one arm of olfactometer. The control glass bottle connected to another arm of olfactometer contained filter paper strip treated with 10 µL hexane only. Airflow through the olfactometer arms was 200 mL/min. To minimize visual distraction for the adults, the Y-tube olfactometer was held vertically and placed inside a white paper box (82 x 82 x 61 cm^3). Illumination was provided by a fluorescent bulb (ca. 100 lux). Chemicals were dissolved in hexane in different concentrations (10^{-2}, 10^{-1}, 1, 10, and 100 µg/µL). A line was drawn on each of the two arms 10 cm from the arm junction. If an adult *M. cribraria* crossed the line within 10 min, it was considered as having made a choice (Bertschy et al. 1997). The positions of the two arms of the olfactometer were systematically switched after testing two insects to avoid positional bias. After completing tests with 5 adults, the olfactometer was washed with soapy water, rinsed with water then ethanol and then dried using a blower. Forty adults were tested individually for each concentration of chemical solutions. The data were generated using 1400 (1200 females, 200 males) different individual adults. The experiments were conducted from 09:00 until 15:00 in the laboratory under 24±3°C and 50-70% RH.

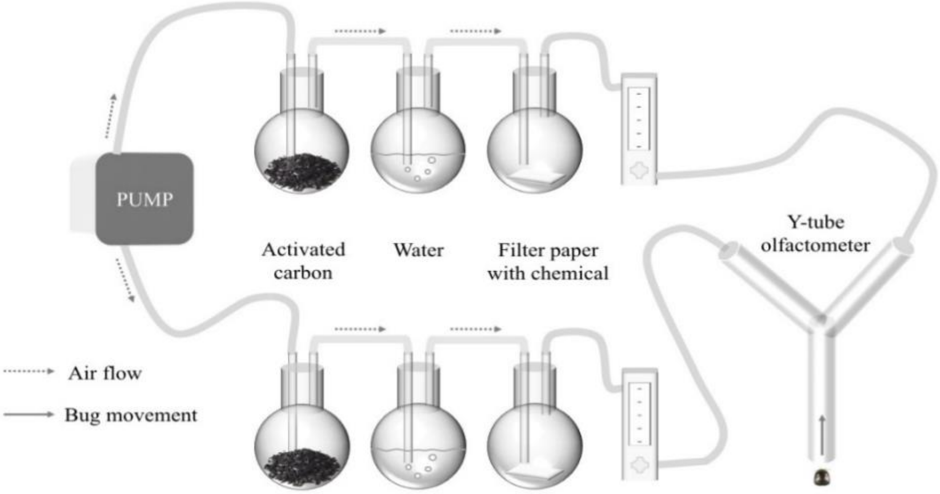

Fig. 4.1 The illustration of Y-tube olfactometer and air deliver system

Data Analysis

Data on EAG responses to synthetic chemical solutions were analyzed by ANOVA Tukey's Honestly Significant Difference (HSD) test ($P < 0.05$) to compare the absolute EAG values among different chemicals, and EAG values among different dose of specific chemicals. Regression analysis was also used for the dose-dependent EAG response data to assess the effect of chemical concentration (SPSS version 22, 2013). The concentration of chemicals was $\log^{10}$-transformed.

Results of Y-tube olfactory bioassays were analyzed with a χ^2 test to test the null hypothesis that there was no preference of adult *M. cribraria* to odors of selected chemicals. The 95% confidence intervals were counted using Agresti & Coull interval (Brown et al. 2002). Individuals that did not make a choice were excluded from the statistical analysis.

Results

GC-EAD and Chemical Analysis

Overall, 24 compounds were identified from extracts of volatiles from kudzu plants, 18 compounds were identified from extracts of volatiles from soybean plants with ten chemicals were common to both plant extracts (Table 4.2). The identified compounds were primarily alcohols, aldehydes, ketones, terpenes and alkanes. Of these chemicals, 8 common chemicals identified from both plant extracts and 7 chemicals identified only from kudzu plant extracts were selected to test for the antennal response by EAG. These chemicals included 1-octen-3-ol, 3-octanol, 3-hexen-1-ol, decanal, methyl salicylate, D-nerolidol, E, E-α-farnesene, tridecane, L-linalool, D-limonene, dodecane, 2-ethyl-1-hexanol, ocimene, benzaldehyde, and nonanal.

No.	Library/ID	Identified in kudzu plant volatiles	Identified in soybean plant volatiles
1	1-octen-3-one		+
2	1-octen-3-ol	+	+
3	3-octanol	+	+
4	2-ethyl-butanal		+
5	3-hexen-1-ol, actate, (Z)-	+	+
6	D-limonene	+	
7	1-hexanol, 2-ethyl	+	
8	Benzaldehyde	+	
9	Cis-pinene		+
10	α-ocimene or β-ocimene	+	+
11	L-linalool	+	
12	Nonanal	+	
13	Methyl salicylate	+	+
14	Dodecane	+	
15	6-undecanone		+
16	Decanal	+	+
17	2-decenal,(E)-	+	+
18	Furan, tetrahydro-2,5-dimethyl-		+
19	Tridecane	+	+
20	α-copaene	+	
21	2-octenal, 2-butyl		+
22	α-gurjunene	+	
23	Aromadenrene	+	
24	α-cubebene	+	
25	E, E-α-farnesene	+	+
26	Cubedol	+	
27	1s, cis-calamene	+	
28	D-neroblidol	+	
29	Cyclododecane		+
30	α-bisabolol	+	+
31	2-ethylhexyl salicylate		+

Table 4.2 Identified compounds from kudzu and soybean plant volatile extracts in GC-MS. “+” means the presence of compounds in plant volatiles.

GC-EAD analysis of kudzu plant extracts consistently showed 6 peaks that corresponded to strong antennal activities (> 0.5 mV) of female *M. cribraria* (Fig. 4.2). Identification of peaks was confirmed by GC-MS. The first two peaks were benzaldehyde (RT 24.12 min) and nonanal

(RT 25.82 min). Peaks 3 and 4 could not be identified in GC-MS because of the low quantities. Peak 5 corresponded to cubedol (RT 53.34) and peak 6 corresponded to 1s, cis-calamenene.

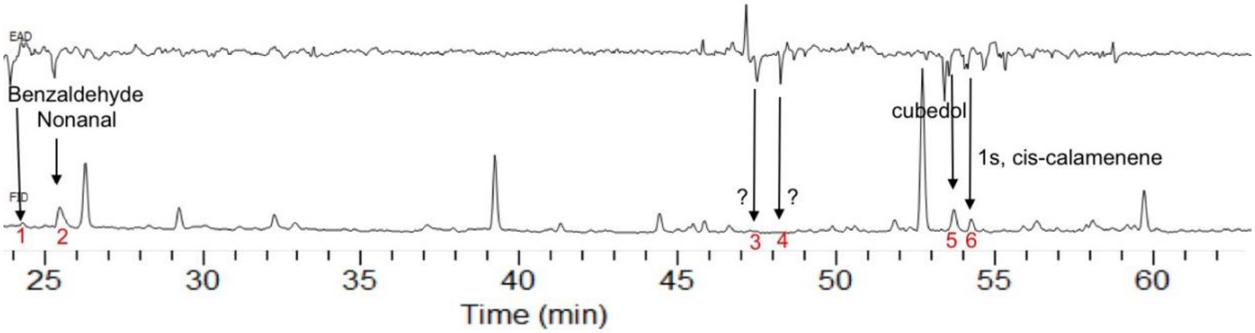

Fig. 4.2 GC-EAD profile of kudzu plant volatile extract on a female *Megacopta cribraria* antenna. Upper race: EAG, lower race: GC-FID.

Consistent responses of female *M. cribraria* were obtained in the GC-EAD analysis to 4 compounds present in soybean plant extracts (Fig. 4.3). Peaks 1 and 2 were identified in GC-MS as 1-octen-3-ol and E, E-α-farnesene, respectively. Peaks 3 and 4 could not be identified in mass spectra libraries.

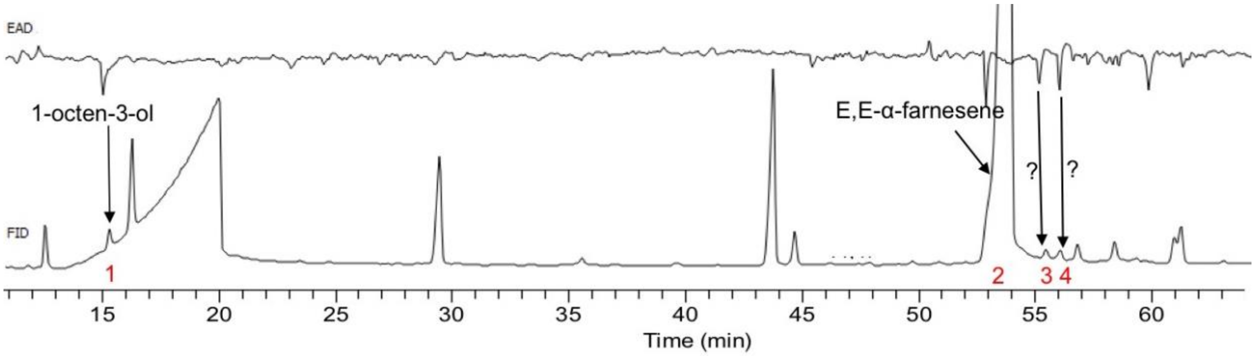

Fig. 4.3 GC-EAD profile of soybean (R1) plant volatile extract on a female *Megacopta cribraria* antenna. Upper race: EAG, lower race: GC-FID.

Comparative EAG Responses to Selected Compounds

There were significant differences in electrophysiological responses to selected chemicals at 10 µg/µL (*F* = 15.264; df =15, 64; *P* < 0.01) (Fig. 4.4). Female *M. cribraria* produced the strongest EAG response to 1-octen-3-ol (0.52 ± 0.02 mV), followed by benzaldehyde (0.36 ± 0.03 mV). Moderate responses were obtained to nonanal (0.29 ± 0.02 mV), ocimene (0.17 ± 0.05 mV), linalool (0.16 ± 0.02 mV) and decanal (0.15 ± 0.04 mV). Responses to other tested chemicals were significantly lower and equivalent to the hexane control.

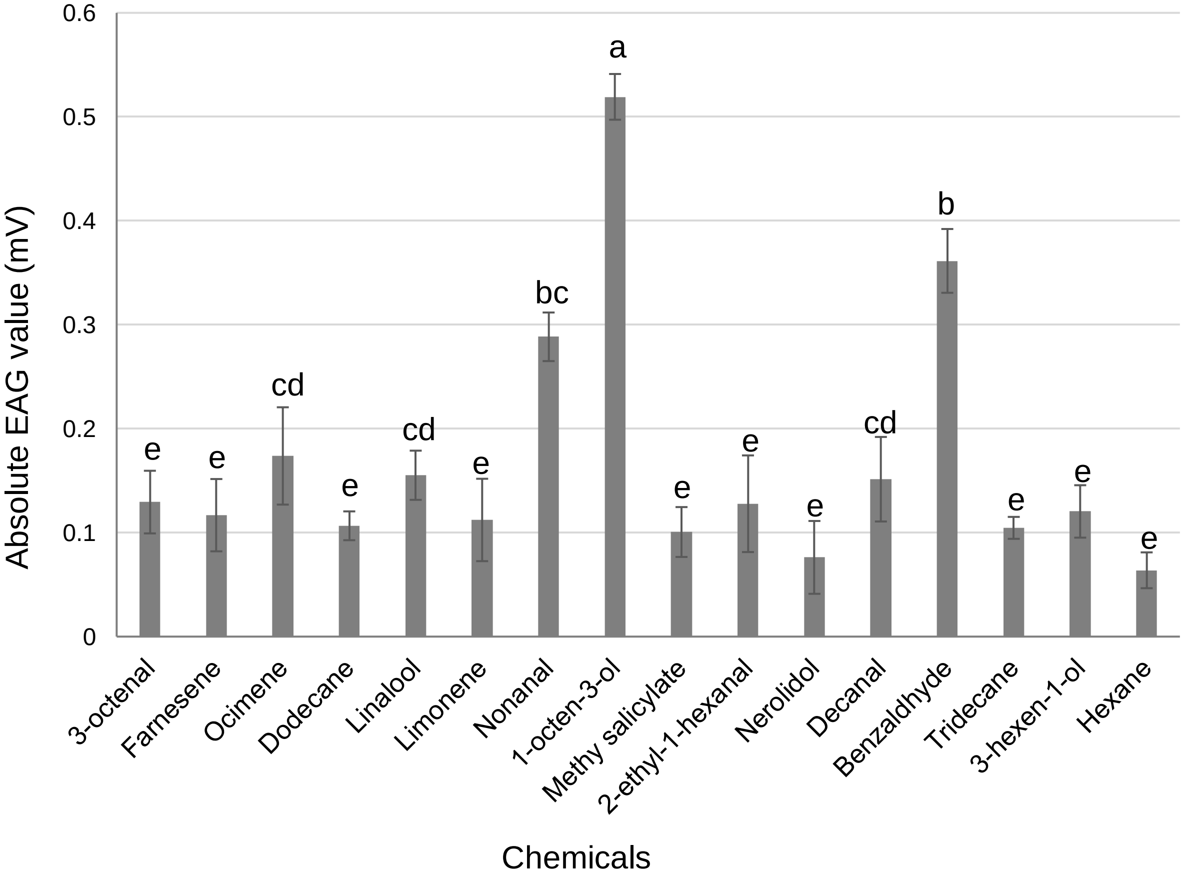

Fig. 4.4 Absolute EAG response (Mean ± SE (mV), n = 5) of female *Megacopta cribraria* antenna to 10 µL of synthetic chemical at 10 µg/µL. Mean EAG responses to the applied doses of the same odor compound were compared by ANOVA, Tukey’s HSD test (P < 0.05). Significant differences are denoted by different letters.

Dose-dependent EAG Response

On the basis of the comparative EAG response results, the four chemicals eliciting the strongest EAG responses were selected to test the dose-dependent response (Fig. 4.5). Over the range 10^{-2} to 10^{2} µg/µL, significantly differences in EAG amplitudes were obtained for 1-octen-3-ol (F = 75.214; df = 5, 24; $P < 0.01$ (Fig. 4.5a), nonanal (F = 11.249; df = 5, 24; $P < 0.01$) (Fig. 4.5b), and benzaldehyde (F = 35.337; df = 5, 24; $P < 0.01$) (Fig. 4.5c), and no significantly difference was observed in ocimene (F = 2.031; df = 5, 24; $P = 0.11$) (Fig. 4.5d).

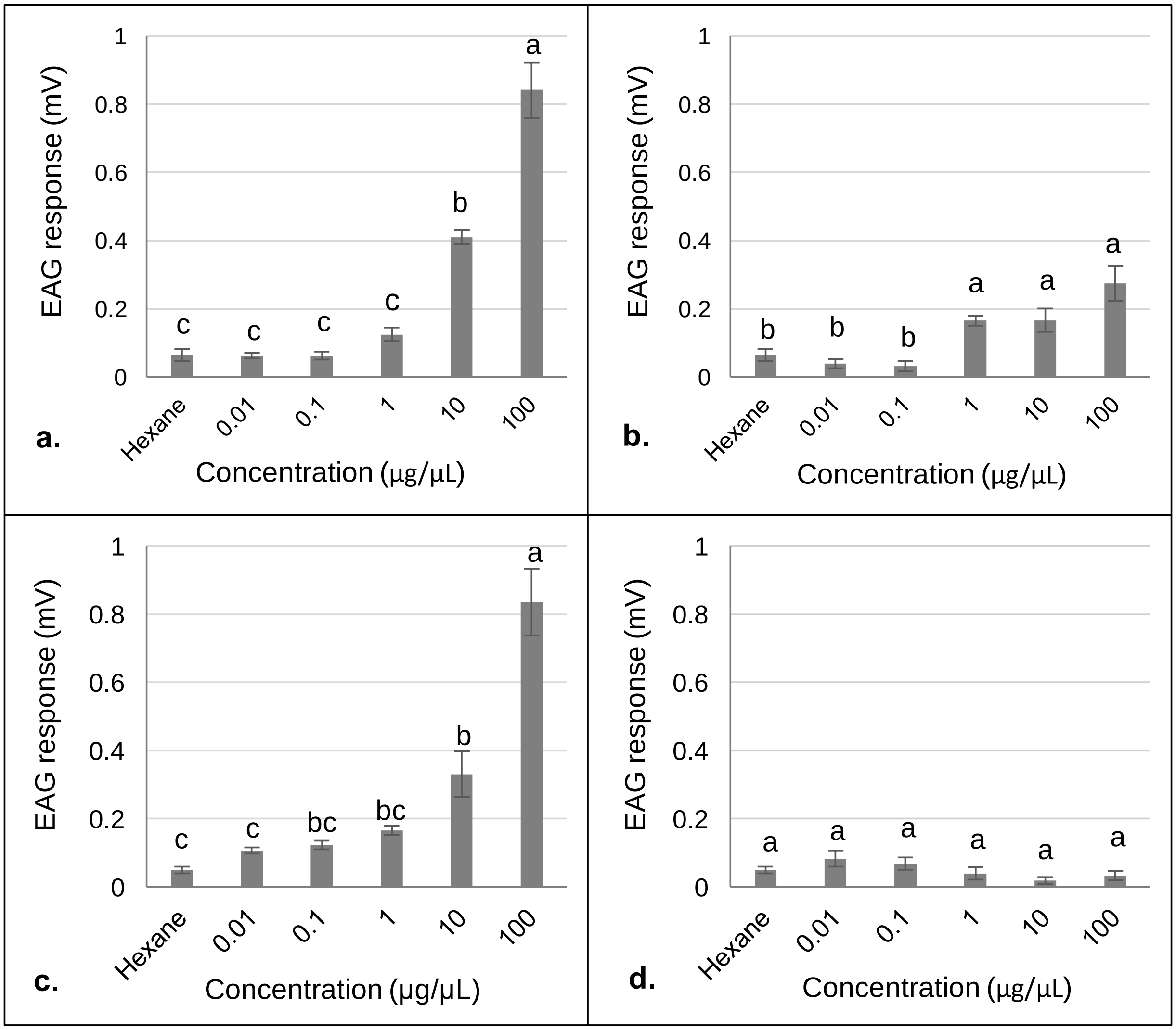

Fig. 4.5 Absolute EAG responses (Mean ± SE (mV), (n = 5)) of female *Megacopta cribraria* to 10 µL of synthetic chemicals (a) 1-octen-3-ol, (b) nonanal (c) benzaldehyde and (d) ocimene at different concentrations (10^{-2}-10^{2}µg/µL). Mean EAG responses to the applied doses of the same odor compound were compared by ANOVA, Tukey's HSD test ($P < 0.05$). Significant differences are denoted by different letters.

Antennal responses of female *M. cribraria* were greatest to 1-octen-3-ol at 100 µg/µL (0.84 ± 0.08 mV) and 10 µg/µL (0.41 ± 0.02 mV) with lower doses producing responses similar to the hexane control. For nonanal, significantly stronger EAG responses were recorded for doses of 1, 10 and 100 µg/µL and responses to 0.01 and 0.1 were no different from the control (hexane). EAG responses of *M. cribraria* to benzaldehyde were also significantly stronger at the higher doses of 100 µg/µL (0.84 ± 0.08 mV) followed by 10 µg/µL (0.41 ± 0.02 mV) with no difference between the lowest dose (0.01 µg) and the hexane control.

Results from the regression analysis of chemical concentration on antennal responses of *M. cribraria* were conducted (Table 4.3). Significant linear responses between antennal activity and chemical concentration were observed on all tested chemicals, 1-octen-3-ol ($F = 71.687$; df = 1, 23; $P < 0.01$), nonanal ($F = 37.037$; df = 1, 23; $P < 0.01$), ocimene ($F = 7.587$; df = 1, 23; $P = 0.01$), and benzaldehyde ($F = 41.277$; df = 1, 23; $P < 0.01$). The EAG responses of *M. cribraria* positively correlated with different dose of these four compounds, indicating that female *M. cribraria* exhibited significantly stronger responses to higher dose of these chemicals. Slopes for 1-oceten-3-ol (Slope = 0.300), benzaldehyde (Slope = 0.167), and nonanal (Slope = 0.135) were positive and indicated an increase in response with increased dose. For ocimene (Slope = 0.048), the slope was very low as there was little increase in response with increased dose.

Chemical	Slope + Intercept[1]	df	*F* value	*P* value
1-octen-3-ol	0.300+0.191	1, 23	71.687	< 0.01
Nonanal	0.135+0.060	1, 23	37.037	< 0.01
Benzaldehyde	0.167+0.026	1, 23	41.277	< 0.01
Ocimene	0.048-0.015	1, 23	7.587	0.011

Table 4.3 Regression equations, person correlations, *F* values, and significance levels for contrasts of slopes describing the effect of chemical concentration (dose) on *Megacopta cribraria* antennal response. Concentrations were $\log^{10}$ transformed. [1]Antennal response = slope (a) + intercept (b).

Y-tube Olfactometer Bioassays

Behavioral responses in a Y-tube olfactometer of female *M. cribraria* (except both sexes were tested for benzaldehyde) to four EAD-active compounds and two previous reported attractants of natural enemies at five different concentrations are presented in Fig. 4.6. Compared with the hexane control arm, female *M. cribraria* were significantly attracted to 1-octen-3-ol at higher concentrations, 1 µg/µL (χ^2 = 4.33; df = 1; *P* = 0.04), 10 µg/µL (χ^2 = 4.33; df = 1; *P* = 0.04), and 100 µg/µL (χ^2 = 5.77; df = 1; *P* = 0.02), but not at the lower concentrations (Fig. 4.6a). For nonanal, the only significantly difference was at 1 µg/µL (χ^2 = 5.77; df = 1; *P* = 0.02) which appeared to be repellent to female *M. cribraria* (Fig. 4.6b). Both female (Fig. 4.6c) and male *M. cribraria* (Fig. 4.6d) were significantly attracted to benzaldehyde at 100 µg/µL (Female: χ^2 = 4.33; df = 1; *P* = 0.04 and male: χ^2 = 4.90; df = 1; *P* = 0.03). Additionally, male *M. cribraria* were attracted to benzaldehyde at the lower doses of 10 µg/µL (χ^2 = 4.33; df = 1; *P* = 0.04) and 1µg/µL (χ^2 = 4.33; df = 1; *P* = 0.04). There was no significantly preference of female *M. cribraria* to methyl salicylate (Fig. 4.6e), farnesene (Fig. 4.6f) and ocimene (Fig. 4.6g) over the range of doses tested.

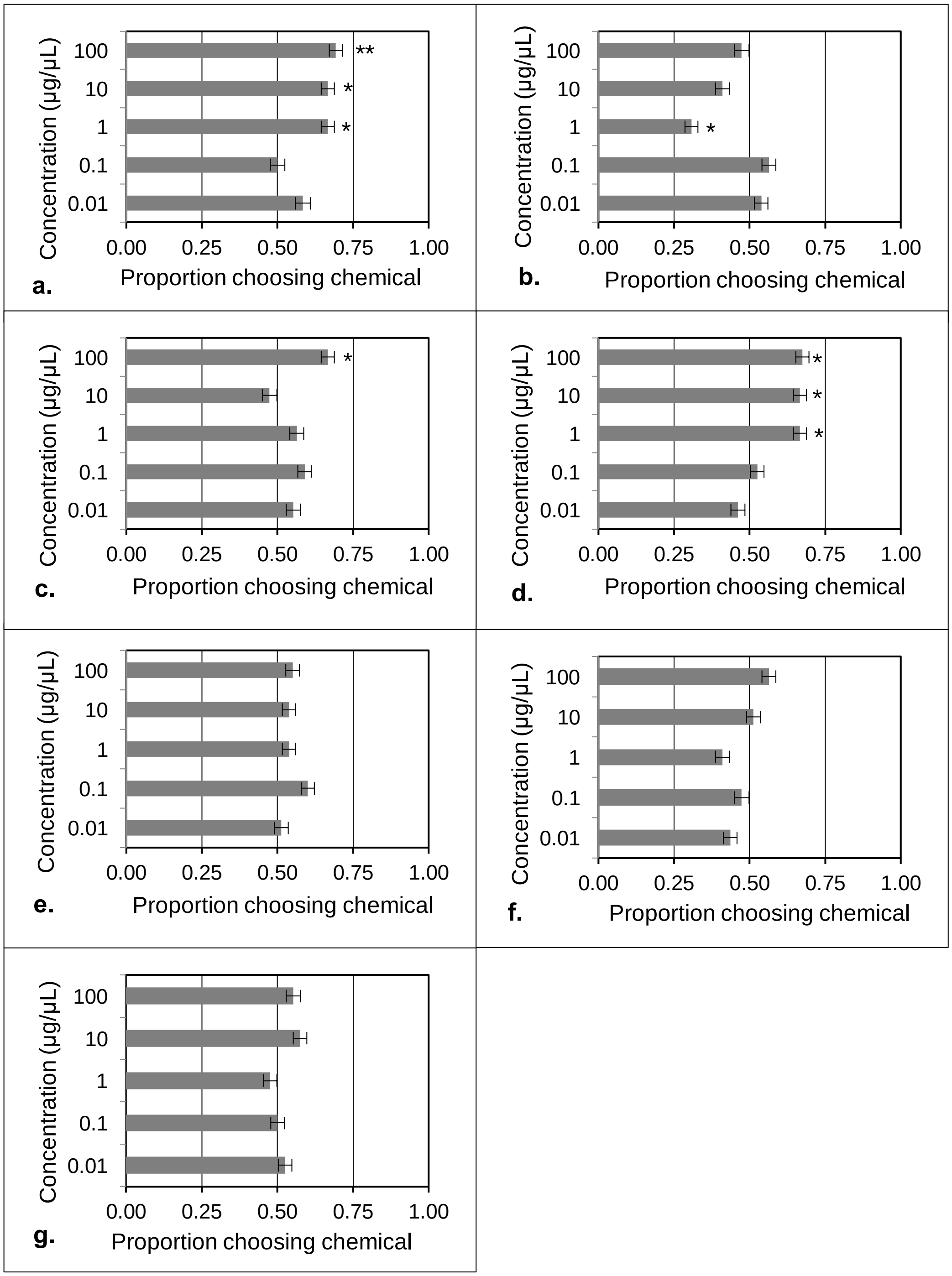

Fig. 4.6 Behavioral responses of adult (n = 40) *Megacopta cribraria* to 10 µL of synthetic chemicals at different concentrations (10^{-2}-10^{2}µg/µL) in olfactometer bioassays. (a) 1-octen-3-ol (female), (b) nonanal (female) (c) benzaldehyde (female), (d) benzaldehyde (male), (e) methyl salicylate (female), (f) ocimene (female), and (g) farnesene (female). Bars represent the proportions of adult Megacopta cribraria choosing synthetic chemicals at different concentrations. Asterisks (*) indicate a significant difference at $P < 0.05$, asterisks (**) indicate a significant difference at $P < 0.01$ (χ^2 test).

Discussion

Analysis of the headspace volatile extracts of the kudzu and soybean plants by GC/EAD revealed the presence of ten EAD-active compounds. Six of them were identified in GC-MS as 1-octen-3-ol, benzaldehyde, nonanal, E, E-α-farnesene, cubedol, and 1s, cis-calamenene. GC-MS analysis of the plant volatile extracts of kudzu and soybean provided identification of 24 compounds from kudzu plant extracts, 18 compounds from soybean plant extracts, and 10 chemicals common from both plant extracts. The volatile compounds produced by soybean plants have been previously investigated (Damiani et al. 2000, Boue et al. 2003, Zhu and Park 2005, Moraes et al. 2008, Michereff et al. 2011). Among them, 3-hexen-1-ol, 3-octenal, 1-octen-3-ol, ocimene, methyl salicylate, as well as E, E-α-farnesene were consistently detected from the volatiles of soybean, and these volatiles are known to elicit strong attractions of a range of insects. For example, methyl salicylate is strongly attractive to *Diaphorina citri* (Hemiptera: Psyllidae) (Mann et al. 2012), and the green leaf compound, 3-hexen-1-ol, is highly attractive to *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) (Li et al. 2014). While a few studies have investigated volatile compounds from kudzu, these were not conducted in detail. In our study, the compounds 1-octen-3-ol, 3-ocetanol, 3-hexen-1-ol, ocimene, methyl salicylate, decanal, E-2-decanal, tridecane, and E, E-α-farnesene were identified as volatiles of both kudzu and soybean. Furthermore, 1-octen-3-ol, ocimene and E, E-α-farnesene elicited significant antennal responses from *M. cribraria* in the GC-EAD analyses. Though other EAD-active compounds nonanal and benzaldehyde, detected in GC-EAD analysis were only identified from kudzu volatiles, they have been reported from soybean plant volatiles in previous studies (de Lumen et al. 1984, Boue et al. 2003, Zhu and Park 2005, Cleveland et al. 2009). The differences between our result and these studies may be due to differences in abundance of plant volatile compounds among plant species and plant growth stages, as well as the quality and quantity of volatiles (Bruce et al. 2005, Schoonhoven et al. 2005, Tasin et al. 2005, Steenhuisen et al. 2010, Ghassemi-Dehkordi et al. 2015). Additional differences may be due to difficulty in detecting low concentrations of some compounds in GC-MS separation and identification (Brattoli et al. 2013), since many key odor compounds often occur at very low concentrations in complex background of volatiles (i.e. background noise) (Mauchline et al. 2008, Sun et al. 2010, Lu et al. 2012, Zhang et al. 2015b). Therefore, the EAG-active compounds associated with some previously reported active chemicals to other insects, which were also detected in kudzu and/or soybean volatiles, were selected for the further electroantennogram analysis.

Previous studies of antennal responses of *M. cribraria* to synthetic chemicals only indicted responses to E-2-hexanal and tridecane (Williams 2016). Our results are consistent with theirs in that no significant antennal responses were detected to tridecane, but 6 more possible chemicals eliciting antennal responses were identified. Among them, 1-octen-3-ol and benzaldehyde elicited higher antennal responses from female *M. cribraria*. Furthermore, the higher slopes of linear regression equations on 1-oceten-3-ol and benzaldehyde may suggest that female *M. cribraria* are more sensitive to the concentration change of these two chemicals. However, the ability of herbivores to detect chemicals via olfactory receptors does not necessarily imply a role for these cues in the behavioral responses (e.g. attraction or repellence) (Bruce et al. 2005, Birkett et al. 2006, Hallem et al. 2006). To address this issue, behavioral bioassays are required to examine how olfactory signals relating to host location in the behavioral ecology of *M. cribraria*.

Our olfactometer bioassay results indicated that attraction of the insect was induced by two ubiquitous volatile compounds (1-octen-3-ol and benzaldehyde). Hence, plant volatile compounds emitted by kudzu and soybean play a key role in host location and attraction of *M. cribraria*. 1-oceten-3-ol, well known as a green leaf compound, has been reported attractive to many insect species, such as *Ahasverus advena* (Waltl) (Coleoptera: Cucujidae) (Pierce et al. 1991), *Callosobruchus maculatus* (Fabricius) (Coleoptera: Chrysomelidae) (Ndomo-Moualeu et al. 2016), green bottle flies, *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) (Brodie et al. 2016) and *Aedes aegypti* (Linnaeus) (Diptera: Culicidae) (Menda et al. 2013). The attractive effects of benzaldehyde to many other insects, such as Cetoniine beetle (Coleoptera: Scarabaeidae) (Steenhuisen et al. 2013), *Chrysopa nigricornis* (Neuroptera: Chrysopidae) (Jones et al. 2011) and *Triatoma infestans* (Hemiptera: Reduviidae) (Fontan et al. 2002) have been reported. Both 1-oceten-3-ol and benzaldehyde have been identified from many other crops, such as cotton (Yu et al. 2007), green bean (De Quirós et al. 2000) and many cultivars of dry bean (i. e. Black bean, pinto bean, and kidney bean) (Oomah et al. 2007). This may explain why *M. cribraria* can be found landing/feeding on many other plants, and the validation in concentration and ratio of key compounds may influent their attractions to insects (Cha et al. 2011). In addition, our results showed that male *M. cribraria* were more sensitive to benzaldehyde than females, perhaps indicating the presence of more odor receptors responding to benzaldehyde on male *M. cribraria* antenna than female antenna. Similar responses were also observed for *Rhopalosiphum padi* (L.) (Park et al. 2000). Further studies on the structure and functions of sensilla on *M. cribraria* could provide an insight into the mechanism of the differences in response between the two sexes *M. cribraria*.

Consistent antennal responses of *M. cribraria* to 1-octen-3-ol and benzaldehyde were recorded in GC-EAD, EAG analysis with attractions in olfactometer bioassays, suggesting that *M. cribraria* has the physiological and behavioral capacity to detect common (1-octen-3-ol) and specific (benzaldehyde) compounds from two different host plants. These two compounds may be the active chemicals in kudzu and soybean plant volatiles responsible for the host location and attraction of *M. cribraria*. However, the antennal responses were not always consistent with behavioral responses. Antennal responses of *M. cribraria* to farnesene and ocimene were different than the behavioral responses. Such differences between antennal responses and behavioral responses have been reported in other insects, such as *Mamestra brassicae* Linnaeus (Lepidoptera: Noctuidae) (Rojas 1999), *Lygus hesperus* Knight (Hemiptera: Miridae) (Williams et al. 2010), and *Maruca vitrata* Fabricius (Lepidoptera: Crambidae) (Wang et al. 2014), which illustrate the importance of using multiple research methods in insect studies. In addition, the effect of isomers β-farnesene and β-ocimene that existed in synthetic chemicals may potentially affect the responses of *M. cribraria*.

Our study provides an insight into the possible host location volatiles used by *M. cribraria* and is the first step towards the identification of host plant-based attractants for semiochemical-based management of *M. cribraria*. The plant volatile chemicals, 1-octen-3-ol and benzaldehyde may have potential as the basis for development of semiochemical-based IPM approaches for *M. cribraria* control. Further field studies with these host associated compounds (single or blend) will provide more insight into the role of these compounds in host plant location by this insect. Much is still to be studied about the interactions between *M. cribraria* and host plants, such as the role of induced volatile emission on *M. cribraria* and its parasitoids. Also, research on the pheromone biology of *M. cribraria* will facilitate the development of semiochemical-based IPM program for this pest.

Conclusion and Future Research Direction

Our study provides the first empirical evidence that plant growth stage affects the attraction of adult kudzu bugs to host soybeans, and that the attractive cue is the constitutive volatiles emitted from whole soybean plants. This growth stage effect was not impacted by soybean planting data or kudzu bug generation. It is therefore way likely that, host-finding and colonization are driven by olfactory sensitive females. Greater attraction at early reproductive stage were also discovered on other legume species. Provided choice of the 6 crops at their most attractive stage, lima bean was the most attractive, followed by soybean and kidney bean; mung bean and the other two showed no attraction. However, the insect completed development only on soybean, mung bean and lima bean. Lima bean is of interest because its greater attractiveness but poor progeny development indicates the characteristics of trap crop. The result also indicates that this insect can use mung bean as host for survival when soybean is unavailable. Further chemical identification of kudzu and soybean plant volatiles showed that 1-octen-3-ol elicited the highest attraction to kudzu bugs in GC-EAD, EAG and olfactometer responses, and it is identified in both kudzu and soybean volatiles. Benzaldehyde elicited higher attraction in male than female, but only identified in kudzu volatile. Nonanal at certain does showed repellence to kudzu bugs rather than attractance.1-octen-3-ol and benzaldehyde may have potential as the basis for development of semiochemical-based IPM approaches for *M. cribraria* control.

Field studies with identified bioactive compounds (single or blend) will provide more insight into the role of these compounds in host plant location by this insect. Much is still to be studied about the interactions between *M. cribraria* and host plants, such as the role of induced volatile emission on *M. cribraria* and its parasitoids. Research on the pheromone biology of *M. cribraria* will facilitate the development of semiochemical-based IPM program for this pest.

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