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„The role of volatiles in the *Piper/Pheidole* association for ant recruitment“

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1 Abstract

Close associations between ants and plants are common throughout the tropics. Several Central American species of *Piper* sect. *Macrostachys* (Piperaceae) have obligate associations with ants, in which the ant partner derives food and shelter from modified plant structures and, in return, protects the plant against fungal infection and herbivory. The major aim of this study was to investigate volatile cues of the damage-induced ant-recruitment of resident *Pheidole bicornis* (Myrmicinae) in myrmecophytic *Piper* species. In the first part of this study, field experiments with *Piper obliquum* and *Piper fimbriulatum* were conducted in the Parque Nacional Piedras Blancas, near the Biological Research Station La Gamba in Costa Rica. Via volatile experiments different terpenes were offered at the second youngest internodes of the plants to artificially induce ant-recruitment by simulating herbivore attack through stem damage. Trials were performed using pure terpenes (ß-caryophyllene, α-copaene, para-cymene and *trans*-3-carene), dilutions and mixtures of terpenes and green-leaf volatiles but none of the treatments did induce more or less the same response as the artificial stem damage did. The most similar ant response was with β-phellandrene and the mixture with ß-caryophyllene and 3-carene 1:100. We conclude that a full ant response depends on the combination of terpenes and environmental conditions as well as on colony size. Via DNA barcoding it was determined that in 18 out of 19 *Piper* plants investigated in this study, the presence of different ant-species can be virtually excluded. It is assumed that all plants investigated are inhabited by *Pheidole bicornis*, their mutualistic ant-partner. To locate oil droplets presumably containing the terpenes which are emitted after stem damage, cross sections of the stems were made and distribution patterns recorded. The cross sections were divided into three regions parenchyma of the bark and sclerenchyma ring, region with phloem and xylem and parenchyma of the pith. Comparisons indicated that the majority of oil cells were located in the pith, fewer were in the phloem and xylem and fewest were found in the bark. Stem-sections and oil cell distribution were compared between myrmecophytic and non-myrmecophytic *Piper* species and no significant differences were found. We believe that oil cells are present in the tissue of the plants for alternative physiological processes and did not evolve specifically to elicit ant responses.

2 Introduction

Myrmecophytism

In tropical ecosystems mutualistic relationships between specialized arboreal ants and plants are common. The degree of the interaction between them vary from facultative to obligate myrmecophytic associations (Fiala et al., 1994; Heil and McKey, 2003). The so called 'myrmecophytes' (ant-plants) are plants, which are living in an obligate symbiosis with specialized plant-ants. Those obligate ant-plant mutualisms have evolved almost exclusively in tropical ecosystems and independently in many lineages (Davidson, 1993). Myrmecophytism is taxonomically widespread and involves 40 genera of ants and more than 100 genera of angiosperms (Davidson, 1993; Heil and McKey, 2003), as they are important elements of tropical communities (Heil and McKey, 2003).

True myrmecophytes provide specialized structures for ants as a nesting space, those are hollow parts of the plant, called 'domatia' (Beattie, 1985). Domatia can be thorns (e.g. *Acacia*), hollow stems (e.g. *Leonardoxa, Macaranga, Cecropia*), leaf pouches (e.g. *Hirtella, Tococa, Maieta*) or petioles (e.g. *Piper*) (Heil and McKey, 2003). Besides nest cavities plants supply ants with food either directly by cellular food bodies (e.g. *Piper, Macaranga, Cecropia*), extrafloral nectar (e.g. *Leonardoxa, Acacia*), or both (Fiala and Maschwitz, 1992), or indirectly through sap-sucking homopteran trophobionts, which ants keep inside the domatia for obtaining honeydew (Gaume et al., 1998; Heil and McKey, 2003). Food bodies are ontogenetically derived from leaflet tips (e.g. *Acacia*) or pearl body-like multicellular or unicellular emergences (e.g. *Macaranga, Piper, Cecropia*) (O'Dowd, 1982). Those epidermal bodies are produced on leaves, petioles, stipules or stems, sometimes even inside the domatia (O'Dowd, 1982; Risch and Rickson, 1981). They contain a high concentration of lipids, proteins and carbohydrates (Fischer et al., 2002; Heil et al., 2004; Heil et al., 1997; Risch et al., 1977) and seem to make up nearly the entire diet of obligate plant-ants (Fischer et al., 2002).

As a benefit for the plant, the resident ants often protect their host plants from herbivory (Janzen, 1967; Vasconcelos, 1991), remove small herbivorous insects and their eggs (Risch and Rickson, 1981), keep them free from overgrowth by encroaching vegetation, clinging vines (Janzen, 1966) and fungal infection (Letourneau, 1998) efficiently. In many cases they supply their host plants with nutrients (Fischer et al., 2003; Gegenbauer et al., 2012).

Herbivory and plant secondary metabolites

Herbivore damage of the host plant is signaled to their resident ants by volatiles. Normally an undamaged plant releases small quantities of volatile chemicals (volatile organic compounds, VOCs) which often include sesquiterpenes, monoterpenes and aromatics (Paré and Tumlinson, 1999).

After herbivore attack, a variety of VOCs is specifically emitted locally from damaged vegetative tissues (Kessler and Baldwin, 2001), which attract natural enemies of herbivores (Dicke, 2000) or – in myrmecophytes – resident ants (Agrawal, 1998; Bruna et al., 2008; Fiala and Maschwitz, 1990; Mayer et al., 2008). These VOCs are derived from terpenoid, fatty-acid and phenolic metabolic pathways and stored in trichomes, specialized glands or vacuoles (Baldwin et al., 2006; Paré and Tumlinson, 1997). Terpenes represent the largest class of plant secondary metabolites, which were released into the air, due to the high vapor pressures at normal atmospheric conditions (Dudareva et al., 2004). However, some of the sesquiterpenes and monoterpenes are only released from mechanically damaged leaves (Rose et al., 1996).

Another group, the so-called 'green leaf volatiles' (GLVs, oxylipins) consist of a composition of saturated and unsaturated C_6-alcohols, esters and aldehydes and are a product of autolytic oxidative breakdown of membrane lipids (Paré and Tumlinson, 1999). They are derived through lipoxygenase cleavage of fatty acids within seconds of injury (Pichersky and Gershenzon, 2002). GLVs are not specific to plants attacked by herbivores (Kessler and Baldwin, 2001).

In obligate mutualisms, ant fitness is strongly tied to host protection, which should be improved by systematic detection of attacking herbivores (Schatz et al., 2009). Patrolling the host and searching for herbivores is inefficient, because herbivore activity is not predictable and the density is usually low (Bruna et al., 2008). A much more efficient and rapid system of ant-recruitment to the damaged tissues is the release of volatile organic compounds following herbivory (Heil and McKey, 2003). This induced ant-recruitment in myrmecophytic plants is quite common (Dyer et al., 2001) and several studies have demonstrated that ants react strongly to the mixture of host-plant chemicals (Agrawal, 1998; Fiala and Maschwitz, 1990; Mayer et al., 2008) released after host-plant damage. Hence, VOCs play an important role in ant-plant communication and ant-recruitment happens via volatiles (Agrawal, 1998; Brouat et al., 2000; Fiala and Maschwitz, 1990). Which volatiles, however, play a central role in ant-plant communication is only poorly investigated.

The system Piper

Good systems to study this question are myrmecophytic *Piper* plants. *Piper* is a genus of the family Piperaceae which has a pantropical distribution. The majority of the species occurs in the neotropical region, where about two-thirds of the described species are found (Burger, 1972; Dyer and Palmer, 2004).

The genus includes more than 1000 species, making it one of the largest genera of basal angiosperms (Kubitzki et al., 1993). The highest diversity of *Piper* species occurs in the American tropics (700 spp.), followed by Southern Asia (300 spp.) (Jaramillo and Manos, 2001). Most species of *Piper* grow in wet, warm lowland evergreen rain forests and are a dominant component of the understory flora. The genus is rich in secondary chemical compounds with oil cells in their tissue (Dyer and Palmer, 2004). About eight species are known to be myrmecophytic.

In myrmecophytic species, associations with ants can range from very loose to facultative, highly specialized or even obligatory (Fischer et al., 2000; Risch et al., 1977). In Central America five species (*P. cenocladum, P. calcariformis, P. obliquum* Ruiz & Pavon*, P. sagittifolium and P. fimbriulatum* C.DC) have obligate associations with ants and they belong to *Piper* section *Macrostachys* (Miq.) C. DC (Tepe et al., 2007). Burger was the first who described in the 70s the myrmecophytic interactions of *Piper*-plants with their *Pheidole bicornis* ant-partner (Burger, 1971; Burger, 1972) and in recent years much research was done.

The myrmecophytic species of *Piper* have characteristic sheathing petioles that become tightly folded into a tube (Letourneau and Dyer, 1998a; Risch et al., 1977). The ant colonies, which inhabit *Piper* species, are housed inside those sheathing leaf bases (Fincher et al., 2008). As the plant increases in size and the ant colony grows, workers apparently excavate the stem pith of the obligate myrmecophytes (Letourneau, 1998; Risch et al., 1977) and the entire plant becomes a domicile (Letourneau, 1983). The stems remain solid until excavated by ant residents (Tepe et al., 2009). Access to the hollow stem is through a round aperture at the base of the leaf-axil within the sheathing base (Letourneau and Dyer, 1998a). This aperture is formed of soft tissue that the ants remove (Burger, 1972). If not maintained by the ants, the entrance holes in all myrmecophytes eventually become sealed by an accumulation of callus (Tepe et al., 2009). A pore between the petiolar and stem cavities allows movement of ants between two domatia (Dyer and Palmer, 2004; Tepe et al., 2009).

Non obligate myrmecophytes have solid stems and petioles that are closed (Dyer and Palmer, 2004; Tepe et al., 2009).

The most important prerequisite for myrmecophytism seems to be the production of single-celled, opalescent food bodies which the plant produces on the adaxial side of the hollow petioles. They are produced only when occupied by *Pheidole bicornis* ants (Risch et al., 1977; Risch and Rickson, 1981), or specialized parasites (Letourneau and others, 1990) are present.

They are rich in lipids and proteins and seem to be the primary source of nutrients for the ants (Fischer et al., 2002) and as *Pheidole bicornis* appears to be specific to these *Piper* species, it seemingly never forages off of its host plants (Letourneau, 1998; Risch et al., 1977). Furthermore the resident ants seem to eat insect eggs, soft-bodied insects and fungal spores found on the leaves and flowers (Letourneau, 1983; Letourneau and Dyer, 1998b; Risch et al., 1977). Ants also remove or kill small herbivorous insects by tossing them off the plant (Letourneau, 1983).

In an earlier study (Mayer et al., 2008) it was shown that the resident ants react with aggressive behavior when the stem of their host plant was injured. Phloem-sucking treehoppers (Hemiptera: Membracidae) pose a serious threat to the stem (Mayer et al., 2008) which might be even more costly to the plant than herbivory to the leaves (Agrawal, 1998). Mayer et al. (2008) suggested that it is of great importance to the resident ants to protect stem tissue, which goes in line with Letourneau (1998) who reported stem protection behavior of *Pheidole bicornis* resident in Costa Rican *Piper* species. In the work of Mayer et al. (2008) 68 VOCs (mainly monoterpenes, sesquiterpenes and GLVs) were identified after stem damage of myrmecophytic and non-myrmecophytic *Piper* species via solid-phase microextraction/gas chromatography–mass spectrometry (Mayer et al., 2008). It was hypothesized, that the reaction of the ants was apparently inducible through certain substances. The question now arises, which substances are responsible for the induced ant-recruitment? Is it a single substance or mixtures? Where are those substances stored? Which roles play GLVs in the ant-plant communication? Do they have a significant impact? Does a difference between myrmecophytes and non-myrmecophytes exist?

Aims of this study

In order to answer the above questions, the present work will focus on interactions of different species of the obligate myrmecophyte understory shrub *Piper* (Piperaceae) with its specialized ant-partner *Pheidole bicornis*.

Aims were as follows:

(1) To make sure that all investigated plants are inhabited by the same ant species, *Pheidole bicornis*, DNA barcoding was conducted.
(2) Field experiments were performed using pure terpenes, dilutions and mixtures of terpenes as well as GLVs, to artificially induce ant-recruitment by simulating herbivore attack through stem damage.
Terpenes used in this work were ß-caryophyllene, α-copaene, para-cymene and *trans*-3-carene. Those chemical compounds were chosen on the basis of results from solid phase microextraction and gas chromatography-mass spectrometry of *Piper* species in earlier studies (Mayer et al., unpublished data).
GLVs comprised of *trans*-2-hexenal and *cis*-3-hexen-1-ol 1:1000. The goal was to experimentally identify whether single substances or a mixture out of the complex blend of VOCs is responsible for the induced ant response.
Furthermore, the influence of GLVs on the intensity of the response was tested by either adding GLVs to a mixture or by excluding them in the experimental settings.
(3) VOCs are very often lipophilic and consequently secreted in essential oil cells (Caissard et al., 2004). To locate oil droplets containing terpenes, cross sections of the stems were made and distribution patterns recorded. Stem-sections and oil cell distribution were compared between myrmecophytic and non-myrmecophytic *Piper* species. Do myrmecophytes possess more oil cells compared to non-myrmecophytes? Do non-myrmecophytes have a different distribution of terpenes in the stems than myrmecophytes? How are oil cells distributed in the stem? Near the bark or yet in the pith? It can be assumed that it would be beneficial if terpenes are located near the bark, so a quicker release can be obtained when the bark is injured via sap-sucking homopterans.

The relationship between *Piper sp.* and their obligate *Pheidole bicornis* is very complex and many aspects of their communication are not clearly understood yet. Hopefully this study is contributing to a better understanding of how volatiles convey between ants and plants.

3 Material and Methods

3.1 Study plants

Myrmecophytic species of *P. obliquum* and *P. fimbriulatum* were used for field experiments with volatiles and for investigations of oil cell distribution via stem sectioning. For the latter experimental setting following species of *Piper* were further used: myrmecophytic *P. cenocladum,* the non-myrmecophytes *P. aduncum* L. and *P. nigrum* L. and two undefined species of non-myrmecophytic *Piper* (later referred to as *P. sp.*_1 and *P. sp.*_2).

P. fimbriulatum is found only on the Pacific coast of Costa Rica and in western Panama (Burger, 1971; Weber and Baumgartner, 2001) and occurs between sea level and approximately 1100 m altitude (Letourneau and Dyer, 1998a). *P. obliquum* has a range from Guatemala over Colombia to Venezuela and Guyana (Weber and Baumgartner, 2001) whereas *P. cenocladum* is endemic to Costa Rica and common in wet forests throughout the Atlantic lowlands (Burger, 1971). *P. aduncum* originates from South America (Rogers and Hartemink, 2000) and is found in tropical habitats of the Caribbean, Southern Mexico and Latin America (Taylor, 2006) and *P. nigrum*, black pepper, is an economically important species, which is native to the humid tropical forests of western coast of South India (Hooker, 1879) but grows wild in Costa Rica.

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Figure 1: a) *Piper obliquum* b) *Piper fimbriulatum* c) Experimental setup for volatile experiments. Volatile substances were applicated on filter paper which was mounted on a tripod and offered at the second youngest internode. d) Petiolus of an inhabited *Piper sp*. plant and two workers of *Pheidole bicornis.* e) Petiolus cut open, containing a minor worker with larvae and pupae and small opalescent food bodies. f) Workers of *Pheidole bicornis* examinating the artificial stem injury.

3.2 Study animals

Pheidole is currently the most species rich genus of ants in the world with a worldwide distribution (Moreau, 2008). All Central American myrmecophytic species are inhabited by their specialized ant-partner *Pheidole bicornis* (Fischer et al., 2000; Letourneau, 1998; Moreau, 2008; Risch et al., 1977), a small (2-3 mm) species (Hymenoptera: Formicidae: Myrmicinae) (Letourneau and Dyer, 1998a). It ranges from the Atlantic and southern Pacific lowlands of Costa Rica to Panama. It has a dimorphic worker caste of a minor and major worker subcaste ((Moreau, 2008) see Figure 2). The big-headed major workers, or so called soldiers, defend their colony, whereas the minor workers are specialized on foraging and performing tasks within the nest (Moreau, 2008). Further *Pheidole bicornis* is an obligate plant ant, which has developed a coevolved association with some *Piper* species and is never found nesting elsewhere.

Ant colonies of *Pheidole bicornis* are often polydomous, which means that they inhabit and maintain multiple nests of the same or even different myrmecophytic *Piper* plants (Risch et al., 1977).

Figure 2: *Pheidole bicornis*. On the left side a minor worker and on the right side a larger soldier.

3.3 DNA-Barcoding

DNA barcoding is an increasingly used method, for non-taxonomists and in difficult taxa, to identify taxa, mostly by amplifying a standardized 658 base pair DNA region (Frézal and Leblois, 2008), the protein coding cytochrome *c* oxidase subunit I (COI) region of the mitochondrial genome. It is maternally inherited, has a rapid mutation rate, does not recombine and is highly conserved.

Therefore it is proposed to serve as a universal and unique marker that every species will most likely have (Frézal and Leblois, 2008) and that "genetic variation between species exceeds variation within species" (Hebert et al., 2003). Hebert et al. established that COI "can serve as the core of a global bio identification system for animals" (Hebert et al., 2003).

Ten ants of each *Piper obliquum* (Riverbed-Trail) and *Piper fimbriulatum* (Ocelot-Trail), which were used in the field experiments, were collected randomly and preserved in 70% ethanol.

For DNA extraction heads of the ants were cut off and each put into a tube with four ceramic pellets in it. Those tubes were mechanically shaken to crush cells of the tissues for homogenization. The peqGOLD Tissue DNA Mini Kit (PEQLAB Biotechnology GmbH, Erlangen, Germany) was used for DNA extractions, according to the manufacturer's specifications. The eluted DNA was frozen until further use for the Polymerase Chain Reaction (PCR, (Mullis et al., 1986)).

The 658bp long barcoding sequence of cytochrome c oxidase subunit 1 (COI) was amplified using the primers Lep F (5'-ATT CAA CCA ATC ATA AAG ATA TTG G-3') and Lep R (5'-TAA ACT TCT GGA TGT CCA AAA AAT CA-3') (Hebert et al., 2004).

All PCR mixes for amplifying the requested part of the mitochondrial gene COI region had a total volume of 25 µl and contained 18.2 µl double-distilled H_20, 2.5 µl 10xPCR buffer (N), 2 µl MgCl2 25mM, 0.1 µl dNTP-mix (2mM), 0.5 µl of each primer (Lep F and Lap R), 0.2 µl Taq Polymerase (Qiagen Inc., Valencia, CA) and 1 µl of double-stranded genomic DNA (Moreau, 2008).

The detailed protocol which was used for the PCR thermo cycler (Mastercycler pro S, Eppendorf, Hamburg, Germany) consisted of initial 4 minutes denaturation at 94°C, 5 cycles of 1 minute denaturation at 94°C, 1min30s annealing at 44°C and 1min30s extension at 72°C followed by 35 cycles of 1 minute denaturation at 94°C, 1min15s annealing at 47°C and 1min15s extension at 72°C and a final cycle of 7 minutes at 72°C. PCR products were electrophoresed in 1.0% TAE agarose gels, stained with 1 µl SybrGold (100x) which is functioning as a fluorescent dye which interacts with DNA and 2 µl LoadingDye for illustrating the migration-progress and afterwards they were visualized under UV light.

To remove all remaining fragments such as primers or deoxy nucleotids the purification process can be achieved by adding 2 µl Exosap (PEQLAB Biotechnologie GMBH, Erlangen, Germany) consisting of Exonuclease III and Shrimp Alkaline Phosphatase (*Pandalus borealis*, Thermo Fisher Scientific - Austria GmbH) to 5 µl PCR products followed by 15 minutes at 37°C for digestion and 15 minutes at 80°C for enzyme deactivation.

The Sanger's chain termination method (Sanger, 1981) was set up with 7 µl ddH2O, 1 µl BigDye™ (PEQLAB Biotechnologie GMBH, Erlangen, Germany), 1 µl template DNA and 1 µl of either forward Lep F or reverse Lep R primer to sequence all gene fragments in both directions. To produce sufficient copies for automated sequencing following PCR protocol was used:
2 minutes at 96°C, 25 cycles of 20 seconds denaturation at 96°C, 20 seconds annealing at 48°C and 4 minutes extension at 60°C and a final 4 minutes extension at 60°C.
The whole automated sequencing process was accomplished with a 3730xl DNA Analyzer (Applied Biosystems, Life Technologies Corporation, Austria). Removing of primers with the program SeqMan (Swindell and Plasterer, 1997) resulted in one contig, the requested barcoding sequence of 658bp. Those sequences were aligned subsequently by hand in BioEdit (Hall, 1999).

3.3.1 Statistical analysis: DNA-barcoding

Sequence divergences among individuals were quantified by using the Kimura-2- Parameter distance model (Kimura, 1980), number of bootstraps: 1000 replicates (Felsenstein, 1985) and pairwise deletion and graphically displayed in a neighbor-joining (NJ) tree (Hebert et al., 2004; Saitou and Nei, 1987). The analysis involved 197 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 42 positions in the final dataset. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5.20 (Tamura et al., 2011).

3.4 Field experiments with volatiles

Field experiments were performed during February and March 2011 in the Parque Nacional Piedras Blancas on the southern pacific slope of Costa Rica, a lowland primary rain forest, near the Biological Research Station La Gamba (N 8°42"61', W 83°12'97"; 70 m a.s.l.), Esquinas Forest.
To test whether certain single terpenes are as efficient in inducing ant response and recruitment as artificial stem damage and to which content GLVs are involved, volatile experiments were conducted.

Ten plants of two myrmecophytic *Piper* species, *P. obliquum* (Riverbed-Trail) and *P. fimbriulatum* (Ocelot-Trail) respectively which were inhabited by their ant-partner *Pheidole bicornis* were chosen randomly. The size of the investigated plants varied between 70 cm and 240 cm and all made a vital impression with a low level of herbivory.

First, experiments with different odors were carried out with 0.2 µL of the following pure terpenes: ß-caryophyllene (Sigma Aldrich), α-copaene (Givaudan SA), para-cymene and *trans*-3-carene (both obtained from Sigma Aldrich). Furthermore dilutions of ß-caryophyllene (1:10 and 1:100), ß-phellandrene (1:10 and 1:100, Givaudan SA) and *trans*-3-carene 1:10 were made with Genapol (used as emulsifier, Sigma Aldrich) and tested accordingly to the experimental design of Mayer et al. (2008). Another experimental trial consisted of a comparison of a mixture (ß-caryophyllene, 3-carene 1:100) with and without green leaf volatiles (*trans*-2-hexenal, *cis*-3-hexenol 1:1000, Acros Organics).

The respective substances were applicated on a piece of filter paper mounted on a tripod and was offered at the second youngest internode. At the end of the experiment the stems were injured at the same internode with a surgical scalpel and the number of ants recorded every 2 minutes for a maximum observation period of 20 minutes. This simulated stem-damage is considered as control simulating natural injuries caused by stem-boring and sap-sucking insects.

3.4.1 Statistical analysis: Field experiments

All of the substances mentioned above were tested against the artificial injury every two minutes for a maximum time span of 20 minutes. Due to a paired experimental setup and not normally distributed data Wilcoxon signed-rank tests for related samples had to be applied (Wilcoxon, 1945). Dilutions were tested separately against each other after the same principle. *P*-values were set at 0.05, if not stated otherwise.

3.5 Oil cells

To investigate whether appearance and distribution pattern of oil cells is different between myrmecophytic and non-myrmecophytic *Piper*, cross-sections of fresh tissue without freezing or embedding were made.

With a Leica VT1200S vibratome using usual razor blades (Gillette® Super Silver™ und Bic® Chrome Platinum) 100 µm thick cross-sections of three youngest internodes from the three myrmecophytic *Piper* species *P. obliquum* (n=3, 131 sections), *P. fimbriulatum* (n=3, 213 sections), *P. cenocladum* (n=1, 48 sections) and four non-myrmecophytic species *P. nigrum* (n=1, 41 sections), *P. aduncum* (n=1, 40 sections), *P. sp*_1 (n=1, 36 sections) and *P. sp*_2 (n=1, 42 sections) were made. The plants sectioned were all cultivated in the greenhouses of the Botanical Garden of the University of Vienna, Austria.

Prior to the cutting process small pieces of the stems were super glued (with cyanoacrylate) onto the 1 cm high specimen holder. The buffer tray was filled with distilled water before the specimen holder was inserted. Amplitude of 0.45 mm and a pace of 0.18 mm/s showed the best results. Sectioning was carried out under water. The fresh sections of the stems were placed on object slides and stained with a mixture of sudan III (Österreichische Heilmittelwerke) and paraffin (0.766g sudan III in 29mL paraffin, dissolved and filtered) which stained the oil containing cells in orange. After five minutes they were washed with glycerin water (1:1, Rotipuran® ≥ 99.5%, p.a., Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and mounted with prewarmed (60°C) Kaiser's glycerol gelatine (Merck KGaA, Darmstadt, Germany).

To investigate the occurrence and distribution of the oil cells an Olympus BX50 microscope was used and pictures were taken with a Nikon Digital Sight (DS-U2). With the software program NIS-Elements D 3.2 pictures were edited.

The sections of the internodes were divided into three different regions: parenchyma of the bark and sclerenchyma ring (A), part with phloem and xylem (I) and parenchyma of the pith (M) (see Figure 3). To measure the area of the stem diameters (and each section) and the density of the oil cells Photoshop CS6 (Adobe Systems; Mountain View, CA) was used. With the function ‘Color Range’ the color of the oil cells was selected, measurement scale was set and measurements recorded (for detailed procedure see Appendix).

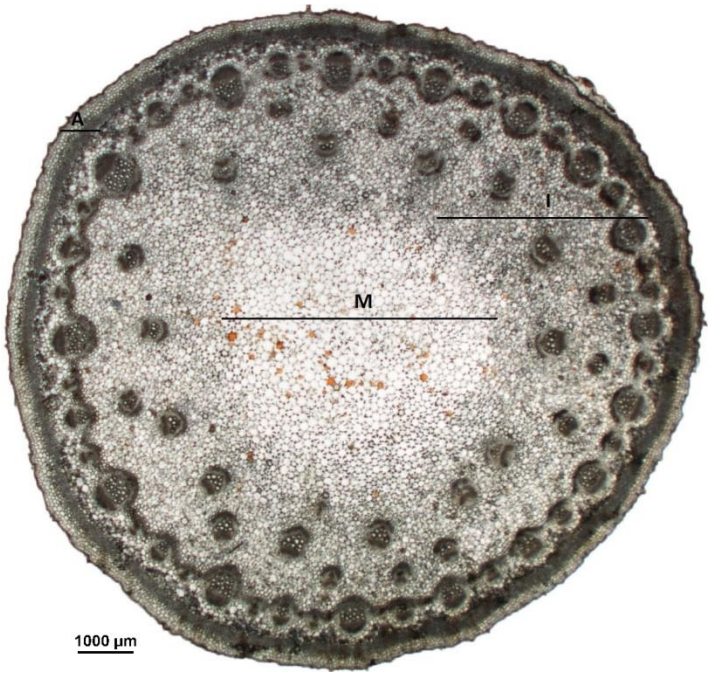


Figure 3: Stem cross-section of *Piper fimbriulatum* divided into three different regions (A), (I) and (M).
A = parenchyma of the bark and sclerenchyma ring, I = phloem and xylem and M = parenchyma of the pith.

3.5.1 Statistical analysis: Oil cells

To look for differences between the three regions (A, I, M, see Figure 3) a test for multiple and related samples had to be conducted. No kind of transformation was able to get the data normally distributed, and therefore instead of parametric repeated measures ANOVAs non-parametric Friedman's tests (Friedman, 1937) were applied. *Post hoc* Wilcoxon signed-rank tests for related samples between each of the three possible pairs of the regions were performed (Wilcoxon, 1945). To respond to the problem of multiple testing and therefore avoiding significances due to multiplicity a Bonferroni-Holms correction (Holm, 1979) was used with α-value set at 0.05.

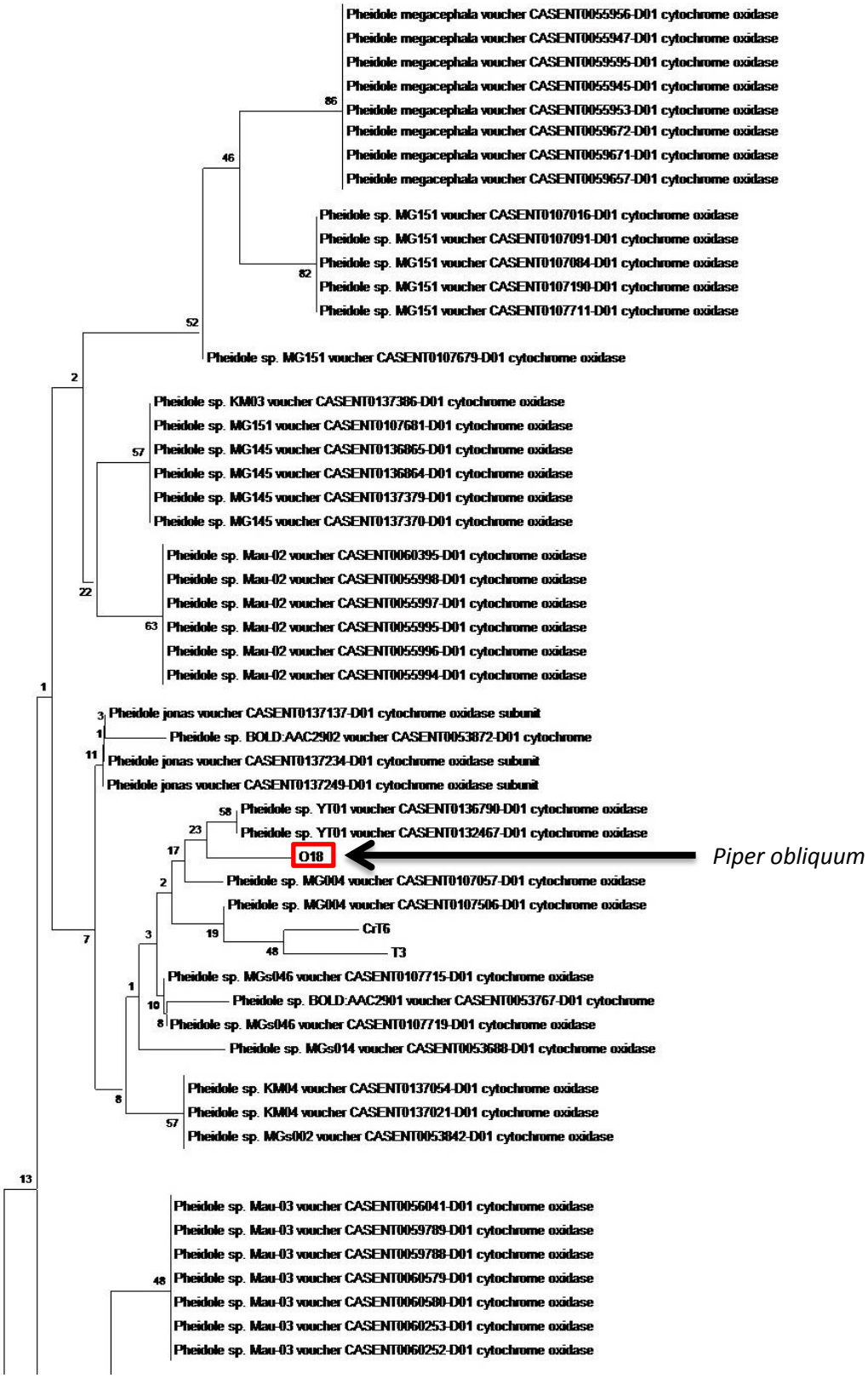
To investigate whether appearance and distribution pattern of oil cells is different between the groups of myrmecophytic and non-myrmecophytic *Piper* species, the mean value of each species for (A), (I) and (M) were calculated. Due to the not normally distributed data, Wilcoxon signed-rank tests were conducted to test for differences between those two groups for (A), (I) and (M) respectively.

3.6 Statistical analysis

All statistical tests were performed with R (R Core Team, 2013) using R Commander (Fox, 2005).

4 Results

4.1 DNA-Barcoding



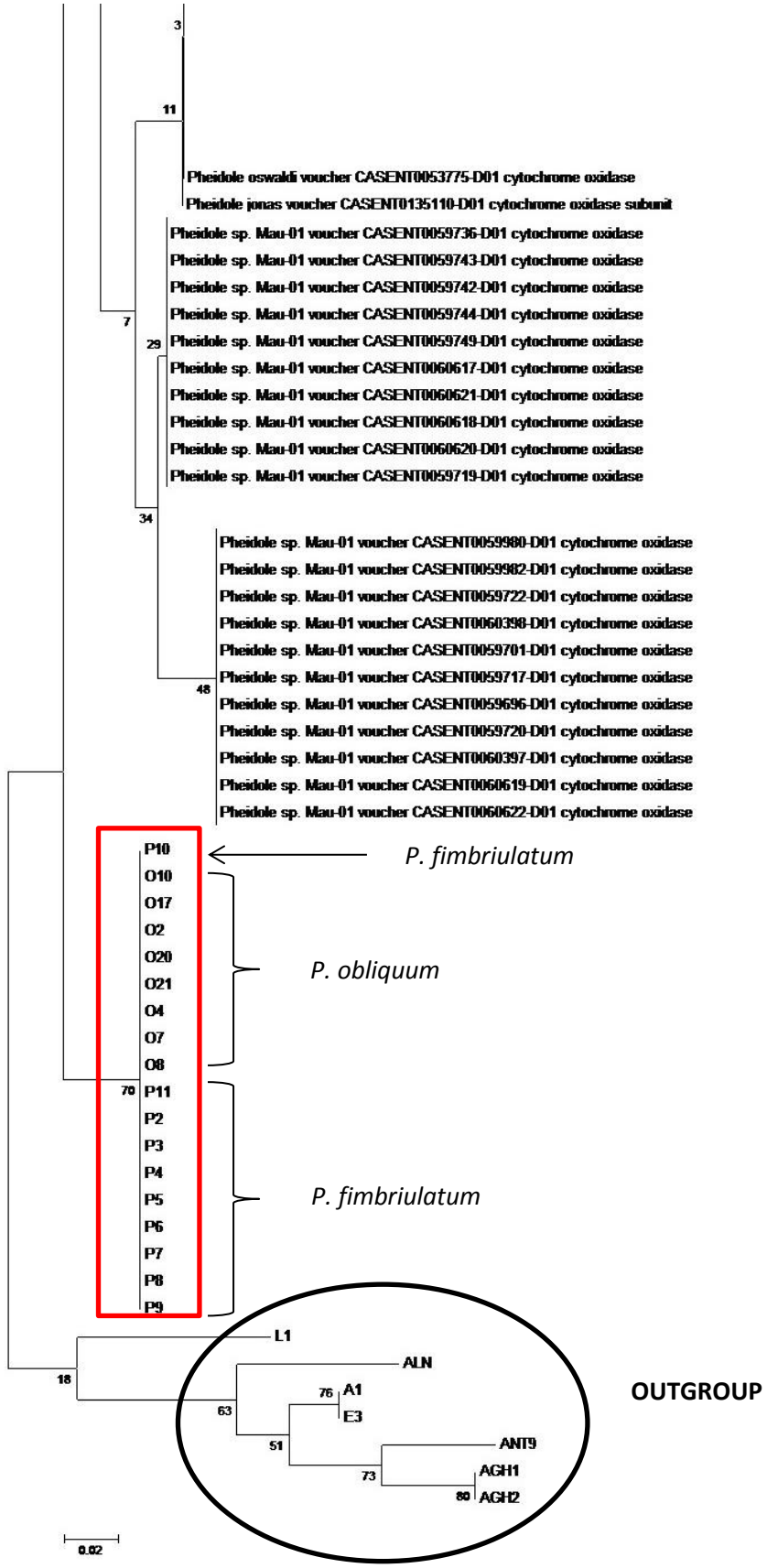


Figure 4: Evolutionary relationships of taxa - To provide a convenient view of the relationships of taxa, the tree was condensed manually.

The two red rectangles show those ant taxa which were collected in the field experiments and investigated through DNA-barcoding (O = *P. obliquum*, n=9; P = *P. fimbriulatum*, n= 10). The optimal tree with the sum of branch length = 1.09726962 is shown. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site.

The Neighbor-Joining tree (see Figure 4) shows that individuals of eight colonies collected from *P. obliquum* and specimen of ten colonies from *P. fimbriulatum* clustered together.
With a high probability all investigated ants belong to the same species. The only exception was the colony from O18, one of the *P. obliquum* plants.

4.2 Field experiments

4.2.1 Piper obliquum

In the experimental settings with *Piper obliquum* (n=10, if not stated otherwise) different chemical compounds were tested to compare the reactions of *Pheidole bicornis* to the real injury (mean values ± SD see Table 1). Chemicals used were ß-caryophyllene, α-copaene, para-cymene and *trans*-3-carene. When tested against the injury, ß-phellandrene 1:100 differed significantly from minute 4 to minute 15, which means that fewer ants responded to the volatiles than to the injury. The 1:10 dilutions of ß-caryophyllene and ß-phellandrene showed a significantly lower ant activity from minute 8 to minute 15, ß-caryophyllene 1:100 only at minute 12 (see Table 2). No significant differences were found when the injury was tested against the 1:10 dilution of *trans*-3-carene, which means that ants responded equally to the injury and to the volatile tested. Comparison between both dilutions of ß-caryophyllene 1:10 and ß-caryophyllene 1:100 showed no significant results. Comparison between ß-phellandrene 1:10 and ß-phellandrene 1:100 as well as between the pure *trans*-3-carene (n=9) and *trans*-3-carene 1:10 resulted in non-significant findings. Apparently, ants react equally to the 1:10 and 1:100 dilutions of ß-caryophyllene and ß-phellandrene and to the pure *trans*-3-carene and the 1:10 dilution of it. The pure substances of *trans*-3-carene and α-copaene (n=9) showed significant differences (fewer ants) from minute 8 to minute 15, pure para-cymene (n=8) yielded only at minute 12 significantly fewer ants than the injury. No significant differences were found when the injury was tested against pure ß-caryophyllene (n=9). Ant activity did not differ between the injury and pure ß-caryophyllene.

To investigate whether green leaf volatiles influence herbivore-induced ant-recruitment, a mixture (consisting of ß-caryophyllene and 3-carene 1:100) was used with and without GLVs (*trans*-2-hexenal, *cis*-3-hexenol 1:1000). Testing the injury against the mixture with GLVs as well as against the mixture without GLVs resulted in non-significant findings which mean that a comparable reaction to the injury was elicited. Comparison between the mixture with GLVs and without GLVs showed no significant results, which means that the ant activity did not differ between the real injury and the mixtures with GLVs and without GLVs.

Table 1: Mean values (MV) and standard deviation (SD) of ant activity following real injury and volatiles respectively. min = minutes, n = number of plants

	Volatiles offered																					
	Injury		beta-caryophyllene		beta-Caryophyllen 1:10		beta-Carypohyllen 1:100		beta-Phellandren 1:10		beta-Phellandren 1:100		trans-3-Caren		trans-3-Caren 1:10		alpha-copaene		para-cymene		Mix with GLVs		Mix without GLVs	
min	MV	SD	MV	SD	MV	SD	MV	SD	MV	SD	MV	SD	MV	SD	MV	SD	MV	SD	MV	SD	MV	SD	MV	SD
0	0.4	0.97	1.78	3.27	0.7	1.34	1	1.76	2.1	5.95	0.4	1.26	0.78	1.09	1.7	3.74	0.33	0.71	0.50	1.07	0.3	0.67	0.2	0.63
2	1.2	1.69	3.11	6.15	1.4	2.17	1.7	3.20	1.6	3.03	0.8	1.62	1.22	1.92	3.4	7.90	0.56	0.88	1.38	3.89	2.3	3.77	1.4	2.12
4	2.1	2.38	3.78	8.38	1.7	2.67	2.3	5.50	1.2	2.53	0.9	1.66	1.00	1.80	4.2	10.94	0.56	0.73	1.38	3.50	1.8	4.02	3.2	5.31
6	2.5	3.17	4.56	9.14	0.7	1.25	2.1	5.13	0.9	2.23	0.5	1.58	0.33	0.71	2.9	7.81	0.33	0.71	1.75	4.17	2.4	4.95	2.4	4.43
8	4.8	4.47	2.44	3.47	1.3	2.11	2	4.74	0.7	1.89	0.3	0.95	0.33	0.71	3.4	7.17	0.44	1.33	1.88	4.55	2.8	5.77	2.3	4.37
10	7.2	8.23	2.56	4.61	1.3	2.87	1.4	3.50	1.3	2.54	0.5	1.58	0.89	1.83	3.3	8.82	0.11	0.33	1.50	4.24	4.6	10.81	2.1	4.46
12	8.4	10.04	1.33	2.50	0.7	1.34	1.6	3.50	1.2	2.30	0.7	1.25	0.22	0.67	3.7	9.43	0.11	0.33	1.25	3.54	3.8	7.61	1.8	4.34
14	7.9	8.86	1.33	2.40	1.1	2.13	1.33	4.00	1.1	2.13	0.3	0.95	0.22	0.67	3.6	10.00	0.11	0.33	1.25	3.54	3.1	5.28	2.8	6.21
15	7.3	8.75	1.33	2.65	0.9	2.18	1.5	4.24	1	2.11	0.3	0.95	0.44	1.33	4.5	11.57	0.11	0.33	1.00	2.83	3.25	5.21	2.55	5.60
n	10		9		10		10		10		10		9		10		9		8		10		10	

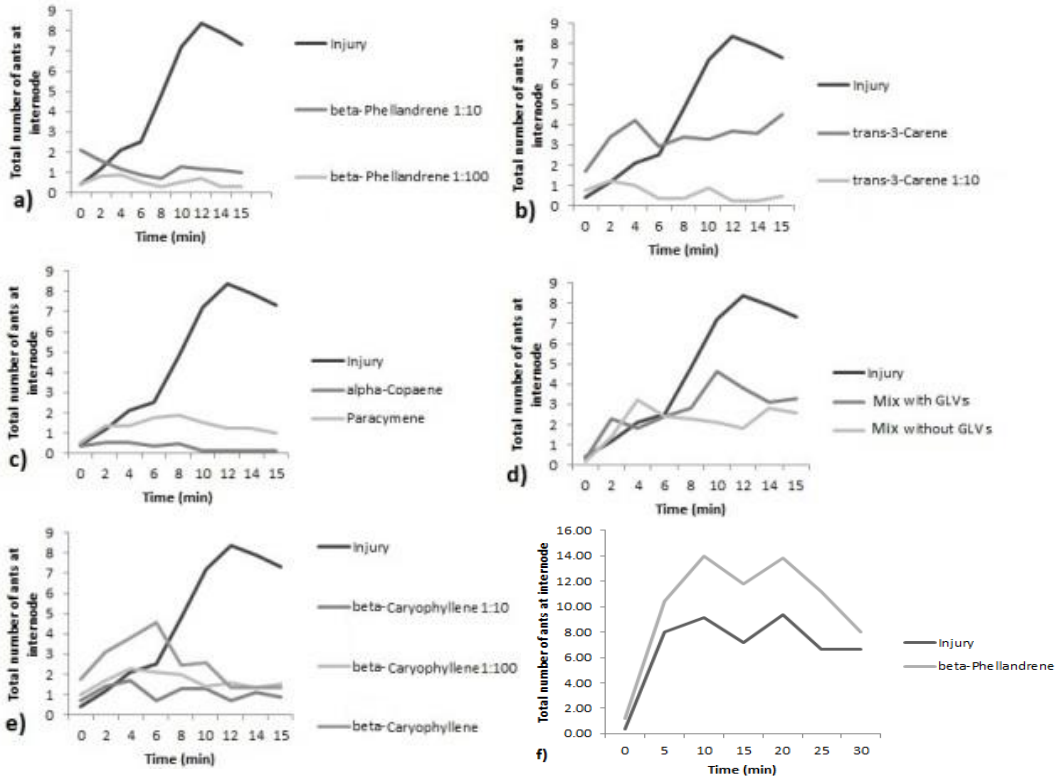


Figure 5: Total number of ants at the internode of *Piper obliquum* (n=10), counted every two minutes for a maximum time span of 15 minutes. a), b), e) Reaction difference correlated with volatile concentration. c) Reaction differences between para-cymene and alpha-copaene. d) Influence of GLVs on ant-recruitment. The mix consisted of ß-caryophyllene and 3-carene 1:100 with and without green leaf volatiles (*trans*-2-hexenal, *cis*-3-hexenol 1:1000). f) Comparison of reaction differences between real injury and beta-phellandrene, ants counted every 5 minutes for a time span of 30 minutes (n=5, unpublished data, Mayer 2008).

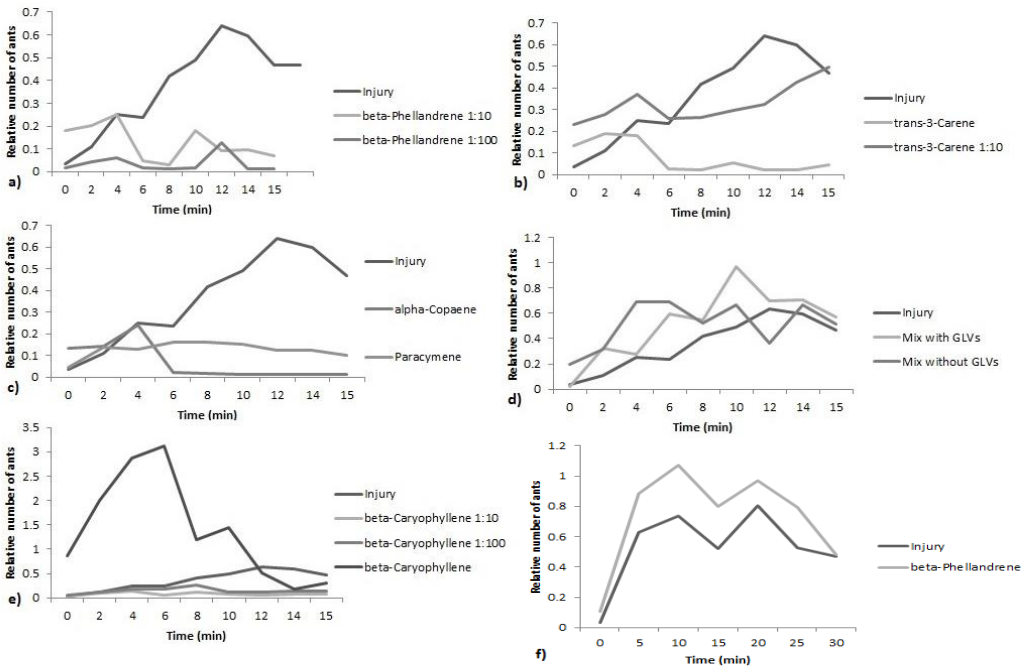


Figure 6: Relative values of ants of *Piper obliquum* (n=10), counted every two minutes for a maximum time span of 15 minutes. a), b), e) Reaction difference correlated with volatile concentration.

c) Reaction differences between para-cymene and alpha-copaene. d) Influence of GLVs on ant-recruitment. The mix consisted of ß-caryophyllene and 3-carene 1:100 with and without green leaf volatiles (*trans*-2-hexenal, *cis*-3-hexenol 1:1000). f) Comparison of reaction differences between real injury and beta-phellandrene, ants counted every 5 minutes for a time span of 30 minutes (n=5, unpublished data, Mayer 2008).

Since it is assumed that colony size has a great influence on herbivore-induced ant-recruitment therefore, to eliminate this influence, the maximum number of ants yield at the artificial injury was set as 100 % and all values form the odor experiments were put into relation to it. Those standardized relative values are not depending on colony size and condition (see Figure 6).

Compared to the total number of ants, relative values show more or less the same results (see Table 2). At minute 14 ß-caryophyllene and ß-caryophyllene 1:100 exhibited statistically significant differences when compared to the injury, which means that fewer ants were yielded in the volatile experiments (mean values ± SD see Table 1). ß-phellandrene 1:10 did not show statistically significant differences at minute 10, which means that ant activity was equal to the injury at that moment. All other results are comparable to those gained with the total numbers of ants.

Table 2: Wilcoxon signed-rank tests for related samples, injury against volatiles offered on *P. obliquum*, tested at minutes (min) 0 to 15, * $p \leq 0.05$, n = number of plants. Bold rectangles show significant absolute values of total numbers of ants, whereas shaded areas just indicate statistical significances, no relative values are shown.

	Volatiles offered										
min	beta-caryophyllene	beta-caryopyllene 1:10	beta-caryophyllene 1:100	beta-phellanderene 1:10	beta-phellanderene 1:100	*trans*-3-carene	*trans*-3-carene 1:10	alpha-copaen	paracymene	Mix with GLVs	Mix without GLVs
0	0.27	1	0.37	0.79	1	0.57	0.10	0.77	1	1	0.79
2	0.79	0.85	0.59	1	0.46	1	0.46	0.17	1	0.20	0.57
4	0.80	0.53	0.60	0.35	0.03 *	0.10	0.83	0.20	0.71	0.60	0.67
6	0.80	0.09	0.39	0.09	0.03 *	0.06	0.55	0.06	0.79	0.93	0.93
8	0.23	0.02 *	0.14	0.03 *	0.02 *	0.04 *	0.50	0.03 *	0.34	0.27	0.23
10	0.24	0.02 *	0.08	0.03 *	0.02 *	0.04 *	0.27	0.04 *	0.28	0.50	0.16
12	0.12	0.01 *	0.02 *	0.02 *	0.02 *	0.02 *	0.15	0.02 *	0.05 *	0.35	0.13
14	0.11	0.01 *	0.06	0.02 *	0.01 *	0.02 *	0.29	0.02 *	0.20	0.15	0.26
15	0.29	0.02 *	0.06	0.01 *	0.01 *	0.02 *	0.27	0.02 *	0.11	0.23	0.26
n	9	10	10	10	10	9	10	9	8	10	10

Wilcoxon signed-rank tests showed no significant differences of ß-caryophyllene, *trans*-3-carene 1:10 and the mixtures with and without GLVs compared to the injury (see Table 2). Standardized values (shaded areas, no genuine values shown) indicate more or less the same significances than absolute values.

Interestingly, ß-caryophyllene 1:10, ß-phellandrene 1:10, *trans*-3-carene and α-copaene exhibited significant differences only after minute eight, ß-caryophyllene 1:100 and para-cymene only at minute 12 and ß-phellandrene 1:100 since minute 4. ß-caryophyllene, *trans*-3-carene 1:10 and the mixtures with and without GLVs did not exhibit any differences compared to the injury.

4.2.2 Piper fimbriulatum

Experiments were performed using the same techniques and treatments as in field experiments with *Piper obliquum*. The mixture of ß-caryophyllene and 3-carene 1:100 with GLVs (*trans*-2-hexenal, *cis*-3-hexenol 1:1000) as well as without GLVs showed significant differences (a weaker reaction of the ants) when tested against the injury, whereas when tested against each other, there were no significant differences (see Table 3), ant activity was equal. No significant differences between the artificial injury tested against single odors and dilutions were detected (n=10, results not shown). Ants responded equally to the injury, the dilutions and the single odors.

Table 3: Wilcoxon signed-rank tests for related samples, mixture with GLVs tested against mixture without GLVs at minutes (min) 0 to 20, showing no significant differences. * $p < 0.05$, n = number of plants.

min	with against without GLVs
0	0.17
2	0.15
4	0.17
6	1
8	0.41
10	1
12	0.85
14	0.58
16	0.37
18	0.59
20	0.18
n	10

4.2.3 Comparison of *Piper fimbriulatum* and *Piper obliquum*

In the experimental setting with *Piper fimbriulatum* the mixtures with and without GLVs yielded a significantly weaker reaction of the ants than the injury. These results are in contrast to the experimental setting with *Piper obliquum*, where the ant activity did not differ between the real injury and the mixtures with and without GLVs. Interestingly, regardless of the other treatments any differences concerning the ant activity in *Piper fimbriulatum* could be found. Obviously, ants responded equally to the injury, the single odors and the dilutions, whereas in *Piper obliquum* a significantly weaker reaction of the ants was recorded.

4.2.4 Cluster analysis for *Piper obliquum*

An agglomerative hierarchical cluster analysis was performed using a Euclidean distance matrix (see Figure 7) as a set of dissimilarities for means of the different treatments of *Piper obliquum* being clustered. This method allows illustrating the differences or similarities of the reaction of the ants between treatments.

```
> Cluster
           1         2         3         4         5         6         7         8         9        10        11
2  14.0385184
3  12.8565591 2.0553453
4  13.9932126 1.7058722 2.4565106
5  15.3879823 1.9416488 3.5647970 2.4413111
6   8.7572827 7.2601653 5.6345166 7.2415468 8.9487429
7   8.5254032 6.0118633 4.8055812 6.1206617 7.4761287 3.6128244
8  11.2112667 3.7218947 2.3459776 4.3304157 5.3275229 4.2664388 3.7229021
9  12.9757609 5.1770147 3.5272484 5.2151844 6.5824964 5.0242007 5.3465028 3.8046146
10 16.0553825 2.5266970 4.1998824 3.0628196 0.8949102 9.6461148 8.1827072 5.9518464 7.1612814
11 15.4483608 1.7239060 3.5177855 2.1426014 0.8753835 8.8085607 7.4236796 5.3271753 6.3722004 1.2619796
12 13.3120012 1.3979896 1.3186430 2.1930287 2.7026607 6.6471328 5.1915195 3.0808887 4.4558664 3.2768066 2.7238870

Cluster method   : complete
Distance         : euclidean
Number of objects: 12
```

Figure 7: The proximity between means of the different treatments was measured as distance matrix with Euclidean distances for complete linkage hierarchical clustering.

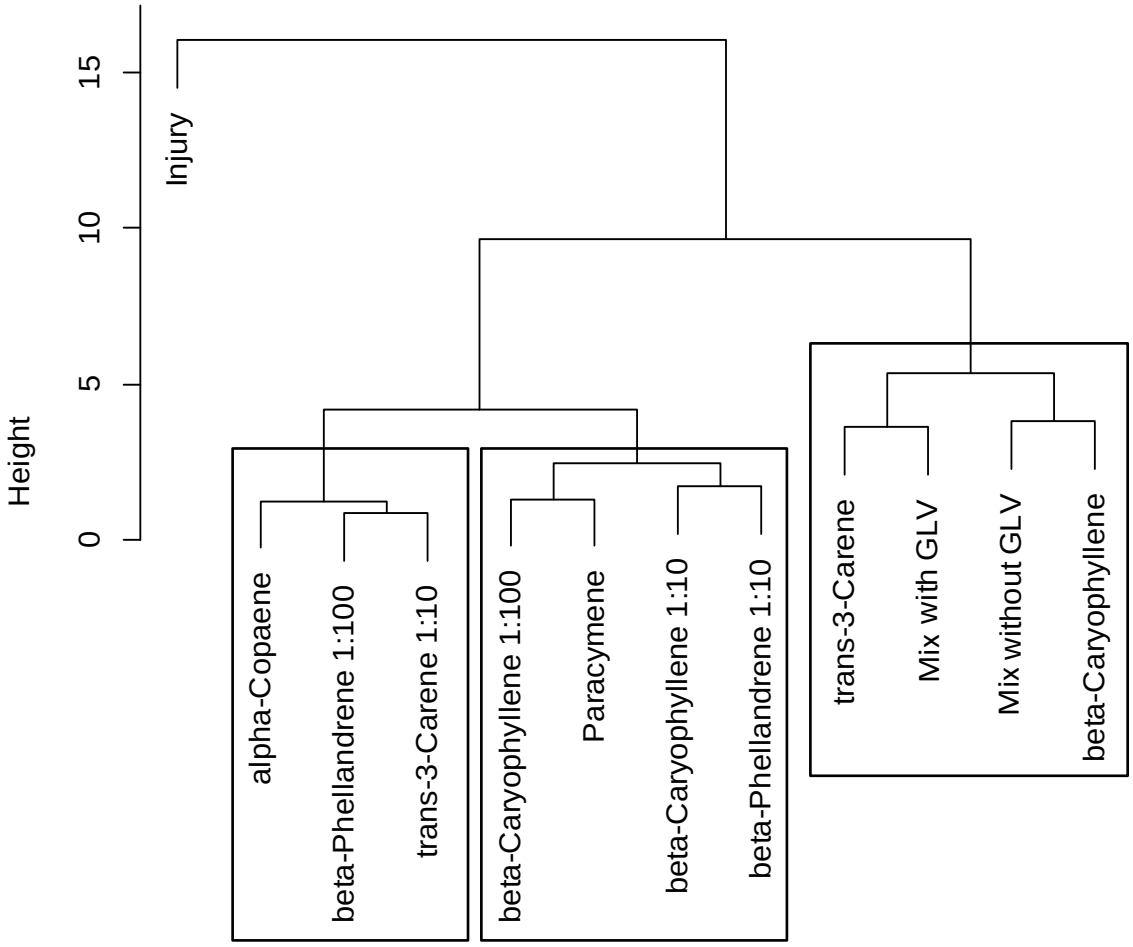


Figure 8: *Piper obliquum*. Dendrogram based on the agglomerative hierarchical clustering samples with Euclidean distance matrix and complete linkage method. Distance between clusters is determined by the two most distant points in the different clusters.

The dendrogram (see Figure 8) clearly shows the great dissimilarity between the artificial injury and all the other treatments with different odors and dilutions. The mix contained ß-caryophyllene and 3-carene 1:100 with and without green leaf volatiles (*trans*-2-hexenal, *cis*-3-hexenol 1:1000). Mainly three clusters can be distinguished, where two of them comprise of pure substances and dilutions and one cluster holds pure substances and the mixture with and without GLVs.

4.2.5 Cluster analysis for *Piper fimbriulatum*

An agglomerative hierarchical cluster analysis was performed using a Euclidean distance matrix (see Figure 9) as a set of dissimilarities for means of the different treatments of *Piper fimbriulatum* being clustered. This method allows illustrating the differences or similarities of the reaction of the ants between treatments.

	1	2	3	4	5	6	7	8	9	10	11
2	23.8042013										
3	22.9543024	1.4212670									
4	21.4372340	1.8894444	2.1725561								
5	22.2310968	2.6901053	2.7178423	1.1547005							
6	20.6967389	1.5716234	1.7088007	1.4142136	1.8257419						
7	21.6968892	2.0420578	2.1954498	0.9128709	0.9128709	1.4719601					
8	21.0007143	1.4124447	1.6568042	0.7905694	1.5138252	1.2747549	0.7905694				
9	19.8060597	1.3583078	0.8031189	2.3717082	2.7003086	1.6201852	2.2638463	1.9364917			
10	20.7781135	2.3653752	2.5777898	2.9368350	3.0482235	2.7613403	2.9368350	2.8722813	2.1213203		
11	20.2479629	1.4124447	0.9721111	1.7677670	2.1889876	1.7677670	1.9039433	1.5000000	1.2247449	2.1213203	
12	18.5931439	1.8894444	1.7663522	3.3911650	4.1432676	3.0822070	3.5355339	2.9368350	2.0310096	3.1819805	2.3717082

```
Cluster method   : complete
Distance         : euclidean
Number of objects: 12
```

Figure 9: The proximity between means of the different treatments was measured as distance matrix with Euclidean distances for complete linkage hierarchical clustering.

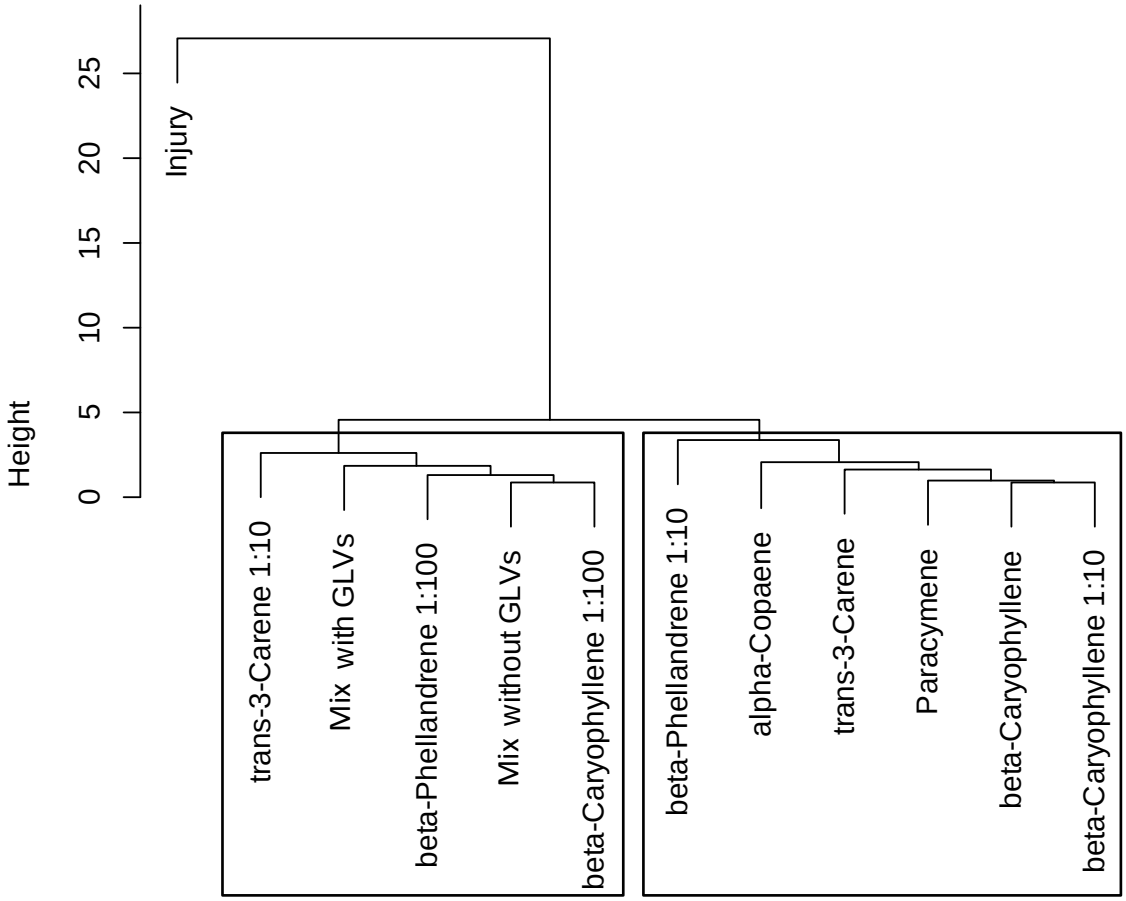


Figure 10: *Piper fimbriulatum*. Dendrogram based on the agglomerative hierarchical clustering samples with Euclidean distance matrix and complete linkage method. Distance between clusters is determined by the two most distant points in the different clusters.

The dendrogram (see Figure 10) clearly shows the great dissimilarity between the artificial injury and all the other treatments with different odors and dilutions. The mix contained ß-caryophyllene and 3-carene 1:100 with and without green leaf volatiles (*trans*-2-hexenal, *cis*-3-hexenol 1:1000). Basically all pure substances clustered together on one side and on the other side mixtures and dilutions formed a second cluster.

4.2.6 Comparison of cluster analyses of *Piper obliquum* and *Piper fimbriulatum*

Both dendrograms (see Figure 8 and Figure 10) clearly show the great dissimilarity between the artificial injury and all the other treatments with different odors and dilutions. This basically means that whatever treatment was applied, ants did not show a reaction similar to the injury. In both dendrograms the mixtures with and without GLVs clustered together. This means that they probably have the same impact on the reactions of the ants. In *P. obliquum* neither the mixture with GLVs nor the mixture without GLVS showed significant differences compared to the artificial injury (see Table 2) whereas in *P. fimbriulatum* mixtures with GLVS as well as without GLVs exhibited significant differences. Para-cymene, ß-caryophyllene 1:10 and ß-phellandrene 1:10 were found in the same clusters of *P. obliquum* and *P. fimbriulatum*. ß-phellandrene 1:100 and *trans*-3-carene 1:10 as well as ß-caryophyllene and *trans*-3-carene clustered together in both dendrograms. There was no interference of ß-caryophyllene 1:100 and α-copaene in the dendrograms of the investigated plants. The results obtained from both cluster analyses cannot be reduced to a common denominator.

4.3 Oil cells

Figure 11: Encompassing diversity of stem cross-sections of the second youngest internodes of a) *P. obliquum*, b) *P. fimbriulatum*, c) *P. cenocladum*, d) *P. nigrum*, e) *P. sp*_2, f) *P.aduncum*, g) *P. sp*_1. a)-c) are representatives of myrmecophytic *Piper* species, d)-g) are representatives of non-myrmecophytic *Piper* species, orange dots are stained oil cells. Note that in these young parts the stem of myrmecophytic *Piper* (a-c) is not yet excavated.

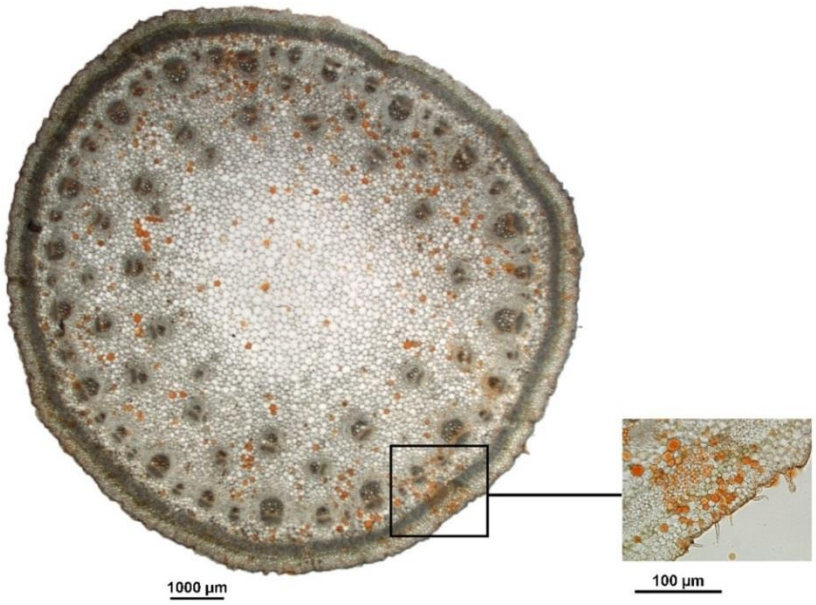


Figure 12: Cross-section of an unexcavated stem of *Piper obliquum* from the second youngest internode, oil cells are stained in orange. The second youngest internode was chosen because it is the most vulnerable part of the plant.

Friedman's tests showed a statistically significant difference of the distribution of oil cells between the three different regions parenchyma of the bark and sclerenchyma ring (A), part with phloem and xylem (I) and parenchyma of the pith (M) in all myrmecophytic and non-myrmecophytic *Piper* species tested (see Table 4).

Table 4: Friedman's tests were performed and showed a significant difference between the three different regions in all *Piper* species tested, * $p < 0.05$, *** $p < 0.001$, n = number of cross-sections per plant.

Piper species	n	Friedman chi-squared	*p*-value	df
P. obliquum A	58	40.37	$p < 0.001$***	2
P. obliquum B	19	6	$p < 0.049$ *	2
P. obliquum C	54	27.15	$p < 0.001$***	2
P. fimbriulatum A	126	145.72	$p < 0.001$***	2
P. fimbriulatum B	50	44.40	$p < 0.001$***	2
P. fimbriulatum C	37	33.43	$p < 0.001$***	2
P. cenocladum	48	49.81	$p < 0.001$***	2
P. nigrum	41	33.51	$p < 0.001$***	2
P. aduncum	40	51.41	$p < 0.001$***	2
P. sp_1	36	36.19	$p < 0.001$***	2
P. sp_2	42	52.11	$p < 0.001$***	2

Post-hoc analyses with Wilcoxon signed-rank tests for related samples between each of the three possible pairs of the regions were conducted with Bonferroni-Holms correction applied.

Table 5: Myrmecophytic *Piper* species. *Post-hoc* analyses with Wilcoxon signed-rank tests showed statistically significant differences between the oil cell distribution in the three regions (A), (I) and (M). In *P. obliquum* B no significant differences were found between (A) and (I) and (A) and (M). In *P. fimbriulatum* C and *P. obliquum* C (I) and (M) did not differ significantly from each other. *** $p < 0.001$, * $p <$ Bonferroni-Holms corrected *p*-value, n = number of cross-sections per plant.

Post-hoc pairs	*P. obliquum* A n=58	*P. obliquum* B n=19	
A<I	$p < 0.001$***	$p = 0.650$	
A<M	$p < 0.001$***	$p = 0.395$	
I<M	$p = 0.024$*	$p = 0.004$*	
Post-hoc pairs	*P. fimbriulatum* B n=50	*P. fimbriulatum* C n=37	*P. cenocladum* n=48
A<I	$p < 0.001$***	$p < 0.001$***	$p < 0.001$***
A<M	$p < 0.001$***	$p < 0.001$***	$p < 0.001$***
I<M	$p = 0.011$*	p = 0,316	$p < 0.001$***
Post-hoc pairs	*P. fimbriulatum* A n=126	*P. obliquum* C n=54	
A<I	$p < 0.001$***	$p < 0.001$***	
A<M	$p < 0.001$***	$p < 0.001$***	
I>M	$p < 0.001$***	$p = 0.1$	

Table 6: Non-myrmecophytic *Piper* species. *Post-hoc* analyses with Wilcoxon signed-rank tests showed statistically significant differences between the oil cell distribution in the three regions (A), (I) and (M). Differences between (I) and (M) in *P. sp*_1 are not significant. *** $p < 0.001$, * $p <$ Bonferroni-Holms corrected p-value, n = number of cross-sections per plant.

Post-hoc pairs	*P. nigrum* n=41	*Post-hoc* pairs	*P. sp*_1 n=36
A<I	$p < 0.001$***	A<I	$p < 0.001$***
A<M	$p = 0.003$*	A<M	$p < 0.001$***
I>M	$p = 0.003$*	I=M	$p = 0.858$
Post-hoc pairs	*P. aduncum* n=40	*P. sp*_2 n=42	
A<I	$p < 0.001$***	$p < 0.001$***	
A<M	$p < 0.001$***	$p < 0.001$***	
I<M	$p < 0.001$***	$p < 0.001$***	

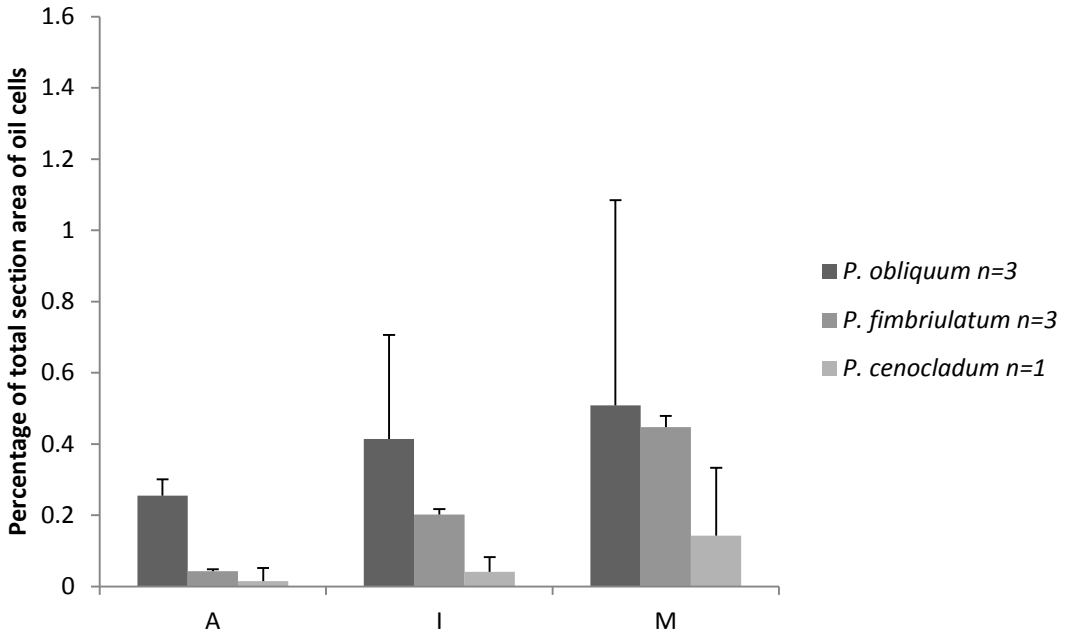


Figure 13: Distribution of oil cells (in percentage of total section area, error bars indicate standard error) ocurring in myrmecophytic *Piper* species, divided into three regions (A), (I) and (M). A = parenchyma of the bark and sclerenchyma ring, I = phloem and xylem and M = parenchyma of the pith (see Figure 1), n= number of plants. For *P. obliquum* and *P. fimbriulatum* mean values are shown.

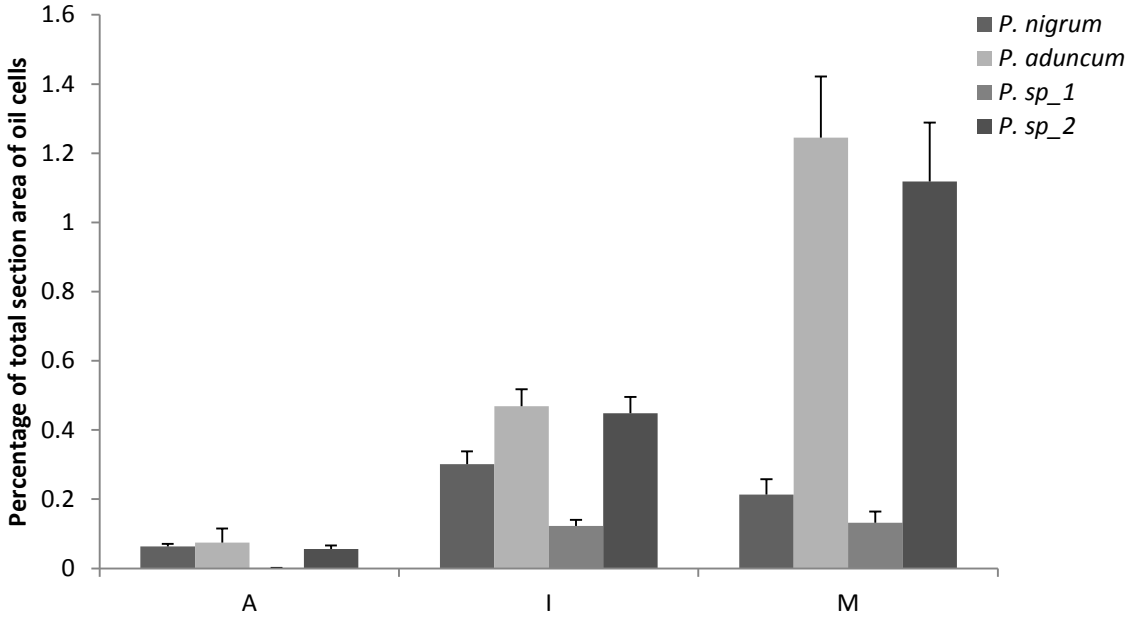


Figure 14: Distribution of oil cells (in percentage of total section area, error bars indicate standard error) ocurring in non-myrmecophytic *Piper* species, divided into three regions (A), (I) and (M). A = parenchyma of the bark and sclerenchyma ring, I = phloem and xylem and M = parenchyma of the pith (see Figure 1), number of plants per species tested=1.

In all species regardless if myrmecophytic or not, percentage amount of oil cells in the parenchyma of the bark and sclerenchyma ring (A) was significantly lower than in the phloem and xylem region (I) and in the parenchyma of the pith (M) (see Table 5 and Table 6). Occurrence of oil cells was significantly higher in (M) than in (I), except for *P.sp*_1, *P. fimbriulatum* A, *P. obliquum* C and *P. nigrum*. In *P.sp*_1 there was no significant difference between the distribution of oil cells in (M) and (I). In *P. fimbriulatum* A and *P. nigrum* (I) had a significantly higher amount of oil cells than (M). In *P. obliquum* B no differences could be found regarding the distribution of oil cells in (A) and (I) as well as (A) and (M). In *P. aduncum* occurrence of oil cells was significantly lower in (A) than in (I) and (M) and in (I) it was significantly lower than in (M).

Wilcoxon signed-rank tests were conducted to look for differences in the distribution of oil cells between myrmecophytic and non-myrmecophytic *Piper* species regarding the three regions (A), (I) and (M). In the parenchyma of the bark and sclerenchyma ring (A) no differences could be found (p = 0.61) as well as in the phloem and xylem region (I, p = 0.11) and in the pith (M, p = 0.48). There is no significant evidence, that the distribution of oil cells between myrmecophytic and non-myrmecophytic *Piper* species is different.

5 Discussion

In this study, we focused on the induced ant-recruitment in *Piper obliquum* and *Piper fimbriulatum* following artificial stem-injuries. We did field experiments with various chemical compounds in order to get an idea on the effect of the components. Via DNA-barcoding it was investigated whether different ant species are present in myrmecophytic *Piper*. As we hypothesize that oil cells store the compounds causing recruiting and alarm, stem sections of myrmecophytic and non-myrmecophytic *Piper* species were made to examine oil cell distribution.

DNA barcoding

Obligate myrmecophytes can be associated with more than one single ant species. To make sure that the influence of different ant species can be excluded in this study, DNA barcoding was performed. DNA barcoding is a method to identify organisms, mostly by amplifying the protein coding cytochrome c oxidase subunit I (COI) region of the mitochondrial genome.

For this purpose, barcoding sequences of unknown specimens are compared with already known ones from libraries. The results showed that in 18 out of 19 myrmecophytic plants the presence of different ant species can be virtually eliminated.

It is assumed that in this study *Pheidole bicornis* inhabited all investigated plants, because *Piper* ant-plants are nearly always occupied by these species (Longino and Cover, 2004). Although the one which does not seem to be *Pheidole bicornis* did not stand out in the course of the experiment, it appears to be a different ant species.

Communication via volatiles

In tropical forests herbivore pressure is high, which results in an evolution of phenological, mechanical and chemical defenses in plants and a diversity of adaptations and interactions between the herbivore-plant relationship (Agrawal, 2007; Coley and Barone, 1996). Herbivory affects plant fitness (e.g. growth, reproduction, competitive ability, photosynthetic rate) negatively and consequently effective anti-herbivore defense strategies are needed (Coley and Barone, 1996).

Plants defend themselves against herbivorous insects directly via physical and chemical defenses and indirectly through attracting natural enemies of herbivores – parasitoids and predators (Kessler and Baldwin, 2001). Those chemical defense mechanisms are constitutive or induced. Many plants are defended by mutualistic ants (Gaume et al., 2005; Heil et al., 2001; Heil and McKey, 2003). The plants release volatiles following herbivory (Heil and Bueno, 2007). Field experiments with *Piper sp.*, *Cecropia sp*., *Macaranga sp.* and *Leonardoxa africana* ssp. *africana* provide strong evidence that communication between the ants and plants runs via those volatile cues (Agrawal, 1998; Brouat et al., 2000; Fiala and Maschwitz, 1990; Fiala et al., 1989; Mayer et al., 2008). Schatz et al. (2009) found that *Petalomyrmex phylax*, which inhabitates the African tree *Leonardoxa africana*, is attracted by one specific compound released by the plant. Methyl salicylate is able to induce a full ant response. It is emitted constitutively only by the young leaves causing patrolling behavior and thus protection of the developing leaves (Brouat et al., 2000). Due to their higher nitrogen and water content and the fast photosynthetic rate, they are often more valuable to the plant (Letourneau, 1983) and are constantly patrolled even if there are no enemies (Heil and McKey, 2003). There are many other examples of ants patrolling younger plant parts, e.g. *Acacia, Macaranga*, *Cecropia* and *Barteria* (Heil et al., 2001), but except for *Leonardoxa* it is not known why.

Apparently ants are able to recognize whether volatiles are released from damaged sites due to herbivory and recruit specifically to the injuries to defend their host plants. In the work of Mayer et al. (2008) it was shown that only mechanical disturbance of myrmecophytic *Piper* stems does not elicit ant-recruitment of their resident ants *Pheidole bicornis* but that volatiles released from the host plant upon damage are responsible.

After stem damage to myrmecophytic and non-myrmecophytic *Piper* species 68 volatile organic compounds (VOCs) were identified (Mayer et al., 2008).

It is known that herbivore-damaged plants emit a wealth of fatty-acid derivatives, terpenes and many other volatile compounds (Pichersky and Gershenzon, 2002). Green-leaf volatiles (GLVs), emerged from the fatty-acid derivatives, are released immediately after general damage due to the breakdown and lipoxygenation of lipid membranes (Caissard et al., 2004).

The question remains, what substances elicit ant-recruitment in the *Piper-Pheidole* association? Are single GLVs sufficient, or are compound mixtures needed to increase the alertness of the ants? Mayer et al. (2008) compared the VOC profiles between myrmecophytic and non-myrmecophytic *Piper* species and found that apart from some terpenes also GLVs occur exclusively in myrmecophytic species. Thus, it is hypothesized that GLVs are elicitors of ant-recruitment.

In this study the influence of green-leaf volatiles on the induced ant-recruitment cannot be approved. The mixture with GLVs showed no significant differences compared to the artificial injury in *P. obliquum* (see Table 2), which means that this treatment might be responsible for the induced ant defense. Unexpectedly, the mixture without GLVs showed no significant differences too, when compared to the injury, which means that the treatment without GLVs could induce ant response as well. Apparently GLVs are not reclusively unique to elicit ant-recruitment, but maybe the mixture of ß-caryophyllene and 3-carene 1:100 is. Further tests are needed to clarify the influence of this mixture to the ant response. Do single compounds yield a full ant-response? In *P. obliquum* β-caryophyllene, β-caryophyllene 1:100, *trans*-3-carene 1:10 and para-cymene exhibited no statistical differences compared to the injury (see Table 2) and might have had influence on the ant-response. Those chemical compounds were chosen on the basis of results from solid phase microextraction and gas chromatography-mass spectrometry of *Piper* species in earlier studies (Mayer et al., unpublished data) and there is a possibility that those substances are elicitors for the ant-recruitment.

β-caryophyllene is a sesquiterpene hydrocarbon, which especially occurs in the chemical composition as a major component of the leaf essential oils from myrmecophytic *P. fimbriulatum*, *P. obliquum* and non-myrmecophytic *P. nigrum* (Martins et al., 1998; Mundina et al., 1998; Scott et al., 2008) and is an inducible substance, emitted after damage in plants caused by herbivores (Holopainen, 2004). In myrmecophytic *Piper* species these substances were proven to be emitted when the stems are damaged. A detailed emission-profile from injured leaves is still needed to prove possible influences on the ant-recruitment.

All other chemical compounds used in this study on *P. obliquum* and *P. fimbriulatum* showed statistical differences when tested against the injury, which means that none of these terpenes yielded an ant-response like the artificial injury did. Ant-response peaked between minute 6 and minute 12 after artificial stem damage (see Figure 5), which is consistent with the findings of Agrawal (1998), who stated that such rapid defense mechanisms are probably "effective against unpredictable and mobile herbivores".

The results presented in this study provide compelling evidence that not a single substance or GLVs are responsible for the herbivore-induced ant-recruitment. As a result of this study it is hypothesized that a bouquet of substances is contributing to a full ant-response.

VOCs and oil cells

Plants produce a vast amount of volatile organic compounds (VOCs) for general and specialized functions (Dudareva et al., 2004) and as they are sedentary, the release of VOCs provides a way of communication with other organisms across distances (Pichersky and Gershenzon, 2002). Generally, those compounds attract species-specific pollinators (Dicke and Baldwin, 2010) and are emitted from undamaged plant parts. One specialized function of VOCs of myrmecophytic *Piper* species may be signaling herbivore activity (e.g. stem injury, leaf damage) to their resident ants. It is beneficial to the plant when herbivore activity is transmitted quickly to the resident ants, so it is very convenient to produce rapidly diffusing compounds (Schatz et al., 2009). In general the VOC-profiles from undamaged plants are noticeably different from those, which are released after mechanical damage to the plant (Paré and Tumlinson, 1999). VOCs are derived from terpenoid, fatty-acid and phenolic metabolic pathways and stored in trichomes, specialized glands or vacuoles (Baldwin et al., 2006; Paré and Tumlinson, 1997). They can be emitted either at the site of damage in leaves or stems or systemically from undamaged parts of affected plants (Dicke and Baldwin, 2010).

Volatiles which are released upon damage of the tissues can be stored in laticifers, precursors in deconjugated forms, whereas other volatiles are stored in conjugated forms in specialized ducts or vacuoles (Baldwin, 2010). In certain cases volatiles are stored under the cuticle (Caissard et al., 2004). Volatile compounds which are released immediately after damage arise from stored pools (Dudareva et al., 2004; Paré and Tumlinson, 1997). The morphology of secreting oil cells is very diverse and includes highly specialized glandular trichomes, osmophores, ducts, conical-papillate cells and sometimes even non-specialized cells (Caissard et al., 2004).

In this study the comparison of the oil cell distribution between myrmecophytes and non-myrmecophytes resulted in non-significant findings. A possible explanation might be, that those volatiles secreted in oil cells did not evolve specifically to elicit ant responses, but were already present in the plant's tissue and used for "alternative physiological processes (i.e. exaptation rather than adaptation)" (Brouat et al., 2000; Bruna et al., 2008). Regarding the distribution between the three different regions parenchyma of the bark and sclerenchyma ring (A), phloem and xylem (I) and parenchyma of the pith (M) (see Figure 3) the results obtained are consistent with Brouat's hypothesis of exaptation (Brouat et al., 2000). In the pith there seems to be the largest number of oil cells aggregated, followed by the phloem and xylem and the least oil cells are found in the bark (see Table 5). The pith gets excavated by the ants, so obviously the oil cells in unexcavated myrmecophytic *Piper* species do fulfill other tasks than signaling herbivore activity. They might be needed for some physiological processes in the tissue of the plants. Before ants excavate the stems of myrmecophytes to create cauline domatia (Risch et al., 1977), they are solid and characterized by heterogeneous pith containing large cells without intercellular crystals (Tepe et al., 2007). Future domatia are restricted to this large-celled region (Tepe et al., 2007), hence the structure of the stem influences the excavation of the pith center directly (Bailey, 1923). This heterogeneous pith with the lack of crystals seems to be an important feature of myrmecophytes of *Piper* sect. *Macrostachys* (Tepe et al., 2007).

The question arises, why oil cells are located in the pith, when it gets excavated? These findings give a strong support for Brouat's hypothesis of exaptation (Brouat et al., 2000) which means that it may be possible that oil cells are present in the tissue of the plants for alternative physiological processes and did not evolve specifically to elicit ant responses.

Influences on the experimental settings

Ants protect their myrmecophytic host plant against a broad range of herbivores (e.g. specialist feeders on *Piper* species are coleopterans and lepidopterans (Heil and McKey, 2003; Letourneau and Dyer, 1998a)), but there are limitations. The influence of a specialized spider, which was present in some of the investigated plants, cannot be excluded and therefore results could be falsified. *Dipoena banksii* (Arachnida: Aranae: Theridiidae) occurs on *Piper* ant-plants and preys on *Pheidole bicornis* (Gastreich, 1999). The spiders build their webs at leaf bases and capture the ants when they are crossing or entering/exiting the petioles (Letourneau and Dyer, 1998a). Gastreich showed, that ants detect and avoid those leaves with the webs and therefore plants suffer from significantly higher rates of folivory when inhabited by *Dipoena banksii* (Gastreich, 1999). Even though we removed spiders before we started the experiments, this "trait-mediated indirect interaction" between ants and spiders could have had great impact on this study.

The occurrence of specialized beetles that exploit *Pheidole* ants on *Piper* was not considered in this study either (Letourneau and Dyer, 1998a). *Phyllobaenus* spp. (Coleoptera: Cleridae) is a parasitic beetle that lives as larva inside the domatia, where it feeds on ants and ant brood (Heil and McKey, 2003; Letourneau and others, 1990). Normally the plants produce food bodies only in the presence of their ant-partners (Risch and Rickson, 1981), but it seems, that this beetle has the ability to stimulate *Piper* myrmecophytes to produce food bodies which it consumes, if ants are absent (Letourneau and others, 1990). By adding *Tarsobaenus letourneauae* to the ant-plant system, Letourneau showed that the average abundance of *Pheidole bicornis* was reduced fivefold and herbivory to the leaves of *Piper cenocladum* increased nearly threefold (Letourneau and Dyer, 1998b). Those specialized beetles thus exploit the mutualistic associations between *Piper* and *Pheidole* (Letourneau and others, 1990). In this study it was not investigated whether plants did suffer from *Phyllobaenus* or any other parasitic beetle, which might have reduced colony size and jeopardized ant fitness, which indirectly influenced volatiles experiments.

Further, it has to be taken into consideration that the size of ant colonies can vary greatly, which certainly has an influence on the number of workers recruited to the site of damage. The study of Mayer et al. (2008) illustrated that colony size can vary between 18 and almost 3000 workers, regarding similar height and condition of the host plants. Rocha and Bergallo (1992) found a positive correlation between colony size and ant-recruitment in the *Cecropia* and *Azteca* association and maybe this holds also true for the *Piper-Pheidole* system.

Inui and Itioka (2007) postulated that ants might respond to damage of their host plant with the release of volatile compounds (e.g. alarm pheromones), in order to recruit others not until they are needed for defense. In the meantime workers can do other tasks (e.g. foraging for food, caring for brood) to enhance colony fitness which in turn indirectly influences the condition of plants positively (Bruna et al., 2008). Gordon (1983), however, stated that "social factors" (e.g. nest maintenance, brood care, feeding) might have an influence on the ant's response to chemical cues and maybe this can explain in part the variability in the ant-recruitment in the experimental settings (see Table 1).

Baldwin found that herbivore-induced release of VOCs differs from the release due to mechanical wounding in many plant species (Baldwin et al., 2006). Obviously a plant can distinguish between herbivore damage and general injuries with the aid of certain components (Paré and Tumlinson, 1999). In this study, plants were injured with a surgical scalpel, so maybe the results got falsified, because of those missing special components which indicate herbivore activity. The emission of volatiles can be also triggered by saliva-derived compounds introduced in the sites of the injury (Baldwin et al., 2006; Halitschke et al., 2001; Paré and Tumlinson, 1999) but a lot of counter-examples show, that those oral regurgitants are not necessarily required (see (Blatrix and Mayer, 2010) and references therein). It is likely, that missing oral exudates did not contribute to the ant-recruitment in this study.

The amount of volatiles and subsequently the composition and yield of essential oils can vary with environmental conditions such as moisture, light and temperature (Dudareva et al., 2004). A variety of terpenes is usually emitted during periods of high temperature, independent of insect feeding (Paré and Tumlinson, 1999). We did not record moisture, lighting conditions or temperature but those factors could have impact on the plant's physiology (Dudareva et al., 2004; Paré and Tumlinson, 1999) and consequently on the released VOC-profile and on the intensity of the ant response in the volatile experiments.

Mutualistic ants contribute a lot to the fitness and survival of their host plants (Beattie, 1985; Heil and McKey, 2003) mainly by reducing herbivore damage and reducing fungal infections (Letourneau, 1998). The loss of an ant-colony affects the host plants tremendously, resulting in higher rates of herbivory, lower vigor, reduced fertility, lower potential seed set and even elevated probability of mortality (Heil and McKey, 2003; Letourneau, 1998). In return, ants are dependent on the fitness of the plant, because they would lose nesting space and food sources if the plant is in a bad condition. It is crucial for plant survival that resident ants respond specifically to stem damage and herbivory and consequently for their own survival on the plants (Inui and Itioka, 2007).

In summary, in this study it was shown that ants did respond to single terpenes and displayed aggressive behavior to protect their host plant but the intensity of the reaction was lower compared to the real injury. Obviously, ants need more than a single chemical compound to recruit to the site of the damaged plant parts.

6 Conclusion

In this project the barcoding method was helpful to assign 18 out of 19 amplified sequences clearly to the same species. Although the one which does not seem to be *Pheidole bicornis* did not stand out in the course of the experiment, it appears to be a different ant species. Field experiments showed that neither GLVs nor single substances do have exclusive impact on the ant recruitment. However, the exact substances which are responsible for ant-recruitment remain to be determined.

The distribution of oil cells between myrmecophytic and non-myrmecophytic Piper species was nearly the same. We conclude that those cells are for alternative physiological processes in the plant and did not evolve specifically to elicit ant responses. Most notably, this is the first study to our knowledge, which investigated oil cell distribution in different *Piper* species. Hence, it is definitely a promising field for future research to come one step closer unraveling the communication between *Piper* and their *Pheidole* ants and the complex mechanisms of volatiles in this association.

7 Zusammenfassung

In den Tropen gibt es eine Reihe von Lebensgemeinschaften von Ameisen und Pflanzen, die voneinander abhängig sind. Einige zentralamerikanische *Piper*-Arten, die zur Sektion *Macrostachys* gehören, sind eine obligatorische Verbindung mit Ameisen eingegangen. Die Pflanzen stellen Wohnräume und Nahrung bereit, während Ameisen die Pflanzen im Gegenzug vor Pflanzenschädlingen und Pilzinfektionen schützen. Das Hauptziel dieser Arbeit bestand darin, die durch Pflanzenschäden induzierte Ameisenansammlung von pflanzenbewohnenden *Pheidole bicornis* (Myrmicinae) in myrmekophytischen *Piper* Arten zu untersuchen. Im ersten Teil dieser Arbeit wurden Feldversuche mit *Piper obliquum* und *Piper fimbriulatum* im Parque Nacional Piedras Blancas, nahe der biologischen Forschungsstadion La Gamba in Costa Rica durchgeführt. Verschiedene flüchtige Duftstoffe (Terpene) wurden auf ein Filterpapier pipettiert und am zweitjüngsten Internodium der Pflanzen angeboten. In verschiedenen Versuchsdurchläufen wurden Reinsubstanzen (ß-Caryophyllen, α-Copaen, Para-Cymen und *trans*-3-Caren), Verdünnungen dieser Reinsubstanzen und Mischungen der Terpene, sowie Green-leaf volatiles verwendet. In der Auswertung wurde ersichtlich, dass weder Green-leaf volatiles noch eine andere chemische Substanz eine mehr oder weniger gleichstarke Ameisenrekrutierung ausgelöst hat, wie die künstliche Stammverletzung mit einem Skalpell. Daraus kann geschlossen werden, dass eine starke Ameisenrekrutierung vermutlich von der Kombination mehrerer Faktoren (Umwelteinflüsse, Koloniegröße, spezifischere Duftstoffe) abhängt. Mittels DNA-barcoding wurde herausgefunden, dass es sich bei 18 von 19 untersuchten Pflanzen immer um dieselbe Ameisenart, nämlich *Pheidole bicornis*, handelt. Das Vorhandensein von mehreren Ameisenarten kann in dieser Studie praktisch ausgeschlossen werden. Um das Vorkommen und die Verteilung von Ölzellen, welche Terpene enthalten, zu untersuchen, wurden Stammquerschnitte diverser myrmekophytischer und nicht-myrmekophytischer *Piper* Arten angefertigt. Diese wurden in drei Bereiche unterteilt: Rindenparenchym mit sklerenchymatischem Ring, Region mit Phloem und Xylem und Markparenchym. Vergleiche zwischen diesen Regionen zeigten, dass sich die meisten Ölzellen im Mark der Stämme und die wenigstens in der Rinde befinden. Weiters wurden die Stammquerschnitte für Vergleiche zwischen myrmekophytischen und nicht-myrmekophytischen *Piper* Arten herangezogen.

Es konnten keine signifikanten Ergebnisse bezüglich der Verteilung der Ölzellen gefunden werden, was uns zu der Annahme führt, dass Ölzellen im Gewebe der Pflanzen möglicherweise für alternative physiologische Prozesse notwendig sind und sich nicht unbedingt spezifisch evolviert haben, um eine Reaktion bei den Ameisen auszulösen.

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9 Appendix

How to measure areas of oil cells in Photoshop CS 6

At first, you need to take a picture of the stem-section with the oil cells stained. Depending on the program you use, it is important to note the scale at the certain magnification you applied. In my case I used an Olympus BX50 microscope and took pictures with a Nikon Digital Sight (DS-U2). For those devices you can extract correction values depending on magnification from Table 7.

Table 7: Magnification (x) and correction values (µm/px)

x	µm/px
M 1.25	5.54
M 4	1.71
M 10	0.68
M 20	0.34
M 40	0.17

After that, you open Photoshop CS 6 and load the picture you want to measure. Now you need to set the measuring scale.

Image → Analysis → Set measurement scale → Custom

Your pixel length is always 1 and the logical length depends on your magnification (e.g. you took a picture with a 4x magnification, so your logical length is 1.17, see Table 7).

With the "Eyedropper Tool" you can absorb the color you want to select. After that, you need to select this color.

Select → Color Range

You can adjust the results by lowering or increasing the tolerance.

With the "Quick Selection Tool" you can remove or add areas. After that you are ready to record and export your measurements.

Image → Analysis → Record measurements

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