Mechanisms enabling specific plant-ant mutualisms:

Acacia-Pseudomyrmex as a model system

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Index

List of Figures 8

List of Tables 10

List of Abbreviations 12

Abstract 15

Deutschsprachige Zusammenfassung 17

Introduction 20

Plant-ant mutualisms 20

Extrafloral nectar (EFN) as a plant reward 23

Ant services to host plants 25

Material and Methods 28

Study system 28

EFN collection and quantification 32

Carbohydrate and amino acid analysis in EFN and ant attraction 32

1. Quantification of carbohydrates and amino acids 32

2. Ant behavioural assays 33

Protein analysis in EFN and protection from microbial infestation 38

1. Quantification of proteins and SDS-PAGE 38

2. Two-dimensional gel electrophoresis and mass spectrometry 40

3. Antifungal protection of EFN in nature 44

4. PR-enzyme activities 45

5. Antifungal effects of EFN 51

Production pattern of EFN, ant defence, and nectar robbers 54

1. Time course of EFN production and ant activity 54

2. Ant-mediated defence of EFN against nectar robbers 55

Ant defence against herbivory and leaf pathogens 56

1. Ant defence against herbivores 56

2. Ant-mediated defence against leaf pathogens 56

3. Volatile analysis of ants 57

Results 59
EFN amino acids and attraction function 59
1. Sugars and amino acids 59
2. Non-Metric Multidimensional Scaling (NMDS) 63
3. Amino acids and ant attraction 66
EFN proteins and protection function from microorganisms 70
1. Total proteins 70
2. Identification and quantification of PR-proteins 73
3. Antifungal protection of EFN in nature 83
4. Pathogenesis-related (PR) enzymes 84
5. Antifungal effects of EFN 87
Temporal pattern of EFN reduces exploitation by nectar robber. 92
1. EFN secretion and ant activity 92
2. Ant-mediated defence against nectar robbers 92
Variations in ant defence against herbivores and pathogens 95
1. Ant defence against herbivores 95
2. Ant defence against leaf pathogens 97
3. Volatile analysis of ants 98
Discussion 103
Attraction function of EFN 103
Protection function of EFN 106
EFN pattern protects exploitation 108
Ant defence effects 109
Conclusion 111
References 113
Curriculum Vitae 122
Erklärung 128

List of Figures

Fig. 1: Myrmecophyte and non-myrmecophyte plant species used in the study 30
Fig. 2: Symbiotic and parasite ant species used in the study 31
Fig. 3: Diagram of the disk diffusion method used for evaluation of EFN inhibitory effects against six fungal species 53
Fig. 4: Sugar quantities in EFNs of myrmecophytes and non-myrmecophytes 60
Fig. 5: EFN secretion rates in myrmecophytes and non-myrmecophytes 61
Fig. 6: Non-metric Multidimensional Scaling (NMDS) ordination diagram of amino acids composition in EFNs 64
Fig. 7: Preferences of symbiotic and non-symbiotic ants to natural EFNs and various EFN mimics with and without amino acids (AAs) 68
Fig. 8: Preferences of symbiotic and non-symbiotic ants to 4AA and 8AA solutions, respectively, with different AA:sugar ratios, and to solutions with different number of AAs 69
Fig. 9: Protein quantities in EFNs 71
Fig. 10: Protein pattern in EFN by SDS-PAGE 72
Fig. 11: Two-dimensional Gel Electrophoresis for *A. cornigera* 74
Fig. 12: Two-dimensional Gel Electrophoresis for *A. hindsii* 75
Fig. 13: Two-dimensional Gel Electrophoresis for *A. collinsii* 76
Fig. 14: Presece of fungi in fresh EFN samples 83
Fig. 15: Activities of three pathogenesis-related (PR) enzymes in EFNs 85
Fig. 16: Acidic and basic chitinase isoforms in EFNs myrmecophyte and non-myrmecophyte species 86
Fig. 17: Acidic and basic glucanase isoforms in EFNs of myrmecophyte and non-myrmecophyte species 86
Fig. 18: Yeast growth in EFNs of myrmecophyte and non-myrmecophyte species 88
Fig. 19: Yeast growth in artificial nectar solutions, with an without chitinase activity 89
Fig. 20: Inhibitory effect of EFN of myrmecophyte and non-myrmecophyte species on growth of the fungal species *P. parasitica* and *F. oxyporum* 91
Fig. 21: Diel patterns in EFN production and activity patterns of the symbiotic ant *P. ferrugineus* on three *Acacia* myrmecophytes 93

Fig. 22: Diel activity patterns of nectar robbers (*F. nigra*) on canopies of three *Acacia* myrmecophytes 94
Fig. 23: Percentage of leaf herbivory and EFN secretion in plants of *A. cornigera* and *A. hindsii* in presence and absence of *P. ferrugineus* ants 96
Fig. 24: Effects of presence and absence of *P. ferrugineus* and *P. gracilis* on bacteria abundance in leaf samples of *A. cornigera* and *A. hindsii* 99
Fig. 25: Effects of presence and absence of *P. ferrugineus* and *P. gracilis* on bacteria diversity in leaf samples of *A. cornigera* and *A. hindsii* 100
Fig. 26: Bacteria present in leaf samples of *Acacia* species 101

List of Tables

Table 1: Composition of sugars - amino acid solutions used for cafeteria experiments 34

Table 2: Composition of SDS-PAGE for nectar protein separation 38

Table 3: Composition of running buffer Tris-Glycine pH 8.3 used for protein separations by SDS-PAGE 39

Table 4: Composition of loading buffer pH 6.8 used ofr protein separation by SDS-PAGE 39

Table 5: Composition of protein staining solution 39

Table 6: Rehydration solution used for gel strips 41

Table 7: Conditions used for Isoelectric focusing (IEF) of gel strips 41

Table 8: Equilibration buffer I for reduction of gel strips 41

Table 9: Equilibration buffer II for alkylation of gel strips 42

Table 10: SDS-PAGE for nectar protein separation. 42

Table 11: Composition for runninf buffer Tris-Glycine pH 8.3 used for protein separation by 2D-SDS-PAGE 42

Table 12: Composition of polyacrylamide gels for determination of acidic / neutral chitinase and glucanase activities 47

Table 13: Composition of running buffer Tris-Glycine pH 8.3 used for acidic / neutral chitinases and glucanases 47

Table 14: Composition of loading buffer pH 6.7 used for acidic / neutral chitinases and glucanases 47

Table 15: Composition of polyacrylamide gels formdetermination of basic chitinase and glucanase activities 49

Table 16: Composition of running buffer Tris-Glycine pH 8.3 for basic chitinases and glucanases 49

Table 17: Composition of loading buffer used for basic chitinases and glucanases 49

Table 18: Composition of the polyacrylamide overlay gel for protein transfers used for basic chitinases and glucanases 50

Table 19: Concentration of single amino acids (AAs), total AAs and total sugars in EFNs of myrmecophyte and non-myrmecophyte species 62

Table 20: Correlations between specific amino acids and the two NMDS axes in EFN of myrmecophyte and non-myrmecophyte species 65

Table 21: Results of MS-BLAST searching using *de novo* peptide sequences for the species *A. cornigera* 77

Table 22: Results of MS-BLAST searching using *de novo* peptide sequences
for the species *A. hindsii* 79
Table 23: Results of MS-BLAST searching using de novo peptide sequences
for the species *A. collinsii* 81
Table 24: Antifungal effects of EFN from myrmecophyte and non-myrmecophyte
species on different fungal species 90
Table 25: Antifungal activity against *Phytophthora parasitica* in different fractions
from EFN from myrmecophyte and non-myrmecophyte species 90
Table 26: Volatiles emitted by *P. ferrugineus* and *P. gracilis* ants 102

List of Abbreviations

'	Minute
%	Percentage
:	Ratio
~	Approximately
2D-PAGE	Two dimensional-polyacrylamide gel electrophoresis
AAs	Amino ccids
AM	Ante Meridiem
ANOVA	Analisis of Variance
APS	Ammonium persulfate
C	Celsius
CFU	Colony forming units
cm	Centimeter
DTT	Dithiothreitol
EFN	Extrafloral nectar
F	Fructose
fmol	Fentomol
G	Glucose
g	Gram
GC	Gas Chromatography
h	Hours
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
I.A.	Internal diameter
IAA	Iodoacetamide
IEF	Isoelectric focusing
JA	Jasmonic acid
K_2HPO_4	Monopotassium phosphate
kD	Kilodalton
KOH	Potassium hydroxide
kV	Kilovolts
L	Liter

LSD	Least significant difference
m	Meters
M	Molar
m/z	mass-to-charge ratio
mA	Miliamper
MALDI-TOF	Matrix Assisted Laser Desorption/Ionisation-Time of Flight
MeCN	Acetonitrile
mL	Mililiter
mM	Milimolar
mm	milimeter
mmoL	Milimol
mmoL	Milimol
MS	Mass Spectrometry
Myrm.	Myrmecophyte
N	North
N	Repetition number
Na-acetate	Sodium acetate
Na-phosphate	Sodium phosphate
ng	Nanogram
nL	Nanoliter
NMDS	Non-Metric Multidimensional Scaling
Non Myrm.	Non myrmecophyte
^{o}	Grade
OD	Optical density
PBS	Phosphate buffered saline
PM	Post Meridiem
rpm	Revolutions per minute
RT	Retention time
S	Sucrose
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
sec.	Second
Sol.	Solution
TCA	Trichloroacetic acid
TEMED	Tetramethylethylenediamine

Tris	Tris(hydroxymethyl)aminomethane
U	Units
uL	Microliter
UV	Ultraviolet
v	Volume
V	Volts
VOCs	Volatile organic compounds
W	West
w	Weight

Abstract

Mutualisms are interactions among different species that lead to net fitness benefits for all partners involved. In plant-ant mutualisms, plants provide to ants an array of rewards, such as extrafloral nectar (EFN), food bodies, or nesting space. Ants are attracted, or completely nourished, by plant-derived food rewards and serve as a means of indirect defence of plants against herbivores. Although these mutualisms can become very specific, the rewards traded among mutualist partners may also be attractive for non-mutualist organisms, i.e., exploiters that make use of the host-derived rewards without reciprocating. Thus, the goal of this study was to investigate mechanisms that drive the specificity of plant-ant interactions, and that stabilize it from exploitation. The mutualism of *Acacia* plants with *Pseudomyrmex* ants was used as a model system, in which we can find different kinds of plant-ant interactions that vary in their specificity: facultative and obligate. Whereas *Acacia* obligate plants (myrmecophytes) secrete EFN at high quantities and constituvely, to house and nourish symbiotic ants of *P. ferrugineus*, facultative ones (non-myrmecophytes) secrete it only in response to damage, attracting generalist ants. These differences in plant-ant interactions make this genus *Acacia* highly suitable to study mechanisms that may determine species-specific interaction. Specifically, I focused my study on the chemistry of EFN (amino acids and proteins) and on the ant behaviour in terms of defence against nectar robbers, herbivores and leaf pathogens.

Amino acid composition of obligate *Acacia* was highly specialized and adapted to the preferences and nutritive requirements of the specialised mutualist ant *P. ferrugineus*. Mutualist ants preferred EFN solutions that contained exactly those amino acids that were quantitatively dominating in myrmecophyte EFN. By contrast, generalist ants preferred sugar solutions with amino acids over mere sugar solutions but were not able to discriminate among different numbers or concentrations of specific amino acids, suggesting, thus, that amino

acids of non-myrmecophyte EFN play an important role in the attraction but less so in the nutrition of ants. On the other hand, EFN of obligate *Acacia* species appeared (bio)chemically protected from microbe infestation. Bioassays demonstrated that fungal growth was inhibited in EFN of myrmecophytes. The identification of proteins in myrmecophyte nectar revealed an abundant presence of PR-proteins, such as glucanases, chitinases and thaumatin- and osmotin like proteins, of which activities were also detected in EFNs. Furthermore, the total amount of proteins was significantly higher in myrmecophyte EFN than in the EFN of non-myrmecophytes. These data, together with the observations that the protein-fraction of myrmecophyte EFN significantly inhibited the growth of various fungi, suggests that nectar proteins are associated with the protection of EFN from microbes.

In parallel to these chemical adaptations on the side of the plant, symbiotic ants of *P. ferrugineus*, unlike the parasite *P. gracilis*, exhibited relevant ecological and chemical adaptations, which contribute to the specificity of the mutualism. *P. ferrugineus* effectively defended their host plants against herbivores and leaf bacteria and protected the EFN from nectar robbers. Nevertheless, the defensive efficiency provided by *P. ferrugineus* was associated with the amounts of rewards provided by the host plant: the host species that invest less in ant rewards received less defence by the symbiotic ant. Thus, *P. ferrugineus* tended to diminish its defensive service when it did not receive the respective pay-off from the host. On the other hand, *P. ferrugineus* had the capacity to induce EFN secretion by myrmecophytes, demonstrating that the host plant also can cease reward production when it does not receive the expected biotic defence. The results of the present study illustrate different chemical and ecological mechanisms that drive the specificity of the *Acacia*-*Pseudomyrmex* mutualism, thus, helping 1) to prevent the mutualism from exploitation and, 2) to stabilize the mutualist interaction.

Deutschsprachige Zusammenfassung

Mutualismen sind Interaktionen verschiedener Arten, bei denen ein Partner einen „Service“ erbringt, welcher von einem anderen Partner „belohnt“ wird. In Pflanzen-Ameisen Mutualismen bieten Pflanzen Ameisen Nahrung in Form von extrafloralem Nektar (EFN) sowie Futterkörperchen und in einigen Fällen auch Nistraum. Im Gegenzug verteidigen Ameisen ihre Pflanze gegen Fraßfeinde, Herbivore und Pathogene. Oft ist es für die Pflanze ein Problem, Nektarkonsumenten fernzuhalten, die keine Gegenleistung erbringen und nur ökologische Kosten verursachen, so genannte „Exploiter“. Ziel meiner Arbeit war die Untersuchung von Mechanismen, die zur Stabilität von Pflanzen-Ameisen Interaktionen führen und vor der Ausnutzung durch solche „Exploiter“ schützen. Der Mutualismus zwischen *Acacia* und *Pseudomyrmex* wurde als Modell verwendet, da verschiedene Spezifitätsgrade innerhalb des Systems auftreten. Während obligate Ameisenpflanzen, so genannte Myrmekophyten, EFN ständig in hohen Raten produzieren, um symbiotische Ameisenkolonien dauerhaft zu ernähren, produzieren die so genannten myrmekophilen Akazienarten EFN erst als eine Antwort auf Herbivorie um Ameisen aus der Umgebung anzulocken. Diese unterschiedlichen Spezifitätsgrade von Pflanzen-Ameisen Interaktionen innerhalb der Gattung *Acacia* erlauben es, artspezifische Interaktionen zu untersuchen. Im Focus meiner Arbeit standen die Untersuchung der chemischen Komponenten des EFNs (Aminosäuren und Proteine) sowie die Untersuchung des Verhaltens von Ameisen im Hinblick auf Verteidigung gegenüber Nektarräubern, Herbivoren und Pathogenen.

Die Aminosäure-Zusammensetzung der myrmekophytischen Akazien war höchst speziell und angepasst an die Präferenzen und Nährstoffbedürfnisse der mutualistischen Ameisen *P. ferrugineus*. Mutualistische Ameisen bevorzugten genau solche EFN-Lösungen, welche die vier quantitativ dominierenden Aminosäuren im Myrmecophyten-EFN enthielten. Im Gegensatz dazu bevorzugten generalistische Ameisen Zuckerlösungen mit Aminosäuren

vor reine Zuckerlösungen. Die Generalisten unterschieden jedoch nicht zwischen Anzahl oder Konzentration spezifischer Aminosäuren. Diese Ergebnisse deuten darauf hin, dass der EFN der myrmekophilen Akazien eine wichtige Rolle für das Anlocken von Ameisen und weniger für deren Ernährung spielt. Weiterhin scheint der EFN der obligaten Akazien (bio)chemisch geschützt vor der Besiedlung durch Mikroben zu sein. In Bioassys war Pilzwachstum durch Myrmekophyten-EFN gehemmt. PR-Proteine (pathogenesis related) wie Glucanasen, Chitinasen, Thaumatin- und Osmotin-Proteine wurden im Myrmekophyten-EFN identifiziert und die entsprechenden Enzymaktivitäten konnten nachgewiesen werden. Zudem war die absolute Menge von Proteinen signifikant höher im EFN der Myrmekophyten als im EFN der myrmekophilen Arten. Diese Ergebnisse deuten zusammen mit der Beobachtung, dass der Proteinanteil des Myrmekophyten-EFNs das Wachstum von verschiedenen Pilzen inhibierte, auf eine Rolle der EFN-Proteine im Schutz vor Mikroben hin.

Parallel zu diesen Anpassungen der Myrmekophyten auf biochemischer Ebene zeigten mutualistischen Ameisen der Art *P. ferrugineus* — im Gegensatz zu der parasitischen Art *P. gracilis* — wichtige ökologische and chemische Anpassunge, welche eine hohe Spezifität des Mutualismus bewirken. *Pseudomyrmex ferrugineus* Ameisen verteidigten die Wirtspflanzen effektiv gegen Herbivores und Pathogene und schützten den EFN vor Nektarräubern. Dennoch war die Effizienz der Verteidigung durch *P. ferrugineus* mit der Menge an Belohnung, welche durch die Pflanze bereit gestellt wurde, verbunden: Wirtspflanzen, die wenig in Belohnungen für die Ameisen investierten, wurden auch weniger effizient durch Ameisen verteidigt. *Pseudomyrmex ferrugineus* verminderte also die Verteidigung, wenn die Ameisen nicht entsprechende Belohnungen von der Pflanze erhielten. Andererseits war die mutualistische Ameisenart in der Lage die EFN-Sekretion durch Myrmeckophyten zu induzieren. Das zeigt, dass die Wirtspflanzen die Nektarproduktion verringern, wenn sie nicht die erwartene Verteidigung der Ameisen erhalten. Insgesamt konnte ich in meiner Arbeit

verschiedene chemische und ökologische Mechanismen identifizieren, die die Spezifizität des *Acacia-Pseudomyrmex* Mutualismus aufrechterhalten, die den Mutualismus vor Ausbeutung schützen und die das mutualistische System stabilisieren.

Introduction

Mutualisms are interactions among members of different species that lead to net fitness benefits for all partners involved. They are based on the exchange of resources and services, which the individual partners can not produce or acquire otherwise (Bronstein 1994). Mutualisms involve organisms of all kingdoms; furthermore, every species on earth appears to be involved in at least one mutualism. Mutualisms have been largely described in nature, and their ecological and evolutionary importance is becoming well recognized. However, how mutualisms are maintained and stabilized in the course of the evolution are questions that still remain to be explored.

Plant-ant mutualisms

Defensive ant-plant interactions are common mutualisms in which plants provide to ants an array of rewards that ranges from extrafloral nectar (EFN, Koptur 2005) to cellular food bodies and domatia (nesting space) (Heil and McKey 2003, Heil 2008). Ants are attracted by plant-derived food rewards and serve as a means of indirect defence of plants against herbivores (Heil 2008; Chamberlain and Holland 2009a). More than 100 genera of angiosperms and 40 species of ants are involved in plant-ant mutualisms, which are widespread in temperate and - particularly - tropical ecosystems, where they play important roles in shaping ecological communities (Heil et al. 2007). Two kinds of interactions can be distinguished within defensive plant-ant mutualisms: facultative and obligate. Facultative are the most common interactions, in which plants offer rewards to attract ants and gain protection from a generalist and opportunistic ant community. Ants benefit from attending plants since they use rewards as nutritive resources; plants in this case are commonly called "myrmecophilic" (i.e., "ant-loving"). Since facultative interactions do not represent highly specialized associations, the partners involved can survive even when the interaction is not

established. In obligate interactions, be contrast, “myrmecophyte plants” are inhabited by specialised ants during major parts of their life (Heil and McKey 2003) and the ants are entirely dependent on the food rewards and nesting space that are provided by the host. These ants, in return, protect efficiently and aggressively their hosts. Such cases represent highly specific and obligate symbioses among plants and ants that cannot survive without each other. A recent meta-analysis of the role of ants as biotic defence showed that ant removal from plants exhibited strong effects on herbivore damage and that these effects are more important in obligate interactions than in facultative ones (Rosumek et al. 2009), as was also previously shown for the *Macaranga* genus (Heil et al. 2001a). This observation suggests that protection provided by ants varies depending on the specificity of the mutualism, being stronger in obligate interactions.

The rewards traded among the mutualist partners can, however, also be attractive to non-mutualist exploiters, which make use of these resources without providing a respective service (Bronstein 2001). Different kinds of exploiters have been described: so-called ‘parasites of mutualisms’ are species that have no evolutionary history as a mutualist but just exploit the resources without returning benefits to either partner; ‘cheaters’, by contrast, are individuals or species that have lost the mutualistic behaviour over evolutionary time but still retain the ability to obtain the benefits from their former partner (Bronstein 2001; Kautz et al. 2009). Particularly well-studied mutualism exploiters of plant-ant interactions include parasitic ants and nectar robbers (Janzen 1975; Letourneau 1990; Raine et al. 2004; Clement et al. 2008). Some ant species are considered parasites of the interaction because they reduce the rate of occupationby the mutualist ant (Raine et al., 2004) and reduce the fitness of the host plant by decreasing its growth rate and/or reproduction (Clement et al. 2008). Other arthropods also exploit plant rewards and may also have detrimental effects on the mutualism. Bees, flies, mites, wasps and beetles have been observed to consume EFN (O'Dowd 1979;

Pemberton 1993; Pemberton and Vandenberg 1993; Van Rijn and Tanigoshi 1999; Röse et al. 2006), and the presence of these nectar robbers can significantly reduce the defensive efficacy provided by ants through competition among nectary-visiting ants and other insects (Heil et al. 2004a; Mody and Linsenmair 2004). Thus, partners involved in mutualisms must present some kinds of mechanisms to maintain and stabilise the the interaction against the exploitation by parasites or the evolution of cheaters. In horizontally transmitted mutualisms is generally assumed that the evolutionary persistence of the interaction is attributed to host sanction mechanisms (Bergstrom and Lachmann 2003; Sachs et al. 2004; Foster and Wenseleers 2006), i.e., host behaviours that direct rewards to reciprocating mutualistic partner, but no to exploiters (Pellmyr and Huth 1994; Kiers et al. 2003). Considering that the transmission of plant-ant mutualism is horizontal (that is, the mutualism has to be established de novo in every generation), it has been suggested that over the course of evolution, myrmecophyte plants have developed some “filter mechanisms”, which exclude exploiters from the mutualism and thus contribute to the stabilisation and specificity of the interaction. In addition, since symbiotic ants are specialised and completely dependent of their plant hosts, they should likely make a more efficient use of host-derived rewards as compared with less specialised parasite ants and they should provide a better protection to the host. Thus, the main objective of this study was to investigate chemical and ecological mechanisms driving the specificity of plant-ant interactions, using the *Acacia*-*Pseudomyrmex* mutualism as a model system.

In the following sections, mechanisms driving the specificity of the *Acacia*-*Pseudomyrmex* system will be studied according to a) the chemistry and secretion of EFN in *Acacia* species, and b) the defence behaviour of *P. ferrugieneus* against nectar robbers, herbivores and leaf pathogens, and its payoff in defence depending on the reward investment by the host plant.

Extrafloral nectar (EFN) as a plant reward

Nectar is an aqueous solution that is secreted by plants to attract and reward animal mutualists. Resulting benefits for plants include protection from herbivores through the attraction of carnivores, such as parasitoidic wasps or of ants (Koptur 1992; Heil 2007, 2008). EFN is usually secreted outside the flowers, and - in contrast to floral nectar – it is not involved in pollination (Bentley 1977; Koptur 1992).

The fraction of soluble solids that can be found in nectar mainly comprises mono- and disaccharides and amino acids. However, other compound classes such as proteins, lipids, phenols, alkaloids and volatile organic compounds (VOCs) have also been reported from various nectars (Kessler and Baldwin 2007; Nicholson and Thornburg 2007). The main function of EFN compounds is related to the attraction of mutualistic ants, and compounds that are mainly regarded responsible for the attraction of ants are sugars (Baker and Baker 1973; Blüthgen and Fiedler 2004; Heil et al. 2005) and amino acids (AAs, Lanza 1988, 1991; Lanza et al. 1993; Blüthgen and Fiedler 2004). Ants generally appear to prefer sugar solutions that contain amino acids over pure sugar solutions (Lanza 1991), but even the detailed identity of amino acids could elicit varying ant responses to artificial EFNs (Blüthgen and Fiedler 2004). Furthermore, preferences for different AAs in nectar can also vary among ant species (Blüthgen and Fiedler 2004). Whereas the attractive function of amino acids to ants in general has been widely studied and demonstrated, no studies have so far investigated whether specific AAs, their concentration, or their mere number have any specific function in shaping plant-ant mutualisms. Both facultative and obligate interactions differ significantly in the specificity of the association between both partners. Whereas in facultative interactions ants are attracted only occasionally to plants, in obligate ones specialised ants inhabit myrmecophyte plants (Heil and McKey 2003). In this last case, there is no need for the plant host to attract ants from the vicinity. Thus, the nutritional importance of EFN appears higher

in the case of mymecophyte plants due to the dependency of the inhabiting ants on the host-derived food rewards. Therefore, a first goal of this study was to determine whether amino acids of nectar are chemically adapted to the ecological requirements of their respective interacting ants.

On the other hand, since EFN is an openly presented resource that contains attractive compounds, EFN requires protection from exploiters. Research on floral nectar has revealed during the last decade that nectar chemical traits are not only related to an attractive function of nectar but also to its protection from non-mutualist organisms. For example, VOCs released from floral nectar have been described as repellants of nectar robbers and florivory (Kessler et al. 2008) and various proteins have been identified and characterised for floral nectar (Lüttge 1961; Baker and Baker 1975; Carter and Thornburg 2000; Carter and Thornburg 2004a; Naqvi et al. 2005), which mainly are enzymes that serve as protection from microbial infection (Carter and Thornburg 2004b; Nicholson and Thornburg 2007). Particularly prominent are the so-called *nectarines*, enzymes in the floral nectar of *Nicotiana sp*. (Carter and Thornburg 2000, 2004b; Naqvi et al. 2005), which are involved in a redox cycle that produces high levels of hydrogen peroxide to maintain the nectar microbe-free (Carter & Thornburg 2004b). This defensive function appears important since nectar composition makes it an excellent medium for microbial growth (Bubán et al. 2003; Raguso 2004). Yeasts are among the species that are most likely present in floral nectar (Sandhu and Waraich 2005; Brysch-Herzberg 2004; Herrera et al. 2008). Moreover, these microorganisms can affect nectar sugar composition (Herrera et al. 2008) and thus reduce the control of the plant over this important nectar trait.

Much less is known on the chemistry of EFN and on the role that particular compounds play in its ecological interactions, even though EFN has been described for plants in more than 300 genera (Bentley 1977; Koptur 1992). Earlier studies suggested that the presence of non-

proteinogenic amino acids in EFN (Baker and Baker 1973) or the invertase-mediated absence of sucrose from EFN might help to defend it from exploiters (Heil et al. 2005). However, no study has so far investigated whether EFN contains also proteins that can protect it from microbe infection, as it has been reported for floral nectar (Carter and Thornburg 2000, 2004a; Naqvi et al. 2005, Nicholson and Thornburg 2007). Thus, I expected that the ecological functions of EFN comprise both the attraction and nutrition of mutualist ants and its protection from microbial infestation, mediated by its protein content.

Ant services to host plants

In mutualist interactions, ants offer to myrmecophyte plants an efficient defence against herbivores (Janzen 1966; Davidson and McKey 1993; Fonseca 1994; Federle et al. 1998; Heil et al. 2001b), neighboring vegetation (Davidson and McKey 1993; Federle et al. 1998), and pathogens (Letourneau 1998). Commonly in obligate interactions, these defence services provided by ants have been associated with plant fitness benefits (Janzen 1966; Vasconcelos 1991; Letourneau and Dyer 1998; Gaume et al. 2005). Among these different ant services to plants, the defence against herbivores has so far most often been investigated. Pruning of neighbouring plants has also been documented, but in a lower number of studies, whereas the role of ants in defence against pathogens represents the least investigated. Therefore, a further objective of this study besides the examination of defence by *Pseudomyrmex* ants against herbivores was to investigate defence against leaf pathogens that is provided by two *Pseudomyrmex* ants to different *Acacia* hosts.

Ant species can differ significantly in the effciciency of the defence that they provide to the plant host (Fraser et al. 2001; Raine et al. 2004; Frederickson 2005; Ness et al. 2006; Miller 2007; Chamberlain and Holland 2009a,b). Multiple ant species are known to co-ocuppy individual plants (Davidson and Mc Key 1993; Raine et al. 2004), thus, mutualist ants

as well as parasites may share the same host plant. It has been reported that parasite ant workers can not effectively reduce the herbivore damage or reduce eggs and larvae of phytophagous insects (Raine et al. 2004; but see Letourneau 1983; De la Fuente and Marquis 1999), thus causing negative effects on plant growth as compared to the mutualist workers (Clement et al. 2008). Although a majority of studies have found a functioning defence in ant-plant mutualisms (Chamberlain and Holland 2009a), there is also a great variation of ant defence in time and space (Bentley 1976); moreover, some studies have not found any defence effect provided by mutualist ants (O'Dowd and Catchpole 1983; Tempel 1983; Rashbrook et al. 1992; Mackay and Whalen 1998; Freitas et al. 2000). Thus, the temporal and spatial pattern of ant activity may be related with variations in the defensive behaviour that is provided by ants.

One important factor that might determine the efficiency of the defence that is provided by the ants is variations in plant reward production. Furthermore, temporal patterns in EFN secretion (see Heil and McKey 2003) have so far been related to the activity pattern of herbivores (Heil et al. 2000) or also by ant visitors (Raine et al. 2004) and it has been hypothesized that the quality and/or quantity of EFN secretion can be related to variation in ant density on plants or also to ant aggressive behaviour (Sobrinho et al. 2002). Thus, EFN-secreting plants can influence the effectiveness of their indirect defence by controlling the amount of the nectar secreted (Heil and McKey 2003). Recently, we demonstrated that different myrmecophyte *Acacia* species produce different amounts of ant rewards, such as EFN production and food bodies (Heil et al. in press). Therefore, in the present study I further aimed to investigate whether rates and patterns of reward investments, specifically EFN secretion, by different *Acacia* myrmecophyte species, pay off in terms of defense against nectar robbers, herbivores and leaf pathogens.

In this *Acacia* genus, non-myrmecophyte species are involved in facultative plant-ant interactions. These species and of the related genera secrete EFN at very low quantities and only in response to herbivore attack (Heil et al. 2004b). In contrast, obligate myrmecophytes secrete EFN constitutively and at high amounts (Heil et al. 2004b). The inhabiting ant species form part of the *Pseudomyrmex ferrugineus* group and obligatorily inhabit particular *Acacia* species on which they are nutritionally dependent (Heil et al. 2004b, 2005; Clement et al. 2008). EFN secretion by Mesoamerican *Acacia* myrmecophytes has been described to occur only as a short, diurnal peak (Raine et al. 2002), and it is not induced in response to damage or JA (Heil et al. 2004c). *Acacia-Pseudomyrmex* obligate interactions are also highly prone to be exploited by the parasite ant *Pseudomyrmex gracilis*, which has been characterized as an exploiter of this mutualism (Clement et al. 2008; Kautz et al. 2009).

These differences in plant-ant interactions make this genus *Acacia* highly suitable to study mechanisms that may determine species-specific interaction. Thus, a comparative approach using a set of related ant-plants that are characterised by these different levels of specificity allows a deeper understanding of the chemical and ecological roles that plant rewards and ant behaviour can play shaping plant-ant interactions as well as in the stabilisation of the mutualism.

Material and Methods

Study system

This study was conducted in the coastal area of the state of Oaxaca, 5 km northwest of Puerto Escondido (Pacific coast; ~15°55′ N and 97°09′ W, elevation 15 m), México. Plants investigated were different species of the *Acacia* genus (Fabaceae). The genus *Acacia* comprises approximately 1350 species (Maslin 2003) and belongs together with the monotypic African genus *Faidherbia* Chev. (Vassal 1972, 1982) to the tribe Acacieae, which forms part of the subfamily Mimosoideae (Fabaceae). All species of *Acacia* included in our study (Fig. 1) are neotropical and assigned to subg. *Acacia*. In detail, *Acacia cornigera* (L.) Willendow, *Acacia hindsii* Benth. and *Acacia collinsii* Saff belong to the myrmecophytes, while *Acacia farnesiana* (L.) Willendow belong to the non-myrmecophytic group of this subgenus. *Prosopis juliflora* Swartz is a closely related and sympatric species of another genus, yet the same subfamily, the Mimosoideae. The phylogeny of the genus *Acacia* and closely related genera indicates that the induction of EFN represents the plesiomorphic or original state within *Acacia*, whereas the constitutive trait in EFN secretion is the derived state. Species were determined following Janzen (1974) and Seigler and Ebinger (1995) and by comparison with specimens held at the Herbario MEXU at UNAM (Mexico City).

Ant species interacting with *Acacia* species in the present system were from the genus *Pseudomyrmex*. The ant genus *Pseudomyrmex* comprises ca. 200 species. Most of these are generalists that nest in dead twigs, but about 40 species are specialized inhabitants of myrmecophytes (Ward and Downie 2005). Ant species considered in this study were the symbiotic ant *P. ferrugineus* and the parasite *P. gracilis* (Fig 2). *P. ferrugineus* F. Smith protect their host from herbivores and encroaching vegetation (Janzen 1966; Janzen 1974), and can not be found nesting outside of the host plant. *P. gracilis* Fabricius is considered a generalist, twig- nesting ant but has been reported that live in thorns of myrmecophyte *Acacia*

species (Skwarra 1935; Wheeler 1942; Ward 1993; Clement et al. 2008). *P. gracilis* can negatively affect the host plant state and growth rate, being also able to exclude the ant mutualist from colonization (Clement et al. 2008). A molecular phylogeny of the *Pseudomyrmex* genus showed that *P. gracilis* did not evolve from former mutualists, and no evidence for cheaters was found (Kautz et al. 2009), thus, is considered as an exploiter species of the mutualism between *Acacia* myrmecophytes and *P. ferrugineus*.

Fig. 1: Myrmecophyte (*A. cornigera, A. hindsii* and *A. collinsii*) and non-myrmecophyte (*A. farnesiana* and *Prosopis juliflora*) plant species used in the study.

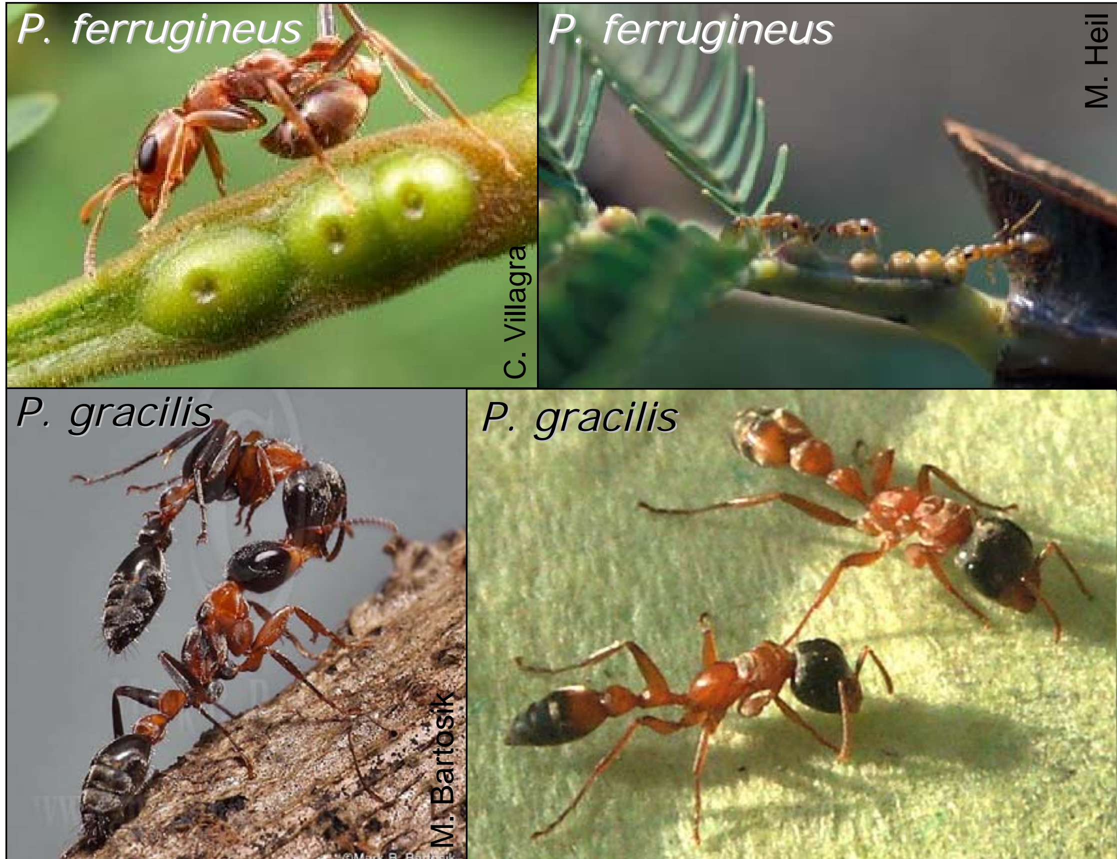


Fig. 2: Symbiotic (*P. ferrugineus*) and parasite (*P. gracilis*) ant species used in the study.

EFN collection and quantification

The collection and quantification of EFN was conducted as follows. Branches of myrmecophytes were deprived of ants and other insects the day before nectar collection by cutting off the inhabited thorns, mechanically removing ants and then placing the branch in a mesh bag after isolating it from the rest of the plant by applying a ring of sticky resin (Tangletrap, The Tanglefoot Corp. Grand Rapids, Mich., USA). Branches of non-myrmecophyte species were induced by applying 1mmol aqueous jasmonic acid solution (Heil et al. 2004c) and then placed in mesh bags. After one day, nectar production rates were quantified as amounts of soluble solids per 24 h and per gram leaf dry mass, by quantifying the nectar volume with micro capillaries (Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany) and the nectar concentration with a refractometer (Atago Co. LTD.) as described previously (Heil et al. 2000, 2001a). The leaves bearing the EFN were then collected and dried (50° C for 48 h). EFN was collected from 5 individuals per species.

Carbohydrate and amino acid analysis in EFN and ant attraction

1. <u>Quantification of carbohydrates and amino acids</u>:

After collection, EFN was stored at -20° until analysis. For carbohydrate analysis, 30 µL of nectar were diluted in 600 µL de-ionised water. After centrifugation and membrane filtration (Vivaspin 500, Vivascience Sartorius Group, Stonehous, UK), sugars were immediately separated by HPLC on an anion exchange column and quantified by pulsed amperometric detection (DIONEX Series 4500 Chromatography System, Dionex, Idstein, Germany). For the analysis of amino acids, 30 µL of nectar were diluted in 200 µL de-ionised water. After centrifugation and membrane filtration, 100 µL of the supernatant were diluted with 20 µL sulfosalicylic acid (12.5%). After incubation at 4° C for 30 min and a second centrifugation, 50 µL of sample buffer were added to 100 µL of the supernatant. Samples were then analysed

using an Amino Acid Analyzer LC 5001 (Biochrom 20 Plus, Cambridge, England). To control for differences in overall nectar concentration, the concentration of each amino acid was related to the sugar content of the respective sample and expressed in mmol amino acid per mmol sugar. Differences in amino acid concentrations among the four species were evaluated with a Kruskal-Wallis ANOVA (N = 5 individual per species). Different individuals were used as replicates to avoid pseudoreplication. Considering that amino acids data were not normally distributed, amino acid composition was evaluated with a Non-Metric Multidimensional Scaling (NMDS), in order to identify putative associations among the species (NMDS allows to reduce a multidimensional data set to two dimensions and thus appeared an appropriate approach for this question) (Borg and Groenen 2005). Ordination was carried out using the following parameters: Bray-Curtis as distance measure, stability criterion of 0.005, 200 iterations, 10 runs with real data y 10 runs with randomized data. The software used for this analysis was PC-ORD v. 4.2 (McCune and Mefford 1999). Values of NMDS axes were compared among species using a univariate ANOVA.

2. Ant behavioural assays:

To study the behavioural responses of ants (symbiotic *vs.* non-symbiotic ants) to EFNs with differing composition, 'cafeteria'-style experiments were carried out under field conditions. Such 'cafeteria'-experiments allow to simultaneously offer different types of food sources to animals that freely can choose among them.

The NMDS of EFN amino acids revealed strongest differences between EFNs of *A. hindsii* and *Prosopis* (see below). We, therefore, focused on these two plant species for the behavioural assays, and evaluated the attraction of obligate *Acacia* symbionts (*Pseudomyrmex ferrugineus* Smith F.) and of non-symbiotic ants to EFNs of these two plant species and to different artificial nectars that mimicked the major differences between the two plant species (see Table 1).

Table 1: Composition of sugars - amino acid solutions used for the "cafeteria experiments". 8 AA, 4 AA and 2 AA means the addition of the respective amino acids as shown in the table to the F (fructose) + G (glucose) sugar solution.

	Sol. 1	Sol. 2	Sol. 3	Sol. 4	Sol. 5	Sol. 6	Sol. 7	Sol. 8
Substances	F+G+S	F+G	F+G +8AA	F+G +4AA	F+G +2 AA	Water	*A. hindsii*	*Prosopis*
Fructose	x	x	x	x	x			
Glucose	x	x	x	x	x			
Sucrose	x		x					
Isoleucine			x					
Leucine			x	x				
Methionine			x					
Phenylalanine			x	x	x			
Proline			x	x	x			
Serine			x					
Threonine			x					
Valine			x	x				
Pure water						x		
EFN *A. hindsii*							x	
EFN *Prosopis*								x

2.1. Experiment High- and Low-AAs. A first field experiment was conducted in March 2007. EFN of *A. hindsii* and *Prosopis* was first collected from several individual plants (N = 3-5) in the field and then pooled to achieve greater nectar volume. Then, EFN collected of *A. hindsii* was adjusted with distilled water to a concentration of 4% (w/v) by using a portable refractometer, which was the highest concentration found in nectar of *Prosopis* in the field. Six nectar mimics were applied at the same concentration (4%): solution (sol.) 1 contained fructose (F) + glucose (G) + sucrose (S) at a ratio of 3:3:1 to mimick sugar ratio as found in the EFN of *Prosopis*, whereas Sol. 2 contained F + G at a relationship of 1:1, mimicking the sugars found in EFN of *A. hindsii*. Three nectar mimics were prepared with different AA compositions: Sol. 3 was a sugar solution (F:G = 1:1) containing methionine, isoleucine, leucine, valine, threonine, phenylalanine, proline and serine (i.e., those AA that were highly correlated with Axis 1, see below, and that most strongly contributed to the chemical difference between EFN of *A. hindsii* and of *Prosopis*). Sol. 4 was a sugar solution (F:G = 1:1) with those four AA that were highly dominant in EFN of *A. hindsii* (see Table 19, result section), and sol. 5 was a sugar solution (F:G = 1:1) containing phenylalanine and proline, which both appear particularly important AA in the physiology of insects (Chapman 1983; Dafni and Kevan 1994; Micheu et al. 2000). Pure water was offered as a control (Sol. 6) (Table 1). These six artificial solutions and fresh EFNs of *A. hindsii* (Sol. 7) and *Prosopis* (Sol. 8) were offered to ants in their natural habitat. Two different AA : sugars ratios were used to evaluate whether ants are able to distinguish among different artificial solutions when these contain different AA : sugar ratios, (i) a ratio of each amino acid to fructose and glucose of 1:50 ('high-AA EFNs', N = 10 cafeterias) and (ii) a ratio of each amino acid to fructose and glucose of 1:1000 ('low-AA-EFNs', n = 17 cafeterias). The ratio 1:50 represents the values that we found in EFN of *Acacia* species (see Table 19, result section).

Independent experiments were conducted for symbiotic and non-symbiotic ants. For *P. ferrugineus*, a 10 µl drop of each of the eight solutions was offered on a horizontal branch of an *A. hindsii* host plant (one cafeteria per plant). For generalist ants, the eight solutions were offered on branches of *Prosopis* that were cut off the plants and placed then on the soil to facilitate the access of generalist ants. In both cases, the individual droplets were offered 10-15 cm apart from each other, and the spatial order varied among the cafeterias. Solutions that had evaporated or that had been entirely consumed were replaced with a new drop of 10 µL. All ants feeding on the droplets were counted 5 times during the morning (between 10:00 AM and 13:00 PM). Each single count lasted 3 min, with an interval of 30-40 min between the individual censuses. Because ant abundance may differ among individual plants, numbers of ants that had been attracted to the individual cafeterias were summed up for every cafeteria to calculate the relative proportion of ants that had been attracted to each individual solution. This percentage of ants was subjected to univariate ANOVA (independent variable: solution type) after arcsine transformation (Sokal and Rohlf 1995). A LSD test was posteriorly applied.

2.2. Experiment Number of AAs. A second "cafeteria experiment" was carried out in January 2009 to examine whether the ratio of AAs to sugars or the number of AAs is most important to determine ant preferences. Given that ants were only able to distinguish among solutions at higher AA concentrations (see Fig. 7, result section), , solutions (4%) at ratios 1:10 and 1:50 of AAs to total sugars were prepared with different number of total AAs (2AA, 4AA and 8AA). The following six solutions were prepared: 1:10-2AA, 1:10-4AA, 1:10-8AA, 1:50-2AA, 1:50-4AA and 1:50-8AA. Solutions were offered in independent experiments to symbiotic (N = 10) and non-symbiotic ants (N = 10). "Cafeteria experiments" were conducted as described above. Differences in the percentage of ants attracted to each solution were

analyzed with univariate ANOVA, after arcsin transformation. LSD was applied then as post hoc test.

2.3. Experiment AAs : sugars ratios. The third experiment was conducted January 2009 to determine which minimal ratio of AAs to total sugars allows ants to differenciate among mimics that contain and that do not contain AAs. Six different 4AA solutions (4%) for symbiotic ants (N = 10) and six different 8AA solutions (4%) for non-symbiotic ants (N = 10) were prepared at different ratios of AAs to total sugars, 1:10, 1:50, 1:100, 1:500 and 1:1000, and tested in "cafeteria experiments". Differences in ant preferences (percentage of ants) among solutions were analyzed with univariate ANOVA, after arcsin transformation. A LSD test was posteriorly applied. "Cafeteria experiments" were conducted in the same way as in both before experiments (see above).

Protein analysis in EFN and protection from microbial infestation

1. Quantification of proteins and SDS-PAGE:

Quantification of total proteins was determined with the Bradford assay (Bradford 1976) in fresh nectar from myrmecophytes *A. cornigera* and *A. hindsii* and from non-myrmecophytes *A. farnesiana* and *Prosopis juliflora*, immediately after the collection in the field. Then, protein quantities were related to the total amounts of secreted soluble solids [mg] and to the dry weight [g] of the respective leaves. Differences in protein quantities among species were analysed with a Kruskal-Wallis test.

Before SDS-PAGE, EFN (stored at -20° C until analysis) of the same 4 plant species (10-20 µL for myrmecophyte species, 150-200 µL for non-myrmecophyte species) was precipitated with 10% TCA (v/v) at 4° C (nectar : TCA = 1 : 2). The mixture was incubated for 1.5 h at 4° C and centrifuged at 13000 rpm for 15 minutes at 4°C. Then, the supernatant was removed and 0.5 mL of absolute ethanol was added. Samples were centrifuged at 7000 rpm for 10 min at 4° C. Finally, proteins (15-20 µg per sample) were separated on a 13% SDS-PAGE Laemmli gel (see Tables 2-4 for SDS-PAGE and buffer composition) and stained with Coomassie Blue solution (Table 5). Electrophoresis running conditions: 130 V for 1.5 h.

Table 2: Composition of SDS-PAGE for nectar protein separation.

	Lower gel (13%)	**Upper gel**
30% Acrylamide 0.8% Methylene bis Acrylamide	12.3 mL	1.5 mL
TrisHCl 1.5 M, pH 8.3 + 0.4% SDS	7 mL	
TrisHCl 0.5 M, pH 6.8 + 0.4% SDS		4 mL
Distilled water	8.3 mL	9.6 mL
APS (10%)	100 µL	150 µL
TEMED	23 µL	15 µL

Table 3: Composition of running buffer Tris-Glycine pH 8.3 - 10x used for protein separations by SDS-PAGE (use 1x).

	Amount
Tris	30.27 g
Glycina	144.13 g
SDS	10 g
Destilled water	Fill up to 1 L

Table 4: Composition of loading buffer pH 6.8 – 4x used for protein separation by SDS-PAGE. Samples were mixed with the loading buffer at a concentration 10:1 (v/v).

	Amount
Tris 1M	0.605 g
Glycine	40 mL
SDS	4 g
Bromophenol blue	5 mg
Destilled water	Fill up to 100 mL

Table 5: Composition of protein staining solution. Distaining solution used had the same composition without coomassie.

	Ratio
Methanol	50%
Acetic Acid	10%
Distilled water	40%
Coomassie R 250	0.25%

2. <u>Two-dimensional gel electrophoresis and mass spectrometry</u>:

Proteins in EFN of the myrmecophytes *A. cornigera*, *A. hindsii* and *A. collinsii* were identified with a Two-Dimensional Gel Electrophoresis and MALDI-TOF/MS (matrix-assisted laser desorption-ionisation – time of flight mass spectrometry. Nectar proteins were extracted with 10% TCA (v/v) (see above). The 2D-PAGE procedure has been described recently (Giri et al. 2006) (see Tables 6-7 for strip rehydration and isoelectric focusing (IEF) conditions; Tables 8-9 for equilibration buffers; and Tables 10-11 for SDS-PAGE and buffer composition). Three replicate gels were used for protein identification. The following modifications have been made to the published procedure. After water removal from the sample wells, the gel plugs were reduced using 20 µl 10mM DTT in 25 mM ammonium bicarbonate for 1h at 56° C, alkylated by 20 µL 55mM IAA at RT in dark for 45 min, and rinsed with 70 µL 50 mM ammonium bicarbonate/50% acetonitrile two times for 20 min to remove the Coomassie stain. The second wash was done with 70 µl 70% acetonitrile for 20 min. The gel plugs were then air-dried for 30 min and overlayed with 15 µl of 50mM ammonium bicarbonate containing 70 ng porcine trypsin (Sequencing grade, Promega). The MTPs were subsequently covered with aluminium foils and the proteins were digested overnight at 37°C. The resulting peptides were extracted from the gel plugs by adding 40 µL 50% acetonitrile in 0.1% trifluoroacetic acid for 20 min and an additional extraction with 70 µL of the same extraction buffer. The extracts were collected in an extraction MTP and vacuum-dried to remove any remaining liquid and the volatile ammonium bicarbonate. A MALDImicro MX mass spectrometer (Waters, Milford, MA, USA) was used in reflectron mode for monitoring of protein digestion and database identification. The tryptic peptides were reconstituted in 6 µL aqueous 0.1% trifluoroacetic acid.

Table 6: Rehydration solution used for gel strips, with an incubation time of 17 h.

	Concentration
Urea	8 M
Chaps	0.5 %
DTT	0.28 %
Carrier ampholites	0.5 %
Bromophenol blue	0.007 %
	Fill up with distilled water

Table 7: Conditions used for Isoelectric focusing (IEF) of gel strips (pH 3-11 NL, 24 cm).

Voltage (V)	Time (h)
500 (Step)	1
1000 (Gradient)	1
10.000 (Gradient)	3
10.000 (Step	2.15

Table 8: Equilibration buffer I for reduction of gel strips (pH 3-11 NL, 24 cm).

	Concentration
Tris-HCl 1.5 M, pH 8.8	50 mM
Urea	6 M
Glycerol	30%
SDS	2%
Bromophenol blue	0.002%
DTT	1%
	Fill up with distilled water

Table 9: Equilibration buffer II for alkylation of gel strips (pH 3-11 NL, 24 cm).

	Concentration
Tris-HCl 1.5 M, pH 8.8	50 mM
Urea	6 M
Glycerol	30%
SDS	2%
Bromophenol blue	0.5%
Iodoacetamide	2.5%
	Fill up with distilled water

Table 10: 2D-SDS-PAGE for nectar protein separation. Electrophoresis running conditions: 500 V for 6 h.

	13% for 100 mL
30% Acrylamide 0.8% Methylene bis Acrylamide	32.5 mL
Tris-HCl 1.5 M, pH 8.8	25 mL
SDS 10%	1 mL
Distilled water	41 mL
APS 10%	500 µL
TEMED	33 µL

Table 11: Composition for running buffer Tris-Glycine pH 8.3 - 4x used for protein separation by 2D-SDS-PAGE (use 4x). For protein staining solution see Table 5.

	Concentration
Tris	100 mM
Glycina	768 mM
SDS	0.4 %
	Fill up with distilled water

Peptides not identified by MALDI-TOF/MS were identified *de novo* using LC/MS/MS (Giri et al. 2006; Pauchet et al. 2008). The aliquots of peptides (1,5-6 µL) were injected on a nanoAcquity nanoUPLC system (Waters, Milford, MA, USA). A mobile phase 400nL/min flow of 0.1% aqueous formic acid (15 µL/min for 1 min) was used to concentrate and desalt the samples on a 20 x 0.180 mm Symmetry C18, 5 µm particle precolumn. The samples were eluted on a 100 mm x 75 µm I.D., 1.7 µm BEH nanoAcquity C18 column, using an increasing acetonitrile gradient in 0.1% aqueous formic acid. Phases A (0.1% formic acid) and B (100% MeCN in 0.1% formic acid) were linearly mixed using a gradient program going up to 5% phase B in A in 0.33 min, increasing to 10% B over 10 min, 40% B over 10 min, and finally increasing to 85% B over 10.5 min, holding at 85%B until the 11th min, and decreasing to to 1%B at 11.1 min. The eluted peptides were transferred to the NanoElectroSpray source of a Synapt HDMS Q-TOF type tandem mass spectrometer (Waters, Milford, MA, USA) through a Teflon capillary union and a metal coated nanoelectrospray tip (Picotip, 50 x 0.36 mm, 10 µm I.D, Waters, Milford, MA, USA). The source temperature was set to 60° C, cone gas flow 20 L/h, and the nanoelectrospray voltage was 3.2 kV. The TOF analyzer was used in reflectron mode. The MS/MS spectra were collected in an 1 s interval in the range of 50-1700 m/z. A mixture of 100 fmol/µL human Glu-Fibrinopeptide B and 80 fmol/µL reserpine in 0.1% formic acid/acetonitrile (1:1 v/v) was infused at a flow rate of 0.9 µL/min through the reference NanoLockSpray source every fifth scan to compensate for mass shifts in the MS and MS/MS fragmentation mode due to temperature fluctuations.

Data were collected by MassLynx v4.1 software and ProteinLynx Global Server Browser v.2.3 software (both Waters, Milford, MA, USA) was used for baseline subtraction and smoothing, deisotoping, de novo peptide sequence identification, and database searches. The peptide fragment spectra were searched against the EBI "planta" specific subdatabase

downloaded on July 22, 2008 from http://www.ebi.ac.uk/. The protein database identification search parameters were: peptide mass tolerance 20 ppm and minimum two peptides found, estimated calibration error 0.005 Da, 1 possible missed cleavage, carbamidomethylation of cysteins and possible oxidation of methionines. A 0.05Da mass deviation was allowed and a calibration error of 0.005 Da for de novo sequencing. The BLAST search was performed internally using the MS-BLAST algorithm (Shevchenko *et al.,* 2001) using minimum one peptide matching at an expect score of 100, with no-gap-hspmax100-sort_by_totalscore -span1 advanced options and PAM30MS search matrix.

In order to obtain a rough impression of the quantitative contribution of chitinases and glucanases to the total amount of proteins in EFN of *A. cornigera*, all spots present in EFN were quantified with the PD Quest 7.3.0 program (2-D Analysis Software, BioRad, 2003) as the volume for each spot (OD x mm^2). First we determined the volume for all spots to represent the total proteins present in the sample. The total volume of glucanases and chitinases was then also determined and related to the total proteins. Spots were considered for quantification only when present in all three replicates.

3. <u>Antifungal protection of EFN in nature</u>:

The occurrence of fungi in EFN under natural growing conditions was investigated by collecting samples from the field and plating them on malt agar plates to quantify numbers of colony forming units (CFU). EFN was adjusted to a concentration of 3% of soluble solids (w/v) by using a portable refractometer. This concentration was chosen since it was not possible to obtain more highly concentrated EFN from *A. farnesiana* (the same criterium was used to adjust EFN in following experiments). 30 µL of EFN (diluted 1:100 in PBS buffer at 0.1 M and pH 7.0) was plated on malt agar plates (20 g malt extract + 15 g agar). The dilution 1:100 was chosen for all treatments after testing a series of different dilutions (1:10, 1:100 and 1:1000). The same procedure was employed for the yeast assay (see below). Plates were

stored at room temperature for 48 hours and then colonies were counted to quantify CFU numbers. Differences in fungal abundance [CFUs 30 µL EFN^{-1}] among the species were analysed with a Kruskal-Wallis ANOVA. The number of replicates was 5 individuals per species.

4. PR-enzyme activities:

4.1. Colorimetric assays: Activities of the pathogenesis-related (PR) enzymes: chitinase, *ß*-1,3-glucanase and peroxidase, were determined in EFN using standard colorimetric assays. EFN was collected from the myrmecophyte species *A. cornigera* and *A. hindsii*, and from the two non-myrmecophytes *A. farnesiana* and *Prosopis juliflora*. Nectar samples were diluted 1:10 with pure water and adjusted to a concentration of 5 % (w/v).

4.1.1. Chitinases: to quantify chitinase activity, assays based on a method of Wirth and Wolf (1990) were conducted in 96-well microplates. A total volume of 100 µL reaction preparation contained 10 µL nectar, 40 µL 50mM Na-acetate buffer (pH 5.0) and 50 µL RBV-chitin (Loewe, München, Germany). Each preparation was replicated 4 times, incubated 2.5 hours at 37° C and stopped with 26 µL 0.05 M HCl. After 5 min incubation at -20° C the plate was centrifuged at 4000 rpm al 4° C. 100 µL of the supernatant were transferred to a new microplate and measured at 550 nm in a spectrophotometer (Smax 190PC, Molecular Devices GmbH, München, Germany).

4.1.2. Glucanases: activity of β-1,3-glucanase was assayed using *Laminaria digitata* laminarin (Sigma) as substrate. The assay mixture contained in a total volume of 135 µL: 5 µL nectar, 10 µL laminarin (20 mg/mL in 50 mM of Na-acetate buffer at pH 5.0), 60 µL copper reactive (175.5 g disodium hydrogen phosphate dihydrate and 200 g potassium sodium tartrate tetrahydrate were dissolved in distilled water and made up to 2.5 L. Then 500 g sodium hydroxide and 40 g copper sulphate pentahydrate were added and mix. Finally, 900 g anhydrous sodium sulphate were added and made up to 5 L with distilled water. The reagent

was stored under dark conditions), 60 µL arsenic reactive (250 g ammonium molybdate tetrahydrate were dissolved in 4.5 L distilled water. Then, 210 mL concentrated H_2SO_4 and 30 g disodium arsenate heptahydrate added, mixed and made up to 5 L with distilled water. The reagent was also stored under dark conditions). The amount of reducing sugars released in the volume was determined by Somogyi-Nelson's method (Somogyi 1952). One unit of activity was defined as the amount of enzyme that catalyzed the release of reducing sugar moieties equivalent to 1µmoL of glucose per minute.

4.1.3. Peroxidases: to quantify peroxidase activity, a total volume of 197 µL reaction solution contained 5 µL nectar, 0.83 µL H_2O_2 (30%), 1 µL guaiacol (99%) and 190 µL 50 mM Na-phosphate buffer at pH 6.0. The oxidation of the substrate was measured spectrophotometrically (Smax 190 PC) at 470 nm as described previously (Hammerschmidt *et al.*, 1982). Kruskal-Wallis ANOVA was used to evaluate differences among species for activities of each enzyme class. The number of replicates was 5 individuals per species.

4.2. Enzyme activities in gel assays: Acidic and basic chitinases and β-1,3-glucanases were determined by native gel assays in order to detect and separate active isoforms in nectar. This was evaluated in EFN of the three myrmecophytes: *A. cornigera*, *A. hindsii* and *A. collinsii*, and of the two non-myrmecophytes: *A. farnesiana* and *Prosopis juliflora*.

4.2.1. Acidic / neutral chitinases and β-1,3-glucanases: 10 µg of proteins per sample were analysed by 15% (w/v) polyacrylamide gel electrophoresis (PAGE) under native conditions, at pH 8.9 according to Davis (1964) (see Table 12-14 for native gel and buffer composition).

4.2.1. Basic chitinases and β-1,3-glucanases: 10 µg of proteins per sample were analysed by 12% (w/v) polyacrylamide gel electrophoresis (PAGE) under native conditions, at pH 4.3 as described by Reisfeld et al. (1962) (see Tables 15-17 native gel and buffer composition).

Table 12: Composition of polyacrylamide gels for determination of acidic / neutral chitinase and β-1,3-glucanase activities.

	Lower gel (15%)	**Upper gel (4%)**
Acrylamide 40%	3.65 mL	0.48 mL
Bis-acrylamide 2%	2 mL	0.26 mL
Tris-HCl 3 M, pH 8.8	1.25 mL	-
Tris-HCl 1.5 M, pH 6.8	-	1.26 mL
Glycol chitin / ß-1,3-glucans	100 µL / 1 mL	-
Distilled water	1.8 mL / 2.8 mL	2.85 mL
APS (10%)	70 µL	50 µL
TEMED	15 µL	5 µL

Table 13: Composition of running buffer Tris-Glycine pH 8.3 - 10x used for acidic / neutral chitinases and β-1,3-glucanases (use 1x).

	Amount
Tris	30 g
Glycina	144 g
Distilled water	Fill up to 1 L

Table 14: Composition of loading buffer pH 6.7 used for acidic / neutral chitinases and β-1,3-glucanases. Samples were mixed with the loading buffer at a concentration 10:1 (v/v).

	Concentration
Tris-HCl pH 6.7	0.5 M
Sucrose	60% (p/v)
Bromophenol blue	0,04% (p/v)
Sodium Azide	0,02% (p/v)

Glycol chitine was embedded in gels at 0.01% (w/v) and used as substrate for chitinase activities. After electrophoresis, chitinase gels were incubated for 4 h at 37° C in sodium acetate buffer 50 mM, pH 5.0. For β-1,3-glucanase activities, a soluble fraction of purified ß-glucans from *Saccharomyces cerevisiae* was used as a substrate (Grenier and Asselin 1993). ß-glucans were incorporated at a final concentration of 0.6 mg mL^{-1} directly in the separation gels. After electrophoresis glucanase gels were incubated for 3 h at 37° C in sodium acetate buffer 50 mM, pH 5.0 as well. Running conditions for electrophoresis of chitinases and glucanases were100 V for 1.5 h. Chitinase activities on gels were revealed by fluorescent staining (10 min) using calcofluor white M2R (0.01% w/v) in 500 mM Tris-HCl (pH 8.9) and visualised after destaining under UV light. β-1,3-glucanase activities on gels were revealed by staining the gels for 15 min with 0.025% (w/v) aniline blue fluorochrome in 150 mM K_2HPO_4, pH 8.6, and visualised under UV light (365 nm).

Table 15: Composition of polyacrylamide gels for determination of basic chitinase and β-1,3-glucanase activities.

	Lower gel (12%)	Upper gel (7.5%)
Acrylamide 40%	2.74 mL	0.73 mL
Bis-acrylamide 2%	1.5 mL	0.4 mL
Acetic acid 22% + KOH 0.6 M	0.75 mL	-
Acetic acid 3.6% + KOH 0.6 M	-	0.5 mL
Distilled water	2.75 mL	2.3 mL
APS (10%)	100 µL	40 µL
TEMED	50 µL	8 µL

For basic activities, substrates of glycol chitin and β-glucans were incorporated in an additional polyacrilamide gel (overlay gel, 7.5%) (see Table 18 for overlay gel composition) to which proteins were transferred.

Table 16: Composition of running buffer Tris-Glycine pH 8.3 - 10x used for basic chitinases and β-1,3-glucanases (use 1x).

	Concentration
ß-alanine	0.3 M
Acetic acid	0.8% (v/v)

Table 17: Composition of loading buffer used for basic chitinases and β-1,3-glucanases. Samples were mixed with the loading buffer at a concentration 5:1 (v/v).

	Concentration
KOH	0.6 M
Acetic acid	3.6% (v/v)
Sucrose	60% (w/v)
Methylene blue	0.04% (w/v)
Sodium azide	0.02% (w/v)

Table 18: Composition of the polyacrilamide overlay gel for protein transfer used for basic chitinases and β-1,3-glucanases.

	Overlay gel (7.5 %)
Acrylamide 40%	1.82 mL
Bis-acrylamide 2%	1 mL
Glycol chitin / ß-1,3-glucans	100 µL / 1 mL
Distilled water	7 mL / 6 mL
APS (10%)	100 µL
TEMED	20 µL

Electrophoresis running conditions for chitinases and β-1,3-glucanases: 35 mA for 3 h with inverse polarity. After electrophoresis, separation gels (attached to a supporting glass plate) were covered with the overlay gel. Bubbles between both gels were eliminated by gently sliding the overlay gel on the top of the separated gel. Both gels together (separating gel + overlay gel), for chitinases and glucanases, were incubated overnight under moist conditions at 37° C with sodium acetate buffer 50 mM, pH 5.0. Chitinase and β-1,3-glucanase activities on overlay gels were revealed and visualised in the same way as for acidic / neutral activities (see above). All electrophoreses were repeated at least three times. All chemical used were purchased from Sigma Chemical Co. (Germany).

5. Antifungal effects of EFN:

5.1. An assay with yeast was carried out in order to evaluate potential effects of EFN enzyme activities on microbial growth. Commercial yeast SK *Saccharomyces cerevisiae* was cultivated on malt extract agar (20 g malt extract + 15 g agar) to isolate a single strain. This single yeast strain was proliferated in liquid medium at 30° C for 24 hours and afterwards centrifuged, resuspended in PBS buffer and stored at 4° C. EFN of all four species was used to evaluate putative effects of nectar enzyme activities on yeast growth. EFNs were adjusted to a concentration of 5% (w/v) by using a portable refractometer, and a 5% sugar solution (fructose : glucose, 1 : 1) was used as a control. 20 µL of each nectar and of the sugar solution was mixed with 20 µL of yeast suspension and incubated for 1 hour at 30° C. 20 µL of a dilution 1:1000 in PBS buffer was plated on malt agar plates (20 g malt extract + 15 g agar) for CFU (colony forming units) determination after 48 hours. Differences among the species were analyzed with a univariate ANOVA. A Tukey test was posterior applied. EFN from eight different plants was used as replicates for each species.

5.2. Another assay was carried out to evaluate effects of chitinase activity as found in EFN of *A. cornigera* and *A. farnesiana* on yeast growth (*Saccharomyces cerevisiae*). Different sugar solutions, with and without *Streptomyces griseus* chitinase (Sigma) were prepared to create mimics of extrafloral nectar (for composition see below). 10 µL of yeast suspension (commercial yeast SK *Saccharomyces cerevisiae*) were incubated with 10 µL of mimic nectar for 1 h at 30° C. Different dilution series of this approach (1:100 and 1:1000) were used for CFU determination on malt agar plates (20 g malt extract + 15 g agar) after 48 hours. Nectar mimics were prepared simulating EFN of one myrmecophyte species (*A. cornigera*) and of one non-myrmecophyte species (*A. farnesiana*). The *A. cornigera* mimic was an aqueous solution of fructose and glucose (1:1 at a concentration of 6% w/v, the EFN concentration usually found for *A. cornigera*) with chitinase activity as found in EFN of this species (0.18

units per mL of sugar solution were used). As controls, pure water solution and chitinase-free sugar solution at the same concentration (6% w/v) were used. Mimics of *A. farnesiana* nectar were prepared with fructose, glucose and sucrose (1:1:1 at a total concentration of 2% w/v) and with chitinase activity as found for this species (0.01 units per mL of sugar solution). Eight repetitions were conducted for each species and differences among treatments were evaluated separately for each species with a univariate ANOVA. A LSD test was posterior applied.

5.3. A third assay was carried out to evaluate a putative inhibitory effect of EFN on six fungal species (*Phytophthora parasitica, Fusarium oxysporum, Verticillium dahliae, Alternaria alternata, Botrytis cinerea, Plectosphaerella cucumerina*) that have been previously described as leaf pathogens for other *Acacia* species (Roux and Wingfield 1997; Kapoor et al. 2004). This assay was performed following the disk diffusion method, which consisted in placing sterile filter paper discs (1 cm diameter; equidistantly separated) impregnated with 10 µL of EFN on the surface of potato destroxe agar (Sigma) plates (see Fig. 3). Then, a slide of each fungal on agarose gel (4 cm^2) was placed on the centre of the agar plate to evaluate the putative inhibition of its growth: lack of growth in the area around the disc means that the respective fungus is susceptible to some antifungal activity present in the EFN. Plates were stored at room temperature for 72 h. Antifungal effects of EFN on the six fungal species were quantified on a relative scale as: +++ (strong effect), ++ (high effect), + (moderate effect), - (no detectable effect). EFNs of five species were used to evaluate their inhibitory effects on the six fungal species. Myrmecophyte EFN (*A. cornigera, A. hindsii* and *A. collinsii*) was adjusted to a concentration of 10% (w/v) by using a portable refractometer, which represents the common EFN concentration found in the field for those species, whereas a 10% sugar solution (fructose : glucose, 1 : 1) was used as a control. Non-myrmecophyte EFN (*A. farnesiana* and *Prosopis juliflora*) was adjusted to a concentration of 3% (w/v) and a 3%

sugar solution (sucrose : fructose : glucose, 1 : 1 : 1) was used as a control. Assays were performed in triplicated for each fungal species.

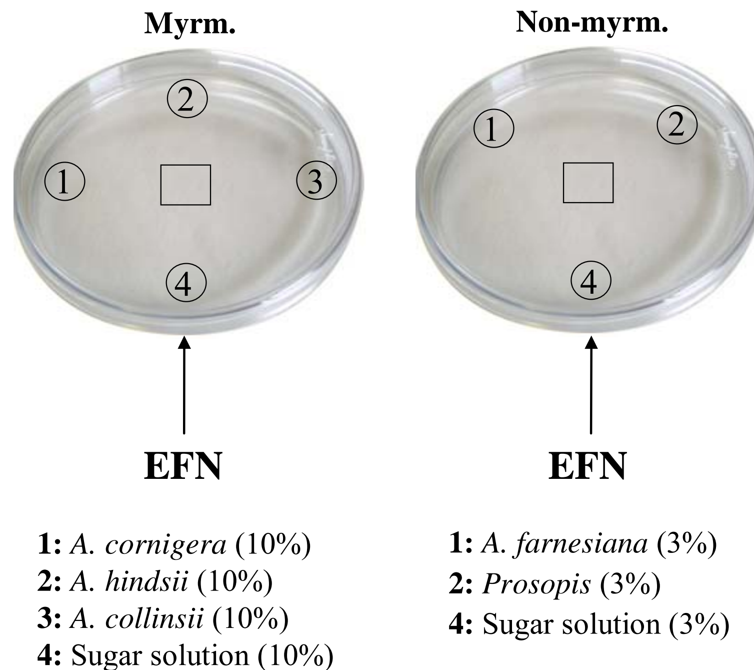


Fig. 3: Diagram of the disk diffusion method used for evaluation of EFN inhibitory effects against six fungal species.

To evaluate which fraction of EFN can be related to its putative antifungal effects, a membrane filtration of 5 kD (Vivaspin 500, Vivascience Sartorius Group, Stonehous, UK) was used to separate the protein fraction (> 20 kD for *Acacia* EFN, see below) from the metabolite fraction (mainly constituted by sugars, < 5 kD) of EFN of the three myrmecophytes *A. cornigera*, *A. hindsii* and *A. collinsii* and of the two non-myrmecophytes *A. farnesiana* and *Prosopis juliflora*. After centrifugation (13.000 rpm for 5 min) both fractions were obtained for each plant species, and the disk diffusion method (see above for methodological description) was carried out on the fungus *Phytophthora parasitica*.

Production pattern of EFN, ant defence, and nectar robbers

Here, I investigated whether specific temporal patterns in reward provisioning by myrmecophyte species can contribute to the defence of the mutualism against exploiters. In order to test this hypothesis I investigated on the same plants the diel EFN secretion patterns of three *Acacia* myrmecophytes together with the activity patterns of resident *P. ferrugineus* ants and of nectar robbers.

1. <u>Time course of EFN production and ant activity</u>

EFN production was quantified for each five plants of every myrmecophyte species: *A. cornigera*, *A. collinsii* and *A. hindsii*. EFN was collected from the three youngest fully developed leaves on the main branch every 2 h from 8.00 AM until 22.00 PM. Before the first nectar collection, nectaries were washed with distilled water to remove any accumulated nectar.

At the same time and on the same individual plants from which EFN was collected, activity of the resident ants (*P. ferrugineus* F. Smith) was determined. Three lines were drawn with a permanent pen along the main stem of each plant. Lines were drawn 24 h before the experiment to exclude any putative effects of odours released from the ink on ant behaviour. Ant activity was evaluated as the number of ants that crossed each line during three minutes. The effect of time of the day on EFN production and on ant activity was evaluated separately for each plant species with Kruskal-Wallis ANOVA, since data did not show homogeneity of variances. The relationship between EFN production and ant activity across the times was then evaluated for each *Acacia* species with a Spearman rank correlation test, using the means for EFN production and ant activity calculated for every time of the day from the values of all five individuals per species.

2. Ant-mediated defence of EFN against nectar robbers

Whether the resident ants can protect EFN from nectar robbers was evaluated through ant exclusion experiments. For these experiments we used the same 5 plant individuals that had been used before. Two treatments, (i) three branches without ants (ant-free) and (ii) three branches with ants (ants present), were applied to each plant. In order to deprive branches of ants thorns were cut off, ants were mechanically removed and branches were then isolated from the rest of the plant by applying a ring of sticky resin (Tangletrap, The Tanglefoot Corp. Grand Rapids, Mich., USA). Activity of EFN robbers was determined as the number of insects landing on leaves of three branches per plant during 60 sec. every 2 h from 08.00 AM to 22.00 PM. The only group of animals showing up as EFN exploiters were determined by Dr. Roubik, Smithsonian Tropical Research Institute, Balboa, Ancon, Republic of Panama.Differences in the activity of EFN robbers between ant-exclusion branches and control branches were evaluated with a Mann-Whitney test, separately for each time of the day.

Ant defence against herbivory and leaf pathogens

Since different myrmecophyte *Acacia* species produce different amounts of ant rewards, such as EFN production and food bodies (Heil et al. 2009, in press), I conducted different field exclusion experiments to investigate whether reward investments by host plants payoff in defence provided by ants against pathogens and herbivores. Furthermore, the importance of the symbiotic ant *P. ferrugineus* as a biotic defence for *Acacia* plants was also evaluated.

1. Ant defence against herbivores: *P. ferrugineus* defence was determined in plants of two myrmecopyhtes: *A. conigera* and *A. hindsii*. *P. ferrugineus* ants were excluded (April 2008) from each one branch of five *A. hindsii* and five *A. cornigera* plants for one month. After this time, leaf damage (quantified as percentage of damaged leaflets) was evaluated in three leaves per branch in excluded branches as well as in control branches to which ants had access. At the same time, EFN secretion was also evaluated (µg g^{-1} dry mass 24 h^{-1}) for *A. cornigera* and *A. hindsii* on those same branches with and without access of *P. ferrugienus* ants using before for herbivory evaluations. Differences in leaf damage and in EFN secretion between treatments were evaluated with a Two-way ANOVA (independent variables: plant species and presence of ants). Percentage of leaf damage was arcsin transformed.

2. Ant-mediated defence against leaf pathogens: An ant-exclusion experiment was carried out in the field in January 2009 with plants of *A. cornigera* and *A. hindsii* to evaluate a putative inhibitory effect of the symbiotic ant *P. ferrugineus* and of the parasitic ant *P. gracilis* on pathogen growth (fungi and bacteria) in leaf tissue of both plant species. Ants were excluded for 1.5 months from each one branch of ten *A. hindsii* and ten *A. cornigera* plants. Control branches were considered those to had ant access. Thus, the following treatments were obtained: 1) *A. cornigera – P. ferrugineus* present, 2) *A. cornigera – P. ferrugineus* absent, 3) *A. cornigera – P. gracilis* present, 4) *A. cornigera – P. gracilis* absent, 5) *A. hindsii – P. ferrugineus* present, 6) *A. hindsii – P. ferrugineus* absent, 7) *A. hindsii – P. gracilis* present, 8)

A. hindsii – P. gracilis absent. After 1.5 months, three leaves were collected per branch and then resuspended in PBS buffer (biphosphat buffer, 0.1 M, pH 7.2) and stored at 4° C for 48 hrs. Bacteria present on leaves were evaluated cultivating 20 µL of a dilution 1:1000 in PBS buffer for each treatment on potato destroxe agar plates (Sigma). Plates were stored at room temperature for 72 h. Differences in fungal abundance [CFU * mg^{-1} dry leaf mass], bacteria abundance [CFU * mg^{-1} dry leaf mass] and bacteria diversity [Index of diversity] among treatments were analysed with a Two-way ANOVA (independent variables: ant species and presence of ants) for each plant species. Fungal and bacteria abundance were log transformed. For diversity analysis, each different colour of bacteria was considered as a different bacterium species. Sequence data for pathogen identification are still in analysis.

Index of diversity (D) was measured with the following formula:

$$D = 1 - \sum_{i=1}^{N} p^2_i$$

p = proportion of individuals for each species.

N = number of species.

3. Volatile analysis of ants: In order whether inhibitory ant effect on pathogens could be related with volatile emission of ants, preliminary analysis were carried out with three colonies of *P. ferrugineus* and with three colonies of *P. gracilis*. Ant colonies were collected in the field in March 2009. Ants of both species (6-8 workers) were placed in a 1.5 mL GC vial. Solid-phase microextraction fibers (50/30 µm divinylbenzene/carboxen/polydimethylsiloxane; Supelco, Bellefont, PA, USA) were exposed to equilibrated headspace for 2.5 h. The equilibrated fibers were analysed by gas chromatography (GC, Gas Chromotograph, 5890, Hewlett Packard) and a MS (Mass Selective Detector, 5972, Hewlett Packard) with a column HP-FAPP of 20 m length and 0.5 mm thickness. The GC was programmed as follow: injector held at 180° C, initial column

temperature at 60° C, and subsequently ramped with 3° C min^{-1} to 80° C and with 8° C min^{-1} to 200° C, held for 15 min. Compounds were identified by comparing mass spectra with spectra of the NIST library. Peak area were integrated and expressed as percentages of total emission per sample. Only peaks that were present in the three colony samples for each ant species were considered for peak area integration.

Results

EFN amino acids and attraction function

1. Sugars and amino acids:

Sucrose, fructose and glucose were the only sugars detected in EFN of *Acacia* and of the closely related *Prosopis*. EFNs of the two non-myrmecophyte species contained all three sugars, while EFNs of the myrmecophytes only contained the monosaccharides, fructose and glucose (Fig. 4). EFN secretion (in µg soluble solids per g leaf dry mass per 24 h) by the myrmecophyte, *A. cornigera*, was significantly higher than for the non-myrmecophyte species ($F_{3,21} = 6.08$; $P < 0.005$; univariate ANOVA) (Fig 5). No significant differences were observed in EFN secretion between *A. cornigera* and *A. hindsii* ($P > 0.05$, Tukey test), and between *A. hindsii* and the non-myrmecophyte species ($P > 0.05$, Tukey test).

Amino acid concentrations varied strongly among the four species, and 'species' was a significant source of variation in the concentrations of 17 of the 19 amino acids investigated (Table 19). The qualitative compositions differed much less, as only two of the four species contained less than 19 amino acids (*A. cornigera*: arginine missing, *Prosopis*: methionine and proline missing), while in EFN of *A. hindsii* and *A. farnesiana* all the 19 amino acids were present.

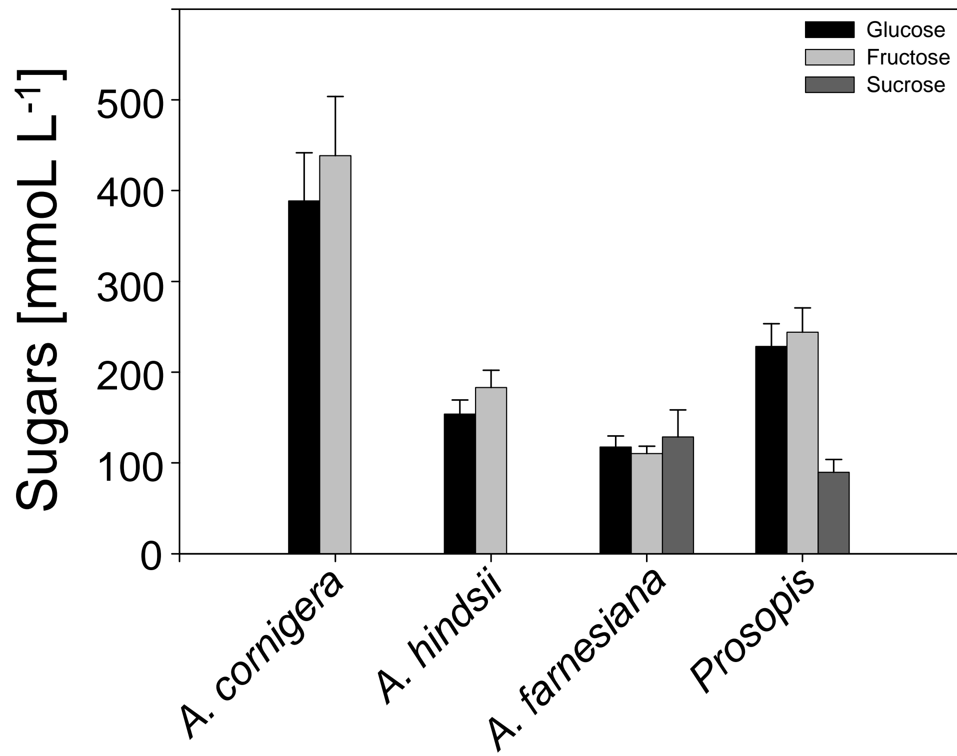


Fig. 4: Sugar quantities in EFNs. Concentrations are depicted in mmol sugars per L EFN as means ± SE. Sample size N = 5 individuals per species.

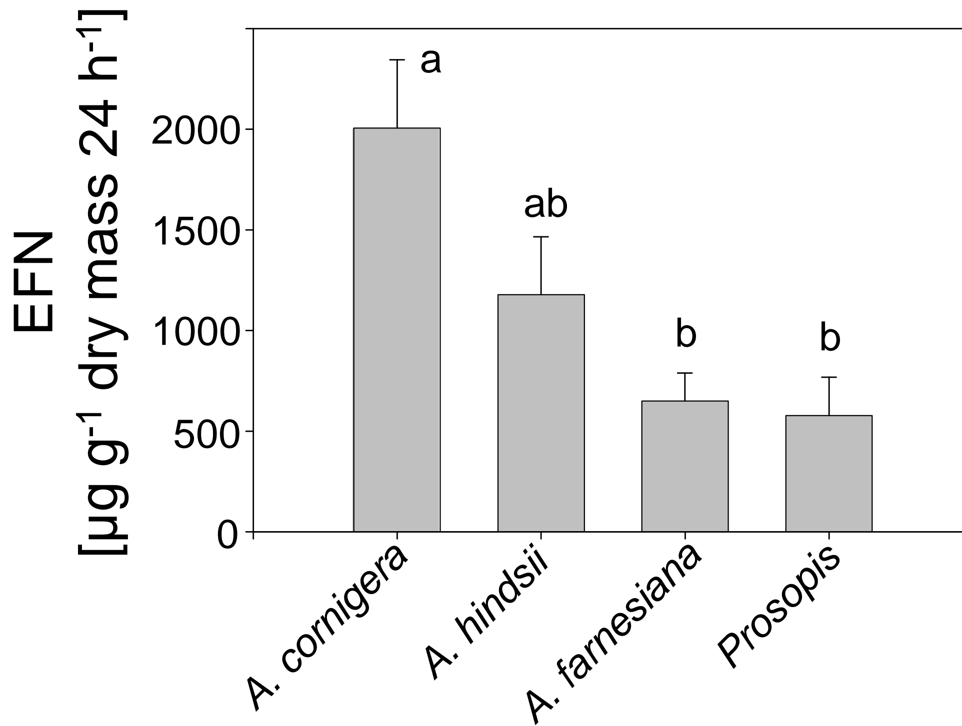


Fig. 5: EFN secretion rates. Amounts of total soluble solids (µg secreted per g leaf dry mass and per 24 h) are depicted for *A. cornigera*, *A. hindsii*, *A. farnesiana* and *Prosopis* as means ± SE. Sample size N = 5 individuals. Different letters indicate significant differences ($P < 0.05$ according to post hoc Tukey test) among the species.

Table 19: Concentration of single amino acids (AAs) (µmol L^{-1}), total AAs (mmol L^{-1}) and total sugars (mmol L^{-1}) in EFN of *A. cornigera*, *A. hindsii*, *A. farnesiana* and *Proposis juliflora*. Statistical differences among the four species were evaluated for each AA with a Kruskal-Wallis ANOVA, and significance levels are indicated: ns P > 0.05, * P < 0.05, ** P < 0.01, and *** P < 0.001. For amino acid names see Table 20. Total AAs refers to the sum of the 19 AAs for each species. Total sugars refer to the sum of fructose and glucose for *A. cornigera* and *A. hindsii*, and of fructose, glucose and sucrose for *A. farnesiana* and *Prosopis* (see Fig. 4).

	A. cornigera	*A. hindsii*	*A. farnesiana*	*Prosopis*
ALA (**)	1846 ± 336	924 ± 102	364 ± 93	178 ± 51
ARG (**)	0 ± 0	10 ± 10	24 ± 14	280 ± 152
ASN (*)	3375 ± 187	581 ± 237	7120 ± 2187	1275 ± 605
ASP (**)	176 ± 15	335 ± 79	496 ± 126	963 ± 355
GLN (**)	1186 ± 170	831 ± 449	1473 ± 408	206 ± 121
GLU (***)	1922 ± 138	2441 ± 848	302 ± 46	294 ± 27
GLY (*)	86 ± 10	209 ± 46	256 ± 32	196 ± 65
HIS (**)	2770 ± 359	1595 ± 158	278 ± 93	469 ± 62
ILE (***)	857 ± 139	1808 ± 207	285 ± 125	7 ± 5
LEU (***)	1405 ± 196	3462 ± 285	56 ± 22	22 ± 7
LYS (ns)	40 ± 17	46 ± 12	38 ± 10	74 ± 21
MET (***)	400 ± 94	1148 ± 93	44 ± 21	0 ± 0
PHE (***)	13127 ± 2672	12738 ± 2085	2809 ± 527	2066 ± 150
PRO (***)	1238 ± 205	912 ± 364	195 ± 96	0 ± 0
THR (*)	450 ± 32	805 ± 83	498 ± 123	124 ± 23
TRP (**)	1489 ± 399	339 ± 86	452 ± 92	938 ± 158
TYR (*)	4606 ± 477	1533 ± 155	1484 ± 300	4816 ± 469
SER (**)	941 ± 141	1001 ± 262	1368 ± 196	381 ± 94
VAL (***)	1712 ± 196	4281 ± 468	620 ± 191	165 ± 36
Total AAs	37 ± 0.6	34 ± 0.6	18 ± 0.3	12 ± 0.2
Total Sugars	827 ± 118	336 ± 34	356 ± 44	562 ± 62

2. <u>Non-Metric Multidimensional Scaling (NMDS)</u>:

Both axes contributed significantly to the variation among the species (Axis 1: $F_{3,16} = 63.0$, $P < 0.001$, univariate ANOVA; Axis 2: $F_{3,16} = 22.4$, $P < 0.001$, univariate ANOVA), allowing a grouping of myrmecophyte vs. non-myrmecophyte species, with *A. hindsii* and *Prosopis* being most distant from each other (Fig. 6). For Axis 1, there were no significant differences among myrmecophyte species and among non-myrmecophytes, but the myrmecophtes differed significantly from the non-myrmecophytes. For Axis 2, *Prosopis* was significantly different from all other three species.

Methionine, isoleucine, leucine, valine, threonine, phenylalanine, proline and serine were the components with the highest contribution to both axes (amino acids with higher correlation coefficients, see Table 20) suggesting that these eight amino acids did increase the C value and thus contributed most strongly to the differentiation among the species. All these eight amino acids where present at much higher concentrations in *A. hindsii* EFN than in EFN of *Prosopis* (see Table 19).

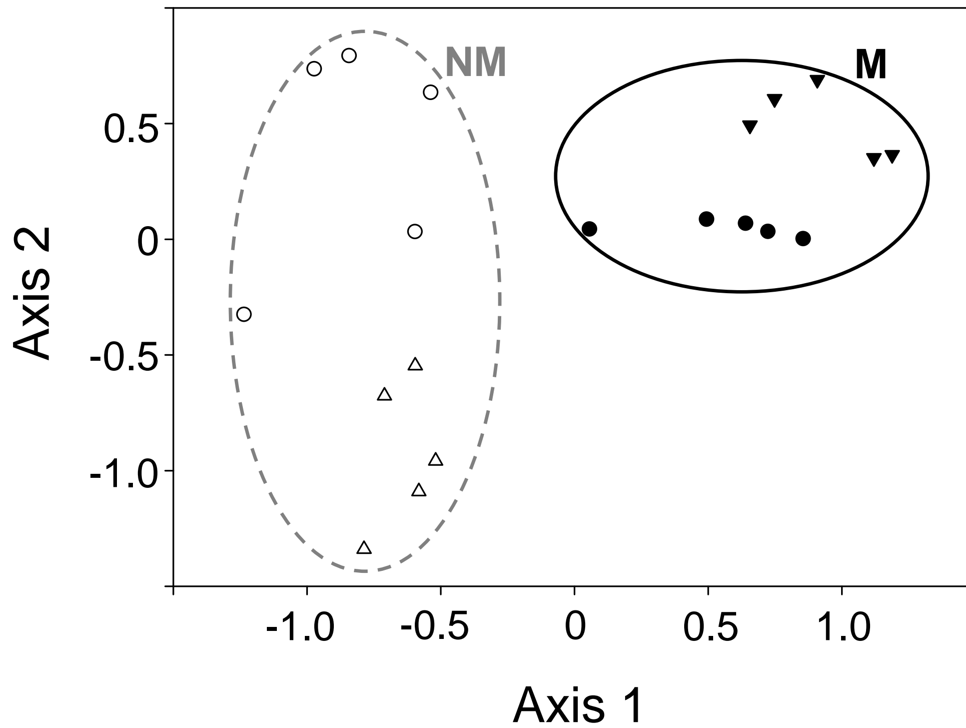


Fig. 6: Non-Metric Multidimensional Scaling (NMDS) ordination diagram of amino acid composition in EFNs. Black circles = *A. cornigera*; black triangles = *A. hindsii*; white circles = *A. farnesiana*; white triangles = *Prosopis*, M = myrmecophytes, NM = non-myrmecophytic species.

Table 20: Correlations between specific amino acids and the two NMDS axes in EFN of three *Acacia* species and *Prosopis*.

	NMS I	NMS II
Amino Acids		
ALA (alanine)	0.64	0.31
ARG (arginine)	-0.32	-0.38
ASN (asparagine)	-0.36	0.45
ASP (aspartic acid)	-0.39	-0.33
GLN (glutamine)	0.08	0.66
GLU (glutamic acid)	0.70	0.40
GLY (glycine)	-0.32	0.23
HIS (histidine)	0.81	0.24
ILE (isoleucine)	0.88	0.66
LEU (leucine)	0.91	0.49
LYS (lysine)	-0.14	-0.18
MET (methionine)	0.87	0.51
PHE (phenylalanine)	0.89	0.35
PRO (proline)	0.67	0.37
THR (threonine)	0.61	0.82
TRP (tryptophan)	0.18	-0.26
TYR (tyrosine)	0.02	-0.65
SER (serine)	0.37	0.74
VAL (valine)	0.87	0.56

3. Amino acids and ant attraction:

In the experiment using low-AA-EFNs (ratio of each AA to fructose = 1:1000 in the artificial mimics), mutualistic ants preferred EFN of *A. hindsii* over EFN of *Prosopis* (Fig. 7a), whereas non-mutualistic ants showed the opposite preference (Fig. 7c). In general, 'solution type' significantly affected the percentage of ants attracted to the different solutions. This remained true both for symbiotic ants ($F_{7,128} = 8.31$; $P < 0.001$; univariate ANOVA) and for non-symbiotic ants ($F_{7,128} = 7.49$; $P < 0.001$; univariate ANOVA). Nevertheless, neither symbiotic nor non-symbiotic ants discriminated among the various AA-containing artificial solutions (Fig. 7a, c). For high-AA-EFNs, the percentages of ants attracted to the different solution types also were significantly different both for symbiotic ants ($F_{7,72} = 10.89$; $P < 0.001$; univariate ANOVA) and non-symbiotic ants ($F_{7,72} = 10.83$; $P < 0.001$; univariate ANOVA) (Fig. 7b, d). Moreover, ants under these conditions distinguished among the artificial solutions, since symbiotic ants significantly preferred the artificial solution with four amino acids (leucine, phenylalanine, proline and valine), while no significant differences were observed among the other artificial solutions. Again, symbiotic ants preferred EFN of *A. hindsii* over the EFN of *Prosopis* (Fig. 7b). On the other hand, non-symbiotic ants significantly preferred the sugar solutions with sucrose over the solution without sucrose, and the sugar-amino acid solutions over sugar-only solutions, although they did not discriminate among the different solutions with amino acids. Consistently with the first experiment, *Prosopis* EFN attracted more non-symbiotic ants than nectar of *A. hindsii* (Fig. 7d).

In the second experiment testing different AA : sugar ratios, significant differences among AA solutions were only observed for symbiotic ants ($F_{5,54} = 6.66$; $P < 0.001$; univariate ANOVA). These symbiotic ants significantly preferred the solution 1:10 over all other solutions, and in fact ant preference decreased continuously with decreasing AA concentration (Fig. 8a). In contrast, non-symbiotic did not differentiate significantly among

solutions with different AA : sugar ratios ($F_{5,54} = 0.27$; $P > 0.05$; univariate ANOVA) (Fig. 8b). Similar results were obtained in the third experiment, where symbiotic ants distinguished among the different solutions ($F_{5,54} = 0.47$; $P > 0.05$; univariate ANOVA, see Fig. 8c) and significantly preferred the solution with 4 AAs over the other solutions at both 1:10 and 1:50 ratios ($F_{5,54} = 4.67$; $P < 0.001$; univariate ANOVA, see Fig. 5c). Again, non-symbiotic ants did not differentiate significantly among solutions ($F_{5,54} = 0.27$; $P > 0.05$; univariate ANOVA) (Fig. 8d)

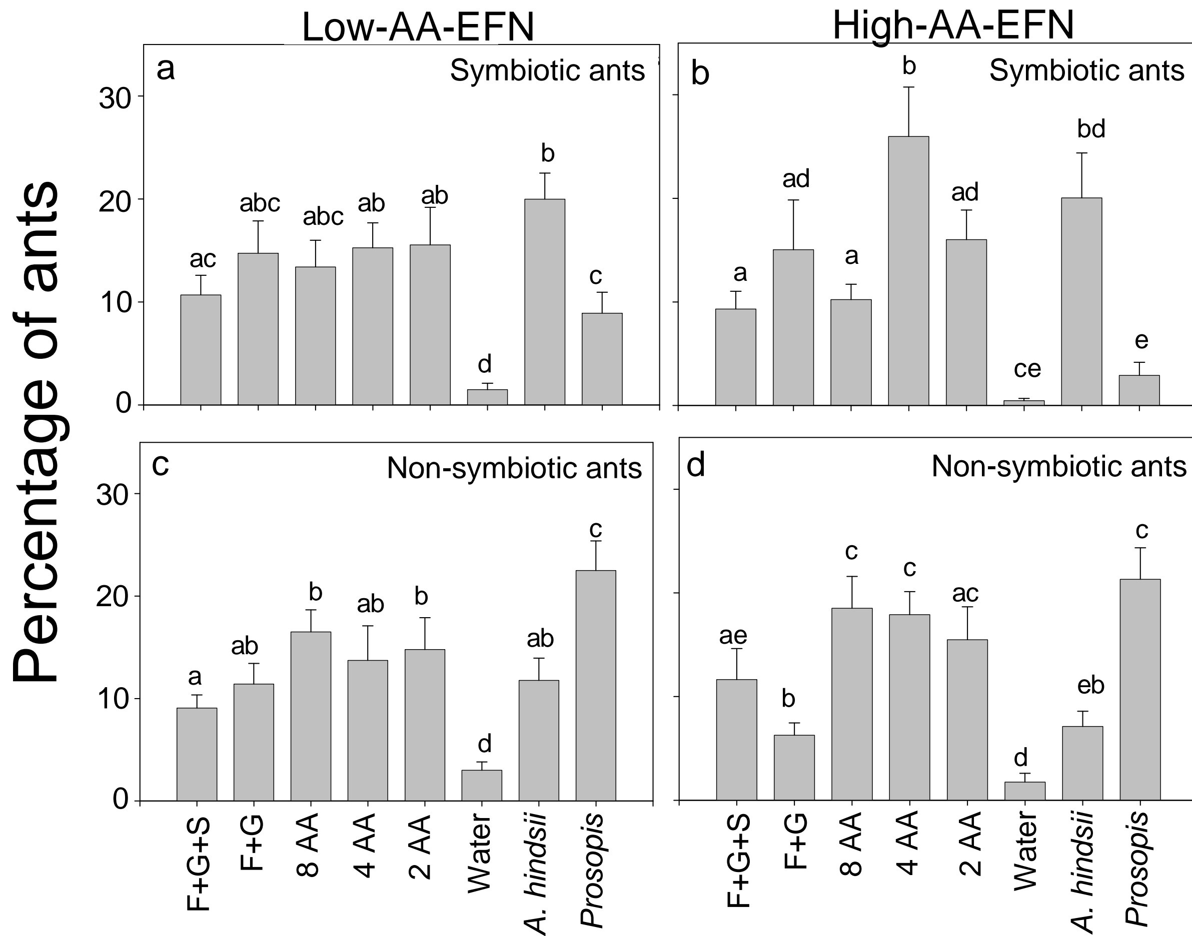


Fig. 7: Preferences of symbiotic and non-symbiotic ants to natural EFNs of *A. hindsii* and *Prosopis* and various EFN mimics with and without amino acids (AA). Solution compositions are indicated in Table 1. Low-AA-EFN (a, b) contained an AA : sugar ratio of 1:1000 (sample size = 17 cafeterias), whereas high-AA-EFNs (c, d) contained a ratio of 1:50 (sample size = 10 cafeterias). Ant preferences are expressed as means + SE of the percentage of all feeding ants that were attracted to each solution. Different letters indicate significant difference in ant attracted among solutions ($P < 0.05$ according to post hoc LSD test).

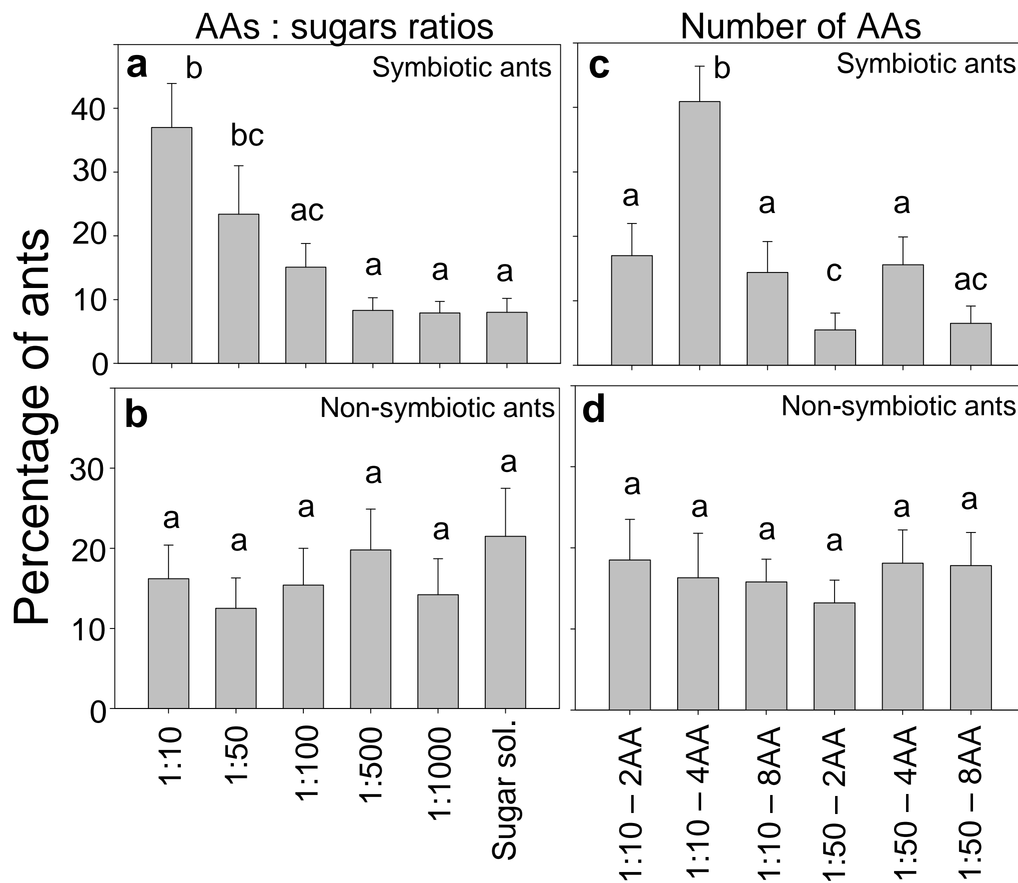


Fig. 8: Preferences of symbiotic and non-symbiotic ants to 4AA and 8AA solutions, respectively, with different AA:sugar ratios (a, b) (sample size = 10 cafeterias), and to solutions with different number of AAs at two different AAs : sugars ratios (c, d) (sample size = 10 cafeterias). Ant preferences are expressed as means + SE of the percentage of all feeding ants that were attracted to each solution. Different letters indicate significant difference in ant attracted among solutions ($P < 0.05$ according to post hoc LSD test).

EFN proteins and protection function against microorganisms

1. Total proteins:

The total amount of proteins as determined with Bradford assays was significantly higher in myrmecophyte EFN than in non-myrmecophyte EFN (Fig. 9a, b), both when expressed per total content of soluble solids (χ^2= 20.0; df = 3; P < 0.001; Kruskal-Wallis) and per leaf dry mass (χ^2= 20.0; df = 3; P < 0.001; Kruskal-Wallis). However, this effect was caused only by gross differences between the two life forms, as there were no significant differences between *A. cornigera* and *A. hindsii*, or between *A. farnesiana* and *Prosopis* (Fig. 9). Similarly, SDS-PAGE analysis showed protein patterns that clearly differed between myrmecophyte and non-myrmecophyte species (Fig. 10). Whereas numerous bands could be observed in EFN of both myrmecophytes, protein bands appeared in much lower numbers and abundances in EFN of *A. farnesiana* and *Prosopis*. For myrmecophyte EFN, molecular weights of the major protein bands ranged between 20 and 50 kDa.

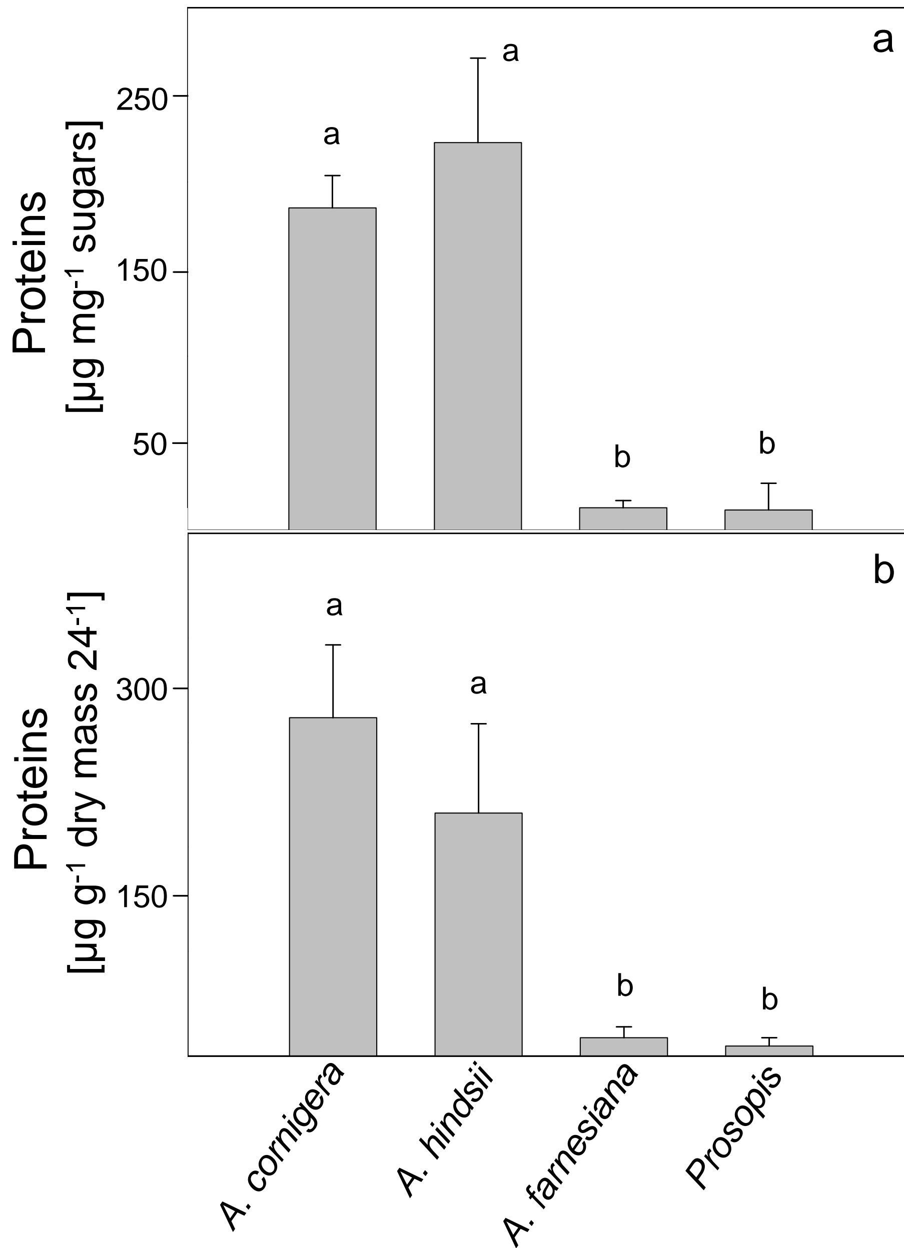


Fig. 9: Protein quantities in EFNs. (a) Relative protein content in EFN [in µg proteins per mg sugars] and (b) investment in EFN proteins per leaf dry mass [in µg proteins per gram leaf dry mass and 24 h] are displayed for *A. cornigera*, *A. hindsii*, *A. farnesiana* and *Proposi*s as means + ES are indicated. Sample size = 7 individuals by species. Different letters indicate significant differences among the species.

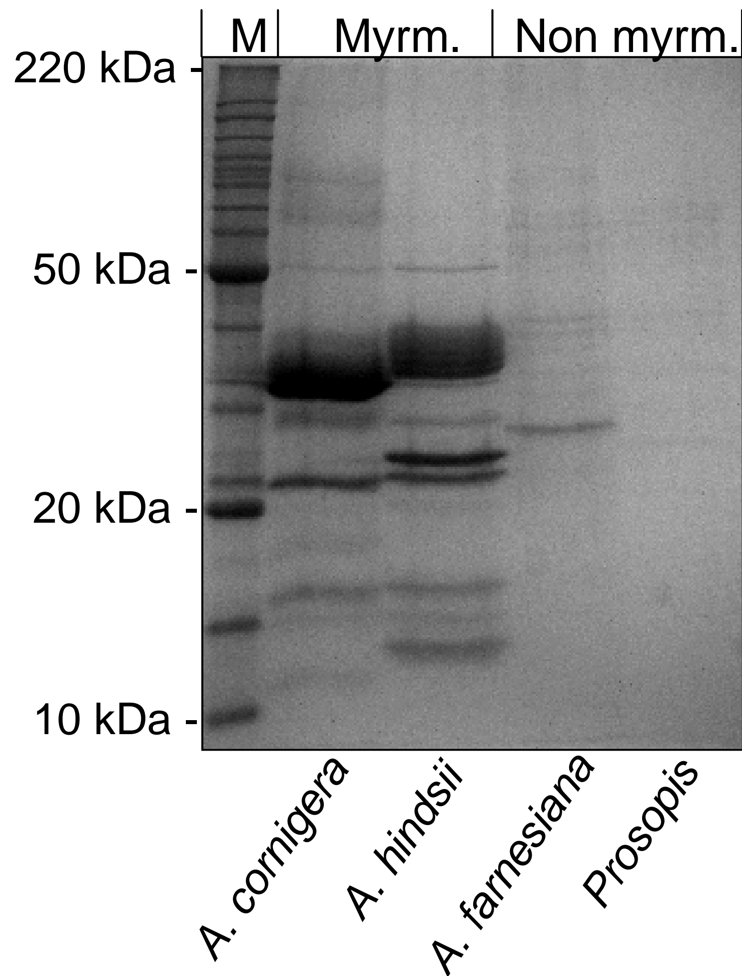


Fig. 10: Protein patterns in EFN. SDS PAGE (13%) profile of EFN proteins from myrmecophyte and non-myrmecophyte species. M indicates the molecular weight markers, Myrm. indicates the myrmecophyte species *A. cornigera* and *A. hindsii*, and Non-myrm. indicates the non-myrmecophyte species *A. farnesiana* and *Prosopis*.

2. Identification and quantification of PR-proteins:

The 2D-gel analysis in EFN proteome of myrmecophyte species revealed a relatively low number of different proteins (see Fig. 11-13). Around 75 % of the proteins for three myrmecophytes ranged in molecular weight between 20 and 37 kDa, which was consistent with the patterns seen in the 1D-gels (Fig. 10). Spots isolated from 2D-gels were analyzed with nanoLC-MS/MS and the fragment spectral data were searched in the Protein Lynx Global Server software against the EBI "planta". The most abundant proteins in EFN of the three myrmecophytes were most similar to chitinases and glucanases (Table 21-23). In order to quantify the extent to which these chitinases and glucanases contributed to the total amount of EFN proteins, we used the PD Quest 7.3.0 program and conducted a relative quantification by determining the volume of each spot as optical density (OD) multiplied with its area [mm^2]. For *A. cornigera*, glucanase proteins contributed ca. 40 % $\pm$ 1.4 (N = 3 gels) to the total proteins in EFN of *A. cornigera*, while chitinase proteins contributed ca. 14 % $\pm$ 1 (N = 3 gels). For *A. hindsii*, glucanase proteins contributed ca. 52 % $\pm$ 2.1 (N = 3 gels) to the total proteins in EFN, while chitinases contributed ca. 16 % $\pm$ 2.6 (N = 3 gels).

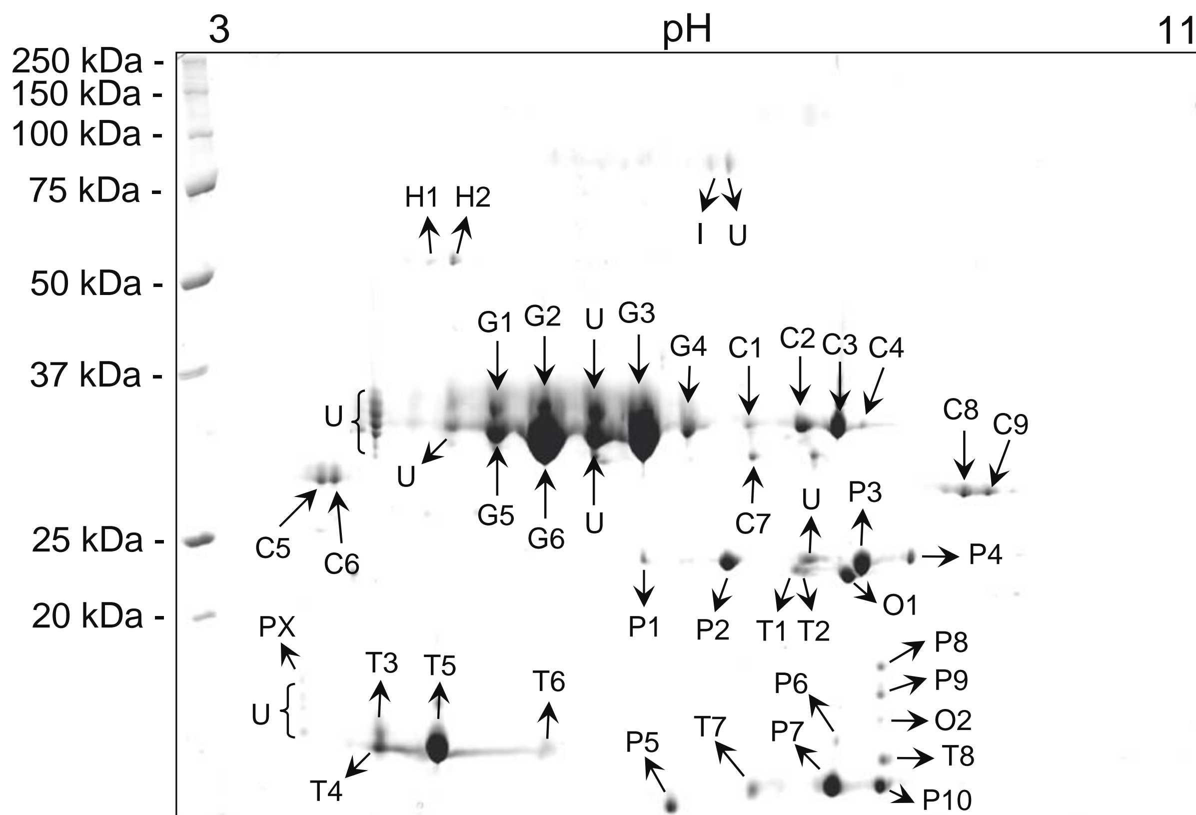


Fig. 11: Two-Dimensional Gel Electrophoresis. Separation of proteins in EFN from *A. cornigera* by 2D-gel (10% SDS-PAGE). C = chitinase proteins, G = glucanase proteins, H = glycoside hydrolase proteins, I = invertase protein, O = osmotin proteins, P = PR-proteins, PX = peroxidase protein, T = thaumatin-like protein, U = unknown proteins.

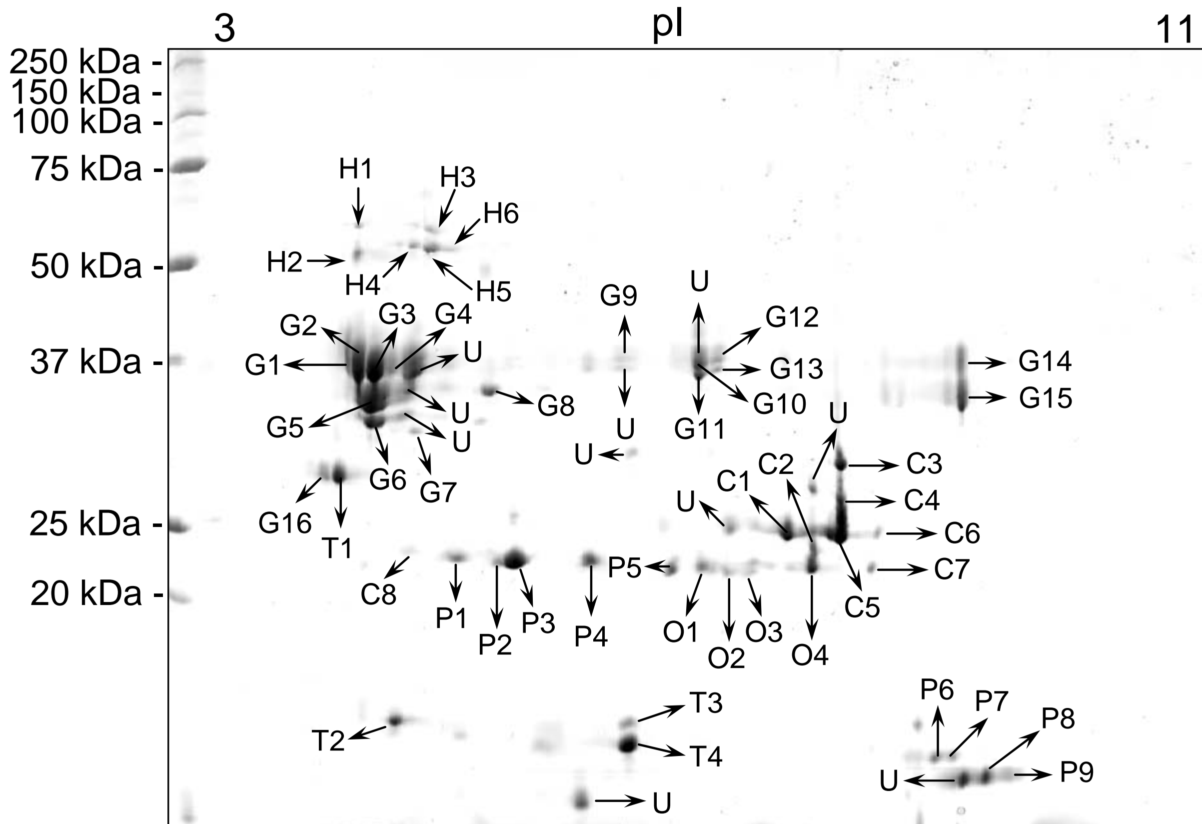


Fig. 12: Two-Dimensional Gel Electrophoresis. Separation of proteins in EFN from *A. hindsii* by 2D-gel (10% SDS-PAGE). C = chitinase proteins, G = glucanase proteins, H = glycoside hydrolase proteins, O = osmotin proteins, P = PR-proteins, T = thaumatin-like protein, U = unknown proteins.

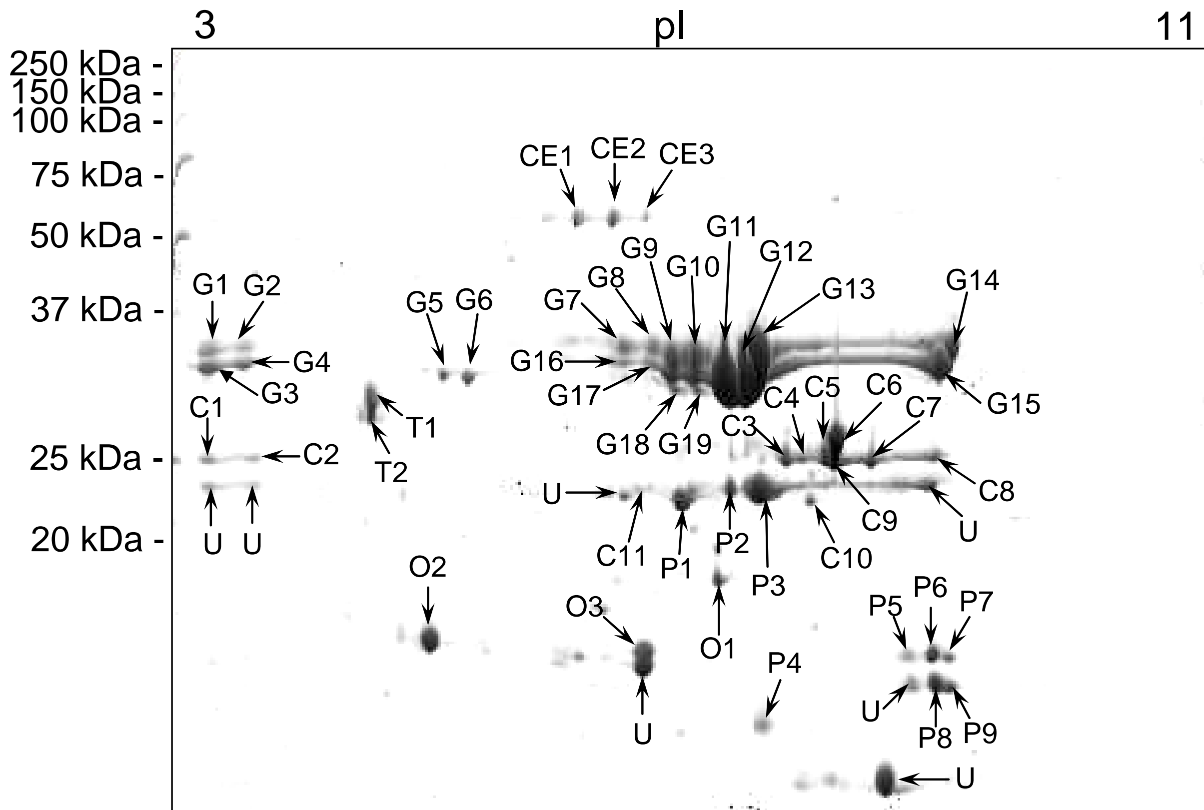


Fig. 13: Two-Dimensional Gel Electrophoresis. Separation of proteins in EFN from *A. collinsii* by 2D-gel (10% SDS-PAGE). C = chitinase proteins, G = glucanase proteins, CE = celullase containing proteins, O = osmotin proteins, P = PR-proteins, T = thamathin-like protein, U = unknown proteins.

Table 21: Results of MS-BLAST searches using *de novo* peptide sequences for the species *A. cornigera*.

Spot	Accession	Description	Plant species	Peptide hits MS-BLAST	MS BLAST score
C1	AAC24807	class I chitinase	*Solanum tuberosum*	2	125
C2	ABD66068	chitinase	*Momordica charantia*	6	289
C3	ABD66068	chitinase	*Momordica charantia*	8	526
C4	O81145	class I chitinase	*Solanum tuberosum*	8	441
C5	CAO78600	endochitinase	*Parkia platycephala*	3	145
C6	Q8LST3	chitinase	*Phytolacca americana*	3	146
C7	1302305A	chitinase	*Nicotiana sp.*	1	107
C8	1302305A	chitinase	*Nicotiana sp.*	1	64
C9	1302305A	chitinase	*Nitoctiana sp.*	2	99
G1	CAJ91137	β-1,3-glucanase	*Platanus x acerifolia*	3	133
G2	ABD85024	β-1,3-glucanase	*Lilium hybrid*	2	85
G3	BAE53384	β-1,3-glucanase	*Sesbania rostrata*	5	88
G4	AAX81590	β-1,3-glucanase	*Fragaria x ananassa*	1	253
G5	AASO9873	endo-β-1,3-glucanase	*Glycine latrobeana*	2	121
G6	AACO4712	β-1,3-glucanase	*Gliycine max*	5	280
H1	ABP03049	glycoside hydrolase	*Medicago trunculata*	4	234
H2	AAB77250	glycoside hydrolase	*Medicago trunculata*	4	234
I	ABB77250	cell wall invertase	*Bambusa oldhamii*	4	262
O1	AAU95238	osmotin-like protein	*Solanum phureja*	8	443
O2	CAC34005	osmotin-like protein	*Capsicum annuum*	2	108
P1	AAO22065	Nt PRp27-like protein	*Solanum tuberosum*	5	314
P2	BAA81904	Nt PRp 27	*Nicotiana tabacum*	5	314
P3	BAA81904	Nt PRp 27	*Nicotiana tabacum*	10	546
P4	BAA81904	Nt PRp 27	*Nicotiana tabacum*	3	188
P5	AAU94913	PR protein 4A	*Arachis hypogaea*	1	67
P6	CAA50596	PR-1a1	*Solanum lycopersicum*	1	81
P7	CAA87071	pathogenesis-related protein, PR-1 type	*Sambucus nigra*	4	275

P8	BAE93153	pathogenesis-related protein 1	*Lolium perenne*	2	145
P9	ABB73064	pathogenesis-related protein PR-1	*Glycine max*	2	117
P10	CAA87071	pathogenesis-related protein, PR-1 type	*Sambucus nigra*	1	98
PX	CAH59427	ascorbate peroxidase	*Plantago major*	1	46
T1	AAD55090	thaumatin	*Vitis riparia*	3	158
T2	AAK59277	thaumatin-like protein	*Sambucus nigra*	4	332
T3	CAA48278	thaumatin-like protein	*Oryza sativa Japonica*	1	84
T4	CAA48278	thaumatin-like protein	*Oryza sativa Japonica*	1	96
T5	AAM15877	thaumatin-like protein	*Triticum aestivum*	2	109
T6	CAA48278	thaumatin-like protein	*Oryza sativa Japonica*	4	230
T7	AAM12886	thaumatin-like protein	*Malus x domestica*	4	154
T8	CAA48278	thaumatin-like protein	*Oryza sativa Japonica*	3	212

Table 22: Results of MS-BLAST searches using *de novo* peptide sequences for the species *A. hindsii*.

Spot	Accession	Description	Plant species	Peptide hits MS-BLAST	MS BLAST score
H1	ABP03050	Glycoside Hydrolase	*Medicao trunculata*	3	196
H2	ABP03050	Glycoside Hydrolase	*Medicao trunculata*	6	280
H4	ABP03050	Glycoside Hydrolase	*Medicao trunculata*	5	275
H3	ABP03050	Glycoside Hydrolase	*Medicao trunculata*	4	206
H5	Q8RU51	Glucan 1,3-β-glucosidase	*Oryza sativa*	12	536
H6	Q8RU51	Glucan 1,3-β-glucosidase	*Oryza sativa*	15	714
G1	BAC15778	Endo-1,3-β-glucanase	*Oryza sativa*	3	191
G2	AAR26001	Endo-1,3-β-glucanase	*Glycine max*	4	222
G3	AAR26001	Endo-1,3-β-glucanase	*Glycine max*	6	326
G4	AAD10380	β-1,3-glucanase precursor	*Oryza sativa*	4	151
G5	Q9CA15	Endo-1,3-β-glucanase	*Arabidopsis theliana*	2	102
G6	CAA10167	Glucan endo-1,3-β-d-glucosidase	*Cicer arietinum*	2	125
G7	AAK97661	β-1,3-glucanase	*Sorghum bicolor*	1	64
G8	BAC15778	Endo-1,3-β-glucanase	*Oryza sativa*	2	118
G9	BAC84500	β-1,3-glucanase	*Oryza sativa*	1	151
G10	Q6S4I9	Endo-β-1,3-glucanase	*Glycine tabacine*	3	136
G11	Q6S9W0	Endo-1,3-β-glucanase	*Glycine max*	5	280
G12	BAC15778	Endo-1,3-β-glucanase	*Oryza sativa*	1	89
G13	BAE53384	β-1,3-glucanase	*Sesbania rostrata*	2	104
G14	BAE53384	β-1,3-glucanase	*Sesbania rostrata*	3	192
G15	BAC15778	Endo-1,3-β-glucanase	*Oryza sativa*	4	202
G16	BAE53384	β-1,3-glucanase	*Sesbania rostrata*	2	154
C1	Q43685	Chitinase class I	*Vigna unguiculata*	4	186
C2	Q8MD06	Chitinase	*Leucaena leucocephala*	9	420
C3	AAM49597	Chitinase	*Leucaena leucocephala*	6	292
C4	Q7X9R8	Chitinase	*Euonymus europaeus*	15	845
C5	ABD66068	Chitinase	*Momordica charantia*	9	561

C6	ABD66068	Chitinase	*Momordica charantia*	4	242
C7	AAG37276	Chitinase	*Fragaria ananassa*	4	237
C8	AAB41324	Class I chitinase	*Medicago sativa*	4	253
O1	ABC55724	Osmotin-like protein	*Fragaria ananassa*	5	310
O2	AAU95243	Osmotin-like protein	*Solanum tuberosum*	4	248
O3	AAF13707	Osmotin-like protein	*Fragaria ananassa*	4	232
O4	AAU95238	Osmotin-like protein	*Solanum phureja*	3	194
T1	AAM00216	Thaumatin-like protein	*Prumus persica*	3	167
T2	CAA48278	Thaumatin-like protein	*Oryza sativa*	1	95
T3	CAA09229	Thaumatin-like protein	*Cicer arietinum*	3	142
T4	Q2QLT4	Thaumatin-like protein	*Oryza sativa*	2	109
P1	AAO22065	NtPRp27-like protein	*Solanum tuberosum*	4	234
P2	AAO22065	NtPRp27-like protein	*Solanum tuberosum*	4	278
P3	AAO22065	NtPRp27-like protein	*Solanum tuberosum*	8	451
P4	BAA81904	NtPRp27	*Nicotiana tabacum*	6	338
P5	BAA81904	NtPRp27	*Nicotiana tabacum*	5	325
P6	AAK30143	Pathogenesis-related protein PR-1	*Capsicum annuum*	2	160
P7	AAK30143	Pathogenesis-related protein PR-1	*Capsicum annuum*	2	172
P8	CAA87071	Pathogenesis-related protein, PR-1 type	*Sambucus nigra*	1	98
P9	CAA52894	PR-1b pathogenesis-related protein	*Hordeum vulgare*	3	186

Table 23: Results of MS-BLAST searches using *de novo* peptide sequences for the species *A. collinsii*.

Spot	Accession	Description	Plant species	Peptide hits MS-BLAST	MS BLAST score
CE1	Q8RU51	Celullase containing protein	*Oryza sativa*	15	803
CE2	Q8RU51	Celullase containing protein	*Oryza sativa*	20	940
CE3	Q8RU51	Celullase containing protein	*Oryza sativa*	11	532
G1	Q84Y06	β-1,3-glucanase	*Fragaria ananassa*	2	143
G2	Q654I9	Endo-β-1,3-glucanase	*Glycine tabacina*	3	176
G3	O49016	β-1,3-glucanase	*Glycine max*	1	77
G4	B2NK62	β-1,3-glucanase	*Lotus japonicus*	4	258
G5	O49012	β-1,3-glucanase	*Glycine max*	6	307
G6	P33157	Endo-1,3-β-glucosidase	*Arabidopsis thaliana*	4	204
G7	Q84Y07	β-1,3-glucanase	*Fragaria ananassa*	3	156
G8	Q6S4J7	Endo-β-1,3-glucanase	*Glycine tabacina*	4	251
G9	Q84Y07	β-1,3-glucanase	*Fragaria ananassa*	1	74
G10	Q6GWG6	β-1,3-endoglucanase	*Glycine soja*	4	230
G11	Q56AP1	β-1,3-glucanase	*Fragaria ananassa*	6	312
G12	Q84Y07	β-1,3-glucanase	*Fragaria ananassa*	7	357
G13	Q6S4I9	Endo-β-1,3-glucanase	*Glycine tabacina*	4	250
G14	B2NK62	β-1,3-glucanase	*Lotus japonicus*	3	214
G15	Q84I07	β-1,3-glucanase	*Fragaria ananassa*	5	260
G16	O49016	β-1,3-glucanase	*Glycine max*	3	149
G17	O49016	β-1,3-glucanase	*Glycine max*	3	146
G18	O49016	β-1,3-glucanase	*Glycine max*	3	182
G19	Q6S4J4	Endo-β-1,3-glucanase	*Glycine latrobeana*	2	84
C1	Q42428	Chitinase	*Castanea sativa*	8	507
C2	Q7X9R8	Chitinase	*Euonymus europaeus*	7	488
C3	Q8MD06	Chitinase	*Leucaena leucocephala*	7	410

C4	Q42428	Chitinase	*Castanea sativa*	8	559
C5	Q7X9R8	Chitinase	*Euonymus europaeus*	9	564
C6	Q9FEW1	Endochitinase	*Nicotiana sylvestris*	13	683
C7	Q207U1	Chitinase	*Momordica charantia*	13	798
C8	Q42428	Chitinase	*Castanea sativa*	11	714
C9	Q42428	Chitinase	*Castanea sativa*	9	623
C10	Q2VAC7	Chitinase	*Ficus pumila*	2	111
C11	Q93WX9	Endochitinase	*Musa acuminata*	2	126
O1	A9QVJ4	Osmotin	*Piper colubrinum*	7	446
O2	Q8S4L2	Osmotin-like protein	*Solanum nigrum*	5	254
O3	Q84MK8	Osmotin	*Solanum tuberosum*	2	104
T1	Q2VC78	Thaumatin-like protein	*Glycine max*	2	125
T2	P83332	Thaumatin-like protein 1	*Prunus persica*	2	133
P1	Q84XQ4	NtPRp27-like protein	*Solanum tuberosum*	6	327
P2	Q84XQ4	NtPRp27-like protein	*Solanum tuberosum*	3	160
P3	Q84XQ4	NtPRp27-like protein	*Solanum tuberosum*	6	317
P4	Q84XQ4	NtPRp27-like protein	*Solanum tuberosum*	6	338
P5	Q84XQ4	NtPRp27-like protein	*Solanum tuberosum*	1	69
P6	Q41359	Pathogenesis-related protein PR-1 type	*Sambucus nigra*	3	181
P7	Q41359	Pathogenesis-related protein PR-1 type	*Sambucus nigra*	2	124
P8	Q2XX51	Pathogenesis-related protein 1	*Zea diploperennis*	5	275
P9	AOMZ69	Pathogenesis-related protein 1	*Musa acuminata*	6	301

3. Antifungal protection of EFN in nature:

No fungi were detected in EFN of the two myrmecophytes, while significantly higher numbers appeared in EFN of non-myrmecophytes (χ^2= 7.2; df = 3; P = < 0.05; Kruskal-Wallis) (Fig. 14). These results suggest that EFN can principally become infested by fungi under natural conditions and that the EFN of myrmecophytes comprises some protection from microorganisms.

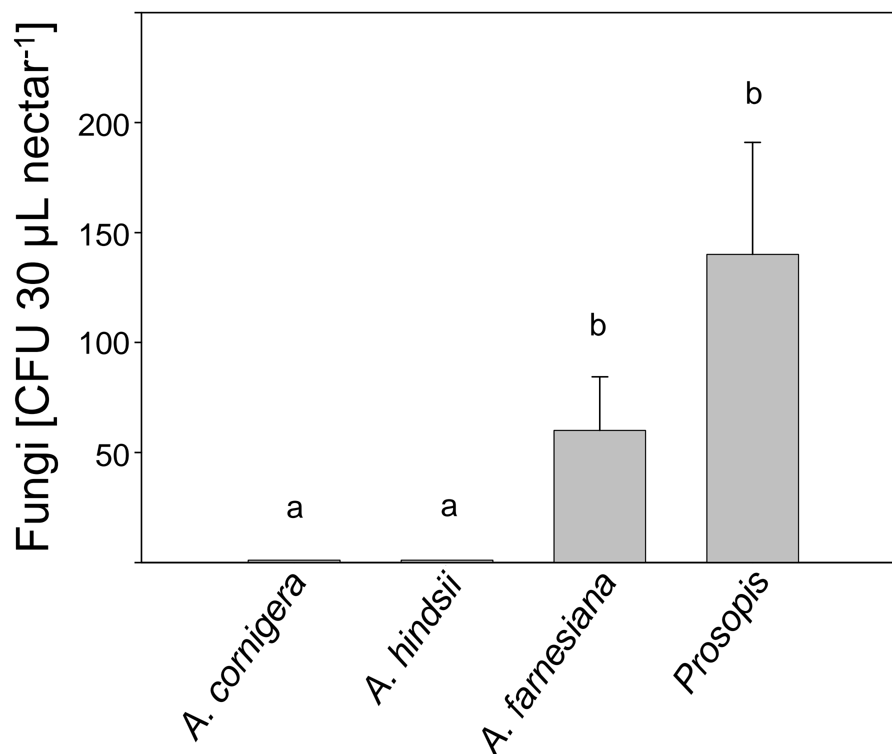


Fig. 14: Presence of fungi in fresh EFN samples. Fungal growth in EFN of the myrmecophytes *A. cornigera* and *A. hindsii* and in EFN of the non-myrmecophytes *A. farnesiana* and *Prosopis*. Fungal growth was evaluated as [CFU 30 µL EFN^{-1}] after 48 h of incubation.

4. Pathogenesis-related (PR) enzymes:

Activities of all three PR-enzymes were detected in EFN of all four species investigated, although differing in dependence on the plant life form. For example, chitinase activity differed significantly among the species (χ^2= 12.78; df = 3; P < 0.01; Kruskal-Wallis), since myrmecophyte EFN in general had higher activities than EFN of non-myrmecophytes (Fig. 15a). Even the two latter species differed significantly, as *A. farnesiana* possessed the lowest activity among all species investigated. Glucanase activity showed the same pattern as chitinase, as it was higher in myrmecophyte than in non-myrmecophyte EFNs (Fig. 15b), and as *A. farnesiana* showed the lowest activity among the four species investigated (χ^2= 11.80; df = 3; P < 0.01; Kruskal-Wallis). In contrast, peroxidase activity did not differ significantly among the four species investigated (χ^2 = 4.00; df = 3; P > 0.05; Kruskal-Wallis) and was much lower than the activities of glucanases and chitinases (Fig. 15c).

PR- enzymes in gel assays indicated that chitinase isoforms, acidic and basic, were abundant in both plant functional groups (Fig. 16a, b), although basic chitinases were lower abundant in EFN of non-myrmecophytes than in myrmecophytes (Fig. 16b). In contrast, glucanase isoforms, both acidic and basic, were abundant but only in myrmecophyte EFN (Fig. 17a, b), from non-myrmecophyte EFN they very almost absent (Fig. 17a, b).

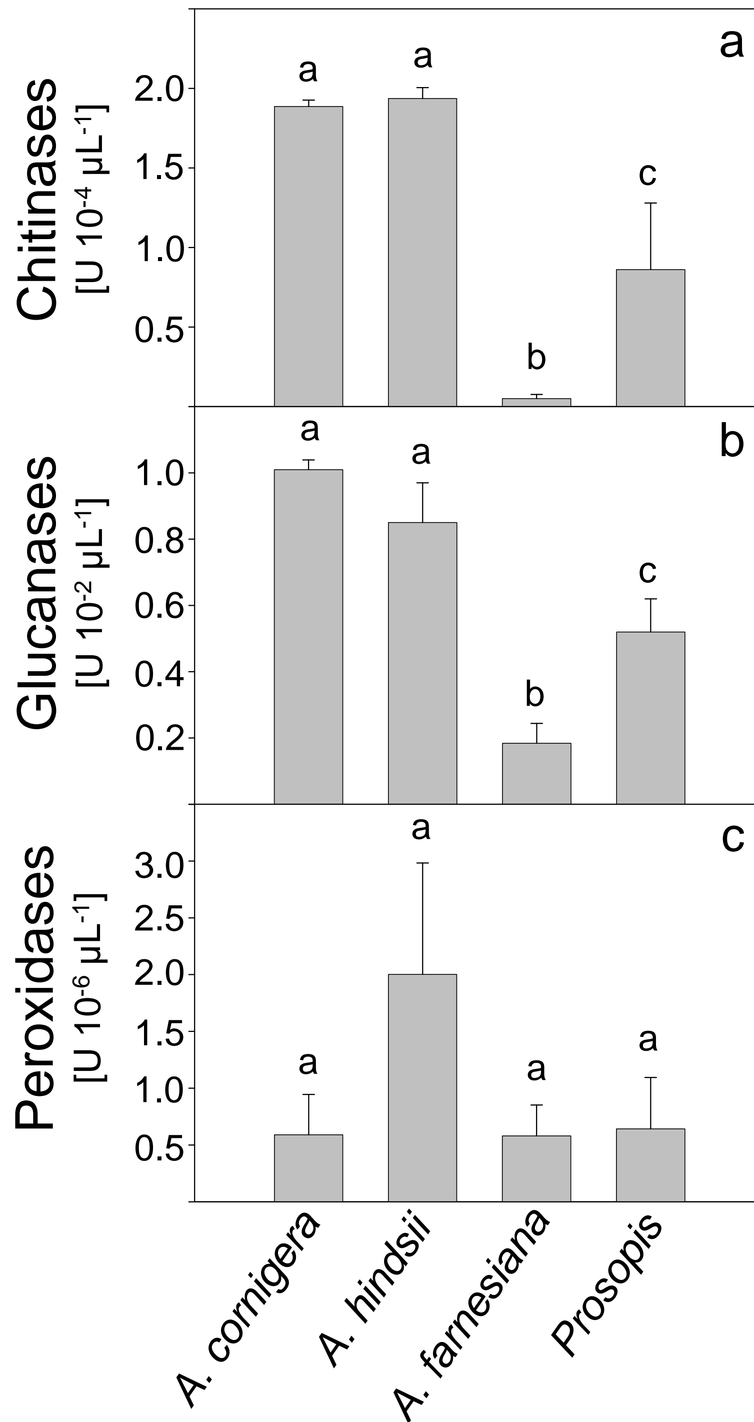
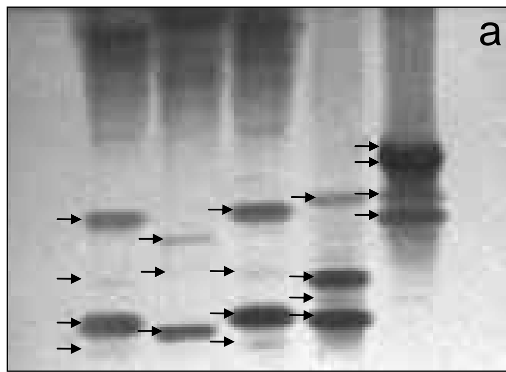


Fig. 15: Activities of three pathogenesis-related (PR) enzymes, (a) chitinase, (b) glucanase and (c) peroxidase in EFN of *A. cornigera*, *A. hindsii*, *A. farnesiana* and *Proposi*s are presented in [units µL EFN^{-1}] as means $\pm$ SE. Sample size = 5 individuals by species. Different letters indicate significant differences among the species.

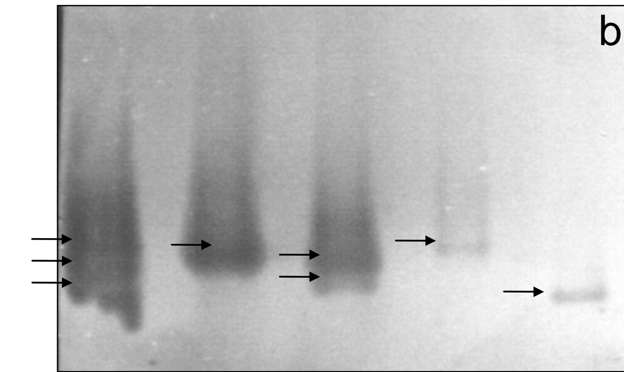
Acidic Chitinases



A. cornigera A. hindsii A. collinsii A. farnesiana Prosopis

Myrm. Non Myrm.

Basic Chitinases

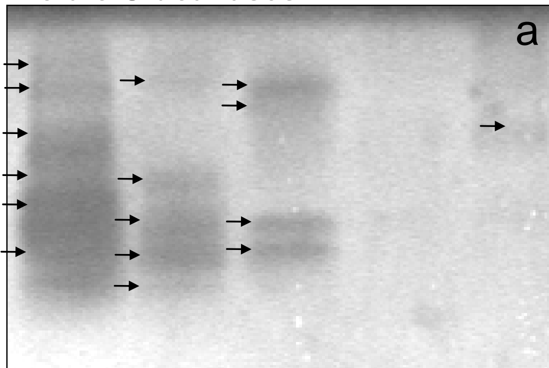


A. cornigera A. hindsii A. collinsii A. farnesiana Prosopis

Myrm. Non Myrm.

Fig. 16: Acidic and basic chitinase isoforms in EFN of myrmecopyhtes (*A. cornigera, A. hindsii* and *A. collinsii*) and non-myrmecophytes (*A. farnesiana* and *Prosopis juliflora*).

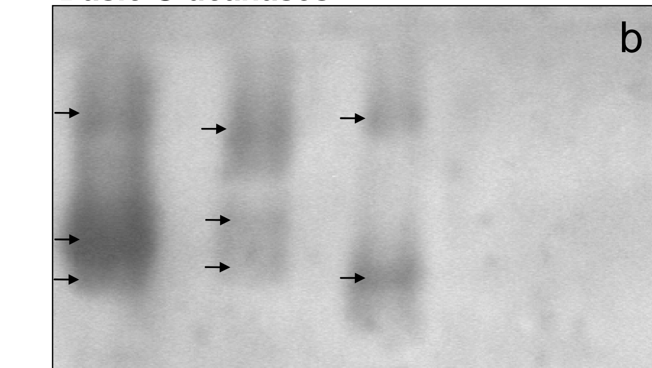
Acidic Glucanases



A. cornigera A. hindsii A. collinsii A. farnesiana Prosopis

Myrm. Non Myrm.

Basic Glucanases



A. cornigera A. hindsii A. collinsii A. farnesiana Prosopis

Myrm. Non Myrm.

Fig. 17: Acidic and basic glucanase isoforms in EFN of myrmecopyhtes (*A. cornigera, A. hindsii* and *A. collinsii*) and non-myrmecophytes (*A. farnesiana* and *Prosopis juliflora*).

5. Antifungal effects of EFN:

Myrmecophyte EFN inhibited the development of yeasts, as significantly less CFUs were found in the EFN of *A. cornigera* and *A. hindsii* as compared to a pure sugar solution (Fig. 18). In contrast, no significant reduction in numbers of CFU was caused by EFN of the two non-myrmecophytes, *A. farnesiana* and *Prosopis*. ‘Species’ was, thus, a significant source of variance in CFU numbers ($F = 5.20$; $df = 4, 35$; $P < 0.01$; univariate ANOVA).

Chitinase activity as found in *A. cornigera* EFN significantly reduced yeast growth ($F = 4.49$; $df = 2, 21$; $P < 0.05$; univariate ANOVA), since a sugar solution without chitinase supported significantly more CFUs than the water control and the nectar mimic with chitinase activity. Therefore, a sugar solution with chitinase activity as found for *A. cornigera* would allow as little microbial growth as a pure water solution (Fig. 19). On the other hand, a sugar solution with chitinase activity as found for *A. farnesiana* did not significantly reduce yeast growth ($F = 0.92$; $df = 2, 21$; $P > 0.05$; univariate ANOVA), although a strong tendency towards a reduction of CFUs was visible, similar to the pattern as found for *A. cornigera* (Fig. 19). Inhibition rates were calculated for each trial as inhibition rate [%] = ((CFU in sugar solution - CFU in sugar solution + chitinase) / CFU in sugar solution)) * 100 and amounted to 36.7 % ± 8 for *A. cornigera* and to 27.5 % ± 18 for *A. farnesiana*. Apparently, chitinase activity as found in *A. farnesiana* EFN was, on average, just not high enough to cause a significant effect.

EFN of myrmecophyte species was also able to inhibit the growth of at least 4 fungal species (Table 24). In contrast, no inhibitory effects were observed by non-myrmecophyte EFN as well as by sugar solutions (Table 24, Fig. 20). On the other hand, only the protein fraction of EFN of three myrmecophytes inhibited the growth of *Phytophthora parasitica* (Table 25). These results support evidence that EFN proteins are the fraction responsible for nectar defence against pathogens in myrmecophyte *Acacia*.

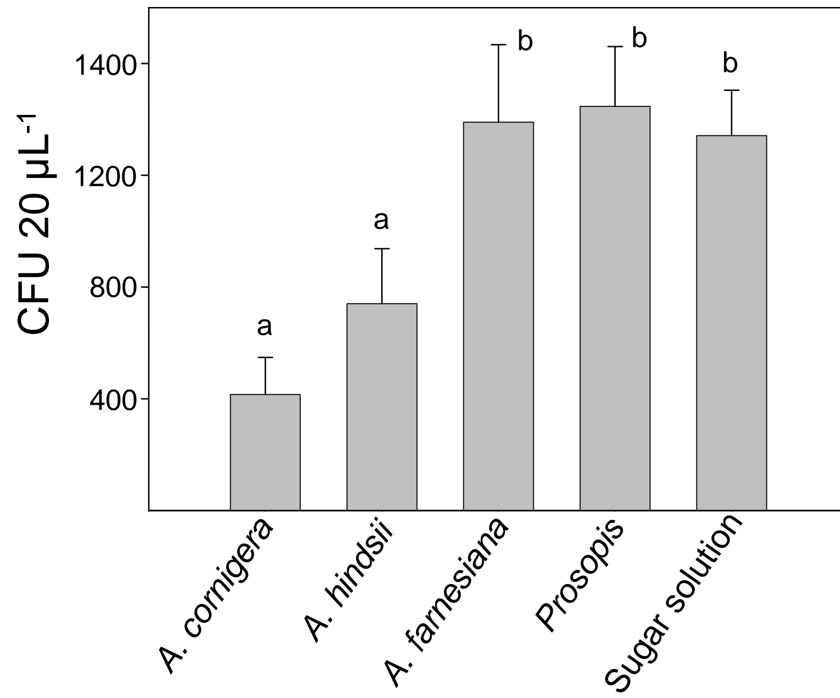


Fig. 18: Yeast growth [CFU 20 μL^{-1}] in EFN of two myrmecophyte (*A. cornigera* and *A. hindsii*) and two non-myrmecophyte species (*A. farnesiana* and *Prosopis*).

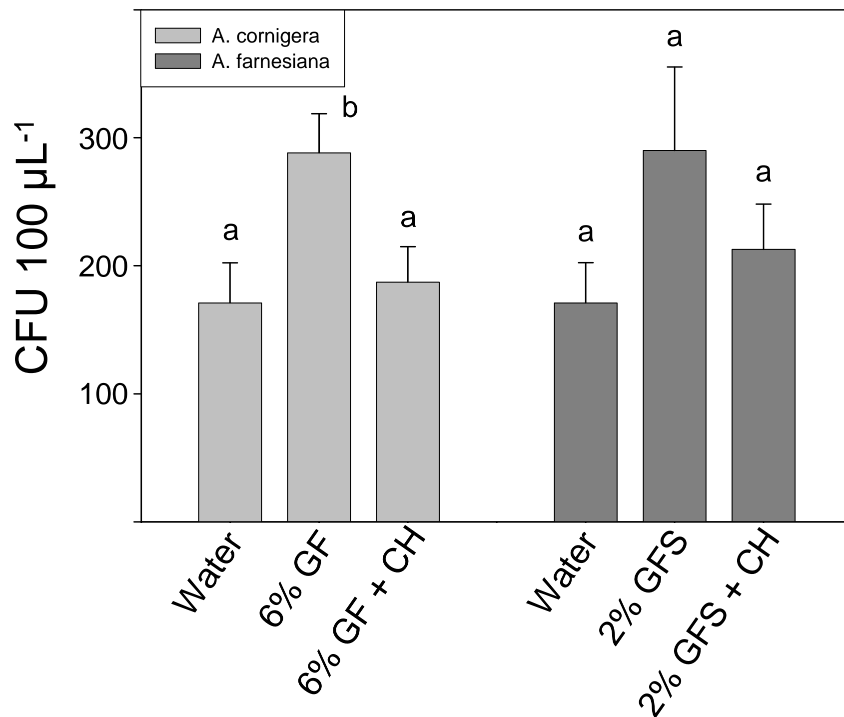


Fig. 19: Yeast growth [CFU 100 μL^{-1}] in artificial nectar solutions, with and without chitinase activity (6% GF = glucose-fructose solution at a concentration of 6%; 6% GF + CH = glucose + fructose solution at a concentration of 6% with chitinase activity as it was found for *A. cornigera*; 2% GFS = glucose-fructose-sucrose solution at a concentration of 2%; 2% GFS + CH = glucose-fructose-sucrose solution at a concentration of 2% with chitinase activity as it was found for *A. farnesiana*).

Table 24: Antifungal effect of EFN from myrmecophyte species (*A. cornigera*, *A. hindsii* and *A. collinsii*) and from non-myrmecophyte species (*A. farnesiana* and *Prosopis*) on different fungal species. +++ indicates a strong effect, ++ a high effect, + a moderate effect, - non effect.

	Phytophthora parasitica	*Plectosphaerella cucumerina*	*Fusarium oxysporum*	*Botrytis cinerea*	*Verticillium dahliae*	*Alternaria alternata*
A. cornigera	+++	-	++	-	++	++
A. hindsii	+++	-	++	-	++	++
A. collinsii	+++	+	++	+	++	++
A. farnesiana	-	-	-	-	-	-
Prosopis	-	-	-	-	-	-
Sugar 10%	-	-	-	-	-	-
Sugar 3%	-	-	-	-	-	-

Table 25: Antifungal activity against *Phytophthora parasitica* in different fractions from the EFN from myrmecophyte species (*A. cornigera*, *A. hindsii* and *A. collinsii*) and from non-myrmecophyte species (*A. farnesiana* and *Prosopis*). +++ indicates a strong effect, - non effect.

	Proteins (> 5kDa fraction)	*Metabolites (< 5kDa fraction)*
A. cornigera	+++	-
A. hindsii	+++	-
A. collinsii	+++	-
A. farnesiana	-	-
Prosopis	-	-

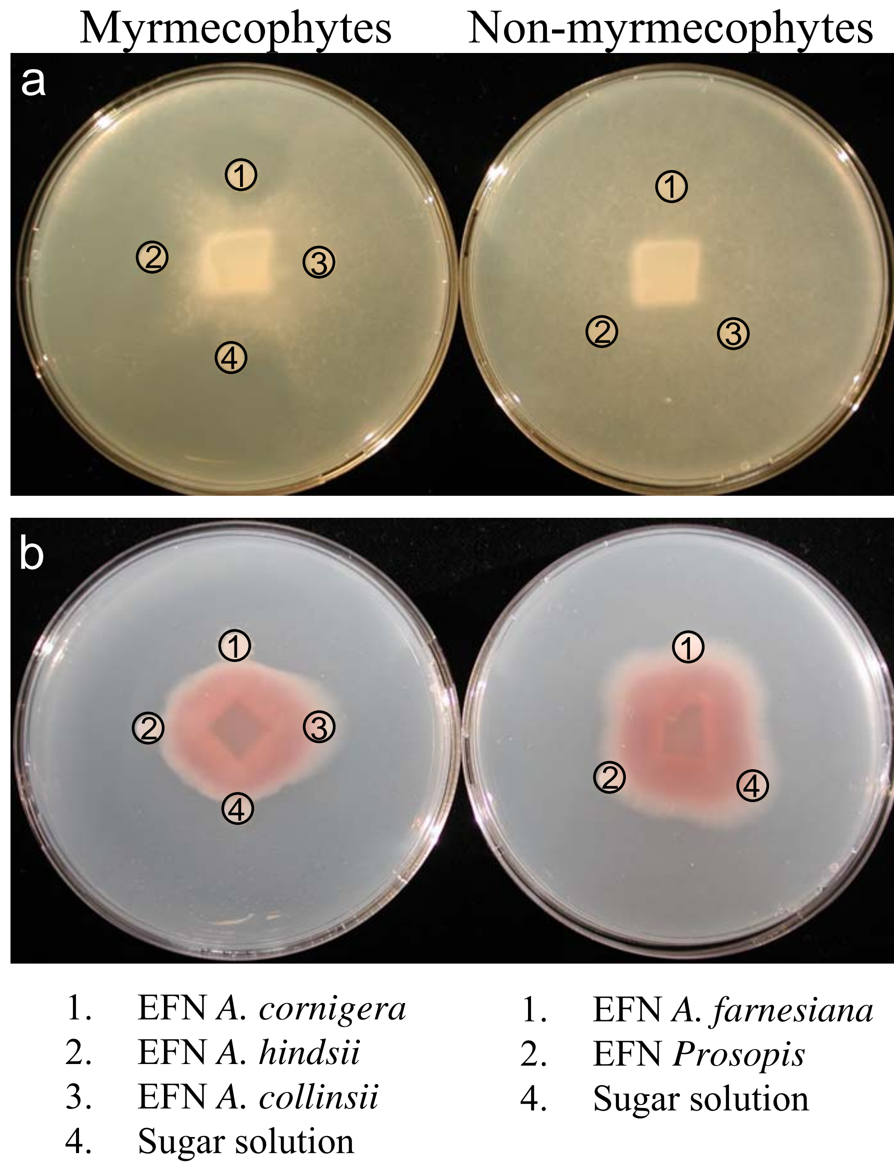


Fig. 20: Inhibitory effect of EFN from three myrmecophyte and two non-myrmecophyte *Acacia* species growth of the fungal species a) *Phytophthora parasitica* and b) *Fusarium oxysporum*. Effect of a sugar solution was evaluated as a control.

Temporal pattern in EFN reduces exploitation by nectar robbers

1. EFN secretion and ant activity:

Time of day had a significant effect on both EFN production and ant activity for all three *Acacia* species: *A. cornigera* (EFN production: $\chi^2 = 40$, df = 7, P < 0.001; Ant activity: $\chi^2 = 17.5$, df = 7, P = 0.014; Kruskal-Wallis test), *A. hindsii* (EFN production: $\chi^2 = 25$, df = 7, P < 0.001; Ant activity: $\chi^2 = 14.4$, df = 7, P = 0.044; Kruskal-Wallis test) and *A. collinsii* (EFN production: $\chi^2 = 17.1$, df = 7, P = 0.017; Ant activity: $\chi^2 = 18$, df = 7, P = 0.012; Kruskal-Wallis test). Also, for all three *Acacia* species, a significant and positive correlation was observed between the amounts of EFN produced and the ant activity on the respective plants (*A. cornigera*: R = 0.58, P < 0.001; *A. collinsii*: R = 0.39, P = 0.014: *A. hindsii*: R = 0.38, P = 0.030, Spearman rank correlation). Moreover, the maximum activity of *P. ferrugineus* on the three *Acacia* hosts coincided with the time of day during which peak EFN secretion could be observed (Fig. 21). For *A. cornigera* and *A. hindsii,* highest values of EFN production and ant activity were observed at 10.00 AM, while for *A. collinsii* EFN production and ant activity reached maximum values at 12.00 PM (Fig. 21).

2. Ant-mediated defence against nectar robbers:

During experiments only one group of insect species was regularly observed as nectar robber on the three *Acacia* species: bees of the genus *Frieseomelitta nigra* (Cresson, 1878) (Apidae). I also found that *P. ferrugineus* ants could protect EFN from visiting *Frieseomelitta nigra* bees; however, the effect of ants was significant only for *A. cornigera* and *A. collinsii* and only at the time of day during which EFN secretion – and thus *P. ferrugineus* activity – was highest (see Fig. 22: difference in *F. nigra* visits between ant-excluded and control branches for *A. cornigera*: Z = 2.402, P = 0.016; for *A. collinsii*: Z = 2.611, P = 0.008, Mann-Whitney test) (Figs 22a, b). During the other censuses of the same plants no significant differences in *F. nigra* visits between branches with and without ants could be observed. Similarly, *F. nigra*

visits to *A. hindsii* were not significantly different between ant-excluded and control branches (Z = 1.148, P = 0.250, Mann-Whitney test), although a tendency towards lower bee numbers on branches with ants became obvious during the time of highest EFN secretion (Fig. 22c).

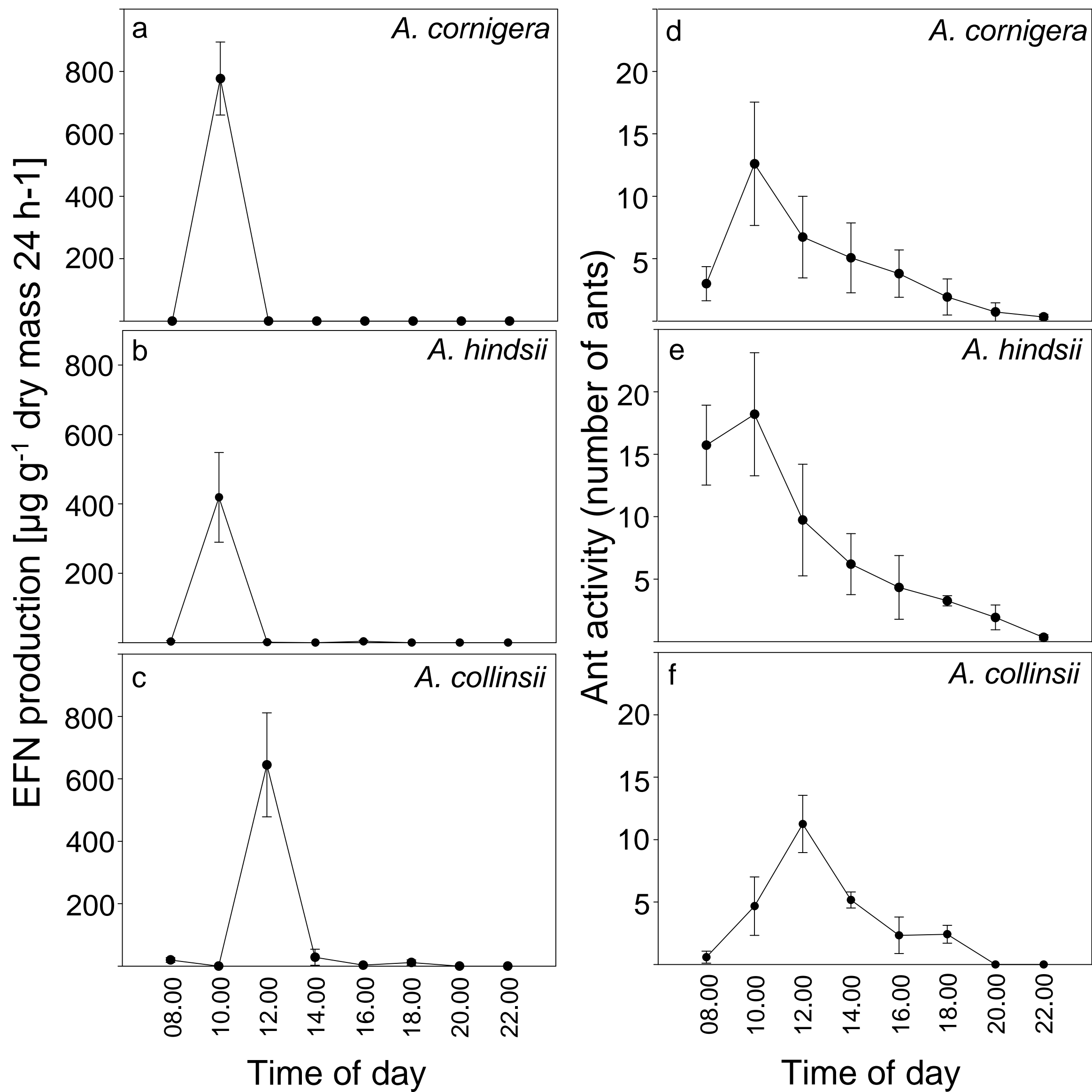


Fig. 21: Diel patterns in EFN production (µg sugar solids g^{-1} dry mass) and activity patterns of the symbiotic-ant *P. ferrugineus* on three *Acacia* myrmecophyte species (a) *A. cornigera*, (b) *A. hindsii*, (c) *A. collinsii*. Ant activity was quantified using counts of ants on three 10 cm branch sections in five individuals for each *Acacia* species.

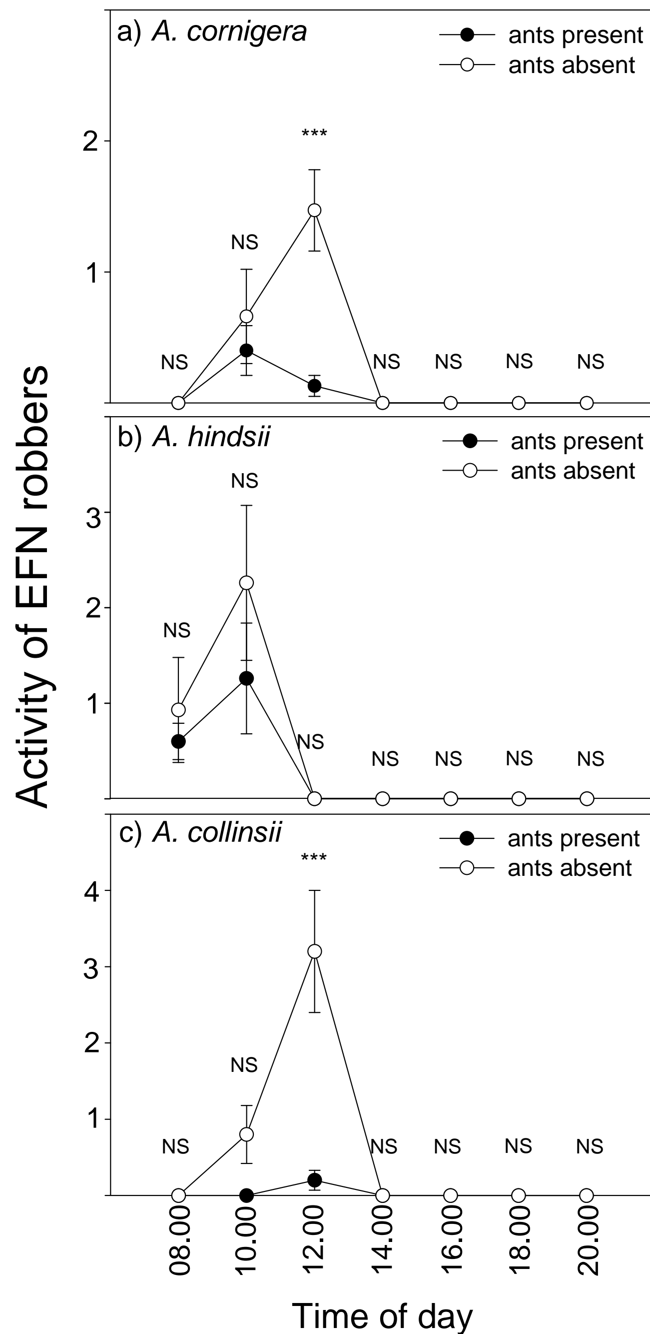


Fig. 22: Diel activity patterns of nectar robbers (*Frieseomelitta nigra.*) on canopies of three *Acacia* myrmecophytes, (a) *A. cornigera*, (b) *A. collinsii*, (c) *A. hindsii*. Activity of nectar robbers was quantified in presence (black circle) and absence (white circle) of resident *P. ferrugineus* ants. Asterisks indicate significant differences in the number of bee visits between branches with and without ants at a certain time (P < 0.05 according to Mann Whitney test), NS = non significant.

Variations in ant defence to hosts against herbivores and pathogens

1. Ant defence against herbivores:

Leaf damage was significantly affected by the plant species ($F_{1,16}$ = 25.65, P < 0.001, Two-way ANOVA) and by *P. ferrugineus* ant presence ($F_{1,16}$ = 28.31, P < 0.001, Two-way ANOVA). Although, a significant interaction between both factors ($F_{1,16}$ = 25.92, P < 0.001, Two-way ANOVA) indicated that plants of *A. cornigera* were strongly affected by herbivore damage under ant absence conditions, whereas plants of *A. hindsii* did not suffer an increase of herbivory when they were deprived from *P. ferrugineus* ants (Fig. 23a). Plant species and presence of ants had also a significant effect on EFN secretion (Two-way ANOVA: plant species: $F_{1,18}$ = 31.14, P < 0.001; ant presence: $F_{1,18}$ = 19.76, P < 0.001; interaction: $F_{1,18}$ = 4.97, P < 0.05) (Fig. 23b). As indicated by the significant interaction, the effect of *P. ferrugineus* ants on EFN secretion differed between the plant species, and symbiotic ants increased EFN production on average by 2.700 µg soluble solids g^{-1} leaf dry mass $24h^{-1}$ in *A. cornigera*, but only by 800 µg g^{-1} $24h^{-1}$ in *A. hindsii*. Symbiotic ants activate EFN secretion, although the effect depends on the host species.

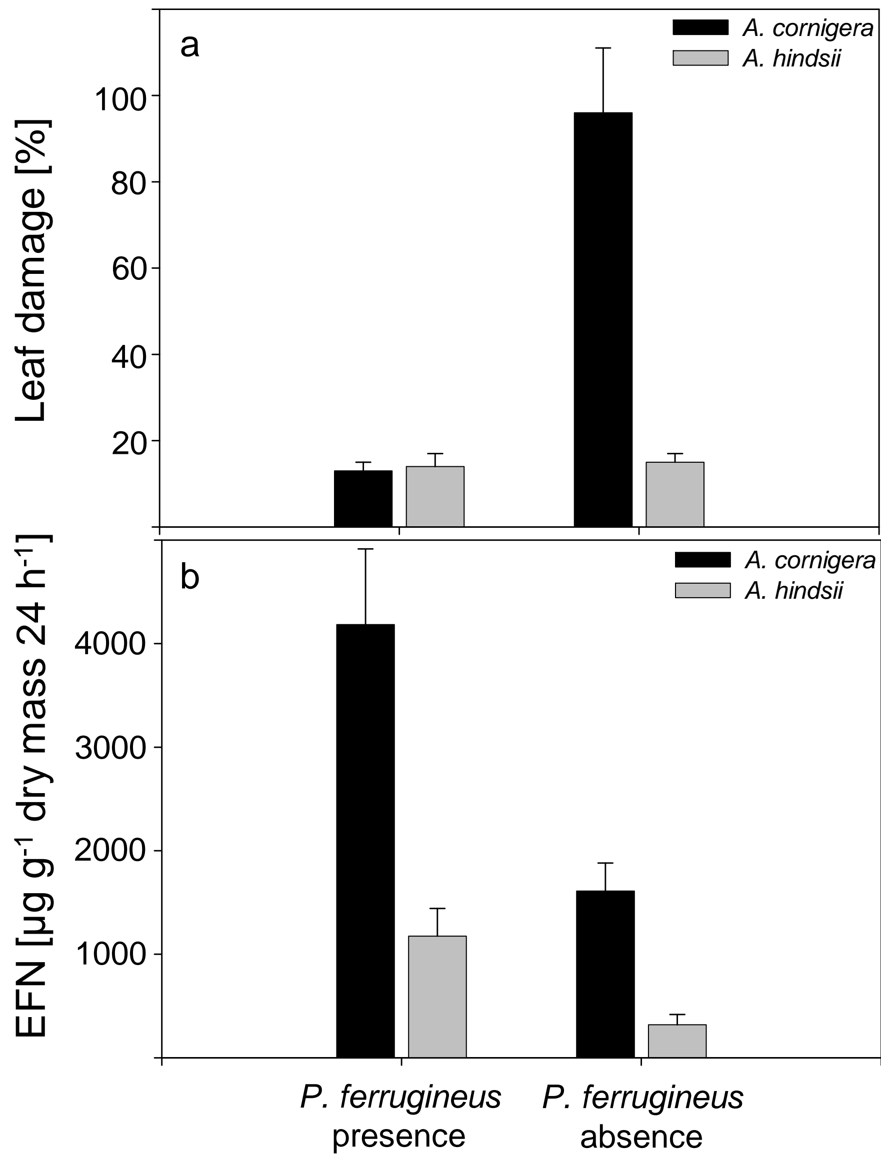


Fig. 23: Percentage of leaf herbivory in plants of *A. cornigera* and *A. hindsii* in presence and absence of the symbiotic ant *P. ferrugineus* (a), EFN secretion (µg of total soluble solids secreted per g leaf dry mass and per 24 h) in plants of *A. cornigera* and *A. hindsii* in presence and absence of the symbiotic ant *P. ferrugineus* (b).

2. <u>Ant defence against leaf pathogens</u>:

Defence of mutualist ants on leaf pathogen growth was observed for bacteria, although not for fungi. No significant effect was observed by ant species, ant presence on fungal abundance on leaves of *A. cornigera* (ant species: $F_{1,36} = 0.26$, $P > 0.05$; ant presence: $F_{1,36} = 1.15$, $P > 0.05$; ant species x ant presence: $F_{1,36} = 2.87$, $P > 0.05$; Two-way ANOVA) and *A. hindsii* (ant species: $F_{1,36} = 0.57$, $P > 0.05$; ant presence: $F_{1,36} = 1.91$, $P > 0.05$; ant species x ant presence: $F_{1,36} = 0.94$, $P > 0.05$; Two-way ANOVA). In contrast, bacteria abundance in leaves of *A. cornigera* and of *A. hindsii* was significantly affected by ant species (*A. cornigera*: $F_{1,36} = 7.45$, $P < 0.005$; *A. hindsii*: $F_{1,36} = 91.59$, $P < 0.001$; Two-way ANOVA) as well as by ant presence (*A. cornigera*: $F_{1,36} = 11.79$, $P < 0.005$; *A. hindsii*: $F_{1,36} = 7.53$, $P < 0.005$; Two-way ANOVA) (Fig. 24). A significant interaction ant species x ant presence, for both hosts (*A. cornigera*: $F_{1,36} = 4.81$, $P < 0.05$; *A. hindsii*: $F_{1,36} = 7.51$, $P < 0.005$; Two-way ANOVA), indicated that ant presence has a different effect on the bacteria abundance depending on the ant species. Presence of the symbiotic ant *P. ferrugineus* decreased significantly bacteria abundance for both plant hosts, whereas no differences were observed on bacteria abundance between treatments with and without presence of the parasitic ant (Fig. 24a, b), i.e., *P. gracilis* does not show an inhibitory effect on bacteria growth in leaves from both plant hosts.

Nevertheless, differences between both *Acacia* hosts were also observed. Although *P. ferrugineus* defended both *Acacia* against leaf bacteria, its defence was greater in *A. cornigera* than *A. hindsii*. These results agree with the differential defence of *P. ferrugineus* against herbivores to different *Acacia* hosts (see above). Furthermore, *A. cornigera* presented more leaf bacteria in plants inhabited by *P. gracilis* than in plants inhabited by *P. ferrugineus*, nevertheless an opposite result was observed for *A. hindsii*, in which bacteria abundance was low in plants inhabited by *P. gracilis* (Fig. 24a, b). On the other hand, bacteria diversity was

also significantly affected by ant species, but only for plants of *A. hindsii* (*A. hindsii*: $F_{1.36}$ = 34.49; P < 0.001; *A. cornigera*: $F_{1.36}$ = 3.39; P > 0.05; Two-way ANOVA) (Fig. 25a, b), in which diversity increased significantly in those plants inhabited by *P. gracilis* (Fig. 26). No significant ant presence effect (*A. hindsii*: $F_{1.36}$ = 0.33; P > 0.05; *A. cornigera*: $F_{1.36}$ = 2.34; P > 0.05; Two-way ANOVA) neither significant interaction of ant species x ant presence (*A. hindsii*: $F_{1.36}$ = 0.28; P > 0.05; *A. cornigera*: $F_{1.36}$ = 0.70; P > 0.05; Two-way ANOVA) was observed on bacteria diversity for both plant hosts.

3. <u>Volatile analysis of ants</u>: There were detected 13 VOCs for *P. ferrugineus* and 16 for *P. gracilis*, being seven compounds common for both ant species. Ants were dominated by alcohols, like hexanol, decanol, octanol, ethanediol, and fatty acids and derivatives, like hexanoic, octanoic, nonanoic, decanoic and benzoic acids (Table 26). Fatty acids contributed ca. 40% to the total VOCs emitted by the symbiotic ant *P. ferrugineus*, whereas they contribute ca. 20% to the total emitted by the parasite ant *P. gracilis*.

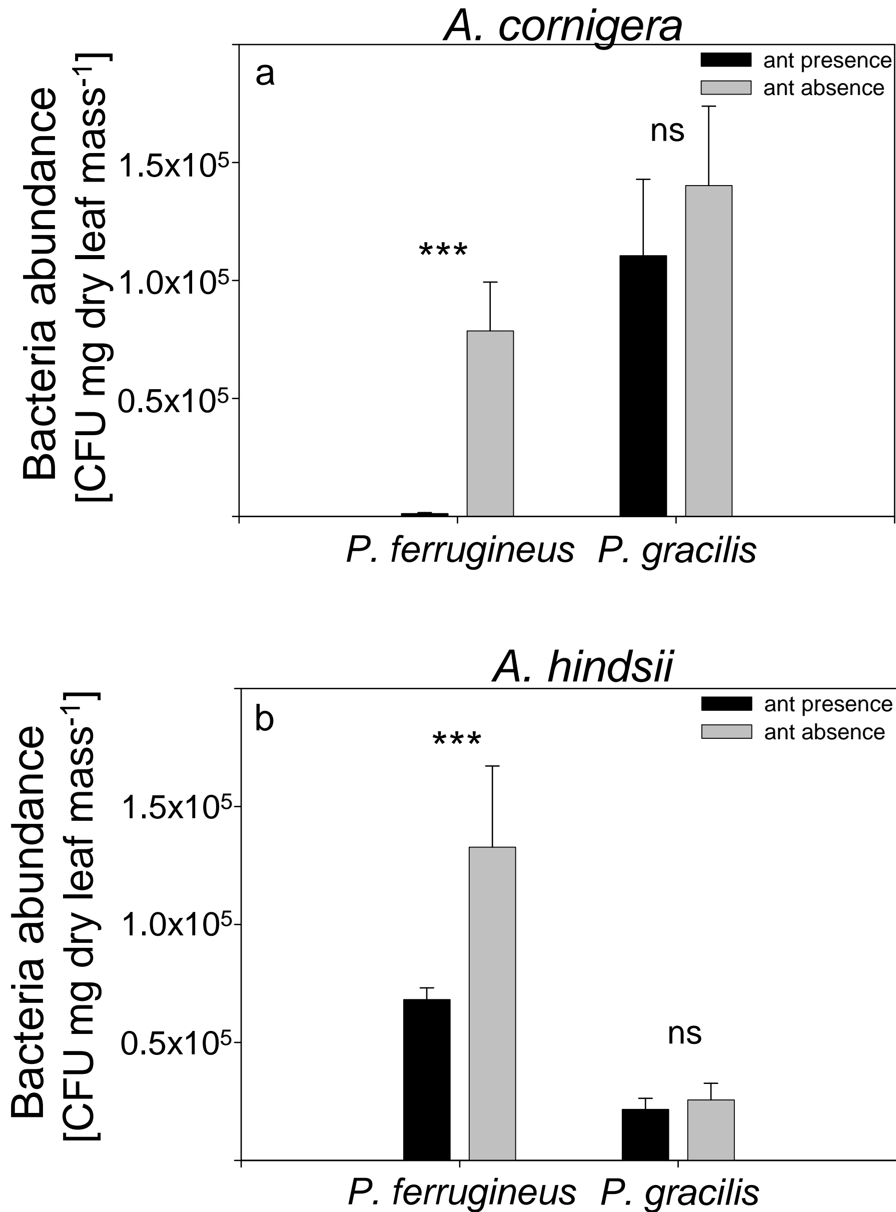


Fig. 24: Effects of presence and absence of *P. ferrugienus* (symbiotic ant) and *P. gracilis* (non-symbiotic ant) on bacteria abundance [CFU mg dry leaf mass^{-1}] in leaf samples of *A. cornigera* (a) and *A. hindsii* (b). Significance levels are indicated: ns P > 0.05, * P < 0.05, ** P < 0.01, and *** P < 0.001 (Two-way ANOVA for each plant species).

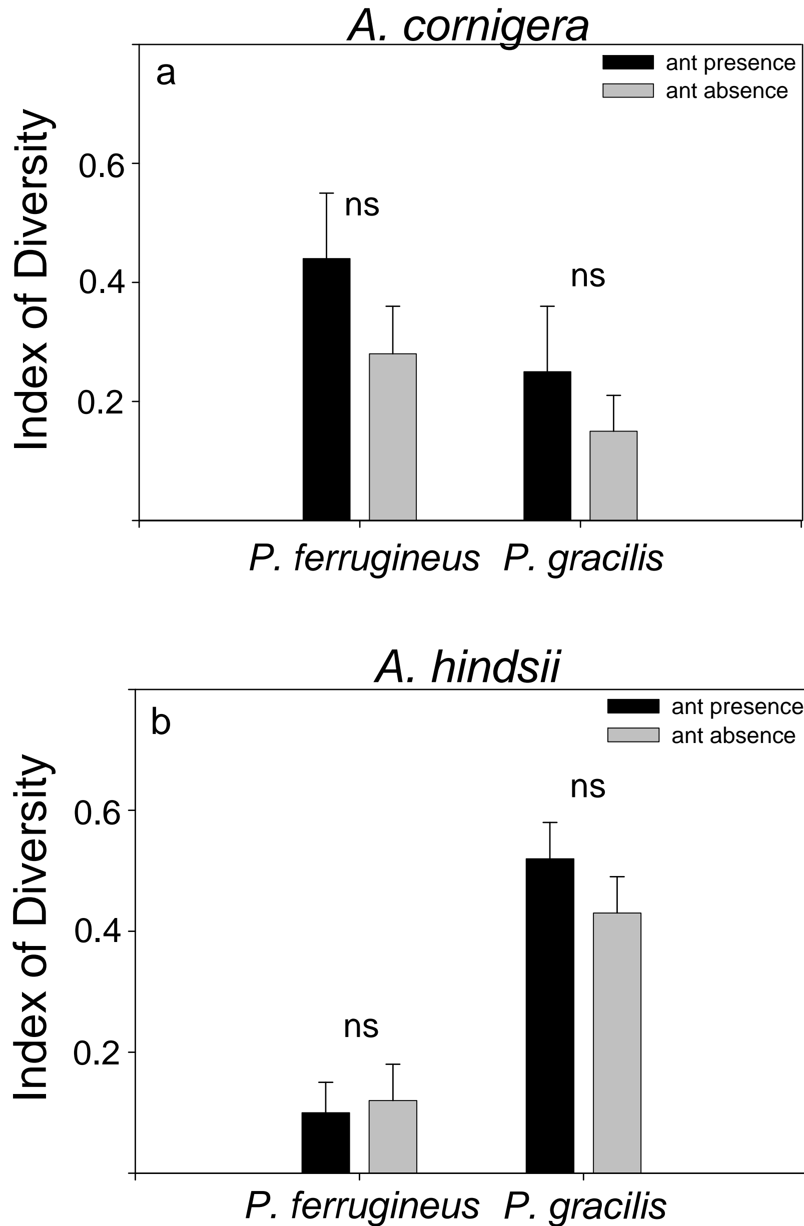


Fig. 25: Effects of presence and absence of *P. ferrugienus* (symbiotic ant) and *P. gracilis* (non-symbiotic ant) on bacteria diversity in leaf samples of *A. cornigera* (a) and *A. hindsii* (b). Significance levels are indicated: ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (Two-way ANOVA for each plant species).

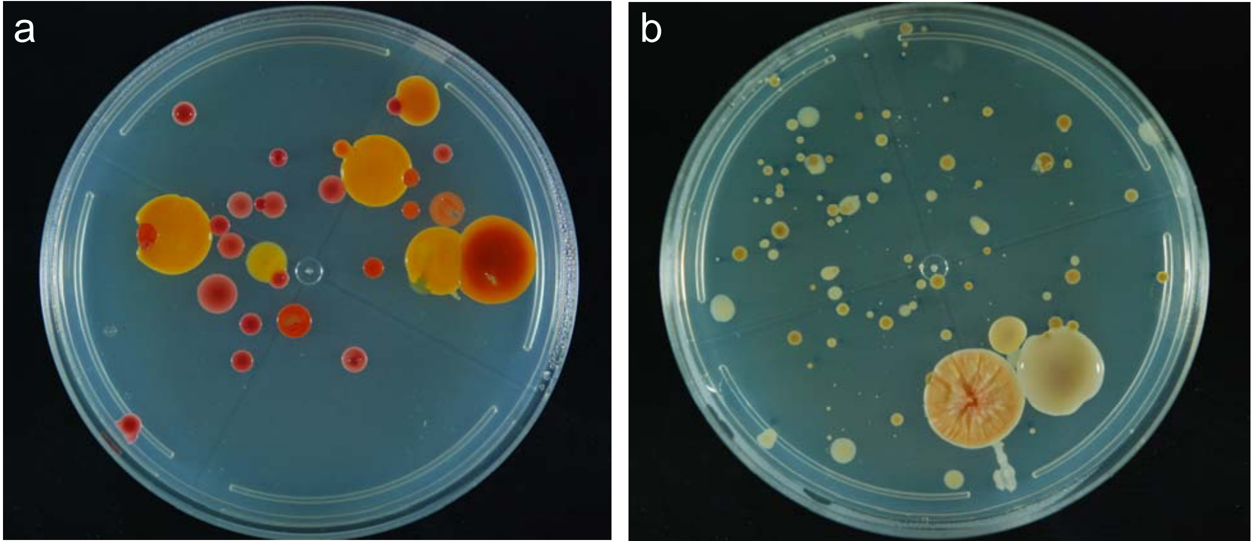


Fig. 26: Bacteria present in leaf samples of *Acacia* species. (a) Sample of *A. hindsii* inhabited by *P. gracilis*, (b) Sample of *A. hindsii* inhabited by *P. ferrugineus*.

Table 26: Volatiles emitted by *P. ferrugineus* and *P. gracilis* ants. RT indicates the retention time of each compound. Abundance of each compound is indicated, and was calculated from the integrated peak areas.

Compound	RT	*P. ferrugineus*	*P. gracilis*
Decane, 2,3,5,8-tetramethyl	5.34	9.70	
Hexanol	9.61		10.04
Acetic acid	13.39	18.89	20.48
Decanal	14.38		2.42
Propanoic acid	15.49		1.83
Octanol	15.8		1.83
2,3-butanadiol	16.71		4.04
Ethanediol	17.31		11.41
Hexanoic acid	20.85	8.40	3.66
Benzyl alcohol	21.43	4.16	
Hexanoic acid, 2-ethyl	22.28		2.9
Heptanoic acid	22.32	2.60	2.7
Octanoic acid	23.78	7.62	3.73
Ethanone	24.28	3.37	
Ethanol, 2-phenoxy	25.02		2.39
Nonanoic acid	25.12	5.22	2.39
Decanoic acid	26.48	6.40	2.86
Diethyl phthalate	28.22	25.20	14.13
Benzoic acid	29.37	8.92	
Dodecanoic acid	30.19		3.65
Butanamide	32.52	1.72	
Tetradecanoic acid	36.79	1.91	

Discussion

The goal of my study was to determine mechanisms that contribute to the specificity and the ecological stability of the *Acacia-Pseudomyrmex* mutualim. The mechanisms investigated concerned 1) the chemical ecology of EFN that is secreted by different *Acacia* species, 2) the defensive behaviour of *Pseudomyrmex* ants and 3) its relationship with the amounts of EFN that are provided by different myrmecophyte *Acacia* hosts. I found that *Acacia* EFN is 1) chemically highly adapted to the nutritive requirements of the symbiotic ant, *P. ferrugineus*, and 2) chemically protected from microbial infestation. Nevertheless, these chemical adaptations were only relevant on myrmecophyte plants, i.e., those *Acacia* species that are involved in obligate and specific mutualisms. Secreted quantities of myrmecophyte EFN and the short peak in its secretion affected the capacity of resident *P. ferrugineus* ants to protect the EFN from exploitation by nectar robbers. *P. ferrugineus* showed also an efficient defence against herbivores and leaf pathogens for two *Acacia* host species. Furthermore, the efficiency of the defence provided by *P. ferrugineus* to *Acacia* hosts was associated with the host plant's investment into rewards, that is, investment in rewards can determine the payoff received.

Attraction function of EFN

The composition of sugars and amino acids varied particularly between the two functionally different types of mutualisms. NMDS analysis demonstrated a separation of myrmecophyte species vs. non-myrmecophytes according to the amino acid composition of their EFN: the myrmecophyte, *A. hindsii,* and the non-myrmecophyte, *Prosopis juliflora*, turned out to be the most distant among the four investigated species (Fig. 6). Interestingly, these chemical distances mirror the phylogenetic relations: a phylogenetic reconstruction based on chloroplast DNA markers (Heil et al. 2004b) also revealed *A. hindsii* and *P. juliflora* to be most distantly related among the species tested here.

I found that the preferences to sugars and amino acids varied among ant species. Behavioural assays with obligate *Acacia* inhabitants (*Pseudomyrmex ferrugineus*) and non-symbiotic ants showed that AA composition affected ant preferences at high but not at low AA:sugar ratios. (Gonzalez-Teuber and Heil 2009a). Several studies have reported interspecific variability in ant preferences to amino acids (Lanza 1988; Lanza et al. 1993; Blüthgen and Fiedler 2004). Our results generally confirm these studies (Fig. 7), nevertheless, differences in ant behaviour were only evident when the relative concentration of single amino acids to sugars was high (1:50), i.e., at concentrations as found in *Acacia* EFN. In contrast, neither symbiotic nor non-symbiotic ants discriminated among artificial mixtures at low amino acid concentrations (1:1000). This result confirms the study by Lanza (1991), who showed that preferences of fire ants were most obvious when nectar mimics contained high levels of amino acids. Therefore, the results of the first part of my study support the general assumption that high concentrations of amino acids in nectar contribute notably to its taste (Gardener and Gillman 2002).

However, ant life history strongly affected whether and how ants responded to certain nectar components, suggesting that the preferences of ants to certain AAs vary according to their respective nutritive needs. AAs that affected the chemical grouping of myrmecophyte-EFNs vs. non-myrmecophyte-EFNs determined to a considerable part the observed behaviour of symbiotic and non-symbiotic ants (González-Teuber and Heil 2009a). As expected, the symbiotic ants specifically preferred the solution containing those four amino acids that are highly concentrated in the EFN of their host plant (*A. hindsii*). Furthermore, symbiotic ants were able to distinguish this specific solution (1:10-4AA) from other solutions (Fig. 8a,c), suggesting that not only AA concentration but also their number and detailed identity determines the preferences that are exhibited by symbiotic ants. By contrast, although non-symbiotic ants preferred the solution with eight amino acids in the first experiment, they did

not distinguish among nectar mimics that differed only in the number or exact concentration of AAs (Fig. 8b,d), while the identity of sugars had a strong and significant effect. Apparently, just the presence of amino acids in the nectar, but not their detailed identity, is important for generalist ants, while symbiotic ants are much more selective. Considering that non-symbiotic ants do not establish an obligate mutualism with plants, they must forage on different plant species, unlike symbiotic ants, which are constitutively nourished by one specific host. This different style of life of symbiotic and non-symbiotic ants affects their preferences and selectiveness with respect to detailed chemical composition of their food sources.

Our results also suggest that those four AAs that contributed most to separate myrmecophyte from non-myrmecophyte EFN and that significantly affected the behaviour of symbiotic ants are particularly important for the nutrition of these ants. Phenylalanine and proline appeared in much lower concentrations in EFN of the two non-myrmecophytes than in EFN of the myrmecophytes, which is in line with the very low concentrations of these two amino acids found in EFN of the non-myrmecophyte, *Macaranga tanarius* (Heil et al. 2000) and in other extrafloral nectars of non-myrmecophytic species (Baker et al. 1978; Inouye and Inouye 1980). These two amino acids were among those that most intensively contributed to the differentiation that NMDS revealed among the EFNs studied here. By contrast, high concentrations of phenylalanine and proline have also been reported for different floral nectars (Carter et al. 2006; Petanidou et al. 2006) and thus might be typical for more important types of nectar-mediated interactions. Phenylalanine is considered one of the ten essential amino acids for honeybees (Chapman 1983; Dafni and Kevan 1994), while proline is preferentially utilized by insect pollinators during the initial phases of insect flight (Micheu et al. 2000). For ants, comparable information is lacking and further physiological studies will be needed to determine the significance of specific amino acids for their metabolism. Thus,

although EFN of both myrmecophyte and non-myrmecophyte *Acacia* species fulfils nutritive functions, an attractive function appeared important only for the non-myrmecophytes, while EFN of myrmecophytes had a higher nutritional importance and appeared to be chemically adapted for nutritive needs of the symbiotic ants.

Protection of EFN from infestation

Freshly field-derived samples of myrmecophyte EFN were free of fungi, unlike the EFNs obtained from the non-myrmecophytes (González-Teuber et al. 2009). As the same remained true for myrmecophytes that had been deprived of their ants (personal observations), myrmecophyte EFN itself must contain compounds that serve its protection from microbial infestation. Furthermore, inhibitory effects against different fungal species were confirmed for the protein fraction of myrmecophyte EFN but not for its metabolite fraction (Table 25). High sugar concentrations might protect nectar from microbial growth (Buban et al., 2003) and secondary compounds have repeatedly been reported from floral nectars (González-Teuber and Heil 2009b). However, the last observation makes a significant role of secondary compounds or sugars in the antifungal activity of EFN highly unlikely and confirms the protective role proteins that are secreted into the EFN of myrmecophytes, as it has already been reported for floral nectar (Thornburg et al. 2003; Carter and Thornburg 2004; Nicholson and Thornburg 2007, see González-Teuber and Heil 2009b).

Indeed, EFN of myrmecophyte *Acacia* species possessed more proteins than the EFN of related non-myrmecophytes, both with respect to overall quantity and to the number of different proteins (Figs. 9-10). Although EFN proteins may serve ant nutrition, the results of the present study demonstrate that at least some of them have another function: the protection of nectar from microbial infection. Chitinase and β-1,3-glucanase proteins were identified (Tables 21-23) and also their functional activity in fresh EFN could be demonstrated (Fig.

15), which is the first description of pathogenesis-related (PR) enzymes in EFN (González-Teuber et al. 2009). A yeast assay underlined that chitinase activity as found in EFN of *Acacia cornigera* could effectively reduce microbial growth rates, whereas the lower activities as found in *A. farnesiana* did not suffice to cause a significant reduction in yeast development (Figs. 18-19).

Chitinases, peroxidases and β-1,3-glucanases are common enzymes in plant pathogen resistance (Van Loon 1999). Chitinases and β-1,3-glucanase exhibit an inhibitory activity against fungi and bacteria (Sela-Buurlage et al. 1993; Fung et al. 2002; Robert et al. 2002), whereas peroxidases normally function via the production of hydrogen peroxide, which then serves as the antimicrobial agent (Orozco-Cardenas and Ryan 1999; Mydlarz and Harvell 2007). In floral nectar of tobacco plants, superoxide dismutase activity and the generation of hydrogen peroxide inhibited microbial growth (Carter and Thornburg 2000).

Overall, PR-proteins made up a major part of the total protein fraction in EFN of myrmecophyte species, with glucanases, chitinases and thaumatin like-proteins being the most abundant classes. Chitinase and glucanase proteins together represented more than the 50 % of the total protein fraction in EFN. Other proteins identified were related to sugar hydrolysis, e.g. invertase (Roitsch and González 2004) and glycoside hydrolase (Zoran 2008). The identification of the invertase protein confirmed earlier results on the presence of this enzyme in *Acacia* EFN (Heil et al. 2005). These enzymes made up, however, a lower proportion, suggesting that the main function of proteins in *Acacia* EFN is related to its protection from microbial infestation.

Although non-myrmecophyte EFN also presented PR-enzyme activity, chitinases were the only active isoforms that could be found in non-myrmecophyte nectar when applying activity gels, whereas acidic and basic β-1,3-glucanases were almost absent from EFN of *A. farnesiana* and *Prosopis*, at least under the conditions studied (Figs. 16-17). Probably, this

absence of active glucanase isoforms explains the differences in the protection from fungi that we observed between EFNs of both functional plant groups: EFNs of non-myrmecophytes exhibited only chitinase activity although several in vitro experiments demonstrated that the antifungal effects of chitinases and β-1,3-glucanases are synergistically enhanced when both enzymes are present (Vogeli et al. 1988; Sela Buurlage et al. 1993; Lawrence et al. 1996; Anfoka and Buchenauer 1997).

In summary, the chemical composition of EFN turned out to be more complex than considered before (González-Teuber and Heil 2009b). The function of EFN components is not restricted to ant attraction but also comprises a protection from microbial infestation, which could be assigned to activities of PR-proteins, a compound class that has been not been reported in earlier studies. Moreover, EFN of myrmecophytes possessed several additional proteins whose identity and physiological functions still remain to be analyzed.

Temporal pattern in EFN secretion reduces exploitation by nectar robbers

Bees compete with the ant mutualists for EFN and the resident ants reduced the numbers of bee visits. The three *Acacia* myrmecophytes that I investigated here showed a diurnal EFN production with highest rates being secreted around noon. This finding agrees with previous reports for *A. hindsii* (Raine et al. 2002) and *A. mayana* (Raine et al. 2004), which underlines the high stability and reproducibility of this temporal pattern. The EFN production by all three species was quantitatively related to the activity of the resident *P. ferrugineus* ants, i.e., the maximal EFN production coincided with the highest ant activity (Fig. 21). We observed the highest activity of nectar robbers during the times of day with maximum EFN production rates, showing that the nectar robbers indeed compete with resident ants for EFN, as has already been reported for stingless bees (O'Dowd 1979) and certain flies (Heil et al. 2004a).

However, many more bees visited ant-free branches as compared to branches on which resident ants were active (Fig. 22), demonstrating that *P. ferrugineus* ants can defend ‘their’ EFN from *Frieseomelitta nigra*, the most abundant nectar robber that was identified in this study system. This defensive effect was significant only when EFN secretion rates were highest, both when comparing different times of the day and the different *Acacia* host species. Thus, defensive effects by *P. ferrugineus* were only evident at the time of the day with the highest ant activity and only for *A. cornigera* and *A. collinsii*, the two ‘high-reward’-species (Heil et al. 2009, in press), which were characterised by higher EFN secretion rates than was *A. hindsii*. Thus, the quantity of EFN and the short pulse in its secretion are two factors that affect the capacity of resident *P. ferrugineus* ants to protect EFN from nectar robbers.

Ant defence against herbivores and pathogens

P. ferrugineus defended its host plant effectively against herbivores and leaf bacteria; nevertheless, these defensive effects differed between the two *Acacia* species. For herbivores, the results indicate a similar trend as observed for the nectar robbers, that is, the ‚high-reward‘-host *A. cornigera* was much more strongly defended by its resident symbiotic ants than was *A. hindsii*. An efficient defence behaviour by *P. ferrugieneus* ants against herbivores has been previously documented (Raine et al., 2004; Clement et al. 2008) for some *Acacia* species. By contrast, the results of the present study represent the first report on an *Acacia*-ant that defends its host against bacterial infections.

Ant-exclusion experiments demonstrated that *Acacia* myrmecophytes quickly reduced EFN secretion when the symbiotic ant was missing and that the strength of this effect differed among the investigated myrmecophyte species (Fig. 23): The presence of *P. ferrugineus* workers increased EFN secretion more on *A. cornigera* than on *A. hindsii*. Most likely, such variations in EFN availability to symbiotic ants are associated with differences in ant

aggressiveness, and therefore, with differences in the defence quality provided by *P. ferrugineus* to its *Acacia* hosts. Differences among hosts in the production of EFN (or other rewards) may, thus, have important impacts on the general protective benefit that resident mutualist ants have for their host plant (Heil et al. 2009, in press).

Bacterial abundances decreased considerably when the plants were inhabited by symbiotic ants, while no such effect was observed on branches inhabited by the parasite, *P. gracilis* (Fig. 24). This ant-mediated defence against the infection by leaf bacteria represents a new function of the ants that was previously unknown. Letourneau (1998) was the first to report an anti-pathogen function of plant-ants and no further studies have been published on this topic since then. How does *P. ferrugineus* mediate this defensive effect? Both ant species emitted several fatty acids derivatives, which have been commonly associated with antimicrobial effects (Sá-Correia 1985; Bergsson et al. 2001; Hismiogullary et al. 2008). Nevertheless, the relative abundances of these VOCs were higher in workers of *P. ferrugineus* than of *P. gracilis*. It has been demonstrated that dodecanoic, decanoic, octanoic and hexanoic acids have effects on a wide range of pathogens, even against bacteria (Petschow et al. 1996; Hismiogullary et al. 2008). Similarly, benzoic acid and diethyl phthalate have been indicated as chemicals with high antimicrobial activity (Morris et al. 1979). Benzoic acid was present only in samples of *P. ferrugienus*, whereas diethyl phtalate was much more abundant in *P. ferrugineus* samples than in *P. gracilis*. This last suggests that fatty acids and derivatives, as VOCs emitted by ants, might contribute to the inhibitory effects that *P. ferrugineus* showed against bacteria on both *Acacia* hosts. Nevertheless, future studies are necessary to test antimicrobial effects of those chemical compounds at realistic concentrations on bacteria isolated from *Acacia* leaves, in order to determine chemical mechanisms underlying this defence against pathogens that is provided by the symbiotic ant, and thus, to understand an

ecologically relevant behaviour of symbiotic ants that contributes to the maintenance of the mutualism.

In summary, my results showed that the presence of the symbiotic ant, unlikely *P. gracilis*, significantly inhibited bacterial growth in leaves of *A. cornigera* and of *A. hindsii*, although the defence service was lower for *A. hindsii*. Thus, this ‚low-reward'-host, *A. hindsii,* received the least effective service by *P. ferrugineus*, both in terms of protection from nectar robber visits and in terms of the defence of the plant against herbivores and pathogenic bacteria. Apparently, symbiotic ants tend to reduce their defence service on those *Acacia* hosts that do not invest highly in ant rewards. Thus, defence provided by ants depends on the payoff received in terms of reward investments.

Conclusions

Myrmecophyte *Acacia* plants secrete EFN as a reward to nourish symbiotic ants. Detailed analyses of this EFN demonstrated that it is chemically adapted to the nutritional requirements of the symbiotic ants and, at the same time, protected from microbial attack. Specific amino acids contributed to the taste and attractiveness of nectars to symbiotic, but much less so to generalist ants, a result that illustrates how strongly the responses of ants to specific nectar components depend on their life style and, thus, on their nutritional requirements. Therefore, amino acids are a chemical component of nectar that likely can shape the structure of ant-plant mutualisms.

On the other hand, EFN of *Acacia* myrmecophytes, unlike that of non-myrmecophytes, turned out to be enzymatically protected from specific exploiters: an invertase keeps the EFN free of sucrose, and therefore unattractive for generalist ants (Heil et al. 2005), and PR-enzymes such as chitinases and glucanases protect EFN from microbial infestations (González-Teuber et al. 2009). Thus, *Acacia* plants employ biochemical strategies

to protect EFN not only from generalist ant exploitation but also from infesting microorganisms.

In parallel to these adaptations on the plant side, the symbiotic ant *P. ferrugineus*, unlike the parasite *P. gracilis*, also exhibits relevant ecological and physiological adaptations, which contribute to the maintenance of the mutualism. *P. ferrugineus* protected *Acacia* host plants effectively from different kinds of enemies and exploiters, that is, herbivores and leaf-infecting bacteria, and nectar robbers that compete with the resident ants for EFN. Nevertheless, the protective efficiency was highly associated with the amounts of rewards provided by the host plant: the host species that invests less in ant rewards, *A. hindsii*, received less defence by the symbiotic ants. The different defensive efficacy exhibited by the same species of symbiotic ant was confirmed for the three types of exploiters studied here, suggesting that *P. ferrugineus* tends to diminish, or cease, its defence service when it does not receive the respective pay-off by the host. On the other hand, the capacity of the mutualist ant to induce EFN secretion – that is not shared by the parasite (Heil et al. 2009, in press) - demonstrates that the plant host also can cease reward production when it does not receive the expected service. In summary, the results of the present study illustrate different chemical and ecological mechanisms that contribute to the specificity and stability of the *Acacia*-*Pseudomyrmex* interaction and, thus, prevent this mutualism from exploitation.

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CURRICULUM VITAE

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EDUCATION

Undergraduate University Education:

1998-2002:	Biologist, University of Concepción, Concepción, Chile.

Postgraduate University Education:

2003-2005:	Master in Botany, University of Concepción, Concepción, Chile.
Currently:	PhD student at University of Duisburg-Essen, Germany

AWARDS AND FELLOWSHIPS

2003: Best Student Prize (2002 generation), Facultad de Ciencias Naturales y Oceanográficas, University of Concepción, Chile.

2003: Master fellowship given by the Graduate School of the University of Concepción, Chile.

2004: Master fellowship given by the Millenium Center for Advanced Studies in Ecology and Research in Biodiversity (CMEB).

2006: PhD fellowship given by the DAAD (Deutscher Akademischer Austausch Dienst).

2008: ISCE's (International Society of Chemical Ecology) Student Travel Award.

RESEARCH EXPERIENCE

2002: Alternate Investigator in the project "Estudio monográfico de las especies con problemas de conservación en el área del Proyecto Ralco (Endesa, CR-326) ("Monographic study of species with conservation problems in the area of Ralco, southern Chile"), Endesa, CR-326. Main Investigator: Dr. Roberto Rodríguez Ríos.

2005: Research Assistant (RA) at the ECOBIOSIS Laboratory, Department of Botany, University of Concepción, Concepción, Chile. FONDECYT project Nº 1030821: "Reclutamiento de especies en ambientes de alta-montaña: un aproximación experimental a la asociación de especies a plantas en cojín" (Species recruitment in high-mountain environments: an experimental approximation to the association with cushion plants: 2003-2006). Employer: Dr. Lohengrin Cavieres.

2006: Scientific in charge of the project "Micropropagation of *Eucalyptus spp*". Employer: Bioforest - Arauco Forest S.A.

PUBLICATIONS

Technical Books:

Rodríguez Ríos, R., Elissetche, J.P. & **M. González-Teuber**. "Monografía Guindo Santo (*Eucryphia glutinosa*): Especie con problemas de conservación en Chile" ("Monography of Guindo Santo (*Eucryphia glutinosa*): A species with conservation problems in Chile"). 2004. Endesa. Santiago, Chile.

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PRESENTATIONS AT SCIENTIFIC MEETINGS

González-Teuber, M., C. Quiroz, M.T.K. Arroyo & L.A. Cavieres, "Relación entre la forma y peso de semillas con la formación de banco de semillas persistentes: una reevaluación" (Relationship between seed shape and seed size with the formation of a persistent seed bank: a reevaluation). XIII Reunión Anual de la Sociedad de Botánica de Chile. September 2001, La Serena, Chile.

González-Teuber, M., C. Quiroz, M.T.K. Arroyo & L.A. Cavieres, "Evaluando la relación entre longevidad y formación de banco de semillas persistentes en especies altoandinas" (Evaluating the relationship between longevity and persistent seed bank formation in high Andean species). XLIV Reunión Anual de la Sociedad de Biología de Chile. November 2001, Pucón, Chile.

González-Teuber, M., A. Jiménez & L.A. Cavieres, "Diversidad de la flora leñosa en la Reserva Nacional Ralco: ¿se conserva lo que se debe conservar? (Diversity of the woody flora in the Ralco National Reserve: do we conserve what we should?). VIII Congreso Latinoamericano de Botánica. October 2002, Cartagena de Indias, Colombia.

González-Teuber, M. & E.Gianoli, "Efectos maternos genéticos y ambientales sobre las respuestas morfológicas de I*pomoea purpurea* frente a la disponibilidad de un soporte físico" (Maternal genetic and environmental effects on morphological responses of *Ipomoea purpurea* to the availability of physical support). XXIX Jornadas Argentinas de Botánica, XV Reunión Anual de la Sociedad de Botánica de Chile. October 2003, San Luis, Argentina.

González-Teuber, M. & E. Gianoli, "Plasticidad fenotípica en tres poblaciones de C*onvolvulus chilensis* en respuesta a condiciones de sequía" (Phenotypic plasticity in three populations of *Convolvulus chilensis* in response to drought conditions). XLVI Reunión Anual de la Sociedad de Biología de Chile. November 2003, Puyehue, Chile.

González-Teuber, M., L.A. Bravo, L.A. Cavieres & L.J. Corcuera, "Intolerancia a la desecación en semillas del ecotipo antártico de *Colobanthus quitensis* (Caryophyllaceae)" (Intolerance to desiccation in seeds of the antarctic ecotype of *Colobanthus quitensis* (Caryophyllaceae)). XLVII Reunión Anual de la Sociedad de Biología de Chile. November 2004, Pucón, Chile.

González-Teuber, M. & E.Gianoli, "Tolerancia en respuesta al daño en dos poblaciones de *Convolvulus chilensis* (Convolvulaceae)" (Tolerance in response to damage in two populations of *Convolvulus chilensis* (Convolvulaceae)). II Reunión Binacional de Ecología. November 2004, Mendoza, Argentina.

González-Teuber M., Suárez L.H., Cavieres L.A. & E. Gianoli, "Hábito trepador como estrategia de escape a la herbivoría en *Convolvulus chilensis* (Convolvulaceae)" (Climbing habit as an strategy to escape from herbivores in *Convolvulus chilensis* (Convolvulaceae)). XLVIII Reunión Anual de la Sociedad de Biología de Chile. November 2005, Pucón, Chile.

Segovia R.A., **González-Teuber M.** & E. Gianoli, "Decisión de oviposición y desempeño de la descendencia en *Megacerus eulophus* (Coleoptera: Bruchidae): efectos de la calidad del hospedero y dieta maternal" (Oviposition decisions and offspring performance in *Megacerus eulophus* (Coleoptera: Bruchidae): effects host quality and maternal diet). XLIX Reunión Anual de la Sociedad de Biología de Chile. November 2006, Pucón, Chile.

Gianoli E., Suárez L.H., Quezada I.M., Molina-Montenegro M.A. & **M. González-Teuber**, "Simulated herbivory constrains plant phenotypic plasticity to abiotic factors in three *Convolvulus* species". 91 Annual Meeting of the Ecological Society of America. August 2006, Memphis, USA.

González-Teuber, M. & M. Heil, "Components of extrafloral nectar (EFN): function in ant attraction and pathogen defence". 25th Anniversary Meeting of the International Society of Chemical Ecology. August 2008, Penn State, USA.

González-Teuber, M., M.J. Pozo & M. Heil, "Antifungal effects of extrafloral nectar (EFN)". 5th Meeting of the IOBC - Induced Resistance in Plants against Insects and Diseases. May 2009, Granada, Spain.

RESEARCH STAYS

- Max Planck Institute for Chemical Ecology, Jena, Germany (1 month).
- CINVESTAV (Centro de Investigación y de Estudios Avanzados), Campus Guanajuato, Mexico (5 weeks).
- Estación Experimental del Zaidín, Granada, Spain (1 month).

INVITED LECTURE

"Attractive and defensive functions of extrafloral nectar (EFN)". Estación Experimental del Zaidín, Granada, Spain.

LANGUAGES

- Spanish (native language)
- English (spoken and written)
- German (spoken and written)

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 6 der Promotionsordnung der Math.-Nat.-Fachbereiche zur Erlangung des Dr. rer. Nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe.

Essen, den 20. Juli 2009 ________________________

Unterschrift des/r Doktoranden/in

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 7 der Promotionsordnung der Math.-Nat.-Fachbereiche zur Erlangung des Dr. rer. Nat., dass ich das Arbeitgebiet, dem das Thema „Mechanisms enabling specific plant-ant mutualisms: *Acacia-Pseudomyrmex* as a model system“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Marcia González-Teuber befürworte.

Essen, den 20. Juli 2009 ________________________

Unterschrift eines Mitglieds der Universität Duisburg-Essen

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 8 der Promotionsordnung der Math.-Nat.-Fachbereiche zur Erlangung des Dr. rer. Nat., dass ich keinen anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner andern Fakultät abgelehnt worden ist.

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