Strategies against herbivory in the carnivorous plant *Nepenthes × ventrata*

Dissertation

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I. Introduction

A. Plant stress ecology and plant defense strategies

Plants are sessile autotrophic organisms. These two characteristics define and constrain their role in ecosystems. Because they are autotrophs, plants are able to use an abiotic source of energy, i.e. light, to produce complex organic compounds. Being sessile means that their physiological functioning is fully dependent on their immediate environment. Their survival is conditional on optimal temperature, humidity, light intensity and availability of nutrients around them, and they cannot move when conditions are not optimal. In this regard, Ingestad, T. (1971) describes key aspects of plant resource requirements. (1) All necessary mineral nutrients are present in the plant in optimal proportions. (2) The nitrogen sources NH_4^+ and NO_3^- are present in the nutrient solution (at the root surface) in an optimal ratio. (3) Total concentration in the solution is optimal.

However, bioavailability of nutrients is relevant. If the resources are not present in the way the plant can make use of them, it is as if that resource did not exist, since the plant cannot assimilate it. The alkalinity or acidity of substrate is determinant for the general bioavailability of nutrients. (Rhodes, D., & Nadolksa-Orczk, A. 2001). There is also abiotic stress caused by population competition (McNickle, G. G., & Dybzinski, R. 2013).

Nutrient requirements are dynamic. They vary between species, between individuals from the same species, and even within the same individual during its lifecycle (Gourley, C. J. P., et al., 1994). Any deviation from these optimal conditions at a given time can generate stress. Thus, the term plant stress is used when there is a substantial and detrimental effect on the physiological functions of the plant. Practically speaking, the term “stress” can be used to describe a situation outside of plant tolerance capability that results in tissue damage, an alteration of plant metabolism, and eventually a negative effect on plant fitness (Rhodes, D., & Nadolska-Orczyk, A. 2001).

Biotic interactions add a level of complexity to these abiotic resource requirements. Many plants require interactions with animals, often insects, for fertilization and seed dispersal. Biological stressors can create plant stress, mostly by damaging plant tissues for their own nutrition. Sources of biotic stress can be herbivores (mammals, reptiles, insects, and nematodes), sap feeders (insects), or pathogens (bacteria, viruses, and fungi).

On this battlefield, plants are not unprotected and have developed efficient strategies to fight against the attacks. In particular, the plant defenses against insect herbivores consist of two strategies, direct and indirect plant defense (Hammerschmidt, R., & Schultz, J. C. 1996). Direct plant defense is a set of features that directly result in a negative impact on the attacker´s fitness (Mithöfer, A., & Boland, W. 2012). Plants can feature a set of morphological modification as physical barriers and/or produce chemical compounds called secondary or specialized metabolites, whose function is to repel, deter, and/or be toxic to herbivores (Belete, T. 2018; Mithöfer, A. et al., 2009). Direct plant defense can also be either constitutive or induced. Zandt, P. A. V. (2007) summarizes constitutive plant defenses as always present, and because they are maintained even in undamaged plants, they are thought to be costlier than induced defenses (Agrawal, A. A., & Karban, R. 1999, Karban, R., & Baldwin, I. T. 1997).

The best examples of morphological constitutive plant defense are physical barriers e.g. thorns, hooks, and trichrome and modified leaves, among other elements (Liu, H. et al., 2017). Chemical compounds serving as constitutive defense are called phytoanticipins. Such stored compounds are often present in the form of inactive glycosylates in the plant tissue and are activated by β-glucosidase during herbivory. This triggers the release of several defensive aglycones. Examples of phytoanticipins are glucosinolates and benzoxazinoids (Belete, T. 2018; Mithöfer, A., & Boland, W. 2012).

In comparison, inducible plant defenses consist mainly of a set of biochemical reactions and compounds that plants produce and release only upon attack. This classification also encompasses the production of secondary metabolites, the phytoalexins. These chemical compounds are generally designed to fight microbes and insects. They are quickly produced in the area under attack. Examples of such compounds are (iso)flavonoids, terpenoids, alkaloids, etc. (Belete, T. 2018).

Another important element of inducible plant defense are pathogenesis related proteins (PR proteins). These proteins are usually absent or found only in very low concentrations, but they are produced in greater quantity upon attack. They can be found in cell walls and in organelle lining. Their induction is mediated mainly by the phytohormones salicylic acid (SA), ethylene (Et), and jasmonic acid (JA) and peptide signals such as systemin (Prasannath, K. 2017). Similarly, protease inhibitors (PIs) affect insect herbivores by impairing digestion. As a consequence, insects cannot assimilate nutrients from the ingested plant tissue (Mithöfer, A., & Boland, W. 2012).

Inducible defense mechanisms can also be physical. A good example is the hardening of plant coatings induced by herbivory, which results in tougher and therefore less palatable plant tissue (Hochuli, D. F. 1996). Similarly, the accumulation of non-organic elements such as calcium oxalates, silica crystals, etc. acts in the same way, causing a significant reduction in larval growth and an increase in herbivorous larvae mortality. Their main function is to act as an abrasive agent and they might interfere with nutrient absorption in chewing insects (Ye, M. et al., 2013; Massey, F. P. et al., 2007; Korth, K. L. et al., 2006; McNaughton, S. J., & Tarrants, J. L. 1983).

In comparison, indirect plant defense also aims at negatively affecting the attacker´s fitness through the involvement of a third trophic level: the attacker´s natural enemies (Kessler, A., & Balwin, I. T. 2001; Arimura, G. I. et al., 2009). As with direct plant defense, it can be constitutive or induced. Induced indirect plant defense consists in recruiting predators or parasitoids of insects upon herbivory. It usually involves the production of volatile organic compounds (VOCs). For example, cotton (Sobhy, I. S. et al., 2015), corn (D'Alessandro, M. et al., 2009), and wild tobacco (Kessler, A., & Baldwin, I. T. 2002) plants produce VOCs to attract parasitoid wasps. Wasps lay their eggs on the herbivore body. When they hatch, wasp larvae feed on the herbivore that carried them (De Moraes, C. M. et al., 1998).

Plants can also attract generalist predators such as ants by producing a food reward. Such a reward often consists in extrafloral nectar (EFN), a sugary solution secreted by specialized glands located outside of flowers. The production of EFN can be induced by herbivory, attract opportunistic or constitutive ants, and result in ants attacking herbivores and patrolling the plant even in the absence of herbivory (Heil, M. 2008).

B. Molecular regulators of stress tolerance strategy.

At least five physiological stages are required for generating a tolerance response to stress. In general, these are: stress perception, signal transduction, gene expression, protein biosynthesis, and biochemical responses. These physiological stages are complex, and each involves a molecular machinery with intricate steps in between (Hrmova, M., & Hussain, S. S. 2021; Singh, K., & Chandra, A. 2021). Among the group of molecules having a signal transduction function are the so-called plant hormones or phytohormones (Meraj, T. A. et al., 2020). Plants use phytohormones as the axis of metabolic logistics. Hence, plant stress responses have their origins from physiological imbalance and are mediated by phytohormones. Thus far, cytokinins, auxins, gibberellins, brassinosteroids, abscisic acid, jasmonates, salicylic acid, ethylene, polyamines and strigolactones are phytohormones that have been identified.

Plant hormones are involved in many physiological processes as command molecules and transduction signals. They are complementary and work in combination, activate as well as deactivate plant defense strategies. Therefore, phytohormones play a leading role in mediating stress tolerance. In the case of abiotic stress like drought, salinity, cold and heat, heavy metals, and osmotic stress, JA, SA, ABA, and Et are involved (Zhao, B. et al., 2021; Zhao, P. et al., 2017). Within this group of hormones, ABA particularly stands out since it also regulates plant development processes such as seed development, dormancy, germination, and stomatal movement (Fujita, M. et al., 2006). Furthermore, under biotic stress circumstances, the same four hormones (JA, SA, ABA, and Et) are involved and have been conferred regulatory properties for resistance (Zhao, B. et al., 2021).

Resistance to herbivorous attack is coordinated primarily by two modes of actions, one lead by the JA-Et complex and the other led by the SA-ABA complex. Within the SA-ABA group, SA exerts a positive action on ABA (Zhao, B. et al., 2021), but not *vice versa* (Audenaert, K. et al., 2002). In contrast, ABA does not have a positive influence on JA´s mode of action (Anderson, J. P. et al., 2004). Both SA and JA are antagonistic (Zhao, B. et al., 2021). Therefore, it has been established that ABA by itself does not confer resistance to herbivorous attack (Fujita, M. et al., 2006; Mauch-Mani, B., & Mauch, F. 2005).

Now, in biotic and abiotic stress situations, ABA has a negative effect on JA (Anderson, J. P. et al., 2004). Therefore, it is reasonable to assume that ABA is affected as a consequence of the activation of the SA-ABA complex, and it has been established that ABA dominates the abiotic response of the plant.

C. Plant carnivory syndrome, *Nepenthes × ventrata*.

Under non-nutrient-limited conditions and from the plant's perspective, the plant-insect interaction has two scenarios, one positive and one negative. From the beneficial aspect, an insect's ecological functions include seed dispersal, pollination, and indirect plant defense. These are examples of ecological services that insects can provide to the plant. From the same plant perspective, the negative aspect of this interaction is the insect's ecological function as consumer, attacking the plant in the form of herbivory.

It is inevitable that plants suffer from abiotic stress. A good example of this is the limit of nutrients in the substrate. World geography is not homogeneous, and this heterogeneity has a direct impact on the life forms of organisms, as well as on their adaptation, survival, and evolution processes. Places with little availability of nutrients are distributed throughout the planet, but plants have been able to colonize a diversity of inhospitable environments.

The nutrient-limitation topic is most often discussed in association with environments that harbor low amounts of biomass, low diversity of organisms, or adverse environments. Most iconic adverse environments associated with these circumstances are wetlands, deserts,

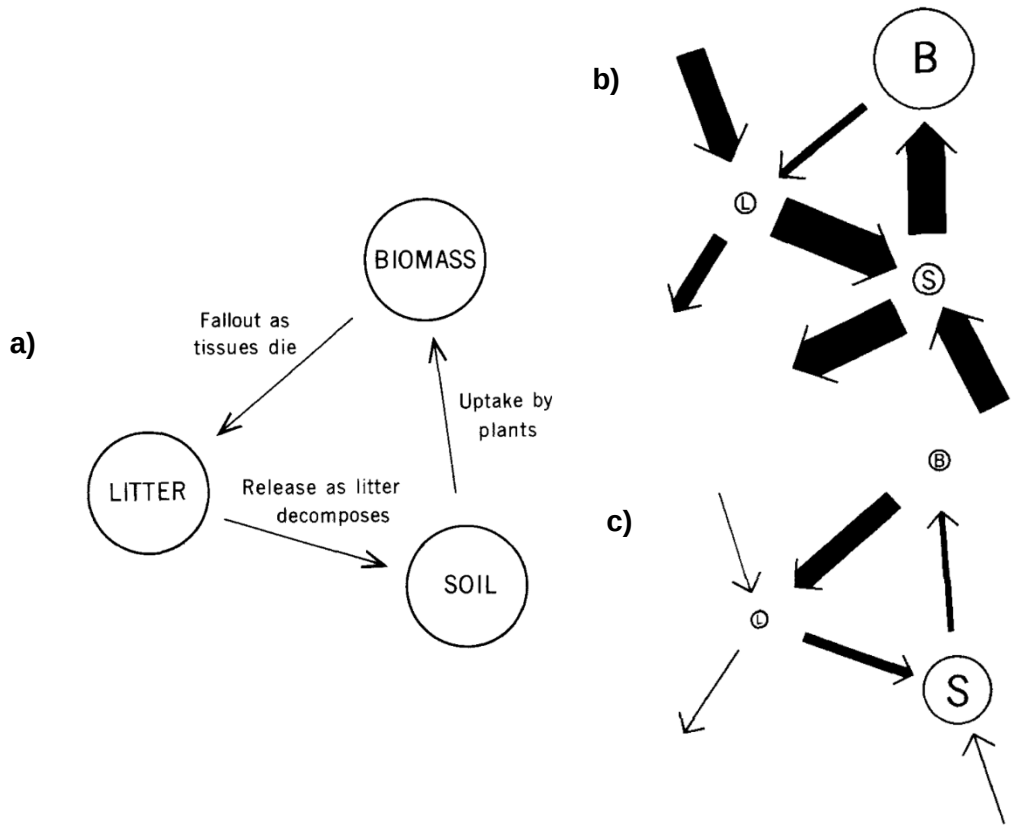

***Figure 1**. Open three-compartment model for hypothetical nutrient circulation in ecosystems, expressed in proportions; Nutrient amount stored in a compartment represented by circles sizes; quantity of nutrient flow in arrows widths; inside circles biomass represented by B, litter represented by L, soil represented by S; general mineral cycle mode in **a)**, biomass nutrients fallout as tissue die, litter nutrients are release as litter decomposes, soil nutrients are absorbed by plants; Nutrient cycling exemplifications in forest ecosystem in **b)** and in desert ecosystem **c)**. Adapted from Gersmehl, P. J. (1976).*

tundra, and glaciers. However, these assumptions are not necessarily the rule and do not apply to tropical rainforests.

We often associate the high rate of biomass production and the exuberance of species as indicators of forest's soil nutrient richness. However, in the case of forests, nutrient richness is not stored in their substrates. Tropical forest's soils are among the poorest in nutrients. This is due to the fact that nutrient deposits in tropical forests are widely distributed in the complex ecosystem's network and its food chain, while the nutrients in deserts are, to a large extent, stored in the substrate. This condition can be appreciated with the diagram created by Gersmehl (**Figure 1**) in which he shows the inter-relationships between nutrients storage and its flows in ecosystems.

There is also the limitation of nutrients due to soil pH. Each nutrient has a different pH-dependent availability (Roques, S. et al., 2013). However, there are certain general trends. Nitrogen, phosphorus, potassium, sulfur, calcium, and magnesium have an availability range that goes from very slightly acidic to slightly alkaline (6.5-8.0). On the other hand, nutrients such as iron, manganese, boron, copper, and zinc are available in major proportion from the soil at acidic pH (5.0-6.5). Molybdenum is somehow an exception, having a wide availability range, from slightly acid (6.5) to strongly alkaline conditions (10.0) in the soil. Nitrogen, phosphorous, and potassium are best known as NPK, the primary nutrients for plant fertilization and the essential and limiting nutrients for plant development (Van Duivenbooden, N., et al., 1995). Specifically, these nutrients are very important due to the key role they play in the production of macromolecules, plant structure, and storage energy, among other functions (Ray, K. et al., 2019).

When there is no nutritional limitation that prevents plant development, plants have the capacity to cope with the ups and downs of biotic and other abiotic stress. In the best case scenario, plants can grow, develop, and reproduce. Even under herbivore attack, plants in no-nutrient-limitation situations have the plasticity to invest in and develop complex and costly defense strategies, without fitness being compromised.

As mentioned above, plants have successfully colonized diverse environments and adapted to the limiting factors offered by each habitat. A good example of this resilience and ability to adapt to nutrient-poor environments is observed in the carnivory syndrome in the plant kingdom. The carnivory syndrome arises as a strategy to respond to a limited nutrient supply given plants' default need of nutrients for multiple physiological and structural functions. For example, *Dionaea*, *Drosera*, *Utricularia*, and *Nepenthes* all share this feature that makes them unique in the plant kingdom.

Their uniqueness in the plant kingdom does not exempt them from being attacked by biological stressors, however. In other words, carnivorous plants must also simultaneously resist biological attack. It is still not known how carnivorous plants are able to resist attack, such as herbivory, by biological aggressors. This will be addressed later in this thesis.

Carnivorous plants are distributed around the globe except for Antarctica. However, there is a higher probability of finding them in certain regions on the planet. Taking the number of species per 10,000 km^2 as a parameter of measurement, Barthlott, W. et al., (2004) maps the distribution of carnivorous plants on the globe (Figure 2). As can be seen from this map, tropical regions have a quite high probability to harbor carnivorous plant species. Within

these regions, the area that shows the highest diversity of carnivorous species is Southeast Asia.

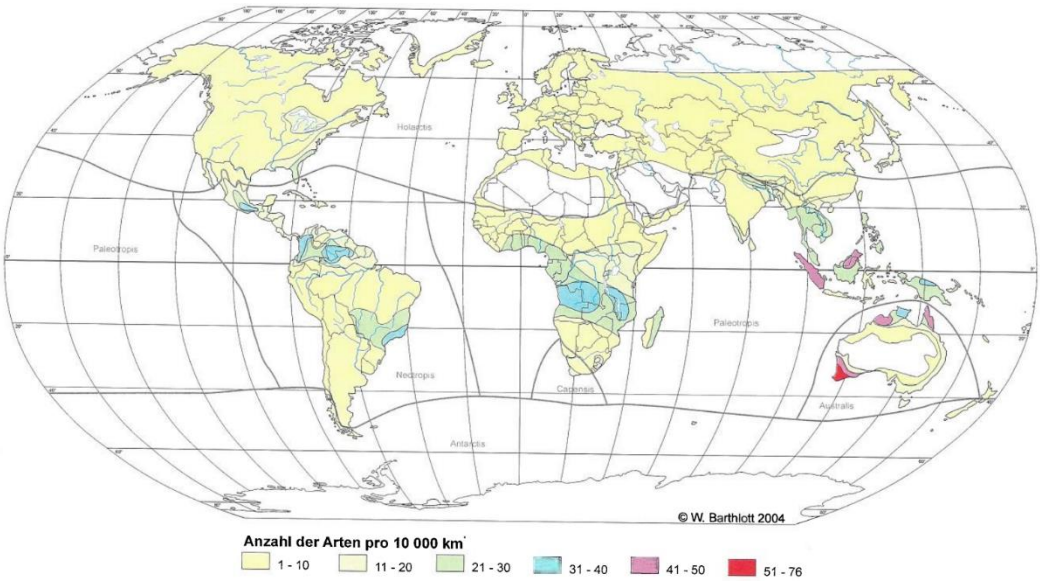

Figure 2. *Distribution of carnivorous plants in the world according to Barthlott, W. et al., (2004)*

Carnivory in the plant kingdom occurs when there is an exception to the rule in the food chain, a reverse energy flow, where the producer has the ability to feed off the consumer. This particular feature, this exception to the rule in the plant kingdom, is the research centerpiece of this investigative work.

The conventional idea is that all plant species take their nutrients in through the roots and their energy in through the leaves. But for every rule there are exceptions. The carnivorous syndrome in the plant kingdom is one of many strategies addressing the need for nutrient assimilation in poor-nutrient substrates (Adamec, L. 1997).

According to Juniper, B. E. et al., (1989), there are many prominent ecological features of carnivorous plants. (1) Carnivorous plants have a weak root system, for example most *Drosera*, *Dionaea*, *Pinguicula*, *Cephalotus*, and most *Nepenthes* species. (2) They are perennial plants with variability in habits, life forms, and longevity, often forming huge clonal colonies by stolons or rhizomes. (3) They are generally tolerant of low-nutrient environments. (4) Carnivory confers an advantage in a poor-nutrient habitat, with insect-scavenging properties at least temporarily offering an advantage. (5) They are generally intolerant of low-light conditions (most *Drosera* species), but there are also examples that do not follow this rule. (6) They are tolerant of temporary or permanent water logging. Unlike many other plants, also, carnivorous plants do not have a mycorrhizal association with their roots (MacDougal, D. T. 1899; Peyronel, B. 1932).

Currently, the following families are the most iconic for representing carnivorous or, to be more exact, insectivorous plants: *Droseraceae*, *Nepenthaceae*, *Drosophyllaceae*, *Cephalotaceae*, *Sarraceniaceae*, *Roridulaceae*, *Lentibulariaceae*, and *Byblidaceae*.

However, the discovery of new species of plants that meet the characteristics of the carnivorous syndrome continues (Lin, Q. et al., 2021; Shaw, P. J., & Shackleton, K. 2011).

As more carnivorous species are discovered, progress has also been made in the understanding of these species' intrinsic processes, including physiological, ecological, their trophic chains, mechanisms of attraction and capture of prey, and phylogeny. This knowledge has facilitated the delineation of the carnivory concept, particularly in plant species that do not exhibit an intrinsic nutrient allocation strategy but share some similarities with carnivorous plants.

The original concept of plant carnivory proposed by Givnish, T. J. et al., (1984) has undergone some adaptations over years due to deeper insights into the syndrome´s characteristics. The most recent concept has been proposed by Ellison, A. M., & Adamec, L. (2018). It brings together the carnivorous plants' characteristics in greater detail. According to this concept, a carnivorous plant must meet these five elements to classify it as such: (1) capturing or trapping prey in specialized, usually attractive, traps; (2) killing the captured prey; (3) digesting the prey; (4) absorbing the metabolites (nutrients) from the killed and digested prey; (5) and use of these metabolites for its own fitness.

Carnivorous plants can be classified into two large groups based on their insect capture strategy: those using active, moving traps based on electric signaling, and those using passive traps without any movements. *Dionaea* and *Drosera* are the typical species examples that belong to the first group. In contrast, *Nepenthes*, *Cephalotus*, and *Sarracenia* are the most distinctive examples in the second group.

The pitfall trap, or pitcher, of the *Nepenthe*s is a metamorphosed leaf. The conventional leaf lamina, or blade, is turned into a pitcher for catching prey, the petiole into a tendril to climb, and the conventional leaf base into a basal leaf-derived leaf blade to ensure photosynthesis (Fleischmann, A. et al., 2018; Owen J, T. P., & Lennon, K. A. 1999). The insects fall into the pitcher due to the slippery surface of the trap opening causing them to drown. Inside the pitcher digestive enzymes are produced (Thorogood, C. J. et al., 2018; Juniper, B. E. et al., 1989) (**Figure 3**).

The pitcher can be divided into three zones: (1) the opening in the upper part, called "peristome", which is involved in the prey capture mechanism. (2) A slippery zone in the mid-inner part of the pitcher, covered by a wax that makes it almost impossible for the insect to escape by climbing out once inside the pitcher. (3) The lowermost part, known as the glandular zone, whose inner walls are covered by bifunctional glands that secrete hydrolytic enzymes into a digestive fluid and at the same time take up the nutrients created by prey degradation (Mithöfer, A. 2011) (**Figure 3**).

The most prominent proteins secreted by the plant into the digestive pitcher fluid are aspartic proteases and other hydrolytic enzymes, as well as proteins with antimicrobial function such as and pathogenesis-related protein-1 (PR1) (Buch, F. et al., 2014; Rottloff, S. et al., 2009). The last example belongs to the classification of pathogenesis-related (PR) proteins acting as plant defense against microbial pathogen attack (in non-carnivorous plants) (van Loon et al., 2006).

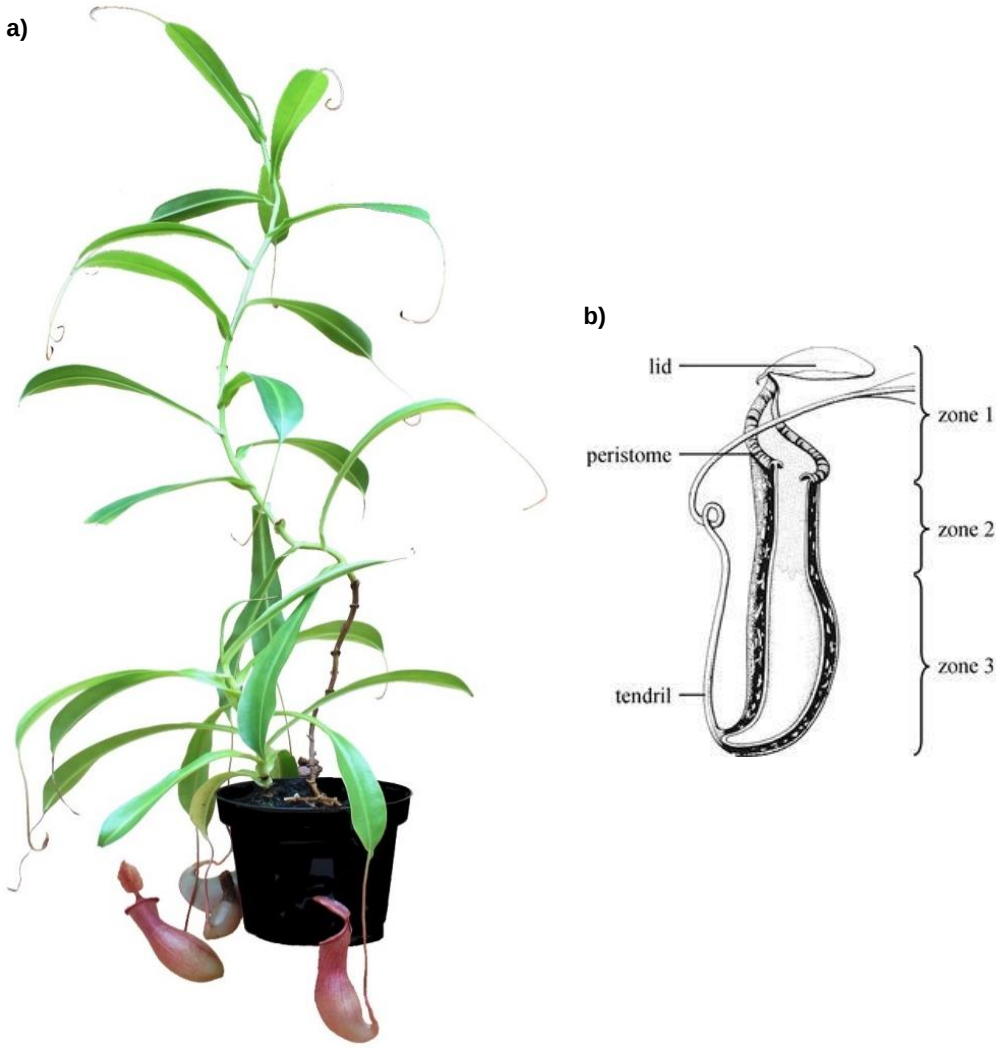

Figure 3: *Illustration of the model organism under study. Photography of the carnivorous climbing vine Nepenthes × ventrata,* ***a)****; Anatomical diagram of the organ with prey capture function, pitcher,* ***b)****. Pitcher consists of the following zones: first, the opening called peristome and a lid; second, is a waxy zone and a third region with digestive function, it hosts the bifunctional glands. Pitcher is connected to the leaf by means of the tendril. Illustrations adapted from: Dávila-Lara, A. et al., (2021a) and Riedel, M. et al., (2003).*

All studies carried out in this work employed *Nepenthes* × *ventrata* as carnivorous plant model-organism. *N.* × *ventrata* is a natural hybrid whose origin is the Philippines. It is a cross between *Nepenthes alata* and *Nepenthes ventricosa*. These species belong to the Caryophyllales and are members of the Nepenthaceae family representing vines and herbs.

According to Flora Malesiana (Cheek, M., & Jebb, M. 2001) most of the about 180 *Nepenthes* species are often found in forest habitats such as disturbed secondary, kerangas, and healthy forests, as well as in swamps. They are usually in open mossy, stunted, ridge-top forest as climbers or epiphytes between 1500-2500 m altitude. However, some species can be found in both lowland and mountain habitats.

Most *Nepenthes* plants in their initial stage consist of a short erect stem with short internodes at its base and a rosette as initial stage. During development, the seedling becomes a bush, producing short stems of 2 m high. In this case, unlike its juvenile stage, the internodes are longer, becoming climbing vines, which are supported by coiling leaf tendrils (Cheek, M., & Jebb, M. 2001).

Nepenthes have been able to survive, develop, and specialize in certain environments depending on nutrient sources, a prominent feature of these species. They have colonized nutrient-deficient habitats throughout their biogeographic area. The area of geographical distribution of *Nepenthes* is quite variable in space and time (van der Ent, A. et al., 2015; Clarke, C., & Moran, J. A. 2016). Possibly, the selective pressure on the morphological traits of *Nepenthes* are due to (relatively) rapid evolutionary processes and, in addition, to the combined circumstances of variations in local and regional climates (Ellison, A. M., & Adamec, L. (2018).

II. Aims of the study.

The pitcher plant *Nepenthes* × *ventrata* has evolved the carnivorous syndrome to solve the nutritional needs that it lacks in the soil. Through this syndrome the plant developed a specialized pitfall-trap to catch prey, the so-called pitcher.

In contrast to most of the studies of these plants, the content of this thesis neither focus on the breakdown process of the prey nor on the assimilation process of the nutrients derived from the prey. Instead, carnivory syndrome is studied not as such, but as a cause of the modifications in the interactions between plant-insect in the face of biotic and abiotic stress.

Therefore, the following aims are proposed:

- To evidence the presume of defense strategies in *Nepenthes* × *ventrata*.
- To demonstrate the nature of defense capability of *N.* × *ventrata*.
- To define the composition of EFN in *N.* × *ventrata.*
- To investigate the function of EFN production in *N.* × *ventrata*.

III. Manuscripts

Manuscript No. 1

Manuscript overview

Manuscript title: Carnivorous *Nepenthes* × *ventrata* plants use a naphthoquinone as phytoanticipin against herbivory

Authors: Alberto Dávila-Lara, Asifur Rahman-Soad, Michael Reichelt, Axel Mithöfer

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Authors' contributions (in %) to the given categories of the publication

Author	Conceptual	Data analysis	Experimental	Writing the manuscript	Provision of material
Alberto Dávila-Lara	50 %	10 %	30 %	30 %	-
Asifur Rahman-Soad	10 %	50 %	70 %	30 %	-
Michael Reichelt	-	20 %	-	10 %	10 %-
Axel Mithöfer	40 %	20 %	-	30 %	90 %-
Total:	100%	100%	100%	100%	100%

Signature candidate

Signature supervisor (member of the faculty)

PLOS ONE

RESEARCH ARTICLE

Carnivorous *Nepenthes x ventrata* plants use a naphthoquinone as phytoanticipin against herbivory

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Abstract

Carnivorous plants feed on animal prey, mainly insects, to get additional nutrients. This carnivorous syndrome is widely investigated and reported. In contrast, reports on herbivores feeding on carnivorous plants and related defenses of the plants under attack are rare. Here, we studied the interaction of a pitcher plant, *Nepenthes x ventrata*, with a generalist lepidopteran herbivore, *Spodoptera littoralis*, using a combination of LC/MS-based chemical analytics, choice and feeding assays. Chemical defenses in *N. x ventrata* leaves were analyzed upon *S. littoralis* feeding. A naphthoquinone, plumbagin, was identified in *Nepenthes* defense against herbivores and as the compound mainly responsible for the finding that *S. littoralis* larvae gained almost no weight when feeding on *Nepenthes* leaves. Plumbagin is constitutively present but further 3-fold increased upon long-term (> 1 day) feeding. Moreover, in parallel *de novo* induced trypsin protease inhibitor (TI) activity was identified. In contrast to TI activity, enhanced plumbagin levels were not phytohormone inducible, not even by defense-related jasmonates although upon herbivory their level increased more than 50-fold in the case of the bioactive jasmonic acid-isoleucine. We conclude that *Nepenthes* is efficiently protected against insect herbivores by naphthoquinones acting as phytoanticipins, which is supported by additional inducible defenses. The regulation of these defenses remains to be investigated.

Introduction

Carnivorous plants have fascinated people, not only scientists, for much more than 150 years; the time when Charles Darwin's pioneer studies on this topic was published in his book about 'Insectivorous Plants' [1]. In plants, insectivory, or in a broader sense carnivory, has evolved as an additional way to compensate for shortages of soil nutrients such as nitrogen and phosphorus [2, 3]. This feature enables carnivorous plants to grow on nutrient-poor soils. The carnivorous syndrome has evolved independently in angiosperms several times as a result of convergent evolution; thus, carnivorous plants represent a polyphyletic group [4, 5]. Three

parameters represent the prerequisites necessary to qualify a plant as carnivorous, i.e. the ability of prey attraction, prey trapping and killing, and prey digestion and nutrients absorption [6]. These prerequisites have been achieved in carnivorous plants by the development of specialized forms, in particular of the trapping mechanisms. Among others, most prominent are the snap-trap of *Dionaea muscipula* (Venus flytrap), the flypaper-traps found in the genera of *Drosera* (sundew) and *Pinguicula* (butterwort), the sucking bladder-traps of *Utricularia spp*. (bladderwort) as well as the pitfall-traps of, for example, *Cephalotus follicularis* and the genera *Sarracenia* and *Nepenthes*, representing new- and old-world pitcher plants. History, systematics and ecology of carnivorous plants has recently been covered by a monograph [7]; newest aspects of molecular evolution and physiology by a review [8].

Nepenthes (S1A Fig) is a tropical plant genus occurring mainly in Southeast Asia. Due to a slippery surface visiting insect prey falls inside the pitcher traps and drown in a digestive fluid [2]. The genus *Nepenthes* has a large chemical diversity and several secondary metabolites were isolated for pharmaceutical, biotechnological and ethnobotanical use especially in traditional medicine [9, 10]. Many reports describe curative effects of *Nepenthes* extracts on various diseases including hypertension, malaria, and oral cancer [11–16]. Among secondary metabolites, carotenoids, flavonoids, sterols, triterpenes, and naphthoquinones (NQ) are described for *Nepenthes* leaves [2, 17, 18]. In particular NQ are described as antimicrobial metabolites. Interestingly, they are also found in the digestive pitcher fluid, e.g. droserone, 5-*O*-methyl droserone in *N. khasiana* [19]; plumbagin, 7-methyl-juglone in *N. ventricosa* [20]. Hence, it was hypothesized that these compounds preserve prey during digestion and provide protection against decomposing microbes [19–22]. NQ derivatives are also described for various tissues of *Nepenthes* species including the pitchers [17, 20, 22, 23]. Recently, an untargeted metabolomics approach was performed in *N. x ventrata* comparing metabolite features of leaves and pitcher tissue before and after prey captures [24]. About 2,000 compounds (MS/MS events) were detected in the two tissues demonstrating enormous metabolome diversity. Besides a huge number of unknown compounds, the common constituents had an aromatic nature [24]. The tissue metabolite specificity could significantly discriminate leaves and pitchers. This suggests that the metabolite compositions might point to their functions and, furthermore, may represent mechanisms that enable the plants to cope with environmental abiotic and biotic challenges [18].

A typical biotic challenge plants have to deal with is the attack of herbivorous insects. Surprisingly, only few observations and studies are published describing the attack of insects on carnivorous pitcher plants. For instance, lepidopteran herbivory was described for some species of the new world pitcher plant *Sarracenia* [25, 26]. For *Nepenthes*, there is only one investigation showing that *N. bicalcarata* plants are infested by the weevil *Alcidodes spec*. [27]. For *N. gracilis*, it has been shown that green pitchers experience more herbivory than red ones [28]. To the best of our knowledge, up to now no other studies have been published that focus on herbivore damage in *Nepenthes*. Seemingly, herbivory on *Nepenthes* tissue is rare. The reason behind is not known yet. However, it is unlikely that all putative herbivores are caught and digested; more likely, *Nepenthes* is equipped with an efficient setting of defensive chemistry, as known for numerous plants [29]. For example, the presence of jasmonates and the related signaling pathway involved in the induction of plant defenses against herbivory is present in carnivorous plants such as *Drosera capensis*; *Dionaea muscipula*; *Nepenthes spp*. as recently reviewed [30]. Moreover, very recently it has been shown that the *Nepenthes* tissue-derived NQ plumbagin has anti-feeding and insect growth-inhibiting properties [23]. However, no *in vivo* feeding experiments on intact *Nepenthes* plants have been done yet and whether or not the NQ plumbagin is a key player in *Nepenthes*' defense remained an open question.

Nevertheless, also in *Nepenthes* chemical defense against feeding insects is most likely established and, maybe, this defense is highly efficient.

In this study, we addressed these issues to learn how a carnivorous *Nepenthes* plant responds to insect attack. We performed choice and feeding experiments employing larvae of the generalist herbivorous moth *Spodoptera littoralis* and *Nepenthes x ventrata* as host plant to investigate the insect's behaviour and growth performance. Feeding-induced quantitative changes in phytohormone and defensive plumbagin levels were analyzed in *N. x ventrata* leaf blades using LC/MS. We further examined the possibility of a phytohormone dependent induction of plumbagin accumulation and other defense-related compounds. Our data suggest that in *Nepenthes* besides prey digestion the jasmonate signaling pathway is also involved in defense induction but increase of constitutive defense is jasmonate independent.

Material and methods

Plants and insects

Nepenthes x ventrata Hort. ex Fleming plants, natural hybrids of *N. alata* Blanco *x N. ventricosa* Blanco, were bought from a company (Gartenbau Carow, Nürtlingen, Germany) about 20 year ago and ever since grown in the MPI greenhouse on peat substrate at 23–25˚C, 80–100% relative humidity, and a 16/8 h light/dark photoperiod. All plants were at least 2 years old. Plant pots were shuffled once in every three months to ensure optimum space and equal support for growth and to avoid any edge effect, caused by differences in micro environmental conditions between the core and at the edge of the samples group. *N. x ventrata* leaves used for the experiments had fully expanded but only contained pitcher buds to focus on the leaf chemistry only without having the influence of the mature pitcher chemistry.

Larvae of the insect *Spodoptera littoralis* Boisd. (Lepidoptera: Noctuidae) were hatched from eggs (provided by Syngenta Crop Protection, Stein, Switzerland) and reared on artificial diet as described [23] at 23–25˚C with a 14/10 h light/dark photoperiod. As a generalist, this moth is frequently used in feeding experiments. It is closely related to *S. litura*, a species that occurs in the same regions as *Nepenthes*.

Feeding and choice assays

Feeding assays. Here, second to third instar *S. littoralis* larvae were used. To avoid cannibalism, all feeding assays were performed with individual larvae. Assays with artificial diet supplemented with plant tissue or plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone, $C_{11}H_8O_3$; Fischer Scientific, Schwerte, Germany) were done as described [23]. The larvae were kept in a controlled temperature cabinet at 25 ± 1˚C and 60–70% relative humidity. Every day fresh diet was provided. For these feeding assays, 15 independent repeats were done.

To analyze weight gain of *S. littoralis* feeding on a *N. x ventrata* leaf, individual 3rd instar larvae were used. The larvae were left to feed on the same leaf for five days in the greenhouse. The whole leaf was covered with an air- and water-permeable polyethylene terephthalate (PET) bag (Toppits, Minden, Germany) to prevent escaping of the larvae and keep them on the particular leaf (S1*B* Fig). Larvae were carefully recovered and re-weighed every day. The number of dead individuals was recorded. For control, larvae growing on artificial diet were placed for the same time and under the same conditions near to the *N. x ventrata* plants.

Feeding-induced defense. For short term herbivory, leaves were infested with a single 3rd instar *S. littoralis* larvae starved for 24 h before starting the experiment. The caterpillar was placed carefully on the *N. ventrata* leaf with a tweezer. Lightweight cage (PET; 10 cm diameter, 1 cm height) was placed on the leaf covering both sides to prevent escaping of the larvae. For

long-term herbivory, PET bags were used instead of cages to cover the entire leaf. All herbivory experiments were performed in the greenhouse.

Choice assay. To measure the preference of larvae towards *N. x ventrata* leaf or pitcher tissue, 15 larvae were confined in a square (12 x 12 cm) petri dish containing a single piece (*c*. 10 cm^2) of leaf and pitcher tissue each in a choice situation. The bioassay was conducted for fixed time period and the choice made by the larvae for a specific tissue was assessed after 30, 60, and 180 min

Phytohormone treatment

For the phytohormone spraying experiment, each treatment group contained five individual plants on a single tray. Trays were placed with sufficient distance to avoid drift of the spray to other treatment groups. Plants were foliar-sprayed (4 mL $plant^{-1}$) twice, at the beginning and after 12 h only with one of the phytohormones jasmonic acid (JA, 500 μM), abscisic acid (ABA, 250 μM), salicylic acid (SA, 250 μM) (all from Sigma-Aldrich, Taufkirchen, Germany) for each treatment group. Spraying solutions were made from 50 mM (JA), and 100 mM (ABA, SA) stock in absolute ethanol and the final volume was adjusted to 20 mL with ddH_2O for each. While spraying, already opened pitcher traps were shaded to avoid droplets entering inside traps. For the control group under the same conditions, plants were sprayed with the same solution without any phytohormone. Leaves were harvested 24 h after first spraying, directly frozen in liquid nitrogen and stored at -80˚C for further analysis.

Phytohormone and plumbagin extraction and quantification

Sampled *N. x ventrata* leaves were finely-ground in liquid nitrogen using mortar and pestle. Approximately 100 mg of finely ground leaf tissue was weighed in 2 mL tubes. The extraction, detection and quantification of phytohormones were performed as previously described [31] with modifications [32] using an LC-MS/MS system. For analysis, each sample was extracted with 1.5 mL methanol containing 60 ng D_6–abscisic acid (Toronto Research Chemicals, Toronto, Canada), 60 ng of D_6–jasmonic acid (HPC Standards GmbH, Cunnersdorf, Germany), 60 ng D_4–salicylic acid (Santa Cruz Biotechnology, Santa Cruz, U.S.A) and 12 ng of D_6-jasmonic acid-isoleucine conjugate (HPC Standards GmbH, Cunnersdorf, Germany) as an internal standard.

Phytohormone and plumbagin analyses were combined and performed by LC-MS/MS as described [31, 32] on an Agilent 1260 series HPLC system (Agilent Technologies, Böblingen, Gemany) with the modification that a tandem mass spectrometer QTRAP 6500 (SCIEX, Darmstadt, Germany) was used. Briefly, chromatographic separation was achieved on a Zorbax Eclipse XDB-C18 column (50 x 4.6 mm, 1.8 μm, Agilent Technologies). Water containing 0.05% formic acid and acetonitrile were employed as mobile phases A and B, respectively. The elution profile was: 0–0.5 min, 10% B; 0.5–4.0 min, 10–90% B; 4.0–4.02 min, 90–100% B; 4.02–4.5 min, 100% B and 4.51–7.0, min 10% B. Flow rate was kept at 1.1 mL min^{-1} and the column temperature was maintained at 25˚C. The mass spectrometer was equipped with a Turbo spray ion source operated in negative ionization mode. The ion spray voltage was maintained at -4,500 eV. The turbo gas temperature was set at 650˚C. Nebulizing gas was set at 60 psi, curtain gas at 40 psi, heating gas at 60 psi, and collision gas was set to "medium". The mass spectrometer was operated in multiple reactions monitoring (MRM) mode, details of the instrument parameters and response factors for quantification can be found in S1 Table. For plumbagin an MRM was added to the method: Q1: m/z 187, Q3: m/z 159, DP: -20, CE: -18. Since we observed that both, the D_6-labeled JA and D_6-labeled JA-Ile standards (HPC Standards GmbH, Cunnersdorf, Germany) contained 40% of the corresponding D_5-labeled compounds, the sum of the peak areas of D_5- and D_6-compound was used for quantification. For

quantification of plumbagin, the internal D_6-JA standard was used applying an experimentally-determined response factor of 164. The response factor was determined by analyzing a mixture of plumbagin (insert supplier of standard here) and D_6-JA at the same concentration.

In addition to using LC-MS/MS for plumbagin quantification, a second method using HPLC-UV was also applied using the same extraction protocol. A 20 μL aliquot of the methanolic extract was separated using high performance liquid chromatography (Agilent 1100 HPLC system, Agilent Technologies) on a reversed-phase C-18 column (Nucleodur Sphinx RP, 250 x 4.6 mm, 5 μm, Macherey-Nagel, Düren, Germany) with a 0.2% formic acid in water (A)-acetonitrile (B) gradient (0 min, 20% B; 0–14 min, 20–76% B; 14–14.1 min, 76–100% B; 14.1-16min 100% B and 16.1–20 min 20% B; flow rate 1.0 mL min^{-1}). Detection was performed with a photodiode array detector, and peaks were integrated at 265 nm. Quantification of plumbagin (Fischer Scientific, Schwerte, Germany) was achieved by generating a plumbagin standard curve in the range of 8 to 250 μg mL^{-1}.

Protease inhibitor assay

Nepenthes x ventrata protein was extracted following the Pierce Plant Total Protein Extraction Kit (ThermoFischer Scientific, Darmstadt, Germany) protocol with minor modifications. For extraction, the native lysis buffer was diluted (1:1) with 50 mM Tris-HCl, pH 7.4 containing 30 mM $CaCl_2$ and 2 mL Eppendorf tubes were used. For each sample, 50 mg freshly ground tissue was mixed with 250 μL extraction buffer. The homogenate was shortly vortexed, and then incubated on ice for 5 min following a centrifugation step for five min at 16,000 x *g* and at 4˚C. The supernatant was recovered for further analysis, i.e. 150 μL extract was recovered and used for protein quantification and trypsin protease inhibitory (TI) activity measurements. The TI's activity was assayed by determining the residual trypsin activity according to [33] using BApNA (Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride; Sigma-Aldrich) as substrate and bovine trypsin (Sigma-Aldrich) as the standard enzyme. The reaction mixture containing 50 μL leaf protein extract, 20 μL trypsin (1 mg mL^{-1} in 50 mM Tris-HCl, pH 7.4 containing 30 mM $CaCl_2$) was incubated at 37˚C for 15 min with shaking (700 rpm). Then, 40 μL of BApNA (10 mg mL^{-1} in DMSO) was added to the assay solution and the reaction mixture was incubated at 37˚C for 20 min with shaking (900 rpm) followed by termination of the reaction by adding 500 μL of 10% (v/v) glacial acetic acid. A blank for each sample was run simultaneously to subtract the absorbance caused by the extract. In blank, acetic acid was added before BApNA. For a positive control, only extraction buffer was added instead of sample extract to avoid any inhibition, representing maximum trypsin activity. The absorbance was recorded at 410 nm alongside the blank with a spectrophotometer.

Statistical analyses

Statistical calculations were performed using GraphPad Prism version 9.0.0 in all cases. Details are indicated in the particular figure legends. For growth experiments, larvae were picked randomly from a large population and all experiments were conducted out under highly standardized conditions to avoid investigator-included bias.

Results

Plumbagin in artificial diet and *Spodoptera littoralis* larva growth

Recently published data on *S. littoralis* larvae feeding on artificial diets that was supplemented with different *Nepenthes* tissue showed that the larvae gained less weight the more tissue was added [23]. We re-analyzed those data (fresh pitcher, 30%; fresh leaf, 30%; dried leaf, 10%,

15%) and calculated the amount of plumbagin that was supplemented with the particular tissue. Two more data sets were added (dried leaf, 1%; plumbagin). Based on these data it can be seen that the increase of plumbagin concentration in the diet negatively correlates with weight gain of the larvae and this effect was independent of the plumbagin source (Fig 1).

Spodoptera littoralis food choice assay

Knowing that the plumbagin concentration in leaf tissue is about 5-times higher than in pitcher tissue and larvae feeding on diet supplemented with pitcher tissue gain more weight [23] (Fig 1), a choice assay was conducted to see if the larvae prefer to feed on leaf or pitcher tissue. Fifteen *S. littoralis* larvae were placed in a petri dish with an equal piece each of *N. x ventrata* leaf and pitcher tissue. After 30, 60, and 180 min the number of larvae feeding on either tissue was noted. As shown in Fig 2, the larvae had a clear and significant preference for the pitcher tissue at all time points. While over the whole period only *ca* 20% of the larvae chose to stay close to or on the leaf, around 50% chose the pitcher after 30 and 60 min. Only after 180 min the number dropped below 40%. Over time also the number of larvae that decided for either tissue dropped. Strikingly, only the pitcher tissue was eaten. The ongoing feeding process was documented to see the remained size of the tissues (S2 Fig).

Spodoptera littoralis feeding on *Nepenthes x ventrata* leaves

Next, we wanted to study the effects of *N. x ventrata* leaves on the feeding behavior of *S. littoralis* larvae when there is no alternative food. Therefore, the larvae were placed on the leaves without the chance to escape. As control, artificial diet was offered close to the plants under the same environmental conditions. While in the latter case larvae showed normal feeding behavior and weight increase, the larvae that fed on the leaves did not gain weight at all, not even after 5 d (Fig 3*A*). This was not due to feeding avoidance as the leaves were eaten and the leaf area was continuously reduced over time (Fig 3*B*). Moreover, if the larvae were taken after 5 d and put on artificial diet, they started growing and developed as normal.

Herbivory and plumbagin accumulation

To further investigate the role of plumbagin in this process, the plumbagin levels were analyzed over time. A constitutive level of this NQ (256 ± 13.5 μg g^{-1} fresh weight; n = 25; mean ± SEM) was detected in the leaves. To examine any additional effect of *S. littoralis* herbivory, both short- and long-term accumulation of plumbagin was determined. Within the first 6 h of feeding, no significant change of plumbagin level was detected in the leaves (Fig 4*A*); however, after day 1 to day 5, a *ca*. 3-fold increase of plumbagin was determined (755 ± 43.0 μg g^{-1} fresh weight; n = 50; mean ± SEM) that stayed at this level during the feeding process (Fig 4B).

Herbivory-changed phytohormone level in *Nepenthes x ventrata* leaves

Because herbivore feeding typically induces an increase of certain phytohormones, in particular of jasmonates, in the infested tissues, various phytohormones were analyzed at the same time points as we analyzed plumbagin. A significant fast and drastic increase of both JA and JA-Ile was found after 1 h, staying high during the short term analyses (Fig 5*A* and 5*C*). In the long-term analyses, the JA level was kept high until day 2 and dropped by half after day 3 to day 5; nevertheless, these JA levels were still significantly higher than in the controls (Fig 5*B*). In case of JA-Ile, the same level (around 60 ng g^{-1} fresh weight) was kept for the 5 d period of the whole feeding experiment (Fig 5*D*). Degradation products of JA-Ile, the bioactive

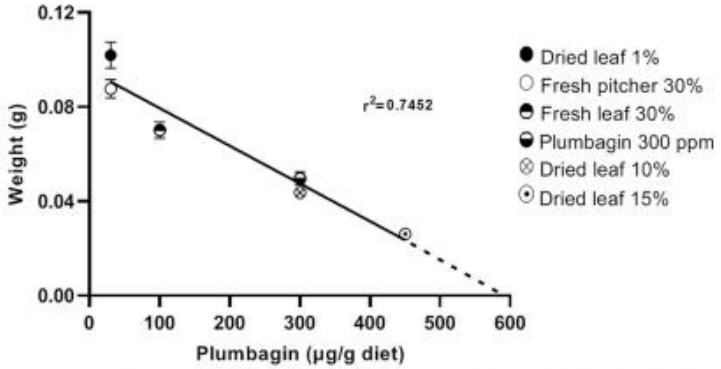

Fig 1. *Spodoptera littoralis* larvae growth *vs.* different plumbagin concentrations in artificial diet. Plumbagin from various sources (represented by different symbols) was included in the diet. In all assays 2nd instar larvae were used; weight was determined at day 5. Regression showed statistical significance with $p < 0.0001$ (estimated $r^2 = 0.745$, $F = 251.5$ (DFn = 1, DFd = 86), alpha level = 0.05). The r^2 value (0.745) indicates a strong relationship between larval development and plumbagin concentrations present in the diet.

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jasmonate, were examined as well. Again, a fast increase of their accumulation was found after 1 h for OH-JA-Ile, which stayed more or less at the same level until day 5 (S3*A* and S3*B* Fig). For COOH-JA-Ile the situation was slightly different. Only after 3 h a significant increase was found and a second 2-fold increase could be detected at day 2 (S3*C* and S3*D* Fig).

Besides jasmonates, the phytohormones SA and ABA were investigated. For SA a slight but significant 2-fold increase was found in the short term analysis that further increased at day 1 (around 5-fold) and stayed at that level for the whole 5 d period (Fig 6*A* and 6*B*). In case of ABA only after 6 h a weak tendency for higher ABA content was recognized that continuously increased and eventually showed a clear accumulation at days 4 and 5 (Fig 6*C* and 6*D*).

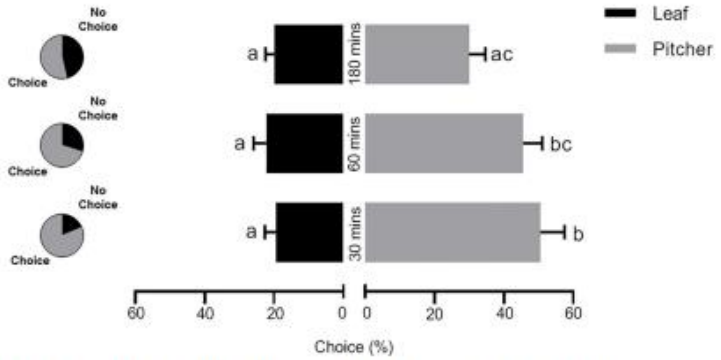

Fig 2. *Spodoptera littoralis* larvae food choice assay for *Nepenthes x ventrata* pitcher and leaf tissue. The proportions of larvae (15 per experiment) making choices are indicated in pie charts. Mean (± SEM), n = 12; two-way ANOVA, Šidák's multiple comparisons test; different letters at the end of each bar indicate significant difference ($p < 0.05$).

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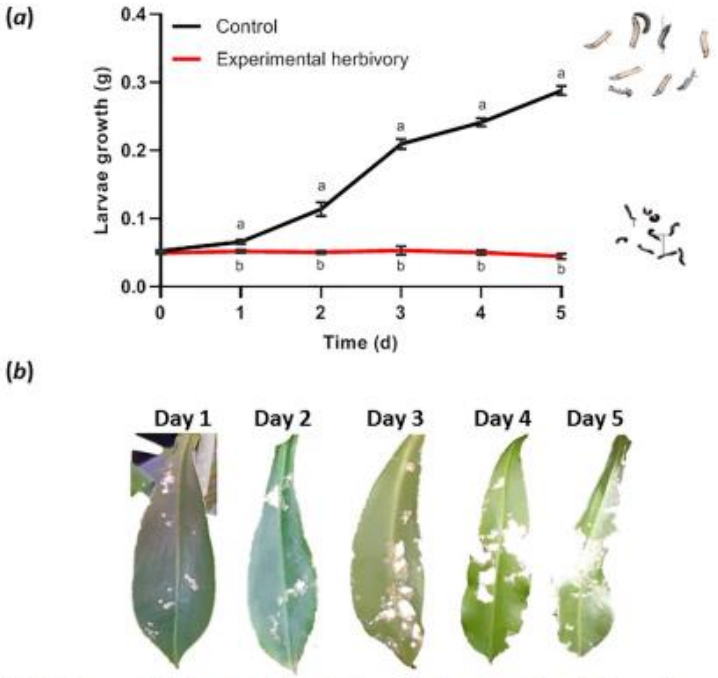

Fig 3. Performance of *Spodoptera littoralis* larvae feeding on *Nepenthes x ventrata* leaves. (a) Change of larvae weight when feeding on leaves (experimental herbivory) or artificial diet (control). Larvae were weighed every day for 5 days. Mean (± SEM), $n = 15$; two-way ANOVA, Šidák's multiple comparisons test; different letters indicate significant difference ($p < 0.05$). Photos of larvae were taken at day 5, squares are 1 cm^2. (*b*) *N. x ventrata* leaf eaten; photos taken at days 1 to 5.

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Phytohormone-mediated accumulation of plumbagin

In order to find out whether or not any phytohormone may be involved in the induction of plumbagin accumulation during long-term feeding, leaves of *N. x ventrata* were treated with phytohormones and plumbagin contents were determined after 24 h. Therefore, solutions of JA, ABA and SA in water were exogenously applied to the leaves of individual plants. No significant difference in the endogenous level of plumbagin was observed in either treatment compared to control or between the treatment groups after 24 h post foliar spray (Fig 7).

Herbivory- and phytohormone-induced protease-inhibitor activity

Besides plumbagin, very likely other defense component are present in *Nepenthes* leaves that might affect the growth of feeding larvae. Thus, we studied the presence of protease (trypsin) inhibitor (TI) activity as example for a wide spread defense mechanism that could also affect the larvae feeding. The TI activity was assayed after 24 h, 48 h and 72 h of *N. x ventrata* leaf infestation by *S. littoralis* larvae. The results showed a significant increase of TI activity in herbivore infested leaves in all treatment groups compared to non-infested leaves as controls at all

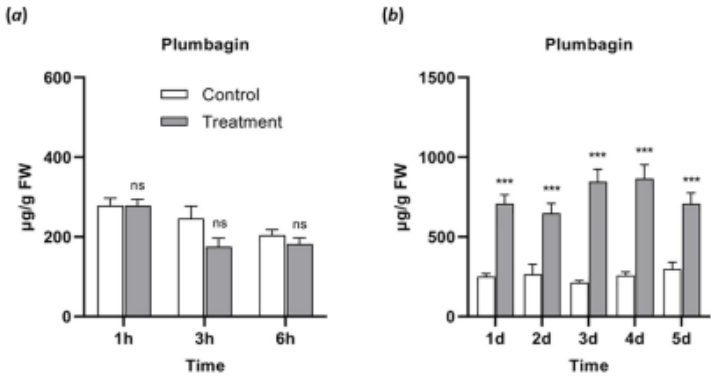

Fig 4. Plumbagin accumulation in *Nepenthes x ventrata* leaves upon *Spodoptera littoralis* larvae feeding. (*a*) Plumbagin content after short term feeding. (*b*) Plumbagin content after long term feeding. Mean (± SEM), $n = 10$; unpaired t-test with Welch correction; ns = not significant; *** $p < 0.001$.

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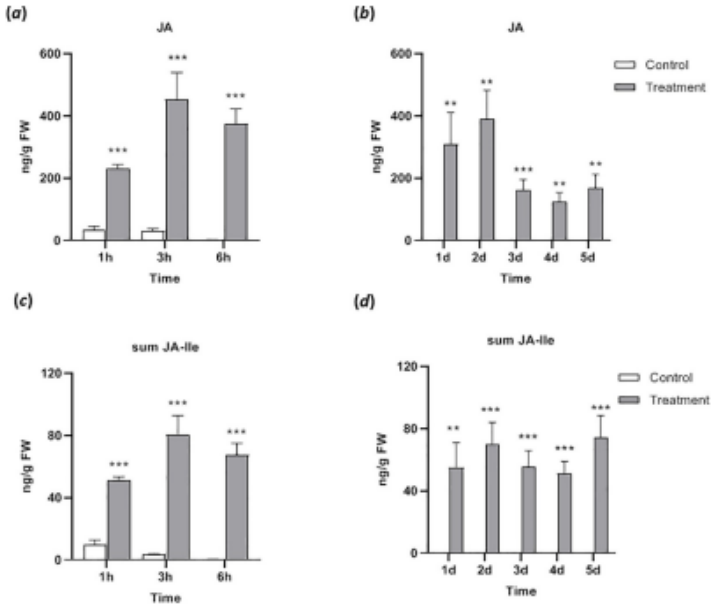

Fig 5. Jasmonates in *Nepenthes x ventrata* leaves upon *Spodoptera littoralis* larvae feeding. (*a*) Jasmonic acid (JA) content after short term and (*b*) long term feeding. (*c*) Jasmonic acid-isoleucine conjugate (JA-Ile) content after short term and (*d*) long term feeding. Mean (± SEM), $n = 10$; unpaired t-test with Welch correction; *** $p < 0.001$; ** $p < 0.01$.

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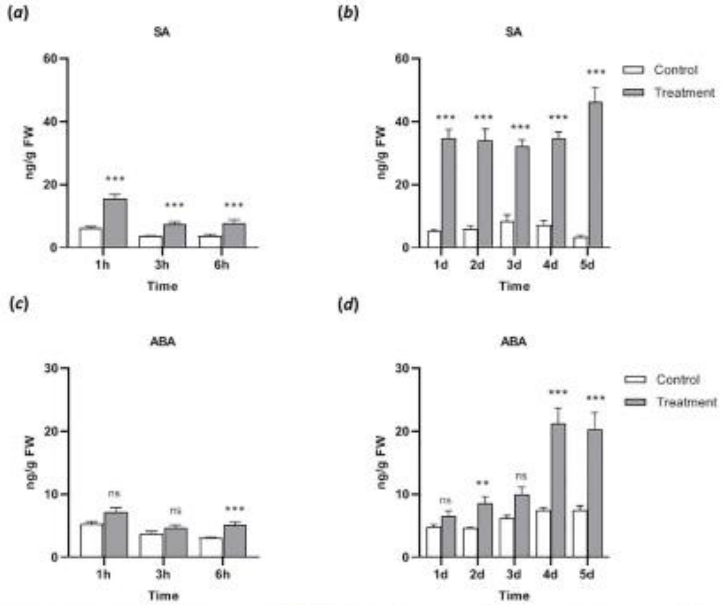

Fig 6. Salicylic acid (SA) and abscisic acid (ABA) in *Nepenthes x ventrata* leaves upon *Spodoptera littoralis* larvae feeding. (*a*) SA content after short term and (*b*) long term feeding. (*c*) ABA content after short term and (*d*) long term feeding. Mean (± SEM), $n = 10$; unpaired t-test with Welch correction; ns = not significant; *** $p < 0.001$; ** $p < 0.01$.

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time points (Fig 8*A*). All OD_{410} values from control groups showed a similar range at all times. This, together with the positive control value suggests the absence of trypsin protease inhibitory activity from protein extracts of undamaged leaves alongside excluding the possibility of the involvement of other metabolites from leaf extract causing trypsin inhibition during the assay. The same samples used for foliar spray experiments to look for plumbagin induction (Fig 7) were analyzed for TI activities as well. Statistically significant differences were observed for all phytohormone-treated samples at 24 h after foliar spray compared to the untreated control groups (Fig 8*B*).

Discussion

While most of the phytochemical studies done on *Nepenthes* and other carnivorous plants mainly addressed carnivorous syndrome-related processes, demonstration of secondary metabolite synthesis and accumulation as a result of herbivory is surprisingly limited. The present study illustrates a defense response in *N. x ventrata* against insect herbivory that is based on NQ, in particular on plumbagin. The presence of NQ has been described for many carnivorous plants [34] belonging to the order Nepenthales [18], a *sensu stricto* sister group to Caryophyllales [35] and including the plant families Droseraceae and Nepenthaceae. This includes species such as *Aldrovanda vesiculosa*, *Dionaea muscipula*, *Drosophyllum lusitanicum*, and *Triphyophyllum peltatum* as well as the genera *Drosera* and *Nepenthes* [34].

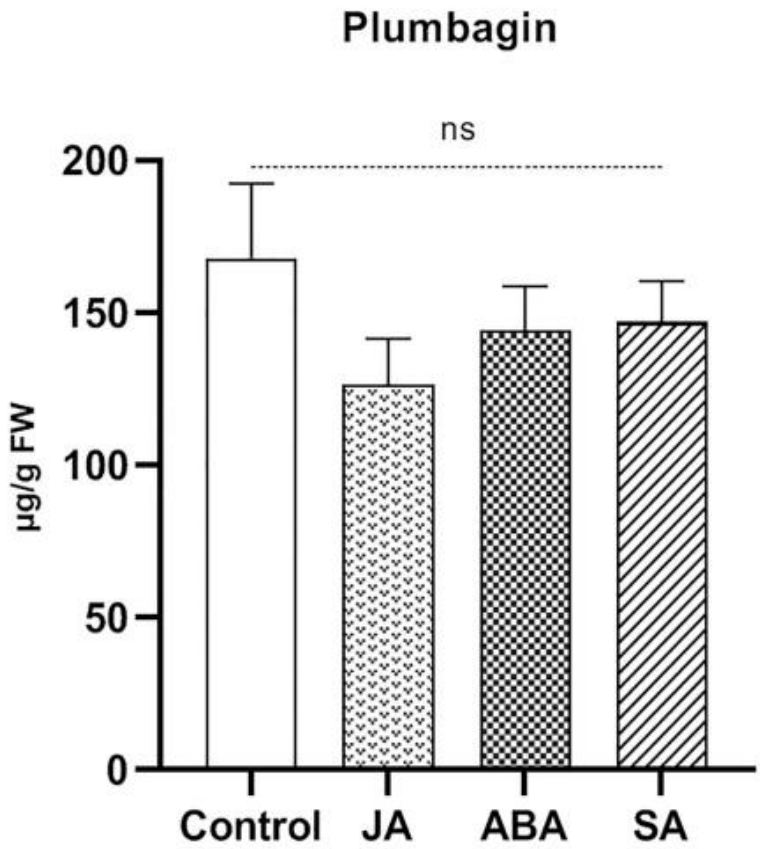

Fig 7. Phytohormone-induced plumbagin accumulation in *Nepenthes x ventrata* leaves. Foliar spray with JA (500 µM), ABA (250 µM), or SA (250 µM) was performed at the beginning (2 mL) and after 12 h (2 mL). Plants were harvested after 24 h. Mean (±SEM), n = 5; one-way ANOVA followed by Tukey's multiple comparisons test; different letters indicate significant difference ($p < 0.05$); ns = not significant.

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The function of plant secondary metabolites often lays in their ecological significance in defense against pathogens and herbivores. The observation that *Nepenthes* plants are rarely infested by insect herbivores could be an example of such phenomenon knowing that NQ are bioactive compounds with insect anti-feeding activity [23]. In that study, plumbagin was found as a dominant secondary metabolite in the tissue of *N. x ventrata* and its function was evaluated by means of the interaction with a generalist insect herbivore, *S. littoralis*.

Experiments with artificial diet containing plumbagin from different sources showed an adverse effect on the growth of the *S. littoralis* larvae due to increasing plumbagin concentrations (Fig 1). This negative effect of high plumbagin concentration on feeding larvae was supported in a choice assay where larvae could choose between *N. x ventrata* leaf and pitcher

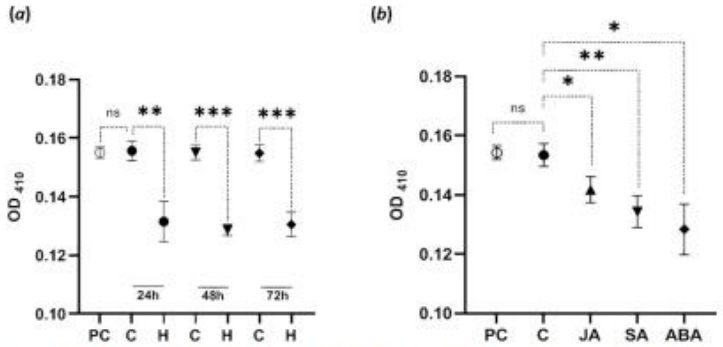

Fig 8. Trypsin protease inhibitor (TI) activities in *Nepenthes x ventrata* leaves. (*a*) TI activities determined after *Spodoptera littoralis* larvae feeding (H) for 24 h, 48 h, and 72 h compared to the respective controls (PC, positive control; C, treatment control). (*b*) TI activities determined after foliar spray with JA (500 μM), ABA (250 μM), or SA (250 μM). Treatment was performed at the beginning (2 mL) and after 12 h (2 mL). Plants were harvested after 24 h. Mean (±SEM), n = 5; unpaired t-test with Welch's correction; asterisks represent difference between individual treatments vs control; ns = not-significant, * $p < 0.05$, ** $p \leq 0.01$; *** $p < 0.001$.

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tissue. Here, a clear preference for pitchers was found independent on exposure time (Fig 2). This corresponds well to the *ca.* 5-fold lower plumbagin content in pitcher tissue [23]. However, artificial diet sometimes comes with the limitation of being suboptimal or super optimal [36]. Compared to most susceptible host plants, artificial diet often fosters more rapid growth [37] thus making insects less susceptible to the tested compound or extract incorporated in diet. This situation was eliminated by letting the larvae feed directly on *N. x ventrata* leaf tissue. Moreover, in a long-term feeding assay on *N. x ventrata* leaves, *S. littoralis* larvae showed no weight gain at all although they fed a lot (Fig 3). For other lepidopteran species such as *S. litura*, *Achaea janata*, and *Trichoplusia ni*, the effect of plumbagin on feeding behavior has also been reported [38–40]. However, the focus of those studies was on the level of feeding avoidance rather than on larval growth and development.

Nevertheless, the finding that *Nepenthes* leaves contain more plumbagin than pitchers and that the latter were preferred by *S. littoralis* in selection tests is somewhat reminiscent of the so-called push-pull strategy in agriculture. In this strategy, a repellent (push) and an attractive (pull) resource are combined to push pests away from crops and attract them elsewhere [41]. It is conceivable that *Nepenthes* takes the same approach, pushing insects away from leaves toward pitcher traps where plumbagin concentrations are lower and an attractive cue awaits the extrafloral nectar [42].

Herbivory causes a series of different reactions in the infested plant. The feeding process leads to the rapid induction of signals including phytohormone (mainly jasmonates) accumulation and finally the activation of wound and herbivory-related defenses [29, 43]. Interestingly, jasmonate signaling is also present in carnivorous plants, as demonstrated for the regulation of the digestive process [30]; for example, in *Dionaea muscipula* [44–47], *Drosera capensis* [48], and *Nepenthes alata* [49]. In addition, in *Drosera capensis* the accumulation of jasmonate upon wounding and treatment with insect-derived oral secretion has been shown [50]. The current study revealed the same jasmonates are also involved in the response of *N. x ventrata* upon *S. littoralis* herbivory. The feeding-stimulated elevation of JA and its biologically active form JA-Ile was rapid and significantly enhanced within the first hour of treatment

(Fig 5). The timing of the jasmonate response is comparable with the situation in other herbivore-infested plants; however, there is a clear discrepancy to the slow accumulation of jasmonates upon prey capture in pitcher tissue of *Nepenthes* that was detectable only after 24 hours [49]. At first glance, the different kinetics of jasmonate accumulation in defense and carnivory suggests a completely different regulation of both processes. However, it might be that the initial signal perception and signaling process leading to jasmonate induction is equally fast but the release of a signal (e.g. chitin) and its presentation might be much slower due to a previously necessary digestive process.

To the best of our knowledge, in almost no previous study done on *Nepenthes*, the role of phytohormones has been extensively investigated outside the carnivorous syndrome context. Only studies on the prey-induced responses in *N. alata* from Yilamujiang et al. [49] found doubling of endogenous SA level after prolonged (48 h) chitin treatment compared to the original level in the pitcher tissue. Slightly similar but again a faster situation was observed in our current study where in short-term herbivory endogenous level of SA nearly doubled at all time points; after 24 hours the SA level further increased and was found to be stable for the next four days (Fig 6*A* and 6*B*). Typically, SA concentrations increase in response to pathogen infection but not as a general wound response [51, 52]. Therefore, one possibility of increased concentration of SA observed upon prolonged herbivory in this study could be a consequence of increased vulnerability of leaves after herbivore damage that may create open passages for pathogens to enter. Considering stress-related hormones such as ABA [53], this study showed elevated endogenous ABA levels upon long term herbivory (Fig 6*D*); a common response known in plants after infestation by natural enemies [54]. However, increases in ABA are not specific to processes that are associated with induction of resistance; many other conditions such as water stress also cause ABA accumulation but do not produce other manifestations of induced resistance. In this investigation long-term herbivory of leaf tissue caused early senescence, which consequently increases dryness of wounded leaves; this is a possible reason for ABA accumulation over time.

A striking result was the finding that larvae feeding on *N. x ventrata* leaves gained almost no weight at all (Fig 3*B*), in contrast to the larvae feeding on enriched diets. This suggested that besides plumbagin an additional factor in the *Nepenthes* tissue might have contributed to this effect. Thus, apart from plumbagin accumulation in *N. x ventrata* as a mode of defense response against herbivores, the presence of other factors might be present, which may affect food digestion in the larvae and cause less or no weight increase. Such a factor and known defensive tools are protease inhibitors [29]. As demonstrated in Fig 8*A*, TI activities were indeed induced upon *S. littoralis* feeding. Although TI of plants are among the best studied proteins in plant biochemistry and biology, there has been no prior studies done on carnivorous plants that showed accumulation of TI upon herbivory. The role of protease inhibitors in plant defense against insects was demonstrated first when Green and Ryan [55] showed the induction of TIs in leaves of potato in response to wounding and insect feeding. Protease inhibitors are polypeptides and proteins that bind to proteolytic enzymes and prevent them from catalyzing [56]. Most of these inhibitors are unique to serine class proteinases, usually found in insects as the main food protein digestive enzymes [57]. Enzyme assays for proteinase inhibitors from our present study showed inhibition of the serine protease trypsin in extracts from herbivory-infested leaves, whereas non-infested leaf extracts showed no significant trypsin activity inhibition. Because TI accumulation occurred upon herbivory and is absent in unstressed leaves, it indicates that in *Nepenthes* TI are part of the inducible defense response.

In their natural environment, plants come across several pests and pathogens. Plant's defense against such a threat involves either a fast consolidation of pre-existing physical and chemical barriers and/or the synthesis of many defensive substances through the induction of

gene expression [29, 58]. In this study, herbivore feeding on *N. x ventrata* leaves enhanced a defense response in the form of plumbagin with a 2 to 3-fold increased endogenous level compared to untreated leaves (Fig 4*B*). Conspicuously, plumbagin was induced only upon long-term feeding and not by treatment of any phytohormones, not even jasmonates (Fig 7). In contrast, all phytohormones induced TI activity indicating that the spray approach was successful (Fig 8*B*). On one hand, the absence of phytohormone inducibility was surprising as jasmonates are rapidly and highly upregulated suggesting that JA-dependent defense reactions and compounds are induced; obviously, in *Nepenthes* various inducible defensive mechanisms remain to be detected. On the other hand, the kinetics of jasmonate or plumbagin accumulation also does not match. It would be worth to elucidate which signals and processes are involved in plumbagin induction upon herbivory; for example, a role for signaling compounds such as reactive oxygen species is conceivable. Although the accumulation of plumbagin in carnivorous plants has been mentioned in both *in vivo* and *in vitro* studies using polysaccharide elicitors [19, 59, 60], also in those cases little is known on the exact molecular mechanism governing the elicitor-induced production and accumulation of this phenolic compound. However, the fact that plumbagin is only slightly induced upon herbivory and all tested *Nepenthes* tissues have a constitutive level [22, 23] classifies this NQ as phytoanticipin rather than a phytoalexin, maybe comparable with glucosinolates in Brassicaceae [61].

Conclusion

In this present study, chemical defenses upon herbivore damage in leaves of the carnivorous plant *N. x ventrata* were identified: the constitutive presence of the NQ plumbagin and the induced trypsin protease inhibitor activities. The latter very likely supports the effect of plumbagin on growth inhibition in *S. littoralis* larvae. Due to the constitutive presence of plumbagin, *Nepenthes* seems to be permanently well protected. Nevertheless, inducible defense responses such as the TI activity can support the first line of defense represented by the phytoanticipins. Strikingly, only TI induction is jasmonate-mediated which raises the question of how the synthesis and accumulation of plumbagin is regulated. From the data obtained so far, it is difficult to infer which signaling molecule may actually be involved in the accumulation of plumbagin in the long term. It will be furthermore interesting to examine if and what kind of other defense responses occur in *Nepenthes* and how those are regulated.

Supporting information

S1 Fig. Setup of *Spodoptera littoralis* feeding experiments on *Nepenthes x ventrata* leaves. (A) *N. x ventrata* (natural hybrid of *N. alata x N. ventricosa)* plant. (B) *S. littoralis* larvae together with the leaf were covered with a PET bag to prevent escaping of the larvae. For control, larvae were placed close to the plant but fed on artificial diet.
(PPTX)

S2 Fig. Choice experiment; *Spodoptera littoralis* feeding on different tissues. (A) Setup in a 12 x 12 cm petri dish containing pieces of *Nepenthes x ventrata* pitcher (red) and leaf (green) tissue on a moist filter paper; an additional filter control was placed as well. (B) Photos of leaf and pitcher pieces with visiting larvae taken at the indicated time points.
(PPTX)

S3 Fig. Jasmonic acid-isoleucine conjugate (JA-Ile) degradation products in *Nepenthes x ventrata* leaves upon *Spodoptera littoralis* larvae feeding. (A) OH-JA-Ile content after short term and (B) long term feeding. (C) COOH-JA-Ile content after short term and (D) long term

feeding. Mean (± SE), $n = 10$; unpaired t-test with Welch correction; *** $p < 0.001$; ** $p < 0.01$.
(PPTX)

S1 Table. Details of phytohormones and plumbagin analyses by LC-MS/MS. HPLC 1260 (Agilent Technologies)-QTRAP6500 (SCIEX)] in negative ionization mode.
(PPTX)

S1 File. Data.
(XLSX)

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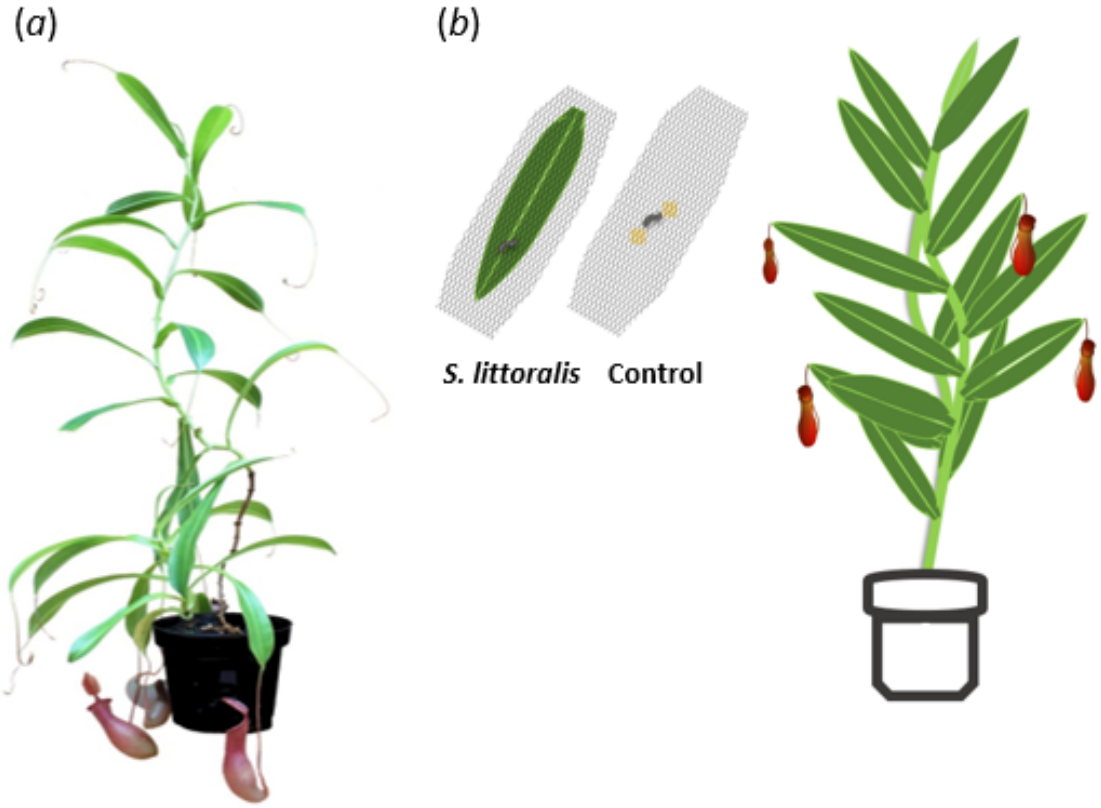
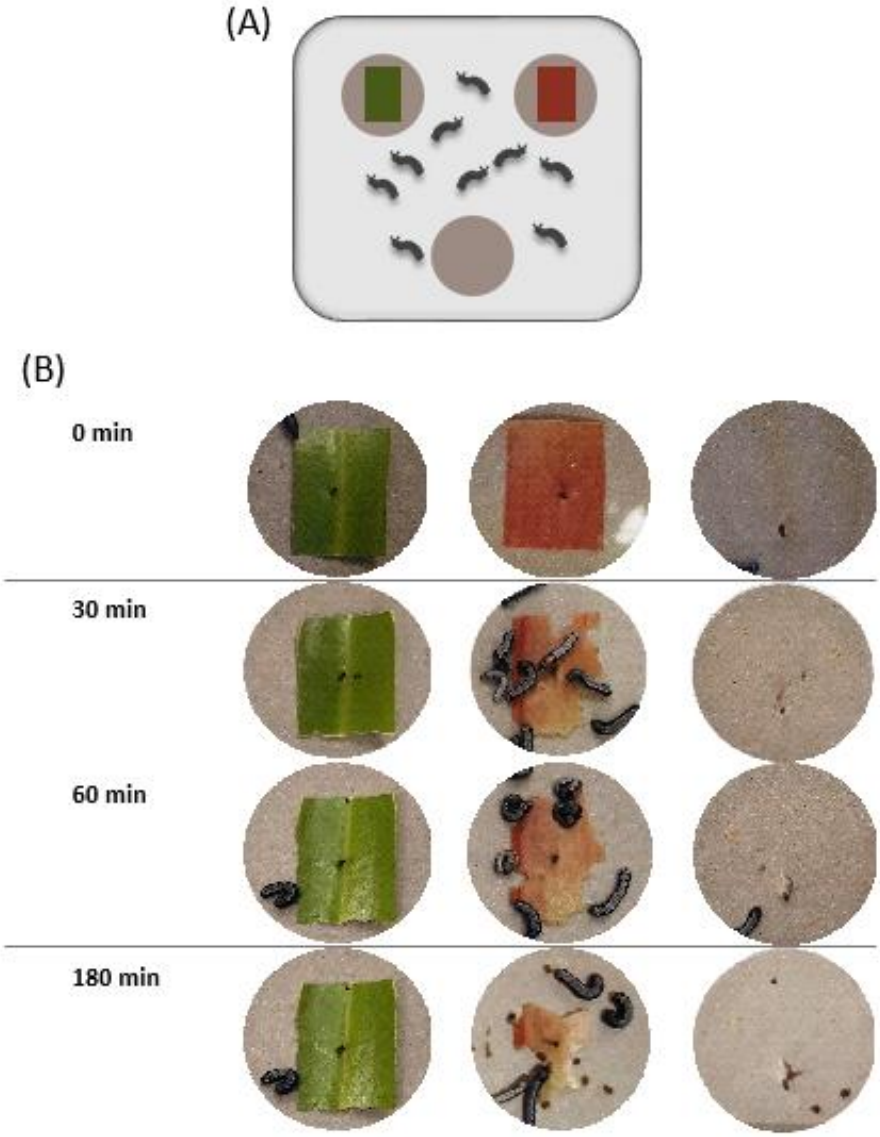
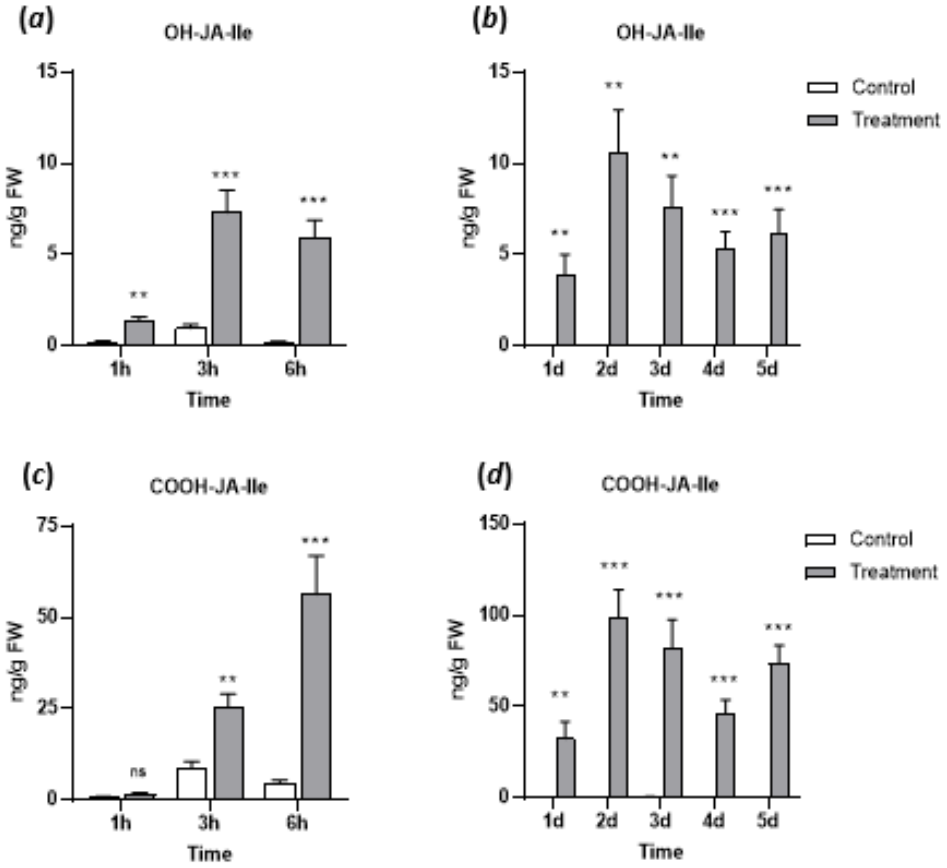
Supplementary material manuscript No. 1



S1 Fig. Setup of *Spodoptera littoralis* feeding experiments on *Nepenthes x ventrata* leaves . (*a*) *N. x ventrata* (natural hybrid of *N. alata x N. ventricosa)* plant. (*b*) *S. littoralis* larvae together with the leaf were covered with a PET bag to prevent escaping of the larvae. For control, larvae were placed close to the plant but fed on artificial diet.



S2 Fig. Choice experiment; *Spodoptera littoralis* feeding on different tissues. (*a*) Setup in a 12 x 12 cm petri dish containing pieces of *Nepenthes x ventrata* pitcher (red) and leaf (green) tissue on a moist filter paper; an additional filter control was placed as well. (*b*) Photos of leaf and pitcher pieces with visiting larvae taken at the indicated time points.



S3 Fig. Jasmonic acid-isoleucine conjugate (JA-Ile) degradation products in *Nepenthes x ventrata* leaves upon *Spodoptera littoralis* larvae feeding. (*a*) OH-JA-Ile content after short term and (*b*) long term feeding. (*c*) COOH-JA-Ile content after short term and (*d*) long term feeding. Mean (± SE), $n = 10$; unpaired t-test with Welch correction; *** $p < 0.001$; ** $p < 0.01$.

S1 Table.
Details of phytohormones and plumbagin analyses by LC-MS/MS [HPLC 1260 (Agilent Technologies)-QTRAP6500 (SCIEX)] in negative ionization mode.

Q1	Q3	RT (min)	Compound	Internal std	RF	DP	CE
136.93	93.00	3.3	SA	D4-SA	1.0	-20	-24
263.00	153.20	3.4	ABA	D6-ABA	1.0	-20	-22
209.07	59.00	3.6	JA	D6-JA	1.0	-20	-24
322.19	130.10	3.9	JA-Ile	D6-JA-Ile	1.0	-50	-30
290.90	165.10	4.6	OPDA	D6-JA	1.0	-20	-24
338.10	130.10	3	OH-JA-Ile	D6-JA-Ile	1.0	-50	-30
225.10	59.00	2.6	OH-JA	D6-JA	1.0	-20	-24
352.10	130.10	3.0	COOH-JA-Ile	D6-JA-Ile	1.0	-50	-30
140.93	97.00	3.3	D4-SA			-20	-24
269.00	159.20	3.4	D6-ABA			-20	-22
215.00	59.00	3.6	D6-JA			-20	-24
214.00	59.00	3.6	D5-JA			-20	-24
328.19	130.10	3.9	D6-JA-Ile			-50	-30
327.19	130.10	3.9	D5-JA-Ile			-50	-30
187.00	159.00	4.3	plumbagin	D6-JA	164.0	-20	-18

S1 Fig. Setup of *Spodoptera littoralis* feeding experiments on *Nepenthes x ventrata* leaves.
(A) *N. x ventrata* (natural hybrid of *N. alata x N. ventricosa)* plant. (B) *S. littoralis* larvae together with the leaf were covered with a PET bag to prevent escaping of the larvae. For control, larvae were placed close to the plant but fed on artificial diet.
https://doi.org/10.1371/journal.pone.0258235.s001
(PPTX)

S2 Fig. Choice experiment; *Spodoptera littoralis* feeding on different tissues.
(A) Setup in a 12 x 12 cm petri dish containing pieces of *Nepenthes x ventrata* pitcher (red) and leaf (green) tissue on a moist filter paper; an additional filter control was placed as well. (B) Photos of leaf and pitcher pieces with visiting larvae taken at the indicated time points.
https://doi.org/10.1371/journal.pone.0258235.s002
(PPTX)

S3 Fig. Jasmonic acid-isoleucine conjugate (JA-Ile) degradation products in *Nepenthes x ventrata* leaves upon *Spodoptera littoralis* larvae feeding.
(A) OH-JA-Ile content after short term and (B) long term feeding. (C) COOH-JA-Ile content after short term and (D) long term feeding. Mean (± SE), $n = 10$; unpaired t-test with Welch correction; *** $p < 0.001$; ** $p < 0.01$.
https://doi.org/10.1371/journal.pone.0258235.s003
(PPTX)

S1 Table. Details of phytohormones and plumbagin analyses by LC-MS/MS.
HPLC 1260 (Agilent Technologies)-QTRAP6500 (SCIEX)] in negative ionization mode.
https://doi.org/10.1371/journal.pone.0258235.s004
(PPTX)

S1 File. Data.
https://doi.org/10.1371/journal.pone.0258235.s005
(XLSX)

Manuscript No. 2

Manuscript overview

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Author	Conceptual	Data analysis	Experimental	Writing the manuscript	Provision of material
Alberto Dávila-Lara	40 %	20 %	100 %	40 %	-
Michael Reichelt	-	10 %	-	10 %	10 %
Ding Wang	-	30 %	-	5 %	-
Heiko Vogel	20 %	30 %	-	5 %	-
Axel Mithöfer	40 %	10 %	-	40 %	90 %
Total:	100%	100%	100%	100%	100%

Signature candidate

Signature supervisor (member of the faculty)

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Proof of anthocyanins in the carnivorous plant genus *Nepenthes*

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Keywords
anthocyanins; betalain; Caryophyllales; *Nepenthes*; plant carnivory

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Yellow to red colored betalains are a chemotaxonomic feature of Caryophyllales, while in most other plant taxa, anthocyanins are responsible for these colors. The carnivorous plant family Nepenthaceae belongs to Caryophyllales; here, red-pigmented tissues seem to attract insect prey. Strikingly, the chemical nature of red color in *Nepenthes* has never been elucidated. Although belonging to Caryophyllales, in *Nepenthes*, some molecular evidence supports the presence of anthocyanins rather than betalains. However, there was previously no direct chemical proof of this. Using ultra-high-performance liquid chromatography-electrospray ionization-high-resolution mass spectrometry, we identified cyanidin glycosides in *Nepenthes* species and tissues. Further, we reveal the existence of a complete set of constitutively expressed anthocyanin biosynthetic genes in *Nepenthes*. Thus, here we finally conclude the long-term open question regarding red pigmentation in Nepenthaceae.

The presence of betalains is a typical phytochemical feature of the plant order Caryophyllales [1]. Betalains are violet to red (betacyanins) and orange to yellow (betaxanthins) pigments that are derived from the amino acid tyrosine. According to the Angiosperm Phylogeny Group classification [2], the occurrence of betalains holds true for the so-called core Caryophyllales, a defined clade of eudicots comprising *c*. 29 families. Only two Caryophyllales families, Caryophyllaceae and Molluginaceae, do not contain betalains but anthocyanins, also red and yellow pigments much more widely distributed in the plant kingdom [3,4]. Within the noncore Caryophyllales, betalains have never been documented. Strikingly, the presence of betalains and anthocyanins exclude each other [1,3,5,6]. Very likely, this is due to two events in the core Caryophyllales, the de-regulation of the tyrosine biosynthesis and gene duplication events [3,6,7]. While betalains are made from tyrosine, anthocyanins are made from phenylalanine. In both cases, the shikimate pathway provides the biosynthetic precursors; that is, the syntheses of tyrosine and phenylalanine compete for prephenate or arogenate as substrate. Whereas during tyrosine synthesis in bacteria and fungi prephenate is converted to 4-hydroxyphenyl pyruvate, in plants prephenate is mainly converted to arogenate. Arogenate is further converted by arogenate dehydrogenases (ADH) into tyrosine. Typically, the ADH is negatively feedback-regulated by tyrosine; however, in betalain accumulating species this regulation is partly lost. Here, a tyrosine-insensitive ADH arose during evolution of the

Abbreviations
ADH, arogenate dehydrogenase; ANS, anthocyanidin synthase; C3′H, 5-*O*-(4-coumaroyl)-D-quinate 3′-monooxygenase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase/flavonone 4-reductase; DODA, DOPA 4,5-dioxygenase; DOPA 3,4-dihydroxyphenylalanine; ESI-MS/MS, electrospray ionization with tandem mass spectrometry; F3′5′H, flavonoid 3′,5′-hydroxylase; F3′H, flavonoid 3′-monooxygenase; F3H, flavanone 3-dioxygenase; HCT, shikimate *O*-hydroxycinnamoyl transferase; UFGT, anthocyanidin 3-*O*-glucosyltransferase; UHPLC–ESI–HRMS, ultra-high-performance liquid chromatography-electrospray ionization-high-resolution mass spectrometry.

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betalain synthetic pathway in the core Caryophyllales that accumulate high amounts of tyrosine and, as a consequence thereof, the substrate for betalain synthesis [7]. In addition, two enzymes downstream of tyrosine synthesis, CYP76AD1 and a 3,4-dihydroxyphenylalanine (DOPA) 4,5-dioxygenase (DODA), underwent gene duplication and concomitant neo-functionalization. These duplications gave rise to DODA-α and CYP76AD1-α isoforms, which seem necessary for betalain synthesis; both new genes arose shortly before the origin of betalain pigmentation [3].

Nepenthales were classified as a noncore group of Caryophyllales [8], which are characterized among other features by lacking betalains [9]. Nepenthales cover noncarnivorous as well as carnivorous lineages. The latter lineage consists of five plant families, including Droseraceae with the genera *Drosera* (sundew) and *Dionaea*, represented by the only species *Dionaea muscipula* (Venus flytrap), and Nepenthaceae with the genus *Nepenthes* (pitcher plants). Almost all of these carnivorous plants have parts with intensive red colors, which are potentially involved in prey attraction. Maybe due to the classification into core and noncore Caryophyllales, there is still confusion about the nature of the red color in carnivorous plants belonging to Caryophyllales. While in *Drosera* spp. anthocyanins, cyanidin and pelargonidin glycosides, have been strongly suggested [5,10], for *D. muscipula* the presence of cyanidin-3-glucoside was demonstrated already in 1966 by chromatographic and spectroscopic (UV, IR) methods in comparison with an authentic standard [11]. Recently, a combination of spectrophotometry, HPLC co-elution and electrospray ionization with tandem mass spectrometry (ESI-MS/MS) proved the presence of delphinidin-3-*O*-glucoside (myrtillin), cyanidin-3-*O*-glucoside (kuromanin), and the cyanidin aglycone in *D. muscipula* [12].

For the genus *Nepenthes*, the situation is not as clear as for *Drosera* and *Dionaea*. There is still no evidence for anthocyanin presence in *Nepenthes*. Nevertheless, there are speculations about the nature of the red coloration in this genus, but no robust data yet. The optical properties of anthocyanins and betalains are very similar, and simple UV/Vis absorption measurements at, for example, 532 nm [13] cannot discriminate both compounds and does not really justify a statement on the pigment's nature. Moreover, some citations are inaccurate and, thus, mere suggestions seem to become true the more often a reference is cited. For example, Moran and Moran [14] are repeatedly cited for the presence of anthocyanins [15,16]. However, with the foliar reflectance analysis used in that study it was not possible to really prove the presence of anthocyanins as the spectral data of anthocyanin and betacyanin are very similar [17]. Also, the study of [18] was listed as a reference for anthocyanins [19] although they only analyzed phenolic compounds and flavonols.

Nevertheless, it has been common sense for many years that also *Nepenthes* species contain anthocyanins, not at least due to molecular studies [1,3,6,7,20]. Unfortunately, the final proof based on reliable chemical analytics as in the case of *Dionaea* [12] is still missing for *Nepenthes*. Here, we aim to evaluate the presence of anthocyanins in *Nepenthes* tissues by employing sensitive analytical techniques, that is, ultra-high-performance liquid chromatography–ESI–high-resolution mass spectrometry (UHPLC–ESI–HRMS). We detected three different cyanidin derivatives and, moreover, found all genes that are necessary for the anthocyanin biosynthetic pathway constitutively expressed.

Materials and methods

Plant material

Nepenthes x ventrata (the natural hybrid of *Nepenthes ventricosa* × *Nepenthes alata*), *Nepenthes thorelii*, *N. ventricosa* plants were grown in the MPI greenhouse at 21–23 °C, 50–60% relative humidity and a 16 h light/8 h dark photoperiod. To keep the plants moistened, they were sprayed with distilled water for 25 s four times per day. Pitchers from *Nepenthes robcantleyi*, *Nepenthes maxima*, *Nepenthes fusca*, and *Nepenthes mirabilis* were provided from the Botanical Garden, Jena, Germany.

Matured and well-developed pitchers were sampled from different plants representing independent biological replicates. Digestive fluid from pitcher was discarded, and pitcher was rinsed three times with sterile ddH_2O. Afterward, tissues of interest (peristome, digestive zone, leaf, branches) were sampled and directly frozen in liquid N_2.

Extraction and quantification of anthocyanins by HPLC-UV

Frozen tissue samples were ground and 100 mg fresh weight powder extracted with 1.0 mL ddH_2O:MeOH (50 : 50 v/v). After mixing, samples were sonicated for 15 min on ice-cold water bath. Therefore, shook for 30 min at 4 °C using Rotator Mixer RM-Multi-1 (STARLAB GmbH, Hamburg, Germany) with the following settings: orbital at 100 r.p.m. for 15 s, reciprocal at 75° for 16 s, and vibro at 3° for 5 s. Samples were centrifuged afterward at 16 000 ***g*** at 4 °C for 30 min, and clear supernatants were collected and used for further analysis.

Anthocyanins were analyzed by reversed-phase HPLC with UV detection using an Agilent 1100 system (Agilent Technologies, Waldbronn, Germany): column used:

Nucleodur Sphinx RP (250 × 4.6 mm, 5 µm; Macherey-Nagel, Düren, Germany); injection volume was 50 µL; flow rate, 1.0 mL·min^{-1}; solvent A, 0.5% (v/v) trifluoroacetic acid; solvent B, acetonitrile. The photodiode array detector was used in the range of 250–650 nm. Samples were analyzed with the following chromatographic gradient: start 5% B, linear gradient from 5% B to 25% B in 20 min followed by a washing cycle. Peaks at 18.1 min and at 18.5 min in the HPLC-UV/Vis chromatograms were identified by match of retention time with commercial standards as cyanidin-3-*O*-galactoside (Extrasynthese, Genay, France) and as cyanidin-3-*O*-glucoside (TransMIT GmbH, Gießen, Germany), respectively. Further identification is based on LC-ESI-HRMS (see below). Quantification was achieved by detection at 520 nm using a calibration curve generated from authentic cyanidin-3-*O*-glucoside.

Identification of anthocyanins by LC-ESI-HRMS

Chemical structures of anthocyanins were determined by UHPLC–ESI–HRMS performed with a Dionex Ultimate 3000 series UHPLC (Thermo Scientific, Schwerte, Germany) and a Bruker timsToF mass spectrometer (Bruker Daltonics, Bremen, Germany). UHPLC was used applying a Zorbax Eclipse XDB-C18 column (100 mm × 2.1 mm, 1.8 µm; Agilent Technologies) with a solvent system of 0.1% (v/v) formic acid (A) and acetonitrile (B) at a flow rate of 0.3 mL·min^{-1}. The elution profile was the following: 0–0.5 min, 5% B; 0.5–11.0 min, 5–60% B; 11.0–11.1 min, 60–100% B, 11.1–12.0 min, 100% B and 12.1–15.0 min 5% B. ESI in positive ionization mode was used for the coupling of LC to MS. The mass spectrometer parameters were set as follows: capillary voltage 4.5 KV, end plate offset of 500 V, nebulizer pressure 2.8 bar, nitrogen at 280 °C at a flow rate of 8 L·min^{-1} as drying gas. Acquisition was achieved at 12 Hz with a mass range from m/z 50 to 1500 with data-dependent MS^2. Fragmentation was triggered at the two most intense peaks applying a target intensity of 20 000 counts, with MS^2 spectra acquisition at 2 Hz, and a limited total cycle time of 2 s. Collision energy was alternated between 20 and 50 to achieve mixed MS^2 spectra.

Search for betalains by HPLC-UV and LC-ESI-HRMS

The HPLC-UV chromatograms at 520 nm from quantification of anthocyanins (see above) where searched for additional peaks that might correspond to betalains. However, in the HPLC-UV chromatograms at 520 nm no other peaks apart from the three described anthocyanins were found. Additionally, betalains were searched for in the raw data from the LC-ESI-HRMS runs in positive ionization mode described above for structure elucidation of anthocyanins. Extracted ion chromatograms for the molecular ion peak $[M + H]^+$ of known betalains [21,22] with an isolation width of m/z 0.002 (Table 1) were inspected for possible peaks. For none of the tested known betalains an $[M + H]^+$ peak could be detected, this means the compound(s) are not there or below the detection limit of the LC-ESI-HRMS system.

Transcriptome analysis: sampling, total RNA extraction, cDNA library preparation, and sequencing

For transcriptome analysis, *N. x ventrata* pitchers were collected first at the opening day of the lid as a reference time point and at the next two consecutive days. To avoid contamination, still-closed pitchers were covered with a mesh as described in [23]. Pitchers were rinsed three times with sterile ddH_2O, digestive zone tissue was dissected sampled in 50 mL polypropylene tubes and directly frozen in liquid N_2. Individual pitchers represent independent biological replicates from different plants. A total of 12 biological

Table 1. Betalains searched in *Nepenthes* spp. peristomes by LC-ESI-HRMS.

Compound	Molecular sum formula	Theoretical m/z for $[M + H]^+$ molecular ion	Detected (D)/not detected (ND)
Amaranthine	$C_{30}H_{34}N_2O_{19}$	727.182853	ND
Isoamaranthine	$C_{30}H_{34}N_2O_{19}$	727.182853	ND
Iresinin I	$C_{36}H_{42}N_2O_{23}$	871.225112	ND
Isoorientin I	$C_{36}H_{42}N_2O_{23}$	871.225112	ND
Celosianin I	$C_{39}H_{40}N_2O_{21}$	873.219633	ND
Isocelosianin I	$C_{39}H_{40}N_2O_{21}$	873.219633	ND
Celosianin II	$C_{40}H_{42}N_2O_{22}$	903.230197	ND
Isocelosianin II	$C_{40}H_{42}N_2O_{22}$	903.230197	ND
Gomphrenin I	$C_{24}H_{26}N_2O_{13}$	551.150765	ND
Isogomphrenin I	$C_{24}H_{26}N_2O_{13}$	551.150765	ND
Gomphrenin II	$C_{33}H_{32}N_2O_{15}$	697.187545	ND
Isogomphrenin II	$C_{33}H_{32}N_2O_{15}$	697.187545	ND
Gomphrenin III	$C_{34}H_{34}N_2O_{16}$	727.198110	ND
Isogomphrenin III	$C_{34}H_{34}N_2O_{16}$	727.198110	ND
Betanin	$C_{24}H_{26}N_2O_{13}$	551.150765	ND
Isobetanin	$C_{24}H_{26}N_2O_{13}$	551.150765	ND
Betanidin	$C_{18}H_{16}N_2O_8$	389.097942	ND
2-Descarboxy-betanidin	$C_{17}H_{16}N_2O_6$	345.108113	ND
Lampranthin II	$C_{34}H_{34}N_2O_{16}$	727.198110	ND
3-Methoxytyramine-betaxanthin	$C_{18}H_{20}N_2O_6$	361.139413	ND
(*S*)-Tryptophan-betaxanthin	$C_{20}H_{19}N_3O_6$	398.134662	ND
Indicaxanthin	$C_{14}H_{16}N_2O_6$	309.108113	ND
Miraxanthin-V	$C_{17}H_{18}N_2O_6$	347.123763	ND
Betalamic Acid	$C_9H_9NO_5$	212.055349	ND

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replicates were used for RNAseq analysis, with four replicates for each time point.

Dissected digestive zones material was finely ground in liquid N_2 using mortar and pestle. Samples were stored at −80 °C until RNA extraction was performed. A 50 mg weighed powdery tissue material was used for total RNA isolation. The extraction was done at room temperature using RP InviTrap® Spin Plant RNA Mini kit (STRATEC Molecular, Berlin, Germany) according to the manufacturer's protocol with some modifications. Total RNA was dissolved in ddH_2O. Each biological sample (digestive zone) was extracted from seven technical replicates and pooled in the final step of RNA elution.

For assessing a rough indicator of quality and yield, A260/A280 and A260/A230 ratios for RNA preparation samples were determined with NanoDrop UV/Vis Spectrophotometer (Thermo Fisher, Schwerte, Germany). To remove any DNA contamination, the isolated RNA was treated with Turbo DNA-free Kit™ (Invitrogen™, Darmstadt, Germany).

Finally, sample quality control was performed using the yield and the assessment of RNA integrity number. This was done based on comparative evaluation of 28S/18S rRNA on an Agilent 2100 Bioanalyzer system following manufacturer's protocol and performed on an Agilent RNA 6000 Nano LabChip® Kit (Agilent Technologies). Transcriptome sequencing was carried out by the Max Planck Genome Center (Cologne, Germany) (https://mpgc.mpipz.de/home/) using poly(A)+ enriched RNA fragmented to an average of 180 nucleotides. Sequencing was done on an Illumina HiSeq3000 Genome Analyzer platform, using standard TruSeq procedures and paired-end (2 × 150 bp) read technology, yielding approximately 15 million reads for each of the 28 *N. x ventrata* samples.

Transcriptome assembly, mapping, and annotation

Quality control measures and *de novo* transcriptome assembly, using the combined RNAseq sequence data was carried out using CLC Genomics Workbench v11.1 (http://www.clcbio.com) To assess transcriptome completeness, we performed a BUSCO (Benchmarking Universal Single-Copy Orthologs; http://busco.ezlab.org) analysis by comparing our assembled transcript sets against a set of highly conserved single-copy orthologs. This was accomplished using the BUSCO v3 pipeline [24], comparing the predicted proteins of the *N. x ventrata* transcriptome to the predefined set of 1614 Embryophyta single-copy orthologs from the OrthoDB v9.1 database. This resulted in 78.7% complete/partial and 21.3% missing BUSCO genes for the pitcher transcriptome assembly. Digital gene expression analysis was carried out using CLC Genomics Workbench v9.1 to generate BAM (mapping) files, and QSEQ Software (DNAStar Inc., Madison, WI, USA) was then used to estimate gene expression levels. Sequence similarity searches of the transcriptome were performed using the NCBI BLAST suite on a Galaxy server against the NCBI nr database. Further sequence annotations were done using Gene Ontology (GO) and InterPro terms (InterProScan, EBI), enzyme classification (EC) codes, and metabolic pathways (Kyoto Encyclopedia of Genes and Genomes, KEGG) implemented in BLAST2GO v5.2 (https://www.biobam.com).

KEGG pathway enrichment analysis

Based on the KEGG orthology (KO), 10 enzymes involved in the anthocyanin biosynthetic pathway were identified: chalcone synthase (K00660); chalcone isomerase (K01859); flavanone 3-dioxygenase (K00475); flavonoid 3′-monooxygenase (K05280); flavonoid 3′,5′-hydroxylase (K13083); bifunctional dihydroflavonol 4-reductase/flavonone 4-reductase (K13082); anthocyanidin synthase, (K05277); anthocyanidin 3-*O*-glucosyltransferase (K12930); shikimate *O*-hydroxycinnamoyl transferase (K13065); 5-*O*-(4-coumaroyl)-D-quinate 3′-monooxygenase (K09754). To verify the putative proteins, all sequences were searched via blastx (*e*-value 1e-3) against NCBI nonredundant database (available from: https://www.ncbi.nlm.nih.gov/; April 23, 2021). Focusing on the essential enzymes, dominantly expressed transcripts with $\log_2$RPKM > 2 (RPKM: reads per kilo base per million mapped reads) at least in one out of three independently on three consecutive days harvested *N. x ventrata* pitcher samples were selected in this study (Table S1).

Results and Discussion

Anthocyanins are a water-soluble group of plant pigments derived from flavonoids. These pigments are produced in the central vacuole of plant cells and occur in reproductive (flowers and fruits) or vegetative (stems, roots, or leaves) plant organs [25]. Still, in vegetative tissues the functional role of anthocyanins remains a controversial topic, ranging from stress response to drought and nutrient deficiency, photoprotection, free radical scavenging, and herbivory defense [26]. In the taxon of core Caryophyllales, anthocyanins are replaced by betalains and the presence of one of these pigments excludes that of the other [1,3,5,6]. Betalains, therefore, have been seen and used as chemotaxonomic markers [27]. Due to their similar locations in plant tissues and cells and their comparable optical features, both pigments are considered to be functional homologues in plant environment interactions [28].

Carnivorous plants of the Droseraceae and Nepenthaceae have been usually assigned to the

Caryophyllales *sensu stricto*, but not to the core group of this order. Thus, for a long time it was not clear which pigments occur in these plants. While for *D. muscipula* (Droseraceae) a recent investigation by Henarejos-Escudero *et al.* [12] eventually demonstrated the presence of the anthocyanins delphinidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside, as well as the aglycone cyanidin, thereby confirming earlier but from a chemical point of view less conclusive results; for Nepenthaceae such study was still missing. As shown in Fig. 1, for *N. x ventrata* peristomes there are only three peaks detected in an HPLC-UV analysis at 520 nm, all three showing the typical UV spectrum of anthocyanins (see inset Fig. 1A). By match of retention time and UV spectra with commercial standards, we proved the presence of cyanidin-3-*O*-glucoside (Fig. 1A, peak #2, major peak; Fig. 1D) and cyanidin-3-*O*-galactoside (Fig. 1A, peak #1; Fig. 1D). The identity was further supported by analysis by LC-ESI-HRMS (Fig. 1C), the molecular ion peak ($[M + H]^+$) of peak #2 at m/z 449.1085 fits the molecular formula of $C_{21}H_{21}O_{11}$(Δ −1.47 p.p.m.), the fragment by collision-induced dissociation at m/z 287.0554 suggests a cyanidin aglycone structure ($C_{15}H_{11}O_6$, Δ −1.34 p.p.m.). The analysis of the commercial cyanidin-3-*O*-glucoside standard resulted in almost identical values (Fig. 1B). The full scan MS data and MS^2 fragmentation pattern data strongly suggest the presence of cyanidin-3-*O*-glucuronide (m/z 463.0874, $C_{21}H_{19}O_{12}$, Δ −0.64 p.p.m.; Fig. 1D), fragment after CID: m/z 287.0553, $C_{15}H_{11}O_6$, Δ −0.99 p.p.m.), represented as peak #3 in the HPLC-UV/Vis chromatogram at 19.2 min (Fig. 1A). These results represent the missing proof for the existence of anthocyanins in Nepenthaceae.

We next analyzed the red colored peristomes and digestive zone of the pitcher of six additional *Nepenthes* species growing from Philippines (*N. x ventrata*; *N. ventricosa*; *N. robcantleyi*, endemic on the island Mindanao), Borneo (*N. fusca*), Sulawesi (*N. maxima*), Vietnam (*N. thorelii*), all together representing the huge area of Southeast Asia. In all species, the dominating anthocyanin was cyanidin-3-*O*-glucoside, followed by cyanidin-3-*O*-galactoside and cyanidin-3-*O*-glucoronide. In all cases, the peristome contained more anthocyanins than the digestive zone, which matches well with the red color of the tissues (Fig. 2). Within the different species, the highest anthocyanin concentration was found in *N. fusca* (*c.* 6.56 $\mu mol \cdot g^{-1}$ fresh weight in peristome and 2.27 $\mu mol \cdot g^{-1}$ fresh weight in digestive zone, respectively). The lowest peristome concentrations were determined in *N. ventricosa* and *N. x ventrata* both with 0.68 $\mu mol \cdot g^{-1}$ fresh weight, the lowest digestive zone concentration in *N. maxima*, *N. mirabilis*, and *N. thorelii* with less than 0.1 $\mu mol \cdot g^{-1}$ fresh weight. In *N. robcantleyi*, no anthocyanins at all could be detected in the digestive zone (Fig. 2). These results indicate that the presence of anthocyanins is widely distributed within Nepenthaceae but also that the different species contain different levels of these pigments.

A more detailed, tissue-specific anthocyanin analysis was performed with branches, leaf blades, pitcher digestive zone, and peristomes of *N. x ventrata* (Fig. 3A). While in the nonred branch and leaf tissues almost no anthocyanins could be found, both the digestive zone and the peristome tissues contained mainly cyanidin-3-*O*-glucoside; the level of cyanidin-3-*O*-galactoside and cyanidin-3-*O*-glucoronide were similar (Fig. 3B). The ratio between the three anthocyanins were constant in the different tissues.

The biosynthetic pathways to anthocyanins are well known, starting with the shikimate pathway, followed by the phenylpropanoid pathway and different possible related routes within the flavonoids biosynthetic pathways leading to the anthocyanidins cyanidin, pelargonidin, and delphinidin and to their respective anthocyanin glycosides [29–31] (Fig. 4A). Most of the biosynthetic enzymes are employed in the generation of the anthocyanidins. This was evident in the transcriptome analysis of the corresponding genes, all of which are constitutively expressed (Fig. 4B), suggesting that the biosynthetic pathways for anthocyanins are active, indicated also by the permanent red coloration. Thus, both molecular evidence (a complete set of constitutively expressed anthocyanin biosynthetic genes) and analytical chemistry-based evidence now demonstrates the existence of anthocyanins in *Nepenthes*.

Although this result is not surprising and was expected, the final proof of anthocyanins in Nepenthaceae was still pending and furthermore supports the recent results for the Droseraceae [12]. We also searched the LC-ESI-HRMS datasets for molecular ion peaks $[M + H]^+$ of known betalains, but did not detect any betalain-corresponding peak (Table 1). Moreover, among the enzymes necessary for betalain biosynthesis we only found three transcripts for two basic enzymes related to DOPA metabolism, for example, an weakly expressed aromatic-*L*-amino-acid/L-tryptophan decarboxylase (K01593; GenBank Acc MZ322092) that may generate dopamine, and two 4,5-DOPA dioxygenase transcripts (*DODA*) (K15777; GenBank Acc MZ322091, MZ322092); no other transcripts of betalain biosynthesis-related genes (*CYP76AD1*, *CYP76AD6*, *5GT*, *6GT*) were detected.

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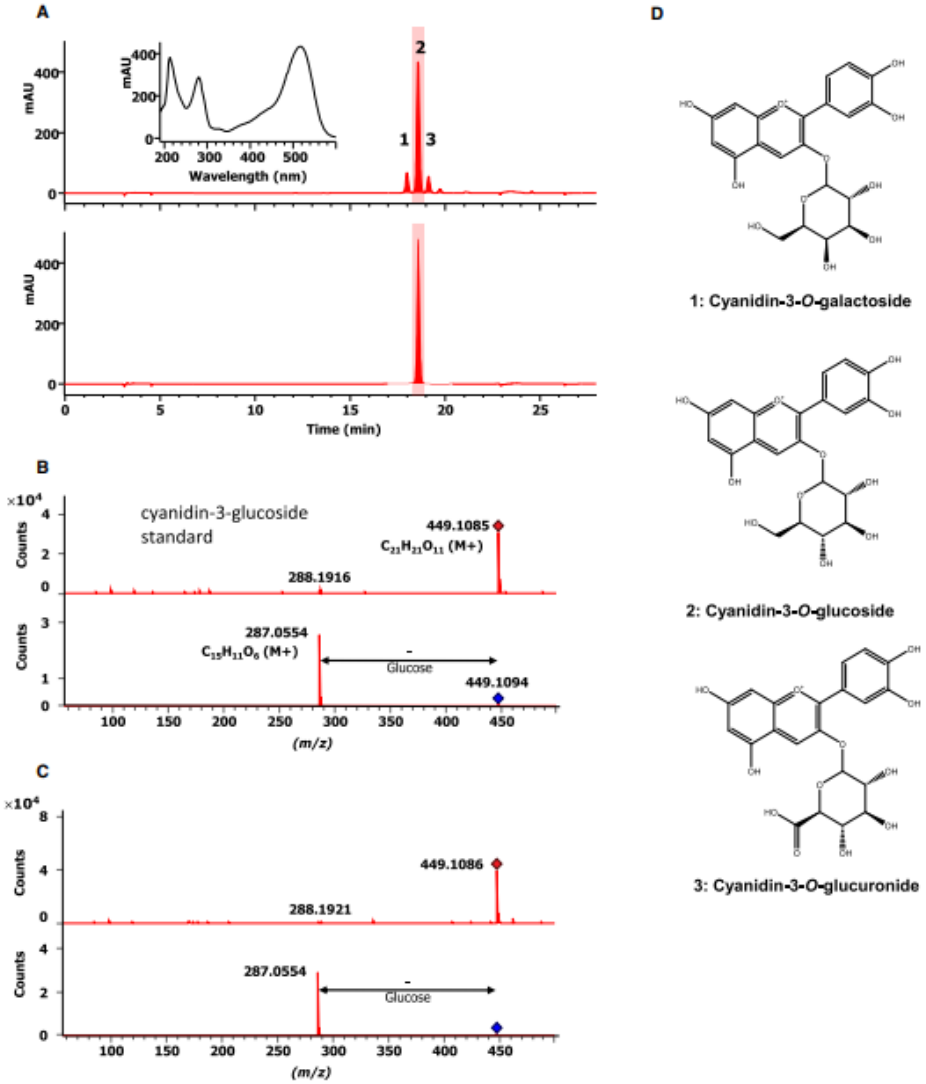

Fig. 1. Identification of cyanidin-3-O-glycosides as the major anthocyanidin compounds in *Nepenthes x ventrata* peristome tissue. (A) HPLC-UV/Vis chromatograms at 520 nm for a *N. x ventrata* peristome extract (upper chromatogram) and a cyanidin-3-*O*-glucoside standard (lower chromatogram). Insert: UV spectrum of (peak #2). Peak #1 was identified as cyanidin-3-*O*-galactoside by comparison to a commercial standard. Peak #3 was tentatively identified by HRMS as cyanidin-3-*O*-glucuronide. (B) Full scan HR-MS spectrum (positive mode) and MS^2 fragmentation spectrum for cyanidin-3-*O*-glucoside standard and (C) peak #2 of *N. x ventrata* peristome extract. (D) Structures of anthocyanins identified from *N. x ventrata* peristome extract. 1. Cyanidin-3-*O*-galactoside corresponding to peak #1 in (A); Chemical formula: $C_{21}H_{21}O_{11}^{+}$; 2. Cyanidin-3-*O*-glucoside corresponding to peak #2 in (A); Chemical formula: $C_{21}H_{21}O_{11}^{+}$; 3. Cyanidin-3-*O*-glucuronide corresponding to peak #3 in (A); Chemical formula: $C_{21}H_{19}O_{12}^{+}$.

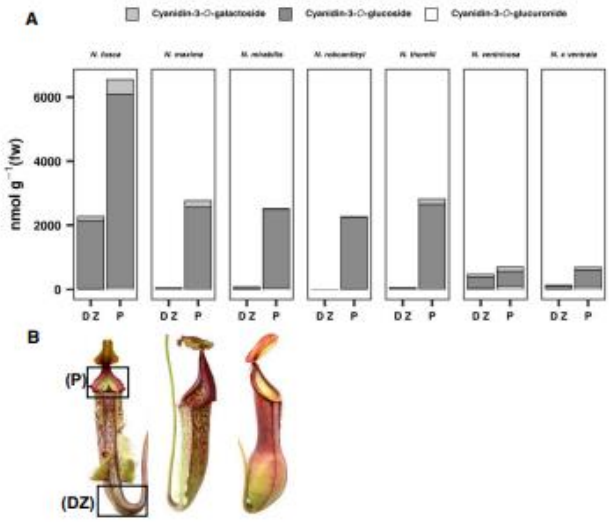

Fig. 2. Proof of principle for the presence of anthocyanins in various species of the genus *Nepenthes*. (A) Distribution and relative amounts of different cyanidin anthocyanins in peristomes and digestive zones of seven *Nepenthes* species. (B) Pitcher tissue pigmentation in three *Nepenthes* species (*Nepenthes fusca*, *Nepenthes maxima*, and *Nepenthes mirabilis*). Black boxes show dissected pitcher tissues harvested for anthocyanin quantification (DZ, digestive zone; P, peristome).

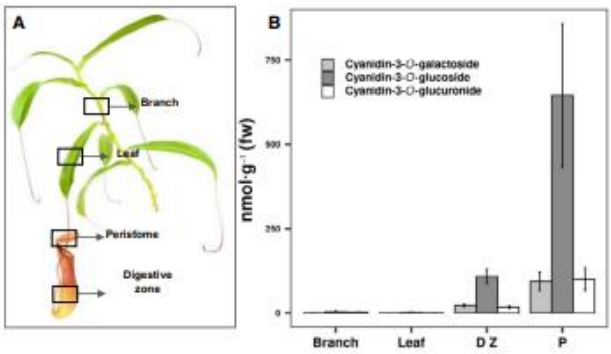

Fig. 3. Distribution and concentrations of anthocyanins in *Nepenthes x ventrata* tissues. (A) Indication of four tissues analyzed for anthocyanin presence: branch, leaf blade, digestive zone, peristome. (B) Determined concentrations of different anthocyanins in *N. x ventrata* tissues ($n = 3$; mean ± SEM).

Although the expression of *DODA* sounds interesting as it may result in the formation of betalamic acid, homologs of *DODA* have been found in many anthocyanin generating taxa within angiosperms [3]. Strikingly, a deep search for betalamic acid was not successful (Table 1) suggesting that the enzyme was not built or it remained inactive.

Hence, it can be postulated that at least all carnivorous plants of the taxon Nepenthales contain anthocyanins rather than betalains. From an ecological and economical point of view, the absence of nitrogen-containing pigments such as betalains makes sense. In particular carnivorous plants that catch insects in order to supplement nutrients with additional nitrogen derived from digested prey [32], should not consume the limited nitrogen for betalain synthesis when anthocyanins might very likely perform similar functions. Nevertheless, not all functions of the red coloration in carnivorous plants are known. The coloration could have initially developed as an adaptive trait, because anthocyanin accumulation is often associated with stress responses [16]. At the same time, it increased

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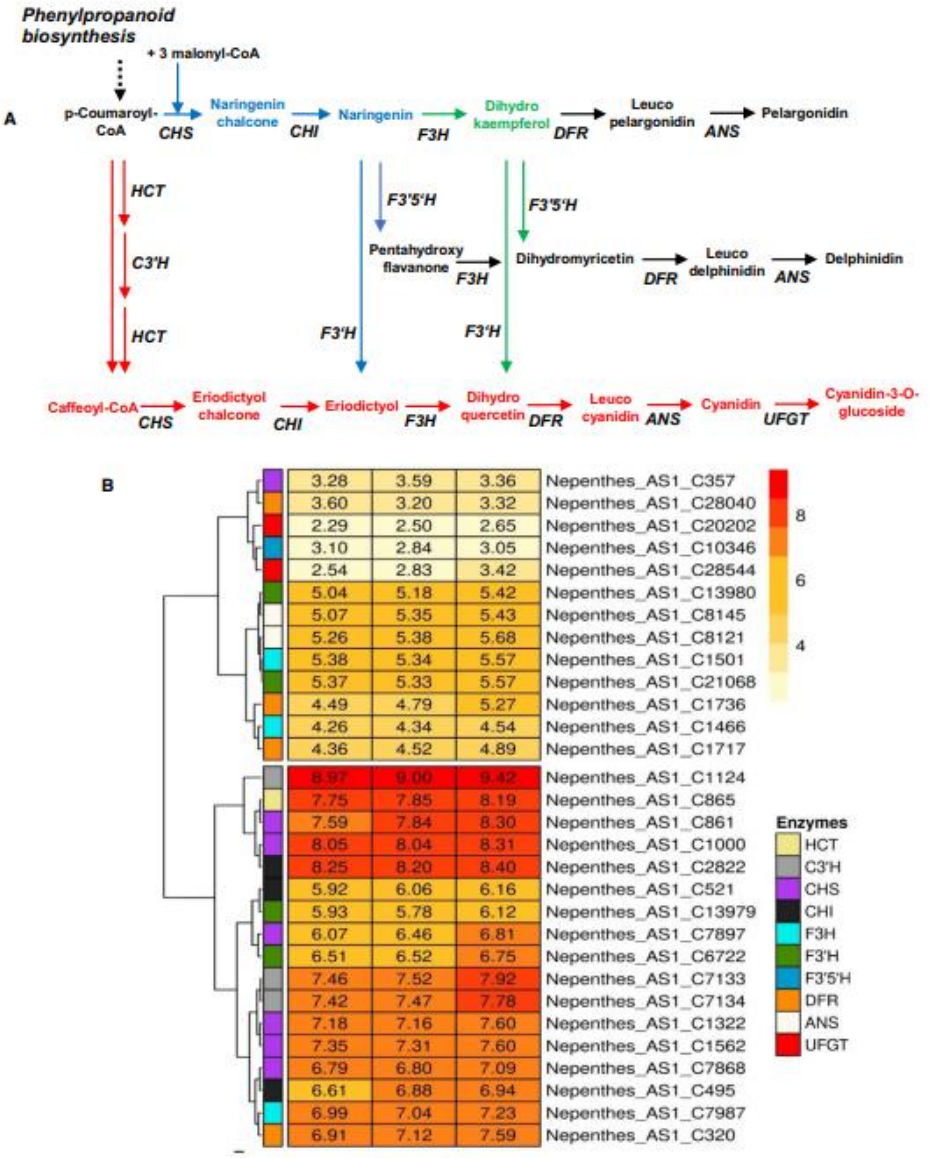

prey capture efficiency of the traps by providing attractive visual signals. As insect prey capture rates positively correlate with levels of red pigmentation, it might enhance the trap efficiency by the red color itself or by providing a special background for better recognition [16]. Concerning herbivores, anthocyanins may

Fig. 4. Molecular evidence for anthocyanin biosynthetic pathways in *Nepenthes x ventrata* pitchers. (A) Anthocyanin biosynthesis pathways. Red, blue, and green colors represent putative pathways for cyanidin 3-*O*-glucoside synthesis in *N. x ventrata*. In black, the metabolic pathway for pelargonidin and delphinidin synthesis; both compounds were not found in this study. Enzymes involved in the pathway are indicated in italics: ANS, anthocyanidin synthase; C3´H, 5-*O*-(4-coumaroyl)-D-quinate 3′-monooxygenase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, bifunctional dihydroflavonol 4-reductase/flavonone 4-reductase; F3′5′H, flavonoid 3′,5′-hydroxylase; F3′H, flavonoid 3′-monooxygenase; F3H, naringenin 3-dioxygenase; HCT, shikimate *O*-hydroxycinnamoyl transferase; UFGT, anthocyanidin 3-*O*-glucosyltransferase. (B) KEGG pathway enrichment analysis. Gene expression profiles of 30 unique transcripts in anthocyanin biosynthetic pathway, based on RPKM values. Candidate genes with $\log_2$RPKM > 2 in at least one sample (columns 1, 2, and 3; independent *N. x ventrata* pitcher samples harvested on three consecutive days) are represented.

protect the tissue from attack by herbivores, which are attracted by green color [19,33]. Moreover, anthocyanins have antioxidant activities which might protect the plant against reactive oxygen species [33]. The strong red pigmentation of the peristome in all *Nepenthes* species supports these hypotheses. Here, more studies need to be done.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

AD-L and AM designed the study. AD-L and MR performed experiments. AD-L, MR, DW, HV, and AM analyzed and interpreted the results. AD-L and AM wrote the manuscript. All authors revised and approved the final version and agree to be held accountable for the article content.

Data Accessibility

Additional supporting information may be found online in the Supporting Information section at the end of the article. GenBank accession numbers are free from Jan 1st, 2022, and available upon request.

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Supporting information

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Table S1. Anthocyanin biosynthesis gene index and GenBank accession numbers.

Supplementary material manuscript No. 2

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Supporting Information

Filename	Description
feb413255-sup-0001-TableS1.xlsx application/excel, 11 KB	**Table S1**. Anthocyanin biosynthesis gene index and GenBank accession numbers.

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Name	Description	IDs
Nepenthes_AS1_C865	shikimate *O* -hydroxycinnamoyl transferase	HCT01
Nepenthes_AS1_C1124	5-O-(4-coumaroyl)-D-quinate 3'-monooxygenase	C3'H01
Nepenthes_AS1_C7133	5-*O* -(4-coumaroyl)-D-quinate 3'-monooxygenase	C3'H02
Nepenthes_AS1_C7134	5-*O* -(4-coumaroyl)-D-quinate 3'-monooxygenase	C3'H03
Nepenthes_AS1_C357	chalcone synthase	CHS01
Nepenthes_AS1_C861	chalcone synthase	CHS02
Nepenthes_AS1_C1000	chalcone synthase	CHS03
Nepenthes_AS1_C1322	chalcone synthase	CHS04
Nepenthes_AS1_C1562	chalcone synthase	CHS05
Nepenthes_AS1_C7868	chalcone synthase	CHS06
Nepenthes_AS1_C7897	chalcone synthase	CHS07
Nepenthes_AS1_C495	chalcone isomerase	CHI01
Nepenthes_AS1_C521	chalcone isomerase	CHI02
Nepenthes_AS1_C2822	chalcone isomerase	CHI03
Nepenthes_AS1_C1466	naringenin 3-dioxygenase	F3H01
Nepenthes_AS1_C1501	naringenin 3-dioxygenase	F3H02
Nepenthes_AS1_C7987	naringenin 3-dioxygenase	F3H03
Nepenthes_AS1_C6722	flavonoid 3'-monooxygenase	F3'H01
Nepenthes_AS1_C13979	flavonoid 3'-monooxygenase	F3'H02
Nepenthes_AS1_C13980	flavonoid 3'-monooxygenase	F3'H03
Nepenthes_AS1_C21068	flavonoid 3'-monooxygenase	F3'H04
Nepenthes_AS1_C10346	flavonoid 3',5'-hydroxylase	F3'5'H01
Nepenthes_AS1_C320	bifunctional dihydroflavonol 4-reductase/flavonone 4-reductase	DFR01
Nepenthes_AS1_C1717	bifunctional dihydroflavonol 4-reductase/flavonone 4-reductase	DFR02
Nepenthes_AS1_C1736	bifunctional dihydroflavonol 4-reductase/flavonone 4-reductase	DFR03
Nepenthes_AS1_C28040	bifunctional dihydroflavonol 4-reductase/flavonone 4-reductase	DFR04
Nepenthes_AS1_C8121	anthocyanidin synthase	ANS01
Nepenthes_AS1_C8145	anthocyanidin synthase	ANS02
Nepenthes_AS1_C20202	anthocyanidin 3-*O* -glucosyltransferase	UFGT01
Nepenthes_AS1_C28544	anthocyanidin 3-*O* -glucosyltransferase	UFGT02

10enzymes_index

Manuscript No. 3

Manuscript overview

Manuscript title: Plumbagin, a Potent Naphthoquinone from *Nepenthes* Plants with Growth Inhibiting and Larvicidal Activities

Authors: Asifur, Rahman-Soad, Alberto Dávila-Lara, Christian Paetz, Axel Mithöfer

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The candidate is:

□ First author, □ Co-first author, □ Corresponding author, ■ Co-author.

Status: Published

Authors' contributions (in %) to the given categories of the publication

Author	Conceptual	Data analysis	Experimental	Writing the manuscript	Provision of material
Asifur Rahman-Soad	30 %	40 %	80 %	30 %	-
Alberto Dávila-Lara	20 %	30 %	10 %	20 %	-
Christian Paetz	10 %	10 %	10 %	10 %	10 %
Axel Mithöfer	40 %	20 %		40 %	90 %
Total:	100%	100%	100%	100%	100%

Signature candidate

Signature supervisor (member of the faculty)

molecules

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Article

Plumbagin, a Potent Naphthoquinone from *Nepenthes* Plants with Growth Inhibiting and Larvicidal Activities

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Abstract: Some plant species are less susceptible to herbivore infestation than others. The reason for this is often unknown in detail but is very likely due to an efficient composition of secondary plant metabolites. Strikingly, carnivorous plants of the genus *Nepenthes* show extremely less herbivory both in the field and in green house. In order to identify the basis for the efficient defense against herbivorous insects in *Nepenthes*, we performed bioassays using larvae of the generalist lepidopteran herbivore, *Spodoptera littoralis*. Larvae fed with different tissues from *Nepenthes x ventrata* grew significantly less when feeding on a diet containing leaf tissue compared with pitcher-trap tissue. As dominating metabolite in *Nepenthes* tissues, we identified a naphthoquinone, plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone). When plumbagin was added at different concentrations to the diet of *S. littoralis* larvae, an EC_{50} value for larval growth inhibition was determined with 226.5 μg g^{-1} diet. To further determine the concentration causing higher larval mortality, sweet potato leaf discs were covered with increasing plumbagin concentrations in no-choice-assays; a higher mortality of the larvae was found beyond 60 μg plumbagin per leaf, corresponding to 750 μg g^{-1}. Plant-derived insecticides have long been proposed as alternatives for pest management; plumbagin and derivatives might be such promising environmentally friendly candidates.

Keywords: naphthoquinones; plumbagin; *Spodoptera littoralis*; insect growth inhibition; carnivorous plants; *Nepenthes*

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1. Introduction

Nepenthes is a tropical plant genus occurring mainly in Southeast Asia. Plants of this genus are carnivorous. They attract, catch, and digest insect prey in order to get additional nutrients, primarily, nitrogen and phosphate [1,2]. Therefore, *Nepenthes* species developed a pitfall trap (Figure 1), called pitcher, where insect prey falls inside due to a slippery surface and drown in a digestive fluid [1,2]. As in many other carnivorous plants, also the genus *Nepenthes* harbors a large chemical diversity; currently, several secondary metabolites are isolated for pharmaceutical, biotechnological, and ethnobotanical use [3,4]. Especially, *Nepenthes* species are well known in traditional medicine. Multiple reports are in the literature describing curative effects of *Nepenthes* extracts on diseases, e.g., on hypertension, cough, fever, urinary system infections [5], malaria [6,7], pain, asthma [7], *Staphylococcus* infection [8], celiac disease [9], and oral cancer cells [10].

However, up to now, most of the chemical analysis in *Nepenthes* has been done for the digestive pitcher fluid. Here, metabolites with antimicrobial properties have been found, e.g., naphthoquinones (NQ; droserone, 5–*O*–methyl droserone in *N. khasiana* [11]; plumbagin, 7-methyl-juglone in *N. ventricosa* [12]). Thus, it is hypothesized that such compounds mediate protection against microbes and preserve prey during digestion [11–14]. NQ derivatives are also described for tissues of various *Nepenthes* species including the pitchers [12,15–17]. In particular, plumbagin is of broad pharmaceutical interest because

it is a candidate that may be used in therapies against various cancers or chronic diseases [18–21]. In addition to NQ, carotenoids, flavonoids, sterols, and triterpenes are described for *Nepenthes* leaves [1,22,23]. Recently, an untargeted metabolomics approach was performed in *N. x ventrata* comparing secondary metabolites of leaves and pitcher tissue before and after prey catches [24]. In that study, about 2000 compounds (MS/MS events) were detected in the two tissues showing enormous metabolome diversity, which was even higher in leaves. Strikingly, the tissue specificity of chemical compounds could significantly discriminate pitchers from leaves. Besides many yet unknown compounds, the common constituents were phenolics, flavonoids, and NQ [24]. These data suggest that the metabolite composition of the tissues can point to their function. In addition, the metabolite composition may represent mechanisms that promote the evolution of plant carnivory as well as enable the plants to cope with environmental challenges [14].

Figure 1. *Nepenthes x ventrata*. A natural hybrid of *N. ventricosa* and *N. alata*. Copyright © A. Rahman-Soad.

(A)biotic challenges include the attack of herbivorous insects. Interestingly, there are only a very few observations and studies published concerning the attack of insects on tissues of carnivorous pitcher plants. Recently, lepidopteran herbivory was described for some species of the new world pitcher plant *Sarracenia* [25,26]. There is only one investigation showing that *N. bicalcarata* plants are attacked by an insect, the weevil *Alcidodes spec*. [27]. Another study shows that in *N. gracilis* red pitchers experience less herbivory than green ones [28]. To the best of our knowledge, no other studies have been published yet that focus on herbivore damage in *Nepenthes*. Obviously, the carnivorous syndrome obtained much more attention. However, herbivory on *Nepenthes* tissue is obviously rare.

The reason for this is not known but it is unlikely that all herbivores are caught and digested. Instead, *Nepenthes* very likely has an efficient setting of defensive chemistry, which is not unusual in many plants [29]. In order to address this hypothesis and gain more insight in the ecological relevance of *Nepenthes* metabolites, we performed bioassays to study the effect of tissue of *N. x ventrata*, a robust natural hybrid of *N. alata* and *N. ventricosa*, on the feeding behavior and larval development of the generalist insect herbivore *Spodoptera littoralis*.

2. Results and Discussion

2.1. Effect of Nepenthes x ventrata Tissue on Insect Larvae Growth

The observation that *Nepenthes* plants are rarely infested by insect herbivores forced us to study this phenomenon. Therefore, freshly harvested tissues from *N. x ventrata* leaves and pitchers were added to an artificial diet and fed to larvae of the generalist herbivore *Spodoptera littoralis*. As can be seen in Figure 2A, starting at day 4 to 5, the presence of leaf but not pitcher tissue significantly affected the performance of the larvae, which gained less weight. At this point, it might be worth to mention that recently in *N. x ventrata* [24] and before in *N. khasiana* [15], the concentration of a NQ, very likely plumbagin, was determined to be significantly higher in leaves compared with pitchers, which may explain the result found in Figure 2A. We also could support these results by comparing plumbagin content in pitcher vs. leaf; by quantitative NMR analysis, we found a 5.2-fold higher plumbagin concentration in leaf compared with pitcher tissue (650 and 125 μg g^{-1} FW, respectively). Although significant, the growth inhibition effect was not very pronounced. Thus, the feeding experiment was repeated with dried leaf tissue in order to add more plant material to the diet, knowing that the water content of *N. x ventrata* tissue is about 90% [24]. Here, the effect of the plant tissue was more distinct (Figure 2B). Both quantities of leaf tissue, 10% and 15% (*w*/*w*), showed clear impairment on the growth and weight of the feeding *S. littoralis* larvae already at day 2. Starting from day 3 on, there was also a significant difference between the larvae feeding on either 10% or 15% of *Nepenthes* tissue that was included in the diet (Figure 2B).

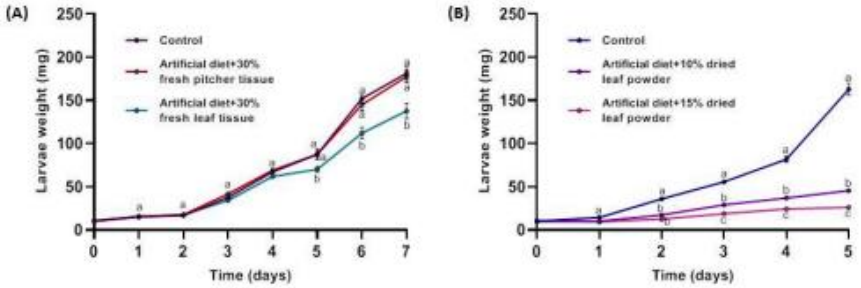

Figure 2. Performance of *Spodoptera littoralis* larvae feeding on artificial diet containing (**A**) fresh leaf powder of *Nepenthes x ventrata* leaf and pitcher (30% (*w*/*w*)) or (**B**) dried *N. x ventrata* leaf powder (10 and 15% (*w*/*w*)) Larvae were weighed every day for 7 days. Mean (± SE) labelled with different letters indicate significant difference ($p < 0.05$); two-way ANOVA, Šidák's multiple comparisons test; $n = 15$.

2.2. Plumbagin in Nepenthes x ventrata Tissue

In many carnivorous plants belonging to the order Nepenthales [14], a *sensu stricto* sister group to Caryophyllales [30] and including the plant families Droseraceae and Nepenthaceae, the presence of NQ has been described [31]. This includes species such as *Aldrovanda vesiculosus*, *Dionaea muscipula*, *Drosophyllum lusitanicum*, as well as the genera *Drosera* and *Nepenthes* [31]. Among their secondary compounds, in particular, plumbagin

is slightly volatile; thus, its presence in plant tissue is often indicated by spontaneous sublimation, thereby staining the tissue surface or plastic material used for storage. We observed this effect with both leaf and pitcher tissue (Figure 3) stored in plastic vials. In order to proof its identity, a part of the compound was removed from the wall of the plastic vial by extraction with dichloromethane.

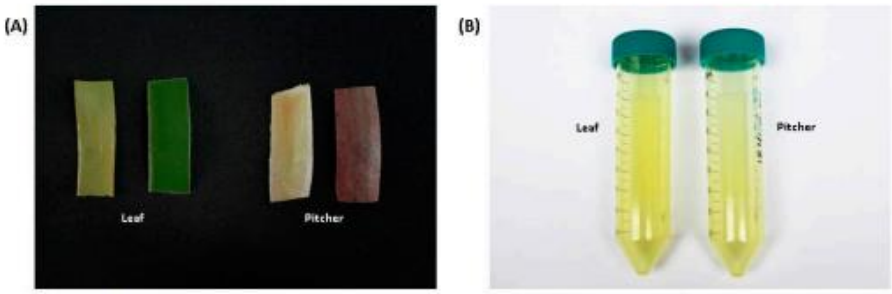

Figure 3. (**A**) Tissues of dry *Nepenthes x ventrata* leaf and pitcher stored for 6 months in a plastic tube. Sublimed compounds cover the dry material with a yellowish color (left) in comparison with freshly cut tissue (right). (**B**) Plastic tubes that stored the different tissue types for 6 months. New tubes do not show any color.

After evaporation of the solvent, the residue was used for NMR analysis. In parallel, leaf extracts from *N. x ventrata* were analyzed by ^{1}H-NMR as well (Figure 4). When compared with a reference, it could be confirmed that the sublimed volatile compound was indeed plumbagin, and this compound could also be proven in leaf material (Figure 4).

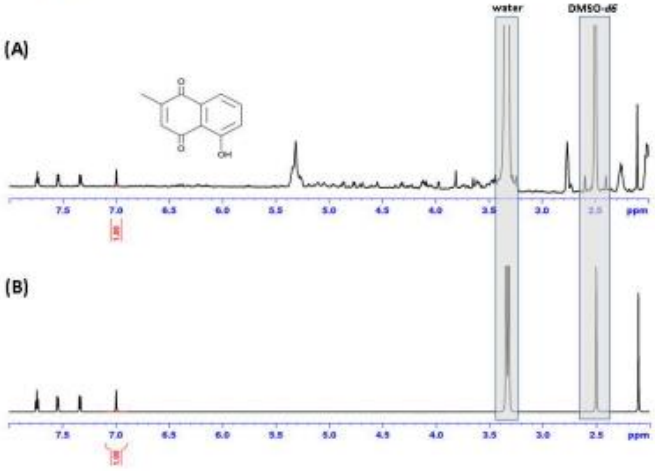

Figure 4. *Cont.*

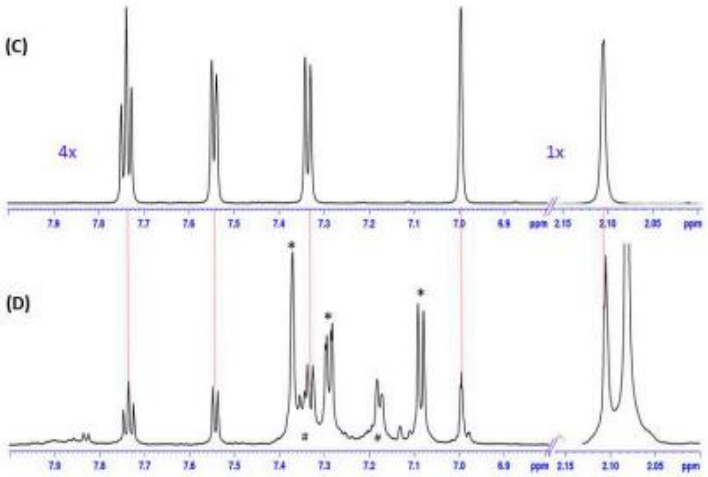

Figure 4. ^{1}H-NMR spectra in DMSO-d_6. (**A**) Plumbagin (see insert) extracted from *Nepenthes x ventrata* leaves and (**B**) a plumbagin reference. (**C**) Details of ^{1}H NMR spectra of a plumbagin reference and (**D**) the volatile exudate emitted by *N. x ventrata* pitcher material. Asterisks (*) indicate the presence of 4-tert-butylcatechol, a polymerization inhibitor probably extracted from the plastic material, and hashes (#) account for an unidentified impurity. The intensity of the aromatic range in (**C**) was increased as indicated by the factor.

These results raised the question of the function of plumbagin and other NQ in carnivorous plants and in *Nepenthes*. In general, NQ are highly bioactive compounds. Besides pharmacological properties against malaria, various cancers, inflammation, and much more [6,19,32–34], they have allelopathic effects as shown for the walnut trees (*Juglans* spp.) releasing the phytotoxin juglone (5-hydroxy-1,4-naphthalenedione) [35,36]. Many defense-related properties are associated with NQ, among them are activities against numerous microbes including human- and phytopathogenic parasites, bacteria, and fungi [31–33]. That means, the NQ might protect the plants from pathogen infection. In addition, for *N. khasiana*, it could be shown that droserone and its derivative 5–*O*–droserone provided antimicrobial protection in the pitcher fluid of [11,37]. Buch and coworkers identified plumbagin and 7-methyl-juglone in the pitcher fluid of *N. ventricosa* [12]. These results suggest a role for NQ in the pitcher fluid in order to control the microbiome in the digestive fluid, together with, e.g., pathogenesis-related proteins such as PR-1 [13,37].

2.3. Growth-Inhibiting and Larvicidal Activities of Plumbagin

Besides the hypothesis that NQ are involved in defense against microbial infection, there are several studies showing that these compounds can also affect insects [31–33,38–43]. We, therefore, performed feeding experiments with plumbagin-supplemented artificial diet and measured the weight of *S. littoralis* larvae every day. Knowing that the amount of plumbagin in *Nepenthes* leaves is about 0.05% of fresh weight [15], we covered a concentration range between 100 and 900 µg g^{-1}, representing 0.01–0.09% fresh mass, respectively. As shown in Figure 5, with increasing plumbagin concentrations, the larvae gained less weight. Based on these data the EC_{50} value was calculated indicating the plumbagin concentration necessary for 50% growth inhibition (weight gain), which was determined as 226.5 µg g^{-1} diet. For some lepidopteran species such as *Spodoptera litura*, *Achaea janata*, and *Trichoplusia ni*, it already has been shown that plumbagin affects the feeding behav-

ior [38–41]. However, in those experiments, the focus of the analysis was on the level of feeding-avoidance rather than on the larval growth.

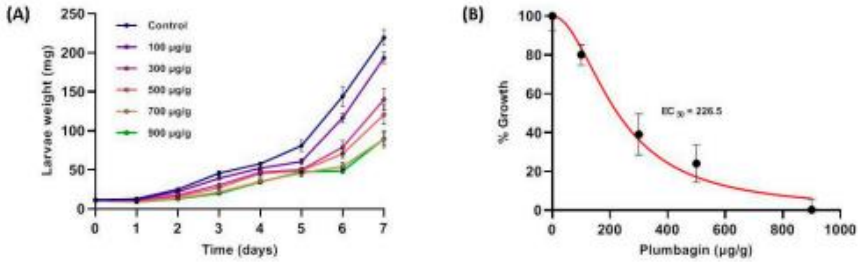

Figure 5. (**A**) Performance of *Spodoptera littoralis* larvae feeding on artificial diet containing various concentrations of plumbagin. Larvae were weighed every day for 7 days. Mean (±SE), $n = 15$. (**B**) Determination of EC_{50} value based on the data obtained in (**A**). EC_{50} was calculated with 226.5 and 1.2 µmol g^{-1} diet, respectively.

In contrast to most other bioassays that analyzed the antifeeding activity of plumbagin, here, the compound of interest was included in the food, not painted on leaves of various plant species. Nevertheless, in order to determine the mortality rate of larvae feeding on plumbagin, we also carried out an experiment using the approach with plumbagin-painted leaves. Therefore, a sweet potato cultivar (Tainong 66) that is known to be susceptible to herbivores and does not induce strong defense response upon attack was selected [44]. In first experiments, we observed that *S. littoralis* larvae even preferred cannibalism than feeding on those leaves. As a consequence, only individualized larvae were used. Up to a plumbagin concentration of 60 μg^{-1} leaf (13.3 µg cm^{-2}, 750 µg g^{-1} leaf) no larvicidal effect was determined for the period analyzed (Figure 6A). With 90 μg^{-1} leaf (20 µg cm^{-2}; 1.125 mg g^{-1} leaf) dead larvae could be found at the end of day 4 and the survival rate drop to 50% at the end of day 5. At 120 μg^{-1} leaf (26.7 µg cm^{-2}; 1.5 mg g^{-1} leaf), dead larvae were detected at day 3 and until the end of day 7, all larvae have died (Figure 6A). For *T. ni* feeding on plumbagin-covered cabbage leaves, an antifeeding effect was also determined in the low microgram per square centimeter range [41]. It also can be seen that the larvae avoided feeding on the leaves covered with high concentrations of plumbagin (Figure 6B,C). With respect to the results shown in Figure 5, it seems that larval growth is heavily affected at higher plumbagin concentrations of around 700 µg plumbagin g^{-1} diet. However, the larvae were affected in growth but still survived at all concentrations tested (up to 900 µg g^{-1}). The plumbagin concentrations used in the no-choice assay also showed no mortality up to 750 µg g^{-1} leaf tissue. Only at the used concentration of 1.125 µg g^{-1} leaf, we found the first larvae dying. This suggests that there might be a threshold of about 1 mg g^{-1} food before the *S. littoralis* larvae begin to die. The experiment is somehow comparable with a recent study by Hu and colleagues [42]. They investigated the mortality of *Pieris rapae* and *Helicoverpa armigera* feeding on cabbage leaves dipped into solutions with different concentrations of plumbagin and juglone, respectively. For plumbagin, IC_{50} values of 11 µg mL^{-1} (*P. rapae*) and 30 µg mL^{-1} (*H. armigera*) were calculated [42]. However, these data are hard to rank as it is not known how much of the compounds of interest was finally on or in the leaf disc. Nevertheless, for all the latter assays, it is difficult to discriminate whether the larvae really die either because of the ingested compounds or of hunger as they consequently avoid feeding. Other studies used topical assays where the compound was added directly onto the insect's (e.g., *S. litura*, *A. janata*, and *Musa domestica*) body to investigate the toxicity of compounds [38,43]. This approach is worth to carry out but not qualified for studies on activities of compounds that are incorporated during herbivory.

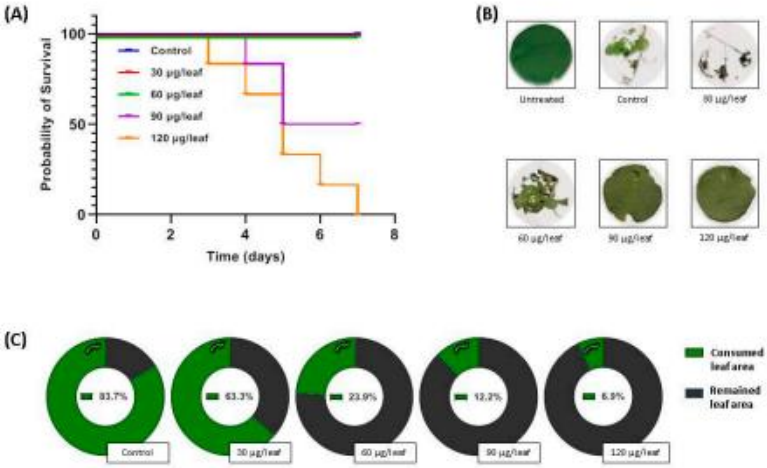

Figure 6. (**A**) Survival rate of *Spodoptera littoralis* larvae feeding on *Ipomoea batatas* (sweet potato) leaf discs painted with various concentrations of plumbagin ($n = 6$). (**B**) Representative leaf discs at the end of the feeding period of day three. Leaf disks were renewed every day. (**C**) Leaf areas consumed by *S. littoralis* larvae (indicated in green) at day 3 depending on the applied plumbagin concentration.

However, the mode of action of NQ is not completely known. In general, NQ are redox-active compounds that can generate oxidative stress [33]; moreover, there are hints for specific inhibition of enzymes and, hence, processes involved in insect development mainly the molting process in insects, e.g., the enzymes phenoloxidase [30], chitin synthetase [45], or ecdysone 20-monooxygenase [46]. The interaction with molting hormone pools is discussed as well [47]. Another study showed that in *Anopheles stephensi*, the level of certain enzymes such as esterases and SOD was decreased significantly in the presence of plumbagin, which also was active as repellent against *A. stephensi* at a concentration of 100 µg mL^{-1}. Further histological investigations showed that muscles, midgut, and hindgut were the most affected tissues [48]. However, most studies suggest that, most likely, the insecticidal activity of plumbagin is based on the inhibition of ecdysis. This also includes a certain specificity against insects compared with neurotoxic insecticidal compounds.

Botanical or plant-derived insecticides have long been touted as environmentally friendly alternatives to synthetic insecticides for pest and disease management [3]; NQ combine the advantage of both low toxicity, compared with conventional pesticides, and restricted environmental contamination and, thus, might be promising candidates for an ecological agriculture.

3. Materials and Methods

3.1. Insects and Plants

Spodoptera littoralis Boisd. (Lepidoptera: Noctuidae) were hatched from eggs kindly provided by Syngenta Crop Protection (Stein, Switzerland) and reared on artificial diet (500 g hackled beans, 9 g ascorbic acid, 9 g 4-ethylbenzoic acid, 9 g vitamin E Mazola oil mixture (7.1%), 4 mL formaldehyde, 1.2 L water, 1 g-sitosterol, 1 g leucine, 10 g AIN-76 vitamin mixture, and 200 mL (7.5%) agar-water solution) at 23–25 °C with a 14 h photoperiod. Sweet potato (*Ipomoea batatas* Lam. cv Tainong 66) scions were grown as described [40] under a 16/8 h light/dark regime at 28/25 °C, respectively, and 70% relative humidity. *Nepenthes x ventrata* (*N. alata x N. ventricosa* hybrid) plants were grown at 21–23 °C, 50–60% relative humidity, and a 16/8 h light/dark photoperiod. Pitcher and

the associated leaf tissues were harvested at the time when the pitchers were just opened, directly frozen in liquid nitrogen and ground with mortar and pestle. Material was used directly (fresh) or freeze-dried before use.

3.2. Feeding Assays

For feeding assays, second to third instar larvae of *S. littoralis* were used. Ground fresh or dried plant material (leaves and pitcher) from *N. x ventrata* was added to the artificial diet with the indicated quantities (*w*/*w*). Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone, $C_{11}H_8O_3$; Fischer Scientific, Schwerte, Germany) was dissolved in acetone and added to the diet. Controls were prepared in the same way without plumbagin. At all the time, it was made sure that acetone was evaporated. For these feeding assays, 15 independent repeats were done. No-choice leaf disks feeding assays according to [34] were further performed on sweet potato. Therefore, leaf discs of 24 mm in diameter were punched out with a cork borer put directly on wet filter paper in a petri dish (5.5 cm diameter). Plumbagin was solved as described before and diluted to the required concentration with 2.5% (*w*/*v*) PEG 2000 (Sigma-Aldrich, Taufkirchen, Germany). That solution was added onto the surface of the discs at the concentrations indicated. For the no-choice assays, 6 independent repeats were performed.

Every day fresh diet or leaf discs were provided. All assays were performed with individual larvae to avoid cannibalism. Larvae were reared for the indicated periods on the particular diets and weighed at the given time.

3.3. Isolation of Plumbagin from Nepenthes x ventrata Leaves

Freshly harvested *N. x ventrata* leaves (7.3 g) were immediately frozen in liquid N_2 and freeze-dried. Dried tissue was ground and extracted with 100 mL dichloromethane (DCM) for 15 min by stirring in Erlenmeyer flasks. After precipitation for 20 min, the clear supernatant (50 mL) was collected and another 50 mL DCM was added to the remaining material for re-extraction, which was repeated six times. Collected supernatants were filtered, combined, and DCM was removed using a rotary evaporator. The dried extract (9.3 mg) was dissolved in 2 mL DCM transferred into a HPLC vial and dried again under N_2 stream. For the whole procedure, only glassware was used. The NQ in the extract was identified by means of NMR spectroscopy by comparing spectral data with those of an authentic standard (plumbagin).

N. x ventrata leaf material was kept in 50 mL polypropylene tubes at room temperature over 6 months during which the NQ sublimed (Figure 4), leaving a yellowish stained plastic material. Absorbed compounds were extracted from closed tubes with DCM (10 mL) for 3 days at room temperature. The extract was transferred into a glass vial and evaporated using N_2 gas. The residue was reconstituted with DMSO-d_6 and subjected to NMR analysis.

Identity of the sublimed and extracted plumbagin was confirmed by ^{1}H-NMR spectroscopy. NMR spectra were measured on a Bruker Avance III HD spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) equipped with a cryoplatform and a TCI 1.7 mm Micro-CryoProbe. Spectra were referenced to the residual solvent signal for DMSO-d_6 at δH 2.50. Spectrometer control and data processing was accomplished using Bruker TopSpin 3.6.1, and standard pulse programs as implemented in Bruker TopSpin 3.6.1 were used.

For a quantitative comparison of ^{1}H NMR spectra of extracts of *N. x ventrata* leaf and pitcher tissue, the spectral intensity was adjusted to equal solvent signal areas. The areas of signals accounting for plumbagin (range: δH 8.00–7.00) were determined and used for calculation based on the respective areas of a plumbagin standard. For preparation of the experiment, 729 mg (FW) of each tissue was ground in liquid N_2 and extracted with 20 mL of dichloromethane in closed vessels at room temperature with shaking. Extracts were filtered through Chromabond PTS phase separation cartridges (Macherey-Nagel, Düren, Germany) and the flow-through was evaporated with N_2 gas at room temperature within 30 min. Afterwards, the residue was reconstituted with 1.2 mL DMSO-*d*6 and subjected to ^{1}H-NMR spectroscopy.

3.4. Statistical Analysis

Statistical calculations were performed using GraphPad Prism version 9.0.0 in all cases. Details are indicated in the particular figure legends. For EC_{50} analysis, the total response was normalized to run between 0% and 100% using control data. For growth experiments, larvae were picked randomly from a large population and all experiments were conducted out under highly standardized conditions to avoid investigator-included bias.

4. Conclusions

Naphthoquinones are known metabolites in several plant species. Among these are various carnivorous plants including the pitcher plant *Nepenthes*. Plumbagin is a prominent NQ in *Nepenthes x ventrata* and it was detected by ^{1}H-NMR in tissues in different concentrations (100 and 650 μg g^{-1} fresh weight in pitcher and leaf, respectively). Plumbagin has known antimicrobial activities and is of pharmaceutical interest. Now, in different feeding assays with *Spodoptera littoralis* larvae the anti-feeding, growth-inhibiting and larvicidal activity of plumbagin or plumbagin-containing tissues was demonstrated at naturally occurring concentrations. Plumbagin as well as other NQ might become alternative compounds as natural insecticides in agriculture.

Author Contributions: A.R.-S., A.D.-L., and A.M. conceived the study and experiments. A.R.-S., A.D.-L., and C.P. performed the experiments and analyzed data. A.R.-S., A.D.-L., C.P., and A.M. discussed the data and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary material manuscript No. 3

3.4. Statistical Analysis

Statistical calculations were performed using GraphPad Prism version 9.0.0 in all cases. Details are indicated in the particular figure legends. For EC_{50} analysis, the total response was normalized to run between 0% and 100% using control data. For growth experiments, larvae were picked randomly from a large population and all experiments were conducted out under highly standardized conditions to avoid investigator-included bias.

4. Conclusions

Naphthoquinones are known metabolites in several plant species. Among these are various carnivorous plants including the pitcher plant *Nepenthes*. Plumbagin is a prominent NQ in *Nepenthes x ventrata* and it was detected by ^{1}H-NMR in tissues in different concentrations (100 and 650 μg g^{-1} fresh weight in pitcher and leaf, respectively). Plumbagin has known antimicrobial activities and is of pharmaceutical interest. Now, in different feeding assays with *Spodoptera littoralis* larvae the anti-feeding, growth-inhibiting and larvicidal activity of plumbagin or plumbagin-containing tissues was demonstrated at naturally occurring concentrations. Plumbagin as well as other NQ might become alternative compounds as natural insecticides in agriculture.

Author Contributions: A.R.-S., A.D.-L., and A.M. conceived the study and experiments. A.R.-S., A.D.-L., and C.P. performed the experiments and analyzed data. A.R.-S., A.D.-L., C.P., and A.M. discussed the data and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Manuscript No. 4

Manuscript overview

Manuscript title: Metabolomics Analysis Reveals Tissue-Specific Metabolite Compositions in Leaf Blade and Traps of Carnivorous *Nepenthes* Plants

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Author	Conceptual	Data analysis	Experimental	Writing the manuscript	Provision of material
Alberto Dávila-Lara	40 %	40 %	70 %	30 %	-
Carlos E. Rodríguez-López	30 %	50 %	30 %	30 %	-
Sarah E. O'Connor	-	5 %	-	10 %	20 %
Axel Mithöfer	30 %	5 %	-	30 %	80 %
Total:	100%	100%	100%	100%	100%

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Metabolomics Analysis Reveals Tissue-Specific Metabolite Compositions in Leaf Blade and Traps of Carnivorous *Nepenthes* Plants

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Abstract: *Nepenthes* is a genus of carnivorous plants that evolved a pitfall trap, the pitcher, to catch and digest insect prey to obtain additional nutrients. Each pitcher is part of the whole leaf, together with a leaf blade. These two completely different parts of the same organ were studied separately in a non-targeted metabolomics approach in *Nepenthes x ventrata*, a robust natural hybrid. The first aim was the analysis and profiling of small (50–1000 *m/z*) polar and non-polar molecules to find a characteristic metabolite pattern for the particular tissues. Second, the impact of insect feeding on the metabolome of the pitcher and leaf blade was studied. Using UPLC-ESI-qTOF and cheminformatics, about 2000 features (MS/MS events) were detected in the two tissues. They showed a huge chemical diversity, harboring classes of chemical substances that significantly discriminate these tissues. Among the common constituents of *N. x ventrata* are phenolics, flavonoids and naphthoquinones, namely plumbagin, a characteristic compound for carnivorous Nepenthales, and many yet-unknown compounds. Upon insect feeding, only in pitchers in the polar compounds fraction, small but significant differences could be detected. By further integrating information with cheminformatics approaches, we provide and discuss evidence that the metabolite composition of the tissues can point to their function.

Keywords: *Nepenthes*; carnivorous plants; UPLC-qToF-MS; metabolomics; tissue specificity; cheminformatics

1. Introduction

Metamorphosis of plant organs is a common feature in higher plants and often an adaptation to the particular environment. Metamorphosis covers genetically fixed changes in both morphology and anatomy leading to new structural or functional modifications. In higher plants, leaves are mainly involved in photosynthesis and transpiration, but many leaf metamorphoses are also known for exhibiting new functions. Examples are spines as protection against herbivores (cacti), needles to reduce water loss (conifers), bulbs for storage of water and nutrients (onion), and tendrils for climbing (pea). Striking structures of leaf metamorphosis are found in many carnivorous plants that live on nutrient-poor soil and catch animal prey to get additional nutrients, such as nitrogen and phosphate [1,2]. Here, the leaves are employed in catching prey, mainly insects. For instance, in Venus flytrap (*Dionaea muscipula*), rapidly closing snap traps are found, in sundew (*Drosera*) species sticky

flypaper traps, and in bladderwort (*Utricularia*) species sucking bladder traps [1,2]. Another type of trap is realized in so-called pitcher traps that can be found in the genus *Nepenthes* (Figure 1), occurring in Southeast Asia.

Figure 1. *Nepenthes x ventrata*. Natural hybrid of *N. ventricosa* and *N. alata*.

These passive traps attract prey to the pitcher opening, the peristome, which is extremely slippery for insects causing them to fall into the pitcher. The lower part of the pitcher is filled with a fluid where the prey drowns. Subsequently, plant-derived hydrolytic enzymes inside the fluid digest the prey and generate absorbable forms of nutrients, which are taken up and delivered further to the plant body through bi-functional glands [2,3]. In *Nepenthes* species, the whole leaf underwent an extensive metamorphosis: the typical leaf lamina (synonym: leaf blade) turned into a pitcher for catching prey, the petiole into a tendril to climb, and the leaf base into a basal leaf-derived leaf blade (from now on: leaf blade) substituting the lamina to ensure photosynthesis (Figure 2) [4,5].

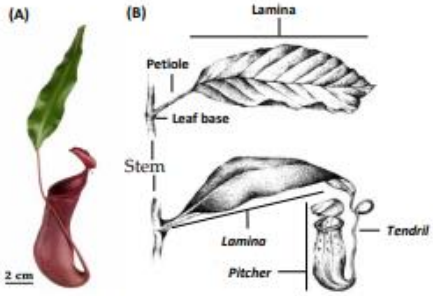

Figure 2. Comparison of leaf morphology. (**A**) *Nepenthes x ventrata* leaf. (**B**) Typical foliage leaves (upper), *Nepenthes* leaf (below). In italics, the leaf parts developed in *Nepenthes* as result of metamorphosis of the typical leaf parts. For further explanation, see the text. Copyright © of drawing (**B**) held by Sarah Zunk.

For many years, scientists studied the different trapping mechanisms in order to understand their function and biomechanics. However, changes and adaptations in leaf morphology and anatomy also come along with changes in the physiology, biochemistry, and molecular biology of carnivorous plants. Thus, in recent years, many studies in carnivorous plants focused more and more on molecular aspects

and "omics" approaches, except metabolomics. Those studies have produced more and deeper insights in the molecular events accompanying the various steps necessary for successful prey hunting and digestion, suggesting, for example, that plant carnivory originates from defense mechanisms [6–12]; however, most studies are still related to the particular traps.

In *Nepenthes*, the pitcher fluid was investigated in detail, including its proteome [13–15] and the composition of organic and inorganic low-molecular-weight compounds [16]. Based on such studies, we learned that the pitcher fluids consist of enzymes necessary for digestion and also defensive proteins belonging to the group of pathogenesis-related proteins [17]. Moreover, the pitcher fluid is poor in inorganic nutrients and contains secondary metabolites with antimicrobial properties, i.e., naphthoquinones; droserone and 5-*O*-methyl droserone are described for *N. khasiana* [18] and plumbagin and 7-methyl-juglon for *N. ventricosa* [16]. These compounds are not widespread in plants but very often occur in carnivorous plants of the order Nepenthales [19], a *sensu stricto* sister group to Caryophyllales [5]. For *Nepenthes*, some of these naphthoquinones were described as inducible by chitin and prey [18,20], suggesting a functional role after prey catch. Naphthoquinones are highly bioactive compounds with defense-related properties [21]. Therefore, it has for a long time been suggested that these compounds are involved in protection against various microbes and pest attack and preserving prey during digestion [16–19]. Plumbagin and some other naphthoquinone derivatives have also been found in various tissues of *Nepenthes* species including the pitchers [16,20,22,23]. In addition, in the literature, the presence of carotenoids, flavonoids, sterols and triterpenes was mentioned for *Nepenthes* leaves [2,24,25].

As many carnivorous plants, including *Nepenthes*, harbor a huge chemical diversity, many secondary metabolites from carnivorous plants are currently isolated for pharmaceutical, biotechnological and pseudo-medical use [2,26,27]. This approach *per se* has led to pharmacologically valuable molecules, and, notably in times of an ongoing pandemic, its value is obvious. However, metabolomics studies to better understand the role of metabolites concerning their ecological function in a carnivorous plant are not available but nevertheless important. As suggested by Hatcher and colleagues [19], the metabolite diversity may represent a mechanism supporting the evolution of carnivory and the ability to cope with new and harsh environments. In addition, regarding the metabolome, carnivorous plants' responses to the assimilation of animal-derived nutrients remain largely unknown. Thus, the examinations of metabolite changes in pitcher and leaf blade tissues before and after prey digestion may also provide insight into dynamic processes in plant metabolism.

In order to address these questions, we used a non-targeted approach to analyze and compare, in *Nepenthes x ventrata*, the ionizable metabolites of specialized tissues; i.e., pitcher traps that are involved in prey catch and (basal) leaf blades involved in photosynthesis. In addition, we analyzed changes in the metabolite composition upon insect prey digestion. Besides these ecological aspects, the unique metamorphosis of a typical leaf organ into highly specialized tissues adds a fascinating developmental aspect.

2. Results

2.1. Metabolomics Reveals a Loss in Metabolite Load and Diversity in the Specialized Pitcher Organ

Drosophila melanogaster-fed and non-fed pitchers and related leaf blades of *N. x ventrata* were subject to independent polar and non-polar extractions. Extracts were analyzed by UPLC-ESI-qTOF in positive mode, with data-dependent fragmentation. Data was acquired in positive mode due to higher sensitivity and the higher quality of fingerprint predictions of SIRIUS+CIS-FingerID in positive as compared to negative mode. Since, in polar extractions, the chromatograms were dominated by a few peaks, to increase the coverage the samples were injected twice; as concentrated extracts and as ten-fold dilution. Using MetaboScape®, in the non-polar extraction 1396 peaks were detected and adducts grouped into 1226 features, 984 of which had at least one MS/MS event. In the polar extracts, 1398 and

560 peaks were detected, grouped in 1250 and 509 features, with 1012 and 383 fragmentation events in concentrated and diluted samples, respectively; both matrices of polar features were concatenated.

To gain an overview of the metabolomics changes, non-supervised analysis was performed separately on both polar and non-polar extracts. For both extractions, a Principal Component Analysis (PCA) showed that the main source of variation is the tissue, separated by the first component, explaining 35% and 25% of the variance in polar and non-polar metabolites, respectively (Figure 3). Interestingly, only the polar features of fed and non-fed pitchers were separated in the PCA (by the third component), explaining around 5% of the variance (Figure 3a). None of the other combinations of PCs, cumulatively explaining up to 95% of the variance, managed to separate samples by feeding status. Remarkably, a consistent trend can be seen in the score plots (Figure S1), where leaf-specific features have a higher *m/z* than pitcher-specific peaks in both polar and non-polar extracts.

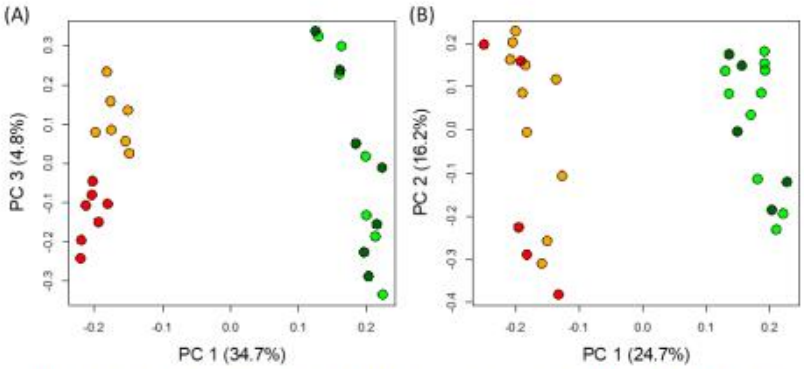

Figure 3. Unsupervised analysis of all detected features. PCA analysis of features detected in polar (**A**) and non-polar (**B**) extracts. Tissue and feeding status are indicated by the colors dark green and light green, showing fed and not-fed leaves, and red and orange, showing fed and not-fed pitchers, respectively.

To complement the non-supervised analysis and to estimate the effect of tissue type and feeding status, two-way ANOVA tests were run on the features. Ratifying the previous observation, only tissue had features that were significantly different (FDR < 0.01). After removing duplicated signals, in the polar fraction 797 differentially accumulated features (DAFs) were found, with the vast majority (634) being highly accumulated in leaf compared to pitcher (163 features; Figure S2). Correspondingly, the non-polar fraction had 449 DAFs that were more balanced, with 272 and 177 over-accumulated in leaf and pitcher, respectively (Figure S3). The DAFs are shown in the cloud plot of Figure 4, where the trend hinted at by the PCA score plots is confirmed: in both polar and non-polar extracts, features over-accumulated in leaf are of higher *m/z* than those over-accumulated in pitcher, with a difference of medians of 122 Th and 121 Th, respectively (Figure S4).

Moreover, besides the finding that leaves show more significantly accumulated features, the fold-change of those features is also remarkably higher (size of the circles in Figure 4) than the features over-accumulated in pitchers (Figure S5).

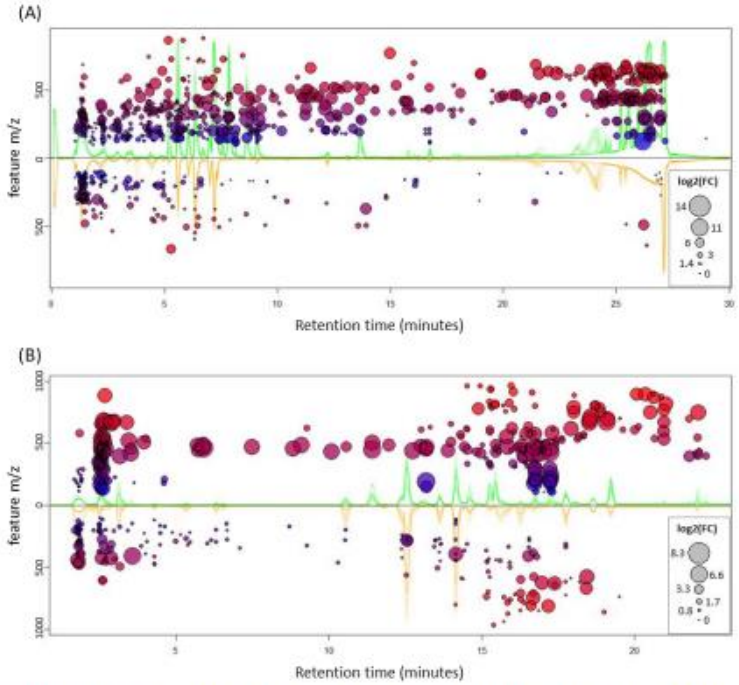

Figure 4. Mirror plots of differentially accumulated features (DAFs). DAFs (FDR < 0.01) in polar (**A**) and non-polar (**B**) extracts are shown for leaf (top) and pitcher (bottom). Circle size depicts the absolute value of the $\log_2$ of the average fold change, on the top if it is over-accumulated in leaf, and on the bottom otherwise. Color and y-axis value depict the *m/z* value of the feature, with blue being low- (100) and red high- (1000) *m/z* features; the further away from the origin, the higher the *m/z*, as indicated by the y-axis. The superimposed, raw base-peak chromatograms (BPC) of all runs are shown in the background, colored accordingly: green, all leaf BPCs; orange, all pitcher BPCs.

2.2. *Database-Independent Spectral Analysis Identifies Key Substructures in DAF*

Assignation of feature identity is a complicated endeavor, which in MS-based metabolomics relies heavily on compound databases. Unequivocal identification of a compound requires isolation and analysis by NMR, and putative identification by fragmentation patterns requires manual curation of candidate lists, generated by algorithms that automate comparisons to databases. Given that *Nepenthes* is an understudied genus, we expect few of the detected compounds to be present in chemical databases; however, some structural information can be directly extracted from the MS/MS spectra.

With that purpose, for every adduct of all DAFs, we collected fragmentation spectra and analyzed it using SIRIUS [28–30] and CSI-FingerID [31], from which the best-predicted fingerprint vectors for each DAF were selected for analysis. In total, 580 DAFs (72%) from the polar and 212 DAFs (47%) from the non-polar fractions were each assigned a vector of chemical fingerprints. For reference, only 11 DAFs (2%) of the non-polar fraction had a hit using the extended database LipidBlast [32]. CSI-FingerID vectors contain 2937 chemical fingerprints [31] to which we assigned one of three values (present, absent, and uncertain) based on their posterior probabilities. We then calculated enrichment

probabilities of the presence and absence of each fingerprint in each tissue, separately for polar and non-polar; the significantly enriched ones (FDR < 0.05) are shown in Tables S1 and S2.

Strikingly, pitcher DAFs have an increased presence of phosphate groups (Figure 5). They also mostly lack tertiary and quaternary carbons and rings, which would point at acyl lipids and phospholipids as those lipids in pitchers that best differentiate them compared with leaves. Accordingly, leaf DAFs have a distinctive annulated ring structure, along with fingerprints of at least two six-carbon rings, ternary carbons and branched fatty acyl chains, all typical fingerprints of sterol lipids. Indeed, analyzing the heatmap of the selected vectors (Figure 5) it can be seen that the right-most clusters, with most of the leaf DAFs, show typical sterol fingerprints. In contrast, the left-most clusters, with the majority of the pitcher DAFs, have at most one ring. In addition, this cluster harbors the prominent PO_2-containing cluster, consisting almost entirely of pitcher DAFs.

Concerning the fingerprints of the polar extracts, there are many more DAFs in leaves than in pitchers. Because structural variability is strikingly higher in polar compounds, interpretation is less straightforward. However, pitcher DAFs are seemingly enriched in compounds with heteroatoms, such as nitrogen or phosphate, and pentose fingerprints. Some diimines are found naturally in purines and ureides—both soluble molecule families that have a high nitrogen load. Given that there are five times more DAFs with fingerprints in leaf than in pitcher, not many characteristic fingerprints can be robustly assigned to be leaf-specific. Nevertheless, one of the main DAFs found in leaf blades, which appears to be 32 times higher in leaf blades than in pitchers, has been tentatively identified as the naphthoquinone plumbagin. In sum, in the corresponding fingerprint heatmap (Figure 6) the enrichment is not as clear cut as in the lipids, given the low abundance of pitcher DAFs. However, it is still noticeable that the right-most cluster concentrates almost exclusively pitcher DAFs: of the 11 DAFs simultaneously having four of these five fingerprints, only one is from leaf. Only one of these compounds had a biologically relevant database hit, resembling a uridine bisphosphate. In addition, interestingly, only five out of the 16 DAFs with a pentose fingerprint are accumulated in leaf.

2.3. Differences in Pitcher Due to Feeding Status

As the PCA suggested that only the polar extract of pitchers had a difference depending on feeding status, and to avoid interference with external variance, a one-way ANOVA was performed specifically in the polar extract of fed and non-fed pitchers. Thus, we found 73 DAFs due to the feeding status, with 27 features accumulating in fed pitchers, and 46 accumulating in non-fed pitchers (Figure S6). Unlike the above-mentioned examples, fold changes appear to be balanced, although the features accumulating in fed pitchers appear to have a higher *m/z* than those in non-fed pitchers (Figure S7). Notably, almost all of the DAFs (69 out of 73) are present only in the concentrated extract, and even there with low intensity.

Since most compounds do not have fragmentation due to low intensity, the full pipeline of SIRIUS+CSI-FingerID was followed, and the candidate list was manually curated. The results are shown in Table S3, where it can be seen that only 11 DAFs had a fragmentation pattern that allowed structural interpretation. Although the largest DAF-containing group is the one of non-fed pitchers (46 DAFs), only four features have assignations. Interestingly, three are nitrogenated: a putative nitrogenated heptose ($C_7H_{15}NO_9$), an unidentified, densely nitrogenated compound ($C_{13}H_{17}N_9O_{12}$), and a third that appears to be a nucleotide phosphate with an either cyclic ($C_{10}H_{17}N_4O_7P$) or acyclic ($C_{10}H_{15}N_4O_6P$) attachment. As for the fed pitcher, seven DAFs were identified, four likely to be phenolic compounds and three nitrogenated compounds. The phenolics were likely three simple phenolics ($C_{10}H_{10}O_3$, $C_{17}H_{22}O_8$, and $C_{13}H_{14}O_{11}$, the latter two glycosylated) and a flavonoid ($C_{17}H_{14}O_7$). The nitrogenated compounds had no hits in biologically relevant databases, only in PubChem; of those, two were compounds with four nitrogen atoms ($C_{22}H_{24}N_4O_7$ and $C_{27}H_{18}N_4O_6$) with very similar fingerprints, with more than two aromatic rings and nitrogen atoms in heterocycles, and the remaining one ($C_{14}H_{16}NO_5$) had a single aromatic ring and a single nitrogen.

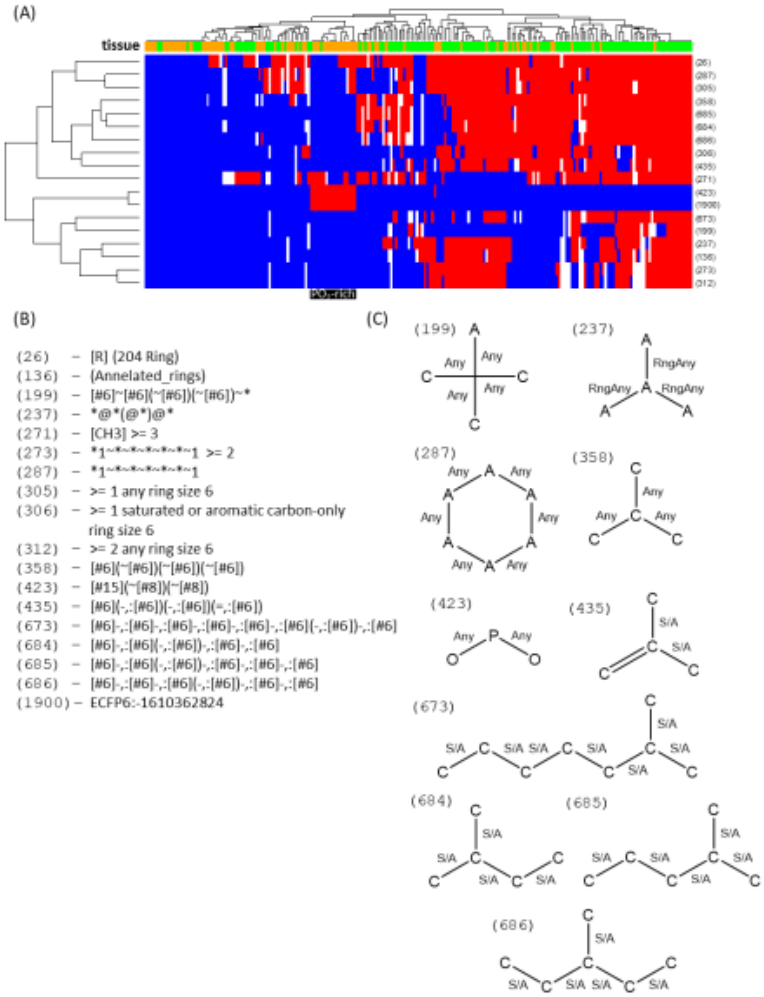

Figure 5. Fingerprint heatmaps of non-polar DAFs. A heatmap (**A**) is shown of the DAFs (columns) that had a fingerprint vector assigned, colored by tissue (green: leaf; orange: pitcher) on the top band. Only the enriched fingerprints (rows) are shown, named by CSI-FingerID relative index position (**A**). Based on posterior probabilities, the fingerprints were determined to be absent (blue), present (red), or uncertain (white). A cluster of DAFs almost exclusively accumulated in pitchers is highlighted in black, with the enriched fingerprints being described in (**B**) and, if graphical representation is possible, in (**C**). Any means it can be any kind of bond, RngAny means the bond is in a ring (of any kind), S/A means it is a single bond that can be anywhere (within a ring or not).

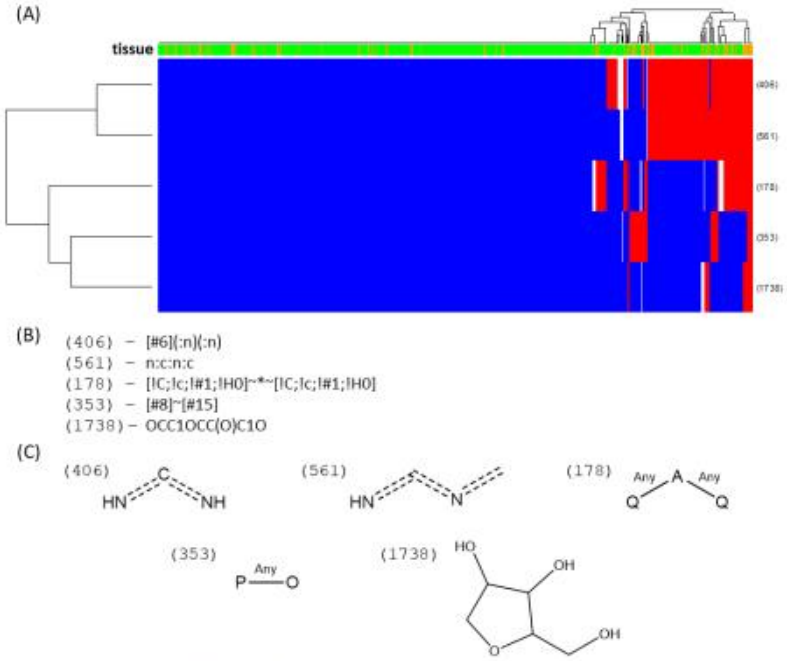

Figure 6. Fingerprint heatmaps of polar DAFs. A heatmap (**A**) is shown of the DAFs (columns) that had a fingerprint vector assigned, with blue cells being present, red being absent, and white being uncertain fingerprints. Given the nature of the sample, being mostly leaf DAFs, only the positive fingerprints enriched in pitcher and absent in leaf are shown. These fingerprints are described in (**B**) and the graphical approximation of their substructure in (**C**). It is important to note that the right-most cluster is unusually enriched in pitcher DAFs, with a high number of positive assignations of most of the selected fingerprints.

3. Discussion

Many low-molecular-weight compounds identified so far in carnivorous plants are volatile compounds suggested to be involved in prey attraction [19,33]. For instance, in *N. rafflesiana*, more than 50 volatiles have been found [34]. Less information is available for non-volatile compounds. Thus, we performed an untargeted metabolomics approach to determine which compounds might be related to carnivory in the metabolism of *Nepenthes x ventrata*, used here as a model plant. Two different questions have been addressed; first, we wanted to see whether or not the leaf blade and the pitcher contain different tissue- and function-specific metabolite patterns; second, we looked for differences in the tissues before and after insect feeding. This is the first study where a metabolomic profiling of the carnivory process in the genus *Nepenthes* is performed. Due to the technical design of this untargeted metabolomics work, the vast majority of primary metabolites fall inside the exclusion range for fragmentation (50–150 *m*/*z*); therefore, no meaningful assignation of identity or fingerprints could be performed on primary metabolites.

3.1. Metabolite Differences in Nepenthes Tissues: Leaf Blade vs. Pitcher

Overall, the number of features observed in leaves was much higher compared with pitcher tissue. In particular, there is a clear trend for the presence of polar compounds with $m/z > 300$ and of non-polar compounds with $m/z > 400$ in leaves. In addition, more over-accumulated features were found in leaves, with higher fold changes compared to pitcher. This means that both metabolite levels and diversity are lower in pitchers.

In the non-polar phase, the DAFs that best discriminate between pitcher and leaf are very likely acyl lipids and phospholipids, which are preferentially found in pitchers, and sterol derivatives, which are preferentially accumulated in leaves. The different membrane composition of these two tissues may be reflective of the differing functions. Sterols affect membrane fluidity and permeability, making the membranes more rigid, and are considered membrane reinforcers [35]. In addition, sterols are critical for the formation of lipid "rafts", which regulate biological processes such as signaling and transport across the membrane [36]. In *Nepenthes*, first, nutrient uptake from the pitcher fluid is performed by the bi-functional glands localized inside the pitcher. Besides carriers, clathrin-mediated endocytosis is involved in this process [37]. Specific for the vesicles of the clathrin-mediated pathway are phospholipids, favoring vesicle formation in contrast to sterols [38]. This might be another point that explains the different distribution of lipophilic metabolites in pitchers and leaves. In addition, a unique feature of *Nepenthes* pitchers is the waxy coating of the inner part of the pitcher, making it slippy for any prey trying to escape. This might also explain the difference in lipophilic metabolites in the pitcher compared with the leaf.

Interestingly, there is a family of polar compounds that simultaneously have a methylene-interrupted heteroatom, diimine-like structure (*~N=C=N~* and *~N=C=N=C-*), and phosphate and pentose fingerprints, and are exclusive to pitchers (10 out of the 11 DAFs with at least four of the five fingerprints). This finding was surprising as the carnivorous plants actually are limited in nitrogen and phosphate, and none of these DAFs are changing significantly due to feeding status. Nevertheless, since pitchers need to be ready for catching and digesting prey, they might be active in transport of both phosphate- and nitrogen-containing compounds. The presence of nucleotide phosphates supports the view at the pitcher as an active tissue ready to start de-novo synthesis of all necessary biosynthetic pathways. As long as no prey or not enough prey has been caught, even the pitcher must be seen as a sink tissue, and transport can occur in any direction. The putative nitrogen- and phosphate-containing glycosylated compounds are not present in biological databases and may hold valuable information on nitrogen and phosphate transport. The nature of these compounds, which might be mobile within the plant, is still an open question. Nitrogenous bases, like ureides, are well known to undergo long-distance transport in rhizobia–legume symbioses [39] as well as in non-nodulated plants [40]. Interestingly, the final enzymatic step to release ammonia from ureides is catalyzed by a urease. Its presence and activity were recently demonstrated for *Nepenthes* and other carnivorous plants [41]. Whether or not this scenario mirrors the nitrogen translocation and distribution that occurs in *Nepenthes* remains to be elucidated.

3.2. Insect Feeding Causes Changes in Polar Metabolite Pattern in Pitchers

In order to better understand the dynamics of the metabolic processes of carnivory in *Nepenthes* plants, immediately after opening, the pitchers were fed with fruit flies or not fed for 72 h. Results of the MS-based untargeted metabolomics analysis determined small but significant changes only in the pitcher tissue and, moreover, only in the fraction containing the polar metabolites. No significant changes in the leaf blade and no changes in the pitchers' non-polar metabolites were found as a result of feeding. Nevertheless, there was a trend showing that fed pitchers accumulated more compounds with higher molecular weight compared with non-fed pitchers, indicating a modulated, increased metabolic activity. Without knowing the exact structures of the compounds, the ecological relevance of changes in metabolite composition remains speculative. It might be due to higher physiological activities, in the sense that mobile compounds are built which can more easily be distributed within the

plant or that the pitcher tissue needs to be more defended against detrimental organisms showing up together with caught prey. This would explain an increase in, for example, some phenolic compounds. For example, in our experiment, the fed pitchers were found having an around four times higher concentration of a flavonoid-related feature (c_331.0809-12.16; $C_{17}H_{14}O_7$; Table S3) compared with non-fed pitchers. It is also suggested that *Nepenthes* is a slowly digesting plant [42]. For example, prey-initiated induction of digestive enzymes such as the protease nepenthesin can take days [43]. Thus, it is conceivable that the selected 72 h of prey digestion were not sufficient to detect more induced metabolites, qualitatively or quantitatively. Following this thread, it may also explain why no effect of feeding was found in the leaf blades. Experiments with *N. hemsleyana*, a coprophagous *Nepenthes* species that does not catch prey any more but feeds on bat feces [44], showed that upon ^{15}N-enriched urea application into pitchers, after only four days, ^{15}N was significantly detectable in protein fractions of leaf blades [41].

These data suggest the lipid composition of pitcher appears to favor vesicle formation, while leaf blade lipids promote rafts and membrane rigidity; pitcher-specific DAFs contain nitrogen and phosphorus, with typical fingerprints of molecules known to undergo long-distance transport; and changes in leaf and pitcher features are weak due to feeding status. We may further speculate that prey-derived nutrients are taken up via vesicles in the pitcher, further degraded, fixed in organic N- and P-rich compounds, and eventually systemically distributed, thereby passing the proximal leaf blades. This is supported by research showing that developing leaves incorporated a higher level of ^{15}N, being preferentially supplied compared with a leaf that carries a fully developed pitcher [45]. Additional future experiments with different time points of harvesting may provide more insight into the dynamics of prey-induced changes in the *Nepenthes* metabolome in different tissues. However, as carnivorous plants mainly hunt for nitrogen and phosphate, it was not surprising to find prey-induced metabolite changes in the fraction containing polar, water-soluble compounds.

LC-MS-based metabolomics is a powerful tool for assessing chemical diversity in an un-biased manner, and is particularly useful for characterizing non-model plants, for which available data is scarce. However, the very nature of understudied plants complicates interpretation of the results, as most methods of putative identification rely heavily on databases, suffering greatly from popularity bias, and require manual curation, hindering analysis of systemic changes, such as those in pools of metabolites. Cheminformatics has long been used to extract information from large databases in an automated manner, but usually requires the existence of a chemical structure. We used a cheminformatics-aided metabolomics approach for characterizing the carnivorous plant *N. x ventrata*, using CSI-FingerID [31] fingerprint vectors directly, entirely bypassing structure assignation, the weakest link in the metabolomics pipeline. This minimizes false positives, and produces a robust, evidence-based approach for exploring systemic changes in metabolites.

In order to elucidate the real structures of the numerous compounds, further analyses are necessary, such as NMR. However, the compounds we found occur at low abundance, and this makes it extremely difficult to isolate enough material for analysis. However, the methods employed in the present study highlight general tissue-specific metabolites and their changes upon prey digestion.

Nevertheless, the fact that many features could not be identified in biologically relevant databases highlights the need to characterize non-model plant species to increase our knowledge of chemical diversity and find still-unknown compounds, which might be biologically or pharmaceutically relevant. In particular, *Nepenthes* species are well known in traditional medicine. Various reports are available describing curative effects of extracts from different *Nepenthes* species and tissues on diseases, for example, on cough, fever, hypertension, urinary system infections [46], malaria [47,48], asthma, pain [48]; *Staphylococcus* infection [49], celiac disease [50], and recently on different kinds of oral cancer cells [51]. Thus, further work on the isolation and structure elucidation of *Nepenthes* metabolites as well as the analysis of their putative pharmaceutical uses seems promising in order to find new structures and therapeutics.

In conclusion, the studied *Nepenthes x ventrata* plant contains a huge variety of different metabolites. We focused on MS-based and data mining approaches to visualize the metabolic differences between leaf and pitcher tissues, and between fed and un-fed plants. Leaf metamorphosis into pitchers and leaf blades generated new tissues that are different in function, which is also clearly represented in their respective DAFs. Surprisingly, insect prey feeding has a much smaller impact on the measured metabolites. Cheminformatics approaches suggest the presence of many structurally unknown compounds which might be of therapeutic interest, bearing in mind that *Nepenthes* species have been long used in traditional medicine. Further research should be carried out addressing the remaining questions of metabolite identification, biosynthetic pathways and the ecological relevance of *Nepenthes* metabolites.

4. Materials and Methods

4.1. Plant Material, Treatment, and Sampling

We used the natural hybrid *Nepenthes x ventrata* (*N. alata x N. ventricosa*) as a model organism. *N. x ventrata* plants were grown in the greenhouse of the MPI for Chemical Ecology at 21–23 °C, 50–60% relative humidity and a 16/8 h light/dark photoperiod. To avoid contamination, still-closed pitchers were covered with a mesh. Once the pitchers opened, they were left untreated for controls or prey degradation was induced by adding 30 wild-type *Drosophila melanogaster*, representing ca. 31 mg fresh weight. Individual pitchers represent independent biological replicates from different plants. After 72 h, pitchers were emptied, i.e., the digestive fluid with or without the remains of fruit flies was discarded, and subsequently rinsed 3 times with sterile distilled water. Next, both the tissue from the glandular zone (lower third part of the pitcher) and the related leaf blade were dissected and sampled in 50-mL Falcon tubes and immediately frozen in liquid nitrogen. The plant material was finely ground in liquid nitrogen using a mortar and pestle. Then, ground material was stored in screw-cap Eppendorf tubes and stored at −80 °C until further processing.

4.2. Metabolomic Extraction

Altogether, 28 individual samples were examined—7 *D. melanogaster*-treated and 7 untreated pitchers—and their corresponding leaf blades harvested after 72 h. Samples were extracted following a procedure derived from [52,53] with some modifications. In short, double extractions of 100 mg FW tissue powder were performed in 2-mL Eppendorf tubes at room temperature, using 500 µL MeOH:ammonium acetate buffer (pH 4.8). Therefore, after 5 min shaking, a 15 min sonication in water bath followed (3× for 5 min and 3 min resting in between). Extracts were centrifuged at 20,000× *g* for 10 min. Clear supernatants were combined and filtrated using a PTFE syringe filter (hydrophilic 0.22 µm pores, 13 mm diameter, Fisherbrand, Cat.# 15161499, Fisher Scientific, Schwerte, Germany). This extract was diluted 1:10 with 75% MeOH and further analyzed.

4.3. Lipidomics Extraction

Here, altogether 30 individual samples were examined: 5 non-treated control pitchers and leaf blades were taken directly after pitcher opening at 0 h; 5 *D. melanogaster*-treated and 5 untreated pitchers and their corresponding leaf blades taken after 72 h. Each sample represents an independent biological replicate. Extractions were done following a procedure derived from Matyash et al. (2008) [54] and Chen et al. (2013) [55] with some modifications. All steps were performed in glass test tubes and kept at room temperature. In short, an adjusted volume of methanol was added to 100 mg FW of tissue powder, based on a ratio of 150:1 *v/w* DW. Milli-Q water was added to a final ratio of 3:1 MeOH:H_2O, taking the water content (87%) of the tissues into consideration, which was determined before. Next, samples were vortexed followed by 5 min sonication in a water bath (5× for 1 min and 1 min resting in between). Thereafter, methyl-*tert*-butyl ether (MTBE) was added to achieve a ratio of 10:3:1 (MTBE:MeOH:H_2O). Samples were vortexed again, sonicated as described and shaken at 100 rpm for 1 h. Afterwards,

milli-Q water was added to reach a total ratio of 20:6:7 (MTBE:MeOH:H_2O). Samples were vortexed, sonicated as previously described, and shaken for 10 min. To separate them into two phases, samples were centrifuged at 100× *g* for 20 min. The organic phase was recovered, while the aqueous phase was extracted again in 2 mL, keeping the ratio of MTBE:MeOH (20:6:7). Both organic phases were combined and evaporated under vacuum at 45 °C. The dry aqueous and organic samples were resuspended in acetonitrile:isopropanol (50:50) to a concentration equivalent to 1 g/L DW and filtrated using a PTFE syringe filter. This extract was diluted 1:10 with acetonitrile:isopropanol (50:50) and further analyzed.

4.4. Metabolic Profiling Using HPLC-qToF-MS

Samples were analyzed using an Elute LC system (Bruker Daltonik, Bremen, Germany) coupled via ESI to a Maxis II q-TOF (Bruker Daltonik, Bremen, Germany). Polar compounds were separated using a Kinetex® XB-C18 column (100 × 2.1 mm, 2.6 µm, 100 Å; Phenomenex, Aschaffenburg, Germany) at 40 °C with a gradient from water to acetonitrile, both modified with 0.1% formic acid, according to [52] with minor modifications. Namely, there was a flow of 0.2 mL/min, a linear gradient from 5% to 75% acetonitrile over 20 min, increased linearly to 95% acetonitrile over 5 min, followed by a 5-min equilibration at the initial conditions. Non-polar compounds were separated using a Luna® Omega PS C18 column (150 × 2.1 mm, 3 µm, 100 Å; Phenomenex, Aschaffenburg, Germany) at 50 °C. Mobile phase A was a mixture of water and acetonitrile (4:1 *v/v*) and mobile phase B was an isopropanol:acetonitrile mixture (9:1 *v/v*); both phases were modified to a final concentration of 10 mM ammonium acetate and 0.1% formic acid. The gradient was as previously published [56] with minor modifications: at a flow of 0.2 mL/min, a linear increase from 40% B to 45% B in 2 min, then to 55% B in 8 min, followed by an immediate step increase to 70% B, then a linear increase to 99% B in 10 min, holding at 99% B for 5 min, and finally returning to the initial conditions for 5 min. For analysis of the extracts, 5 µL of a 10-fold dilution was injected, and, for the polar extracts, a second batch of 5 µL of concentrated extract was injected. Injections in each of these three batches were randomized, with 5 evenly interleaved quality control injections of pooled samples, preceded by 4 "dummy" injections of pooled quality control samples to passivate the column, which was extensively washed after each batch. Analyses of the quality control samples are shown in Figures S8–S10.

Acquisition of MS data was done using the same conditions for both polar and non-polar compounds. Ionization was performed via pneumatic-assisted electrospray ionization in positive mode (ESI+) with a capillary voltage of 4.5 kV and an end plate offset of 500 V; a nebulizer pressure of 3 bar was used, with nitrogen at 350 °C and a flow of 12 L/min as the drying gas. Acquisition was done at 12 Hz following a mass range from 50 to 1000 *m/z*, with data-dependent MS/MS and an active exclusion window of 0.2 min, a reconsideration threshold of 1.8-fold change, and an exclusion range of 50–150 *m/z*. Fragmentation was triggered on an absolute threshold of 400 and acquired on the most intense peaks using a target intensity of 20,000 counts, with MS/MS spectra acquisition between 12 and 20 Hz, and limited to a total cycle time range of 0.5 s. Collision energy was determined automatically by the software depending on *m/z* value. At the beginning of each run, an injection of 20 µL of a sodium formate–isopropanol solution was performed in the dead volume of the injection, and the *m/z* values were re-calibrated using the expected cluster ion *m/z* values.

4.5. Feature Detection

Peak detection was done using Metaboscape software (Bruker Daltonik, Bremen, Germany) with the T-Rex 3D algorithm for qTOF data. For the non-polar runs, parameters for detection were an intensity threshold of 500 with a minimum of 7 spectra, and features were kept if they were detected in at least 3 replicates of the same treatment, tissue and time (60% of n). Adducts of $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, and $[M+NH_4]^+$ were grouped as a single feature if they had an EIC correlation of 0.8. For the polar runs, the intensity threshold was set to 1000, the features were kept if detected in at least 5 replicates of the same treatment and tissue (70% of n), and adducts were grouped in the same manner, only excluding the ammonium adduct, which was not expected in the polar runs.

4.6. Spectral Analysis

Proprietary MS Bruker files were re-calibrated with cluster ions of sodium formate in the dead-volume injection time and converted to mzXML [57–59] using Bruker DataAnalyst software (Bruker Daltronik, Bremen, Germany). Access to the raw data in mzXML files was done in R with the aid of the *mzR* library [60]. MS/MS data was extracted for selected features using an in-house built code that searched in all samples for fragmentation events triggered in a window of 0.5 min within the feature retention time (RT). To avoid misassignation of closely eluting isobaric compounds within the RT window, the maximum of intensity in the MS1 extracted ion chromatogram (XIC) of the feature *m/z* (with 5 ppm error) that was closest to the feature RT was searched. Only contiguous peaks decreasing in intensity from the previous point in the MS1 XIC and with intensity higher than 10% of the maximum were kept. The new RT window was determined by the time in the first and last events. Within this new RT window, all fragmentation events whose parent ions matched the feature *m/z* within a 5 ppm error were stored. The fragmentation events of the most abundant 5 (non-polar) and 7 (polar) peaks for each feature adduct were merged using previously published in-house binning algorithm [61], and saved as MASCOT generic format (MGF) files.

Candidate structures and database-independent fingerprint vectors were obtained by loading the above-mentioned MGF files into the SIRIUS [28–30] and CSI-FingerID [31] pipeline. Candidate structures for the DAFs of fed and non-fed pitchers were obtained by searching the top hit of CSI-FingerID in all databases and manually curating the results; for all the other analyses, fingerprint vectors of the top 10 candidates of all predicted formulas were exported and loaded in R. When more than one adduct was present in a feature, only the formulas that matched the formulas of the adducts were kept. Then, only fingerprints that explained more than 3 peaks and more than one third of the intensity were kept. The final selection of the fingerprint vectors was made by collapsing all the adducts per feature, only keeping the fingerprint vectors corresponding to the top-scoring candidate and those that were less than 30% different. Fingerprints were assigned as present if the highest posterior probability of fingerprint vectors and adducts was greater than 0.75, as absent if the lowest posterior probability was less than 0.25, and as uncertain otherwise. Enrichment for presence and absence were calculated via a hypergeometric test, with uncertain assignations not being considered in the probability calculations as either hits or fails. The p-values of the hypergeometric tests were corrected for multiple testing.

4.7. Statistical Analysis

All statistical analyses were performed using the R 3.6.1 *base* package [62] and graphics using a combination of the *ggplot2* [63] and *gplots* [64] libraries, unless otherwise specified. Analysis of polar and non-polar fractions was done separately, given the nature of the experiments. Since the maximum signal-to-noise ratio was assumed to be 1/3, the zeroes in the matrices were replaced by their respective minimum measured area, divided by three, and then $\log_{10}$-transformed. The resulting matrices, estimated as Normal by Q-Q plots, were used for ANOVAs. For principal component analysis, these $\log_{10}$-transformed matrices were z-scaled by subtracting the mean and dividing by the standard deviation in a feature-wise manner. For the non-polar analysis, a two-way ANOVA was done on samples after 72 h, taking tissue and treatment as factors, and blocking by extraction batch. Since no difference was found by treatment, the 0 h control was added to analysis discriminating tissue, blocking by all other variables. For the polar analysis, a two-way ANOVA was done on the concatenated matrix of concentrated and diluted injections, taking tissue and treatment as factors. The features were de-duplicated only after statistical testing and false discovery rate correction, and this deduplication was only performed on significantly different peaks. Features were considered duplicated if they shared the same *m/z* (within 10 ppm or an absolute 0.0025 difference) and retention time (within 0.15 min) and were not detected as different features in either the concentrated or diluted injections. That is, if 3 (significantly different) features were detected in the concentrated batch within that window (10 ppm, 0.15 min), and 2 (significantly different) features were detected in the diluted sample, the deduplication

would keep all 3 (significantly different) features in the concentrated sample because, even when they share *m/z* and RT, they were detected as different features by MetaboScape. This is a conservative approach for calculating both FDR and fold change. All statistical testing was controlled for multiple testing by Benjamini and Hochberg's (1995) [65] false discovery rate correction.

Supplementary Materials: The following are available online at http://www.mdpi.com/1422-0067/21/12/4376/s1, Figure S1: PCA Scores vs. *m/z*; Figure S2: Heatmap of polar DAFs; Figure S3: Heatmap of non-polar DAFs; Figure S4: The *m/z* plots; Figure S5: Fold-change density plot; Figure S6: Heatmap of polar DAFs in pitchers due to feeding status; Figure S7: Feeding fold-change density plots; Figure S8: Quality control injections in the lipidomics experiment; Figure S9: Quality control injections in the polar experiment, injecting the raw extracts; Figure S10: Quality control injections in the polar experiment, injecting the ten-fold diluted extracts; Table S1: Polar fingerprints; Table S2: Non-polar fingerprints; Table S3: Features. Raw data was deposited in Metabolights Study MTBLS1783, as well as in the EDMOND database (DOI: https://dx.doi.org/10.17617/3.42).

Author Contributions: A.D.-L., C.E.R.-L. and A.M. conceived the study and experiments. A.D.-L. and C.E.R.-L. performed the experiments and analyzed data. A.D.-L., C.E.R.-L., S.E.O. and A.M. discussed the data and wrote the manuscript. All authors read and agreed to the present version of the manuscript.

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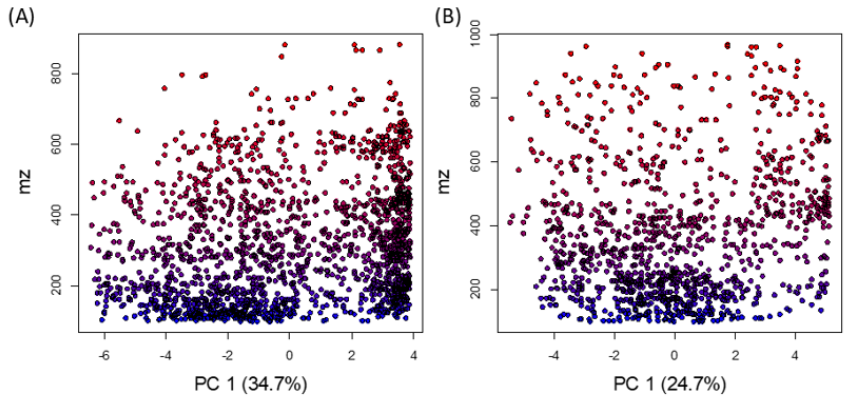
Supplementary material manuscript No. 4

Metabolomics analysis reveals tissue specific metabolite compositions in leaf blade and traps of carnivorous *Nepenthes* plants

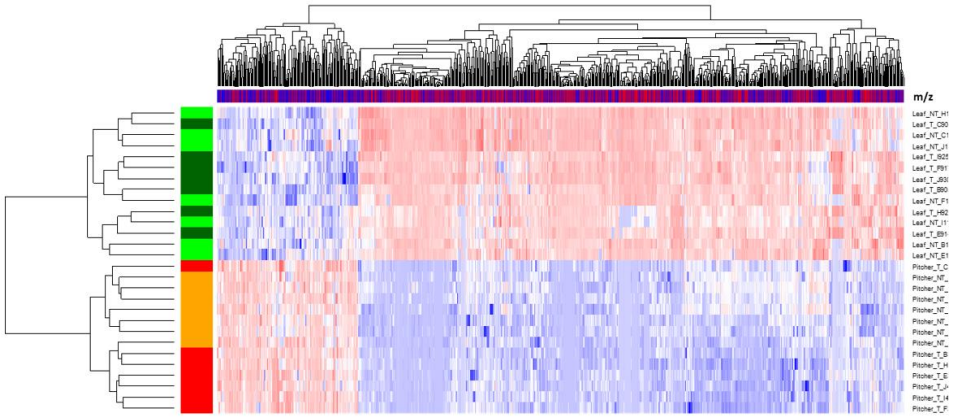
Alberto Davila-Lara, Carlos E. Rodriguez Lopez, Sarah E. O'Connor, Axel Mithöfer

7 Supplementary Figures

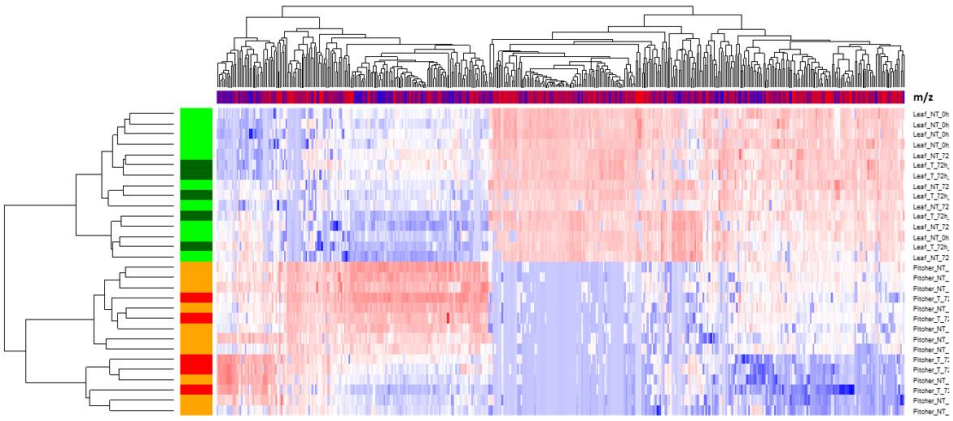
3 Supplementary Tables (Excel file)



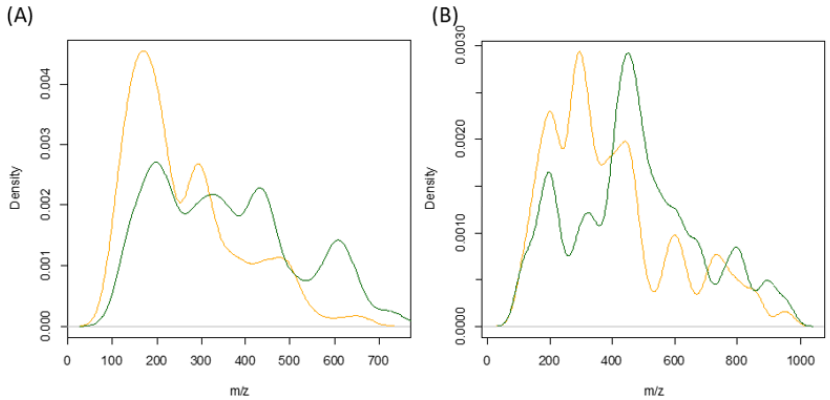
Supplementary Figure 1. PCA Scores vs *m/z*. All features for polar (A) and non-polar (B) extracts are shown, plotted by their main component score and the *m/z*. Circles are colored by *m/z*, from low (blue) to high (red) *m/z* value of the feature, to aid visualization.



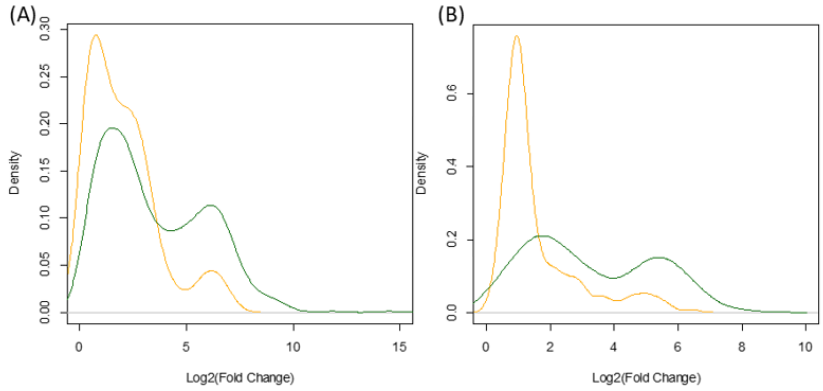
Supplementary Figure 2. Heatmap of polar DAFs. A heatmap for all DAFs (FDR<0.01) of the polar extracts, with low abundance being blue and high abundance in red. The band on the top is colored by *m/z* from low (blue) to high (red) *m/z*, and the left band by tissue and feeding status: dark green and light green being fed and not-fed leaves; and red and orange, fed and not fed pitcher, respectively.



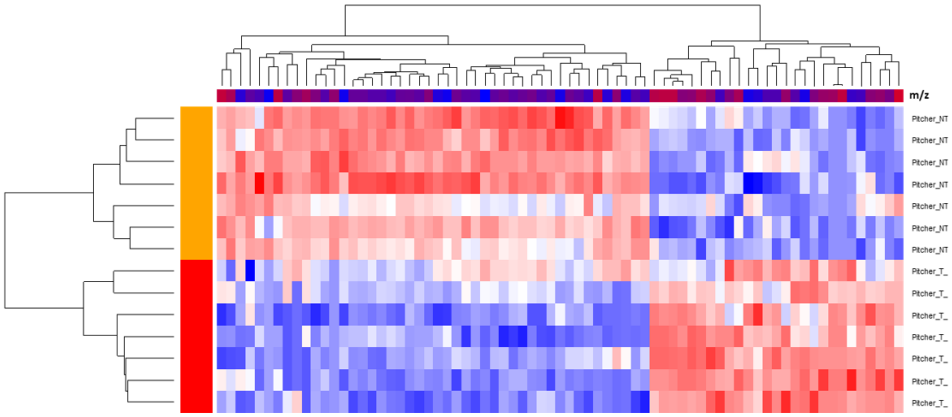
Supplementary Figure 3. Heatmap of non-polar DAFs. A heatmap for all DAFs (FDR<0.01) of the non-polar extracts, with low abundance being blue and high abundance in red. The band on the top is colored by *m/z* from low (blue) to high (red) *m/z*, and the left band by tissue and feeding status: dark green and light green being fed and not-fed leaves; and red and orange, fed and not fed pitcher.



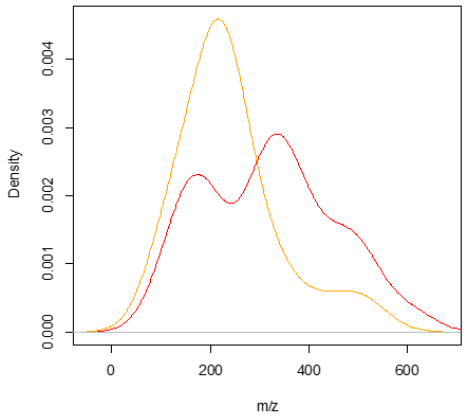
Supplementary Figure 4. The *m/z* density plots. A plot of kernel density estimates for all polar (A) and non-polar (B) DAFs' (FDR<0.01) measured *m/z*, colored by tissue: Orange being all fed and not fed pitcher, and dark green all fed and not fed leaves. The lines depict the distribution of *m/z* values in the corresponding samples.



Supplementary Figure 5. Fold-change density plots. A plot of kernel density estimates for all polar (A) and non-polar (B) DAFs' (FDR<0.01) absolute $\log_2$ fold-change value. In orange, pitcher, and in dark green, all leaves fold-change distributions.



Supplementary Figure 6. Heatmap of polar DAFs in pitchers due to feeding status. A heatmap for all DAFs (FDR<0.05) of the polar extracts of pitchers, with low signal being blue and high signal in red. The band on the top is colored by *m/z* from low (blue) to high (red) *m/z*, and the left band representing fed (red) and not-fed (orange) pitcher.



Supplementary Figure 7. Feeding fold-change density plots. A plot of kernel density estimates for *m/z* of polar DAFs' (FDR<0.05) due to feeding status of pitchers. In red, fed, and in orange non-fed pitcher *m/z* distributions.

Manuscript 5 (unpublished data)

"Two distinct extrafloral nectars in *Nepenthes × ventrata*"

Introduction

Nepenthes × *ventrata* lives on nutrient-poor soils. As other carnivorous plants, it can survive despite the lack of nutrients by capturing insect preys: thanks to its traps called "pitchers", it can catch and kill its prey, digest them, and acquire their nutrients. Pitchers are passive capture mechanisms: prey are captured without the intervention of electrical signals (active capture mechanisms, of *Drosera* and *Dionaea* traps, for example, require electrical signaling to activate complex capture apparatus). Other examples of carnivorous plants with passive capture traps are the pitchers of *Sarracenia*, *Darlingtonia*, *Cephalotus* and *Heliamphora* and the sticky leaves of *Genlisea*. In *N.* × *ventrata*, pitchers contain a liquid with digestive enzymes such as chitinases, proteases and other hydrolases (Fukushima, K. et al., 2017). Exploring insects fall when walking on the pitcher's slippery peristome, or mouth they drown, and get digested (Bauer, U. et al., 2008).

The mechanisms of prey capture, digestion, and assimilation have been extensively studied (Mithöfer, A., 2011). Prey specificity has also been documented in several *Nepenthes* species: in *N. rafflesiana*, pitchers from the upper part of the vine capture mostly winged insects, while pitchers from the lower strata of the vine capture more non-winged insects such as ants (Di Giusto, B. et al., 2010). *N. ampullaria* captures besides insect prey also leaf litter; *N. albomarginata* mainly catches termites (Mithöfer, A. 2022). However, specific prey-attraction mechanisms in *Nepenthes* have received little attention.

Nepenthes species produce a sugary solution on branches and peristomes (**Figure 1**). This type of solution is called extrafloral nectar (EFN) and plays a crucial role in attracting ants in ant-plants, for example. In these cases, EFN attracts bodyguard ants to vulnerable organs under attack and/or detracts foraging ants from other resources such as pollen (Rico-Gray, V., & Oliveira, P. S. 2008).

The present study aims at testing two non-exclusive hypotheses regarding the attractant role of EFN in *Nepenthes*:

- EFN might attract prey to pitchers;
- EFN might attract ant bodyguards to protect leaves against herbivores.

Given that *N.* × *ventrata* resources are extremely constrained by its environment, the composition of EFN must be optimized to balance insect attraction with energy investment and nutrient waste. Therefore, examining the composition of *N.* × *ventrata* EFN can provide clues about its ecological role. If the main role of EFN is to attract ant bodyguards, its composition should be similar to the composition of EFN in ant plants, i.e. rich in sugars and other nutrients such as amino acids. If the main role of EFN is to attract prey, it should be rich in sugars but poor in nutrients, to avoid the waste of feeding amino acids to insects only to reabsorb them later from their digested tissues.

We conducted a targeted metabolite analysis in *N.* × *ventrata* EFN, from branches and from pitchers separately, to characterize their composition. Using LC/GC-MS, we found that the

main components of *N.* × *ventrata* EFN are three sugars: glucose, fructose and sucrose. We also detected the presence of amino acids, although their concentration was a lot higher in branch EFN than in pitcher EFN. Thirdly, we found evidence for phytohormones in EFN, indicating that EFN production might be related to stress. Finally, we conducted the same analyses in several other plant species exhibiting a spectrum of facultative-obligate interactions with ants. The comparison of the composition of *N.* × *ventrata* EFN to other EFN with well-described ecological roles provided evidence that *N.* × *ventrata* EFN serves to attract bodyguards to branches and prey to pitchers.

Figure 1. *Illustration indicating two types of nectar from different sites of production within Nepenthes* × *ventrata. Enlarged areas of* ***a****) branch and* ***b****) pitcher's peristome with nectar droplets on the surface of the producing tissue that denotes its origin. Copyright © of drawing held by Ronny Zimpel.*

Results

Biochemical characterization of *Nepenthes × ventrata* extrafloral nectars and tissues.

Aiming at nutritional profiling of the nectars, amino acids, and vitamins were measured. Recently described compounds with protective properties for Nepenthes were also sought. In addition, phytohomones, molecules that are commonly associated with herbivory-induced responses, were also measured. In total, we analyzed 35 metabolites and 48 samples (12 samples of branch EFN, 12 samples of peristome EFN, 12 samples of branch tissue, and 12 samples of pitcher tissue). These 35 metabolites were organized into three functional categories:

- Nutrition-related metabolites: B-vitamins (B1, B2, B3, and B5) and amino acids (Thr, Trp, His, Tyr, Leu, Phe, Val, Ile, Met, Ala, Pro, Ser, Lys, Asn, Arg, and Gln).

- Stress-regulation-related metabolites or phytohormones (SA, JA, ABA, JA-Ile, *cis*-OPDA, OH-JA, OH-JA-Ile, and COOH-JA-Ile)

- Defense-related metabolites: anthocyanins (cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, and cyanidin-3-*O*-glucuronide), plumbagin and γ-aminobutyrate (GABA).

Intra-category variability was much lower than inter-category variability (**Figure 2**). EFN samples were more similar to each other than they were to tissue samples, and EFN samples from branches were more similar to each other than to EFN samples from peristomes.

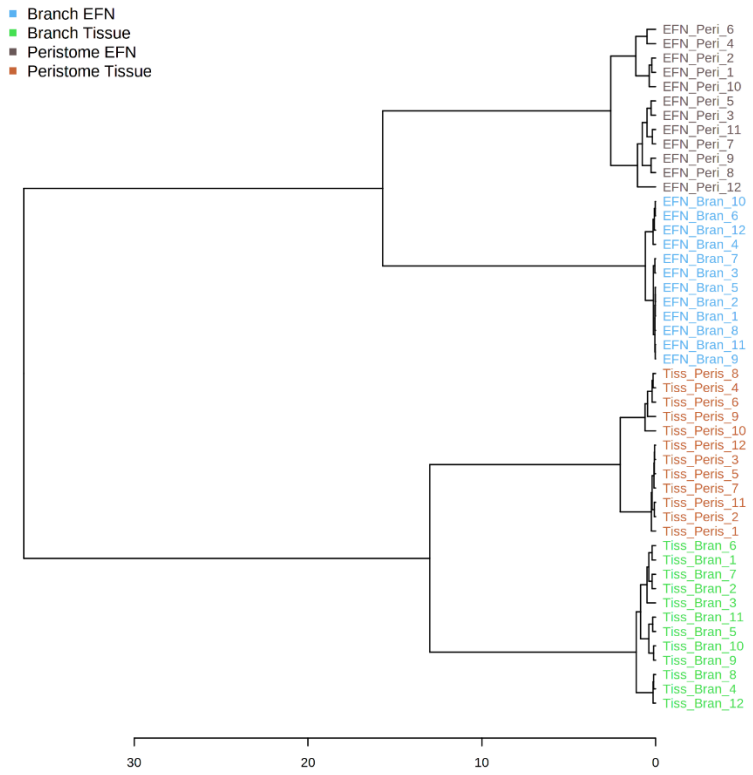


***Figure 2**: Hierarchical clustering dendrogram of 12 peristome EFN, 12 branch EFN, 12 peristome tissues, and 12 branch tissues of Nepenthes × ventrata based on 35 metabolites. The dendrogram was built using Spearman metric distance and Ward linkage. Dendrogram end's branches indicate independent biological samples. Sample collection was randomly performed over one year. Four robust groupings are observed according to the sample nature.*

To look into more detail of the composition of each sample category, we built a clustered heat map (**Figure 3**).

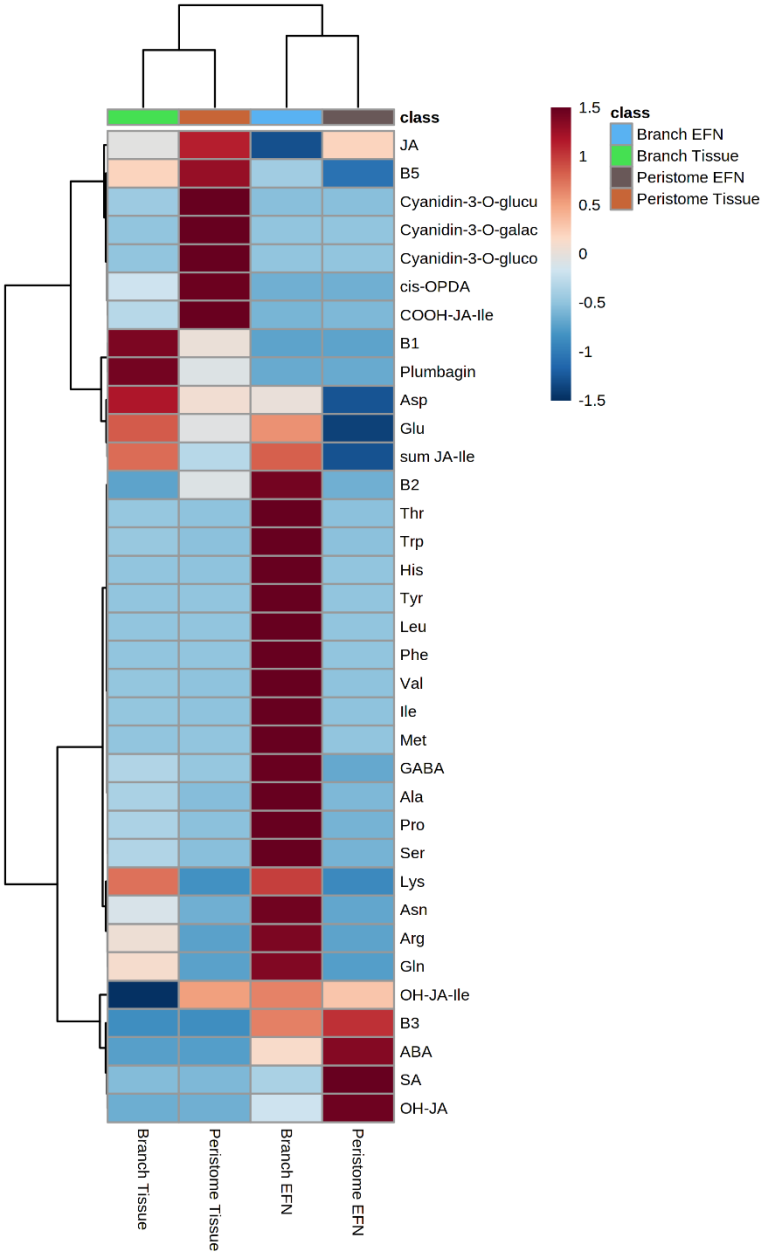


***Figure 3**: Clustered heat map for 35 metabolites analyzed in tissues and EFNs in Nepenthes × ventrata (Pearson distance and Ward clustering algorithm, generated using MetaboAnalyst). The color scale represents the scaled abundance of each metabolite, red hues indicate relative high abundance, blue hues indicate relative low abundance.*

Branch EFN was rich in amino acids. In fact, branch EFN showed the highest concentration in 16 amino acids, even higher than branch and peristome tissues. Given that EFN is very unlikely to be a prime location for metabolic activity, we can hypothesize that these amino acids are destined for consumption by ant bodyguards. This can be confirmed by the quasi-absence of metabolites conferring protection to plant tissues, such as plumbagin.

In contrast, peristome EFN is very poor in amino acids and most other metabolites. This means that peristome EFN has a very poor nutritional value, especially compared to branch EFN.

Branch tissues showed the highest concentration in metabolites associated with direct defense. Similarly, peristome tissues were also very rich in defense-related metabolites. The arsenal differed between the two types of tissues, branch tissues are rich in plumbagin, whereas peristome tissues are rich in anthocyanins. However, GABA concentration was the highest in Branch EFN (**Figure 4**).

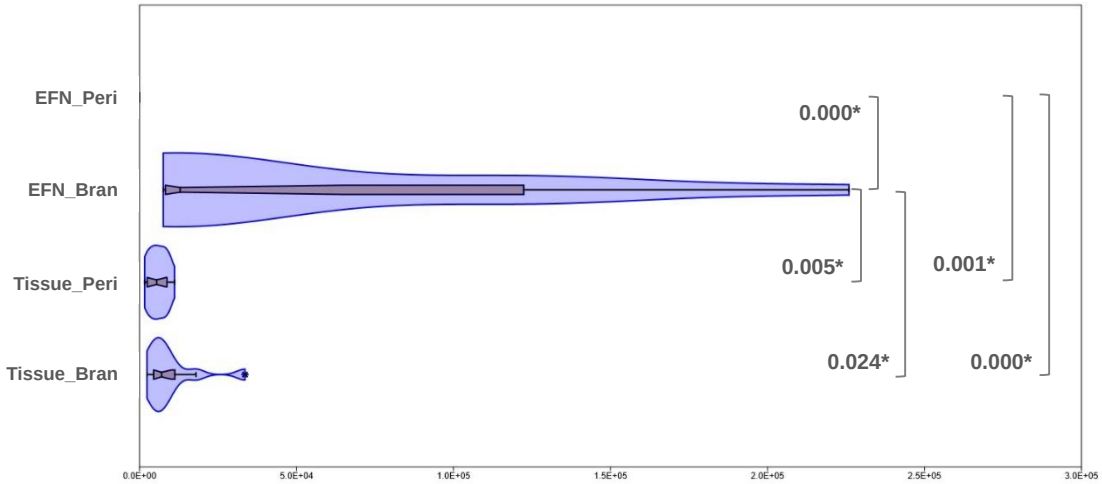


***Figure 4**: Violin plots of the quantification of GABA in tissues and EFN of Nepenthes × ventrata. Boxplots show the 25% percentile, the median and the 75% percentile; whiskers show the minimum and maximum values. Values are given in ng/g. P-values and asterisks indicate statistical significance of pairwise comparisons using Dunn test.*

Biochemical comparison of *Nepenthes × ventrata* EFNs with other species: amino acids

We compared the biochemical composition of *N. × ventrata* EFN to other EFN with a well-described ecological role. We sampled EFN from two plant species engaged in an obligate and specific mutualistic relationship with ants (*Vachellia chiapensis* and *V. colinsii)* and from two plant species engaged in obligate non-specific mutualistic relationships with ants (*Tococa guianensis* and *T. quadrialata*) (Mesquita-Neto, J. N. et al., 2020; Michelangeli, F. A. 2010; González-Teuber, M., & Heil, M., 2009a). We also included EFN samples from two plant species engaged in facultative interactions with ants (*Vachellia farnesiana* and *Leucaena leucocephala*). In all samples, we measured the concentration of 18 amino acids: His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val, Arg, Gln, Pro, Tyr, Ala, Asp, Asn, Glu, and Ser. The rest of the species analyzed were chosen as all of them are EFN producing and for their availability in the greenhouse.

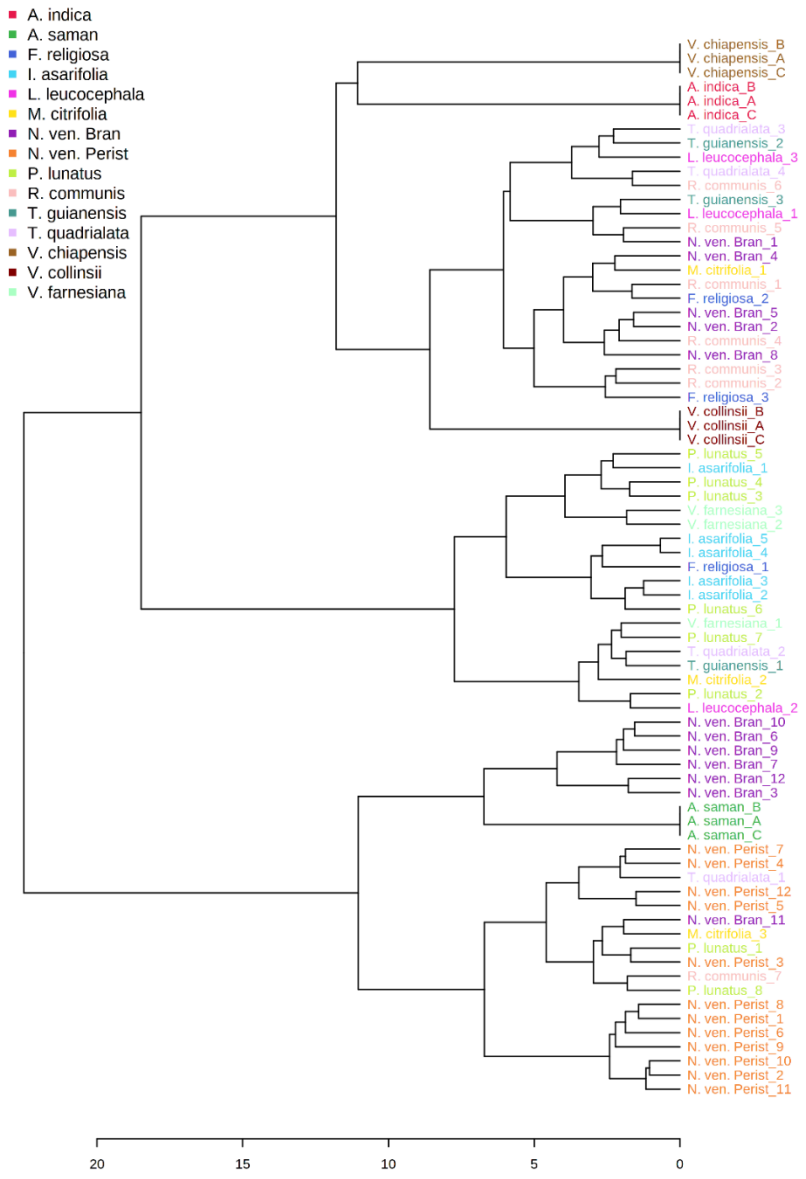


Figure 5*: Hierarchical clustering dendrogram of 67 EFN samples from 14 species based on amino acid content. N. × ventrata samples include EFN from branches and peristomes. The dendrogram was built using Spearman metric distance and Ward linkage.* Nepenthes *nectar samples (12 from branch and 12 from peristome) are reanalyzed from the previous dendrogram.*

Three main clusters emerged from the analysis. The first clusters comprised almost all samples from plants engaged in obligate interaction with ants, some from plants involved in facultative interactions with ants, and from *N. × ventrata* branch samples. The second cluster comprised mostly samples from plants involved in facultative interactions with ants, and no

N. × *ventrata* samples. The third cluster has two subclusters, one with only *N.* × *ventrata* samples from branches and *A. saman* samples, the other with all *N.* × *ventrata* samples from peristomes, one *N.* × *ventrata* sample from a branch, and a few other species. These results show, first, that *N.* × *ventrata* branches and peristome EFNs fall in different ecological categories, and second, that *N.* × *ventrata* branch EFN is not closer to the EFN from obligate ant associates than to facultative ant associates.

We explored the differences and similarities between EFNs a bit further with a Principal Component Analysis (PCA; **Figure 6**). Branch and peristome *N.* × *ventrata* EFNs were well

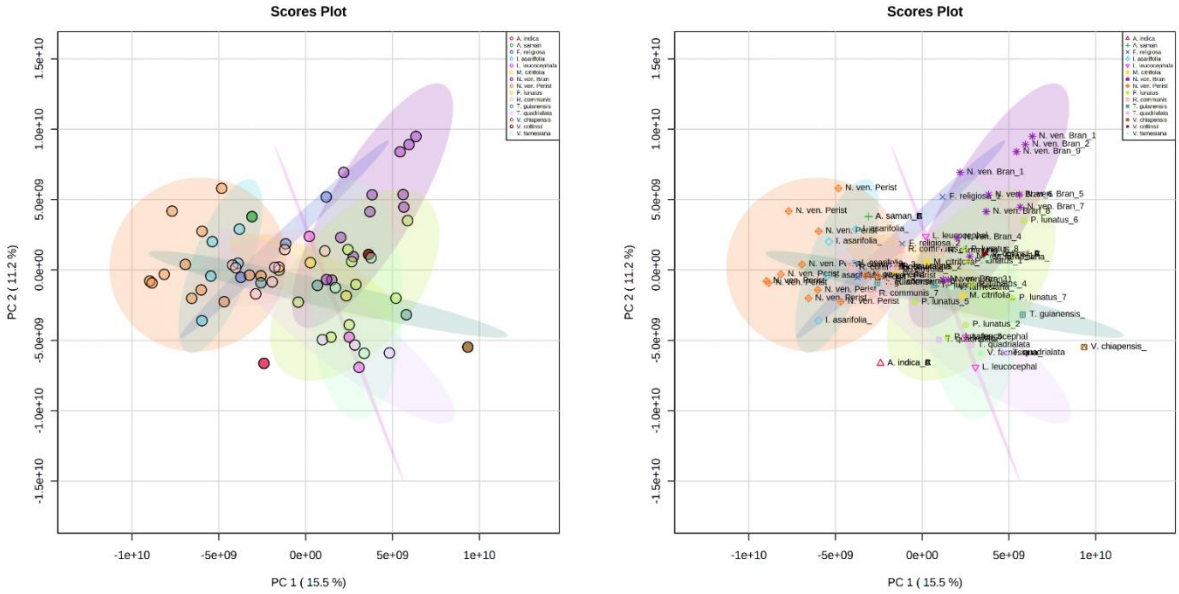


***Figure 6**: Principal component analysis using MetaboAnalyst. Data produced from 14 plant species (67 EFN samples), generated based on the content in 18 amino acids. Nepenthes × ventrata samples include EFN from branches and from peristomes. Chart without labels on the left, with labels on the right.*

differentiated along the first principal component, which accounts for 15.5% of the variability. Confirming the results of the previous analysis, there was a clear overlap between *N.* × *ventrata* branch EFN and other plant species, whereas *N.* × *ventrata* peristome EFN samples were almost isolated along with samples from *I. asarifolia*. Interestingly, *N.* × *ventrata* branch samples showed a wide distribution along the second principal component, which accounts for 11.2% of the variability, with some samples being isolated from the rest.

We next looked at the amino acids with the highest contribution to the first principal component (Asp, Lys, Val, and Glu) (**Figure 7)**.

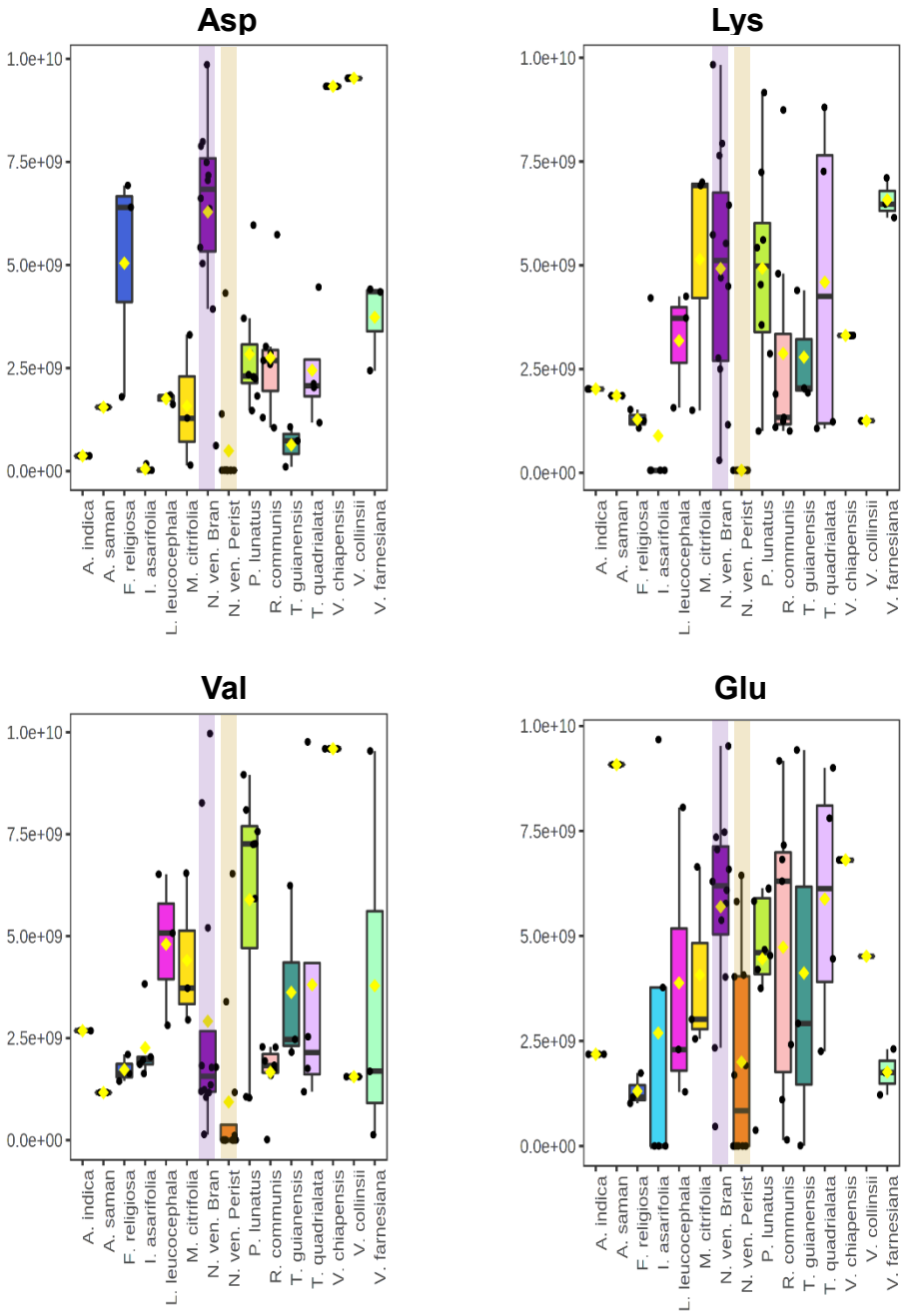


Figure 7*: Quantification of the four amino acids with the highest contribution to the first principal component of the PCA. The purple column represents samples of N. × ventrata branch EFN; the orange column represents samples of N. × ventrata peristome EFN. Y-axis scale is in ng/g.*

Three of these amino acids (Asp, Lys, and Val) showed a very low (nearly null) concentration in *N. × ventrata* peristome EFN, while Glu concentration was among the lowest. Conversely, Asp, Lys and Glu concentration were among the highest in *N. × ventrata* branch EFN.

Biochemical comparison of *Nepenthes* × *ventrata* EFNs with other species: sugars

We also compared the sugar composition of *N.* × *ventrata* EFNs to other EFNs of the same other plant species. In all samples, we measured the concentration of three sugars: fructose, glucose, and sucrose. We built a dendrogram based on a hierarchical clustering analysis (**Figure 8**).

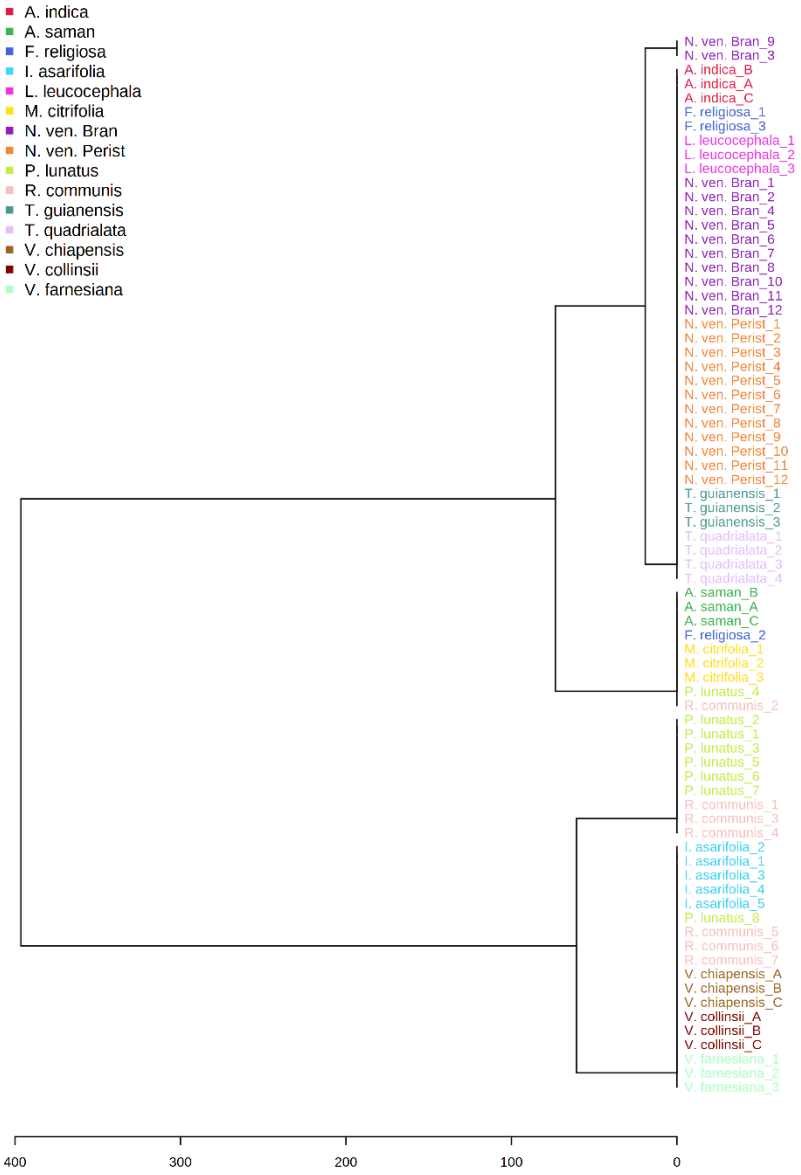


***Figure 8**: Hierarchical clustering dendrogram of 67 EFN samples from 14 species based on sugar content. N. × ventrata samples include EFN from branches and peristomes. The dendrogram was built using Spearman metric distance and Ward linkage and generated using MetaboAnalyst.*

Two main clusters emerged from the analysis. The first clusters comprised all samples from the two *Vachellia* species engaged in obligate interaction with ants, some from plants involved in facultative interactions with ants, and no *N.* × *ventrata*. The second cluster comprised all samples from the two *Tococa* species engaged in obligate interaction with ants, some from plants involved in facultative interactions with ants, and all samples of *N.* × *ventrata*, regardless of whether they come from branches or peristomes.

These results show that, when it comes to sugars, there is no clear pattern differentiating plants involved in obligate relationships with ants from plants involved in facultative relationships with ants. There is also no clear difference between *N.* × *ventrata* branch EFN and *N.* × *ventrata* peristome EFN.

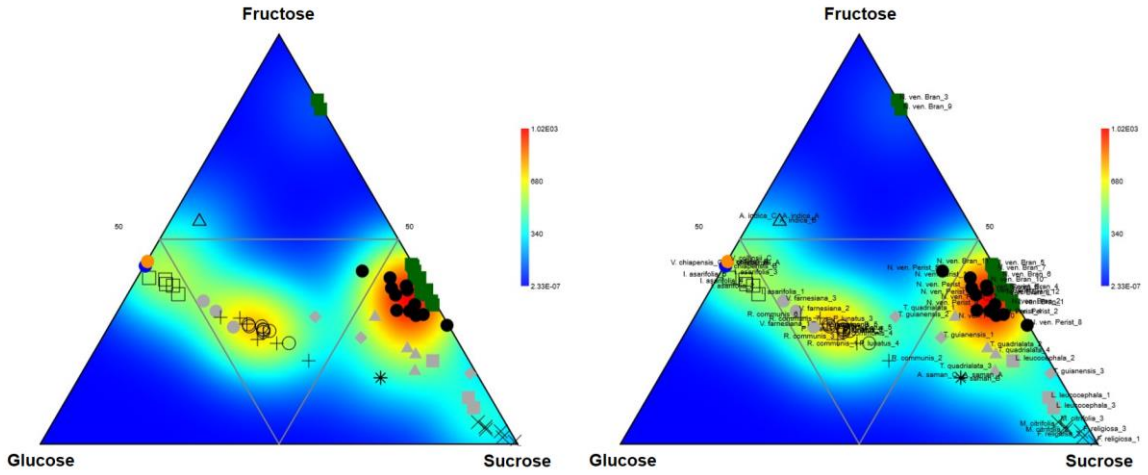


Figure 9*: Ternary plot of 67 EFN samples from 14 species based on sugar content (fructose glucose and sucrose). N. × ventrata samples include EFN from branches and peristomes. Color map of point density computed with kernel density method produced on PAST. Green squares, branch from N. × ventrata; black filled dots, peristome from N. × ventrata; orange and blue filled dots, obligate symbiotic relationship species; gray filled dots, rhombus, triangles and squares, facultative symbiotic relationship species.*

In order to understand the distribution of the ecological roles of the different species according to the three sugars that constitute the EFN, a ternary plot was carried out to decipher the grouping patterns of the species.

Nepenthes EFN are grouped at the opposite side from species having obligate symbiotic relationship. Still, they form a distinct and separate group from the facultative species. No species has only a single sugar type. Obligate species (*V. chiapensis* and *V. collinsii*) have no sucrose. *Nepenthes* is one of the species with the lowest glucose in its EFN (**Figure 9**).

Discussion

Our characterization of *N.* × *ventrata* EFN provides unique and crucial insights into their ecological role. Since the literature lacks a general description of EFN composition in carnivorous plants of the genus *Nepenthes*, the first step was to identify and quantify metabolites present and measurable in *N.* × *ventrata* EFN. This step allows for the classification of EFN types, and their comparison with EFN of other plant species. Through this comparison with EFN, whose functions are already described in non-carnivorous plants, we can gain insights into the ecological role that the two types of *N.* × *ventrata* EFN can play. It is important to interpret our results in the context of nutrient scarcity, in order to understand the implications of the plant's investing in potentially costly bait. On one hand, *N.* × *ventrata* peristome EFN might help the plant attracting more prey and therefore acquiring more resources. On the other hand, producing EFN probably comes at a cost, that might be detrimental to the plant fitness if prey are too rare. Our results show that the plant allocation of resources to direct defense, maybe indirect defense, and attraction strategies is optimized.

Only *N.* × *ventrata* tissues contained anthocyanins and plumbagin; such direct defense metabolites were absent from *N.* × *ventrata* EFN. This is not surprising since EFN is produced specifically for insect consumption. However, surprisingly, branch EFN showed a very high concentration in GABA (Figure 3). The concentration was six-fold higher in branch EFN than in branch tissues. In contrast, in addition to lacking defense metabolites, peristome EFN lacks GABA entirely, suggesting that the accumulation of GABA in branch EFN specifically might be important for *N.* × *ventrata*. Furthermore, GABA is an amino acid, meaning that its synthesis and excretion must be costly for the plant.

There is strong evidence for GABA as an important part of the direct plant defense arsenal, for example in *Phaseolus vulgaris*, *Solanum lycopersicon*, *Arabidopsis thaliana*, and *Glycine max*, to mention just a few (Wang, G. et al., 2019; Copley, T. R. et al., 2017; O'Leary B. M. et al., 2016; MacGregor, K. B. et al., 2003; McLean, M. et al., 2003). Both local and systemic accumulation of GABA has been observed in plants exposed to tissue damage generated by larvae, and the fitness of insects fed on GABA is significantly decreased (Scholz, S. S. et al., 2017; Scholz, S. S. et al., 2015). Why would *N.* × *ventrata* produce an insecticide compound in such high concentration in a resource destined to be consumed by insects? A conceivable answer is that GABA is not used here as an insecticide, but it might play a role in the protection of the plant by ants. Bees fed on GABA-supplemented diets showed a heightened level of locomotion (Felicioli, A. et al., 2018). If this effect is also true in other hymenopterans, GABA might cause ants to be more active on the plant and therefore increase the chance that they would find the pitcher and be caught. Therefore, the role of GABA in branch EFN might be more related to prey capture than to direct plant defense. In addition, as previously described, the higher plumbagin concentration in leaf compared with pitcher tissue (Davila-Lara, A. et al., 2021a,b) could also drive the insect prey towards the pitcher.

Of the stress-related phytohormones measured, JA was mainly present in peristome tissue and moderate levels JA-Ile were present in branch tissue. Surprising was the finding of phytohormones in *Nepenthes* EFN. JA-Ile showed up mainly in the branch nectar, while the rest of the hormones were more abundant in the peristome nectar (**Figure 2**). The presence

of extracellular phytohormones has already been reported. Yilamujiang, A. et al., (2016) described that *Nepenthes*' pitcher fluid contains JA-Ile. However, the presence of phytohormones in EFN has never been described. Jasmonates are involved in plant adaptation to stress: JA-Ile activates plant's physiological defense mechanisms (Vadassery, J. et al., 2014; Staswick, P. E., & Tiryaki, I. 2004). A variety of stressors can induce a jasmonate-mediated physiological response: herbivory, some microbial infections, physical damage, extreme temperatures, drought, hypersalinity, or even heavy metal stresses can trigger the release of JA-Ile (Zander, M., 2021; Marquis, V. et al., 2020; Howe, G. A. et al., 2018; Li, Q. et al., 2017). JA-Ile binds to the co-receptor complex formed by coronatine insensitive 1 (COI1) protein, SCF E3 ubiquitin ligase, and jasmonate zinc-finger inflorescence meristem (JAZ). This generates a ubiquitination and subsequent degradation of JAZ. The absence of JAZ protein (repressor degradation) results in the activation of transcription factors that modulate plants' response gene expression (Takeuchi, J. et al., 2021; Raza, A. et al., 2021; Ali, M., & Baek, K. H., 2020; Wasternack, C., & Song, S., 2017; Wasternack, C., & Hause, B., 2013). Since phytohormones perform their mechanism of action in the cell nucleus, finding non-negligible amounts of genetic regulators outside the cell is intriguing.

Unlike branch tissues, peristome tissues had higher concentrations of the jasmonate metabolites cis-OPDA, JA (biosynthetic precursors of the bioactive JA-Ile), as well as COOH-JA-Ile, and OH-JA-Ile (inactivation products). This pattern could be explained by the optimal defense theory, given that branches already possess defense mechanisms that are absent from peristomes. The optimal defense theory predicts that plant defenses should be concentrated in the most valuable and vulnerable parts of a plant (Rhoades, D. F., 1979; McKey, D., 1974). Given that *N.* × *ventrata* branches are protected by constitutive chemical defenses (e.g. plumbagin) and most probably by patrolling ants that are collecting EFN, they have little need for costly inducible defenses. Conversely, pitchers show only anthocyanins and there is no evidence that ants patrol there. Thus, pitchers might have a higher need for JA-Ile-related inducible defenses.

The presence of jasmonates in EFN is more surprising. However, EFN production in lima bean (*Phaseolus lunatus*) has been shown to be selectively induced by JA-Ile (Radhika, V. et al., 2010). The ability for *N.* × *ventrata* to produce EFN only when necessary (i.e. under herbivory stress and when there are ants in the vicinity to attract) would be crucial to prevent waste in resources and energy (Bixenmann, R. J. et al., 2011). The potential cascade relationship between herbivore stress, jasmonate production, induction of EFN secretion, and attraction of ant bodyguards, remains to be studied in *N.* × *ventrata*. High concentrations of salicylic acid (±868 ng/g) were also found in peristome EFN. SA regulates many plant physiological processes and is involved in adaptive responses to abiotic stress such as responses to salinity, osmosis, drought, and heat Khan, M. I. R. et al., 2015; Nazar, R. et al., 2015; Khan, M. I. R., & Khan, N. A. 2013; Hayat, Q. et al., 2010). In addition, SA is involved in plant basal defense and resistance against pathogens (Liu, H. et al., 2016; Lu, M. et al., 2016). It is still unclear how any of these physiological mechanisms relate to SA presence in peristome EFN.

Our results show that *N.* × *ventrata* EFN also have a different nutrient composition depending on the producing organ. Branch EFN is rich in all amino acids and vitamins B2 and B3. Conversely, peristome EFN showed almost no amino acid and was only rich in vitamin B3. Sugar composition was similar between the two types of EFN. This major

difference in nutritional value of EFNs hints at different ecological roles. To attract ant bodyguards, ant-plants produce EFN packed with nutrients such as amino acids (Heil, M., 2011; 2008). Branch EFN seems to match this description of bodyguard-attracting nectar in ant-plants. Moreover, insects cannot synthesize the B2-vitamin, which is also present in high concentration in *N.* × *ventrata* branch EFN. This crucial vitamin that acts as a coenzyme in various enzymatic reactions can only be acquired by insects through their diet (or supplied by symbiotic microorganisms in some specific cases; Douglas, A. E., 2017). In contrast, the nutritional value of peristome EFN is very low, consisting almost exclusively of sugars. It is tempting to speculate that its function is only to keep insects on the pitcher and to guide them to the pitcher mouth, which will increase the probability of prey capture. An investment of costly nutrients such as nitrogen-containing amino acids would be useless. There is no reason for *N.* × *ventrata* to provide costly nutrients to insects, that will be immediately killed and digested. Similar EFN is also found in plants involved in facultative unspecific interactions with ants such as *I. asarifolia*. Antagonistic interactions with ants were described in another *Ipomoea* species: in *I. carnea*, patrolling ants destroy flowers and prevent pollination (Martins, J. et al., 2019). It's possible that the role of EFN in *I. carnea* is not to attract ants to the plant but to detract them from flowers. Therefore, the plant does not really provide a service to the ants and the EFN is nutrient poor. So far, it is not possible to rule out that *Nepenthes* uses ants as a protective agent against herbivores. Still, the fact that branch EFN is very nutritious is a good reason to ensure a continuous supply of ants on the plant. Probably some ants are distracted away from the branches and are captured as prey in the pitcher. Therefore, catching a few ants once in a while is sufficient to maintain the nutritional need of the plant.

Another scenario is conceivable as well. The EFN on branches may be involved in the attraction of protective ants in a situation similar to that known from ant-plants. In this case, a protection-predation strategy is accomplished that is not dissimilar to the push-pull cropping system of agricultural pest management, in which rows of cereals are interspersed between rows of repellent crops (push), while rows of attractant crops are planted at the periphery of the plantation (pull) Eigenbrode, S. D. et al., 2016). In *N.* × *ventrata*, the "push" agents are the combination of direct and indirect defense arsenals (plumbagin, predatory ants attracted by branch EFN), while the "pull" agents are the peristome EFN and the pitchers. This framework offers an attractive explanation for the lack of evidence for herbivore damage in *Nepenthes* species in natural conditions. However, there is no convincing evidence for the recruitment of bodyguard ants in *Nepenthes* except for *N. bicalcarata*, which lives together with protective ant of the species *Camponotus schmitzi* (Merbach, M. A. et al, 2007).

Conclusion

In *Nepenthes*, EFN is a mediator of the predatory carnivorous lifestyle: in the end attracted and fed insects are consumed by the plant. Our results show for the first time that the same individual plant can produce two types of EFN with different chemical compositions, each optimized for a unique ecological function. This unexpected differentiation between two types of EFN is the result of the evolution of the carnivory syndrome in plants growing on nutrient-poor soils. It presents the double advantage of both attracting putative insect prey and guiding them to the traps where they are captured to be killed and digested. The presence of compounds that push insects away from leaves towards the pitcher trap supports this strategy. Again, as seen in other examples such as the adaptation of jasmonate signaling and the employment of defensive proteins, here carnivorous plants use existing features of plants involved in defensive strategies and coopt them for their particular needs. Here, *Nepenthes* reveal a subtle strategy to capture their insect prey, a highway to hell.

Materials and methods

Plant material

The carnivorous plant *Nepenthes* × *ventrata* (Hort. *ex* Fleming) is a natural hybrid of *N. alata* (Blanco) and *N. ventricosa* (Blanco). Plants were bought from the company (Gartenbau, Carow, Nürtingen, Germany) and were kept at the Max Planck Institute for Chemical Ecology for 20 years. Growing conditions were maintained as close as possible to natural conditions: 21-23°C during the day and 19-21°C during the night with a relative humidity varying from 50 to 60% and a photoperiod of 16 hours light and eight hours darkness.

Phaseolus lunatus (L.) were grown in the same conditions. *Albizia saman* (Jacq.) and *Ricinus communis* (L) plants were also grown under the same temperature and relative humidity conditions as mentioned before, but with a different photoperiod: 14 hours light and 10 hours darkness. *Azadirachta indica* (A.Juss.), *Ficus religiosa* (L.), *Ipomea asarifolia* (Desr.), *Leucaena leucocephala* (Lam.), *Morinda citrifolia* (L), *Tococa guianensis* (Aubl.), *Tococa quadrialata* (Naudin), *Vachellia chiapensis* (Saff.), *Vachellia collinsii* (Saff.), *Vachellia farnesiana* (L.) plants were also grown at a 22-24°C day temperature and 16-18°C night temperature, with a 65-70% relative humidity.

EFN and tissue collection

We collected EFN and harvested tissues from 12 *N.* × *ventrata* individuals, from branches and peristomes separately. We also collected EFN samples from the following plant species (number of individuals between brackets): *A. indica* (one), *A. saman* (two), *F. religiosa* (three), *I. asrifolia* (five), *L. leucocephala* (three), *M. citrifolia* (three), *P. lunatus* (eight), *R. communis* (seven), *T. guianensis* (three), *T. quadrialata* (four), *V. chiapensis* (one), *V. collinsii* (one), *V. farnesiana* (three).

EFN collection was done with spatulas. Much care was taken to avoid contamination by scratching tissue. Samples were store in Eppendorf tubes. Due to the minute volume of EFN produced by each plant, it took an entire year to collect EFN volumes large enough to be analyzed. Samples were kept at -20°C until used for analyses. Plants already producing EFN were chosen for tissue sampling (24 independent biological replicates). Subsequently, tissue was washed with *dd*H_2O until nectar was removed. Then, both tissue (branches and peristomes) were sampled and stored in 50-mL polypropylene tubes and immediately frozen in liquid nitrogen. Tissues were finely ground in liquid nitrogen using mortar and pestle. 100 mg of tissue was stored in screw-cap Eppendorf tubes and stored at -80° until processing.

Extractions

We measured the concentration of phytohormones, amino acids, B-vitamins, plumbagin and anthocyanins from a single extract per *N.* × *ventrata* sample. Sugar quantification was conducted on a subsample extract. Similarly, amino acid and sugar concentrations were measured in two separate EFN samples in every other plant species. The extraction, detection and quantification of phytohormones, amino acids, B-vitamins, plumbagin and anthocyanins were performed as described in Dávila-Lara, A. et al., (2021a,b), Heyer, M. et al., (2018), Khaksari, M. et al., (2018), Crocoll, C. et al., (2016), and Vadassery, J. et al., (2012).

Approximately 100 mg of *N.* × *ventrata* branch or peristome tissues were finely-ground in liquid nitrogen using mortar and pestle and weighed in 2 mL tubes. We added 1.0 mL extraction buffer to each tube: *dd*H_2O:MeOH (50:50 v/v) containing standards for phytohormones and B-vitamins. Samples were then sonicated for 15 min in an ice-cold water bath (3 rounds of 5 min sonication with 3 min rest in between). They were then agitated for 30 min at 4°C using a Rotor Mixer RM-Multi-1 (STAR-LAB GmbH, Hamburg, Germany) with the following settings: orbital at 100 rpm for 15 s, reciprocal at 75° for 16s, and vibro at 3° for 5 s. Finally, samples were centrifuged at 16,000 *g* at 4°C for 30 min. Clear supernatants were collected and used for further analysis. The following phytohormone standards were used: 60 ng of D_6-abscisic acid (Toronto Research Chemicals, Toronto, Canada), 60 ng of D_6-jasmonic acid (HPC Standards GmbH, Cunnersdorf, Germany), 60 ng of D_4-salicylic acid (Santa Cruz Biotechnology, Santa Cruz, U.S.A), and 12 ng of D_6-jasmonic acid isoleucine conjugate (HPC Standards GmbH, Cunnersdorf, Germany). For B-vitamin analyses 100 ng of each of the following internal standards were used: D_3-thiamine, ^{13}C,$^{15}N_2$-riboflavin, D_4-nicotinic acid, and $^{13}C_3$,^{15}N-pantothenic acid (all Toronto Research Chemicals, Toronto, Canada). Similar extractions were conducted in EFN samples to quantify sugars, at a ratio of 1 mg of EFN/10 µL extraction buffer, and without internal standards.

Phytohormone and plumbagin quantification

LC-MS/MS quantification of phytohormones and plumbagin were carried out as described in Heyer, M. et al., (2018); Vadassery, J. et al., (2012) on an Agilent 1260 series HPLC system (Agilent Technologies, Böblingen, Germany) coupled to a tandem mass spectrometer QTRAP 6500 (AB SCIEX, Darmstadt, Germany). Chromatographic separation was achieved on a Zorbax Eclipse XDB-C18 column (50 x 4.6 mm, 1.8 µm, Agilent

Technologies). Here, *dd*H_2O containing 0.05% formic acid and acetonitrile as mobile phases A and B, respectively, was used. The elution profile was: 0-0.5 min, 10% B; 0.5-4.0 min, 10-90% B; 4.0-4.02 min, 90-100% B; 4.02-4.5 min, 100% B and 4.51-7.0, min 10% B. Flow rate was kept at 1.1 mL min^{-1} and the column temperature was maintained at 25 °C. The mass spectrometer (QTRAP 6500 (AB SCIEX, Darmstadt, Germany)) was equipped with Turbo spray ion source operated in negative ionization mode. The ion spray voltage was maintained at -4,500 eV. The turbo gas temperature was set at 650 °C. Nebulizing gas was set at 60 psi, curtain gas at 40 psi, heating gas at 60 psi, and collision gas set to "medium". The mass spectrometer was operated in multiple reaction monitoring (MRM) mode.

For plumbagin, an MRM was added to the method: Q1: m/z 187, Q3: m/z 159, DP: -20, CE: -18. Retention time: 4.3 min. RF relative to D_6-jasmonic acid: 164.0. Since we observed that both the D_6-labeled JA and D_6-labeled JA-Ile standards (HPC Standards GmbH, Cunnersdorf, Germany) contained 40% of the corresponding D_5-labeled compounds, the sum of the peak areas of D_5- and D_6-compound was used for quantification.

B-vitamin analysis:

Water soluble B-vitamins quantification was done by LC-MS/MS. Thiamine (B1); riboflavine (B2); niacine (B3); and pantothenic acid (B5) were quantified using the method described by Khaksari, M. et al., (2018). With the exception of the elution profile, the method and instrumentation was the same as used for phytohormone quantification. The elution profile was: 0–3.0 min, 0% B; 3.0–6.0 min, 0–80% B; 6.0–6.01 min, 80–100% B; 6.01–7.0 min, 100% B and 7.1–10.0, min 0% B. Flow rate was kept at 1.1 mL/min^{-1} and column temperature was maintained at 25°C. The mass spectrometer (QTRAP 6500 (AB SCIEX, Darmstadt, Germany)) was equipped with a Turbo spray ion source operated in positive ionization mode. The ion spray voltage was maintained at 5,500 eV. The turbo gas temperature was set at 620°C. Nebulizing gas was set at 60 psi, curtain gas at 40 psi, heating gas at 60 psi, and collision gas was set to "medium". The mass spectrometer was operated in multiple reaction monitoring (MRM) mode with the following parameters and responses factors: niacin: Q1: m/z 124, Q3: m/z 80, DP: 20, CE: 25, Retention time: 1.2 min, RF: 1.0; pantothenic acid: Q1: m/z 220, Q3: m/z 90, DP: 20, CE: 19, Retention time: 5.1 min, RF: 1.0; thiamin: Q1: m/z 265, Q3: m/z 122, DP: 20, CE: 17, Retention time: 0.43 min, RF: 1.0; riboflavin: Q1: m/z 377, Q3: m/z 243, DP: 20, CE: 31, Retention time: 5.45 min, RF: 1.0.

Amino acid analysis:

For tissue samples, an aliquot of the liquid extraction was diluted to a 1:10 ratio (v:v) in *dd*H_2O containing U-[^{13}C, ^{15}N] labeled algal AA (Isotec, Miamisburg, US) at a concentration of 10 µg mL^{-1} as internal standards. For EFN samples, 15-70 mg of sample were dissolved in *dd*H_2O and brought to a 1 mg:10 µl concentration. We mixed 10 µl of the obtained EFN solution with 90 µl of *dd*H_2O containing internal standards mix U-[^{13}C, ^{15}N] labeled algal amino acids. For amino acids quantification in the diluted extracts LC-MS/MS according to (Crocoll, C. et al., 2016) with a QTRAP6500 mass spectrometer (AB Sciex, Darmstadt, Germany) coupled to the LC system was used. The analysis involved the coupling of the reversed-phase LC by electrospray ionization (ESI) in positive ionization mode to the

tandem mass spectrometer operated in MRM mode. Individual amino acids were quantified in respect to the U-^{13}C, ^{15}N-labeled amino acid internal standard, except for Trp and Asn. Trp was quantified using ^{13}C, ^{15}N-Phe applying a response factor of 0.42, and Asn was quantified using ^{13}C, ^{15}N-Asp applying a response factor of 1.0.

Anthocyanin analysis

Anthocyanins were analyzed following the method described by Dávila-Lara, A. et al., (2021b) using a reversed-phase HPLC with UV detection using an Agilent 1100 system (Agilent Technologies, Waldbronn, Germany), Nucleodur Sphinx RP columns (250 x 4.6 mm; µm; Macherey-Nagel, Düren, Germany). The injection volume was 50 µL, the flow rate was 1.0 mL min^{-1}, solvent A was 0.5% (v/v) trifluoroacetic acid, and solvent B was acetonitrile. The photodiode array detector was used in the 250-650 nm range. Samples were analyzed with the following chromatographic gradient: start 5% B, linear gradient from 5% B to 25% B in 20 min followed by a washing cycle. Peaks at 18.1 min and at 18.5 min in the HPLC-UV/Vis chromatograms were identified by match of retention time with commercial standards as cyanidin-3-*O*-galactoside (Extrasynthese, Genay, France) and as cyanidin-3-*O*-glucoside (TransMIT GmbH, Gieβen, Germany), respectively. Quantification was achieved by detection at 520 nm using a calibration curve generated from authentic cyanidin-3-*O*-glucoside.

Sugar analysis

For each EFN sample, an EFN stock solution diluted in *dd*H_2O at a 1 mg ml^{-1} concentration was prepared. 100 µl of stock solution was dried and derivatized using 20 µl pyridine (Sigma-Aldrich, Munich, Germany) and 20 µl MSTFA (Macherey-Nagel, Düren, Germany) for 30 min at 60°C. 10 µl of the solution 1:100 was diluted in dichloromethane and 1 µl was injected. Measurements were carried out using a Thermo-Scientific Trace 1310 ISQ Lt GC-MS system equipped with a Zebron™ ZB -5 column (30 m x 0.25 mm x 0.25 µm, plus a 10 m guardian end from Phenomenex, Aschaffenburg, Germany). The following instrument settings were used: injector temperature at 230°C, helium as carrier gas, 1.5 ml min^{-1} flow rate, and split ratio at 1:10. The initial temperature was 120°C, for 2 min, heating up 10°C min^{-1} up to 280°C. Measurements were done in positive mode at 70 eV. Ion source temperature was set at 280°C and MS transfer line temperature at 250°C. For identification, fructose, glucose and sucrose standards were used and treated same way as samples.

Statistical analysis, including principle component analysis, heatmap, dendrograms, and ternary plot:

Statistical analyses were performed using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca) a free online platform from Xia Lab at McGill University, Canada. Measures were normalized by converting concentrations into ng g^{-1} magnitudes based on the MW of each metabolite. Data were not scaled. The biochemical comparison between the tissues and EFN from *N.* × *ventrata* branches and peristomes was done by building a hierarchical clustering dendrogram using Spearman metric distance and Ward

linkage. The comparison was furthered using an unsupervised hierarchical clustering heatmap based on Pearson distances and the Ward clustering algorithm (Ishak, N. A. et al., 2021; Yuan, M. et al., 2012). In order to obtain general information on the variation of the data, principal component analysis (PCA) was used to assess the targeted metabolites data. The advantage of using PCA is that it seeks explanations for a maximum of variation in a multivariate data set without the need for a priori information on the sample group. Thus, it is an unsupervised dimension reduction method (Want, E., & Masson, P., 2011). The biochemical comparison of *N.* × *ventrata* EFN to the EFN of other species was also done using hierarchical clustering dendrograms with Spearman metric distance and Ward linkage. The comparison was furthered using principal component analyses. The ternary plot comparing the sugar content among all EFN samples was computed with a kernel density method. Ternary plot as well as the bar plots for GABA concentrations were generated in the freeware data analyzer PAST 4.03 (PAleontological STatistics) (Hammer, Ø. et al, 2001). The multiple comparison of the concentration of GABA between tissues and EFN were done using Kruskal-Wallis test followed by Dunn test for pairwise comparisons. These analyses were conducted in R v 4.1.1 (R Core Team, 2021).

IV. Discussion

The work presented in this thesis addressed and analyzed three major points:

- Metabolome changes in *Nepenthes* × *ventrata* tissues after insect prey digestion.
- The direct defense strategy against herbivory in *N.* × *ventrata*: isolation and identification of toxic compounds.
- The role of extrafloral nectar in *N.* × *ventrata:* attraction and capture of prey as consequence of the plant´s indirect defense strategy.

Although plant carnivory is rare, this phenomenon has been well studied. In contrast, the study of herbivory on carnivorous plants is very limited. It's likely that *Nepenthes* experiences herbivory, but this phenomenon has not been reported or described in detail, except for *N. bicalcarata* and the attack of a weevil (*Alcidodes spec.*) (Merbach, M. A. et al., 2007).

In order to better understand herbivory in *N.* × *ventrata*, the first step in my approach was to evaluate any putative toxicity of *N.* × *ventrata* leaves. We showed that larvae of the generalist herbivore *Spodoptera littoralis* fed on a diet containing *N.* × *ventrata* tissues grew less than larvae from the control group (Rahman-Soad, A. et al., 2021). I found that the compound responsible for this effect was plumbagin, a member of the naphthoquinone family. Our earlier metabolomic study had already provided hints of the presence of plumbagin in *N.* × *ventrata* (Dávila-Lara, A. et al., 2020), and we confirmed this by comparing tissue extracts and standard spectral data by ^{1}H-NMR spectroscopy (Rahman-Soad, A. et al., 2021). To further our understanding, an experimental herbivory set up was created with controlled conditions in which herbivores could feed on *N.* × *ventrata*. Odorless PET foil (Toppits' Bratschlauch, Germany) was used to enclose *S. littoralis* larvae in the entire leaf of *Nepenthes*. As a control condition, the same setup was created but providing artificial diet as the only food source for larvae. The experiment was done under greenhouse conditions and lasted for five days (Dávila-Lara, A. et al., 2021a). Given that plumbagin is constitutively present in *Nepenthes* tissues, this demonstrates that plumbagin serves as a phytoanticipin involved in plant defense against herbivores. Strikingly, larvae feeding on leaves gained almost no weight but did not die; thus maybe the *Nepenthes* leaf diet is kind of "slim diet food".

N. × *ventrata* constitutively stores this chemical compound at a concentration of ± 250 µg g^{-1} fresh weight in leaves, but its accumulation can be further induced by herbivory (Dávila-Lara, A. et al., 2021a). After 24h herbivore attack, *N.* × *ventrata* can accumulate up to ± 755 µg g^{-1} fresh weight of plumbagin in leaves. This high concentration can remains constant over at least five days of constant herbivory. The concentration necessary for 50% growth inhibition (weight gain) for those larvae is 226.5 µg g^{-1} in artificial diet (Rahman-Soad, A. et al., 2021). However, larvae can still survive up to 900 µg g^{-1} concentration of plumbagin in their diet. Kubo, I., et al. (1983) also demonstrated that plumbagin impacts insect ecdysis. The concentration of plumbagin was higher in leaves than in pitcher tissues (Dávila-Lara, A. et al., 2022; Rahman-Soad, A. et al., 2021; Dávila-Lara, A. et al., 2020). This explains why larvae feeding on leaf tissue were growing less than those fed on pitcher's tissue. Moreover, in a choice assay, regardless of the exposure time, *S. littoralis* larvae also showed a preference for feeding on *N.* × *ventrata* pitchers compared to leaves (Rahman-Soad, A. et

al., 2021; Dávila-Lara, A. et al., 2021a). Taken together, these results suggest that herbivores can detect the presence of plumbagin before tasting the leaves. Indeed, there is robust evidence that plumbagin is a slightly volatile compound (Rahman-Soad, A. et al., 2021). The constitutive presence of plumbagin in high concentration probably results in herbivores being repelled from leaves. Moreover, the rising concentration of plumbagin in case of attack increases the deterrent effect and intoxicates the herbivore. This phenomenon probably creates a "push" effect that detracts insects from leaves and redirects their attention toward other more palatable parts of the plants, in this case, the pitchers.

Another finding that confirms the high defensive capacity of *Nepenthes* is the *de novo* induction of trypsin protease inhibitor (TI) accumulation observed in herbivory experiments in *Nepenthes* (Dávila-Lara, A. et al., 2021a). Protease-inhibitors (PIs), or antiproteases, are molecules that inhibit protease functions by breaking down the digestive target protein (Avilés-Gaxiola, S. et al., 2018; Rawlings, N. D. et al., 2004). Trypsin inhibitors are part of the direct plant arsenal against herbivory: they reduce the biological activity of trypsin found in the digestive tract of animals and therefore prevent them from digesting the plants as food (Engelking, L. R. 2015; Rawlings, N. D., & Barrett, A. J. 1994). Interestingly, in *N.* × *ventrata* PI enzymatic activity was increased by different topically applied phytohormones, in contrast to plumbagin (Dávila-Lara, A. et al., 2021a).

Contrary to what we saw with plumbagin, I found a higher concentration of anthocyanins in pitcher tissues than in branch tissue (Dávila-Lara, A. et al., 2022 **Manuscript 5 (*unpublished data)***; Dávila-Lara, A. et al., 2021b). The presence of anthocyanins has been reported in other carnivorous plants such as *Dionaea muscipula* (cyanidin-3-glucoside) (Di Gregorio, G. J., & Dipalma, J. R. (1966) and (delphinidin-3-*O*-glucoside; cyanidin-3-*O*-glucoside; cyanidin aglycone) (Henarejos-Escudero, P. et al., 2018), but never before in *Nepenthes* species. In non-carnivorous plants, anthocyanins have been shown to play important roles in protecting leaf tissues against a range of biotic and abiotic stressors, including herbivores and pathogens (Liu, Y. et al., 2018; Gould, K. S. 2004).

Visual factors such as the variability in the size of dark speckles on the plant, movement caused by wind, and the spot arrangement can simulate the presence of active ants and deter other insects. Interestingly, Lev-Yadun, S., & Inbar, M. (2002) suggest that dark speckles observed on the stem and petioles of *Xanthium trumarium* (Asteraceae) and *Arisrum vulgare* (Araceae) might mimic ant swarms. A similar effect can be suggested for anthocyanins for *N.* × *ventrata*'s pitchers. Linear peristome patterning may further contribute to the illusion of swarms of ants in motion. Anthocyanins (cyanidin-3-*O*-galactoside: $C_{21}H_{21}O_{11}^{+}$; cyanidin-3-*O*-glucoside: $C_{21}H_{21}O_{11}^{+}$; cyanidin-3-*O*-glucuronide: $C_{21}H_{19}O_{12}^{+}$) are cheap to produce for the plant, since they don't contain nitrogen. Ant mimicry could be a cost-effective way to benefit from the repellent effect of ants without having to produce costly rewards for ant bodyguards (Lev-Yadun, S., & Gould, K. S. 2008; Lev-Yadun, S., & Inbar, M. 2002). Note that ants themselves are probably not affected by ant morphological mimicry in plants, since they explore their environment primarily through smell and touch instead of vision. Conversely, it's also possible that anthocyanins are involved in insect attraction in *N.* × *ventrata* rather than in protection from herbivores. Gilbert, K. J. et al. (2018) showed in a field study that the redder pitchers, the higher the degree of herbivory. However, this hypothesis has been discarded in other carnivorous plants, whose traps also contain anthocyanins, such as *Sarracenia leucophylla* (Rodenas, Y. J. 2012), *Pinguicula planifolia*

(Annis, J. 2016), and *Drosera rotundifolia* (Foot, G. et al., 2014). More research is needed to elucidate the role of anthocyanins in *N.* × *ventrata* pitchers and in carnivorous plants in general.

The role of anthocyanins as a toxin in direct defense in *Nepenthes* has not yet been demonstrated such that its role may be only related to direct defense against herbivores, although it might to have the function of attracting certain types of insects. Research into the combined potentiating, synergistic and antagonistic effects of plumbagin, anthocyanins, GABA, and protease inhibitors are needed in order to provide further insight into the modes and mechanisms of biochemical compounds. Further, toxicological experiments should be done with *Nepenthes*' natural herbivore enemies.

Questions arise from the data presented here related to the volatile feature of plumbagin. What is the volatile function of plumbagin? Will it be an attractant or repellent for ants? Will it serve as a means of plant-plant communication to transmit a warning message to other neighboring plants about herbivore attack?

As with many other plants *N.* × *ventrata* is capable of producing extrafloral nectar (EFN) (González-Teuber, M., & Heil, M., 2009b). Nevertheless, data reported in this work showed there are two types of EFN in this species, one with high nutritional value and produced in the branches of the plant, and the other with lower nutritional qualities and produced on the peristome. The role of EFN in non-carnivorous plants is focused on directly attracting ants. It is highly plausible that EFN in *Nepenthes* has this same function. Compounds that contribute to the nutritional value of EFN, serve as an exchange currency in a partnership between non-carnivorous plants and ants. It is a fee for the bodyguard-service role provided by ants, who attack herbivores that try to consume the plant (Heil, M., 2015; 2011; 2008). However, some questions arise, including: why do *Nepenthes* plant invest valuable resources in developing both direct and indirect defense strategies? which ecological functions are involved in the production of the two different types of nectars in the same individual?

Nepenthes is in constant need of capturing prey. Therefore, it is not efficient for the plant to instantly kill its aggressor at a distant site and waste potential prey. For this reason, it is reasonable to think that *Nepenthes* may be implementing "push-pull" strategy as a derivation of direct defense, attracting prey while guiding them in stepping-stone fashion to their capture. This "pull" effect is achieved in two steps. First, ants are attracted to branches by high-nutrient EFN, ant scouts actively recruiting other workers after finding a nutritious and accessible food resource in branch EFN. Local ant colonies might even rely on repeated exploitation of this resource. Then, volatile plumbagin would exert the "push" force pushing ants away from leaves, either to stay on branches or to climb on pitchers. The second "pull" force, occurs once ants are on the pitcher, EFN from the peristome guides exploring ants towards the pitcher mouth, where, due to the pitcher's efficiency, the ants become a captured prey. As consequence, the plant may be taking advantage of the ants as an indirect defense resource, but at the same time, using them as a prey resource.

Regarding attraction of prey, a proteome analysis of EFN has not been performed, but information on this would provide valuable data on the investment that *Nepenthes* puts into resources to defend the nectar or simply how nutritious the nectar is. It would also be important to analyze which type of the two EFN produced by *Nepenthes* might have a

greater attracting effect on ants. In this area, fieldwork needs to be done to identify natural enemies and natural symbionts. It will also be necessary to assess whether ants have a preference of choice for EFN produced by *Nepenthes* over a sugar solution.

Experiments on the ant's ability to attack *Spodoptera littoralis* larvae can be studied in laboratory conditions, observing the ants' behavior in the presence of larvae. Then results should be compared in greenhouse conditions, exposing a plant to herbivory by *S. littoralis* larvae and then analyzing the ants' ability to attack the larvae. Another way to analyze would be to expose a *Nepenthes* plant with a colony of ants, and then analyze the response capacity of the ants to the introduction of an herbivore in that microenvironment. Will the ant be able to deter or kill the larvae, or will they rather drive the larvae towards the opening of the pitcher? Ideally all experimentation should be validated under natural conditions. This mean, not only *in situ*, but also using the plant's natural herbivores as well as the ants living in the same ecosystem where *Nepenthes* occur.

This brings us to other major problem, *Nepenthes*' natural enemies are unknown, nor is the feeding habit of this plant known at the species level. Further comparative toxicological studies with herbivorous species that are natural enemies of the plant are needed in order to understand the thresholds levels of tolerance.

Last but not least, *Nepenthes,* in natural conditions faces abiotic stress (nutrient limitation), biotic stress (herbivory), and at the same time a prey digestion and nutrient absorption. Which will be prioritized by the plant if has to face all three situations? To defend itself from attack or to nourish itself? A differential relationship with insects is expected to happen, since the interaction is at different levels of complexity. Therefore, it is most likely to find that the plant developed a certain strategy to modulate/moderate the different types of biological interaction (herbivores, prey, pollinators, other trophic levels).

V. Conclusions

1. During this thesis, we learned that *Nepenthes* × *ventrata* has a complex defense arsenal including constitutive and inducible defenses against herbivores.

2. This arsenal is influenced by the carnivory habits of the plant, since organs involved in prey capture have a different set of defenses compared to the rest of the plant.

3. Plant defense may play a role in carnivory, for example plumbagin (and chemical compounds in general) potentially providing a “push” away from leaves and towards pitchers.

4. Further, *Nepenthes* produces extrafloral nectar (EFN) as do non-carnivorous plants.

5. These two different *Nepenthes* EFN have different chemical components and different sites of production.

6. The plant’s existing features are implemented to meet both plant’s defense and prey capture.

VI. Outlook

The carnivore syndrome in the plant kingdom is a phenomenon that continues to intrigue many generations of scientist. The understanding of this phenomenon is not fully elucidated. Considerable progress has recently been made in understanding the chemical and biochemical components of these plant.

While the enzymatic composition of the *Nepenthes*' digestive cocktail has been studied in detail, there was previously no knowledge of the metabolic compounds induced during the digestive process. Data presented here provides precedent for a new field of research on the ecology of *Nepenthes*. Many ecological questions remain unanswered, however, such as: What happens belowground? What is the contribution of the root to the plants' nutrition, with and without captured prey? Are there signals moving between pitchers and roots? Are there pathogens? Why does the plant lack a symbiotic relationship with nitrogen-fixing microorganisms in its root system? Why instead established *de novo* a syndrome that incurs such a high resource investment? Above ground, the tactic of mimicking ant swarms on the plant by means of anthocyanin concentration as a deterrent mechanism for herbivores should be also studied. One way can be recording the degree of damage caused by herbivory in areas of the pitcher with high amounts of anthocyanins. Another possibility would be to compare the degree of herbivory in red pitchers using different backgrounds aiming to analyze whether color contrast plays a role in attracting or deterring herbivory.

Study on the metabolome shows that *Nepenthes* has a great diversity of compounds, but no doubt many other metabolites are yet to be discovered and described for *Nepenthes*. One major limitation for this is the reference library for compounds. Nevertheless, other stress conditions in the plant may trigger the accumulation of completely new metabolites that have not yet drawn the attention of the scientific community. Other screenings should be put under scrutiny on different stressors, with the aim of discovering metabolites produced in a variety of circumstances. Knowledge gained from these studies has great potential for applications, not only for a better understanding of ecological strategies, but also for more environmental friendly applications that can be used, for example, as an inspiration for integrated pest management. *Nepenthes* has a great potential as bioprospecting resource and further experiments should be conducted under a variety of stressful conditions. In terms of applied sciences, implementation of plumbagin as a pest control product is one of the potential applications that can be used in the agricultural industry. In order to achieve a commercial use of plumbagin, first requires further study; for instance, concentrations and toxicity levels in the rest of the tropic chain need to be analyzed. How and where does plumbagin accumulates in the different trophic strata, what are the detoxification processes, what is the duration of toxicity: these are among other factors that need further elucidation. Due to plumbagin's toxic properties, there is also great potential for its use as an antibiotic and chemotherapeutic. Antimicrobial resistance is currently a global public health issue, and new chemical compounds are needed for study. Regarding basic science questions, plumbagin's toxic mode of action is not yet known. Probably the observed impact on the insect is due to the fact that plumbagin acts on the microbiota of the larvae and, as a consequence, the larva experiences underdevelopment. Studies on the effect of plumbagin in the absence of insect microbiota should be tested.

VII. Summary

Although they are sessile organisms, plants have colonized a wide variety of environments over time. Such a distribution is feasible only due to their high adaptability to the limiting factors of their habitat. Carnivory in the plant kingdom is an adaptation to nutrient limitation in the substrate. The “pitcher plant” *Nepenthens* is a carnivorous genus native to South-East Asia. Previous research on carnivory syndrome in *Nepenthes* focuses largely on the characterization of the biomolecular components involved in the digestion process of caught prey inside the pitcher, a specialized organ. Their unique features do not protect the plants from attacks of biotic stressors. Nevertheless, the defense mechanisms of these plants are hardly known. I use the hybrid *Nepenthes* × *ventrata* as model organism to study the plant’s response capacity to biotic stressors.

Leaves of *Nepenthes* contain a high concentration of the compound plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), which can be classified as a phytoanticipin. When plants are attacked for 24 hours by larvae of the generalist herbivore *Spodoptera littoralis*, an increase of the plumbagin concentration is induced. This process is accompanied by *de novo* production of other substances as trypsin (proteases) inhibitors.

Insect-wise, it is shown that the larvae prefer to eat the pitcher tissue over the leaf tissue of *Nepenthes*. Using LC-MS and NMR analysis, it was demonstrated that pitcher tissue has a lower plumbagin concentration compared to leaves. In fact, larvae fed on artificial food containing leaf tissue displayed a lower weight than larvae consuming artificial food containing a comparable amount of pitcher tissue. It was also found that larvae achieve 50% growth inhibition (EC_{50}) if they consume 226.5 µg g^{-1} plumbagin supplied in artificial food, as well as a significant higher mortality rate of larvae when plumbagin exceed 750 µg g^{-1} in artificial food.

The detection of plumbagin is also a confirmation of results obtained from the first analysis of the metabolome in this species. In this screening study, it is affirmed that leaves and pitchers of *Nepenthes* × *ventrata* have different metabolic profiles and a high tissue-specific chemical diversity. In addition, when digestion in the pitcher is induced in *N.* × *ventrata*, only polar compounds from the pitcher were significantly different. Further, it was possible to identify anthocyanins, which provide red coloration, in *Nepenthes*. For the first time it is shown that the genus *Nepenthes,* although belonging to the Caryophyllales*,* produces anthocyanins instead of betalains. The majority of anthocyanins is produced in the opening of the pitcher, the so-called peristome, while leaves and branches produce lower amounts.

Nepenthes × *ventrata*, like many non-carnivorous plants, produce a sugary solution, extrafloral nectar (EFN). In non-carnivorous plants, EFN often has a rewarding function for ecological service provided by ants as indirect defense. Here, I report a comprehensive analysis of EFNs: I show that *Nepenthes* produces two very different types of EFN on the same plant, nutrient poor at the pitcher peristome and nutrient rich on the branches. From these discoveries, it is possible to argue about the possibility of a “push-pull strategy” implemented by *Nepenthes*. Where the push effect may be represented by the plant’s direct defense strategies (plumbagin, anthocyanins, protease inhibitors), while the pull effect is exerted by the EFN produced on the tendrils and later on at the peristome.

VII. Zusammenfassung

Obwohl sie sessile Organismen sind, haben Pflanzen im Laufe der Zeit eine große Vielfalt verschiedener Milieus besiedelt. Eine solche Verbreitung ist nur dank ihrer hohen Anpassungsfähigkeit an die begrenzenden Faktoren des Habitats möglich. Karnivorie im Pflanzenreich ist eine Anpassung an Nährstoffmangel im Substrat. Die “Kannenpflanze” *Nepentes* ist eine Gattung karnivorer Pflanzen, die aus Südostasien stammt. Die bisherige Forschung über Karnivorie bei *Nepenthes* fokussiert sich größtenteils auf die Beschreibung der biomolekularen Bestandteile, die am Verdauungsprozess der Beute in der sogenannten Kanne, einem darauf spezialisierten Organ, beteiligt sind. Ihre einzigartigen Eigenschaften schützen die Pflanzen nicht vor Angriffen biotischer Stressoren. Dennoch sind die Verteidigungsmechanismen dieser Pflanzen kaum bekannt. Als Modellorganismus zur Untersuchung des Reaktionsvermögens der Pflanzen auf biotische Stressoren verwende ich die Hybride *Nepenthes* × *ventrata*.

Blätter von *Nepenthes* enthalten eine hohe Konzentration der Verbindung Plumbagin (5-Hydroxy-2-methyl-1,4-naphthochinon), die als Phytoantizipin eingestuft werden kann. Wenn Pflanzen 24 Stunden lang von Larven des generalistischen Herbivoren *Spodoptera littoralis* angegriffen werden, wird eine Steigerung des Plumbagingehalts hervorgerufen. Diesen Prozess begleitet die De-novo-Bildung anderer Stoffe wie Trypsin-(Proteasen-)Inhibitoren.

Bei den Insekten sieht man, dass die Larven das Kannengewebe gegenüber dem Blattgewebe der *Nepenthes* bevorzugt fressen. Unter Verwendung von LC/MS- und NMR-Analysen zeigt sich, dass das Kannengewebe im Vergleich zu den Blättern eine niedrigere Plumbaginkonzentration aufweist. Tatsächlich entwickeln Larven, die mit blattgewebehaltigem künstlichen Futter ernährt werden, ein niedrigeres Gewicht als Larven, deren Futter eine vergleichbare Menge Kannengewebe enthält. Es zeigt sich auch, dass die Larven 50% Wachstumshemmung (EC_{50}) erreichen, wenn sie 226,5 µg g^{-1} Plumbagin über die künstliche Nahrung konsumieren, sowie eine signifikant höhere Sterberate der Larven, wenn das Plumbagin 750 µg g^{-1} in der Nahrung überschreitet.

Das Auffinden von Plumbagin ist auch eine Bestätigung der Ergebnisse aus der ersten Metabolomanalyse dieser Spezies. In dieser Screening-Studie wird bestätigt, dass Blätter und Kannen von *Nepenthes* × *ventrata* unterschiedliche Stoffwechselprofile und eine hohe gewebespezifische chemische Diversität aufweisen. Darüber hinaus unterscheiden sich nur die polaren Verbindungen der Kannen signifikant, wenn die Verdauung in den Kannen von *N.* × *ventrata* einsetzt. Außerdem ist es möglich Anthocyane zu identifizieren, die für eine rötliche Färbung bei *Nepenthes* sorgen. Erstmals zeigt sich, dass die Gattung *Nepenthes* trotz ihrer Zugehörigkeiten zu den Nelkenartigen (*Caryophyllales*) Anthocyane statt Betalainen produziert. Die meisten Anthocyane werden in der Kannenöffnung, dem sogenannten Peristom, gebildet, während Blätter und Stiele geringere Mengen produzieren.

Wie auch viele nichtkarnivore Pflanzen produziert *Nepenthes* × *ventrata* extrafloralen Nektar (EFN), eine zuckerhaltige Lösung. Bei nichtkarnivoren Pflanzen hat EFN häufig eine Belohnungsfunktion für ökologische Dienste, die als indirekte Verteidung von Ameisen erbracht werden. Hier stelle ich eine umfassende Analyse von extrafloralen Nektaren vor: Ich zeige, dass zwei stark abweichende Arten von EFN von einer Pflanze produziert werden – nährstoffarm an den Kannen und nährstoffreich an den Ranken. Von dieser Entdeckung ausgehend lässt sich über die Möglichkeit einer Push-Pull-Strategie diskutieren, die *Nepenthes* umsetzt. Dabei könnte der Push-Effekt von den direkten Abwehrstrategien der Pflanze (Plumbagin, Anthocyane, Protease-Inhibitoren) dargestellt werden, während der Pull-Effekt vom EFN auf den Blättern und weiter auf dem Peristom ausgeht.

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IX. Appendix

Own contribution description

Manuscript No. 1

Short reference [Dávila-Lara, A. et al., (2021), PLoS ONE]

Contribution of the doctoral candidate

Contribution of the doctoral candidate to figures reflecting experimental data (only for original articles):

Figure(s) # 1, 2, 3, 8,	☐	100% (the data presented in this figure come entirely from experimental work carried out by the candidate)
	☐	0% (the data presented in this figure are based exclusively on the work of other co-authors)
	■	Approximate contribution of the doctoral candidate to the figure: 50 % Brief description of the contribution: *(Experimental optimization set up, Data analysis, and Design and edition of the figure)*

Figure(s) # 4, 5, 6, 7,	☐	100% (the data presented in this figure come entirely from experimental work carried out by the candidate)
	☐	0% (the data presented in this figure are based exclusively on the work of other co-authors)
	■	Approximate contribution of the doctoral candidate to the figure: 30 % Brief description of the contribution: *(Experimental optimization set up, and Data analysis)*

Figure(s) # S1 Fig, S2 Fig, S3 Fig, S1 Table	☐	100% (the data presented in this figure come entirely from experimental work carried out by the candidate)
	☐	0% (the data presented in this figure are based exclusively on the work of other co-authors)
	■	Approximate contribution of the doctoral candidate to the figure: 30 % Brief description of the contribution: *(Experimental optimization set up, Data analysis, and Design and edition of the figure)*

_____ _____

Signature candidate Signature supervisor (member of the Faculty)

Manuscript No. 2

Short reference [Dávila-Lara, A. et al., (2021), Federation of European Biochemical Societies, Open Bio]

Contribution of the doctoral candidate

Contribution of the doctoral candidate to figures reflecting experimental data (only for original articles):

Figure(s) # Table 1, Figure 1, 2, 3, 4	☐	100% (the data presented in this figure come entirely from experimental work carried out by the candidate)
	☐	0% (the data presented in this figure are based exclusively on the work of other co-authors)
	■	Approximate contribution of the doctoral candidate to the figure: 85 % Brief description of the contribution: *(Experimental optimization set up, Literature search, Design and edition of the figure)*

Figure(s) # Table S1	☐	100% (the data presented in this figure come entirely from experimental work carried out by the candidate)
	☐	0% (the data presented in this figure are based exclusively on the work of other co-authors)
	■	Approximate contribution of the doctoral candidate to the figure: 50 % Brief description of the contribution: *(Experimental design, Literature search, Contribution of the transcriptome original data)*

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Signature candidate Signature supervisor (member of the Faculty)

Manuscript No. 3

Short reference [Rahman-Soad, A. et al., (2021), Molecules, Multidisciplinary Digital Publishing Institute, MPDI]

Contribution of the doctoral candidate

Contribution of the doctoral candidate to figures reflecting experimental data (only for original articles):

Figure(s) # 1, and 3	■	100% (the data presented in this figure come entirely from experimental work carried out by the candidate)
	☐	0% (the data presented in this figure are based exclusively on the work of other co-authors)
	☐	Approximate contribution of the doctoral candidate to the figure: Brief description of the contribution:

Figure(s) # 2, and 6	☐	100% (the data presented in this figure come entirely from experimental work carried out by the candidate)
	☐	0% (the data presented in this figure are based exclusively on the work of other co-authors)
	■	Approximate contribution of the doctoral candidate to the figure: 50 % Brief description of the contribution: *(Experimental design, Literature search, Data interpretation, and Literature search)*

Figure(s) # 4, and 5	☐	100% (the data presented in this figure come entirely from experimental work carried out by the candidate)
	☐	0% (the data presented in this figure are based exclusively on the work of other co-authors)
	■	Approximate contribution of the doctoral candidate to the figure: 30 % Brief description of the contribution: *(Experimental design, Literature search, Data presentation, and Data interpretation).*

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Signature candidate Signature supervisor (member of the Faculty)

Manuscript No. 4

Short reference [Dávila-Lara, A. et al., (2020), International Journal of Molecular Sciences, Multidisciplinary Digital Publishing Institute, MPDI]

Contribution of the doctoral candidate

Contribution of the doctoral candidate to figures reflecting experimental data (only for original articles):

Figure(s) # 1	■	100% (the data presented in this figure come entirely from experimental work carried out by the candidate)
	☐	0% (the data presented in this figure are based exclusively on the work of other co-authors)
	☐	Approximate contribution of the doctoral candidate to the figure: Brief description of the contribution:

Figure(s) # 2, 3, 4, 5, 6. Figure S1, Figure S2, Figure S3, Figure S4, Figure S5, Figure S6, Figure S7, Figure S8, Figure S9, Figure S10, Table S1, Table S2, Table S3.	☐	100% (the data presented in this figure come entirely from experimental work carried out by the candidate)
	☐	0% (the data presented in this figure are based exclusively on the work of other co-authors)
	■	Approximate contribution of the doctoral candidate to the figure: 50 % Brief description of the contribution: *(Experimental design, Experiment optimization, Sample processing, Data pruning, Data interpretation, Literature search, Contribution of original data).*

Figure(s) # 4, and 5	☐	100% (the data presented in this figure come entirely from experimental work carried out by the candidate)
	☐	0% (the data presented in this figure are based exclusively on the work of other co-authors)
	■	Approximate contribution of the doctoral candidate to the figure: 30 % Brief description of the contribution: *(Experimental design, Literature search, Data presentation)*

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Signature candidate Signature supervisor (member of the Faculty)

X. Eigenständigkeitserkärung

Entsprechend der geltenden, mir bekannten Promotionsordnung der Fakultät für Biowissenschaften der Friedrich-Schiller-Universität Jena erkläre ich, dass ich die vorliegende Dissertation eigenständig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe. Personen, die bei der Durchführung der Experimente, Auswertung sowie der Fertigstellung der Manuskripte mitgewirkt haben sind vor den jeweiligen Publikationen sowie im Anhang aufgeführt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht. Weiterhin wurde keine ähnliche oder andere Abhandlung als Dissertation anderswo eingereicht.

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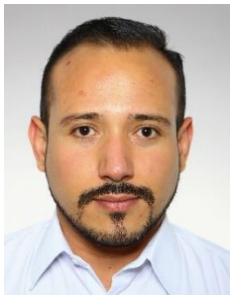
Alberto Xavier Dávila Lara

XI. Curriculum Vitae



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Personal information

Name: Alberto Xavier Dávila-Lara.

Nationality: Nicaraguan.

Place and date of birth: México city. México, 24-10-1988.

Studies 2008 - present

- **Licenciado** in biology, a 5-years study program. **2008 – 2013**.
  Faculty of Science and Technology.
  Universidad Nacional Autónoma de Nicaragua, León. (**UNAN-León**).

- Master Module in Molecular Biodiversity Research 2 (Nr. MOD319). **2014**.
  Paris Lodron University of Salzburg (**PLUS**), **Austria**.

- **PhD student**. **2016 – present**.
  IMPRS (International Max Planck Research School) member at **Max Planck Institute for Chemical Ecology. Jena, Germany**, https://www.ice.mpg.de/person/111987/2824.
  Friedrich-Schiller-University Jena, Biological Pharmaceutical Faculty. Jena, Germany. Thesis title: Strategies against herbivory in the carnivorous plant *Nepenthes × ventrata*.
  Research and experimental approaches executed at Max Planck Institute for Chemical Ecology, Jena, Germany.

Professional experiences 2014 - 2022

- **Researcher.** Max Planck Institute for Chemical Ecology. Jena, Germany. October 2016 – Current time.

  My PhD involved the following areas:
  - Biochemical and molecular ecology.
  - Chemical analysis.
  - Protein chemistry.
  - Molecular biology.
  - Insect (ant) behavior.
  - Metabolomics.
  - Plant physiology.
  - Toxicology.

  Master thesis co-supervisor: *Md. Asifur Rahman-Soad.* Max Planck Institute for Chemical Ecology. Jena, Germany. **January 2019 – December 2020.**
  Field: Plant defense, Insect herbivory, toxicology, carnivorous plants.

- **Assistant Professor.** Science and Technology Faculty at "Universidad Nacional Autónoma de Nicaragua-León" (**UNAN-León)** Department of Biology. **April 2016 – April 2018.**

- **Research Assistant** in the Molecular Biology Center at Central American University (**CBM-UCA**). Managua, Nicaragua.

  Tasks:
  - GMO (Genetic Modified Organisms) detection in imported crops by inmuno assays and PCR.
  - Plant pathogen detection by PCR in *Burkholderia glumae* in rice.
  - Design of the research project: *Genomic biorepository for native microbiota in water and substrates samples from the construction zone of the canal route through Nicaragua*.

  Researcher:
  - Collaboration in the research project: *Cloning, purification and characterization of the RNAse R from E. coli.* Service offered to the company New England Biolabs (**NEB**) Ipswich, MA. USA. Molecular Biology Center (CBM-UCA). Managua, Nicaragua.

Participation in Scientific Committees

- Member of the National Biotechnology Commission (**CNB**) organized by the Nicaraguan Science and Technology Council (**CONICYT**) completing the national biotechnology catalog **2015**, and discussing means to promote research in Nicaragua.
- Member of the selection tribunal for the National Prize of Nicaragua in Biotechnology awarded by the Nicaraguan Science and Technology Council (**CONICYT**) in **2014**.

Organization of Scientific Meetings

- Workshop: *Innocuous and Microbial quality control in the use of Medical plants.* Training in microbiology techniques in quality control methodology. By MSc.: Oscar Rodríguez Villavicencio. Workshop sponsored as part of the Partnerships for Enhanced Engagement in Research (**PEER**) project of the Molecular Biology Center at Central American University (**CBM-UCA**) **2015**.
- *International workshop to identify potential unintended impacts associated with the interoceanic canal project through Nicaragua*. Science Academy of Nicaragua (**ACN**), International Council for Science Regional Office for Latin America and the Caribbean (**ICSU**), and InterAmerican Network of Academies of Sciences (**IANAS**). Managua, Nicaragua **2014.**

Others

- Updating the database of the Nicaraguan genetic profile as part of paternity test services offered in the Molecular Biology Center at Central American University (**CBM-UCA**). Managua, Nicaragua **2015**.
- Received Molecular methods' training in the research project: *Allelic frequency of 17 microsatellites from chromosome Y in the Nicaraguan population.* The Molecular Biology Center (**CBM-UCA**) Managua, Nicaragua **2014.**

Teaching 2011 - 2020

- Co-supervisor for master research thesis. Md. Asifur Rahman Soad. Master thesis title: Insect herbivory-induced defense responses in the carnivorous plant *Nepenthes*. Master student from Friedrich-Schiller-University of Jena. Biological Pharmaceutical Faculty. Research developed at the Max Planck Institute for Chemical Ecology. Jena, Germany. **January 2019 – December 2020**.
- Internship instructor for a bachelor student from the Friedrich-Schiller-University of Jena, Biochemistry Faculty. Training at the Max Planck Institute for Chemical Ecology. Jena, Germany. **July 2020**.
- Instructor in professional practices for six students of the 3rd, 4th, and 5th year of Biology in the "Licenciatura" program: Field sampling techniques, methods for preservation and transportation of samples, molecular procedures. UNAN-León, Nicaragua. **2014**.
- Assistant instructor in laboratory classes of the Genetic Management Sources course (MRG). UNAN, León, Nicaragua. **2010 – 2014**.

Leader of Research Teams 2010 - 2015

- Spatial genetic structure of *Calycophyllum candidissimum* in natural populations of Nicaragua. As part of my Diploma research. León-Nicaragua, Salzburg-Austria **2014-2015.**
- *"Application of SIG in the study: Distribution and abundance of Hypostomus plecostomus in Ometepe Island".* XXX University Day of Scientific Development (**JUDC**), León, 23th-24th October **2012.**

- *"Preliminary study abundance and composition of zooplankton in Utricularia gibba at the Palo Verde-Costa Rica wetland"*. Organization for Tropical Study (**OTS**). Costa Rica. August 27th-September 11th **2012**.
- *"Preliminary study determination of distribution and abundance of Utricularia gibba at a management plot in Palo Verde, National Park, Costa Rica".* Organization for Tropical Study (**OTS**). Costa Rica. August 27th-September 11th **2012**.

Papers

- **Dávila-Lara, A.**, Rahman-Soad, A., Reichelt, M., & Mithöfer, A. (**2021a**). Carnivorous *Nepenthes* × *ventrata* plants use a naphthoquinone as phytoanticipin against herbivory. *Public Library of Science*, **16**, e0258235. https://doi.org/10.1371/journal.pone.0258235.
- **Dávila-Lara, A.**, Reichelt, M., Wang, D., Vogel, H., & Mithöfer, A. (**2021b**). Proof of anthocyanins in the carnivorous plant genus *Nepenthes*. *Federation of European Biochemical Societies, Open bio*, **11**, 2576-2585. https://febs.onlinelibrary.wiley.com/doi/10.1002/2211-5463.13255.
- Rahman-Soad, A., **Dávila-Lara, A.**, Paetz, C., & Mithöfer, A. (**2021**). Plumbagin, a potent naphthoquinone from *Nepenthes* plants with growth inhibiting and larvicidal activities. *Molecules*, **26**, 825. https://www.mdpi.com/1420-3049/26/4/825.
- **Dávila-Lara, A.,** Rodríguez-López, C. E., O'Connor, S. E., & Mithöfer, A. (**2020**). Metabolomics analysis reveals tissue-specific metabolite compositions in leaf blade and traps of carnivorous *Nepenthes* plants. *International Journal of Molecular Sciences*, **21**, 4376. https://www.mdpi.com/1422-0067/21/12/4376.
- **Dávila-Lara, A.**, Affenzeller, M., Tribsch, A., Díaz, V., & Comes, H. P. (**2017**). AFLP diversity and spatial structure of *Calycophyllum candidissimum* (Rubiaceae), a dominant tree species of Nicaragua's critically endangered seasonally dry forest. *Heredity*, **119**, 275-286. https://www.nature.com/articles/hdy201745.

Scientific Presentations

Oral presentations:

- Herbivory on a carnivorous plant (*Nepenthes* × *ventrata*). Presented at the Institute Symposium, Max Planck Institute for Chemical Ecology. Jena, Germany. November, 4th – 5th **2020**.
- *Nepenthes* × *ventrata* extrafloral nectar chemistry is tailored for prey attraction. Presented at the 19th International Max Planck Research School (IMPRS) Symposium. Jena, Germany. June, 30th – July 1st **2020**.
- Spatial genetic structure of natural populations of *Calyophyllum candididdimum* (Vahl.) DC. (*Rubiaceae*) in Nicaragua by AFLPs. Presented at the Regional Botany Congress. San Salvador, El Salvador. October, 28th – 30th **2015**.

Poster Presentations:

- Herbivory on a carnivorous plant: A phytoanticipin mediates defense. Presented at the 20th International Max Planck Research School (IMPRS) Symposium, Max Planck Institute for Chemical Ecology. Jena, Germany. November, 4th – 5th **2021**.
- Perception, signaling, and defense regulation in plant-insec interaction. Presented at the Scientific Advisory Board Meeting, Max Planck Institute for Chemical Ecology. Jena, Germany. June, 14th – 16th **2021**.
- Challenging the ecological role of *Nepenthes* × *ventrata* extrafloral nectar. Presented at the Institute Symposium, Max Planck Institute for Chemical Ecology. Jena, Germany. September, 23th – 24th **2019**.
- Chemical characterization of *Nepenthes* × *ventrata* extrafloral nectar. Presented at the 18th International Max Planck Research School (IMPRS) Symposium. Dornburg, Germany. March, 13th – 14th **2019**.
- Molecular approaches to understand carnivory syndrome in *Nepenthes*. Presented at the 17th International Max Planck Research School (IMPRS) Symposium. Dornburg, Germany. March, 13th – 14th **2018**.
- Detection of blindness for colors in students of biology, UNAN-León. XXIX University Day of Scientific Development (JUDC). National Autonomous University of Nicaragua-León (UNAN-León). León, Nicaragua. November, 15th **2011**.

- Diversity of macroscopic algae in Juan Venado Island and Peña El Tigre in Las Peñitas. XXVIII University Day of Scientific Development (JUDC). National Autonomous University of Nicaragua-León (UNAN-León). León, Nicaragua. Novembre, 4th **2010**.

Awards and Scholarships

- *"Outstanding achievement and dedication in the study of the career of Biology, obtaining the highest score in the Faculty of Science and Technology in **2012** UNAN-León."*

- *German Academic Exchange Service* (**DAAD**) *fellowship.* Research Grant – Doctoral Programmes in Germany, 2016/17. Four years founding scholarship. **2016- September 2020.** Followed by financing through the **Max Planck Institute for Chemical Ecology, Jena, Germany.**

Advanced training and workshops

Selection of courses attended:

- Mini lecture series on "Chemical ecology of plant-herbivore coevolution". Online. May 17th – 21st **2021**.
- From science to data science. Webinar. IMPRS-CE/BGC. August 21st **2020**.
- Research data management. Online seminar. MPI-CE. May 19th **2020**.
- IMPRS-IC/BGC: Communication. MPI for Biogeochemistry. Jena, Germany. January 29th - 30th **2020**.
- Plant Transformation Workshop. MPI-CE. Jena, Germany. November 14th – 15th **2019**.
- Introduction to Ecometabolomics for Ecologists. IDiv. Leipzig, Germany. August 12th – 16th **2019**.
- How to plan your science career. MPI for Biogeochemistry. Jena, Germany. May 13th **2019**.
- Plant morphometry. MPI-CE. Jena, Germany. January 15th – 18th **2019**.
- How to finish you PhD. MPI-CE. October 22nd **2018**.
- Grant Proposal Writing. MPI-CE/Biogeochemistry. Jena, Germany. September 27th – 28th **2018**.
- de.NBI/de.STAIR Training Course: A primer for RNA-Seq processing, interpreting and visualization: Leibniz Institute on Aging (FLI) & Faculty of Biosciences, Friedrich Schiller Univesity Jena. Jena, Germany. June 27th – 29th **2018**.
- NMR spectroscopy. MPI-CE. Jena, Germany. June 13th – 15th **2018**.
- Introduction in basic statistic and R. MPI-CE. Jena, Germany. April 11th – May 5th **2018**.
- Academic writing: How to create good texts. MPI-CE. Jena, Germany. March 20th – 21st **2018**.
- Instroductory R Course. MPI-CE. Jena, Germany. November 21st – 22nd **2017**.
- Leadership skills. MPI-CE/Biogeochemistry. Jena, Germany. November 14th – 15th **2017**.
- Presentation – Talks & Posters. MPI-CE. Jena, Germany. October 23rd – 24th **2017**.
- The Basics of Light and Fluorescence Microscopy. MPI-CE. Jena, Germany. August 21st – 24th **2017**.
- Black Forest Summer School 2017 on next generation sequencing and phylogenetics: Leistungszentrum Herzogenhorn. July 24th – 27th **2017**.
- Good Scientific Practice in the Doctoral Training Phase: Max Planck Institute for Chemical Ecology (MPI-CE). Jena, Germany. May 4th **2017**.

Communications Skills

Selection of public relations activities:

- Candidate parenting. Supervision at the IMPRS Recruitment. August 25th – 26th **2020**.
- Article presented at Medienarbeit PLUS/CE Biannual MPI-CE newsletter. May 1st **2020**.
- Presentation at the 7 Lange Nacht der Wissenschaften. Wie wohnst du? Die Anpassung von Ameisen an extreme Lebensräume. Jena, Germany. November 22nd **2019**.
- Medienarbeit Wissenschaftsmagazin der Max-Planck-Gesellschaft: Max Planck Forschung. Das Wissenschaftsmagazin der Max-Planck-Gesellschaft. February **2019**.
- Presentation at the 6 Lange Nacht der Wissenschaften. Fleischfressende Pflanzen – Fakten un Mythen. Jena, Germany. November 24th **2017**.

Interpreter and translator in the following events:

- *"Good practices of management in aquaculture"* course with emphasis on food security and national and international legislation*:* León, November 28th-29th **2011.**
- Course on *"Comparative Immunology of invertebrates and lower vertebrates (fish and amphibians)"*. Department of Biology - **DAAD** German Academic Exchange Service*:* León, May 9th-June 17th, **2011.**

Language Skills

English

- Successfully completed all the levels in English as a foreign language**.**

German:

- "Deutsch für Ausländer", Level B1.1: S.P.E.A.K German Course. Marburg, Germany **2016.**

Computer Skills

Word, Power Point, Excel, Access, Structure, GenAlEx, Arlequin, SplitTREE, AFLPdat, PAST, DivaGis, QGis, Bruker DataAnalyst, SIRIUS, CSI:FingerID, MetaboAnalyst, Origin, Adobe Photoshop, Adobe Illustrator, Inkscape.

Professional References

- ***PD. Dr. Axel Mithöfer. PhD.*** Group leader, Research Group Plant Defense Physiology. Associate professor at the **Friedrich-Schiller University** Jena. **Max Planck Institute for Chemical Ecology**. Hans-Knöll-Straβe 8. 07745 **Jena, Germany**. Tel.: +49 (0) 3641 57-1263.amithoefer@ice.mpg.de
- ***Dr. Andreas Tribsch, Assoc.Prof.*** Head of the Working Group Ecology, Biodiversity and Evolution of Plants. Head of the Herbarium, **University of Salzburg**. Hellbrunnerstr. 34, A-5020 Salzburg, Austria. Tel.: +43 (0) 662 / 8044-5504. andreas.tribsch@plus.ac.at
- ***Univ.-Prof. Dr. Hans-Peter Comes.*** Working Group Leader, Deputy Head of Botanical Garden, **University of Salzburg**. Hellbrunnerstr. 34, A-5020 Salzburg, Austria. Tel.: +43 (0) 662 / 8044-5505. Fax: +43 (0) 662 / 8044-142. Hans-Peter.Comes@plus.ac.at

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Signature **Date**

XII. Acknowledgement

First, I would like to thank my supervisor, Axel Mithöfer, who has been a guiding light in my learning process for the past five years. I learned a lot from his scientific and professional experience. To the DAAD for the scholarship, I was able to continue my research thanks to the support they showed in times of emergency health crises. Without their assisting, today's reality would not be possible. I could pursue my dream of doing research thanks to the backup I received from Waltraud. To the Max Planck Society and to the Institute for Chemical Ecology, for encouraging scientific research, for guaranteeing a fair and quality work environment. Certainly, their support in the moment to global crisis was fundamental to continue my work despite the complexity of the situation. To the Plant Defense work group: Andrea, (M)Andrea, Ding, Liza, and also to my former colleagues: Anja, Monika, Marilia, Valeria, Laura and specially Lucía. I learned many techniques with you and it was a pleasure to share experiences, dinners and talks together.

I would like to thank Pierre-Jean, who has been not just a mentor, but a good friend. I enjoyed the process of discussing and reflecting on the experimental designs. Thanks for your time, your comments, guiding me in the learning process and also for listening to me at times when I felt I was giving up. My collaborators: Prof. O'Connor, Christian, Heiko, Carlos, Michael, and Kerstin for willingness to work with me, thank you for guiding me on the right path. To the IT department of the institute, greenhouse team, and workshop team, in particular to Martin, Birgit, and Daniel without your time and interest, I would not have achieved the results. Without a doubt, Asifur's impetus was key to the findings achieved. It was an honor to share experiences with such a talented young man. To Anna, Maricela and Simona in whom I found sincere, honest, and transparent friends.

I want to thank my family, my dad who always had that unbreakable bond of support that transcends time and distance barriers. Video calls on weekends motivated me to continue with my personal goal. To my grandmothers and grandfathers, some of them are no longer able to see my achievements, but I am more than conscious their company never left me and they were the silent voice guiding me in the right direction. To Eugenio and Belén, who have been friends, confidants and a great emotional support. Thank you for accompanying me through my challenges and life plans. Thank you for supporting me in the midst of my anguish and teaching me to enjoy the daily routine of life. I am very fortunate to have both of you as my siblings. To Sarah and Greg, who have been an unconditional family to me. Thank you for your attention, emotional support, and for looking out for me. Transforming the way, I see family ties and supporting me in my moments of crisis.

To my best friend, Mayra, the long phone calls were a fundamental support in my psychological health and encouraging me to keep going. A very special thanks to Ronny, who in addition to bring light and happiness to my days, has also been a life-changer in my way of thinking. Thank you for teaching me that life has a large gray scale between black and white.

And finally, I want to thank and dedicate this work to my great love, my mother, who has been the unconditional support I have always had. Thank you for never doubting me, even when I wanted to give up. Thank you for being the shelter in turbulent days, thank you for being the always support in my life!