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Insect herbivory-induced defense responses in the carnivorous plant *Nepenthes*

Master’s Thesis

Submitted by

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1 ZUSAMMENFASSUNG

Wenn es um die Untersuchung der Interaktion zwischen fleischfressenden Pflanzen und Insekten geht, konzentriert sich fast die gesamte Literatur zu diesem Thema auf das so genannte "carnivory Syndrom". Der Befall solche Pflanzen durch herbivore Insekten, ist dagegen noch nie gründlich untersucht worden. Hier untersuchen wir die Reaktion der fleischfressenden Pflanze, *Nepenthes x ventrata*, auf den Angriff eines generalistischen Pflanzenfressers, *Spodoptera littoralis*. Mit Hilfe von Fütterungsversuchen, Wahlversuchen, Phytohormon- und Sekundärmetabolit-Analysen sowie Protease-Inhibitor-Aktivitätsversuchen zeige ich, dass *N. x ventrata* effizient gegen Herbivoren-Angriffe verteidigt wird. So wuchsen in dieser Studie Larven von *S. littoralis*, die mit verschiedenen *Nepenthes x ventrata* Gewebetypen gefüttert wurden, signifikant schlechter, wenn sie eine Blattgewebe-Diät statt einer Diät mit Kannenfallen-Gewebe bekamen. Als dominierenden Metaboliten in Nepenthes-Geweben identifizierten wir hier ein Naphthoquinone, Plumbagin (5-Hydroxy-2-methyl-1,4-naphthochinon), das als erste konstitutive Verteidigungslinie gegen Herbivorenbefall dient. Durch Zugabe von verschiedenen Konzentrationen Plumbagin zur künstlichen Nahrung von *S. littoralis*-Larven , wurde für die Hemmung des Larvenwachstums ein EC_{50}-Wert von 226,5 µg g^{-1} Nahrung ermittelt. Zur weiteren Bestimmung der Konzentration, die eine höhere Larvenmortalität verursacht, wurden Süßkartoffel (*Ipomoea batatas*) Blattscheiben in No-Choice-Assays mit ansteigenden Plumbagin-Konzentrationen belegt; eine höhere Mortalität der Larven wurde jenseits von 60 µg Plumbagin pro Blattscheibe, entsprechend 750 µg g^{-1}, festgestellt. Choice-Assays mit Nepenthes-Geweben deuteten darauf hin, dass die Gewebe bei höherem Plumbagin-Gehalt besser geschützt sind. Nach Herbivorie an der Pflanze durch *S. littoralis*-Larven stieg der Plumbagin-Gehalt um das Dreifache, während die Trypsin-Protease-Inhibitor-Aktivität (PI) *de novo* induziert wurde. Unter den abwehrbezogenen Phytohormonen waren die Jasmonate nach Herbivorenschäden stark erhöht. Im Gegensatz zu PI zeigte sich bei Blattsprühexperimenten, dass die Akkumulation von Plumbagin nicht durch Phytohormone induzierbar war. Soweit mir bekannt ist, gibt es keine anderen detaillierten Untersuchungen zu Insekten Herbivor-bezogenen Abwehrreaktionen in einer fleischfressenden Pflanze.

2 SUMMARY

When it comes to the study of carnivorous plant-insect interaction, almost all literature on this topic focuses on what is known as the 'carnivory syndrome'. The attack of herbivorous insects that infest such plants, on the other hand, have never been thoroughly studied. Here, we investigate the response of a carnivorous plant, *Nepenthes x ventrata*, upon attack of a generalist herbivore, *Spodoptera littoralis*. Using feeding assays, choice assays, phytohormone and secondary metabolite analyses, as well as protease inhibitor activity assays, here *N. x ventrata* is demonstrated to be efficiently defended against herbivore attack. In this study, *S. littoralis* Larvae fed with different tissues from *Nepenthes x ventrata* grew significantly less when feeding on a diet containing leaf tissue than pitcher-trap tissue. As dominating metabolite in *Nepenthes* tissues, we identified a naphthoquinone, plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), serving as a first constitutive defense line against herbivore infestation. When plumbagin was added at different concentrations to the artificial 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 (*Ipomoea batatas*) leaf discs were covered with increasing plumbagin concentrations in no-choice-assays; higher mortality of the larvae was found beyond 60 µg plumbagin per leaf disc, corresponding to 750 µg g^{-1}. Choice assays with *Nepenthes* tissues indicated that tissues are better protected with higher plumbagin level. Upon herbivory on the plant by *S. littoralis* larvae, plumbagin levels increased three-fold, while trypsin protease inhibitor activity (PI) was *de novo* induced. Among the defense-related phytohormones, jasmonates were strongly increased after herbivore damage. In contrast to PI, foliar spray experiments revealed that plumbagin accumulation was not inducible by phytohormones. As far as we are concerned, no other detailed research is available regarding insect herbivory-related defense responses in carnivorous plants.

3 INTRODUCTION

3.1 Carnivory syndrome in plants

Plants have adapted to utilize all available habitats, from tropical to arctic climates, arid and semi-arid zones, damp and waterlogged areas, maximum sunshine to full shade. Therefore, carnivorous plants' evolution can be defined as an adaptation to nutrient-poor environments. Catching and digesting animal prey, mainly insects is considered an alternate mechanism for acquiring supplemental nutrients such as nitrogen and phosphorus (Mithöfer, 2011)

The attributes that differentiate carnivorous plants are also present in plants not considered carnivorous (Porembski & Barthlott, 2006), such as visual and odiferous lures, directional guides, secreting glands, trapping, absorbing glands and rapid movement. For example, to influence pollination, some non-carnivorous plants capture insects (Bröderbauer et al., 2013). Some leaves display fast motion in plants like *Mimosa pudica* and *Desmodium* (Paudel & Shrestha, 2018). A sticky material that falls on the ground is secreted from some tree leaves (Galloway et al., 2020). While all the individual features of carnivorous plants can be seen in other plants separately, the organism is completely special when incorporated in the same plants: a carnivorous plant whose adapted leaves can attract, capture and digest prey as additional nutrient source to eventually derive some benefit resulting in growth, survival, or reproduction (Brownlee, 2013).

The fascination with carnivorous plants stems partially from these plants' potential to overturn the order that we expect to see in nature. The "carnivorous syndrome" refers to development by changes in anatomical structure, glandular structure, gene expression, and evolutionary characteristics (Pavlovič et al., 2007). About 1000 of the one-quarter of a million flowering plants species are carnivorous, mostly belonging to Caryophyllales and Lamiales (Biswal et al., 2017). Carnivorous syndrome in plants has developed independently in the plant kingdom at least nine times (Albert et al., 1992; Givnish et al., 2015) with distinct ways of prey capturing abilities. They can be divided into active and passive traps based on the potential for the mobility of developed trapping mechanisms (Król et al., 2012). An active trap is one in which accelerated trapping motion is an integral feature of the trapping process i.e., the snap-trap of *Dionaea muscipula* (Venus flytrap), the flypaper-traps of *Drosera* (sundew) and *Pinguicula* (butterwort), the sucking bladder-traps of *Utricularia spp.* (bladderwort); whereas in passive traps, accelerated movement is absent in the trapping mechanism surface (Pietropaolo & Pietropaolo, 2001) i.e. the pitfall traps of

Cephalotus follicularis and the genera *Sarracenia* and *Nepenthes* are among the most prominent ones.

Carnivorous plants demonstrate general concepts of coevolution and convergent evolution (Ellison, 2006; Ellison & Gotelli, 2009); in turn, a greater understanding of these mechanisms in carnivorous plants have contributed to the notion of convergent evolutionary associations that reveal new symbiotic relationships (Hedrich & Fukushima, 2021). Many carnivorous plants have unique ecological relationships with other organisms that contribute to prey capture and digestion and/or plant defenses against herbivores (digestive mutualism, myrmecophily, coprophagy) (Anderson et al., 2003). Several of the carnivorous plants' morphological modifications and many of the genes triggered in the trap leaves seem to have developed or been repurposed from herbivore defense pathways (Bemm et al., 2016). The ties between several carnivorous plants and their non-carnivorous ancestors have been identified in the last two decades in molecular phylogenetics (Porembski & Barthlott, 2006). As a result, we are now in a much better role to clarify the evolution of carnivorous plants based on interpretation of time and place of divergence from non-carnivorous ancestors including the discovery of ancestral carnivorous drivers acting on ecological discrepancies between carnivorous plants and their non-carnivorous relatives.

3.2 Nepenthes as a model species

The genus *Nepenthes* includes approximately 120 species that are likely to be the most studied pitcher plants (Miguel et al., 2018). *Nepenthes*' distribution is limited to the Old World's tropical regions (Gilbert et al., 2020). They develop in an area bordered by Madagascar, north to the islands of Seychelles, Sri Lanka and northeastern Australia, including the Malay Archipelago (Indonesia and Malaysia) (Jebb & Cheek, 1997; Murphy et al., 2020). The environments of *Nepenthes* are also very complex. They flourish on continuously moist limestone rocks, in sand fields with both a rainy and dry season, in swamps that are most of the year underwater, on seashores, as epiphytes growing on the plants and as creepers on the soil surface (Pietropaolo & Pietropaolo, 2001).

***Figure 1**: Nepenthes x ventrata. A natural hybrid of N. ventricosa and N. alata.*

Nepenthes are herbaceous perennials whose stems are very rugged, with a diameter of more than two inches. The stem length exceeds up to 20 meters in some species (Pietropaolo & Pietropaolo, 2001). The long leaf blades are green to yellow, with midribs reaching past the leaf blade apex to form a cylindrical tendril that is as long or sometimes longer than the leaf blade. The tips of yellow-green tendrils grow into a pitcher in a suitable climate. For decades, their pitchers' variety in scale, form and colour pattern has drawn much attention. Independent of the variety, the pitcher traps exhibit three distinct functional areas to trap insects successfully (Mithöfer, 2011). Attracting and capturing prey- includes the peristome referring to a vivid collar-shaped structure circling and overhanging the pitcher's opening. The slippery region- covered with wax that favours capture and prohibits prey from fleeing (Gaume et al., 2002). The digestive region- the bottom end of the pitcher covered by bifunctional glands (able to secrete and absorb) that create an acidic viscoelastic, digestive fluid essential to assimilate nutrients obtained from preys (Gorb & Gorb, 2009; Gaume & Forterre, 2007). The peristome's anisotropy properties enable pitcher traps to draw attention; for the slippery properties, insects that fall into pitcher traps are submerged (Gaume et al., 2002; Bohn & Federle, 2004) and eventually digested by the enzyme mixture found in pitcher fluid. However, the pitcher lid is a genus-specific trait and is not present in any other closely related genera. The carnivorous syndrome in *Nepenthes* is a significant expenditure in resources for plants. In order for the benefits derived from digested prey to outweigh the expense of carnivory, the pool of digestive enzymes must be adequate (Ellison, 2006; Ellison & Gotelli, 2009). A remarkable

adaptation of pitcher fluid involved proteins helping to digest prey or organic compounds (Miguel et al., 2018). These enzymes became interesting candidates for biotechnological and medicinal applications due to their biochemical properties. Besides the digestive fluid, the genus *Nepenthes* also harbours a wide chemical diversity with ethnobotanical uses. As with many other carnivorous plants, several secondary metabolites are being isolated for medicinal and biotechnological use (Moran & Clarke, 2010). In specific, the species of *Nepenthes* are well known in traditional medicine. Throughout studies, the curative effects of *Nepenthes* extract on diseases such as hypertension, cough, fever, urinary system infections, malaria, pain, asthma, infection with Staphylococcus, celiac disease, and oral cancer cells has been mentioned several times (Rahman-Soad et al., 2021).

These plants' features provide insights for many applications ranging from healthcare solutions to environmentally sustainable management of agricultural pests and allowing the plant to be a fitting model for evaluating theories relevant to the evolution and diversification of advanced plant organs.

3.2.1 Metabolite composition of Nepenthes

As of now, much of the chemical research performed on *Nepenthes* has focused on its digestive fluids, including its proteome and low-molecular-weight biomolecules (Rottloff et al., 2016). Based on available data, it is known that the pitcher fluid consists of digestive enzymes and even defensive proteins belonging to the pathogenesis-related protein group (Rottloff et al., 2016; Yilamujiang, 2017). Moreover, the pitcher fluid is low in inorganic nutrients and contains secondary metabolites with antimicrobial properties, e.g., naphthoquinones (NQ; droserone, 5–O–methyl droserone in *N. khasiana*; Plumbagin, 7-methyl-juglone in *N. ventricosa*) (Buch et al., 2013). Thus, it is hypothesized that such compounds mediate protection against microbes and preserve prey during digestion (Dávila-Lara et al., 2020).

As all carnivorous plants, *Nepenthes* plants have developed strategies to lure insects to traps. Many low-molecular-weight compounds identified so far in carnivorous plants are volatile compounds suggested being involved in prey attraction. For instance, in *N. rafflesiana*, more than 50 volatiles have been found (Di Giusto et al., 2010). However, for non-volatile chemicals, less detail is available. The presence of anthocyanins in *Nepenthes* causes red pigmentation (Kováčik et al., 2012), with some studies reporting this coloration as a visual signal for carnivorous plants

and some have hypothesized that contrast between red and a green foliage background may be attractive (Schaefer & Rolshausen, 2005; Bennett & Ellison, 2009; El-Sayed et al., 2016). However, from the studies of Gilbert et al., 2018 on *N. gracilis* showed, color does not correspond with the amount of prey caught in, but observation revealed that red pitchers are subjected to less herbivory. Recently an untargeted metabolomics approach was performed in *N. x ventrata* comparing secondary metabolites in leaves and pitcher tissue before and after prey catching (Dávila-Lara et al., 2020). In that study, around 2000 metabolites were detected in the two tissues showing very high metabolome diversity, which was even higher in leaves, indicating a tissue specificity of chemical compounds may have a major function in the plant. Among the various compounds, common constituents found were flavonoids, phenolics, and NQs (Dávila-Lara et al., 2020; Rahman-Soad et al., 2021). The metabolite profile may provide clues as to the processes that underlie the evolution of carnivory as well as the means by which plants are able to cope with environmental challenges.

3.3 Basic skeleton of naphthoquinones

Naphthoquinones are widespread in nature as one of the large groups of secondary metabolites. Many higher plant families produce NQ, including Nepenthaceae (Devi et al., 2016), Droseraceae (Didry et al., 1998; Budzianowski, 2000), Avicenniaceae (Ito et al., 2000), Bignoniaceae (Schmeda-Hirschmanna & Papastergioub, 2003), Boraginaceae (Özgen et al., 2004), Ebenaceae (Dzoyem et al., 2007), Juglandaceae (Hirakawa et al., 1986), Drosophyllaceae (Bringmann et al., 1998) and Plumbaginaceae (De Paiva et al., 2003). In plants, they commonly occur as reduced and glycosidic forms (Babula et al., 2006). For certain species, NQ are present as monomers such as plumbagin, juglone, naphthazarin, or dimers and even trimers (Crosby et al., 2010; Olímpio Da Silva et al., 2012; Babula et al., 2009). NQ's chemical composition is based upon the bi-cyclic framework – a substitution for naphthalene skeleton in positions C1 and C4 (1,4-NQ), or C1 and C2 (1,2-NQ). The biosynthetic pathways suggested for NQ biosynthesis are the shikimate and acetate–malonate (polyketide) pathways (Babula et al., 2009; Widhalm & Rhodes, 2016). NQ are typically coloured, particularly yellow or brown; therefore, they play significant roles in pigmentation, e.g., Zhu et al., 2016 showed the production and localization of red coloured bioactive NQ in the roots of *Echium plantagineum*. In certain metabolic processes, NQ act as functional constituents. In addition, they hinder the growth of insect larvae and may have a sedative or toxic effect on aquatic species and livestock (Wright et al., 2007). Their antimicrobial, anti-parasitic, and cytotoxic properties derive

from their ability to suppress electron transfer, uncouple oxidative phosphorylation, intercalate in the DNA double helix, alkylate biomolecules, and use redox cycling to generate reactive oxygen radicals (Babula et al., 2009; Eilenberg et al., 2010; Raj et al., 2011). NQ, especially juglone, are widely studied with regards to allelopathy (Altikat et al., 2013) and also mentioned as phytoalexin against parasitic plants including seed germination inhibition from other species (Spencer et al., 1986).

3.3.1 Common naphthoquinones from Nepenthes

Phytochemically, NQ are important characteristics of *Nepenthes*, distributed in all plant tissue and chemo-taxonomic markers within the Nepenthales (Bringmann et al., 2000; Aung et al., 2002). From the leaves of *Nepenthes gracilis*, plumbagin, isoshinanolone, epishinanolone, shinanolone, were isolated (Aung et al., 2002), from roots of *N. rafflesiana* droserone, hydroxydroserone, plumbagin and the nepenthones A-C (Cannon et al., 1980; Rizzacassa & Sargent, 1987). Roots of *N. thorelli* contain plumbagin, droserone, 2-methylnaphthazarin and isoshinanolon (Likhitwitayawuid et al., 1998). Interestingly, they are also found in the digestive pitcher fluid, Eilenberg and coworkers (2010) identified two derivatives of plumbagin, droserone (3,5-dihydroxy-2-methyl-1,4-naphthoquinone) and its derivative 5-O-methyldroserone (2-methyl-3-hydroxy- 5-methoxy-1,4-naphthoquinone) in the pitcher fluid of *N. khasiana*. Raj et al., 2011 reported the same NQ in *N. khasiana* upon chitin induction and first to detect the biologically active NQ plumbagin in *N. khasiana.* Buch and coworkers identified plumbagin and 7-methyl-juglone in the pitcher fluid of *N. ventricosa*. As of now, plumbagin (5-hydroxy-2-methyl-1,4-naphthalenedione) is the most described and reported molecule with its derivatives from *Nepenthes*.

3.3.2 Plumbagin as a potent Naphthoquinone with bioefficacy against insects

Naphthoquinone derivatives are commonly distributed in different carnivorous plant species (Devi et al., 2016). The most widely studied NQ among them is plumbagin, which belongs to a class of phytochemicals that provides natural protection for plants against insects and is proposed to be extremely useful in developing potent agrochemicals because of the high abundance and relatively non-toxic nature (Pavela, 2013). *In vitro* experiments from Rischer et al. (2002) showed the availability of plumbagin in *N. insignis* via *de novo* synthesis, which might have further enhanced the plants' fitness against phytophagous and pathogenic organisms. Many reports

indicate that plumbagin can manifest its chemotoxic effects by targeting central mechanisms likely to be evolutionarily retained in eukaryotes (Kapur et al., 2018). Plumbagin works as repellent, even for adapted Lepidoptera (Villavicencio and Perez-Escandon, 1992). Insects feeding on plants covering a vital dose of plumbagin die either immediately or during the next ecdysis due to inhibition of ecdysteroid (Joshi and Sehnal, 1989) and chitin synthetase (Kubo et al., 1983) production. Plumbagin stimulates the mitochondrial electron transfer mechanism by intercepting electrons from the respiratory chain (Kapur et al., 2018). Reactive oxygen species that lead to proteolytic cell degradation can also be produced by plumbagin when interacting with electron-transfer flavin enzymes such as NAD(P)H-dehydrogenases and/or oxidases located in the cell membrane (Galek et al., 1990; Eilenberg et al., 2010). The compound's volatility with a relatively high redox potential makes it a strong anti-feeding agent with low toxicity (Tokunaga et al., 2004).

3.4 Spodoptera littoralis as a model organism

Spodoptera littoralis, commonly known as the Egyptian cotton leafworm, is a generalist herbivore and has a host range of at least 87 plant species over 40 plant families (Brewer & Landis, 2010). In the eastern Mediterranean, it is a major pest of cotton, maize, tomato, and tobacco (Oepp & Bulletin, 2015). A large number of older larvae may defoliate plants, destroy crops and migrate to neighbouring fields. Chemical control, especially concerning cotton in Egypt, has been extensively reported (Brewer & Landis, 2010). In some cases, various organophosphorus, synthetic pyrethroids and other insecticides have been used, with tolerance and cross-resistance (Shoaib et al., 2014). The moths have highly specialized chemoreceptors on the ventral surface of the tarsi and the distal portion of the proboscis and respond to a certain number of sugars present in nectar (CABI, 2021). The moths are also strong flyers able to scatter

Figure 2: *Spodoptera littoralis caterpillar. Copyright © Matthias Held, University of Neuchâtel*

over a long distance to pursue new/suitable host plants. The female lays eggs in clusters of 20 to 1000 on lower leaf surfaces, often on the plant's lower parts (Sullivan, 2007). Depending on the temperature, the eggs hatch in 3-5 days (Simmons et al., 2017), and initial larval feeding induces leaf skeletonization, an early infestation sign (Brewer & Landis, 2010). In warm climates six larval instars of *S. littoralis* occurs over three weeks, with an additional larval stage and longer development times in cooler regions and overwinters as pupae in the soil. In warm climates, up to 8-9 generations are possible (Brewer & Landis, 2010; Simmons et al., 2017). The devastating impacts caused by these pests have made them an important experimental model in ecological and physiological studies.

3.5 Aims of the study

In some plant species herbivore infestation is less common than in others. While the exact cause is often unknown, it is most likely due to a well-balanced composition of defensive secondary plant metabolites. Herbivory on carnivorous plants is significantly less observed although these plants are highly attractive to insects. Despite a comprehensive analysis and publication of the carnivorous syndrome, records of herbivores feeding on carnivorous plants or associated plant defenses are surprisingly limited. Therefore, to investigate the carnivorous plant's defense chemistry, in this study we used the pitcher plant, *Nepenthes x ventrata*, as a model species when challenged with a generalist lepidopteran herbivore *Spodoptera littoralis*.

4 EXPERIMENTAL SETUP

4.1 Insect rearing

Spodoptera littoralis used in this study were collected as egg mass from 'Syngenta Crop Protection' (Stein, Switzerland). All attained eggs were reared from hatch to pupation on an artificial diet consisting of 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 agar-water solution (7.5%). Eggs were kept in plastic boxes with artificial diet as food and placed in a climate-controlled chamber at 26 ± 1 °C, 70 ± 10% RH, and a photoperiod of 12:12 h until hatched. Neonate larvae were moved in a different climate-controlled chamber at 23–25 °C, 70 ± 10% RH, and with a 14 h photoperiod. Multiple plastic boxes with constant air access (30 × 40 × 8 cm) were used throughout the procedure for each batch of eggs and larvae, and the rearing time was ~20–22 days.

4.2 Plant growth conditions

Nepenthes x ventrata (a natural hybrid of *N. ventricosa* and *N. alata*) plants were grown in the greenhouse on a mixed substrate (sphagnum/bark/leaves/moss) with an average temperature of 23-25 °C and 80-100 % humidity. The photoperiod was at least 12 h of light per day. The plants were watered with rainwater only. For ease of the experiment, the total number of plants were divided into two groups. The first group consisted of fully grown six years old plants with multiple branches, and the other group consisted of small yet mature plants and at least 2 years old all of the size. Both groups were used for the study with regular records. Plant pots were shuffled once in every three months to ensure optimum space and equal support for growth.

4.3 Selecting leaf number

Nepenthes x ventrata leaves used for the experiments had fully expanded leaf blades but only contained pitcher buds with the highest probability of having functional pitchers later. However, leaves containing mature pitcher were avoided to focus only on the leaf chemistry without having the influence of the pitcher chemistry. To determine a specific leaf number of mentioned criteria, all greenhouse plants were monitored for 30 days, and based on the observation, only leaf numbers 4-6 were used for harvesting and herbivore infestation that lack already developed pitcher.

4.4 Isolation of plumbagin from Nepenthes x ventrata leaves

Plumbagin was isolated from *N. x ventrata* leaves that had been freshly harvested, immediately frozen in liquid N2, and freeze-dried. An amount of 7.3 g of pulverized dried tissue was extracted with 100 mL dichloromethane (DCM) for 15 min with stirring. After standing 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 supernatant were filtered, combined and DCM was removed using a rotary evaporator. Dried extract (9.3 mg) was dissolved in 2 mL DCM transferred into HPLC vial and dried again under N_2 stream and further subjected to ^{1}H NMR spectroscopy.

4.4.1 ^{1}H NMR spectroscopy for structural confirmation of plumbagin

Identity of 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-cryo probe. Spectra were referenced to the residual solvent signal for DMSO-d6 at δH 2.50. Spectrometer control and data processing were 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 to 7.00) were determined and used for calculation based on the respective areas of a plumbagin standard. 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-d6 and subjected to ^{1}H NMR spectroscopy.

4.5 Insect feeding bioassay

4.5.1 Feeding bioassay with plant tissue

Insect feeding bioassays were conducted on 2nd instar larvae (mean weight 10 ± 2 mg) of *S. littoralis*. The required amount of grinded leaf tissue either fresh or freeze-dried was incorporated into artificial diet at different concentrations to study the effect on larvae growth and development. Newly molted 2nd instar larvae were taken from rearing boxes and divided into

control and treatment groups. 15 larvae were placed on the control diets individually, and the same numbers of larvae were placed on the plant tissue containing diet at different concentrations in small plastic cups, ensuring minimal variation between populations. Later the trays containing larvae were kept in a controlled temperature cabinet at 25 ± 1 °C and 60-70% relative humidity. The diet was replaced every day to avoid getting it dehydrated. Weight of each larva was measured at alternate days, and the mean weight ± SE was calculated.

4.5.2 Dose-response bioassay

The chronic growth bioassay was carried out by incorporating a series of five different concentrations of synthetic plumbagin into the artificial diet of *S. littoralis* larvae. At first, different plumbagin amounts for the wanted final concentrations were weighed into separate glass vials and dissolved with 2 mL of acetone per 100 g of artificial diet. The dissolved plumbagin was mixed directly to prepared artificial diet at room temperature and left open for some time to let the acetone evaporate. The control diets were prepared with carrier solvent alone. The setup was made as same as the insect feeding bioassays and development was monitored.

4.5.3 Leaf herbivory of Nepenthes by S. littoralis larvae

A day before introducing the leaf herbivore in the green house, *N. x ventrata* leaves were gently cleaned by spraying distilled water on the surface. *S. littoralis* 3rd instar larvae of were weighed at the beginning of the experiment using microbalance. For all herbivory experiments leaves were infested with a single larva that starved for 24 h before the experiment was started. For short term herbivory, lightweight cage was placed on the leaf covering both sides to prevent escaping of the larvae. For long-term herbivory, nylon bags were used instead of cages to cover the entire leaf and increase herbivory surface area. The larvae were left to feed on *N. x ventrata* leaves for five days in the green house, recovered and re-weighed every day to measure weight gain. The number of dead larvae was recorded. Larvae that could not be retrieved were considered dead.

4.6 Feeding deterrence

4.6.1 Antifeedant Bioassay

Besides being tested on the artificial diet, effect of feeding deterrence against plumbagin was also confirmed in host plants of *S. littoralis* larvae. An antifeedant bioassay was performed with sweet potato (*Ipomoea batatas*) leaves grown at 28/25 °C under a 16:8 h light: dark regime, respectively and 70% relative humidity. To cover leaf surface, plumbagin was solved using a minimum volume

of acetone and diluted to the required concentration with 2.5% (w/v) PEG 2000 in distilled water (Sigma-Aldrich, Taufkirchen, Germany). Leaf discs of 24 mm in diameter were punched out with plastic construct, covered with known plumbagin concentration in carrier solvent and placed in round small petri dishes on a wet filter paper. Every day new leaf discs were provided. All assays were performed with individual larvae to avoid cannibalism.

4.6.2 Choice assay

To measure the preference of *S. littoralis* larvae towards leaf or pitcher tissue of *N. x ventrata*, larvae were confined in a square petri dish of 120 x 120 mm in diameter containing single piece of leaf and pitcher tissue in a choice situation. The bioassay was conducted for fixed time periods and the choice made by the larvae for a specific tissue was assessed over time. The remaining tissue area was measured to calculate the area consumed from each tissue using the software ImageJ.

4.7 Phytohormone and plumbagin analysis

4.7.1 Phytohormone and plumbagin quantification using LC-MS/MS

Sampled *N. x ventrata* leaves were grinded to a fine powder in liquid nitrogen using mortar and pestle, approximately 100 mg of finely ground leaf tissue was weighed in 2 mL tubes. The extraction and detection were performed as previously described by Vadassery et al. (2012) with minor modification. For analysis, each sample was mixed with 1.5 mL methanol containing 60 ng D_6–abscisic acid (Santa Cruz Biotechnology, Santa Cruz, U.S.A.), 60 ng of D_6–jasmonic acid (HPC Standards GmbH, Cunnersdorf, Germany), 60 ng D_4–salicylic acid (Sigma-Aldrich, Taufkirchen, Germany) and 12 ng of jasmonic acid-$^{13}C_6$-isoleucine conjugate as an internal standard. After brief mixing, samples were placed in a Rotator Mixer RM-Multi 1 (STARLAB GmbH, Hamburg, Germany) using the program 100 rpm: 15 s, 75°: 16 s, 3°: 5 s shook for 30 min at 4 °C. Samples were centrifuged afterwards at 13,000 x *g* at 4 °C for 20 min, the supernatant was collected and the remaining pellet resuspended in 500 µL methanol. The resuspended pellet was shaken and centrifuged again as previously described. The supernatants were combined and concentrated for 3 h for every 30 samples using Eppendorf Concentrator plus (Eppendorf AG, Hamburg, Germany). The concentrate was resuspended in 500 µL methanol and centrifuged at 16,000 x *g* at 4 °C for 10 min. Lastly, 400 µL of supernatant were used for LC-MS/MS measurements.

Phytohormone and plumbagin analysis were performed by LC-MS/MS as in Heyer et al. (2018) on an Agilent 1260 series HPLC system (Agilent Technologies) with the modification that a tandem mass spectrometer QTRAP 6500 (SCIEX, Darmstadt, Germany) was used. 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 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 reaction monitoring (MRM) mode, details of the instrument parameters and response factors for quantification can be found in Table S1. 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.

4.7.2 Plumbagin quantification using HPLC-UV

Apart from using LC-MS/MS for plumbagin quantification, a second method using HPLC-UV was also used 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, 5µm, Macharey-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). Detection was performed with a photodiode array detector, and peaks were integrated at 265 nm. Quantification of plumbagin was achieved by generating a plumbagin standard (Sigma, P7262) curve in the range of 7.8 to 250 µg/mL.

4.8 Phytohormone spraying

For the phytohormone spraying experiment, each treatment group contained 5 individual *N. x ventrata* plants on a single tray. Trays were placed having sufficient distance to avoid drift of the spray to other groups of treatment. Plants were foliar-sprayed (4 mL/plant) at two different time intervals with 12 h gap interval within 24 h and only with one of the phytohormone JA (500 µM), ABA (250 µM), SA (250 µM) for each treatment group. Spraying solutions were made from 50 mM (JA), and 100 mM (ABA and 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 condition, plants were sprayed with the same solution but without any phytohormone. Leaves were harvested after 24 h of starting the experiment.

4.9 Protein extraction from Nepenthes x ventrata leaves

Trypsin inhibitor protein from *N. x ventrata* plant was extracted following Pierce Plant Total Protein Extraction Kit (Pierce™, A44056) protocol with minor modifications. The native lysis buffer was diluted (1:1) with 50 mM Tris-HCl containing 30 mM $CaCl_2$, pH 7.4 for extraction and instead of supplied kit filter cartridges, standard 2 mL Eppendorf tubes were used. For each sample, 250 µL of extraction buffer was added to 50 mg grinded fresh tissue. The mixture was vortexed shortly, and after 5 min incubation on ice, the sample was centrifuged at 16,000 x *g* for 5 min. After centrifugation, 150 µL extract was recovered and used for protein quantification and trypsin inhibitory activity.

4.9.1 Estimation of protease inhibitor activity

Protease inhibitor (PI) activity was assayed by determining the residual trypsin activity following the slightly modified Kakade et al. (1974) method using benzoyl-D,L-4-arginine-p-nitroanilide (BApNA) (Sigma, B4875-1G) as the substrate and bovine trypsin (Sigma, T-8128) as the standard enzyme. The reaction mixture containing 50 µL leaf protein extract, 30 µL trypsin (1mg trypsin dissolved in 5 mL of 50 mM Tris-HCl containing 30 mM $CaCl_2$, pH 7.4) was incubated at 37 °C for 15 min with gentle shaking (Thermomixer; 700 rpm). Then, 40 µL of BApNA (5 mg/mL in DMSO) was added to the assay solution, incubating the reaction mixture at 37 °C for 20 min with gentle shaking (Thermomixer; 900 rpm) followed by termination of the reaction by adding 500 µL of 10% (*v/v*) glacial acetic acid. To subtract the absorbance caused by the extract, a blank for each sample using the same amount of protein extract was used. In addition, for positive control representing maximum trypsin activity, a reaction without extract was used. In blank, acetic acid was added before BApNA, and in trypsin control, only extraction buffer was added in place original sample extract. The absorbance was recorded at 410 nm in 96 well plates using spectrophotometer.

4.10 Statistical analysis

Statistical calculations were performed using GraphPad Prism version 9.0.0 in all cases. Details are indicated in the particular figure legends.

5 RESULTS

5.1 Larvae performance on plant tissue diet

5.1.1 Toxicity assay with plant tissue

In order to determine any toxicity of *N. x ventrata* leaf and pitcher tissue to *S. littoralis* larvae an experiment was carried out by supplementing grinded fresh tissue (30%, *w/w*) of leaf and pitcher separately with artificial diet. Second instar larvae of *S. littoralis* were fed for seven days on the particular diets (Fig. 3). Larval developmental time was significantly affected from day 5 onwards by leaf tissue supplementation to the artificial diet compared to pitcher tissue supplemented diet and control diet. Larvae fed on pitcher tissue supplemented diet had no significant difference than the larvae that fed on the control diet.

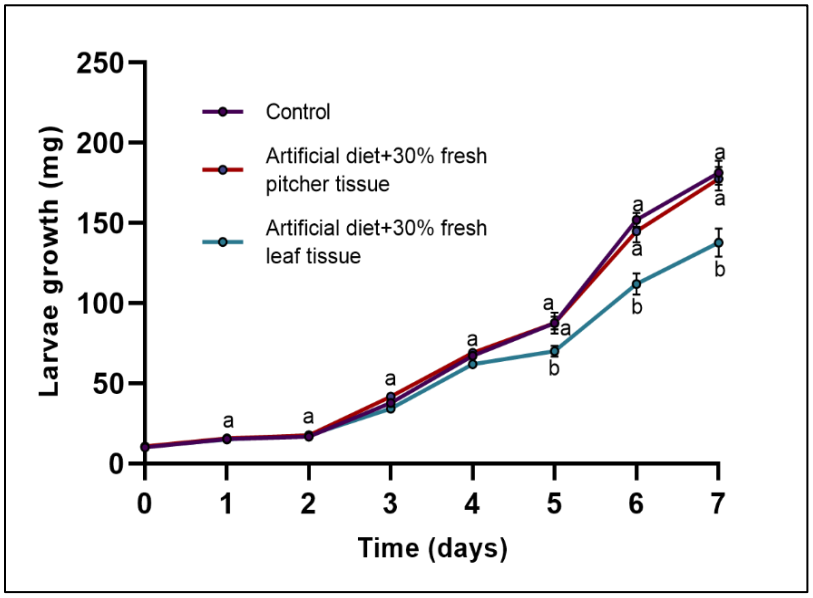


Figure 3: *Larvae weights recorded upon feeding of 2nd instar Spodoptera littoralis on artificial diet containing fresh leaf tissue of Nepenthes x ventrata leaf and pitcher (30% (w/w)). Individual weights of each larvae were recorded every day. Mean (± SEM) labelled with different letters indicate significant difference (p<0.05); Two-way ANOVA, Šidák's multiple comparisons test; n=15/group.*

To incorporate higher amount of leaf tissue for more significant growth effect of larvae, harvested leaf tissue was freeze-dried to remove water content and incorporated into the artificial diet as 10% and 15% (w/w) dry weight after grinding into a fine powder (Fig. 4). The larvae's growth and weight were strongly affected starting from 2nd day, and there was also a substantial variation between the larvae feeding on either 10% or 15% dried leaf tissue containing diet from day 3 onwards.

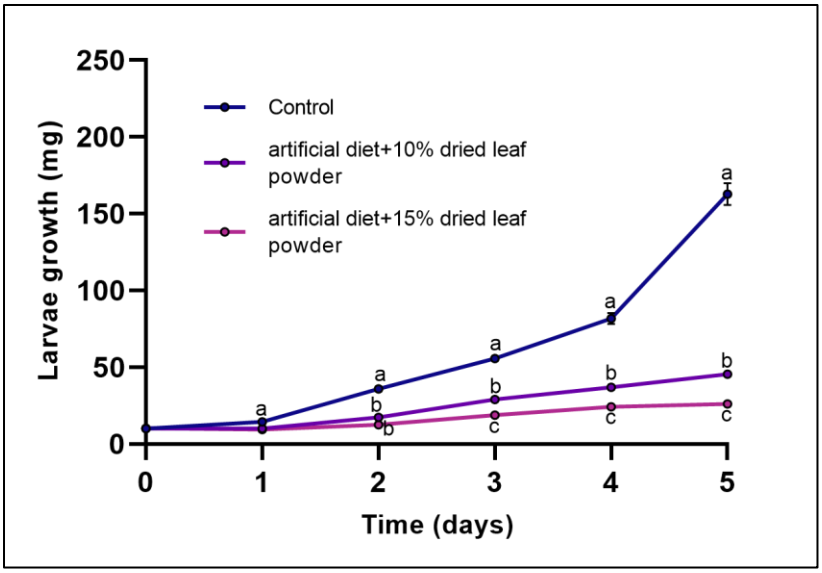


***Figure 4**: Larvae weights recorded upon feeding of 2nd instar Spodoptera littoralis on artificial diet containing dried Nepenthes x ventrata leaf powder (10% and 15 % (w/w)). Individual weights of each larvae were recorded every day. Mean (± SEM) labelled with different letters indicate significant difference (p<0.05); Two-way ANOVA, Šidák's multiple comparisons test; n=15/group.*

5.1.2 Leaf herbivore performance

To study the impact of *N. x ventrata* leaves on the feeding behavior of *S. littoralis* larvae when there is no alternative food, the larvae were place on the leaves without the chance to escape. In the same environmental conditions, artificial diets were offered in close proximity to the plants to another group of larvae as control. Although larvae fed on the artificial diet exhibited normal feeding behavior and weight gain in the latter case, larvae fed on the leaves gained no weight at all within 5 days. (Fig. 5).

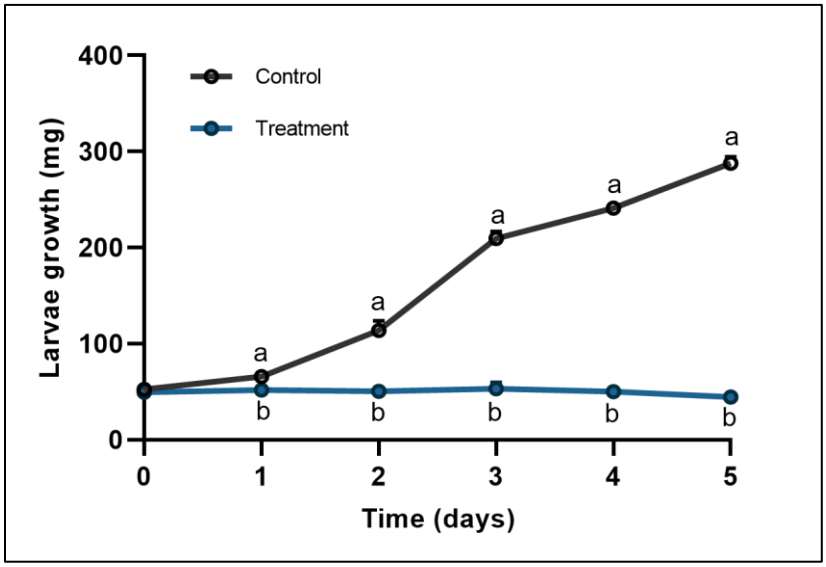


***Figure 5:** Larvae weights recorded upon herbivory by 3rd instar Spodoptera littoralis on a single leaf of Nepenthes x ventrata leaf in the green house. Individual weights of each larvae were recorded every day. Mean (± SEM) labelled with different letters indicate significant difference (p<0.05); Two-way ANOVA, Šidák's multiple comparisons test; n=10/group.*

5.2 *Structural confirmation of plumbagin*

Following the hypothesis that NQ are involved and responsible for the feeding assay results, the presence of NQ in *N. x ventrata* tissues was examined. A total amount of 8.55 mg of NQ-containing material was isolated from 7.32 g of the dried leaf tissue after dichloromethane (DCM) extraction and used for ^{1}H-NMR Spectroscopy. Based on chemical shift and integration in comparison to a reference, plumbagin could be identified in the sample (Fig. 6).

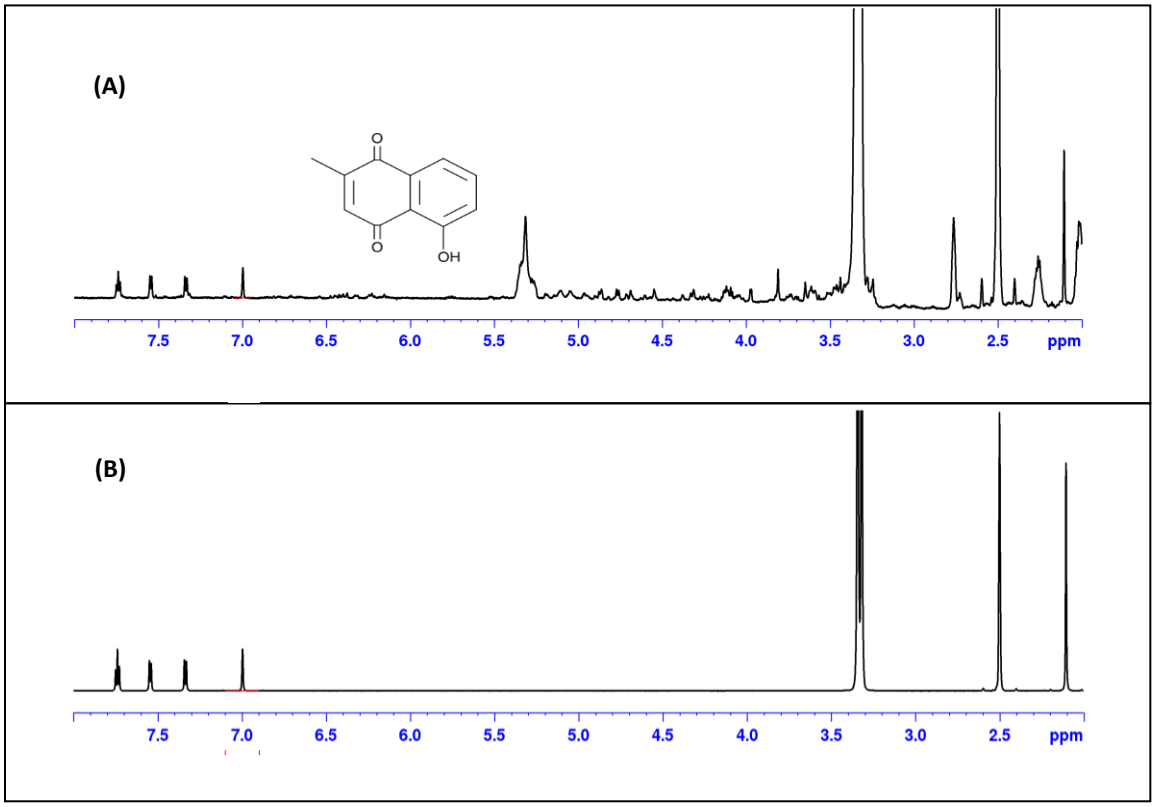


Figure 6: *^{1}H NMR analysis of leaf extract in DMSO-d6. (A) Plumbagin extracted from Nepenthes x ventrata leaves and (B) a plumbagin reference.*

Interestingly, plastic tubes in which the leaf and pitcher tissue from *N. x ventrata* were stored turned yellowish during prolonged storage, which could be due to slow but continuous sublimation of volatile compounds released from the tissue thereby staining the tube (Fig. 7).

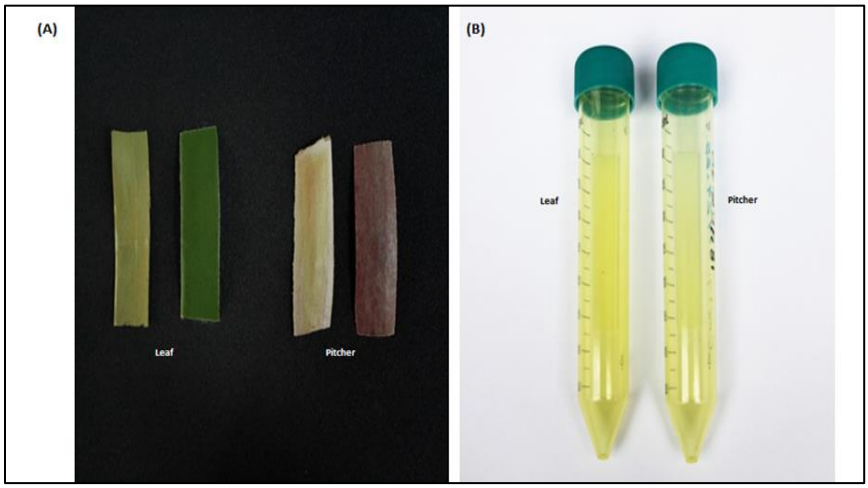


***Figure 7**: Tissues of Nepenthes x ventrata leaf and pitcher stored for 6 months in a plastic tube. (A) Sublimed compounds cover the dry material with a yellowish color (left) in comparison with freshly cut tissue (right). (B) Plastic tubes that stored different tissue types for 6 months.*

To proof the identity of the sublimed compound, part of the compound was removed from the wall of the plastic vial by extraction with dichloromethane. After evaporation of the solvent, the residue was used for ^{1}H-NMR analysis. When compared with a reference, it could be confirmed that the sublimed volatile compound was indeed plumbagin (Fig. 8).

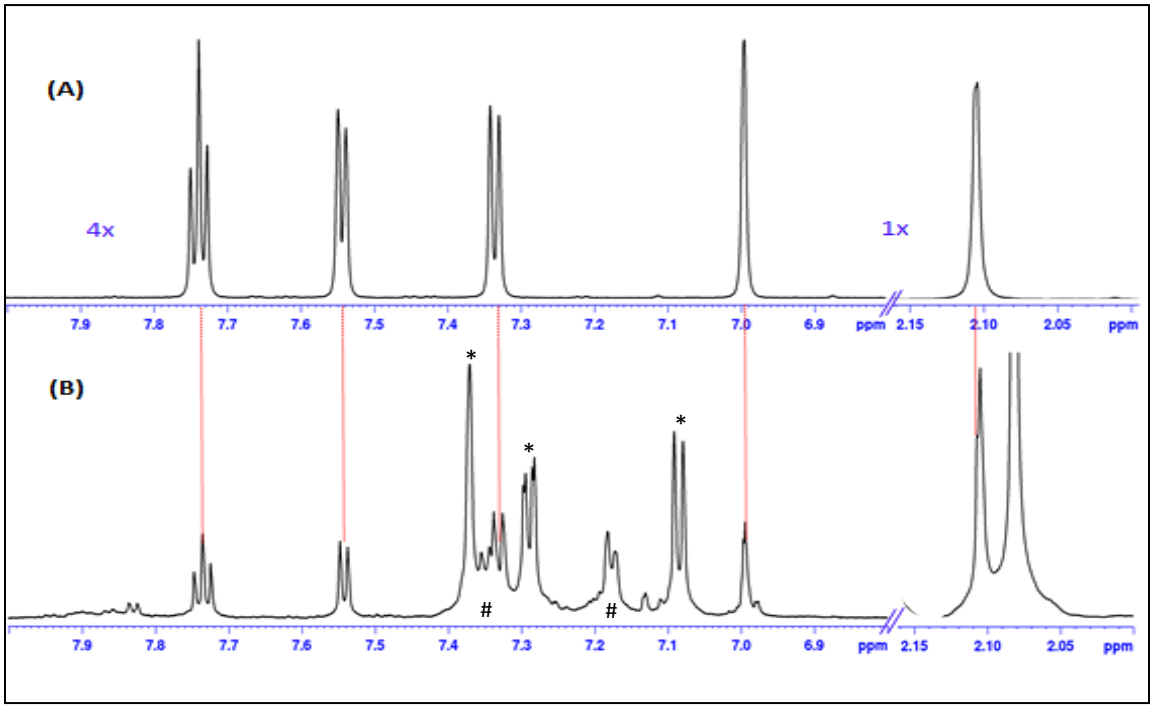


***Figure 8:** ^{1}H NMR analysis in DMSO-d6 for sublimed volatile compound. (A) Details of ^{1}H NMR spectra of a plumbagin reference and (B) the volatile exudate emitted by Nepenthes x ventrata pitcher material. Asterisks (*) indicate the presence of 4-tert-butylcatechol, a polymerization inhibitor probably extracted from the plastic material, and hashes (#) account for an unidentified impurity. The intensity of the aromatic range in (B) was increased as indicated by the factor.*

5.3 Plumbagin mediated bioassays

5.3.1 Dose-response bioassays

After the identification of plumbagin, the pure compound was further tested for the inhibition of larval growth in an inverse dose-dependent manner when added to the artificial diet. After seven days of feeding, clear difference can be seen between the different concentrations as well as in comparison with artificial diet only (Fig. 9). However, no cases of mortality were observed for any group feeding on plumbagin containing diet.

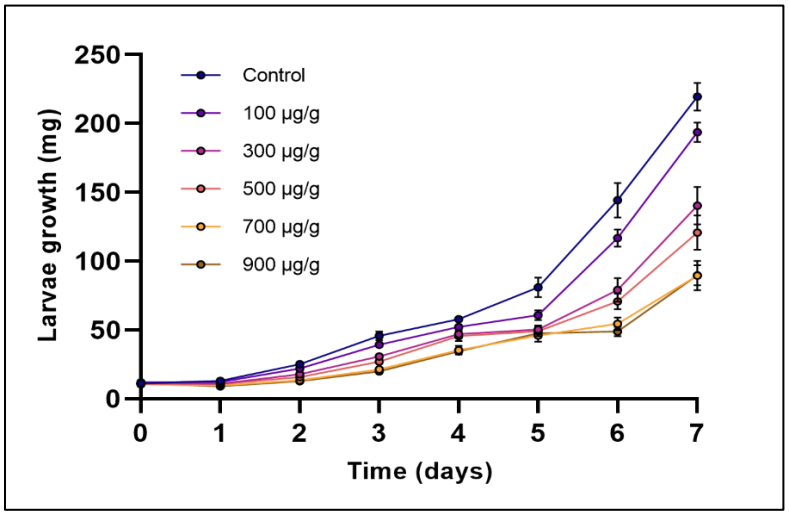
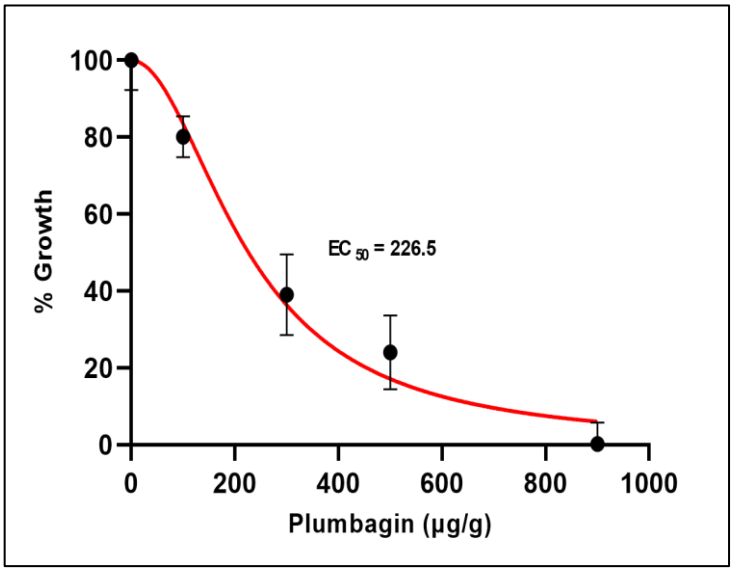


***Figure 9**: Larvae weights recorded upon feeding of 2nd instar Spodoptera littoralis on artificial diet containing various concentrations of plumbagin. Individual weight of each larvae was recorded every day for seven days. Mean (± SEM), n=15/group.*

From the observed growth of larvae on the artificial diet containing different plumbagin concentrations, an EC_{50} value was calculated as 226.5 µg g^{-1} and 1.2 µmol g^{-1} diet, respectively (Fig. 10).

***Figure 10:** The EC_{50} for 2nd instar Spodoptera littoralis feeding on artificial diet containing various plumbagin concentrations is shown as a function of growth (weight) after 7 days of feeding. The best-fit sigmoidal curve calculated by non-linear regression analysis is plotted in the figure. EC_{50} of 226.5 µg/g was calculated by non-linear regression fitting of dose response curves, the total response was normalized to run between 0% and 100% using control data.*



5.3.2 Choice bioassay

In all former experiments, the feeding larvae had only one offered food source. Thus, in a choice situation, the leaf and pitcher tissue from *Nepenthes* were provided to see whether larvae prefer one tissue to the next. The larvae showed constant preference towards pitcher tissue compared to leaf tissue (Fig. 11). The avoidance of leaf tissue is statistically significant at all tested time points. The decrease in the larvae's choice-making over time may result from less tissue availability due to constant feeding.

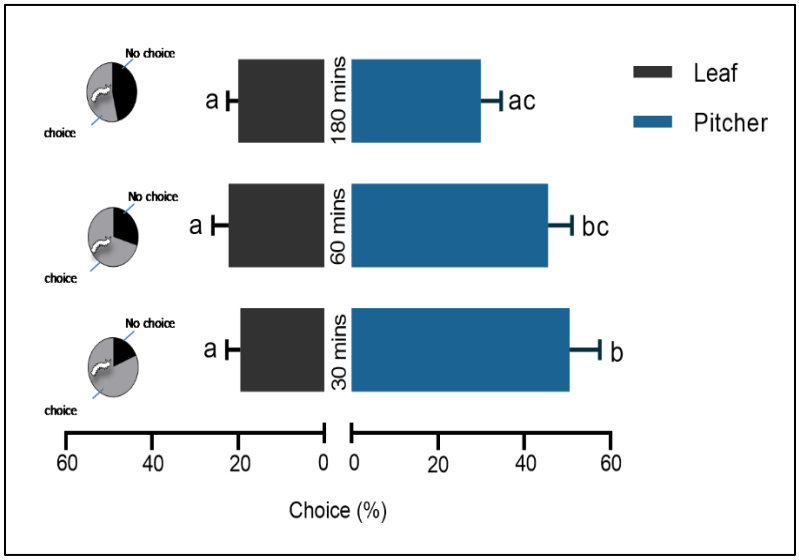


***Figure 11**: Preference of Spodoptera littoralis larvae between leaf and pitcher tissue of Nepenthes x ventrata at three different time intervals. The proportions of larvae making choices are indicated in pie charts. Mean (± SEM) labelled with different letters at the end of each bar indicate significant difference ($p<0.05$); Two-way ANOVA, Šidák's multiple comparisons test, n=180.*

5.3.3 No-choice bioassay

In order to exclude *N. x ventrata* specific effects on the feeding behaviour, a feeding deterrence bioassay with no-choice method was conducted with *Ipomoea batatas* (sweet potato) leaf discs. Discs were painted with different concentrations of plumbagin. A larval mortality of 100% was found at 120 µg plumbagin/leaf. Negative control using only the carrier solvent of plumbagin showed no mortality among the individuals tested within 7 days (Fig. 12).

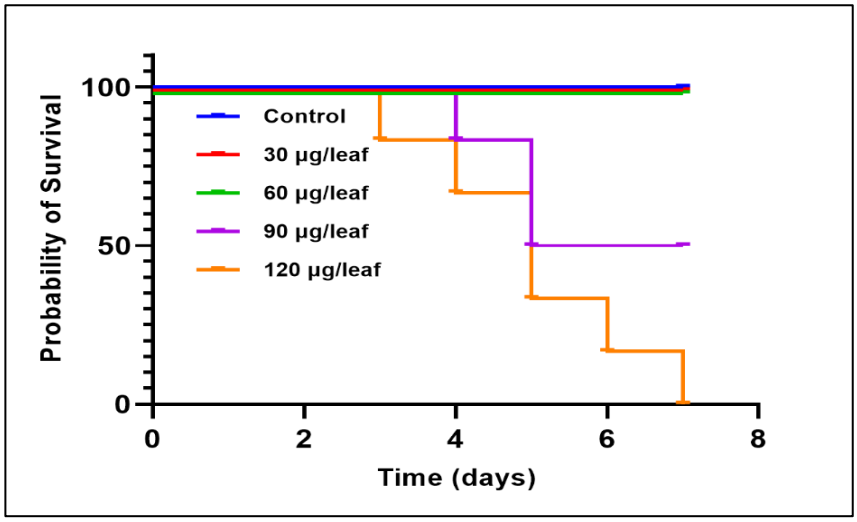


Figure 12: *Survival rate of Spodoptera littoralis larvae feeding on Ipomoea batatas leaf discs painted with various concentrations of plumbagin.*

There was also a relationship between the concentration of plumbagin and feeding avoidance. With higher plumbagin concentration, the consumption of larvae reduced greatly (Fig. 13).

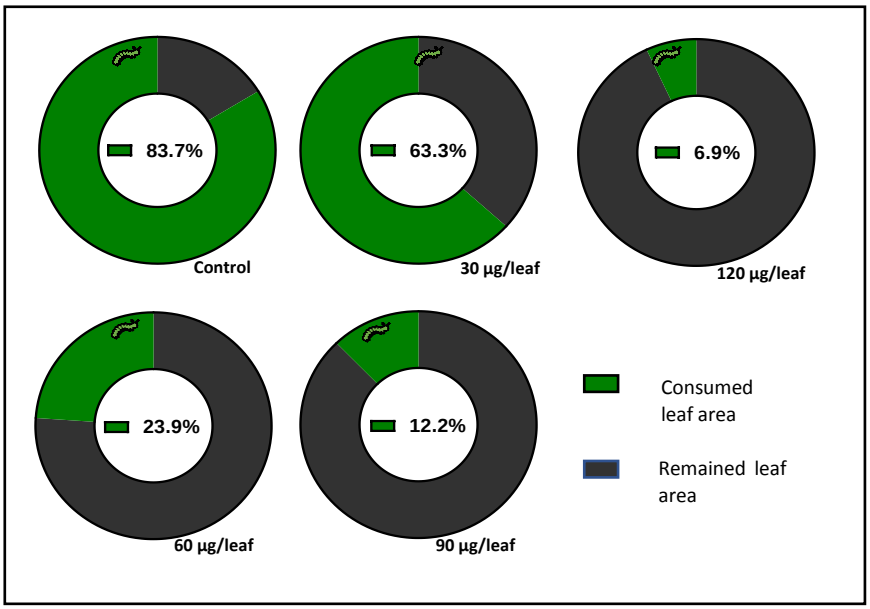


Figure 13*: Leaf area consumed by 3rd instar Spodoptera littoralis larvae. Ipomoea batatas leaf discs were painted with different plumbagin concentrations. Each pie represents mean area loss in percentage for different groups at a one day. Each group represents the mean of 6 leaf disk consumed at day 3.*

5.4 Induced plant defense response upon herbivory

5.4.1 Phytohormone levels upon short term herbivory

Next, the effect of *S. littoralis* feeding on typical plant defense responses in *N. x ventrata* was investigated. As shown in Figure 14, herbivory induced phytohormone accumulation. JA levels were significantly higher than the respective controls. The levels of ABA and SA appeared to decrease marginally after sustained infestation, though mostly not crucial in comparing treatments. The active JA isoleucine conjugate (sum-JA-Ile) level displayed a similar pattern as JA in herbivore infested leaves for all time points. Jasmonate degradation products such as OH-JA-Ile and COOH-JA-Ile were also found to be substantially elevated after herbivore infestation. However, the degradation product OH-JAs displayed a lower degree of accumulation than control at the start of infestation, which increased steadily, resulting in a substantial difference compared to control after 6 h of herbivorous infestation. The findings suggest that JA acts as the core regulator of short-term leaf infestation by herbivores, while ABA and SA are less involved.

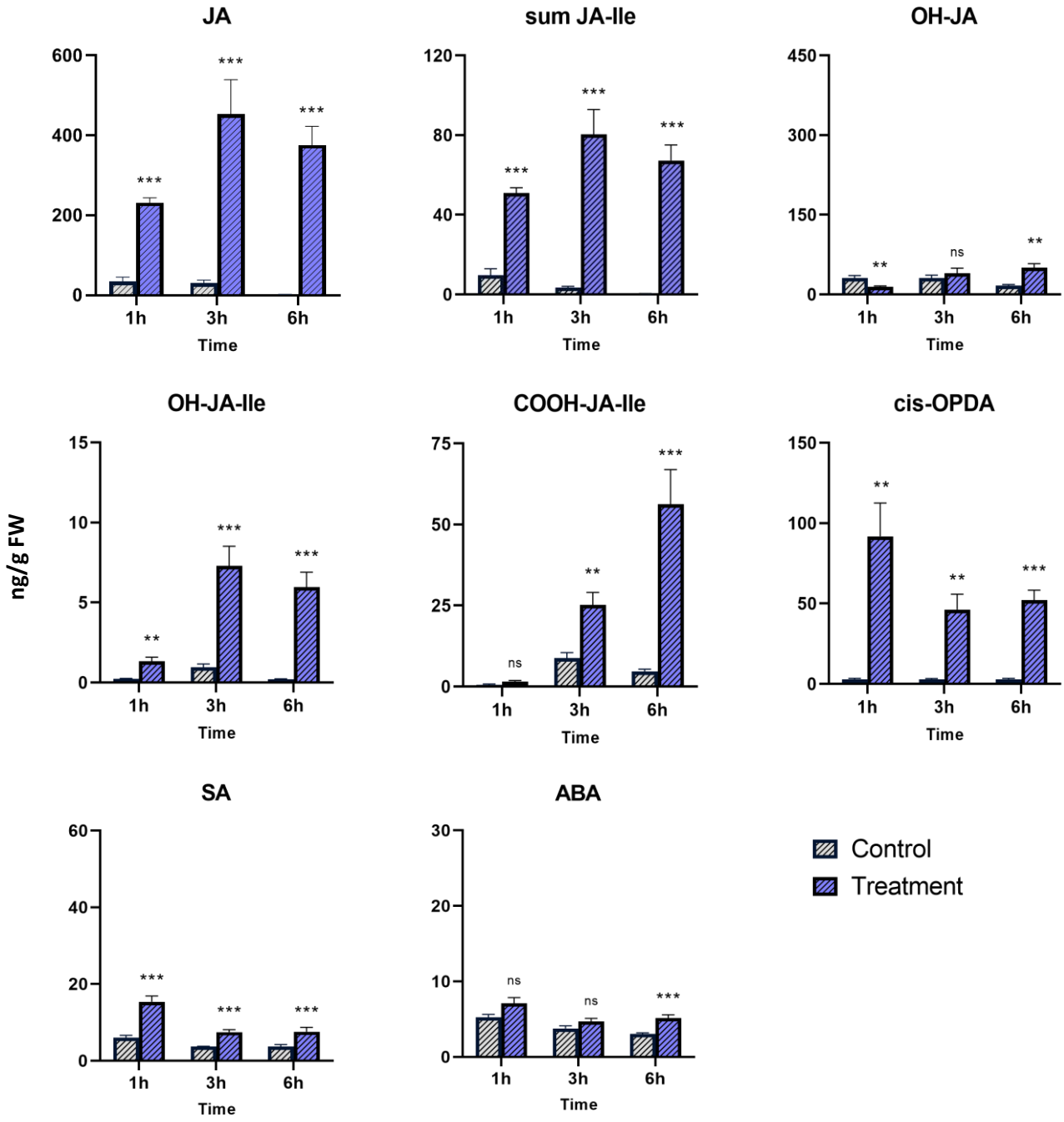


***Figure 14**: Phytohormone levels in Nepenthes x ventrata leaf subjected to short term herbivory by 3rd instar Spodoptera littoralis larvae. Control and herbivore-damaged plants were collected at different time points within 24 h (1, 3 and 6 h after herbivore feeding). Absolute levels of JAs, SA and ABA were measured by LC-MS/MS and are presented in terms of ng/g fresh weight of the samples. Each bar represents the mean (±SEM) of 10 biological replicates (individual plants). Asterisks represent difference between treatment and control within same groups at p>0.05 as non-significant, ns; ** ≤0.01; *** ≤0.001 by an unpaired t-test with Welch's correction. (JA= Jasmonic acid; sum JA-Ile= jasmonoyl-isoleucine conjugates; SA= Salicylic acid; ABA= Abscisic acid)*

5.4.2 Plumbagin levels upon short term herbivory

The same samples from short term leaf infestation by herbivores have also been tested for plumbagin levels to check if plumbagin accumulation occurs after short-term herbivory. While all treatments resulted in a remarkable accumulation of JA, its conjugates, precursor and other metabolites (Fig. 14), no significant difference was observed for the same treatments in plumbagin accumulation thereby indicating no correlation in between (Fig. 15).

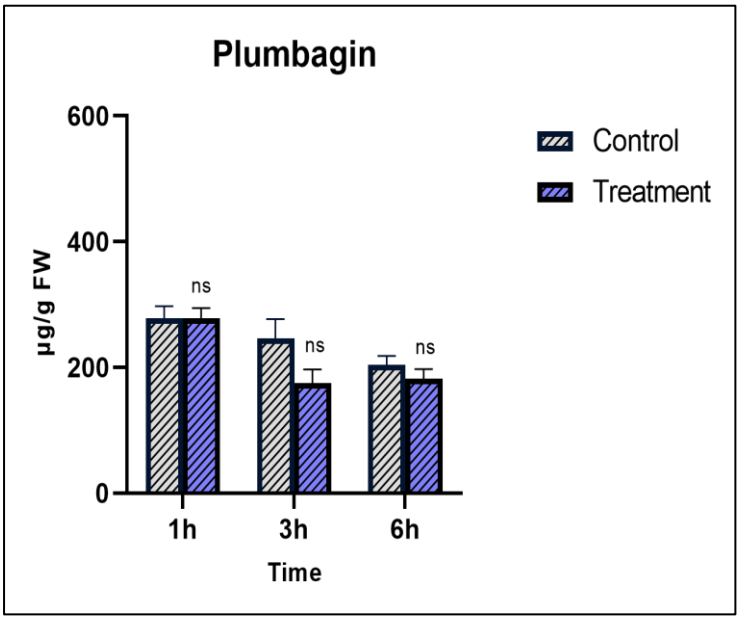


Figure 15: *Plumbagin accumulation in Nepenthes x ventrata leaf upon long term herbivory. Levels of plumbagin was measured by LC-MS/MS alongside phytohormone analysis and are presented in terms of ng/g fresh weight of the samples. Each bar represents the mean (±SEM) of 10 biological replicates for each treatment (individual plants) and controls. Difference between treatment and control within same groups were calculated by an unpaired t-test with Welch's correction (p>0.05); non-significant, ns.*

5.4.3 Phytohormone levels upon long term herbivory

To investigate if prolonged treatment of leaves by herbivore follows the same pattern of phytohormone up-regulation, leaves were infested by herbivore for days instead of hours (Fig. 16). The concentration of JA and its degradation products showed a general upward trend but fluctuated between treatment days. There is a large drop in jasmonic acid content after day 2, which remained in almost similar concentration between day 3 and day 5. Compared to short term herbivory, levels of OH-JA seem heavily upward starting from day 2 to day 5 including other degradation products such as OH-JA-Ile and COOH-JA-Ile. Increased SA concentrations may result from leaves becoming prone to pathogen attack upon constant wounding by herbivore for an extended period. The increase of ABA concentration in a time-dependent manner is also noticeable due to prolonged herbivory of leaves.

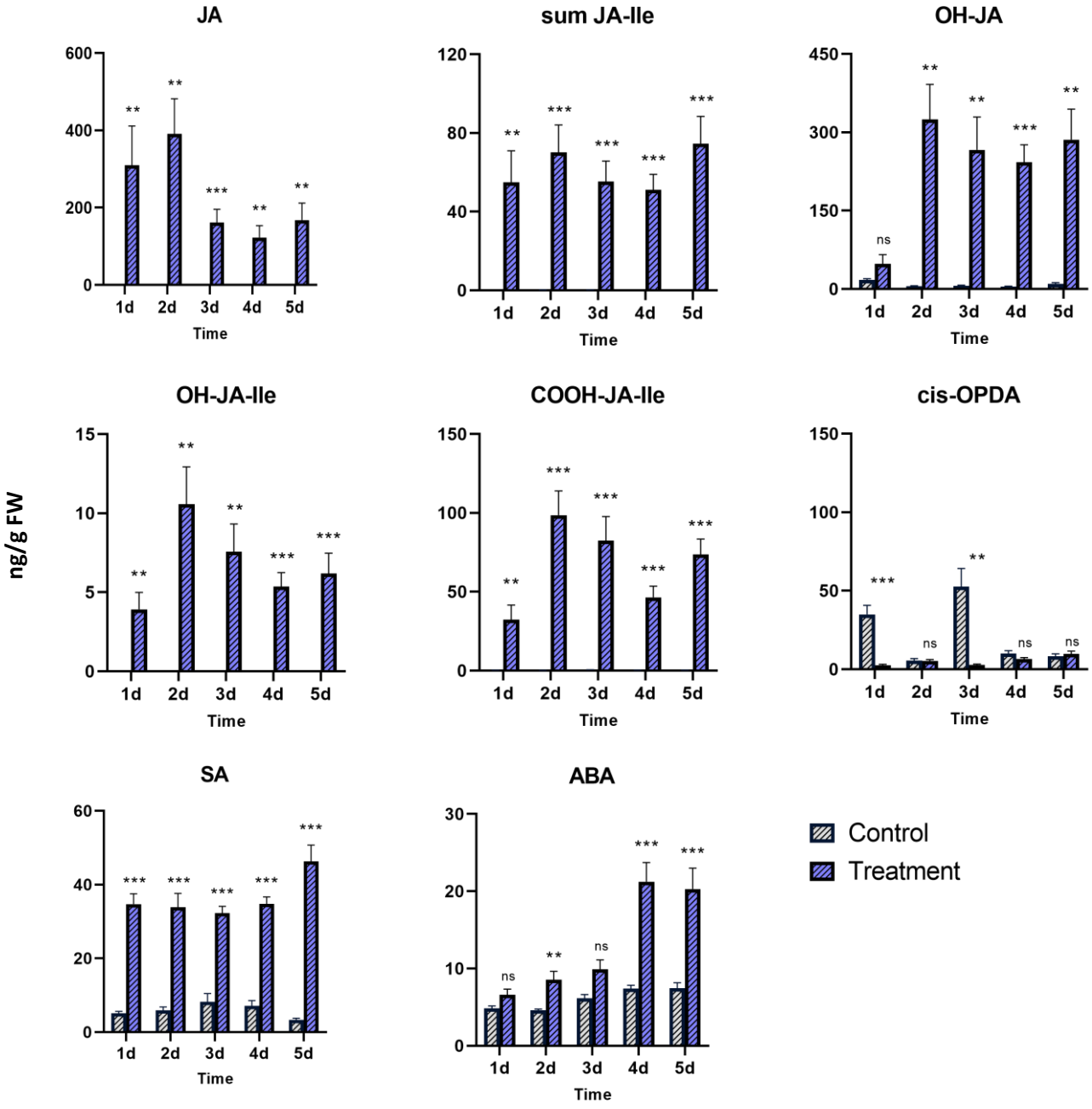


Figure 16: *Phytohormone levels in Nepenthes x ventrata leaf subjected to long term herbivory by 3rd instar Spodoptera littoralis larvae. Control and herbivore-damaged plants were collected at different days with 24 h time interval for 5 days upon herbivory. Absolute levels of JAs, SA and ABA were measured by LC-MS/MS and are presented in terms of ng/g fresh weight of the samples. Each bar represents the mean (±SEM) of 10 biological replicates (individual plants). Asterisks represent difference between treatment and control within same groups at p>0.05 as non-significant, ns; ** ≤0.01; *** ≤0.001 by an unpaired t-test with Welch's correction. (JA= Jasmonic acid; sum JA-Ile= jasmonoyl-isoleucine conjugates; SA= Salicylic acid; ABA= Abscisic acid)*

5.4.4 *Plumbagin levels upon long term herbivory*

Long-term herbivore infestation of the leaves had apparent treatment results at all days in terms of plumbagin accumulation (Fig. 17). While the plumbagin concentration ranged slightly from treatment to treatment, a minimum two-fold increase in cumulative plumbagin level can be observed compared to the control groups. No direct correlation was observed between the increased level of phytohormones and upward plumbagin concentration. However, compared to short-term herbivory, a substantial increase in plumbagin accumulation is evident from herbivores' long-term infestation of the leaves.

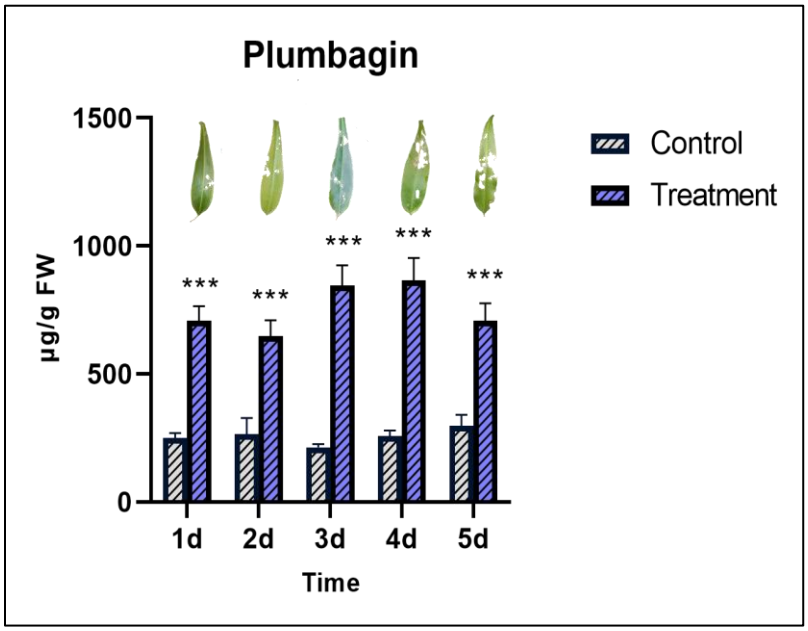


***Figure 17**: Plumbagin accumulation in Nepenthes x ventrata leaf upon long term herbivory. Levels of plumbagin was measured by LC-MS/MS and are presented in terms of ng/g fresh weight of the samples. Each bar represents the mean (±SEM) of 10 biological replicates for treatments (individual plants) and 5 biological replicates for controls (individual plants). Data were analyzed using unpaired t-test with Welch's correction. Asterisks represent difference between treatment and control within same groups at p>0.05 as, *** ≤0.001 by an unpaired t-test with Welch's correction.*

In order to investigate the possibility that plumbagin induction during long term feeding is mediated by phytohormones, an additional experiment using foliar spray. Therefore, solutions of JA, ABA and SA in water were exogenously applied to the leaves of individual *N. x ventrata* plants growing in the greenhouse. No major 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. 18).

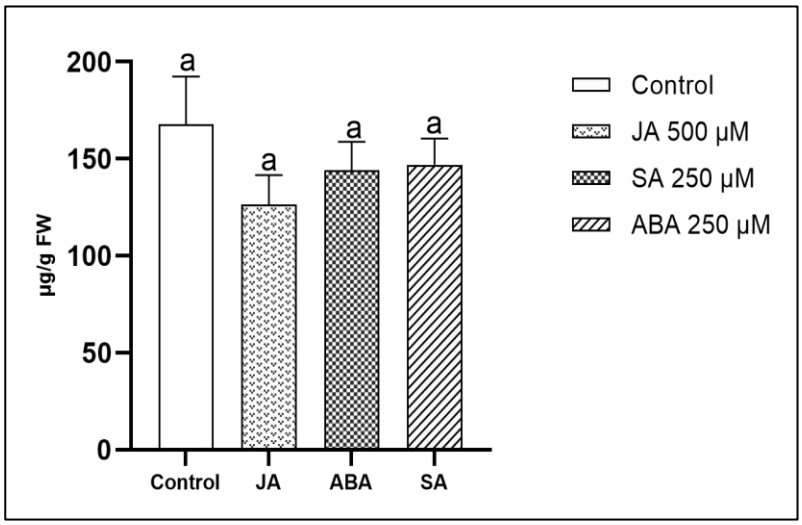


Figure 18: *Effect of foliar spray with phytohormones (JA, ABA & SA) on plumbagin accumulation. Vertical bars represent means of 5 biological replicates (individual plants) (±SEM). Statistical significance at p<0.05 between treatment and control groups; one-way ANOVA followed by Tukey's multiple comparisons test.*

5.4.5 Accumulation of trypsin inhibitor

Beside plumbagin, other compounds might be involved in herbivore defense in *N. x ventrata.* Therefore, the existence of trypsin inhibitors (TI) was investigated as an example of a wide spread defense mechanism that also could affect the feeding of larvae. 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 uninfested leaves as controls at all time points (Fig. 19). Resulting OD_{410} from control groups also showed a similar range at all times and the positive control suggesting the absence of trypsin 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.

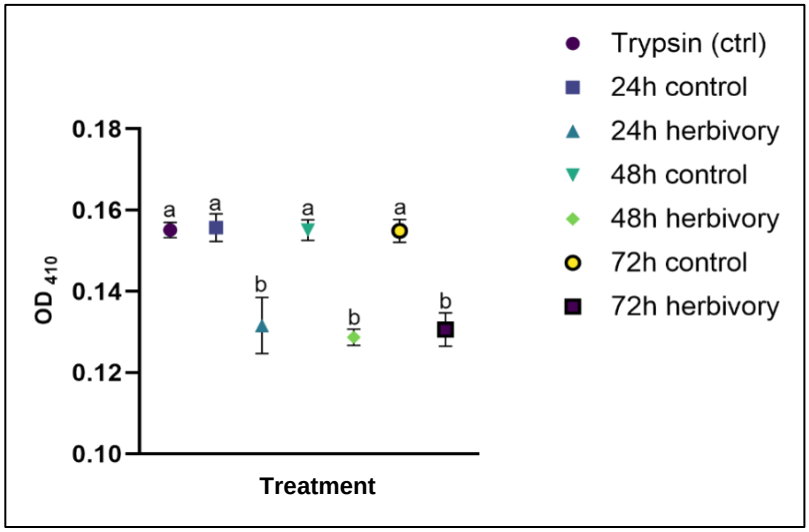


Figure 19: *Herbivory induced trypsin inhibitor activity in Nepenthes x ventrata leaves. Each symbol represents means of 5 biological replicates (individual plants) (± SEM). Different letters indicate significant differences (p<0.05) according to one-way ANOVA followed by Tukey's multiple comparisons test.*

Strikingly, when the same samples that were used for foliar spray experiments to look for plumbagin induction were checked for proteinase inhibitors, significant trypsin inhibition activities were observed for all phytohormone-treated samples upon 24 h of foliar spray compared to trypsin control and untreated control groups (Fig. 20).

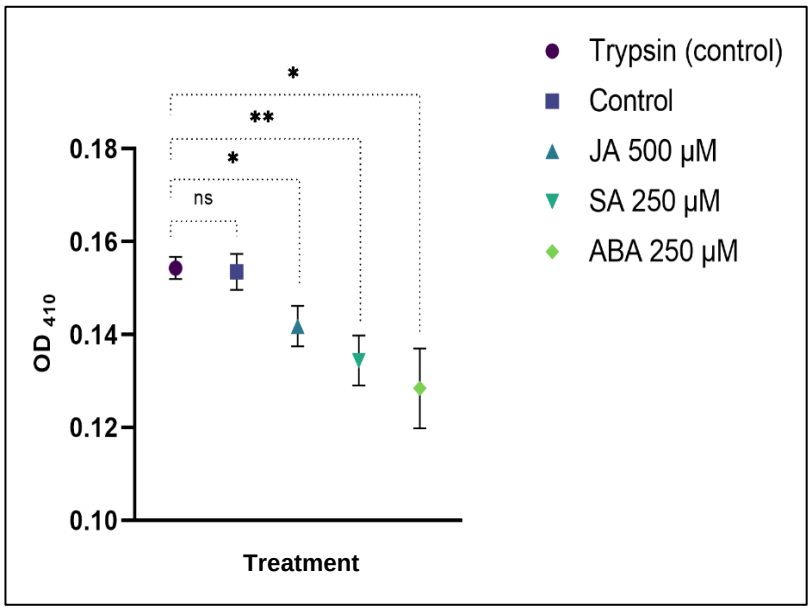


Figure 20: *Effect of foliar spray with phytohormones (JA, ABA & SA) on trypsin inhibition activity. Vertical bars represent means of 5 biological replicates (individual plants) (±SEM). Asterisks represent difference between individual treatments vs control at p>0.05 as non-significant, ns; * p<0.05, ** ≤0.01; unpaired t-test with Welch's correction test.*

6 DISCUSSION

6.1 Plumbagin as a defense compound in Nepenthes x ventrata

The presence of naphthoquinones has been described for many carnivorous plants (Devi et al., 2016) belonging to the order Nepenthales (Hatcher et al., 2020), a *sensu stricto* sister group to Caryophyllales (Fleischmann et al., 2017) and including the plant families Droseraceae and Nepenthaceae. This includes species such as *Aldrovanda vesiculosus*, *Dionaea muscipula*, *Drosophyllum lusitanicum*, as well as the genera *Drosera* and *Nepenthes* (Devi et al., 2016). The meanings of such plant secondary metabolite often lay in their ecological significance in defense setup of the plant by keeping at bay the herbivores and also be necessarily integrated into the total metabolic scheme. Often multiple functions are then to be expected to the benefit of the plant (Kariñho-Betancourt, 2018). The observation that *Nepenthes* plants are rarely infested by insect herbivores could be an example of such phenomenon knowing that NQ are highly bioactive compounds with strong insect antifeedant activity (Rahman-Soad et al., 2021). In this study, the NQ plumbagin was found as a dominant secondary metabolite in the tissue of *Nepenthes x ventrata* and its function was evaluated by means of the interaction of a generalist insect herbivore *S. littoralis*.

Experiments with artificial diet containing harvested tissues from *N. x ventrata* leaf and pitcher incorporated in fresh or dried form showed an adverse effect on the growth of the *S. littoralis* larvae. Interestingly, larvae fed with leaf tissue containing diet did gain less weight while the pitcher tissue diet fed larvae performed well (Figs. 1 & 2). The resulting development of the larvae could be an indication of post ingestive effects. This may include deterrence emerging from the changed food chemistry due to plant tissue incorporation and the level of the responsible metabolite(s) present. However, artificial diet sometime comes with the limitation of being suboptimal or super optimal (Wolfson, 1988). Compared to most susceptible host plants, artificial diet often fosters more rapid growth (Resse and Field, 1986) thus making insects less susceptible to the tested compound or extract incorporated in diet. This situation was eliminated by letting the larvae feed on the leaf tissue of *N. x ventrata* directly where larvae showed almost no development in terms of gaining weight (Fig. 3). Nevertheless, the better growth performance of *S. littoralis* feeding on pitcher compared with leaf tissue (Fig. 1) can also be traced back to the presence of plumbagin. Recently, in *N. x ventrata* (Dávila-Lara et al., 2020) and before in *N.*

khasiana (Raj et al., 2011), the concentration of a NQ, very likely plumbagin, was determined to be significantly higher in leaves compared with pitchers. These data was supported from the comparison of plumbagin content in *N. x ventrata* in pitcher vs. leaf; by quantitative NMR analysis, showing 5.2-fold higher plumbagin concentration in leaf compared with pitcher tissue (650 and 125 µg g^{-1} FW, respectively) (Rahman-Soad et al., 2021). These finding may also explain the preference of *S. littoralis* for pitcher over leaf tissue in a choice assay (Fig. 9).

There are several studies showing that NQ can affect the growth of insects (Babula et al., 2006; Devi et al., 2016; Widhalm & Rhodes, 2016) and in this study artificial diet feeding experiments with plumbagin containing plant tissue from *Nepenthes* supported the same. However, the likelihood of other allelochemical(s) from plant tissue alongside plumbagin possibly being involved in the artificial diet negatively affecting the larval growth cannot be eliminated entirely. Therefore, we performed feeding experiments with only synthetic plumbagin-supplemented diet knowing exact food chemistry and measured the weight of *S. littoralis* larvae every day. Knowing that *Nepenthes* leaves have approximately 0.05% of their fresh weight as plumbagin (Raj et al., 2011), a concentration range between 100 and 900 µg g^{-1} was covered, representing 0.01–0.09% fresh mass, respectively. As shown in Figure 7, the larvae's weight decreased with higher plumbagin concentrations. The EC_{50} value was calculated based on the data, indicating the plumbagin concentration needed to inhibit larvae growth by 50% which was determined as 226.5 µg g^{-1} diet.

Since the endogenous levels of plumbagin were already known from leaf and pitcher tissue analysis of *N. x ventrata* including the water content of leaf tissue (Dávila-Lara et al., 2020), it was possible to calculate the available amount of plumbagin that was present in the plant tissue incorporated diets Figure 21.

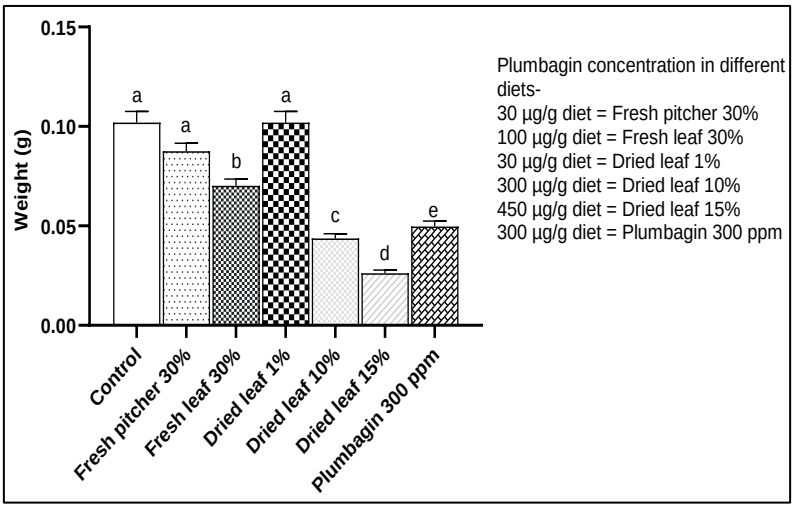
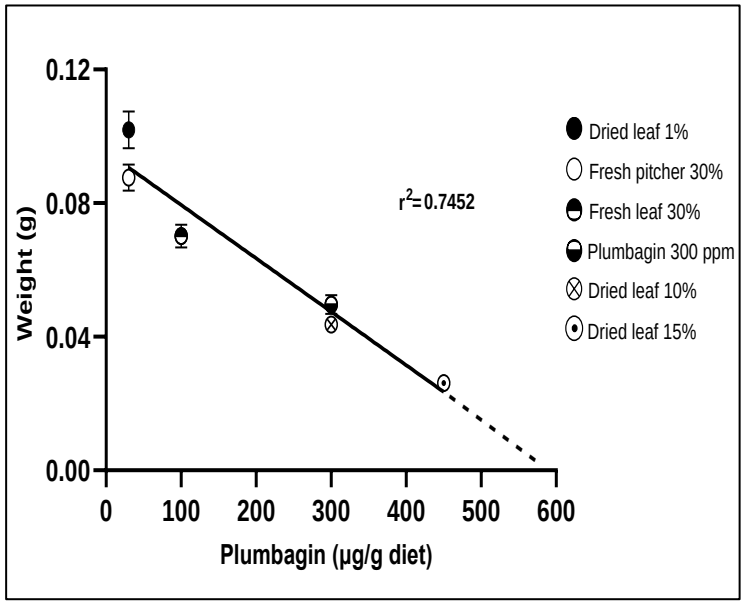


Figure 21: *Growth comparison of Spodoptera littoralis larvae upon 5 days feeding on different artificial diets. Each vertical bar represent means of 15 biological replicates (individual larvae) (± SEM) and different letters indicate significant difference (p<0.05) according to one-way ANOVA followed by Tukey's multiple comparisons test.*

Upon comparison of larvae growth on different artificial diet representing various plumbagin concentrations from different sources, a regression analysis showed a clear link between the larval weight gain and the amount of available plumbagin in the artificial diet (Fig. 21).

Figure 22: *Plot of weight gain vs. plumbagin concentration in different artificial diet for 2nd instar Spodoptera littoralis larvae after 5 days feeding. Each symbol represents the gained weight of larvae feeding on different diets with varied plumbagin concentration. Regression showed statistical significance with p<0.0001 (estimated r^2= 0.7452, F= 251.5 (DFn = 1, DFd = 86), alpha level = 0.05). The r^2 value (0.7452) also indicates a strong relationship of larval development with concentration of plumbagin present in the artificial diet.*



In fact, this could show that the weight gain from the artificial diet by *S. littoralis* larvae is highly depending on plumbagin concentration. For some lepidopteran species such as *S. litura*, *Achaea janata*, and *Trichoplusia ni*, the effect of plumbagin on feeding behavior has already been demonstrated (Akhtar et al., 2012; Sreelatha et al., 2009; Tokunaga et al., 2004). However, the focus of the study in those experiments was on the level of feeding avoidance rather than larval growth and development.

Contrary to most bioassays that investigated the antifeedant activity of plumbagin, in this study an experiment was carried out following leaf disk assay method in order to determine larval mortality feeding on plumbagin painted leaf. Therefore, a cultivar of sweet potato (Tainong 66) that is vulnerable to herbivores and does not provoke strong defensive response upon herbivore attack was chosen (Meents et al., 2019). In first experiments, we observed that *S. littoralis* larvae even preferred cannibalism than feeding on those leaves. Therefore, only individualized larvae were used. Up to a plumbagin concentration of 60 μg^{-1} leaf (13.3 $\mu g\ cm^{-2}$, 750 $\mu g\ g^{-1}$ leaf) no larvicidal effect was determined for the period analyzed *(*Fig. 10). With 90 μg^{-1} leaf (20 $\mu g\ cm^{-2}$; 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 $\mu g\ cm^{-2}$; 1.5 mg g^{-1} leaf), dead larvae were detected at day 3 and until the end of day 7, all larvae have died (Fig. 10). For *T. ni* feeding on plumbagin-covered cabbage leaves, an antifeeding effect was also determined in the low microgram per square centimeter range (Akhtar et al., 2012). The larvae can also be seen to avoid feeding on the leaves coated with higher plumbagin concentrations (Fig. 11). With respect to the results shown in Figure 7, 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 $\mu g\ g^{-1}$). The plumbagin concentrations used in the no-choice assay also showed no mortality up to 750 $\mu g\ g^{-1}$ leaf tissue. Only at the used concentration of 1.125 $\mu g\ g^{-1}$ 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 very similar to Hu and colleagues' (2018) recent study. 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 $\mu g\ mL^{-1}$ (*P. rapae*) and 30 $\mu g\ mL^{-1}$ (*H. armigera*) were calculated (Hu et al., 2018). 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 (Pavela, 2013; Sreelatha et al., 2009). This approach is worth to carry out but not qualified for studies on activities of compounds that are incorporated during herbivory.

6.2 *Phytohormone signaling upon herbivory of Nepenthes x ventrata leaf*

While most of the phytochemical studies done on *Nepenthes* plant is mostly concerning the carnivorous process, demonstration of phytochemical synthesis and accumulation as a result of herbivory is surprisingly limited for the same plant. This study illustrates the phytochemical defense response of *N. x ventrata* upon herbivore wounding.

Herbivory causes different kinds of damage to the plants. Wounding following insect attack leads to the rapid (minutes to several hours) induction of a series of events, including the generation and release of specific signals, the subsequent perception and transduction of those signals followed by phytohormone biosynthesis (Gfeller et al., 2010) and finally, activation of wound related defenses. Often jasmonates relay this information and lead to induction of gene expression (Kazan & Manners, 2008). Expression of a particular gene can be regulated transcriptionally by a high number of transcription factors that bind to the promoter regions of a gene upon hormonal changes (Singh et al., 2002). Salicylic acid as well as jasmonates such as jasmonic acid, are closely correlated with defense-related induction of gene expression such as genes of pathogenesis related (PR) proteins (van Loon et al., 2006, Bari and Jones, 2009) that are induced as a response of various attacks (van Loon et al., 2006, Sinha et al., 2014).

From prior phytochemical studies, it is known that JAs can induce the formation of digestive cavities and regulate enzyme production in response to different stimuli in carnivorous plants (Pavlovič & Mithöfer, 2019) thus coopting existing signaling pathways actually involved in defense against herbivore attack. The involvement of JAs in initiation of carnivorous response first came from the studies of Ueda et al. (2010) while looking trap closing factors in the Venus flytrap (*D. muscipula*) where coronatine, a molecular mimic of JA-Ile, found to be highly effective in the induction of trap closing. Later, Yilamujiang et al. (2016) showed elevated endogenous concentrations of JA and JA-Ile in the pitchers of the genus *Nepenthes* upon prey induction. However, closer look from current study revealed the same JAs involved in the defense response of *N. x ventrata*. The wound-stimulated elevation of JA with its biological active form is rapid, which was observed from the short-term herbivory experiments in this study (Fig. 12). However, with prolonged herbivore infestation (long-term herbivory, Fig. 13) a much higher accumulation of JA catabolites can be seen in comparison with short-term herbivory ultimately confirming the fate of JA and JA-Ile, which has been described by Wasternack & Hause (2013).

However, the action of JA can be more subtle than a simple quantitative change, as is commonly detected in whole plant organs. The elevation of JA often follows a transient manner, suggesting modulation of JA level may play an inducing role, but continued high levels are not always required for sustained activity (Ruan et al., 2019).

In previous studies done on *Nepenthes*, the role of signaling hormones together with JAs has not been extensively discussed outside the carnivorous process context. Studies on the prey-induced responses in *Nepenthes* from Yilamujiang et al. (2016) found doubling of endogenous SA level after prolonged (48 h) chitin treatment compared to the level observed after 24 h of the same treatment in the pitcher tissue. Slightly similar situation was observed from our current studies where long-term herbivory showed nearly doubled endogenous level of SA shorty after 24h compared to short-term herbivory in leaf tissue of *Nepenthes* (Fig. 14).

SA of plant origin has been recognized for its healing properties since antiquity. Of the signal molecules that are being considered, production of SA is probably the most specific; concentrations increase in response to pathogen infection but not as a general wound response (Enyedi et al. 1992; Malamy et al. 1990). Therefore, one possibility of increased concentration of SA observed upon prolonged herbivory in this study could be a consequence of elicitors of *S. littoralis* oral secretions, which can modulate plant defense (Guo et al. 2013). On the other hand, increased vulnerability of leaf upon herbivore damage may create open passages for pathogens to enter. SA induces transcriptional production of a variety of chemicals thought to be involved in plant defense such as the pathogenesis-related proteins and oxidative enzymes.

Considering stress-related hormones such as abscisic acid (ABA) (Tuteja 2007), this study showed elevated endogenous ABA levels upon long term herbivory; a common response known in plants after infestation by natural enemies (Weldegergis et al. 2015). 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 study long-term herbivory of leaf tissue causes early senescence, which consequently increases dryness of wounded leaves; this is also a possible reason for ABA accumulation over time.

6.3 *Plumbagin is inducible upon herbivory*

In the 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 (Freeman, 2008).

In this study, induced defense response by herbivore feeding on *N. x ventrata* leaves showed plumbagin accumulation in wounded leaves with a minimum of 2-fold increased endogenous level compared to untreated leaves. A very similar response was found from the carnivorous plant *Dionaea muscipula* where Tokunaga et al. (2004) showed accumulation of plumbagin (0.5% weight of fresh trap lobes) upon defense response against predation. Although the accumulation of plumbagin has been mentioned several times in both *in vivo* and *in vitro* studies using polysaccharides as elicitors (Marczak et al., 2005; Nahálka et al., 1998; Eilenberg et al., 2010), little is known on the exact molecular mechanism governing the herbivory-induced production and accumulation of this phenolic compound when it comes to carnivorous plants.

Wound-induced production of plant secondary metabolites is often mediated by signaling-molecules such as JA, reactive oxygen species (ROS) and ethylene (ET) (Jacobo-Velázquez et al., 2015). From our current study, knowing plumbagin as a phenolic compound with strong antioxidant activity, there could be strong relation between ROS production in damaged leaf upon herbivory and plumbagin accumulation. From the studies of wound induced production of phenolics in carrots Jain et al. (2014) showed ROS play a key role in such accumulation. On the other hand, ET and JA are suggested as essential modulators of ROS levels (Jacobo-Velázquez et al., 2015) supporting the same involvement of ROS upon wounding.

ROS are well-known secondary messengers involved in a number of cellular processes, including stress tolerance (Sharma et al., 2012). The delicate balance between ROS development and scavenging determines whether ROS can act as a damaging or signaling molecule. It is important for cells to tightly regulate ROS levels in order to prevent oxidative damage, rather than fully eliminating them because of their multifunctional roles which is controlled by efficient antioxidative system comprising of the nonenzymic as well as enzymic antioxidants (Noctor & Foyer, 1998). Among non-enzymic systems ascorbate (AsA), glutathione (GSH), carotenoids, tocopherols, and phenolics serve as potent antioxidants within the cell (Sharma et al., 2012).

Several studies until now have reported increased activities of many enzymes of the antioxidant defense system in plants to combat oxidative stress induced by various environmental stresses. Maintenance of a high antioxidant capacity to scavenge the toxic ROS has been linked to increased tolerance of plants to these environmental stresses (Clemens & Weber, 2016; Zaefyzadeh et al., 2009). Based on this, further experiments to elucidate a putative role of ROS in *Nepenthe*s carnivory and under herbivore attack should be done.

6.3.1 Proposed resource allocation for plumbagin production

Plants with naturally slow growth rates are often preferred over those with fast growth rates in areas where resources are limited; slow growth rates, in turn, encourage major investments in defense compounds (Coley et al., 1985).

The primary metabolism of plants makes the carbon skeletons available that are required for the biosynthesis of distinct phenolic metabolites, which are involved in resistance to environmental stress, as well as in various aspects of signaling and defense (Caretto et al., 2015). Primary metabolism, however, depends on a substantial amount of available plant resources. Thus, when the growth rate is high, phenolic compounds are depleted by the substrates' shortage (Lattanzio et al., 2009). The general premise is that primary metabolism cannot afford to produce secondary protection compounds because it must balance its needs to survive. On the other hand, limited resources in plants available for the production of defense compounds are needed for self-preservation, and this requires putting the plants in a position where they have to allocate their carbon skeletons to self-defense, which in turn diverts carbon from primary to secondary metabolism (Coley et al., 1985; Herms & Mattson, 1992).

Therefore, the observed plumbagin in *Nepenthes* could be a result of the activation of the phenylpropanoid metabolism together with those metabolic pathways involved in the supplementation of carbons skeletons needed for phenolics biosynthesis such as the shikimate route; a known pathway for plumbagin biosynthesis (Widhalm & Rhodes, 2016). With the shikimate pathway as the central unit focused on the few intermediates, the general phenylpropanoid metabolism generates a vast range of secondary metabolites (Vogt, 2010). Besides, phenylpropanoid pathway products participate in various aspects of plant growth, structural support and stimuli reaction (Biała & Jasiński, 2018). They partake in plant response to

light and mineral depletion as well as have a critical role in interactions with other organisms (Yang et al., 2018; Clemens & Weber, 2016; Naoumkina et al., 2010).

6.4 Accumulation of proteinase inhibitors upon herbivory

A striking result was the finding that larvae feeding on *N. x ventrata* leaves gained almost no weight at all, 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, in this study, apart from plumbagin accumulation in *N. x ventrata* as a mode of defense response against herbivores, proteinase inhibitors (PI) were also investigated, which may affect the digestion of food in the larvae and cause less or no weight increase. As demonstrated in Figure 17, PI activities were indeed induced upon herbivory. Although PI 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 PIs upon herbivory.

PI are polypeptides and proteins that bind to proteolytic enzymes and prevent them from catalyzing (Clemente et al., 2019). Most of these inhibitors are unique to serine class proteinases, usually found in insects as the main food protein digestive enzymes. (Jamal et al., 2013). Enzyme assays for proteinase inhibitors from our present study showed a clear inhibition of trypsin (a serine protease) from herbivory infested leaf protein extracts, whereas uninfested leaf protein extracts showed no significant inhibition of trypsin indicating the accumulation rather happened as response to herbivory wounding that is absent in unstressed leaf (Fig. 16).

The understanding that induced PI in plants may function as protective substances against subsequent insect attack (e.g., herbivory) originated with studies by Mikel and Standish (1947) where larvae of certain insects were observed being unable to develop normally on soybean products. Subsequently, the trypsin inhibitors found in soybeans were later discovered to be toxic to the larvae of the flour beetle, *Tribolium confusum* (Lipke et al., 1954). The role of PIs in plant defense against insects was demonstrated more directly when Green and Ryan (1972) showed the induction of PIs in leaves of potato in response to wounding and insect feeding.

6.5 Effect on Plumbagin level and PI activity upon foliar spray of signaling hormones.

In this present study, the chemical defenses observed upon herbivore damage were: plumbagin accumulation and increased protease inhibitor activities in *N. x ventrata* leaves. To address the

question which endogenous, herbivory-related signals were involved, phytohormones as the obvious and induced candidates (Figs. 12 & 14) were further tested. Upon external foliar spray application of JA, SA, and ABA no response to plumbagin accumulation was detected within 24 h of treatment (Fig. 16). In contrast, all phytohormones induced PI activity (Fig. 18) indicating that the spray approach was successful. Moreover, in contrast to PI, plumbagin levels are constitutive, at least not phytohormone inducible. From the data obtained, it is difficult to infer which signaling molecule may actually be involved in the accumulation of plumbagin in long terms. As mentioned earlier in the possible relationship between ROS and plumbagin accumulation, it would be worth trying to see if exogenous ROS i.e. H_2O_2 might really have an effect on endogenous plumbagin levels.

7 Conclusion and outlook

In this present study, based on the observed effects of the naphthoquinone plumbagin on herbivore's growth inhibition and antifeedancy, it is reasonable to assume that this compound is an effective chemical defense of *Nepenthes*. Although the effect of naphthoquinones on ecologically relevant herbivores was previously mentioned in different studies, the circumstantial evidence that accumulation of naphthoquinone can occur due to herbivory is impressive. Furthermore, additional inducible defense responses in *Nepenthes,* such as the PI activity, can support the first line of defense represented by the naphthoquinone phytoanticipins. The findings in this study have so far been the first thorough analysis of any carnivorous plant's defense response. Among the herbivore defenses of *Nepenthes* described in this report, only PI induction is phytohormone-mediated, raising the question of how plumbagin synthesis and accumulation is orchestrated. It is difficult to deduce from the data obtained yet what signaling molecule may actually be involved in plumbagin build-up in long-term periods but following earlier suggestions to observe ROS levels alongside plumbagin accumulation could be worthwhile. It is also important to look at whether and what other defensive responses are taking place in *Nepenthes* by looking into shuffling expression of genes synthesizing alkaloids, terpenoids or flavonoids and how they are controlled. We almost certainly understood only a small part of the story but opening doors for further exploration.

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Declaration

I declare here that this thesis has been produced by myself. I did not use any of published sources and data than those that are specified as reference. I also confirm that this master's thesis has not been use as part of an earlier course achievement or examination procedure.

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