

Ecophysiological Effects Induced by an Oligophagous Insect Herbivore in an Early Spring Geophyte

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Abstract

Insect herbivores, whose larval stages live and feed inside a plant leaf, are expected to have an impact on the leaf beyond the actual tissue damage. They might influence leaf photosynthesis. Here, we provide a brief insight by studying a simple model relationship between the spring geophyte ramsons *Allium ursinum* with short-lived green leaves and its oligophagous, leaf-mining hoverfly *Cheilosia fasciata*. We expected that the effect of leaf miners on short-lived leaves could interfere with resource allocation for the next season. Measurements of leaf greenness and chlorophyll fluorescence were performed to evaluate the stress impact of leaf miner damage upon photosynthesis during the short vegetative season of ramsons. Results showed that the onset of leaf senescence occurred earlier in infested leaves, which was also indicated by lower maximum efficiency of photochemistry compared to non-infested leaves. There was no evidence that infested leaves were able to compensate for the damage by increasing the rate of photosynthesis. Given the short lifespan of leaves and the earlier onset of senescence in infested leaves, we discuss two differential hypotheses: negative effects for the plant if the larvae have already left the mine, and negative effects for the leaf miner if the larvae are still developing inside the leaf.

Introduction

Insect-plant interactions represent an enormous diversity of interactions, which range from beneficial to damaging for the plant (Bernays 1992, Kergoat et al. 2017). Insect herbivores cause damage, which plants recognize through molecular patterns associated with damage or/and with elicitors, resulting in defense mechanisms aiming at intoxication and deterrence of insects and triggering the plant's resistance (Giron et al. 2018). Leaf miners and other insect herbivores like leaf grazers have an impact on the leaf beyond the actual tissue damage. They could influence leaf photosynthesis (Lombardini et al. 2013). This is difficult to estimate because plants respond in various ways: they increase allelochemical production, decrease photosynthesis (Raimondo et al. 2003; Wagner et al. 2008), compensate for the loss of tissue by an increased rate of photosynthesis (Welter 1989) or in some cases continue to photosynthesize without a change (Wagner et al. 2008). Moreover, a plant's responses inevitably influence interaction with other biotic partners – other herbivores, mutualistic and parasitic insects, microbes and fungi (Giron et al. 2018 and references therein).

The majority of studies dealing with leaf miner effects on host plants are performed on woody plants, mainly trees, and agricultural plants and focus on plant species that occur the whole year round (Liu et al. 2015). According to published studies, it seems that the effect on photosynthesis is highly dependent on the life-form of the plant and the morphology and anatomy of the leaf, e.g. whether it is a deciduous tree or a herbaceous plant, whether the leaf lamina is pinnate or not etc. Although many plants have a relatively long growing period, spring geophytes in the understory have a short time to develop green leaves, flowers, fruits/seeds and to allocate sufficient resources to the bulb for the next spring before the canopy closes. The effect of leaf miners could be very serious because of interference with photosynthesis and chlorenchyma damage during this time, possibly causing a reduction in resources for the bulb, which might have an effect in the next season.

In order to investigate the effect of leaf miners on their host plants in spring understory vegetation, we used the model system of the geophyte ramsons *Allium ursinum* (L.) and its oligophagous hoverfly *Cheilosia fasciata* Schin. & Egg. (Diptera, Syrphidae). Ramsons is a species that grows throughout large parts of temperate Europe and flourishes mainly in April and May/June, depending on the latitude and altitude. Adults of *C. fasciata* laying their eggs on ramsons leaves (Speight 2017). From these, larvae emerge and mine the leaves. Most of the time, a larva remains on the same leaf but might sometime change leaves (Hövmeyer 1992). Studying such a system in the natural environment reflects actual interactions more efficiently, additionally considering the climatic conditions and resource availability (Hövmeyer 1987, 1995). A key resource for *A. ursinum*, a spring geophyte of the deciduous forest understory, is the photosynthetically active radiation (PAR) defining photosynthesis efficiency, which decreases through the vegetative season as the tree canopies develop overhead. This represents a very narrow timeframe for *A. ursinum* in spring to develop green leaves, flowers, fruits/seeds and to allocate sufficient resources to the bulb for the next spring.

We were therefore interested how damage inflicted by the leaf miner affects the leaves of a spring geophyte. As has been established, green *A. ursinum* leaves are short-lived and respond to decreasing PAR availability by starting to senesce and at least partly translocating nutrients to the bulb. The most obvious sign of leaf senescence is a loss of green coloration, caused by the breakdown of chlorophyll. Additionally, in more detail, the chlorophyll fluorescence parameters, obtained by non-invasive measurement of photosystem II (PSII) activity are frequently used to indicate functional changes in photosynthesis under various stressors (Murchie and Lawson 2013). We estimated that the greenness of the leaf and the operational photosynthesis during growth conditions could elucidate the effects of the leaf miner, if we compared healthy and infested leaves. We hypothesized that, in the case of larval leaf miners attacking short-lived *A. ursinum* leaves, a compensatory increase in photosynthesis would mean more food for the leaf miner and would not apply to *A. ursinum*. Instead, we expected the larval leaf miner to decrease photosynthesis to such an extent as to enable detection by chlorophyll fluorescence, which would further indicate an adaptive response of the plant to its biotic environment.

Materials And Methods

Study area

The study area is located in the central part of Slovenia near the town of Laško (46°09'01.9"N, 15°13'55.8"E; 230 m a.s.l.) on the elevated western bank 75 m from the Savinja river (mean monthly discharge 30 m³/s). This area has a temperate climate, with a mean annual temperature of 10.9°C in 2011 and 11.3°C in 2012 (data from a private meteorological station Laško – Lahomšek) and mean annual precipitation of about 1230 mm (ARSO METEO). The precipitation peak occurs in summer (34%), while in spring and fall the precipitation is very similar (23% and 26%, respectively) (Nadbath 2012). In the winter of 2011/2012, the snow cover lasted 18 days, 17 of which were in February (ARSO METEO).

This moderately hilly region is partly overgrown by submontane beech forest remnants on sandstones and marl. We selected a dense *A. ursinum* population, covering approximately 800 m² within such a forest belonging to the association *Hacquetio-Fagetum* Košir 1962, a common forest type in lowlands and on hills and mountains up to 600 m a.s.l. (Marinček 1987). The location had an eastern exposure. The dominant tree species were *Fagus sylvatica* L., *Quercus petraea* (Matt.) Liebl., and *Acer pseudoplatanus* L. In the herb layer, besides *A. ursinum*, several spring geophytes were abundant, like *Anemone nemorosa* L., *Asarum europaeum* L., *Dentaria enneaphyllos* (L.) Crantz, *Hacquetia epipactis* (Scop.) DC., *Helleborus niger* L., *Hepatica nobilis* L., *Lamium orvala* L., *Polygonatum multiflorum* (L.) All., *Pulmonaria officinalis* L., *Symphytum tuberosum* L. and various other herbaceous species with peak development later in the vegetation season.

Survey protocol

In 2012, four permanent 25cmx25cm plots within the *A. ursinum* population were monitored weekly from 22nd of April until the 6th of May. In these plots infested and non-infested plants were chosen. Measurement of chlorophyll fluorescence (fluorometer Handy PEA, Adsotech, Kings Lynn, UK) within each plot were performed on 10 healthy leaves of random plants and on all infested leaves (between 16 and 21 leaves). On the infested leaves, the non-injured portion of the leaf lamina was measured. Chlorophyll fluorescence parameters were measured in actual field conditions, providing the following light-adapted fluorescence parameters: minimal fluorescence (F_0') equaling the steady-state fluorescence in light (F'), maximal fluorescence in the light-adapted state (F_m'), and maximum efficiency of PS II photochemistry in the ambient light (F_v'/F_m'). The photosynthetic photon flux density (photosynthetically active radiation – PAR) was measured to evaluate site conditions. For the same leaves, the green color of the leaf was obtained (SPAD value) as a measure of chlorophyll content. The measurement was performed with the SPAD-502 chlorophyll meter (Konica – Minolta, Osaka, Japan). Five consecutive measurements were performed on the upper lamina of each selected leaf, and as the final value, an average, calculated by the device, was included in the analysis.

Statistical analysis

For the data analysis, the data was first explored regarding outliers, homogeneity, normality and possible interactions (Zuur et al. 2010). A general linear mixed model (GLMM) was used to analyze the data. The dependent variables were the green coloration of the leaf (SPAD-value), light-adapted minimum (F_0'), maximal (F_m') fluorescence and PSII maximum efficiency (F_v'/F_m'). The independent variables were the date of sampling and whether the leaves were infested or not. Every statistical analysis started with a full model where the variables were selected with a stepwise backwards approach. The model selection was done with the Akaike Information Criterion (Burnham and Anderson 2002).

Results

A difference was found in chlorophyll content (average 28.86), measured through the green color of the leaf, between infested and non-infested leaves over time (Fig 1a). In general, there was no difference between infested and non-infested leaves ($t=0.94$, $P=0.350$). The chlorophyll content dropped over the following two weeks compared to day 113 (day 120: $t=-4.37$, $P<0.001$; day 127: $t=-13.61$, $P<0.001$). However, there was an interaction found between the days and the infested leaves. Compared to day 113, the infested leaves had a lower chlorophyll content on day 120 compared to day 113 ($t=5.34$, $P<0.001$), and an even lower one on day 127 ($t=12.33$, $P<0.001$).

The initial fluorescence in ambient light conditions (F_0') was higher in non-infested leaves compared to infested leaves ($t=3.015657$, $P=3e-03$) (Fig 1b) and increased over time (120: $t=3.79$, $P<0.001$; day 127: $t=4.69$, $P<0.001$).

There was a difference found in maximal fluorescence (F_m) in the light-adapted state between infested and non-infested leaves over time (Fig 1c). In general, there was no difference between infested and non-infested leaves ($t=1.65$, $P=0.1018$). Over time, the maximal fluorescence dropped over the following two weeks compared to day 113 (day 120: $t=-2.73$, $P=0.0071$; day 127: $t=-8.24$, $P<0.001$). There was an interaction found between the days and the infested leaves. Compared to day 113, the infested leaves had an equal F_m on day 120 compared to day 113 ($t=1.48$, $P=0.1405$), and even lower on day 127 ($t=6.35$, $P<0.001$).

There was a difference found in PSII maximum efficiency between infested and non-infested leaves over time (Fig 1d). In general, there was no difference between infested and non-infested leaves ($t=1.91$, $P=0.0576$). Over time, the PSII maximum efficiency dropped over the following two weeks compared to day 113 (day 120: $t=-4.45$, $P=0.0000$; day 127: $t=-6.22$, $P<0.001$). There was an interaction found between the days and the infested leaves. Compared to day 113, the infested leaves had an equal PSII maximum efficiency on day 120 compared to day 113 ($t=1.92$, $P=0.0565$), and even lower on day 127 ($t=3.40$, $P<0.001$). A correlation was observed between the SPAD and the PSII maximum efficiency ($Rho=0.62$, $P<0.001$).

Discussion

The results showed that the mining behavior of the hoverfly larvae of *C. fasciata* accelerated the demise of the ramsons leaves. Additionally, we found a decrease in the amount of chlorophyll (Fig.1 a); there was also a decrease in the photosynthetic activity of the leaves. Chlorophyll *a* fluorescence parameters, which are sensitive to age and senescence (Desotgiu et al 2012), showed changes over time accordingly, but differentially. Initial fluorescence (F_0') remained constant over time and remained at the same level even when the leaves were infested; however, there was an indication of some small increase over time. Experiments have shown that under mild stress F_0' does not change, while under high stress, this parameter increases (Lichtenthaler et al. 2005). According to our measurements, infested and non-infested leaves experienced increased stress over time. Because there was no significant difference between the two leaf types, we cannot establish how much of the stress might be attributed to the leaf

miner damage and how much merely to senescence. The enhancement of F_0' reflects the damage level to the PSII (Jiao et al. 2003), which is also reflected by the decrease in the PSII maximum efficiency (F_v/F_m) in accounting for the operational photosynthesis. The decrease was detected over time in both leaf types (Fig.1 d), which coincides with decreasing PAR and simultaneous aging of the leaves. However, the F_v/F_m of infested leaves declined more severely, and measured values had greater variability, with some values below 0.6, figures which were never measured in non-infested leaves. As with the decrease in chlorophyll content, the results of PSII maximum efficiency indicate greater senescence of infested leaves, as well. What is more, a significant correlation was observed between the chlorophyll content and the operational photosynthesis, in the given environmental conditions. In *A. ursinum* the decrease in operational photosynthesis was observed for the whole non-injured portion of the infected leaf, not only for the mines, which is often not the case for tree leaves (Raimondo et al. 2003; Lombardini et al. 2013).

The onset of senescence would have differential effects, as discussed below, which are negative for the plant if the larvae have already left the mine, or negative for the leaf miner if the larva is still developing inside the leaf. The physiological effects of leaf mining on plants was mostly studied on insects that consume the leaf mesophyll of tree leaves (Wagner et al. 2008). Herbaceous species are rarely considered, especially those with short-lived leaves like spring geophytes. Leaf mining of *C. fasciata* had an effect detectable by measurement of the greenness of the *A. ursinum* leaf. and as expected, there was no indication that *A. ursinum* leaves could compensate for leafmining with increased photosynthesis of the infested leaf. According to observations in the field, not all plant leaves are infected with the leaf miner (de Groot and Kogoj 2015), thus reassuring that plants will photosynthesize and still allocate some nutrients to the bulbs for survival until the next year.

However, faster onset of senescence could go beyond the damage inflicted by leaf miners and might represent a response by the plant as well. The chlorophyll content of infested and non-infested leaves declined over time; however, infested leaves had lower values and started senescing earlier. We believe that the reduction in PSII maximum efficiency of *A. ursinum* infected leaves, indicated by loss of chlorophyll as well, could be strong enough to affect the leaf miner if still present in the leaf. We could hypothesize that rushing the senescence of a leaf, which is destined to wither soon anyway, could be an adaptation to starve the leaf miner, or at least, to slow its development. A similar hypothesis was proposed for tree species (Owen 1978); however, it was not sufficiently supported by data, since trees exhibit a variety of leaves depending on leaf age and heliomorphosis, which influence the leaf fall that often happens too late to have an effect on leafminer larvae survival (Pritchard and James 1984). Leaves of herbaceous vernal species in the forest understory differ significantly from tree leaves in morphological features, chemical composition, phenology and longevity. Additionally, short-lived leaves enable univoltine miner species. Therefore, for univoltine *C. fasciata* larvae, rapid development would be beneficial, since the infected *A. ursinum* leaves wither more rapidly than healthy leaves; however, further research is needed to confirm this hypothesis. We could elaborate this hypothesis further by stating that the herbaceous vernal species *A. ursinum* might profit by early senescing infected leaves and in such way that at least part of the nutrients can be still translocated to the bulb and not lost to the leaf miner.

In conclusion, the reaction of plants to the leafminer is withering of the leaves, and a decrease in photosynthesis and chlorophyll quantity. As established, this strategy to decrease photosynthesis could have a strong negative effect for both plant and leafminer, for the plant by decreasing energy storage in the bulb and therefore potentially decreasing growth in the following year, and for the leafminer by potential resource limitation. In order to better understand the pros and cons of this strategy, more research should be done into the survival of the leafminer and the regeneration of the plants.

Declarations

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Conflicts of interest/Competing interests (include appropriate disclosures): We declare no conflict of interest.

Availability of data and material: Data is available on demand.

Code availability: Freely available software R was used with general code described in the Methods section and the software is included in the Reference list.

Authors' contributions: NS designed the study, supervised the field work and co-wrote the manuscript, TŠ performed the field work, MG performed the statistical analyses and co-wrote the manuscript.

Ethics approval (include appropriate approvals or waivers): We declare the presented manuscript reports about our own and original results. The manuscript is not submitted, considered for publication or published (neither partly nor fully) in any other journal. Work of others is properly acknowledged and included in the reference list. No copyrighted material is used. All measurements were performed *in situ in vivo* on plant leaves. No animals were collected. No species or habitat under study is protected.

Consent to participate

All listed authors contributed sufficiently to be listed as authors and agree to be included as authors.

Consent for publication

All listed authors have approved the manuscript before submission, including the names and order of authors.

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Figures

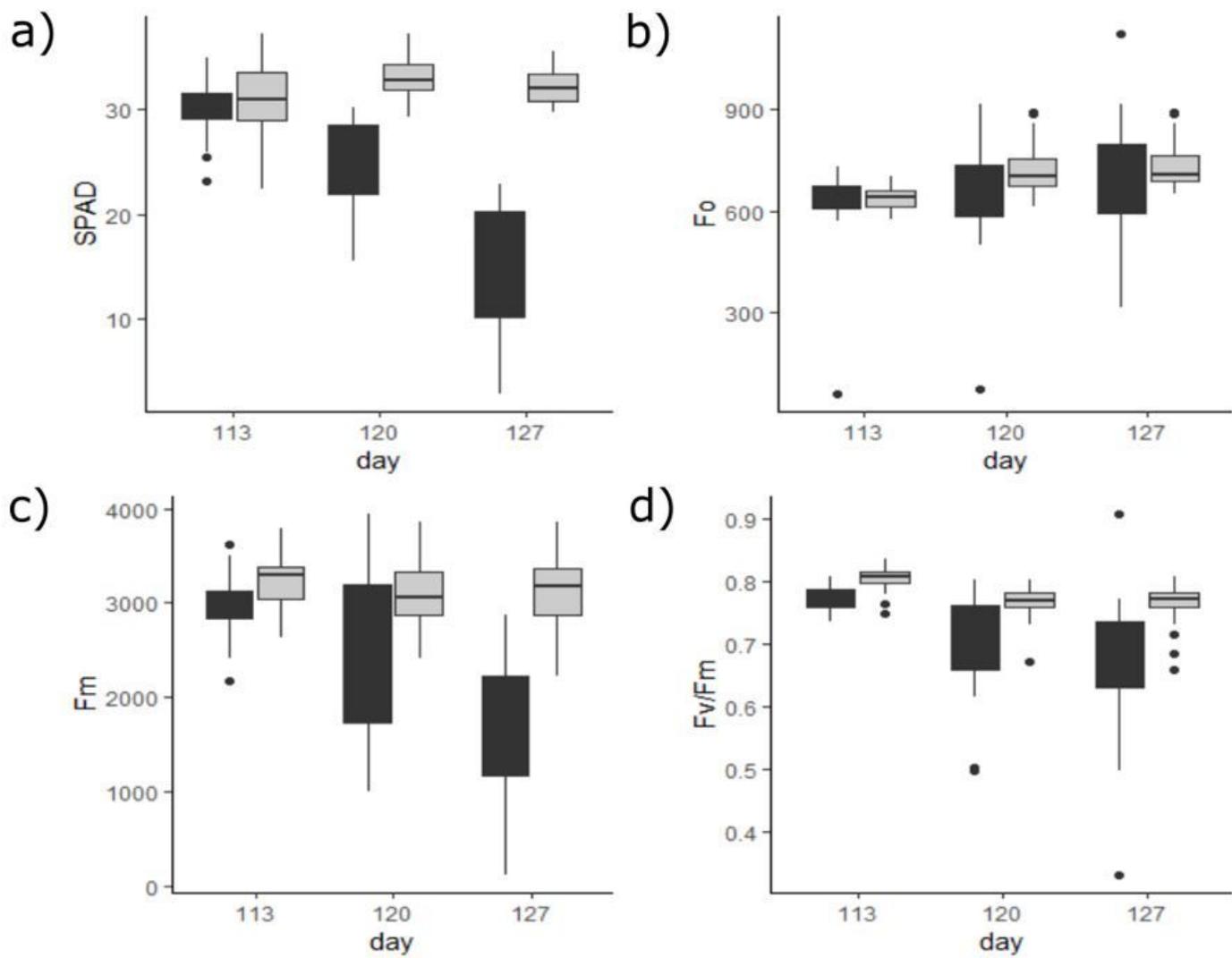


Figure 1

Effect of insect herbivory on different physiological attributes on the leaves of *Alium ursinum*: a) SPAD, b) F₀, c) F_m, d) F_v/F_m