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Herbivory mitigation through increased water-use efficiency in a leaf-mining moth–apple tree relationship

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ABSTRACT

Herbivory alters plant gas exchange but the effects depend on the type of leaf damage. In contrast to ectophagous insects, leaf miners, by living inside the leaf tissues, do not affect the integrity of the leaf surface. Thus, the effect of leaf miners on CO_2 uptake and water-use efficiency by leaves remains unclear. We explored the impacts of the leaf-mining moth *Phyllonorycter blancardella* (Lepidoptera: Gracillariidae) on light responses of the apple leaf gas exchanges to determine the balance between the negative effects of reduced photosynthesis and potential positive impacts of increased water-use efficiency (WUE). Gas exchange in intact and mined leaf tissues was measured using an infrared gas analyser. The maximal assimilation rate was slightly reduced but the light response of net photosynthesis was not affected in mined leaf tissues. The transpiration rate was far more affected than the assimilation rate in the mine integument as a result of stomatal closure from moderate to high irradiance level. The WUE was about 200% higher in the mined leaf tissues than in intact leaf portions. Our results illustrate a novel mechanism by which plants might minimize losses from herbivore attacks; via trade-offs between the negative impacts on photosynthesis and the positive effects of increased WUE.

Key-words: *Phyllonorycter blancardella*; insect plant interactions; intercellular CO_2 conductance; leaf miner; photosynthesis; stomatal conductance; transpiration.

INTRODUCTION

Stomatal behaviour is of great interest to plant physiologists and ecologists because the stomata play a central role in transpiration and photosynthesis. Stomata provide the main short-term control of all gas exchanges (CO_2, water vapour, O_2) taking place at the plant–atmosphere interface (Jones 1998). Plants constantly face a trade-off between stomatal closure, which prevents water loss, and stomatal opening, which favours CO_2 uptake for fixation in leaf tissues (Ziegler 1987). Moreover, the opening level of the stomata directly affects the leaf temperature (Nobel 1999). While many studies have explored the impact of abiotic factors such as irradiance and atmospheric CO_2 concentration on transpiration and photosynthesis at the leaf scale (Jarvis 1976; Willmer & Fricker 1996; Campbell & Norman 1998), comparatively much less is known on the mechanisms by which herbivores alter the unbalance between water loss and CO_2 assimilation. Thus, the effects of herbivory on leaf physiology depend on the environmental conditions and on the type of damage, that is, the feeding guild of the herbivore (Welter 1989; Macedo *et al*. 2005).

Studies reporting the impacts of herbivory on photosynthesis mainly dealt with ectophagous insects. The effects are highly variable and depend on the exact insect–plant interaction. Most of the time, the loss of photosynthetic tissues following feeding induces an increase in photosynthetic rate per unit area in the remaining leaf tissues, allowing the plant to compensate partially for herbivory (Welter 1989). In other cases, herbivory has been found to induce a decrease in assimilation rate in the remaining leaf tissues. The area showing a reduced photosynthesis can be up to threefold larger than the area removed by the insect (Zangerl *et al*. 2002). Large reductions in photosynthesis were also measured on leaves attacked by spider mites, which are mesophyll feeders (Welter 1989; Haile & Higley 2003). In apple trees, the European red mite reduces both leaf and whole-canopy net CO_2 exchange rates (Francesconi *et al*. 1996).

The effects of herbivory on stomatal conductance and transpiration rate have received much less attention than its impacts on photosynthesis. Recently, the study reported by Aldea *et al*. (2005) showed that injuries from ectophagous insects induce a large increase in water loss through the perimeter of the damaged tissues. Both net photosynthesis and stomatal conductance in the remaining leaf tissues were not affected. By contrast, Tang *et al*. (2006) indicated that both water stress, induced by the increased rate of water loss near the damaged tissues, and the reduced stomatal conductance in the tissues away from the injuries contributed to the inhibition of photosynthesis in the remaining leaf tissues. The general conclusion that can be

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drawn from such studies is that either assimilation and transpiration rates are affected in a similar way or photosynthesis is reduced while water loss is increasing. In the first case water-use efficiency (WUE) (i.e. ratio between the amount of CO_2 assimilated and amount of water loss) is reduced, and in the second case it remains at best constant if the plant compensates for the loss of tissues. Full compensation is, however, rather rare (Welter 1989).

Endophagous insects such as leaf miners do not disturb the integrity of the leaf surface. Leaf-mining herbivores are living within the leaf tissues by creating a structure called a mine. Water can be lost only through the stomata or the epidermis of the mine as well as in intact leaf tissues. The few studies reporting the impact of leaf mining on leaf gas exchanges indicate that photosynthesis is reduced (Johnson *et al*. 1983; Welter 1989; Whittaker 1994; Schaffer *et al*. 1997; Raimondo *et al*. 2003). Raimondo and colleagues (2003) found that the reduction in leaf photosynthesis equals the portion of the leaf surface which is actually mined. They also report a strong decrease in stomatal conductance in mined leaves. These authors suggested therefore that the actual damage of leaf mining on leaf physiology might be less than previously estimated. Li, Nyrop & Lakso (2003) detected no effects on leaf and canopy photosynthesis when tissues mined by the spotted tentiform leaf miner correspond to less than 10% of leaf areas. These results suggest that leaf mining causes less damages in terms of plant gas exchange than ectophagous herbivores do.

Besides the biochemical control of plant gas exchanges, the rate of leaf transpiration and photosynthesis is limited by the resistance pathways including the stomata and internal space (Nobel 1999). The stomatal pores are the only way of communication between ambient air and the leaf atmosphere. Stomata were suggested to be still functional in the lower mine integument (Whittaker 1994; Raimondo *et al*. 2003). They might therefore be able to react to the leaf miner presence and/or activity. Leaf-mining larvae release significant amount of respiratory CO_2 within a mine (Pincebourde & Casas 2006), and the opening level of the stomata might be adjusted as a function of this internal CO_2 source. Moreover, leaf miners profoundly alter the internal structure of the leaf by feeding on mesophyll tissues, and might therefore affect the internal resistance for CO_2 diffusion from the substomatal cavity to mesophyll cells. The response of the stomata to these factors may be a way for a leaf to minimize the negative impacts of herbivory.

Our objective was to determine precisely the balance between the negative and potential positive impacts of leaf mining on gas exchanges, through the effects on photosynthesis and WUE. We explored the effects of the spotted tentiform leaf miner, *Phyllonorycter blancardella* F. (Lepidoptera: Gracillariidae), on the stomatal conductance, transpiration and photosynthetic responses of apple leaves (*Malus domestica* Borkh) to changes in leaf irradiance. We measured the gas exchange occurring in the integument of both mines occupied by a larva and empty mines in order to dissociate the effects resulting from the mine structure and larval presence. Then we quantified the intercellular air space conductance for CO_2 within mines to test whether the leaf miner facilitates the diffusion of CO_2 through the mine. We also measured the amount of CO_2 released by larvae within its mine to be compared with the assimilation rate of leaf tissues remaining within a mine. We calculated the WUE of mined leaf tissues, allowing us to estimate the net result of the effects of herbivory on all gas exchanges.

MATERIALS AND METHODS

Organisms and study system

The study was conducted in September 2004 on a set of 52 1-year-old apple seedlings (*M. domestica*), on which several mature leaves were infested with *P. blancardella* miners. Seedlings were grown within a greenhouse under temperate conditions [daily natural variations: air temperature, T_{air} = 15–29 °C; relative humidity, RH = 40–85%; and photosynthetically active radiation (PAR) irradiance, Q, up to 1600 μmol m^{-2} s^{-1}] and were watered daily with a commercial nutritive solution. As leaf age affects stomatal conductance (Field 1987; Jones 1992), the emergence date of each leaf was recorded and leaves of the same age were chosen throughout the study (mean ± SD, 32.1 ± 2.5 d).

P. blancardella is a microlepidopteran for which the larval development is divided into five stages. During the first three stages, larvae are sap feeders. During the fourth and fifth stages, larvae are tissue feeders and their feeding behaviour causes the formation of feeding windows on the upper leaf side (Pottinger & LeRoux 1971; Djemai, Meyhöfer & Casas 2000; Pincebourde & Casas 2006). Feeding windows are translucent patches remaining after chlorophyll-containing tissues have been consumed (Fig. 1a). The lower mine integument is a white, sometimes greenish, and very thin leaf tissue (Fig. 1a). By contrast to other leaf-miner species, the upper and lower mine integuments are not suberized. The mine also looks like a protuberance at the upper leaf surface. The height of this protuberance is about 10 times higher than the leaf thickness, leading to a large air space within the mine as compared with leaf thickness (Fig. 1b).

In this study, the physiological measurements were performed on leaves containing one or two mines of the last stage. Empty mines were obtained after an ectoparasitoid larva *Pnigalio pectinicornis* (Hymenoptera: Eulophidae) had fed the leaf miner and left the mine through a small circular hole (0.5 mm in diameter) in the lower mine integument (Pottinger & LeRoux 1971).

Leaf and mine gas exchanges

Leaf gas exchanges were measured with an infrared gas analyser equipped with a 2 × 3 cm leaf chamber system (LI-6400, Li-Cor Inc., Lincoln, NE, USA). Stomatal conductance for water vapour (g_s: mol m^{-2} s^{-1}), transpiration rate (Tr: mmol m^{-2} s^{-1}) and net CO_2 assimilation rate

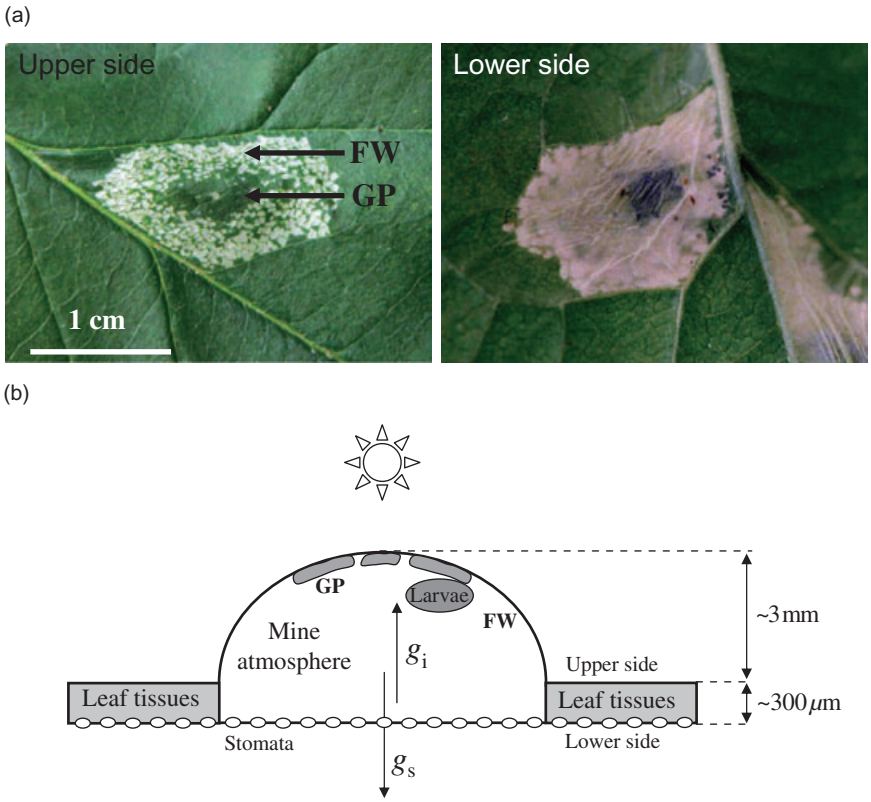

Figure 1. (a) The upper (left) and lower (right) surfaces of a mine (× 2.8). The feeding windows (FW) result from the feeding activity of the larva within the mine, whereas green patches (GP) correspond to the chlorophyll-containing tissues remaining in the mine. A single mine always contains only one larva. The upper mine surface (or integument) is therefore made from both feeding windows and palisade cells (green patches). The lower mine integument corresponds to the lower, thin-translucent leaf epidermis containing stomata. The dark area in the middle of the mine corresponds to the larval dejections. The upper and the lower mine integuments are not suberized. (b) A schematic cross section of a mine, indicating that the mine protuberance height is higher than leaf thickness, creating a very large air space inside the leaf tissues. Legend: g_s, stomatal conductance for water vapour; g_i, internal (intercellular) air space conductance for CO_2 diffusion.

(A: μmol m^{-2} s^{-1}) were calculated by the LI-6400 data analysis program using the general formula of von Caemmerer & Farquhar (1981). Measurements were performed on three categories of fully exposed apple leaves: intact leaves, leaves with occupied mines and leaves with empty mines. A thin layer of vegetal oil was used to coat the intact lower face of the leaves to measure gas exchange occurring only in the mine integument (empty and occupied mines). Transpiration rate in intact leaves having both lower and upper surfaces fully coated was null (mean ± SD = –0.018 ± 0.034 mmol m^{-2} s^{-1}, n = 10). In the case of empty mines, the small hole made by the parasitoid in the lower mine integument was filled with a droplet of vegetal oil.

For each category, g_s, Tr and A were measured at nine irradiance levels, from 1500 down to 0 μmol m^{-2} s^{-1} under standard conditions (ambient CO_2 concentration 35 Pa, leaf temperature 25 °C and leaf water vapour pressure deficit 1 kPa). Leaf irradiance was controlled by varying the distance between the sample and a 250 W metal iodide bulb (City Plante, Tours, France). Leaves were allowed to equilibrate at a given irradiance level for 20–30 min before any measurements were taken and data were discarded if stomatal conductance was not stable after 45 min. Stomatal conductance, transpiration rate and net assimilation rate were expressed as a quantity per unit of transpiring leaf area (i.e. per unit of area uncovered by vegetal oil). Gas exchange in mines was compared with that in intact leaf tissues having 2 cm^2 free of vegetal oil. Preliminary experiments showed that the size of the uncovered intact leaf area (patches of 0, 1, 2 and 4 cm^2) does not affect stomatal conductance, transpiration rate and net assimilation rate when expressed per unit transpiring area (Pincebourde 2005).

In the case of mines, net assimilation rate was also expressed per unit of photosynthetically active surface (i.e. surface occupied by green patches), which is lower than the mine surface. For this purpose, we measured the mine surface occupied by green patches. Leaves with empty and occupied mines were sampled after gas exchange measurements and numerical images of the mine surfaces were obtained using a scanner. Scans were analysed using the Scion Image software (Scion Corporation, Frederick, MD, USA), which quantified the portion of the total mine surface occupied by green patches and feeding windows.

We also verified that the conductance for water vapour of the upper integument (which does not contain stomata) of mines is not significantly increased in comparison with intact leaf tissues. Mean conductance was 0.009 ± 0.010 and 0.003 ± 0.003 mol m^{-2} s^{-1} in mines (n = 10) and intact leaves (n = 10), respectively, corresponding to less than 8% of maximal conductance in the two cases.

WUE

The WUE value indicates the number of CO_2 moles assimilated by leaf tissues per 100 H_2O moles lost. The WUE was calculated from WUE = (A/Tr) × 100, where both A and Tr are expressed in mol m^{-2} s^{-1}. We extrapolated the WUE calculation to the whole leaf surface for a typical apple leaf (surface 30 cm^2) having 1 or 20 mines. We simplified the extrapolation by assuming that the assimilation and

transpiration rates are homogeneous over the whole intact leaf surface and over the whole mined leaf surface. We estimated the WUE at the whole leaf scale by summing the total amounts of water transpired and CO_2 assimilated by both the intact leaf surface and the mined leaf surface containing feeding windows and green patches.

Intercellular air space CO_2 conductance of leaves and mines

The intercellular air space conductance to CO_2 diffusion (g_i) of mines and intact leaves was estimated according to Syvertsen *et al*. (1995) and Piel *et al*. (2002) as

$$g_i = \frac{\left(\frac{f_{ias}}{1-\varepsilon}\right)^{1.55}}{\alpha L(1-\varepsilon)}, \quad (1)$$

where L is the leaf thickness (mm), ε is the fraction of leaf thickness occupied by epidermis, f_{ias} is the fraction of total leaf or mine volume occupied by air space, and α is a fitted constant (= 1.63 m s μmol^{-1}, see Syvertsen *et al*. 1995). The term $f_{ias}/(1-\varepsilon)$ corresponds to the fraction of mesophyll volume occupied by intercellular space. The 1.55 power accounts for the tortuosity in the diffusion pathway (Ball 1981). Leaf thickness and the fraction occupied by epidermis were measured using optical microscopy with hand-made leaf sections on 10 leaves from five different seedlings. The mean leaf thickness L was 267 ± 25 μm and the mean fraction of leaf thickness occupied by epidermis ε was 10 ± 2%.

The fraction of mine (containing a larva) and intact leaf volume occupied by air space were estimated according to Piel *et al*. (2002) by vacuum infiltration using a solution of distilled water and 0.1% Triton X-100 (Sigma Chemicals, Perth, WA, Australia). Ten leaf discs of about 1 cm^2 and 10 leaf-mined sectors were collected. Each disc and mine was individually weighed (Supermicro, Sartorius, Richmond, UK) immediately before and after infiltration. The intercellular air space fraction was calculated from

$$f_{ias} = 1 - \frac{FW}{IW}, \quad (2)$$

where FW is the fresh weight before infiltration and IW is the weight after infiltration.

Amount of leaf tissues removed at the end of larval development

The mine surface occupied by feeding windows and green patches at the end of the larval development was measured on 23 mines. A scaled digital image of the mine surface was taken during the nymphal stage using a camera (Super Dynamic WV-GP4560; Panasonic, Matthews, NC, USA). Images were treated using the Scion Image software to calculate the total mine surface and the surface made of green patches.

Insect respiration

The amount of CO_2 released by larvae was measured using an LI-6400 infrared gas analyser-leaf chamber system at 10 PAR irradiance levels ranging from 1500 to 0 μmol m^{-2} s^{-1}. A slow flow rate (52 μmol s^{-1}) was used to improve the signal-to-noise ratio of the system. However, as respiration of a single larva was too low to be measured individually, a group of 10 larvae was used for each measurement. Mines were excised and living larvae were collected and placed into the LI-6400 leaf chamber in individual perforated open plastic capsules (9 mm in length and 6 mm in diameter) mimicking mine conditions. The RH was 80–90% and T within capsules was 25 °C. Larvae were not directly irradiated; the upper mine integuments containing the feeding windows were cut out, dried (to ensure that they do not respire anymore) and placed above each capsule with the external surface facing the light source. The irradiance level was altered by changing the distance between the sample and the 250 W metal iodide bulb as before. The amount of CO_2 released by larvae (mol m^{-2} s^{-1}) was measured on four groups of 10 larvae, and for each group 10 measurements were taken every 10 s at each irradiance level after 20 min of acclimation to chamber conditions. Dimensions of each larva (length and diameter) were measured and areas were estimated by considering their bodies to be a cylinder in order to quantify the gas exchange surface of the insect. Knowing the relative surfaces of the green patch areas and larval body, the respiration rate was converted in μmol per unit of green patch area to be compared with the assimilation rate of leaf tissues.

Statistical analysis

Means were provided with their SD. Maximum values of stomatal conductance, transpiration rate and net assimilation rate were compared using the Mann–Whitney statistical test. The stomatal conductance and assimilation rate responses to varying irradiance were analysed using non-linear models under TableCurve 2D (SYSTAT Software Inc., Richmond, CA, USA). To test whether these non-linear models fitted the data adequately, we performed a least squares analysis (Johnson & Omland 2004) and the residuals were controlled for their uniform distribution. F-statistic analysis was used to test for differences between two datasets by comparing entire curves having the same equation. Probabilities were compared to a threshold value (α) of 0.05.

RESULTS

Mined areas

The mine surface occupied by feeding windows at the end of larval development, that is, the total surface fed by a larva, was positively correlated to the whole surface of the mine (linear regression: $R^2 = 0.64$, $P < 0.001$) (Fig. 2). The linear relationship indicates that a larva fed only 47 ± 8% on average of the total surface of tissues available within a mine, whatever the mined surface.

The mean total area of occupied and empty mines, on which gas exchange measurements were made,

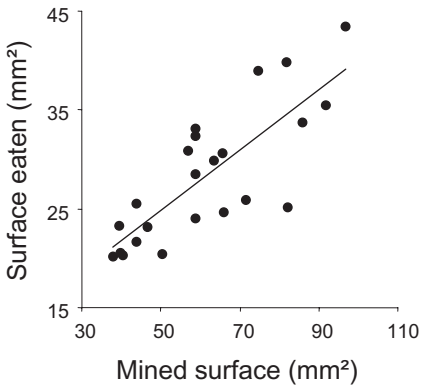

Figure 2. Surface occupied by feeding windows (i.e. surface eaten) at the end of larval development as a function of the total mined surface.

was 1.13 ± 0.17 cm^2 and 0.89 ± 0.17 cm^2, respectively, with 0.74 ± 0.23 cm^2 and 0.71 ± 0.18 cm^2 being green patches (Fig. 3). The portion of mined surface made from green patches is larger in empty mines ($80 \pm 10\%$) than in occupied mines ($64 \pm 12\%$) because the parasitoid wasp had killed the leaf miner before it ate most chlorophyll-containing tissues.

Miner effects on intercellular air space conductance and maximal levels of gas exchanges

The volume fraction of leaf tissues occupied by air space was increased by a factor two in mines containing a larva as compared with intact leaf tissues (Table 1). This difference led to a threefold higher intercellular air space CO_2 conductance in mines compared with intact leaf tissues (Table 1). The intercellular conductance measured on occupied mines also applies to empty mines as the two categories have the same morphology.

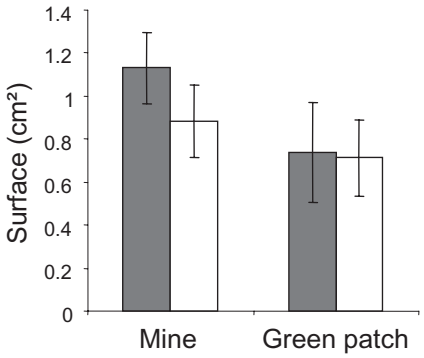

Figure 3. Mean mined surface and mean surface occupied by green patches in occupied (grey bars) and empty mines (white bars) used during the gas exchange measurements.

Table 1. The intercellular air space

	Intact leaf	Occupied mine
f_{ias}	0.38 ± 0.08a	0.75 ± 0.03b
g_i (mol m^{-2} s^{-1})	0.43 ± 0.14a	1.21 ± 0.07b

The table shows the mean volume fraction of leaf thickness occupied by air space (f_{ias}) and the mean intercellular air space CO_2 conductance (g_i) of intact leaf tissues and occupied mines. Significant differences between means in a row are indicated by letters (Mann–Whitney test, $\alpha = 0.05$).

The maximal stomatal conductance and transpiration rate were strongly reduced in occupied and empty mines as compared with intact leaves (Table 2). Occupied mines exhibited significantly lower assimilation values than intact leaves and empty mines when expressed per unit total mined surface (Table 2). However, the maximal net assimilation rate of occupied mines expressed per unit green patch area was not significantly different from intact leaves (Table 2).

Miner effects on light responses of stomatal conductance, transpiration and photosynthesis

For intact leaves, the stomatal conductance for water vapour and the transpiration rate increased over the irradiance range tested (Fig. 4). By contrast, stomatal conductance and transpiration rate reached a maximal value at a mean irradiance level 571 ± 156 μmol PAR m^{-2} s^{-1} in empty and occupied mine integuments. Occupied mines always exhibited significantly lower stomatal conductance and transpiration rate than empty mines (stomatal conductance: $F_{1,63} = 24.8$, $P < 0.001$; transpiration rate: $F_{1,63} = 17.2$, $P < 0.001$) (Fig. 4). At high irradiance, stomatal conductance of occupied mines represented only 19% of mean maximal stomatal conductance of intact leaves. In empty mines, slightly higher values in comparison with intact leaves were measured at low irradiance (0–300 μmol PAR m^{-2} s^{-1}), thereafter at high irradiance stomatal conductance decreased to about 40% of that of intact

Table 2. Mean maximal stomatal conductance (g_s), transpiration rate (Tr) and assimilation rate of intact apple leaves, empty mines and occupied mines obtained during the light response measurements

	Intact leaf tissue	Empty mines	Occupied mines
g_s (mol m^{-2} s^{-1})	0.348 ± 0.034a	0.198 ± 0.034b	0.134 ± 0.038b
Tr (mmol m^{-2} s^{-1})	3.073 ± 0.350a	2.006 ± 0.330b	1.427 ± 0.493b
A (μmol m^{-2} s^{-1})	9.135 ± 1.684a	7.801 ± 0.474a	6.155 ± 0.639b
$A_{green\ patch}$ (μmol m^{-2} s^{-1})	(9.135 ± 1.684)a	9.009 ± 0.856a	7.889 ± 1.474a

Net assimilation rate for occupied and empty mines was expressed per unit mine area (A) as well as per unit green patch area ($A_{green\ patch}$). Significant differences between means in a row are indicated by letters (Mann–Whitney test, $\alpha = 0.05$).

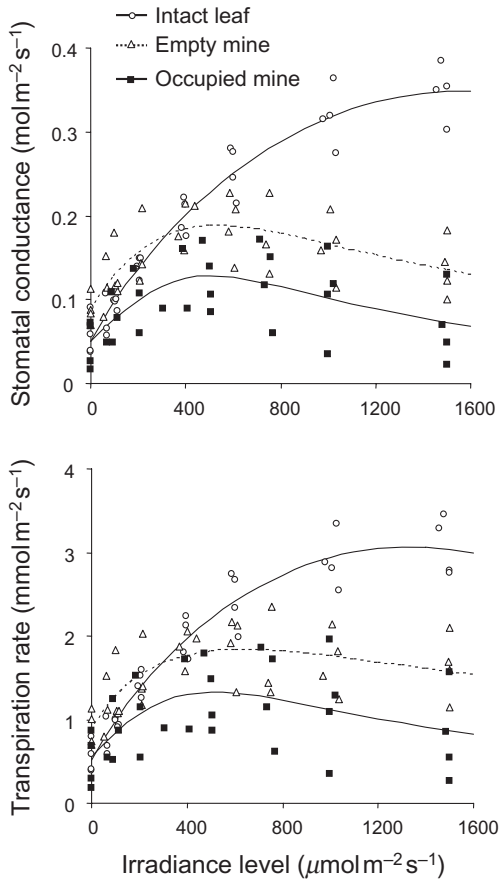

Figure 4. Stomatal conductance and transpiration rate responses to irradiance level in intact leaf tissues, occupied mines and empty mines. Non-linear models for stomatal conductance responses: intact leaf, $F_{2,28} = 277.5$, $P < 0.001$; empty mine, $F_{3,30} = 11.5$, $P < 0.001$; occupied mine, $F_{3,27} = 5.2$, $P = 0.006$. Non-linear models for transpiration rate responses: intact leaf, $F_{2,28} = 219.6$, $P < 0.001$; empty mine, $F_{3,30} = 9.7$, $P < 0.001$; occupied mine, $F_{3,27} = 3.4$, $P = 0.031$.

leaves (Fig. 4). A similar pattern was observed for the transpiration rates (Fig. 4).

Net assimilation rates of intact leaves, empty mines and occupied mines increased non-linearly with irradiance up to the saturation level (Fig. 5a). At high irradiance, empty mines exhibited slightly lower assimilation values than intact leaves but the shape of the response curve of empty mines and intact leaves were not statistically different ($F_{1,63} = 0.3$, $P = 0.61$). The shape of the net assimilation rate response curve of occupied mines was not different from that of empty mines ($F_{1,63} = 1.1$, $P = 0.30$), despite the maximal values differed significantly (Fig. 5a). The shape of the net assimilation rate response expressed per unit green patch surface in occupied mines was similar to that of empty mines and intact leaf tissues ($F_{1,63} = 2.3$, $P = 0.10$) (Fig. 5b).

Miner effects on WUE

The net assimilation rate was linearly correlated to the transpiration rate in intact leaf tissues (linear regression: $R^2 = 0.85$, $P < 0.0001$) (Fig. 6). The relationship between the transpiration rate of the mine integument and the net assimilation rate of the photosynthetically active surface was only weakly significant in empty mines (linear regression: $R^2 = 0.19$, $P = 0.02$) (Fig. 6). However, the net assimilation rate of green patch surface was not correlated to the transpiration rate in occupied mines (linear regression: $P = 0.38$) (Fig. 6). Indeed, the highest values of net assimilation rate in mine integuments were obtained at lower transpiration rate values than in intact leaf tissues (Fig. 6). This suggests that the photosynthetically active tissues remaining in the mines are able to maintain the net assimilation rate while water losses are considerably lowered.

The mean WUE at high irradiance level (Q = 1500 μmol PAR m^{-2} s^{-1}) in empty mines was about 66% higher than that in intact leaf tissues (Mann–Whitney test: $P = 0.02$) (Fig. 7). Moreover, the mean WUE of occupied mines at high irradiance level was increased by 78% when compared with that of empty mines (Mann–Whitney test:

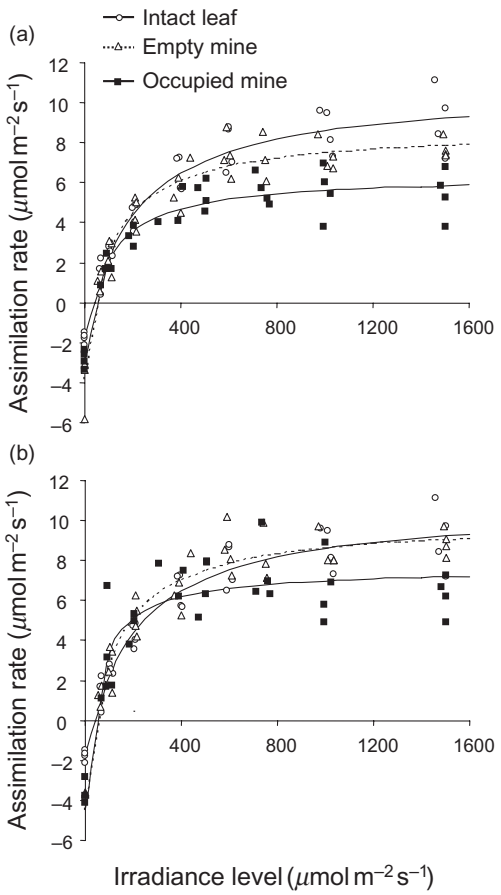

Figure 5. Assimilation rate response to irradiance level in intact leaf tissues, occupied mines and empty mines. Assimilation rate of mined tissues was expressed (a) per unit total mine surface and (b) per unit photosynthetically active tissues (green patches). Non-linear model for intact leaf: $F_{2,28} = 242.3$, $P < 0.001$. Non-linear models for assimilation rate expressed per unit mine surface and per unit green patch surface, respectively: empty mine, $F_{3,30} = 283.4$, $P < 0.001$ and $F_{3,30} = 281.4$, $P < 0.001$; occupied mine, $F_{3,27} = 183.5$, $P < 0.001$ and $F_{3,27} = 89.8$, $P < 0.001$.

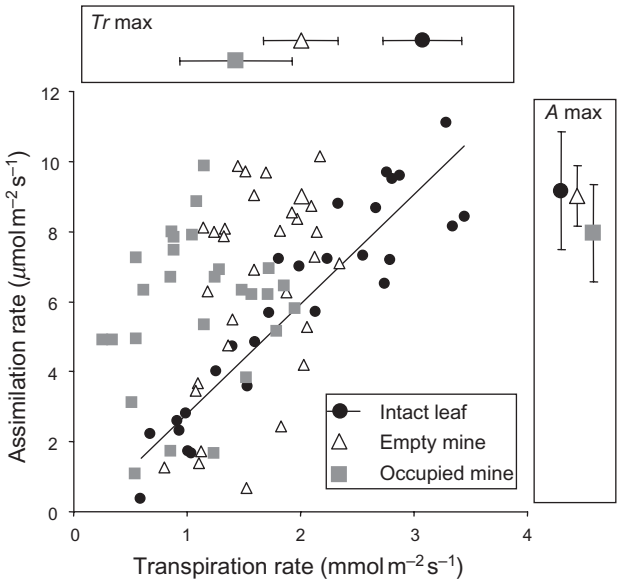

Figure 6. Relationship between the assimilation rate of photosynthetically active tissues and transpiration rate in intact leaf tissues, occupied mines and empty mines. The enlarged symbols within boxes are the mean maximal assimilation rate *A* (right box) and maximal transpiration rate *Tr* (upper box) for the three categories and indicate that the green patches in mines maintain a high assimilation rate but at a lower transpiration rate than in intact leaf tissues.

$P = 0.02$) (Fig. 7). This indicates that the maximal WUE in occupied mines is significantly increased by 196% as compared with intact leaf tissues (Mann–Whitney test: $P < 0.005$).

By extrapolating the results to the whole leaf scale, we found that the presence of a single mine on a typical apple leaf only weakly affected the leaf photosynthesis and that the impact on leaf WUE tended to positive (Table 3).

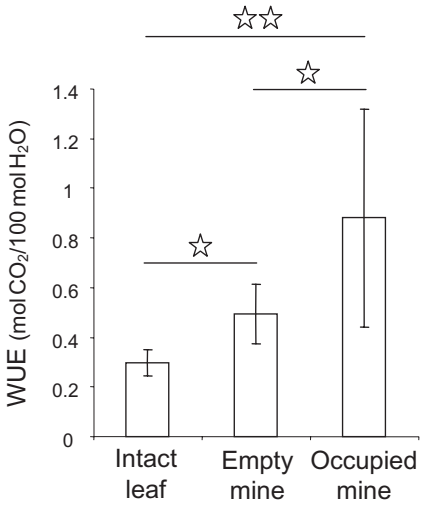

Figure 7. Mean water-use efficiency (WUE) (mol CO_2/100 mol H_2O) at irradiance level 1500 μmol m^{-2} s^{-1} in intact leaf tissues, occupied mines and empty mines. Stars indicate the level of statistical significance (one star: $P < 0.05$; two stars: $P < 0.005$).

Table 3. The balance between photosynthetic and water loss at high irradiance level ($Q = 1500$ μmol m^{-2} s^{-1})

	% reduction or increase at $Q = 1500$ μmol m^{-2} s^{-1}		
	Measured at mine scale	Predicted at whole leaf scale	
		1 mine	20 mines
Net assimilation rate	−32.6	−1.2	−24.6
Transpiration rate	−53.6	−2.1	−42.9
WUE	+196.0	+2.9	+34.5

The reduction in net assimilation rate and transpiration rate in a single mine (mine scale) were calculated from values in Table 1. The reduction or increase (in comparison to intact leaf tissues) in net assimilation rate, transpiration rate and water-use efficiency (WUE) were extrapolated for a typical apple leaf (area 30 cm^2, whole leaf scale) containing (1) a single mine and (2) 20 mines, assuming that gas exchange in the intact leaf tissues adjacent to the mines are not altered (but see Proctor *et al*. 1982 and Raimondo *et al*. 2003).

However, the presence of 20 mines on the leaf surface induced the assimilation rate to be one quarter lower than that of the intact leaf, and the transpiration rate was so much lowered that the leaf WUE was increased by about 35% (Table 3).

Insect CO_2 release in response to increasing irradiance

A positive linear relationship was observed between larval respiration and irradiance at a constant air temperature around larval body (Fig. 8). The amount of CO_2 released by a larva increased from about 0.4 μmol m^{-2} s^{-1} in the dark, to 1.8 μmol m^{-2} s^{-1} at 1500 μmol m^{-2} s^{-1} (these values are expressed per unit of green patch area). Larval respiration rate corresponds therefore to up to 23% of maximal assimilation rate of green patches in occupied mines.

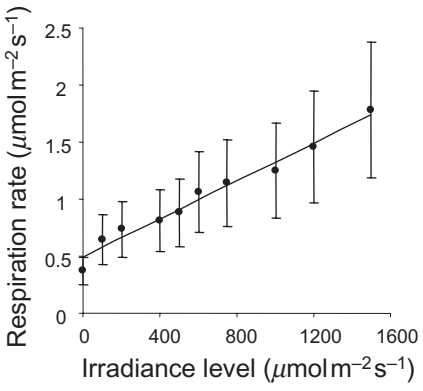

Figure 8. Mean respiration rate of larvae as a function of the irradiance level above the mine surface. The respiration rate of larvae was expressed in μmol CO_2 per unit of green patch area per second. Each dot represents the mean larval respiration rate over the four groups of 10 larvae each.

DISCUSSION

Leaf mining alters photosynthesis rate

The feeding activity of the leaf-mining moth *P. blancardella* leads to a 33% lower maximal net assimilation rate in occupied mines as compared with intact leaves. The impact of the leaf miner on plant physiology is only local, that is at the mine location (Proctor *et al*. 1982; Raimondo *et al*. 2003). However, these impacts, when integrated over the leaf surface, can have significant consequences to the physiological performance of the plant, but only when many mines are present. The loss of photosynthetic activity is related to the mine area, which is actually eaten up as the photosynthetic activity of green patches is not significantly altered. This result implies that the impact of folivory on photosynthesis equals the sum of its holes (feeding windows). Moreover, the negative impact on the leaf assimilation rate is less than the total mined (i.e. attacked) surface because some photosynthetically active tissues remain within the mines. About half of the mine surface is still made of green patches at the end of larval development. This portion might vary according to the nutritional quality of leaf tissues. By contrast, Raimondo *et al*. (2003) found that the reduction in assimilation rate matched the total mined surface in a leaf miner (*Cameraria ohridella*), which did not create green patches. Therefore, the loss of photosynthetic activity was generally strictly proportional to the area fed by the insect in the leaf-mining guild. From this conclusion we predict that the presence of only one mine of *P. blancardella* would lead to a very weak decrease in the whole apple leaf assimilation rate, whereas 20 mines would cause a decrease of about 25%. These extrapolations are consistent with the measurements reported elsewhere. Proctor *et al*. (1982) recorded a decrease of only 23% in leaf net assimilation rate despite the presence of 20 mines on apple leaves. Li *et al*. (2003) measured a drop of about 13% in apple leaf photosynthesis because of the presence of 10 mines. The response of leaf photosynthesis to leaf miners thus strongly contrasts with the case of ectophagous herbivores, for which the decrease in assimilation rate could be three times larger than the area removed by feeding (Zangerl *et al*. 2002), and with the case of galling insects, for which the leaf assimilation rate could be increased by up to 36% (Fay, Hartnett & Knapp 1993).

We cannot exclude the hypothesis that the respiratory CO_2 released by the leaf miner inside the mine is assimilated by the photosynthetically active patches remaining in the mine. The infrared gas analyser we used measures the apparent net assimilation rate, that is, the ambient CO_2 which is actually assimilated, but does not take into account the CO_2 that might be produced inside the mine and instantaneously assimilated. We show that a larva releases significant amounts of CO_2 within a mine. Recently, Pincebourde & Casas (2006) indicated that the respiration rate of larvae linearly increases with the irradiance level above the mine because of the high amount of radiation transmitted by the feeding windows, which causes their body temperature to warm. The slight negative impact of the leaf miner on assimilation rate, as deduced from our measurements, might actually be lower than expected. As a consequence, the increase in WUE at the mine location would be also underestimated. It would be quite interesting to measure the changes in the stable carbon isotope within photosynthetically active tissues remaining in the mine to test if the green patches actually derive their CO_2 from larval respiration.

Leaf mining alters stomatal behaviour

The light response curves we report for stomatal conductance and transpiration rate in an intact leaf are consistent with those generally found for C_3 plants (e.g. Morison 1987; Jones 1992; Huxman & Monson 2003). Our results indicate clearly that stomata in the mined leaf tissues respond to a change in irradiance level differently than they do in the intact leaf tissues, but also that they remain functional, as previously suggested (Whittaker 1994; Raimondo *et al*. 2003). A lower stomatal conductance value was obtained at high irradiance level in the mined tissues compared with intact leaf tissues. This makes sense as the stomata may adapt their opening level to the slightly lowered assimilation rate of CO_2 through the mined surface. However, moderate to high irradiance levels induce the stomata to close in the mine integument. Stomatal closure was not the consequence of horizontal CO_2 diffusion from the tissues covered by the vegetal oil to the uncovered and transpiring leaf portions because the stomatal conductance of intact leaf tissues surrounded by covered tissues was still similar to that of a leaf uncovered by vegetal oil (Pincebourde 2005).

The mechanisms responsible for the stomatal closure from moderate to high radiation level are still unknown but we formulated three non-mutually exclusive hypotheses. First, the feeding windows were shown recently to transmit about 40% of incoming radiation in the red wavelength range within a mine (Pincebourde & Casas 2006). Talbott *et al*. (2003) showed that isolated stomata from a wild type of *Arabidopsis* are closing when the red light flux is above the saturation level. This stomatal closure at high red light levels was suggested to be a result of photoinhibition occurring within the guard cells. The physical structure of the mine is one of the determinants of the stomatal closure because it occurred in empty mines as well. The second hypothesis involves the CO_2 released by a larva within its mine. The mine integuments are not suberized, suggesting that gas exchange between the lower side of a mine and the upper side is still possible. Our results, showing that green patches are photosynthetically active and that the stomata are still able to adjust their opening level, reinforce this interpretation. Stomata are known to adjust their opening level as a function of the intercellular CO_2 concentration, allowing photosynthesis mechanism to be optimized (Morison 1987; Assmann 1999; Huxman & Monson 2003; Tuzet, Perrier & Leuning 2003). Stomata might sense a temporary increase in the intercellular CO_2 concentration caused by the insect respiration from

moderate to high irradiance levels, leading to the closure of the stomata and to the stabilization of the intercellular CO_2 concentration at lower transpiration levels. The third hypothesis we propose is related to the strong modification in the CO_2 conductance of the intercellular air space induced by the feeding activity of the leaf miner. By eliminating some mesophyll tissues within the leaf, a larva induces an increase in the intercellular CO_2 conductance leading to a faster diffusion of CO_2 from the substomatal cavity to the green patches. The intercellular air space CO_2 conductance is a controlling factor of both the intercellular air space CO_2 concentration and the assimilation rate (Parkhurst 1994). CO_2 diffusion inside the leaf tissues is therefore less limited within a mine than within an intact leaf. This mechanism might allow net photosynthesis to be unaffected despite the stomatal closure.

Leaf mining decouples transpiration from photosynthesis

A functional coupling between photosynthesis and stomatal conductance in the intact leaf is usually observed in C_3 plants (e.g. Wong, Cowan & Farquhar 1979; Morison 1987; Jarvis & Davies 1998; Roberntz & Stockfors 1998). The linear relationship we report between transpiration and assimilation rates confirms this coupling in intact apple leaves. Nevertheless, we found no functional coupling between assimilation and stomatal conductance or transpiration in mines occupied by a larva. This coupling was very weak in empty mines. The intercellular CO_2 concentration is thought to be the signal allowing the stomata to adapt their opening level as a function of the assimilation rate, inducing therefore the functional coupling between stomata and photosystems (Assmann 1999). Zeiger & Field (1982) also revealed the existence of a photocontrol on the functional coupling between photosynthesis and stomatal conductance. They showed that the spectral composition of incoming radiation can alter independently photosynthesis and stomatal conductance. There is coherence here with the hypotheses we are proposing to explain the stomatal closure in the mined tissues. The CO_2 released by the leaf miner might disturb the intercellular CO_2 concentration signal and the red-enriched light transmitted by feeding windows may be sensed by the photosystem present within the guard cells. Therefore, the two mechanisms could lead to a decoupling between photosynthesis and transpiration rate.

The stomatal conductance, and hence the transpiration rate, was far more affected than the assimilation rate in the mined tissues. Our results indicate clearly that the green patches remaining within the mines are still able to maintain a photosynthesis rate close to that of intact leaves, but the maximal assimilation rates are reached at a transpiration rate which is roughly 50% lower than that of intact leaves. The WUE is therefore positively affected. This positive impact on WUE might even be underestimated if we assume that the CO_2 released by larvae is actually assimilated within the green patches.

CONCLUSION

Ectophagous insects often cause a large increase in the transpiration rate while photosynthesis is decreased, inducing therefore a drop in WUE in the leaf tissues remaining after the herbivore attack (Welter 1989; Aldea *et al*. 2005). Mesophyll feeders such as spider mites induce a decrease in both photosynthesis and transpiration rate (Welter 1989; Haile & Higley 2003; Reddall *et al*. 2004). Our study reports a nearly reverse mechanism for leaf miners. Their apparent negative impact on photosynthesis is mitigated by their positive effects on WUE.

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