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**Nitrogen nutrition of tomato plant alters leafminer dietary intake dynamics**

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**ABSTRACT**

The leafminer *Tuta absoluta* (Meyrick) is a major pest of the tomato crop and its development rate is known to decline when nitrogen availability for crop growth is limited. Because N limitation reduces plant primary metabolism but enhances secondary metabolism, one can infer that the slow larval development arises from lower leaf nutritive value and/or higher plant defence. As an attempt to study the first alternative, we examined the tomato-*T. absoluta* interaction in terms of resource supply by leaves and intake by larvae. Tomato plants were raised under controlled conditions on N-sufficient vs. N-limited complete nutrient solutions. Plants were kept healthy or artificially inoculated with larvae for seven days. Serial harvests were taken and the N, C, dry mass and water contents were determined in roots, stems and leaves. Leaf and mine areas were also measured and the N, C, dry mass and water surface densities were calculated in order to characterize the diet of the larvae. The infestation of a specific leaf lessened its local biomass by 8-26%, but this effect was undetectable at the whole plant scale. Infestation markedly increased resource density per unit leaf area (water, dry mass, C and N) suggesting that the insect induced changes in leaf composition. Nitrogen limitation lessened

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*Abbreviations*: C, carbon; dat, days after transplantation; DW, dry weight; EWT, equivalent water thickness; FW, fresh weight; HN, high nitrogen nutrition; LN, low nitrogen nutrition; N, nitrogen; SLC, specific leaf carbon content; SLN, specific leaf nitrogen content; SLW, specific leaf weight

whole plant growth (by 50%) and infested leaflet growth (by 32-44%). It produced opposite effects on specific resource density per unit area, increasing that of dry mass and C while decreasing water and N. These changes were ineffective on insect mining activity, but slowed down larval development. Under N limitation, *T. absoluta* consumed less water and N but more dry mass and C. The resulting consequences were a 50-70% increase of C:N stoichiometry in their diet and the doubling of faeces excretion. The observed limitation of larval development is therefore consistent with a trophic explanation caused by low N and/or water intakes.

**Keywords:**

*Solanum lycopersicum*, *Tuta absoluta*, hydroponics, faeces, nitrogen, frass, insect development

## 1 Introduction

The leafminer *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is thought to originate from Central America and since 2006 it has been spreading rapidly throughout Europe and the Mediterranean area (Desneux et al., 2010). It is regarded as a major threat to the sustainability of tomato (*Solanum lycopersicum* L.) production on a global scale (Desneux et al., 2011). Female adults of *T. absoluta* lay eggs on various plant parts, and after hatching the larvae penetrate these tissues. Once within the plant, they feed mainly on the mesophyll and develop depending on *in situ* factors, including the nitrogen (N) status of the tomato crop. Indeed, immature herbivore insects respond to poor nutritional quality of their host plant by decreasing rates of development and/or lowering pupal and adult weights (Chen et al., 2004; Cornelissen and Stiling, 2006; Stiling et al., 1999; Uesugi, 2015). Considering the higher carbon to nitrogen (C:N) ratio in plant than animal tissues, nitrogen strongly limits herbivore development (Anderson et al., 2004; Hövemeyer, 1995; Kagata and Ohgushi, 2012; Mattson, 1980) thus making the concentration of this element an important criterion of food quality. Furthermore, N fertilization not only increases plant N content, but also alters the content of other plant primary metabolites such as carbohydrates, which are necessary for the nutrition of insects (Hermans et al., 2006). Moreover, food quality also depends on the presence of allelochemicals (Scriber and Slansky, 1981) whose concentration often relates to plant N status (Herms and Mattson, 1992).

*T. absoluta* responds to varying N status of the tomato crop in accordance with these general principles. For instance, low N inputs that restrict tomato growth reduce survival rate of insect larvae, decrease their pupal weights and increase the time needed to reach maturity (Han et al., 2014; Lariat et al., 2016). Two reasons may explain these effects. Firstly, the chemical defence of the tomato crop is strengthened by low N nutrition as a consequence of enhanced synthesis and concentration of a variety of C- and N-based allelochemicals (Hoffland et al., 2000; Lariat et al., 2012a, 2012b, 2016; Royer et al., 2013; Wilkens et al., 1996a). Secondly, reducing the nitrogen supply to plants reduces the nutritive value of the tissue for herbivores. This trophic effect results from changes induced in the C:N status of plant tissues in which *T. absoluta* larvae feed and develop. It is well established that tomato tissues respond to N fertilization by increasing organic-N and water contents while lowering carbohydrate contents (Cárdenas-Navarro et al., 1999; Huanosto Magaña et al., 2009; Royer et al., 2013; Urbanczyk-Wochniak and Fernie, 2005). Thus, fertilization markedly changes the nutritive quality of tomato for *T. absoluta*, especially by altering the balance between C and N.

In the present study, we tested the hypothesis of Han et al. (2014) who proposed that reduced tomato N and water contents may impair and/or slow down *T. absoluta* development through a sub-optimal intake of food *e.g.* fewer N-based nutrients in host plant (*Nitrogen limitation hypothesis*: White, 1993; Schoonhoven et al., 2005) and/or food quality (*Plant vigor hypothesis*: Price, 1991; White, 2009). In order to assess this trophic effect on the leafminer, we placed *T. absoluta* larvae on a specific developing leaf and manipulated the N status of tomato plants growing hydroponically, using a well established automatized system (Adamowicz and Le Bot, 2008; Adamowicz et al., 2012). Traits of insect, leaf and plant development were assessed from subsequent serial harvests of the plants.

## 2 Materials and methods

### 2.1 Plant material and growth conditions

Tomato seeds (*Solanum lycopersicum* L. cv. Better Bush VFN Hybrid, breeder: Tomato Growers) were sown and grown using a hydroponic system under controlled conditions. For more details concerning the cultivar and germination conditions see Larbat et al. (2012a) and Royer et al. (2016). Eleven days after sowing, the seedlings were transplanted into two independent Nutrient Film Technique systems set in twin growth chambers (16 h photoperiod, 23 °C / 18 °C day/night in air and 23 °C in nutrient solutions, 60 % air humidity). Plants were left undisturbed until harvest in order to avoid any mechanical stress. Before the experiment, all temperature sensors (air and solution) were calibrated in a water bath with 0.01 °C precision (Haake model C35/F6, Karlsruhe, Germany).

The twin climatic chambers were used to impose two contrasting N nutrition regimes. Half the plants received a full nutrient solution supplying N as 1.0 mM NO<sub>3</sub>, a concentration previously shown to be non-limiting for tomato growth (Adamowicz and Le Bot, 2008) and presently referred to as "High N" (HN). The solution was prepared with deionized water and pure salts to produce the following mM concentrations: Ca(NO<sub>3</sub>)<sub>2</sub> 0.5; KH<sub>2</sub>PO<sub>4</sub> 1.0; K<sub>2</sub>SO<sub>4</sub> 1.0; MgSO<sub>4</sub> 1.5; CaSO<sub>4</sub> 3.0. Trace elements were provided as EDTA-Fe (43 µM) and 0.3 ml l<sup>-1</sup> of a stock solution with the following mM concentrations: Mo 0.94; Mn 38.8; Zn 10.8; Cu 1.6; B 68.7; Fe 35.8. The remaining half of the plants also received a full nutrient solution, with a lower N concentration previously shown to limit plant growth without affecting photosynthesis or producing N deficiency symptoms (Adamowicz and Le Bot, 2008; Le Bot et al., 2009; Royer et al., 2016), and referred to here as "Low N" (LN). The initial NO<sub>3</sub> concentration in this solution was 30 µM (as Ca(NO<sub>3</sub>)<sub>2</sub> 0.015 mM and all other salts as in HN, except CaSO<sub>4</sub> 3.485 mM). This

N concentration set-point was adjusted daily so that the LN plants absorbed only 1/3<sup>rd</sup> of the nitrate taken up by HN plants. Nitrogen concentration was regulated hourly in all solutions using the automatic system *Totomatix* (Adamowicz et al., 2012) by additions of a maintenance solution containing (M): KNO<sub>3</sub> 0.411; Ca(NO<sub>3</sub>)<sub>2</sub> 0.206; Mg(NO<sub>3</sub>)<sub>2</sub> 0.0885. The pH was also monitored and regulated hourly by *Totomatix* to 5.5.

## 2.2 *Plant infestation by T. absoluta larvae*

*T. absoluta* adults were reared on caged tomato plants in climatic chambers. Females laid eggs and after hatching, stage 3 larvae (scale 1-4) were selected for the experiment. Half of the HN and LN plants were infested 14 days after transplantation (dat) with 12 larvae laid on the three terminal leaflets of the 4<sup>th</sup> true leaf. This specific larvae number was chosen because in a preliminary experiment, it was shown to trigger plant response to larval attacks with only limited damage to the whole plant (Supplementary material, Fig. S1). Control plants were subjected to mock depositions of larvae, performed with a paintbrush. We decided not to constrain the larvae in cages on the leaflets because leaf disturbance is known to elicit plant response defences (Braam, 2005; Gigolashvili et al., 2007; Savatin et al., 2014).

## 2.3 *Plant harvesting and sample preparation*

The first harvest (H1, 12 HN and 11 LN plants) was taken 14 dat, prior to *T. absoluta* deposition. The second (H2, 9 plants per treatment) and the third (H3, 9 plants per treatment) harvests occurred 3 (17 dat) and 7 days (21 dat) after larvae deposition. At harvest, leaves, stems and roots were separated. The three terminal and the two adjacent leaflets of the infested leaves were sampled separately, their areas were measured and the larvae were collected. Thus, weight measurements and elemental analyses were performed on infested leaves devoid of larvae but containing their excrements. Roots were rinsed in deionised water. Organ fresh weights (FW, 0.1 mg precision) were measured and samples were frozen in liquid N<sub>2</sub> and stored at -80 °C until freeze-drying. Dry weights (DW, 0.1 mg precision) were determined and the samples were ground to a fine powder and stored at -20 °C.

## 2.4 *Traits of T. absoluta damage and development*

On each infested leaf, larvae and pupae were counted at H2 and H3 and at H3, their global FW (0.01 mg precision) was also determined. At each harvest, control and damaged leaves were weighed, then laid flat close to a ruler on a light table, and azimuthal digital pictures were taken to determine the areas of leaflets, mines (*i.e.* transparent areas) and excrements (*i.e.* black areas).

The leaflet images were delineated using Photoshop CS4 extended (Adobe systems Software, Ireland) and processed using ImageJ (Schneider et al., 2012) with the macro given in Appendix B.

## 2.5 Elemental analysis

Total C and N were determined by an online continuous flow elemental analyzer (Carlo Erba - NA 1500, Thermo Scientific, Lakewood, NJ USA) using the Dumas method.

## 2.6 Data processing

### 2.6.1 Plant nitrogen intake and uptake

At H2 and H3, the nitrogen concentration was measured in all plant organs. Thus, the mean plant nitrogen intakes (mmol N per plant) were computed for each nutrition and infestation treatment by summing their respective organ N-contents. Global N absorption from the nutrient solution was computed continuously from transplantation to the end of the experiment by applying hourly the unbiased balance sheet of Adamowicz and Le Bot (2013) to the data collected by the *Totomatix* system. For each treatment, the result was divided by the number of plants present during this interval to calculate the mean plant N uptake (mmol N per plant).

### 2.6.2 Leaf resource densities and larval resource intake

For control plants, the specific leaf weights (SLW, mg DW cm<sup>-2</sup>, also named LMA leaf mass per area), specific leaf C contents (SLC, mg C cm<sup>-2</sup>), specific leaf nitrogen contents (SLN, mg N cm<sup>-2</sup>) and equivalent water thicknesses (EWT, mg H<sub>2</sub>O cm<sup>-2</sup>) of the mock treated leaflets were calculated as usual:

$$SLW = \frac{DW}{A}; SLC = \frac{QC}{A}; SLN = \frac{QN}{A}; EWT = \frac{FW - DW}{A}$$

with *FW*, *DW*, *QC*, *QN* and *A* being the fresh weights, dry weights, C and N contents and areas of leaflets, respectively. In infested leaflets, the mines dug by *T. absoluta* induce some mathematical uncertainties in these parameters. Thus, in order to determine their lower and upper limits, two calculations were applied to the above formulae, where *A* represents either the whole leaflet areas as above (*i.e.* including the mine areas), or only the green areas (*i.e.* excluding the mine areas). Both limits were used to estimate the most likely dry masses, C, N and water ingested by *T. absoluta* larvae, as  $A_m \times SLW$ ,  $A_m \times SLC$ ,  $A_m \times SLN$  and  $A_m \times EWT$ , respectively,  $A_m$  being the mine areas.

### 2.6.3 Statistics

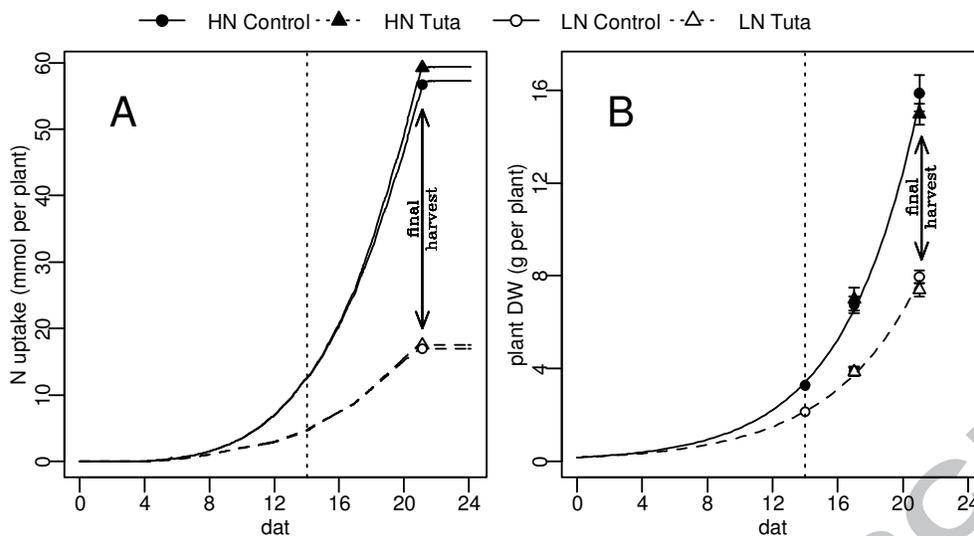
Computations and plots were performed using the R software (R Core Team, 2016) and statistical significance was set at  $P < 0.05$ . Non-linear regressions were performed using the `nls` procedure. Chi-squared tests were applied to contingency tables using the `chisq.test` procedure. Analyses of variance (ANOVA) and multivariate analyses of variances (MANOVA) were performed using the `lm` procedure with harvest, nutrition and infestation as fixed factors. (M)ANOVAs were restricted to harvests H2 and H3 where the experimental design was crossed, balanced and complete. However, missing observations resulted in unbalanced data sets and type II or III (M)ANOVAs were calculated (library `car`). The minimal adequate (M)ANOVA models were determined by stepwise deletion of higher order non-significant interactions. The effects of nutrition and instar on insect mean masses at H3 were determined by weighted ANOVA, the weights being the number of individuals in each insect pool. Post hoc tests were performed using the `pairs` procedure of the package `lsmeans`. Box-plots, Normal Q-Q plots and correlation between variance and mean, assessed the data distribution and homoscedasticity. When necessary, Box and Cox transformations were applied to the data.

## 3 Results

### 3.1 Whole plant responses to nutrition and herbivory

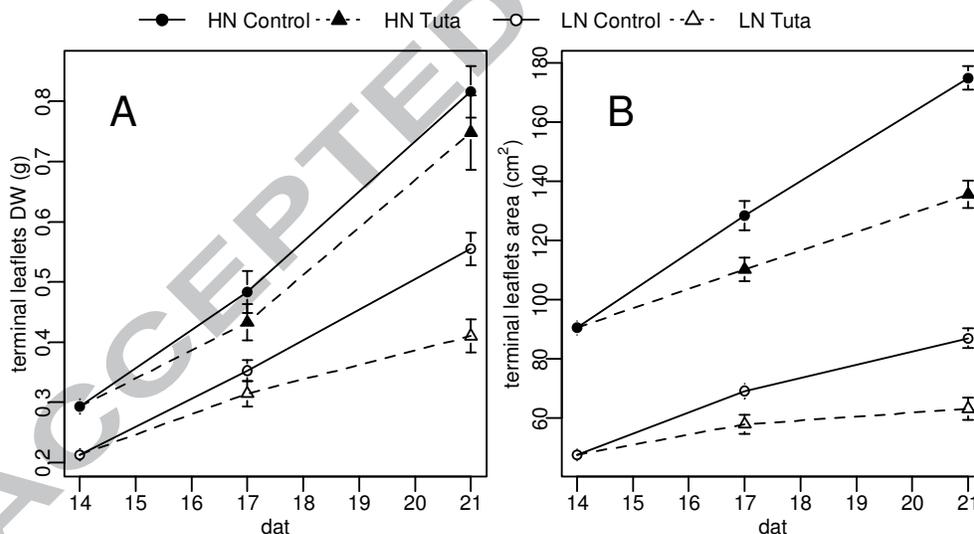
Nitrogen uptake (mmol N per plant) increased exponentially over time, and LN plants absorbed 1/3rd of the amount taken up by the HN plants, as planned in our methodology (Fig. 1A). After the final harvest (H3), N uptake ceased completely, confirming that all measurements prior to H3 were due only to plant absorption activity. Furthermore, there was an excellent match between plant N uptake on the whole crops as calculated from the measurements of the *Totomatix* setup and N analysed in sampled plants (Supplementary material, Fig. S2). Control and infested plants showed only slight differences in N uptake within the HN and LN treatments (4 and 3 % differences at H3, respectively, but a statistical significance cannot be assessed).

Plant dry weights (g per plant) increased exponentially over time (Fig. 1B), with significantly higher relative growth rates for HN than LN plants (0.217 and 0.185 days<sup>-1</sup>, respectively;  $t(92) = 5.5$ ,  $P < 0.001$ ). At H3, HN plants were twice the dry weight of LN plants ( $t(64) = 18$ ,  $P < 0.001$ ). There was no significant effect of *T. absoluta* herbivory on relative growth rates and harvested plant biomasses.



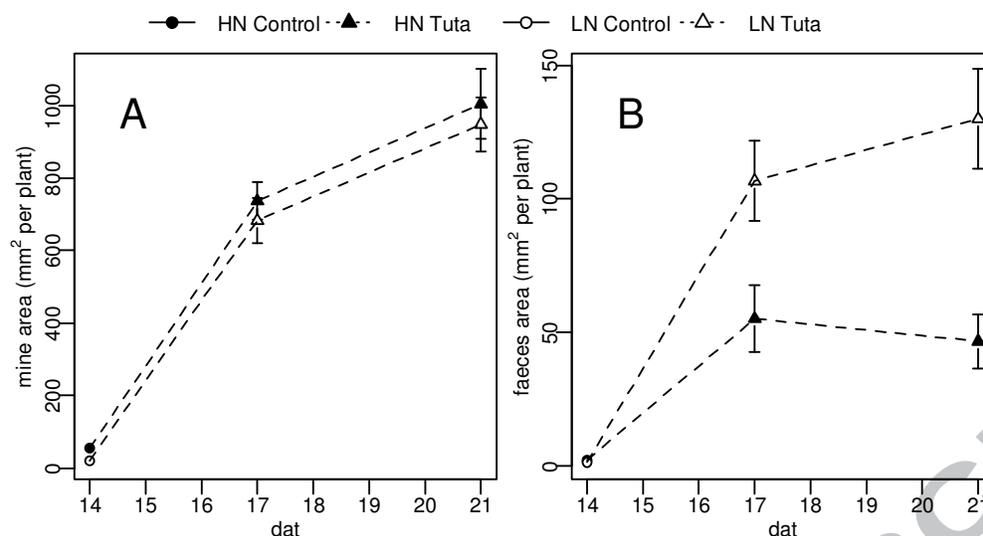
**Fig. 1. Tomato nitrogen uptake and growth.**

Following transplantation (0 dat, days after transplantation), the plantlets were supplied with either adequate (HN) or low N (LN) full nutrient solutions. At 14 dat (vertical dotted line), half the plants were infested by *T. absoluta* larvae. In [A], the lines represent hourly measurements of whole crop uptakes (mmol N per plant). Symbols at the final harvest identify control and infested plants. In [B], symbols and vertical bars are means  $\pm$  s.e.m. of the whole plant dry weights (g per plant) at the three harvests (11-12 replicates at 14 dat, 8-9 at 17 and 21 dat). Differences were significant between HN and LN plants ( $F_{1,64} = 154$ ,  $P < 0.001$ ), but not between control and infested plants. Thus, the lines represent the mean exponential growths of all HN (solid line) and LN (dashed line) plants:  $DW = DW_0 \times \exp(\mu \times \text{dat})$ , with  $DW_0 = 0.161$  (plant dry weight at 0 dat, forced to be the same on HN and LN) and relative growth rate  $\mu = 0.217$  (HN) and  $0.185$  (LN)  $\text{days}^{-1}$ .



**Fig. 2. Terminal leaflet growth and expansion.**

Control and *T. absoluta*-infested plants were raised under high (HN) or low (LN) nitrogen nutrition. At 14 dat, 12 larvae were laid on the three terminal leaflets of the fourth true leaf. Control plants received a mock inoculation using a paintbrush. Symbols and vertical bars are means  $\pm$  s.e.m. of 8-9 (17 and 21 dat) to 11-12 (14 dat) harvested plants. All treatments affected significantly the leaflet biomass ( $F_{1,67} = 68$ ,  $P < 0.001$  for nutrition, and  $F_{1,67} = 12$ ,  $P < 0.001$  for infestation) and area ( $F_{1,65} = 502$ ,  $P < 0.001$  for nutrition, and  $F_{1,65} = 16$ ,  $P < 0.001$  for infestation). In [A], dry weights are expressed in g, and in [B], areas in  $\text{cm}^2$  include the mines.



**Fig. 3. Areas of mines and faeces on terminal leaflets infested by *T. absoluta* larvae.**

Experimental conditions and symbols are as in Fig. 2. In [A], the mine areas (mm<sup>2</sup> per plant) did not change significantly with N treatments while in [B], the mine area occupied by faeces (mm<sup>2</sup> per plant) responded significantly to N ( $F_{1,32} = 23$ ,  $P < 0.001$ ). Notice that at 14 dat, the plants were not yet infested but the measured areas in [A] and [B] are slightly positive due to uncertainty of the automatic image processing in delineating the mines or faeces.

### 3.2 Leaflet responses to nutrition and herbivory

Control (*i.e.* mock treated) and *T. absoluta*-infested leaflets responded significantly to N nutrition for biomass accumulation (Fig. 2A;  $F_{1,67} = 68$ ,  $P < 0.001$ ) and area expansion (green areas + mines, Fig. 2B;  $F_{1,65} = 502$ ,  $P < 0.001$ ). Compared with controls, *T. absoluta*-infested leaflets had a significantly lower biomass (by 8 and 26 % in HN and LN, respectively;  $F_{1,67} = 12$ ,  $P < 0.001$ ) and area (by 27 and 22 % in HN and LN, respectively;  $F_{1,65} = 16$ ,  $P < 0.001$ ).

### 3.3 Herbivory intensity and *T. absoluta* development

The area of mines increased significantly from 3 to 7 days after the larvae were placed on the leaves (*i.e.* between H2 and H3; Fig. 3A;  $F_{1,32} = 15$ ,  $P < 0.001$ ), without any significant difference between N treatments. On average, the mine area was around 980 mm<sup>2</sup> per plant at H3.

At H2 and H3, we recovered around 60% of the larvae laid at H1 and there was no significant effect of N nutrition (Table 1). The missing larvae did not succeed in infesting the leaflets and died on the leaf surface during the first hours following the artificial infestation. At H3, the recovered insect contingent included larvae and pupae. The number of pupae was significantly higher under HN than LN (Table 1;  $\chi^2(1) = 7.4$ ,  $P < 0.01$ ), and the status of N nutrition significantly affected the average larval and pupal fresh weights (Table 2;  $F_{1,31} = 8.8$ ,  $P < 0.01$ ).

The mine area occupied by faeces was significantly influenced by N nutrition, the area at H3 being nearly three-fold higher under LN than HN (Fig. 3B;  $F_{1,32} = 23$ ,  $P < 0.001$ ). The apparent changes between H2 and H3 were not significant.

**Table 1. Contingency tables of larvae laid and recovered on infested tomato plants.**

Immediately after the first harvest (H1), 12 larvae were laid on each of 18 remaining plants per nutritional treatment (HN and LN). Half of these were harvested three days later (H2) and the other half seven days later (H3). The data from one plant (HN/H3) were excluded from the analyses because larvae were recovered on the third instead of the fourth true leaf.

	Deposited at H1			Recovered at H2 and H3								
	Larvae			Larvae			Pupae			Larvae + Pupae		
	H2	H3	Sum	H2	H3	Sum	H2	H3	Sum	H2	H3	Sum
HN	108	96	204	64	31	95	0	34	34	64	65	129
LN	108	108	216	65	52	117	0	15	15	65	67	132
Sum	216	204	420	129	83	212	0	49	49	129	132	261

**Table 2. Larval and pupal fresh weights (mg per insect) measured at the last harvest (H3) on HN and LN nutritional treatments.**

The larvae and pupae of each harvested plant were counted (see Table 1) and pooled. On each plant, the pools of larvae or pupae were weighed and the average insect weights were calculated. The table summarizes the number of measured pools (n), their mean values and standard errors of means (s.e.m.). The effect of nitrogen nutrition was significant (weighted ANOVA;  $F_{1,31} = 8.8$ ,  $P < 0.01$ ), but differences between larvae and pupae were not significant.

Larvae	n	Mean	s.e.m.	Pupae	n	Mean	s.e.m.
HN	8	4.40	0.94	HN	8	4.43	0.27
LN	9	3.05	0.31	LN	9	3.62	0.40

### 3.4 Leaf traits and resource intake by larvae

In order to determine the likelihood of some leaf traits in infested plants (specific leaf weight, SLW; equivalent water thickness, EWT; specific leaf carbon and nitrogen contents, SLC and SLN, respectively), calculations were made following two extreme hypotheses, where the leaflet DW, H<sub>2</sub>O, C and N spread (i) uniformly across the whole area, or (ii) only across the green area (*i.e.* excluding mines). The resulting probable values are shown as shaded areas in Fig. 4.

The specific leaf weight of control and infested leaflets increased significantly during leaf ageing (Fig. 4A;  $F_{1,66} = 46$ ,  $P < 0.001$ ) and was consistently higher under LN nutrition ( $F_{1,66} = 63$ ,  $P < 0.001$ ). Herbivory appeared to increase SLW as the values for control plants were always below the probable values (shaded areas) of infested plants. The difference was not significant with the lower boundary but it was very highly significant with the upper one ( $F_{1,66} = 21$ ,

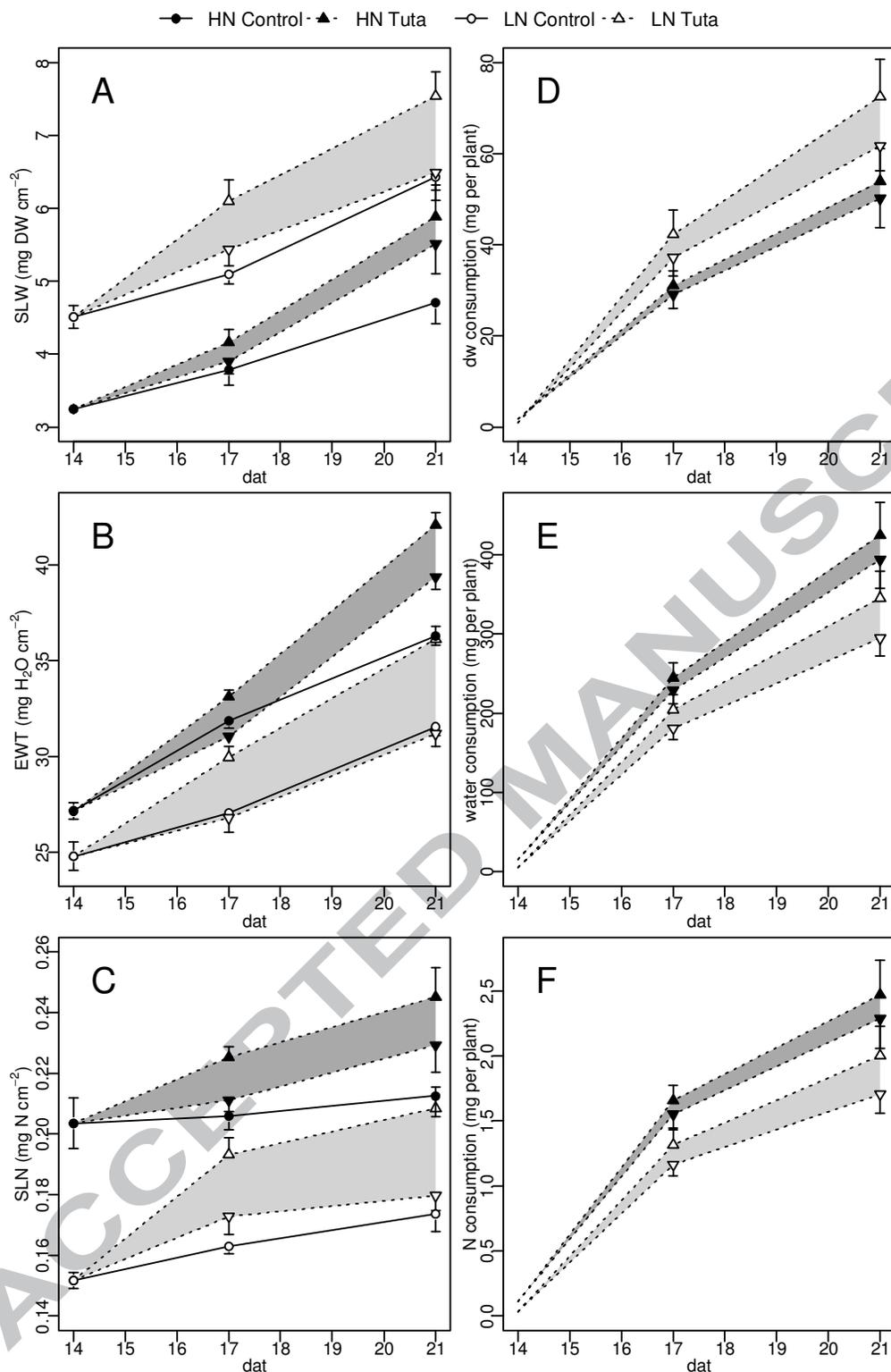
$P < 0.001$ ). The leaflet carbon concentration (37.91 %, s.e.m. = 0.15) was unaffected by nutrition and infestation. The specific leaf carbon content (not shown) followed the SLW patterns shown in Fig. 4A, with higher SLC under LN nutrition ( $F_{1,66} = 51$ ,  $P < 0.001$ ). Herbivory increased SLC similarly to SLW ( $F_{1,66} = 16$ ,  $P < 0.001$  for the difference between control and upper boundary).

The equivalent water thickness of control and infested leaflets increased significantly during leaf ageing (Fig. 4B;  $F_{1,62} = 38$ ,  $P < 0.001$ ) but was consistently lower under LN nutrition ( $F_{1,66} = 40$ ,  $P < 0.001$ ), contrary to SLW. Herbivory affected significantly EWT at H3 ( $F_{1,31} = 81$ ,  $P < 0.001$  upper boundary, and  $F_{1,30} = 9.7$ ,  $P < 0.01$  for the interaction with nutrition at lower boundary). Indeed, the increase was more obvious under HN nutrition.

The specific leaf nitrogen content of control and infested leaflets increased significantly during leaf ageing (Fig. 4C;  $F_{1,66} = 7.8$ ,  $P < 0.01$ ) and was consistently higher under HN than under LN nutrition ( $F_{1,66} = 126$ ,  $P < 0.001$ ). Herbivory increased SLN, as values for control plants were always significantly below the probable values of infested plants (shaded areas;  $F_{1,66} = 6.8$ ,  $P < 0.05$  lower boundary, and  $F_{1,66} = 55$ ,  $P < 0.001$  upper boundary).

The larvae intake of specific resources (DW, water, C and N, mg per plant) was calculated as the products of mine areas and upper & lower resource densities (SLW, EWT, SLC and SLN). The shaded areas in Fig. 4D-F, therefore, denote the zones for which likelihood could be evaluated. The difference between HN and LN treatments was statistically assessed by MANOVA.

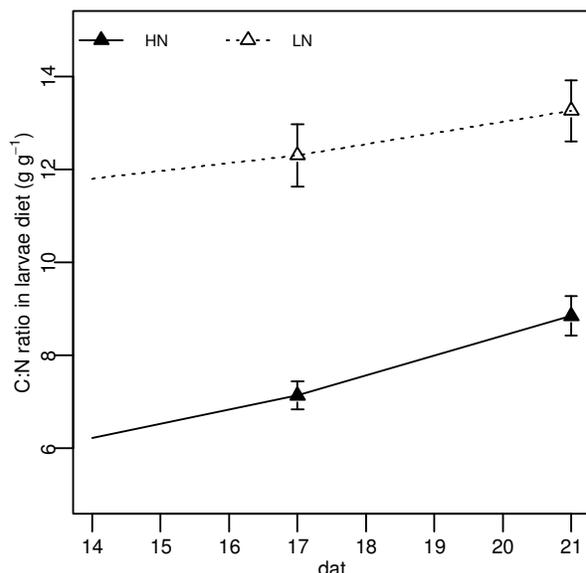
The larvae DW intake increased significantly (Fig. 4D;  $F_{2,31} = 18$ ,  $P < 0.001$ ) from H2 (17 dat, 3 days after larvae deposition) to H3 (21 dat) and was consistently higher under LN nutrition ( $F_{2,31} = 9.4$ ,  $P < 0.001$ ). The larvae carbon intakes followed DW intake patterns and therefore were not plotted. They increased significantly from H2 to H3 ( $F_{2,31} = 14$ ,  $P < 0.001$ ) and were consistently higher under LN than HN nutrition ( $F_{2,31} = 9.4$ ,  $P < 0.001$ ). At H3, larvae C intake (mg per plant) laid in between 18.6-20.0 for HN plants, increasing to 22.5-26.4 for LN plants. The larvae water consumption increased significantly from H2 to H3 (Fig. 4E;  $F_{2,30} = 28$ ,  $P < 0.001$ ) and was consistently lower under LN nutrition ( $F_{2,30} = 38$ ,  $P < 0.001$ ). The larvae nitrogen intake increased significantly from H2 to H3 (Fig. 4F;  $F_{2,30} = 14$ ,  $P < 0.001$ ) and was consistently lower under LN nutrition ( $F_{2,30} = 45$ ,  $P < 0.001$ ). The C:N ratio in larvae diet was up to 1.5-1.7 times higher in LN than HN nutrition (Fig. 5;  $F_{1,67} = 248$ ,  $P < 0.001$ ) and increased significantly with leaf age ( $F_{1,67} = 18$ ,  $P < 0.001$ ).



**Fig. 4. Resource area densities and resource consumption by larvae.**

Experimental conditions and symbols are as in Fig. 2. [A], specific leaf weight (SLW, mg DW cm<sup>-2</sup>); [B], equivalent water thickness (EWT, mg H<sub>2</sub>O cm<sup>-2</sup>); [C], specific leaf nitrogen (SLN, mg N cm<sup>-2</sup>). In infested plants, these parameters were calculated according to two extreme hypotheses considering either that the mines contain no resource (*i.e.* densities are null), or, on the opposite that mines have the same resource densities as green areas. These calculations (dashed lines) delimit the likely density values (shaded areas, dark grey under HN, light under LN). In [D-F], these likely values were multiplied by the

mine areas to calculate the corresponding likely resource consumptions by *T. absoluta* larvae, also shown as shaded areas (HN in dark, LN in light). All resource consumptions responded significantly to N treatments ( $P < 0.001$ ).



**Fig. 5. Dynamic changes of C:N ratio in larvae diet.**

Experimental conditions and symbols are as in Fig. 2. The symbol absence at 14 dat indicates that plants were not infested yet.

#### 4 Discussion

Our results demonstrate that following infestation, *T. absoluta* induces changes in the nutritive value (C, N and water contents) of the infested leaves under both conditions of high and low nitrogen availability. This study also confirms that when N fertilization is limited, the *T. absoluta* larvae need more time to reach maturity because of the lower leaf nutritive value. Prior to discussing these findings, it is important to question the efficiency of the experimental setup in ensuring fine-tuning of tomato leaf N status and in producing unbiased data. Specific conditions are indeed required to study the interaction between N nutrition and the leafminer *T. absoluta*, particularly since nitrogen is a main determinant of the nutritive value of food source for herbivores (Fagan et al., 2002; Scriber and Slansky, 1981; Slansky and Feeny, 1977).

##### 4.1 Suitability of experimental conditions

In each treatment, the N content of sampled plants matched the N uptake value measured continuously by the *Totomatix* setup on the whole crop, inferring that the harvested plants were representative of the crop (Supplementary material, Fig. S2).

Throughout the growth period until the final harvest, plant N uptake was markedly restricted under the low N treatment compared with the high N treatment (Fig. 1A). Likewise, dry biomass accumulation was continuously limited under the low N treatment as compared with the high N

plants (Fig. 1B). Limitation of N was less effective in decreasing biomass ( $\sim 1/2$ ) than N uptake ( $\sim 1/3$ ), confirming the observation of Royer et al. (2016). As a consequence, N concentration declined in LN plant tissues making them of a lower nutritive value for leafminers. Plant differentiation between low and high N treatments was significant at the onset of infestation by larvae (represented on Fig. 1 by the vertical dashed line), with low N plants showing no evidence of N deficiency (not shown).

Artificial insect inoculation is a commonly used technique to study local plant responses. In our experiment, the load of 12 larvae produced only limited damage to the whole plant, as insect consumption represented only 0.9 % (LN) and 0.3 % (HN) of plant dry mass at the final harvest. Neither N uptake nor whole plant DW accumulation were noticeably affected by the presence of herbivory (Fig. 1). Larval mortality was 38 %, but it was evenly distributed among treatments (Table 1), implying that mortality was not a source of bias in data interpretation.

#### 4.2 Nitrogen nutrition strongly influenced nutrient status of infested leaves

The leaves used for inoculation grew actively during the experiment (Fig. 2) but the LN treatment efficiently reduced their growth and expansion, implying, therefore, important quality changes in leaf food source at the infestation site.

The specific leaf weight (or its inverse, the specific leaf area) is a growth-related trait (Lambers and Poorter, 1992) highly responsive to environmental conditions such as nutrient availability (Poorter et al., 2009). In our experiment, the LN treatment was even more effective in restricting area expansion (Fig. 2B) than mass accumulation (Fig. 2A), resulting in higher SLW in the LN treatment compared with HN, at any of the harvest dates (Fig. 4A). Since the LN treatment increased SLC similarly to SLW, it is unlikely that C limited the performance of larvae under LN.

It has been reported that leaf water content responds markedly to nitrate nutrition (Cárdenas-Navarro et al., 1999) and that water may limit insect performance through bottom-up effects (Han et al., 2014, 2015a, 2015b; Scriber and Slansky, 1981). In this experiment, we measured both the specific leaf nitrogen content (SLN, Fig. 4C) and the leaf water content per unit area (*i.e.* the equivalent water thickness EWT, Fig. 4B). We found that SLN and EWT increased over time, but they were both consistently lowered by LN nutrition, rendering nitrogen and water plausible factors for limiting the performance of larvae in the LN treatment.

### 4.3 Herbivory modified nutrient status of infested leaves

Strong local effects of herbivory were observed on the attacked leaflets, which were not fully mature and still growing (Fig. 2) while the mines were being actively dug (Fig. 3A). At the final harvest, the dry biomass of the infested leaflets was 79 mg (HN) and 145 mg (LN) lower than their respective controls. These deficits cannot be fully explained by insect consumption because they would be expected to approximately match larval weights since excrements (frass) remained inside the mines. Considering the number of insects recovered per plant, these leaflet dry mass deficits represent 11 (HN) or 20 (LN) mg DW per insect. This is well above presumed larval dry mass, because their measured FWs were lower than 5 mg per individual larva (Table 2). We infer, therefore, that herbivory restricted leaflet growth. This may be the outcome of lower photosynthesis or a lowering of resource allocation to growing tissues. This restriction in growth could be the result of either the disruption of vascular vessels by the larvae, or the resource reallocation to chemical defence (Nabity et al., 2009; Trumble et al., 1993) leading to more intense dark respiration (Schmidt et al., 2009).

Nevertheless, herbivory enhanced all traits under study (SLW, EWT, SLC and SLN; Fig. 4A-C) implying that the abundance of the major resources (C, N and H<sub>2</sub>O) increased where the insect was present. Rapid changes in SLW (and thus SLC) are known to result from variation in reserve compounds (mainly non-structural carbohydrates), caused by the imbalance of source and sink activities at leaf level (Bertin and Gary, 1998). Thus, from a mere trophic viewpoint, we may hypothesize that because *T. absoluta* decreased leaflet growth (Fig. 2), the use of C and N for growth was locally restricted, allowing these resources to accumulate and thus to be at the disposal of the larvae. From this trophic viewpoint, the miner improved the leaf nutritive value.

Such improvements of the C and N status at the infestation site have been mostly studied in plants infested by galling insects (Giron et al., 2016). They have also been found in wheat infested by Hessian flies (Zhu et al., 2008). In the case of leafminers, however, little information is available. It has been reported that in mined leaves, C and N concentrations are the same (*Eriocrania* spp. Zeller on *Betula pendula*, Johnson et al., 2002) or are lower (*Cerodontha iridiphora* on *Iris hexagona*, Schile and Mopper, 2006) than healthy controls. It has also been shown that *Phyllonorycter blancardella* maintains sugar and soluble protein concentrations at the level of healthy controls (Body et al., 2013; Giron et al., 2007). Concerning water, leaves mined by *P. blancardella* exhibited lower stomatal conductance and transpiration rate (Pincebourde et al., 2006). However, none of these studies calculated resource area densities or reported resource ingestion of larvae. Thus, to our knowledge, this is a first report that a

leafminer (*T. absoluta*) concomitantly improves the C, N and water status at infection sites compared with healthy controls.

#### 4.4 *Tuta absoluta* responded to N treatments

There are reports in the literature of large variation in the mean *T. absoluta* pupal weight (from 2.8 to 4.6 mg, Ecole et al., 2001; Pereyra and Sánchez, 2006) and our measurements are in agreement with these findings (Table 2). Insect weights were significantly lower under LN than HN as in our previous findings (Han et al., 2014; Larbat et al., 2016). Furthermore, plant N limitation significantly retarded insect development as assessed by the lower number of pupae recovered in LN leaves at the final harvest (Table 1), thus confirming previous results for the same leafminer (Han et al., 2014; Larbat et al., 2016) and two others, *Acrocercops albinatella* and *Brachys tessellatus*, respectively (Cornelissen and Stiling, 2006). However, *T. absoluta* differed from these latter miners, because the slower development under LN was not found to reduce mining activity. Indeed, *T. absoluta* dug their mines at the same rate in HN and LN treatments (fig. 3A). Moreover, much more frass accumulated in mines under LN than HN treatment (fig. 3B), confirming that the larvae maintained an active feeding activity. As a consequence, this finding questions the influence of food source quality on insect performance, which we attempted to evaluate.

In order to quantify food quality and performance of insect feeding, it is necessary to measure the masses of ingested food, excreted frass and insects (Scriber and Slansky, 1981). These measurements are tricky with miners because of technical limitations, which explains the rarity of such data for this guild (Stiling and Cornelissen, 2007). Thus, as was carried out in other studies (Hövmeyer, 1995; Mansfield et al., 1999; Uesugi, 2015), we evaluated larvae ingestion through mine areas and resource surface densities (Fig. 4D-F). The outcome of this evaluation showed that larvae dug mines at the same rate for both N treatments but they ingested more dry biomass and more C in the LN treatment as already reported for two other miners (Mansfield et al., 1999; Uesugi, 2015).

Insect herbivores developing in greenhouses are deprived of the typical food mix diversity found in natural environments because cultivation of a single crop and nitrogen fertilization practice produce tissues of relatively fixed C:N ratios. Therefore, when *T. absoluta* feed on tomato leaves, it is essential that the amount of ingested biomass provides the larvae with a minimum mix of nutrients vital for growth and development into mature adults capable of reproduction. From this viewpoint, the nutriment balance in insect diets (*e.g.* carbohydrates, amino acids, water and allelochemicals) underpins many parts of their fitness (Behmer, 2009).

This makes the so-called "geometrical framework" (GF), originally proposed by Raubenheimer and Simpson (see review by Behmer, 2009), a salient theory from which to interpret our results.

In the concept of GF, the assumption is made that animals ingest an optimal amount of food to fulfil their gross demand. Depending on food composition (C and N, for instance), this optimal amount determines their "*food intake target*", and the extent to which this is different from actual intake triggers important fitness costs. Our experiment shows that *T. absoluta* food intake was greater when larvae developed on LN compared with HN tomatoes (Fig. 4D). The strategy of eating more food of low N content increased the C:N ratio in the insect diet (Fig. 5), this being the only possible strategy by which the larvae could meet their intake target. Indeed *T. absoluta* larvae remained on the infested leaf, even though in the field they are mobile and capable of migrating from young to older leaves (*i.e.* they produce a silk thread to descend from upper to the lower parts of the foliage, Torres et al., 2001; Urbaneja et al., 2013). Had such migration occurred in our experiment, however, it would have worsened insect diet in the LN plants, as N concentration also decreases with leaf ageing (Minkenberg and Ottenheim, 1990; Wilkens et al., 1996b). This explanation suggests that larvae ingested food until their N requirement was covered and thereby suffered an excess of carbohydrate-C intake, which had to be removed. The logical consequence of eating more biomass is the production of larger amounts of excrement in the LN treatment as observed in Fig. 3B. In accordance with the GF theory, there is a fitness cost associated with such behaviour and our observations of lower pupa weight (Table 2) and greater time length from larva to pupa in the LN treatment (Table 1) could be an indication of such cost. It should be noted that larvae in the LN treatment also ingested less water (Fig. 4E) since N limitation strongly reduced leaf water content (Fig. 4B). No definite conclusion, however, can be drawn concerning whether N or water was the major limiting factor for the insect.

This paper adds key insights into previous plant defence studies, mainly focused on tomato allelochemicals, showing that moderate N limitation increases various C-based defensive compound concentrations (Larbat et al., 2012a, 2012b, 2014, 2016; Le Bot et al., 2009; Royer et al., 2013; Stout et al., 1998) as well as the N-based glycoalkaloid tomatine (Royer et al., 2013). As far as we are aware, our paper provides the first experimental evidence to support the recent proposal of Han et al. (2014) that the slower development rate of *T. absoluta* larvae on low N tomatoes results from both increased plant defence and lower nutritive value.

**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

CR and VC conceived the idea of the experiment; all authors helped to design the experiment; JLB and SA conducted the nutrition of tomatoes; ND provided the leafminers; JLB, CR, RL, SA and VC performed the harvests; RL and VC performed the chemical analyses; JLB processed all digital images and determined the areas of leaflets, mines and faeces; SA processed the data and drafted the manuscript; and all authors revised the manuscript.

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## Appendix A. Supplementary data

## Appendix B. ImageJ macro to determine leaf, mine and faeces areas

The macro requires ImageJ version 1.50c. It has been developed to analyse plane azimuthal pictures of detached leaves attacked by the tomato leafminer *Tuta absoluta*. This pest generates specific symptoms: (i) the larvae dig mines which appear as transparent areas; (ii) the insect excretes faeces at the mine's end, appearing as black areas. This allows for automatic delineation of specific areas by image analysis.

The ImageJ macro delineates total leaf area (green surface), mine area (white surface) and faeces area (black surface). The macro performs on good quality pictures, which implies a sharp focus and homogeneous (clear) background making a good contrast with the green zones. Pictures taken on a light table suit this requirement. The macro expects a scale in the

measurement routine, for example, a black line of 50-100 mm that can be selected *via* the wand tool of ImageJ. Images should have a good resolution (typically > 20 pixels per mm).

To use the macro, save the text below as a text file into the plugins folder of ImageJ and restart the software. The macro will become available in the plugins menu of ImageJ under the specified name. Follow the on-screen instructions to perform the analyses. You can contact the authors to get a suitable test image.

```
//Beginning of Macro
// suppressing the old ROIs
nombre=roiManager("count");
if (nombre>0) {roiManager("Select
All");roiManager("Delete");}
print("Start macro: "+ nombre + " old ROIs
suppressed");
//Opens an image file & stores the file "name"
path = File.openDialog("Select File");
print(path);
open(path); // open the file
run("Scale to Fit");
name=getTitle();
print(name);
index = lastIndexOf(path, ".");
if (index!=-1) newpath = substring(path, 0, index);
// records image sizes (L & H) and stores values in 2
variables
run("Select All");
getSelectionBounds(x, y, width, height);
img_large=width;
img_haut=height;
// name1 is the Working File for All leaflets area
measurement
run("Duplicate...", "title=leaf_area_"+name);
run("Scale to Fit");
name1=getTitle();
print(name1);
// get the scale from the image
setTool("wand");
title = "WaitForUser";
msg = "Use the Wand Tool to select the scale bar,
then press \"OK\".";
waitForUser(title, msg);
getSelectionBounds(x, y, width, height);
//print("x value = " + x, "y value = " + y, "width= " +
width, "height= " + height);
makeLine(x, y, x+width, y);
pixscale=width;
scale=getNumber("Indicate the scale's length (in
mm), then press \"OK\".", 76.2);
run("Set Scale...", "distance=pixscale known=scale
unit=mm");

print(width + " pixels for ", scale+" mm");
// Set the working layer as "Blue"
run("Split Channels");
selectWindow(name1+" (red)");
close();
selectWindow(name1+" (blue)");
run("Scale to Fit");
run("Duplicate...", "title=Total_"+name1);
// Select the areas to remove from the analyses
// Save their coordinates for later use
setTool("rectangle");
waitForUser("Use the tool Rectangle, to select the
unwanted zone with the labels, then press \"OK\".");
getSelectionBounds(x, y, width, height);
label_x=x;
label_y=y;
label_width=width;
label_height=height;
setColor(255,255,255);
fill();
// Threshold the total leaf area
setAutoThreshold("Default");
setThreshold(0, 250);
//title = "WaitForUser";
//msg = "If necessary use \"Threshold\" to adjust the
threshold, then press \"OK\".";
//waitForUser(title, msg);
run("Convert to Mask");
run("Options...", "iterations=1 count=1 do=[Fill
Holes]");
run("Options...", "iterations=6 count=1 do=Erode");
run("Options...", "iterations=4 count=1 do=Dilate");
// Identify the leaflets as the first ROI
setTool("wand");
title = "WaitForUser";
msg = "select the leaflets with the wand tool (shift +
click for multiple selection), then press \"OK\".";
waitForUser(title, msg);
Roi.getBounds(x, y, width, height);
roiManager("Add");
run("Set Scale...", "distance=pixscale known=scale
pixel=1 unit=mm");
```

```

run("Set Measurements...", "area perimeter limit
redirect=None decimal=1");
run("Analyze Particles...", "size=1-Infinity display
include summarize");
roiManager("Select", 0);
// make a black working image to measure damages
// Paste the leaflets ROI
newImage("mine_area_"+name, "8-bit black",
img_larg, img_haut, 1);
// Separate the mines from the faeces areas, which
// are not white, since they contain black faces
selectWindow(name1+" (green)");
run("Duplicate...", "title=faeces_area_"+name);
makeRectangle(label_x, label_y, label_width,
label_height);
setColor(255);
fill();
setAutoThreshold("Default");
setOption("BlackBackground", false);
setThreshold(0, 47);
title = "WaitForUser";
msg = "If necessary use \"Threshold\" to adjust the
threshold, then press \"OK\".";
waitForUser(title, msg);
run("Convert to Mask");
run("Options...", "iterations=1 count=1 do=[Fill
Holes]");
run("Options...", "iterations=6 count=1 do=Dilate");
run("Options...", "iterations=6 count=1 do=Erode");
run("Options...", "iterations=1 count=1 do=[Fill
Holes]");
run("Set Scale...", "distance=pixscale known=scale
unit=cm");
run("Analyze Particles...", "size=0.1-Infinity
circularity=0.0-1 display include summarize");
// Threshold mine areas of white color
selectWindow(name1+" (green)");
roiManager("Select", 0);
run("Copy");
selectWindow("mine_area_"+name);
roiManager("Select", 0);
setSelectionLocation(x, y);
run("Paste");
setAutoThreshold("Default");
setOption("BlackBackground", false);
setThreshold(180, 255);
title = "WaitForUser";
msg = "If necessary use \"Threshold\" to adjust the
threshold, then press \"OK\".";
waitForUser(title, msg);
run("Convert to Mask");
selectWindow(name1+" (green)");
close();
selectWindow(name1+" (blue)");

```

```

close();
// combine the two areas previously thresholded
imageCalculator("OR",
"mine_area_"+name,"faeces_area_"+name);
selectWindow("mine_area_"+name);
run("Options...", "iterations=1 count=1 do=[Fill
Holes]");
run("Options...", "iterations=6 count=1 do=Dilate");
run("Options...", "iterations=6 count=1 do=Erode");
run("Options...", "iterations=1 count=1 do=[Fill
Holes]");
run("Set Scale...", "distance=pixscale known=scale
unit=cm");
run("Analyze Particles...", "size=0.5-Infinity
circularity=0.0-1 display include summarize add");
run("Tile");
// Display the ROIs of Impacts in Magenta on initial
image
// (warning, not the first ROI which contains the
leaflets)
selectWindow(name);
count=roiManager("count");
for (i=1; i<count; i++) {
roiManager("Select", i);
Roi.setFillColor("Magenta");
}
roiManager("update");
// Save file ROI next to analyzed image
roiManager("Save", newpath+"_ROI_.zip");

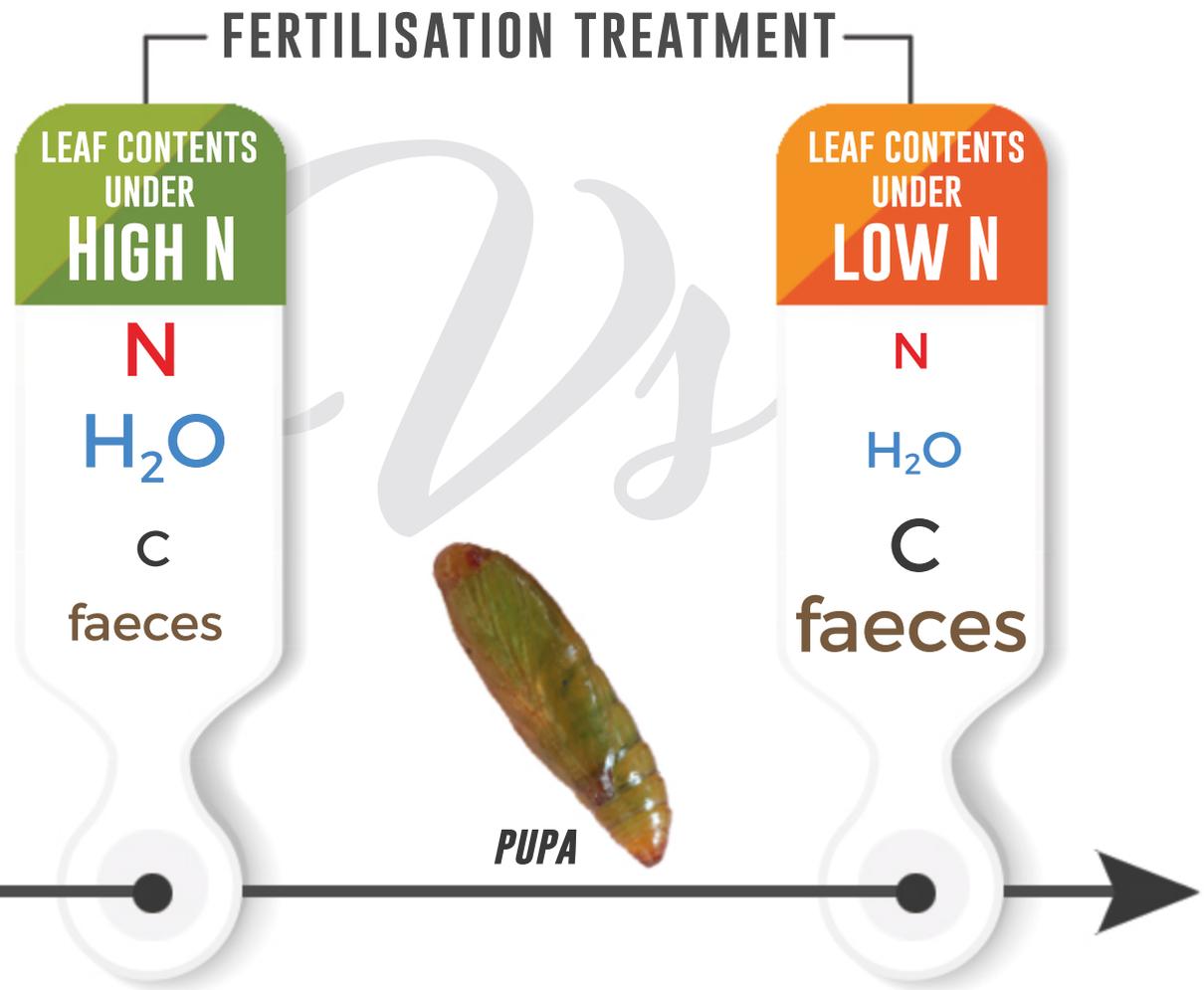
```

# TOMATO X *TUTA ABSOLUTA*



LARVA

DEVELOPMENTAL TIME



- *T. absoluta* larvae improved C, N and water status of mined tomato leaves
- An original method evaluated miner's resource ingestion and frass production
- N limitation forced larvae to ingest less N and water, but more C and biomass
- Under N limitation, larvae produced more frass
- Under N limitation, time needed for larvae to reach pupae was delayed

ACCEPTED MANUSCRIPT