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How do plants defend themselves? : Study of the Tomato - Tuta absoluta pathosystem in interaction with nitrogen fertilization

Victoire Coqueret

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Victoire Coqueret. How do plants defend themselves? : Study of the Tomato - Tuta absoluta pathosystem in interaction with nitrogen fertilization. Agronomy. Université de Lorraine, 2017. English. NNT : 2017LORR0234 . tel-01924665

HAL Id: tel-01924665

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DOCTORAT INRA - RÉGION LORRAINE

THÈSE

présentée et soutenue publiquement pour l'obtention du titre de docteur
de L'Université de Lorraine
spécialité doctorale « Sciences Agronomiques »

par

VICTOIRE COQUERET
LE 12 DÉCEMBRE 2017

HOW DO PLANTS DEFEND THEMSELVES?

STUDY OF THE
TOMATO - *Tuta absoluta*
PATHOSYSTEM IN
INTERACTION WITH
NITROGEN FERTILIZATION

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FONCTIONNEMENT DE LA PLANTE AGRESSÉE:

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TOMATE - *Tuta absoluta*
EN INTERACTION AVEC LA
FERTILISATION AZOTÉE

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REMERCIEMENTS

Je remercie, tout d'abord, les financeurs de ce projet de thèse, l'**INRA** et la **région Lorraine**, ainsi que la **DAAD** qui m'ont permis de réaliser ce travail.

Je souhaite aussi remercier mon directeur et mon co-directeur de thèse, **Dr Christophe ROBIN** (DR) et **Dr Romain LARBAT** (CR) pour m'avoir confié ce projet de thèse et m'avoir encadrée au cours de ces quatre années. Je vous remercie pour l'entière confiance que vous m'avez accordée et pour vos conseils, avis et suggestions pour mener à bien ce projet.

Je tiens également à remercier **Dr Jacques LE BOT** (CR), **Dr Stéphane ADAMOWICZ** (CR) et **Dr Sybille UNSICKER** qui m'ont encadrée pendant tout ou partie de ma thèse, pour leurs conseils, leur aide et leur gentillesse. Je remercie de nouveau Jacques pour les nombreuses analyses d'images qu'il a effectuées et Stéphane pour son aide précieuse en statistique. Je remercie aussi **Pr Jonathan GERSHENZON** de m'avoir accueilli pendant 3 mois et demi au sein de son département de recherche, Max Planck Institut for Chemical Ecology.

Je remercie aussi tous les membres de mon comité de thèse **Dr Didier LE THIEC** (DR), **Dr Nicolas DESNEUX** (CR), **Dr Corinne ROBERT** (CR), **Dr Loïc PAGES** (CR) et **tous mes encadrants** pour leur expertise et leurs conseils. De même, je remercie **Pr Sylvain PLANTUREUX**, **Pr Alain HEHN**, **Dr Jean VILLERD** (IR), **Dr Nadia MICHEL** (MC), **Dr Helmut MEISS** (MC), **Dr Alice MICHELOT** (MC) et **Dr Christian BOCKSTELLER** (IR) pour leur participation active aux exercices de réflexion sur mon projet de thèse. Je remercie chaleureusement les membres du jury qui ont accepté de réviser mon travail avec bienveillance, notamment les rapporteurs **Pr Marco TREVISAN** et **Pr François VERHEGGEN**, mais aussi les examinateurs **Dr Sybille UNSICKER** et **Pr Murielle ADRIAN**.

Je remercie également **Dr Ernest KIRKBY** pour la révision de l'anglais de mon tout premier article et je remercie **Valerie** et **Jean-Luc GENIEUX** pour la révision de l'anglais de ce manuscrit.

Je tiens aussi à remercier toutes les personnes qui m'ont apporté leur aide pour la mise en place et le déroulement

des expérimentations, pour la réalisation et l'interprétation des diverses analyses réalisées. Je cite : **Aude FAUVET**, **Dr Jean VILLERD**, **Claude GALLOIS**, **Jérémy GROSJEAN**, **Dr Michaël REICHEL**, **José FABRE**, **Guillaume GARCIA**, **Valérie SERRA** et **Philippe BEAREZ**. Merci à vous tous.

Je remercie également **Christine FIVET** et le directeur de l'Ecole Doctorale RP2E **Pr Stéphane DESOBRY**, pour leur travail, leur efficacité, leur confiance et parfois même, leur patience. Je remercie également les personnes qui m'ont encadrée au sein de l'EIR-Agreenium : **Cathy MEJEAN** et **Mireille MOURZELA**. Je remercie aussi **Etienne HAOUY** et **Justine GALET** pour leurs conseils lors du concours "Ma Thèse en 180 secondes" .

Je remercie sincèrement **Dr Dorothea THOLL**, **Dr Martin HEIL**, **Pr Harro BOUWMEESTER**, **Pr Florian SCHIESTL**, **Dr Sybille UNSICKER** et **Dr Michael GUTENSOHN** pour l'organisation du Gordon Research Conference et Gordon Research Seminar et pour m'avoir permis de présenter un poster, en 2016.

Je remercie également mes ex-collègues de travail du LAE, **Dr Célia KRIEGER**, **Dr Aline CHANSEAUME**, **Dr Nicolas ROMILLAC** et **Elodie WARISSE** pour leur joie de vivre et j'ai une pensée particulière à **Béatrice NOIRTIN**, qui était une femme merveilleuse et avec qui j'aimai discuter des choses de la vie.

Je remercie également tous les étudiants du MPI qui m'ont aidé à m'intégrer très rapidement au laboratoire : Sandra LACKNER, Franziska EBERL, Carlos SANCHEZ ARCOS, Jan GÜNTHER, Erica PERRECA, Simone FROMMEYER, Dineshkumar KANDASAMY, mais aussi Andrew, Marcos, Aileen, Aleksa, Diego, Vinzenz, Thomas, Andria, Maria, Ross, Selina, Chhana, Felipe, Verena, Anton...

Enfin, je remercie mes parents **Marie-Odile** et **Xavier** ainsi que mes frères et sœurs **Charles**, **Elise**, **Clémence** et **Martin** et ma belle-sœur **Marie** pour leur soutien et leurs encouragements. Je remercie plus particulièrement ma sœur Elise pour les travaux graphiques qu'elle a réalisés pour ma thèse (Figure 21 page 89, le poster présenté au GRC et la mise en page de ce manuscrit).



ACKNOWLEDGEMENTS

First of all, I would like to thank the sponsors of this thesis project, **INRA** and the **Lorraine region**, as well as the **DAAD**, which enabled me to carry out this work.

I would also like to thank my supervisor and co-supervisor, **Dr Christophe ROBIN** (DR) and **Dr Romain LARBAT** (CR) for having entrusted me with this Ph.D. project and to have supervised me during these four years. I thank you for the confidence you have placed in me and for your advice and suggestions in order to carry out this project.

I would also like to thank **Dr Jacques LE BOT** (CR), **Dr Stéphane ADAMOWICZ** (CR) and **Dr Sybille UNSICKER** for giving me advice, help and kindness during all or part of my thesis. I thank Jacques again for the many analyses of leaf surfaces that he made and Stéphane for his precious help in statistics. I would also like to thank **Pr Jonathan GERSHENZON** for hosting me for 3 months and a half in his research department, Max Planck Institute for Chemical Ecology.

I also thank all the members of my thesis committee, **Dr Didier LE THIEC** (DR), **Dr Nicolas DESNEUX** (CR), **Dr Corinne ROBERT** (CR), **Dr Loïc PAGES** (CR) and **all my supervisors** for their expertise and advice. I would like to thank **Pr Sylvain PLANTUREUX**, **Pr Alain HEHN**, **Dr Jean VILLERD** (IR), **Dr Nadia MICHEL** (MC), **Dr Helmut MEISS** (MC), **Dr Alice MICHELOT** (MC) and **Dr Christian BOCKSTELLER** for their active contributions during the reflection exercises on my Ph.D. project.

I warmly thank the members of the jury who agreed to review my work with sympathy, notably the reviewers **Pr Marco TREVISAN** and **Pr François VERHEGGEN**, but also the examiners **Dr Sybille UNSICKER** and **Pr Murielle ADRIAN**.

I also thank **Dr Ernest KIRKBY** for the revision of the English of my very first article and I thank **Valerie** and **Jean-Luc GENIEUX** for the revision of the English of this manuscript.

I would also like to thank all the people who helped me with the set-up and running of the experiments, for the realization and interpretation of the various analyses carried out: **Aude FAUVET**, **Dr Jean VILLERD**, **Claude**

GALLOIS, **Jérémy GROSJEAN**, **Dr Michaël REICHEL**, **José FABRE**, **Guillaume GARCIA**, **Valérie SERRA** and **Philippe BEAREZ**. Thank you to all of you.

I would also like to thank **Christine FIVET** and the director of the RP2E Doctoral School, **Pr Stéphane DESOBRY**, for their work, their efficiency, their confidence and sometimes even their patience. I would also like to thank the people who helped me with the EIR-Agreenium: **Cathy MEJEAN** and **Mireille MOURZELA**. I also thank **Etienne HAOUY** and **Justine GALET** for their advice during the "*Ma thèse en 180 secondes*" competition.

I sincerely thank **Dr Dorothea THOLL**, **Dr Martin HEIL**, **Dr Harro BOUWMEESTER**, **Pr Florian SCHIESTL**, **Dr Sybille UNSICKER** and **Dr Michael GUTENSOHN** for organizing the Gordon Research Conference and Gordon Research Seminar and for affording me a participation, in 2016.

I would also like to thank my ex-AEA colleagues, **Dr Célia KRIEGER**, **Dr Aline CHANSEAUME**, **Dr Nicolas ROMILLAC**, **Elodie WARISSE** and **Aude FAUVET** for their joy of life and their moral support and I have a special thought to **Béatrice NOIRTIN** who was a wonderful woman and with whom I liked to discuss the things of life.

I am also grateful to all the students who were/are working at the MPI and who facilitated my integration into the group: Sandra LACKNER, Franziska EBERL, Carlos SANCHEZ ARCOS, Jan GÜNTHER, Erica PERRECA, Simone FROMMEYER, Dineshkumar KANDASAMY, and Andrew, Marcos, Aileen, Aleksa, Diego, Vinzenz, Thomas, Andria, Maria, Ross, Selina, Chhana, Felipe, Verena, Anton... Finally, I thank my parents **Marie-Odile** and **Xavier** and my brothers and sisters **Charles**, **Elise**, **Clémence**, **Martin** and **Marie**, my sister in law, for their moral support and encouragements. In particular, I would like to thank my sister Elise for the graphic work she did for my thesis (Figure 21 page 89, the poster presented to the RCMP and the layout of this manuscript).

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**4TH
COVER SUMMARY**

ABBREVIATIONS

Api : Apio furanosyl rutin	ISA : Laboratory UMR 1355 INRA – 7254 CNRS - Nice Sophia Antipolis University, Sophia Agrobiotech Institute
C : Carbon	KR : Kaempferol rutinoside
CGA : Chlorogenic acid	L : Local
CHA : Caffeic hexaric acid	LAE : Laboratory UMR 1121 Université de Lorraine-INRA Agronomy and Environment Nancy-Colmar
CNBH : Carbon nutrient balance hypothesis	LN : Low nitrogen
CNRS : Centre national de la recherche scientifique (National center for scientific research)	LOX : Lipoxydases
CO₂ : carbon dioxide	MC : Maître de conférence
COP : Parts conference (conférence des parties)	MPI : Max Planck Institute
CoQA : p-coumaroyl quinic acid	N : Nitrogen
CP : Caffeoyl putrescine	NFT : Nutrient Film Technique
CR : Chargé de recherche	Pi : <i>Phytophthora infestans</i>
DAAD : Deutscher Akademischer Austauschdienst	PI : Protein inhibitors
Das : Days after sowing	POD : Peroxydases
Dat : Days after transplantation	PPO : Polyphenol oxydases
DR : Directeur de recherche	PSH : Laboratory UR 1115 INRA of Avignon Plants and Horticultural Systems
DW : Dry weight	Pst : <i>Pseudomonas syringae</i> PV tomatata
EPPO : European and Mediterranean plant protection and organisation	Rut : Rutin
EWT : Equivalent water thickness	S : Systemic
FA : Formic acid	SLC : Specific leaf carbon
FQA : Feruloyl quinic acid	SLN : Specific leaf nitrogen
Fol 2 : <i>Fusarium oxysporum</i> sp. lycopersici	SLW : Specific leaf weight
For : <i>Fusarium oxysporum</i> sp. radice lycopersici	T : Temperature
FW : Fresh weight	TMV : Tobacco mosaic virus
GDBH : Growth differentiation balance hypothesis	TSWV : Tomato spot wilt virus
HN : High nitrogen	UMR : Unité mixte de recherche
Hx, x = 1, 2 or 3 : Harvest number 1, 2 or 3	UR : Unité de recherche
INRA : Institut national de la recherche agronomique (French national institute for agricultural research)	VOC : Volatile organic compound
IR : Ingénieur de recherche	

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ENGLISH VERSION

1.1

FOREWORD

About 10 years ago, the global society became aware of the dangers of plant protection products on environment and human health. To regulate the use of these products, plans have been established in France and in Europe, and agreements have been signed between more than 200 countries. In this context, agriculture must find alternative methods for controlling pests and maintaining the crop production. The use of insect predators, sexual confusion or the stimulation of natural plant defences are some biological methods on which agronomists work. My research work falls within this agro-environmental logic with the goal to reduce the use of plant protection product in agriculture and to promote a plant's natural defences, once known. This work emerged from a collaborative project between the laboratory UMR 1121 Université de Lorraine-INRA Agronomy and Environment Nancy-Colmar (LAE), the laboratory UR 1115 INRA of Avignon Plants and Horticultural Systems (PSH) and the laboratory UMR 1355 INRA - 7254 CNRS - Nice Sophia Antipolis University, Sophia Agrobiotech Institute (ISA). This collaboration seeks to understand the effect of nitrogen availability on tomato ecophysiological responses. The previous Ph.D. student, Mathilde ROYER, studied the trade-off between plant growth and defence in response to nitrogen limitation and biotic stresses such as *Pseudomonas*, *Botrytis* and *Tuta absoluta*. She showed that C:N ratio is a reliable

indicator for this trade-off, that resources allocation between different metabolites varies with pests and each pest reacts specially to changes of nitrogen availability for the plant. She made a first experiment showing that *Tuta absoluta* herbivory did not induce the synthesis of tomato a number of defence compounds. The hypothesis was that the damage intensity was too low to induce a plant response.

Hence, my Ph.D. project contributes to the identification of plant ecophysiological responses to herbivore stress in the context of tomato-*Tuta absoluta* pathosystem. This project has been financed by INRA and the Lorraine Region, from October 2013 to September 2016. I conducted my work in the LAE where I have been supervised by Christophe ROBIN and Romain LARBAT. My work benefited from the expertise of: Jacques LE BOT and Stéphane ADAMOWICZ, from PSH, Nicolas DESNEUX from ISA and of Sybille UNSICKER from the Chemical Ecology laboratory, a research unit of the Max Planck Institute, in Jena. Around this project, we gathered scientific partners from INRA of Avignon, INRA of Sophia Antipolis and the Max Planck Institute of Jena.

Enrolled at the RP2E Doctoral School, I have also benefited from the training courses offered by the Agreenium International School of Research (EIR-Agreenium). Agreenium is a French consortium of research centers and universities that promotes

excellence in doctoral studies in the field of life sciences. Notably thanks to the EIR-Agreenium, INRA, MPI and DAAD (Deutscher Akademischer Austauschdienst), I carried out a three and a half month internship in the Chemical Ecology laboratory of MPI in Jena, Germany. The work carried out during this internship is an integral part of my Ph.D. project. In this respect, I ask for obtaining of two labels: the EIR-Agreenium label of excellence and the European Doctorate label.

During my thesis, I benefited from two meetings with a thesis committee. The first one took place in June 27, 2014 and brought together Christophe ROBIN, Romain LARBAT, Jacques LE BOT, Stéphane ADAMOWICZ, Didier LE THIEC (INRA EEF - Nancy), Nicolas DESNEUX and Corinne ROBERT (INRA Ecosys - Grignon). The second one took place on September 10, 2015; Sybille UNSICKER and Loïc PAGES (PSH) joined this committee.

Following this brief foreword on my doctoral path, I suggest going through the heart of the scientific project by reading this manuscript based on the scheme of scientific publications. In this first chapter, you will find a bibliographical introduction and the presentation of the goals and issues of my Ph.D. and the experimental strategy.

BIBLIOGRAPHIC REVIEW

1.2.1

AGRICULTURE AND ENVIRONMENT: A NEW WAY TO THINK

Agriculture, that is to say the cultivation of plants and the breeding of animals, began about 10,000 years ago. The domestication of plants required an understanding of their developmental and reproductive cycle which allowed the selection of phenotypic characters from wild plants. Characteristics were selected, such as the small size of the wheat tillers to avoid lodging or the brittle peduncle facilitating fruit harvesting. However, the main characteristics selected for increasing yields were fruit size, number of grain per ear, and nutritional richness of fruits (Doré *et al.*, 2006). Therefore, most of the selected plants favour the allocation of resources towards fruits, seeds, tubers, ... to the detriment of the defence mechanisms. This selection, coupled with the development of cultivation methods (clearing, ploughing, triennial rotation, *etc.*), made possible the control of crops. During the 20th century, the development of chemical inputs, agricultural machinery and plant genetic improvements enabled a sharp increase in agricultural yields. Indeed, nitrogen, an essential element for the growth of plants, is present in many molecules enabling cells' proper functioning; it is found in DNA molecules (carrier of genetic information) or in proteins (catalysts of chemical reactions). Nitrogen fertilization, combined with phosphorus and potassium, promotes plant growth and significantly increases yields (Chorley, 1981; Adekiya and Agbede, 2009, Olaniyi and Ajibola,

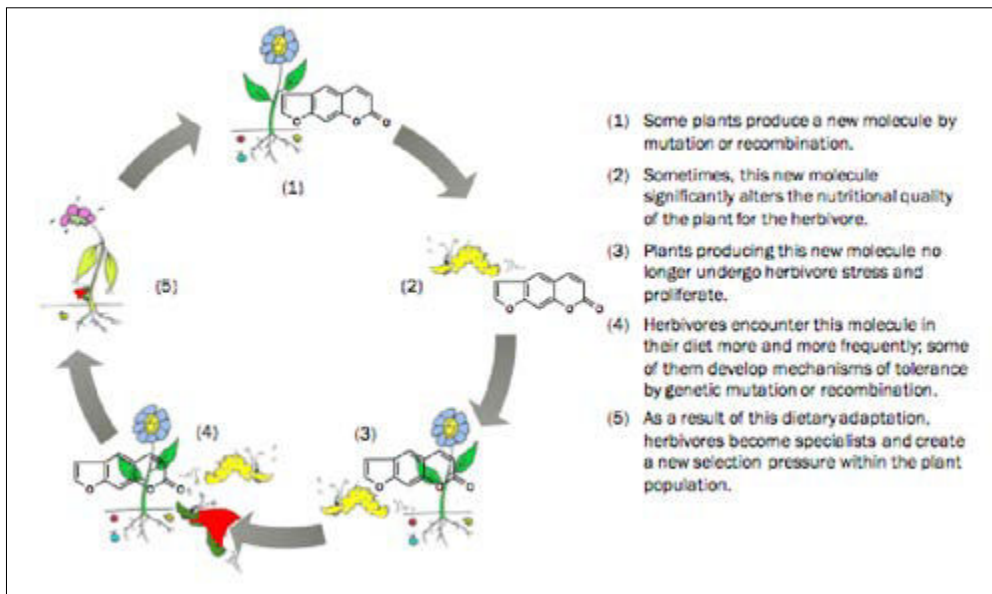
2008). Moreover, because of their toxic properties, many natural defence compounds have been counter-selected in wild species, making crops more vulnerable to biotic attack and requiring more and more pesticide applications. But, for the first time, in 1962, Rachel Carson describes the harmful effects of chemical inputs such as insecticides, fungicides and nitrogenous amendments, on population and on the environment (Carson, 1962). Indeed, the application of massive doses of nitrogenous fertilizers led to a sharp increase in the nitrate content of groundwater. In these waters, excess of nitrate modifies the biological equilibrium which favours the development of certain fungi and bacteria which making the groundwater impure. Ingested in too greater quantity, this polluted water has an adverse effect on human health (Bouchard *et al.* 1992). Moreover, recent studies sounded the alarm bell by highlighting the effect and the risk of pesticide applications on human health and non-vertebrate biodiversity. Indeed, some studies have shown the presence of pesticide residues in the fatty tissue of tumors and incriminate these chemicals (Falck *et al.*, 1992; Aronson *et al.*, 2000). Other studies correlate the time and frequency of pesticide exposure to the presence of tumors in the population (Hardell and Erikson, 1999; Alavanja *et al.*, 2004). More recently, studies show the pesticide threat for non-vertebrate diversity and recall the essential role played by pollinators or

insect predators such as bees or ladybirds, for crops (Brittain *et al.*, 2010; Geiger *et al.*, 2010; Tschardtke *et al.*, 2012). Thus, important projects led to the development of pesticide risk evaluation tools on agro-ecosystem and on human health, in particular the HAIR (HARmonised environmental Indicators for pesticide Risk) project (Calliera *et al.*, 2006 and 2008).

For the last ten years, France and many other countries around the world have been trying to limit the use of these products by legislating on their use and by encouraging the search for alternative methods such as sustainable cultivation methods,

bio-pesticides or biological control. The decision-support tools, which have been developing over the last ten years, make it possible to predict the risks of infestation and / or infection and to reasonably apply plant protection products. The Ecophyto II plan reaffirms the French government's objective to halve the use of these products in agriculture (Plan Ecophyto II, 2015). As agronomist, our role is to provide answers to limit the use of chemical inputs and maintain good crop productions. But for that, we have first to understand plant defence mechanisms.

1.2.2 PLANT DEFENCE



■ **FIGURE 1** : PLANT-INSECT CO-EVOLUTION SCHEME. ADAPTED FROM BERENBAUM, 1983.

• A CO-EVOLUTION STORY

The first terrestrial plants appeared during the Upper Ordovician period, about 450 million years ago (Steemans *et al.*, 2009). Since then, plants have adapted to abiotic constraints and have co-evolved with the pathogens and herbivores that would have appeared at the end of the Silurian period (Labandeira, 2007). Co-evolution is a concept that was described by Ehrlich and Raven in 1964. Twenty years later, Berenbaum described a hypothetical scenario of co-evolution between plants producing specific defence compounds (coumarins) and herbivorous insects. The progress of a co-evolution phase is based on 5 steps, see Figure 1.

(1) Some plants produce a new molecule by natural genetic mutation or recombination.

(2) Sometimes, this new molecule significantly alters the nutritional quality of its tissues for the herbivore.

(3) Thus, plants producing this molecule no longer undergo herbivore stress and are able to proliferate.

(4) Herbivores encounter this molecule in their diet more and more frequently. Some of them develop tolerance mechanism by genetic mutation or recombination.

(5) As a result, herbivores become specialists and create a new selection pressure within the plant population. These five steps have been repeated in a loop, leading today to a diversity of defence molecules in plants (Berenbaum, 1983).

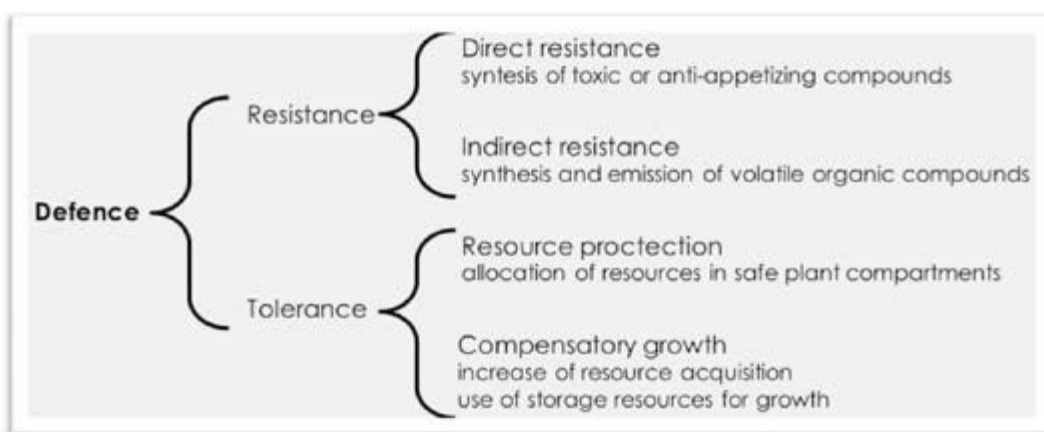
Plants' adaptation required the establishment of defensive mechanisms, thanks to genetic mutations that allowed the creation of new enzymes. Enzymes are actors of all chemical reactions in cells. These chemical reactions allow the synthesis of molecules and an indefinable number of genetic mutations and recombinations led to plant macroscopic changes such as the rosebushes' spines (Lev-Yadun, 2001) or trichomes (Schillmiller, 2010) in addition to the hundreds of thousands of defence compounds estimated in plant kingdom (Schuman and Baldwin, 2016).

• A COMBINATION OF RESISTANCE AND TOLERANCE

Defence mechanisms have been classified according to two strategies: resistance, which is directly reducing the “fitness”, *i.e.* the “reproductive success” of the aggressor and tolerance, decreasing the impact of the aggressor on the plant (Figure 2). Resistance involves the synthesis of specific defence compounds that may be present in the plant tissues or may be emitted in the air due to their volatility. These defence compounds can be toxic, anti-appetizing, repellent to herbivorous insects (Dicke *et al.*, 2003) or attract predatory insects or parasites of the herbivore (Halitschke *et al.*, 2008). More than 70.000 defence compounds have been recorded to date (Fürstenberg-Hägg *et al.*, 2013). The three main chemical classes to which these molecules belong are terpenoids, phenols and alkaloids.

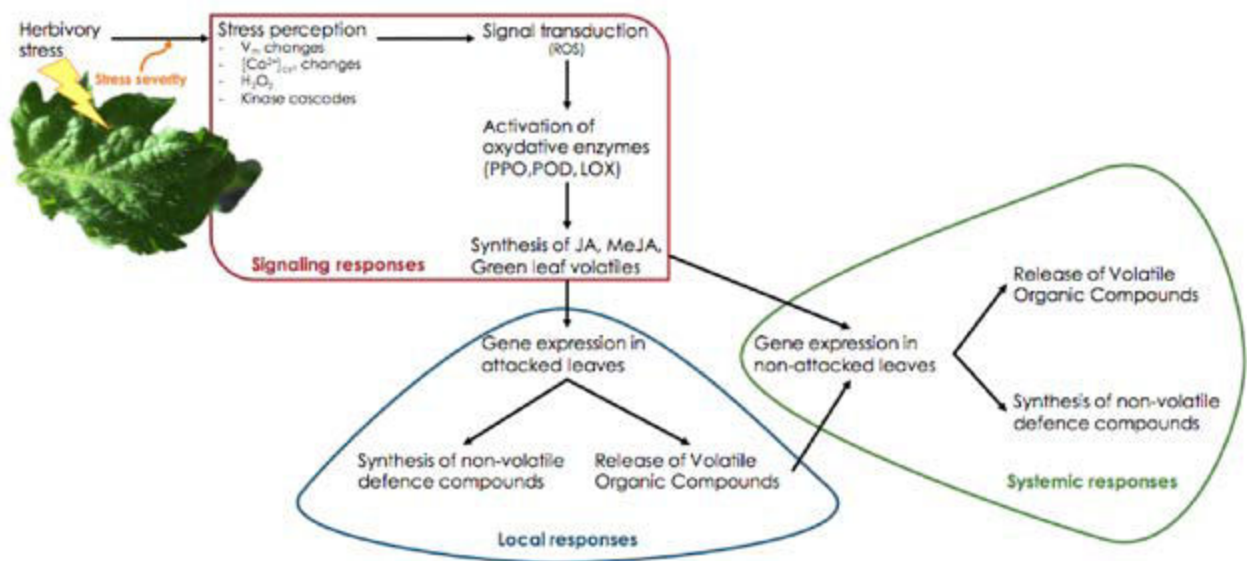
Tolerance includes all the mechanisms that limit the herbivory impact on plant fitness. Ecological and evolutionary implications of plant tolerance to herbivory are still under study (Fornoni, 2011). Two theories have been formulated following observations of a number of tolerance mechanisms:

resource protection and compensatory growth. During aggression, maize is able to move its carbohydrate resources (Holland *et al.*, 1996) and tomato to move amines (Gomez *et al.*, 2010) to compartments far from the infection zone and which will allow the plant to continue its growth and development as soon as the assault is over. The theory of compensatory growth is based on the ability of a plant to increase carbon uptake by photosynthesis (Thomson *et al.*, 2003) and / or nitrogen by root absorption (Newingham *et al.*, 2007), allowing the plant to compensate the tissue loss. Compensatory growth is however not a systematic tolerance response, since in contrast, some plants subjected to leaf herbivory were shown to reduce the photosynthetic capacity or carbon fixation in the neighbouring tissues of the wound, such as *Datura* subjected to *Manduca sexta* herbivory and the tomato subjected to a methyl-jasmonate treatment, a defence elicitor (Barron-Gafford *et al.*, 2012; Gomez *et al.*, 2010).



■ **FIGURE 2** : PLANT DEFENCE STRATEGIES

Plant defence strategies are resistance, involving direct resistance by producing toxic and anti-appetizing compounds and indirect resistance by producing and emitting volatile organic compounds, and tolerance involving resource protection by allocating resources in safe plant compartments and compensatory growth involving the resource acquisition and promoting plant regrowth.



■ **FIGURE 3** : PLANT DEFENCE INDUCTION

Bites create cellular lesions that result in an electrical potential inversion of the membrane interface. This electrical signal rapidly produces the liberation of calcium ions, the formation of hydrogen peroxide and a cascade of enzymatic phosphorylations. Following this perception, the plant produces hyperreactive oxidative species (ROS), activates oxidative enzymes (LOX, PPO, POD) and synthesizes phytohormones such as jasmonic acid or methyl jamonate and even green leaf volatiles (GLV). These phytohormones and volatiles allow activation of local responses near the aggression site and systemic responses. These local and systemic responses result in the activation of gene expressions which allow the synthesis of many non-volatile and volatile defence compounds.

• DEFENCE INDUCTION

Plants naturally have a constitutive level of defence that limits first infections (Wittstock and Gershenzon, 2002). But in case of attack, plants are able to reinforce their defences thanks to perception, recognition and signalling mechanisms.

STRESS PERCEPTION AND RECOGNITION

When a herbivore attacks a plant, bites or piercings create cell damages and generate an electrochemical disturbance of the cell membrane, followed by a cytoplasmic release of the Ca^{2+} ions (Lecourieux *et al.*, 2006) and causing oxidative stress expressed by the production of reactive oxidative species (ROS), which are deleterious to the insect (Figure 3). This phenomenon is called hypersensitive reaction.

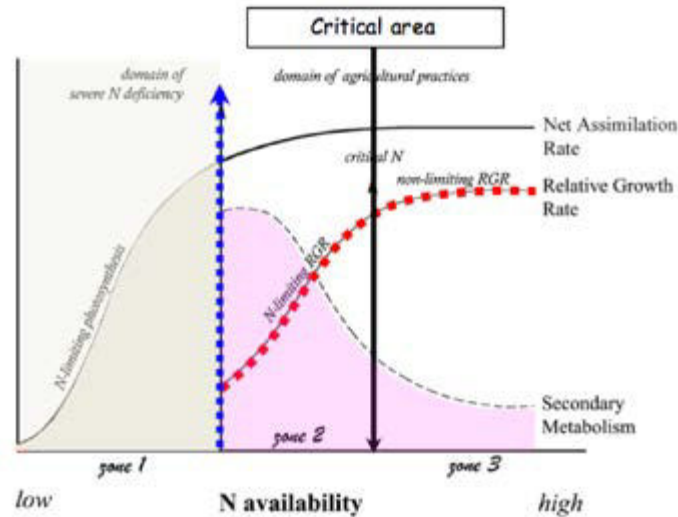
In parallel to the perception of physical damage, plants are able to recognize a number of the insect salivary molecules, acting as plant defence elicitors (Alborn *et al.*, 1997, Halitschke *et al.*, 2001, Musser *et al.*, 2006 and Maffei *et al.*, 2007). After this recognition, plants adapt the signalling pathway specifically to the aggressor and activate specific biosynthetic pathways.

ENZYMATIC ACTIVATION AND HORMONAL SIGNAL PROPAGATION

Following the stress perception and recognition, calcium ion influx activates calcium-dependent enzymes that promote phosphorylation reactions and modify transcriptional activities (Kanchiswamy *et al.*, 2010; Figure 3). Antioxidant enzymes are then synthesized and activated (POD: peroxidase, LOX: lipoxygenase, PPO: polyphenoloxidase) to produce toxins and reduce the quality and digestibility of plant tissues. The synthesis proportion of each enzyme is specific to the pathosystem (Stout *et al.*, 1994; War *et al.*, 2012).

Many hormones, such as salicylic, jasmonic acids and ethylene, have been identified playing a role in the stress propagation. These hormones, synthesized in the aggression area, are transmitted to the rest of the plant *via* the vascular system or emitted into the air. They act alone, in synergy or antagonistically, in the activation of specific defence biosynthetic pathways against the pest, locally to the aggression site and in the other compartments of the plant (Onkokesung *et al.*, 2010).

NITROGEN LIMITATION : A LEVER TO IMPROVE NATURAL PLANT DEFENCE



■ **FIGURE 4** : NORMS OF REACTION FOR NET ASSIMILATION RATE, RELATIVE GROWTH RATE AND SECONDARY METABOLISM IN RESPONSE TO CHANGING RESOURCE AVAILABILITY, LE BOT *ET AL.* 2009, ADAPTED FROM HERMS & MATTSON, 1992)

CO₂ net assimilation, relative growth rate and the secondary metabolism are severely and negatively affected by nitrogen deficiency. For nitrogen availability above the critical value, the CO₂ net assimilation and the relative growth rate reach an optimal high, while the intensity of secondary metabolism is lower. Between these two levels of nitrogen, defence metabolism reaches its optimum intensity, whereas CO₂ assimilation has already reached its optimal high and the relative growth rate is slightly lower than its optimal.

A theory correlated the availability of resources and the defence level of plants. This theory, developed in 1983 and called Carbon - Nutrients Balance Hypothesis (CNBH) predicts that carbon will be more easily allocated to the synthesis of carbonaceous compounds in plants that grow on nitrogen - poor soils. Conversely, for plants that grow on low carbon soils, the allocation of resources will promote the synthesis of nitrogen defence compounds (Bryant *et al.*, 1983). Herms and Mattson (1992) expanded this theory on all factors that slow plant growth more actively than photosynthesis, and called it GDBH (for Growth Differential Balance Hypothesis). Thus, nitrogen availability can be used to enhance plant defence metabolism, indeed,

for a limited nitrogen availability (zone 2, Figure 4), defence metabolism reaches its optimum intensity, whereas CO₂ assimilation has already leveled off and the relative growth rate is slightly lower than its optimal.

This phenomenon has been observed for *Pentaclethra macroloba*, a tropical tree, presenting a foliar concentration of saponins increasing with a decrease of nitrogen availability, in contrast to the concentration of flavanes (Massad *et al.*, 2012). In tomato plants, another plant specie (*Solanum lycopersicum*), nitrogen limitation increased the foliar concentration of simple carbohydrates and defence compounds, such as chlorogenic acid, rutin or kaempferol-rutinoside (Le Bot *et al.*, 2009).

• TOMATO

Tomato, *Solanum lycopersicum*, is a dicotyledone specie of the Solanaceae family, originally from South America. The tomato has become a model plant for the genetic improvement of vegetable plants (Kimura and Sinha, 2008). Scientific advances have made it possible to create tomato varieties adapted to different climatic regions, to different modes of cultivation and whose organoleptic characteristics of the fruit correspond to the demand. According to FAO statistics (2014), tomatoes are the first vegetable production in the world and are now widely grown on every continent. Originally, the cultivation of tomatoes was practiced in soil, although, it has been gradually replaced by the greenhouse and / or soil-less cultivations. Greenhouse tomato cultivation limits the impact of adverse climate, but it is often subjected to biotic aggression and therefore requires the growers' utmost vigilance. Most of the bio-aggressors belong to the reign of fungi, virus, bacteria and animals (insects). Tomato pathogenic fungi include *Oidium neolycopersici*, the causal agent of the powdery mildew; *Phytophthora infestans*, the mildew agent; *Botrytis cinerea*, agent of the gray rot. These fungi directly extract the nutrients necessary for their development in the cells of the host plant (Watterson, 1986; Corbaz, 1990). *Clavibacter michiganensis* and *Pseudomonas syringae* are

two pathogens of bacterial canker and speckle, respectively, highly dreaded by tomato growers. These bacteria develop into the intercellular spaces and the cells around it are killed and used as a nutrient medium (Corbaz, 1990; Blancard, 2009). Insects damages caused to the tomato are specific according to the insect feeding guild. Sucking insects such as aphids (*Aphis gossypii* or *Myzus persicae*) or whiteflies cause indirect damages, since aphids can transport and transmit viruses to the plant. They also produce honeydew on leaf surface, and this promotes fungal and bacterial development (Berlinger, 1986; Csizinszky *et al.*, 2005; Nayka *et al.*, 2005). Many caterpillars, such as *Helicoverpa armigera* or *Chrysodeixis chalcites*, feed on the foliage and sometimes even attack the fruits with their grinding jaws. Other larvae described as leafminers, such as *Tuta absoluta*, only feed on the parenchyma, digging galleries between the two foliar epidermis (Canadian Food Inspection Agency, 2012). Whatever the attack, tomato crops suffer direct productivity losses due to fruit damage or indirect ones, due to the foliage decline, thereby reducing the production of photosynthetic assimilates and hence the development of the plant and fruits.

• ***Tuta absoluta*: A TOMATO PEST, MORE AND MORE DIFFICULT TO CONTROL**

Tuta absoluta is a microlepidoptera since its wingspan is about 1 cm (Figure 5). This moth is native of South America and was first described by Meyrick in 1917. *Tuta absoluta* larva is described as a leafminer because it feeds mainly on foliar

parenchyma after penetration into the leaf. Its particular mode of nutrition makes its detection quite easy (Figure 6). The presence of this pest in a tomato crop can cause a complete loss of the crop.



■ **FIGURE 5** : *Tuta absoluta* MOTH (MEYRICK, 1917)



■ **FIGURE 6** : TOMATO FOLIOLE PRESENTING A MINE FORMED BY A *Tuta absoluta* LARVA

Its development is extremely fast, since its development cycle lasts only 29 days at 30 °C and a female can lay up to 260 eggs (Marcano, 1995; Table 1). Therefore, *Tuta absoluta* develops more rapidly in tropical and subtropical climates. Nevertheless, it is now also present in regions of temperate climate.

Tuta absoluta was first described in Spain, in 2006 and spread over Europe and North Africa in the years 2006-2012 (Blancard, 2014; Figure 7). It first appeared in France, in the region of *Languedoc-Roussillon* at the end of 2008. This pest continues its progression towards Asia, which is the world biggest tomato producer.

Developmental stages		15°C	20°C	25°C	30°C
	Egg	10 days	7 days	4 days	3 days
	Larva	36 days	23 days	15 days	11 days
	Pupa	21 days	12 days	7 days	6 days
	Adult	23 days	17 days	13 days	9 days
	Total	90 days	59 days	39 days	29 days

■ **TABLE 1** : TIMING OF THE DIFFERENT DEVELOPMENTAL STAGES OF *Tuta absoluta* AT 15, 20, 25 AND 30 °C, ADAPTED FROM MARCANO, 1995



■ **FIGURE 7** : PROPAGATION OF *Tuta absoluta*, A TOMATO PEST
Adapted from EPPO reports (European and Mediterranean plant protection and organisation), 2016.

The intensive use of pesticides, in South America, since the 1970's, has led to the appearance of resistance. The active ingredients for which this pest has been shown to be resistant are: metamidophos and cartap which are thiocarbamates, abamectin, pyrethroid permethrin (Siqueira *et al.*, 2000) and indoxacarb and spinosad which are inhibitors of the synthesis of chitin (Lietti *et al.*, 2005). Other control methods exist: male trapping using female pheromones, is an extremely expensive technique and is often used to detect the presence of the insect or to estimate the density of its population. The introduction of predators or parasitoids is a very effective technique if these are native to the area to be treated.

More and more studies are published on the impact of various factors (temperature, predator, pesticide, *etc.*) and on the development of *Tuta absoluta* (Leite *et al.*, 1999; De Medeiros *et al.*, 2009; Krechmer and Foerster, 2015; Roditakis *et al.*, 2015; Silva *et al.*, 2015; Yalcin *et al.*, 2015; Bawin *et al.*, 2016; Han *et al.*, 2016; Martins *et al.* 2016; Ozigokce *et al.*, 2016; Perdikis and Arvaniti, 2016; Polat *et al.*, 2016; Marchioro *et al.*, 2017; Van Lenteren *et al.*, 2017), but very few investigate the defence mechanisms developed by tomatoes facing this pest.

• CURRENT UNDERSTANDING OF TOMATO DEFENCE AGAINST *Tuta absoluta*

Facing this uncontrollable pest development, an active search was conducted for the creation of resistant tomato varieties. Most tomatoes develop hairs on the surface of leaves and stems (Figure 8). These hairs, called trichomes, are fine cellular excrescences sequestering a large number of defence molecules (Schillmiller *et al.*, 2010). It has been shown that the higher the density of trichome on leaf surface, the higher was the plant resistance against *Tuta absoluta* (Oliveira *et al.*, 2012). The breakdown of these trichomes caused by the insect movements on the leaf surface generates on the one hand the release of these defence molecules and on the other hand induces the synthesis of jasmonic acid and the formation of hydrogen

peroxide (H₂O₂), a reactive oxygen species (Peiffer *et al.*, 2009). In 2006, De Resende *et al.* showed a negative correlation between the constitutive content of acylated sugars in trichomes of two tomato varieties and the survival of *Tuta absoluta* larvae. However, these varieties have a very low yield and / or fruit quality. Jasmonic acid plays an important role in plant defence. Indeed, jasmonic acid is a phytohormone that signals the presence of a bio-aggressor to the rest of the plant, *via* the plant vascular system, and activate the synthesis of defence compounds. This molecule also contributes to the pubescence of new trichomes on the leaf surface (Campos *et al.*, 2009).



■ **FIGURE 8** : PICTURE OF A TOMATO PLANT HIGHLIGHTING TRICHOMES ON LEAVES AND STEMS SURFACES.

Insect bites cause cell damage and result in a hypersensitive reaction. It has been shown that *Tuta absoluta* increases the activity of polyphenol oxidases (Larbat *et al.*, 2016) whereas tomatoes react to the leafmining herbivory of *Liriomyza trifolii*, by activation of peroxidases (Stout *et al.*, 1994). Thus, we suppose that tomato perception is specific even through leafmining herbivores. In addition, the herbivory of two newly born larvae of *Tuta absoluta* did not affect the concentration of 14 polyphenols and tomatine in tomato leaf tissues, after several days of herbivory (Larbat *et al.*, 2016). This study also revealed that *Tuta absoluta* induces no change in protein content nor in plant nitrogen distribution, therefore raising questions: does *Tuta absoluta* herbivory go unnoticed with regard to the tomato plant? Does *Tuta absoluta* inhibit the mechanisms of tomato defences? Or: is the larval load too low to activate tomato defences? In contrast with the report of Larbat

et al. (2016), the study of tomato volatile compounds shows that *Tuta absoluta* herbivory increases the emission of 38 compounds, mostly peptidic fatty acids (C-18) (Silva *et al.*, 2017). In this study, the infestation load was estimated at 245 newborn larvae per plant and herbivory lasted for 3 days. The difference in larval load in the last two studies mentioned is striking and suggests that a preliminary study is required to determine what is the minimum load able to induce local and systemic tomato defences.

In addition, a recent study by Han *et al.* (2014) showed that the mortality rate of *Tuta absoluta* larvae increased when the insect was feeding on plants suffering nitrogen limitation and their development slowed down. This nitrogen limitation decreased the tissue nitrogen content (Han *et al.*, 2014) and it has been shown that similar limitation increased defence compound concentration in tomatoes (Royer *et al.*, 2013; Larbat *et al.*,

2012). Some tomato phenolic compounds and tomatine are known to be toxic to the tomato fruitworm *Heliothis zea* (Isman and Duffey, 1982). Two hypotheses can thus be considered: (i) nitrogen limitation leads to a low nutritional quality of the foliar tissues, due to low nitrogen content and / or (ii) nitrogen limitation increases plant resistance to the development of *Tuta absoluta*, via enhanced concentrations of defensive compounds.

Finally, all these findings raise several questions that this study will attempt to answer: what are the defence compounds elicited by *Tuta absoluta* herbivory? What role does nitrogen limitation play in the tomato defences against *Tuta absoluta*? Does tomato plant induce tolerance mechanisms towards *Tuta absoluta*?

OBJECTIVES AND ISSUES OF THE Ph.D., EXPERIMENTAL STRATEGY AND MANUSCRIPT STRUCTURE

The analysis of the scientific literature, briefly summarized above, showed that there are few information available on the response traits of the tomato to the infestation by its enemy, *Tuta absoluta*. In the frame of resistance mechanisms, we do not have extensive knowledge of the specific compounds elicited in infested tomatoes and of their spatiotemporal patterns in the hours and days following infestation. It appears from very recent works that the VOC cocktail is modified under herbivory by *Tuta absoluta*. In terms of tolerance mechanisms, response traits of infested tomatoes remains to be investigated, in order to assess the capacity of tomato to adapt to this herbivore. Thus, the objective of my Ph.D. project was to investigate the response traits of tomato under herbivory by *Tuta absoluta* larvae, in order to assess local and systemic responses, in terms of soluble and volatile compounds. We investigated these responses under two contrasted nitrogen availabilities, regarding the fact that nitrogen limitation is a lever to increase the concentration of defence-related compounds in plant tissues. The set of data acquired from the different experiments will help to get an integrated overview of the tomato - *Tuta absoluta* interactions and will contribute to build-up a mechanistic model of these interactions, a project at the scale of the research team. The identification of compounds, whose synthesis in plant tissues is stimulated or elicited by *Tuta absoluta*, is crucial.

Indeed, these compounds can attract predators or parasitoids and / or repel females before egg deposition. The biocontrol is an important issue of my work. Nitrogen nutrition being easily driven by the growers at the production scale, an issue of my Ph.D. is to propose new cultural practices in order to reduce the use of inputs.

Our experimental strategy was based on vegetative stage tomato plants, cultivated in hydroponics in air-conditioned rooms, facilitating the regulation of nitrogen nutrition, the control of external factors such as temperature and brightness and avoiding undesired biotic stresses. The tomato cultivar chosen, *Solanum lycopersicum* L. cv. Better Bush VFN Hybrid (breeder: Tomato Growers), is easy to control since it is an early and dwarf variety that grow well on hydroponics. Its defence mechanism is elicited by scarification (Royer, 2013) and its polyphenols concentration increased under nitrogen limitation (Larbat *et al.*, 2012). In order to get a good infestation success, we decided to infest tomatoes using 3rd instar larvae (on a scale of 4) that are less susceptible to die when they are manipulated than eggs or younger larvae.

Additionally, we wanted to induce the plant defence mechanisms without too much alteration of its growth, and observe plant defence on local and systemic zones. Thus, larvae were laid on the three terminal leaflets of the 4th true leaf, starting from the bottom of the plant, except for one experiment, in which younger plants were needed.

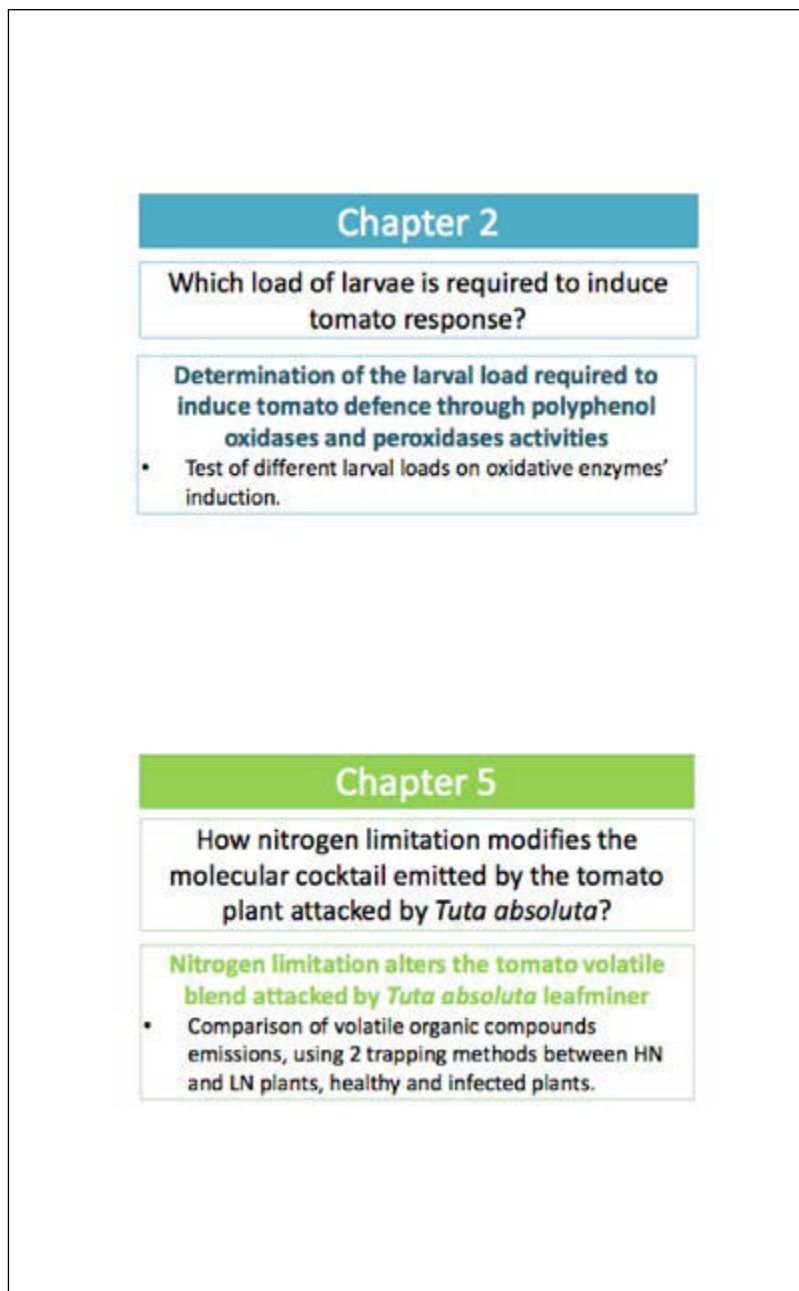
My thesis manuscript is structured into six chapters, the contents of which are briefly described Figure 9.

First, considering preliminary results obtained in our team (Royer *et al.*, 2013) showing that neither polyphenols nor tomatine concentrations were influenced by *Tuta absoluta* herbivory and the literature showing that tomato volatile blend was affected by this pest (Silva *et al.*, 2017), it seems to be essential to determine a minimal larval load which induces tomato responses. We have therefore tested different larval loads on the elicitation of oxidative enzymes known to react to herbivory. This study is presented in Chapter 2.

Secondly, it was shown that nitrogen limitation altered the development of *Tuta absoluta* larvae, reducing larval mass gain and slowing their development (Han *et al.*, 2014, Larbat *et al.*, 2016). Therefore, we tested the “trophic hypothesis” that nitrogen limitation decreases *Tuta absoluta* development because of a decline in the nutritional quality of leaf tissues. The results of the nitrogen impact on the diet of *Tuta absoluta* are presented in Chapter 3.

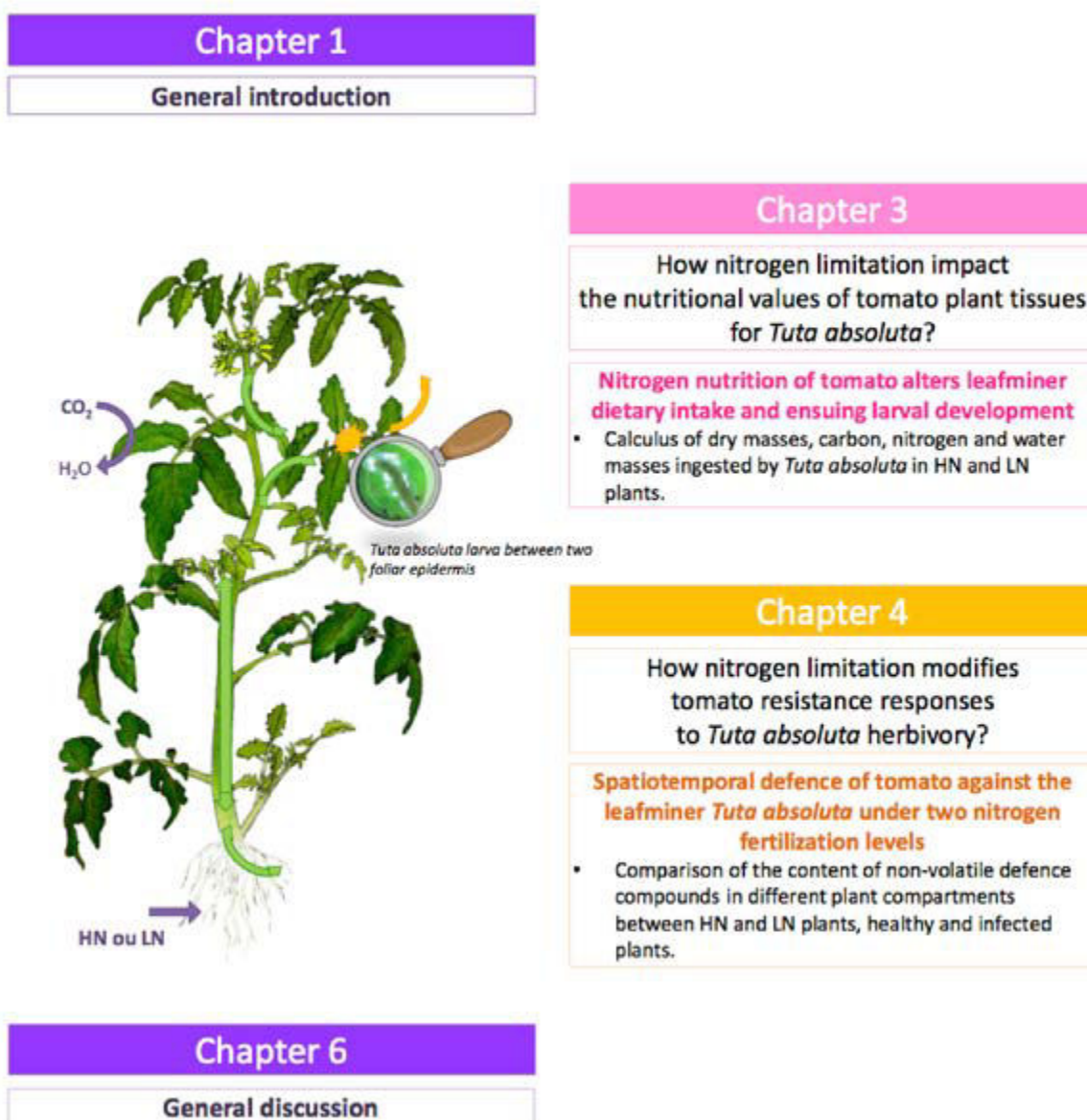
In Chapter 4, we discussed the impacts that nitrogen limitation and herbivory produced on

the tomato defence compounds. This section embraces the constitutive and inducible defence on *Tuta absoluta* development, which cannot be denied. Hence, this study focuses on leaf tissue defence compounds and their distribution within the tomato plant.



Chapter 5 is devoted to indirect defence. In this section, we compared qualitative results from two trapping methods and analysed the quantitative impact of nitrogen limitation and *Tuta absoluta* herbivory on tomato VOC emission rate. Finally, I propose a general discussion to summarize

the important results of this project, clarify the difficulties of assessing the costs and benefits of defence and put forward innovative ideas in the search for plant-insect interactions.



■ **FIGURE 9** : SCHEMATIC REPRESENTATION OF THE MANUSCRIPT, RESEARCH QUESTIONS, ARTICLES AND MAIN EXPERIMENTAL STRATEGIES

2.1

AVANT-PROPOS

Depuis une dizaine d'année, la société mondiale prend conscience des dangers de certains produits phytosanitaires sur l'environnement et la santé. Afin de réglementer l'utilisation de ces produits, des plans ont été établis en France, en Europe, et des accords ont été signés entre plus de 200 pays du monde. Dans ce contexte, l'agriculture doit trouver des méthodes alternatives pour lutter contre les ravageurs et maintenir un rendement de production. L'utilisation d'auxiliaires, la confusion sexuelle ou encore la stimulation des défenses naturelles sont quelques méthodes de lutte biologique sur lesquelles les agronomes travaillent. Mon travail de recherche s'inscrit dans cette logique agro-environnementale visant à réduire l'usage des produits phytosanitaire en agriculture, en favorisant les défenses naturelles de la plante, une fois celles-ci connues. Ce travail de recherche a émergé à partir d'un projet collaboratif entre l'UMR 1121 Université de Lorraine - INRA Agronomie et Environnement Nancy-Colmar (LAE), l'UR 1115 INRA d'Avignon Plantes et Systèmes de cultures Horticoles (PSH) et l'UMR 1355 INRA - 7254 CNRS - Université Nice Sophia Antipolis, Institut Sophia Agrobiotech (ISA). Cette collaboration cherche à comprendre l'effet de la disponibilité en azote sur les réponses écophysiologicals de la tomate. La précédente étudiante en doctorat, Mathilde ROYER, a étudié le compromis entre la croissance et la défense de

la tomate en réponse à la limitation azotée et aux contraintes biotiques telles que *Pseudomonas*, *Botrytis* et *Tuta absoluta*. Elle a montré que le ratio C:N est un indicateur fiable de ce compromis, que l'allocation des ressources entre différents métabolites varie en fonction du ravageur et que chaque ravageur réagit spécifiquement à la disponibilité en azote de la plante. L'une de ces observations était que l'herbivorie de *Tuta absoluta* n'induisait pas la synthèse de composés de défense chez la tomate. Nous pensons que ce résultat provient d'une trop faible intensité de dégâts pour induire une réponse de la plante.

Par conséquent, mon projet de doctorat contribue à la compréhension des réponses écophysiologicals des plantes au stress herbivore dans le contexte du pathosystème tomate - *Tuta absoluta*. Ce projet a été financé par l'INRA et la région Lorraine, d'octobre 2013 à septembre 2016. J'ai mené mon travail au LAE où j'ai été supervisé par Christophe ROBIN et Romain LARBAT. Mon travail a bénéficié de l'expertise de : Jacques LE BOT et Stéphane ADAMOWICZ, de PSH, Nicolas DESNEUX de l'ISA et de Sybille UNSICKER du laboratoire d'écologie chimique, une unité de recherche de l'Institut Max Planck, à Jena. Dans le cadre ce projet, nous avons ainsi réuni des partenaires scientifiques de l'INRA d'Avignon, de l'INRA de Sophia Antipolis et de l'Institut Max Planck de Jena.

Inscrite à l'école de doctorat RP2E, j'ai également bénéficié des cours de formation offerts par l'École internationale de recherche Agreenium (EIR-Agreenium). Agreenium est un consortium de centres de recherche et d'universités français qui favorise l'excellence en études doctorales dans le domaine des sciences de la vie. Notamment grâce à l'EIR-Agreenium, INRA, MPI et DAAD (Deutscher Akademischer Austauschdienst), j'ai effectué un stage de trois mois et demi au laboratoire d'Ecologie Chimique du MPI à Jena, en Allemagne. Le travail effectué au cours de ce stage fait partie intégrante de mon projet de doctorat. En cela, je postule à l'obtention de deux labels : le label d'excellence EIR-Agreenium et le label Doctorat européen. Au cours de ma thèse, j'ai bénéficié de deux

rencontres avec mon comité de pilotage de la thèse. Le premier a eu lieu le 27 juin 2014 et a rassemblé Christophe ROBIN, Romain LARBAT, Jacques LE BOT, Stéphane ADAMOWICZ, Didier LE THIEC, Nicolas DESNEUX et Corinne ROBERT. La seconde a eu lieu le 10 septembre 2015 et Sybille UNSICKER et Loïc PAGES ont rejoint les membres de mon comité.

Suite à ce bref avant-propos sur mon parcours doctoral, je vous propose de rentrer au cœur du projet scientifique en lisant ce manuscrit basé sur le schéma de publications scientifiques. Dans ce premier chapitre, j'ai réalisé une synthèse bibliographique et je vous dévoile les enjeux et objectifs de ma thèse ainsi que la stratégie expérimentale instaurée.

SYNTHÈSE BIBLIOGRAPHIQUE

2.2.1

AGRICULTURE ET ENVIRONNEMENT : UNE NOUVELLE MANIÈRE DE PENSER

L'agriculture, c'est-à-dire la mise en culture de plantes et l'élevage d'animaux, a débuté il y a environ 10 000 ans. La domestication des plantes a nécessité la compréhension de leur cycle de développement, de reproduction et a permis de sélectionner des caractères phénotypiques à partir des plantes sauvages. Des caractères, tels que la petite taille des talles de blé pour éviter la verse ou encore le pédoncule cassant facilitant la récolte des fruits, ont été sélectionnés. Mais, les principaux caractères sélectionnés, qui ont permis d'augmenter les rendements, sont la grosseur des fruits, le nombre de grains par épi ou encore la richesse nutritionnelle des fruits (Doré *et al.*, 2006). La plupart des plantes sélectionnées favorisent donc l'allocation des ressources vers les fruits, graines, tubercules... au détriment des mécanismes de défense. Cette sélection associée au développement des méthodes de culture (défrichage, labour, rotation triennale...) a permis de maîtriser les cultures. Au cours du XX^{ème} siècle, le développement des intrants chimiques, du machinisme agricole et les améliorations génétiques des plantes ont permis une forte hausse du rendement agricole. En effet, l'azote, un élément indispensable à la croissance des plantes, est présent dans de nombreuses molécules permettant le bon fonctionnement des cellules ; on le retrouve dans les molécules d'ADN (support de l'information génétique) ou encore dans les

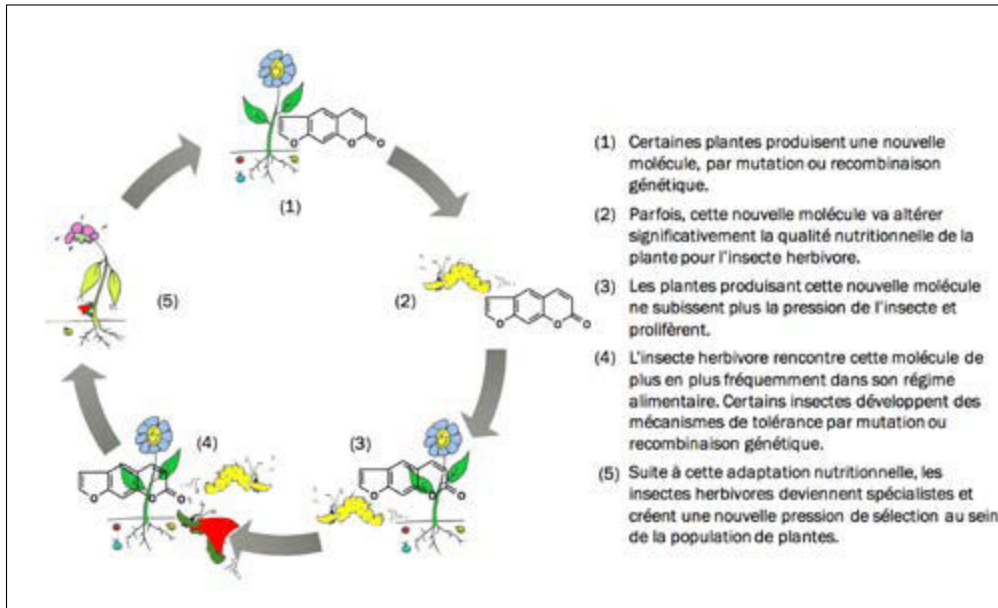
protéines (catalyseurs des réactions chimiques). La fertilisation azotée, associée au phosphore et au potassium, favorise la croissance des plantes et augmente considérablement les rendements (Chorley, 1981 ; Adekiya and Agbede, 2009 ; Olaniyi et Ajibola, 2008). En outre, en raison de leurs propriétés toxiques, de nombreux composés de défense ont été contre-sélectionnés chez les espèces sauvages, rendant les cultures plus vulnérables aux attaques biotiques et de plus en plus demandeurs en pesticides. Mais, en 1962, Rachel Carson dénonce pour la première fois les méfaits des intrants chimiques tels que les insecticides, les fongicides et des amendements azotés, sur la population et l'environnement, dans son livre intitulé "*Silent spring*" (Carson, 1962). En effet, les épandages de doses massives d'engrais azotés ont conduit à une forte hausse de la teneur en nitrate des eaux souterraines. Dans ces eaux, les excès de nitrate modifient l'équilibre biologique, favorisant le développement de certains champignons et bactéries et rendant les eaux souterraines impures. Ingré en trop grande quantité, le nitrate a un effet néfaste sur la santé humaine (Bouchard *et al.*, 1992). De plus, des études récentes tirent la sonnette d'alarme en mettant en évidence les effets et les risques des épandages de pesticides sur la santé humaine et la biodiversité des invertébrés. En effet, des études ont montré la présence de résidus de pesticides dans les tissus adipeux des tumeurs

et incriminent ces composés chimiques (Falck *et al.*, 1992 ; Aronson *et al.*, 2000). D'autres études corrélient le temps et la fréquence des expositions aux pesticides à la présence de tumeurs au sein d'une population (Hardell et Erikson, 1999 ; Alavanja *et al.*, 2004). Plus récemment, des études dévoilent l'effet néfaste des pesticides sur la diversité des invertébrés, en rappelant le rôle essentiel que jouent les pollinisateurs ou les insectes prédateurs dans les cultures, tels que les abeilles ou les coccinelles (Britain *et al.*, 2010 ; Geiger *et al.*, 2010 ; Tschardtke *et al.*, 2012). Ainsi, d'importants projets ont abouti à la mise au point d'outils d'évaluation des risques liés aux pesticides sur les agro-écosystèmes et sur la santé humaine, en particulier le projet HAIR (*HARmonised environmental Indicators for pesticide Risk*) (Calliera *et al.*, 2006 et 2008).

Depuis une dizaine d'année, la France, l'Europe et bien d'autres pays du monde entier tentent de

limiter l'utilisation de ces produits en légiférant sur leur mode d'utilisation et en favorisant la recherche de méthodes alternatives telles que les méthodes de cultures durables, les bio-pesticides ou la lutte biologique. Les outils informatiques d'aide à la décision, qui se développent depuis une dizaine d'année, permettent de prédire les risques d'infestation et / ou d'infection et de traiter les cultures de manière raisonnée. Le plan Ecophyto II réaffirme la volonté du gouvernement français à réduire de moitié l'utilisation de ces produits en agriculture (Plan Ecophyto II, 2015). En tant qu'agronome, il est notre devoir de fournir des réponses pour limiter l'utilisation des intrants chimiques tout en maintenant un bon rendement agricole. Mais, pour cela, nous devons d'abord comprendre les mécanismes de défense des plantes.

LES DÉFENSES DES PLANTES



■ **FIGURE 10** : SCHÉMA DE LA CO-ÉVOLUTION ENTRE LES PLANTES ET LES INSECTES HERBIVORES. D'APRÈS BERENBAUM, 1983.

• UNE HISTOIRE DE CO-EVOLUTION

Les premiers végétaux terrestres seraient apparus à l'époque de l'Ordovicien supérieur, il y a environ 450 millions d'années (Stemans *et al.*, 2009). Depuis, les végétaux se sont adaptés aux contraintes abiotiques (environnemental et climatique) et ont co-évolué avec les agents pathogènes et les herbivores qui seraient apparus à la fin du Silirien (Labandeira, 2007). La co-évolution est un concept qui a été décrit par Ehrlich et Raven, en 1964. Vingt ans plus tard, Berenbaum décrit un scénario hypothétique de co-évolution entre les plantes produisant des composés spécifiques de défense : les coumarines et les insectes herbivores.

Il décrit le déroulement d'une phase de co-évolution en 5 étapes, voir Figure 10. (1) Certaines plantes produisent une nouvelle molécule par mutation ou recombinaison génétique naturelle. (2) Parfois, cette nouvelle molécule modifie de manière significative la qualité nutritionnelle de ses tissus pour les insectes herbivores. (3) Ainsi, les plantes produisant cette molécule ne subissent plus la pression imposée par l'herbivorie et prolifèrent. (4) Les herbivores rencontrent alors, de plus en plus fréquemment, cette molécule dans leur alimentation. Certains d'entre eux vont développer un mécanisme de tolérance par mutation ou recombinaison génétique naturelle. (5) Suite à cette adaptation alimentaire, les herbivores deviennent spécialistes et créent une nouvelle pression de sélection au sein de la population de plantes. Ces cinq étapes se répètent en boucle conduisant aujourd'hui à une diversité de molécules de défense chez les plantes (Berenbaum, 1983).

L'adaptation des végétaux a requis la mise en place de mécanismes de défenses grâce à des modifications génétiques, qui ont permis la création de nouvelles enzymes. Les enzymes sont les acteurs de toutes réactions chimiques dans les cellules. Ces réactions chimiques permettent la synthèse de molécules, et un nombre indéfinissable de mutations et de recombinaisons génétiques ont conduit à des modifications macroscopiques, telles que les épines du rosier (Lev-Yadun, 2001) ou encore les trichomes (Schilmiller, 2010), en plus des estimés centaines de milliers de molécules de défense présentes dans le règne végétal (Schuman et Baldwin, 2016).

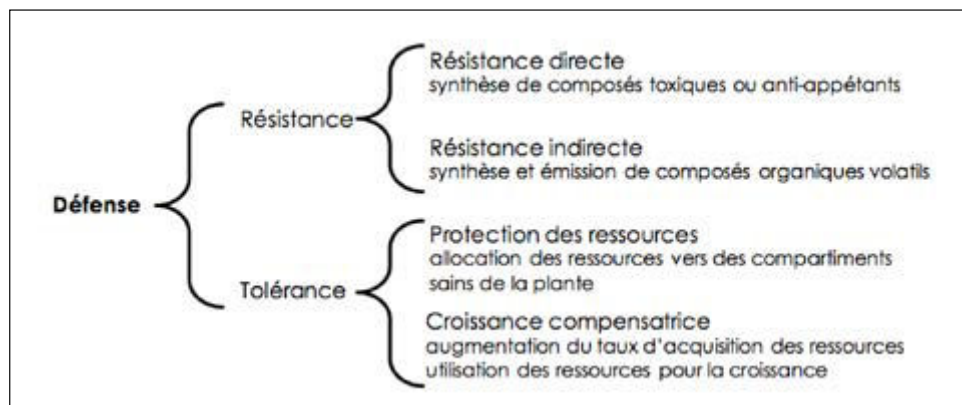
• UNE COMBINAISON ENTRE LA RESISTANCE ET LA TOLERANCE

Les mécanismes de défense ont été classés selon deux stratégies : la résistance, réduisant directement la "fitness", classiquement traduit par "valeur sélective", "succès reproducteur" ou "valeur adaptative", de l'herbivore agresseur et la tolérance, diminuant l'impact de l'agresseur sur la plante.

La résistance implique la synthèse de molécules spécifiques de défense qui peuvent être présentes dans les tissus de la plante ou encore être émises dans l'air en raison de leur volatilité (Figure 11). Ces molécules de défense peuvent être toxiques, anti-appétantes, répulsives envers les insectes herbivores (Dicke, 2003) ou encore attirer des insectes prédateurs ou des parasites de l'herbivore (Halitschke, 2008). Plus de 70 000 molécules de défense ont été identifiées à ce jour (Fürstenberg-Hägg *et al.*, 2013). Les trois principales classes chimiques auxquelles appartiennent ces molécules sont les terpénoïdes, les phénols et les alcaloïdes.

La tolérance regroupe les mécanismes qui visent à limiter l'impact de l'herbivorie sur la fitness de la plante (Figure 11). L'intérêt écologique et évolutif de la tolérance à l'herbivorie chez les végétaux nécessite encore des investigations (Fornoni, 2011). Cependant, deux théories ont

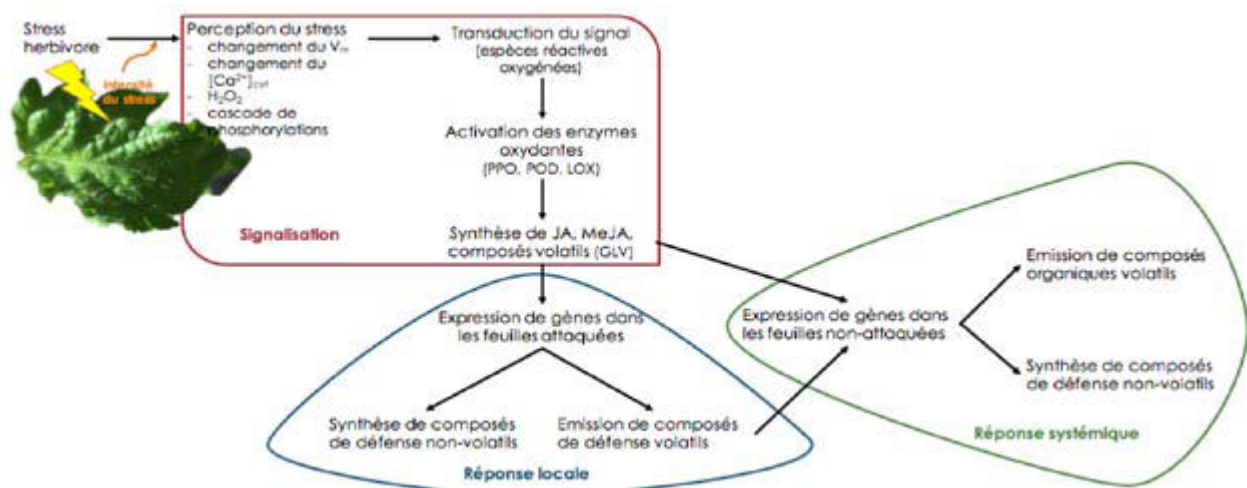
été formulées suite aux observations de certains mécanismes de tolérance : la protection des ressources et la croissance compensatoire. Au cours d'une agression, certaines plantes sont capables de déplacer leurs ressources glucidiques (Holland *et al.*, 1996) et / ou aminées (Gomez *et al.*, 2010) vers des compartiments éloignés de la zone d'infection et qui permettront à la plante de continuer sa croissance et son développement dès que l'agression sera terminée. La théorie de la croissance compensatoire repose sur la capacité d'une plante à augmenter l'assimilation du carbone, par photosynthèse (Thomson *et al.*, 2003) et / ou de l'azote par absorption racinaire (Newingham *et al.*, 2007), permettant ainsi de compenser la perte de tissus liée à l'herbivorie. Cependant, la croissance compensatoire n'est pas une réponse de tolérance universelle. En effet, certaines plantes soumises à l'herbivorie montrent une baisse de la capacité photosynthétique ou de la fixation du carbone, telles que le *Datura* soumis à l'herbivorie de *Manduca sexta* et la tomate soumise à un traitement au méthyl-jasmonate, un éliciteur des défenses (Barron-Gafford, 2012 ; Gomez *et al.*, 2010).



■ FIGURE 11 : STRATÉGIES DE LA DÉFENSE DES PLANTES

• INDUCTION DES DEFENSES

Les plantes possèdent un niveau de défense constitutif qui limite les premières infections (Wittstock et Gershenzon, 2002). Mais en cas d'attaque, les plantes peuvent renforcer leur niveau de défense de base grâce aux mécanismes de perception, de reconnaissance et de signalisation.



■ **FIGURE 12 :** INDUCTION DE LA DEFENSE DES PLANTES

Les morsures créent des lésions cellulaires qui se traduisent par une inversion du potentiel électrique à l'interface membranaire. Ce signal électrique engendre très rapidement la libération d'ions calcium, la formation de peroxyde d'hydrogène et une cascade de phosphorylations enzymatiques. Suite à cette perception, la plante produit des molécules oxydantes hyper réactives (ROS), active des enzymes oxydantes (LOX, PPO, POD) et synthétise des phytohormones telles que l'acide jasmonique ou le méthyl jasmonate et des composés volatils de feuilles vertes (GLV). Ces phytohormones et ces volatils permettent l'activation des réponses locales à l'agression et systémiques. Ces réponses locales et systémiques se traduisent par l'activation de l'expression de gènes qui permet la synthèse de nombreux composés de défense non-volatils et volatils.

PERCEPTION ET RECONNAISSANCE DU STRESS

Lorsque qu'un herbivore s'attaque à une plante, les lésions cellulaires créées par les morsures ou les piqûres engendrent une perturbation de l'équilibre électrochimique de la membrane cellulaire, suivi d'une libération cytoplasmique de l'ion Ca^{2+} (Lecourieux *et al.*, 2006 ; Figure 12) et provoquant un stress oxydatif s'exprimant par la production de composés oxydatifs réactifs (ROS : reactive oxidative species), délétères pour l'insecte herbivore. C'est ce

que l'on appelle la réaction hypersensible.

En parallèle à la perception du dommage physique, la plante est capable de reconnaître certaines molécules salivaires de l'insecte, jouant alors le rôle d'éliciteurs de la défense (Alborn *et al.*, 1997 ; Halitschke *et al.*, 2001 ; Musser *et al.*, 2006 and Maffei *et al.*, 2007). Grâce à cette reconnaissance, la plante adapte la voie de signalisation du stress et active des voies de synthèse spécifiques de l'agresseur.

ACTIVATION ENZYMATIQUE ET DIFFUSION DU SIGNAL PAR LA VOIE HORMONALE

Suite à la perception et la reconnaissance du stress, l'afflux d'ion calcium active des enzymes calcium-dépendantes qui favorisent les réactions de phosphorylation et modifient les activités de transcription (Kanchiswamy *et al.*, 2010 ; Figure 12). Des enzymes anti-oxydantes sont alors synthétisées et activées (POD : peroxydase, LOX : lipoxygénase, PPO : polyphénoloxydase) pour produire des toxines et réduire la qualité et la digestibilité des tissus de la plante (Stout, 1994 ; War *et al.* 2012).

De nombreuses hormones, telles que l'acide salicylique, l'acide jasmonique ou l'éthylène, ont été identifiées jouant un rôle dans la propagation du stress. Ces hormones, synthétisées dans la zone d'agression, sont transmises au reste de la plante *via* le système vasculaire ou émises dans l'air. Elles interviennent seules, en synergie ou de manière antagoniste dans l'activation de voies de synthèses spécifiques du métabolisme de défense dans les autres compartiments de la plante (Onkokesung, 2010).

2.2.3

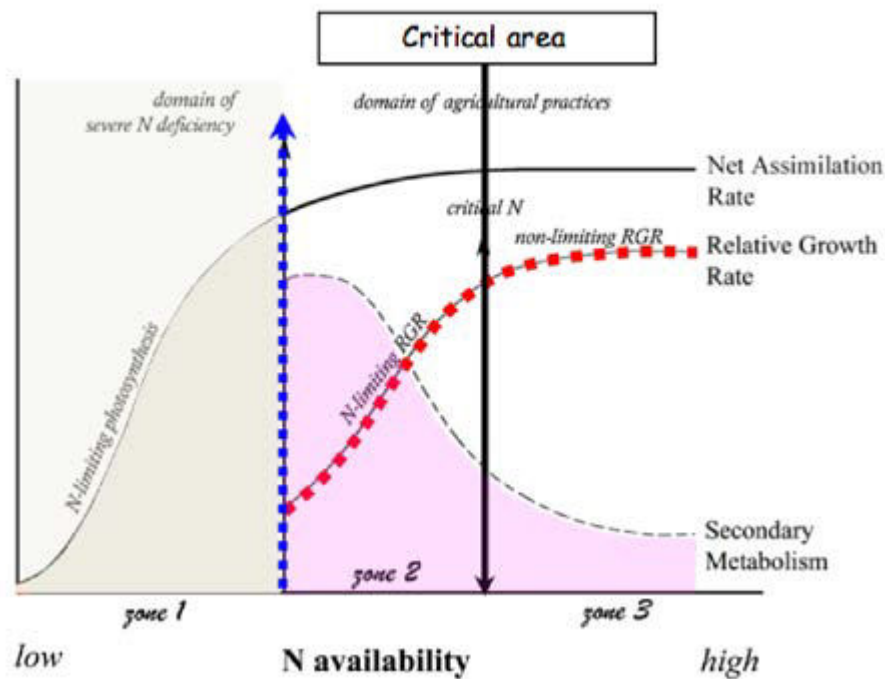
LA LIMITATION DE LA FERTILISATION AZOTÉE : UN LEVIER POUR AMÉLIORER LES DÉFENSES NATURELLES DES PLANTES

Une théorie corrèle la disponibilité des ressources et le niveau de défense des plantes. Cette théorie, développée en 1983 et intitulée "Carbon - Nutrients Balance hypothesis (CNBH), prédit que le carbone sera plus facilement alloué à la synthèse de composés de défense carbonés chez les plantes poussant sur des sols pauvres en azote. À l'inverse, pour les plantes qui poussent sur des sols pauvres en carbone, l'allocation des ressources favorisera la synthèse de composés de défense azotés (Bryant *et al.*, 1983). Herms et Mattson (1992) ont élargi cette théorie à tous les facteurs qui

ralentissent la croissance des plantes plus fortement que la photosynthèse, augmentent l'allocation des ressources pour le métabolisme de la défense et l'ont nommé GDBH pour Growth differential balance hypothesis. Ainsi, la disponibilité en azote peut être utilisée pour améliorer le métabolisme de défense des plantes. En effet, pour une disponibilité en azote limitante (zone 2, Figure 13), le métabolisme de défense atteint son intensité maximale, alors que l'assimilation du carbone a déjà atteint son plateau optimal et le taux de croissance relatif est légèrement inférieur à son

optimum.

Ce phénomène a été observé chez *Pentaclethra maculosa*, un arbre tropical, présentant une concentration foliaire des saponines qui augmente avec la baisse de la disponibilité en azote, contrairement à la concentration en flavanes (Massad *et al.*, 2012). Chez la plante de tomate (*Solanum lycopersicum*), la limitation azotée engendre une augmentation de la concentration foliaire des glucides simples et des composés de défense, tels que l'acide chlorogénique, la rutine ou le kaempférol-rutinoside (Le Bot *et al.*, 2009).



■ **FIGURE 13** : DIAGRAMME REPRÉSENTANT L'IMPACT DE LA DISPONIBILITÉ EN AZOTE SUR LE TAUX D'ASSIMILATION NET DE CO_2 , LE TAUX RELATIF DE CROISSANCE ET LE MÉTABOLISME DE DÉFENSE. LE BOT *et al.* (2009), ADAPTÉ À PARTIR HERMS & MATTSON (1992)

L'assimilation nette du CO_2 , le taux de croissance relative des plantes et le métabolisme secondaire sont affectés sévèrement et négativement par la carence en azote. Pour des disponibilités en azote supérieures à la valeur critique, l'assimilation nette de CO_2 et le taux relatif de croissance atteignent un plateau optimal, alors que l'intensité du métabolisme secondaire est moins forte. Entre ces deux niveaux d'azote, le métabolisme de défense atteint son intensité optimale alors que l'assimilation du CO_2 a déjà atteint son plateau optimal et le taux relatif de croissance diminue légèrement.

• LA TOMATE

La tomate de nom latin *Solanum lycopersicum* est une dicotylédone de la famille des Solanacées, originaire d'Amérique Centrale et du Sud. La tomate est devenue une plante modèle pour l'amélioration génétique des plantes légumières (Kimura et Sinha, 2008). Les avancées scientifiques ont permis de créer des variétés de tomate adaptées à différentes régions du monde, à différents modes de culture et dont les caractéristiques organoleptiques du fruit correspondent à la demande. D'après les données statistiques de la FAO (2011), la tomate est la première production légumière au monde et est aujourd'hui largement cultivée sur tous les continents. Originellement, la culture de tomate se pratiquait en sol, bien que toujours présente, elle a été petit à petit remplacée par la culture sous serre et / ou hors-sol. La culture de tomate sous serre permet de limiter l'impact des intempéries, mais elle reste souvent sujette à des agressions biotiques et nécessite donc la plus grande vigilance des producteurs. Les

nombreux bio-agresseurs appartiennent au règne des champignons, des bactéries ou encore des animaux (insectes). Parmi les champignons pathogènes de la tomate, on retrouve *Oidium neolycopersici*, agent responsable des tâches poudreuses blanches ; *Phytophthora infestans*, agent du mildiou ; *Botrytis cinerea*, agent de la pourriture grise. Ces champignons prélèvent les nutriments nécessaires à leur développement directement dans les cellules de la plante hôte (Watterson, 1986 ; Corbaz, 1990). *Clavibacter michiganensis* et *Pseudomonas syringae* sont deux agents pathogènes du chancre bactérien et de la moucheture bactérienne, respectivement, très redoutées par les producteurs de tomates. Ces bactéries se développent dans les espaces intercellulaires tuant les cellules aux alentours qui leur servent de milieu nutritif (Corbaz, 1990 ; Blancard, 2009). En ce qui concerne les insectes, les dégâts causés sur la tomate sont de différents types. En effet, les insectes piqueurs - suceurs, comme les pucerons (*Aphis*

gossypii ou *Myzus persicae*) ou les aleurodes causent des dégâts de manière indirecte (Csizinszky *et al.*, 2005 ; Nayka *et al.*, 2005). Les pucerons peuvent véhiculer et transmettre des virus à la plante et le miellat, que déposent les pucerons et les aleurodes à la surface des feuilles, est propice au développement fongique et bactérien (Berlinger, 1986). De nombreuses chenilles, telles que *Helicoverpa armigera* ou *Chrysodeixis chalcites*, dévorent le feuillage et parfois même les fruits grâce à leurs mâchoires broyeuses. D'autres larves qualifiées de mineuses, comme *Tuta absoluta*, ne se nourrissent que du parenchyme laissant apparaître des galeries entre les deux épidermes foliaires (Canadian Food Inspection Agency, 2012). Quel que soit l'agression, les cultures de tomate subissent des pertes de productivités liées à des dommages causés sur les fruits, directement, ou sur le feuillage réduisant ainsi la production photosynthétique et donc le développement de la plante et de ses fruits.

• ***Tuta absoluta*: UN RAVAGEUR DE LA TOMATE DE PLUS EN PLUS DIFFICILE À MAITRISER**

Tuta absoluta est un micro lépidoptère puisque son envergure est d'environ 1 cm (Figure 14). Ce papillon originaire d'Amérique du Sud a été décrit pour la première fois par Meyrick en 1917. La larve de *Tuta absoluta* est qualifiée de mineuse car elle se nourrit principalement du parenchyme foliaire après

pénétration à l'intérieur de la feuille (Figure 15). Son mode de nutrition particulier rend sa détection assez facile. La présence de ce ravageur dans une culture de tomate peut provoquer une perte intégrale de la récolte.






■ **FIGURE 14** : PAILLON DE NUIT, *Tuta absoluta* (MEYRICK, 1917)



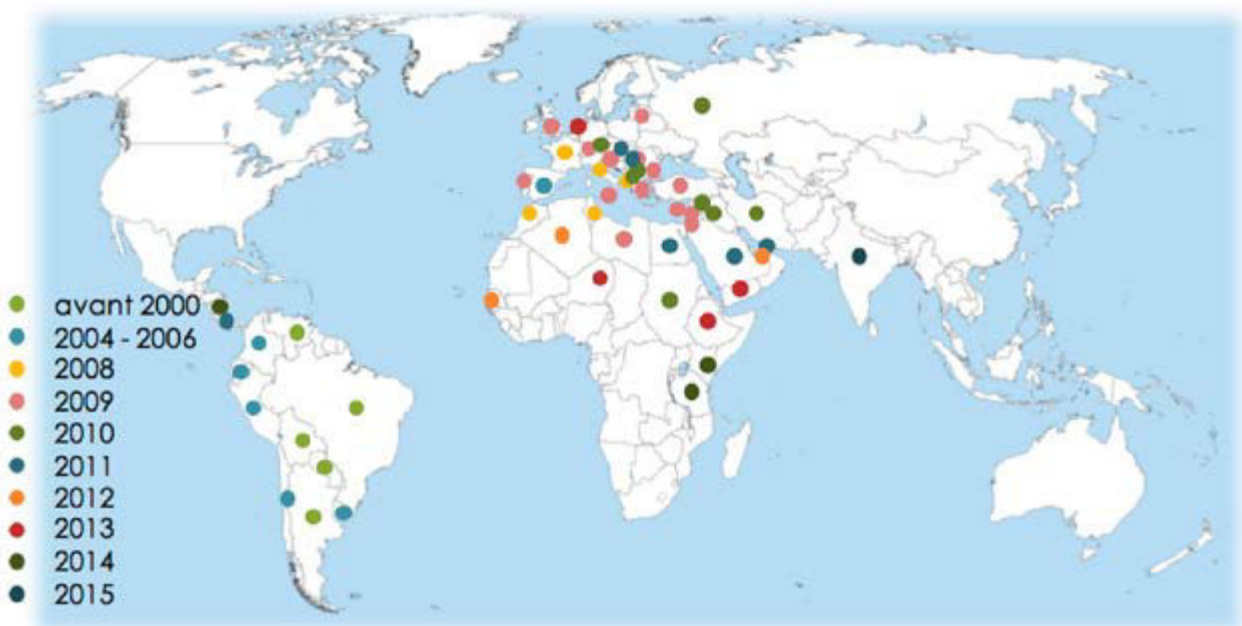
■ **FIGURE 15** : FOLIOLE DE TOMATE PRÉSENTANT UNE GALERIE FORMÉE PAR L'HERBIVORIE D'UNE LARVE DE *Tuta absoluta*

Son développement est extrêmement rapide, puisque son cycle de développement ne dure que 29 jours à 30°C et qu'une femelle peut pondre jusqu'à 260 œufs (Marcano, 1995 ; Table 2). *Tuta absoluta* se développe donc plus rapidement sous les climats tropicaux et subtropicaux. Néanmoins, il est maintenant présent dans les régions de climat tempéré.

Tuta absoluta est arrivée en Europe, par l'Espagne, en 2006 et s'est propagé en l'Europe, en Amérique du Nord et en Afrique du Nord dans les années 2006-2012 (Blancard, 2014 ; Figure 16). Il a fait sa première apparition en France, dans la région du Languedoc-Roussillon, fin 2008. Ce ravageur continue sa progression vers l'Asie, qui est le plus grand producteur de tomate.

Stades de développement		15°C	20°C	25°C	30°C
	Oeuf	10 jours	7 jours	4 jours	3 jours
	Larve	36 jours	23 jours	15 jours	11 jours
	Pupe	21 jours	12 jours	7 jours	6 jours
	Adulte	23 jours	17 jours	13 jours	9 jours
Total		90 jours	59 jours	39 jours	29 jours

■ **TABLE 2** : DURÉE DES STADES DE DÉVELOPPEMENT DE *Tuta absoluta* À 15, 20, 25 ET 30 °C



■ **FIGURE 16** : PROPAGATION DE *Tuta absoluta*, UN RAVAGEUR DE LA TOMATE, Adapté des données de l'EPP0 (European and Mediterranean plant protection and organisation), 2016.

L'utilisation intensive de certains pesticides, en Amérique du Sud, depuis les années 1970, a engendré l'apparition de résistance chez *Tuta absoluta*. Les matières actives pour lesquelles ce ravageur a été montré résistant sont : le métamidophos et le cartap qui sont des thiocarbamates, l'abamectine, la perméthrine pyréthroïde (Siqueira *et al.*, 2000) et l'indoxacarbe et le spinosad qui sont des inhibiteurs de la synthèse de chitine (Lietti *et al.*, 2005). D'autres méthodes de lutte existent. Le piégeage des mâles grâce aux phéromones femelles est une technique extrêmement coûteuse et est souvent utilisée pour détecter la présence de l'insecte ou pour estimer la densité de sa population. L'introduction d'un

prédateur ou d'un parasitoïde est une dernière technique assez efficace si ceux-ci sont natifs de la zone à traiter.

De plus en plus d'études sur l'impact de divers facteurs (température, prédateur, pesticide...) sur le développement de *Tuta absoluta* sont publiées (Leite *et al.*, 1999 ; De Medeiros *et al.*, 2009 ; Krechemer et Foerster, 2015 ; Roditakis *et al.*, 2015 ; Silva *et al.*, 2015 ; Yalcin *et al.*, 2015 ; Bawin *et al.*, 2016 ; Han *et al.*, 2016 ; Martins *et al.* 2016 ; Ozgokce *et al.*, 2016 ; Perdakis et Arvaniti, 2016 ; Polat *et al.*, 2016 ; Marchioro *et al.*, 2017 ; Van Lenteren *et al.*, 2017), cependant très peu d'entre elles s'interrogent sur les mécanismes de défense que développe la tomate face à ce ravageur.

• ÉTAT ACTUEL DES CONNAISSANCES SUR LES DÉFENSES DE LA TOMATE VIS-A-VIS DE *Tuta absoluta*

Face au développement incontrôlable de ce ravageur, une recherche active est menée pour créer des variétés de tomates résistantes. La plupart des tomates développent des poils à la surface des feuilles et des tiges (Figure 17). Ces poils, nommés trichomes, sont de fines excroissances cellulaires séquestrant un grand nombre de molécules de défense (Schillmiller *et al.*, 2010). Il a été démontré que plus la densité de ces trichomes était élevée, plus les plants de tomate étaient résistants à *Tuta absoluta*. La cassure de ces trichomes, provoquée par le déplacement des insectes à la surface des feuilles, engendre d'une part la libération de ces molécules de défense et d'autre part induit la synthèse d'acide jasmonique et la formation de peroxyde d'hydrogène (H_2O_2), une espèce réactive d'oxygène (Peiffer *et al.*, 2009). En 2006, De Resende *et al.* ont montré une corrélation négative entre le contenu constitutif des sucres acylés dans les trichomes de deux variétés de tomates et la survie des larves de *Tuta absoluta*. Cependant, ces variétés ont un rendement et / ou une qualité de fruit très faible. L'acide jasmonique joue un rôle important dans la défense des plantes. En effet, l'acide jasmonique est une molécule qui signale la présence d'un bio-agresseur au reste de la plante *via* le système vasculaire et active la synthèse de composés de défense. Cette phytohormone contribue également à la pubescence de nouveaux trichomes à la surface des feuilles (Campos *et al.*, 2009).

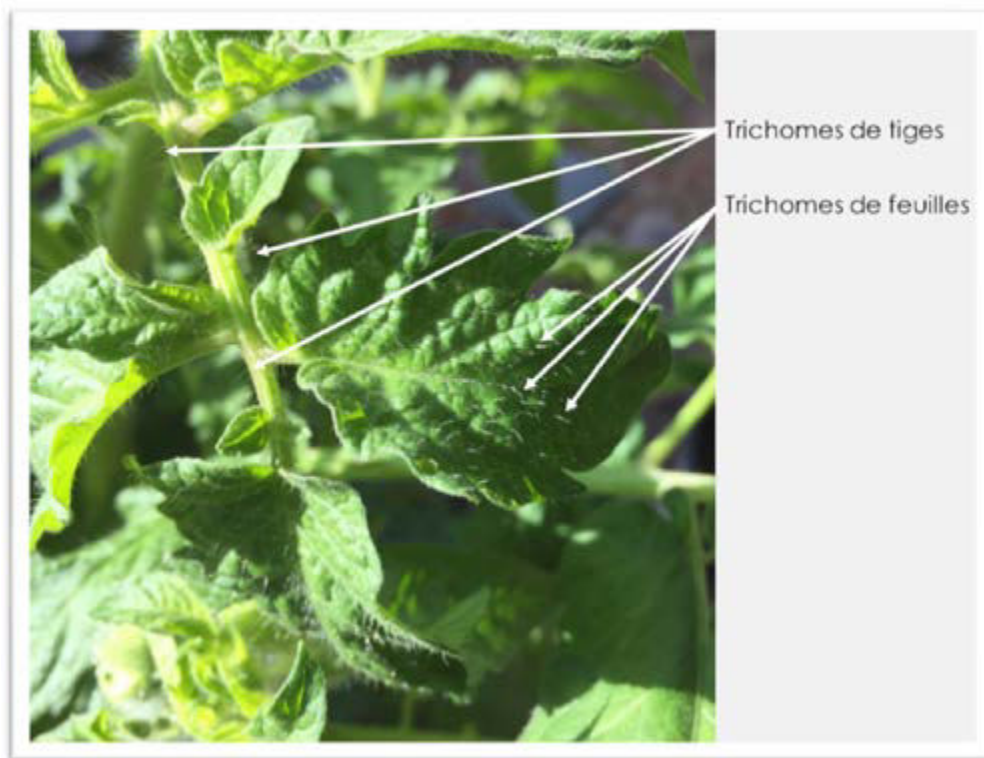
Les morsures des insectes provoquent des lésions cellulaires et engendrent une réaction hypersensible. Il a été montré que *Tuta absoluta*

augmente l'activité des polyphénol oxydases (Larbat *et al.*, 2016) tandis que la tomate réagit à l'herbivorie d'une mineuse, *Liriomyza trifolii*, par l'activation des peroxydases (Stout *et al.*, 1994). Ainsi, nous supposons que la perception de l'agression par la tomate est spécifique même parmi les différentes espèces de larves mineuses. En outre, l'herbivorie de deux larves nouvellement-nées de *Tuta absoluta* n'a pas affecté la concentration de 14 polyphénols et de la tomatine dans les tissus de la tomate, après plusieurs jours d'herbivorie (Larbat *et al.*, 2016). Cette étude a également révélé que *Tuta absoluta* n'engendre pas de modification de la teneur en protéines ni de la distribution de l'azote des plantes, ce qui soulève des interrogations : l'herbivorie de *Tuta absoluta* passe-t-elle inaperçue à l'égard de la plante de tomate ? *Tuta absoluta* inhibe-t-elle les mécanismes de défenses de la tomate ? Ou, la charge en larves est-elle trop faible pour activer les défenses de la tomate ? Contrairement aux travaux de Larbat *et al.* (2016), l'étude des composés volatils de la tomate montre que l'herbivorie de *Tuta absoluta* augmente l'émission de 38 composés dont principalement des acides gras peptidiques (C-18) (Silva *et al.*, 2017). Dans cette étude, la charge utilisée pour l'infestation des plants de tomate a été estimée à 245 larves nouvellement nées par plante et l'herbivorie a duré trois jours. La différence de la charge en larves utilisée dans les deux dernières études mentionnées est frappante et suggère qu'une étude préliminaire est nécessaire pour déterminer quelle est la charge minimale capable d'induire les défenses locales et systémiques de la tomate.

De plus, une étude récente de Han *et al.* (2014) a montré que le taux de mortalité des larves de *Tuta absoluta* a augmenté lorsque l'insecte se nourrissait de plante souffrant d'une limitation en azote et que leur développement ralentissait. Cette limitation en azote a diminué la teneur en azote des tissus (Han *et al.*, 2014) et il a été démontré qu'une limitation similaire augmentait la concentration des composés de défense dans les tomates (Larbat *et al.*, 2012 ; Royer *et al.*, 2013). Certains composés phénoliques et la tomatine sont connus pour être toxiques pour la noctuelle de la tomate, *Heliothis zea* (Isman et Duffey, 1982). Deux hypothèses peuvent donc être envisagées : (i) la limitation de

l'azote conduit à une faible qualité nutritionnelle des tissus foliaires, en raison de la faible teneur en azote et / ou (ii) la limitation azotée augmente la résistance des plantes au développement de *Tuta absoluta*, via des concentrations accrues en composés de défense.

Finalement, tous ces résultats soulèvent plusieurs interrogations auxquelles cette étude tentera de répondre : quels sont les composés de défense élicités par l'herbivorie de *Tuta absoluta* ? Quel rôle joue la limitation azotée dans les défenses de la tomate vis-à-vis de *Tuta absoluta* ? La plante de tomate induit-elle des mécanismes de tolérance vis-à-vis de *Tuta absoluta* ?



■ **FIGURE 17** : PHOTO D'UN PLANT DE TOMATE METTANT EN VALEUR DES TRICHOMES PRÉSENTS SUR LES FEUILLES ET SUR LES TIGES.

OBJECTIFS ET ENJEUX DE LA THÈSE, STRATEGIE EXPERIMENTALE ET STRUCTURE DU MANUSCRIT

L'analyse de la littérature scientifique, brièvement résumée ci-dessus, a montré qu'il y a peu d'informations disponibles sur les traits de réponse de la tomate à l'infestation par le ravageur, *Tuta absoluta*. Dans le cadre des mécanismes de résistance, nous n'avons pas une connaissance approfondie des composés spécifiques induits chez les tomates infestées et de leurs profils spatiotemporels dans les heures et les jours qui suivent l'infestation. Des travaux récents montrent que le bouquet de COVs est modifié sous herbivorie de *Tuta absoluta*. En termes de mécanismes de tolérance, les traits de réponse des tomates infestées restent à étudier, afin d'évaluer la capacité de la tomate à s'adapter à cet herbivore. Ainsi, l'objectif de mon projet de thèse était d'étudier les traits de réponse de la tomate attaquée par des larves de *Tuta absoluta* afin d'évaluer les réponses locales et systémiques en termes de composés solubles et volatils. Nous avons étudié ces réponses sous deux disponibilités d'azote contrastées, sachant que la limitation de la disponibilité de l'azote est un levier pour augmenter la concentration des composés liés à la défense dans les tissus végétaux. L'ensemble des données acquises à partir des différentes expériences permettra d'obtenir une vue d'ensemble intégrée des interactions tomate - *Tuta absoluta* et contribuera à construire un modèle mécaniste de ces interactions, c'est un projet à l'échelle de l'équipe de recherche. L'identification

des composés, dont la synthèse dans les tissus végétaux est stimulée ou déclenchée par *Tuta absoluta*, est cruciale. En effet, ces composés peuvent attirer des prédateurs ou des parasitoïdes et / ou repousser les femelles pondueuses. Le biocontrôle est une question importante de mon travail.

La nutrition azotée étant facilement pilotée par les producteurs à l'échelle de la production, l'enjeu de ma thèse est de proposer de nouvelles pratiques de cultures qui permettraient la réduction d'utilisation des intrants.

Notre stratégie expérimentale repose sur l'utilisation de plants de tomate au stade végétatif, cultivés en hydroponie dans des chambres climatisées, facilitant ainsi la régulation de la nutrition azotée, le contrôle des facteurs externes tels que la température, la luminosité et limitant fortement l'entrée de contraintes biotiques indésirables. Le cultivar de tomate choisis, *Solanum lycopersicum* L. cv. Better Bush VFN Hybrid (producteur : Tomato Growers), est une variété de tomates faciles à contrôler, puisqu'il s'agit d'une variété précoce, naine qui se développe bien en culture hydroponique. De plus, son mécanisme de défense est induit par la scarification (Royer, 2013) et sa concentration de polyphénols augmente sous limitation de la nutrition azotée (Larbat *et al.*, 2012). Afin d'obtenir un bon succès d'infestation, nous avons décidé d'utiliser des larves au 3^{ème}

stade larvaire (sur une échelle de 4) qui sont moins susceptibles de mourir lorsqu'elles sont manipulées que des œufs ou des larves plus jeunes. En outre, nous voulions induire les mécanismes de défense sans altérer la croissance des plantes et observer leurs défenses sur des zones locales et systémiques. Ainsi, les larves ont été déposées sur les trois folioles terminales de la 4^{ème} feuille de la plante, en comptant à partir du bas, à l'exception d'une expérimentation, dans laquelle nous avons besoin de plantes plus jeunes.

Mes travaux de thèse sont structurés en six chapitres dont le contenu est brièvement décrit ci-après, Figure 18.

En premier lieu, compte tenu des résultats préliminaires obtenus dans notre équipe (Royer, 2013), montrant que la concentration en polyphénols et en tomatine n'était pas influencée par l'herbivorie de deux larves de *Tuta absoluta* et que dans la littérature, une étude montre un effet de ce ravageur, à l'aide d'une charge beaucoup plus importante, sur la composition du bouquet de volatils émis par la tomate (Silva *et al.*, 2017), il semble donc essentiel de déterminer la charge larvaire minimale qui permette d'induire des réponses de la tomate.

Nous avons donc testé différentes charges en larves sur l'élicitation d'enzymes oxydatives connues pour répondre à l'herbivorie. Cette étude est présentée dans le chapitre 2.

Dans un deuxième temps, il a été montré que la limitation azotée altérait le développement des larves de *Tuta absoluta*, en réduisant le gain de masse des larves et en ralentissant leur développement (Han *et al.*, 2014 ; Larbat *et al.*, 2016). Par conséquent, nous avons testé "l'hypothèse trophique" selon laquelle la limitation azotée altérerait le développement de *Tuta absoluta* en raison d'une baisse de la qualité nutritive des tissus foliaires. Les résultats de l'impact de la nutrition azoté de la plante sur le régime alimentaire de *Tuta absoluta* sont présentés dans le chapitre 3.

Dans le chapitre 4, nous avons discuté des répercussions de la limitation de l'azote et de l'herbivorie sur les composés de défense de la tomate. Cette section englobe la défense constitutive et inductible, dont le rôle ne peut être nié, à l'encontre du développement de *Tuta absoluta*. Par conséquent, cette étude se concentre sur les composés de défense des tissus foliaires et leur distribution dans la plante de tomate.

Le chapitre 5 est consacré à la défense indirecte. Dans cette section, nous avons comparé les résultats qualitatifs de deux méthodes de

Chapitre 2

Quelle charge en larves est nécessaire pour induire une réponse de la tomate?

Determination of the larval load required to induce tomato defence through polyphenol oxidases and peroxidases activities

- Test de différentes charges en larves sur l'induction d'enzymes oxydantes.

Chapitre 5

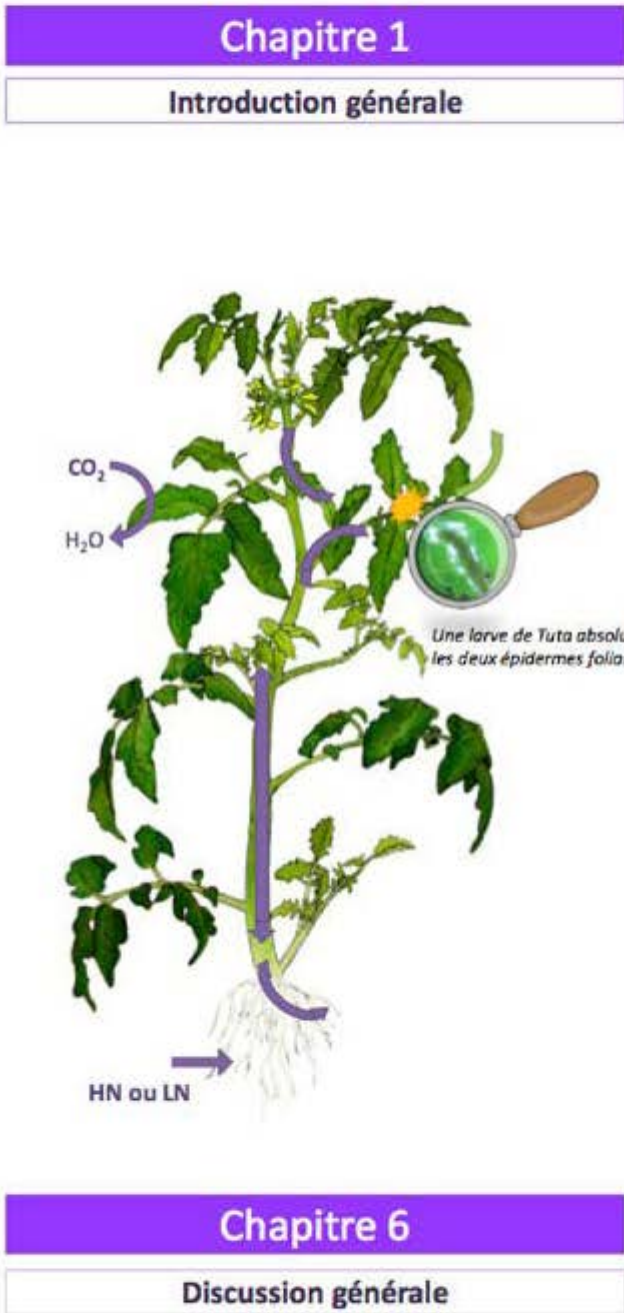
Comment la limitation azotée modifie le cocktail moléculaire émis par la tomate agressée par *Tuta absoluta*?

Nitrogen limitation alters the tomato volatile blend attacked by *Tuta absoluta* leafminer

- Comparaison des émissions de composés volatils en utilisant deux méthodes de piégeages entre les plants HN et LN, sains et infectés.

piégeage et analysé l'impact quantitatif de la limitation de l'azote sur les réponses de la tomate vis-à-vis de *Tuta absoluta*. Enfin, je vous propose une discussion générale pour résumer les résultats

importants de ce projet, clarifier les difficultés d'évaluer les coûts et bénéfices de la défense et proposer des idées novatrices dans la recherche des interactions plante-insecte.



■ **FIGURE 18** : ORGANISATION DU MANUSCRIPT EN CHAPITRE, QUESTIONS DE RECHERCHE ET STRATÉGIES EXPÉRIMENTALES

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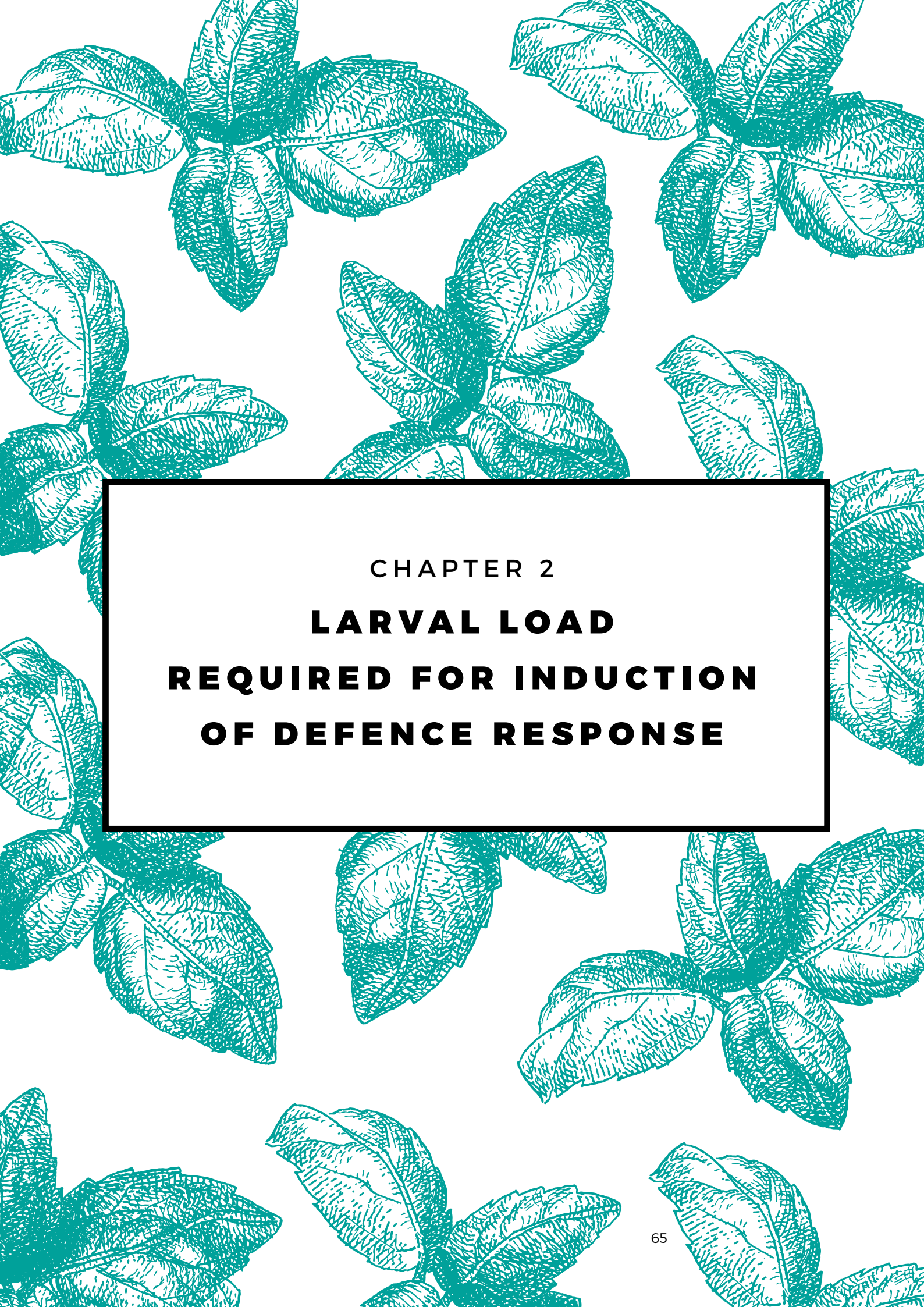
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CHAPTER 2

**LARVAL LOAD
REQUIRED FOR INDUCTION
OF DEFENCE RESPONSE**

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FOREWORD

An experimental prerequisite to determine tomato responses to the larval herbivory of the *Tuta absoluta* microlepidoptera was to investigate the larval load required for induction of a plant response. We then analysed the activity of oxidative enzymes, known to be induced by herbivory. We have thus determined the effect of different larval loads on the induction of these enzymes. For the logic of the thesis manuscript, the results of this work are presented in this first chapter, in the form of a scientific paper. However, the obtained results have been incorporated as additional data in the first article published in my thesis, in the Journal of Insect Physiology in March 2017 and entitled "Nitrogen Nutrition of Tomato Plant Alters Leafminer Dietary Intake Dynamics", (Coqueret *et al.*, 2017).

PRÉAMBULE

Un prérequis aux expérimentations visant à déterminer les réponses de la tomate à l'herbivorie de la larve mineuse du microlépidoptère *Tuta absoluta* était d'étudier quelle charge en larve était nécessaire pour induire une réponse de la plante. Nous avons alors analysé l'activité d'enzymes oxydantes, connues pour être induites lors d'un stress herbivore. Nous avons ainsi déterminé l'effet de différentes charges en larves sur l'induction de ces enzymes. Pour la logique du manuscrit de thèse, ce travail est présenté dans ce premier chapitre de résultats, sous la forme d'un article scientifique. Cependant, les résultats obtenus ont été incorporés en tant que données supplémentaires au premier article publié sur ma thèse, intitulé "Nitrogen Nutrition of Tomato Plant Alters Leafminer Dietary Intake Dynamics" , publié dans le Journal of Insect Physiology, en mars 2017 (Coqueret *et al.*, 2017).

1

ABSTRACT

Following a herbivory attack, plants induce a group of reactions leading to specific defence. In order to determine the number of larvae required to induce tomato plant defence and avoid severe herbivory damage on leaf tissues, we tested the effect of 3 loads of larvae (1, 6 and 12 larvae) on oxidative enzymes, namely polyphenol oxidases (PPO) and peroxidases (POD). These loads were compared to a negative and a positive control (no larva and manual scarification). Larva deposit and scarification were performed on the three terminal leaflets of the 4th leaf. Harvest and sampling were done 48 hours later. Herbivory did not severely change plant fresh weight nor total leaf surface. Within the 2 days, mined areas

per larvae were not different between larval loads, and were around 0.5 cm². Polyphenol oxidase activity was locally induced in the infested leaves of plants subjected to 12 larvae and was systemically induced whatever the larval load. Peroxidase activity was progressively induced with the larval load in the terminal leaflets and was also induced in the compartments below the infected leaf which, unlike upper leaves, showed no difference with both controls.

KEY WORDS :

Solanum lycopersicum / *Tuta absoluta* / defence induction / polyphenol oxidase / peroxidase

2

INTRODUCTION

During the evolutionary process, plants have developed defence mechanisms against herbivores (Ehrlich and Raven, 1964). Insect herbivore bites create two types of induction. The first one comes from the physical damage to the plant whose signal is rapidly launched as a cell electrochemical potential difference (Hilker and Meiners, 2010). The second one is a specific recognition of a protein of the insect saliva by fixation on a plant pattern recognition (Maffei *et al.*, 2007; Mithöfer and Boland, 2008). Both inductions tend towards the release of Ca^{2+} in cell cytoplasm, leading to an oxidative burst and activating oxidative enzymes such as peroxidases or polyphenol oxidases (Stout *et al.*, 1994; Kudla *et al.*, 2010; Niinemets *et al.*, 2013). These oxidative enzymes have been described in many plant species (Mayer, 2006) and POD participate in the elimination of the reactive oxidative species within plant tissues under attack, and in the synthesis of toxins that

directly deters the insect feeding (War *et al.*, 2012). Moreover, when PPOs come in contact with phenolic substrates, for example when leaf tissue is damaged by insect feeding, they rapidly oxidize phenolics into orthoquinones. Thus, the digestibility of protein and the bioavailability of amino acids for the herbivore are reduced (Felton *et al.*, 1989). PPOs can also be combined with specific phenolic substrates in glandular trichomes to produce a “super glue” trap for insects (Falco *et al.*, 2001). Additionally, this signal transduction allows phytohormone syntheses, such as jasmonate, salicylate or derivatives, activating various defence metabolism pathways in the whole plant (Onkokesung *et al.*, 2010).

At different stages of defence induction, it has been shown that plant responses are specific to the feeding guilds. Indeed, chewing insects such as caterpillars induce plant jasmonic acid signalling, piercing and sucking insects such as aphids induce

salicylic acid and leafminers would induce both plant phytohormones (reviewed by Felton and Korth, 2000 and Arimura *et al.*, 2011). The caterpillar *Helicoverpa zea* induces polyphenol oxidases in tomato leaves whereas the leafminer *Liriomyza trifolii* and the mite *Aculops lycopersici* stimulate peroxidase activities (Stout *et al.*, 1994). Howe and Jander (2008) indicated that plant defence responses are commensurate with the intensity and duration of the attack. The effect of increasing aphid density on cotton lead to an increase of peroxidase concentration (Ramirez and Eubanks, 2016), while Kant *et al.* have shown an increase of tomato response with increasing duration of spider mite attack (Kant *et al.*, 2004). But this hypothesis has been rarely tested (Niinemets *et al.*, 2013) and not tested for the tomato - *Tuta absoluta* pathosystem.

A recent study has shown no effect of *Tuta absoluta* on the concentration of 14 polyphenols and tomatine in tomato leaf tissues, after 7 days of herbivory of two newly born larvae per plant (Larbat *et al.*, 2016). But, another study on tomato volatile compounds, shows a positive effect of the herbivory of *Tuta absoluta* on the emission of 38 compounds, mostly peptidic fatty acids (C-18) (Silva and al., 2017). The infection load was estimated at 245 newborn larvae per plant and the herbivory lasted 3 days. The difference in larval load in the last-mentioned studies, is unquestionable. Thus, our objective was to test and determine which load of *Tuta absoluta* larvae would induce local and / or systemic defence on vegetative tomato plants. Enzymatic activities (PPO and POD) are suitable indicators of plant defence responsiveness to herbivory and were measured in local and systemic plant compartments.

3

MATERIALS AND METHODS

3.1

TOMATOES AND INSECTS

Tomato seeds (*Solanum lycopersicum* L. cv. Better Bush VFN Hybrid, grower: Tomato Growers) were sown and after germination, plantlets were grown using a hydroponic system under controlled conditions. Better Bush is an early and dwarf variety, easy to manage in a hydroponic growth system and showing good responses to scarification elicitation (Royer *et al.*, 2013). When this cultivar is grown under nitrogen limitation, it has been reported that plant

polyphenol concentration is enhanced (Larbat *et al.*, 2012).

Tuta absoluta was provided by Dr. N. DESNEUX (Institut Sophia Agrobiotech UMR INRA 1355, Sophia-Antipolis) and adults reared on tomato plants in an insectarium set in a climatic chamber. Then, 3rd instar (scale 1 to 4) larvae were selected for infestation from the insectarium.

3.2

PLANT GROWTH CONDITIONS

Eleven days after sowing, the seedlings were transplanted onto a hydroponic system set in a growth chamber (16 h photoperiod, 24 °C / 18 °C day / night in air and 25 °C in nutrient solutions, 60 % air humidity). Plants received a full nutrient solution supplying nitrogen at a high concentration for tomato growth. Nitrogen was provided as NO₃⁻ and its concentration was adjusted once a day at 7 mM. The pH was also adjusted between 5 and 6.

3.3

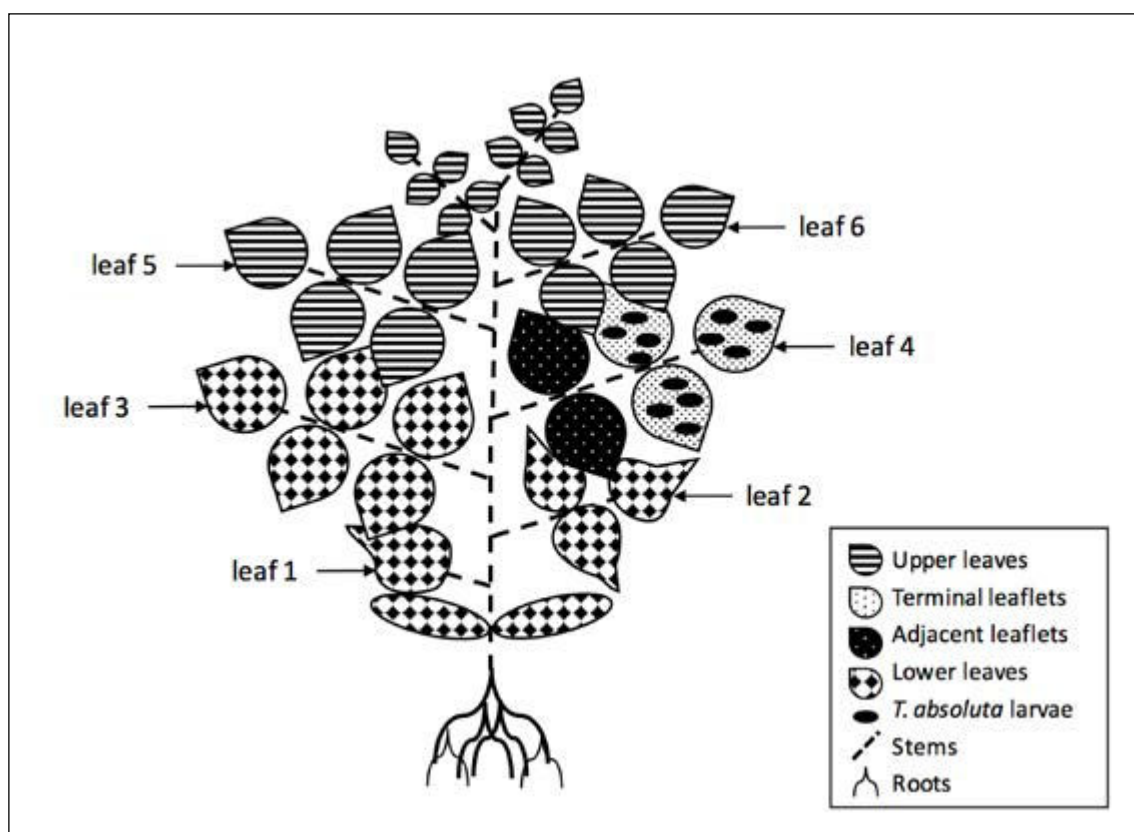
TREATMENTS

Treatments started when the plants had 4 fully developed leaves. One, 6 or 12 larvae were deposited on the three terminal leaflets of the 4th true leaf from the collar using 6 plant replicates. Larvae, that did not penetrate the leaf following 2 hours,

were replaced in order to optimize the number of replicates with effective herbivory. Three plants were lightly touched with a paintbrush to mimic larval deposit (mock inoculation) and three other plants were scarified using an orienteering control punch.

HARVEST AND SAMPLING

Forty-eight hours after the onset of the treatment, plants were harvested. Larvae were counted and retrieved from the plants. Roots, stem and leaves were sampled separately (Figure 19) and freeze-dried. Samples were then weighted using a 0.01 g precision balance.



■ **FIGURE 19** : SCHEME OF PLANT COMPARTMENTATION USED DURING THE HARVEST

3.5

POLYPHENOL OXIDASE AND PEROXIDASE ENZYMATIC ACTIVITIES

Fresh frozen samples were ground using pestle and mortar. Fifty milligrams of fresh matter were mixed in 2 mL Eppendorf and 500 µL of extraction buffer (potassium phosphate buffer 0.1 M pH 7, containing 7 % of PVPP and 1 % of Triton X-100). Using a pellet pestle, the mixture was blended. After 30 s of vortex, samples were centrifuged at 10.000 x g during 10 min.

In a 96 wells' microplate, 300 µL of activity buffer (for PPO: potassium phosphate buffer at 0.1 M pH 7, containing 3 mM of caffeic acid / for POD: potassium phosphate at 0.1 M pH 7, containing 0.25 % of guaiacol

and 0.125 % of H₂O₂) were transferred in a well. The plate was set for 2 min in the microplate lector to warm the buffer at 25 °C. Fifteen microliters of extract were added in an empty hole for PPO activity and 10 µL for POD activity. Using a multichannel pipette, 240 µL of the activity buffer were mixed with the extracts. Absorbance of the mixture was read at 470 nm every 10 sec for 8 min with the spectrophotometer Cary® 300 Scan (Varian, Palo Alto, USA) and Cary Win UV 3 software. Enzymatic activities were determined using initial speed.

3.6

STATISTICS

Non-parametric tests were performed using XLSTAT (v18.07.38968, Addinsoft) and statistical significance was set at $P < 0.05$. Multiple comparisons were

performed using Kruskal-Wallis test and pairwise ranking comparisons were performed using Dunn's procedure.

■ **TABLE 3:** PLANT FRESH WEIGHT (G), MEAN AND S.E.M.

Larvae were deposited and scarification was done on the three terminal leaflets 48 h before harvest. Larvae were retrieved and counted, plant samples were weighted and leaf surfaces were measured using Li-3100 C area meter (Li-Cor, Lincoln Nebraska, USA). Different letters indicate significant differences between treatments (Pairwise ranking comparisons using Dunn's procedure, $P < 0.05$).

treatment	n	Plant FW (g)		Leaves FW (g)		Stems FW (g)		Roots FW (g)	
		mean	s.e.m.	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.
0 larva	3	a 29.7	2.3	a 11.6	0.9	a 12.4	1.1	a 5.7	0.5
1 larva	6	a 36.4	5.5	a 14.3	2.2	a 14.0	2.0	a 8.1	1.3
6 larvae	6	a 31.6	5.4	a 12.7	2.1	a 12.3	2.3	a 6.7	1.1
12 larvae	6	a 32.0	6.7	a 12.9	2.5	a 12.0	2.6	a 7.1	1.7
scarification	3	a 35.1	2.4	a 14.1	1.1	a 13.5	1.1	a 7.5	0.3

4

RESULTS

4.1

PLANT FRESH WEIGHT

Forty-eight hours after larval deposit or scarification, leaf, stem, root and entire plant fresh weights did not derogate from control ones (Table 3). However, it is worth noticing that the mean standard error (s.e.m.) increased with the larval load.

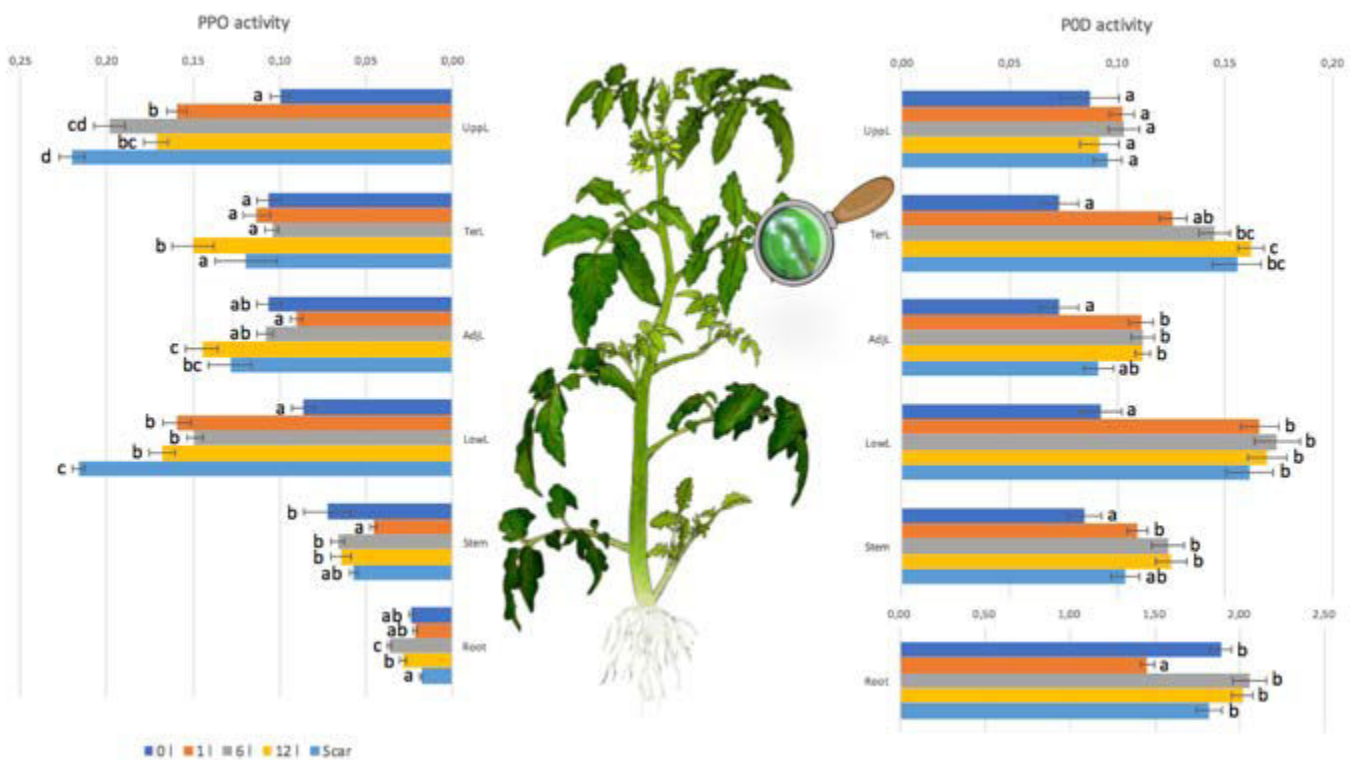
4.2

TOTAL PLANT LEAF AREA AND LEAF AREA OF INFESTED LEAF

Mean mine area dug by one larva was not different between the 3 loads (around 0.5 cm² of leaf consumption per larva after 2 days of herbivory). Thus, mine areas increased linearly with the number of larvae deposited on the leaves and the higher loads ate significantly higher leaf area. This effect was negligible at the whole leaf scale as total leaf area was not significantly affected by the treatments (Table 4).

■ **TABLE 4 :** LEAF, MINE AREAS AND MINE AREA PER LARVA (cm²), MEAN AND S.E.M. Larvae were deposited and scarification was done on the three terminal leaflets of the 4th leaf, 48 h before harvest. Larvae were retrieved and counted, plant samples were weighted and leaf surfaces were measured using Li-3100 C area meter (Li-Cor, Lincoln Nebraska, USA). Nb larvae refers to the average of live larvae found during harvest. Different letters indicate significant differences between treatments (Pairwise ranking comparisons using Dunn's procedure, $P < 0.05$).

treatment	n	Nb larvae	Leaf Area (cm ²)		Mine Area (cm ²)		Mine Area / larva (cm ²)				
			mean	s.e.m.	mean	s.e.m.	mean	s.e.m.			
0 larva	3	0	a	485	39	a	0.00	0.00	N/A	N/A	
1 larva	6	1.3	a	532	72	ab	0.63	0.17	a	0.53	0.17
6 larvae	6	5.3	a	474	78	ab	2.91	0.69	a	0.53	0.27
12 larvae	6	11.2	a	465	94	b	5.95	1.13	a	0.53	0.09
scarification	3	0	a	529	32	a	0.00	0.00	N/A	N/A	



■ **FIGURE 20** : PLANT PEROXIDASES (POD) AND POLYPHENOL OXIDASES (PPO) RESPONSES TO THE LOAD OF ARTIFICIALLY INOCULATED *Tuta absoluta* LARVAE.

These enzymatic activities (arbitrary units, *i.e.* spectrophotometric absorbance changes per minute and per mg FW) are suitable indicators of herbivore attacks (Duffey and Stout, 1996; Larbat *et al.*, 2016). They were measured on tomato leaves, stems and roots using the method described in Stout *et al.* (1994). Larvae were deposited and scarification was done on the three terminal leaflets 48 h before harvest. Samples were separated, frozen in liquid nitrogen and kept at -80°C before measurement. Numbers under the bars denote the larval load (0-12) and “scar” stands for scarification. Bars are means \pm s.e.m. of 3 - 6 replicates. Different letters indicate significant differences between treatments for each plant compartment separately (Multiple comparisons using Kruskal-Wallis test and Dunn’s procedure, $P < 0.01$).

OXIDATIVE ENZYME ACTIVITIES

The presence of larvae led to an increased polyphenol oxidase (PPO) activity (Figure 20). This activity induction was dependent on the load. In the terminal and adjacent leaflets of the infected leaf, the PPO activity was significantly stimulated under herbivory with 12 larvae (almost 50 % higher compared to the negative control), whereas this induction was significant from 1 larva in the upper and lower leaves (from 0.6 to 2.2 times higher compared to the negative control). In stems, there was no impact of herbivory except for the 1 larva load which showed a lower PPO activity than the negative control (about 1/3rd lower). In roots, PPO activity was around 4 times lower than in leaves. Only the 6 larvae load showed a significantly higher PPO activity than the control (about 2/3rd higher). For the other treatments (1, 12 larvae and scarification), root PPO activity showed no significant differences with the control.

Peroxidase (POD) activity was increased by *Tuta absoluta* herbivory (Figure 20). In the terminal leaflet, 6, 12 larvae and scarification treatments showed higher POD activities than the control (2, 2.2 and 2.1 times higher, respectively). Larvae treatments (1, 6 and 12) induced an increase of POD activity in the adjacent leaflets and the stems compared to the control. POD activities in upper leaves were not significantly different between the treatments. In lower leaves, POD activity was higher for all the treatments compared to the control (from 75 to 90 % higher). POD activity was almost 10 times higher in roots than in leaves or stems. In roots, only the one larva treatment led to a significant modification of POD activity (decreasing) with regard to control.

5

DISCUSSION

5.1

PLANT PHYSIOLOGY AND *Tuta absoluta* HERBIVORY

Tuta absoluta larvae hatch on the leaf surface and penetrate immediately the tissue. Once within the plant, they feed mainly on the mesophyll and develop. Within the 2 days, each 3rd instar larva ate about 0.5 cm² of leaf mesophyll and this did not depend on the larval load. Thus, a potential competitiveness for food between larvae did not happen. Furthermore, the tissue loss caused by *Tuta absoluta* herbivory did not impact total leaf surface nor plant fresh weight.

5.2

OXIDATIVE ENZYME INDUCTIONS

The constitutive level of PPO activity in Better Bush cultivar was around 3 times lower than the PPO activity of Santa Clara tomato variety for high nitrogen - control plants (Larbat *et al.*, 2016). The concentration of PPO, encoded by at least 7 different genes in tomato, may differ even between varieties of the same specie (Thipyapong and Steffens, 1997). Moreover, for the reason that experimental conditions are likely to be different between studies, the comparison of the enzymatic activities are tricky. We showed that the enzymatic activities were different between plant compartments. The leaves and stems had a stronger PPO activity than roots and *vice versa* for POD activities. Peroxidases are able to polymerize phenol with the H₂O₂ cofactor and thus reduce the digestibility of plant tissues for herbivores. But in roots, they have been shown to contribute to the elimination of bacterial phenols

and to the production of lignin and suberin for the cell wall (Quiroga *et al.*, 2000; Wevar Oller *et al.*, 2005), which explains their high concentration in roots compared to the other plant compartments.

Tomato defence induction has been observed *via* PPO and POD activities. Thus, PPO and POD activities were induced in systemic leaves from the attacked zones with at least 1 larva, but the local response started with a 12 larvae load for PPO and 6 larvae load for POD. It is well-known that these oxidative enzymes are reactive to herbivory attack (Stout *et al.*, 1994; War *et al.*, 2012), and it has already been shown that *Tuta absoluta* induces PPO activity in tomato leaves with a 2 young larvae load (Larbat *et al.*, 2016) but to our knowledge, this is the first report to give the spatial distribution of PPO and POD enzymatic activities within tomato plants attacked by *Tuta absoluta*.

CONTRIBUTION AND ACKNOWLEDGEMENTS

CR, VC and RL conceived the idea of the experiment and helped to design the experiment. ND provided the leafminers. VC, Aude Fauvet (INRA), CR and RL performed the harvests; VC and AF performed the analyses and VC processed the data. We acknowledge the technical assistance of AF and Claude Gallois (Université de Lorraine).

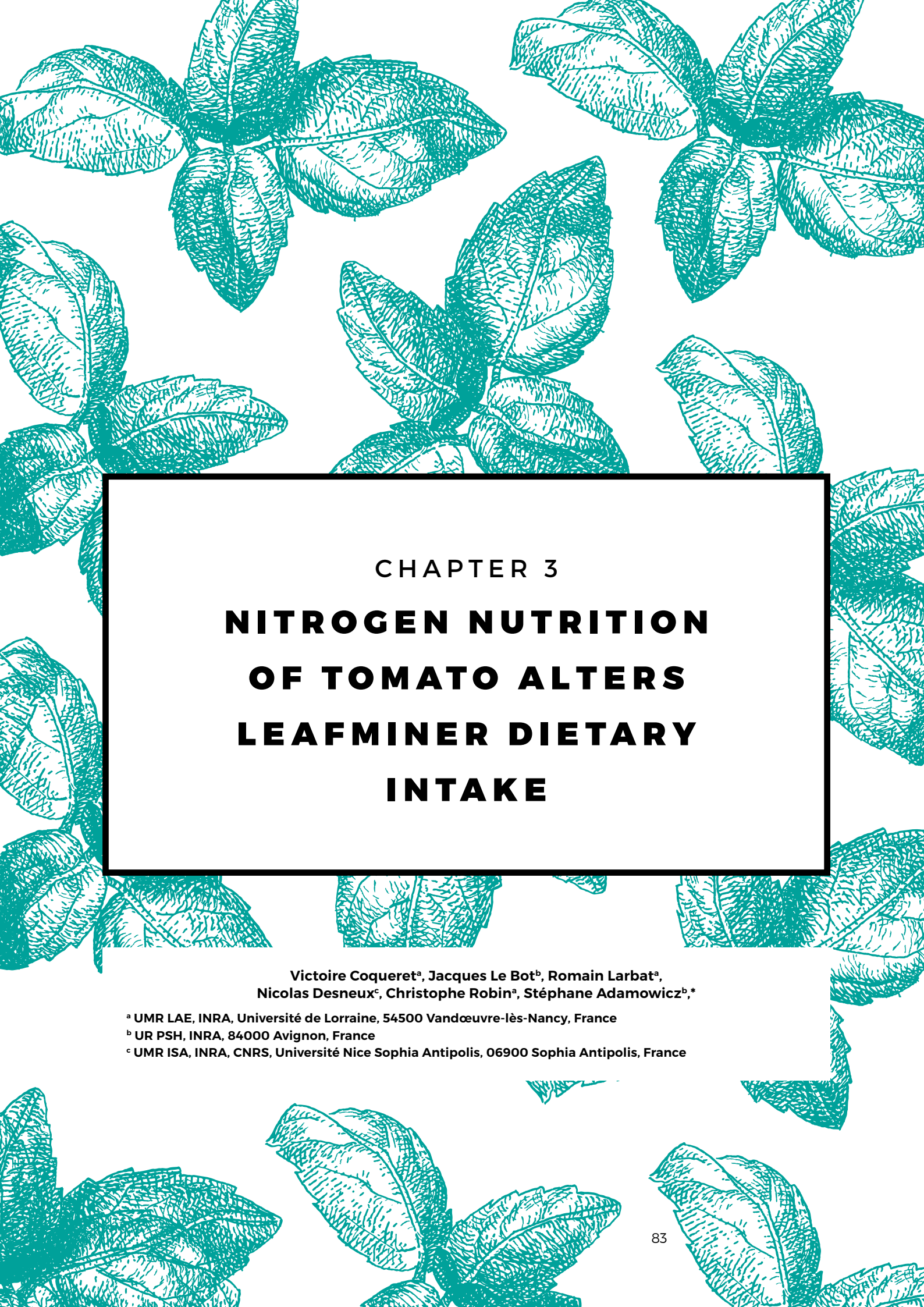
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CHAPTER 3

**NITROGEN NUTRITION
OF TOMATO ALTERS
LEAFMINER DIETARY
INTAKE**

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FOREWORD

The intensive use of inputs in agriculture ensures high yields but is nevertheless controversial. The excessive use of fertilizers and plant protection products has undesirable effects on the quality of harvest yields, and on water, modifying their biotic balance and equally on human health. Agricultural practices, based on recent recommendations and legislations, tend towards a sharp decrease or even a ban on phytosanitary treatments. In some cases, no effective alternative methods are available. Plants have developed defence mechanisms against pests. In the case of the tomato - *Tuta absoluta* pathosystem, the literature review indicates that little knowledge is available about the responses of the plant to the herbivorous aggressor. The preceding chapter indicates that, soon after the infestation, a low larval load, located on a few leaflets, activated locally and systemically the enzymes involved in the oxidative burst. Recent research shows that limiting nitrogen availability increases the mortality rate of these pests and slows their growth (Han *et al.*, 2014, Larbat *et al.*,

2016). Two hypotheses that have to be tested, can explain the impact of this nitrogen limitation on the herbivore: i) the “trophic” hypothesis: a decrease in the nutritional values of the larval food bowl (tissues consumed) and / or ii) the “defence” hypothesis: an increase in the concentration of defence compounds and the presence of more defence compounds in the tissues of plants infested and limited in nitrogen. In this chapter, we tested the first hypothesis, knowing that the second will be tested in the next chapter. We have implemented an experiment at INRA, Avignon, in culture chambers with strict control and regulation of nitrogen supply to tomatoes. This chapter was published in the *Journal of Insect Physiology*, April 2017 (vol.91, pp. 130-138).

PRÉAMBULE

L'usage intensif d'intrants en agriculture permet d'obtenir des rendements élevés mais il est remis en question. En effet, l'utilisation excessive de fertilisants et produits phytosanitaires entraîne des effets indésirables sur la qualité des produits récoltés, sur les eaux, modifiant leurs équilibres biotiques et sur la santé humaine. Les pratiques agricoles, s'appuyant sur des recommandations et législations récentes, tendent vers une forte diminution voire une interdiction des traitements phytosanitaires. Dans certains cas, aucune alternative efficace n'est disponible. Les végétaux ont développé des mécanismes de défense contre les bioagresseurs. Dans le cas du pathosystème tomate - *Tuta absoluta*, l'examen de la littérature indique que peu de connaissances sont disponibles sur les réponses de la plante à l'agresseur herbivore. Le chapitre précédent indique que rapidement après l'infestation, une faible charge en larves, localisée sur quelques folioles, active localement et de manière systémique des enzymes impliquées dans le stress

oxydatif. Des travaux récents montrent que la limitation de la disponibilité en azote augmente le taux de mortalité de ce ravageur et ralentit sa croissance (Han *et al.*, 2014; Larbat *et al.*, 2016). Deux hypothèses à tester peuvent expliquer l'impact de cette limitation en azote sur l'herbivore i) hypothèse "trophique" d'une diminution de la valeur nutritive du bol alimentaire des larves (tissus consommés) et / ou ii) hypothèse "défense" de l'enrichissement en nombre et en concentration des composés de défense dans les tissus des plantes infestées et limitées en azote. Dans ce chapitre, nous avons testé la première hypothèse, sachant que la seconde sera testée dans le chapitre suivant. Nous avons mis en œuvre une expérimentation à l'INRA d'Avignon dans des chambres de culture avec un contrôle et une régulation strictes de l'alimentation en azote des tomates. Ce chapitre a fait l'objet d'une publication dans *Journal of Insect Physiology*, en avril 2017 (vol. 99, pp. 130-138).

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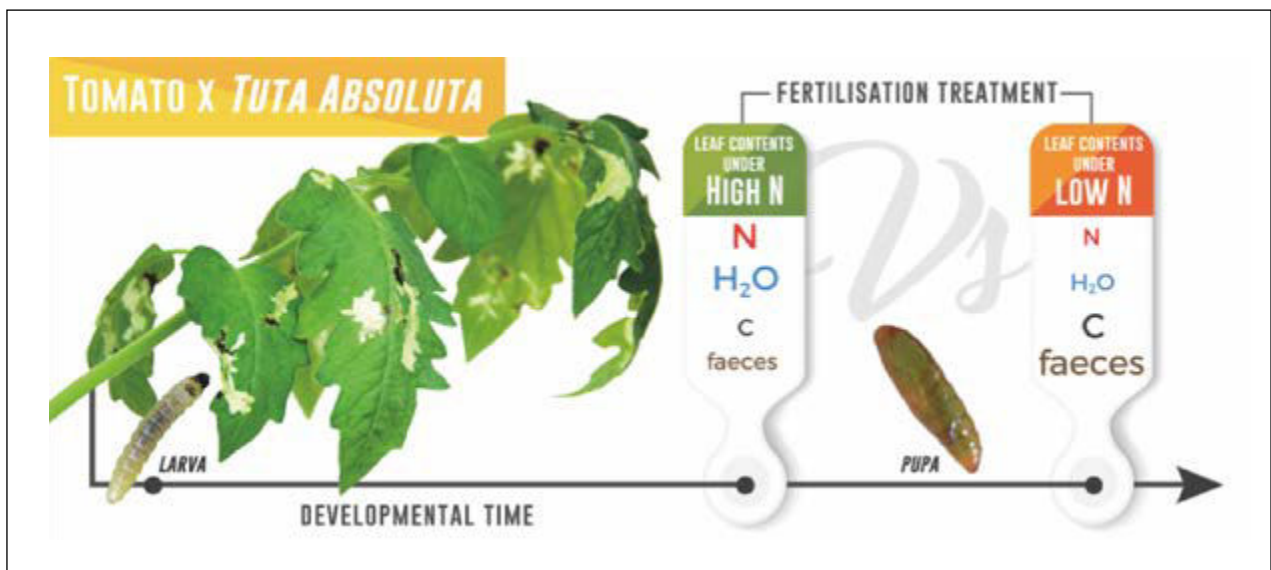
ABSTRACT

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 nitrogen limitation reduces plants' primary metabolism but enhances their 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-*Tuta absoluta* interaction in terms of resource supply by leaves and intake by larvae. Tomato plants were raised under controlled conditions on nitrogen-sufficient *versus* nitrogen-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 on 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 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 nitrogen limitation, *Tuta absoluta* consumed less water and nitrogen 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 nitrogen and / or water intakes.

KEY WORDS :

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



■ **FIGURE 21** : GRAPHICAL ABSTRACT: NITROGEN NUTRITION OF TOMATO ALTERS LEAFMINER DIETARY INTAKE.

2

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 *Tuta 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 the 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 plants as opposed to 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, nitrogen fertilization not only increases plant nitrogen 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 nitrogen status (Herms and Mattson, 1992).

Tuta absoluta responds to varying nitrogen status of the tomato crop in accordance with these general principles. For instance, low nitrogen 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 nitrogen 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 *Tuta absoluta* larvae feed and develop. It is well established that tomato tissues respond to nitrogen fertilization by increasing organic-nitrogen 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 tomatoes for *Tuta 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 nitrogen and water contents may impair and/or slow down *Tuta 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 *Tuta absoluta* larvae on a specific developing leaf and manipulated the nitrogen status of tomato plants growing hydroponically, using a well-established automated 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.

3

MATERIALS AND METHODS

3.1

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 nitrogen nutrition regimes. Half the plants received a full nutrient solution supplying nitrogen as 1.0 mM NO₃⁻, a concentration previously shown to be non-limiting for tomato growth (Adamowicz and Le Bot, 2008) and presently referred to as “High Nitrogen” (HN). The solution was prepared with deionized water and pure salts to produce the following mM concentrations: Ca(NO₃)₂ 0.5; KH₂PO₄ 1.0; K₂SO₄ 1.0; MgSO₄ 1.5; CaSO₄ 3.0. Trace elements were provided as EDTA-Fe (43 μM) and 0.3 mL·L⁻¹ 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 nitrogen concentration previously shown to limit plant growth without affecting photosynthesis or producing nitrogen deficiency symptoms (Adamowicz and Le Bot, 2008; Le Bot *et al.*, 2009; Royer *et al.*, 2016), and referred to here as “Low Nitrogen” (LN). The initial NO₃⁻ concentration in this solution was 30 μM (as Ca(NO₃)₂ 0.015 mM and all other salts as in HN, except CaSO₄ 3.485 mM). This nitrogen concentration set-point was adjusted daily so that the LN plants absorbed only 1/3rd 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): KNO₃ 0.411; Ca(NO₃)₂ 0.206; Mg(NO₃)₂ 0.0885. The pH was also monitored and regulated hourly by *Totomatix* to 5.5.

3.2

PLANT INFESTATION BY *Tuta absoluta* LARVAE

Tuta absoluta adults were reared on caged tomato plants in climatic chambers. Females laid eggs and after hatching, 3rd instar larvae (scale 1 to 4) were selected for the experiment. Half of the HN and LN plants were infested 14 days after transplantation (dat) with 12 larvae deposited on the three terminal leaflets of the 4th 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 (Supplemental data, Figure 27; Chapter 2). Control plants were subjected to mock inoculations of larvae, performed with a paintbrush. We decided not to constrain the larvae to cages on the leaflets because leaf disturbance is known to elicit plant response defences (Braam, 2005; Gigolashvili *et al.*, 2007; Savatin *et al.*, 2014).

3.3

PLANT HARVESTING AND SAMPLE PREPARATION

The first harvest (H1, 12 HN and 11 LN plants) was taken 14 dat, prior to *Tuta 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 N₂ 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.

3.4

TRAITS OF *Tuta absoluta* DAMAGE AND DEVELOPMENT

On each infested leaf, larvae and pupae were counted at H2 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, page 114.

3.5

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.

3.6

DATA PROCESSING

3.6.1

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 nitrogen absorption from the nutrient solution was computed continuously

from transplantation to the end of the experiment by hourly applying 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 nitrogen uptake (mmol N per plant).

3.6.2

LEAF RESOURCE DENSITIES AND LARVAL RESOURCE INTAKE

For control plants, the specific leaf weights (**SLW**, mg DW cm⁻², also named LMA leaf mass per area), specific leaf C contents (**SLC**, mg C cm⁻²), specific leaf nitrogen contents (**SLN**, mg N cm⁻²) and equivalent water thicknesses (**EWT**, mg H₂O cm⁻²) 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 *Tuta 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 *Tuta absoluta* larvae, as **Am x SLW**, **Am x SLC**, **Am x SLN** and **Am x EWT**, respectively, Am being the mine areas.

3.6.3

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.

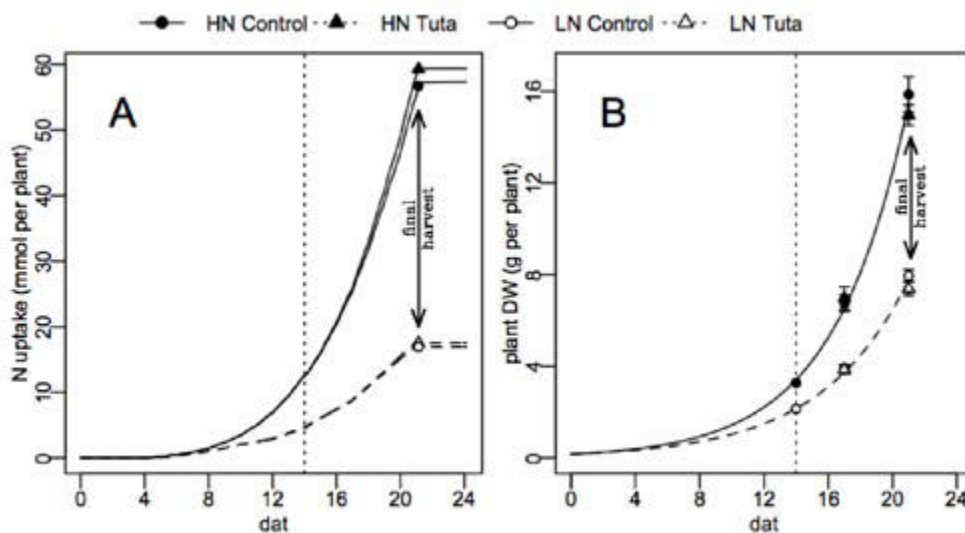
4

RESULTS

4.1

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 method (Figure 22.A). After the final harvest (H3), nitrogen 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 nitrogen uptake on the whole crops as calculated from the measurements of the *Totomatix* setup and nitrogen analysed in sampled plants (Supplemental data, page 113). Control and infested plants showed only slight differences in nitrogen uptake within the HN and LN treatments (4 and 3 % differences at H3, respectively, but a statistical significance cannot be assessed).



■ **FIGURE 22**: TOMATO NITROGEN UPTAKE AND GROWTH.

Following transplantation (0 dat, days after transplantation), the plantlets were supplied with either adequate (HN) or low nitrogen (LN) full nutrient solutions. At 14 dat (vertical dotted line), half the plants were infested by *Tuta 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 $\mu = 0.217$ (HN) and 0.185 (LN) days⁻¹.

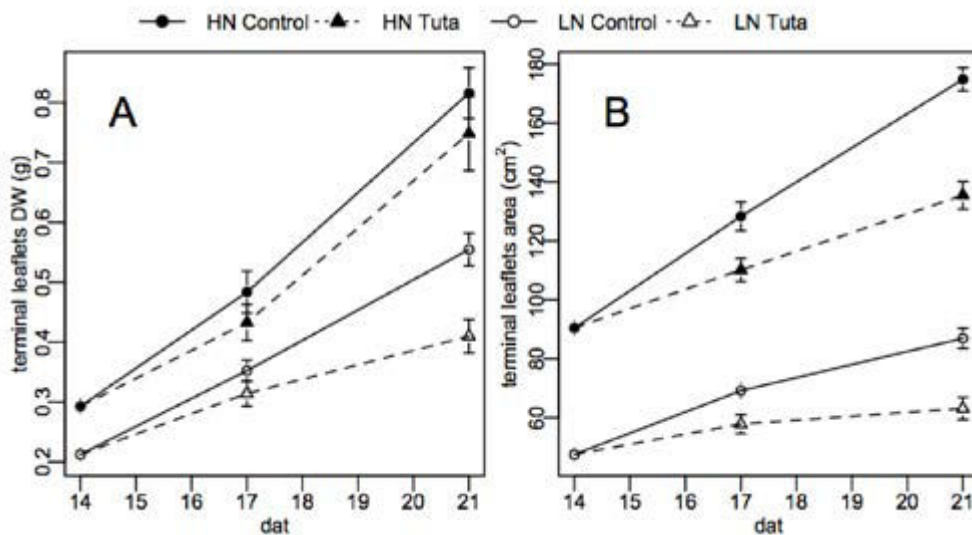
Plant dry weights (g per plant) increased exponentially over time (Figure 22.B), with significantly higher relative growth rates for HN than LN plants (0.217 and 0.185 days⁻¹, respectively;

$t_{(92)} = .5, P < 0.001$). At H3, HN plants were twice the dry weight of LN plants ($t_{(64)} = 18, P < 0.001$). There was no significant effect of *Tuta absoluta* herbivory on relative growth rates and harvested plant biomasses.

4.2

LEAFLET RESPONSES TO NUTRITION AND HERBIVORY

Control (*i.e.* mock treated) and *Tuta absoluta*-infested leaflets responded significantly to nitrogen nutrition for biomass accumulation (Figure 23.A; $F_{1,67} = 68, P < 0.001$) and area expansion (green areas + mines, Figure 23.B; $F_{1,65} = 502, P < 0.001$). Compared with controls, *Tuta absoluta*-infested leaflets had a significantly lower biomass (by 8 and 26 % in HN and LN, respectively; $F_{1,67} = 12, P < 0.01$) and area (by 27 and 22 % in HN and LN, respectively; $F_{1,65} = 16, P < 0.001$).



■ **FIGURE 23** : TERMINAL LEAFLET GROWTH AND EXPANSION.

Control and *Tuta absoluta*-infested plants were raised under high (HN) or low (LN) nitrogen nutrition. At 14 dat, 12 larvae were deposited 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 the leaflet biomass significantly ($F_{1,67} = 68, P < 0.001$ for nutrition, and $F_{1,67} = 12, P < 0.01$ for infestation) and area ($F_{1,65} = 502, P < 0.001$ for nutrition and $F_{1,65} = 16, P < 0.0001$ for infestation). In [A], dry weights are expressed in g, and in [B], areas in cm² include the mines.

HERBIVORY INTENSITY AND *Tuta 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; Figure 24.A; $F_{1,32} = 15$, $P < 0.001$), without any significant difference between nitrogen treatments. On average, the mine area was around 980 mm² per plant at H3.

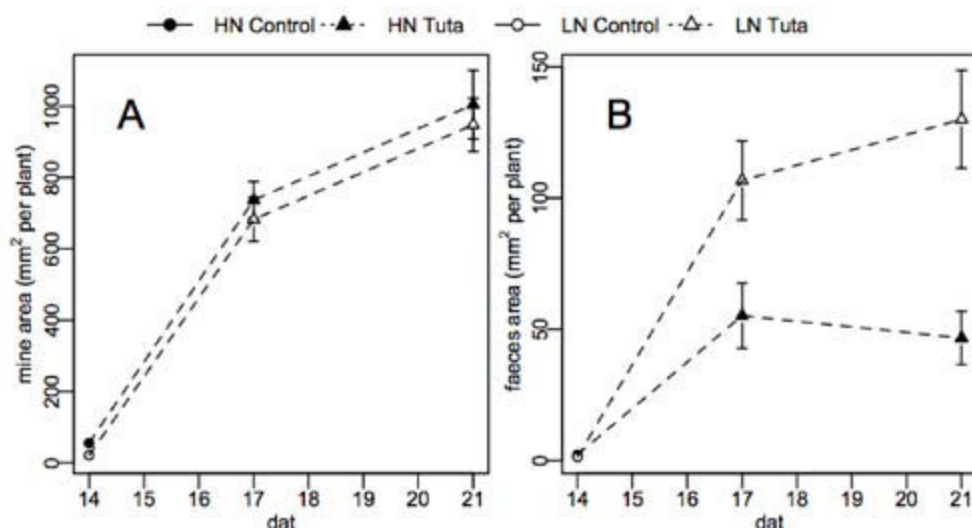
At H2 and H3, we recovered around 60 % of the larvae laid at H1 and there was no significant effect of nitrogen nutrition (Table 5). The missing larvae did not succeed in infesting the leaflets and died on the leaf surface during the first hours following

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 5; $\chi^2(1) = 7.4$, $P < 0.01$), but the status of nitrogen nutrition significantly affected the average larval and pupal fresh weights (Table 6; $F_{1,37} = 8.8$, $P < 0.01$).

The mine area occupied by faeces was significantly influenced by nitrogen nutrition, the area at H3 being nearly three-fold higher under LN than HN (Figure 24.B; $F_{1,32} = 23$, $P < 0.001$). The apparent changes between H2 and H3 were not significant.

■ **TABLE 5** : CONTINGENCY TABLES OF LARVAE DEPOSITED AND RECOVERED ON INFESTED TOMATO PLANTS. Immediately after the first harvest (H1), 12 larvae were deposited 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



■ **FIGURE 24** : AREAS OF MINES AND FAECES ON TERMINAL LEAFLETS INFESTED BY *Tuta absoluta* LARVAE. Experimental conditions and symbols are as in Figure 22. In [A], the mine areas (mm² per plant) did not change significantly with nitrogen treatments while in [B], the mine area occupied by faeces (mm² per plant) responded significantly to nitrogen ($F_{1,32} = 23, P < 0.001$). Notice that at 14 dat, the plants were not yet infected 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.

■ **TABLE 6** : 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 5) 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; $F_{1,37} = 8.8, P < 0.01$), 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

LEAF TRAITS AND RESOURCE INTAKE BY LARVAE

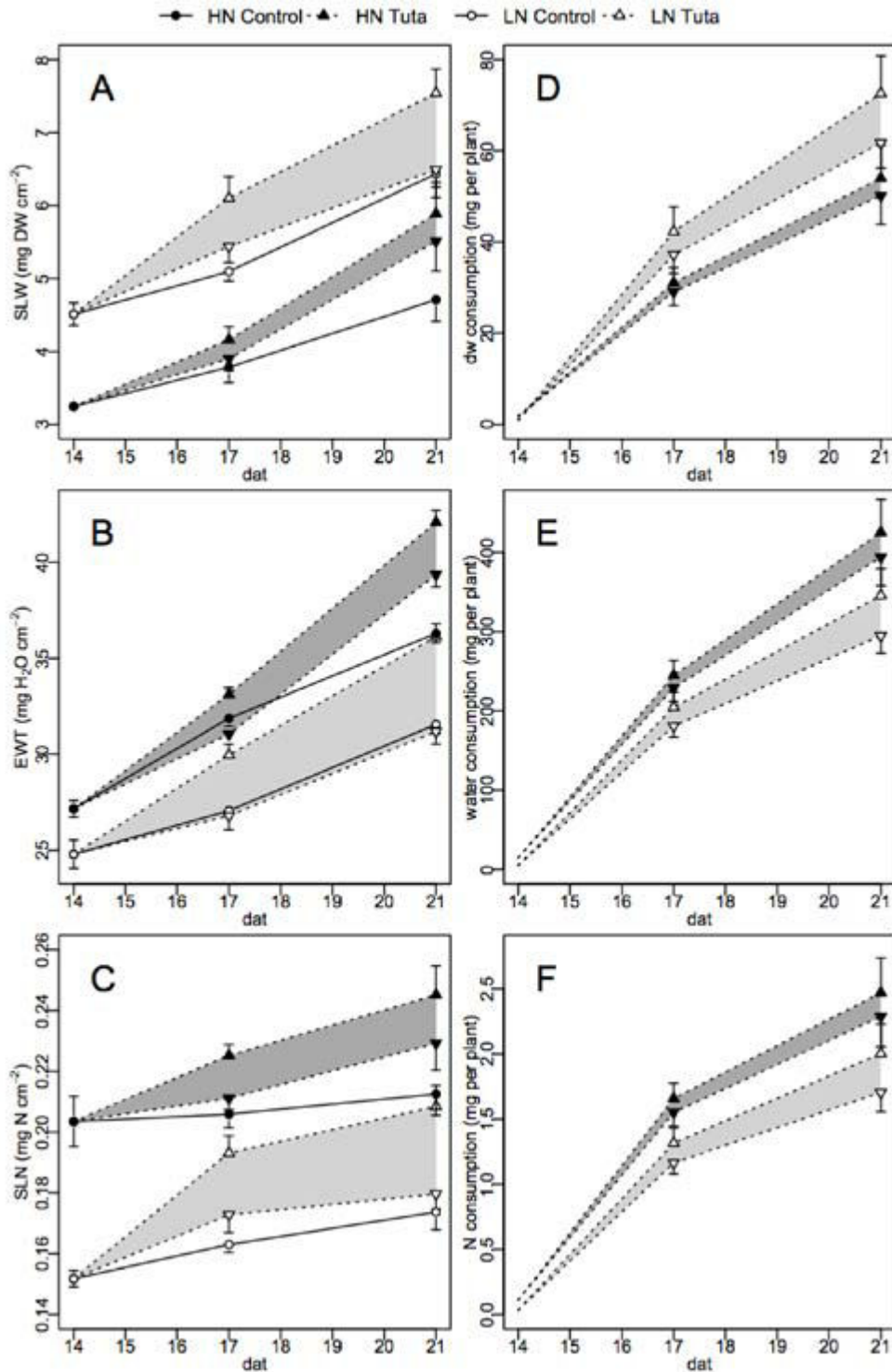
In order to determine the likelihood of some leaf traits in infested plants (specific leaf weight, SLW; equivalent water thickness, EWT; specific leaf carbon and nitrogen contents, SLC and SLN, respectively), calculations were made following two extreme hypotheses, where the leaflet DW, H₂O, C and N 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 Figure 25.

The specific leaf weight of control and infested leaflets increased significantly during leaf ageing (Figure 25.A; $F_{1,66} = 46$, $P < 0.001$) 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 Figure 25.A, 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 (Figure 25.B; $F_{1,62} = 38$, $P < 0.001$) but was consistently

lower under LN nutrition ($F_{1,66} = 40$, $P < 0.001$), contrary to SLW. Herbivory significantly affected 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 (Figure 25.C; $F_{1,66} = 7.8$, $P < 0.01$) 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).



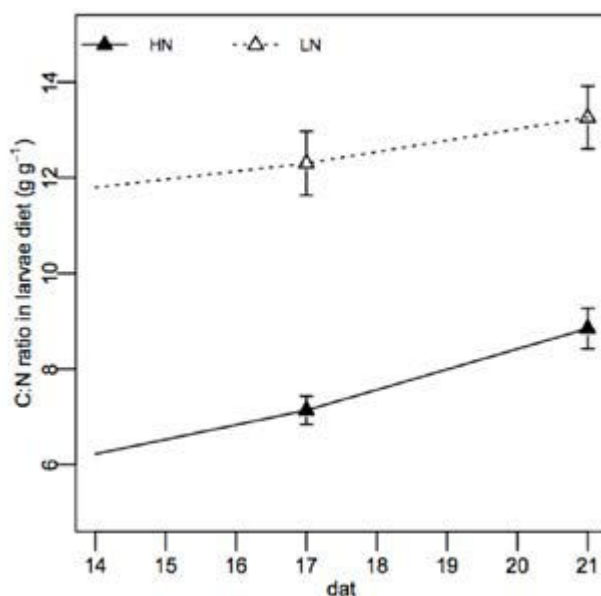
■ **FIGURE 25**: RESOURCE AREA DENSITIES AND RESOURCE CONSUMPTION BY LARVAE.

Experimental conditions and symbols are as in Figure 22. [A], specific leaf weight (SLW, mg DW cm⁻²); [B], equivalent water thickness (EWT, mg H₂O cm⁻²); [C], specific leaf nitrogen (SLN, mg N cm⁻²). 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 other hand 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 *Tuta absoluta* larvae, also shown as shaded areas (HN in dark, LN in light). All resource consumptions responded significantly to nitrogen treatments ($P < 0.001$).

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 Figure 25.D - 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 (Figure 25.D; $F_{2,31} = 18$, $P < 0.001$) 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, larvaecarbon intake (mg per plant) deposited in between 18.6 - 20.0 for HN plants, increased to 22.5 - 26.4 for LN plants. The larvae water consumption increased significantly from H2 to H3 (Figure 25.E; $F_{2,30} = 28$, $P < 0.001$) and was consistently lower under LN nutrition ($F_{2,30} = 38$, $P < 0.001$). The larvae nitrogen intake increased significantly from H2 to H3 (Figure 25.F; $F_{2,30} = 14$, $P < 0.001$) 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 (Figure 26; $F_{1,67} = 248$, $P < 0.001$) and increased significantly with leaf age ($F_{1,67} = 18$, $P < 0.001$).



■ **FIGURE 26** : DYNAMIC CHANGES OF C:N RATIO IN LARVAE DIET. Experimental conditions and symbols are as in Figure 22. The symbol absence at 14 dat indicates that plants were not yet infested.

5

DISCUSSION

Our results demonstrate that following infestation, *Tuta 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 nitrogen fertilization is limited, the *Tuta 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 by ensuring fine-tuning of tomato leaf nitrogen status and by producing unbiased data. Specific conditions are indeed required to study the interaction between nitrogen nutrition and the leafminer *Tuta 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).

5.1

SUITABILITY OF EXPERIMENTAL CONDITIONS

In each treatment, the nitrogen content of sampled plants matched the nitrogen uptake value measured continuously by the *Totomatix* setup on the whole crop, inferring that the harvested plants were representative of the crop (Supplemental data, Figure 28).

Throughout the growth period until the final harvest, plant nitrogen uptake was markedly restricted under the low nitrogen treatment compared with the high nitrogen treatment (Figure 21.A). Likewise, dry biomass accumulation was continuously limited under the low nitrogen treatment as compared with the high nitrogen plants (Figure 22.B). Limitation of nitrogen was less effective in decreasing biomass (~ 1/2) than nitrogen uptake (~ 1/3), confirming the observation of Royer *et al.* (2016). As a consequence, nitrogen concentration declined in LN plant tissues making them of a lower nutritive value for leafminers.

Plant differentiation between low and high nitrogen treatments was significant at the onset of infestation by larvae (represented on Figure 22 by the vertical dashed line), with low nitrogen plants showing no evidence of nitrogen 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 the plants' dry mass at the final harvest. Neither nitrogen uptake nor whole plant DW accumulation were noticeably affected by the presence of herbivory (Figure 22). Larval mortality was 38 %, but it was evenly distributed among treatments (Table 5), implying that mortality was not a source of bias in data interpretation.

NITROGEN NUTRITION STRONGLY INFLUENCED NUTRIENT STATUS OF INFESTED LEAVES

The leaves used for inoculation grew actively during the experiment (Figure 23) 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 (Figure 23.B) than mass accumulation (Figure 23.A), resulting in higher SLW in the LN treatment compared with HN, at any of the harvest dates (Figure 25.A). Since the LN treatment increased SLC similarly to SLW, it

is unlikely that carbon limited larvae performance 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, Figure 25.C) and the leaf water content per unit area (*i.e.* the equivalent water thickness EWT, Figure 25.B). 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 larvae performances in the LN treatment.

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 (Figure 23) while the mines were being actively dug (Figure 24.A). 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 6). 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; Figure 25.A - C) implying that the abundance of the major resources (C, N and H₂O) 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 *Tuta absoluta* decreased leaflet growth (Figure 23), 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 *Phyllonorycter 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 (*Tuta absoluta*) concomitantly improves the C, N and water status at infection sites compared with healthy controls.

5.4

***Tuta absoluta* RESPONDED TO NITROGEN TREATMENTS**

There are reports in the literature of large variation in the mean *Tuta 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 6). Insect weights were significantly lower under LN than HN as in our previous findings (Han *et al.*, 2014; Larbat *et al.*, 2016). Furthermore, plant nitrogen limitation significantly retarded insect development as assessed by the lower number of

pupae recovered in LN leaves at the final harvest (Table 5), 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, *Tuta absoluta* differed from these latter miners, because the slower development under LN was not found to reduce mining activity. Indeed, *Tuta absoluta* dug their mines at the same

rate in HN and LN treatments (Figure 24.A). Moreover, much more frass accumulated in mines under LN than HN treatment (Figure 24.B), confirming that the larvae maintained an active feeding activity. As a consequence, these findings question 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, 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 (Figure 25. D - F). The outcome of this evaluation showed that larvae dug mines at the same rate for both nitrogen 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 practices produce tissues of relatively fixed C:N ratios. Therefore, when *Tuta 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 nutrient 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 *Tuta absoluta* food intake was greater when larvae developed on LN compared with HN tomatoes (Figure 25.D). The strategy of eating more food of low nitrogen content increased the C:N ratio in the insect diet (Figure 26), this being the only possible strategy by which the larvae could meet their intake target. Indeed, *Tuta 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 nitrogen concentration also decreases with leaf ageing (Minkenbergh and Ottenheim, 1990; Wilkens *et al.*, 1996b). This explanation suggests that larvae

ingested food until their nitrogen 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 Figure 24.B. In accordance with the GF theory, there is a fitness cost associated with such behaviour and our observations of lower pupa weight (Table 6) and greater time length from larva to pupa in the LN treatment (Table 5) could be an indication of such cost. It should be noted that larvae in the LN treatment also ingested less water (Figure 25.E) since nitrogen limitation strongly reduced leaf water content (Figure 25.B). No definite conclusion, however, can be drawn concerning whether nitrogen 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 nitrogen 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 *Tuta absoluta* larvae on low nitrogen 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.

FUNDING

The Ph.D. grant of Victoire Coqueret was funded by INRA (Département Environnement et Agronomie) and the Regional Council of Lorraine.

ACKNOWLEDGEMENTS

We acknowledge the technical assistance of Aude Fauvet, Valérie Serra, José Fabre, Guillaume Garcia and Philippe Bearez (INRA) for conducting the experiments and helping with harvests and analyses. We thank Dr. E.A. Kirkby (University of Leeds, UK) for revising the English and improving the manuscript, and Elise Coqueret for designing the graphical abstract.

6

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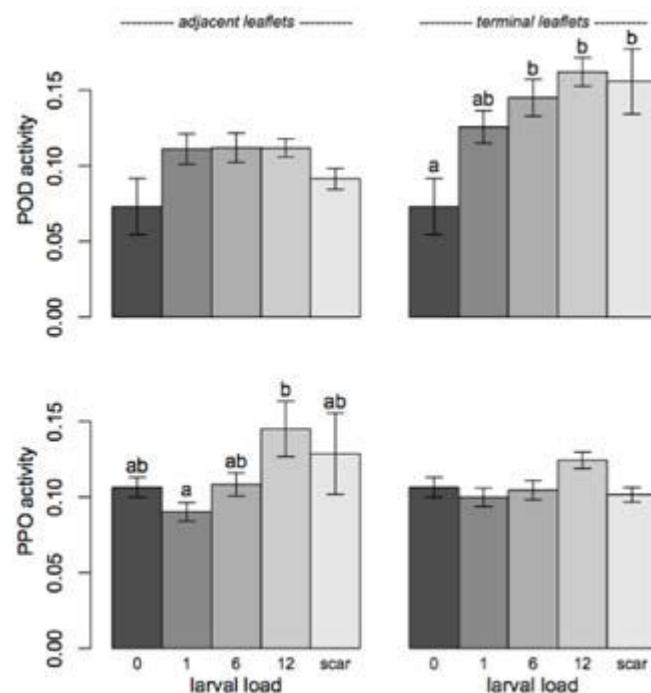
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7

SUPPLEMENTAL DATA AND APPENDIX

7.1

PLANT POLYPHENOL OXIDASE AND PEROXIDASE RESPONSES TO THE LOAD OF ARTIFICIALLY INOCULATED *Tuta absoluta* LARVAE

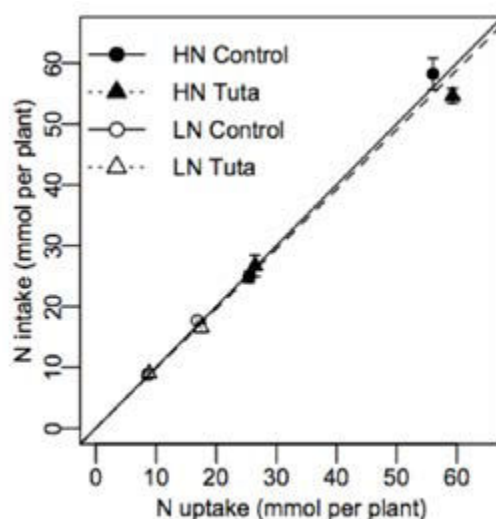


■ **FIGURE 27**: PLANT PEROXIDASES (POD) AND POLYPHENOL OXIDASES (PPO) RESPONSES TO THE LOAD OF ARTIFICIALLY INOCULATED *Tuta absoluta* LARVAE.

These enzymatic activities (arbitrary units, *i.e.* spectrophotometric absorbance changes per minute and per mg FW) are suitable indicators of herbivore attacks (Duffey and Stout, 1996; Larbat *et al.*, 2016). They were measured in HN tomatoes on the fourth true leaves using the method described in Stout *et al.* (1994). Larvae were laid on the three terminal leaflets 48 h before harvest. The leaflets of infected leaves were separated, frozen in liquid nitrogen and kept at -80°C before measurement. The adjacent leaflets, which remained free of larvae, reveal a possible systemic response. Numbers under the bars denote the larval load (0-12) and "scar" stands for scarification. Bars are means \pm s.e.m. of 3 - 6 replicates. Only when necessary, different letters indicate significant differences between treatments (Tukey HSD test, $P < 0.05$).

7.2

MEASUREMENT AGREEMENT BETWEEN NITROGEN INTAKE AND NITROGEN UPTAKE



■ **FIGURE 28** : MEASUREMENT AGREEMENT BETWEEN NITROGEN INTAKE (INFERRED FROM PLANT CHEMICAL ANALYSES AT HARVESTS) AND NITROGEN UPTAKE (INFERRED FROM NUTRIENT SOLUTION ANALYSES).

The solid line indicates when intake equals uptake; the dashed line is the regression through the origin ($R^2 = 0.997$, $P < 0.001$), and its slope (0.98, 95% confidence interval [0.93, 1.03]) is not significantly different from unity. Symbols and bars are means \pm s.e.m. of 8-12 harvested plants.

IMAGEJ MACRO TO DETERMINE LEAF, MINE AND FAECES AREAS

The macro requires ImageJ version 1.50 c. 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»);roi-
Manager («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_larg=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);

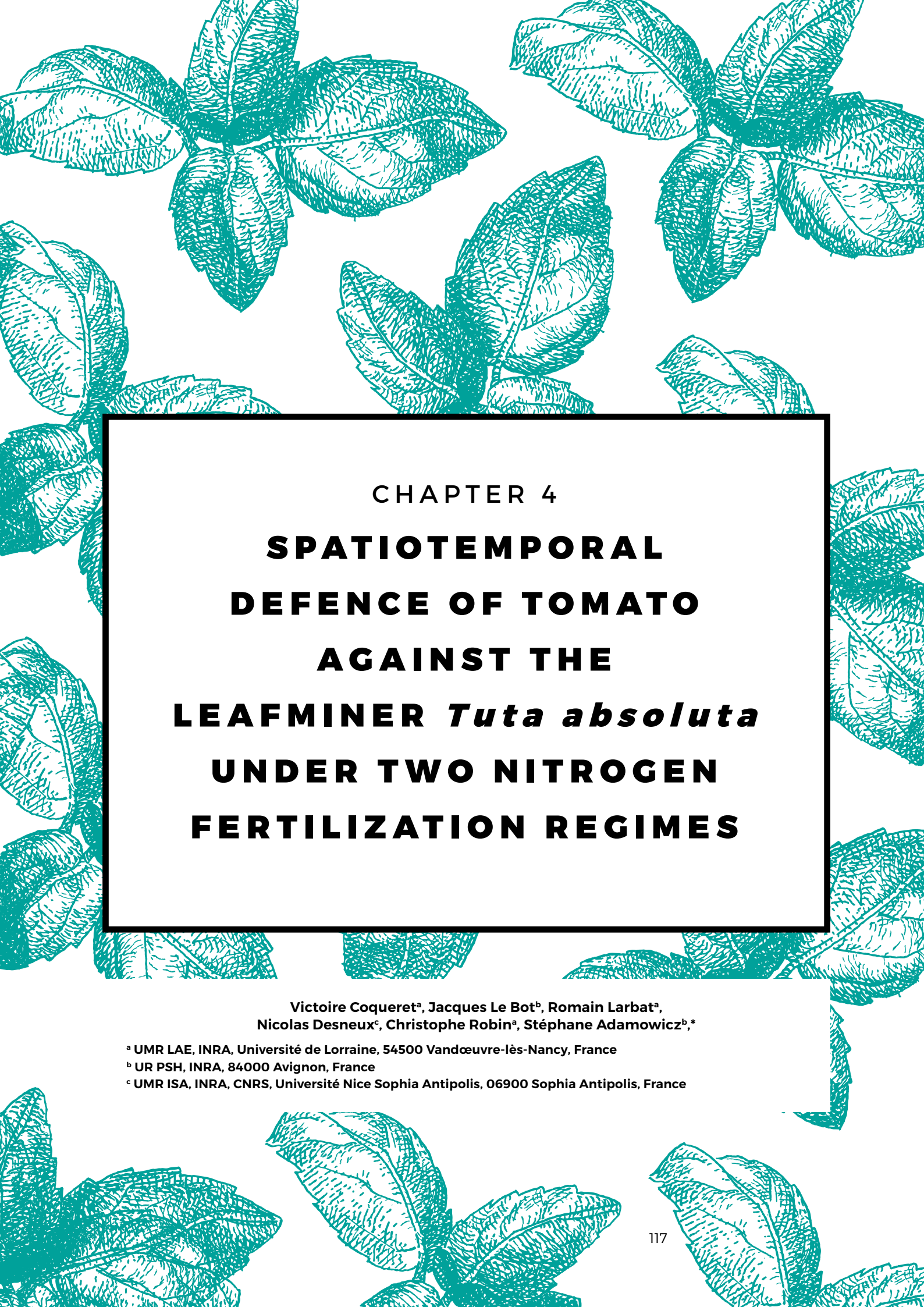
// get the scale from the image
setTool («wand»);
title = «WaitForUser»;
msg = «Use the Wand Tool to select the scale
bar, then press \»OK\».);
waitForUser (title, msg);
getSelectionBounds (x, y, width, height);
//print («x value = « + x, «y value = « + y,
«width= « + width, «height= « + height);
makeLine (x, y, x+width, y);
pixscale=width;
scale=getNumber («Indicate the scale's length
(in mm), then press \»OK\».);
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 ana-
lyses
// Save their coordinates for later use
setTool («rectangle»);
waitForUser («Use the tool Rectangle, to se-
lect 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 selec-
tion), 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=0.5-In-
finity 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.setFill(«Magenta»);
}
roiManager(«update»);
// Save file ROI next to analyzed image
roiManager(«Save», newpath+»_ROI_.zip»);

```





CHAPTER 4

**SPATIOTEMPORAL
DEFENCE OF TOMATO
AGAINST THE
LEAFMINER *Tuta absoluta*
UNDER TWO NITROGEN
FERTILIZATION REGIMES**

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FOREWORD

In the previous chapters, we have shown that a small load of larvae, localized on a few leaflets, locally and systemically activates enzymes involved in the oxidative burst in tomatoes. The nitrogen limitation modifies the foliar tissues composition. Thus, the nutritional quality of the larval food bowl is reduced. The herbivore activity is unaffected but larval development is slowed. The larvae consume leaves whose C:N ratio is increased by nitrogen limitation. In addition, recent work from the team has shown that nitrogen limitation increases the concentration of phenolic compounds and tomatine in tomato leaves, compounds known for their deleterious role on pests. One of the hypothesis of the larval development slowing is the presence of secondary compounds whose syntheses are stimulated by herbivory and which concentrations are increased by nitrogen limitation. At the beginning of the

thesis, there was no work describing the effects of tomato herbivory by *Tuta absoluta* on the induction of defence compound synthesis in tissues. The logical consequence of my Ph.D. work consisted in identifying the consequences of *Tuta absoluta* herbivory on the concentration of defence compounds in the different organs of the tomato under two conditions of nitrogen nutrition. These compounds may be toxic or anti-appetant to the insect. In this context, we have implemented an experiment at the INRA in Avignon. The research will soon result in a patent, then will be valued by a publication.

PRÉAMBULE

Nous avons montré dans les chapitres précédents, qu'une faible charge en larves, localisée sur quelques folioles, active localement ou de manière systémique chez la tomate, des enzymes impliquées dans le stress oxydatif. La limitation azotée modifie la composition des tissus foliaires. Ainsi, la qualité nutritionnelle du bol alimentaire des larves est réduite. L'activité herbivore des larves n'est pas affectée, mais le développement larvaire est ralenti. Les larves consomment des feuilles dont le ratio C:N est augmenté par la limitation en azote. De plus, les travaux récents de l'équipe ont montré que la limitation en azote augmente la concentration en composés phénoliques et en tomatine des feuilles de tomate, composés connus pour leur rôle délétère sur des bioagresseurs. Une des causes du ralentissement de développement larvaire est en effet la présence de composés secondaires dont les

synthèses sont stimulées par l'herbivorie et dont les concentrations sont augmentées par la limitation azotée. Au lancement de la thèse, il n'y avait pas de travaux décrivant les effets de l'herbivorie de la tomate par *Tuta absoluta* sur l'induction de la synthèse de composés de défense dans les tissus. La suite logique de mon travail de thèse a donc consisté à identifier les conséquences de l'herbivorie de *Tuta absoluta* sur la concentration en composés de défense dans les différents organes de la tomate sous deux conditions de nutrition azotée. Ces composés peuvent être toxiques ou anti-appétant pour l'insecte. Dans ce cadre, nous avons mis en œuvre une expérimentation à l'INRA d'Avignon. Les résultats de ce chapitre seront valorisés dans un premier temps par le dépôt d'un brevet, puis par une publication.

1

ABSTRACT

The spatio-temporal localization of defence-related compounds is important to consider regarding the survival strategy of plants facing pests. A systemic response rapidly obtained is vital for the plant in order to limit the infestation. The leafminer *Tuta absoluta* (Meyrick) is a major pest of the tomato crop. Its development rate declines when nitrogen availability is limited for the host-plant growth. We examined specific tomato local and systemic soluble defence compounds from phenolics, flavonoids and glyalkaloids chemical classes, after 3 and 7 days of herbivory. Two sets of healthy *versus* infested were raised under controlled conditions on nitrogen-sufficient *versus* nitrogen-limited complete nutrient solutions. Locally to the infestation, feruloyl quinic acid, rutin and kaempferol rutinoside concentrations transitorily decreased, while caffeoyl putrescine concentration, for both sets, strongly increased. Tomato plants responded to herbivory by rapidly increasing caffeoyl putrescine concentration systemically while chlorogenic acid and tomatine concentrations increased after 7 days. Nitrogen limitation increased the concentration of defence

compounds in control and in infested plants, which might limit plant infestation and improve its resistance against pests such as *Tuta absoluta*.

KEY WORDS :

Herbivory - Nitrogen limitation - *Solanum lycopersicum* - Systemic *versus* local induced responses - *Tuta absoluta*

2

INTRODUCTION

Plants have evolved with insect herbivores for hundreds of millions of years, leading to complex resistance mechanisms. Among them, plant-induced defence involves stress detection, signalling with enzyme activation and phytohormone distribution within plant organs and defence compound synthesis (Fu and Xinnian, 2013).

There is ample literature showing that plant responses to herbivore attacks are highly insect specific (Stout, 1998; Walling, 2000; Ali and Agrawal, 2012) because insect feeding strategies and plant recognition of oral secretions lead to several signal induction balances and interactions (Fürstenberg-Hägg *et al.*, 2013). Thus, piercing and chewing herbivores trigger different profiles of oxidative enzyme activities, phytohormone concentrations and secondary metabolite accumulations in plants (Stout *et al.* 1994; Olson and Roseland, 1991; Mewis *et al.*, 2005). In the group of herbivores, leafminers have the ability to feed and develop within the plant tissues. This feeding strategy protects these insects from natural enemies and from epicuticular defence barriers (Connor and Taverner, 1997). Plant defence mechanisms against leafminers are still not well understood, in particular the signalling and metabolic pathways that are elicited by the attacks (Inbar *et al.*, 2001; Stam *et al.*, 2014), rendering this

model valuable to study.

Nitrogen is one of the most important elements for all biological tissues due to its role in metabolic processes and genetic coding. Plants and herbivorous insects are sensitive to nitrogen variations in their environments. On the one hand, crops' nitrogen fertilization improves plant growth and yield (Chorley, 1981) but decades of excessive inputs have caused environmental pollution leading to compelling legislations (Ecophyto, COP21; Spiertz, 2009). On the other hand, limiting nutrient resources, particularly nitrogen, enhances plants' constitutive defences against pathogens and pests and perhaps reduces the cost of plant-induced defences (Mattson, 1980; Han *et al.*, 2016). Tomato plants do not derogate from these general principles and it has been shown that limitation of nitrogen nutrition reduces plant growth and tissue nitrogen concentration (Adamowicz and Le Bot, 2008) and increases concentration of constitutive defence compounds such as phenolics, flavonoids and glycoalkaloids (Fritz *et al.*, 2006; Le Bot *et al.*, 2009; Larbat *et al.*, 2012 a & b, 2014; Royer *et al.*, 2013). Thus, studying the trade-off between plant growth and defence balance, valuably contributes to improving crops' management.

The tomato pinworm *Tuta absoluta* Meyrick

(Lepidoptera: Gelechiidae) is native of South America (Guillemaud *et al.*, 2015). This microlepidoptera has invaded Europe, Africa and the Middle East since its first appearance in Spain in 2006 (Desneux *et al.* 2010, 2011). *Tuta absoluta* is considered as one of the major tomato pests causing large losses in production. In the EU-27, the export value loss from a *Tuta absoluta* outbreak has been estimated at ca. € 11.5 million per year (EU Document 52013SC0169, 2013). This tomato leafminer developed resistance to multiple pesticides and thus its control on crops is threatened (Siqueira *et al.*, 2000; Lietti *et al.*, 2005). It becomes urgent to find alternative methods for controlling this pest, and to understand how tomato plants specifically defend themselves against this pest is a first step. De Oliveira *et al.* (2012) have shown that tomato strains rich in 2-tridecanone, zingiberene and acyl sugars were more resistant than control tomato strains containing “normal” concentrations of the three allelochemicals. Indeed, these allelochemicals are present in a lower concentration in cultivated tomato plants. Moreover, recent studies have described that nitrogen limitation and deficiency have an impact on the development and survival of *Tuta absoluta* leafminer larvae (Han *et al.*, 2014; Coqueret *et al.*, 2017). Such effects are possibly due to changes in soluble defence compounds in

tomato plant tissues (Larbat *et al.*, 2016) and tomato volatile organic compound emissions affecting *Tuta absoluta* attraction and oviposition (Profitt, 2011).

In the present study, our objectives were (i) to identify defence-related molecules elicited in the first days following larval infestation of tomatoes by *Tuta absoluta*, (ii) to determine how herbivory changed the spatiotemporal pattern of these compounds in the plant and (iii) how nitrogen fertilization affects their constitutive and inducible levels. Our strategy was to grow plants hydroponically in climatic chambers in order to control environmental factors and plant nitrogen nutrition. We studied responses of vegetative plants facing *Tuta absoluta* herbivory, under two regimes of plant nitrogen nutrition, one adequate for maximum growth (high nitrogen: HN) and the other limiting plant growth (low nitrogen: LN). We measured defence compounds (phenolics and tomatine) on the local site of attack and in distant vegetative parts. We considered that studying these plant parts would reflect local and systemic responses of HN *versus* LN tomato plants subjected to *Tuta absoluta* attack or maintained insect-free, 3 and 7 days after larval infestation.

3

MATERIALS AND METHODS

3.1

PLANT AND INSECT MATERIAL

Tomato seeds (*Solanum lycopersicum* L. cv. Better Bush VFN Hybrid, breeder: Tomato Growers) were sown and grown using a hydroponic system under controlled conditions.

Better Bush is an early and dwarf cultivar, well adapted to hydroponic culture and responsive to scarification elicitation (Royer *et al.*, 2013). Moreover, the polyphenol content of cv. Better Bush is mediated by nitrogen availability (Larbat *et al.*, 2012a and 2012b).

Tuta absoluta adults, provided by INRA-ISA of Sophia-Antipolis were reared on tomato plants grown under insectarium conditions in a climatic chamber. After mating, the females laid eggs on leaves and larvae hatched out. Third instar larvae were selected to infest young tomato plants for the experiment.

3.2

GROWTH CONDITIONS

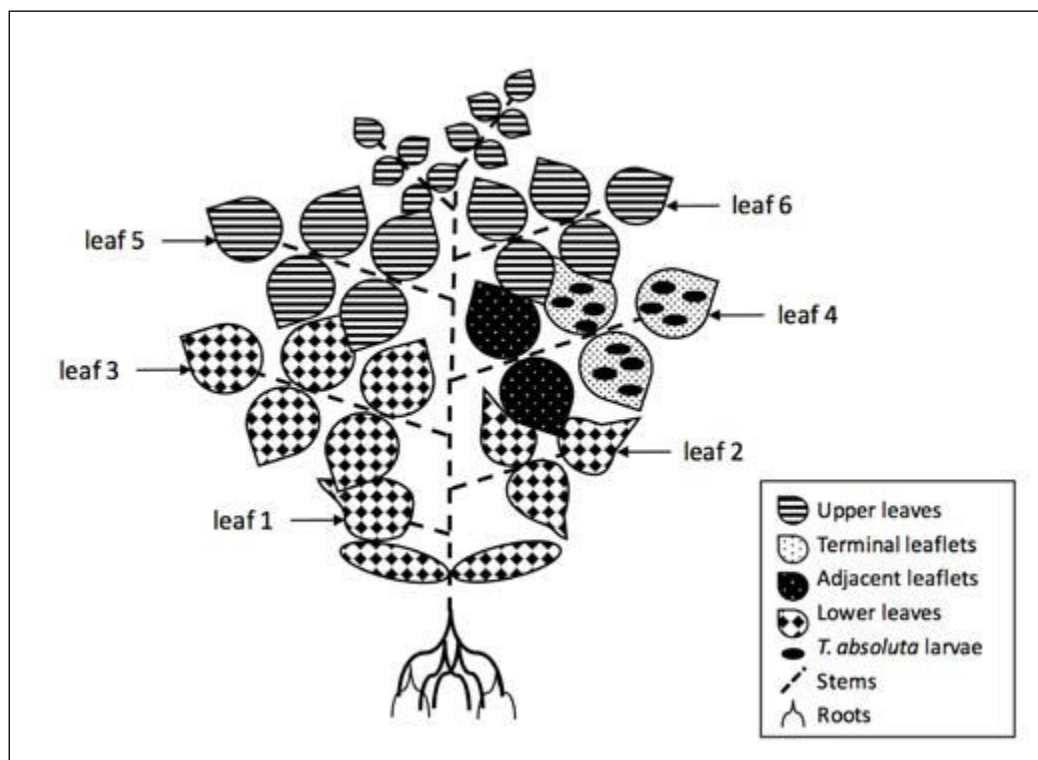
Eleven days after sowing, the seedlings were transplanted into two independent Nutrient Film Technique systems set in twin growth chambers (16 h photoperiod, 23 °C/18 °C day/night in air and 23 °C in nutrient solutions, 60 % air humidity). Before the experiment, all temperature sensors (air and solution) were calibrated in a water bath with 0.01 °C precision (Haake model C35/F6, Karlsruhe, Germany). The twin chambers were used to impose two contrasting nitrogen nutrition regimes. Half the plants received a full nutrient solution supplying N-NO₃⁻ at a concentration of 1 mM, which has been shown to be non-limiting for tomato growth (Adamowicz and Le Bot, 2008) and presently referred to as «High Nitrogen» (HN, for details see Chapter 3, page 100

and Royer *et al.*, 2016). The remaining plants received a full nutrient solution supplying nitrogen at a much lower concentration, known to limit plant growth (Adamowicz and Le Bot, 2008), called hereafter “Low Nitrogen” (LN). The initial NO₃⁻ concentration in this solution was 30 μM but this set-point was adjusted daily in order to allow the LN plants to absorb only 1/3rd of the nitrate taken up by HN plants. Both HN and LN concentrations were continuously (*i.e.* hourly) regulated at their respective set-points using the automatic system *Totomatix* (Adamowicz *et al.*, 2012). The pH was monitored and regulated by *Totomatix* to 5.5. The detailed composition of the nutrient solutions is given in Chapter 3, page 92.

3.3

PLANT INFESTATION BY *Tuta absoluta* LARVAE

For each nitrogen regime, half the plants were infested 14 days after transplantation (dat) with 12 larvae deposited on the three terminal leaflets of the 4th true leaf (leaf 4 on Figure 29). The other half constituted the group of control plants, which were subjected to mock depositions of larvae, performed with a paintbrush.



■ **FIGURE 29** : SCHEME OF PLANT COMPARTMENTATION USED DURING THE HARVEST

3.4

PLANT HARVEST AND SAMPLE PREPARATION

Plants were sampled 3 days (17 dat) and 7 days (21 dat) after larvae deposit. Leaves, stems and roots were separated (Figure 29). The three terminal and the two adjacent leaflets of the infested leaves were sampled separately. Leaf areas were measured and the larvae were retrieved and collected. Roots were

rinsed with deionized water. Organ fresh weights (FW, 0.1 mg precision) were measured and samples were frozen in liquid N₂ 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.

3.5

ANALYSES OF SOLUBLE DEFENCE COMPOUNDS

Phenolics and tomatine were extracted from 30 mg dry powder of leaf, stem and root plant materials as described in Royer *et al.* (2013). The extraction protocol was adapted from Bénard *et al.* (2009) and all steps were carried out at 4 °C. The dry powder was extracted in 1 mL 60 % aqueous methanol and 50 µL taxifolin (internal standard, 2 mg.mL⁻¹ methanol), then blended (1 min) and centrifuged (10 min, 2 800 × g). The extraction was repeated and the supernatants pooled and vacuum-dried. The residue was dissolved (500 mL 70 % methanol) and filtered (0.45 µm) before analysis. Phenolic compounds were analysed on this extract. For tomatine quantification, the extracts were diluted 50 times in 70 % MeOH containing 2 µM solanine as internal standard. Both undiluted and diluted extracts were then analysed using an U-HPLC system (Prominence, Shimadzu, Japan) equipped with a binary solvent delivery pump connected to a diode array detector. An aliquot of 3 µL extract was separated on a C18 Zorbax Eclipse Plus (150 × 2.1 mm, 1.8 µm) column (Agilent, USA) using a gradient elution from 1 to 50 % MeOH 0.1 % formic acid (FA) in 7.1 min, then 99 % MeOH 0.1 % FA in 0.8 min, with a flow rate of 430 µL.min⁻¹. The column was rinsed for 2 min with 99 % MeOH 0.1 % FA and re-equilibrated to the initial conditions for 2 min prior to the next run. Quantification was based on the area under the peak determined at 320 nm and expressed relative to calibration curves. Tomatine was detected by mass analysis carried out in ESI positive ion mode (ESI+) by following the major ion at m/z 529. The internal standard, solanine, was followed at m/z 868. Tomatine was quantified relative to a tomatine calibration curve (0.2-10.0 µM). Mass spectrometric conditions were previously described in Royer *et al.* (2013).

3.6

DATA PROCESSING AND STATISTICS

Compound concentration C was calculated using the following equation :

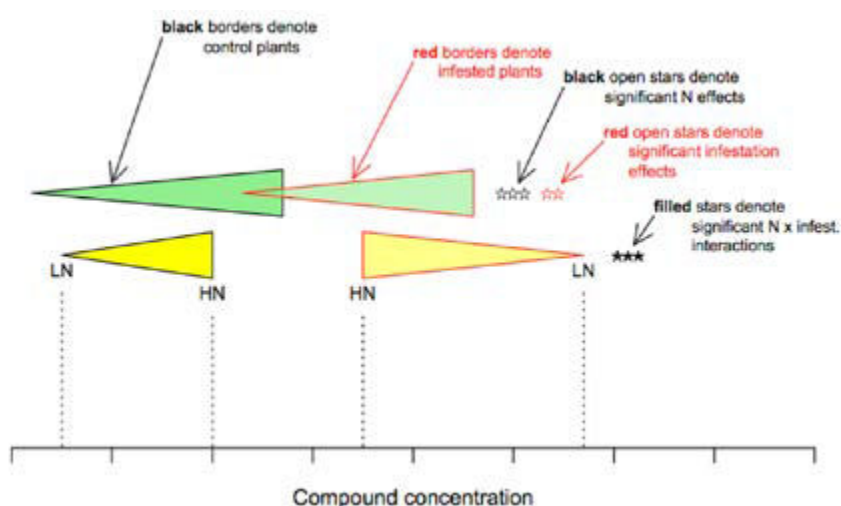
$$C = RF \times PA \times \frac{M}{m_e} \times \frac{V_d}{V_i}$$

where RF is the response factor, PA the peak area, M the molar mass, m_e the mass of the extract, V_d the dilution volume and V_i the injected volume.

Computations and plots were performed using the R software (R Core Team, 2016) and statistical significance was set at $P < 0.05$. Analyses of variance (ANOVA) were performed using the `lm` procedure with harvest, nutrition and infestation as fixed factors. Missing observations resulted in unbalanced data sets and type II or III ANOVAs (`library car`) were calculated. The minimal adequate ANOVA models were determined by stepwise deletion of higher order non-significant interactions. Box-plots, Normal Q-Q plots and correlation between variance and mean, assessed the data distribution and homoscedasticity. When necessary, Box and Cox or logarithmic transformations were applied to the data.

4 RESULTS

Figure 30 shows the graphical chart in order to facilitate reading of the diagrams and is available as a loose sheet, below.



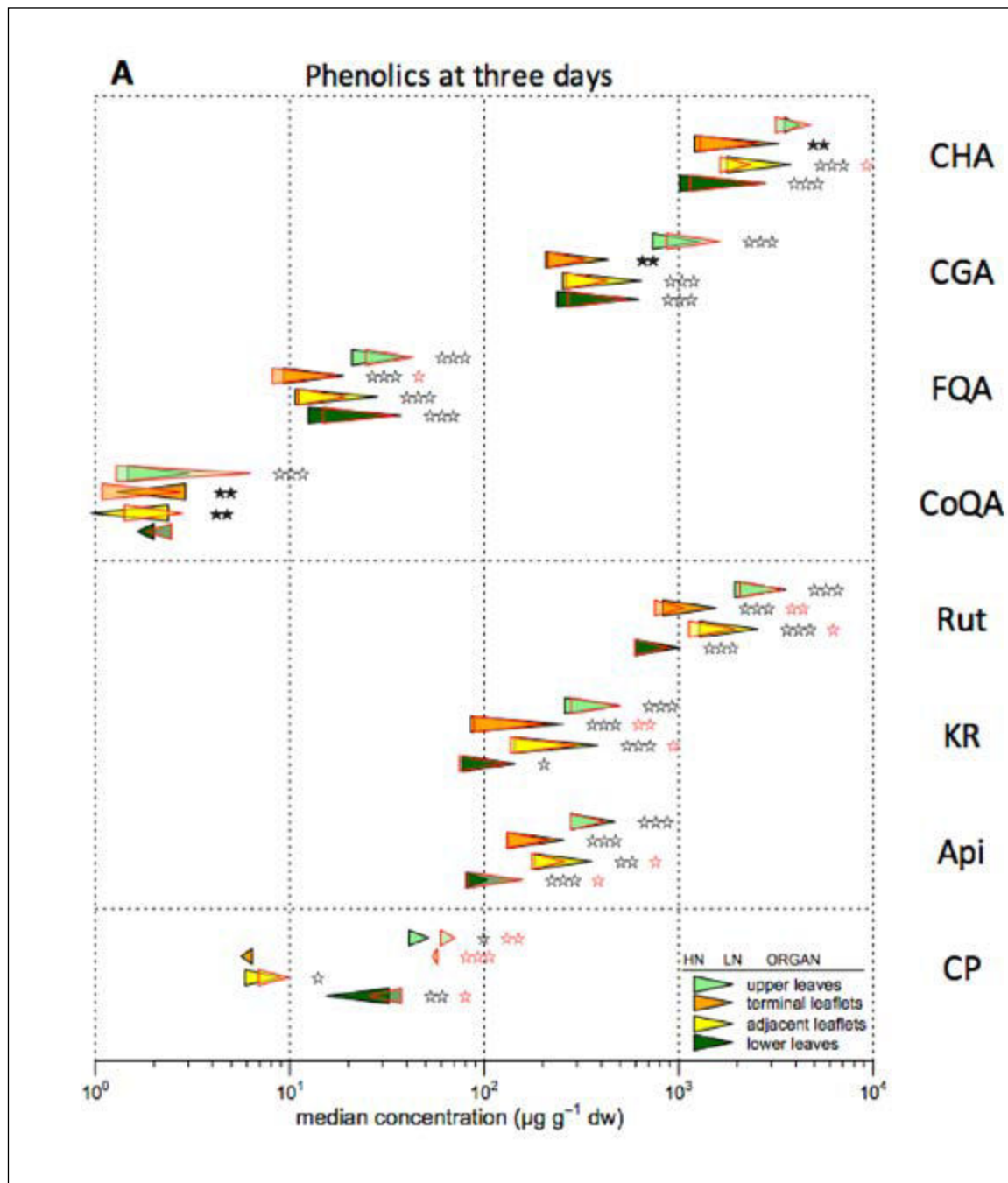
■ FIGURE 30 : GRAPHICAL INTERPRETATION CHART.

Each coloured triangle represents the change of median compound concentration between LN (left or right sharp end) and HN (the opposite edge) treatments. At a given ordinate, the black-bordered triangle represents control plants, while the red characterizes the infested plants. Each colour filling and ordinate is typical of the studied organ or chemical, depending on the case. The level of statistical significance is coded by the star number (1, 2, 3 for $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively), no star meaning no effect of the treatments. Filled stars code for statistically significant interactions, and by convention their presence prevents the plotting of single factor significance.

4.1

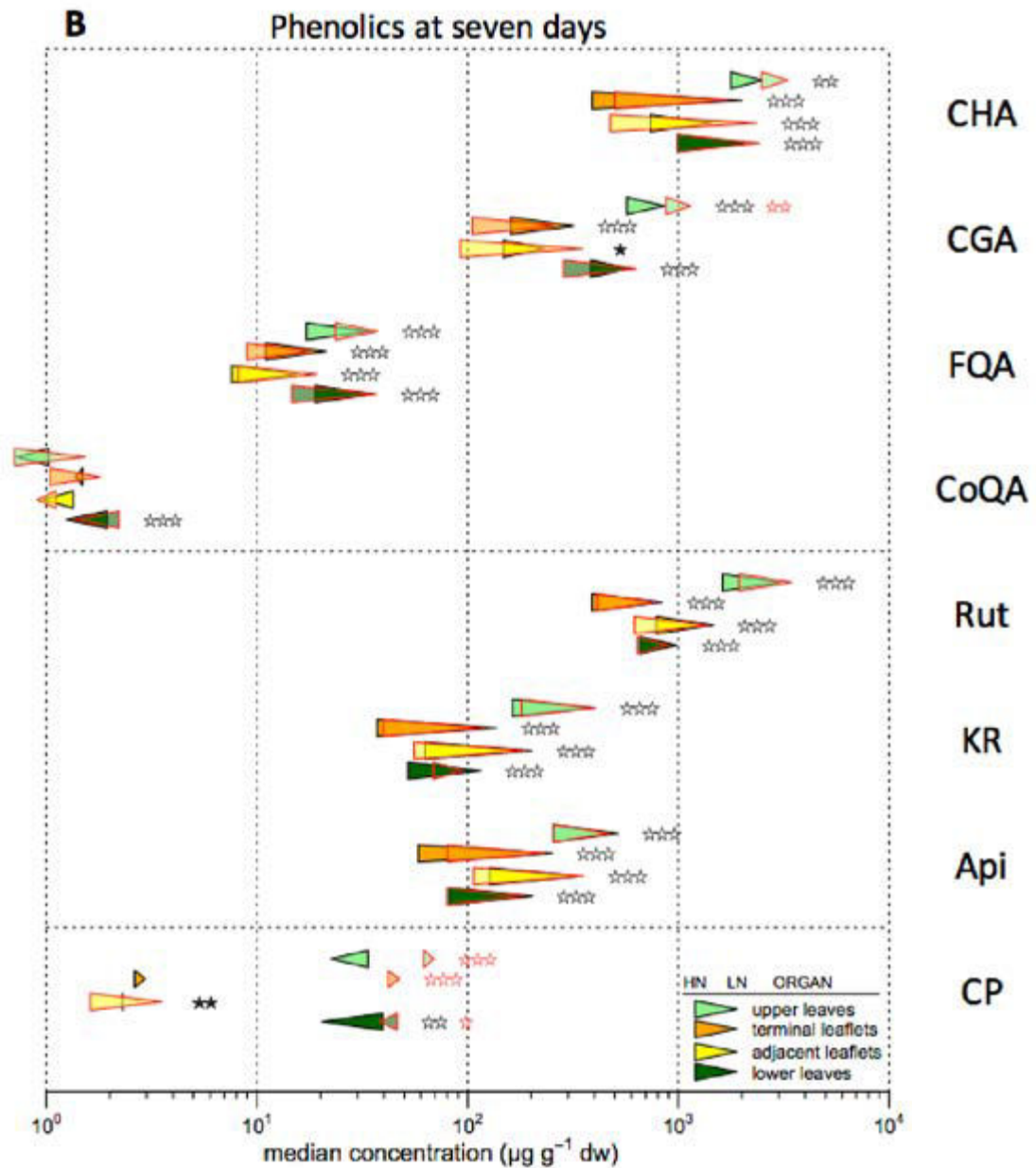
***Tuta absoluta* HERBIVORY AND NITROGEN LIMITATION ALTERED PHENOLIC CONCENTRATIONS IN PLANT ORGANS**

The analysis of leaf tissues revealed fifteen phenolic compounds, which were identified and classified into 3 chemical groups. The hydroxycinnamic acid derivatives included 6 caffeoyl hexaric acids isomers (CHA-1 to -6), 3 chlorogenic acid isomers (3- to 5-CGA), coumaroyl quinic acid (CoQA) and feruloyl quinic acid (FQA). Three flavonoids were identified: rutine (Rut), kampferol rutinoside (KR) and apio-furanosyl rutin (Api). For phenolamides, caffeoyl putrescines (CP1 and CP2) were also identified.



■ **FIGURE 31**: PHENOLICS MEDIAN CONCENTRATION ($\mu\text{g g}^{-1}$ OF DW) IN TOMATO LEAVES COMPARTMENTS AND ROOTS AFTER 3 (A) AND 7 (B) DAYS OF HERBIVORY.

Larvae were deposited on the three terminal leaflets 3 and 7 days before plant harvest. Phenolics were extracted from samples of 4 leaf compartments (upper leaves, terminal leaflets, adjacent leaflets and lower leaves) using the method described in Royer *et al.* 2013. Concentrations were calculated from peak areas, response factors, molar masses, mass of the extract, dilution and injected volumes. Black outlined triangles refer to control plants and red outlined triangles refer to infested plants. Nitrogen impact is shown using this symbol ☆, herbivory impact using this symbol ☆☆ and their interaction ☆☆☆. Significant differences are represented using ☆☆☆ if $P < 0.001$; ☆☆☆☆ if $P < 0.01$ and ☆☆☆ if $P < 0.05$.



• **AFTER 3 DAYS OF HERBIVORY**

After 3 days of herbivory (Figure 31.A), herbivory increased the concentration of CP in all leaves. In the upper leaves, it was 1.3 and 1.5 time higher in infected plants and control ones, respectively for HN and LN plants. In the terminal leaflets, CP concentration strongly increased (10 and 8 times in HN and LN plants, respectively). In the adjacent leaflets, herbivory also increased CP concentration

by 13 % and 84 % in HN and LN plants, respectively. Finally, in the lower leaves, herbivory increased CP concentration by 31 % in HN and by 57 % in LN plants. In these old leaves, herbivory increased also Api concentration by 9 % and 38 % in HN and LN plants, respectively. By contrast, herbivory decreased FQA, Rut and KR concentrations in the terminal leaflets of both HN and LN plants by about 14 % and 20 %, respectively. In the adjacent leaflets, herbivory

decreased CHA, Rut, KR and Api concentrations by about 11 % and 27 % for HN and LN plants, respectively.

With few exceptions, nitrogen limitation almost doubled the phenolic concentrations in most compartments of control and infested plants. It had no impact on CHA in upper leaves, on CoQA in lower leaves and on CP in the terminal leaflets. Nitrogen limitation declined CP concentration in lower leaves by about 50 % and 40 %, in control and infested plants, respectively.

There were few nitrogen x herbivory interactions; for LN plants, herbivory increased CoQA concentration of the infested leaf, whereas its concentration decreased in the case of HN plants.

• AFTER 7 DAYS OF HERBIVORY

After 7 days of herbivory (Figure 31.B), in upper leaves of infested plants, CGA concentration was higher than in controls by 39 % and 31 % and CP concentration was higher by 71 % and 187 % in HN and LN plants respectively. In the terminal leaflets of infested leaves, CP concentration was strongly higher compared to controls, by a factor of 12 and 8 times respectively in HN and LN plants. CP concentration was also higher in lower leaves of infested plants by 29 % and 94 % in HN and LN plants, respectively. With few exception, nitrogen limitation multiplied by about 2.3 the phenolic concentrations in almost all compartments for control and infested plants. With few exceptions, nitrogen limitation more than doubled phenolic concentrations in almost all

compartments for control and infested plants. CoQA and CP concentrations of LN tomatoes declined in lower leaves while the limitation had no impact on CoQA concentrations in upper leaves, terminal and adjacent leaflets, and CP concentrations in upper leaves and terminal leaflets.

There were few nitrogen x herbivory interactions. Nitrogen limitation increased the impact of herbivory in the adjacent leaflets, since CP concentration was 74 % lower in HN infested plants and 238 % higher in LN infested plants than their respective controls.

■ **FIGURE 32** : TOMATINE MEDIAN CONCENTRATION ($\mu\text{g}\cdot\text{g}^{-1}$ OF DW) IN TOMATO LEAVES COMPARTMENTS AND ROOTS AFTER 3 AND 7 OF HERBIVORY.

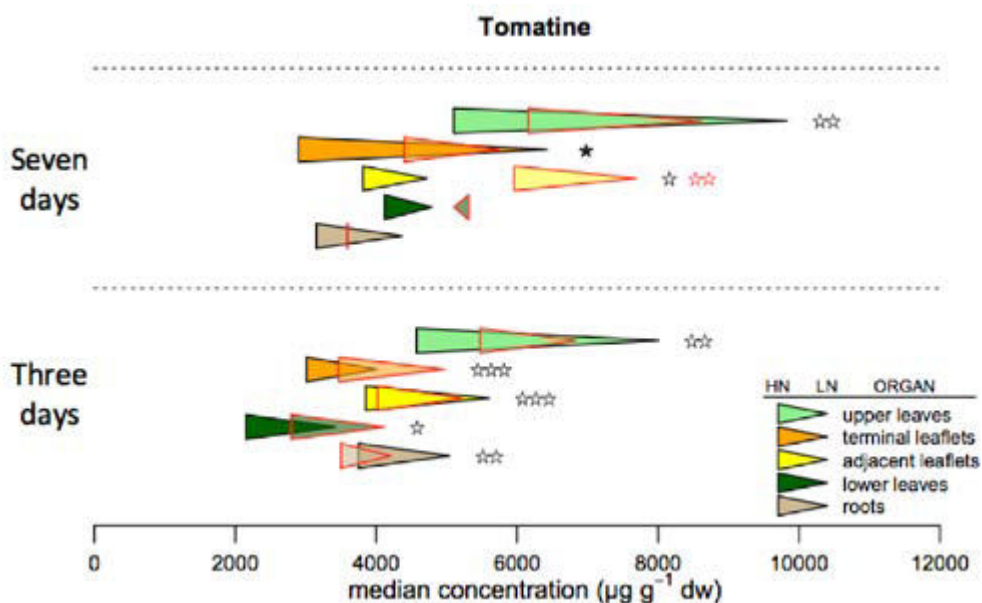
Larvae were deposited on the three terminal leaflets 3 and 7 days before plant harvest. Tomatine was extracted from samples of 4 leaf compartments (upper leaves, terminal leaflets, adjacent leaflets and lower leaves) using the method described in Royer *et al.* 2013. Concentrations were calculated from peak areas, response factors, molar masses, mass of the extract, dilution and injected volumes. Black outlined triangles are control plants and red outlined triangles are infested plants. Significant nitrogen impact is shown using this symbol ☆, herbivory impact using this symbol ★ and their interaction is signalled by this symbol ★. Significant differences are coded by stars * if $P < 0.001$, ** if $P < 0.01$ and * if $P < 0.05$.**

4.2

***Tuta absoluta* HERBIVORY AND NITROGEN LIMITATION ALTERED TOMATINE CONCENTRATIONS IN PLANT ORGANS**

Tomatine concentration was determined in leaves, stems or roots compartments (Figure 32). After 3 days of herbivory, tomatine concentration was not affected in leaves nor in roots by *Tuta absoluta*. After 7 days, tomatine concentration increased in the terminal leaflets by a factor of 1.5 in HN plants only. This was not significant for LN plants. In the adjacent leaflets, tomatine concentration increased by a factor of 1.75 and 1.5, respectively for HN and LN plants. Herbivory had no effect on the other compartments.

Four days after the onset of infestation, nitrogen limitation increased tomatine concentration in all compartments, being about 1.5 and 1.35 times higher in control and infested plants, respectively. After 7 days, this effect no longer existed in the lower leaves and roots. But in the other compartments (upper leaves, terminal and adjacent leaflets) it persisted and even increased in infested plants since tomatine concentration was about 1.9 times higher for LN than HN plants.



5

DISCUSSION

It has been reported that upon attack of 12 larvae of the leafminer *Tuta absoluta*, tomatoes proactively trigger oxidative enzymes (Coqueret *et al.*, 2017) and we show here that this larval load changes the concentration of defence compounds in various tomato plant compartments. Some of these

defence compounds can be ascribed to new elicited compounds of tomato responding to herbivory by the leafminer *Tuta absoluta*. Our study also confirms previous observations that limiting nitrogen fertilization increases the concentration of defensive compounds (Royer *et al.*, 2013; Larbat *et al.*, 2015).

5.1

SUITABILITY OF EXPERIMENTAL CONDITIONS

Suitable plant growth conditions were required to study the interaction between nitrogen availability and the leafminer *Tuta absoluta*, in particular for nitrogen, which is a lever capable to modulate constitutive plant defence (Herms and Mattson, 1992; Fritz *et al.*, 2006). Thus, an appropriate and efficacious experimental setup was a prerequisite to the acquisition of unbiased data.

The key elements of our practice during the experiment were mentioned in the previous article (Coqueret *et al.*, 2017) and they referred (1) to the correlation between nitrogen content of sampled plants and nitrogen uptake measured on the whole crop by the *Totomatix* setup, (2) to the absence of nitrogen deficiency symptom in LN plants indicating that nitrogen slowed growth without plant injury, (3) to the no noticeable change in nitrogen uptake or plant DW accumulation during *Tuta absoluta* herbivory and (4) to the absence of bias in data interpretation since larval mortality was evenly distributed among treatments.

HERBIVORY AND NITROGEN LIMITATION AFFECT SPATIOTEMPORAL PHENOLIC CONCENTRATION IN LEAVES

Leaf phenolic concentration became higher under herbivory. Investigations on the impacts of phenolic compounds on chewing insects have shown contrasting effects. In artificial diets, pure phenolics and usually additional oxidative substrates generate antinutritive effects leading to growth decline and survival reduction (Elliger *et al.*, 1981; Isman and Duffey, 1982, Felton *et al.*, 1992). *In planta* experimentations, however, revealed no deterrent impact of phenolics on phytophagous insects (Bi *et al.*, 1997; Eichenseer *et al.*, 1997; Ali *et al.*, 1999). We assume that activated PPO oxidised phenolics led the reactive oxygen species and antinutritive compound concentrations to increase, in particular quinones (Appel, 1993). In this study, we are not able to confirm that oxidative enzymes effectively reduced phenolic compounds into quinones. However, we hypothesize that PPO, POD and phenolics play an important role in early defence against the tomato leafminer allowing the advent of a systemic defence and thus increasing phenolic concentration in the whole leaf compartment.

Nitrogen limitation enhanced constitutive plant defence by increasing leaf phenolic compound concentrations. A nitrogen x herbivory interaction was observed after 4 days of herbivory, when indeed phenolic concentration dropped faster in LN than HN leaves, suggesting that phenolic oxidations and their deterrent effects on herbivores became greater.

A previous study demonstrated the deterrent effect of nitrogen limitation on *Tuta absoluta* larval survival (Larbat *et al.*, 2016), although the load of two 1st instar larvae did not affect phenolic concentration in plant tissues. Therefore, damage severity seems to play an important role in the level of plant defence.

A more carefully investigation into the compounds analysed after 3 days of herbivory, showed that CP concentration increased in the young leaves and in the infested terminal leaflets, while CHA, CGA, Rut and KR concentrations declined in the infested leaf. After 7 days of herbivory, at the site of the attack, only CP concentration remained higher than in control plants. In parallel, a systemic defence was set in younger leaves where CP and CGA concentrations increased. CP appears to be an important reactive compound involved in tomato defence against *Tuta absoluta*. Thus, to our knowledge, this is a first report on the elicitation of CP and more generally of phenolamides, by the leafminer (*Tuta absoluta*). These compounds provide antimicrobial activities against fungus and bacteria (Edreva *et al.*, 2007), and phenolamide concentrations have already been reported to increase in tomato plants, subjected to wounding (Pearce *et al.*, 1998).

HERBIVORY AND NITROGEN LIMITATION AFFECT TOMATINE CONCENTRATION IN LEAVES

After 3 days of herbivory, leaf tomatine concentration increased ($P < 0.01$). After 7 days of herbivory, tomatine concentration in adjacent leaflets of infested plants, was higher than in the control plants. No other herbivory impact was noticed. Alkaloids are the prominent compounds showing deterrent effects on herbivorous insects (Levin, 1976) and tomatine, which is the most representative alkaloid in tomatoes, is known to affect larval survival, growth and development (Bentley *et al.*, 1984). Our finding that tomatine concentration was higher in LN than in HN plants, agree with previously published results (Royer *et al.*, 2013; Han *et al.*, 2016). This behaviour may seem odd because tomatine is a N-based compound and one could expect its synthesis to be proportional to nitrogen tissue concentration. Indeed, the Carbon - Nutrients Balance Hypothesis (CNBH) predicts that carbon will be more easily allocated to the synthesis of carbonaceous compounds in plants that grow on nitrogen - poor soils. And conversely, for plants that grow on low carbon soils, the allocation of resources will promote the synthesis of nitrogen defence compounds. Once again, nitrogen limitation appeared to enhance defence compound concentration.

The present study provides new insights in the spatiotemporal tomato plant defence response against the leafminer *Tuta absoluta*. Early stage responses led to a local chemical distribution with an increase in CP and a decrease in CHA, CGA, Rut and KR. Then, the response became systemic with an increase of CP and CGA in leaves. In parallel to phenolic changes, tomatine concentration became higher in the infested leaf. Moreover, nitrogen limitation increased the concentration of defence compounds, which may improve plant resistance against pests such as *Tuta absoluta*.

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; SA processed the data. VC drafted the manuscript; and all authors revised the manuscript.

ACKNOWLEDGEMENTS

The Ph.D. grant of Victoire Coqueret was co-funded by INRA (Département Environnement et Agronomie) and the Regional Council of Lorraine. All our thanks to the Regional Council of Lorraine for its financial support through the "projet émergent: Identification des réactions de défense de la tomate à l'herbivorie par la larve mineuse *Tuta absoluta* par une étude de transcriptomique et analyses de composés organiques volatils". (grant 15P03127). Larvae were provided by N. Desneux (INRA ISA Sophia Antipolis). We thank Aude Fauvet, Valérie Serra, José Fabre, Guillaume Garcia and Philippe Bearez (INRA) for their important technical contribution to this work.

6


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CHAPTER 5

**NITROGEN LIMITATION
ALTERS THE VOLATILE
BLEND OF TOMATO
ATTACKED BY
*Tuta absoluta***

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FOREWORD

In the previous chapters, we have shown that a small load of larvae, localized on a few leaflets, locally and systemically activates enzymes involved in the oxidative burst in tomatoes. The nitrogen limitation modifies the foliar tissues composition. Thus, the nutritional quality of the larval food bowl is reduced. The herbivore activity is unaffected but larval development is slowed. Therefore, the “trophic” hypothesis partly explains the observed effect on the larval development. However, we could not exclude the “defence compounds” hypothesis. Indeed, we have shown that nitrogen-limited plants contained defence compounds in greater concentrations, in their tissues than plants whose nitrogen was plethorically supplied. This already known result is complemented by a major phenomenon in the tomato against *Tuta absoluta*. Indeed, we have shown that tomatoes responded

to herbivory by eliciting the synthesis of caffeoyl putrescine and tomatine. These compounds are known for their deleterious role on bio-aggressors. Another component of the tomato resistance that has to be investigated to strengthen tomato defence mechanisms in relation with *Tuta absoluta*, is the emission of volatile organic compounds, which may be repellent to the pest or attract predatory insects. We have set up an experiment in our laboratory in cooperation with the Department of Biochemistry of the Max Planck Institute for Chemical Ecology in Jena. This work, described in the second part of this chapter, will be submitted for publication shortly.

PRÉAMBULE

Nous avons montré dans les chapitres précédents, qu'une faible charge en larves, localisée sur quelques folioles, active localement ou de manière systémique chez la tomate, des enzymes impliquées dans le stress oxydatif. La limitation azotée modifie la composition des tissus foliaires. Ainsi, la qualité nutritionnelle du bol alimentaire des larves est réduite. L'activité herbivore des larves n'est pas affectée, mais le développement larvaire est ralenti. L'hypothèse "trophique" explique donc en partie l'effet observé sur le développement des larves. Cependant, nous ne pouvons pas exclure l'hypothèse "composés de défense". En effet, nous avons montré que les plantes sous limitation azotée contenaient dans leurs tissus des composés de défense en plus grandes concentrations que les plantes dont l'azote était apporté de manière pléthorique. Ce résultat déjà connu est complété

par un phénomène majeur dans la défense de la tomate vis-à-vis de *Tuta absoluta*. En effet, nous avons montré que les tomates répondaient à l'herbivorie en élicitant la synthèse de la caffeoyl putrescine et de la tomatine. Ces composés sont connus pour leur rôle délétère sur des bio-agresseurs. Une autre composante de la résistance de la tomate à approfondir est l'émission de composés organiques volatils par la tomate vis-à-vis de *Tuta absoluta*, ces composés pouvant être répulsifs pour le ravageur ou attirer les insectes prédateurs. Nous avons mis en place une expérimentation dans notre laboratoire en coopération avec le Département de Biochimie du Max Planck Institute for Chemical Ecology à Jena. Ce travail, décrit dans ce chapitre, sera soumis pour publication prochainement.

1

ABSTRACT

Upon attack by herbivorous insects, plants emit volatiles; some compounds will be specifically deleterious to the pest while others will attract natural enemies. It is well known that nitrogen limitation increases the concentration of defence compounds in plant tissues, such as phenolics or alkaloids. Its impact on volatile defence compounds is not documented at all. Here, we identified volatile organic compounds (VOCs) induced by larvae of the leafminer *Tuta absoluta*, an economically important pest of tomato (*Solanum lycopersicum*), under two contrasted nitrogen regimes. Plants were grown hydroponically, in climatic chambers. Tomato VOCs were collected at different intervals, from 2 to 54 hrs following infestation of leaves by *Tuta absoluta* larvae. VOCs were then collected using PDMS tubes (qualitative data) and a push-pull system with Poropak Q filters (qualitative and quantitative data). Tomato plants VOCs belonged to different chemical classes which the most prominent are monoterpenes, sesquiterpenes and alkanes. From qualitative results, upon attack, plants emitted lower proportions of alkanes and 4-methyl-1-decene and higher proportions of humulene compared to control plants. Whereas, plants under nitrogen limitation emitted higher proportions of aldehydes, 2 terpenoid alcohols and lower proportions of

2 monoterpenes. According to the quantitative data, the emission of 9 VOCs was stimulated by *Tuta absoluta* herbivory (β -caryophyllene, humulene, m-cymene, 1,3,8-menthatriene, β -cis-ocimene, β -phellandrene, β -pinene, terpinolene and an unidentified compound). Whereas, the emission of the methyl-salicylate phytohormone, denderolasine and nine other compounds was higher under nitrogen limitation than for plants under high nitrogen regime. We revealed here new VOCs (not documented up to now) whose emission by tomato leaves was mediated by *Tuta absoluta* herbivory and influenced by nitrogen availability. Further investigations are necessary to assess the ecological role of these VOCs and the impact of nitrogen availability on the tomato-*Tuta absoluta*-natural enemies' interactions.

KEY WORDS :

Herbivory - Methyl-salicylate - Nitrogen limitation - *Solanum lycopersicum* - Terpenes - *Tuta absoluta* - VOCs

2

INTRODUCTION

Chemical communication is one mean for plants to interact with their environment. Indeed, plants constitutively synthesize and emit in their vicinity many volatile organic compounds (VOCs) belonging to a large panel of chemical classes (Pichersky *et al.*, 2006; Baldwin, 2010). VOCs are stored and emitted from specialized glands or trichomes present at the surface of leaves and stems (Paré and Tumlinson, 1997) and play, therefore, a role in passive defence. Following a mechanical damage, for example artificial defoliation, insect herbivory or egg deposition, stored VOCs are immediately released and the syntheses of other classes of VOCs, called herbivore-induced plant volatiles (HIPV), are activated *via* intra-plant signalling (War *et al.*, 2011). Some HIPVs have been found to directly deter the pest from feeding (Laothawornkitkul *et al.*, 2008) or to act as “alarm calls”, allowing within plant, inter-plant and plant-insect communications (Heil and Karban, 2010). Thus, VOCs play a prominent role in plant chemical defence against pests; thanks to specific olfactory imprints, they induce systemic

plant defence, attract predators or parasitoids and repel egg-laying females (De Moraes *et al.*, 2001; Hoballah and Turlings, 2001; Halitschke *et al.*, 2008; Godard *et al.*, 2008).

Tomato produces a large array of VOCs (Buttery and Ling, 1993) whose emission in terms of diversity and amount is mediated by genotypic or biotic factors such as the herbivore species (Proffitt *et al.*, 2011; Silva *et al.*, 2017). Many abiotic factors, such as drought, temperature, light intensity, CO₂ and O₃ partial pressure, nutrient deficiency, mediate also the emission of VOCs (Holopainen and Gershenzon, 2010). However, the impact of nitrogen limitation on VOC emission is very poorly documented, although nitrogen fertilisation is the main factor used by growers to stimulate plant growth and fruit production. The unique paper found indicates that acetic acid (an antifungal volatile) and several lipoxygenase pathway products were emitted at higher levels from nitrogen-treated *Brassica napus* (Veromann *et al.*, 2013). Literature is more documented for other defence-related compounds;

thus, nitrogen limitation was shown to enhance tomato constitutive defence, such as phenolic and tomatine concentrations, and to alter *Tuta absoluta* larvae development and survival (Han *et al.*, 2014; Larbat *et al.*, 2016; Coqueret *et al.*, 2017).

Among the herbivores that affect crops, the tomato pinworm *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae), native of South America (Guillemaud *et al.* 2015), is considered as one of the major pests causing large losses in tomato production. Newly born larvae hatch at the leaf surface and penetrate across the epidermal leaf tissue to consume leaf parenchyma, thus creating mines. The insect causes serious damages to growers. In the EU-27, the export value loss from *Tuta absoluta* outbreak has been estimated at *ca.* €11.5 million per year (EU Document 52013SC0169, 2013). Moreover, this pest has been found to be resistant to some pesticides. Thus, alternative issues are necessary to develop and regarding biocontrol issues, it is relevant to improve the identification of VOCs and evaluate the impact of nitrogen limitation on the composition of the

volatile blend emitted by tomatoes. Indeed, these compounds can attract parasitoids, predators and may repel *Tuta absoluta* females from laying eggs on leaves.

The aim of our study is to assess whether or not nitrogen limitation affects the composition of the volatile blend emitted by tomato foliage subjected to *Tuta absoluta* herbivory. Our strategy was to grow tomato plants hydroponically in growth chambers to control environmental growth conditions (climate, nitrogen availability). We characterized VOC response to *Tuta absoluta* herbivory, from tomatoes raised under two nitrogen nutrition regimes, one adequate for maximum plant growth (high nitrogen: HN) and the other limiting plant growth (low nitrogen: LN). VOC characterization was conducted from 2 to 54 hours after larval infestation in order to decipher short and medium-term plant response.

3

MATERIALS AND METHODS

3.1

PLANT MATERIAL AND GROWTH CONDITIONS

Tomato seeds (*Solanum lycopersicum* L. cv. Better Bush VFN Hybrid, breeder: Tomato Growers) were sown on humid paper filter in Petri dishes. Five days after sowing (das) seedlings were transferred for six days on a hydroponic system adapted to young germinating plantlets, in a growth chamber, with the following conditions: 16 h photoperiod, 23 °C / 18 °C day / night and 25 °C in nutrient solutions, 60 ± 20 % air humidity. Better Bush is an early and dwarf cultivar, easy to manage and well adapted to hydroponics. It is responsive to scarification elicitation (Royer *et al.*, 2013) and polyphenol concentration is enhanced by nitrogen limitation (Larbat *et al.*, 2012 a).

Eleven days after sowing, the plantlets were transferred to NFT systems (Nutrient Film Technique) set in the same growth chamber. The entire experiment comprised 32 plants. Half of the plants (16 plants) received a full nutrient solution supplying N-NO₃⁻ at high concentration (HN) and the other half received a full nutrient solution supplying N-NO₃⁻ at a low concentration (LN), limiting for tomato growth (Figure 33; Adamowicz and Le Bot, 2008). Nitrate concentration and pH were measured and rectified manually with an increasing frequency as growth proceeded, up to once a day for HN and twice a day for LN. For more details here see Royer *et al.* (2013).

However, all the plants could not be measured at the same time because of limited space inside the growth room and of limited number VOC trapping systems. Therefore, the experiment was run over 4 successive periods, each period considering only HN or LN plants, *i.e.* [4 infected HN plants and 4 healthy HN plants] or [4 infected LN plants and 4 healthy LN plants].

3.2

***Tuta absoluta* LARVAE AND LEAF INFESTATION**

Prior to the experiment, *Tuta absoluta* adults provided by INRA Sophia-Antipolis were reared on tomato plants in an insectarium set in a climate chamber neighbouring of the main experiment. A large population of larvae developed on the leaves. Third instar larvae (larval development being divided into 4 stages) were then selected from this population

and deposited on targeted leaves, twenty-eight days after transplantation (twelve 3rd instar larvae laid on the three terminal leaflets of the 4th true leaf (8 HN plants and 8 LN plants), while control plants were slightly touched with a paintbrush to simulate larval deposit. Larvae were allowed to feed up to plant harvest.

3.3

COLLECTION OF PLANT VOLATILES AND ANALYSIS

Volatiles were collected using a dynamic push-pull system (Tholl *et al.*, 2006; McCormick *et al.*, 2014). One day before the larval deposit (27 dat), plants were set in PET-bags (polyethylene terephthalate / Albal[®], Cofresco Frischhalteprodukte GmbH & Co. KG; 31 cm diameter and 55 cm height). A metal wire, set inside the bag around each plant, prevented contacts between the bag and the leaves. Charcoal-filtered air was pumped (Thomas pump, Gardner Denver, USA) into the PET bags at a flow rate of 1.5 L.min⁻¹. At the same time, a second pump flushed the air out of the bag at a flow rate of 1 L.min⁻¹. The pumps were installed in a Lock & Lock[®] plastic box to protect them from water and dirt. An air regulator was set at each pump to adjust airflow rates. Since trapping materials have different affinities for VOCs, we used two kinds of traps and sorbents (PDMS (polydimethylsiloxane) tubes and Porapak-Q filters; ARS Inc., Gainesville Florida, USA) to maximise volatile trapping.

3.3.1 PDMS TUBES

PDMS trap consists of 2 mm diameter and 5 mm long metal tube filled with PDMS (Kallenbach, 2014). Trapping the VOCs started 28 days and comprised seven times of collection, respectively after 2, 5, 9, 23, 33, 47 and 54 hours of herbivory (Figure 33). PDMS trap was set on the metal string set inside the bag. During VOC collection, the bags were sealed with a cable binder at their bottom and top. PDMS were let for 40 min to trap VOCs passively. After this short collection period, PDMS traps were stored in glass vials at -20 °C until analysis.

Analysis was carried out using gas chromatography mass spectrometry using Ultra Thermo desorption unit TD20 connected to a quadrupole GC-

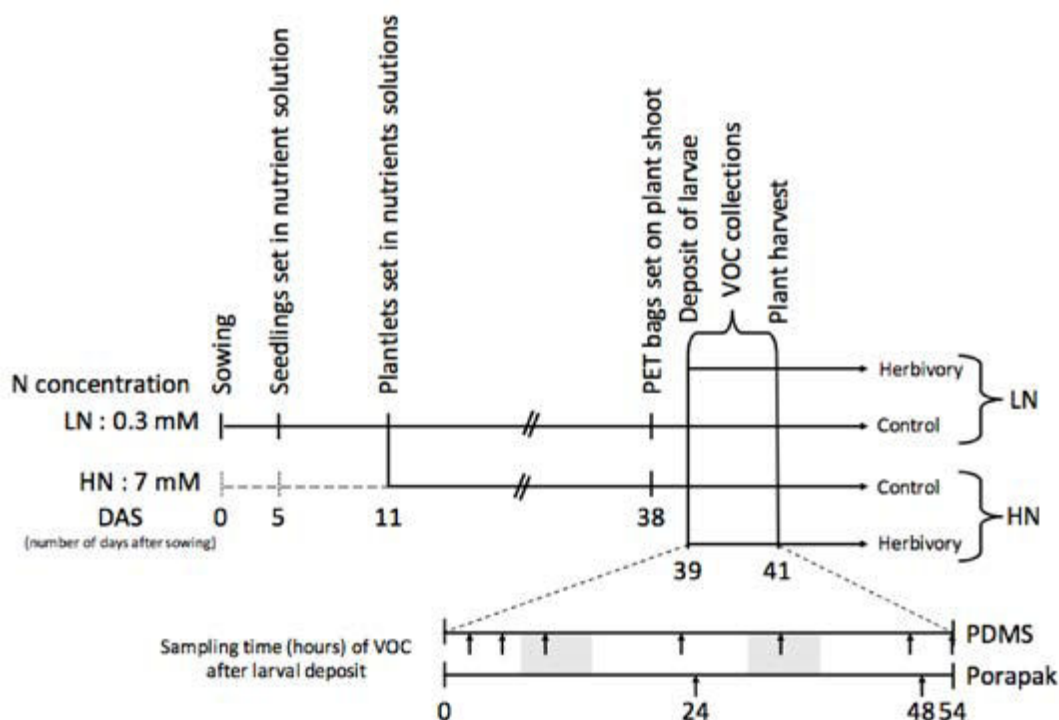
MS-QP2010Ultra (Shimadzu; transfer line temperature: 240 °C, ion source temperature: 220 °C, mass range: 33-400 m/z). Volatiles were desorbed from PDMS tubes placed in 89 mm glass TD tubes (Supelco), through a liquid nitrogen flow at a rate of 60 mL.min⁻¹ at 200 °C for 8 min. Molecules were separated with helium at a flow rate of 40 cm.s⁻¹ through a 30 m x 250 µm, 0.25 µm Rtx-5MS column. The oven temperature was held at 40 °C for 5 min and then increased to 185 °C at a 5 °C.min⁻¹ rate. At the end of the run, the temperature was increased to 280 °C at a rate of 30 °C.min⁻¹ for 0.83 min in order to clean the column. Every ten samples, a blank tube was included. Data processing was

performed using the program Shimadzu GC-MS solution software (v2.72) provided by the manufacturer and exported *via* its export function into MS Excel macro file. Compounds were identified by comparison of retention times and mass spectra from Wiley and National Institute of Standards Technology libraries. Only the compounds presenting more than 85 % identity with a match were named.

The relative proportion of each molecule in the volatile blend was computed by dividing its peak area by the cumulated peak area of all identified molecules. Chemical class percentages were calculated adding each considered compound percentage.

3.3.2 PORAPAK-Q FILTERS

Porapak-Q filter consists of a glass tubes containing 30 mg Porapak-Q (ARS, Florida, USA). A push-pull system was used (Tholl *et al.*, 2006). The collection of VOCs using Porapak-Q filters was done twice, 24 and 48 hours after larval deposit. Porapak-Q filter was set on the pull-out PTFE tube and the air passed through at rate 0.5 L.min⁻¹. The inlet air-flow was set at 1.5 L.min⁻¹. During VOC collection, the bags were closed at their bottom and top with a cable binder. After 2 hours of volatiles collection, glass tubes were removed and volatiles were eluted using 200 µL of dichloromethane containing 10 ng.µL⁻¹ of internal standard nonylacetate. Eluates were then stored in glass vials at -20 °C, until analysis.



■ **FIGURE 33** : TIME LINE OF THE EXPERIMENTAL PROTOCOL FOR THE CAPTURE OF VOCs FROM SHOOTS OF TOMATOES GROWN IN FULL NUTRIENT SOLUTIONS EITHER AT 7 mM (HN) OR LIMITED 0.3 mM NO₃ (LN).

Half the plants were infested with 12 larvae of *T. absoluta* set on the 4th fully developed leaf. Plastic bags were set on shoots 24 h before larval deposit and VOCs were collected 2, 9, 23, 33, 47 and 54 h after larval deposit with PDMS tubes, or 24 and 48 hours after larval deposit with Porapak-Q sorbent. Plant were sampled immediately after the last VOC sampling, 55 h after the beginning of the infestation.

Analysis was performed using gas chromatography (Agilent 6890 series, Agilent Santa Clara, CA, USA) coupled either to a quadrupole mass selective detector (Agilent 5973, interface T 270 °C, quadrupole T 50 °C, source T 230 °C; electron energy 70 eV) or a flame ionization detector (FID, T 300 °C). Molecules were separated in a 30 m x 250 μm x 0.25 μm DB5-MS column (Wilcom GmbH, Heppenheim, Germany) through He (mass detector) or H₂ (FID) carrier gases. One microliter of the sample was injected without splitting at an initial oven temperature of 40 °C (2 min hold) followed by a ramp to 155 °C (1 °C min⁻¹), a ramp to 300 °C (60 °C min⁻¹), and a hold for 3 min. Compounds were identified by mass comparison of retention times and mass spectra from Wiley and National Institute of Standards Technology libraries. Only the compounds presenting more than 85 % identity with a match were given a name. The amount of all compounds was determined using their flame ionization detection peak area reported to the area of the internal standard, using the same method than GC-MS.

Emission rates (X) were calculated in pg h⁻¹:

$$X = RF \times \frac{PA(x)}{PA(IS)} \times \frac{\overline{v}_{in}}{2 \times \overline{v}_{out}} \times 1000,$$

with RF the response factor corresponding to the compound ($RF=1$, when unknown), $PA(x)$ the compound X peak area, $PA(IS)$ the internal standard peak area, the factor "2" corresponds to the trapping time (hours), \overline{v}_{in} and \overline{v}_{out} , the bag inlet and Porapak Q filter outlet flowrates, respectively. Class compounds emissions were calculated by adding the emission rates of each considered class.

3.3.3 PLANT HARVEST

Plants were harvested 55 hours after the onset of herbivory. Leaves, stems and roots were sampled and weighed for fresh mass. Plant samples were dried at 50 °C for four days and weighed for dry mass.

3.4

STATISTICS

Computations were performed using the XLSTAT software (v18.07.38968, Addinsoft) and statistical significance was set at $P < 0.05$.

3.4.1 PLANT BIOMASS

Analysis of variance (ANOVA) were performed between variance and mean, assessed the data distribution and homoscedasticity. Box and Cox transformations were applied to the data.

3.4.2 QUALITATIVE ANALYSIS OF VOCs

Repeated measures ANOVA were performed to assess the impact of nitrogen availability, herbivory and their interactions. Mauchly's sphericity test was performed to attest that the covariance matrix was spherical. When the sphericity of the covariance matrix was violated, Greenhouse-Geisser and Huynh-Feldt corrections were performed on the data (Schneier and Gurevitch, 2001).

3.4.3 QUANTITATIVE ANALYSIS OF VOCs

Mixed model for repeated measures ANOVA were performed to assess the impact of nitrogen availability, herbivory and their interactions, using the relative maximal likelihood (REML) estimation.

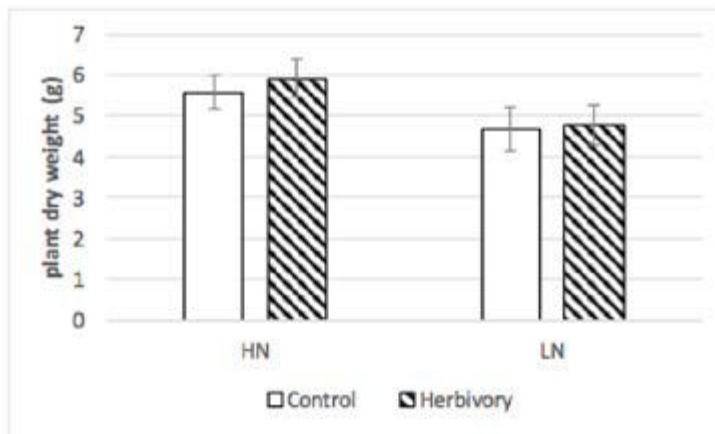
4

RESULTS

4.1

PLANT BIOMASS

Mean plant dry weights were almost 1 g higher for HN control than LN control plants. Statistics showed that nitrogen limitation induced a significant slowing in plant mass gain. Whereas, control and infested plants dry weights were not significantly different (Figure 34). Thus, *Tuta absoluta* herbivory did not induce severe damage to plants.



	WILK'S LAMBDA		
	VALUE	F	P-VALUE
NITROGEN	0.889	4.514	0.041
HERBIVORY	0.995	0.185	0.670
N X H	0.998	0.068	0.796

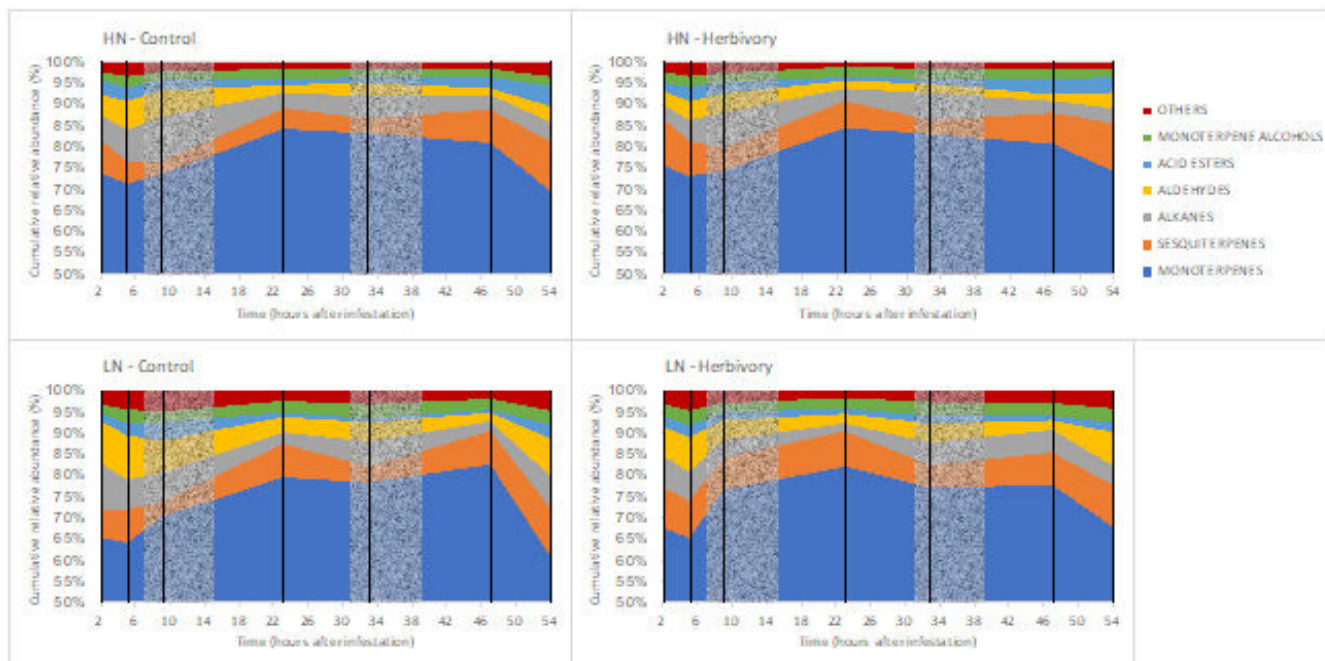
■ **FIGURE 34** : PLANT DRY MASS (g).

Control and *Tuta absoluta*-infested plants were raised under high (HN) or limited (LN) nitrogen nutrition. Twenty-eight days after transplantation, 12 larvae were deposited on the three terminal leaflets of the 4th leaf. Control plants received a mock inoculation using a paintbrush. Vertical bars are s.e.m. ANOVA was done using Wilk's lambda test, Wilks values, *F* and *p-values* are shown on the table.

4.2

QUALITATIVE ANALYSIS OF TOMATO VOLATILE BLEND OVER TIME

Plant volatile composition was qualitatively analysed, from 2 to 54 h after plant infestation. Figure 35 is a representation of volatile profile bouquet to help the reader to visualize changes. The volatile bouquet evolved along plant growth, day/night periods, and showed diel patterns.



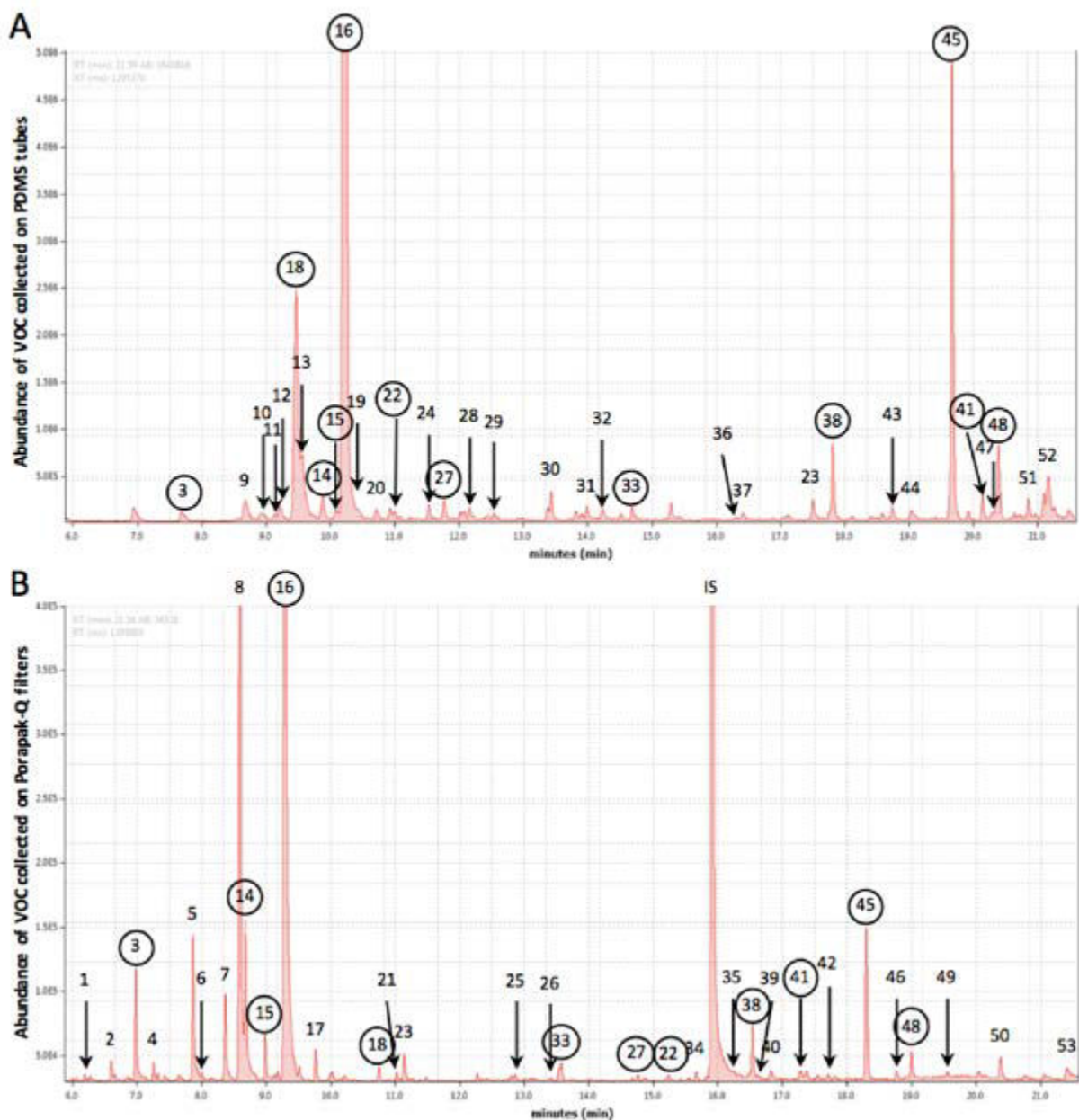
■ **FIGURE 35** : RELATIVE COMPOSITION OF THE VOLATILE BLEND EMITTED BY TOMATO SHOOT.

Volatiles were grouped into compound classes (see caption). Abscissa and ordinate are time evolved after larval deposit (h) and compound emission (relative scale), respectively. Vertical lines denote trapping times. Volatiles were trapped in PDMS tubes and analysed using TD-GCMS. Means of 8 replicates were calculated as the sum of each compound relative proportion in a same class. The shaded areas represent night periods.

The proportion of each compound was analysed at each trapping times. Means and standard errors are shown in supplementary data (Table 8, pages 168-173). Twenty-eight compounds were categorized into 6 classes: monoterpenes (more than 60 % of the volatile blend), sesquiterpenes, terpenoid alcohols, alkanes, aldehydes, acid esters and five other undetermined compounds (Figure 36.A).

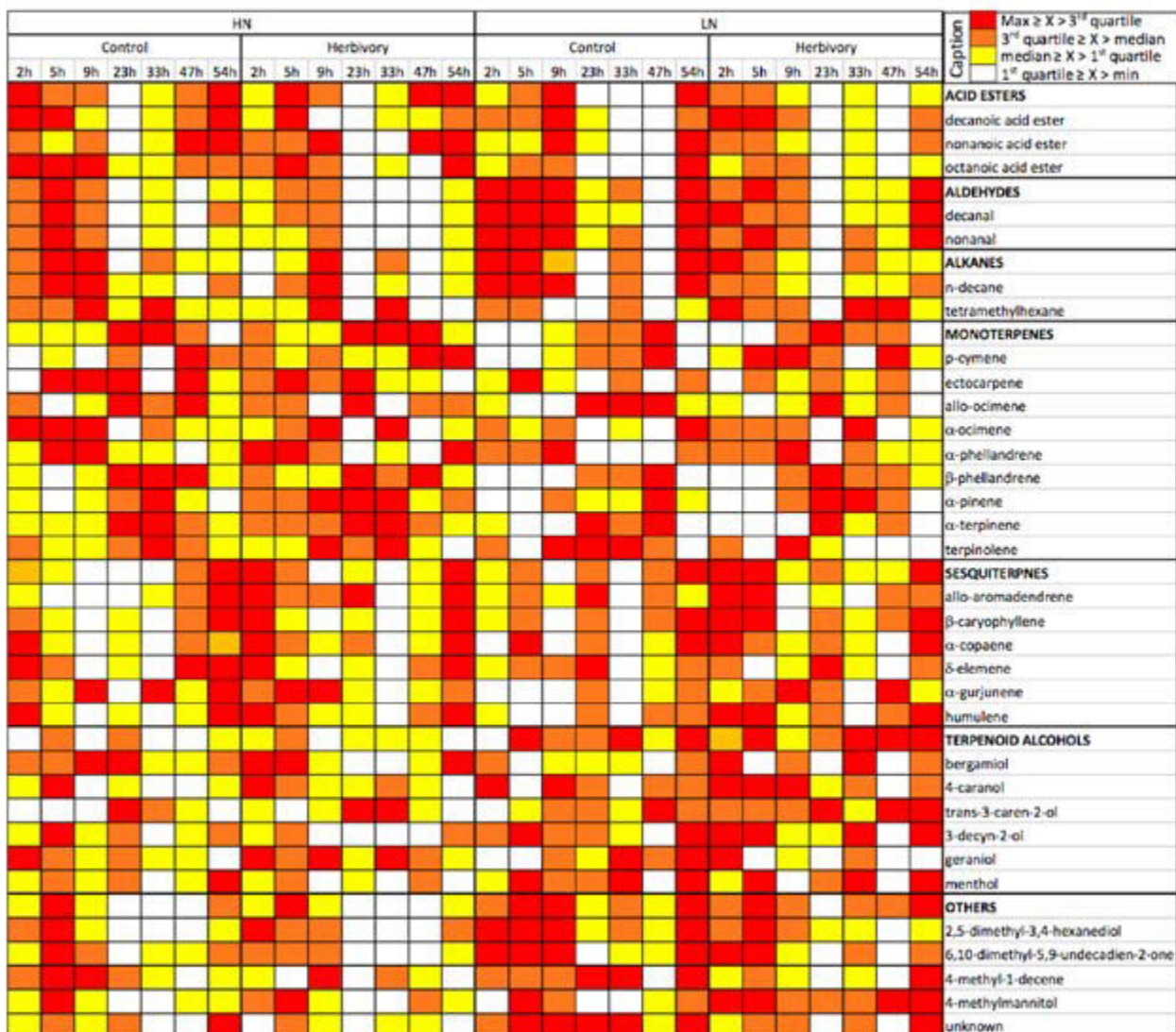
Repeated measures ANOVA showed effects of nitrogen availability and herbivory on the proportion of VOCs in the plant headspace (Table 8, Figure 37,

page 158). Compared to HN plants, LN plants' headspace had higher proportions of both aldehydes (decanal and nonanal) and of 2 terpenoid alcohols (3-decyn-2-ol and menthol), whereas proportions of monoterpenes (α - terpinene, terpinolene and near the significance α -pinene) were lower. The proportion of alkanes and 4-methyl-1-decene decreased in the presence of *Tuta absoluta*, whereas the proportions of the sesquiterpene, humulene, were higher in infected plants' headspace than control ones.



■ **FIGURE 36** : TOMATO VOLATILE PROFILES FROM (A) TD-GC-MS OF PDMS SAMPLING AND (B) GC-MS OF PORAPAK Q SAMPLING.

Compounds are numbered from 1 to 54. 1: cyclohexanone; 2: cyclopentane; 3: α -pinene; 4: propanoic acid ester 1; 5: m-cymene; 6: 1,3,8-p-menthatriene; 7: β -pinene; 8: 2-carene; 9: trans-3-carene-2-ol; 10: 4-methylmannitol; 11: 2,2,5,5-tetramethylhexane; 12: geraniol; 13: α -phellandrene; 14: α -terpinene; 15: p-cymene; 16: β -phellandrene; 17: β -cis-ocimene; 18: terpinolene; 19: bergamiol; 20: α -ocimene; 21: unknown 1; 22: dimethyl-decane; 23: nonanal; 24: allo-ocimene; 25: unknown 2; 26: methyl-salicylate; 27: methyl-1-decene; 28: Unknown 3; 29: 4-caranol; 30: ectocarpene; 31: menthol; 32: 3-decyn-2-ol; 33: decanal; 34: ascaridole; 35: an alkane; 36: nonanoic acid ester; 37: octadecanoic acid ester; 38: δ -elemene; 39: putative cyclohexanone; 40: propanoic acid ester 2; 41: α -copaene; 42: β -elemene; 43: decanoic acid ester; 44: 2,5-dimethyl-3,4-hexanediol; 45: β -caryophyllene; 46: aristolene; 47: 6,10-dimethyl-5,9-undecanedien-2-one; 48: humulene; 49: heneicosane; 50: pentanoic acid ester; 51: allo-aromadendrene; 52: α -gurjunene; 53: denderolasine; 1S: nonylacetate.



■ **FIGURE 37** : HEATMAP OF EACH VOLATILE COMPOUND PERCENTAGE IN THE HEADSPACE OF PLANTS SUPPLIED WITH HN OR LN AND INFESTED BY *Tuta absoluta* OR NOT.

Control and *Tuta absoluta*-infested plants were raised under high (HN) or limited (LN) nitrogen nutrition. Twenty-eight days after transplantation, 12 larvae were deposited on the three terminal leaflets of the 4th leaf. Control plants received a mock inoculation using a paintbrush. Volatiles were trapped in PDMS tubes, after 2, 5, 9, 23, 33, 47 and 54 h after the larval deposit and analysed using TD-GCMS. Relative proportion of each volatile compound was calculated. Volatiles were grouped into compound classes. For each VOC or chemical class, its proportion was separated into quartile between treatments (see caption).

4.3

QUANTITATIVE ANALYSIS OF VOLATILE EMISSIONS, AFTER 24 AND 48 h OF HERBIVORY

Thirty-three compounds were separated by GC-MS and GC-FID (Figure 36.B, page 157) and 29 of them were identified using MS spectra and retention time. Compounds were categorized into 7 groups: 3 acid esters, 2 aldehydes, 3 alkanes, 3 cetones, 11 monoterpenes, 7 sesquiterpenes and 4 uncategorized.

Means ($\text{pg}\cdot\text{h}^{-1}$), standard errors and ANOVA results of plant volatile emission 24 h and 48 h after larval deposit are shown in Table 7.

Nitrogen limitation did not alter the total VOC emission rate, but the emission of eleven VOCs was significantly enhanced in LN plants compared to HN ($P < 0.05$). Methyl salicylate emission rate was up to 3-5 times for LN than HN plants. A similar increase was observed for several other compounds (propanoic acid ester 1, both aldehydes, heneicosane, the 3 cetones, denderolasine, undecene and the unidentified compound named Unknown 1), which emission rates were 20 to 150 % higher for LN than HN plants.

Tuta absoluta herbivory enhanced by 20 to 90 % ($P < 0.1$) the VOC emission of 5 monoterpenes (m-cymene, 1,3,8-menthatriene, β -cis-ocimene, β -phellandrene and terpinolene), two sesquiterpenes (β -caryophyllene and humulene) and the unidentified compound Unknown 2.

	Sig.	24h of herbivory							
		High Nitrogen				Low Nitrogen			
		Control		Herbivory		Control		Herbivory	
		mean	s.e.m.	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.
TOTAL VOC	.	3475 ± 576	3749 ± 1191	5459 ± 936	7316 ± 1037				
ACIDESTERS		36.77 ± 4.31	34.19 ± 2.22	54.32 ± 8.49	43.21 ± 2.03				
propanoic acid ester1	**	7.13 ± 0.67	6.63 ± 0.34	11.50 ± 1.38	11.45 ± 1.94				
propanoic acid ester2		27.94 ± 4.25	20.05 ± 2.97	28.56 ± 5.91	18.40 ± 2.86				
pentanoic acid ester		6.64 ± 1.33	8.76 ± 2.78	19.01 ± 4.75	13.37 ± 2.01				
ALDEHYDES	**	16.60 ± 2.06	9.96 ± 1.30	31.65 ± 3.62	33.88 ± 4.12				
decanal	**	5.30 ± 0.94	2.68 ± 0.73	9.96 ± 1.21	9.92 ± 1.11				
nonanal	**	12.06 ± 1.31	8.43 ± 0.65	21.69 ± 2.68	23.96 ± 3.38				
ALKANES		33.88 ± 4.49	34.42 ± 5.96	125.73 ± 37.41	76.18 ± 14.26				
dimethyl-decane		15.92 ± 1.94	15.27 ± 2.23	78.18 ± 31.99	31.72 ± 5.21				
alkane1		4.38 ± 0.70	3.54 ± 0.74	8.23 ± 0.79	7.13 ± 0.96				
heneicosane	*	14.20 ± 2.07	16.12 ± 3.12	39.33 ± 6.89	37.34 ± 8.43				
CETONES	**	38.95 ± 3.76	34.75 ± 3.18	69.77 ± 8.61	70.22 ± 12.01				
cyclohexanone	*	9.72 ± 1.71	6.14 ± 1.57	16.18 ± 2.80	17.47 ± 3.28				
cyclopentanone	**	17.33 ± 1.47	16.08 ± 0.92	29.71 ± 3.23	28.80 ± 4.87				
putative cyclohex-2-enone	**	13.29 ± 1.36	12.54 ± 0.80	25.90 ± 3.44	23.95 ± 4.08				
MONOTERPENES	.	3150 ± 548	3342 ± 1077	4529 ± 893	6314 ± 943				
ascaridole		4.18 ± 0.79	6.14 ± 1.96	10.48 ± 2.41	8.53 ± 1.21				
2-carene		635 ± 107	624 ± 186	842 ± 166	1149 ± 170				
<i>m</i> -cymene	.	95.51 ± 16.32	103.81 ± 34.37	148.71 ± 30.52	204.24 ± 33.82				
<i>p</i> -cymene		15.73 ± 3.58	12.59 ± 3.01	23.10 ± 4.10	32.33 ± 6.82				
menthatriene	.	18.36 ± 4.06	21.97 ± 7.29	28.37 ± 5.50	39.26 ± 7.63				
β-cis-ocimene	.	4.82 ± 0.77	7.08 ± 2.47	11.86 ± 3.28	16.59 ± 3.05				
β-phellandrene	.	2094 ± 367	2275 ± 749	3068 ± 604	4302 ± 631				
α-pinene		79.46 ± 13.70	76.96 ± 22.61	97.77 ± 19.68	131.05 ± 19.32				
β-pinene	.	23.23 ± 4.25	27.37 ± 8.34	43.74 ± 11.00	60.06 ± 13.21				
α-terpinene		177.23 ± 32.38	185.39 ± 59.72	247.31 ± 46.79	353.21 ± 61.67				
terpinolene	.	8.63 ± 1.59	11.55 ± 3.23	11.81 ± 2.09	17.48 ± 2.52				
SESQUITERPENES	***	181.89 ± 37.93	274.55 ± 98.71	602.42 ± 91.05	730.38 ± 97.52				
aristolene		5.57 ± 1.19	5.69 ± 1.56	7.83 ± 1.62	7.69 ± 1.09				
β-caryophyllene	*	65.67 ± 17.18	96.10 ± 33.36	117.39 ± 24.41	176.43 ± 24.20				
copaene		7.57 ± 1.75	14.50 ± 3.99	8.63 ± 1.58	11.95 ± 3.29				
denderolasine	***	90.93 ± 24.26	138.82 ± 51.80	440.89 ± 87.09	490.09 ± 77.51				
β-elemene		N/A ± N/A	2.08 ± 0.69	5.04 ± 1.05	6.18 ± 1.14				
δ-elemene		7.09 ± 1.66	9.50 ± 3.48	11.99 ± 2.73	13.96 ± 2.98				
humulene	*	10.27 ± 2.47	17.39 ± 5.60	18.75 ± 3.92	28.50 ± 3.94				
OTHERS	**	15.88 ± 2.72	17.96 ± 5.95	45.03 ± 6.56	45.82 ± 6.00				
methyl-salicylate	**	N/A ± N/A	5.27 ± 1.75	18.26 ± 5.26	10.32 ± 2.95				
unknown1	**	5.54 ± 1.39	4.03 ± 1.11	10.00 ± 1.65	11.41 ± 1.49				
unknown2	.	7.11 ± 1.39	10.36 ± 3.14	9.72 ± 1.49	13.67 ± 2.44				
undecene	**	5.83 ± 0.60	5.42 ± 0.47	10.58 ± 1.25	10.42 ± 1.86				

48h of herbivory							
High Nitrogen				Low Nitrogen			
Control		Herbivory		Control		Herbivory	
mean	s.e.m.	mean	s.e.m.	mean	s.e.m.	mean	s.e.m.
6618 ± 532		9656 ± 2575		3793 ± 819		5537 ± 1136	
56.98 ± 8.65		44.71 ± 3.90		36.18 ± 4.19		49.77 ± 6.93	
8.39 ± 1.37		8.56 ± 0.78		11.82 ± 1.71		13.61 ± 2.72	
35.25 ± 8.89		22.82 ± 4.71		17.12 ± 3.06		19.06 ± 3.08	
13.34 ± 1.05		13.33 ± 2.36		15.00 ± 3.22		19.95 ± 5.27	
23.69 ± 2.65		17.11 ± 2.28		22.41 ± 4.99		27.76 ± 6.74	
8.51 ± 1.19		6.78 ± 1.18		7.83 ± 1.47		8.71 ± 2.23	
15.18 ± 1.59		11.30 ± 1.27		15.56 ± 3.64		19.04 ± 4.65	
51.72 ± 10.61		102.10 ± 51.80		71.41 ± 13.55		83.57 ± 19.77	
22.30 ± 3.96		52.65 ± 31.60		29.16 ± 4.99		34.55 ± 7.81	
6.38 ± 1.16		11.59 ± 5.76		7.11 ± 1.19		7.02 ± 1.32	
23.96 ± 5.71		37.86 ± 14.86		36.03 ± 7.59		42.01 ± 10.66	
51.69 ± 8.15		55.00 ± 6.89		64.08 ± 10.51		80.21 ± 15.79	
14.80 ± 2.10		15.14 ± 2.66		15.71 ± 3.66		20.71 ± 4.65	
20.70 ± 3.15		20.69 ± 2.05		28.71 ± 3.60		32.73 ± 5.89	
16.19 ± 3.42		19.16 ± 3.39		21.63 ± 3.46		26.76 ± 5.45	
6066 ± 523		8953 ± 2489		2977 ± 714		4563 ± 968	
6.89 ± 1.27		13.08 ± 3.41		6.33 ± 1.47		8.04 ± 1.97	
1157 ± 104		1671 ± 450		559 ± 136		830 ± 170	
169.98 ± 15.62		269.78 ± 82.93		90.51 ± 20.45		148.88 ± 37.45	
33.15 ± 5.27		40.77 ± 11.37		14.90 ± 3.59		25.50 ± 6.57	
32.43 ± 3.49		51.61 ± 15.11		19.89 ± 6.33		34.57 ± 9.44	
12.65 ± 1.17		13.93 ± 3.73		9.85 ± 2.98		15.63 ± 4.62	
4092 ± 346		6080 ± 1704		2009 ± 479		3076 ± 648	
137.54 ± 12.83		202.25 ± 54.01		68.51 ± 15.73		102.56 ± 20.95	
50.49 ± 3.56		66.22 ± 16.11		30.65 ± 7.93		52.36 ± 13.13	
357.37 ± 37.38		522.08 ± 150.55		169.29 ± 43.03		263.15 ± 58.43	
17.26 ± 1.43		24.42 ± 7.42		8.62 ± 2.10		13.35 ± 3.32	
338.74 ± 46.79		439.43 ± 92.39		579.88 ± 158.42		690.74 ± 148.03	
7.93 ± 0.78		7.99 ± 1.15		8.47 ± 1.70		11.59 ± 2.78	
144.22 ± 20.39		163.23 ± 31.12		113.23 ± 23.33		202.04 ± 49.34	
14.61 ± 2.85		20.50 ± 4.00		9.57 ± 2.86		14.97 ± 3.62	
128.43 ± 41.27		201.84 ± 50.66		424.07 ± 131.56		413.50 ± 82.60	
4.14 ± 0.86		6.75 ± 1.44		4.39 ± 0.87		5.91 ± 1.29	
16.77 ± 2.23		15.13 ± 2.64		15.54 ± 3.51		22.89 ± 6.19	
25.01 ± 3.02		26.89 ± 5.22		18.62 ± 4.20		33.21 ± 8.01	
28.40 ± 3.89		39.05 ± 9.52		41.85 ± 10.47		42.02 ± 10.76	
4.48 ± 0.64		7.08 ± 1.80		29.19 ± 7.40		7.25 ± 1.47	
8.15 ± 1.40		7.04 ± 1.23		9.76 ± 2.00		15.36 ± 4.84	
12.71 ± 2.05		22.40 ± 6.27		6.50 ± 1.12		13.67 ± 3.40	
6.90 ± 1.15		7.58 ± 0.75		9.38 ± 1.48		10.68 ± 2.20	

■ **TABLE 7**: VOLATILE EMISSION (pg h⁻¹) OF HEALTHY (CONTROL) AND INFESTED (HERBIVORY) TOMATO PLANTS UNDER HIGH AND LIMITING NITROGEN AVAILABILITY.

Mean, standard error mean and statistics of 8 replicates. Italics indicate significant herbivory effect and bold nitrogen effect, with *P-values* < 0.001 (***), 0.001 < 0.01 (**), 0.01 < 0.05 (*), 0.05 < 0.1 (.).

5

DISCUSSION

5.1

COMPARISON OF BOTH VOC SAMPLING METHODS

We used two different VOC sampling methods. The first one consisted of solid phase microextraction made of PDMS (polydimethylsiloxane). This sorbent was recently and commonly used for plant volatiles sampling in field experimentation (Van Pinxteren *et al.*, 2010; Seethapathy and Górecki, 2012). The method is easy and rapid to set up (Kallenbach, *et al.*, 2014). The second method consisted of a push and pull air system. The circulating atmosphere reduces the impact of the bags on plant atmospheric environment, such as the atmospheric CO₂ partial pressure, the humidity of the air... We used Porapak Q, a sorbent reported to have a low water retention and a good recovery in the adsorption of plant VOCs (Berezkin and Drugov, 1991; Pichersky and Gershenzon, 2002). Both methods appeared robust in terms of diversity of compounds trapped, but are employed with different goals. PDMS tubes are easy to use and give semi-quantitative “snapshots” of the volatile blend of the plant headspace whereas the more time-consuming push - pull system provides quantitative data of volatile emission rates from

plant leaves (Chang *et al.*, 1986; Bruce *et al.*, 2010; Miresmailli *et al.*, 2010). Only 13 out of 53 compounds were found common to both methods, most likely attributable to the matrix affinity difference for the volatiles compounds. β -phellandrene was the most emitted VOC by Better bush tomato plant, accounting for more than half of the total VOC blend (a result obtained from both methods). PDMS tubes allowed the trapping of terpenoid alcohols that were not present in the Porapak Q sampling analyses (Figure 36, page 157). The Porapak Q trap revealed different terpenoids and the phytohormone methyl salicylic acid (Figure 36, page 157). These two methods are complementary in order to optimize the identification of the VOC blend composition.

In comparison to other methods used to determine tomato VOCs, our results showed a similar main composition of the *Solanum lycopersicum* L. cv. Better Bush VFN Hybrid than found in literature for different tomato varieties and VOC collection methods (Farag and Paré, 2002; Profitt *et al.*, 2011; Strapasson *et al.*, 2014).

5.2

NITROGEN LIMITATION MODIFIED TOMATO VOC BLEND

It has been established that nitrogen limits pest infestation and development (Herms and Mattson, 1992) and increases plant defence compounds' concentration in leaf tissues (Larbat *et al.*, 2012 b; 2014 and 2016). However, studies about the effect of nitrogen limitation on plant VOC emission are lacking. We have shown here that LN had no significant impact on the total amount of VOCs emitted per plant (Table 7), but we highlighted 11 compounds whose proportion (^a) or emission rate (^b) increased under nitrogen limitation (methyl salicylate phytohormone^b, the sesquiterpene

dendrolasine^b, 3-decyn-2-ol^a, menthol^a, aldehydes^{a,b}, cetones^b and few other compounds) and 2 compounds whose proportion decreased under LN conditions (α -terpinene^b and terpinolene^{a,b}). No interaction was found between nitrogen availability and *Tuta absoluta* herbivory. Among the nitrogen-inducible compounds, methyl salicylate was shown to play an important role by mediating predatory mite *Phytoseiulus persimilis* attraction (Ament *et al.*, 2010). Thus, the role of these nitrogen-induced compounds must be investigated for pest repellence or predatory attraction.

5.3

Tuta absoluta LEAFMINING ENHANCED THE EMISSION OF SIX MONOTERPENES AND TWO SESQUITERPENES

Upon herbivory attack, plants change the composition of VOC emissions (Dicke and Baldwin, 2010). A recent study on the pathosystem tomato - *Tuta absoluta*, showed that *Tuta absoluta* leafmining increased the emission of volatile alcohols, aldehydes, acid esters, 5 monoterpenes and 4 sesquiterpenes (Silva *et al.* 2017). This study used a different sorbent matrix (Tenax TA (20/35

mesh; CAMSCO, Houston, TX, USA), rendering uneasy the comparison with our results. Our experiments highlighted 9 VOCs whose proportion (^a) or emission rate (^b) were influenced by *Tuta absoluta* leafmining: β -caryophyllene^b, humulene^{a,b}, m-cymene^b, menthatriene^b, β -cis-ocimene^b, β -phellandrene^b, β -pinene^b, terpinolene^b and n-decane^a. β -caryophyllene and

β -cis-ocimene are the only two compounds in common with Silva *et al.* (2017).

Four roles are attributed to the herbivore-induced VOCs: (1) toxicity for the herbivores (Maffei, 2010), (2) contribution to intra and interplant communication (Kost and Heil, 2006), (3) predators or parasitoids attraction (Tumlinson *et al.*, 1993) and (4) repellency of the pest female limiting oviposition (Kessler and Baldwin, 2004). Recent studies have shown the predators or parasitoids' attractiveness and pest repellency of few specific herbivore-induced VOCs such as β -caryophyllene, β -cis-ocimene and humulene (Pichersky and Gershenzon, 2002; De Moraes *et al.*, 2001). The present study provides new insight in the VOC emission following infestation, by identifying tomato VOCs that are induced by nitrogen limitation and *Tuta absoluta* leafmining. We highlighted new chemical compounds potentially involved in plant-defence mechanisms. In 2015, De Backer *et al.* emit the hypothesis that monoterpenes are involved in the attraction of *Macrolophus pygmaeus*, a natural enemy of *Tuta absoluta* and to demonstrate the attraction properties of the *Tuta absoluta*-induced volatiles for parasitoids or predators. The role of these new molecules on *Tuta absoluta* parasitoids, such as *Necremnus artynes*, *Stenomestus japonica* and *Neochrysocharis formosa* attractiveness (Gabarra *et al.*, 2014) and pest repellence, whose emission is stimulated by nitrogen limitation, needs to be investigated in order to assess their impact on pest control management.

AUTHOR CONTRIBUTIONS

CR and VC conceived the idea of the experiment; all authors helped to design the experiment; VC and Aude Fauvet conducted the nutrition of tomatoes; ND provided the leafminers; CR, AF and VC performed the harvests; VC performed the chemical analyses, processed the data and drafted the manuscript; and all authors revised the manuscript.

ACKNOWLEDGEMENTS

The Ph.D. grant of Victoire Coqueret was co-funded by INRA (Département Environnement et Agronomie) and the Regional Council of Lorraine. All our thanks to the Regional Council of Lorraine for its financial support "projet émergent" (grant 15P03127). Our thanks go to the EIR-Agreenium (International Research School) and the Deutscher Akademischer Austauschdienst, DAAD, for their financial support to VC during her period at the MPI Jena. Larvae were provided by Philippe Bearez and Nicolas Desneux (INRA ISA Sophia Antipolis). We thank Aude Fauvet (INRA), Claude Gallois (Université de Lorraine), Michael Reichelt, Sandra Lackner, Franziska Eberl and Carlos Sanchez Acros (MPI for Chemical Ecology) for their advice on various practical aspects of this study.

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SUPPLEMENTAL DATA

■ **TABLE 8** : MEAN, STANDARD ERROR OF THE PROPORTION OF EACH COMPOUND (%) AND P-VALUES FROM REPEATED MEASURES ANOVA FOR NITROGEN LIMITATION (**BOLD**), *Tuta absoluta* HERBIVORY (*ITALIC*) AND TIME FACTORS.

Volatiles were trapped using PDMS tubes and were analysed using TD-GCMS. Means \pm s.e.m. of proportions were calculated from 8 replicates.

	h+2			
	HN		LN	
	Control	Herbivory	Control	Herbivory
	mean \pm s.e.m.	mean \pm s.e.m.	mean \pm s.e.m.	mean \pm s.e.m.
ACID ESTERS	3.555 \pm 0.786	2.176 \pm 0.287	2.089 \pm 0.329	2.627 \pm 0.560
Decanoic acid ester	0.711 \pm 0.106	0.366 \pm 0.067	0.653 \pm 0.114	0.674 \pm 0.189
Nonanoic acid ester	1.088 \pm 0.407	0.788 \pm 0.178	0.744 \pm 0.213	1.069 \pm 0.510
Octanoic acid ester	1.755 \pm 0.339	1.168 \pm 0.168	0.773 \pm 0.115	0.885 \pm 0.163
ALDEHYDES	4.649 \pm 0.442	3.010 \pm 0.394	9.912 \pm 2.233	6.728 \pm 0.557
<i>Decanal</i>	<i>1.628 \pm 0.262</i>	<i>1.031 \pm 0.126</i>	<i>3.093 \pm 0.843</i>	<i>2.295 \pm 0.357</i>
<i>Nonanal</i>	<i>3.022 \pm 0.286</i>	<i>1.980 \pm 0.318</i>	<i>6.819 \pm 1.408</i>	<i>4.433 \pm 0.304</i>
ALKANES	5.988 \pm 1.100	3.517 \pm 0.644	11.199 \pm 1.766	7.386 \pm 1.516
n-decane	5.301 \pm 0.877	3.139 \pm 0.606	10.291 \pm 1.621	6.143 \pm 1.139
Tetramethylhexane	1.374 \pm 0.285	0.756 \pm 0.144	1.816 \pm 0.437	2.486 \pm 0.639
MONOTERPENES	73.827 \pm 1.711	75.502 \pm 1.585	65.420 \pm 4.541	67.461 \pm 1.440
P-cymene	0.113 \pm 0.019	0.207 \pm 0.031	0.134 \pm 0.032	0.178 \pm 0.046
Ectocarpene	0.112 \pm 0.035	0.481 \pm 0.273	0.144 \pm 0.047	0.129 \pm 0.029
Allo-ocimene	0.280 \pm 0.044	0.269 \pm 0.057	0.250 \pm 0.065	0.201 \pm 0.056
α -ocimene	0.417 \pm 0.098	0.258 \pm 0.057	0.289 \pm 0.106	0.321 \pm 0.089
α -phellandrene	4.409 \pm 0.903	5.059 \pm 0.567	4.448 \pm 0.675	4.934 \pm 0.752
β -phellandrene	52.262 \pm 1.624	53.507 \pm 1.791	44.240 \pm 4.678	45.860 \pm 1.660
α -pinene	0.448 \pm 0.083	0.459 \pm 0.061	0.315 \pm 0.048	0.363 \pm 0.068
<i>α-terpinene</i>	<i>0.377 \pm 0.099</i>	<i>0.543 \pm 0.096</i>	<i>0.311 \pm 0.093</i>	<i>0.262 \pm 0.056</i>
<i>Terpinolene</i>	<i>15.452 \pm 1.218</i>	<i>14.811 \pm 0.740</i>	<i>15.308 \pm 0.556</i>	<i>15.345 \pm 0.941</i>
SESQUITERPENES	7.744 \pm 0.805	10.976 \pm 1.094	6.446 \pm 0.914	9.672 \pm 1.208
Allo-aromadendrene	0.095 \pm 0.023	0.215 \pm 0.044	0.100 \pm 0.023	0.166 \pm 0.037
β -caryophyllene	5.467 \pm 0.629	7.564 \pm 0.846	4.944 \pm 0.900	7.118 \pm 0.940
α -copaene	0.364 \pm 0.065	0.479 \pm 0.039	0.193 \pm 0.035	0.353 \pm 0.042
δ -elemene	0.376 \pm 0.073	0.807 \pm 0.091	0.157 \pm 0.039	0.271 \pm 0.113
α -gurjunene	0.243 \pm 0.035	0.326 \pm 0.067	0.191 \pm 0.038	0.229 \pm 0.047
Humulene	1.199 \pm 0.098	1.626 \pm 0.159	0.921 \pm 0.081	1.562 \pm 0.153
TERPENOID ALCOHOLS	1.954 \pm 0.151	2.319 \pm 0.145	1.775 \pm 0.149	2.795 \pm 0.503
Bergamiol	0.036 \pm 0.010	0.051 \pm 0.011	0.047 \pm 0.013	0.108 \pm 0.067
4-caranol	0.076 \pm 0.030	0.147 \pm 0.055	0.134 \pm 0.043	0.189 \pm 0.125
Trans-3-carene-2-ol	0.613 \pm 0.088	0.943 \pm 0.151	0.725 \pm 0.143	0.930 \pm 0.202
<i>3-decyn-2-ol</i>	<i>0.295 \pm 0.037</i>	<i>0.250 \pm 0.031</i>	<i>0.346 \pm 0.070</i>	<i>0.533 \pm 0.198</i>
Geraniol	0.826 \pm 0.103	0.951 \pm 0.107	0.428 \pm 0.077	0.861 \pm 0.134
<i>Menthol</i>	<i>0.128 \pm 0.024</i>	<i>0.094 \pm 0.018</i>	<i>0.134 \pm 0.068</i>	<i>0.224 \pm 0.119</i>
OTHERS	2.283 \pm 0.275	2.500 \pm 0.426	3.275 \pm 0.732	3.137 \pm 0.311
2,5-dimethyl-3,4-hexanediol	0.580 \pm 0.105	0.808 \pm 0.314	0.842 \pm 0.274	0.475 \pm 0.089
6,10-dimethyl-5,9-undecadien-2-one	0.473 \pm 0.080	0.607 \pm 0.089	0.941 \pm 0.353	0.935 \pm 0.232
4-methyl-1-decene	0.130 \pm 0.040	0.098 \pm 0.025	0.112 \pm 0.030	0.098 \pm 0.032
4-methylmannitol	0.581 \pm 0.210	0.669 \pm 0.108	0.496 \pm 0.125	1.010 \pm 0.248
Unknown	0.608 \pm 0.215	0.478 \pm 0.162	0.946 \pm 0.359	0.619 \pm 0.253

h+5				h+9			
HN		LN		HN		LN	
Control	Herbivory	Control	Herbivory	Control	Herbivory	Control	Herbivory
mean ± s.e.m.	mean ± s.e.m.	mean ± s.e.m.	mean ± s.e.m.	mean ± s.e.m.	mean ± s.e.m.	mean ± s.e.m.	mean ± s.e.m.
3.058 ± 0.503	3.327 ± 0.570	2.494 ± 0.479	2.756 ± 0.792	2.624 ± 0.576	2.859 ± 0.569	4.534 ± 1.579	2.051 ± 0.321
0.831 ± 0.197	0.805 ± 0.200	0.671 ± 0.128	0.775 ± 0.125	0.333 ± 0.067	0.186 ± 0.076	1.428 ± 0.596	0.552 ± 0.120
0.860 ± 0.208	1.020 ± 0.253	0.741 ± 0.275	0.780 ± 0.394	0.872 ± 0.337	1.355 ± 0.325	1.295 ± 0.413	0.560 ± 0.180
1.578 ± 0.258	1.501 ± 0.262	1.082 ± 0.220	1.201 ± 0.352	1.419 ± 0.282	1.341 ± 0.341	1.811 ± 0.630	0.938 ± 0.156
6.827 ± 1.081	4.587 ± 0.598	10.370 ± 2.177	8.043 ± 1.387	6.007 ± 1.033	4.530 ± 0.588	7.618 ± 0.966	4.585 ± 0.474
2.355 ± 0.373	2.084 ± 0.341	3.228 ± 0.693	2.222 ± 0.777	1.763 ± 0.259	1.514 ± 0.228	2.240 ± 0.464	1.032 ± 0.174
4.471 ± 0.824	2.503 ± 0.369	7.143 ± 1.496	5.822 ± 0.820	4.244 ± 0.790	3.016 ± 0.397	5.378 ± 0.567	3.553 ± 0.343
7.215 ± 1.441	4.728 ± 0.960	7.242 ± 1.642	6.958 ± 1.615	10.739 ± 1.935	8.398 ± 1.619	6.871 ± 0.735	4.309 ± 0.736
6.733 ± 1.251	4.322 ± 0.776	6.718 ± 1.542	6.200 ± 1.308	9.519 ± 1.606	6.639 ± 1.369	6.871 ± 0.735	3.852 ± 0.894
1.926 ± 0.376	1.623 ± 0.276	1.397 ± 0.259	1.516 ± 0.358	2.440 ± 0.585	2.814 ± 0.630	N/A ± N/A	1.828 ± 0.385
71.154 ± 2.905	72.942 ± 2.027	63.946 ± 3.179	65.202 ± 3.660	73.598 ± 3.204	74.379 ± 1.919	70.064 ± 4.460	76.504 ± 2.005
0.139 ± 0.052	0.158 ± 0.034	0.131 ± 0.028	0.229 ± 0.058	0.106 ± 0.029	0.218 ± 0.044	0.188 ± 0.034	0.257 ± 0.076
1.556 ± 0.737	2.557 ± 0.960	3.391 ± 1.845	1.209 ± 0.588	1.222 ± 0.698	0.900 ± 0.430	0.152 ± 0.032	0.145 ± 0.039
0.140 ± 0.024	0.294 ± 0.080	0.168 ± 0.043	0.185 ± 0.050	0.257 ± 0.117	0.120 ± 0.034	0.203 ± 0.068	0.218 ± 0.057
0.613 ± 0.187	0.305 ± 0.041	0.342 ± 0.129	0.370 ± 0.063	0.571 ± 0.244	0.685 ± 0.218	0.388 ± 0.206	0.335 ± 0.089
5.697 ± 1.099	5.386 ± 0.758	4.623 ± 0.496	4.755 ± 0.611	5.326 ± 0.757	4.789 ± 0.399	6.146 ± 0.878	5.141 ± 0.826
49.760 ± 2.026	50.381 ± 2.203	42.717 ± 3.411	46.951 ± 3.478	51.464 ± 3.444	50.445 ± 1.986	46.564 ± 3.245	53.821 ± 2.482
0.364 ± 0.063	0.467 ± 0.109	0.356 ± 0.074	0.340 ± 0.058	0.392 ± 0.087	0.901 ± 0.258	0.554 ± 0.154	0.614 ± 0.144
0.384 ± 0.085	0.441 ± 0.095	0.238 ± 0.038	0.126 ± 0.033	0.359 ± 0.109	0.431 ± 0.114	0.214 ± 0.065	0.282 ± 0.064
14.067 ± 2.311	13.911 ± 1.656	14.350 ± 2.029	11.536 ± 1.955	14.501 ± 1.550	16.255 ± 0.865	15.768 ± 2.287	15.788 ± 1.293
5.525 ± 0.514	8.350 ± 0.841	7.729 ± 1.732	8.728 ± 1.050	2.867 ± 0.751	5.113 ± 1.339	3.565 ± 0.654	7.555 ± 1.388
0.070 ± 0.012	0.169 ± 0.048	0.158 ± 0.044	0.184 ± 0.028	0.065 ± 0.014	0.153 ± 0.085	0.095 ± 0.033	0.061 ± 0.012
3.891 ± 0.393	5.411 ± 0.673	6.190 ± 1.765	6.319 ± 0.837	1.324 ± 0.107	3.105 ± 0.999	2.358 ± 0.475	3.292 ± 0.497
0.228 ± 0.049	0.234 ± 0.056	0.348 ± 0.066	0.273 ± 0.056	0.138 ± 0.029	0.224 ± 0.060	0.170 ± 0.033	0.178 ± 0.029
0.270 ± 0.103	0.221 ± 0.048	0.264 ± 0.090	0.066 ± 0.017	0.157 ± 0.046	0.082 ± 0.022	0.284 ± 0.129	0.226 ± 0.053
0.222 ± 0.064	1.224 ± 0.937	0.158 ± 0.034	0.283 ± 0.059	0.866 ± 0.734	0.780 ± 0.634	0.118 ± 0.033	2.922 ± 1.458
0.934 ± 0.074	1.113 ± 0.137	0.703 ± 0.172	1.655 ± 0.167	0.362 ± 0.088	0.807 ± 0.190	0.611 ± 0.148	0.876 ± 0.113
2.727 ± 0.232	2.463 ± 0.140	3.593 ± 0.575	3.685 ± 0.706	1.680 ± 0.167	2.137 ± 0.368	2.541 ± 0.372	2.189 ± 0.253
0.050 ± 0.016	0.076 ± 0.016	0.017 ± 0.004	0.025 ± 0.006	0.050 ± 0.013	0.054 ± 0.015	0.050 ± 0.013	0.061 ± 0.019
0.134 ± 0.072	0.131 ± 0.049	0.055 ± 0.013	0.418 ± 0.172	0.088 ± 0.021	0.075 ± 0.028	0.138 ± 0.045	0.257 ± 0.113
0.714 ± 0.128	0.650 ± 0.113	0.833 ± 0.192	0.973 ± 0.276	0.605 ± 0.068	0.800 ± 0.190	0.973 ± 0.330	0.951 ± 0.182
0.435 ± 0.092	0.344 ± 0.114	0.699 ± 0.289	0.411 ± 0.077	0.198 ± 0.043	0.161 ± 0.035	0.314 ± 0.066	0.272 ± 0.063
0.815 ± 0.078	0.766 ± 0.139	0.576 ± 0.127	0.577 ± 0.081	0.663 ± 0.105	1.005 ± 0.194	0.790 ± 0.133	0.644 ± 0.112
0.625 ± 0.207	0.539 ± 0.114	1.422 ± 0.463	1.339 ± 0.485	0.136 ± 0.044	0.088 ± 0.022	0.329 ± 0.120	0.091 ± 0.018
3.495 ± 0.486	3.447 ± 0.479	4.419 ± 1.478	4.629 ± 0.825	2.486 ± 0.440	2.584 ± 0.652	4.807 ± 1.986	2.807 ± 0.328
0.837 ± 0.262	0.565 ± 0.113	0.816 ± 0.216	0.625 ± 0.131	0.496 ± 0.096	0.481 ± 0.069	0.747 ± 0.222	0.465 ± 0.075
0.749 ± 0.148	0.736 ± 0.225	0.836 ± 0.170	1.689 ± 0.390	0.693 ± 0.190	0.613 ± 0.289	1.084 ± 0.510	0.441 ± 0.110
0.147 ± 0.045	0.118 ± 0.036	0.162 ± 0.045	0.105 ± 0.029	0.342 ± 0.142	0.253 ± 0.091	0.138 ± 0.038	0.109 ± 0.022
1.227 ± 0.404	1.304 ± 0.523	1.417 ± 0.614	1.441 ± 0.578	0.521 ± 0.132	0.644 ± 0.377	0.860 ± 0.259	0.975 ± 0.266
0.688 ± 0.248	0.916 ± 0.320	1.385 ± 0.960	0.950 ± 0.543	0.582 ± 0.296	0.657 ± 0.213	1.995 ± 1.202	1.074 ± 0.370

■ TABLE 8 - PART 2

	h+23			
	HN		LN	
	Control	Herbivory	Control	Herbivory
	mean ± s.e.m.	mean ± s.e.m.	mean ± s.e.m.	mean ± s.e.m.
ACID ESTERS	1.162 ± 0.239	0.972 ± 0.061	1.184 ± 0.385	1.201 ± 0.273
Decanoic acid ester	0.149 ± 0.048	0.206 ± 0.056	0.487 ± 0.129	0.167 ± 0.048
Nonanoic acid ester	0.369 ± 0.129	0.304 ± 0.065	0.446 ± 0.201	0.440 ± 0.157
Octanoic acid ester	0.645 ± 0.124	0.461 ± 0.039	0.434 ± 0.092	0.594 ± 0.157
ALDEHYDES	2.034 ± 0.298	1.595 ± 0.339	3.463 ± 1.327	2.240 ± 0.578
Decanal	0.475 ± 0.103	0.315 ± 0.069	0.736 ± 0.287	0.459 ± 0.134
Nonanal	1.559 ± 0.260	1.280 ± 0.285	2.727 ± 1.056	1.781 ± 0.468
ALKANES	3.474 ± 0.776	2.997 ± 0.288	2.853 ± 0.552	1.919 ± 0.522
n-decane	3.242 ± 0.767	2.814 ± 0.296	2.795 ± 0.510	1.844 ± 0.479
Tetramethylhexane	0.462 ± 0.103	0.366 ± 0.069	0.464 ± 0.058	0.201 ± 0.047
MONOTERPENES	84.288 ± 1.374	84.450 ± 0.609	79.464 ± 3.284	82.155 ± 1.530
p-cymene	0.209 ± 0.029	0.152 ± 0.035	0.198 ± 0.034	0.216 ± 0.047
Ectocarpene	1.990 ± 0.738	1.147 ± 0.431	0.024 ± 0.005	1.133 ± 0.547
Allo-ocimene	0.367 ± 0.057	0.404 ± 0.038	0.383 ± 0.053	0.355 ± 0.064
α-ocimene	0.142 ± 0.033	0.195 ± 0.037	0.182 ± 0.055	0.151 ± 0.053
α-phellandrene	5.138 ± 0.988	4.216 ± 0.790	3.947 ± 0.759	3.630 ± 0.618
β-phellandrene	61.648 ± 1.285	62.881 ± 1.123	58.850 ± 3.834	61.785 ± 1.169
α-pinene	0.481 ± 0.119	0.796 ± 0.101	0.447 ± 0.096	0.632 ± 0.090
α-terpinene	0.863 ± 0.030	0.927 ± 0.028	0.723 ± 0.088	0.691 ± 0.073
Terpinolene	15.376 ± 1.241	15.457 ± 0.691	16.693 ± 0.869	14.601 ± 1.376
SESQUITERPENES	4.829 ± 0.421	6.421 ± 0.618	7.800 ± 1.137	8.305 ± 0.528
Allo-aromadendrene	0.096 ± 0.021	0.182 ± 0.050	0.171 ± 0.092	0.112 ± 0.029
β-caryophyllene	3.526 ± 0.318	4.680 ± 0.465	5.678 ± 0.786	6.228 ± 0.359
α-copaene	0.177 ± 0.033	0.239 ± 0.033	0.260 ± 0.074	0.230 ± 0.027
δ-elemene	0.168 ± 0.041	0.186 ± 0.047	0.395 ± 0.136	0.331 ± 0.058
α-gurjunene	0.170 ± 0.034	0.232 ± 0.047	0.284 ± 0.096	0.228 ± 0.027
Humulene	0.746 ± 0.073	0.931 ± 0.078	1.012 ± 0.111	1.174 ± 0.106
TERPENOID ALCOHOLS	2.682 ± 0.195	2.393 ± 0.182	2.675 ± 0.309	2.537 ± 0.222
Bergamiol	0.070 ± 0.041	0.022 ± 0.006	0.024 ± 0.014	0.023 ± 0.005
4-caranol	0.078 ± 0.021	0.076 ± 0.014	0.092 ± 0.038	0.063 ± 0.012
Trans-3-carene-2-ol	1.166 ± 0.157	1.231 ± 0.215	1.065 ± 0.144	1.377 ± 0.328
3-decyn-2-ol	0.301 ± 0.040	0.190 ± 0.049	0.382 ± 0.147	0.275 ± 0.044
Geraniol	0.735 ± 0.082	0.713 ± 0.101	0.598 ± 0.102	0.554 ± 0.124
Menthol	0.360 ± 0.081	0.190 ± 0.057	0.515 ± 0.231	0.248 ± 0.058
OTHERS	1.532 ± 0.262	1.172 ± 0.205	2.396 ± 1.093	1.643 ± 0.349
2,5-dimethyl-3,4-hexanediol	0.317 ± 0.056	0.274 ± 0.062	0.390 ± 0.092	0.343 ± 0.044
6,10-dimethyl-5,9-undecadien-2-one	0.218 ± 0.041	0.249 ± 0.080	0.288 ± 0.153	0.218 ± 0.055
4-methyl-1-decene	0.123 ± 0.056	0.060 ± 0.016	0.284 ± 0.218	0.057 ± 0.015
4-methylmannitol	0.210 ± 0.040	0.240 ± 0.058	0.256 ± 0.074	0.954 ± 0.334
Unknown	0.691 ± 0.219	0.437 ± 0.184	1.214 ± 0.664	0.234 ± 0.098

h+33				h+47			
HN		LN		HN		LN	
Control	Herbivory	Control	Herbivory	Control	Herbivory	Control	Herbivory
mean \pm s.e.m.	mean \pm s.e.m.	mean \pm s.e.m.	mean \pm s.e.m.	mean \pm s.e.m.	mean \pm s.e.m.	mean \pm s.e.m.	mean \pm s.e.m.
<u>1.451 \pm 0.225</u>	<u>1.414 \pm 0.255</u>	<u>1.129 \pm 0.189</u>	<u>1.703 \pm 0.254</u>	<u>2.487 \pm 0.802</u>	<u>3.556 \pm 1.375</u>	<u>0.833 \pm 0.165</u>	<u>1.012 \pm 0.235</u>
0.344 \pm 0.154	0.341 \pm 0.085	0.177 \pm 0.044	0.283 \pm 0.106	0.392 \pm 0.097	0.270 \pm 0.075	0.142 \pm 0.036	0.208 \pm 0.082
0.448 \pm 0.110	0.441 \pm 0.150	0.383 \pm 0.077	0.669 \pm 0.149	1.288 \pm 0.850	2.933 \pm 1.404	0.213 \pm 0.059	0.312 \pm 0.072
0.659 \pm 0.178	0.674 \pm 0.179	0.705 \pm 0.166	0.750 \pm 0.152	1.106 \pm 0.217	0.564 \pm 0.154	0.478 \pm 0.116	0.607 \pm 0.175
<u>2.823 \pm 0.286</u>	<u>2.272 \pm 0.296</u>	<u>4.579 \pm 1.057</u>	<u>4.468 \pm 0.632</u>	<u>1.709 \pm 0.455</u>	<u>1.537 \pm 0.404</u>	<u>2.144 \pm 0.430</u>	<u>2.399 \pm 0.396</u>
0.702 \pm 0.139	0.548 \pm 0.099	0.926 \pm 0.154	0.958 \pm 0.123	0.403 \pm 0.111	0.463 \pm 0.114	0.437 \pm 0.059	0.601 \pm 0.099
<u>2.121 \pm 0.211</u>	<u>1.724 \pm 0.221</u>	<u>3.653 \pm 0.932</u>	<u>3.511 \pm 0.609</u>	<u>1.306 \pm 0.355</u>	<u>1.074 \pm 0.303</u>	<u>1.707 \pm 0.411</u>	<u>1.798 \pm 0.309</u>
5.455 \pm 1.081	6.231 \pm 2.088	5.831 \pm 1.319	5.391 \pm 0.812	3.534 \pm 0.751	2.797 \pm 0.533	2.182 \pm 0.432	5.235 \pm 1.808
3.569 \pm 0.470	3.607 \pm 0.928	4.624 \pm 1.328	4.176 \pm 0.850	3.186 \pm 0.630	2.616 \pm 0.494	2.065 \pm 0.407	3.397 \pm 0.617
2.515 \pm 0.955	2.623 \pm 1.584	1.931 \pm 0.727	1.620 \pm 0.457	0.697 \pm 0.143	0.363 \pm 0.082	0.233 \pm 0.052	2.941 \pm 1.693
<u>83.247 \pm 1.089</u>	<u>82.961 \pm 2.149</u>	<u>78.257 \pm 2.523</u>	<u>76.913 \pm 1.639</u>	<u>80.638 \pm 2.742</u>	<u>80.673 \pm 1.914</u>	<u>82.362 \pm 1.358</u>	<u>77.906 \pm 2.476</u>
0.098 \pm 0.021	0.161 \pm 0.043	0.221 \pm 0.116	0.093 \pm 0.025	0.271 \pm 0.038	0.227 \pm 0.026	0.298 \pm 0.028	0.379 \pm 0.135
0.072 \pm 0.017	0.159 \pm 0.032	1.090 \pm 0.633	0.147 \pm 0.031	1.226 \pm 0.585	0.154 \pm 0.041	0.113 \pm 0.029	1.160 \pm 0.657
0.310 \pm 0.044	0.210 \pm 0.042	0.366 \pm 0.080	0.240 \pm 0.048	0.439 \pm 0.044	0.325 \pm 0.043	0.327 \pm 0.049	0.316 \pm 0.056
0.343 \pm 0.070	0.399 \pm 0.153	0.214 \pm 0.080	0.444 \pm 0.089	0.220 \pm 0.072	0.166 \pm 0.040	0.191 \pm 0.088	0.165 \pm 0.032
5.793 \pm 1.150	5.770 \pm 1.196	3.422 \pm 0.648	5.552 \pm 1.391	4.419 \pm 0.846	4.622 \pm 0.865	3.785 \pm 0.647	4.059 \pm 0.535
58.969 \pm 1.674	56.569 \pm 2.882	56.358 \pm 3.411	57.307 \pm 1.264	59.302 \pm 2.508	61.337 \pm 1.969	62.224 \pm 1.365	58.531 \pm 2.376
2.184 \pm 1.144	3.202 \pm 2.330	0.509 \pm 0.132	0.729 \pm 0.133	0.432 \pm 0.057	0.512 \pm 0.124	0.644 \pm 0.092	0.597 \pm 0.088
0.682 \pm 0.085	0.677 \pm 0.093	0.486 \pm 0.119	0.284 \pm 0.092	0.659 \pm 0.095	0.588 \pm 0.067	0.719 \pm 0.062	0.577 \pm 0.083
<u>16.364 \pm 1.632</u>	<u>17.383 \pm 1.935</u>	<u>16.552 \pm 1.450</u>	<u>12.969 \pm 2.081</u>	<u>15.329 \pm 0.951</u>	<u>14.558 \pm 1.137</u>	<u>15.050 \pm 1.107</u>	<u>12.557 \pm 1.298</u>
3.437 \pm 0.345	3.312 \pm 0.561	3.899 \pm 0.326	5.482 \pm 0.741	7.722 \pm 1.588	7.304 \pm 1.273	7.945 \pm 1.116	7.526 \pm 1.033
0.100 \pm 0.045	0.067 \pm 0.025	0.057 \pm 0.014	0.039 \pm 0.012	0.138 \pm 0.048	0.130 \pm 0.044	0.178 \pm 0.044	0.154 \pm 0.048
2.282 \pm 0.206	2.430 \pm 0.410	3.027 \pm 0.299	4.211 \pm 0.635	5.912 \pm 1.336	5.435 \pm 0.899	6.050 \pm 0.932	5.464 \pm 0.790
0.120 \pm 0.029	0.178 \pm 0.034	0.157 \pm 0.038	0.228 \pm 0.059	0.283 \pm 0.054	0.196 \pm 0.045	0.227 \pm 0.044	0.179 \pm 0.033
0.104 \pm 0.028	0.059 \pm 0.015	0.164 \pm 0.050	0.198 \pm 0.069	0.336 \pm 0.084	0.309 \pm 0.082	0.163 \pm 0.035	0.139 \pm 0.027
0.405 \pm 0.269	0.097 \pm 0.025	0.114 \pm 0.024	0.118 \pm 0.039	0.224 \pm 0.058	0.215 \pm 0.043	0.250 \pm 0.085	0.672 \pm 0.427
0.467 \pm 0.068	0.589 \pm 0.102	0.503 \pm 0.102	0.693 \pm 0.167	0.864 \pm 0.090	1.020 \pm 0.192	1.130 \pm 0.124	1.060 \pm 0.218
<u>2.025 \pm 0.290</u>	<u>2.328 \pm 0.364</u>	<u>3.270 \pm 0.532</u>	<u>3.294 \pm 0.535</u>	<u>1.979 \pm 0.170</u>	<u>2.457 \pm 0.305</u>	<u>2.422 \pm 0.294</u>	<u>3.121 \pm 0.721</u>
0.024 \pm 0.009	0.031 \pm 0.008	0.034 \pm 0.006	0.062 \pm 0.021	0.028 \pm 0.006	0.035 \pm 0.009	0.018 \pm 0.004	0.021 \pm 0.007
0.047 \pm 0.012	0.073 \pm 0.016	0.033 \pm 0.006	0.083 \pm 0.025	0.068 \pm 0.017	0.064 \pm 0.014	0.074 \pm 0.016	0.095 \pm 0.020
1.071 \pm 0.198	1.185 \pm 0.204	0.855 \pm 0.163	0.826 \pm 0.147	0.772 \pm 0.141	0.835 \pm 0.194	1.387 \pm 0.269	2.323 \pm 0.697
0.165 \pm 0.043	0.169 \pm 0.030	0.237 \pm 0.055	0.491 \pm 0.279	0.300 \pm 0.039	0.185 \pm 0.044	0.163 \pm 0.028	0.216 \pm 0.034
0.757 \pm 0.130	0.932 \pm 0.173	1.381 \pm 0.687	0.744 \pm 0.158	0.621 \pm 0.075	0.786 \pm 0.121	0.743 \pm 0.168	0.568 \pm 0.098
0.076 \pm 0.018	0.088 \pm 0.027	0.896 \pm 0.338	1.099 \pm 0.398	0.235 \pm 0.115	0.636 \pm 0.334	0.045 \pm 0.013	0.056 \pm 0.013
<u>1.563 \pm 0.190</u>	<u>1.556 \pm 0.256</u>	<u>3.098 \pm 0.773</u>	<u>2.750 \pm 0.495</u>	<u>1.634 \pm 0.271</u>	<u>1.676 \pm 0.321</u>	<u>1.928 \pm 0.388</u>	<u>2.802 \pm 0.778</u>
0.247 \pm 0.026	0.249 \pm 0.046	0.499 \pm 0.114	0.361 \pm 0.073	0.235 \pm 0.045	0.231 \pm 0.062	0.352 \pm 0.094	0.215 \pm 0.047
0.373 \pm 0.108	0.245 \pm 0.046	0.504 \pm 0.146	0.695 \pm 0.274	0.322 \pm 0.144	0.237 \pm 0.051	0.318 \pm 0.120	0.199 \pm 0.039
0.104 \pm 0.040	0.107 \pm 0.055	0.280 \pm 0.171	0.093 \pm 0.033	0.086 \pm 0.023	0.107 \pm 0.034	0.104 \pm 0.058	0.067 \pm 0.011
0.484 \pm 0.140	0.388 \pm 0.224	0.514 \pm 0.154	0.831 \pm 0.531	0.635 \pm 0.276	0.676 \pm 0.372	0.624 \pm 0.276	2.216 \pm 0.814
0.415 \pm 0.172	0.629 \pm 0.208	1.364 \pm 0.606	0.769 \pm 0.224	0.357 \pm 0.146	0.467 \pm 0.157	0.542 \pm 0.198	0.468 \pm 0.151

■ TABLE 8 - PART 3

	h+54			
	HN		LN	
	Control	Herbivory	Control	Herbivory
	mean ± s.e.m.	mean ± s.e.m.	mean ± s.e.m.	mean ± s.e.m.
ACID ESTERS	4.764 ± 1.445	3.579 ± 0.995	3.189 ± 0.475	2.469 ± 0.401
Decanoic acid ester	0.835 ± 0.244	0.569 ± 0.090	0.630 ± 0.191	0.560 ± 0.059
Nonanoic acid ester	2.894 ± 1.557	1.547 ± 0.631	1.101 ± 0.304	1.017 ± 0.323
Octanoic acid ester	1.139 ± 0.223	1.463 ± 0.357	1.537 ± 0.275	0.891 ± 0.122
ALDEHYDES	3.818 ± 0.953	3.635 ± 0.827	9.017 ± 1.775	7.786 ± 1.792
Decanal	1.041 ± 0.171	0.900 ± 0.204	2.539 ± 0.562	2.226 ± 0.549
Nonanal	2.777 ± 0.832	2.735 ± 0.685	6.478 ± 1.237	5.560 ± 1.273
ALKANES	4.695 ± 0.718	3.587 ± 0.542	7.560 ± 1.194	4.664 ± 0.880
n-decane	4.340 ± 0.611	3.252 ± 0.430	7.307 ± 1.144	4.425 ± 0.847
Tetramethylhexane	0.710 ± 0.138	0.672 ± 0.152	0.674 ± 0.143	0.638 ± 0.133
MONOTERPENES	69.459 ± 2.356	73.977 ± 1.679	60.657 ± 5.714	67.959 ± 3.348
P-cymene	0.195 ± 0.035	0.267 ± 0.052	0.130 ± 0.023	0.143 ± 0.042
Ectocarpene	0.123 ± 0.037	0.097 ± 0.022	0.208 ± 0.073	0.125 ± 0.025
Allo-ocimene	0.211 ± 0.047	0.301 ± 0.061	0.226 ± 0.053	0.207 ± 0.055
α-ocimene	0.232 ± 0.094	0.287 ± 0.069	0.510 ± 0.275	0.280 ± 0.064
α-phellandrene	3.910 ± 0.677	5.145 ± 0.678	5.048 ± 1.026	4.977 ± 0.875
β-phellandrene	49.853 ± 2.983	53.346 ± 1.154	48.140 ± 4.556	50.403 ± 1.930
α-pinene	0.390 ± 0.054	0.501 ± 0.072	0.430 ± 0.087	0.312 ± 0.067
α-terpinene	0.299 ± 0.058	0.364 ± 0.074	0.429 ± 0.106	0.263 ± 0.082
Terpinolene	14.245 ± 1.391	13.822 ± 1.527	7.396 ± 2.527	11.970 ± 2.248
SESQUITERPENES	11.439 ± 1.714	11.373 ± 0.930	11.588 ± 3.141	9.759 ± 0.926
Allo-aromadendrene	0.230 ± 0.072	0.210 ± 0.037	0.118 ± 0.021	0.152 ± 0.033
β-caryophyllene	7.418 ± 1.047	8.443 ± 0.862	9.431 ± 2.914	7.160 ± 0.727
α-copaene	0.283 ± 0.071	0.354 ± 0.047	0.285 ± 0.057	0.314 ± 0.079
δ-elemene	0.408 ± 0.102	0.428 ± 0.084	0.308 ± 0.078	0.276 ± 0.083
α-gurjunene	2.131 ± 1.625	0.327 ± 0.047	0.398 ± 0.149	0.256 ± 0.048
Humulene	1.315 ± 0.172	1.651 ± 0.086	1.087 ± 0.184	1.667 ± 0.228
TERPENOID ALCOHOLS	2.289 ± 0.290	1.732 ± 0.163	3.421 ± 0.743	3.285 ± 0.925
Bergamiol	0.042 ± 0.011	0.060 ± 0.018	0.060 ± 0.017	0.049 ± 0.013
4-caranol	0.071 ± 0.016	0.058 ± 0.014	0.143 ± 0.049	0.105 ± 0.025
Trans-3-carene-2-ol	0.584 ± 0.063	0.566 ± 0.125	1.109 ± 0.437	1.169 ± 0.287
3-decyn-2-ol	0.378 ± 0.090	0.339 ± 0.055	0.564 ± 0.206	0.699 ± 0.408
Geraniol	0.593 ± 0.135	0.674 ± 0.106	0.900 ± 0.432	0.481 ± 0.081
Menthol	0.720 ± 0.226	0.082 ± 0.018	0.877 ± 0.156	0.815 ± 0.405
OTHERS	3.365 ± 0.445	1.999 ± 0.385	4.568 ± 0.789	4.079 ± 0.667
2,5-dimethyl-3,4-hexanediol	0.448 ± 0.076	0.509 ± 0.078	0.860 ± 0.260	0.512 ± 0.122
6,10-dimethyl-5,9-undecadien-2-one	0.747 ± 0.177	0.383 ± 0.106	1.229 ± 0.471	0.657 ± 0.140
4-methyl-1-decene	0.097 ± 0.023	0.061 ± 0.014	0.397 ± 0.169	0.196 ± 0.064
4-methylmannitol	0.612 ± 0.224	0.713 ± 0.321	0.997 ± 0.398	1.392 ± 0.466
Unknown	1.518 ± 0.632	0.422 ± 0.175	1.318 ± 0.449	1.784 ± 0.562

N	H	NxH	T
0.08287	0.51112	0.92356	< 0.0001
0.30908	0.17823	0.93247	< 0.0001
0.05682	0.80812	0.77597	0.00806
0.14901	0.17703	0.80730	< 0.0001
0.00118	0.07542	0.66565	< 0.0001
0.00530	0.06580	0.46720	< 0.0001
0.00098	0.09083	0.75660	< 0.0001
0.39121	0.02713	0.89327	< 0.0001
0.20145	0.00411	0.86166	< 0.0001
0.42768	0.21849	0.44466	0.00510
0.01586	0.39250	0.84141	< 0.0001
0.18608	0.16367	0.83722	0.00045
0.44330	0.33474	0.83877	0.00215
0.18614	0.32394	0.62425	< 0.0001
0.59179	0.91106	0.85812	0.00034
0.94806	0.46873	0.71677	< 0.0001
0.14887	0.42366	0.68867	< 0.0001
0.05572	0.32889	0.49467	0.00725
0.00101	0.92491	0.17168	< 0.0001
0.02435	0.72246	0.56484	0.00072
0.19685	0.02376	0.86579	< 0.0001
0.61718	0.23771	0.27549	0.00109
0.09351	0.13601	0.40708	< 0.0001
0.95931	0.17310	0.70031	< 0.0001
0.22000	0.69638	0.36462	< 0.0001
0.71075	0.45654	0.19619	0.04141
0.33317	0.00015	0.41370	< 0.0001
0.01349	0.63799	0.84319	0.00253
0.59317	0.60324	0.34956	0.35726
0.08344	0.06223	0.26347	0.05603
0.09427	0.29445	0.59102	0.00012
0.03232	0.77156	0.44424	0.00696
0.48743	0.82817	0.13651	0.44642
0.00667	0.59950	0.84033	< 0.0001
0.03237	0.54765	0.85649	< 0.0001
0.26767	0.22723	0.25645	< 0.0001
0.05962	0.66598	0.97503	< 0.0001
0.44716	0.04921	0.44568	0.32497
0.24296	0.33180	0.43100	0.00150
0.18887	0.32726	0.59884	0.02652



The background of the page is a repeating pattern of green leaves, likely mint, rendered in a detailed, engraved style. The leaves are scattered across the page, with some overlapping. A central white square with a black border contains the chapter title.

CHAPTER 6
**GENERAL
DISCUSSION**

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ENGLISH VERSION

1.1

REMINDER OF THE CHALLENGES AND OBJECTIVES OF MY THESIS

Over the last decade, agriculture has been blamed for the adverse effects of some agricultural practices, while there is an increasing need to optimize agricultural production due to the world population growth. How to reconcile environmental and ecological agricultural practices and agrosystem productivity? Public authorities in Europe and, in particular in France have implemented corrective measures to reduce the impact of agriculture on soils, aquifers, biodiversity and climate change. The ambition of the measures adopted by the National Environmental Health Plan (2009, Ecophyto 2018 and Ecophyto 2) is to reduce the use of crop protection products by 50 % by 2025. To achieve this very ambitious objective, we need to revolutionize practices and promote agro-ecology to design production practices based on the functionality offered by ecosystems. In this context, agronomic research is essential to find production alternatives that integrate biodiversity, landscape, mineral and water resources, while maintaining high productivity. It is necessary in some cases to invent new strategies since producers face technical dead-ends due to banning of chemicals with noticeable adverse effects. New knowledge on the interactions between plants and biotic / abiotic factors, is essential to offer producers alternatives to chemical inputs.

My Ph.D. work contributes to overcome these major challenges. It was aimed at identifying responses of the tomato against a bioagressor, *Tuta absoluta*, which causes very significant yield losses in an increasing number of countries around the world. We have varied the amount of nitrogen available to the plant for examining its responses. Nitrogen fertilization is a simple agronomic lever to implement with strong impact on the concentration of secondary metabolites involved in plant defence (Larbat *et al.*, 2012; Royer *et al.*, 2013). We have focused our study on resistance mechanisms (oxidizing activities, soluble and volatile defence compounds) and have quantified the impact of herbivory on simple plant growth traits (leaf area, R / S ratio, biomass ...). Having identified the interactions between the tomato and its bioagressor, one of the immediate issue of my Ph.D. was to evaluate the costs and benefits of tomato defence against *Tuta absoluta*, in interaction with the nitrogen fertilization. My results enable for the quantification of these costs for the plant and will be used, in future investigations conducted by the research team 'Secondary metabolites' of our department (LAE) for modelling the fluxes of carbon resources within the infested tomato.

SUMMARY OF THE RESULTS

In order to quantify the costs and benefits of defence, it is essential to precisely determine the physiological responses of the plant, to quantify the impact of aggression on growth traits and plant development, and to quantify the impact of plant's defences on the insect pest (traits of life, population dynamics). Original results on the resistance mechanisms of tomato to its herbivorous bioaggressor emerged from our experiments.

Tomatoes established herbivore-induced resistance resulting in an increase of the concentration of chlorogenic acid, caffeoyl putrescine and tomatine, locally at the level of the parazited zone, and / or systemically in the other non-infested foliar compartments. These three molecules are characteristic of tomatoes, but are also produced by a great diversity of plant species. These molecules are known to be elicited by herbivory, fungal or bacterial stress (Sempruch *et al.*, 2012; Wojciechowska *et al.*, 2014). In the laboratory, we showed that they were also elicited in tomato infected with pathogenic bacteria *Pseudomonas syringae* (Royer *et al.*, 2016); the response thus appears to be generic in the tomato defence. Our study brings the first evidence that *Tuta absoluta* stimulates the synthesis of caffeoyl putrescine in tomatoes. The role of these molecules in tomato defence against *Tuta absoluta* has yet to be defined. However, previous studies on grass herbivores

indicate that these three molecules reduce the palatability and digestibility of foliar tissues, slowing larval development (Isman and Duffey 1982, Stamp and Yang, 1996, Kaur *et al.*, 2010).

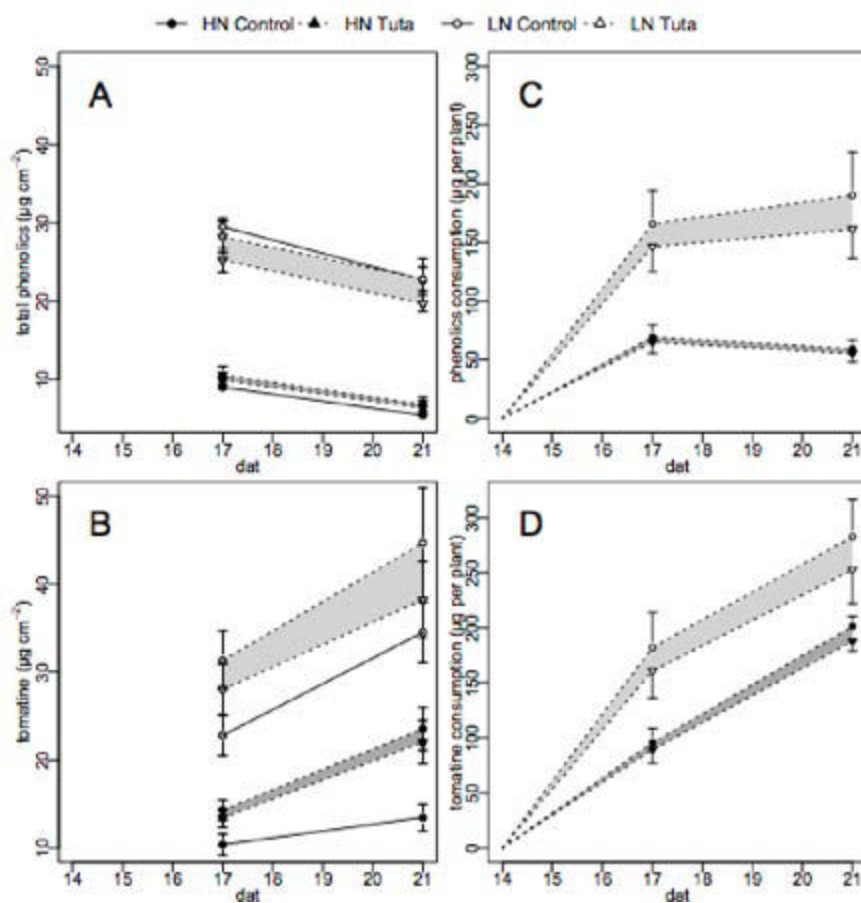
We have also shown that tomatoes set up an indirect defence against *Tuta absoluta*, consisting in the emission of volatile compounds in the vicinity of plants. We have identified several new VOCs synthesized in *Tuta absoluta* infested plants (m-cymene, menthatriene, β -cis-ocimene, β -phellandrene, terpinolene, β -caryophyllene, humulene). Among all the VOCs that we have identified, two monoterpenes, β -cis-ocimene and β -phellandrene, as well as two other sesquiterpenes, β -caryophyllene and humulene, are known to be emitted in greater quantities during herbivore attack (Degenhardt *et al.*, 2010, Irmisch *et al.*, 2014; Silva *et al.*, 2017). In addition, β -caryophyllene, β -cis-ocimene and humulene have been shown to attract predatory insects, parasitoids or repel insect with respect to the pest (De Moraes *et al.*, 2001, Pichersky and Gershenzon, 2002). Thus, their elicitation seems to be generic to herbivory and not specific to *Tuta absoluta*. The other compounds that we have identified are potentially specific to tomato - *Tuta absoluta* interaction, but only a more detailed analysis would enable us to confirm this specificity and to test the impact of these molecules on this pathosystem.

Moreover, the specificity of tomato - *Tuta absoluta* pathosystem can be due to the qualitative and quantitative composition of the odorous bouquet, even if it contains common volatile molecules. The specificity of tomato - *Tuta absoluta* interactions could be enhanced by further analyzes in metabolomics, transcriptomics (expression of RNAseq genes) and by investigating the induced signaling pathways.

Contrary to what was expected, our results showed small effects of nitrogen limitation on the observed responses. Nevertheless, as documented in literature, nitrogen restriction affects larval development. My work brings additional evidences to the “trophic” hypothesis of slowing larval development by nitrogen limitation. Thus, by strongly modifying the stoichiometry of the food bowl, the larvae have to consume more

matter to acquire the nitrogen necessary to their development. Nevertheless, we can not exclude the hypothesis of “defence compounds”. Indeed, we have shown that the concentrations of phenolic compounds and tomatine (Figure 38.A & B) and of the VOCs hypothetically involved in the constitutive defence, are stronger in the case of nitrogen limitation.

We estimate that the amount of defence compounds ingested by larvae within a period of 7 days (Figure 38.C & D). Larvae deposited on nitrogen-limited tomatoes, ingested nearly 3 times more phenolic compounds and 1.5 times more tomatine than the larvae deposited on nitrogen-rich plants. We can expect that this increased consumption of defence compounds has an impact on larval development.



■ **FIGURE 38** : DEFENCE COMPOUND DENSITY AND CONSUMPTION BY THE LARVAE.

Experimental conditions and symbols are as in Figure 23. [A], total phenolics concentration ($\mu\text{g cm}^{-2}$); [B], tomatine concentration ($\mu\text{g cm}^{-2}$); [C], phenolic consumption by larvae ($\mu\text{g per plant}$) and [D], tomatine consumption by larvae ($\mu\text{g per plant}$). 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 other hand 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 [C-D], these likely values were multiplied by the mine areas to calculate the corresponding likely resource consumptions by *Tuta absoluta* larvae, also shown as shaded areas (HN in dark, LN in light). Phenolics and tomatine consumptions responded significantly to nitrogen treatments ($P < 0.001$).

CRITICAL ANALYSIS OF THE EXPERIMENTAL STRATEGY

The methodological choices made during my thesis allowed us to evidence original behaviors based on new data (see previous section), which contribute to enrich our knowledge on the interactions between tomato and *Tuta absoluta*, depending on the environment. It is worth recalling here the relevance of these choices, but also their limitations. Our choice of tomato-*Tuta absoluta* pathosystem was based on the following arguments. *Tuta absoluta* is a tomato pest in full expansion and difficult to control in crops. This positions our work in an agro-economic reality offering new options for bio-control. Moreover, the feeding guild of this pest is little documented in the scientific literature compared to the chewing or piercing insects, whereas it has been shown that leafmining does not activate the same mechanisms of defence in plants. Indeed, Kawazu *et al.* (2012) showed that the *Liriomyza sativae* leafminer sequentially induces in tomatoes salicylic acid defence pathways, known to be activated by piercing insects, and jasmonic acid defence pathways, known to be activated by chewing insects. In *Arabidopsis*, the leafminer *Liriomyza huidobrensis* induces a response profile similar to the one obtained by the *Pieris rapae*,

a chewing larva, when considering signaling pathways; but at the same time closer to the one generated by the bacteria *Pseudomonas syringae* considering the secondary metabolites (Zhang *et al.*, 2013). Thus, it has been observed that the cascade of events in plant subjected to *Tuta absoluta* is poorly understood and may involve several phytohormones.

Our choice to directly deposit larvae on tomato leaf and not eggs, as what naturally happens, was justified by the need of repeatability during experimentation. Indeed, experience gained by our team says that it is not possible to ensure sufficient repeatability if the eggs are manually deposited because of their small size and fragility (personal communication). This method of deposition may have an impact on the response of tomato against *Tuta absoluta*, as it has been shown in *Arabidopsis* following the oviposition of *Pieris rapae* (Little *et al.*, 2007), and oviposition amplified the plant response to herbivory for the pathosystem *Nicotiana attenuata* / *Spodoptera exigua* (Bandoly *et al.*, 2015). We made the choice for Better Bush dwarf tomato cultivar which allowed us working with more individuals and therefore more replications during

our experiments, using culture chambers.

Moreover, this cultivar has been used for several years in the laboratory, it has been well characterized in terms of its phytochemistry and its response to nitrogen limitation (Larbat *et al.*, 2012, 2014). This cultivar is nevertheless quite different from industrial production varieties, which would need to validate our results on this aspect. Nevertheless, the recent work by Han *et al.* (2016) seems to show that the observed effects of nitrogen restriction on the development of *Tuta absoluta* are not dependent on the cultivar.

On the one hand, we sought to optimize the control of all parameters during plant growth and development throughout the experiments. Thus, the nutrition regulation of plants enabled optimal and reliable nitrogen concentrations in the nutrient solutions. On the other hand, we have faced troubles with the functioning of culture chambers, which did not make it possible to make a complete evaluation of all the experiments that were carried out.

Experiments were conducted under controlled environmental conditions. However, we are aware that these conditions are somewhat far from the

agronomic and climatic reality. Reproducing at the lab scale multi-trophic interactions (plant - insect herbivores - insect predators - microorganisms - climate) would be very interesting but experimentally unrealistic. Thus, we focused our investigation on short- and medium-term responses (a few hours to a few days after the larvae were deposited), which are reasonable periods of time for monitoring resistance and tolerance responses. A survey over longer time scales would allow a better understanding of resistance and tolerance mechanisms on plant development, fruit yield and insect attraction or repulsion.

A promising path for future research would be to investigate the pathosystem in more complex environments by integrating bacterial or fungal microorganisms. Infestation would be carried out sequentially, or in series, to observe the effect of microorganism on the tomato defences against *Tuta absoluta* (control strategy by natural defence stimulation).

OUTLOOK

The biological material that we produced under well defined conditions during my Ph.D. has been characterized by analysing a selection of physiologic and biochemical descriptors. However, we have preserved samples for additional analysis by our team.

1.4.1

NON-TARGETED ANALYZES OF TOMATO RESPONSES TO *Tuta absoluta* HERBIVORY

In this context, analyzes are currently carried out on samples to identify :

- a greater diversity of defence molecules whose concentration is mediated by the herbivory of *Tuta absoluta* and / or by nitrogen fertilization ;
- the metabolic pathways regulated at the transcriptome scale during the aggression of *Tuta absoluta* (RNAseq data).

1.4.2

TOLERANCE OF TOMATO IN RELATION TO *Tuta absoluta*

Besides the resistance mechanisms, we also intended to evaluate the plasticity of tomatoes to *Tuta absoluta* by the study of some tolerance mechanisms (leaf loss compensation by photosynthesis stimulation, modification of carbon resource distribution patterns and / or biochemical compartments of reserve). We have carried out the corresponding experiments, but experimental uncertainties have not made it possible to make a coherent and robust presentation in the manuscript. Nevertheless, some of the results obtained are important to mention here as they provide some initial features on the ability of tomato to react with *Tuta absoluta*.

Carbon assimilation was investigated by precise measurements of CO₂ influx (assimilation during photoperiods) and efflux (respiration at night) at the scale of plant shoot, immediately after infestation and for a period of three days. The carbon

partitioning was investigated by determination of dry masses partitioning, RGR, and by the investment of carbon in biochemical compartments.

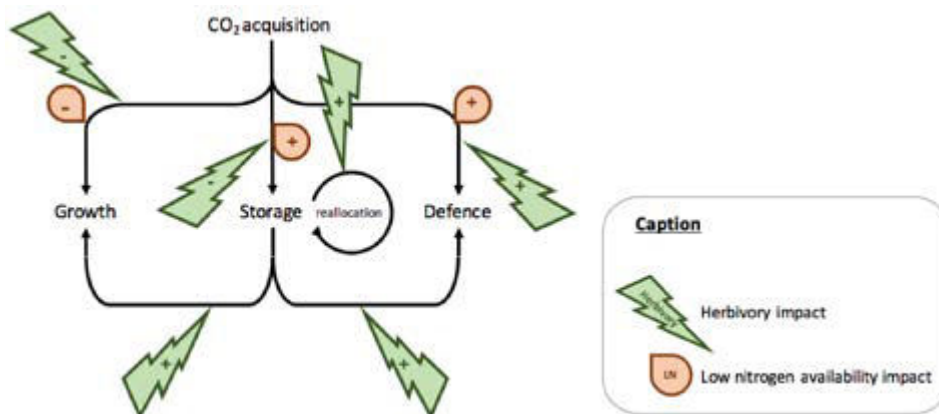
We have shown that tomato attacked by *Tuta absoluta* modifies the distribution of its biomass. It is not new to observe changes in the distribution of biomass due to herbivorous stress, particularly between the aerial and the root parts (Mihaliak and Lincoln, 1989). However, we have shown that infested tomato favors the allocation of its biomass to the growth of the young leaves with respect to the older leaves, which is an original result. In addition, we showed that *Tuta absoluta* herbivory does not disturb the rate of CO₂ acquisition, even if the leaf loss eventually leads to a reduction in global CO₂ acquisition. Tomato therefore does not modify its photosynthetic capacity. This result is similar to what was obtained on tomato attacked by the tobacco sphinx, a chewing caterpillar (Welter, 1991),

contrarily to common observations in plants under biotic stress (Bilgin *et al.*, 2010) and especially in tomatoes subjected to methyl-jasmonate treatment (Gomez *et al.*, 2010). In addition, we have shown that the resource feedstock measured by starch concentration, is lower in the infected leaflets than in its homologue in healthy plants. At this stage, we can not conclude on the use of this resource, but we propose several hypotheses: (i) a carbon limitation in attacked leaf might result

from an export of carbon resources to the growing areas (observed through a higher proportion of biomass in growing areas than in older ones), (ii) the plant would use this resource to increase its defence level. Indeed, starch can be remobilized by the activation of hydrolases in plants under sucrose starvation due to some herbivore stress (Lu *et al.*, 2007). Additional assays on simple glucosides, amino acids and proteins are in progress.

1.4.3

TOWARDS A MECHANISTIC MODEL OF TOMATO - *Tuta absoluta* INTERACTIONS



■ **FIGURE 39 :**
SIMPLIFIED MODEL
OF THE IMPACT OF
HERBIVORY AND
NITROGEN LIMITATION
ON PLANT RESOURCES
DISTRIBUTION
BETWEEN GROWTH /
STORAGE / DEFENCE.

My results provides data for feeding a model describing the reactive defence as a function of nitrogen nutrition. The interest of such a model based on all the obtained results would result in an accurate simulation of resource partitioning between major functions (growth, defence, storage). This model requires megadata on the acquisition of resources and on the intra-plant distribution of the primary molecules (carbohydrates, amino acids ...), defence molecules and enzymatic activities. My data enable to develop a first, very simple, model (Figure 39). Nitrogen restriction induces changes in the equilibrium of resource partitioning. In attacked plants, the storage of the carbonaceous resources as well as the defence metabolism are favored with respect to the growth of the plant. Herbivory causes changes in the distribution of resources. The growth of young leaves is favored with respect to the older ones. Newly acquired resources are dedicated to defence pathways. The stored resources are delocalized to healthy areas of the plant and / or used for the direct or indirect defence of the plant.

TOWARDS A BIOCONTROL SOLUTION

We have shown that the herbivory of *Tuta absoluta* induces the synthesis of some defence metabolites stored in leaves or emitted in the air. A continuation of my Ph.D. work would consist in testing the effects of each one of these molecules, individually or as a mixture, on the development and mortality of *Tuta absoluta* larvae, with the objective of formulating a biopesticide against this pest.

Moreover, it seems possible to test volatile compounds on the attraction of predatory insects and on the repulsion of *Tuta absoluta* females. These findings could lead to the development of a new method for biocontrol similar to sexual confusion, but resulting in pest repulsion or predators attraction.

RAPPEL DES ENJEUX ET OBJECTIFS DE MA THÈSE

Depuis une dizaine d'années, l'agriculture est pointée du doigt en raison des effets néfastes de certaines pratiques agricoles alors que les besoins d'optimiser la production agricole sont croissants du fait de l'augmentation de la population mondiale. Les pouvoirs publics en Europe et en France, en particulier, ont mis en œuvre des mesures correctives visant à réduire l'impact de l'agriculture sur la biodiversité et le changement climatique. L'ambition des mesures adoptées par le Plan National Santé Environnement (2009 ; Ecophyto 2018 et Ecophyto II) est de réduire de 50 % l'utilisation des produits phytosanitaires sur les cultures, d'ici 2025. Pour être atteignable, cet objectif très ambitieux nécessite de révolutionner les pratiques et de promouvoir l'agro-écologie pour concevoir des systèmes de production s'appuyant sur les fonctionnalités offertes par les écosystèmes. Dans ce cadre, la recherche agronomique est indispensable pour trouver des alternatives de pratiques agricoles qui intègrent la biodiversité, le paysage et les ressources minérales, tout en maintenant un objectif de production élevée. Il faut dans certains cas, développer de nouvelles stratégies, car les producteurs se trouvent devant des impasses techniques, du fait de l'interdiction de certains produits phytosanitaires. De nouvelles connaissances sur les interactions entre les plantes et leurs environnements biotiques et abiotiques, sont indispensables pour proposer aux producteurs

des alternatives aux intrants chimiques.

Au sein de ces grands enjeux, mes travaux de thèse visaient à identifier des réponses de la tomate à l'herbivorie de *Tuta absoluta*, un bioagresseur qui cause des pertes de rendements très importantes dans plus en plus de pays, dans le monde. Nous avons fait varier la disponibilité en azote pour la plante afin d'examiner les conséquences sur ses réponses. La fertilisation en azote est un levier agronomique simple à mettre en œuvre et dont la limitation influence les concentrations en métabolites secondaires impliqués dans la défense des plantes (Larbat *et al.*, 2012). Notre étude a ciblé des mécanismes de résistance (activités enzymatiques oxydantes, composés de défense solubles et volatils) et permis de quantifier l'impact de l'herbivorie sur des traits simples de croissance de la plante (surfaces foliaires, ratio R/S, biomasses...). Après avoir identifié les interactions entre la tomate et son bioagresseur, un des enjeux immédiats de ma thèse est d'établir les coûts et bénéfices de la défense de la tomate vis-à-vis de *Tuta absoluta*, en fonction de la fertilisation azotée de la plante. Mes résultats vont permettre de quantifier le coût de cette défense et ils seront utilisés dans la démarche de l'équipe de recherche 'Métabolites Secondaires' de notre unité dont un des objectifs est de modéliser le fonctionnement de la plante agressée.

SYNTHÈSE DES RÉSULTATS

Pour pouvoir quantifier les coûts et les bénéfices de la défense, il est indispensable de déterminer précisément les réponses physiologiques de la plante, de quantifier les impacts de l'agression sur les traits de croissance et développement de la plante, et de quantifier l'impact de la mise en place des défenses de la plante sur l'insecte ravageur (traits de vie, dynamique des populations). Nos expérimentations ont permis d'obtenir des résultats souvent originaux sur des mécanismes de résistance de la tomate à son bioagresseur herbivore.

La plante de tomate met en place une résistance induite par l'herbivore, se traduisant par une augmentation de la concentration en acide chlorogénique, caffeoyl putrescine et tomatine localement au niveau de la zone parasitée et /ou de manière systémique dans les autres compartiments foliaires non infestés. Ces trois molécules sont caractéristiques de la tomate, mais sont aussi produits par une grande diversité d'espèces. Ces molécules sont connues pour être synthétisées dans le cas d'un stress herbivore, fongique ou bactérien (Sempruch *et al.*, 2012 ; Wojciechowska *et al.*, 2014). Au laboratoire, nous avons montré qu'elles étaient également élicitées chez la tomate infectée par la bactérie pathogène *Pseudomonas syringae* (Royer *et al.*, 2016) ; elles apparaissent donc génériques de la défense de la tomate. Notre étude est la 1^{ère} montrant que *Tuta absoluta* stimule la synthèse de caffeoyl putrescine chez la tomate. Le rôle de ces molécules dans la défense de la tomate vis-à-vis de *Tuta absoluta* doit encore

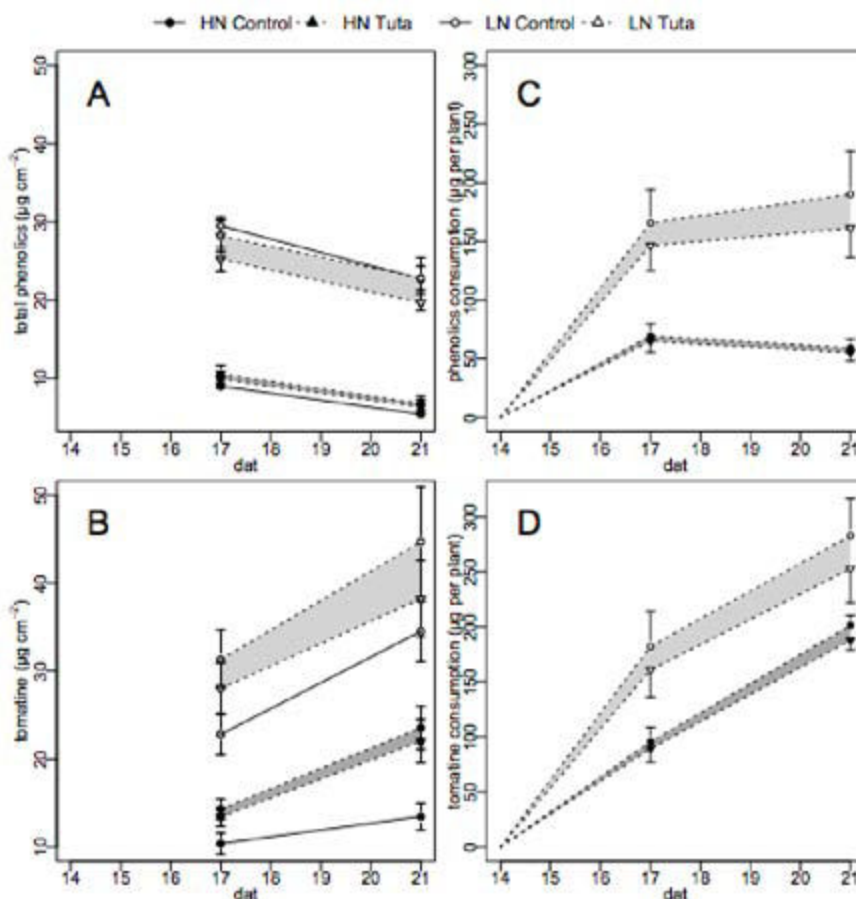
être confirmé. Néanmoins, des études précédentes portant sur des herbivores broyeurs, indiquent que ces trois molécules réduiraient l'appétence et la digestibilité des tissus foliaires, ralentissant alors le développement des larves (Isman et Duffey, 1982 ; Stamp et Yang, 1996 ; Kaur *et al.*, 2010).

Nous avons montré aussi que la tomate met en place une défense indirecte contre *Tuta absoluta*, basée sur l'émission de composés volatils au voisinage des plantes. Nous avons identifié plusieurs nouveaux COVs synthétisés lors de l'herbivorie par *Tuta absoluta* (m-cymène, menthatriène, β -cis-ocimène, β -phellandrene, terpinolène, β -caryophyllène, humulène). Parmi tous les COVs que nous avons identifiés, les monoterpènes β -cis-ocimène et β -phellandrène, ainsi que les sesquiterpènes β -caryophyllène et humulène sont connus pour être émis en plus grande quantité lors d'une attaque herbivore (Degenhardt *et al.*, 2010 ; Irmisch *et al.*, 2014 ; Silva *et al.*, 2017). De plus, le β -caryophyllène, le β -cis-ocimène et l'humulène ont un rôle attractif pour les insectes prédateurs et les parasitoïdes ou répulsif contre les insectes ravageurs (De Moraes *et al.*, 2001 ; Pichersky et Gershenzon, 2002). Ainsi, leur élicitation semble générique de l'herbivorie et pas spécifique de *Tuta absoluta*. Les autres composés que nous avons identifiés sont potentiellement spécifiques de l'interaction tomate - *Tuta absoluta* mais seule une analyse plus fouillée nous permettrait de tester cette spécificité et de tester l'impact de ces molécules sur ce pathosystème. Par ailleurs, la spécificité du pathosystème tomate - *Tuta absoluta* peut tenir à la composition qualitative et

quantitative du bouquet odorant même si celle-ci renferme des molécules communes. La spécificité des interactions tomate - *Tuta absoluta* pourrait être renforcée par des analyses plus poussées en métabolomique, transcriptomique (expression de gènes RNAseq) et en s'intéressant aux voies de signalisation induites.

Contrairement à ce qui était attendu, nos résultats montrent peu d'effets de la limitation en azote sur les réponses observées. Néanmoins, comme ce qui a été démontré précédemment, la limitation azotée impacte le développement larvaire. Mes travaux ont permis de renforcer l'hypothèse "trophique" du ralentissement du développement larvaire par la limitation en azote. Ainsi, la stoechiométrie du bol alimentaire est fortement modifiée et les larves doivent consommer plus de matières pour acquérir l'azote nécessaire à leur développement. Néanmoins nous ne pouvons pas

écarter l'hypothèse "composés de défense" . En effet, nous avons montré que les concentrations en composés phénoliques et en tomatine (Figure 40.A & B) et des COVs supposés être impliqués dans la défense constitutive sont plus fortes en cas de limitation en azote. Nous avons estimé la quantité en composés de défense que les larves ont ingérés au cours des 7 jours (Figure 40.C & D), à partir des concentrations mesurées et des surfaces foliaires (surface totale des feuilles et surface des mines). Les larves déposées sur les tomates limitées en azote ont ingéré près de 3 fois plus de composés phénoliques et 1.5 fois plus de tomatine que les larves déposées sur les plants bien pourvus en azote. Il est probable que cette consommation accrue en ces composés a impacté le développement larvaire.



■ **FIGURE 40** : DENSITÉ EN COMPOSÉS DE DÉFENSE ET CONSOMMATION DES COMPOSÉS DE DÉFENSE PAR LES LARVES.

Les conditions expérimentales et les symboles sont donnés dans la Figure 25, page 101. [A], concentration en composés phénoliques totaux ($\mu\text{g}\cdot\text{cm}^{-2}$); [B], concentration en tomatine ($\mu\text{g}\cdot\text{cm}^{-2}$); [C], la consommation des composés phénoliques par les larves (μg par plante) and [D], consommation de tomatine par les larves (μg par plante). Chez les plants infestés, les paramètres ont été calculés à partir de deux hypothèses extrêmes considérant soit que les mines ne contiennent aucune ressource (*i.e.* que les concentrations sont nulles) soit que les mines contiennent autant de ressources que les parties vertes de la feuille. Ces calculs (lignes en pointillés) délimitent les valeurs de concentration les plus probables (aires grisées, gris foncé pour HN, gris clair pour LN). [C-D], ces valeurs probables ont été multipliées par la surface des mines afin de calculer la consommation de ressource par *Tuta absoluta*, la plus probable. De même, cette consommation est indiquée à l'aide de zones grisées (HN: gris foncé et LN: gris clair). La consommation de composés phénoliques et de tomatine répond de manière significative au traitement azoté ($P < 0.001$).

ANALYSE CRITIQUE DE LA STRATÉGIE EXPÉRIMENTALE

Les choix méthodologiques opérés au cours de ma thèse, ont permis de mettre en évidence un certain nombre de données nouvelles, résumées dans la section précédente, qui contribue à enrichir nos connaissances sur les interactions plantes herbivores, en interaction avec l'environnement. Il convient ici de rappeler l'intérêt de ces choix, mais aussi leurs limites. Le choix du pathosystème tomate - *Tuta absoluta* nous est apparu pertinent à plusieurs titres. *Tuta absoluta* est un ravageur de la tomate en pleine expansion et difficile à contrôler dans les cultures ce qui positionne ces travaux dans une réalité agro-économique avec la possibilité d'offrir des pistes de recherches pour le bio-contrôle de ce ravageur. De plus, le mode de nutrition de ce ravageur est peu documenté dans la littérature scientifique comparé aux insectes broyeur ou piqueurs, alors qu'il est démontré qu'il n'active pas les mêmes mécanismes de défense chez la plante. En effet, chez la tomate, Kawazu *et al.* (2012) ont montré que la mouche mineuse *Liriomyza sativae* engendre séquentiellement une activation des voies de défense de l'acide salicylique, connues pour être activées par les insectes piqueurs, et des voies de défense de l'acide jasmonique, connues pour être activées par les insectes broyeurs. Chez *Arabidopsis*, la mouche

mineuse *Liriomyza huidobrensis*, induit un profil de réponse à la fois proche de celui engendré par la larve broyeur *Pieris rapae* si on considère les voies de signalisation, mais en même temps plus proche de celui engendré par la bactérie *Pseudomonas syringae* si on considère les métabolites secondaires (Zhang *et al.*, 2013). Ainsi, on a fait le constat que la cascade d'évènements dans la plante soumise à l'herbivorie par *Tuta absoluta* n'est pas connue et que potentiellement, plusieurs phytohormones interviennent.

Notre choix de mettre les tomates directement en présence des larves de *Tuta absoluta* et non pas des œufs, comme ce qui se passe de manière naturelle, s'est justifié par le besoin de répétabilité lors des différentes expérimentations. En effet, l'expérience de l'équipe a montré qu'on ne peut pas envisager un taux de répétabilité suffisant des expérimentations si l'on dépose manuellement les œufs, du fait de leur faible taille et de leur fragilité (communication personnelle). Ce mode de dépôt pourrait avoir un impact sur la réponse de la tomate à *Tuta absoluta*, comme cela a été montré chez *Arabidopsis* suite à l'oviposition de *Pieris rapae* (Little *et al.*, 2007), mais également chez la pathosystème *Nicotiana attenuata* / *Spodoptera exigua* où il a été montré que l'oviposition avait pour effet d'amplifier la réponse

de la plante à l'herbivorie de la larve (Bandoly *et al.*, 2015).

Nous avons fait le choix du cultivar de tomate nain Better Bush qui nous permettait de travailler avec plus d'individus et donc plus de répétitions, lors de nos expérimentations en chambre de culture. Par ailleurs, ce cultivar est utilisé depuis plusieurs années au laboratoire et a été bien caractérisé sur le plan de sa phytochimie et de sa réponse à la limitation de la fertilisation azotée (Larbat *et al.*, 2012, 2014). Ce cultivar est néanmoins assez différent des variétés de production, ce qui nécessiterait de valider nos résultats en considérant ces dernières. Néanmoins, les travaux récents de Han *et al.* (2016) semblent montrer que l'effet observé de la limitation azotée sur le développement de *Tuta absoluta* ne dépend pas du cultivar.

Nous avons cherché à optimiser le contrôle de tous les paramètres de croissance et de développement des plantes tout au long des expérimentations. Ainsi, la régulation de la nutrition de la plante a permis d'obtenir des concentrations optimales et fiables d'azote dans les solutions nutritives. En revanche, nous avons rencontré des aléas de fonctionnement des chambres de culture, ce qui n'a pas permis de valoriser toutes les expérimentations réalisées.

Nous avons mené nos expérimentations dans

des conditions environnementales contrôlées. Cependant, nous sommes conscients que nos conditions de culture sont éloignées de la réalité agronomique et climatique. La reproduction en laboratoire des interactions multi-trophiques (plante-agresseurs-microorganismes-auxiliaires-climat) serait très intéressante mais expérimentalement irréaliste. Enfin, nous nous sommes intéressés aux réponses à court et moyen termes (quelques heures à quelques jours après le dépôt des larves), délais connus pour l'établissement de la résistance et de la tolérance. Une étude intégrant des échelles de temps plus longues permettrait de mieux appréhender les conséquences des mécanismes de résistance et de tolérance sur le développement de la plante, le rendement en fruit et sur l'attraction ou la répulsion d'insectes.

Une voie prometteuse de recherche consisterait à complexifier le pathosystème en intégrant un micro-organisme bactérien ou fongique, dont l'infestation serait réalisée de manière séquentielle ou en série pour observer l'effet du micro-organisme sur les défenses de la tomate vis-à-vis de *Tuta absoluta* (stratégie de lutte par stimulation des défenses naturelles).

PERSPECTIVES

Le matériel biologique que nous avons produit sous des conditions expérimentales bien définies, au cours de ma thèse, a été caractérisé par des analyses physiologiques et biochimiques. Cependant, nous avons préservé des échantillons pour des analyses supplémentaires pour notre équipe.

2.4.1

ANALYSES NON-CIBLÉES DES RÉPONSES DE LA TOMATE À L'HERBIVORIE DE *Tuta absoluta*

Dans ce cadre, des analyses sont en cours pour identifier :

- une plus grande diversité de molécules de défense dont la concentration répond à l'herbivorie de *Tuta absoluta* et / ou à la limitation azotée ;
- les voies métaboliques régulées à l'échelle du transcriptome lors de l'agression de *Tuta absoluta* (données RNAseq).

2.4.2

TOLÉRANCE DE LA TOMATE VIS-À-VIS DE *Tuta absoluta*

Au-delà de ces mécanismes de résistance, nous avons aussi l'intention d'évaluer la plasticité de la tomate à l'agression par *Tuta absoluta* par l'étude de mécanismes de tolérance (compensations de pertes de surfaces foliaires par stimulation de photosynthèse, modification des schémas de répartition des ressources carbonées vers des organes hors d'atteinte du bioagresseurs et / ou dans des compartiments biochimiques de réserves). Nous avons mis en œuvre des expérimentations dans ce cadre mais des aléas expérimentaux n'ont pas permis d'en faire une présentation cohérente et robuste dans le manuscrit de thèse. Néanmoins, certains des résultats acquis sont importants à mentionner ici, car ils apportent des premiers éléments sur la capacité de la tomate à réagir vis-à-vis de *Tuta absoluta*.

L'assimilation du carbone a été étudiée par des

mesures précises d'afflux de CO₂ (assimilation pendant les photopériodes) et d'efflux (respiration nocturne) à l'échelle de la partie aérienne, immédiatement après l'infestation et pendant 3 jours. La répartition du carbone a été étudié par détermination de la répartition de la biomasse, du taux relatif de croissance et par l'investissement de carbone dans les compartiments biochimiques.

Nous avons mis en évidence que la tomate agressée par *Tuta absoluta* modifie la répartition de sa biomasse. Il n'est pas nouveau d'observer des modifications dans la répartition de la biomasse suite à un stress herbivore, notamment entre la partie aérienne et la partie racinaire (Mihaliak et Lincoln, 1989), cependant, nous avons montré que la tomate agressée tend à favoriser l'allocation de sa biomasse à la croissance des jeunes feuilles au lieu des feuilles plus âgées, ce qui est un résultat

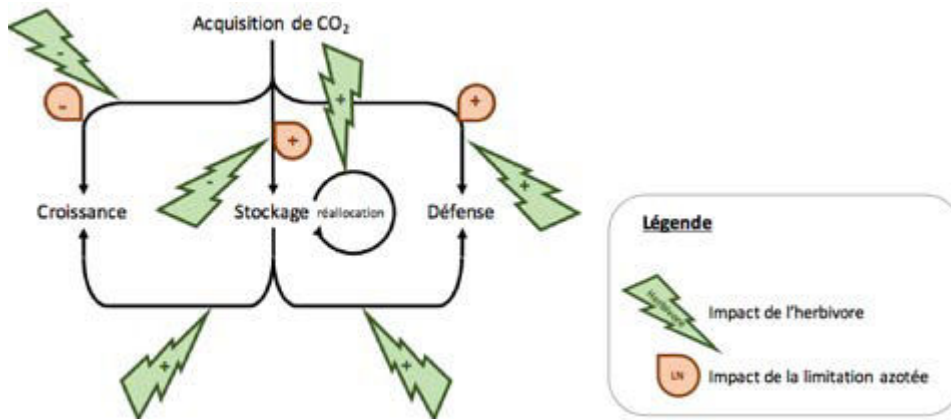
innovant. De plus, nous avons mis en évidence que l'herbivorie de *Tuta absoluta* ne perturbait pas le taux d'acquisition du CO₂ de la plante, même si la perte de surface foliaire engendre finalement une baisse de l'acquisition de CO₂. La plante de tomate ne modifie donc pas sa capacité photosynthétique et ce résultat est similaire à celui de la tomate agressée par le sphinx du tabac, une chenille broyeurse (Welter, 1991), mais il va à l'encontre de ce qui est "généralement" observé chez les végétaux sous un stress biotique (Bilgin *et al.*, 2010) et notamment chez la tomate soumise à un traitement de méthyl-jasmonate (Gomez *et al.*, 2010).

De plus, nous avons montré que la concentration en amidon est plus faible dans les folioles infestées par *Tuta absoluta* que leurs homologues dans la plante saine. À ce stade, nous ne pouvons pas

conclure sur l'utilisation de cette ressource, mais nous faisons plusieurs hypothèses : (1) une limitation du carbone dans les feuilles attaquées résulte d'une exportation des ressources en carbone vers les zones de croissance (observé à travers une plus grande proportion de la biomasse allouée aux zones de croissance par rapport aux zones plus âgées) (2) la plante utilise cette ressource pour augmenter son niveau de défense. En effet, l'amidon peut être remobilisée par l'activation d'hydrolases chez les plantes en carence glucodique liée à l'herbivorie (Lu *et al.*, 2007). Des dosages complémentaires des sucres simples, des acides aminés et des protéines sont en cours de réalisation. Ces résultats complémentaires nous permettrons d'avancer des hypothèses sur le compromis croissance-défense et sur la protection des ressources de la plante.

2.4.3

VERS UNE MODÉLISATION MÉCANISTE DES INTERACTIONS TOMATE - *Tuta absoluta*



■ **FIGURE 41 :**
MODÈLE SIMPLIFIÉ
DE L'IMPACT DE
L'HERBIVORIE ET
DE LA LIMITATION
AZOTÉE SUR LA
REPARTITION DES
RESSOURCES ENTRE
LA CROISSANCE /
LE STOCKAGE / LA
DÉFENSE.

Mes résultats apportent de nombreuses données qui permettront d'alimenter un modèle décrivant le fonctionnement de la défense réactive à la nutrition azotée. L'intérêt de ce modèle sera d'assembler les résultats obtenus et faire des simulations sur le partage des ressources entre des fonctions majeures (croissance, défense, mise en réserve). Cette modélisation nécessite des mégadonnées sur l'acquisition des ressources et sur la répartition intra-plante des molécules primaires (glucides, acides aminés...), des molécules de défense et des activités enzymatiques. Mes données permettront d'élaborer un premier modèle très simple (Figure 41). La limitation azotée induit des

changements dans l'équilibre du partage des ressources. Chez les plantes attaquées, le stockage des ressources carbonées ainsi que le métabolisme de défense sont favorisés par rapport à la croissance de la plante. L'herbivorie engendre des modifications dans la distribution des ressources. La croissance des étages foliaires situés au-dessus de l'attaque sera favorisée par rapport aux étages inférieurs. Les ressources nouvellement acquises sont détournées pour la défense. Les ressources stockées sont délocalisées vers des zones saines de la plante et / ou utilisées pour la défense directe ou indirecte de la plante.

2.4.4

VERS UNE SOLUTION DE BIOCONTRÔLE

Nous avons mis en évidence que la synthèse de certains composés de défense, présents dans les feuilles ou émis dans l'air, était élicitée par l'herbivorie de *Tuta absoluta*. Une poursuite de mon travail de thèse sera donc de tester l'effet de chacune de ces molécules ou en mélange sur le développement et la mortalité des larves de *Tuta absoluta*, dans l'objectif de formuler un biopesticide à l'encontre de ce ravageur.

De plus, il serait possible de tester des composés volatils sur l'attraction d'insectes prédateurs et sur la répulsion des femelles pondueuses de *Tuta absoluta*. Ces résultats pourraient permettre la création d'une nouvelle méthode de lutte biologique dont la forme serait similaire aux méthodes de confusion sexuelle, mais dont le rôle serait de faire fuir les ravageurs ou d'attirer des prédateurs.

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PUBLICATION LIST

This thesis enabled to write and to present the following papers and communications :

PUBLISHED ARTICLE

Coqueret V., J. Le Bot, R. Larbat, N. Desneux, C. Robin and S. Adamowicz. "Nitrogen Nutrition of Tomato Plant Alters Leafminer Dietary Intake Dynamics." *Journal of Insect Physiology*, April 2017.

ARTICLES TO BE SUBMITTED

Coqueret V., R. Larbat, J. Le Bot, S. Adamowicz, N. Desneux and C. Robin. "Spatiotemporal defence of tomato against the leafminer *Tuta absoluta* under two nitrogen fertilization" Chapter 4.

Coqueret V., S. Unsicker, R. Larbat, J. Le Bot, S. Adamowicz and C. Robin. "Nitrogen limitation alters the tomato volatile blend attacked by *Tuta absoluta* leafminer" Chapter 5.

Coqueret V., R. Larbat, J. Le Bot, S. Adamowicz, N. Desneux and C. Robin. "Tomato carbon uptake and distribution in response to *Tuta absoluta* leafmining, under two nitrogen conditions" Discussion.

ORAL COMMUNICATIONS

Robin C., V. Coqueret, R. Larbat and J. Le Bot. « Fonctionnement de la plante sous contraintes biotiques : vers un nouvel équilibre fonctionnel ? », Séminaire : Fonctionnement de la plante sous contraintes biotiques, Avignon FR, October 2, 2014.

Coqueret V. "Impact de l'herbivorie de *Tuta absoluta*

sur les défenses de la tomate", 5ème rencontre MétaSpé, Nancy FR, January 21 and 22, 2016.

Communications for the general public

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RÉSUMÉ

FONCTIONNEMENT DE LA PLANTE AGRESSÉE:

ETUDE DU PATHOSYSTÈME TOMATE - *Tuta absoluta*
EN INTERACTION AVEC LA FERTILISATION AZOTÉE

L'identification des réponses écophysiological des plantes est un préalable pour améliorer les défenses naturelles des plantes et réduire l'usage d'intrants chimiques dans les cultures. Les réponses des plantes vis-à-vis d'une agression herbivore fait intervenir des mécanismes de résistance et de tolérance. Les réponses à une agression d'une larve mineuse sont peu documentées contrairement aux insectes piqueurs ou broyeur. L'objectif de ma thèse a été de caractériser les traits de réponse de la tomate vis-à-vis de *Tuta absoluta* afin de déterminer les réponses locales et systémiques en termes de composés solubles et volatils. Nous avons regardé les réponses de la tomate soumise à deux niveaux contrastés de la disponibilité en azote, sachant que l'azote est un levier sur lequel il est possible de s'appuyer pour modifier le niveau de défense des plantes. Nous avons travaillé sur la tomate, une solanacée cultivée en hydroponie et sujette à un ravageur *Tuta absoluta*. Notre stratégie de recherche s'est portée sur l'identification et la quantification des composés de défense solubles et volatils, et sur la détermination du pouvoir nutritionnel des tissus foliaires.

Nous avons montré que la plante de tomate met en place une résistance induite par l'herbivore se traduisant par une augmentation de la concentration en acide chlorogénique, caffeoyl putrescine et tomatine localement au niveau de la zone parasitée et / ou de manière systémique dans les autres compartiments foliaires non infestés. Le rôle de ces molécules dans la défense de la tomate vis-à-vis de *Tuta absoluta* doit encore être confirmé. Nous avons montré aussi que la tomate met en place une défense indirecte contre *Tuta absoluta*, basée sur l'émission de composés volatils au voisinage des plantes. Nous avons identifié plusieurs nouveaux COVs synthétisés

lors de l'herbivorie par *Tuta absoluta* (m-cymène, menthatriène, β -cis-ocimène, β -phellandrène, terpinolène, β -caryophyllène, humulène). Parmi tous les COVs que nous avons identifiés, certains sont connus pour être émis en plus grande quantité lors d'une attaque herbivore et jouer un rôle dans l'attraction des insectes prédateurs ou la répulsion des femelles pondeuses. Ainsi, leur élicitation semble générique de l'herbivorie et pas spécifique de *Tuta absoluta*. La spécificité des interactions tomate - *Tuta absoluta* pourrait être renforcée par des analyses plus poussées en métabolomique, transcriptomique (expression de gènes RNAseq) et en s'intéressant aux voies de signalisation induite.

Contrairement à ce qui était attendu, nos résultats montrent peu d'effets de la limitation en azote sur les réponses observées. Néanmoins, comme ce qui a été démontré précédemment, la limitation azotée impacte le développement larvaire. Mes travaux ont permis de renforcer l'hypothèse "trophique" du ralentissement du développement larvaire par la limitation en azote. Ainsi, la stoechiométrie du bol alimentaire est fortement modifiée et les larves doivent consommer plus de matières pour acquérir l'azote nécessaire à leur développement. Néanmoins nous ne pouvons pas écarter l'hypothèse "composés de défense". En effet, nous avons montré que les concentrations en composés phénoliques et tomatine et des émissions de COVs supposés être impliqués dans la défense constitutive, sont plus fortes en cas d'une limitation en azote.

La diversité et la complémentarité des expérimentations permettront aux données acquises d'alimenter un modèle de fonctionnement de la plante agressée qui pourrait comporter une fonction de défense réactive à la nutrition azotée.



HOW DO PLANTS DEFEND THEMSELVES?

STUDY OF THE
TOMATO - *Tuta absoluta*
PATHOSYSTEM IN
INTERACTION WITH
NITROGEN FERTILIZATION

SUMMARY

The identification of ecophysiological responses of plants is a prerequisite for improving the natural defences of plants and reducing the use of chemical inputs in crops. Plant responses to herbivorous attack involve defence and tolerance mechanisms. Plant responses to a leafminer larva are poorly documented, in contrast to chewing and piercing insects. The objective of my Ph.D. was to characterize the tomato trait responses to *Tuta absoluta* herbivory in order to determine the local and systemic responses in terms of soluble and volatile compounds. We looked at the responses of tomatoes subjected to two contrasting levels of nitrogen availability, knowing that nitrogen is a lever to promote the basal plant defence level. We worked on tomato, a Solanaceae, grown in hydroponics and subjected to *Tuta absoluta*. Our research strategy has focused on identifying and quantifying soluble and volatile defence compounds and determining the nutritional value of foliar tissues. We have shown that tomatoes establish an induced-resistance against the herbivore, which results in a concentration increase of chlorogenic acid, caffeoyl putrescine and tomatine locally at the level of the parasite zone and / or systemically in the others non-infested foliar compartments. The defence role of these molecules on *Tuta absoluta* has yet to be confirmed. We have also shown that tomatoes set up an indirect defence against *Tuta absoluta*, based on the emission of volatile compounds in the vicinity of plants. We have identified several new herbivore-induced VOCs (m-cymene, menthatriene,

β -cis-ocimene, β -phellandrene, terpinolene, β -caryophyllene, humulene). Some of the identified VOCs are known to be emitted in greater quantities during a herbivorous attack and to play a role in attracting predatory insects or repelling egg-laying females. Thus, their elicitation seems generic to herbivory and not specific to *Tuta absoluta*.

The specificity of tomato - *Tuta absoluta* interactions could be enhanced by further analysis in metabolomics, transcriptomics (expression of RNAseq genes) and by investigating the induced signaling pathways.

Contrary to what was expected, our results show little effect of the nitrogen limitation on the observed responses. Nevertheless, as demonstrated previously, nitrogen restriction affects larval development.

My work has reinforced the "trophic" hypothesis of slowing larval development by a limited nitrogen availability. Thus, the stoichiometry of the food bowl is highly modified and the larvae have to consume more material to acquire the nitrogen necessary for their development. Nevertheless, we can not exclude the hypothesis "compounds of defence". Indeed, we have shown that the concentrations of phenolic compounds and tomatine and of the VOC emission supposed to be involved in the constitutive defence are increased in the case of nitrogen limitation.

The diversity and complementarity of the experiments will allow the acquired data to feed a functional model of the attacked plant. This model might include plant nitrogen regimes as a reactive plant defence factor.

« TOUT EST POSSIBLE À QUI RÊVE, OSE, TRAVAILLE
ET N'ABANDONNE JAMAIS. »

XAVIER DOLAN