



# Bottom-up effects of fertilization and irrigation on plant-herbivorous insect-natural enemy tri-trophic interactions in agroecosystems

Peng Han

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Peng Han. Bottom-up effects of fertilization and irrigation on plant-herbivorous insect-natural enemy tri-trophic interactions in agroecosystems. Agricultural sciences. Université Nice Sophia Antipolis, 2014. English. NNT : 2014NICE4057 . tel-01665481

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Thèse de doctorat l'Université de Nice-Sophia Antipolis

Mention : Biologie des Interactions et Ecologie

Ecole Doctorale Sciences de la Vie et de la Santé

Pour l'obtention du grade de Docteur de l'Université de Nice-Sophia Antipolis

Présenté par **Peng HAN**

# **Effets bottom-up et top-down des variations de fertilisation et d'irrigation sur des réseaux tri-trophiques en agroécosystèmes**

Soutenue le 23 Septembre 2014

Devant le jury composé de:

Dr. Eric Wajnberg, Directeur de Thèse

Dr. Nicolas Desneux, co-Directeur de Thèse

Dr. Anne-Violette Lavoie, co-Directeur de Thèse

Prof. Philippe Giordanengo, Rapporteur

Dr. Yvan Rahbé, Rapporteur

Dr. Christine Cousteau, Examineur

Dr. Claire Lavigne, Examineur

Cette thèse a été effectuée au sein de l'INRA (Institut National de la Recherche Agronomique), de l'équipe TEAPEA (Ecologie théorique et appliquée aux agrosystèmes semi-confinés).

Institut Sophia Agrobiotech

400 Route des Chappes

06903 Sophia-Antipolis

France

## Acknowledgments

*This PhD thesis was supported by a fellowship from the Chinese government and funds from the French National Institute for Agricultural Research. I would like to thank all the persons who have contributed to the development of my project: Dr. Nicolas Desneux, Dr. Anne-Violette Lavoit (INRA, Sophia-Antipolis) and Dr. Eric Wajnberg (INRA, Sophia-Antipolis). This project would not have been a success without the collective efforts.*

*I first would like to express my gratitude to my supervisor, Dr. Nicolas Desneux, researcher at INRA Sophia-Antipolis, who initiated and supervised this thesis, who has been my tutor since 2009 when I was a Master student in China. He has encouraged and helped my work with enthusiasm. During these past years, he brought me the scientific support and made me benefit from his experience. I would like also thanking Dr. Anne-Violette Lavoit who helped me during the course of the PhD, both for the experiments and the writing of the thesis; I greatly benefited from her logic thinking in writing manuscripts. I also thank Dr. Eric Wajnberg for agreeing to be the director of my PhD, for his time and for his valuable advices on data analyses. Finally, I thank Dr. Jacques Le Bot for his help during the process of the experiments, and Dr. Adamowicz Stéphane (INRA, Avignon) who helped me during the revisions of the PhD manuscript.*

*I thank Dr. Claire Lavigne (INRA Avignon), Dr. Christine Cousteau (INRA Sophia Antipolis), Prof. Philippe Giordanengo (Université de Picardie Jules Verne) and Dr. Yvan Rahbé (INRA Lyon) for agreeing to be the members of my PhD thesis jury. Especially, Philippe Giordanengo and Yvan Rahbé who agreed to evaluate this work as Rapporteurs. I am especially grateful to them.*

*I thank all those who participated in the experiments. Big thanks to Philippe Bearez, Edwige Amiens-Desneux, Lionel Salvy, Sylvain Nuée for their technical support, encouragement and good humor. Also, I would thank some other colleagues: Dr. Antonio Biondi (University of Catane, Italy), Yongcheng Dong and Zhijian Wang (Huazhong Agricultural University) who have been actively involved in my experiments.*

*I also thank my former tutor, Dr. Changying Niu, who helped and encouraged me to study abroad, and to Anaïs, Yuxiang, Anthony, Laura, Luciana, Cylla and all the other colleagues and friends for the general good ambience of the working environment.*

*Finally, I thank my parents and other family members who encouraged me to pursue this higher education abroad. Big thanks to my partner who has supported and helped me during the stress of my PhD! I love all of you more than you can ever imagine.*

*Thanks to all!*

*Sophia-Antipolis, France*

## Résumé

Le système 'plante-ravageur-ennemi naturel' fournit un modèle de base idéal pour comprendre comment les communautés d'arthropodes sont structurées et comment les interactions (directes et indirectes) entre les différents acteurs contribuent à façonner la structure des communauté. Dans les agro-écosystèmes, les forces "*top-down*" correspondent aux effets de contrôle que les organismes arthropodes des niveaux trophiques supérieurs (par exemple, les prédateurs) ont sur les espèces des niveaux inférieurs (par exemple, leurs proies). Les communautés d'arthropodes peuvent également être influencées par des forces "*bottom-up*" induites par des variations dans les régimes de fertilisation ou d'irrigation ou par des variations de certains traits des plantes (résistance aux herbivores ou adaptations aux stress environnementaux). De plus, les forces "*bottom-up*" peuvent affecter l'impact "*top-down*" des ennemis naturels sur les herbivores soit directement (par exemple, les effets sur prédateur omnivore) ou médiées par les insectes herbivores intermédiaires. Dans ce contexte, les objectifs de cette thèse ont été de comprendre comment les variations de l'apport des ressources dans les agro-écosystèmes (disponibilité en azote et en eau) peuvent influencer les interactions entre les plantes, les herbivores et les ennemis naturels. Cette question a été étudiée aussi bien à l'échelle individuelle (traits d'histoire de vie des insectes) que populationnelle (dynamique des populations).

Les études ont été réalisées sur deux agro-écosystèmes basés sur les cultures de la tomate et du coton. Sur tomate, le système "*Solanum lycopersicum* L - mineuse *Tuta absoluta* - prédateur omnivore *Macrolphus pygmaeus*" a été utilisé dans des conditions de laboratoire et en serre en France. Nous avons démontré des effets "*bottom-up*" des intrants d'azote et/ou d'eau sur les herbivores et leur prédateur omnivore. La préférence alimentaire du prédateur a été également fortement influencée par la disponibilité en l'eau apporté à la plante. Les études sur le coton (*Gossypium arboreum* L) ont été réalisées dans des conditions de terrain en Chine centrale. Nous avons identifié les principales forces de régulation "*top-down*", notamment via des coccinelles prédatrices (*Propylaea japonica*) et des parasitoïdes de pucerons, sur le puceron *Aphis gossypii* Glover. Dans une seconde étude sur le coton transgénique Bt (*Bacillus thuringiensis*), nous avons démontré qu'une fertilisation azotée moindre diminue les populations du puceron *A. gossypii*, sans pour autant compromettre l'effet "*top-down*" issu de la guildes des ennemis naturels. Enfin, cette thèse souligne l'importance de la manipulation de ces différentes forces dans les stratégies de gestion des espèces nuisibles dans le cadre de la lutte intégrée.

## Abstract

The “Plant-herbivorous insect-natural enemy” system provides an ideal basic model to understand how the plant-inhabiting arthropod communities are structured and how various mechanisms (i.e. direct and indirect interactions) contribute to shape the community structure. In agro-ecosystems, top-down forces encompass the controlling effects that arthropod organisms of the higher trophic level (e.g., predators) have on species at the next lower level (e.g., prey). Arthropod communities may also be influenced by bottom-up forces induced by environmental variations (e.g. fertilization or irrigation regimes) or plant traits (plant insect-resistance or plant-adaptive traits). Furthermore, bottom-up forces may affect top-down forces on herbivores either directly (e.g., effects on omnivorous predator) or mediated by the intermediate herbivorous insects. In this context, the aims of the PhD study were to disentangle how variations in resource inputs (i.e. nitrogen and water availability) affect interactions among plant, herbivores and their natural enemies at both the individual (life-history traits) and population (population dynamic) levels.

The studies were carried out on two agrosystems based on tomato and cotton. On tomato, the system '*Solanum lycopersicum* L - leafminer *Tuta absoluta* - omnivorous predator *Macrolphis pygmaeus*' was used under laboratory and greenhouse conditions in France. We found strong evidence of bottom-up effects of nitrogen and/or water inputs on the herbivore and the omnivorous predator. Feeding ecology of the predator was also strongly influenced by water availability. Studies on cotton (*Gossypium arboreum* L) were carried out under field conditions in central China. We identified the key top-down regulation forces from ladybirds *Propylaea japonica* and aphid parasitoid Aphidiines on cotton aphid *Aphis gossypii* Glover. In another study in transgenic Bt (*Bacillus thuringiensis*) cotton, we found that a lower nitrogen fertilization may support lower *A. gossypii* populations, but not compromise top-down regulation since its key natural enemy guild was generally not affected. Moreover, this work highlights how the manipulation of these forces could favor the management of a given pest species in the framework of Integrated Pest Management.

## Résumé de la thèse en français

Dans la nature, les plantes couvrent la plupart des surfaces terrestres. Dans la plupart des écosystèmes terrestres, ce sont des producteurs primaires et forment ainsi la base des réseaux trophiques de ces écosystèmes. Le groupe des consommateurs primaires, organismes hétérotrophes consommant les plantes, est largement dominé par les insectes (appartenant au taxon des arthropodes). Plantes et Arthropodes sont les deux taxons d'organismes vivants les plus importants, à la fois en termes d'abondance des espèces et de quantité de biomasse (Schoonhoven et al. 2005). Ainsi, les relations entre les insectes et les plantes sont d'une importance cruciale.

L'étude des interactions plantes-insectes dans les écosystèmes terrestres a été particulièrement développé. Notamment, le système 'plante-ravageur-ennemi naturel' fournit un modèle de base idéal pour comprendre comment les communautés d'arthropodes sont structurées et comment les interactions (directes et indirectes) entre les différents acteurs contribuent à façonner la structure de la communauté. Les interactions entre plantes, "ravageurs" et "ennemis naturels" peuvent être influencées par divers facteurs environnementaux biotiques et/ou abiotiques. Dans ce contexte, les forces "*bottom-up*" "*top-down*" vont largement influencer le niveau intermédiaire i.e. les herbivores. Les forces "*bottom-up*" peuvent être dirigées par divers facteurs qu'ils soient intrinsèques au système (e.g., les caractéristiques des plantes en termes de résistance aux ravageurs ou de tolérance aux stress environnementaux) ou externes au système (e.g., facteurs abiotiques de l'environnement tels que la disponibilité des ressources) (Hunter et Price 1992).

La variation des facteurs environnementaux est susceptible de provoquer des modifications de l'état physiologique des plantes (notamment en termes de qualité nutritionnelle et de capacité de défense) déclenchant ainsi des effets de bas en haut ("*bottom-up*") sur l'herbivore, voire sur le niveau trophique supérieur (i.e. les ennemis naturels) par effet de cascade. De plus, L'herbivore peut être influencé indirectement par des interactions indirectes entre le premier et le troisième niveau trophique (e.g., "l'appel à l'aide" qui consiste à une reconnaissance des signaux volatiles de la plante

attaquée par les ennemis naturels des ravageurs). En revanche, les forces "*top-down*", s'exerçant de haut en bas, englobent les effets de contrôle que les organismes arthropodes du niveau trophique supérieur (e.g., les prédateurs ou parasitoïdes) ont sur les espèces du niveau immédiatement inférieur (ici une proie ou hôte). Ces deux forces "*bottom-up*" et "*top-down*" agissent de concert déterminant ainsi un contrôle complexe des populations d'herbivores.

La raison pour laquelle nous nous concentrons sur les conséquences de ces deux forces sur les insectes herbivores provient de la nécessité de la gestion de ces insectes qui causent souvent de graves dommages sur les cultures. Pour l'Homme, ces insectes herbivores sont des insectes ravageurs. Dans les agro-écosystèmes, la lutte antiparasitaire intégrée (LAI) vise à contrôler les populations d'insectes ravageurs pour que la qualité des cultures reste en-dessous du niveau de préjudice économique. La lutte biologique est l'un des éléments les plus importants du programme. Cependant, la réussite/l'efficacité de ces stratégies peut être fortement influencée par les interactions mentionnées ci-dessus et les forces qui s'y ajoutent. En mettant l'accent sur le contrôle biologique d'un insecte ravageur donné, l'étude du rôle des deux forces "*bottom-up*" et "*top-down*" sur la régulation des insectes ravageurs répond à la fois à des questions écologiques fondamentales, mais aussi appliquée car concernant l'efficacité des stratégies de Lutte Biologique Intégrée. Dans ce contexte, les objectifs de cette thèse sont de comprendre comment les variations de disponibilités en ressources (azote et eau) d'un système donné peut influencer les interactions entre les plantes, les herbivores et les ennemis naturels, aussi bien à l'échelle individuelle (traits d'histoire de vie) que populationnelle (dynamique des populations). Au vu de l'importance de ces questions dans les agro-systèmes, cette étude s'est basée sur deux modèles de plantes agricoles: tomate (*Solanum lycopersicum* L) et coton (*Gossypium arboreum* L). Pour les études sur plants de tomate, des expériences ont été menées en laboratoire pour évaluer la performance individuelle des insectes et en conditions semi-contrôlées (système serre) pour les aspects populationnels. Pour les études sur le coton, les expériences ont été menées sur le terrain, à l'échelle populationnelle



Dans le chapitre 1, une synthèse bibliographique décrit les interactions et les forces s'exerçant dans un système tri-trophique. Ensuite le chapitre 2, décrit le contexte agro-systémique dans lequel s'inscrit cette étude en mettant l'accent sur la lutte biologique dans le cadre des stratégies de lutte intégrée. Dans les chapitres 3 et 4, nous avons inclus toutes les études individuelles et populationnelles respectivement. Enfin le dernier chapitre (5), propose une discussion générale sur ce travail de thèse qui a été réalisé.

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## **List of abbreviations**

HIPVs: Herbivore-Induced Plant Volatiles

IGP: Intraguild Predation

HIPVs: Herbivore-Induced Plant Volatiles

TMIIs: Trait-mediated Indirect Interactions

DMIIIs: Density-mediated Indirect Interactions

PPSH: Pulsed Plant Stress Hypothesis

ABS: Access and Benefit Sharing

CBD: Convention of Biological Diversity

GDBH: Growth-differentiation Balance Hypothesis

IRGM: Insect-Resistance Genetically Modified

## **General introduction**

## **General introduction**

In nature, plants cover most of the terra firma on planet Earth. They serve as the primary producers in most terrestrial ecosystems and form the basis of food web in those ecosystems. Arthropod insect is one of the most dominant group among plant consumers. These two groups represent the most two extensive taxa of living organisms, both in abundance of species and in amount of biomass (Schoonhoven et al. 2005). Thus, the relationships between insects and plants (plant-arthropod interactions) are of crucial importance.

Plant-arthropod interactions in terrestrial ecosystem have received considerable research interests. Notably, the “Plant-herbivorous insect-natural enemy” provides an ideal basic model to understand how the plant-inhabiting arthropod communities are structured and how various mechanisms (i.e. direct and indirect interactions) contribute to shaping the community structure. The “Plant-herbivorous insect-natural enemy” interactions may be influenced by various biotic and/or abiotic factors. In this context, both “Bottom-up” and “Top-down” forces largely influenced the intermediate herbivorous insect. “Bottom-up” forces can be mediated by various factors: intrinsic (e.g., plant traits in terms of insect resistance or environmental stress tolerance), or environmental (e.g., resource availability). These forces cause variations in plant status (e.g. plant quality for herbivorous insects in terms of nutritional quality and defensive level), and thus trigger bottom-up effects on the herbivorous insect, often cascading up to the higher trophic level (i.e. natural enemies). In contrast, “Top-down” forces encompass the controlling effects that arthropod organisms of the higher trophic level (e.g. predators or parasitoids) have on species on the adjacent lower level (e.g. prey or host which appear to be also the herbivorous pests). However, the natural enemies (themselves as the source of “Top-down” forces) may be enhanced or weakened by the “Bottom-up” forces. Therefore, these two forces act in concert and influence the individual performance and the population dynamic of the herbivorous insects.

The reason that we focus on the consequences of both forces on herbivorous



insects comes from the necessity to manage these insects which often cause serious damage on cultivated plants/crops. From human's perspective, these herbivorous insects are pest insects. In agro-ecosystems, Integrated Pest Management (IPM) aims to suppress pest insect populations below the economic injury level. Biological control is one of the most important components of the program. However, the performance of natural enemies (i.e. fitness and biocontrol efficacy) may be greatly influenced by the various bottom-up effects mentioned above, such as variation in resource inputs and intrinsic traits in plant/crop cultivars. Understanding the role of both bottom-up and top-down forces in influencing pest insects provide insights into fundamental ecological questions, as well as help guide IPM.

In this context, the aims of the PhD was to disentangle how variation in resources input (nitrogen and water availability) may influence the interactions among plant, herbivores and natural enemies; both at the individual (life-history traits) and population (population dynamic) levels. Thus, in chapter 1, we discussed the “Bottom-up” and “Top-down” forces in the typical tri-trophic systems. Then, in chapter 2, we defined our research scope that all the studies were confined within the context of agro-ecosystems, focusing on the biological control within the IPM frameworks. For the studies on tomato plant, experiments were conducted in order to assess the insect individual performance under laboratory conditions as well as the populations in greenhouse conditions (Chapter 3 and 4). For the studies on cotton plant, insect field populations were monitored (Chapter 4). In the last chapter 5, a general discussion of this PhD work was performed and prospects of the current study were tentatively proposed. All these studies were expected to provide a better understanding of mechanisms underlying the “Plant-herbivorous insect-natural enemy” tri-trophic interactions, but also theoretical foundation for biological control within IPM programs.

## **Chapter 1: Bottom-up and top-down forces in community ecology**

## **D) Community ecology: direct and indirect interactions**

Community ecology is the study of the interactions of a set of species co-occurring at a given time and space (McGill et al. 2006). By definition, an individual ecosystem is composed of populations of organisms, interacting within communities, and contributing to the cycling of nutrients and the flow of energy (Constantin de Magny et al. 2014). Lawton et al. (1999) have stated that “Community ecology is a mess” insinuating that communities are too complex and contingent to allow general laws. In response, Simberloff (2004) has argued that after all community ecology is not a weak science: Traditional community ecological research, often local, experimental, and reductionist, is crucial in understanding and responding to many key conservation and environmental issues, such as threatened species and communities, ecosystem management of invasive introduced species and global change. Among these issues, community ecology has contributed considerably to address critical applied problems, i.e., management of invasive or endemic pests (Ohgushi et al. 2012).

The difficulties of these studies can be largely attributed to the food web complexity in which species are directly, and more often, indirectly interconnected with other species. The relationship between the two organisms may arise from direct interaction mainly including predation, parasitism, competition and mutualism. But more often, the impacts of one species on another are influenced by one or more intermediate species, which is termed as “indirect interactions” (**Table 1**).

In a food web context, the consumer-resource interaction is the basic direct interaction between pairs of species, mainly including herbivory, predation, parasitism, (**Table 1**). Aside from the inter-specific interactions, direct interactions may also occur at intra-specific level, such as cannibalism and interference competition. All these direct interactions have been considered to play a major role in shaping the ecological and evolutionary patterns in ecological communities (Wootton 1994).

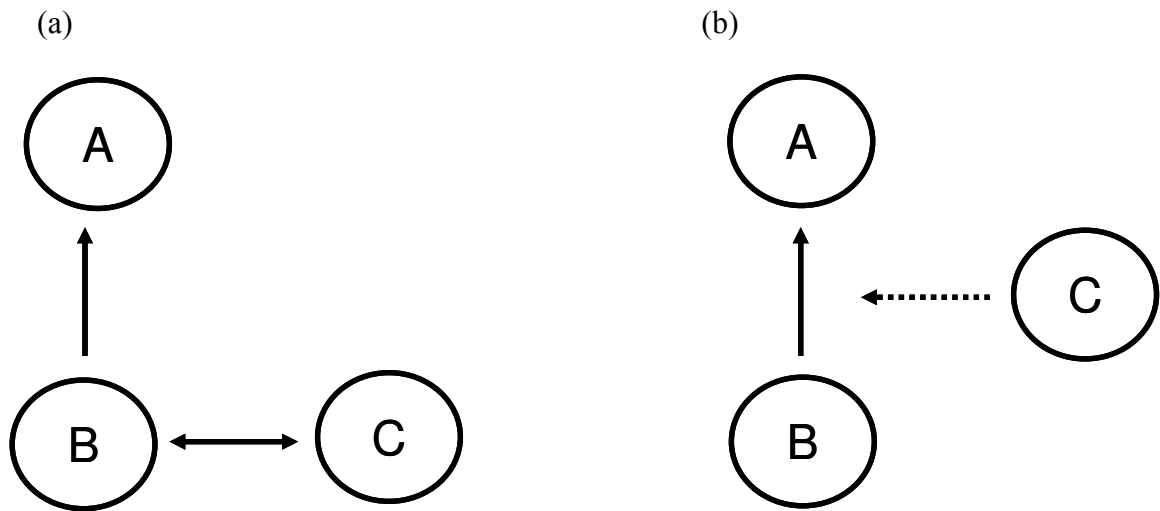
Indirect interactions occur when the impact of one species on another depends on the presence of a third species. There are two basic ways in which one species can

**Table 1:** A summary of commonly studied direct or indirect interactions:

Direct interactions		Indirect interactions
<i>Inter-specific</i>	<i>Intra-specific</i>	<ul style="list-style-type: none"> <li>- Apparent competition</li> <li>- Exploitative competition</li> <li>- Trophic cascade</li> <li>- Apparent predation</li> <li>- Intraguild predation</li> </ul>
Herbivory	Cannibalism	
Predation	Interference competition	
Parasitism		
Competition		
Mutualism		
Commensalism		

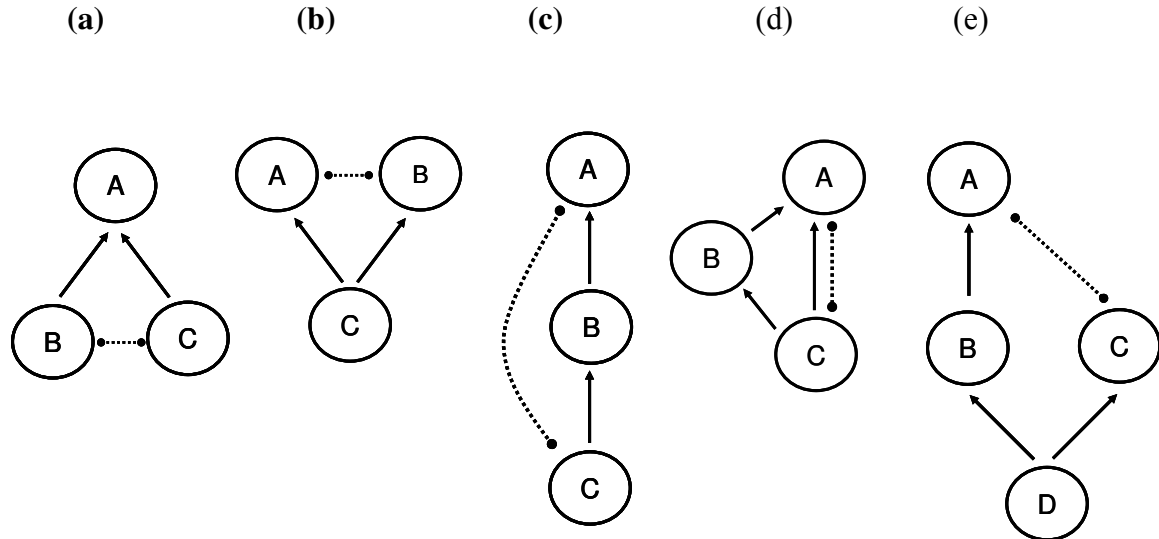
indirectly affect another: interaction chain and interaction modification (**Fig. 1**). (Wootton 1994). Basically, indirect interactions may arise when there are at least three interacting species, with a pair of them (species A and B in **Fig. 1**) that interact directly. The third species (species C in **Fig. 1**) interacts indirectly with a member of the pair either by interacting directly with the other member (**Fig. 1a**) or by influencing the interaction within the pair (**Fig. 1b**). These two basic types of way that interact with each other are pervasive in the following indirect interactions.

**Apparent competition** occurs when one species is shared by two species (**Fig. 2a**), and they can be classified depending on the time period. In “**Short-term apparent competition**”, indirect interactions occur within a generation and are primarily due to the change in behavior of natural enemy. In this case, one species of natural enemy may benefit from an increase in density of the alternative host or prey since the natural enemy may switch to this host or prey (thus also called “**Apparent mutualism**” (Abrams and Matsuda 1996). However, in the long term, such indirect interactions that occur a number of generations due to the changes in densities of one species may harm the other species that shared by a natural enemy (i.e., “**Long-term apparent competition**”) (Holt 1977; Holt and Kotler 1987; van Veen FJF et al. 2006).



**Figure 1:** Two basic patterns in which one species can indirectly interact with another. (a) Interaction chain: species C affects species A through direct interactions with species B (i.e., changes in abundance or traits in species B); (b) Interaction modification: species C affects species A through influencing how species B interacts with species A. Adapted from Wootton 1994.

**Exploitative competition** involves two predators share one prey species (**Fig. 2b**; Wootton 1994) where they negatively affect each other because prey resource is often limited. Another form of indirect interactions, called as **tri-trophic cascade**, concerns the top species eating the middle species which eats the bottom species, then the top species could have an indirect interaction with the bottom species) (**Fig 2c**). This model is basic for the plant-based arthropod communities. “**Intraguild predation**” (**IGP**) (**Fig. 2d**) occurs when the consumers not only prey on the primary consumer (e.g., the key herbivores) but also on other predators (Rosenheim et al. 1993). In this case, the suppression on prey by the secondary predator may harmed by the intraguild predation by the primary predator. The more complex “**Apparent predation**” (**Fig. 2e**) occurs when one species may benefit another specie through trophic cascade as well as the action of exploitative competition. All these basic models have substantially contributed to our understanding of complex web-based community structures and the population dynamics of a given species (Holt and Lawton 1994; Polis et al. 2000; Werner and Peacor 2003).



**Figure 2:** Five commonly investigated indirect interactions. Solid arrows represent trophic links (i.e., consumer-resource interactions) and the arrow points toward the direction of energy flow. Dashed lines connect impacted species. Subfigures: **(a) Apparent competition**: species B and C are in apparent competition since they are consumed by species A; **(b) Exploitative competition**: species C is shared by species A and B (i.e., exploitative effect on limited resources); **(c) Tri-trophic cascade**: species A may trigger positive trophic cascade on species C by attacking species B; **(d) Introguid predation**: Species A to some extent positively affect species C by predation on species B; **(e) Apparent predation**: species A may benefit species C via the positive trophic cascade effect on species D.

Indirect interactions can be also classified as either (1) density-mediated (Abrams 1995) or (2) trait-mediated (Werner and Peacor 2003; Ohgushi et al. 2012). **Density-mediated indirect interactions (DMIIs)** occur when one species affect another species through the changes in density of the intermediate species population density in the trophic cascade. For instance, an eagle may attack and kill rabbits and thus reduce rabbit population size, which in turn relaxes herbaceous plants from herbivory by rabbits. Hence, the eagle's positive indirect effect on plants is somehow mediated by density decrease in rabbits (i.e., density-mediated indirect interactions). In another way, **trait-mediated indirect interactions (TMIIs)** refer to the consequences of interactions mediated by changes in behavior or other trait plasticity

in species across a wide range of systems. In the same context of the previous example, the rabbits may change their behavior to avoid predation risk by eagles; for instance, they can hide more when in the presence of eagles. Thus for plants, the feeding pressure from rabbits is somehow alleviated by the presence of eagles. In this case, it is a change in rabbit's trait (i.e., behavior change to avoid predation risk) that influences the plant-rabbit interactions (i.e., trait-mediated indirect interactions).

While DMIs have been acknowledged historically in natural communities (Abrams 1995), the importance of TMIs in ecological communities has become recognized over the last two decades (Gastreich 1999; Relyea 2000; Bolker et al. 2003; Schmitz et al. 2004; Mouritsen and Poulin 2005; Preisser et al. 2005; Peckarsky et al. 2008). Yet there are considerable debates over their nature, strength and overall importance in trophic cascades. Schmitz et al (2004) have suggested a powerful conception named “landscapes of fear” to view ecological systems (Brown et al. 1999) in which all indirect interactions are primarily trait-based. The authors postulated a hypothesis that different predators just create varying rules of predation based on their hunting modes and habitat domains and that this set of rules determines whether DMIs or TMIs will dominate.

## **II) Tri-trophic interactions mediated by plants**

In nature, plants cover most of the *terra firma* and serve as the primary producers in most terrestrial ecosystems where they form the basis of the food web. Among arthropods, arthropod insects constitute a dominant group among plant consumers. Plants and insects represent the two most extensive taxa of living organisms, both in abundance of species and in amount of biomass (Schoonhoven et al. 2005). Thus, the relationships between insects and plants are of the utmost importance.

Within plant-insect interactions, the basic tri-trophic cascade (**Fig 2c**) is a “**plant-herbivorous insect-natural enemy**” interaction. This model is composed by a first trophic level (plants), a second one (herbivorous insects) and a third one (parasitoids/predators). In plant-inhabiting arthropod communities, such tri-trophic

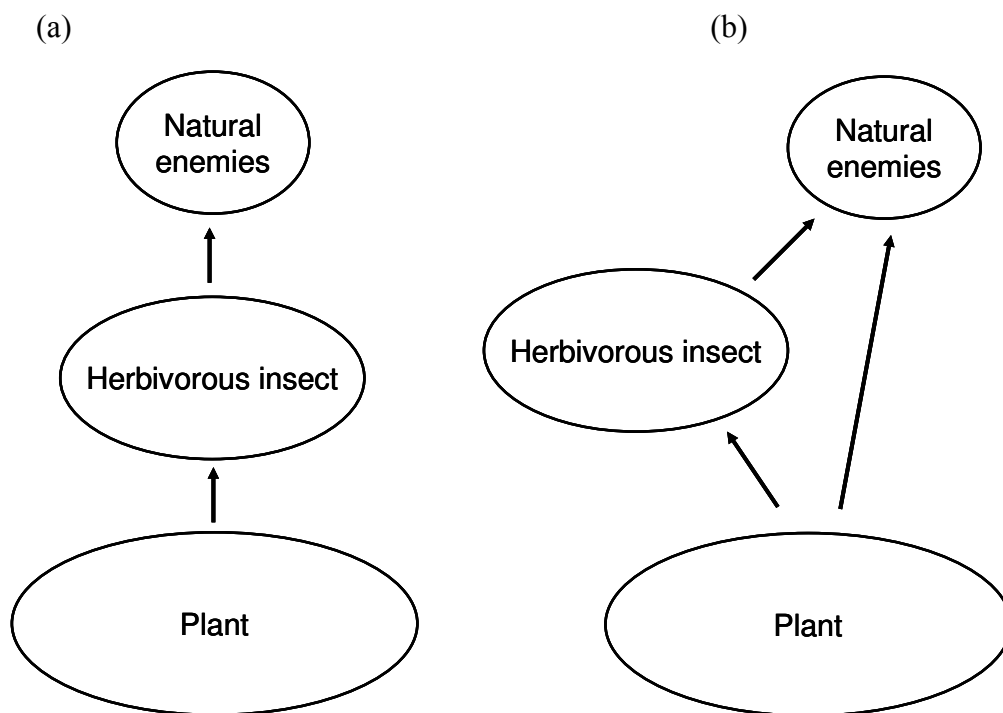
interactions may occur in two basic patterns (**Fig. 3**): 1) the arthropod natural enemies attack herbivorous insects and release plant from herbivory (**Fig. 3a**); 2) the arthropod natural enemies are both phytophagous and predatory (**Fig. 3b**); as occurs with some species from the Heteroptera family. Obviously, in both cases we cannot understand the interactions between predators and herbivorous insect without understanding the role of plants (Price et al. 1980). The main reason is that plants serve as a conduit of energy flow and as connection between the abiotic environment and the plant-inhabiting insect communities. Any perturbation and modification of environmental conditions may induce changes in plant physiology, biochemistry, morphology and thus trigger bottom-up effects on herbivorous insect-natural enemy interactions (Walker and Jones 2001). Likewise, it is difficult to understand plant-herbivorous insect interactions without considering natural enemies. Herbivorous insects are attacked and fed upon by a range of diverse arthropods. However, the impact of natural enemies on herbivorous insects can be influenced by plant-herbivore interactions. For example, in response to herbivore attacks, plants may produce semiochemicals called herbivore-induced plant volatiles (HIPVs) which act to repel pests and attract their natural enemies (Dicke et al. 2009; Khan et al. 2008)

Overall, the tri-trophic “Plant-herbivorous insect-natural enemy” interactions provides an ideal basic model to understand how the plant-inhabiting arthropod community are structured and how various mechanisms (i.e., direct and indirect interactions) contribute to shaping the community structure. As discussed by Ohgushi et al. (2012), this holistic, “plant-herbivorous insect-natural enemy” tri-trophic perspective contributes to the rise of at least three research areas: First, it expands our view on plant defense from direct defense (e.g., herbivore-induced defense or constitutive defense) to indirect defense (e.g., HIPVs to recruit natural enemies of herbivores) (Howe and Jander 2008; Dicke et al. 2009). Second, it promotes our understanding of the forces influencing the evolution of the host range and the diet breadth of herbivores by considering the dual effects of host plant traits and predation/parasitism risk by their natural enemies (Singer and Stireman 2005). Third,



it allows us to predict the influences on population of herbivores by modifying plant traits through various biotic (e.g., plant cultivars or genotypes) or abiotic factors (e.g., water, nutrients etc.). Furthermore, from an applied point of view, understanding “plant-herbivorous insect-natural enemy” interactions helps to improve the pest insects management.

The nature of direct interactions and indirect interactions within the ecological community were discussed above. However, to better understand how the middle trophic-level species such as herbivores are influenced either at individual life-history or population abundance level, the two major forces (i.e., bottom-up and top-down forces) mediated by various biotic (e.g., plant traits, natural enemies) or abiotic factors (e.g., resources availability) deserve further discussion.



**Figure 3:** Two basic patterns for tri-trophic interactions in which plant- inhabiting insect communities are structured: (a): the plant is attacked by a herbivorous insect which is attacked and fed upon by a predator/parasitoid; (b) the plant is attacked by a herbivorous insect which is attacked and fed upon by a predator/parasitoid i.e., at the same time, the plant is the food source of the predator (i.e., omnivorous predator) or other omnivorous natural enemies. Solid arrows represent trophic links (i.e., consumer-resource interactions) and the arrow points toward the direction of energy flow.

### **III) Bottom-up vs. top-down forces influencing plant-mediated tri-trophic interactions**

The relative importance of both forces on the population dynamics of herbivorous insects has been long debated (see review by Hunter et al. 1997). The debate started with the proposition “world is green” which predicted that natural enemies regulate the populations of most herbivores below a certain density at which significant defoliation would occur (Hairston et al. 1960). It prompted numerous theoretical and empirical studies on the effects of bottom-up and top-down forces on community structures (White 1978; Hunter and Price 1992; Power 1992; Spiller and Schoener 1994; Dyer and Letourneau 1999; Polis et al 2000; Denno et al 2002, 2003; Costamagna and Landis 2006; Costamagna et al 2007). Top-down and bottom-up forces have been widely acknowledged to act in concert to shape arthropod communities in both aquatic (Strong 1992; Pace et al. 1999) and terrestrial ecosystems (Hunter and Price 1992; Power 1992; Hunter et al. 1997; Zaugg et al 2013), and particularly in agroecosystems (Snyder and Wise 2001; Cardinale et al. 2003; Lang 2003; Rypstra and Marshall 2005; Costamagna and Landis 2006; Costamagna et al. 2007; Chen and Ruberson 2008). Agro-ecosystems often consist of simplified food webs with strong reciprocal impacts between species occupying adjacent trophic levels, leading to ideal systems to manipulate bottom-up vs. top-down forces (Denno et al. 2003; Costamagna and Landis 2006).

In agro-ecosystems, arthropod communities can be largely structured by bottom-up forces including various intrinsic factors (i.e., plant traits in terms of insect resistance or environmental stress tolerance), or abiotic factors (i.e., resource / nutrition availability) (Hunter and Price 1992). Improper resource inputs to plants may induce physiological changes in plants that are linked to their nutritional value and chemical defenses (Chen et al. 2010; Gutbrodt et al 2011). These changes may trigger bottom-up effects (either positive or negative) on pests at individual and/or population levels. For instance, Inbar et al (2001) found that tomato plants suffering nitrogen deficiency and/or water stress showed higher levels of chemical defense and

had a lower nutritional value for insects, and thus affected performances in herbivorous insects. In addition, these physiological changes in plants may influence top-down forces on herbivores, e.g., when their natural enemies are omnivorous predators (i.e., plant feeding) or if toxic plant compounds spill over from herbivores to natural enemies throughout the food chain (Eubanks and Denno 2000; Janssen et al 2003).

Top-down forces encompass the controlling effects that arthropod organisms at the higher trophic level (e.g., predators or parasitoids) have on species at the adjacent lower level (e.g., prey or host). It has been considered a primary force in regulating herbivorous arthropods (Pace et al. 1999; Shurin et al. 2010; Costamagna and Landis 2006; Zaugg et al 2013). However, top-down forces may be weakened by the factors that potentially reduce the predation or parasitism efficiency (Power 1992). For instance, IGP (**Fig. 2d**) may disrupt the regulation of primary natural enemy agent on herbivores (Snyder and Wise 2001; Symondson et al 2002).

Although it is well accepted that these two forces act in concert in influencing the herbivorous populations, one of the forces may dominate compared to the other. For instance, studies have been performed in the field where bottom-up forces have been shown to exert the primary regulation on herbivore populations (Stiling and Rossi 1997; Denno et al 2002), whereas other studies supported the prevalence of top-down forces (Dyer and Letourneau 1999; Walker and Jones 2002; Costamagna and Landis 2006), or neither of force exerted significant effect on herbivore populations (Cornelissen and Stiling 2009). Furthermore, it is important to recognize that the bottom-up and top-down forces do not act solely, but interact with each other to determine their overall effects on herbivorous insect communities particularly via changes in plant traits (Ohgushi et al. 2012). Bottom-up forces may modify the sign and strength of top-down cascading effects, and vice versa. A study on oak-herbivore community showed that bottom-up forces influence the impact of top-down forces, especially during the early stages of oak establishment and their colonization by herbivores and predators (Forkner and Hunter 2000). Bottom-up forces that vary with changes in plant quality or growing status strongly influence the prey-predator

interactions (Denno et al. 2002; Pearson 2010). Moreover, the relative strength of bottom-up (basal resources) and top-down (predation or parasitism) could be influenced by the specific insect behavioral characteristics, such as escape/defensive behavior in response to predation risk (Denno et al. 2003). Also, the life-history strategy in herbivorous insects has been considered as another important mediator of the relative strengths of bottom-up and top-down forces (Denno et al. 2002).

### **III.1) Bottom-up forces**

Arthropod herbivores populations can be markedly shaped by bottom-up effects (Mattson 1980; Hunter and Price 1992; Hunter et al 2001). The factors that may trigger cascade influence on arthropod community mainly include variation in resource inputs (either in quality or quantity) to plants (White 1993; Fischer and Fiedler 2000), plant insect-resistance traits (e.g., insecticidal toxins or insect enzyme inhibitors) (Ferre and van Rie 2002; Han et al. 2012), or plant-adaptive traits against various abiotic stresses (e.g., drought resistance) (Galmés et al 2013). These factors not only act solely, but also can generate crossing-effects on the plant-herbivorous insect interactions.

Phytophagy is a widespread phenomenon in terrestrial plant-inhabiting arthropods. It concerns not only herbivorous insects, but also omnivorous insects (e.g., broadly in the Heteropteran family) (Coll and Guershon 2002). The plant-feeding arthropods need plants to obtain nutrients and/or water to survive and develop (Schoonhoven et al. 2005). But in nature, they forage in environments where plants show contrasted palatability levels in terms of nutritional value (e.g., nitrogen richness and water content) and toxicity (e.g., plant chemical defense) (Inbar et al 2001; Schoonhoven et al. 2005; Howe and Jander 2008; Kaplan and Jennifer 2011). Variation in quality and quantity of nutritional resource inputs to plants (e.g., fertilization regime) may trigger bottom-up effects on herbivorous insect as well as on their natural enemies. For instance, experiments showed that improving the host-plant nutritional value enhanced the development and fertility of some herbivorous insects

(Awmack and Leather 2002), and other that it enhances also the performance and population density of some predators and parasitoids (Hunter and Price 1992; Hunter 2003). Furthermore, a wide range of secondary compounds which act as **constitutive chemical defenses** (toxins and other defensive barriers that are produced even when herbivores are absent) or as **induced chemical defenses** (toxins and other defensive traits that are expressed only in response to herbivory) (Howe and Jander 2008) may also vary with the quality and quantity of resources offered to plants (Couture et al. 2010).

### **III.1.1) Bottom-up forces mediated by abiotic factors**

Any variation in plant nutritional values and/or plant defense profiles may have bottom-up effects on herbivorous insects (Inbar et al. 2001). Variation in such plant traits may result from changing abiotic environments, such as light, water, and nutrients (Turtola et al. 2013; Gutbrodt et al 2011; Larbat et al. 2012).

Among these abiotic factors, nitrogen and water have deserved a major historical focus. Nitrogen and water are crucial resources required for optimal plant growth and reproduction (Schoonhoven et al. 2005). Thus, variations in nitrogen (excessive or deficient) and/or water input (drought or water logging) alter physiological, biochemical, and/or morphological plant traits, resulting in bottom-up effects on arthropod communities, such as herbivores and their natural enemies (**Fig. 4**). For the plant-inhabiting arthropods (herbivorous insects and their natural enemies), three main interactions are involved when the resources (i.e., nitrogen and water) vary: (1) from a resource viewpoint, plants offer shelter and supply food or other extra-food such as pollen and nectar to insects; (2) from a defense viewpoint, plants express a variety of chemical and mechanical deterrents in the presence and absence of herbivory; (3) from a morphological or architectural viewpoint, plants influence the feeding/foraging cues of arthropod insects.



organic compounds and all the energy required to living beings, nitrogen has also been viewed as a central element because of its crucial role in forming basic living materials such as proteins and nucleic acid (Novoa and Loomis 1981). Thus, nitrogen fertilizers are often required to enhance plant production in agriculture. Adequate nitrogen supplies are fundamental to maximize crop yields, but excessive nitrogen inputs can contaminate groundwaters (Jaynes et al. 2001). Therefore, an appropriate nitrogen fertilizer management is required to balance the nitrogen demand by crops with the need to minimize environmental perturbations.

However, it is worth noting that the average N concentration is substantially lower in plants (2-4%) than in insects (8-14%), which suggests that herbivorous insects need to eat large amounts of plant material to obtain the nitrogen required for their growth (Schoonhoven et al. 2005). When the nitrogen content (that correlates to protein content) of plant food increases, herbivores become more efficient in converting plant material into insect tissue (McNeill and Southwood 1978). However, despite the essential role of nitrogen for insect growth, the total plant nitrogen content has been considered as a poor index of its nutritional value (Schoonhoven et al. 2005). This is mainly due to the fact that high nitrogen levels may coincide also with higher concentration of nitrogen-containing defensive compounds, such as alkaloids, that restrict the efficiency of food utilization by insects (Schoonhoven et al. 2005). Therefore, plant nitrogen inputs can exert a variety of bottom-up effects and potentially alter significantly tri-trophic interactions (for review, see Chen et al. 2010).

In plant-herbivorous insect interactions, the variation in nitrogen input to plants may trigger changes in plant nutritional quality (from an herbivore's perspective) and in profiles of defensive traits (Inbar et al. 2001; Royer et al. 2013), which in turn lead to bottom-up effects on herbivorous insect performance (e.g., survival, development, fertility etc.). For instance, the development of Lepidoptera herbivores has been found positively correlated with plant nitrogen concentration (Cates et al 1987; Estiarte et al. 1994; Hunter and McNeil 1997; Grundel et al 1998; Inbar et al. 2001), and the similar phenomenon was found in other insect groups (Ohmart et al. 1985; Obermaier and

Zwöfler 1999).

Then, herbivorous insects are limited by low nitrogen concentrations in food plants (White 1984; Ravenscroft 1994), which was termed as the “**Nitrogen limitation hypothesis**” (White 1993). This effect can be explained by two aspects: (1) Insect herbivores may suffer a nutritional deficit when plants experience nitrogen shortage since insects need to transform the inorganic nitrogen forms present in plant tissues and/or utilize plant-derived amino acids (Schoonhoven et al. 2005); (2) insect herbivores may suffer from higher concentrations of some defensive compounds when soil nitrogen is not adequate (Inbar et al. 2001). For example, nitrogen limitation in the rooting medium has been shown to induce higher levels of phenolics in all tomato organs (Inbar et al. 2001; Le Bot et al 2009; Larbat et al 2012; Royer et al 2013). Although such positive correlation between the plant N concentration and the performance of herbivores has been often observed, several studies related the inverse, (Joern and Behmer 1998; Fischer and Fiedler 2000) which somehow undermined the general application of the *Nitrogen limitation hypothesis*.

In the context of plant-herbivorous insect-natural enemy tri-trophic interactions, the overall effect of plant nitrogen nutrition on insect herbivores (either at individual or population scale) is even more complex because there are indirect and direct effects of plant nitrogen on herbivore natural enemies. (1) indirect effects result from the trophic cascade mediated by the herbivores (Li and Jackson 1997; Francis et al 2001). For instance, the development time of immature parasitoids is typically positively related to host sizes, which are related to plant nutritive values (Chen et al. 2010). Adult parasitoids are also likely indirectly responsive to the plant nitrogen status through their fecundity that depends on the hosts on which they feed (Thompson 1999). Thus, the fitness of predators depends on the quality of preys, which in turn depends on plant nutritive value. For instance, the spider *Portia fimbriata* had greater survival when provided with preys composed of intraguild spiders, compared to those provided with nitrogen-poor herbivorous insects (Li and Jackson 1997). (2) direct effects of plant N on the fitness of enemies occurs when plants offer food (sugar, pollen, nectar etc.) and shelter to natural enemies (Wäckers and vanRij 2005; Portillo



et al. 2012). Indeed, the enhancement of many life-history parameters such as longevity, fecundity and movement, was observed when natural enemies fed on such plant foods (Rahat et al. 2005; Sivinski et al. 2006; Irvin and Hoddle 2007). Thus, any variation in the production of these foods due to plant nitrogen fertilization is likely to affect these life-history parameters. However, the effects of nitrogen inputs on nectar production vary among plant species, and may interact with other nutrients (Burkle and Irwin 2009). At last direct plant N effects on predation/parasitism rates can result from (1) changes in plant morphological variables (plant size, trichome density and distribution etc.) that affect the foraging behavior of enemies, or (2) changes in HIPV profiles likely to repel pests and attract their natural enemies (for review, see Chen et al. 2010).

### **III.1.1.2) Water**

Insects, like other animals, need water to ensure survival and normal growth and reproduction. They may utilize plant-derived water as well as dew drops or other sources of free liquid water (Schoonhoven et al. 2005). Plants exposed to insufficient water (drought) may not only restrict water inputs to herbivorous insects, but may also undergo physiological changes that make them unsuitable for insect feeding (Inbar et al. 2001). Likewise, plants exposed to excessive water (water logging) have reduced growth due to nutritional deficit (Steffens et al. 2005), and have sub-optimal quality as food for herbivorous insects.

An increase of the frequency of drought events has been predicted due to climate changes (Dai 2013). However, drought influence on plant-herbivorous insect interactions remains poorly understood. Plant water content has been considered an important nutritional quality index for many Lepidopteran larvae (Gutbrodt et al. 2011). Drought-mediated changes in plant nutritive value and defense chemical concentration might have contradictory effects on insect herbivores. Firstly, the edibility of plant tissues decreases when the foliage tends to wilt, because insects face difficulties in obtaining enough water from wilted tissue (Schoonhoven et al. 2005).

Furthermore, water not only acts as an indispensable ingredient for herbivore metabolism but it also transports other nutrients (amino acids, soluble carbohydrates etc.) (Douglas 2003; Huberty and Denno 2004). Therefore, herbivores feeding on drought stressed plants might suffer a nutrient deficit. Secondly, drought can markedly alter the resistance to insect herbivory through decreases in the concentration of defense metabolites in plant tissues (Gutbrodt et al 2011).

Generally, the effects of drought depend primarily on insect feeding strategies and feeding specialization as well as on the modalities of drought (Inbar et al. 2001; Huberty and Denno 2004; Mody et al, 2009; Gutbrodt et al. 2011). Feeding strategies (sucking, chewing, mining etc.) show different responses to drought. Generally, leaf miners and chewers are considered more sensitive than phloem feeders to drought (Larsson 1989; Inbar et al. 2001). The **Plant vigor hypothesis** (Price 1991) and the **Plant stress hypothesis** (White 1993) were proposed to explain this difference. The former predicts that herbivores perform better on rapidly-growing, i.e., vigorous, plants (Price 1991), and this hypothesis has been supported particularly in leafminer and leaf chewer (Inbar et al. 2001; Han et al. 2014). The latter predicts that water-stressed plants are more suitable hosts for senescence-feeders that feed preferentially on overmature and senescing tissues (White 2009). Drought modalities are also determinants of the consequences on herbivorous insects. The **Pulsed Plant Stress Hypothesis (PPSH)** has been proposed to explain discrepancies in the effect of plant water stress on herbivores, an outbreak *in situ* (White 1969; Mattson and Haack 1987) versus depression in the laboratory (Huberty and Denno 2004). PPSH predicts that sap-feeder populations (e.g., aphids or whiteflies) respond positively to plants that are under discontinuous stress where bouts of stress and recovery of turgor allow sap-feeders to benefit from stress-induced increases in plant nitrogen, however, sap-feeders may be negatively affected by continuous water stress. At last, the outcomes of drought on herbivorous insects also depend on feeding specialization even if they adopt the same feeding strategy (Gutbrodt et al. 2011). A lower feeding speciality in insects (i.e., generalist herbivores) may benefit their performance when plants suffer drought.

According to this context, this PhD study will focus on some questions that remain partially answered. => QUESTIONS: **How some variations in fertilization and irrigation processes may affect the performance of herbivorous insect? Do herbivorous insects from different feeding guild (e.g., phloem-feeder and leafminer) respond differently to these variations? Which mechanisms underlie the observed bottom-up effects of nitrogen and water input on herbivorous insect?**

### **III.1.2) Bottom-up forces mediated by intrinsic factors**

#### **III.1.2.1) Plant tolerance traits to environmental stresses**

Environmental stresses to plants/crops have been predicted to become more severe and widespread (Dickie et al. 2014). Plants are subject to various abiotic stresses mainly including drought, flood, salt, cold, heat and even pollution (Kozłowski and Pallardy 2002). However, they have evolved various responses and adaptations these deleterious environmental stresses during their development and growth. These plant adaptive traits in plants are involved in the consequences of environmental stresses on plant-herbivorous insect interactions.

Among stresses, water deficit (i.e., drought) attracted most attention due to global climate changes. Water deficit is a factor that restricts carbon availability to plants, through limited assimilation by photosynthesis and through assimilates partitioning (Chaves 1991). However, plants have the capacity to overcome moderate water limitations. In the long term, many species are able to adapt the tissue structures involved in water uptake and transportation, or to enhance the water use efficiency (WUE) in response to water limitation (Chaves 2009; Galmés et al. 2013). Furthermore, the reduced growth rates due to water limitation result in lower plant heights and restricted leaf areas that limit transpiration and help plant resisting water restrictions. Indeed, Osório et al (1998) found that water deficits are more important in delaying plant growth than in changing patterns of carbon allocation in an

evergreen tree species.

Nowadays, the improvement of drought tolerance traits in crops has become a crucial objective. For instance, genome editing technology has been recently utilized to allow modifying the regulation of key genes involved in drought tolerance while maintaining productivity (Shan et al. 2013).

Nitrogen deficiency is another limiting factor to plant growth (Colnenne et al. 2002; Zhao et al. 2005). In agriculture, nitrogen fertilizers have been widely applied to crops in adequate quantities. The use of nitrogen fertilizers continues to increase worldwide but has remained relatively constant for the past 15 years in Western Europe and United States. However, the over-use of nitrogen fertilizers can increase nitrate concentrations in surface and ground waters, which likely lead to unwanted environmental problems such as eutrophication (Yang et al. 2008). This over-use has decreased the nitrogen use efficiency (NUE, expressed as grain production per unit of applied nitrogen) in all countries (Hatfield and Prueger 2004). Thus, improvements of the nitrogen-deficiency tolerance (NDT) and NUE traits are considered important objectives for breeding research. For instance, QTL (Quantitative Trait Loci) mapping techniques were used to highlight the genetic mechanism involved in the observed correlations between NDT and NUE traits in rice, and could be used as targets to improve NDT and NUE traits in future breeding programs (Wei et al. 2012).

Nevertheless, in modern agriculture, diverse plant cultivars (hereafter referred as *resistant cultivars*) have already been selected to cope with sub-optimal environments. Plant-adaptive traits against various abiotic stresses (e.g., water and N limitations) involve complex physiological mechanisms (Cattivelli et al. 2008; Farooq et al. 2009; Feng et al. 2010). For instance, enormous efforts have been directed to improve the drought-adaptation in plants by increasing the water-use efficiency (Galmés et al. 2013). Acclimation to drought conditions may comprise long-term adjustments involving gene expression and modification of plant physiology, biochemistry, morphology and anatomy (Chaves et al. 2002; Flexas et al. 2006; Chaves et al. 2009).

However, to date, it is unclear how these adaptations in resistant cultivars might affect the bottom-up effects of drought on herbivorous insects; in contrast, in

susceptible cultivars, drought generally compromises herbivorous insects (Inbar et al. 2001). Therefore, the use of plant cultivars with drought-resistance or nitrogen-deficiency tolerance trait, may be a valuable to understand the bottom-up effects of water and N inputs on plant-herbivorous insect interactions.

=> QUESTIONS: **If we found bottom-up effects of nitrogen and water on a given herbivorous insect species on the plant cultivar that susceptible to nitrogen- and water- related stresses, what could happen to these bottom-up effects on the plant cultivar that tolerate these stresses? For example, if drought affected the performance of a leafminer on plant cultivar A (i.e., susceptible to drought), will the same effect be observed on the cultivar B (i.e., drought tolerant)?**

### **III.1.2.2) Plant resistance to herbivorous insects**

Herbivorous insects utilize various feeding strategies to obtain the nutrients or useful compounds they require. Meanwhile, plants respond to herbivory using various mechanical defenses interfering with insect behaviors, and also chemical defenses with the production of a set of toxins and defensive compounds intended for disrupting insect physiological processes (Howe and Jander 2008). Herbivory-challenged plants can also produce and emit volatile compounds that attract herbivore natural enemies and thus enhance their resistance level. Such interactions have served as important selective forces in the evolution of herbivore diet breadth as well as plant anti-herbivory mechanisms (Schoonhoven et al. 2005; Agrawal 2006). Overall, these traits in plants can be classified according to the mechanisms involved in the resistance to herbivory.

*Mechanical vs. chemical defense* - Mechanical defenses consist mainly in physical barriers, such as spines, thorns, waxy cuticle, trichomes and sticky glands that hinder feeding and other herbivore behaviors. For example, trichomes occur on plant surfaces and thus are the first direct protection against herbivores (Simmons and Gurr 2004). Trichomes were classified as glandular and non-glandular (Luckwill

1943). The former have glandular heads that secrete and accumulate 1) sticky materials to hinder insect walking behaviors and 2) toxic chemicals as insecticides. The latter are devoid of heads and serve as mere mechanical obstructers to insect movements (Simmons and Gurr 2004). Plants accumulate a large range of toxic secondary metabolites as chemical defense against herbivory. A great variety of small molecules with toxic and antifeedant effects have been revealed, such as terpenoids, alkaloids, as well as other plant secondary metabolites including furanocoumarins, cardenolides, tannins, saponins etc. (Howe and Jander 2008). Alkaloids form one of the most widely distributed secondary metabolite group notably represented in Solanaceae family, and have been considered highly efficient against a wide range of herbivorous insects (Levinson 1976; Vrieling and Macel 2002). Aside from secondary metabolites, plants synthesize also defensive proteins that exert direct effects on herbivorous attackers. For example, proteinase inhibitors (PIs) impair various classes of digestive proteases in the insect midgut, resulting in amino acid deficiencies that impair herbivore growth and development (Green and Ryan 1972; Zavala et al. 2004; Han et al. 2012).

*Constitutive vs. induced defense* - By definition, constitutive defenses are always existent, irrespective of herbivore presence (Howe and Jander 2008). For instance, reproductive plant tissues typically accumulate large amounts of defensive proteins and metabolites irrespective of the herbivory threat. These traits can be either chemical or mechanical. In contrast, induced defenses are triggered by herbivory. The ability of plants to recognize and respond defensively to insect attack was discovered in the 1970s (Green and Ryan 1972; Haukioja and Hakala 1975). It relies on the innate ability of each cell to perceive danger and to transmit signals systemically in order to defend against the ongoing and possible future attacks (Howe and Jander 2008). Responses to herbivory may be mediated by the jasmonic acid (JA) or salicylic acid (SA) signaling pathways (Kessler and Baldwin 2002). However, these chemical defenses have long been thought to be costly for plants because of the resources consumed in their biosynthesis (i.e., allocation cost) (Herms and Mattson 1992; Purrington 2000; Wittstock and Gershenzon 2002). So many plants employ induced

defense probably because such defense has various benefits including it is a means of minimizing allocation costs (Agrawal and Karban 1999).

*Direct vs. indirect defense* - It is widely accepted that plant defense can be classified as direct or indirect defense (Karan and Baldwin 1997; Lou et al. 2006). The mechanical and chemical defenses evoked above are clearly direct defenses against herbivores, but plants can also protect themselves indirectly through the recruitment of parasitoids and predators (Thaler 1999; Kessler and Baldwin 2001). Indeed, some plant species produce constitutively volatile organic compounds that attract herbivore enemies (Khan et al. 1997). Indirect defense has received increasing interests, with a promising focus on induced indirect defence. Indeed, plants may synthesize specific semiochemicals called **herbivore-induced plant volatiles** (HIPVs) that act as repellents for herbivorous pests and as attractants for organisms antagonistic to these pests (Khan et al. 2008, Dicke et al 2009). Several studies revealed the existence of herbivore-specific elicitors of the production of HIPVs (Mattiacci et al. 1995; Alborn et al. 1997; Halitschke et al. 2001).

In addition to naturally-occurring plant defenses, insect-resistance traits have been artificially introduced into plants since the eighties, using modern transgenic techniques. *Insect-resistance genetically modified* (IRGM) crops are mainly based on *Bacillus thuringiensis* (*Bt*) endotoxins, but other modifications include proteinase inhibitors, such as bovine trypsin inhibitor (Christeller et al. 2002), snowdrop agglutinin (Hilder et al. 1995) and cowpea trypsin inhibitor (CpTI; Felton and Gatehouse 1996). IRGM is the second most widespread trait after herbicide resistance that has been introduced into GM crops (O'Callaghan et al. 2005). The adoption of *Bt* crops has been shown efficient against given pests of certain crops and to reduce considerably the use of pesticides for these crops (Lu et al. 2012). Thus, *Bt* crops have been viewed as cost-efficient and environmentally sound (Romeis et al 2006), even though their wide adoption might result in environmental concerns. Indeed, the potential non-target effects of *Bt* protein on beneficial organism is viewed as a major concern (O'Callaghan et al. 2005; Andow and Zwahlen 2006; Romeis et al. 2008; Duan et al. 2010).

=> QUESTIONS: **What are the roles of plant defensive traits, either natural-occurring or genetically-modified, that play in predicting the bottom-up effects of nitrogen and water inputs to plants on herbivorous insects as well as their natural enemies?**

### **III.2) Top-down forces**

These forces relate to the effects exerted by organisms from the higher trophic levels on herbivorous insects. Top-down regulation of herbivores by predators, parasitoids or entomopathogens, is pervasive and can lead to trophic cascades that release plants from herbivory (Costmagna et al 2007). The top-down forces are influenced by a set of biotic factors including **interspecific interactions** and **habitat management**.

**Intraguild predation (IGP)**, i.e., trophic interactions among members of a same functional guild,, has been considered important for the natural control of a given herbivorous species (Polis et al. 1989). The attack of generalist parasitoids by coccinellids is the most common IGP case. Overall, IGP can enhance (Cardinale et al. 2003), disrupt (Persad and Hoy 2004; Philpott et al. 2004; Rosenheim et al. 2004a), or be neutral (Lang 2003; Snyder and Ives 2003; Rosenheim et al. 2004b; Costamagna et al. 2007) on naturally occurring biological control. **Hyperparasitism** is another important specific interaction that may weaken top-down forces. It occurs when the parasite of a target herbivorous insect is the host of another parasite (Schooler et al. 2011).

Provision and manipulation of shelter habitat features within the agroecosystems may also significantly influence the top-down forces (Landis et al. 2000). The goal of **habitat management** (or biological control services). is to create a suitable ecological infrastructure within the managed agricultural ecosystems by providing food sources such as alternative preys or hosts, and shelter to natural enemies (Landis et al. 2000), with the aim to enhance the top-down forces that control herbivorous insects. The



selection criteria for choosing plants in habitat management programs mainly rely on their attractiveness for natural enemies, on the production and accessibility of pollen and/or nectar, on seed availability and also on the occurrence of previous successes (for review, see Fiedler et al. 2008). For instance, two tussock-forming grasses, *Dactylis glomerata* and *Holcus lanatus*, were highlighted as best overwinter habitat for aphid predators (Thomas et al. 1991; Wratten et al. 1998).

### **III.2.1) Predation**

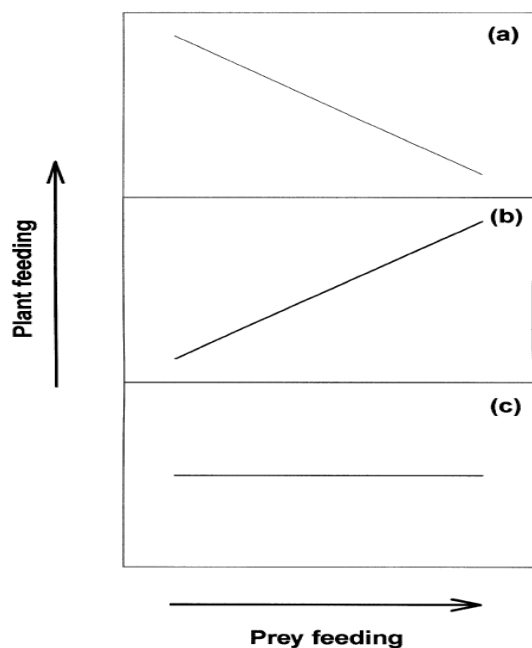
Most arthropod predators are generalist, which means that they have a wide range of preys. **Generalist predators** can play an important role in controlling herbivore populations. Compared to the specialist natural enemies used in agriculture for biological control, especially parasitoids, the importance of generalist predators in biological control programs (e.g., conservation or augmentative biological control) is increasingly acknowledged (Symondson et al. 2002). There is no doubt that generalist predators can be efficient against indigenous and exotic pests (Rosenheim et al. 1993; Snyder and Ives 2003). This is why inter- and intraspecific interactions between generalist predators and interactions between generalists and specialists need to be fully investigated to ensure a success of biological control (Symondson et al. 2002). Intraguild predation exerted by generalist predators on parasitized larvae or eggs as well as other predatory species may disrupt the efficacy of parasitism (Michaud 2004; Gagnon et al. 2011). For instance, *Tamarixia radiate*, a highly host-specific parasitoid species to the Asian citrus psyllid *Diaphorina citri*, has been showed to suffer heavy mortality from intraguild predation by Coccinellids (Michaud 2004).

Omnivory plays an important role in community dynamics (McCann et al. 1998; Eubanks and Denno 2000; Sinia et al. 2004). Indeed, omnivorous predators prey on herbivorous insects and also feed on plants which can cause damage (Janssen et al. 2003). Most Heteroptera species are considered as **omnivorous predators** which need to acquire nutrients and energy from their host plants as well as from insect preys (Janssen et al. 2003). Omnivorous predators are increasingly exploited for

biological control in the sustainable integrated pest management (IPM) because of their high efficacy in the suppression of pest populations (Castañé et al. 2011). Many Heteroptera species seem omnivorous predators that need to acquire nutrients and energy from both host plants and insect preys (Janssen et al. 2003), but the frequency of this behavior has not been precisely assessed (Naranjo and Gibson 1996; Coll 1998; Kaplan and Thaler 2011). Before the release and augmentation of omnivorous predators use in biological control programmes, establishing to which extent they may damage crops appears crucial (Castañé et al. 2011).

Because omnivorous predators might put crops at risk, it is essential to fully understand the functional relationships between prey and plant feedings. These relationships founded three hypothetical models, namely the *switching*, *facilitating* and *independence* models (Gillespie and McGregor, 2000). In the switching model, plants and preys are alternative foods for predators. Thus, the prey feeding should increase when plant feeding decreases due to low plant quality, resulting in inverse relationships between plant and prey feedings (**Fig. 5a**). In the facilitating model, plants contain key components (nutrients or water) that facilitate prey feeding through digestion or assimilation and thus, the prey feeding rate increases as plant feeding rate increases. It was suggested that omnivorous Heteroptera need plant water to produce the saliva required for the extra-oral digestion of preys (Cohen 1995, 1998; Sinia et al 2004). Thus, in the facilitating model, positive relationships between plant and prey feedings are expected (**Fig. 5b**). In the independence model, by definition, there is no relation between prey and plant feedings (**Fig. 5c**).

The performance and feeding ecology of omnivorous predators may be influenced by bottom-up effects mediated by resources input. => QUESTIONS: **What are the bottom-up effects of nitrogen and water inputs to plants on the performance of omnivorous predators as well as their feeding ecology (i.e., plant feeding vs. prey feeding)?**



**Figure 5:** Three models of feeding behavior in predatory Heteroptera according to Gillespie and McGregor (2000): **(a)** The amount/frequency of plant feeding decreases with increased prey feeding (Switching hypothesis). This may occur when lower plant quality do not fill predator needs; **(b)** The amount/frequency of plant feeding increases with prey feeding (Facilitating hypothesis). This may occur when plant feeding is required to facilitate prey feeding; **(c)** The amount/quantity of plant feeding has no relation with prey feeding (Independence hypothesis).

### III.2.2) Parasitism

Parasitoids are defined as organisms that spend a significant portion of their life history attached to or within a single host organism and ultimately kill the host. Insect parasitoids (hereafter referred to *parasitoids*) occur in five orders of holometabolous insects: Hymenoptera, Diptera, Coleoptera, Lepidoptera, and Neuroptera (Eggleton and Belshaw 1992). Among these orders, Hymenoptera parasitoids account for nearly 78% of the estimated number of total parasitoid species (Feener and Brown 1997). Parasitism has a great diversity of forms and it has been proposed to categorize parasitoids into two major groups according to their effects on host development (Askew and Shaw 1986). **Koinobionts** allow the host to develop whereas **idiobionts** stop host development and larvae usually feed externally. Moreover, koinobionts can be subdivided further into **endoparasitoids** and **ectoparasitoids** which develop within and outside the host body, respectively. In addition, it is common for a parasitoid itself to serve as host to another parasitoid's offspring, which is thus termed **hyperparasitism** (Sullivan and Völkl 1999). Another type of parasitism is

**superparasitism** which refers to a female parasitoid laying an egg in a host already parasitized by a conspecific (Hamelin et al. 2007).

Due to the strong top-down forces they exert on herbivorous insect populations, parasitoids have been extensively used as efficient biological control agents against a wide range of pest insects in the augmentative or classical biological control programmes (van Lenteren 2000; Boivin et al. 2011; Ragsdale et al. 2011; Chailleux et al. 2013 a,b; Biondi et al. 2013). From a pest biological control perspective, the purchase and release of natural enemies to target pest insects is often called *augmentation*. Augmentation has been applied notably with the egg parasitoid *Trichogramma* sp. for controlling various types of moths. However, parasitoid use is limited by their high host specificity. This is especially true when various pest insects occur simultaneously. In such case, the combined use of several parasitoid species has been recommended (Salin et al. 2011), but attention should be paid to the occurrence of inter-specific competition (Sidney et al. 2010). Another strategy targeting multiple pests suggests the combined use of parasitoids with generalist predators (Chailleux 2013a).

### **III.2.3) Disease/infestation**

In addition to the use of predators and parasitoids, it is also possible to use specific micro-organisms for the biological control of arthropod pests. Named **entomopathogens**, they include entomopathogenic nematodes, fungi, bacteria and viruses, which all contribute substantially to the natural suppression of arthropod populations.

*Entomopathogenic fungi* –This major group of natural enemies can develop dramatic epizootics that lead to rapid declines in arthropod host populations (Pell et al. 2010). Thus they contribute substantially to the natural regulation of many insect and mite species. For example, the gypsy moth, *Lymantria dispar* (Lepidoptera: Erebidæ) populations in North America are regularly controlled by *Entomophaga maimaiga* (Entomophthorales: Entomophthoraceae) (Hajek 1999), the cotton aphid, *Aphis*

*gossypii* Glover (Hemiptera; Aphididae) populations by *Neozygites fresenii* (Abney et al. 2008) and multiple aphid populations by *Pandora neoaphidis* (Entomophthorales: Entomophthoraceae) (Pell et al. 2001). However, to increase the top-down forces of these organisms on herbivorous arthropods, further investment on conservation biological control by manipulation of both the crop environment and also habitats outside the crop is required (Pell et al. 2010). Factors of interest include biotic environment (e.g., humidity), pesticide applications, burning of crop residues, occurrence of alternative hosts or interaction with wider natural enemy communities.

*Entomopathogenic bacteria* – Most insect bacterial pathogens belong to Bacillaceae, Pseudomonadaceae, Enterobacteriaceae, Streptococcaceae and Micrococcaceae. Among Bacillaceae, *Bacillus thuringiensis* (*Bt*), a widespread soil bacteria, can kill a wide range of insect orders. The crystal-forming  $\delta$ -endotoxins produced by *B. thuringiensis* are the most widely used biologically produced insecticides (Cannon 1996; Schnepf et al. 1998; De Maagd et al. 1999). However, evolution of resistance by pest insects threatens the continued effectiveness of *Bt* protein in sprays and transgenic crops (Ferre and van Rie 2002; Wu and Guo 2005; Gassmann et al. 2009; Tabashnik et al. 2009).

*Entomopathogenic nematodes* – Two families, Steinernematidae and Heterorhabditidae, aroused most of interest in nematode use for the biological control of arthropod herbivores. The free-living, non-feeding infective juveniles of these nematodes possess the characters of both insect parasitoids or predators and microbial pathogens: they have chemoreceptors and are locomotive as predators and parasitoids; they are virulent. As pathogens, nematodes can be cultured easily *in vitro* and they have a high reproductive potential (Kaya and Gaugler 1999).

*Entomopathogenic virus* – Baculoviruses have received the most research interest and commercial development (Huber 1986; Miller 1997; Lacey and Shapiro-Ilan 2008). The narrow host ranges of most entomopathogenic viruses can be both a limitation and advantage depending on the community structure of arthropod pests in a given crop, as well as on the regulation exerted by insect predators and parasitoids and broad-spectrum insecticides applications (Lacey and Shapiro-Ilan 2008).

#### **IV) Objectives**

The objectives of this thesis are

- i. to highlight the interest of IPM to control pests in agro-ecosystems (chapter 2);
- ii. to study the bottom-up effects of nitrogen/water inputs to crops, on the biological traits of herbivorous insects (chapter 3);
- iii. to further study how the environmental stress tolerance traits (nitrogen and water limitations) in plants influence bottom-up effects on herbivorous insect species from different feeding guilds (chapter 3);
- iv. to examine further how they affect the performance of omnivorous predators, through their feeding behavior, i.e., plant vs. prey feeding (chapter 3) ;
- v. to study how the combined effects of bottom-up forces (i.e., nitrogen/water inputs) and top-down forces (i.e., natural enemies) affect the population dynamics of herbivorous insects under both greenhouse and field conditions (chapter 4).

## **Chapter 2: Integrated Pest Management (IPM) in agro-ecosystems**

## I) IPM in agro-ecosystems

The integrated pest control concept appeared for the first time in a publication by Stern et al. (1959). It served as foundation for the *integrated pest management* (IPM) today. IPM is the core part of integrated production (IP) that involves pest, disease and weed managements (Boller et al. 2004), and it is also part of the sustainable agriculture concept which replaces potentially polluting inputs by the use of natural resources and regulations (Wijnands et al. 2012; Biondi 2013).

Among the many definitions of IPM, we present below the technical definition proposed by the University of California (UC Davis, Statewide Integrated Pest Management Program. <http://www.ipm.ucdavis.edu/GENERAL/whatisipm.html>).

*“Integrated pest management (IPM) is an ecosystem-based strategy that focuses on long-term prevention of pests or their damage through a combination of techniques such as biological control, habitat manipulation, modification of cultural practices, and use of resistant varieties. Pesticides are used only after monitoring indicates they are needed according to established guidelines, and treatments are made with the goal of removing only the target organism. Pest control materials are selected and applied in a manner that minimizes risks to human health, beneficial and non-target organisms, and the environment.”*

### I.1) Biological control

Under the IPM framework, the biological control of agricultural endemic or invasive pests, has developed as an independent goal since the nineties (van Driesche 1994). Such natural pest control services are delivered using a wide community of arthropod predators, parasitoids and entomopathogens that naturally occur in the agro-ecosystems or are artificially introduced. Depending on the features of the target pests and on the way biological control is implemented, three types of pest biological control programmes are widely accepted:

*Classical biological control* – It relies on the artificial introduction and



augmentation of biocontrol agents issued from the area where invasive species originate. Such control often requires long periods (5-20 years) to induce changes in the arthropod population and for the community responses to reach a stable end point. However, when successful, the classical biological control brings long-term protection to native biodiversity and ecosystems without recurrent costs (van Driesche et al. 2010).

*Conservation biological control* – It is the deliberate manipulation of agro-ecosystems to enhance the survival, fitness, and behavioral performances of natural enemies, and thus improve their efficacy against pests (Barbosa 1998; Landis et al. 2000). This strategy can be accomplished by habitat management in which plant-based resources in the landscape are manipulated to facilitate the natural pest control by natural enemies (Landis et al. 2000). The actions involve the establishment of specific plants that provide natural enemies with extra resources (pollen, nectar, alternative hosts) and shelters (Landis et al. 2000; Fiedler et al 2008). More and more attention is being paid worldwide to conservation biological control, because it can control primary and secondary pests, and reduces the likelihood of pest outbreaks and resurgence (Naranjo and Ellsworth 2009). Similarly to classical biological control, it is a sustainable strategy that does not contribute to insecticide resistance, does not put human health at risk, and is environmentally friendly (Wyckhuys et al 2013).

*Augmentative biological control* – It refers to all forms of biological controls in which natural enemies are periodically re-introduced to increase the natural population. It usually relies on the commercial mass production and release of selected biological control agents (Biondi 2013; van Lenteren 2006). There are two main approaches to augmentation under greenhouses: inundative and inoculative releases. Inundation consists in the release of large numbers of natural enemies for the immediate regulation of pests. Inoculation consists in the periodic release of small enemy numbers throughout the crop growing season, starting when the pest population is low. The released natural enemies are expected to reproduce themselves to exert long-term pest management by their offsprings (van Lenteren 2012).

## **I.2) Compatibility of Insect-Resistance Genetically Modified (IRGM) crops with biological control**

Interactions between biological control agents (insect predators, parasitoids, and pathogens) and IRGM crops have involved not only the complex toxicological relationships, but also the prey/host quality mediated indirect effects on performance of natural enemies (Lundgren et al. 2009a). Hence, to meet the goal of IPM, the compatibility of IRGM crops and biological control agents or natural enemies become a critical subject. Interactions between IRGM crops and natural enemies can be additive (neutral), synergistic (positive) or antagonistic (negative), and obviously, the antagonistic effect should be avoided for IPM implementation (Bernal et al. 2004).

When considering toxicological relationships, these effects could be due to toxicity exposure via feeding on plant materials, or toxin accumulation in tri-trophic plant-herbivore-parasitoid/predator interactions (Poppy and Sutherland 2004; Romeis et al. 2006). Carnivorous insects (most predatory insects) may only encounter the latter pathway, but omnivorous predators, due to their phytophagy, may be confronted with both pathways. Numerous studies have examined the potential impact of IRGM crops on natural enemies using various approaches: toxicity-based, tri-trophic interaction-based, community-based and meta-data based (reviewed in Lundgren et al. 2009a). An overall consensus has been achieved that IRGM crops can be very compatible with biological control if IRGM crops have been employed properly as part of an IPM philosophy. On the one hand, natural enemies must be preserved to control the population dynamics of the pest species that are not targeted by IRGM crops. On the other hand, if natural enemies properly preserved, these agents may delay the onset of resistance to IRGM crops in target pest species.

## **II) IPM: endemic and invasive pest insects**

**Endemic pest insects** are the species that are historically established in an area where the crop damage by this species has reached Economic Injury Level (EIL).

With the extensive commercial growth of IRGM crops worldwide, special attention should be paid to the non-target pest, generally referred to the “secondary pests”, which are likely to increase with time because of two factors: (1) the general non-targeting by transgenic endotoxins; (2) the decreased pesticide application. For instance, in Bt cotton field, secondary pests could potentially evolved into primary pests if there is no addition control strategies (Xu et al. 2008; Men et al. 2008; Lu et al. 2010).

**The cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphidiae) (Fig. 6)**, which is a pest non-targeted by *Bt* protein (as other aphids, e.g., see Ramirez-Romero et al. 2008, Romeis and Meissle 2011), is considered as secondary pest insect in the Yangtze River Valley Cotton-growing region in central China. Despite that cotton aphid populations have shown continuous decline in density in cotton fields in the past 15 years in Northern China (Lu et al. 2012), cotton aphid outbreaks may occur and reach economically damaging levels (Wu and Guo 2005) owing to specific weather conditions (e.g., less rainfall during aphid population-growing season) or resistance to pesticides (Yi et al. 2012).

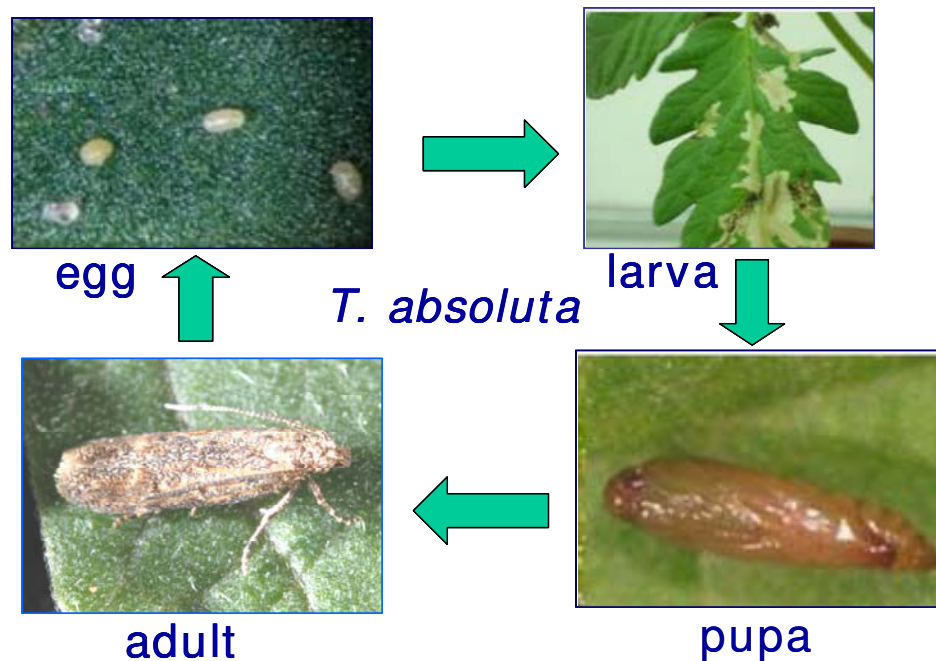
**Invasive** (i.e., Exotic, alien) **pest species** are organisms (plants, animals, and microorganisms) that are not native to a particular region, and are able to rapidly colonize a new area and become serious pests, often because they are no longer under natural control of natural enemies in their new habitat. Generally, invasive arthropod pest may cause various environmental and economic problems: serious damages to the agricultural production, environmental pollution due to overuse of insecticide, increased management costs and increased administrative costs for the exportation of products from this area. The management of invasive pests has largely relied on classical biological control programs (van Drische 1994; van Drische et al. 2010), or though inoculative or inundative releases of biological control agents into the system in an augmentative biological control program (Obrycki and Kring 1998; van Lenteren 2006; van Lenteren 2012).

**Figure 6:** *Aphis gossypii* infestation on cotton young leaves. (Photo by HP)



**The tomato leafminer, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) (Fig. 6)** is an invasive insect species from South America. Its life-cycle comprises four development stages: egg, larva, pupa and adult (see **Fig. 7**). Adults usually lay eggs on the leaves or stems, and to a lesser extent on fruits. After hatching, young larvae penetrate leaves, aerial fruits (like tomato) or stems, on which they feed and develop (Desneux et al. 2010). It has been considered as a major pest threatening the tomato production worldwide, and it has rapidly invaded the Mediterranean basin since it first appeared in Spain in 2006 (Desneux et al. 2010, 2011; Chailleux et al. 2013a, b; Biondi et al. 2013). At the arrival of this species, the management tactics have largely relied on extensive pesticide applications, which ultimately lead to the disruption of existing Integrated Pest Management (IPM) systems (Desneux et al. 2010). Thus, the ongoing invasion of *T. absoluta* and its devastating outcomes on tomato production has prompted applied research on integrated management of this invasive species, e.g., biological control (Biondi et al. 2013; Chailleux et al. 2013a, 2013b; Ingegno et al. 2013; Zappalà et al. 2013; Mollá et al. 2014; Parolin et al. 2014).

**Figure 7:** Life cycle of *Tuta absoluta*. The larvae infest mainly leaves, but also stems and fruits. (follow JENDs poster by Han et al. 2013)



*Tuta absoluta* is the major pest species that studied in the current PhD study. Recently, the new **Access and Benefit Sharing** (ABS) procedures under the **Convention of Biological Diversity** (CBD) have already made it difficult to collect and introduce alien biological control agents for biological control research and/or commercial use (Cock et al. 2010). Thus, prior to the studies on bottom-up effects of nitrogen and water inputs on *T. absoluta*, or interactions between *T. absoluta* and their natural enemies (chapter 3 and 4), it is necessary to firstly identify indigenous natural enemies targeting this pest in Southern France. Here, we present our work regarding the field sampling and identification of key natural enemies in Southern France (**Article 1**).

# Article 1

## **Indigenous natural enemies attacking *Tuta absoluta* (Lepidoptera: Gelechiidae) in southern France.**

Biondi A <sup>1,2\*</sup>, Chailleux A <sup>1,3</sup>, Lambion J <sup>4</sup>, Han P <sup>1</sup>, Zappalà L <sup>2</sup>, Desneux Nicolas<sup>1</sup>

<sup>1</sup> French National Institute for Agricultural Research (INRA), UMR1355, 400 Route des Chappes, 06903, Sophia-Antipolis, France

<sup>2</sup> Department of Agri-food and Environmental Systems Management, University of Catania, Via Santa Sofia 100, 95123 Catania, Italy

<sup>3</sup> InVivo Agro-Solutions, 83 Avenue de la Grande-Armée, 75582 Paris 16, France

<sup>4</sup> Groupe de Recherche en Agriculture Biologique (GRAB), BP 11283, 84911 Avignon, Cedex 9, France

Corresponding author: [antonio.biondi@unict.it](mailto:antonio.biondi@unict.it)

## Indigenous Natural Enemies Attacking *Tuta absoluta* (Lepidoptera: Gelechiidae) in Southern France

Biondi<sup>\*,\*\*</sup>, A.; A. Chailleux<sup>\*,\*\*\*</sup>; J. Lambion<sup>\*\*\*\*</sup>; P. Han<sup>\*</sup>; L. Zappalà<sup>\*\*</sup> and N. Desneux<sup>\*</sup>

<sup>\*</sup>French National Institute for Agricultural Research (INRA), 400 Route des Chappes, 06903 Sophia-Antipolis, France

<sup>\*\*</sup>Department of Agri-food and Environmental Systems Management, University of Catania, Via Santa Sofia 100, 95123 Catania, Italy. E-mail: antonio.biondi@unict.it

<sup>\*\*\*</sup>In Vivo Agro-Solutions, 83 Avenue de la Grande-Armée, 75582 Paris 16, France

<sup>\*\*\*\*</sup>Groupe de Recherche en Agriculture Biologique (GRAB), BP 11283, 84911 Avignon, Cedex 9, France.

(Received: April 24, 2013 and Accepted: May 22, 2013)

### ABSTRACT

The South American tomato leafminer, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is a serious worldwide threat to tomato industry. Since it has spread to the Afro-Eurasia, it was responsible of extensive damage and its control mainly relied on chemical insecticide applications. In this context, a survey of natural enemies attacking *T. absoluta* spontaneously was conducted in Southern France. Infested tomato leaves were collected from protected tomato crops in seasons 2011 and 2012. Three species of mirid predators [*Macrolophus pygmaeus* (Rambur), *Nesidiocoris tenuis* (Reuter) and *Dicyphus* sp.] were found preying on eggs and young larvae of the moth in both seasons of sampling. Eggs were also attacked by two trichogrammatid species, *Trichogramma achaeae* Nagaraja & Nagarkatti and *Trichogramma* sp. Whereas, *T. absoluta* larvae were parasitized by four parasitoid species belong to the families; Braconidae (*Bracon nigricans* Szépligeti), Eulophidae [*Neochrysocharis formosa* (Westwood), *Stenomesus* sp. near *japonicus* and *Necremnus* sp. near *artynes*]. Although further studies are needed to assess the actual role of these species in *T. absoluta* control, obtained results represent the first step towards developing biological and integrated management strategies against this pest in France. Furthermore, these results stress the need of conservation strategies of indigenous natural enemies to control the exotic pest species.

**Key words:** *Tuta absoluta*, invasive pest, predators, parasitoids, biological control, France.

### INTRODUCTION

Alien invasive pests represent a serious global threat to agronomic ecosystems and their economic and ecological effects on agricultural producers are widely recognized (Desneux, *et al.* 2011 and Ragsdale, *et al.* 2011). Indeed, invasive species can notably reduce yields and increase costs related to their management, generally resulting in increased reliance on pesticides (Liang, *et al.* 2012 and He, *et al.* 2013). The tomato leafminer, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is an invasive pest, native to South America, where it is considered one of the most devastating pests of tomato (Desneux, *et al.* 2010 and Gontijo, *et al.*, 2013). Although it is also able to attack and cause damage on different genera and species of the Solanaceae plants, this species has a particular preference for tomato (*Solanum lycopersicum* L.) (Tropea Garzia, *et al.* 2012). The larvae of this pest feed and develop in the tomato leaves and the moth has a very high reproductive potential (Caparros Megido, *et al.* 2012). It commonly causes complete yield loss in greenhouse and open-field tomatoes in infested areas, particularly if no control measures are adopted (Desneux, *et al.* 2011).

*T. absoluta* was reported for the first time outside its native continent in eastern Spain in late 2006 and has subsequently spread throughout many countries of the Mediterranean basin and central and northern

Europe (Desneux, *et al.* 2011). The impact of *T. absoluta* on tomato crops, during the first years after its detection, has led European and North African growers to use insecticides extensively (Desneux, *et al.* 2010 and Cagnotti, *et al.* 2012). Nevertheless, chemical approach may disrupt previous successful Integrated Pest Management (IPM) procedures adopted during the last decades for other pests in tomato cultivations (Arnò and Gabarra, 2011 and Biondi, *et al.* 2012a, b). Indeed, this approach may disturb the role of natural enemies because of multiple side effects (Desneux, *et al.* 2007) and may lead to pest resistance (Liang, *et al.* 2012; Shad, *et al.* 2012 and Gontijo, *et al.* 2013).

Lack of co-evolved natural enemies in the new invaded areas may contribute to explain why *T. absoluta* rapid diffusion in the Palaearctic looks so explosive in comparison to the native area in which biological control agents are frequent (Luna, *et al.* 2012). In this context, biological control may be an environmentally and economically-sound tool for the management of *T. absoluta* in Afro-Eurasia (Desneux, *et al.* 2010 and Chailleux, *et al.* 2012). In particular, conservation biological control strategies that imply on the use of indigenous biological control agents could play a key role against invasive pests (Pons, *et al.* 2011 and Ragsdale, *et al.* 2011). Various ecological measures are implemented to enhance abundance or activity of natural enemies,

including creation of refuges, increasing the availability of alternative hosts and preys, providing essential food resources such as flowers and applying only pesticides that are selective towards biocontrol agents (Gardiner, *et al.* 2009; Lu, *et al.* 2012 and Seagraves and Lundgren, 2012). Thus, knowledge on the indigenous natural enemies that are adapted to the new invasive pest is crucial when developing sustainable pest control strategies. Moreover, the European Directive 2009/128/EC (EEC/CEE, 2009) aims to make European agriculture less dependent on the use of pesticides.

The present study aimed to identify indigenous predator and parasitoid species complex of *T. absoluta* in Southern France after few years of the pest arrival.

## MATERIALS AND METHODS

The survey was conducted in some of the French areas where *T. absoluta* was first detected, namely Provence-Alpes-Côte d'Azur and Languedoc-Roussillon (EPPO, 2009), by sampling infested leaves on protected tomato crops. All the fields were selected since representative of the typical Southern France commercial protected tomato cultivations and because they had been infested by *T. absoluta* during the previous cropping cycle. Sampling of infested tomato leaves concerned with all the tomato cultivation area in the farms where the survey took place. At least 50 infested (by *T. absoluta*) leaves were sampled randomly and no more than two leaves per infested plant were collected. Samplings were carried out bi-weekly or occasionally during late spring, summer and early fall of 2011 and 2012. Table (1) presents sampling sites and timing.

The samples were placed in sealed plastic bags, and transferred to the laboratory. Then, the leaves were carefully checked, using a stereomicroscope in order to exclude the presence of any other pests and to assess the presence of predators feeding on the pest. Predatory adults were immediately killed in alcohol 96% for identification, using the taxonomic key of Goula and Alomar (1994). Whereas, to facilitate their specific identification, predators' immature stages were reared until adulthood on tomato sprouts, maintained into plastic glasses, following the procedure of Biondi *et al.* (2012a). Afterwards, sampled leaves and isolated immature predators were then maintained in the laboratory and reared at 25±1°C, 60±10% R.H. and L14:D10 photoperiod.

Some of the newly emerged parasitoid adults were used to start laboratory colonies for further

biological and ecological studies. Whereas, other wasp specimens were killed and stored to be identified later using the taxonomic keys of Doult and Viggiani (1968), Burks (2003), and Yu and van Achterberg (2010).

## RESULTS AND DISCUSSION

Three mirid predatory species, belong to the subfamily Dicyphinae, were found feeding on *T. absoluta* during the two sampling seasons. The first in term of abundance was *Macrolophus pygmaeus* (Rambur). It was found in four sampling sites and in both seasons of study (Table 2). Another close mirid species, *Nesidiocoris tenuis* (Reuter) was found in the site of Cannes in August 2011. Both species showed relative potential to feed effectively on eggs and young larvae of *T. absoluta* soon after the pest arrival to Europe (Urbaneja, *et al.* 2009 and Desneux, *et al.* 2010). *Nesidiocoris tenuis*, was also recorded in Egypt (El-Arnaouty, *et al.* 2012). The third predatory species, *Dicyphus* sp. was collected on tomato infested leaves (Table 2). These bugs are important polyphagous predators that regulate arthropod populations such as; whiteflies, and other pests like aphids, mites and leafminers. They are known for colonizing spontaneously on various open and protected crops (Lucas and Alomar, 2002 and Ingegno *et al.* 2009). Efficient activities of *M. pygmaeus* and *Dicyphus* spp. were well recognized. The status of *N. tenuis*, as a beneficial and/or pest species, was controversial for a long time, since it could feed on tomato plants causing necrotic rings when preys were less available (Calvo, *et al.* 2009). On the other hand, both *M. pygmaeus* and *N. tenuis* were marketed and successfully used in inoculative biological control programs in combination with selective pesticides and/or egg parasitoids (Zappalà, *et al.* 2012b; Bompard, *et al.* 2013 and Chailleux, *et al.* 2013).

Two egg parasitoid species; *Trichogramma achaeae* Nagaraja & Nagarkatti and unidentified *Trichogramma* sp. (Hymenoptera: Trichogrammatidae) emerged from the sampled *T. absoluta* eggs (Table 2). *Trichogramma* spp. have been released extensively on millions of hectares all over the world for biological control against eggs of lepidopterous pests (Pizzol, *et al.* 2012). *Trichogramma achaeae* is a cosmopolitan egg parasitoid attacking eggs of several pests (Chailleux, *et al.* 2012). This species has been recently introduced into Europe to be involved in the tomato IPM programs through inundative releases (Cabello, *et al.* 2012 and Chailleux, *et al.* 2013).

*Tuta absoluta* larvae were found parasitized by three hymenopterous ectoparasitoids species;



Table (1): Sampling sites and frequency for natural enemies of *Tuta absoluta* on tomato in Southern France, seasons 2011-2012

No.	Department	Location	Sampling period and frequency
1	Bouches du Rhône	Mollégès	Bi-weekly samplings from August to October 2011, 2012
2	Bouches du Rhône	Eyragues	Bi-weekly samplings from August to October 2011
3	Vaucluse	Monteux	Bi-weekly samplings from August to October 2011, 2012
4	Hérault	Mudaison	Bi-weekly samplings from August to October 2011, 2012
5	Gard	La Caillar	Occasional sampling May 2011
6	Gard	Jonquières-Saint-Vincent	Occasional sampling May 2011
7	Alpes Maritimes	Cannes	Occasional samplings from August to October 2011
8	Alpes Martitime	La Broc	Occasional samplings from August to September 2012
9	Alpes Martitime	Carros	Occasional samplings from August to September 2012

Table (2): Predatory species found on sampled tomato leaves associated with *Tuta absoluta* in Southern France, seasons 2011-2012

Order	Family	Species	Site(s)	Month(s)
Hemiptera	Miridae	<i>Macrolophus pygmaeus</i>	1,2,3,7	October 2011; July, August, September 2012
		<i>Nesidiocoris tenuis</i>	7	August 2011
		<i>Dicyphus</i> sp.	2,4,7,8	October 2011; August 2012

Table (3): Parasitoid species recovered from sampled tomato leaves infested with *Tuta absoluta* in Southern France, seasons 2011-2012

Order:	Family	Species	Site(s)	Month(s)
Hymenoptera	Eulophidae	<i>Necremnus</i> sp. near <i>artynes</i>	1,3,4,7,8,9	August, October 2011; August, September 2012
		<i>Neochrysocharis formosa</i>	2,7	August 2011
		<i>Stenomesus</i> sp. near <i>japonicus</i>	6	May 2011
Hymenoptera	Braconidae	<i>Bracon nigricans</i>	7,8	August 2011; July, August, September 2012
Hymenoptera	Trichogrammatidae	<i>Trichogramma</i> sp.	6,7,8	June, July 2011; October 2012
		<i>Trichogramma achaeae</i>	7,8	September 2011

*Bracon nigricans* Szépligeti (Braconidae), *Stenomesus* sp. near *japonicus* (Ashmead) and *Neochrysocharis formosa* (Westwood) (Eulophidae). *B. nigricans* was found in the Alpes Maritimes department in both sampling seasons (Table 3). It is a generalist ectoparasitoid and widely distributed in the whole Palaearctic region (Yu and van Actherberg, 2010). It was found by Biondi, *et al.* (2013) as an idiobiont ectoparasitoid of *T. absoluta* mature larvae. The eulophid, *N. formosa* is a parasitoid of various leaf-mining and stem-boring species. It has a cosmopolitan distribution and is the only species recorded attacks *T. absoluta* in Europe and in South America (Luna, *et al.* 2012 and Zappalà, *et al.* 2012a). Finally, the most abundant larval parasitoid species recovered from the samples in this study was *Necremnus artynes* Ashmead (Hymenoptera: Eulophidae). It was found parasitizing *T. absoluta* larvae in almost all the sampled crops and in both seasons (Table 3). According to this result and those obtained by Zappalà, *et al.* (2012a) and Abbes, *et al.* (2013), this species has a good potential to be included in tomato IPM programs, since it was able to build up its population in insecticide treated crops and performed a parasitism rate up to 25.5%.

Obtained results provided important and useful basic information which may help in development of new IPM strategies against *T. absoluta* in France. The whole recovered natural enemy complex was composed of three generalist predators and four parasitic species. These biological control agents may feed on some other pest species and they are widely acknowledged as providing valuable levels of regulation of several pests in agro-ecosystems all over the world. They are able to build up their populations in highly disturbed ecosystems by using alternative host species in order to establish their populations on the crop before the pest arrival (Desneux and O'Neil, 2008, and Yu and van Actherberg, 2010). Thus, sustainable rational habitat management strategies with particular reference to the functional biodiversity (Gardiner, *et al.* 2009 and Lambion, 2011) and with use of selective pesticide(s) are strongly encouraged to preserve this indigenous resource (Desneux, *et al.* 2007; Biondi, *et al.* 2012a, and Lu, *et al.* 2012). On the other hand, in order to better understand the biology and the ecology of these generalist natural enemies, other aspects should be further investigated. This is particularly a true for those species with an uncertain taxonomy, such as *N. near artynes* and *S. near*

*japonicus*, since different biological and ecological traits can be highlighted among different cryptic species (Desneux, *et al.* 2009).

## ACKNOWLEDGEMENTS

The authors wish to thank: U. Bernardo (CNR – IPP, Naples, Italy) for confirming the identification of *Necremnus* sp. near *artynes*; ANRT and *InVivo* for funding the AC (Ph.D. fellowship); University of Catania for founding AB (Ph.D. fellowship). This work was partially funded by the Plant Health and Environment Department of INRA and by the French Ministry of Agriculture (CASDAR Project 10063).

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This previous study (**Article 1**) showed that three species of mirid predators [*Macrolophus pygmaeus* (Rambur), *Nesidiocoris tenuis* (Reuter) and *Dicyphus* sp.] were found preying on eggs and young larvae of the moth in both seasons of sampling. Eggs were also attacked by two trichogrammatid species, *Trichogramma achaeae* Nagaraja & Nagarkatti and *Trichogramma* sp. Whereas, *T. absoluta* larvae were parasitized by four parasitoid species belong to the two families Braconidae and Eulophidae.

However, it is essential to test the effectiveness of these identified natural enemies against *T. absoluta* under greenhouse conditions if an augmentative biological control program is expected. Therefore, tests of effectiveness of *Trichogramma* parasitoids and its combined use with a generalist predator *M. pygmaeus* in regulating *T. absoluta* was further preformed under greenhouse conditions (**Article 2**). The experiment was conducted in the greenhouses located at INRA Sophia-Antipolis, France.

## Article 2

# **Suitability of the Pest-Plant System *Tuta absoluta* (Lepidoptera: Gelechiidae)-Tomato for *Trichogramma* (Hymenoptera: Trichogrammatidae) Parasitoids and Insights for Biological Control.**

Chailleux A<sup>1,2\*</sup>, Biondi A<sup>1,3</sup>, Han P<sup>1</sup>, Tabone E<sup>4</sup>, Desneux N<sup>1</sup>

<sup>1</sup> French National Institute for Agricultural Research (INRA), UMR1355, 400 Route des Chappes, 06903, Sophia-Antipolis, France

<sup>2</sup> InVivo Agro-Solutions, 83 Avenue de la Grande-Armée, 75582 Paris 16, France

<sup>3</sup> Department of Agri-food and Environmental Systems Management, University of Catania, Via Santa Sofia 100, 95123 Catania, Italy

<sup>4</sup> French National Institute for Agricultural Research (INRA), UEFM, 90 Chemin Gustave Raymond, 06160 Antibes, France

\*Corresponding author: [chailleux.anais@gmail.com](mailto:chailleux.anais@gmail.com)

# Suitability of the Pest–Plant System *Tuta absoluta* (Lepidoptera: Gelechiidae)–Tomato for *Trichogramma* (Hymenoptera: Trichogrammatidae) Parasitoids and Insights for Biological Control

ANAÏS CHAILLEUX,<sup>1,2,3</sup> ANTONIO BIONDI,<sup>1,4</sup> PENG HAN,<sup>1</sup> ELISABETH TABONE,<sup>5</sup>  
AND NICOLAS DESNEUX<sup>1</sup>

J. Econ. Entomol. 106(6): 2310–2321 (2013); DOI: <http://dx.doi.org/10.1603/EC13092>

**ABSTRACT** The South American tomato leafminer, *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae), is a major pest that has recently invaded Afro-Eurasia. Biological control, especially by *Trichogramma* parasitoids, is considered to be promising as a management tool for this pest. However, further development of *Trichogramma*-based biocontrol strategies would benefit from assessing the impact of released parasitoid offspring on the pest. Under laboratory conditions, we 1) compared the parasitism of five *Trichogramma* species-strains on the pest–plant system *T. absoluta*–tomato, and 2) assessed various biological traits of parasitoids, mass-reared on a factitious host (*Ephesia kuehniella* Zeller), when developing on *T. absoluta*. In addition, we evaluated the overall efficiency of two specific *Trichogramma* species when released under greenhouse conditions in combination with a common natural enemy in tomato crop, the predator *Macrolophus pygmaeus* Rambur. Parasitoids emerging from *T. absoluta* on tomato showed lower parasitism rates and poor biological traits, for example, wing deformations, reduced longevity, when compared with the control reared on the factitious host. Under greenhouse conditions, the parasitoids that developed on *T. absoluta* after initial releases contributed little to biological control of *T. absoluta*, and parasitism tended to be lower when the predator was present. However, a slightly higher *T. absoluta* control level was achieved by combining the predator and release of the parasitoid *Trichogramma achaeae* Nagaraja and Nagarkatti. This study shows that *Trichogramma* parasitoids may not build up populations on the *T. absoluta*–tomato system, but that *Trichogramma* parasitoids can be used in combination with *M. pygmaeus* to enhance biological control of the pest in tomato crops.

**KEY WORDS** fitness, generalist predator, invasive species, inundative release, mass-rearing

The South American tomato leafminer, *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae), is one of the most devastating tomato pests in South America (Gontijo et al. 2013). The pest has recently invaded and spread in the Afro-Eurasian continent, and in few years has become a major pest in tomato crops (Desneux et al. 2010, 2011a). Appearance of *T. absoluta* has led to extensive insecticide use by tomato growers, potentially causing a multitude of undesired side effects on nontarget organisms (Desneux et al. 2007; Arnó and Gabarra 2011; Biondi et al. 2012, 2013a). Among possible management methods currently studied (Amer et al. 2012, Cagnotti et al. 2012, Caparros Megido et al. 2012, Cocco et al. 2013), bio-

logical control is considered for the development of environmentally and economically sound pest management tools for this pest in Europe (Desneux et al. 2010, Zappalà et al. 2013). Agricultural pests are largely controlled via top–down influences of natural enemies in a wide range of agro-ecosystems (van Driesche and Bellows 1996, Hawkins et al. 1997, Symondson et al. 2002, Lu et al. 2012). However, their use as biocontrol agents, either through augmentation or conservation biological control, is often slowed down because their effectiveness in the field is difficult to predict.

Parasitoids belonging to the *Trichogramma* genus (Hymenoptera: Trichogrammatidae) are generalist egg parasitoids, mainly of Lepidoptera. They are frequently used in biological control programs, notably through inundative releases (Smith 1996, Pintureau 2009, Mills 2010). In addition, inoculative releases of Trichogrammatids have been tested on agricultural pests in various crops (Thomson et al. 2003, Hoffmann et al. 2006, Mills 2010). In South America, *T. absoluta* is parasitized by *Trichogramma pretiosum* Riley under laboratory conditions (Pratissoli and Parra 2000), and

<sup>1</sup> French National Institute for Agricultural Research (INRA), ISA, 400 Route des Chappes, 06903 Sophia-Antipolis, France.

<sup>2</sup> InVivo AgroSolutions, 83 avenue de la Grande Armée, 75782 Paris, France.

<sup>3</sup> Corresponding author, e-mail: [chailleux.anais@gmail.com](mailto:chailleux.anais@gmail.com).

<sup>4</sup> Department of Agri-Food and Environmental Systems Management, University of Catania, Via Santa Sofia 100, 95123 Catania, Italy.

<sup>5</sup> French National Institute for Agricultural Research (INRA), UEFM, 90 Chemin Gustave Raymond, 06160 Antibes, France.

Miranda et al. (1998) reported that nearly 10% of *T. absoluta* mortality occurring at the egg stage is because of natural parasitism by this parasitoid. Trichogrammatids have been used in tomato crops against *T. absoluta* through inundative releases both in the native (mainly with *T. pretiosum*; Parra and Zucchi 2004, Pratissoli et al. 2005) and the invaded areas (with *Trichogramma achaeae* Nagaraja and Nagarkatti; Cabello et al. 2012, Calvo et al. 2012). Considering the effectiveness of these programs and the natural parasitism of this pest by Trichogrammatids in South America and in Europe (Desneux et al. 2010, Zappalà et al. 2012, Biondi et al. 2013b), screenings for more effective species in the invaded area are ongoing (Chailleux et al. 2012, Khanh et al. 2012).

Various factors can influence the impact of inundative releases of mass-reared parasitoids. For instance, host selection by parasitoids may be influenced by the characteristics of both the host and host plant (Chau and Mackauer 2001, Desneux and Ramirez-Romero 2009). In addition, host preference in parasitoids tends to correlate with the fitness gained from the host (van Alphen and Vet 1986, Driessen et al. 1991, Chau and Mackauer 2001), this being defined as the "preference-performance" hypothesis (Jaenike 1978, Desneux et al. 2009b). However, very-low quality hosts can also be parasitized (Janssen 1989; Heimpel et al. 2003; Desneux et al. 2009a, 2012). Studies on acceptance of *Trichogramma* reared on factitious host species toward natural hosts yielded variable results. El-Wakeil (2007) showed differences in pest parasitism, which depended on the rearing host. The size of the natal host affected natural host parasitism rates in the field, and female parasitoids usually accepted host eggs of the same size or larger as their natal host (Salt 1940, Nurindah et al. 1999). By contrast, Kolliker-Ott et al. (2003) reported no preference-performance relationship for *Trichogramma brassicae* Bezdenko mass-reared on the factitious host *Ephesia kuehniella* Zeller when parasitizing the target host, the European corn borer *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae).

The effectiveness of *Trichogramma* releases may depend not only on the biological characteristics of the parasitoid species or strains used, but also on their interactions with a specific pest-plant system (Tabone et al. 2010, Andrade et al. 2011, Yuan et al. 2012). Depending on the affinity of the parasitoids with the pest-plant system, the biocontrol services provided by the released parasitoids most likely vary. *Trichogramma* are mainly used as biocontrol agent through inundative releases, but the impact of *Trichogramma* generations developing within a crop can play a crucial role in the success of biological control programs (Mills 2010). For example, use of *T. brassicae* for biological control of the European corn borer in maize takes into account the effect of parasitoids developing in the field for the long-term control of the pest (Pintureau 2009). Additional mass-releases may be reduced and overall crop protection costs can be lowered.

To document the possible biocontrol impact of five *Trichogramma* species or strains, hereafter named "strains," when developing on *T. absoluta* eggs in the tomato crops after inundative releases, we evaluated their parasitism and the subsequent development of their offspring. Thus, in a first experiment, in the laboratory, we compared 1) a "released generation": developed in *E. kuehniella* eggs and offered *T. absoluta* eggs, with 2) a "field generation": developed in *T. absoluta* eggs and offered *T. absoluta* eggs, and a control treatment: parasitoids developed in *E. kuehniella* eggs and offered *E. kuehniella* eggs. This aimed at assessing possible reduced parasitoid performance when parasitoids switched from the mass-rearing host to the targeted pest-plant system. In a second experiment, in greenhouse, we tested the overall efficiency of caged release and the subsequent generation (that corresponds to the field generation) of two *Trichogramma* strains against *T. absoluta*. In addition, to assess the usefulness of *Trichogramma* parasitoids within the framework of current biocontrol programs in tomato crops in Europe, we included the predator *Macrolophus pygmaeus* Rambur (Hemiptera: Miridae) in the greenhouse experiment because it is widely used for biocontrol of whiteflies on tomato, and is also released against *T. absoluta* because it preys on eggs (Desneux et al. 2010, Bompard et al. 2013).

## Materials and Methods

**Biological Materials.** The plants used in the experiments were tomato plants, *Solanum lycopersicum* L. 'Marmande,' 5 wk old for the laboratory experiment and 7 wk old for the cage experiment. Plants were grown in climatic chambers ( $25 \pm 1^\circ\text{C}$ , 65% relative humidity [RH], and a photoperiod of 16:8 [L:D] h); pesticide applications were strictly avoided, and a nutrient solution was applied daily. A colony of *T. absoluta* was set up using greenhouse-collected individuals in July of 2009 (initial number of individuals = 190) at the French National Institute for Agricultural Research (INRA), Alenya, France. *T. absoluta* was reared in growth chambers ( $25 \pm 1^\circ\text{C}$ ,  $70 \pm 10\%$  RH, and a photoperiod of 16:8 [L:D] h) in mesh netting cages (55 by 75 by 80 cm) containing potted tomato plants (see Biondi et al. 2013c). Adult moths were fed on honey placed on the mesh inside the cages and on the plant. The parasitoids used for the experiments originated from collections (Table 1). Dr. B. Pintureau (INRA, Lyon, France) identified the species before the experiments, and voucher specimens were deposited at the INRA (Sophia-Antipolis, France). *Trichogramma euproctidis* Girault, and both *Trichogramma evanescens* Westwood strains were chosen because they were shown to be promising strains for biocontrol of *T. absoluta* during a previous study (29 strains tested, Chailleux et al. 2012). *T. pretiosum* was considered because it is mass reared and released to control this pest in South America. *T. achaeae* was tested because it has already been commercialized in Europe and Africa against *T. absoluta*. Colonies of parasitoids were reared on ultraviolet-irradiated eggs of a substi-



Table 1. Country of origin, initial host plant, and host and year of collection of the five *Trichogramma* strains tested

Species	Geographic origin	Host plant	Most species	Year of collection
<i>T. achaeae</i> Nagaraja and Nagarkatti, 1969	Canaries islands	Tomato	<i>Chrysodeixis chalcites</i>	2010
<i>T. euproctidis</i> Girault, 1911	Switzerland	—	—	—
<i>T. evanescens</i> Westwood, 1833 (1)	Northern France	Vine	<i>Lobesia botrana</i>	1990
<i>T. evanescens</i> Westwood, 1833 (2)	Northern France	Cauliflower	<i>Argyrotaenia sphaleropa</i>	2002
<i>T. pretiosum</i> Riley, 1876	Uruguay	Vine	<i>Argyrotaenia sphaleropa</i>	1995

tute host, *E. kuehniella* ( $18 \pm 1^{\circ}\text{C}$ ,  $70 \pm 10\%$  RH, and a photoperiod of 12:12 [L:D] h), until used in the experiments. Rearing was carried out in glass tubes (4.5 cm in length and 0.7 cm in diameter), and the parasitoids were fed on honey droplets. *E. kuehniella* eggs were glued on a piece of cardboard (3 by 10 mm) with 10% arabic gum (Pizzol et al. 2010). Parasitoids were maintained for at least three generations at the temperature of  $25^{\circ}\text{C}$  on *E. kuehniella* eggs before starting experiments.

In the laboratory trials, the *T. absoluta* eggs used were 0–12 h old, and the parasitoids were 0–24 h old (as both host and parasitoid ages can play a role in development of the *Trichogramma* offspring, see Pizzol et al. 2012). To obtain parasitoid females that had developed in *E. kuehniella* eggs, females from the colonies were released on *E. kuehniella* eggs in the same conditions as described for the rearing, but female emergence date was checked precisely. Parasitoid females developed on *T. absoluta* eggs were obtained by releasing parasitoids from the colonies on *T. absoluta* eggs on tomato leaflets for 12 h inside ventilated Plexiglas tubes (4 cm in diameter and 14 cm in length). The parasitoids used in all the experiments were collected daily from these tubes, namely those containing parasitized eggs of *E. kuehniella* or *T. absoluta*. *M. pygmaeus* specimens for the greenhouse experiment were reared on *E. kuehniella* eggs on artificial substrate by the private company Biotop (Valbonne, France) and after being purchased, they were reared for at least two generations in the laboratory. Rearing was carried out in cages (55 by 75 by 80 cm) covered with a mesh in climatic chambers ( $23 \pm 1^{\circ}\text{C}$ ,  $70 \pm 5\%$  RH, and a photoperiod of 16:8 [L:D] h) on tobacco plant, and *E. kuehniella* eggs were provided ad libitum. New eggs were added twice a week, and tobacco plants were changed monthly. Adult predators used in the experiments were 1–3 d old.

**Impact of *T. absoluta*–Tomato Pest–Plant System on Parasitism and Offspring Development.** The experiment was conducted in growth chambers ( $25^{\circ}\text{C}$ ,  $70 \pm 10\%$  RH, and a photoperiod of 16:8 [L:D] h). Two different combinations were tested mimicking the development of two generations of *Trichogramma* and the rearing situation (control treatment): 1) the released generation (developed in *E. kuehniella* eggs on cardboard and offered *T. absoluta* eggs on tomato leaflets), 2) the field generation (developed in *T. absoluta* eggs on tomato leaflets and offered *T. absoluta* eggs on tomato leaflets), and 3) parasitoids in the rearing situation were used as a control. Control individuals developed on *E. kuehniella* eggs and were offered *E. kuehniella* eggs on cardboard.

**Control (Treatment 3) and Released Generations (Treatment 1).** Females emerging from *E. kuehniella* eggs (obtained as described in the “Biological Materials” section) were used both for the control treatment, when releasing the females on *E. kuehniella* eggs, and for the released generation treatment, when releasing the female on *T. absoluta* eggs. Therefore, young mated females were released individually in glass tube containing 30–40 eggs of *E. kuehniella* (the same technique as previously described for the rearing) and fed with honey droplets during 24 h to measure the control (“rearing generation”) parameters. However, for the released generation, young mated females were presented individually with 30–40 *T. absoluta* eggs on a tomato leaflet in ventilated Plexiglas tubes (4 cm in diameter and 14 cm in length) with honey during 24 h. The stems of leaflet, sticking out of the tube, were planted into floral foam for watering.

**Field Generations (Treatment 2).** To evaluate the field generation parameters, young *Trichogramma* females emerging from *T. absoluta*–parasitized eggs (obtained as described in the “Biological Materials” section) were presented individually to 30–40 *T. absoluta* eggs on a tomato leaflet in the ventilated tube (with honey droplets as food source) during 24 h. Thus, we used the same oviposition conditions as the released generation, except that females had previously developed in *T. absoluta* eggs vs. *E. kuehniella* eggs.

For the three treatments, tubes containing parasitized eggs were kept in a climatic chamber ( $25^{\circ}\text{C}$ ,  $70 \pm 10\%$  RH, and a photoperiod of 16:8 [L:D] h) and maintained until offspring emergence for 15 d. For each treatment, the number of parasitized eggs (black eggs) and of aborted eggs (white and unhatched eggs) was counted after 5 d of parasitization. The sex ratio (females per total) and the wings deformation occurrence (absent, stump, or crumple wings) of offspring individuals (F1) were recorded 2 d after emergence, namely 15 d after the parasitization. Ten to 15 replicates were carried out per parasitoid strain and per treatment.

**Impact of the Host on the Longevity.** We assessed the longevity of female parasitoids that had developed in *T. absoluta* eggs (=the field generation) and in *E. kuehniella* eggs as a control. Females were obtained as described in the “Biological Materials” section. These were placed in glass tubes (4.5 cm in length and 0.7 cm in diameter), closed with a cotton ball and provided with honey or left without in climatic chambers ( $25^{\circ}\text{C}$ ,  $70 \pm 10\%$  RH, and a photoperiod of 16:8 [L:D] h). Twenty to 25 females per strain and per rearing host were tested. The survival was checked daily with a binocular microscope, and parasitoids were consid-



ered dead when they did not react after being touched by a fine paint brush.

**Cage Experiment.** A cage experiment was carried out with the aim of testing the efficiency of the field generation under realistic conditions. The experiment was carried out in cages (100 cm in height, 70 cm in width, and 100 cm in length, made with insect-proof mesh) that were placed in a greenhouse at the INRA Sophia AgroBiotech Institute (Sophia-Antipolis, France). The greenhouse was similar to the ones used by many tomato growers in France and more broadly in Southern Europe (semicontrolled temperatures,  $\min < \text{mean temperature} < \text{max}$ :  $15.5 < 24.2 < 37.4^\circ\text{C}$ ;  $\min < \text{mean RH} < \text{max}$ ,  $18.0 < 63.0 < 90.0\%$ ; natural ambient light: mid-July mid-August 2011). The two *Trichogramma* species tested, *T. achaeae* and *T. euproctidis*, which showed the highest parasitism at the field generation in the laboratory (see "Results" section), were chosen for this experiment. Both species were tested alone and in combination with the predator *M. pygmaeus*. The predator was also tested alone and a control with the pest alone was carried out. The following six treatments or combinations were studied: 1) *T. absoluta*, 2) *T. absoluta* + *M. pygmaeus*, 3) *T. absoluta* + *T. achaeae*, 4) *T. absoluta* + *T. euproctidis*, 5) *T. absoluta* + *T. achaeae* + *M. pygmaeus*, and 6) *T. absoluta* + *T. euproctidis* + *M. pygmaeus*. The experiment lasted 16 d to allow first field generation observation, and six replicates (cages) were carried out per treatment.

Eight tomato plants (development stage from seven to eight fully expanded leaves) were placed into the cages. First, 22-d-old *T. absoluta* adults (10 males and 10 females) were released per cage. After 24 h, predators, parasitoids, or both, were introduced into the cages. Depending on the treatment, 400 *Trichogramma* adults and eight pairs of *M. pygmaeus* (1:1, male/female) were released. These release rates were chosen according to the recommendations of producers of natural enemies. To release *Trichogramma* parasitoids, we mimicked a field release similar to what is recommended by the producers. One open glass tube containing a small cardboard strip bearing 400 parasitized *E. kuehniella* eggs was placed in the center of each cage. Honey droplets were provided as food source to the parasitoids. A quality control carried out on a sample of 1,000 parasitized eggs per parasitoid strain (randomly selected from the stock of parasitized eggs used in this experiment) showed that parasitoid emergence was always  $>90\%$ . Individuals showed a sex ratio of  $\approx 0.70$  (females/total) for both species. Therefore, we assumed that releasing 400 parasitized eggs corresponded to a release of  $\approx 252$  parasitoid females.

To facilitate the parasitization monitoring, we added four sentinel tomato leaflets bearing 30 *T. absoluta* eggs (15 on adaxial and 15 on abaxial surfaces of each leaflet) before the parasitoids release. These leaflets were placed on the top of a stake so that they always touched a tomato leaf in the upper plant part. The stems of these leaflets were put inside a tube full

of water. Five days later, leaflets were collected and parasitized eggs (black eggs) and predated eggs (empty crumpled eggs) were counted under a binocular microscope and percent parasitism and percent predation rates were calculated. Then, the leaflets were put back inside their respective cages.

To evaluate parameters in the same way for the field generation, produced by the released parasitoid, we provided new sentinel leaflet at the field generation emergence time. The *Trichogramma* juveniles (egg to adult) developmental time is 8 d at  $30^\circ\text{C}$  (Foerster and Foerster 2009); therefore, the new leaflets were placed in each cage 8 d after the *Trichogramma* releases, following the same procedure previously described. The leaflets were collected 7 d later to enable an effective assessment of overall parasitism undertaken by the second-generation parasitoid females, as they emerged for up to 3 d and adults longevity in greenhouse conditions is  $\leq 3$  d (A. C. and N. D., unpublished data). Parasitized eggs and predated eggs were counted under a binocular microscope as for the released generation. Plants were destructively sampled at the end of the experiment (after 16 d). The number of *T. absoluta* larvae and *M. pygmaeus* juveniles were counted in each cage on four plants chosen randomly.

**Statistical Analyses.** All statistical analyses were performed using the R software with the *multcomp* and *agricolae* packages. For the laboratory experiments, the number of parasitized and aborted eggs were analyzed using a generalized linear model (GLM) based on Poisson distributed data with a log link function. Sex ratio (females per total) and wing malformations were analyzed using a GLM designed for modeling binomial data with a logit link function. For these four parameters the effect of the factors Rearing host (host egg used to rear parasitoid), Oviposition host (host egg parasitized by the females in the experiments), and *Trichogramma* strain were tested. Longevity was also analyzed using a GLM, but it was designed for modeling gamma data with an inverse link function. The effect of the factors Rearing host, Honey supply, and *Trichogramma* strain were tested.

For the cage experiments, the percentages of parasitism, aborted eggs, and predated eggs were analyzed using a GLM designed for modeling binomial data. For the parasitism and aborted eggs, the effect of the factors, *M. pygmaeus*, *Trichogramma* generation (released or field generations) and *Trichogramma* strain factors were tested. For the predated eggs the effect of the factors *Trichogramma* strain and *Trichogramma* generation were tested. A GLM designed for Poisson distributed data were used to analyze the number of *T. absoluta* larvae and *M. pygmaeus* juveniles. For *T. absoluta* larvae, the factor *Trichogramma* strain and *M. pygmaeus* were tested, and for the *M. pygmaeus* juvenile, the *Trichogramma* strain factor was tested. Finally, multiple comparisons were performed using a Fisher least significant difference (LSD) post hoc test for both laboratory and cage experiments.

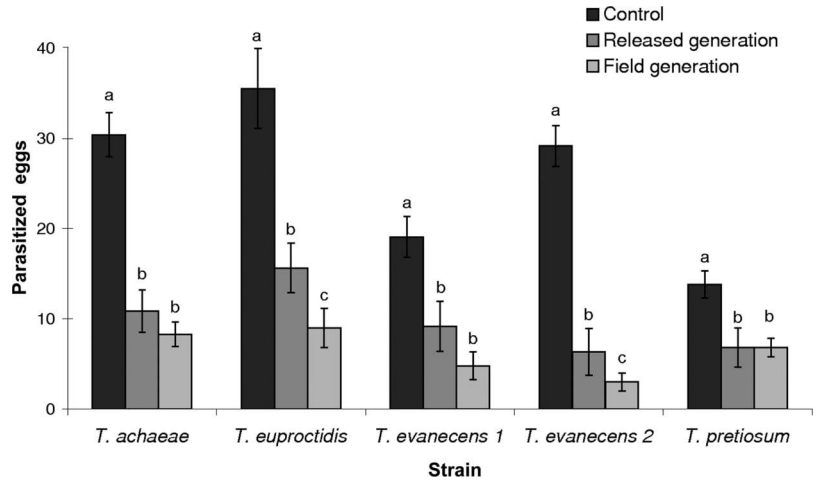


Fig. 1. Mean ( $\pm$ SEM) number of parasitized eggs per parasitoid female for the five *Trichogramma* strains tested. Released generation: developed in *E. kuehniella* eggs and offered *T. absoluta* eggs; field generation: developed in *T. absoluta* eggs and offered *T. absoluta* eggs; control: developed in *E. kuehniella* eggs and offered *E. kuehniella* eggs. Post hoc tests (Fisher LSD test) were carried out separately for each strain tested. For each generation tested (released and field generations) and for the control, the histograms bearing the same letter are not significantly different at  $P < 0.05$ .

Results

**Impact of *T. absoluta*-Tomato Pest-Plant System on Parasitism and Offspring Development.** There was a significant effect of the Rearing host (host in which ovipositing parasitoid developed;  $F_{(1,181)} = 80.980$ ;  $P < 0.001$ ) and of the Oviposition host (host parasitized in the experiment;  $F_{(1,182)} = 91.780$ ;  $P < 0.001$ ) on the number of parasitized eggs. Parasitism also varied significantly among the *Trichogramma* strains ( $F_{(4,177)} = 8.902$ ;  $P < 0.001$ ). The number of parasitized eggs ranged from  $35.5 \pm 4.4$  for the rearing generation of *T. euproctidis* to  $3.0 \pm 1.0$  for the field generation of *T.*

*evanescens 2* (Fig. 1). Parasitism of all the strains was significantly lower when females were offered *T. absoluta* (released and field generations) than *E. kuehniella* eggs (control). Parasitism rates of *T. euproctidis* and *T. evanescens 2* were also lower when females had developed on *T. absoluta* (field generation) than when females had developed on *E. kuehniella* eggs (control and released generation; Fig. 1).

The host egg species in which the females developed (Rearing host) significantly affected the sex ratio of their offspring (Fig. 2;  $F_{(1,127)} = 5.149$ ;  $P < 0.025$ ). Indeed, the *T. achaeae* field generation was more male-

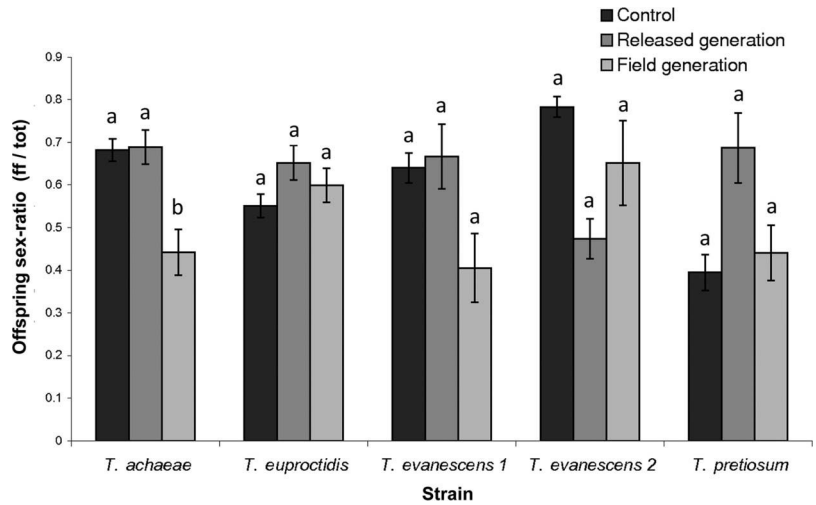


Fig. 2. Mean sex ratio ( $\pm$ SE; females per total) of offspring for the five *Trichogramma* strains tested. Released generation: females developed in *E. kuehniella* eggs and offered *T. absoluta* eggs; field generation: females developed in *T. absoluta* eggs and offered *T. absoluta* eggs; control: females developed in *E. kuehniella* eggs and offered *E. kuehniella* eggs. Post hoc tests (Fisher LSD test) were carried out separately for each strain tested. For each generation tested (released and field generations) and for the control, the histograms bearing the same letter are not significantly different at  $P < 0.05$ .

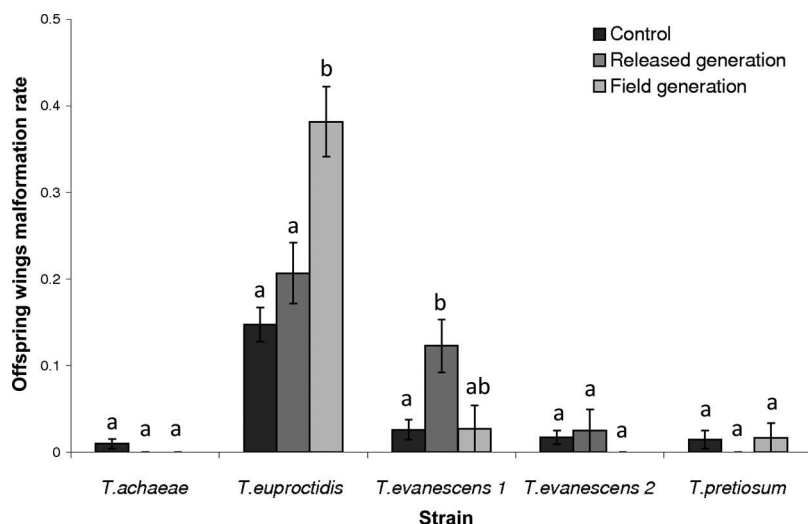


Fig. 3. Proportion ( $\pm$ SE) of offspring with wings malformation: absent, stump, or crumple wings, for the five *Trichogramma* strains tested. Released generation: females developed in *E. kuehniella* eggs and offered *T. absoluta* eggs; field generation: females developed in *T. absoluta* eggs and offered *T. absoluta* eggs; control: females developed in *E. kuehniella* eggs and offered *E. kuehniella* eggs. Post hoc tests (Fisher LSD test) were carried out separately for each strain tested. For each generation tested (released and field generations) and for the control, the histograms bearing the same letter are not significantly different at  $P < 0.05$ .

biased than its generations developing on *E. kuehniella* eggs. By contrast, the Oviposition host factor did not significantly affect the sex ratio ( $F_{(1,126)} = 0.0736$ ;  $P < 0.787$ ). The sex ratio of the offspring also varied significantly among strains tested (significant *Trichogramma* strain factor;  $F_{(4,122)} = 4.937$ ;  $P < 0.001$ ). The sex ratio significantly differed among generations only in the case of *T. achaeae* when strains were tested individually (Fig. 2; Fisher LSD test). The sex ratio of offspring from the field generation was significantly lower than in the two other treatments (control and released generation). We observed similar trends for *T. evanescens* 1 (Fig. 2), but differences among treatment were not significant either for *T. evanescens* 1 or the three other strains (most likely owing to a low number of offspring individuals in the field generation).

*Trichogramma* strain, Rearing host, and Oviposition host factors all had a significant impact on wing malformations occurring in offspring ( $F_{(4,122)} = 39.107$ ;  $P < 0.001$ ;  $F_{(1,127)} = 24.765$ ;  $P < 0.001$ ;  $F_{(1,126)} = 7.479$ ;  $P = 0.007$ , respectively). The strain presenting the highest proportion of offspring with malformed wings was *T. euproctidis* (Fig. 3), the highest proportion was observed for the *T. euproctidis* field generation, with  $38 \pm 4\%$  of offspring with wing malformations (Fig. 3).

**Impact of the Host System on the Longevity.** The effects of the Rearing host ( $\chi^2 = 173.319$ ;  $df = 1$ ;  $P < 0.001$ ) and the Honey supply factors ( $\chi^2 = 173.319$ ;  $df = 1$ ;  $P < 0.001$ ) were significant on female parasitoid longevity (Fig. 4). The *Trichogramma* strain factor also had a significant effect on *Trichogramma* longevity ( $\chi^2 = 28.530$ ;  $df = 4$ ;  $P < 0.001$ ), with a maximum of 18 d on average for *T. evanescens* 1 developed in *E. kuehniella* egg and supplied with honey (Fig. 4A). Without

honey, longevity did not exceed 4 d (for *T. pretiosum* emerged from *E. kuehniella* eggs; Fig. 4B).

**Cage Experiment.** *Trichogramma* strain and *M. pygmaeus* had significant effects on the percentage of parasitism observed on the sentinel leaflets (Fig. 5;  $F_{(1,29)} = 22.648$ ;  $P < 0.001$  and  $F_{(1,30)} = 8.981$ ;  $P = 0.007$ , respectively). The parasitism of the field generation was significantly lower than of the released generation ( $F_{(1,28)} = 33.542$ ;  $P < 0.001$ ). The highest level of percent parasitism was obtained with *T. achaeae* alone. On the contrary, no parasitism was observed for the field generation of *T. euproctidis* in the presence of *M. pygmaeus* (Fig. 5).

In the three treatments with *M. pygmaeus*, the number of eggs eaten did not vary significantly depending on the *Trichogramma* strain (*T. achaeae* or *T. euproctidis* or absence) ( $F_{(2,21)} = 0.080$ ;  $P = 0.923$ ). No significant effect of the *Trichogramma* generation factor (released, field generations) was observed ( $F_{(1,20)} = 0.876$ ;  $P = 0.363$ ). *T. absoluta* eggs were preyed on an average  $71.7 \pm 3.6\%$ , ranged between 25 and 96.6%. The factors *Trichogramma* strain (*T. achaeae*, *T. euproctidis* or absence), *M. pygmaeus*, and *Trichogramma* generation had a significant effect on the number of aborted eggs ( $F_{(2,44)} = 3.918$ ;  $P = 0.029$ ;  $F_{(1,46)} = 12.429$ ;  $P = 0.001$ ; and  $F_{(1,43)} = 12.429$ ;  $P = 0.001$ , respectively). However, the overall percentages of aborted eggs were low throughout the course of the experiments in cages ( $\leq 5\%$ ). The maximum values were observed in groups with *Trichogramma* parasitoids without *M. pygmaeus* (released generation).

At the end of the experiment, an overall average of  $2.12 \pm 0.50$  *M. pygmaeus* juveniles per plant was recorded, ranging from one to three per plant. No significant differences related to the *Trichogramma* pres-

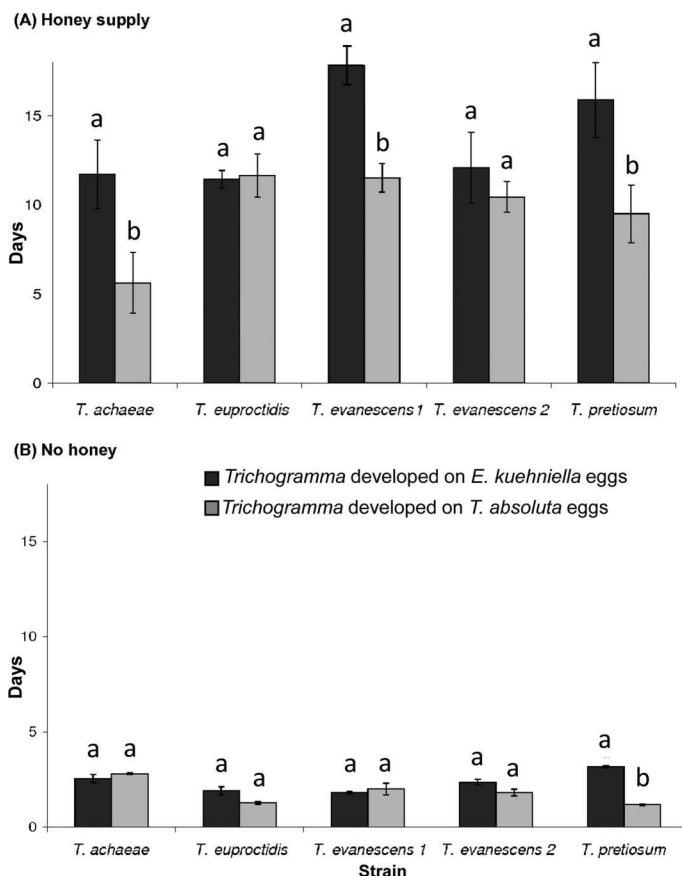


Fig. 4. Mean ( $\pm$ SE) longevity of the five *Trichogramma* strains tested for females developed in *E. kuehniella* eggs (equivalent to the control and the released generation); and for females developed in *T. absoluta* eggs (equivalent to the field generation); (A) in presence of honey as food source and (B) without any food sources. Post hoc tests (Fisher LSD test) were carried out separately for each strain tested. For each emergence host tested the histograms bearing the same letter are not significantly different at  $P < 0.05$ .

ence were observed ( $F_{(2,9)} = 0.022$ ;  $P = 0.978$ ). The number of *T. absoluta* larvae was influenced by *Trichogramma* presence, though only numerically different (Fig. 6;  $F_{(2, 21)} = 2.870$ ;  $P = 0.086$ ), and it was significantly affected by *M. pygmaeus* ( $F_{(1,20)} = 9.178$ ;  $P = 0.008$ ). The highest number of larvae was obtained in the control (*T. absoluta* alone) and the lowest infestation was observed in the treatment combining *T. achaeae* and *M. pygmaeus*, although it was not significantly different from the treatments with *T. achaeae* alone and *M. pygmaeus* alone and with *T. euproctidis* (Fig. 6).

### Discussion

The parasitism of five *Trichogramma* strains, belonging to four species, on *T. absoluta* was evaluated in the laboratory by testing two different parasitoid generations, particularly the ones potentially occurring in tomato crop after inundative releases of parasitoids. The parasitism of *T. absoluta* eggs on tomato by parasitoids from the released and field generations was related to the parasitism observed in individuals from

a control treatment, which is reared in the laboratory on the factitious host *E. kuehniella*. The highest parasitism was recorded for the generations under the standard rearing conditions on the factitious host. Parasitism decreased when oviposition occurred on the novel pest-plant system. Among the three treatments, the lowest parasitism was recorded for the field generation although it was not consistent for *T. euproctidis* and *T. pretiosum*, which showed same parasitism levels in both released and field generations.

Overall, parasitism efficiency decreased on the *T. absoluta*-tomato system, so that this pest-plant system appeared unsuitable for the *Trichogramma* strains tested. Our results showed a decrease of female progeny sex ratio, increased malformations of individuals (deformed wings), and reduced longevity (for *T. achaeae*, *T. evanescens* 1, and *T. pretiosum*, with up to a 6-d reduction) in the pest-plant system studied compared with rearing conditions.

Plausibly, differences among parasitoid efficiency recorded for the control and the released and field generations are linked to host physiological suitability for the *Trichogramma* strains tested. Although the host

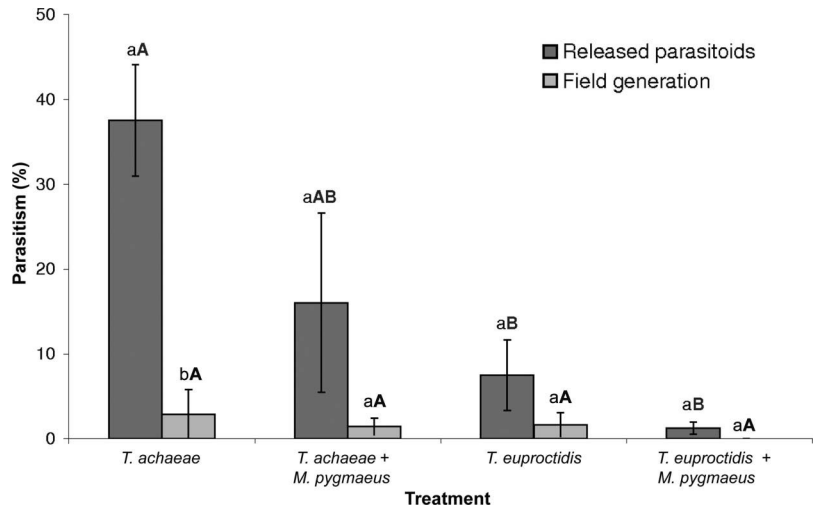


Fig. 5. Mean ( $\pm$ SE) percent parasitism of *T. absoluta* eggs obtained on sentinel leaflets placed in greenhouse cages for the four treatments with *Trichogramma* releases, for the released generation (females developed in *E. kuehniella* eggs) and the field generation (females developed in *T. absoluta* eggs). Two types of post hoc tests (Fisher LSD test) have been done separately 1) each strain separately, generations bearing the same lowercase letters are not significantly different for  $P < 0.05$ ; and 2) each generation separately, strains bearing the same capital letters are not significantly different at  $P < 0.05$ .

size–parasitoid fitness relationship is not always consistent among parasitoids species (Harvey et al. 2013), parasitoids largely depend on the quality and quantity of resources provided by the host for their larval development (Mackauer 1996, Pennacchio and Strand 2006). A close relationship has been generally observed between the size of the host parasitized and the size and fitness of parasitoid offspring, for example, egg load of emerging females (Harvey et al. 2013). In addition, offspring sex ratio and longevity can be linked to the size of parasitoid individual (Fidgen et al. 2000) and *Trichogramma* parasitoids commonly show preference for relatively large host eggs (Roriz et al. 2006). *T. achaeae* is reared on *E. kuehniella* eggs by producers of natural enemies, and eggs of this host are

threefold larger than those of *T. absoluta*. Therefore, the decrease in parasitoids quality (parasitism efficiency, sex ratio, longevity, and wing malformations) that developed in *T. absoluta* eggs may result from the small size of the host eggs, for example, smaller parasitoids emerged from *T. absoluta* eggs (A. C., unpublished data). Wing malformations in *Trichogramma* have been documented in other studies. Wings and abdominal malformations were observed in *Trichogramma* parasitoids reared on artificial diet (Consoli and Parra 1996), and sexual dimorphism (apterous males and fully winged females) of *T. semblidis* was described by Salt (1937) when the parasitoids developed in small eggs (*Sialis lutaria* L. [Megaloptera: Sialidae]) vs. *E. kuehniella* eggs. Finally, we cannot

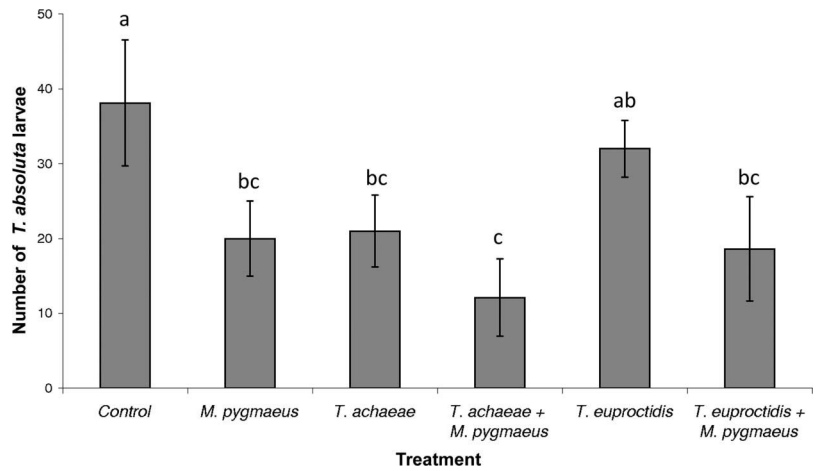


Fig. 6. Mean ( $\pm$ SE) number of *T. absoluta* larvae per plant 16 d after insect releases in greenhouse cages for the six treatments tested. Values marked with the same letter do not differ significantly at  $P < 0.05$  (Fisher LSD test).



exclude that parasitoid rearing, at least concerning *Trichogramma* for many generations on a factitious host, could have affected parasitoids capacity to attack other hosts (Hopper and Roush 1993, Hoffmann et al. 2001, Chailleux et al. 2012).

Understanding the suitability of the pest–plant system that underpins the parasitism pattern in the laboratory has important practical implications, as highlighted by our greenhouse cage experiments, where the parasitism activity of the field generation of both *Trichogramma* species was quite low in cages under greenhouse conditions. However, overall *T. absoluta* control achieved by *T. achaeae* was similar to those achieved with *M. pygmaeus* when these natural enemies were used alone ( $\approx 46\%$  decrease in *T. absoluta* larvae per plant, see Fig. 6), though the experiment was carried out over a short period as infestation levels were measured 16 d after the pest was introduced into the cages. As opposed to the results under laboratory conditions, *T. euproctidis* both released and field generations (with or without predator) showed the lowest parasitism rates in the greenhouse cage experiments. This species was not able to control *T. absoluta* populations; there was no significant difference in *T. absoluta* density compared with the control (see Fig. 6). These results indicate that populations of parasitoids released in the tomato crop may disappear soon after the releases. Therefore, the impact of *Trichogramma* field generation(s) on *T. absoluta* population growth could be considered negligible for biological control, at least with strains tested in our study.

This study further documents the possible impacts of *Trichogramma* generations when parasitoids emerged in the greenhouse from *T. absoluta*-parasitized eggs. Poor effectiveness of the field generation parasitoid in *T. absoluta* parasitism may be because of various factors. First, continuous tomato plant growth during the course of the cage experiment has led most likely to an increasing plant architecture complexity between the beginning of the experiment, in which the parasitoids were first released (released generation), and the end of the experiment, when parasitoids developing in *T. absoluta*-parasitized eggs emerged. Increased habitat complexity is known to negatively affect efficiency (host searching, dispersal ability, or both) of natural enemies in finding hosts or prey (Rutledge and O'Neil 2005, Tabone et al. 2012). Second, parasitism effectiveness may have decreased because of 1) the small size of the parasitoids emerging from *T. absoluta* eggs (such small host eggs make parasitoid females mature fewer eggs, Kazmer and Luck 1995), and 2) increased sex ratio in offspring produced (more males in the population). Third, parasitoid mobility may have been reduced on tomato plants because of the high pubescence of tomato. Foraging ability was consequently reduced (Carrillo et al. 2008). *Trichogramma* parasitoids are known to be poor flyers and to forage primarily by walking and jumping on substrates (Keller 1987, Olson and Andow 2006). In addition, previous studies reported that tomato trichomes have negative effects on *Tricho-*

*gramma* efficiency (Kauffman and Kennedy 1989, Farar et al. 1994).

Our results suggest that the pest–plant system *T. absoluta*–tomato shows unfavorable characteristics for the effective establishment of *Trichogramma* parasitoids in tomato crops. This contrasts with other pest–plant systems in which *Trichogramma* field parasitoid generations provide biocontrol services to some extent after the initial release in the crop, for example, on the European corn borer in maize crop (Thomson et al. 2003, Hoffmann et al. 2006, Pintureau 2009). Thus, the expected biocontrol service provided by the *Trichogramma* field generations in tomato crop against *T. absoluta* should be considered low. *Trichogramma*-based biocontrol programs targeting *T. absoluta* on tomato crop therefore may need to rely on periodical inundative releases of the parasitoid, at least for *Trichogramma* strains so far identified as potential natural enemies of *T. absoluta* in Europe.

We also evaluated the effect of the generalist predator *M. pygmaeus* presence on the effectiveness of *T. achaeae* and *T. euproctidis* releases against *T. absoluta*. *M. pygmaeus* decreased the abundance of parasitoids hence their effectiveness on *T. absoluta*, both through direct competition for the resource (moth eggs), and direct predation on *Trichogramma*-parasitized eggs (Desneux et al. 2011b, Chailleux et al. 2013). Parasitism exerted by the released and field generations on both parasitoid strains was lower when the predator was present. The highest *T. absoluta* control level ( $<68\%$  larvae per plant than in the control) was achieved when combining the parasitoid *T. achaeae* with the predator, although it was not significantly different from *T. achaeae* or *M. pygmaeus* when tested alone. In addition, the presence of *M. pygmaeus* nymphs suggested effective predator establishment after introduction into cages. Previous studies reported that supplementary releases of *T. achaeae* in tomato crops, in which another mirid predator (*Nesidiocoris tenuis* Reuter) was released during the cropping cycle, improved the biological control of *T. absoluta* (Desneux et al. 2010; Cabello et al. 2012). Our results back up these conclusions. However, additional releases of *T. achaeae* did not improve control of *T. absoluta* when *N. tenuis* was inoculated by preplant releases in nurseries (Calvo et al. 2012), namely when the predator populations were already well established in the tomato crop before arrival of *T. absoluta*. Nevertheless, such a strategy based on very early establishment of *N. tenuis* in nursery plants often leads to use of insecticides when the omnivorous predator reaches high densities. Indeed, this predator is known to inflict damage to tomato plants, causing necrotic rings on stems and flowers and punctures in fruits (Calvo et al. 2009, Castañé et al. 2011).

Our study suggests that the integration of *Trichogramma* parasitoids for biological control strategies against *T. absoluta* in tomato crops should rely on the use of several inundative releases and should also take into account the population levels of generalist predators either artificially released, naturally occurring in the tomato crop, or both.

## Acknowledgments

We thank Cécile Thomas, Philippe Bearez, and Eddy-Amre Colombel (INRA Sophia-Antipolis) for technical assistance; Méline Beal and Jacques Frandon (Biotop, InVivo AgroSolutions) for providing biological materials; ANRT (National Association of Research and Technology) and InVivo AgroSolutions for funding to A. C. (Ph.D. fellowship); the University of Catania for funding to A. B. (Ph.D. fellowship); the French ministry of agriculture for funding to N. D. (CASDAR [Account for Agricultural and Rural Development] project 10063); and the anonymous referees as well as the handling editor for their helpful comments.

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Received 21 February 2013; accepted 10 September 2013.

As shown from the **Article 2**, the parasitoids that developed on *T. absoluta* after initial releases contribute little to biological control of *T. absoluta*, and parasitism tended to be lower when the predator *M. pygmaeus* was present (**Fig. 8**). However, a slightly higher *T. absoluta* control level was achieved by combining the predator and release of the parasitoid *Trichogramma achaeae* Nagaraja and Nagarkatti. The findings suggested that *Trichogramma* parasitoids can be used in combination with *M. pygmaeus* to enhance the biological control of *T. absoluta* in tomato-producing greenhouses.

In conclusion, the works presented in **Article 1 and 2** provide valuable information of promising indigenous natural enemies against *T. absoluta*. One of the important species, *M. pygmaeus*, will be included into our testing models in the following studies presented in **chapter 3 and 4**.

**Figure 8:** Predation behavior of the omnivorous predator *M. pygmaeus* adult: (a) Predator foraging for prey eggs; (b) Prey eggs sucked (or not) by *M. pygmaeus*



**Chapter 3: Nitrogen and water inputs mediated  
plant-arthropods Interactions: studies at individual  
scale**

Herbivorous insects have obtained the capacity of acquiring nutrients from plants while protecting themselves from the harm of plant defense during the long-history of plant-herbivorous insect co-evolution (Agrawal 2006; Howe and Jander 2008). Herbivorous insects need to obtain nutrients and water from their host plants to survive, develop and reproduce. Because of such tight link between them, life-history parameters of herbivorous insects could be strongly influenced by the bottom-up forces, either biotic factors (e.g., plant insect-resistance trait) (Kaplan and Jennifer 2011) or abiotic factors (e.g., resource input) (Chen et al. 2010). Variation in resource input to plants is likely to trigger bottom-up effects on the survival and development of herbivorous insects feeding on. Suboptimal resource inputs (i.e., excessive or insufficient) may induce changes in nutritional value and chemical defense profile in host plants that are closely related to the insects that feed on them (Inbar et al. 2001). Overall, the effect of resource input on herbivorous insects may be related to four main factors: (i) type and intensity of stress (e.g., we define the improper resource input as “stress”), (ii) plant cultivar and (iii) insect species (Inbar et al. 2001; Johnson 2008; Mody et al. 2009).

(i) Nitrogen input to plants is considered as an important factor that may trigger bottom-up effects on plant-feeding insects (Chen et al. 2010). Nitrogen content in plant may be a limiting factor to the performance of herbivorous insects because the shortage of nitrogen content in plant tissues could not meet the nutrient requirement by insects (i.e., “Nitrogen limitation hypothesis”) (White 1993). However, the results from several other studies have questioned this hypothesis (Joern and Bechmer 1998; Fischer and Fielder et al. 2000). Overall, nitrogen status in plants may alter the suitability of plants as host for herbivorous insects, the suitability of herbivorous insects as prey/host of natural enemies, and the plant indirect defense by recruitment of natural enemies (Chen et al. 2010).

Water is another potentially important resource which could mediate plant-herbivorous insect interactions (Schoonhoven et al. 2005). The bottom-up effects of drought on insect performance have been attributed to a combination of

changes in plant nutritional value and chemical defenses (Inbar et al. 2001; Gutbrodt et al. 2011). The theoretical consequences of drought events for plants and associated herbivorous insects have been predicted in several hypotheses. These include the “Plant stress hypothesis” which predicted that drought enhance the performance of senescence-feeders (i.e., insects that feed on mature plant materials) (White 1969; 2009), the “Plant vigor hypothesis” which predicted that optimal water input may enhance the performance of flush-feeders (i.e., herbivores insects preferring to feed on young buds or leaves) when the plants are vigorous (i.e., unstressed) (Price 1991), the **“Growth-differentiation balance hypothesis”** (GDBH) which predicted that the production of defensive compounds in plant shows a parabolic response to the increasing intensity of drought, with the highest level as intermediate drought (Herms and Mattson 1992), the “Pulsed stress hypothesis” which predicted that the phenology of drought is a key factor for predicting the effects of drought on sap-feeders, with enhanced performance when the plant is under pulsed/intermittent drought conditions, vice versa (Huberty and Denno 2004), and an integrated insights which highlight the importance of herbivore food specialization (Gutbrodt et al. 2011). Furthermore, Intensity of drought is also an important factor influencing the consequences on herbivores (Mody et al. 2009).

(ii) Variation in resource inputs to plants may cause physiological changes that are relevant to insect performance. However, these physiological changes may be different in the plant cultivars that have the capacity to tolerate or to adapt to these environmental variations (hereafter referred to “Resistant plant cultivars”) compared to the susceptible ones (Cattivelli et al. 2008; Farooq et al. 2009; Feng et al. 2009). In modern agriculture, diverse plant cultivars have been bred to cope with various sub-optimal growing conditions. Thus, the environmental stress tolerance traits in these resistant plant cultivars may mitigate the possible negative bottom-up effects on herbivorous insects generated by the corresponding environmental stress. For instance, drought has been proved to reduce the larval relative growth rate of the corn earworm *Heliothis zea* (Lepidoptera: Noctuidae) on a drought-susceptible tomato cultivar

(Inbar et al. 2001), in contrast, on a drought-resistance tomato cultivar, it is expected that drought resistance traits may mitigate the negative effects of drought on the performance of this species.

(iii) The process of phytophagy in these species is one of the major conduits of energy flow between plants and the rest of the food web (Agrawal 2006). Phytophagy is common in arthropod insects with some species acting solely plant-feeding (Schoonhoven et al. 2005) or acting both plant feeding and prey feeding (i.e., omnivory) (Naranjo and Gibson 1996; Coll 1998). The former is called as herbivorous insects and later as omnivorous insects. Herbivorous insects employ various feeding strategies such as chewing, sucking, and mining. Differences in feeding strategies in insects may result in different responses to the resources variations (Inbar et al. 2001). Furthermore, omnivorous insect species is considered as a special group because they also act as predators of other insects. Among this group, the predatory Heteroptera has been considered as a special form of omnivory (Coll 1998; Lalonde et al. 1999). They show plastic feeding behavioural responses depending on the foraging environment, such as prey densities (Agrawal et al. 1999; Montserrat et al. 2004), plant quality (Eubanks and Denno 1999, 2000; Janssen et al. 2003) and other abiotic factors (Gillespie and McGregor 2000; Sinia et al. 2003). Numerous Heteroptera species have been exploited as biological control agents against a wide range of agricultural pests such as aphids, thrips or Lepidoptera eggs (Coll 1998; Margaritopoulos et al. 2003; Desneux et al. 2006; Castañé et al. 2011; Chailleux et al. 2013a). Hence, modifying feeding ecology of omnivorous Heteroptera by manipulating their foraging environment may be a useful option for enhancing their role in biological control.

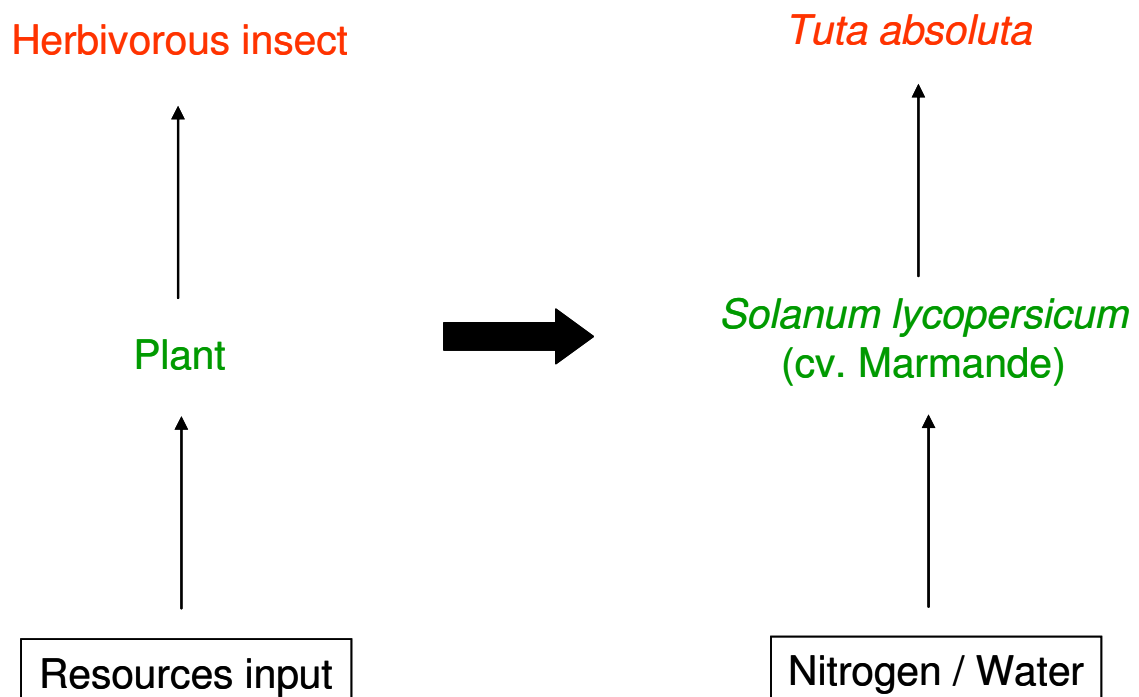
=> **In this chapter**, we presented 4 studies which examined the bottom-up effects of plant resource inputs (nitrogen and water) on plant-arthropods interactions. These bottom-up effects were characterized for plant traits including plant growth, physiological functions, nutritional quality and chemical defenses, as well as for insect life-history parameters.

### I) Bottom-up effects of nitrogen and water on a leafminer

Plant-herbivorous insect interactions can be influenced by various biotic or abiotic factors (Hunter et al. 1992; 2001; Inbar et al. 2001; Mody et al. 2009; Gutbrodt et al. 2011). Among the abiotic factors, resource inputs such as nitrogen and water to plants may trigger bottom-up effects on herbivorous insects that feeding on (White et al. 1993; Huberty and Denno 2004).

In the article 3, the study model was present in **Fig. 1**. We studied the bottom-up effects of various levels of nitrogen and water inputs to tomato plants on survival and development of an invasive tomato leafminer, *Tuta absoluta* (Meytick) (Lepidoptera; Gelechiidae). **Figure 2** shows the experimental design under laboratory conditions.

**Figure 1:** The study model of article 3. The left diagram shows the general model, the right shows the biological model used in this study



**Figure 2:** Experimental setup on *T. absoluta* under laboratory conditions:



(A) Tomato plant growth under laboratory conditions before the *T. absoluta* infestation



(B) *Tuta absoluta* larvae develop on the tomato leaves trapped by the mesh bags



## Article 3

### **Nitrogen and water availability to tomato plants triggers bottom-up effects on the leafminer *Tuta absoluta*.**

Peng Han<sup>1</sup>, Anne-Violette Lavoie<sup>1,2</sup>, Jacques Le Bot<sup>3</sup>, Edwige Amiens-Desneux<sup>1</sup>,  
Nicolas Desneux<sup>1\*</sup>

<sup>1</sup> French National Institute for Agricultural Research (INRA), UMR1355, 400 Route  
des Chappes, 06903, Sophia-Antipolis, France

<sup>2</sup> University of Nice Sophia-Antipolis (UNS), UMR1355, 400 Route des Chappes,  
06903, Sophia-Antipolis, France

<sup>3</sup> French National Institute for Agricultural Research (INRA), UR 1115 Plant and  
Horticultural Cultivating Systems, 84000 Avignon, France

\* Corresponding author: [nicolas.desneux@sophia.inra.fr](mailto:nicolas.desneux@sophia.inra.fr)



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SUBJECT AREAS:  
INVASIVE SPECIES  
ECOSYSTEM ECOLOGY

Received  
14 January 2014

Accepted  
5 March 2014

Published  
28 March 2014

Correspondence and  
requests for materials  
should be addressed to  
N.D. (nicolas.desneux  
@sophia.inra.fr)

# Nitrogen and water availability to tomato plants triggers bottom-up effects on the leafminer *Tuta absoluta*

Peng Han<sup>1</sup>, Anne-Violette Lavoit<sup>1,2</sup>, Jacques Le Bot<sup>3</sup>, Edwige Amiens-Desneux<sup>1</sup> & Nicolas Desneux<sup>1</sup>

<sup>1</sup>French National Institute for Agricultural Research (INRA), UMR1355, 400 Route des Chappes, 06903, Sophia-Antipolis, France, <sup>2</sup>University of Nice Sophia-Antipolis (UNS), UMR1355, 400 Route des Chappes, 06903, Sophia-Antipolis, France, <sup>3</sup>French National Institute for Agricultural Research (INRA), UR1115 PSH (Plantes et Systèmes de culture Horticoles), 84000 Avignon, France.

This study examined the effects of various levels of nitrogen inputs (optimal, insufficient and excessive) and water inputs (optimal, low drought and high drought) to tomato plants (*Solanum lycopersicum*) on survival and development of an invasive tomato leafminer, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). Plant growth i.e. plant height and the number of nodes declined under insufficient or excessive nitrogen treatment. Compared to optimal N, insufficient N treatment decreased leaf N content and increased the carbon/nitrogen ratio (C/N) whereas an excess of N had no effect on both leaf N content and leaf C/N ratio. Sub-optimal nitrogen supplies, water treatments and their interactions, significantly reduced the leafminer survival rate and slowed down its development. Together with the findings from three recent companion studies, we assumed that a combination of changes in nutritional value and chemical defense could explain these observed effects. Furthermore, our findings supported both the “Plant vigor hypothesis” and the “Nitrogen limitation hypothesis”.

Plant-arthropod interactions can be markedly shaped by bottom-up forces<sup>1–4</sup>. Phytophagous arthropods need to obtain nutrients from their host plants in order to survive and develop. However, they usually forage in environments where developing plants show contrasted palatability levels in terms of nutritional value (nitrogen richness or water content) as well as toxicity related to defensive traits (through secondary metabolites)<sup>1,5</sup>. Plant nutritional value and plant defenses are highly variable owing to factors such as plant species, presence of competitors, light, temperature, as well as water and nutrient availability<sup>6–10</sup>. Such variations in plant quality can lead to trophic cascades such as bottom-up effects on phytophagous arthropods (e.g. on development, fecundity, etc.)<sup>2–3,5</sup>.

Nitrogen is one of the most commonly applied mineral fertilizers in crop production due to its importance in the composition of plant tissues. Plant feeding insects thus need to transform and utilize the different inorganic forms of nitrogen that occur in plant tissues<sup>1,5</sup>. Indeed, the development of the lepidopterans has been positively correlated to plant nitrogen content<sup>5,7,11–15</sup>. By contrast, a shortage of nitrogen input to the plants was shown to impair the performance of herbivore insects, which was termed the “Nitrogen limitation hypothesis”<sup>16</sup>. The explanation could be that (1) insects need to transform the inorganic nitrogen forms present in plant tissue and/or utilize directly plant-derived amino acids to synthesize structural proteins and enzymes<sup>5</sup>; (2) nitrogen deficient plants have a higher accumulation of plant allelochemicals which can be toxic to herbivorous insects. For instance, tomato plants subjected to nitrogen limitation accumulate more phenolic compounds in their organs<sup>17–20</sup>. Although many studies reported the existence of positive correlations between insect performance and host plant nitrogen content as mentioned above, several studies however have documented negative effects due to high nitrogen content in leaves and undermined the general application of the “Nitrogen limitation hypothesis”<sup>15,21,22</sup>.

Water is another potentially important abiotic factor which could mediate plant-herbivore insect interactions<sup>5,21</sup>. Water-availability mediated interactions between herbivorous insects and their host plants are affected by various factors including herbivore feeding specialization, herbivore species and the seasonality of water availability<sup>10,23</sup>. The plant water content is a useful index of its nutritional value for many lepidopterous larvae<sup>5</sup>. In addition, drought may influence plant chemical defense level leading to either enhanced or reduced resistance to herbivores<sup>7,10</sup>. Historically, drought periods have been considered a major factor underlying outbreaks of herbivorous insects<sup>24–26</sup>. However, the consequences of water-availability on plant-insect interactions have been predicted in several hypotheses primarily in the “Plant stress hypothesis” which assumes that stressed plants are



better hosts for senescence-feeders<sup>24,27</sup> or in the “Plant vigor hypothesis” which suggests that herbivorous insects prefer and perform better on rapidly-growing plants i.e. vigorous ones<sup>28</sup>. Within a given feeding guild, such as phloem-feeders, aphids do respond positively to intermittently stressed plants but negatively to continuously stressed ones. The “Pulsed stress hypothesis” assumes that the outcomes depend on the occurrence of water stress: continuous or pulsed drought<sup>23</sup>. In general, insect feeding guild (phloem feeder, leaf miner or leaf chewer) and feeding specialization (contrasting feeding preferences of two Lepidoptera species of the same feeding guild) have been used to explain various herbivore responses to water stressed plants<sup>7,10,23</sup>.

In this context, we studied how nitrogen and water inputs and/or their interactions may induce potential plant physiological changes, either in terms of nutritional value or chemical defenses and how, in turn, they would affect herbivorous insect survival and development. The tomato plant *Solanum lycopersicum* L. and the herbivorous insect *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) were used to set up a “nutrient & water input-plant-herbivore insect” testing system to address these questions. The tomato leafminer *T. absoluta* is a devastating tomato pest from South America which has rapidly invaded the Mediterranean basin since it first appeared in Spain in 2006<sup>29,30,49</sup>. *Tuta absoluta* adults usually lay eggs on the leaves and stems, and to a lesser extent on fruits. After hatching, young larvae penetrate and enter the plant tissues to feed and develop<sup>29</sup>. Moreover, leafminers have the most intimate relationships with their host plants and thus are considered the most appropriate to test the hypotheses mentioned above<sup>7</sup>. Our aims were (1) to test whether the altered nitrogen and water inputs to tomato plants could trigger a bottom-up effect on biological traits of *T. absoluta*; (2) to evaluate the plant physiological changes that could explain the effects observed on *T. absoluta*. Attempts to answer these questions may help to provide theoretical support to optimize IPM (Integrated Pest Management) of *T. absoluta*.

## Results

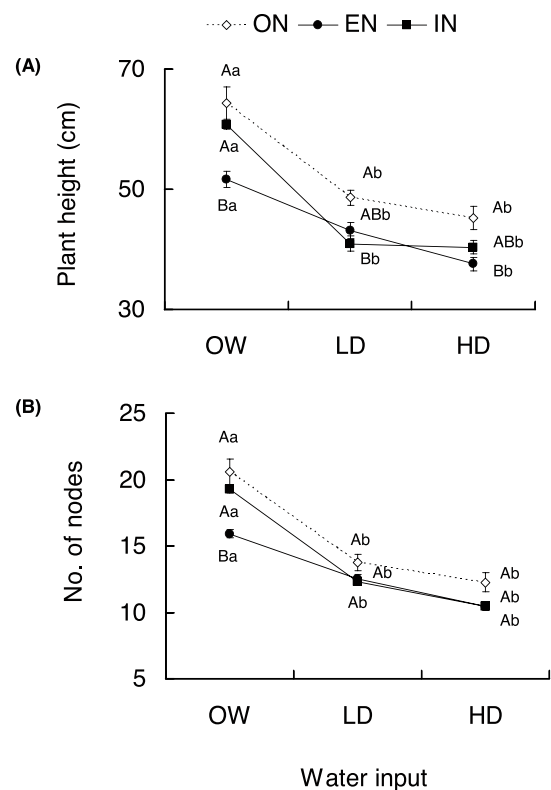
**Plant growth.** Statistical results on plant growth dataset are summarized in Table 1. Nitrogen and water inputs affected plant height significantly on the three measurement dates (58 days after seeding [DAS 58], Fig. 1; DAS 32 and DAS 40 not shown) whereas effect of *nitrogen* × *water* interaction was only found at DAS 58. The number of nodes per plant was affected differently by nitrogen and water inputs and their interaction was significant (Fig. 1).

Plants supplied with the optimal water input increased in height i.e. about 30% higher compared to those subjected to low or high drought. For the optimal water treatment, the plants fertilised with the optimal nitrogen regime attained significantly greater height than those treated with excessive nitrogen input ( $P < 0.001$ ), but their height was similar to that of those treated with insufficient nitrogen input ( $P = 0.767$ ). The number of nodes per plant followed a similar pattern as plant height since only excessive nitrogen treatment significantly reduced the number of nodes compared to optimal and insufficient N treatments (both  $P < 0.001$ ).

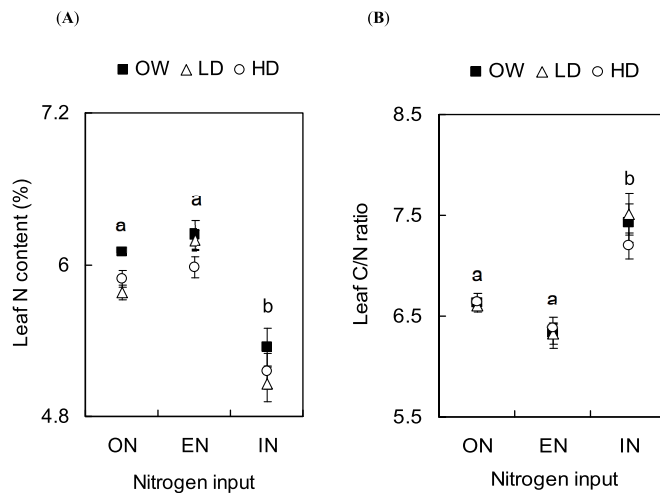
**Leaf N content and C/N ratio.** Leaf N content (dry mass percent) differed significantly among the nitrogen treatments ( $F_{2, 135} = 47.556$ ,  $P < 0.001$ ), but not among water treatments ( $F_{2, 135} = 0.230$ ,  $P = 0.795$ ) (Fig. 2). There was no interaction *nitrogen* × *water* on the leaf N content ( $F_{4, 135} = 2.392$ ,  $P = 0.054$ ). The plants treated with optimal nitrogen showed a leaf N content similar to those treated with excessive nitrogen input. Nevertheless the leaf N content was significantly higher than in those treated with insufficient nitrogen input ( $P < 0.001$ ). Leaf C/N ratio differed significantly among the nitrogen treatments ( $F_{2, 135} = 43.487$ ,  $P < 0.001$ ), but not among the water treatments ( $F_{2, 135} = 2.668$ ,  $P = 0.073$ ) (Fig. 2). There was no

**Table 1 | Factorial ANOVA used to analyze plant traits: (A) Plant height at DAS 32 (32 days after seeding), DAS 40 and DAS 58; (B) Number of nodes at DAS 58. Main factors tested were the “nitrogen” and “water”**

A - Plant height			
Source of variation	Df	F	P values
DAS 32			
Nitrogen	2	18.34	<0.001
Water	2	38.69	<0.001
Nitrogen × water	4	1.32	0.268
DAS 40			
Nitrogen	2	23.43	<0.001
Water	2	50.74	<0.001
Nitrogen × water	4	0.60	0.662
DAS 58			
Nitrogen	2	23.41	<0.001
Water	2	114.93	<0.001
Nitrogen × water	4	3.47	0.011
B - Number of plant nodes at DAS 58			
Source of variation	Df	F	P values
Nitrogen	2	21.32	<0.001
Water	2	188.20	<0.001
Nitrogen × water	4	4.48	0.002



**Figure 1 | (A) Plant height (mean ± SEM, n = 12 plants) and (B) Number of nodes (mean ± SEM, n = 12 plants) at 58 days after seeding (DAS 58) for tomato plants treated with different nitrogen and water inputs. (ON: optimal nitrogen; EN: excessive nitrogen; IN: insufficient nitrogen; OW: optimal water; LD: low drought; HD: high drought). Capital letters indicated the comparison among different nitrogen inputs within a given water input level; Lower case letters indicated the comparison among different water inputs within a given nitrogen input level. Different letters indicate significant difference at  $P < 0.05$ .**



**Figure 2 |** (A) Leaf N content (in dry mass percent, mean  $\pm$  SEM,  $n = 6$ ) and (B) Leaf C/N ratio (unit-less, mean  $\pm$  SEM,  $n = 6$ ) of tomato leaf before the artificial infestation of *T. absoluta*. (OW: optimal water; LD: low drought; HD: high drought; ON: optimal nitrogen; EN: excessive nitrogen; IN: insufficient nitrogen). Comparisons were done only among different “nitrogen input” treatments because no effect of “water input” was found. Different Letters indicate significant difference at  $P < 0.05$ .

interaction *nitrogen*  $\times$  *water* on leaf C/N ( $F_{4, 135} = 2.282$ ,  $P = 0.064$ ). The leaf C/N ratio was similar between optimal and excessive N treatments whereas it was significantly higher on plants treated with insufficient nitrogen input ( $P < 0.001$ ).

***T. absoluta* survival.** Both water and nitrogen inputs significantly affected *T. absoluta* survival from egg to pupa or to adult (nitrogen:  $\chi^2 = 23.89$ ,  $df = 2$ ,  $P < 0.001$ ; water:  $\chi^2 = 17.68$ ,  $df = 2$ ,  $P < 0.001$ ; interaction:  $\chi^2 = 4.71$ ,  $df = 4$ ,  $P = 0.318$ ) (Table 2). *Tuta absoluta* survival also showed various, different responses to nitrogen and water inputs between both pupa and adult stages ( $\chi^2 = 12.96$ ,  $df = 1$ ,  $P < 0.001$ ). For the optimal nitrogen input, *T. absoluta* had a significantly lower survival rate (proportion of eggs reaching pupa or adult stages) when the plants suffered high drought (ON-OW vs. ON-HD, Table 2). For the excessive nitrogen input, the pattern was similar to those observed for optimal nitrogen. However, significantly fewer *T. absoluta* reached adulthood when the plants received optimal water input (EN-OW vs. ON-OW, Table 2). For the insufficient nitrogen input, the rate of *T. absoluta* survival significantly declined (for both pupae and adults) in all water treatments when compared to the ON-OW condition (ON-OW vs. IN-treatments, Table 2).

***T. absoluta* development. Pupal weight.** Both nitrogen and water inputs and their interaction had significant effect on *T. absoluta* pupal weight (Fig. 3A, nitrogen:  $F_{2, 399} = 66.851$ ,  $P < 0.001$ ; water:  $F_{2, 399} = 13.815$ ,  $P < 0.001$ ; interaction:  $F_{4, 399} = 5.583$ ,  $P < 0.001$ ). For both the optimal water input and the low drought, pupal weight was significantly higher under optimal nitrogen input than under excessive or insufficient nitrogen inputs (all  $P < 0.05$ ). However, in the high drought treatment, pupal weight was equally low at the three nitrogen levels (IN vs. ON:  $P = 0.460$ ; EN vs. ON:  $P = 0.500$ ).

**Development time from egg to pupa or to adult.** Both nitrogen and water and their interaction significantly affected *T. absoluta* development time (Fig. 3B “from egg to pupa”: nitrogen:  $F_{2, 399} = 109.96$ ,  $P < 0.001$ ; water:  $F_{2, 399} = 118.72$ ,  $P < 0.001$ ; interaction:  $F_{4, 399} = 6.172$ ,  $P < 0.001$ ; Fig. 3C “from egg to adult”: nitrogen:  $F_{2, 299} = 33.059$ ,  $P < 0.001$ ; water:  $F_{2, 299} = 33.126$ ,  $P < 0.001$ ; interaction:  $F_{4, 299} = 3.328$ ,  $P = 0.011$ ).

Under the optimal water and the low drought treatments, the development time from egg to pupa was significantly extended when

**Table 2 |** Survival proportions of *T. absoluta* individuals reaching pupae (from egg to pupa) or adults (from egg to adult) when feeding on tomato plants supplied with various nitrogen and water inputs (ON: optimal nitrogen; EN: excessive nitrogen; IN: insufficient nitrogen; OW: optimal water; LD: low drought; HD: high drought). ON-OW was considered as control group and other treatments were compared to this control group. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  (significantly different from values of individuals developing on plants subjected to optimal nitrogen [ON] and optimal water [OW], in *italics*) (permuted Fisher exact test)

Nitrogen input	Water input	Proportion of individuals reaching pupae	Proportion of individuals reaching adults
ON	OW	0.70	0.57
ON	LD	0.58	0.44
ON	HD	0.51 **	0.39 *
EN	OW	0.58	0.42 *
EN	LD	0.60	0.44
EN	HD	0.43 ***	0.36 **
IN	OW	0.54 *	0.39 *
IN	LD	0.38 ***	0.26 ***
IN	HD	0.28 ***	0.22 ***

the plants received either excessive or insufficient nitrogen inputs (all  $P < 0.05$ , Fig. 3B). Under high drought, the development time was only prolonged significantly by the insufficient nitrogen input ( $P = 0.017$ ). Besides, the development time from egg to pupa increased on the plants subjected to high drought at any nitrogen input level (all  $P < 0.05$ , Fig. 3B).

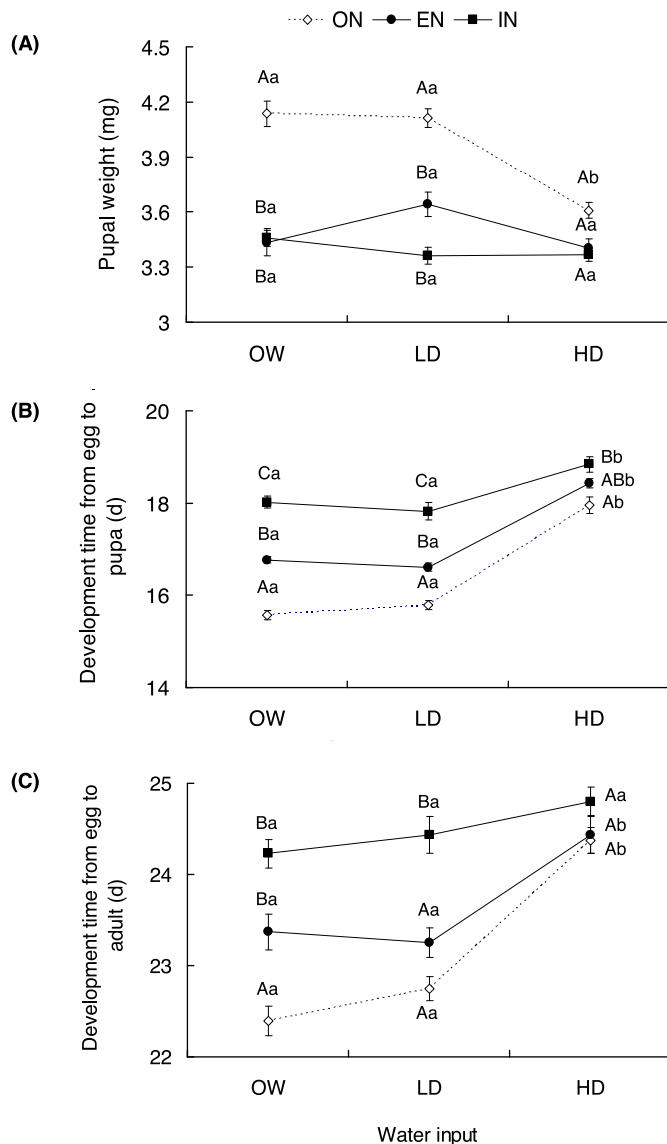
Under the optimal water input, the development time from egg to adult was significantly extended when the plants were under excessive or insufficient nitrogen inputs (all  $P < 0.05$ , Fig. 3C). Under the low drought treatment, this development time was only significantly delayed by the insufficient nitrogen input ( $P < 0.001$ , Fig. 3C). Under high drought, there was no difference in development time among the three nitrogen input levels (IN vs. ON:  $P = 0.950$ ; EN vs. ON:  $P = 0.980$ ). For optimal and excessive nitrogen treatment, the delay in the development time from egg to adult was significantly prolonged under high drought (all  $P < 0.05$ ).

#### Relationship between *T. absoluta* development and leaf N content.

Table 3 shows the regression relationship between leaf N content and *T. absoluta* development i.e. development time either from egg to pupa or from egg to adult and pupal weight. The development time was negatively correlated to leaf N content and pupal weight was positively correlated to leaf N content. The degree of linear correlation and the correlation coefficient ( $R^2$ ) varied depending on the water treatments. The linear correlation was stronger in the case of optimal water input than in that of low or high drought for the three parameters.

#### Discussion

Our data demonstrated that variation of nitrogen and water inputs to tomato plants significantly affected some biological traits of *T. absoluta*. Firstly, nitrogen limitation and drought reduced the survival rate from egg to pupa or adult stages. Secondly, nitrogen limitation and drought reduced pupal weight and slowed down the development of *T. absoluta*. These results therefore fulfill the first aim of this paper by making it clear that altered nitrogen and water inputs to tomato plants do trigger a bottom-up effect on the biological traits of *T. absoluta*. Thus, two questions remain to be answered: (i) what are the physiological changes in the host plants that could explain the effects on the biological traits of *T. absoluta*? (ii) Did our results



**Figure 3** | (A) Pupal weight (mean  $\pm$  SEM,  $n = 25-63$ ); (B) Development time (mean  $\pm$  SEM,  $n = 25-63$ ) from egg to pupa; (C) Development time (mean  $\pm$  SEM,  $n = 20-51$ ) from egg to adult of *T. absoluta* feeding on tomato plants with different nitrogen and water inputs. (ON: optimal nitrogen; EN: excessive nitrogen; IN: insufficient nitrogen; OW: optimal water; LD: low drought; HD: high drought). Capital letters indicate the comparison among different nitrogen inputs at a given water input level; Lower case letters indicate the comparison among different water inputs at a given nitrogen input level. Different Letters indicate significant difference at  $P < 0.05$ .

support or refute the “Nitrogen limitation hypothesis”, the “Plant stress hypothesis” and the “Plant vigor hypothesis”?

In this section we will discuss how the water/nitrogen treatments influenced plant physiology and in turn affected the survival and development of *T. absoluta*. Drought was seen to negatively affect *T. absoluta* survival and development. In low or high drought treatments, the foliage tended to wilt thus probably becoming less edible for Lepidoptera larvae<sup>10</sup>. Here, *T. absoluta* showed a lower survival rate when fed on the host plants subjected to drought. We assumed that larvae faced difficulty in obtaining enough water for optimal development thus causing a slowdown in their development. In addition, water not only acted as an indispensable ingredient for herbivore metabolism but also mediated nitrogen availability for plants<sup>23</sup>. It was generally agreed that nitrogen and water content are positively correlated<sup>31</sup>. *T. absoluta* larvae therefore may suffer a deficit of N-based nutrient in the plants subjected to drought.

Insufficient nitrogen input to the tomato plants triggered negative bottom-up effects on *T. absoluta* which was consistent with the “Nitrogen limitation hypothesis”<sup>16</sup>. We may infer that *T. absoluta* larvae could be affected by changes in nutritional value and chemical defense in tomato plants. Firstly, N-limited tomato plants might have lower leaf nutritional value for *T. absoluta* larvae. Indeed, nitrogen was considered as a limiting nutrient factor for the growth of many Lepidoptera herbivores because larvae may experience lack of organic nitrogen, i.e. specific protein and/or amino acids, leading to reduced or impaired metabolism during their critical growth period and even to premature death<sup>13,14,32</sup>. Moreover, concentration of non-nitrogenous compounds in leaves could also be negatively affected by insufficient nitrogen input, such as starch in tomato leaves as well as soluble carbohydrate in young tomato leaves<sup>20</sup>. *T. absoluta* suffered a higher mortality rate during the larvae stage. In addition, lower leaf nutritional value may influence the feeding behaviour of the larvae<sup>3</sup>; in our study, we observed *T. absoluta* had a longer development time i.e. delayed development. One possible explanation could be that larvae must compensate for the insufficient N-based food by increasing daily food intake or switching foraging activity toward N-richer plant organs such as young leaves or buds<sup>33-35</sup>. In our study, *T. absoluta* larvae were confined in a mesh cage with only one leaf as food so they could not forage other areas richer in nitrogen. Therefore, the larvae development might be slower due to sub-optimal food quality and/or quantity e.g. fewer N-based nutrients.

Secondly, N-limited tomato plants could have higher levels of chemical defense against *T. absoluta*. GDBH (Growth Differentiation Balance Hypothesis) predicts that moderate growth limitations caused by external factors such as low nutrient availability will result in an accumulation of carbohydrates and, subsequently, in increased concentrations of constitutive secondary compounds<sup>36</sup>. Indeed, we assumed that the N-limited tomato plants produced more insect-defensive soluble phenolics (C-based defensive compounds) i.e. chlorogenic acid, rutin, kaempferol-rutinoside since their concentration is positively correlated with C/N ratio in tomato leaves<sup>7,18-20,37</sup>. Moreover, the concentration of constitutive tomatine,

**Table 3** | Linear correlation between (1) development time of *T. absoluta* from egg to pupa, (2) development time of *T. absoluta* from egg to adult or (3) *T. absoluta* pupal weight and plant leaf N content under various water inputs to the plants (OW: optimal water; LD: low drought; HD: high drought)

	OW	LD	HD
Development time from egg to pupa	$y = -2.50x + 31.17$ $R^2 = 0.715$	$y = -2.09x + 28.18$ $R^2 = 0.667$	$y = -0.46x + 20.99$ $R^2 = 0.080$
Development time from egg to adult	$y = -2.09x + 35.48$ $R^2 = 0.735$	$y = -2.20x + 35.63$ $R^2 = 0.630$	$y = -0.39x + 26.80$ $R^2 = 0.099$
Pupal weight	$y = 0.74x - 0.40$ $R^2 = 0.624$	$y = 0.62x + 0.41$ $R^2 = 0.372$	$y = 0.17x + 2.54$ $R^2 = 0.174$





a key N-based glycoalkaloid defensive compound in tomato, is also positively correlated with leaf C/N ratio<sup>20</sup>.

Excessive nitrogen input to the tomato plants also triggered negative bottom-up effects on *T. absoluta*. This study reported for the first time that excessive nitrogen input slowed down the development of a lepidoptera herbivore and induced a lower pupal mass. The negative effects on *T. absoluta* could only be attributed to higher chemical defense because the leaf nutritional value was similar between optimal and excessive nitrogen treatments; leaf N content and leaf C/N ratio are equal. We assumed that plants excessively supplied with nitrogen could allocate their surplus of nutrient resources to the synthesizing of N-containing compounds such as glycoalkaloids which may deter the insect metabolism and thus slow down their development. For example, Johnson *et al.*<sup>38</sup> observed on N<sub>2</sub>-fixing plant (lupines) that nitrogen-rich plants may benefit from faster and higher alkaloid induction than nitrogen-limited plants under herbivore attack.

To conclude, we suggest that the negative impact of insufficient nitrogen treatment on *T. absoluta* survival and development might be due to both the leaf nutritional value decrease and the leaf chemical defense increase. Furthermore, the negative impact of excessive nitrogen treatment could only be linked to higher chemical defense levels. Finally, there is an interaction between both factors on *T. absoluta* development showing that either factor is limiting with regard to the life-history parameters examined (Fig. 3A: “nitrogen” as a limiting factor for pupal weight; Fig. 3B, 3C: “water” as a limiting factor for development time from egg to pupa or to adult).

Two main hypotheses have so far been acknowledged to explain the effects of plant growth related stresses on insect herbivores, namely the “Plant stress hypothesis” and the “Plant vigor hypothesis”. The former assumes that stressed plants serve as a more suitable host for herbivore insects because of increased nutritional value i.e. amino acid and reduced plant defensive compound syntheses<sup>25,32</sup>. Larsson<sup>39</sup> modified this hypothesis and emphasized the idea that the effects of stressed plants on herbivore performances greatly depend on the insect feeding strategy, such as leaf mining, leaf chewing or sap feeding. The latter theory, an opposing one called the “Plant vigor hypothesis”, predicted that the best plant hosts for insect herbivores are vigorous plants<sup>28</sup>. Our findings support the second hypothesis since *T. absoluta* survived better and developed

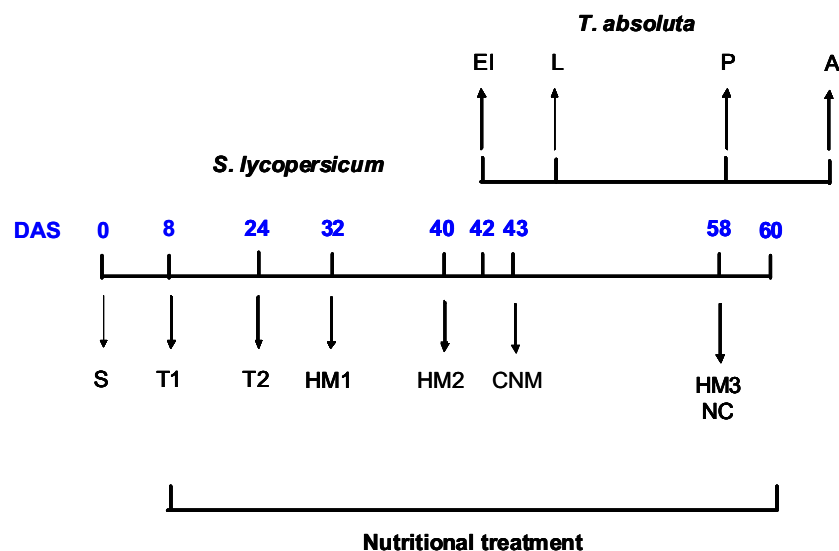
faster on the tomato plants with better growth status e.g. a larger plant growth rate in terms of height and node numbers (Fig. 1). By contrast, the plants subjected to non-optimal nitrogen and drought treatments were less suitable hosts for *T. absoluta*; larvae showed a lower survival rate and sub-optimal development compared to those developing on plants under optimal treatment.

Among the various stress types, the role of nitrogen limitation has received important attention in predicting plant-herbivore insect interactions. Our data also supported the “Nitrogen limitation hypothesis” since insufficient nitrogen input significantly lowered leaf N content (see Fig. 2) which in turn compromised the survival and development performances of *T. absoluta*. In particular, *T. absoluta* development was found to be negatively correlated to the leaf N content (see Table 3). The leafminer needed more time to develop in order to compensate for the nitrogen deficiency; despite longer development time, optimal pupal weight was not reached.

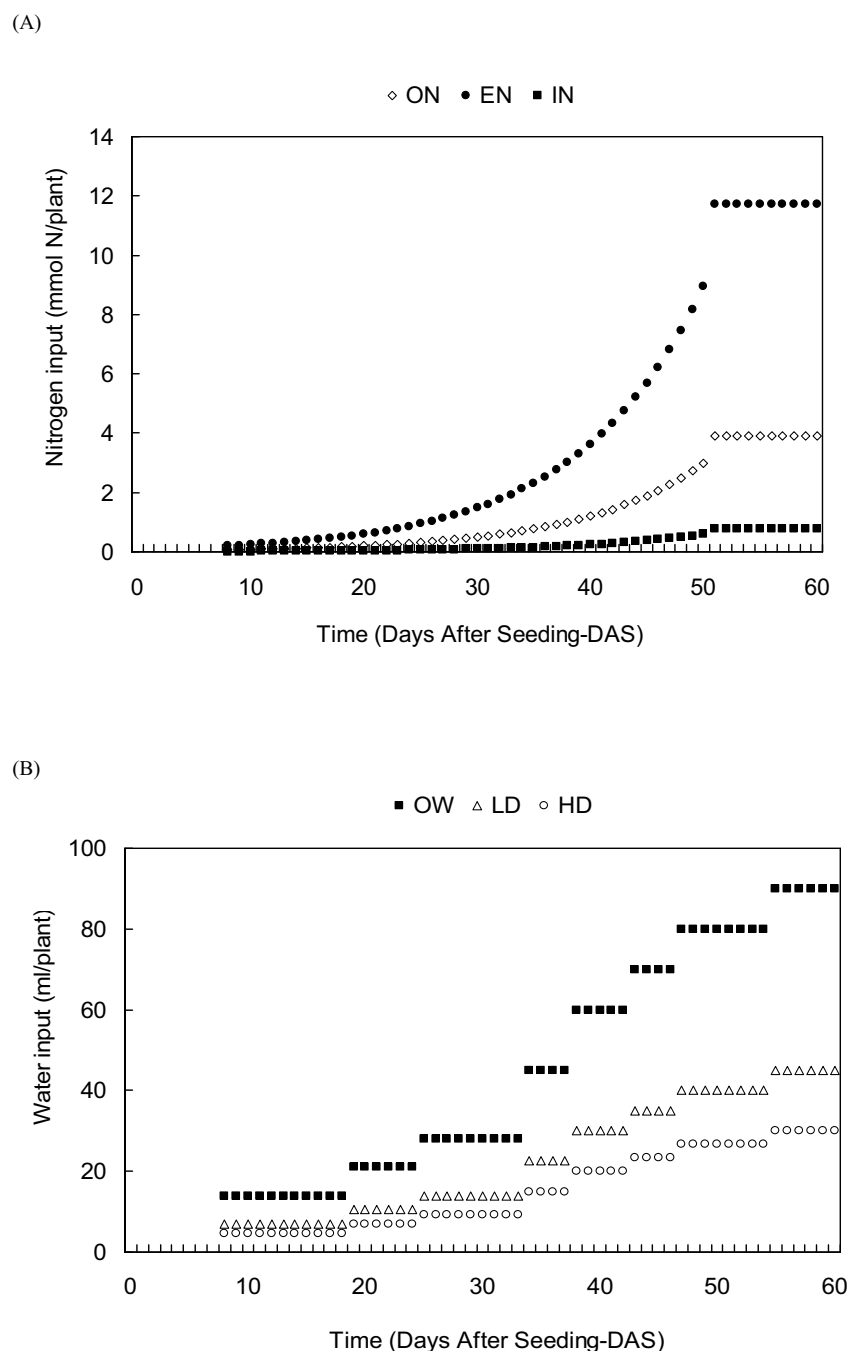
In conclusion, various nitrogen and water inputs to tomato plants affected the biological traits of the leafminer *T. absoluta* and these effects were probably due to a combination of lower plant nutritional value and higher chemical defenses. Our findings support both the “Plant vigor hypothesis” and the “Nitrogen limitation hypothesis” which are well reported for Lepidoptera pests. The manipulation of fertilizing and irrigating regimes may help to optimize agricultural practices by promoting negative effects on herbivorous insects. This could actually be achieved with little damage to plants and negligible crop yield losses<sup>40–42</sup>. Our findings show that sub-optimal nitrogen input, excessive or insufficient, and drought to tomato plants are unfavorable to *T. absoluta* but also appear unfavorable to plants resulting in lower growing status. Future work should aim at quantifying the trade-off between the negative impact on herbivorous pests and plant yield, as well as including arthropods from the higher trophic level i.e. natural enemies.

## Methods

**Biological material.** Tomato plants cv. Marmande were grown from seeds in a climatic chamber (12 h light, 24 ± 1 °C, 65 ± 5% RH) in cubic plastic pots (7 × 7 × 6.5 cm). The plants were then grown under laboratory conditions (16 h light, 24 ± 1 °C, 65 ± 5% RH). At DAS 8 (Days After Seeding) the seedlings were washed to remove soil and then transferred to new pots containing limestone grains (Perlite Italiana srl, Corsico, Italy) (see schedule Fig. 4). At DAS 24, the plants were



**Figure 4** | Sampling protocol schedule including plant growth (S: plant seeding; T1/T2: routine plants transfer), *T. absoluta* infestation and development throughout the plant development stages (EI: egg incubation on plant leaves; L: larvae; P: pupae; A: adults) and plant sampling events (HM1, HM2 and HM3: 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> plant height measurement, respectively; CNM: carbon/nitrogen content measurement in tomato leaves before *T. absoluta* larvae infestation; NC: node counting of tomato plants); DAS: days after seeding; Nutritional treatments lasted from DAS 8 to DAS 60 (also see Figure 1).



**Figure 5 | (A) Amount of daily nitrogen input (mmol) per pot throughout the plant growth period; (B) Input of water volume (ml of solution) per pot throughout the growth period.**

transferred to larger pots (diameter: 10 cm, height: 9 cm) filled with the same substrate.

The *T. absoluta* colony was maintained in regular chambers using tomato plants ( $25 \pm 1^\circ\text{C}$ , RH  $70 \pm 10\%$ , 16 h light). It was kept in cages measuring  $55 \times 75 \times 80$  cm and containing tomato plants (as described by Biondi *et al.*<sup>43</sup>). Food (honey) and water were provided *ad libitum* to adults in rearing cages. Newly-oviposited *T. absoluta* eggs ( $\leq 24$  h) were used to infest the plants. To obtain these eggs, we used the method described by Chailleux *et al.*<sup>44</sup>. Ten couples of *T. absoluta* adults were placed inside a plastic tube containing a fresh tomato leaf to enable mating and oviposition.

**Nitrogen and water input.** At DAS 8, nine treatments were initiated on the potted seedlings using a fully crossed design with three nitrogen inputs (ON: optimal nitrogen; EN: excessive nitrogen; IN: insufficient nitrogen) and three water inputs (OW: optimal water; LD: low drought; HD: high drought) (see Fig. 5).

The amounts of nutrient inputs and volumes of feeding solution supplied to plants increased daily depending on the plant development stage. Indeed, the amount of nitrogen taken by plants was controlled by the increase in their daily dry mass<sup>45,46</sup>.

Moreover, when growth reached steady-state i.e. exponential growth phase, plant N concentration remained constant<sup>45</sup>. We therefore calculated the daily amounts of nitrogen required to produce optimal dry mass during approximately 52 days of growth (ON treatment). To proceed, we used data of tomato growth previously obtained in our laboratory conditions<sup>47</sup>. We used 3 stock solutions to provide nitrate, phosphate and sulphate salts independently (+ Fe & micronutrients). The following concentrations were used (in M:  $\text{NO}_3^- = 1$ ;  $\text{H}_2\text{PO}_4^- = 0.21$ ;  $\text{SO}_4^{2-} = 0.055$ ;  $\text{K} = 0.641$   $\text{Ca} = 0.215$ ;  $\text{Mg} = 0.114$ ). Kanieltra trace elements (Hydro Azote, France) were given as well as formula 6 Fe and EDTA-Fe at the following concentrations (in  $\mu\text{M}$  in the  $\text{SO}_4^{2-}$  stock solution: Mo 20; Mn 815; Zn 227; Cu 33; B 1444; Fe 3760). To differentiate N inputs, three different doses, namely  $v_s$  (ON),  $3v_s$  (EN) and  $v_s/5$  (IN) of the nitrate stock solution were used daily to fertilize the plants. These doses were added to the water intake (see below) of each pot in order to set the optimal, excessive and insufficient nitrogen inputs, respectively (Fig. 5A).

We applied a “step increase” pattern to daily water inputs (Fig. 5B). Optimal daily water input (hereafter named “v” in volume) was determined as the amount capable of fully-saturating the perlite substrate without visible drainage, i.e. field capacity.



Based on v, complementary volumes of v/2 and v/3 were applied daily to set the low and high drought treatments per plant respectively. All nutrient solutions were adjusted to pH 5.5 by using H<sub>2</sub>SO<sub>4</sub> (0.2 M).

**Sampling protocols.** Figure 2 shows the schedule of all the events recorded during the experiment with regard to leaf sampling and the period of pest artificial infestation on plants.

**Plant infestation.** At DAS 42 (see Fig. 4), one leaf, the 3<sup>rd</sup> fully-developed leaf (with 5 leaflets) from the top, from each plant was selected to be infested with one newly-oviposited *T. absoluta* egg ( $\leq 24$  h). The eggs were checked daily until larvae hatched. If an egg failed to hatch, a newly-hatched larva ( $< 6$  h old) was released on the leaf. To avoid larvae escaping, each infested leaf was then bagged with a nylon mesh (0.2 mm, 30 × 24 cm).

**Biological traits of *T. absoluta*.** Survival and development time were recorded for each insect. After pupation, all the pupae (pupated within 48 h) were weighed individually and then kept in a small petri dish (4.2 cm in diameter) until the adult emerged.

**Leaf sampling and plant measurements.** Plant height was measured individually on three dates (DAS 32, 40, 58). For each plant, the node number was counted at DAS 58. To assess the effects of the treatments on leaf nitrogen and carbon contents, we randomly selected six plants from each treatment and sampled their 4<sup>th</sup> fully-developed leaf from the top. Leaf samples were taken at DAS 43 before *T. absoluta* larvae infestation and were harvested and dried at 60°C for 72 h.

**Leaf N content and C/N ratio measurement.** To analyse the nitrogen and carbon contents (dry mass percent), the dried leaf samples were ground into fine powder using a ball mill MM301 (Retsch, Germany) and stored individually in small tubes. Leaf nitrogen and carbon contents were measured with an elemental analyser (Flash EA1112 Series, ThermoFinnigan, Milan, Italy) at INRA-PSH Avignon. The leaf C/N ratio was calculated from the mass data (in g).

**Data analyses.** We performed two-way ANOVA, followed by Tukey's post hoc test for multiple comparisons when necessary, in order to determine the effects of nitrogen and water inputs on plant height, number of nodes per plant, leaf N content, leaf C/N ratio, *T. absoluta* pupal weight, development time from egg to pupa and from egg to adult. Shapiro and Bartlett tests were used to assess variance homogeneity and normality, respectively. In addition, survival of *T. absoluta* was fitted to a log-linear model (using "water", "nitrogen" and "stage" as factors), and further analyzed with permuted Fisher exact test. In order to test the "Nitrogen limitation hypothesis", insufficient and optimal nitrogen data were used to test the relationships between leaf N content and *T. absoluta* development traits i.e. development time and pupal weight by using regression analysis. The excessive nitrogen dataset was not included in this analysis because nitrogen provided in excess may induce various effects (notably negative ones) beyond the actual "nitrogen concentration - plant growth" relationship<sup>17</sup>. All these data were analysed using the R software<sup>48</sup>.

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## Acknowledgments

This work was supported by funds from the *Environment and Agronomy* department of INRA and from the Chinese government (PhD fellowship to P.H.). We thank Philippe Bearez for technical assistance and Antonio Biondi for comments on an earlier version of the manuscript.

## Author contributions

P.H., A.L.V. and N.D. scoped and designed the study; P.H., A.L.V. and E.A.D. performed the experiments and analyzed data; P.H., A.L.V., J.L.B. and N.D. interpreted results and wrote the manuscript.

## Additional information

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Han, P., Lavoit, A.-V., Le Bot, J., Amiens-Desneux, E. & Desneux, N. Nitrogen and water availability to tomato plants triggers bottom-up effects on the leafminer *Tuta absoluta*. *Sci. Rep.* **4**, 4455; DOI:10.1038/srep04455 (2014).



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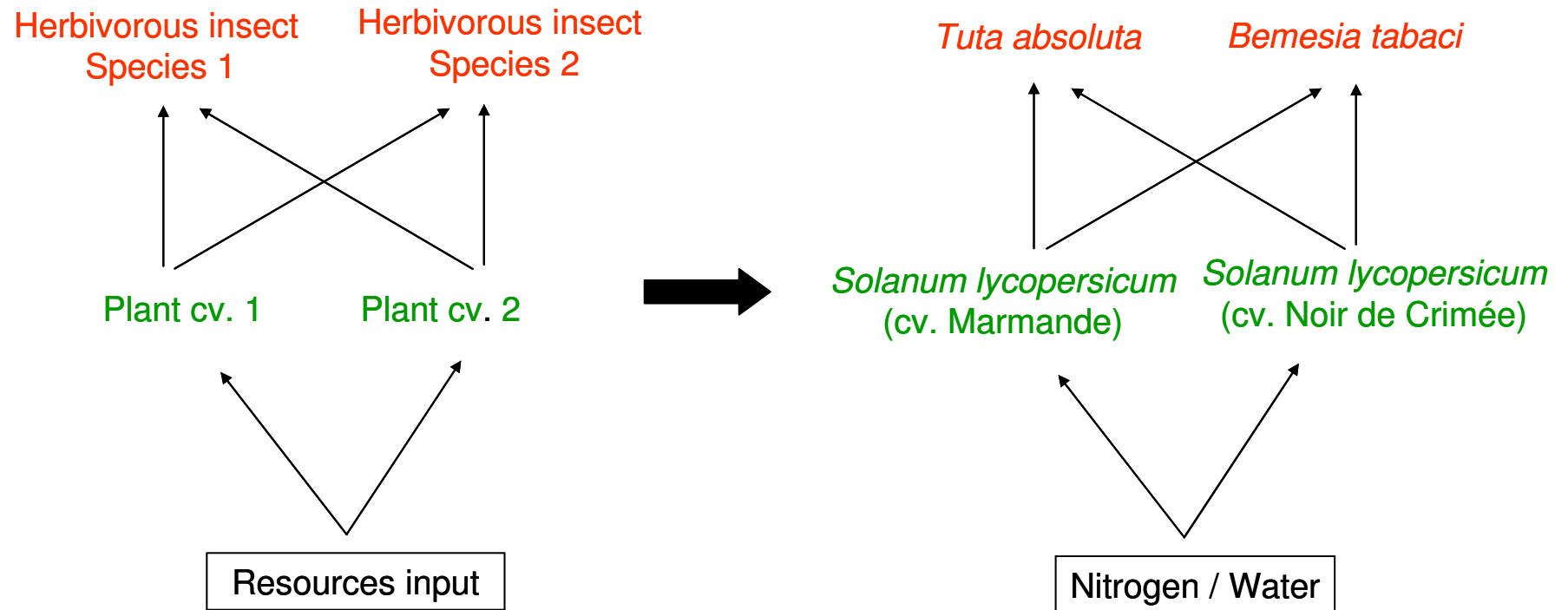
## II) Bottom-up effects of nitrogen and water on a leafminer and a sap-feeder on different plant cultivars

We concluded from the article 3 that sub-optimal nitrogen inputs (either excessive or insufficient), drought and their interactions, significantly reduced *T. absoluta* survival and slowed down its development. However, consequences of water and nitrogen stress on plant-insect interactions is highly diverse depending on the plant cultivar, insect feeding strategy, stress type and intensity (Inbar et al. 2001; Johnson 2008; Mody et al. 2009). For example, plants with drought tolerance traits may mitigate the negative bottom-up effects of drought on insects that feeding on. Moreover, insects from different feeding guild (leaf chewer, phloem-feeder, or leafminer etc.) may respond differentially to the plants that were under environmental stress (Inbar et al. 2001).

In this context, two questions were raised: (i) Do the bottom-up effects of resource-based stress (i.e., nitrogen deficiency and/or drought) on herbivore insect mitigate if the plants have the capacity to tolerate or to adapt to these stresses? (ii) Do the insect species from distinct feeding guild respond in the same way to the related stresses? And do they perform differentially on these cultivars?

To answer these questions, we used the study model presented in **Fig. 3**. Hence, in article 4, we studied the bottom-up effects of various levels of nitrogen and water inputs to tomato plants on survival and development of an invasive tomato leafminer, *Tuta absoluta*, and on survival of phloem feeder whitefly *Bemesia tabtaci* on two different tomato cultivars “Marmande” and “Noir de Crimée”.

**Figure 3:** The study model of article 4. The left diagram shows the general model, the right shows the biological model used in this study



## Article 4

### **Plant adaptations to abiotic constraints do not mitigate negative bottom-up effects in herbivorous insects.**

Han Peng<sup>1</sup>, Lavoit Anne-Violette<sup>2</sup>, Michel Thomas<sup>3</sup>, Le Bot Jacques<sup>4</sup>, Seassau  
Aurelie<sup>1</sup>, Amiens-Desneux Edwige<sup>1</sup>, Desneux Nicolas<sup>1\*</sup>

<sup>1</sup> French National Institute for Agricultural Research (INRA), UMR1355, 400 Route  
des Chappes, 06903, Sophia-Antipolis, France

<sup>2</sup> University of Nice Sophia-Antipolis (UNS), UMR1355, 400 Route des Chappes,  
06903, Sophia-Antipolis, France

<sup>3</sup> University of Nice Sophia-Antipolis (UNS), UMR7272, Faculty of Sciences, 28,  
Avenue de Valrose, 06108 Nice, France

<sup>4</sup> French National Institute for Agricultural Research (INRA), UR 1115 Plant and  
Horticultural Cultivating Systems, 84000 Avignon, France

\* Corresponding author: [nicolas.desneux@sophia.inra.fr](mailto:nicolas.desneux@sophia.inra.fr)

**Abstract:** Consequences of plant-insect interactions may vary depending on nutritional status of the plants as well as the plant cultivars. We hypothesize that (i) drought adaptation and nitrogen-deficiency regulation capacity in tomato cultivars may mitigate the bottom-up effects of drought and nitrogen deficiency on herbivorous species, and (ii) the herbivorous insects from different feeding guild may respond differently depending on the stress type (i.e. drought or nitrogen deficiency) and plant cultivar. Here, we evaluated the performance of two herbivorous insects from distinct feeding guilds, the leaf miner *Tuta absoluta* and the phloem feeder *Bemisia tabaci*, feeding on the two tomato cultivars (cv. Marmande (M) vs. cv. Noir de Crimée (NC)) under optimal/insufficient nitrogen and optimal/insufficient water inputs (i.e., drought). We found that (i) cv. NC appears less sensitive to drought stress (i.e more drought tolerant) than cv. M in terms of several leaf gas-exchanges traits as well as glycoalkaloids, whereas cv. NC is more sensitive to nitrogen deficiency than cv. M in terms of leaf nitrogen status and glycoalkaloids; (ii) Drought adaptation in cv. NC did not help mitigate the negative bottom-up effects of drought on *T. absoluta*. Similarly, a stronger capacity of regulating nitrogen deficiency in cv. M did not help mitigate the bottom-up effects of nitrogen limitation on *T. absoluta* and *B. tabaci*. (iii) Irrespective of plant cultivar, both nitrogen and water deficit significantly affected *T. absoluta* survival and development. However, *B. tabaci* survival was only negatively affected by the nitrogen deficiency, not the water deficit. The patterns of these negative responses of the insects were similar on both cultivars. Therefore, our results reject our first hypothesis, but partially support the second.

**Key words:** Plant-insect interaction, Agrosystem, Irrigation, Nitrogen fertilization, Plant defenses, Glycoalkaloid, *Solanum lycopersicum*, Insect feeding guilds

## **Introduction**

In terrestrial ecosystems, various biotic (i.e. environmental stress tolerance in plants and insect resistance in plants etc.) or various abiotic factors (e.g. nutrients, resource quality and quantity etc.) may act as crucial bottom-up forces in influencing natural communities (Hunter and Price 1992). Among these factors, plant nutritional conditions (i.e. resource quality and quantity) act as one of the most important bottom-up forces influencing plant-insect interactions (Hunter and Price 1992; Denno et al. 2002; Costamagna and Landis 2006), such as nitrogen and water inputs (Inbar et al. 2001). For instance, when plants were grown under drought or non-optimal nitrogen input conditions, plant nutritional quality as well as defensive compounds (both quantity and quality) may vary (Inbar et al. 2001; Gutbrodt et al. 2011; Royer et al. 2013), and consequently trigger bottom-up effects on herbivorous insects that feed on them. However, these bottom-up effects of resources input/availability to plants are highly variable and may depend on various factors: type of resource, intensity of stress, plant cultivar, feeding strategy of feeding species as well as feeding specialization (Inbar et al. 2001; Huberty and Denno 2004; Mody et al, 2009; Gutbrodt et al. 2011).

Water is one of the crucial resources for both plants and insects that feed on them. Varying water availability to plants may substantially induced physiological changes in terms of nutritional values and chemical defense in plants, thus affecting herbivores insects (Koricheva et al. 1998; Gutbrodt et al. 2011; Tariq et al. 2012). Effects of drought on plant-herbivorous insect interactions is primarily dependent on insect feeding strategy, insect feeding specialization as well as modalities of drought (Inbar et al. 2001; Huberty and Denno 2004; Mody et al, 2009; Gutbrodt et al. 2011). Firstly,

different insect feeding strategies (i.e. phloem-feeding, chewing, or leaf-mining etc.) may influence the insect responses to drought. Generally, leafminer and leaf chewers are considered to be more sensitive to drought than phloem feeders (Larsson 1989; Inbar et al. 2001). Thus two primary hypotheses *Plant vigor hypothesis* (Price 1991) and the *Plant stress hypothesis* (White 1993) were developed to explain such discrepancy. The former predicts that herbivorous insects perform better on rapidly-growing plants i.e. vigorous ones (Price 1991), and this hypothesis has been supported particularly in leafminer and leaf chewer (Inbar et al. 2001; Han et al. 2014). The latter predicts that water-stressed plants are more suitable hosts for senescence-feeders that feed preferentially on overmature and senescing tissues (White 2009). Secondly, the modalities of drought event are also important in determining the consequences of drought on herbivorous insects. Pulsed Plant Stress Hypothesis (PPSH) has been described to explain discrepancies between phloem feeder outbreaks observed *in situ* on water-stressed plants and the negative effects on phloem feeder detected in laboratory studies (Huberty and Denno 2004). PPSH predicts that phloem feeder populations (e.g. aphids or whiteflies) respond positively to plants that are under discontinuous stress where bouts of stress and recovery of turgor allow phloem feeders to benefit from stress-induced increases in plant nitrogen, however, phloem feeders may be negatively affected by continuous water stress. Thirdly, the outcomes of drought on herbivorous insects also depend on insect feeding specialization even if they adopt the same feeding strategy (Gutbrodt et al. 2011). Drought event may increase susceptibility of plants particularly to generalist herbivorous insects.

Nitrogen availability for plants is also a key factor influencing herbivorous insect performance (Mattson 1980). Generally no consensus has been reached on the

bottom-up effects of nitrogen inputs on herbivorous insects. The *Nitrogen limitation hypothesis* (White 1993) predicts that reduced availability of nitrogen to plants may impair the performance of Lepidoptera insects and this hypothesis has been supported by numerous studies (White 1993; Hunter and McNeil 1997; Grundel et al 1998; Inbar et al 2001; Cornelissen et al 2009; Han et al. 2014). However, several other studies reported contradicting results that high nitrogen content level in leaves compromised the biological traits in herbivores such as pupal survival, adult survival and adult body size (Joern and Behmer 1998; Fischer and Fiedler 2000). For phloem feeders, increase in nitrogen input and optimal plant nitrogen status generally enhanced the performance of phloem-feeding insects, such as whiteflies, thrips etc. (Jauset et al. 1998; Bentz et al. 1995 a, b; Bi et al 2001; 2003; Inbar et al 2001; Chau et al. 2005; Hogendorp et al. 2006).

However, in modern agriculture, diverse plant cultivars have been selected to cope with sub-optimal growing conditions (hereafter referred as “resistant cultivar”). Plant adaptation to various abiotic stresses (i.e. drought and nitrogen deficiency) involve complex physiological mechanisms (Cattivelli et al. 2008; Farooq et al. 2009; Feng et al. 2009). For instance, enormous effects have been directed to improve the drought-adaptation in plants by increasing the water-use efficiency (Galmés et al. 2013). Acclimation to drought conditions may comprise long-term adjustments involving gene expression and modification of plant physiology, biochemistry, morphology and anatomy (Chaves et al. 2002; Flexas et al. 2006; Chaves et al. 2009). However, to date, it is unclear how all these changes occurred in resistant cultivars may influence the potential bottom-up effects of drought on herbivorous insects; in contrast, drought generally compromises herbivorous insects that feed on drought-susceptible cultivars (Inbar et al. 2001; Han et al. 2014). Therefore, it may be



a valuable attempt to consider plant cultivars with drought-resistance or nitrogen-deficiency tolerance traits in better understanding the bottom-up effects of water and nitrogen inputs on plant-herbivorous insect interactions.

In order to understand the bottom-up effects of variable environments on plant-insect interaction, a “nitrogen / water-tomato plant-herbivorous insects” testing system was set up. The cultivar 'Marmande' (referred as cv. M in the following) is a commonly-used cultivar in local farming and organic farming under greenhouse in France and is also our plant model, often used in our previous studies on plant-insect interactions (Chailleux 2013a, 2013b; Jaworski et al. 2013; Han et al 2014). The cultivar “Noire de Crimée” (hereafter referred as cv. NC) is also a commonly used cultivar in France and is sold as "drought-tolerant cultivar" by numerous seed sellers. Thus in the present study, three main questions were proposed: (i) Do cv. M and cv. NC respond differently to drought and nitrogen deficiency in terms of chemistry, physiological traits as well as chemical defense against herbivores (i.e. with one cultivar more tolerant to drought/nitrogen deficiency than the other)? (ii) Since the negative bottom-up effects of drought and nitrogen deficiency on a Lepidoptera *Tuta absoluta* (Meytick) (Lepidoptera: Gelechiidae) has been identified on drought-susceptible tomato cultivar (cv. M) (Han et al. 2014), do drought-tolerance and nitrogen-regulation traits in either cv. M or cv. NC mitigate such bottom-up effects on this Lepidoptera? (ii) If the bottom-up effects do not mitigate on these resistant cultivars, do the insect species from distinct feeding guild (i.e. leafminer and phloem feeder) respond differently to the related stresses? We hypothesize that (i) drought tolerance and nitrogen regulation traits in tomato cultivars may mitigate the bottom-up effects of drought and nitrogen deficiency on herbivorous species, and (ii) the herbivorous insects from different feeding guild may respond differently

depending on the stress type (i.e. drought or nitrogen deficiency) and plant cultivar.

## Material and Methods

### *Biological material*

Both cultivars ('Marmande', cv. M and 'Noire de Crimée' cv. NC) of Tomato (*Solanum lycopersicum* L.) were grown from seeds in a climatic chamber (12 h light,  $24\pm1^{\circ}\text{C}$ ,  $65\pm5\%$  RH) in cubic plastic pots ( $7\times7\times6.5$  cm). The plants were then grown under laboratory conditions (16 h light,  $24\pm1^{\circ}\text{C}$ ,  $65\pm5\%$  RH). At DAS 8 (Days After Seeding) the seedlings were washed to remove soil and then transferred to new cubic pots containing limestone grains (Perlite Italiana srl, Corsico, Italy) (see schedule Fig. 1). At DAS 24, the plants were transferred to larger pots (diameter: 10 cm, height: 9 cm) filled with the same substrate.

The *T. absoluta* colony was maintained in regular chambers using tomato plants ( $25 \pm 1^{\circ}\text{C}$ , RH  $70 \pm 10\%$ , 16 h light) (Han et al. 2014). Honey and water were provided *ad libitum* to adults in rearing cages. Newly-oviposited *T. absoluta* eggs ( $\leq 24$  h) were used to infest the plants at DAS 40 (see Fig. 1). To obtain these eggs, we used the method described by Chailleux et al (2013). Ten couples of *T. absoluta* adults were placed inside a plastic tube containing a fresh tomato leaf to enable mating and oviposition.

The *B. tabaci* colony was maintained in regular chambers using young tobacco plants ( $25 \pm 1^{\circ}\text{C}$ , RH  $70 \pm 10\%$ , 16 h light). Honey and water were provided *ad libitum* to adults in rearing cages. Two tobacco plants were put inside the cage at DAS

35 to obtain 1<sup>st</sup> stage nymphs (crawlers) for tomato plants infestation at DAS 45 (see Fig. 1).

### *Nitrogen and water input*

We used the same nitrogen and water inputs regime that used in our previous study (Han et al. 2014), but only two levels of nitrogen inputs (ON: optimal nitrogen; IN: insufficient nitrogen) and two levels of water inputs (OW: optimal water; DT: drought, i.e. the “HD”-“high drought” in Han et al. 2014). The treatments started at DAS 8 and ended at DAS 60 (Figure S2).

The amounts of nutrient inputs and volumes of feeding solution supplied to plants increased daily in an exponential way depending on the plant development stage (Ingestad and Agren, 1995; Le Bot et al. 1998). Three stock solutions to provide nitrate, phosphate and sulphate salts independently (+ Fe & micronutrients) were mixed and applied to the plants (see the detailed formula in Han et al. 2014). To differentiate N inputs, two different doses,  $v_n$  and  $v_n/5$  of the nitrate stock solution were used daily to fertilize the plants. These doses were added to the water intake (see below) of each pot in order to set the optimal nitrogen (ON) and insufficient nitrogen (IN) inputs respectively (Figure S2 A).

A “step increase” pattern was used for daily water inputs (Figure S2 B). Daily optimal water (OW) input was determined empirically as the amount required to fully-saturate the perlite substrate without visible drainage. Based on  $v_w$ , complementary volume  $v_w/3$  was applied daily to set the drought (DT) treatment per plant. All nutrient solutions were adjusted to pH 5.5 by using H<sub>2</sub>SO<sub>4</sub> (in 0.2 M). In our study, DT was considered as “pulsed water stress” because the plants received

solution daily i.e. sporadically and a tutor recovery was expected in plant leaves since no evident symptom of wilting was observed (Huberty and Denno 2004).

### *Experimental setup*

Figure S1 shows the schedule of all the events recorded during the experiment. This schedule repeated for two times (2 trials) except that the leaf gas-exchange measurement (LGE) was only performed in the second trial.

*Leaf gas-exchange measurement* - CO<sub>2</sub> assimilation, transpiration and stomatal conductance measurements were performed on three well-developed terminal leaflets (one leaflet per plant) per treatment at DAS 38. They were measured with a portable photosynthesis systems (Li-6400, Li-Cor, Lincoln, NE, USA) equipped with a light source (6200-02B LED, Li-Cor). Leaves were first acclimated in the chamber for more than 20 min under controlled conditions: Leaf temperature of 27°C, air CO<sub>2</sub> concentration of 400  $\mu\text{mol CO}_2 \text{ mol}^{-1}$  air and saturating photosynthetic photon flux density (PPFD) of 700  $\mu\text{mol hv m}^{-2} \text{ s}^{-1}$ .

*Leaf sampling* - At DAS 43, the 4<sup>th</sup> fully-developed leaf from the top from each plant were sampled. These leaves were immediately dried at 60°C for 72 h for the quantification of leaf N and glycoalkaloids content.

*Leaf N content and C/N ratio quantification* - To analyse the nitrogen and carbon contents (dry mass percent), the dried leaf samples were ground into fine powder using a ball mill MM301 (Retsch, Germany) and stored individually in small tubes. Leaf nitrogen and carbon contents were measured with an elemental analyser (Flash EA1112 Series, ThermoFinnigan, Milan, Italy) at INRA-PSH Avignon. The leaf C/N ratio was calculated as the ratio between leaf carbon content (%) and leaf nitrogen content (%).

*Leaf glycoalkaloid quantification* - We used the same powder samples from dried leaves for glycoalkaloid analysis as those used for leaf N content quantification. Glycoalkaloids were extracted from dried tomato leaves (5 g) mixed with 2 mL of 5% acetic acid (CH<sub>3</sub>COOH) in water (v/v). The suspension was first mixed with a vortex and then extracted twice for 30 min using an ultrasonic assisted extractor (Reus) at room temperature. After the extraction, the supernatant was filtered through a 0.45 µm PVDF Puradisc™ filters (Whatman, GE Healthcare). All samples were kept at -20°C until the LC analyses. Glycoalkaloid standards ( $\alpha$ -tomatine and tomatidine) were also diluted in 5% CH<sub>3</sub>COOH solution.

All analyses were performed on an Ultimate 3000 Rapid Separation LC (RSLC) systems (Thermo Scientific) equipped with a PDA detector and hyphenated to an ESI-Q-TOF mass spectrometer (microTOFQII, Bruker Daltonique). Separation was carried out on an Ascentis Express Fused-Core™ C18 column (100 × 2.1mm i.d., 2.7 µm; Supelco) with its corresponding guard column (Ascentis express, 2.1 mm id x 50 mm, 2.7 µm, Supelco). After optimisation, a gradient elution program was developed enabling separation of glycoalkaloids. The flow rate was set at 400µL/min and the solvent system was (A) water (H<sub>2</sub>O) 0.1% acid formic (AF, 0.1% v/v) and (B) acetonitrile (ACN) 0.1% AF (v/v). The elution program was: 2% B for 5 min, 50% B for 35 min, 100% B for 5 min and hold for 3 min, back to 2% B in 5 min and conditioning for 2.5 min. The column oven was thermostated at 35°C and the autosampler at 6°C. The injection volume was set at 5 µL.

Before analyses, the mass spectrometer was calibrated in an external mode using a mix of known mass (ESI-L Low concentration Tuning Mix, Agilent Technologies). HRMS data were acquired in positive ionisation and in MS scan modes. The source temperature was set at 195°C, the capillary voltage at 3.8kV, nebuliser gas (nitrogen)

at 2.8 bar and dry gaz (nitrogen) at 9 L/min. Mass spectra acquisition was set at 5000 spectra/sec on a mass range of 50-2000 m/z. LC-MS raw data are processed using Data Analysis 4.1 software (ESI Compass 1.5, Bruker Daltonique). An ion extraction method using a mass window of 0.01 Da was used for the quantification of glycoalkaloids. To obtain the corresponding quantity, the measured ion abundance was reported on a standard curve (for  $\alpha$ -tomatine and tomatidine) obtained in the same condition of analysis and reprocessing.

*Plant infestation* - At DAS 40 (see Fig. 1), one leaf, the 3<sup>rd</sup> fully-developed leaf (with 5 leaflets) from the top, from each plant was selected to be infested with one newly-oviposited *T. absoluta* egg ( $\leq 24$  h). The eggs were checked daily until larvae hatched. If an egg failed to hatch, a newly-hatched larva ( $< 6$  h old) was released on the leaf. To avoid larvae escaping, each infested leaf was then bagged with a nylon mesh (0.2 mm, 30  $\times$  24 cm). The *T. absoluta* infestation was performed on 96 plants. At DAS 45 (see Fig. 1), the other group of 96 plants were infested by *B. tabaci* crawlers. Six crawlers were released on the 3<sup>rd</sup> fully-developed leaf for each plant. They were checked until all of them started walking and piercing leaf tissues.

*Biological traits of T. absoluta and B. tabaci* - For *T. absoluta*, survival and development time of each life stage were recorded. Pupa mass were recorded by weighing the pupae. For *B. tabaci*, mortality of crawlers and numbers reaching 2<sup>nd</sup> nymph stage were recorded after 7 days of infestation.

#### *Data analyses*

Multivariate analysis of variance (MANOVA) test were performed to analyze the effects of trial, nitrogen, water and cultivar on glycoalkaloids quantifications in leaf dry mass. Factorial ANOVA (“nitrogen”, “water” and “cultivar”) were performed to

test the effects of nitrogen, water and cultivar on plant traits including leaf N content, leaf C/N ratio, glycoalkaloid compounds and insect traits including *T. absoluta* pupal weight, development time from egg to pupa and from egg to adult, *B. tabaci* mortality and survival rate reaching 2<sup>nd</sup> nymph stage. Shapiro and Bartlett tests were used to assess variance homogeneity and normality, respectively. Independent samples t-test was used to examine the difference in response of CO<sub>2</sub> assimilation, leaf transpiration rate and stomatal conductance to drought among the two cultivars. Only the data collected from “ON” (optimal nitrogen) were used in impact of order to focus on the impact of drought. Independent samples t-test was used to examine the response of leaf N content and C/N ratio between optimal nitrogen (ON) and insufficient nitrogen (IN) (i.e. ON vs. IN) for both water treatments. Tukey post hoc test for multiple comparisons were performed to examine the effect of nitrogen, water and cultivar on all the glycoalkaloid compounds, as well as development of *T. absoluta* and survival of *B. tabaci*. Survival of *T. absoluta* was fitted to a log-linear model using the permuted Fisher exact test (using “water”, “nitrogen” and “stage” as factors). All these data were analysed using the R software (R Core Team 2009).

## Results

### *Impact of variable water input on leaf-gas exchange parameters*

Figure 1 showed the effect of drought on photosynthesis rate (CO<sub>2</sub> assimilation), transpiration rate and stomatal conductance at leaf level for both cultivars. Cv. M showed decreased photosynthesis rate, transpiration rate and stomatal conductance under drought compared those measured under optimal water input (photosynthesis:

$P = 0.007$ ,  $df = 20$ ,  $t = 1.649$ ; transpiration:  $P = 0.001$ ,  $df = 20$ ,  $t = 4.113$ ; stomatal conductance:  $P = 0.008$ ,  $df = 20$ ,  $t = 2.934$ ). In contrast, cv. NC responded similarly among optimal water input and drought in terms of transpiration rate and stomatal conductance (transpiration:  $P = 0.394$ ,  $df = 18$ ,  $t = 0.873$ ; stomatal conductance:  $P = 0.278$ ,  $df = 18$ ,  $t = 1.119$ ). But, the photosynthesis rate significantly decreased under drought compared to optimal water input ( $P = 0.003$ ,  $df = 18$ ,  $t = 3.494$ ).

#### *Impact of nitrogen, water and cultivars input on plant traits*

Table 2 shows the MANOVA analysis of effects of various factors (i.e. trial, nitrogen, water, cultivar) on the quantifications of glycoalkaloids. A significant effect of “trial” was found. However, we did not find strong interaction between the factor “trial” with the factor “nitrogen”, “water” or “cultivar” (Figure S3, S4, S5). This indicated that a similar effect pattern of these factors on glycoalkaloids between the two trials. Thus, we pooled the data of glycoalkaloids from the two trials for further analysis. Also, data of leaf N content and C/N ratio did not differed between the two trials ( $F_{1, 176} = 0.298$ ;  $P = 0.586$ ), thus the data from two trials were pooled. Factorial ANOVA on various plant traits were summarized in Table 1.

*Leaf N content and C/N ratio* –Both leaf N content and C/N ratio were affected by nitrogen inputs, but not the water inputs. A strong interaction between nitrogen and cultivar on these two parameters was found (Table 1). Leaf N content in cv. NC was significantly lower than that in cv. M (Fig. 3; IN-OW:  $P = 0.001$ ,  $df = 46$ ,  $t = 3.411$ ; IN-DT:  $P < 0.0001$ ,  $df = 46$ ,  $t = 5.102$ ). Leaf C/N ratio in cv. NC was significantly higher than that in cv. M (Fig. 3;  $P < 0.0001$ ,  $df = 46$ ,  $t = -5.207$ ; IN-DT:  $P < 0.0001$ ,  $df = 46$ ,  $t = -3.928$ ).

*Glycoalkaloid* – Table S shows the concentrations of tomatidine, tomatine 1 and



tomatine 2 quantified from the leaf dry mass on both tomato cultivars (except for “Ddhydrotomatine”; no absolute value was available because of lack of standard samples). Depending on the factorial ANOVA results (Table 1), we only present the compounds that were significantly affected by the interaction effect of “nitrogen  $\times$  water”, “nitrogen  $\times$  cultivar” and “water  $\times$  cultivar”. Irrespective of cultivar, concentration of tomatidine differed significantly among the treatments (ON-OW < ON-DT = IN-OW < IN-DT) (Fig. 3). However, concentration of dehydrotomatine was significantly lower only in ON-DT than other treatments (Fig. 3). Regarding the “nitrogen  $\times$  cultivar” effect on the four alkaloid compounds, the two cultivars responded differently to the nitrogen treatment. Insufficient nitrogen input induced higher concentrations of tomatidine, dehydrotomatine and tomatine 2 in cv. NC (all  $P < 0.0001$ ), but not in cv. M. Also, these three compounds were found higher in cv. M than those in cv. NC (Fig. 4). Similarly, when examining “water  $\times$  cultivar” effect on alkaloids concentrations, the two cultivars responded differently to water treatments. Drought induced higher concentrations of tomatidine and tomatine 1 in cv. M, but not in cv. NC (Fig. 5).

#### *Impact of nitrogen, water and cultivars input on insect biological traits*

Factorial ANOVA on various insect biological traits were summarized in Table 3.

*T. absoluta* survival and development - Both nitrogen and water inputs significantly affected *T. absoluta* survival from egg to pupa or to adult (nitrogen:  $\chi^2 = 9.55$ ,  $df = 1$ ,  $P = 0.002$ ; water:  $\chi^2 = 7.64$ ,  $df = 1$ ,  $P = 0.006$ ; nitrogen  $\times$  water:  $\chi^2 = 0.00$ ,  $df = 1$ ,  $P = 0.986$ ) (Table 4). *Tuta absoluta* also showed different responses between pupa and adult stages (stage:  $\chi^2 = 9.18$ ,  $df = 1$ ,  $P = 0.002$ ). However, similar pattern

was observed on both cultivars regarding the response of *Tuta absoluta* survival to various treatments (cultivar:  $\chi^2 = 0.02$ ,  $df = 1$ ,  $P = 0.900$ ). On both cultivars, *T. absoluta* had a significantly lower survival rate in reaching any development stage when the plants suffered insufficient nitrogen and drought treatment (IN-DT vs. ON-OW, Table 4). On cv. M, drought to the plants resulted in a lower survival rate of individuals reaching pupa even when the plants received optimal nitrogen (ON-DT vs. ON-OW, Table 4). On cv. NC, sufficient nitrogen input to the plants resulted in a lower survival rate of individuals reaching pupa even when the plants received optimal water input (IN-OW vs. ON-OW, Table 4).

Table 3 and Fig. 6 showed that both nitrogen and water inputs significantly affected *T. absoluta* development time from egg to pupa, from egg to adult and for pupa weight. All the development-related traits had the similar pattern on both cultivars in response to various treatments ( $P = 0.755$ ,  $P = 0.724$ ,  $P = 0.693$  respectively). The development time from egg to pupa or to adult was significantly extended when the plants suffered drought and/or received insufficient nitrogen input (all  $P < 0.05$ ) (other treatments vs. ON-OW, Fig. 6).

*B. tabaci* survival- Only nitrogen significantly affected the mortality and number of individuals reaching 2<sup>nd</sup> nymph (Table 3 and Fig. 7). The two traits had the similar pattern on both cultivars in response to various treatments ( $P = 0.768$  and  $P = 0.690$ , respectively). Regardless of water input, insufficient nitrogen input significantly increased *B. tabaci* mortality and decreased number of individuals reaching 2<sup>nd</sup> stage (all  $P < 0.05$ ) (IN-OW vs. ON-OW and IN-DT vs. ON-OW, Fig. 7).

## Discussion

Our current results provide clear answers to the questions proposed earlier in the introduction. Firstly, we found strong evidence of plant resistance traits against drought and nitrogen deficiency. Cultivar M was more tolerant to nitrogen deficiency than cv. NC, whereas cv. NC was more tolerant to drought than cv. M. Secondly, drought-tolerance and nitrogen-regulation traits in a resistance tomato cultivar did not help to mitigate the negative bottom-up effects of drought and nitrogen deficiency on the herbivorous insect *T. absoluta*. These negative bottom-up effects were retained on both tomato cultivars even though either of the cultivar indeed showed drought tolerance or nitrogen deficiency regulation. Thirdly, the herbivorous insects from different feeding guild responded differently to the two types of stresses (i.e. drought and nitrogen-deficiency), with *T. absoluta* negatively affected by both factors, but *B. tabaci* only affected by nitrogen deficiency. However, their responses appeared similar on two cultivars. Therefore, the current findings did not support the first hypothesis, but partially support the second.

### **Cultivar M and cv. NC respond differently to drought and nitrogen deficiency in terms of chemistry, physiological traits as well as chemical defense against herbivores**

Our findings suggested that the two tomato cultivars cv. M and cv. NC indeed respond differently to drought and nitrogen deficiency. Firstly, we found strong evidence that cv. NC was more tolerant to drought than cv. M.. Cultivar Marmande was sensitive to drought with significantly decreased photosynthesis, transpiration rate and stomatal conductance observed, but only photosynthesis rate was affected

under drought cv. NC (Fig. 1). It may indicate that cv. M need to adjust water flux under drought conditions while cv. NC need not (Farooq et al. 2009). Moreover, drought induced higher concentrations of defensive compounds: tomatidine and tomatine 1 in cv. M (Fig. 5). This agree with the fact that moderate drought often causes an increase in the content of defensive compounds (Gutbrodt et al. 2011). However, such effect was not found on cv. NC where the contents of defensive compounds remained at equally low levels irrespective of water input. Secondly, in contrast, cv. NC appeared more sensitive to nitrogen deficiency than cv. M because leaf N content and leaf C/N in cv. NC vary with the nitrogen deficiency when cv. M did not (Fig. 2). Also, nitrogen deficiency caused an increase in contents of tomatidine, dehydrotomatine and tomatine 2 in cv. NC (Fig. 4). Such positive relationship between glycoalkaloids content and leaf C/N ratio was consistent with the previous report (Royer et al. 2013). However, since cv. M was less sensitive to nitrogen deficiency, thus these defensive compounds did not differ among the nitrogen treatments (Fig. 4). Overall, these results suggested that cv. M is somehow nitrogen deficiency adaptive and cv. NC appears drought tolerance.

#### **Bottom-up effects of drought and nitrogen deficiency retained on the resistant cultivar**

Negative bottom-up effects of drought on *T. absoluta* was found on cv. M in our previous study (Han et al 2014), as well as the other Lepidoptera pests on other drought-susceptible plants (White 1993; Inbar et al. 2001; Schoonhoven et al 2005; Gutbrodt et al. 2011). These consequences have been attributed to the lower nutritional value (from insect's perspective) and higher defensive level. As the glycoalkaloid was the main defense chemical in all organs (Royer et al. 2013), increase in level of defensive compounds including tomatidine and tomatine 1 under

drought observed in the current study may well explain such consequence (Fig. 3 and Fig. 5). However, induction of higher levels of these defensive compounds was only found in cv. M, not in cv. NC. In other words, lower chemical defense in cv. NC may benefit the performance of *T. absoluta*. Despite this, it did not help cv. NC to mitigate the negative bottom-up effects of drought on *T. absoluta* in comparison to cv. M (Table 3, 4 and Fig. 6).

Likewise, nitrogen-regulation trait did not help cv. M to mitigate the negative bottom-up effects of nitrogen deficiency on *T. absoluta* (Table 3, 4 and Fig. 6). Specifically, the plant defensive level in term of glycoalkaloids was not increased by nitrogen deficiency (Fig. 4). Thus, the negative effect of nitrogen deficiency could only be linked to decreased nutritional value of leaves as food for *T. absoluta*. Compared to optimal nitrogen input, the leaf N content in cv. M significantly decreased due to nitrogen deficiency even though this value is still higher than that in cv. NC. Hence, such sub-nutritious food may explain that the negative effects of nitrogen deficiency on *T. absoluta*.

### **Insects from different feeding guilds responded differently to drought and nitrogen deficiency**

Herbivorous insects from different feeding guilds have different plant feeding strategy and they may respond differentially to the source-based stressed plants (Inbar et al. 2001; Huberty and Denno 2004). In the current study, both nitrogen and water deficiency significantly affected *T. absoluta* survival and development on both tomato cultivars.

However, the phloem feeder *B. tabaci* was only affected by the nitrogen deficiency instead of water deficit. This agreed with the reports on negative bottom-up effects of nitrogen-limited plants on whiteflies (Bentz et al. 1995 a, b; Inbar et al 2001;

Bi et al 2001, 2003). We assumed that the whiteflies may face a lack of N-based nutrient uptake on the nitrogen-limited plants because phloem feeders need to ingest a diet rich in soluble carbohydrates as well as relatively high levels of free amino acids from plants (Buchanan et al, 2000; Crafts-Brandner 2001).

However, water input variation did not affect the *B. tabaci* survival (see table 1 and Fig. 6). Thus, our data did not support the “PPSH” hypothesis which predicted a positive effects of pulsed water stressed plants on phloem feeders (Huberty and Denno 2004). We hypothesize a trade-off theory to explain such neutral response. Firstly, pulsed water stress induced an increase in phloem nitrogen predicted from the PPSH theory (Huberty and Denno 2004; Mody et al, 2009). Thus, *B. tabaci* may benefit from the increase in leaf phloem nitrogen content i.e. protein and free amino acid. Secondly, however, the plants under drought conditions may contain less phloem nutrients for *B. tabaci* because we found that drought treatment induced a significantly lower foliar photosynthetic rate (Fig.1). Thus, these plants may synthesize and accumulate less primary metabolites i.e. soluble carbohydrates in plant phloem sap and the deficiency in primary metabolite-based nutrients may not be able to meet the nutritional need of *B. tabaci* young nymphs. To conclude, these two contrasting nutrient related forces may exert an offset effect of pulsed water stress on quality of plant nutrients, which eventually resulted in a non-significant i.e. neutral effect on *B. tabaci*.

The consistent negative effects of nitrogen deficiency on both insect species were found. “Nitrogen limitation hypothesis” (White 1993) predicted that the herbivorous insect performance was positively correlated with plant leaf N content. Our current findings provide further evidence for this insect-plant interaction theory which has been previously supported (Ravenscroft 1994; Obermaier and Zwölfer 1999; Inbar et

al 2001; Han et al. 2014). Aside from the nitrogen-based nutrients, a suitable P: C ratio (plants' protein to carbohydrate ratio) of plant food is critical to growth and development of many herbivorous insect (Simpson and Raubenheimer 1993; Bede et al, 2007; Chen et al 2008). It was assumed that nitrogen limitation may reduce P;C ratio in tomato leaves even though such ratio for *T. absoluta* and *B. tabaci* are still unknown. Therefore, we conclude that negative effects of nitrogen limitation on the herbivores may be attributed to the decrease in P:C ratio, or increase in concentrations of plant chemical defensive compounds such as tomatidine, dehydrotomatine and tomatine 2 (Figure 4; Royer et al. 2013) as well as phenolics (Le Bot et al. 2009; Larbat et al. 2012; Royer et al. 2013), or a combination of the two attributes.

### **Acknowledgments:**

This work was supported by funds from the *Environment and Agronomy* department of INRA and from the Chinese government (PhD fellowship to PH). We thank Philippe Bearez and Edwige Amiens-Desneux for technical assistance during the experiment and the platform of analytical biochemistry (Sophia-Agrobiotech Institute Research; INRA).

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**Figure legends:**

**Figure 1:** Leaf photosynthesis rate, transpiration rate (mean  $\pm$  SEM,  $n = 9-12$ ) and stomatal conductance (mean  $\pm$  SEM,  $n = 9-12$  plants) measured on the two tomato cultivars cv. M and cv. NC treated with optimal water input and drought. “Ns” indicates “the difference is not significant”, whereas “\*\*” indicates significantly difference at  $P < 0.001$  using independent samples t-test)

**Figure 2:** Leaf N content (in dry mass percent, mean  $\pm$  SEM,  $n = 24$ ) and leaf C/N ratio (unit-less, mean  $\pm$  SEM,  $n = 24$ ) of the two tomato cultivars cv. M and cv. NC treated with different nitrogen and water inputs (ON: optimal N; IN: insufficient N; OW: optimal water; DT: drought). (“\*\*” and “\*\*\*\*” indicates significantly difference at  $P < 0.001$  and  $P < 0.0001$  respectively using independent t-test)

**Figure 3:** Effects of nitrogen and water inputs on concentration of defensive compounds tomatidine ( $\mu\text{g}/\text{mg}$  leaf dry mass (LDM)) and dehydrotomatine (relative content: ion abundance/mg LDM) in tomato plants. (ON: optimal N; IN: insufficient N; OW: optimal water; DT: drought). Columns with different letters are significantly different from each other (Tukey post hoc test for multiple comparisons,  $P < 0.05$ )

**Figure 4:** Effects of nitrogen input on concentration of defensive compounds tomatidine ( $\mu\text{g}/\text{mg}$  leaf dry mass (LDM)), dehydrotomatine (relative content: ion abundance/mg LDM) and tomatine 2 ( $\mu\text{g}/\text{mg}$  LDM) in two tomato cultivars cv. M and cv. NC. (ON: optimal N; IN: insufficient N); Columns with different letters are significantly different from each other (Tukey post hoc test for multiple comparisons,  $P < 0.05$ )

**Figure 5:** Effects of water input on concentration of defensive compounds tomatidine ( $\mu\text{g}/\text{mg}$  leaf dry mass (LDM)) and tomatine 1 ( $\mu\text{g}/\text{mg}$  LDM) in two tomato cultivars

cv. M and cv. NC. (ON: optimal N; IN: insufficient N); Columns with different letters are significantly different from each other (Tukey post hoc test for multiple comparisons,  $P < 0.05$ )

**Figure 6:** *T. absoluta* mean development time (mean  $\pm$  SEM, n = 30-59) from egg to pupa (d), development time (mean  $\pm$  SEM, n= 21-47) from egg to adult (d), and pupa weight (mean  $\pm$  SEM, n= 30-59) (mg) on the two tomato cultivars cv. M and cv. NC treated with different nitrogen and water inputs (ON: optimal N; IN: insufficient N; OW: optimal water; DT: drought). Different letters indicate significant difference at  $P < 0.05$  by Tukey post hoc test for multiple comparisons

**Figure 7:** *B. tabaci* mortality and individuals reaching the 2<sup>nd</sup> nymph stage (mean  $\pm$  SEM, n = 6) on the two tomato cultivars cv. M and cv. NC treated with different nitrogen and water inputs (ON: optimal N; IN: insufficient N; OW: optimal water; DT: drought). The comparisons were performed within the groups with either capital letters or small letters. Different letter indicate significant difference at  $P < 0.05$  by Tukey post hoc test for multiple comparisons

**Table 1:** Factorial ANOVA analysis on plant traits: plant leaf N content, leaf C/N ratio, glycoalkaloids (tomatidine, dehydrotomatine, tomatine 1 and tomatine 2). Main factors tested were the “nitrogen”, “water”, “cultivar”.

Plant traits		Leaf N content		Leaf C/N ratio		Tomatidine		Dehydrotomatine		Tomatine 1		Tomatine 2	
Source of variation	Df	F	<i>P</i>	F	<i>P</i>	F	<i>P</i>	F	<i>P</i>	F	<i>P</i>	F	<i>P</i>
Nitrogen	1	<b>705.23</b>	<b>&lt;0.001</b>	<b>498.89</b>	<b>&lt;0.001</b>	<b>19.71</b>	<b>&lt;.0001</b>	<b>19.69</b>	<b>&lt;.0001</b>	<b>4.58</b>	<b>0.0338</b>	<b>7.14</b>	<b>0.0083</b>
Water	1	3.64	0.058	0.30	0.586	<b>48.99</b>	<b>&lt;.0001</b>	0.85	0.3581	<b>17.25</b>	<b>&lt;.0001</b>	<b>9.29</b>	<b>0.0027</b>
Cultivar	1	<b>7.11</b>	<b>0.008</b>	<b>6.11</b>	<b>0.014</b>	<b>67.09</b>	<b>&lt;.0001</b>	<b>38.41</b>	<b>&lt;.0001</b>	<b>7.18</b>	<b>0.0081</b>	<b>14.21</b>	<b>0.0002</b>
Nitrogen × Water	1	0.07	0.788	<b>4.07</b>	<b>0.045</b>	<b>8.85</b>	<b>0.0034</b>	<b>4.78</b>	<b>0.0303</b>	0.19	0.6634	1.17	0.2807
Nitrogen × Cultivar	1	<b>23.48</b>	<b>&lt;0.001</b>	<b>21.97</b>	<b>&lt;0.001</b>	<b>5.15</b>	<b>0.0246</b>	<b>6.10</b>	<b>0.0145</b>	0.01	0.9059	<b>10.46</b>	<b>0.0015</b>
Water × Cultivar	1	0.32	0.571	0.10	0.751	<b>13.48</b>	<b>0.0003</b>	0.43	0.5128	<b>7.81</b>	<b>0.0058</b>	2.23	0.1376



**Table 2:** Multivariate analysis of variance (MANOVA) test of the effects of trial, nitrogen, water and cultivar on concentrations of glycoalkaloids (tomatidine, dehydrotomatine, tomatine 1 and tomatine 2) quantified from the leaf dry mass of tomato plants.

MANOVA results				
Source	Wilks' Lambda	Df	<i>F</i>	<i>P</i>
Trial	0.382	4, 160	64.73	< 0.0001
Nitrogen	0.668	4, 160	19.92	< 0.0001
Trial × Nitrogen	0.865	4, 160	6.23	0.0001
Water	0.516	4, 160	37.59	< 0.0001
Trial × Water	0.855	4, 160	6.77	< 0.0001
Nitrogen × Water	0.877	4, 160	5.61	0.0003
Cultivar	0.596	4, 160	27.16	< 0.0001
Trial × Cultivar	0.881	4, 160	5.39	0.0004
Nitrogen × Cultivar	0.931	4, 160	2.98	0.0209

Water × Cultivar	0.888	4, 160	5.04	0.0007
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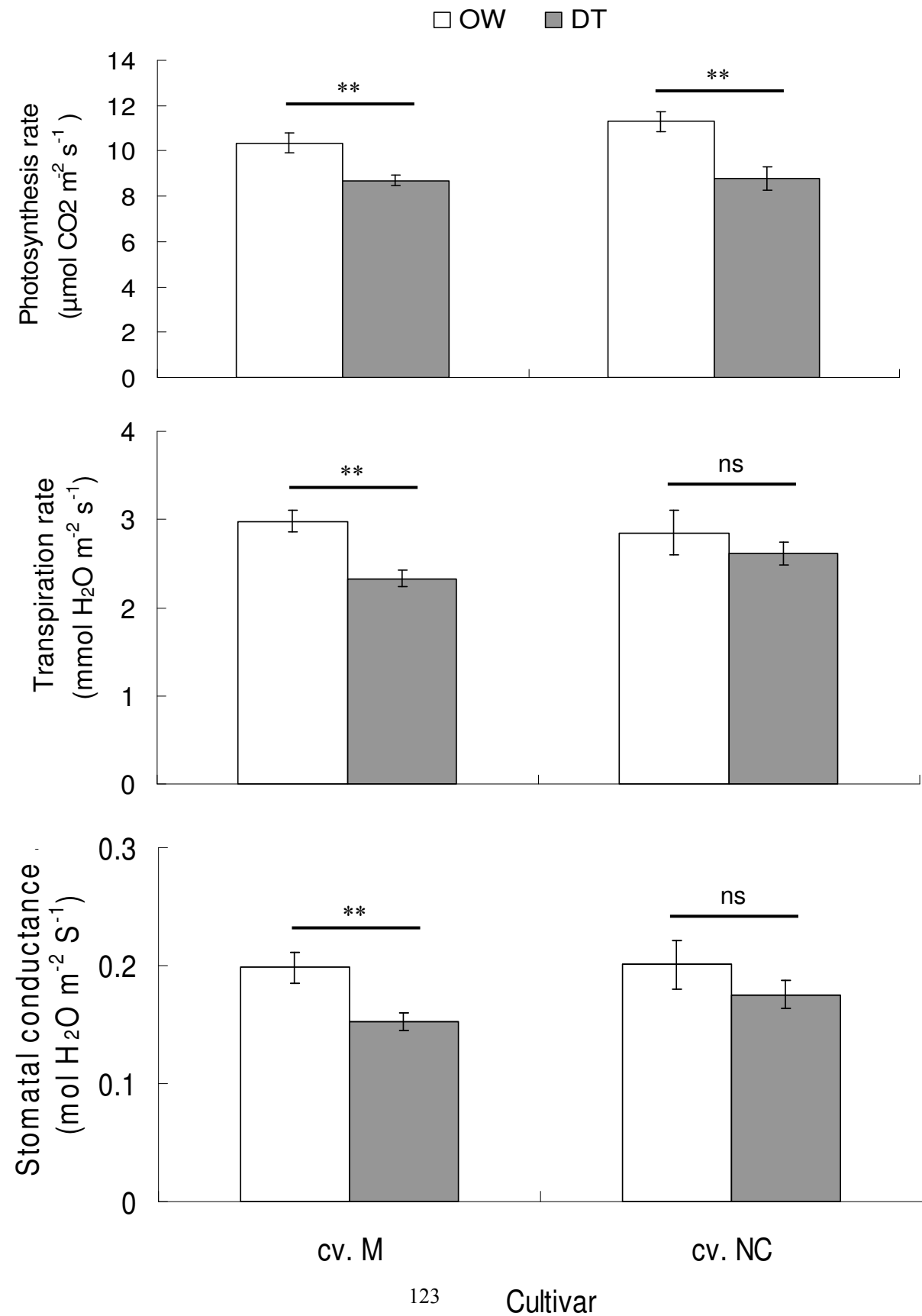
**Table 3:** Factorial ANOVA analysis on insect biological traits: *T. absoluta* development time from egg to pupa, *T. absoluta* development time from egg to adult, pupa weight, *B. tabaci* mortality and individuals reaching the 2<sup>nd</sup> nymph stage. Tested factors were the nitrogen input named as “nitrogen”, water input named as “water” and tomato cultivars named as “cultivar”

Insect traits		<i>T. absoluta</i> development time from egg to pupa		<i>T. absoluta</i> development time from egg to adult		<i>T. absoluta</i> pupa weight		<i>B. tabaci</i> mortality		<i>B. tabaci</i> individuals reaching 2 <sup>nd</sup> nymph	
Source of variation	Df	F	P	F	P	F	P	F	P	F	P
Nitrogen	1	<b>171.72</b>	<b>&lt;0.001</b>	<b>97.08</b>	<b>&lt;0.001</b>	<b>99.10</b>	<b>&lt;0.001</b>	<b>19.91</b>	<b>&lt;0.001</b>	<b>15.072</b>	<b>&lt;0.001</b>
Water	1	<b>67.28</b>	<b>&lt;0.001</b>	<b>47.90</b>	<b>&lt;0.001</b>	<b>36.03</b>	<b>&lt;0.001</b>	1.42	0.241	0.89	0.354
Cultivar	1	0.10	0.755	0.13	0.724	0.16	0.693	0.09	0.768	0.16	0.690
Nitrogen × Water	1	<b>10.21</b>	<b>0.002</b>	2.62	0.107	<b>14.45</b>	<b>&lt;0.001</b>	0.80	0.377	0.88	0.354
Nitrogen × Cultivar	1	3.16	0.076	0.77	0.382	0.00	0.960	0.00	1.000	0.02	0.894
Water × Cultivar	1	1.10	0.295	1.17	0.280	0.15	0.698	0.09	0.768	0.02	0.894

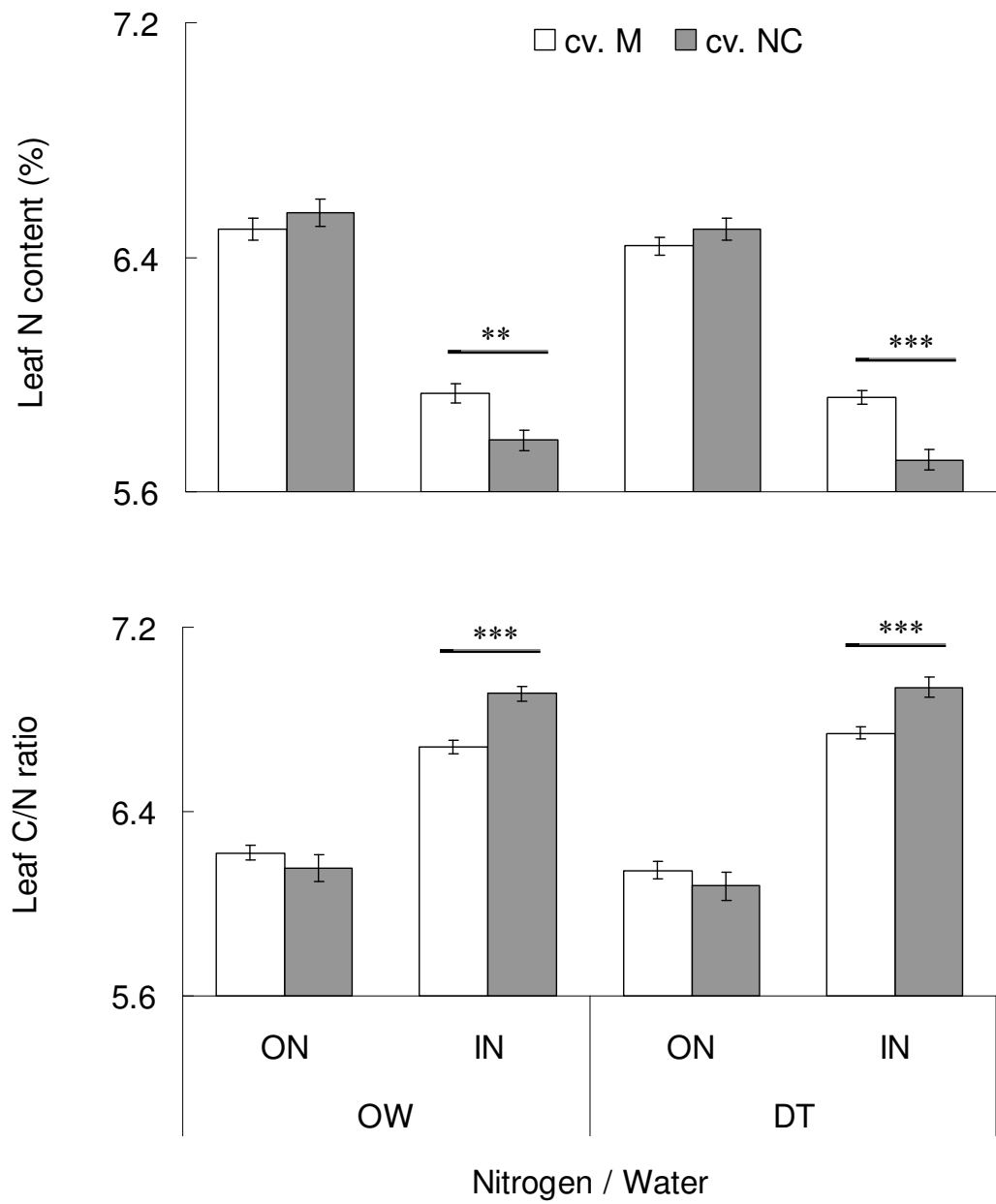
**Table 4:** Survival proportions of *T. absoluta* individuals reaching pupae (from egg to pupa) or adults (from egg to adult) when feeding one of the two tomato cultivars cv. M and cv. NC treated with different nitrogen and water inputs (ON: optimal N; IN: insufficient N; OW: optimal water; DT: drought). ON-OW was considered as control group and other treatments were compared to this control group. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  (significantly different from values of individuals developing on plants subjected to optimal nitrogen [ON] and optimal water [OW], in italics) (permuted Fisher exact test). Comparisons were performed within the cultivar.

Cultivar	Nitrogen	Water	Proportion of individuals reaching pupae	Proportion of individuals reaching adults
“M”	<i>ON</i>	<i>OW</i>	0.64	0.51
		DT	0.47 *	0.40
	IN	OW	0.51	0.41
		DT	0.42 **	0.29 **
“NC”	<i>ON</i>	<i>OW</i>	0.69	0.52
		DT	0.57	0.40
	IN	OW	0.47 **	0.40
		DT	0.39 ***	0.32 *

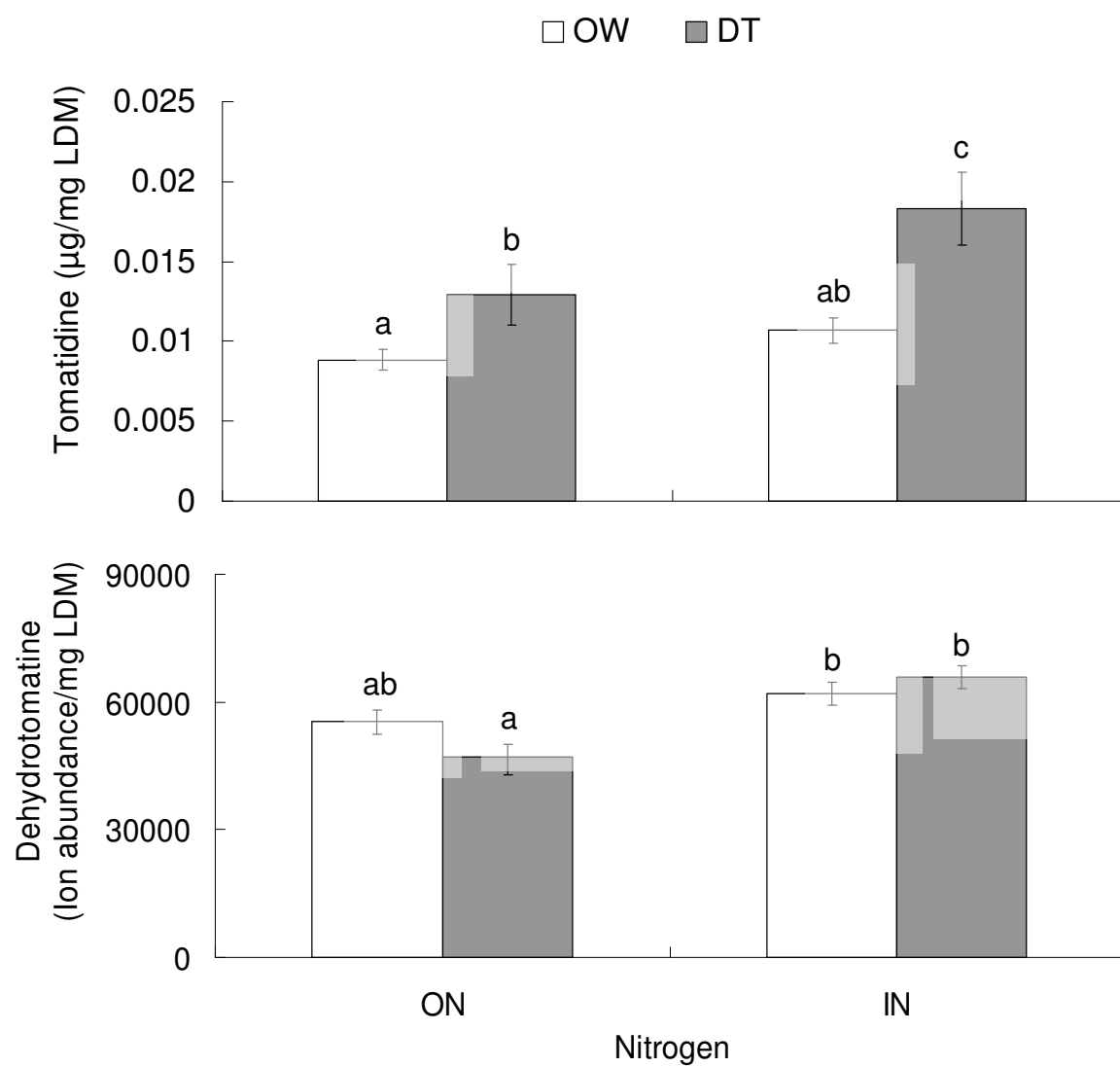
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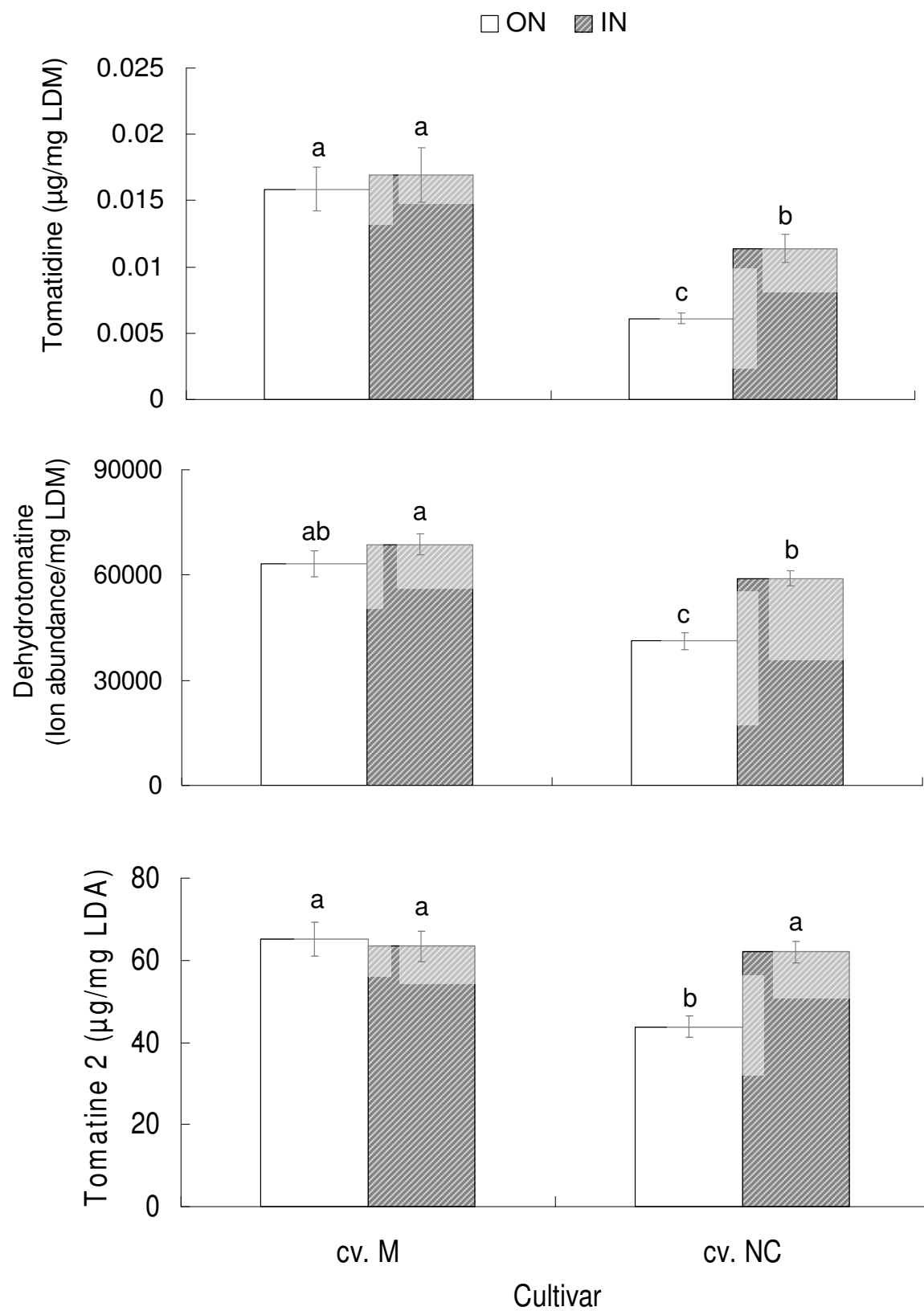
**Figure 2:**



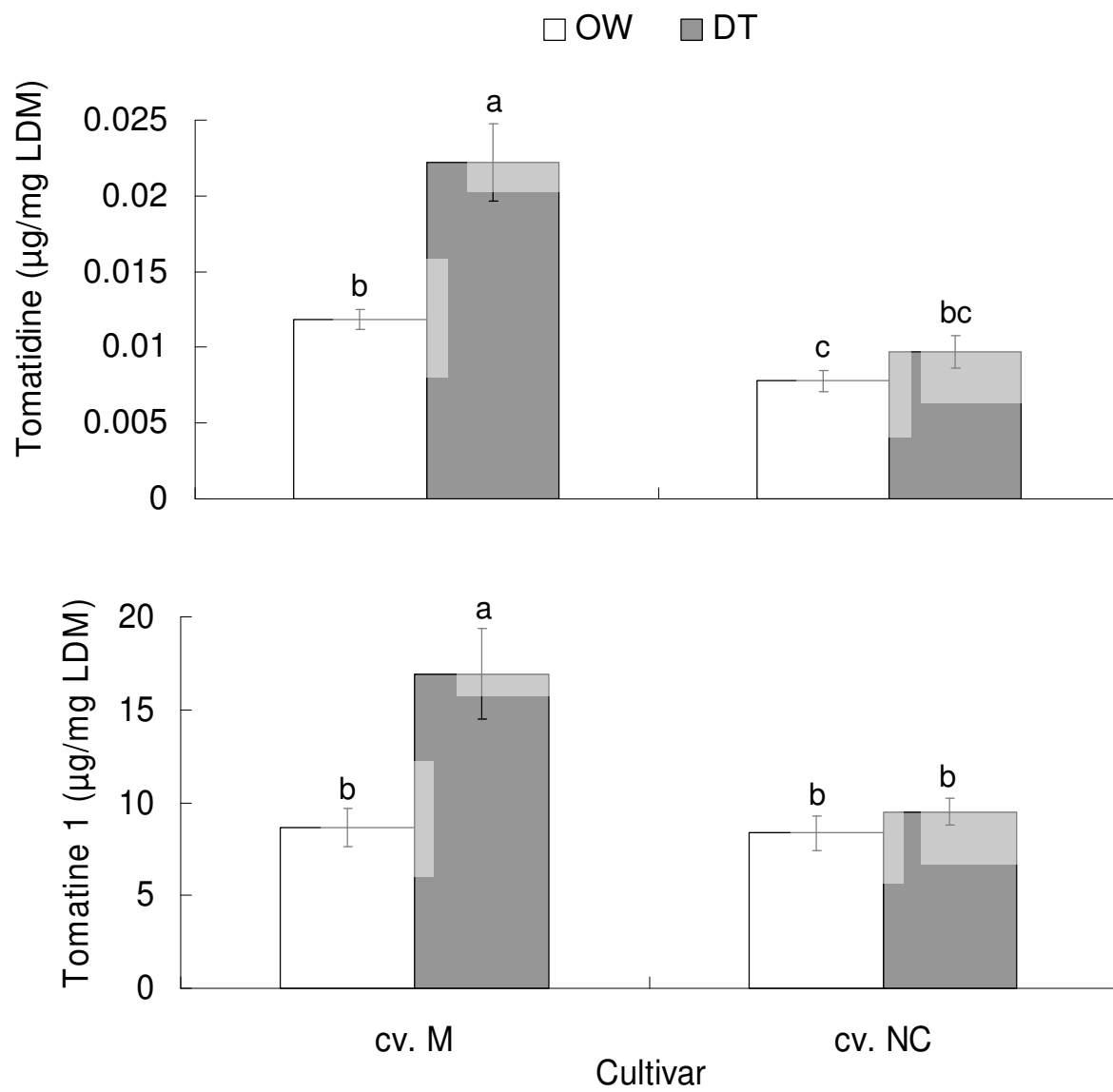
**Figure 3:**



**Figure 4**

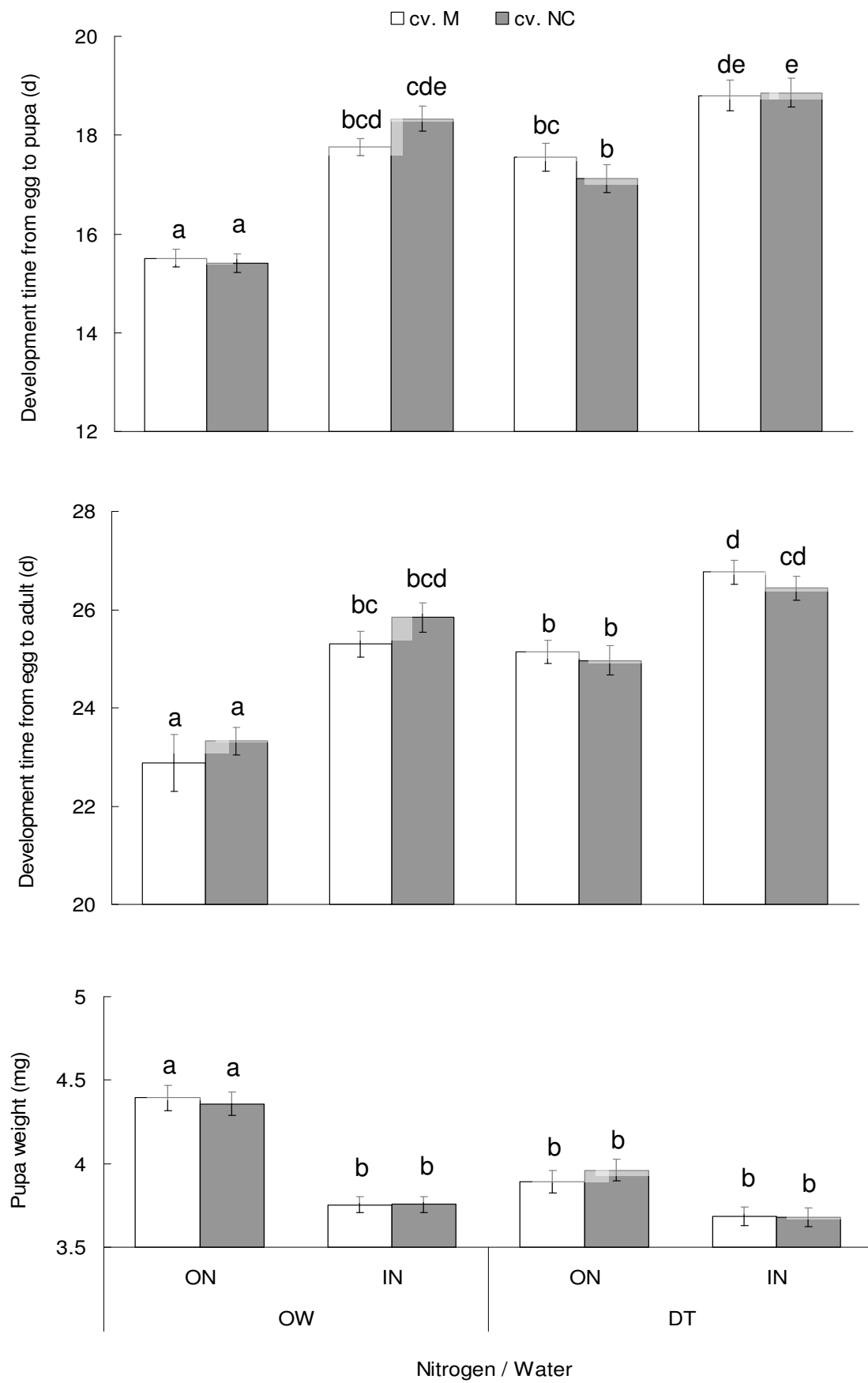


**Figure 5:**

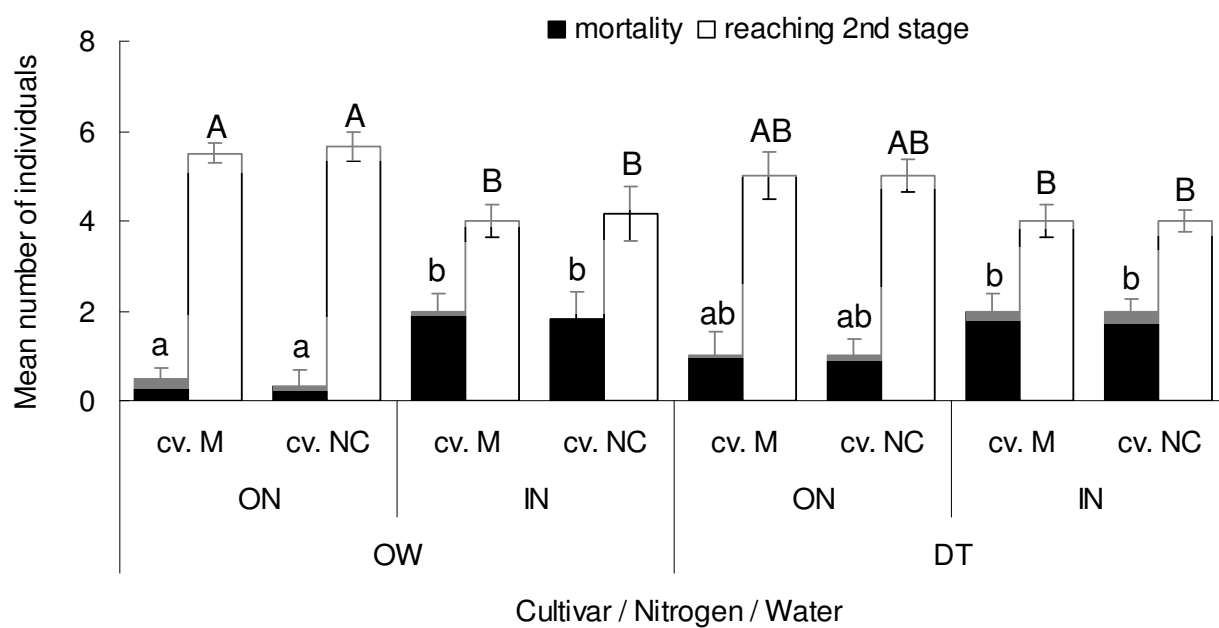




**Figure 6:**



**Figure 7**



## Supplementary materials

**Table S:** Mean concentration ( $\pm$  SEM) of glycoalkaloids (tomatidine, tomatine 1 and tomatine 2, in  $\mu\text{g}/\text{mg}$  leaf dry mass) quantified from the leaf dry mass of the two tomato cultivars (cv. M and cv. NC); ON: optimal nitrogen; IN: insufficient nitrogen; OW: optimal water; DT: drought

Cultivar	cv. M				cv. NC			
Nitrogen	ON		IN		ON		IN	
Water	OW	DT	OW	DT	OW	DT	OW	DT
Tomatidine	0.012 $\pm$ 0.001	0.021 $\pm$ 0.003	0.012 $\pm$ 0.001	0.023 $\pm$ 0.004	0.006 $\pm$ 0.001	0.006 $\pm$ 0.001	0.010 $\pm$ 0.001	0.014 $\pm$ 0.002
Tomatine 1	9.753 $\pm$ 1.74	19.19 $\pm$ 3.89	7.600 $\pm$ 1.09	14.77 $\pm$ 3.04	9.175 $\pm$ 1.28	11.25 $\pm$ 0.41	7.544 $\pm$ 1.29	7.612 $\pm$ 1.32
Tomatine 2	68.14 $\pm$ 4.35	61.37 $\pm$ 7.65	64.61 $\pm$ 5.26	61.96 $\pm$ 5.25	51.87 $\pm$ 4.13	35.09 $\pm$ 2.41	65.99 $\pm$ 3.97	57.24 $\pm$ 3.22

**Figure S1:** Experimental schedule including plant growth (**S**: plant seeding; **T1/T2**: routine plants transfer), plants measurements: leaf gas exchange (**LGE**); plant sampling for leaf N content and C/N ratio (**CNQ**), and glycoalkaloid quantification (**GAQ**) and insects infestation: *T. absoluta* eggs infestation; *B. tabaci* 1<sup>st</sup> nymph stage infestation; **DAS**: days after seeding; Nutritional treatments were applied from DAS 8 to DAS 60

**Figure S2:** Nitrogen and water treatments to the plants: (**A**) Amount of daily nitrogen input (mmol) per pot throughout the plant growth period; (**B**) Input of water volume (ml of solution) per pot throughout the growth period

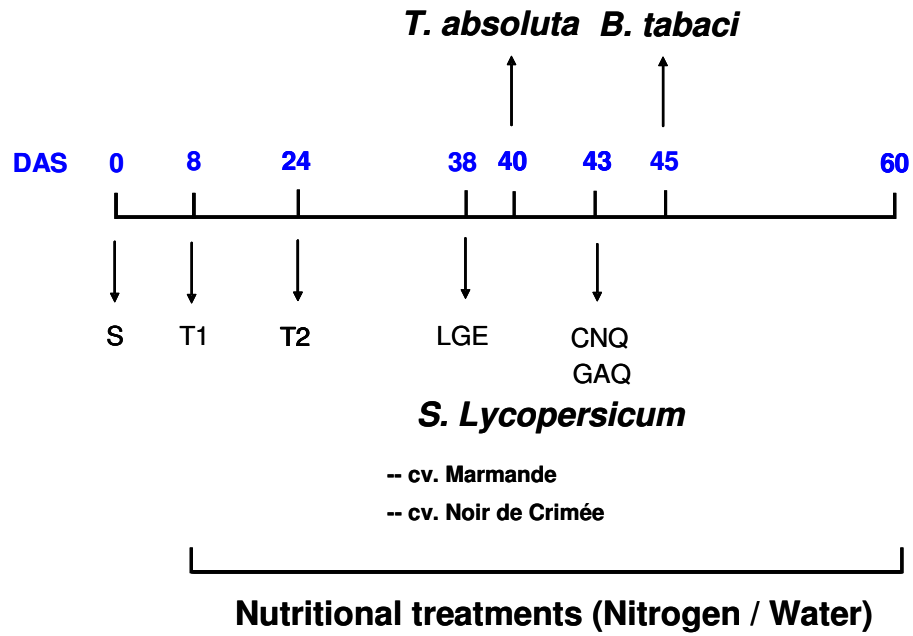
**Figure S3:** Effects of nitrogen input (ON: optimal nitrogen; IN: insufficient nitrogen) on a proxy of glycoalkaloids amount (relative abundance (means numbers of ions) per Leaf Dry mass in mg<sup>-1</sup>) for tomatidine, dehydrotomatine, tomatine 1 and tomatine 2 and for two trials (1 circles and solid line and 2 triangles and dotted lines). Data from two tomato cultivars (cv. M and cv. NC) subjected to various conditions of water input (optimal water or drought conditions) were pooled together (n=48).

**Figure S4:** Effects of water input (OW: optimal water; DT: Drought Treatment) a proxy of glycoalkaloids amount (relative abundance (means numbers of ions) per Leaf Dry mass in mg<sup>-1</sup>) for tomatidine, dehydrotomatine, tomatine 1 and tomatine 2 and for two trials (1 circles and solid line and 2 triangles and dotted lines). Data from two tomato cultivars (cv. M and cv. NC) subjected to various conditions of nitrogen input (optimal or insufficient) were pooled together (n=48)..

**Figure S5:** Effects of tomato cultivar (M: 'Marmande'; NC: 'Noire de Crimée') on a proxy of glycoalkaloids amount (relative abundance (means numbers of ions) per Leaf Dry mass in mg<sup>-1</sup>) for tomatidine, dehydrotomatine, tomatine 1 and tomatine 2 and for two trials (1 circles and solid line and 2 triangles and dotted lines). Data from

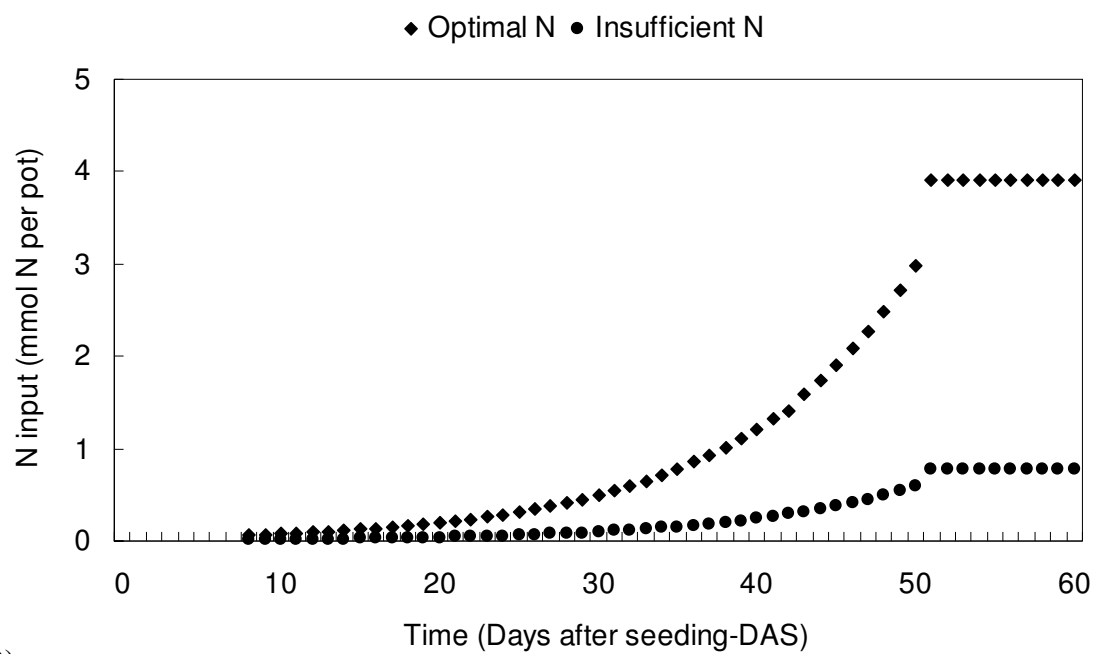
individuals subjected to various conditions of water input (optimal water or drought conditions) and nitrogen input (Optimal or Insufficient) were pooled together (n=48).

**Figure S1:**

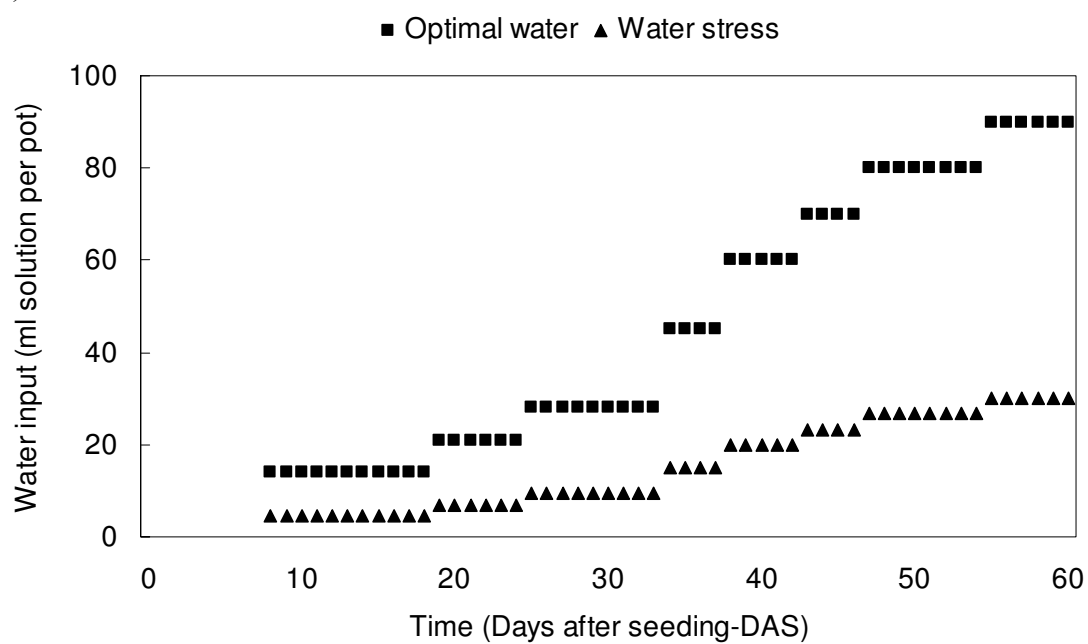


**Figure S2:**

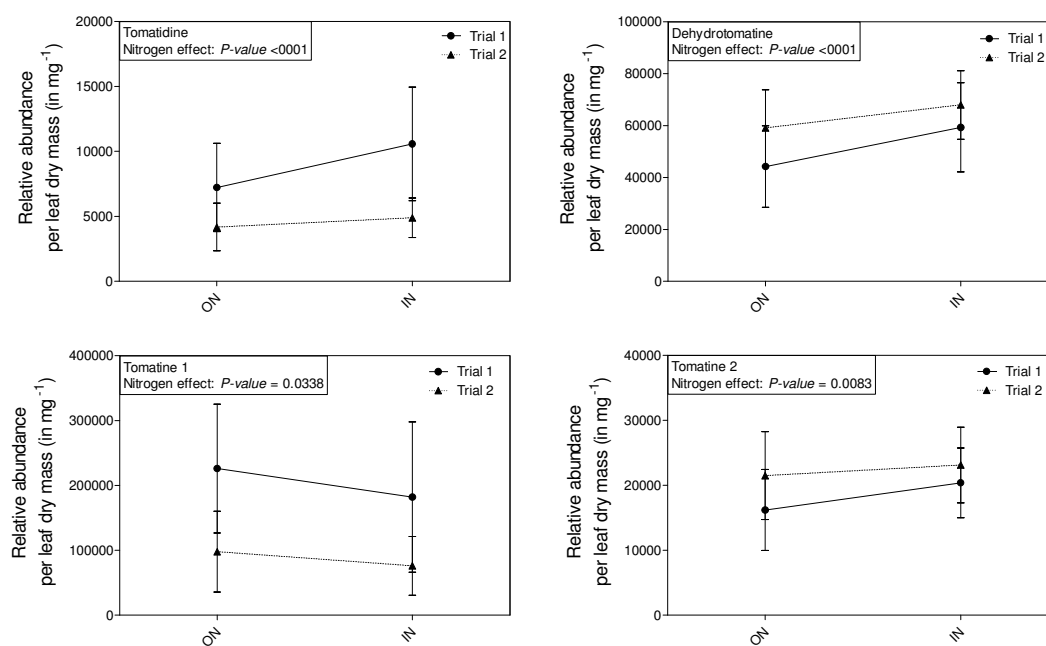
(A)



(B)

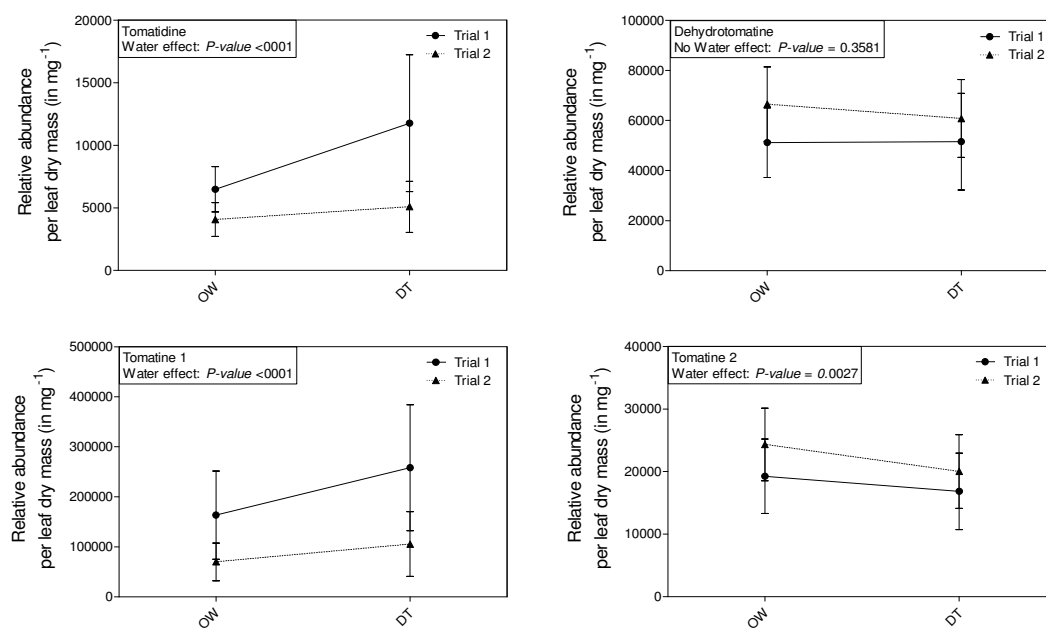


**Figure S3**

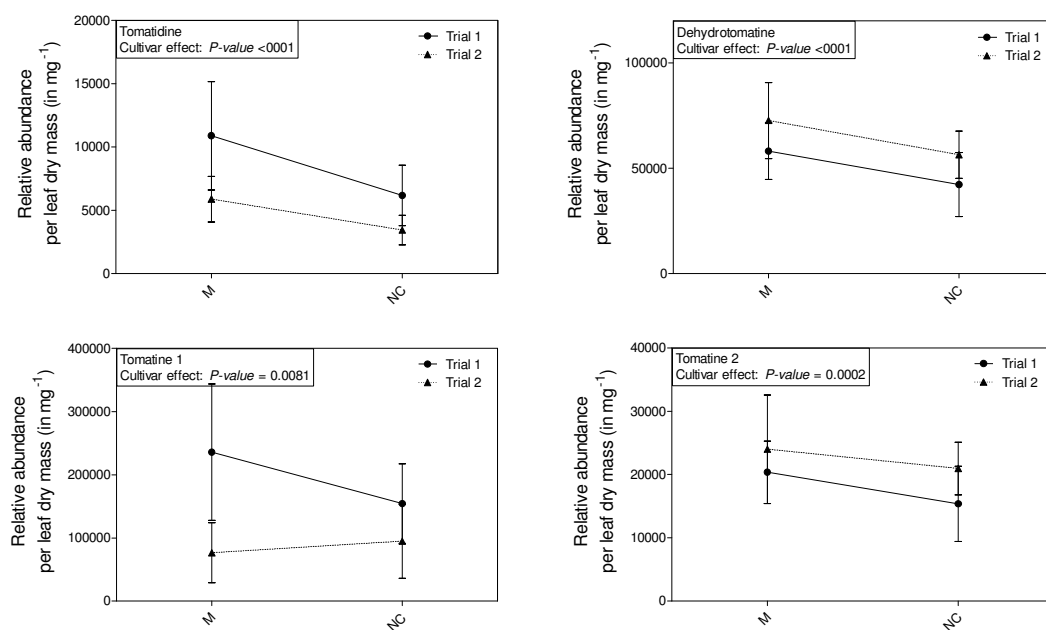




**Figure S4**



**Figure S5**



### III) Bottom-up effects of nitrogen and water on predation performance of an omnivorous predato

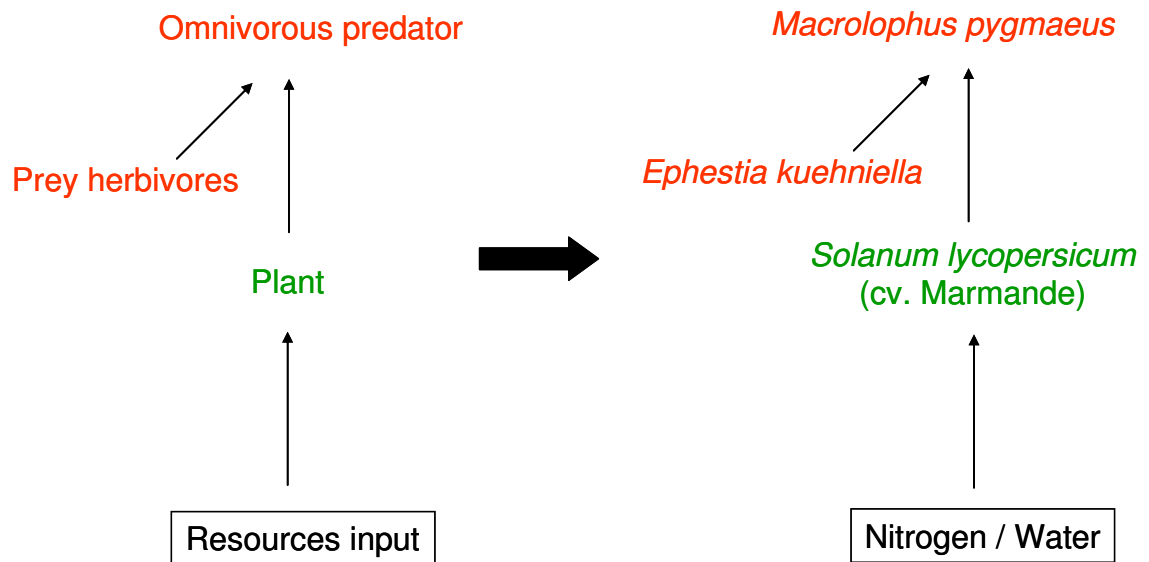
Our previous studies on plant-arthropod interactions have been limited within the first two trophic levels: plants and herbivorous insects. However, it is also important to include the organisms from level 3 (i.e., natural enemies) into our study, especially when we include the omnivorous predator into the model.

Omnivorous feeding strategy i.e., omnivory plays an important role in community dynamics (McCann et al. 1998; Eubanks and Denno 2000; Sinia et al. 2004). Among these organisms, we intend to consider the species family Heteroptera because omnivory is common in most of Heteroptera predators (Naranjo and Gibson 1996; Coll 1998; Kaplan and Thaler 2011). Furthermore, many species from this family have been largely used as predators against a wide range of pests in biological control programs (Coll 1998; Sinia et al. 2004). At least, some of them are omnivores which not only target prey animals, but also damage plants (Janssen et al. 2003). The omnivory adopted by these predators makes the study modeling more complex.

Heteroptera omnivorous predators acquire nutrients from prey animals, but also water by feeding on plants. Numerous studies have showed that lower plant nutritional quality increased the predation on herbivorous insects by Heteroptera predators (Agrawal et al. 1999; Eubanks and Denno 2000; Janssen et al 2003). To date, however, no study has considered modifying the foraging environment to increase the biological control efficiency on prey (i.e., often the target pests) by manipulating the resource inputs to the plants, such as nitrogen and water. We hypothesized that the enhanced plant growing status may facilitate the predation on prey items by Heteroptera predators.

Thus, to test this hypothesis, the study model was presented in **Fig. 4**. In the article 5, we examined the effects of nitrogen and water inputs to tomato plants (*Solanum lycopersicum* L.) on the predation strength of the omnivore *Macrolophus pygmaeus* Wagner (Hemiptera: Miridae) on egg of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) used as prey (**Fig. 5**). The study had theoretical implications since it allows testing our hypothesis and thus helps understand the predation strength of Heteroptera predators on plants suffering resources input. Practically, the findings from the current study may help optimize biological control efficiency of Heteroptera predators.

**Figure 4:** The study model of article 5. The left diagram shows the general model, the right shows the biological model used in this study



**Figure 5:** Bioassays on *Macrolophus pygmaeus* inside the mesh bags



## Article 5

### **Nitrogen and water limitation in tomato plants triggers negative bottom-up effects on the mirid omnivorous predator *Macrolophus pygmaeus***

Han Peng<sup>1</sup>, Bearez Philippe<sup>1</sup>, Adamowicz Stephane<sup>2</sup>, Lavoie Anne-Violette<sup>1,3</sup>,  
Desneux Nicolas<sup>1\*</sup>

<sup>1</sup> French National Institute for Agricultural Research (INRA), UMR1355, 400 Route  
des Chappes, 06903, Sophia-Antipolis, France

<sup>2</sup> French National Institute for Agricultural Research (INRA), UR1115 PSH (Plantes  
et Systèmes de culture Horticole), 84000 Avignon, France

<sup>3</sup> University of Nice Sophia-Antipolis (UNS), UMR1355, 400 Route des Chappes,  
06903, Sophia-Antipolis, France

\* Corresponding author: [nicolas.desneux@sophia.inra.fr](mailto:nicolas.desneux@sophia.inra.fr)

**Abstract:** Omnivorous Heteroptera play important roles in biological control of a wide range of pest insects. Their predation could be influenced by various biotic or abiotic factors. Here we examined the effects of nitrogen and water inputs to tomato plants (*Solanum lycopersicum* L.) on the predation strength of the omnivore *Macrolophus pygmaeus* Wagner (Hemiptera: Miridae) on eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) used as prey. We tested the hypothesis that a change in plant status, manipulated by sub-optimal nutrients and water inputs, may change predation on *E. kuehniella* by the predator *M. pygmaeus*. Nitrogen and water restrictions depressed the predation of *E. kuehniella* eggs by *M. pygmaeus*. Moreover, we observed a 30% reduction in the predator longevity on plants under low water inputs. Therefore, the negative bottom-ups effect of nitrogen and water limitation (in tomato plants) toward *M. pygmaeus* may be detrimental for mirid predator-based IPM programs in tomato crops.

**Key words:** omnivory, tri-trophic interactions, predation, longevity, plant quality, biocontrol

## Introduction

Arthropod is one of the most diverse groups in nature and this diversity could be characterized by the wide range of feeding strategies they adopt. Among them, omnivorous feeding strategy, i.e. omnivory plays an important role in community dynamics (McCann et al. 1998; Eubanks and Denno 2000; Sinia et al. 2004). Omnivorous predators can feed and damage plants while acting as predators of herbivorous insects (Janssen et al. 2003). Omnivorous arthropods are increasingly exploited for biological control in the sustainable integrated pest management (IPM) because of their high efficacy in the suppression of pest populations (Coll 1998; Margaritopoulos et al. 2003; Castañé et al. 2011; Chailleux et al. 2013a; Mollá et al. 2014). However, their efficacy may vary with biotic and abiotic variables (Symondson et al. 2002).

Several Heteroptera species are omnivorous predators which need to acquire nutrients and energy exploiting host plants and insect preys as well (Janssen et al. 2003). Phytophagous behaviour is common even if the frequency has not been precisely assessed (Naranjo and Gibson 1996; Coll 1998; Kaplan and Thaler 2011). Incidentally, changes in the plant nutritional status may affect the performance of omnivorous Heteroptera. Lower plant quality induced by the manipulation of plant organs (Eubanks and Denno 2000) or phytoalexins induction by earlier insect attacks (Agrawal et al. 1999; Agrawal and Klein 2000) increased the predation on herbivores by omnivores. In contrast, the improvement of plant quality by supplementing high-quality food type, such as pollen, decreased the predation rate (Janssen et al. 2003). However, little is known regarding how omnivorous predator could be influenced by the variation in plant quality mediated by the manipulation of



source-based nutrients, such as water and nitrogen inputs. Understanding this could be valuable for optimizing the IPM strategies by manipulating the agricultural practices.

In this study, we hypothesized that a change in plant status, through manipulating nutrient (N) and water inputs, may change predation strength on a given prey by an omnivorous Heteroptera predator. The study was carried out using the biological system: tomato (*Solanum lycopersicum* L.) as plant, the mirid *Macrolophus pygmaeus* Wagner (Hemiptera: Miridae) as omnivorous predator, and eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) as food prey for the mirid *Macrolophus pygmaeus* is well known to feed on plant vegetative organs (Castañé et al. 2011) and is used as an efficient biological control agent against a wide range of pests, including whiteflies, thrips, leafminers, aphids, mites and eggs of Lepidoptera pests (Barnadas et al. 1998; Riudavets and Castañé 1998; Hansen et al. 1999; Margaritopoulos et al. 2003; Zappalà et al. 2013). It is also a predator of the Lepidopteran pest, *Tuta absoluta* Meyrick (Biondi et al. 2013, Bompard et al. 2013, Chailleux et al. 2013a, 2013b; Jaworski et al. 2013), a major invasive pest threatening the tomato production worldwide (Desneux et al. 2010, 2011; Zappalà et al. 2013). Indeed, in a companion study, we showed that variation in plant quality manipulated by various levels of nitrogen and water inputs to the tomato plants did affect *T. absoluta* survival and development (Han et al. 2014). Thus, understanding how the plant nutritional inputs may also affect the predation of *M. pygmaeus* on tomato Lepidoptera pests may provide an overview on *S. lycopersicum* - *T. absoluta* - *M. pygmaeus* tri-trophic interactions to optimize IPM programme of this major pest.

## Materials and methods

### *Biological material*

Tomato plants (*Solanum lycopersicum* L. cv. Marmande), were grown from seeds in a climatic chamber (L:D 12:12, 24±1°C, 65±5% RH) as reported by Han et al. (2014). Germination was carried out in small plastic pots (7×7× 6.5 cm, TEKU, Rixheim, France) filled with compost (Tonusol, Draguignan, France). In order to control nutrition, the plantlets were transferred after rinsing the roots to pots containing an inert substrate (perlite from Perlite Italianasrl, Corsico, Italy) the first time (T1) at DAS 8 (DAS - days after sowing, Fig. S1), and the second time (T2) to larger pots (diam. 10 cm, height 9cm) at DAS 24.

Seven-day old *Macrolophus pygmaeus* female adults (BIOTOP, Valbonne, France) were reared on tobacco plants in a mesh cage in a climatic chamber (L:D 16:8, 25 ± 1°C, 70 ± 10% RH). UV-irradiated *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) eggs are commonly used as factitious prey for the mass-rearing of *M. pygmaeus*, and a recent study by Mollà et al. (2014) demonstrated how the food regime composed by this prey and tomato plants, is suitable for the successful *M. pygmaeus* population development. Therefore, in the present study, UV-irradiated *E. kuehniella* eggs were used as prey for *M. pygmaeus* during all the bioassays. The eggs were provided by BIOTOP (Valbonne, France) and were stored at 4°C until the predation trials.

### *Plant nutrition: nitrogen and water inputs*

Nutritional treatment design was adapted from Han et al (2014). From DAS 8, a full nutrient solution adjusted to pH 5.5 was supplied daily to plants in a fully crossed

design combining two nitrogen levels (ON vs. IN, optimal vs. insufficient nitrogen respectively) with two water levels (OW vs. DT, optimal water vs. drought resp.). Fifteen plants were subject to each treatment, i.e. 60 plants in total.

Nitrogen nutrition was determined *a priori* according to the principles of programmed nutrient additions (Asher and Blamey 1987; Ingestad and Ågren 1988, 1992): ON inputs (Fig. S2A, closed symbols) were calculated on a daily basis as the N amount required to obtain the maximum tomato growth and the highest leaf [N], as previously determined by Han et al. (2014) under the same experimental conditions. IN inputs (Fig. 2S A, open symbols) were calculated as ON/5. Water supplies followed a “step increase” pattern (Fig. 2SB). OW was determined by empirical evidence that the perlite substrate was fully-saturated and without leakage of nutrient solution. DT was calculated as OW/3.

### *Bioassays*

Plant growth status was evaluated non-destructively by monitoring plant height at three dates (DAS 32, 40 and 58) and the number of nodes (DAS 58) (Fig.S1 showed the schedule of plant sampling and predation trials).

The predation behavior of *M. pygmaeus* females was assessed by comparing the average daily consumption of *E. kuehniella* eggs on the plants treated with various nitrogen and water treatments. Three successive predation trials (P1, P2 and P3) were performed (see Fig. S1). In each trial, two randomly selected leaves were taken from all plants and provided with 80 fresh *E. kuehniella* eggs (ten eggs on the abaxial and adaxial surfaces of the four top leaflets). Each leaf was covered with a mesh-bag with one 7-day old *M. pygmaeus* mated female, which were starved for 24 hrs prior to the trials. The numbers of preyed eggs were counted as depleted egg shells after 48 hrs.

Then, the egg shells were removed and 80 new *E. kuehniella* eggs were deposited for the next trial. At last, 10 eggs were offered daily until the death of each predator in order to estimate their longevity. Predators were considered dead when they remained completely immobile after being touched with a fine paint brush.

### *Data processing*

Shapiro and Bartlett tests were used to test variance homogeneity and normality of residuals, respectively. Factorial two-way ANOVAs (Nitrogen x Water) were performed with R software (R Development Core Team 2010) on plant heights (measured at DAS 32, 40 and 58), node numbers (measured at DAS 58), total number of eggs preyed by *M. pygmaeus* during the three trials and *M. pygmaeus* longevity. The predation data from the three trials were summed.

## **Results**

### *Plant growth*

As expected from previous experiments in similar conditions (Han et al. 2014), Table 1A shows that nitrogen treatments and interaction between nitrogen and water had no significant effects on both criteria. Instead, the effects of water treatments were significant ( $P < 0.001$ ).

Figure 1 shows indeed that at DAS 58, similar plant heights and numbers of nodes were observed under ON and IN, while the DT treatment restricted strongly

both the plant heights (Fig. 1A) and the number of nodes compared to the OW (Fig. 1B).

#### *Predation activity and longevity of *M. pygmaeus**

Both the nitrogen and water treatments had significant effects on the predation (Table 1B). No interaction effect “Nitrogen  $\times$  Water” was found on both parameters. Indeed, Fig. 2 shows that applying insufficient nitrogen and/or low water inputs to tomato plants restricted strongly the predation of *M. pygmaeus* on the *E. kuehniella* eggs. However, the predator longevity was only sensitive to the plant water status (Table 1B), with a marked reduction only under the DT treatment (Fig. 3).

### **Discussion**

This experiment was designed to test the hypothesis that the changes in plant status may influence the predation activity of an omnivorous Heteroptera. Indeed, we found that the plants under sub-optimal nitrogen and water inputs did allow omnivorous predators to consume significantly less preys (Fig. 2). Moreover, drought treatment induced a reduction in longevity of the predator by nearly 30% compared to the optimal water input (Fig.3). Overall, sub-optimal nitrogen and water input had a negative effect on the omnivorous predators.

The effect of drought on plant growing status, i.e. smaller height and fewer numbers of nodes, was clearly observed in present study (Fig. 1) and in our earlier study as well (Han et al. 2014). In addition, *M. pygmaeus* may suffer a water limitation on their predation behavior because they faced difficulties to acquire water.

Among various benefits, omnivorous Heteroptera feed on plants to acquire the water that required for the saliva production needed for the extra-oral digestion (Cohen 1998; Sinia et al 2004; Gillespie and McGregor 2000). Thus, we could assume that in our experiment, a water deficit in plant tissues restricted the predator ability to digest eggs, which resulted in a longevity decrease (Fig. 3).

The nitrogen content of host plants often limits the performance of herbivorous insects according to the “*Nitrogen limitation hypothesis*” (White 1993; Joern and Behmer 1998). We confirmed this hypothesis with *Tuta absoluta* on tomato treated with insufficient nitrogen input showing a decrease in leaf nitrogen content and an increase in C/N ratio (Han et al. 2014). Here we observed that it concerns the omnivorous predator *M. pygmaeus* as well, since significantly fewer eggs were preyed on the plants treated with insufficient nitrogen input (Fig. 2). Unfortunately, little is known about the feeding habits of omnivorous Heteroptera (Kaplan and Thaler 2011): types of food (plant vs. prey) they prefer, plant parts they use, types of source they exploit (cell content, phloem or xylem saps). Neither the injuries (feeding marks on leaves or stems) done by *M. pygmaeus* have been identified yet (Castañé et al., 2011). Thus, experiments allowing the direct observation of *M. pygmaeus* feeding behaviour (probing frequency, duration of feeding bouts, probing site selection) are clearly required (Sinia et al. 2004).

At least two scenarios can be evoked to explain nitrogen effects: the first one is trophic hypothesis where *M. pygmaeus* needs to feed on plants because they can provide some nutrients that the prey animals cannot provide. The concentration/composition of these nutrients may vary depending on the nitrogen input, even if they do not contain nitrogen itself, as nutrition alters the concentration of a large range of organic and mineral compounds in tomato (Huanosto Magaña et al.

2009). The second hypothesis is related to the plant chemical defense (see review by Kaplan and Thaler 2011). Indeed, the concentration of many carbon based defence compounds increases in the vegetative parts of tomatoes treated with low nitrogen treatment (Le Bot et al. 2009; Larbat et al. 2012, 2014), thus restricting the performances of herbivores and omnivores as well.

Taking as a whole the results of the present research and those of our companion study (Han et al. 2014), we can conclude that optimal nitrogen and water inputs to the tomato plants favored survival and development of the herbivore *T. absoluta*, and the predation activity and longevity of an omnivorous predator *M. pygmaeus*. These results certainly questioned the IPM strategies in terms of pest and natural enemy population dynamics. More comprehensive works at a broader field scale on arthropods population levels and on crop yields are required to definitely evaluate the effects of nitrogen and water manipulations.

## **Acknowledgments**

This work was supported by funds from the Environment and Agronomy department of INRA and from the Chinese government (PhD fellowship to PH). We thank Edwige Amiens-Desneux for technical assistance during the experiments.

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## Figure legends

**Figure 1** Mean plant heights (**A**) and mean number of nodes (**B**) of tomato plants at DAS 58 under different nitrogen and water inputs. ON and IN represent optimal and insufficient nitrogen inputs, resp.; OW and DT represent optimal water input and drought, resp. Vertical bars denote standard errors (n=15). Capital letters (A, B) indicated the comparison among different nitrogen inputs overall; Lower case letters (a, b) indicated the comparison among different water inputs within a given nitrogen input level. Different letters indicate significant difference at  $P < 0.05$ .

**Figure 2:** Mean predation (cumulative number of *E. kuehniella* eggs preyed within the three predation trails) by *M. pygmaeus* adults on tomato plants under different nitrogen and water inputs. ON and IN represent optimal and insufficient nitrogen inputs, resp.; OW and DT represent optimal water input and drought, resp. Vertical bars denote standard errors (n = 30). Capital letters (A, B) indicated the comparison among different nitrogen inputs overall; Lower case letters (a, b) indicated the comparison among different water inputs within a given nitrogen input level. Different letters indicate significant difference at  $P < 0.05$ .

**Figure3:** Mean longevity (days) of *M. pygmaeus* female adults reared on tomato plants under different nitrogen and water inputs. ON and IN represent optimal and insufficient nitrogen inputs, resp.; OW and DT represent optimal water input and drought, resp. Vertical bars denote standard errors (n = 30). Capital letters (A, B) indicated the comparison among different nitrogen inputs overall; Lower case letters (a, b) indicated the comparison among different water inputs within a given nitrogen input level. Different letters indicate significant difference at  $P < 0.05$ .

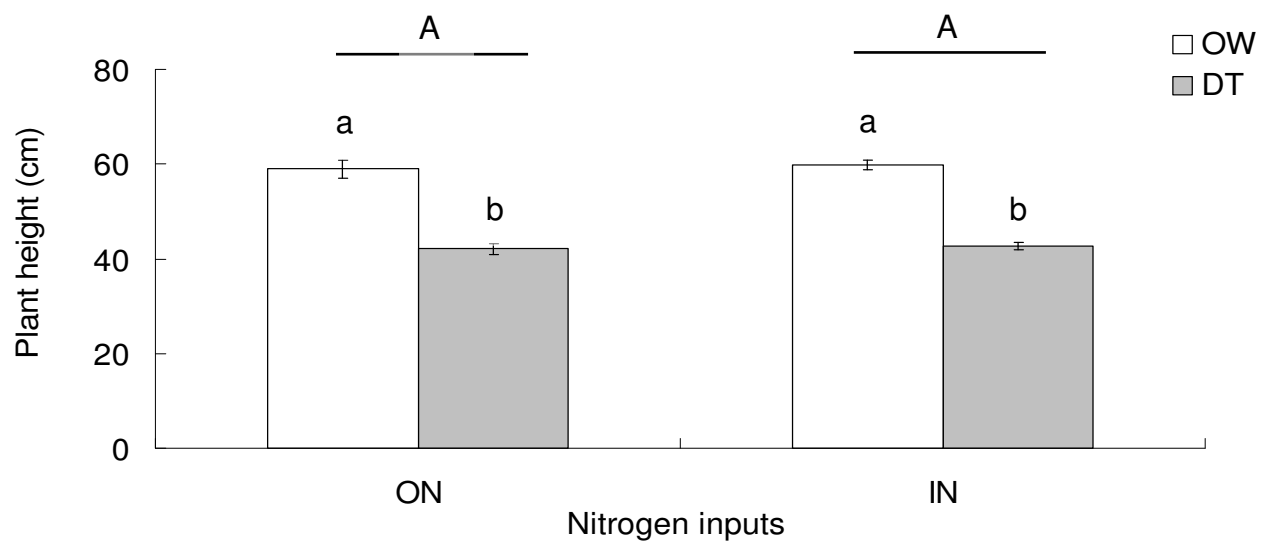
**Table 1:** Factorial ANOVA of nitrogen and water treatments on **(A) plant growth:** plant height at DAS 32 (DAS - days after sowing), 40 and 58, and number of nodes per plant at DAS 58; **(B) predator traits:** prey (*E. kuehniella* eggs) predation activity and longevity of the predator *M. pygmaeus*.

<b>A - plant growth</b>		Plant height (DAS 32)		Plant height (DAS 40)		Plant height (DAS 58)		nodes per plant (DAS 58)	
Source of variation	Df	F	<i>P</i> values	F	<i>P</i> values	F	<i>P</i> values	F	<i>P</i> values
nitrogen	1,56	0.001	0.975	0.002	0.968	0.33	0.567	3.93	0.052
water	1,56	<b>139.84</b>	<b>&lt; 0.001</b>	<b>132.34</b>	<b>&lt; 0.001</b>	<b>168.88</b>	<b>&lt; 0.001</b>	<b>249.84</b>	<b>&lt; 0.001</b>
nitrogen × water	1,56	0.009	0.926	1.33	0.253	0.004	0.949	1.91	0.172

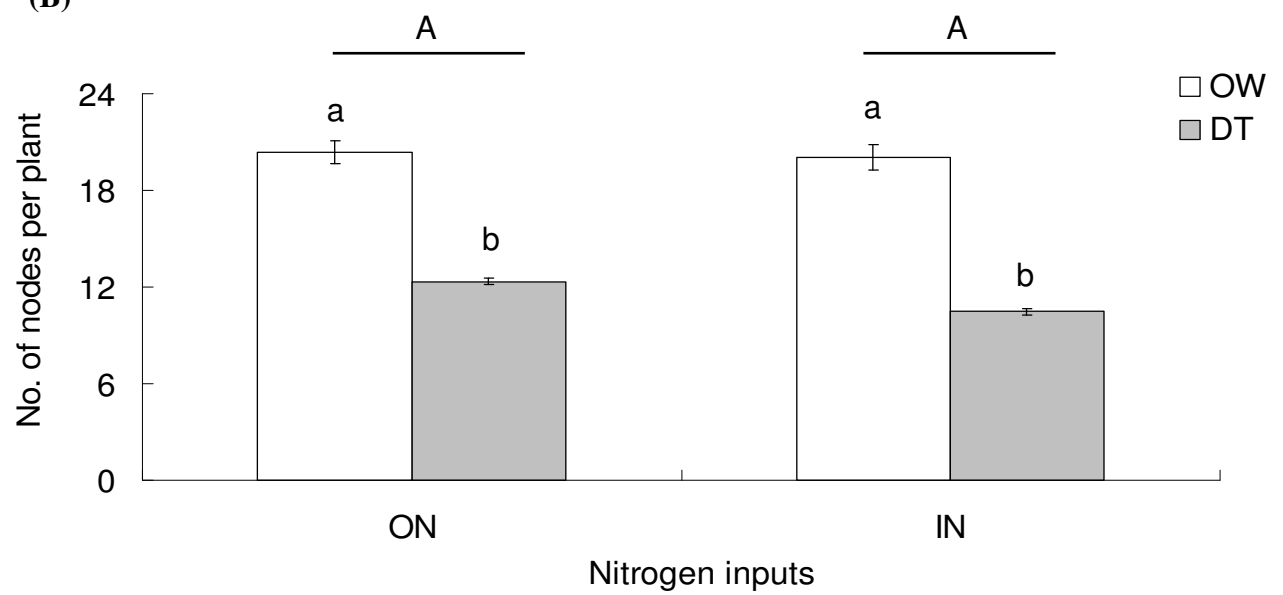
<b>B - predator traits</b>		consumption rate		Longevity	
Source of variation	Df	F	<i>P</i> values	F	<i>P</i> values
nitrogen	1,116	<b>7.75</b>	<b>0.006</b>	0.000	1.000
water	1,116	<b>46.67</b>	<b>&lt; 0.001</b>	<b>59.92</b>	<b>&lt; 0.001</b>
nitrogen × water	1,116	0.42	0.708	1.39	0.241

**Figure 1**

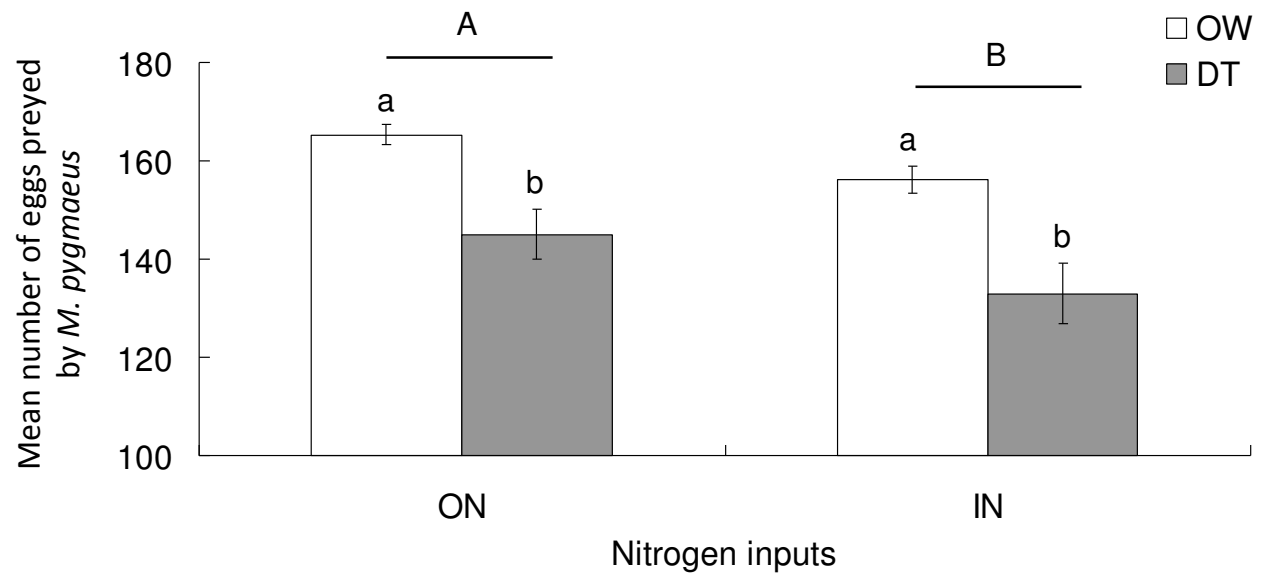
**(A)**



**(B)**

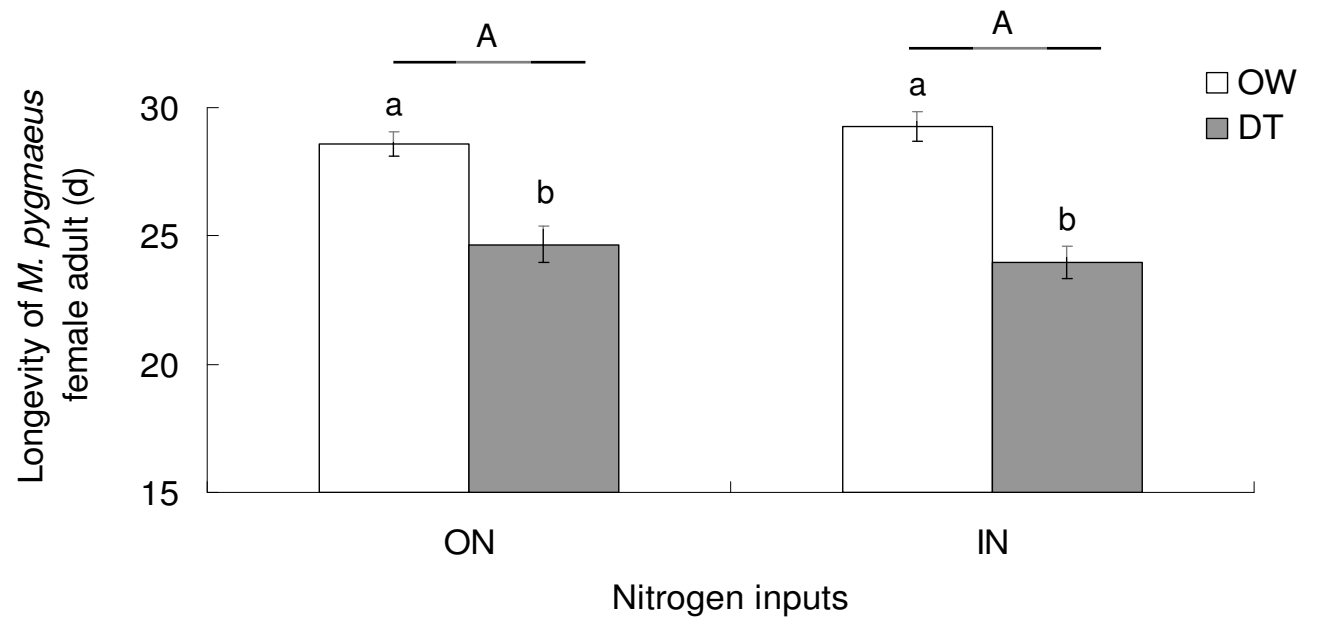


**Figure 2**





**Figure 3**

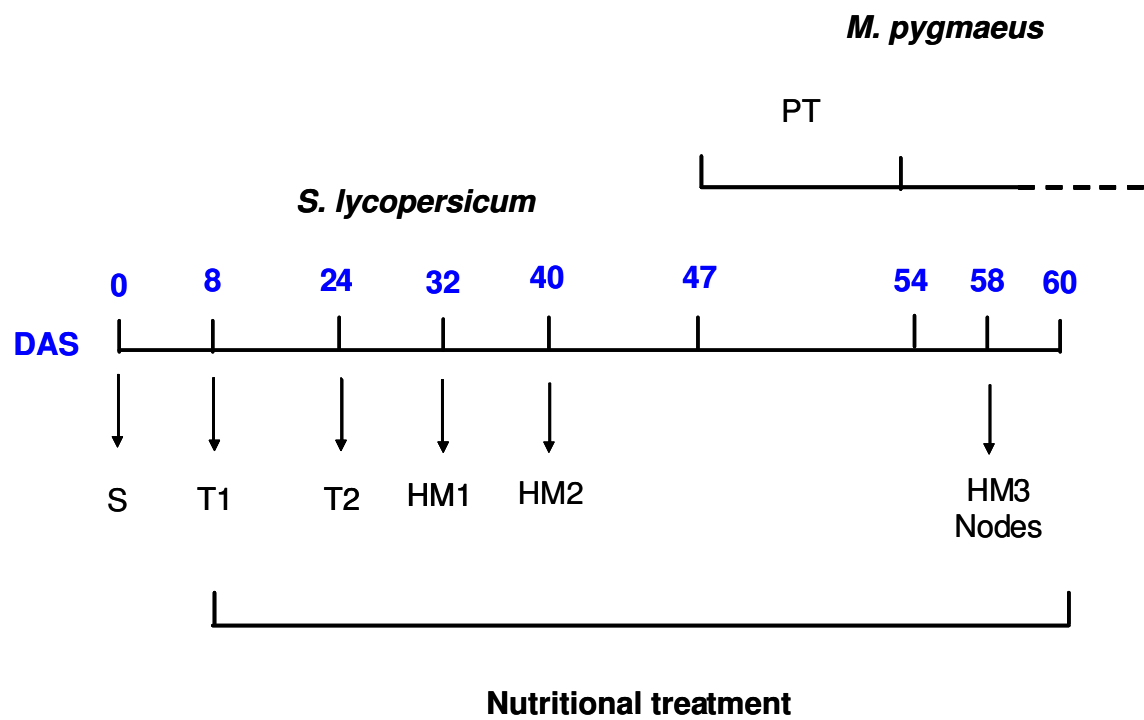


### Supplementary materials

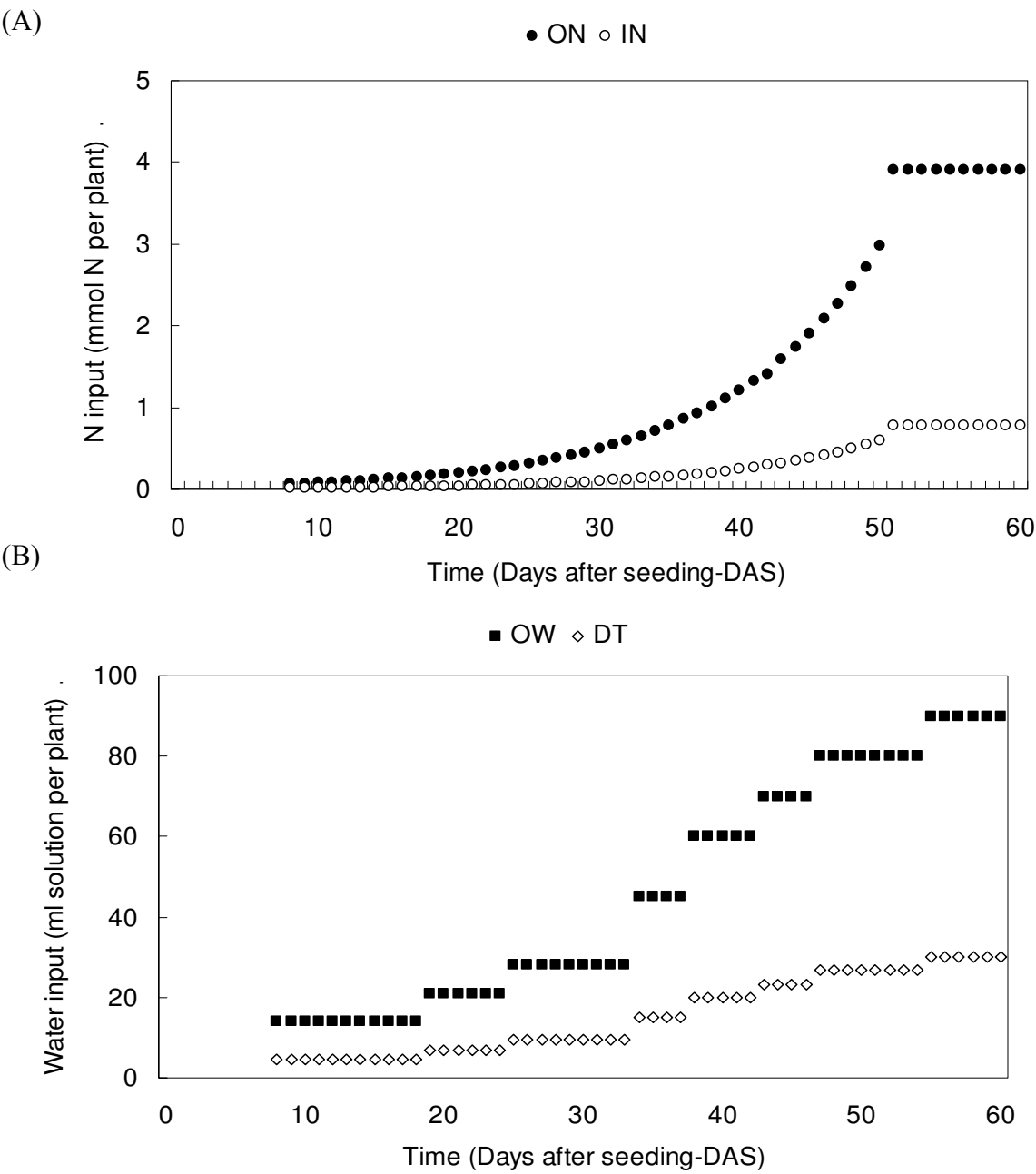
**Figure S1:** Experimental calendar (in day) of plant cultivation, nutritional treatment, all plant sampling events and predation trails by *M. pygmaeus* throughout the plant developmental stage; **S:** plant seeding; **HM1/HM2/HM3:** 1<sup>st</sup> /2<sup>nd</sup> /3<sup>rd</sup> plant height measurement; **NC:** nodes counting; **T1/T2:** routine plants transfer; **PT:** three predation trails: 1<sup>st</sup>: DAS 48-50; 2<sup>nd</sup>: DAS 50-52; 3<sup>rd</sup>: DAS 52-54. **DAS:** days after seeding

**Figure S2:** Nitrogen and water treatments to the tomato plants: **(A)** Amount of daily nitrogen input (mmol) per pot, and **(B)** Input of water volume (ml of solution) per pot throughout the growth period (ON: optimal nitrogen; IN: insufficient nitrogen; OW: optimal water; DT: drought)

Suppl. Figure 1



Suppl. Figure 2



#### IV) Bottom-up effects and nitrogen and water on feeding ecology of an omnivorous predator

Our previous study (**Article 5**) suggested that both nitrogen deficiency and drought had a negative effect on predation strength of an omnivorous Heteroptera species *M. pysmaeus*. Moreover, drought significantly reduced their longevity. We concluded that plants under optimal growing status may enhance the performance of omnivorous predators. In practice, optimal fertilization practices should be adopted to ensure an optimal biological control effectiveness exerted by the predator. However, it is still unclear how these resource based manipulations may affect their feeding ecology, i.e., the functional relationship between plant feeding and prey feeding (Gillespie and McGregor 2000). Moreover, little is known about the feeding habits of omnivorous Heteroptera (Kaplan and Thaler 2011): types of food (plant vs. prey) they prefer and plant parts they use. For example, further experiments should be designed to allow direct observations on their plant and prey feeding behavior (i.e., frequency, duration of feeding bouts) (Sinia et al. 2014). The quantification of plant and prey feeding in omnivorous Heteroptera has not been performed in the context of manipulation of plant growing status through various levels of nitrogen and water inputs. Unlike other omnivorous arthropods, the predatory bugs from Heteroptera exhibit a relatively high incidence of omnivory. Nitrogen is an essential nutrient necessary to the synthesis of digestive enzymes, and water is required for the production of the saliva and for extracting the maximum nutrients from their prey while avoiding indigestible prey structures (Cohen et al. 1995; 1998). Therefore, we predicted that the sub-optimal inputs of nitrogen and water to plants may reduce the plant feeding by the omnivore, and in turn reduce its prey feeding (i.e., “Facilitation hypothesis”).

Thus, to test this hypothesis, we the same study model iadopted in the previous study (Fig. 3). The study in article 6 is a companion study to the one in article 5. We examined the effects of variation in plant growing status, manipulated by contrasting levels of nitrogen (optimal N vs. insufficient N) and water (optimal water vs. drought) inputs, combined with the presence/absence of prey items, on (i) the feeding behaviour of a Heteroptera omnivore (plant feeding vs. prey feeding) (**Fig. 6**), (ii) plant preference behavior, and (iii) its life-history parameters: fertility and longevity.

**Figure 6:** Macrocosm setup for observation of feeding behavior of *M. pygmaeus*.



(A) Feeding behavior observation of a *M. pygmaeus* in a microcosm



(B) A *M. pygmaeus* adult feeds on the tomato stem

## Article 6

### **Obligatory phytophagy in an omnivorous predatory bug: a case study supporting the facilitation hypothesis**

Han Peng<sup>1†</sup>, Dong Yong-cheng<sup>1,2†</sup>, Lavoir Anne-Violette<sup>1,3</sup>, Adamowicz Stéphane<sup>4</sup>,  
Bearez Philippe<sup>1</sup>, Wajnberg Eric<sup>1</sup>, Desneux Nicolas<sup>1\*</sup>

<sup>1</sup> French National Institute for Agricultural Research (INRA), UMR1355, 400 Route des Chappes, 06903, Sophia-Antipolis, France

<sup>2</sup> Hubei Insect Resources Utilization and Sustainable Pest Management Key Laboratory, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, 430070, China

<sup>3</sup> University of Nice Sophia-Antipolis (UNS), UMR1355, 400 Route des Chappes, 06903, Sophia-Antipolis, France

<sup>4</sup> French National Institute for Agricultural Research (INRA), UR1115 PSH (Plantes et Systèmes de culture Horticole), 84000 Avignon, France

\* Corresponding author: [nicolas.desneux@sophia.inra.fr](mailto:nicolas.desneux@sophia.inra.fr)

† These two authors contributed equally to the study.

**Abstract:** Omnivorous arthropods make dietary choices, i.e., feeding on plants vs. on prey, according to the characteristics of the environment in which they forage (e.g., availability of prey, plant and prey qualities, etc.). Such decision, and subsequent impact on key life history traits of the predators, may be affected by the availability of nutrients and water to plants. It may occur through bottom-up forces impacting their prey, direct and indirect effects occurring owing to food uptake on the plants, and/or a combination of both. In the present study, we examined the impacts of prey presence (Lepidoptera eggs) and availability of nitrogen (N) and water to host tomato plants on the omnivorous predator *Macrolophus pygmaeus* Wagner (Heteroptera: Miridae). We studied the feeding behavior of the predator (plant vs. prey feeding) and the life history traits (fertility and longevity) on individual plants subjected to contrasted nitrogen fertilizing and watering regimes. In the absence of prey, the predator fed equally on the plants treated with contrasting levels of nitrogen and water. By contrast, the feeding rate on the plant decreased in presence of prey when plants suffered low water input. In addition, the feeding rate on prey was positively correlated with that on plant and the predator showed a strong preference toward plants bearing prey and subjected to high water regime. The presence of prey as food enhanced fertility and longevity of *M. pygmaeus*, but the plants under low water input actually decreased predator longevity. Our results support the *Facilitation Hypothesis*: the use of water taken from plants facilitates the prey feeding activity (e.g., digestion) by omnivorous predators from Heteroptera family. Phytophagous activity in such predators is not a choice but a need for high fitness.

**Key words:** Omnivory, predator, longevity, fertility, nitrogen, water



## Introduction

Omnivorous feeding strategy, i.e., absence of carnivore or herbivore feeding specialization, plays an important role in arthropod community dynamics (Polis 1991; Naranjo and Gibson 1996; McCann et al. 1998; Eubanks and Denno 1999; Coll and Guershon 2002; Eubanks 2005; Lundgren 2009; Chubaty et al. 2014). Feeding on more than one trophic level balances the diets of omnivores when alternative foods provide complementary nutrients (Bjorndal 1991; Polis and Strong 1996; Agrawal et al. 1999). Omnivorous arthropods play a major role in regulating herbivore arthropod populations in various natural and managed ecosystems (McMurty and Croft 1997; Coll 1998; Eubanks and Denno 2000; Coll and Guershon 2002; Symondson et al. 2002). However, their fitness (and their efficiency in some extent) is strongly dependent on characteristics of the environment in which they forage (Agrawal et al. 1999; Eubanks and Denno 1999; Eubanks and Denno 2000; Sina et al. 2003).

By definition, omnivorous predators need to acquire nutrients from both their host plants and their prey (Janssen et al. 2003); their dietary choice (e.g., feeding on plants vs. on prey) may depend on habitat characteristics, notably the availability and density of prey (Agrawal et al. 1999; Montserrat et al. 2004), the plant and prey qualities (Agrawal et al. 1999; Eubanks and Denno 1999, 2000; Janssen et al. 2003), and abiotic factors such as water availability (Gillespie and McGregor 2000; Sinia et al. 2003). For example, a poor plant quality can promote consumption of prey eggs in western flower thrips (Agrawal et al. 1999; Janssen et al. 2003) and in big-eyed bugs (Eubanks and Denno 1999; 2000). Such dependence on plant quality implies that the feeding behaviour of these omnivores should be affected by the actual availability of nutrients and water of the host plants through cascading effects. Several studies assessed the effects of contrasting levels of water and nutrients applied to host plants, on the performances of various herbivorous insects (Fischer and

Fielder 2000; Inbar et al. 2001; Han et al. 2014), and of some omnivorous bugs (Groenteman et al. 2006; Seagraves et al. 2011). However, the impact of plant quality variation mediated by resource inputs (e.g., water and nutrients) on both the feeding behavior and life-history traits of key omnivorous predators have been scarcely documented.

A theoretical framework with three contrasting hypotheses was proposed by Gillespie and McGregor (2000) to describe the main relationships between plant and prey feeding activities of omnivorous predators: (i) *Switching hypothesis* - the omnivorous predators switch between plant and prey foods and thus, the prey feeding rate increases as plant feeding rate decreases (e.g., when the plant quality is low); (ii) *Facilitation hypothesis* - plants contain key components (nutrients or water) that facilitate prey feeding through digestion or assimilation and thus, the prey feeding rate increases as plant feeding rate increases; (iii) *Independence hypothesis* - the prey and plant feeding rates are independent. Most studies support the *Switching hypothesis* when plant quality is low (i.e., lower palatability and higher plant defense level) (Coll 1996; Agrawal et al. 1999; Eubanks and Denno 1999; Jassen et al. 2003; Eubanks 2005; Kaplan and Thaler 2011), but one study supports the *Facilitation hypothesis* (Sinia et al. 2004) by showing that water acquisition through plant feeding by an omnivorous predator is essential for its complete feeding on prey.

Phytophagous activity is common in the omnivorous predators belonging to the Heteroptera sub-order. However, the prevalence of such activity in most groups has not been precisely studied (Naranjo and Gibson 1996; Coll 1998; Kaplan and Thaler 2011). Unlike other omnivorous arthropods, the predatory bugs from Heteroptera exhibit a relatively high incidence of omnivory. It relates likely to their complex ingestion mechanism involving an extra-oral enzymatic digestion process termed “solid-to-liquid feeding” (Cohen 2000). In this respect, nitrogen is an essential nutrient necessary to the synthesis of digestive enzymes, and

water is required for the production of the saliva and for extracting the maximum nutrients from their prey while avoiding indigestible prey structures (Cohen et al. 1995; 1998). We may hypothesize that Heteroptera predators face difficulties in ingesting and/or digesting prey items on plants suffering drought and/or nitrogen deficiencies, thus resulting in lower prey feeding activity. Assessing the frequency of plant vs. prey feeding with changes in plant quality mediated by the manipulation of water and nitrogen inputs, may help understanding the feeding ecology of such omnivores. Furthermore, it may be helpful for optimal use of Heteroptera predators in biological control programs.

Therefore, we studied the combined impacts of contrasted host plant quality, by manipulating levels of nitrogen (high vs. deficiency) and water (high vs. low), and prey availability (presence vs. absence), on (i) the feeding activity of an Heteropteran omnivorous predator, (ii) plant preference, and (iii) key predator life-history traits: fertility and longevity. We hypothesized that plants subjected to sub-optimal nitrogen and water inputs may prompt a decrease in plant utilization (i.e., feeding) by a Heteroptera omnivore, and in turn reduce its prey feeding (i.e., facilitation hypothesis). The tri-trophic system “tomato (*Solanum lycopersicum*) - eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) - mirid predator *Macrolophus pygmaeus* Wagner (Hemiptera: Miridae)” was employed to test this hypothesis. *Macrolophus pygmaeus* is well-known to feed on plant vegetative organs (Castañé et al. 2011) and is used as an efficient biological control agent against a wide range of pests, including whiteflies, thrips, leafminers, aphids, mites and eggs of lepidopteran pests (Margaritopoulos et al. 2003; Castañé et al. 2011; Chailleux et al. 2013).

## Material and Methods

### *Study organisms*

Tomato plants (*Solanum lycopersicum* L. cv. Marmande), were grown from seeds in a climatic chamber (L:D 12:12,  $24 \pm 1^\circ\text{C}$ ,  $65 \pm 5\%$  RH) as reported by Han et al. (2014). Germination was performed in small plastic pots ( $7 \times 7 \times 6.5$  cm, TEKU, Rixheim, France) filled with compost (Tonusol, Draguignan, France). In order to control nutrition, the plantlets were transferred after rinsing the roots to pots containing an inert substrate (perlite from Perlite Italiana srl, Corsico, Italy) the first time (T1) at 8 DAS (DAS - days after seeding. see Fig. 1) and the second time (T2) to larger pots (diam. 10 cm, height 9cm) at 24 DAS.

On average seven-day old omnivorous predator *Macrolophus pygmaeus* female adults in an average of seven-day old (BIOTOP Company, Valbonne, France) were reared on tomato plants in a climatic chamber (L:D 16:8,  $25 \pm 1^\circ\text{C}$ ,  $70 \pm 10\%$  RH). *Ephestia kuehniella* (Zeller) eggs were commonly used as prey for the mass-rearing of *M. pygmaeus*. UV-irradiated *E. kuehniella* eggs were used as prey for *M. pygmaeus* in the present study. The eggs were provided by the BIOTOP Company (Valbonne, France) and were stored at  $4^\circ\text{C}$  prior to the experiment.

### *Plant nutrition: nitrogen and water inputs*

Nutritional treatment design was adapted from Han et al (2014). From 8 DAS, a full nutrient solution adjusted to pH 5.5 was supplied daily to plants in a fully crossed design combining two nitrogen levels (HN vs. LN, high vs. low nitrogen respectively) with two water levels (HW vs. LW, high water vs. low water respectively). Each treatment was applied to 33 plants (total 132). Among these, 24 plants (six per treatment) were free of insect as control.

The nitrogen nutrition protocol was followed the principles of programmed nutrient additions (Asher and Blamey 1987; Ingestad and Ågren 1988, 1992): HN inputs were calculated on a daily basis as the nitrogen amount required to obtain the maximum tomato growth and the highest leaf nitrogen concentration, as previously determined by Han et al. (2014) under the same experimental conditions. LN inputs were calculated as HN/5. Water supplies followed a “step increase” pattern. HW was determined by empirical evidence that the perlite substrate was fully-saturated and without leakage of nutrient solution. LW was calculated as OW/3 (see Fig S1).

#### *Plant feeding vs. prey feeding behavior*

Figure 1 shows the schedule of all the experimental events.

The feeding observation of *M. pygmaeus* females was conducted by confining them separately in a microcosm system i.e., small Petri dishes (diameter: 10 cm) with a lateral ventilation hole (diameter: 7 cm) covered with fine nylon mesh screen. The females and the terminal three leaflets of a tomato plant were enclosed in the Petri dish. Two treatments on prey availability were performed: with and without *E. kuehniella* eggs as prey diet. The behavior of *M. pygmaeus* was observed and recorded as: (i) plant feeding, (ii) prey feeding, (iii) walking (or searching) and (iv) resting (or grooming). The piercing of leaf tissues with the insect stylets was considered as “plant feeding”, while the piercing and sucking behavior on eggs were recorded as “prey feeding”. The “walking” behavior mainly included searching for food or shelter. The “resting” behavior mainly involves grooming of stylets or remaining still. When a behavior was observed during 10 seconds, it was recorded and the next predator was observed. The direct observation of each predator was performed twice daily (10h00 -11h00 and 15h00 -16h00) during one week from 45 to 51 DAS (see Figure 1). After the data

collection, the plant feeding rate was calculated as the proportion of records where *M. pygmaeus* was observed as plant-feeding. The prey feeding rate was calculated following the same logic.

### *Plant preference*

The plant preference trial was conducted in a mesh cage (length  $\times$  width  $\times$  height: 2 $\times$ 1 $\times$ 1 m) under greenhouse conditions ( $27 \pm 3^\circ\text{C}$ ,  $65 \pm 10\%$  RH). About 80 *M. pygmaeus* adults (sexual ratio nearly 1:1, starved for 24 h) were released into a cage containing plants treated with various nitrogen and water treatments. In each trial, eight plants were used with one plant from each treatment ( $2 \times 2 \times 2$ : two nitrogen levels, two water levels and two prey availabilities: presence or absence of *E. kuehniella* eggs). The plants were arranged in a circle and the tested adults were released in the centre. The adults were allowed 30 mins for choosing their plant habitat. Then, the numbers of adults found on each plant were recorded. Ten trials (replicates) were performed. To avoid a bias resulting from space preference of flying adults, the position of each plant treatment was randomly affected in each trial. All trials were performed between 15h and 17h in a period of 4 days (52-55 DAS).

### *Predator fertility & longevity*

Emerging nymphs within the microcosms were counted and removed daily in order to assess the fertility of *M. pygmaeus* females. To estimate longevity, the predators were considered dead when they remained completely immobile.

### *Data processing*

Factorial two-way ANOVAs (nitrogen  $\times$  water) were performed on plant feeding rate in the absence of prey, and on plant and prey feeding rate in the presence of prey in *M. pygmaeus*. Factorial three-way ANOVAs (nitrogen  $\times$  water  $\times$  prey) were performed on plant preference, fecundity and longevity of *M. pygmaeus*. Linear regression analysis was conducted on plant vs. prey feeding rates. Independent sample t-test assessed the effect of “water” and/or “prey” on the plant preference, fertility and longevity when necessary. Shapiro and Bartlett tests were used to assess variance homogeneity and normality of residuals, respectively. All the datasets were analyzed using the R software (R Core Team, 2010).

## **Results**

### *M. pygmaeus feeding behavior*

Table 1A shows that in the presence of prey, the plant and prey feeding rate were affected only by the water treatment. Furthermore, in this condition, Fig. 2A shows a significant positive correlation between the plant and prey feeding rates ( $R^2 = 0.935$ ,  $P = 0.034$ ). On the contrary, Table 1A and Fig. 2B show that in the absence of prey, the plant feeding rate was neither affected by N or water inputs.

### *M. pygmaeus plant preference*

*Macrolophus pygmaeus* plant preference was strongly affected by the water input and the presence of prey, but not by the nitrogen input (Table 1B). Indeed, Fig. 3 shows that plants suffering low water inputs attracted significantly less *M. pygmaeus* adults in both nitrogen inputs (ON-OW vs. ON-DT:  $F = 0.686$ ,  $t = 0.413$ ,  $P = 0.002$ ; IN-OW vs. IN-DT:  $F = 0.567$ ,  $t$

= 5.504,  $P < 0.001$ ). Furthermore, irrespective the nitrogen or water treatment, *M. pygmaeus* adults moved significantly more towards plants bearing prey than plants without prey (Table 1B and Fig. 3).

#### *M. pygmaeus* fertility and longevity

The effect of the presence of prey was significantly positive on *M. pygmaeus* fertility (Table 1B). Indeed, Fig. 4A shows that the fertility was much lower in the absence of prey. On the contrary, neither the nitrogen or water treatments had significant effects on the predator longevity (Table 1B and Fig. 4A).

Table 1B shows that the effect of the presence of prey was also very highly significant on longevity, and that, in addition, the water treatment was also significant on longevity. Furthermore, Table 1B reveals a highly significant water  $\times$  prey interaction. Indeed, Fig. 4B shows that the presence of prey provokes a larger increase in the predator longevity on plants that receive sufficient watering, than on plants suffering water limitation.

## **Discussion**

This experiment was designed to test the “Facilitation hypothesis” which predicts that sub-optimal nitrogen and water inputs to plants (i.e., limiting plant growth) reduce the plant feeding rate by an omnivore, and consequently reduce also its prey feeding rate. Indeed, we found that the prey feeding rate by *M. pygmaeus* was positively correlated with the plant feeding rate (Fig. 1A). Water limitation applied to plants strongly limited both the plant and the prey feeding rates by the omnivore. Additional findings showed that (i) in the presence of



prey, water limitation induced a lower plant preference by *M. pygmaeus* and a lower longevity, but did not affect fertility; (ii) the presence of prey strongly increased the fertility and the longevity of *M. pygmaeus*. In addition, the predator preferred significantly more the plants bearing prey to plants without prey. Thus, our results clearly support the predictions of the “Facilitation hypothesis”. In the absence of prey, *M. pygmaeus* fed equally on the plants treated with contrasting N and water inputs. In the presence of prey, we observed a positive correlation between plant and prey feeding by *M. pygmaeus*. The water limitation treatment decreased both the plant and prey feeding rates by the predator, which is consistent with our previous study (Han et al., unpublished data) showing also a negative effect of drought on the predation of prey eggs. Furthermore, our findings agree with the assumption by Sinia et al (2004) that water acquired through plant feeding is an essential resource required for prey predation. Among various benefits, omnivorous Heteroptera feed on plants to acquire the water necessary to produce the saliva required for the extra-oral digestion of prey (Cohen 1998; Sinia et al 2004; Gillespie and McGregor 2000).

The low water treatment applied to plants affected the biological traits of *M. pygmaeus*. It reduced significantly the predator longevity on plants bearing prey (Table 1B, Fig. 4B). Since the predator needs water for prey digestion (Cohen 1995), drought may disturb this physiological process and thus lead to decreased fitness and longevity. Also, the significant prey  $\times$  water interaction suggests that prey feeding increased the water stress in *M. pygmaeus*, as also reported by Sinia et al (2004). Instead of being affected by nitrogen and water treatments, *M. pygmaeus* fertility was dramatically and positively responsive to the presence of prey (Fig 4A). Prey feeding has been proved to enable *M. pygmaeus* to achieve full reproduction (Vandekerkhove and De Clercq 2010). Other predaceous bugs also need prey as food sources to achieve optimal reproduction rates (Richards and Schidt 1996; Cocuzza et al.

1997). *Macrolophus pygmaeus* adults chose much less plants under low water regime and plants without prey (Table 1B, Fig. 3). This is probably because plants under low water regime were smaller (not shown), and thus, had less chances to attract the omnivores. Likewise, predators that were starved for 24 h were more likely to select the plants supplemented with prey.

Besides ecological implications related to the stability of predator-prey population dynamics (Eubanks and Denno 1999, 2000; Lalonde et al. 1999; Gillespie and McGregor 2000; Sinia et al. 2004), the facilitation hypothesis has applied implications because omnivorous Heteroptera are biological control agents used in agriculture. Taking *M. pygmaeus* as an example, such agents need to acquire water by feeding on plant tissue or sap to facilitate prey predation and digestion, and, potentially, this may cause injury to plants (Castañé et al. 2011). Thus, injuries might increase with increased densities of predators and prey. In this context, plants face a trade-off between the costs (i.e., increase in injuries caused by predators) and the benefits (i.e., decrease in damages caused by prey). Thus, special attention should be paid to such trade-off before the application of these predators in an augmentative biological control program.

In conclusion, our case study with the omnivorous Heteroptera *M. pygmaeus* clearly supports the facilitation hypothesis stating that omnivorous Heteroptera derive water from plants to facilitate prey feeding, and that phytophagy in omnivorous Heteroptera seems not a choice but a necessity. Our study provides insights for future studies on feeding ecology and into the suitability of use of omnivorous predators in biological control of pests: (1) Studying the feeding ecology of omnivorous predators by manipulating the plant nutritional status (through contrasting levels of nutrients, CO<sub>2</sub>, water) may be valuable for other promising omnivorous predators; (2) Direct observation of the feeding behaviour of omnivorous

predators in confined microcosms can be used as a useful tool for understanding their feeding ecology.

### **Acknowledgments**

We thank Edwige Amiens-Desneux for technical assistance during the experiments. This work was supported by funds from the Plant Health and Environment department of INRA, from the Chinese government (PhD fellowship to PH) and funds from FP7-PEOPLE-2012-IRSES, project ASCII (grant number: 318246).

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## Figure legends

**Figure 1** Experimental calendar (in day) of plant cultivation, nutritional treatment, and all the experimental trails of *M. pygmaeus* throughout the plant developmental stage; **S**: plant seeding; **T1/T2**: routine plants transfer; **Feeding trail in microcosm**:

DAS45 – DAS51; **Plant preference trail**: DAS52 – DAS55; **DAS**: days after seeding

**Figure 2** Plant feeding rates (mean  $\pm$  SE) of *M. pygmaeus* female adults observed in the microcosm feeding trials, in relation to **(A)** prey feeding rates (mean  $\pm$  SE, the solid line is a linear regression); **(B)** plant nutritional status, with HN and LN optimal and insufficient nitrogen inputs to tomato plants, respectively and HW and LW optimal and insufficient water inputs, respectively. Feeding rates were defined as the relative frequency (0 to 1) of the feeding behavior.

**Figure 3** Plant preference (mean no. of individuals  $\pm$  SEM) by *M. pygmaeus* adults between plants treated with two nitrogen nutrition levels (HN: high nitrogen; LN: low nitrogen), combined to two watering regimes (HW: high water; LW: low water). PP: presence of prey; AP: absence of prey. In each trial, 80 adults were released in the cage, and 10 trials were performed.

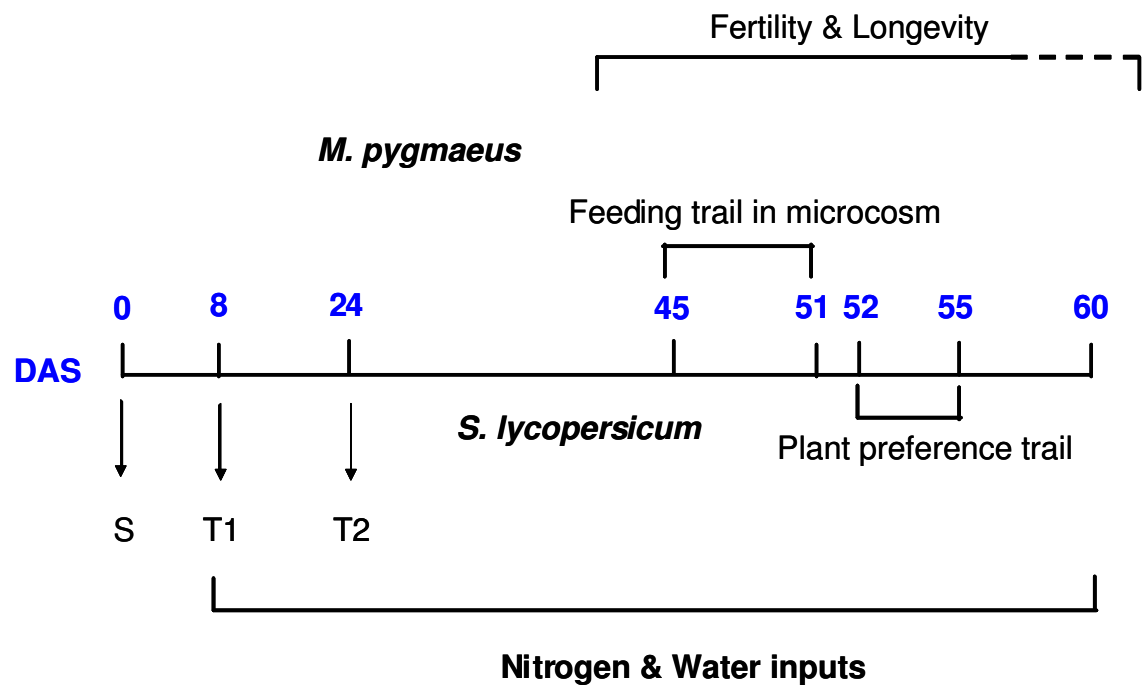
**Figure 4** Effects of the tomato plant nutritional status and of the presence of prey on **(A)** the fertility (mean no. of nymphs  $\pm$  SE,  $n = 14-27$ ) and **(B)** the longevity (mean no. of days  $\pm$  SE;  $n = 14-27$ ) of *M. pygmaeus* females. The plants were submitted to two N nutritions (HN: high nitrogen; LN: low nitrogen), combined to two watering regimes (HW: high water; LW: low water). PP: presence of prey; AP: absence of prey.

**Table 1:** Factorial ANOVAs on **(A)** plant feeding rate and prey feeding rate in the presence of prey, plant feeding rate in the absence of prey, and **(B)** plant preference, fertility and longevity of *M. pygmaeus*. Main factors nitrogen and water were tested for (A), and nitrogen, water and prey were tested for (B). Bold characters denote significant effects.

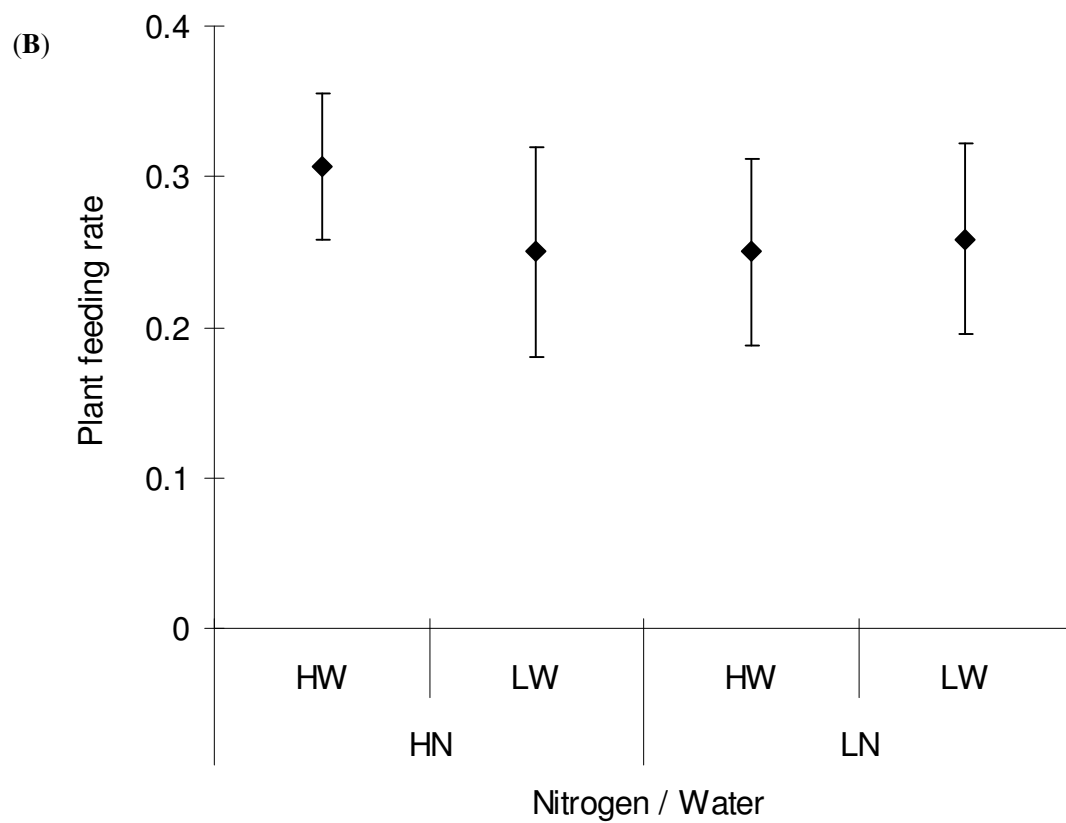
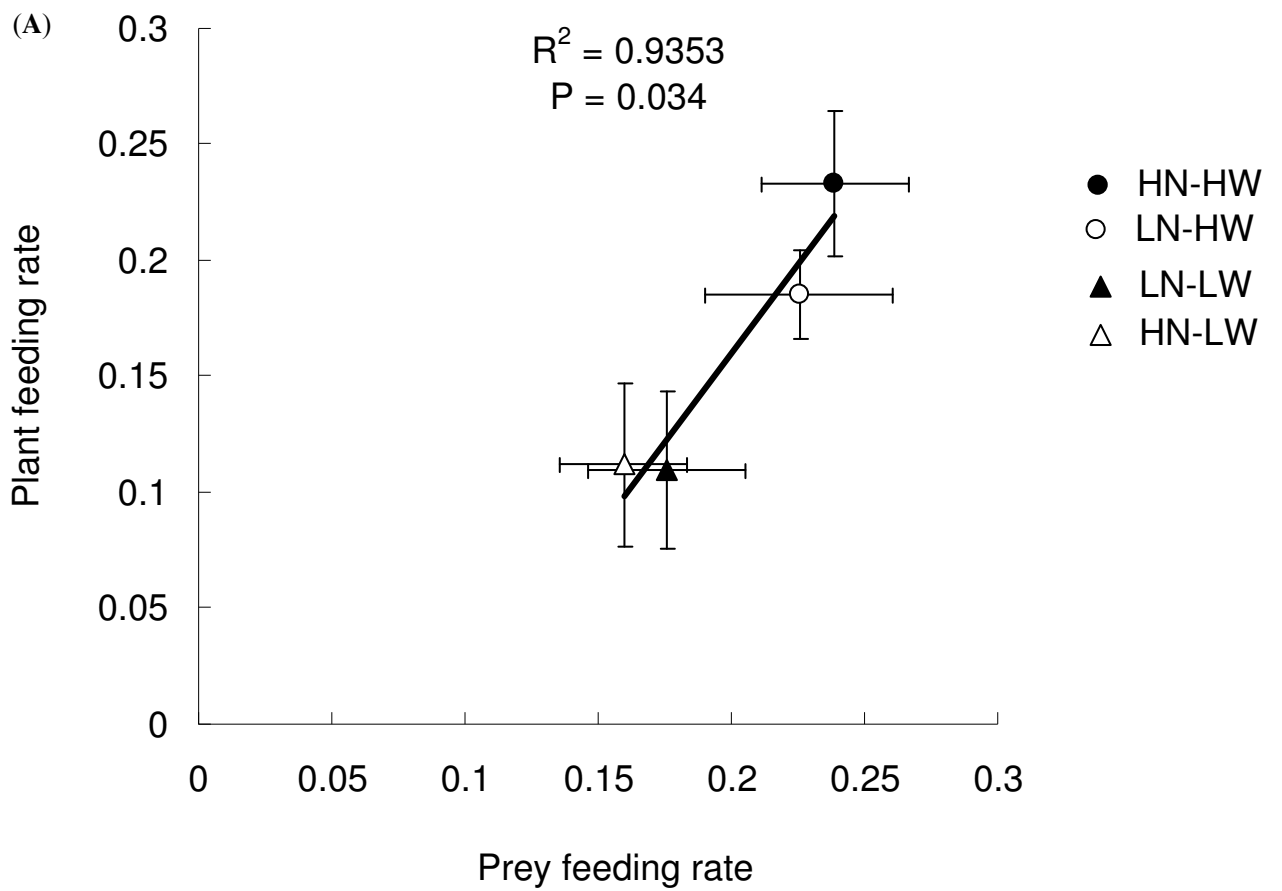
A – Microcosm feeding trial	Presence of prey				Absence of prey	
	Plant feeding rate		Prey feeding rate		Plant feeding rate	
Source of variation	F <sub>1,16</sub>	<i>P</i> values	F <sub>1,16</sub>	<i>P</i> values	F <sub>1,16</sub>	<i>P</i> values
nitrogen	0.648	0.433	0.002	0.965	0.155	0.699
water	<b>10.496</b>	<b>0.005</b>	<b>4.877</b>	<b>0.042</b>	0.153	0.701
nitrogen × water	0.535	0.475	0.254	0.621	0.275	0.607

<b>B – Biological traits</b>	Plant preference		Fertility		Longevity	
Source of variation	F <sub>1, 72</sub>	<i>P</i> values	F <sub>1, 152</sub>	<i>P</i> values	F <sub>1, 152</sub>	<i>P</i> values
nitrogen	0.222	0.639	0.843	0.360	0.422	0.517
water	<b>57.531</b>	<b>&lt; 0.0001</b>	0.012	0.911	<b>5.918</b>	<b>0.016</b>
prey	<b>40.769</b>	<b>&lt; 0.0001</b>	<b>112.01</b>	<b>&lt; 0.0001</b>	<b>227.30</b>	<b>&lt; 0.0001</b>
water × nitrogen	1.765	0.188	0.372	0.543	0.396	0.530
water × prey	0.222	0.639	0.340	0.561	<b>9.423</b>	<b>0.003</b>
nitrogen × prey	0.222	0.639	2.536	0.113	0.399	0.529

Figure 1



**Figure 2**



**Figure 3**

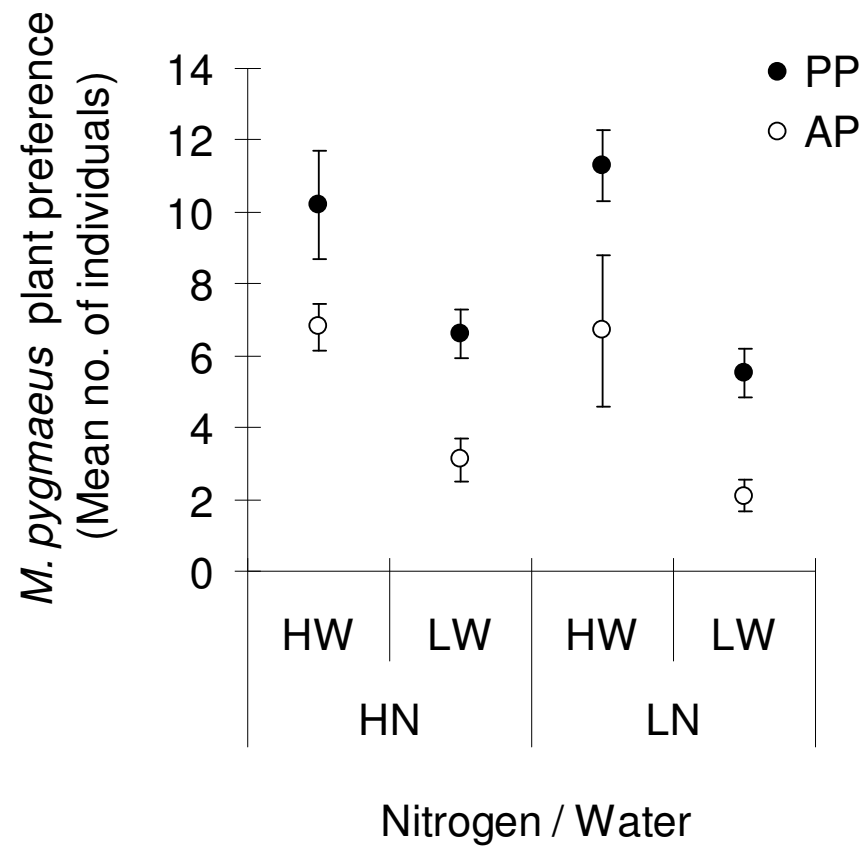
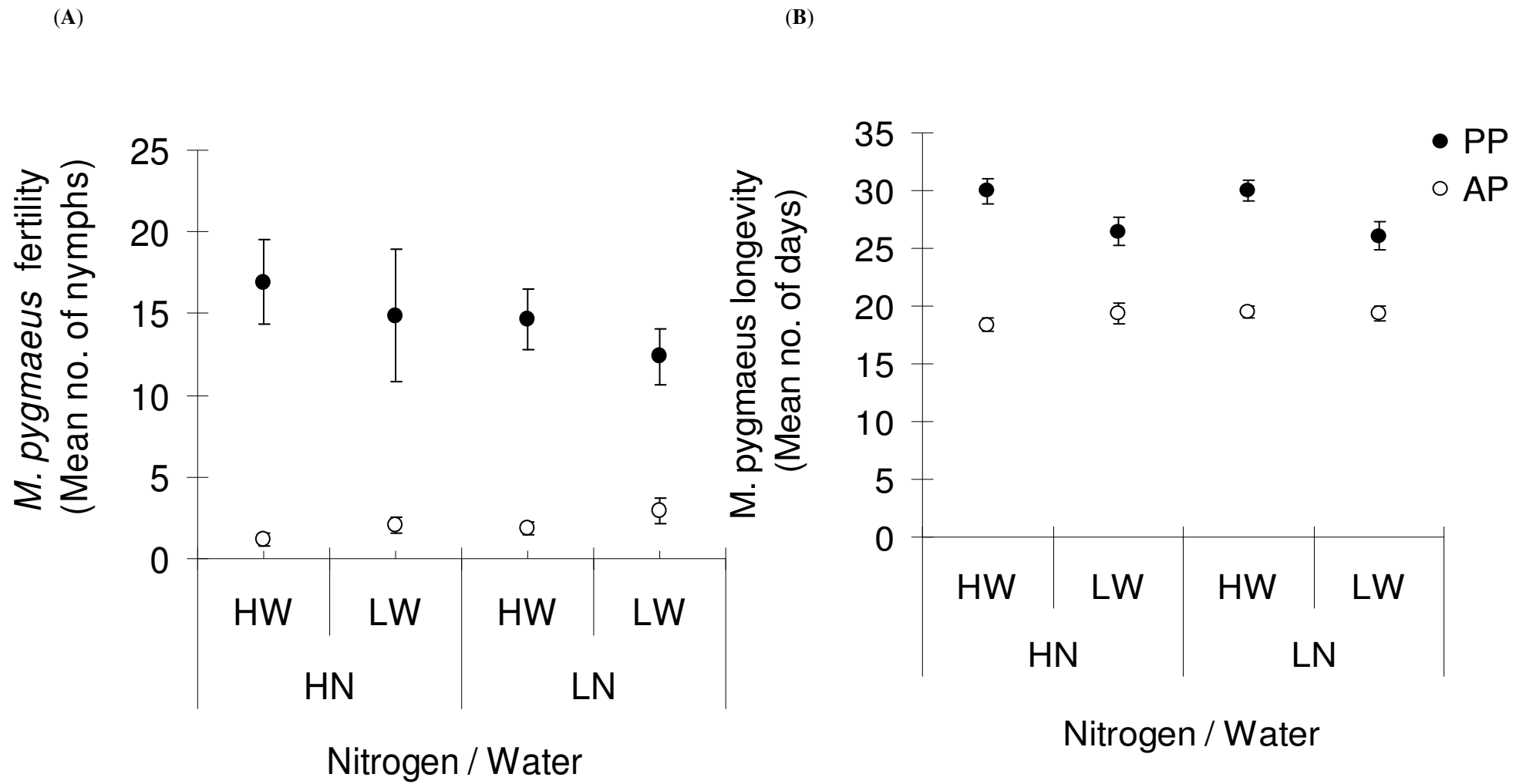


Figure 4





### Conclusion of chapter 3

In summary, the following major conclusions can be drawn:

- (i) Improper nitrogen and water inputs triggered strong negative bottom-up effects on both herbivorous insects and omnivorous predator. *Tuta absoluta*, *B. tabaci* and *M. pygmaeus* performance survival/development were negatively affected with improper resources input to plants. The data supported “Nitrogen limitation hypothesis” and “Plant vigor hypothesis”. Nitrogen availability restricted survival and development of *T. absoluta*. Plants treated with optimal nitrogen and water inputs showed optimal growing status and favor the performance of *T. absoluta*.
- (ii) Other factors such as insect feeding strategy and plant cultivars (with resistant traits against nitrogen deficiency and drought) played important role in determining the consequences of these bottom-up effects. *Tuta absoluta* (leafminer) was affected by both excessive/insufficient nitrogen input and drought, but *B. tabaci* (phloem-feeder) was only affected by nitrogen deficiency-challenged plants.
- (iii) Plant resistance traits (i.e., drought adaptive and nitrogen deficiency regulation) did not necessarily mitigate the bottom-up effects of these stresses on herbivorous insects described in (i) and (ii).
- (iv) The omnivorous predator *M. pygmaeus* showed a sub-optimal predatory performance when plants were under nitrogen deficiency and/or drought.
- (v) The omnivorous predator *M. pygmaeus* showed a positive correlation between plant feeding and prey feeding. The data supported “Facilitation hypothesis” that the plant feeding positively correlates with the prey feeding by omnivorous predators. Water acquired through plant feeding could be a limiting factor for facilitating prey feeding (i.e., predation) for them.

## **Chapter 4: Nitrogen and water inputs mediated plant-arthropods Interactions: studies at population scale**

In the previous chapter, the bottom-up effects of nitrogen and water on plant-insects interactions have been examined at individual level. We found strong bottom-up effects of nitrogen and water inputs on performances of the tomato leafminer *T. absoluta* and whitefly *B. tabaci*, and omnivorous predator *M. pygmaeus* (conclusion of chapter 3). However, bottom-up effects by resource inputs and top-down forces by the natural enemies on herbivorous insects observed at the individual level do not always translate into population effects (Zaugg et al 2013). Thus, to completely understand how bottom-up and top-down forces interact on the herbivorous insects, it is necessary to design experiments at larger scales which allowed monitoring the population dynamics of both herbivorous insect and natural enemies.

In chapter 4, we presented several studies which examined the bottom-up effects of plant resource inputs (i.e., nitrogen and water) and top-down effects of natural enemies on a target herbivorous pest species, either on tomato (*Solanum lycopersicum* L) or cotton (*Gossypium arboreum* L). The study on tomato was performed under greenhouse conditions while study on cotton (Bt cotton vs. conventional cotton) was conducted in the open field.

In the case of *T. absoluta*, the test system “Nitrogen / Water – *Solanum lycopersicum* – *T. absoluta* – *M. pygmaeus*” are expected to set up under greenhouse conditions. The herbivorous insect and predators were introduced into the system with tomato plants under various nitrogen and water inputs. Then the population dynamics of these two species can be monitored for predicting the relative strength of bottom-up forces vs. top-down forces (**Study 7**).

Our study was not confined to studying the tomato agro-system. We performed larger-scale experiments on cotton under field conditions. Cotton (genus *Gossypium*) is an ecologically important crop widely grown in China (Wu and Guo 2005). The widespread adoption of insect-resistant genetically modified (IRGM) Bt cotton has led to decreased use of chemical insecticides and enhanced biocontrol services provided by natural enemies in Northern China (Lu *et al.* 2012). The Yangtze River Valley Cotton-planting Zone (YRZ) is one of the largest cotton-growing regions (Wan et al. 2008). In this region, several insect-resistant GM cotton cultivars, notably the transgenic cultivar that combines the two genes *CryIAc* (Bt endotoxin) and *CpTI* (Cowpea Trypsin Inhibitor), have been widely

commercially used during the past decade (Han *et al.* 2010a, 2010b; Chen *et al.* 2011). Secondary pest insects are the species less well-known, but of great concern and may cause economic consequences on plant production when the external conditions change. The cotton aphid *Aphis gossypii* Glover (Hemiptera: Aphididae), non-targeted by Bt endotoxin, is considered as a secondary pest insect in this planting region. Cotton aphid may reach a high population density that causes serious damage to cotton (Wu and Guo 2005) owing to specific weather conditions (e.g., drought) or resistance to pesticides (Yi et al. 2012). Therefore, under the framework of IPM on *A. gossypii*, two primary research need to be performed:

First, it is crucial to identify the guild of natural enemies potentially attacking *A. gossypii* in the Bt cotton agro-ecosystems in YRZ (**Article 8**). Identifying the key natural enemies against the targeted pest(s) in any cropping system is the first step for effectively implementing IPM programs (Norris et al. 2003; Liu et al. 2004; Desneux et al. 2010; Ragsdale et al. 2011). For example, identification of top-down forces (i.e., key natural enemies) in a given agro-ecosystems may help further research on how these natural enemies may be promoted for enhanced conservation biological control (Landis et al. 2000; Lundgren et al. 2009b; Wratten et al. 2012).

Second, it is also important to study the bottom-up effects of resource input as well as top-down effects of natural enemy guild on the target pest *A. gossypii* (**Article 9**) based on the information of natural enemy guild obtained from previous studies. Furthermore, another source of bottom-up forces on *A. gossypii* population may come from plant resistance (Bt insecticidal proteins) via an indirect effect, i.e., the altered community structure of beneficial natural enemies in Bt cotton compared to a susceptible (i.e., conventional) cultivar (Lu et al. 2012).

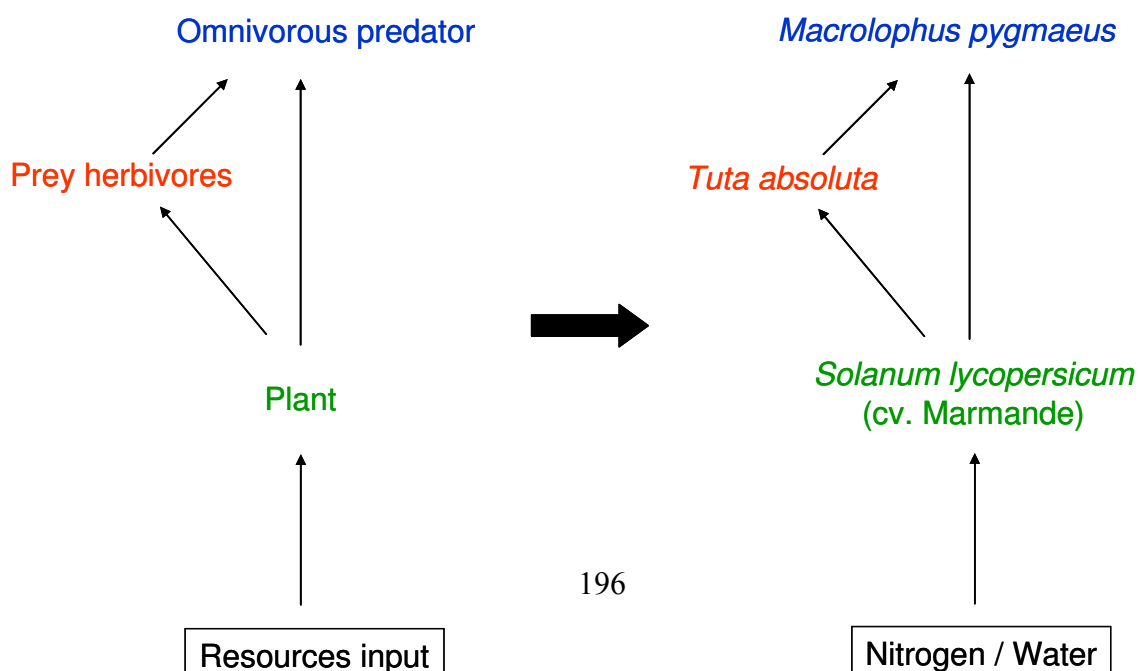
Thus, understanding the combined effects of all these forces in regulating the herbivorous pest has the potential to provide insights into fundamental ecological questions, as well as help guide the IPM programs of the target pest (Costamagna and Landis 2006; Costamagna et al. 2007).

## I) Bottom-up and top-down effects regulating a leafminer population

Our previous chapter presented strong bottom-up effects of nitrogen and water inputs on a herbivorous insect as well as an omnivorous predator. Because of the trophic link between herbivorous insect and the predator, it is difficult to predict the overall effects of these resources manipulations on the herbivorous insect. One recommended option is setting up a test system including plants, herbivorous insect and omnivorous predator. This design allows predicting the consequences of their interactions among them by monitoring their population dynamics.

Thus, in **study 7**, the test model “water/nitrogen – *Solanum lycopersicum* – *T. absoluta* – *M. pygmaeus*” was set up under greenhouse conditions (**Fig. 6**). Water (high vs. low) and nitrogen (high vs. low) inputs are expected to be controlled by the automatic irrigation system. *Tuta absoluta* and *M. pygmaeus* were introduced into the system with tomato plants under four different treatments: HW-HN, HW-LN, LW-HN, LW-LN. The two species were firstly introduced into the cages with arranged plants for population establishment. Then, the population dynamics of the two species were monitored by counting the numbers of insects at various stages (**Fig. 7**).

**Figure 7:** The study model of study 7. The left diagram shows the general model, the right shows the biological model used in this study



**Figure 8:** Experimental set-up use for population monitoring of herbivore *T. absoluta* and predator *M. pygmaeus* on tomato plants treated with high vs. low nitrogen fertilization and high vs. low water input under greenhouse conditions, at INRA Sophia-Antipolis, France; Four plants were cultivated in each cage.



## **Study 7**

### **Effects of water and nitrogen availability on the plant-leafminer-omnivorous predator tri-trophic interactions**

Han P<sup>1</sup>, Dong YC<sup>1,2</sup>, Lavoit AV<sup>1,3</sup>, Bearez P<sup>1</sup>, Desneux N<sup>1</sup>

<sup>1</sup> French National Institute for Agricultural Research (INRA), UMR1355, 400 Route des Chappes, 06903, Sophia-Antipolis, France

<sup>2</sup> Hubei Insect Resources Utilization and Sustainable Pest Management Key Laboratory, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, 430070, China

<sup>3</sup> University of Nice Sophia-Antipolis (UNS), UMR1355, 400 Route des Chappes, 06903, Sophia-Antipolis, France

## Introduction

The tri-trophic “Plant-herbivorous insect-natural enemy” interactions provides an ideal basic model to understand how the plant-inhabiting arthropod community are structured by various mechanisms (i.e. direct and indirect interactions) (Polis et al. 2000; Schmitz and Suttle 2001; Shurin et al. 2002; van Veen et al. 2005). However, such interactions may be influenced by plant-mediated bottom-up effects via plant genotypes (Johnson 2008), host plant resistance (Walker and Jones 2001), and notably resources availability i.e. fertilization to plants (Denno et al. 2002; Chau et al. 2005; Stiling and Moon 2005; Costamagna and Landis 2006; Couture et al. 2010; Chen et al. 2010; Chow et al. 2012). Among the resources required by plants, nitrogen and water availability have received most interests as they can exert a variety of bottom-up effects and potentially alter tri-trophic interactions via various mechanisms (Huberty and Denno 2004; Schoonhoven et al. 2005; Gutbrodt et al. 2011). Variations in plant nutritional quality and plant defensive profile due to nitrogen/water input manipulations have induced strong bottom-up effects on predator-prey (or parasitoid-host) relationships (Mattson and Haack 1987; Inbar et al. 2001).

Insects, like other animals, need obtain nitrogen-based nutrients (e.g. amino acid) and water by plant feeding to ensure survival and growth (Schoonhoven et al. 2005). First, the plant-inhabiting insects may suffer from water shortage and nitrogen limitation when the plants are under water deficit (i.e. drought) (Han et al. 2014). First, plant tissues may become less nutritious for insects when plants suffering water and nitrogen deficiency. In this case, herbivorous insects may face difficulty in obtaining adequate water as the plant leaves tend to



wilt, but also it restrict the uptake of nutrients as water acts as an indispensable ingredient for mediating other nutrient availability (e.g. amino acid, soluble carbohydrates, lipid etc.) (Douglas 2003; Huberty and Denno 2004). Second, water limitation to plants may allow them produce higher defensive compounds such as secondary defense metabolites (Gutbrodt et al 2011). Likewise, nitrogen deficiency in the rooting medium has been shown to induce higher levels of phenolics in plants such as tomato (Inbar et al. 2001; Le Bot et al 2009; Lariat et al 2012; Royer et al 2013). Hence, water/nitrogen deficiency may act in combination and induce negative bottom-up effects on plant-feeding insects.

The effects of water/nitrogen limitation on herbivorous insects (Price 1991; White 1993; Fischer and Fielder 2000; Inbar et al. 2001; Huberty and Denno 2004; Mody et al, 2009; Gutbrodt et al. 2011), as well as on natural enemies (Gillespie and McGregor 2000; Sinia et al. 2003; Groenteman et al. 2006; Seagraves et al. 2011) have been extensively studied. However, little is known regarding the influences of water/nitrogen availability on plant- herbivorous insect-natural enemy tri-trophic interactions. It becomes more necessary to investigate this topic when the natural enemy is an omnivorous predator due to the fact that they may be influenced by altered prey quality/quantity (i.e. trophic cascades), as well as by altered plant quality (Agrawal et al. 1999; Eubanks and Denno 2000; Kaplan and Thaler 2011).

In our previous studies, the testing model “Tomato - *Tuta absoluta*” as well as “Tomato - Lepidoptera eggs - *Macrolophus pygmaeus*” were used to study the bottom-up effects of water availability on the performances of the herbivore (Han et al. 2014) as well as the omnivorous predator (Article 5 and 6). We found that nitrogen and water deficit negatively affected the performance of both *T. absoluta* and *M. pygmaeus* at individual scale. Thus, in

the current study, an integrated model “Tomato – *T. absoluta* – *M. pygmaeus*” was set up to examine the population effects of these two variables by monitoring the population dynamics of the two species in one system, and notably to identify the overall impacts on *T. absoluta* populations. We hypothesize that (i) two-fold negative effects of low water input on the omnivorous predator were expected: (1) the decreased quantity/quality of *T. absoluta* as prey, and (2) the decreased suitability of plant food (i.e. lower plant quality), and that (ii) such two-fold negative effect on the predator may benefit *T. absoluta* by offsetting partially the negative bottom-up effects from water deficit.

## **Material and Methods:**

### *Study organisms*

Tomato plants (*Solanum lycopersicum* L. cv. Marmande) were grown from seeds in a climatic chamber (L:D 12:12, 24±1°C, 65±5% RH). Germination was performed in small plastic pots (7×7× 6.5 cm, TEKU, Rixheim, France) filled with compost (Tonusol, Draguignan, France). One week after sowing, the seedlings were transferred individually with each kept in a plastic box containing an inert substrate (perlite from Perlite Italianasrl, Corsico, Italy). Routine nutrient solutions were applied to each potted seedlings daily to ensure optimal growth until 5-week old (35 DAS, DAS: days after sowing).

A *Tuta absoluta* colony was reared on tomato plants in a climatic chamber (L:D 16:8, 25 ± 1°C, 70 ± 10% RH). *Macrolophus pygmaeus* female adults (BIOTOP, Valbonne, France)

were reared on tomato plants under the same conditions. It has been proved to be efficient predator on *T. absoluta* larvae and eggs (Chailleux et al. 2013; Mollá et al. 2014). UV-irradiated *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) eggs that commonly used as prey for the mass-rearing of *M. pygmaeus* were provided by BIOTOP and kept in 4 °C to maintain fresh prior to the experiment.

### *Nitrogen/Water treatments*

The experiment was carried out in one of the compartments in the greenhouse located at Sophia Antipolis INRA center in France. Ambient temperature, relative humidity inside the greenhouse and solar radiation outside the greenhouse were monitored throughout the experiment (see Figure 1). The fertilization/irrigation regimes were applied by an automatically-controlled irrigation system. Starting from DAS 35, we manipulated the nitrogen and water inputs to the tomato plants at two levels: high water (HW) vs. low water (LW) inputs and high nutrient (HN) vs. low nutrient (LN) (Figure 2). Thus we have four treatments: HW-HN, HW-LN, LW-HN and LW-LN. Compared to HW treatment, we manipulate the LW by cutting off the irrigation manually every 24 hrs. Differences between HN and LN were controlled by the system. For each irrigation treatment, six replicates (cages/plots) were set up with four tomato plants in each cage.

### *Insect introduction*

The experiments were initiated by the introduction of the predator *M. pygmaeus* and herbivore *T. absoluta* into the cages with plants inside (Figure 2). For *M. pygmaeus*, the first

introduction (randomly selected two males and four females from the *M. pygmaeus* colony) and the second (two males and five females) were performed at 41 DAS and 49 DAS respectively. For *T. absoluta*, the first introduction (randomly selected one male and two females from the *T. absoluta* colony) and the second (two males and one female) were performed at 49 and 56 DAS respectively. To ensure their population establishment, UV-irradiated *E. kuehniella* eggs and well-grinding pollen were provided as food for *M. pygmaeus* once per week during the two weeks (from 41 DAS to 56 DAS). On the two dates of *T. absoluta* release, honey diluents (10% honey) were sprayed onto the plants as food source for *T. absoluta*. On 61 DAS, i.e. one week after the second *T. absoluta* release, the numbers of *T. absoluta* and *M. pygmaeus* were checked and the populations were adjusted by supplement the insects when necessary. In addition, heights of all the plants were measured at 66 DAS.

#### *Population monitoring*

Starting from 61 DAS, the population dynamics of the two species were monitored in each cage during the following 7 weeks until DAS 110 (Figure 2). Adults and nymphs of *M. pygmaeus*, adults and larvae of *T. absoluta* were counted throughout the four plants in each cage. *Tuta absoluta* adults were first counted because of their phototactic behavior. To avoid the re-counting of flying *M. pygmaeus* adults, counting was conducted with minimal disturbance of the plants.

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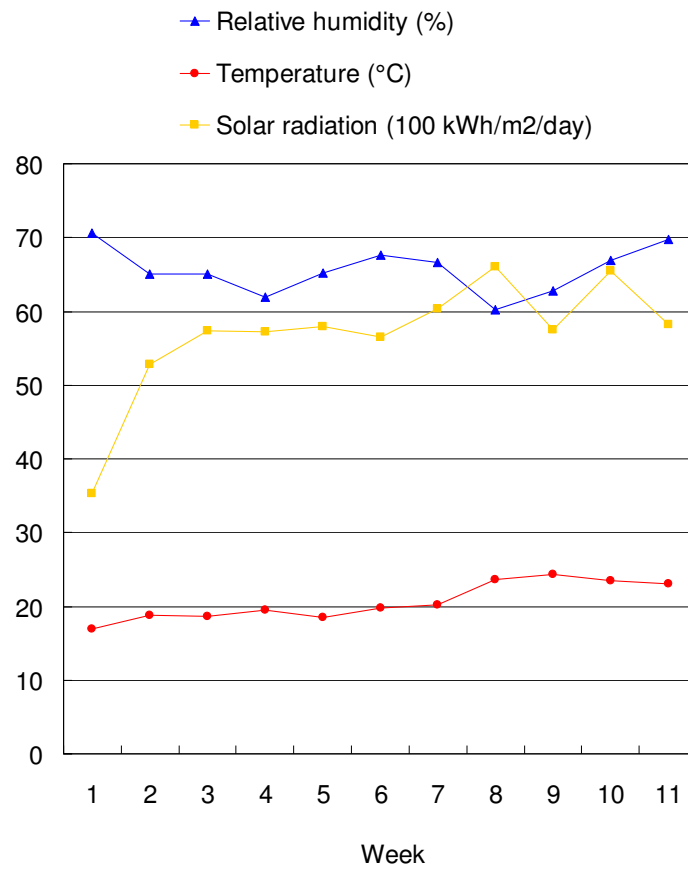
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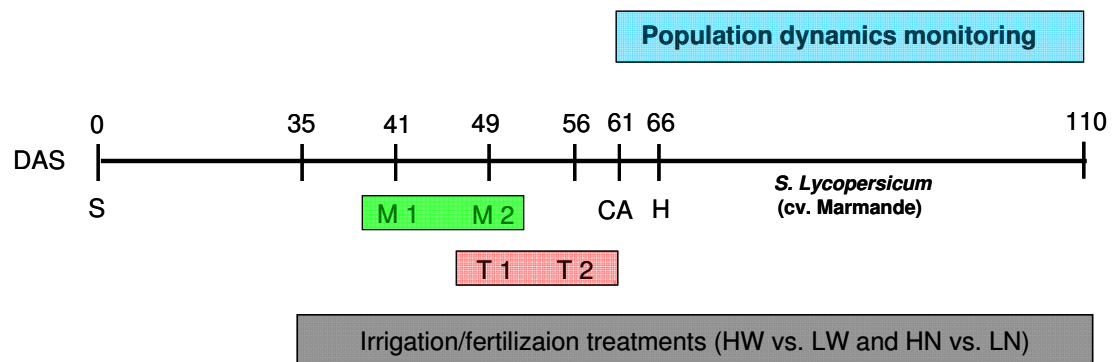
**Figure 1:** Monitoring of ambient temperature, relative humidity inside the compartment and solar radiation outside the greenhouse during the 11 weeks from 16<sup>th</sup> April to 2<sup>nd</sup> July in 2014, INRA Sophia-Antipolis, France

**Figure 2:** The experimental schedule: **DAS**: days after seeding; **S**: seeding; **M1/M2**: the first and second introduction of *M. pygmaeus* adults into the cages; **T1/T2**: the first and second introduction of *T. absoluta* adults into the cages; **H**: plant height measurements; **CA**: populations checking and adjustment before population monitoring; **HW/LW**: high and low water inputs; **HN/LN**: high and low nutrient inputs.

**Figure 1:**



**Figure 2**

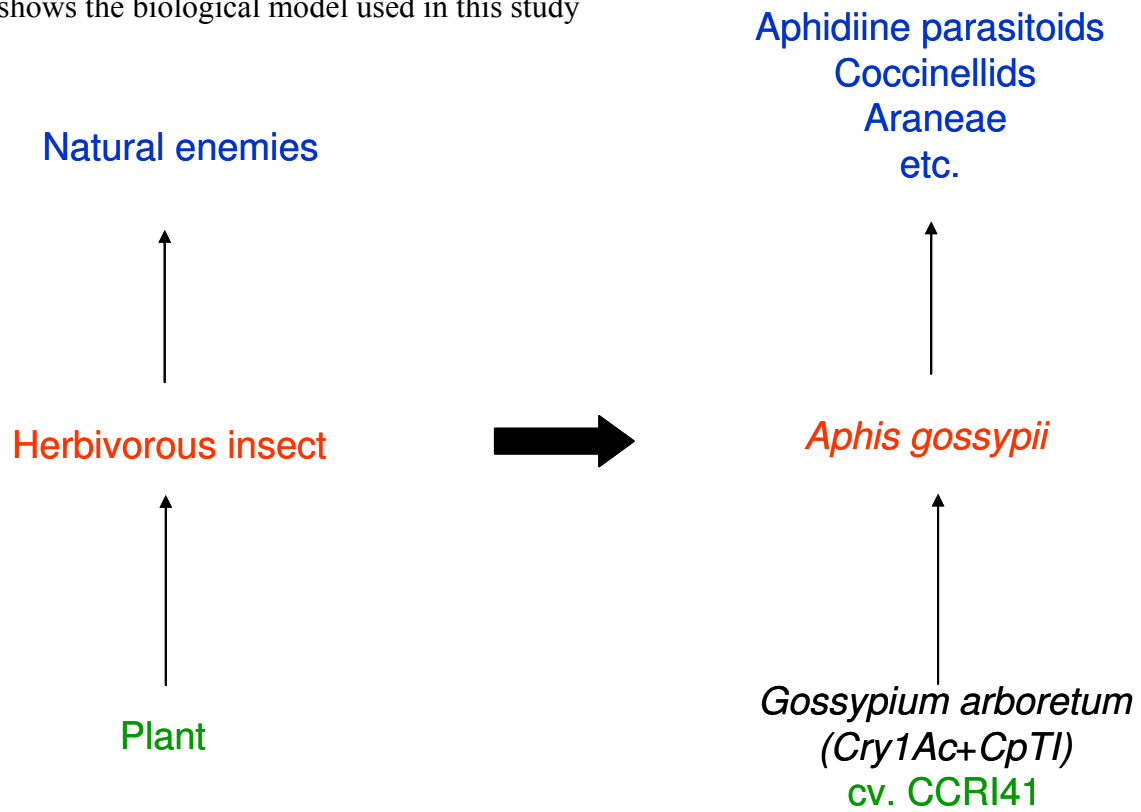


## II) Identification of top-down forces regulating cotton aphid population in field

Our previous studies have focused on examining the bottom-up effects of resource input on plant-arthropods interactions. The strong effects of water and nitrogen inputs to the plants have been observed on herbivorous insects, omnivorous predator and their population dynamics. However, in agro-ecosystems, top-down forces regulating herbivorous insects has been strongly debated since it was postulated as a dominant force shaping natural communities by Hairston et al. (1960). Several herbivorous pest species have been suggested to be primarily controlled via top-down influences of their natural enemy guild (Rosenheim et al. 2004a; Costamagna and Landis 2006; Costamagna et al. 2007). Top-down forces exerted by natural enemy guild can lead to trophic cascades that release plants from herbivory (Polis et al. 2000), which forms the theoretical foundation of biological control. Therefore, these key natural enemies (i.e., one or a group of arthropod species which primarily contribute to the reduction of herbivore population) were expected to be identified.

Thus, in **article 8**, we monitored the cotton aphid *A. gossypii* population dynamics and identified the effect of natural enemies on cotton aphid population growth using various exclusion cages in transgenic Cry1Ac+CpTI cotton field (**Fig. 8**). Exclusion cages with fine mesh netting (various sizes of openings) manipulate the entry of various types of natural enemies (i.e., predators, parasitoids or both). The experiment was conducted in a cotton field located in Ezhou, Hubei Province, China (**Fig. 9**).

**Figure 9:** The study model of article 8. The left diagram shows the general model, the right shows the biological model used in this study



**Figure 10:** Field exclusion cage setups to create various levels of exposure to cotton aphid to natural enemies. The red circle indicates one plot (or cage exclusion).



## Article 8

### **Identification of top-down forces regulating cotton aphid population growth in transgenic Bt cotton in central China**

Peng Han<sup>1,2</sup>, Chang-ying Niu<sup>1\*</sup>, Nicolas Desneux<sup>2\*</sup>

<sup>1</sup> Hubei Key Laboratory of Insect Resources Application and Sustainable Pest Control, Plant Science & Technology College, Huazhong Agricultural University, Wuhan, China

<sup>2</sup> French National Institute for Agricultural Research (INRA), Sophia-Antipolis, France

**\* Corresponding authors:**

e-mail: [nicolas.desneux@sophia.inra.fr](mailto:nicolas.desneux@sophia.inra.fr)

e-mail: [niuchangying88@163.com](mailto:niuchangying88@163.com)



# Identification of Top-Down Forces Regulating Cotton Aphid Population Growth in Transgenic Bt Cotton in Central China

Peng Han<sup>1,2</sup>, Chang-ying Niu<sup>1\*</sup>, Nicolas Desneux<sup>2\*</sup>

**1** Hubei Key Laboratory of Insect Resources Application and Sustainable Pest Control, Plant Science & Technology College, Huazhong Agricultural University, Wuhan, China, **2** French National Institute for Agricultural Research (INRA), Sophia-Antipolis, France

## Abstract

The cotton aphid *Aphis gossypii* Glover is the main aphid pest in cotton fields in the Yangtze River Valley Cotton-planting Zone (YRZ) in central China. Various natural enemies may attack the cotton aphid in Bt cotton fields but no studies have identified potential specific top-down forces that could help manage this pest in the YRZ in China. In order to identify possibilities for managing the cotton aphid, we monitored cotton aphid population dynamics and identified the effect of natural enemies on cotton aphid population growth using various exclusion cages in transgenic Cry1Ac (Bt)+CpTI (Cowpea trypsin inhibitor) cotton field in 2011. The aphid population growth in the open field (control) was significantly lower than those protected or restricted from exposure to natural enemies in the various exclusion cage types tested. The ladybird predator *Propylaea japonica* Thunberg represented 65% of Coccinellidae predators, and other predators consisted mainly of syrphids (2.1%) and spiders (1.5%). The aphid parasitoids Aphidiines represented 76.7% of the total count of the natural enemy guild (mainly *Lysiphlebia japonica* Ashmead and *Binodoxys indicus* Subba Rao & Sharma). Our results showed that *P. japonica* can effectively delay the establishment and subsequent population growth of aphids during the cotton growing season. Aphidiines could also reduce aphid density although their impact may be shadowed by the presence of coccinellids in the open field (likely both owing to resource competition and intraguild predation). The implications of these results are discussed in a framework of the compatibility of transgenic crops and top-down forces exerted by natural enemy guild.

**Citation:** Han P, Niu C-y, Desneux N (2014) Identification of Top-Down Forces Regulating Cotton Aphid Population Growth in Transgenic Bt Cotton in Central China. PLoS ONE 9(8): e102980. doi:10.1371/journal.pone.0102980

**Editor:** Guy Smagghe, Ghent University, Belgium

**Received:** January 14, 2014; **Accepted:** June 25, 2014; **Published:** August 29, 2014

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**Funding:** This study was financially supported by the National Natural Science Foundation of China (Grant IBN-31071690, 31371945), and International Atomic Energy Agency (via Research Contract No. 16015, 17153 to CYN). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** Dr. Nicolas Desneux is currently an Academic editor of PLOS ONE, but this does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

\* Email: niuchangying88@163.com (CYN); nicolas.desneux@sophia.inra.fr (ND)

## Introduction

The widespread adoption of insect-resistant genetically modified (GM) Bt cotton has led to decreased use of chemical insecticides and enhanced biocontrol services provided by natural enemies in Northern China [1,2]. The Yangtze River Valley Cotton-planting Zone (YRZ), which located in central China, is one of the largest cotton-growing regions nationwide [3]. In this region, several insect-resistant GM cotton cultivars, notably the transgenic cotton that combines the two genes *Cry1Ac* (Bt endotoxin) and *CpTI* (Cowpea Trypsin Inhibitor), have been widely adopted during the past decade [4–6]. The cotton aphid *Aphis gossypii* Glover (Hemiptera: Aphididae), a pest not targeted by Bt endotoxin (as is the case with other aphids, e.g. see [7,8]), is considered a secondary insect pest in the YRZ. Although cotton aphid populations have shown continuous decline in seasonal density in cotton fields in the past 15 years in Northern China [2], cotton aphid outbreaks may occur and reach economically damaging levels [1] owing to particular weather conditions (e.g. less rainfall during the aphid population-growth season) or pesticide resistance [9].

In agro-ecosystems, natural enemies play an important role in controlling arthropod pest populations [2,10]. For example, Hawkins and Marino [11] reported that insect parasitoids caused the highest mortality among the biotic factors for many pest species (mortality compiled for 78 pest species). Symondson *et al.* [10] stressed the importance of generalist predators in regulating pest populations. Various studies have documented top-down forces regulating herbivore populations and crop biomass yield [12–15] and also identified key natural enemies of predators involved in pest suppression in specific crops [14,16–18]. Indeed, it is crucial to characterize the guild of potential natural enemies capable of attacking targeted pest(s) for developing a sustainable Integrated Pest Management (IPM) program in any cropping system [19–21]. For example, identifying key natural enemies in a given agro-ecosystem may orient further research on how these natural enemies may be promoted to enhance biological control [22–25]. Therefore, studies documenting top-down forces in agro-ecosystems are crucial for developing effective IPM programs.

In Bt-cotton cropping systems in the YRZ, no systematic study has been carried out to characterize *A. gossypii* population dynamics and to identify the specific top-down forces that may help managing this pest in Bt cotton fields. In the present study,



using various types of natural enemy exclusion cages and artificially released aphid populations, we aimed to (i) monitor aphid population dynamics in open field, (ii) assess specific effects of natural enemies on *A. gossypii* population dynamics, and (iii) identify the key natural enemies of *A. gossypii* in Bt cotton. The results of the present study will help optimize integrated management of *A. gossypii* in Bt-cotton cropping systems in central China.

## Materials and Methods

### Cotton field and aphid colony

Experiments were conducted during the summer of 2011 at Ezhou experimental station (Huazhong Agricultural University), Ezhou, Hubei province, China (114.7 E, 30.3 N). The GM cotton cultivar CCRI41 (Zhongmian 41) which produces insecticidal proteins Cry1Ac (*Bt* endotoxin) and CpTI (Cowpea trypsin inhibitor) [4,26] was used during the study. The CCRI41 seeds were provided by the Institute of Cotton Research of Chinese Academy of Agricultural Sciences (CAAS), Anyang, China. Seeds were sowed on April 27<sup>th</sup> in a 1.5-ha cotton field with 1-m spacing between rows and the cotton was cultivated using standard agronomic techniques except that no pesticides were applied. The field had been used for cotton cultivation for several years. The area surrounding the field consisted of mainly cotton (55%), rice (30%), sweet potato (10%), other minor cropping plants, and natural habitats.

Naturally occurring *A. gossypii* were collected in May from a cotton field at Huazhong Agricultural University (Wuhan, China) which had been cropped without insecticide applications. These aphids were used to establish a colony in the laboratory (on cotton) at the university and used as the source of aphids for infesting the plants during the field study.

### Experimental setup

Four different degrees of predator exclusion were tested using various exclusion designs in the Bt cotton field: (i) Exclusion cages with 530×530 μm openings in which aphids were fully protected from all insect natural enemies. (ii) Restriction cages with 3×3 mm openings in which aphids were partially protected. This size of openings restricted entry by large predators i.e. Coccinellids, but allowed small predators to enter [13,27]. (iii) Sham cages built with 530×530 μm mesh netting but included a 40 cm high opening in the middle and the bottom respectively (modified from [15]). This treatment was used to assess possible disruptive effect of caging (e.g. mesh, wood sticks, etc.) on the activity of natural enemies and aphid population growth within the plots. (iv) No cage, a completely open area (named “open field” hereafter), which used four wood sticks standing upright into the ground and a tape surrounding them as guidance for sampling range and plot size and position.

The four different treatments were established on July 28<sup>th</sup> (Fig. 1a) using a completely randomized block design (Fig. 1b). The distance between treatments inside each block was 3 m and between blocks was 10 m. The field cages were made of wood frames (2×2×2 m, length×width×height) covered by fine nylon mesh netting with openings of 530 μm or 3 mm according to the various designs used, see above). Four plants were enclosed in each cage with a distance of 1 m between plants. We used 2×2×2 m cages because the cotton cultivar used could grow up to 1.8 m height and 1 m width during the season [4,5]. One side of each cage was equipped with a zipper to enable sampling.

Prior to the artificial aphid infestation, any resident aphids and other insects were removed by hands, brushes and mouth

aspirators in all of the cages and plants in the open field plots (20 plots total). On July 28<sup>th</sup>, ten aphids were released on each plant of the four different treatments. Aphids were placed on the highest central leaf of the plants using a camel's hair brush. From August 4<sup>th</sup> to Sept 30<sup>th</sup>, all arthropod pests and natural enemies on the four plants within each plot were recorded and identified to family or species level. In the case of aphid parasitoids, the non-emerged parasitoid mummies (pupae stage of the parasitoid) were counted (with black- and tan-colored mummies assigned to the Aphelinidae and Aphidiinae parasitoid families, respectively). The field survey was carried out on a weekly basis (every 7–8 days) from noon to 6 pm for each date of survey. Mummy samples were collected from the various plots during the course of the study (mainly from Aug 20<sup>th</sup> to Sept 7<sup>th</sup> when parasitoid densities were at high levels) for further identification of parasitoids using appropriate identification keys by [28–31]. The collected mummies (n = 119) were brought back to the laboratory and placed in Petri dishes in a Climatic Chamber (25°C, 65% RH and 16:8 h/L:D) until parasitoid adults emerged.

### Statistical analysis

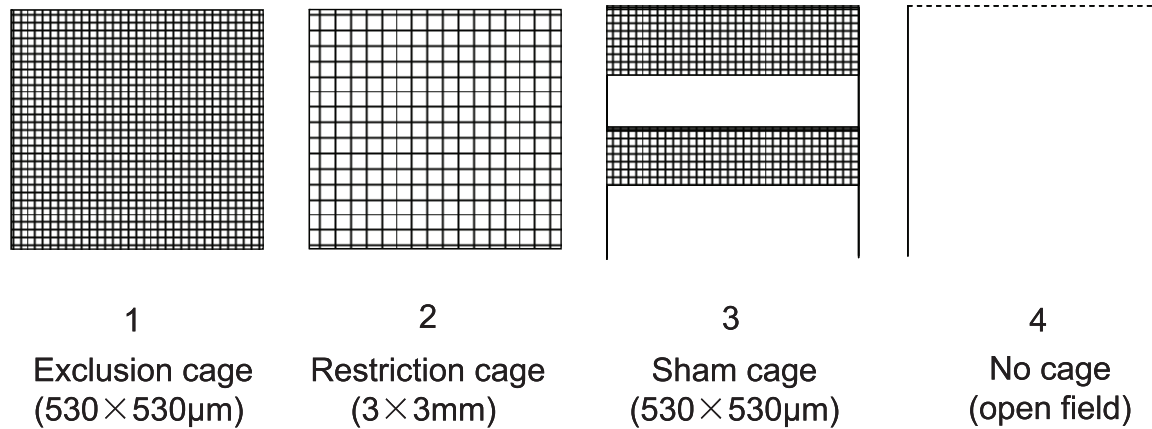
We tested the effect of predator exclusion degree (factor: cage type), as well as the effect of the date (factor: date) on the aphid counts and on numbers of main natural enemies recorded (see below) using a generalized linear model based on a Poisson distribution and a log-link function (Proc Genmod in the SAS statistical package, SAS Institute, NC, USA).

## Results

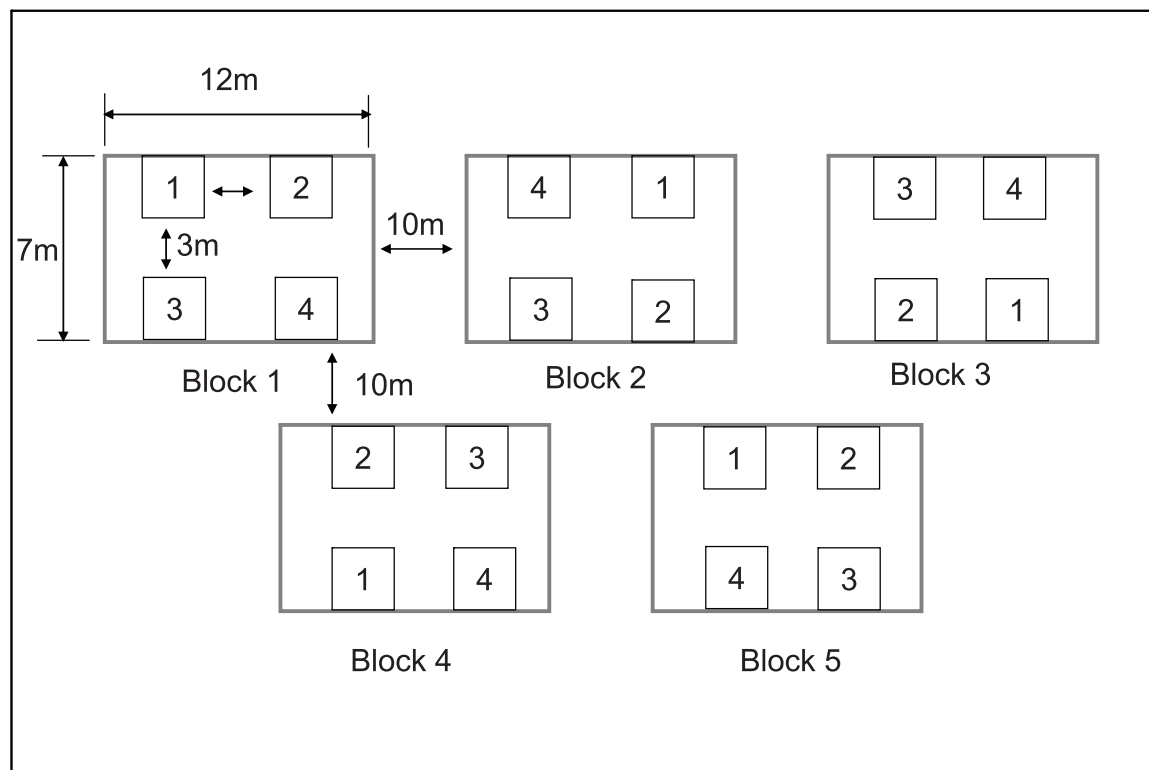
Overall, three dominant arthropod guilds were identified during the surveys: (i) pest insects, (ii) natural enemies of *A. gossypii* and (iii) omnivorous insects (Table 1). *Aphis gossypii* accounted for 85.1% of total pest insects recorded; the other three main pest species were the leafhopper *Empoasca biguttula* Shiraki, the whitefly *Bemisia tabaci* Gennadius and the common cutworm *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae). The natural enemy guild was largely dominated by the aphid parasitoids which accounted for 76.7% of all natural enemies recorded during the study. Aphidiines (tan-colored mummies) were most commonly observed; only 3 Aphelinidae mummies were found during the study. The parasitoids identified (i.e. those emerged from mummies brought back to the laboratory) were primarily *Lysiphlebia japonica* Ashmead and *Binodoxys (Trioxys) indicus* Subba Rao & Sharma (51.8% and 37.7% of samples collected, respectively). Two other species were also identified at lower rates: *Aphidius gifuensis* Ashmead and *B. near communis* (8.8% and 1.8%, respectively). Coccinellids represented 11.2% of all natural enemies observed, with *Propylaea japonica* Thunberg being the dominant species belonging to this group of predators (65.03%). *Harmonia axyridis* Pallas (20.04%) and *Coccinella septempunctata* Linnaeus (14.92%) were also observed as less common coccinellid species. The other natural enemies belonged to the syrphid, spider and lacewing predator groups. Omnivorous insects were also observed, mainly Hemipteran piercing-sucking bugs belonging to the Miridae, Nabidae and Anthocoridae families.

*Aphis gossypii* densities we recorded differed significantly among cage types (Fig. 2, cage type factor:  $\chi^2 = 12.20$ , df = 3,  $P = 0.007$ ) and as function of the dates when the aphid populations were surveyed during the season (date factor:  $\chi^2 = 17.73$ , df = 7,  $P = 0.013$ ). The two factors did not interact significantly when analyzing aphid counts ( $\chi^2 = 20.07$ , df = 21,  $P = 0.521$ ). More aphids were found in exclusion cages and restriction cages than in sham cages or open field plots. There was a 180-fold aphid

(A)



(B)



**Figure 1. Design of the field study.** (A) The four different cage treatments, i.e. natural enemy exclusion degree, in the field study; Exclusion cages: prevented natural enemy (predators and aphid parasitoids) movement, Restriction cage: prevented predator movement but allowed aphid parasitoids to colonize the plants, Sham cage and No cage: allowed free access to the plants for all natural enemies. (B) Within- and among block design: the distance among treatments within a block was 3 m, and among blocks was 10 m. The experimental cotton field was 70 m  $\times$  30 m.  
doi:10.1371/journal.pone.0102980.g001

**Table 1.** Dominant arthropods, per guild, found during the surveys.

Guild	Taxonomy	Total counts	Percentage within guild (%)
Pest insects	<i>Aphis gossypii</i> Glover	30611	85.1
	<i>Empoasca biguttula</i> Shiraki	1693	4.7
	<i>Bemisia tabaci</i> Gennadius	2727	7.6
	<i>Spodoptera litura</i> Fabricius	924	2.6
Natural enemies	Coccinellids <sup>a</sup>	449	11.2
	Aphid parasitoids (Aphidiines)	3081	76.7
	Syrphidae	83	2.1
	Araneae <sup>b</sup>	60	1.5
	Chrysopa (lacewings)	52	1.3
Omnivorous insects	Hemiptera (bugs) <sup>c</sup>	124	

Total counts of dominant arthropods per guild in the experimental blocks during the field survey from August 4<sup>th</sup> to September 30<sup>th</sup>, 2011, in Ezhou (China).

<sup>a</sup>mainly *Propylaea japonica* Thunberg (292, 65.03%), *Harmonia axyridis* Pallas (90, 20.04%) and *Coccinella septempunctata* Linnaeus (67, 14.92%).

<sup>b</sup>mainly *Erigonidium graminicolum* Sundevall.

<sup>c</sup>mainly from Miridae, Nabidae and Anthocoridae families.

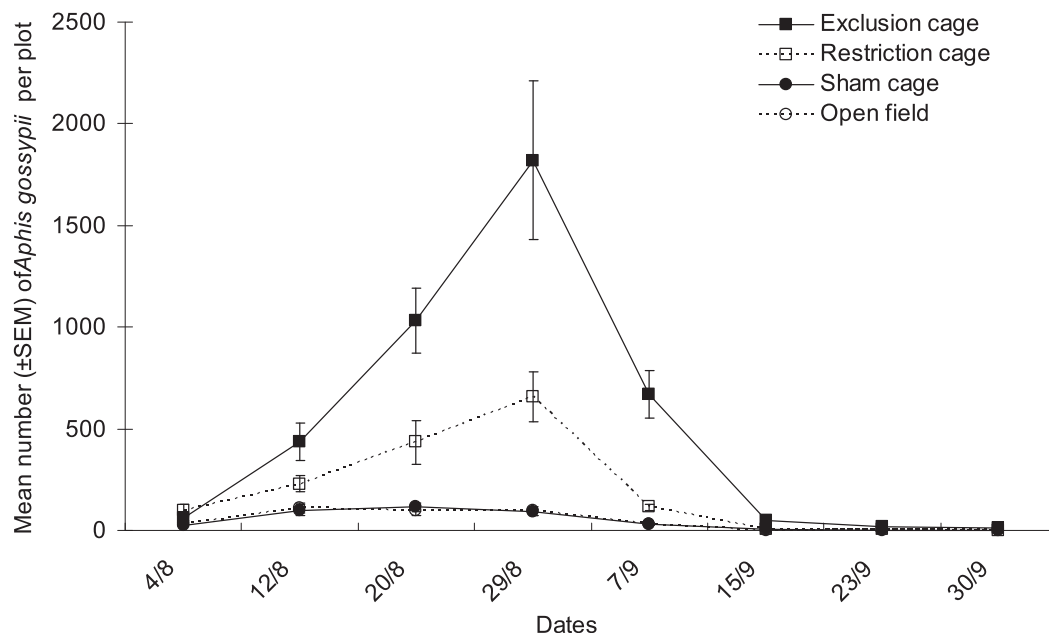
doi:10.1371/journal.pone.0102980.t001

population growth by August 29<sup>th</sup> from the initial aphid count at the July 28<sup>th</sup> release in the exclusion cages, whereas there was only a 10-fold aphid population increase in the sham cages and open field plots. Sham cages and open field plots showed no difference in aphid numbers during the course of this study (Fig. 2).

The numbers of coccinellids recorded also differed significantly among cage types (Fig. 3A, cage type factor:  $\chi^2 = 18.20$ ,  $df = 3$ ,  $P < 0.001$ ) and among dates of sampling (date factor:  $\chi^2 = 19.52$ ,  $df = 7$ ,  $P = 0.007$ ). There was no significant interaction between the two factors ( $\chi^2 = 19.87$ ,  $df = 21$ ,  $P = 0.134$ ). Many more coccinellids were recorded in sham cages and open field plots than in exclusion cages and restriction cages; however no difference in coccinellids was observed between sham cages and open field plots. *Propylaea japonica* was the dominant species

among the Coccinellidae family during the survey (Fig. 3B). The counts for this species followed the same trends as were observed for the coccinellid group as a whole: more *P. japonica* were found in sham cages and open field plots (significant cage type factor:  $\chi^2 = 19.00$ ,  $df = 3$ ,  $P < 0.001$ , and date factor:  $\chi^2 = 19.89$ ,  $df = 7$ ,  $P = 0.006$ , no significant interaction:  $\chi^2 = 23.12$ ,  $df = 21$ ,  $P = 0.333$ ).

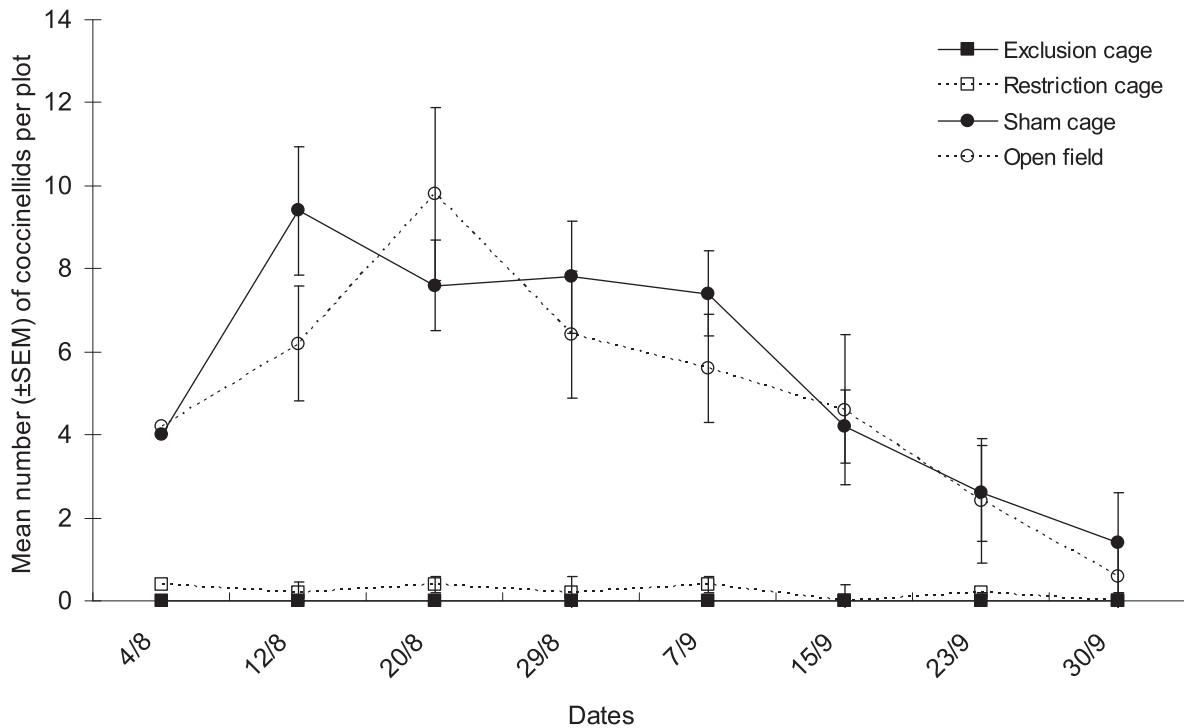
The numbers of Aphidiine mummies differed significantly between cage types (Fig. 4, cage type factor:  $\chi^2 = 8.91$ ,  $df = 3$ ,  $P = 0.031$ ) and dates (date factor:  $\chi^2 = 19.03$ ,  $df = 7$ ,  $P = 0.008$ ), but the two factors did not interact significantly overall ( $\chi^2 = 22.89$ ,  $df = 21$ ,  $P = 0.274$ ). Overall, many more Aphidiine parasitoids were found in restriction cages than in the other three cage treatments on Aug 20<sup>th</sup>, Aug 29<sup>th</sup> and Sept 7<sup>th</sup> (Fig. 4); the



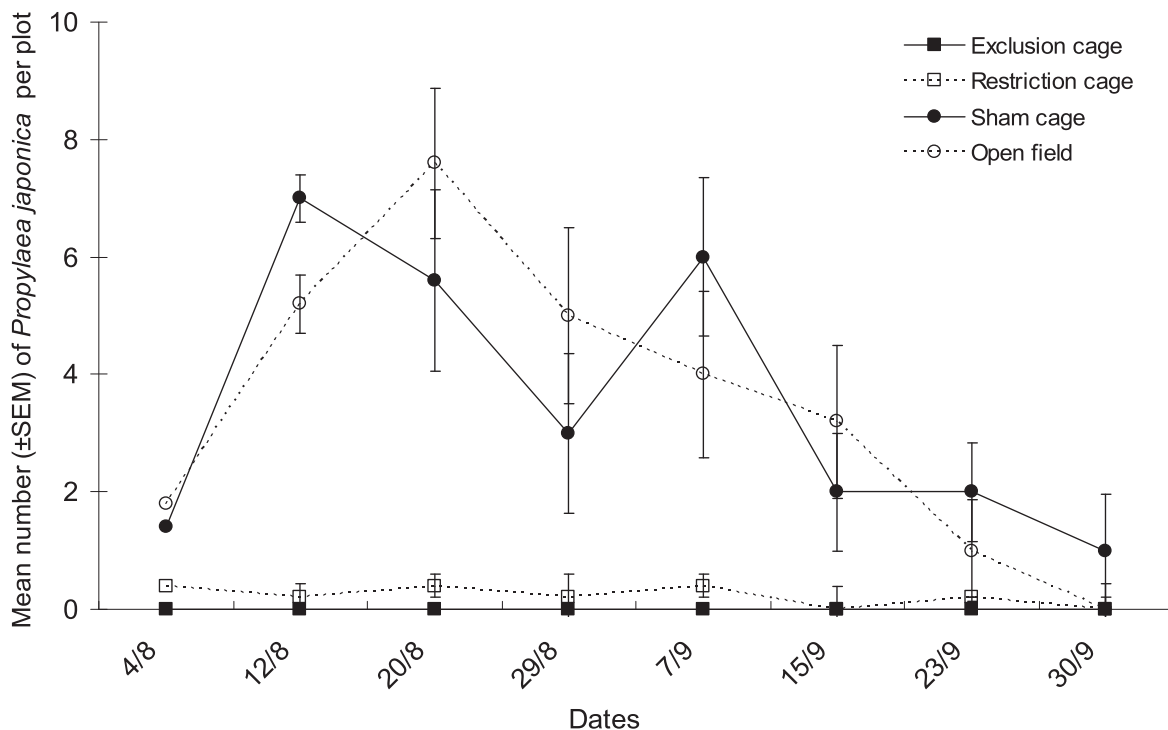
**Figure 2. Cotton aphid population dynamics.** Mean numbers ( $\pm$ SEM) of *A. gossypii* per plot in the various natural enemy exclusion treatments from early August to end of September in Bt cotton in Ezhou (China).

doi:10.1371/journal.pone.0102980.g002

(A)



(B)



**Figure 3. Population dynamics of coccinellid predators.** Mean numbers ( $\pm$ SEM) of (A) all Coccinellids and (B) *P. japonica* per plot in the various natural enemy exclusion treatments from early August to end of September in Bt cotton in Ezhou (China). doi:10.1371/journal.pone.0102980.g003

parasitoid density increased markedly by 30- to 40-fold beginning Aug 12<sup>th</sup> and reached a peak on Aug 29<sup>th</sup>.

The numbers of other natural enemies differed significantly among cage types as well (Fig. 5, cage type factor:  $\chi^2 = 10.16$ ,  $df = 3$ ,  $P = 0.017$ ) and dates (date factor:  $\chi^2 = 18.61$ ,  $df = 7$ ,  $P = 0.046$ ), no significant interaction was observed between the two factors ( $\chi^2 = 25.67$ ,  $df = 21$ ,  $P = 0.458$ ).

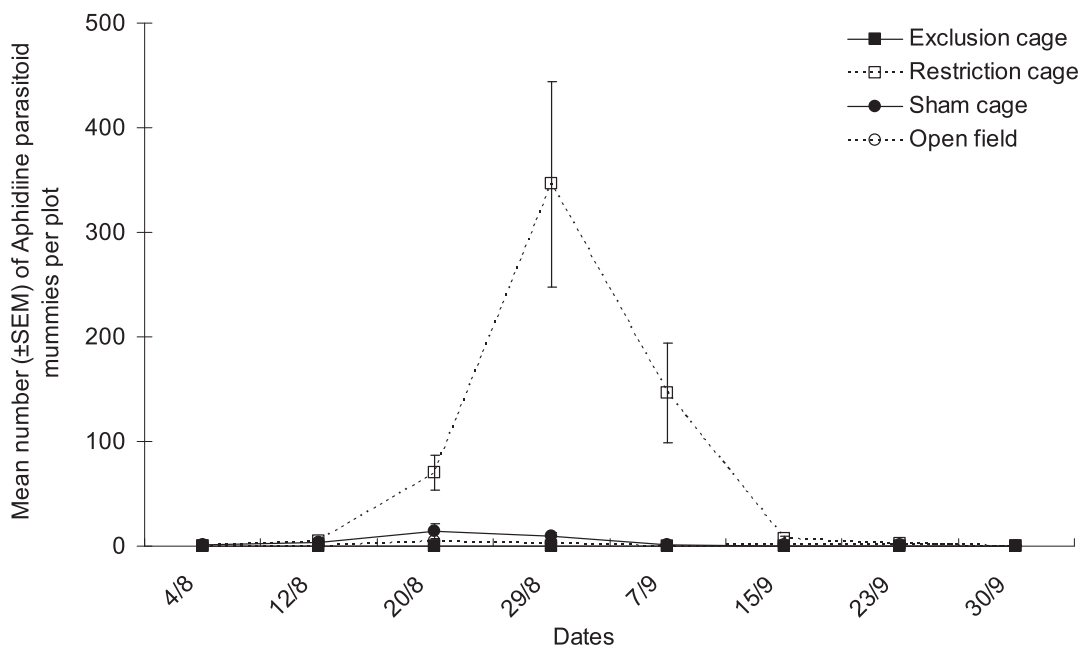
## Discussion

Our study demonstrated the contribution of natural enemies (predators and parasitoids) on cotton aphid population growth in Bt+CpTI cotton field. In the absence of predators and parasitoids resulting from exclusion cages, cotton aphid populations increased up to maximum of 180-fold from aphid density at the initial release date, while in the presence of natural enemies (open field plots or sham cages) aphid populations showed a maximum 10-fold increase. These major differences in aphid population dynamics show the importance of top-down forces on this pest infesting Bt cotton. We identified the coccinellid *P. japonica* and the Aphidiine parasitoids as the predominant natural enemies in the cotton field, with distinct but additive effects on cotton aphid population growth. The best control of aphid populations was obtained when both natural enemy types had access to the aphids in open field plots or sham cages.

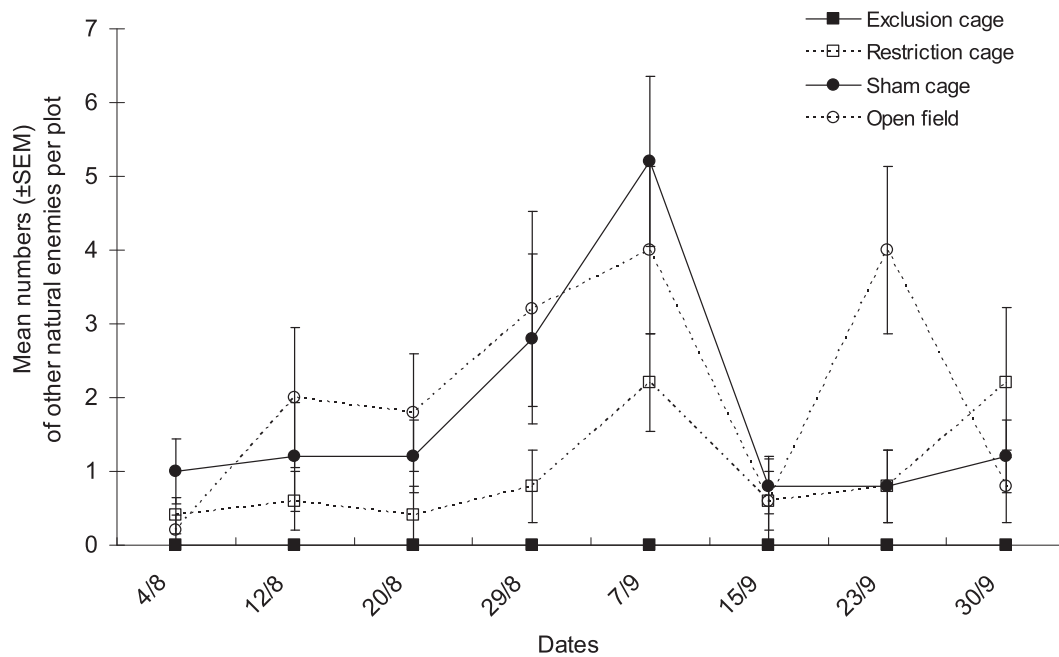
The coccinellid *P. japonica* proved to be an important natural enemy for suppressing cotton aphid population growth in Bt cotton fields in the YRZ in China. *Propylaea japonica* is a well-known predator of *A. gossypii* [32,33] and its life history characteristics and phenology make it a good candidate biocontrol agent for management of the aphid in Bt cotton. This predator colonizes cotton fields early in the cotton seedling stage, at the same time as the aphid population starts infesting the cotton field. Being a generalist predator, it can feed on a variety of prey including spider mites, thrips, white flies and other small species [34,35], including those observed during our study (e.g. whiteflies

and leafhoppers, see Table 1). Alternative prey can help promote establishment of predators early in the season when the targeted pest is scarce (e.g. see [36]). Therefore, *P. japonica* can effectively delay the establishment and subsequent population growth of aphids early in the growing season. Such characteristics often make, generalist predators useful in the strategy of conservation biological control (e.g. see [10,37,38]).

The Aphidiine parasitoids, mainly *L. japonica* and *B. indicus*, were also found to suppress cotton aphid population growth. In the restriction cages, when coccinellid predators did not have access to the aphid populations, the parasitoids reduced aphid peak population by nearly 2/3 (see aphid densities in exclusion cages vs. restriction cages, Fig. 2). However, Aphidiines alone could not totally prevent aphid population growth, as aphid density reached ~600 aphids per plot by Aug 29<sup>th</sup>. In these restriction cages there was a rapid early season aphid population growth because predators known to limit pest population increase early in the season were excluded [10]. However, as aphid density increased in these plots, aphid parasitoid adults were attracted and this resulted in abundant parasitized mummies in the following weeks. When predators were present (in the sham cages and open field plots) the parasitoid populations remained at low densities throughout the season, either because of possible intraguild predation [39,40] of parasitoid mummies by coccinellids (e.g. see [41]), or through resource competition of aphid parasitoids (aphids) with the generalist predators in the plots [42,43]. In this instance, the aphid parasitoids may help reduce aphid densities primarily when aphid populations have already reached a certain density. Previous surveys of natural enemies of cotton aphid carried out in different regions of China produced variable collections of species records. Sun *et al.* [34] reported that the predator guild in cotton fields near Beijing (Xibeiwang) was dominated by *Chrysoperla sinica* Tjeder, *P. japonica*, various spiders and *Orius minutus* L. The same authors also reported that *Lysiphlebia japonica* was the dominant aphid parasitoid; the parasitoid guild in Xibeiwang region may be similar to the one



**Figure 4. Population dynamics of aphid parasitoids.** Mean numbers ( $\pm$ SEM) of Aphidiine per plot in the various natural enemy exclusion treatments from early August to end of September in Bt cotton in Ezhou (China). doi:10.1371/journal.pone.0102980.g004



**Figure 5. Population dynamics of other natural enemies.** Mean numbers ( $\pm$ SEM) of other natural enemies per plot in the various natural enemy exclusion treatments from early August to end of September in Bt cotton in Ezhou (China). doi:10.1371/journal.pone.0102980.g005

recorded in YRZ. Zhou *et al.* [44] reported similar findings to ours as *L. japonica* and *P. japonica* were dominant in Hebei province. However, in contrast to our results, they reported very low biodiversity in coccinellid species (we found that *H. axyridis* and *C. septempunctata* were well represented in our plots) and that *C. sinica* and heteropteran predators (mainly *O. minutus* and the mirid predator *Campylomma diversicornis* Reuter) were quite abundant (as much as coccinellids). In the Xinjiang region, Xu *et al.* [45] conducted surveys on predators and found them, in order of importance in terms of density, coccinellids>spiders>lacewings>heteropteran predators. These contrasting results among geographic regions highlight the need to identify the specific natural enemies at play in a given region when developing conservation biological control programs.

When examining the aphid parasitoid group, it is worth mentioning that Aphelinid parasitoids were nearly absent from the field (as reported in other agro-ecosystems, e.g. in *Brassicae* crops [46,47]). When considering Aphidiine parasitoids, *L. japonica* proved to be a key natural enemy of cotton aphid in Northern China [44,48]. This species is also a natural enemy of phylogenetically closely related aphid species [49] e.g. the soybean aphid *Aphis glycines* Matsumura in Japan and Indonesia [50] and the brown citrus aphid *Toxoptera citricida* Kirkaldy [51,52]. Several species from the *Binodoxys* genus are known to efficiently attack *A. gossypii* [31,53,54] and *B. indicus* may be an important natural enemy of this aphid pest in the YRZ region as well as other regions not extensively surveyed.

Ecological compatibility of GM crops and natural enemies is a key issue for implementing biological control programs within GM cropping systems [55–57]. Previous studies suggested that Bt+CpTI cotton might not affect population dynamics of natural enemies [34,45]. No effect was observed on the fitness of *P. japonica* when fed with *A. gossypii* on Bt cotton ([33,58], but see [59]). In addition, aphid parasitoids may not be exposed to Bt toxins [7,8]. However, they can be negatively affected by Trypsin Inhibitors [60] e.g. CpTI. Zhou *et al.* [44] reported a 44%

decrease in *L. japonica* population density in Bt-CpTI cotton fields. Although we did not carry out a formal comparison between non Bt and Bt cotton cultivar, we highlighted a strong top-down effect on cotton aphid populations. Therefore natural enemies, as a whole group, are effective in limiting aphid population growth in Bt-CpTI cotton fields.

Our study demonstrated the importance of the top-down force exerted by natural enemies, mainly coccinellids and Aphidiine parasitoids, on cotton aphid in Bt cotton field in China. However, the relative strength of top-down vs. bottom-up forces on *A. gossypii* still needs to be studied in order to develop IPM including such forces in a sustainable and comprehensive way, especially since various studies have already identified the importance of bottom-up forces (e.g. fertilization regime) on herbivore population dynamics [61–64]. Developing such optimized IPM would help manage secondary pests that may show population outbreaks in Bt cotton since its wide spread adoption in China. For example, *S. litura* larvae were found in relatively high density during our surveys and this species can cause considerable damage to cotton crops. This finding is consistent with the reported low susceptibility of this pest species to current Bt cotton cultivars [3]. Secondary pests may promote applications of insecticides in Bt cotton with potential associated multiple negative effects on human health and non-target organisms [65–67]. Highly selective chemical pesticides may be required at times [68,69] but limiting the application of pesticides and promoting more sustainable pest management strategies should be prioritized. For example, optimized IPM may aim at combining biocontrol agents as top-down force [70,71] with bottom-up forces like fertilization regimes and/or cultural practices [13,72–75] for efficient management of pests. In addition, the sustainable use of GM crops can lead to drastic reduction in pesticide usage at the wide scale [2]; developing optimized IPM in Bt crops such Bt cotton would help capitalize on the benefits provided by transgenic methods in cropping systems.

## Acknowledgments

We thank Professor Kongming Wu (CAAS) and Tim Oppenheim for comments on the manuscript and Jintao Wang, Zhenzhong Chen, Jian Zhu, Xuejian Huang for support during the field experiments.

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## Author Contributions

Conceived and designed the experiments: PH CYN ND. Performed the experiments: PH. Analyzed the data: PH ND. Contributed reagents/materials/analysis tools: CYN. Wrote the paper: PH CYN ND.

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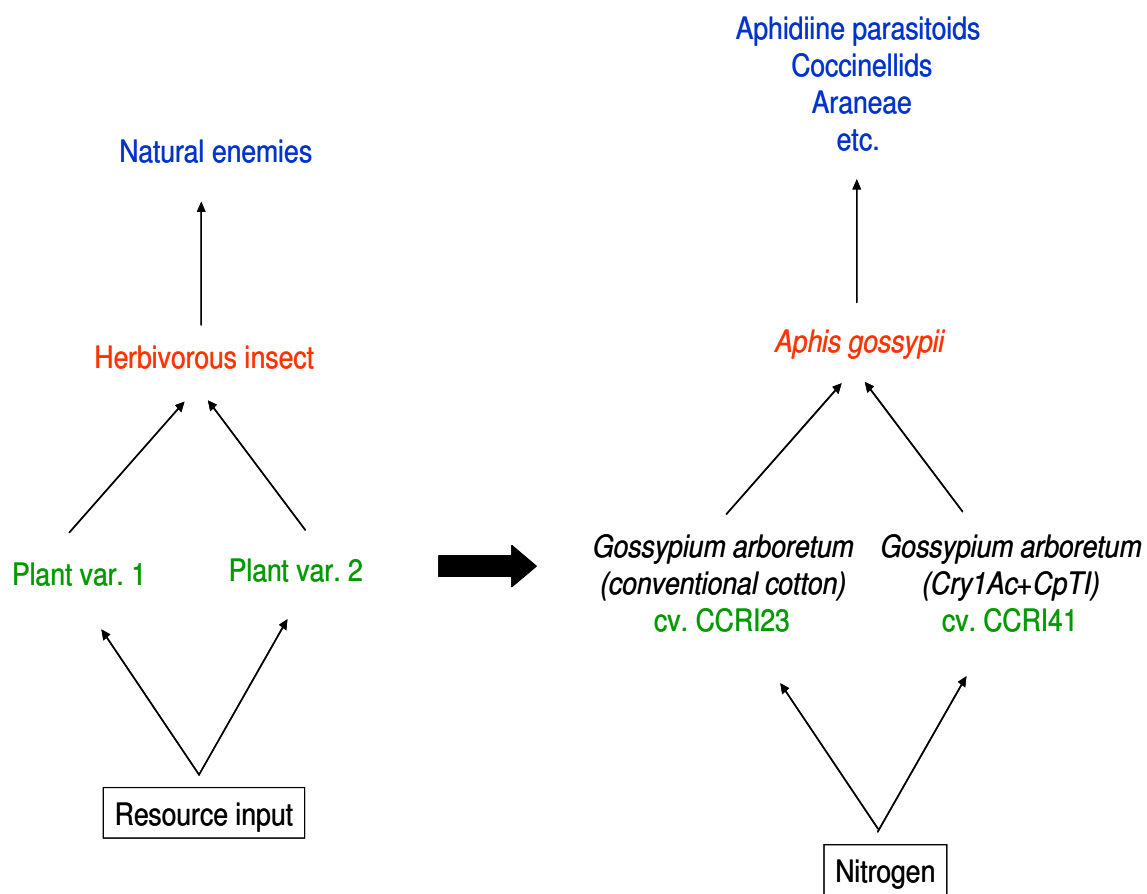
### III) Bottom-up and top-down effects regulating cotton aphid population in field

Our previous study (**Article 8**) demonstrated the importance of the top-down forces exerted by natural enemies, mainly the predator coccinellids and aphid parasitoids, on cotton aphid *A. gossypii* in Bt cotton field in China. However, the relative strength of top-down vs. bottom-up forces on *A. gossypii* still needs to be studied in order to develop IPM including such forces in a sustainable and comprehensive way, especially since various studies have already identified the importance of bottom-up forces (e.g., fertilization regime) on herbivore population dynamics (Stiling and Rossi 1997; Denno et al. 2002; Huberty and Denno 2004; Ai et al. 2011). Nitrogen fertilization and water have been showed to have strong bottom-up effects on arthropod communities (Chen et al. 2010; Gutbrodt et al. 2011).

Moreover, plant genotype has been proved an important ecological factor shaping tri-trophic communities (Underwood and Rausher 2000; Johnson 2008; Grinnan et al. 2013). The plant resistance against a given group of herbivorous pests (e.g., transgenic *Bt* insecticidal protein) in plants is another potential factor influencing the complex interaction between plants and arthropod communities.

Thus, in article 9, the study model was present as bellow (**Fig. 10**). The study in article 9 is a companion study with that reported in article 8. To manipulate top-down forces, various exclusion cages were employed to manipulate the entries of various types of natural enemies (i.e., allowing entries of predators, parasitoids or both). Two types of bottom-up forces were manipulated in the field experimental design: plant cultivar (Bt cotton and conventional cotton), nitrogen fertilization (no input, low input and high input).

**Figure 11:** The study model of article 8. The left diagram shows the general model, the right shows the biological model used in this study



## Article 8

### **Combined bottom-up and top-down forces regulating the cotton aphid in conventional versus Bt cotton in central China**

Peng Han<sup>1</sup>, Yong-sheng Yao<sup>2,3</sup>, Chang-ying Niu<sup>2</sup>, Xi-wu Gao<sup>3</sup>, Nicolas Desneux<sup>1</sup>

<sup>1</sup> French National Institute for Agricultural Research (INRA), UMR1355, 400 Route des Chappes, 06903, Sophia-Antipolis, France

<sup>2</sup> Hubei Insect Resources Utilization and Sustainable Pest Management Key Laboratory, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, 430070, China

<sup>3</sup> Department of Entomology, China Agricultural University, Beijing, 100193, China

**Abstract:** In natural and agricultural ecosystems, bottom-up and top-down forces shape arthropod communities. Although various studies have documented the strength of these forces in many agro-ecosystems, very few have described their relative strength in regulating agricultural pest populations. This is notably true when considering transgenic crops that do not target secondary pests, e.g. *Bacillus thuringiensis* (*Bt*) toxins from *Bt* crops do not target aphid pests, despite that primary and secondary of cotton plant metabolisms may be affected by introduction of *Bt* gene. We investigated the relative strength of top-down and bottom-up forces in regulating cotton aphid, *Aphis gossypii* Glover, in conventional and *Bt* cotton fields in central China. We manipulated the bottom-up forces by contrasting the fertilization regimes (no, low or high nitrogen) and the top-down forces by selectively restricting activity of aphid natural enemies (using exclusion cages). We observed strong evidence for top-down control of cotton aphid population growth; coccinellids, spiders and Aphidiine parasitoids were abundant during the survey. Bottom-up effect through fertilization regime was also observed on the pest; reduced nitrogen inputs supported smaller aphid populations. Similar effect was observed also, in some extent, on natural enemies in conventional but *Bt* cotton; reduced nitrogen inputs in conventional cotton supported smaller populations of aphid natural enemies (notably specific ones like Aphidiines). In overall, aphid population growth was impaired through a combined effect of top-down and bottom-up forces (through nitrogen fertilization); manipulating nitrogen fertilization regimes to support smaller cotton aphid populations while not compromising biocontrol services provided by natural enemies may help managing this secondary pest in the context of the high prevalence of *Bt* cotton in China.

**Key words:** transgenic cotton, cotton aphid, biological control, nitrogen, fertilization

## Introduction

In terrestrial ecosystems, the strength of bottom-up vs. top-down forces in structuring arthropods communities has received increasing attention during the two past decades (Dyer and Letourneau 1999; Denno et al. 2003; Gruner et al. 2004; Costamagna and Landis 2006; Cornelissen and Stiling 2009; Asiimwe et al. 2013; Rzanny et al. 2013; Zaugg et al. 2013). Top-down force is characterized by the mortality of herbivorous arthropods (second trophic level) owing to the assemblage of consumers from the third trophic level i.e. natural enemies (Liu et al. 2004; Costamagna and Landis 2006, 2007; Han et al. 2014b). Herbivorous arthropod communities are also structured through bottom-up forces e.g. fertility regimes and host plant resistances (Walker and Jones 2001; Denno et al. 2002; Johnson 2008; Chen and Ruberson 2008; Ai et al. 2009; Han et al. 2014a). However, few studies have documented simultaneous manipulations of both forces in agro-ecosystems (Dyer and Stireman 2003; Costamagna et al. 2006). Characterizing the relative strength of top-down and bottom-up forces in agro-ecosystems may help optimizing pest management practices and ultimately prompt development of more sustainable integrated pest management (IPM) programs. This is notably true when considering the increasing use of transgenic *Bt* crops worldwide; *Bt* toxins do not target secondary pests like aphids. Bottom-up effects on aphids owing to varying fertilizing regimes may show different trends according to cultivars used, i.e. *Bt* vs. non *Bt*, because nitrogen-related primary and secondary of plant metabolisms can be affected by introduction of *Bt* gene(s) (e.g. in cotton, Chen et al. 2005; Ma et al. 2014), with potential effects on pests (Ma et al. 2014). Such effects on metabolisms might also affect top-down

forces if they result in trophic cascades on natural enemies.

The Yangtze River Valley Cotton-planting Zone (YRZ) is one of the largest cotton-planting regions (Wan et al 2008) where several insect-resistant transgenic cultivars (Han et al 2010; 2014b). The cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae) is considered a secondary pest in YRZ and is not targeted by the *Bt* cotton (as is the case with other aphids, e.g. see Ramirez-Romero et al. 2008; Romeis and Meissle 2011). Although cotton aphid populations have shown continuous decline in seasonal density in cotton fields in the past 15 years in China (Lu et al. 2012), cotton aphid outbreaks may occur and reach economically damaging levels (Wu and Guo 2005) owing to particular weather conditions (e.g. less rainfall during the aphid population-growth season) or pesticide resistance (Yi et al 2012). We documented significant impacts of natural enemies on cotton aphid in YRZ (Han et al 2014b); such top-down forces involved generalist predators, mainly coccinellids, and specialist natural enemies with mainly Aphidiine parasitoids (hereafter referred to “parastioids”). In cotton cropping systems, bottom-up effects of nitrogen fertilization regime have been identified on cotton aphid population growth (Chen et al 2008; Ai et al. 2011), as well as on other arthropods (Bi et al 2001, 2003; Chen et al. 2008; Ma et al. 2014). No study documented combined manipulation of both top-down forces and fertilization regimes in *Bt* cotton yet.

In cotton agro-system, we manipulated (i) bottom-up forces by varying nitrogen fertilization applied to cotton; we used three contrasted levels of urea-based fertilizers (high, low and no nitrogen input), and (ii) top-down forces by selectively restricting activity of aphid natural enemies (using exclusion cages) (Costamagna and Landis 2006, 2007; Han et al

2014b). We assessed such effects in conventional cotton as well as in *Bt* cotton, the transgenic Cry1Ac+CpTI cotton and the its non-transgenic near-isoline were used. This transgenic cotton that combines the two genes Cry1Ac (*Bt* endotoxin) and CpTI (Cowpea Trypsin Inhibitor) has been the most adopted *Bt* cotton cultivar in the YRZ. We aimed to (i) estimate the strength of the bottom-up and top-down forces on cotton aphid population dynamics, and (ii) assess how nitrogen fertilization may differentially impact natural enemies of cotton aphid in *Bt* and conventional cotton.

## **Material and Methods**

### *Cotton varieties*

Two cotton cultivars were used for the study. One variety “CCRI41” expressed the two genes *Cry1Ac* (*Bt* endotoxin) and *CpTI* (Cowpea Trypsin Inhibitor) (named “*Bt* cotton” thereafter). The second variety “CCRI23” is the parent variety of “CCRI41” (named “conventional cotton” thereafter). Both varieties were extensively used as model cultivars in previous studies (Han et al 2010, 2012, 2014b).

### *Aphid colony*

Naturally occurring cotton aphids were collected in May 2013 from a cotton field at the Experimental Station of Huazhong Agricultural University (HAU), Wuhan, China. Aphids were used to establish a colony under laboratory conditions ( $25 \pm 2^{\circ}\text{C}$ , RH  $70 \pm 10\%$ , 16 h

light). This colony was used as the source for artificial infestations of cotton plants in the field study.

#### *Field experiment setup*

The field experiment was conducted at the Experimental Station of HAU. This field was previously cultivated free of insecticide application and did not receive nitrogen fertilizer for three years prior our study. Bt cotton and conventional cotton seedlings were planted in a 1.43-ha field on May 12<sup>th</sup>. The two blocks (i.e. one block for each cultivar) had a distance of 50 meters (see Fig. 1B). On May 20<sup>th</sup>, the foliar fertilization was uniformly applied using urea and monopotassium phosphate (active gradient with 750 g/ ha). On June 12<sup>th</sup>, three levels of urea nitrogen treatments were applied to cotton seedlings: high nitrogen with 150 kg/ha, medium nitrogen with 75 kg/ha and low nitrogen with 0 kg/ha.

The cage restriction was designed following the methods described in the previous studies (Costamagna and Landis 2006; Costamagna et al. 2007; Han et al. 2014b). Four different treatments, i.e. different degree of predator exclusion, were applied using restriction cages with various enter restrictions (see Fig. 1A): (i) exclusion cages (mesh with openings of 530×530μm) in which aphids were fully-protected from all natural enemies (predators and parasitoids). (ii) restriction cages (openings of 3×3mm) in which aphids were partially protected. This size of openings prevented entry by large predators i.e. coccinellids, but allowed small predators to enter (Fox et al. 2005; Han et al 2014b). (iii) sham cages that were built with the mesh of openings 530×530μm but included a 40-cm high opening in the middle and the bottom respectively. This treatment was set up to assess the possible disruptive effect



of cage (e.g. mesh, bamboo sticks, etc.) on the activity of natural enemies and aphid population growth. (iv) no cage (i.e. open field), i.e. fully opened area (named “open field” thereafter) which used four bamboo sticks standing upright into the ground and a tape surrounding them as a cue for sampling range. To avoid the effects of ground-dwelling predators, a plastic barrier (around 15cm buried in the soil) were connected to the nettings of exclusion cages and restriction cages (after Costamagna et al. 2007).

The four treatments were established between June 18<sup>th</sup> and June 20<sup>th</sup> using a completely randomized block design (Fig. 1B). For either Bt cotton or conventional cotton field, the distance of 10 meters were set among the area treated with three levels of urea nitrogen. For each nitrogen treatment, four blocks (replicates) were done with a distance of 10 meters. Within each block, one of each cage treatment type (plot) was included. Four plants were covered within each cage type (2×2×2 m in length × width × height) (Han et al. 2014b). The plants were cultivated with a distance of 1m among each other. One side of each cage (except for “sham” cages and “open field”) was sewed with a zipper to allow observations on plants.

#### *Arthropod population dynamics*

Prior to artificial infestation of aphids, any resident aphids and other insects were removed by hands and aspirators for whole plots. On June 21<sup>th</sup> and 22<sup>nd</sup>, twenty aphids were artificially released onto one randomly chosen leaf from each plant. From June 28<sup>th</sup> to August 25<sup>th</sup>, aphids and their natural enemies within each plot were recorded. All the recorded species were identified to family or species level depending on arthropod species. In the case of parasitoids, the un-merged parasitoid mummies, i.e. pupae stage of the parasitoid, were

counted (with black- and tan-colored mummies belonging to the Aphelinidae and Aphidiinae parasitoid families, respectively). The field survey was conducted every 5-7 days from noon to 6 pm for each date of survey. Mummy samples were collected for further identification of parasitoids following the identification keys previously reported (Stray 1966; 1979; Stary and Schlinger 1967; Desneux et al. 2009b).

### *Data analyses*

We tested the effect of cotton cultivar (factor “cultivar”; two levels: Bt cotton and conventional cotton), various exclusion cage treatments (factor “cage type”; four levels: exclusion cage, restriction cage, sham cage and open field), nitrogen treatments (factor “nitrogen”; three levels: high, low and no nitrogen input) as well as the dates (factor “date”; 12 sampling dates) on the density of cotton aphid and main natural enemies. The datasets were analyzed using a generalized linear model based on a Poisson distribution and a log-link function (Proc Genmod in the SAS statistical package, SAS Institute, NC, USA).

## **Results**

Cotton aphid densities differed significantly as function of the sampling dates (Fig. 2; factor “date”:  $\chi^2 = 92.89$ ,  $df = 11$ ,  $P < 0.001$ ) and among cage types (factor “cage type”:  $\chi^2 = 90.19$ ,  $df = 3$ ,  $P < 0.001$ ). Nitrogen input manipulation also significantly influenced *A. gossypii* densities (factor “nitrogen”:  $\chi^2 = 54.71$ ,  $df = 2$ ,  $P < 0.001$ ), and their densities decreased with

the decrease in nitrogen input. Such effect was found on both Bt and conventional cotton since no significant interaction effect was found (“cultivar x nitrogen”:  $\chi^2 = 1.64$ ,  $df = 2$ ,  $P = 0.441$ ). The effect of cage type on *A. gossypii* densities depended on nitrogen input level (“cage type x nitrogen”:  $\chi^2 = 39.21$ ,  $df = 6$ ,  $P < 0.001$ ) as the extent of population growth sheltered by exclusion cage decreased with lowered nitrogen input (e.g. “exclusion cage” on conventional cotton: 56-, 40- and 22- fold growth in *A. gossypii* densities for high, low and no-nitrogen input respectively). Furthermore, a significant interaction effect (“cultivar x cage type”:  $\chi^2 = 13.10$ ,  $df = 3$ ,  $P = 0.004$ ) was also found since the extent of population growth sheltered by both exclusion cage and restriction cage differed between cotton cultivars (e.g. conventional cotton vs. Bt cotton with high nitrogen input: exclusion cage and restriction cage induced 56- and 30- fold growth in *A. gossypii* densities on conventional cotton, whereas 43- and 20- fold were observed on Bt cotton).

The densities of coccinellids differed significantly as function of the sampling dates (Fig. 3; factor “date”:  $\chi^2 = 45.88$ ,  $df = 11$ ,  $P < 0.001$ ) and among cage types (factor “cage type”:  $\chi^2 = 91.30$ ,  $df = 3$ ,  $P < 0.001$ ). Nitrogen input manipulation also significantly influenced coccinellids densities (factor “nitrogen”:  $\chi^2 = 20.17$ ,  $df = 2$ ,  $P < 0.001$ ), and their densities decreased with the decrease in nitrogen input. Such effect was found on both Bt and conventional cotton (no significant interaction “cultivar x nitrogen”:  $\chi^2 = 0.10$ ,  $df = 2$ ,  $P = 0.953$ ). The effect of cage type on coccinellids densities was similar on both cotton cultivars (“cultivar x cage type”:  $\chi^2 = 1.74$ ,  $df = 3$ ,  $P = 0.627$ ). However, such effect was dependent on nitrogen input levels (“cage type x nitrogen”:  $\chi^2 = 17.69$ ,  $df = 6$ ,  $P = 0.007$ ).

The densities of predatory spiders recorded differed significantly as function of the

sampling dates (Fig. 4; factor “date”:  $\chi^2 = 42.59$ ,  $df = 11$ ,  $P < 0.001$ ) and among cage types (factor “cage type”:  $\chi^2 = 81.91$ ,  $df = 3$ ,  $P < 0.001$ ). Nitrogen input manipulation also significantly influenced spider densities (factor “nitrogen”:  $\chi^2 = 8.42$ ,  $df = 2$ ,  $P < 0.015$ ), and their densities decreased with the decrease in nitrogen input. Such effect was found on both Bt and conventional cotton since no significant interaction effect was found (“cultivar x nitrogen”:  $\chi^2 = 0.59$ ,  $df = 2$ ,  $P = 0.746$ ). The effect of cage type on spider densities was similar on both cotton cultivars (“cultivar x cage type”:  $\chi^2 = 2.38$ ,  $df = 3$ ,  $P = 0.498$ ). Also, such effect was similar on all the nitrogen input levels (“cage type x nitrogen”:  $\chi^2 = 8.23$ ,  $df = 6$ ,  $P = 0.221$ ).

Aphidiines (tan-colored mummies) were most commonly observed; Aphelinidae mummies were barely found (six in total) during the whole study. The densities of parasitoids (mummies) differed significantly as function of the sampling dates (Fig. 5; factor “date”:  $\chi^2 = 59.99$ ,  $df = 11$ ,  $P < 0.001$ ), among the cage types (factor “cage type”:  $\chi^2 = 40.24$ ,  $df = 3$ ,  $P < 0.001$ ), among the nitrogen input levels (factor “nitrogen”:  $\chi^2 = 28.45$ ,  $df = 2$ ,  $P < 0.001$ ), as well as between the cultivars (“cultivar”:  $\chi^2 = 54.49$ ,  $df = 1$ ,  $P < 0.001$ ). The effect of nitrogen input on densities of the parasitoids depended on cotton cultivar type (“cultivar x nitrogen”:  $\chi^2 = 16.78$ ,  $df = 2$ ,  $P < 0.001$ ) since the decreased tendency in densities of parasitoids in restriction cages due to the decrease in nitrogen input was only found on conventional cotton (e.g. conventional cotton: around 280, 200 and 120 mummies recorded for high, low and no input, whereas in Bt cotton: around 120, 100 and 80 mummies recorded for high, low and no input respectively). The effect of cage manipulations on the densities of these mummies interacted strongly with cotton cultivar (“cultivar x cage type”:  $\chi^2 = 16.78$ ,  $df = 2$ ,  $P < 0.001$ )

as the extent of population growth sheltered by restriction cages decreased differed significantly between the two cultivars (i.e. higher growth of mummies in conventional cotton compared to Bt cotton). Furthermore, the effect of cage manipulations on the densities of these mummies interacted strongly with nitrogen inputs (“nitrogen x cage type”:  $\chi^2 = 18.21$ ,  $df = 2$ ,  $P < 0.006$ ) as the number increase in mummy densities in restriction cages compared to other treatments significantly decreased with the lowered nitrogen input.

The densities of other natural enemies recorded differed significantly as function of the sampling dates (Fig. 6; factor “date”:  $\chi^2 = 44.60$ ,  $df = 11$ ,  $P < 0.001$ ) and among cage types (factor “cage type”:  $\chi^2 = 83.82$ ,  $df = 3$ ,  $P < 0.001$ ). Nitrogen input manipulation also significantly influenced the abundance of these natural enemies (factor “nitrogen”:  $\chi^2 = 11.58$ ,  $df = 2$ ,  $P < 0.003$ ), and their densities decreased with the decrease in nitrogen input. Such effect was found on both Bt and conventional cotton since no significant interaction effect was found (“cultivar x nitrogen”:  $\chi^2 = 1.76$ ,  $df = 2$ ,  $P = 0.415$ ). The effect of cage type on spider densities was similar on both cotton cultivars (“cultivar x cage type”:  $\chi^2 = 0.45$ ,  $df = 3$ ,  $P = 0.930$ ). Also, such effect was similar on all the nitrogen input levels (“cage type x nitrogen”:  $\chi^2 = 11.83$ ,  $df = 6$ ,  $P = 0.066$ ).

## Discussion

Our study provided evidences of combined bottom-up (through various levels of nitrogen inputs) and top-down regulations (biocontrol services provided by predators and parasitoids)

on *A. gossypii* population in both conventional and Bt cotton. Firstly, *A. gossypii* population growth was strongly suppressed by the natural enemy guild (mainly coccinellids, spiders and parasitoids). However, their populations were positively influenced by increased nitrogen input, and this pattern was similar in both Bt and conventional cotton. Secondly, we found strong positive bottom-up effects of nitrogen input on population dynamics of members of the natural enemy guild surveyed, except that the positive effect of increased nitrogen on parasitoids was only found in conventional cotton.

Cotton aphid population was strongly affected by generalist predators and parasitoids: (i) *Aphis gossypii* population increased significantly with the exclusion of natural enemies, (ii) cotton aphid population remained at low levels in the open field during the cotton growing season where a relatively high number of natural enemy guild (i.e. coccinellids and predatory spiders etc.) were found. Apparently, generalist predators responded numerically to the seasonal population dynamic of *A. gossypii*. The roles of these top-down forces in regulating aphid populations were consistent with those reported in our previous study (Han et al. 2014b), and also agreed with the findings on other aphid species in other cropping systems (Liu et al. 2004; Costamagna and Landis 2006; Costamagna et al. 2007). Moreover, high densities of the parasitoids, mainly *L. japonica* and *B. indicus*, were found in the restriction cages where all the natural enemies were excluded but parasitoids. The parasitoids reduced aphid population by nearly 50% from all the plots (restriction cages vs. exclusion cages, Fig. 2). However, the parasitoids alone were not able to control the aphid population at relatively low levels (restriction cages vs. open field, Fig. 2). A combined impact of both the generalist predators and specialist parasitoids on aphid population was expected, even though the

intraguild predation pressure on the parasitoids mummies was likely to occur in the open field (e.g. see Chacón and Heimpel 2010).

Cotton aphid population was strongly influenced by variations in nitrogen input, but not cotton cultivar (Bt vs. conventional). Higher nitrogen inputs supported higher *A. gossypii* populations (i.e. total numbers of aphids and peak population density) when natural enemy was partially or completely excluded in field plots (Fig 2. exclusion and restriction cages). We assumed that plants fertilized with low or no nitrogen input may induce nitrogen deficiency in plants and had lower suitability for phloem-sucking insects like aphids. The positive correlation between *A. gossypii* population and nitrogen fertilization levels has been reported in several previous studies (Slosser et al. 1997; Cisneros and Godfrey 2001; Chen and Ruberson 2008), and such positive correlation was also evident in other sap-deeding herbivores, such as leafhoppers and whiteflies, as well as plant-feeding Hemipteran species such as Miridae bugs (Jauset et al. 1998; Bentz et al. 1995 a, b; Bi et al 2001; 2003; Inbar et al 2001; Chau et al. 2005). Furthermore, the pattern of *A. gossypii* population dynamics as well as the peak density did not differ between Bt and conventional cotton plots even though Bt cotton may produce higher levels of free amino acids under the higher nitrogen fertilization (Chen et al. 2005). In instance, our results are in concordance with previous studies showing no evidence that *Bt* toxin itself is inducing any direct effect on non-target pest species population dynamics (Head et al. 2005; Naranjo 2005; Whitehouse et al. 2005; Naranjo 2011).

Parasitoid population was affected by variation in nitrogen, but such effect was only found in conventional cotton. Higher nitrogen input induced higher densities of parasitoids in conventional cotton (Fig. 5). Such effect may result from the fact that the parasitoids have

high requirements for quality hosts (Chau and Mackauer, 2000; Ode and Heinz 2002). Host developing on plants suffering nitrogen deficiency may show lower suitability for parasitoid development. Host aphids showing sub-optimal physiological suitability for parasitoid development are usually less selected for oviposition (Desneux et al. 2009a). In our case, parasitoids may have preferred high-quality hosts, i.e. cotton aphids, developing on the plants treated with higher nitrogen. However, such effect of nitrogen on the parasitoid population was not found in Bt cotton (Fig. 5).

Irrespective of nitrogen inputs, parasitoids showed higher population levels in Bt cotton than in conventional cotton plots (conventional vs. Bt cotton, Fig. 5) even though similar population densities of host aphid was observed between the two cultivars (conventional vs. Bt cotton, Fig. 2). Varying nitrogen input may affect plant quality in a way that affects aphid ability to prevent parasitism; this may differ between conventional and Bt cotton plants because nitrogen-related primary and secondary of plant metabolisms can be affected by introduction of *Bt* gene(s) (e.g. in cotton, Chen et al. 2005; Ma et al. 2014). In addition, bacterial symbionts inside aphids can markedly reduce the parasitism rate by parasitoids (Oliver et al. 2005). However, the impact of host plant quality on aphid endosymbionts is unknown.

Population dynamics of predators coccinellids and spiders as well as other natural enemies were significantly affected by the variation in nitrogen fertilization, but did not vary between cultivars (Bt vs. conventional) (Fig. 3, 4, 6). The lack of effect of *Bt* on the generalist predators matched with previous results on risk assessment of *Bt* crops on generalist natural enemies (see Romeis et al. 2006). Interaction between “cotton cultivar” and “nitrogen



fertilization” was only found on coccinellids. Decreased nitrogen input supported lower coccinellid densities in conventional cotton plots but not in *Bt* cotton ones (Fig. 3). Coccinellids rely primarily on aphids (Wu and Guo 2005) and we assumed that the variation in nitrogen input may trigger trophic cascades via altered nutritional quality of aphids (Chen et al. 2010).

In addition, the population of species from “other natural enemies” mainly including Syrphidae, Chrysopa (lacewings), Hemiptera (Miridae, Nabidae and Anthocoridae) did not vary between cotton cultivars (*Bt* vs. conventional) (Fig. 6). Some of species from “the other natural enemies” are omnivorous predators, e.g. Hemiptera sub order. Even though the plant feeding by omnivorous Hemiptera predators occurs by inserting their stylets randomly into plant tissues and sucking liquid content and liquefied materials by enzymatic degradation in salivary (Cohen et al. 1995, 1998), the Cry proteins was not detectable in bodies of this type of arthropods (Torres and Ruberson 2006). Hence, the impact of Cry protein presence in *Bt* cotton on these predators was negligible (Fig. 6).

In conclusion, our data demonstrated compatibility of fertilization (i.e. nitrogen)-based bottom-up forces and top-down regulation by natural enemies in regulating cotton aphid in *Bt* cotton. Besides the evident top-down effects from natural enemies observed from our current and past studies (Han et al. 2014b), *A. gossypii* population was strongly affected by reduced nitrogen inputs. Moreover, the similar impact of nitrogen was also found on all the natural enemies. However, lower nitrogen input supported lower abundance of two key natural enemy guild predatory coccinellids and parasitoids in conventional cotton, but this effect was not found in *Bt* cotton. Thus, in *Bt* cotton crops, lower nitrogen fertilization regimes may

support lower *A. gossypii* populations, but not compromise top-down regulation since its key natural enemy guild were not affected; manipulating nitrogen fertilization regimes to support smaller cotton aphid populations while not compromising biocontrol services provided by natural enemies may help managing this secondary pest in the context of the high prevalence of *Bt* cotton in China.

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### Figure legends:

**Figure 1:** (A) Design of the four different treatments, i.e. natural enemy exclusion degree, in the field study; Exclusion cages (no arthropod can enter into the cage), Restriction cage (parasitoid adults can enter into the cage), Sham cage and No cage (i.e. open field) (free access of all the arthropods); (B) Within- and among block design: the distance among treatments within a block was 4m, and among blocks was 10m. A distance of 10m was set up among blocks that treated with various levels of nitrogen. A distance of 50m was set up between the block either cultivated with Bt or conventional cotton.

**Figure 2:** Mean numbers ( $\pm$ SE) of *A. gossypii* per plot in the various natural enemy exclusion treatments with varying nitrogen inputs (i.e. high, low and no) in both Bt and conventional cotton from late June to the end of August in HZAU experimental station, Wuhan, China.

**Figure 3:** Mean numbers ( $\pm$ SE) of coccinellids per plot in the various natural enemy exclusion treatments with varying nitrogen inputs (i.e. high, low and no) in both Bt and conventional cotton from late June to the end of August in HZAU experimental station, Wuhan, China.

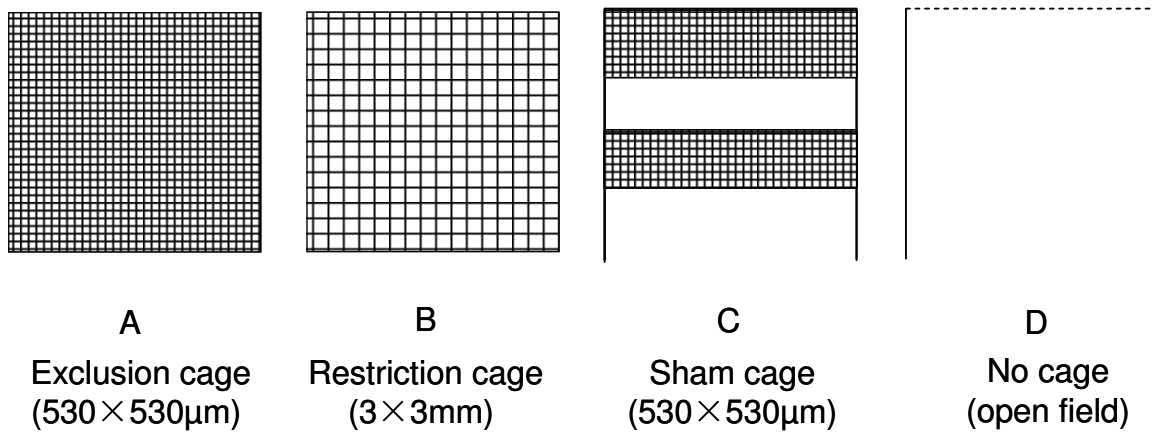
**Figure 4:** Mean numbers ( $\pm$ SE) of predatory spiders per plot in the various natural enemy exclusion treatments with varying nitrogen inputs (i.e. high, low and no) in both Bt and conventional cotton from late June to the end of August in HZAU experimental station, Wuhan, China.

**Figure 5:** Mean numbers ( $\pm$ SE) of parasitoids per plot in the various natural enemy exclusion treatments with varying nitrogen inputs (i.e. high, low and no) in both Bt and conventional cotton from late June to the end of August in HZAU experimental station, Wuhan, China.

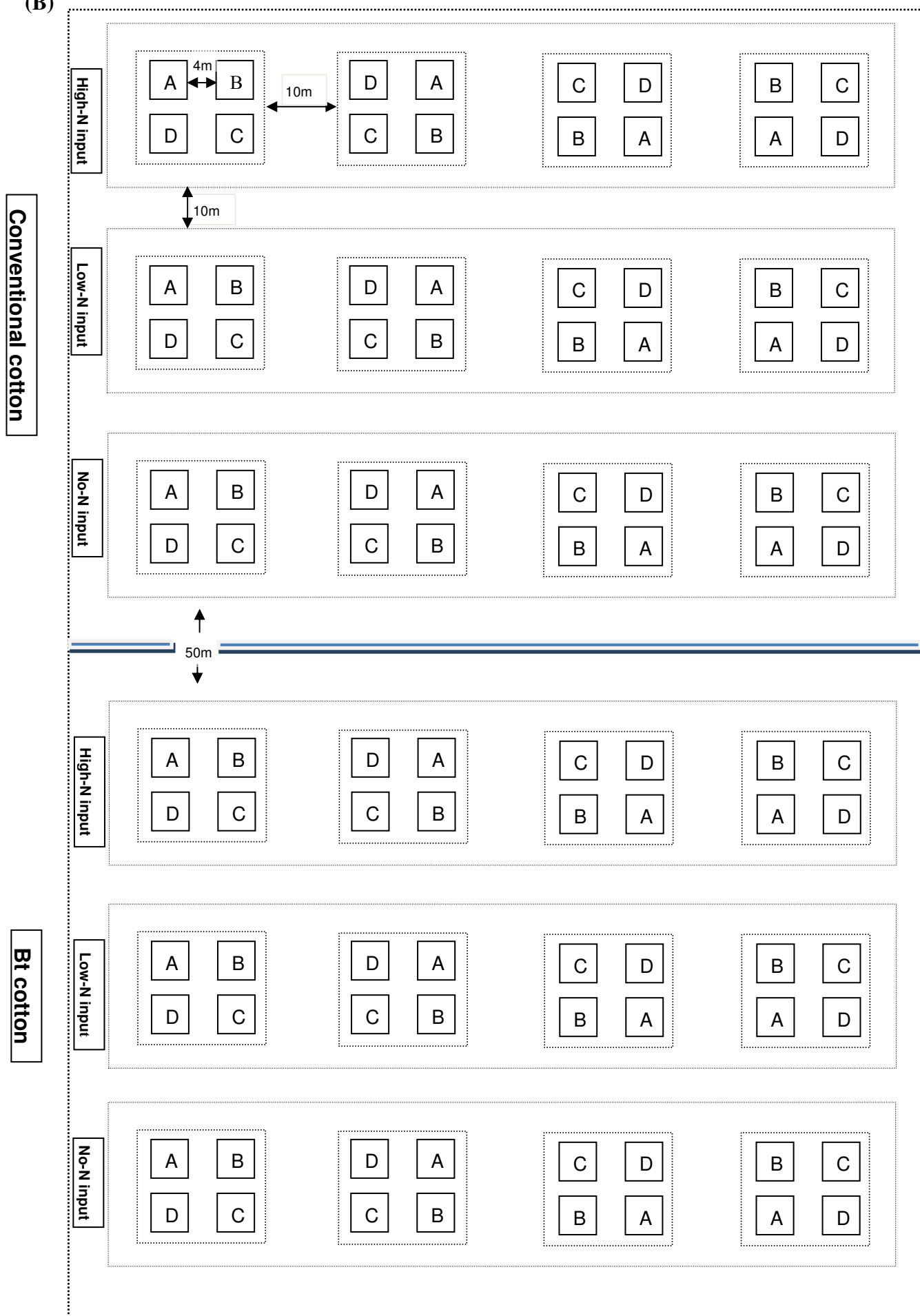
**Figure 6:** Mean numbers ( $\pm$ SE) of other natural enemies per plot in the various natural enemy exclusion treatments with varying nitrogen inputs (i.e. high, low and no) in both Bt and conventional cotton from late June to the end of August in HZAU experimental station, Wuhan, China.

**Figure 1**

**(A)**

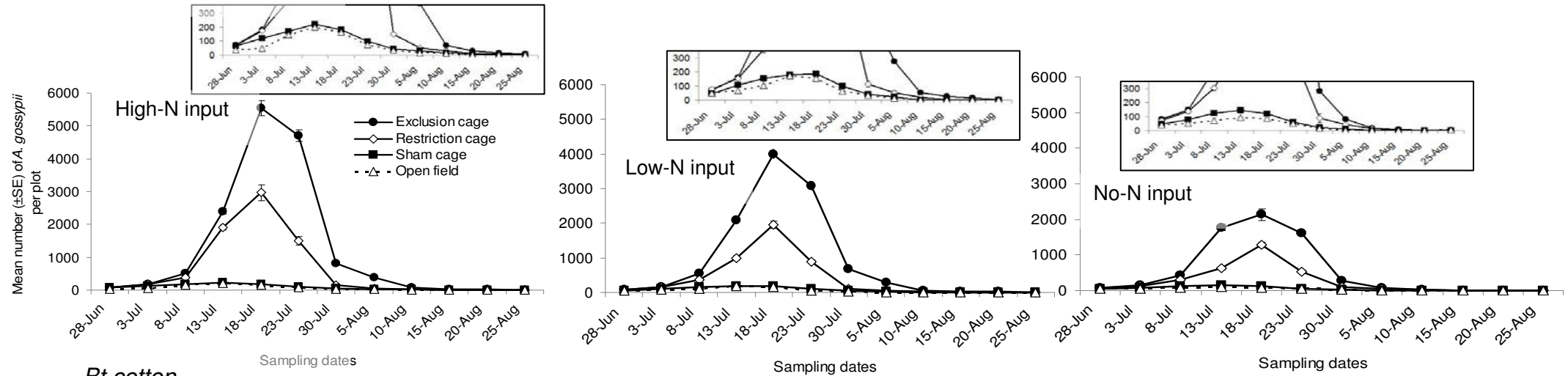


**(B)**

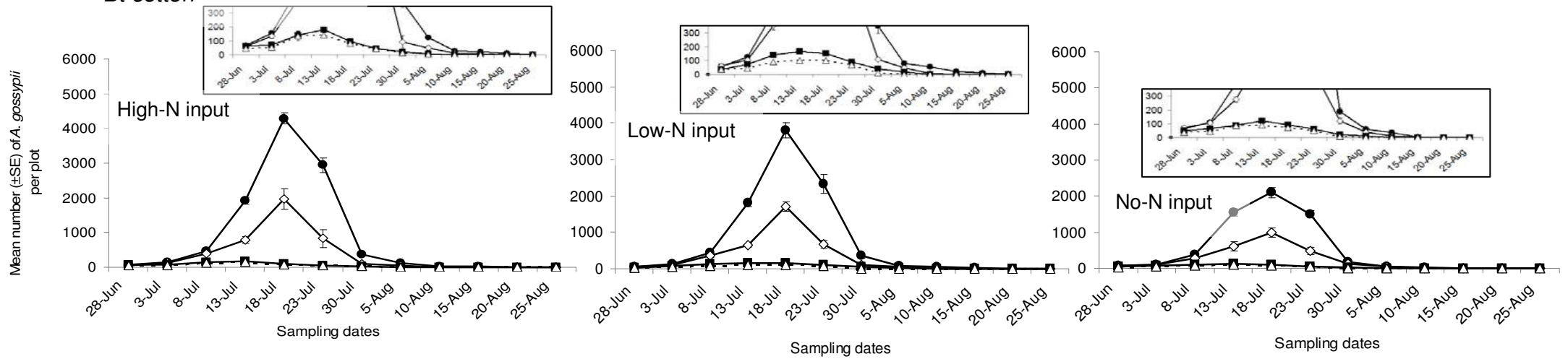


**Figure 2**

*Conventional cotton*

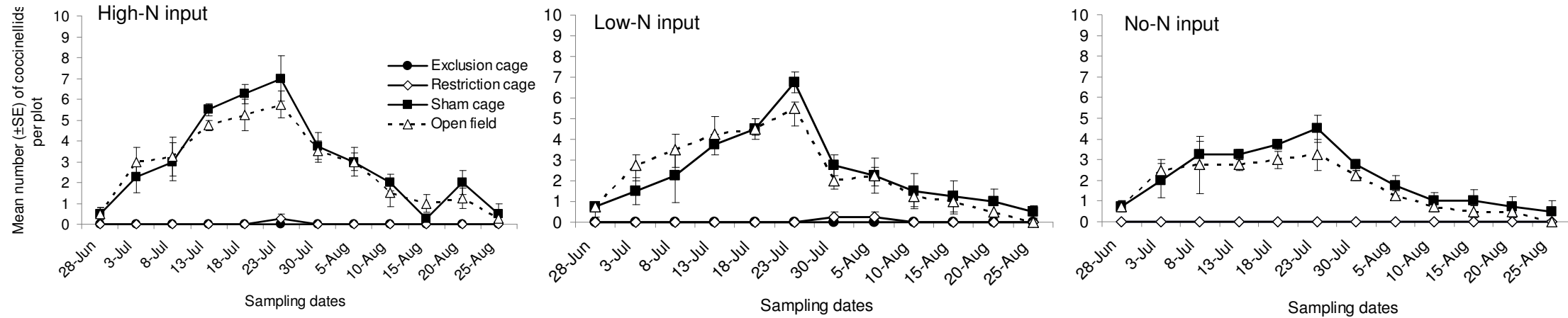


*Bt cotton*

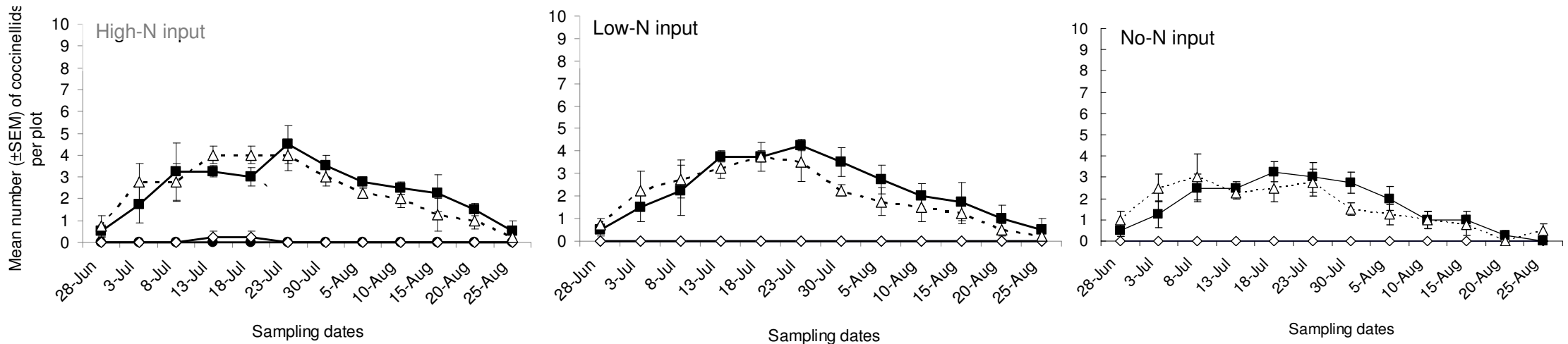


**Figure 3**

*Conventional cotton*



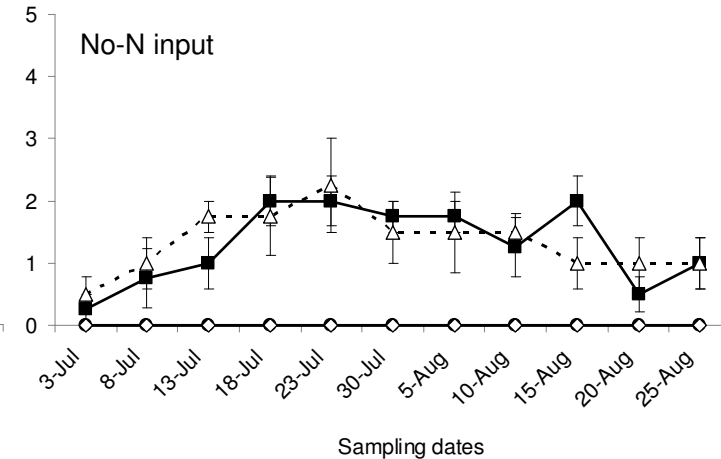
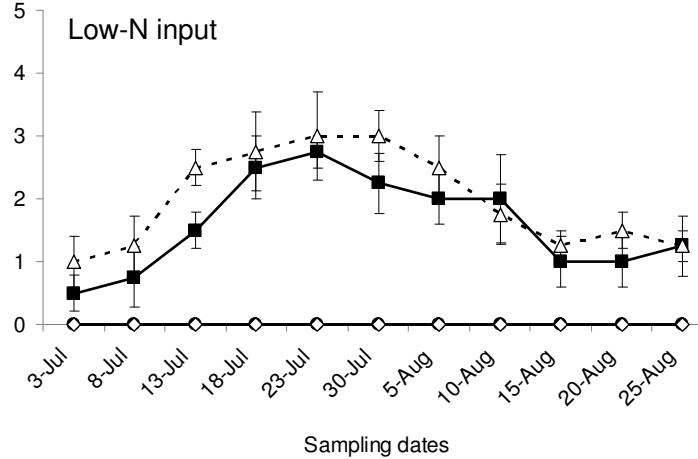
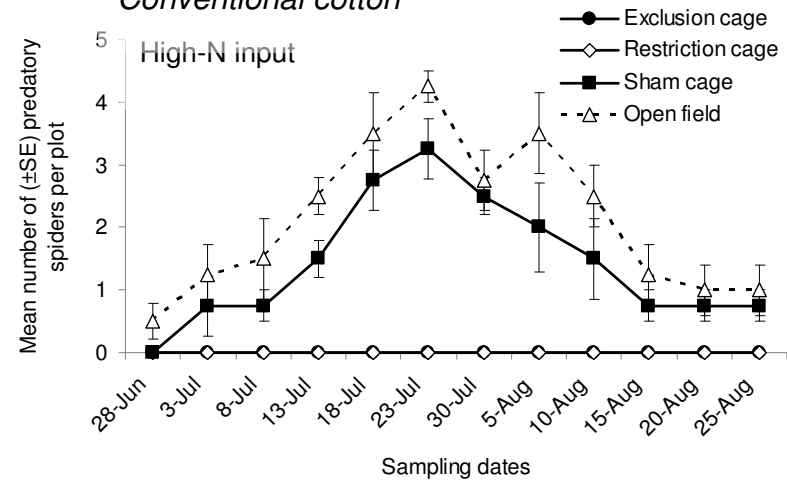
*Bt cotton*



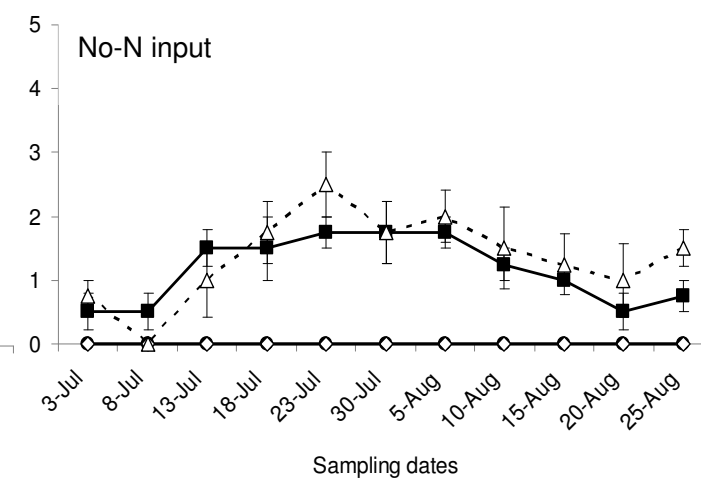
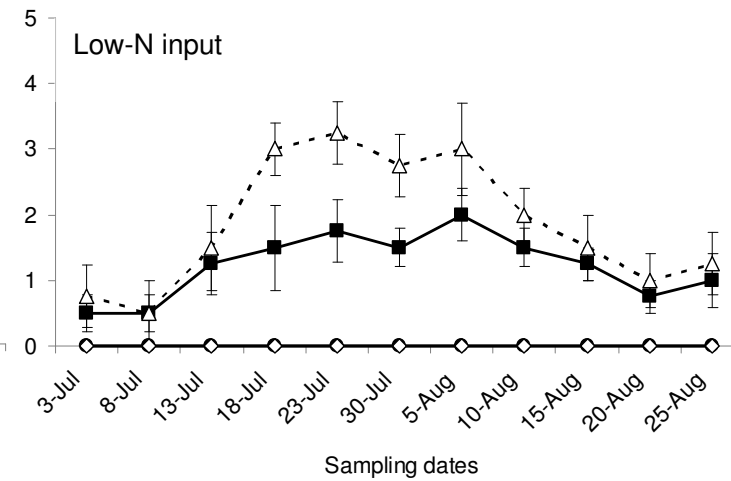
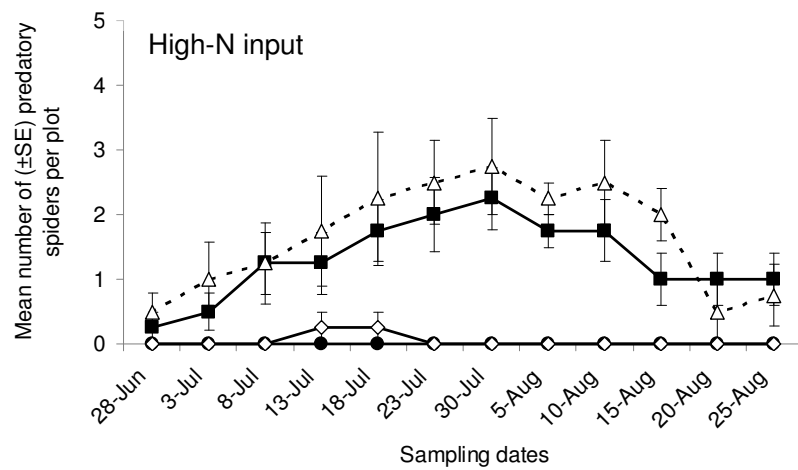


**Figure 4**

*Conventional cotton*

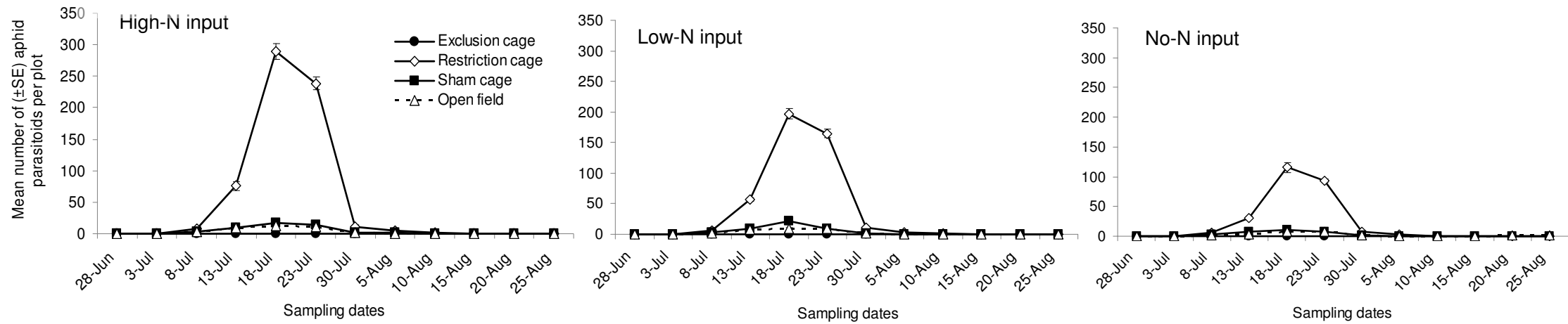


*Bt cotton*

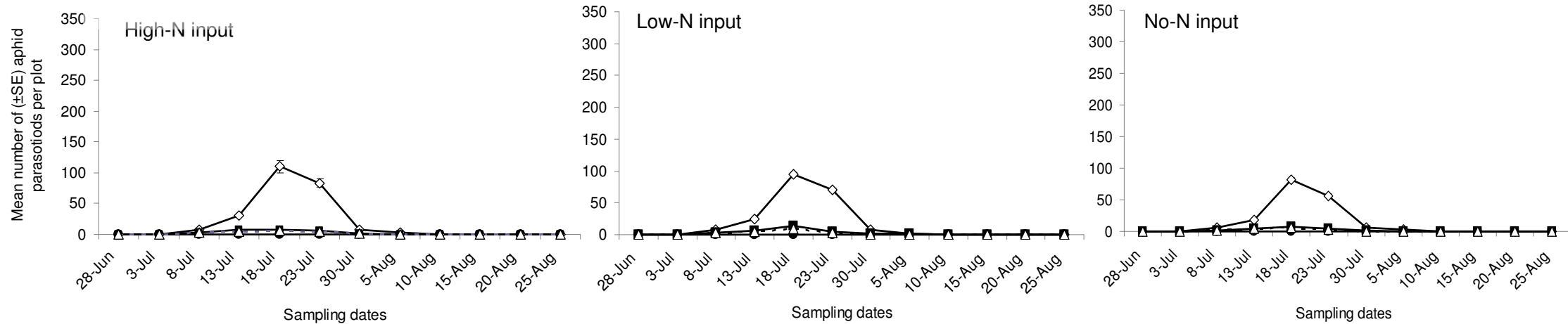


**Figure 5**

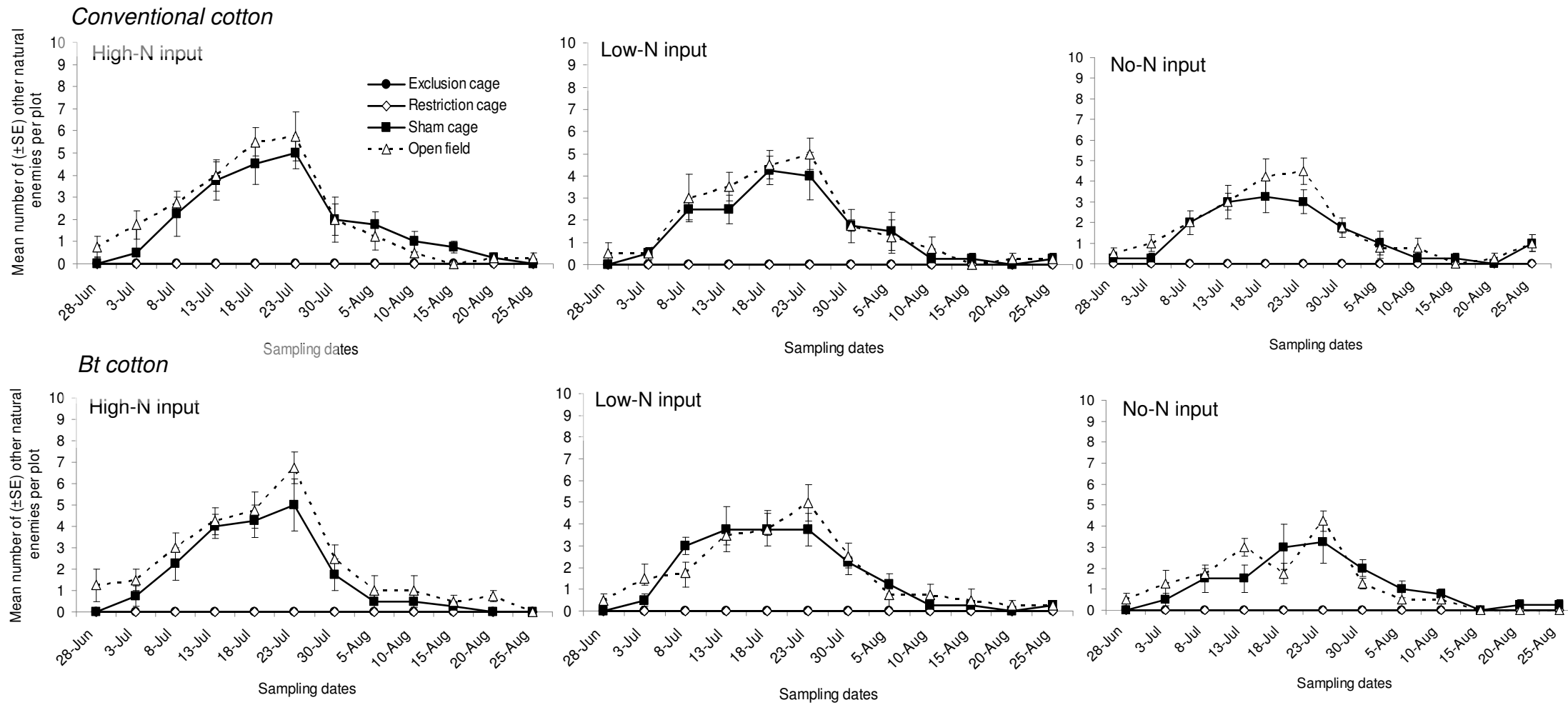
*Conventional cotton*



*Bt cotton*



**Figure 6**



#### IV) Conclusion of chapter 4

In summary, the following major conclusions can be drawn:

- (i) We identified the strong top-down forces exerted by natural enemies, mainly the predator coccinellids and aphid parasitoids, on cotton aphid *A. gossypii* in Bt cotton field in central China.
- (ii) We observed strong evidence for top-down control of cotton aphid population growth. Coccinellids, spiders and Aphidiine parasitoids were equally abundant during the survey in both conventional and Bt cotton. Bottom-up effect through fertilization regime was also observed on the pest; reduced nitrogen inputs supported smaller aphid populations. Lower nitrogen fertilization also supported smaller populations of the key natural enemies (i.e., coccinellids and parasitoids), but such effect was only significant in non-Bt cotton. The key natural enemies in Bt cotton remained constant with varying levels of nitrogen input.
- (iii) By integrating the two studies on cotton, manipulating nitrogen fertilization regimes to support smaller cotton aphid populations while not compromising biocontrol services provided by natural enemies may help managing this secondary pest in the context of the high prevalence of Bt cotton in China.

## **General discussion**

## General discussion

Within “Plant-Herbivorous insect-Natural enemy” tri-trophic interactions, my PhD research investigated the bottom-up and top-down forces regulating herbivorous insects at both individual (life-history parameters) and population levels. Sources of bottom-up forces that were examined in our study mainly include three factors: (i) resource inputs (i.e. nitrogen and water), (ii) plant adaptive traits to environmental stresses (i.e., nitrogen deficiency and drought), and (iii) plant resistance to herbivore attack. Top-down forces are controlling effects of natural enemy guild (i.e., arthropod, predators and parasitoids) herbivorous insects. Seven biological models were set up under laboratory or field conditions to test various hypotheses, ranging from basic ones regarding plant-arthropod interactions to applied ones concerning biological control of herbivorous insects. I discuss the main findings below.

### **(I) Nitrogen and water availability triggered strong bottom-up effects on both herbivorous insect and omnivorous predator**

Results showed that variation in nitrogen and water inputs to tomato plants significantly affected survival and development of *T. absoluta* (Han et al. 2014a), as well as of the omnivorous predator *M. pygmaeus* (**Article 5**). Firstly, sub-optimal nitrogen inputs (either nitrogen deficiency or excessive nitrogen input) and drought reduced their survival rates and impaired their development i.e., reduced pupal weight and delayed development time in *T. absoluta*. Overall, these negative effects may result from a combination of (i) lower plant nutritional quality and/or (ii) higher level of chemical defenses. Secondly, insufficient nitrogen and water inputs to plants depressed the predation rate of *M. pygmaeus* on prey *E. kuehniella* eggs. In addition, predator longevity was significantly decreased when the plants suffered drought.

**Drought** was observed to negatively affect survival and development of *T. absoluta*. During the experiment, the tomato foliage tended to wilt, thus probably

becoming less edible for Lepidoptera larvae (Gutbrodt et al. 2011). *Tuta absoluta* larvae may face difficulties in obtaining enough water on such leaves thus causing a slowdown in their development. In addition, water mediated nitrogen availability for plants (Huberty and Denno 2004). Shortage of water may limit the nitrogen assimilation by plants, and *T. absoluta* larvae therefore may suffer a deficit of N-based nutrient derived from the plants (e.g., amino acids). Furthermore, *T. absoluta* larvae may suffer from higher chemical defense since we observed higher concentration of glycoalkaloids in leaves from the plants treated with water limitation (Fig. 5, Article 4).

The negative impact of **insufficient nitrogen** treatment on *T. absoluta* survival and development may be due to a combination of decrease in leaf nutritional value and increase in plant chemical defense. Firstly, leaf nitrogen content, an important indication of plant nutritional value for Lepidoptera larvae (Hunter and McNeil 1997; Fischer and Fiedler 2000; Inbar et al. 2001), significantly decreased in the plants suffering from nitrogen deficiency (Han et al. 2014a). In this case, *T. absoluta* larvae may experience a lack of organic nitrogen, i.e., specific proteins and/or amino acids, leading to reduced or impaired metabolism during their critical growth period and even to premature death (before reaching adulthood). Moreover, insufficient nitrogen treatment may influence feeding behavior of *T. absoluta*. We assumed that *T. absoluta* experienced a longer development time i.e., delayed development because the larvae need to compensate for insufficient-N based food by increasing their feeding time. Secondly, higher plant chemical defense against herbivore attack was expected in tomato plant suffering from nitrogen deficiency. Nitrogen-limited tomato plants may produce more insect-defensive soluble phenolics (C-based defensive compounds such as chlorogenic acid, rutin, kaempferol-rutinoside) since their concentration has been proved to be positively correlated with C/N ratio in leaves (Stout et al. 1998; Inbar et al. 2001; Le Bot et al. 2009; Larbat et al. 2012; Royer et al. 2013).

In addition, we found that **excessive nitrogen** input to tomato plants also affected survival and development of *T. absoluta*. Such negative effect could only be attributed

to higher chemical defense because the leaf nutritional value was not reduced compared to the optimal nitrogen input; leaf N and content and leaf C/N ratio were equal (Han et al. 2014a). The mechanisms underlying the negative effects of excessive nitrogen inputs to plants on *T. absoluta* are still unknown. At least, the mechanism involved is not the same as that in the case of insufficient nitrogen.

Because phytophagy activity occurs also in omnivorous predators, they may be influenced by the variation in plant quality mediated by the manipulation of source-based nutrients, such as water and nitrogen inputs. We found that the tomato plants under sub-optimal nitrogen and water inputs (i.e., nitrogen deficiency and drought) lead the omnivorous predator *M. pygmaeus* to consume significantly less prey. Moreover, **drought** treatment applied to host plant (tomato) induced a reduction in longevity of the predator by nearly 30% compared to the optimal water input (**Article 5**). We assumed that drought in plants limit predation rate because predators faced difficulties to obtain water from the plants. Omnivorous Heteroptera need to acquire water for the production of saliva required for the extra-oral digestion of prey food (Cohen 1998; Sinia et al. 2004; Gillespie and McGregor 2000). Consequently, decreased predation on prey food due to water shortage might result in a shorter longevity because of a deficit of nutrients uptake; this likely happens even if predators could feed on plant tissues to try acquiring complementary nutrients (Sinia et al. 2004). Such plant feeding behavior was studied during the course of the PhD (Article 6).

In addition, the predator *M. pygmaeus* consumed significantly less prey on the plants treated with **nitrogen deficiency**, but their longevity was not affected (Article 5). At least two possible scenarios can be proposed to explain such contrasted effects of nitrogen deficiency in host plants: the first one is a trophic hypothesis where *M. pygmaeus* needs to feed on plants because plant materials provide key nutrients that prey food does not provide. The concentration / composition of these nutrients may vary depending on the nitrogen input, even if they do not contain nitrogen themselves, as nutrition alters the concentration of a large range of organic and



mineral compounds in tomato (Huanosto Magaña et al. 2009). The second hypothesis is related to plant chemical defenses (see review by Kaplan and Thaler 2011). Indeed, the concentration of many carbon-based defensive compounds increases in the vegetative parts of tomatoes treated with low nitrogen input (Le Bot et al. 2009; Larbat et al. 2012, 2014) thus restricting the performances of herbivores and omnivores as well.

The findings from our studies also contribute to the growing body of literature which has been linked to the various hypotheses that proposed to predict the bottom-up effects of contrasting levels of nitrogen and water inputs to plants on herbivorous as well as on omnivores. Regarding nitrogen input, the *Nitrogen limitation hypothesis* (White 1993) predicts that reduced availability of nitrogen to plants generally impairs the performance of Lepidoptera insects and this hypothesis has been supported by numerous studies (White 1993; Hunter and McNeil 1997; Grundel et al 1998; Inbar et al 2001; Cornelissen and Stiling 2009; Han et al. 2014a), but this support is not unanimous (Joern and Behmer 1998; Fischer and Fiedler 2000). The inconsistency from the later was contributed to the variation in species and life0history parameters measured.

Similarly, a majority of case studies on the phloem feeders also supported the fact that increased nitrogen input to plants generally enhanced the performance of phloem-feeding insects that feed on them, such as whiteflies and thrips (Jauset et al. 1998; Bentz et al. 1995 a, b; Bi et al 2001; 2003; Inbar et al 2001; Chau et al. 2005; Hogendorp et al. 2006), as well as in our case study on the whitefly *B. tabaci* (Article 4).

To date, almost all water input mediated plant-herbivore interactions have focused on drought event as drought events have been predicted to increase in frequency and intensity due to global climate change (Christensen et al. 2007). As detailed in the chapter 1 of the PhD, effects of drought on plant-herbivorous insect interactions is primarily dependent on insect feeding strategy, insect feeding specialization, and strength of drought applied (Inbar et al. 2001; Huberty and Denno

2004; Mody et al, 2009; Gutbrodt et al. 2011). Our findings from Article 3 suggested that *T. absoluta* had an optimal survival and development on the tomato plants with better growth status (i.e., vigorous plants) treated with optimal water and nitrogen input, and thus supported the “*Plant vigor hypothesis*”. We also found evidence of negative impact of drought on the omnivorous predator *M. pygmaeus* (Article 5, 6). On water-limited tomato plants, *M. pygmaeus* had decreased frequency of plant-feeding and shorter longevity.

Before going to the section IV discussing the bottom-up effects of nitrogen and water on plant-herbivorous insect-natural enemy interactions at population levels, two sections (II and III) are developed below to discuss the importance of variations in plant cultivar and insect feeding strategy in predicting and understanding the bottom-up effects of nitrogen and water inputs on plant-arthropods relationships.

## **(II) Variation in plant cultivars and herbivorous insect feeding strategy played important role in determining the consequences of the bottom-up effects**

Despite the pervasive bottom-up effects of resource input (nitrogen and water) on arthropod herbivores, these effects may vary owing to various other factors such as plant cultivar and insect feeding strategy (i.e., the feeding guild that insects belong to; e.g., leaf chewer, leaf miner, phloem feeder etc.). Studying combinations of different plant cultivars different insects and measurements of physiological and biochemical plant traits (i.e., nutritional value and chemical defense) may provide a thorough understanding of the possible mechanisms involved.

In modern agriculture, diverse plant cultivars have been selected to cope with sub-optimal growing conditions, such as drought and nitrogen deficiency adaptive traits in plants (Chaves et al. 2002; Flexas et al. 2006; Cattivelli et al. 2008; Chaves et al. 2009; Farooq et al. 2009; Feng et al. 2009). Taking drought as an example, acclimation to drought conditions may include long-term adjustments involving gene expression(s) and modification(s) of plant physiology, biochemistry and morphology

(Chaves et al. 2002; Flexas et al. 2006; Chaves et al. 2009). Two questions were considered in the PhD work: (1) it is unclear how these changes occurring in drought-adaptive cultivars could influence potential bottom-up effects of drought (either negative or positive) on herbivorous insects; by contrast, drought generally impairs herbivorous arthropods feeding on drought-susceptible cultivars (Inbar et al. 2001; Han et al. 2014a); (2) it is also unknown whether the insects from different feeding guild respond differently on these various cultivars. The same questions should be documented on cultivars that may be less sensitive to nitrogen deficiency. We hypothesize that (i) drought tolerance and nitrogen regulation traits in tomato cultivars may mitigate the bottom-up effects of drought and nitrogen deficiency on herbivorous species, and (ii) the herbivorous insects from different feeding guilds may respond differently depending on the stress types (i.e., drought or nitrogen deficiency) and plant cultivars.

The hypotheses mentioned above were tested in the **Article 4**. Prior to the discussion of impacts on herbivorous insects, the variation in plant cultivar responses to resource-based stress (i.e., nitrogen deficiency and drought) should be checked. We found strong evidences that cv. Noir de Crimée (NC) is more tolerant to drought than cv. Marmande (M), whereas cv. M is more adapted (i.e., less sensitive) to nitrogen deficiency than cv. NC. Firstly, drought induced decreases in photosynthesis and transpiration rates as well as in stomal conductance in cv. M. By contrast, only decreased photosynthesis rate in cv. NC was observed (Fig. 1, Article 4). Moreover, drought caused increases in defensive compounds tomatidine and tomatine 1 in cv. M, but such effects was not found in cv. NC (Fig. 5, Article 4). Secondly, and by contrast, leaf N content and leaf C/N ratio were affected in both cultivars, but cv. NC proved to be much more sensitive to nitrogen deficiency than cv. M, i.e., cv NC. showed much lower leaf N content and higher leaf C/N ratio than cv. M under drought conditions (Fig. 2, Article 4). Similarly, nitrogen deficiency significantly decreased contents of tomatidine, dehydrotomatine and tomatin 2 in cv. NC compared to optimal nitrogen input, but such effects were not found on cv. M (Fig. 4, Article 4). Overall, these

results suggested that cv. M is nitrogen deficiency adapted and cv. NC appears drought-tolerant.

Our study showed that negative responses of each insect to drought and/or nitrogen deficiency did not differ between cultivars (Table 3 and 4, Article 4), thus rejecting our first hypothesis and these negative effects were similar on both cultivars despite two facts: (i) either of both showed a certain degree of adaptation to drought or nitrogen limitation and (ii) cv. NC generally showed a higher chemical defense in terms of glykaloids concentrations in leaves compared to cv. M. Our data supported the second hypothesis. Both nitrogen and water deficit significantly affected *T. absoluta* survival and development (Table 4 and Fig. 6, Article 4). By contrast *B. tabaci* survival was only negatively affected by the nitrogen deficiency, but drought (Fig. 7, Article 4). Furthermore, the response pattern did not differ between the two cultivars.

### **(III) Omnivorous predator feeding ecology: a positive correlation between plant feeding and prey feeding**

In Article 5, we evaluated the effects of variations in plant growing status (mediated by nitrogen and water inputs) on predation and longevity of the omnivorous predator *M. pygmaeus*. Our data suggested that nitrogen and water restrictions reduced the predation of *E. kuehniella* eggs by the omnivorous predator *M. pygmaeus*. However, the mechanisms involved in these effects still remain unclear. We predicted that these effects might be linked to the feeding ecology of *M. pygmaeus*. Plant feeding and prey feeding by Heteroptera predators are often linked each other and such functional relationships could vary with environments in which predators forage, notably the availability and density of prey (Agrawal et al. 1999; Montserrat et al. 2004), plant and prey quality (Agrawal et al. 1999; Eubanks and Denno 1999, 2000; Janssen et al. 2003), as well as abiotic factors such as water (Gillespie and McGregor 2000; Sinia et al. 2003). Therefore, we investigated plant feeding behavior, as well as

the relationships between plant feeding and prey feeding by *M. pygmaeus* when plants were receiving various levels of resources (nitrogen and water) (**Article 6**).

In the absence of prey, the predator fed equally on the plants treated with contrasting levels of nitrogen and water (Fig. 2B, Article 6). In contrast, the feeding rate on the plant decreased in the presence of prey when plants suffered drought (Table 1, Article 6). The feeding rate on prey was positively correlated with the feeding rate on the plant (Fig. 2A, Article 6), and the predator showed a strong preference toward plants bearing prey and subjected to optimal water input (Fig. 3, Article 6). The presence of prey as food enhanced fertility and longevity of *M. pygmaeus*, though drought applied to the plant actually decreased predator longevity (Fig. 4, Article 6). Our results supported the "Facilitation Hypothesis": omnivorous predators (like from the Heteroptera family) use water taken from plants to facilitate the prey feeding activity. Phytophagous activity in such predators is likely not a choice but a need for relatively high fitness. In this case, water is considered as a limiting factor because water is required for the production of the saliva used for extra-oral digestion and to enable omnivorous predators to extract the maximum nutrients from their prey while avoiding indigestible prey structures (Cohen et al. 1995; 1998). Furthermore, shortage of water in plants may also affect predators' plant feeding behavior; this is an effective way of feeding that could provide Heteroptera predators a wide and balanced source of nutrients (Coll and Guershon 2002; Portillo et al. 2012). During the study, we have repeatedly observed that *M. pygmaeus* inserted their stylets into the stems of tomato leaves. They probed the unknown nutrients and stayed for sucking if they found the right site. Also, we have observed *M. pygmaeus* females inserting their ovipositor into the tomato stem for oviposition.

#### **(IV) Combined effects of bottom-up and top-down forces in determining the population dynamics of herbivorous insects.**

The fact that bottom-up (driven by resources availability) or top-down forces

(driven by natural enemies behavior), or both, determine the population abundance, have long been a controversial subject (Hunter and Price 1992; Walker and Jones 2001; Singer and Stireman 2005). To address this point, the first step is often studying performances of individual herbivores under various levels of bottom-up and top-down regulations, followed by studies at population level. However, these effects observed at individual level (if any) do not necessarily translate at population-level effect. For example, Zaugg et al. (2013) showed that bean quality affected individual bean weevil performances under laboratory and field conditions, but no correlations between measured performance under laboratory conditions and weevil infestation levels or parasitism rates in the field were found. When considering “Plant-herbivorous insect-natural enemy” tri-trophic interactions, plant quality either mediated by resources input or by different intrinsic genotypes may trigger cascading effects up to the higher trophic levels (Teder and Tammaru 2002; Johnson 2008; Sarfraz et al. 2009; Stenberg 2012, but see Zaugg et al. 2013). Bottom-up forces may affect top-down regulation (through natural enemies) of herbivores either directly (e.g., effects on omnivorous predators) or mediated by the intermediate herbivorous insects (e.g. modification in prey/host quality or quantities). Hence, the overall population effect of the bottom-up forces on the herbivorous insect is becoming difficult to predict. One option is to include the organisms from the three trophic levels in one system.

In resource input-mediated “plant-herbivore-omnivore” interactions, nitrogen and water availability have triggered strong bottom-up effects on the individual performance of *T. absoluta* and the omnivorous predator *M. pygmaeus* in our test models (**Article 3, 4, 5 and 6**). More accurately, when integrating the organisms in one system, plant quality may not only directly affect the herbivore *T. absoluta*, but also indirectly affect the top-down regulation of *T. absoluta* by influencing the omnivorous predators. Thus, we should consider simultaneously the impact of resource input-mediated plant quality on both *T. absoluta* and *M. pygmaeus* population dynamics (chapter IV), as well as the trophic links between *T. absoluta*

and *M. pygmaeus*. Notably, water and nitrogen deficits have been considered as a limiting factor for predation behavior in *M. pygmaeus* (Article 5, 6). An experimental model “*S. Lycopersicum* – *T. absoluta* – *M. pygmaeus*” was set up to examine the effects of water/nitrogen availability on population dynamics of these two insects, and notably the overall impacts on *T. absoluta* populations. We thus proposed two hypotheses. First, two-fold negative effects of low water and/or water input on the omnivorous predator were expected: (1) the decreased quantity/quality of *T. absoluta* as prey, and (2) the decreased suitability of plant food due to lower plant quality. Second, such two-fold negative effect on the predator may benefit *T. absoluta* by offsetting partially the negative bottom-up effects from water/nitrogen deficit. To test these hypotheses, we carried out a study under greenhouse conditions (study 7). We included *T. absoluta* and *M. pygmaeus* in one system and monitored their population dynamics on plants treated with contrasting levels of nitrogen and water inputs. The experiment and analyses could not be completed before submitting the PhD manuscript.

Like in other crop field (e.g. soybean; Costgama and Landis 2006; Ragsdale et al. 2011), the highly simplified food webs and interactions between adjacent trophic levels in cotton field (Wu and Guo 2005) make it an ideal system to study bottom-up and top-down forces. Hence, under the framework of this current PhD work, we also conducted studies in cotton-based agro-ecosystem. Within the “cotton - cotton aphid - natural enemies” tri-trophic interactions in the field, we first identified the key natural enemies of cotton aphid *A. gossypii* in central China (Han et al. 2014b). Using exclusion cage techniques, we identified that coccinellids predators and aphid parasitoids have strong top-down regulations on *A. gossypii*. Among the coccinellids, *Propylaea japonica* Thunberg represented 65 % of coccinellids predators. *Propylaea japonica* is a well-known predator of *A. gossypii* (Zhang et al. 2006) and its life history characteristics and phenology make it a good biocontrol agent candidate for the management of the aphid in cotton in China. This predator colonizes cotton fields early in the cotton seedling stage when the aphid population starts infesting the

seedlings. Other predators including predatory spiders and Chrysopae (lacewings) also contributed to the population regulation of *A. gossypii*. The aphid parasitoids, mainly *L. japonica* and *B. indicus*, were also found to limit cotton aphid population growth. *L. japonica* proved to be a key natural enemy of cotton aphid in Northern China (Hou et al. 1997). The aphid parasitoids may help reduce aphid densities primarily when aphid populations have already reached a certain density.

Overall, identification of key natural enemies (key top-down forces) is the first step of an IPM program. Optimized IPM may aim at combining biocontrol agents as top-down force (Landis et al. 2000; Costamagna and Landis 2006; Costamagna et al. 2007; Ragsdale et al. 2011) with bottom-up forces like fertilization regimes and/or cultural practices (Walker and Jones 2001; Denno et al 2002; Chen and Ruberson 2008; Ai et al 2009; Chen et al. 2010) for efficient management of pests. Effects of nitrogen fertilization on the key natural enemies of *A. gossypii*, as well as the combined impacts of nitrogen fertilization and natural enemies were examined (**Article 9**). To minimize environmental perturbations by nitrogen fertilizer especially the contamination of groundwater (Jaynes et al. 2001), efforts have been currently made to increase the nitrogen use efficiency (NUE) (i.e., plant production unit per nitrogen input unit) by crops (Xu et al. 2012). Our findings showed combined effects of bottom-up and top-down forces in regulating cotton aphid. Their population was strongly reduced by lower nitrogen inputs (Fig. 2, Article 9). This finding was consistent with the study by Chen and Ruberson (2008) where the cotton aphid was found most abundant in highest nitrogen fertilization treatments (even though only in one year of the study), as well as in several other earlier studies on cotton aphid (Slosser et al. 1997; Cisneros and Godfrey 2001). Moreover, the similar impact of nitrogen was also found on all the natural enemies, but generally this impact was only evident in non-Bt cotton, i.e. lower nitrogen input supported lower population of key natural enemies including coccinellids and aphid parasitoids in non-Bt cotton (Fig. 3 and 5, Article 9). Such effects were not strong in Bt cotton. Thus, in Bt cotton, a lower nitrogen fertilization supported lower *A. gossypii* populations, but did not compromise



top-down regulation of *A. gossypii* since the key natural enemy guilds were not affected. These findings convey the important message that a compatibility of bottom-up (i.e. nitrogen regime and insect-resistance trait “Bt”) and top-down forces (i.e. natural enemies) in regulating cotton aphid can be expected.

Since the sustainable use of IRGM crops can lead to a drastic reduction in pesticide usage at the wide scale (Naranjo 2005, 2011; Lu et al. 2012), biological control agents should help to suppress outbreaks of secondary pests not targeted by IRGM crops (Romeis et al. 2006; Lundgren et al. 2009). In the context of Bt cotton adoption in China, compatibility between biological control and Bt cotton may provide opportunities to improve sustainable IPM systems. Furthermore, such top-down forces in regulating secondary pests can be combined with the bottom-up forces via manipulation of fertilization regimes. However, a thorough risk assessment of any new IRGM cultivars are required before the its release into the environment (Romeis et al. 2008).

## Conclusions and Prospects

In conclusion, our studies at both individual and population levels provided several basic and applied implications. Our current work provided a thorough assessment of bottom-up forces (resources input) in influencing the individual performance and populations of herbivorous insects and their natural enemies, as well as the combined effects of both bottom-up and top-down forces on herbivorous insects. Firstly, we obtained strong evidence supporting the “Nitrogen limitation hypothesis” and “Plant vigor hypothesis” when predicting the effects of resources input (e.g., nitrogen and water) on plant-insect interactions (Article 3).

Taking as a whole our findings on *M. pygmaeus* (Article 5, 6) and on *T. absoluta* (Han et al. 2014a), we can conclude that optimal nitrogen and water inputs to the tomato plants favored survival and development of the herbivore *T. absoluta*, as well as the predation activity, longevity and fertility of the omnivorous predator *M. pygmaeus*. However, the overall bottom-up effects of nitrogen and water inputs and top-down regulation by *M. pygmaeus* on *T. absoluta* at population level remain unknown. A systemic model allowing identifying population effects is further needed. Secondly, these bottom-up effects could be largely influenced by various biotic factors such as insect feeding strategies and plant adaptive traits against environmental stresses (Article 4). Thirdly, the study with omnivorous predators provided strong evidence of “Facilitation hypothesis”; we emphasized the positively correlated relationship between plant feeding and prey feeding (i.e., predation). Water has been proved a crucial resource that limits both behaviors in omnivorous predators (Article 5 and 6). Lastly, both bottom-up (resources inputs) and top-down forces (natural enemies) act in concert to influence populations of herbivorous insects in terrestrial arthropods communities (Study 7, Article 9 and Han et al. 2014b).

These studies have the potential not only to provide insights into basic ecological questions, but also help developing optimized IPM programs. From applied perspectives, the manipulation of fertilization and irrigation regimes may help to

optimize agricultural practices by promoting negative effects on herbivorous insects (often the pests) and enhancing performances of their natural enemies, while not compromising the crop yields.

Unfortunately, our exploration of potential mechanisms underlying the observed plant-arthropods interactions are still in progress. Additional studies that will encompass more parameters (e.g., plant chemical defenses) as well as insect feeding strategies (both for herbivorous insects and omnivorous predators) are needed to provide more explanatory power. Future studies in resource input-mediated plant-herbivore insect-natural enemy tri-trophic interactions would benefit from:

(1) Providing rigorous measurements of nutritional values that are relevant to the plant-feeding insects. Numerous studies provided the quantification of defensive compounds when plants were treated with contrasting water or nitrogen inputs (Inbar et al. 2001; Gutbrodt et al. 2011; Larbat et al. 2012; Hagenbucher et al. 2014; Royer et al. 2013; Larbat et al. 2014). However few works have considered the variation in nutrients required by insect during their critical development and growing periods. This part of work would require an insect physiology approach, e.g., digestion and circulatory insect systems. In addition, investigations of insect food breadth may provide valuable information for better understanding their nutrients requirements.

(2) Identifying the phyto-compounds that omnivorous predators (notably heteropterans) ingest during their feeding: To date, little is known about the feeding ecology of Heteroptera predators (Coll and Cuershon 2002; Kaplan and Thaler 2011) even though various models have been proposed to understand the relationship between plant and prey feeding (i.e., predation) (Gillespie and McGregor 2000). Plant feeding has been proved to provide Heteroptera predators with key nutritional resources that can not be obtained from prey only (Eubanks and Styrsky 2005). However, it is still unknown what key resources are really needed and where they derive from, i.e., phloem, xylem, cell-content, etc. Also, it is unclear how predators obtain nutrients from plant tissues when attempting to avoid defensive toxins. In all cases, this point is likely linked to the first point.

(3) Designing experiments that allow a long-term monitoring of population dynamics in the field during multiple consecutive years: several field studies have examined the bottom-up effects of fertilization regime and/or agricultural practices as well as top-down effects (natural enemies) on the key herbivorous insects, but mostly only during a single year (Liu et al. 2004; Costamagna and Landis 2006; Costamagna et al. 2007; Han et al. 2014a), however, data may vary between years since the impact of a given factor (fertilization and/or natural enemy) on herbivore populations may vary on a yearly basis (Chen and Roberson 2008). Therefore a field survey lasting several years is recommended (Desneux et al. 2006; Ai et al. 2011; Lu et al. 2012). Furthermore, effects of fertilization and/or irrigation manipulations on plant production (i.e., yield) should be assessed in future studies.

(4) Integrating the manipulation of bottom-up forces (fertilization and irrigation) in the augmentative and/or conservation biological control programs: More applied studies are needed for characterizing the best combination of (i) manipulation of resource inputs with (ii) release (or conservation) of biological control agents under greenhouse or field conditions. It may provide useful data helping developing more optimal IPM packages.

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Poster presentation. 13<sup>th</sup> JEDN, University of Nice, Sophia-Antipolis, France  
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