AGRICULTURAL UNIVERSITY OF ATHENS
SCHOOL OF PLANT SCIENCES
DEPARTMENT OF CROP SCIENCE
LABORATORY OF AGRICULTURAL ZOOLOGY & ENTOMOLOGY

PhD Thesis

Interactions of the herbivorous insect *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) and tomato and their importance in the control of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) and other important enemies of tomato

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ATHENS
2023

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Αλληλεπιδράσεις του ζωοφυτοφάγου εντόμου *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) και της τομάτας και η σημασία τους στην αντιμετώπιση του *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) και άλλων σημαντικών εχθρών της τομάτας

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Interactions of the herbivorous insect *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) and tomato and their importance in the control of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) and other important enemies of tomato

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Abstract

Tomato, *Solanum lycopersicum* L. (Solanaceae) is one of the most valuable cultivated crops worldwide. However, it is infested by several insect pests that reduce quantity and quality of the product. Recently, one of the most devastating insect pests of tomato, the South American pinworm *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) has caused serious damages and substantial increase in the tomato production cost, worldwide. Due to the shortcomings of its chemical control, biological control has been prioritized with positive results. *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) is a generalist predator and one of the major biological control agents of *T. absoluta* and other pests of tomato. In addition to this, recent studies have shown that its phytophagy can trigger defense responses in tomato plants against pests, such as two spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) and *T. absoluta*. Knowledge on the plant defence induction effects on the expression of related genes offers important information in pest control because certain genes may be associated with the activation of the defence pathways and act locally or have a systemic effect. Furthermore, plant feeding by arthropods causes several changes in the metabolomic profile of the plant. Individual metabolites produced after defence induction may be used as sources of new insecticides. However, the gene expression levels and metabolite profiling have been little studied in plants punctured by mirid predators. Finally, information is given regarding the RNA interference, suppression or silencing (RNAi) technique which very recently showed positive results in the control of *T. absoluta* but its possible effect on a natural enemy of *T. absoluta* has not been examined.

In the second chapter of presentstudy, the expression of genes governing Jasmonic Acid (JA) biosynthesis pathway activated by plant feeding of *N. tenuis*and the fluctuations in the levels of underlying metabolites have been studied. Fifteen 3rdinstar nymphs of *N. tenuis* were caged on each top and lower leaf of tomato plants for 4 d to induce plant defense; after this period the predators were removed. *T. absoluta*, oviposition preference, larval period, and pupal weight were significantly reduced in *N. tenuis* punctured plants. *T. urticae* adults exhibited a significantly higher escape tendency and reduced survival on punctured plants.

Metabolomics indicated such observations revealing substantial differences between *N. tenuis*-punctured and unpunctured (control) plants. Metabolites directly associated with the activation of the JA defense pathway, such as the precursor α-linolenic acid, had increased concentrations. The expression of the defense-related genes *PI-II*, *MYC2*, *VSP2*, and *HEL* was increased in the top leaves whereas only *VSP2* and *MBP2* in the lower leaves; interestingly, in the middle (unpunctured) leaves *VSP2*, *HEL*, and *MBP2* were also upregulated, indicating systemic signaling. Therefore, phytophagy of *N. tenuis* caused adverse effects on *T. absoluta* and *T. urticae*, whereas the multi-omics approach (phenomics, metabolomics, and genomics) offered valuable insights into the nature of the plant defense responses and provided useful evidence for future applications in integrated pest management.

In the third chapter of this study, the density threshold of *N. tenuis* nymphs per plant for induction of defence on *T. absoluta* was searched together with the persistence of the plant defence effects. For this reason, 3, 6 or 10 nymphs were enclosed on each of the top and bottom leaf of young tomato plants for 4 days. The results showed that oviposition by *T. absoluta* females was significantly reduced only on the plants punctured by the highest *N. tenuis* density used. Our findings showed that plants punctured by the highest predator density were more repellent to *T. absoluta* for either period of 7 or 14 days after the removal of predators, compared to control plants. The systemic nature of the induced defences was also expressed in all periods post treatment. Those results provide valuable information in critical aspects for possible practical use of plant defence effects induced by *N. tenuis* on *T. absoluta* such as the average number of predators required per plant, systemic nature of the effects and the persistence of the effects induced.

In the last chapter, the possible compatibility of dsRNA-mediated pest management of *T. absoluta* with its biological control agent *N. tenuis* was examined. In fact, RNAi-mediated insect pest management has recently shown promising results against *T. absoluta,*however its compatibility with the natural enemies of this pest has not been assessed. In particular, this study aimed to investigate whether dsRNA (dsTa-αCOP) designed to target the *T. absoluta*-αCOP gene could cause adverse effects to its major biocontrol agent, the predator, *N. tenuis*. Oral exposure of *N. tenuis* to dsRNA (dsNt-αCOP) designed to target *N. tenuis*-αCOP resulted in a 61%, 67% and 55% reduction in its transcript level in comparison to the sucrose, dsGFP and dsTa-αCOP treatments, respectively. In addition, significantly higher mortality of 57% was recorded in dsNt-αCOP-treated *N. tenuis* when compared to the sucrose (7%),

dsGFP (10%) and dsTa-αCOP (10%) treatments. Similarly, oral exposure of *T. absoluta* to dsTa-αCOP resulted in 50% mortality. Moreover, the predation rate of ~33-39 *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs by *N. tenuis* adult dramatically reduced to almost half in the surviving dsNt-αCOP-treated *N. tenuis*. This worst-case exposure scenario confirmed for the first time that the RNAi methodis functional in *N. tenuis* and that the risk of exposure through the oral route is possible. However, dsTa-αCOP did not cause lethal or sub-lethal effects to *N. tenuis* upon oral exposure. In the context of a biosafety risk assessment of RNAi-mediated insect management, investigating the effects on non-target organisms is essential in order to include this method as part of an integrated pest management strategy. Based on our laboratory assays, RNAi-mediated control is compatible with the biological control of *T. absoluta* by its natural enemy *N. tenuis*, adding the RNAi approach in the armoire of integrated pest management of *T. absoluta*. In conclusion,this Thesis focuses on advanced eco-friendly management of *T. absoluta* and *T. urticae* employing several innovative pest management techniques. Additionally indicates necessary bioassay methodologies and new approaches useful for the implementation of these new pest management strategies. Thus, the findings add knowledge to the upcoming IPM methodologies which can be implemented in near future in tomato crop.

Scientific area: Integrated Pest Manegement

Keywords: *Tuta absoluta, Tetranychus urticae*, *Nesidiocoris tenuis*, Integrated Pest Manegement, IPM, pest, predator, tomato, mirid, plant defence induction, defense related genes, jasmonic acid pathway, *MPB2* gene, *MYC2*gene, *PI-II*gene, *VSP2* gene, *HEL* gene, metabolites, RNAi, αCOP gene

Αλληλεπιδράσεις του ζωοφυτοφάγου εντόμου *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) και της τομάτας και η σημασία τους στην αντιμετώπιση του *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) και άλλων σημαντικών εχθρών της τομάτας

Τμήμα: Επιστήμης Φυτικής Παραγωγής
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Περίληψη

Η καλλιέργεια της τομάτας, *Solanum lycopersicum* L. (Solanaceae) είναι μια από τις καλλιέργειες με τη μεγαλύτερη οικονομική σημασία παγκοσμίως. Ωστόσο, η τομάτα προσβάλλεται από διάφορα έντομα που μειώνουν την ποσότητα και την ποιότητα της παραγωγής. Πρόσφατα, ένα από τα πιο καταστρεπτικά έντομα της τομάτας, το *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) προκαλεί σοβαρές ζημιές και μεγάλη αύξηση στο κόστος παραγωγής. Η αντιμετώπισή του βασίζεται σε σημαντικό βαθμό και στη βιολογική αντιμετώπιση καθώς με τη χημική μέθοδο απαιτούνται επαναλαμβανόμενοι ψεκασμοί που μεταξύ άλλων, οδηγούν σε ανάπτυξη ανθεκτικότητας, αυξάνουν το κόστος και τον κίνδυνο υπολειμμάτων. Το *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) είναι ένα ζωοφυτοφάγο αρπακτικό, κύριος βιολογικός παράγοντας ελέγχου του *T. absoluta* και άλλων εντόμων-εχθρών της τομάτας. Επιπλέον, πρόσφατες μελέτες έχουν δείξει ότι η φυτοφαγία του μπορεί να προκαλέσει την επαγωγή της άμυνας του φυτού της τομάτας με σημαντικές αρνητικές επιδράσεις στο *Tetranychus urticae* Koch (Acari: Tetranychidae) και στο *T. absoluta*. Ωστόσο, η γνώση σχετικάμε τηνέκφραση γονιδίων άμυνας του φυτού ως αποτέλεσμα της φυτοφαγίας των αρπακτικών είναι πολύ περιορισμένη, αν και μπορεί να προσφέρει σημαντικές πληροφορίες στον έλεγχο των εντόμων-εχθρών, καθώς ορισμένα γονίδια που μπορεί να σχετίζονται με την ενεργοποίηση ορισμένων αμυντικών μονοπατιών να δρουν τοπικά ή να έχουν συστημική επίδραση, ενεργοποιώντας την άμυνα σε ολόκληρο το φυτό. Επιπλέον, η φυτοφαγία των αρπακτικών προκαλεί αλλαγές στο μεταβολικό προφίλ του φυτού ενώ μεμονωμένοι μεταβολίτες που παράγονται μετά από επαγωγή της άμυνας μπορούν να χρησιμοποιηθούν ως πηγές νέων εντομοκτόνων για την ενεργοποίηση της άμυνας του φυτού.Ωστόσο, τα επίπεδα γονιδιακής έκφρασης και το μεταβολικό προφίλ έχουν πολύ λίγο μελετηθεί.Τέλος, δίνονται στοιχεία σχετικά με την τεχνική RNA παρέμβασης, καταστολής ή σίγασης (RNAi) η οποία πολύ πρόσφατα έδειξε θετικά αποτελέσματα στον έλεγχο του *T. absoluta* αλλά δεν έχει εξετασθεί η τυχόν επίδρασή της σε κάποιον φυσικό εχθρό του *T. absoluta*.

Στο δεύτερο κεφάλαιο αυτής της διατριβής, μελετήθηκε η έκφραση των γονιδίων που διέπουν το μονοπάτι βιοσύνθεσης του Ιασμονικού Οξέος (ΙΟ) που ενεργοποιείται από τη διατροφή του *N. tenuis* σε φυτά τομάτας και οι διακυμάνσεις στα επίπεδα των μεταβολιτών που προκαλούνται μετά την επαγωγή της άμυνας. Δεκαπέντε νύμφες 3^{ης} ηλικίας του *N. tenuis* εγκλωβίστηκαν σε κάθε κορυφαίο και κατώτερο φύλλο φυτών τομάτας για 4 ημέρες για να προκληθεί η επαγωγή της άμυνας των φυτών. Μετά από αυτή την περίοδο τα αρπακτικά απομακρύνθηκαν. Όσον αφορά το *T. absoluta*, η προτίμηση ωοτοκίας, η διάρκεια ανάπτυξης της προνύμφης και το βάρος της νύμφης μειώθηκαν σημαντικά στα φυτά με *N. tenuis* σε σχέση με το μάρτυρα. Επίσης, τα ενήλικα *T. urticae* εμφάνισαν σημαντικά υψηλότερη τάση διαφυγής και μειωμένη επιβίωση σε σχέση με τα φυτά-μάρτυρες. Η μεταβολομική ανάλυση επιβεβαίωσε τα αποτελέσματα αποκαλύπτοντας ουσιαστικές διαφορές μεταξύ των φυτών της επέμβασης και του μάρτυρα. Οι μεταβολίτες που σχετίζονται άμεσα με την ενεργοποίηση του μονοπατιού του ΙΟ, όπως η πρόδρομος ουσία α-λινολενικό οξύ, είχαν αυξημένες συγκεντρώσεις. Η έκφραση των σχετιζόμενων με την άμυνα γονιδίων *PI-II, MYC2, VSP2* και *HEL* αυξήθηκε στα επάνω φύλλα ενώ τα *VSP2* και *MBP2*μόνοστα κάτω φύλλα. Είναι ενδιαφέρον ότι στα μεσαία (μη εκτεθειμένα στο *N. tenuis*) φύλλα, τα*VSP2, HEL* και *MBP2*υπερεκφράστηκαν, υποδεικνύοντας συστημική ενεργοποίηση της άμυνας του φυτού. Συμπερασματικά, η φυτοφαγία του *N. tenuis* προκάλεσε δυσμενείς επιπτώσεις στην ωοτοκία και στην ανάπτυξη του*T. absoluta* και στην επιβίωση και συμπεριφορά του*T. urticae*, ενώ η ανάλυση των φαινομένων που σχετίζονται με την επαγωγή της άμυνας του φυτού μέσω της φαινολογίας, της γενομικής και της μεταβολομικής του (phenomics, genomics και metabolomics) πρόσφερε πολύτιμες γνώσεις για την κατανόηση της φύσης των αμυντικών αντιδράσεων των φυτών και παρείχε χρήσιμα στοιχεία για πιθανές μελλοντικέςεφαρμογές στην ολοκληρωμένη αντιμετώπιση των εντόμων-εχθρών των καλλιεργειών.

Στο τρίτο κεφάλαιο αυτής της μελέτης, ερευνήθηκε το κατώτερο όριοπληθυσμιακής πυκνότητας νυμφών του *N. tenuis* ανά φυτό τομάταςπου απαιτείται για να επάγει την άμυνα του φυτού στο *T. absoluta*καθώς και το διάστημαπου η άμυνα του φυτού παραμένει ικανή να προκαλεί αρνητικές επιδράσεις στο *T. absoluta*. Για το λόγο αυτό, 3, 6 ή 10 νύμφες του *N. tenuis* εγκλωβίστηκαν σε κάθε κορυφαίο και κατώτερο φύλλο νεαρών φυτών τομάτας με τρία φύλλα συνολικά, για 4 ημέρες. Τα αποτελέσματα έδειξαν ότι η ωοτοκία του*T. absoluta* μειώθηκε σημαντικά μόνο στα φυτά που χρησιμοποιήθηκε η υψηλότερη πυκνότητα του *N. tenuis*. Περαιτέρω πειράματα έδειξαν ότι στα φυτά (με την υψηλότερη πυκνότητα

αρπακτικών)τα θηλυκά του*T. absoluta*εναπόθεσαν σημαντικά λιγότερα ωά σε σχέση με φυτά-μάρτυρες, 7 και 14 ημέρες μετά την απομάκρυνση των αρπακτικών. Η συστημική φύση τηςεπαγόμενηςάμυνας των φυτών επιβεβαιώθηκε επίσης σε όλες τις περιόδους καθώς και στο μεσαίο φύλλο υπήρχε μείωση της εναπόθεσης ωών όπως και στα νέα φύλλα που εκπτύχθηκαν κατά τη διάρκεια των πειραμάτων. Αυτά τα αποτελέσματα προσφέρουν πολύτιμες πληροφορίες για την πιθανή πρακτική εφαρμογήτης μεθόδου στην αντιμετώπιση του *T. absoluta* σε κρίσιμες πτυχέστης, όπως ο αριθμός των αρπακτικών που απαιτούνται ανά φυτό, η συστημική ενεργοποίηση και η διάρκεια διατήρησης των επιδράσεων που προκαλούνται.

Στο τελευταίο κεφάλαιο της διατριβής, εξετάστηκε η πιθανή συμβατότητα της διαχείρισης του *T. absoluta*με τη μέθοδο dsRNA με την παράλληλη χρήση του *N. tenuis*. Η διαχείριση εντόμων με τη μέθοδο RNAi έδειξε πρόσφατα υποσχόμενα αποτελέσματα και κατά του *T. absoluta*, ωστόσο η συμβατότητά της με τους φυσικούς εχθρούς του *T. absoluta* δεν έχει αξιολογηθεί. Συγκεκριμένα, αυτή η μελέτη είχε στόχο να διερευνήσει εάν το dsRNA (dsTa-αCOP) που σχεδιάστηκε για να στοχεύει το γονίδιο *T. absoluta*-αCOP θα μπορούσε να προκαλέσει δυσμενείς επιπτώσεις στον κύριο παράγοντα βιολογικού ελέγχου του, το αρπακτικό, *N. tenuis*. Η έκθεση του *N. tenuis*μέσω της στοματικής οδού σε dsRNA (dsNt-αCOP) που σχεδιάστηκε για να στοχεύει το *N. tenuis*-αCOP, είχε ως αποτέλεσμα 61%, 67% και 55% μείωση του επιπέδου μεταγραφής του σε σύγκριση με τις επεμβάσεις όπου χρησιμοποιήθηκε σακχαρόζη, dsGFP και dsTa-αCOP, αντίστοιχα. Επιπλέον, σημαντικά υψηλότερη θνησιμότητα 57% του *N. tenuis* καταγράφηκε στιςεπεμβάσειςμε dsNt-αCOP σε σύγκριση με σακχαρόζη (7%), dsGFP (10%) και dsTa-αCOP (10%). Επίσης, η ημερήσια αρπακτική ικανότητα(33-39 ωά)*Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) ανά ενήλικο*N. tenuis* μειώθηκε δραματικά σχεδόν στο μισό σε άτομα*N. tenuis* που επέζησαν μετά την επέμβαση με dsNt-αCOP. Αυτά τα πειράματα έδειξαν για πρώτη φορά ότι ο μηχανισμός RNAi είναι λειτουργικός στο *N. tenuis* και ότι είναι δυνατός ο κίνδυνος έκθεσής του μέσω της στοματικής οδού. Αντίθετα, το dsTa-αCOP δεν προκάλεσε θανατηφόρα και υποθανατηφόρα αποτελέσματα στο *N. tenuis* κατά την από του στόματος έκθεση. Αντιθέτως, όπως αναμενόταν, η έκθεση του *T. absoluta* στο dsTa-αCOP είχε ως αποτέλεσμα 50% θνησιμότητα. Στο πλαίσιο της αξιολόγησης του κινδύνου βιοασφάλειας της διαχείρισης εντόμων με τη μέθοδο RNAi, η διερεύνηση των επιπτώσεων σε οργανισμούς μη-στόχους είναι απαραίτητη προκειμένου να συμπεριληφθεί αυτή η μέθοδος σε προγράμματα ολοκληρωμένης αντιμετώπισης. Με βάση τα αποτελέσματα αυτής της εργασίας, ο έλεγχος με

τη μέθοδο RNAi μπορεί να είναι συμβατός με το βιολογικό έλεγχο του *T. absoluta* από τον φυσικό εχθρό του *N. tenuis*, υποδεικνύοντας ότι η προσέγγιση RNAi θα μπορούσε να ενταχθεί σε στρατηγικές ολοκληρωμένης αντιμετώπισης του *T. absoluta*.

Συμπερασματικά, η διατριβή αυτή εστιάζει στην ανάπτυξη φιλικών προς το περιβάλλον μεθόδων διαχείρισηςτου*T. absoluta* και του*T. urticae* χρησιμοποιώντας καινοτόμες τεχνικές και υποδεικνύει μεθοδολογίες βιοδοκιμών. Τα ευρήματα προσθέτουν χρήσιμη γνώση για την ανάπτυξη νέων στρατηγικών διαχείρισης του *T. absoluta* καιτου*T. urticae*.

Επιστημονική περιοχή: Ολοκληρωμένη αντιμετώπιση

Λέξεις κλειδιά: *Tuta absoluta, Tetranychus urticae, Nesidiocoris tenuis,* Ολοκληρωμένη Αντιμετώπιση,έντομο-εχθρός, αρπακτικό, τομάτα, επαγωγή άμυνας του φυτού, γονίδια, Ιασμονικό Οξύ, *MPB2, MYC2, PI-II, VSP2, HEL*,μεταβολίτες, RNAi, αCOP

ACKNOWLEDGEMENT

Encountering the completion of my Thesis work has made me feel immense happiness to show my gratitude towards many people who have made this experience an unforgettable and pleasant journey. Their contribution is exceptionally admirable and after reaching the destination here I am to thank all of them who made this journey a successful one.

At this moment of accomplishment, I pay homage to my esteemed mentor, Dr. Dionysios Perdikis, Associate Professor, Laboratory of Agricultural Zoology & Entomology, Department of Crop Science, Agricultural University of Athens (AUA), Athens, Greece for his keen involvement, guidance and constant supervision throughout the entire period of my research work till the completion of my Thesis. This work would not have been possible without his guidance and persistent encouragement.

Merely an acknowledgement is never enough to show my cordial respect towards Indian Council of Agricultural Research (ICAR), New Delhi for providing me with the international doctoral fellowship.

I would like to show my high gratitude to Dr. Andreas Voloudakis, Assistant Professor, Laboratory of Plant Breeding and Biometry, Department of Crop Science, Agricultural University of Athens, Greece, from whom I have received valuable suggestions and advice's during my research and thesis work. I shall ever be grateful to him for providing the scholarship for MSc Agriculture at AUA, Greece through ERASMUS MUNDAS programme which further provided me the opportunity to pursue my PhD at AUA, Greece.

I want to extend my gratitude to Prof. Argyro Fatinou, Laboratory of Ecology and Environmental Science, AUA, Athens, Greece from whom I learned to overcome many difficulties.

Here I want to extend my deepest sense of gratitude to Dr. Thanasis Kaldis for his constant guidance and training during my research and publications.

I wish to offer my deep sence of gratitude to the examination committee for my PhD apart from my supervisor and co-supervisors: Research Professor Alberto Urbaneja, Center at the Valencian Institute of Agricultural Research (IVIA), Associate Professor Maria Pappas, Democritus University of Thrace, Assistant Professor Konstantinos Aliferis, A.U.A. and Associate Professor Nickolas Kavallieratos, A.U.A.

Words of thanks would be less to express my special gratitude to Prof. Ir. Guy Smagghe who have provided me the opportunity to train in the advanced Laboratory of Bioscience Engineering, Ghent University, Belgium. I offer my deepest gratitude to iPlanta, European Coorporation of Science and Technology for offering the short term scientific mission and allowing me to learn about dsRNA mediated pest management. My words will fall short to show my gratitude to my post doctoral fellow and mentor Dr. Clauvis Niji Taning at Ghent University. I am also grateful to my lab mates Rohit, Deise, Christine, Xieping, Wenxin, Jacqueline for their humble gesturesthroughout my stay in Ghent.

I am equally thankful to Dr. George Papadoulis, Head and Professor, Laboratory of Agricultural Zoology and Entomology AUA, Athens for his valuable suggestions and comments prior and during the progress of my work.

I also extend my sincere appreciation to Dr. Konstantinos Aliferis, Laboratory of Pesticide science, AUA, Athens for training me to use metabolomic tools for phytoanalytical studies.

I wish to thank my PhD colleague and friend Sofia Dervisoglou for helping me throughout my study period in AUA in an extraordinary way.

I am grateful to Dr. A. Tsagkarakis, Assistant Professor, and Dr. Lena Panou, Laboratory of Agricultural Zoology and Entomology, AUA, Athens for all the encouragement during the progress of my work.

I wish to thank my laboratory mates Zoe, Eleni, Kostas, Vasilis, Vagelis, Evangelia, Vassiliki for sharing a healthy laboratory environment that helped me a lot to perform well .

Special mention to my beloved friends Sofia Kondrali, Marta and Vassiliki for encouraging me and bringing positivity into my life during the doctoral study.

During my stay in Athens, I have seeked Embassy of India for many instances which is why they deserve special mention. His excellency Mr. Amrit Lugun, Ambassador of India, Embassy of India, Athens and Mr. Krishnanand Jha, Second Secretary (Consular), Embassy of India, Athens who did not only help me during COVID PANDEMIC but exceptionally assited me for DOATAP recognition of my home university, Assam Agricultural University (AAU), Jorhat, India in 2021. I have the previledge to meet Her Excellency, Dr. Shamma Jain Ambassador of India, Embassy of India, Athens during 2018-19, who have been my constant inspiration throughtout the study period.

Regarding my DOATAP certification, which is a recognition of foreign degree by Greek National Council of Education, I have been immensely supported by His Excellency, Vice Chancellor Dr. Bidyut kumar Deka, and Dr. Anup Kumar Das, Joint Registrar, AAU, Jorhat who promptly responded to my requests and connected with the National Greek Council of Education. Special thanks are also due to Professor Effie Tsakalidou from AUA for her constant support throughout the DOATAP procedure and Professor Epameinondas Paplomatas from AUA for his helping me approach the procedure correctly.

I wish to mention the name of my master's supervisor Dr. Anjumoni Devee, Associate Professor AAU, who never failed in guiding me throughout my academic career including master's research, application of my PhD fellowship and procedures of DOATAP recognition of my degrees.

My vocabulary utterly fails in expressing my accolade to my mother Smt Swapna Devi for her constant support and trust. I dedicate this work to my beloved father Late Druna Sarmah who is blessing me from heaven. This work wouldn't have seen the light of the day, had I not been constantly inspired by my sister Momi and brother Vishal. Their enormous support have always been my strength. I wish to thank my supportive husband

Ankur, and my parents in law Smt. Bulbuli Sharma and Shree Dwijen Sharma for their encouraging words throughout the study. I am grateful for their kind blessings, love, patience, overwhelming support and inspiration being so far from me.

Behind every big achievement lies the help, guidance and support of many people. Acknowledgement is the simplest way to show our utmost gratitude towards them. Here I take this great opportunity to thank all those whose name could not find a separate place but have helped me directly or indirectly in the present investigation.

Last but not the least, I thank almighty, for guiding me through my life, giving me opportunities, and fulfilling my dreams.

With my permission this work has been checked for plagiarism

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“I dedicate my thesis to my beloved father Late Druna Sarmah who is blessing me from heaven in every step of my life”.

CHAPTER 1

General Introduction

1. 1.Tomato cultivation and its widespread economic importance

Tomato,*Lycopersicon esculentum* (Mill) (Solanaceae) is one of the most important food and cash crops with fresh and processed consumption worldwide. The genus *Lycopersicon* is divided into two groups, namely the *Eulycopersicon* and *Eriopersicon*. The cultivated *L. esculentum* belongs to *Eulycopersicon.*The first ample description of tomato was made by Miller in 1768 (Rick et al. 1978; Díez & Neuz 2008; Caruso et al. 2022; Van Andelet al. 2022). The origin of tomato is South America and was introduced in Europe by Spanish explorers during the 16th century. It was not grown as edible food crop till 17th century. Soon after, it was spread in several parts of the world including Asian countries like India and Japan, and others of the Mediterranean region in the 18th century (Jenkins et al. 1948; Díez & Neuz 2008; Boccia et al. 2020).

Tomato is the most consumed vegetable in the world. As per a recent study 100 g of ripe tomato contains 17.71 g protein, 4.96 g lipid, 5.96 g carbohydrates, 50.60 g total sugar and pH of 3.83 with energy of 34.67 kcal (Ali et al. 2009). It is rich in vitamin A and vitamin C, with moderate levels of folate and potassium along with vitamin E, iron, minerals, β-carotene, lycopene, flavonoids, organic acids, phenolics, and several water-soluble vitamins (Giovanelli & Paradise 2002; Coyago-Cruz et al. 2018). In addition, it is rich in antioxidants such as chlorogenic acid, rutin, plastoquinones, tocopherol, and xanthophylls (Bhargava & Srivastava 2019). Red tomatoes contain lycopene, which is one of the most important anti-oxidant sources (Strati & Oreopoulou 2011; Arain et al. 2018). It is helpful in prevention against chronic diseases, like cancer (Perveen et al. 2015; Melfi et al. 2018). In addition, tomato exhibits a bright color which contributes in the aesthetics of the meal and stimulates the appetite (Sainju & Dris 2006; Lagerkvist et al. 2023). According to FAO, tomato is overall a nutritious source of food for a balanced diet for children and adults (Arata et al.2020; Herforth et al. 2020).

The cultivated tomato can be grown as short or long duration vegetable crop, covered or uncovered, for direct consumption or processing. Commercial cultivation of tomatoes can be divided into two major systems depending on the cultivation method. Cost-intensive glasshouse structures facilitated with modern technology, open-field fresh market tomatoes, and open-field tomatoes for processing and traditional farming systems (Lang 2004; Heuvelink 2005). In 2016, tomato was the sixth most valuable cultivated crop of worth US$ 87.9 billion. In global basis, tomato is cultivated over an area of 4 million hectares with a

total production of 180 million tons and average productivity of 27,202kg/ha. Production of 182,256,458 MT was recorded in 2018, where Europe accounted for 23,291,126 MT (http://www.fao.org/faostat/en/#data/Q). In terms of tomato production, Greece is ranked 7th in Europe with a volume of 885,150 tons in 2018 (EUROSTAT 2020). In India, tomato is cultivated on 0.52 million hectares with production of 7.42 million tons (productivity 14,269 kg/ha) (FAO 2020). The most important tomato-producing countries are China, India, Turkey, USA, Egypt, Italy, Iran, Spain, Mexico, and Brazil (FAO 2020). Tomato cultivation brings forth one of the major sources of livelihood in many parts of the world, providing great potential for local employment opportunities because it can be processed into purées, juices, ketchup or sold as canned and dried tomatoes (Singh 2004; Robinson et al. 2013; Padilla-Bernal et al. 2015; Pankaj et al. 2022).

Tomato is highly susceptible to several pests and diseases (Cortez et al. 2002; Fuentes et a. 2017). About 200 species of arthropods attack tomatoes (Lange & Bronsen 1981; Perdikis et al. 2008). Tomatoes are prone to insect pests from the seedling stage until harvest and further during storage in warehouses. At a global level, insect pests may incur about 50% of yield losses in case that crop protection measures are not taken (https://www.actahort.org/index.htm) (Zalom 2003; Silva et al. 2017). The main insect pests are the silverleaf whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) , the corn earworm *Helicoverpa zea* (Boddie) and the cotton bollworm *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), tomato fruit borer moth *Neoleucinodes elegantalis* (Guenée) (Lepidoptera: Crambidae), the South American tomato pinworm *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), the aphids*Myzus persicae* (Sulzer) and *Macrosiphum euphorbiae* (Thomas) (Hemiptera: Aphididae), the two spotted spider mite *Tetranychus urticae*Koch (Acari: Tetranychidae) and the thrips (Thysanoptera: Thripidae) (Perdikis et al. 2008; Zeist et al. 2018). The honey dew produced by aphids and whiteflies is the substrate for the secondary developmentof sooty mold which deteriorates the photosynthetic capacity of the plant and reduce the commercial value of the fruits (Messelink 2020). Moreover, aphids, whiteflies, and thrips are vectors of tomato viruses. Among all the arthropods, *T. absoluta* is the most devastating pest of tomato (Lopes Filho 1990; Picanço 2000; Campos & Biondi 2019). Finally, the two spotted spider mite is also reported as a key pest of tomato (Vacante 2015; Wang et al. 2016).

2. 2.The South American tomato pinworm *Tuta absoluta* (Meyrick)(Lepidoptera: Gelechiidae)

Tuta absoluta was originally described as *Phthorimaea absoluta* by Meyrick in 1917 based on specimens collected in Huancayo, Peru. After its description, this species was placed by different researchers in three differentgenera. Firstly, it was placed in the genus*Gnorimoschema* Busck 1900 by Clarke (1962). In 1967, Povolny, placed it in the genus *Scrobipalpula* (Povolny 1964). Finally, after revision of the Gnorimoschenini (Gelechiinae), the genus *Scrobipalpuloides* was placed into *Tuta* (Povolny 1987) (https://www.cabi.org/isc/).

T. absoluta is a small moth with silvery grey scales and black spots on the fore wings. It follows a complete metamorphosis and undergoes four stages, namely, egg, larva, pupa and adult. The egg length ranges from 0.65 mm ~ 1 mm, whereas that of larvae ranges from 0.5 mm to 7.8 mm. Adult body is 5–7 mm long with a wingspan of 10–14 mm with brown-grey-silver speckled wings (Imenes et al. 1990).

This pest was first described outside Peru during 1960s, when its distribution expanded to other south American counties (Barrientos et al. 1998; Gonzalez-Cabrera et al. 2011; Li et al. 2020). During early 1980s, it was already spread into Argentina, Bolivia, Brazil, Chile, Colombia, Ecuador, Paraguay, Uruguay, and Venezuela (Barrientos et al. 1998; Estay 2000; Desneux et al. 2010; Campos et al. 2017). According to Biondi & Desneux (2019) agricultural trade had facilitated the spread of the pest in S. America. Entry into Europe took place through eastern Spain in 2006 (Urbaneja et al. 2007). In 2008 it was recorded in The Netherlands (Potting 2009) and in 2009 in UK (Campos et al. 2017). In mainland Greece and Crete, it was reported in 2009 (Roditakis et al. 2010). According to EPPO, *T. absoluta* was recorded in Cyprus and southern parts of Germany in 2010. In 2014, its distribution was expanded eastwards reaching India (Sridharet al. 2014). In Africa, *T. absoluta* invasion initiated from northern countries in 2007 and within few years the pest was recorded in South Africa (Biondi et al. 2018; Mansour et al. 2018). In conclusion, *T. absoluta* is distributed in Europe, Africa, the Middle East, and parts of Asia (Desneux et al. 2010; Campos et al. 2017; Biondi et al. 2018); it was recently reported in China in 2020 (Zhang et al. 2020) (Fig. 1).

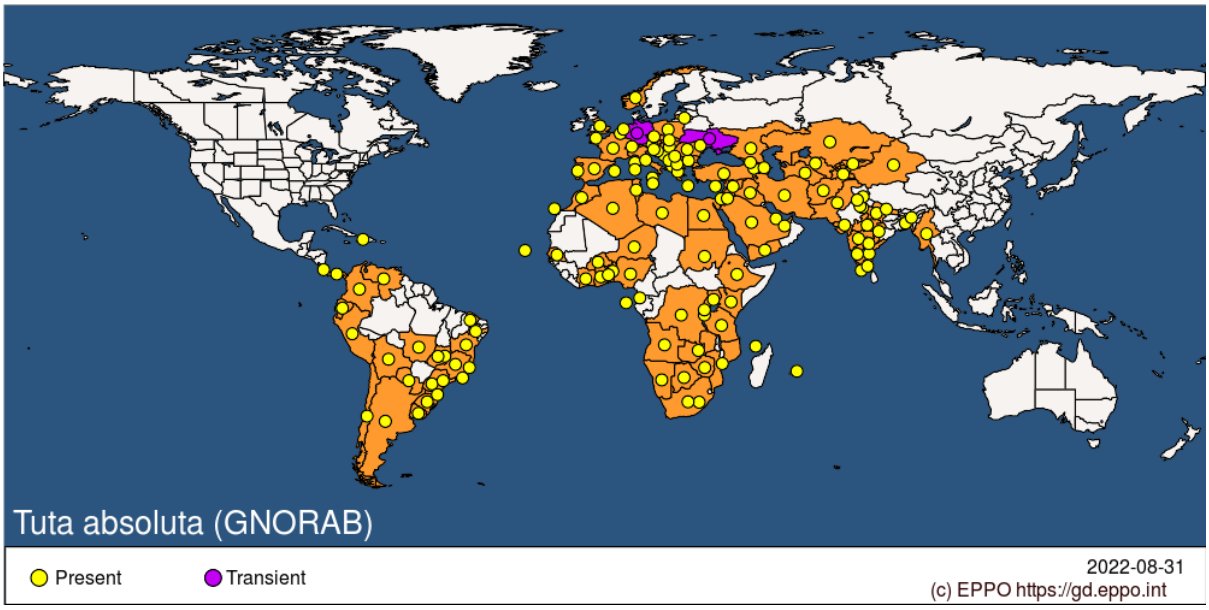


Fig. 1.1. Current distribution of *Tuta absoluta*. Yellow dots represent records of its presence in the various countries and purple dots represent the transient phase of the pest in these locations (Source: European and Mediterranean Plant Protection Organization: EPPO).

T. absoluta adapted well in new habitats of tomato production system worldwide (Desneux et al. 2011; Campos et al. 2017). Multivoltinism characteristic, short generation time, wide availability of its main host plant and relatively wide thermal adaptability greatly favour its invasive nature (Desneux et al. 2010; Garzia et al. 2012; Guedes & Picanço 2012; Biondi et al. 2018). Estimations show that *T. absoluta* increased its range radius by an average of 600 km per year (Campos et al. 2017). Global trade and transportation facilitate the rapid dispersal and spread of this invasive pest (Biondi & Desneux 2019; Guimapi et al. 2020).

T. absoluta host plant range includes species from the Solanaceae family such as eggplant (*Solanum melongena* L.), potato (*Solanum tuberosum* L.), tobacco (*Nicotiana tabacum* L.), jimson weed (*Datura stramonium* L.), the African eggplant (*Solanum aethiopicum*L.), and the European black nightshade (*Solanum nigrum* L.) (Sylla et al. 2019). *T. absoluta* is also recordedin members of the Amaranthaceae family such as wild spinach (*Chenopodium album* L.), sugar beet (*Beta vulgaris* L.), and common bean (*Phaseolus vulgaris* L.) (EPPO 2009).

The eggs are laid singly on leaves of the upper part of the canopy, on the young stems and on sepals (Garzia et al. 2012; Sylla et al. 2019). The underside of the leaf is preferred in plants before flowering. However, adult females prefer both sides for oviposition in plants

after flowering (Torres et al. 2001). The most preferred part is young, expanded leaves (73%), stems (21%), sepals (5%), and finally green fruits (1%) (Estay 2000). The 3rd and 4th leaves were often preferred by females for egg laying (Dervisoglou et al. 2022). Time required for egg hatching is3.90 ±0.13days at 27 °C (Torres et al. 2001). According to Silva et al. (2015) the larval developmental period is 8.91 ± 0.09 days (d) at 25 ± 1 °C, and 9.87 ± 0.29 d at 30 ± 1 °C. Pupal period lasted for 7.08 ± 0.23d at 25 ± 2°C (Gharekhaniet al. 2014) and 9.84 ± 0.16d at 26 ± 0.5°C (Rostami et al. 2017). A single female lays 260 eggs during lifetime at 27°C (Uchoa-Fernandes et al. 1995). The life cycle completion from egg to adult of *T. absoluta* required 23.8± 0.65 d at 25°C (Gharekhani & Ebrahimi 2014), 19.06 ± 0.56 d at 25°C (Silva et al. 2015), and 26±0.50 d at 33°C (Martins et al. 2016). The optimum temperature for egg, larval and pupal development were 26, 28, and 30°C, respectively (Martins et al. 2016). Temperature thresholds for development of egg, larva and pupa were estimated to be 6.9 ± 0.5, 7.6 ± 0.1 and 9.2 ± 1°C, respectively (Urbaneja et al. 2013). The thermal constant from egg to adult has been estimated to be 453.6-degree days (DD). Accordingly, thermal constants for egg, larva and pupa were 103.8 ± 1.4, 238.5 ± 0.5 and 117.3 ± 5.3 DD, respectively (Barrientos et al. 1998). *T. absoluta* may complete up to 10 generations annually in South America (Korycinska & Moran 2009; Desneux et al. 2010) in tomatoes. In Italy, 9 generations per year (Sannino &Espinosa 2010) and in Spain 13 generations per year (Vercher et al. 2010) have been reported in tomato crop systems.

*T. absoluta*larvae mine into the leaf feeding on the mesophyll tissue, creating galleries leaving the epidermis intact (Fig.1.2).According to Garzia et al. (2012), photosynthetic capacity is highly reduced by the larval phytophagy, reducing the production levels in open field and protected tomato crops (Shashank et al. 2015).The larvae cause damage on tomato fruits creating small holes beneath the sepals (Garzia et al. 2012). The damaged fruits loose their market value, leading to reduced farmers'profit. Apart from this, the infested fruits are difficult to be located and removed from the produce, making post-harvest procedures cost-ineffective for the farmer (De Castro et al. 2013). Larvae also feed on the flower buds (Biondi et al. 2018). Although the most devastating damage is done through the leaf mesophyll feedingand fruit boring, the larva stem mining deters the growth and development of the tomato plant (Torres et al. 2001). Such infestations lead to commercial downgrading of the produce and also affect the market chain of tomato (USDA 2011).

(a)

(b)

(c)

(d)

Fig.1.2. (a) Damage on tomato leaflet by larvae of *Tuta absoluta**, (b) View of mesophyll feeding by larvae of *T. absoluta*, (c) Damage on tomato fruit by *T. absoluta* larvae (Photo: D. Perdikis) (d) Damage by *T. absoluta* at field (Photo: D. Perdikis)

Without control measures, *T. absoluta* causes serious damage to the crop that has led to shortage of supply and affects market price of tomatoes (Gharekhani & Ebrahimi 2013; Zekeya et al. 2017; Negeri and Getu 2018;Tadele& Emana 2018). *T. absoluta* may cause up to 100% damage of crop without control methods beingtaken (Lopez 1991; Desneux 2010; Hamidattu 2020). Therefore, the management of the American pinworm should be carefully

**The photos without a reference are prototypes taken by the author.*

administered. One of the first approaches to control this pest was the development of less susceptible oradministered. One of the first approaches to control this pest was the development of less susceptible or resistant cultivarsfrom wild tomatoes (de Azevedo et al. 2003; Guedes & Picanço 2012). Cultivars such as Raha, Quintini, ES9090F1(Sohrabi et al. 2016), and Grandella from Iran (Rostami et al. 2017) are table tomatoes, and moderately resistant against *T. absoluta*. In the armoire of control methods, the preventive agronomic ones such as ploughing, manuring, optimal irrigation, crop rotation, and soil solarization and sanitation are highly recommended. Removal of infested plant tissues should be strictly followed in order to avoid any contamination in the upcoming crop or reduce the pest dispersal (Guedes & Picanço 2012). The succession of a solanaceous crop should be avoided for a tomato crop system (Baldwin et al. 2016; Sylla et al. 2019). Removal and destruction of damaged fruits and plant tissues should be routinely followed as well as avoiding growing tomatoes in late dry season (Guedes et al. 2012; Mansour et al. 2018; Sylla et al. 2018). Insect-proof screens have been shown to limit and/or prevent *T. absoluta* infestations (Biondi et al. 2016; Mansour et al. 2018; Han et al. 2019). Biondi et al. (2015) reported α-cypermethrin-treated insect-proof nets to be more effective than the non-treated ones, against *T. absoluta* adults.

Monitoring of *T. absoluta* populations in tomato crops is performed by trapping males in delta traps baited with female pheromone (Witzgall et al. 2010). For monitoring, traps are recommended at the rate of 1 trap in greenhouses smaller than 2,500 m^2 and as a general rule 2–4 traps per ha. In addition, in open field, the placement of 5 traps per ha have been recommended (Al-Zaidi 2009; Caparros et al. 2013).

Mating disruption has been proved effective in glasshouse conditions and contribute to control the moth population (Vacas et al. 2011; Caparros et al. 2013). The ability of *T. absoluta* to reproduce parthenogenetically, although at a low rate, may reduce the efficacy of mating disruption (Silva 2008; Caparros et al. 2012; Grant et al. 2021).The use of light traps 1 trap/ 0.05 ha limited the leaf damage significantly at low or moderate population density of *T. absoluta* during the summer-winter season, while they were ineffective in winter-summer, as the density of tomato leafminer increased by the end of the tomato cultivation season (Cocco et al. 2012). Mass trapping mating disruption using baited pheromone have been reported to be effective in reducing the population of *T. absoluta* (Witzgall et al. 2010) in greenhouse tomato crops (Caparros et al. 2013; Cocco et al. 2013; Megido et al. 2013; Desneux et al. 2021). Sex pheromone water traps at a density of at 20 to 25 traps/ha in

glasshouse condition or 40 to 50 traps/ha in open fields effectively contribute in the control this pest (Bolckmans 2009).

Economic threshold levels of *T. absoluta* are considered as 1-3% damaged fruits or 1 mine per leaf or >25 males per trap per week (Desneux et al. 2012, Urbaneja 2013). The trap monitoring records have been used to estimate economic threshold such as that reported by Benvenga et al. (2007) (i.e. 45±19 males/trap/day). Long term use of the commercially available pesticides has led to resistancedevelopment against organophosphate and pyrethroid insecticides detected in Chile, Brazil, Argentina, and Europe (Salazar & Araya 2001; Lietti et al. 2005;Haddi et al. 2012; Silva et al. 2015). Resistance has been also recorded against abamectin (Silva et al. 2016), indoxacarb (Silva et al. 2011; 2016) and chlorantraniliprole (Silva et al. 2019). In Greece, resistance to diamides, which acts on insect ryanodine receptors,is reported for *T. absoluta* (Roditakis et al. 2015), and similarly in UK (Grant et al. 2019) and in Brazil (Silva et al. 2016b). Therefore, prevention of resistance development is a key aspect of any IPM strategy against *T. absoluta* (Guedes et al. 2019; Desneux et al. 2021).

A high number of natural enemies, i.e. more than 160 taxa, have been reported to supress *T. absoluta* populations (Zappala et al. 2013; Ferracini et al. 2019). Biological control of *T. absoluta*greatly relies on the augmentation and conservation of omnivorous mirid predators such as *Nesidiocoris tenuis* (Reuter) and *Macrolophus pygmaeus*(Rambur) (Hemiptera: Miridae), *Dicyphus bolivari* Lindberg and *Dicyphus errans* (Wolff) (Hemiptera: Miridae) and conservation of parasitoids like *Diadegma pulchripes* (Kokujev) (Hymenoptera:Ichneumonidae), *Bracon osculator* (Nees) (Hymenoptera: Braconidae) and *Necremnus* sp. (Hymenoptera: Eulophidae) (Desneux et al. 2010; Zappala et al. 2013; Ingegno et al. 2019; Soares et al. 2019). Recently, the mirid predators *N. tenuis* and *M. pygmaeus*have been reported as highly efficient biocontrol agents against *T. absoluta* in Europe (Biondiet al. 2013). *Bacillus thuringiensis* (Bacillales: Bacillaceae) is widely used whereas microbial organisms such as *Beauveria bassiana* (Hypocreales: Cordycipitaceae) has proven to be effective (Tsoulnara et al. 2016; Pan et al. 2020; Biondi et al. 2020). Entomopathogenic nematodes such as *Steinernema carpocapsae* (Weiser) (Rhabditida: Steinernematidae), *Steinernema feltiae* Filipjev (Rhabditida: Steinernematidae), and *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae) may also be effectiveagainst *T. absoluta* (De Luca et al. 2015; Amizadeh et al. 2019).

1.3. The two spotted sider mite *Tetranychus urticae* Koch (Acari: Tetranychidae)

Another key pest of tomato is the two-spotted spider mite *T.urticae* (Lange& Bronson1981; Ilias et al. 2017; Schmidt - Jeffris & Cutulle 2019). The great morphological variability of *T. urticae* led to a long list of synonyms of this species (Geijskes 1938; Gasser 1951). Bolland et al. (1998) comprehend the most widely used synonyms such as *Acarus telarius*(L.), 1758,*Tetranychus telarius* (L.) Duges, 1834.

Theexternal morphologyof *T. urticae*was first studied by McGregor (1950), and summarized by Bolland et al. (1998) and later revised by Migeon & Flechtmann (2004). The body of the *T. urticae*female adult is ca 0.5 mm long and oval in shape with reddish-orange or greenish yellow color. The adult has distinctively one visible large spot on each side of its idiosoma because of accumulation of body waste; for this reason, the spots are not visible in the larval stage. Females have elliptical body with 12 pairs of dorsal setae. During overwintering females are mostly orange colored. Males are much smaller than females. The male's body is also elliptical, but has tapering caudal end (Fasulo & Denmark 2000; Vacante 2015).

Eurasiahas been reported as *T. urticae*'sarea of origin (Navajas 1998). *T. urticae*was found in deciduous fruit trees of USA (Tuttle & Baker 1968). It wasrecorded in Polandin 1985, Netherlands in 1961, Switzerland in 1984, Italy and France in 1986, and Greece in 1988 (Migeon 2005; Xue et al. 2020). During 1970's this spider mite was recorded in Africa (Carey 1982). In Asia it was reported during 2004 (Ho et al. 2004; Xue et al. 2020). Currently it is recorded inmany countries in Africa, Asia, Australasia, Europe, North, Central and South America (Fig. 1.3). Its establishment in new areas is facilitated by its adaptability into different climatic conditions and its dispersal by walking or by mechanical transfer through wind, plants, tools and people (Zhang 2003).

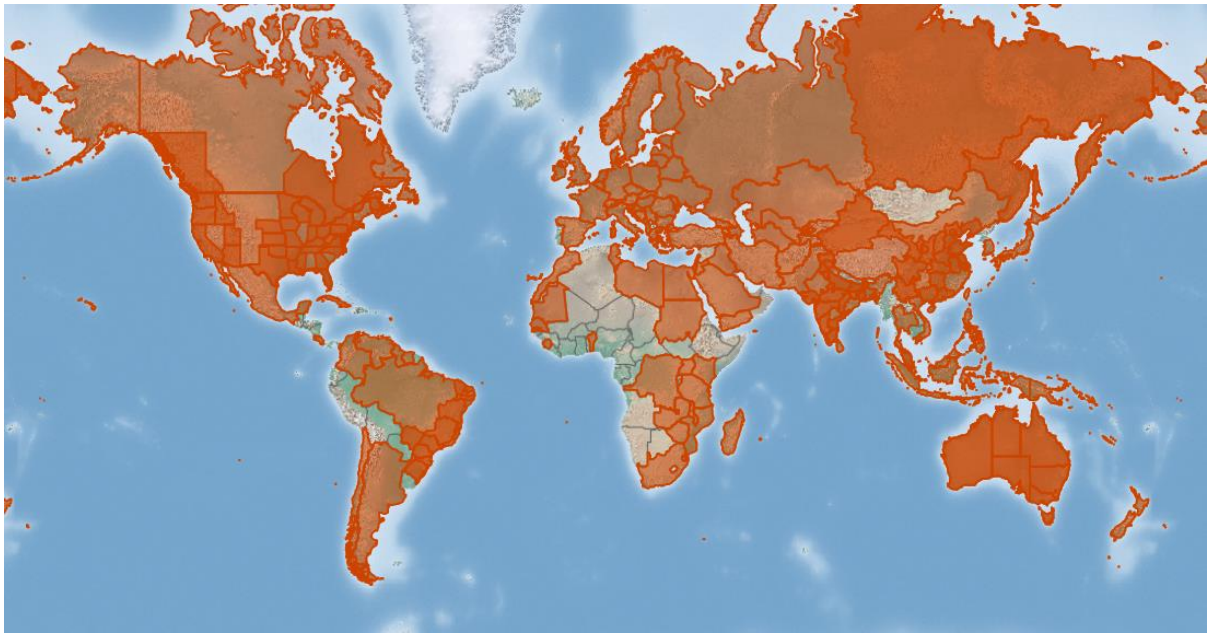


Fig.1.3. Current distribution of spider mite*Tetranychus urticae*worldwide.
Source: Centre for Agriculture and Bioscience International (CABI) accessed on 03/09/2022.

The life cycle of *T. urticae*consistsof egg, larva, two nymphal instars and adult. The two nymphal instars are named protonymph and deuteronymph. The eggs hatch in 28.8 ± 0.35 days at 12.5±0.01°C (Bayu et al. 2017), 2.3±0.01 d at 27±1°C (Shih et al. 1976), 3.50±0.13 d at 33±1°C (Riahi et al. 2013).According to Kasap (2004), the female developmental period is 15.5±0.15 d at 20±1°C and 6.5±0.18 d at 35±1°C, respectively. The female longevity is 12.91± 1.65 d at 25±1°C, and 3.56±0.54 daysat 30± 1°C. Male longevity is 6.80±1.13 d at 25±1°C and 3.20±1.66 d at 30±1°C (Riahi et al. 2013). A female may lay 100 eggs at 25 ±1°C (Sabelis 1982) and 143.9 eggs at 29±1°C(Shih et al. 1976). According to Sarmento et al. (2011a) a single female may lay 5 to 15 eggs per day indicating its high reproduction potential. The estimated lower thermal threshold for egg development was 12°C, 12.5°C for nymphal development, and 11.63°C from egg to adult. The thermal constantfor egg to adult development was estimated to be 127.81DD (Bayu et al. 2017).

T. urticae has a very wide range of host plants (>1000 species) including vegetables, trees, ornamentals plants (Van Leeuwen 2013; Papapostolou 2020). Among the most preferred host plants for *T. urticae* are tomato, pepper, cucumber, strawberry, soy, apple, grape, and citrus (Grbić et al. 2011; Sousa et al. 2019), but it has been reported on French beans (Maniania 2008), watermelon (Afsoon et al. 2019), ornamental plants such as chrysanthemum, rose and orchids (Razmjou et al. 2009; Sousa et al. 2019), maize (Bui et al. 2018; Martin & Latheef 2019) and other species (https://www.cabi.org/isc/datasheet/53366#REF-DDB-74664).

T. urticae inserts its stylets into the leaf tissue and suck out the contents of the mesophyll cells. Mostly they feed on the abaxial surface of the leaf where they are protected from UV rays. *T. urticae* produces mechanical injury by damaging chlorophyll which produces the grey spots which may later become darkas the number of necrotic cells increases (Nachman & Zemek, 2002; Agutet al. 2018). The feeding activity may lower the concentrations of nitrogen, phosphorous, and protein and as a result disrupts cell physiology, reducing photosynthesis and,at the same time, injects phytotoxic compounds (Tomczyk & Kropczynska 1985; Meck et al. 2013; Agutet al. 2018). Such attack may incur significant yield losses and may also reduce the market value of the produce due to aesthetic damage (Attia et al. 2013)

For cultural control high humidity levels may reduce the reproductive potential of females of *T. urticae* (Sabelis 1986; Duso et al. 2004; Attia et al. 2013) but this technique may not benefit the crop. The economic threshold of this pest has been estimated to be 50 mites per leaflet on strawberry (Greco et al. 2005), and 50 mites per leaflet on tomato (Meck et al. 2013). Commercially available acaricides are extensively used to control *T. urticae*. Carbamates, organophosphates, pyrethroids, organotin miticides, benzoylureas, METI-acaricides, hydrazine carbazate, macrocyclic lactones, N-substituted halogenated pyrrole,abamectin, fenpyroximate, and spirocyclic compounds like spirodiclofenhave beenthe most widely used synthetic acaricides against *T. urticae* (Van Leeuwen et al. 2007; Attia et al. 2013; Pavela 2015; Reddy & Dolma 2018; Guedes 2019). In organic crops, the use of nicotine extracted from tobacco, rotenone from *Derris elliptica* (Fabaceae), and pyrethrum from *Chrysanthemum* (Asteraceae) has shown positive results (Attia et al. 2013). However, repetitive use of the acaricides leads to the development of pest resistance. The resistance against organophosphates was identified in 1950's. Since then, *T. urticae* has developed resistance to carbamates, avermectins, milbemycins, clofentezine, hexythiazox, diflovidazin, Oganotin miticides and in more than 90 different compounds (https://www.pesticideresistance.org/) (Sparks & Nauen 2015; Papapostolou et al. 2020). Males of *T. urticae* may develop resistance to carbamates that can be transferred to future generations. As a result resistance in males is of great concern (Attia et al. 2013). Because of its resistance to many pesticides, the two-spotted spider mite has been considered the "most resistant pest species" in many areas worldwide (Martínez-Huasancheet al. 2019;Wuet al. 2019).

Biological control is the most promising control strategy against *T. urticae* (Sarmento et al. 2011b; Cabello et al. 2012; Navajas et al. 2013; Liu et al. 2020). Notably,

biological control of spider mite is commonly practiced by using predatory mites such as *Phytoseiulus persimilis* (Athias-Henriot) (Mesostigmata: Phytoseiidae) and *Neoseiulus californicus* (McGregor) (Mesostigmata: Phytoseiidae) (Knapp et al. 2020). *P. persimilis*is very efficient against *T. urticae* and feeds on all stages of *T. urticae* even on densely packed webs (Oliveira et al. 2007). *N. californicus* performs well in less densely packed web. The shortcomings of using *P. persimilis* include its sensitivity to environmental conditions such as temperature fluctuations and relative humidity lower of 60% as well as quality and quantityof its prey *T. urticae* (Abad-Moyanoet al. 2010; Ditilloet al. 2016). Additionally, *P. persimilis* is impeded by molecules released by the glandular trichomes on tomato leaves and then its effectiveness may not be satisfactory (Kennedy 2003; Sharma et al. 2017). *N. californicus* is less voracious and has lower reproductive rate than *P. persimilis* (Attia et al. 2013; Kazak et al. 2015). *Feltiella acarisuga* (Vallot) (Diptera: Cecidomyiidae) is an active predator of *T. urticae* and is used commercially. *F. acarisuga* consumes more spider mitesthan *P. persimilis* (Mo et al. 2007).*P. persimilis* lacks compatibility with *F. acarisuga* as it may consumeeggs of *F. acarisuga*if prey is limited (Gillespie et al. 1998). Chemical applications disrupt natural enemies of *T. urticae* and thus, use of selective chemicals have to be prioritized in IPM programs (Van Leeuwen et al. 2010; Ferreira et al. 2015; Liu et al. 2020).

Taking into consideration the above-mentioned information about the pest status of*T. absoluta* and *T. urticae*it becomes clear their high economic importance, however, their chemical control is associated with rapidresistance and health risks. Therefore, recruitment of their natural enemies can be a useful tool in IPM programs and provide satisfactory results and sustainability in the management of these two serious crop pests.

One of the most effective natural enemies of *T. absoluta* and *T. urticae*is the predator *N. tenuis* (i.e. Urbaneja et al. 2009; Desneux et al. 2011; Naselli et al. 2016; van Lenteren et al. 2018).

1.4. The zoophytophagous predator *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) its geographical distribution, biology and its utility as promising biological control agent

The zoophytophagous predator *N. tenuis* is one of the most promising biological control agents, colonizing tomato crops and suppressing key pests like *T. absoluta* (Arnó et al. 2009; Mollá et al. 2009, 2010; Urbaneja et al. 2009; Desneux et al. 2010; Siscaro et al. 2019), *B. tabaci* and *T. vaporariorum* or thrips such as *Frankliniella occidentalis* (Pergande) (Goula

1985; Calvo et al. 2009; Mirhosseini et al. 2019; Soares et al. 2019), and mites such as spider mite *T. urticae* (Sanchez et al. 2009; Naselli et al. 2016; Gavkare et al. 2017).

N. tenuis was first described as *Cyrtopeltis tenuis* by Reuter (1895). Researchers have given several names to this mirid species; the *Nesidiocoris volucer* by Kirkaldy (1902), *Cyrtopeltis javanus* by Poppius (1914), *Cyrtopeltis ebaeus* by Odhiambo (1961), and *Nesidiocoris tenuis* by Kerzhner (1988) were most referred.

*N. Tenuis*life cycle consists of egg, nymph, and adult stages. The egg is inserted in the plant tissue, mostly in the stem of the plant. The nymph passes through 5 instars to reach adulthood. The greenish-grey-brown adult possesses scattered black-grey spots on the wings, the antennae and the legs and a transverse black band behind its head in pronotum. Its length ranges from 3.01–3.08 mm for males and 3.22–3.26 mm for female (Kim et al. 2016), whereas Sylla et al. (2016) and Adeleye & Seal (2021) describes the adult of *Nesidiocoris tenuis* to be 6 -10 mm in length.

*N. tenuis*is a cosmopolitan mirid bug widely distributed in Mediterranean region (Kerzhner & Josifov 1999). Conclusively, according to CABI (https://www.cabi.org/isc/datasheet/16251#todistributionDatabaseTable) it has been reported from Europe, Australia, Africa, Japan, Middle East, and South America (Fig. 1.4). Recently it has been reported in USA (Esparza-Diaz et al. 2021) where its populations may effectively contribute in the control of tomato pests (Roda et al. 2020).

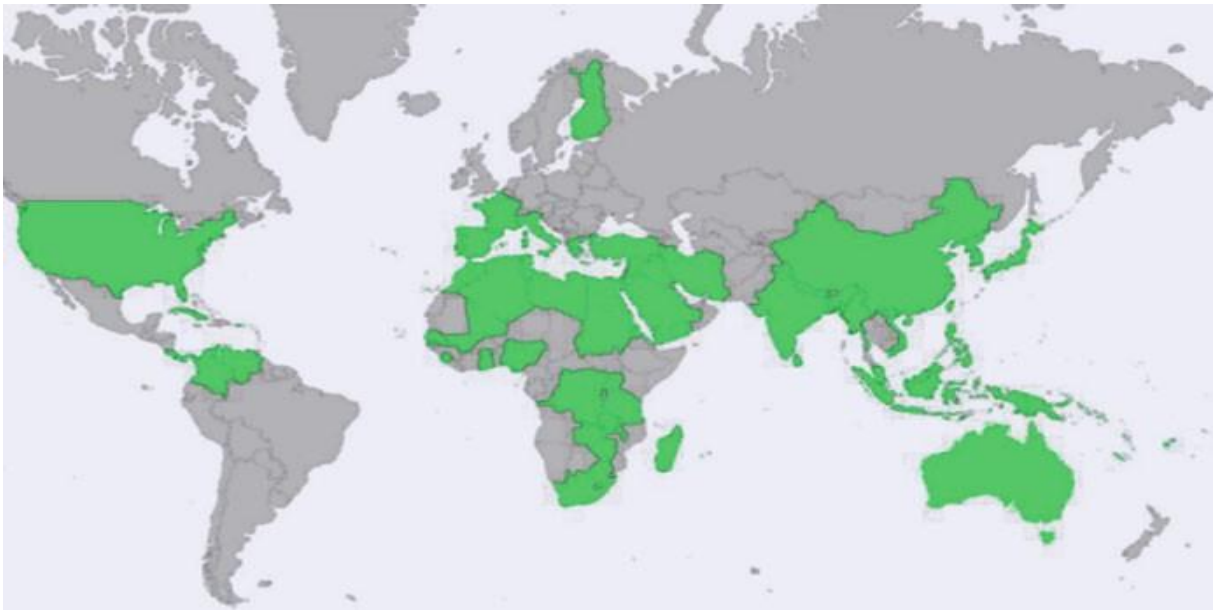


Fig.1.4. Current distribution of mirid bug *Nesidiocoris tenuis* worldwide.
Source: Centre forAgriculture and Bioscience International (CABI) accessed on 03/09/2022.

This polyphagous mirid has a wide range of host plants from Solanaceae family such as tomato, tobacco, eggplant, potato, *S. nigrum*and the Cucurbitaceae family such as *Lagenaria siceraria* L.(CAB International 1971; Alomar & Albajes 1996; Bhatt & Patel 2018).

*N.tenuis*is a zoophytophagous predator able to feed both on prey and planttissue (Naranjo & Gibson 1996; Coll 1998). The phytophagy nature of *N. tenuis* aids in fulfilling the predator's requirements of water but also of nutrients (Thomine et al. 2020). This behavior supports establishment and persistence of its populations on the crop with positive impacts on its efficacy as a biological control agent (Biondi et al. 2016). Plant feeding also facilitates the predator to establish itself in sweet pepper crops (Calvo & Urbaneja 2004; Urbaneja et al. 2005). However, several studies have reported that high populations of *N. tenuis* in periods of prey absence may impede plant development and cause abortion of flowers (Arnó et al. 2006; Calvo et al. 2008; Castañe et al. 2011) together with punctures on fruits (Arnó et al. 2010; Chinchilla-Ramírez et al. 2021). Sanchez & Lacasa (2008) reports that plant feeding results into visible necrotic rings along with flower abortion, but interestingly they argued that such abortion increases the weight of the remaining fruits, which finally may compensate the yield loss to a certain level. In Northern Europe this predator is considered as a tomato pest under certain cases (Pérez-Hedo et al. 2016; Moerkens et al. 2020). In fact its economic damage vastly varies among geographical regions (Carnero et al. 2000; Sanchez et al. 2006; Siscaro et al. 2019), temperature and tomato variety (Siscaro et al. 2019). In Greece, caged experiments have shown that 32 individuals per tomato plant did not cause flower abortion or plant damage (Perdikis et al. 2009). Castañé et al. (2011) stated that such damages are related to predator-to-prey abundance. In fact,the phytophagy behaviour of *N. tenuis* makes it the most controversial biocontrol agent (Chinchilla-Ramírez et al. 2021). Provision of sugars, genetic selection for strains of less phytophagy or use of companion plants have been recently reported as means of reducing the damage potential of *N. tenuis* populations (Urbaneja-Bernatet al. 2019; Chinchilla-Ramírez et al. 2020; Konan et al. 2021, respectively).

Average egg hatching time is 30.8±0.19 daysat 15±1°C, 17.0±0.19d at 20±1°C and 6.3±0.02 d at 35±1°C. The nymph requires 55.9±0.37 dat 15±1 °C, 21.2±0.39 d at 20±1°C and 8.6±0.49 d at 35±1°C to complete its development. The thermal constant for the egg development was 148.6±12.3 DD and for its nymphal development was estimated to be 182.3±8.9 DD (Sanchez et al. 2009). Duration of life cycle was estimated to be 94.2±0.3 days at 15±0.5 °C, 17.8 ± 0.2 dat 27±0.5°C and 14.0 ± 0.2 d at 32±0.5°C (Hughes et al. 2009).

N. tenuis has been successfully incorporated into IPM programs of *T. absoluta* and is released in greenhouses with the first appearance of its target pest (Calvo et al. 2012). The pre-plant release of the predator *N. tenuis* contributes to its establishment on the crop and effectively control *T. absoluta* in greenhouses (Perdikis et al. 2015). In a study by Calvo et al. (2009) inoculative release of *N. tenuis* at the rate of 4 individuals/ plant released once effectively controlled *B. tabaci* in greenhouse tomatoes in Murcia (southeast Spain). Release of *N. tenuis* at a rate of 4.5 mirids per plant in the presence of *T. absoluta* has been recommended by Mollá et al. (2011) as a threshold that guarantees satisfactory pest control for the rest of the season. In addition, *N. tenuis* may naturally colonize open field or greenhouse tomato crops and its populations can efficiently control pests (Sanchez et al. 2008; Perdikis et al. 2011; Pérez-Hedo et al. 2016).

1.5. Induced resistance by phytophagy of *N. tenuis* and upregulation of expression of defense related genes

In addition to its efficacy in reducing pest numbers and ability to sustain its populations in the absence of prey, recently it has been revealed that *N. tenuis* can have another beneficial role in pest control by inducing plant defense responses. In fact, earlier studies have shown thatplant-feeding behaviour of zoophytophagous predators activates defence mechanisms in plants (Kessler & Baldwin 2004; Inbar & Gerling 2008; Lundgren 2009). According to Han et al. (2018) upon pest attack, the plant produces antibiotic and antixenotic compounds which negatively affect the herbivores, whereas systemic signals alarm the rest parts of the plant (Davis et al 1991; Zhang and Baldwin 1997; Koch et al. 2016; Malook et al. 2019). This is achieved through the activation of plant defence pathways. Induced resistance in plants against biotic stresses is mainly attributed to the phenylpropanoid and octadecanoid pathways mediated by salicylic acid (SA) and jasmonic acid (JA), respectively (Zhao et al. 2009; Scott et al. 2010; He et al.2011). These pathways produce a number of plant defensive secondary metabolites in intermediate steps, which affect the growth and development of phytophagous insects and also activate the release of volatiles which repel the pests and may attract the pests' natural enemies (Howe & Jander 2008; He et al.2011). JA mediated plantdefenseresponses act against phloem feeders (i.e. whiteflies, aphids), mites, and insects with chewing mouth parts (Walling 2000; Schaller 2008). JA mediates octadecanoidpathways during activation of induced resistance, whereas the salicylic acid (SA) mediates phenylpropanoid pathway (Zhao et al. 2009; Scott et al. 2010; He et al.2011). In most cases, JA and SA defense signaling pathways are mutually antagonistic in dicotyledonous species (Bari et al. 2009). The induction

of defence pathways is triggered by elicitors (Walling 2000; Bonaventure et al. 2011). This may be confined to the site of trigger called as locally acquired resistance (LAR) or it may be expressed systematically known as systemic acquired resistance (SAR), or it may be induced systemic resistance (ISR) (Karban & Myers 1989; Walling 2000; Aljbory & Chen 2018). In contrast to SAR which is triggered by the accumulation of salicylic acid, ISR instead relies on signal transduction pathways activated by jasmonate and ethylene(Conrath 2006).In regards to pest control, systemic resistance offers protection of the entire plant including parts not directly punctured by the predator and this isan important attribute for success in pest management.

Recent studies have shown that plant defenses triggered by *N. tenuis* on tomato plants could cause significant adverse effects on insect and mite pests. Tomato plants pre-punctured by *N. tenuis* caused 35 % reduction in infestation by *T. urticae* (Pérez-Hedo et al. 2018). Pappas et al. (2015) reported reduced oviposition of *T. urticae* due to pre-exposure of plants to theclosely related mirid bug *M. pygmaeus*. Pérez-Hedo et al. (2015) demonstrated that tomato plants punctured by *N. tenuis* released Herbivore Induced Plant Volatiles (HIPVs) which made them less attractive to the whitefly *B. tabaci* andmore attractive to its parasitoid *Encarsia formosa*(Gahan) (Hymenoptera: Aphelinidae). Naselli et al. (2016) demonstrated that pre-exposure of tomato plant to all mobile stages of *N. tenuis* can be less attractive to whitefly *B. tabaci* andmore attractive to *E. formosa*. So, the zoophytophagous mirid bugs benefit the plants by directly feeding on the pest as well as by inducing plant mediated resistance against the invading pest. Furthermore, HIPVs released after plant defense induction by *N. tenuis*may trigger defenses in nearby healthy tomato plants and this has been recently explored as a new finding in pest control (Pérez-Hedo et al. 2021).

Plant defence induction causes high level of expression in defence related genes. This is important information because certain genes may be associated with the activation of certain pathways and act locally or have a systemic effect. The most widely studied gene *Proteinase Inhibitor* (PI) shows higher level of expression due to injury in the plant tissue and is regulated by the methyl ester of JA (MeJA) and its metabolic precursor, 12-oxo-phytodienoic acid (Walling 2000; Stintzi et al. 2001; Zhang et al. 2018). A conjugate of JA and ammino acids are formed by the enzyme Jasmonoyl-isoleucine conjugate synthase 1 (JAR1) which forms JA-Ile (Staswick & Tiryaki 2004; Howe et al. 2018).JA-Ile binds to the F-box protein Coronatine-insensitive 1 (COI1); the jasmonate ZIM domain (JAZ) repressor proteins are degraded with the help of functional modules including a repertoire of COI1–JAZ (Coronatine Insensitive1–Jasmon Atezim Domain) coreceptors by coupling with jasmonoyl-L-isoleucine; JAZ binds to

transcriptional activators such as MYC2 and as a result repress JA signalling (Thines et al. 2007; Howe et al. 2018). There are two branches of signalling pathways for JA associated pathways. MYC2 is one of the branches,which regulates defence against herbivory, and the second branch ethylene response factor (ERF), which regulates plant defence against necrotrophic pathogens (Pieterse et al. 2012; War et al. 2018). Vegetative storage protein (VSP) is induced upon injury (Dombrecht et al.2007; Kinoshita & Betsuyaku 2018) but has also been reported to be involved in defence against insect herbivory in *Arabidopsis thaliana* (L.) (Liu et al. 2005; Kinoshita & Betsuyaku 2018). The MYC2 branch of the JA signalling pathway regulates the *VSP2* and *lipoxygenase 2* (*LOX2*) genes. The ERF branch is associated with ERF 1 octadecanoid-responsive *Arabidopsis* 59 (*ORA59*).

Pérez-Hedo et al. (2015b) demonstrated that tomato plants punctured by *N. tenuis* showed higher expression of the genes *ASR1* (abscisic acidstress ripening protein 1) and (*PIN2*) wound-induced proteinase inhibitor II precursor In addition, Naselli et al. (2016) demonstrated that all motile stages of *N. tenuis* can trigger defensive responses in tomato plants and equally upregulate the expression of the genes *ASR1* and *PIN2*. Pappas et al. (2015) reported reduced performance of *T. urticae* and upregulated expression of the gene *Proteinase inhibitors (PI)* due to phytophagy of *M. pygmaeus*.

The plant defence induction modifies plant physiology and may cause extended changes in the production of secondary metabolites. For this reason, precursors of JA mediated defence pathway, JA and cis-(+)-12-oxo-phytodienoic acid (OPDA) have been foundas expected in high levels in plants punctured by mirid bug *N. tenuis* (Pérez-Hedo et al. 2015b; Bouagga et al. 2018; Zhang et al. 2018). However, the changes in the individual metabolites produced after defence induction by *N. tenuis* have not been studied.

1.6. Metabolomic profiling as a tool to study plant defense mechanism

The metabolites responsible for the induced resistance against a pest can be exploited to offer a deeper insight of the changes in the phytohormones within a plant (Ku et al. 2016; De Falco et al. 2019). Such information may be helpful in gene editing, via e.g. CRISPR (clustered regularly interspaced short palindromic repeat) which is a family of DNA sequences found in the genomes of prokaryotic organisms such as bacteria and archaea, to produce more insect-resilient crop varieties (Han et al. 2019; Zhao et al. 2020). In addition, precise knowledge of the metabolites may offer valuable information to develop biopesticides based on selected metabolites as a new pest management method to activate plant defense and developresilient

and environmentally compatible pest control methods. Furthermore, in depth knowledge of the metabolite profile changes due to feeding damage in the plant may contribute in pest management through classical plant breeding. For example, a wild tomato species with higher level of identified secondary metabolite that is associated with a defense mechanism, may be crossed with a high yielding cultivar in order to achieve a more tolerant variety.

The study of comprehensive qualitative and quantitative profile of a large number of small molecules (less than or equal to about 1500 daltons present in the biological system) is called “Metabolomics” (Trethewey et al. 1999; Fiehn et al. 2000; Kalaiselvi et al. 2019). Oliver et al. (1998) first used the term ‘metabolome’ to define the metabolites adjunct to living tissues.The metabolome may be considered as the chemical phenotype of a plant that interacts with its environment (van Dam & Meijden 2011). Metabolomics is an interdisciplinary approach which combines chemical analyses, data from experimental design and pre-processing analyses for biological interpretation of phenotypical observations (Brown et al. 2005; Aliferis et al. 2011; Kalaiselvi et al. 2019). Metabolites are considered as the final response of a biological system to environmental changes or gene regulations. The metabolome is, therefore, the most predictive of phenotypical records (Dettmer et al. 2007; Kuehnbaum et al. 2013).

Metabolomic studies are mostly comprised of Gas Chromatography (GC), Liquid Chromatography (LC), Nuclear Magnetic Resonance (NMR) Spectroscopy,and Mass Spectrometry (MS) (Krishnan et al. 2005; Ward et al. 2010; Yang et al. 2020). In NMR, interaction of resonances of magnetic nuclei and the external magnetic field is estimated (Hatada & Kitayama 2004; Aliferis et al. 2011). NMR provides structural analysis of metabolites produced in cell fluids, crude extraction, intact tissues or in an organism (Manzo 2016). One of the pioneer works by Kirk et al. (2005) suggested that NMR metabolomic analyses can be employed for distinguishing the metobolome of two species of *Senecio* from their hybrids. On the other hand, MS is an analytical technique that measures the mass-to-charge ratio of ions. MS is highly sensitive and facilitates the identification of the compounds present in a system and detect the components it is comprised of. According to Villas-Boas et al. (2005), the combined approach of chromatography with the mass-spectrometry enables us to gain a clear chemical analysis of a composite biological system. The masses of ionised molecules and its unit atoms are simply combined and exhibited by the spectrum. MS is a rather user-friendly procedure and the output is interpreted with available software.

In general, the metabolites are distinguished inprimary metabolites and secondary metabolites. The primary metabolites are directly involved in the vital functions of a plant

such as growth, development, and reproduction. Secondary metabolites are produced in response to plant-to-plant interactions (i.e. competition for light and nutrients, environmental adaptation, odors, herbivores, pollinator attractors, and defense stimuli of the plant) (Seigler 1998; Buchanan et al. 2015). A plant is capable of producing a large number of secondary metabolites to reciprocate to the pests and pathogens as well as the beneficial arthropods. These secondary metabolites play important role in the signaling pathways (War et al. 2018). Barah et al. (2013) suggested that any change in the secondary metabolites also influences the primary metabolites.

Plant feeding by arthropods causes several changes in the metabolomic profile of the plant (Aliferis et al. 2011; Yang et al. 2020). In fact, the defence mechanisms governed by the defence related genes results into upregulation or downregulation of the metabolites participating in the respective activated biosynthetic pathways changing the metabolome of the plant (Zhang et al. 2018). Marti et al. (2013) reported changes in concentration levels of at least 30 metabolites of maize plants when infested by cotton leafworm *Spodoptera littoralis* (Boddie) (Lepidoptera: Noctuidae). Another interesting study revealed that upon attack of *Manduca sexta* (L.) (Lepidoptera: Sphingidae) or *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae), the damaged plant tissues of tomato plant were caused several changes in their metabolomic profile, quantitatively and qualitatively, which also variedamong the different insect pests used (Steinbrenner et al. 2011). Widarto et al. (2006) reported that the chewing by caterpillars of *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) and *Spodopteraexigua* (Hübner)(Lepidoptera: Noctuidae) in *Brassica rapa* L. change the metabolomic profile of the plant. Bouagga et al. (2018) mentioned that herbivory produces a conjugate of volatiles referred as herbivore-induced plant volatiles (HIPVs) (Mumm et al. 2010; Naselli et al. 2017), which are a blend of aldehydes, esters, alcohols, terpenes and many other aromatic compounds.The study by AlJabr et al. (2017) showed the potential of using secondary metabolites as insecticides since secondary metabolites such as phenylpropanoids and coumarin, mixed with artificial diet, exhibited toxicity to thered palm weevil *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae).

1.7. RNAi mediated gene silencing of *Tuta absoluta* and its possiblecompatibility with biological control

Within the efforts to increase the efficacy of IPM packages in the control of serious insect pests, RNAi based pest management methods have shown promising results. Substantial

amount of lethal effects were recorded by RNAi-mediated methods on insect pests belonging to Diptera, Coleoptera, Hymenoptera, Orthoptera, Blattodea, Lepidoptera and Hemiptera (Yang et al.2011; Christiaens et al. 2020; Mezzetti et al. 2020).

RNA interference (RNAi) is a post-transcriptional gene silencing pathway, triggered by double-stranded RNA (Lindbo et al. 1993; Jorgensen 1995; Hammond 2005; Liu et al. 2020) (Fig. 1.5). DsRNA acts as an elicitor molecule and is processed into small RNAs by Dicers or Dicer-like proteins [DCLs from the dicer gene identified in *Drosophila melanogaster*Meigen (Drosophilidae: Diptera) (Bernstein et al. 2001; Baulcombe 2004; Lewsey et al. 2018)]. Small interfering RNAs (21-24 nt) bind to Agronuate (AGO) protein and other related proteins (Rnase H enzymes) to form RNA-induced silencing complex (RISC) which contains an RnaseH-like domain responsible for target degradation (Huvenne & Smagghe 2010; Zhu &Palli 2020) (Fig. 5). Fire et al. (1998) were the first to demonstrate the application of exogenous dsRNA that silenced the homologous endogenous mRNA in the rhabditid nematode *Caenorhabditis elegans* and along with Coll (1998) coined this phenomenon as RNA interference (RNAi). In recent decades, RNAi has been considered as a feasible mechanism for silencing gene expression in several organisms, and it can be employed in selective agricultural pests, including insect pests (Huvenne & Smagghe 2010; Kaldis et al. 2018).

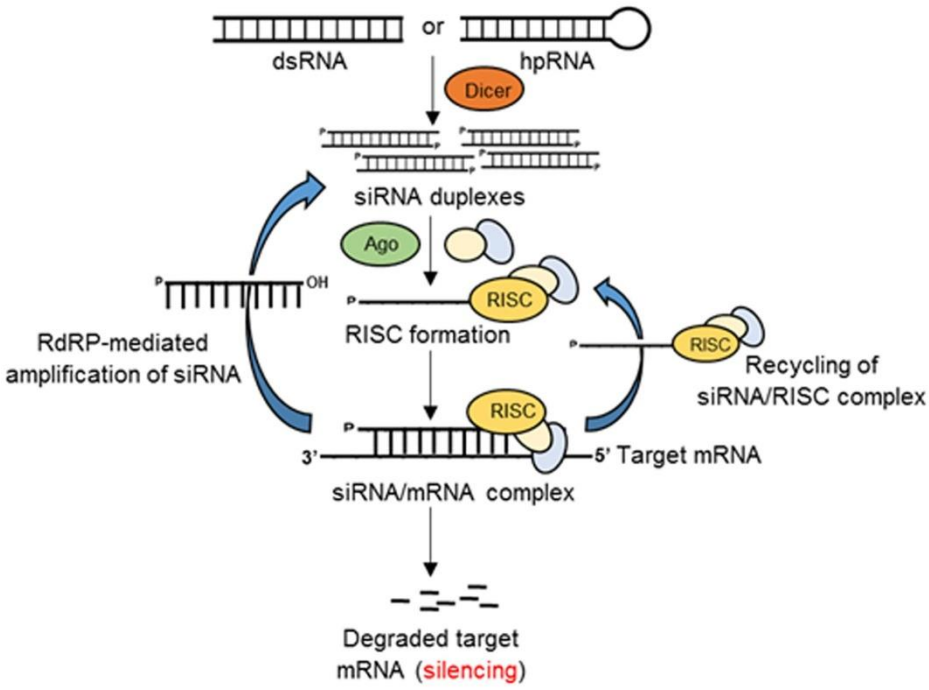


Fig. 1.5. Mechanism of the RNAi pathway (Source: Majumdar et al. 2017).

In recent studies RNAi mediated pest management of *T. absoluta* has come to light. Camargo et al. (2016) demonstrated that*T. absoluta* larvae exposed to dsRNA targeting the genes *V-ATPase* (Vacuolar ATPase-A) and *AK* (Arginine kinase) increased mortality, reduced damage on leaf tissues and caused ~60% reduction in the respective gene transcript accumulationwhich indicates the impact of gene silencing in *T. absoluta*. They delivered dsRNA through leaflet petioles immersed in an aqueous solution mixed with dsRNA or through *in planta*-induced transient gene silencing where the dsRNA molecule was uptaken by plant immersed in the solutionand via plant the larvae of *T. absoluta* acquired the dsRNA. Bento et al. (2019), delivered dsRNA targeting *juvenile hormone inducible protein* (*JHP*), *juvenile hormone epoxide hydrolase protein* (*JHEH*), *ecdysteroid 25-hydroxylase* (*PHM*), *chitin synthase A* (*CHI*), *carboxylesterase* (*COE*), and *arginine kinase* (*AK*) through expression in *Escherichia coli* HT115 (DE3) and via artificial diet in larvae of *T. absoluta* and observed larval mortality and significant decrease in transcript accumulation. In another study by Rahmani & Bandani (2020), dsRNA targeting *V-ATPase* was applied on the surface of the tomato leaf which were fed by *T. absoluta* larvae. This resulted into larval mortality of 40% and gradual reduction in gene expression. Therefore, RNAi is a method effective against *T. absoluta*; however, RNAi-mediated control of *T. absoluta* may cause side effects on *N. tenuis*, its major biocontrol agent,which effects however have not been investigated so far. On the other hand, *N. tenuis* is very effective in the control of *T. absoluta* but its use is limited by its long establishment period (i.e. up to 12 weeks according to Pérez-Hedo et al. 2020) and it may cause damage on the crop (Castañe et al. 2011). This being the case, the combined use of *N. tenuis*with alternative methods such as RNAi may improve the efficacy of sustainable management programs against *T. absoluta.* Therefore, any side-effects of RNAitools effective in the control of *T. absoluta* should be searched on *N. tenuis*.

In fact, RNAi based pest management has beenassociated with the biosafety of beneficial organisms.The application of dsRNA should not harm the natural enemies that feed on the target or other pests of the plant and contribute in the their control in augmentative or conservation biological control systems. Ecological risk assessment of exposure of double-stranded RNA transcripts of Vacuolar-sorting protein SNF7, against western corn rootworm, have been tested to be safe for non-target biocontrol agents such as *Pediobius foveolatus* (Crawford), *Coleomegilla maculata* (DeGeer), *Poecilus chalcites* (Say), *Aleochara bilineata* Gyllenhal, *Chrysoperla carnea* (Stephens), and *Orius insidiosus* (Say) at field level (Bachman et al. 2015). Interestingly, lady bird individuals of *Hippodamia convergens* Guérin-Méneville, *Harmonia axyridis* Pallas, *C.maculata*, and *Coccinella*

septempunctata (L.) were administered with dietary RNAi toxicity assay for dsRNA of the *v-ATPase A* designed for western corn root borer *Diabrotica virgifera virgifera*LeConte. The results showed that RNAi caused adverse effects on the beneficial insects such as mortality (Pan et al. 2020). Therefore, biosafety risk assessment is an essential step to investigate the level of any effects on non-target organisms and has to be searched much more intensively in order to developRNAi based bio-pesticides (Papadopoulou et al. 2019; Christiaens et al. 2021).

SCOPE OF STUDY

Tomato is one of the most important crops worldwide but in the last two decades its productivity and sustainability is seriously threatened by the invasive and devastating insect pest *Tuta absoluta*. Reliance on chemical control has caused several drawbacks and therefore, more resilient control methods have been investigated. In this regard, biological control is the most widely used. *Nesidiocoris tenuis* is a zoophytophagous mirid predator that has been considered as the major natural enemy of *T. absoluta*in augmentative and conservation biological control schemes, almost worldwide. Apart from its efficacy in reducing *T. absoluta* populations, recent studies have shed light on innovative and little searched aspects of its role as a biological control agent. First to note, it has recently been proved that its phytophagy can trigger tomato plant defenses which cause adverse effects on tomato pests such as whiteflies or mites. Activation of plant defenses apart of its theoretical interest may be used by pest management practitioners as an innovative alternative method in pest control. Aiming to gain thorough knowledge on the plant defense effects triggered by *N. tenuis* on *T. absoluta,* the following research efforts have been implemented under the frame of this work:

1. The effects of plant defense activation were investigated on several biological traits of *T. absoluta* such as larval period, pupal weight, oviposition preference, and period of initiation of larval mines by neonate larvae.
2. The systemic nature of the defense effects were always searched due to their importance in offering protection of the entire plant i.e. including not punctured parts or plant tissues developed after puncturing by *N. tenuis*
3. The expression of sixgenes *VSP2, PI, MYC2, HEL, AOS* and *MBP2* associated with the jasmonic acid-related biosynthetic defense pathway was studied to identify genes associated with the effects produced
4. The metabolomic profile of *N. tenuis*-punctured plants in comparison to unpunctured plants was searched to reveal the effect of treatments on various metabolites related to essential biosynthetic pathways.
5. The above mentionedeffects were also examined in the case of another serious tomato pest, *Tetranychus urticae,* for comparison reasons because its feeding mode differs to that of *T. absoluta*.
6. The adverse effects on *T. absoluta,* their systemic nature, the gene-expression and the quantification and mapping of the metabolomic profile of tomato plants were finally

integrated to explore their associations and extract outcomes for further research efforts.

The potential of plant defense activation by *N. tenuis* through puncturing tomato plants has been suggested as avaluable innovative method in pest control. However, our knowledge on the density of the predator individuals required to initiate plant defense has not been investigated. This is particularly important in the case of *N. tenuis* since it may cause damage on the plant and thus it should have been used in as low as possible number per plant to prevent increase of its population. In addition, the period of persistence of the plant defense effects has not been searched against *T. absoluta* and this is also useful for potential practical use of the method. For these reasons, the density threshold of *N. tenuis* for plant defense activation against *T. absoluta* and the persistence of the effects have been searchedin a separate chapter.

Finally, the potential to combine *N. tenuis* with RNAi-mediated management of *T. absoluta*was investigated. Recent studies have revealed that RNAi can be effective in *T. absoluta* control; however, it has not been searched whether RNAi may cause any side-effects on natural enemies, including *N. tenuis*. A set of laboratory assays was designed and executed to assess whether RNAi-mediated control can be compatible with the biological control of *T. absoluta* by its natural enemy *N. tenuis*.

All these research efforts and outcomes, contribute original knowledge and cover recent research needs which can be valuable in the development of further research activities but also of other initiatives for their practical use.

The experiments with insects were carried out in the laboratoryof Agricultural Zoology and Entomology, Division of Crop Science, AUA, Athens. The cultivation of host plants and rearing of predator and preywere maintained in the glasshouse of Agricultural Zoology and Entomology Laboratory, AUA, Athens. The experiments related to the expression of defense related gene were performed at Laboratory of Plant Breeding and Biometry, Faculty of Crop Science, AUA, Athens. The metabolomic profiling of the samples were conducted at Laboratory of Pesticide Science, Faculty of Crop Science, AUA, Athens. RNAi based experiments were conducted at Laboratory of Plant Breeding and Biometry, Faculty of Crop Science, AUA, Athens in collaboration with Laboratory of Agrozoology, Department of Plants and Crops, Faculty of Bioscience Engineering, Ghent, Belgium.

CHAPTER 2

Metabolomic and genomic approach to study defense induction by *Nesidiocoris tenuis* against *Tuta absoluta* and *Tetranychus urticae* in tomato plants*

2.1. Abstract

Tomato, *Solanum lycopersicum* L. (Solanaceae) is one of the high value crops worldwide and currently, one of the most devastating insect pests limiting tomato production is South Americanpinworm *Tuta absoluta* (Meyrick). The larvae feed on mesophyll tissues, stems and fruitsof and may cause up to 100% yield loss. The phytophagous nature of one of its most widely used biological control agents, thepredatory mirid bug *Nesidiocoris tenuis* (Reuter), can trigger the defense responses in tomato plants, which however have been very littleassessed against *T. absoluta*. In addition, the expression of genes associated with the jasmonic acid-related biosynthetic defense pathway and the underlying fluctuations of metabolite levels have been largely unexplored. In the present study, fifteen 3rdinstar nymphs of *N. tenuis* were caged on each top and lower leaf of tomato plants for 4d to induce plant defense; after this period the predators were removed. In regards to *T. absoluta*, oviposition preference, larval period and pupal weight were significantly reduced in *N. tenuis*-punctured plants.In addition, *Tetranychus urticae* Koch adults exhibited a significantly higher escape tendency and reduced survival on *N. tenuis*-punctured plants. The results indicated a systemic nature of the protective effect. In the top leaves *PI-II*, *MYC2*, *VSP2*, and *HEL* genes were upregulated, while in the lower leaves only *VSP2* and *MBP2*; interestingly, in the middle (unpunctured) leaves *VSP2*, *HEL*, and *MBP2* were upregulatedindicating systemic signaling. Metabolomic profiling of *N. tenuis*-punctured plant in comparison to unpunctured plants revealed the substantial effect of treatments on various metabolites related to essential biosynthetic pathways. Metabolites directly associated with the activation of the JA defense pathway such as α-linolenic acid had increased concentrations. Collectively, our results demonstrate that phytophagy of *N. tenuis* caused adverse effects on *T. absoluta* and *T. urticae*, whereas the multi-omics approach (phenomics, genomics, and metabolomics) offeredvaluable insights into the nature of the plant defense responsestriggered by omnivorous predators and provide evidence useful for future applications in the pest control.

**Based on the publication: "Sarmah N, Kaldis A, Kalampokis I, Aliferis KA, Voloudakis A, Perdikis D (2022) Metabolomic and genomic approach to study defense induction by Nesidiocoris tenuis against Tuta absoluta and Tetranychus urticae in tomato plants. Metabolites 12: 838.*

2.2. Introduction

Plant and insect have co-evolved in a dynamic relationship. Plants exhibit an array of defensive strategies against pest attacks (Zhao et al.2009; Karban 2011; War et al. 2012) by developing various biochemical and morphological traits (Howe and Jander 2008; War et al. 2012). Induced resistance is qualitative or quantitative enhancement of plant's defense mechanism against pests in response to external physical or chemical stimuli (Maffei et al.2012; Pieterse et al. 2012; Pérez-Hedo et al. 2015). Plant allele chemical production is induced by the injury to the plant or by mechanical disruption of plant tissue. These mechanical disruptions evoke phytochemical responses such as cellular chemical changes, changes in cell adjacent to the damaged tissues andgeneralized changes in plant part or entire plant (War et al.2013). Wounding plant tissue may induce changes in protein, lipid and phenol metabolism. Phytochemicals produced by the damaged plant may be detrimental to insects (Howe & Jander 2008; War et al. 2012).

American pinworm tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is one of the key pests of tomato and may cause up to 100 % yield loss in absence of control measures (Desneux et al. 2010; Biondi et al. 2018). Control of *T. absoluta* widely relies on intensive insecticide spraying, which could lead to insecticide resistance development (Roditakis et al. 2017; Guedes et al. 2019; Richardson et al. 2020). Due to shortcomings of chemical based control methods, biological control has been developed asan alternative. Zoophytophagous insect predators *Nesidiocoris tenuis* (Reuter) and *Macrolophus pygmaeus* (Rambur) (Hemiptera: Miridae) are frequently employed against *T. absoluta*, whiteflies, aphids and spider mites (Feraccini et al. 2019). These predators have several attributes making them effective such as their polyphagy, plant feeding ability, establishment prior to pest infestation and efficient prey searching ability (Perdikis & Lykouressis 2000; Perdikis et al. 2011; Biondi et al. 2018; Sarmah et al. 2019).

Recent studies have shown that their phytophagy triggers defense rendering plants less prone to subsequent pest attacks. Due to their phytophagous habits, mirid predators wound plant tissues by inserting their mouthparts to ingest plant sap and other nutrients. This wounding process triggers the production of secondary metabolites that are toxic to herbivores and the release of herbivore-induced plant volatiles (HIPVs) which repel pests and/or attract their natural enemies. Tomato plants punctured by *N. tenuis* were less attractive to whiteflies and spider mites (Pérez-Hedo et al. 2015b; Pérez-Hedo et al. 2018a). Similarly, previous exposure to *M. pygmaeus* has negatively affected the reproduction of *Tetranychus*

urticae Koch (Acari: Tetranychidae) and thrips (Pappas et al. 2015; Zhang et al. 2018). This finding of the indirect beneficial effect of omnivorous predators, through their phytophagy has attracted the scientific interest aiming at developing new approaches in pest control, such as through the use of predator-pretreated plants in the nursery prior to their field transplantation for sweet pepper plants (also called pre-plant release method) (Bouagga et al. 2018).

Plant defense responses are induced after herbivore attack and function by producing phytochemicals, such as secondary metabolites or toxins, which make plants less attractive and their tissues less nutritious to pests providing defense (War et al. 2018). The induced plant resistance is based primarily on the activation of a network of defensive signal transduction pathways. Such resistance is initiated by the biosynthesis of several phytohormones such as jasmonic acid (JA), cis-(+)-12-oxo-phytodienoic acid (OPDA) (i.e. a precursor of JA), salicylic acid (SA), abscisic acid (ABA), gibberellic acid (GA), and ethylene (ET) (Howe & Jander 2008; Pieterse et al. 2012). A plethora of bibliographic references indicates that JA and OPDA play the most prominent role in plant response to herbivory. They belong to the wider family of oxylipins, which are oxygenated compounds, produced in chloroplasts, originating from α-linolenic acid (α-LeA) (Stintzi et al. 2001; Wasternack 2015). JA and OPDA accumulated in *M. pygmaeus* or *N. tenuis* infested plants (Pérez-Hedo et al.2015a; Bouagga et al. 2018; Zhang et al. 2018).

The production of plant defense metabolites and the activation of signaling pathwaysare governed by the expression of defensive genes. Proteinase Inhibitors (Pis) are the most studied defense-related proteins in plants (War et al. 2018), making plant tissues undesirable for herbivory by inhibiting proteolytic function in the mid gut of herbivores. *PI genes*are marker genes for JA and have been found to be upregulated in tomato or pepper plants previously exposed to *N. tenuis* or *M. pygmaeus* (Pappas et al. 2015; Pérez-Hedo et al. 2015b; Bouagga et al. 2018; Pérez-Hedo et al. 2018a). In addition to *Pi*s, the measurement of the transcriptional response of selected JA-induced marker genes could offer valuable information in searching the anti-herbivore plant responses. In fact, plants synthesize a variety of secondary metabolites in response to herbivory (Van Dam and Van der Meijimden 2011; Zaynab et al. 2018) and therefore, different genes may be responsible for differential metabolic responses triggered in plants. Studies on *Arabidopsis thaliana* and tomato elucidated the mode-of-action (MoA) of JA, of the JA receptor, and of other factors that trigger downstream signal transduction pathways, leading to the induction of defense-related genes (Reymond et al. 2000; Stintzi et al. 2001). Among them, vegetative storage protein 2

(*VSP2*), myrosinase-binding protein 2 (*MBP2*), hevein-like peptide (*HEL*), and allene oxide synthase (*AOS*) could be explored. *VSP2* exhibits anti-insect activities regulated by the JA-activated transcriptional factor *MYC2* (Liu et al. 2005; Verhage et al. 2011). *MBP2* is involved in producing toxins by affecting the metabolism of glucosinolates (Burow et al. 2009). *HEL* is an anti-microbial peptide, highly induced by insect pests that feed by chewing (Reymond et al. 2000). *AOS* is a component of the JA biosynthetic pathway (Ziegler et al. 2001). The above-mentioned genes could also be induced by OPDA, although its mode-of-action is not completely understood (Reymond et al. 2000; Stintzi et al. 2001; Taki et al. 2005).

Defense responses may occur in the plant organ originally attacked by a pest (local response) but may also be present in unexposed parts of the same plant (systemic response). Knowledge on the intensity level of these systemic effects and whether they may offer adequate protection against pests at the different plant parts is essential for future applications in pest control (War et al. 2012). Systemic response activation by *M. pygmaeus* and *N. tenuis* herbivory has been reported (Pappas et al. 2015; Zhang et al. 2018). Zhang et al. (2018) concluded that metabolites likely have a major role in the systemic effects. In addition, the increases in JA levels, triggered by phytophagy may be transient (Schittko et al. 2000), but changes in other metabolites may last for much longer periods (Karban & Baldwin 1997; Agrawal 1998). To approach an in-depth understanding of consequence of exposure of plants to insects, which results into the changes in molecular and biochemical mechanisms, a combined analysis of the transcriptome and metabolome reconfiguration may provide a comprehensive illustration. Metabolome represents a chemical phenotype of the plant that participates in physiological processes as a result of the plant-environment interaction. However, the chemical nature of omnivorous predators' plant feeding induced responses is still unknown. Based on the potentially essential role of metabolites in plant defense responses (Mhlongo et al. 2018), their levels in exposed and non-exposed plant parts were taken into consideration.

Metabolomics is the study of the small molecules identified and quantified in the cell fluid, tissue, biological system or an organism. The advance combination of nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) allows detection and comparison of chemical entities presented through chemometric software (Idle & Gonzalez 2007). Plants are capable of producing plethora of secondary metabolites likephenolics, terpenoids, flavonoids, alkaloids, glucosinolates in response to attack of pest and pathogen (Wu & Baldwin 2010). As defence mechanism, secondary metabolites used by plants act as

feeding deterrents or toxins that decrease food intake or food-utilization efficiency (Gabrys &Tjallingii 2002; Kim et al. 2008) or reduce chances of survival by attracting natural enemies (Behmer et al. 2011; Unsicker et al. 2009). The identifications and concentration levels of the such metabolites can indicate the potential defensive capabilities of plant metabolites on insect herbivores. Targeted analyses are helpful if previous knowledge suggests that a specific metabolite or metabolite class like flavonoids or glucosinolates (Krastanov 2010).

Furthermore, the exploitation of the associations between gene expression and metabolite levels in punctured vs unpunctured plant parts will offer the means to develop resilient and environmentally compatible control means in pest management, i.e. through classical plant breeding or gene editing technologies (Ku et al. 2016; de Falco et al. 2019; Han et al. 2019; Zhao et al. 2020), or by seed treatment and/or spraying the plants with metabolites proved to activate systemic effects (Strapasson et al. 2014; Esmaeily et al. 2020). However, the changes caused by omnivorous predators on the metabolome of exposed plants are still largely unknown.

Within this context, the aim of the present work was to comprehensively investigate the systemic defensive effects, the gene-expression of a set of genes involved in the JA defense pathway activation and the quantification and mapping of the metabolomic profile of tomato leaf. The local expression dynamics of genes were investigated and furthermore, the metabolite profiles of punctured and unpunctuired leaves were recorded using *T. absoluta* as the target organism due to its importance. To our knowledge, this is the first time that the defense induced by mirid-feeding-response against *T. absoluta* has been investigated in depth.*T. urticae* was also tested as a target pest since it has a different feeding mode than *T. absoluta*, a feature that may affect its response to induced plant defenses (Thaler et al. 2012; Zhang et al. 2018).

2. 3.Materials and methods

2.3. (i) Tomato plants grown in glasshouse condition and rearing of insects

Plant material

Tomato plants (cv.Ace 55, Optima, and Elpida [Spirou House of Agriculture, Athens, Greece]) and bean plants (cv.Barbouni [Spirou House of Agriculture, Athens, Greece]) were grown in a ventilated glasshouse with constant conditions of 25±2ºC and 65 ± 10% RHat the Agricultural University of Athens.

Tomato plants were developed from seeds sown individually in plastic seed trays in the glasshouse and transplanted after five weeks into 11 cm diam. Bean plants had been grown from seeds individually in plastic pots with Bas Van Buuren Potting Compost (Bas Van Burren B.V, Netherlands) substrate. Plants were not sprayed with any pesticide and were kept free from pests and diseases. They were kept in wooden cages of 75cm x 68cm x 68 cm (Fig. 2.1a).

Insects rearing

Tuta absoluta rearing was initiated from adults collected from a tomato crop located in Marathon, Greece(38° 8’24.87”N 23°58’6.65”E). The colony was kept on tomato plants (cv. Elpida (Fig. 2.1b). *T. urticae* rearing was initiated from adults collected from a tomato crop located in the area of Chalkis, Greece. Bean plants were used for rearing of *T. urticae* (Fig. 2.1c). Rearing of *N. tenuis* (Nesibug, Koppert, The Netherlands) was kept in tomato plants (cv. Elpida) with “Entofood” (Koppert B.V., The Netherlands) offered *ad libitum*. Entofood consists of supplementary food containing dead eggs of flour moth *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) and brine shrimp cysts *Artemia* spp.

Fig. 2.1a. Tomato plants cv Optima and cv Ace 55 grown in wooden cages maintained in glasshouse.

Fig. 2.1b. Rearing of *Tuta absoluta* maintained on tomato plant cv Elpida

Fig. 2.1c. Rearing of *Tetranychus urticae* maintained on bean plants cv.Barbouni

Fig. 2.1d. Top and bottom leaves of tomato plant covered with organdy bag with *N. tenuis*. Middle leaf received cages without any predator to prevent any feeding by *N. tenuis*

Fig. 2.1e. Plants caged to assure no escape or pest/disease damage.

Fig. 2.1f. Cages placed in environment chambers

2.3. (ii) Tomato plants punctured by *N. tenuis*

Five-week-old tomato plants (cv. Ace 55 and cv. Optimahaving three fully expanded leaves, were used. The use of two, instead of a single cultivar was prioritized because we tested the hypothesis mirid bug punctured tomato plants of any cultivar exhibit defense responses with activation of *PI* genes that have been reported in previous studies (i.e., cv. Optima by Pérez-Hedo et al. (2015) and cv. Ace 55 by Pappas et al. (2015)), additionally effects of such defense responses on *T. absoluta* and *T. urticae* were investigated. These results would be

also valuable for the potential wider applicability of the results, if there was not an effect of cultivar. Fifteen 3rd instar nymphs of *N. tenuis* were placed on each top and bottom leaf of each plant (Naselli et al. 2016) (Fig. 2.1c). Then, each leaf with the nymphs or not, was enclosedinto an organdy Cloth (12x15 cm). No food for *N. tenuis* was added. The middle leaf of each plant was enclosed in a cage without any predator introduced (Fig. 2.1d). Each leaf of the control tomato plants was caged individually without predator. All the experiments were conducted at 25±1°C, 65±5% RH, and 16:8 h L:D photoperiod. After four days of exposure, the predators were removed and their survival was always found higher than 80%. The plant damage, caused by the predators, was estimated by counting the brown necrotic rings in 10 top and 10 bottom leaves of each tomato cultivar (Fig.2.1 e,f).

2.3. (iii) Effects on oviposition, larval development period and pupal weight of *T. absoluta* due to plant feeding by *N. tenuis*

After the removal of the predators, a punctured plant was introduced in a cage (35x35x60 cm) (BioQuip CA, USA) together with an unpunctured tomato one, both of either the cv. Ace 55 or cv. Optima. Plants were kept without touching either each other or the cage walls. Then, three pairs of *T. absoluta* adults (less than 48 h old) were introduced into the cage using a mechanical aspirator and were allowed to oviposit for the next 24 h. Then, the number of eggs oviposited on each leaf of both plants was recorded visually. Ten replications (cages) were used per cultivar.

Ready-to-hatch (4d-old)eggs of *T. absoluta* were placed carefully on each top, middle, and bottom leaf (one egg per leaf) of *N. tenuis*-treated anduntreated plants. The mortality was monitored daily for each larva during its development on each leaf. Upon moulting into pupa, the pupal weight was recorded using an analytical balance (KERN ACS 80-4, Germany). In this case, only one cultivar (cv. Ace 55) was used because the effect of cultivar was not found significant on the oviposition rate of *T. absoluta*. Ten punctured and ten control plants were used.

2.3.(iv) Effects on escape tendency and survival of *T. urticae* due to plant feeding by *N. tenuis*

Following the removal of *N. tenuis*, 10 (5 female and 5 male) young adults (3-6 days old) of *T. urticae* were placed on a leaflet of each tomato leaf of *N. tenuis*-treated and untreated plants using a fine brush. *T. urticae* escape tendency was recorded by counting the number of

adults remained on the leaflet 1, 2, and 5 hrs later. The effects on the survival rate of the *T. urticae* adults were assessed using 5 female and 5 male adults of less than 24 h in the adult stage and previously starved for 2 h. The adults were confined within a circular area of 3 cm in diameter on the adaxial surface of a tomato leaflet of each leaf of a tomato plant by the aid of entomological glue (Temo-O-Cid Glue, Verde Vivo Company, Vigonovo, Italy). After 48 h their survival was recorded based on the dead adults found in the circular area. Ten punctured and 10 control plants were used as replicates for each cultivar.

2.3.(v) Sampling and sample preparation for GC/EI/MS metabolomics

Fifteen 3rd instar nymphs of *N. tenuis* were released on each top and lower leaf of five-week old tomato plants of cv. Ace 55 for four days. Top, middle, and bottom leaves of six biological replicates per punctured and unpunctured plants were harvested and immediately flash frozen in liquid N_2 for metabolism quenching in 50 mL falcon tubes. The extraction of the tomato leaf metabolome was performed as previously described with minor modifications (Kostopoulou et al. 2020). Briefly, tissues were pulverized to a fine powder in a mortar using a pestle in liquid nitrogen. A portion (40 mg) was transferred into 2 mL Eppendorf tubes and the extraction was performed by adding 500 μL of a methanol-ethyl acetate (50:50 v:v) mixture. The resulting suspensions were sonicated, stirred, and filtered through PTFE filters (0.2 μm diameter pore, Macherey-Nagel, Duren, Germany). Filtered extracts were spiked with 20 μL of a ribitol solution (0.2 mg.mL^{-1} in methanol) (Sigma-Aldrich Ltd, Steinheim, Germany), which was used as an internal standard. The extracts were evaporated to dryness and derivatized by applying a two-step process (Kalampokis et al. 2018; Kostopoulou et al. 2020) using a solution of methoxylamine hydrochloride in pyridine (98% w/w) (Sigma-Aldrich Ltd) for methoxymation and N-Trimethylsilyl-N-methyl trifluoroacetamide (MSTFA) for silylation. Blank samples were similarly analyzed to monitor metabolite features not related to the analyzed biological material. Furthermore, analytical standards were analyzed for the absolute identification of selected metabolites (Sigma-Aldrich Ltd).

2.3.(iv) GC/EI/MS metabolite profiling of of N. tenuis-punctured tomato plants or unpunctured tomato plants and data pre-processing

The metabolite profiling of the samples was performed using an Agilent 6890 analytical platform (Agilent Technologies Inc.,USA), equipped with a 5973 series mass selective detector and a 7683 autosampler. The settings of analyses have been previously described

(Kalampokis et al. 2018; Kostopoulou et al. 2020). Briefly, 1 μL of the samples were injected on a column [HP-5MS, length; 30 m, i.d.; 0.25 mm, film thickness 0.25 μm (Agilent Technologies Inc., USA)] with the injector set to a 10:1 split mode. Helium was used as the carrier gas at a flow rate of 1 $mL.min^{-1}$and full scan mass spectra were acquired at the mass range of 50-800 Da (scan rate of 4 scans.s-1). The oven temperature initally was set at 70 °C for 5 min, followed by a 5 °C min^{-1} increase to 295 °C and kept for 2 min. The temperature for the MS source was 230 °C and for the quadrupole 150 °C. The ionization was positive electron ionization at 70 eV and full scan mass spectra were acquired at the mass range of 50-800 Da at a rate of 4 $scans.s^{-1}$. The acquired total ion chromatograms were deconvoluted using the software AMDIS v.2.66 (NIST, Gaithersburg, USA) and data pre-processing was performed by the bio-informatics software MSDIAL v.4.38 (Tsugawa et al. 2015; Lai et al. 2017). The aligned data were exported to MS Excel® for curation and further examined for inconsistencies (Kalampokis et al. 2018; Kostopoulou et al. 2020). Metabolite features present in less than 50% among replicates were excluded from further analyses. Additionally, metabolite features detected also in the experimental blank samples, were removed during matrix curation and were excluded from further processing.

Tentative metabolite identification was based on matching their mass spectra and retention times to reference entries of the Golm Metabolome Database (Kopka et al. 2005) and the National Institute of Standards and Technology library ’08 (NIST 08, Gaithersburg, USA) (mass spectra similarity >95%), and for selected metabolites absolute identification was performed using analytical standards. For the biological interpretation of the results and the discovery of trends and the corresponding biomarkers using the software SIMCA-P v.13.0 (Umetrics, Sartorius Stedim Data Analytics AB, Sweden), a previously described approach was adopted (Aliferis et al. 2013; Kalampokis et al. 2018) with minor modifications. Briefly, multivariate analysis was performed, and the discovery of tomato metabolites-biomarkers was based on OPLS-DA regression coefficients (Coeffs) ($P<0.05$). Standard errors were calculated using Jack-knifing. Metabolites with values of Co-effs>1 and Coeffs<-1 were considered as biomarkers.

2.3.(vii) RNA extraction of *N. tenuis*-punctured tomato plants or unpunctured tomato plants and plant gene expression analysis of defense related gene

To investigate the systemic effect caused by *N. tenuis* phytophagy, the upregulation of anti-herbivory genes *PI-II, MYC2, VSP2, HEL, AOS,* and *MBP2* was investigated. Fifteen 3rd instar nymphs of *N. tenuis* were released on each top and lower leaf of five-week-old tomato

cv. Ace 55 plants for four days. Six *N. tenuis*-punctured tomato plants and six unpunctured (untreated) plants were employed for gene expression analysis.Upon completion of the 4-day treatment, bulk samples of 200 mg tomato leaf tissue from three plants per treatment (two independent biological replicates) were collected separately from the top, middle, or lower leaves of*N. tenuis*-punctured and unpunctured plants. Leaf tissue was flash frozen in liquid nitrogen to stop gene expression and total RNA isolation was performed using TRIzol by adapting the Yoo et al. (2004) procedure. RNA concentration was measured spectrometrically and it was adjusted to 100 ng.µL-1 with Rnase-free water. 1-2 µg RNA from each sample was electrophoresed at 135 V for 35 minutes in 1.5 % agarose gel. The integrity of the ribosomal bands confirmed the quality of RNA. Reverse transcription (RT) was performed employing an oligo-dT primer and FIREScript Reverse Transcriptase, Solis BioDyne, Estonia, following standard pro-tocol (Yoo et al. 2004).

Several genes, involved in the JA and OPDA pathways, were selected for expression analysis (Table 2.1). Gene sequence information on the respective genes in *A. thaliana* and tomato was retrieved from TAIR (https://www.arabidopsis.org/) and SGN (https://solgenomics.net/), respectively. Specific primers were designed employing Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/) (Table 2.1). Expression of the selected genes was analyzed initially by semi-quantitative PCR (30 cycles). PCR products were gel electrophoresed in a 1.5 % agarose gel, followed by EtBr staining and gel image recording under UV. For quantification of the expression levels of the selected genes, quantitative PCR (qPCR) reactions were performed with the help of 5X HOT FIREPol EvaGreen qPCR Supermix (Solis BioDyne, Estonia), in a StepOnePlus Real-Time PCR System (Applied Biosystems, USA). For the relative quantification, the 2-ΔΔCT method was employed (Schmittgen &Livak 2008), using TIP41 as the housekeeping gene for normalization purposes (Expósito-Rodríguez et al. 2008).

Table 2.1.Names of the genes with their respective selection criteria and primers used.

Gene name		Criteria for selection	Primers Forward (F) and Reverse I	Refs
Proteinase inhibitor II	PI-II	Induced by the JA pathway, harmful for the digestive system of insects	F: GGATATGCCCAGGTTCAGAAGGAA R: AATAGCAACCCTTGTACCCTGTGC	Bosch et al. 2014
Vegetative storage protein 2	VSP 2	Acid phosphatase and anti-insect activity, specific to JA, induced by MYC2	F: CTGGTTATGCAGTCCCACAAT R: ACGTCGATATTGTTTGCCAAG	This study
Myrosinase -binding protein 2	MBP2	May contribute to the production of toxins protecting against herbivory	F: CACAAACATCAGAGGCCATTT R: TGCACCATGTTTTACTGACCA	This study
Allene oxide synthase	AOS	Component of the JA-biosynthesis pathway, coi1-dependent	F: GATTTCGTTGTGATGGTTTCG R: TCGACGTTGAGTGTACCGTAA	This study
Hevein-like peptide	HEL	Antimicrobial peptide, highly induced by herbivory	F: TGTTGATTATATCCGCGATTG R: TTGGAAGGTGAACAAAATTCG	This study

Myelocytomatosis oncogene transcription factor 2	MYC2	Major transcription factor, component of the JA pathway, anti-insect activity	F: GATGATCCAACAAGCCACAGT R: CGATGTCAACGCTACCCTAAG	This study
TAP4 interacting protein of 41 kDa	TIP41	Housekeeping gene	F: GCTGCGTTTCTGGCTTAGG R: ATGGAGTTTTTGAGTCTTCTGC	Expósito-Rodríguez et al. 2008

2.3. (viii) Statistical analysisof data collected

The number of eggs laid by *T. absoluta* were analyzed with a mixed model with fixed factors being the "treatment" (i.e., *N. tenuis*-punctured vs. unpunctured plant), the "cultivar" (Ace 55 vs. Optima), and the "leaf position" (i.e., top, middle and lower). Then, aiming to control for plant and leaf position variation it wasincluded in the model "plant" X "leaf position" as nested random effects. Data of its larval developmental period and the pupal weight were similarly analyzed with fixed factors being the "treatment" (i.e., *N. tenuis*-punctured vs. unpunctured plant) and the "leaf position" (i.e. top, middle, and lower). Raw data were log transformed prior to the analysisto meet the assumptions(Aitchison 1982). The escape tendency of *T. urticae* individuals was estimated as the percentage of individuals escaped from the leaflet at each time interval. The data were arcsine transformed and compared within each tomato cultivar with fixed factors being the "treatment", the "leaf position", and the "time interval" and "plant" X "leaf position" as nested random effects. The data of *T. urticae* adults' survival 48 h after their release were analyzed following the same methodology with factors being the "cultivar", the "treatment", and the "leaf position" after data were arcsine transformed (Aitchison 1982). In all cases means were compared using the Tukey's HSD test ($P < 0.05$).

For gene expression analysis, significant differences were determined with a Student's t-test performed in a pairwise manner by comparing the gene expression levels in plants punctured by *N. tenuis* or unpunctured on the same strata (top, middle, or lower leaves). The

results are presented as the mean values from two biological replicates ± standard error (SE). Statistical analyses were performed with JMP 14.0 (SAS Institute Inc., 2016).

2. 4. Results

2. 4. (i) Oviposition Preference of *T. absoluta*

The oviposition of *T. absoluta* was significantly affected by the "treatment" (F = 47.08, df = 1,81, P < 0.001) and the "leaf position" (F = 60.63, df=2,81, P < 0.001), with their interaction being significant too (F = 5.28, df=2,81, P <0.007). The interaction was due to the significantly lower number of eggs laid on the middle and top leaves of the punctured plants in comparison to the respective leaves of the unpunctured plants (Fig.2.2a). This indicates a systemic effect since the middle leaves were not punctured by the predator.

2.4. (ii) Effects of *N. tenuis*-punctured tomato plants on larval development and pupal weight of *T. absoluta*

No larval mortality of *T. absoluta* was recorded on *N. tenuis*-punctured plants. However, the larval development period was significantly longer on punctured plants (cv. Ace 55) (F =166.34, df=1,27, P< 0.001) (Fig. 2.2b). The effect of leaf position and their interaction were not significant (F=0.25, df=2,27, P = 0.77 and F=0.21, df=2,27, P = 0.82, respectively). The pupal weight was significantly affected by the "treatment" and the "leaf position", with their interaction not being significant (F = 98.76, df = 1,27, P< 0.001, F = 12.85, df = 2,27, P< 0.001 and F = 0.13, df = 2,27, P = 0.88, respectively). The pupal weight was significantly larger for the larvae developed in the top, middle, and lower leaves of the unpunctured plants than the *N. tenuis*-punctured plants (Fig.2.2c). The pupal weight was significantly larger in the top than the lower leaves in the control plants.

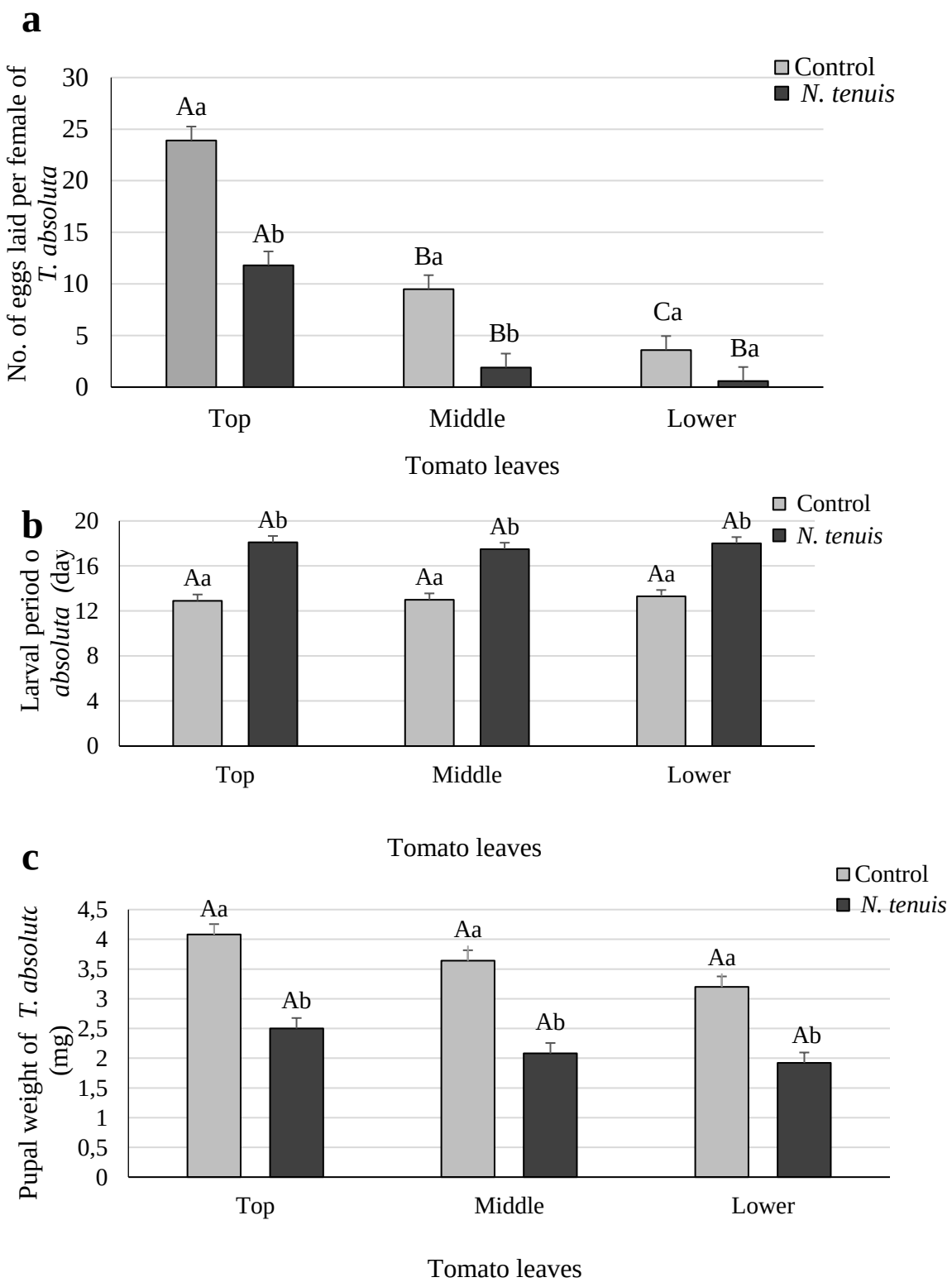
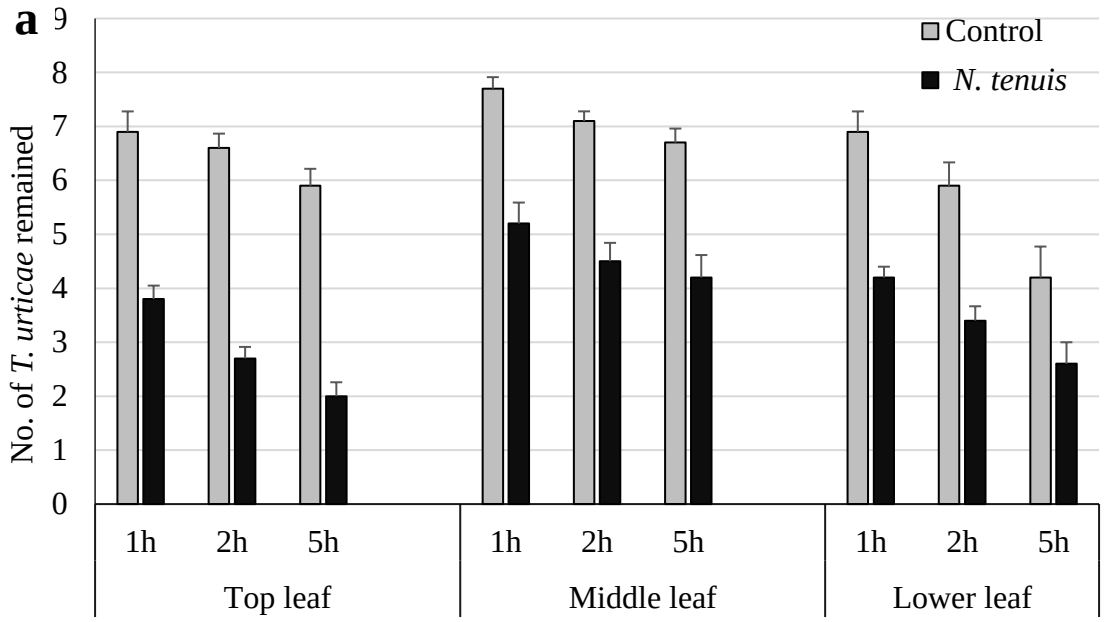


Fig. 2.2. Number (mean ± SE) of eggs oviposited (a), duration of larval period (b), and pupal weight (c) of *T. absoluta* on top, middle, and lower leaves of tomato plants punctured by *N. tenuis* in comparison to unpunctured (control) tomato plants. Columns with the same capital letter are not significantly among leaves within each treatment, and columns followed by the same small letter are not significantly different between treatments within each leaf category (ANOVA, Tukey HSD, $P < 0.05$, n=10).

2. 4. (iii).*N. tenuis*-punctured tomato plants induced escape tendency of *T. urticae*

The escape tendency of *T. urticae* adults was significantly higher on the *N. tenuis*-punctured than the unpunctured plants in each cultivar (F = 29.30, df=1,135, P <0.001 and F = 33.95, df =1,135, P < 0.001, for cv Ace and cv Optima, respectively) (Fig.2.3a,b). It was significantly higher on the top or the lower leaves than the middle leaves (F = 11.77, df =2,135, P < 0.001 and F = 12.33, df=2,135, P < 0.001, for cv Ace and Optima, respectively) and was significantly higher 1 h post treatment (F = 36.11, df =2,135, P < 0.001 and F = 39.02, df =2,135, P < 0.001, for cv. Ace 55 and Optima, respectively). The interaction between the "leaf position" and the "time interval" was significant for cv Ace (F = 3.32, df =4,135, P < 0.01) since the escape tendency was significantly higher onthe middle leaf, 5 hrs after release of the mites than on the other leaves (Fig.2.3a,b).



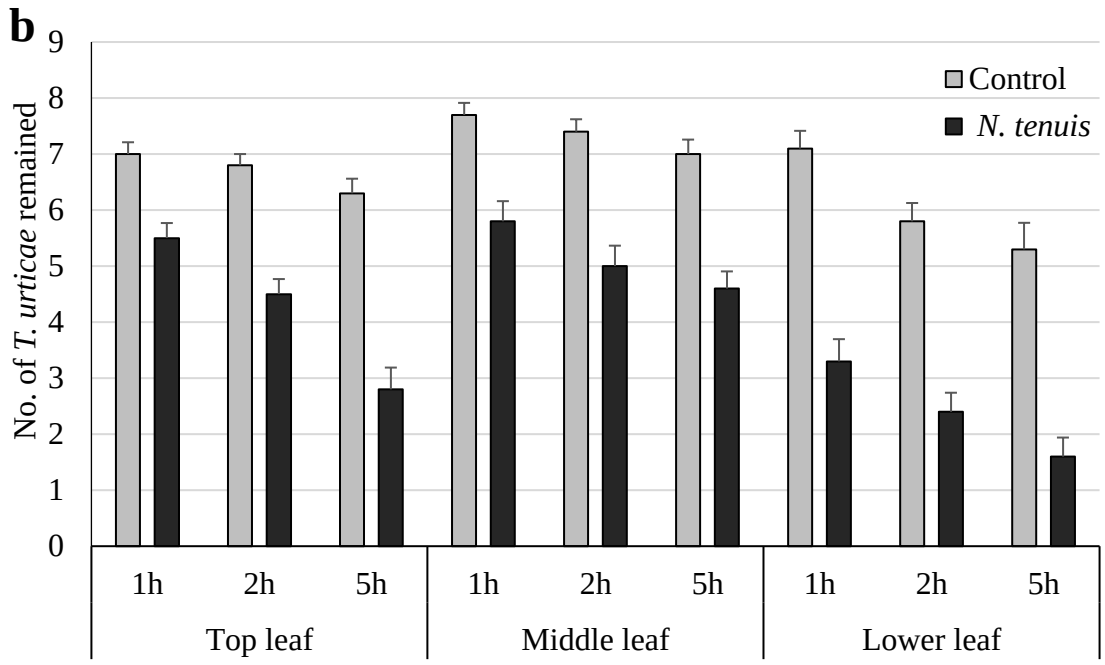


Fig. 2.3. Number of *T. urticae* adults (mean ± SE) remained on leaflets of top, middle or lower leaves of tomato plants punctured by *N. tenuis* in comparison to unpunctured plants after 1, 2, and 5 hours on two tomato cultivars cv. Ace 55 (a) and cv. Optima (b). In all cases a significantly higher number remained on the control that the treated leaf in each time interval in both cultivars. A significantly higher number of adults remained 1h than at 2h and 5h post treatment on the control leaf, in the case of cv. Optima.

2.4. (iv) *N.tenuis*-punctured tomato plants affectedsurvival of *T. urticae*

The survival of *T. urticae* females was significantly affected by the "treatment" and the "leaf position" (F = 54.03, df = 1,81, P<0.001 and F = 7.11, df = 2,81, P < 0.003, respectively). A significantly higher survival rate was recorded on the top and the lower leaves of unpunctured plants as compared to the punctured plants (Fig. 2.4). The survival rate was significantly higher in the middle than the lower leaves of the untreated plants, whereas in the punctured plants it was significantly higher in the middle than the top leaves.

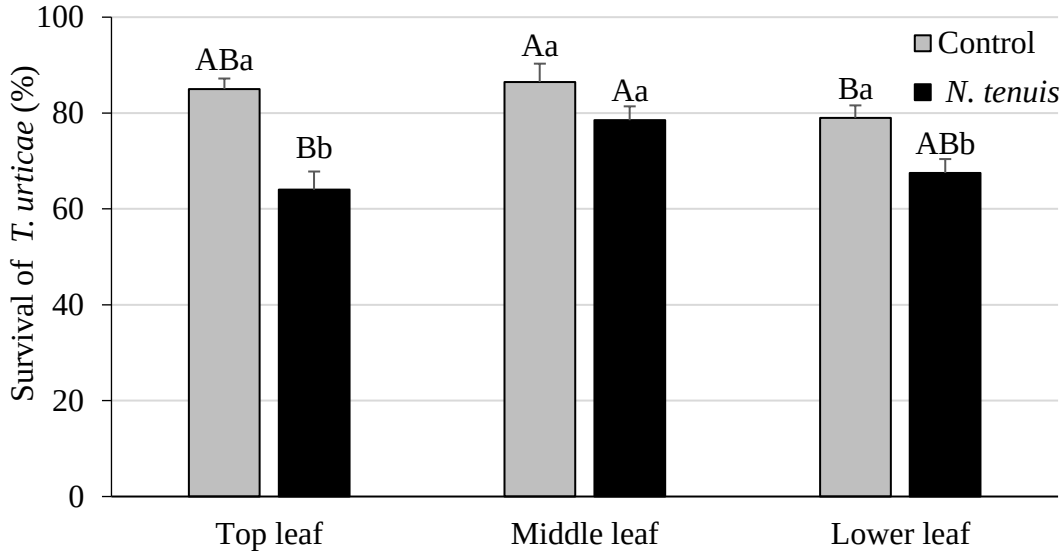


Fig. 2.4.Survival rate (% of alive individuals ± SE) of *T. urticae* 48h after their release on tomato plants punctured with *N. tenuis* in comparison to unpunctured plants on top, middle, and bottom leaves of tomato plants. Columns with the same capital letter are not significantly different among leaves within each treatment, and columns followed by the same small letter are not significantly different between the treatments in each leaf category (ANOVA, Tukey HSD, $P < 0.05$, n=10).

2.4.(v) Overview of the metabolomics analyses of *N. tenuis*-punctured tomato plants

In total, 149 metabolite features were reproducibly recorded, and metabolomics analyses revealed the substantial effect of treatments on 59 metabolites that are related to 43 essential biosynthetic pathways. The differences in the metabolite profiles re-vealed a strong discrimination among the various treatments (Fig.2.5) and the leverage of annotated metabolites are displayed in the corresponding OPLS coefficient plot (Fig.2.5b).

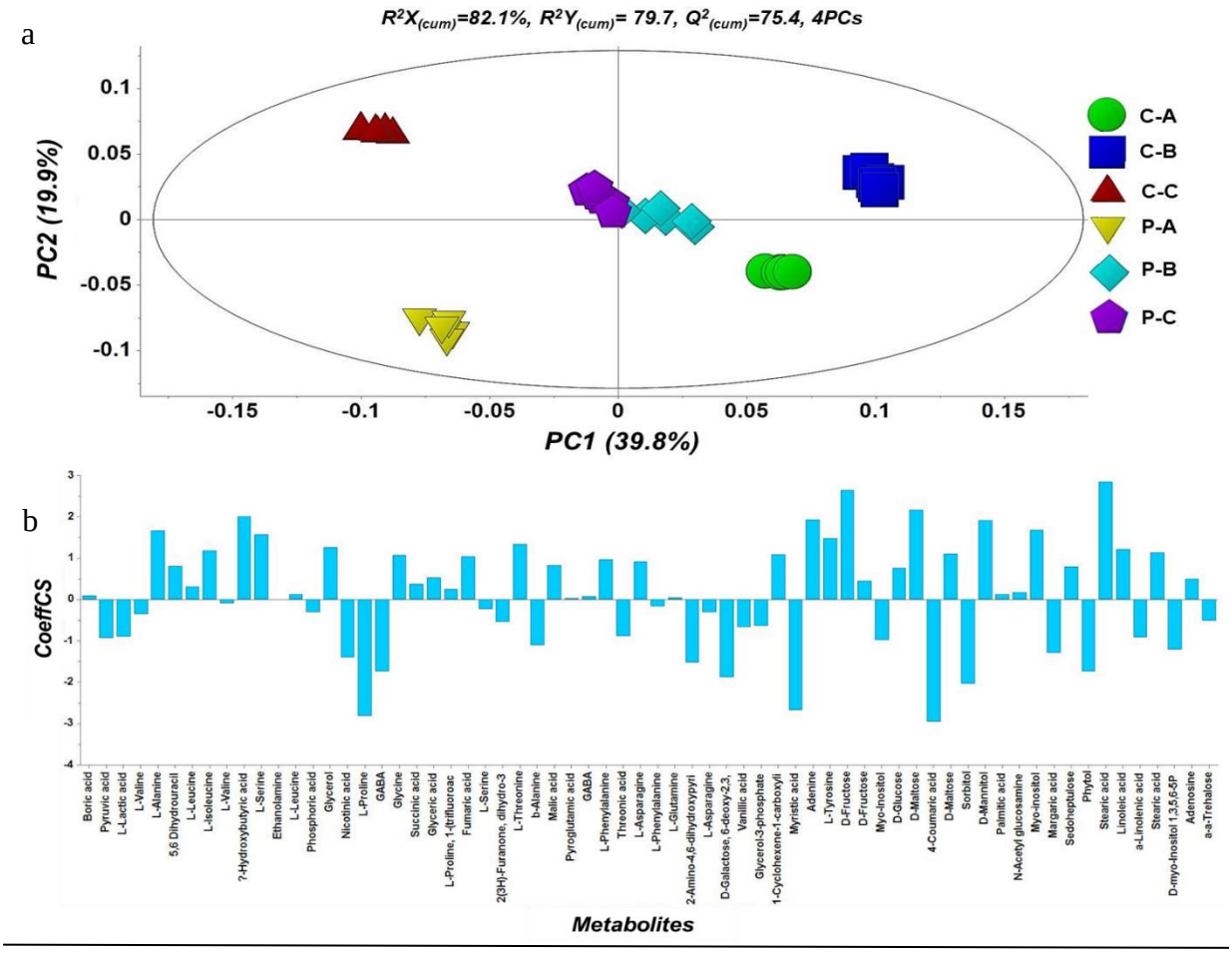


Fig. 2.5. Orthogonal partial least squares-discriminant analysis (OPLS-DA) PC1/PC2 score plot for the GC/EI/MS metabolite profiles of *N. tenuis*-punctured tomato top, middle, and lower leaves in comparison to the respective leaves of the untreated plants. The ellipse represents the Hotelling's T^2 with 95% confidence interval. Six pooled samples were analyzed per treatment (Initial C; control or unpunctured plants, P; plants punctured by predator, A; top, B; middle, C; lower leaves) (a), and OPLS coefficient plot with values of scaled and centered PLS regression coefficients (Coeffs) for the selected Y variables for the whole dataset.

The *de novo* construction of the metabolic network of *N. tenuis*-treated plants was based on the data retrievedfrom the Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.genome.jp/43xp) (Fig. 2.6).

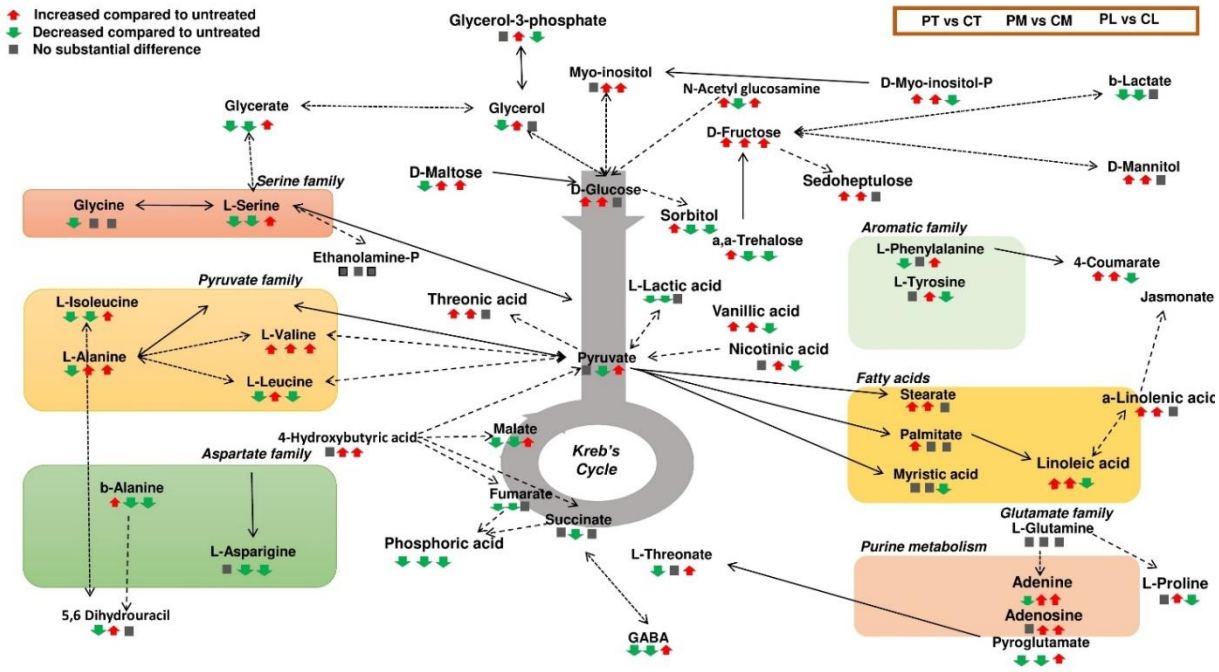
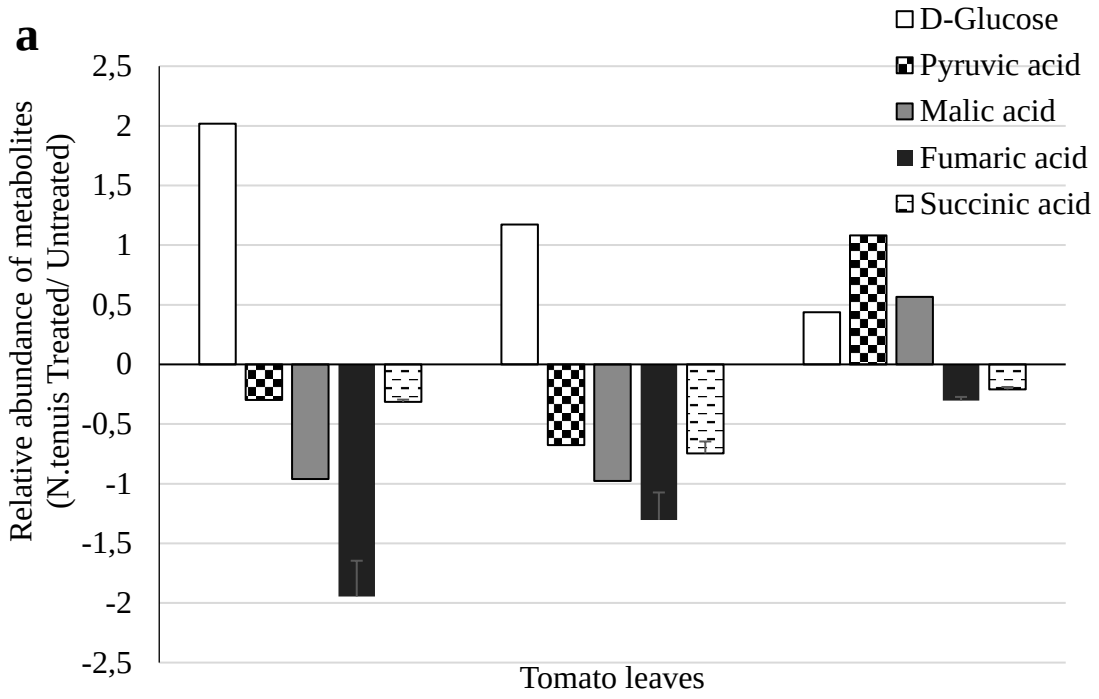


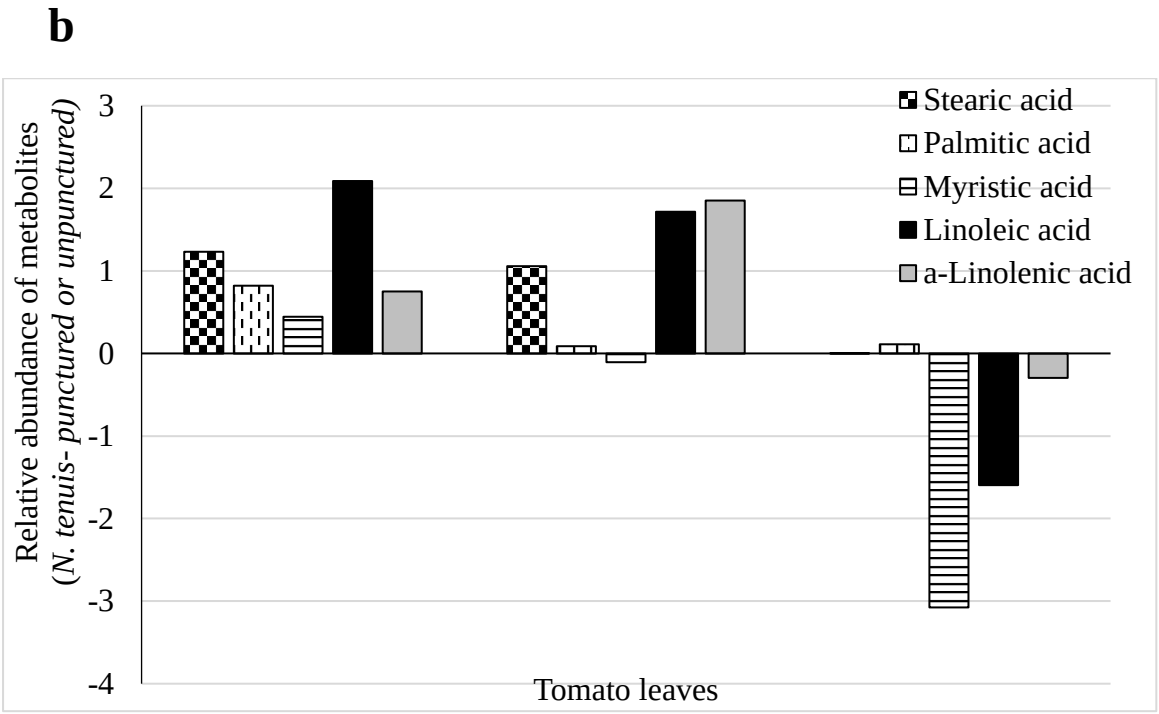
Fig. 2.6. Metabolic network of tomato leavesdisplaying the differences between the metabolic composition of unpunctured and *N. tenuis*-puncturedleaves. Single-headed or double-headed arrows indicate one or two-way reactions between metabolite pools, respectively.Solid lines symbolize one-step consecutive metabolites in a biosynthetic pathway and dashed lines multi-step or not fully elucidated biosynthetic pathway sections. Three different symbols below each metabolite represents their relative abundance in *N. Tenuis*-punctured tomato plant tissues (top, middle, and bottom leaf) as compared to those in the unpunctured plants. Red upward arrow indicates increased abundance in *N. tenuis*-punctured compared to unpunctured. Green downward arrow indicates decreased abundance in *N. tenuis*-treated compared to untreated. Gray squares indicate no substantial differences in the two treatments (PT; predator-top, CT; control-top, PM; predator-middle, CM; vs control-middle, PL; predator-bottom, CL; control-bottom leaves).

An interesting finding regarding Krebs' cycle was that top (punctured) and middle (unpunctured) leaves of *N. tenuis*-treated plants exhibited a similar fluctuation in their metabolome as compared to the bottom leaves (Fig. 2.6). Glucose was detected in high amounts in top and middle leaves. This contrasts with the observed levels of Krebs cycle intermediates like pyruvic, malic, fumaric and succinic acids, where much lower levels were detected in the top and middle leaves (Fig. 2.7a).

Fatty acids such as stearic, myristic, linoleic, and α-linolenic acids were recorded in higher levels in the top and middle leaves, compared to the bottom leaves (Fig. 2.7b). On the other hand, except for the top leaves, palmitic acid amounts did not differ between the middle and bottom leaves (Fig. 2.7b). Significantly higher levels of 4-coumaric acid were detected in the top and middle leaves of *N. tenuis*-treated plants (Fig. 2.7c). Given the fact that L-phenylalanineis the precursor for 4-coumaric acid, it is noteworthy that this metabolite

showed downregulation and upregulation in the top and bottom leaves, respectively (Fig. 2.7c).





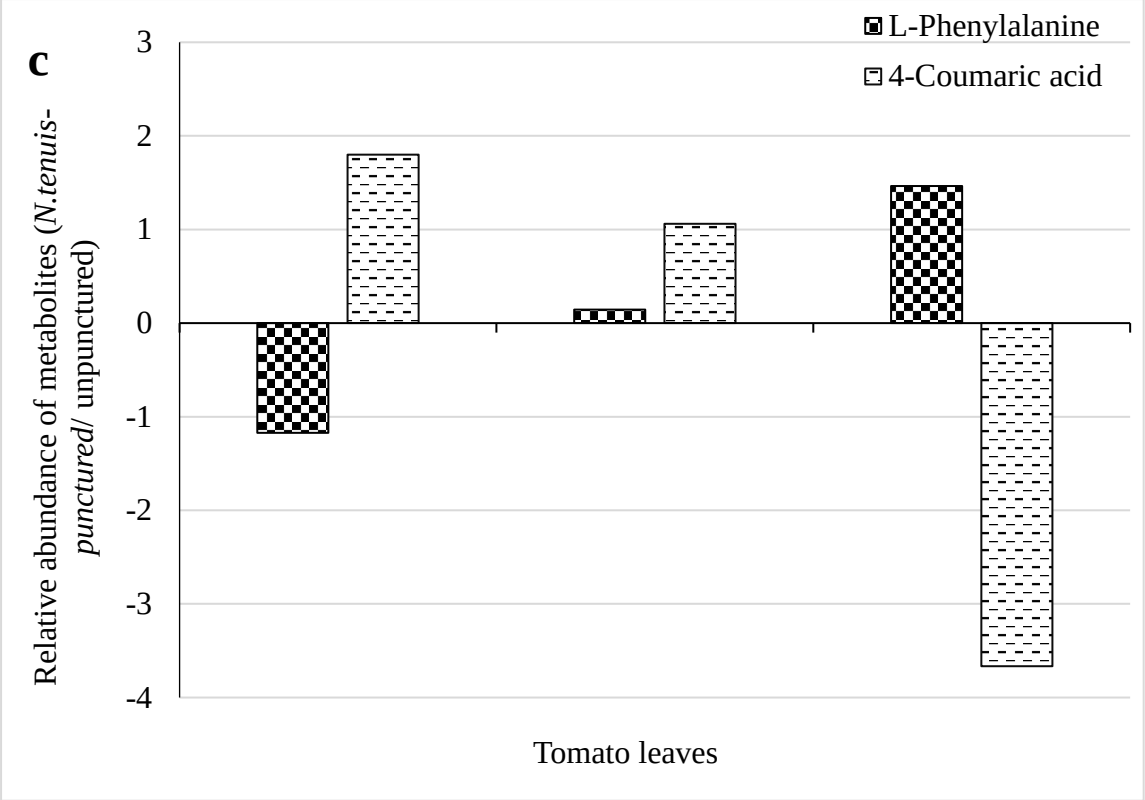


Fig.2.7. Effect of the *N. tenuis*-treatment in metabolites related to glycolysis and Krebs's cycle (a), Fatty acids metabolism (b), and related to shikimate pathway (c). Positive or negative values indicate upregulation or downregulation of the selected metabolites in *N. tenuis*-puncturedin comparison to unpunctured plants, respectively.

2.4.(vi) Quantification of plant gene expression

A pronounced upregulation of *PI-II*, MYC*2*, *VSP2*,and *HEL* in the top (treated) leaves as compared to untreated top leaves was observed. Slight upregulation recorded in the treated top leaves for *AOS* and in the treated bottom leaves for *MBP2*. Notably in treated plants *VSP2* exhibited upregulation in the middle as well as lowerbottom leaves (Fig. 2.8).

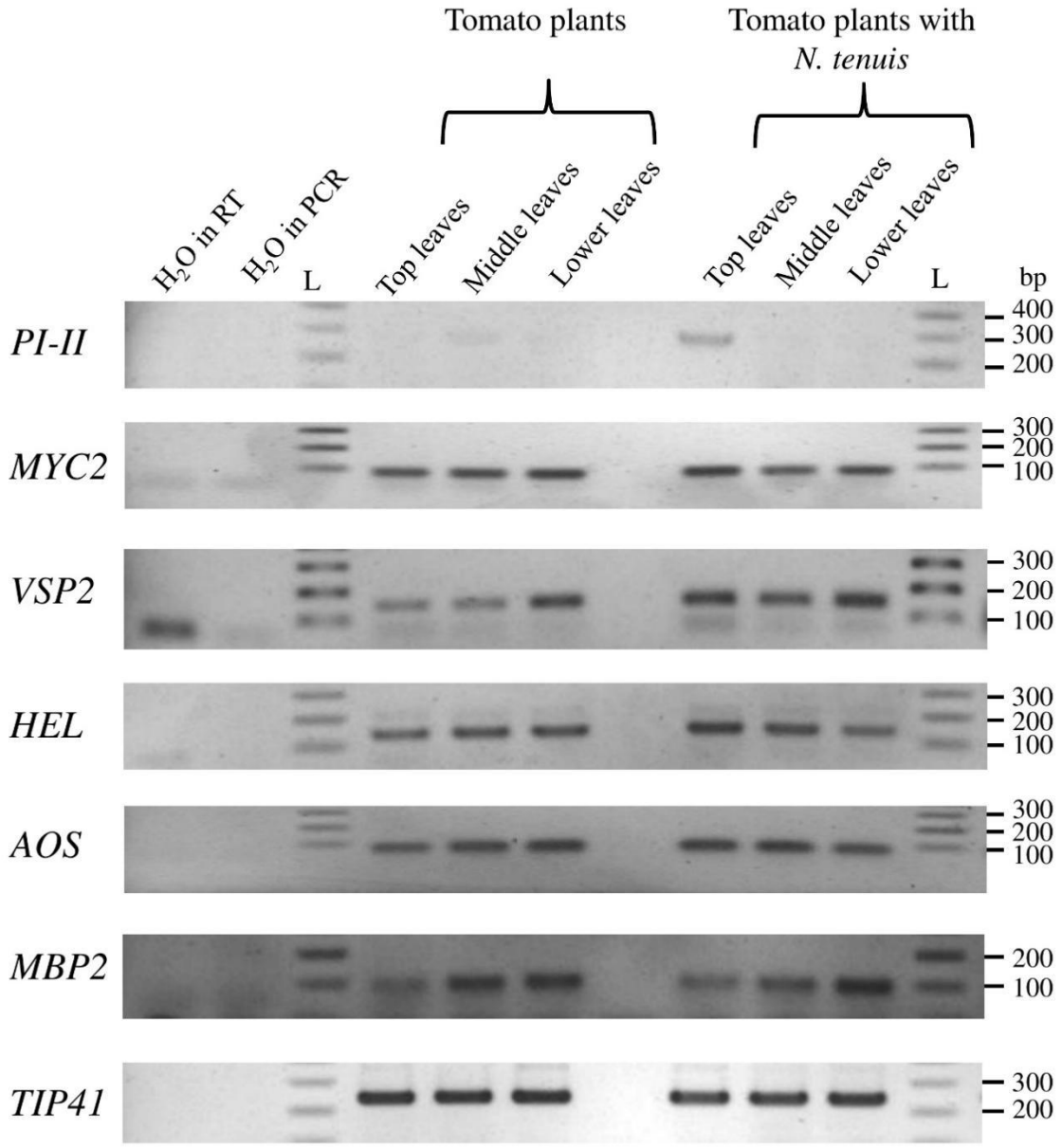


Fig. 2.8. Expression analysis of defense-related genes on different strata of tomato plants punctured or unpunctured by*N. tenuis*. The semi-quantitative RT-PCR results after gel electrophoresis are presented showing the abundance levels of selected tomato genes: proteinase inhibitor II (*PI-II*), *MYC2* transcription factor, vegetative storage protein 2 (*VSP2*), hevein-like peptide (*HEL*), allene oxide synthase (*AOS*), myrosinase-binding protein 2 (*MBP2*). *TIP41* was used as the internal housekeeping gene, whose expression does not significantly change. In all panels, L is a 100-bp DNA ladder (New England Biolabs, USA).H_2O-RT: Water was used as a template for the RT reaction. H_2O-PCR: Water was used as a template for the PCR reaction.

qPCR analysis results of the selected genes indicated a high upregulation (11.84X) for *PI-II* and a significant upregulation for *MYC2* (3.55X) and for *HEL* (3.26X) in the top leaves of treated plants as compared to control plants (Fig. 2.9). Interestingly, *VSP2* showed a

significant upregulation in all leaves (2.1X, 4.22X, and 1.8X in top, middle, and bottom leaves, respectively). Significant upregulation in the middle leaves was also observed for *HEL* (4.37X). A significant upregulation was also recorded for *MBP2* in middle (2.1X) and bottom (2.27X) leaves of treated plants (Fig. 2.9).

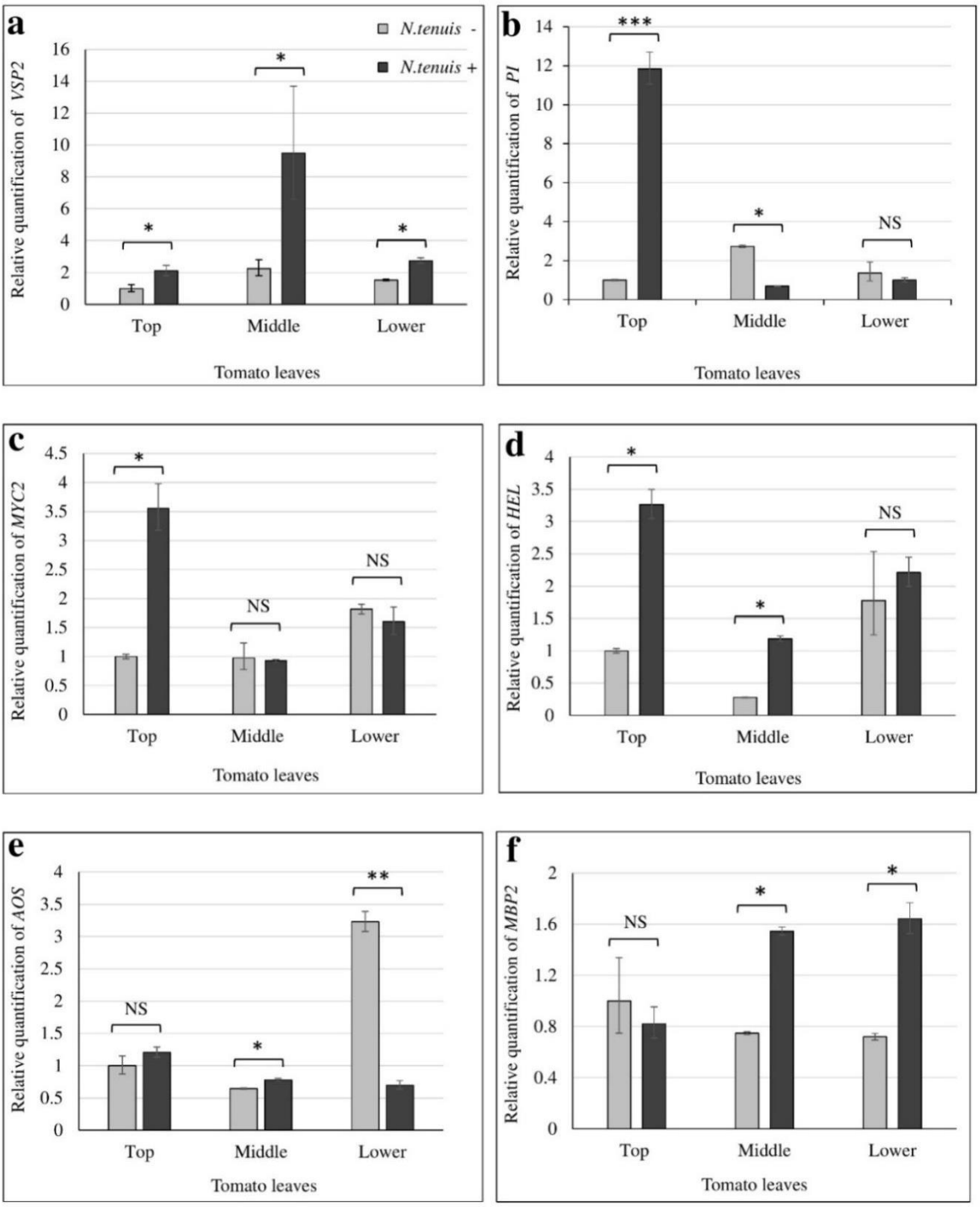


Fig.2.9.Quantification of defense-related genes affected by the phytophagy of *N. tenuis*on different strata of tomato plants. The relative quantification levels of selected defense-related genes, obtained by RT-quantitative PCR, are shown. Quantification was performed for (a)*PI*, (b)*VSP2*, (c)*MYC2*, (d)*HEL*, *AOS*, and (f)*MBP2*. For normalization purposes *TIP41* was used as the internal control. The $2^{-\Delta\Delta CT}$ method was employed for the quantification. The value obtained for top leaves of unpunctured tomato plants was arbitrarily set as 1. Values for all other samples are relative to this. Results were obtained from two biological replicates. Error bars at graphs represent the standard error. Asterisks indicate that the mean expression value in plants punctured by*N. tenuis* is significantly different from unpunctured (control) plants (*$P < 0.05$, ** $P < 0.005$, *** $P < 0.001$). NS indicates no statistically significant differences.

2.5. Discussion

To our knowledge, this is the first report of the induction of tomato defensive responses against the egg laying and larval development of *T. absoluta* following exposure to *N. tenuis*. *T. absoluta* adults were less attracted to *N. tenuis*-treated than untreated tomato plants by an olfactometer bioassay (Pérez-Hedo et al. 2015b), most likely due to the emission of unfavorable volatiles (Pérez-Hedo et al. 2018b). Similarly, *N. tenuis* treatment reduced the survival rate of *T. urticae* but not its oviposition, a result that is in accordance with Pérez-Hedo et al. (2018a) but further induced escape tendency of *T. urticae* adult. On the other hand, previous studies have concluded that additional research is required to clarify whether spider mites disperse more from plants previously puncturedby*M. pygmaeus* (Zhang et al. 2018) and whether antixenosis effects, due to plant priming by *M. pygmaeus*, are significant for spider mites (Pappas et al. 2015). Furthermore, *T. urticae* females, in a Y-tube bioassay, equally selected tomato plants punctured by*N. tenuis* or not (Perez-Hedo et al. 2018a). In the present work the behaviour of *T. urticae* adults was recorded in short intervals to clarify that the pre-exposure to *N. tenuis* induces a strong avoidance behavior of the mite. The effect of *N. tenuis* treatment on both pests was similar on the two tomato cultivars used, suggesting that this approach could have a general applicabilityin tomato. Taken together, the results indicate that the tomato defense mechanism triggered by *N. tenuis* has a potential to contribute to the control of *T. absoluta* and *T. urticae.*

The systemic nature of the protective effects was observed, since the unpunctured middle (systemic) leaf of the *N. tenuis*-treated plants negatively impacted *T. absoluta*, similarly to the leaves directly punctured by *N. tenuis*. Such observation is of paramount importance since the quest for the whole plant protection is desirable. However, regarding the *T. urticae* survival rate, there was less adverse effect on the systemic leaf as compared to the top (local) leaf. This may be due to the different feeding mode between the two pests (*T. absoluta* and *T. urticae),* which might differentially affect the uptake or the differential transport of the deleterious substance for the two pests' metabolites.

The observedsystemic resistance response against *T. abolutaand T. urticae* in the middle leaves of *N. tenuis*-punctured plants, correlate well with the metabolomics analysis; e.g. the increased levels of metabolites,such as α-linolenic and linoleic acids, could be directly associated with the exhibited negative effects. Similarly, in the top leaves of *N. tenuis*-treated plants increased levels of *α*-linolenic acid were recorded. It has been proposed that the JA pathway is regulated by substrate availability of *α*-linolenic acid (Wasternack

2015). The identification of high amounts of the metabolite in the middle leaves of *N. tenuis*-treated plants, which were not directly punctured bythe predators, indicates the existence of a systemic signal that could trigger the JA pathway in remote plant parts. Hydroperoxide fatty acids, originating from linoleic acid catabolism, may act as long-distance mobile signals that trigger *de novo* JA biosynthesis in distant parts in cotton (Sun et al. 2014). Analyses suggest that the high amounts of linoleic acid in top and middle leaves of *N. tenuis*-treated plants might constitute a source of long-distance mobile signals. We propose a model, in which, oxylipin intermediates produced in the top leaves move to the middle leaves, triggering the JA biosynthetic pathway and also the generation of new mobile signals from linoleic acid catabolism, thus, contributing to the amplification of the defensive response. In line with the proposed model is the observation of relatively high amounts of glycerol-3-phosphate in the middle leaves of the *N. tenuis*-treated plants, which is an important precursor of numerous metabolites. Chanda et al. (2011) presented evidence that glycerol-3-phosphate or a glycerol-3-phosphate-associated factor contribute to systemic immune responses by facilitating the movement of the lipid-transfer protein.

The major effects that the *N. tenuis*phytophagy exerts on plant metabolism are indicated by the increased amount of glucose in the top and middle tomato leaves. Glucose is a substrate for glycolysis, which in association with the Krebs cycle, produces the necessary energy to fuel the growth processes of the plant. The reduced glucose turnover in these tissues, is in line with the lower amounts of the intermediary products of the Krebs cycle indicate that the metabolism in the top and middle leaves is redirected from growth and development to defense, as a result of the *N. tenuis* treatment. In line with this, metabolomics revealed the activation of the shikimate pathway through phenylalanine, which could be converted to coumarate, i.e. a precursor of anthocyanins which act as anti-oxidant compound in response to various stresses (Wasternack et al. 2019). Taken together, the results suggest that a strong link between JA pathway activation and secondary metabolite production is induced in tomato as a result of pre-exposure to *N. tenuis,* leading to effective local and systemic defense responses against two important tomato pests.With regard to the JA pathway, Pérez-Hedo et al. (2015) demonstrated that phytophagy of *N. tenuis* results into high levels of OPDA and isoleucine conjugate of JA (JA-Ile). Zhang et al. (2018) reported similar effects when sweet pepper plants were attacked by *M. pygmaeus*. Pérez-Hedo et al. (2018)reported upregulated expression of *PIN2* and higher concentration of plant protein inhibitors *PI-II1* and *PI-II2* via activation of the JA pathway. Similarly, Pappas et al. (2015)

reported higher accumulation of transcripts of *PI* genes in tomato plants punctured by *M. pygmaeus.*

Previous studies on *A.thaliana* have shown that *PI-II, VSP2,* and *MBP2* are mainly induced by exogenous application of JA, *AOS* is induced by JA and OPDA, while *HEL* is induced in a higher level by OPDA than by JA (Reymond et al. 2000; Stintzi et al. 2001). Therefore, the upregulation of *PI-II,VSP2, MBP2,* and *HEL*, observed in our study, indicates the activation of both JA and OPDA signaling pathways by *N. tenuis*.

The current study extended our knowledge regarding the array of defense-related genes whose expression is affected by *N. tenuis* punctures in tomato. Previous studies on tomato have focused phytohormones *JA, ABA* and *SA* and quantified the transcript levels of *PIN2, ASR1* (Pérez-Hedo et al. 2015) and *PI*-related genes (Pérez-Hedo et al. 2018) related genes. Since in our study *PI* was upregulated only in the top leaves, *PI* may have a limited contribution to defense against *T. absoluta*. In contrast, the level ofupregulation varied among the other genes tested depending on the plant leaf position and if punctured or not. In the top leaves (punctured) *MYC2, VSP2,* and *HEL* were upregulated and interestingly, in the middle leaves (unpunctured) *VSP2, HEL,* and *MBP2* were upregulated while in the lower leaves (punctured) *VSP2* and *MBP2* were upregulated.This is an important finding since the understanding of the mechanisms of systemic resistance may facilitate advance pest management systemswhereas identification of genes whichare responsible for the systemic activation of the JA pathway and the protection of the entire plant consists of a key knowledge to develop effective pest control methods. The JA- or OPDA-induced gene expression results align well with that of metabolomics data previously discussed.

Our metabolomics results suggested no induction of the JA pathway in the bottom (older) leaves, although these leaves were punctured by *N. tenuis*. In agreement, four out of six defense-related genes (*PI-II, MYC2, HEL, AOS*) studied were not up-regulated in lower leaves punctured by *N. tenuis*, suggesting that the JA pathway is not induced. In contrast, in younger leaves, which showed the highest photosynthetic capacity, the responses to herbivory were stronger than in older leaves (Van Dam et al. 2001; Anderson et al. 2005; Van Dam et al. 2011).

Altogether, the results offer valuable information on the selection of the appropriate genes to offer whole plant protection or long-lasting protective effects in tomato. Future experiments could aid in the production of more resistant plants through gene editing technologies. CRISPR-dCas9 could be used to activate transcription, in a non-transgenic manner, of an endogenous gene, e.g. the genes that are upregulated by *N. tenuis* treatment

(Piatek et al. 2015). Delayed development and increased mortality of beetles and the houseflywere achieved by incorporating *VSP2* protein into their diets (Liu et al. 2005). Additionally, the use of defense-related secondary metabolites, detected in the present study, may open the potential for novel bio-active compounds to become future plant-derived ecofriendly insecticides (Isman& Akhtar 2007; Ulrich-Merzenich et al. 2007).

Overall, plant feeding by *N. tenuis* can deteriorate the performance of *T. absoluta* and *T. urticae*, through the activation of the JA pathway, alterations in metabolite levels and systemic responses. The multi-omics approach (phenomics, transcriptomics, and metabolomics) enabled us to identify genes and metabolites important in plant priming. The results contribute to a growing image of how priming of plant defense is activated and function, and shape new approaches for future practical applications in pest control. Further studies may follow investigating the activation of other non-JA-mediated pathways.

CHAPTER 3

Tomato plant defense activation by *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) and persistence of its effects against *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae)*

3.1. Abstract

Nesidiocoris tenuis (Reuter) (Hemiptera: Miridae) is an omnivorous predator widely used in the control of tomato pests. Its plant feeding can induce tomato plant defenses with significant adverse effects on mites and whiteflies, however, their effects on the serious tomato pest *Tuta absoluta* have not been searched in detail. In this study, the density threshold of *N. tenuis* nymphs per plant for activation of defense against *T. absoluta* was searched by enclosing 3, 6 or 10 nymphs on each of the top and bottom leaf of young tomato plants for 4d. After the removal of the nymphs, oviposition rates of *T. absoluta* females were recorded. The results showed that repellence recorded only when 20 nymphs were used per plant. Following a similar methodology, the persistence of the effects was tested for a period of 7d and 14d after the removal of the predators. In both periods the punctured tomato plants were less preferred than control plants. Furthermore, the systemic nature of the defenses was also confirmed on leaves not directly punctured by the predator and on the newly emerged leaves. The results offer information valuable towards the assessment of the plant defense effects induced by *N. tenuis* on *T. absoluta* and on critical aspects for their practical application.

3.2. Introduction

Nesidiocoris tenuis (Reuter) (Hemiptera: Miridae) is an effective predator of several key pests of tomato. This predator is often released in protected tomato crops against whiteflies, aphids and the devastating tomato pest *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) (Urbaneja et al. 2012; Zappalà et al. 2012; Soares et al. 2019). Plant feeding has been recognized as a key factor enhancing *N. tenuis* efficacy in biological control allowing its populations to persist in the crop during prey scarcity periods and control the pests when they emerge in the crop and before increase in numbers (Castañé et al. 2004; Perdikis et al. 2011).

**Based on the publication: "Sarmah N, Voloudakis A, Dervisoglou S, Fantinou A, Perdikis D (2022) Tomato plant defence activation by Nesidiocoris tenuis andpersistence of its effects against Tuta absoluta. Bulletin of Insectology 75: 239-246."*

Besides the visible efficacy of *N. tenuis* in pest control, plant feeding has recently attracted the scientific interest due to the evidence gained that it induces tomato plant defense against insect and mite pests. Plant feeding by *N. tenuis* activates defense pathways related to jasmonic acid (JA) and abscisic acid (ABA). Tomato plants previously punctured by *N. tenuis* were less attractive to adults of *Bemisiatabaci* (Gennadius) (Hemiptera: Aleyrodidae) but more attractive to its parasitoid*Encarsia formosa* (Gahan) (Hymenoptera: Aphelinidae) (Pérez-Hedo et al. 2015; 2017; 2021) and caused 35% reduction in the population of *Tetranychus urticae* Koch (Acari: Tetranychidae) (Pérez-Hedo et al. 2018). Survival and oviposition of *T. urticae* were significantly reduced on tomato plants punctured by females of another closely related mirid predator *Macrolophus pygmaeus* (Rambur) (Pappas et al. 2015). Sweet pepper plants punctured by adults of *M. pygmaeus* significantly reduced reproduction of the two-spotted spider mite but not that of *Myzus persicae* (Sulzer) (Hemiptera: Miridae) (Zang et al. 2018).

These encouraging results indicate that defense activation by *N. tenuis* or *M. pygmaeus* may be exploited as a new strategy in the management of pest infestations on tomato plants (Pappas et al. 2015, Perez-Hedo et al. 2017; Bouagga et al. 2020; Sarmah et al. 2021; Silva et al. 2021). Towards this direction a fundamental aspect to be considered is the duration of persistence of the plant defense activation against pests. Repellency against *B. tabaci* and attraction to the parasitoid *E. formosa*were reported after 7 and 14 days of tomato plants punctured by *N. tenuis*, respectively (Bouagga et al. 2018). A significantly reduced oviposition and survival rate of spider mites on tomato plants punctured by *M. pygmaeus*14 days earlier have been reported, too (Pappas et al. 2015).

An additional aspect critical in the evaluation of this pest control method is the potential of these plant defense responses to occur not only at or near the site of plant feeding by *N. tenuis* but to occur throughout the plant including the plant parts not punctured by the predator and including also, the new plant leaves developed post treatment. These responses have been reported as systemic effects and have been considered essential for protecting plants by activating the defence, before insects relocate to other parts of the plant.Their persistence has been confirmed against *T. urticae* by *M. pygmaeus*for 14 days (Pappas et al. 2015) and *B. tabaci* 7 days after plant feeding by *N. tenuis* (Bouagga et al. 2018).

Aiming to exploit the application of the activation of plant defense responses as a sustainable new strategy in pest control it should be considered that *N. tenuis* plant feeding

may cause plant damage by the production of brown necrotic rings on stems and leaf or flower petioles (Castañe et al. 2011; Chinchilla-Ramírez et al. 2021). The damage severity depends on the population density of the *N. tenuis* per plant (Calvo et al. 2009; Chinchilla-Ramírez et al. 2021). Therefore, it is essential to determine the lowest density of predator capable to initiateeffective defense responses in tomato plantsagainst the target pests achieving effective control levels in order to simultaneously minimize the plant damage risk.

Tuta absoluta is a serious pest of tomatoes worldwide. However, little attention has been paid to the effects of plant defence responses on its performance. Pérez-Hedo et al. (2018) reported non-preference effect on *T. absoluta* due to phytophagy of *N. tenuis*. Sarmah et al. (2022), showed that oviposition preference, larval period and pupal weight of *T. absoluta* were reduced significantly on *N. tenuis*-punctured tomato plants. Despite of the encouraging evidence gained, the duration of the persistence of the defense effects including thepersistence of their systemic nature and the lowest density of *N. tenuis* per plant required to induce tomato plant defense have not been assessed against *T. absoluta*. However, this is essential for any further steps into the development of this method as an alternative strategy in pest control particularly because this pest uses a different feeding mode than all the other pests tested so far and the feeding mode has been reported as potentially involved in the plant defense effects (Thaler et al. 2012; Zhang et al. 2018). Therefore, the specific objectives of the present study were to: a) compare the potential of several densities of *N. tenuis* in thedefense induction of tomato plant b) assess their systemic nature and c) assess the persistence of the plant defense effects against *T. absoluta*.

3.3.Materials and methods

3.3.(i) Tomato plants grown in glasshouse condition and rearing of insects

Plant material

Tomato plants (cv Ace 55 and cv Elpida) [Spirou House of Agriculture, Athens, Greece]), were developed from seeds sown individually in plastic seed trays in the glasshouse. The seedlings were transplanted in plastic pots with compost (Bas Van Burren B.V, Netherlands) substrate. The potted plants were maintained in wooden cages of 75 x 68 x 68 cm kept in a ventilated glasshouse at the Agricultural University of Athens. Plants were kept pest-free and were not sprayed with any pesticide.

Insect rearing

Tuta absoluta rearing was initiated from adults collected from a tomato crop located in Marathon, Greece and maintained on tomato plants (cv. Elpida). Rearing of *N. tenuis* (Nesibug, Koppert, The Netherlands) was maintained on tomato plants (cv. Elpida) with "Entofood" (Koppert B.V., The Netherlands), offered *ad libitum*.

3.3.(ii). Puncturing of tomato plant by predator, *N. tenuis* for activation of defense

Five-week-old tomato plants (cv. Ace 55) with three fully expanded leaves were used in the experiments. The plant defense activation was investigated using 3rd instar nymphs of *N. tenuis* (less than 12h in that instar). Before their use, the nymphs were deprived of Entofood for 2 hrsby placing them individually with tomato leaflets in petri dishes (Pérez-Hedo et al. 2015). On each bottom and the top leaf of each plant nymphs were placed at the following densities: 1) three, 2) five and 3) ten. No food for *N. tenuis* was added and each leaf was enclosed into an organdy bag (12x15 cm). The middle leaf of each plant was enclosed in organdy bag without any predator inside. Each leaf of the control (unpunctured) tomato plants was caged individually without any predator. All the experiments were conducted at 25±1°C, 65±5% RH, and 16:8 h L:D photoperiod. After four days of exposure, the predators were removed and their survival was found to be always higher than 80%. The plant damage caused by the predators was recorded as a surrogate of their phytophagy rate by counting the brown necrotic rings in bottom and top leaves of each punctured tomato plant.Ten replications (plants) were used for each predator density and control and the experiments were repeated twice in 2 months intervals.

3.3.(iii). Predator's density effects on life traits of *T. absoluta* through plant defense induction

After the removal of the predators (0-day post treatment, 0 dpt), a *N. tenuis*-punctured plant was introduced in a cage (35x35x60 cm) (BioQuip CA, USA) together with an unpunctured (control) tomato plant. Then, three pairs of *T. absoluta* adults (less than 48 h old) were introduced into the cage and were allowed to oviposit for the next 24 h. *T. absoluta* adults were provided with sugar solution (10% sucrose) via a piece of cotton in one plastic cup (30 mL) placed inside the cage. After 24h, the number of eggs oviposited on each leaf of both plants was recorded. Experiments were conducted at 25±1°C, 65±5% RH, and 16:8 h L:D

photoperiod. Ten replications (cages) were used for each predator density and the experiments were repeated twice in 2 months intervals. In addition, on 0dpt, the time required by newly emerged 1st instar larvae of *T. absoluta* to initiate tunnelling was also recorded on the bottom, middle and top leaf of plants punctured by the highest density of *N. tenuis,* as described above. This was done by placing an egg with a ready to hatch larva on bottom, middle and top leaf and recording under stereomicroscope the time required by the emerged larva to insert its cephalic capsule in the leaf tissue and initiate a mine, at 25±2.0 ºC. Ten replications (larvae) were used per leaf category for the punctured and unpunctured (control) plants.

3.3.(iv) Persistence of the plant defense induction effects

The oviposition preference of *T. absoluta* was further tested on plants punctured by the highest nymphal density (10 nymphs of *N. tenuis* enclosed per bottom and top leaf), 7 and 14dpt to record the persistence of the induced resistance against *T. absoluta*. For this reason, the same procedure was followed as above described, but after the removal of the predators at 0dpt, the punctured plants were maintained at 25±1°C, 65±5% RH, and 16:8 h L:D photoperiod without exposure to any insect for either 7dpt or 14dpt. After 7dpt, the plants had four fully expanded leaves. In this case, the leaves were designated counting from the bottom as L1, L2, L3 and L4. Three pairs of *T. absoluta* adults (less than 48 h old) were introduced into a cage with a punctured and a unpunctured plant and were allowed to oviposit for the next 24 h. After this period, the number of eggs oviposited on each leaf of both plants was recorded. Similarly, another group of plants 14 days post treatment (14 dpt) was exposed to the pest, under the same conditions. Fourteen days post treatment the plants had five fully expanded leaves designated from the bottom as L1, L2, L3, L4 and L5. Ten replications (cages) were used for each post treatment period. The experiments were repeated twice in 2 months interval.

3.3.(v) Statistical analysis

The number of necrotic rings produced by *N. tenuis* per leaf at 0dpt were analyzed with a Generalized Linear Model with normal distribution and identical function because the data did not folllow the assumptions of ANOVA. The set of data was not significant (χ^2=0.008, *df*=1,118, *P*=0.88), and thus the data were pooled and compared with factors the “predator

density" (i.e. *N. tenuis*-punctured with 6, 10 or 20 nymphs per leaf) and the "punctured leaf position" (bottom vs top tomato leaves).

The data of total number of eggs laid per plant at 0dpt were analyzed with factors the "data set", the "predator density" and the "treatment, punctured and control plant". The effect of the data set was not significant (F = 2.69, df = 1,108, P = 0.10) and thus data were pooled and compared with a 2-way ANOVA with factors the "predator density" and the "treatment". The data of the number of *T. absoluta* eggs laid per leaf at 0dpt were not significantly affected by the set of the replications (F = 0.09, df = 1,358, P = 0.75) and analyzed with a 3-way ANOVA with factors the "predator density", the "treatment" (punctured and unpunctured plant) and the "leaf position" (bottom, middle or top leaf).

The time required by each *T. absoluta*larva to initiate tunnel mining in plants punctured by 10 nymphs were analyzed with a Generalized Linear Model with normal distribution and identical function because the data did not follow the assumptions of ANOVA. The effect of the set of data was not significant ($\chi 2$=0.001, df=1,118, P=0.98), and the data were pooled and compared with with factors the "leaf position" and the "treatment".

The data on the total number of eggs laid per cage with a control and a punctured plant on 0dpt, 7dpt and 14dpt were not affected by the set of data (F = 0.08, df =1,58, P = 0.78), and analyzed with one way ANOVA with factor the "period after the treatment".

The data on the number of eggs laid per plant were not affected by the set of data (F =0.1, df =1,118, P =0.82) and were analyzed with factors the treatment (punctured or control plant) and the period after treatment (0, 7, 14 dpt).

The data of the eggs laid per leaf of punctured or unpunctured plants were analyzed separately for each period with factors the "treatment" (puntuted or unpunctured plant) and the "plant leaf" (L1, L2, L3 and L4 at 7dpt and L1, L2, L3, L4 and L5 at 14dpt). The set of data was not significant in both cases (F = 0.023, df = 1,198, P = 0.87 and F =0.023, df =1,198, P = 0.87 for the 7dpt and the 14dpt, respectively).

In all cases where ANOVA was used the means were compared using the Tukey's HSD test ($\alpha = 0.05$). All the analyses were performed by statistical package JMP 14.1.0.

3.4. Results

3. 4.(i) Damage caused on tomato plants by *N. tenuis*

The effect of the "predator density" (χ^2 = 94.02, df = 2,114, P< 0.0001), the "leaf position" (bottom vs top tomato leaves) (χ^2 = 52.63, df=1,114, P< 0.0001), and their interaction (χ^2 =

48.37, *df*=2,114, *P*< 0.0001) were significant on the number of necrotic rings recorded per leaf. The interaction was due to the fact that the mean number of necrotic rings was significantly higher in the top than the bottom leaves at the highest predator density (i.e., 20 *N. tenuis* per plant), however, at the lower predator densities there was no significant difference (Fig. 3.1).

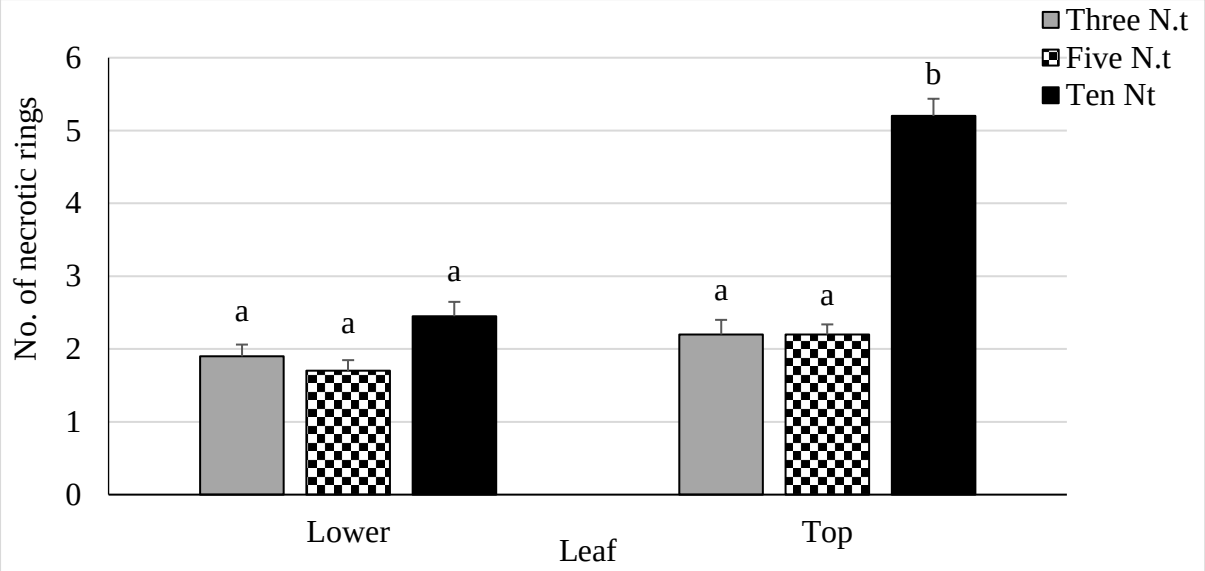


Fig. 3.1.Number (mean ± SE) of necrotic rings produced by different densities of *N. tenuis*3rd instar nymphs enclosed on L1 (bottom) and L3 (top) leaves of tomato plants for 4d. Columns with the same letter are not significantly different (ANOVA, Tukey HSD, P < 0.05).

3.4. (ii).Oviposition preference of *T. absoluta* between unpunctured and punctured tomato plants by different *N. tenuis* densities

According to the statistical analysis, the total number of eggs laid per plant by each *T. absoluta* female at 0dpt was significantly affected by the predator density (*F*= 18.46, *df*= 2,114, *P*<0.001 format), but the effects of treatment and their interaction were not significant (*F*= 0.04, *df*= 1,114, *P*=0.83 and *F*= 1.02, *df*= 2,114, *P*=0.36, respectively). The number of eggs laid on the untreated and the treated plant was significantly reduced at the highest density in comparison to that recorded at the lowest predator density (Fig.3.2). The number of eggs laid was not different between the treated and the untreated plant in each predator density level.

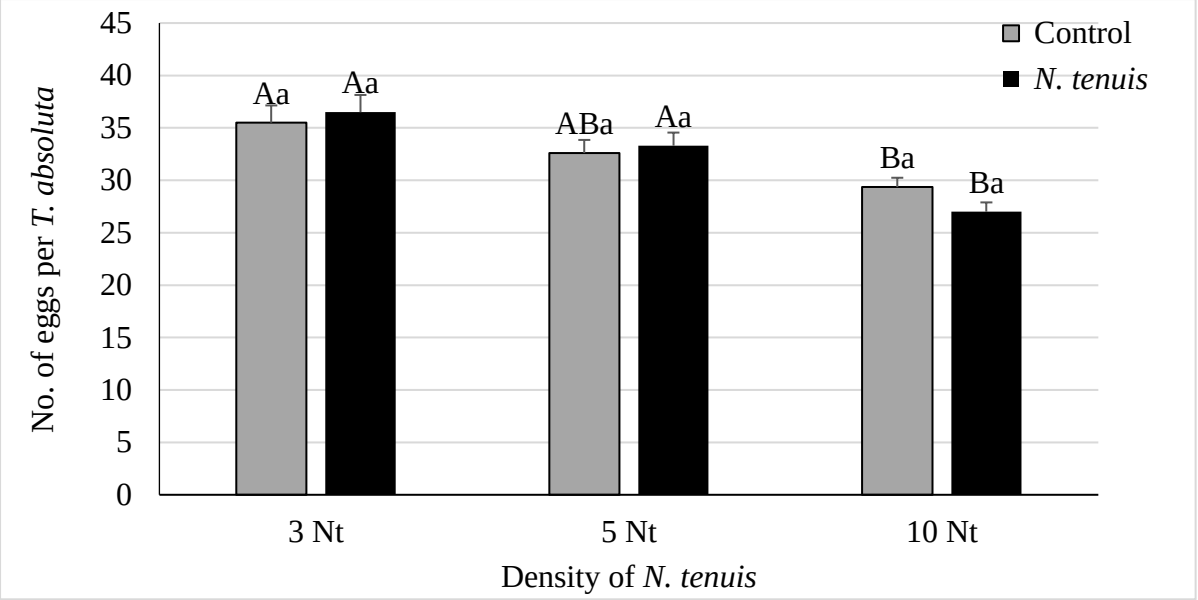


Fig. 3.2. Number (mean ± SE) of *T. absoluta* eggs per female of *T. absoluta* oviposited on tomato plants punctured by three, five and ten *N. tenuis* in comparison to unpunctured (control) tomato plants at 0 dpt. Columns with the same capital letter are not significantly different among predator density levels at each treatment and columns followed by the same small letter are not significantly different between treatments at each predator density (ANOVA, Tukey HSD, $P< 0.05$).

Regarding leaf oviposition preference of *T. absoluta* females at 0dpt, a significant interaction was recorded among the factors "predator density" (i.e. *N. tenuis*-punctured with 6, 10 or 20 nymphs and control) and "leaf position" (bottom vs middle vs top leaf) ($F = 2.69$, $df = 4, 342$, $P< 0.03$). In all cases a significantly higher number of eggs had been oviposited on the top in comparison to the middle and the bottom leaf. Comparisons showed that a significantly reduced number of eggs of *T. absolut*a laid in the top leaves when plants were punctured by 20 3rd instar of *N. tenuis* as compared to the corresponding leaf of unpunctured plants (Fig. 3.3).

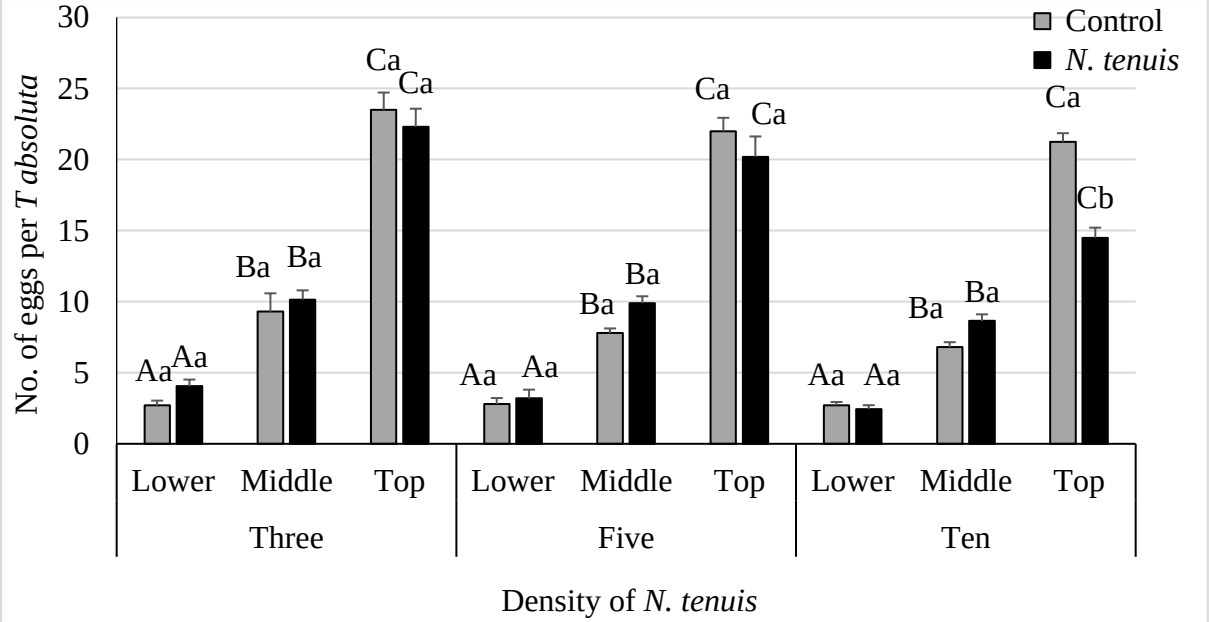


Fig. 3.3.Number (mean ± SE) of *T. absoluta* eggs oviposited on tomato plants punctured with different densities of *N. tenuis* in comparison to unpunctured tomato plants on bottom, middle and top leaves of tomato. Three, five or ten nymphs of *N. tenuis* had been enclosed for 4d on each bottom and top leaf of each plant, while middle leaves remained without *N. tenuis*. On the leaves of the control plants no *N. tenuis* had been enclosed. Columns followed by the same capital letter are not significantly different among leaves in each treatment (i.e. density of *N. tenuis*) and columns followed by the same small letter are not significantly different between treated and control plants in each leaf category within each treatment (ANOVA, Tukey HSD, $P< 0.05$).

3.4.(iii) Period for larval tunneling initiation of *T. absoluta* on *N. tenuis*-punctured and unpunctured tomato plants

The time required by a 1st instar larva of *T. absoluta* at 0dpt to locate a suitable location on the tomato leaflet to insert its cephalic capsule and initiate feeding on plants punctured by 20 *N. tenuis* nymphs was significantly affected by the factor "treatment" (χ^2= 89,51, *df* =1, 114, $P< 0.0001$) but not by the "leaf position" (bottom vs middle vs top leaf) (χ^2 =1.75, *df* = 2, 114, P =0.41) and their interaction (χ^2 =0.32, *df* = 2, 114, P =0.85). The amount of time required on bottom, middle and top leaf of unpunctured plants was always significantly lower than that in the treated plants and did not differ significantly among leaves in the treated or the untreated plants (Fig. 3.4).

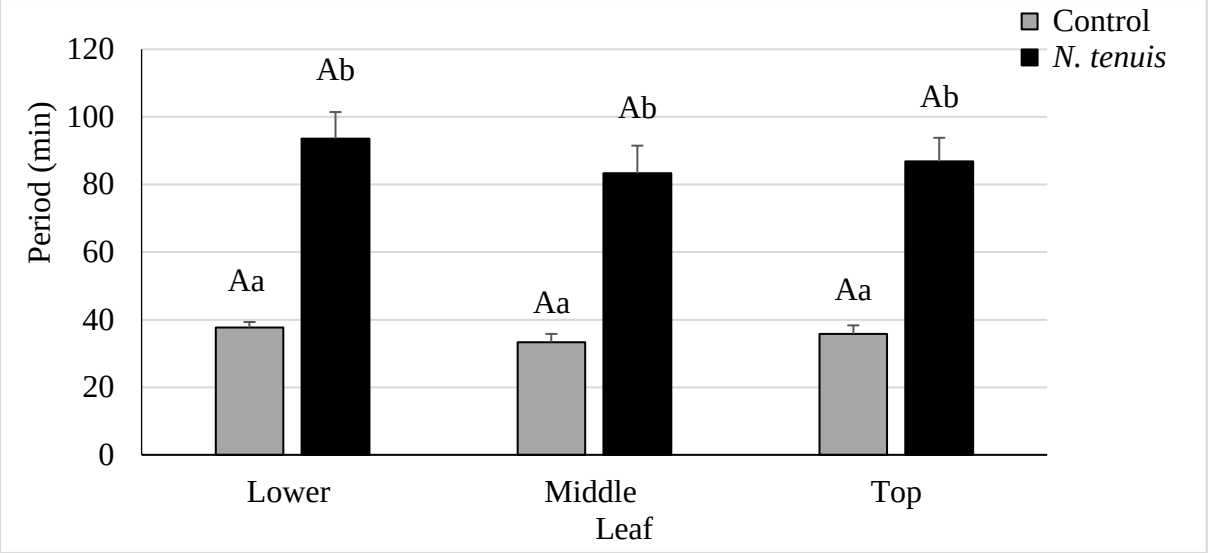


Fig. 3.4. The length of the period required (mean ± SE) by 1st instar larvae of *T. absoluta* to initiate tunnel miningon "punctured by *N. tenuis*" tomato plants in comparison to unpunctured tomato plants. Ten nymphs of *N. tenuis*had been enclosed for 4d on each bottom and top leaf of the punctured plants, while the middle leaf remained without *N. tenuis*. On the leaves of the control plants no *N. tenuis* had been enclosed. Columns with the same capital letter are not significantly different among bottom, middle and top leaves at each treatment, and columns followed by the same small letter are not significantly different between treatments within each leaf category (ANOVA, Tukey HSD, $P< 0.05$).

3.4.(iv) Persistence of the plant induction effects

The total number of eggs of *T. absoluta* laid per cage with a plant punctured by 20 *N. tenuis* nymphs and a control plant was significantly higher on 0dpt than the longer periods post treatment, without significant difference between the latter (57.85 ± 0.74, 48.80±0.89 and 48.95±0.83 eggs per cage on 0, 7, 14dpt, respectively, $F = 39.11$, $df = 2, 57$, $P< 0.001$).

The total number of eggs laid on each plant (punctured or unpunctured) in the cage on 0dpt, 7dpt and 14dpt, was affected by a significant interaction between "treatment" and "post treatment period"($F = 4.95$, $df = 2, 114$, $P < 0.0001$) whereas the main effects of "treatment" and "post treatment period" were significant, too ($F = 207.90$, $df = 1,114$, $P < 0.0001$ and $F = 40.64$, df = 2,114, $P < 0.0001$, respectively) (Fig. 3.5). In all periods, the number of eggs laid on the control plant was significantly higher than in the punctured plant and the respective percentage of reduction was 15%, 26% and 28%. The number of eggs laid on 7 dpt and 14 dpt in either the unpunctured or the punctured plants was significantly reduced in comparison to the respective number laid on 0dpt.

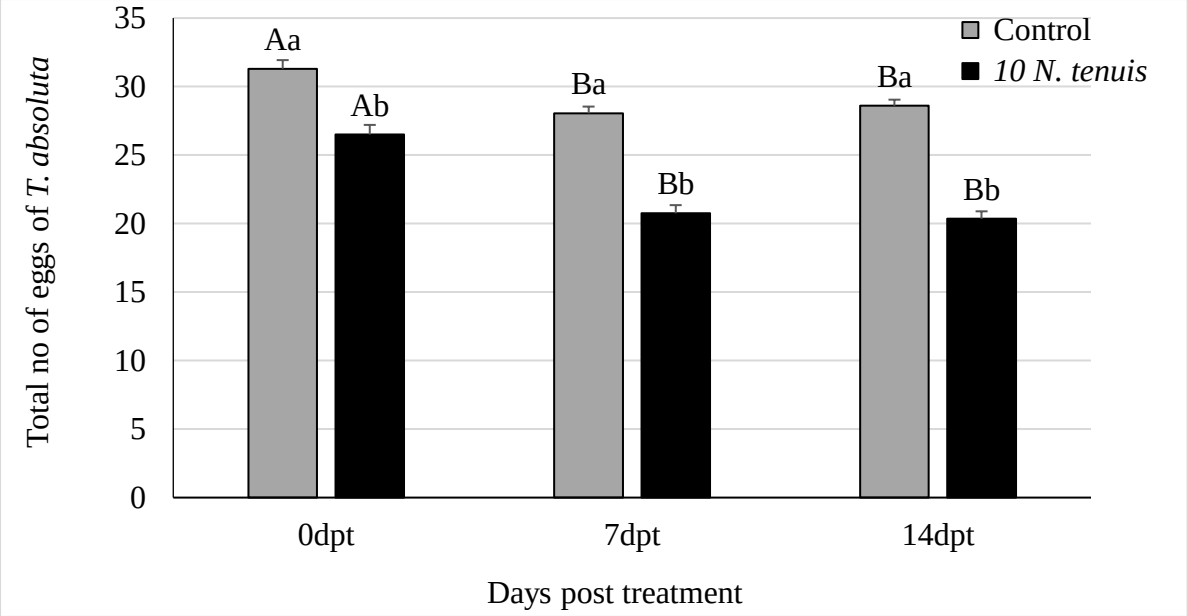


Fig. 3.5. Number (mean ± SE) of *T. absoluta* eggs oviposited on a tomato plant at 0, 7 and 14 days after punctured by *N. tenuis* and a control (unpuctured) plant, when placed together in a cage. Columns with the same capital letter are not significantly different among the three post treatment intervals within each treatment (puntured or unpunctured plant) and columns followed by the same small letter are not significantly different between the two treatments within each time interval (ANOVA, Tukey HSD, $P < 0.05$).

The factor "treatment" (i.e. 20 nymphs of *N. tenuis* or without nymphs), the "leaf position" and their interaction had significant effects on persistence of resistance after one week period from predators' removal (F = 48.76, df = 1, 152, P< 0.0001, F = 467.52, df = 3, 152, P< 0.0001 and F = 5.39, df = 4, 152, P = 0.0015, respectively). Significantly reduced number of eggs were laid by *T. absoluta* on top (L4) and L3 leaf of punctured plants in comparison to their corresponding leaves of unpunctured plants. There was no significant difference in number of eggs laid in L1 or L2 (Table 3.1,2).

Table 3.1. Number of eggs laid by *T. absoluta* (mean ± SE) on each leaf of tomato plants punctured by *N. tenuis* 7-days earlier, in comparison to unpunctured plants. Ten nymphs of *N. tenuis* had been enclosed for 4d on each L1 (bottom) and L3 (top) leaves of the plants, while L2 (middle) leaves remained without *N. tenuis*. The newly grown leaves were designated as L4 which were also not punctured by *N. tenuis.* Means followed by the same capital letter are not significantly different in a row and means followed by the same small letter are not significantly in each column (Tukey HSD, $P< 0.05$).

Treatment	**Leaf position**			
	L1	**L2**	**L3**	**L4** (top leaf)
Unpunctured plant	2.82±0.24 Aa	3.05±0.26 Ba	9.40±0.38 Ca	13.35±0.41 Da
***N. tenuis*-punctured plant**	1.75±0.20 Aa	2.61±0.25 Ba	7.55±0.36 Cb	10.60±0.33 Db

The factor "treatment" (i.e. 20 nymphs of *N. tenuis* or without nymphs) and the "leaf position" had significant effect on persistence of resistance after two-week time lag and their interaction was also significant ($F = 51.73$, $df = 1, 190$, $P< 0.0001$, $F = 580.54$, $df = 4,190$, $P<0.0001$ and $F = 6.34$, $df = 4, 190$, $P< 0.0001$). The number of eggs laid was significantly higher on the top leaves in both punctured and unpunctured plants. Significantly reduced number of eggs were laid on the L3, L4 and L5 leaves in comparison to their corresponding leaves of unpunctured plant (Table 3.2).

Table 3.2.Number of eggs laid by *T. absoluta* (mean ± SE) on each leaf of tomato plants punctured by *N. tenuis* 14-days earlier, in comparison to unpunctured plants. Ten nymphs of *N. tenuis* had been enclosed for 4d on each L1 (bottom) and L3 (top) leaves of the plants, while L2 (middle) leaves remained without *N. tenuis*. The newly grown leaves were designated as L4 and L5 which were also not punctured by *N. tenuis.* Means followed by the same capital letter are not significantly different in a row and means followed by the same small letter are not significantly in each column (Tukey HSD, $P< 0.05$).

Treatment	**Leaf position**				
	L1	**L2**	**L3**	**L4**	**L5** (top leaf)
Unpunctured plant	0.65±0.16 Aa	1.5±0.18 Ba	5.4±0.35 Ca	7.95±0.33 Da	12.0±0.36 Ea
***N.tenuis*Punctured plant**	0.85±0.16 Aa	1.05±0.15 Ba	3.95±0.25 Cb	6.75±0.21 Db	10.15±0.36 Eb

3.5. Discussion

The results showed that the defence induction of tomato plants against *T. absoluta* by the phytophagy of *N. tenuis* was dependent on *N. tenuis* density per plant. Tomato plants punctured by 6 or 10 nymphs of 3rd instar in total, for 4d were not able to repel adults of *T. absoluta*. However, *T. absoluta* females laid significantly reduced number of eggs on tomato plants punctured by 20 nymphs of *N. tenuis*. Pérez-Hedo et al. (2018) showed that exposure of a tomato plant to 20 adults of *N. tenuis* for 24h induced defense against *T. urticae*. Naselli et al. (2016) reported that 20 individuals of nymphs and adults of *N. tenuis* enclosed for 24hon a tomato plant were able to repel *B. tabaci* adults. Pappas et al. (2015) reported significantly lower oviposition and survival rate of spider mites on tomato plants punctured by 2 females of *M. pygmaeus* for 4 days, but such an effect was not recorded in the case of whiteflies.Zhang et al. (2018) reported that on tomato plants punctured by 10 adults of *M. pygmaeus* for 4 days, *T. urticae* reproduction was significantly reduced but not that of *M. persicae*. These results show that plant defence responses against different pests may be dependent on the level of density of the predator and the period of its plant feeding, likely depending on the pest species under study. Our results showed that a higher number of *N. tenuis* may be required for the induction of plant defence against *T. absoluta* compared to that against mites or whiteflies. This could be associated with different feeding mode of *T. absoluta* in comparison to mites or whiteflies and suggests that species specific studies should be undertaken.

The adverse effects on the oviposition of *T. absoluta* recorded at 14dpt after the exposure of plants to the predator, indicate a long persistence of the plant mediated response effects. Pérez-Hedo et al. (2018) showed that at 14dpt the number of *T. urticae* individuals was reduced by 35% on tomato plants punctured by twenty 4th instar nymphs of *N. tenuis* for 24h. Pappas et al. (2015) showed that the number of eggs laid and the survival of spider mites were adversely affected at 14dpt by 41% and 20% respectively, when two young females of *M. pygmaeus* had been used per plant for a period of 4 days. Bouagga et al. (2018) using a Y-tube olfactometer assay showed that sweet pepper plants punctured by 25 *N. tenuis* adults per plant for 24hsignificantly repelled *B. tabaci* 7dpt, however, this effect was raised 14dpt. Therefore, it seems that the persistence of the effects depends on the target pest species, the density and the length of the period of predators' plant feeding. In our study, the adverse effects on the oviposition of *T. absoluta* were comparable at 7dpt and 14dpt indicating that their potential to last longer than 14dpt should be searched by future research.

Our results provide evidence that the persistence of plant defence response against *T. absoluta* lasted for a long period not only locally but also systemically in the plant since the adverse effects against *T. absoluta*oviposition**w**ere recorded on leaves not punctured but also on the new leaves grown during the post treatment period.This persistence of systemic defensive response may have important practical implications due to the effective protection against *T. absoluta* on entire plants, including the new leaves. Pappas et al. (2015) reported analogous to our results systemic adverse effects on the oviposition and survival of *T. urticae*on tomato plants exposed to only two virgin females of *M. pygmaeus* for a period of 4 days. These effects lasted for a similarly long post treatment period (14dpt). It is likely that systemic defence response and its persistence may be associated with different predator density and plant feeding period against different pest species.

The period required for a newly hatched larva of *T. absoluta*to mine into leaves was significantly increased at 0dpt in all the leaves of a punctured tomato plant in comparison to the control. Such an effect may increase larval susceptibility to predation or insecticides indicating another aspect of the protective effect of the plant defence activation by *N. tenuis* (Sarmah et al. 2021).In addition, considering that the middle leaf was not punctured by the predator, this confirms effective systemic plant defence responses against young larvae of *T. absoluta*.

The number of eggs laid by *T. absoluta* females on an unpunctured (control) plant was significantly reduced at the 7dpt and 14dpt in comparison to that recorded at the 0dpt, in a cage where it was placed together with a punctured plant. Similarly, the total number of eggs laid per cage with one punctured and one control plant was reduced at 7dpt and 14dpt. These findings suggest that the induced defense effects produced by plant feeding of *N. tenuis* may provide protection to nearby tomato plants increasing the potential of this method in pest control. This supports the results of Pérez-Hedo et al. (2021) reported that the release of herbivore induced plant volatiles (HIPV) in greenhouse environment could activate defense mechanisms in healthy tomato plants.

The density of 20 *N. tenuis* nymphs per plant, where plant defense effects against *T. absoluta* were recorded, produced 7.65 necrotic rings per plant after 4 days of exposure. This number of rings noted is comparable to that reported by Urbaneja-Bernat et al. (2019) (i.e. 7.8 necrotic rings per 30 cm high plant exposed to twelve 3rd instar nymphs of *N. tenuis* for seven days). Further research is needed to determine whether these effects may cause significant adverse effects on plant growth.

In parallel, persistence of plant defense responses induced by spraying with metabolites or by releasing volatiles may be investigated, too (Esmaeily et al. 2020; Pérez-Hedo et al. 2021).The present study explored the density level of the predator *N. tenuis* required to induce tomato plant defense response against the serious tomato pest, *T. absoluta*, and the persistence of the protective effects produced. The results showed that the effects depend on the density of the predator and persisted at least as long as 14dpt. During this period, protection of unpunctured leaves was effective, showing the potential of the plant induction to protect the entire plant. Therefore, plant defense induced by plant feeding of *N. tenuis* may have a long persistence and should be assessed for the control of *T. absoluta*. Further studies may explore the persistence of the effects for longer periods, their parallel efficacy against other pests as well as their persistence in the field towards a more sustainable pest management.

CHAPTER 4

DsRNA-mediated pest management of *Tuta absoluta* is compatible with its biological control agent *Nesidiocoris tenuis**

4.1. Abstract

RNAi interference (RNAi) is a conserved post-transcriptional regulation mechanism found in all eukaryotes which is initiated by double-stranded RNA in the cytoplasm, leading to a homology-dependent gene silencing effect. This prevents protein synthesis of the target gene. Orally deliverable RNAi is being employed to develop species-specific biopesticides. RNAi-mediated insect pest management has recently shown promising results against one of the most serious pest of tomato, the tomato leafminer *Tuta absoluta*. This study aimed to investigate whether dsRNA (ds*Ta-αCOP*) designed to target *T. absoluta-αCOP* genecould cause adverse effects to its biocontrol agent, the mirid predator *Nesidiocoris tenuis.* Oral exposure of *N. tenuis* to dsRNA (ds*Nt-αCOP*) designed to target *N. tenuis-αCOP* resulted in a 61%, 67% and 55% reduction in its transcript level in comparison to the sucrose, ds*GFP*, and ds*Ta-αCOP* treatments, respectively. In addition, significantly higher mortality of 57% was recorded in ds*Nt-αCOP*-treated *N. tenuis* when compared to the sucrose (7%), ds*GFP* (10%), and ds*Ta-αCOP* (10%) treatments. Moreover, the predation rate of ~33-39 *Ephestia kuehniella* eggs per *N. tenuis* adult dramatically reduced to almost half by the surviving of the ds*Nt-αCOP*-treated *N. tenuis*. This worst-case exposure scenario confirmed for the first time that the RNAi machinery is functional in *N. tenuis* and that the risk of exposure through the oral route is possible. In contrast, ds*Ta-αCOP*did not cause any sub-lethal effects to *N. tenuis* upon oral exposure. Oral exposure of *T. absoluta*to ds*Ta-αCOP* resulted in 50% mortality. In context of biosafety risk assessment of RNAi-mediated insect management, investigating the effects on non-target organisms, is essential in order to include this method as part of an integrated pest management strategy. Based on our laboratory assays, RNAi-mediated control is compatible with the biological control of *T. absoluta* by its natural enemy *N. tenuis*, adding the RNAi approach in the armoire of integrated pest management of *T. absoluta.*

**Based on the publication: "Sarmah N, Kaldis A, Taning CNT, Perdikis D, Smagghe G, Voloudakis, A (2021)dsRNA-Mediated pest management of Tuta absoluta is compatible with its biological control agent Nesidiocoris tenuis.Insects 12:274*

Key words: RNAi, biosafety, integrated pest management, IPM, biological control, oral droplet feeding

4.2. Introduction

The fast growing world population demands advanced agricultural technology to increase productivity in order to satiate the need of food requirement. But along with the productivity, the losses by pest, pathogens and weeds have also been expanded. Insect pest management has become a priority in research planning due to the huge loss of 18–20% of the annual crop production worldwide (Sharma et al. 2017). Researchers are working on sustainable, environment friendly and cost-effective alternative pest control methods.The breakthrough discovery of RNAi, defined as sequence-specific silencing of target geneby Fire et al. (1998) where the substantial decline of an endogenous mRNA transcript through the injection of homologous dsRNA in *Caenorhabditis elegans* (Maupas) (Rhabditida:Rhabditidae) was recorded,has revolutionized molecular entomology. It has emerged as an excellent reverse genetic tool in various insect ordersand as a next generation pest management technology. The high species-selectivity and a short environmental persistence of the active ingredient are the main advantages of this approach (Christiaens et al. 2020; Sharma et al. 2021). In RNAi dsRNA-specific endonucleases, such as Dicer-2, enable in the production of small interfering RNAs (siRNAs) from the dsRNA. In turn, siRNAs are loaded to the RNA-induced silencing complexes (RISCs), having as a central component the Argonaute proteins which also possess endonucleolytic capacity. Argonautes use the siRNAs as guides for the recognition of mRNAs with matching sequence identity, finally causing the degradation or the translational inhibition of these mRNAs.The RNAi pathway in insects which is triggered as a response to exogenous dsRNA is mediated through Dicer-2, Argonaute-2 and the insect-specific dsRNA-binding protein R2D2. Phylogenetic analysis in insects revealed that the aforementioned core components of RNAi might have been duplicated or lost during the evolutionary history. This could explain the observed variability in the efficacy of RNAi across different insect lineages (Dowling et al. 2016).

The first RNAi-mediated effects in insects were recorded in the fruit fly *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) (Kulkarni et al. 2006). Since then, application of RNAi mechanism has been extensively studied in insects (Zhang et al. 2015; San Miguel et al. 2016). In insect pest control, RNAi-mediated gene knockdown effect has been demonstrated in Diptera, Coleoptera, Hymenoptera, Orthoptera, Blattodea, Lepidoptera, and Hemiptera (Baum et al. 2007; Mao et al. 2007; Price et al. 2008; Dias et al. 2019). Among Lepidoptera, satisfactory RNAi results have been reported in *Spodoptera exigua* (H.) (Noctuidae) (Tian et a. 2009), *Helicoverpa armigera* (H.) (Noctuidae) (Yu et al. 2009),

Plutella xylostella (L.) (Plutellidae) (Chaitanya et al. 2017), and *Mythimna separata* (W.) (Noctuidae) (Ganbaatar et al. 2017).

The delivery of dsRNA plays great role to achive successful results. The silencing efficiency varies depending on the species and method of its delivery like ingestion, microinjection or soaking. Microinjection is more frequently used method under laboratory conditions because it can indicate the known dose and thus increases its effectiveness (Yu et al. 2013). However, it rather requires expensive equipments and skilled technicians expanding the cost incurred. Microinjection is also not a practical approach for screening a large number of target genes for pest management (Rodrigues et al. 2017). Ingestion or soaking can be more appropriate for screening of target genes for future control strategies at field level (Yu et al. 2013; Taning et al. 2016). Oral delivery of dsRNA is applicable for a high-throughput screening of genes. It is a practical approach for small insects and their early instars and itrequires less investment (Zhu et al. 2011).For effective implementation of RNAi, a regular and an autonomous take-up of the dsRNA by the insect is essential. The mid gut is the best region for dsRNA uptake because it is the main site of absorption in insects (Hakim et al. 2010).

Tomato leafminer, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is one of the most invasive and destructive insect pests of Solanaceous crop tomato being the major host globally (Biondi et al. 2018). Biological control is a promising alternative to control *T. absoluta*(Desneux et al. 2010). One such predatory natural enemy of *T. absoluta* is *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae). It is widely employed against thrips, whiteflies, spider mites, aphids, leaf miners and moths as a generalist zoophytophagous predator (Urbaneja et al. 2012; Zappalá et al. 2013; Perez-Hedo et al. 2017). In the absence of prey,plant feeding behaviour of *N. tenuis* may cause necrotic rings on the stem and in many cases flower abortion is also reported (Arnó et al. 2006). For this reason, the release of a lower number of *N. tenuis* is highly recommended (Molla et al.2011). Another drawback of using *N. tenuis* is the need of extended establishment period on the crop (Pérez-Hedo et al. 2021). Although, *N. tenuis* is a reliable contributor to *T. absoluta* management, such limitations may hamper its efficacy and further its acceptance and adoption by the farmers. Consequently, aiming to sustain the benefits of the biological control, the possibility to combine biological control with another pest control method such as RNAi may be explored.

In search for an environmentally friendly approach to manage *T. absoluta*, the RNA interference (RNAi)-based control strategy has recently been employed with encouraging results (Camargo et al. 2016; Bento et al. 2020). Functional RNAi has been achieved in *T.*

absoluta in several genes such as *Juvenile hormone inducible protein* (*JHP*), *Juvenile hormone epoxide hydrolase protein* (*JHEH*), *Ecdysteroid 25-hydroxylase* (*PHM*), *Chitin synthase A* (*CHI*), *Carboxylesterase* (*COE*) (Bento et al. 2020), *Arginine kinase* (*AK*) (Camargo et al. 2016; Bento et al. 2020), and *Vacuolar ATPase-A* which exhibited significant larval mortality in this pest (Bento et al. 2020; Rahmani et al. 2021).

Besides its efficacy, integrating RNAi Integrated Pest Management (IPM) strategies, requires the assessment of RNAi's effects on the natural enemies of the target pests (Neumeier et al. 2021; Taning et al. 2020). In a study by Pan et al. (2020), the effects of dietary RNAi toxicity assay of *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) active dsRNA for the gene *V-ATPase* proved to be detrimental to four non-target lady bird species (Coleoptera:Coccinellidae). Castellanos et al. (2019) reported that the combined use of RNAi-mediated gene silencing against the Neotropical brown stink bug *Euschistus heros* (F.) (Hemiptera: Pentatomidae), together with eggs of parasitoid *Telenomus podisi* Ashmead (Hymenoptera: Scelionidae), proved to be a feasible approach. These studies denote the critical need to evaluate the lethal or sub-lethal effects of RNAi approach on natural enemies as an essential aspect for decision of incorporating RNAi in IPM programs.

The main objective of the present study is to investigate whether the RNAi-based control of *T. absoluta* may cause lethal or sub-lethal effects on its major natural enemy, namely the predator *N. tenuis*. We present, for the first time, evidence that the performance of the predator *N. tenuis* is not negatively affected by the dsRNA targeting the ortholog *αCOP* gene of *T. absoluta*. This raises the possibility to combine RNAi-mediated management with the biological controlof *T. absoluta*, for an effective protection of tomato crops from this notorious pest.

4.3. Materials and methods

4.3.(i). Tomato plants grown in glasshouse condition and rearing and maintenance of insects

Tomato plants (cv. Elpida) were developed from seeds without applying any pesticide (Fig. 4.1). They were inspected daily, and any pest found was removed. *Nesidiocoris tenuis*(Nesibug™, Koppert, The Netherlands) were reared on tomato plants andwere provided with a mixture of *E. kuehniella* eggs and dried cysts of *Artemia* spp. (Entofood™, Koppert) *ad libitum*, twice per week (Fig. 4.2). *Tuta absoluta* initially collected from Marathon, Greece (Latitude: 38° 09' 11.41" N, Longitude: 23° 57' 46.01" E) were reared on tomato plants (Fig.

4.3 and Fig. 4.4). Adult moths were fed with sugar solution (10% sucrose) (Tarusikirwa et al. 2021); a piece of cotton was half-dipped in the sugar solution in each of four plastic cups (30 mL), placed inside a rearing cage. Adult moths could feed from the piece of cotton half-hanging outside each cup. Each rearing was maintained separately in wooden entomological cages (80×80×70 cm) in a glasshouse at temperature of 25±2.5ºC and natural lighting at the Agricultural University of Athens, Greece.

Fig. 4.1. Tomato plants grown from seeds in the greenhouse.

Fig. 4.2. Rearing of *N. tenuis* in the greenhouse.

Fig. 4.3. Rearing of *T. absoluta* cultured separately in insectary.

Fig. 4.4. *T. absoluta* provided with fresh tomato plants.

4.3.(ii). Target gene selection and dsRNA synthesis

As a target gene for RNA silencing in *T. absoluta* the *alphaCOP* (*αCOP*) (Coatomer subunit alpha protein) was selected. *αCOP* is an essential eukaryotic gene responsible for the mediation of biosynthetic protein transport from the endoplasmic reticulum to the Golgi network. To investigate the risk of exposure of *N. tenuis* to dsRNA targeting *T. absoluta*, namely *Ta-aCOP*, a worst-case scenario was considered where *N. tenuis* was directly exposed to dsRNA designed to specifically target *Nt-aCOP*.To identify the nucleotide sequence of *αCOP* in *N. tenuis*, the *αCOP* sequence from *Halyomorpha halys* (Stål) (Hemiptera:Pentatomidae) (Accession no. XM_014431769.2) was used as a "query" against the whole-genome shotgun contig database of NCBI via BLASTN analysis. A corresponding genome shotgun sequence from *N. tenuis* (Accession no. CADCXU010020467.1) was used to design primers for the synthesis of dsRNA molecules of *Nt-aCOP* (Table 4.1). To produce ds*Nt-αCOP*, total RNA was extracted from 5th instar nymphs of *N. tenuis*using a TRIzol-based method essentially as described by Yoo et al. (2004). A number of pooled nymphs were frozen in liquid nitrogen and then added in an Eppendorf tube containing 500 µl TRIzol reagent (Ambion, Austin, YX, USA). Quickly, we grinded the insect tissue inside TRIzol using a homogenizer with suitable pestles for 10 min. Then we placed the tube at 4^{o}C for 1 h. We added 100 µl chloroform, mixed well and centrifuged at 15000 g for 15 min. RNA was then precipitated using isopropanol and sodium acetate. After drying, the RNA pellet was diluted in Rnase-free H_2O. The optical density (OD) at 260 nm was measured spectrometrically by a Fisher Scientific Multiskan FC Reader. Residual genomic DNA from the extracted RNA was removed by Turbo DNAfree kit (Ambion, Austin, USA), after which cDNA was prepared from the RNA, using oligo-dT and Superscript IV (Thermo Fisher Scientific, Carlsbad, USA). A selected fragment of *Nt-αCOP* was amplified by PCR (95 °C – 2 min, 35 cycles of 95°C – 30 s, 60°C – 30 s, 72°C – 45 s, followed by 72°C – 5 min)with *Taq* DNA polymerase (Invitrogen, Waltham, USA), using 500 ng of cDNA as a template and *Nt-αCOP* specific primers flanked at their 5' ends by a T7 promoter sequence (5'-TAATACGACTCACTATAGGG-3') (Table 4.1). The identity of the *Nt-αCOP* fragment was verified by sequencing. *In vitro* synthesis of dsRNA targeting the *αCOP* gene of *N. tenuis* (ds*Nt-αCOP*) was then performed using the MEGAscript RNAi kit (Thermo Fisher Scientific) essentially as described by Voloudakis et al. (2015). The length of ds*Nt-αCOP*was 391 nt (Fig. 4.5). As a negative control, dsRNA targeting the *GFP* (Green Fluorescent Protein of jellyfish *Aequoreavictoria* gene) (ds*GFP*), a gene that is absent in insects, was also

synthesized using a plasmid containing a GFP insert (NC_011521.1) and GFP-specific primers (5'-TACGGCGTGCAGTGCT-3', 5'-TGATCGCGCTTCTCG-3'). The length of ds*GFP* was 455 nt (Fig.4.5).

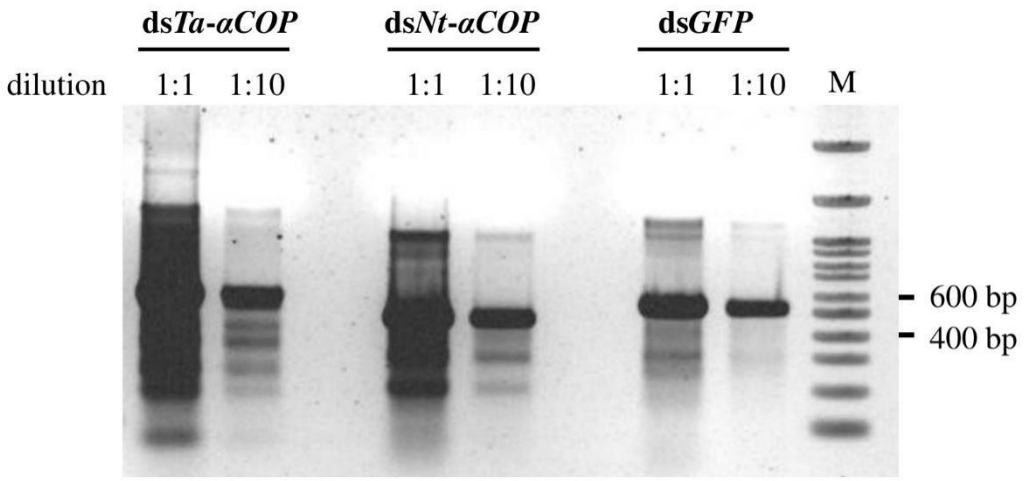


Fig.4.5.*In vitro* synthesis of dsRNA for application in*T. absoluta* and *N. tenuis*. DsRNA molecules derived from a 505-nt fragment of the *αCOP* gene of *T. absoluta* (ds*Ta-αCOP*), a 391-nt fragment of the *αCOP* gene of *N. tenuis* (ds*Nt-αCOP*) and a 455-nt fragment of the *GFP* gene (ds*GFP*) were produced (see Materials and methods). To check their quality, 1 μl from undiluted and from 1:10 diluted dsRNA was electrophoresed on 1.5% agarose gel. M is a 100-bp DNA ladder (New England Biolabs, Beverly, MA, USA).

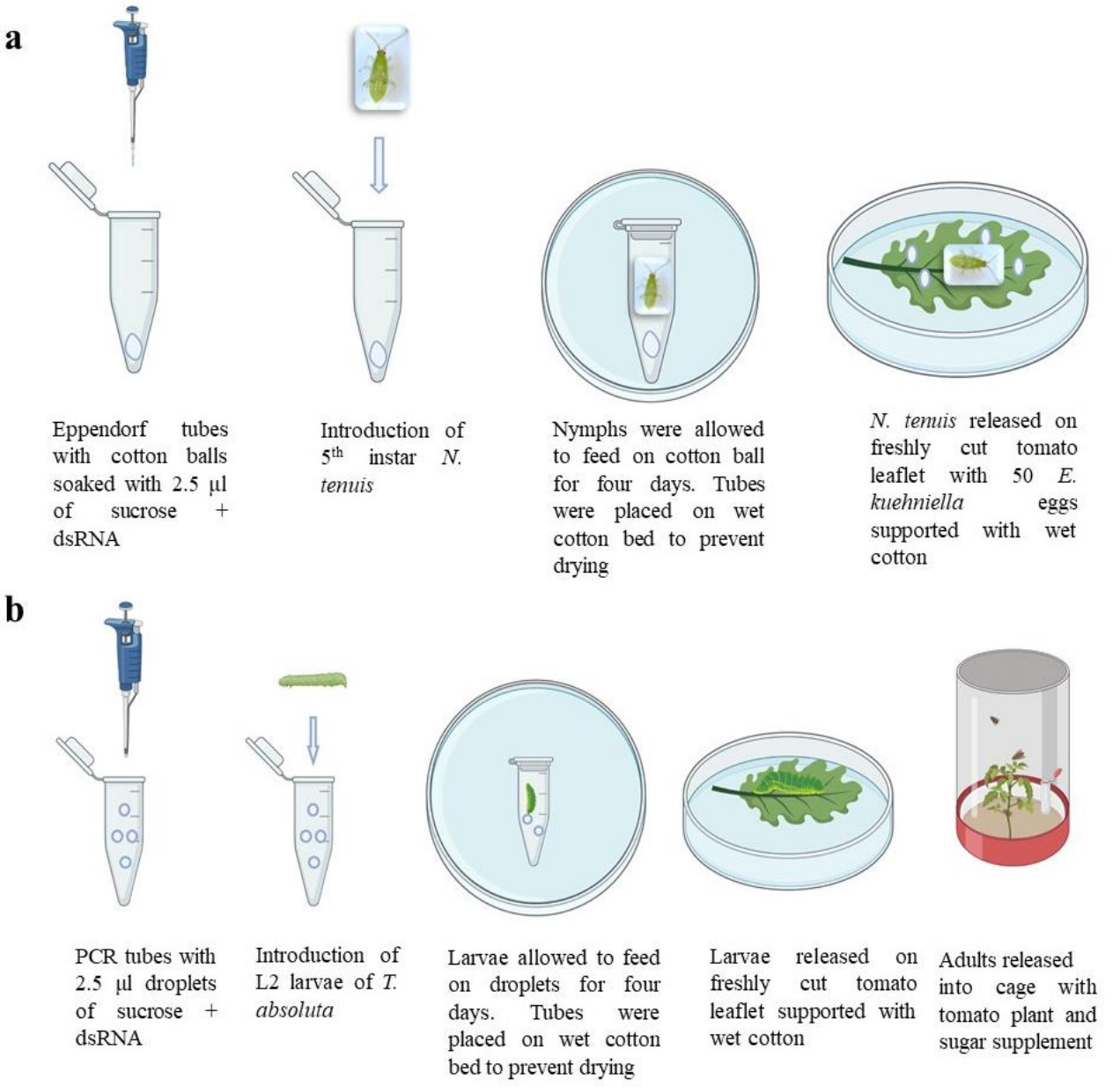


Fig. 4.6. Experimental set up for oral delivery of dsRNA via sucrose. (a) For *N. tenuis* set up, cotton balls were soaked with a solution composed of 0.5 M sucrose + 0.5 µg/µl dsRNA in an Eppendorf tube. A single 5th instar nymph was introduced in each Eppendorf tube for 4 d. Three independent experiments employing 14 Eppendorf tubes were performed for each treatment. Eppendorf tubes containing sucrose only (without dsRNA) were used as the control treatment. (b) in *T. absoluta*, ten 0.25 µl droplets (total amount 2.5 µl) of a solution composed of 0.5 M sucrose + 0.5 µg/µl dsRNA were placed on the inner wall of the PCR tube. A single L2 larva was introduced in each PCR tube, remaining there for 4 d. Three independent experiments employing 14 PCR tubes were performed for each treatment. PCR tubes containing sucrose only (without dsRNA) were also included as control treatment. Image was created using biorender.com.

Table 4.1. Primers designed in this study for *in vitro* production of dsRNA and gene expressionanalysis in *Nesidiocoris tenuis* and *Tuta absoluta*.

Name	Type	Sequence (5´-3´)	Product size (nt)	Target species	Purpose
ds*Nt-αCOP* (joined to T7 promoter)	F	TAATACGACTCACTATAGGG CACACTGCCCCTGATCGTAT	391	*N. tenuis*	dsRNA production
	R	TAATACGACTCACTATAGGG GTCGAGTTTACGCAGGAAGC			
qPCR-*Nt-αCOP*	F	GGGAGGACTCGAAGAACATTT	95	*N. tenuis*	qPCR
	R	GATCGTGCCCTTCCAAGAC			
qPCR-*Nt-ATPB*	F	CATACGCCAAGGGAGGTAAA	356	*N. tenuis*	qPCR
	R	CTGGGTGAAACGGAAAATGT			
ds*Ta-αCOP* (joined to T7 promoter)	F	TAATACGACTCACTATAGGG CCGTTTTCATCACAGGTCT	505	*T. absoluta*	dsRNA production
	R	TAATACGACTCACTATAGGG CGGTCATGGCCACTAAGAAT			

To identify the nucleotide sequence of *Ta-αCOP*, the *αCOP* sequence from *Papilioxuthus*(L.) (Lepidoptera:Papilionidae)(Accession no. XM_013305848.1) was used for BLASTN analysis. A corresponding genome shotgun sequence from *T. absoluta* (Accession no. SNMR01042501) was used to design primers for dsRNA production. DsRNA targeting *Ta-αCOP* (ds*Ta-αCOP*) was synthesized as described above for *Nt-αCOP*. The length of ds*Ta-αCOP* was 505 nt (Fig.4.5). The concentration of all dsRNAs produced was brought to 1 µg/µl using a Fisher Scientific Multiskan FC Reader (Thermo Fisher Scientific).

4.3.(iii). Oral delivery of dsRNA to *N. tenuis* and *T. absoluta*

The oral feeding method (Turner et al. 2006; Taning et al. 2016; Pan et al. 2020) was used to deliver ds*Nt-αCOP*, ds*GFP*, ds*Ta-αCOP*, and sucrose solution to *N. tenuis*. In brief, a 1.2 mg

cotton ball was inserted into a sterile 1.5 ml Eppendorf tube and was soaked with a sucrose droplet containing either 2.5 µl of sucrose solution (0.5 M) or 2.5 µl of sucrose solution (0.5 M) mixed with specific dsRNA molecules (0.5 µg/µl) (Fig. 4.6). Second or third instar nymphs of *N. tenuis* were transferred from the rearing cage to caged tomato plants with eggs and cysts offered *ad libitum*, and kept at 25±1°C, 65 ± 5% RH and 16 h light. Fifth (5^{th}) instar nymphs of *N. tenuis* of 24-48 hrs in that instar were collected from the caged plants and placed individually in Petri dishes for 2 hrs before their use in the experiments having access only to a tomato leaflet in order to reduce variation in their hunger level. Then each nymph was introduced individually into an Eppendorf tube (Fig. 4.6). The tube opening was covered with parafilm and pin holes were made on it to allow adequate ventilation (Fig. 4.7).A preliminary experiment with 30 *N. tenuis* individuals feeding on sucrose only for 4 d was performed. Monitoring for 14 days showed that all individuals survived and moulted to the next instar, indicating that this set up was suitable for the normal development of the predator. The next step was to expose *N. tenuis* to four treatments: a) sucrose only, b) sucrose+ds*GFP*, c) sucrose+ds*Ta-αCOP*, d) sucrose+ds*Nt-αCOP*. *N. tenuis*nymphs, inside the Eppendorf tubes, could feed on the cotton balls for 4 d (25 ±1ºC, 65±5% RH, and 16 L:8 D photoperiod). The tubes were placed on a wet cotton bed in a 12-cm-diameter Petri dish to prevent the soaked cotton from drying out during the exposure period (Fig. 4.10). The Petri dishes had 2 mesh-covered holes (3 cm diameter each) on their lids to reduce the accumulation of humidity.

Upon completion of the 4-day-feeding period all the nymphs (n = 10) from each treatment had moulted to adults. After removal from the tubes, the adults were immediately introduced into individual Petri dishes with a tomato leaflet supported by wet cotton. On the leaflet, 50 eggs of *E. kuehniella* (Epheggs™, Bioinsecta, Thessaloniki, Greece)were offered as prey (Fig. 5a) under the above mentioned conditions. The number of eggs consumed by *N. tenuis* were counted under a stereoscopic microscope after 24 hrs, i.e., at 1 dpt (day post treatment). Then, adults were kept individually in a Petri dish where they were provided daily with a fresh tomato leaflet and Entofood *ad libitum*. Their predation rate on *E. kuehniella* eggs was further assessed at 4 dpt following the above mentioned methodology. The mortality of *N. tenuis* individuals was monitored every day up to 10 dpt. The experiment was repeated thrice. 10 nymphs/treatment were used in each experiment (Fig. 4.11).

In *T. absoluta*, the dsRNA molecules were administered by sucrose droplet feeding (Pan et al. 2020; Turner et al. 2006) (Fig. 4.7). A single 2^{nd} instar larva of *T. absoluta* (starved

for 2 h) was carefully placed in a sterile 0.2 ml PCR tube, in which ten 0.25 µl droplets (1.5 mm in size is appropriate for larval feeding) had been placed (Fig. 4.7). According to our preliminary experiments this quantity of sucrose solution could support the development of the larva for a period of 4 d (Fig. 4.8). The solutions used were: a) sucrose only, b) sucrose+ds*GFP* and c) sucrose+ds*Ta-αCOP*. The final concentration of sucrose in the administered droplets was 0.5 M and that of dsRNA was 0.5 µg/µl. The PCR tube opening was covered with parafilm (Fig. 4.9) and ten pin holes were made on it to allow adequate ventilation. These PCR tubes were placed on a wet cotton bed in a 12-cm-diameter Petri dish (Fig. 4.10) and kept at 25±1ºC, 65±5% RH, and 16 L:8 D photoperiod (Fig. 4.11). Larvae were allowed to feed for 4 d in the PCR tubes (Fig. 4.8); upon completion of the 4-day-feeding period, they were carefully placed individually, with the help of a fine brush, on a freshly-cut tomato leaflet supported by a wet sterile cotton, in a Petri dish (Fig. 4.11).

Fig. 4.7. Droplets of sucrose solution placed on inner wall of PCR tube

Fig. 4.8. L2 larva of *T. absoluta* feeding on droplet of sucrose mixture

Fig. 4.9. Parafilmed Eppendorf tube with *N. tenuis* inside

Fig. 4.10. Eppendorf tubes placed on wet cotton bed

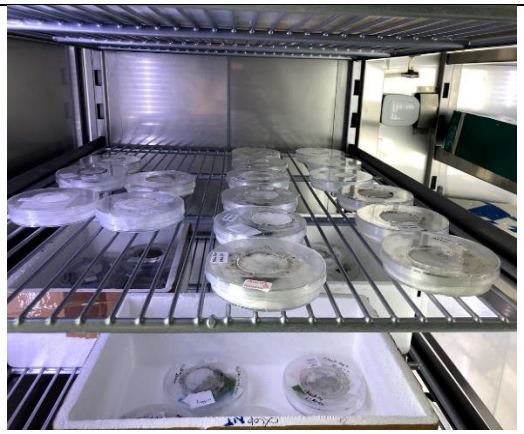


Fig. 4.11. Petri dishes with *N. tenuis* provided with 50 *E. kuehniellla* eggs placed inside the growth chamber

The amount of time required for each larva to find a suitable feeding site on the tomato leaflet and insert its head in the mesophyll was recorded over time. Then, each larva was allowed to feed on the leaflet in the dish. The effect of the dsRNA application on larval survival was recorded daily till pupation. A larva was considered dead when it was not able to move back to a ventral position after being placed on its dorsum within one minute (Reyes et al. 2012). In addition, the developmental period and pupal weight for each larva were recorded. Each pupa was weighed using an analytical balance (Kern ACS 80-4, Balingen, Germany). Finally, adult survival was monitored for one week after emergence from the pupa. During this period the adults were kept in cages with tomato plants and were provided with sugar solution as food supplement. The experiment was repeated thrice. 14 larvae/ treatment were used in each experiment.

4.3.(iv). Gene expression analysis

Three independent biological replicates were employed for gene expression analysis. Four *N. tenuis* adult individuals per treatment were selected immediately after the completion of the 4-day-feeding period and RNA was extracted as described above. Reverse transcription (RT) was performed by employing oligo-dT and random primers. In brief, 2 µl from each RNA sample was mixed with 0.5 µl of oligo-dT (100 µM), 0.5 µl of random primers (100 µM) and 12 µl of Rnase-free water. The PCR tubes were placed at 65 ºC for 5 min and quickly transferred on ice. Then 5 µl of hot mix (2 µl 10X RT buffer with DTT, 1 µl dNTPs [10 mM], 0.5 µl FIREScript reverse transcriptase [Solis BioDyne, Tartu, Estonia], 0.25 µl Rnase inhibitor and 1.25 µl of Rnase-free water) were added in each PCR tube. RT was performed

at 27 ºC for 10 min followed by 60 min at 37 ºC to produce cDNA, then the enzyme was inactivated at 85 ºC for 5 min and the tubes were stored at -20 ºC. Semi-quantitative PCR reactions were carried out using the KAPA Taq DNA polymerase or the KAPA high fidelity (HiFi) DNA polymerase (Kapa Biosystems, Cape Town, South Africa) following the manufacturer's instructions. Quantitative PCR (qPCR) analysis was performed employing the 5X HOT FIREPol EvaGreen qPCR Supermix (Solis BioDyne), in a StepOnePlus Real-Time PCR System (Applied Biosystems, Forster City, USA). Relative quantification of gene expression was carried out using the $2^{-\Delta\Delta CT}$ method, as described by Schmittgen & Livak (2008).

4.3.(v). Statistical analysis of data

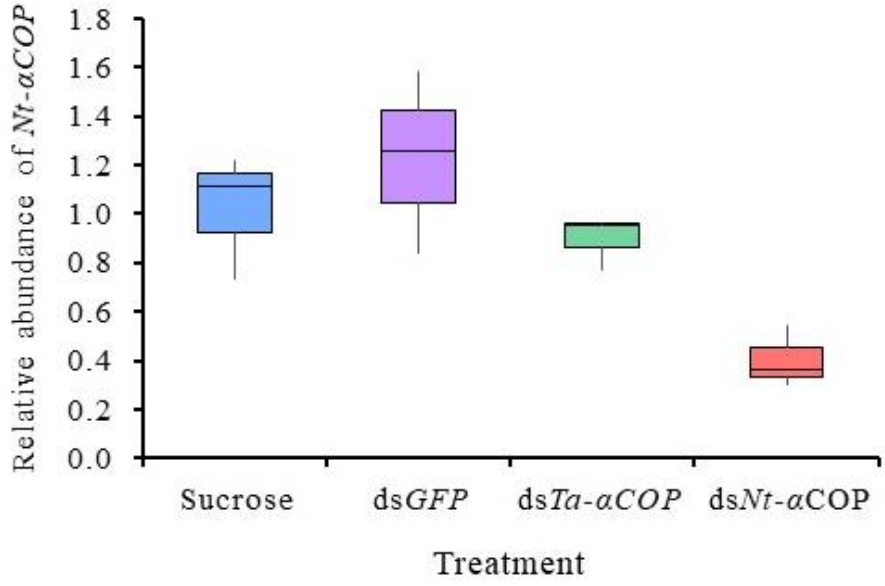
Significant differences in the quantity of the endogenous *αCOP* gene expression level in *N. tenuis* nymphs fed with sucrose only, sucrose+ds*GFP*, sucrose+ds*Ta-αCOP*, or sucrose+ds*Nt-αCOP* were determined by Student's t-test ($p< 0.05$) performed in a pairwise manner. For normalization purposes, *ATPB* was used as the internal control. The mean survival curves of *T. absoluta* larvae and *N. tenuis* individuals on the different treatments were compared by log-rank (Mantel-Cox) test ($p< 0.0001$) with GraphPad Prism version 8.2.0 software (GraphPad Software, San Diego, USA). For the study of sublethal effects of dsRNAs, each *N. tenuis* nymph was considered as one replicate. The data of the three independent experiments were pooled and one-way ANOVA was performed to analyse the predation rate of *N. tenuis*. In a similar manner, the time required by each *T. absoluta* larva to initiate tunnel mining was investigated. The mean values of all treatments were compared using the Tukey's HSD test ($\alpha = 0.05$) by the statistical package JMP 14.1.0.

4.4. Results

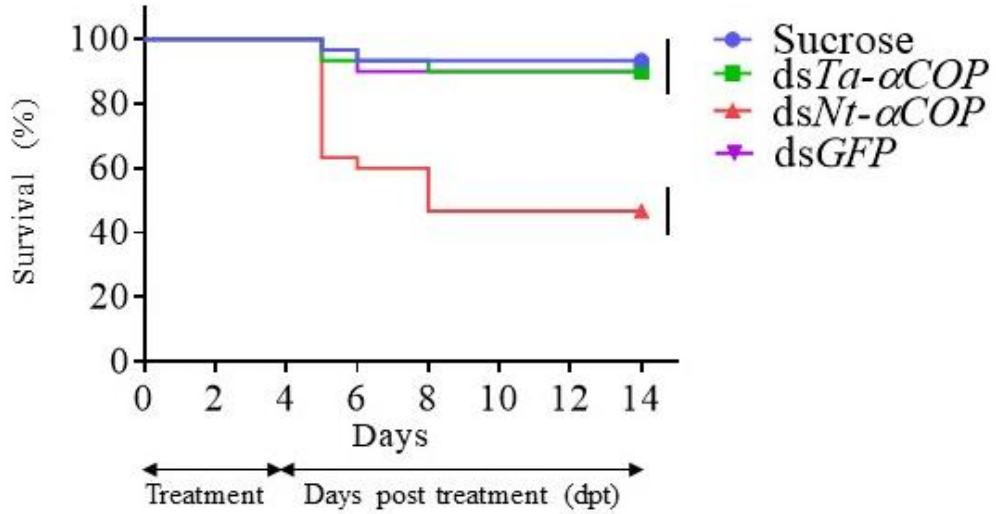
4.4.(i). Effects of oral feeding of ds*Nt-αCOP* in *N. tenuis*

αCOP, an essential eukaryotic gene, was selected as the gene target in the present study. DsRNA (ds*Nt-αCOP*) targeting the *αCOP* gene (*Nt-αCOP*) in *N. tenuis* was produced and fed to *N. tenuis* nymphs. ds*Nt-αCOP*-treated *N. tenuis* showed a mean reduction of *Nt-αCOP* transcript levels by 61% and 67% in comparison to the sucrose- and the dsGFP-treated controls, respectively (sucrose-treated: $p = 0.010$; ds*GFP*-treated: $p = 0.011$) (Fig. 4.12).This confirmed that the oral exposure of *N. tenuis* to gene-specific dsRNA can lead to RNAi-mediated gene knockdown.

a


Relative abundance of Nt-αCOP
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sucrose
dsGFP
dsTa-αCOP
dsNt-αCOP
Treatment

b


Survival (%)
100
80
60
40
20
0
0 2 4 6 8 10 12 14
Days
Treatment
Days post treatment (dpt)
Sucrose
dsTa-αCOP
dsNt-αCOP
dsGFP

c

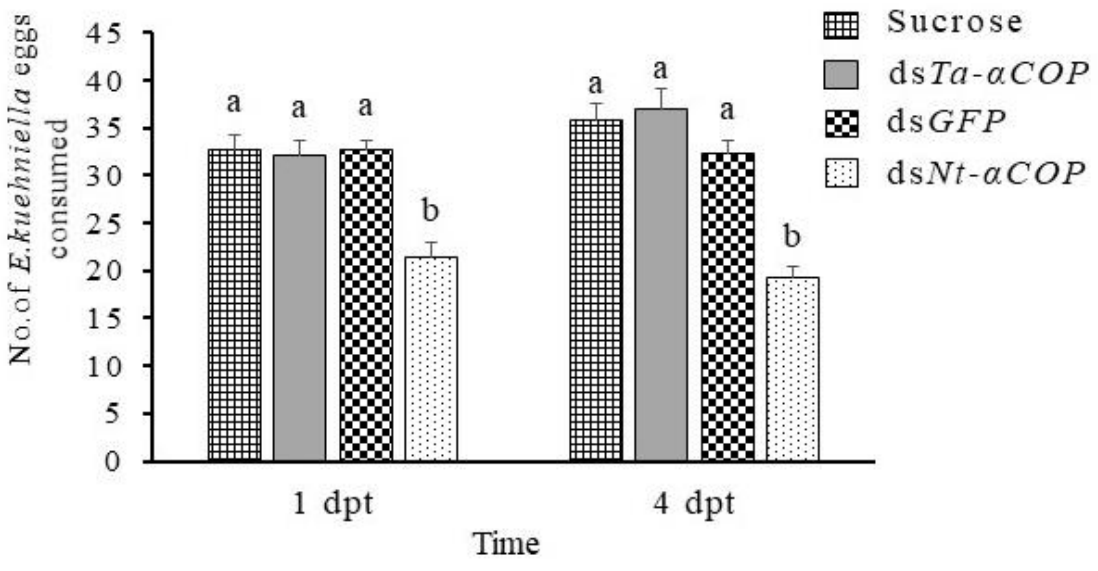

No. of E.kuehniella eggs consumed
45
40
35
30
25
20
15
10
5
0
a a a b
a a a b
1 dpt
4 dpt
Time
Sucrose
dsTa-αCOP
dsGFP
dsNt-αCOP

Fig. 4.12. Effect of homologous and non-homologous dsRNA on survival and predation rate of *N. tenuis*. (a)Relativequantification of the endogenous *Nt-αCOP* gene expression by RT-qPCR. The treatments tested were sucrose, sucrose + ds*GFP*, sucrose + ds*Ta-αCOP*, and sucrose + ds*Nt-αCOP*. Results were obtained from three biological replicates. For normalization, *ATPB* was used as the internal control. Relative expression values were obtained using the $2^{-\Delta\Delta CT}$ method. For statistical analysis, the Student's t-test was employed. Theexpressionn levels of *Nt-αCOP*in ds*Nt-αCOP*treatmentsignificantly differ ($p < 0.05$) in comparison to the other three treatments. (b)Mean survival curves of *N. tenuis* fed from 0 to 4 d on sucrose, sucrose+ds*GFP*, sucrose+ds*Ta-αCOP*,sucrose+ds*Nt-αCOP* and subsequently fed with *E. kuehniella* eggs for 10 days on a tomato leaflet. Curves terminating at the different vertical bars are significantly different according to the log rank test ($p < 0.0001$). (c) Number of *E. kuehniella* eggs consumed per *N. tenuis* individual at 1 and 4 dpt, after feeding for 4 d on sucrose, sucrose+ds*GFP*, sucrose+ds*Ta-αCOP*,sucrose+ds*Nt-αCOP*. Columns followed by different letter differ significantly ($p < 0.05$).

4.4.(ii). No significant cross-silencing effects of ds*Ta-αCOP* on *N. tenuis*

Ds*Nt-αCOP*-treated *N. tenuis* showed a mean reduction of *Nt-αCOP* transcript levels by 55% in comparison to ds*Ta-αCOP*-treated *N. tenuis* ($p = 0.004$) (Fig. 4.12a). In addition, no significant reduction in the transcript levels of *Nt-αCOP* was observed ($p > 0.05$) in ds*Ta-αCOP*-treated *N. tenuis* when compared to either of the controls (ds*GFP*- or sucrose only-treated *N. tenuis*) (Fig. 4.12a). These observations correlated with the low level of sequence complementarity between the selected *Ta-αCOP* region (used for ds*Ta-αCOP* production) and its ortholog *Nt-αCOP* in *N. tenuis* (Fig. 4.13), suggesting that ds*Ta-αCOP* does not trigger any significant cross-silencing effect against the ortholog *αCOP* gene in *N. tenuis.*

Moreover, no significant difference ($p > 0.78$) in the mortality was recorded at 10 dpt in *N. tenuis* that were treated with ds*Ta-αCOP* (10% mortality) when compared to the ds*GFP* (10% mortality) or sucrose (7% mortality) controls (Fig. 4.12b). This result correlated with the observation that the transcript level of *Nt-αCOP* did not significantly change in these treatment groups. On the other hand, the 10% mortality in ds*Ta-αCOP*-treated *N. tenuis* is significantly lower *(*$p = 0.0001$) than the 57% mortality recorded in ds*Nt-αCOP*-treated *N. tenuis.* This indicates that the exogenous application of ds*Ta-αCOP* might not cause lethal effects in *N. tenuis.*

Lastly, ds*Ta-αCOP*-treated *N. tenuis* showed no significant difference in predation rate at 1 and 4 dpt when compared to any of the controls (ds*GFP*- and sucrose-treated groups) (Fig. 4.12c). Most importantly, the predation rate of ds*Ta-αCOP*-treated *N. tenuis* was significantly higher (32.1 and 37 eggs consumed/adult on 1 dpt and 4 dpt, respectively) than in ds*Nt-αCOP*-treated *N. tenuis* (21.5 and 19.3 eggs consumed/adult on 1 dpt and 4 dpt, respectively). Taken all these together, our data could support that the performance of the

predator *N. tenuis* was not negatively affected by the dsRNA targeting of the prey *T. absoluta*.

Fig. 4.13.DNAsequence alignment between the region of *αCOP* from *T. absoluta* used for dsRNA production and the respective region of *αCOP* from *N. tenuis*. Alignment was carried out using MUSCLE (Multiple Sequence Comparison by Log- Expectation). Highlighted in blue capital letters indicate the identical nucleotides between the two sequences.

4.4.(iii). Oral delivery of ds*Ta-αCOP* to *T. absoluta* can cause lethal to sublethal effects

A significantly higher mortality was observed in *T. absoluta* larvae that were fed with ds*Ta-αCOP* when compared to either the sucrose only- or the ds*GFP*-treated larvae. By 5 dpt, mortality had reached an average of 50% for the ds*Ta-αCOP*-treated larvae when compared to the ds*GFP*-treated control ($df = 2$, $F = 85.69$, $p < 0.0001$) (Fig. 4.14a). No significant difference in survival was recorded between the sucrose only- and the ds*GFP*-treated groups. In addition to the survival, the amount of time required by *T. absoluta* larvae to penetrate the leaf tissue was also evaluated. Our results indicated that ds*Ta-αCOP*-treated larvae required a significantly ($p < 0.0001$) longer period of about 41.7 min to penetrate the leaf tissue when compared to the ds*GFP*-treated control that required 25.7 min (Fig. 4.14b). This was about double the time required for tunnel initiation for the ds*Ta-αCOP*-treated larvae, indicating

possible sublethal effects following oral exposure to gene-specific dsRNA. Nevertheless, 100% of the surviving larvae (sucrose: n = 39; ds*GFP*: n = 40; dsTa-αCOP: n = 21) successfully pupated and emerged as adults in all treatments and further survived for 7 d after emergence.

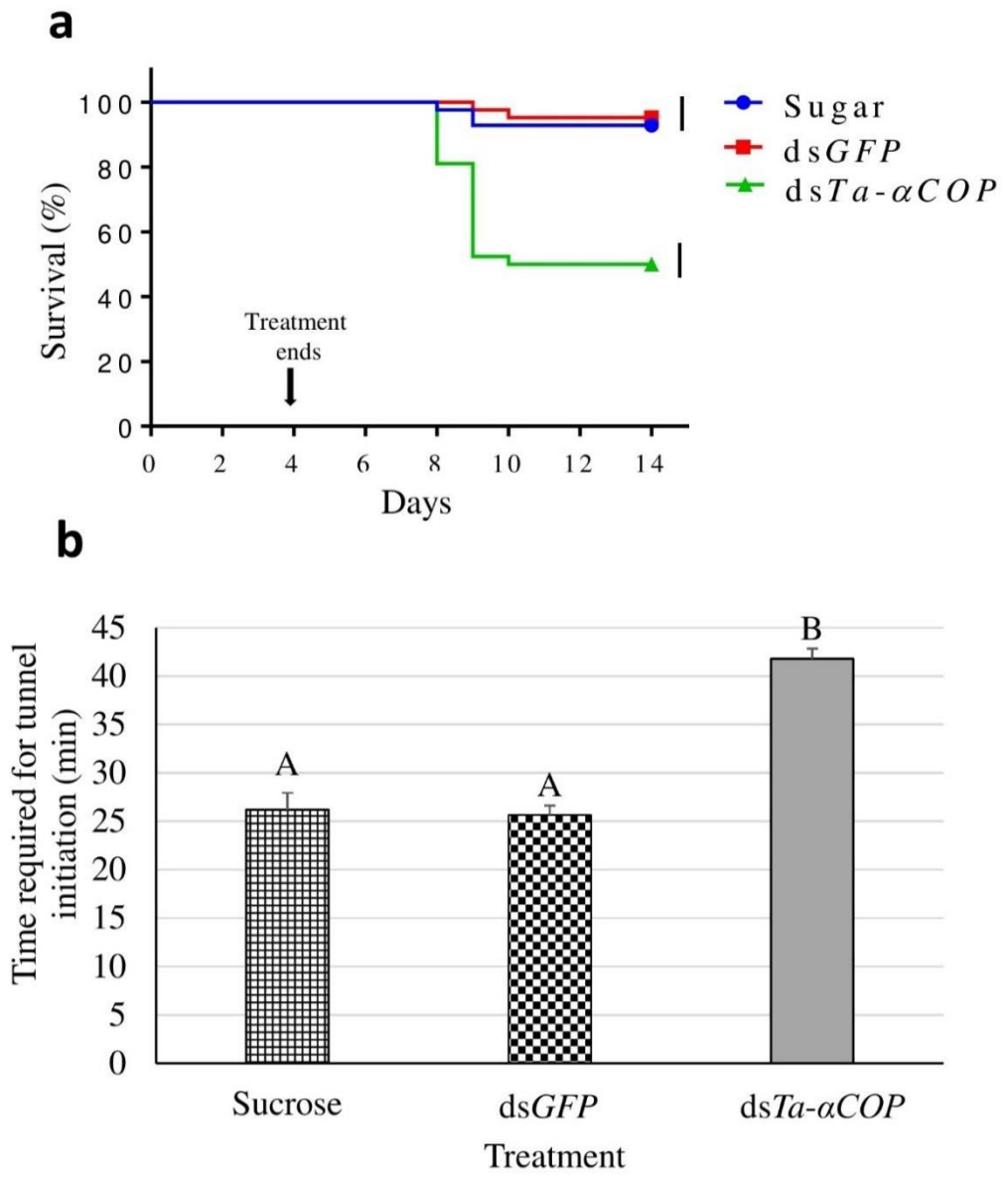


Fig. 4.14. Effect of ds*Ta-αCOP* application on L2 larvae of *T. absoluta* through sucrose droplet oral delivery.(a)Mean survival curves of second-instar larvae of *T. absoluta* fed for 4 d on sucrose, sucrose+ds*GFP*, sucrose+ds*Ta-αCOP*,and subsequently fed for 10 days on a tomato leaflet. Curves terminating at the different vertical bars are significantly different according to the log rank test ($p < 0.0001$). (b)Time required (mean ± SE) to initiate tunnel mining by second-instar larvae of *T. absoluta* fed for 4 d on sucrose, sucrose+ds*GFP*, and sucrose+ds*Ta-αCOP*.Columns followed by different letter differ significantly ($p < 0.05$).

4.5. Discussion

Our data indicated that dsRNA targeting *Ta-αCOP* of the pest, *T. absoluta*, did not cause any lethal nor sublethal effects in its biological control agent, *N. tenuis*. Furthermore, thepredatory behaviour of *N. tenuis* was not affected at 1 and 4 dpt after four days of exposureto ds*Ta-αCOP*.

The successful gene-silencing of *αCOP*, via RNAi, has been reported to significantly reduce survival in other insects such as *Nezara viridula* (L.) (Hemiptera: Pentatomidae) (Sharma et al. 2021), *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) (Taning et al. 2016), and *Brassicogethes aeneus* (Fabricius) (Coleoptera: Nitidulidae) (Willow et al. 2020). In the present, significantly higher mortality of 57% was recorded at 4 dpt in ds*Nt-αCOP*-treated *N. tenuis* when compared to the ds*GFP* (10% mortality), and the sucrose (7% mortality) controls.

For the first time, it was shown that the application of dsRNA targeting the *Ta-αCOP*gene caused 50% mortality in *T. absoluta*. Camargo et al. (2016) reported 50% and 43%mortality in *T. absoluta* through silencing of the genes *V-ATPase* and *AK*, respectively.Mortality percentages of 45%, 46%, 49% and 72% were observed when larvae were exposed

to dsRNA designed to target the genes *CHI*, *JHP*, *COE* and *AK*, respectively (Bento et al. 2020). Therefore,the gene *αCOP* can cause relatively significant mortality in *T. absoluta* within a week. Thisagrees with the lethal effects of dsRNA targeting the gene *αCOP* designed for other pestinsects such as the sweet potato weevil, *Cylas formicarius* (F.) (Coleoptera: Brentidae) (Prentice et al. 2017) and pollen beetle *Brassicogethes aeneus* F. (Coleoptera: Nitidulidae) (Willow et al. 2020). In addition, itscompatibility with *N. tenuis* indicates that *Ta-αCOP* is a potentially valuable gene to beemployed in further developments of RNAi-based strategies against *T. absoluta*.Along with the mortality in ds*Ta-αCOP*-fed larvae of *T. absoluta*, the time required toinitiate feeding into the mesophyll of the tomato leaflet was nearly twice when comparedto the control (ds*GFP*-treatment), which demonstrated an RNAi-mediated sub-lethal effecton the larvae. In IPM such delays are of particular importance because the treated larvaewould be exposed for a much longer period either to predation risk by the foragingbiological control agents or to residues of contact insecticides applied on the leaf. In thelatter case, there may be a reduction of pesticide use due to the increased efficacy of thepesticide application. Another important parameter that was examined at the organism level was the predation rate (proxy for sublethal effect) of *N.*

tenuis. At 1 and 4 dpt, a significantly lower predation rate (21.5 and 19.3 eggs consumed/adult, respectively) was recorded in *N. tenuis* adults fed with ds*Nt-αCOP* when compared to the sucrose- and ds*GFP*-treated controls.Therefore, the evaluation of non-lethal effects of RNAi on *T. absoluta*may also reveal synergies with the other IPM tactics and their significance in *T. absoluta*control should be investigated in future studies.The advantages of RNAi-mediated pestcontrol indicate its potential for future field application (Li et al. 2015; Mamta et al. 2017; Christiaens et al. 2020). However, a fundamentalstep in this direction is the development of appropriate risk assessment tools to generatereliable data for registration purposes (Arpaia et al. 2020, 2021; Mezzetti et al. 2020; Papadopoulou et al. 2020).

Notably, in previous studies (Singh et al. 2017; Grover et al. 2019; Tayler et al. 2019). RNAi was reported as ineffective or of lower effectiveness in insects belonging to several orders,including hemipteran. Therefore, here,we targeted an endogenous gene in *N. tenuis* and demonstrated, for the first time, that RNAi is functional in *N. tenuis*. This is an important finding, showing that future effortsmade in the direction of RNAi-based pest management should consider possible nontargeteffects on *N. tenuis*. This is particularly relevant because *N. tenuis* is a generalist predator since, apart from *T. absoluta*, it is also widely recruited against key hemipteran pests of tomato such as the whiteflies *Trialeurodes vaporariorum* (Westwood) or *Bemisia tabaci* (Gennadius) (Soares et al. 2019). Our study confirms that the oral delivery method is practical with a chewing insect,namely *T. absoluta*, and a piercing insect such as *N. tenuis*. Therefore, this method canbe used in laboratory risk assessment studies of RNAi on non-target insects (Taning et al. 2016; Mezzetti et al. 2020). Itseffectiveness also reflects its potential ease in applicability considering that field applicationof tailor-made dsRNA pesticides most likely will be administered through spraying infuture agricultural practices.Taken together, our findings offer new insights in the potential of RNAi as an advancedmethod for sequence-based pest management strategies against *T. absoluta*. The applicationof ds*Ta-aCOP* causes mortality to *T. absoluta* and is safe for its biological control agent *N. tenuis*. Therefore, RNAi has the potential to be compatible with the utilization of *N. tenuis*in an efficient IPM strategy against *T. absoluta*; however, more field-realistic studies shouldelaborate this further.

GENERAL CONCLUSION

The current study aimed to shed light on the following innovative and limited studied aspects related to *N. tenuis* plant defence activation. Firstly, knowledge on the effects of tomato defence against *T. absoluta* has been only shown by repellency, however in the present study adverse effects on oviposition and development were recorded. The latter indicate the importance of plant defense induction in the management of *T. absoluta* proving thatits basic biological traits are affected.

- The systemic plant defence activation has beenalso confirmed for other pests, however, the upregulated expression of several genes among upper, middle (unpunctured) and lower leaves offer for first time a thorough knowledge to assess the contribution of genes in plant defence induction.
- The identification of genes that are responsible for the plant induction phenomena offers key knowledge to develop effective pest control methods to produce of more resistant plants i.e. through gene-editing technologies after the selection of suitable genes to offer whole plant protection in tomato.
- The metabolomics analysis showed the high correlation of the systemic resistance against *T. aboluta* and *T. urticae* with increased levels of certain metabolites. In this regard, α-linolenic and linoleic acids, could be directly associated with the exhibited resistance in middle leaves and a-linolenic acid in the top leaves. This indicates the basis of the systemic signal that triggers the JA pathway in remote plant parts. Therefore, linoleic acid may be characterized as a source of long-distance mobile signals in the plant contributing to the amplification of the defensive response in plant tissues. A similar function may have been exhibited by glycerol-3-phosphate in the middle leaves of the *N. tenuis*-punctured plants.
- Effects on the plant metabolism were also showed by the reduced glucose turnover in the top and middle tomato leaves, indicating a shift from growth and development to defence, as a result of the *N. tenuis* treatment. The results confirm our hypothesis that there is variability in the expression levels of genes activated and there should be a strong link between JA pathway activation and secondary metabolite production responsible for the plant defence induction of tomato plants punctured by *N. tenuis*.Several studies have stressed that the plant defence induced by *N. tenuis* constitutes a valuable new strategy in the control of mites, whiteflies and other tomato pests. This perspective is limited by the damage potential of *N. tenuis.*

- On the other hand, it is also required for effective control of *T. absoluta* to identify the length of the period that effective defence lasts. Our results verified the lowest density level of *N. tenuis* to activate plant defence. The results also showed that the effects lasted for at least 14 days together with their systemic nature protecting also the newly emerged leaves during the course of the experiments. All these outcomes offer knowledge useful to practitioners to design application methods of this new pest control approach in a less costly and more effective way.
- The use of dsRNA-mediated management of *T. absoluta* and other pests have been suggested by several publications. As an integral part of any effort to include this method as part of an integrated pest management strategyit has always been reported the need forits biosafety risk assessment. However, its compatibility with biological control has been rarely studied and no information existed for *N. tenuis*.
- Our studies confirmed for the first time that the RNAi is functional also against *N. tenuis* through the oral route. However, dsTa-αCOP that was effective against *T. absoluta* did not cause any lethal or sub-lethal effects to *N. tenuis* upon oral exposure.
- In addition, the present study also confirm success of the oral feeding by droplet method which can be widely utilized for future studies as it is less expensive than artificial diets. The record of the distance between the egg and the mine of the neonate larvae of *T. absoluta* revealed significant variation among treatments indicating that this parameter is sensitive and may be useful in future experiments.
- In general, these results indicated that RNAi-mediated control can be compatible with the biological control of *T. absoluta* by its natural enemy *N. tenuis*. Based on this, it could be possible to add the RNAi approach in the armoire of integrated pest management tactics of *T. absoluta*. Furthermore, its combination with the use of *N. tenuis* may benefit efficacy of biological control strategies against *T. absoluta*.

The results gave important insights in several little studied aspects of *N. tenuis* efficacy and use in pest control. However, the results indicated the need for future research developments so that to explore futher the associations between *N. tenuis*-*T. absoluta*, *T. urticae* - tomato plant and their practical application such as:

1. Develop plants with genes responsible for plant defence and assess their efficacy in pest control

2. Use plant metabolites to activate plant defence and assess their efficacy in pest control, together with any impacts in plant productivity
3. Study the persistence of induced plant defence effects in longer periods
4. Extend the compatibility tests of ds-RNA method on the development, reproduction and behavior of *N. tenuis*
5. Study the plant defence effects under semi-field or field conditions

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