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Tritrophic interactions: Possible host-plants effects on the resistance of *Diabrotica* pests to natural enemies

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Pamela Bruno

Thesis director:
Prof. Ted C. J. Turlings
Thesis committee:
Dr. Carla C. M. Arce, University of Neuchâtel, CH
Dr. Ricardo A. R. Machado, University of Neuchâtel, CH
Dr. Stefan Toepfer, CABI, Delémont, CH
Dr. Ivan Hiltpold, Agroscope, Nyon, CH

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UNIVERSITÉ DE NEUCHÂTEL

Faculté des Sciences
Secrétariat-décanat de Faculté
Rue Emile-Argand 11
2000 Neuchâtel – Suisse
Tél : + 41 (0)32 718 21 00
E-mail : secretariat.sciences@unine.ch

IMPRIMATUR POUR THESE DE DOCTORAT

La Faculté des Sciences de l'Université de Neuchâtel autorise l'impression de la présente thèse soutenue par

Madame Pamela BRUNO

Titre:

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sur le rapport des membres du jury composé comme suit:

- Prof. Ted Turlings, directeur de thèse, Université de Neuchâtel, Suisse
- Dre Carla Cristina Marques Arce, Université de Neuchâtel, Suisse
- Dr Ricardo Machado, Université de Neuchâtel, Suisse
- Dr Ivan Hiltpold, Agroscope, Changins, Suisse
- Dr Stephan Toepfer, CABI, Delémont, Suisse

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Le Doyen, Prof. A. Bangerter

Summary

The subtribe Diabroticina of chrysomelid beetles includes many important pests of extensive and horticultural crops in the Americas, such as maize and cucumber. One of the most devastating species, the western corn rootworm Diabrotica virgifera virgifera (WCR), is also an invasive pest in Europe. For this and related Diabrotica pests, the development of efficient biological control is urgently needed, and for this further knowledge on tritrophic interactions of Diabrotica, their host plants and their natural enemies is required.

In this thesis, I explored the adaptations of Diabrotica pests to their host plants and the plants' defensive compounds, and evaluated the possible protection that Diabrotica larvae derive from ingesting these plant secondary metabolites. During field surveys in Mexico, I observed that parasitism of adult Diabroticina beetles by parasitoids is minimal (Chapter I), but found that the larvae can be successfully infected by adapted entomopathogenic nematodes (EPN) (Chapter II). Highly-infective EPN isolates collected in Mexican maize fields could be promising candidates to control the maize-specialist WCR in Europe. These isolates and/or their endosymbiotic bacteria may have adapted to the defense arsenal deployed by WCR larvae, which includes the sequestration of maize benzoxazinoids, but additional unknown strategies seem to be also involved. Larvae of the generalist Diabrotica balteata do not sequester benzoxazinoids after feeding on maize and seem to be less able to resist EPN infections than WCR. However, they are able to sequester cucurbitacins after feeding on cucumber plants (Chapter III). I provided strong evidence that D. balteata larvae can transformation and sequester cucurbitacins from cucumber plants. However, I found no evidence that the sequestered cucurbitacins can protect the larvae from infection by EPN, nor from the attack by insect predators, entomopathogenic fungi or bacteria. Larval performance was also not positively affected by cucurbitacin contents in plant tissues that they consumed, hence the adaptive significance of cucurbitacin sequestration remains to be unraveled. Surprisingly, the D. balteata larvae were observed to actively forage on aboveground tissues, which prompted us to study whether this behavior provides any benefits and if other Diabrotica species do the same (Chapter IV). Larvae of D. balteata, D. undecimpunctata and D. virgifera were all found to be able to feed on leaves as well as roots from different agricultural plants that contain distinct secondary metabolites. Yet, their performance and the susceptibility against EPN infection were not differentially impacted by the tissue that the larvae had fed on.

Taken together, these results contribute to a better understanding of the impact of host plant secondary metabolites ingested by insect herbivores on the third trophic level. The success of Diabrotica species as crop pests seems to be related to their unique traits to survive in an unpredictable environment by feeding on different plants and tissues, successfully coping with a wide variety of plant defense chemicals, which in some cases may provide protection against natural enemies. I hypothesize that maize-

feeding Diabrotica larvae may be able to detoxify benzoxazinoids, and discuss the origin and adaptive significance of cucurbitacin sequestration by Diabrotica species, which appears not to be related to defense against natural enemies. Finally, the isolates of EPN obtained, as well as the new insights on the aboveground feeding behavior by Diabrotica larvae reported in this thesis, will hopefully contribute to the development of novel and effective biocontrol strategies against Diabrotica pests.

Keywords

benzoxazinoids – *Celatoria compressa* – *Centistes diabroticae* – cucumber – cucurbitacins – *Diabrotica* – Diabroticina – entomopathogenic nematodes – *Heterorhabditis* – maize – natural enemies – plant secondary metabolites – sequestration – tritrophic interactions – root-feeding – western corn rootworm.

Résumé

La sous-tribu Diabroticina de chrysomèles comprend de nombreux ravageurs importants des cultures agricoles et horticoles dans les Amériques, tels que le maïs et le concombre. L'une des espèces les plus dévastatrices, la chrysomèle des racines de maïs *Diabrotica virgifera virgifera* (WCR), est également un ravageur envahissant en Europe. Pour ce ravageur et les espèces de *Diabrotica* apparentées, le développement d'une lutte biologique efficace est nécessaire de toute urgence, et pour cela, des connaissances supplémentaires sur les interactions tri-trophiques entre *Diabrotica*, leurs plantes hôtes et leurs ennemis naturels sont nécessaires.

Dans cette thèse, j'ai exploré les adaptations des ravageurs *Diabrotica* à leurs plantes hôtes avec leurs composés défensifs, et évalué l'éventuelle protection que les larves de *Diabrotica* obtiennent après l'ingestion de ces métabolites secondaires des plantes. Au cours d'échantillonnages sur le terrain au Mexique, j'ai observé que le parasitisme des coléoptères Diabroticina adultes par des parasitoïdes est minime (Chapitre I), mais j'ai constaté que les larves peuvent être infectées avec succès par des nématodes entomopathogènes (EPN) adaptés (Chapitre II). Des isolats d'EPN hautement infectieux collectés dans des champs de maïs mexicains pourraient être des candidats prometteurs pour contrôler WCR en Europe. Ces isolats et/ou leurs bactéries endosymbiotiques pourraient être adaptés à l'arsenal de défense déployé par les larves de WCR, qui comprend la séquestration des benzoxazinoïdes du maïs, mais des stratégies inconnues supplémentaires semblent également être impliquées. Les larves du généraliste *Diabrotica balteata* ne séquestrent pas des benzoxazinoïdes après s'être nourries des plantes de maïs et semblent moins capables de résister aux infections à EPN que les WCR. Cependant, elles sont capables de séquestrer des cucurbitacines après avoir mangé des plantes de concombre (Chapitre III). J'ai fourni des preuves solides que les larves de *D. balteata* peuvent transformer et séquestrer les cucurbitacines des plants de concombre. Cependant, je n'ai trouvé aucune preuve que les cucurbitacines séquestrées puissent protéger les larves de l'infection par des EPN, ni de l'attaque par des insectes prédateurs, des champignons entomopathogènes ou des bactéries. La performance des larves n'a pas non plus été affectée positivement par la teneur en cucurbitacines des tissus végétaux qu'elles ont consommées, d'où la signification adaptative de la séquestration de cucurbitacines reste à élucider. Curieusement, j'ai observé que les larves de *D. balteata* se nourrissent activement de tissus au-dessus de la surface, tels que tiges, cotylédons et feuilles, ce qui m'a incité à étudier si ce comportement procure des avantages et si d'autres espèces de *Diabrotica* ont un comportement similaire (Chapitre IV). Les larves de *D. balteata*, *D. undecimpunctata* et *D. virgifera*, normalement décrites comme larves de racines, se sont toutes avérées capables de se nourrir de feuilles ainsi que de racines de différentes plantes agricoles contenant des métabolites secondaires distincts. Pourtant, leurs performances et leur sensibilité à l'infection par des EPN n'ont pas été affectées différemment par le tissu que les larves avaient consommées.

Ensemble, ces résultats contribuent à une meilleure compréhension de l'impact des métabolites secondaires des plante hôte ingérés par des insectes herbivores sur le troisième niveau trophique. Le succès des espèces de *Diabrotica* en tant que ravageurs des cultures semble être lié à leurs caractéristiques uniques pour survivre dans un environnement imprévisible en se nourrissant de différentes plantes et tissus, faisant face avec succès à une grande variété de composés de défense des plantes, qui dans certains cas peuvent fournir une protection contre les ennemis naturels. J'émet l'hypothèse que les larves de *Diabrotica* se nourrissant de maïs sont capables de détoxifier les benzoxazinoïdes, et discute de l'origine et de l'importance adaptative de la séquestration de cucurbitacines par les espèces de *Diabrotica*, qui ne semblerait pas liée à la défense contre les ennemis naturels. Enfin, les isolats d'EPN obtenus, ainsi que les nouvelles connaissances sur le comportement alimentaire des larves de *Diabrotica* rapportées dans cette thèse, contribueront, espérons-le, au développement de stratégies de biocontrôle nouvelles et efficaces contre les ravageurs du genre *Diabrotica*.

Mots clés

benzoxazinoïdes, *Celatoria compressa*, *Centistes diabroticae*, concombre, cucurbitacines, *Diabrotica*, Diabroticina, ennemis naturels, *Heterorhabditis*, maïs, interactions tri-trophiques, métabolites secondaires des plantes, nématodes entomopathogènes, séquestration

Contents

General introduction and thesis outline

Tritrophic interactions

Through coevolution, plants have developed numerous and ingenious adaptations to defend themselves against herbivory (Núñez-Farfán *et al.*, 2007). Direct defenses, that specifically target the herbivores and aim at impeding their feeding, can include physical barriers such as vegetative structures with hard cuticules, trichomes and thorns, as well as chemicals like the production of toxic molecules designed to deter herbivory (Schoonhoven *et al.*, 2005). Indirect defenses, on the other hand, are designed to attract natural enemies of the herbivores, which can include the release of volatile cues to recruit parasitoids or predators (Turlings *et al.*, 1990; Birkett *et al.*, 2000; Freeman & Beattie, 2008; Heil, 2008; van Dam, 2009; Aljbory & Chen, 2018). In response, insects and other herbivores have developed counter-measures to circumvent the plant defenses (Ehrlich & Raven, 1964; Berenbaum, 1983; Heidel-Fischer & Vogel, 2015). For instance, generalist insects feed on plants from different families, and therefore need to deal with a broad range of plant defense chemicals (van der Meijden, 1996). As a consequence, they tend to have evolved avoidance and tolerance traits (Dussourd & Eisner, 1987; Stotz *et al.*, 2000), as well as strategies like selecting plant ages and tissues with lower levels of toxic compounds (Köhler *et al.*, 2015). In contrast, specialist insects feed on plants from the same genus (monophagous) or from different, but related genera (olyphagous) (Schoonhoven *et al.*, 2005; Bernays & Chapman, 2007). For these adapted herbivorous insects, defensive plant metabolites from plants can be beneficial by providing a protective niche, an advantageous oviposition site or a food source with chemicals that will render the insect less prone to predator attack or parasitism (Boppré, 1986). Some insects can even detoxify or sequester these chemicals from the plants, which can protect them from their own natural enemies (Nishida, 2002; Glauser *et al.*, 2011; Petschenka & Agrawal, 2016; Robert *et al.*, 2017). In addition, the protective chemicals can be passed on to the offspring as part of a “chemical legacy” (Dussourd *et al.*, 1988; Tallamy *et al.*, 2000; Opitz & Müller, 2009). However, some natural enemies have adapted to the plant defenses coopted by the insects they feed on (Zhang *et al.*, 2019), and are therefore excellent candidates for biological control of certain specialized pests.

Study system

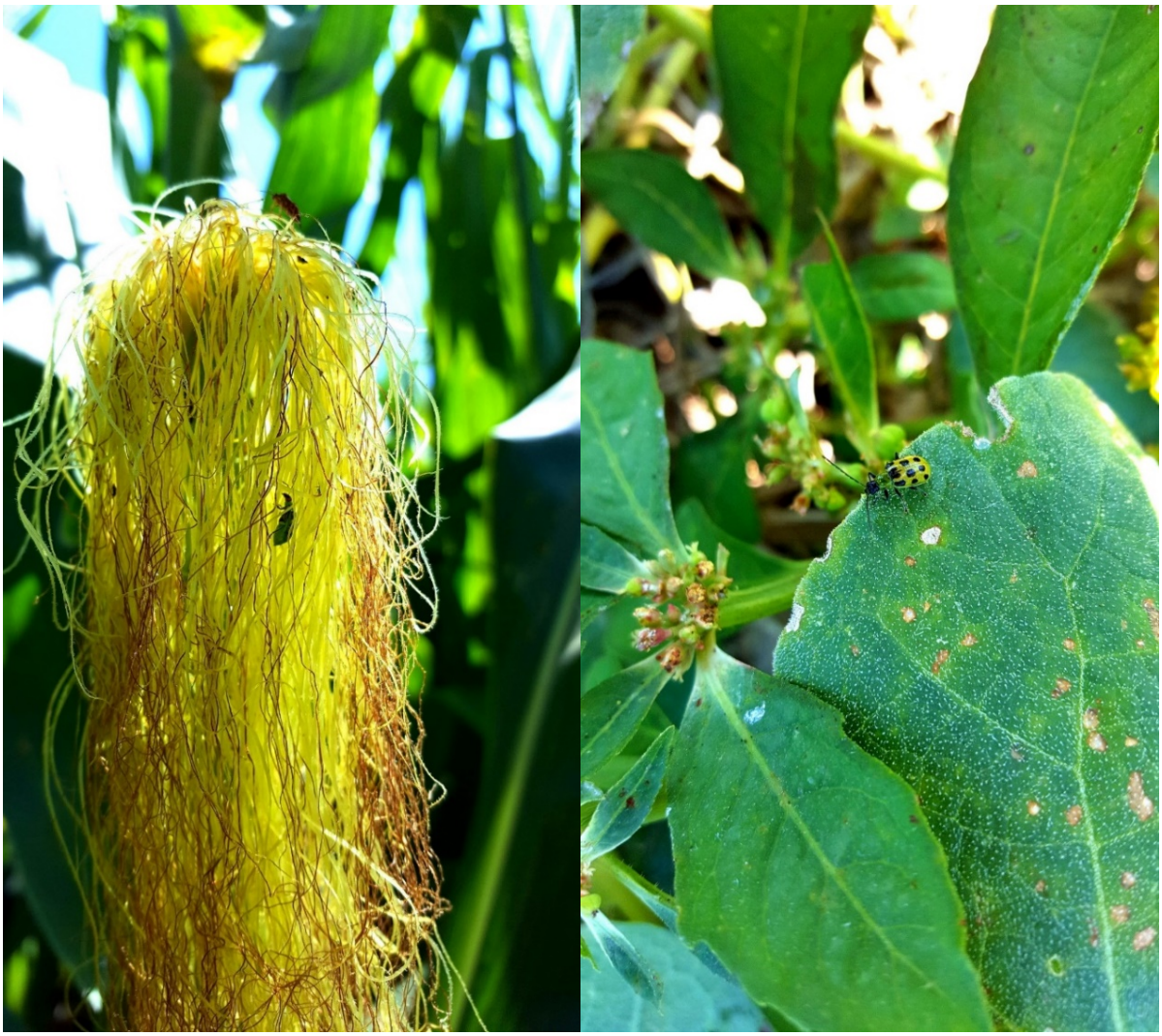
The subtribe Diabroticina (Coleoptera: Chrysomelidae: Galerucinae: Luperini) (Seeno & Wilcox, 1982) comprises more than 900 species of leaf beetles, the majority with Mesoamerican origin (Webster, 1895). Among them are various pests, mainly belonging to the genus *Diabrotica*, *Acalymma* and *Cerotoma* (Krysan, 1986), whose adults are leaf feeders. The larvae are called rootworms because of their root-feeding behavior, however their ecology is poorly described. The genus *Diabrotica* is the largest in the Diabroticina subtribe, with more than 400 species of Neotropical lineage

(Branson & Krysan, 1981), and is divided in three subgroups: signifera, fucata and virgifera (Krysan & Smith, 1987a). The small subgroup signifera originates from South America and has little economic significance. The fucata subgroup comprises multivoltine non-diapausing polyphagous species that can be found through the Americas, such as the banded cucumber beetle *Diabrotica balteata* LeConte and the spotted cucumber beetle / southern corn rootworm *D. undecimpunctata howardi* Barber (Krysan, 1986; Marín-Jarillo, 2012; Cabrera Walsh *et al.*, 2020). Finally, the subgroup virgifera comprises univoltine species from North America, which overwinter as diapausing eggs, and are olyphagous or monophagous as larvae and polyphagous as adults (Krysan *et al.*, 1977; Krysan, 1982). Other genera of agricultural importance from the Diabroticina subtribe are the cucumber beetles of the genus *Acalymma* Barber, specialized on cucurbits both as larvae and adults (Munroe & Smith, 1980), and the bean leaf beetles of the genus *Cerotoma* Chevrolat, which mostly feed on legumes (Koch *et al.*, 2005) but also on cucurbits (Koch *et al.*, 2004).

The western corn rootworm (WCR, *Diabrotica virgifera virgifera* LeConte, Coleoptera: Chrysomelidae) is one of the most economically-important species of the virgifera subgroup in the *Diabrotica* genus (Krysan & Smith, 1987b). The larvae are specialists on maize, but the adults can feed on many plants (Branson & Ortman, 1967). WCR most likely originates from Mexico, from where it moved northward, being today a major belowground pest in the US Corn Belt (Gray *et al.*, 2009). In the early nineties, WCR was introduced in Europe and is spreading causing tremendous yield losses in several regions of Central Europe every year (Bača, 1993; Kiss *et al.*, 2005; Gray *et al.*, 2009; Bažok *et al.*, 2021). The successful spread of WCR in Europe could be explained by three main factors: first, the invasion of this alien species took place in a territory where the crop is also not native, thus it is likely to lack indigenous natural enemies that control the pest. Secondly, WCR is highly adapted to maize as a host plant and is immune to its main chemical defense (Robert *et al.*, 2012), which is based on benzoxazinoids (BXs) (Niemeyer, 1988). WCR larvae even use the BXs as feeding stimulants (Robert *et al.*, 2012) and they can sequester them for their own defense (Robert *et al.*, 2017). Lastly, an invasive bridgehead effect, meaning that the individuals of WCR from North America that invaded Europe belonged to a particularly successful population that possesses traits that may have contributed to the establishment of WCR in European countries (Guillemaud *et al.*, 2011; Bermond *et al.*, 2021).

Apart from BXs from maize plants, polyphagous Diabroticina species need to deal with other plant secondary metabolites. As well as the other members of the Luperine tribe, Diabroticina adults are known to compulsively feed on Cucurbitaceae plants that contain highly toxic cucurbitacins (Metcalf & Lampman, 1989; Gillespie *et al.*, 2003; Eben & Espinosa de Los Monteros, 2013). These compounds are non-volatile bitter oxygenated tetracyclic triterpenoids (Rehm *et al.*, 1957; Lavie & Glotter, 1971; Metcalf *et al.*, 1980; Chen *et al.*, 2005) that can protect the plants against herbivores and pathogens (David & Vallance, 1955; Da Costa & Jones, 1971; Nielsen *et al.*, 1977;

Bar-Nun & Mayer, 1990; Sachdev-Gupta *et al.*, 1993). However, and as a result of an evolutionary arms race, cucurbitacins are not effective as defense against all organisms, as is the case for Diabroticina beetles, many of which are closely associated to Cucurbitaceae plants (Metcalf, 1979; Metcalf, 1986; Eben & Espinosa de Los Monteros, 2013). Cucurbitacins are strong attractants and phagostimulants both for the larvae (DeHeer & Tallamy, 1991) and adults (Chambliss & Jones, 1966; Metcalf *et al.*, 1980; Nishida & Fukami, 1990). Nevertheless, the consumption of cucurbitacins by Diabroticina can have negative effects on larval growth, oviposition and survival of adults (Ferguson *et al.*, 1985; Hirsh & Barbercheck, 1996). In addition, cucurbitacins have no substantially nutritional value, and have only been mentioned in relation to steroid nutrition (Halaweish *et al.*, 1999). It has been reported that adults of several Diabroticina species, such as *Diabrotica undecimpunctata*, *D. balteata*, *D. virgifera virgifera, D. speciosa, D. cristata, Cerotoma arcuata* and *Acalymma vittatum*

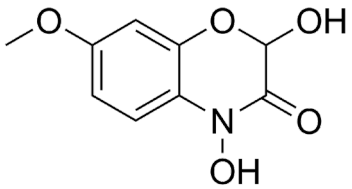


Left: Beetles of the western corn rootworm *Diabrotica virgifera* feeding on maize silks.

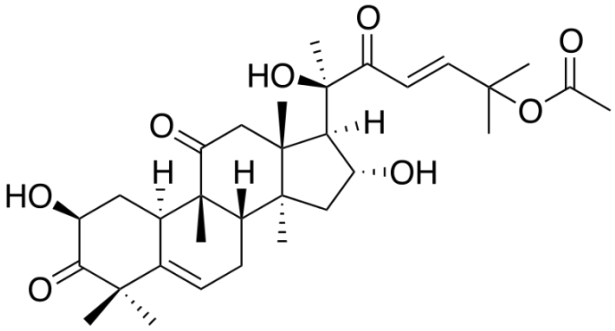
Right: Spotted cucumber beetle *D. undecimpunctata* feeding on leaves of wild squash *Cucurbita foetidissima* (right). Photos: P. Bruno

can sequester and detoxify cucurbitacins (Ferguson *et al.*, 1985; Andersen *et al.*, 1988; Nishida *et al.*, 1992). It is thought that the larvae can also sequester cucurbitacins (Ferguson & Metcalf, 1985; Tallamy *et al.*, 1998), but the mechanisms, costs and benefits of this capacity are not clear and more research is needed.

Traditional management efforts to control Diabroticina rootworms included crop rotation, soil insecticides and seed treatments (Sivčev *et al.*, 2012). Particularly for WCR, crop rotation is one of the most effective strategies used, but it has been reported that certain populations of WCR have adapted to this practice and can overwinter as diapausing eggs on soybean and other crops used for the rotation, and then hatch on the next season and colonize the new maize crop (Levine *et al.*, 2002; Pierce & Gray, 2006; Gray *et al.*, 2009). WCR has also evolved resistance to different insecticides that are commonly applied (Ball & Weekman, 1962; Meinke *et al.*, 1998; Pereira *et al.*, 2015). Another control strategy against rootworms is the planting of transgenic Bt maize which produce insecticidal Crystal proteins (Cry) derived from the bacterium *Bacillus thuringiensis*. It has been available in the US market since 2003 and it is broadly used in countries where such crops are allowed, but this is not the case for most European countries. Moreover, in several US regions WCR has evolved resistance to different Bt toxins (Meihls *et al.*, 2008; Gassmann *et al.*, 2016). As an alternative, Integrated Pest Management is being considered as a long-term approach, in particular for WCR in European countries, including the application of biological control agents (Kuhlmann & Toepfer, 2005). Unfortunately, no effective indigenous natural enemies have been found in Europe for any life stage of WCR (Toepfer & Kuhlmann, 2004).



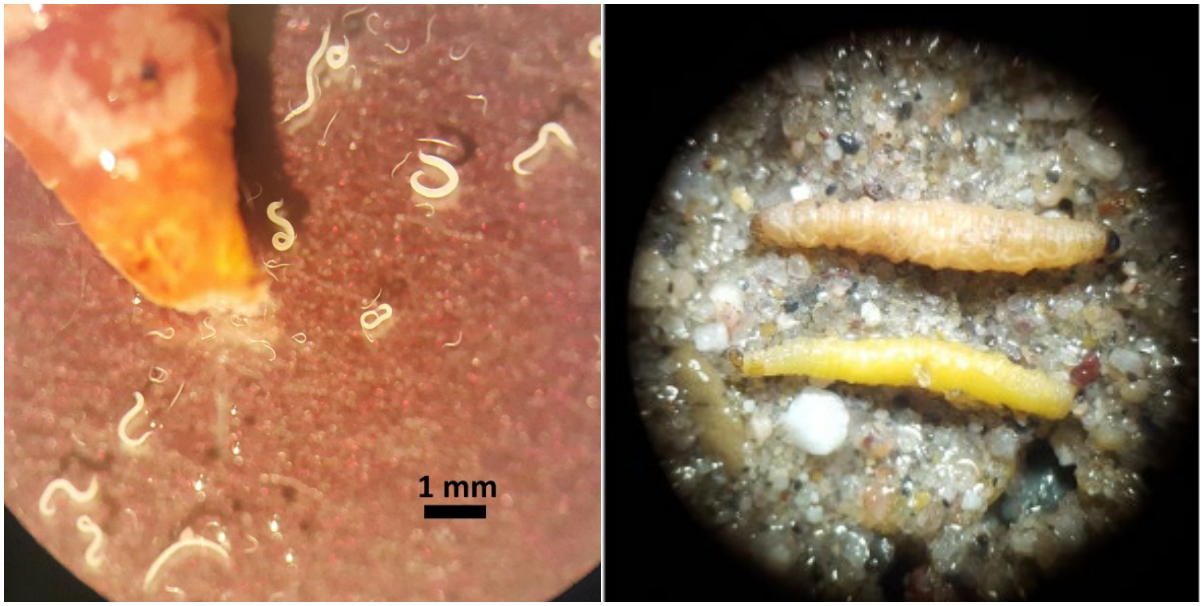
benzoxazinoid DIMBOA



Cucurbitacin B

For the development of classical biological control, entomopathogenic nematodes (EPN) are the most promising candidates against Diabroticina rootworms (Kuhlmann & van der Burgt, 1998; Toepfer *et al.*, 2008). These are soil-dwelling insect parasitic roundworms (Nematoda), commonly used in Integrated Pest Management programs as biocontrol agents (Kaya & Gaugler, 1993; Kaya *et al.*, 2006). The ability of EPN to colonize and kill their insect hosts is enabled by their endosymbiotic bacteria, which are released into the insect hemocel after EPN penetration through natural openings (Boemare *et al.*, 1996). The bacteria produces toxins that kill the host and digest their internal tissues, providing EPN with a food source and a niche for reproduction. Once

the resources are depleted, the EPN produce so- called “infective juveniles” (IJs), which leave the insect cadaver in search of a new host (Kaya & Gaugler, 1993). Most species used for biological control belong to the genera *Steinernema* and *Heterorhabditis*. *Steinernema* EPN are associated with symbiotic bacteria of the genus *Xenorhabditis*, and *Heterorhabditis* species have bacterial symbionts from *Photorhabdus* spp., a genus characterized for the production of bioluminescence (Boemare *et al.*, 1997; Boemare, 2002; Machado *et al.*, 2018). The successful application of EPN in the field is subjected to many obstacles, such as soil temperature and humidity, seasonal persistence of the nematodes, desiccation, etcetera (Koppenhöfer & Fuzy, 2006; Shapiro-Ilan *et al.*, 2006; Toepfer *et al.*, 2010; Jaffuel *et al.*, 2016; Kim *et al.*, 2021).



Left: Entomopathogenic nematodes (EPN) and their infective juveniles (IJs) leaving the cadaver of an infected larva of the greater wax moth *Galleria mellonella*. **Right:** *Diabrotica virgifera* infected by EPN (top) *versus* a non-infected larva (bottom). Photos: P. Bruno

Other natural enemies of Diabroticina that could be used for biological control of the adults are the tachinid fly *Celatoria compressa* Wulp and the braconid wasp *Centistes (Syrrhizus) diabroticae* Gahan (Kuhlmann & van der Burgt, 1998; Kuhlmann & Toepfer, 2005). These parasitoids insert eggs inside the adults of different Diabroticina species, such as *Acalymma* sp., *Cerotoma* sp. and *Diabrotica* sp. (Zhang *et al.*, 2003; Toepfer *et al.*, 2008). Further research is needed to assess the potential of these parasitoids to control WCR in Europe, as well as of other Diabroticina species in the American continent. In particular, a better understanding of the tritrophic interactions between *Diabrotica* pests, their host plants’ defenses and their natural enemies, seems essential for the development of effective biological control. My thesis aims to contribute to this lack of knowledge.

To this end, the following questions were addressed:

Chapter I: Are parasitoids of Diabroticina beetles good candidates for the development of biological control against WCR in Europe?

The tachinid fly *Celatoria compressa* and the braconid wasp *Centistes* (*Syrrhizus*) *diabroticae* are parasitoids of Diabroticina beetles that occur in Mexico, Central and South America. These species have been suggested as good candidates to control *D. virgifera* adults in Europe, as they only parasitize species of the Diabroticina subtribe, which are not present in Europe with the exception of invasive WCR (Toepfer *et al.*, 2008). Therefore, we surveyed parasitized beetles at different agricultural fields on four states of Mexico, and assessed the parasitism levels on the different Diabroticina species. The insects collected were further identified. The aim of the study was to establish parasitoid rearings, to then study whether these species are well-suited for the development of biocontrol strategies against the invasive WCR in Europe.

Chapter II: Are EPN from Mexico adapted to BXs sequestered by WCR from maize roots?

A recent study reported that a commercial strain of entomopathogenic nematodes and their bacterial endosymbionts were negatively affected by benzoxazinoids sequestered by *D. virgifera* larvae (Robert *et al.*, 2017). It can be expected that the natural enemies of WCR in Mexico are well adapted to the defenses of WCR and that they have the potential to be highly effective biocontrol agents (Kuhlmann & van der Burgt, 1998). In this study, we conducted surveys in Mexican maize fields to obtain various species and strains of entomopathogenic nematodes. We established a range of lab colonies and molecularly identified the different isolates. To evaluate the adaptation of the isolates to WCR defenses, in particular to BXs sequestration, we compared their infection capacities of the isolates on WCR compared to larvae of *D. balteata*, who do not sequester benzoxazinoids, as well as on WCR fed on maize lines with and without benzoxazinoids.

Chapter II, Annex: Which are the bacterial symbionts of the EPN *Heterorhabditis atacamensis* and *H. mexicana*?

As a result of our survey, we obtained isolates of EPN species whose bacterial symbionts had not been studied yet. In a collaborative project, we identified and reported the bacterial endosymbiont of *Heterorhabditis atacamensis* and *Heterorhabditis mexicana*.

Chapter III: Do larvae of *D. balteata* sequester cucurbitacins? What are the costs of the sequestration? Do sequestered cucurbitacins protect *D. balteata* larvae against natural enemies?

Diabroticina beetles have coevolved with Cucurbitaceae plants (Metcalf, 1986; Eben & Espinosa de Los Monteros, 2013), whose main secondary metabolites are the

extremely bitter cucurbitacins (Rehm *et al.*, 1957). Adult beetles of Diabroticina species are thought to sequester cucurbitacins (Ferguson *et al.*, 1985; Andersen *et al.*, 1988; Nishida *et al.*, 1992), which has been assumed to provide protection against the attack of natural enemies (Ferguson & Metcalf, 1985). It is unclear whether the larvae are also capable of sequestering cucurbitacins, and whether this protects them against the attack of natural enemies. In this project, we extensively studied the sequestration of cucurbitacins from cucumber plants by *D. balteata* larvae, and explored whether the sequestration impacts larval performance and/or protects the larvae against a wide range of common natural enemies. We also observed an aboveground novel feeding behavior for *D. balteata* larvae, which prompted us to study with in more detail.

Chapter IV: Are *Diabrotica* larvae able to feed on aboveground tissues? Does this behavior have costs and/or benefits for the larvae?

Our observation of *D. balteata* larvae feeding on aboveground tissues led us to investigate whether this behavior could have an impact on their development and protection. This feeding behavior has been reported in the past, but little is known (Cardona *et al.*, 1982; Canales Santos, 1989; Jiménez Martínez, 2014; Sosa-Baldivia, 2016). To get more insight into this, we compared the larval behavior and performance of *D. balteata, D. undecimpunctata* and WCR when fed on leaves or roots of six different plants, and profiled the main secondary metabolites present in the different tissues and plant species. In a second step, we explored whether the tissues and plants consumed would have an effect on the protection against infection by entomopathogenic nematodes.

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1. New insights into the host range of the tachinid fly *Celatoria compressa* and the braconid wasp *Centistes* (*Syrrhizus*) *diabroticae*, parasitoids of Diabroticina beetles

Pamela Bruno[1], Angela Köhler[2], Julio Bernal[3], Rebeca Alvarez-Zagoya[4], Juan Francisco Pérez-Domínguez[5], Antonio Marín-Jarillo[6], Stefan Toepfer[7], Christelle A. M. Robert[8], Matthias Erb[8], Carla CM Arce[1] & Ted CJ Turlings[1]

1. Laboratory of Fundamental and Applied Research in Chemical Ecology, Institute of Biology, University of Neuchâtel, Neuchâtel, Switzerland.

2. Current address: Friedrich Schiller University Jena, Jena, Germany

3. Current address: Department of Entomology, Texas A&M University, Texas, United States of America

4. Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional, Unidad Durango, Instituto Politécnico Nacional, Durango, México

5. Departamento de Entomología, INIFAP Instituto Nacional de Investigaciones Forestales Agricolas y Pecuarias, Sagarpa, Jalisco, Mexico

6. Centro de Investigación Regional del Centro, Campo Experimental Bajío, INIFAP Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias, Celaya, Guanajuato, México

7. CABI, c/o Plant Protection and Soil Conservation Directorate, Hódmezővásárhely, Hungary

8. Institute of Plant Sciences, University of Bern, Bern, Switzerland

*Corresponding author: ted.turlings@unine.ch

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Abstract

For the biological control of beetles of the western corn rootworm, promising candidates are the parasitoids *Celatoria compressa* and *Centistes* (*Syrrhizus*) *diabroticae*. However, further studies of their biology and ecology are needed. With the aim of establishing rearings, we surveyed 25 fields of different agricultural crops across Central Mexico. We obtained 2124 beetles and a parasitism level of 0.56 % for *C. diabroticae* and 1.51 % for *C. compressa*. These results suggest that the parasitoids are not very successful at parasitizing *Diabrotica* and related species in nature, and thus might not be suited for the development of biological control.

Main Body

The tachinid fly *Celatoria compressa* Wulp and the braconid wasp *Centistes* (*Syrrhizus*) *diabroticae* Gahan have been suggested for biological control of the western corn rootworm *Diabrotica virgifera virgifera* (WCR) and related species (Eben & Barbercheck, 1996; Kuhlmann & van der Burgt, 1998; Kuhlmann & Toepfer, 2005; Toepfer *et al.*, 2008). The host range of these parasitoids seems to be restricted to the Diabroticina subtribe (Luperini), to which WCR belongs (Toepfer *et al.*, 2008). This fact makes them excellent candidates against the invasive WCR in Europe, since no other Diabroticina are present in this continent (Fischer, 1981; Fischer, 1983; Zhang *et al.*, 2003; Zhang *et al.*, 2004; Smyth & Hoffmann, 2010). There is little knowledge about the biology and ecology of these parasitoids, and deeper knowledge is needed for the development of successful biological control strategies.

A survey across 25 sites in the Mexican states of Durango, Guanajuato, Zacatecas and Jalisco was carried out in October 2016 to obtain beetles of the subtribe Diabroticina from natural and cultivated areas, with the aim of establishing rearings and to study the chemical ecology and interactions of the parasitoids with different *Diabrotica* species. To this end, we collected adults from fields with the traditional Mexican growing system 'milpa' (maize in association with squash and/or bean) as well as from monocultures of bean, sorghum and alfalfa, and from surrounding and native weeds (mostly *Cucurbita foetidissima* and *Amaranthus* spp). The beetles were collected with aspirators and nets and kept individually, fed with zucchini and pollen, in order to link the eventual emergence of parasitoids to the beetle species and the site of collection. In case of death, the food was taken away and a piece of moist cotton was added to the container.

We obtained a total of 2124 beetles across 25 sampling sites (Table I). The species of beetles and parasitoids were identified visually by Dr. Rebeca Alvarez-Zagoya and with morphological keys by Dr. Antonio Marín-Jarillo (INIFAP Celaya) (Figure 1). The emergence of parasitoids from beetle cadavers per sampling site is presented in Table I, and per beetle species in Table II. We obtained 12 *Centistes diabroticae* wasps and 32 *Celatoria compressa* flies out of 2124 beetles collected, which represents a parasitism level of 0.56 % for *C. diabroticae* and 1.51 % for *C. compressa*. After the

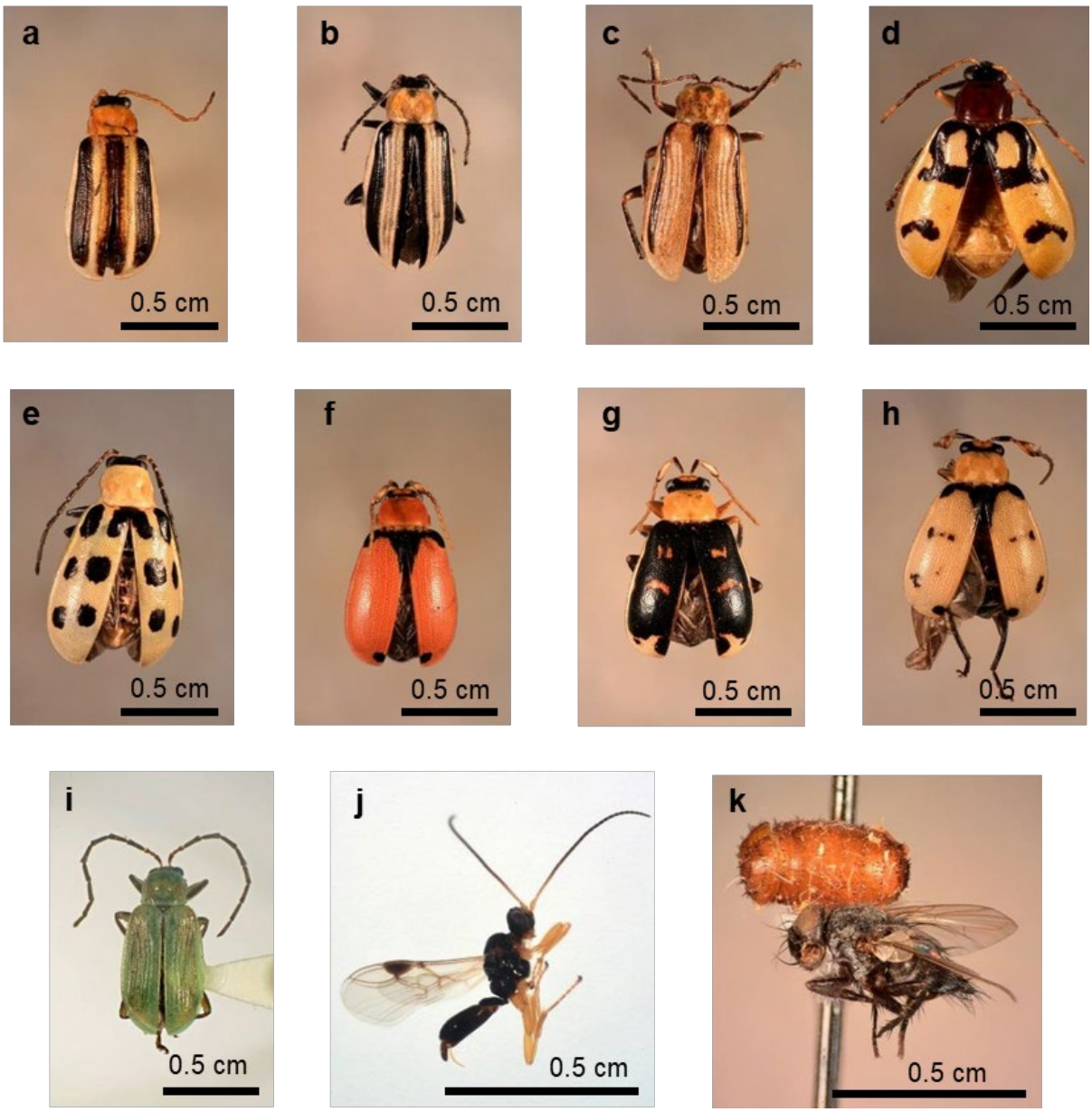

Figure 1. General aspect and identification of the Diabroticina beetles collected across Mexican fields and of their parasitoids. a) *Acalymma* sp., b) *A. trivittata*, c) *Diabrotica virgifera virgifera*, d) *D. adelpha*, e) *D. undecimpunctata howardi*, f, g) *Cerotoma* spp., h) *Cerotoma ruficornis*., i) *D. virgifera zeae*, j) *Centistes diabroticae*, and k) *Celatoria compressa*. Scale bars represent 0.5 cm. Other species such as *D. balteata*, *D. longicornis* and *D. cristata* were also obtained but images of fixed insects are no available. Insect fixation, photos and species identification were performed by Dr. Antonio Marín-Jarillo (INIFAP Celaya, México).

emergence of parasitoids, they were fed with a mixture of honey, sugar and brewers' yeast (1:1:1) in moist cotton (Fischer, 1983). The reproduction of the emerged parasitoids and the establishment of rearings were attempted but not accomplished due to the low rate of emergence, lack of male/female ratios, lack of suitable age ratios and low survival of the flies and wasps. Previous attempts to rear these parasitoids have been only been reported twice (Fischer, 1983; Zhang *et al.*, 2003). Zhang et al.

based their work in the previous knowledge by Fischer and from studies on related species (Cabrera Walsh *et al.*, 2003).

The extremely low levels of natural control of Diabroticina beetles by these two parasitoids suggest that they might not be as suitable as previously suggested for the biological control of *Diabrotica*, *Acalymma* and *Cerotoma* pests. However, our sampling was carried out in October, and many of the maize crops we sampled might have been too advanced in their phenology for Diabrotica beetles, which prefer to feed on silks. Other studies might have better success if the sampling is performed earlier in the season.

Table I. Location of sampling sites for the Diabroticina beetle collection, and level of beetle parasitism per sampling site found by *Centistes diabroticae* and *Celatoria compressa.*

Site	Latitude	Longitude	State	Location	Plant host	Diabroticina beetles	*Centistes diabroticae*	*Celatoria compressa*
1	24.005875	-104.616050	Durango	Durango	Maize + squash	29	0	2
2	23.954327	-104.626355		Durango	Maize + squash	55	0	0
3	23.946972	-104.595483		Durango	Maize + squash	18	0	0
4	23.947391	-104.580000		Durango	Squash	68	0	2
5	23.988212	-104.626044		Durango	Squash	177	1	4
6	24.011935	-104.474856		Durango	Maize	29	0	1
7	23.942488	-104.464062		Colonia Minerva	Maize	75	1	1
8	23.931359	-104.486387		Colonia Minerva	Bean	58	0	0
9	24.475820	-104.716430		Canatlán	Maize	22	0	0
10	24.024097	-104.554791		Nombre de Dios, Los Encinos	Wild squash	196	0	3
11	23.931359	-104.486387		Colonia Minerva	Bean	46	6	1
12	23.757260	-103.844965		Villa Insurgentes	Maize + squash	44	0	1
13	23.557698	-103.262134	Zacatecas	Saín Alto, Sombrerete	Maize + bean	73	1	0
14	23.602799	-103.321977		Saín Alto, Sombrerete	Maize + pepper	54	0	0
15	23.208545	-103.045665		Fresnillo	Maize	8	0	0
16	23.222819	-103.040881		Fresnillo	Squash	55	1	10
17	22.700386	-103.036563		Jerez	Maize + squash	76	0	0
18	20.914769	-101.218718	Guanajuato	Guanajuato	Maize	81	0	2
19	20.906431	-101.095279		San Martín de Terreros	Maize	3	0	2
20	20.859019	-101.035061		San Martín de Terreros	Maize	2	0	0
21	19.698528	-104.351968	Jalisco	Autlán	Maize + Squash	261	1	2
22	19.787049	-104.350630			Sorghum	168	0	0
23	19.787049	-104.350630		Autlán	Squash	10	0	0
24	19.787049	-104.350630			Alfalfa	328	1	0
25	20.330021	-102.544488		Tepatitlán	Maize	188	0	1
					TOTAL	2124	12	32
					PARASITISM (%)		0.56	1.51

Table II. Levels of parasitism by *Centistes diabroticae* and *Celatoria compressa* per beetle species of the subtribe Diabroticina sampled across several fields in Mexico.

Diabroticina beetle species	total	*Centistes diabroticae*		*Celatoria compressa*	
		Count	(%)	Count	(%)
Diabrotica virgifera virgifera	193	7	3.63	0	0.00
Diabrotica virgifera zeae	215	1	0.47	4	1.86
Diabrotica undecimpunctata	324	0	0.00	11	3.40
Diabrotica balteata	244	0	0.00	0	0.00
Diabrotica adelpha	49	0	0.00	0	0.00
Diabrotica semiopaca	16	0	0.00	0	0.00
Diabrotica longicornis	1	0	0.00	0	0.00
Diabrotica cristata	6	0	0.00	0	0.00
Diabrotica sp.	63	1	1.59	0	0.00
Cerotoma spp. (possibly *ruficornis)*	420	0	0.00	2	0.48
Acalymma vittattum	199	0	0.00	0	0.00
Acalymma trivittatum	394	3	0.76	15	3.81
TOTAL	2124	12	0.56	32	1.51

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2. Entomopathogenic nematodes from Mexico that can overcome the resistance mechanisms of the western corn rootworm

Pamela Bruno[1], Ricardo AR Machado[2], Gaétan Glauser[3], Angela Köhler[1,4], Raquel Campos-Herrera[5], Julio Bernal[6], Stefan Toepfer[7], Matthias Erb[2], Christelle AM Robert[2], Carla CM Arce[1]* and Ted CJ Turlings[1]*

1. Laboratory of Fundamental and Applied Research in Chemical Ecology, University of Neuchâtel, Emile-Argand 11, 2000 Neuchâtel, Switzerland.

2. Institute of Plant Sciences, University of Bern, Bern, Switzerland.

3. Neuchâtel Platform of Analytical Chemistry, University of Neuchâtel, Neuchâtel, Switzerland.

4. Current address: Friedrich Schiller University Jena, Jena, Germany.

5. Instituto de Ciencias de la Vid y del Vino (Universidad de La Rioja, CSIC, Gobierno de La Rioja), Logroño, La Rioja, Spain.

6. Department of Entomology, Texas A&M University, Texas, United States of America.

7. CABI, c/o Plant Protection and Soil Conservation Directorate, Hódmezővásárhely, Hungary.

*Corresponding authors: carla.marques@unine.ch, ted.turlings@unine.ch

Carla CM Arce and Ted CJ Turlings. Laboratory of Fundamental and Applied Research in Chemical Ecology, University of Neuchâtel, Emile-Argand 11, 2000 Neuchâtel, Switzerland.

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Abstract

Natural enemies of herbivores are expected to adapt to the defence strategies of their preys or hosts. Such adaptations may also include their capacity to cope with plant metabolites that herbivores sequester as a defence. In this study, we evaluated the ability of Mexican entomopathogenic nematodes (EPN) to resist benzoxazinoids that are sequestered from maize roots by the western corn rootworm (WCR, *Diabrotica virgifera virgifera;* Coleoptera: Chrysomelidae), an important maize pest in America and Europe. From maize fields throughout Mexico, we retrieved 40 EPN isolates belonging to five different species, with a majority identified as *Heterorhabditis bacteriophora*. In the laboratory, all nematodes readily infected non-sequestering larvae of the banded cucumber beetle (*D. balteata*), while infectivity varied strongly for WCR larvae. While some *H. bacteriophora* isolates seemed negatively affected by benzoxazinoids, most showed to be resistant. Thus, EPN from Mexican maize fields are resistant to these plant defence metabolites, but the results also indicate that WCR larvae possess other mechanisms that help to resist EPN. This work contributes to a better understanding of the capacity of herbivore natural enemies to resist plant defence metabolites. Furthermore, it identifies several benzoxazinoid-resistant EPN isolates that may be used to control this important maize pest.

Introduction

Biological control is a key element of integrated pest management. For the control of root feeding insect pests, some of the most effective natural enemies are entomopathogenic nematodes (EPN), which are generalist parasites of insects that inhabit the soils of most regions of the world (Kaya & Gaugler, 1993; Hominick, 2002; Kaya *et al.*, 2006; Campos-Herrera, 2015). They enter their hosts and regurgitate symbiotic bacteria that kill the insects by septicaemia and toxaemia (Kaya & Gaugler, 1993; Campos-Herrera, 2015). The main EPN species used for biological control belong to the genus *Heterorhabditis* and *Steinernema* (Kaya *et al.*, 2006), respectively associated to *Photorhabdus* and *Xenorhabdus* bacteria (Adams *et al.*, 2006; Machado *et al.*, 2018; Machado *et al.*, 2019). Both the EPN and their bacterial partners need to overcome the host defences for successful infection and propagation (Brivio *et al.*, 2002; Brivio *et al.*, 2004; Eleftherianos *et al.*, 2010; Castillo *et al.*, 2011; Castillo *et al.*, 2013; Toubarro *et al.*, 2013). As a result of an evolutionary arms race with their natural enemies, insects have evolved a variety of defensive strategies to resist the attack of these enemies, one of which is to sequester secondary metabolites from the plants on which they feed (Opitz & Müller, 2009; Agrawal *et al.*, 2012; Erb & Robert, 2016; Petschenka & Agrawal, 2016; Heckel, 2018). The fact that the sequestration of plant-produced toxins by root-feeding insects can have a negative impact on the performance of EPN and their symbiotic bacteria has only recently become evident (Barbercheck *et al.*, 1995; Barbercheck & Wang, 1996; Robert *et al.*, 2017).

The western corn rootworm (WCR, *Diabrotica virgifera virgifera* LeConte, Coleoptera: Chrysomelidae) is one of the pests that could be subject of biological control through

EPN (Kuhlmann & van der Burgt, 1998). WCR is one of the main pests of maize in the United States Corn Belt (Kiss *et al.*, 2005; Gray *et al.*, 2009) and has been accidently introduced and spread throughout Europe since the 1980s (Szalai *et al.*, 2011). The control potential of EPN is limited by the fact that WCR larvae are able to sequester benzoxazinoids (Robert *et al.*, 2017), the most abundant defence metabolites found in young maize tissue (Frey *et al.*, 2009; Robert *et al.*, 2017). Contrary to generalist herbivores, which are negatively affected by benzoxazinoids (Wouters *et al.*, 2016), WCR larvae themselves are immune and even attracted to benzoxazinoids (Robert *et al.*, 2012; Hu *et al.*, 2018) and can convert and store them in their bodies (Robert *et al.*, 2017). Sequestration of benzoxazinoids has been shown to provide WCR larvae with resistance towards a commercial EPN strain and its symbiotic bacteria (Robert *et al.*, 2017).

Recent research found that EPN strains collected from US maize fields in regions where WCR has been present for at least 50 years were more effective in infecting WCR than EPN from other regions (Zhang *et al.*, 2019). These EPN were less affected by benzoxazinoids sequestered by WCR, suggesting that EPN may be locally adapted to deal with the defence mechanisms of their insect hosts (Zhang *et al.*, 2019). An artificial selection experiment found that benzoxazinoid resistance in EPN increases strongly when the nematodes were selected on WCR, and also increases significantly, but less strongly, when selected on the banded cucumber beetle (BCB, *D. balteata*), which does not sequester benzoxazinoids (Zhang *et al.*, 2019). This latter result suggests that benzoxazinoid resistance in EPN may also increase in the absence of a benzoxazinoid sequestering host, possibly via exposure to residual benzoxazinoid levels in the herbivores or the rhizosphere.

WCR likely originated in Mexico (Lombaert *et al.*, 2018), where maize was domesticated *circa* 9000 years ago from teosinte (*Zea mays parviglumis*) (Matsuoka *et al.*, 2002). Although WCR is present in Mexico, other root herbivores such as the larvae of the Mexican corn rootworm (*Diabrotica virgifera zeae*), the southern root cornworm (*D. undecimpunctata*), the banded cucumber beetle (*D. balteata*) and white grubs, such as *Phyllophaga* spp., are often abundant in maize fields (Álvarez-Zagoya *et al.*, 2008; Marín-Jarillo & Bujanos-Muñiz, 2008; Marín-Jarillo, 2012). No information is currently available on the capacity of Mexican maize root pests other than WCR to sequester benzoxazinoids. In this study, we explored the capacity of EPN strains that were isolated from Mexican corn fields to infect BCB and WCR and resist benzoxazinoids. We hypothesized that Mexican EPN should have an enhanced capacity to resist the benzoxazinoid-dependent defences of WCR due to the fact that they evolved in a benzoxazinoid-rich environment and may have shared an evolutionary history with benzoxazinoid containing root herbivores. To this end, we isolated and identified 40 EPN isolates from maize fields throughout Mexico. We then assessed their ability to infect WCR and BCB in the laboratory. In a second step, we tested the impact of benzoxazinoids on the resistance of WCR towards the different EPN isolates by feeding WCR larvae on benzoxazinoid-containing or benzoxazinoid-

deficient maize seedlings. Taken together, these experiments allowed us to assess the capacity of different Mexican EPN to infect and kill two different rootworm species and to gain insights into the prevalence and relative importance of benzoxazinoid resistance in these EPN isolates.

Results

Mexican maize fields host several EPN species

In total, 40 EPN isolates were retrieved by baiting method from mixed soil samples of 16 out of 114 maize fields (14%) from the states of Oaxaca, Jalisco, Michoacán, Guanajuato, Zacatecas and Durango (Fig. 1, Table 1). Durango state had the highest percentage of EPN-hosting maize fields (44%, 4 out of 9). The other locations had 8 to 18% positive samples, except Querétaro locations, from which no EPN were collected. According to 18S, D2/D3 and concatenated 18S-D2/D3 rRNA gene sequences comparisons, the recovered EPN belong to the species *Heterorhabditis indica*, *H. atacamensis*, *H. mexicana*, *H. bacteriophora, Steinernema riobrave* and a potentially new species closely related to *H. bacteriophora* (Fig. 2, Fig. 3, Supplementary Fig. S1-S8). Independently of the evolutionary inference method (Maximum Likelihood or Neighbour Joining) or the sequence used, similar taxonomic conclusions can be drawn (Fig. 2, Fig. 3, Supplementary Fig. S1-S8). *Heterorhabditis bacteriophora* was the most dominant species (21 of 40 isolates, 52.5%) and its distribution was the broadest, being recovered in Durango, Zacatecas, Jalisco and Guanajuato states. Three isolates, MEX-39, MEX-40, MEX-41, form a clearly distinct cluster from the close *H. bacteriophora*, and might therefore be a new species (Fig. 2, Supplementary Fig. S1-S2 and S3-S4). Eight isolates of *H. indica* were recovered, all from Oaxaca state, a single *S. riobrave* isolate was obtained from Michoacán state, and a single *H. atacamensis* isolate and most of the *H. mexicana* isolates were recovered from Guanajuato state.

Contrary to BCB, WCR larvae sequester benzoxazinoids from maize seedlings

To test whether WCR larvae sequester benzoxazinoids under our experimental conditions, we quantified the benzoxazinoids present in seedlings of maize and in larvae fed on those seedlings (Figure 4 a and b). We first quantified the benzoxazinoids in the seedlings of DFI 45321 maize, which is the hybrid used as insect food in our *in-house* rearing, as well as in WCR larvae that fed on that maize. The total amount of benzoxazinoids found was 489 ± 125 µg/g fresh weight (FW) in DFI 45321 maize seedlings, of which 84% was DIMBOA-Glc and 11% was HDMBOA-Glc (Fig. 4 c). WCR larvae fed on seedlings of this maize contained 489 ± 66 µg/g FW of benzoxazinoids, mainly composed of HDMBOA-Glc (91%) (Fig. 4 d, Supplementary Table MBOA-Glc was not present in plants, though it was present in larvae, which confirms previous findings (Maag *et al.*, 2014) (Robert *et al.*, 2017). BCB larvae fed on DFI 45321 maize, on the other hand, had <1% of the total amount of benzoxazinoids found in WCR larvae (Fig. 4 d, Supplementary Table S3, $F_{2,10}=65.585$, $p<0.001$).

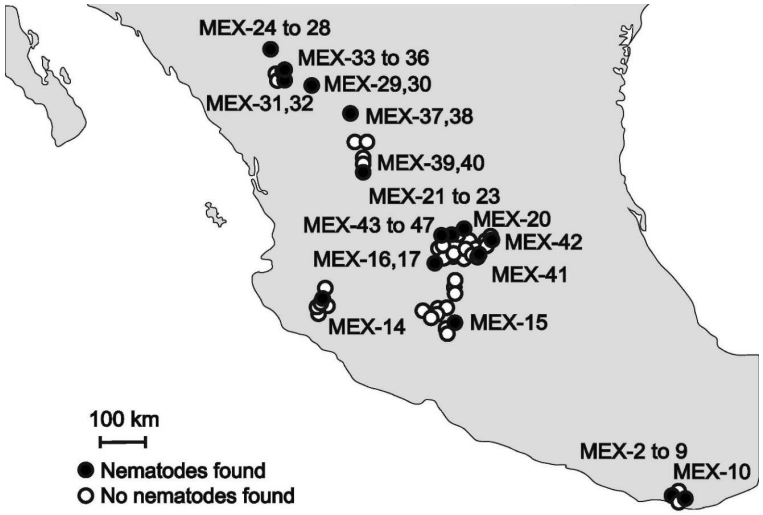

Figure 1. Location of soil samples and origin of entomopathogenic nematode isolates (EPN) obtained from 16 Mexican maize fields out of 114 maize fields sampled. One mixed soil sample was taken per maize field location and baited with *Galleria mellonella* larvae. Black filled dots indicate sites where EPN isolates were collected. Empty dots indicate soil samples from which no nematodes were obtained. Original map by FreeVectorMaps.com, modified with Adobe Illustrator CC 2018.

We then measured benzoxazinoids in maize seedlings of wild type B73 and in a corresponding benzoxazinoid-deficient *bx1* mutant line. We found 586 ± 127 µg/g FW of benzoxazinoids in wild type B73 seedlings, of which, DIMBOA-Glc represented 65% and HDMBOA-Glc 27% (Fig. 4 e, Supplementary Table S4). In contrast, the maize seedlings of the *bx1* mutant had 95% less total benzoxazinoids content compared to wild type B73 maize (Fig. 4 e, Supplementary Table S4, $F_{2,10}$=19.922, p=0.002). WCR larvae that were fed on wild type B73 maize seedlings had a total benzoxazinoid content of 612 ± 115 µg/g FW, 75% of which consisted of HDMBOA-Glc, whereas WCR larvae fed on *bx1* mutant seedlings had only 0.05% of the total benzoxazinoids found in WCR larvae fed on the wild type B73 maize (Fig. 4 f, Supplementary Table S6, $F_{2,9}$= 45.328, p<0.001). Together, these results confirm earlier findings that WCR, but not BCB, sequesters benzoxazinoids.

Table 1. Isolates obtained per entomopathogenic nematode-infected sample and origin of soils of Mexican maize fields. Each *Galleria mellonella* larva from the '*Galleria* baiting" soil extraction method was kept separately and the emerging EPN formed one isolate.

State	Sampled fields	EPN infected fields	Entomopathogenic nematodes			
			Latitude (°)	Longitude (°)	Altitude (masl)	Isolates
Oaxaca	19	2	15.886541	-97.116045	1	MEX-2, MEX-4, MEX-5, MEX-6, MEX-7, MEX-8, MEX-9
			15.920639	-97.12642	20	MEX-10
Jalisco	13	1	19.893941	-104.04043	903	MEX-14
Michoacán	13	1	19.627714	-101.41115	2277	MEX-15
Guanajuato	45	6	20.738882	-101.33939	1760	MEX-16, MEX-17
			20.963636	-100.83139	1869	MEX-20
			20.924801	-100.94449	1993	MEX-21, MEX-22, MEX-23
			20.470774	-100.59571	1926	MEX-41
			20.565299	-100.77389	1766	MEX-42
			20.914769	-101.21872	1913	MEX-43, MEX-44, MEX-45, MEX-46, MEX-47
Querétaro	4	0	-	-	-	-
Zacatecas	11	2	23.208545	-103.04567	2080	MEX-37, MEX-38
			22.161371	-102.88794	1743	MEX-39, MEX-40
Durango	9	4	24.006255	-104.61634	1789	MEX-24, MEX-25, MEX-26, MEX-27, MEX-28
			24.000478	-104.61628	1878	MEX-29, MEX-30
			23.954223	-104.62634	1885	MEX-31, MEX-32
			23.988059	-104.62605	1880	MEX-33, MEX-34, MEX-35, MEX-36
TOTAL	114	16				

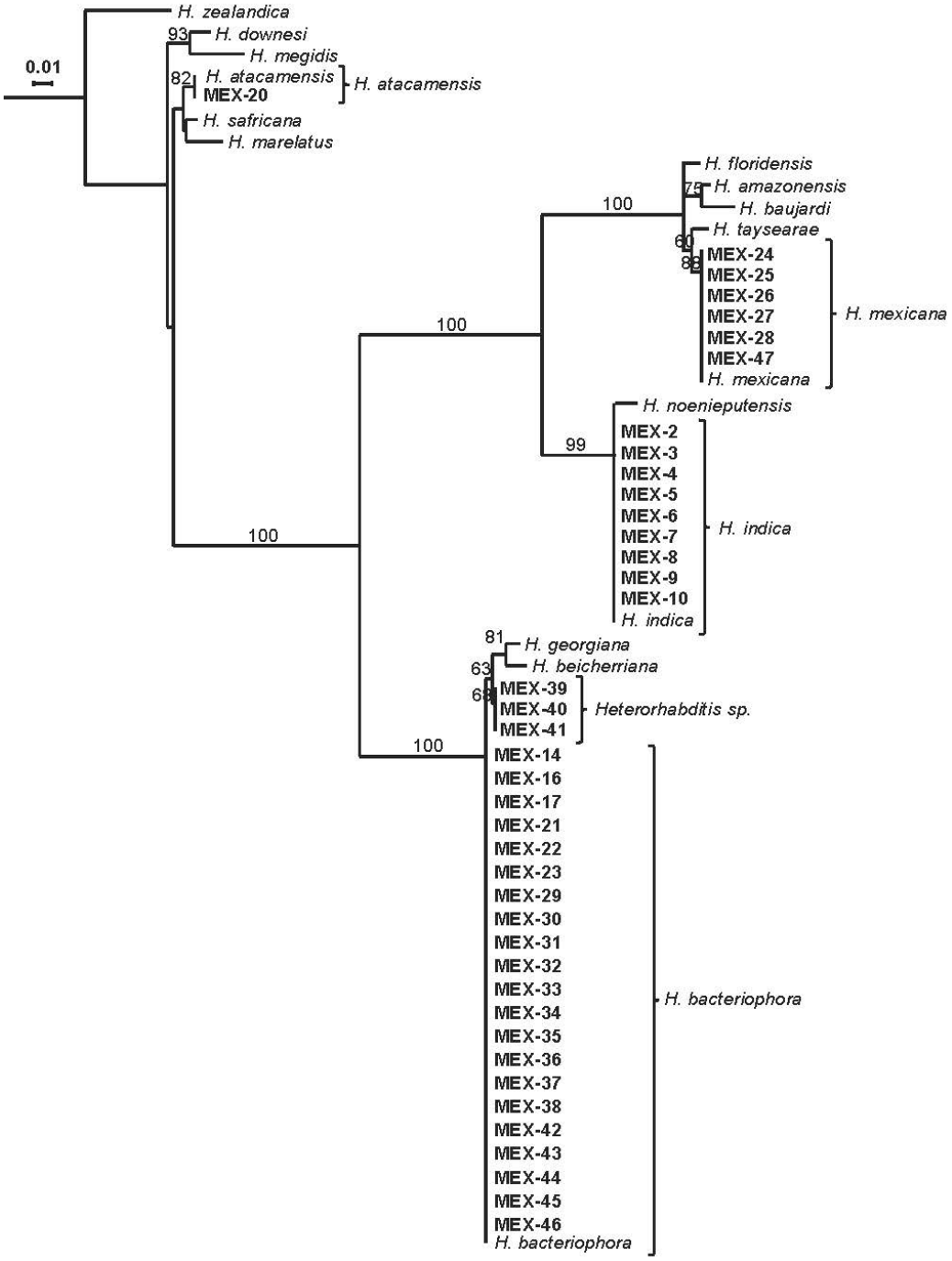

Figure 2. Maximum-likelihood phylogenetic tree of *Heterorhabditis* nematodes based on 18S ribosomal RNA gene sequences. The evolutionary distances were computed using the the Hasegawa-Kishino-Yano model. The tree with the highest log likelihood (-3422.19) is shown. Numbers at nodes represent bootstrap values higher than 70% based on 100 replications. The rate variation among sites was modeled with a gamma distribution (5 categories (+G, parameter = 0.4803)). Bar represents 0.01 nucleotide substitutions per sequence position. Accession numbers of the gene sequences used for the reconstructions and those obtained in this study are available in Table S1 and S2, respectively.

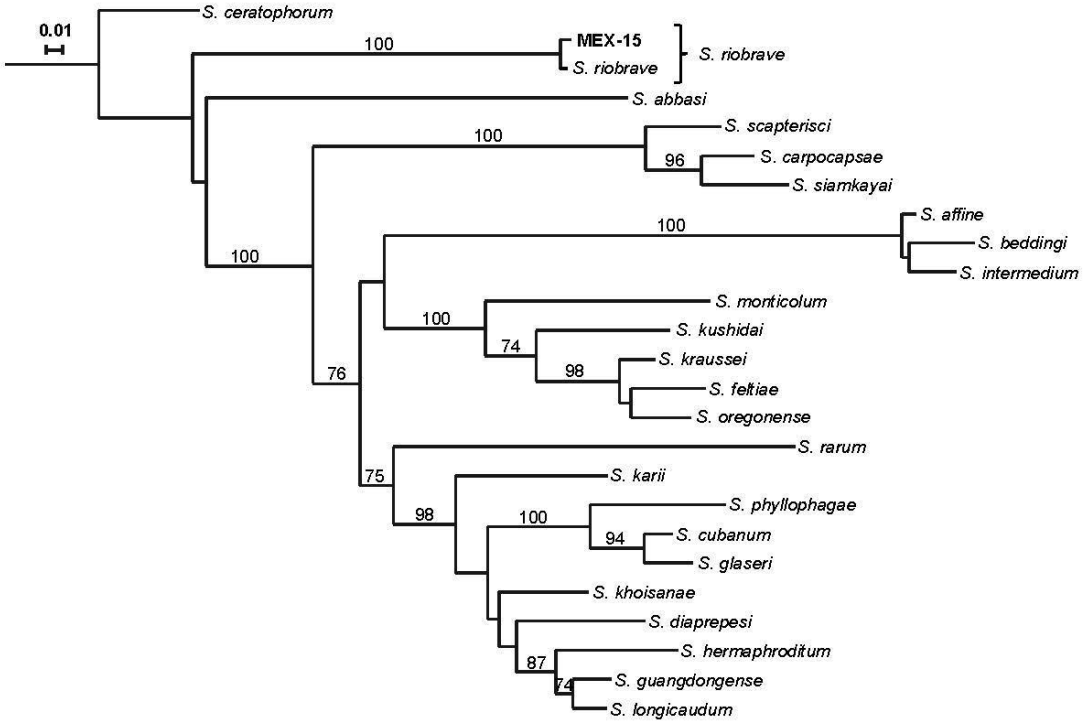

Figure 3. Maximum-likelihood phylogenetic tree of *Steinernema* nematodes based on 18S ribosomal RNA gene sequences. The evolutionary distances were computed using the General Time Reversible model. The tree with the highest log likelihood (-7942.12) is shown. Numbers at nodes represent bootstrap values higher than 70% based on 100 replications. The rate variation among sites was modeled with a gamma distribution (5 categories (+G, parameter = 0.4233)). Bar represents 0.01 nucleotide substitutions per sequence position. Accession numbers of the gene sequences used for the reconstructions and those obtained in this study are available in Table S1 and S2, respectively.

Mexican EPN show high variability in infecting WCR but not BCB larvae

In order to screen the capacity of the collected EPN isolates to infect the benzoxazinoid-sequestering WCR larvae, we compared the infection capacity of each EPN isolate towards benzoxazinoid-sequestering WCR larvae and non-sequestering BCB larvae. Infection capacity across different EPN isolates appeared highly variable on WCR larvae, but was generally high and homogeneous on BCB larvae (Fig. 5, $F_{2,\ 2111} = 543.57$, $p < 2.2E\text{-}16$). *Steinernema riobrave* nematodes infected few WCR (9% ± 4) but all BCB larvae (100%) (Fig. 5 a). In contrast, *H. indica* and *H. atacamensis* nematodes were highly infective on both, WCR (90 ± 4% and 95 ± 2%, respectively) and BCB larvae (99% ± 1% and 89 ± 4% respectively) (Fig. 5 a). *Heterorhabditis mexicana* isolates were moderately infective on WCR larvae (51 ± 5%), but strongly infective on BCB larvae (95 ± 3%) (Fig. 5 a). The most variable infection rates were found in *H. bacteriophora* isolates on WCR larvae, which varied from 35 ± 6% to 90 ± 4% (Fig. 5 b). Thus, WCR larvae are more resistant to most Mexican nematode isolates than BCB larvae. Results of the statistical analyses are shown in Supplementary Table S6.

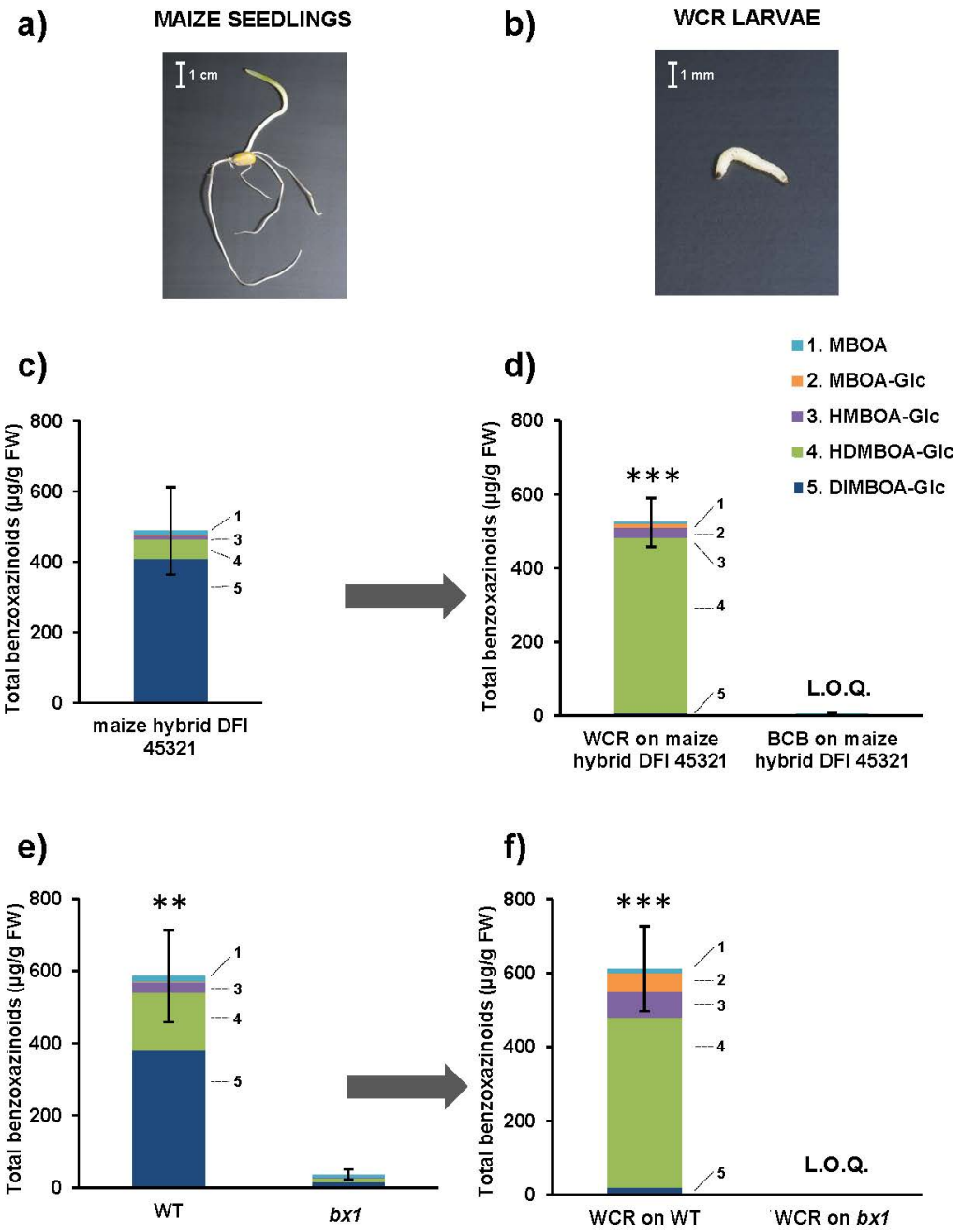

Figure 4. Contents of benzoxazinoids in maize seedlings and sequestration of benzoxazinoids by western corn rootworm larvae. a) Maize seedlings used for the rearings of WCR and BCB. b) Larvae of WCR used in the experiments. c) Benzoxazinoids concentrations in maize seedlings of the hybrid DFI 45321 (n=5). d) Benzoxazinoids concentrations in dissected larvae (n=5) of WCR and BCB fed on hybrid maize seedlings DFI 45321. e) Benzoxazinoids concentrations in maize seedlings (n=5) of the benzoxazinoid-containing wild type (WT) B73, and the benzoxazinoid-deficient *bx1* mutant maize. f) Benzoxazinoids concentrations in dissected WCR larvae fed on the WT B73 (n=4) or on the benzoxazinoid-deficient *bx1* mutant (n=5) since hatching. Bars indicate average (± SE). Stars indicate significant differences between treatments: ***p<0.001, **p<0.01.

Most *Heterorhabditis bacteriophora* isolates are adapted to the benzoxazinoid-dependent defences of WCR

The high variation in the infection capacity on WCR larvae across EPN isolates, compared to the overall-high infection on BCB, suggested that the nematode isolates differed in their capacities to resist benzoxazinoid-dependent WCR defence. In order to test this hypothesis, we evaluated the infection capacity of all *H. bacteriophora* isolates on benzoxazinoid-containing and benzoxazinoid-free WCR larvae. To produce benzoxazinoid-free WCR larvae, we fed them on benzoxazinoid-deficient *bx1* mutant maize seedlings. The infection capacity was uniform across all *H. bacteriophora* isolates against both benzoxazinoid-containing and benzoxazinoid-free WCR larvae, except the isolates MEX-37, MEX-23 and MEX-40, which showed a significantly lower infection rate on benzoxazinoid-containing larvae than their benzoxazinoid-free counterparts (Fig. 6). Surprisingly, the infection of benzoxazinoid-containing WCR larvae by the isolate MEX-42 was higher than of benzoxazinoid-free WCR larvae. Results of the statistical analyses are shown in Supplementary Table S7.

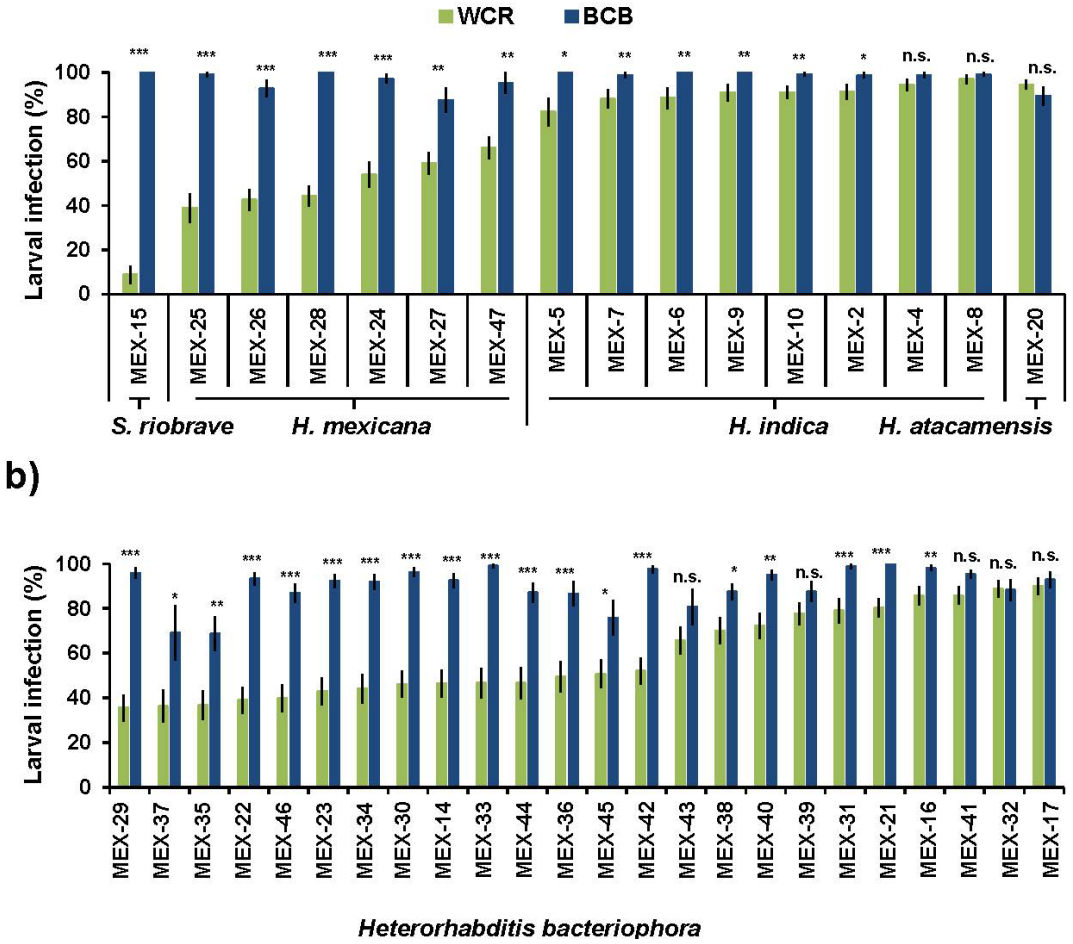

Figure 5. Infection rates of all Mexican EPN isolates on WCR larvae (green) and BCB (blue) seven days after EPN inoculation. (a) *Steinernema riobrave*, *Heterorhabditis mexicana*, *H. indica* and *H. atacamensis*, and (b) *Heterorhabditis bacteriophora* EPN isolates (n~20). Bars indicate average (± SE). Stars indicate significant differences between treatments for each EPN isolate: *p<0.05, **p<0.01, ***p<0.001.

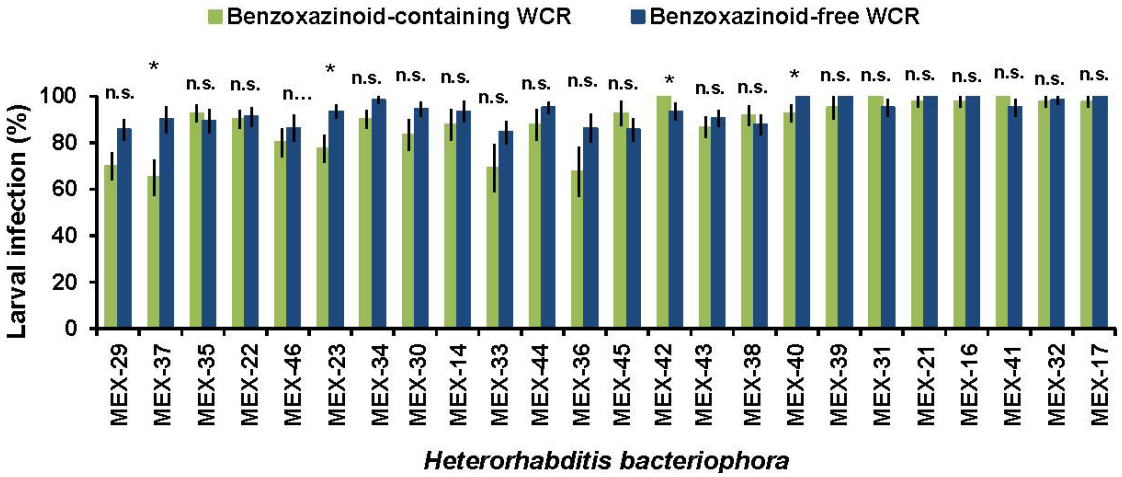

Figure 6. Infection rates of *Heterorhabditis bacteriophora* isolates on WCR larvae fed on benzoxazinoids-containing (green) or benzoxazinoid-free (blue) maize seedlings seven days after EPN inoculation. Bars indicate average (± SE). Stars indicate significant differences between treatments for each EPN isolate: *p<0.05, **p<0.01, ***p<0.001.

Discussion

For the present study, 40 novel EPN isolates were obtained from 114 maize fields from across seven Mexican states. Using phylogenetic reconstructions based on rRNA gene sequences, they were identified as *Heterorhabditis indica*, *H. atacamensis*, *H. mexicana*, *H. bacteriophora,* and *Steinernema riobrave*. Interestingly, the *H. bacteriophora*-classified MEX-39, MEX-40 and MEX-41 isolates form a slightly, but clearly distinct group from *H. bacteriophora*. Traditionally, isolates sharing more than 97% similarity in rDNA sequences are classified as same species, which applies to MEX-39, MEX-40 and MEX-41. However, it is increasingly recognized that the 97% threshold is rather conservative and recent studies suggest that the threshold for species delimitation can be greater than 99% (Tang *et al.*, 2012; Borg Dahl *et al.*, 2018; Vu *et al.*, 2019).Therefore, we should be open to the possibility that the isolates MEX-39, MEX-40 and MEX-41 represent a new species. Further evidence based on cross-breeding analyses should be obtained to test this possibility [36]. Similar results with Rwandan isolates close to or the same as *H. bacteriophora* were recently reported by Fallet et al.(Fallet *et al.*, in press). Plant chemical defences can be exploited by adapted herbivores (Boppré, 1986). A prime example is the sequestration of plant defence compounds by herbivorous insects, which help them to protect themselves against their natural enemies (Ferguson & Metcalf, 1985; Nishida & Fukami, 1990; Opitz & Müller, 2009; Petschenka & Agrawal, 2016; Robert *et al.*, 2017). However, natural enemies may adapt to overcome the toxic effect of plant secondary metabolites that they frequently encounter in their environment. It has recently been postulated that, although EPN are often generalists (Hazir *et al.*, 2004), their limited mobility confines populations to small geographic areas, and that this confinement exerts sufficient pressure to each population to adapt to local hosts and associated plant secondary metabolites (Zhang *et al.*, 2019). Thus, EPN populations that have coexisted with benzoxazinoids for a long

time are expected to have evolved some tolerance or resistance to these compounds. Indeed, when exposed to WCR fed either on benzoxazinoid-containing or benzoxazinoid-deficient maize seedlings (Fig. 6), almost all of our Mexican *H. bacteriophora* isolates were equally capable to kill both types of larvae. This is in agreement with the findings of Zhang et al. (Zhang *et al.*, 2019) and implies that most of *H. bacteriophora* are adapted to benzoxazinoid-dependent defences of WCR. Interestingly however, we still observed substantial variability among the isolates in their capacity to infect WCR, suggesting the presence of other WCR defence mechanisms to which not all EPN are equally well adapted. This variability may also be due to the fact that some isolates are somewhat distinct from *H. bacteriophora*, and may even represent a new species.

As WCR is usually not very abundant in Mexican maize fields (Marín-Jarillo, 2012), EPN populations may vary in their ability to overcome WCR defence mechanisms that are independent of benzoxazinoids. Evolved resistance to sequestered benzoxazinoids might only be one of the strategies that renders some but not all the EPN isolates highly infective on WCR. Our results imply that EPN and/or their symbiotic bacteria have also evolved other traits to overcome WCR resistance. They may, for instance, be able to suppress the humoral immune response of the host insect (Carper *et al.*, 2019). This ability often depends on the EPN-bacteria association and indeed has been reported to differ even among conspecific EPN strains (Hallem *et al.*, 2007; Li *et al.*, 2007). Additionally to the effects of sequestered benzoxazinoids, symbiotic bacteria may also be affected by other factors in the host's diet, to which they could evolve resistance (Barbercheck & Wang, 1996; Robert *et al.*, 2017). It should be noted that WCR larvae in this study were fed with different maize genotypes in two experiments, and nutritional or chemical aspects of DFI 45321 and B73 maize may have affected the susceptibility of WCR larvae to EPN infection(Barbercheck *et al.*, 1995; Coley *et al.*, 2006). Possibly, WCR larvae acquired a resistance factor from DFI 45321 maize that is absent in B73 maize, which could help explain the overall high infection of WCR larvae in the second infectivity experiment. Our work indicates that WCR larvae possess effective defence strategies, much more than BCB larvae, that allow them to escape certain EPN which have overcome benzoxazinoid sequestration.

The potential of *H. bacteriophora* as a biological control agent of WCR has been widely recognized and studied, and commercial products based on this EPN are registered in a number of European countries. It is considered as one of the most effective EPN species in the biological control of WCR larvae, particularly in light of the recent phase-out of neonicotinoid-based seed coatings and highly toxic soil pesticides such as tefluthrin (Kurtz *et al.*, 2008; Hiltpold *et al.*, 2010; Geisert *et al.*, 2018). Nevertheless conventional EPN application methods have various limitations such as being knowledge-intense for the farmer (Georgis *et al.*, 2006), or sometimes not cost-effective for low-value crops like maize. But novel, more effective EPN application methods are under development, such as application in alginate beads (Hiltpold *et al.*, 2012; Jaffuel *et al.*, 2020; Kim *et al.*, under review). In addition, our results show that selecting the *H.*

bacteriophora most adapted to benzoxazinoid-based defences of WCR or breeding for such traits may improve the biocontrol product efficacy. It may also be wise to include the so far little-considered *H. indica* or *H. atacamensis* in research and development on the biological control of WCR. In conclusion, readily mass-produced (Han & Ehlers, 2000), highly-infectious EPN that are well adapted to overcome WCR resistance could be the best candidates for further improving biocontrol strategies against this exceedingly important maize pest.

Materials and methods

Biological resources

Maize (*Zea mays mays* L.) of the inbred line B73 (wild type) and the near-isogenic line *bx1* (Maag *et al.*, 2016) (*bx1* mutant) were provided by the laboratory of Biotic Interactions (University of Bern, Switzerland). Seeds were germinated in humid vermiculite (Greendoor, Switzerland) and 5-day-old seedlings were used for the rearing of the larvae.

WCR eggs were originally provided by CABI (Hódmezővásárhely, Hungary) from the WCR non-diapause colony of USDA ARS (Brookings, SD, USA). BCB eggs were provided by Syngenta Crop Protection (Stein, Switzerland). WCR and BCB were reared on the maize hybrid DFI 45321 (DSP, Delley, Switzerland) as previously described (Erb *et al.*, 2011). Briefly, neonate larvae were placed on 5-day-old germinated maize seedlings in rearing boxes (BugDorm, USA) filled with potting soil (Einheitserdewerke Patzer Gebrüder Patzer GmbH & Co. KG, Germany), and the plant material was refreshed every three days. The adults were kept in cages (BugDorm, USA) and fed with 10-day-old maize plants. Neonates of WCR and BCB were fed on either the maize hybrid DFI 45321, the wild type B73 or the mutant line *bx1* until the larvae reached the third instar and then they were used for the experiments. All experiments were performed using third instar larvae.

Survey of entomopathogenic nematodes

Entomopathogenic nematodes were surveyed in 114 maize fields in the Mexican states of Durango, Zacatecas, Guanajuato, Querétaro, Michoacán, Jalisco and Oaxaca, during July and August 2012 (Table 1). Four locations were randomly selected in each field to collect 500 g at 20 to 40 cm depth. The four samples were homogenized and pooled to obtain one 2 kg representative soil sample per field. EPN were recovered using the "*Galleria* baiting" method (Bedding & Akhurst, 1975; Campos-Herrera *et al.*, 2015). Briefly, two plastic pots of 250 ml were filled with soil from each soil sample and ten larvae of the greater wax moth *Galleria mellonella* L. (Lepidoptera: Pyralidae) were added. The pots were covered and kept at room temperature in the dark for four days, after which the mortality of the larvae was evaluated. Dead and living larvae were removed, and the soil was baited for a second time with 10 fresh larvae. The dead larvae were individually placed onto paper in nematode traps, consisting of islands of small Petri-dishes in a water filled larger Petri-

dish (White, 1927) . Emerging infective juveniles from each larva were separately collected from the water. *In vivo* colonies of EPN were established under quarantine conditions at the University of Neuchâtel, Switzerland (nematode storage in darkness at 12ºC).

Identification of entomopathogenic nematodes

Genomic DNA was extracted using the QIAamp DNA Mini Kit, following manufacturer's instructions (Qiagen Ltd., Valencia, CA). For this, 20,000 freshly emerged infective juveniles were centrifuged for ten minutes at 4 ºC and 10,000 G. The supernatant was removed, and the nematode were resuspended in 200 µl of milli-Q water in a 1.5 ml microcentrifuge tube. The nematodes were then ground for 15 seconds with a pestle motor, 180 µl of buffer ATL and 20 µl of proteinase K (600mg/ml) were added, and the tubes were incubated at 56°C for 2 hours. The extracted DNA was eluted in 50 µl of milli-Q water and stored at -20 °C until analysis. The quality of the DNA was evaluated with nanodrop and electrophoresis migration. PCR was performed to amplify the 18S rRNA gene using primers 18S (5'-TTGATTACGTCCCTGCCCTTT-3') and 28S (5'-TTTCACTCGCCGTTACTAAGG-3') (Vrain *et al.*, 1992) and the D2/D3 region of the 28S rRNA gene using primer 536 (5'-CAGCTATCCTGAGGGAAAC-3') (Stock *et al.*, 2001). PCR products were purified using the QIAquick gel purification Kit (Qiagen Ltd., Valencia, CA) and sequenced by Sanger sequencing (Microsynth AG, Balgach, Switzerland). The evolutionary histories were inferred from either the 18S, the D2/D3 or both sequences concatenated using the Neighbour-joining method (Saitou & Nei, 1987) and the Maximum-Likelihood method (Murshudov *et al.*, 1997). For Maximum-Likelihood method-based phylogenies, best-fit substitution model analysis were carried out prior to inferring evolutionary histories (Nei & Kumar, 2000; Kumar *et al.*, 2016). All trees are drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of trees in which the associated taxa clustered together is shown next to the branches. In all cases, initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The Maximum-likelihood *Heterorhabditis* 18S-based evolutionary history was inferred based on the Hasegawa-Kishino-Yano model (Hasegawa *et al.*, 1985). The tree with the highest log likelihood (-3422.19) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4803)). There were a total of 849 positions in the final dataset (Fig. 2). The Maximum-likelihood *Heterorhabditis* D2/D3-based evolutionary history was inferred based on the Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (-1812.68) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.0500)). There were a total of 845 positions in the final dataset (Supplementary Fig. S1). The Maximum-likelihood *Heterorhabditis* concatenated 18S-D2/D3-based evolutionary history was inferred based on the Kimura 2-parameter model (Kimura,

1980). The tree with the highest log likelihood (-6209.06) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.2726)). There were a total of 1892 positions in the final dataset (Supplementary Fig. S2). The Maximum-likelihood *Steinernema* 18S-based evolutionary history was inferred based on the General Time Reversible model (Nei & Kumar, 2000). The tree with the highest log likelihood (-7942.12) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4233)). There were a total of 832 positions in the final dataset (Fig. 3). The Maximum-likelihood *Steinernema* D2/D3-based evolutionary history was inferred based on the Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (-4491.90) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.2931)). There were a total of 879 positions in the final dataset (Supplementary Fig. S3). The Maximum-likelihood *Steinernema* concatenated 18S and D2/D3-based evolutionary history was inferred based on the Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (-16814.12) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3552)). There were a total of 2126 positions in the final dataset (Supplementary Fig. S4). For Neighbour-Joining based phylogenies, the evolutionary distances were computed using the Kimura 2-parameter model (Kimura, 1980), with gamma-distributed rate variation among sites. The trees were constructed with the bootstrap test (1000 replicates) showing the percentage of replicate trees in which the associated taxa clustered together (Felsenstein, 1985) (Supplementary Fig. S5-S8). Interactive Tree of Life v3.5.1 (Chevenet *et al.*, 2006; Letunic & Bork, 2016) was used to edit and represent the phylogenetic trees. All evolutionary analyses were conducted in MEGA7 (Kumar *et al.*, 2016). All the sequences obtained were deposited into the GenBank. Accession numbers of the gene sequences used for the reconstructions and those obtained in this study are available in Table S1 and S2, respectively.

Benzoxazinoids profiling in insects and plants

To assess benzoxazinoid sequestration by the insect larvae, we measured the content of benzoxazinoids in larval tissues of third instar WCR and BCB larvae fed on hybrid DFI 45321 maize seedlings, as well as WCR larvae fed either on the wild type B73 or on *bx1* mutant maize. As previously reported, the sequestration occurs when the chemical compounds are transferred from the gut to other body tissues, such as muscles and haemolymph (Opitz & Müller, 2009; Erb & Robert, 2016; Heckel, 2018). Therefore, the benzoxazinoids were measured from larvae from which the gut was removed (n=5). Eight to ten larvae were pooled per biological replicate. We also quantified the amount of benzoxazinoids in the maize seedlings of the genotypes that were used in the experiments (n=5, five days old). Maize seedlings or larvae (guts removed) were flash frozen in liquid nitrogen and ground into a fine powder. To 20 mg of plant or larval tissue per sample, 1 ml or 400 µl of the extraction buffer (MeOH: H_2O: formic acid (FA); 50: 50: 0.5%) were added, respectively. Extracts were vortexed for 1

min and centrifuged at 20,000 g for 20 min at 4°C. Supernatants were used for further analyses. Plant samples of the hybrid DFI 45321 and the wild type B73 were diluted 50 times, and samples of the the maize mutant *bx1* were diluted 20 times. Larval extracts were directly used without dilution for the analyses. We measured the main benzoxazinoids that had previously been found in maize plants and in WCR larvae: 2-(2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one)-β-d-glucopyranose (HDMBOA-Glc), 2-(2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one)-β-d-glucopyranose (DIMBOA-Glc), 2-β-d-glucopyranosyloxy-7-methoxy-1,4-benzoxazin-3-one (HMBOA-Glc), 6-methoxy-2-benzoxazolinone N-glucoside (MBOA-Glc) and 6-methoxy-2-benzoxazolinone (MBOA) (Cambier *et al.*, 2000; Oikawa *et al.*, 2002; Glauser *et al.*, 2011; Robert *et al.*, 2012; Robert *et al.*, 2017). The quantification was performed by ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS). The equipment was composed of an Ultimate 3000 RSLC (Dionex, Thermo Fisher Scientific) interfaced to a 4000 QTRAP (AB Sciex) through an electrospray probe. Benzoxazinoids were separated on an Acquity UPLC BEH C18 column (50x2.1mm, 1.7 µm, Waters) in gradient mode using H_2O+formic acid 0.05% and acetonitrile + formic acid 0.05% as phases A and B, respectively. The flow rate was set at 0.4 ml/min and the column temperature at 30°C. An injection of 2.5 µl was made. The gradient started at 2% B, linearly increased to 40% B in 3 min, then to 100% B in 2 min, and the column was finally washed at 100% B for 2 min and reconditioned at 2% B for 4 min. The mass spectrometer was operated in MRM mode using specific transitions for each benzoxazinoid compound. The instrument was run in negative ionization from 0.0 – 4.28 min for the detection of DIMBOA-Glc HMBOA-Glc and MBOA-Glc, and in positive ionization from 4.29 – 5.0 min for the detection of HDMBOA-Glc and MBOA. The gas temperature in the MS source (TEM) was set to 550°C and the nebulizing (GS1), drying (GS2) and curtain (CUR) gas flows set to 55, 50 and 15 psi, respectively. Quantification was done by external calibration using standards purified from plants at 1, 5, 20, 100, 500 and 2000 ng/ml. The lowest limits of quantification on pure standards were 0.5 – 1.0 ng/ml for benzoxazinoid glucosides and 2.0 ng/ml for benzoxazinoid aglucones.

EPN infectivity tests

Two different experiments were conducted with the newly isolated Mexican EPN. In the first experiment, the capacity of infection of the 40 EPN isolates was evaluated to test whether WCR larvae are more resistant against EPN attack than BCB larvae. Here, both WCR and BCB larvae were fed on the hybrid DFI 45321, which contains benzoxazinoids. The second experiment aimed to test whether the differences between the infection of WCR and BCB larvae could be explained by the sequestration of benzoxazinoids by WCR larvae. For this, we assessed the infection capacity of the 24 collected *H. bacteriophora* isolates on larvae of WCR that were fed either on the benzoxazinoid-containing wild type B73 line or on its near-isogenic *bx1* mutant line. Both experiments were carried out according to Zhang et al., (Zhang *et al.*, 2019). Briefly, four WCR or four BCB larvae were placed in a Ø55 mm Petri-dish (VWR International, LLC, Switzerland), that was half-filled with moist autoclaved sand (Ø 1 –

4 mm) (Migros, Switzerland). Approximately five hundred newly emerged infective juvenile nematodes suspended in 500 µl of tap water were applied to each Petri-dish (concentration of 21 IJs/cm^2). Controls were treated with 500 µl of tap water. All Petri-dishes were sealed with parafilm to prevent larvae from escaping and to maintain a high level of humidity. The dishes were kept in the dark at 22 ºC and EPN infection was verified 7 days after inoculation by recording colour changes (Supplementary Fig. S9) or by direct dissection. A total of 10 – 20 dishes for each EPN isolate were prepared for each experiment.

Statistical analyses

All data were analysed in R (R Development Core Team 2015). Differences in benzoxazinoids contents in plants and larvae were assessed with One-way ANOVA under Gaussian error distribution. Larval infections were compared within each EPN for the different type of larvae using Generalized Linear Models (GLM) under binomial distribution. All analyses were followed by residual analysis to verify the suitability of the error distribution and model fitting when necessary to quasibinomial models.

Data availability

The data supporting the findings of this study are available in the supplementary materials.

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Author contributions

M.E, C.A.M.R. and T.C.J.T conceived the original project. P.B., J.B. and A.K. conducted nematode surveys. P.B., C.C.M.A., R.A.R.M., G.G., S.T. and R.C.H. performed experiments. P.B. and C.C.M.A. carried out statistical analyses. P.B. and C.C.M.A. wrote the first draft of the manuscript. All authors contributed to the final manuscript.

Additional information

Accession numbers and supplementary information can be retrieved in the online version.

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Supplementary material

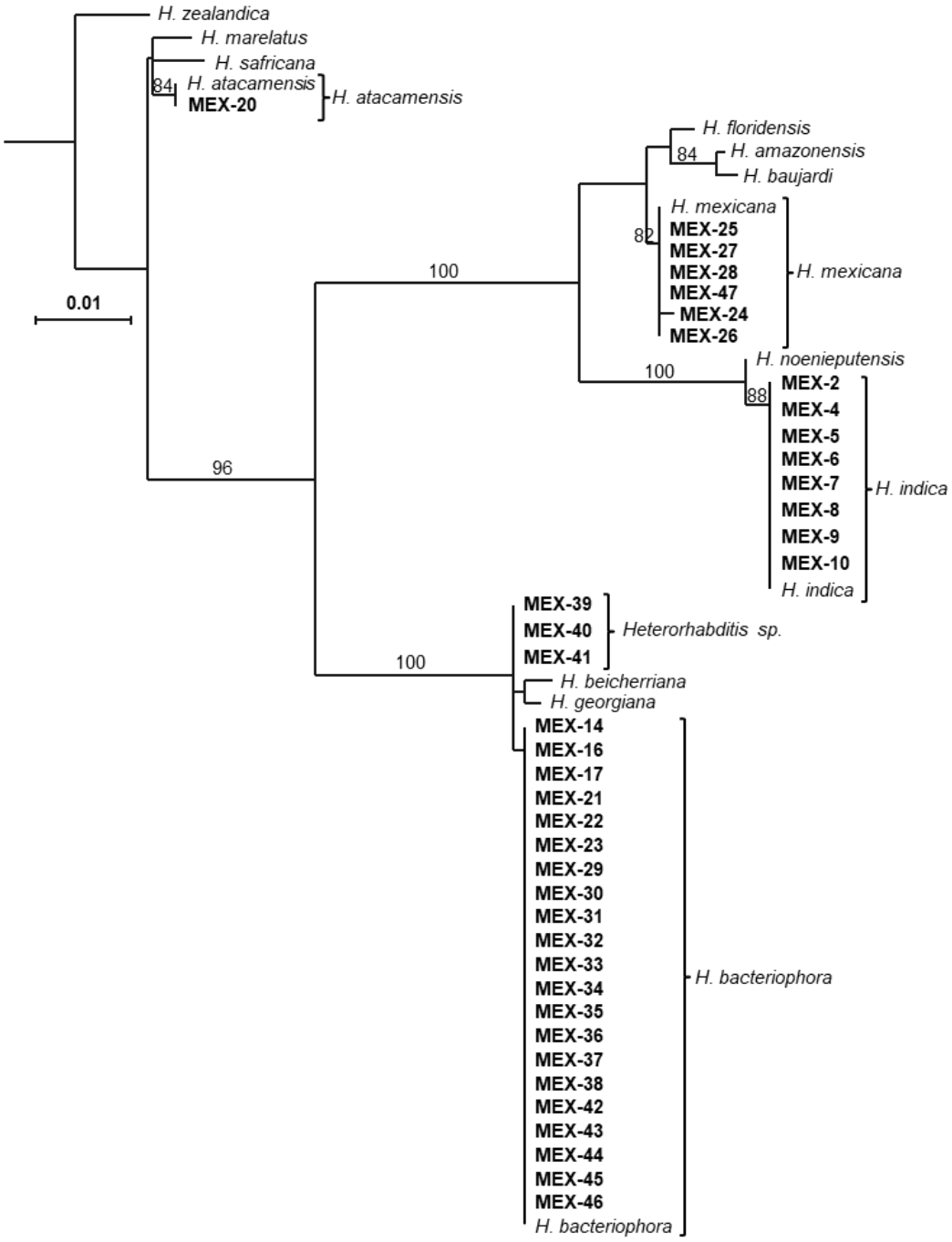

Figure S1. Maximum-likelihood phylogenetic tree of 14 species of *Heterorhabditis* nematodes based on D2/D3 ribosomal RNA gene sequences. The species *H. downesi* and *H. taysearae* could not be included since there are no available sequences in the GenBank. The evolutionary distances were computed using the Kimura 2-parameter model. The tree with the highest log likelihood (-1812.68) is shown. Numbers at nodes represent bootstrap values higher than 70% based on 100 replications. The rate variation among sites was modeled with a gamma distribution (5 categories (+G, parameter = 0.0500)). Bar represents 0.01 nucleotide substitutions per sequence position. Accession numbers of the gene sequences used for the reconstructions and those obtained in this study are available in Table S1 and S2, respectively.

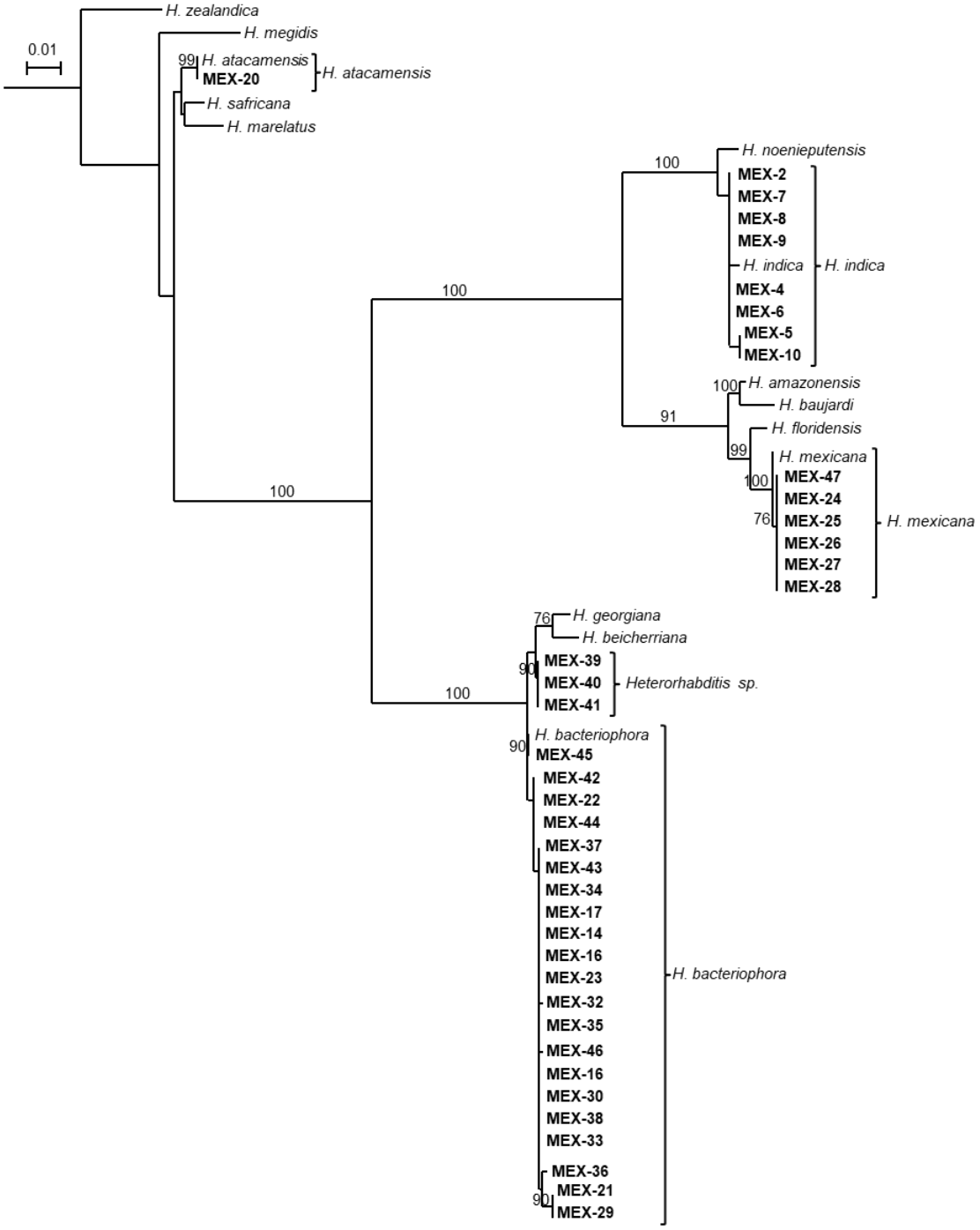

Figure S2. Maximum-likelihood phylogenetic tree of 14 species of *Heterorhabditis* nematodes based on concatenated 18S and D2/D3 ribosomal RNA gene sequences. The species *H. downesi* and *H. taysearae* could not be included since there are no available D2/D3 sequences in the GenBank. The evolutionary distances were computed using the Kimura 2-parameter model. The tree with the highest log likelihood (-6209.06) is shown. Numbers at nodes represent bootstrap values higher than 70% based on 100 replications. The rate variation among sites was modeled with a gamma distribution (5 categories (+G, parameter = 0.2726)). Bar represents 0.01 nucleotide substitutions per sequence position. Accession numbers of the gene sequences used for the reconstructions and those obtained in this study are available in Table S1 and S2, respectively.

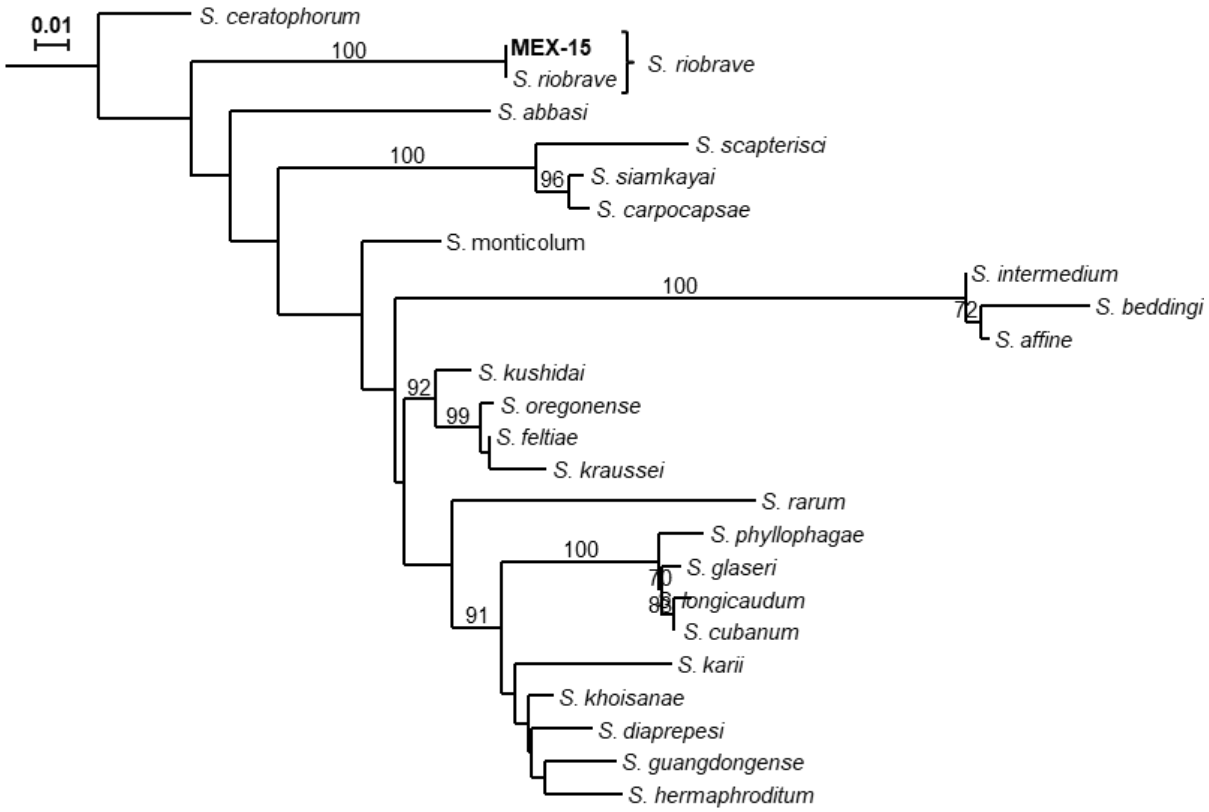

Figure S3. Maximum-likelihood phylogenetic tree of 24 species of *Steinernema* based on D2/D3 ribosomal RNA gene sequences. The evolutionary distances were computed using the Kimura 2-parameter model. The tree with the highest log likelihood (-(-4491.90) is shown. Numbers at nodes represent bootstrap values higher than 70% based on 100 replications. The rate variation among sites was modeled with a gamma distribution (5 categories (+G, parameter = 0.2931)). Bar represents 0.01 nucleotide substitutions per sequence position. Accession numbers of the gene sequences used for the reconstruction and those obtained in this study are available in Table S1 and S2, respectively.

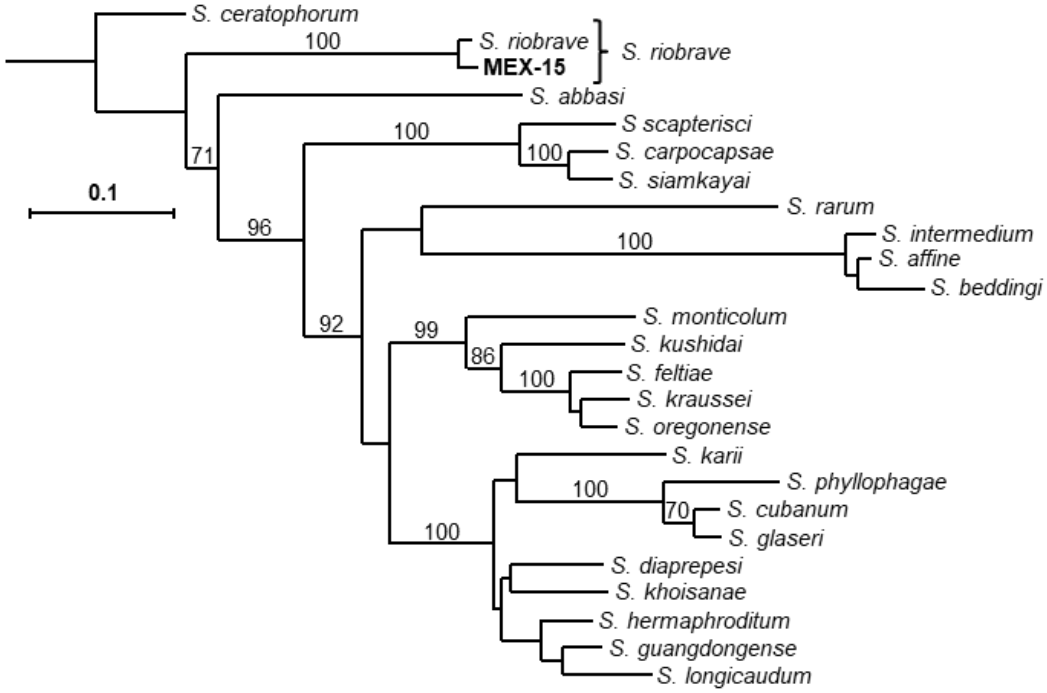

Figure S4. Maximum-likelihood phylogenetic tree of 24 species of *Steinernema* nematodes based on concatenated 18S and D2/D3 ribosomal RNA gene sequences. The evolutionary distances were computed using the Kimura 2-parameter model. The tree with the highest log likelihood (-16814.12) is shown. Numbers at nodes represent bootstrap values higher than 70% based on 100 replications. The rate variation among sites was modeled with a gamma distribution (5 categories (+G, parameter = 0.3552)). Bar represents 0.01 nucleotide substitutions per sequence position. Accession numbers of the gene sequences used for the reconstructions and those obtained in this study are available in Table S1 and S2, respectively.

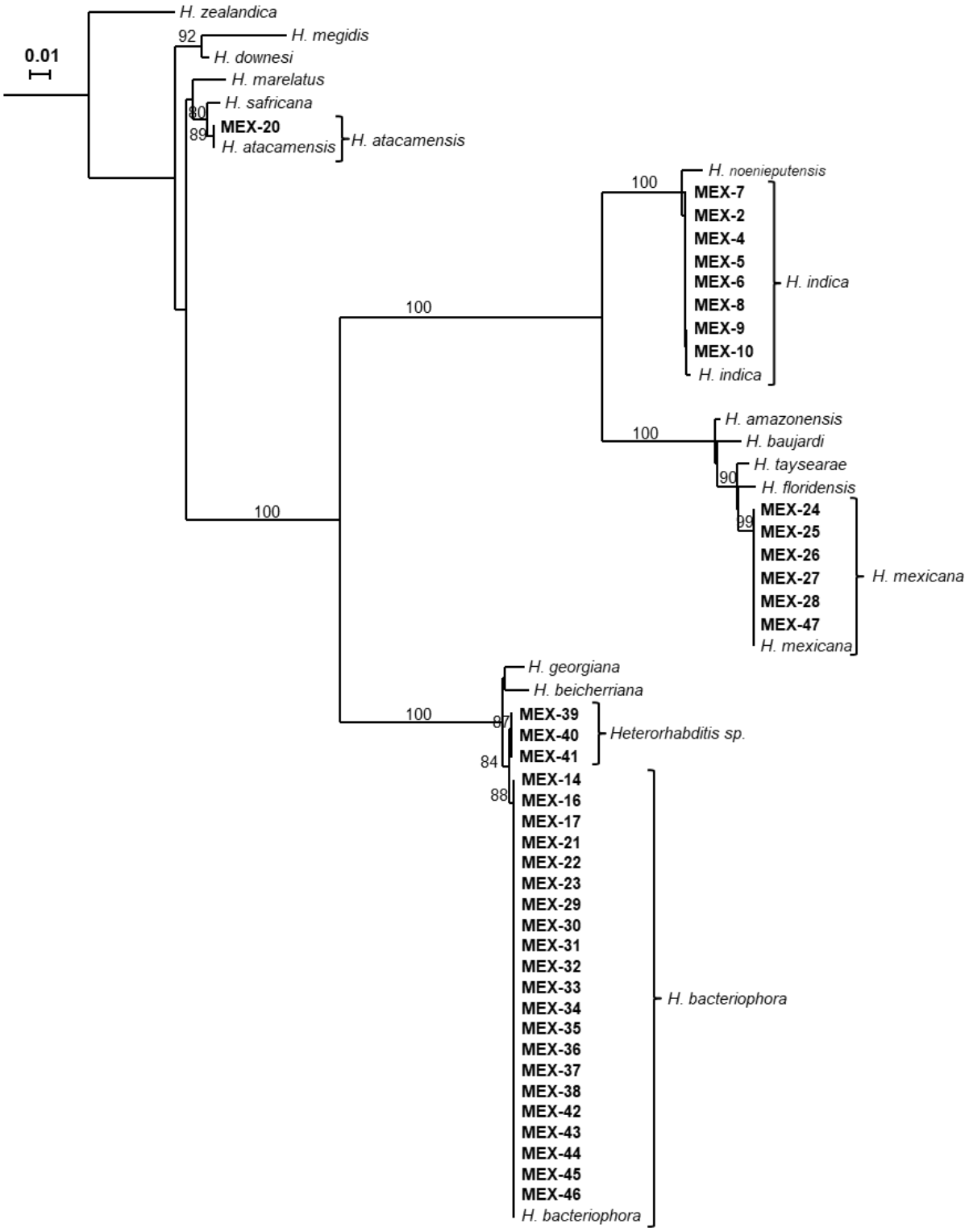

Figure S5. Neighbour-Joining phylogenetic tree of 16 species of *Heterorhabditis* nematodes based on 18S ribosomal RNA gene sequences. The evolutionary distances were computed using the Kimura 2-parameter model. Numbers at nodes represent bootstrap values higher than 70% based on 1000 replications. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). Bar represents 0.01 nucleotide substitutions per sequence position. Accession numbers of the gene sequences used for the reconstructions and those obtained in this study are available in Table S1 and S2, respectively.

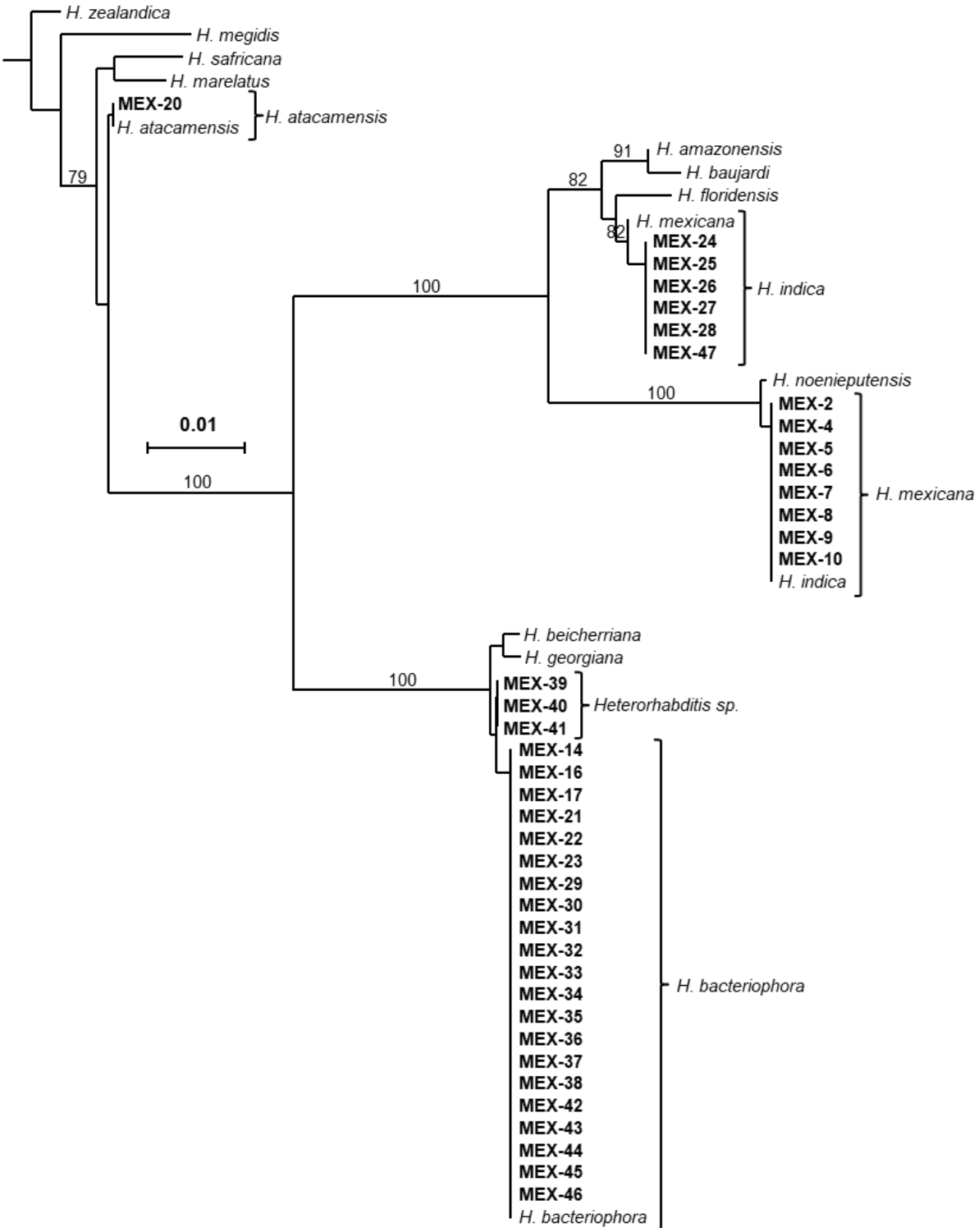

Figure S6. Neighbour-Joining phylogenetic tree of 14 species of *Heterorhabditis* nematodes based on D2/D3 ribosomal RNA gene sequences. The species *H. downesi* and *H. taysearae* could not be included since there are no available sequences in the GenBank. The evolutionary distances were computed using the Kimura 2-parameter model. Numbers at nodes represent bootstrap values higher than 70% based on 1000 replications. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). Bar represents 0.01 nucleotide

substitutions per sequence position. Accession numbers of the gene sequences used for the reconstruction and those obtained in this study are available in Table S1 and S2, respectively.

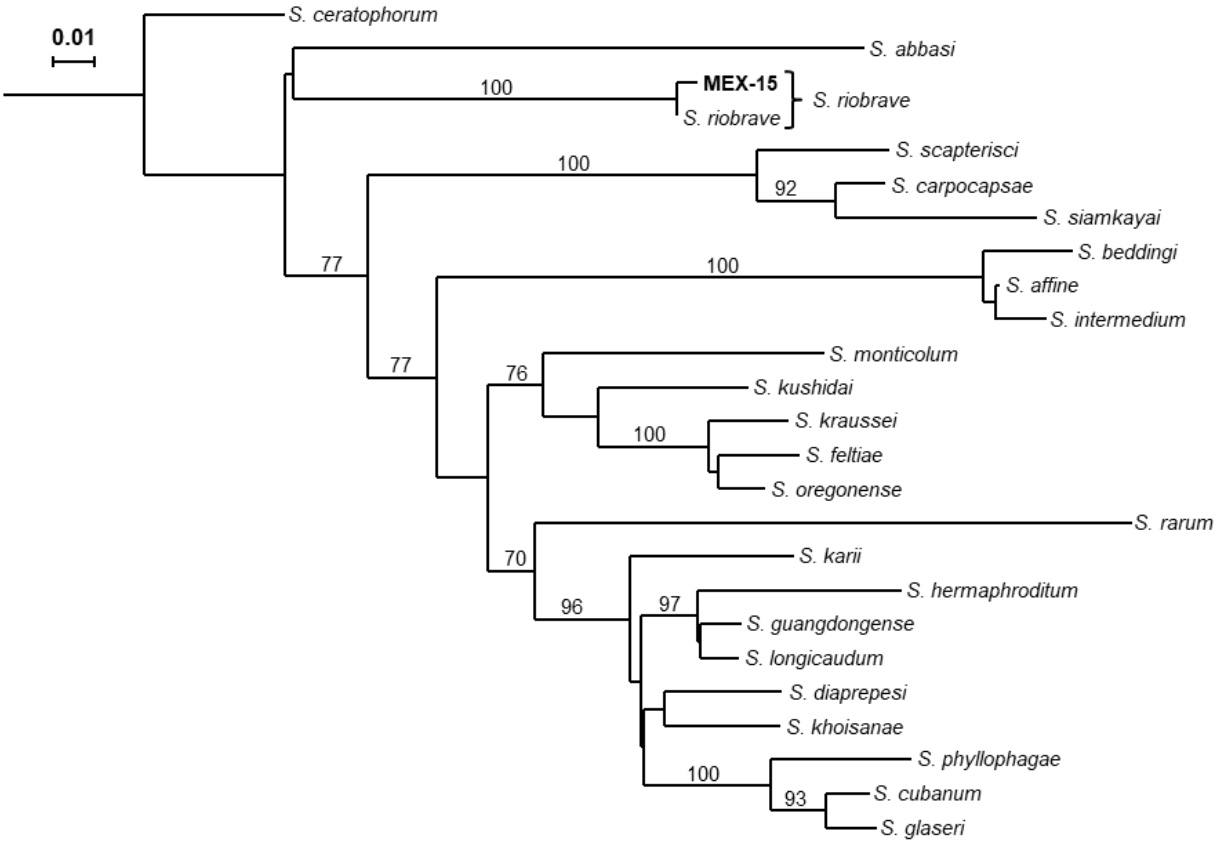

Figure S7. Neighbour-Joining phylogenetic tree of 24 species of *Steinernema* nematodes based on 18S ribosomal RNA gene sequences. The evolutionary distances were computed using the Kimura 2-parameter model. Numbers at nodes represent bootstrap values higher than 70% based on 1000 replications. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). Bar represents 0.01 nucleotide substitutions per sequence position. Accession numbers of the gene sequences used for the reconstruction and those obtained in this study are available in Table S1 and S2, respectively.

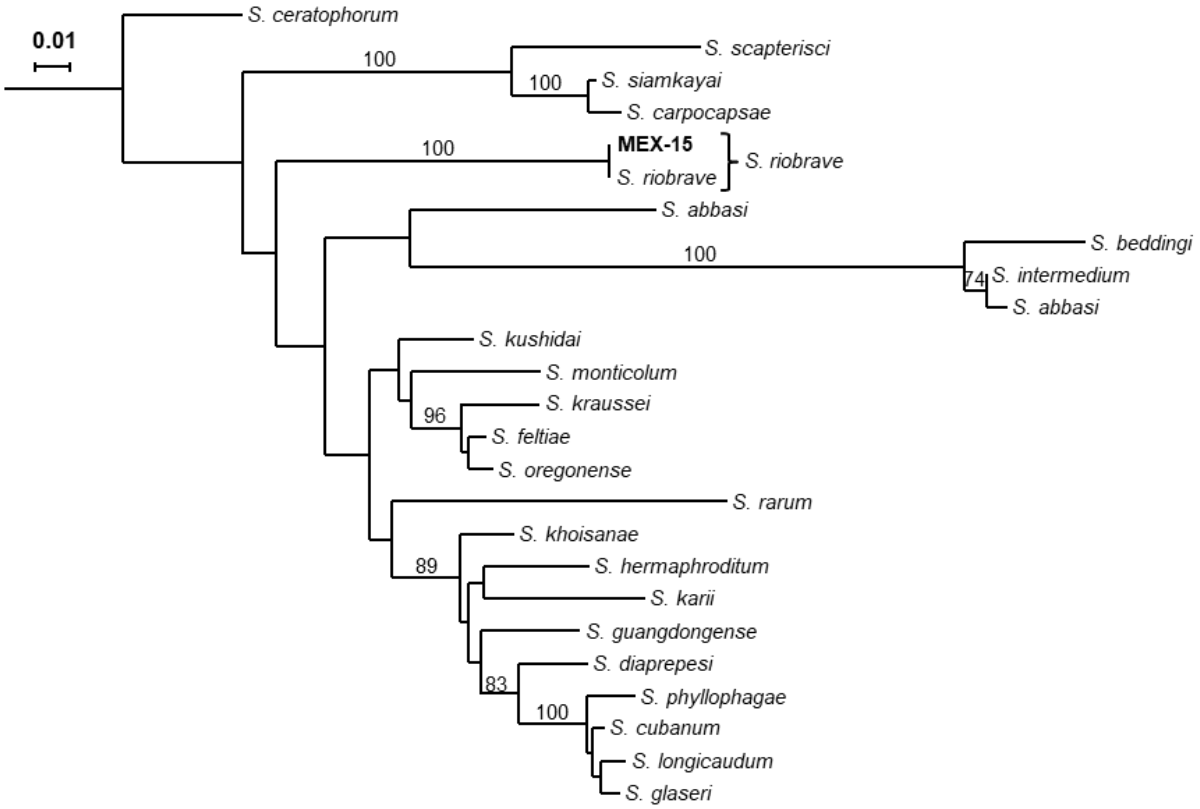

Figure S8. Neighbour-Joining phylogenetic trees of 24 species of *Steinernema* nematodes based on D2/D3 ribosomal RNA gene sequences. The evolutionary distances were computed using the Kimura 2-parameter model. Numbers at nodes represent bootstrap values higher than 70% based on 1000 replications. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). Bar represents 0.01 nucleotide substitutions per sequence position. Accession numbers of the gene sequences used for the reconstruction and those obtained in this study are available in Table S1 and S2, respectively.

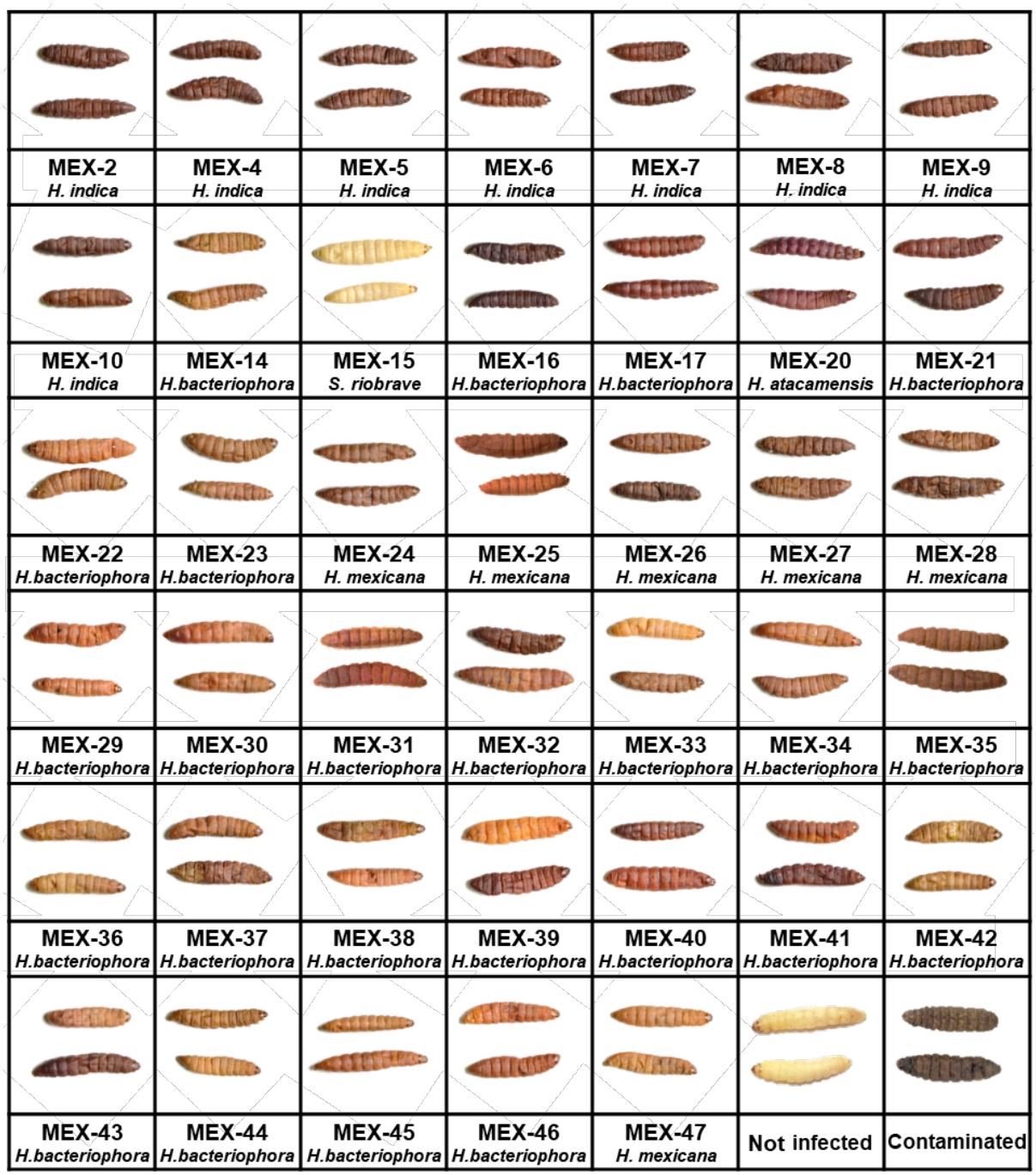

Figure S9. Coloration pattern of *Galleria mellonella* larvae infected with Mexican EPN. These colors were used as a reference for the evaluation of the infectivity tests.

Table S1. Accession numbers of the sequences used for the phylogenetic reconstructions in this study.

Reference species	Accession number	
	18s	D2/D3
Heterorhabditis amazonensis	KU870321.1	EU099036.1
Heterorhabditis atacamensis	HM230723.1	HM230724.1
Heterorhabditis bacteriophora	EU716335.2	EU313541.1
Heterorhabditis baujardi	EU363039.1	MF621013.1
Heterorhabditis beicherriana	HQ896630.2	HQ896631.2
Heterorhabditis downesi	EF043442.1	N/A
Heterorhabditis floridensis	DQ372922.1	EU099034.1
Heterorhabditis georgiana	HQ225885.1	EU099033.1
Heterorhabditis indica	MK990223.1	EU100415.1
Heterorhabditis marelatus	AY321479.1	EU100412.1
Heterorhabditis megidis	AY293284.1	EU100413.1
Heterorhabditis mexicana	EF043444.1	EU100414.1
Heterorhabditis noenieputensis	FJ235075.1	JX624110.1
Heterorhabditis safricana	FJ473361.1	EU100416.1
Heterorhabditis taysearae	EF043443.1	N/A
Heterorhabditis zealandica	GU174010.1	EU099035.1
Steinernema abbasi	KF573496.1	MG198916.1
Steinernema affine	KY818705.1	KC287226.2
Steinernema beddingi	AY603397.1	AY603396.1
Steinernema carpocapsae	KC571265.1	KJ950293.1
Steinernema ceratophorum	AY230165.1	AF331888.1
Steinernema cubanum	AY230166	AF331889.1
Steinernema diaprepesi	AF122021.1	DQ849320.1
Steinernema feltiae	AY230169.1	JF728855.1
Steinernema glaseri	AY230171.1	AF331908.1
Steinernema guangdongense	AY170341.1	AY169558.1
Steinernema hermaphroditum	JQ687355.1	JQ687356.1
Steinernema intermedium	AY230172.1	JN808125.1
Steinernema karii	GQ497742.1	AF331902.1
Steinernema khoisanae	DQ314287.1	DQ314289.1
Steinernema kraussei	AY230175.1	JN683826.1
Steinernema kushidai	AB243440.1	AF331897.1
Steinernema longicaudum	AY230177.1	AF331894.1
Steinernema monticolum	AF122017.1	HM778114.1
Steinernema oregonense	AY230180.1	AF331891.1
Steinernema rarum	DQ221117.1	KT378447.1
Steinernema riobrave	GU174001.1	AF331893.1
Steinernema scapterisci	AF122020.1	GU395646.1
Steinernema siamkayai	KX886343.1	KY311815.1
Steinernema phyllophagae	FJ410327.1	FJ666054.1

Table S2. Accession numbers of the sequences obtained in this study and deposited to the GenBank.

Isolate	Proposed classification	Accession number	
		18s	D2/D3
MEX-2	*Heterorhabditis indica*	MK421471	MK421431
MEX-4	*Heterorhabditis indica*	MK421473	MK421433
MEX-5	*Heterorhabditis indica*	MK421474	MK421434
MEX-6	*Heterorhabditis indica*	MK421475	MK421435
MEX-7	*Heterorhabditis indica*	MK421476	MK421436
MEX-8	*Heterorhabditis indica*	MK421477	MK421437
MEX-9	*Heterorhabditis indica*	MK421478	MK421438
MEX-10	*Heterorhabditis indica*	MK421479	MK421439
MEX-14	*Heterorhabditis bacteriophora*	MK421482	MK421440
MEX-15	*Steinernema riobrave*	MK421550	MK421555
MEX-16	*Heterorhabditis bacteriophora*	MK421483	MK421441
MEX-17	*Heterorhabditis bacteriophora*	MK421484	MK421442
MEX-20	*Heterorhabditis atacamensis*	MK421485	MK421443
MEX-21	*Heterorhabditis bacteriophora*	MK421486	MK421444
MEX-22	*Heterorhabditis bacteriophora*	MK421487	MK421445
MEX-23	*Heterorhabditis bacteriophora*	MK421488	MK421446
MEX-24	*Heterorhabditis mexicana*	MK421489	MK421447
MEX-25	*Heterorhabditis mexicana*	MK421490	MK421448
MEX-26	*Heterorhabditis mexicana*	MK421491	MK421449
MEX-27	*Heterorhabditis mexicana*	MK421492	MK421450
MEX-28	*Heterorhabditis mexicana*	MK421493	MK421451
MEX-29	*Heterorhabditis bacteriophora*	MK421494	MK421452
MEX-30	*Heterorhabditis bacteriophora*	MK421495	MK421453
MEX-31	*Heterorhabditis bacteriophora*	MK421496	MK421454
MEX-32	*Heterorhabditis bacteriophora*	MK421497	MK421455
MEX-33	*Heterorhabditis bacteriophora*	MK421498	MK421456
MEX-34	*Heterorhabditis bacteriophora*	MK421499	MK421457
MEX-35	*Heterorhabditis bacteriophora*	MK421500	MK421458
MEX-36	*Heterorhabditis bacteriophora*	MK421501	MK421459
MEX-37	*Heterorhabditis bacteriophora*	MK421502	MK421460
MEX-38	*Heterorhabditis bacteriophora*	MK421503	MK421461
MEX-39	*Heterorhabditis bacteriophora*	MK421504	MK421462
MEX-40	*Heterorhabditis bacteriophora*	MK421505	MK421463
MEX-41	*Heterorhabditis bacteriophora*	MK421506	MK421464
MEX-42	*Heterorhabditis bacteriophora*	MK421507	MK421465
MEX-43	*Heterorhabditis bacteriophora*	MK421508	MK421466
MEX-44	*Heterorhabditis bacteriophora*	MK421509	MK421467
MEX-45	*Heterorhabditis bacteriophora*	MK421510	MK421468
MEX-46	*Heterorhabditis bacteriophora*	MK421511	MK421469
MEX-47	*Heterorhabditis mexicana*	MK421512	MK421470

Table S3 ANOVA results for the comparisons of benzoxazinoids present in WCR and BCB larvae fed on hybrid maize DFI 45321 (*n*=5). Stars indicate significant differences between treatments: *p<0.05, **p<0.01, ***p<0.001.

Benzoxazinoids	WCR larvae fed on hybrid maize DFI 45321		BCB larvae fed on hybrid maize DFI 45321		F	p-value	Signif. codes
	Mean (µg/g FW)	±SE	Mean (µg/g FW)	±SE			
MBOA	2.75	0.74	0.12	0.03	12.591	7.53E-03	**
MBOA-Glc	11.04	0.77	1.56	0.59	95.521	1.01E-05	***
HMBOA-Glc	27.33	3.99	1.87	0.57	40.000	2.27E-04	***
HDMBOA-Glc	476.53	58.07	1.03	0.63	67.044	3.69E-05	***
DIMBOA-Glc	7.09	2.32	0.34	0.09	8.430	1.98E-02	*
TOTAL	**524.73**	**65.89**	**4.92**	**1.90**	**65.585**	4.00E-05	***

Table S4 ANOVA results for the comparisons of benzoxazinoids present in maize seedlings (*n*=5). Stars indicate significant differences between treatments: *p<0.05, **p<0.01, ***p<0.001.

Benzoxazinoids	WT B73		Mutant *bx1*		F	p-value	Signif. codes
	Mean (µg/g FW)	±SE	Mean (µg/g FW)	±SE			
MBOA	15.11	2.09	6.46	0.59	15.871	4.04E-03	**
MBOA-Glc	1.14	0.20	0.40	0.01	13.165	6.70E-03	**
HMBOA-Glc	29.13	7.74	3.69	0.54	10.747	1.12E-02	*
HDMBOA-Glc	159.84	43.78	9.04	3.08	11.806	8.88E-03	**
DIMBOA-Glc	380.74	73.41	16.72	2.17	24.570	1.11E-03	**
TOTAL	**585.96**	**127.22**	**36.31**	**6.40**	**19.922**	**2.10E-03**	**

Table S5 ANOVA results for the comparisons of benzoxazinoids present in benzoxazinoid-containing (*n*=4) and benzoxazinoid-free (*n*=5) WCR larvae. Stars indicate significant differences between treatments: *p<0.05, **p<0.01, ***p<0.001.

Benzoxazinoids	Benzoxazinoid-containing WCR larvae		Benzoxazinoid-free WCR larvae		F	p-value	Signif. codes
	Mean (µg/g FW)	±SE	Mean (µg/g FW)	±SE			
MBOA	10.88	3.27	9.69E-04	3.58E-04	14.313	6.86E-03	**
MBOA-Glc	50.96	9.54	1.14E-02	1.19E-03	36.997	5.00E-04	***
HMBOA-Glc	70.70	16.85	1.65E-02	4.31E-03	22.804	2.02E-03	**
HDMBOA-Glc	459.11	76.98	2.75E-01	4.40E-02	46.054	2.56E-04	***
DIMBOA-Glc	20.00	8.14	2.44E-03	1.61E-03	7.820	2.67E-02	*
TOTAL	**611.64**	**114.78**	**0.306**	**0.051**	**45.328**	**2.69E-04**	***

Table S6 ANOVA results for the comparisons of Mexican EPN infection on WCR and BCB larvae seven days after inoculation. Stars indicate significant differences between treatments for each EPN isolate: *p<0.05, **p<0.01, ***p<0.001.

EPN isolate	Species	WCR larvae			BCB larvae			Test	p-value	Signif. code
		n	Mean (µg/g FW)	±SE	*n*	Mean (µg/g FW)	±SE			
MEX-2	*H. indica*	23	91.30	3.37	20	98.75	1.25	Chisq= 31.320	0.01896	*
MEX-4	*H. indica*	22	94.32	2.82	20	98.75	1.25	Chisq= 25.244	0.0963	n.s.
MEX-5	*H. indica*	23	82.25	6.40	10	100.00	0.00	F= 7.316	0.011	*
MEX-6	*H. indica*	23	88.41	4.74	20	100.00	0.00	Chisq= 42.333	0.001389	**
MEX-7	*H. indica*	23	88.04	4.25	20	98.75	1.25	Chisq= 36.581	0.004506	**
MEX-8	*H. indica*	25	97.00	2.20	26	99.04	0.96	Chisq= 23.308	0.2572	n.s.
MEX-9	*H. indica*	23	90.94	3.81	20	100.00	0.00	Chisq= 27.865	0.01235	**
MEX-10	*H. indica*	25	91.00	2.84	26	99.04	0.96	Chisq= 29.283	0.00358	**
MEX-14	*H. bacteriophora*	35	46.43	6.20	20	92.50	3.19	F= 29.879	1.261E-06	***
MEX-15	*S. riobrave*	23	8.70	4.04	20	100.00	0.00	Chisq= 35.799	<2.2E-16	***
MEX-16	*H. bacteriophora*	37	85.59	4.30	26	98.08	1.33	F= 7.4	0.008486	**
MEX-17	*H. bacteriophora*	20	90.00	3.80	21	92.86	3.51	F= 0.282	0.5985	n.s.
MEX-20	*H. atacamensis*	38	94.52	1.99	26	89.42	4.20	F= 1.471	0.2297	n.s.
MEX-21	*H. bacteriophora*	25	80.33	4.22	16	100.00	0.00	Chisq= 31.225	4.68E-06	***
MEX-22	*H. bacteriophora*	37	38.96	6.00	26	93.27	2.96	F= 46.469	4.779E-09	***
MEX-23	*H. bacteriophora*	37	43.02	6.17	26	92.31	3.03	F= 36.599	9.642E-08	***
MEX-24	*H. mexicana*	37	53.83	5.84	26	97.12	2.12	F= 38.689	4.972E-08	***
MEX-25	*H. mexicana*	27	38.89	6.58	20	99.00	1.00	F= 60.065	7.982E-10	***
MEX-26	*H. mexicana*	37	42.57	4.83	26	92.63	3.90	F= 44.044	9.724E-09	***
MEX-27	*H. mexicana*	37	59.01	5.22	26	87.50	5.59	F= 11.606	0.001169	**
MEX-28	*H. mexicana*	37	44.14	4.66	26	100.00	0.00	Chisq= 57.004	<2.2E-16	***
MEX-29	*H. bacteriophora*	37	35.59	5.98	26	95.83	2.41	F= 54.740	4.775E-10	***
MEX-30	*H. bacteriophora*	35	46.19	6.07	20	96.25	2.05	F= 40.817	4.331E-08	***
MEX-31	*H. bacteriophora*	27	79.01	5.54	20	98.75	1.25	F= 12.468	0.0009686	***
MEX-32	*H. bacteriophora*	20	88.75	3.84	22	88.26	4.92	F= 0.002	0.9693	n.s.
MEX-33	*H. bacteriophora*	37	46.62	6.58	26	99.04	0.96	F= 60.083	1.179E-10	***
MEX-34	*H. bacteriophora*	37	44.23	6.50	26	91.99	3.41	F= 31.128	5.877E-07	***
MEX-35	*H. bacteriophora*	35	36.67	6.58	20	68.75	7.67	F= 9.543	0.003193	**
MEX-36	*H. bacteriophora*	35	49.52	7.04	20	86.58	5.62	F= 12.850	0.0007348	***
MEX-37	*H. bacteriophora*	23	36.23	7.33	10	69.17	12.24	F= 6.063	0.01956	*
MEX-38	*H. bacteriophora*	20	70.00	5.90	22	87.50	3.58	F= 7.225	0.01043	*
MEX-39	*H. bacteriophora*	37	77.70	4.96	26	87.50	4.65	F= 1.558	0.2167	n.s.
MEX-40	*H. bacteriophora*	34	72.30	5.70	20	95.00	2.29	F= 11.209	0.00152	**
MEX-41	*H. bacteriophora*	36	85.88	3.94	26	95.19	1.97	Chisq= 65.364	0.5557	n.s.
MEX-42	*H. bacteriophora*	35	51.19	6.04	20	97.50	1.72	F= 40.419	4.86E-08	***
MEX-43	*H. bacteriophora*	35	65.71	6.28	20	80.83	8.11	F= 2.2622	0.1385	n.s.
MEX-44	*H. bacteriophora*	35	46.67	7.09	20	87.08	4.34	F= 17.010	0.0001317	***
MEX-45	*H. bacteriophora*	35	50.71	6.36	20	75.83	7.81	F= 6.307	0.01511	*
MEX-46	*H. bacteriophora*	37	39.77	6.13	26	86.86	4.36	F= 27.438	2.126E-06	***
MEX-47	*H. mexicana*	34	65.93	4.98	16	95.31	4.69	F= 12.050	0.001106	**

Table S7 ANOVA results of *H. bacteriophora* isolates infection on benzoxazinoid-containing and benzoxazinoid-free WCR larvae seven days after inoculation. Stars indicate significant differences between treatments for each EPN isolate: *p<0.05, **p<0.01, ***p<0.001.

EPN isolate	Benzoxazinoid-containing WCR larvae			Benzoxazinoid-free WCR larvae			Test	p-value	Signif. code
	n	Mean (µg/g FW)	±SE	*n*	Mean (µg/g FW)	±SE			
MEX-14	10	87.50	6.72	15	93.33	4.54	Chisq= 24.166	0.212	n.s.
MEX-16	10	97.50	2.50	15	100.00	0.00	Chisq= 4.696	0.163	n.s.
MEX-17	10	97.50	2.50	15	100.00	0.00	Chisq= 4.854	0.181	n.s.
MEX-21	10	97.50	2.50	15	100.00	0.00	Chisq= 4.854	0.174	n.s.
MEX-22	10	90.00	4.08	15	91.11	4.18	Chisq= 22.895	0.796	n.s.
MEX-23	10	77.50	5.83	15	93.33	2.95	Chisq= 20.466	0.022	*
MEX-29	10	70.00	5.85	15	85.56	4.41	Chisq= 22.005	0.108	n.s.
MEX-30	10	83.33	6.80	15	94.44	3.01	Chisq= 21.804	0.100	n.s.
MEX-31	10	100.00	0.00	15	95.00	3.62	Chisq= 13.675	0.078	n.s.
MEX-32	10	97.50	2.50	15	98.21	1.79	Chisq= 10.337	0.796	n.s.
MEX-33	10	69.17	10.40	15	84.44	4.87	F= 2.012	0.169	n.s.
MEX-34	10	90.00	4.08	15	98.33	1.67	Chisq= 13.403	0.062	n.s.
MEX-35	10	92.50	3.82	15	89.29	5.05	F= 0.266	0.611	n.s.
MEX-36	10	67.50	10.57	15	86.11	6.12	F= 3.014	0.096	n.s.
MEX-37	10	65.00	7.64	15	89.88	5.81	F= 7.715	0.011	*
MEX-38	10	91.67	4.30	15	87.78	4.29	Chisq= 22.631	0.410	n.s.
MEX-39	10	95.00	5.00	15	100.00	0.00	Chisq= 6.422	0.163	n.s.
MEX-40	10	92.50	3.82	15	100.00	0.00	Chisq= 7.815	0.025	*
MEX-41	10	100.00	0.00	15	95.00	3.62	Chisq= 10.128	0.145	n.s.
MEX-42	10	100.00	0.00	15	93.33	3.83	Chisq= 14.849	0.047	*
MEX-43	10	86.67	4.51	15	90.56	3.62	Chisq= 18.781	0.559	n.s.
MEX-44	10	87.50	6.72	15	95.00	2.67	Chisq= 22.547	0.367	n.s.
MEX-45	10	92.50	5.34	15	85.56	5.04	F= 0.521	0.478	n.s.
MEX-46	10	80.00	6.24	15	86.11	5.73	F= 1.224	0.280	n.s.

2.1. Description and characterization of *Photorhabdus khanii* subsp. *guanajuatensis* subsp. nov. isolated from *Heterorhabditis atacamensis* and *Photorhabdus luminescens* subsp. *mexicana* subsp. nov. isolated from *Heterorhabditis mexicana* entomopathogenic nematodes

Ricardo AR Machado[1]*, Pamela Bruno[2], Carla CM Arce[2], Nicole Liechti[3], Angela Köhler[2,4], Julio Bernal[5], *Rémy* Bruggmann[2], Ted CJ Turlings[2]

1. Institute of Plant Sciences, University of Bern, Bern, Switzerland.

2. Laboratory of Fundamental and Applied Research in Chemical Ecology, University of Neuchâtel, Neuchâtel, Switzerland.

3. Interfaculty Bioinformatics Unit, University of Bern, Bern, Switzerland.

4. Leibniz-Institut für Naturstoff-Forschung und Infektionsbiologie, Jena, Germany.

5. Department of Entomology, Texas A&M University, College Station, Texas, United States of America.

*Corresponding author:

Ricardo AR Machado (ricardo.machado@ips.unibe.ch, Institute of Plant Sciences, Altenbergrain 21. Postal Code: 3013. University of Bern, Bern, Switzerland. +41 (0) 31 631 8814.

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Abstract

Two gram-negative, rod-shaped, non-spore-forming bacteria, MEX20-17^T and MEX47-22^T, were isolated from the digestive system of *Heterorhabditis atacamensis* and *H. mexicana* entomopathogenic nematodes, respectively. 16S rRNA-gene sequences suggest that strains MEX20-17^T and MEX47-22^T belong to the γ-Proteobacteria and to the *Photorhabdus* genus. Deeper analyses using housekeeping gene-based and whole genome-based phylogenetic reconstruction suggest that MEX20-17^T is closely related to *P. khanii* and that MEX47-22^T is closely related to *P. luminescens*. Sequence similarity scores confirm these observations: MEX20-17^T and *P. khanii* DSM 3369^T share 98.9% nucleotide sequence identity of concatenated housekeeping genes (NSI), 70.4% *in silico* DNA-DNA hybridization (*is*DDH) and 97% orthologous average nucleotide identity (orthoANI); and MEX47-22^T and *P. luminescens* ATCC 29999^T share 98.9% NSI, 70.6% *is*DDH and 97% orthoANI. Physiological characterization indicates that both strains differ from all validly described *Photorhabdus* species and from their more closely related taxa. We therefore propose to classify MEX20-17^T and MEXT47-22^T as new subspecies within *P. khanii* and *P. luminescens*, respectively. Hence, the following names are proposed for these strains: *Photorhabdus khanii* subsp*. guanajuatensis* subsp. nov. with the type strain MEX20-17^T (=LMG 30372^T =CCOS 1191^T) and *Photorhabdus luminescens* subsp. *mexicana* subsp. nov. with the type strain MEX47-22^T (=LMG 30528^T =CCOS 1199^T). These propositions automatically create *Photorhabdus khanii* subsp. *khanii* subsp. nov. with DSM 3369^T as the type strain (currently classified as *P. khanii*), and *Photorhabdus luminescens* subsp. *luminescens* subsp. nov. with ATCC 29999^T as the type strain (currently classified as *P. luminescens*). Accession numbers of whole genome sequences were deposited in the NCBI (MEX20-17^T: PUJY00000000; MEX47-22^T PUJX00000000).

Keywords

Photorhabdus spp. – *Heterorhabditis* spp. – whole genome sequencing – *is*DDH – polyphasic classification.

Main body

Heterorhabditis entomopathogenic nematodes are soil-inhabiting organisms that parasitize and reproduce inside small arthropods (Poinar & Veremchuk, 1970, Khan et al., 1976). They enter their preys by penetrating through the cuticle or natural openings such as the mouth, spiracles, or the anus, and crawl towards the haemocoel where they regurgitate their *Photorhabdus* symbiotic bacterial partners upon the perception of unknown chemical cues (Kaya & Gaugler, 1993). *Photorhabdus* cells multiply within the haemocoel, cause septicemia and eventually kill the colonized organisms (Lacey & Georgis, 2012). The genus *Photorhabdus* was described by Boemare *et al*. in 1993 and includes symbiotic bacteria of *Heterorhabditis* entomopathogenic nematodes (Boemare et al., 1993). Since then, several species and subspecies of *Photorhabdus* that are associated with *Heterorhabditis* nematodes have been described (Boemare et

al., 1993, Akhurst et al., 2004, An & Grewal, 2011, An & Grewal, 2010, Ferreira et al., 2013, Ferreira et al., 2014, Fischer-Le Saux et al., 1999, Glaeser et al., 2017, Hazir et al., 2004, Orozco et al., 2013, Szallas et al., 1997, Tailliez et al., 2010, Thomas & Poinar, 1979, Tóth & Lakatos, 2008, Machado et al., 2018). However, the taxonomic identity of the symbiotic bacterial partners of *Heterorhabditis atacamensis* and *H. mexicana* nematodes have not yet been reported in the current literature (Maneesakorn et al., 2020).

Usually, 16S rRNA and housekeeping gene sequences are used to reconstruct the phylogeny of the *Photorhabdus* genus Glaeser et al., 2017, Glaeser & Kämpfer, 2015). Recently, however, these methods were shown to provide insufficient information to resolve the phylogenetic relationships of this bacterial group, particularly of very closely related species (Machado et al., 2018). To fully meet the guidelines of the *ad hoc* Committee on Reconciliation of Approaches to Bacterial Systematics (Wayne et al., 1987) and based on *in silico* DNA-DNA hybridization isDDH scores as the gold standard for bacterial species circumscription, the reclassification of the several species/subspecies of the *Photorhabdus* genus was recently proposed (Machado et al., 2018). Hence, the *Photorhabdus* genus currently contains the following 19 species with validly published names: *P. akhurstii, P. asymbiotica, P. australis, P. bodei*, *P. caribbeanensis*, *P. cinerea*, *P. hainanensis*, *P. heterorhabditis*, *P. kayaii*, *P. khanii*, *P. kleinii*, *P. laumondii*, *P. luminescens*, *P. namnaonensis*, *P. noenieputensis*, *P. stackebrandtii*, *P. tasmaniensis, P. temperata* and *P. thracensis*. *Photorhabdus laumondii* is further divided into two subspecies: *P. laumondii* subsp. *laumondii* and *P. laumondii* subsp. *clarkei* (Machado et al., 2018).

During the characterization of microorganisms isolated from the intestines of entomopathogenic nematodes that were recovered from Mexican soil samples, two bacterial strains were isolated on Luria-Bertani agar plates at 28°C: MEX20-17^T isolated from *Heterorhabditis atacamensis,* and MEX47-22^T isolated from *H. mexicana* (Table S1).

Entomopathogenic nematodes were isolated by the 'Galleria baiting' method (Bedding & Akhurst, 1975). Briefly, *Galleria mellonella* larvae (Lepidoptera: Pyralidae) were exposed to 100 nematode infective juveniles. Three to four days later, insect cadavers were surface-sterilized and cut open with a blade. Bacteria-digested internal organs were spread onto Luria–Bertani (LB) agar plates (Sigma-Aldrich, Switzerland) and incubated at 28°C for 24–48 h. *Photorhabdus*-like colonies were subcultured until monocultures were obtained. A single primary form colony was then selected and used for further experiments. Bacteria primary forms were determined by examining colony characteristics on NBTA plates (LB agar plates supplemented with 25 mg ml^{-1} bromothymol blue and 4 mg ml^{-1} triphenyl-2,3,5-tetrazolium chloride). The strains were further subcultured and maintained on Luria-Bertani (LB) agar plates at 28°C. To test for taxonomic diversity of the symbiotic bacteria harbored by the nematode populations, we amplified 16S rRNA bacterial genes from DNA extracted individually

from twenty nematode specimens by polymerase chain reaction (PCR). Bioluminescence was determined from liquid cultures using a TriStar LB 942 Multimode Microplate Reader (Berthold Technologies, Switzerland). The analysis of cellular fatty acids was performed by using the Sherlock MIS (MIDI Inc, Newark, USA) system as described (Miller, 1982, Kuykendall et al., 1988, Kämpfer & Kroppenstedt, 1996). The above-mentioned tests were carriedout only for MEX20-17^T and MEX47-22^T. API 20E strips were used to carry additional physiological tests according to manufacturer's instructions (bioMérieux, Inc. Durham, NC). DNA was extracted by heat shock. PCR conditions were set as described (Marchesi et al., 1998). Cell morphology was observed under a Zeiss light microscope at a magnification of 1000X, with cells grown for 5 days at 28°C on LB agar plates. Motility was tested on soft agar as described (Tremblay & Déziel, 2008). Cytochrome oxidase production was tested on discs containing *N,N*-dimethyl-p-phenylenediamine oxalate and α-naphthol according to manufacturer's conditions (Sigma-Aldrich, Switzerland). Catalase activity was determined by adding a drop of 10% (v/v) H_2O_2 into 50 µl of a liquid LB-grown, 24-h-old bacterial culture. The ability of bacterial strains to absorb dye was tested by growing the cells on NBTA agar containing bromothymol blue and triphenyl-2,3,5-tetrazolium chloride (Sigma- Aldrich, Switzerland) (Akhurst et al., 1996).

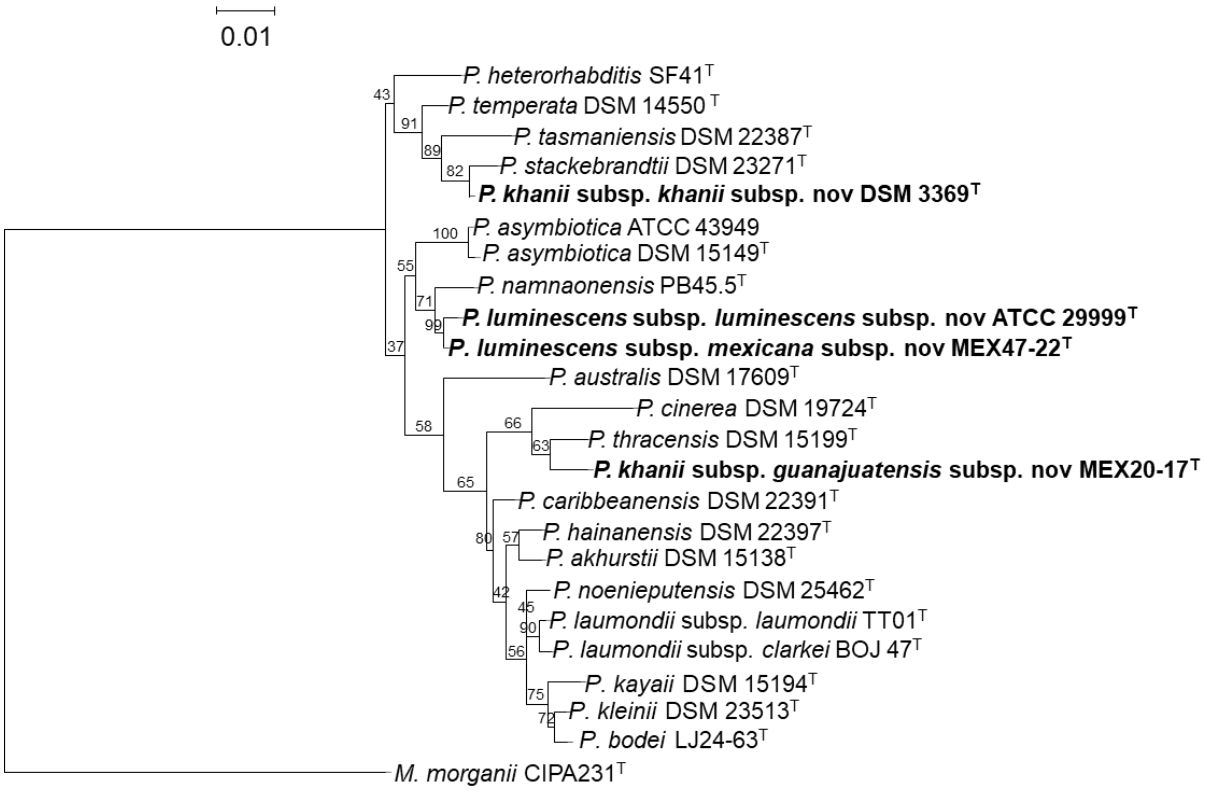

Fig. 1. Maximum-likelihood phylogenetic tree of *Photorhabdus* bacterial strains reconstructed from 1354 nucleotide positions of 16S ribosomal RNA gene sequences. Numbers at nodes represent bootstrap values based on 100 replications. Bar represents 0.01 nucleotide substitutions per sequence position. Accession numbers of gene sequences used for the reconstruction are shown in Table S2.

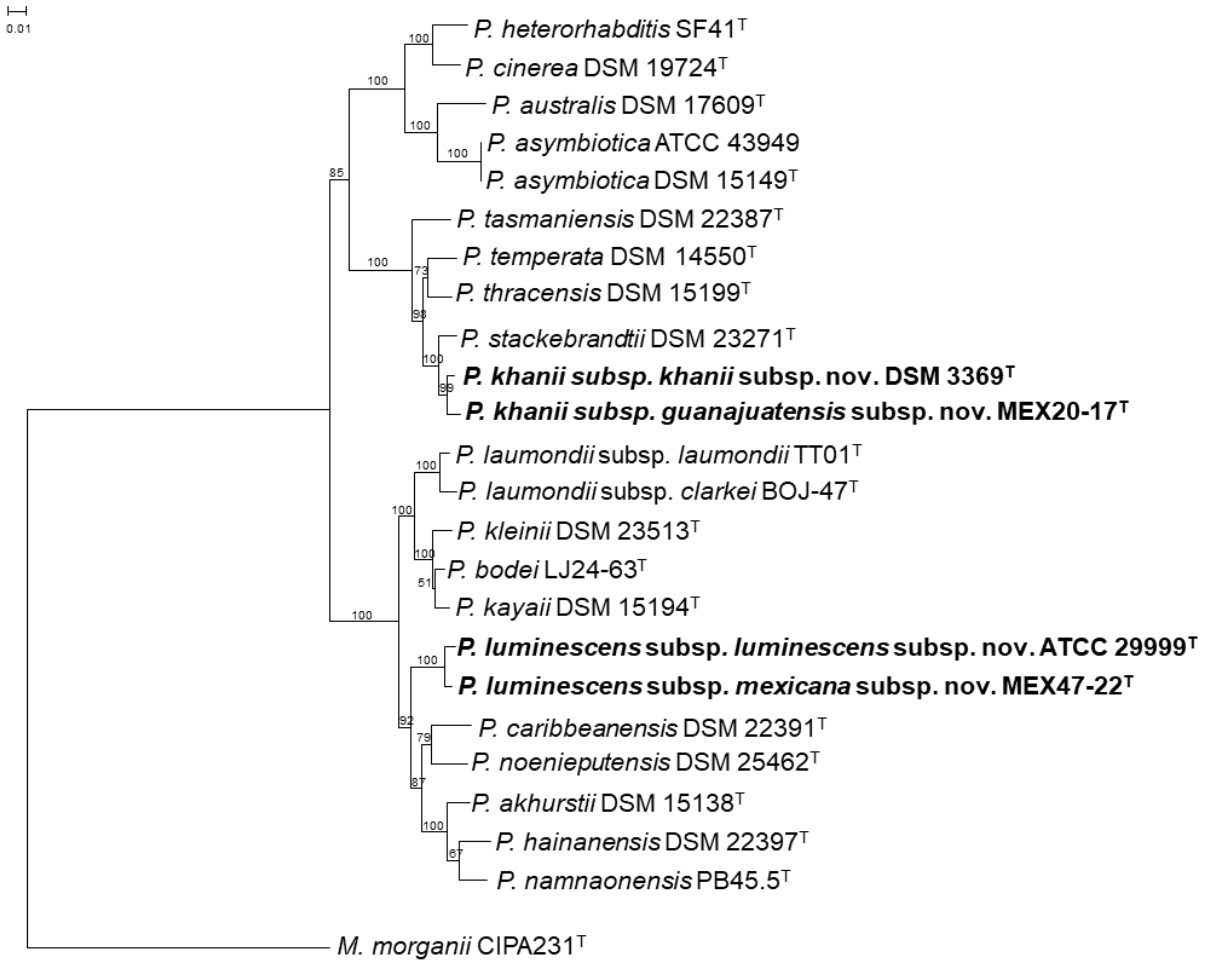

Fig. 2. Maximum-likelihood phylogenetic tree of *Photorhabdus* bacterial strains reconstructed from concatenated nucleotide sequences of five protein-coding genes (*dnaN*, *gltX*, *gyrB*, *infB* and *recA*). For the analysis, 4317 nucleotide positions were considered. Numbers at nodes represent bootstrap based on 100 replications. Bar represents 0.01 nucleotide substitutions per sequence position. Accession numbers of gene sequences used for the reconstruction are shown in Table S2.

In this case, strains MEX20-17^T and MEX47-22^T were tested in parallel and the experiments were conducted two independent times with the type strains of all *Photorhabdus* taxa with validly published names: *P. akhurstii* DSM 15138^T, *P. asymbiotica* DSM 15149^T, *P. australis* DSM 17609^T, *P. bodei* LJ24-63^T, *P. caribbeanensis* DSM 22391^T, *P. cinerea* DSM 19724^T, *P. hainanensis* DSM 22397^T, *P. heterorhabditis* SF41^T, *P. kayaii* DSM 15194^T, *P. khanii* DSM 3369^T, *P. kleinii* DSM 23513^T, *P. laumondii* subsp. *clarkei* BOJ-47^T, *P. laumondii* subsp. *laumondii* TT01^T, *P. luminescens* subsp. *luminescens* ATCC 29999^T, *P. namnaonensis* PB45.5^T, *P. noenieputensis* DSM 25462^T, *P. stackebrandtii* DSM 23271^T, *P. tasmaniensis* DSM 22387^T, *P. temperata* DSM 14550^T, *P. thracensis* DSM 15199^T. Bacterial genomic DNA was extracted using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, Switzerland) following manufacturer's instructions.To obtain genomic sequences, the TruSeq DNA PCR-Free LT Library Prep (FC-121-3003) was used for library

preparation. Indexed libraries were then pooled at equimolar concentrations and sequenced (2 x 150 bp) on an Illumina HiSeq 3000 instrument. The raw Illumina reads were quality trimmed using Trimmomatic 0.36 (Options: SLIDINGWINDOW:4:8 MINLEN:127) (Bolger et al., 2014). The resulting reads were assembled with SPAdes 3.10.1 (*k*-mer sizes of 21, 33, 55, 77, 99, 127 bp) (Bankevich et al., 2012). Scaffolds with a mean read-depth smaller than 20% of the median read-depth of the longer scaffolds (≥ 5,000 bp) as well as scaffolds that were shorter than 200 bp were removed. The final assemblies were polished using Pilon 1.22 (Walker et al., 2014). Genome sequences were deposited in the National Center for Biotechnology Information. Accession numbers are listed in Table S2. Genome sequence data are given in Table S3. Whole genome-based phylogenetic relationships were reconstructed using Roary 3.6.2 from the core genomes of eight related species whose genome sequences were obtained in this study (*P. tasmaniensis* DSM 22387^T, *P. stackebrandtii* DSM 23271^T, *P. cinerea* DSM 19724^T, *P. akhurstii* DSM 15138^T, *P. caribbeanensis* HG29^T, *P. hainanensis* DSM 22397^T, *P. noenieputensis* DSM 25462^T, and *P. heterorhabditis* SF41^T) and from the core genomes of several *Photorhabdus*

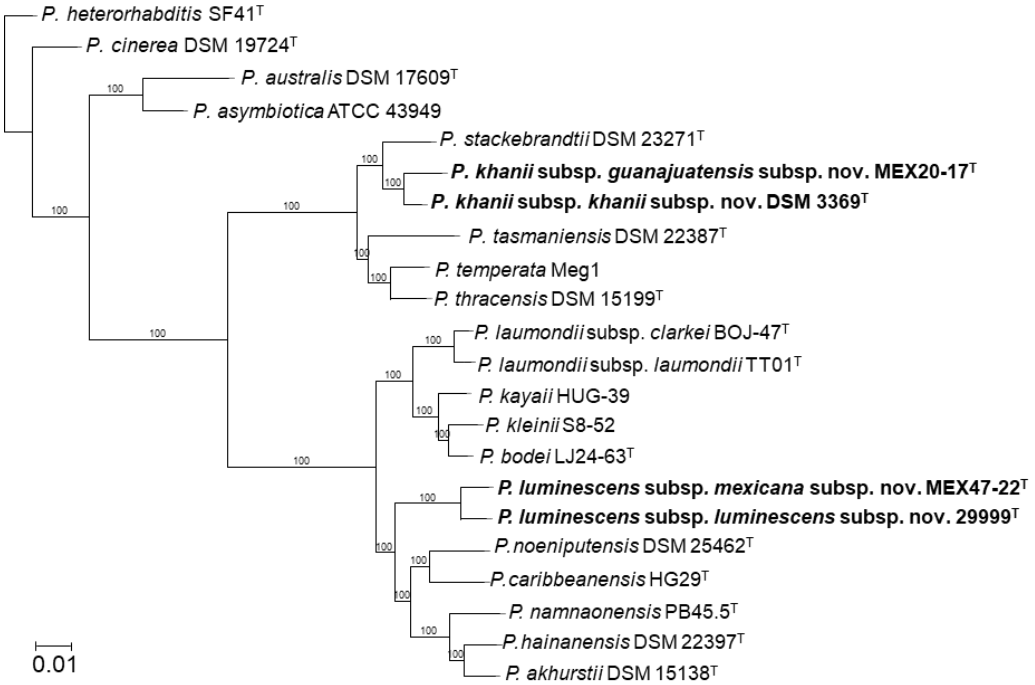

Fig. 3. Phylogenetic reconstruction based on core genome sequences of *Photorhabdus* bacterial strains. 1662 open reading frames were analyzed. Numbers at the nodes represent SH-like branch supports. Bar represents 0.01 nucleotide substitutions per sequence position. Accession numbers of the genome sequences used for the reconstruction are shown in Table S2.

	P. heterorhabditis SF41^T	*P. cinerea* DSM 19724^T	*P. australis* DSM 17609^T	*P. asymbiotica* ATCC 43949	*P. stackebrandtii* DSM 23271^T	***P. khanii* subsp. *guanajuatensis* MEX20-17^T**	***P. khanii subsp. khanii* DSM 3369^T**	*P. tasmaniensis* DSM 22387^T	*P. temperata* Meg1	*P. thracensis* DSM 15199^T	*P. laumondii* subsp. *clarkei* BOJ-47^T	*P. laumondii subsp. laumondii* TT01^T	*P. kayaii* HUG-39	*P. kleinii* S8-52	*P. bodei* LJ24-63^T	***P. luminescens* subsp. *mexicana* MEX47-22^T**	***P. luminescens subsp. luminescens* ATCC 29999^T**	*P. noenieputensis* DSM 25462^T	*P. caribbeanensis* HG29^T	*P. namnaonensis* PB45.5^T	*P. hainanensis* DSM 22397^T	*P. akhurstii* DSM 15138^T
P. heterorhabditis SF41^T	ID	57.9	44.2	42.4	32.4	31.9	31.8	32	32.4	33	31.7	32.8	32.2	31.7	32.4	30.4	30.8	31.8	30.7	30.7	31.1	30.8
P. cinerea DSM 19724^T	57.9	ID	43.5	42.4	32.5	31.7	32	31.8	33.3	32.7	31.3	31.8	31.7	31.3	32.3	30.1	30.4	30.6	30.3	30.2	30.6	30.6
P. australis DSM 17609^T	44.2	43.5	ID	48.8	31	30.7	30.6	31	31	31.7	31	31.3	31.3	31.1	31.5	30.3	30.2	30.9	30.3	30.1	30.9	30.6
P. asymbiotica ATCC 43949	42.4	42.4	48.8	ID	31.7	31.3	31.2	30.7	31.2	31.8	30.8	31	31.1	31.2	31.3	30.6	30.7	30.2	30.1	30	30	30.2
P. stackebrandtii DSM 23271^T	32.4	32.5	31	31.7	ID	57	64.2	47.5	52.7	53.1	32.7	33.2	33.3	33.6	33.4	31.8	31.7	31.7	31.1	31.3	31.3	31.3
***P. khanii* subsp. *guanajuatensis* MEX20-17^T**	31.9	31.7	30.7	31.3	57	ID	70.4	47.9	52.2	52.2	32.3	32.5	32.8	33.2	32.9	31.9	31.7	31.6	31.1	31.3	31.4	31.3
***P. khanii subsp. khanii* DSM 3369^T**	31.8	32	30.6	31.2	64.2	70.4	ID	47.8	51.9	52.2	32.5	32.6	32.8	33.2	32.8	31.8	31.8	31.4	31.3	31.2	31.4	31.3
P. tasmaniensis DSM 22387^T	32	31.8	31	30.7	47.5	47.9	47.8	ID	51.4	51.3	32.3	32.5	32.9	32.2	32.8	30.9	31	31.3	30.7	31.1	31.1	31.2
P. temperata Meg1	32.4	33.3	31	31.2	52.7	52.2	51.9	51.4	ID	67.2	32.3	32.4	32.8	32.3	33.1	31.2	31.4	31.4	31.1	31.2	31.4	31.3
P. thracensis DSM 15199^T	33	32.7	31.7	31.8	53.1	52.2	52.2	51.3	67.2	ID	33.1	33.9	35.2	33.9	34.2	31.8	31.9	32.4	31.6	32	32.4	32.1
P. laumondii subsp. *clarkei* BOJ-47^T	31.7	31.3	31	30.8	32.7	32.3	32.5	32.3	32.3	33.1	ID	76.5	59.2	54.4	59.8	44.6	44.4	48.3	46.8	46	47.5	48.1
P. laumondii subsp. laumondii TT01^T	32.8	31.8	31.3	31	33.2	32.5	32.6	32.5	32.4	33.9	76.5	ID	58.4	53	57.7	44	44.2	49.1	47.3	44.9	46	46.3
P. kayaii HUG-39	32.2	31.7	31.3	31.1	33.3	32.8	32.8	32.9	32.8	35.2	59.2	58.4	ID	63.6	69.3	44.9	45.1	48	47	45.5	46.5	46.6
P. kleinii S8-52	31.7	31.3	31.1	31.2	33.6	33.2	33.2	32.2	32.3	33.9	54.4	53	63.6	ID	69.2	46.8	47.9	46.7	45.7	44.5	45.3	45.5
P. bodei LJ24-63^T	32.4	32.3	31.5	31.3	33.4	32.9	32.8	32.8	33.1	34.2	59.8	57.7	69.3	69.2	ID	45.1	45.2	48.1	46.9	46.8	47.9	48.1
***P. luminescens* subsp. *mexicana* MEX47-22^T**	30.4	30.1	30.3	30.6	31.8	31.9	31.8	30.9	31.2	31.8	44.6	44	44.9	46.8	45.1	ID	70.6	46	45.7	43.9	44.4	44.6
***P. luminescens subsp. luminescens* ATCC 29999^T**	30.8	30.4	30.2	30.7	31.7	31.7	31.8	31	31.4	31.9	44.4	44.2	45.1	47.9	45.2	70.6	ID	46.3	45.7	44.1	44.8	44.9
P. noenieputensis DSM 25462^T	31.8	30.6	30.9	30.2	31.7	31.6	31.4	31.3	31.4	32.4	48.3	49.1	48	46.7	48.1	46	46.3	ID	56.8	48.3	50.1	51
P. caribbeanensis HG29^T	30.7	30.3	30.3	30.1	31.1	31.1	31.3	30.7	31.1	31.6	46.8	47.3	47	45.7	46.9	45.7	45.7	56.8	ID	49.1	50.9	51.7
P. namnaonensis PB45.5^T	30.7	30.2	30.1	30	31.3	31.3	31.2	31.1	31.2	32	46	44.9	45.5	44.5	46.8	43.9	44.1	48.3	49.1	ID	61.3	59.7
P. hainanensis DSM 22397^T	31.1	30.6	30.9	30	31.3	31.4	31.4	31.1	31.4	32.4	47.5	46	46.5	45.3	47.9	44.4	44.8	50.1	50.9	61.3	ID	68.7
P. akhurstii DSM 15138^T	30.8	30.6	30.6	30.2	31.3	31.3	31.3	31.2	31.3	32.1	48.1	46.3	46.6	45.5	48.1	44.6	44.9	51	51.7	59.7	68.7	ID

Fig. 4. Pairwise comparison of *in silico* DNA-DNA Hybridization (*is*DDH) scores (%) of *Photorhabdus* strains.

species with publicly available genomes and validly published names as described elsewhere (Machado et al., 2018). The resulting core genome was aligned using MAFFT 7.187 (Katoh & Standley, 2013). Based on this alignment, a maximum likelihood tree was constructed using RAxML 8.2.9 (options: -m GTRGAMMA -# 1000 -f a) (Stamatakis, 2006). To reconstruct 16S rRNA gene-based phylogenetic relationships, we obtained 16S rRNA (*rrs*) sequences of all *Photorhabdus* species/subspecies with validly published names either from whole genome sequences or from public repositories. Sequences extracted from whole genome sequences of MEX20-17^T and MEX47-22^T were confirmed by Sanger sequencing and are given in supplementary material. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (-3765.08) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches.

Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4295)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 80.44% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Codon positions included were 1st, 2nd, 3rd, and noncoding. All positions containing gaps and missing data were eliminated. There was a total of 1340 positions in the final dataset. To reconstruct housekeeping gene-based phylogenetic relationships, sequences of the following genes were obtained either from whole genome sequences or from public repositories: the recombinase A (*recA*), the DNA polymerase III beta subunit (*dnaN*), the glutamyl-tRNA synthetase (*gltX*), the gyrase beta subunit (*gyrB*) and the translation initiation factor IF-2 (*infB*) (Dereeper et al., 2010, Dereeper et al., 2008). Sequences were aligned with MUSCLE (v3.8.31) (Edgar, 2004). The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (-20639.60) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.5407)). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 40.38% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 4280 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Tamura et al., 2013). Graphical representation and edition of the phylogenetic tree were performed with TreeDyn (v198.3) and with Interactive Tree of Life (v3.5.1) (Chevenet et al., 2013, Letunic & Bork, 2016). Final alignments included sequences of all type strains of the genus *Photorhabdus*. *Morganella morganii* subsp. *morganii* SC01 was used as outgroup. Accession numbers of all genes used are listed in Table S2. Whole genome sequences were used to calculate, with formula 2, the *in silico* DNA-DNA hybridization (*is*DDH) scores using the genome-to-genome distance calculator (GGDC) web service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) (Meier-Kolthoff et al., 2013, Auch et al., 2010a, Auch et al., 2010b, Lee et al., 2016). Nucleotide sequence identities (NSI) of five concatenated housekeeping genes were calculated in BioEdit (Hall, 1999). Orthologous average nucleotide identities (OrtoANI) were calculated in OAT (Lee et al., 2016).

16S rRNA gene sequence similarity calculations indicated that the closest relatives of strain MEX20-17^T are *P. cinerea* DSM 19724^T (97.7 %) and *P. thracensis* DSM 15199^T (98.2 %) and that the closest relatives of strain MEX47-22^T are *P. luminescens* ATCC 29999^T (99.7%) and *P. namnaonensis* PB45.5^T (98.9%) (Figure 1, Supplementary Table S4). Lower sequence similarities were found with all validly described species of the genus *Photorhabdus*. Due to the high 16S rRNA gene sequence similarity scores observed, we extracted housekeeping gene sequences (*recA*, *dnaN*, *gltX*, *gyrB* and *infB*) from whole genome sequences and from public repositories of all *Photorhabdus* type strains, reconstructed their phylogenies using the maximum likelihood method and calculated their nucleotide sequence identity scores (NSI) (Figure 2, Supplementary Table S5). MEX20-17^T clusters together with *P. stackebrandtii* DSM 23271^T and *P. khanii* DSM 3369^T and shares 98.3% and 98.9% nucleotide sequence identity of housekeeping genes with these species, respectively (Figure 2, Supplementary Table S5). MEX47-22^T clusters together with *P. luminescens* ATCC 29999^T and shares 98.9% nucleotide sequence identity of housekeeping genes with this species. To confirm the inferred phylogenetic relationships, we constructed phylogenetic trees based on core genomes (1652 genes) obtained from the newly acquired genomic sequences of *P. tasmaniensis* DSM 22387^T, *P. stackebrandtii* DSM 23271^T, *P. cinerea* DSM 19724^T, *P. akhurstii* DSM 15138^T, *P. caribbeanensis* HG29^T, *P. hainanensis* DSM 22397^T, *P. noenieputensis* DSM 25462^T, and *P. heterorhabditis* SF41^T and from several *Photorhabdus* species with validly published names and with publicly available genomes and calculated *in silico* DNA-DNA hybridization scores (*is*DDH) and orthologous average nucleotide identity values (orthoANI). MEX20-17^T again clusters together with *P. stackebrandtii* DSM 23271^T and *P. khanii* DSM 3369^T while MEX47-22^T clusters together with *P. luminescens* ATCC 29999^T (Figure 3). *In silico* DNA-DNA hybridization scores between MEX20-17^T and most *Photorhabdus* species were lower than 70% and between MEX20-17^T and *P. khanii* DSM 3369^T was 70.4% (CI: 67.4-73.3%), while orthoANI value was 97% (Figure 4, Supplementary Table S6). Similarly, *in silico* DNA-DNA hybridization scores between MEX47-22^T and most *Photorhabdus* species were lower than 70% and between MEX47-22^T and *P. luminescens* ATCC 29999^T was 70.6% (CI: 67.6-73.4%), while orthoANI value was 97% (Figure 4, Supplementary Table S6). Taken together, MEX20-17^T and *P. khanii* DSM 3369^T share 98.9% NSI, 70.4% *is*DDH and 97% orthoANI, and MEX47-22^T and *P. luminescens* ATCC 29999^T share 98.9% NSI, 70.6% *is*DDH and 97% orthoANI. Given that the thresholds for species delimitation are 97% NSI, 70% isDDH and 95-96% orthoANI, we propose to classify MEX20-17^T as a new subspecies within the species *P. khanii*, and MEX47-22^T as a new subspecies within the species *P. luminescens* (Machado et al., 2018, Glaeser & Kämpfer, 2015, Wayne et al., 1987, Meier-Kolthoff et al., 2014). It is noteworthy to mention that the isDDH scores between these strains fall close to the species delimitation boundaries, we therefore calculated additional overall genome relatedness indexes to support our taxonomic conclusions, and suggest to follow a similar approach in future studies. Biochemical characterization supports the status of MEX20-17^T and MEX47-22^T as new taxa since they exhibit

unique biochemical profiles, which differ from the profiles of other strains from other taxa (Table 1). Based on the results of this polyphasic approach, we propose the creation of *P. khanii* subsp. *guanajuatensis* subsp. nov. with MEX20-17^T (=LMG 30372^T =CCOS 1191^T) as the type strain and the automatic creation of *P. khanii* subsp. *khanii* subsp. nov. with DSM 3369^T as type strain (currently classified as *P. khanii*), and the creation of *P. luminescens* subsp. *mexicana* subsp. nov. with MEX47-22^T (=LMG 30528^T =CCOS 1199^T) as the type strain with the automatic creation of *P. luminescens* subsp*. luminescens* subsp. nov. with ATCC 29999^T as the type strain (currently classified as *P. luminescens*).

Table 1. API 20E-based phenotypic characters to differentiate all *Photorhabdus* species/subspecies with validly published names. The presented results were obtained by testing the type strain of all the listed taxa in parallel. The experiment was conducted two independent times. Results obtained in this study were compared with the results presented in the literature from the original description of some of the taxa. The presented results are therefore a compilation of the original descriptions and of this study, and were therefore conducted at least two independent times and with at least the type strain for each taxon. For further information refer to the original studies (Boemare et al., 1993, Akhurst et al., 2004, An & Grewal, 2011, An & Grewal, 2010, Ferreira et al., 2013, Ferreira et al., 2014, Fischer-Le Saux et al., 1999, Glaeser et al., 2017, Hazir et al., 2004, Tailliez et al., 2010, Thomas & Poinar, 1979, Tóth & Lakatos, 2008, Machado et al., 2018). 1: *P. akhurstii*, 2: *P. asymbiotica*, 3: *P. australis*, 4: *P. bodei*, 5: *P. caribbeanensis*, 6: *P. cinerea*, 7: *P. hainanensis*, 8: *P. heterorhabditis*, 9: *P. kayaii*, 10: *P. khanii* subsp. *khanii* subsp. nov., 11: *P. khanii* subsp. *guanajuatensis* subsp. nov., 12: *P. kleinii*, 13: *P. laumondii* subsp. *laumondii*, 14: *P. laumondii* subsp. *clarkei*, 15: *P. luminescens* subsp. *luminescens* subsp. nov., 16: *P. luminescens* subsp. *mexicana* subsp. nov., 17: *P. namnaonensis*, 18: *P. noenieputensis*, 19: *P. stackebrandtii*, 20: *P. tasmaniensis*, 21: *P. temperata*, 22: *P. thracensis*. "+": more than 80% of strains and/or conducted tests showed a positive reaction, "-": more than 80% of strains (n) and/or conducted tests (t) showed a negative reaction, "v": variable, about 50% of strains/conducted tests showed a positive reaction, "w": weak, more than 80% of strains and/or conducted tests showed a weak, positive reaction.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
β-Galactosidase	-	-	w	-	-	-	-	-	v	-	-	-	-	+	-	-	-	-	-	-	-	v
Arginine dihydrolase	-	-	-	-	-	-	-	-	-	v	-	-	-	-	-	-	-	-	+	-	-	-
Lysine decarboxylase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ornithine decarboxylase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrate utilization	+	+	+	+	+	+	+	-	+	v	-	v	-	-	+	-	+	+	-	-	+	v

H_2S production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urease	+	+	-	-	v	-	-	-	+	v	-	-	+	-	v	-	-	v	-	-	-	+
Tryptophan deaminase	-	-	v	+	+	-	+	+	+	+	+	+	-	+	-	+	+	+	+	v	-	+
Indole production	+	-	-	+	+	-	+	-	+	-	+	+	+	-	+	-	v	v	v	+	-	-
Acetoin production	+	-	-	-	-	-	-	-	+	-	-	+	-	+	+	-	-	w	-	-	-	-
Gelatinase	+	+	+	+	-	v	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+
Glucose oxidation	+	+	w	+	+	+	w	w	-	+	+	-	-	-	-	-	-	-	+	+	+	v
Mannitol oxidation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol oxidation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol oxidation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose oxidation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Saccharose oxidation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Melibiose oxidation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Amygdalin oxidation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arabinose oxidation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(Cytochrome) oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NO_2 production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NO_2 reduction to N_2 gas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	v	-	-	v	-	-	-	-

Protologues

Emended description of *Photorhabdus khanii* (Taillez *et al*. 2010) Machado *et al*. 2018.

(kha'ni.i. N.L. gen. masc. n. *khanii* in honour of A. Khan, who described the nematode *Chromonema heliothidis* Khan *et al*. 1976 =*Heterorhabditis bacteriophora* Poinar, 1975 recovered from prepupal and pupal specimens of the corn earworm, *Heliothis zea*, from Clayton, North Carolina, 1971). Cells are motile, non-spore-forming rods (approx. 1 µm wide, 1.5-2 µm long), gram-negative, oxidase negative, catalase positive. Growth in LB broth is stopped by temperatures above 35-36°C. Colonies are mucoid, circular, slightly irregular margins, yellow or orange in color with a diameter of approximately 2 mm after 48h growth on LB agar, and produce light. β-Galactosidase negative. Indole and urease variable. Gelatinase and tryptophan deaminase positive. The type strain is symbiotically associated with *Heterorhabditis bacteriophora* nematodes. Strains Meg and Habana are also representatives of this species. The type strain is C1^T (=NC19^T =CIP 109947^T =DSM 3369^T).

Description of *Photorhabdus khanii* subsp. *khanii* subsp. nov.

(kha'ni.i. N.L. gen. masc. n. *khanii* in honour of A. Khan, who described the nematode *Chromonema heliothidis* Khan *et al*. 1976 =*Heterorhabditis bacteriophora* Poinar, 1975 recovered from prepupal and pupal specimens of the corn earworm, *Heliothis zea*, from Clayton, North Carolina, 1971). Cells are motile, non-spore-forming rods (approx. 1 µm wide, 1.5-2 µm long), gram-negative, oxidase negative, catalase positive. Growth in LB broth is stopped by temperatures above 35-36°C. Colonies are mucoid, circular, slightly irregular margins, yellow or orange in color with a diameter of approximately 2 mm after 48h growth on LB agar, and produce light. Acid is produced from salicin and aesculin but not from L-fucose. *Myo*-inositol is assimilated but cis-aconitate is not. Indole negative. Urease variable. Gelatinase positive. The lack of indole production allows to differentiate it from its close relative *P. khanii* subsp. *guanajuatensis*. The type strain is symbiotically associated with *Heterorhabditis bacteriophora* nematodes. Strains Meg and Habana are also representatives of this subspecies. The type strain is C1^T (=NC19^T =CIP 109947^T =DSM 3369^T). Whole genome sequences of this strain are available in the National Center for Biotechnology Information data bank under accession number NZ_AYSJ01000002.1.

Description of *Photorhabdus khanii* subsp. *guanajuatensis* subsp. nov.

(gua.na.jua.ten'sis N.L. fem. adj. *guanajuatensis* from Guanajuato, Mexico, where the nematodes hosting the type strain were isolated). Cells are motile, non-spore-forming rods (approx. 1 µm wide, 1.5-2 µm long) and gram-negative. Good growth occurs on LB at 28-30°C. Colonies are mucoid, yellow or orange in color, circular, convex and margin are slightly irregular, with a diameter of approximately 2 mm after 48h growth on LB agar. Catalase positive, indole positive and produce bioluminescence. Oxidase, β-Galactosidase, citrate and urease negative. Gelatinase positive. Fatty acid profile is

composed of $C_{14:0}$, $C_{16:0}$, iso-$C_{15:0}$, $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH and $C_{18:1}\omega7c$. The type strain was isolated from *Heterorhabditis atacamensis* MEX20 entomopathogenic nematodes. The type strain is MEX20-17^T (=LMG 30372^T =CCOS 1191^T). Whole genome sequences of this strain are available in the National Center for Biotechnology Information data bank under accession number PUJY00000000.

Emended description of *Photorhabdus luminescens* (Thomas and Poinar 1979) Boemare *et al*. 1993 emend. Machado *et al*. 2018.

(lu.mi.nes'cens. N.L. part. adj. *luminescens* luminescing; for its bioluminescence). Colonies are mucoid, circular, slightly irregular margins, yellow or orange in color, sometimes brownish, with a diameter of approximately 1-2 mm after 48h growth on LB agar, and produce light. Cells are rods. Maximum temperature for growth in nutrient broth is 38-39°C. Indole, urease and tryptophan deaminase reaction variable. Citrate, mannitol and DL-lactate are used as a sole carbon and energy source by some strains/subspecies. Annular haemolysis of sheep-blood agars. The natural habitat is the intestinal lumen of entomopathogenic nematodes such as *Heterorhabditis bacteriophora* and *H. mexicana* and insects infected by these nematodes. The type strain is Hb^T (=ATCC 29999^T =DSM 3368^T).

Description of Photorhabdus luminescens subsp. luminescens

(lu.mi.nes'cens. N.L. part. adj. *luminescens* luminescing; for its bioluminescence). Colonies are mucoid, circular, slightly irregular margins, yellow or orange in color, sometimes brownish, with a diameter of approximately 1-2 mm after 48h growth on LB agar, and produce light. Cells are large rods (2x0.5-6x1.4 µm). Bioluminescence is more than 100-fold greater in phase one than in phase two. Maximum temperature for growth in nutrient broth is 38-39°C. Indole reaction positive or weak in at least one phase for most strains. Aesculin hydrolysis-positive. Citrate and mannitol are used as a sole carbon and energy source. DL-lactate is not used as sole source of carbon. Acid is produced from inositol and maltose by most strains. Negative for DNase and tryptophan deaminase. Variable for urease. Annular haemolysis of sheep- and horse-blood agars. Inclusion bodies occur in phase one cell protoplasm and are poorly produced in phase two cultures. The natural habitat is the intestinal lumen of entomopathogenic nematodes such as *Heterorhabditis bacteriophora* and insects infected by these nematodes. Type strain is Hb^T (=ATCC 29999^T =DSM 3368^T). Whole genome sequences of this strain are available in the National Center for Biotechnology Information data bank under accession number FMWJ01.

Description of *Photorhabdus luminescens* subsp. *mexicana* subsp. nov.

(me.xi.ca'na. N.L. fem. adj. *mexicana* from Mexico, where the nematodes hosting the type strain were isolated). Cells are motile, non-spore-forming rods (approx. 1 µm wide, 1.5-2 µm long) and gram-negative. Good growth occurs on LB at 28-30°C. Colonies are mucoid, yellow or orange in color with a diameter of approximately 2 mm after 48h

growth on LB agar and luminescent. Catalase positive, oxidase negative. Annular hemolytic activity on sheep-blood agar plates. Negative for β-galactosidase, urease, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and hydrogen sulphide production. The lack of citrate utilization, gelatinase, acetoin and indole production, and the presence of tryptophan deaminase activity allow to differentiate it from its close relative *P. luminescens* subsp. *luminescens*. Fatty acid profile is composed of $C_{14:0}$, $C_{16:0}$, iso-$C_{15:0}$, $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH and $C_{18:1}\omega7c$. The type strain was isolated from *Heterorhabditis mexicana* MEX47 nematodes collected from Guanajuato, Mexico. The type strain is MEX47-22^T (=LMG 30528^T =CCOS 1199^T). Whole genome sequences of this strain are available in the National Center for Biotechnology Information data bank under accession number PUJX00000000.

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Conflicts of Interest and Ethical Statement

The Authors declare no conflict of interest and that no humans or animals were used for experimental purposes.

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Supplementary Material

Table S1. Place and date of collection of nematodes hosting the symbiotic bacteria described in this study.

Nematode strain	Place of collection	Symbiotic bacteria	Isolation date
Heterorhabditis mexicana MEX47	Guanajuato, Mexico. 20.914769 N; 101.218718 W	*P. luminescens* subsp. *mexicana* subsp. nov. MEX47-22^T	January 2017
Heterorhabditis atacamensis MEX20	Guanajuato, Mexico. 20.963636 N; 100.831401 W	*P. khanii* subsp. *guanajuatensis* subsp. nov. MEX20-17^T	July 2017

Table S2. Accession numbers of the sequences used in this study.

Current Classification	Proposed Classification	Whole genome	16S	infB	dnaN	recA	gyrB	gltX
P. akhurstii DSM 15138^T	*P. akhurstii* DSM 15138^T	RCWE00000000	MK039086	JQ901840.1	FJ831503.1	FJ862005.1	EU930347.1	FJ844913.1
P. asymbiotica DSM 15149^T	*P. asymbiotica* DSM 15149^T	Not available	MK039071	KF740643.1	FJ862017.1	FJ862017.1	AY278494.1	FJ844929.1
P. asymbiotica ATCC 43949	*P. asymbiotica* ATCC 43949	NC_012962.1	Z76752.1	NC_012962.1	NC_012962.1	NC_012962.1	NC_012962.1	NC_012962.1
P. australis DSM 17609^T	*P. australis* DSM 17609^T	NZ_KL543985.1	AY280572	JQ723517	FJ831489	FJ862018	AY278496	FJ844930
P. bodei LJ24-63^T	*P. bodei* LJ24-63^T	NSCM00000000	MK039080	NSCM00000000	NSCM00000000	NSCM00000000	NSCM00000000	NSCM00000000
P. caribbeanensis DSM 22391^T	*P. caribbeanensis* DSM 22391^T	RCWB00000000	MK039083	JQ901842.1	FJ831499.1	FJ862003.1	EU930360.1	FJ844916.1
P. cinerea DSM 19724^T	*P. cinerea* DSM 19724^T	PUJW00000000	MK039069	KF740645.1	KF740659.1	KF740654.1	KF740662.1	KF740651.1
P. hainanensis DSM 22397^T	*P. hainanensis* DSM 22397^T	RCWD00000000	MK039085	JQ901843.1	FJ831502.1	FJ862004.1	AY278498.1	FJ844914.1
P. heterorhabditis SF41^T	*P. heterorhabditis* SF41^T	RCWA00000000	MK039068	KF418145.1	KF418142.1	KF418141.1	KF418144.1	KF418143.1
P. kayaii DSM 15194^T	*P. kayaii* DSM 15194^T	Not available	MK039081	JQ901845	FJ831494	FJ861996	EU930348	FJ844909.1
P. kayaii HUG-39	*P. kayaii* HUG-39	NSCN00000000	MK053914	NSCN00000000	NSCN00000000	NSCN00000000	NSCN00000000	NSCN00000000
P. khanii DSM 3369^T	*P. khanii* subsp. *khanii* DSM 3369^T	NZ_AYSJ01000002.1	MK039076	KF740642.1	FJ831486.1	FJ862011.1	AY278497.1	FJ844921.1
MEX20-17^T	*P. khanii* subsp. *guanajuatensis* MEX20-17^T	PUJY00000000	MK053912	PUJY00000000	PUJY00000000	PUJY00000000	PUJY00000000	PUJY00000000
P. kleinii DSM 23513^T	*P. kleinii* DSM 23513^T	Not available	MK039079	JQ901849.1	JQ901854.1	JQ901853.1	JX5134077.1	JQ901855.1
P. kleinii S8-52	*P. kleinii* S8-52	NSCL00000000	MK053915	NSCL00000000	NSCL00000000	NSCL00000000	NSCL00000000	NSCL00000000
P. laumondii subsp. *clarkei* BOJ-47^T	*P. laumondii* subsp. *clarkei* BOJ-47^T	NSCI00000000	MK039078	NSCI00000000	NSCI00000000	NSCI00000000	NSCI00000000	NSCI00000000
P. laumondii subsp. *laumondii* TT01^T	*P. laumondii* subsp. *laumondii* TT01^T	NC_005126.1	MK039077	NC_005126.1	NC_005126.1	NC_005126.1	NC_005126.1	NC_005126.1
P. luminescens ATCC 29999^T	*P. luminescens* subsp. *luminescens* ATCC 29999^T	FMWJ01	MK039082	FMWJ01	FMWJ01	FMWJ01	FMWJ01	FMWJ01
MEX47-22^T	*P. luminescens* subsp. *mexicana* MEX47-22^T	PUJX00000000	MK053913	PUJX00000000	PUJX00000000	PUJX00000000	PUJX00000000	PUJX00000000
P. namnaonensis PB45.5^T	*P. namnaonensis* PB45.5^T	LOIC01	MK039087	LOIC01	LOIC01	LOIC01	LOIC01	LOIC01
P. noenieputensis DSM 25462^T	*P. noenieputensis* DSM 25462^T	RCWC00000000	MK039084	JQ424885.1	JQ424882.1	JQ424881.1	JQ424884.1	JQ424883.1
P. stackebrandtii DSM 23271^T	*P. stackebrandtii* DSM 23271^T	PUJV00000000	MK039075	KF740646.1	KF740658.1	KF740655.1	KF740661.1	KF740650.1
P. tasmaniensis DSM 22387^T	*P. tasmaniensis* DSM 22387^T	PUJU00000000	MK039072	KF740641.1	FJ831478.1	FJ862008.1	EU930356.1	FJ844926.1
P. temperata DSM 14550^T	*P. temperata* DSM 14550^T	Not available	MK039073	JQ723516.1	FJ831485.1	FJ862012.1	AY278517.1	FJ844918.1
P. temperata Meg1	*P. temperata* Meg1	NZ_JGVH01000003.1	NZ_JGVH01000003.1	NZ_JGVH01000003.1	NZ_JGVH01000003.1	NZ_JGVH01000003.1	NZ_JGVH01000003.1	NZ_JGVH01000003.1
P. thracensis DSM 15199^T	*P. thracensis* DSM 15199^T	NZ_CP011104.1	MK039074	KF740640.1	FJ831481.1	FJ862015.1	EU930351.1	FJ844927.1
M. morganii SC01^T	*M. morganii* SC01^T	-	AJ301681.1	AMWL02	AMWL02	AMWL02	AMWL02	AMWL02

Table S3. Genome data of the genome sequences obtained in this study.

Bacterial species	Assembly size (bp)	GC content (%)	Number of contigs	N50 (bp)	Genome coverage
P. khanii subsp. *guanajuatensis* MEX20-17^T	5512522	43.51	267	103995	829
P. luminescens subsp. *mexicana* MEX47-22^T	5827786	42.53	170	214657	537
P. cinerea DSM 19724^T	4899918	42.26	171	193242	461
P. stackebrandtii DSM 23271^T	4846662	43.12	224	163732	687
P. tasmaniensis DSM 22387^T	5163014	43.56	280	82847	578
P. akhurstii DSM 15138^T	5546433	42.78	183	177142	1006
P. hainanensis DSM 22397^T	5486928	42.83	167	114556	804
P. noenieputensis DSM 25462^T	5364989	42.54	233	106369	950
P. caribbeanensis HG29^T	5356249	42.43	181	107369	712
P. heterorhabditis SF41^T	5046526	42.23	226	109067	884

Table S4. Pairwise nucleotide similarities (%) of 16S rRNA gene sequences of *Photorhabdus* strains. 1354 nucleotide positions were analyzed.

	P. heterorhabditis SF41^T	*P. australis* DSM 17609^T	*P. asymbiotica* DSM 15149^T	*P. asymbiotica* ATCC 43949	*P. namnaonensis* PB45.5^T	***P. luminescens* subsp. *mexicana* MEX47-22^T**	***P. luminescens subsp. luminescens* ATCC 29999^T**	*P. temperata* DSM 14550^T	*P. tasmaniensis* DSM 22387^T	***P. khanii* subsp. *khanii* DSM 3369^T**	*P. stackebrandtii* DSM 23271^T	*P. cinerea* DSM 19724^T	***P. khanii* subsp. *guanajuatensis* MEX20-17^T**	*P. thracensis* DSM 15199^T	*P. hainanensis* DSM 22397^T	*P. akhurstii* DSM 15138^T	*P. caribbeanensis* DSM 22391^T	*P. noenieputensis* DSM 25462^T	*P. laumondii subsp. clarkei* BOJ-47^T	*P. laumondii* subsp. *laumondii* TT01^T	*P. kleinii* DSM 23513^T	*P. bodei* LJ24-63^T	*P. kayaii* DSM 15194^T
P. heterorhabditis SF41^T	ID	97.1	97.3	97.4	97.0	97.2	97.1	97.5	96.5	97.4	96.8	96.6	96.5	96.2	95.9	95.9	96.5	96.0	95.9	96.2	95.7	95.7	95.4
P. australis DSM 17609^T	97.1	ID	97.4	97.5	96.4	96.8	96.6	96.5	95.7	96.3	95.9	96.8	96.2	96.0	96.8	96.5	97.3	96.8	96.7	96.9	96.8	96.6	96.6
P. asymbiotica DSM 15149^T	97.3	97.4	ID	99.8	97.9	98.2	98.2	97.7	96.5	97.5	96.9	96.0	95.7	96.0	96.5	96.5	97.1	96.9	96.7	96.9	96.8	96.8	96.8
P. asymbiotica ATCC 43949	97.4	97.5	99.8	ID	98.1	98.4	98.3	97.9	96.7	97.7	97.1	96.1	95.9	96.2	96.6	96.7	97.2	96.9	96.7	96.9	97.0	96.9	96.8
P. namnaonensis PB45.5^T	97.0	96.4	97.9	98.1	ID	98.9	98.7	97.7	96.6	96.9	96.8	95.7	96.0	96.4	97.9	97.8	97.4	97.5	97.1	97.2	97.3	97.2	97.1
***P. luminescens* subsp. *mexicana* MEX47-22^T**	97.2	96.8	98.2	98.4	98.9	ID	99.7	97.9	96.6	97.3	97.0	95.8	96.2	96.4	97.9	97.3	97.7	97.4	97.2	97.3	97.3	97.4	97.2
***P. luminescens subsp. luminescens* ATCC 29999^T**	97.1	96.6	98.2	98.3	98.7	99.7	ID	97.9	96.6	97.2	96.9	95.8	96.2	96.4	97.7	97.1	97.5	97.1	96.9	97.0	97.0	97.2	97.1
P. temperata DSM 14550^T	97.5	96.5	97.7	97.9	97.7	97.9	97.9	ID	97.7	98.4	98.1	96.8	97.1	97.4	96.8	96.3	96.9	96.6	96.3	96.4	96.5	96.8	96.2
P. tasmaniensis DSM 22387^T	96.5	95.7	96.5	96.7	96.6	96.6	96.6	97.7	ID	98.0	97.6	95.4	96.0	96.5	95.6	95.6	95.7	95.4	95.0	95.4	95.1	95.3	95.4
***P. khanii* subsp. *khanii* DSM 3369^T**	97.4	96.3	97.5	97.7	96.9	97.3	97.2	98.4	98.0	ID	99.4	96.0	97.0	97.2	96.1	95.9	96.5	96.2	95.7	96.0	95.9	95.9	95.7
P. stackebrandtii DSM 23271^T	96.8	95.9	96.9	97.1	96.8	97.0	96.9	98.1	97.6	99.4	ID	96.2	97.3	97.2	96.4	95.9	96.5	96.5	96.0	96.0	96.2	96.2	95.8
P. cinerea DSM 19724^T	96.6	96.8	96.0	96.1	95.7	95.8	95.8	96.8	95.4	96.0	96.2	ID	97.7	97.0	96.8	96.8	97.0	97.1	96.8	96.8	96.9	97.1	96.8
***P. khanii* subsp. *guanajuatensis* MEX20-17^T**	96.5	96.2	95.7	95.9	96.0	96.2	96.2	97.1	96.0	97.0	97.3	97.7	ID	98.2	97.4	97.1	97.7	97.7	97.2	97.3	97.3	97.4	97.0
P. thracensis DSM 15199^T	96.2	96.0	96.0	96.2	96.4	96.4	96.4	97.4	96.5	97.2	97.2	97.0	98.2	ID	97.8	97.8	97.8	97.5	97.0	97.4	97.4	97.6	97.1
P. hainanensis DSM 22397^T	95.9	96.8	96.5	96.6	97.9	97.9	97.7	96.8	95.6	96.1	96.4	96.8	97.4	97.8	ID	98.7	98.7	98.5	98.2	98.3	98.5	98.7	98.1
P. akhurstii DSM 15138^T	95.9	96.5	96.5	96.7	97.8	97.3	97.1	96.3	95.6	95.9	95.9	96.8	97.1	97.8	98.7	ID	98.4	98.4	98.2	98.5	98.2	98.5	97.9
P. caribbeanensis DSM 22391^T	96.5	97.3	97.1	97.2	97.4	97.7	97.5	96.9	95.7	96.5	96.5	97.0	97.7	97.8	98.7	98.4	ID	98.5	98.5	98.7	98.3	98.6	97.9
P. noenieputensis DSM 25462^T	96.0	96.8	96.9	96.9	97.5	97.4	97.1	96.6	95.4	96.2	96.5	97.1	97.7	97.5	98.5	98.4	98.5	ID	98.9	99.0	98.7	98.5	98.4
P. laumondii subsp. clarkei BOJ-47^T	95.9	96.7	96.7	96.7	97.1	97.2	96.9	96.3	95.0	95.7	96.0	96.8	97.2	97.0	98.2	98.2	98.5	98.9	ID	99.6	98.8	98.6	98.5
P. laumondii subsp. *laumondii* TT01^T	96.2	96.9	96.9	96.9	97.2	97.3	97.0	96.4	95.4	96.0	96.0	96.8	97.3	97.4	98.3	98.5	98.7	99.0	99.6	ID	98.7	98.5	98.5
P. kleinii DSM 23513^T	95.7	96.8	96.8	97.0	97.3	97.3	97.0	96.5	95.1	95.9	96.2	96.9	97.3	97.4	98.5	98.2	98.3	98.7	98.8	98.7	ID	99.4	98.8
P. bodei LJ24-63^T	95.7	96.6	96.8	96.9	97.2	97.4	97.2	96.8	95.3	95.9	96.2	97.1	97.4	97.6	98.7	98.5	98.6	98.5	98.6	98.5	99.4	ID	98.8
P. kayaii DSM 15194^T	95.4	96.6	96.8	96.8	97.1	97.2	97.1	96.2	95.4	95.7	95.8	96.8	97.0	97.1	98.1	97.9	97.9	98.4	98.5	98.5	98.8	98.8	ID

Table S5. Pairwise comparison of nucleotide sequence identities (NSI) of concatenated protein-coding genes (*dnaN*, *gltX*, *gyrB*, *infB* and *recA*) of *Photorhabdus* strains. 4317 nucleotide positions were analyzed.

	P. heterorhabditis SF41^T	*P. cinerea* DSM 19724^T	*P. australis* DSM 17609^T	*P. asymbiotica* ATCC 43949	*P. asymbiotica* DSM 15149^T	*P. tasmaniensis* DSM 22387^T	*P. temperata* DSM 14550^T	*P. thracensis* DSM 15199^T	*P. stackebrandtii* DSM 23271^T	***P. khanii* subsp. *khanii* DSM 3369^T**	***P. khanii* subsp. *guanajuatensis* MEX20-17^T**	*P. laumondii* subsp. *laumondii* TT01^T	*P. laumondii subsp. clarkei* BOJ-47^T	*P. kleinii* DSM 23513^T	*P. bodei* LJ24-63^T	*P. kayaii* DSM 15194^T	***P. luminescens* subsp. *luminescens* ATCC 29999^T**	***P. luminescens* subsp. *mexicana* MEX47-22^T**	*P. caribbeanensis* DSM 22391^T	*P. noenieputensis* DSM 25462^T	*P. akhurstii* DSM 15138^T	*P. hainanensis* DSM 22397^T	*P. namnaonensis* PB45.5^T
P. heterorhabditis SF41^T	ID	96.7	93.4	93.4	93.5	90.1	90.3	90.3	89.9	90.1	89.9	89.5	89.3	89.4	89.7	89.4	89.7	89.9	89.1	88.9	89.0	88.9	88.8
P. cinerea DSM 19724^T	96.7	ID	93.2	93.4	93.5	91.0	91.7	91.8	91.3	91.5	91.5	89.6	89.4	89.7	89.8	89.6	89.9	90.2	89.4	89.1	89.3	89.2	89.1
P. australis DSM 17609^T	93.4	93.2	ID	95.1	95.1	89.7	89.8	90.0	89.8	90.0	89.8	88.6	88.6	89.3	89.6	89.3	89.0	89.3	88.8	88.5	89.0	88.6	88.6
P. asymbiotica ATCC 43949	93.4	93.4	95.1	ID	99.9	89.4	89.9	89.9	89.6	89.8	89.5	89.0	88.7	89.5	89.7	89.4	89.2	89.6	88.8	88.9	88.9	88.6	88.5
P. asymbiotica DSM 15149^T	93.5	93.5	95.1	99.9	ID	89.5	89.9	90.0	89.6	89.8	89.5	89.1	88.8	89.5	89.7	89.4	89.2	89.6	88.8	88.9	88.9	88.6	88.6
P. tasmaniensis DSM 22387^T	90.1	91.0	89.7	89.4	89.5	ID	95.7	96.0	95.7	96.0	95.9	89.2	88.9	89.6	89.4	89.4	89.5	89.7	88.9	89.3	89.1	89.2	89.1
P. temperata DSM 14550^T	90.3	91.7	89.8	89.9	89.9	95.7	ID	97.0	96.6	97.0	97.1	89.5	89.1	90.2	89.9	89.8	89.7	90.0	89.2	89.5	89.5	89.8	89.5
P. thracensis DSM 15199^T	90.3	91.8	90.0	89.9	90.0	96.0	97.0	ID	96.9	96.7	96.9	90.1	89.6	90.4	90.2	90.0	89.9	90.2	89.7	89.9	89.9	90.0	89.7
P. stackebrandtii DSM 23271^T	89.9	91.3	89.8	89.6	89.6	95.7	96.6	96.9	ID	98.3	98.0	89.7	89.5	90.4	90.0	90.0	89.7	90.0	89.4	89.8	89.7	89.7	89.4
***P. khanii* subsp. *khanii* DSM 3369^T**	90.1	91.5	90.0	89.8	89.8	96.0	97.0	96.7	98.3	ID	98.9	89.7	89.5	90.4	90.0	90.0	89.9	90.2	89.3	89.6	89.6	89.6	89.3
***P. khanii* subsp. *guanajuatensis* MEX20-17^T**	89.9	91.5	89.8	89.5	89.5	95.9	97.1	96.9	98.0	98.9	ID	89.5	89.3	90.3	90.0	89.9	89.8	90.2	89.3	89.6	89.7	89.7	89.5
P. laumondii subsp. *laumondii* TT01^T	89.5	89.6	88.6	89.0	89.1	89.2	89.5	90.1	89.7	89.7	89.5	ID	98.5	96.2	96.5	96.5	94.8	94.9	94.6	95.2	94.5	94.3	94.6
P. laumondii subsp. clarkei BOJ-47^T	89.3	89.4	88.6	88.7	88.8	88.9	89.1	89.6	89.5	89.5	89.3	98.5	ID	95.9	96.6	96.1	94.6	94.8	94.3	94.6	94.3	94.2	94.5
P. kleinii DSM 23513^T	89.4	89.7	89.3	89.5	89.5	89.6	90.2	90.4	90.4	90.4	90.3	96.2	95.9	ID	98.3	98.1	94.7	95.0	94.1	94.4	94.0	94.0	94.1
P. bodei LJ24-63^T	89.7	89.8	89.6	89.7	89.7	89.4	89.9	90.2	90.0	90.0	90.0	96.5	96.6	98.3	ID	98.7	95.1	95.4	94.8	95.1	94.8	94.6	94.9
P. kayaii DSM 15194^T	89.4	89.6	89.3	89.4	89.4	89.4	89.8	90.0	90.0	90.0	89.9	96.5	96.1	98.1	98.7	ID	95.1	95.3	94.6	94.8	94.5	94.3	94.5
***P. luminescens* subsp. *luminescens* ATCC 29999^T**	89.7	89.9	89.0	89.2	89.2	89.5	89.7	89.9	89.7	89.9	89.8	94.8	94.6	94.7	95.1	95.1	ID	98.9	95.0	95.3	94.8	94.5	94.6
***P. luminescens* subsp. *mexicana* MEX47-22^T**	89.9	90.2	89.3	89.6	89.6	89.7	90.0	90.2	90.0	90.2	90.2	94.9	94.8	95.0	95.4	95.3	98.9	ID	95.1	95.5	95.0	94.7	94.6
P. caribbeanensis DSM 22391^T	89.1	89.4	88.8	88.8	88.8	88.9	89.2	89.7	89.4	89.3	89.3	94.6	94.3	94.1	94.8	94.6	95.0	95.1	ID	95.9	95.6	95.0	95.2
P. noenieputensis DSM 25462^T	88.9	89.1	88.5	88.9	88.9	89.3	89.5	89.9	89.8	89.6	89.6	95.2	94.6	94.4	95.1	94.8	95.3	95.5	95.9	ID	95.5	95.0	95.1
P. akhurstii DSM 15138^T	89.0	89.3	89.0	88.9	88.9	89.1	89.5	89.9	89.7	89.6	89.7	94.5	94.3	94.0	94.8	94.5	94.8	95.0	95.6	95.5	ID	96.8	96.9
P. hainanensis DSM 22397^T	88.9	89.2	88.6	88.6	88.6	89.2	89.8	90.0	89.7	89.6	89.7	94.3	94.2	94.0	94.6	94.3	94.5	94.7	95.0	95.0	96.8	ID	96.8
P. namnaonensis PB45.5^T	88.8	89.1	88.6	88.5	88.6	89.1	89.5	89.7	89.4	89.3	89.5	94.6	94.5	94.1	94.9	94.5	94.6	94.6	95.2	95.1	96.9	96.8	ID

Table S6. Pairwise comparison of orthologous average nucleotide identities (OrtoANI) scores (%) of *Photorhabdus* strains.

	P. heterorhabditis SF41^T	*P. cinerea* DSM 19724^T	*P. australis* DSM 17609^T	*P. asymbiotica* ATCC 43949	*P. stackebrandtii* DSM 23271^T	***P. khanii* subsp. *guanajuatensis* MEX20-17^T**	***P. khanii subsp. khanii* DSM 3369^T**	*P. tasmaniensis* DSM 22387^T	*P. temperata* Meg1	*P. thracensis* DSM 15199^T	*P. laumondii* subsp. *clarkei* BOJ-47^T	*P. laumondii subsp. laumondii* TT01^T	*P. kayaii* HUG-39	*P. kleinii* S8-52	*P. bodei* LJ24-63^T	***P. luminescens* subsp. *mexicana* MEX47-22^T**	***P. luminescens subsp. luminescens* ATCC 29999^T**	*P. noenieputensis* DSM 25462^T	*P. caribbeanensis* HG29^T	*P. namnaonensis* PB45.5^T	*P. hainanensis* DSM 22397^T	*P. akhurstii* DSM 15138^T
P. heterorhabditis SF41^T	ID	94.9	91.6	91.0	86.9	86.8	86.8	86.9	86.9	87.1	86.5	86.6	86.6	86.3	86.7	85.8	86.0	86.4	85.9	86.0	86.2	86.0
P. cinerea DSM 19724^T	94.9	ID	91.5	91.2	87.0	86.9	86.9	86.8	87.5	87.1	86.4	86.5	86.5	86.3	86.8	86.0	86.0	86.1	86.0	85.9	86.2	86.1
P. australis DSM 17609^T	91.6	91.5	ID	93.0	86.1	86.0	86.0	86.1	86.1	86.4	86.0	86.0	86.1	85.9	86.0	85.7	85.6	85.8	85.6	85.7	86.0	85.9
P. asymbiotica ATCC 43949	91.0	91.2	93.0	ID	86.5	86.2	86.3	86.0	86.2	86.5	86.0	85.9	85.9	86.0	86.0	85.9	85.9	85.8	85.6	85.6	85.6	85.6
P. stackebrandtii DSM 23271^T	86.9	87.0	86.1	86.5	ID	94.8	96.1	92.5	93.7	93.7	86.8	86.9	87.1	87.3	87.2	86.7	86.8	86.6	86.5	86.5	86.5	86.5
***P. khanii* subsp. *guanajuatensis* MEX20-17^T**	86.8	86.9	86.0	86.2	94.8	ID	97.0	92.8	93.6	93.6	86.9	86.9	87.0	87.2	87.1	86.7	86.8	86.7	86.3	86.4	86.5	86.5
***P. khanii subsp. khanii* DSM 3369^T**	86.8	86.9	86.0	86.3	96.1	97.0	ID	92.6	93.6	93.8	87.0	87.0	87.1	87.3	87.0	86.8	86.8	86.7	86.5	86.5	86.7	86.6
P. tasmaniensis DSM 22387^T	86.9	86.8	86.1	86.0	92.5	92.8	92.6	ID	93.7	93.6	86.7	86.6	86.9	86.7	87.1	86.2	86.4	86.3	86.2	86.3	86.3	86.3
P. temperata Meg1	86.9	87.5	86.1	86.2	93.7	93.6	93.6	93.7	ID	96.4	86.6	86.7	86.9	86.8	87.1	86.3	86.5	86.6	86.4	86.3	86.4	86.6
P. thracensis DSM 15199^T	87.1	87.1	86.4	86.5	93.7	93.6	93.8	93.6	96.4	ID	87.2	87.3	87.7	87.5	87.7	86.8	86.8	87.0	86.7	86.7	86.8	86.8
P. laumondii subsp. *clarkei* BOJ-47^T	86.5	86.4	86.0	86.0	86.8	86.9	87.0	86.7	86.6	87.2	ID	97.7	95.0	94.1	95.0	91.9	91.9	92.9	92.6	92.3	92.6	92.7
P. laumondii subsp. laumondii TT01^T	86.6	86.5	86.0	85.9	86.9	86.9	87.0	86.6	86.7	87.3	97.7	ID	94.9	93.9	94.7	91.7	91.7	93.1	92.5	91.9	92.2	92.3
P. kayaii HUG-39	86.6	86.5	86.1	85.9	87.1	87.0	87.1	86.9	86.9	87.7	95.0	94.9	ID	95.8	96.7	92.0	91.9	92.6	92.5	92.1	92.3	92.4
P. kleinii S8-52	86.3	86.3	85.9	86.0	87.3	87.2	87.3	86.7	86.8	87.5	94.1	93.9	95.8	ID	96.7	92.4	92.6	92.3	92.1	91.8	91.9	92.1
P. bodei LJ24-63^T	86.7	86.8	86.0	86.0	87.2	87.1	87.0	87.1	87.1	87.7	95.0	94.7	96.7	96.7	ID	91.9	92.0	92.7	92.4	92.5	92.5	92.7
***P. luminescens* subsp. *mexicana* MEX47-22^T**	85.8	86.0	85.7	85.9	86.8	86.7	86.8	86.2	86.3	86.8	91.9	91.7	92.0	92.4	91.9	ID	96.9	92.4	92.3	91.7	91.9	92.0
***P. luminescens subsp. luminescens* ATCC 29999^T**	86.0	86.0	85.6	85.9	86.8	86.8	86.8	86.4	86.5	86.8	91.9	91.7	91.9	92.6	92.0	96.9	ID	92.4	92.3	91.8	91.8	91.9
P. noenieputensis DSM 25462^T	86.4	86.1	85.8	85.8	86.6	86.7	86.7	86.3	86.6	87.0	92.9	93.1	92.6	92.3	92.7	92.4	92.4	ID	94.8	93.0	93.5	93.6
P. caribbeanensis HG29^T	85.9	86.0	85.6	85.6	86.5	86.3	86.5	86.2	86.4	86.7	92.6	92.5	92.5	92.1	92.4	92.3	92.3	94.8	ID	93.2	93.5	93.8
P. namnaonensis PB45.5^T	86.0	85.9	85.7	85.6	86.5	86.4	86.5	86.3	86.3	86.7	92.3	91.9	92.1	91.8	92.5	91.7	91.8	93.0	93.2	ID	95.6	95.3
P. hainanensis DSM 22397^T	86.2	86.2	86.0	85.6	86.5	86.5	86.7	86.3	86.4	86.8	92.6	92.2	92.3	91.9	92.5	91.9	91.8	93.5	93.5	95.6	ID	96.6
P. akhurstii DSM 15138^T	86.0	86.1	85.9	85.6	86.5	86.5	86.6	86.3	86.6	86.8	92.7	92.3	92.4	92.1	92.7	92.0	91.9	93.6	93.8	95.3	96.6	ID

3. Sequestration of cucurbitacins from cucumber plants by *Diabrotica balteata* larvae provides no protection against natural enemies

Pamela Bruno[1], Carla CM Arce[1]*, Ricardo AR Machado[2], Gaia Besomi[1], Anna Spescha[3], Monika Maurhofer[3], Gaétan Glauser[4], Charlyne Jaccard[5], Betty Benrey[5] & Ted CJ Turlings[1]*

1. Laboratory of Fundamental and Applied Research in Chemical Ecology, Institute of Biology, University of Neuchâtel, Switzerland.

2. Experimental Biology group, Institute of Biology, University of Neuchâtel, Switzerland.

3. Plant Pathology Group, Institute of Integrative Biology, ETH Zürich, Switzerland.

4. Neuchâtel Platform of Analytical Chemistry, University of Neuchâtel, Switzerland

5. Laboratory of Evolutionary Entomology, Institute of Biology, University of Neuchâtel, Switzerland.

*Corresponding authors: carla.marques@unine.ch & ted.turlings@unine.ch

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Abstract

- Cucurbitacins are bitter triterpenoids produced by Cucurbitaceae plants that protect them against various insects and pathogens, including the banded cucumber beetle, *Diabrotica balteata*. The banded cucumber beetle sequesters cucurbitacins, potentially as a putative defensive mechanism against natural enemies. Whether the larvae sequester and use cucurbitacins as defenses in unknown.
- We profiled cucurbitacin levels in four varieties of cucumber, *Cucumis sativus*, with contrasting biosynthetic capacities, and in larvae fed on these plants, and correlated this with larval performance. Finally, we evaluated whether the sequestered cucurbitacins helped to protect the larvae against insect predators, entomopathogenic nematodes, fungi and bacteria.
- We found considerable qualitative and quantitative differences in cucurbitacin contents in the four cucumber varieties and tissues. The larvae accumulated and transformed cucurbitacins when fed on cucumber plants. While the larvae fed extensively on both belowground and aboveground tissues, the sequestered cucurbitacins mainly came from the roots. Cucurbitacin content had no effect on larval performance and, surprisingly, did not provide protection against the natural enemies tested.
- The results show that *D. balteata* larvae can indeed sequester and transform cucurbitacins, but that this does not confer any protection against typical natural enemies and its significance remains to be determined.

Keywords

Diabroticina – entomopathogenic – plant defense – predators – rootworms – tritrophic interactions

Introduction

Plants rely on a diverse array of chemical defenses to protect them against herbivore attacks. These mostly involve secondary metabolites with repellent, toxic and/or deterrent effects (Steppuhn *et al.*, 2004; Gershenzon & Dudareva, 2007; Opitz & Müller, 2009; Agrawal *et al.*, 2012; Wouters *et al.*, 2016). Upon herbivory, plant cells are ruptured, which can trigger a process of chemical modification of metabolites, e.g. non-toxic glucosylated molecules are hydrolyzed to toxic aglucones. Such is the case for benzoxazinoids, the main defense compounds of Poaceae plants, as well as for glucosinolates, the principal secondary metabolites of the Brassicaceae family (Rask *et al.*, 2000; Frey *et al.*, 2009; Wouters *et al.*, 2016). For the Cucurbitaceae family, which include many species of melons, cucumbers, pumpkins and gourds, the defense against herbivory is primarily mediated by a group of extremely bitter tetracyclic triterpenoids, the cucurbitacins (Rehm *et al.*, 1957; Lavie & Glotter, 1971; Metcalf *et al.*, 1980; Chen *et al.*, 2005). These compounds are also present in a few species of

other plant families such as Brassicaceae (Curtis & Meade, 1971; Nielsen *et al.*, 1977; Sachdev-Gupta *et al.*, 1993), and even in some fungi and bacteria (reviewed in Chen et al., 2005). Cucurbitacins have been shown to be effective in protecting leaves and fruits against the attack of several arthropods and pathogens (Da Costa & Jones, 1971; Nielsen *et al.*, 1977; Bar-Nun & Mayer, 1990; Sachdev-Gupta *et al.*, 1993). In addition, when these compounds are extracted and applied to non-producing plants or diets, similar negative effects have been observed against beetles and birds (Mason & Turpin, 1990; Nishida *et al.*, 1992; Tallamy *et al.*, 1997). Cucurbitacins are also highly toxic to vertebrates (David & Vallance, 1955), and there are several reports of unfortunate human poisonings (Ferguson *et al.*, 1983; Rymal *et al.*, 1984; Aldous *et al.*, 1994; Verma & Jaiswal, 2015), with even a reference of intoxication by gourds written in the Old Testament ("Death in the pot", 2 Kings 4:38-41, written around the year 600 BC). Although biological effects of cucurbitacins have been observed for a long time, the chemical complexity and the toxicity of cucurbitacins are still not entirely understood.

Several insects have evolved diverse mechanisms to circumvent plant defenses strategies, thus being capable of actively exploiting the plants (Zhu-Salman *et al.*, 2005; Felton & Tumlinson, 2008; Ali & Agrawal, 2012). This is also the case for beetles of the subtribe Diabroticina (Coleoptera: Chrysomelidae), which are known to feed on Cucurbitaceae plants despite the cucurbitacins that they contain (Metcalf *et al.*, 1980; Eben *et al.*, 1997b; Eben *et al.*, 1997a; Eben, 2012; Eben & Espinosa de Los Monteros, 2013). In fact, cucurbitacins even act as strong attractants (kairomones) and phagostimulants for these beetles (Howe *et al.*, 1976; Metcalf *et al.*, 1980). In addition, it has been reported that adults of some Diabroticina species can sequester and detoxify cucurbitacins (Ferguson *et al.*, 1985; Andersen *et al.*, 1988; Nishida *et al.*, 1992). The beetles excrete most of the ingested cucurbitacins, but also conjugate a small proportion that is distributed between the hemolymph, gut and the rest of the body (Ferguson *et al.*, 1985). This accumulation seems to be persistent (Ferguson *et al.*, 1985; Andersen *et al.*, 1988) and the sequestered cucurbitacins are even transferred to the eggs (Ferguson & Metcalf, 1985; Ferguson *et al.*, 1985; Tallamy *et al.*, 1998). Regarding the feeding behavior of the larvae, known as rootworms, the literature is scarce, although it has been observed that they prefer cucurbitacin-containing plants (DeHeer & Tallamy, 1991) and it is thought that they also sequester cucurbitacins (Ferguson *et al.*, 1985; DeHeer & Tallamy, 1991; Barbercheck *et al.*, 1995; Tallamy *et al.*, 1998).

The sequestration of plant secondary metabolites by herbivorous insects often confers protection against natural enemies (Nishida, 2002; Opitz & Müller, 2009; Agrawal *et al.*, 2012; Robert *et al.*, 2017), a trait that could be linked to their success as pests of many economically important crops (Robert *et al.*, 2017). Ferguson and Metcalf (1985) observed that mantids refused to prey on several beetles belonging to the Diabroticina subtribe, including *Diabrotica balteata* and *Acalymma vittatum*, when they had been fed on "bitter" squash (assumed to contain cucurbitacins). Because the mantids also

rejected a large number of *Acalymma* beetles that had fed on a non-bitter diet, it was concluded that these rejected beetles had sequestered the cucurbitacins at their larval stages. This research was considered as the first evidence of the sequestration of cucurbitacins by Diabroticina beetles and larvae for their defense against natural enemies. This protection hypothesis is controversial, however, because later research has been inconsistent, and did not always find similar effects for Diabroticina beetles (Howe *et al.*, 1976; Gould & Massey, 1984; Nishida & Fukami, 1990), eggs (Brust & Barbercheck, 1992; Tallamy *et al.*, 1998) and larvae (Barbercheck, 1993; Barbercheck *et al.*, 1995; Eben & Barbercheck, 1997; Barbercheck *et al.*, 2003). Hence, there is need for more knowledge on the phenomenon of cucurbitacin sequestration by Diabroticina larvae.

In this study, we extensively investigated the sequestration of cucurbitacins from cucumber plants (*Cucumis sativus* L) by larvae of the banded cucumber beetle *Diabrotica balteata* LeConte and its effects on natural enemies. To this end, we profiled the main cucurbitacins from four commercial cucumber varieties in different tissues and analyzed the cucurbitacins accumulated over time by gut-dissected *D. balteata* larvae fed on these plants. To test for costs of the sequestration, we evaluated the weight gain and mortality of larvae that were fed on cucumber plants with or without cucurbitacins. The larvae were found to not only feed on roots, but also extensively on the aboveground plant parts. We therefore assessed if their performance and the sequestration of cucurbitacins were dependent on which tissues they had fed on. Subsequently, we determined the mortality of *D. balteata* larvae fed on cucurbitacin-containing and cucurbitacin-free cucumber plants when exposed to insect predators, entomopathogenic nematodes, fungi and bacteria. The results show that *D. balteata* larvae can indeed accumulate considerable amounts of cucurbitacins, mainly from root tissues, which they convert into other forms. This sequestration was not deleterious to their development but also did not offer them any protection against natural enemy attack.

Materials and methods

Biological resources

Plants

Four cucumber (*Cucumis sativus* L.) commercial varieties with contrasting cucurbitacin biosynthetic capacities were used for experiments: Tanja (“T”, Zollinger Bio, CH), Sonja F1 EKO (“S”, Koeman Flowerbulbs, NL), Hokus EKO (“H”, Koeman Flowerbulbs, NL) and Marketmore 76 (“M76”, Southern Exposure Seed Exchange, USA). Maize (*Zea mays* L.) seedlings of the hybrid DFI 45321 (DSP, Delley, CH) were used to rear *D. balteata*. Cucumber plants were grown under phytotron conditions (400 µmol/m^2/s – LD 16:8 – 28:26 °C, RH 60%) in soil (Einheitserdewerke Patzer Gebrüder Patzer GmbH & Co. KG, DE), watered every other day and fertilized twice a week (8-8-6 NPK Maag Garden, CH). The plants used in the experiments were grown either individually or in group of 5 to 10 plants per pot (2 l), to profile cucurbitacins and to grow larvae,

respectively. Experiments were performed with cucumber plants with two expanded leaves (15 – 20 days). For assays with entomopathogenic bacteria, 3-days old seedlings of cucumber varieties were used instead.

Insects, entomopathogenic nematodes, fungi and *bacteria*

Diabrotica balteata eggs were provided by Syngenta Crop Protection (Stein, CH). Since neonate *D. balteata* larvae have extremely high mortality rates on cucumber plants, neonates were first fed on seedlings of maize hybrid DFI 45321 for 5 – 7 days and then were fed on cucumber plants of the different varieties.

The predators used were adults of the greenhouse rove beetle *Atheta coriaria* Kraatz (Coleoptera: Staphylinidae), larvae of the common green lacewing *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae) and adults of the minute pirate bug *Orius laevigatus* Fieber (Heteroptera: Anthocoridae). They were purchased from Andermatt Biocontrol Suisse AG (Grossdietwil, CH) and kept under controlled conditions (LD 16:8 – 22 °C, RH 60%) and fed on *Ephestia kuehniella* eggs (Andermatt Biocontrol Suisse AG, CH) until used for experiments (1 – 2 days). The Heterorhabditis bacteriophora entomopathogenic nematodes (EPN) were collected in Mexico as described (Bruno *et al.*, 2020). The commercial *H. bacteriophora* strain was obtained from e-Nema (Schwentinental, DE). All EPN isolates were reared in last instar *Galleria mellonella* larvae using White traps as described and stored at 12 °C in the dark (White, 1927; Bedding & Akhurst, 1975; Campos-Herrera *et al.*, 2015; Bruno *et al.*, 2020).

The strains of entomopathogenic fungi *Metarhizium brunneum* BIPESCO5 (strain F52, EFSA, 2012; available as “Met52 Granular” by Novozymes Switzerland AG, CH) and *Beauveria bassiana* Naturalis (strain ATCC 74040, EFSA, 2013; available as “Naturalis-L” by Andermatt Biocontrol AG, CH) were provided by Agroscope Reckenholz (Zurich, CH). The entomopathogenic fungi were cultured on larvae of *Galleria mellonella*, re.isolated and cultured on selective SM agar at 24 °C in the dark (Strasser *et al.*, 1996). Conidia from the third generation were collected from culturing agar plates by suspending them in 1 ml 0.01% aqueous Tween80 (Sigma-Aldrich, CH). To collect the fungal spores, the resulting conidia suspensions were passed through medical gaze and centrifuged for 10 min at 2,700 g and 15 °C. Supernatants were discarded and the pellets were re-suspended in distilled water. For experiments, the concentrations were adjusted to 10^3, 10^5, and 10^7 spores per gram of sand. Bacterial strains of *Pseudomonas protegens* CHA0 (Stutz *et al.*, 1986; Ruffner *et al.*, 2013) and *Pseudomonas chlororaphis* PCL1391 (Chin-A-Woeng *et al.*, 1998; Ruffner *et al.*, 2013) were cultured from glycerol stocks at the Plant Pathology Group (ETH Zurich, CH). The bacteria strains from the stocks were streaked with an inoculation loop on King's B (King *et al.*, 1954) agar supplemented with ampicillin (40 µg/ml), cycloheximide (100 µg/ml) and chloramphenicol (13 µg/ml) (KB^{+++}) and grown for two days at 24 °C. These colonies were used to inoculate LB (Bertani, 1951) liquid cultures and incubated overnight at 24 °C and 180 rpm. The cultures were centrifuged at 2700 rpm for 10 min at 4 °C, the supernatants discarded and the pellets re-suspended in NaCl 0.9% by

vortexing. For experiments, the concentration was adjusted to 10^8 cfu/ml (OD_{600} ≈ 0.125).

Cucurbitacin measurements

To profile plant cucurbitacins, we collected roots, stems, cotyledons, and leaves of the four plant varieties separately, harvested from undamaged plants or plants damaged by 15 second-instar *D. balteata* larvae that freely fed on them for five days (n=5). Plant tissues were flash frozen in liquid nitrogen and ground into a fine powder. To profile insect-sequestered cucurbitacins, second instar *D. balteata* larvae were fed freely on whole cucumber plants for three, five or seven days (n=5–10), or were fed only on roots or shoots for five days (n=5). Then, the insects were collected, dissected and their guts removed to specifically quantify cucurbitacins stored in insect tissues and hemolymph. Eight to ten dissected larvae were pooled per biological replicate. Insect samples were flash frozen in liquid nitrogen and ground into a fine powder. To extract cucurbitacins, 100 mg of plant material or 20 mg of larval tissue per sample were dissolved in 1 ml or 500 µl of pure MeOH, respectively. Then, the samples were vortexed for 1 min and centrifuged at 20,000 g for 20 min at 4 °C. All the supernatants were filtered (13 mm Syringe filter, PTFE hydrophilic, 0.22 µm, BGB, CH) before analysis by liquid chromatography-mass spectrometry. The quantification of cucurbitacins was performed using a Waters Acquity UPLC™ system equipped with an Acquity UPLC BEH C18 column (Waters, 2.1 × 50 mm, 1.7 µm particle size) and connected to a Synapt G2 QTOF mass spectrometer (Waters, Milford, MA, USA). The entire system was controlled by Masslynx 4.1. The mobile phases consisted of formic acid (0.05 %) in water (A) and in acetonitrile (B). A linear gradient from 5 – 100 % B in 7 min was performed, following by washing at 100% B for 2 min and requilibration at 5 % B for 2 min. The flow rate was 0.4 ml/min and the injection volume was 2.5 µl. The mass spectrometer was operated in negative electrospray ionization over a mass range of 85 – 1200 Da. Accurate mass measurements (< 2 ppm) were obtained by infusing a solution of leucine-enkephalin (0.5 µg/ml) throughout the run. Cucurbitacins were putatively identified based on their exact masses (allowing for molecular formula determination) and retention times and compared with those of the standard Cucurbitacin B as well as with available databases such as the Dictionary of Natural Product (CRC Press). In some cases, the presence of several possible cucurbitacin isomers prevented full identification. Cucurbitacin B solutions at 0.02, 0.08, 0.4, 2, 5 and 10 µg/ml were used to establish a calibration curve for quantitation and all other cucurbitacins were determined as cucurbitacin B equivalents. Data for quantitation were processed in TargetLynx XS (Waters).

Performance of *Diabrotica balteata* larvae on cucumber plants

To evaluate the performance of *D. balteata* larvae on the different plant varieties, we fed *D. balteata* larvae on whole plants, on only roots, or only shoots and measured insect weight and mortality. Insects were released in 250 ml plastic pots containing 10 g of moist potting soil (Einheitserdewerke Patzer Gebrüder Patzer GmbH & Co. KG,

DE) and either roots, shoots, or whole plants. Five independent pots with fifteen first-instar larvae per treatment were evaluated (n=5). In the case of detached roots, the roots were carefully covered with the soil to avoid desiccation. Pots were maintained in a plant growth chamber (LD 16:8 – 22 °C, RH 60 %). The weight and mortality of the larvae were recorded for five days. At the end of the experiment, the surviving larvae were dissected to measure cucurbitacins as described above.

Bioassays with natural enemies

All experiments with natural enemies were assessed on *D. balteata* larvae previously fed on cucumber varieties (T, S, H and M76, see biological resources) for five days. Experiments performed testing entomopathogenic nematodes, fungi and bacteria were carried out in 30 ml plastic cups with lids (Frontier Scientific Services, Inc., USA) with either moist filter paper on the bottom (predators) or with a 5 mm layer of moist autoclaved washed sand (Ø 1 – 4 mm) (Migros, CH) for entomopathogenic nematodes.

Predation assays were carried out with *Atheta coriaria* adults, *Chrysoperla carnea* larvae or *Orius laevigatus* adults (n=30). One *D. balteata* larva and one predator were released in a 30 ml plastic cup with a moist filter paper on the bottom of the cup. The cups were closed with lids and stored in the dark at 24 ºC. The number of larvae eaten by each predator was determined after 48 hours. The infectivity of 25 *H. bacteriophora* nematode isolates was carried out according to Zhang et al. (2019) and Bruno et al. (2020) (n=10-20). Approximately 25 newly emerged EPN suspended in 500 µl of tap water were applied to each plastic cup. Each cup contained four *D. balteata* larvae. All cups were closed with lids and kept in the dark at 24 ºC. Infection by EPN was verified after 5 days.

To evaluate EPN reproductive potential, four *D. balteata* larvae were infected with 125 newly emerged EPN as described above (n=10). Five days after, nematode infectivity was recorded. Five days later, 2 ml of tap water were added to each of the plastic cups that contained at least one infected larva (2 – 10 cups per variety and EPN strain). Five days late, this is 15 days after EPN infection, cups were gently shaken, 5 drops of 20 µl were taken from each sample, and the number of IJs were counted under a microscope. The average number of IJs counted in the five drops were then used to extrapolate the concentration of IJs in the sample, and this was divided by the number of infected larvae per sample.

Bioassays with entomopathogenic fungi (EPF) were performed with *Metarhizium brunneum* BIPESCO5 and *Beauveria bassiana* Naturalis at 10^3, 10^5 and 10^7 conidia/ml (n=30). One ml of conidia suspension was applied to each plastic cup with moist sand and four *D. balteata* larvae. The cups were closed with lids and stored in the dark at 24 °C. Mortality of the larvae by EPF was regularly evaluated for ten days.

The mortality of *D. balteata* larvae by entomopathogenic bacteria was assessed with *Pseudomonas protegens* CHA0 and *Pseudomonas chlororaphis* PCL1391. Experiments were carried out in plastic cups with moist sand and four *D. balteata* larvae

(n=30). As the bacteria had to be ingested to colonize the midgut of the larvae (Kupferschmied *et al.*, 2013), 3-day seedlings of the corresponding cucumber varieties were bathed in 10^8 cfu/ml bacterial suspensions for 30 min and then placed on the sand in the plastic cups, where they were rapidly eaten by the larvae. The cups were closed with lids and stored in the dark at 24 °C. Mortality of the larvae by EPP was regularly evaluated for seven days.

Tasting of cucumber leaves

The threshold of bitterness perception of aqueous dilutions of cucumber leaf extracts was assessed by a tasting panel of 40 untrained people. Cucumber plants were grown as previously described (see plant material). Leaves were cut from the plants and ground at room temperature with a mortar and pistil. The leaf extracts were diluted at 0.001, 0.002, 0.004 and 0.02 g/ml with tap water. Each person in our panel tasted four subsequent drops of 250 µl of the increasing concentrations of one cucumber variety and the limit of response (LR) to bitterness was recorded. Water, apple slices and crackers were offered between each tasting. Considering that the leaves of the cucurbitacin-containing cucumber varieties tested (H and M76) have up to 65 µg/g of total cucurbitacins, the highest concentration of leaf extract would be of 1.3 µg/ml, and the highest amount of cucurbitacin tasted per person would be 0.44 µg. Studies on toxicity of cucurbitacins on mice report LD50 from 1.1 to 340 µg/kg for different cucurbitacins (reviewed in Fan, 2021).

Statistical analyses

Statistical tests were carried out in R statistical software (v. 4.0.0; R Development Core Team, 2019) using Analysis of Variance (ANOVA), followed by residual analyses to verify suitability of distributions of the tested models. Differences in cucurbitacins contents in plants and larvae and the larval weight were analysed using Generalized Linear Models (GLM) with a Gaussian distribution followed by pairwise comparisons of Least Squares Means (*LSMeans*). Orthogonal partial least squares discriminant analyses (OPLS-DA) and hierarchical heatmaps were carried out using MetabolAnalyst 5.0 (Chong et al., 2019) to check for differences among cucurbitacins in plant and larval tissues. Larval mortality was compared within each natural enemy for the different food sources using Generalized Linear Models (GLM) under binomial distribution followed by pairwise comparisons of Least Squares Means (*LSMeans*).

Results

Diabrotica balteata larvae feed on above and belowground tissues of cucumber plants.

We observed that *Diabrotica balteata* larvae dwell mainly on the base of the plants, feeding on the stems, causing plant wilting and stem bending. When the plant aboveground tissues are in contact with the soil, the larvae rapidly feed on them, but avoid the trichomes (Fig. 1, Fig. S1). When the larvae feed on aboveground tissues,

they become intense yellow, whereas when they only feed on roots, their color is light-cream (Fig. S2).

Cucumber varieties have different cucurbitacins levels in their tissues, and *Diabrotica balteata* larvae sequester cucurbitacins from cucumber plants mainly from the roots.

Cucurbitacins were quantified from roots, stems, cotyledons and leaves of cucumber plants from four commercial varieties (Tanja "T", Sonja F1 EKO "S", Hokus EKO "H" and Marketmore 76 "M76"), from undamaged plants or plants damaged by 15 second-instar *D. balteata* larvae (Fig. 2 b-e). We found nine main cucurbitacins, which were putatively identified according to their molecular formulas (Table S1). The varieties T and S had very low contents of cucurbitacins in all the measured tissues, with less than 1 µg/g of total cucurbitacins, whereas the cucurbitacin levels for the varieties H and M76 were 100 times higher. For H and M76, the cucurbitacins profile were composed of three metabolites that were up to 99.5% of the total contents: dihydro-cucurbitacin B-glucoside (77 – 85 %, respectively), cucurbitacin IIa (12 – 14%, respectively) and cucurbitacin C (4 – 9%, respectively). For the four varieties, 71 to 99 % of the total cucurbitacin contents were found in aboveground tissues, and overall the cucurbitacin contents were very similar for undamaged and damaged plants, showing no induction of cucurbitacins (Fig. 2 b-e, Fig. 3 a, b). However, stems of H plants had slightly higher levels of cucurbitacin C in damaged plants compared to undamaged plants: 0.04 and 0.31 µg/g, respectively ($F_{2,8\text{-}9}$=5.2159, p=0.04) (Fig. 2 c, Fig. 3 b). Similarly, the variety M76 showed an increase in the amounts of two cucurbitacins when the tissues were damaged by *D. balteata* feeding, with 32 µg/g in undamaged and 37 µg/g in damaged cotyledons, 45 µg/g in undamaged and 60 µg/g in damaged leaves. These changes were mainly due to higher contents in cucurbitacin IIa in damaged cotyledons ($F_{2,8}$=5.6049, p=0.03) (Fig. 2 d, Fig. 3 b), and higher amounts of cucurbitacin C and cucurbitacin IIa in damaged leaves (cucurbitacin C: $F_{2,8}$=5.6623, p=0.03, cucurbitacin IIa: $F_{2,8}$=10.0630, p=0.006) (Fig. 2 e, Fig. 3 b).

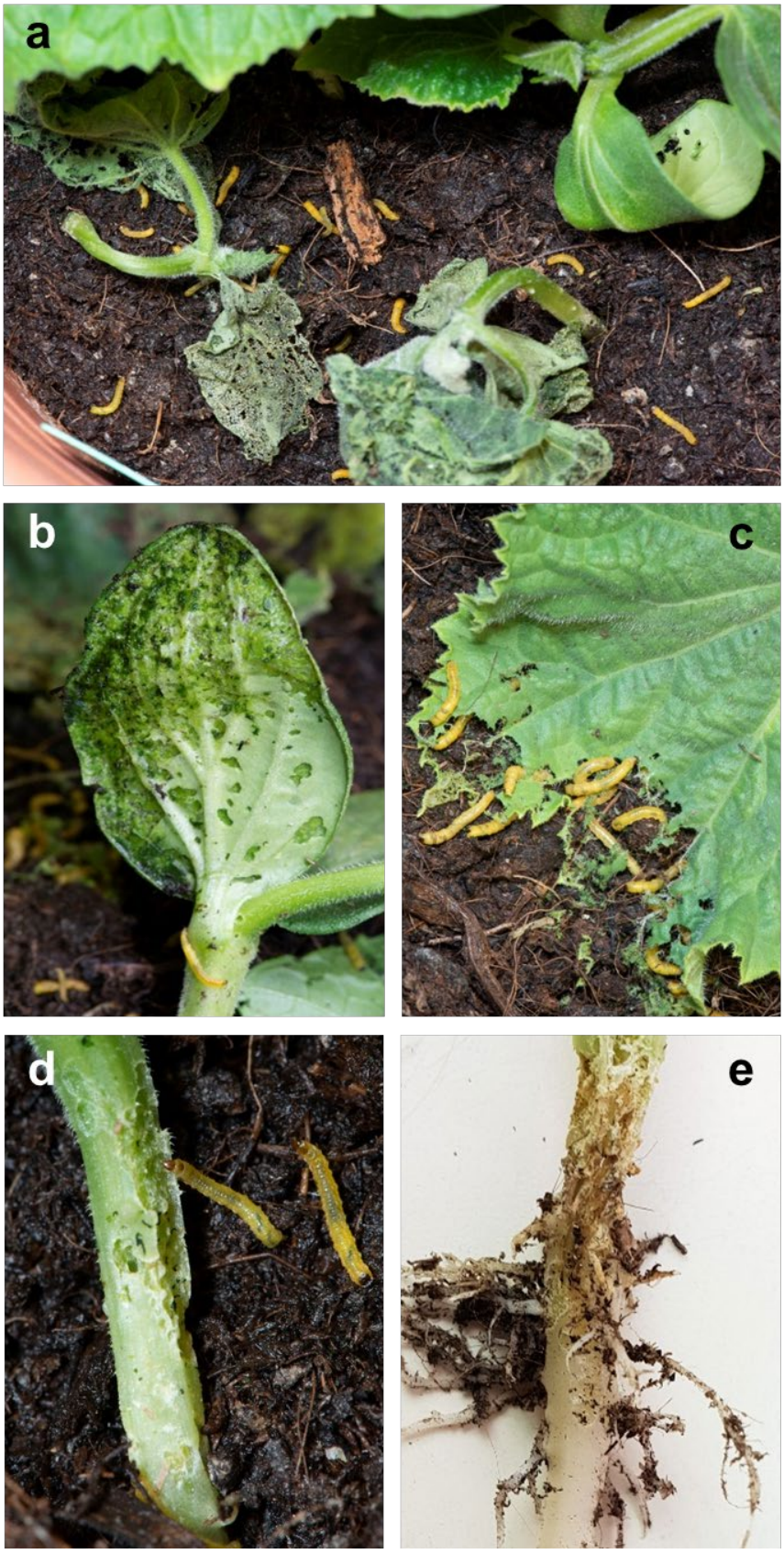

Figure 1. *Diabrotica balteata* larvae feed on above and below ground tissues of cucumber plants. (a) General view of the feeding pattern. Cotyledons (b), leaves (c), stems (d) and roots (e) are attacked by larvae. Photos: © Neil Villard & Pamela Bruno, FARCE.

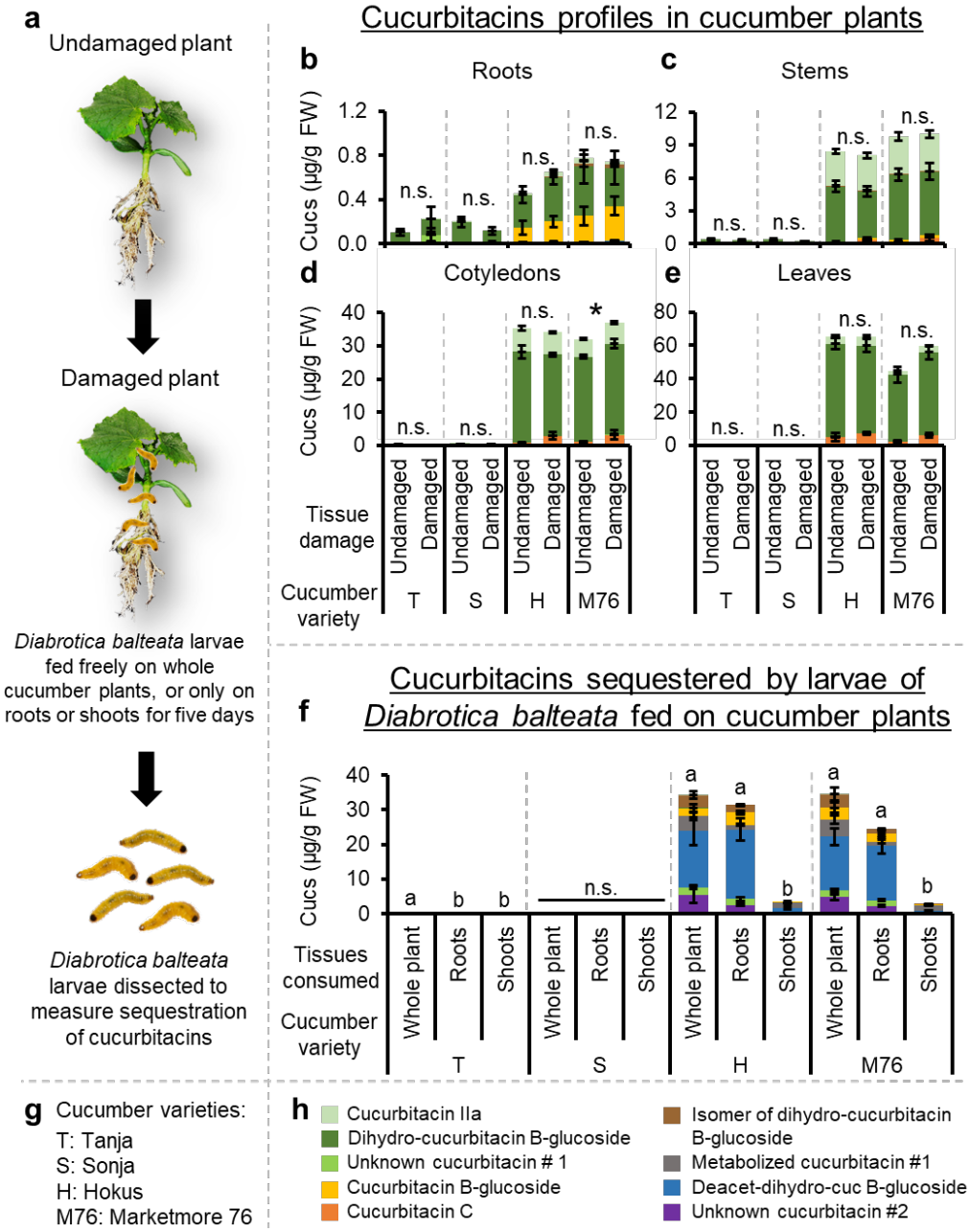

Figure 2. Cucumber varieties have different cucurbitacins levels in their tissues, and *Diabrotica balteata* larvae sequester cucurbitacins from cucumber plants mainly from the roots. (a) Graphic representation of the experimental setup. (b-e) Cucurbitacins present in cucumber roots (b, n=7-8), stems (c, n=7-9), cotyledons (d, n=7-8) or leaves (e, n=7-8) of healthy plants (Undamaged) or after 15 second-instar *D. balteata* larvae freely fed on the plants (Damaged) for five days. (f) Sequestered cucurbitacins in *D. balteata* larvae after freely feeding for five days on whole cucumber plants, or only on roots or shoots (n=5). (g) Commercial varieties of cucumber used for the experiments: "T" (Tanja), "S" (Sonja), "H" (Hokus) or "M76" (Marketmore 76). (h) Putative identification of the main cucurbitacins found in plant and larval tissues. Bars indicate average (± SE). *P* values are given for treatments [generalized linear model (family, Gaussian)] followed by pairwise comparisons of Least Squares Means (LSMeans). Not significant (n.s., *p<0.05). Different letters indicate significant differences among plant tissues within each cucumber variety, p<0.05. Photos: © Neil Villard & Pamela Bruno, FARCE.

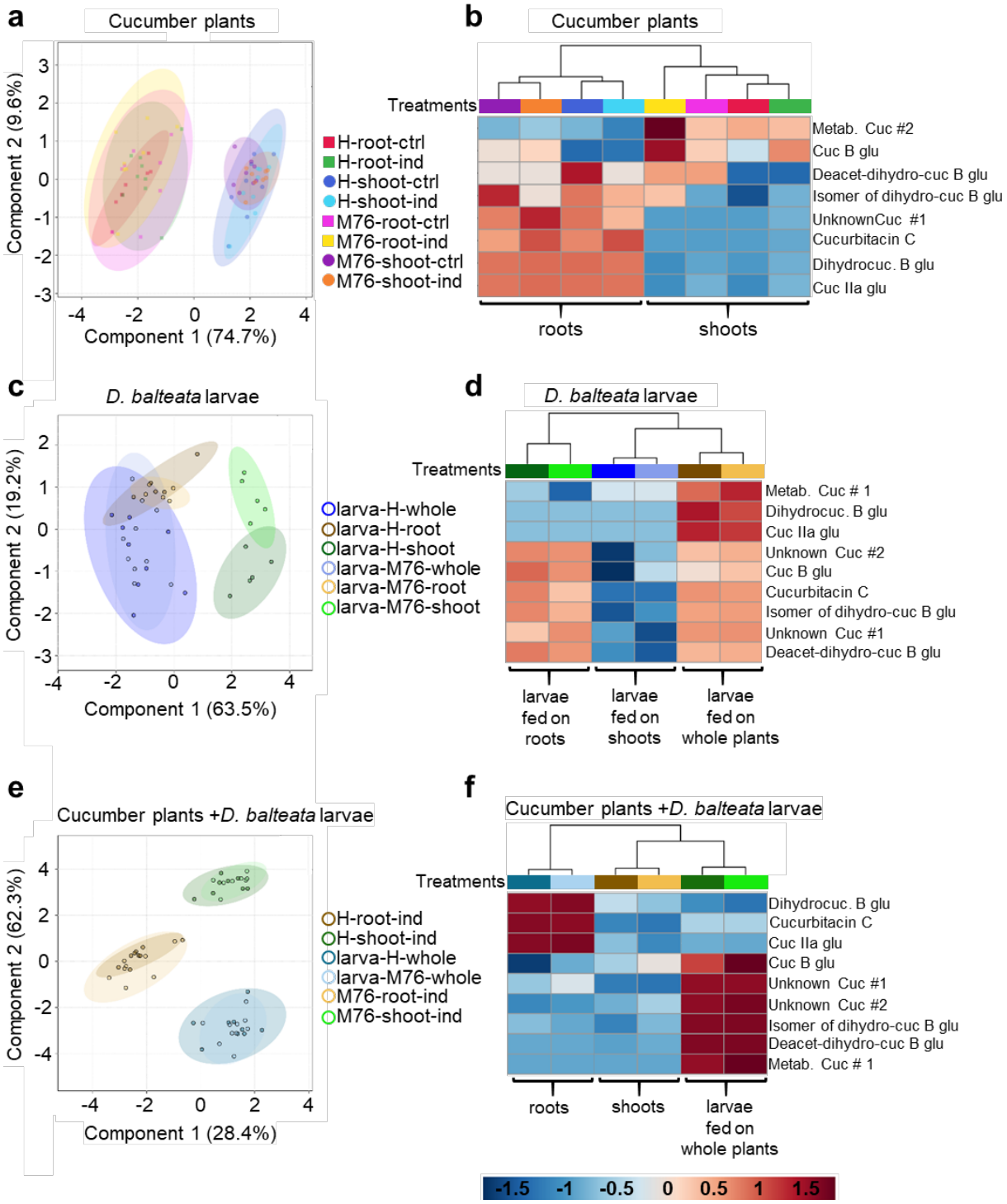

Figure 3. Differences in cucurbitacins in cucumber plants and sequestered by *Diabrotica balteata* larvae fed on those plants. Results of a discriminant analysis (OPLS-DA) and hierarchical clustering heatmaps of the contents of cucurbitacins in (a, b) cucumber roots or shoots damaged by *D. balteata* larvae, in (c, d) *D. balteata* larvae fed on cucumber roots, shoots or whole plants, and (e, f) comparison of cucurbitacins in cucumber roots or shoots and in *D. balteata* larvae fed on those tissues.

After *D. balteata* larvae fed for five days on whole plants or only on roots or shoots of the four cucumber varieties, sequestered cucurbitacins were profiled in dissected

larvae (Fig. 2 f, Fig. 3 c, d). Regardless of the tissues they had fed on, larvae from T or S plants accumulated negligible contents of cucurbitacins in their bodies (<0.3 µg/g). On the contrary, larvae fed on whole plants of the varieties H and M76 accumulated up to 34 µg/g of total cucurbitacins and when they were fed only on roots they accumulate up to 31 µg/g.

The profiles of cucurbitacins from larvae that fed either on whole plants or roots were very similar. Surprisingly, the larvae that fed only on shoots had ten times less cucurbitacins (up to 3.1 µg/g) sequestered (Fig. 2 f).

The accumulation dynamics of cucurbitacins in the larvae increased gradually over time after feeding on whole plants of H and M76 for three, five or seven days, and the qualitative composition of cucurbitacins were similar over time (Fig. S3). The similar profiles for cucurbitacins from larvae fed on roots and those fed on whole plants indicate that *D. balteata* larvae accumulate cucurbitacins mainly from the roots (Fig. 3 c, d). The larvae metabolize the cucurbitacins sequestered from the plants and accumulate transformed forms of cucurbitacins in their bodies (Fig. 3 e, f). Statistical results are available in the Supplementary tables S2 – S6.

Cucurbitacins from cucumber do not affect the performance of *Diabrotica balteata* larvae

To determine whether cucurbitacin sequestration influence larval growth, we recorded the weight gain and mortality of *D. balteata* larvae three and five days after they fed on whole cucumber plants or only or roots or shoots of the varieties T, S (CUC -), H or M76 (CUC +) (Fig. 4). After three days on the different tissues and varieties, there were some small differences in the weight gain of the larvae, with higher values for larvae fed on whole plants of S compared to the other varieties (Fig. 4 a), as well as for larvae fed on roots of T and S, in comparison with their counterparts fed on H and M76 (Fig. 4 c). On the contrary, larvae fed on shoots of S plants had slightly lower weight gain than larvae fed on H plants (Fig. 4 e). Nevertheless, after five days all these differences disappeared, and there were no differences in weight gain for larvae fed on varieties with or without cucurbitacins, regardless of whether they fed on whole plants, or only on roots or shoots (Fig. a, c, e). Similarly, the larvae fed on any of the cucumber varieties had no differences in mortality after three and five days (Fig. 4 b, d, f), regardless of the tissues consumed and the contents of cucurbitacins of each variety. All these data suggest that cucurbitacins do not affect larval performance of *D. balteata* on cucumber plants. Statistical results are available in the Supplementary tables S7 – S8.

Cucurbitacins do not protect *Diabrotica balteata* larvae against common natural enemies.

In order to assess if sequestered cucurbitacins protect *D. balteata* larvae against natural enemies, larvae fed on whole plants of cucumber varieties with or without cucurbitacins for five days (CUC - : T, S, CUC + : H, M76) were tested against a wide

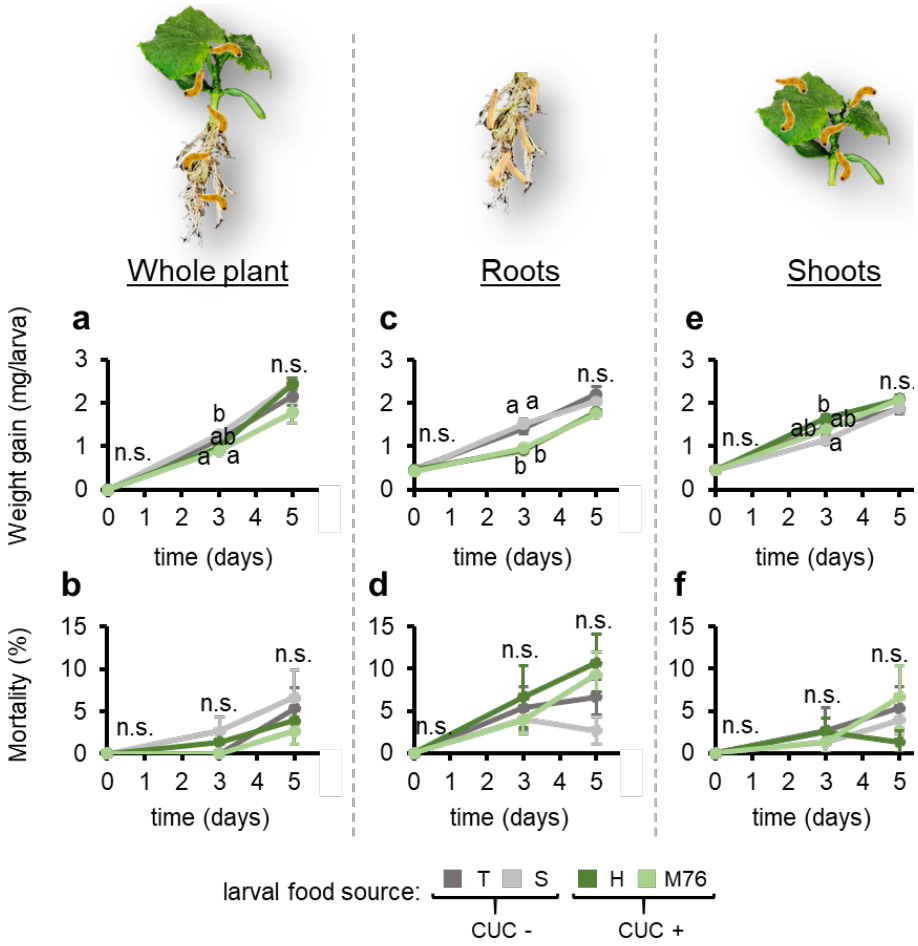

Figure 4. *Diabrotica balteata* larvae do not perform well on cucumber plants. Individual weight gain (mg/larva) and mortality (%) of 15 second-instar *D. balteata* larvae fed on whole cucumber plants (a, b), or only on roots (c, d) or shoots (e, f) for five days (n=5). Commercial varieties of cucumber used for the experiments: "T" (Tanja, dark grey), "S" (Sonja, light grey), "H" (Hokus, dark green) or "M76" (Marketmore 76, light green). Lines indicate average (± SE). Lines indicate average (± SE). *P* values are given for treatments [generalized linear model (family, Gaussian (weight gain) or Binomial (mortality))] followed by pairwise comparisons of Least Squares Means (LSMeans). Not significant (n.s., p>0.05). Different letters indicate significant differences among plant tissues within each cucumber variety, p<0.05. Photos: © Neil Villard & Pamela Bruno, FARCE.

range of common natural enemies, including insect predators, entomopathogenic nematodes (EPN), fungi and bacteria (Fig. 5). The cucurbitacin content in the larvae did not show any effect on the predation rates after 48 hours by *Atheta coriaria* ($F_{4,30}$=0.1189, p=0.94) (Fig. 5 a), *Chrysoperla carnea* (Chisq=119.26, df=26, p=0.84) (Fig. 5 b), and *Orius laevigatus* ($F_{4,30}$=0.2603, p=0.85) (Fig. 5 c). In a similar manner,

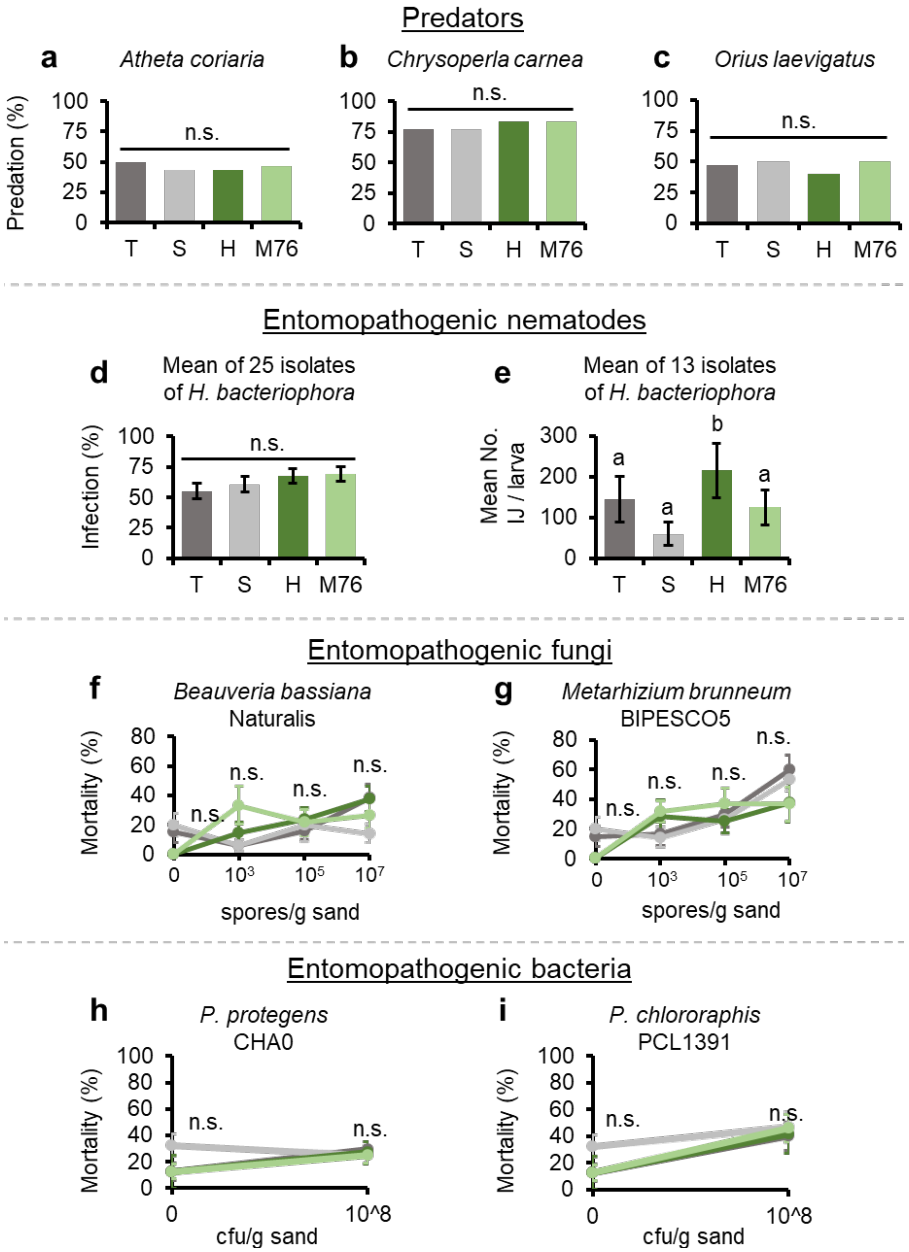

Figure 5. Cucurbitacins do not protect *Diabrotica balteata* larvae against natural enemies. Second-instar *D. balteata* larvae fed for five days on cucumber plants of the commercial varieties "T" (Tanja, dark grey), "S" (Sonja, light grey), "H" (Hokus, dark green) or "M76" (Marketmore 76, light green) and then were exposed to natural enemies. (a-c) Predation (%) of one *D. balteata* larva 48 hours after being exposed to one (a) *Atheta coriaria* adult, (b) *Chrysoperla carnea* larva or (c) *Orius laevigatus* adult (n=30). (d) Mean infection rates (%) of 25 *Heterorhabditis bacteriophora* isolates five days after inoculation on four *D. balteata* larvae (n=15-20). (e) Mean number of infected juveniles produced by 13 isolates of *H. bacteriophora* (n=2-10). Mortality of four *D. balteata* larvae seven days after infection with *B. bassiana* (f, n=30), *M. brunneum* (g, n=30), or five days after infection with *P. protegens* (h, n=30) and *P. chlororaphis* (i, n=30). Bars indicate percentage (a, b, c, f, g, h, i) or average (d and e) (± SE). *P* values are given for treatments [generalized linear model (family, Binomial)] followed by pairwise comparisons of Least Squares Means (LSMeans). Not significant (n.s., $p>0.05$). Different letters indicate significant differences among plant varieties, $p<0.05$.

the mean infection percentage of 25 EPN isolates on the larvae did not differ for on any of the cucumber varieties that *D. balteata* had fed on (Chisq=37.941, df=96, p=0.24) (Fig. 5 d). For 19 of the 25 isolates there were no differences among treatments, and in the isolates where the infections varied significantly, the differences are not related to cucurbitacin contents (Fig. S4). In order to determine whether the EPN were indirectly affected by the cucurbitacins sequestered by their host larvae, we compared the progeny production of 13 of the 25 isolates previously evaluated (Fig. 5 e, Fig. S5). The mean progeny production of the 13 isolates was significantly higher for EPN that had infected larvae fed on cucumber plants of the variety H, compared to the other three varieties ($F_{4,2\text{-}10}$=8.8908, p<0.001) (Fig. 5 e). This was in particular the case of two of the EPN isolates; MEX-21 and MEX-23, and for all the other isolates we did not observe differences in the number of infective juveniles (IJs) produced by the EPN that had killed larvae fed on varieties with or without cucurbitacins (Fig. S5). The mortality of *D. balteata* larvae seven days after inoculation with different concentrations of the entomopathogenic fungi *Beauveria bassiana* Naturalis and *Metarhizium brunneum* BIPESCO5 did not differ among the larval food sources (Fig. 5 f, g). The mortality of the larvae was generally very low and rapidly increased after seven days, especially for higher fungal concentrations, but this was unrelated to the cucumber varieties that the larvae had fed on (Fig. S6). Similarly, the mortality of *D. balteata* larvae five days after ingestion of *Pseudomonas protegens* CHA0 or *P. chlororaphis* PCL 1391 entomopathogenic bacteria was not significantly different for the four larval food sources (Fig. 5 h, i). In general, the mortality increased after seven days for all the larvae, but this was not consistent for all varieties with or without cucurbitacins (Fig. S7). Overall, cucurbitacins sequestered by *D. balteata* larvae did not provide protection against the wide range of natural enemies evaluated. Statistical results are available in the Supplementary tables S9 – S14.

An intense vegetal taste can be confused with bitterness.

A tasting panel determined the threshold of bitterness perception for four increasing concentrations of leaf extracts of cucumber plants of the varieties T, S (CUC -), H or M76 (CUC +) (Fig. S8). The highest concentration tasted (0.02 g/ml) of the varieties T and S was thought to be bitter for 30% of the tasting panel. On the contrary, the varieties H and M76 were already recognized to be bitter at the lowest concentrations of 0.002 g/ml, and at 0.02 g/ml, the totality of the panel indicated bitterness perception. This could suggest a possible confusion between bitterness and a strong herbal taste, for the highly concentrated leaf extracts.

Discussion

In this study, the ability of *Diabrotica balteata* larvae to sequester cucurbitacins was confirmed after feeding on cucumber plants. We profiled the cucurbitacins in plants of four commercial cucumber varieties, and found that some varieties have up to 27 µg/g of total cucurbitacins, whereas others have less than 1 µg/g of total cucurbitacins. For cucurbitacin-containing varieties, more than 99% of the cucurbitacins are accumulated

in the shoots, in particular in the leaves. The cucurbitacins from cucumber plants show scarcely any induction after *D. balteata* feed on the plants for five days. Although *D. balteata* larvae feed extensively on aboveground cucumber tissues, they sequester and accumulate the cucurbitacins mainly from roots, transforming some of them into different cucurbitacins. The sequestration of cucurbitacins did not show a measurable effect on larval performance, and provided no protection against the evaluated insect predators, entomopathogenic nematodes, fungi and bacteria.

Diabroticina larvae are called rootworms, in reference to their soil-dwelling behavior. We had therefore expected the larvae to only feed on roots, and we were surprised to observe that most of the larvae foraged on the soil surface around the stem base, and that they extensively fed on aboveground tissues. This behavior is not commonly reported, but has been observed for Diabroticina larvae feeding on maize (Canales Santos, 1989), beans (Cardona *et al.*, 1982), cucurbits (Jiménez Martínez, 2014), and chia (Sosa-Baldivia, 2016). Related to this behavior, we observed that when *D. balteata* larvae fed on aboveground tissues, their light-cream color changes to an intense yellow, which does not happen when they feed only on roots. Larval color changes after feeding on different plant parts has already been observed in Lepidoptera (Greene, 1989; Yamasaki *et al.*, 2009) and Diptera (Zettler *et al.*, 1998), and has been suggested to provide camouflage against predators (Eacock *et al.*, 2017). Whether the color change provides protection to *D. balteata* larvae remains to be tested.

The biosynthesis of cucurbitacins from cucumber is driven by a Mendelian gene Bi (bitter foliage) and also by a “bitter fruit” gene Bt dependent on Bi dominance (Andeweg & De Bruyn, 1959; Pierce & Wehner, 1990). However, abiotic stresses such as drought and heat can increase bitterness (Andeweg & De Bruyn, 1959; Kano & Goto, 2000), and more genes seem to be involved in the biosynthesis and regulation of cucurbitacins (Shang *et al.*, 2014). It has been traditionally postulated that cucurbit plants are either entirely “bitter” (with cucurbitacins), or they are “sweet” or “non-bitter” (without cucurbitacins), and that the taste of cotyledons (or even insects that fed on them) can be correlated with presence or absence of cucurbitacins (Howe *et al.*, 1976; DeHeer & Tallamy, 1991; Brust & Barbercheck, 1992; Dhillon, 1993; Agrawal *et al.*, 1999). Our own tasting panel could easily identify solutions with cucurbitacins even at very diluted concentrations, but most tasters were confused by the most concentrated samples derived from “non-bitter” plants, which was likely due to the strong vegetal taste. Untrained people might have trouble distinguishing this from the bitterness of cucurbitacins. Diabroticina beetles perceive different cucurbitacins at different concentrations (Metcalf *et al.*, 1980; Metcalf & Lampman, 1989; Tallamy *et al.*, 2005), and the degree of bitterness might not have a linear correlation to the amount of cucurbitacins, a fact that could be masked when evaluated by taste. The chemical identities of many of the compounds found in our analyses have not been reported before, including several cucurbitacins that were were glucosylated. Hence, pure standards are not available commercially and their bitterness remains unknown.

Although all cucurbitacins are thought to be extremely bitter and to confer toxicity (Chen *et al.*, 2005), the quantitative variation in bitterness and toxicity between aglucone and glucosylated cucurbitacins has only recently been explored (Zhong *et al.*, 2017), and further studies are necessary. Our analyses also showed that cucurbitacins are distributed differently among the plant tissues, and plants are not exclusively "bitter" or "sweet". For rootworm studies, tasting the aboveground tissues by humans might not be an accurate way to assess the presence of cucurbitacins, since the gene Bi seems to be only linked to the bitterness in the foliage.

We found hardly any induction of cucurbitacins after tissue damaged by larval feeding. Contents in undamaged and damaged tissues were generally very similar, with the exception of cucurbitacin C levels in stems of the variety H and leaves of M76, and cucurbitacin IIa glucoside in cotyledons and leaves of M76 cucumber plants, which were higher in damaged plants. Agrawal *et al.* (1999), had previously reported the induction of cucurbitacin C in the cucumber variety M76, after spider mites fed on the leaves. Similarly, Tallamy (1985) observed an induction of cucurbitacin B and D in mechanically damaged squash leaf tissues. On the other hand, Apriyanto and Potter (1990), did not find an induction of cucurbitacin production on cucumber leaves after inoculation with tobacco necrosis virus (TNV).

Other possible effects of induced resistance might include changes in trichomes, such as trichome density and induction of toxic compounds in the trichome cells (Pullin & Gilbert, 1989). The trichomes in cucumber are multicellular and contain several secondary metabolites, such as flavonoids (Pan *et al.*, 2021). All the cucumber varieties tested in our experiments had large trichomes on their stems, leaves (mostly on the nerves) and cotyledons. We observed that the larvae clearly avoided the trichomes, feeding around the leaf nerves and in the under sides of leaves and cotyledons, where the trichomes are smaller. It appears that in cucumber plants the trichome density does not change after leaf herbivory (Agrawal *et al.*, 1999). Whether flavonoids or other secondary metabolites in the trichomes are inducible, though, has not been yet tested.

The sequestration and metabolization of cucurbitacins by Diabroticina has been previously studied for adult beetles that either only consumed purified aglucone cucurbitacin B (Ferguson *et al.*, 1985), or fed on squash fruits known to have cucurbitacin B and D (Andersen *et al.*, 1988). Since the beetles only supposedly ingested aglucone cucurbitacins, it was assumed that they hydrogenated, acetylated, desaturated, and glucosylated the cucurbitacins in their bodies. Our analyses found hydrogenated, deacetylated and glucosylated cucurbitacins both in plant and larval tissues. Therefore, we cannot hypothesize as previously suggested, that those molecules are stable products of cucurbitacin detoxification by *Diabrotica* insects (Tallamy *et al.*, 2000). Concerning sequestration by larvae of *Diabrotica*, Barbercheck *et al.* (1995) and Tallamy *et al.* (1998) quantified cucurbitacins in full bodies of *D. undecimpunctata howardi* larvae fed on squash. This strongly indicated sequestration,

but the quantified cucurbitacins in those analyses could have been from the plant tissues in the larval guts at the moment of quantification. By dissecting the larval guts before the analyses, we could conclusively demonstrate the sequestration of these compounds by *D. balteata* larvae and their accumulation in the body tissues.

Although the highest amount of cucurbitacins were found in aboveground plant tissues (in particular for the varieties H and M76), we were surprised to observe that larvae accumulated and metabolized cucurbitacins mostly when they fed on roots. Apart from this and the different coloration of the larvae, we did not detect other differences between larvae fed on below versus aboveground tissues. The larvae did not obtain an evident nutritional advantage after feeding on roots or shoots, as the weight gain and mortality were very similar for all tissues (and varieties). However, we did not analyze the primary metabolism of the plants used in the experiments, and other nutritional aspects of *D. balteata* larvae fed on roots versus shoots might be different, which could help explain why they feed on aboveground tissues.

Whether cucurbitacins protect Diabroticina species against natural enemies is a controversial subject (Tallamy & Krischik, 1989; Tallamy *et al.*, 2005). Adult beetles of *D. undecimpunctata* fed on cucurbits have been shown to be rejected by mantids (Ferguson & Metcalf, 1985), but not by birds, mice and frogs (Gould & Massey, 1984). Eggs from beetles fed on bitter or non-bitter cucumber are equally predated by carabid larvae, mites or centipedes (Brust & Barbercheck, 1992), but are partially protected against entomopathogenic fungi infection (Tallamy *et al.*, 1998). It is less clear if there are any effects on entomopathogenic nematodes (Barbercheck *et al.*, 1995) and entomopathogenic fungi (Tallamy *et al.*, 1998).

In addition to assessing the direct effects of cucurbitacins sequestered by the larvae on their vulnerability to natural enemies, we also evaluated possible long-term effects on natural enemy fitness. For this, we compared the progeny of the entomopathogenic nematodes that succeeded in killing larvae that had fed on cucumber plants with and without cucurbitacins. Although we observed differences in the number of infective juveniles (IJs) among treatments, this was no correlated to the cucurbitacin contents in the plants (Fig. S6). As it has been previously hypothesized, this effect could be related to the nutritional state of the larvae as food sources for the nematodes (Barbercheck *et al.*, 1995). All of our larvae had similar weight after feeding on cucumber plants, but other aspects of the nutrition could have been influenced by feeding on the different varieties. We could not assess the survival and performance of predators fed on *D. balteata* larvae due to the reflex bleeding of the larvae. The hemolymph of *Diabrotica* species is extremely coagulant, and quickly glues the mouthparts of predators together. They eventually die unable to clean themselves despite vigorous attempts. This defense trait has been well described for larvae of *Diabrotica* species (Wallace & Blum, 1971; Lundgren *et al.*, 2009).

Our results provide first conclusive evidence for the ability of *D. balteata* larvae to sequester cucurbitacins from their host plants, but the function of this remains

somewhat of a mystery. The association of Diabroticina beetles and cucurbitacins might have other effects than protection. Further ecological studies, including abiotic stresses and a wider range of natural enemies, might provide new insights into the unclear reasons for pharmacophagy of cucurbitacins by *Diabrotica* larvae.

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Supplementary Material

Figure S1. *Diabrotica balteata* larvae avoid trichomes from cucumber plants. Third-instar larvae of *D. balteata* after feeding on cucumber plants for five days. (a) Detail of the upper leaf side of a cucumber leaf attacked by *D. balteata* larvae. (b) Cucumber leaf nerves and stem after *D. balteata* larvae fed on the leaf. (c) Detail of the lower leaf side being consumed, whereas the upper leaf side is mostly avoided. Photos: © Neil Villard & Pamela Bruno, FARCE.

Figure S2. *Diabrotica balteata* larvae change color when fed on aboveground tissues. Third-instar larvae of *D. balteata* after feeding on (a) shoots or (b) roots of cucumber plants for five days. Larvae fed on shoots are dark yellow, whereas larvae fed only on roots stay light cream. Photos: © Neil Villard & Pamela Bruno, FARCE.

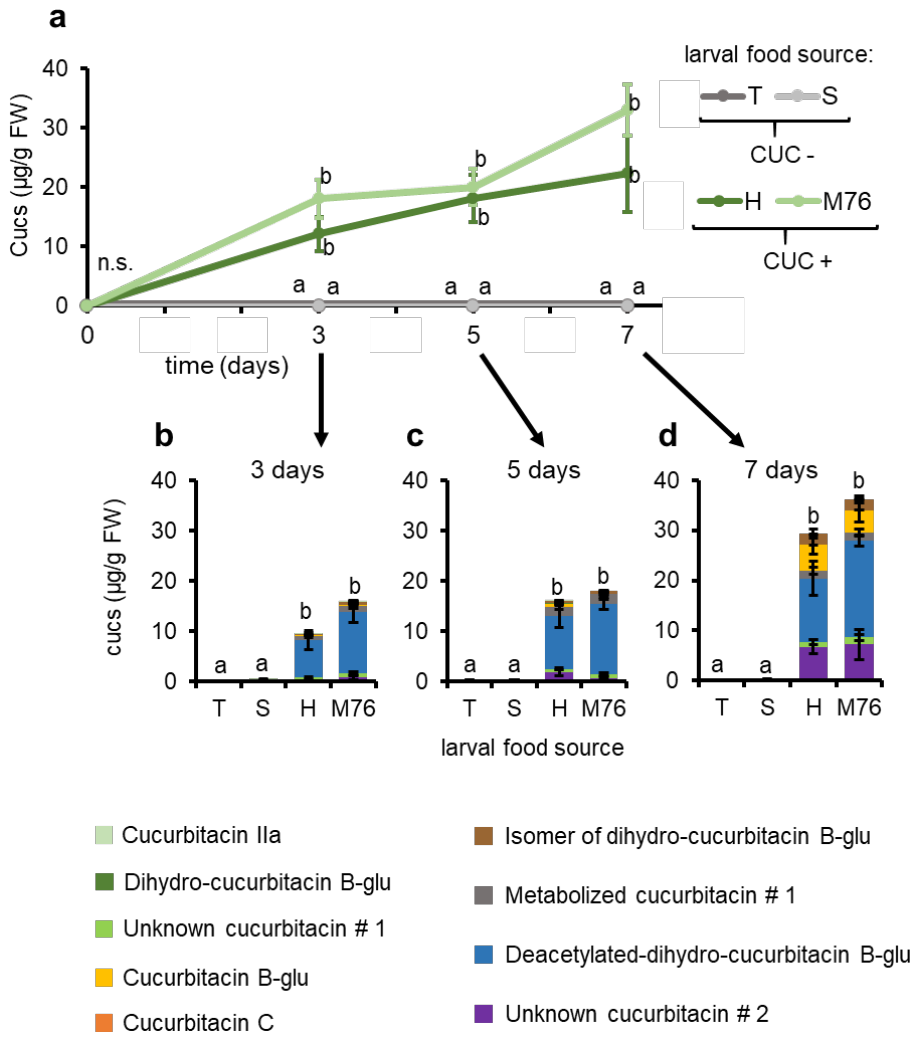

Figure S3. The accumulation of sequestered cucurbitacins by *Diabrotica balteata* larvae increases over time. Total sequestered cucurbitacins (a) and main cucurbitacins (b-d) in gut-dissected *D. balteata* larvae after freely feeding on cucumber plants of the commercial varieties "T" (Tanja), "S" (Sonja), "H" (Hokus) or "M76" (Marketmore 76) for three (b, n=5-9), five (c, 7=10) or seven days (d, n=5). Bars indicate average (± SE). *P* values are given for treatments [generalized linear model (family, Gaussian)] followed by pairwise comparisons of Least Squares Means (LSMeans). Different letters indicate significant differences among plant tissues within each cucumber variety, $p<0.05$.

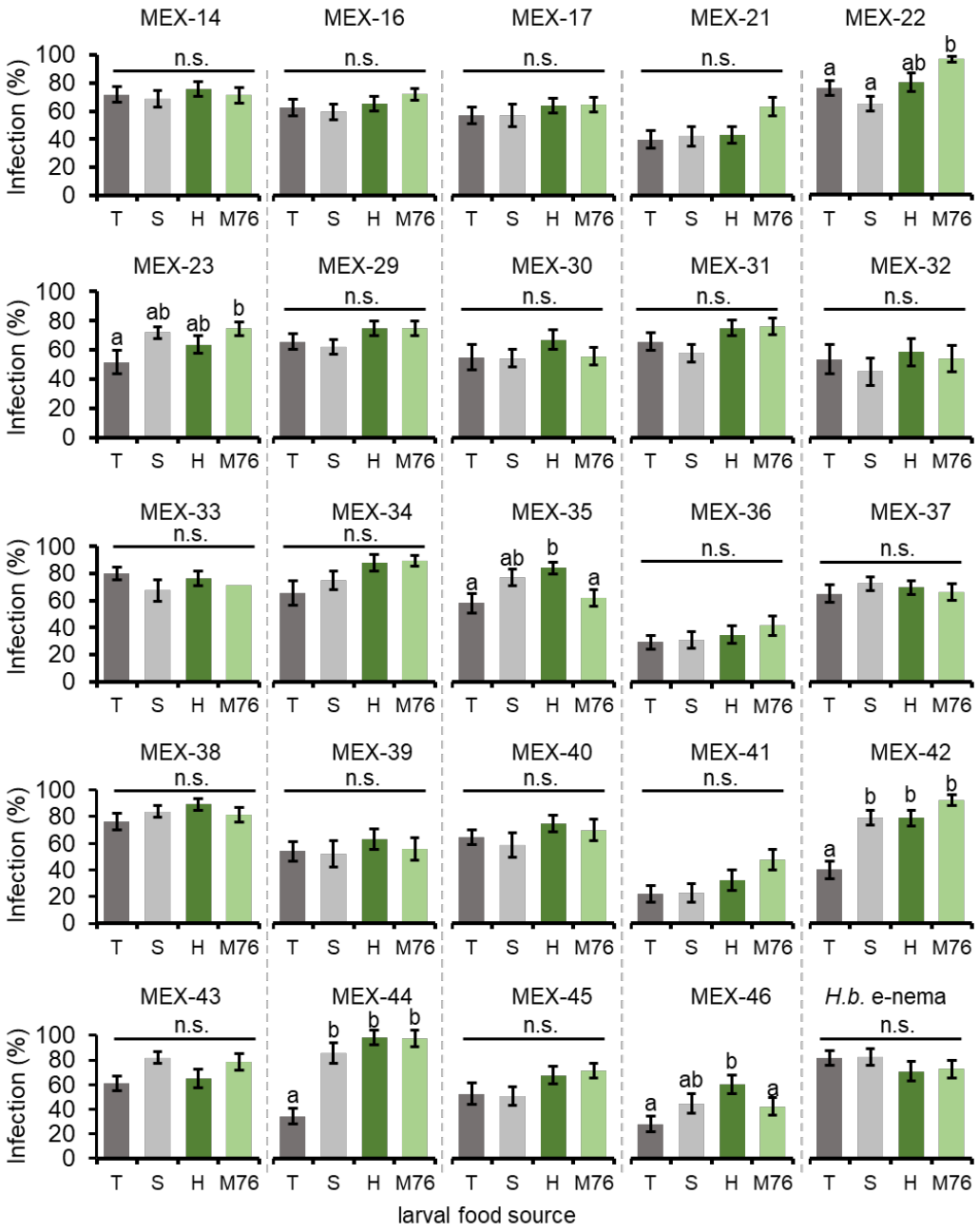

Figure S4. Cucurbitacins do not protect *Diabrotica balteata* larvae against several isolates of *Heterorhabditis bacteriophora* entomopathogenic nematodes. Mean infection rates (%) of 25 isolates of *Heterorhabditis bacteriophora* entomopathogenic nematodes five days after inoculation with 500 ml *H. bacteriophora* (50 nematodes/ml) on four second-instar *D. balteata* larvae previously fed on cucumber plants of the commercial varieties "T" (Tanja, dark grey), "S" (Sonja, light grey), "H" (Hokus, dark green) or "M76" (Marketmore 76, light green) for five days (n=15-20) . Bars indicate average (± SE). P values are given for treatments [generalized linear model (family, Binomial)] followed by pairwise comparisons of Least Squares Means (LSMeans). Not significant (n.s., $p>0.05$) and letters indicate significant differences between treatments ($p<0.05$).

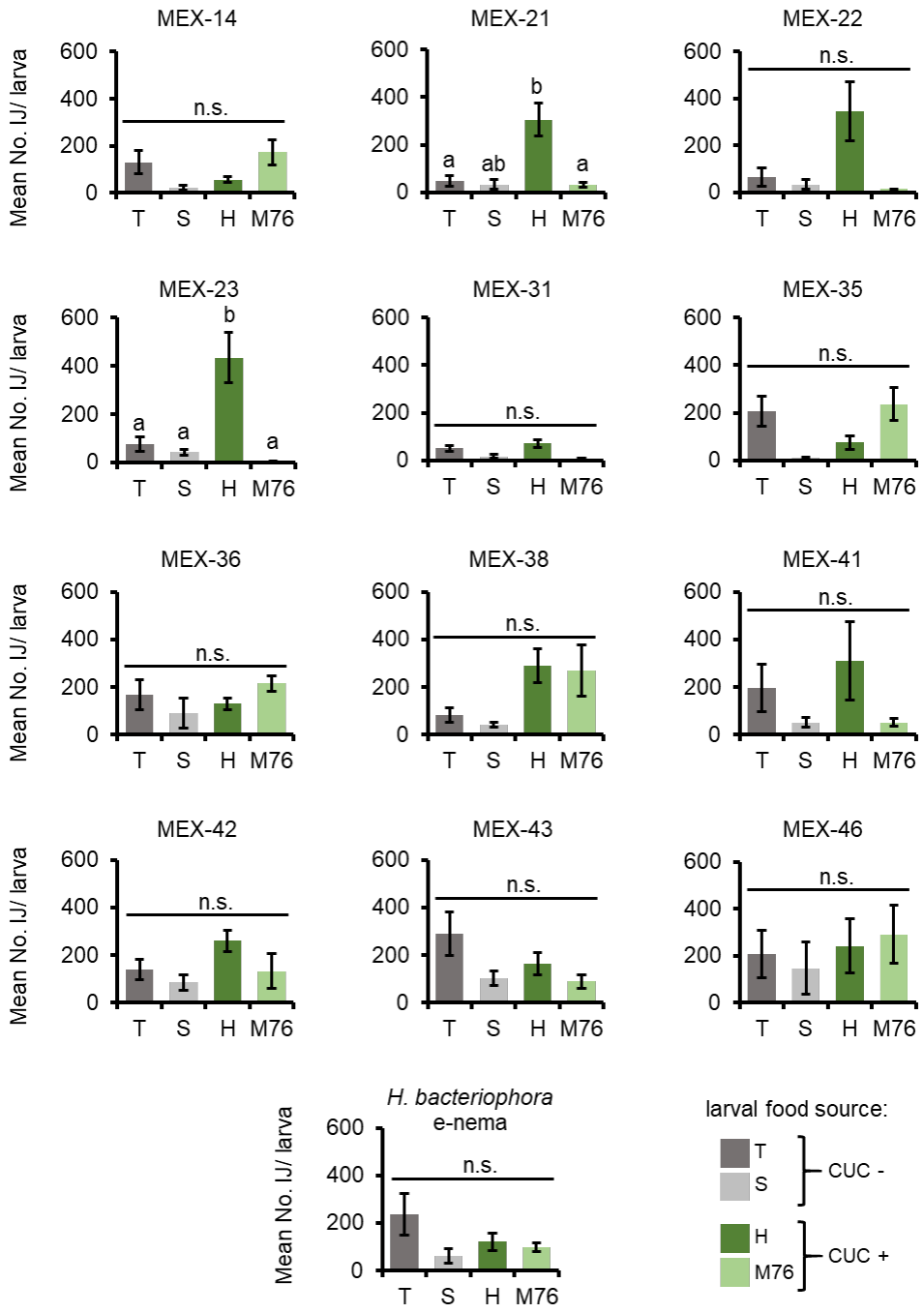

Figure S5. Progeny of *Heterorhabditis bacteriophora* entomopathogenic nematodes from *Diabrotica balteata* larvae fed on cucumber plants. Mean number (%) of Infective Juveniles (IJ) per *D. balteata* larva infected by 13 isolates of *Heterorhabditis bacteriophora* entomopathogenic nematodes 15 days after inoculation with 500 ml of a suspension with *H. bacteriophora* (250 nematodes/ml) on four second-instar *D. balteata* larvae (n=2-10). Prior inoculation (n=10), the larvae fed for five days on cucumber plants of the commercial varieties "T" (Tanja, dark grey), "S" (Sonja, light grey), "H" (Hokus, dark green) or "M76" (Marketmore 76, light green). Bars indicate average (± SE). P values are given for treatments [generalized linear model (family, Binomial)] followed by pairwise comparisons of Least Squares Means (LSMeans). Not significant (n.s., $p>0.05$) and letters indicate significant differences between treatments ($p<0.05$).

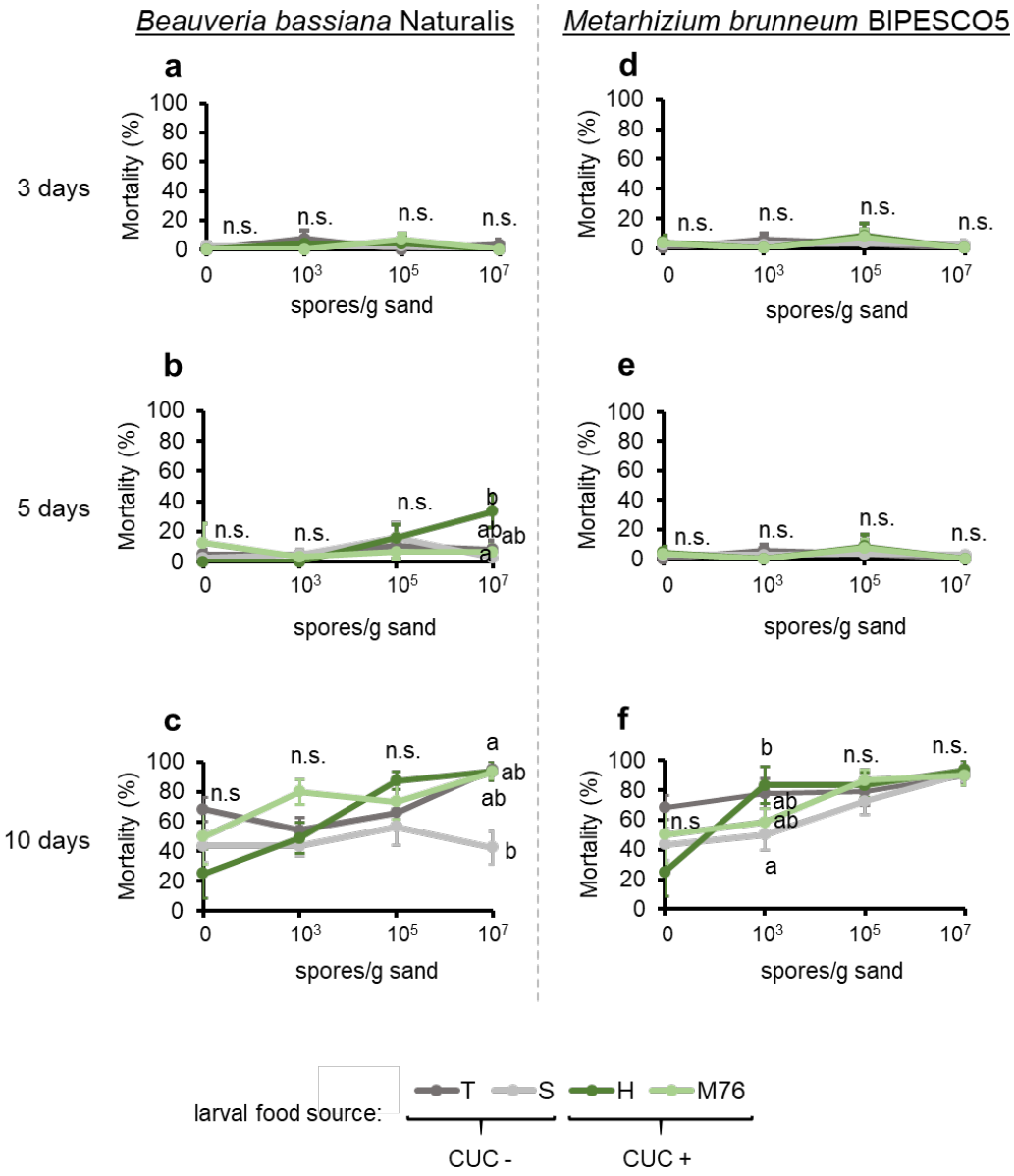

Figure S6. Cucurbitacins do not protect *Diabrotica balteata* larvae against entomopathogenic fungi. Second-instar *D. balteata* larvae fed for five days on cucumber plants of the commercial varieties “T” (Tanja, dark grey), “S” (Sonja, light grey), “H” (Hokus, dark green) or “M76” (Marketmore 76, light green) and then were exposed to entomopathogenic fungi. Mortality (%) of four *D. balteata* larvae infected with *B. bassiana* Naturalis (a-c) or *M. brunneum* BIIPESCO5 (d-f, n=30) after three (a, d), five (b, e) or ten days (c, f) (n=30). Bars indicate average (± SE). *P* values are given for treatments [generalized linear model (family, Binomial)] followed by pairwise comparisons of Least Squares Means (LSMeans). Not significant (n.s., $p>0.05$). Different letters indicate significant differences among plant varieties, $p<0.05$.

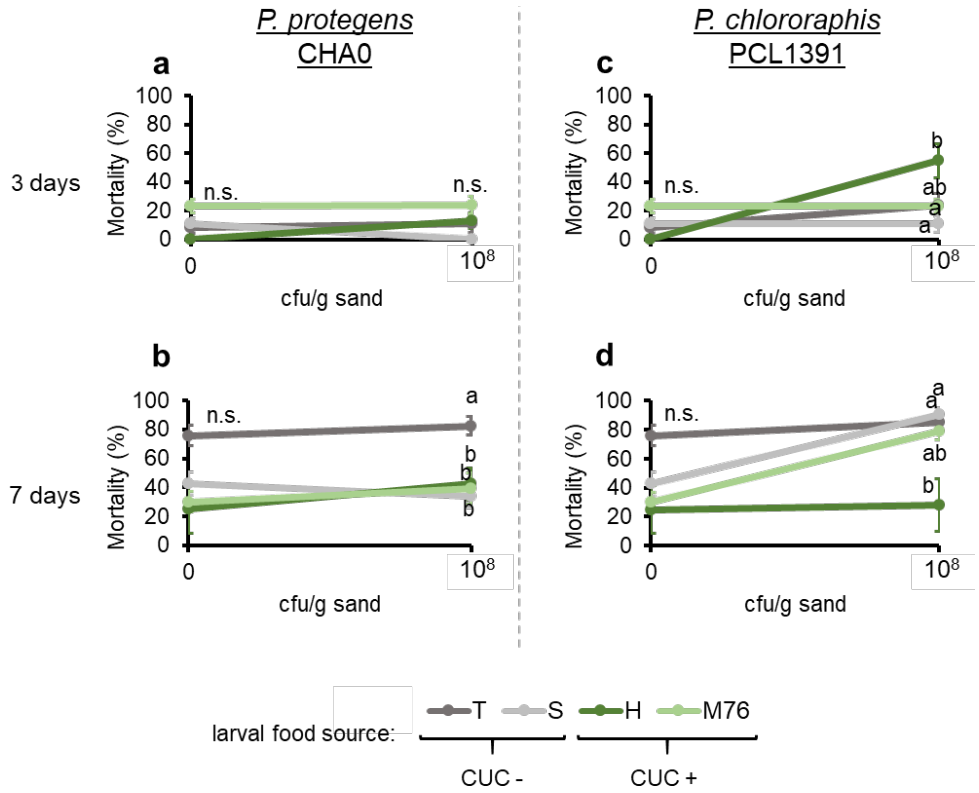

Figure S7. Cucurbitacins do not protect *Diabrotica balteata* larvae against entomopathogenic bacteria. Second-instar *D. balteata* larvae fed for five days on cucumber plants of the commercial varieties "T" (Tanja, dark grey), "S" (Sonja, light grey), "H" (Hokus, dark green) or "M76" (Marketmore 76, light green) and then were exposed to entomopathogenic bacteria. Mortality (%) of four *D. balteata* larvae infected with *P. protegens* CHA0 (a, b) or *P. chlororaphis* PCL1391 (c, d) after three (a, c) or seven days (b, d) (n=30). Bars indicate average (± SE). *P* values are given for treatments [generalized linear model (family, Binomial)] followed by pairwise comparisons of Least Squares Means (LSMeans). Not significant (n.s., $p>0.05$). Different letters indicate significant differences among plant varieties, $p<0.05$.

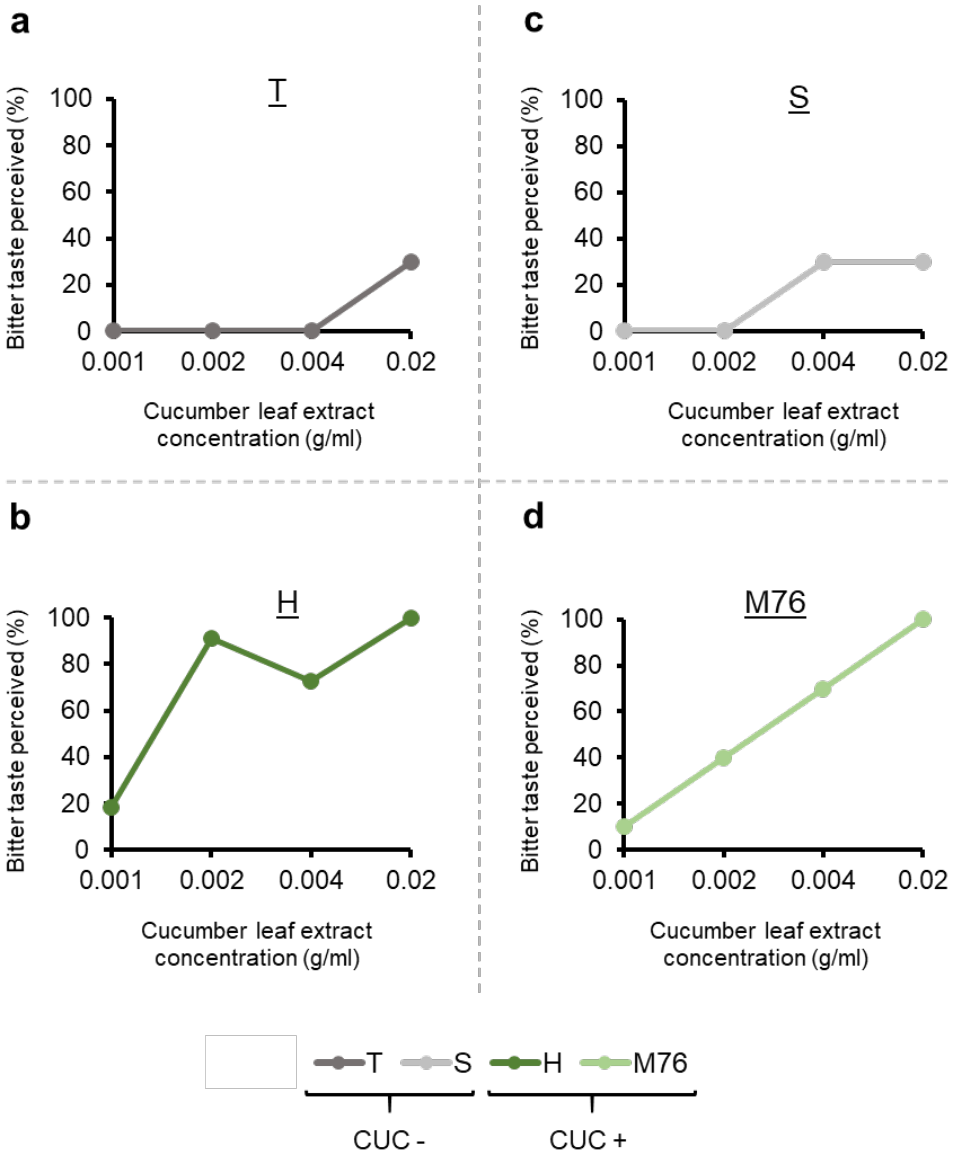

Figure S8. An intense vegetal taste can be confused with bitterness. Threshold of bitterness perception by a tasting panel after tasting 250 µL of four increasing concentrations of aqueous leaf extracts of cucumber commercial varieties “non-bitter” (without cucurbitacins) (a) “T” (Tanja) or (b) “S” (Sonja), or “bitter” (with cucurbitacins) (c) “H” (Hokus) or (d) “M76” (Marketmore 76) (n=10). Lines indicate percentage.

Table S1. Putative identification of cucurbitacins found in plant and larval tissues. Cucurbitacins were putatively identified based on their exact masses (allowing for molecular formula determination) and retention times and compared with those of the standard Cucurbitacin B as well as with available databases such as the Dictionary of Natural Product (CRC Press).

RT (min)	$(M+HCOO)^-$	$(M-H)^-$	MF	Putative identification
2.52	769.4002	723.3948	C38H60O13	Cucurbitacin IIa glucoside
2.44	767.3840	721.3792	C38H58O13	Dihydrocucurbitacin B glucoside
2.16	725.3730	679.3730	C36H56O12	Unknown Cucurbitacin #1
3.02	765.3694	719.3640	C38H56O13	Cuc B glucoside
3.02	605.3328	559.3328	C32H48O8	Cucurbitacin C
3.08	767.3834	721.3779	C38H58O13	Isomer of dihydro-cucurbitacin B glucoside
2.44	727.3903	681.3848	C36H58O12	Metabolized Cucurbitacin # 1
2.49	725.3757	679.3695	C36H56O12	Deacetylated-dihydro-cucurbitacin B glucoside
2.52	723.3651	677.3601	C36H54O12	Unknown Cucurbitacin # 2

Table S2. ANOVA results for the comparisons of cucurbitacin content in cucumber tissues of the variety T, undamaged or damaged by fifteen *D. balteata* larvae freely feeding on the plants for five days. Not significant (n.s., p>0.05).

Plant tissue	Putative cucurbitacin	Cucumber variety T								
		Undamaged			Damaged			F	p-value	symbol
		n	Mean (µg/g FW)	±SE	*n*	Mean (µg/g FW)	±SE			
roots	Cuc C	8	0.00	0.00	7	0.01	0.01	0.82	0.37	n.s.
	Unknown Cuc # 1	8	0.00	0.00	7	0.00	0.00	0.82	0.33	n.s.
	Unknown Cuc # 2	8	0.00	0.00	7	0.06	0.06	1.02	0.32	n.s.
	Deacetylated Dihydro Cuc B glu	8	0.00	0.00	7	0.00	0.00	14.00	0.00	n.s.
	Metabolized Cuc # 1	8	0.00	0.00	7	0.00	0.00	0.00	0.00	n.s.
	Cuc B glu	8	0.00	0.00	7	0.00	0.00	1.00	0.33	n.s.
	Dihydro Cuc B glu	8	0.10	0.03	7	0.14	0.11	1.30	0.33	n.s.
	Isomer of Dihydro Cuc B glu	8	0.00	0.00	7	0.00	0.00	1.00	0.33	n.s.
	Cuc II a glu	8	0.00	0.00	7	0.00	0.00	1.00	0.33	n.s.
	TOTAL	**8**	**0.10**	**0.03**	**7**	**0.22**	**0.19**	**1.81**	**0.19**	**n.s.**
stems	Cuc C	8	0.00	0.00	8	0.00	0.00	0.36	0.55	n.s.
	Unknown Cuc # 1	8	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Unknown Cuc # 2	8	0.00	0.00	8	0.01	0.01	1.00	0.33	n.s.
	Deacetylated Dihydro Cuc B glu	8	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Metabolized Cuc # 1	8	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Cuc B glu	8	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Dihydro Cuc B glu	8	0.36	0.12	8	0.24	0.11	0.56	0.46	n.s.
	Isomer of Dihydro Cuc B glu	8	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Cuc II a glu	8	0.01	0.01	8	0.01	0.01	0.03	0.87	n.s.
	TOTAL	**8**	**0.37**	**0.13**	**8**	**0.26**	**0.13**	**0.43**	**0.51**	**n.s.**
cotyledons	Cuc C	8	0.00	0.00	8	0.00	0.00	1.00	0.33	n.s.
	Unknown Cuc # 1	8	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Unknown Cuc # 2	8	0.01	0.01	8	0.00	0.00	1.00	0.33	n.s.
	Deacetylated Dihydro Cuc B glu	8	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Metabolized Cuc # 1	8	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Cuc B glu	8	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Dihydro Cuc B glu	8	0.19	0.06	8	0.12	0.03	0.81	0.38	n.s.
	Isomer of Dihydro Cuc B glu	8	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Cuc II a glu	8	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	TOTAL	**8**	**0.20**	**0.07**	**8**	**0.12**	**0.03**	**1.20**	**0.29**	**n.s.**
leaves	Cuc C	7	0.00	0.00	7	0.00	0.00	2.39	0.14	n.s.
	Unknown Cuc # 1	7	0.00	0.00	7	0.00	0.00	0.00	0.00	n.s.
	Unknown Cuc # 2	7	0.00	0.00	7	0.01	0.01	0.758	0.40	n.s.
	Deacetylated Dihydro Cuc B glu	7	0.00	0.00	7	0.00	0.00	1.00	0.33	n.s.
	Metabolized Cuc # 1	7	0.00	0.00	7	0.00	0.00	0.00	0.00	n.s.
	Cuc B glu	7	0.00	0.00	7	0.00	0.00	0.00	0.00	n.s.
	Dihydro Cuc B glu	7	0.14	0.04	7	0.15	0.07	0.01	0.89	n.s.
	Isomer of Dihydro Cuc B glu	7	0.02	0.02	7	0.00	0.00	1.00	0.33	n.s.
	Cuc II a glu	7	0.00	0.00	7	0.00	0.00	0.60	0.45	n.s.
	TOTAL	**7**	**0.16**	**0.06**	**7**	**0.17**	**0.08**	**0.002**	**0.95**	**n.s.**

Table S3. ANOVA results for the comparisons of cucurbitacin content in cucumber tissues of the variety S, undamaged or damaged by fifteen *D. balteata* larvae freely feeding on the plants for five days. Not significant (n.s., p>0.05) and stars indicate differences between treatments: *p<0.05.

Plant tissue	Putative identification of the cucurbitacin	Cucumber variety S								
		Undamaged			Damaged			F	p-value	symbol
		n	Mean (µg/g FW)	±SE	*n*	Mean (µg/g FW)	±SE			
roots	Cuc C	8	0.00	0.00	8	0.00	0.00	1.00	0.33	n.s.
	Unknown Cuc # 1	8	0.00	0.00	8	0.00	0.00	1.00	0.33	n.s.
	Unknown Cuc # 2	8	0.00	0.00	8	0.01	0.01	1.00	0.33	n.s.
	Deacetylated Dihydro Cuc B glu	8	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Metabolized Cuc # 1	8	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Cuc B glu	8	0.00	0.00	8	0.00	0.00	1.00	0.33	n.s.
	Dihydro Cuc B glu	8	0.19	0.05	8	0.11	0.03	0.01	0.90	n.s.
	Isomer of Dihydro Cuc B glu	8	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Cuc II a glu	8	0.01	0.01	8	0.00	0.00	0.24	0.24	n.s.
	TOTAL	**8**	**0.21**	**0.06**	**8**	**0.12**	**0.04**	**0.02**	**0.88**	**n.s.**
stems	Cuc C	7	0.00	0.00	9	0.00	0.00	1.15	0.30	n.s.
	Unknown Cuc # 1	7	0.00	0.00	9	0.00	0.00	0.00	0.00	n.s.
	Unknown Cuc # 2	7	0.00	0.00	9	0.00	0.00	0.00	0.00	n.s.
	Deacetylated Dihydro Cuc B glu	7	0.00	0.00	9	0.00	0.00	0.00	0.00	n.s.
	Metabolized Cuc # 1	7	0.00	0.00	9	0.00	0.00	0.00	0.00	n.s.
	Cuc B glu	7	0.00	0.00	9	0.00	0.00	0.00	0.00	n.s.
	Dihydro Cuc B glu	7	0.33	0.13	9	0.18	0.07	0.85	0.00	n.s.
	Isomer of Dihydro Cuc B glu	7	0.00	0.00	9	0.00	0.00	0.00	0.00	n.s.
	Cuc II a glu	7	0.00	0.00	9	0.01	0.01	0.55	0.55	n.s.
	TOTAL	**7**	**0.00**	**0.13**	**81**	**0.01**	**0.07**	**0.77**	**0.39**	**n.s.**
cotyledons	Cuc C	8	0.01	0.00	8	0.00	0.00	2.97	0.10	n.s.
	Unknown Cuc # 1	8	0.00	0.00	8	0.00	0.00	0.004	0.94	n.s.
	Unknown Cuc # 2	8	0.00	0.00	8	0.12	0.12	0.88	0.36	n.s.
	Deacetylated Dihydro Cuc B glu	8	0.00	0.00	8	0.00	0.00	1.13	0.30	n.s.
	Metabolized Cuc # 1	8	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Cuc B glu	8	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Dihydro Cuc B glu	8	0.37	0.08	8	0.16	0.04	7.30	0.01	*
	Isomer of Dihydro Cuc B glu	8	0.03	0.03	8	0.00	0.00	1.13	0.30	n.s.
	Cuc II a glu	8	0.01	0.01	8	0.00	0.00	2.31	0.14	n.s.
	TOTAL	**8**	**0.42**	**0.12**	**8**	**0.28**	**0.16**	**1.43**	**0.24**	**n.s.**
leaves	Cuc C	7	0.00	0.00	8	0.01	0.01	1.14	0.30	n.s.
	Unknown Cuc # 1	7	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Unknown Cuc # 2	7	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Deacetylated Dihydro Cuc B glu	7	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Metabolized Cuc # 1	7	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Cuc B glu	7	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Dihydro Cuc B glu	7	0.24	0.09	8	0.25	0.09	0.001	0.97	n.s.
	Isomer of Dihydro Cuc B glu	7	0.00	0.00	8	0.00	0.00	0.86	0.36	n.s.
	Cuc II a glu	7	0.00	0.00	8	0.01	0.00	4.34	0.05	n.s.
	TOTAL	**7**	**0.24**	**0.10**	**8**	**0.27**	**0.10**	**0.02**	**0.87**	**n.s.**

Table S4. ANOVA results for the comparisons of cucurbitacin content in cucumber tissues of the variety H, undamaged or damaged by fifteen *D. balteata* larvae freely feeding on the plants for five days. Not significant (n.s., p>0.05) and stars indicate differences between treatments: *p<0.05.

Plant tissue	Putative identification of the cucurbitacin	Cucumber variety H								
		Undamaged			Damaged			F	p-value	symbol
		n	Mean (µg/g FW)	±SE	*n*	Mean (µg/g FW)	±SE			
roots	Cuc C	8	0.00	0.00	8	0.01	0.00	0.86	0.36	n.s.
	Unknown Cuc # 1	8	0.01	0.00	8	0.01	0.00	0.00	0.95	n.s.
	Unknown Cuc # 2	8	0.00	0.00	8	0.00	0.00	0.88	0.36	n.s.
	Deacetylated Dihydro Cuc B glu	8	0.00	0.00	8	0.00	0.00	0.88	0.36	n.s.
	Metabolized Cuc # 1	8	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Cuc B glu	8	0.13	0.06	8	0.18	0.05	0.39	0.53	n.s.
	Dihydro Cuc B glu	8	0.30	0.08	8	0.40	0.07	1.00	0.33	n.s.
	Isomer of Dihydro Cuc B glu	8	0.00	0.00	8	0.01	0.01	1.96	0.18	n.s.
	Cuc II a glu	8	0.02	0.01	8	0.04	0.01	2.87	0.11	n.s.
	TOTAL	**8**	**0.46**	**0.15**	**8**	**0.65**	**0.15**	**1.11**	**0.30**	**n.s.**
stems	Cuc C	8	0.04	0.01	9	0.31	0.11	5.21	0.037	*
	Unknown Cuc # 1	8	0.00	0.00	9	0.00	0.00	0.96	0.34	n.s.
	Unknown Cuc # 2	8	0.00	0.00	9	0.00	0.00	1.63	0.22	n.s.
	Deacetylated Dihydro Cuc B glu	8	0.01	0.00	9	0.01	0.00	0.25	0.61	n.s.
	Metabolized Cuc # 1	8	0.00	0.00	9	0.00	0.00	0.00	0.00	n.s.
	Cuc B glu	8	0.15	0.03	9	0.19	0.04	0.83	0.37	n.s.
	Dihydro Cuc B glu	8	5.02	0.50	9	4.26	0.43	1.32	0.26	n.s.
	Isomer of Dihydro Cuc B glu	8	0.07	0.02	9	0.06	0.02	0.17	0.68	n.s.
	Cuc II a glu	8	3.15	0.22	9	3.20	0.28	0.01	0.89	n.s.
	TOTAL	**8**	**8.44**	**0.78**	**9**	**8.04**	**0.88**	**0.14**	**0.71**	**n.s.**
cotyledons	Cuc C	7	0.72	0.29	7	2.87	1.15	3.30	0.09	n.s.
	Unknown Cuc # 1	7	0.00	0.00	7	0.00	0.00	0.00	0	n.s.
	Unknown Cuc # 2	7	0.04	0.02	7	0.02	0.02	0.97	0.34	n.s.
	Deacetylated Dihydro Cuc B glu	7	0.00	0.00	7	0.00	0.00	0.00	0.00	n.s.
	Metabolized Cuc # 1	7	0.00	0.00	7	0.00	0.00	0.00	0.00	n.s.
	Cuc B glu	7	0.00	0.00	7	0.00	0.00	0.00	0.00	n.s.
	Dihydro Cuc B glu	7	27.41	2.03	7	24.47	0.63	1.92	0.19	n.s.
	Isomer of Dihydro Cuc B glu	7	0.00	0.00	7	0.00	0.00	1.00	0.33	n.s.
	Cuc II a glu	7	7.13	0.63	7	6.73	0.37	0.30	0.59	n.s.
	TOTAL	**7**	**35.30**	**2.96**	**7**	**34.09**	**2.16**	**0.17**	**0.68**	**n.s.**
leaves	Cuc C	7	4.90	2.60	8	7.09	0.95	0.62	0.447	n.s.
	Unknown Cuc # 1	7	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Unknown Cuc # 2	7	0.09	0.05	8	0.09	0.09	0.0006	0.98	n.s.
	Deacetylated Dihydro Cuc B glu	7	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Metabolized Cuc # 1	7	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Cuc B glu	7	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Dihydro Cuc B glu	7	55.79	3.35	8	52.70	4.14	0.33	0.57	n.s.
	Isomer of Dihydro Cuc B glu	7	0.00	0.00	8	0.00	0.00	1.00	0.33	n.s.
	Cuc II a glu	7	4.62	0.80	8	5.29	0.63	0.05	0.81	n.s.
	TOTAL	**7**	**65.40**	**6.80**	**8**	**65.17**	**5.80**	**0.02**	**0.87**	**n.s.**

Table S5. ANOVA results for the comparisons of cucurbitacin content in cucumber tissues of the variety M76, undamaged or damaged by fifteen *D. balteata* larvae freely feeding on the plants for five days. Not significant (n.s., p>0.05) and stars indicate differences between treatments: *p<0.05, **p<0.01.

Plant tissue	Putative identification of the cucurbitacin	Cucumber variety M76								
		Undamaged			Damaged			F	p-value	symbol
		n	Mean (µg/g FW)	±SE	*n*	Mean (µg/g FW)	±SE			
roots	Cuc C	8	0.00	0.00	8	0.00	0.00	0.05	0.81	n.s.
	Unknown Cuc # 1	8	0.01	0.00	8	0.02	0.01	1.29	0.27	n.s.
	Unknown Cuc # 2	8	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Deacetylated Dihydro Cuc B glu	8	0.00	0.00	8	0.00	0.00	0.0005	0.99	n.s.
	Metabolized Cuc # 1	8	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Cuc B glu	8	0.24	0.09	8	0.32	0.09	0.41	0.53	n.s.
	Dihydro Cuc B glu	8	0.44	0.15	8	0.34	0.15	0.22	0.64	n.s.
	Isomer of Dihydro Cuc B glu	8	0.03	0.03	8	0.03	0.02	0.01	0.89	n.s.
	Cuc II a glu	8	0.06	0.02	8	0.02	0.01	1.62	0.22	n.s.
	TOTAL	**8**	**0.78**	**0.29**	**8**	**0.74**	**0.28**	**0.01**	**0.90**	**n.s.**
stems	Cuc C	7	0.10	0.03	8	0.53	0.31	1.64	0.22	n.s.
	Unknown Cuc # 1	7	0.00	0.00	8	0.01	0.01	0.34	0.56	n.s.
	Unknown Cuc # 2	7	0.01	0.01	8	0.00	0.00	1.15	0.30	n.s.
	Deacetylated Dihydro Cuc B glu	7	0.00	0.00	8	0.00	0.00	0.56	0.46	n.s.
	Metabolized Cuc # 1	7	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Cuc B glu	7	0.24	0.05	8	0.23	0.06	0.02	0.88	n.s.
	Dihydro Cuc B glu	7	5.95	0.57	8	5.81	0.77	0.02	0.88	n.s.
	Isomer of Dihydro Cuc B glu	7	0.06	0.01	8	0.03	0.01	4.06	0.06	n.s.
	Cuc II a glu	7	3.46	0.38	8	3.40	0.33	0.01	0.91	n.s.
	TOTAL	**7**	**9.82**	**1.06**	**8**	**10.00**	**1.49**	**0.01**	**0.9**	**n.s.**
cotyledons	Cuc C	8	0.98	0.36	8	3.09	1.36	2.25	0.15	n.s.
	Unknown Cuc # 1	8	0.00	0.00	8	0.00	0.00	1.00	0.33	n.s.
	Unknown Cuc # 2	8	0.07	0.03	8	0.20	0.11	1.32	0.26	n.s.
	Deacetylated Dihydro Cuc B glu	8	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Metabolized Cuc # 1	8	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Cuc B glu	8	0.00	0.00	8	0.00	0.00	0.12	0.72	n.s.
	Dihydro Cuc B glu	8	25.65	0.50	8	27.34	1.38	1.32	0.26	n.s.
	Isomer of Dihydro Cuc B glu	8	0.02	0.02	8	0.00	0.00	1.00	0.33	n.s.
	Cuc II a glu	8	5.28	0.32	8	6.48	0.40	5.60	0.03	*
	TOTAL	**8**	**31.99**	**1.22**	**8**	**37.11**	**3.25**	**3.40**	**0.08**	**n.s.**
leaves	Cuc C	8	2.09	1.00	8	6.21	1.42	5.66	0.03	*
	Unknown Cuc # 1	8	0.00	0.00	8	0.00	0.00	1	0.33	n.s.
	Unknown Cuc # 2	8	0.04	0.02	8	0.11	0.06	1.05	0.32	n.s.
	Deacetylated Dihydro Cuc B glu	8	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Metabolized Cuc # 1	8	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Cuc B glu	8	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Dihydro Cuc B glu	8	40.24	4.70	8	49.43	4.02	2.20	0.15	n.s.
	Isomer of Dihydro Cuc B glu	8	0.00	0.00	8	0.00	0.00	0.00	0.00	n.s.
	Cuc II a glu	8	2.28	0.34	8	4.00	0.42	10.06	0.006	**
	TOTAL	**8**	**44.65**	**6.06**	**8**	**59.75**	**5.92**	**3.80**	**0.07**	**n.s.**

Table S6. ANOVA results for the comparisons of content of cucurbitacin sequestered by *D. balteata* larvae fed on cucumber full plants (shoots + roots), or only on roots or shoots for five days. P values are given for treatments [generalized linear model (family, Gaussian)] followed by pairwise comparisons of Least Squares Means (LSMeans). Letters indicate significant differences between treatments (p<0.05).

Putative identification of the cucurbitacin	Shoots + Roots				Roots				Shoots				F	p-value
	n	Mean (µg/g FW)	±SE	group	*n*	Mean (µg/g FW)	±SE	group	*n*	Mean (µg/g FW)	±SE	group		
T-fed *D. balteata* larvae														
Cuc C	5	0.01	0.01	a	5	0.00	0.00	a	5	0.00	0.00	a	2.64	0.11
Unknown Cuc # 1	5	0.00	0.00	a	5	0.00	0.00	a	5	0.00	0.00	a	1.00	0.39
Unknown Cuc # 2	5	0.00	0.00	a	5	0.00	0.00	a	5	0.00	0.00	a	0.00	0.00
Deacetylated Dihydro Cuc B glu	5	0.01	0.01	a	5	0.00	0.00	a	5	0.00	0.00	a	2.66	0.11
Metabolized Cuc # 1	5	0.00	0.00	a	5	0.00	0.00	a	5	0.00	0.00	a	1.00	0.39
Cuc B glu	5	0.02	0.00	a	5	0.00	0.00	b	5	0.00	0.00	b	15.75	0.001
Dihydro Cuc B glu	5	0.14	0.03	a	5	0.04	0.04	b	5	0.01	0.01	b	5.60	0.01
Isomer of Dihydro Cuc B glu	5	0.00	0.00	a	5	0.00	0.00	a	5	0.00	0.00	a	1.00	0.39
Cuc II a glu	5	0.04	0.01	a	5	0.00	0.00	b	5	0.00	0.00	b	12.38	0.001
TOTAL	5	0.23	0.07	a	5	0.04	0.04	b	5	0.01	0.01	b	10.76	0.002
S-fed *D. balteata* larvae														
Cuc C	5	0.02	0.01	a	5	0.00	0.00	b	5	0.00	0.00	b	5.72	0.01
Unknown Cuc # 1	5	0.01	0.01	a	5	0.00	0.00	a	5	0.00	0.00	a	1.00	0.39
Unknown Cuc # 2	5	0.01	0.01	a	5	0.00	0.00	a	5	0.00	0.00	a	2.39	0.13
Deacetylated Dihydro Cuc B glu	5	0.01	0.01	a	5	0.00	0.00	a	5	0.00	0.00	a	2.56	0.11
Metabolized Cuc # 1	5	0.00	0.00	a	5	0.00	0.00	a	5	0.00	0.00	a	0.00	0.00
Cuc B glu	5	0.01	0.01	a	5	0.00	0.00	a	5	0.00	0.00	a	2.65	0.11
Dihydro Cuc B glu	5	0.07	0.01	a	5	0.08	0.08	a	5	0.00	0.00	a	0.89	0.43
Isomer of Dihydro Cuc B glu	5	0.01	0.01	a	5	0.00	0.00	a	5	0.00	0.00	a	2.51	0.12
Cuc II a glu	5	0.03	0.00	a	5	0.00	0.00	b	5	0.00	0.00	b	83.23	<0.001
TOTAL	5	0.17	0.06	a	5	0.08	0.08	a	5	0.00	0.00	a	2.73	0.10
H-fed *D. balteata* larvae														
Cuc C	5	0.03	0.00	a	5	0.00	0.00	b	5	0.00	0.00	b	45.23	<0.001
Unknown Cuc # 1	5	5.45	2.48	a	5	2.48	0.27	ab	5	0.00	0.00	b	3.57	0.06
Unknown Cuc # 2	5	2.12	0.49	a	5	1.74	0.56	a	5	0.05	0.04	b	6.53	0.01
Deacetylated Dihydro Cuc B glu	5	16.26	4.02	a	5	20.08	3.26	a	5	1.44	0.40	b	10.76	0.002
Metabolized Cuc # 1	5	4.26	1.01	a	5	1.31	0.26	b	5	1.67	0.44	b	6.05	0.01
Cuc B glu	5	2.40	0.98	a	5	3.61	0.48	a	5	0.01	0.01	b	8.49	0.005
Dihydro Cuc B glu	5	0.07	0.02	a	5	0.00	0.00	b	5	0.00	0.00	b	11.48	0.002
Isomer of Dihydro Cuc B glu	5	3.59	1.09	a	5	2.13	0.31	ab	5	0.00	0.00	b	7.66	0.007
Cuc II a glu	5	0.02	0.01	a	5	0.00	0.00	a	5	0.00	0.00	a	1.68	0.22
TOTAL	5	34.20	10.10	a	5	31.34	5.15	a	5	3.18	0.89	b	8.15	0.006
M76-fed *D. balteata* larvae														
Cuc C	5	0.02	0.00	a	5	0.00	0.00	b	5	0.00	0.00	b	111.01	<0.001
Unknown Cuc # 1	5	4.73	0.85	a	5	2.22	0.23	b	5	0.16	0.06	c	20.09	0.00
Unknown Cuc # 2	5	1.93	0.31	a	5	1.64	0.25	a	5	0.00	0.00	b	20.78	0.00
Deacetylated Dihydro Cuc B glu	5	15.56	2.43	a	5	15.78	2.27	a	5	0.69	0.16	b	20.25	0.00
Metabolized Cuc # 1	5	4.99	1.21	a	5	1.17	0.44	b	5	1.48	0.15	b	8.00	0.006
Cuc B glu	5	3.32	1.46	a	5	2.42	0.73	a	5	0.27	0.11	a	2.75	0.10
Dihydro Cuc B glu	5	0.03	0.02	a	5	0.00	0.00	a	5	0.00	0.00	a	3.62	0.05
Isomer of Dihydro Cuc B glu	5	3.97	1.76	a	5	1.13	0.16	ab	5	0.04	0.02	b	3.96	0.04
Cuc II a glu	5	0.01	0.01	a	5	0.00	0.00	b	5	0.00	0.00	b	5.34	0.02
TOTAL	5	34.57	8.04	a	5	24.36	4.07	a	5	2.65	0.50	b	12.02	0.001

Table S7. ANOVA results for the comparisons of weight gain by *D. balteata* larvae fed on cucumber full plants (shoots + roots), or only on roots or shoots for five days. P values are given for treatments [generalized linear model (family, Gaussian)] followed by pairwise comparisons of Least Squares Means (LSMeans). Letters indicate significant differences between treatments ($p<0.05$).

Treatment		T-fed *D. balteata* larvae				S-fed *D. balteata* larvae				H-fed *D. balteata* larvae				M76-fed *D. balteata* larvae				F	p-value
Plant part consumed	**Time (days)**	*n*	(mg/ larva)	±SE	group	*n*	(mg/ larva)	±SE	group	*n*	(mg/ larva)	±SE	group	*n*	(mg/ larva)	±SE	group		
Full plants	3	5	1.14	0.09	ab	5	1.27	0.06	b	5	0.97	0.09	a	5	0.90	0.03	a	5.35	0.009
	5	5	2.15	0.21	a	5	2.43	0.12	a	5	2.44	0.13	a	5	1.78	0.25	a	2.75	0.07
	total	5	3.28	0.30	ab	5	3.70	0.18	b	5	3.41	0.22	ab	5	2.68	0.28	a	4.32	0.02
Roots	3	5	0.95	0.11	a	5	1.07	0.10	a	5	0.45	0.02	b	5	0.53	0.06	b	13.90	<0.001
	5	5	0.79	0.15	a	5	0.52	0.14	a	5	0.89	0.04	a	5	0.81	0.07	a	1.97	0.15
	total	5	1.75	0.26	a	5	1.60	0.25	ab	5	1.34	0.06	b	5	1.34	0.13	b	4.91	0.01
Shoots	3	5	0.98	0.16	ab	5	0.69	0.09	a	5	1.19	0.09	b	5	0.94	0.07	ab	3.65	0.03
	5	5	0.43	0.08	a	5	0.75	0.13	a	5	0.45	0.11	a	5	0.69	0.05	a	2.93	0.06
	total	5	1.41	0.24	a	5	1.44	0.21	a	5	1.64	0.20	a	5	1.63	0.12	a	1.63	0.22

Table S8. ANOVA results for the comparisons of mortality rates of *D. balteata* larvae fed on cucumber full plants (shoots + roots), or only on roots or shoots for five days. P values are given for treatments [generalized linear model (family, binomial)] followed by pairwise comparisons of Least Squares Means (LSMeans). Letters indicate significant differences between treatments ($p<0.05$).

Treatment		T-fed *D. balteata* larvae				S-fed *D. balteata* larvae				H-fed *D. balteata* larvae				M76-fed *D. balteata* larvae				Chisq	p-value
Plant part consumed	Time (days)	*n*	Mortality (%)	±SE	group	*n*	Mortality (%)	±SE	group	*n*	Mortality (%)	±SE	group	*n*	Mortality (%)	±SE	group		
Full plants	3	5	0.00	0.00	a	5	2.67	1.63	a	5	1.33	1.33	a	5	0.00	0.00	a	2.56 E-10	1.00
	5	5	5.33	2.49	a	5	6.67	2.11	a	5	3.92	1.60	a	5	2.67	1.63	a	7.02	0.20
	total	5	5.33	2.49	a	5	9.33	3.74	a	5	5.25	2.93	a	5	2.67	1.63	a	17.65	0.23
Roots	3	5	5.33	2.49	a	5	4.00	1.63	a	5	6.67	3.65	a	5	3.92	1.60	a	2.56 E-10	1.00
	5	5	6.67	2.11	a	5	2.67	1.63	a	5	10.67	3.40	a	5	9.33	2.67	a	18.03	0.85
	total	5	12.00	4.60	a	5	6.67	3.27	a	5	17.33	7.05	a	5	13.25	4.27	a	15.70	0.201
Shoots	3	5	2.67	2.67	a	5	1.25	1.25	a	5	2.58	1.58	a	5	1.33	1.33	a	2.56 E-10	1.00
	5	5	5.33	2.49	a	5	3.92	1.60	a	5	1.33	1.33	a	5	6.67	3.65	a	16.82	0.86
	total	5	8.00	5.16	a	5	5.17	2.85	a	5	3.92	2.92	a	5	8.00	4.98	a	18.40	0.35

Table S9. ANOVA results for the comparisons of predation rates of *D. balteata* larvae 48 hours after exposure to insect predators. Previous exposure to predators, the larvae fed freely on cucumber plants of the varieties T, S, H or M76 for five days. P values are given for treatments [generalized linear model (family, binomial or quasibinomial)] followed by pairwise comparisons of Least Squares Means (LSMeans). Letters indicate significant differences between treatments ($p<0.05$).

Natural enemies	T-fed *D. balteata* larvae			S-fed *D. balteata* larvae			H-fed *D. balteata* larvae			M76-fed *D. balteata* larvae			Test	p-value
	n	Predation (%)	group	*n*	Predation (%)	group	*n*	Predation (%)	group	*n*	Predation (%)	group		
Atheta Coriaria	30	50	a	30	43.33	a	30	43.33	a	30	46.67	a	F=0.12	0.948
Chrysoperla carnea	30	76.67	a	30	76.67	a	30	83.33	a	30	83.33	a	Chisq= 119.26	0.841
Orius laevigatus	30	46.66	a	30	50	a	30	40	a	30	50	a	F=0.26	0.854

Table S10. ANOVA results for the comparisons of infection rates of *D. balteata* larvae five days after inoculation with 25 isolates of *Heterorhabditis bacteriophora* entomopathogenic nematodes (EPN). Previous exposure to predators, the larvae fed freely on cucumber plants of the varieties T, S, H or M76 for five days. P values are given for treatments [generalized linear model (family, binomial or quasibinomial)] followed by pairwise comparisons of Least Squares Means (LSMeans). Letters indicate significant differences between treatments ($p<0.05$).

EPN isolate	T-fed *D. balteata* larvae				S-fed *D. balteata* larvae				H-fed *D. balteata* larvae				M76-fed *D. balteata* larvae				Test	p-value
	n	Infection (%)	±SE	group	*n*	Infection (%)	±SE	group	*n*	Infection (%)	±SE	group	*n*	Infection (%)	±SE	group		
MEX-14	20	71.67	5.65	a	20	68.75	5.98	a	20	76.00	5.24	a	20	71.25	5.81	a	F=0.31	0.81
MEX-16	15	62.22	5.80	a	17	59.41	5.62	a	20	65.00	5.26	a	20	71.75	4.45	a	Chisq= 74.02	0.46
MEX-17	15	61.67	6.39	a	15	61.67	8.56	a	15	69.44	5.90	a	14	70.24	5.78	a	F=0.39	0.75
MEX-21	20	39.58	6.31	a	20	41.67	7.05	a	20	42.50	5.89	a	20	62.92	6.55	a	F=2.84	0.04
MEX-22	15	76.11	5.20	a	18	65.28	5.36	a	17	80.39	6.66	ab	20	97.08	2.03	b	Chisq= 66.97	<0.001
MEX-23	15	51.67	8.26	a	17	71.86	4.26	ab	20	63.75	5.87	ab	20	74.50	4.62	b	F=2.78	0.04
MEX-29	20	65.83	5.20	a	20	62.08	4.93	a	20	75.00	5.13	a	20	75.00	5.13	a	F=1.66	0.18
MEX-30	15	55.00	8.86	a	17	54.41	6.15	a	20	67.08	6.55	a	20	55.50	6.00	a	F=0.78	0.51
MEX-31	20	65.83	6.07	a	20	57.92	5.94	a	20	75.00	5.44	a	20	76.25	5.58	a	F=2.02	0.12
MEX-32	15	52.78	9.95	a	15	44.44	9.47	a	15	57.78	9.18	a	15	53.33	8.90	a	F=0.23	0.87
MEX-33	20	80.00	4.66	a	20	67.50	7.93	a	20	76.25	5.58	a	20	71.25	5.81	a	F=0.77	0.51
MEX-34	15	65.56	9.23	a	18	75.00	6.84	a	17	87.75	6.19	a	20	89.58	3.91	a	F=2.55	0.06
MEX-35	15	55.56	6.84	a	17	73.82	5.87	ab	20	80.50	4.13	b	20	59.00	5.86	a	F=3.94	0.01
MEX-36	20	30.00	5.45	a	20	31.67	6.24	a	20	35.83	6.40	a	20	42.50	7.50	a	F=0.99	0.40
MEX-37	20	65.00	6.64	a	20	72.50	5.10	a	20	69.58	5.00	a	19	66.23	5.96	a	F=0.33	0.79
MEX-38	15	76.67	6.20	a	18	83.80	4.49	a	17	89.22	4.67	a	20	81.67	5.38	a	F=1.13	0.34
MEX-39	15	53.33	7.44	a	15	51.67	9.80	a	15	62.22	7.84	a	15	55.00	8.40	a	F=0.56	0.64
MEX-40	15	63.89	5.37	a	14	57.74	8.89	a	14	73.81	5.92	a	15	68.89	7.78	a	F=1.29	0.28
MEX-41	20	22.08	6.14	a	20	22.50	6.51	a	19	32.02	7.60	a	20	47.08	7.39	a	F=3.02	0.03
MEX-42	20	39.58	6.31	a	18	78.24	5.52	b	17	77.94	5.80	b	20	90.83	3.87	b	F=14.15	<0.001
MEX-43	15	61.11	5.85	a	18	81.94	4.87	a	16	65.10	7.50	a	15	78.33	6.84	a	F=2.53	0.06
MEX-44	20	30.00	5.45	a	18	74.54	7.02	b	17	85.78	5.04	b	20	85.00	6.50	b	F=15.14	<0.001
MEX-45	15	52.22	8.59	a	18	50.46	7.75	a	16	67.19	7.11	a	15	71.11	5.91	a	F=1.98	0.12
MEX-46	20	26.67	6.00	a	20	42.50	7.60	ab	20	57.50	7.15	b	20	40.42	6.85	ab	F=4.04	0.01
eNema EF	15	71.11	5.39	a	18	71.76	6.03	a	16	61.67	6.95	a	15	63.33	8.11	a	F=0.77	0.51
TOTAL	**25**	**54.90**	**6.24**	**a**	**25**	**60.72**	**6.29**	**a**	**25**	**67.46**	**5.88**	**a**	**25**	**68.95**	**5.82**	**a**	**Chisq= 37.94**	**0.242**

Table S13. ANOVA results for the comparisons of content of cucurbitacin sequestered by *D. balteata* after the larvae fed freely on full cucumber plants for 3, 5 and 7 days. P values are given for treatments [generalized linear model (family, Gaussian)] followed by pairwise comparisons of Least Squares Means (LSMeans). Letters indicate significant differences between treatments ($p<0.05$).

Time (days)	Putative cucurbitacin	T-fed *D. balteata* larvae				S-fed *D. balteata* larvae				H-fed *D. balteata* larvae				M76-fed *D. balteata* larvae				F	p-value
		n	Mean (µg/g FW)	±SE	group	*n*	Mean (µg/g FW)	±SE	group	*n*	Mean (µg/g FW)	±SE	group	*n*	Mean (µg/g FW)	±SE	group		
3	Cuc C	9	0.00	0.00	a	8	0.06	0.03	a	8	0.02	0.02	a	9	0.03	0.02	a	1.62	0.21
	Unknown Cuc # 1	9	0.00	0.00	a	8	0.04	0.02	a	8	0.33	0.16	a	9	0.74	0.43	a	2.05	0.13
	Unknown Cuc # 2	9	0.02	0.02	a	8	0.02	0.02	a	8	0.45	0.15	ab	9	0.85	0.36	b	3.84	0.02
	Deacetylated Dihydro Cuc B glu	9	0.04	0.02	a	8	0.05	0.03	a	8	7.50	1.93	b	9	12.25	2.03	b	18.66	<0.001
	Metabolized Cuc # 1	9	0.02	0.02	a	8	0.04	0.03	a	8	0.72	0.23	ab	9	1.14	0.49	b	3.92	0.02
	Cuc B glu	9	0.01	0.01	a	8	0.04	0.02	a	8	0.15	0.09	a	9	0.25	0.10	a	2.56	0.07
	Dihydro Cuc B glu	9	0.02	0.02	a	8	0.02	0.02	a	8	0.03	0.02	a	9	0.03	0.02	a	0.20	0.90
	Isomer of Dihydro Cuc B glu	9	0.02	0.02	a	8	0.10	0.03	a	8	0.33	0.17	a	9	0.53	0.24	a	2.43	0.09
	Cuc II a glu	9	0.02	0.02	a	8	0.08	0.03	a	8	0.02	0.02	a	9	0.08	0.03	a	2.73	0.06
	TOTAL	**9**	**0.13**	**0.12**	**a**	**8**	**0.44**	**0.23**	**a**	**8**	**9.55**	**2.79**	**b**	**9**	**15.91**	**3.73**	**b**	**18.57**	**<0.001**
5	Cuc C	8	0.00	0.00	a	10	0.03	0.02	a	8	0.06	0.03	a	8	0.00	0.00	a	2.37	0.09
	Cuc C	8	0.00	0.00	a	10	0.03	0.02	a	8	1.70	0.56	b	8	0.60	0.25	a	7.54	<0.001
	Unknown Cuc # 1	8	0.04	0.02	a	10	0.09	0.02	a	8	0.78	0.21	b	8	0.84	0.27	b	7.17	<0.001
	Unknown Cuc # 2	8	0.08	0.03	a	10	0.08	0.03	a	8	10.56	2.34	b	8	13.95	1.03	b	35.43	<0.001
	Deacetylated Dihydro Cuc B glu	8	0.04	0.02	a	10	0.04	0.02	a	8	1.77	0.56	ab	8	1.95	0.69	b	6.43	0.002
	Metabolized Cuc # 1	8	0.00	0.00	a	10	0.02	0.02	a	8	0.62	0.39	a	8	0.07	0.07	a	2.47	0.08
	Cuc B glu	8	0.08	0.03	a	10	0.03	0.02	a	8	0.08	0.03	a	8	0.00	0.00	a	2.70	0.06
	Dihydro Cuc B glu	8	0.06	0.03	ab	10	0.02	0.02	a	8	0.45	0.16	ab	8	0.55	0.23	b	4.15	0.01
	Isomer of Dihydro Cuc B glu	8	0.02	0.02	a	10	0.04	0.02	a	8	0.05	0.03	a	8	0.00	0.00	a	1.42	0.26
	TOTAL	**8**	**0.30**	**0.15**	**a**	**10**	**0.37**	**0.18**	**a**	**8**	**16.07**	**4.30**	**b**	**8**	**17.96**	**2.53**	**b**	**55.21**	**<0.001**
7	Cuc C	5	0.00	0.00	a	5	0.03	0.03	a	5	0.00	0.00	a	5	0.00	0.00	a	0.02	0.41
	Cuc C	5	0.03	0.03	a	5	0.00	0.00	a	5	6.75	1.41	b	5	7.19	2.96	b	6.01	0.01
	Unknown Cuc # 1	5	0.03	0.03	a	5	0.00	0.00	a	5	0.89	0.43	ab	5	1.39	0.58	b	3.55	0.04
	Unknown Cuc # 2	5	0.00	0.00	a	5	0.04	0.04	a	5	12.76	3.38	b	5	19.36	1.16	c	29.15	<0.001
	Deacetylated Dihydro Cuc B glu	5	0.00	0.00	a	5	0.06	0.04	a	5	1.52	0.76	a	5	1.62	0.77	a	2.73	0.08
	Metabolized Cuc # 1	5	0.00	0.00	a	5	0.07	0.04	a	5	5.20	1.92	a	5	4.45	2.38	a	3.31	0.05
	Cuc B glu	5	0.00	0.00	a	5	0.00	0.00	a	5	0.00	0.00	a	5	0.00	0.00	a	16.00	0.00
	Dihydro Cuc B glu	5	0.03	0.03	a	5	0.04	0.04	a	5	2.20	0.88	b	5	2.18	0.72	b	4.80	0.01
	Isomer of Dihydro Cuc B glu	5	0.00	0.00	a	5	0.00	0.00	a	5	0.00	0.00	a	5	0.00	0.00	a	16.00	0.00
	TOTAL	**5**	**0.08**	**0.08**	**a**	**5**	**0.23**	**0.18**	**a**	**5**	**29.33**	**8.77**	**b**	**5**	**36.19**	**8.58**	**b**	**18.20**	**<0.001**

Table S14. ANOVA results for the comparisons of EPN progeny of infected *D. balteata* larvae 15 days after inoculation with 13 isolates of *Heterorhabditis bacteriophora* entomopathogenic nematodes (EPN). The progeny is expressed as average number of infective juveniles (Ijs) per infected *D. balteata* larva. Previous EPN inoculation, the larvae fed freely on cucumber plants of the varieties T, S, H or M76 for five days. P values are given for treatments [generalized linear model (family, binomial or quasibinomial)] followed by pairwise comparisons of Least Squares Means (LSMeans). Letters indicate significant differences between treatments ($p<0.05$).

Treat.	T-fed *D. balteata* larvae				S-fed *D. balteata* larvae				H-fed *D. balteata* larvae				M76-fed *D. balteata* larvae					
EPN isolate	*n*	Average IJs/ infected larva	±SE	group	*n*	Average IJs/ infected larva	±SE	group	*n*	Average IJs/larva	±SE	group	*n*	Average IJs/ infected larva	±SE	group	**F**	**p-value**
MEX-14	8	129.31	49.96	a	4	20.83	10.03	a	10	55.89	12.00	a	7	173.33	53.37	a	2.71	0.07
MEX-21	7	46.83	21.55	a	2	33.33	20.00	ab	10	305.44	70.57	b	6	33.33	10.18	a	6.30	0.00
MEX-22	7	65.24	40.47	a	6	74.44	28.31	a	10	345.44	124.96	a	5	13.33	0.00	a	2.98	0.05
MEX-23	10	76.22	29.10	a	7	42.38	10.95	a	10	434.44	104.81	b	2	20.00	6.67	a	7.30	0.00
MEX-31	8	50.14	12.11	a	5	17.33	6.22	a	10	70.33	17.23	a	3	5.56	1.70	a	3.09	0.05
MEX-35	8	205.83	62.79	a	3	13.33	0.00	a	5	75.11	28.41	a	10	236.89	70.81	a	1.85	0.17
MEX-36	10	168.00	63.10	a	3	91.11	62.58	a	10	129.11	23.97	a	10	214.67	33.76	a	0.97	0.42
MEX-38	8	81.81	29.58	a	6	42.22	9.38	a	9	288.89	72.48	a	8	267.50	108.04	a	2.98	0.05
MEX-41	7	196.19	100.07	a	6	50.37	19.82	a	9	310.37	164.98	a	9	50.37	16.78	a	1.47	0.25
MEX-42	10	138.89	41.20	a	8	85.28	33.29	a	10	261.00	44.73	a	7	132.70	73.30	a	2.60	0.07
MEX-43	9	290.86	90.98	a	10	103.22	30.00	a	10	164.67	47.34	a	8	88.61	29.91	a	2.69	0.06
MEX-46	8	206.67	100.29	a	6	146.67	112.24	a	10	241.89	116.40	a	9	290.37	123.74	a	0.24	0.87
eNema EF	4	236.67	87.67	a	8	62.50	30.31	a	10	120.67	37.08	a	10	96.89	18.36	a	2.66	0.07
TOTAL	**13**	**145.59**	**56.07**	**a**	**13**	**60.23**	**28.70**	**a**	**13**	**215.64**	**66.53**	**b**	**13**	**124.89**	**42.05**	**a**	**8.89**	**<0.001**

4. Feeding behavior of *Diabrotica* larvae: long-thought "rootworms" that feed on leaves

Pamela Bruno[1], Eléonore Gindroz[2], Gaétan Glauser[3], Ted CJ Turlings[1], Ricardo AR Machado[4]* & Carla CM Arce[1]*

1. Laboratory of Fundamental and Applied Research in Chemical Ecology, Institute of Biology, University of Neuchâtel, Neuchâtel, Switzerland.

2. École polytechnique fédérale de Lausanne - EPFL, Lausanne, Switzerland.

3. Neuchâtel Platform of Analytical Chemistry, University of Neuchâtel, Switzerland

4. Experimental Biology Research Group, Institute of Biology, University of Neuchâtel, Neuchâtel, Switzerland.

*Corresponding authors: carla.marques@unine.ch & ricardo.machado@unine.ch

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Abstract

The subtribe Diabroticina (Coleoptera: Chrysomelidae) includes important pests of agricultural crops in North and South America. The adult beetles feed on aboveground plant tissues, whereas the larvae are assumed to exclusively feed on roots. During preliminary experiments with cucumber plants, *Diabrotica balteata* larvae were observed to also extensively feed on leaves. This prompted us to explore whether feeding on leaves provides these so-called "rootworms" any benefits, and if this feeding behavior extends to other *Diabrotica* species. We profiled the main secondary metabolites of six agricultural plants from five different families; maize, cucumber, soybean, red kidney bean, white radish and red beet, and compared the weight gain, biomass consumption and mortality of *D. balteata*, *D. undecimpunctata* and *D. virgifera* larvae, seven days after they fed on roots or on leaves of these plant species. In addition, surviving larvae were exposed to the entomopathogenic nematode *Heterorhabditis bacteriophora* and infection rates were evaluated after five days. All three *Diabrotica* species readily fed on leaves as well as roots, but larval performance varied considerably depending on plant tissue and plant species. Nematode infection rates were similar for larvae fed on leaves or roots of the same plant, but different among larvae fed on different plant species. This could be related to the ability of the nematodes to cope with the secondary metabolites of the plants that the larvae had ingested. A deeper understanding of the feeding behavior of *Diabrotica* larvae might help to develop effective and targeted management strategies for these agricultural pests.

Keywords

banded cucumber beetle – southern corn rootworm – western corn rootworm – entomopathogenic nematodes– larval performance – plant defenses

Introduction

The genus *Diabrotica* Chevrolat (Coleoptera: Chrysomelidae) of leaf beetles encompasses many pests of a wide variety of extensive crops, such as maize and soybean, but also of horticultural productions like cucurbits (Krysan *et al.*, 1986; Marín-Jarillo, 2012; Cabrera Walsh *et al.*, 2020). Most species are polyphagous both as larvae and adults, such as the banded cucumber beetle *D. balteata* LeConte and the spotted cucumber beetle/ southern corn rootworm *D. undecimpunctata howardi* Barber (Krysan, 1986). *Diabrotica virgifera*, on the other hand, is a specialist on maize during its immature stages (Moeser & Hibbard, 2005). *Diabrotica* beetles feed on foliage, pollen and flowers, and in the case of maize plants, also on silk and kernels (Branson & Krysan, 1981). The larvae are called rootworms and dwell in the rhizosphere of the plants tunneling the root system (Branson, 1986), which reduces water and nutrient intake (Godfrey *et al.*, 1993) and can favor pathogen infections (Kurtz *et al.*, 2010).

It is commonly thought that the larvae of *Diabrotica* spp. feed exclusively on roots (Godfrey *et al.*, 1993). However, Cardona *et al.* (1982) observed that larvae of *D.*

balteata and of a closely related species, *Cerotoma fascialis*, could forage on all parts of young bean plants. The larvae tunneled not only the roots, but continued upwards inside of the stems and also consumed hypocotyls and cotyledons, ultimately killing the small plants (Cardona *et al.*, 1982). Similar behavior has been reported for *Diabrotica* spp. on maize plants (Canales Santos, 1989), cucurbits (Jiménez Martínez, 2014) and chia (Sosa-Baldivia, 2016).

Plants often allocate their nutrients and defensive compounds differentially between roots and shoots (Gómez *et al.*, 2012; Machado *et al.*, 2013; Robert *et al.*, 2014; Köhler *et al.*, 2015). Therefore, the access to both below and aboveground tissues of the plant hosts by root-feeding larvae might have adaptive advantages in terms of optimizing diet requirements. For instance, insects can benefit from feeding on more nutritionally-rich tissues and/or modulate the intake of toxic secondary metabolites by choosing among different organs (Kelley *et al.*, 2002; Erb *et al.*, 2013; Köhler *et al.*, 2015). In some cases, specialist species even opt to feed on tissues with higher contents of plant defenses, accessing a unique feeding niche (Bowers & Puttick, 1988). Such is the case of *Diabrotica virgifera* larvae, that preferentially forage on highly-nutritious crown roots of maize, with elevated contents of benzoxazinoids, in comparison with primary and secondary roots (Robert *et al.*, 2012). Unfortunately, there is scarce knowledge about *Diabrotica* larvae feeding on aboveground tissues, thus the drivers of this behavior are still unexplained.

In this study, we explore whether *Diabrotica* larvae can indeed feed on above as well as belowground tissues of different agricultural plants, and whether the leaf-feeding behavior provides the larvae with metabolites that may protect them against natural enemy attack. As a first step, we compared the larval performance and plant biomass consumption of *D. balteata*, *D. undecimpunctata*, and *D. virgifera*, after they fed leaves or roots of maize, cucumber, soybean, red kidney bean, white radish or red beet. Subsequently, we assessed the infection rates of the larvae by a commercial strain of entomopathogenic nematodes. In addition, we profiled the main secondary metabolites of the leaves and roots of the six studied plants. We observed that the larvae are capable of feeding on leaves of most of the plants evaluated, and that the performance varied considerably among the different insect species, plants and tissues. Yet, the larvae did not differ in their susceptibility to entomopathogenic nematodes after feeding on different tissues of the same plant species.

Materials and methods

Biological resources

Plant materials

Six species of agricultural plants were used for the experiments: maize (*Zea mays* L.) var. Delprim (DSP, Delley, CH), cucumber (*Cucumis sativus* L.) var. Marketmore 76 (Southern Exposure Seed Exchange, USA), soybean (*Glycine max* L.) var. Bohnen bio (Rapunzel, DE), red kidney bean (*Phaseolus vulgaris* L.) (Vivien Paille, FR), white

radish, also known as Japanese radish or Japanese daikon (*Raphanus sativus* Bailey var. *longipinnatus*) cv. Zürcher Markt (Samen Mauser, BioLine, CH), and red beet (*Beta vulgaris* Alefeld subsp. *vulgaris* var. *conditiva*) cv. Rote Kugel (Samen Mauser, BioLine, CH). Maize, soybean and red kidney bean seeds were germinated in moist vermiculite (Greendoor, CH) for three days. Cucumber, white radish and beetroot seeds were directly sown in soil. All plants were grown in 300 ml plastic pots with a mixture of 1:1 of potting soil (Einheitserdewerke Patzer Gebrüder Patzer GmbH & Co. KG, DE) and washed sand (Ø 1 – 4 mm) (Migros, CH). Maize plants were grown under greenhouse conditions (LD 16:8 – 26:24°C, RH 60%). The other species were grown under phytotron conditions (400 µmol/m^2/s – LD 16:8 - 28:26 °C, RH 60%). All plants were watered every other day and fertilized twice a week (8-8-6 NPK Maag Garden, CH). Experiments were performed with plants with three expanded leaves (10-12 days for maize plants and 15-20 days for the other species).

Insects and nematodes

Diabrotica balteata (Coleoptera: Chrysomelidae) eggs were provided by Syngenta Crop Protection (Stein, CH). *Diabrotica undecimpunctata* eggs were purchased from Crop Characteristics, Inc. (Farmington, MN, USA). *Diabrotica virgifera* eggs were originally provided by CABI (Hódmezővásárhely, Hungary) from the non-diapause colony of USDA ARS (Brookings, SD, USA). Because neonate larvae have very high mortality, all larvae were initially fed on maize hybrid DFI 45321 (DSP, Delley, CH) for five to seven days as previously described (Erb *et al.*, 2011) before being used for experiments. A commercial strain of *Heterorhabditis bacteriophora* entomopathogenic nematodes was obtained from e-Nema (Schwentinental, DE). The nematodes were reared *in vivo* with the "*Galleria* baiting" method (Bedding & Akhurst, 1975) using White traps as described (White, 1927; Campos-Herrera *et al.*, 2015; Bruno *et al.*, 2020) and stored at 12 °C in the dark.

Performance of the larvae

To determine whether the three species of *Diabrotica* larvae perform differently when fed on leaves or roots, we measured the weight gain, mortality and tissue consumption of *Diabrotica balteata* and *D. undecimpunctata* larvae after feeding on of maize, cucumber, soybean, bean, white radish or beetroot (see details in biological resources), these plants and their tissues. *Diabrotica virgifera* were only offered leaves or roots of maize, cucumber and soybean, due to the low number of individuals. Briefly, 15 first-instar *D balteata* or *D. undecimpunctata* larvae, or ten first-instar *D. virgifera* larvae were placed in 250 ml plastic pots with 20 g of moist potting soil (Einheitserdewerke Patzer Gebrüder Patzer GmbH & Co. KG, DE). One or two detached and pre-weighed leaves or roots of one of the six species was placed in each pot (n=6) and they were closed with lids and kept at room temperature for 7 days. The amount of plant tissue needed was calculated by pre-tests to ensure *ad libitum* feeding by the larvae (data not shown), and it was found to be less than 1 g of fresh weight per pot for two days. Plant material was replaced every two to three days, and after

removal, it was dried at 40 °C for 48 hr to have the dry weight (DW) of all the “eaten” tissues. The average of water content of each type of plant tissue was calculated with control samples with no larvae in them. The biomass consumption by the larvae was calculated as the difference between the initial dry weight (estimated with the water content) and the final dry weight. After 7 days, all larvae were retrieved, counted and individually weighed.

Larval infection with entomopathogenic nematodes

The infection capacity of a commercial strain of *Heterorhabditis bacteriophora* entomopathogenic nematodes (for details see biological resources) was determined on the larvae that were recovered after the 7-day performance experiment. The infectivity by *H. bacteriophora* nematodes was carried out according to Bruno *et al.* (2020). Three to four larvae were placed in 30 ml plastic cups (Frontier Scientific Services, Inc., USA) that had a 5 mm layer of moist autoclaved washed sand (Ø 1 – 4 mm) (Migros, CH). Approximately 25 newly emerged nematodes suspended in 500 µl of tap water were applied to each cup. All cups were closed with lids and kept in the dark at 24 ºC. The infection was verified after 5 days by color evaluation (see Bruno *et al.* (2020) for details).

Analyses of plant secondary metabolites

We profiled the main secondary metabolites of the roots and leaves of the six plant species. For maize plants, we quantified the main benzoxazinoids, for cucumber the main cucurbitacins, for soybean and red kidney bean polyphenols and cyanogenic compounds, for white radish glucosinolates, and for red beet betanins. Due to the overall similar tissue consumption per plant by the three larval species, we only quantified secondary metabolites after *D. balteata* larvae had fed on the leaves and roots, as well as on undamaged tissues. Roots or leaves of the six plant species were placed in plastic pots with soil and left undamaged or with ten first-instar *D. balteata* larvae, under the same conditions as in the performance experiment (n=5). After 48 hours they were harvested, flash frozen in liquid nitrogen and ground into a fine powder. The secondary metabolites of maize, cucumber, soybean, red kidney bean and white radish plants were analyzed using liquid chromatography and mass spectrometry.

To extract the different secondary metabolites using liquid chromatography, to 50 mg of plant material was placed in 500 µl of extraction buffer were added. The buffers used were: MeOH:H_2O:formic acid (50:50:0.5) for benzoxazinoids, 100% MeOH for cucurbitacins, MeOH:H_2O:formic acid (70:30:0.1) for flavoinoids and also for glucosinolates, and :H_2O (50:50) for betanin. The samples were vortexed for 1 min and centrifuged at 20,000 g for 20 min at 4 °C. In the case of soybean and red kidney bean plants, samples were first heated at 90 ºC for 10 min, sonicated for 5 min on ice and then centrifuged. The supernatants were transferred into vials (for glucosinolates analyses diluted 10 times) and analyzed as detailed below.

Benzoxazinoids from maize plants were analyzed as described in Meihls *et al.* (2013).

Glucosinolate quantification from white radish plants was done as described (Glauser *et al.*, 2012) using sinigrin as reference standard for the calibration curve. Flavonoids from soybean and red kidney bean plants were analyzed according to Moreira et al. (Moreira *et al.*, 2018). In total, eight glucosides of quercetin, kaempferol and isorhamnetin were identified and quantified in bean samples. Cucurbitacins from cucumber plants and betanin from red beet plants were measured by UHPLC-PDA-QTOFMS using an Acquity UPLC coupled to an eLambda PDA and a Synapt G2 mass spectrometer. For cucurbitacins, a linear gradient from 5 – 100 % B in 7 min was performed, following by washing at 100% B for 2 min and requilibration at 5 % B for 2 min. The flow rate was 0.4 ml/min and the injection volume was 2.5 µl. The mass spectrometer was operated in negative electrospray ionization over a mass range of 85 – 1200 Da. Accurate mass measurements (< 2 ppm) were obtained by infusing a solution of leucine-enkephalin (0.5 µg/ml) throughout the run. Cucurbitacins were putatively identified based on their exact masses (allowing for molecular formula determination) and retention times and compared with those of the standard Cucurbitacin B as well as with available databases such as the Dictionary of Natural Product (CRC Press). In some cases, the presence of several possible cucurbitacin isomers prevented full identification. Cucurbitacin B solutions at 0.02, 0.08, 0.4, 2, 5 and 10 µg/ml were used to establish a calibration curve for quantitation and all other cucurbitacins were determined as cucurbitacin B equivalents. For betanin analysis from red beet plants, a linear gradient of 2-21.2% B in 2 min at a flow rate of 0.4 mL/min was applied and in these conditions betanin eluted at 1.20 min. The temperature of the column was set to 45 °C. The volume of injection was 1 µL. Betanin was monitored at 534 nm with the PDA detector and using the (M+H)+ ion at m/z 551.151 with the TOFMS. Since no pure standard of betanin was available, we performed a relative quantification by integration of peak areas in the different samples. All data for quantitation were processed in TargetLynx XS (Waters).

Statistical analyses

Statistical tests were carried out with R statistical software (v. 4.0.0; R Development Core Team 2019) using Analysis of Variance (ANOVA), followed by residual analysis to verify suitability of distributions of the tested models. Differences in plant secondary metabolites, weight gain and tissue consumption were analyzed using Generalized Linear Models (GLM) with a Gaussian distribution. Differences in larval mortality and infection by entomopathogenic nematodes were analyzed using Generalized Linear Models (GLM) under binomial distribution, with model fitting to quasibinomial when necessary.

Results

Diabrotica larvae can feed on leaves and roots with no apparent differential effect on their performance.

In order to evaluate whether *Diabrotica* larvae can feed on aboveground tissues as well as on roots, we offered *D. balteata* and *D. undecimpunctata* detached leaves or

roots of maize, cucumber, soybean, red kidney bean, white radish or red beet. In the case of *D. virgifera*, larvae were given leaves or roots of maize, cucumber or soybean. In all cases, we evaluated larval performance after seven days of feeding on these tissues (Fig. 1, Fig. S1).

The larval performance of *D. balteata* larvae is shown in Figure 2. When feeding on maize or on red beet tissues, the larvae ate larger amounts of roots than leaves (Fig. 2 a), and consistently gained more weight on roots (Fig. 2 b). Contrarily, when *D. balteata* were offered cucumber and white radish tissues, they consumed larger amounts of leaf biomass than root biomass (Fig. 2 a) and as a result they gained more weight on leaves, resulting in the highest weight gains among the different plant species and tissues evaluated (up to 8 mg/larva, Fig. 2 b). Larvae of *D. balteata* consumed equal amounts of leaves and roots of soybean and red kidney bean (Fig. 2 a), but gained significantly higher weight on roots compared to leaves (Fig. 2 b). Finally, the mortality of *D. balteata* on leaves and roots was lower than 20% and similar among all plants tested, except for red kidney bean, on which mortality was as high as 57 % for leaf-fed larvae (Fig. 2 c). After feeding on leaves or roots of all six plant species, the performance of *D. undecimpunctata* larvae after seven days showed that they consumed equal amounts of leaves and roots of maize, cucumber and soybean, larger amounts of roots of red kidney bean and red beet, and larger amounts of leaves of white radish (Fig. 3 a). The weight gain of *D. undecimpunctata* larvae showed the same pattern as *D. balteata* larvae, with higher gain for larvae fed on roots of maize, soybean, red kidney bean and red beet, and higher weight gain for larvae fed on cucumber and white radish leaves (Fig. 3 b). Larvae fed on soybean leaves had a negligible weight gain change, and in some samples their weight even decreased over seven days. The mortality of *D. undecimpunctata* larvae ranged between 23 and 75 % for all plants and tissues, with similar results on leaves and roots for maize, soybean, red kidney bean and red beet, and higher mortality of larvae fed on roots of cucumber and white radish- fed larvae, compared to their counterparts fed on leaves (Fig. 3 c). Larvae of *D. virgifera* were only offered leaves and roots of maize, cucumber and soybean due to the low number of individuals (Fig. 4). Similarly to *D. balteata, D. virgifera* larvae consumed more root tissues than leaves of maize (Fig. 4 a) and they gained more weight on roots, but had similar mortality on both tissues (Fig. 4 b, c). *Diabrotica virgifera* larvae ate larger amounts and gained more weight on cucumber leaves than roots, (Fig. 4 a, b), but their mortality was similar on both tissues. Surprisingly, the mortality of *D. virgifera* larvae was less than 20 % after seven days of feeding on cucumber tissues, which was comparable to the mortality observed on maize tissues which they have specialized. The statistical results for the larval performance of *D. balteata*, *D. undecimpunctata* and *D. virgifera* are available in the Supplementary Tables S1-S3.

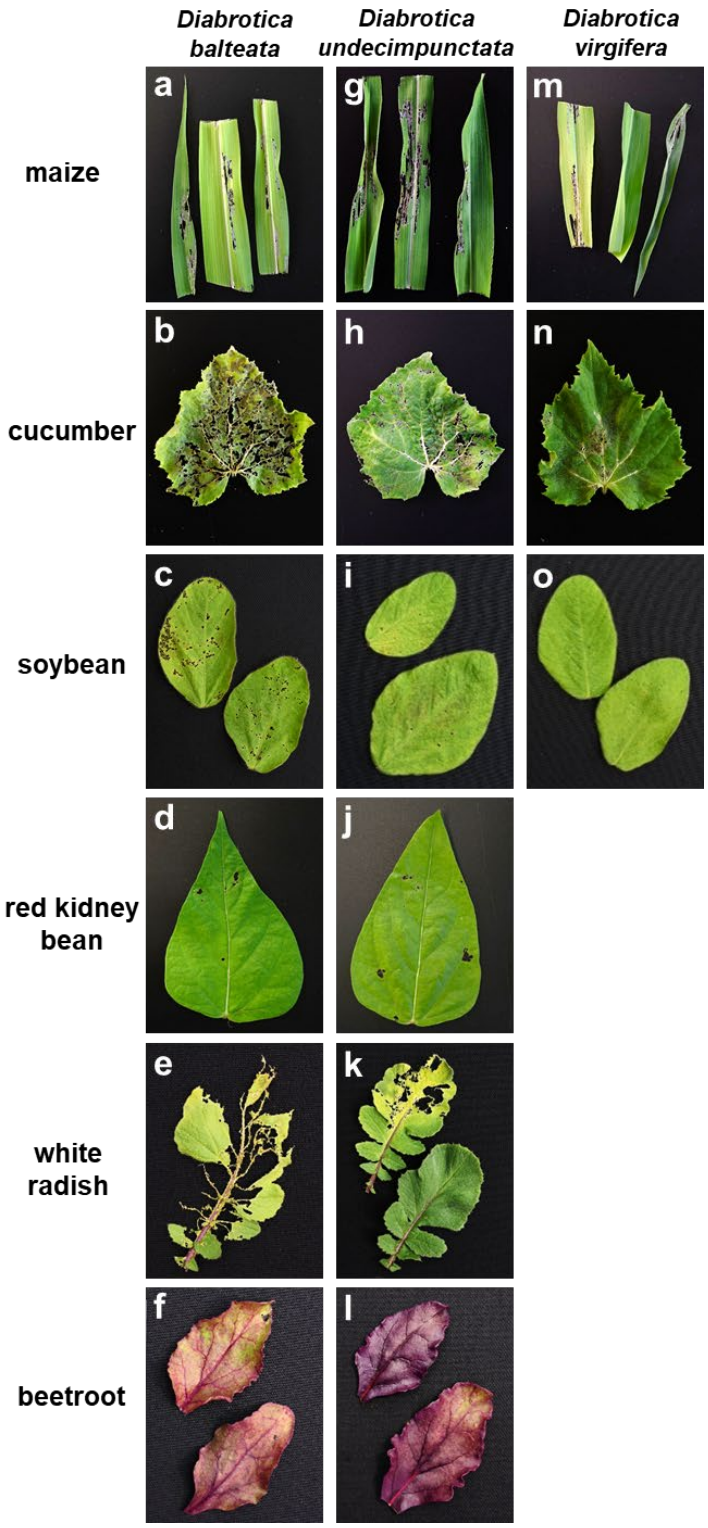

Figure 1. *Diabrotica balteata*, *D. undecimpunctata* and *D. virgifera* larvae can feed on leaves. Damage on leaves after fifteen second-instar *Diabrotica balteata* or *D. undecimpunctata* larvae, or ten second-instar *D. virgifera* larvae fed on leaves for 48 hours. *Diabrotica balteata* larvae fed on (a) maize, (b) cucumber, (c) soybean, (d) red kidney bean, (e) white radish or (f) beetroot. *Diabrotica undecimpunctata* larvae fed on (g) maize, (h) cucumber, (i) soybean, (j) bean, (k) white radish or (l) beetroot. *Diabrotica virgifera* larvae fed on (m) maize, (n) cucumber, or (o) soybean.

Larval infection rates by entomopathogenic nematodes did not differ among *Diabrotica* larvae fed on leaves or roots of the same plant.

After seven days feeding on leaves or roots, surviving larvae of *D. balteata*, *D. undecimpunctata* and *D. virgifera* were inoculated with entomopathogenic nematodes of a commercial strain of *Heterorhabditis bacteriophora*. The infection rates were evaluated five days after inoculation and the results are shown in Figure 5. For all three *Diabrotica* species there were no differences in the infection percentages of larvae that had previously fed on leaves or on roots of any of the plants evaluated. However, in some cases there were striking differences among plant species. Of the *D. balteata* larvae that had fed on red beet only around 10 % were infected, whereas the infection rate reached 80 % for larvae fed on soybean (Fig. 5 a). For *D. undecimpunctata* larvae, the infection rates were lowest after the larvae fed on cucumber and soybean, but the differences among plants were less distinct (Fig. 5 b). Finally, for *D. virgifera*, the infection was always lower than 60 %, with no significant differences among the larvae fed on the three plants, maize, cucumber and soybean (Fig. 5 c). The statistical results are available in the Supplementary Tables S4-S6.

The evaluated plants have distinctive profiles of secondary metabolites in their leaves and roots.

The main secondary metabolites of each of the six plants studied were analyzed under the same conditions as in the performance experiment; for leaves or roots damaged by *D. balteata* larvae for 48 hours or for leaves and roots left undamaged (Fig. 6, Supplementary Tables S7-S18). For maize, cucumber, soybean and red kidney bean, the roots contained much lower amounts of secondary metabolites than the corresponding leaves tissues. Overall, we did not observe a clear induction of the metabolites after *D. balteata* larvae fed on the tissues. For maize plants, we quantified the main benzoxazinoids (BXs, Fig. 6 a, Supplementary Table S7, S8) and the total of BXs content did not change after the larvae fed on the leaves, but in roots the amounts were slightly higher for roots with larval damage compared to control roots. In the leaves, the levels of two compounds changed dramatically after the larval damage: MBOA (four times higher, $F_{2,5}$=15.36, p=0.004;) and HDMBOA-Glc (ten times higher, $F_{2,5}$=104.30, p<0.001). In contrast, the amount of DIMBOA-Glc was lower in damaged leaves compared to control leaves ($F_{2,5}$=4.24, p=0.07). For cucumber plants, we profiled the contents of cucurbitacins (Cucs, Fig. 6 b, Supplementary Tables S9, S10). The total levels of Cucs did not change after herbivory neither in the leaves nor in the roots, but the leaves contained up to ten times more cucurbitacins than the roots, which had less than 1 µg/g. For soybean (Fig. 6 c, Supplementary Tables S11, S12) and red kidney bean (Fig. 6 d, Supplementary Tables S13, S14), we analyzed the main flavonoids present in the tissues, and in both cases the amounts in leaves were much higher than in the roots, which had negligible amounts. Soybean leaves did not increase the production of flavonoids after larval feeding. In the case of red kidney bean (Fig. 6 d), the total amounts of flavonoids in leaves reached 290 µmol/g for leaves

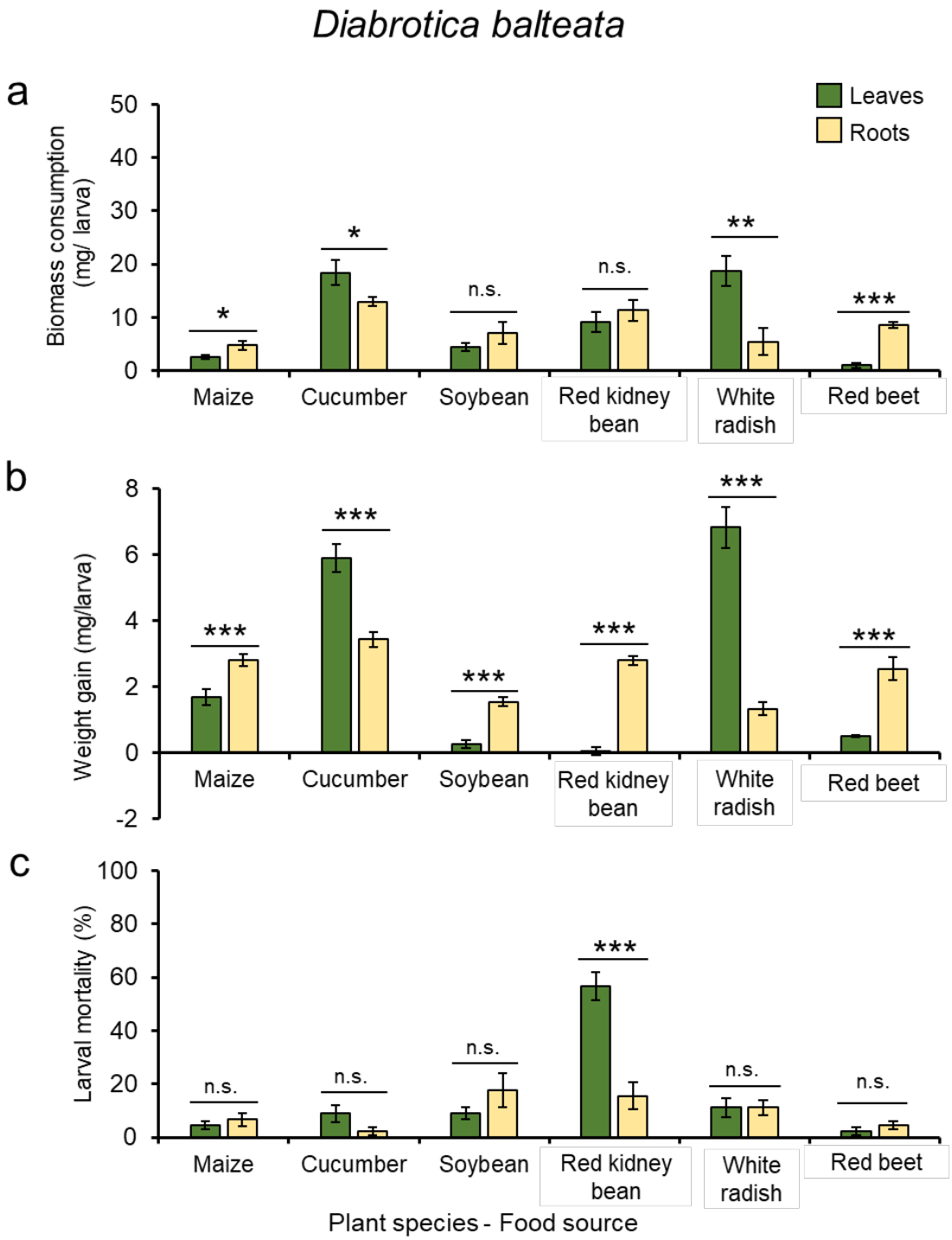

Figure 2. Larval performance of *Diabrotica balteata* fed on leaves or roots of different plant species for seven days. (a) Tissue consumption, (b) weight gain, and (c) mortality rate of fifteen second-instar *D. balteata* larvae fed on leaves (green) or roots (yellow) of maize, cucumber, soybean, red kidney bean, white radish or red beet for seven days (n=6). Bars indicate the average of weight gain per larva (mg ± SE), weight of tissues consumed per larva (mg of dry matter ± SE) or larval mortality (%). Not significant (n.s., $p>0.05$) and stars indicate significant differences between treatments for each plant species: *$p<0.05$, **$p<0.01$,***$p<0.001$.

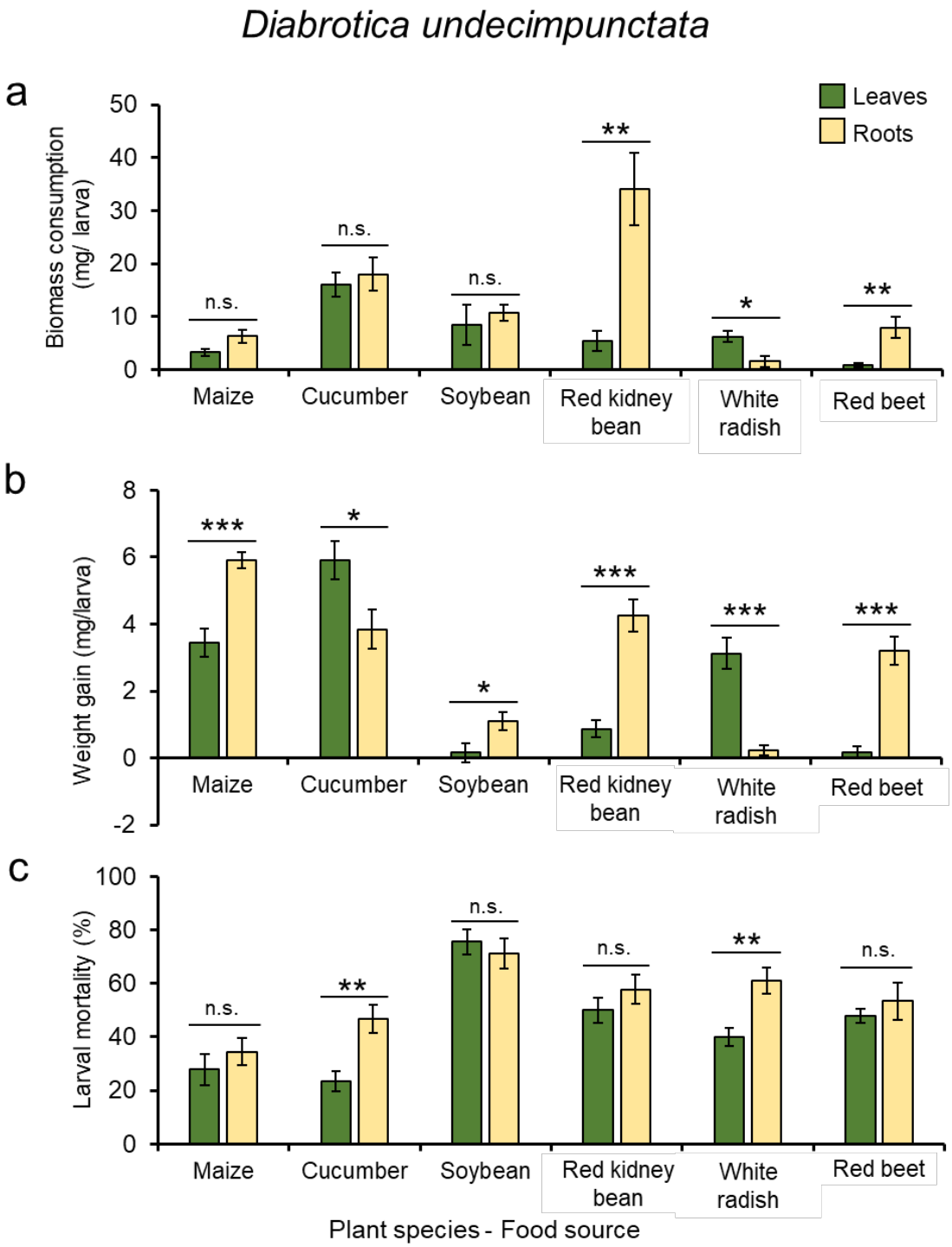

Figure 3. Larval performance of *Diabrotica undecimpunctata* fed on leaves or roots of different plant species for seven days. (a) Tissue consumption, (b) weight gain, and (c) mortality rate of fifteen second-instar *D. balteata* larvae fed on leaves (green) or roots (yellow) of maize, cucumber, soybean, red kidney bean, white radish or red beet for seven days (n=6). Bars indicate the average of weight gain per larva (mg ± SE), weight of tissues consumed per larva (mg of dry matter ± SE) or larval mortality (%). Not significant (n.s., $p>0.05$) and stars indicate significant differences between treatments for each plant species: *$p<0.05$, **$p<0.01$,***$p<0.001$.

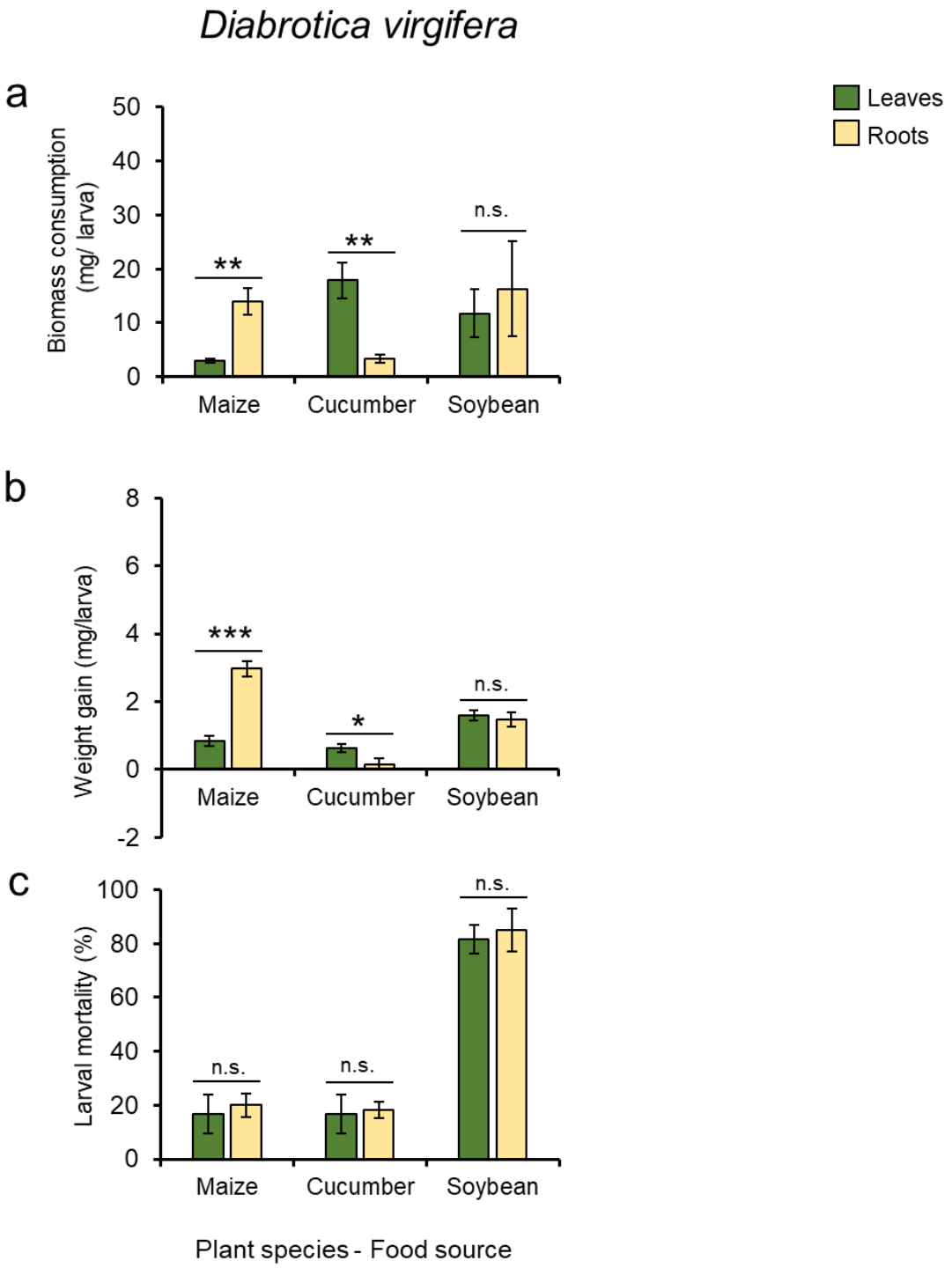

Figure 4. Larval performance of *Diabrotica virgifera* fed on leaves or roots of different plant species for seven days. (a) Tissue consumption, (b) weight gain, and (c) mortality rate of fifteen second-instar *D. balteata* larvae fed on leaves (green) or roots (yellow) of maize, cucumber or soybean for seven days (n=6). Bars indicate the average of weight gain per larva (mg ± SE), weight of tissues consumed per larva (mg of dry matter ± SE) or larval mortality (%). Not significant (n.s., $p>0.05$) and stars indicate significant differences between treatments for each plant species: *$p<0.05$, **$p<0.01$,***$p<0.001$.

with larval damage, which was not significantly different from undamaged leaves ($F_{2,5}$=0.105, p=0.153). However, the flavonoid rutin was only found on control leaves. The main glucosinolates present in white radish leaves and roots are shown in Fig. 6 e and Supplementary Tables S15 and S16. For tissues eaten by *Diabrotica* larvae, there was no significant difference between the total amount of glucosinolates found in leaves and roots ($F_{2,5}$=1.98, p=0.19), although the composition was drastically different between both tissues: for leaves, 60 % of total glucosinolates corresponded to glucoraphenin and 35 % to 4-methylthio-3-butenyl, however for roots 4-methylthio-3-butenyl glucosinolate accounted for 94 % of the total amount of glucosinolates. Interestingly, white radish had larger amounts of glucosinolates in the roots than in the leaves ($F_{2,5}$=41.09, p<0.001), which was opposite to the pattern found in the other plant species and could be the result of selection during domestication. The profiles for leaves and roots were similar with and without herbivory (leaves: $F_{2,5}$=0.33, p<0.58; roots: $F_{2,5}$=0.36, p<0.56). Betanin relative contents from red beet tissues are shown in Fig. 6 f and Supplementary Tables S17 and S18. Leaves with larval damage had almost three times the amount of betanin than damaged roots ($F_{2,5}$=7.25, p=0.03), but similar level than undamaged leaves ($F_{2,5}$=0.06, p=0.811). Similarly, the amount of betanin in undamaged roots was more than three times higher than roots with larval damage ($F_{2,5}$=10.46, p=0.012).

Discussion

In this study we described in detail the behavior of rootworms feeding on aboveground tissues and the consequences for their performance. Larvae of three *Diabrotica* species were found to readily feed on both leaves and roots of maize, cucumber, soybean, red kidney bean, white radish and red beet, but their weight gain greatly depended on the type of tissue and the identity of the plant consumed. However, larval mortality did not or only marginally differ between larvae that had fed on leaves or roots of the same plant. Moreover, the tissue that the larvae fed on had no effect on their susceptibility to infection by a commercial strain of entomopathogenic nematodes. The capacity to feed on different host plants and tissues might help the larvae to survive in an unpredictable and changing environment, but it does not seem to provide them protection against common soil natural enemies.

Food quality is one of the first aspects evaluated by insects when making a foraging decision (Erb *et al.*, 2013). Larvae of *D. virgifera*, for instance, use sugar contents in maize roots during host plant finding and acceptance, as well as to identify the most nutritious tissues to feed on (Machado *et al.*, 2021). *Diabrotica* larvae may also rely on such chemical cues when offered different plant species, allowing them to selectively feed on plants on which they perform best. The performance of *Diabrotica* larvae on different plant hosts has been previously studied for *D. virgifera* (Branson & Ortman, 1967; Moeser & Vidal, 2004; Oyediran *et al.*, 2004) and *D. undecimpunctata* (Barbercheck, 1993; Hirsh & Barbercheck, 1996), and it was generally observed that

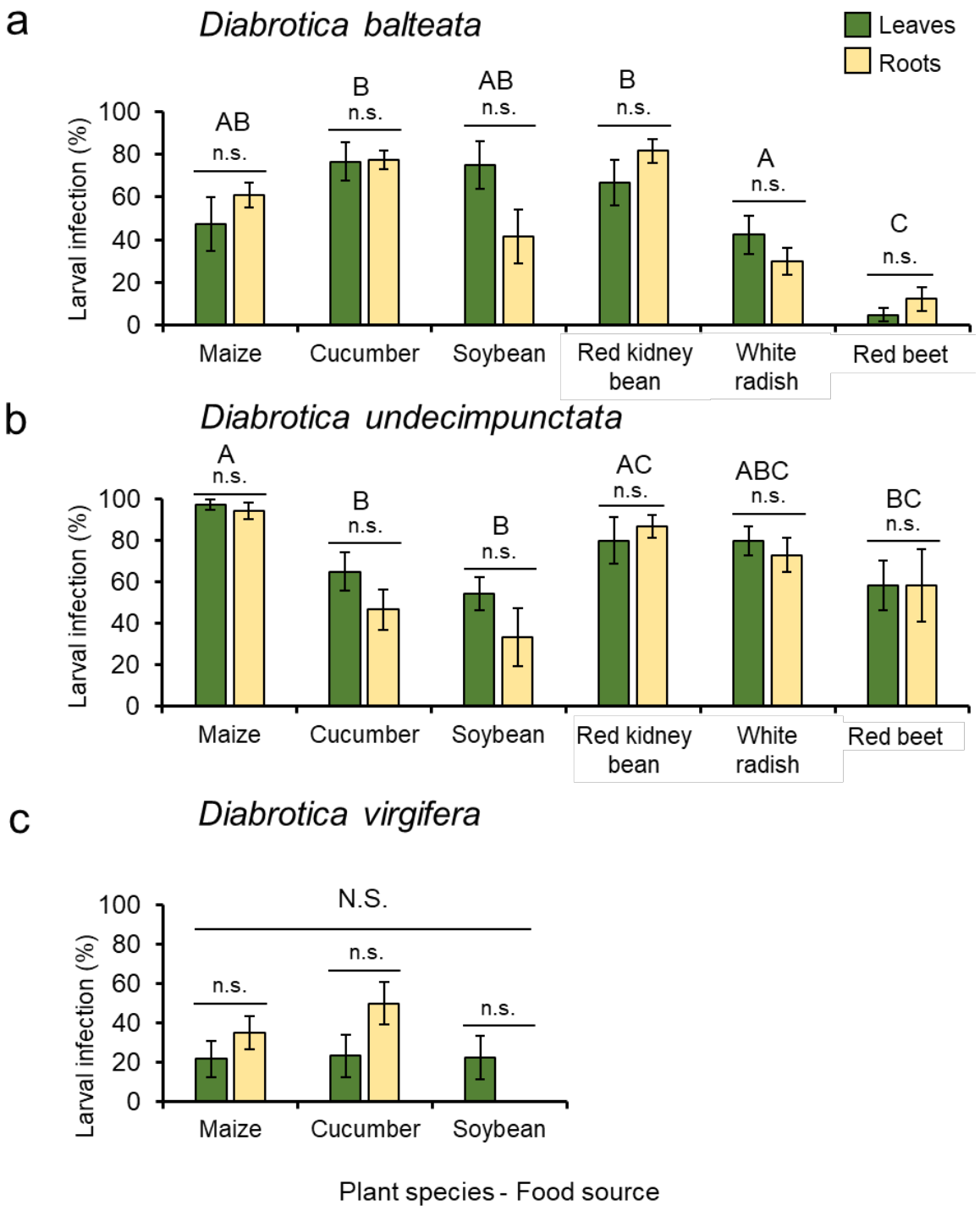

Figure 5. Infection rates (%) of *Diabrotica* larvae five days after inoculation with *Heterorhabditis bacteriophora* entomopathogenic nematodes. Prior to the infection, second instar (a) *D. balteata* and (b) *D. undecimpunctata* fed for seven days on leaves (green) or roots (yellow) of maize, cucumber, soybean, red kidney bean, white radish or red beet (n=4-10). (c) *D. virgifera* larvae fed for seven days on leaves (green) or roots (yellow) of maize, cucumber, soybean (n=2-10). Bars indicate average infection (± SE). Differences between tissues for each plant: not significant (n.s., $p>0.05$). Letters indicate significant differences between plants, regardless of the tissue: $p<0.05$.

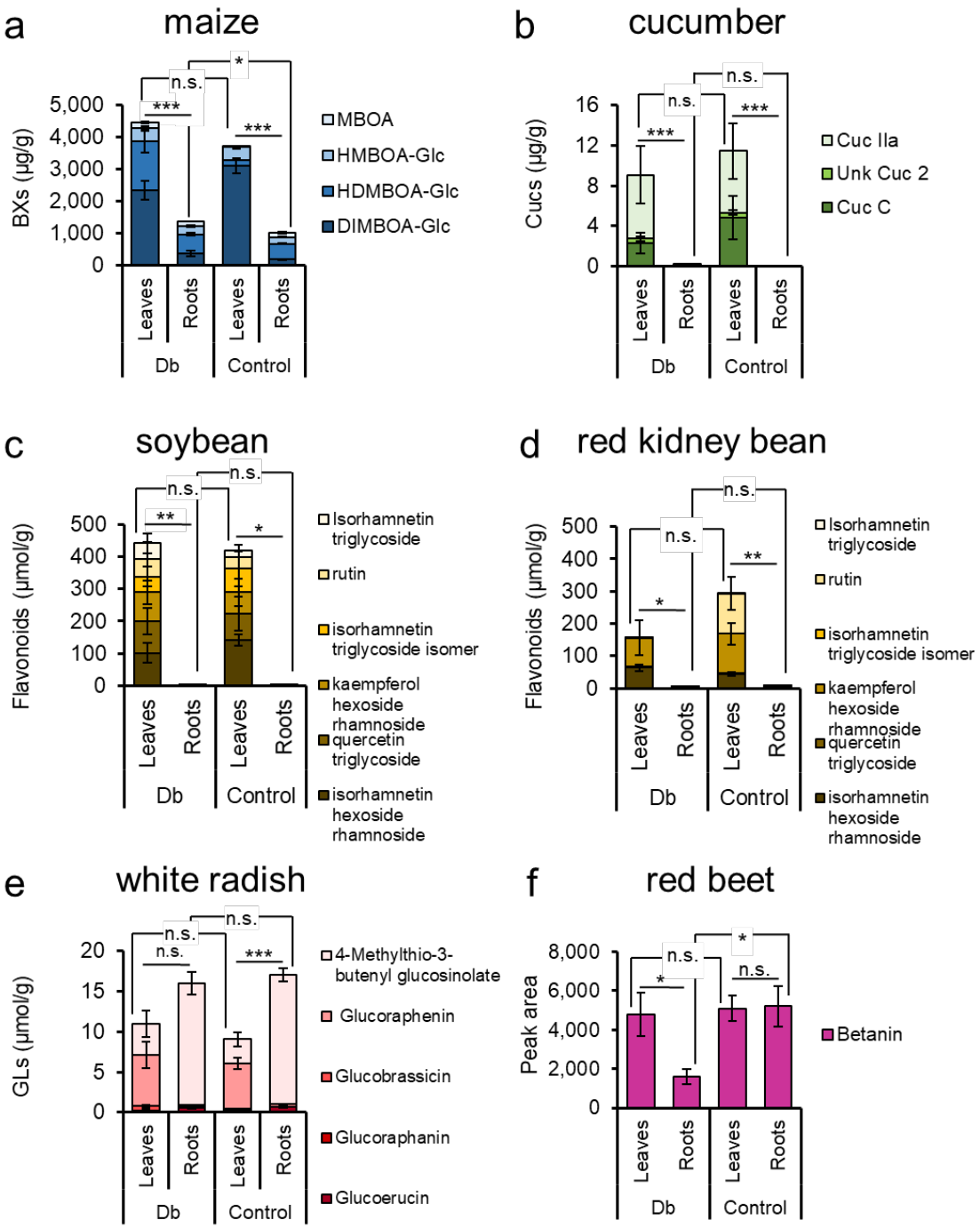

Figure 6. Main secondary metabolites present in the leaves and roots of the plant species used for experiments. The main defense compounds of each plant species used in the experiments were profiled in leaves and roots, 48 hours after *Diabrotica balteata* larvae fed on the tissues (Db) or on undamaged tissues (Control) (n=5). (a) Benzoxazinoids (BXs) from maize, (b) cucurbitacins (Cucs) from cucumber, flavonoids from (c) soybean and (d) red kidney bean, (e) glucosinolates (GLs) from white radish and (f) betanin from red beet. Not significant (n.s., $p>0.05$) and stars indicate significant differences between treatments for each plant species: *$p<0.05$, **$p<0.01$,***$p<0.001$.

the larvae gained more weight after feeding on plants with low nitrogen and high sugars and starch levels, such as maize. However, as there is no report about *Diabrotica* larvae feeding on aboveground tissues, the performance of the larvae is only known for roots of different plant species. Contrarily to maize, soybean and common bean plants contain relatively large amounts of nitrogen due to their nitrogen-fixating capacities (Mylona *et al.*, 1995), which might help to explain the poorer development on both tissues of these plants by the *Diabrotica* species we evaluated. Cucumber plants are heavily consumed by *Diabrotica* adults but do not provide a good source of nutrients for the larvae (Tanemura *et al.*, 2008), which gain little weight on both roots and shoots of different cucumber varieties (Bruno *et al.*, *unpubl.*). There is only scarce information about the nutritional quality of leaves and roots of small plants of white radish and red beet, with most research focused on the edible roots and their secondary metabolites (Kumar & Brooks, 2018; Gamba *et al.*, 2021). However, one study evaluated the mortality of larvae of *D. balteata* after feeding for seven days on extracts of wild radish, and there the mortality was variable but lower than 60 % for the most concentrated extracts (McCutcheon *et al.*, 2009), which is comparable to what we observed for larvae fed on leaves and roots of white radish. To our knowledge there is no report of *Diabrotica* larvae feeding on red beet roots or their leaves, but *Diabrotica virgifera* larvae have been observed to feed on other Amaranthaceae plants, such as *Amaranthus* weeds (Moeser & Vidal, 2004). Just as in our study, despite the high tissue consumption on these plants the larvae did not gain weight. Future studies should also take into consideration possible differences in pupation and adult emergence for *Diabrotica* that, as larvae, fed on leaves and roots of different plants.

While trying to choose the best-quality food, generalist and specialist species have evolved different strategies to cope with plant secondary metabolites (van der Meijden, 1996; Despres *et al.*, 2007; Ali & Agrawal, 2012; Erb *et al.*, 2013). Generalists, such as *D. balteata* and *D. undecimpunctata,* tend to tolerate the toxicity of the plant defense compounds, but dilute the negative effects by choosing among a wider host range than specialist species (Moreau *et al.*, 2003). Meanwhile, specialists like *D. virgifera* larvae have evolved highly-tuned mechanisms of detoxification and sequestration, that allow them to exploit the plants' resources efficiently (Petschenka & Agrawal, 2016; Robert *et al.*, 2017). For instance, caterpillars of the specialist *Spodoptera frugiperda* can detoxify benzoxazinoids (Glauser *et al.*, 2011) and prefer to forage on young maize leaves, whereas the generalist *S. littoralis* preferentially chooses to feed on older leaves, which are not as nutritious but less inducible for the production of benzoxazinoids (Köhler *et al.*, 2015). In a similar manner, *D. virgifera* larvae choose to feed on crown roots of maize, which are richer in sugars and iron but also in contents of benzoxazinoids, that they are able to tolerate and sequester (Robert *et al.*, 2012; Robert *et al.*, 2017; Hu *et al.*, 2018). However, in our experiments, larvae of the generalist *D. undecimpunctata* showed greater weight gain and *D. balteata* lower mortality on both maize tissues than *D. virgifera*, which might be explained by the adaptation of the former two laboratory strains to maize plants, on which they have

been reared for many generations. The chemical profiling of the plants showed that overall they have much higher amounts of secondary metabolites in leaves than in roots, with the exception of glucosinolates in white radish, of which the roots contained very high levels of 4-methylthio-3-butenyl glucosinolate, a strongly-pungent glucosinolate, specific for white radish (Ishida *et al.*, 2012). Glucosinolates are known to have antimicrobial and antiherbivore activity after hydrolyzing to isothiocyanates and other toxic aglucones upon tissue damage, a process commonly known as the mustard oil bomb (Bones & Rossiter, 1996). As generalist insects, *D. balteata* and *D. undecimpunctata* might not be adapted to cope with glucosinolates. In contrast, *Diabrotica* larvae are known to sequester cucurbitacins, yet our own research shows that *D. balteata* larvae do not perform well on cucumber shoots or roots, regardless of the cucurbitacin content (Bruno et al., *unpublished*). We also included red beets in our study, which are characterized by the presence of the intensely colored pink betalains such as betanin, especially in the roots. These indole-derived glycosides with known antimicrobial activity (Skalicky *et al.*, 2020) may have affected the capacity of entomopathogenic nematodes to infect larvae fed on red beet plants. The infection rates were particularly low for red beet-fed larvae of *D. balteata*, which revealed a clear effect of diet on vulnerability of EPN. However, for none the plants and insects evaluated did the larvae obtain differential protection against entomopathogenic nematode infection after feeding on leaves or roots.

The ability of *Diabrotica* larvae to feed on aboveground tissues might influence the potential of survival and even of colonization of new host plants (Moeser & Hibbard, 2005; Marchioro & Krechemer, 2018). It facilitates diet selection and optimization, but also exposes the insects to additional biotic and abiotic risk factors. Further research of this behavior is important for the accurate assessment of a potential invasion risk of *Diabrotica* species. Furthermore, a deeper understanding of the insects' feeding strategies could contribute to the development of more efficient integrated pest management strategies such as crop rotation, chemical and biological control of *Diabrotica* pests.

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Supplementary Material

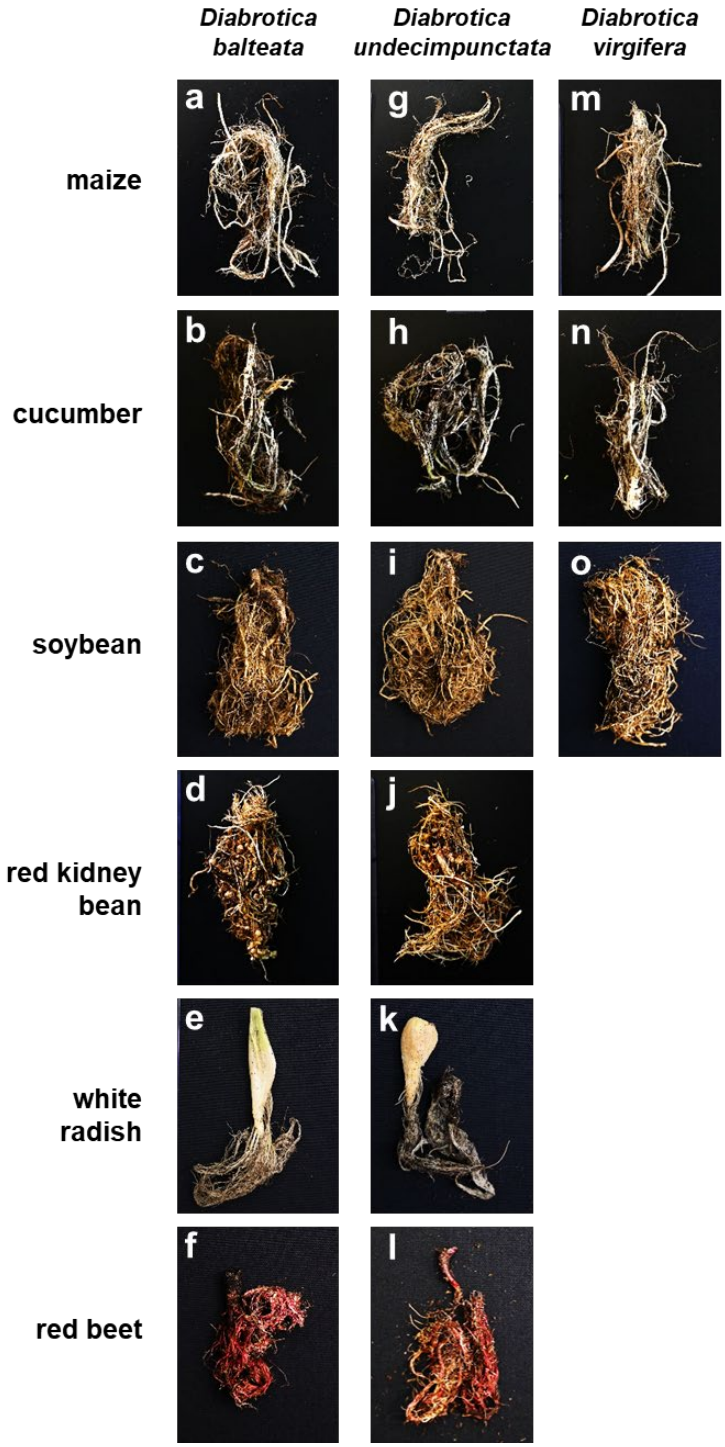


Figure S1. *Diabrotica balteata*, *D. undecimpunctata* and *D. virgifera* larvae feed on roots. General aspect of roots after fifteen second-instar *Diabrotica balteata* or *D. undecimpunctata* larvae, or ten second-instar *D. virgifera* larvae fed on roots for 48 hours. *Diabrotica balteata* larvae fed on (a) maize, (b) cucumber, (c) soybean, (d) red kidney bean, (e) white radish or (f) red beet. *Diabrotica undecimpunctata* larvae fed on (g) maize, (h) cucumber, (i) soybean, (j) red kidney bean, (k) white radish or (l) red beet. *Diabrotica virgifera* larvae fed on (m) maize, (n) cucumber, or (o) soybean.

Table S1. ANOVA results for the comparisons of dry biomass of leaves and roots of different plants consumed by larvae of *Diabrotica balteata*, *D. undecimpunctata* and *D. virgifera* for seven days. Not significant (n.s., p>0.05). Stars indicate significant differences between treatments: *p<0.05, **p<0.01,***p<0.001.

Treatment		larvae fed on leaves			larvae fed on roots			F	p-value	Signif. code
Larva	Plant	*n*	Mean biomass (mg/ larva)	±SE	*n*	Mean biomass (mg/ larva)	±SE			
Diabrotica balteata	maize	6	2.56	0.40	6	4.69	0.79	5.86	0.036	*
	cucumber	6	18.40	2.37	6	12.92	0.85	4.72	0.055	n.s.
	soybean	6	4.34	0.79	6	7.02	2.02	1.52	0.246	n.s.
	red kidney bean	6	9.11	1.84	6	11.40	1.96	0.73	0.414	n.s.
	white radish	6	18.68	2.78	6	5.41	2.60	12.17	0.006	**
	red beet	6	0.92	0.43	6	8.57	0.55	120.97	6.60E-07	***
Diabrotica undecimpunctata	maize	6	3.29	0.66	6	6.25	1.18	4.82	0.053	n.s.
	cucumber	6	16.04	2.24	6	18.00	3.14	0.26	0.621	n.s.
	soybean	6	8.39	3.79	6	7.93	2.99	0.01	0.926	n.s.
	red kidney bean	6	5.46	1.89	6	34.09	6.84	16.29	0.002	**
	white radish	6	6.25	1.10	6	1.54	1.01	9.88	0.010	*
	red beet	6	0.91	0.40	6	7.97	1.98	12.23	0.006	**
Diabrotica virgifera	maize	6	2.95	0.36	6	13.94	2.53	18.45	0.002	**
	cucumber	6	17.86	3.38	6	3.28	0.74	17.73	0.002	**
	soybean	6	11.75	4.39	6	16.34	8.89	0.21	0.654	n.s.

Table S2. ANOVA results for the comparisons of weight gain of larvae of *Diabrotica balteata*, *D. undecimpunctata* and *D. virgifera* after feeding on leaves or roots of different plants for seven days. Not significant (n.s., p>0.05). Stars indicate significant differences between treatments: *p<0.05,***p<0.001.

Treatment		larvae fed on leaves			larvae fed on roots					
Larva	Plant	*n*	Mean weight gain (mg/ larva)	±SE	*n*	Mean weight gain (mg/ larva)	±SE	F	p-value	Signif. code
Diabrotica balteata	maize	6	1.69	0.25	6	2.80	0.19	56.80	1.98E-05	***
	cucumber	6	5.91	0.42	6	3.42	0.22	34.35	0.0002	***
	soybean	6	0.27	0.12	6	1.54	0.14	52.18	2.85E-05	***
	red kidney bean	6	0.04	0.12	6	2.79	0.15	197.76	6.49E-08	***
	white radish	6	6.83	0.63	6	1.33	0.20	88.47	2.78E-06	***
	red beet	6	0.50	0.04	6	2.54	0.36	41.22	7.63E-05	***
Diabrotica undecimpunctata	maize	6	3.45	0.42	6	5.90	0.24	21.90	8.69E-04	***
	cucumber	6	5.91	0.56	6	3.84	0.59	4.46	0.061	*
	soybean	6	0.16	0.29	6	1.10	0.27	7.65	1.99E-02	*
	red kidney bean	6	0.87	0.25	6	4.27	0.49	38.43	1.02E-04	***
	white radish	6	3.13	0.47	6	0.23	0.16	50.26	3.34E-05	***
	red beet	6	0.17	0.19	6	3.19	0.42	64.19	1.16E-05	***
Diabrotica virgifera	maize	6	0.85	0.14	6	2.97	0.22	64.94	1.10E-05	***
	cucumber	6	0.63	0.12	6	0.15	0.16	5.79	0.0369	*
	soybean	6	1.59	0.15	6	1.46	0.21	0.25	0.634	n.s.

Table S3. ANOVA results for the comparisons of mortality of larvae of *Diabrotica balteata*, *D. undecimpunctata* and *D. virgifera* after feeding on leaves or roots of different plants for seven days. Not significant (n.s., p>0.05). Stars indicate significant differences between treatments: **p<0.01,***p<0.001.

Treatment		larvae fed on leaves			larvae fed on roots			Test	p-value	Signif. code
Larva	Plant	*n*	Mean mortality (%)	±SE	*n*	Mean mortality (%)	±SE			
Diabrotica balteata	maize	6	4.44	1.41	6	6.67	2.43	Chisq=9.167	0.514	n.s.
	cucumber	6	8.89	3.30	6	2.22	1.41	Chisq=4.015	0.0729	n.s.
	soybean	6	8.89	2.22	6	17.78	6.36	F=2.057	0.182	n.s.
	red kidney bean	6	56.67	5.09	6	15.56	5.07	F=25.462	0.0005	***
	white radish	6	11.11	3.72	6	11.11	2.81	F=0	1	n.s.
	red beet	6	2.22	1.41	6	4.44	1.41	Chisq=7.822	0.4019	n.s.
Diabrotica undecimpunctata	maize	6	27.78	5.82	6	34.44	4.99	F=0.7442	0.408	n.s.
	cucumber	6	23.33	3.75	6	46.67	5.16	Chisq=9.038	0.0009	n.s.
	soybean	6	75.56	4.77	6	71.11	5.62	F=0.3652	0.559	n.s.
	red kidney bean	6	50.00	4.79	6	57.78	5.35	Chisq=9.482	0.295	n.s.
	white radish	6	40.00	3.44	6	61.11	4.99	Chisq=7.0042	0.005	**
	red beet	6	47.78	2.68	6	53.33	7.10	Chisq=10.665	0.456	n.s.
Diabrotica virgifera	maize	6	16.67	7.15	6	20.00	4.47	F=0.1507	0.706	n.s.
	cucumber	6	16.67	7.15	6	18.33	3.07	F=0.0446	0.837	n.s.
	soybean	6	81.67	5.43	6	85.00	8.06	F=0.1134	0.743	n.s.

Table S4. ANOVA results for the comparisons between the infection rates per tissue five days after inoculation with *Heterorhabditis bacteriophora* entomopathogenic nematodes on larvae of *Diabrotica balteata*, *D. undecimpunctata* and *D. virgifera* previously. Prior inoculation, the larvae fed on leaves or roots of different plants for seven days. Not significant (n.s., p>0.05).

Treatment		larvae fed on leaves			larvae fed on roots			Test	p-value	Signif. code
Larva	Plant	*n*	Mean infection (%)	±SE	*n*	Mean infection (%)	±SE			
Diabrotica balteata	maize	10	47.50	12.61	10	60.83	5.83	F=0.5789	0.457	n.s.
	cucumber	10	76.67	8.94	10	77.50	4.49	F=0.3399	0.568	n.s.
	soybean	10	81.67	10.67	10	54.17	13.45	F=3.7156	0.069	n.s.
	red kidney bean	10	66.67	10.54	10	81.67	5.53	F=1.2882	0.271	n.s.
	white radish	10	42.50	8.91	10	30.00	6.24	F=14.869	0.077	n.s.
	red beet	10	5.00	3.33	10	12.50	5.59	Chisq=17.436	0.202	n.s.
Diabrotica undecimpunctata	maize	10	97.50	2.50	10	94.17	3.94	Chisq=11.109	0.533	n.s.
	cucumber	10	65.00	9.28	10	46.67	9.88	F=1.222	0.284	n.s.
	soybean	4	54.17	7.98	5	33.33	13.94	Chsiq=5.024	0.504	n.s.
	red kidney bean	10	80.00	11.33	10	86.67	5.44	F=0.049	0.827	n.s.
	white radish	10	80.00	6.94	8	72.92	8.30	Chisq=15.219	0.489	n.s.
	red beet	8	58.33	11.79	8	58.33	17.54	F=0.289	0.599	n.s.
Diabrotica virgifera	maize	10	21.67	9.31	10	35.00	8.41	F=0.852	0.368	n.s.
	cucumber	10	23.33	10.89	10	50.00	10.83	F=1.724	0.206	n.s.
	soybean	3	22.22	11.11	2	0.00	0.00	Chisq=0.737	0.201	n.s.

Table S5. ANOVA results for the comparisons between the infection rates per plant five days after inoculation with *Heterorhabditis bacteriophora* entomopathogenic nematodes on larvae of *Diabrotica balteata* or *D. undecimpunctata* previously fed on six different plants for seven days. Not significant (n.s., $p>0.05$). Letters indicate significant differences between plants: $p<0.05$.

Treatment	maize				cucumber				soybean			
Larva	*n*	Mean infection (%)	±SE	Group	*n*	Mean infection (%)	±SE	Group	*n*	Mean infection (%)	±SE	Group
Diabrotica balteata	20	54.17	6.93	ab	20	77.08	4.87	b	20	58.33	9.09	ab
Diabrotica undecimpunct.	20	95.83	2.30	ab	20	55.83	6.92	b	9	42.59	8.83	b

red kidney bean				white radish				red beet				Test	p-value
n	Mean infection (%)	±SE	Group	*n*	Mean infection (%)	±SE	Group	*n*	Mean infection (%)	±SE	Group		
20	74.17	6.04	b	20	36.25	5.48	a	20	8.75	3.28	c	F=17.606	7.24E-13
20	83.33	6.17	ac	20	76.67	5.04	abc	16	58.33	10.21	bc	F=7.663	4.27E-06

Table S6. ANOVA results for the comparisons between the infection rates per plant five days after inoculation with *Heterorhabditis bacteriophora* entomopathogenic nematodes on larvae of *Diabrotica virgifera* previously fed on three different plants for seven days. Not significant (n.s., $p>0.05$). Letters indicate significant differences between plants: $p<0.05$.

Treatment	maize				cucumber				soybean				Test	p-value
Larva	*n*	Mean infection (%)	±SE	Group	*n*	Mean infection (%)	±SE	Group	*n*	Mean infection (%)	±SE	Group		
Diabrotica virgifera	20	28.33	6.29	a	20	36.67	8.07	a	5	13.33	8.16	a	F=0.6474	0.529

Table S7. ANOVA results for the comparisons of the main benzoxazinoids in detached leaves compared to roots of maize plants after *Diabrotica balteata* larvae fed for 48 hours, or in undamaged tissues (control). Not significant (n.s., p>0.05). Stars indicate significant differences between treatments: *p<0.05, **p<0.01,***p<0.001.

Treatment	Compound	leaves			roots			F	p-value	Signif. code
		n	Mean (µg/g)	±SE	*n*	Mean (µg/g)	±SE			
eaten by larvae	MBOA	5	165.70	25.12	5	140.40	10.15	0.8723	0.3776	n.s.
	DIMBOA-Glc	5	2339.48	294.46	5	378.65	81.73	41.17	0.000206	***
	HDMBOA-Glc	5	1519.24	339.88	5	596.53	37.43	7.2818	0.02714	*
	HMBOA-Glc	5	427.32	58.58	5	259.03	26.46	6.8541	0.03074	*
	TOTAL	**5**	**4451.75**	**718.04**	**5**	**1374.62**	**155.77**	**72.44**	**2.79E-05**	***
control	MBOA	5	40.00	6.63	5	136.22	21.97	17.585	0.003023	**
	DIMBOA-Glc	5	3107.36	229.13	5	194.90	16.92	160.7	1.41E-06	***
	HDMBOA-Glc	5	185.17	19.16	5	488.30	17.88	133.76	2.84E-06	***
	HMBOA-Glc	5	383.47	38.99	5	206.87	18.12	16.868	0.003404	**
	TOTAL	**5**	**3716.00**	**293.92**	**5**	**1026.29**	**74.89**	**104.30**	**7.25E-06**	***

Table S8. ANOVA results for the comparisons of the main benzoxazinoids in detached leaves or roots of maize plants after *Diabrotica balteata* larvae fed for 48 hours compared to undamaged tissues (control). Not significant (n.s., p>0.05). Stars indicate significant differences between treatments: *p<0.05, **p<0.01.

Tissue	Compound	eaten by larvae			control			F	p-value	Signif. code
		n	Mean (µg/g)	±SE	*n*	Mean (µg/g)	±SE			
leaves	MBOA	5	165.70	25.12	5	40.00	6.63	23.417	0.001	**
	DIMBOA-Glc	5	2339.48	294.46	5	3107.36	229.13	4.236	0.074	n.s.
	HDMBOA-Glc	5	1519.24	339.88	5	185.17	19.16	15.358	0.004	**
	HMBOA-Glc	5	427.32	58.58	5	383.47	38.99	0.388	0.551	n.s.
	TOTAL	**5**	**4451.75**	**718.04**	**5**	**3716.00**	**293.92**	**2.994**	**0.122**	**n.s.**
roots	MBOA	5	140.40	10.15	5	136.22	21.97	0.030	0.867	n.s.
	DIMBOA-Glc	5	378.65	81.73	5	194.90	16.92	4.847	0.059	n.s.
	HDMBOA-Glc	5	596.53	37.43	5	488.30	17.88	6.809	0.031	*
	HMBOA-Glc	5	259.03	26.46	5	206.87	18.12	2.645	0.143	n.s.
	TOTAL	**5**	**1374.62**	**155.77**	**5**	**1026.29**	**74.89**	**6.287**	**0.037**	*

Table S9. ANOVA results for the comparisons of the main cucurbitacins in detached leaves compared to roots of cucumber plants after *Diabrotica balteata* larvae fed for 48 hours, or in undamaged tissues (control). Not significant (n.s., p>0.05). Stars indicate significant differences between treatments: ***p<0.001.

Treatment	Compound	leaves			roots			F	p-value	Signif. code
		n	Mean (µg/g)	±SE	*n*	Mean (µg/g)	±SE			
eaten by larvae	Cuc B	5	0.00	0.00	5	0.00	0.00	1.000	0.347	n.s.
	Cuc C	5	2.30	1.03	5	0.00	0.00	45.086	1.50E-04	***
	Unknown Cuc # 1	5	0.00	0.00	5	0.00	0.00	N/A	N/A	N/A
	Unknown Cuc # 2	5	0.45	0.20	5	0.00	0.00	600.860	8.19E-09	***
	Deacetylated Dihydro Cuc B glu	5	0.00	0.00	5	0.00	0.00	N/A	N/A	N/A
	Metabolized Cuc # 1	5	0.00	0.00	5	0.00	0.00	N/A	N/A	N/A
	Cuc B glu	5	0.00	0.00	5	0.01	0.01	1.000	0.347	n.s.
	Dihydro Cuc B glu	5	0.00	0.00	5	0.20	0.09	1.635	0.237	n.s.
	Isomer of Dihydro Cuc B glu	5	0.00	0.00	5	0.00	0.00	N/A	N/A	N/A
	Cuc II a glu	5	6.32	2.83	5	0.00	0.00	369.060	5.59E-08	***
	TOTAL	**5**	**9.07**	**4.06**	**5**	**0.22**	**0.10**	**191.900**	**7.13E-07**	***
control	Cuc B	5	0.00	0.00	4	0.00	0.00	N/A	N/A	N/A
	Cuc C	5	4.85	2.17	4	0.00	0.00	26.042	1.40E-03	***
	Unknown Cuc # 1	5	0.00	0.00	4	0.00	0.00	N/A	N/A	N/A
	Unknown Cuc # 2	5	0.48	0.21	4	0.00	0.00	93.558	2.66E-05	***
	Deacetylated Dihydro Cuc B glu	5	0.00	0.00	4	0.00	0.00	N/A	N/A	N/A
	Metabolized Cuc # 1	5	0.00	0.00	4	0.00	0.00	N/A	N/A	N/A
	Cuc B glu	5	0.00	0.00	4	0.05	0.02	1.296	0.292	n.s.
	Dihydro Cuc B glu	5	0.00	0.00	4	0.00	0.00	1.296	0.292	n.s.
	Isomer of Dihydro Cuc B glu	5	0.00	0.00	4	0.00	0.00	N/A	N/A	N/A
	Cuc II a glu	5	6.09	2.72	4	0.00	0.00	691.960	2.94E-08	***
	TOTAL	**5**	**11.41**	**5.10**	**4**	**0.05**	**0.02**	**108.55**	**1.63E-05**	***

Table S10. ANOVA results for the comparisons of the main cucurbitacins in detached leaves or roots of cucumber plants after *Diabrotica balteata* larvae fed for 48 hours compared to undamaged tissues (control). Not significant (n.s., p>0.05). Stars indicate significant differences between treatments: *p<0.05, **p<0.01, ***p<0.001.

Tissue	Compound	eaten by larvae			control			F	p-value	Signif. code
		n	Mean (µg/g)	±SE	*n*	Mean (µg/g)	±SE			
leaves	Cuc B	5	0.00	0.00	5	0.00	0.00	N/A	N/A	N/A
	Cuc C	5	2.30	1.03	5	4.85	2.17	26.042	0.001	**
	Unknown Cuc # 1	5	0.00	0.00	5	0.00	0.00	N/A	N/A	N/A
	Unknown Cuc # 2	5	0.45	0.20	5	0.48	0.21	93.558	2.66E-05	***
	Deacetylated Dihydro Cuc B glu	5	0.00	0.00	5	0.00	0.00	N/A	N/A	N/A
	Metabolized Cuc # 1	5	0.00	0.00	5	0.00	0.00	N/A	N/A	N/A
	Cuc B glu	5	0.00	0.00	5	0.00	0.00	1.296	0.292	n.s.
	Dihydro Cuc B glu	5	0.00	0.00	5	0.00	0.00	1.296	0.292	n.s.
	Isomer of Dihydro Cuc B glu	5	0.00	0.00	5	0.00	0.00	N/A	N/A	N/A
	Cuc II a glu	5	6.32	2.83	5	6.09	2.72	691.960	2.94E-08	***
	TOTAL	**5**	**9.07**	**4.06**	**5**	**11.41**	**5.10**	**108.550**	**1.63E-05**	***
roots	Cuc B	5	0.00	0.00	4	0.00	0.00	N/A	N/A	N/A
	Cuc C	5	0.00	0.00	4	0.00	0.00	7.935	0.023	*
	Unknown Cuc # 1	5	0.00	0.00	4	0.00	0.00	N/A	N/A	N/A
	Unknown Cuc # 2	5	0.00	0.00	4	0.00	0.00	0.269	0.618	n.s.
	Deacetylated Dihydro Cuc B glu	5	0.00	0.00	4	0.00	0.00	N/A	N/A	N/A
	Metabolized Cuc # 1	5	0.00	0.00	4	0.00	0.00	N/A	N/A	N/A
	Cuc B glu	5	0.01	0.01	4	0.05	0.02	N/A	N/A	N/A
	Dihydro Cuc B glu	5	0.20	0.09	4	0.00	0.00	N/A	N/A	N/A
	Isomer of Dihydro Cuc B glu	5	0.00	0.00	4	0.00	0.00	N/A	N/A	N/A
	Cuc II a glu	5	0.00	0.00	4	0.00	0.00	0.571		n.s.
	TOTAL	**5**	**0.22**	**0.10**	**4**	**0.05**	**0.02**	**4.204**	**0.074**	**n.s.**

Table S11. ANOVA results for the comparisons of the main flavonoids in detached leaves compared to roots of soybean plants after *Diabrotica balteata* larvae fed for 48 hours, or in undamaged tissues (control). Not significant (n.s., p>0.05). Stars indicate significant differences between treatments: *p<0.05, **p<0.01, ***p<0.001.

Treatment	Compound	leaves			roots			F	p-value	Signif. code
		n	Mean (µmol/g)	±SE	*n*	Mean (µmol/g)	±SE			
eaten by larvae	rutin	5	54.04	54.04	5	0.41	0.11	0.985	0.350	n.s.
	quercetin triglycoside	5	97.01	40.32	5	0.33	0.02	5.750	0.043	*
	kaempferol triglycoside	5	0.13	0.08	5	0.18	0.07	0.209	0.660	n.s.
	Isorhamnetin triglycoside	5	49.62	30.59	5	0.34	0.02	2.596	0.146	n.s.
	isorhamnetin triglycoside isomer	5	48.91	30.12	5	0.27	0.07	2.608	0.145	n.s.
	kaempferol hexoside rhamnoside	5	89.65	36.90	5	0.31	0.08	5.861	0.042	*
	isorhamnetin hexoside rhamnoside	5	102.17	31.86	5	0.42	0.03	10.200	0.013	*
	kaempferol-glucuronide	5	0.41	0.14	5	0.35	0.10	0.15	0.71	n.s.
	TOTAL	**5**	**441.94**	**224.04**	**5**	**2.61**	**0.50**	**12.45**	**0.01**	**
control	rutin	5	36.33	36.33	5	0.40	0.12	0.978	0.352	n.s.
	quercetin triglycoside	5	80.28	52.38	5	0.24	0.06	2.335	0.165	n.s.
	kaempferol triglycoside	5	0.07	0.07	5	0.12	0.08	0.294	0.603	n.s.
	Isorhamnetin triglycoside	5	18.54	18.54	5	0.26	0.07	0.972	0.353	n.s.
	isorhamnetin triglycoside isomer	5	73.11	53.55	5	0.32	0.01	1.848	0.211	n.s.
	kaempferol hexoside rhamnoside	5	66.80	43.17	5	0.08	0.08	2.389	0.161	n.s.
	isorhamnetin hexoside rhamnoside	5	142.15	17.67	5	0.30	0.08	64.472	4.25E-05	***
	kaempferol-glucuronide	5	0.32	0.09	5	0.56	0.19	1.357	0.278	n.s.
	TOTAL	**5**	**417.60**	**221.79**	**5**	**2.30**	**0.67**	**8.948**	**0.017**	*

Table S12. ANOVA results for the comparisons of the main flavonoids in detached leaves or roots of soybean plants after *Diabrotica balteata* larvae fed for 48 hours compared to undamaged tissues (control). Not significant (n.s., p>0.05).

Tissue	Compound	eaten by larvae			control			F	p-value	Signif. code
		n	Mean (µmol/g)	±SE	*n*	Mean (µmol/g)	±SE			
leaves	rutin	5	54.04	54.04	5	36.33	36.33	0.074	0.793	n.s.
	quercetin triglycoside	5	97.01	40.32	5	80.28	52.38	0.064	0.807	n.s.
	kaempferol triglycoside	5	0.13	0.08	5	0.07	0.07	0.330	0.581	n.s.
	Isorhamnetin triglycoside	5	49.62	30.59	5	18.54	18.54	0.755	0.410	n.s.
	isorhamnetin triglycoside isomer	5	48.91	30.12	5	73.11	53.55	0.155	0.704	n.s.
	kaempferol hexoside rhamnoside	5	89.65	36.90	5	66.80	43.17	0.162	0.698	n.s.
	isorhamnetin hexoside rhamnoside	5	102.17	31.86	5	142.15	17.67	1.205	3.04E-01	n.s.
	kaempferol-glucuronide	5	0.41	0.14	5	0.32	0.09	0.296	0.601	n.s.
	TOTAL	**5**	**441.94**	**224.04**	**5**	**417.60**	**221.79**	**0.017**	**0.899**	**n.s.**
roots	rutin	5	0.41	0.11	5	0.40	0.12	0.001	0.975	n.s.
	quercetin triglycoside	5	0.33	0.02	5	0.24	0.06	1.915	0.204	n.s.
	kaempferol triglycoside	5	0.18	0.07	5	0.12	0.08	0.258	0.625	n.s.
	Isorhamnetin triglycoside	5	0.34	0.02	5	0.26	0.07	1.398	0.271	n.s.
	isorhamnetin triglycoside isomer	5	0.27	0.07	5	0.32	0.01	0.620	0.454	n.s.
	kaempferol hexoside rhamnoside	5	0.31	0.08	5	0.08	0.08	4.276	0.072	n.s.
	isorhamnetin hexoside rhamnoside	5	0.42	0.03	5	0.30	0.08	2.068	0.188	n.s.
	kaempferol-glucuronide	5	0.35	0.10	5	0.56	0.19	1.039	0.338	n.s.
	TOTAL	**5**	**2.61**	**0.50**	**5**	**2.30**	**0.67**	**0.949**	**0.359**	**n.s.**

Table S13. ANOVA results for the comparisons of the main flavonoids in detached leaves compared to roots of red kidney bean plants after *Diabrotica balteata* larvae fed for 48 hours, or in undamaged tissues (control). Not significant (n.s., p>0.05). Stars indicate significant differences between treatments: *p<0.05, **p<0.01, ***p<0.001.

Treatment	Compound	leaves			roots			F	p-value	Signif. code
		n	Mean (µmol/g)	±SE	*n*	Mean (µmol/g)	±SE			
eaten by larvae	rutin	5	0.00	0.00	5	0.58	0.06	91.711	1.17E-05	***
	quercetin triglycoside	5	4.64	1.56	5	0.30	0.08	7.763	0.024	*
	kaempferol triglycoside	5	0.13	0.08	5	0.24	0.06	1.270	0.292	n.s.
	Isorhamnetin triglycoside	5	0.33	0.09	5	0.20	0.08	1.130	0.319	n.s.
	isorhamnetin triglycoside isomer	5	0.22	0.09	5	0.20	0.08	0.022	0.886	n.s.
	kaempferol hexoside rhamnoside	5	87.05	54.25	5	3.35	0.84	2.379	0.162	n.s.
	isorhamnetin hexoside rhamnoside	5	64.02	9.97	5	0.44	0.05	40.679	2.14E-04	***
	kaempferol-glucuronide	5	0.00	0.00	5	0.89	0.28	9.930	0.014	*
	TOTAL	**5**	**156.39**	**66.05**	**5**	**6.19**	**1.53**	**9.334**	**0.016**	*
control	rutin	5	123.45	51.11	5	0.79	0.17	5.760	0.043	*
	quercetin triglycoside	5	4.73	0.82	5	0.28	0.07	29.571	6.18E-04	***
	kaempferol triglycoside	5	0.17	0.07	5	0.00	0.00	5.790	0.043	*
	Isorhamnetin triglycoside	5	0.18	0.07	5	0.25	0.07	0.452	0.520	n.s.
	isorhamnetin triglycoside isomer	5	0.18	0.07	5	0.17	0.07	0.026	0.876	n.s.
	kaempferol hexoside rhamnoside	5	120.38	33.74	5	4.73	0.84	11.744	0.009	**
	isorhamnetin hexoside rhamnoside	5	43.96	4.15	5	0.45	0.05	109.810	5.98E-06	***
	kaempferol-glucuronide	5	0.00	0.00	5	1.69	0.92	3.335	0.105	n.s.
	TOTAL	**5**	**293.06**	**90.03**	**5**	**8.36**	**2.20**	**15.921**	**0.004**	**

Table S14. ANOVA results for the comparisons of the main flavonoids in detached leaves or roots of red kidney bean plants after *Diabrotica balteata* larvae fed for 48 hours compared to undamaged tissues (control). Not significant (n.s., p>0.05). Stars indicate significant differences between treatments: *p<0.05, **p<0.01.

Tissue	Compound	eaten by larvae			control			F	p-value	Signif. code
		n	Mean (µmol/g)	±SE	*n*	Mean (µmol/g)	±SE			
leaves	rutin	5	0.00	0.00	5	123.45	51.11	5.835	0.042	*
	quercetin triglycoside	5	4.64	1.56	5	4.73	0.82	0.003	0.961	n.s.
	kaempferol triglycoside	5	0.13	0.08	5	0.17	0.07	0.136	0.722	n.s.
	Isorhamnetin triglycoside	5	0.33	0.09	5	0.18	0.07	1.464	0.261	n.s.
	isorhamnetin triglycoside isomer	5	0.22	0.09	5	0.18	0.07	0.087	0.776	n.s.
	kaempferol hexoside rhamnoside	5	87.05	54.25	5	120.38	33.74	0.272	0.616	n.s.
	isorhamnetin hexoside rhamnoside	5	64.02	9.97	5	43.96	4.15	3.450	0.100	n.s.
	quercetin-glucuronide	5	0.00	0.00	5	0.00	0.00	N/A	N/A	N/A
	kaempferol-glucuronide	5	0.00	0.00	5	0.00	0.00	N/A	N/A	N/A
	TOTAL	**5**	**156.39**	**66.05**	**5**	**293.06**	**90.03**	0.105	**0.153**	**n.s.**
roots	rutin	5	0.58	0.06	5	0.79	0.17	1.361	0.277	n.s.
	quercetin triglycoside	5	0.30	0.08	5	0.28	0.07	0.035	0.856	n.s.
	kaempferol triglycoside	5	0.24	0.06	5	0.00	0.00	15.938	3.99E-03	**
	Isorhamnetin triglycoside	5	0.20	0.08	5	0.25	0.07	0.267	0.619	n.s.
	isorhamnetin triglycoside isomer	5	0.20	0.08	5	0.17	0.07	0.094	0.767	n.s.
	kaempferol hexoside rhamnoside	5	3.35	0.84	5	4.73	0.84	1.335	0.281	n.s.
	isorhamnetin hexoside rhamnoside	5	0.44	0.05	5	0.45	0.05	0.048	0.832	n.s.
	quercetin-glucuronide	5	0.00	0.00	5	0.00	0.00	N/A	N/A	N/A
	kaempferol-glucuronide	5	0.89	0.28	5	1.69	0.92	0.682	0.433	n.s.
	TOTAL	**5**	**6.19**	**1.53**	**5**	**8.36**	**2.20**	**0.879**	**0.376**	**n.s.**

Table S15. ANOVA results for the comparisons of the main glucosinolates in detached leaves compared to roots of white radish plants after *Diabrotica balteata* larvae fed for 48 hours, or in undamaged tissues (control). Not significant (n.s., p>0.05). Stars indicate significant differences between treatments: *p<0.05, **p<0.01, ***p<0.001.

Treatment	Compound	leaves			roots			F	p-value	Signif. code
		n	Mean (µmol/g)	±SE	*n*	Mean (µmol/g)	±SE			
eaten by larvae	Glucoraphanin	5	0.18	0.05	5	0.20	0.03	11.043	0.010	*
	Glucoraphenin	5	6.35	1.62	5	5.60	0.72	14.221	0.005	**
	Glucoerucin	5	0.10	0.03	5	0.11	0.03	27.515	7.78E-04	***
	4-Methylthio-3-butenyl glucosinolate	5	3.81	1.61	5	2.97	0.92	27.618	7.69E-04	***
	Glucobrassicin	5	0.51	0.16	5	0.15	0.04	6.433	0.035	*
	TOTAL	**5**	10.95	3.48	**5**	9.03	1.74	**1.988**	**0.196**	**n.s.**
control	Glucoraphanin	5	0.02	0.00	5	0.03	0.00	28.364	7.06E-04	***
	Glucoraphenin	5	0.23	0.03	5	0.33	0.06	53.315	8.38E-05	***
	Glucoerucin	5	0.60	0.09	5	0.65	0.07	52.231	9.00E-05	***
	4-Methylthio-3-butenyl glucosinolate	5	15.04	1.40	5	15.98	0.81	111.940	5.56E-06	***
	Glucobrassicin	5	0.08	0.04	5	0.01	0.01	13.784	0.006	**
	TOTAL	**5**	15.96	1.56	**5**	17.00	0.95	**41.090**	**2.07E-04**	***

Table S16. ANOVA results for the comparisons of the main glucosinolates in detached leaves or roots of white radish plants after *Diabrotica balteata* larvae fed for 48 hours compared to undamaged tissues (control). Not significant (n.s., p>0.05). Stars indicate significant differences between treatments: *p<0.05, **p<0.01.

Treatment	Compound	eaten by larvae			control			F	p-value	Signif. code
		n	Mean (µmol/g)	±SE	*n*	Mean (µmol/g)	±SE			
leaves	Glucoraphanin	5	0.18	0.05	5	0.02	0.00	0.080	0.784	n.s.
	Glucoraphenin	5	6.35	1.62	5	0.23	0.03	0.179	0.683	n.s.
	Glucoerucin	5	0.10	0.03	5	0.60	0.09	0.081	0.784	n.s.
	4-Methylthio-3-butenyl glucosinolate	5	3.81	1.61	5	15.04	1.40	0.202	0.665	n.s.
	Glucobrassicin	5	0.51	0.16	5	0.08	0.04	4.676	0.063	n.s.
	TOTAL	**5**	10.95	3.48	**5**	15.96	1.56	0.329	**0.582**	**n.s.**
roots	Glucoraphanin	5	0.20	0.03	5	0.03	0.00	1.973	0.198	n.s.
	Glucoraphenin	5	5.60	0.72	5	0.33	0.06	2.264	0.171	n.s.
	Glucoerucin	5	0.11	0.03	5	0.65	0.07	0.201	0.666	n.s.
	4-Methylthio-3-butenyl glucosinolate	5	2.97	0.92	5	15.98	0.81	0.340	0.576	n.s.
	Glucobrassicin	5	0.15	0.04	5	0.01	0.01	3.308	0.106	n.s.
	TOTAL	**5**	9.03	1.74	**5**	17.00	0.95	**0.360**	**0.565**	**n.s.**

Table S17. ANOVA results for the comparisons of betanin in detached leaves compared to roots of red beet plants after *Diabrotica balteata* larvae fed for 48 hours, or in undamaged tissues (control). Not significant (n.s., p>0.05). Stars indicate significant differences between treatments: *p<0.05, **p<0.01, ***p<0.001.

Compound	Treatment	leaves			roots			F	p-value	Signif. code
		n	Mean (peak area)	±SE	*n*	Mean (peak area)	±SE			
Betanin	eaten by larvae	5	4777.24	1110.98	5	1597.33	401.29	7.25	0.027	*
	control	5	5094.38	638.59	5	5215.34	1044.43	0.01	0.924	n.s.

5. Conclusions and Outlook

The results presented in this thesis provide novel insights into the interactions between several cultivated plants, larvae of *Diabrotica* beetles and the natural enemies of these pests.

The questions addressed in the thesis are briefly answered below, and outlooks for each research project are proposed:

Are parasitoids of Diabroticina beetles good candidates for the development of biological control against WCR in Europe? (Chapter I)

The extremely low number of parasitic flies and wasps obtained during our survey in Mexico might indicate that Diabroticina adult beetles are not readily controlled by these organisms. This would suggest that they are not very promising candidates for the development of classical biological control against WCR in Europe. However, the short time window available to collect beetles that are alive but that have already been parasitized might have led us to underestimate real parasitism rates. This is due to the fact that parasitized beetles die within 24-48 hours after parasitization (Fischer, 1981; Fischer, 1983) and the retrieval in field conditions of beetle cadavers containing parasitoid eggs is practically unfeasible. Future surveys could target other regions and at other moments in the crops' phenology, for instance during maize and squash pollination, and might thus be more successful at obtaining large amounts of living parasitized beetles (Zhang, F., Alvarez-Zagoya, R., *personal communications*). A sampling trip of three months might be the minimum necessary to have enough collection time and two generations of the parasitoids, which would allow the second generation of pupae to be transported to new rearing facilities. In addition, after the field collection of parasitized beetles, specific laboratory conditions are needed. These include facilities with controlled humidity, air current, temperature and photoperiod, as well as plants inside the cages. Laboratory personnel might help feed the beetles and daily retrieve and maintain the pupae at high humidity conditions, until emergence of adults inside cages with good ventilation and available honey drops. Ventilation and temperature control are particularly important at sunrise and early morning, when virgin females emerge and are rapidly fecundated. These observations might help future researchers increase their chances of successfully establishing rearings for parasitic flies and wasps of Diabroticina beetles, to explore their biocontrol potential against *Diabrotica* and related pests.

Are EPN from Mexico adapted to benzoxazinoids sequestered by WCR from maize roots? (Chapter II)

Form field surveys in Mexico we obtained several EPN isolates that might be adapted to WCR defenses and showed high capacity to infect WCR that contains high amounts of BXs. These highly-infective EPN isolates could potentially be used as effective biocontrol agents in the Integrated Pest Management of WCR in Europe. However, WCR appears to also rely on different strategies other than the sequestration of benzoxazinoids to resist EPN infection. Further research could help elucidate the mechanisms of WCR defense against EPN and/or their symbiotic bacteria. The immune response, in particular the secretion of antimicrobial-peptides (AMP), might be different between WCR and *D. balteata* larvae. To better understand this, the transcriptional activation of AMP genes (Ffrench-Constant *et al.*, 2007), their expression with GFP reporter constructs (Hallem *et al.*, 2007), as well as the encapsulation of EPN with hemocytes or melanin (Poinar, 1974), might be compared between WCR and *D. balteata* larvae fed on benzoxazinoid-free maize seedlings and inoculated with EPN.

To which species belong the symbiotic bacteria of *Heterorhabditis atacamensis* and *H. mexicana*? (Annex to Chapter II)

The molecular analyses of the mutualistic bacteria collected from *Heterorhabditis atacamensis* and *H. mexicana* revealed that these bacteria belonged to new subspecies of *Photorhabdus khanii* and *Photorhabdus luminescens*, respectively. In a fruitful collaboration with the University of Bern, we described and reported the new subspecies, which were thereupon named *Photorhabdus khanii* subsp. *guanajuatensis* subsp. nov. (isolated from *Heterorhabditis atacamensis)*, and *Photorhabdus luminescens* subsp. *mexicana* subsp. nov. (isolated from *H. mexicana)*.

Do larvae of *D. balteata* sequester cucurbitacins? What are the costs of the sequestration? Do sequestered cucurbitacins protect *D. balteata* larvae against natural enemies? (Chapter III)

This study provides solid evidence that the larvae of *D. balteata* are able to sequester cucurbitacins from different plant tissues, including leaves and stems. Overall, we found no induction of cucurbitacin production after *D. balteata* larvae had fed on cucumber plants, but the identities of the cucurbitacins found in plants and larvae were considerably different. This implies that the larvae transform the molecules and mostly accumulate transformed cucurbitacins in their bodies. Importantly and surprisingly, we did not observe an apparent impact of sequestered cucurbitacins on the larval performance of *D. balteata* larvae, nor did they provide protection against attack from natural enemies. Although a wide range of natural enemies was tested, it remains possible that the sequestration of cucurbitacins by *D. balteata* larvae target other natural enemies, such as birds, rodents or pathogens not evaluated in this study.

Further research is also needed to unravel the physiological mechanisms of the sequestration of cucurbitacins by *D. balteata* larvae and its significance for the the insect. In order to do so, the first step would be to isolate and purify the main cucurbitacins in cucumber plants. With pure cucurbitacins, performance experiments could evaluate full development of the larvae on cucurbitacin-free and cucurbitacin-containing diets, and other factors like time for pupation, pupation rates, emergence of adults and fitness, could also be investigated. In addition, the different purified cucurbitacins might be used in feeding experiments to directly evaluate their toxicity on *D. balteata* larvae, on other *Diabrotica* species, and even on the survival of natural enemies. Feeding experiments using these purified cucurbitacins might also help evaluate the nutritional value and the molecular changes in the plant-cucurbitacins after consumption by *D. balteata* larvae, *i. e.* metabolization, accumulation in body tissues, and excretion (Waldbauer, 1968; Friedrichs *et al.*, 2020). After gaining this knowledge, specific gene families involved in particular chemical changes produced by the larvae to transform plant cucurbitacins into those accumulated in their bodies, could be silenced to study the toxicity of the different molecules. This would help to better understand the role of cucurbitacin metabolic transformations by the larvae, which might be related to the avoidance of toxicity of plant-cucurbitacins.

Another aspect of this chapter that could be furtherly investigated is the lack of induction of cucurbitacins after *D. balteata* larvae fed on the plants, which might be particular to the adapted *Diabrotica* species, that do not seem to be negatively affected by cucurbitacins, contrary to other herbivores (Metcalf, 1986). To explain this, one hypothesis could be that *Diabrotica* larvae might avoid the induction of cucurbitacins by benefitting of suppressors of the jasmonic acid signally pathway, which could be present in bacteria from their oral secretion, as has been previously observed in larvae of the Colorado potato beetle (*Leptinotarsa decemlineata*) (Chung *et al.*, 2013). To evaluate this, contents of cucurbitacins after mechanical wounding, or after feeding by larvae treated or not with antibiotics might be compared. In addition, bacteria could be isolated from the oral secretion of *D. balteata* larvae and then applied to mechanically-wounded plants, after which cucurbitacin analyses could be performed. These experiments might help gain insight on the adaptation of Diabroticina species to feed on the Cucurbitaceae family.

Are *Diabrotica* larvae able to feed on aboveground tissues? Does this behavior have costs and/or benefits for the larvae? (Chapter IV)

In this study, we described a peculiar aboveground feeding-behavior of *Diabrotica* larvae that has been largely ignored in the literature. We observed that larvae of *D. balteata*, *D. undecimpunctata* and *D. virgifera* (WCR) can readily survive feeding on leaves as well as on roots of different plants. Feeding on the different tissues did not affect the larval susceptibility to EPN infection. Other aspects of performance (such as time to pupate), as well as the preference for different tissues and risk of predation for larvae feeding above or belowground, as well as exposure to new abiotic stressors,

remain to be investigated. Feeding experiments that study the larval performance on different tissues until full development, choice experiments between leaves and roots, and field observations of the larvae under natural conditions, would be essential to better understand this unique feeding behavior of these so-called rootworms.

Other questions that have arisen from this thesis and that could be addressed in future research are:

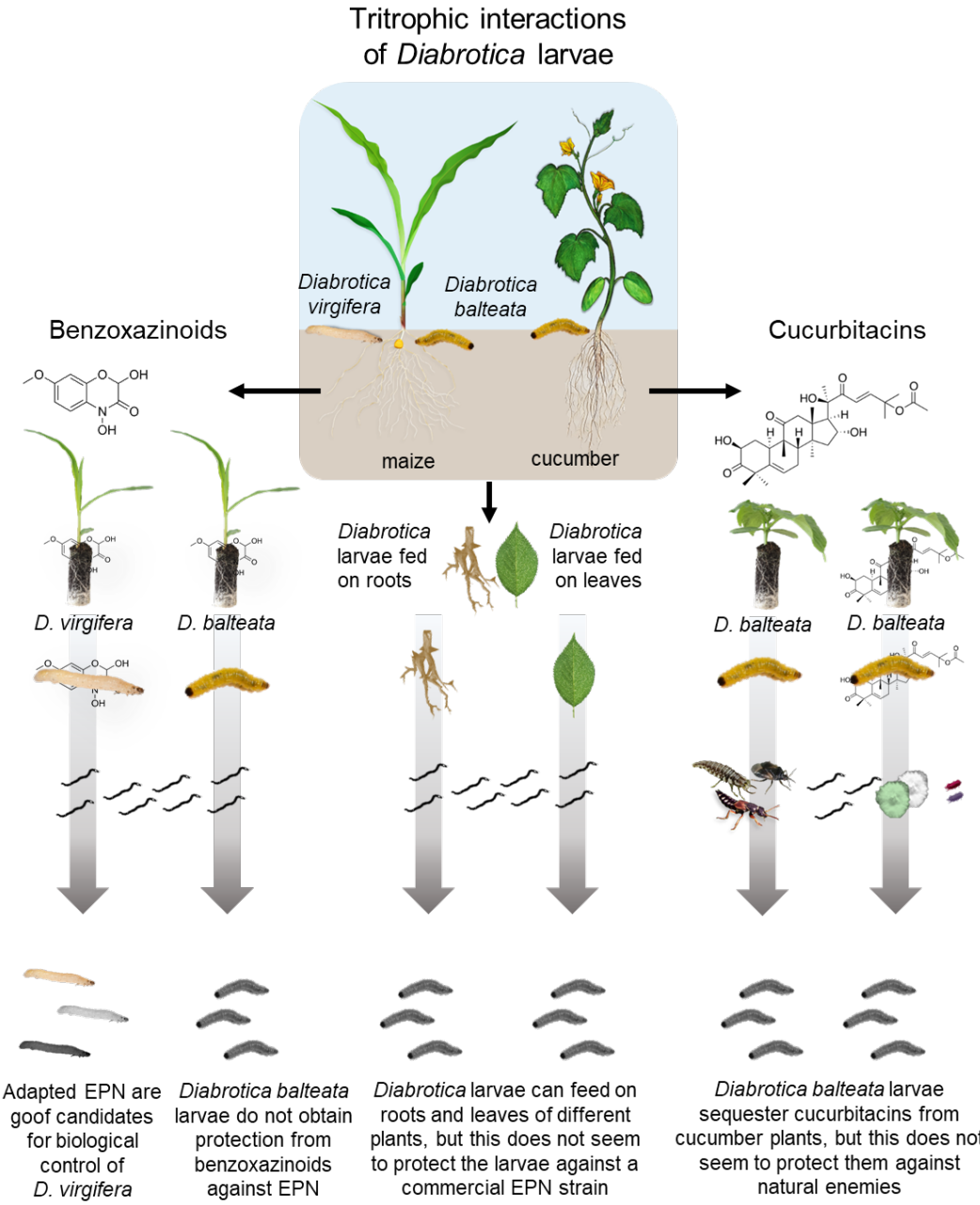
- **Which evolutionary forces have led *D. balteata* larvae to sequester cucurbitacins instead of benzoxazinoids?**

In our experiments, we observed that larvae of *D. balteata* perform well on maize seedlings, just as well as *D. virgifera*, and better than when fed on cucumber plants (regardless of the contents of cucurbitacins). Therefore, we ask ourselves why they choose to feed on apparently less suitable plants and why the larvae sequester cucurbitacins, which do not seem to protect them against natural enemies (Chapter III)? BXs, in contrast, have been shown to protect WCR against EPN (Chapter II). We hypothesize that *D. balteata* larvae are also capable of detoxifying BXs, but the mechanisms are not known. Isotopically-labeled BXs and cucurbitacins could be used to explore the physiological mechanisms of sequestration of cucurbitacins and detoxification of BXs by *D. balteata* larvae, as well as in complementation tests to determine the costs and benefits of both processes. The ability of *Diabrotica* larvae to cope with cucurbitacins, unlike many other herbivores, may provide them with a unique niche that cannot be exploited by others. This niche advantage may outweigh any ecological and physiological costs associated with feeding on Cucurbitaceae.

- **Which factors could explain the association between cucurbits and Diabroticina species?**

The voracious consumption of Cucurbitaceae plants by Diabroticina and other Luperini beetles, including the "Old World" Aulacophorina subtribe of pumpkin beetles, has been historically considered as a pharmacophagy (Nishida & Fukami, 1990). The term implies that the insects only feed on a particular plant for their secondary metabolites as medicine, instead of for their nutritional value (Boppré, 1984). This would suggest that Cucurbitaceae are not the current hosts of the larval stages of Luperines, but rather their ancestral hosts, and that these plants are still sought by the adults to complement the lack of cucurbitacins in their diets, mainly for a defensive advantage (Boppré, 1984; Boppré, 1986). However, the ancestral host theory is being challenged by newer experimental and phylogenetic research (Gillespie *et al.*, 2003; Eben, 2012; Eben & Espinosa de Los Monteros, 2013). As a consequence, an alternative hypothesis has been proposed by Tallamy et al., called the "loose receptor" theory (Tallamy *et al.*, 1999; Gillespie *et al.*, 2003; Tallamy *et al.*, 2005). This scenario

hypothesizes that the Luperine beetles, which feed mainly on pollen (Metcalf *et al.*, 1980), have evolved an association to cucurbits due to the molecular similarity between cucurbitacins and sterols present in pollen, added to the fact that receptor sites on the beetles' taste sensilla have unspecific or "loose" bindings (Tallamy *et al.*, 2005). Therefore, the search for pollen would have also selected towards an attraction to cucurbitacins. This "physiological coincidence" would have first favored the tolerance to cucurbitacins, and the advantages obtained by the defensive properties of cucurbitacins would have ultimately derived in the evolution of cucurbitacin sequestration (Tallamy *et al.*, 2005; Eben, 2012). As our own results did not show any protective benefit for larvae fed on cucurbitacin-containing plants, we cannot support either of these two hypotheses. Nevertheless, it cannot be discarded that other cucurbitacins, belonging to plants that evolved with the beetles in their center of origin, i. e. *Cucurbita* spp. with *Diabrotica* and *Acalymma* spp. in Mesoamerica, or *Cucumis* with *Aulacophora* spp. in Asia, might have different effects than those found in this study (Ramdas Menon *et al.*, 1972; Lewis & Metcalf, 1996; Benrey & Jaccard, 2018; Jaccard *et al.*, 2021). Future research might shed new light either on the adaptive significance of feeding on Cucurbitaceae by *Diabrotica* species, or perhaps different protective values of the sequestered compounds.



Graphic representation of the results and conclusions derived from this thesis.

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6. Acknowledgements

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The life in Neuchâtel during my PhD is something I will always treasure in my memory. The intense lab work was easily forgotten at the beautiful lake. The uni and the third-Friday apéros, the movie nights, the sushi / tacos / curry nights, the Pubquiz at Café du Cerf, hiking in Switzerland, ski lessons and lots of sport classes. Insect-themed patisserie. Learning German. A bit of singing, a bit of guitar. And so much more. All those moments shared with my friends inside and outside of the lab made the life during my PhD so special: Ottilie, mi mejor amiga argentina, tu amistad fue una de las mejores cosas de Suiza. Marine et Gaia, merci pour tous les thés et les conversations enrichissantes, et pour votre grande amitié. Mary, it was a huge pleasure meeting you, we should have had more chats outside. All the LEF members, specially my dearest Moe and Adrienne, Sarah and Ludo. Nos chers amis Marie et Francesco, Sonia and James. Yosra, Diane et Ilham, ça a été super de vous rencontrer ! Tom et les mathématiciens, tous uniques, rêveurs et randonneurs: Luc et Aïsha, Ana et les dessins de coasters aux Brasseurs, Valentin et ses grand feus, Thiebout, Joé, Radu et beaucoup d'autres qui passent par l'institut…Tom, tu es une de mes personnes préférées dans ce monde, merci pour tous ces moments des discussions agréables, musique, randonnées, et pour tes histoires surréalistes magnifiques. And Tobias, who changed my life forever and now we continue the adventure together. Thank you for your company, support and love.

Finally, thanks to my family, who accompanied me throughout these past years and enjoyed a bit of the beautiful Switzerland with me.

7. Curriculum Vitae: Pamela Bruno

Hermann-Föge-Weg 4, 37073 Göttingen, Germany │ pamela.bruno.hurth@gmail.com

EDUCATION

2016-Aug 2021 **PhD in Chemical Ecology** at the laboratory of Fundamental and Applied Research in Chemical Ecology (FARCE) Neuchâtel, Switzerland, under the supervision of Prof. Ted Turlings and Dr. Carla Marques Arce. Title: "Tritrophic interactions: Possible host plants effects on the resistance of *Diabrotica* pests to naturel enemies".

2016 **M. Sc. Plant Biology**, specialization in Plant Pathology. University of Poitiers, France.

2014 **Agronomic Engineer** (M. Sc. equivalent level), specialization in Viticulture and Enology. National University of Cuyo, Argentina

RESEARCH & ACADEMIC EXPERIENCE

Next project:

2021-2024 Postdoctoral position at the Laboratory of Agricultural Entomology, Prof. Michael Rostás. Department for Crop Sciences, Georg-August-Universität Göttingen, Germany. https://www.uni-goettingen.de/de/3240.html?id=6257

Publications

Bruno, P., Machado, R.A., Glauser, G., Kohler, A., Campos-Herrera, R., Bernal, J., Toepfer, S., Erb, M., Robert, C.A.M., Arce, C.C. and Turlings, T.C., 2020. Entomopathogenic nematodes from Mexico that can overcome the resistance mechanisms of the western corn rootworm. *Scientific reports*. https://www.nature.com/articles/s41598-020-64945-x

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kill strategy against 3 wireworms combining semiochemicals and entomopathogenic nematodes, 2021. *Turkish Journal of Zoology*. doi:10.3906/zoo-2106-38

Manuscripts in preparation

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Bruno, P., Gindroz, E., Glauser, G., Turlings, T. C. J., Machado, R. A R. & Arce, C. C. M. Feeding behavior of *Diabrotica* larvae: Long-thought rootworms that feed on leaves.

Bruno, P., de Vries Reilingh, S. M., Carrea, A., Bouvier, J. Eggs of the brown dog tick *Rhipicephalus sanguineus* are avoided by common predatory insects.

Bruno, P.*, Di Caprio, L.*, Jaccard, C., Ye, W., Arce, C. C. M., & Benrey, B. Induced resistance of squash plants to the banded cucumber beetle *Diabrotica balteata*. * equally contributing authors.

Arce, C. C. M, Machado, R. A . R, Mamin, M., **Bruno, P.**, Benrey, B., Glauser, G., Erb, M., Robert, C. A. M., & Turlings, T. C. J. *Diabrotica virgifera virgifera* females can sequester multiple plant toxins to protect their eggs against natural enemies.

Jaccard, C.* Marguier, N. T.*, Arce, C. C. M., **Bruno, P.,** Glauser, G., Turlings, T. C. J., & Benrey, B. The effects of domestication of squash, *Cucurbita argyrosperma*, on a belowground tritrophic interaction. * equally contributing authors (*to be submitted to Plant, Cell & Environment*)

Machado, R. A. R, Bhat, A. H.*, Abolafia, J.*, Müller, A.*, **Bruno, P.**, Fallet, P., Arce, C. C. M., Turlings, T. C. J., Bernal, J. S., Kajuga, J., Waweru, B., & Toepfer, S. Multi-locus phylogenetic analyses uncover species boundaries and revealed the occurrence of two new entomopathogenic nematode species, *Heterorhabditis ruandica* n. sp. and n. sp. * equally contributing authors.

Oral presentations

2020 Virtual Meeting of the American Society of Entomology (ESA 2020). **First place award** at the graduate 10-minute paper oral presentations in Chemical Ecology.

2019 Annual PhD Student Meeting 2019, University of Neuchâtel, Switzerland. Sequestration of cucurbitacins by *Diabrotica balteata* larvae and their possible role in providing protection against entomopathogenic nematodes. **First place award.**

2018 Fifth Congress of the Latin American Association of Chemical Ecology, Valparaíso, Chile. Impact of benzoxazinoid sequestration by the western corn rootworm on entomopathogenic nematodes. **Second place award.**

2018 Biology 18, University of Neuchâtel, Switzerland: Surveying Mexican fields for natural enemies of the Western Corn Rootworm.

Poster presentations

2019 Gordon Research Conference Plant-Herbivore Interactions, Ventura, California, US: **BRUNO, Pamela**, GLAUSER G., TURLINGS, T.C.J. and ARCE C.C.M. Sequestration profiles of cucurbitacins from cucumber by *Diabrotica balteata* larvae and their possible role in providing protection against entomopathogenic nematodes.

2018 34th Meeting of the International Society of Chemical Ecology, Budapest, HU: **BRUNO, Pamela**, ARCE, C., MACHADO, R., KOEHLER, A., CAMPOS-HERRERA, R., BERNAL, J., ERB, M., ROBERT, C.A.M. and TURLINGS, T.C.J. Surveying Mexican fields for natural enemies of the Western Corn Rootworm.

2018 Biology 18, University of Neuchâtel, CH: **BRUNO, Pamela**, ARCE, C., MACHADO, R., KOEHLER, A., BERNAL, J., ALVAREZ-ZAGOYA, R., PEREZ-DOMINGUEZ, J. F., ERB, M. and TURLINGS, T.C.J. Surveying Mexican fields for natural enemies of the Western Corn Rootworm (Poster presentation and flash talk).

2017 Insect Chemical Ecology 17, Penn State, US: **BRUNO, Pamela**, KOEHLER, A. and TURLINGS, T.C.J. Survey of natural enemies of the Western Corn Rootworm in Central Mexico.

2017 IWGO Beijing 2017, Beijing, CN: **BRUNO, Pamela**, KOEHLER, A. and TURLINGS, T. Survey of natural enemies of the Western Corn Rootworm in Central Mexico (Poster presentation, **student travel grant**).

2017 Annual PhD Student Meeting 2017, University of Neuchâtel, CH: **BRUNO, Pamela**, KOEHLER, A. and TURLINGS, T.C.J. Survey of natural enemies of the Western Corn Rootworm in Central Mexico (Poster presentation, **best poster award**).

UNDERGRADUATE INTERNSHIPS

GEVES Beaucouzé, France

2016 Master 2 Internship "Determination of an Extraction and a Multiplication Method for a Phytopathogenic Nematode" as part of a confidential R&D Project aiming to create a Resistance Test for the registration of resistant varieties. Development of the project, greenhouse and in vitro essays, data analysis, written & oral presentation of results.

École d'Ingénieurs de Purpan, Toulouse, France

2013-2014 Internship in Molecular Biology and Microbiology Laboratory. Samples preparation, lyophilization, DNA extraction, PCR, RT-PCR, data analysis and presentation, fungi culture, microbiological essays.

National University of Cuyo, Mendoza, Argentina

2013 Salinity in Vineyards. Chemical analyses of plant material and creation of a Practical Guide for winegrape growers for salinity management. Agricultural Chemistry laboratory.

2012-2013 Dynamics of phosphorus in soils of arid areas with tomato culture. Production of greenhouse tomato plants, soil preparation, soil analyses, plant analyses, roots extraction for analyses of mycorrhizae. Agricultural Chemistry and Edaphology laboratories.

2012 Influence of water and saline stress of rootstocks and varieties of grafted vines of cv. Malbec. Greenhouse essay and chemical analyses. Agricultural Chemistry laboratory, in collaboration with INTA Luján de Cuyo, Mendoza, Argentina.

2012 Evaluation of Boron content in leaves and its association with the content in soil and in irrigation water from vineyards of the main irrigated oasis of Mendoza, Argentina. Chemical analyses. Agricultural Chemistry laboratory.

2012 Topinambur tubers quality and conservation properties, innovation with utilization of *Aloe vera* and *Aloe saponaria* gel. Experiment installation, measurements, data analyses. CODE: 06/A537. Biannual Project 2011-2013-SECTYP-UNCUYO.

2012 Internship in wine production in Bodegas Norton, S.A. Mendoza, Argentina. Industrial wine making.

INTA Luján de Cuyo, Mendoza, Argentina (National Institute of Agricultural Technology)

2009 Adaptation of cherry tree rootstocks and varieties to agro-meteorological conditions of Mendoza, Argentina. Field and laboratory essays. EEA Luján de Cuyo, Mendoza, Argentina.

Teaching

2017-2021 Integrated Pest Management. Spring semester of the Master in Biology, specialization Sustainable Agriculture University of Neuchâtel, Switzerland.

Practical classes (TP) of Chemical Ecology. Bachelor in Biology, 3rd year, University of Neuchâtel, Switzerland.

Student mentoring

2021 Master thesis of Leandro di Caprio in collaboration with Dr. Betty Benrey from the laboratory of Evolutionary Entomology, University of Neuchâtel, Switzerland.

2020 Master thesis of Eléonore Gindroz (EPFL) at University of Neuchâtel, Switzerland.

2019-2020 Master thesis of Anthony Pignal in collaboration with Dr. Betty Benrey from the laboratory of Evolutionary Entomology, University of Neuchâtel, Switzerland.

2017-2020 Internships of bachelor students (1-3 months): Clément Etter, Tobias Or, Johnny Wing-Moreira, Gaia Besomi, Galien Flückiger, Candice Dulong.

Community and scientific contribution

2018 Organization committee member SeeDS Annual PhD meeting of the doctoral program in Evolutionary Ecology – CUSO, Neuchâtel, CH.

SKILLS

Laboratory Analytical techniques: sample preparation and extraction of metabolites (LC-MS, UPLC-Q-TOF-MS). Molecular techniques: DNA extraction, PCR, RT-qPCR. Others: development of setups to study arthropods' behavior, rearing of insects, rearing of phytopathogenic and entomopathogenic nematodes, culture of phytopathogenic fungi and bacteria.

Administration Collaboration with management and administration of the laboratory of Chemical Ecology (FARCE) at the University of Neuchâtel: acquisition of laboratory materials, laboratories' organization,

quarantine permits, website of the FARCE laboratory (https://www.unine.ch/farce)

Marketing Work social media management (Twitter, website, Facebook), newsletters, tradeshows. https://twitter.com/FARCE_lab

Software Microsoft Office package, R. Phylogeny: BioEdit, GeneSnap, MEGA

PROFESSIONAL EXPERIENCE

2016 Six-month laboratory R&D internship at the French official organization for variety evaluation and seed testing (GEVES). Project: Development of a resistance test for the registration of resistant plant varieties against a phytopathogenic nematode.

2015 Seasonal Inspector-auditor for the National Institute of Viti-viniculture (INV), Mendoza, Argentina. Control and consulting mission for wine grape growers during winemaking season, inspection of wineries and vineyards.

2014-2015 Commercial representative and Frost Analyst for SHuR FARMS Frost Protection® (Colton, CA, United States). Commercial and technical contact in Argentina and USA. Consulting and Education mission for fruit and grapevine growers about Frost Protection. Commercial development, GIS, Frost Analysis, social media management. Contact with international clients in Spanish, French and English.

LANGUAGES

Spanish mother tongue

English fluent

French fluent

German intermediate

Italian basic

OTHER INTERESTS

Handicrafts Painting, drawing, sowing, embroidering, creation of decorative objects.

Patisserie 10+ years of creation and commercialization of cakes, cupcakes, macarons, chocolates, bonbons, Easter eggs, etc.

Music Choir singing: 10+ years of experience on academic/ symphonic/ traditional/ popular music (events, tours). Basic guitar.