

Insight into the host-specificity of a native and a newly introduced parasitoid of *Tuta absoluta* and prospect for biological control

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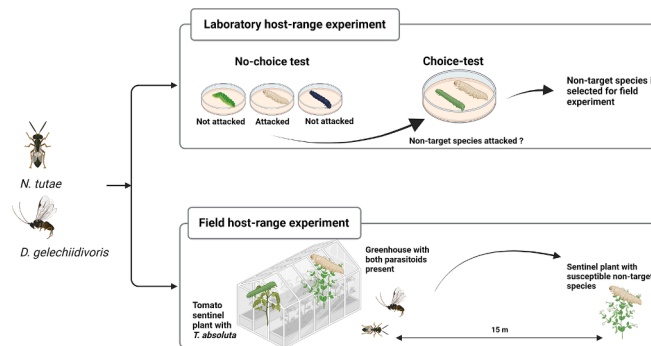
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HIGHLIGHTS

- We evaluate host specificity of a native and an accidentally introduced parasitoid.
- The native parasitoid attacks several non-target species in the laboratory.
- The introduced species shows affinity for the target pest and a related non-target.
- The parasitism rate of each parasitoid was evaluated in three greenhouses.
- Both species show potential as biological control agents against *Tuta absoluta*.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:

Host-range
Accidental introduction
Sentinel plant
Classical biological control
Host-parasitoid community
Environmental risk assessment

ABSTRACT

New strategies are urgently needed to control *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), one of the most destructive pests of tomato crops. Originating from South America, it is spreading rapidly and has evolved resistance to most common insecticides making it highly challenging to control. Regarding biological control, the parasitoid *Necremnus tutae* Ribes & Bernardo (Hymenoptera: Eulophidae) is widely present around the Mediterranean basin and has adapted to the invader playing a substantial role in its control. In addition, the neotropical parasitoid *Dolichogenidea gelechiidivoris* Marsh (Hymenoptera: Braconidae) has established accidentally following its host in Spain and Algeria and a classical biological control program using this species is ongoing in Sub-Saharan Africa. Understanding the trophic connections between *D. gelechiidivoris*, *N. tutae*, the targeted host *T. absoluta* and potential non-target hosts is essential to assess their value as augmentative or classical biological control agents and to promote their establishment in conservation biocontrol. We conducted host-specificity tests in the laboratory using four European leafminer species to evaluate the specificity of these two parasitoids to *T. absoluta*. We then assessed their affinity for *T. absoluta* by exposing sentinel plants with the alternative host *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae) inside and around greenhouses and measuring each parasitoid species' field parasitism rate. Our results show that of the four non-target species tested in the laboratory, *N. tutae* attacked three, while *D. gelechiidivoris* attacked only *P. operculella*. In the greenhouse, *N. tutae* did not prefer *P. operculella* or *T. absoluta*, whereas *D. gelechiidivoris* preferred the latter. The mean parasitism rate of *T. absoluta* over the three-month monitoring period in the greenhouses reached 27% for

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<https://doi.org/10.1016/j.biocontrol.2024.105464>

Received 26 May 2023; Received in revised form 9 February 2024; Accepted 11 February 2024

Available online 16 February 2024

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N. tutae and 35 % for *D. gelechiidivoris*. We discuss the importance of host affinity for understanding potential non-target effects and the value a natural enemy can have as biological control.

1. Introduction

Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae), recently reinstated as "*Phthorimaea absoluta*" (Chang and Metz, 2021), is a serious threat to worldwide tomato production (Campos et al., 2017; Desneux et al., 2011, 2010; Han et al., 2019; Mansour et al., 2018). *Tuta absoluta* is very difficult to control due to the cryptic behavior of its larvae, its increasing resistance to synthetic insecticides, and its high reproduction rate (Guedes et al., 2019; Sylla et al., 2019). Outside of South America, its endemic region (EPPO, <https://gd.eppo.int>), the pest has already spread to more than 90 countries, including all Mediterranean countries. In 2017, *T. absoluta* reached China, the world's biggest tomato producer causing severe damage (Zhang et al., 2020, 2021). North America and the Oceanian continent are at high risk of being invaded.

In the Mediterranean region, *Necremnus tutae* Ribes & Bernardo (Hymenoptera: Eulophidae) is among the most abundant larval parasitoids found to attack *T. absoluta* (Gabarra et al., 2014; Zappalà et al., 2013). It shows an average parasitism rate of about 18 % in tomato crops (Arnó et al., 2021) that can reach 75–80 % in some cases (Giorgini et al., 2019 and references within). To date, *N. tutae* is promoted in Europe only through conservation biocontrol methods (Desneux et al., 2022). The native hosts of *N. tutae* are unknown because this species (formerly reported as *N. sp. nr. artynes*) has only recently been described (Gebiola et al., 2015). The species appears to be polyphagous, targeting lepidopteran leafminer species and even some coleopteran species, although it is reported to parasitize only host larvae encased in mines (Chailleux et al., 2014). In 2015, *N. tutae* was found parasitizing the almond bark beetle *Scolytus amygdali* Geurin-Meneville (Coleoptera: Scolytinae) in Tunisia (Zeiri et al., 2015). *Cosmopterix pulchrimella* Chambers (Lepidoptera: Cosmopterigidae), a leafminer of upright pellitory, is the third host reported to be parasitized by *N. tutae* (Bodino et al., 2016). In addition, the closely related parasitoid species *N. artynes*, often mistaken for *N. tutae* in previous surveys on *T. absoluta* (Gebiola et al., 2015), has a wide host range. Besides *T. absoluta*, *N. artynes* is also reported on *Aproaerema anthyllidella* Hübner and *Vulcaniella pomposella* (Zeller) (Lepidoptera: Cosmopterigidae).

Recently, another larval parasitoid of *T. absoluta*, the braconid *Dolichogenidea gelechiidivoris* Marsh (Hymenoptera: Braconidae) (Syn.: *Apanteles gelechiidivoris* Marsh), has been reported from the Mediterranean, i.e., Spain and Algeria (Denis et al., 2022; Krache et al., 2021). Native to Colombia, Chile, and Peru (Fernandez-Triana et al., 2020; Yu et al., 2016), it is considered the most important parasitoid for natural and augmentative biological control in Colombian tomato crops (Bajonero and Parra, 2017). The host range of *D. gelechiidivoris* seems confined to lepidopteran hosts belonging to the Gelechiidae family and related to solanaceous plants (Salas Gervassio et al., 2019). Although no risk assessment study has been published to date, *D. gelechiidivoris* has been widely used in classical biocontrol programs against *T. absoluta* in Easter Island, Kenya, Uganda, and Ethiopia, as well as against *Phthorimaea operculella* (Zeller) in North America and *Keiferia lycopersicella* (both Lepidoptera: Gelechiidae) in Hawaii, (Desneux et al., 2010; Aigbedion-Atalor et al., 2020; Aigbedion-Atalor et al., 2021, Nakao and Funasaki, 1979).

With the ongoing spread of *T. absoluta*, both parasitoids have gained increased attention. They could be fostered for their control function in areas where they are already present or introduced as classical biological control agents in countries where they are not present. Assessing the host-specificity of these parasitoids is the first step for evaluating potential environmental risks resulting from their introduction. High host-specificity enhances the parasitoid's efficiency in finding and attacking the target pest species (Kimberling, 2004; Rossinelli and Bacher, 2015)

and reduces the risk of ecological disturbance, such as reducing populations of non-target species (McEvoy, 1996; Collatz et al., 2021). Moreover, studying the network ecology in the field could help understand trophic interactions in both the native and the invaded ranges, complement laboratory-based host-specificity tests, and provide insights into potential interactions of biological control agents. Therefore, we evaluated the specificity for *T. absoluta* of both parasitoids, first in the laboratory and then in the field. We (1) assessed whether parasitism is restricted to leafminers; (2) assessed host-specificity in the laboratory with different leafminer species; (3) evaluated the parasitism rate from cultivated tomato plants in different Spanish greenhouses, on *T. absoluta* on commercially grown tomato and tomato and potato sentinel plants infested with *T. absoluta* and *P. operculella*, respectively; and (4) surveyed the parasitoid complex/community on other leafminer species around the greenhouses.

2. Material and methods

2.1. Laboratory experiment

2.1.1. Insect and plant material

The laboratory experiments were conducted in a quarantine facility at Agroscope in Switzerland and were approved by the Federal Office for the Environment (order: A22004600). All experiments and insect rearing were conducted in climate chambers at 25 ± 1 °C, 70 ± 10 % RH, and a 16:8 h L:D photoperiod.

The rearing of *T. absoluta* was established with individuals provided by Andermatt Biocontrol, Switzerland. Adult moths were kept in mesh cages and provided with cotton soaked in honey/water solution (10 % v/v) placed on the top of the cage and tomato plants (*Solanum lycopersicum* cv. Rentita) for egg-laying. After seven days, plants with eggs and young larvae were moved to another cage to start a new generation. Additional plant material was added regularly.

Colonies of the parasitoids *N. tutae* and *D. gelechiidivoris* were initiated with individuals collected in commercial tomato fields in El Maresme county (Barcelona, Spain). Adults were kept in mesh cages (50 × 50 × 50 cm) (bug dorm, MegaView Science Co., Ltd., Taiwan) and provided cotton soaked in honey/water (10 % v/v) and tomato plants with *T. absoluta* second to third instar larvae for parasitism. After emergence, adult parasitoids were collected and stored at 12 °C with honey/water solution provided on a cotton pad. At ten-day-intervals, a new parasitoid generation was initiated by releasing about 30 adult parasitoids of mixed sexes into a newly prepared cage. All female parasitoids used in the experiments were naive, mated (stored with males for at least two days), and less than a week old.

Four species of leafminers were selected as non-target species: i) The potato tuber moth *P. operculella* as a closely related species from the same clade as *T. absoluta* (Chang and Metz, 2021), ii) the horse chestnut leafminer, *Cameraria ohridella* Deschka & Dimic (Lepidoptera: Gracillariidae) and iii) the pear blister leaf miner *Leucoptera malifoliella* Costa (Lepidoptera: Lyonetiidae) as lepidopteran leafminers from two different families than *T. absoluta*, and iv) *Liriomyza bryoniae* (Kaltenbach) (Diptera: Agromyzidae) as a leafminer from another order but feeding on tomato.

Rearing of *P. operculella* was established with individuals provided by Andermatt Biocontrol, rearing of *L. malifoliella* and *L. bryoniae* with individuals collected in a commercial apple orchard and a tomato field, respectively, in the Zurich region. After emergence, adults were kept in mesh cages and provided cotton soaked in honey/water solution placed on the top of the cage. The species were provided with potato tubers (cv. Red Pontiac), apple branches (cv. Jonagold), and tomato plants (cv.

Rentita), respectively, for egg-laying. After seven days, plant materials with larvae were moved to another cage to start a new colony. Because of the short life of cut horse chestnut branches, it was impossible to rear *C. ohridella*. Therefore, leaves with mines of this sole leafminer species on horse chestnut in Switzerland were collected in the Berne region and used directly for the experiment after checking that those larvae were alive and not previously ectoparasitized. This was possible without opening the mines by observing each leaf with light from underneath ensuring that the larva was moving and thus alive.

2.1.2. Preliminary experiments: Are parasitoids attacking larvae outside the mines?

Experiments were conducted to evaluate if *D. gelechiidivoris* restricts its parasitism behavior to hosts inside mines and to confirm this observation for *N. tutae*, which has previously been reported (Chaillieux 2014). For each parasitoid species, four treatments with only the target host *T. absoluta* were conducted: **A** Two larvae of *T. absoluta* within their mine in the presence of a female parasitoid (positive control); **B** Two larvae outside of the mine in the presence of a female parasitoid (test); **C** Two larvae within their mine without parasitoid (mortality control inside the mines); **D** Two larvae outside the mine without parasitoid (mortality control outside the mines). Per treatment, 20 replicates were conducted accounting for a total of 40 larvae tested with 20 female parasitoids in treatment A and B.

The setup consisted of a Petri dish (10 cm dia.) covered with a mesh with a wet cotton disc on the bottom to keep the leaf humid and a droplet of honey as a food source for the parasitoid. Per Petri-dish, two larvae were offered to single female parasitoids for 36 h. Third instar larvae were used for *N. tutae* and second instar larvae for *D. gelechiidivoris*, corresponding to the parasitoid's preferred host instar (Aigbedion-Atalor et al., 2020; Calvo et al., 2013). For treatments A and C, larvae collected with a fine brush were placed on tomato leaflets (5 ± 2 cm in length, freshly cut) to allow time to burrow into a mine 24 h prior to each experiment; for treatments B and D, larvae were collected just prior to the experiment.

Every mine was opened under a stereomicroscope three days later, and each larva was categorized as dead or alive. For *N. tutae*, ectoparasitism was assessed three days later, and for *D. gelechiidivoris*, 12 days later, when the parasitoid larvae left the host larvae for pupation. An approximation of non-reproductive host mortality, which results from host feeding or host killing (Zhang et al., 2022, hereafter referred to as "hosts killed"), was calculated by subtracting the natural mortality found in the absence of a parasitoid (treatment C and D) from the total dead larvae found in the presence of a parasitoid (treatment A and B).

2.1.3. Host-specificity testing of *Necremnus tutae*

In a no-choice test, single female parasitoids were offered non-target host larvae of *L. bryoniae*, *L. malifoliella*, *C. ohridella*, and *P. operculella* or larvae of *T. absoluta* as control. As in the preliminary experiment, both treatments were repeated without parasitoids to control for natural mortality, resulting in four treatments: **A** Target host *T. absoluta* with the addition of a female parasitoid (positive control); **B** Non-target host with the addition of a parasitoid (test); **C** Target host *T. absoluta* without parasitoid (mortality control with target host); **D** Non-target host without parasitoid (mortality control with the non-target host). The same process and methods were used as in the preliminary experiment. Per Petri dish, two larvae (third instar *T. absoluta* larvae or larvae of the alternative host corresponding in size) were offered to single females for 36 h. Leaves with mines containing larvae were selected and cut (1–2 cm²). Per treatment, at 20–25 replicates were conducted accounting for at least a total of 40 larvae tested for 20 female parasitoids in treatment A and B.

2.1.4. Host-specificity testing of *Dolichogenideia gelechiidivoris*

Because *D. gelechiidivoris* is a koinobiont endoparasitoid, host larvae continue to feed after parasitization and the parasitism can be assessed

only after 12 days when the parasitoid immature leaves the host larva for pupation. Since the experimental set-up used did not allow to keep the pieces of cut leaves containing the non-target larvae in a good state for longer than a few days, behavioral observations were conducted for this parasitoid. Single parasitoid females ($n = 20\text{--}32$) were offered two mines of the alternative host in a no-choice setup. Leaves with mines were collected and cut (1–2 cm²) to select mines containing larvae similar in size to the second instar *T. absoluta* larvae. Per Petri-dish, two pieces of leaf with second instar *T. absoluta* larvae or alternative hosts were offered at an equal distance to single females. Subsequently, each female was offered a leaf with about five *T. absoluta* mines for one minute to assess its responsiveness. Observations of females not responsive during this time were discarded.

When females stung the alternative host, behavioral observations were repeated in a choice setup. Per Petri dish, two larvae of each host were offered, totaling four larvae per dish ($n = 30$ observations). Observations in no-choice and choice tests were noted using Observer XT11 (Noldus, Netherlands). Behaviors were recorded for seven minutes and categorized as cleaning, resting, walking on the Petri dish, antennating/searching the leaf (all: continuous behaviors), and stinging (event). Due to the noticeable attraction of female *D. gelechiidivoris* reared on *T. absoluta* on tomato plants for *P. operculella* in the choice setup, observations were repeated with females reared on *P. operculella* on potato plants ($n = 30$).

2.2. Field experiment

The field experiments were conducted in and around three commercial tomato greenhouses in El Maresme and La Selva counties (Northeast of Spain), and samples were evaluated at IRTA's Research Center (Cabrils, Barcelona, Spain). The greenhouses were located within a 10 km radius distance and less than 3 km from each other. Location A (41°40'16" N, 2°46'00" E) was a 1'600 m² low-cost tunnel, completely open at the sides (up to 3 m height) surrounded by arable land. Location B (41°41'48" N, 2°46'30" E) was a 600 m² semi-closed tunnel with meshes on the side wall (allowing insects to move in and out) surrounded by a forest on one side and other greenhouses on the other side. Location C (41°37'22" N, 2°39'12" E) was a 1'136 m² greenhouse completely closed beside the roof opening and surrounded by similar greenhouses (see Fig. S2). Wild herbaceous vegetation occurred around each greenhouse. The greenhouses were selected based on the presence of *T. absoluta* and both parasitoids.

2.2.1. Host preference in the field

Infested tomato and potato sentinel plants were placed inside and around the three greenhouses to assess the preference of both parasitoids for *T. absoluta* and *P. operculella*. Sentinel plants were planted in pots (15 cm dia., 20 cm height) and were used when they were about 30 cm high with at least five fully developed leaves. Before the experiment, plants were offered for ten days to newly emerged adult moths (10 per plant, sex ratio 0.5) to obtain infestation with L1 to L3 instar larvae at the beginning of the experiment. *T. absoluta* and *P. operculella* were obtained from an established laboratory colony that originated from field-sampled adults from El Maresme County and were reared as described above. Adult moths were fed with honey/water solution during the entire infestation period. Sentinel plants were then covered with a mesh (mesh size 0.4 mm) to avoid predation by adult mirids and larger animals, and the pots were sprayed with insect glue (Soveurode®, Plantin SARL) to avoid predation by soil-dwelling insects (see Fig. S1 of sentinel plant).

Three consecutive runs were conducted between September and November 2022 (starting on 29.09, 20.10, or 10.11), during which the plants stayed for four days in each location. Twenty-four sentinel plants were placed in each location: seven tomato and eight potato plants inside and nine potato plants outside the greenhouses. Sentinel plants inside were placed 15 m or more from each other and were distributed

equally. The potato plants outside were placed to assess the preference when the target host, *T. absoluta*, was not close. They were placed at five to 15 m distance to the greenhouse wall and 15 m or more from each other (see Fig. S3, S4, S5).

After each run, each sentinel plant was individualized in a plastic container (20 cm dia., 30 cm height) covered with a mesh and incubated at 22 °C in the lab until moths or parasitoids emerged. Additional plant material was added when needed (sentinel plant completely consumed). After emergence, all insects were aspirated for counting. The parasitism rate for each parasitoid was assessed as follows:

$$\text{For } D. \textit{gelechiidivoris} = \frac{\text{Total number of } D. \textit{gelechiidivoris} \text{ emerging}}{\text{Total number of insects emerging}}$$

$$\text{For } N. \textit{tutae} = \frac{\left(\frac{\text{Total number of } N. \textit{tutae} \text{ emerging}}{1.41} \right)}{\text{Total number of insects emerging}}$$

In addition, mines of *T. absoluta* were collected during each run to evaluate the parasitism rate from cultivated tomato plants already occurring in the greenhouse. Three persons collected mines for 20 min or up to 150 mines.

2.2.2. Survey of non-target species in the surroundings

Three persons searched for 20 min the surroundings (up to 100 m) of the greenhouse to collect any leafminer species. All samples were individualized and incubated at 22 °C in the lab until the leafminers or parasitoids emerged.

2.3. Statistical analysis

The software NCSS (2023) (NCSS, LLC, US) was used for statistical analysis of the laboratory experiments. Data were tested for normal distribution using the Shapiro–Wilk test, and visual inspections were made using Q–Q plots. The data were mostly not normally distributed (Shapiro–Wilk test, $P < 0.05$). To compare the ratio of the four categories (Parasitized, Host killed, Dead, and Alive) between hosts inside and outside of mines (experiment 2.1.3) and target and non-target hosts (2.1.3), a χ^2 test on contingency tables (2×4) was used. A Mann-Whitney *U* test was used to compare the time spent searching on mines of different non-target hosts under no-choice conditions. Bonferroni correction was used for the multiple comparisons, and a value of $P < 0.05/3 = 0.016$ was considered statistically significant. Wilcoxon signed-rank test was used to compare the searching time between target and target non-target host in the choice setup. The proportion of females stinging the mines was compared with a χ^2 test (experiment 2.1.4).

For experiment 2.2.1, all computations were carried out with the R 4.2.2 software (R Development Core Team 2022). Differences in parasitism rate between sentinel plants were assessed with a generalized linear mixed-effects model (glmm) with binomial data distribution (parasitism yes/no) and Month*Greenhouse as a random effect in all models to take into account pseudoreplication (3 greenhouses \times 3 months = 9 replicates). We tested all models for evidence of overdispersion (based on the ratio of residual deviance to residual degrees of freedom being close to 1, as in (Bolker et al., 2009)). Since we had relatively large counts (>5) in all groups, it was justified to use glmmPQL to account for overdispersion (Bolker et al., 2009). Therefore, we refitted overdispersed models using penalized quasi-likelihood (function glmmPQL from R package MASS). Two different models were run for each parasitoid. **A**) Experiment with potato plants infested with *P. operculella* and tomatoes infested with *T. absoluta* inside the greenhouse. The influence of host type on the parasitism rate was evaluated with the host (*T. absoluta*/*P. operculella*) as a fixed effect. **B**) Experiment with potato plants infested with *P. operculella* inside and outside the greenhouse. The influence of the sentinel plant location on the parasitism rate was evaluated with location (inside/outside) as a fixed effect. Finally, the parasitism rate of *T. absoluta* from cultivated

tomato plants in the greenhouses was evaluated with a generalized linear model where the months were set as a fixed factor.

3. Results

3.1. Laboratory experiments

3.1.1. Preliminary experiments: Are parasitoids attacking larvae outside the mines?

In the control group, where host larvae ($n = 40$) were offered within the mines, females of *N. tutae* ($n = 20$) and *D. gelechiidivoris* ($n = 20$) each parasitized 17.5 % of the *T. absoluta* larvae. However, when offered outside of the leaf ($n = 40$), no larva was parasitized by either parasitoid species. Non-reproductive host mortality referred to as “hosts killed” was found for both parasitoids, regardless of whether the larvae were inside or outside the mines. For *N. tutae*, however, a significantly higher percentage of larval hosts were killed when they were in the mines, 47.5 %, vs. outside the mines, 22.5 % ($X^2 = 13.2$, $df = 2$, $P = 0.001$). *D. gelechiidivoris* killed the same percentage of hosts, 32.5 %, inside or outside the mines.

3.1.2. Host-specificity testing of *Necremnus tutae*

The proportions of larvae parasitized, host-killed, alive and dead of the non-target hosts *L. bryoniae*, *L. malifoliella*, and *C. ohridella* differed significantly from those of the target host *T. absoluta* (Chi-square test; all: $\chi^2 < 21.6$, $df = 3$, $P < 0.001$; Fig. 1). No significant difference was detected for *P. operculella* ($\chi^2 = 6.71$, $df = 3$, $P = 0.082$). None of the female *N. tutae* parasitized the dipteran leafminer *L. bryoniae* ($n = 20$). Conversely, females parasitized all three non-target lepidopteran species, ranging from 5 % for *L. malifoliella* ($n = 20$) to 7 % for *P. operculella* ($n = 20$) and 16 % for *C. ohridella* ($n = 25$) (see Fig S6). The parasitism rate did not significantly differ from the target host *T. absoluta* (6 to 10 %) except for *C. ohridella*, where parasitism was significantly higher ($\chi^2 = 30.45$, $df = 3$, $P = 0.001$). A high percentage of hosts killed ranging from 35 to 46 % was found in all the control groups with *T. absoluta*. For the non-target host *L. bryoniae*, a low percentage of 5 % was witnessed. With 42.5 % of *P. operculella* larvae killed, the percentage appeared to be slightly higher than for the target host (35 %). No killed larvae of *C. ohridella* and *L. malifoliella* were found.

3.1.3. Host-specificity testing of *Dolichogenidea gelechiidivoris*

The time spent searching by female *D. gelechiidivoris* ($n = 20$) in no-choice tests on mines of the non-target hosts was low, ranging from 12.5 ± 12.53 (mean seconds \pm SE) for *L. bryoniae* to 65.2 ± 19.13 for *C. ohridella* (Fig. 2A). Females spent significantly more time searching the mines of *C. ohridella* than those of *L. malifoliella* ($U = 206$, $P = 0.001$) and *L. bryoniae* ($U = 121.5$, $P = 0.001$). No differences were found between the times spent searching the mines of *L. bryoniae* and *L. malifoliella* ($U = 293$, $P = 0.602$; Mann-Whitney *U* test). None of the females stung larvae of the dipteran leafminer *L. bryoniae*. However, a small proportion of female parasitoids stung *L. malifoliella* (3.3 %) and even more *C. ohridella* (12.5 %) (see Video S1). Since all females stung *T. absoluta* mines during the observation, no individuals were rejected as non-responsive.

When given a choice, females ($n = 30$) spent significantly more time searching the mines of the target host *T. absoluta* than the mines of *L. malifoliella* or *C. ohridella* (Wilcoxon signed-rank test: all $Z > 3.84$, $P < 0.001$; Fig. 2B). A proportion of females stung the mines of *L. malifoliella* (3.3 %) and *C. ohridella* (15.4 %), but significantly more (≥ 93.3 %) stung the mines of *T. absoluta* (Chi-square test; $\chi^2 > 42$, $df = 1$, $P < 0.001$).

Female *D. gelechiidivoris* reared from *T. absoluta* ($n = 30$) spent similar time searching the mines of *P. operculella* and *T. absoluta* (Wilcoxon signed-rank test; both $Z > 1.11$, $P < 0.264$), and those females did not show any stinging preference (Chi-square test, all; $\chi^2 < 1.06$, $df = 1$, $P > 0.302$). When reared on *P. operculella* for one generation, females (n

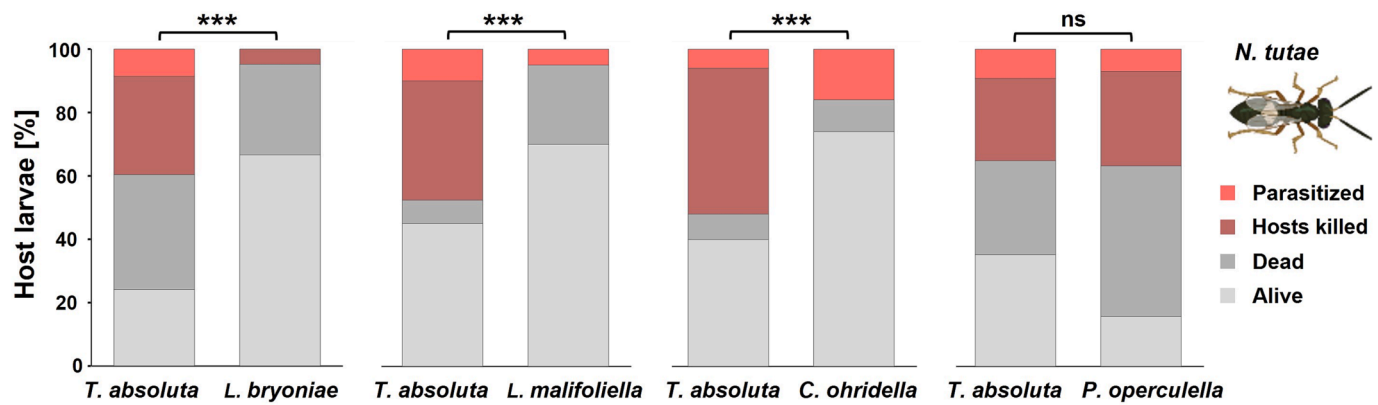


Fig. 1. Result of no-choice experiment with the parasitoid *Necremnus tutae* and from left to right the different non-target hosts (right bars) *Liryomiza bryoniae*, *Leucoptera malifoliella*, *Cameraria ohridella*, and *Phtorimaea operculella*. The target host *Tuta absoluta* was used as a control in each no-choice assay (left bars). An approximation of the number of host killed larvae was calculated by subtracting the calculated natural mortality of the control from the total dead larvae found in the treatment. The ratio of the four categories (Parasitized, Hosts killed, Dead, and Alive) between target and non-target hosts was compared with a χ^2 test on contingency tables (2×4); ns $P > 0.05$; *** $P < 0.001$. Per treatment, 20 replicates or more were conducted, accounting for at least 40 larvae tested for 20 female parasitoids.

= 30) spent significantly more time searching the mines of *P. operculella* (Wilcoxon signed-rank test; both $Z > 3.81$, $P < 0.001$), but more females (56.6 %) stung the larvae of *T. absoluta* (Chi-square test; $\chi^2 = 5.55$, $df = 1$, $P = 0.018$) than *P. operculella* (26.6 %).

3.2. Field experiment

3.2.1. Host preference in the field

The infestation levels of the sentinel plants were similar, with a mean of 75.6 and 76.9 individuals (pest + parasitoids) emerging per potato and tomato sentinel plants, respectively. Sentinel plant type or location also did not affect the parasitism rate by *N. tutae* (Both $P > 0.175$, Fig. 3B, Table S1). On potato sentinel plants outside the greenhouse, parasitism rate for *D. gelechiidivoris* was significantly higher in comparison to potato sentinel plants inside ($P = 0.001$, Fig. 3D, Table S1). Inside the greenhouse, *D. gelechiidivoris* caused significantly higher parasitism levels on tomato sentinel plants with *T. absoluta* when compared to potato sentinel plants with *P. operculella* ($P = 0.004$, Fig. 3B, Table 1 SI).

A higher discovery rate of sentinel plants was found for *D. gelechiidivoris* (49.4–60.3 %) than for *N. tutae* (15.5–33.3 %; Table 1). Over the three months, the mean parasitism rate from cultivated tomato plants by *D. gelechiidivoris* reached 35.1 %, whereas *N. tutae* parasitism rate reached 27 % (Fig. 4). The mean of the added parasitism rate of both parasitoid species reached 65.2 % in September, 48.8 % in October, and 72.2 % in November.

The number of host insects collected to assess parasitism from cultivated tomato plants strongly differed between greenhouses due to different pest densities. The number of insects emerging from collected mines ranged from 348 to 1234 individuals (pest + parasitoids) for the open greenhouse, 13 to 76 for the semi-open greenhouse, and 5 to 83 for the closed (besides the roof opening) greenhouse. In September and October, a significantly lower parasitism rate of *N. tutae* was found in the greenhouses (both $P < 0.008$; Table. S2).

3.2.2. Survey of non-target species in the surroundings

Only one leafminer species was found during the sampling around the greenhouses: *Chrysoesthia sexguttella* (Thunberg) (Lepidoptera: Gelechiidae) on *Chenopodium album*. It was present around all three greenhouses during September and October, the plant being in senescence in November. More than 50 mines were sampled during the two first monitorings. No parasitism by *N. tutae* or *D. gelechiidivoris* was recorded. However, 15 parasitoid individuals of *Pnigalio soemius* (Walker) (Hymenoptera: Eulophidae) emerged from the collected samples, and one individual of the Pteromalidae family.

4. Discussion

This study investigated host-specificity and parasitism on cultivated tomatoes of two promising parasitoids of *T. absoluta* in Catalonia, thereby giving a first insight into their trophic interactions with the wider insect community. We found that parasitism of both species is restricted to leafminers and that the host-range of *N. tutae* is likely much broader than the one of *D. gelechiidivoris*. Both species demonstrated high natural parasitism rates on cultivated tomato plants in greenhouses.

The preliminary experiment revealed that both parasitoid species did not parasitize any *T. absoluta* larvae offered outside of the mine; however, both displayed non-reproductive host-killing, regardless if the larvae were inside or outside of the mines. For a parasitoid, the host is a crucial resource with distinctive physical, chemical, and behavioral characteristics, which determine the host's recognition and acceptance (Rehman and Powell, 2010). The results here indicate that mine recognition is highly important for successful parasitism by these two species. A previous study highlighted that the leafminer parasitoid *Symplexis serieicornis* Nees (Hymenoptera, Eulophidae) already detects the mine during flight (Casas, 1989). A host within the mine may provide additional protection for the parasitoid offspring, particularly for an ectoparasitoid such as *N. tutae*. It might also be that the host larvae in the mines are less mobile and likely less able to defend themselves through aggressive or evasive behavior (Greeney et al., 2012) than outside the mine, which could explain why a higher host-killing rate for *N. tutae* on *T. absoluta* larvae was found inside the mine. However, in the case of *D. gelechiidivoris*, the host-killing rate was the same for larvae outside and inside the mines. Notably, the host-killing rate for *D. gelechiidivoris* was higher than 30 %, which represents a significant contribution to the control of *T. absoluta* by this parasitoid. To our knowledge, this is the first quantification of non-reproductive host-killing by this Braconidae.

N. tutae, as a native species, was present in the region before *T. absoluta* invasion in 2006 and has a pre-existing host range beyond *T. absoluta*. Under no-choice conditions, *N. tutae* attacked all three lepidopteran species tested in the host specificity assays, but not the dipteran species, and even parasitized more than twice as many larvae of the non-target host *C. ohridella* as the target host, confirming a broad host spectrum. Interestingly, the non-reproductive host-killing behavior of *N. tutae* was restricted to *T. absoluta*, the closely related species *P. operculella*, and although quite low, to the dipteran non-target host. This could be explained by the fact that larvae of these species are relatively mobile and, once stung, sometimes leave the mines and die outside (J.

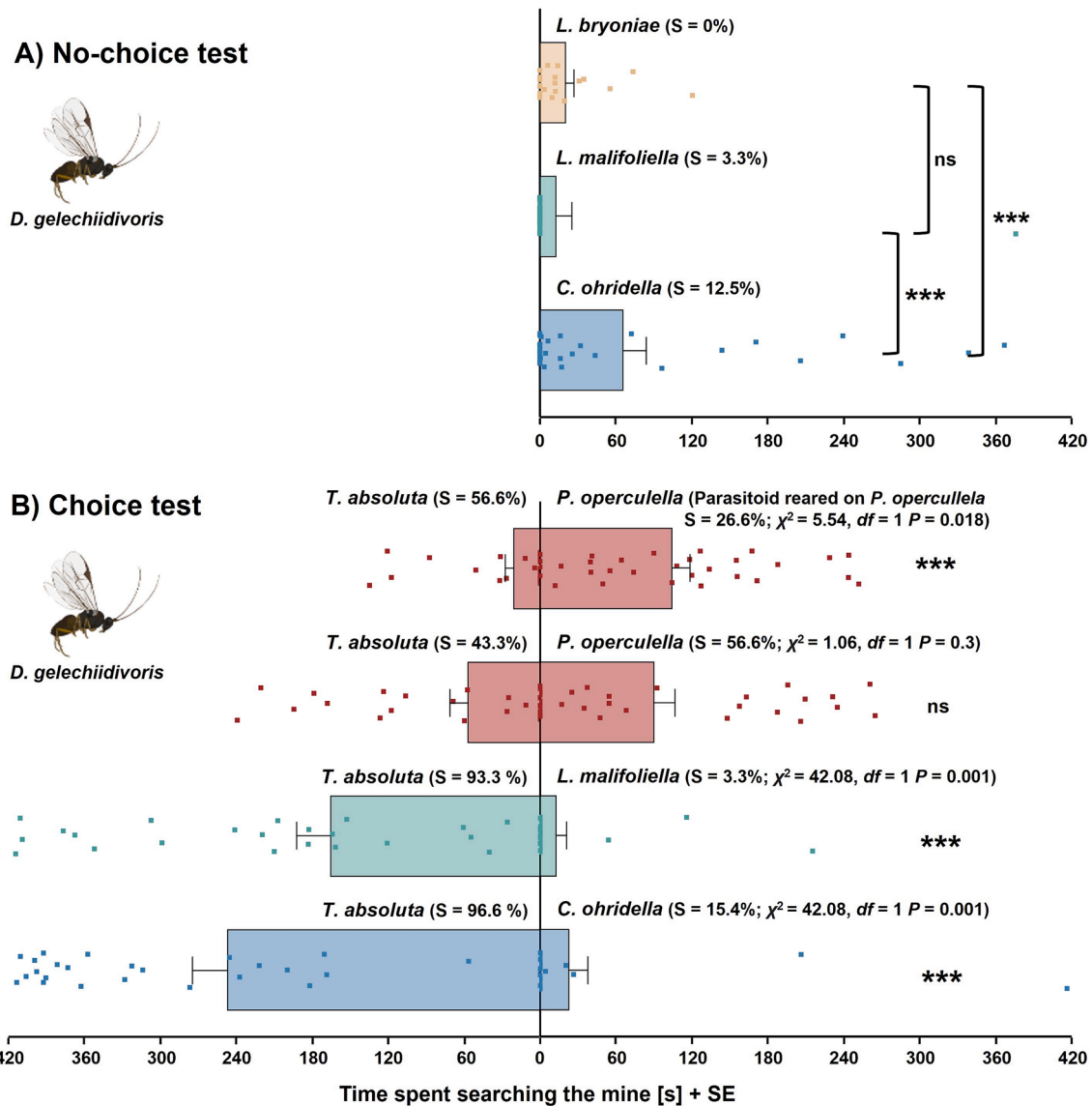


Fig. 2. Mean time spent searching the host mine by female *Dolichogenidea gelechiidivoris*. Observations were conducted for 7 min. Each female stinging the mine was also recorded (S = proportion of female stinging). The sample size of observed females ranged from 20 to 32. **A)** No-choice experiment with the non-target host *Liriomyza bryoniae*, *Leucoptera malifoliella*, and *Cameraria ohridella*. A Mann-Whitney U test was used to compare the time spent searching. **B)** Choice experiment with the target host *T. absoluta* and the non-target host *P. operculeella*, *L. malifoliella*, and *C. ohridella*. A χ^2 test was used to compare the proportion of females stinging a larva (results given within brackets) and a Wilcoxon signed-rank test was used to compare the searching time; ns $P > 0.05$; *** $P < 0.001$.

Gonthier personal observation), preventing *N. tutae* from laying eggs. In contrast, the non-target hosts *C. ohridella* and *L. malifoliella* are obligate leafminers (Gross and Price, 1988), which cannot leave the mines, allowing more time for *N. tutae* for oviposition. Despite this, *N. tutae* accepts many species for oviposition, raising concerns for non-target lepidopteran leafminer species. In the field, *N. tutae* showed no preference for *T. absoluta* on tomato-sentinel plants compared to *P. operculeella* on potato-sentinel plants.

Because of the evolutionary history with *T. absoluta* (Salas Gervasio et al., 2019), *D. gelechiidivoris* has likely evolved a strong affinity for this pest. In the choice experiment, female *D. gelechiidivoris* seemed mainly attracted to the mines of *T. absoluta* on tomato leaves. It is worth mentioning that this species is known to display probing behavior and reject many larvae after “stinging” (Gonthier et al., 2023a). The females were possibly only probing the non-target host; unfortunately, it was impossible to rear the stung host to assess parasitism since the larvae suffered high mortality due to the decaying leaves. Finally, time spent searching and stinging seems not to be solely determined by the plant

species (tomato) as no female *D. gelechiidivoris* were recorded stinging the mine of *L. bryoniae*, and the time spent searching the tomato leaves containing this host was very short in the no-choice test. Females spent the same time searching the mines of *P. operculeella* as the mines of *T. absoluta*, and no difference was found between the proportions of females stinging either host. Interestingly, when reared for one generation on *P. operculeella*, females spent significantly more time searching the mines of *P. operculeella*. This behavior change confirms previous studies demonstrating the influence of the rearing host on parasitoid preferences (Bodino et al., 2016; Boycheva-Woltering et al., 2019; Gonthier et al., 2023b).

There are several ways in which biosafety aspects, i.e., host specificity, can be measured and addressed (Barratt, 2011). Monitoring the realized host range post-introduction can help to verify predictions made by host-specificity testing under confined conditions (Louda et al., 2003). Here, no preference for *P. operculeella* or *T. absoluta* was observed in the laboratory experiments for *D. gelechiidivoris*, but a preference for the latter was visible in the field. This discrepancy could be explained by

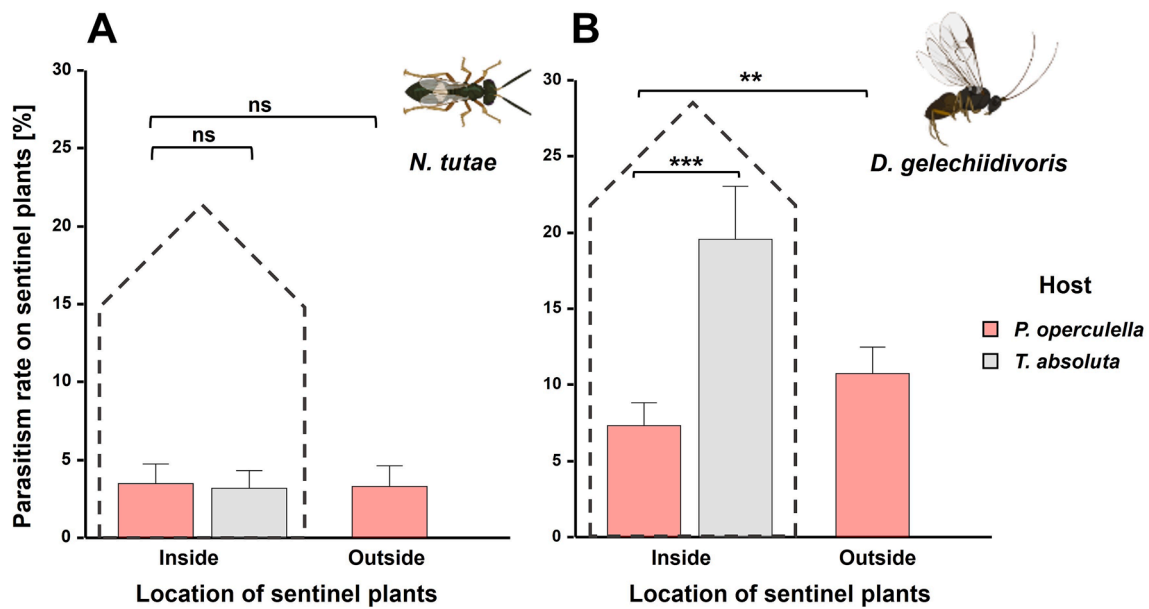


Fig. 3. A) Influence of host identity (*Phthorimaea operculella* on potato plant or *Tuta absoluta* on tomato plant) and sentinel plant location (inside or outside of the greenhouse) on parasitism rate by *Necremnus tutae*. Parasitism rates on sentinel plants + SE are shown. B) influence of host identity and plant location on parasitism rate by *Dolichogenidea gelechiidivoris*. Single parasitism rates on sentinel plants + SE are shown. (** $P < 0.01$, *** $P < 0.001$, a summary of the statistics is presented in Table 1 SI).

Table 1

Summary of mean discovery rate (at least one host of the sentinel plant parasitized) found over three months on the different sentinel plants placed inside and outside the three greenhouses for *Necremnus tutae* and *Dolichogenidea gelechiidivoris*.

Sentinel plants/greenhouses	Discovery rate	
	<i>D. gelechiidivoris</i>	<i>N. tutae</i>
<i>T. absoluta</i> on tomato plants inside (n = 49)	60.3 %	33.3 %
<i>P. operculella</i> on potato plants inside (n = 56)	53.8 %	15.5 %
<i>P. operculella</i> on potato plants outside (n = 53)	49.4 %	32.1 %

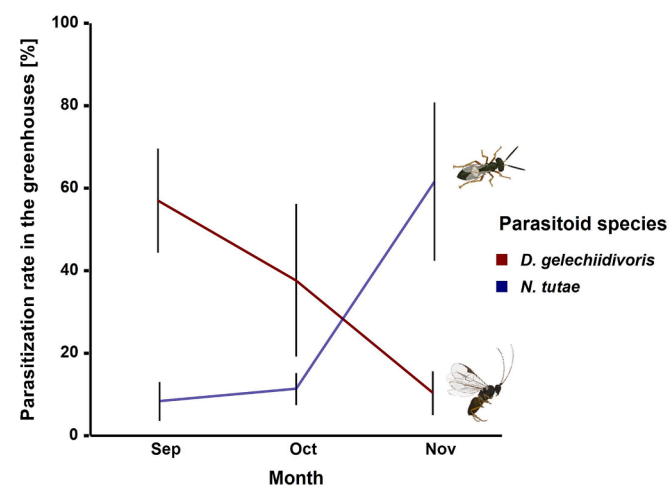


Fig. 4. Mean parasitism rate + SE (black bar) of *Tuta absoluta* by the parasitoids *Dolichogenidea gelechiidivoris* and *Necremnus tutae* over time in three greenhouses (n = 3). The sample size of hosts collected ranged in September from 9 to 1234, October from 16 to 508, and November from 5 to 348.

the fact that the olfactory cues of a cut leaf with only two larvae are much weaker than a whole plant, as *D. gelechiidivoris* attraction for infested plants increased with increasing infestation rate (Ayelo et al.,

2022). This is an encouraging result regarding host specificity and risk assessment since both species belong to the same clade (Chang and Metz, 2021).

Flying natural enemies, such as parasitic wasps, can move quickly and cover a large area (Schellhorn et al., 2014), making them impossible to contain. Both parasitoids intensely parasitized hosts on the potato plants inside and outside the greenhouses, and they are thus moving in and out of the greenhouses and potentially impacting non-target species. In the case of *D. gelechiidivoris*, parasitism levels were higher on sentinel plants outside the greenhouse, likely due to the lower density of the target host outside.

Understanding the seasonal dynamics of both the pest and the natural enemies is critical for promoting natural enemies for biological control. The establishment and success of natural enemies can be heavily influenced by the season due to environmental conditions and host availability (Welch and Harwood, 2014). Our goal was to assess the situation towards the end of the tomato season. The parasitism rate of *N. tutae* increased towards the end of the season, whereas the rate of *D. gelechiidivoris* decreased. Interestingly, a previous study in 2020 (Denis et al., 2022) recorded a steadily increased parasitism rate for *D. gelechiidivoris* from May (2.7 %) until October (21.8 %). Why we found a decrease from September to November 2022 remains to be determined, but it is likely that the population of *D. gelechiidivoris* reaches its peak between September and October. The findings raise the need for further investigation due to the limited scope of our single-year study with a limited sample size, potentially missing broader seasonal patterns. The neotropical *D. gelechiidivoris* may be less adapted to the colder temperatures at the end of the growing season (mean temperature in Malgrat de Mar in November = 14.2 °C, minimum recorded 4.4 °C, see Fig. S7) than the native *N. tutae*. Exposing pupae of *D. gelechiidivoris* to 4 °C for seven days significantly lowered parasitism abilities (Morales-Perdomo et al., 2018). Recent studies on the performance of *D. gelechiidivoris* at 10 °C found that the net reproductive rate decreases drastically (Agboka et al., 2022), displaying even a negative intrinsic rate of increase (Aigbedion-Atalor et al., 2022). Finally, because *D. gelechiidivoris* is a koinobiont parasitoid, the immature stages' survival depends on the host's cold tolerance (Bajonero et al., 2008). Larvae of *T. absoluta* are not very cold-tolerant; 50 % of larvae died after 12.4 days at 5 °C (Van Damme et al.,

2015). If *D. gelechiidivoris* can overwinter via an alternative host remains to be determined. Consequently, successful overwintering and establishment of *D. gelechiidivoris* in countries with colder climates appear unlikely, and *N. tutae* is likely mainly responsible for pest reduction during winter in non-heated greenhouses.

Although both parasitoid species preferably target different host instars of *T. absoluta*, interspecific competition (Feng et al., 2015; Karlsson et al., 2018; Savino et al., 2017) could explain the contrasting population dynamic at the end of the season. Denis et al. (2022) reported that 11 % of larvae ectoparasitized by *N. tutae* yielded a *D. gelechiidivoris* adult. Several factors can determine which parasitoid will prevail in a competition targeting the same host. For instance, the venom of idiobiont ectoparasitoids may affect the development of endoparasitic koinobionts (Harvey et al., 2013). Thus, when an ectoparasitoid and an endoparasitoid engage in multiple parasitisms, the ectoparasitoid usually emerges as the winner (Mitsunaga and Yano, 2004). For a similar situation, a recent study in Kenya evaluated the interaction between *D. gelechiidivoris* and the native ectoparasitoid *Stenomesus japonicus* (Ashmead) (Hymenoptera: Eulophidae), which like *N. tutae* targets the third instar of *T. absoluta* (Mama Sambo et al., 2022). The authors found that both species coexisted and recorded a low multiparasitism level of 5 % or less. Even though *N. tutae* is still present in high density six years after the arrival of *D. gelechiidivoris* in Spain (Denis et al., 2021), the outcome of within-host competition remains to be studied, and the overall interaction deserves further investigation.

When surveying alternative hosts around the greenhouse, the leafminer *C. sexguttella* belonging to the same family as the target host *T. absoluta* was found repeatedly during the experiment on *C. album*. Even though no *N. tutae* or *D. gelechiidivoris* emerged from any of the *C. sexguttella* collected, the leafminer was found to be parasitized by *P. soemius*. This species has been recorded parasitizing *T. absoluta* and might contribute to its natural control (Ferracini et al., 2012; Zappalà et al., 2012; Gabarra et al., 2014). Hence, *C. album* could be a plant to promote the presence of *P. soemius* - although its role in controlling the pest is questionable since no parasitism of *T. absoluta* by *P. soemius* was recorded during our experiments. Another potential alternative host species is the micro-Lepidoptera *C. pulchrimella*, which was reported to be parasitized by *N. tutae* in the laboratory (Bodino et al., 2016). While its host plant, *Parietaria officinalis* (Pellitory-of-the-wall), was recorded repeatedly in all three greenhouses, no mines of this lepidoptera species were found. While our study provides valuable insights into the parasitoid community within the immediate vicinity of greenhouses, it's essential to acknowledge that the radius of exploration was restricted to 100 m, potentially overlooking the broader landscape effects on parasitism due to the wide-ranging flight capabilities of parasitoids. A larger area surveyed beyond the immediate vicinity of the greenhouses might yield different insights.

The results presented here show that the release of *D. gelechiidivoris* could help control *P. operculella* as much as *T. absoluta*. This finding is highly relevant for China, for instance, since *T. absoluta* is causing severe damage (Zhang et al., 2021), and *P. operculella* is the major constraint to the commercial production of potatoes in the country (Gao, 2018). *D. gelechiidivoris* could also benefit the control of future invasions, such as of the tomato pinworm *K. lycopersicella* (Aigbedion-Atalar et al., 2022), whose distribution is now restricted to South and North America. *D. gelechiidivoris* could potentially attack the Guatemalan potato tuber, *Tecia solanivora* Povolny (Lepidoptera: Gelechiidae), since it is from the same family and feeds on Solanaceae, which could help to control the ongoing outbreak of the pest in Spain (Jeger et al., 2018). Finally, based on these results, *P. operculella* could be a suitable alternative host for rearing both parasitoid species. The ease of rearing the *P. operculella* on potato tubers (Gui and Li, 2003) makes it a more convenient and cheaper option than rearing on *T. absoluta*. Nevertheless, as shown by our result and the study of Bodino et al. (2016), the rearing host strongly influenced the preference of the offspring for the future host. This characteristic should therefore be taken into account.

The research presented here is the first to examine the host-specificity in the laboratory and in the field of *N. tutae* and *D. gelechiidivoris*, which play an important role in the biocontrol of *T. absoluta* in the native area of the pest and around the Mediterranean basin (Desneux et al., 2022; Denis et al., 2022). Overall, our study provides clear evidence for the polyphagy of *N. tutae* and the oligophagy of *D. gelechiidivoris* in the study area. The high host-specificity and attraction of *D. gelechiidivoris* towards *T. absoluta* and related species such as *P. operculella* make it an ideal candidate for augmentative or classical biological control. In contrast, the polyphagous nature of *N. tutae* poses a potential risk for non-target species. The study suggests that countries interested in classical biocontrol should prioritize *D. gelechiidivoris* and conduct host-specificity tests, focusing on leafminers of the Gelechiidae family. Nevertheless, the Gelechiidae family is incredibly large, consisting of 865 species from 109 genera in Europe alone (Huemer and Karsholt, 2020). Therefore, when selecting species for host-specificity testing, it is advisable first to evaluate those belonging to the same genera as *T. absoluta* (Gelechiinae) feeding on plants in the Solanaceae family or other closely related families. Moreover, further research is needed to understand the potential intraguild competition of both parasitoids and the overwintering capacity of *D. gelechiidivoris*, which could predict the establishment, spread, and effectivity and be relevant to assess the risk posed in the case of augmentative biocontrol in the northern countries. Last, countries at high risk of being invaded by *T. absoluta* where *P. operculella* is already present, such as New Zealand, Australia, or North America, could develop preemptive biocontrol programs (Caron et al., 2021) using *P. operculella* as an alternative host to screen for native parasitoids.

Credit authorship contribution statement

Jérémy Gonthier: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft. **Judit Arnó:** Methodology, Supervision, Validation, Writing – review & editing. **Jörg Romeis:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Jana Collatz:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We would like to thank Andermatt Biocontrol AG for providing eggs of *T. absoluta* and *P. operculella* to start a colony and Julien Kambor-Prieur for helping us to collect mines of *L. malifoliella*. We wish to thank Carmen Denis for her substantial help in identifying parasitoids. We also want to acknowledge Aleix Teruel, Victor Muñoz, and Pilar Hernández from the Sustainable Plant Protection of IRTA in Spain for outstanding technical support. We thank the Federació d'ADV's SELMAR, who provided the list of possible farms to sample and facilitated our access to the greenhouses as well as the farmers for allowing us to carry out your experiment on their properties. JA was funded by the Horizon Europe project ADOPT-IPM (Project 101060430) and from the CERCA Program of the Generalitat de Catalunya.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biocontrol.2024.105464>.

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