Host range testing of *Tamarixia dryi* (Hymenoptera: Eulophidae) sourced from South Africa for classical biological control of *Trioza erytreae* (Hemiptera: Psyllidae) in Europe

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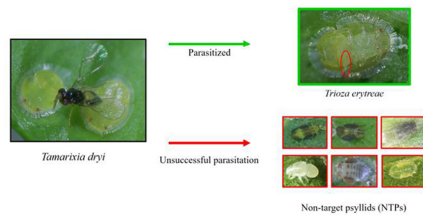[b] Grupo TRAGSA (Empresa de Transformació Agraria, S.A), Spain
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GRAPHICAL ABSTRACT

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ABSTRACT

The African citrus psyllid, *Trioza erytreae*, vectors citrus greening or huanglongbing (HLB) disease. The psyllid has been reported from mainland Europe, where it is rapidly spreading from the northwest to the southwest of the Iberian Peninsula. In order to reduce its spread and population levels, a classical biological control program with the parasitoid *Tamarixia dryi* is under development in Spain. We evaluated the host specificity of *T. dryi* using 11 non-target psyllid (NTP) species, including five species of the genus *Trioza*. The psyllids were selected based on phylogenetic and ecological criteria. *Tamarixia dryi* exhibited a high host specificity. Females did not parasitize any of the 11 NTPs tested, except for one nymph of a gall-forming *Trioza* species closely related to *Trioza montanetana*. *Tamarixia dryi* only laid one egg on a nymph when it was removed from the gall on *Convolvulus canariensis* and exposed directly to the parasitoid. However, the immature parasitoid died before emerging. We further confirmed that *T. dryi* did not parasitize a representative triozid species, *Trioza laurisilvae,* of the endemic Canarian fauna after long time exposure. Our results demonstrate that *T. dryi* is a highly specific parasitoid and its introduction, release and establishment in Europe within the classical biological control program of *T. erytreae* should not affect other psyllid species. Therefore, no significant environmental impact is expected.

1. Introduction

The African citrus psyllid, *Trioza erytreae* (Del Guercio) (Hemiptera: Triozidae), is the latest citrus key pest recorded from mainland Europe. It was detected in the northwest of Spain in 2014 (Pérez-Otero et al., 2015; Cocuzza et al., 2017) and, since then, has spread towards the southwest of the Portuguese coast reaching Lisbon (DGAV, 2018). *Trioza erytreae* heavily infests citrus trees in private gardens in Galicia and Portugal. This psyllid transmits the citrus greening or huan-glongbing (HLB) disease, the most devastating citrus disease in the world (Bové, 2006; Gottwald, 2007). The disease is associated with three phloem α-proteobacterias, "*Candidatus* Liberibacer *asiaticus*" (CLas), "*Ca*. Liberibacter *americanus*" (CLam) and "*Ca*. Liberibacter *africanus*" (CLaf). *Trioza erytreae* is associated with CLaf (Bové, 2006). Since its first record from South Africa in 1929 (Jagoueix et al., 1994; Li et al., 2006), HLB has been reported from Sub-Saharan Africa, Yemen and a few Atlantic Ocean islands (Bové, 2006; Duran-Vila et al., 2014). Although HLB has not yet been detected in European countries (Pérez-Otero et al., 2015; Siverio et al., 2017; Cocuzza et al., 2017), the mere presence of the psyllid is a major threat for the Mediterranean citrus industry.

Since *T. erytreae* has already spread through the west of the Iberian Peninsula (Galicia-Spain and Portugal), its eradication is impossible and consequently a series of strategies need to be implemented urgently. Nowadays, classical biological control is the most feasible approach because no native parasitoids have been recovered from *T. erytreae* during different surveys carried out in both the Atlantic islands and the Iberian Peninsula (Fernandes and Aguiar, 2002; Cocuzza et al., 2017). *Tamarixia dryi* (Waterson) (synonym *Tetrastichus dryi*) (Hymenoptera: Eulophidae) is the most abundant and effective biological control agent of *T. erytreae* in its area of origin (Catling, 1969; Mc Daniel and Moran, 1972; Van den Berg and Greenland, 2000; Tamesse and Messi, 2002). It is a solitary koinobiont ectoparasitoid parasitizing 3rd to 5th nymphal instars of the psyllid. *Tamarixia dryi* has been used successfully in classical biological control programs in Reunion Island and Mauritius (Etienne and Aubert, 1980; Aubert and Quilici, 1985). In these islands, *T. dryi* is able to control the psyllid populations effectively (Etienne and Aubert, 1980). Taking into consideration these successes and in order to reduce the spread of the psyllid, the Instituto Valenciano de Investigaciones Agrarias (IVIA) in collaboration with the Instituto Canario de Investigaciones Agrarias (ICIA) applied for the permits to introduce *T. dryi* into the Canary Islands and mainland Spain. The parasitoid was collected in several citrus orchards from Pretoria (South Africa) (Pérez-Rodríguez et al., submitted).

In the past, several introductions of parasitoids have undesirable negative effects on non-target insects (Heimpel and Mills, 2017). In order to avoid negative effects of parasitoid introductions, environmental agencies and scientists have developed ecological risk assessments (EPPO, 2014). Testing host specificity is the most common method used to determine whether non-target species are attacked by the proposed parasitoid and to assess ecological risks (Hokkanen et al., 2003; Van Lenteren et al., 2006; Heimpel and Mills, 2017).

One of the key points in host-specificity tests is to define the list of non-target species to be evaluated. Since parasitoid species tend to parasitize and develop in or on hosts phylogenetically close to their target host (Percy et al., 2018), the phylogenetic pattern is a major determinant in host-specificity tests (Dijkerman, 1990; Godfray, 1994; Brodeur et al., 1996; Van Driesche et al., 2004; Kuhlmann et al., 2005; Desneux et al., 2012). Moreover, host-specificity tests should also include a list of additional non-target species for ecological and biological control reasons (Heimpel and Mills, 2017).

The main aim of this study was to test the host specificity of the parasitoid *T. dryi*. For this purpose, 11 non-target psyllids (NTPs) were selected based on their phylogenetic proximity to *T. erytreae* and for ecological reasons such as sharing similar host resources. Several endemic and representative psyllids of the Canarian fauna were included

in the test. Since some psyllid nymphs are protected by galls, the nymphs both within the galls and removed from their galls were offered to *T. dryi* females. Finally, behavioral and long-time exposure experiments were carried out using *Trioza laurisilvae* Hodkinson (Hemiptera: Triozidae) as NTP representative of the endemic Canarian fauna. This work presents the results from mandated host specificity testing of *T. dryi* in quarantine before its release and establishment in the Canary Islands and mainland Europe for the classical biological control of *T. erytreae*.

2. Material and methods

2.1. Insect cultures and collections

Host range tests were carried out at the University of Pretoria (South Africa) and ICIA (Tenerife, Spain). The collection and management of the plants and insects varied slightly between the two places.

2.1.1. Trioza erytreae

For the establishment of two *T. erytreae* cultures, one in South Africa and one in the Canary Islands, approximately 40 adults were collected from 12 pesticide-free lemon trees at the Pretoria University Farm (25°44′52.1″S 28°15′33.6″E) (South Africa) and in different orchards of pesticide-free lemon trees in the Canary Islands (10 km around 28°31′29″N 16° 22′12″W) (Spain), respectively. Adult psyllids were collected using a manual aspirator from young flushes and placed in 120 ml plastic tubes with a muslin cover. They were transported to the laboratory and identified using a dissecting microscope. Once identified to species level, adult psyllids were released into corresponding species-specific cultures on citrus. Citrus plants with nymphs suitable for parasitism (3rd to 5th instar) were collected 12 days after *T. erytreae* adults were released. The plants were used for both maintaining the *T. dryi* cultures and for experiments.

In South Africa, one-year-old pesticide-free grapefruit plants (*Citrus paradisi* Macf.) were used as host plants for *T. erytreae*. Plants were kept in 20 cm in diameter pots in bug dorms in a 60 × 60 × 60 cm plastic cage (BugDorm-2 insect tents; MegaView Science Co., Ltd., Taichung, Taiwan) in a climate chamber at 25 ± 2 °C, 60 ± 10% relative humidity (RH) and 14L: 10D h photoperiod. The plants were watered twice per week.

In the Canary Islands, two-year-old pesticide-free lemon plants (*Citrus limon* (L). Burm.f.) were used as host plants for *T. erytreae*. Plants were grown on vermiculite and peat (1:3; vol:vol) in 20 cm diameter pots and were kept in a greenhouse at 23 ± 2 °C, 70 ± 10% RH and natural photoperiod. Plants were watered twice per week and fertilized once per week using a modified Hoagland's solution.

In both countries, young trees were pruned to obtain homogeneous young flushes. The optimal state for the introduction of these plants into the *T. erytreae* rearing units was reached when the oldest leaves of the new flushes measured about 4 cm. This aspect is critical because *T. erytreae* females lay their eggs in the axis of very young leaves (Moran and Buchan, 1975).

2.1.2. Non target psyllid (NTP) for host specificity testing

Multiple criteria were used for the selection of 11 representative NTP species for host specificity testing (Table 1). NTPs were collected from their respective host plant species by cutting the twigs or leaves where they were settled. The infested plant material was enclosed in plastic bags with tissue paper to absorb excess moisture and transported to the laboratory where psyllids were identified using a dissecting microscope. Twigs and leaves with nymphs were used in experiments.

2.1.3. Tamarixia dryi

In South Africa, parasitoids were obtained directly from parasitized 3rd- to 5th-instar nymphs of *T. erytreae* collected in a citrus orchard at the Experimental Farm of the University of Pretoria (25°44′52.1″S

Table 1
Selection criteria and psyllid species used for host specificity testing of *Tamarixia dryi* together with host plant species, location of psyllid collection and experiments carried out.

Selection criteria	Psyllid species and family	Host plant species	Location of collection and experiments	Host range experiment
Target pest species	*Trioza erytreae* (Triozidae)	*Citrus limon*	Canary Island, South Africa	Natural[*]
Close phylogenetic relatedness to *T. erytreae*	*Trioza alacris* (Triozidae)	*Laurus nobilis*	Canary Island	Natural and exposed[**]
Close phylogenetic relatedness to *T. erytreae* and native psyllid to Canary Island	*Trioza laurisilvae* (Triozidae)	*Laurus azorica*	Canary Island	Natural and exposed
	Trioza sp. I (Triozidae)	*Withania aristata*	Canary Island	Natural
	Trioza sp. II (Triozidae)	*Convolvulus floridus*	Canary Island	Natural and exposed
	Trioza sp. III (Triozidae)	*Convolvulus canariensis*	Canary Island	Natural and exposed
Native host plant related to citrus	*Agonoscena* sp. (Psyllidae)	*Ruta pinnata*	Canary Island	Natural and exposed
High probability of occurrence in native vegetation surrounding citrus groves	*Bactericera tremblayi* (Triozidae)	*Allium ampeloprasum*	Canary Island	Natural
	Euphyllura olivina (Psyllidae)	*Olea europea*	South Africa	Natural
	Ctenarytaina eucalypti (Psyllidae)	*Eucalyptus globulus*	Canary Island	Natural
	Glycaspis brimblecombei (Psyllidae)	*Eucalyptus camaldulensis*	Canary Island	Natural and exposed
	Spondyliaspis plicatuloides (Psyllidae)	*Eucalyptus* spp.	South Africa	Natural

* Naturally settled on leaves/galls.
** Removed from galls and exposed to parasitoids on the leaves.

28°15′33.6″E). Individual parasitized psyllid nymphs were placed singly in 1 ml microtubes with one drop of sucrose (catalogue number: S0389; Sigma-Aldrich®, UK) 1 M provided *ad libitum* and the microtubes were then closed with a cotton bud. The microtubes were kept in an incubator under controlled conditions at 25 ± 2 °C, $60 \pm 10\%$ RH and 14 L: 10D h photoperiod and checked daily until parasitoids emerged. Once emerged, *T. dryi* parasitoids were identified and the sex recorded. Individuals were kept singly in the same microtubes and maintained under the same conditions as before.

In the Canary Islands, individuals of *T. dryi* sourced from South Africa were used (Pérez-Rodríguez et al., submitted). In order to reduce the risk of accidental importation of the pathogen associated with citrus greening into quarantine, only adult parasitoids were introduced into Spain to initiate cultures. In order to maintain the cultures, detached citrus leaves infested with nymphs suitable for parasitism by *T. dryi* (3rd to 5th instar) were collected from the culture of *T. erytreae* and offered to adult parasitoids in 120 ml plastic tubes (57×73 mm) closed with muslin to allow for ventilation. Leaf petioles were attached to a piece of wet cotton wool to keep their turgidity. Males and females of *T. dryi* were introduced with a supplementary drop of honey inside the tubes for 48 h. After removing *T. dryi*, tubes were kept at 23 ± 1 °C, $65 \pm 2\%$ RH, and L16: D8 photoperiod. After ten days, tubes were checked daily until parasitoids emerged. Once emerged, *T. dryi* parasitoids were sexed and transferred to a 2 ml glass vial with one drop of honey added on the inside and closed with cotton wool.

In both South Africa and Spain, one newly emerged female and male less than 24 h old were introduced into a 2 ml glass vial, with a cotton wool cover and one drop of sucrose (South Africa) or honey (Canary Islands). After 24 h, males were removed from the vial. Females were kept in the vials for an additional 2 to 3 days before they were used in the experiment.

2.2. Experiment 1: Host range experiment on naturally settled nymphs

To determine whether *T. dryi* parasitizes or kills the selected NTPs, psyllid nymphs were offered to single female parasitoids over a period of 48 h (Tables 2 and 3). A single female was released into a 100 ml glass vial with one drop of honey and leaves infested with psyllids before closing the opening of the vial with muslin. Each glass vial contained between one and three leaves infested with the corresponding NTPs. Leaf petioles were inserted with a piece of wet cotton wool to keep them turgid. The number of live, dead and parasitized psyllids were recorded after 48 h. Hosts from the 3rd to the 5th instar

were considered as suitable for parasitism because these are the preferred instars of *T. dryi* (Mc Daniel and Moran, 1972). Females of *T. dryi* lay their eggs underneath the body of their hosts, between the abdomen and the hind legs (see graphical abstract). Hosts were recorded as parasitized when they had a parasitoid egg underneath their body. Parasitism was calculated as:

Rate of parasitism

$$= \text{parasitized 3rd to 5th instar psyllids}/(\text{parasitized 3rd to 5th instar psyllids} + \text{alive 3rd to 5th instar psyllids})$$

Mortality was calculated as:

$$\text{Mortality} = \text{dead 3rd to 5th instar psyllids/all 3rd to 5th instar psyllids}$$

Natural mortality of psyllids under prevailing experimental conditions was assessed with control treatments without the parasitoid being present for all the NTPs. These controls were not included for *Trioza alacris*, *Euphyllura olivina* and *Spondyliaspis plicatuloides*. All replicates were kept in an incubator under controlled conditions (25 ± 2 °C, $60 \pm 10\%$ of RH and L14:D10 photoperiod). The mean number of nymphs in the 3rd to 5th instar exposed to parasitoids ranged between six and 23 nymphs and experiments were replicated between four and 20 times per season (Table 2.)

2.3. Experiment 2: Host range experiment on nymphs removed from their galls and exposed to T. dryi

Experiments with psyllid species that settled and rolled leaves or produced galls that were apparently inaccessible to parasitoids were carried out as described above with healthy leaves (Table 3). To avoid breaking their fragile mouthparts, we opened the rolled leaves or galls and carefully transferred nymphs in the 3rd to 5th instar that were walking after being gently disturbed with a fine hair paint brush to new healthy leaves. Nymphs were left for 24 h to settle and feed on the new leaf. These new leaves were kept in 100 ml glass tubes with one drop of honey and closed with a muslin cover for ventilation. We then followed the same methodology as described for the naturally exposed psyllid nymphs. The mean number of nymphs exposed to parasitoids ranged between 10 and 23 nymphs and the experiment was replicated between 15 and 20 times (Table 3). Experiments 1 and 2 were carried out in parallel and the same controls were therefore used for both experiments (Tables 2 and 3).

Table 2

Number of psyllid nymphs in the 3rd to the 5th instar per replicate, number of parasitized psyllid nymphs and percent mortality (mean ± SE) after exposure to the parasitoid *Tamarixia dryi* for 48 h. The mortality of exposed and unexposed (control) psyllid nymphs to *T. dryi* was compared for each host species. The results of the statistical analyses are provided in the Supplementary materials.

Location and season	Psyllid host	Treatment	Replicates	Psyllids/plant	% Psyllid mortality	Parasitized psyllids
Canary Island (winter)	*Trioza erytreae*	Control	20	11.3 ± 0.2	37.07 ± 7.7	–
		T. dryi	20	11.2 ± 0.4	46.47 ± 8.8	1.9 ± 0.5
	Trioza alacris	Control	–	–	–	–
		T. dryi	10	8.8 ± 1.9	65.7 ± 9.1	0
	Bactericera tremblayi	Control	15	10.0 ± 0.0	16.7 ± 2.9	–
		T. dryi	18	8.8 ± 0.5	13.3 ± 2.7	0
	Glycaspis brimblecombei	Control	12	7.2 ± 0.5	27.2 ± 4.2	–
		T. dryi	12	7.8 ± 0.5	22.5 ± 2.3	0
	Aganoscesna sp.	Control	8	14.3 ± 1.3	2.5 ± 1.9	–
		T. dryi	8	12.8 ± 1.5	0	0
Canary Island (spring)	*Trioza erytreae*	Control	15	23.3 ± 1.5	37.4 ± 6.9	–
		T. dryi	15	18.1 ± 1.2	39.4 ± 4.2	2.1 ± 0.4
	Trioza laurisilvae	Control	20	17.1 ± 1.7	54.8 ± 6.9	–
		T. dryi	20	15.6 ± 1.6	50.5 ± 4.6	0
	Ctenarytaina eucalypti	Control	20	10.8 ± 0.9	48.2 ± 0.4	–
		T. dryi	20	15.8 ± 0.9	43.6 ± 3.8	0
Canary Island (fall)	*Trioza erytreae*	Control	15	18.2 ± 2.4	29.7 ± 4.2	–
		T. dryi	15	17.4 ± 2.3	36.8 ± 4.3	2.0 ± 0.4
	Trioza sp. I	Control	9	11.8 ± 2.8	16.2 ± 0.9	–
		T. dryi	9	12.6 ± 6.3	17.9 ± 1.3	0
	Trioza sp. II	Control	15	12.7 ± 0.9	24.8 ± 5.2	–
		T. dryi	15	11.0 ± 0.9	23.1 ± 2.7	0
	Trioza sp. III	Control	15	11.6 ± 5.2	4.6 ± 2.2	–
		T. dryi	15	10.4 ± 2.9	4.2 ± 2.0	0
South Africa (spring)	*Trioza erytreae*	Control	–	–	–	–
		T. dryi	6	6.8 ± 0.4	0	1.5 ± 0.3
	Euphyllura olivina	Control	–	–	–	–
		T. dryi	4	6.0 ± 0.0	0	0
	Spondyliaspis plicatuloides	Control	–	–	–	–
		T. dryi	10	6.5 ± 0.2	0	0

2.4. Experiments 3 and 4: Trioza laurisilvae as an alternative host for T. dryi

Since *T. laurisilvae* is an endemic psyllid species from the laurisilva (*Laurus azorica* (Lauraceae)), also known as laurel forest, from the Canary Islands, we carried out detailed experiments at the facilities of ICIA. In Experiment 3, we recorded and compared parasitism and time spent by parasitoids searching for and ovipositing in the first nymph encountered. Experimental arenas consisted of a Petri dish (5 cm in diameter) with a 1 cm in diameter muslin-covered hole on top to allow for air exchange. One leaf of the respective host plant was placed upside down in an arena and infested with 15 3rd- to 5th-instar nymphs.

Leaves were infested following the same methodology described for Experiment 2. A single adult female *T. dryi* was introduced into each arena with a drop of honey on the wall of the Petri dish. To prevent parasitoids escaping from the experimental arena, the Petri dishes were sealed with Parafilm® (Pechiney Plastic Packaging, Menasha, WI, USA). Parasitoids were observed for 40 min, or until they laid their first egg. We considered that a female had laid an egg, i.e. parasitzed a nymph, when it stung the nymph for more than two minutes. After the observation, the female was removed and parasitized nymphs were examined under a dissecting microscope to confirm oviposition. Fifteen *T. dryi* females per psyllid species were evaluated.

In Experiment 4, *T. dryi* females were exposed to either *T. laurisilvae*

Table 3

Number of psyllid nymphs in the 3rd to 5th instar per replicate, number of parasitized psyllid nymphs and percent mortality (mean ± SE) after removal from galls and exposure to the parasitoid *Tamarixia dryi* for 48 h. The mortality of the psyllids exposed and unexposed (Control) to *T. dryi* was compared for each host species. The results of the statistical analyses are provided in the Supplementary materials.

Location and season	Psyllid host	Treatment	Replicates	Psyllids/ plant	% Psyllid mortality	Parasitized psyllids
Canary Island (winter)	*Trioza erytreae*	Control	20	11.3 ± 0.2	37.1 ± 7.7	–
		T. dryi	20	11.2 ± 0.4	46.5 ± 8.8	1.9 ± 0.5
	Aganoscesna sp.	Control	16	9.8 ± 0.5	2.5 ± 2.3	–
		T. dryi	16	10.0 ± 0.0	1.9 ± 1.0	0
Canary Island (spring)	*Trioza erytreae*	Control	15	23.3 ± 1.5	37.4 ± 6.9	–
		T. dryi	15	18.1 ± 1.2	39.5 ± 4.2	2.1 ± 0.4
	Trioza laurisilvae	Control	20	10.0 ± 0.0	54.0 ± 6.3	–
		T. dryi	20	10.0 ± 0.0	56.0 ± 4.9	0
Canary Island (fall)	*Trioza erytreae*	Control	15	18.2 ± 2.4	29.7 ± 4.2	–
		T. dryi	15	17.4 ± 2.3	36.8 ± 4.3	2.0 ± 0.4
	Trioza sp. II	Control	15	10.0 ± 0.0	59.5 ± 5.6	–
		T. dryi	15	10.0 ± 0.0	62.0 ± 5.8	0
	Trioza sp. III	Control	15	10.0 ± 0.0	69.5 ± 4.4	–
		T. dryi	15	10.0 ± 0.0	66.5 ± 5.1	0.05 ± 0.05

or *T. erytreae* nymphs on their respective host plants for seven days. We used the same experimental arena as in Experiment 3 but leaves were infested with 10 nymphs. Leaves, wet cotton wool and nymphs were replaced daily. A single mated adult female of *T. dryi* was introduced into each arena provisioned with a droplet of honey in the Petri dish. Nymph mortality and parasitism were checked daily under a dissecting microscope. Natural mortality of psyllids under prevailing experimental conditions was assessed without the parasitoid being present as control. Ten parasitoid females (replicates) per each psyllid species were evaluated.

In both experiments, *Tamarixia dryi* females were obtained following the same procedure as in Experiments 1 and 2. The experiments were carried out in a climate chamber under same conditions as in Experiment 1.

2.5. Statistical analysis

We used a generalized linear model (GLM) assuming a quasi-Poisson error distribution to analyze the number of nymphs parasitized by *T. dryi* in Experiments 1 and 2 by comparing the explanatory factor "location and season". NTP species that were not parasitized by *T. dryi* were excluded from the analysis. We used GLMs assuming a quasibinomial error distribution to analyze the percentage of mortality. For Experiments 1 and 2, we compared the mortality of nymphs exposed to *T. dryi* and unexposed nymphs for each experiment and psyllid species. In Experiment 4, the presence of parasitoids, the day and their interaction were the explanatory factors. The analysis was conducted for each host separately: *T. erytreae* and *T. laurisilvae*. The statistical software package 'R' (http://www.R-project.org) was used for these analyses.

3. Results

3.1. Experiment 1: Host range experiment on naturally settled nymphs

When *T. dryi* females had access to their natural host *T. erytreae*, they parasitized between 1.5 and 2.1 nymphs in the 3rd to the 5th instar in 48 h. The mean number of parasitized nymphs was similar in the four "locations and seasons" analyzed ($F_{3,\ 55} = 5.15$, $P = 0.85$) (Table 2). No *T. dryi* eggs were observed on *T. erytreae* nymphs in the control treatments, indicating no accidental contamination.

Tamarixia dryi females did not parasitize any of the 11 NTPs tested in the four experiments (Table 2). Their presence did not affect the mortality of the NTPs settled within the galls (Table 2: for each NTPs we compared the mortality in treatment "control" *vs* treatment "*T. dryi*"; statistical values are provided in Supplementary materials).

3.2. Experiment 2: Host range experiment of psyllid nymphs exposed to T. dryi

Tamarixia dryi females parasitized only one out of the 150 nymphs of *Trioza* sp. III on *C. canariensis* (Table 3). However, the immature parasitoid died in the first larval instar just after hatching. *Tamarixia dryi* females did not parasitize any of the other NTPs in the three experiments and their presence did not influence the mortality of the NTPs when removed from their galls and exposed to the parasitoid directly (Table 3 and Supplementary materials).

3.3. Experiment 3 and 4: T. laurisilvae as an alternative host of T. dryi

No *T. laurisilvae* nymphs were parasitized during the 40 min *T. dryi* females searched in the arena, whereas females parasitized 14 of the 15 *T. erytreae* nymphs offered (93.3% parasitism). Female *T. dryi* took 280 ± 50 s to locate and parasitize the first *T. erytreae* nymph.

Tamarixia dryi females parasitized *T. erytreae* nymphs but not *T. laurisilvae* nymphs during seven days of exposure (Fig. 1a, b). Parasitism

rate of *T. erytreae* increased significantly 2 days after *T. erytreae* was exposed to *T. dryi* ($F_{6,\ 63} = 3.23$, $P = 0.008$).

Trioza erytreae mortality was influenced neither by the day of the experiment ($F_{6,\ 139} = 0.07$, $P = 0.78$) nor by the presence of *T. dryi* ($F_{1,\ 139} = 3.16$, $P = 0.078$). The interaction between day and parasitoid presence was not significant ($F_{6,\ 139} = 0.2$, $P = 0.66$) (Fig. 1a). The mortality of *T. laurisilvae* nymphs varied among days ($F_{6,\ 139} = 4.77$, $P = 0.031$) but was independent of parasitoid presence ($F_{1,\ 139} = 0.32$, $P = 0.57$) and the interaction of both factors ($F_{6,\ 139} = 0.01$, P = 0.92) (Fig. 1b).

4. Discussion

Our risk assessment demonstrates that *T dryi* is a highly specific parasitoid. The risk assessment included, among others, five non-target *Trioza* species, *T. alacris*, *T. laurisilvae* and three new *Trioza* species, which develop on *Withania aristata* (Solanaceae)*, Convolvulus floridus* and *Convolvulus canariensis* (Convolvulaceae). *Tamarixia dryi* females only parasitized the target species *T. erytreae* and one individual of the non-target *Trioza* sp. III, which develops on *C. canariensis*. However, it is unlikely that *T. dryi* is going to parasitize this species in the field because *Trioza* sp. III produces lignified galls on leaves that prevent the access of parasitoids and no parasitism of nymphs inside the galls was recorded. Only removal of the nymphs from the galls resulted in parasitism and then only of a single nymph and the immature parasitoid did not develop in this psyllid but died after a few days. Aubert and Quilici (1985) observed that *T. dryi* parasitizes *Trioza litseae* Bordage (Hemiptera: Triozidae) but this species has not been recorded in the Canary Islands or mainland Europe (www.hemiptera-databases.org).

Tamarixia dryi could be adapted to parasitize psyllids that develop on Rutaceae or on plants within the same niche. We, therefore, included the psyllid *Aganoscesna* sp., which develops on *Ruta pinnata* (Rutaceae), a bush native to the Canary Islands (mainly in Tenerife island) (Ginovés et al., 2010). However, *T. dryi* did not lay any eggs on *Aganoscesna* nymphs. Our results are in agreement with those obtained by Hoddle and Pandey (2014) who evaluated the host range of the congeneric *Tamarixia radiata* (Waterson) (Hymenoptera: Eulophidae), a parasitoid of the Asian citrus psyllid *Diaphorina citri* Kuwayama (Hemiptera: Liviidae). These authors concluded that releasing *T. radiata* in southern California poses a negligible environmental risk. In fact, to our knowledge, no impacts have been reported in any of the numerous areas where either *T. radiata* or *T. dryi* have been released.

The presence of *T. dryi* did not increase the mortality of the NTPs. Parasitoids can cause the mortality of their host without parasitizing them via host feeding and other nonreproductive mechanisms (Abram et al., 2019). These behaviors have often gone unnoticed and should be considered in evaluation risk assessments in classical biological control programs (Abram et al., 2019). Although *T. dryi* host feeds, stings and rejects *T. erytreae* nymphs (i.e. mutilation) (Urbaneja-Bernat et al., in prep), we did not observe these behaviors when the parasitoid was exposed to the NTPs. Therefore, the risk caused by these behaviors is likely to be negligible.

In the second part of our study, we analyzed the relationship between *T. dryi* and *T. laurisilvae* in detail because this psyllid is associated with the macaronesian laurisilva forest in the Canary Islands (Aguiar, 2001; Moya et al., 2004). For this, a long exposure no choice test and direct observations were carried out. The results of these experiments confirmed the high specificity of this parasitoid without adverse effects on one of the most important endemic psyllid species from the Laurisilva.

Overall, our results demonstrate that *T. dryi* is a highly specific parasitoid and its introduction, release and establishment in Europe within the classical biological control program of *T. erytreae* should not affect other psyllid species. Therefore, no significant environmental impact is expected. On the other hand, the potential benefits of its establishment are very high, especially since the psyllid has not yet
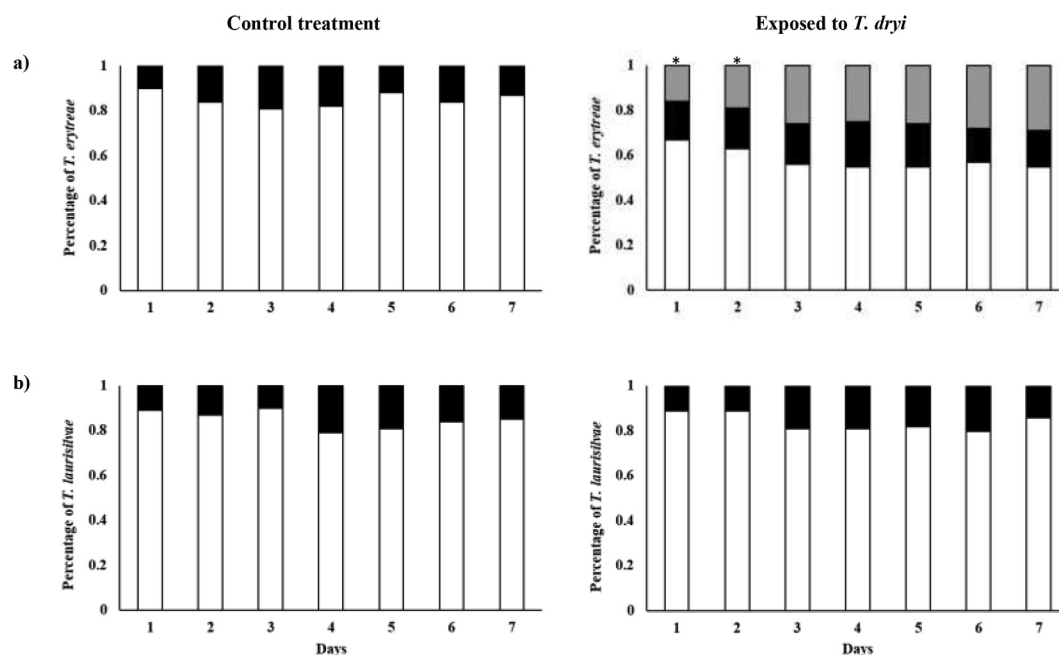
Fig. 1. Percentage of *Trioza erytreae* (a) and *T. laurisilvae* (b) nymphs alive (white), dead (black) and parasitized (grey) without (left panels) and with (right panels) *Tamarixia dryi* seven days after exposure to the parasitoid. * Shows statistically significant differences between days in the percentage of parasitized psyllids.

arrived in the main citrus producing areas (Mediterranean basin) and HLB has not been detected. If *T. dryi* is as efficient as described from Reunion Island, the parasitoid might delay the spread of *T. erytreae* in Europe. The high specificity of *T. dryi* is a limiting factor not only for its establishment and spread but also for its rearing in areas where the psyllid has not arrived. Since *T. dryi* is host-specific, it should be reared only in those areas already colonized by the psyllid to avoid the accidental spread of *T. erytreae* via potential escapes from rearing facilities. Therefore, in Spain, the only country that has applied for permits to introduce and release *T. dryi* in mainland Europe, it could only be reared in Galicia, where citrus is present only in backyards, and in the Canary Islands, from where it should be transported to mainland Europe. Taking this into consideration, as well as the presence of *T. erytreae* in mainland Portugal, where it spread up to Lisbon (DGAV, 2018), the introduction, mass-production and release of *T. dryi* in Portugal should also be considered. Our study supports the release of *T. dryi* as a biological control agent in classical biological control programs for *T. erytreae* in Europe.

Author statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2019.04.018.

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