INSTITUTO POLITÉCNICO DE BRAGANÇA Escola Superior Agrária

Spread patterns of *Trioza erytreae* on citrus orchards and the potential role of natural enemies as biocontrol agents

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“In a gentle way, you can shake the world”

(Mahatma Gandhi)

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Abstract

Citrus production is a relevant economic activity around the world. In Portugal, Algarve is the leading citrus fruits producing region, it produces the famous and so-called "Orange of the Algarve". Huanglongbing(HLB), also known as citrus greening disease, is the biggest problem of citrus cultivation in the world. This disease reduces citrus production, causes the loss of colors of fruits, turns them greenish, increases fruit acidity, and reduces sugars. HLB is caused by the group of bacteria *Candidatus* Liberibacter, being propagated by two vectors, the African Citrus Psylla *Trioza erytreae* (Del Guercio, 1908) (Hemiptera: Triozidae) and the Asian Citrus Psylla *Diaphorina citri* (Kuwayama, 1908) (Hemiptera: Psyllidae). *Trioza erytreae* is currently present in the Iberian Peninsula, which raises a threat to the citrus industry. Nowadays, the geographic distribution of *T. erytreae* ranges from the Basque Country (Spain) to Algarve (Portugal). In Portugal, *T. erytreae* already reached the Algarve region. Although the vector is widely dispersed, HLB was not currently detected in the Iberian Peninsula. This work aimed to study environmentally friendly alternatives to chemical control of *T. erytreae*. The application of kaolin clay sprayed on citrus plants was found to significantly reduce the landing and settlement of flying adults of *T. erytreae* on lemon plants in the field. Moreover, a kaolin film applied on lemon plants in laboratory significantly reduced the ability of *T. erytreae* to successfully access the floematic tissues, thus reducing the risk of infection by HLB on lemon plants. This suggests that kaolin could be an efficient tool to manage *T. erytreae*, especially in the context of sustainable citrus agriculture. Understanding the trophic interactions between prey and its predators is vital for both population ecology and integrated pest management (IPM). In IPM, knowledge of how an ecosystem's trophic interactions affect prey densities is exploited to improve pest management strategies. The predatory bug *Anthocoris nemoralis* (Fabricius, 1794) (Hemiptera: Anthocoridae) is an abundant predator in fruit orchards. This species is considered a generalist predator, though it has been shown to prefer psyllids over other prey. To assess the potential of *A. nemoralis* as natural enemy in portuguese citrus orchards, field samples were collected from a lemon orchard in northwestern Portugal. Molecular gut-content analysis was performed in the laboratory to verify the presence of prey DNA. A PCR-based approach was used for detecting DNA of *T. erytreae* in the gut

of *A. nemoralis*. Accordingly, 15 primer sets designed within rrnL gene (16S), the rrnS gene (12S), and the COI genes were tested for specificity, sensibility, and feasibility in detecting *T. erytreae* in the gut content of *A. nemoralis*. Overall, one primer set targeting rrnL gene (16S), pair LSU_F1/LSU_R1 showed specificity and sensitivity for *T. erytreae* so that this PCR-based diagnostic assay may help in the implementation of sustainable management tools aimed to limit the spread of the pathogen transmitted by the psyllid.

Keywords: Huanglongbing; *T. erytreae*; kaolin; natural enemies.

Resumo

Os citrinos são fruteiras com elevada importância económica no mundo. Em Portugal, o Algarve, é a região com maior expressão e onde se produz a famosa e bem conhecida Laranja do Algarve. Um dos maiores problemas que a citricultura enfrenta a nível mundial é a doença de Huanglongbing(HLB) também conhecida como "greening" dos citrinos. Esta doença é característica pela diminuição da produção, redução da coloração dos frutos, tornando-os esverdeados, aumento da acidez assim como a redução de açucares, tornando inviável o seu consumo em fresco. O HLB é causado pelo grupo de bactérias *Candidatus* Liberibacter e é propagado através de dois vetores, a Psila Africana dos Citrinos *Trioza erytreae* (Del Guercio, 1908) (Hemiptera: Triozidae) e a Psila Asiática dos Citrinos *Diaphorina citri* (Kuwayama 1908) (Hemiptera: Psyllidae). *Trioza erytreae* está presente na Península Ibérica, o que suscita uma grande ameaça para a citricultura. Atualmente, a distribuição geográfica de *T. erytreae* vai desde o País Basco (Espanha) até ao Algarve (Portugal). Apesar do vector estar amplamente disperso, ainda não foi detetada nenhuma planta infetada com HLB na Península Ibérica. Este trabalho visa estudar alternativas sustentáveis e amigas do ambiente, em alternativa à luta química contra *T. erytreae*. A aplicação de caulino nos limoeiros em ensaios de campo aberto mostrou uma redução significativa na aterragem e colonização de adultos de *T. erytreae* em plantas de limoeiro pulverizadas com caulino. Além disso, em laboratório foi possível observar que plantas de limoeiros onde foi aplicado caulino, reduziu significativamente a capacidade de *T. erytreae* aceder com sucesso aos tecidos floemicos da planta, reduzindo assim o risco de infeção por HLB em limoeiros. Tal sugere que o caulino pode ser uma ferramenta eficiente para gerir *T. erytreae*, especialmente num contexto de produção sustentável. Compreender as interações tróficas entre pragas e inimigos naturais é da maior importancia tanto para compreender a ecologia das populações como para a proteção integrada das pragas (PI). Em PI, o conhecimento das interações tróficas num ecossistema contribui para a melhoria da proteção integrada contra pragas. *Anthocoris nemoralis* (Fabricius, 1794) (Hemiptera: Anthocoridae) é um predador abundante em pomares, esta espécie é considerada um predador generalista, embora tenha sido demonstrado que prefere as psilas entre outras presas. Para avaliar a importância do *A. nemoralis* como predador em pomares de citrinos em Portugal, foram recolhidos indivíduos de um pomar de limoeiros. A análise molecular do conteúdo do trato intestinal

foi realizada em laboratório para verificar a presença de ADN de *T. erytreae*, utilizando uma abordagem baseada na PCR como forma de detetar ADN de *T. erytreae* no trato intestinal de *A. nemoralis*. Consequentemente, foram testados 15 conjuntos de primers, do gene rrnL (16S), do gene rrnS (12S), e dos genes do COI para averiguar a especificidade, sensibilidade e viabilidade na deteção de *T. erytreae* no conteúdo intestinal de *A. nemoralis*. O conjunto de primers do gene rrnL (16S), o par LSU_F1/LSU_R1 mostrou alta especificidade e sensibilidade para a deteção de *T. erytreae*, de modo que esta técnica de diagnóstico baseado em PCR pode ajudar na implementação de ferramentas de de proteção sustentável destinadas a limitar a propagação do patógeno transmitido pelo psilídeo.

Palavras-Chave: Huanglongbing; *T. erytreae*; caulino; inimigos naturais.

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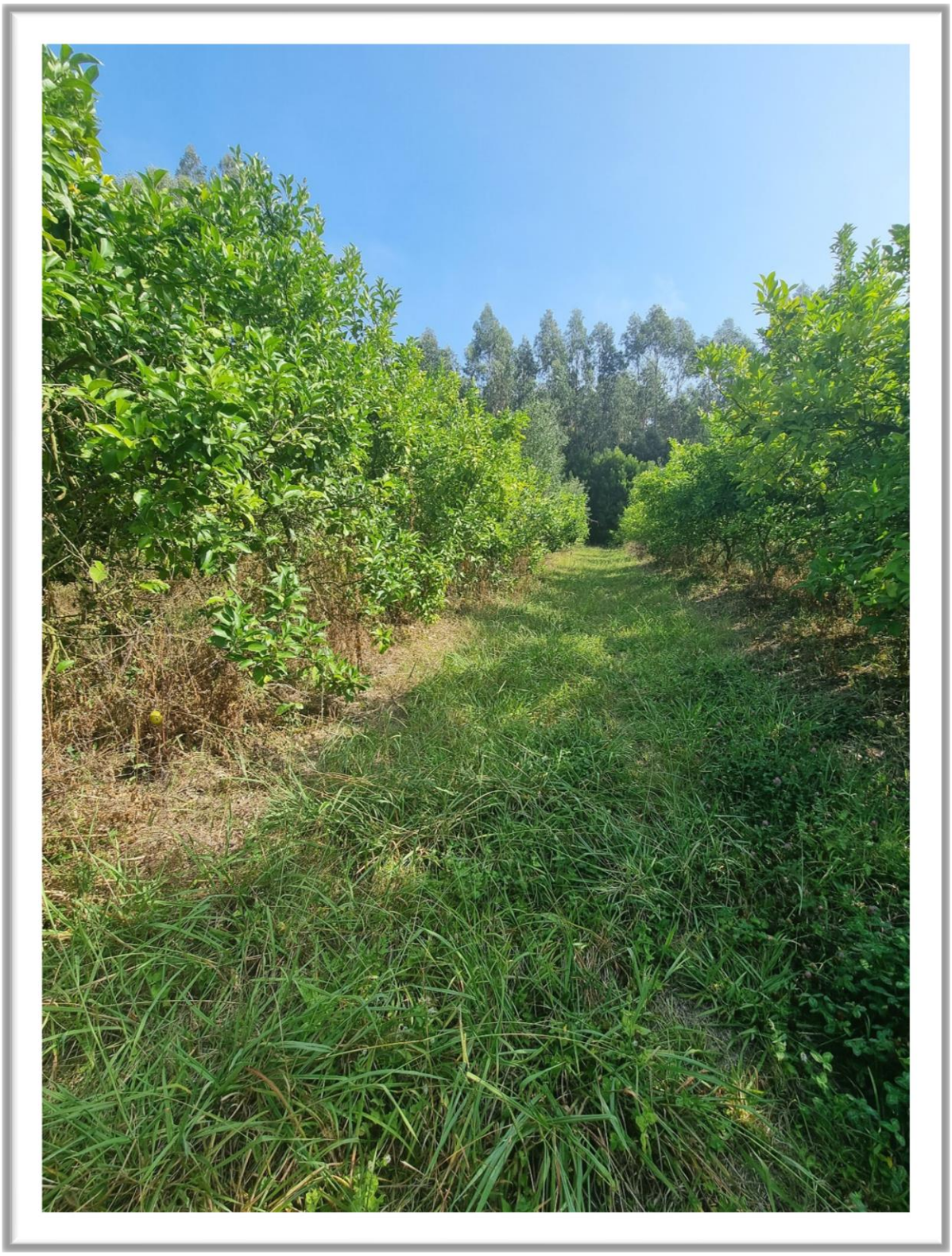
Chapter 1. Framework and objectives



1. Framework and objectives

The most important threat of the citrus industry worldwide is the huanglongbing (HLB), or citrus greening disease, which is responsible for billions of euros of losses and jobs every year. Two vectors of this disease are the African citrus Psyllid, *Trioza erytreae* (Del Guercio), and the Asian citrus Psyllid, *Diaphorina citri* (Kuwayama). The Mediterranean region is one of the largest citrus producers worldwide. HLB has not been reported yet in the region, although the vector *T. erytreae* is already present in mainland Portugal and Spain. Fortunately, *T. erytreae* did not reach the major citrus production areas of both countries yet. However, research on the spread ability of *T. erytreae* and knowledge on the efficiency of its natural enemies is still scarce. Accordingly, the specific objectives of this dissertation are:

1. To assess the effect of the application of kaolin clay on the landing, spread, settlement, and probing behavior of *T. erytreae* on lemon plants in the field and laboratory, respectively;

2. To study the potential pest suppression of selected naturally occurring predators.

Chapter 2. Introduction

2.1. Huanglongbing (HLB) or Citrus greening disease

A wide range of diseases caused by bacteria, fungi, virus, nematodes, oomycetes, and viroids attack citrus (da Graça et al., 2016). But, the disease that is most concerned on a global scale is Huanglongbing (HLB), also known as citrus greening disease, or in China, as yellow bud disease (Figure 1) (Bové, 2006; Alquezar et al., 2021). HLB is one of the most complicated citrus diseases, with relations between the pathogen, vector, hosts, and the environment in its broader definition (climate, soils, plant nutrition, presence of other pathogenic pests, etc.). Furthermore, it is associated with a long latent period, incapacity thus far to culture the causal organism, and the lack of known sources of natural resistance, making it a major challenge for researchers, regulatory agencies, and the citrus industry (Cocuzza et al., 2017).

Figure 1. A: Citrus fruit infected with HLB. B: Citrus leaves with HLB (fount: Unsplash. www.unsplash.com).

The five major citrus production areas are Asia, the Mediterranean basin, North America, South America, and Africa (Figure 2). Mediterranean basin, Australia, and New Zealand are the only HLB-free regions. Until now, there is no cure for this disease. Although the history of HLB began more than a century ago, only in the last decade has the disease reached very worrying proportions in the production of citrus in the western hemisphere (Gottwald, 2010; Cocuzza et al., 2017). Over time, HLB weakens the citrus tree until fruit production and quality are finally minimal, making its production impracticable due to low yield. In the last 15 years, HLB and accounted for substantial economic losses in Asia, Africa, and the American continent (Gottwald, 2010).

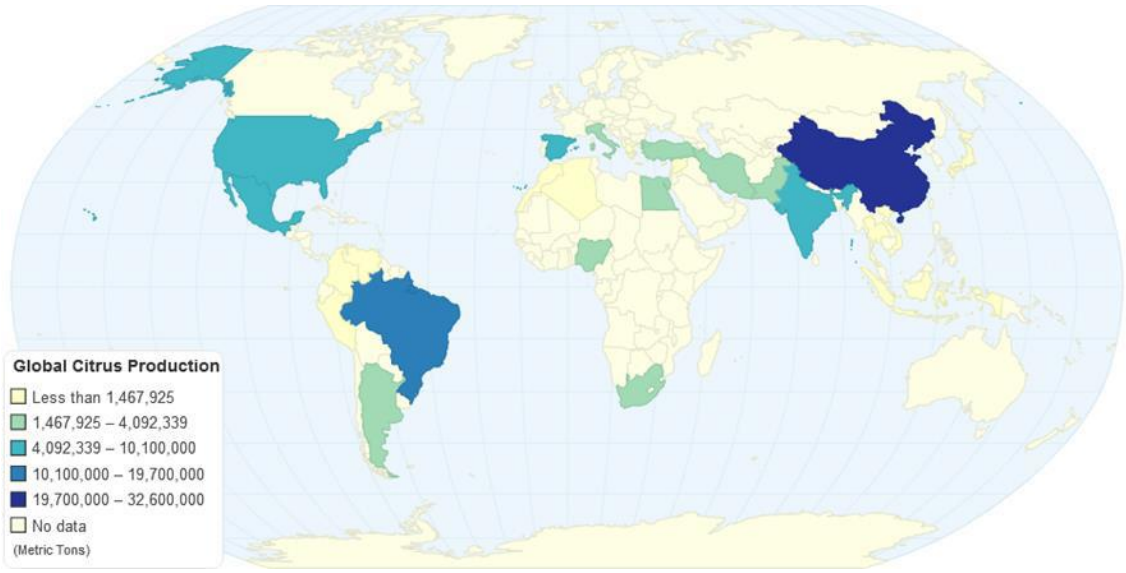


Figure 2. The citrus-producing countries around the world (FAO, 2015).

HLB is related to three species of the genus *Candidatus*: *Candidatus* Liberibacter asiaticus (CLas), occurring in Asia and the Americas, *Candidatus* Liberibacter africanus (CLaf), documented in Africa, and *Candidatus* Liberibacter americanus (CLam) in Brazil (Bové, 2006). After Bové (2006), additional four subspecies of CLaf have also been recognized: *Candidatus* Liberibacter africanus subsp. capensis (CLafC), *Candidatus* Liberibacter africanus subsp. clausenae (CLafCl), *Candidatus* Liberibacter africanus subsp. zanthoxyli (CLafZ) and *Candidatus* Liberibacter africanus subsp. vepridis (CLafV) (Roberts et al., 2015) (Figure 3).

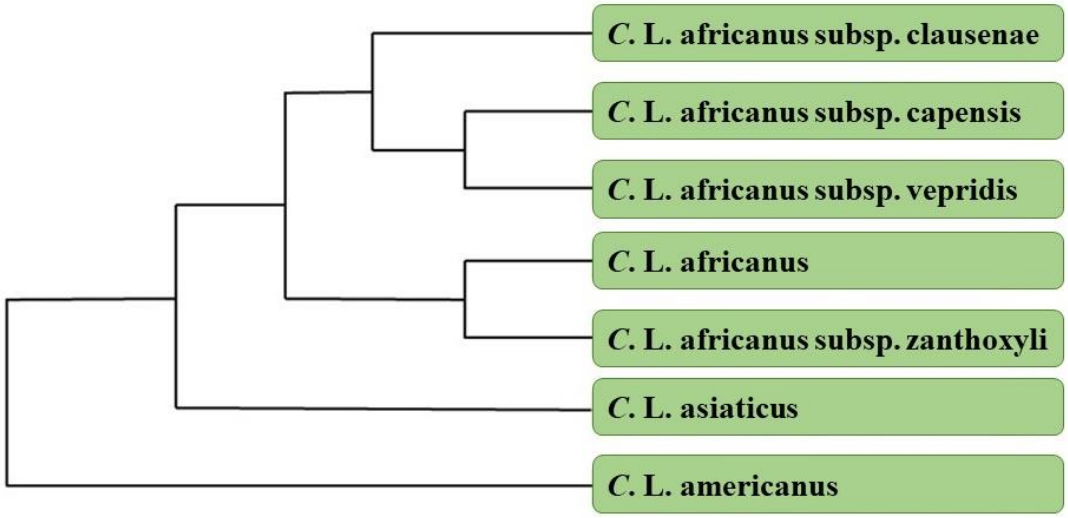


Figure 3. Phylogeny of *Candidatus* Liberibacter spp. associated with Huanglongbing of citrus and other Rutaceae based on rplJ sequences (redrawn from Roberts et al., 2015).

All *Candidatus* Liberibacter spp. are Gram-negative alpha (a)-proteobacteria (family *Rhizobiacea*). Two species of citrus psyllids, *Diaphorina citri* (Asian citrus psyllid) and *Trioza erytreae* (African citrus psyllid) transmit the bacteria (Bové, 2006) (Figure 4).

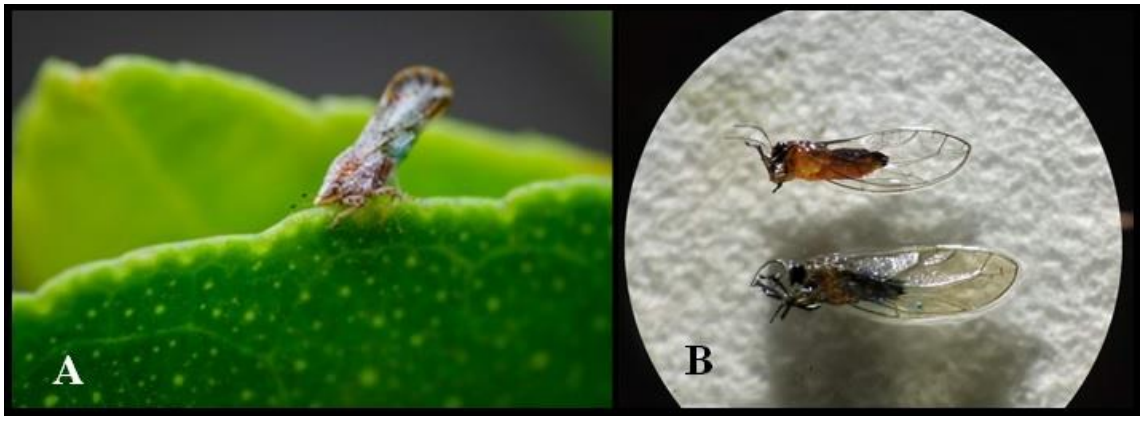


Figure 4. A: Adult of *Diaphorina citri* (fount: Unsplash. www.unsplash.com). B: Male and female adults of *Trioza erytreae.*

The African citrus psyllid transmits cLaf occurring in cool and moist regions in highlands (mostly above 900 m), with temperatures below 30 ºC (optimum 22–25 °C). Its vector, *T. erytreae* is sensitive to temperatures above 32 ºC (Da Graca & Kortsen, 2004). The Asian form, CLas, is transmitted by the Asian citrus psyllid, is more tolerant to heat and can withstand temperatures of 30–35 °C, so that the optimal temperature range for *D. citri* is 25–28 °C (Da Graca & Kortsen, 2004). This work focuses on the African citrus psyllid, *T. erytreae.*

2.2. African citrus psyllid *T. erytreae*

One of the vectors of HLB, the African citrus psyllid *T. erytreae* (Del Guercio, 1908) (Hemiptera: Triozidae), recently reached in mainland Europe, being firstly reported at Madeira Island (Portugal), where it was found in 1994 (Carvalho and Aguiar, 1997) and the Canary Islands (Spain) in 2002 (González-Hernández, 2003). *Trioza erytreae* apparently remained confined to these islands until it was reported in August 2014 in Vilanova de Arousa (northwestern mainland Spain (Pérez-Otero et al., 2015). Shortly after, *T. erytreae* was found in two other Corunha province locations and six Pontevedra

locations (Galicia, mainland Spain). In 2015, *T. erytreae* was found in mainland Portugal in the district of Porto (Pérez-Otero et al., 2015). Since its introduction, the expansion of *T. erytreae* could be facilitated by the tradition of the local population having citrus plants with ornamental purposes. For example, the lemon tree blooms several times a year, thus allowing lemons in the garden throughout the year; however, the fresh green leaves are immediately exploited by *T. erytreae* for feeding and breeding. The population of this vector of HLB constantly spread to southern areas of Portugal (Figure 5) and northern areas of Spain, threatening the whole citrus industry of the Mediterranean basin (Benhadi-Marín et al., 2020).

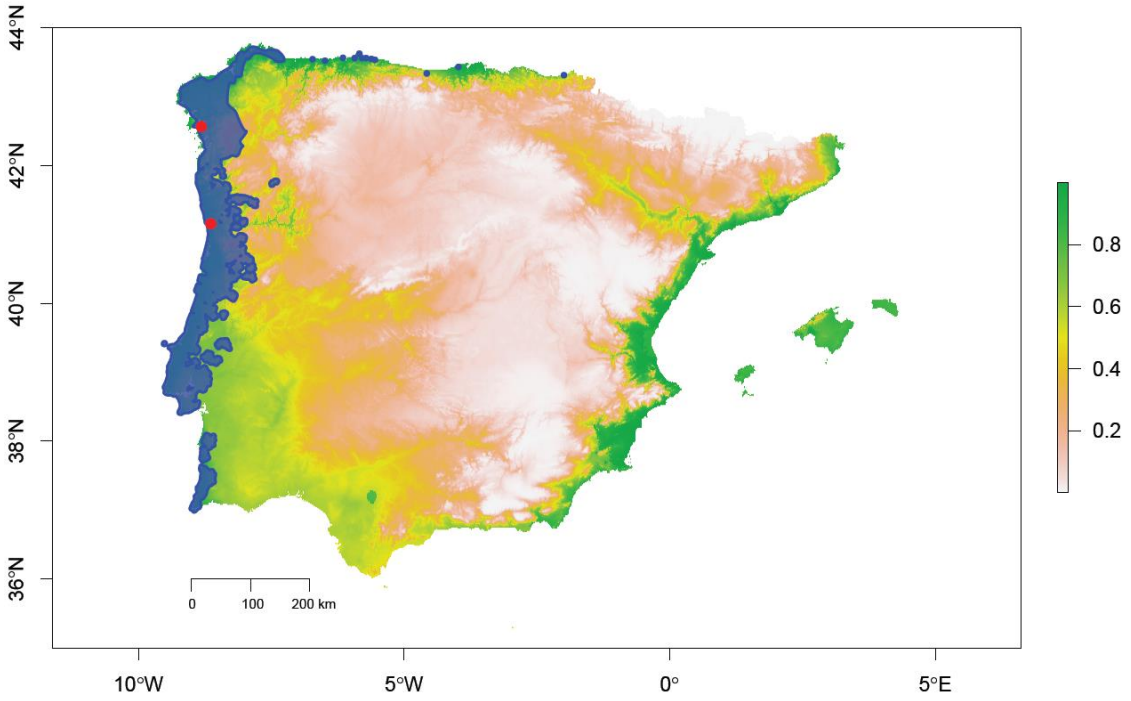


Figure 5. Spread of *Trioza erytreae* throughout the Iberian Peninsula in October 2021. Red dots are the entry points of *Trioza erytreae* in Pontevedra (Spain) and Porto (Portugal), respectively. Blue areas and dots represent areas of occurrence of *Trioza erytreae*. Greenish areas correspond to climatic suitability (p) of citrus plants.

2.2.1. Taxonomy, morphology, and geographical distribution of *T. erytreae*

Triozidae is one of the largest families of Psylloidea, encompassing almost 1000 species distributed in nearly 70 genera (Cocuzza et al., 2017). Its members are easily identifiable in the adult stage by the structure and venation of the forewings, lacking both

a costal break and the pterostigma, and having the vein R+M+Cu, which branches into its component veins at approximately the same point. The fifth instar nymphs are characterized by the presence of differently shaped wax producing sectasetae forming a complete marginal fringe in the head, wing pads, and abdomen.

Trioza erytreae is most likely native to Southeastern Africa. The species was initially described in 1918 from samples of *Citrus limon* collected in Eritrea. Presently, the species is mainly distributed throughout the Afrotropic ecozone, including the sub-Saharan Africa and the islands of St. Helena, Mauritius, Réunion and Madagascar, and Saudi Arabia and Yemen (Cocuzza et al., 2017) (Figure 6)

Figure 6. Global geographical distribution of *Trioza erytreae*. Red dots represent presence. Adapted from EPPO (2021).

The ideal conditions for *T. erytreae* are a cool and humid climate, although populations can also develop in hot and dry climates or in climates characterized by high temperatures and frequent rainfall. The altitudinal range of the species is 100 to 1300 m (Cocuzza et al., 2017).

The eggs of *T. erytreae* (0.3 mm long) are smooth with a sharp end and a short posterior stalk, corresponding to the insertion point in the vegetable tissues to aid in leaf fixation and serve to maintain moisture. The color of the eggs varies according to maturity, yellow to dark orange, and dark orange in the final phase (Cocuzza et al., 2017).

The post-embryonic development encompasses five nymph instars o stages, of which the fifth presents relevant diagnostic characteristics. During the five instars, *T. erytreae* is ventrally downward, slightly elongated, with visible marginal waxy white filaments or with another color going from pale yellow after hatching to olive green or dark grey in a final stage, with a pair of dark patches occurring in some populations dorsally on the abdomen, the characteristic galls on an infested citrus leaf are made by the nymphs (Figure 7A). The first four stages have on average 0.25-0.41, 0.44-0.56, 0.63-0.75 and 0.94-1.13 mm in length, respectively (Moran & Blowers, 1967). The fifth and final phase is 1.38-1.66 mm long and 0.87-1.12 mm wide, without dorsal sectasetae, but 25-30, 98-112, 6-9, and 74-85 truncated tubular sectasetae forming dense marginal fringes on each side of the head, bow cushions, rear wing, and abdomen cushions, respectively (Moran & Blowers, 1967).

Adult males and females are about 4 mm in length (Figure 7B), although females are more robust than males (Moran & Blowers, 1967). Usually, after emerging as adults, they have a green lettuce color, and over time they darken until dark brown after completing sclerotization (five days approximately). The head is dark, up to black. *T. erytreae* has a characteristic feeding pattern that causes the abdomen to rise with the body about 35 degrees high. The head has well-developed and elongated gene cones. Forewings are hyaline, with clearly visible dark veins. Legs are brown hind. The sexes are easily distinguished by the shape of the abdomen and the structure of the genitalia. The male genitalia has two projections in the form of a wrench. The female genitalia has an elongated shape.

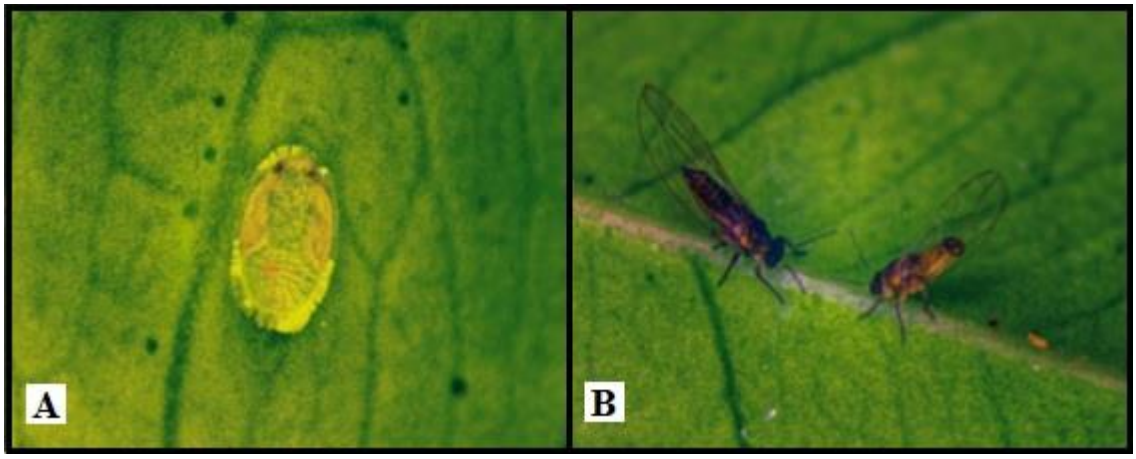


Figure 7. A: *Trioza erytreae* nymph, B: *Trioza erytreae* adults female at right male at left.

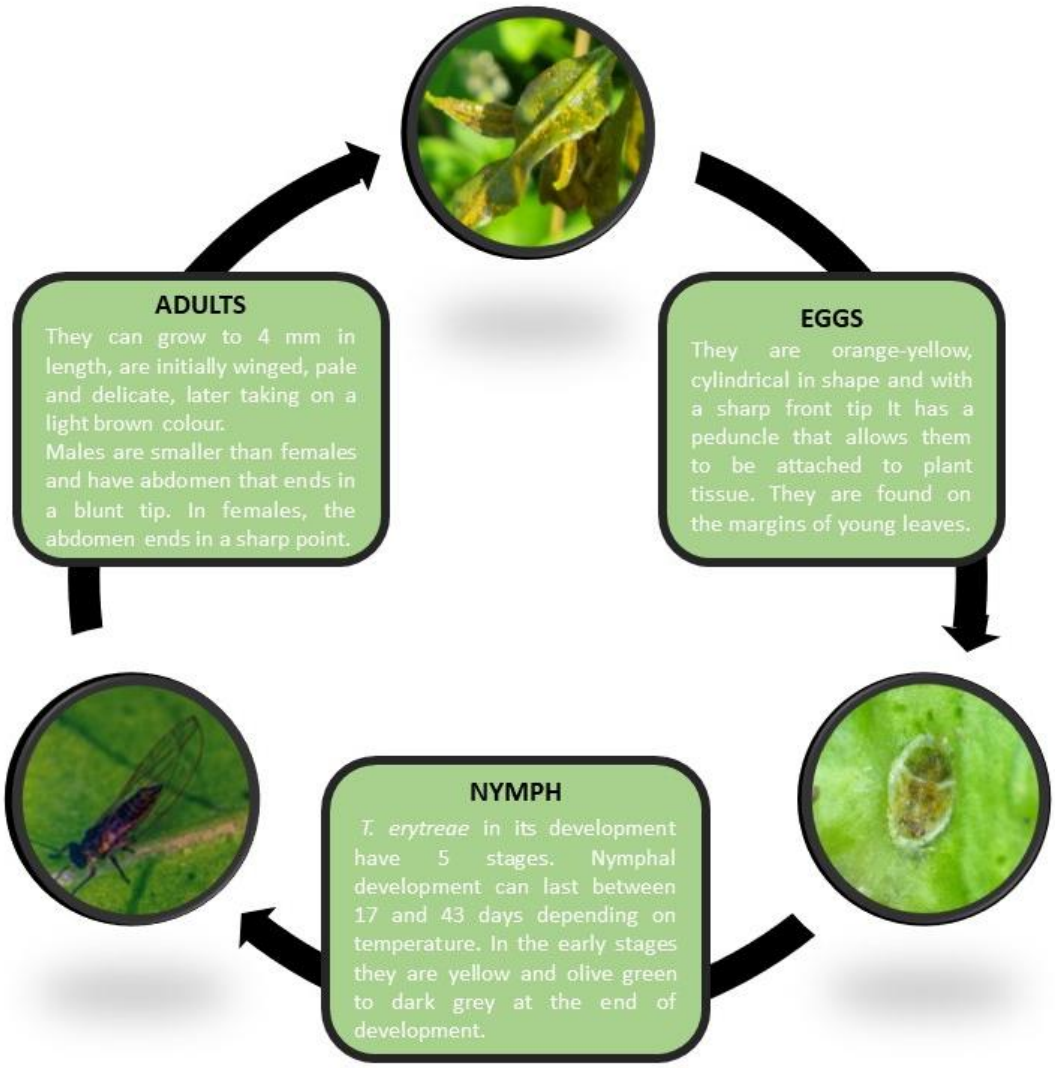


Figure 8. Life cycle of *Trioza erytreae*.

2.2.2. Invasion and subsequent spread of *T. erytreae* in Europe: the case of the Iberian Peninsula

Citrus production in the Iberian Peninsula occupies an area of 328626 ha, of which 307560 ha correspond to mainland Spain (MERCASA, 2020) and 21482 ha to mainland Portugal (INE, 2020). These crop areas result in a production of 6137 and 398 thousand tons in Spain and Portugal, respectively. In both countries, the most representative crops are oranges and tangerine varieties (mandarins, clementine's, and satsumas) followed by lemons (FAOSTAT, 2020).

In recent years, the area for citrus production in Spain decreased; however, it remains the most important fruit crop in the country.

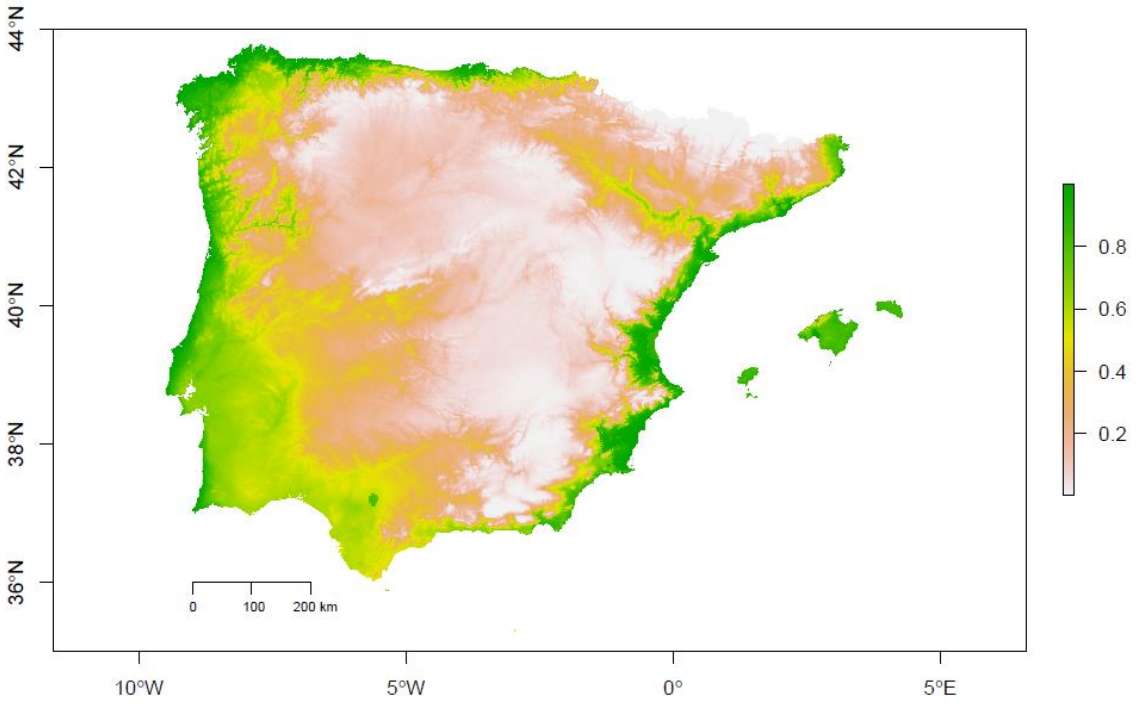


Figure 9. Climatic suitability for *Citrus* cultivation throughout the Iberian Peninsula. Reproduced from Benhadi-Marín et al. (2020) with permission of authors. Greenish areas correspond to the best habitat suitability.

In 2019 there were 307560 hectares dedicated to *Citrus* cultivation (Figure 9), 1% more than in 2020. The production areas correspond to the east and south-west coastal areas of the peninsula, namely in Valencia (60%), Andalusia (25%), Murcia (10%), and Catalunya (3%) (MERCASA, 2020). The orange tree still has the highest production area, 149909 hectares in 2019 of sweet orange and 275 hectares of bitter orange, followed by mandarins with 104498 ha in 2019 and lemons with 49,074 ha. In addition to these crops, grapefruit and other citrus fruits are also grown in Spain, with an area of 2422 ha and 1382 ha respectively (MERCASA, 2020). The citrus agroecosystem in Portugal encompassed 21482 ha in 2020, of which 17221 ha corresponded to orange trees, 2483 ha to tangerines, and 1644 ha to lemons (INE, 2020). Most of the citrus production takes place in southernmost Portugal in the Algarve region.

2.2.3. Management of *T. erytreae*

There are two main sustainable pest management strategies, integrated pest management (IPM) and biological control. Due to the potentially harmful effects derived

from classical pest control for ecosystems and human health, legislation is becoming more stringent regarding the chemical control of pests. Moreover, the producers, processors, and final users are increasingly claiming for chemical-free products. Accordingly, pest control strategies such as IPM and biological control can be used as an alternative to classical control.

Insect pest control with chemical pesticides (Figure 10) resulted in several problems, including resistance to insecticides, secondary pest outbreaks usually controlled by natural enemies, safety risks to humans and animals, contamination of groundwater tables, and biodiversity decline, among others environmental concerns. These problems and the need for new alternatives to the predominance and dependence on conventional insecticides have stimulated a growing interest in integrated pest management. As a result, sustainable agriculture in the 21st century will increasingly depend on new methods to make environmentally friendly pest management possible and reduce human contact with chemical pesticides as much as possible (Lacey et al., 2001).

Figure 10. Examples of chemical pesticides application methods. A: by tractor, B: by backpack mechanical sprayer and C: by Plane (fount: Unsplash. www.unsplash.com).

Since the late 1950s, IPM has evolved quite slowly in Europe. In the early 1980s, a significant development took place in countries such as Germany, Switzerland, and certain regions of Italy (Cavaco et al., 2006). In Portugal, although the initiatives to develop the IPM practices were initiated in the 1980s and continued in the following decade. Nevertheless, until the 1990s the implementation of IPM in the field was minimal. Although, in 1994s, with the implementation of agri-environmental measures, the practice of IPM increased (Cavaco et al., 2006).

In Portugal, some works were developed to identify natural enemies of lemon pests that potentially exert natural biological control. In 2006, Silva et al. (2006) developed works in lemon orchards in the region of Mafra (Portugal) to measure the impact of natural parasitoids on citrus leaf miners. Eight parasitoids species were identified, and the parasitism rates were estimated. Silva et al. (2006) concluded that the diversity of parasitoid species varied according to the phenology of the host and the lemon plant. Nevertheless, other organisms also have an important role as natural enemies of lemon pests. Predators (Figure 11), like spiders, chrysopids, and ladybirds, are among the most important groups, but fungi and bacteria also have importance (Lacey et al., 2001).

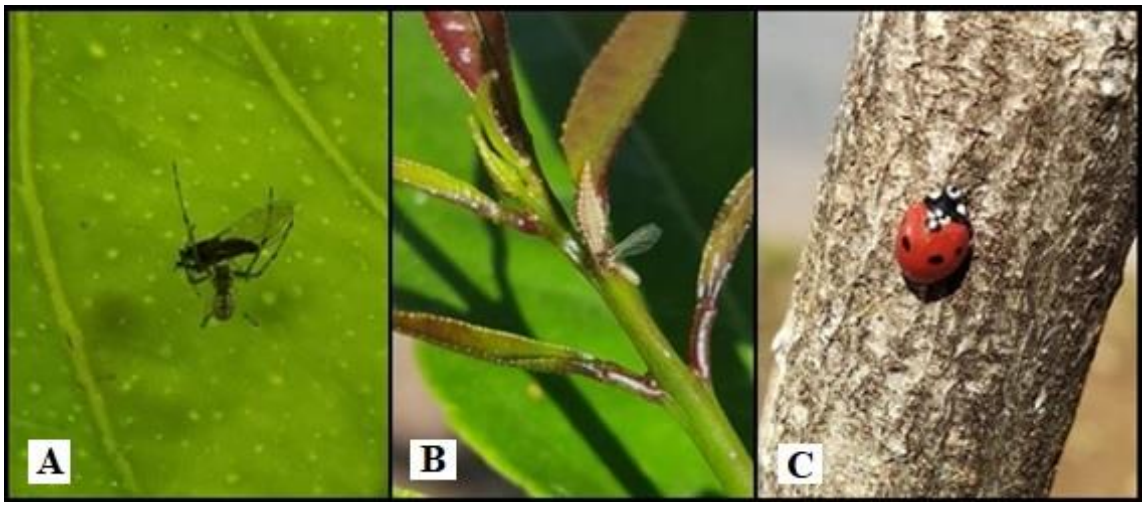


Figure 11. Examples of predators. A: Spider eating an adult of *Trioza erytreae.* B: Larvae of Chrysopidae eating an adult of *Trioza erytreae*. C: An adult for of Coccinellidae.

Biological control aims to reduce the pest population to an economically acceptable level avoiding harmful effects to the ecosystem such as ecotoxicological effects. Consequently, the assemblage of predators can exert pressure on pest populations, for example, impeding significant pest outbreaks. However, their efficiency level depends on careful monitoring and the use of selective insecticides for other pests. *Tamarixia dryi* (Waterston, 1922) (Hymenoptera: Eulophidae) was been released in Spain and was having great results parasitizing *T. erytreae* in Canária Islands, due to its effectiveness, are currently doing mass releases of this wasp (Figure 12) on citrus orchards (Hernández-Suárez et al., 2020).

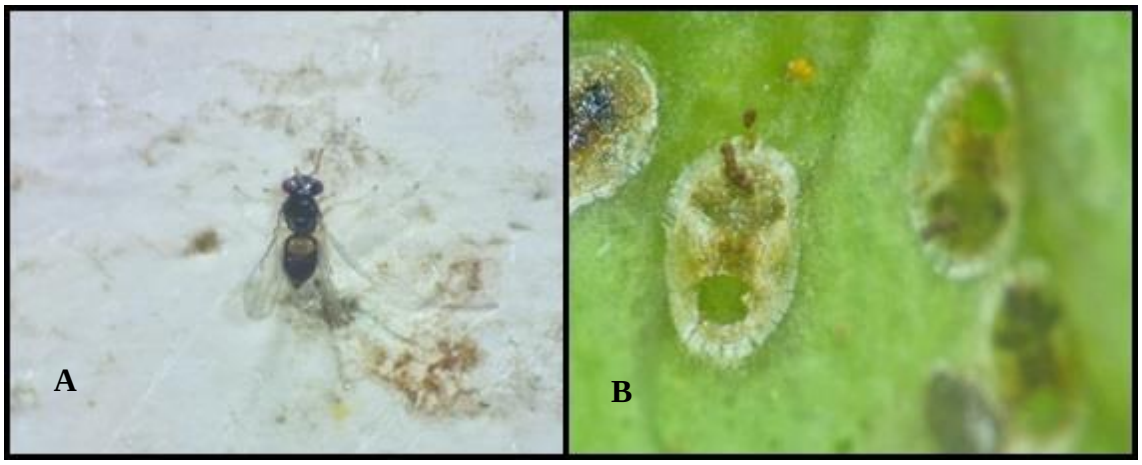


Figure 12. A: Adult of *Tamarixia dryi* B: Nymph of *Trioza erytreae* parasitized by *Tamarixia dryi.*

2.3. References

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Chapter 3. Effect of kaolin on the landing, settlement, probing, and feeding behaviour of *T. erytreae*

3.1. Introduction

Due to rising health concerns, the concept of Good Agricultural Practices has emerged toward sustainable agriculture. Accordingly, reducing the large-scale use of chemical pesticides results in the bioaccumulation of toxic compounds in the crop, derived products, and the whole ecosystem. The primary goal of sustainable agriculture relies on improving agriculture to meet the increasing demand for food production while preserving natural resources for future generations, thus aligning with the number two of the Sustainable Development Goals (2030 Agenda) (United Nations, 2021). In this context, several modern tools have been developed and implemented, such as the case of particle film technologies (PFT).

Particle film technologies are aqueous formulations made from chemically inert clay or mineral particles formulated explicitly for coating. These clays have been widely used to reduce the damage caused by insects, diseases, and solar injury (Sharma et al., 2015). For example, kaolin is an aluminosilicate mineral clay, chemically inert over a wide pH range. White kaolin has been used as protection against pests in a wide range of crops such as apple, almond, cabbage, cotton, olive, pear, pecan, tomatoes, walnut, and wine grape (Puterka et al., 2000; Knight et al., 2001; Cottrell et al., 2002; Friedrich et al., 2003, Showler, 2003; Daniel et al., 2005; Rosati et al., 2006; Kahn & Damicone, 2008; Glenn et al., 2010; Pascual et al., 2010; Alavo & Abagli, 2011; Silva & Ramalho, 2012).

In general, a pool of studies reported kaolin as an effective substance in reducing the population of pests encompassing Coleoptera, Diptera, Hemiptera, and Lepidoptera (Showler, 2003; Mazor & Erez, 2004; Daniel et al., 2005; Sackett et al., 2007; Pascual et al., 2010; Alavo & Abagli, 2011; Silva & Ramalho, 2012; Martinou et al., 2014) acting as a repellent or barrier for pests and affecting the recognition and attractiveness of host plants (Showler, 2002). Regarding the citrus agroecosystem, the effect of kaolin on the biology, behavior, and *D. citri* populations has been tested on *Citrus sinensis* L. (sweet orange) in Florida (USA) (Hall et al., 2007).

The other vector of HLB, *T. erytreae*, is currently present in the Iberian Peninsula, which raises a threat to the citrus industry. Currently, the geographic distribution of this triozid ranges from the Basque Country (Spain) to the Algarve region (Portugal). However, no studies have been yet conducted on the African Citrus Psyllid, *T. erytreae*, regarding the potential effect of kaolin as a tool to manage the pest.

Trioza erytreae is a sap-sucking insect, and its feeding behavior can be monitored using electrical penetration graphs (EPG) (Tjallingii, 1978). This method consists of an electric circuit formed by the insect, the plant to be tested, and the pot soil. Once the insect inset the stylet into the plant tissue, release a signal, and a resistor device detects a wave, and the sign is registered as a graph. This system discriminates when the insect is probing on each tissue type (Tjallingi, 1978). EPGs have been widely used to investigate aphids' feeding behavior and food preference (e.g. van Helden & Tjallingii, 1993; Garzo et al., 2002), and psyllids such as *Bactericera cockerelli* (Sandanayaka et al., 2019). The feeding preference of *T. erytreae* between lemon and orange plants was successfully assessed by Benhadi-Marín et al. (2021). Notwithstanding, the feeding behavior response of *T. erytreae* to plants sprayed with kaolin is still unknown.

The objectives of this chapter were: (1) to study the effect of the application of kaolin clay on the landing and settlement of adult individuals of *T. erytreae* in selected lemon orchards using open field mark-recapture experiments, and (2) to assess the impact of kaolin particle films on the probing and feeding behaviour of *T. erytreae* on lemon plants.

3.2. Material and methods

3.2.1. Open field mark-recapture assays

3.2.1.1 Study Area

Two lemon orchards, *Citrus limon* (L.) (variety "Lunario") were selected, one located in Vale (Alvarelhos, Trofa, Porto district, 41°18'03.3"N 8°36'28.0"W) (Figure 13), and the other located in Ribela (Vila Nova de Famalicão, Braga district, 41°26'39.7"N 8°30'15.7"W) (Figure 14). The first one is a newly planted orchard with one-year-old plants, and the other the plants had two years old. Both study areas are currently located within the demarcated area of *T. erytreae* (DGAV, 2016–2021). According to the Köppen & Geiger classification, the region's climate is of the Csb type (Peel et al., 2007), characterized by warm summers of Mediterranean climate. The prevailing wind is Northeast-East and West-East in Vale and Ribela, respectively.

Figure 13. Panorâmic view of the experimental orchard located in Vale, Trofa (2020).

Figure 14. Panorâmic view of the experimental orchard located in Ribela, Vila Nova de Famalicao (2021).

3.2.1.2. Description of orchards and experimental design

The orchard in Vale encompasses an area of 0.7 ha and presents flat topography. Corn crops and open grassland fields surround it. The lemon plants were distributed in 14 rows of the plantation. Plants were interspaced by 3 m with a distance of 4 m between rows. Lemon plants were one year old at the moment of the release. A total of 90 plants (70 cm in height in average) were initially selected, and a series of three rows of quadrats were defined throughout the orchard according to the total number of plants per row of plantation. The schematic design is present in Figure 15.

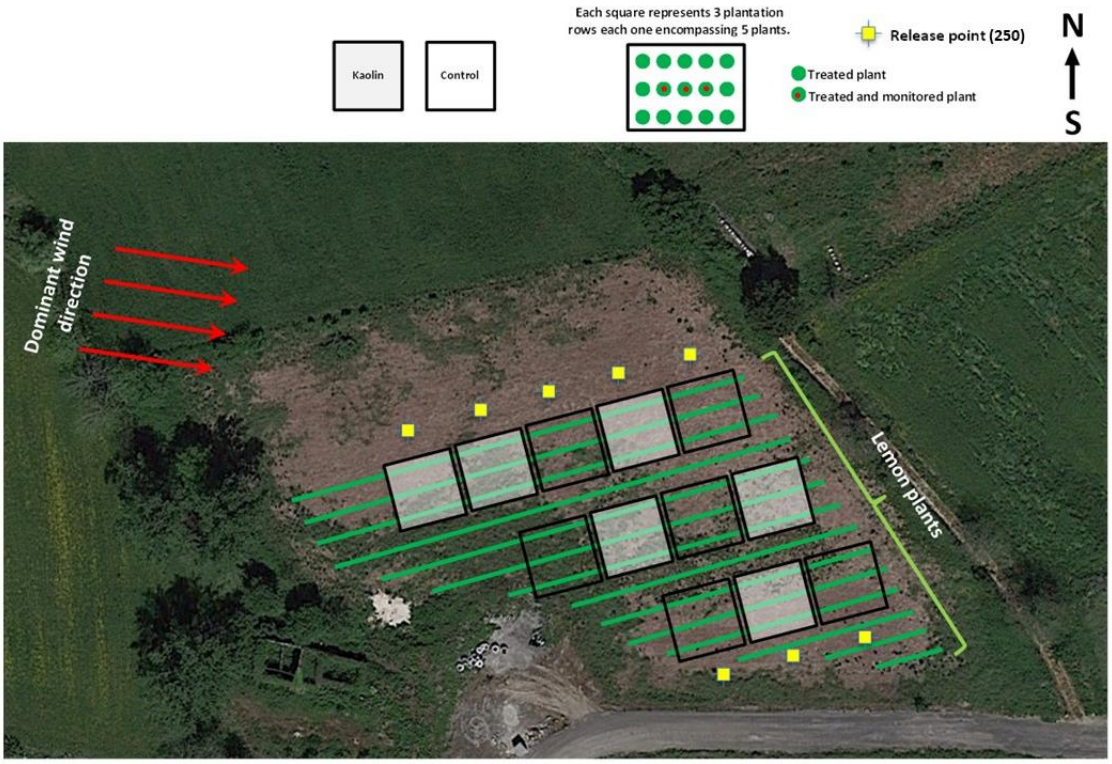

Figure 15. Schematic experimental design of experiment developed in Vale (Trofa)

Each row of quadrats were separated by one row of the plantation. Each quadrat consisted of three rows of five plants following the plantation rows. Each row of quadrats encompassed a total of five, four, and three quadrats, respectively. In turn, the three rows of quadrats encompassed three, two, and one quadrat treated with kaolin, whereas the remaining quadrats were used as control (i.e. two control quadrats per row). The control quadrats were sprayed only with water. The central row of plants within each quadrat was

initially planned to be inspected for marked individuals; however, due to the low number of recaptured individuals after the first time-lapse (i.e. 8 h after the release, see below), all the lemon plants within the orchard were inspected. The release of *T. erytreae* was made in 10th September 2020.

In Ribela, the selected plot has an area of 0.4 ha and is part of a broader extension of citrus cultivation with a total area of 3.4 ha. All the plants within the area were two years old at the moment of the assay. The selected orchard has a flat topography, and plants are interspaced by 2 m arranged in a total of 16 rows with a between-row distance of 6 m. A total of 60 plants (1.20 m in height in average) were selected throughout the central area of the orchard, encompassing a grid of 4 rows with 15 plants each (Figure 16).

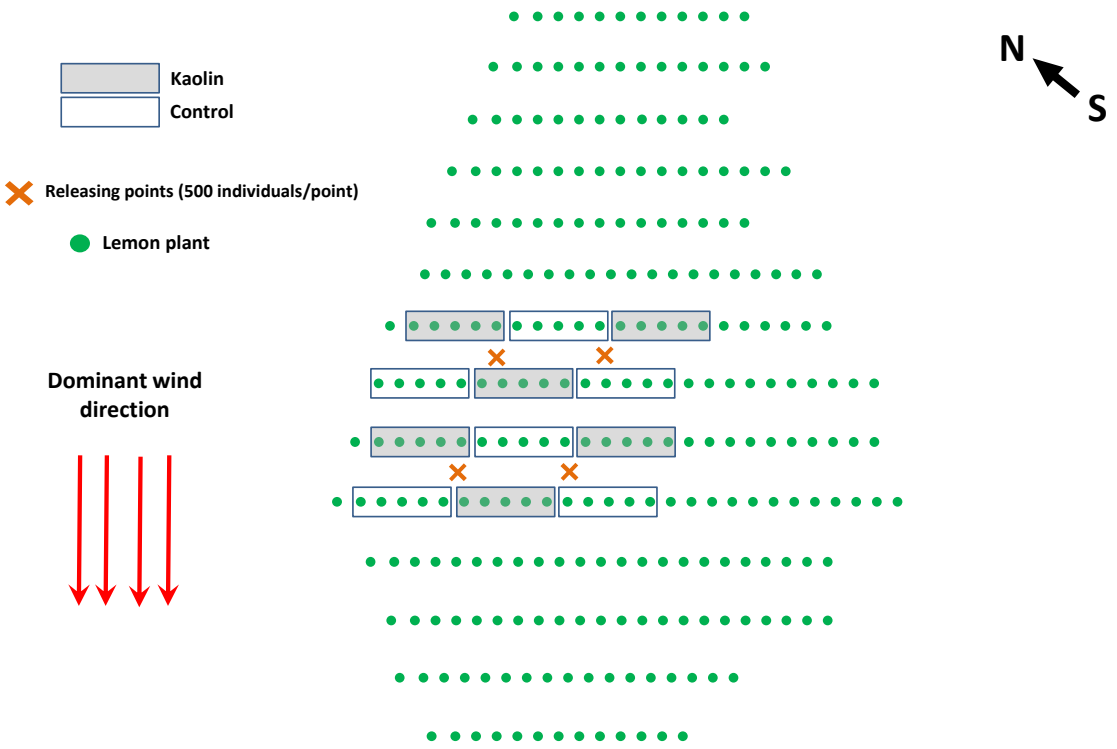

Figure 16. Schematic experimental design of experiment developed in Ribela (Vila Nova de Famalicão).

In each row, three blocks were delimited of five plants each, and the blocks were alternatively defined as control or treated with kaolin (i.e. a total of six treated and six control blocks). Thus, the first and third row encompassed two control and one treated

blocks, whereas the second and fourth row encompassed two control and one treated blocks.

Two assays were conducted in Ribela to assess the effect of the phenological state of the plants on the number of recaptured individuals. A first release was made on 8th July 2021 when the lemon plants were mostly sprouting (plants with sprouts hereafter), and a second release on 22th July 2021 when the sprouts were already mature (plants without sprouts hereafter).

3.2.1.3. Application of commercial kaolin

Kaolin (Surround® WP, Tessenderlo Kerley Inc., USA) was applied using a standard 20 L backpack mechanical sprayer (Figure 17) firstly at 3% and subsequently at 2%. In order to ensure the kaolin effect, the reflected UVA light was measured at solar zenith in five plants (20 cm above the canopy) using an ALMEMO 25904S radiometer (Ahlborn GmbH, Holzkirchen, Germany) for UV spectrum so that the experiments were conducted with an UVA light reflection of 4.17 ± 0.07 (mean ± SE) in treated plants and 0.78 ± 0.03 (mean ± SE). In Vale, the first application of kaolin (3%) was made in 18th July 2020. Since then, subsequent applications of kaolin (2%) were made in a biweekly basis until 9th September 2020. In each application, the kaolin was sprayed on the 15 plants of the corresponding quadrat. In Ribela, a total of three applications of kaolin were done in 2th, 8th, and 22th July 2021, respectively.

Figure 17. Kaolin application using a standard 20 L backpack mechanical sprayer.

3.2.1.4. Origin, preparation, and method of release of adult individuals of *T. erytreae*

For the release assay developed in Vale, in 2020, the adults of *T. erytreae* were obtained in the experimental farm "Quinta do Crasto" of the Agrarian Campus of Vairão (University of Porto) (41°19'38.5"N 8°40'32.4"W). A total of 2000 individuals were captured on lemon and tangerine trees and placed in eight Falcon tubes (50 mL) (250 individuals per tube) (Figure 18).

Figure 18. Falcon tube (50 mL) containing 250 individuals of *Trioza erytreae* .

Once the individuals were distributed among the tubes, a small amount of fluorescent blue dust (Day-Glo Color Corp. Cleveland, OH, USA) was deposited into each Falcon tube (3 mg per tube). Each tube was then carefully rotated by hand for 30 seconds to sprinkle the marker on the insects. Marking insects with fluorescent dust is a commonly used method to assess dispersal in the field through release-recapture experiments (e.g. Lago et al., 2021) (Figure 19).

Figure 19. Individuals of the *Trioza erytreae* marked in the Falcon tubes ready to be released.

Once marked, the individuals were placed in eight lemon seedlings (i.e. 250 individuals per plant) for 48 hours to feed. The release day (9th September 2020), the seedlings were transported to the field and placed at ground level at eight release points (i.e. 250 marked individuals per point). The release points were located eight meters away from the border rows of the plantation and distributed along a single row so that the northernmost and southernmost row encompassed five and three release points, respectively. At the moment of release (10:00 am), each seedling was cut and fixed horizontally on a plastic support (12.5 cm in height) to avoid the soil heat and allow the marked individuals to fly.

The individuals used for the first release conducted in Ribela in 2021 were obtained from an established rearing culture in a greenhouse at the experimental farm of the Agrarian Campus of Vairão, and from a commercial orchard located near Vairão (Caracoi, 41°18'24.57"N 8°38'36.96"W) for the second release. In this case, the individuals were distributed among 4 Falcon tubes (500 mL) with 500 individuals per

tube (i.e. a total of 2000 individuals each release), marked as depicted before, and immediately transported in the Falcon tubes to the field to be released. Four releasing points were established within the grid of plants, two points between the first and second row of plantation and two points between the third and fourth row of plantation coinciding with the change of blocks. At each releasing point, a fine wood stick (1 m in height, 7 mm in diameter) (Figure 20) was vertically nailed to the ground, and a Falcon tube was fixed vertically at the tip of the stick with the opening facing up. The four tubes were opened at 10:00 am, allowing individuals to fly.

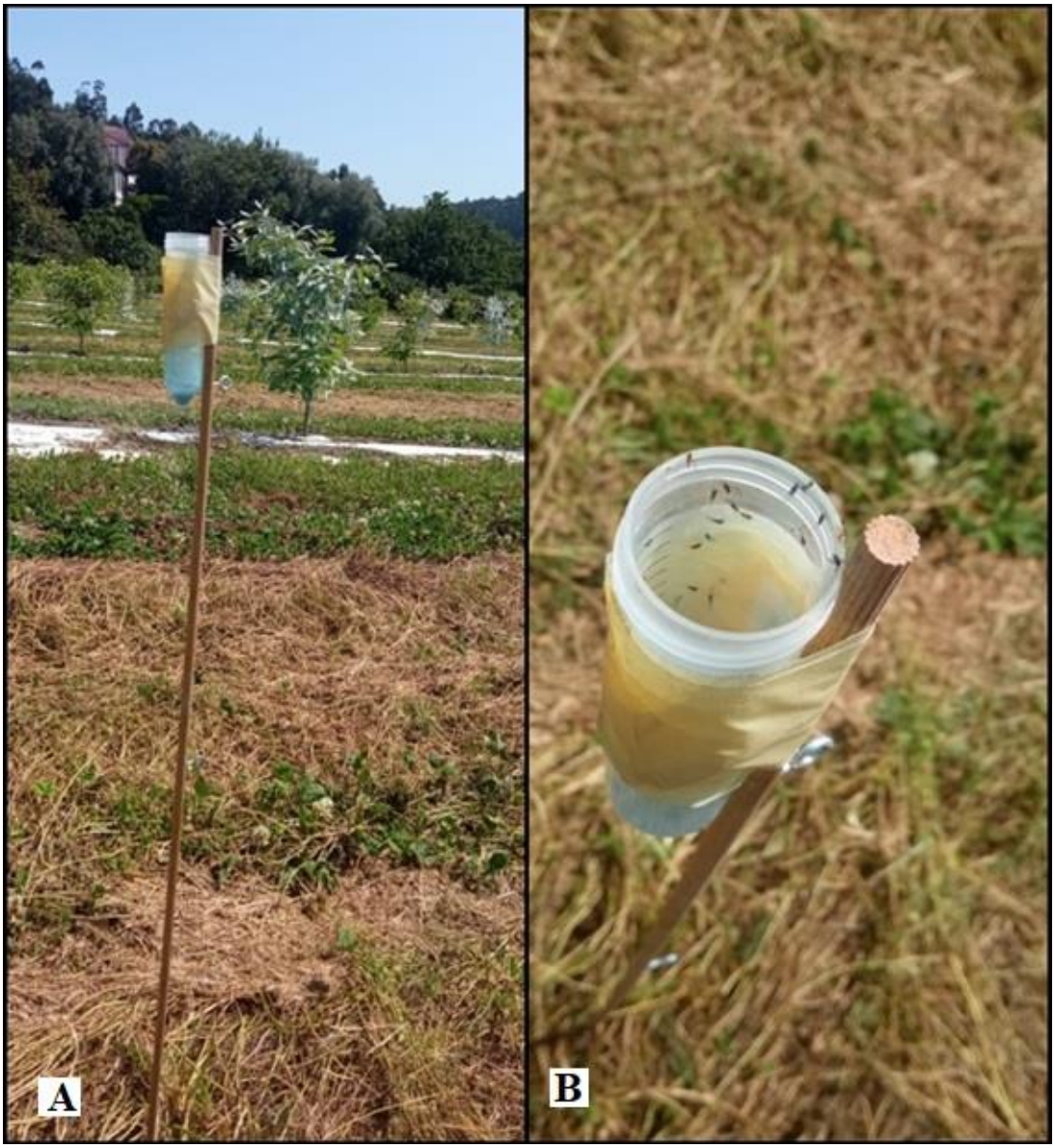

Figure 20. A: Picture of one releasing point of marked *Trioza erytreae* . B: Marked *Trioza erytreae* ready to leave the falcon tube.

The evaluation of the recapture of individuals was made by direct visual search of marked insects on the plants. In Vale, the observations were made at 8, 24, 72, and 120 h after the release, whereas in Ribela, observations occurred at 6, 12, 24, and 48 h after release. Despite the released individuals were marked with color, the presence of symptoms (i.e. galls, nymphs, and eggs) and naturally occurring adults of *T. erytreae* were monitored before each release assay.

3.2.2. Effect of kaolin on the probing and feeding behavior of *T. erytreae*

The electrical penetration graph (EPG) technique was used to evaluate the probing and feeding behavior of *T. erytreae* on lemon (cv. Eureka) seedlings (at the 4-leaf stage) previously sprayed with kaolin (Surround® 3%). Plants sprayed with water were used as an untreated control. The methodology used followed Bonani et al (2010). Plants were sprayed with kaolin 24 h before starting experiments.

The EPG recordings were obtained using a DC monitor, GIGA-8d model (EPG Systems, the Netherlands) (Tjallingii, 1978), adjusted to 100× gain. Young adult psyllids were maintained in a refrigerator at 4 ºC for 30 s to reduce their activity and immediately immobilized by a vacuum device under a dissection microscope. Then a gold wire (2 cm long and 18.5 μm of diameter; Sigmund Cohn, Mount Vernon, NY, USA), previously connected to a copper electrode (3 cm long and 1 mm in diameter), was attached to the psyllid prothorax using water-based silver conductive paint glue (EPG Systems, the Netherlands). Then the insects were connected to the EPG device and placed on the abaxial surface of the last expanded lemon leaf on control and treated kaolin plants. Another electrode (copper, 10 cm long × 2 mm wide) was inserted into the pot substrate containing the lemon seedling. The EPG recordings lasted 8 h and were conducted inside a Faraday cage (100 × 110 × 90 cm) to avoid electrical noise. Only those EPG recordings with an optimal signal quality were considered for analysis. The EPG recordings were acquired and analyzed using Stylet+ software for Windows (EPG Systems, the Netherlands). A total of 15 and 14 EPGs recordings (8 h) (replicates) on control lemon plants (treated with water) and plants treated with kaolin were recorded and analyzed, respectively. Recordings in which psyllids exhibited aberrant behavior (total duration of

non-probing (Np) >75%) were discarded. A single psyllid and plant combination were used for each replicate. Each plant was used only once for each insect.

The EPG waveforms associated with specific stylet tip positions and insect activities were characterized according to previous EPG studies with the psyllids *D. citri* (Bonani et al., 2019) and *Bactericera trigonica* (Hodkinson, 1981) (Hemiptera: Triozidae) (Antolinez et al., 2017). The EPG waveforms considered were "np" representing non-probing behavior (stylets are not inserted into the leaf tissue), and waveform "C" representing the stylet intracellular penetration into the leaf tissue. Three waveforms related to phloem activity were recorded: waveform "D" which represents phloem contact, waveform "E1," which represents salivation into phloem sieve elements, and waveform "E2", which is associated with passive phloem sap ingestion. Furthermore, waveform "G" was considered since it represents active xylem sap ingestion.

To analyse the impact of kaolin treatment on probing and feeding behaviour of *T. erytreae*, a selected set of EPG non-sequential variables (Table 1) were calculated: the number of waveform events for each insect (the number of times that a waveform occurs for each insect), the total waveform duration for each insect (the sum of overall occurrences of a waveform for each insect) and the mean duration of waveform events for each insect (the total waveform duration divided by the number of waveform events for each insect). For the calculation of the mean duration of G, E1, and E2 the following criteria was assumed: (1) if there was only a single bout and it was truncated by the end of the recording, then the value of the truncated bout was included in the calculation of the mean, (2) if there were two bouts of the waveform and the final bout was truncated, this bout was included in the calculation of the mean if the value is greater in duration than the non-truncated bout. However, if the value of the truncated bout was less than the non-truncated bout, it was excluded. Finally, (3) if there were three or more bouts of the waveform and the final bout was truncated, and the duration of the truncated bout was greater than the median of the non-truncated bouts, then the truncated value was included in the calculation of the mean bout duration. However, if it was less than the median of the non-truncated bouts, then the truncated bout was deleted. The following sequential variables was calculated: time to first probe from start of EPG, time from the start of EPG to first sustained E2 (> 10 min), and time to the 1st E2 from the start of 1st probe. If a particular waveform event did not exist, then the value was considered missing data.

3.3. Data analysis

3.3.1. Open field mark-recapture assays

The number (N) of recaptured individuals (Figure 21) was counted in each plant at each time-lapse after release. Due to the low number of recaptures in Vale no further data analysis was conducted for this orchard. For Ribela, a Welch *t*-test for unequal variances was used to compare the number of recaptured individuals in plants with and without sprouts.

Figure 21. Example how a recaptured individual of *Trioza erytreae* looks like.

The effect of the application of kaolin on the landing and settlement of *T. erytreae* was assessed using generalized estimating equations (GEEs). This method is an extension of the generalized linear models (GLMs) used to analyze low replicated count data as an alternative to more complex generalized mixed models (Zuur et al., 2009; Pekár et al., 2018). GEEs can be used to estimate the within-subject correlation (ρ). If this correlation is found to be low, then GLMs can be used for modeling purposes regardless of replication (Zuur et al., 2009). An interchangeable correlation structure was assumed (i.e.,

a single correlation parameter, ρ). The six blocks were used as replicates (random term) whereas treatment (kaolin vs. control), and time-lapse were used as explanatory variables with Poisson-like distribution and logarithmic link function. Since the correlation parameter was low (ρ = 0.003), GLMs with negative binomial distribution to deal with potentially overdispersed data were used hereafter so that two models were developed, one for plants with sprouts, and one for plants without sprouts. For each model a *post-hoc* analysis was conducted to compare the different time lapses. Finally, for each assay and time lapse, Welch *t*-tests for unequal variances were used to compare the number of recaptured individuals between treated and control plants. All the analyses were conducted in R (R Core Team, 2018).

3.3.2. Effect of kaolin on the probing and feeding behaviour of *T. erytreae*

EPG variables were processed for each given insect with the help of an Excel Data Workbook CSIC-UAL elaborated by Garzo E. (Institute of Agricultural Sciences, CSIC, Spain) and Alvarez A.J. (University of Almería, Spain). The data obtained for each of the EPG variables and the two treatments (kaolin-treated and untreated plants) were analyzed using R (R Core Team, 2020) at a 0.05 significance level. First, a series of transformations of the data obtained were carried out using the Ln (× + 1) transformation. Then, a Shapiro-Wilk test was used to check for normality and a Bartlett test to check for the homoscedasticity of the data. The results of these tests indicated that normality or homoscedasticity of the variances was not fulfilled. Therefore, a non-parametric Mann-Whitney *U*-test was used to compare each of the EPG variables between the two treatments. The proportion of individuals that produced a specific waveform type (PPW) was compared between treatments using a χ^2-test followed by the Fisher's Exact test if the expected values were lower than 5. This analysis was done using Statview 4.0 software (Abacus Concepts, Berkeley, CA, USA).

3.4. Results

3.4.1. Open field mark-recapture assays

A total of 40 marked individuals were recaptured at 23 control plants at the end of the assay in Vale in 2020. After each time-lapse (8, 24, 72, and 120 h), 14, 14, 9, and 3 individuals were recaptured in eight, nine, eight, and two control plants, respectively. No individuals were found on treated plants (Figure 22).

In 2021 in Ribela, 192 individuals were recaptured during the first assay (plants with sprouts), whereas 41 individuals were recaptured during the subsequent assay (plants without sprouts). The mean number of recaptured individuals per plant was significantly higher ($t = 4.37$, $P < 0.001$) in the assay with plants with sprouts compared with plants without sprouts (Figure 23). During the assay with sprouts, 92, 50, 30, and 20 individuals were recaptured after 6, 12, 24, and 48 h, respectively. On the other hand, 12, 12, 12, and 5 individuals were recaptured after 6, 12, 24, and 48 h, respectively, during the assay without sprouts.

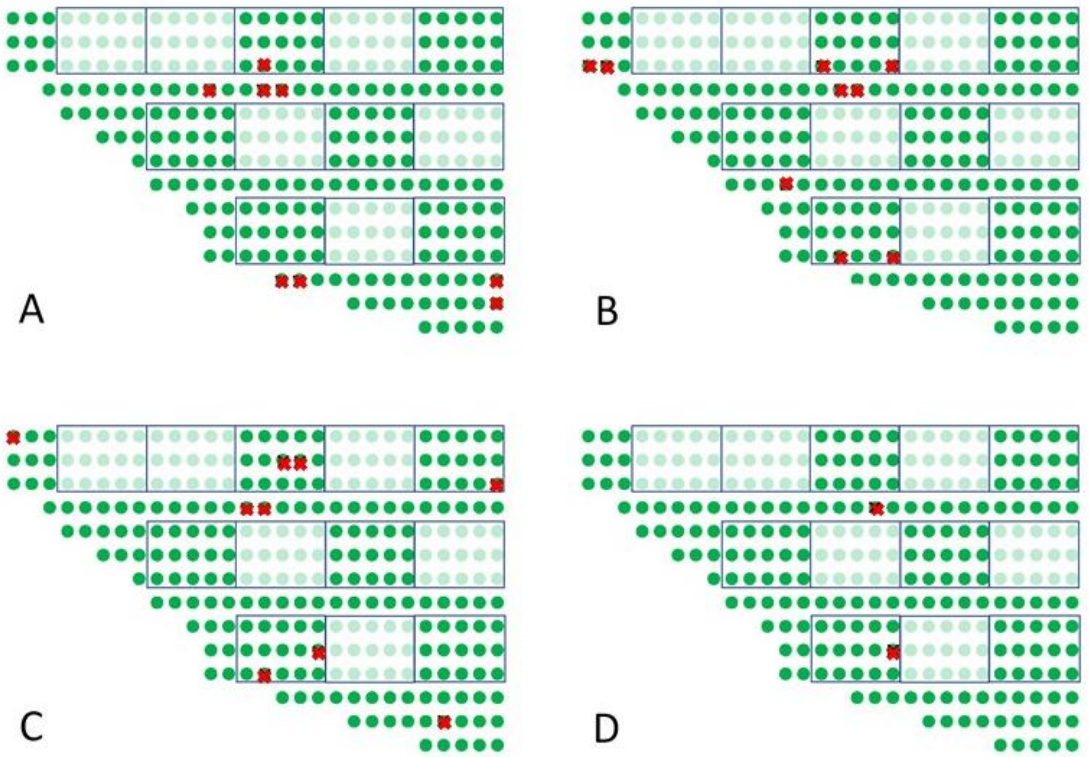

Figure 22. Position where *Trioza erytreae* individuals were recaptured within the orchard 8 h (A), 24 h (B), 72 h (C), and 120 h (D) after release. Squares represent the initially selected quadrats of plants. Green dots represent untreated (control) plants. Light green dots represent sprayed (kaolin) plants. Crosses indicate the plants were at least one individual was found at each time lapse after release.

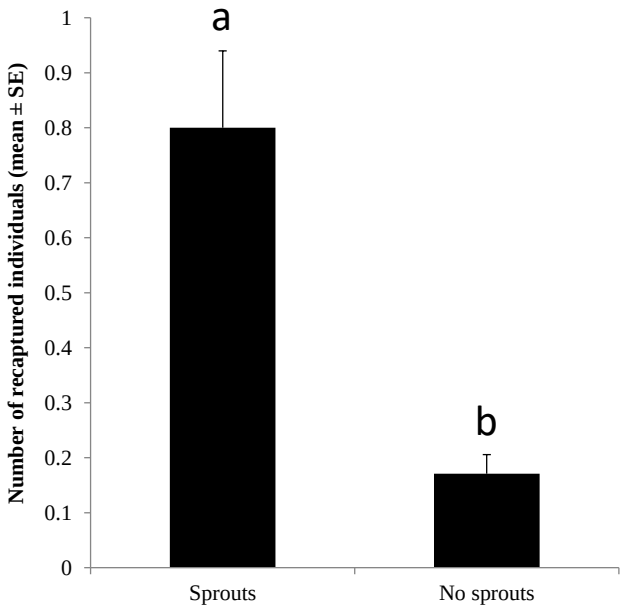

Figure 23. Marked individuals of *Trioza erytreae* recaptured on lemon plants at different phenological states (plants with and without sprouts) in open field in 2021 (Ribela, Portugal). Different letters above bars indicate a significantly different number of individuals recaptured for each time-lapse ($\alpha = 0.001$).

The number of individuals recaptured in control plants was significantly higher compared with treated plants at each time lapse in the experiment with plants with sprouts ($\chi2 = 113.0$, df = 1, $P < 0.001$) (Figure 24 A). The same pattern was found for plants without sprouts except after 48 h after release ($\chi2 = 47.6$, df = 1, $P < 0.001$) (Figure 24 B). The number of recaptured individuals significantly decreased with time during the assay with sprouts ($\chi2 = 23.0$, df = 3, $P < 0.001$) (Figure 24 A), whereas no significant differences were found during the assay without sprouts ($\chi2 = 2.9$, df = 3, $P < 0.41$) (Figure 24 B).

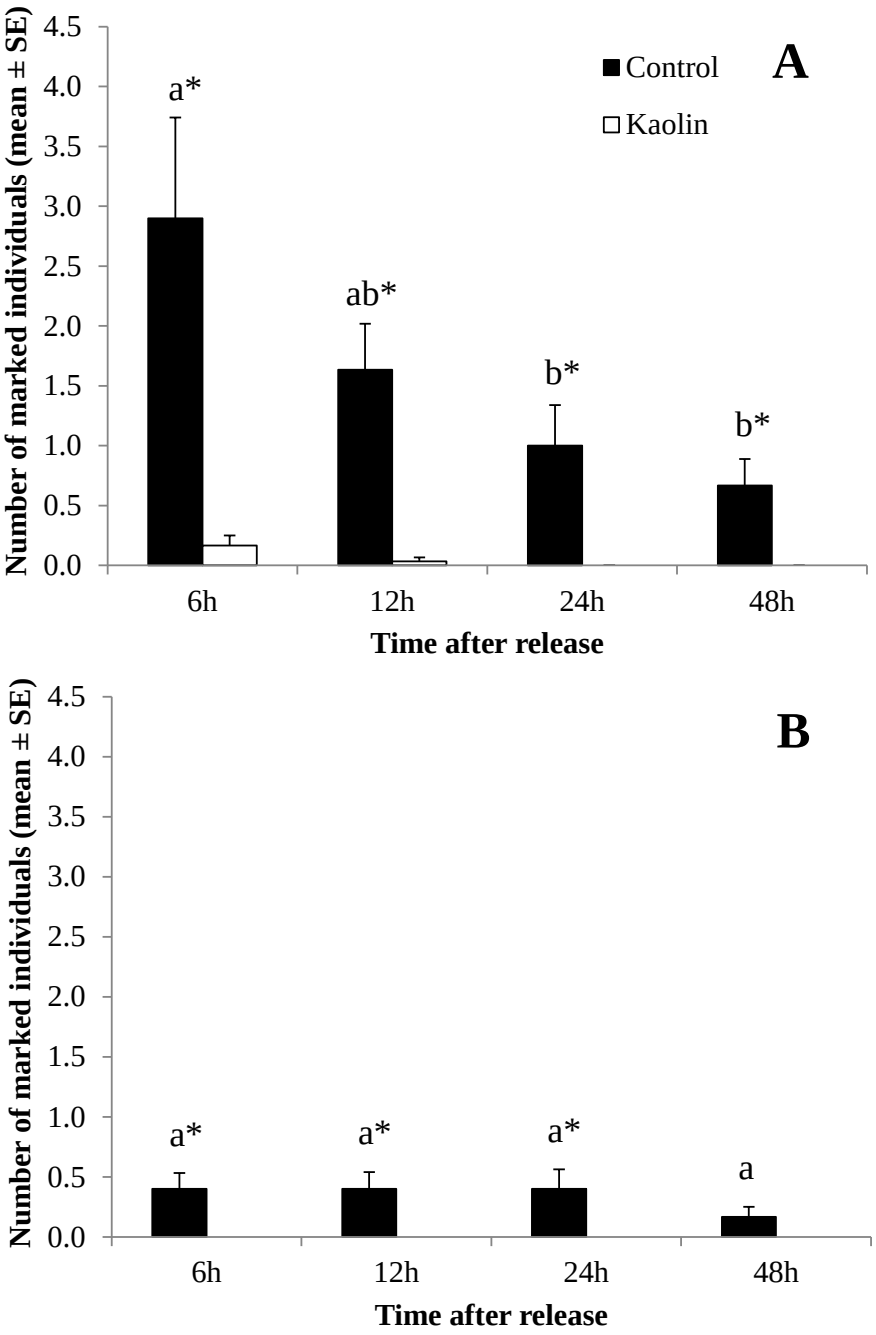

Figure 24. Number of marked individuals of *Trioza erytreae* recaptured after release on lemon plants at different time-lapses and phenological states in open field (Ribela, Portugal). A: plants with sprouts (8th July 2021); B: plants without sprouts (22nd July 2021) (no individuals were found in treated plants in this assay). Different letters above bars indicate a significantly different number of individuals recaptured for each time-lapse ($\alpha = 0.001$). Asterisks above bars indicate significant differences between treatments for each time-lapse ($\alpha = 0.05$).

3.4.2. Effect of kaolin on the probing and feeding behavior of *T. erytreae*

The effect of kaolin on the probing and feeding behavior of *T. erytreae* on lemon plants is shown in figures 25 & 26 and table 1. The results indicated that *T. erytreae* prefers to feed on the untreated control than on kaolin-treated plants. The percentage of individuals that showed phloem-related activities (waveforms "D"," E1" & "E2") over time increased on control plants compared to kaolin-treated plants (Figure 25). The main difference observed between treatments was the percentage of time spent in phloem and xylem ingestion. Psyllids spent longer periods in xylem ingestion and less time in phloem ingestion when exposed to kaolin-treated plants (Figure 25). A higher percentage of individuals remained in phloem ingestion (E2) on the control than on the kaolin-treated plants, mainly after the 3rd hour of exposure (Figure 26: control 6.67%-26.67%; kaolin 7.14%-14.29%). However, a high percentage of individuals exposed to kaolin-treated plants spent their time in xylem ingestion activities throughout the recording period compared to the control plants (Figure 26: control 0%-26.67%; kaolin 0%-50%).

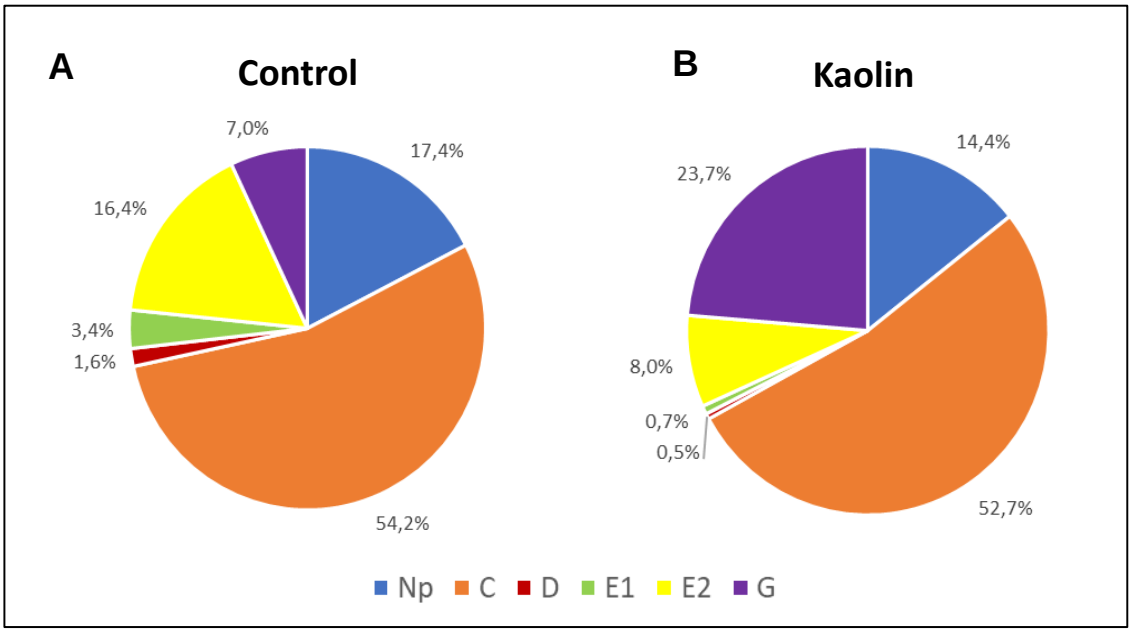

Figure 25. Percentage of the total duration of each waveform event (Waveform Np, Non-probe; Waveform C, Pathway; Waveform D, phloem contact; Waveform E1, phloem salivation; Waveform E2, phloem sap ingestion, and Waveform G, xylem sap ingestion) according to the EPG record of *Trioza erytreae* on untreated lemon plants and plants treated with kaolin.

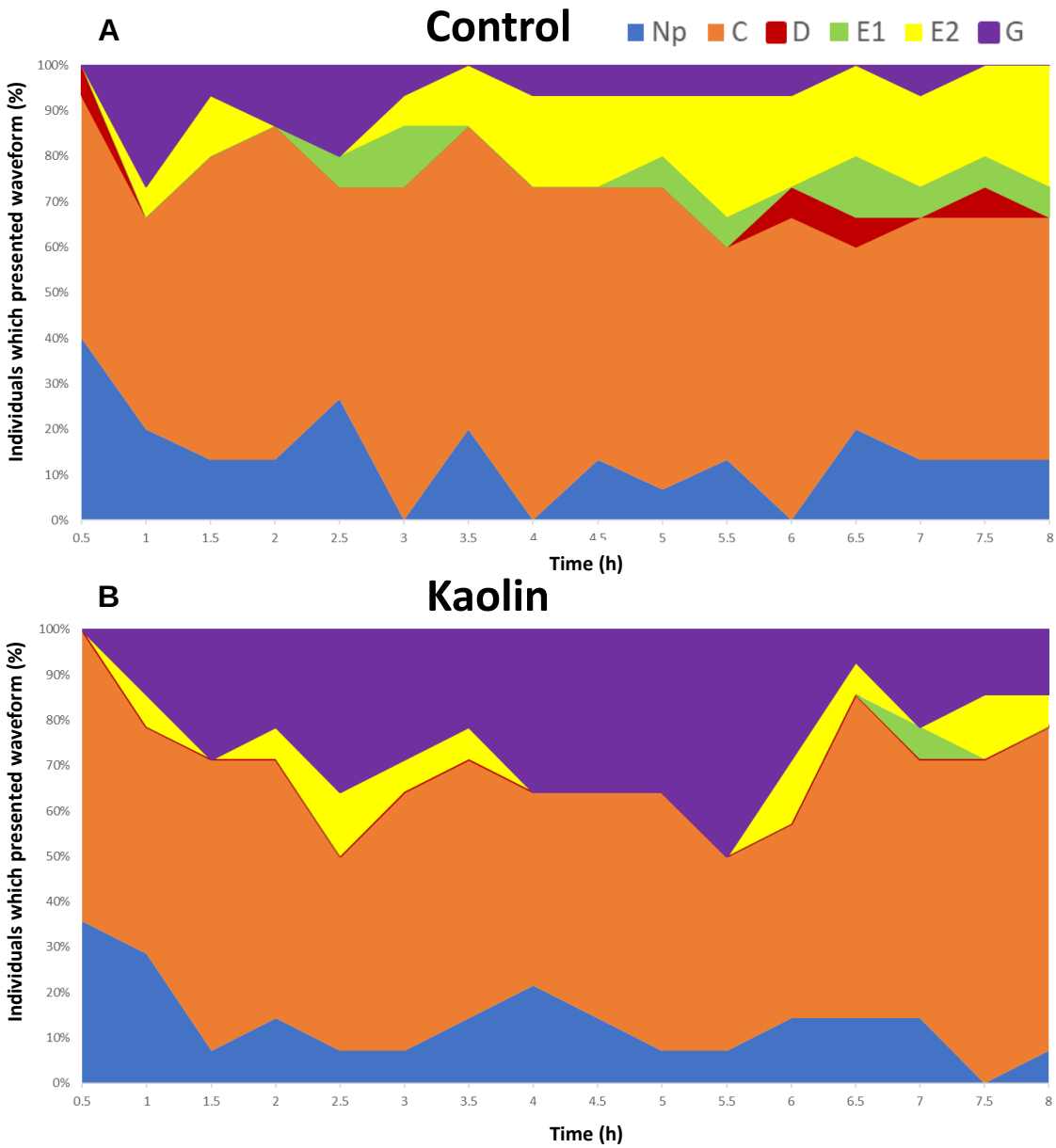

Figure 26. Percentage of individuals of *Trioza erytreae* exhibiting specific waveforms at 30-min intervals over a total recording time of 8 h on untreated lemon plants and plants treated with kaolin.

Thirty three EPG variables were considered for analysis of the probing and feeding behaviour of *T. erytreae* when exposed to kaolin treated plants (Table 1).

Table 1. EPG variables of *Trioza erytreae* exposed to untreated and treated kaolin lemon plants during eight hours of recording. PPW: proportion of individuals that produced a specific waveform type period on lemon plants. Time is expressed in minutes. Means within a row followed by different letters are significantly different. Np: Non-probe; C: Pathway; D: phloem contact; E1: phloem salivation; E2: phloem sap ingestion; G: xylem sap ingestion.

	Kaolin-treated plants		Control plants		P
Non-Sequencials Variables	**PPW**	**Mean ± SE**	**PPW**	**Mean ± SE**	
Np	14/14	4.93±0.84 a	15/15	11.87±1.68 b	0.003
Probe	14/14	4.86±0.83 a	15/15	11.73±1.63 b	0.002
C	14/14	9.86±1.54 a	15/15	22.73±2.34 b	<0.001
G	12/14	1.71± 0.35 a	10/15	1.00+/-0.22 a	0.172
D	8/14	3.50±1.37 a	15/15	10.33±1.89 b	0.003
E1	8/14	3.79±1.46 a	15/15	11.60±1.98 b	0.002
E2	7/14	2.50±0.94 a	14/15	6.67±1.17 b	0.007
Sustained E2>10 min	5/14	0.71±0.32 a	12/15	1.73±0.42 b	0.031
Total duration (min.)					
Np	14/14	68.90±19.24 a	15/15	83.67±10.87 a	0.070
Probe	14/14	411.10±19.24 a	15/15	396.33±10.87 a	0.070
C	14/14	253.00±21.00 a	15/15	260.16±14.14 a	0.747
G	12/14	113.61±23.82 a	10/15	33.70±7.41 b	0.006
D	8/14	2.33±0.88 a	15/15	7.61±1.54 b	0.004
E1	8/14	3.53±1.56 a	15/15	16.37±3.38 b	0.001
E2	7/14	38.64±18.66 a	14/15	78.50±20.03 b	0.023
Sustained E2 >10min	5/14	32.82±16.49 a	12/15	64.57±20.14 b	0.039
Mean duration (min.)					
Np	14/14	16.72±3.36 a	15/15	9.93+/-2.47 a	0.133
Probe	14/14	153.12±39.12 a	15/15	74.85±29.66 b	0.002
C	14/14	34.48±6.17 a	15/15	13.79±2.28 b	<0.001
G	12/14	85.32±18.85 a	10/15	36.25±5.68 b	0.017
E1	8/14	0.95±0.28 a	15/15	1.38±0.21 a	0.155
E2	7/14	12.25±4.42 a	14/15	24.00±13.85 a	0.971
sustained E2 >10min	5/14	42.38±15.29 a	12/15	40.83±15.52 a	0.646
Mean duration of E2 per phloem phase	7/14	10.22±4.35 a	14/15	12.24±12.24 a	1.000
Duration of the 1st E2 in the recording	7/14	2.64±1.24 a	14/15	22.59±13.36 b	0.018
Sequential variables					
Time to first probe from start of EPG	14/14	21.91±5.64 a	15/15	13.82±3.63 a	0.234
Time from start of EPG to first sustained E2 >10 min	5/14	386.65±43.21 a	12/15	283.33±35.47 b	0.049
Time to the 1st E2 from start of 1st probe	7/14	282.75±53.53 a	14/15	154.87±35.69 a	0.102
Indices (% of time on a given waveform)					
Percentage of probing spent in C	14/14	61.76±4.29 a	15/15	66.10±3.68 a	0.477
Percentage of probing spent in E1	8/14	0.804±0.35 a	15/15	4.098±0.86 b	<0.001
Percentage of probing spent in E2	7/14	8.39±3.98 a	14/15	19.02±4.75 b	0.016
Percentage of number of E2s that are sustained	5/14	21.80±6.57 a	12/15	31.63±7.54 a	0.524
Percentage of probing spent in G	12/14	28.52±5.76 a	10/15	8.84 ±1.94 b	<0.001

No significant differences were observed in the total duration of no-probes (Np) and probes between treatments, but the number and mean duration of probes and no-probes were significantly different. Psyllids exhibited significantly less number of non-probing and probing events on kaolin-treated plants than on control plants (Table 1). However, the mean duration of the probes was significantly longer on plants treated with kaolin (153.12 ± 39 min) than on control plants (74.85 ± 29.66 min) (U = 37, P = 0.002). Similarly, the mean duration of time that psyllids spent in intercellular stylet pathway (waveform C) was significantly longer on kaolin-treated plants (34.48±6.17 min) than on control plants (13.79 ± 2.28 min) (P < 0.001). The EPG results did not show evidence of delay in the initialization of the first probe when psyllids were placed on kaolin-treated plants. In both treatments, kaolin-treated and untreated plants, the psyllids started probing soon after the start of the recording and with no significant differences between treatments (time to first probe from start of EPG: 21.91 ± 5.64 and 13.82 ± 3.63 min, respectively, P = 0.234).

The number and total duration of the phloem activities ("D" "E1","E2" & "sE2") was clearly affected by the kaolin treatment. *T. erytreae* showed significantly less number and shorter duration of phloem-related events on kaolin-treated than on the untreated plants (Table 1). Also, the psyllid spent less time in phloem sap ingestion (duration of the 1st E2 in the recording & Percentage of probing spent in E2) on the kaolin-treated than on the control plants (Table 1). Furthermore, kaolin was able to delay sustained phloem sap ingestion by *T. erytreae* as shown by the time spent from start of EPG to first sustained phloem ingestion (E2 > 10 min), which was significantly longer on plants treated with kaolin (386.65 ± 43.21 min) than on the untreated control (283.33 ± 35.47 min) (P = 0.049).

However, no significant differences in the mean duration of phloem activities were found between kaolin-treated and control plants (Table 1). However, a significantly lower proportion of individuals were able to engage in phloem ingestion activities when exposed to kaolin. In fact, only half of the individuals (PPW = 7/14) exposed to kaolin-treated plants were able to ingest from the phloem while almost all were able to engage in phloem sap ingestion when exposed to untreated control plants (PPW = 14/15) (χ^2 = 6.807; P = 0.014). Moreover, only 5 out of 14 individuals exposed to kaolin-treated plants were able to show a sustained phloem sap ingestion compared to 12 out of 15 individuals on the untreated control (χ^2 = 5.854; P = 0.0252). Furthermore, no significant differences were found in the number of psyllids that showed xylem ingestion events between

treatments (Table 1: kaolin 1.71 ± 0.35; control 1.00 ± 0.22; P = 0.172) (PPW = 10/15 control; 12/14 kaolin; χ^2 = 1.435; P = 0.390). However, the total duration and mean duration of time that psyllids spent in xylem ingestion was significantly higher and more than twice than those exposed to the untreated control. Also, the percentage of probing time spent in xylem sap ingestion was significantly higher on kaolin than on control plants (kaolin 28.52 ± 5.76; control 8.84 ± 1.94; P < 0.001) (Table 1).

3.5. Discussion

In this work, the application of kaolin clay was found to significantly reduce the landing and settlement of *T. erytreae* in lemon plants in the field. In Vale, no individuals were found on treated plants; however, the total number of recaptured individuals was extremely low (2.00 %). In Ribela, 9.60 % of individuals were recaptured after the assay in plants with sprouts, whereas 2.05 % of individuals were recaptured in plants without sprouts. These results agree, in general, with Tomaseto et al. (2016) that found a significantly higher amount of fluorescent-marked individuals of the other vector of HLB, *D. citri*, in plants with the presence of young citrus leaves in São Paulo (Brazil). Also, Lewis-Rosenblum et al. (2015), using an *in situ* protein marking technique, found that the marked psyllids captured increased with the density of emerging young leaves of sweet orange in Florida (USA).

The difference between the number of individuals recaptured in plants with and without sprouts in Ribela can be explained in terms of *T. erytreae* bioecology. Females of *T. erytreae* show a strong preference for plants with sprouts since the oviposition is restricted to tender shoots (Cocuzza et al., 2017). In fact, Martini et al. (2016) found that young emerging leaves were the major factor driving the population of *D. citri* during winter in commercial groves in Florida when the number of shoots is extremely reduced, and the available sites for oviposition significantly decrease.

The low number of recaptured individuals in Vale could also result from this behavior in *T. erytreae*. Moreover, the citrus grove in Vale is unprotected from wind, and according to Martini et al. (2015), windbreaks significantly influence the spread pattern of *D. citri.* Moreover, although wind direction may not be correlated with the number of marked psyllids recaptured (Lewis-Rosenblum et al., 2015), psyllids, including species

of *Trioza*, present remarkable migration capabilities being able to travel distances of several kilometers (Greenslade et al., 2021).

Regarding the application of kaolin, the number of recaptured individuals in this work was always significantly higher in control plants. Indeed, individuals who landed in kaolin sprayed plants were only found in plants with sprouts and during the first 12 h. This suggests that the clay particles may have a deterrent effect on *T. erytreae* regarding the selection of the landing site and the site tenacity. These results agree with the results obtained by Miranda et al., (2021) in Araraquara (Brazil), which recorded a significantly lower number of *D. citri* in plants sprayed with kaolin every 7 and 14 days with plants control. Ramírez-Godoy et al. (2018) in Apulo (Colombia) observed a reduction in the adult, nymph, and egg populations of *D. citri* due to foliar applications of kaolin compared to untreated plants. In Florida, Pierre et al. (2021) found lower numbers of individuals of *D. citri* in sweet orange plants treated with red and white kaolin than in plants in which foliar insecticide was applied. On the other hand, Hall et al. (2007) observed lower adult movement, settling, and oviposition on kaolin-treated citrus trees compared with control trees.

A kaolin particle film creates a physical barrier on the plant surface, affecting the behavior and fitness of arthropods. *Trioza* spp. are not the exception and the numbers of eggs and nymphs of *T. apicalis* Först. was found to be reduced when kaolin was applied in carrots (*Daucus carota* ssp. *sativus* L.) in Finland (Nissinen et al., 2020). In fact, kaolin has also been used against *Ceratitis capitata* (Wiedemann, 1824) (Diptera: Tephritidae) in citrus orchards (D'Aquino et al., 2011). The effectiveness of kaolin is related to mechanical-derived aspects such as more difficult attachment and probing (Miranda, 2018; Salerno, 2020), wavelength-dependent interactions such as interference on visual cues (Antignus, 2000). Nonetheless, the use of kaolin may lead to non-desired side effects, such as reducing the life span of beneficial organisms (see Benhadi-Marín et al., 2016).

Regarding the EPG assay, the results clearly showed that kaolin modified psyllids' probing and feeding behavior by reducing the time spent in phloem sap ingestion but increasing the xylem sap time. Miranda et al. (2018) demonstrated that the application of kaolin reduced up to 50% of the proportion of individuals of *D. citri* that were able to reach the phloem compared to control plants. The changes in the feeding behavior of *T. erytreae* could be related to dehydration of individuals when exposed to kaolin, which may lead individuals to spend long periods in xylem sap ingestion and a much shorter

time in phloem-related activities. These results suggest that processed kaolin could be an efficient tool to reduce HLB infection in lemon orchards.

In conclusion, the application of kaolin clay showed to be helpful to prevent the landing and settlement of *T. erytreae* on lemon plants in the field. As an alternative to synthetic insecticides, kaolin is an efficient and widely used tool for sustainable management and deserves to be implemented in the lemon agroecosystem to reduce or prevent the landing of *T. erytreae*. This is especially important after flushing since during this period; citrus plants are more susceptible to the attack of *T. erytreae*. Moreover, even if the individuals were able to settle, the kaolin clay film had a deterrent effect on the feeding of *T. erytreae*, thus preventing the transmission of HLB to *C. limon* plants. However, further research must be conducted on the most efficient dose, the way of application (e.g. different models of backpack sprayer, tractor application), the effect of different kaolin types (e.g. red kaolin), number of applications, the season of application, and side effects on potential natural enemies. Notwithstanding, kaolin clay represents a promising tool for limiting or preventing the spread of the HLB disease throughout Europe.

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Chapter 4. The use of molecular tools for *Trioza erytreae* predators detection

4. Introduction

The African Citrus Psyllid *Trioza erytreae* (Hemiptera, Triozidae), a pest recently arrived in Europe, is one of the main vectors of the causal agents of the Huanglongbing disease (HLB) in citrus (Cocuzza et al., 2017). This disease has been associated to three Liberibacter species, being *T. erytreae* reported to have the capacity to transmitted two species, namely *Candidatus* Liberibacter asiaticus (Jagoueix, Bové and Garnier) and *Candidatus* Liberibacter africanus (Jagoueix, Bové and Garnier) (Bové, 2006; Ajene et al., 2019). These gram-negative fastidious bacterial species are phloem-limited, affecting the plant's vascular system (Munir et al., 2018). The most characteristic symptoms of HLB are observed in the leaves, which show a pattern of yellow and green areas lacking clear limits between the colors. Fruits from HLB-infected trees remain green and become deformed and taste much more acidic and bitter (Cocuzza et al., 2017). In more severe cases, fruits drop prematurely, and trees develop extensive twig dieback, leading to the death of the plant and thus causing significant yield losses (Deng et al. 2019; Silva et al. 2016). The presence of *T. erytreae* in Europe was reported for the first time in 1994, on the Madeira island (Portugal) (Carvalho and Aguiar, 1997), and later, in 2002, in the Canary Islands (Spain) (González-Hernández, 2003). *Trioza erytreae* remained confined to these islands until its report in Vilanova de Arousa (north-western mainland Spain) in August 2014 (Pérez-Otero et al., 2015). In 2015, *T. erytreae* was founded in mainland Portugal, in the district of Porto (Pérez-Otero et al., 2015). Since then, the population of *T. erytreae* has been spread throughout the western Atlantic coast, either to southern Portugal or northern Spain, threatening all citrus farming in the Mediterranean basin (Benhadi-Marín et al., 2020). So far, the presence of HLB in the Iberian Peninsula was not detected (Wang, 2020). Therefore, the containment and control of *T. erytreae* are extremely important to prevent its spreading and thus to reduce the risks of a possible outbreak of HLB (Urbaneja-Bernat et al., 2020).

Nowadays, the control of *T. erytreae* is mainly based on the use of chemical insecticides (Cocuzza et al., 2017). However, the active ingredient most effective against this pest was banned from agriculture in European Union countries (Regulation 2018/785 of 29 May). This poses a challenge for the Mediterranean citrus sector due to the lack of alternatives for the *T. erytreae* control. Moreover, the high frequency of insecticides that must be apply to reach the whish level of efficacy associated to their broad-spectrum, may impair the integration of chemical and biological controls within an integrated pest

management (IPM) program, as most natural enemies are highly susceptible to pesticides (Jacas and Urbaneja, 2010). The exclusive use of insecticides may also lead to several environmental problems (Sharma et al., 2020) and has been revealed to be insufficient due to small orchards and private gardens/backyards representing reservoirs of *T. erytreae* (Cocuzza et al., 2017). Therefore, there is an urgent need to develop more effective and environmentally friendly tools to control this pest. This strategy has long been promoted by the EU (Directive 2009/128/EC), and more recently, it forms part of the European Green Deal, in particular of the Farm to Fork Strategy, which aims to reduce pesticide use in the EU by 50% by 2030.

The exploitation of natural enemies of *T. erytreae*, such as predators, could be one strategy to reach this demand. Indeed, several insects belong to families Coccinellidae, Anthocoridae, Miridae, Chrysopidae, Hemerobiidae, Syrphdiae and Formicidae, and the order Arachnida, have been reported to be potential predators of *T. erytreae* (Gonzalez-Hernandez, 2003; Catling, 1970; van den Berg et al., 1987). However, predator-prey relationships cannot always be identified by direct observation in field conditions (Symondson, 2002). In this regard, the molecular identification of prey in predator diets by using polymerase chain reaction (PCR)-based techniques have been proven to be highly effective in identifying natural enemies of several insect pests (King et al., 2008; Sint et al., 2011; Rejili et al., 2016; Albertini et al., 2018). Using taxon-specific primers, PCR techniques allow the detection of specific prey ingested in the predator's diet since their DNA remains in the predator's gut before being eliminated by the digestion process (Symondson, 2002). Thus, the choice of target DNA region and the length of the sequence region to amplify are two important issues that must be taken into account during the design of taxon-specific primers. The majority of recent studies have used an approach targeting multiple-copy, small and abundant DNA fragments in cells, such as mitochondrial DNA (mtDNA), as the degradation of prey DNA is expected to occur during the digestion process (Sousa et al., 2019). In insects, mtDNA is typically a small double-stranded circular molecule encoding 13 protein-coding genes, two rRNA genes (12S rRNA and 16S rRNA), and 22 tRNA genes required for mitochondrial protein synthesis (Wolstenholme, 1992). The mitochondrial protein-coding gene cytochrome oxidase subunit I (COI) has been the most commonly targeted DNA barcoding markers in studies of gut contents (e.g., King et al., 2008; Sint et al., 2011; Rejili et al., 2016; Albertini et al., 2018). In contrast, the mitochondrial 12S and 16S rRNA genes have been less explored as genetic markers to assess predator-prey interactions (Cho et al., 2019;

Ajene et al., 2020; Song et al., 2019). These two rRNA genes have slower substitution rates and are generally better for the design of group-specific primers than the COI gene (Ballard 2000; Mueller 2006). Moreover, they have many indels (insertion/deletion mutations), making 12S rRNA and 16S rRNA useful species-specific markers (Harper et al., 2005). Accordingly, the main goal of this work was to design and evaluate taxon-specific primers targeting in the mitochondrial COI, 12S rRNA, and 16S rRNA genes, to be used for a PCR-based diagnostic method, to detect *T. erytreae* within potential predators. Feeding experiments were performed to evaluate the effectiveness of this DNA-based diagnostic tool. Specifically, this work analysed: (i) the suitability of the molecular marker selected regions and the specificity and sensitivity of the designed primers on *T. erytreae* detection; (ii) and the prey detectability over time in the *Anthocoris nemoralis* (Fabricius, 1794) (Hemiptera: Anthocoridae) using DNA extracts from their body.

4.1. Material and Methods

4.1.1. Collection and molecular identification of the arthropods

To evaluate the ability of primer pairs designed to specifically amplify the DNA of *Trioza*, adults of *T. erytreae* were collected in Caracoí, Portugal (41°18'26.7"N 8°38'39.5"W) to be used as a positive control. *Aphis gossypii* (Glover, 1877) (Aphididae), *Aphis ruborum* (Börner, 1932) (Aphididae), *Bactericera tremblayi* (Wagner, 1961) (Triozidae), *Bactericera trigonica* (Hodkinson, 1981) (Triozidae), *Bactrocera oleae* (Rossi, 1790) (Tephritidae), *Psylla pyri* (Linnaeus, 1758) (Psyllidae), *Drosophila melanogaster* (Linnaeus, 1758) (Drosophilidae) and *Euphyllura olivina* (Costa, 1839) (Liviidae), were used as non-target species. *A. gossypii*, *A. ruborum*, *B. tremblayi* and *B. trigonica* were obtained from the insect collection of the Consejo Superior de Investigaciones Científicas (CSIC) of Madrid (Spain), while *D. melanogaster* was obtained from the insect collection of the Polytechnic Institute of Bragança (Portugal). *C. pyri* specimens were collected with an entomological sweep net (38 cm in diameter), from the natural soil vegetation, on the Campus of the Polytechnic Institute of Bragança (41 47' 53.2" N, 6 45' 51.5" W, Portugal), between July and August 2019. The same procedure was used to collect *B. oleae* and *E. olivina* specimens in olive orchards located at

Mirandela (41°33'15.0"N 7°07'06.8"W), between July and August 2019. All the arthropods were initially identified to the genus/species level using a binocular stereoscopic, preserved in absolute ethanol, and stored at −20 °C, until subsequent DNA extraction. Their identification was further confirmed based on sequencing of a portion of mitochondrial COI sequence. Briefly, after specimens' homogenization in liquid nitrogen, the genomic DNA was extracted using the Speedtools tissue DNA extraction kit (Biotools, Spain), following the instructions given by the manufacturer. The barcode region of the mitochondrial COI gene was then amplified using the universal primers LCO1490/HCO2198 (Folmer et al., 1994), in a thermocycler (Bio-Rad) using 20 µl PCR reaction, containing 1x buffer, 2.5 mM of $MgCl_2$, 200 µm of dNTPs, 0.2 µm of each primer, and 1.25 U of Taq DNA polymerase (BIORON, Gmbh). Cycling conditions included: initial denaturing at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 20 s, with a final extension of 72 °C for 7 min. Identification of PCR products (~710 bp) were performed at 1% (v/v) agarose gel stained with 1X Gel Red™ nucleic acid gel dye (Biotium, California, USA), and amplified products were then purified and sequenced at Macrogen Inc. (Madrid, Spain). The DNA sequences were analysed and edited with MEGA v10.1.8 (Kumar et al., 2018) and the identification of each specimen was confirmed by querying the GenBank database using the Nucleotide Basic Local Alignment Search Tool (BLASTn) in NCBI's website (www.ncbi.nlm.nih.gov).

4.1.2. Design of *T. erytreae*-specific primers and development of diagnostic PCRs

A taxon-based search was performed in the NCBI database to check for sequences availability, particularly the eventual existence of (nearly) complete nuclear and/or mitochondrial genome sequences. Along with a preliminary literature survey, this allowed to recognize that the mostly used molecular marker for *Trioza* spp. and their relatives is the mitochondrially encoded cytochrome c oxidase I (COI). Sixty partial or complete COI sequences from psyllids taxa were selected, comprising (1) *T. erytreae* specimens from different geographical origins (including from Madeira and Canary Islands), (2) other *Trioza* spp., and (3) phylogenetically close relatives, i.e., representatives from Group D and sister clades (Group A, B and C) from *Triozidae*, as defined in the study of Percy et al. (2018). Also, it was possible to select and retrieve 24 mitochondrial genome sequences from the NCBI GenBank database from the superfamily

Psylloidea, as illustrated by Percy et al. (2018). It included the mitogenomes from *T. erytreae* (NC_038142), from three other *Trioza* spp., and from several close relatives in the Triozidae. Multiple alignments either for COI or mitogenome sequences were then obtained using the ClustalW algorithm in MEGA X (Kumar et al., 2018). To evaluate the relationship between selected organisms, those alignments were also subjected to phylogenetic analyses. Neighbor-Joining (NJ) trees with 5000 bootstrap replicates were constructed to each multiple-alignment using the MEGA X. Phylogenetic trees were edited with Inkscape 0.92 (www.inkscape.org). The phylogenetic trees in (supplementary material) Figures S1 and S2 summarize the psyllids included in each alignment used for the design of the primers and showed the phylogenetic relationship among these organisms.

The design of the primers was performed according to basic primer design rules, e.g., oligonucleotides length, G/C content, melting temperatures, self-dimer and hairpin formation, identification of highly conserved regions for the target organism, among other parameters (Dieffenbach et al., 1993). Oligonucleotide candidates were submitted to in silico analyses to ascertain their specificity by using Primer-BLAST (Ye et al., 2012). The thermodynamic properties' suitability of the primers was tested by using the online softwares OligoEvaluator (www.oligoevaluator.com) and PrimerSuite (www.primer-suite.com), allowing to choose the better candidates. A shortlist of potentially suitable oligonucleotide candidates was thus obtained, with four of them targeting the rrnL gene (16S), three the rrnS gene (12S), and six the COI gene (Table 2 and Figure 27). The primers were then synthesized at Frilabo (Portugal).

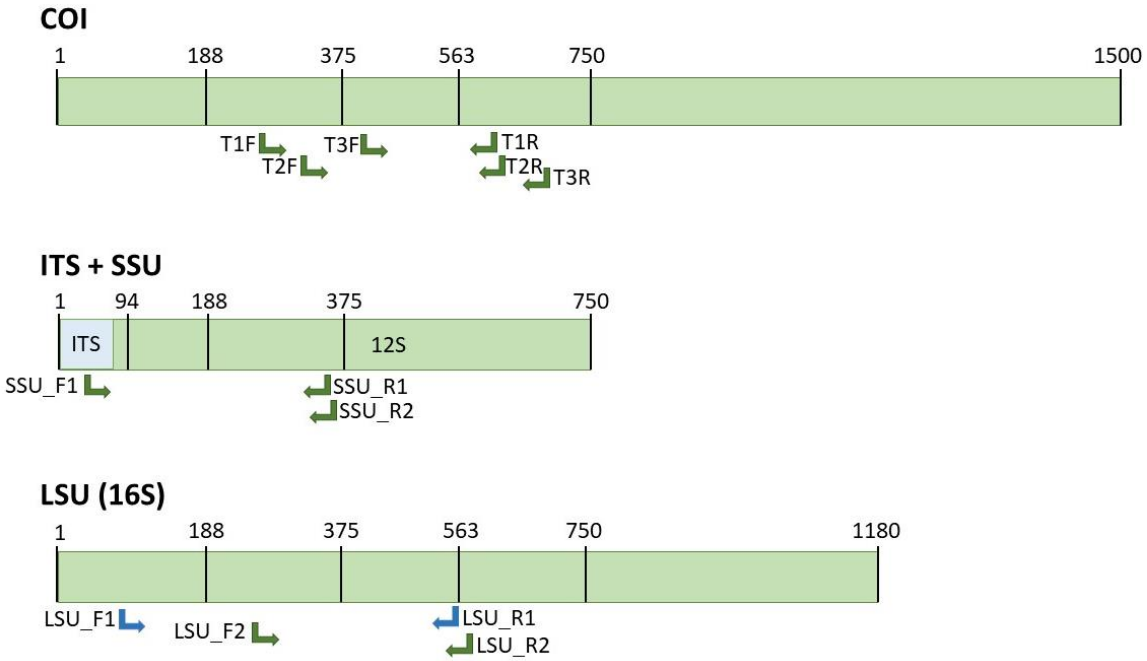

Figure 27. Schematic diagram of COI and rrnL and rrnS gene regions showing the binding sites of the candidate PCR primers developed and tested in this study. Nucleotide positions according to the loci retrieved from the complete mitochondrial genome of *Trioza erytreae* (NC_038142.1); COI depicted from positions 3056-4588 (1533 nt), rrnL (16S) from 13830-14978 (1186 nt), and ITS + rrnS (12S) from 14979-15735 (74 nt, ITS + 684 nt, 12S).

Table 2. Candidate primers designed and developed in this study to specifically amplify *Trioza erytreae*

Target loci[&]	**Primer name**	**Positions [#]**	**Sequence 5´- 3´**
mt rrnL: 16S	LSU_F1	140-158	GACTTTATACATAAACACC
mt rrnL: 16S	LSU_F2	268-284	CTAAAATAGGATCTTCC
mt rrnL: 16S	LSU_R1	543-559	CCTGCTCAATGCTTAGG
mt rrnL: 16S	LSU_R2	543-561	AACCTGCTCAATGCTTAGG
mt ITS-rrnS: 12S	SSU_F1	56-77	GCTATTCATTTTCTTTCCAGGC
mt ITS-rrnS: 12S	SSU_R1	333-350	GTATGCCGCCGTCATGAG
mt ITS-rrnS: 12S	SSU_R2	333-358	ATTTACTTGTATGCCGCCGTCATGAG
mt COI	T1F	256-282	GCTCCAGATATAGCATTTCCTCGACTT
mt COI	T2F	335-356	AGTTGGGACAGGATGAACAGTT
mt COI	T3F	388-409	GAGGATATTCAGTAGATACTGC
mt COI	T1R	577-596	GCTAAAACAGGTAATGCCAA

mt COI	T2R	579-603	TGCTCCTGCTAAAACAGGTAATGCC
mt COI	T3R	635-656	AGGATCTCCTCCCCCGGCTGGA

[&] *mt*: mitochondrial; *rrnL* and *rrnS*: large (16S) and small (12S) subunit rRNA genes, respectively; *ITS*: internal transcribed spacer; *COI*: cytochrome c oxidase subunit I

[#] Positions according to the mitochondrial complete genome sequence of *Trioza erytreae* (NC_038142.1)

Primer combinations (see Table 3 for a list) were tested, their PCR conditions optimized, and their specificity and sensitivity evaluated. The specificity of the primers for *T. erytreae* was evaluated by using as template genomic DNA (gDNA) extracted from seven specimens from this species and from the 8 non-target species (*A. gossypii*, *A. ruborum*, *B. tremblayi*, *B. trigonica*, *B. oleae*, *C. pyri*, *D. melanogaster* and *E. olivina*). The sensitivity of the primers was assessed using gDNA concentrations from *T. erytreae* at 10 ng/μL and 0.1 ng/μL.

Table 3. All primers combination tested in this study and their estimated PCR product sizes (bp).

Primer sets	**Length (bp)**
LSU_F1 - LSU_R1	419
LSU_F2 - LSU_R1	291
LSU_F1 - LSU_R2	421
LSU_F2 - LSU_R2	293
SSU_F1 - SSU_R1	294
SSU_F1 - SSU_R2	302
T1F - T1R	343
T1F - T2R	350
T1F - T3R	419
T2F - T1R	245
T2F - T2R	252
T2F - T3R	321
T3F - T1R	192
T3F - T2R	199
T3F - T3R	268

For all PCR assays, each primer pair was used in 20 µL reactions, containing 14.7 µM of water, 2 µM of buffer, 0.4 µM of dNTP's, 0.4 µM of each primer, and 0.5 µM of Taq DNA polymerase (BIORON, GmbH). The PCR program was optimized by varying the annealing temperature (from 48ºC to 64ºC through gradient PCR). Primer sets showing good performance at higher annealing temperatures were then subjected to further tests and optimizations to improve the specificity and sensitivity by varying the time of denaturation, annealing and extension. Optimized cycling protocols for the selected primer pairs are indicated in the results section.

4.1.3. Post feeding detection period of *T.erytreae* in *A. nemoralis*

Predation assays were conducted to observe how long it is possible to detect the presence of *T. erytreae* DNA within the intestinal tract of *Anthocoris nemoralis* (Fabricius, 1794) (Figure 28) after ingestion. To perform this test, *A. nemoralis* was collected from lemon branches with nymphs of *T. erytreae*, in an orchard in Caracoí (41°18'45.8"N 8°38'10.4"W), Vila do conde, in mid-July and August 2019. Adults of *A. nemoralis* were captured by shaking the branches to a plastic tray, followed by their collection with the help of a mouth aspirator.

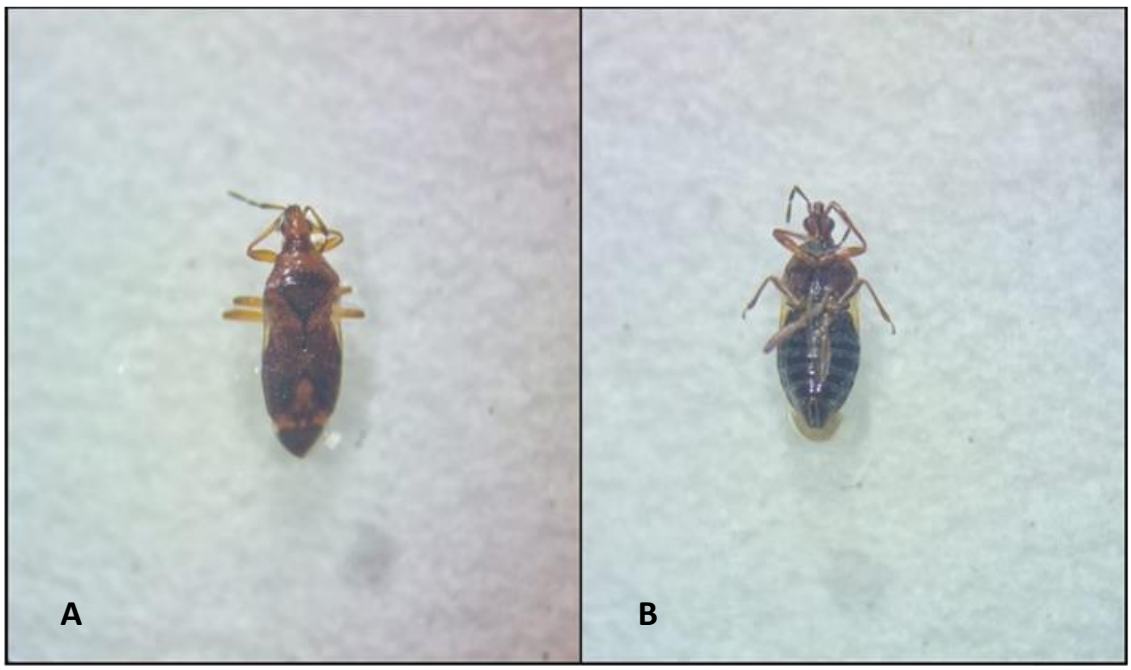

Figure 28. Adult of *Anthocoris nemoralis* A: back side, B: front side.

A. nemoralis specimens were transported to the Agrobiotechnology laboratory of the Escola Superior Agrária de Bragança, where they were identified by sex and placed individually in Petri dishes (7 cm in diameter) to be starved for 24 hours. During this period, the *A. nemoralis* were kept at 21°C with 55% relative humidity and 16:8 h (L:D) photoperiod with water supply. At the end of the starvation period, one leaf with approximately 20 nymphs (at N4 and N5 stages) of *T. erytreae*, collected in Caracói, was given to each *A. nemoralis*. Predators were left to feed for 2 hours on the leaf with the nymphs of *T. erytreae*, and after this period, *A. nemoralis* were sacrificed at 1, 2, 4, 6, 8, 10, 12, 18, 24, 36, 42 and 48 hours after the 2 h of feeding. As controls were used starving *A. nemoralis* specimens (for 24 hours, time 0) and specimens sacrificed after 2 hours of feeding. The individuals of *A. nemoralis* were sacrificed by placing them in 96% ethanol and frozen at -20 °C. For each time, 8 specimens, 4 of each sex, were used as replicate. After being macerated in liquid nitrogen, DNA from each *A. nemoralis* was extracted using the SpeedTools DNA Tissue Extraction kit (Biotools, Spain), according to manufacturer instructions. PCR amplifications of *T. erytreae* DNA from the gut of *A. nemoralis* was performed by using the primer set LSU_F1-LSU_R1, and the respective optimized PCR condition which is described in results section.

4.2. Results and Discussion

In this work, was tested the effectiveness of 15 primer pairs designed for *T. erytreae* (Table 2). In total, five primer pairs, one from rrnL (16S) region LSU_F1/LSU_R1 (expected size: 419 bp), another from ITS + rrnS (12S) region SSU_F1/SSU_R1 (expected size: 294 bp) and four from COI region T1F/T1R (expected size: 343 bp), T3F / T1R) (expected size: 192 bp) and T3F/T2R (expected size: 199 bp), successfully yielded DNA fragments of the expected size, although a few amplicons showed double bands (Figure 4.3). These five pair primers showed to be specific for *T. erytreae* among several non-target arthropod species, including species belonging to the same family of *T. erytreae* (i..e., Triozidae), namely *B. tremblayi* and *B. trigonica* (data not showed). Among these 5 pair primers, LSU_F1/LSU_R1, T1F/T1R, T3F/T1R and T3F/T2R, revealed to be highly sensitive to *T. erytreae*, being able to amplified DNA of this insect at concentration of 1 ng/µL (Figure 4.3). The remaining 10 pair primers showed

no specificity and/or no sensibility towards *T. erytreae* (data not shown). Amplifications with high annealing temperatures were then performed for the pair primers LSU_F1/LSU_R1, T1F/T1R, T3F/T1R and T3F/T2R, in order to minimize cross-amplification of nontargets and primer annealing to false priming sites (King et al., 2008). Results indicated that the primer pair LSU_F1/LSU_R1 provides more consistent PCR than the tested primer sets. Its high specificity and sensitivity for *T. erytreae*, was achieved with the following PCR cycle conditions: an initial denaturation for 3 min at 94°C, followed by 35 cycles at 94°C for 30 s, 57°C for 30 s, 72°C for 20 s and a final extension at 72°C for 7 min (Figure 29).

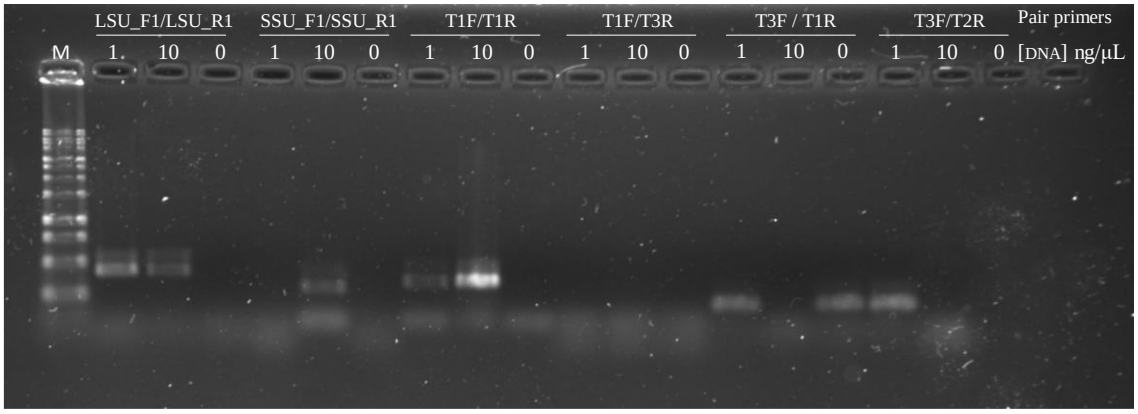

Figure 29. Primer's sensitivity for *Trioza erytreae.* Agarose gel electrophoresis of amplification products of the PCR using the primers pairs LSU_F1/LSU_R1 (expected size: 419 bp), SSU_F1/SSU_R1 (expected size: 294 bp), T1F/T1R (expected size: 343 bp), T1F/T3R (expected size: 419 bp), T3F / T1R) (expected size: 192 bp) and T3F/T2R (expected size: 199 bp), and DNA of *T. erytreae* at 1 and 10 ng/µL. Water was used as negative control (0 ng/µL DNA). Lane M: Molecular Weight Marker 1000 bp DNA Ladder (BIORON, GmbH).

The primer pair LSU_F1/LSU_R1 targets one region from 16S ribosomal RNA and generates a PCR product of 419 bp. COI has been the most widely used marker to detect prey DNA within predator's guts (King et al., 2008; Sint et al., 2011; Rejili et al., 2016; Albertini et al., 2018). However, in the present work, 16S showed to be a possible and even better alternative to COI, as this marker minimizes primer bias and provides more consistent PCR than COI for detecting *T. erytreae* DNA. Similarly, (Muilenburg et al., 2008) found that the 16S rRNA gene was more useful than COI when designing

primers for species-specific detection of predation of *Enaphalodes rufulus* (Haldeman) (Coleoptera: Cerambycidae) by ant species.

Hence, the primer pair LSU_F1/LSU_R1 was selected, and its suitability to detect *T. erytreae* DNA in feeding assays (Figure 30) was evaluated. The predation bioassay is not yet complete, but the results get so far showed that our diagnostic PCR assay allows the detection of *T. erytreae* DNA in *A. nemoralis* at least up least up to 18 h post-feeding (Figure 4.4). Moreover, the reduction in band intensity over the digestion process suggests that digestion time is an important aspect for the detectability of *T. erytreae* DNA, as previously reported for other insects (Juen & Traugott, 2007; King et al., 2008). The detectability of prey DNA following consumption by predator did not differ between males and females, suggesting that both genders are important predators of *T. erytreae*. *A. nemoralis*, a small-size (3-4 mm length) anthocorid species, is one of the most abundant predators in fruit orchards in Europe (Sigsgaard, L. 2010, Solomon et al. 1989; 2000), and have been shown to be important generalist predators in fruit orchards. *Anthocoris nemoralis* has been the focus of many studies in Europe, as it shows a clear preference for psyllids (Anderson 1962b; Dempster 1963; Sigsgaard 2010; Solomon et al. 2000). Thus, *A. nemoralis* may realize two parallel types of seasonal development, depending on the habitat and the kind of prey (Saulich & Musolin, 2009), and the peak of the abundance of the overwintered individuals was recorded in mid-April (Anderson, 1962b). During digestion, prey DNA molecules are broken into smaller fragments and thus, their detection in the gut it is likely to be enhanced by targeting short DNA fragments (< 300 bp) (Hoogendoorn & Heimpel, 2001; Chen et al., 2000). However, PCR efficiency is also determined by factors such as PCR reagents, the quality of the template DNA extract, efficiency of the primers used, and the cycle conditions. Therefore, optimized PCR assays will sometimes allow detection of larger prey DNA fragments, even up to 500 bp (Agusti et al., 1999; Hoogendoorn & Heimpel, 2001; Juen & Traugott, 2007). Moreover, short PCR products (up to 100 bp) might hinder species identification because multiple similar sequences exist in closely related species (Yang et al., 2014). Here, the primer pair LSU_F1/LSU_R1 was able to detect larger fragments (419 bp) of *T. erytreae* DNA during the digestion process. Similarly, this pair of primers was suitable to detect *T. erytreae* on DNA samples extracted from the whole body of the prey *A. nemoralis*. In general, molecular prey detection requires the dissection of the predator gut (Agustí et al., 2003; Næss, 2016; Batuecas et al., 2021) to reduce the predator DNA and thus potentially improve sensitivity. However, this procedure is laborious, time-consuming, and requires

experience. On the contrary, DNA extraction from the whole body is faster and more user-friendly.

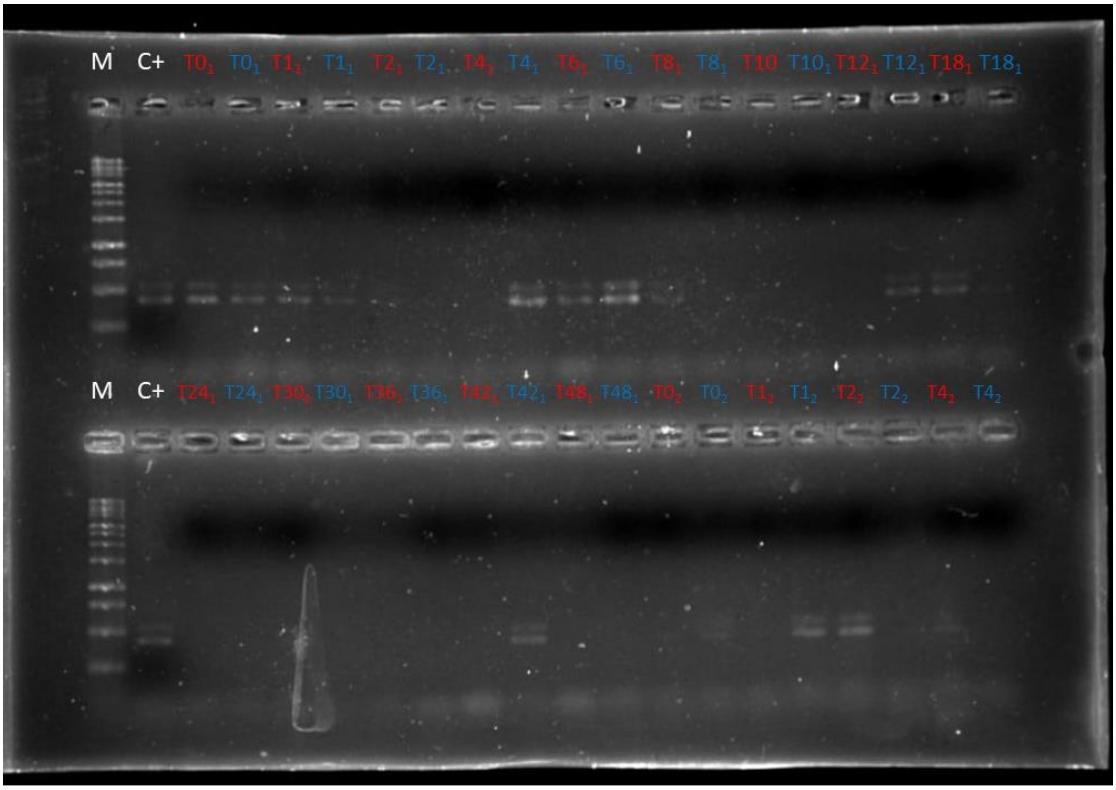

Figure 30. PCR amplification products obtained from DNA samples extracted in different post-feeding times, by using the primer pair LSU_F1/LSU_R1 (expected size: 419 bp). Lane M: Molecular weight marker: 1000 bp DNA Ladder (BIORON, GmbH); Lane C+: *Trioza erytreae*; Lanes with T0, T1, T2, T4, T6, T8, T10, T12, T18, T24, T30, T36, T42 and T48 are the post-feeding times, the red ones represent *Anthocoris nemoralis* females, the blue ones represent *A. nemoralis* males.

In conclusion, although preliminary, the findings suggest that the *T. erytreae* primers here designed and the optimized PCR-based diagnostic assay can provide an effective and sensitive method for detecting potential predators of the main vector of *Candidatus* Liberibacter spp. This PCR-based diagnostic assay can help in the implementation of more sustainable measures to limit the spread of this vector-borne pathogen.

4.3. References

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Final conclusions

The application of kaolin clay proved to be effective to prevent the landing and colonization of adults of *T. erytreae* on lemon plants in the selected orchard. As an alternative to synthetic insecticides, kaolin clay could be an efficient and widely used tool for sustainable management and deserves to be implemented in the citrus agroecosystem to reduce or prevent *T. erytreae* landing. This is especially important after flushing since citrus plants are more susceptible to be attacked by *T. erytreae*. Furthermore, even if the individuals of *T. erytreae* were able to land on the plants, the kaolin clay film had a deterrent effect on the feeding behaviour of *T. erytreae*. This could be an efficient way to prevent HLB transmission on lemon plants. Accordingly, further research should be conducted on the most efficient dose, the manner of application, the effect of different types of kaolin, the frequency and timing of application, and potential side effects on natural enemies. Thus, kaolin clay is a promising tool to limit or prevent the spread of HLB disease throughout Europe.

The final part of this work deals with the use of a naturally occurring predator in lemon orchards to limit *T. erytreae* population in Portugal. The results obatined through the assay conducted using *A. nemoralis* as model predator suggests that the designed primers for *T. erytreae* and the optimized PCR-based diagnostic assay may provide an effective and sensitive method to detect potential predators of the main vector of *Candidatus* Liberibacter spp in Europe. Accordingly, this PCR-based diagnostic assay may help in the implementation of sustainable management tools aimed to limit the spread of the pathogen transmitted by the psyllid.

In summary, after the experiments conducted both in the field and in the laboratory, the control of *T. erytreae* through sustainable management as an alternative to synthetic insecticides, seems to be a suitable approach to limit the populations of *T. erytreae* in Portugal.

Supplementary material

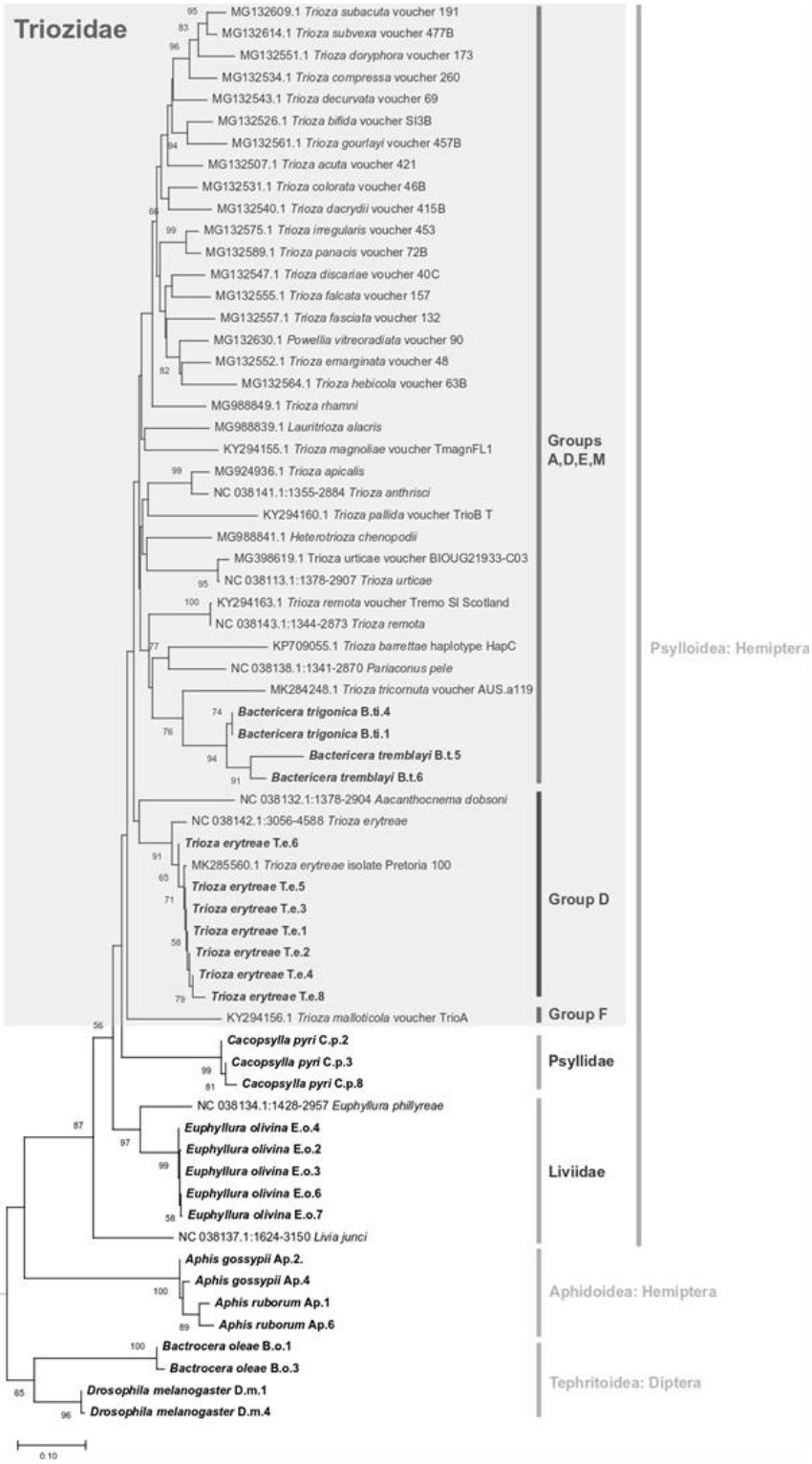

Figure S1. NJ phylogenetic tree of COI sequences from psyllids and other insects used in this work, either in the primer designing (sequences not bolded, with accession numbers) or in the following primer testing (in bold). Groups of Triozidae according to Percy et al. (2018).

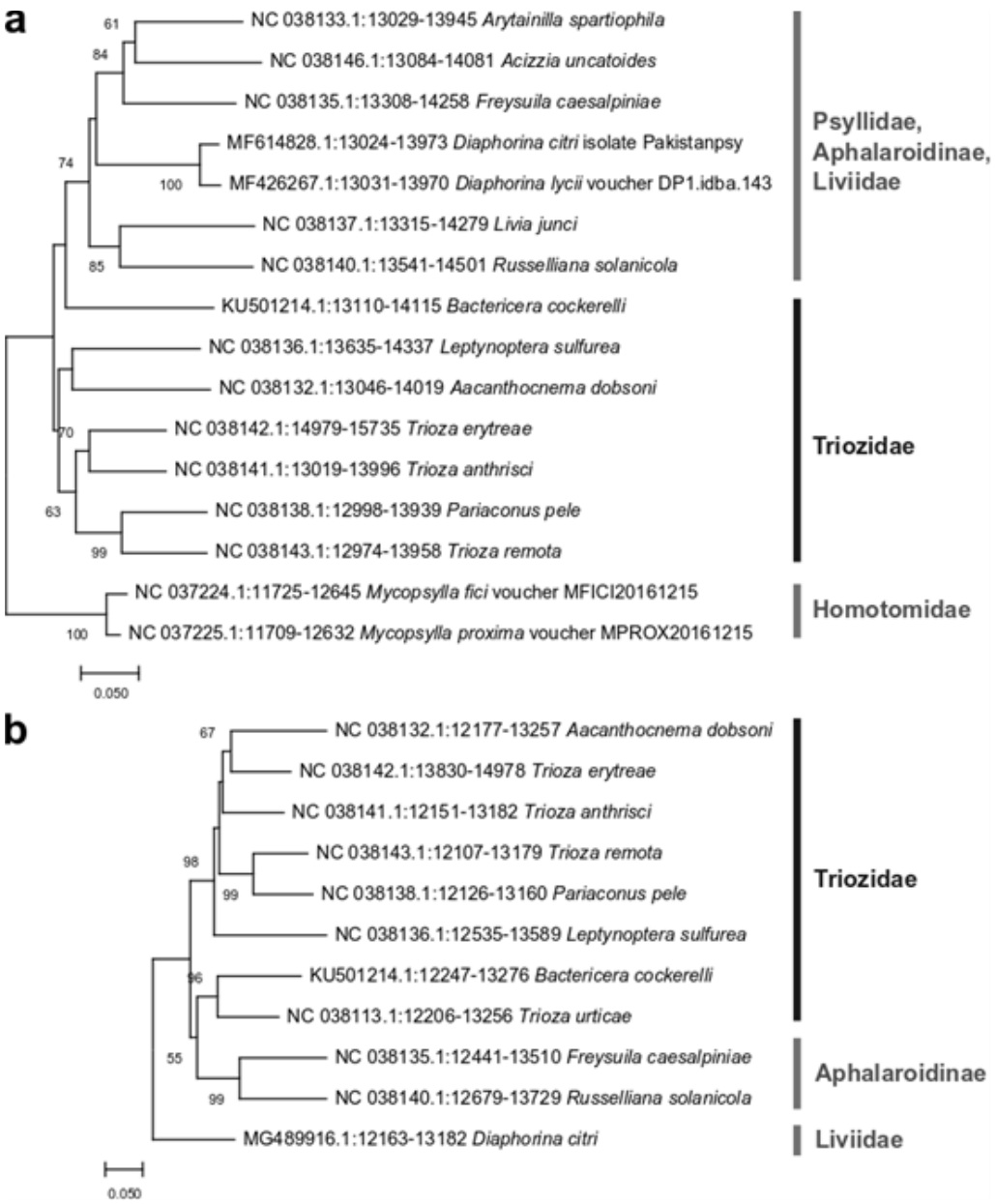

Figure S2. NJ phylogenetic trees for complete ITS+SSU (a) and LSU (b) sequences from the family Triozidae and related psyllids used for the primers designing. Sequences were retrieved from complete mitogenomes; nucleotide positions are indicated after accession numbers.