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# Current distribution and status of the Persea mite *Oligonychus perseae* infesting avocado trees in Morocco

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**Summary** Surveys to assess the current distribution and status of the Persea mite *Oligonychus perseae* Tuttle, Baker and Abatiello (Acari: Tetranychidae) and its natural enemies were conducted in 85 avocado orchards in north-west Morocco between October 2023 and October 2025. The mite was recorded on leaves in several orchards with low to high infestation while it did not occur on the fruits. A morphological identification of *O. perseae* is provided, based on Moroccan specimens. Currently, *O. perseae* is not causing economic damage to avocado fruits in Morocco, but its establishment in new areas within the country and the increase of its population on leaves may constitute a threat to fruit and yield in commercial avocado cultivation in the future.

*Additional Keywords:* Hass variety, Integrated pest management, monitoring, Morocco

## Introduction

In the Mediterranean region, Morocco is a significant producer and exporter of fresh fruits of avocado (*Persea americana* Mill.). In the last decade, hectares of avocado cultivation area in the country have significantly increased due to rising consumer demand, mainly from European Union countries. Despite water scarcity in the country, avocado production area expanded to approximately 11,770 ha with an annual production of 116,207 tonnes of fresh avocados in 2024 (FAOSTAT, 2026).

Many sap-sucking pests attack this crop, including mite species. Tetranychidae species, can cause direct feeding injuries by piercing and sucking out plant cell contents from leaf and fruit surfaces. *Oligonychus* is the largest genus in the family and based on the orientation of the male aedeagus; it consists of two subgenera: *Oligonychus* Berlese and *Reckiella,* Tuttle and Baker (Mushtaq *et al.*, 2023). *Oligonychus* Berlese is one of the most economically important and largest subgenus, with 211 species (Mushtaq *et al.*, 2021, 2024).

The Persea mite *Oligonychus perseae* (Tuttle, Baker and Abatiello), is a known major pest of avocado, especially in Central America (EFSA *et al.*, 2022). It is polyphagous, feeding on approximately 20 different hosts in 17 botanical families (EFSA *et al.*, 2022; Torres *et al.*, 2024; EPPO, 2026). Native to Central America, it was first described in 1975 on intercepted avocado plants from Mexico at a quarantine facility in Texas (USA). *Oligonychus perseae* is the main cause of economic damage in major avocado-producing regions worldwide (Torres *et al.*, 2024). It is now widespread and has been reported from North America (California, Florida and Mexico), Hawaii, and Central America (Costa Rica) (EPPO, 2026). Within the European Plant Protection Organization (EPPO) region, *O. perseae* has been reported from France (Auger *et al.*, 2023), Greece (Bitsakis *et al.*, 2026), Israel (Maoz *et al.*, 2011a), Italy (Zappala *et al.*, 2015), Morocco (Smaili and Benyahia, 2018a), Portugal and Madeira Island (Ferreira *et al.*, 2006; Boieiro *et al.*, 2013) and Spain (mainland and Canary Islands) (Torres *et al.*, 2024). The Persea mite was included in the EPPO alert list in 2003 and was

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then removed in 2008 (EFSA *et al*., 2022).

In Morocco, until late in the 2010s, avocado groves were relatively free from major insect pests. Since then, avocado production has expanded into several new areas within the country and subsequently several new pests were recorded starting in 2018 (Smaili and Benyahia, 2018 a,b; Boutaleb-Joutei, 2019; Ait-Baddou *et al*., 2024; Smaili *et al*., 2024; Haddad *et al*., 2025 a,b). *Oligonychus perseae* was first detected infesting avocado leaves at Mnasra (Kenitra province) in October 2018 (Smaili and Benyahia, 2018a). Thereafter, no further studies or additional details have been published, so very little is known about its distribution within the country. This study aims to provide a description of the current distribution and status of *O. perseae* on avocado in Morocco. It provides morphological diagnosis of the species based on Moroccan specimens.

## Materials and Methods

The survey for *O. perseae* was conducted in western and northern region of Morocco, where most avocados are grown, between October 2023 and October 2025. In total, 85 avocado orchards were surveyed across nine provinces (Fig. 1): Berkane and Oujda (north eastern, Morocco), El Jadida (western Morocco), Kenitra, Khemisset, Larache, Sale, Sidi Slimane and Rabat (north western Morocco). Monitoring was conducted two to three times in each orchard where ten trees were randomly selected, and 20 leaves and eight fruits were observed and examined from each tree. Avocado leaves were collected in small plastic bags and then carefully transferred to the laboratory for further identification of pest presence. In addition, the beating method (10 branches were beaten) was performed four times from each orchard (for each visit), using a white plastic bag connected to a plastic funnel (20 cm in diameter) to capture insects.

Natural enemies observed were also collected for further identification to species level. Total mites on the second major vein from ten avocado leaves that were randomly picked from ten trees from each orchard. The infestation levels (%) were estimated using the same formula as that used in a previous study on avocado (Smaili *et al*., 2024; Haddad *et al*., 2025b), based on the ratio of insect-infested leaves (or fruits) to the total number of leaves (and fruits) examined, multiplied by 100. Infestation levels were categorized into four classes: absent or rare when very few or no avocado mites were found (0–<1%); low (1–<5%); moderate (5–<10%); and high (10–100%). The average number of *O. perseae* on leaves was estimated using Machlitt's method (Machlitt, 1998). Samples of avocado leaves containing *O. perseae* were labeled and kept at 4°C for subsequent identification.

*Oligonychus perseae* was identified by Smaili and Benyahia (2018a). Identification of *O. perseae* was conducted under a stereomicroscope (Olympus SZX7, Japan) based on the, morphological characters by Torres *et al.* (2018), Mushtaq *et al.* (2021) and Auger *et al.* (2023). Information on the pattern of infested avocado leaves on which the mite has been found facilitated species identification. Photographs of mite and leaves infested with *O. perseae* were taken in the laboratory using a digital camera attached to the same stereomicroscope.

## Results and Discussion

### Identification of *Oligonychus perseae* (Tuttle, Baker and Abbatiello)

Macroscopically, *O. perseae* adult and immatures are light-yellowish. Adult female with oval-shaped body, slightly flattened and elongated with two or three black spots to metasternum (Fig. 2).

Microscopic specimens of *O. perseae* (n= 20) were identified with accurate description by Mushtaq *et al.* (2024). Female with 9 and 6 or 7 tactile setae on tibia I and II, respectively (Fig. 2). Male ventrally bent part of aedeagus short, with pointed tip. Female with dorsal body setae slender, setae c1 and d1 about three-quarters as long as distance

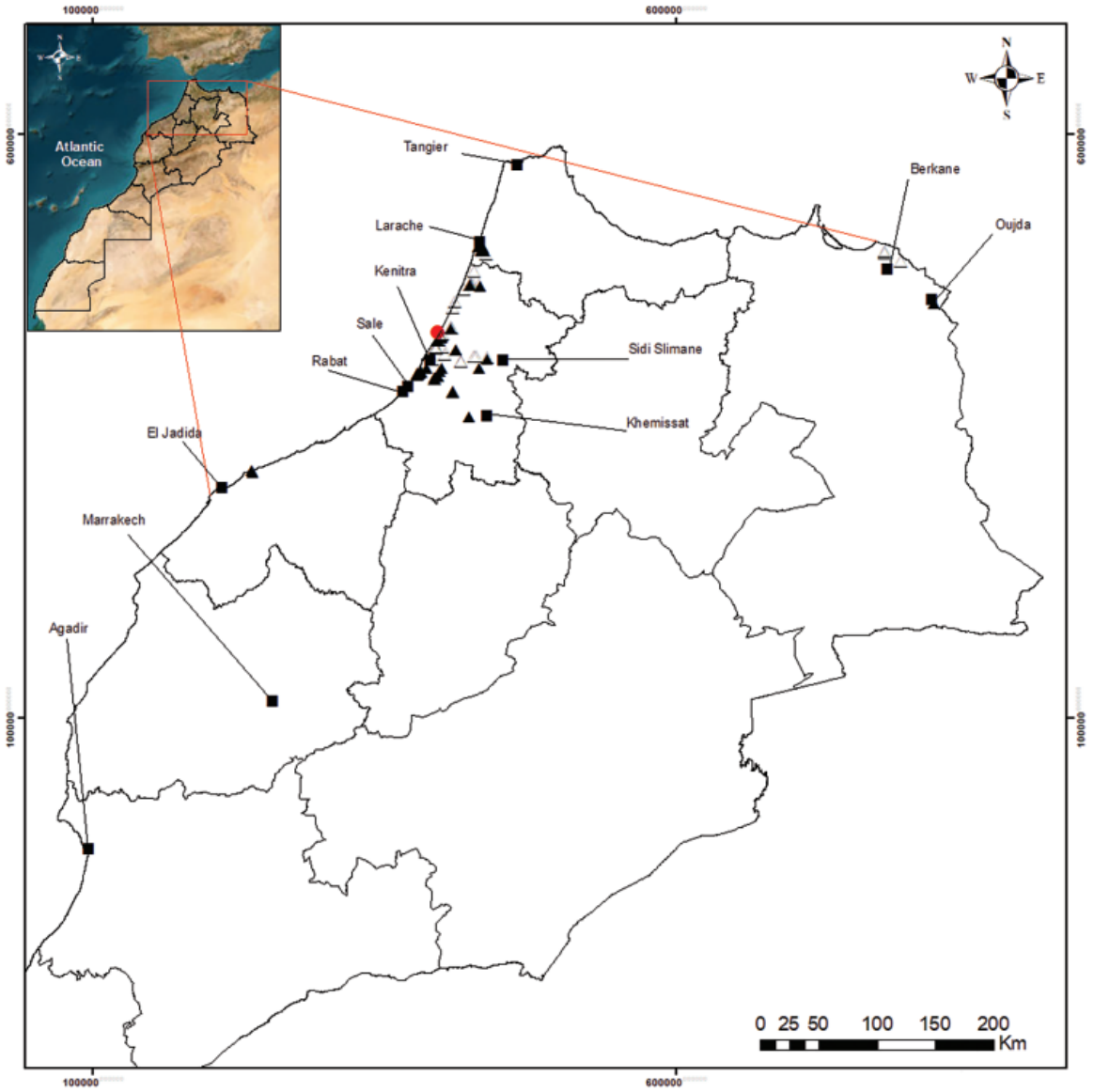


**Figure 1**. Map of Morocco with inset showing sites studied between October 2023 and October 2025. Provinces (grey solid square) sampled avocado sites (empty triangle), avocado sites with the current occurrence of *Oligonychus perseae* (Tuttle, Baker and Abatiello) (black solid triangle), avocado site with the first occurrence of the Persea mite during October 2018 (red solid circle). Credit O. Tounia

between them, e1 reaching to bases of f1; striae forming inverted V-pattern between d1-f1 setae.

## Biology

As other Tetranychidae species, *O. perseae* has five developmental stages (egg, larva, protonymph, deutonymph and adult male and female) (Fig. 2). Feeding and reproduction activities occur in the characteristic nests. Aponte and McMurtry (1997) reported that the life cycle of the Persea mite ranges from 34.9 days (at 15°C) to 9.8 days (at 30°C). In this study, the average number of nests built by a female was 12.17 at 20°C and the greatest number of eggs per female per nest was 5.20 at 25°C.

## Occurrence, dispersal, damage, and economic importance

*Oligonychus perseae* was recorded at several of the 85 avocado surveyed groves in

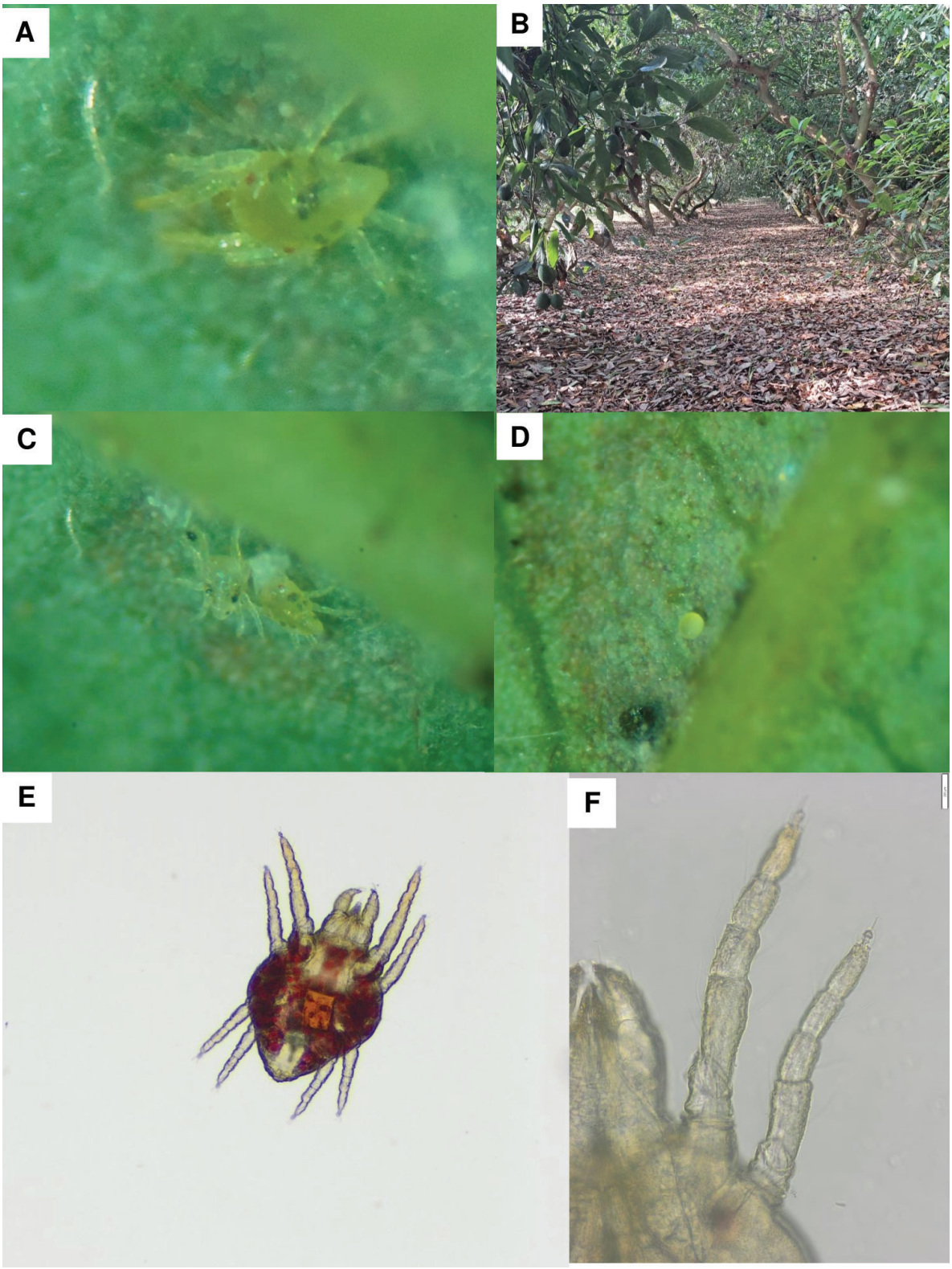


**Figure 2.** *Oligonychus perseae* (Tuttle, Baker and Abatiello) in avocado. A. Habitus of adult; B. Habitat: avocado orchard; C. Nymphal instars; D. egg; E. Adult dorsal view in habitus with sclerotized area; F. View of the tibia and tarsus of the first and second legs. Photographs by O Tounia and MC Smaili.

north and west Morocco (Fig. 1). This geographical distribution practically indicates an expansion of several Km since the Persea mite was first observed with restricted

distribution at avocado orchards in Mnasra (34.469535N, -6.532931W) (Kenitra province) between October and December 2018 and later in Laouamra zone (Larache province) (Smaili and Benyahia, 2018a). In this survey, population expansion was documented near Azemmour city (Azemmour province) and near Oujda city (Oujda province), about 199 km southwest and 425 km northeast of the original detection site of Mnasra (Fig. 1, red circle). In addition, *O. perseae* was newly recorded in several other orchards between 12 to 79 km away from the original detection site supporting also potential further spread of the mite considering the rising number of the avocado growers in Morocco.

Infestation levels found on the leaf samples collected in the present study (October 2023 to October 2025) at these orchards are provided in Table 1. *Oligonychus perseae* populations were found only on avocado leaves, at low to high infestation levels. Leaf damage was observed where *O. perseae* had settled on the leaf underside along the midrib and other larger veins to feed on phloem sap. When population levels were high, damage occured also in the lamina area (between two veins) of leaves. On the underside of leaves, feeding damage showed minute necrotic and webbing spots, called "nests" of about 1-2 $mm^2$ at each damaged area (Fig. 3). Feeding from the underside appeared as circular yellow chlorotic spots on the upper side of the leaf. Feeding damage was to cells of the epidermal and spongy and palisade parenchyma

**Table 1**: Infestation levels of *Oligonychus perseae* (Tuttle, Baker and Abatiello) in avocado orchards in Morocco: provinces sampled and leaf infestation levels [number of *O. perseae* on leave according to Machlitt's method] recorded between October 2023 and October 2025.

| Provinces | GPS coordinates | Infestation on avocado leaf |
|---|---|---|
| El Jadida province (Azemmour) | 33,363699N, -8,228033W | High 45.00% [51.60] (02/05/2025) |
| El Jadida province (Azemmour) | 33.374793 N, -8.234502W | High 52.50% [66.72] (02/05/2025) |
| Berkane (Madagh-Laatamna) | 34.981877 N, -2.199860W | Absent 0.00 % [0.00] (13/07/2025) |
| Oujda province (near Oujda city) | 34.651576 N, -1.899311W | High 50.00% [64.80] (07/06/2025) |
| Kenitra province (Bouknadel) | 34.1417222 N, -6.7241389W | High 48.75% [67.32] (12/04/2025) |
| Kenitra province (Mnasra, first reported site) | 34.469535 N, -6.532931W | High 40.00% [58.32] (02/11/2024) |
| Kenitra province (Had Oulad Jelloul) | 34.502650 N, -6.411888W | High 16.25% [57.00] (12/04/2025) |
| Kenitra province (Mnasra) | 34.415837 N, -6.532931W | High 38.75% [53.64] (16/05/2024) |
| Khemisset province (Tiflet) | 34.017926 N, -6.394414W | Low 3.75% [47.28] (12/06/2025) |
| Khemisset province (Tiflet) | 33.825041 N, -6.237855W | Moderate 6.25% [44.64] (12/06/2025) |
| Larache province (near Larache city) | 35.108592N, -6.128303W | High 58.75% [66.48] (27/12/2024) |
| Larache province (near Larache city) | 35.101394N, -6.133627W | High 41.25% [51.24] (10/11/2024) |
| Sidi Slimane province (Dar Belamri) | 34,264994N, -6,078167W | High 17.50% [48.48] (19/04/2025) |

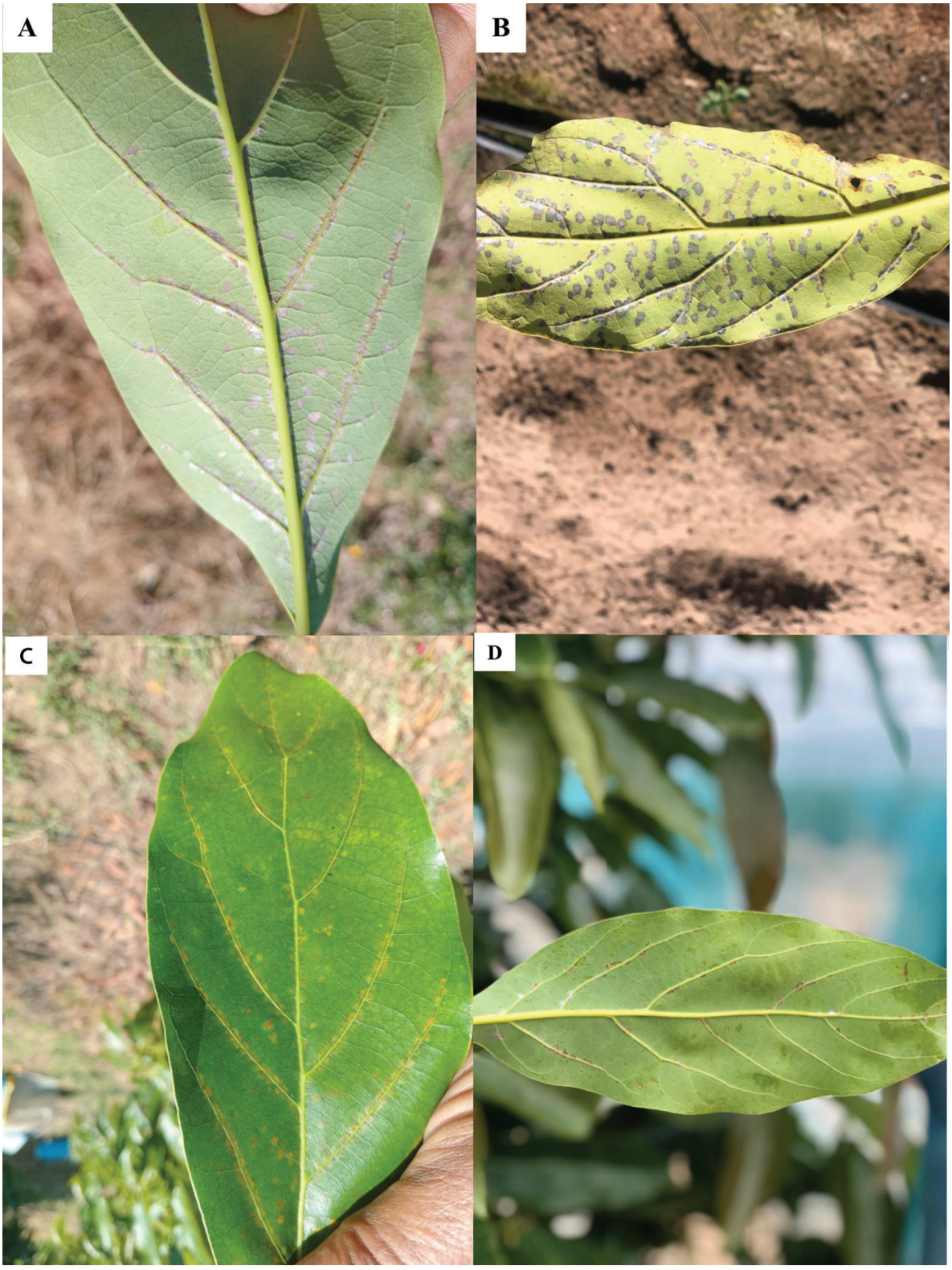


**Figure 3.** *Oligonychus perseae* (Tuttle, Baker and Abatiello) infestation on avocado leaf. A. Tiny nest on veins; B. Heavy infestation and discoloration of leaf; C. Low infestation on veins limited by unfavorable climate condition; D. Low infestation. Photographs by O Tounia and MC Smaili.

(Fig. 3). Heavy mite damage can lead to discolored leaf tissue and leaf drop. The occurrence of *O. perseae* did not affect the fruits. In Spain, *O. perseae* has been reported as an

economically important foliar pest of avocados (Torres *et al.*, 2023). High populations (> 500 mites per leaf) can cause partial or total tree defoliation and increase the risk of sunburn to young fruit, leading to premature fruit drop may (EFSA, 2022).

In the coastal production areas in the northwest of Morocco (Larache and Kenitra provinces), *O. perseae* population may be observed between September to May, with their numbers and associated nest-picked leaves becoming more noticeable from October until December. In those coastal provinces (especially Mnasra, province Kenitra) significant damages were recorded on avocado leaves for a long portion in the growing seasons as humid air conditions and more moderate temperatures can favour the mite. Additionally, the micro-sprinkler mounted within each tree's canopy makes the environment in tree canopies more suitable for mite development and persistence. In Greece, the Persea mite rapidly spread across nearly all regions where avocados are cultivated in the western and coastal part of Crete (Bitsakis *et al.*, 2026). In drier regions, far from the coast, with high temperatures and low humidity throughout more of the growing season, *O. perseae* did not persist (significant numbers between October and November) and less damage was observed.

Other recorded pests included the yellow cottony cushion scale, *Icerya seychellarum* (Westwood) (Hemiptera: Coccomorpha: Monophlebidae) and the citrus leafhopper, *Penthimiola bella* (Stål) (Hemiptera, Cicadellidae) were found infesting the same area of leaf with *O. perseae* (Fig. 4).

### Natural enemies

Natural enemies of *O. perseae* recorded during this study included *Euseius stipulatus* (Athias-Henriot) and *Typhlodromus phialatus* (Athias-Henriot) (Acari: Phytoseiidae) and *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae), with very low numbers on both on *O. perseae*-infested leaves and non-infested leaves. Smaili *et al.* (2020, 2025) listed divers beneficial species, including Phytoseiidae species, in Morocco and gave insights into their potential use as biocontrol agents. In Morocco, most avocado orchards are planted on sandy soil and are overcrowded, with insufficient light for supporting wild herbaceous ground cover that support natural enemy populations. This is likely one reason natural enemy species diversity and abundance within herbaceous ground covers is often very low.

In Israel and Spain, maize pollen that supports natural enemies was sprayed on avocado leaves and contributed to increased *E. stipulatus* and *Euseius scutalis* (Athias-Henriot) populations and reduced *O. perseae* infestations (Maoz *et al.*, 2011b; Torres *et al.*, 2024). In addition, Maoz *et al.* (2011b) reported that in Israel, field trials of Rhodes grass (*Chloris gayana* Kunth) cover crop provided windborn pollen that helped conserve native *E. scutalis* populations was a highly effective and sustainable approach to *O. perseae* management. Similarly, in Spain, González-Fernández *et al.* (2009) planted maize plants between rows of avocado trees to support natural enemies of *O. perseae*. The same authors reported that laboratory experiments demonstrated that *Neoseiulus californicus* (McGregor) (Mesostigmata: Phytoseiidae) feeds inside prey nests, whereas *E. stipulatus* does not, thus reducing the competition among predators.

## Conclusions

*Oligonychus perseae* was found at several avocado orchards in Morocco at low to high leaf infestation levels, mainly between October and May. Infestation was observed only on leaves especially in coastal avocado production areas in north and west Morocco. The expansion of Persea mite to new regions and higher infestation levels may constitute a threat to fruit and yield in these commercial avocado orchards. Further research is necessary to detect and prevent the spread of *O. perseae* to new avocado growing areas in Morocco, as the impact of the climatic change and avocado farming norms that reinforce overcrowded orchards

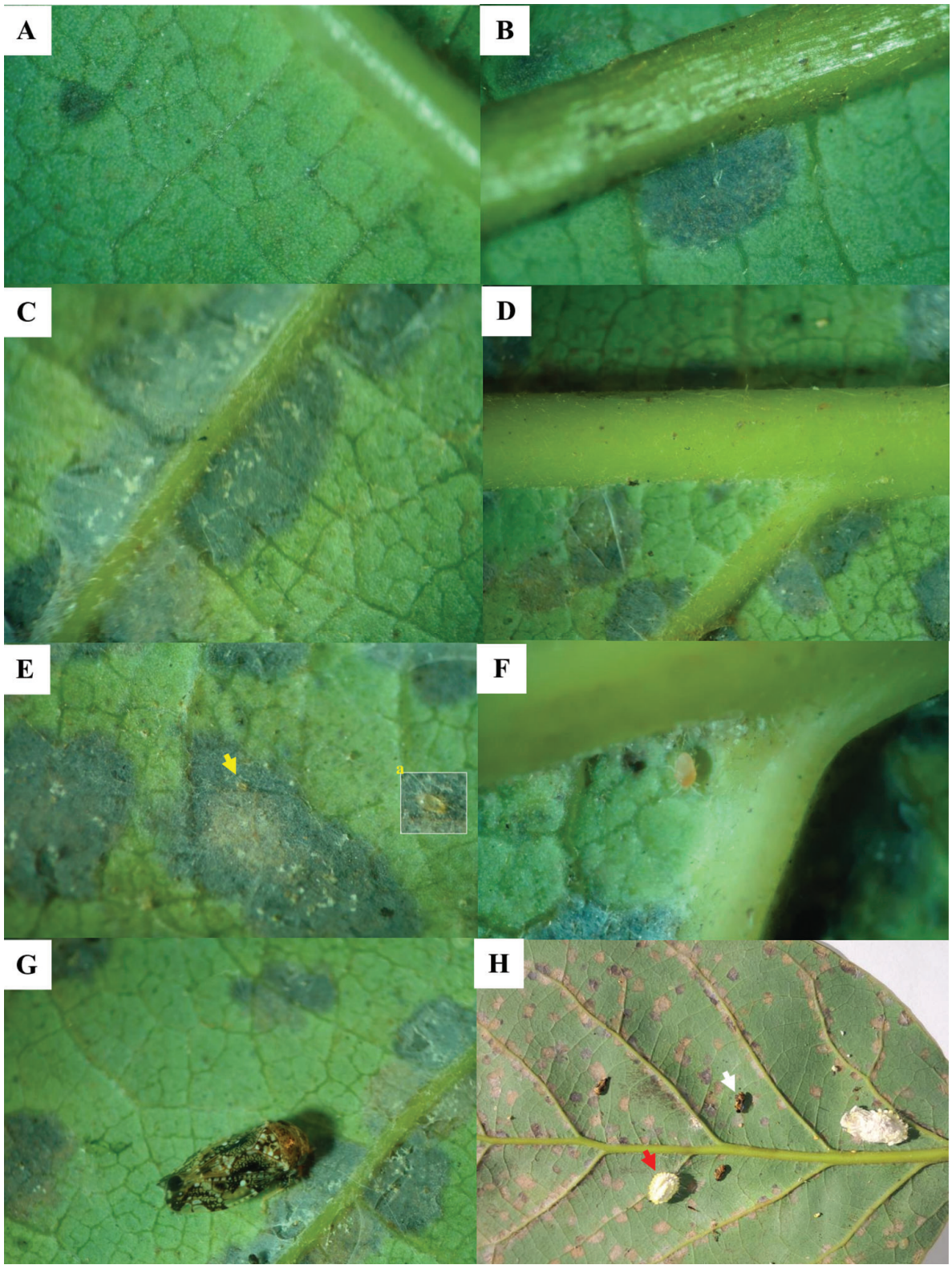


**Figure 4.** *Oligonychus perseae* (Tuttle, Baker and Abatiello). A. Tiny nest on underside Leaf; B. Nest next to the midrib of leaf; C. Webb on nest; D. Nest next to the second vein; E. Presence of mite Tarsonemidae species (a) in the nest between two veins (yellow arrow); F. *Lorryia formosa* (Cooreman) (Acari: Tydeidae) next to nest of the Persea mite. G. High *O. perseae*-infested avocado leaf with the presence of the citrus leafhopper *Penthimiola bella* (Stål). H. Leaf underside with the presence of the yellow cottony cushion scale, *Icerya seychellarum* (Westwood) (red arrow), *P. bella* adult (white arrow) and nests of the Persea mite (red arrow). Photographs by O Tounia and MC Smaili.

and associated high humidity microclimatic conditions might support increased pest population. Research should include spacing, pruning, and use of cover crops to support natural enemies in developing an integrated pest management program against the Persea mite.

***Conflict of Interest***

*The authors declare no conflict of interest.*

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# Υφιστάμενη κατάσταση και διασπορά του ακάρεως *Oligonychus perseae* σε προσβεβλημένα δένδρα αβοκάντο στο Μαρόκο

O. Tounia, A.Boutaleb-Joutei, N. Haddad, A.El Gada, R. Lahlali και M.C. Smaili

**Περίληψη** Επισκοπήσεις για την αξιολόγηση της υφιστάμενης κατάστασης και διασποράς του ακάρεως *Oligonychus perseae* Tuttle, Baker και Abatiello (Acari: Tetranychidae) και των φυσικών εχθρών του διεξήχθησαν σε 85 οπωρώνες αβοκάντο στο βορειοδυτικό Μαρόκο μεταξύ Οκτωβρίου 2023 και Οκτωβρίου 2025. Το άκαρι καταγράφηκε σε φύλλα σε αρκετούς οπωρώνες με χαμηλή έως υψηλή προσβολή, ενώ δεν εντοπίστηκε σε καρπούς. Πραγματοποιήθηκε μορφολογική ταυτοποίηση του *O. perseae* με βάση δείγματα από το Μαρόκο. Προς το παρόν, το άκαρι δεν προκαλεί οικονομική ζημιά στους καρπούς αβοκάντο στο Μαρόκο, αλλά η εγκατάστασή του σε νέες περιοχές εντός της χώρας και η αύξηση του πληθυσμού του στα φύλλα μπορεί να αποτελέσει απειλή για τους καρπούς και την απόδοση στην εμπορική καλλιέργεια του αβοκάντο στο μέλλον.

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# Behavioral responses of a herbivorous mite and its potential predatory mirid to synthetic plant volatiles

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**Summary** Herbivore-induced plant volatiles (HIPVs) mediate tritrophic interactions among plants, herbivores and their natural enemies. Using Y-tube olfactometer bioassays, we evaluated the behavioral responses of the zoophytophagous predator *Macrolophus pygmaeus* (Hemiptera: Miridae) and the herbivorous mite *Tetranychus urticae* (Acari: Tetranychidae) to ten synthetic volatile compounds associated with HIPV blends emitted by pepper (Capsicum annuum) plants under herbivore attack. Each compound (methyl salicylate [MeSA], farnesene, [mix of isomers] decanal, (+)-3-carene, (–)-β-pinene, (1S)-(–)-α-pinene, nonanal, hexanal, (Z)-3-hexenyl acetate, and 1H-indole) was tested at three concentrations (1, 10 and 100 ppm) against a hexane solvent control. At 100 ppm, *M. pygmaeus* was significantly attracted to MeSA and farnesene and significantly repelled by (+)-3-carene; neither attractant elicited a response at 10 ppm. *Tetranychus urticae* showed no attraction to any compound but was strongly repelled by (+)-3-carene at all three concentrations tested. Results demonstrate that HIPVs can differentially influence predator and herbivore behaviour in a dose-dependent manner. The identification of (+)-3-carene as a potent repellent for *T. urticae* and of MeSA and farnesene as attractants for *M. pygmaeus* provides a basis for volatile-based IPM strategies that integrate pest deterrence with natural enemy attraction within the crop, which could represent a practical tool supporting conservation biological control in pepper.

*Additional Keywords*: herbivore-induced volatiles, integrated pest management, predator recruitment, tritrophic interactions

## Introduction

Plants have developed a wide range of defense strategies against herbivores, generally categorised as direct or indirect. Direct defenses include physical barriers, such as trichomes and thickened cuticles, as well as chemical traits, including secondary metabolites such as alkaloids and phenolic compounds, and defense proteins such as protease inhibitors, all of which decrease plant palatability or impair herbivore fitness. Indirect defenses operate by producing and emitting chemical signals that recruit natural enemies of herbivores, primarily volatile organic compounds (VOCs) released into the surrounding environment (Dicke and Sabelis, 1988; Vet and Dicke, 1992). These defenses can be constitutive (always present at baseline levels) or induced in response to damage (Karban and Baldwin, 1997; Heil and Baldwin, 2002).

Plants emit a complex mixture of VOCs, including terpenoids, green leaf volatiles (GLVs), benzenoids, and amino acid derivatives, whose composition is influenced by plant species, developmental stage, and environmental factors (Dudareva *et al*., 2006; Holopainen and Gershenzon, 2010). During herbivore attack, plants produce a specific subset of these compounds, designated herbivore-induced plant volatiles (HIPVs), generated via active biosynthetic pathways triggered by elicitors in herbivore secretions and saliva (Hare, 2011).

HIPVs serve as informational signals within tritrophic interactions, transmitting cues regarding plant condition and the presence and nature of attacking herbivores to natural enemies such as parasit-

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oids, predatory mites, and insects (Dicke *et al*., 1990; Turlings *et al*., 1990). Through these tritrophic interactions, plants can indirectly reduce herbivore pressure by facilitating the location and recruitment of natural enemies (Turlings and Erb, 2018; Otto *et al*., 2025). Prior exposure to HIPVs can prime plant defenses, enabling faster and stronger responses to subsequent attack (Riahi *et al*., 2022). Nevertheless, the composition of HIPV blends exhibits substantial variability: both the plant species and the identity of the attacking herbivore influence the qualitative and quantitative composition of the emitted blend (Geervliet *et al*., 1997; Van den Boom *et al*., 2004), and infestation intensity can further modify the chemical profile (Ayelo *et al*., 2021). This specificity means that responses observed in a particular plant-herbivore pairing cannot be broadly generalised, and that HIPVs may not always confer an indirect defensive advantage, at times attracting generalist herbivores or failing to elicit the expected responses in natural enemies (Peñaflor and Bento, 2013).

GLVs are key categories within HIPV blends, which are rapidly emitted upon cell membrane disruption via the lipoxygenase pathway and form part of the general wound response, as well as monoterpenes and sesquiterpenes whose biosynthesis is more specifically linked to herbivore recognition (Matsui, 2006). The production of GLVs is primarily regulated through the jasmonic acid (JA) and salicylic acid (SA) signaling pathways, JA predominantly activated by tissue-damaging herbivores such as chewing insects and cell-content feeders, while SA more strongly associated with responses to phloem-feeding insects and biotrophic pathogens, though neither pathway is exclusive to a single attacker type and substantial overlap occurs (Dicke and-Baldwin, 2010; Thaler *et al*., 2012). Crosstalk between these pathways enables plants to fine-tune their volatile emission profile in response to the identity of specific attackers, producing qualitatively distinct HIPV blends that reflect the nature of the biotic challenge (Zhang *et al*., 2020).

Pepper (*Capsicum annuum*) is an economically important vegetable crop cultivated worldwide and is susceptible to a broad range of herbivorous pests, making it a relevant system for studying arthropod-induced plant defense responses (Florencio-Ortiz *et al*., 2018). Among the key pests affecting pepper, the two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) is a polyphagous herbivore of major economic significance. In tomato, *T. urticae* feeding has been shown to activate both the JA and SA pathways and to trigger the emission of volatile terpenoids and methyl salicylate, components of indirect defense (Kant *et al*., 2004; Alba *et al*., 2015). Analogous responses have been directly demonstrated in pepper: *T. urticae* infestation of *C. annuum* induced both JA and SA signaling, substantially altered the volatile emission profile, including a pronounced increase in methyl salicylate emission, and enhanced the attractiveness of infested plants to predatory arthropods (Zhang *et al*., 2020).

The zoophytophagous predatory mirid *Macrolophus pygmaeus* (Rambour) (Hemiptera: Miridae) is a commercially deployed biological control agent widely used in greenhouse cropping systems, known to exploit volatile cues to locate herbivore-infested plants and orient toward suitable prey (Lins *et al*., 2014; Pérez-Hedo *et al*., 2018a). Beyond direct predation, the phytophagy of *M. pygmaeus* also triggers the release of plant volatiles that can recruit additional natural enemies, generating cascading tritrophic effects that amplify its biocontrol value (Zhang *et al*., 2022). Moreover, the volatile blend induced by mirid phytophagy has been shown to influence the colonization behavior of subsequent herbivores on the same plant (Silva *et al*., 2021a), suggesting that these predators contribute to crop protection through multiple, interconnected chemical mechanisms. However, whereas the responses of *M. pygmaeus* to complex plant volatile blends have been characterised in tomato-based systems (Pérez-Hedo *et al*., 2018b), its behavioral responses to individually tested synthetic HIPV com-

pounds from pepper remain poorly understood. The volatile blend emitted by *Capsicum annuum* plants under arthropod attack has been characterised in response to multiple herbivore guilds, including zoophytophagous mirids (Bouagga *et al*., 2018), the spider mite *T. urticae* (Zhang *et al*., 2020; Zhang *et al*., 2023), thrips (Esmaeily *et al*., 2025), aphids (Ali *et al*., 2022), and lepidopteran larvae (Cardoza and Tumlinson, 2006, Yuan *et al*., 2022), revealing a chemically diverse HIPV repertoire spanning green leaf volatiles, monoterpenes, sesquiterpenes, aliphatic aldehydes, and aromatic compounds. Whether individual constituents of this blend differ in their capacity to attract or repel *M. pygmaeus* and *T. urticae* remains to be established.

Interest in using HIPVs as tools for integrated pest management (IPM) has grown significantly. Based on push-pull principles, where stimuli are employed to make the protected resource less attractive to pests while guiding them toward alternative, appealing sources (Cook *et al*., 2007), the strategic use of synthetic HIPVs to both repel herbivorous pests and attract natural enemies is seen as a promising volatile-based IPM strategy (Peñaflor and Bento, 2013). Methyl salicylate (MeSA) has been shown in field tests to attract beneficial arthropods, including predatory insects and parasitoids (James, 2003), whereas (E)-*β*-farnesene, an aphid alarm pheromone, acts as a kairomone, drawing predators and parasitoids to aphid-infested plants (Francis *et al*., 2004). However, behavioral responses to individual VOCs depend heavily on dose and context and can differ markedly between single compounds and complex blends (Kaplan, 2012, Holopainen and Blande, 2013). Therefore, systematic testing of these compounds under controlled conditions is crucial for their effective and informed use in volatile-based IPM approaches.

This study aimed to analyse how *M. pygmaeus* and *T. urticae* respond to ten plant-derived volatile compounds chosen from the pepper HIPV blend, tested with Y-tube olfactometer bioassays at three different concentrations. We hypothesised that specific compounds in the pepper HIPV blend would affect the two species differently, acting as attractants for the predator *M. pygmaeus* and as repellents for the herbivore *T. urticae*, and that these effects would depend on concentration. This could inform targeted use of individual volatiles in biological control and volatile-based IPM strategies based on push-pull principles.

## Materials and Methods

### Insects

The *M. pygmaeus* colony was established from approximately 250 individuals obtained from the commercially available product MACROLOPHUS (Bio-insecta, Greece). The colony was maintained on young tomato plants (*Solanum lycopersicum* L., cv. Ace 55, 2-3 weeks old) in 200 mL plastic pots with a peat substrate (Klasmann-TS2), housed in insect-proof cages (47.5 × 47.5 × 47.5 cm, BugDorm, MegaView Science Co., Ltd.) under controlled conditions: 25 ± 2°C, 16:8 h photoperiod (L:D), and 65 ± 5% relative humidity (RH). A constant supply of *Ephestia kuehniella* (Lepidoptera: Pyralidae) eggs on posted strips was provided as a food supplement, with bee pollen added weekly.

In all experiments, young adult females, aged 7 to 10 days, were used. Before the bioassays, females were individually starved for 24 hours in 4 cm-diameter plastic containers with mesh lids, each containing a small piece of moistened cotton to maintain humidity. During starvation, the insects were maintained at 25 ± 2°C, with a 16:8-hour light: dark cycle and a relative humidity of 60 ± 5%.

### Spider mites

The *T. urticae* colony was established from approximately 700 mites collected from field-infested tomato plants and maintained in the laboratory for several years. A sub colony of the spider mite population was established and maintained on pep-

per plants (*C. annuum* L., cv. П13) for several generations before the bioassays. The colony was maintained on intact pepper plants potted in 12 cm diameter plastic pots with peat substrate (Klasmann-TS2), in insect-proof cages (47.5 × 47.5 × 47.5 cm, BugDorm, MegaView Science Co., Ltd.) under: 25 ± 2°C, 16:8 h L:D, 60–70% RH.

Adult inseminated females approximately 5 days old were used in the bioassay. A starvation period of 24 hours before bioassays was used, during which the adult females were individually placed in 1.5 mL Eppendorf tubes. The opening of the Eppendorf was covered with thin mesh to facilitate proper ventilation, whereas at the tip of the tube, a small hole was drilled to allow contact with a water-soaked cotton wool layer used to maintain proper humidity.

## Synthetic compounds

Ten compounds were selected to represent the major chemical classes documented in the volatile blend of *Capsicum annuum* plants under arthropod attack (aliphatic aldehydes, green leaf volatile esters, aromatic compounds, monoterpenes, and sesquiterpenes) on the basis of their occurrence in pepper HIPV profiles characterised in response to mirid phytophagy (Bouagga *et al*., 2018), *T. urticae* infestation (Zhang *et al*., 2020; Zhang *et al*., 2023), *Spodoptera litura* (Lepidoptera: Noctuidae) herbivory (Yuan *et al*., 2022), *Frankliniella occidentalis* (Thysanoptera: Thripidae) infestation (Esmaeily *et al*., 2025), and *M. persicae* infestation (Ali *et al*., 2022), with the exception of nonanal, for which a direct pepper-specific herbivore-induced reference was not identified in the available literature; its inclusion reflects its chemical class representativeness as a C9 aliphatic aldehyde produced via the lipoxygenase pathway in herbivore-damaged Solanaceous plants (Matsui, 2006, Hare, (2011). The compounds belong to five chemical groups: (1) aliphatic aldehydes: hexanal (≥98%; TCI, Japan), nonanal (≥97%; Sigma-Aldrich, Germany), and decanal (≥97%; TCI, Japan); (2) green leaf volatiles: (Z)-3-hexenyl acetate (≥98%; Sigma-Aldrich, Germany); (3) aromatic compounds: methyl salicylate (MeSA; ≥99%; Sigma-Aldrich, Germany) and 1H-indole (≥98%; Sigma-Aldrich, Germany); (4) monoterpenes: (+)-3-carene (≥90%; TCI, Japan), (–)-*β*-pinene (≥94%; TCI, Japan), and (1S)-(–)-*α*-pinene (≥97%; TCI, Japan); and (5) sesquiterpenes: farnesene, mixture of isomers (≥80%; Sigma-Aldrich, Germany). Details of all compounds are summarised in Table 1.

For each liquid compound, a primary stock solution was prepared by dissolving 10 µL of the neat compound (measured using a calibrated 100 µL glass pipette) in hexane to a final volume of 10 mL. Given its higher density and to achieve a tractable working range, farnesene was prepared using 100 µL of neat compound in 10 mL hex-

**Table 1**. Synthetic volatile compounds tested in Y-tube olfactometer bioassays with their chemical group, purity, and supplier.

| Compound | Chemical group | Purity | Supplier |
|---|---|---|---|
| Hexanal | Aliphatic aldehyde | ≥98% | TCI, Japan |
| Nonanal | Aliphatic aldehyde | ≥97% | Sigma-Aldrich, Germany |
| Decanal | Aliphatic aldehyde | ≥97% | TCI, Japan |
| (Z)-3-Hexenyl acetate | Green leaf volatile ester | ≥98% | Sigma-Aldrich, Germany |
| Methyl salicylate (MeSA) | Aromatic compound | ≥99% | Sigma-Aldrich, Germany |
| 1H-Indole | Aromatic compound | ≥98% | Sigma-Aldrich, Germany |
| (+)-3-Carene | Monoterpene | ≥90% | TCI, Japan |
| (–)-β-Pinene | Monoterpene | ≥94% | TCI, Japan |
| (1S)-(–)-α-Pinene | Monoterpene | ≥97% | TCI, Japan |
| Farnesene (mixture of isomers) | Sesquiterpene | ≥80% | Sigma-Aldrich, Germany |

ane. Resulting stock concentrations, calculated from compound density, ranged from approximately 800 to 1174 mg/L for most compounds and 8790 mg/L for farnesene. 1H-indole, being a solid at room temperature, was prepared by dissolving 1 mg in 10 mL of hexane to give a stock concentration of 100 mg/L. Working solutions at 1, 10, and 100 ppm (mg/L) were prepared by serial dilution of the respective stock solutions in hexane to a final volume of 10 mL. These concentrations were selected to span a biologically relevant range enabling characterization of dose-dependent behavioral responses, consistent with concentration ranges used in comparable olfactometer studies (Silva *et al*., 2021b; Ayelo *et al*., 2021), rather than to replicate specific field residue levels, which vary considerably with plant species, herbivore load, and environmental conditions (Holopainen and Blande, 2013). All solutions were stored in sealed glass vials at 4°C in the dark until use and freshly prepared on the day of each experiment. The quantity of active ingredient deposited per replicate corresponded to 1000, 100, and 10 ng for the 100, 10, and 1 ppm working solutions, respectively, calculated from 10 µL of the applied working solution. The hexane control consisted of 10 µL of pure hexane applied under identical conditions.

## Olfactometer assays

Behavioral responses of *M. pygmaeus* and *T. urticae* to the ten synthetic volatile compounds were assessed in a glass Y-tube olfactometer (4 cm internal diameter; main arm 25 cm long, lateral arms 17 cm long, 60° angle between lateral arms). Each lateral arm was connected via Teflon tubing to a 4 L glass container serving as an odor-source chamber: one containing the test compound and the other containing the hexane control. Compressed air was supplied by a low-pressure compressor, first passing through an activated carbon filter and a humidification flask to remove background contaminants, before entering the glass chambers and the olfactometer. Airflow was adjusted to 1.2 L/min per arm using a flowmeter (KYTOLA, Finland). For each odor source, 10 µL of the test compound or hexane control was applied by pipette onto a filter paper strip (1 × 2 cm) placed in a glass Petri dish on a sheet of aluminum foil. The loaded strip was left to stand for 2 min in the open air to allow solvent evaporation before being transferred to the bottom of the respective glass chamber. All Y-tube experiments were conducted between 10:00 and 17:00 h, at 25 ± 2 °C and 70 ± 10% RH.

A single female individual was introduced at the base of the main arm downwind using an aspirator (*M. pygmaeus*) or a fine brush (*T. urticae*) and observed for up to 10 min, following the general procedure described by Lins *et al.* (2014). Individuals were considered to have made a choice when they moved at least 3 cm into one of the lateral arms. Individuals who did not choose a lateral arm within 10 min were classified as unresponsive and excluded from analysis. Each individual was tested only once. After every 10 replicates, the lateral arm positions were switched to minimize positional bias. After every 20 replicates, the Y-tube and glass chambers were washed with unscented soap and acetone and left to dry completely, and the filter paper sources were replaced. For each treatment (compound and concentration), at least 40 individuals were tested.

Bioassays began at the highest concentration (100 ppm). Bioassays at lower concentrations (10 and 1 ppm) were conducted only when a statistically significant response had been observed at the preceding higher concentration. For *M. pygmaeus*, sequential concentration testing was applied exclusively to compounds eliciting statistically significant attraction at the preceding concentration; repellent responses were not evaluated at lower concentrations, as the sequential protocol was designed specifically to characterise the concentration threshold of predator attraction.

## Data analysis

Data were analysed using the chi-square goodness-of-fit test ($\chi^2$) to assess whether

the proportion of individuals choosing each arm differed significantly from a 50:50 random distribution (α = 0.05). Analyses were performed independently for each compound, concentration, and species. Statistics were performed using IBM Corp. (2020).

## Results

Responses of *M. pygmaeus*

At 100 ppm, female *M. pygmaeus* showed a significant preference for two volatile compounds, farnesene and MeSA, over the hexane control, and a significant avoidance of (+)-3-carene (farnesene vs. hexane: $\chi^2$ = 4.26, P = 0.039; MeSA vs. hexane: $\chi^2$ = 5.33, P = 0.021; (+)-3-carene vs. hexane: $\chi^2$ = 17.36, P < 0.001) (Fig. 1). In accordance with the sequential testing protocol, which applied to attractant responses in *M. pygmaeus* only, the repellent response to (+)-3-carene was not evaluated at lower concentrations. No significant positive or negative responses were observed for the remaining seven volatile compounds.

Consequently, only the two compounds that elicited a positive response at 100 ppm (MeSA and farnesene) were further evaluated at a lower concentration (10 ppm). However, at this concentration, female *M. pygmaeus* did not show a significant preference for either compound (MeSA vs. hexane: $\chi^2$ = 0.45, P = 0.501; farnesene vs. hexane: $\chi^2$ = 0.20, P = 0.654) (Fig. 2). Therefore, testing was not extended to 1 ppm for these compounds.

Responses of *T. urticae*

At 100 ppm, female *T. urticae* showed no attraction to any of the ten compounds tested. A significant avoidance response was observed for (+)-3-carene relative to the hexane control ((+)-3-carene vs. hexane: $\chi^2$ = 28.06, P < 0.001) (Fig. 3). No other compound elicited a significant positive or negative response.

Given this result, (+)-3-carene was further evaluated at lower concentrations to characterise the dose-response relationship. The avoidance effect persisted at 10

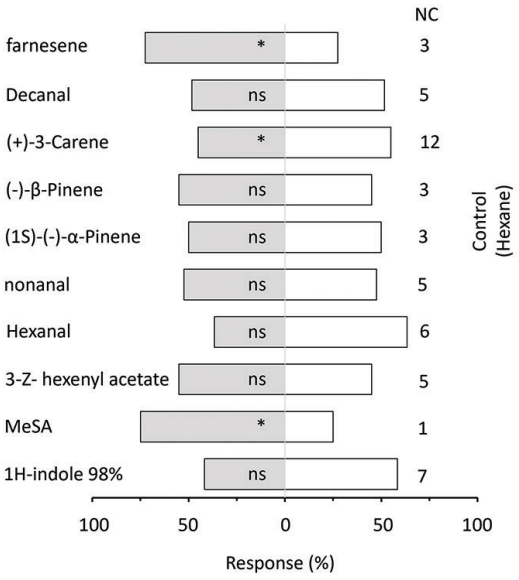


**Figure 1**. Behavioral responses of *Macrolophus pygmaeus* adult females in a Y-tube olfactometer to ten synthetic volatile compounds associated with the herbivore-induced plant volatile (HIPV) blend of *Capsicum annuum*, each tested at 100 ppm against a hexane solvent control. Grey bars represent the percentage of responding females choosing the test compound arm; white bars represent the percentage choosing the hexane control arm. An asterisk indicates significant preference ($\chi^2$ test: P<0.05). NC= indicates the number of mirids that did not make a choice; ns= nonsignificant; MeSA= methyl salicylate.

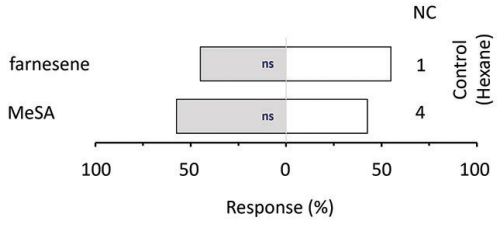


**Figure 2**. Behavioral responses of *Macrolophus pygmaeus* adult females in a Y-tube olfactometer to two synthetic volatile compounds associated with the herbivore-induced plant volatile (HIPV) blend of *Capsicum annuum*, each tested at 10 ppm against a hexane solvent control. Grey bars represent the percentage of responding females choosing the test compound arm; white bars represent the percentage choosing the hexane control arm. An asterisk indicates significant preference ($\chi^2$ test: P<0.05). NC= indicates the number of mirids that did not make a choice; ns= nonsignificant; MeSA= methyl salicylate.

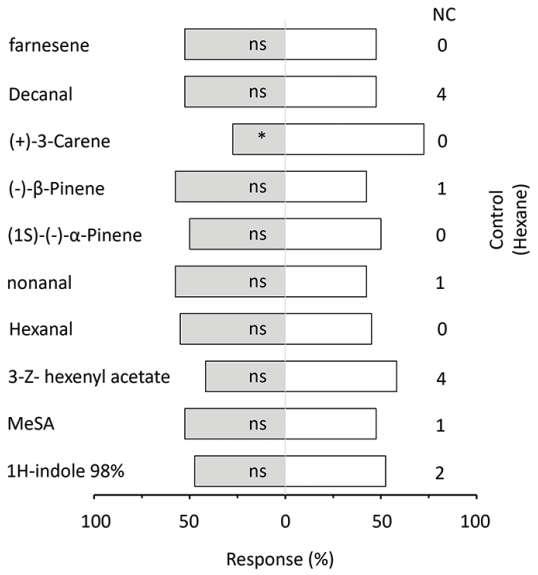


**Figure 3**. Behavioral responses of *Tetranychus urticae* adult females in a Y-tube olfactometer to ten synthetic volatile compounds associated with the herbivore-induced plant volatile (HIPV) blend of *Capsicum annuum*, each tested at 100 ppm against a hexane solvent control. Grey bars represent the percentage of responding females choosing the test compound arm; white bars represent the percentage choosing the hexane control arm. An asterisk indicates significant preference ($\chi^2$ test: P<0.05). NC= indicates the number of spider mites that did not make a choice; ns= nonsignificant; MeSA= methyl salicylate.

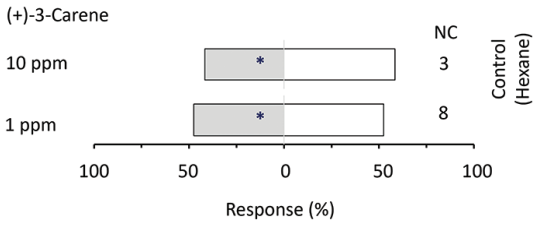


**Figure 4**. Behavioral responses of *Tetranychus urticae* adult females in a Y-tube olfactometer to (+)-3-Carene, tested at 10 and 1 ppm against a hexane solvent control. Grey bars represent the percentage of responding females choosing the tested compound arm; white bars represent the percentage choosing the hexane control arm. An asterisk indicates significant preference ($\chi^2$ test: P<0.05). NC= indicates the number of spider mites that did not make a choice; ns= nonsignificant; MeSA= methyl salicylate.

ppm ($\chi^2$ = 28.63, P < 0.001) and remained significant at 1 ppm ($\chi^2$ = 16.15, P < 0.001) (Fig. 4), demonstrating consistent repellent activity across the entire concentration range tested.

## Discussion

The present study demonstrated that *M. pygmaeus* and *T. urticae* respond differently to individual volatile compounds. *Macrolophus pygmaeus* was significantly attracted to MeSA and farnesene and repelled by (+)-3-carene at 100 ppm, whereas *T. urticae* showed no attraction to any compound tested but was consistently repelled by (+)-3-carene across all three concentrations evaluated. These results are consistent with the established role of HIPVs as mediators of tritrophic plant-herbivore-natural enemy interactions, first demonstrated for parasitic wasps by Turlings *et al.* (1990) and subsequently extended to a broad range of arthropod natural enemies (Turlings and Erb, 2018). They also add to existing knowledge by characterizing the behavioral responses of the zoophytophagous mirid predator and its mite prey to individually tested synthetic volatiles related to the pepper HIPV blend, underscoring the importance of evaluating individual compounds in isolation rather than inferring behavioral activity from blend composition alone.

The attraction of *M. pygmaeus* to MeSA is consistent with its well-established role as a semiochemical for natural enemy recruitment across a broad range of arthropod communities (James, 2003; James and Price, 2004; Mallinger *et al*., 2011). MeSA emission is typically triggered by activation of the shikimate pathway following phytophagy by zoophytophagous predators, herbivore feeding, or pathogen attack (Pérez-Hedo *et al*., 2018a; Silva *et al*., 2021b). Characterization of HIPVs induced by *M. pygmaeus* and *Nesidiocoris tenuis* (Hemiptera: Miridae) phytophagy in tomato identified MeSA as one of the major differential volatile peaks alongside several GLVs, confirming its relevance as a chemical signal associated with mirid activity on Solanaceous crops (Pérez-Hedo *et al*., 2018a). Silva *et al*. (2021b) further

demonstrated through Y-tube olfactometer trials with synthetic compounds that MeSA as well as (Z)-3-hexenyl propanoate, and (Z)-3-hexenyl acetate attracted *M. pygmaeus*, suggesting that both aromatic and fatty acid-derived volatile esters function as attractants for predatory mirids. The present results support MeSA's attractiveness to *M. pygmaeus* in the pepper system. The absence of a significant response to (Z)-3-hexenyl acetate in pepper may reflect plant species effect/differentiation and/or concentration-dependent threshold effects, given that the two studies used different dilution protocols, or intrinsic differences between the predator populations tested, including possible effects of colony origin and rearing conditions on olfactory sensitivity. Both studies used naïve, previously unexposed adult females, so differential prior experience cannot account for the discrepancy.

To the best of the authors' knowledge, no prior study has demonstrated that a mirid predator is attracted to farnesene as an individually tested synthetic compound. Maselou *et al*. (2019) reported that *M. pygmaeus* nymphs were significantly attracted to *Myzus persicae*-(Hemiptera: Aphididae) infested aubergine plants whose headspace contained (E)-*β*-farnesene among other induced volatiles; however, the individual behavioral contribution of farnesene was not evaluated in isolation. This is noteworthy given previous findings showing that the monoterpenes ocimene and limonene, and the sesquiterpene caryophyllene, did not attract *M. pygmaeus*, *N. tenuis*, or *Dicyphus bolivari* (Hemiptera: Miridae) in olfactometer assays with tomato-derived HIPVs (Silva *et al*., 2021b), suggesting that terpene attractiveness in mirid predators is highly compound-specific rather than a chemical class-level property. The commercial farnesene preparation used in the present study consists of a mixture of sesquiterpene isomers rather than a single geometric form (Wang *et al*., 2024). Identification of the specific isomers responsible for the observed attractant activity will be necessary before practical conclusions can be drawn, and comparison with the kairomonal activity of (E)-*β*-farnesene, which functions as the aphid alarm pheromone and has been reported to attract the coccinellid *Adalia bipunctata* (Coleoptera: Coccinellidae) (Francis *et al*., 2004), may help clarify the molecular basis of this response.

Attraction to both MeSA and farnesene was not sustained at 10 ppm, indicating that the behavioral responses of *M. pygmaeus* to these compounds are dose-dependent and may occur only above certain threshold concentrations. Dose-dependent responses to mirid volatiles have been reported by Ayelo *et al.* (2021), who found that individual monoterpenes attracted *N. tenuis* only at doses 100-fold above the natural emission rate of a single *T. absoluta*-infested tomato plant, equivalent to the simultaneous release from 100 plants, whereas lower doses were ineffective. This threshold effect aligns with the broader literature, which shows that arthropod behavioral responses to plant volatiles are strongly concentration-dependent, with both the magnitude and direction of responses varying with dose (Ayelo *et al*., 2021). The absence of a response at 10 ppm does not preclude the ecological relevance of MeSA and farnesene, as natural HIPV concentrations in the field vary considerably with plant species and herbivore load (Geervliet *et al*., 1997; Van den Boom *et al*., 2004), as well as with environmental conditions, including wind speed, and temperature that govern volatile plume dispersion and persistence (Holopainen and Blande, 2013). Additionally, the inexperienced adult females used in this study may represent a conservative estimate of responsiveness. Lins *et al.* (2014) demonstrated that *N. tenuis* individuals were attracted to HIPVs only after prior experience with the odor source, and similar learning effects have been documented in anthocorid predators (Drukker *et al*., 2000) and predatory mites (de Boer *et al*., 2005).

The most consistent finding of this study was the strong, persistent avoidance of (+)-3-carene by both *M. pygmaeus* and *T. urticae*, with the repellent effect in *T. urticae* maintained across all three concentra-

tions evaluated (100, 10, and 1 ppm). This response is of particular ecological interest given that 3-carene has been reported to attract other mirid predators under different experimental conditions. Ayelo *et al*. (2021) found that 3-carene attracted *N. tenuis* only at a dose approximately 100-fold above the natural emission rate of a single *T. absoluta*-infested tomato plant, with lower doses ineffective. The contrasting responses, i.e. repellency in *M. pygmaeus* and *T. urticae* in the present study versus attraction in *N. tenuis* in Ayelo *et al.* (2021), may reflect species-specific differences in olfactory receptor sensitivity and behavioral ecology among mirid predators or differences in the plant-herbivore system. It should also be noted that both the present study and Ayelo *et al*. (2021) evaluated compounds individually, and responses to single compounds do not necessarily predict behavioral outcomes when the same compounds are presented as components of complex volatile mixtures (Mumm and Dicke, 2010). Nevertheless, Ayelo *et al.* (2021) found a repellence effect from (E)-*β*-caryophyllene to *N. tenuis* at the highest dose tested, demonstrating that terpenoids can elicit avoidance responses in mirid predators and that the deterrent potential of this chemical class is compound- and species-specific.

The consistent avoidance of (+)-3-carene by *T. urticae* across a two-orders-of-magnitude range of concentrations suggests a robust, dose-persistent deterrent signal, potentially indicating a plant chemical cue associated with unsuitable or overexploited host tissue. Considering that (+)-3-carene was detected in the headspace volatile profile of *C. annuum* leaves infested by *T. urticae* (Zhang *et al*., 2023), its repellent effect in the present study supports the hypothesis of an induced volatile direct defense function against the attacking herbivore in pepper, although this interpretation requires direct experimental validation under plant-based conditions. Additionally, the repellent effect of 3-carene on *T. urticae* has been demonstrated through contact-based leaf-disc assays with essential oil components (Da Camara *et al*., 2015, Tak and Isman, 2017). These studies used enantiomerically unresolved material under contact conditions rather than in a volatile phase. Although not directly comparable, they still support the current result that this monoterpene acts as a repellent against *T. urticae*.

*Tetranychus urticae* showed no attraction to any of the ten compounds tested individually, including (1S)-(-)-*α*-pinene. This contrasts with Mérida-Torres *et al.* (2023), who reported that α-pinene at 10 ng (equivalent to the amount deposited on the filter paper at the lowest concentration tested in the present study) attracted *T. urticae* in olfactometer bioassays using a colony collected from strawberry plants. Several methodological and biological differences may account for this discrepancy. First, Mérida-Torres *et al.* (2023) did not specify the enantiomeric form of *α*-pinene used, suggesting a racemic or commercially unspecified preparation, whereas the present study employed the specific (1S)-(-) enantiomer, which differs substantially in biological activity. Second, the colony used in the present study was reared on pepper, whereas the Mérida-Torres *et al.* (2023) colony originated from strawberry. *Tetranychus urticae* populations adapted to different host plants are known to differ in their volatile-mediated responses (Maeda *et al.*, 2001). More broadly, the absence of attraction to any individually tested compound is consistent with the view that *T. urticae* host orientation is driven by the integrated perception of complex volatile blends rather than responses to single constituents (Mumm and Dicke, 2010).

Overall, the results demonstrate that individual HIPVs associated with pepper differ substantially in their capacity to influence the behaviour of *T. urticae* and *M. pygmaeus*, and that these effects are concentration-dependent and species-specific. The identification of MeSA and farnesene as attractants for *M. pygmaeus* at higher concentrations, combined with the consistent, dose-persistent repellency of (+)-3-carene toward *T. urticae*, provides a preliminary basis for ex-

ploring volatile-based strategies within integrated pest management. In the context of greenhouse pepper production, where *M. pygmaeus* is routinely introduced as a biological control agent, MeSA and farnesene could function as within-crop attractants to recruit and retain the released predator, analogous to the attract-and-reward concept in conservation biological control, in which synthetic HIPVs are used to draw natural enemies into crop environments and complementary resources sustain their populations (Simpson *et al*., 2011; Kaplan, 2012). Simultaneously, (+)-3-carene may serve as a deterrent volatile that reduces *T. urticae* colonisation pressure, creating a chemical push component that complements the predator-attracting pull. This combined push-attract approach, drawing on both push-pull principles (Cook *et al*., 2007; Peñaflor and Bento, 2013) and conservation biological control strategies, represents a conceptually coherent framework for the targeted deployment of individual pepper HIPVs in sustainable pest management.

Finally, it is important to consider a number of limitations of the current study. First, Y-tube olfactometer bioassays present stimuli under simplified, controlled conditions that do not capture the complexity of field exposure, including the heterogeneity of volatile plumes, the presence of competing background odors, and the influence of wind and temperature on volatile dispersion and persistence (Holopainen and Blande, 2013). Second, behavioral responses were assessed to individually tested compounds, whereas natural HIPV exposure in the field involves complex multicomponent blends; responses to single compounds do not necessarily predict outcomes in complex mixtures (Mumm and Dicke, 2010). Third, naive, previously unexposed females were used, and prior olfactory experience can substantially alter responsiveness in both mirid predators and predatory mites (de Boer *et al*., 2005; Lins *et al*., 2014). These considerations underscore the importance of validating the present findings under semi-field and field conditions. Specifically, future studies should evaluate whether MeSA and farnesene can recruit and retain *M. pygmaeus* within greenhouse pepper crops, and whether (+)-3-carene can reduce *T. urticae* colonisation under realistic agronomic conditions, accounting for volatile plume dynamics, competing stimuli, natural enemy dispersal, and the influence of repeated or prolonged exposure on behavioral responses.

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# Συμπεριφορικές αποκρίσεις ενός φυτοφάγου ακάρεως και του δυνητικού θηρευτή του Miridae σε συνθετικές πτητικές φυτικές ενώσεις

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**Περίληψη** Οι πτητικές ενώσεις που επάγονται από τα φυτά ως απόκριση στην τροφική δραστηριότητα φυτοφάγων εχθρών (HIPVs) επηρεάζουν τις τριτροφικές αλληλεπιδράσεις μεταξύ φυτών, φυτοφάγων και των φυσικών εχθρών τους. Στην παρούσα εργασία αξιολογήσαμε σε βιοδοκιμές με ολφακτομέτρο τύπου Y, τις συμπεριφορικές αποκρίσεις του ζωοφυτοφάγου αρπακτικού *Macrolophous pygmaeus* (Hemiptera: Miridae) και του φυτοφάγου ακάρεως *Tetranychus urticae* (Acari: Tetranychidae) σε δέκα συνθετικές πτητικές ενώσεις που σχετίζονται με μίγματα HIPVs που εκλύονται από φυτά πιπεριάς (*Capsicum annuum*) μετά την προσβολή τους από φυτοφάγους εχθρούς. Κάθε ένωση (Μεθυλικός εστέρας του σαλικυλικού οξέος [MeSA], φαρνεσένιο [μίγμα ισομερών], δεκανάλη, (+)-3-καρένιο, (–)-*β*-πινένιο, (1S)-(–)-*α*-πινένιο, νονανάλη, εξανάλη, οξικό (Z)-*3*-εξενυλ εστέρας του οξικού οξέος και 1H-ινδόλιο) αξιολογήθηκε σε τρεις συγκεντρώσεις (1, 10 και 100 ppm) έναντι του μάρτυρα (εξάνιο, διαλύτης των πτητικών ενώσεων). Στα 100 ppm, το *M. pygmaeus* έδειξε σημαντική προσέλκυση προς το MeSA και το φαρνεσένιο και σημαντική αποφυγή του (+)-3-καρενίου. Κανένα από τα δύο δεν προκάλεσε απόκριση στα 10 ppm. Ο κοινός τετράνυχος *T. urticae* δεν έδειξε προσέλκυση προς καμία ένωση, αλλά έντονη αποφυγή του (+)-3-καρενίου και στις τρεις συγκεντρώσεις που εξετάστηκαν. Τα αποτελέσματα δείχνουν ότι οι HIPVs μπορούν να επηρεάσουν διαφορετικά τη συμπεριφορά των αρπακτικών και των φυτοφάγων εχθρών με τρόπο που εξαρτάται από τη δόση εφαρμογής . Ο εντοπισμός του (+)-3-καρενίου ως ισχυρού απωθητικού για τον τετράνυχο *T. urticae*, και του MeSA και του φαρνεσενίου ως ελκυστικών για το *M. pygmaeus* διαμορφώνει πλαίσιο για στρατηγικές Ολοκληρωμένης Φυτοπροστασίας μέσω της εφαρμογής πτητικών ενώσεων, συνδυάζοντας την απώθηση φυτοφάγων με την προσέλκυση του φυσικού εχθρού τους εντός της καλλιέργειας, κάτι που θα μπορούσε να αποτελέσει ένα πρακτικό εργαλείο που υποστηρίζει τη βιολογική αντιμετώπιση μέσω της διατήρησης φυσικών εχθρών στην πιπεριά.

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# Winter survival of *Culex pipiens* biotype *pipiens* adults in Central Greece

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**Summary** Winter survival is a key life-history trait of insect vectors in temperate ecosystems, shaping early seasonal population growth and vector-borne disease epidemiology. For West Nile virus infection in Europe, including Greece, *Culex pipiens* is the principal vector. We conducted overwintering experiments in three areas in Central Greece, to assess winter survival of adult *Culex pipiens* biotype *pipiens* under coastal (Nea Anchialos and Volos) and inland (Kalamaki) conditions. A proportion of females survived winter at all sites, whereas males did not overwinter. Female survival reached 23.9% in Kalamaki and 25.3% in Nea Anchialos, while in Volos it ranged from 9.4 to 15.7% across treatments. Surviving females were able to blood-feed and oviposit in spring, producing viable offspring. The proportion of fertile females was 52.3% in Kalamaki and 47.4% in Nea Anchialos, whereas in Volos it ranged from 20.8 to 47.2%. Survival varied among locations and treatments, suggesting an effect of local environmental conditions on overwintering success. The results provide evidence that females of *Cx. pipiens* biotype *pipiens* can survive winter conditions in the study areas and subsequently initiate reproduction in spring. This study contributes to improving the understanding of the overwintering ecology of an important vector and highlights the need for further studies in different habitats.

*Additional keywords:* longevity, mosquitoes, population dynamics, Thessaly, vector borne diseases

## Introduction

In temperate and cold regions, mosquitoes have evolved a variety of overwintering strategies that allow populations to persist through unfavorable environmental conditions. Depending on the species, overwintering may occur at the egg, larval, or adult stage (Bale *et al.,* 2010; Nelms *et al.,* 2013; Denlinger and Armbruster, 2016; Becker *et al.,* 2020). Low temperatures and precipitation are among the most important environmental drivers determining the duration and intensity of overwintering, with effects that vary across latitudinal gradients and local climatic regimes (Gill *et al.,* 2017; ECDC, 2020; Rozsypal *et al.,* 2021; Siperstein *et al.,* 2023). In most temperate areas, winter conditions suppress mosquito reproduction and population growth, leading to substantial population declines prior to the onset of the spring generation (Siperstein *et al.,* 2023). From an epidemiological perspective, overwintering survival has been increasingly recognized as a critical determinant of early-season vector abundance and the re-establishment of arbovirus transmission cycles in temperate regions (Brugman *et al.,* 2018; Bellone and Failloux, 2020; Sauer *et al.,* 2022). The proportion of vectors that successfully overwinters can influence the timing and magnitude of population growth in spring, with important implications for the persistence and amplification of mosquito-borne pathogens.

The mosquito species *Culex pipiens* (Diptera: Culicidae) is considered the principal vector of West Nile virus (WNV) in Europe (Kang *et al.,* 2016; Brugman *et al.,* 2018; Rozsypal *et al.,* 2021), including Greece, where repeated outbreaks have been reported

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over the past two decades (Patsoula *et al.,* 2016, 2020; ECDC, 2021; Vakali *et al.,* 2023). The *Cx. pipiens* complex comprises two morphologically indistinguishable but ecologically distinct biotypes, "*pipiens*" (Linnaeus, 1758) and "*molestus*" (Forskal, 1775), as well as their hybrids. These biotypes differ in behavior, physiology, and overwintering biology, traits that may influence their seasonal dynamics and epidemiological importance (Becker *et al.,* 2012; Papadopoulos *et al.,* 2016; Koenraadt *et al.,* 2019; Vinogradova, 2000; Ioannou *et al.,* 2021).

In temperate regions, *Cx. pipiens* biotype *pipiens* overwinters as inseminated adult females that enter facultative reproductive diapause (Mitchell and Briegel, 1989; Denlinger and Armbruster, 2016; Dörge *et al.,* 2020; Rozsypal *et al.,* 2021). Diapause induction is primarily driven by decreasing temperature and photoperiod during late larval and pupal development (Denlinger and Armbruster, 2014; Field *et al.,* 2022). Adults' diapause triggers a chain reaction of molecular changes, including reduced insulin signaling and juvenile hormone deficiency, leading to ovarian arrest and increased metabolic lipid accumulation (Zhou and Miesfeld, 2009; Sharma *et al.,* 2019; Meuti, 2026). Diapausing females suppress host-seeking and reproductive activity and rely on accumulated lipid reserves to survive the winter months, whereas males do not enter diapause and typically fail to survive winter conditions (Denlinger and Armbruster, 2014; Vinogradova, 2000). With increasing temperatures in spring, females terminate diapause, resume blood feeding, and initiate oviposition, giving rise to the first seasonal generation. Optimal overwintering temperatures for adults' range between 2 and 6°C. Temperatures below 0°C are generally lethal to overwintering adults if they last for several days. Temperatures above 6°C lead to a gradual increase in metabolic activity, causing depletion of stored lipids and reduced winter survival (Dörge *et al.,* 2020; Bergman *et al.,* 2020; Kampen *et al.,* 2021; Rozypal *et al*., 2021).

Overwintering survival is strongly influenced by microclimatic conditions within hibernation sites, particularly temperature stability and humidity (Koenraadt *et al.,* 2019; Rozsypal *et al.,* 2021; Sauer *et al.,* 2022). Shelters that remain relatively frost-free, such as underground structures, barns, and other protected habitats, are commonly used by overwintering *Cx. pipiens* females. Mild winter conditions or frequent temperature fluctuations may increase metabolic activity and accelerate depletion of lipid reserves, thereby reducing survival, whereas cooler and more stable environments tend to enhance overwintering success (Koenraadt *et al.,* 2019; Rozsypal *et al.,* 2021; Sauer *et al.,* 2022). In southern temperate regions, including the Mediterranean basin, evidence of adult mosquito overwintering has been increasingly documented, highlighting the importance of local microclimatic conditions and human-made shelters (Ravasi *et al.,* 2022; Beleri *et al.,* 2023; Lührsen *et al.,* 2023).

In the present study, we investigated the overwintering capacity of *Cx. pipiens* biotype *pipiens* adults in three regions of Thessaly, Central Greece, encompassing both coastal and inland environments. Using field-based overwintering experiments, we aimed to (i) assess survival patterns of adult mosquitoes during the winter months, and (ii) determine whether overwintering females are capable of resuming blood feeding and oviposition in spring, thereby contributing to the initiation of the first seasonal generation. By providing field data, this study contributes to a better understanding of the overwintering ecology of *Cx. pipiens* biotype *pipiens* in southern temperate regions and informs future investigations on vector population dynamics and disease risk.

## Materials and Methods

### Study areas and mosquito colonies

Overwintering experiments with *Cx. pipiens* biotype *pipiens* were conducted in the region of Thessaly, Central Greece, at three locations representing different environ-

mental settings: (a) Kalamaki, an inland village adjacent to Lake Karla, characterized by extensive agricultural activity and wetland-associated vegetation (b) Nea Anchialos, a coastal settlement, and (c) Volos, a coastal urban area (Fig. 1). Nea Anchialos and Volos influenced by maritime climatic conditions, with milder winter temperature fluctuations and urban/peri-urban vegetation. The three sites differ in their microclimatic characteristics, particularly regarding humidity and thermal stability during winter. The experiments were designed as semi-field studies aiming to assess adult overwintering survival under local winter conditions.

Two overwintering experiments were performed. Experiment 1 took place during the winter of 2012–2013 at Kalamaki and Nea Anchialos. Experiment 2 was conducted during the winter of 2013–2014 in Volos and included additional experimental treatments to explore potential effects of organic material present in overwintering shelters.

Adult mosquitoes used in all experiments originated from laboratory colonies of *Cx. pipiens* biotype *pipiens* maintained at the Laboratory of Entomology and Agricultural Zoology, University of Thessaly (Volos, Greece). Colonies were established from egg rafts laid by laboratory-reared adults and maintained for experimental use.

## Rearing of immature stages and adult pre-exposure conditions

Egg rafts (~70 per experiment) were placed in plastic containers containing 12 L of water and reared outdoors at the premises of the Department of Agriculture, Crop Protection and Rural Environment, University of Thessaly, Volos, in protected locations shielded from direct rainfall. Larvae were provided with artificial food (Purina Adult cat food, Friskies) and both food and water were replenished at regular intervals to ensure adequate developmental conditions.

Immature development occurred under natural autumn photoperiod and ambient temperature conditions. Upon pupation, pupae of both sexes were transferred to plastic containers containing 250 mL of water and placed inside Plexiglas cages (20 × 20 × 20 cm, bearing four 15 × 15 cm mesh covered windows to facilitate ventilation) for adults' emergence.

Newly emerged adults were maintained for 10–12 days in a temperature-controlled storage room (15 ± 2°C) under natural light conditions. During this period, adults were provided with a 10% sucrose solution to allow mating and accumulation of lipid re-

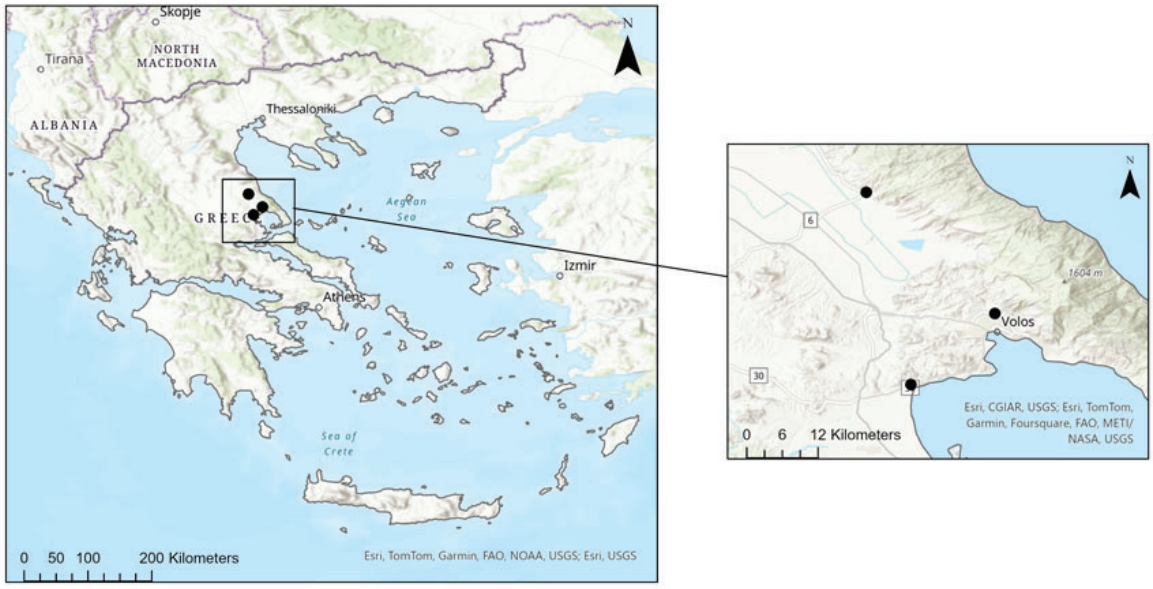



**Figure 1**. Map showing the locations (Kalamaki, Nea Anchialos, Volos), where the experimental study took place.

serves necessary for overwintering. After this pre-exposure period, sucrose was replaced with plain water prior to transfer to overwintering sites.

**Overwintering experimental procedures**

For Experiment 1, five cages (~100 adults per cage) were transferred to Kalamaki on December 30, 2012 and to Nea Anchialos on January 4, 2013. Cages were placed in dark, humid, and sheltered storage areas protected from rain and wind, simulating typical overwintering environments. Cages were placed on shelves at 1-1,5 m above the ground and 20-30 cm from the nearest wall.

For Experiment 2 in Volos, ten cages (~ 100 adults per cage) were transferred to the overwintering site on December 1, 2013. Cages were randomly assigned to one of the following treatments and placed in the overwintering site as above:

- Treatment A: plain water only
- Treatment B: plain water plus organic-rich infusion (a vial containing liquid collected from a composting bin, as a proxy of natural detritus and microbial environment)
- Treatment C: plain water only, with adults originating from a later egg-hatching cohort (October 15, 2013), serving as a temporal control

Each treatment consisted of five cages, with one cage representing the experimental unit. No additional food sources were provided during the overwintering period.

At the end of winter (early to mid-March), a 10% sucrose solution was reintroduced into all cages, and cages were transferred back to a semi-outdoor area at the University of Thessaly. Transfer dates were March 7, 2013 (Kalamaki), March 14, 2013 (Nea Anchialos), and March 19, 2014 (Volos).

**Blood feeding, oviposition and egg hatch**

Surviving females from Kalamaki and Nea Anchialos were pooled by location and offered a two-hour blood meal on April 11, 2013, using an artificial feeding device. The mosquitoes were fed certified human blood maintained at 38°C using two custom-made feeders connected to a circulating warm-water bath (Ioannou *et al.,* 2021).

The mating status of females was supported by published validation assays showing that 1-day-old *Culex pipiens* females with males under laboratory conditions achieve 100% insemination within 5 days, confirmed by spermathecal dissection (Fytrou *et al.*, 2022). Oviposition containers (plastic cups containing 250 mL of water) were placed in the cages from April 20 to April 22, 2013.

In Volos, surviving females were offered a blood meal on April 23, 2014, and oviposition containers were provided on April 28 to April 30, 2014. Percentages of female fertility in all cases were determined by dividing the total number of single egg rafts laid with the total number of surviving females. Egg rafts were collected and examined under a stereomicroscope to assess hatching success. An egg raft was considered as succesfully hatched when more than 50% of its total eggs yielded neonate larvae. Since Experiment 1 revealed hatching rates more than 90% (see results) for laid egg rafts, this parameter was not recorded in Experiment 2.

**Survival monitoring**

Adult survival was monitored at regular intervals throughout the overwintering period. Dead individuals were removed from cages and recorded. Sex was determined based on morphological characteristics. Accidental losses were recorded and excluded from survival analyses where appropriate.

**Meteorological data**

Ambient temperatures throughout the experimental period, from immature development to oviposition, were recorded with the help of special electronic devices (HOBO, Onset, USA). Temperature data corresponding to each location are presented in Figures 2–4, together with indications of exposure periods, feeding events, and oviposition.

**Statistical analysis**

Survival analyses were performed using

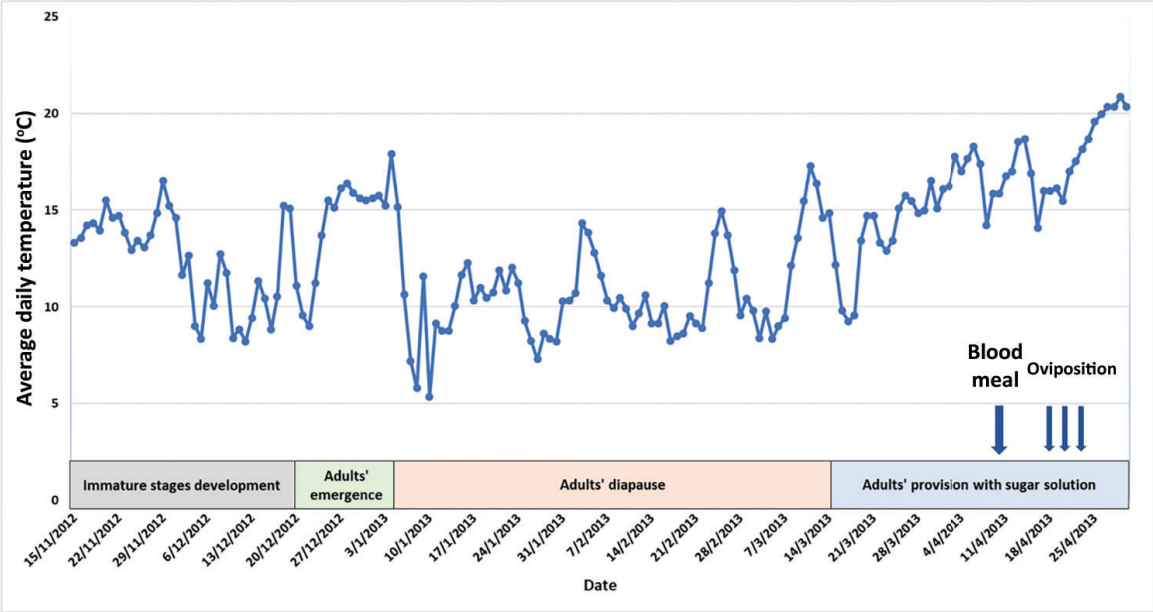



**Figure 2**. Average daily temperatures that prevailed throughout the experimental study of winter survival of *Culex pipiens* biotype *pipiens* in Nea Anchialos (a coastal area) Thessaly, during the period 2012-2013.

Kaplan–Meier survival curves and log-rank tests to compare survival between sexes and among treatments. Cox regression was employed to examine whether the sex of adults and the overwintering site were significant predictors of adult mortality rates. Statistical analyses were conducted using R software (version 4.3.2; R Foundation for Statistical Computing, Vienna, Austria). Statistical significance was set at $\alpha = 0.05$.

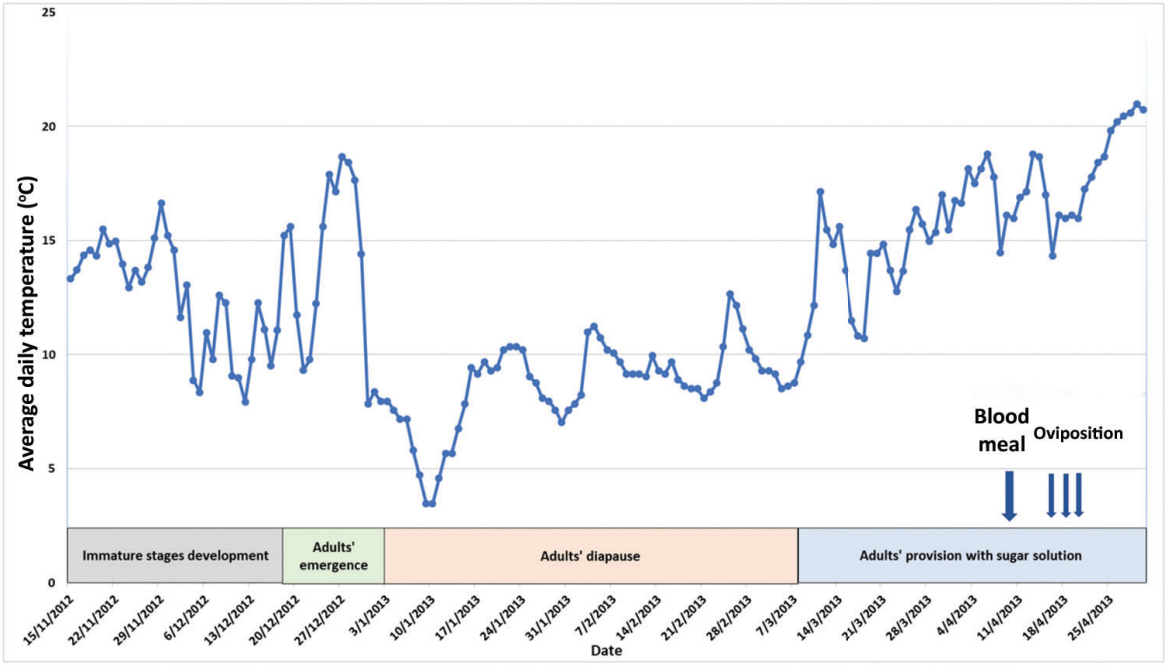



**Figure 3**. Average daily temperatures that prevailed throughout the experimental study of winter survival of *Culex pipiens* biotype *pipiens* in Kalamaki (a mainland village), Thessaly, during the period 2012-2013.

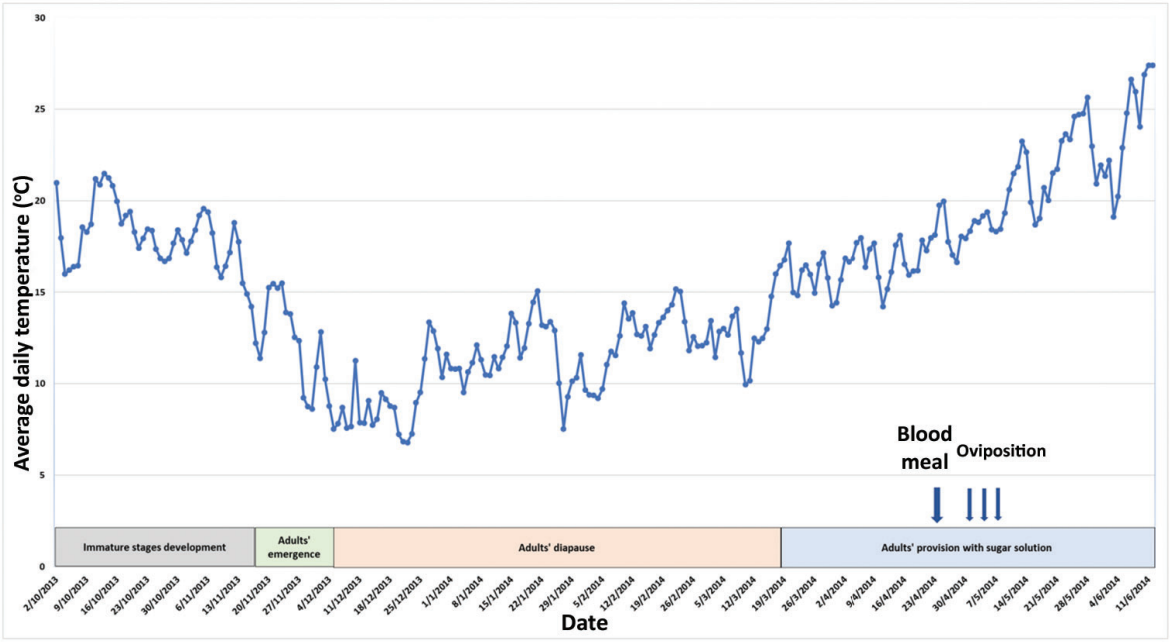



**Figure 4.** Average daily temperatures that prevailed throughout the experimental study of the winter survival of *Culex pipiens* biotype *pipiens* in Volos, Thessaly, during the period 2013-2014.

## Results

### Winter survival of *Culex pipiens* biotype *pipiens* in Kalamaki and Nea Anchialos

According to the average daily temperatures during the experimental period in Nea Anchialos and Kalamaki, from immature development to oviposition of overwintering females, (Figs 2 and 3, respectively), in both locations, temperatures remained relatively high until late December, while the colder winter period began in early January. An increase in temperature was observed in early March, marking the end of the winter period. Overall, winter temperatures were lower in the inland location of Kalamaki compared to the coastal site of Nea Anchialos.

In Kalamaki, a total of 450 adults (90 per cage; 173 females, i.e. 39% of exposed individuals) were exposed to overwintering conditions, while in Nea Anchialos 456 adults were exposed (91.2 per cage; 294 females, i.e. 64.7% of exposed individuals), as determined at the end of the experimental procedure considering also accidental loses. No males survived until the end of the overwintering period at either site (Fig. S1). Male survival was generally longer in Kalamaki than in Nea Anchialos.

Female mortality in Nea Anchialos increased progressively throughout the winter period (Fig. S1). In contrast, female survival in Kalamaki remained relatively high until late February, followed by a significant decline thereafter (Fig. S1). The reintroduction of sucrose solution in early March coincided with a stabilization of female mortality in both locations. At the onset of oviposition (April 20), the proportion of surviving females reached 23.9% in Kalamaki (44 individuals in total) and 25.3% in Nea Anchialos (76 individuals in total). Of these females, 52.3 and 47.4% oviposited in Kalamaki and Nea Anchialos, respectively, while 95.7 and 91.7% of the laid egg rafts hatched.

Mortality rates of males and females at Kalamaki and Nea Anchialos were compared with the Kaplan Meier curves and the Cox proportional hazards model. Overall, and in both locations, male longevity was shorter than that of females with males failing to overwinter (Fig. 5; $p < 0.01$). The hazard ratio of males compared to females was 12.9 (95%CI: 9.69, 17.2) in Kalamaki and 78.9 (95%CI: 47.4, 131.0) in Nea Anchialos.

Cox regression analysis considering lo-

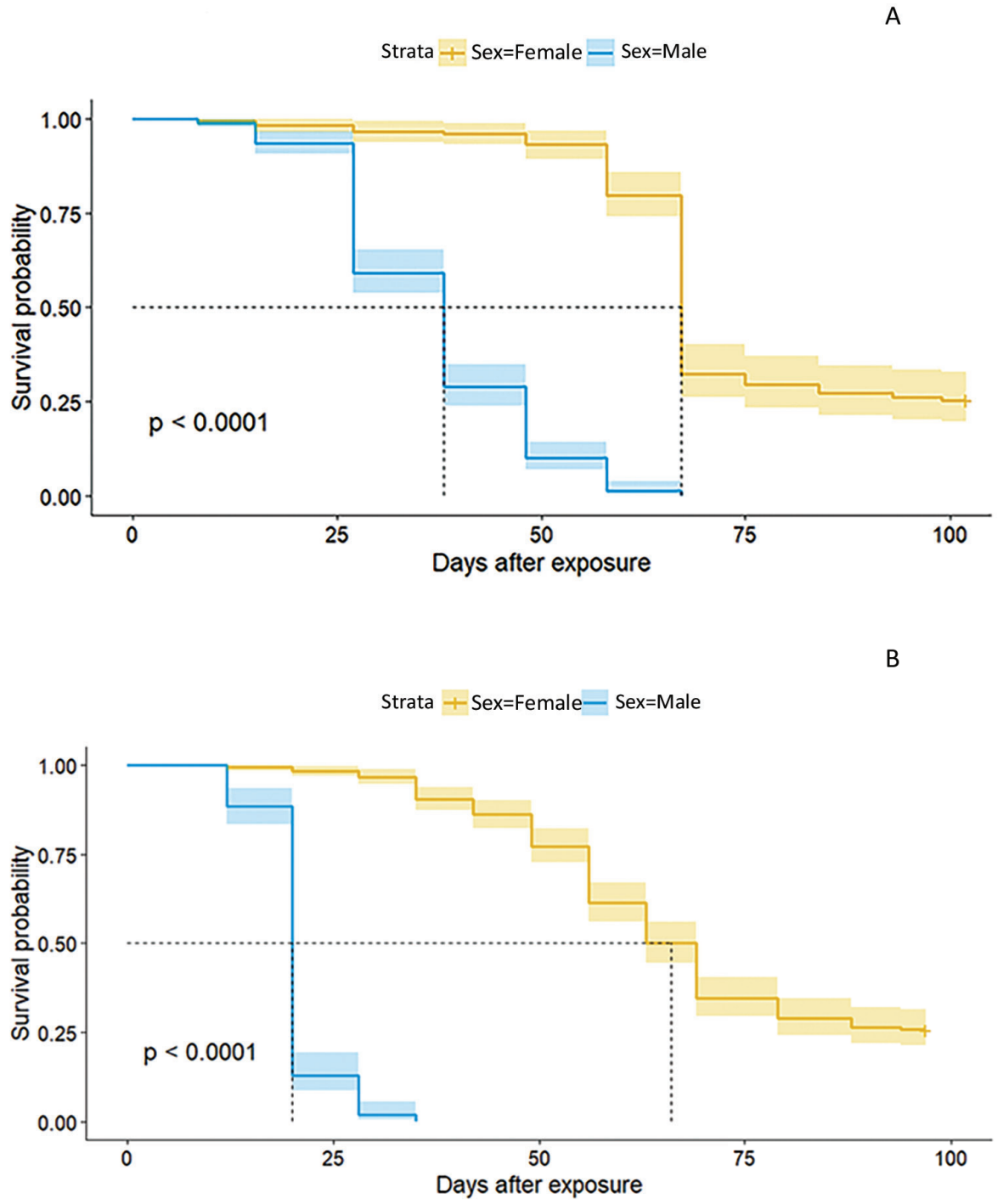



**Figure 5**. Kaplan Meier curves (with 95%CI) including log-rank test (shown p-value) for the survival of males and females in Kalamaki (A) and Nea Anchialos (B). Dashed lines represent the median survival for males (20 days) and females (66 days).

cation of exposure, sex and their interaction as predictors revealed that males have a significant higher hazard rate compared to females ($p < 0.001$), adjusted for location, and mortality rates were higher in Kalamaki compared to Nea Anchialos ($p < 0.001$) (Fig. 6, Fig. S2). Overall hazard rates considering both males and females were similar between the two locations ($p = 0.425$). The significant interaction between sex and location is associated with higher mortality rates for male in Nea Anchialos compared to Ka-

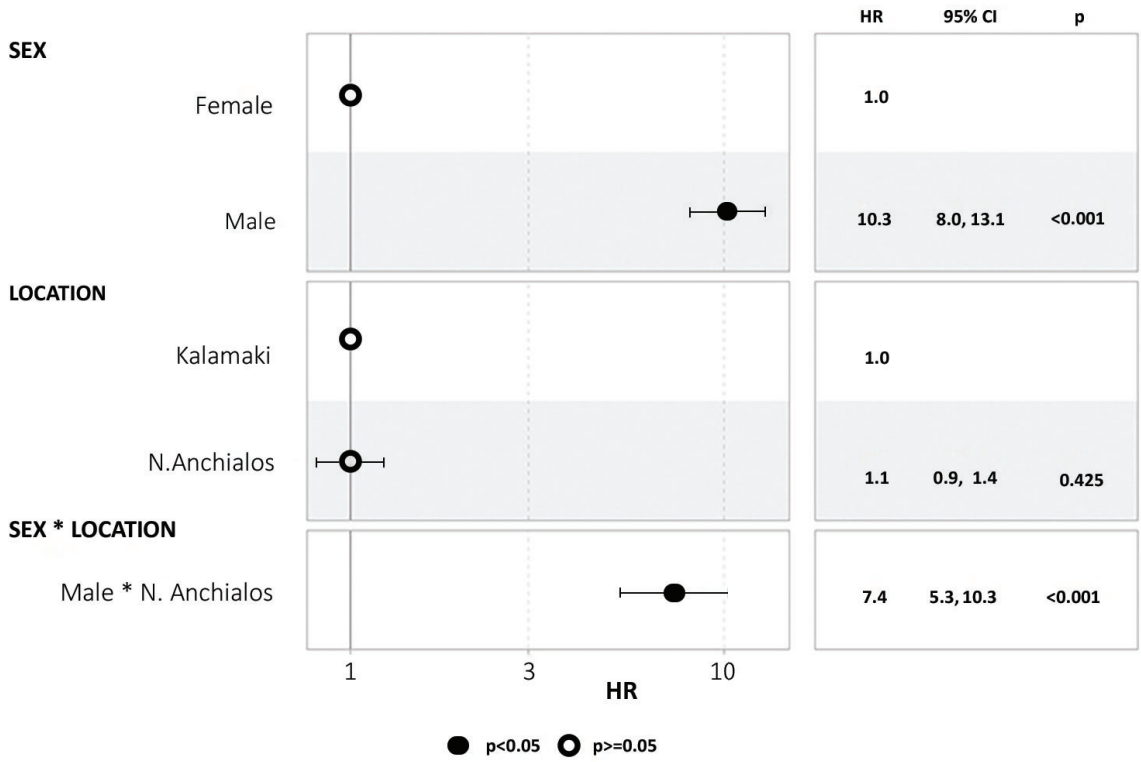



**Figure 6.** Forest plot depicting the hazard ratio for males and females exposed to Kalamaki and Nea Anchialos.

lamaki. Indeed, comparing survival patterns of females in the two location no significant differences were found (Fig. 6; log-rank test, $p = 0.16$).

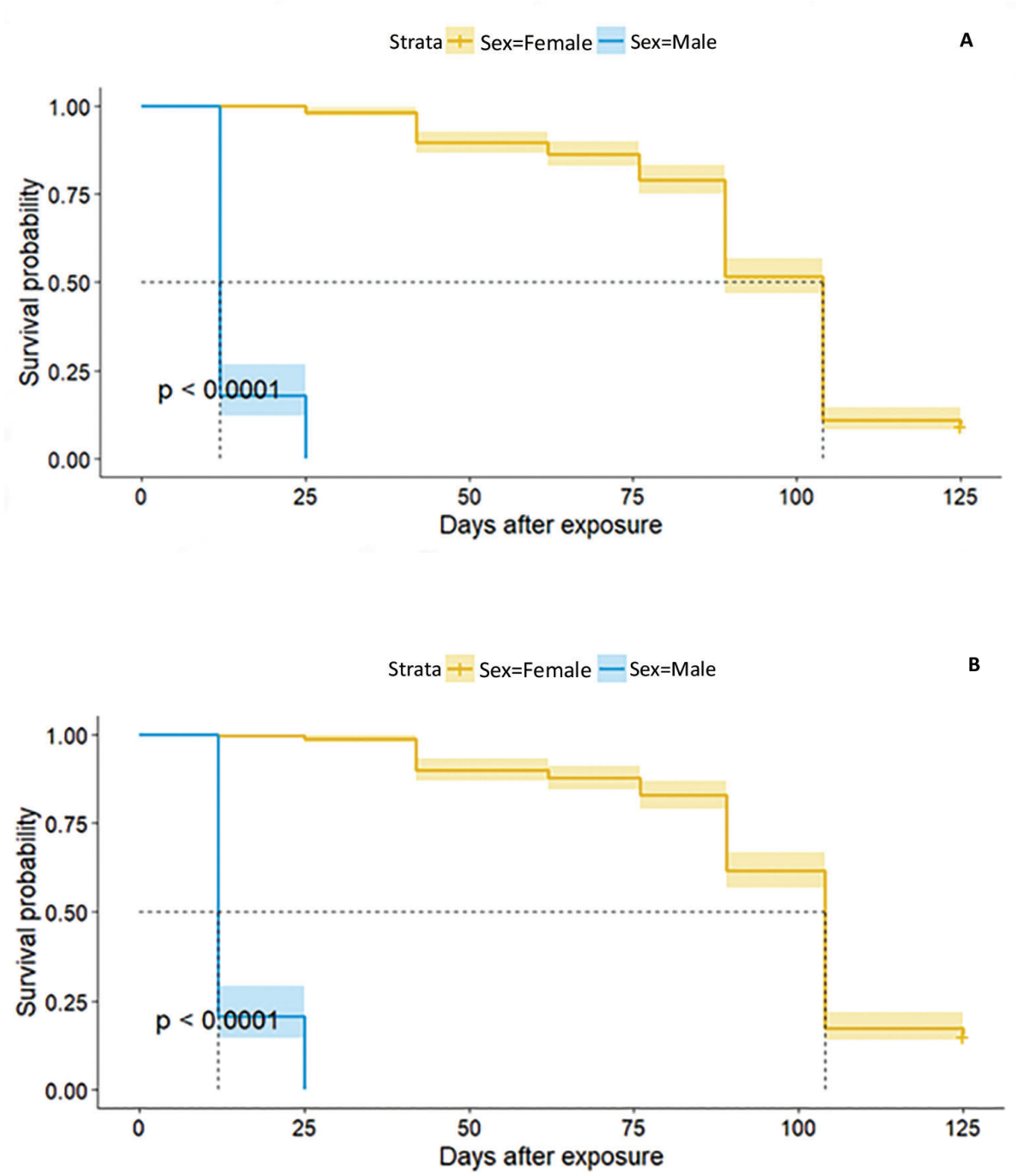
## Winter survival of *Culex pipiens* biotype *pipiens* in Volos

The average daily temperatures recorded during the overwintering experiment in Volos are shown in Figure 4. A decrease in temperature in early December marked the onset of winter conditions, while temperatures increased again in mid-March. The total number of adults exposed to winter conditions was 482 (average per cage 96.4; 371 females, i.e. 76.9%), 475 (average per cage 95; 349 females, i.e. 73.5%) and 509 (average per cage 101.8; 388 females, i.e. 76.2%) for the treatments A, B and C respectively. In all treatments, male survival was markedly lower than female survival, and no males survived beyond late December. Details of female and male survival patterns in each cage are given in Figure S3.

Among cage variation was low in Treatment A and B and quite higher in Treatment C (Figs S3-S5). Overall at the end of the winter period 9.4, 15.7 and 12.4% of the females managed to survive in Treatments A, B and C respectively. Among cage variation in male survival was minimal within the same treatment and overall survival patterns among treatments negligible as well (Fig. S4). Comparing the survival of the two sexes of *Cx. pipiens*, the ability of females (Fig. S5a) to overwinter in all three treatments and the inability of males (Fig. S5b) to survive are evident. In all three treatments provision of sugar solution on March 16, 2014 reduced mortality rates. At the start of oviposition (April 28) in Treatment A, Treatment B and Treatment C, the number of females alive was 35, 53, and 48, of which 13, 25, and 10 oviposited, with the oviposition rate reaching 37.1, 47.2, and 20.8% respectively.

Kaplan–Meier survival analysis followed by the log-rank test revealed the higher mortality of males compared to that of fe-

males within each treatment (Fig. 7; log-rank test, $p < 0.01$). Cox regression analysis including treatment, sex and their interaction as predictors confirmed the overall higher hazard rates of death for males compared to females ($p < 0.001$) and differences among the three treatments (Fig. 8). The hazard ratio of Treatment B and C compared to baseline A was slightly but significantly lower and higher respectively (Fig. 8; $p < 0.05$). The interaction between sex and treatment was not statistically significant. Kaplan Meier analysis comparing survival patterns of the three female groups followed by log-rank test revealed significant differences among the three treatments (Fig. 7; log-rank test, $p < 0.01$). Nevertheless, the proportion of surviving females at the end of the exposure period was similar among the three treatments (chi-square test, $p > 0.05$).



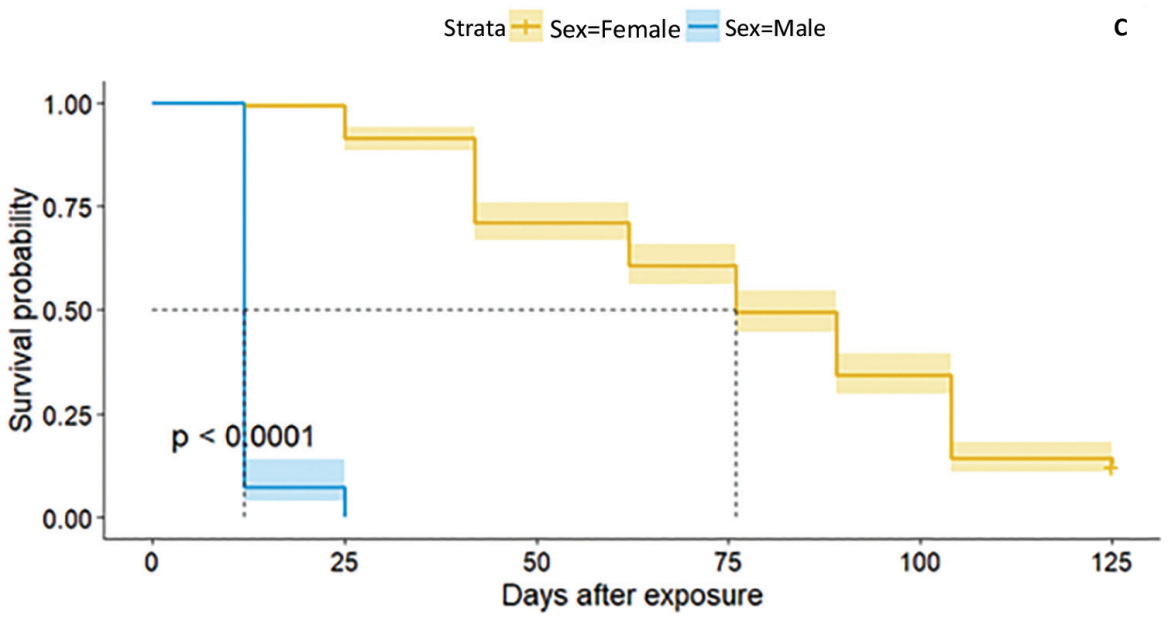



**Figure 7.** Kaplan Meier curves (with 95%CI) including log-rank test (shown p-value) for the survival of males and females in treatment A (Plain water plus organic-rich infusion exposed to the wild on October 1, 2013), treatment B (Plain water only, exposed to the wild on October 1, 2013), and treatment C (Plain water only exposed to the wild on October 15, 2013). Black dashed lines represent the median survival for males (12 days) and females (76 days).

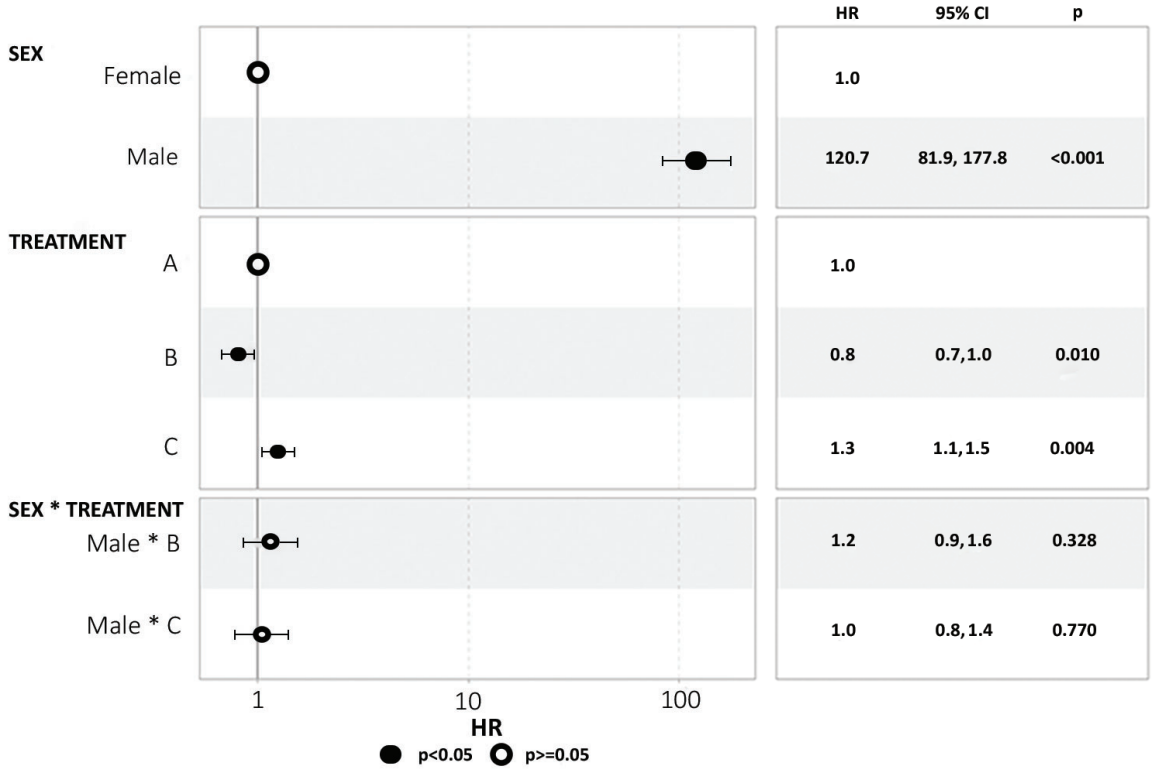



**Figure 8**. Forest plot depicting the hazard ratios (HR) for different sex, treatment, and interaction between sex and treatment groups exposed to Volos, during the winter 2012-13. Treatment A (Plain water, exposed to the wild on October 1, 2013), treatment B (Plain water plus organic-rich infusion, exposed to the wild on October 1, 2013), and treatment C (Plain water exposed to the wild on October 15, 2013).

## Discussion

The overwintering experiments conducted in three regions of Thessaly provide indicative evidence that a proportion of *Cx. pipiens* biotype *pipiens* females are capable of surviving winter conditions in Central Greece and subsequently initiating reproduction in spring. Despite being initiated relatively late in the season, the experiments consistently showed successful overwintering of females at all sites, while males failed to survive until the end of the cold period. These findings are in agreement with the well-established diapause strategy of *Cx. pipiens* biotype *pipiens*, whereby inseminated females overwinter as adults while males do not enter diapause and typically perish during winter.

Female survival patterns differed among locations and treatments, although successful overwintering was observed in both coastal and inland areas. Differences in male longevity between sites may reflect variation in local microclimatic conditions, particularly temperature, with lower temperatures potentially reducing metabolic rates and delaying mortality. In contrast, female survival curves were broadly comparable among locations, suggesting that the overwintering capacity of *Cx. pipiens* biotype *pipiens* females is relatively robust across the range of winter conditions encountered in the study area. Similar patterns have been reported in other temperate regions, where female survival during winter is closely linked to the stability of microclimatic conditions within overwintering shelters (Koenraadt *et al*., 2019; Rozsypal *et al*., 2021; Sauer *et al*., 2022).

A significant finding of the present study is that a proportion of overwintering females successfully blood fed, oviposited, and produced viable offspring in spring, demonstrating their potential contribution to the initiation of the first seasonal generation. This observation is epidemiologically relevant, as overwintering females may play a critical role in shaping early-season population dynamics and, consequently, the risk of arbovirus transmission (Brugman *et al*., 2018; Bellone and Failloux, 2020). The observed variation in oviposition rates among locations and treatments highlights the importance of pre- and post-winter conditions in determining reproductive success. Although the presence of organic matter in the water did not significantly influence female survival during overwintering, it appeared to have a modest positive effect on survival. In addition, females exposed to this treatment exhibited higher post-overwintering oviposition rates, with the proportion of ovipositing females increasing by approximately 10% relative to Treatment A and 27% relative to Treatment C. The reduction in mortality following the reintroduction of sugar solution in early spring further underscores the importance of energy availability during the post-diapause period, as previously reported for diapausing *Cx. pipiens* females (Mitchell and Briegel, 1989; Denlinger and Armbruster, 2014).

Temperature is widely recognized as a primary driver of overwintering survival in mosquitoes, influencing metabolic rates, activity levels and depletion of lipid reserves. Although the present study was not designed to identify specific thermal thresholds, the observed survival patterns are consistent with previous work indicating that relatively mild and stable winter conditions can support adult overwintering of *Cx. pipiens* biotype *pipiens* (Rozsypal *et al*., 2021; Sauer *et al*., 2022). Differences between coastal and inland sites likely reflect microclimatic variation rather than fundamental differences in overwintering strategy, emphasizing the importance of local environmental conditions in shaping overwintering outcomes.

While the ecological and epidemiological roles of *Cx. pipiens* biotypes and their hybrids are well documented, the present study focused exclusively on *Cx. pipiens* biotype *pipiens*. Consequently, no conclusions can be derived from the current data regarding differences among biotypes or the role of hybrids in overwintering and pathogen transmission. Nevertheless, the demonstrated ability of *Cx. pipiens* biotype *pipi-*

*ens* females to survive winter and reproduce in spring supports the notion that overwintering adults may contribute to the persistence of vector populations and potentially facilitate early-season pathogen circulation, as suggested by studies detecting WNV in overwintering *Culex* females in Europe (Brugman *et al*., 2018; Kampen *et al*., 2021).

Several limitations should be considered when interpreting the present findings. The experiments were initiated relatively late in the season, which may have influenced diapause establishment and precluded assessment of mortality earlier in winter. Since the experiments started in December-January, the initial phase of diapause induction and the associated mortality rate are likely to have been incompletely recorded. Subsequently, the reported overwintering survival rates may represent conservative estimates of survival following the initial transition to winter conditions. In addition, the limited number of cages per site and the use of laboratory-reared mosquitoes restrict the extent to which the results can be generalized to natural populations. Interannual climatic variability, which was not captured in the present study, may further influence overwintering success. Despite these limitations, the results provide valuable field-based evidence of overwintering survival of *Cx. pipiens* biotype *pipiens* in a southern temperate region, where experimental data remain comparatively scarce. The use of laboratory-reared mosquitoes is likely to limit the ability to directly generalize the results in natural populations, as laboratory colonies could differ from wild populations regarding the diapause expression, stress tolerance, and energy reserve dynamics. Nevertheless, the use of laboratory populations permitted greater standardization of the experiments and reduced uncontrolled biological variability among treatments (Nelms *et al*., 2013; Ioannou *et al*., 2021; Siperstein *et al*., 2023).

Overall, the findings highlight the importance of overwintering ecology for understanding seasonal population dynamics of *Cx. pipiens* and their implications for vector-borne disease risk. Future studies that will include multiple winters, additional biotypes, and detailed characterization of natural overwintering shelters will be essential to improve estimates of overwintering survival and to elucidate the role of local environmental conditions in shaping mosquito population dynamics and pathogen transmission.

***Supplementary Materials***

*Figure S1: Winter survival of Culex pipiens biotype pipiens in Kalamaki (a mainland village) (A) and in Nea Anchialos (a coastal area) (B), Thessaly, 2012-2013.*

*Figure S2: Kaplan Meier curves (with 95%CI) including log-rank test (shown p-value) for the survival of females in Nea Anchialos and Kalamaki. Black dashed lines represent the median survival for females in Nea Anchialos (66 days) and females in Kalamaki (67 days).*

*Figure S3: Survival rate of Culex pipiens biotype pipiens female adults regarding treatment 1 (a), treatment 2 (b), and treatment 3 (c), in each cage, in Volos, Thessaly, 2013-2014.*

*Figure S4: Survival rate of Culex pipiens biotype pipiens male adults regarding treatment 1 (a), treatment 2 (b), and treatment 3 (c), in each cage, in Volos, Thessaly, 2013-2014.*

*Figure S5: Survival rate of Culex pipiens biotype pipiens adults (a) females, (b) males, regarding the three treatments in Volos, Thessaly, 2013-2014.*

***Author Contributions***

*Conceptualization, C.I., C.H. and N.T.P.; Methodology, N.T.P. and C.I.; Software, C.I and P.T.; Validation, X.X., Y.Y. and Z.Z.; Formal analysis, C.I., S.B. and N.T.P.; Investigation, C.I., P.T.; Resources, C.H., E.P., A.M. and N.T.P.; Data curation, C.I. and P.T.; Writing—original draft preparation, S.B., A.M., E.P. and N.T.P.; Writing—review and editing, S.B., A.M., E.P. and N.T.P.; Visualization, A.M., E.P.; Supervision, N.T.P.; Project administration, N.T.P.; Funding acquisition, C.H. and N.T.P. All authors have read and agreed to the published version of the manuscript.*

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***Competing interests***
*The authors declare no competing interests.*

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# Χειμερινή επιβίωση των ενηλίκων του *Culex pipiens* biotype *pipiens* στην Κεντρική Ελλάδα

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**Περίληψη** Η επιβίωση κατά τη χειμερινή περίοδο αποτελεί κρίσιμο στοιχείο της βιολογίας των εντόμων-διαβιβαστών σε εύκρατα οικοσυστήματα, καθώς συνδέεται άμεσα με την έναρξη και τη δυναμική αύξηση των πληθυσμών κατά την επόμενη αναπαραγωγική περίοδο, με σημαντικές επιπτώσεις στην επιδημιολογία των μεταδιδόμενων νοσημάτων. Το είδος *Culex pipiens* αποτελεί τον κύριο διαβιβαστή του ιού του Δυτικού Νείλου στην Ευρώπη, συμπεριλαμβανομένης της Ελλάδας. Στην παρούσα μελέτη παρουσιάζονται πειράματα διαχείμασης που πραγματοποιήθηκαν σε τρεις περιοχές της Κεντρικής Ελλάδας, με στόχο την αξιολόγηση της ικανότητας διαχείμασης των ενηλίκων του *Cx. pipiens* biotype *pipiens* υπό φυσικές, τοπικές χειμερινές συνθήκες. Διεξήχθησαν δύο πειραματικές προσεγγίσεις σε παράκτιες περιοχές (Νέα Αγχίαλος και Βόλος) και σε ηπειρωτική περιοχή (Καλαμάκι). Τα αποτελέσματα έδειξαν ότι ένα ποσοστό των θηλυκών του *Cx. pipiens* biotype *pipiens* επέζησε επιτυχώς της χειμερι-

νής περιόδου σε όλες τις μελετώμενες περιοχές μελέτης, ενώ τα αρσενικά δεν παρουσίασαν ικανότητα διαχείμασης. Τα ποσοστά επιβίωσης των θηλυκών στο τέλος της χειμερινής περιόδου έφτασαν το 23,9% στο Καλαμάκι, το 25,3% στη Νέα Αγχίαλο, ενώ στο Βόλο κυμάνθηκε από 9,4% έως 15,7%. Τα θηλυκά που επέζησαν μετά τη χειμερινή περίοδο ήταν ικανά να τραφούν με αίμα και να ωοτοκήσουν κατά την άνοιξη, παράγοντας βιώσιμους απογόνους. Σημαντικός αριθμός θηλυκών που επιβίωσαν έδωσαν αυγά (σχεδίες αυγών) τόσο στο πείραμα στο Καλαμάκι όσο και στη Νέα Αγχίαλο (52,3% και 47,4% αντίστοιχα). Το ποσοστό των θηλυκών που ωοτόκησαν στο πείραμα στον Βόλο κυμάνθηκε από 20,8% έως 47,2%. Η επιβίωση των θηλυκών παρουσίασε διαφοροποιήσεις μεταξύ των περιοχών και των πειραματικών μεταχειρίσεων, υποδηλώνοντας πιθανή επίδραση των τοπικών περιβαλλοντικών συνθηκών στη δυναμική της διαχείμασης. Τα ευρήματα παρέχουν ενδεικτικά στοιχεία ότι τα θηλυκά του *Cx. pipiens* biotype *pipiens* μπορούν να διαχειμάζουν επιτυχώς στις περιοχές μελέτης και στη συνέχεια να ξεκινήσουν την αναπαραγωγική τους διαδικασία την άνοιξη. Τα αποτελέσματα της παρούσας μελέτης συμβάλλουν στη βελτίωση της κατανόησης της οικολογίας της διαχείμασης ενός σημαντικού διαβιβαστή παθογόνων και αναδεικνύουν την ανάγκη για περαιτέρω μελέτες σε διαφορετικά ενδιαιτήματα.

## Supplementary material

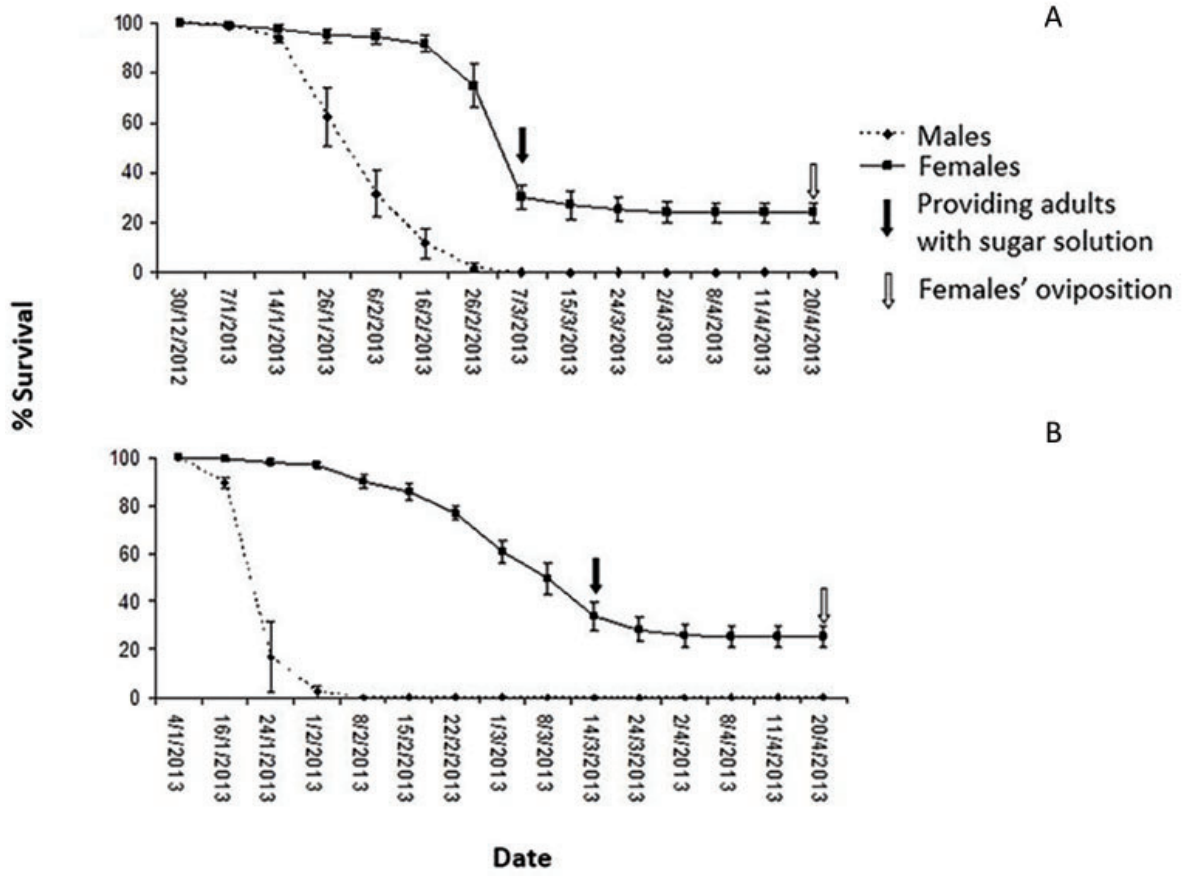



**Figure S1**: Winter survival of *Culex pipiens* biotype *pipiens* in Kalamaki (a mainland village) (A) and in Nea Anchialos (a coastal area) (B), Thessaly, 2012-2013.

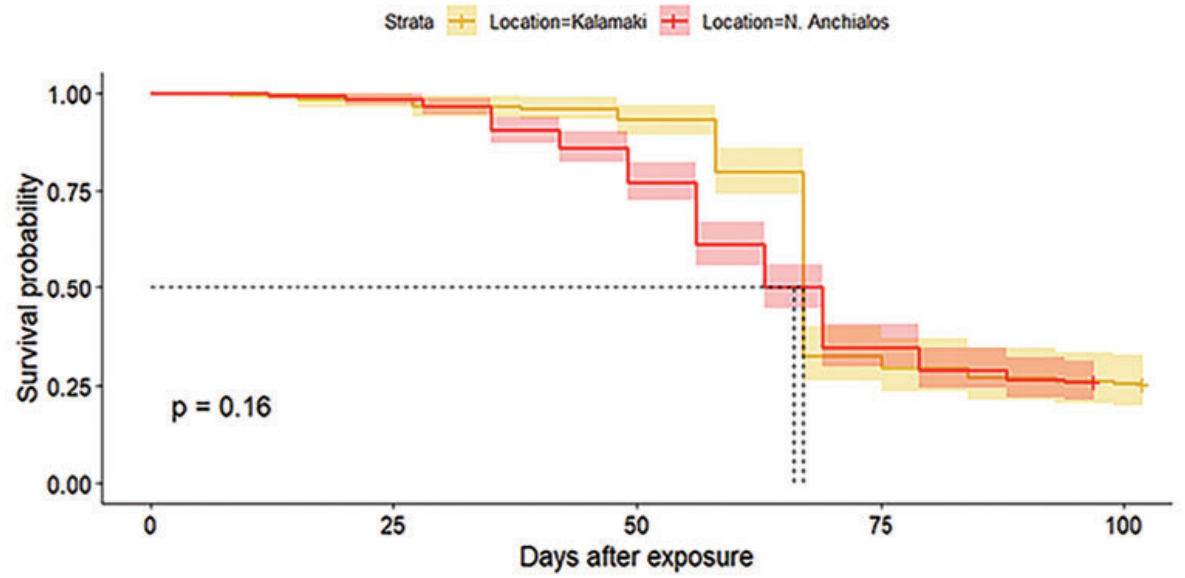
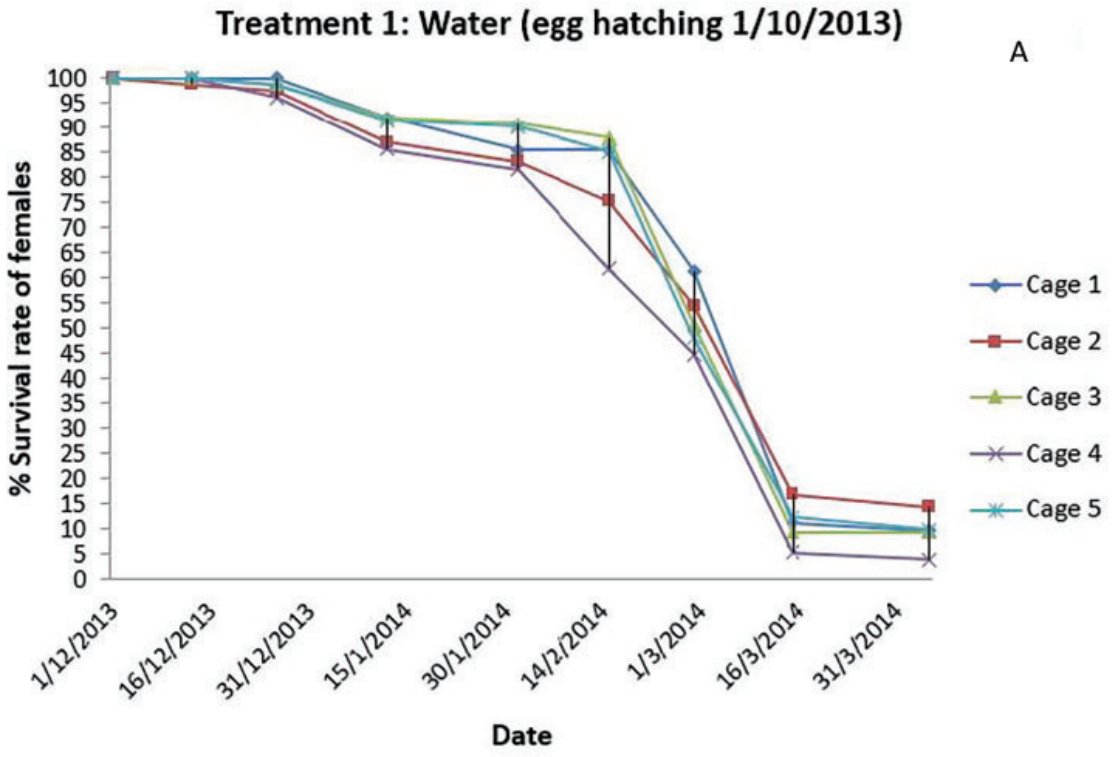
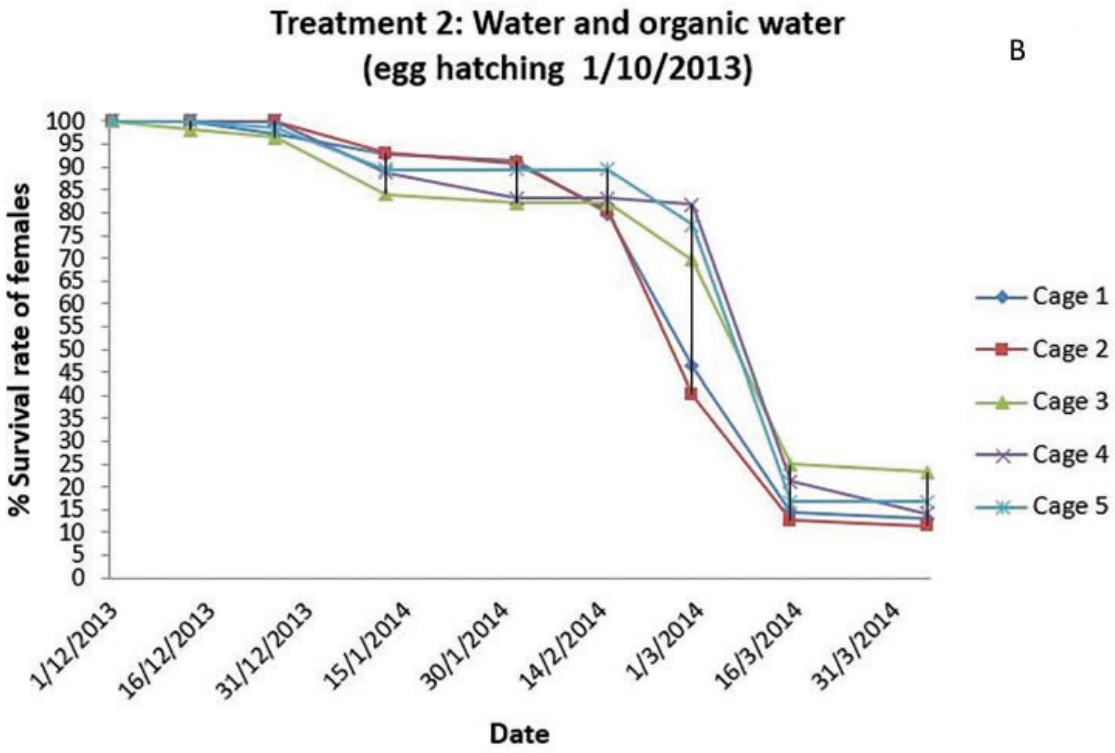



**Figure S2**: Kaplan Meier curves (with 95%CI) including log-rank test (shown p-value) for the survival of females in Nea Anchialos and Kalamaki. Black dashed lines represent the median survival for females in Nea Anchialos (66 days) and females in Kalamaki (67 days).



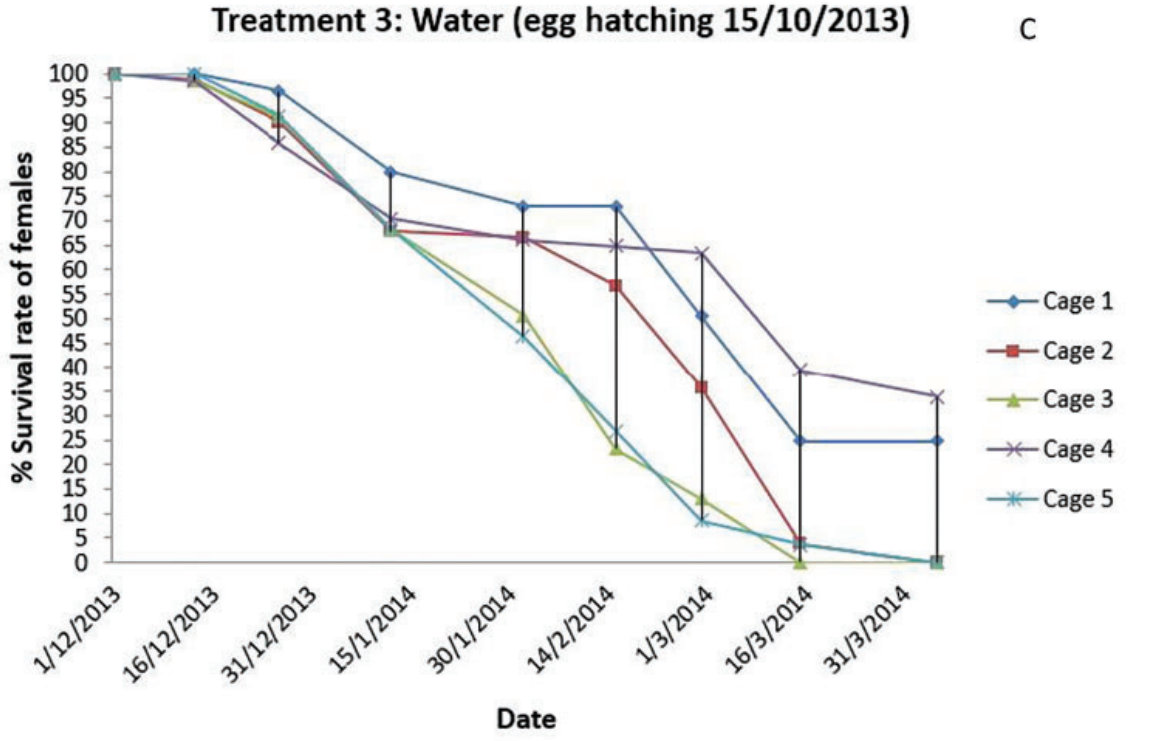
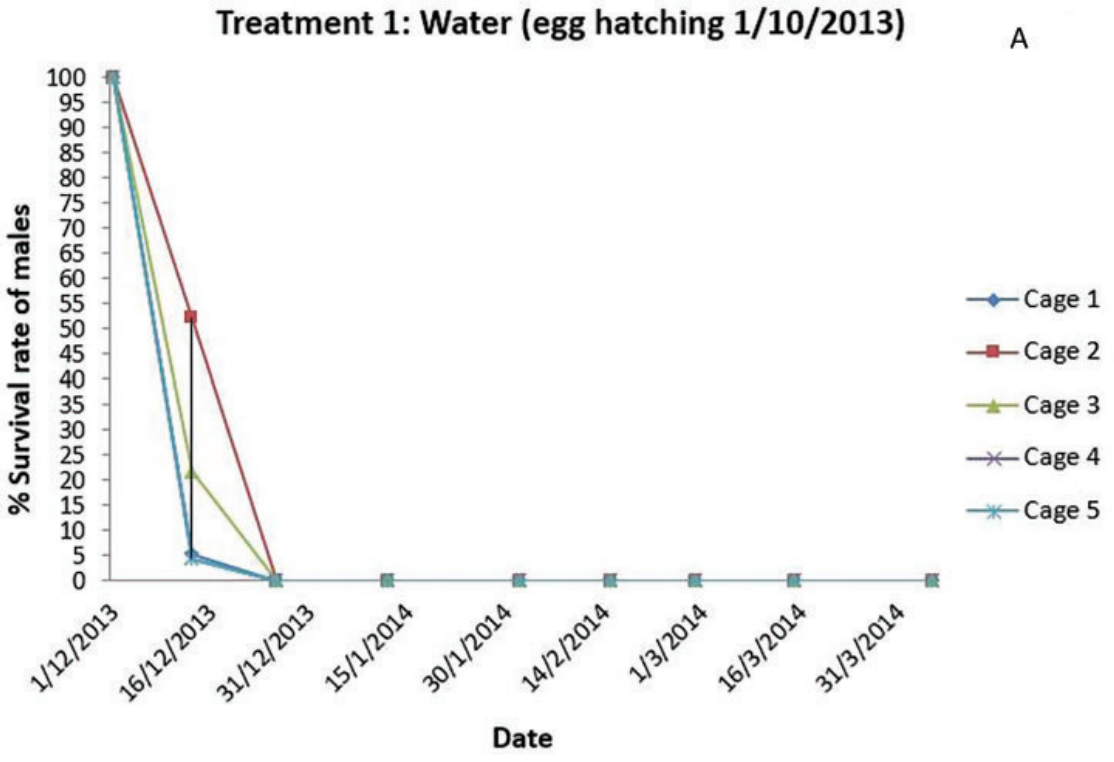
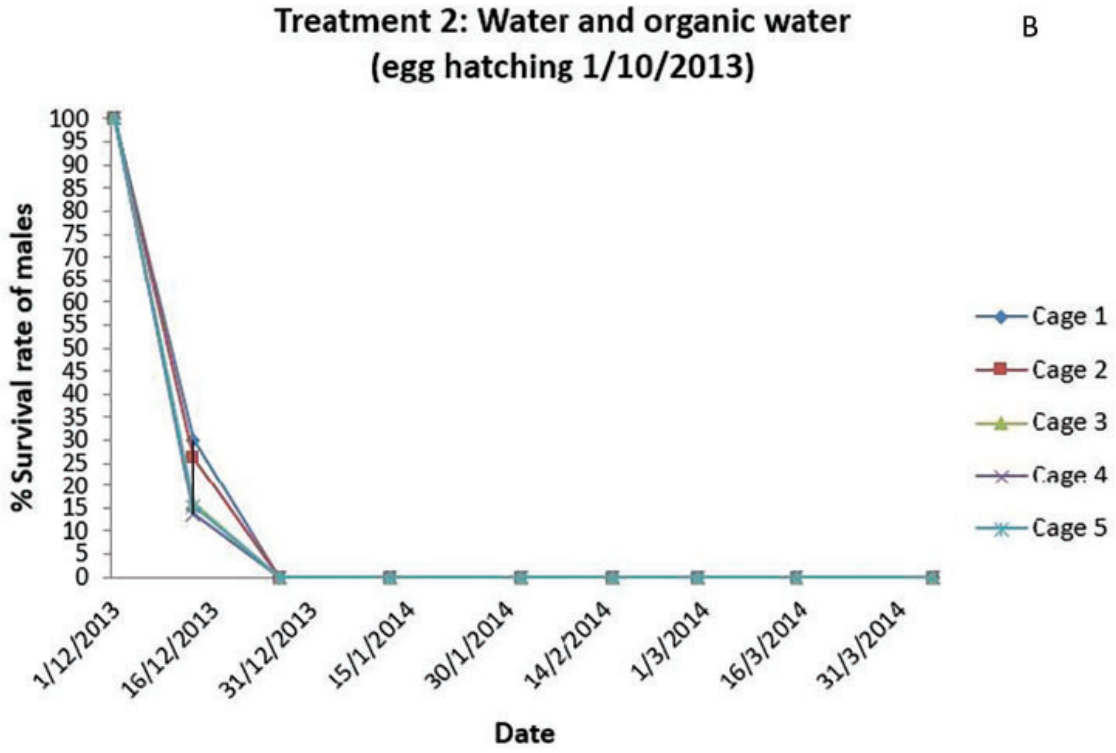



**Figure S3**: Survival rate of *Culex pipiens* biotype *pipiens* female adults regarding treatment 1 (A), treatment 2 (B), and treatment 3 (C), in each cage, in Volos, Thessaly, 2013-2014.





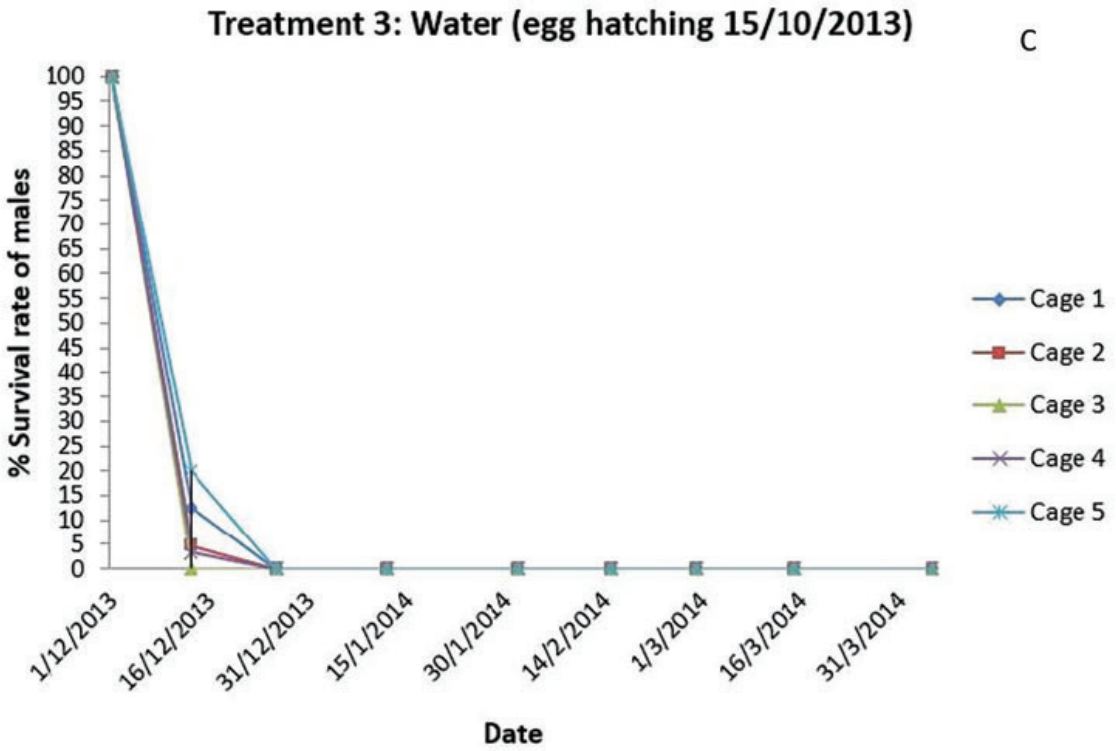




**Figure S4.** Survival rate of *Culex pipiens f. pipiens* male adults regarding treatment 1 (A), treatment 2 (B), and treatment 3 (C), in each cage, in Volos, Thessaly, 2013-2014.

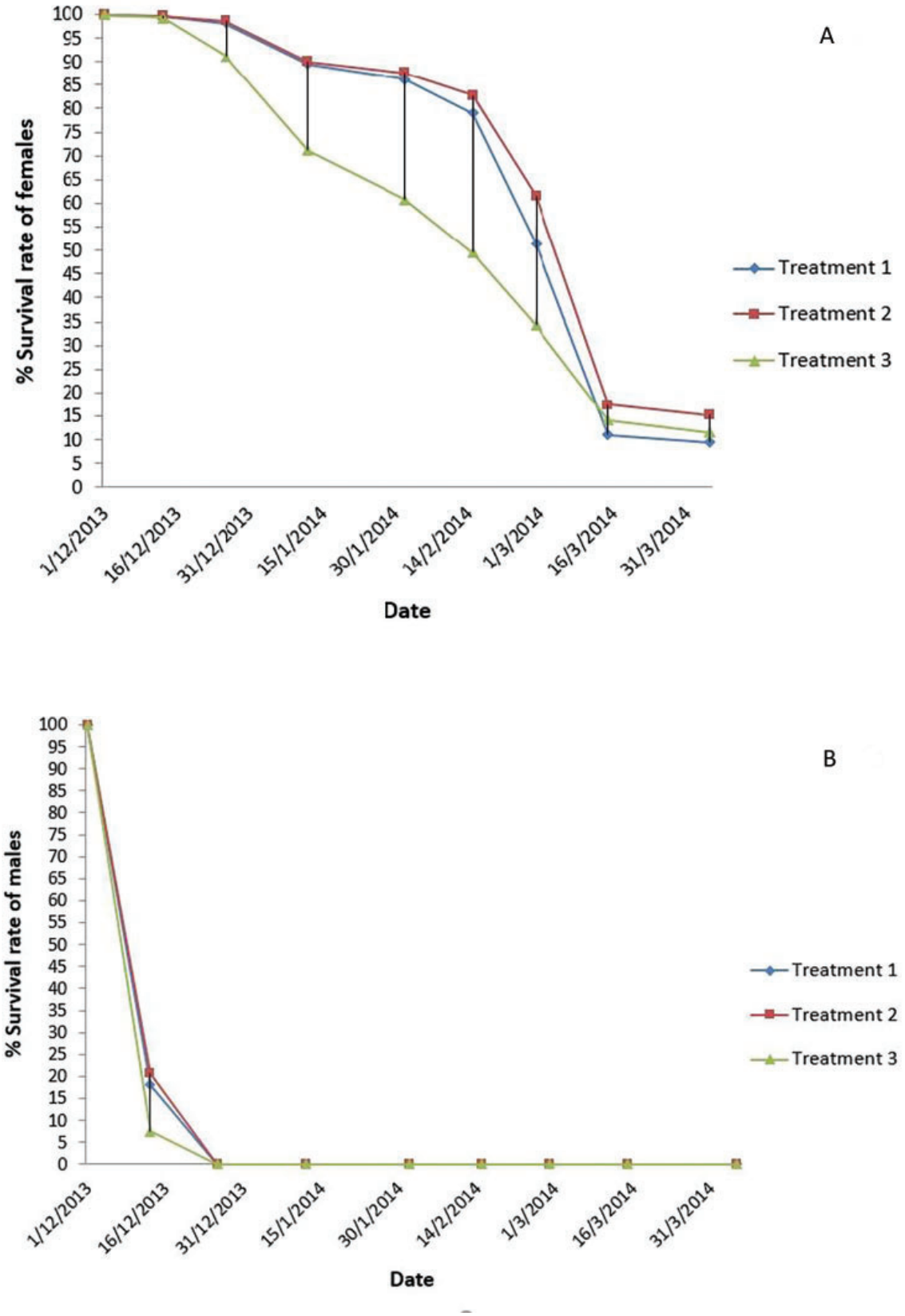



**Figure S5.** Survival rate of *Culex pipiens f. pipiens* adults (A) females, (B) males, regarding the three treatments in Volos, Thessaly, 2013-2014.

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# Low energy portable industrial radiography X-ray machine of irradiation of *Phthorimaea operculella* sterilization

I. Idris[1*] and W. Harara[2]

**Summary** Gamma ray's irradiation has long widely utilized in medical, industrial and agricultural research and applications. Many countries have approved its use as an effective tool for the sterile insect technique (SIT) within integrated pest management programs. The actual study evaluates the potential of using low-energy portable industrial radiography (LEPIR) X-ray machine (typically employed for nondestructive testing of materials) to sterilize the potato tuber moth (PTM) pupae and adult moths rather than gamma radiation that is routinely used in SIT programs. Our data are consistent with previous finding and reveal clear dose-dependent damaging detrimental effects on key biological parameters of the pest, including adult emergence, longevity, fecundity, fertility and $F_1$ egg hatchability at a dose of 150 Gy. These findings indicate that LEPIR X-ray machine may serve as a practical and alternative to gamma irradiation in any SIT program targeting the potato tuber moth.

*Additional Keywords*: low energy, potato tuber moth, radiation, SIT, X-ray

## Introduction

Several studies have revealed that the sterile insect technique (SIT) is a safe, effective, and environmentally friendly technique for controlling or even eradicating pests. Furthermore, SIT has become a regular component of area-wide integrated pest management programs against various agricultural insect pests (Hendrichs *et al*., 2009; Marec and Vreysen, 2019; Dyck *et al*., 2021). Multiple pests have been effectively controlled using this technique, including fruit flies, tsetse flies, and potato tuber moth (Makee and Saour, 1999; Hendrichs *et al*., 2009; Idris and Hussian, 2021). Basically, SIT depends on releasing sterile males of the insect of interest into a wild population, which decrease reproduction rates (Knipling, 1985).

The potato tuber moth (PTM) (*Phthorimaea operculella* Zeller) causes crop losses of up to 100% in over 90 countries in the world (Chandel *et al*., 2020; Adhikari *et al*., 2022). During the growing season, *Phthorimaea operculella* (Zeller) larvae damage host foliage; however, as senescence approaches, the adult females deposit the eggs within soil cracks or directly on exposed tubers (Rondon, 2010). There is a resilient pupal stage in the pest's life cycle, which provides a critical bridge for population survival; concurrently, the highly mobile, nocturnal adults ensure continuity and expansion of infestations by dispersing spatially and oviposition precisely (Fenemore, 1979). Syria cultivates about 24,789 hectares of potatoes with estimated yield of 486,605 tons per year, and potato production depends on an excessive usage of chemical insecticides to control *P. operculella* infestation. It is well documented that these insecticides are expensive, nonselective, and pose an environmental threat. Moreover, *P. operculella* has developed resistance to most insecticides, reducing their effectiveness (Idris and Shoaib, 2019; Idris *et al*., 2021).

Several studies have demonstrated that gamma radiation and X-rays can be potentially used in SIT programs to induce sterility among insect pests (Saour and Makee, 1997; Makee and Saour, 1999; 2003; Idris and Shoaib, 2019; Wang *et al*., 2023; Cho *et al*., 2026). There are, however, some significant disadvantages of using gamma radi-

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ation over X-ray technology, particularly when it comes to application logistics and regulatory restrictions (Kaboré *et al*., 2023). A common method of radiation delivery in SIT programs is the Cobalt-60 isotope, which is strictly regulated with plenty of security concerns, radioactive disposal problems, complicating procurement and management (Mastrangelo *et al*., 2010; Urquidi *et al*., 2015). On the other hand, X-rays are more flexible practically because they are not subject to the same regulatory restrictions, as it requires fewer shielding and safety measures, making them more appropriate for pest control applications (Liang *et al*., 2019; Abdelhafiz *et al*., 2024). In addition, X-ray technology has become increasingly popular as a safer, more efficient alternative that induces pest sterility without affecting commodity quality due to recent advancements. In particular, low-dose X-ray treatments provide a biosecurity barrier against cryptic pests, thus bypassing the regulatory and environmental challenges associated with chemical fumigants (Follett *et al*., 2013; Praveen *et al*., 2025).

Even though X-ray treatments are capable of achieving high levels of sterility, they tend to preserve almost all insects' biological functions such as longevity, flight, and mating behavior better than gamma radiation. In SIT programs, this is crucial for achieving successful pest control by producing sterile males that fly farther, live longer, and court females more effectively (Wang *et al*., 2023; Huang *et al*., 2025). A study on *Cydia pomonella L*. combining SIT with X irradiation found that X-ray treatment effectively sterilized male moths without significantly compromising their mating competitiveness, but 366 Gy of X-ray caused complete sterility in males. Compared to non-irradiated males, X-rays reduced mating competitiveness and lifespan, but gamma radiation is more damaging to flight muscles, wings, and overall vigor, making X-rays less damaging. Also, both 91.2 Gy of X-rays and 124.9 Gy of gamma radiation caused 99% sterility in male Mediterranean fruit fly (*Ceratitis capitata*), but X-rays required lower doses and caused less biological damage (Mastrangelo *et al*., 2009; Zhang *et al*., 2023). Moreover, in Cotton bollworm (*Helicoverpa armigera*), 200 Gy of X-ray caused inherited sterility, while 400 Gy caused complete egg infertility, showing dose flexibility (Chu *et al*., 2026).

Photonic ionizing radiation, including X-rays and gamma rays, has been extensively used in insect pest management and control programs. However, despite their widespread application, limited information is currently available regarding the potential advantages and effectiveness of low-energy X-ray radiation within Sterile Insect Technique (SIT) programs (Pernicka and McLean, 2007; Russel and Bardley, 2015). On the other hand, in the traditional academic research and nondestructive testing of materials (NDT), the low-energy, portable industrial radiography (LEPIR) X-ray machines are utilized. Low-energy portable industrial radiography X-ray machine, with anode water-cooling and 100% duty cycle, is usually used to perform research and services in nondestructive testing of materials. However, it can be also considered as a powerful tool to provide irradiation for research works in phytosanitary, in stored product pest control and in SIT pest management programs. This type of X-ray machine is capable to provide irradiation doses up to 400 Gy at once, without interruption, for the selected kV and FPID that situated between 40 and 100 cm. In addition, these types of X-ray machines are portable and require little shielding to comply with the safety protection regulations from ionizing radiation, thus, the irradiation can be performed in both research laboratory or in nondestructive testing laboratory (Martin, 2006; Maharaj, 2008; Bushong and Goerner, 2012; Kwon *et al*., 2022). Moreover, this type of X-ray machine, due to its light safety protection shielding, can be used as an integral part of low-production commercial system for irradiation treatment of single layers of grains, seeds or any agriculture products moving over a belt (Hallman, 2013; Indiarto *et al*., 2020; Zhang and Zhou, 2022). Consequently, this system will overcome the public concerns of consum-

ing irradiated agriculture products such as the one that commercially irradiated using Cobalt-60 radioisotope due to the believe that these commodities may have been polluted and become radioactive (Fox, 2002; Gunes and Tekin, 2006; Maherani *et al*., 2016; Castell-Perez and Moreira, 2021).

Thus, the study aimed to investigate the potential of low-energy X-ray irradiation as a substitute for conventional irradiation techniques, while also assessing whether the sterilization process negatively affected important biological and performance parameters of *P. operculella* pupae and adult moths instead of using gamma rays' radiation.

## Materials and methods

### Characterizations of the Smart 160W industrial X-ray machine

A directional beam portable industrial radiography X-ray machine with anode water cooling and 100% duty cycle at 20°C was used to irradiate *P. operculella* at pupae and adult moth stages. The type of the X-ray machine that has been recruited in this study is YXLON International- Smart 160W. The working range of this equipment can be adjusted from 10 kV to160 kV. The operation current of the tube of this X-ray machine is fixed and equal to 4 mA. The conical shape of the radiation beam has an angle of 40°. In order to obtain a circular shape of the field of radiation, the axe of the radiation beam had been adjusted to be vertical on the irradiation plane. The diameter and the area of each circle with homogeneous irradiation field depend on the vertical distance between the focal spot of the X-ray tube and the plane of irradiation (Bryant and Mcintire, 1985; Halmshow, 1995).

The paragraph output radiation doses of the X-ray machine were measured in the radiography laboratory of the Atomic Energy Commission of Syria by using an ionization chamber that was calibrated in the Dosimetry laboratory of the International Atomic Energy Agency (IAEA). The measurements were performed for the whole operation kV rang, starting from 20 kV to 160 kV with 20 kV increment, by positioning the thin spherical sensor of the ionization chamber at the horizontal plan of irradiation at a vertical distance equal to 40 (cm), between the focal spot of the X-ray machine to the horizontal plan of irradiation distance (FPID), and exposing the sensor to X-ray radiation at every cited kV for one minute. The result of these measurements was taken as an accumulated electric charge in (Coulomb/min) unit and it was transformed to the (Gray/h) unit to draw the exposure curve of the relation between the irradiation doses in (Gray/h) for every applied kV at the cited FPID.

To respond to the different irradiation needs, the sensor of the ionization chamber was exposed for another FPID that equals to 50, 60, 75, and 100 cm respectively and the results of these exposures are shown in Table 1 and in Figure 1 (Ma *et al.*, 2001; Pernicka and McLean, 2007). The air kerma rate was measured by a reference ionization chamber PTW 34069 and determined using the following equation:

$$K_{air} = M_0.\ N_k.\ K_{T,P}.\ K_H.\ K_{pol}.\ K_s$$

Where:
$K_{air}$: air kerma rate (Gy/min)
$M_0$: average reading of the ionization chamber in (nC/min).
$N_k$: calibration factor of the ionization chamber in term of air kerma rate in (Gy/nC).
$K_{T,P}$: correction factor for air temperature and air pressure
$K_H$: correction factor for humidity
$K_{pol}$: correction factor for polarity
$K_s$: correction factor for recombination

### Insects stock culture

The insects used in this study were selected from our laboratory stock culture, which is renewed each year with a field collection of PTM individuals. They were reared on wax coated potato slices, maintained at a constant temperature of 25±1°C, with 70±5% relative humidity, and 12-hour light-darkness cycle as described by Saour and Makee (1997). The larvae were reared on

**Table 1**. Irradiation doses of the X- ray machine at the applied kV and for five values of FPID distances performed at X-ray irradiation of the potato tuber moth *Phthorimaea operculella*.

| Operating potential (KV) | Dose (Gy/h) at FPID= 40 cm | Dose (Gy/h) at FPID= 50cm | Dose(Gy/h) at FPID=60cm | Dose(Gy/h) at FPID=75cm | Dose(Gy/h) at FPID= 100cm |
|---|---|---|---|---|---|
| 20 | 87.8 | 56.2 | 39.0 | 25.0 | 14.0 |
| 40 | 125.8 | 80.5 | 55.8 | 35.8 | 20.1 |
| 60 | 170.1 | 108.9 | 75.6 | 48.4 | 27.2 |
| 80 | 201.9 | 129.2 | 89.6 | 57.4 | 32.3 |
| 100 | 221.6 | 141.8 | 98.4 | 63.0 | 35.4 |
| 120 | 237.6 | 152.1 | 105.5 | 67.6 | 38.0 |
| 140 | 247.2 | 158.2 | 109.8 | 70.3 | 39.6 |
| 160 | 253.6 | 162.3 | 112.6 | 72.1 | 40.6 |

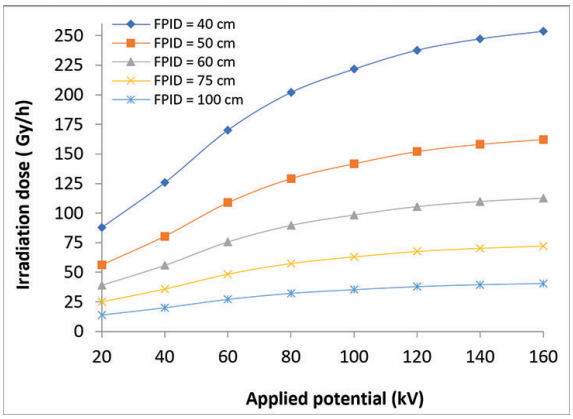


**Figure 1**. Curves of the irradiation doses as a function of the applied kV at five values of FPID performed at X-ray irradiation of the potato tuber moth *Phthorimaea operculella*.

wax-coated potato slices, which were laid over a layer of sand in plastic containers (40 cm x 25 cm x 10 cm) in which they pupated. As new adults emerged, they were collected and kept in 800ml transparent plastic jars (10-12 pairs per jar); a filter paper band was added to the bottom of every jar to promote oviposition, and 10% sucrose solution was provided as a food source. In addition to keeping females and males together until death, the eggs were collected daily, counted, and left until they hatched on new wax-coated potato slices. In the experiments, all the eggs, pupae and adults (male and female) used were randomly collected from the lab. colony at ≤24 h old. The stage (pupae, adult) that did not meet IAEA standards for quality control, such as undersized or malformed, were discarded. All experiments were repeated three times.

### Effect of X-ray irradiation of *P. operculella* pupae on adult emergence, longevity, fecundity and hatchability

Pupae n=120 were divided into four groups for X-ray irradiation with 0, 100, 150, and 200 Gy doses (each pupae group placed in plastic Petri dishes 9 cm diameter). Irradiation of the PTM pupae and adults was conducted at FPID equal to 50 cm at potential difference equal to 100 kV. After adult insect emergence, irradiated males and female moths in each group were paired individually in a 350 mL transparent plastic box provided with a band of filter paper for oviposition and 10% sucrose solution as a food source. At each studied dose, the adult males and female moths were counted as they emerged and kept for oviposition until death. Eggs laid at each dose were collected, counted, and incubated for hatching for determining the hatchability.

### Effect of X-ray irradiation of *P. operculella* adults on adult longevity, fecundity and hatchability

A group of 160 newly emerged females and males were exposed to X-ray irradiation doses (0, 100, 150, 200 Gy). The irradiated males and female moths of each dose were paired individually in the same above-mentioned 350 mL transparent plastic boxes and were kept to oviposition until they died; laid eggs were counted and set aside for determining the hatchability.

### Effect of X-ray irradiation of adults on the fecundity and hatchability of *P. operculella* after crosses of irradiated with non-irradiated males and females

N=400 pupae were individually placed in transparent plastic tubs (7 x 1.2 cm), and after adults' emergence, 80 females and males were chosen and exposed to X-ray irradiation different doses (0, 100, 150, 200 Gy). Irradiated females were divided into four groups (different dose exposure) and paired with80 non-irradiated males (20 couples/group). Similarly, 80 emerged males exposed to X-ray irradiation ate different doses (0, 100, 150, 200 Gy), were divided into four groups, and paired with 80 non-irradiated females. During each treatment, twenty pairs of males and females were placed in a breeding box containing cotton soaked in a sugar solution (10%) and filter paper surrounded for oviposition. After each dose treatment, the adults of both males and females were kept for oviposition until death. Eggs were collected, separated and counted daily to determine egg hatch.

### Statistical analysis

All statistical analyses were performed at $P < 0.05$, using SAS Institute, Cary, North Carolina, USA's P Stat View statistical software (version 5.0). ANOVA-Tukey HSD tests were used to determine the statistical significance of differences in means in emergence, adult longevity, fecundity, and hatchability data, and a Student's T-test was used to examine the variance in fecundity and hatchability between crosses of irradiated with non-irradiated males and females.

## Results

### Values of the measured output radiation doses of the X-ray machine

The values of the measured output radiation doses of the X-ray machine taken for eight values of kV that are, (20, 40, 60, 80, 100, 120, 140, 160) kV at every one of the five values of FPID that are 40, 50, 60, 75, and 100 cm are consigned in Table 1. The curves of the measured irradiation doses for the applied kV on the X-ray tube at each of the five cited values of FPID in Figure 1 reveal that the exposure dose increases by increasing the applied kV between the anode and the cathode of the X-ray tube, and the irradiation dose increases by decreasing the applied vertical value of FPID.

### Diameter of the circular areas of irradiation

The diameter of each one of the five circles that have homogenous irradiation dose taken at the five values of FPID is presented in Table 2. The recorded values were calculated by using the defined angle of the radiation beam divergence of the X-ray tube and the applied values of FPID. The diameters of the circular areas of irradiation vs the FPID values where the centers of all circles are located at the central axis of the radiation beam are shown in Figure 2. The optical photo during X-ray irradiation of *P. operculella* pupae and adult moths is presented in Figure 3.

### Effect of X-ray irradiation of *P. operculella* pupae on adult emergence, longevity, fecundity and hatchability

A significant, dose-dependent decreasing effects has been observed on many critical biological parameters including adult emergence, male and female moths longevity and fecundity (Table 3). A complete adult emergence was observed at the highest dose of 200 Gy. Furthermore, $F_1$ egg hatch-

**Table 2**. Diameter of the circular areas of the homogenous Irradiation doses at five FPID distances performed at X-ray irradiation of the potato tuber moth *Phthorimaea operculella*.

| FPID (cm) | Diameter of the circular area of irradiation(cm) |
|---|---|
| 20 | 14.6 |
| 40 | 29.1 |
| 60 | 43.7 |
| 80 | 58.2 |
| 100 | 72.8 |

ability has been completely inhabited at the dose of 150 Gy.

### Effect of X-ray irradiation of *P. operculella* adults on adult longevity, fecundity and hatchability

Adult longevity was not significantly impacted by any of the test doses when compared to the untreated control group. Conversely, both fecundity and $F_1$ egg hatchability confirmed a dose-dependent decreasing as the irradiation dose increased. Remarkably, $F_1$ egg hatch was almost completely inhibited by 150 Gy (Table 4).

### Effect of X-ray irradiation of adults on the hatchability and fecundity of *P. operculella* after crosses of irradiated with non-irradiated males and females

Fecundity and $F_1$ egg hatchability of offspring from all treatment crosses (normal females X treated males, treated females X normal males) declined with increasing X-ray dose. A notable sex-specific effect of irradiation was observed: crosses involving irradiated females paired with normal males exhibited significantly higher fecundity and $F_1$ egg hatchability compared to the reciprocal crosses in which irradiated males were paired with normal females (Table 5).

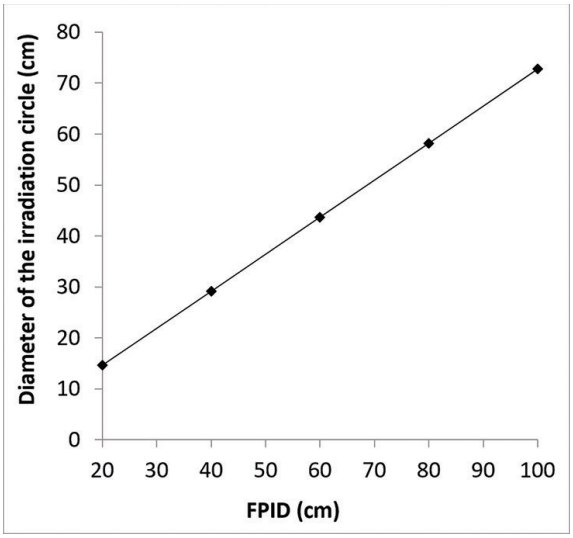


**Figure 2**. Diameters of the circular area of the homogeneous irradiation dose vs the vertical values of FPID performed at X-ray irradiation of the potato tuber moth *Phthorimaea operculella*.

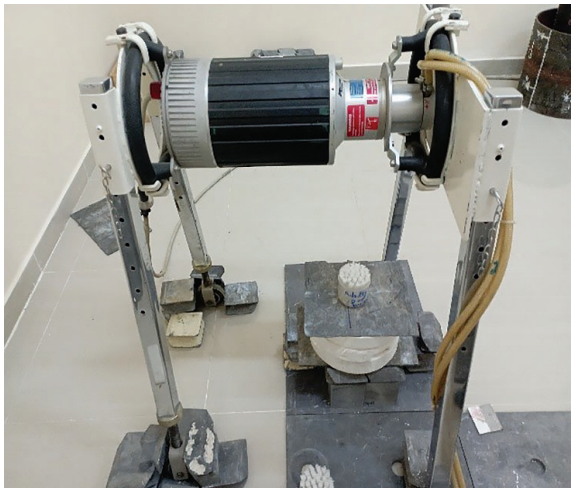

**Figure 3**. YXLON International - Smart 160W X-ray machine during irradiation for sterilisation of *Phthorimaea operculella* pupae and adults.

**Table 3**. Effect of X-ray irradiation of *Phthorimaea operculella* pupae on adult emergence, longevity, fecundity, and hatchability.

| Dos (Gy) | n | Emergence (%) (Mean ± SD) | Adult Longevity (♀, day) (Mean ± SD) | Adult Longevity (♂, day) (Mean ± SD) | No. Eggs (♀/total) (Mean ± SD) | Hatchability (%) (F1) (Mean ± SD) |
|---|---|---|---|---|---|---|
| 0 | 30 | 93 ± 2.5 A | 10 ± 0.9 A | 9.3 ± 1.3 A | 58.9 ± 4.5 A | 85.1 ± 8.6 A |
| 100 | 30 | 30 ± 4.6 B | 5.4 ± 1.1 B | 6.4 ± 1.5 B | 4.3 ± 2.9 B | 1.1 ± 0.7 B |
| 150 | 30 | 6.7 ± 2.6 C | 1.4 ± 1.2 C | 1.7 ± 0.7 C | 1.3 ± 1.2 C | 0 B |
| 200 | 30 | 0 C | 0 D | N/A D | N/A C | N/AB |
| Df | | 2 | 2 | 2 | 2 | 2 |
| F- value | | 62.875 | 629.459 | 481.588 | 2158.897 | 1926.132 |
| p | | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |

Means ($\bar{x}$ ± SD) followed by different capital letters in columns are significantly different at $p < 0.05$ (Tukey HSD test).

**Table 4**. Effect of X-ray irradiation of *Phthorimaea operculella* adults on adult longevity, fecundity, and hatchability.

| Dose (Gy) | n | Adult Longevity (♀, day) (Mean ± SD) | Adult Longevity (♂, day) (Mean ± SD) | No. Eggs (♀/total) (Mean ± SD) | Hatchability (%) (Mean ± SD) |
|---|---|---|---|---|---|
| 0 | 20 | 9.6 ± 1.3 A | 9.85 ± 1.3 A | 56.85 ± 5.7 A | 93.6 ± 5.03 A |
| 100 | 20 | 9.3 ± 1.2 A | 9.65 ± 1.6 A | 15.2 ± 4.3 B | 6.65 ± 2.04 B |
| 150 | 20 | 6.25 ± 1.3 A | 9.6 ± 1.4 A | 5.1 ± 1.2 C | 0 C |
| 200 | 20 | 6.35 ± 1.0 A | 9.2 ± 1.5 A | 0.15± 0.3 D | 0 C |
| Df | | 3 | 3 | 3 | 3 |
| F- value | | 0.335 | 0.777 | 1007.147 | 5699.981 |
| p | | 0.8036 | 0.5105 | <0.0001 | <0.0001 |

Means ($\bar{x}$ ± SE) followed by different capital letters in columns are significantly different at $p < 0.05$ (Tukey HSD).

**Table 5**. The effect of X-ray irradiation of *Phthorimaea operculella* adults on the fecundity and hatchability after crosses of irradiated with non-irradiated males and females.

| Dose (Gy) | n | (T♀ X N♂) | | (N♀X T♂) | |
|---|---|---|---|---|---|
| | | No. Eggs (Mean ± SD) | Hatchability (%) (Mean ± SD) | No. Eggs (Mean ± SD) | Hatchability (%) (Mean ± SD) |
| 0 | 20 | 72.9 ± 5.07 Aa | 77.4 ± 4.7 Aa | 78 ± 5.1 Aa | 73.9 ±4.7 Aa |
| 100 | 20 | 38.8 ± 7.9 Bb | 25.55 ± 4.6 Bb | 52.2 ± 8.9 Ba | 43.1 ±5.9 Ba |
| 150 | 20 | 26.8 ± 5.7 Cb | 8.2 ± 1.6 Cb | 38.8 ±8.3 Ca | 32.3 ±5.9 A Ca |
| 200 | 20 | 10.2 ± 2.4 Db | 0 Db | 18 ± 3.8 Da | 14 ±3.14 A Da |
| Df | | 3 | 3 | 3 | 3 |
| F-value | | 440.527 | 2078.554 | 268.187 | 281.993 |
| p | | <0.0001 | <0.0001 | <0.0001 | <0.0001 |

Means ($\bar{x}$ ± SD) followed by different capital letters in columns are significantly different at $p < 0.05$ (Tukey HSD test). Means ($\bar{x}$ ± SD) followed by different small letters in rows (between no. of eggs and hatchability in each cross with opposite) are significantly different at $p < 0.05$ (two-proportion z-test).

## Discussion

X-irradiation produced dose-dependent biological effects that varied by life stage and sex. When pupae were irradiated, it decreased adult emergence, longevity, and fecundity, completely preventing emergence at 200 Gy and fully inhibiting $F_1$ egg hatch at 150 Gy. In adults, longevity was unaffected, but fecundity and egg hatch declined with increasing dose, with total inhibition at 150 Gy. Cross-mating experiments revealed females are more strongly sterilized by irradiation than males, as irradiated females × normal males produced fewer offspring than the reciprocal cross.

Our study revealed that *P. operculella* pupae irradiated with X-rays decreased adult emergence and lifespan as well as fecundity, ending with a complete inhibition of $F_1$ egg hatchability at 150 Gy. There has been evidence that X-ray irradiation or gamma radiation has a significant negative impact on a variety of biological parameters in *P. operculella* (Saour and Makee, 1997; Cho *et al*., 2021). Accordingly, several studies on lepidopteran and dipteran pests including *Ephestia elutella* (Hübner) (Lepidoptera: Pyralidae), *Cydia pomonella* (Lepidoptera: Tortricidae), *Spodoptera litura* (Fabricius) (Lepidoptera:

Noctuidae), *Aedes aegyp*ti L. (Diptera: Culicidae), and *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae) showed that X-ray irradiation at definite doses resulted in a decrease in adult emergence, longevity, sterility, fecundity, and a zero $F_1$ egg hatch (Chang *et al*., 2016; Zhao *et al*., 2022; Jiang *et al*., 2022; Wang *et al*., 2023; Zhang *et al*., 2023; Chen *et al*., 2025). Thus, many studies on diverse insect species support these findings, and highlight the biological effects of X-ray radiation. Some of the comparative studies of X-ray and gamma irradiation indicated that X-ray can be as effective as or, in some cases, more effective than gamma irradiation depending on the applied dose, the insect species and the developmental stage (Hendrichs *et al.,* 2009; Yamada *et al*., 2023).

The results indicate that X-ray irradiation of adults could be a promising phytosanitary measure against *P. operculella* because as the dose increases, fecundity and egg hatch are clearly reduced, even though adult longevity is not significantly affected (Hallman and Hellmich, 2009). Morover, due to the higher sensitivity of females than males, treatment success depends on achieving complete sterility, especially in females, rather than causing immediate death; this finding is in accordance with international phytosanitary evidence, which indicates that irradiation can be beneficial when it induces sterility or $F_1$ sterility rather than direct death (Follett, 2024). As a result of cross-mating results, X-ray irradiation is an effective technique for supporting SIT programs, since irradiated adults still mate but produce fewer viable offspring; this makes sterile males especially important, since they can compete with wild males and reduce reproduction without killing them immediately (Saour and Makee, 1997; Dyck *et al*., 2021).

Regarding the target developmental stage of PTM for SIT, fertility was strongly suppressed after X-ray irradiation of both pupal and adult stages, but survival effects were different; whereas pupal irradiation reduced adult emergence more strongly, adult irradiation caused only sterility, with little effect on longevity; overall, the study suggests that male-only release of irradiated *P. operculella* would be the most practical approach to SIT (Makee and Saour, 1999; 2003; Marec and Vreysen, 2019).

The current research indicates that X-ray irradiation doses cause sterilization of *P. operculella*, imparting the same sterilization properties as gamma in earlier studies (Saour and Makee, 1997). However, other studies have shown that X-rays can be equally effective, if not superior, to gamma rays for sterilizing many other species. For example, X-ray irradiation produced male sterility comparable to gamma rays in *A. aegypti* pupae and adults in comparison to gamma rays (Wang *et al*., 2023). Low-rate of irradiation in the smart 160W industrial X-ray machine could explain why insect sterility is higher at high doses as compared to gamma radiation doses. However, when used in SIT at the same radiation dose, there is a clear difference between the effects of high and low radiation dose rates on insect reproduction. In accordance with the results of LEPIR X-ray machine irradiation (low-energy), Bakri *et al*. (2021) reported that the application of high doses at low dose rates results in higher sterility, which explains why high doses may be more effective when low dose rates are applied.

## Conclusion

The findings of this study have particularly important implications for the SIT on the PTM control because they demonstrate that low-energy LEPIR X-ray machine irradiation can be an effective substitute for gamma radiation in inducing PTM sterility. Observed reductions in reproductive capacity demonstrate that this approach has the potential to suppress pest populations effectively. Furthermore, the dose-dose rate relationship provides valuable insight into optimizing irradiation protocols, which can increase the efficiency and cost-effectiveness of SIT programs. Further research should explore the potential of X-ray-based sterilization for use as an alternative to gamma radiation in the SIT against *P. operculella*.

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# Φορητή βιομηχανικής χρήσης συσκευή ακτίνων-Χ χαμηλής ενέργειας για τη στείρωση της φθορίμαίας *Phthorimaea operculella*

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**Περίληψη** Η ακτινοβολία γ χρησιμοποιείται εδώ και πολλά χρόνια σε ευρεία κλίμακα στην ιατρική, βιομηχανική και γεωργική έρευνα και τις εφαρμογές τους. Πολλές χώρες έχουν εγκρίνει τη χρήση της ως αποτελεσματικό εργαλείο για την τεχνική εξαπόλυσης στείρων εντόμων (Sterile Insect Technique, SIT) στο πλαίσιο ολοκληρωμένων προγραμμάτων διαχείρισης επιβλαβών οργανισμών. Η παρούσα μελέτη αξιολογεί τη δυνατότητα χρήσης μιας φορητής βιομηχανικής συσκευής ακτίνων Χ χαμηλής ενέργειας (Low-Energy Portable Industrial Radiography, LEPIR), η οποία χρησιμοποιείται συνήθως για μη καταστροφικούς ελέγχους υλικών, για τη στείρωση νυμφών και ακμαίων της φθορίμαίας της πατάτας (*Phthorimaea operculella*), ως εναλλακτική της ακτινοβολίας γ που χρησιμοποιείται συνήθως στα προγράμματα SIT. Τα αποτελέσματα μας συμφωνούν με προηγούμενα ευρήματα και καταδεικνύουν σαφείς, δοσοεξαρτώμενες επιζήμιες επιδράσεις σε βασικές βιολογικές παραμέτρους του εντόμου, όπως η έξοδος των ακμαίων, η μακροβιότητα, η γονιμότητα, η αναπαραγωγική ικανότητα, καθώς και η εκκολαψιμότητα των αυγών της πρώτης θυγατρικής γενιάς (F1), σε δόση 150 Gy. Τα ευρήματα αυτά υποδεικνύουν ότι η συσκευή ακτινών X LEPIR μπορεί να αποτελέσει μια πρακτική και αξιόπιστη εναλλακτική λύση έναντι της ακτινοβόλησης με ακτίνες γ σε προγράμματα SIT που στοχεύουν στον σκώρο της πατάτας.

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REVIEW ARTICLE

# Herbicide-resistant weeds in Greek rice cultivation systems: A Review of the status, drivers, and Integrated Management Implications

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**Summary** Herbicide resistance has emerged as a major constraint to sustainable rice (*Oryza sativa* L.) production in Greece, where intensive water-seeded monoculture systems rely heavily on chemical weed control. This review synthesizes current knowledge on rice production systems, herbicide use patterns, and the evolution of herbicide-resistant weeds in Greek agroecosystems. Rice cultivation is concentrated in northern Greece under flooded conditions that favor the proliferation and persistence of competitive weed species. The dominant resistant weeds include *Echinochloa* spp., *Cyperus difformis*, weedy rice (red rice) and *Leptochloa fusca* subsp. *fascicularis*. Resistance has been documented to multiple herbicide modes of action, including propanil, acetolactate synthase (ALS) inhibitors, acetyl-CoA carboxylase (ACCase) inhibitors, synthetic auxins, and glyphosate, with increasing occurrence of cross- and multiple-resistant biotypes. Resistance evolution is driven by prolonged herbicide reliance, continuous monoculture, seedbank persistence, and dispersal through irrigation networks. The review further evaluates species-specific resistance mechanisms and highlights integrated weed management strategies based on preventive, tactical, and long-term Best Management Practices. This baseline assessment provides a consolidated reference for researchers, advisors, and policymakers and supports the development of coordinated resistance management frameworks aligned with EU Integrated Pest Management principles and the specific challenges of Greek rice production.

*Additional keywords*: ALS inhibitors, cross-resistance, integrated weed management, paddy fields, resistance mechanisms, water seeded rice

## 1. Introduction

Rice cultivation in Greece is geographically concentrated in a few major delta and basin systems, namely the Axios–Aliakmonas–Gallikos Delta, the Strymonas/Serres Basin, and smaller production areas in Kavala/Drama and western Greece in Amvrakikos region, (Vasilakoglou *et al*., 2018). These regions account for the national rice production and are characterized by intensive, monocultural, flooded paddy systems. Such production conditions create an ideal environment for the evolution and establishment of herbicide-resistant weed populations, turning Greek rice agroecosystems into key hotspots of herbicide resistance (Vasilakoglou *et al*., 2000; Kaloumenos *et al*., 2013a; Kaloumenos *et al*., 2013b).

Over the past three decades, numerous cases of resistance have been documented among the dominant weed species, including *Echinochloa phyllopogon, E. oryzicola, E. crus-galli, E. colona*, *Cyperus difformis*, *Oryza sativa*, and *Leptochloa* sp. (Heap, 2026). The weed flora in Greek paddies is dominated by highly fecund and persistent species, such as *Echinochloa* spp., *Cyperus difformis*, and weedy/red rice (*Oryza sativa* f. *spontanea*); those species biology, including high seed output, prolonged seed persistence, and adaptability to alternating moisture regimes, amplifies the risk of resistance selection under repeated use of narrow-spec-

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trum herbicides (Damalas *et al*., 2008; Osca *et al.,* 2021).

A special perspective on herbicide resistance is necessary in Greece since water-seeded rice systems in the country present a unique combination of agronomic, ecological, and socio-economic features that distinguish them from upland or Asian and American rice systems. Key contributors in the accelerated evolution of herbicide resistance in Greek rice systems include the long-term reliance on a limited range of herbicide modes of action, which has created a highly selective environment favoring the proliferation of resistant biotypes, minimal crop rotation, and the occurrence of weed mimicry, particularly in *Echinochloa phyllopogon* (Papapanagiotou *et al*., 2025a). Additional drivers involve the re-infestation of paddies through irrigation and drainage canals, which act as reservoirs of resistant biotypes, and the biological stability and persistence of resistant phenotypes, which allow their propagation across seasons without significant fitness penalties.

Greek specific constraints, including the limited herbicide toolbox, EU regulatory restrictions, and environmental protection requirements, shape both the choice and timing of chemical interventions, making stewardship and Best Management Practices (BMPs) context-dependent. Evidence from other regions shows that BMPs succeed only when tailored to local biological, chemical, and socio-economic conditions (Norsworthy *et al*., 2012).

The present baseline report consolidates available knowledge on the geographic distribution of resistance, mechanisms underlying herbicide adaptation, agronomic drivers, and potential management strategies in Greek rice production systems. By synthesizing these data, the report aims to provide a comprehensive foundation for understanding current resistance dynamics and informing future integrated weed management interventions. This work has been conducted within the framework of the Hellenic Foundation for Research & Innovation (H.F.R.I) research project named ARTS-Rice project (www.arts-rice.gr), which aims to enhance sustainable rice production in the Mediterranean through integrated weed management and the mitigation of herbicide resistance.

## 2. Rice production systems in Greece

### Production systems

Rice cultivation in Greece is concentrated primarily in Central Macedonia, including the prefectures of Thessaloniki, Imathia, and Pella, which collectively represent the largest production zone (>70%) and focus on intensive commercial varieties, both japonica and indica types. The second major area is the Serres/Strymonas Basin (~20% of national production), while Eastern Macedonia–Thrace, encompassing Kavala and Drama, supports smaller but historically significant areas for the emergence and study of herbicide-resistant weeds. Finally, the Arta/Amvrakikos region represents a minor production area. Across these regions, rice agroecosystems are highly specialized, with heavy alluvial soils, permanent irrigation infrastructure, and intensive management practices.

Continuous rice monoculture dominates Greek rice cultivation, with minimal crop rotation and uniform management practices (Papapanagiotou *et al.,* 2025a). Soils are generally heavy-textured and fertile, allowing high yields under flooded conditions. Key weed species that thrive under these agro-ecological conditions include *Echinochloa* spp., *Cyperus difformis*, *Alisma plantago-aquatica*, and weedy rice populations, many of which have developed herbicide resistance over time (Vasilakoglou *et al*., 2018). The combination of monoculture, uniform herbicide application, and the stability of resistant phenotypes has contributed to the persistence and expansion of problematic weed populations.

### Herbicide use

Weed control strategies rely heavily on a rather narrow portfolio of herbicides, which

has intensified selection pressure and contributed to resistance evolution. During the last 5 years, commonly used modes of action; data retrieved from the Greek Ministry of Rural Development and Food's Plant Protection Products database on January 8, 2026. The list is as follows:

- ALS inhibitors (Group 2). penoxsulam, bispyribac-sodium, halosulfuron-methyl and imazamox; only in Clearfield systems
- ACCase inhibitors (Group 1): cyhalofop-butyl, profoxydim, propaquizafop, and clethodim; only in Provisia systems
- Auxinic herbicides (Group 15): MCPA, triclopyr, florpyrauxifen
- PS-II Inhibitors (Group 5): bentazone
- EPSPS inhibitots (Group 9): glyphosate
- Microtubule Polymerization Inhibitor (MPI) (Group 3): pendimethalin
- DOXP inhibitor (Group 12): clomazone
- HPPD inhibitor (Group 27): benzobicyclon

In addition to fully authorized plant protection products, several herbicides have been authorized in Greek rice systems under emergency authorizations, as permitted under Article 53 of Regulation (EC) No 1107/2009 (European Parliament and Council of the European Union, 2009). These include benzobicyclon, profoxydim, propanil, quinclorac, and pretilachlor, all granted for periods not exceeding 120 days. The recurring reliance on such emergency authorizations reflects the structural limitations of the fully registered herbicide portfolio in Greek rice and highlights the regulatory vulnerability of the sector's weed management toolbox, particularly in the context of escalating herbicide resistance.

## 3. Herbicide resistance in rice

### Propanil Resistance

Historically, the first documented cases of herbicide resistance in Greek rice occurred in the 1980s with Echinochloa crus-galli exhibiting reduced susceptibility to propanil (Giannopolitis and Vassiliou, 1989). Metabolic detoxification mechanisms were identified as key contributors to early resistance, signaling the vulnerability of Greek rice systems to adaptive responses among dominant weeds (Giannopolitis and Vassiliou, 1989; Vasilakoglou et al., 2000). These early observations highlighted the limitations of relying solely on a single mode of action for weed control and set the stage for future resistance monitoring.

### ALS Resistance Establishment

This emergence coincided with the intense reliance on ALS inhibitors (i.e. during the 2000-2010 period), reflecting the direct link between herbicide use patterns and the evolution of target-site resistance. As such, ALS-inhibitor resistance became increasingly widespread. Resistance was confirmed in Echinochloa oryzoides and E. phyllopogon (Damalas et al., 2006, 2008), while Cyperus difformis developed ALS resistance via specific point mutations in the ALS gene (Ntoanidou et al., 2016).

### Cross- and Multiple Resistance

Between 2010 and 2020, multiple cases of cross-resistance to acetolactate synthase (ALS)-inhibiting herbicides were confirmed in Greek rice agroecosystems, involving active ingredients such as penoxsulam, bispyribac-sodium, and imazamox. During the same period, the emergence of acetyl-CoA carboxylase (ACCase) inhibitor resistance was also reported, further constraining chemical control options (Damalas et al., 2006; Vasilakoglou et al., 2018). In addition, documented herbicide antagonistic interactions reduced field-level efficacy, exacerbating control failures. Collectively, these developments reflect the cumulative effects of prolonged and repetitive herbicide selection pressure and expose the structural limitations of conventional herbicide rotation schemes in managing genetically diverse and adaptable weed populations.

Since 2020, resistance patterns have be-

come increasingly complex, with stacked and multi-mechanism resistance phenotypes now being reported. Notably, multiple resistance to ALS inhibitors, ACCase inhibitors, and auxinic herbicides has been identified in *Echinochloa phyllopogon* populations (Papapanagiotou *et al*., 2025a), while glyphosate resistance has been documented in *Echinochloa colona* (Travlos *et al*., 2020). Importantly, resistant biotypes appear to exhibit no detectable fitness penalties under non-treated conditions (Papapanagiotou *et al.,* 2023), facilitating their persistence, spread, and competitive dominance within rice production systems.

This advanced resistance phase represents a critical challenge for sustainable weed management in Greek rice systems and underscores the urgent need for integrated weed management (IWM) approaches. Such strategies must move beyond herbicide-centric paradigms and incorporate chemical diversification, cultural practices, ecological regulation, and system-level redesign to effectively mitigate resistance evolution and ensure long-term agroecosystem resilience.

## 4. Weed species-by-species resistance profile to herbicides in rice

### *Echinochloa phyllopogon* (late watergrass)

***Resistance status.*** *Echinochloa phyllopogon* has become one of the most problematic weed species in Greek rice systems due to its widespread and robust resistance profile. ALS inhibitor resistance is strong and prevalent across major rice-growing zones (Vasilakoglou *et al*., 2018), while ACCase resistance has also been confirmed in multiple field populations (Papapanagiotou *et al*., 2025a). Resistance to auxinic herbicides, including dicamba and MCPA, has emerged more recently (Papapanagiotou *et al*., 2025a). Greece represents one of the earliest European cases where multiple resistance to ALS, ACCase, and auxinic herbicides has been documented (Papapanagiotou *et al*., 2025a).

Global studies indicate emerging resistance and reduced sensitivity of *Echinochloa* species to florpyrauxifen-benzyl, driven by target-site mutations and metabolic detoxification mechanisms (Zhang *et al*., 2023; Deng *et al*., 2025). Although only empirical reports of reduced efficacy currently exist in Greece, the confirmation of similar metabolic resistance mechanisms in Greek *Echinochloa* populations suggests a high risk of cross-resistance and necessitates proactive monitoring to ensure long-term herbicide efficacy.

***Mechanism.*** Resistance in *E. phyllopogon* is driven by a combination of target-site and non-target-site mechanisms. ALS target-site mutations, including Trp574 and Pro197 variants, are commonly found. Additionally, enhanced metabolic detoxification, likely mediated through cytochrome P450 enzymes, contributes to herbicide breakdown. Importantly, these resistant biotypes exhibit no measurable fitness penalty, allowing them to persist and spread under continuous selection pressure.

### *Echinochloa oryzicola*

***Resistance status.*** *Echinochloa oryzicola* is considered one of the most noxious weeds in rice fields in Northern Greece (Kaloumenos *et al*., 2013b). Several studies have been conducted in order to confirm resistance to ALS -inhibiting herbicides. Specifically, it has been found that *E. oryzicola* indicates cross resistance to penoxsulam, bispyribac-sodium, imazamox, foramsulfuron, nicosulfuron and rimsulfuron. It is suggested that cross resistance to ALS herbicides is imposed by continuous use of them and highlights the herbicide selection pressure caused by rice monoculture.

***Mechanism.*** *Echinochloa oryzicola* exhibits well-documented ALS inhibitor resistance, primarily associated with the Trp-574-Leu point mutation (Kaloumenos *et al*., 2013b).

***Echinochloa crus-galli* (barnyard grass)**
***Resistance status.*** *Echinochloa crus-galli* was the first Greek rice weed reported to develop herbicide resistance, specifically to propanil (Giannopolitis and Vassiliou, 1989; Vasilakoglou *et al*., 2000). Subsequent surveys have noted reduced susceptibility to ALS-inhibiting herbicides, suggesting ongoing adaptation under continued selection pressure.

***Mechanism.*** Metabolic resistance, potentially involving enhanced detoxification pathways, is likely responsible for reduced herbicide sensitivity (Kanatas, 2020).

***Echinochloa colona* (jungle rice)**
***Resistance status.*** Glyphosate resistance has been confirmed in *E. colona* populations (Travlos *et al.,* 2020). While this species primarily affects levee and canal systems, its seeds can be transported via water into paddies, leading to reinfestation and potential establishment within cultivated fields.

***Mechanism.*** No EPSPS target-site mutations have been detected, suggesting that resistance is driven by metabolic mechanisms that degrade glyphosate or reduce its uptake.

***Echinochloa oryzoides (early watergrass)***
***Resistance status.*** A major challenge with *E. oryzoides* is its morphological similarity to cultivated rice during early growth stages (Altop *et al*., 2014). This mimicry allows seedlings to escape pre-emergence and early post-emergence herbicide applications, resulting in significant contributions to the soil seedbank and making management particularly difficult. Early signs of ACCase tolerance were also observed in some populations (Damalas *et al.,* 2006), suggesting the potential for future multiple-resistance development.

***Mechanism.*** No further evidence is available regarding the underlying resistance mechanisms implying that a genetic-based classification between tolerant and susceptible accessions cannot be defined (Altop *et al.,* 2014).

**High-Risk Traits in *Echinochloa* spp**. *Echinochloa* spp. possess a set of biological traits that facilitate resistance evolution and persistence. The key biological traits that confer high resistance risk to each *Echinochloa* species present in Greek rice systems are summarized in Table 1.

***Cyperus difformis* (rice sedge)**
***Resistance status.*** Control of rice sedge has declined sharply in areas where ALS herbicides dominate weed management programs. This trend underscores the necessity for diversified weed control strategies combining chemical, cultural, and mechanical measures to prevent further expansion of resistant populations.

***Mechanism.*** ALS resistance in *Cyperus difformis* is widely documented across Greece (Ntoanidou *et al*., 2016; Vasilakoglou *et al*., 2018). Point mutations at Pro197 and Trp-574-Leu confer cross-resistance to multiple ALS-inhibiting herbicides, complicating conventional control approaches. Cross-resistance in Greek populations extends specifically to azimsulfuron and halosulfuron-methyl: Ntoanidou *et al.* (2016) demonstrated that applications of up to 64 times the recommended field rate of both herbicides failed to reduce growth of resistant *C. difformis* populations from the Thessaloniki area by 50%, while a susceptible reference population was controlled at one quarter of the recommended rate. This high-level cross-resistance across multiple ALS-inhibiting active ingredients further restricts the chemical control options available for sedge management in Greek rice andreinforces the need for herbicide diversification incorporating non-ALS modes of action such as bentazone, benzobicyclon, and florpyrauxifen-benzyl (Tehranchian *et al*., 2015).

**Weedy rice (red rice)**
***Resistance status.*** Weedy rice exhibits variable sensitivity to imazamox (Vasilakoglou

**Table 1.** Biological traits of *Echinochloa* spp. that increase the risk of herbicide resistance evolution and persistence in Greek rice paddies.

| Species | Trait Increasing Resistance Risk |
|---|---|
| *E. phyllopogon* | High fecundity, prolonged emergence period, extensive genetic variability, water-dispersed seeds facilitating movement across paddies |
| *E. oryzoides* | Rice mimicry during early growth, allowing escape from pre- and early post-emergence management, contributes to large soil seedbanks |
| *E. crus-galli* | Rapid metabolic adaptation, enabling early development of herbicide tolerance and potential cross-resistance |
| *E. colona* | Thrives in levee and canal margins, seeds easily reinvade paddies via water movement, facilitating regional dispersal |
| *E. oryzicola* | |

and Dhima, 2005). Although full resistance has not yet developed in Greek rice fields, the species poses a high evolutionary risk due to potential gene flow with imidazolinone-tolerant rice varieties cultivated elsewhere. If such gene transfer occurs, rapid emergence of resistant weedy rice could pose serious threats to production, necessitating close monitoring and proactive management strategies.

***Mechanism*.** The resistance mechanisms include: a) Target-site mutations: ALS (acetolactate synthase) gene in weedy rice often harbors amino acid substitutions. For example, in studies from other regions, the S653N substitution a) key mutation conferring imidazolinone resistance) has been found (Unan *et al.,* 2024); b) Gene flow: The same S653N mutation is found in IMI-resistant cultivated rice, suggesting that resistance in weedy rice may arise via cross-pollination (Unan *et al*., 2024); c) Non-target site mechanisms: Some resistant weedy rice individuals do not show ALS mutations, which implies possible metabolic resistance, or other mechanisms (e.g., enhanced herbicide detoxification) (Unan *et al*., 2024).

**Evolution through selection pressure:** Repeated use of imazamox (IMI) creates strong selective pressure, favoring both spontaneous mutants and geneflow events over time.

### *Leptochloa fusca* spp. *fascicularis* (bearded sprangletop)

***Resistance status*.** Populations of *Leptochloa fusca* spp. *fascicularis* have developed resistance mechanisms to ALS- inhibiting -herbicides in rice monoculture systems in northern Greece (Papapanagiotou *et al*., 2025b). It has been spotted on cross-resistance to ACCase inhibitors, including profoxydim, propaquizafop, cyhalofop + penoxsulam ACCase inhibitor cross resistance restricts the efficacy of available chemical control methods bearded sprangletop in rice cultivation.

***Mechanism*.** Resistance to *Leptochloa fusca* spp. *fascicularis* is related to target-site mutation of the ACCase enzyme. Specifically, this point mutation leads to substitution of tryptophan (Trp) by cysteine at position 1999 in the CT domain of the ACCase gene (Papapanagiotou *et al*., 2025a).

## 5. Management implications and recommendations

### Herbicide resistance management

Chemical control strategies must evolve to mitigate resistance. Immediate diversification of herbicide modes of action is critical, incorporating compounds such as florpyrauxifen, early-residual products (such as clomazone) and HPPD inhibitors (such as benzobicyclon). Herbicide rotation plans should be informed by population-specific resistance profiles, alternating ALS, ACCase, and auxinic herbicides where effective. In areas where ALS resistance is confirmed, ALS herbicides should be avoided to prevent further selection pressure.

## Introduction of new herbicides in rice systems

The increasing prevalence of herbicide-resistant weed populations in rice has intensified the need for new herbicide sites of action that can be integrated into existing weed management programs. In water-seeded rice systems, long-term reliance on acetolactate synthase (ALS) and acetyl-CoA carboxylase (ACCase) inhibitors has resulted in widespread resistance among key species such as *Echinochloa* spp., *Cyperus difformis*, and other aquatic weeds.

In response to these challenges, the recent introduction of benzobicyclon, a 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitor, and florpyrauxifen-benzyl, a synthetic auxin of the arylpicolinate group, has expanded the herbicide portfolio available for rice production. These herbicides provide alternative modes of action, improved crop selectivity under flooded conditions, and new opportunities for rotating and diversifying chemical weed control strategies, thereby supporting integrated approaches to herbicide resistance management in modern rice systems (Norsworthy *et al*., 2012; Duy *et al*., 2018; Poole, 2020).

***Benzobicyclon.*** Benzobicyclon introduces a mode of action distinct from ALS and ACCase inhibitors, which dominate weed control programs in Greek rice systems. Its importance lies in providing an effective alternative for managing weed populations resistant to these commonly used herbicide groups (Papapanagiotou *et al*., 2025a). From a resistance-management perspective, benzobicyclon is particularly valuable for controlling ALS-resistant sedges, broadleaf weeds, and *Echinochloa* spp., and has also demonstrated some activity against resistant weedy rice populations. Its integration into rice weed management programs allows rotation of herbicide sites of action and reduces selection pressure on ALS and ACCase inhibitors, provided it is used within diversified chemical and cultural strategies (Liu *et al*., 2021).

***Florpyrauxifen-benzyl.*** Florpyrauxifen-benzyl represents a novel auxinic chemistry for rice weed management. Unlike older synthetic auxins, florpyrauxifen-benzyl primarily interacts with the AFB5 auxin receptor, resulting in a distinct physiological response in susceptible weeds (Epp *et al.,* 2016).

The herbicide is characterized by excellent crop selectivity in rice, allowing both pre-emergence and post-emergence applications without significant phytotoxicity when applied according to label recommendations (Epp *et al*., 2016). This selectivity, combined with systemic activity, enables effective control of a broad spectrum of problematic weeds in flooded rice systems, including *Echinochloa* spp., *Cyperus difformis*, *Schoenoplectus* spp., *Heteranthera* spp., and *Alisma* spp., many of which have developed resistance to ALS inhibitors.

From a resistance-management standpoint, florpyrauxifen-benzyl offers a critically important alternative site of action for Mediterranean rice systems heavily reliant on ALS and ACCase inhibitors. Its use in sequential or rotational programs, either alone or in combination with other herbicides, contributes to diversified selection pressure and helps delay further resistance evolution. However, indications of reduced sensitivity to florpyrauxifen-benzyl have been documented in *Echinochloa* spp. populations in other rice-producing regions. Lim *et al*., (2021) and Takano *et al*., (2023) reported that CYP450-mediated metabolic detoxification can confer reduced sensitivity to florpyrauxifen-benzyl in *Echinochloa* populations with a prior history of ALS inhibitor exposure. This finding is of particular relevance to Greek rice systems, where CYP450-mediated metabolic resistance to ALS and ACCase inhibitors has already been confirmed in *E. phyllopogon* populations (Papapanagiotou *et al*., 2025a), raising the possibility that cross-resistance to florpyrauxifen-benzyl may emerge under continued selection pressure.

This cross-resistance risk is particularly relevant in Greek rice systems, where repeated exposure to ALS inhibitors has progres-

sively selected for enhanced CYP450-mediated detoxification, the same mechanism already confirmed in *E. phyllopogon* populations from Greek rice fields (Papapanagiotou *et al*., 2025a). Given the critical role of florpyrauxifen-benzyl in modern weed management, its long-term viability depends on a shift away from exclusive reliance toward its strategic integration within diversified management frameworks. To safeguard its efficacy and ensure the sustainability of rice production, it is essential to combine this chemistry with alternating modes of action and robust cultural control tactics (Wright *et al*., 2021). Such proactive stewardship is not merely a recommendation but a necessity to prevent the onset of resistance and preserve this vital tool for future use.

## Combination of chemical control with rotational crops

In Greek rice systems, when rotational crops are used, the following could be applied: a) Crop rotation with cotton and the use of soil-applied herbicides (benfluralin, pendimethalin) or post-emergence grass herbicides (propaquizafop, quizalofop, cycloxydim, clethodim). These herbicides have a different mode of action than ALS inhibitors and therefore offer effective alternatives against imazamox-resistant populations; b) Crop rotation with maize, combined with soil-applied herbicides either single or in mixtures such as (dimethenamid + terbuthylazine, or pethoxamid + terbuthylazine or mesotrione + terbuthylazine or sulcotrione + terbuthylazine). Application of pre-emergence herbicides with different modes of action also helps manage ALS-resistant populations; c) Crop rotation with cereals, particularly barley (*Hordeum vulgare* L.), is an effective complementary strategy for managing herbicide-resistant weeds in Greek rice-based systems, especially in coastal and saline soils where barley is well adapted. The cereal phase disrupts the life cycle of rice-adapted weeds, reduces the soil seedbank of *Echinochloa* spp. and weedy rice. The shift to autumn sowing further alters weed emergence patterns and enables the use of cultural practices that contribute to seedbank depletion. From a resistance-management perspective, cereals provide both chemical diversification and ecological disruption, reducing selection pressure on rice herbicides and limiting the carryover of resistant weed populations into subsequent rice crops (Buhler *et al*., 1997; Beckie, 2006).

***Herbicide-Tolerant Rice Systems.*** The management of herbicide-resistant weeds in water-seeded rice systems has been particularly challenging in Mediterranean environments, where continuous rice monoculture, limited crop rotation options, and prolonged flooding constrain the diversity of available weed control tactics. Under these conditions, herbicide-tolerant (HT) rice systems have emerged as important tools within integrated weed management frameworks, especially for controlling weedy rice (*Oryza sativa* f. *spontanea*) and herbicide-resistant grass weeds such as *Echinochloa crus-galli*.

***The Clearfield® Rice System.*** The Clearfield® rice system, based on tolerance to imidazolinone (IMI) herbicides, was first commercialized in the early 2000s and represents a major milestone in rice weed management. Clearfield rice varieties are non-genetically modified and were developed through induced mutation and conventional breeding, carrying point mutations in the acetolactate synthase (ALS) gene that confer resistance to IMI herbicides such as imazamox and imazethapyr (Tan *et al*., 2006). These mutations typically involve amino acid substitutions at key positions of the ALS enzyme (e.g. Ala122, Ser653), reducing herbicide binding without compromising crop fitness.

In Mediterranean rice systems, including Greece, Clearfield technology has enabled effective post-emergence control of weedy rice populations and *Echinochloa* species that are difficult or impossible to manage selectively in conventional rice. Yield stability and harvest quality improvements following Clearfield adoption have been widely documented (Burgos *et al.,* 2008;

Kaloumenos *et al*., 2013b). However, the intensive and repeated use of IMI herbicides has also resulted in rapid selection pressure, leading to the evolution of IMI-resistant weedy rice through both target-site resistance and gene flow from Clearfield cultivars (Busconi *et al*., 2012; Goulart *et al*., 2014).

Hybridization between Clearfield rice and weedy rice has been reported in several rice-growing regions within only a few years of adoption, highlighting the need for strict stewardship (Shivrain *et al*., 2007; Norsworthy *et al*., 2012). In Mediterranean environments, where water-seeded systems favor synchronous emergence and flowering, the risk of outcrossing may be particularly high if volunteer rice and escaped weedy rice plants are not rigorously controlled. Consequently, Clearfield rice cannot be considered a standalone resistance solution but rather a transitional tool that must be embedded within rotation schemes, non-ALS herbicide use, and cultural practices such as stale seedbeds and volunteer management.

***The Provisia® Rice System.*** To address the increasing prevalence of IMI-resistant weedy rice and grass weeds, the Provisia® rice system was developed as a complementary HT technology. Provisia rice varieties are tolerant to ACCase-inhibiting herbicides (Group 1), particularly cycloxydim, and were likewise developed through conventional breeding rather than genetic engineering (Camacho *et al*., 2019). Resistance is conferred by a specific point mutation in the ACCase enzyme (Ile1781→Leu), which reduces herbicide sensitivity while maintaining normal lipid biosynthesis in the crop.

The Provisia system provides effective control of IMI-resistant weedy rice, *Echinochloa* spp., and other problematic grasses, offering a critically needed alternative mode of action in rice systems dominated by ALS resistance. Field evaluations have demonstrated high levels of efficacy when cycloxydim is applied sequentially at early growth stages of target weeds, particularly under low-stress crop conditions (Webster *et al*., 2021).

In Greece, the recent introduction of Provisia rice represents an important opportunity to diversify chemical weed control in water-seeded rice, where ACCase inhibitors were previously unavailable for selective in-crop use (Papapanagiotou *et al*., 2025a). However, as with Clearfield, the sustainability of Provisia depends on strict stewardship. ACCase resistance in grass weeds is well documented globally, and over-reliance on cycloxydim would likely result in rapid resistance evolution if used continuously or without integration of residual herbicides and non-chemical tactics (Webster *et al*., 2021).

***Stewardship and integration in rice systems.*** International experience clearly demonstrates that HT rice systems extend their useful lifespan only when alternated with each other and with conventional rice production. In Greek rice systems, best management practices include rotating Clearfield and Provisia technologies across years, avoiding consecutive planting of the same HT trait, integrating pre-emergence residual herbicides with different modes of action, and enforcing zero tolerance for volunteer rice and weedy rice escapes.

A rotational approach combining Clearfield and Provisia systems, complemented by crop rotation where feasible (e.g. maize, cotton, winter cereals), is particularly relevant for Greece, where rice is often grown continuously in deltaic environments. Such alternation reduces selection pressure on both ALS and ACCase target sites and limits the accumulation of resistant weedy rice biotypes. Importantly, HT rice systems must remain embedded within broader integrated weed management strategies that include water management, competitive cultivars, precise fertilization, and mechanical or manual removal of escapes.

Overall, Clearfield and Provisia technologies represent powerful but finite tools for resistance management in Mediterranean water-seeded rice. Their value lies not in replacing integrated weed management, but

in restoring chemical diversity and enabling strategic rotations that delay resistance evolution while maintaining crop productivity.

## Preventive measures and strategies

The use of clean, certified, and weed-free crop seed is one of the most cost-effective and preventive strategies against the introduction and spread of weedy rice (*Oryza sativa* f. *spontanea*) and other problematic weed species. In Mediterranean rice ecosystems, contaminated seed lots represent a primary vector for herbicide resistance spread and weed re-infestation, especially where farmers rely on saved seed. Studies across Greece, Italy, and Spain have shown that even minimal contamination (<1%) can perpetuate weedy rice populations for multiple seasons, due to seed dormancy and genetic mimicry (Ferrero *et al*., 2021).

***Avoiding contamination of rice fields: importance of using clean seed.*** The weed spreads from contaminated to clean areas through infested seed. Animals and machinery can also disperse wild rice. Some national laws allow small numbers of wild rice seeds in certified seed lots. Early detection and prevention of establishment are the first steps to reduce infestation and spread, especially in direct-seeded systems. Systematic monitoring is essential to prevent invasion and to enable timely management. Some farmers use saved seed from the previous year, which is often contaminated. Piveta *et al*. (2021) estimated that sowing seed contaminated with just 2 red rice seeds per kg can, after three years, produce 10 kg of red rice seed per acre. In Sri Lanka (Chauhan *et al*., 2014), using clean seed increased yield by 29–41% compared to farmer-saved contaminated seed.

***Harvest, drying, and storage sanitation.*** Physical contamination can also occur post-harvest, when weedy rice seeds are inadvertently mixed during combine harvesting, drying, or storage. Cleaning harvesters, transport augers, and storage bins between rice varieties or fields is critical. Studies in the U.S. Midsouth (Norsworthy *et al*., 2013) show that combines not cleaned between fields can transfer up to 10,000 weedy rice seeds per hectare. European best practices now recommend a cleaning protocol involving (i) removal of residual grain from headers and elevators, (ii) operation of the threshing system for 5–10 minutes to expel residual seeds, and (iii) physical washing of key components between seed lots (Gómez de Barreda *et al*., 2021). Additionally, segregated storage and drying facilities prevent the mixing of different seed lots. In Italy and Spain, regional cooperatives have adopted traceability systems for drying and packaging to maintain seed purity (Vidotto *et al*., 2021; Osca *et al*., 2021).

## Use of false seeding to manage weeds in paddy fields

Greek rice producers have been able to voluntarily participate in the "false seeding" program under the CAP Rural Development Program, receiving financial support of €200/ha/year. Beneficiaries who opt in are required to implement false seeding (preparation of a seedbed without actual rice sowing) for five consecutive years across all enrolled rice plots. After standard pre-sowing practices, certain obligations must be followed: Flood the plots lightly in April (about one month before normal rice sowing, depending on the region) to stimulate weed seed germination; Drain the water into nearby water bodies (rivers, wetlands, drainage canals); Mechanically destroy the emerged weeds by surface soil treatment before rice sowing.

False seeding can effectively control both resistant and *Ammannia* spp., sedges (Ferrero *et al*., 2021). Proper seedbed preparation, rational fertilizer use, competitive variety selection, dense sowing, and proper water management enhance the efficiency of false seeding. Using certified weed-free rice seeds is equally important to prevent introducing new weeds. Pre-plant soil treatment to 3–4 cm depth when weed seedlings have 2–3 leaves further improves false seeding efficiency. Deep plowing every 3–4

years during primary field preparation helps bury weed seeds to depths where they cannot germinate or rot due to excess moisture. Although effective against red rice and other grasses, false seeding has some challenges: a) Rice sowing occurs 2–3 weeks later than normal, reducing the feasibility of long-season, high-yielding varieties, and decreasing yields by 100–200 kg/ha. Yield is also affected by soil-climatic conditions and agricultural practices; b) Increased water supply duration by irrigation authorities raises water costs; c) It is difficult to implement on wet, heavy soils.

## 6. Knowledge gaps, monitoring needs, Best Management Practices

### Knowledge Gaps

Despite extensive research documenting herbicide resistance in Greek rice systems, several critical knowledge gaps remain. Firstly, there is no national resistance monitoring scheme, which limits the ability to detect emerging resistant populations in a timely manner or to coordinate mitigation efforts across regions. Secondly, systematic molecular characterization for ACCase and auxinic and to a lesser extent of ALS inhibitor resistance remains rather limited, constraining our understanding of the specific mutations, metabolic pathways, and potential for cross-resistance. Thirdly, high-resolution mapping of soil seedbanks is largely absent, leaving uncertainties regarding the spatial distribution and density of resistant seeds within and between paddies. Finally, long-term datasets evaluating fitness costs of resistant biotypes across diverse environmental conditions are lacking, which hampers predictive modeling of resistance spread and the development of sustainable management strategies.

### Monitoring Priorities

To address the aforementioned knowledge gaps, the following monitoring activities should be prioritized which are essential for establishing a coordinated, evidence-based approach to herbicide resistance management in Greece:

- Annual in-field and regional screening in high-risk regions such as the Thessaloniki Delta region to track emerging resistance trends.
- Molecular diagnostics targeting *Echinochloa* spp. to identify key ALS, ACCase, and auxinic resistance mutations, enabling early detection and informed management decisions.
- Canal network surveillance to monitor the spread of glyphosate-resistant *E. colona* and other water-dispersed weed populations, which serve as major reinfestation pathways.
- Seedbank resistance mapping to quantify the density, distribution, and temporal dynamics of resistant seeds, supporting strategic interventions at field and landscape scales.

### Best Management Practices

Considering the reviewed status, gaps and challenges, Best Management Practices (BMP) that could be applied at various stages of the crop and represent various targets in the Greek rice systems, incorporate preventive, tactical and long-term strategies (Table 2). In particular, preventive measures, including assessment of weed biology (BMP 1), multifaceted weed management strategies (BMP 2), maintenance of weed-free fields (BMP 3), use of certified weed-free seed (BMP 4), and routine scouting (BMP 5), are essential to reduce initial weed pressure and prevent the introduction of resistant biotypes such as Echinochloa spp. and weedy rice. Tactical BMPs, encompassing the use of herbicides with multiple modes of action (BMP 6), adherence to labeled application rates (BMP 7), and integration of crop competitiveness and cultural control measures (BMP 8), target early-season escapes, optimize herbicide efficacy, and minimize selection for resistance. Long-term measures, including mitigation of weed seed movement and crop residue management (BMP 9), harvest-time seed management

**Table 2**. A detailed concept of an Integrated Weed Management guide linking rice growth stages to key Best Management Practices (BMPs) for sustainable weed control and herbicide resistance management in Greek rice systems.

| Rice Growth Stage | Preventive BMPs (BMP 1–5) | Tactical BMPs (BMP 6–8) | Long-Term BMPs (BMP 9–11) | Key Notes / Targets |
|---|---|---|---|---|
| Pre-Planting / Field Prep | Start clean (stale seedbed, preplant flooding) Ensure weed-free seed (certified seed) | — | — | Reduce initial weed pressure; prevent introduction of resistant seeds. Target *Echinochloa* and weedy rice first flushes. |
| Sowing / Early Emergence (0–3 leaf) | — | Apply PRE herbicides with multiple MOAs (BMP 6) Follow label rates and proper calibration (BMP 7) | — | Ensure early weed control; avoid under-dosing and late applications that select for resistance. |
| Seedling / Vegetative Growth (3–5 leaf) | — | Sequential POST herbicides (multiple MOAs) Apply at optimal growth stage | — | Maintain selective pressure, prevent escapes; monitor weed size and coverage. |
| Canopy Development / Mid-Season | — | Competitive cultivars, narrow row spacing, water management (BMP 8) | Weed seed movement mitigation, residue management begins (BMP 9) | Reduce late-season weed growth and suppress seed set. |
| Heading / Pre-Harvest | — | — | Harvest-time management (BMP 10: header adjustments, chaff collection) | Prevent seed return to soil seedbank; reduce spread of resistant biotypes. |
| Post-Harvest / Field Rest | — | — | Post-harvest flooding, winter cover crops, field border management (BMP 11) | Destroy remaining seeds, reduce reinfestation; coordinate with neighboring farms. |

(BMP 10), and management of field borders to prevent reinfestation (BMP 11), are crucial to suppress late-season weed growth, reduce seedbank replenishment, and limit the spread of resistant populations.

This framework emphasizes the importance of timing, integration, and farmer coordination for sustainable weed control and resistance management in Mediterranean rice systems. Without systematic monitoring, diversification of herbicide modes of action, and implementation of integrated weed management, resistance levels are expected to continue expanding, posing a substantial threat to the economic viability and sustainability of Greece's rice sector. Strategic interventions combining field, landscape, and policy-level actions are essential to maintain long-term productivity and prevent further escalation of herbicide-resistant weed populations.

## 7. Conclusion

Greek rice systems represent one of the most thoroughly documented herbicide resistance hotspots in Europe. Five major

weed species-*Echinochloa phyllopogon, E. oryzoides, E. crus-galli, E. colona, Cyperus difformis*, and weedy rice-exhibit confirmed resistance, including multiple and cross- resistance mechanisms, with *E. phyllopogon* being particularly problematic. ALS resistance is now widespread, frequently accompanied by failures in ACCase and auxinic herbicides. Glyphosate resistance is emerging in adjacent canal ecosystems, highlighting the importance of water-dispersed pathways. Resistance traits show minimal fitness costs, ensuring persistence across seasons and complicating control. Future of integrated weed management (IWM) in Greek rice systems will increasingly depend on the coordinated implementation of preventive, tactical, and long-term BMPs throughout the rice growth cycle.

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ΑΡΘΡΟ ΑΝΑΣΚΟΠΗΣΗΣ

# Ζιζάνια ανθεκτικά στα ζιζανιοκτόνα στα Ελληνικά συστήματα καλλιέργειας ρυζιού: Ανασκόπηση της υφιστάμενης κατάστασης, των αιτιών και των επιπτώσεων στην Ολοκληρωμένη Διαχείριση

Α. Μοτσενίγου, Δ. Βλότσος, Ν. Βιδάλη, Α. Πετράκη και Δ. Χάχαλης

**Περίληψη** Η ανθεκτικότητα στα ζιζανιοκτόνα αποτελεί σημαντικό περιοριστικό παράγοντα για τη βιώσιμη παραγωγή ρυζιού (*Oryza sativa* L.) στην Ελλάδα, όπου τα εντατικά συστήματα υδροσποράς βασίζονται κυρίως στη χημική καταπολέμηση ζιζανίων. Η ανασκόπηση συνθέτει τις υπάρχουσες γνώσεις για τα συστήματα παραγωγής ρυζιού, τα πρότυπα χρήσης ζιζανιοκτόνων και την εξέλιξη ανθεκτικών ζιζανίων στα ελληνικά αγροοικοσυστήματα. Τα κυρίαρχα ανθεκτικά είδη περιλαμβάνουν τα *Echinochloa* spp., *Cyperus difformis*, Weedy rice (red rice) και *Leptochloa fusca* subsp. *fascicularis*. Η ανθεκτικότητα έχει τεκμηριωθεί σε πολλαπλούς τρόπους δράσης, συμπεριλαμβανομένων αναστολέων ALS, ACCase, συνθετικών αυξινών και γλυφοσάτης, με αυξανόμενη εμφάνιση βιοτύπων με πολλαπλή ανθεκτικότητα. Η εξέλιξή της οφείλεται στη μακροχρόνια χρήση ζιζανιοκτόνων, στη μονοκαλλιέργεια και στη διασπορά μέσω δικτύων άρδευσης. Αξιολογούνται οι μηχανισμοί ανθεκτικότητας ανά είδος και προτείνονται στρατηγικές ολοκληρωμένης διαχείρισης ζιζανίων βασισμένες σε βέλτιστες πρακτικές διαχείρισης. Η εργασία αποτελεί αναφορά για ερευνητές, συμβούλους και υπεύθυνους πολιτικής και είναι εναρμονισμένη με τις αρχές Ολοκληρωμένης Φυτοπροστασίας της Ε.Ε.

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