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Epidemiological role of *Orientus ishidae* in Apple Proliferation and implications for sustainable orchard management

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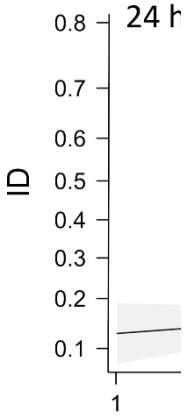

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LIST OF SYMBOLS AND ABBREVIATIONS

AAP	Acquisition Access Period
AP	Apple Proliferation
BS	Baseline scenario
CI	Confidence interval
CP	Cull price (non-marketable fruits)
EPG	Electrical penetration graph
FD	Flavescence dorée
IAP	Inoculation access period
IPM	Integrated pest management
LP	Latency period
MLH	Mosaic leafhopper (common name of *Orientus ishidae*)
MS1	Management scenario 1 (removal without replacing)
MS2	Management scenario 2 (removal followed by replacing)
NCI	Net change in income
PB	Partial budgeting
PBA	Partial budget analysis
PD	Production of affected trees (before uprooting)
PH	Production of healthy trees
PR	Production of replanted trees
Rc	Replacing cost
RNQP	Regulated non-quarantine pest
TP	Tree density (trees/ha)
UNS	Unmanaged scenario (no management)

YH	Yield of healthy trees
YST	Yellow sticky trap(s)
ΔCt	Delta Ct (comparative threshold cycle)

ABSTRACT

Phytoplasmas are obligate, phloem-limited plant pathogenic bacteria responsible for numerous economically damaging diseases worldwide. Among phytoplasma-associated diseases, Apple Proliferation (AP), caused by '*Candidatus* Phytoplasma mali' (16SrX-A), represents a major threat to apple production in Europe, particularly in Trentino (Northern Italy), where it induces typical symptoms, such as witches' broom formation, enlarged stipules and the production of small fruits, leading to severe losses in both yield and income.

Since 2019, a renewed AP outbreak has been recorded in Trentino, despite the fact that monitored populations of the confirmed psyllid vectors *Cacopsylla picta* and *C. melanoneura* remained low and unevenly distributed across the region, population levels that, based on historical epidemiological data, would not be expected to sustain the observed disease incidence. This discrepancy raised the hypothesis that one or more additional vector species might be contributing to AP spread. Concurrently, unusually high populations of the invasive mosaic leafhopper *Orientus ishidae* were detected in apple orchards throughout the region. This East Asian species, now widespread in Europe, is a polyphagous phloem-feeder and a confirmed vector of the 16SrV-C and D phytoplasmas, causal agents of Flavescence dorée (FD) disease. While the molecular detection of '*Ca.* P. mali' in field-collected specimens, coupled with the concurrent increase in both AP incidence and *O. ishidae* population levels, strengthened the hypothesis of its involvement in AP epidemiology, no experimental evidence on its actual transmission competence existed, leaving its AP epidemiological role entirely unresolved.

Beyond the transmission question, several fundamental aspects of the biology and landscape ecology of *O. ishidae* in northern Italian agroecosystems remained unaddressed: its diffusion and

population dynamics in apple orchards, its ability to complete development on apple, and whether orchard populations could function as epidemiological bridges toward adjacent vineyards. Notably, the suitability of cultivated grapevine as a developmental host had never been experimentally assessed, a gap that left unexplained the consistently low abundance of *O. ishidae* in vineyards despite its confirmed FD vector status, and that carries direct implications for FD risk assessment in mixed apple–vineyard landscapes.

Monitoring conducted across northern Italy (2022–2023) revealed that *O. ishidae* is widespread and abundant in apple orchards, particularly under organic management, reaching mean weekly captures of up to 8.4 individuals per trap. Field-collected individuals were screened by PCR, with 16–18% testing positive for '*Ca.* P. mali', confirming natural acquisition. Laboratory and semi-field trials demonstrated that apple is a suitable reproductive host: nymphs completed development within 25–30 days and adults survived 23–28 days, while feeding activity induced characteristic yellowing symptoms affecting up to 23% of leaves.

Transmission experiments (2022–2024) were conducted using different phytoplasma strains (AT1, AT2), acquisition access periods, and inoculum sources. Although 4–22% of insects tested positive after exposure, qPCR quantification revealed extremely low phytoplasma titers, and no transmission to healthy test plants occurred. Infection dynamics experiments showed a progressive decline in phytoplasma detection following acquisition, with positivity decreasing from 85% immediately after a 5-day acquisition period to 0% after 15 latent days, indicating transient passive acquisition rather than propagative infection. These results demonstrate that *O. ishidae* is not a competent AP vector and functions as a dead-end host for '*Ca.* P. mali'.

Given the spatial interspersion of apple orchards and vineyards in Trentino, additional experiments evaluated the species' potential role in FD epidemiology. Survival assays testing nymphs development

and adult survival on different host plant revealed that cultivated grapevine is an unsuitable host: no nymphs completed development, and adult median survival ranged from 1–3 days across cultivars, compared to 23–28 days on apple. Field transect studies at apple–vineyard interfaces showed that adult abundance declined by an order of magnitude beyond orchard boundaries, indicating limited spillover. Collectively, these findings identify apple orchards as demographic sources while demonstrating that host plant constraints restrict establishment in vineyards, thereby limiting the epidemiological relevance of *O. ishidae* in mixed agricultural landscapes.

Since *O. ishidae* was excluded as an AP vector, attention was redirected to quantifying the economic implications of the epidemic and evaluating management strategies for confirmed epidemiological drivers. To quantify the practical implications of these findings, a 20-year partial budget analysis was conducted to estimate the economic impact of AP and evaluate alternative management strategies. Results demonstrated that the unmanaged scenario caused the greatest economic damage, with gross return reductions approaching 30%. Removal of symptomatic trees followed by systematic replacing proved most effective despite higher short-term costs, reducing net income losses to -2.1% (IPM) and -4.0% (organic) compared to -2.9% and -6.0% for removal without replacing. Sensitivity analyses revealed that profitability was more responsive to epidemiological than economic parameters, emphasizing that improved disease monitoring and modeling would enhance decision-making more effectively than refined cost estimates.

In conclusion, this work demonstrates that *O. ishidae*, despite its abundance in apple orchards and ability to acquire *'Ca.* P. mali', does not function as an AP vector and plays no significant role in AP epidemiology in Trentino. Therefore, further research should focus on its FD transmission risk, while AP management remains centered on vector control, tree removal, and the long-term research objective of developing resistant rootstocks.

CHAPTER 1. INTRODUCTION

1.1 What is a phytoplasma?

Phytoplasmas are obligate parasitic bacteria of the class Mollicutes (phylum Tenericutes) characterized by the absence of a rigid cell wall (Bertaccini et al., 2022; IRPCM, 2004). Delimited only by a trilaminar membrane, these single, sub-microscopic cells (typically ranging from 200 to 800 nm in diameter) exhibit marked pleomorphism, a trait that enables them to adopt highly variable shapes (Doi et al., 1967; Wang et al., 2024)

These highly specialized bacteria are characterized by reduced genome sizes and by an obligate parasite life cycle, dependent on two biological hosts: the phloem sieve elements of vascular plants and the tissues of their phloem-feeding insect vectors (Hogenhout et al., 2008; Trivellone and Dietrich, 2021). Following their discovery in the 1960s, these pathogens were initially referred to as mycoplasma-like organisms (MLOs), due to their morphological resemblance to animal and human mycoplasmas (Doi et al., 1967). Subsequent molecular investigations revealed their distinct evolutionary lineage, leading to their current recognition as phytopathogenic Mollicutes (Davis and Lee, 1993; Lee et al., 2000).

Phytoplasma infections cause an extensive range of plant diseases, with more than 1,000 documented disorders in diverse plant families, resulting in major economic losses to global agriculture (Bertaccini, 2022).

1.2 Taxonomy and classification

Phytoplasmas represent a monophyletic group within the Mollicutes class and belong to the Acholeplasmataceae family (Hogenhout et al., 2008). Due to their unculturable nature under standard

laboratory conditions, the provisional taxon *'Candidatus* Phytoplasma*'* was formally proposed in 2004 by the IRPCM Working Team (IRPCM, 2004). Taxonomic classification relied primarily on the 16S rRNA gene, allowing the definition of 16Sr groups and subgroups through restriction fragment length polymorphism (RFLP) analyses (Davis and Lee, 1993; Wei et al., 2007). However, the limited resolution of this single locus soon became apparent, prompting the development of more robust multilocus sequence typing (MLST) and multilocus sequence analysis (MLSA) systems (Duduk and Bertaccini, 2011; Wang et al., 2024). Multilocus approaches employ a combination of ribosomal and non-ribosomal genes, such as secY, tuf, and groEL*,* as well as membrane protein genes (imp, vmpA) that are subject to strong selective pressures and therefore informative for population-level differentiation (Alessio et al., 2025; Kakizawa et al., 2006; Wang et al., 2024; Wei et al., 2021) In response to this growing genomic complexity, updated guidelines for phytoplasma taxonomy and nomenclature were published in 2022 (Bertaccini et al., 2022; Wei and Zhao, 2022). This progressive refinement of phytoplasma taxonomy, from morphology-based designations to multilocus and genome-based frameworks, reflects a broader paradigm shift in prokaryotic systematics and currently, the classification includes 48 recognized *'Candidatus* Phytoplasma*'* species and nearly 300 ribosomal subgroups (Bertaccini et al., 2022; Wei et al., 2024; Wei and Zhao, 2022).

1.3 Symptoms induced by phytoplasma infection

Phytoplasma infections cause a wide and complex range of physiological and developmental abnormalities in host plants, as recently reviewed by Bertaccini (2022). The most characteristic manifestations are developmental disorders that profoundly alter plant morphology. These symptoms include witches' broom (abnormal shoot proliferation), phyllody (transformation of floral organs into leaf-like structures), and virescence (greening of normally pigmented floral tissues) (Bertaccini, 2022). Other frequent symptoms, although not specific to phytoplasma diseases, include stunting or

dwarfism, general chlorosis, and phloem necrosis, often culminating in progressive plant decline or an overall negative impact on production (Bertaccini, 2022). Despite their highly reduced genomes, these bacteria exert a remarkable influence on plant growth, morphology, and metabolism, primarily through the secretion of specialized effector proteins and disruption of nutrient and hormonal homeostasis (Wang et al., 2024). At the molecular level, phytoplasma pathogenicity largely depends on secreted effector proteins, which can be broadly classified into two major functional categories (Wang et al., 2024).

The first category includes effectors that manipulate plant growth, development, and defense by altering hormonal signaling and transcriptional regulation (Janik et al., 2017; Mittelberger et al., 2024; Wang et al., 2024). The second category comprises effectors that modulate plant–insect interactions, enhancing vector attraction, feeding, and fecundity (MacLean et al., 2014; Sugio et al., 2011).

In addition to these secreted effectors, membrane-associated adhesion proteins such as the immunodominant membrane protein (Imp) and antigenic membrane protein (Amp) mediate attachment to plant actin filaments and to insect midgut components, determining vector specificity and transmission capability (Kakizawa et al., 2006; Trivellone et al., 2019).

1.4 Phytoplasmas transmission: a hemipteran affair

Phytoplasmas spread through a combination of biotic and abiotic pathways. While vegetative propagation, grafting, and seed transmission contribute to local persistence and long-range spread (Caglayan et al., 2019), the primary mechanism of natural diffusion remains transmission by sap-sucking insects. All confirmed phytoplasma vectors belong exclusively to the order Hemiptera (Trivellone and Dietrich, 2021; Weintraub and Beanland, 2006). However, the detection of phytoplasmas in phloem-feeding insects does not guarantee transmission capability and only a limited subset of these species are competent vectors able to spread the pathogen between plants, while many

others act as dead-end hosts, in which the pathogen can persist temporarily without being transmitted further (Wei et al., 2021). To date, approximately 200 hemipteran species, distributed across 13 families of Auchenorrhyncha, Fulgoromorpha, Cicadomorpha, and Psylloidea, have been associated with phytoplasmas, but fewer than half are confirmed as competent transmitters (Trivellone and Dietrich, 2021). Most vectors belong to the Cicadellidae, while relatively fewer are found among Fulgoroidea, and only three psyllid genera (*Cacopsylla*, *Bactericera and Diaphorina*) are known to include proven phytoplasma vectors (Weintraub et al., 2019). Known phytoplasma vectors are not evenly distributed across insect lineages: they are clustered in a few subfamilies and genera, suggesting that vector competence is evolutionarily rare but has emerged independently multiple times (Dietrich, 2013; Trivellone and Dietrich, 2021; Wei et al., 2021). The fact that several genera include multiple vector species further supports the idea that vector competence can be an evolutionarily conserved trait, maintained through speciation events (Dietrich, 2013; Trivellone and Dietrich, 2021; Wei et al., 2021). Although vectors remain unidentified for many phytoplasma strains, well-studied examples reveal strong specificity between pathogen lineages and insect groups and only a few groups, such as 16SrI and 16SrXII, display broader vector ranges, with up to 70 competent species across several families (Trivellone and Dietrich, 2021). This uneven distribution reflects the complex biological interaction between phytoplasmas and their insect hosts. Successful transmission requires the pathogen to overcome multiple anatomical and molecular barriers within the vector, while failure at any of these steps results in transient acquisition without transmission, distinguishing competent vectors from incidental carriers.

1.5 Infection cycle in insect vectors and phytoplasma-vector interactions

Phytoplasmas are transmitted in a persistent–propagative manner, remaining within the insect throughout its lifetime and replicating in various tissues (Weintraub and Beanland, 2006). Successful infection of a new plant occurs only if the three key phases of the transmission cycle are completed.

The first phase, the Acquisition Access Period (AAP), defines the duration of feeding required for the insect to ingest a sufficient phytoplasma titer from the phloem of an infected plant (**Figure 1**). The AAP can range from a few minutes to several hours, with longer periods generally increasing acquisition efficiency, and is significantly influenced by phytoplasma concentration within the host plant (Bosco and Tedeschi, 2013). Critically, the initial colonization of the vector midgut during the AAP is not a passive process but depends on specific molecular recognition between phytoplasma surface-exposed proteins and insect cell components. The immunodominant membrane protein (Imp), immunodominant membrane protein A (IdpA), and antigenic membrane protein (Amp) interact with insect cytoskeletal proteins such as actin and myosin, facilitating adhesion to and translocation across midgut epithelial cells (Kakizawa et al., 2006). This interaction underlies vector specificity at the midgut level: only insect species presenting compatible receptor configurations allow productive colonization, whereas incompatible interactions prevent crossing of the midgut barrier and preclude systemic infection (Hogenhout et al., 2008; Trivellone et al., 2019).

The second phase, the Latency Period (LP), spans the interval between initial acquisition and the insect's ability to transmit the pathogen. During this phase, phytoplasmas that have successfully crossed the midgut epithelium, potentially within vesicles or via transcellular routes, enter the hemocoel and circulate in the hemolymph (Bosco and Tedeschi, 2013; Pagliari et al., 2019). They subsequently replicate in various internal tissues, including the Malpighian tubules, fat bodies, brain, and reproductive organs, before ultimately colonizing the salivary glands (Koinuma et al., 2020)

(**Figure 1**). The duration of the LP is highly variable, ranging from a few days to over 80 days, and is significantly affected by factors such as phytoplasma species/strain, vector species, and environmental conditions like temperature (Bosco and Tedeschi, 2013; Pagliari et al., 2019). This systemic dissemination is not passive: phytoplasmas must actively evade the vector's innate immune defenses, and infection has been shown to modulate the expression of immune-related genes in competent vectors, suggesting active suppression or subversion of immune surveillance as a prerequisite for successful colonization (Galetto et al., 2018). The colonization of the salivary glands represents a second molecular bottleneck, dependent on specific protein–receptor interactions at the salivary gland barrier analogous to those operating at the midgut (Rashidi et al., 2015). The duration of the LP is highly variable, ranging from a few days to over 80 days, and is affected by phytoplasma species and strain, vector species, and environmental conditions such as temperature (Bosco and Tedeschi, 2013; Pagliari et al., 2019).

The third phase, the Inoculation Access Period (IAP), is the feeding duration during which the infective insect inoculates a healthy plant (**Figure 1**). This occurs when phytoplasmas, having reached sufficient concentration in the salivary glands, are ejected within the insect's watery saliva into phloem sieve elements (Koinuma et al., 2020). The IAP can last only a few hours, though longer periods generally increase transmission efficiency (Bosco and Tedeschi, 2013).

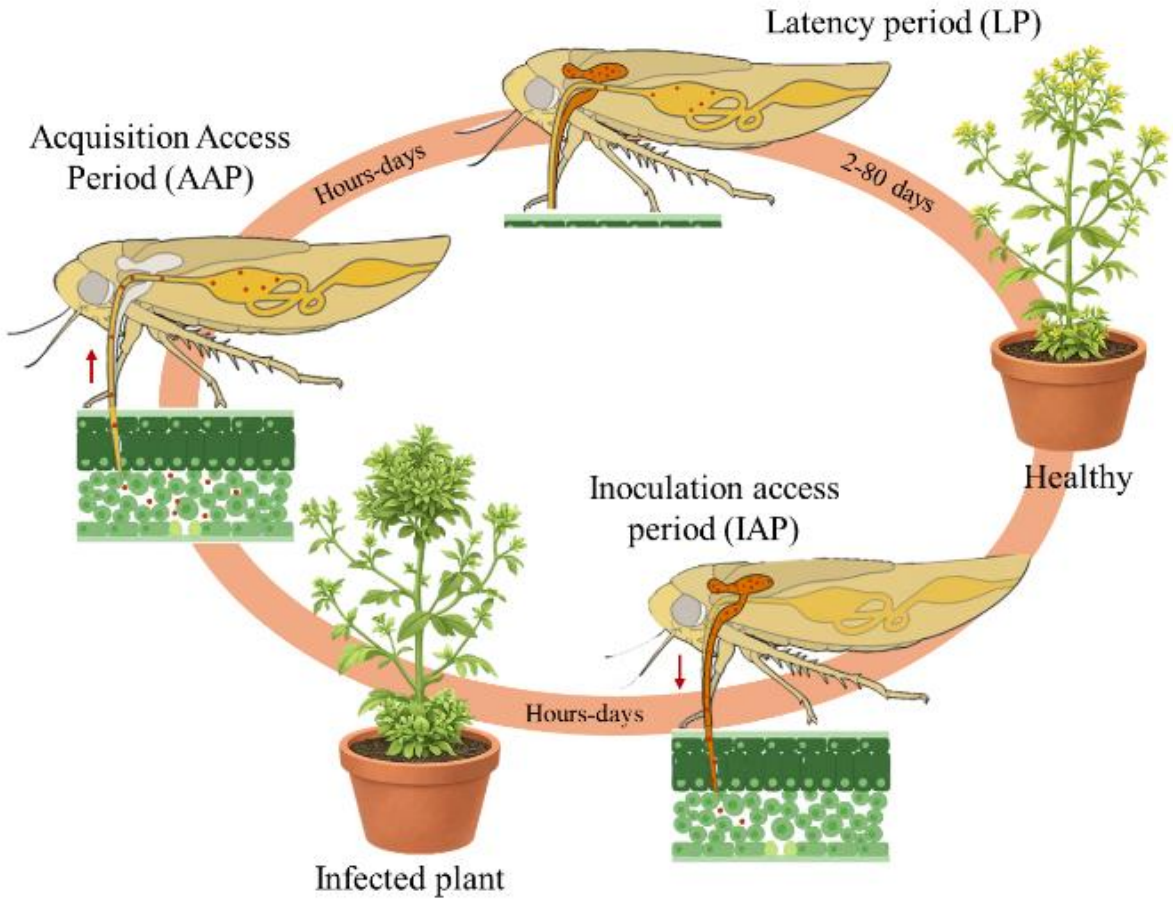

Figure 1 Schematic representation of the phytoplasma transmission cycle involving a leafhopper vector and a host plant. During the acquisition access period (AAP), the insect acquires phytoplasma cells while feeding on an infected plant. Phytoplasmas subsequently cross the midgut epithelium, circulate in the hemolymph, and colonize the salivary glands during the latent period (LP), which may last from several days to weeks. Following the LP, the insect becomes infective and transmits phytoplasmas to a healthy plant during the inoculation access period (IAP). Illustration adapted from Canola Council of Canada (https://www.canolacouncil.org/canola-encyclopedia/insects/aster-leafhopper/)

The sequential nature of these molecular and anatomical barriers means that vector competence is not a binary trait but the cumulative outcome of multiple compatibility checkpoints between pathogen and host. When one or more of these checkpoints fails the insect acts as a dead-end host: phytoplasma DNA may be detected by molecular assays shortly after acquisition feeding, reflecting transient presence in gut tissues, but the pathogen fails to establish propagative infection and is progressively cleared. This pattern, characterized by initial detection followed by rapid decline in prevalence and titer over time, is mechanistically distinct from the stable or increasing titers observed in competent

vectors and indicates passive ingestion rather than active colonization (Weintraub and Beanland, 2006; Wei et al., 2021). The distinction between transient passive acquisition and true propagative infection is therefore critical for interpreting molecular screening data: detection of phytoplasma DNA in a field-collected insect is insufficient to establish vector status, and must be complemented by experimental transmission trials and quantitative titer dynamics (Bosco and Tedeschi, 2013).

Beyond anatomical colonization, persistent–propagative infection involves physiological and molecular interactions between phytoplasma and vector. This intimate association is accompanied by extensive reprogramming of insect gene expression, including immune pathways, and can result in either beneficial or detrimental effects on vector fitness. For example, *Macrosteles quadrilineatus* (Forbes) infected with aster yellows phytoplasma (*'Candidatus* Phytoplasma asteris') lives longer than uninfected conspecifics, and females infected with the 'Bolt' strain show higher fecundity (Beanland et al., 2000). By contrast, 'flavescence dorée' phytoplasma (FDp, 16SrV-C, D) significantly reduces the fitness of its vectors, decreasing adult lifespan, fecundity and egg hatching rate in both the natural vector *Scaphoideus titanus* (Ball) and the experimental vector *Euscelidius variegatus* (Kirschbaum) (Bosco and D'Amelio, 2009; Bressan et al., 2005). This direct manipulation of vector biology is often complemented by indirect, plant-mediated effects, where phytoplasmas manipulate plant physiology to indirectly benefit vector populations (Gross et al., 2022; Wang et al., 2024). Effector proteins such as SAP11 and SAP54 reshape plant morphology and volatile emission profiles, increasing attractiveness to insect vectors and improving feeding and oviposition behavior (MacLean et al., 2014; Sugio et al., 2011).

In addition to horizontal transmission via plant feeding, several phytoplasmas achieve vertical transmission via female vector's ovaries, a process known as transovarial transmission (TOT). This mechanism allows the bacteria to persist across insect generations, establishing the vector as a long-

term reservoir (Tedeschi and Bertaccini, 2019) and it has been demonstrated in several species, including *Matsumuratettix hiroglyphicus* (Matsumura) (Hanboonsong et al., 2002), *Cacopsylla pruni* (Scopoli) (Tedeschi et al., 2006) and *Cacopsylla picta* (Foerster) (Mittelberger et al., 2017a). Although relatively rare, this form of transmission appears to occur primarily in long-established vector–phytoplasma associations (e.g., 16SrI, 16SrX, and 16SrXI groups) and represents a major evolutionary step toward symbiotic persistence within insect lineages.

1.6 Apple proliferation phytoplasma

Apple Proliferation (AP) is one of the most damaging plant diseases of cultivated apple (*Malus domestica* Borkh.) in Europe and is associated with *'Candidatus* Phytoplasma mali*'* (Seemüller and Schneider, 2004). The disease causes substantial yield and quality losses in affected orchards and during severe epidemic years such as 2003–2004, economic losses in Italy alone were estimated at approximately €100 million (Strauss, 2009). Owing to its impact on fruit production and trade, '*Ca.* P. mali' is listed as a regulated non-quarantine pest (RNQP) in the European Union under Regulation EU 2016/2031 (EPPO, 2018).

'*Candidatus* Phytoplamsa mali' is assigned to ribosomal group 16SrX (apple proliferation group), specifically to subgroup 16SrX-A, and is closely related to *'Candidatus* Phytoplasma pyri' (16SrX-C) and *'Candidatus* Phytoplasma prunorum' (16SrX-F), the causal agents of pear decline and European stone fruit yellows, respectively (Lee et al., 2000; Seemüller and Schneider, 2004).

The genome of '*Ca.* P. mali' is highly reduced and consists of a single linear chromosome of about 750 kb that differs from the other phytoplasmas for the low GC-content (Kube et al., 2008). Early molecular methods for classifying AP phytoplasmas relied on sequencing a non-ribosomal DNA fragment containing three putative ORFs. This approach enabled researchers to define the initial

genetic structure, identifying at least three distinct subtypes designated AT-1, AT-2, and AP-15 (Jarausch et al., 2000). Later, a more detailed view of genetic variation within '*Ca.* P. mali' was provided through the analysis of genes coding for ribosomal proteins L22 and S3, which suggested greater heterogeneity and resulted in the establishment of four rpX subtypes (rpX-A, rpX-B, rpX-C, and rpX-D) (Martini et al., 2008). Subsequent integrated studies, combining both ribosomal and non-ribosomal markers, further refined this classification. For example, work conducted on '*Ca*. P. mali' populations from northwestern Italy corroborated the established AT-1, AT-2, and AP-15 subtypes while simultaneously isolating two new genetic lineages, AT-1a and AT-1b, within the AT-1 subtype (Casati et al., 2010). Together, these molecular tools provide the basis for tracing introduction pathways, understanding regional epidemiology and relating specific strain groups to particular vector species or ecological contexts.

1.6.1 Symptomatology

Infection by AP triggers a broad and highly variable range of symptoms across *Malus* species, with expression often modulated by cultivar and environmental factors (Schmid, 1989, 1975). The disease is primarily recognized through two highly characteristic, diagnostically relevant symptoms: the formation of witches' brooms (abnormal, clustered growth of shoots) and the development of enlarged, dentate stipules (Bisognin et al., 2007; Jarausch et al., 2007a) (**Figure 2**). Importantly, the most economically relevant consequence of infection is not plant mortality, which is rare and mostly observed under experimental conditions (Kartte and Seemüller, 1991), but rather the production of small, poorly colored, and low-flavor fruits, often bearing elongated peduncles (**Figure 2**). These changes reduce marketability and can lead to substantial financial losses (Herzog et al., 2012; Strauss, 2009).

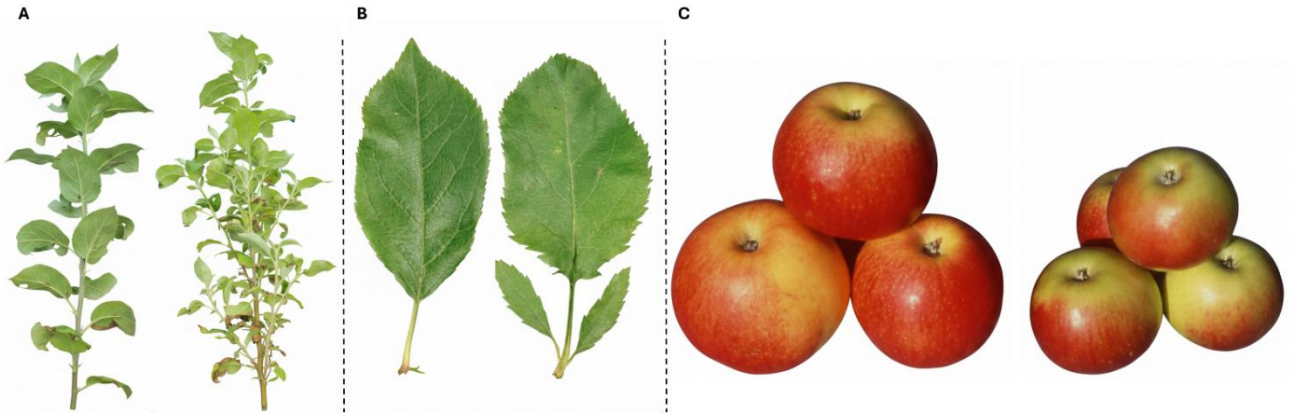

Figure 2 Asymptomatic organs (left) compared with AP-symptomatic organs (right): (A) healthy and infected branches showing witches' broom; (B) leaves showing enlarged stipules compared with a healthy leaf; (C) normal-sized fruit compared with small, poorly colored fruits. Photos retrieved from the EPPO Global Database.

Quantitative studies have reported marked reductions in fruit size (–27% to –49%) (Lemoine, 1990; Schmid, 1989), weight (-5% to -79%) (Kaminska and Zawadzka, 1970a; Zawadzka, 1976) and discoloration (Herzog et al., 2012). Consequently, the proportion of apples falling below the commercial diameter threshold (6 cm) has been reported to range from 16% to 80% (Herzog et al., 2012; Lemoine, 1990) and yield reduction (measured as kg/tree) can vary widely (0% to 91%) (Kaminska and Zawadzka, 1970a; Lemoine, 1990; Schmid, 1989; Trifonov, 1965; Zawadzka, 1976).

Beyond these primary effects, AP may also induce secondary, less specific symptoms such as early autumn leaf reddening, early bud break and stunted branch growth. When expressed concurrently, these symptoms can reliably support an AP diagnosis (Jarausch et al., 2024). While traditionally considered a secondary symptom, premature leaf reddening has been recently reappraised as a robust indicator of AP infection, particularly in field surveys conducted in Germany (Jarausch et al., 2024). Extensive monitoring has demonstrated the high reliability of this visual manifestation, where specific AP symptoms (e.g., witches' brooms) show a 100% correlation with the late-season leaf reddening (Jarausch et al., 2024). Molecular analyses further underscore its diagnostic value: trees exhibiting reddening as the sole symptom tested positive for '*Ca.* P. mali' by PCR in 71% to 97% of cases (Jarausch et al., 2024). This symptom is triggered by specific climatic conditions, notably cold

night temperatures (around 5°C) followed by warm daytime temperatures (around 20°C) in September (Jarausch et al., 2024).

Finally, AP presents the distinctive phenomenon of recovery, characterized by the temporary or permanent cessation of above-ground symptoms (Carraro et al., 2004). However, recovery is not curative, as phytoplasmas may persist in the root system, maintaining the tree as a reservoir and enabling spontaneous recolonization of the canopy with possible symptom recurrence (Osler et al., 2000). Complete eradication of the pathogen from infected trees has yet to be achieved.

1.6.2 Pathogenicity

The molecular mechanisms underlying symptom development are not fully elucidated, and current research mainly focuses on two aspects: disruption of host primary metabolism - chloroplast function, and manipulation of plant hormonal regulation through effector proteins. AP infection significantly impairs carbohydrate translocation to sink tissues, leading to growth inhibition (stunting) and accumulation of sugars in source tissues, which has been associated with leaf chlorosis (Lepka et al., 1999). This is accompanied by altered expression of genes involved in the Calvin cycle and photosynthesis (Aldaghi et al., 2012; Siewert et al., 2014) and by changes in sugar profiles, with reduced glucose, fructose and sorbitol and increased sucrose and starch, which may act as signaling molecules (Giorno et al., 2013). AP also accelerates chlorophyll degradation via the same pathway used in seasonal senescence, resulting in reduced chlorophyll content and modified catabolite production (Mittelberger et al., 2017b).

More recently, heterologous expression of the AP effector $SAP11_{CaPm}$ in *Nicotiana occidentalis* revealed broad reprogramming of host gene expression, with downregulation of defense, phytohormone, chloroplast related genes and upregulation of oxidative phosphorylation and ATP biosynthesis, suggesting that AP effectors simultaneously suppress photosynthesis and defense while

boosting host energy metabolism (Mittelberger et al., 2024). In addition, AP strongly interferes with plant hormonal balance, and this is thought to underlie many of the characteristic symptoms (Siewert et al., 2014; Zimmermann et al., 2015). A central role is played by the SAP11-like effector encoded by ATP_00189 (called $SAP11_{CaPm}$), which binds key regulators of growth, defense and hormone signaling (Janik et al., 2017; Sugio et al., 2011). Recent work has shown that it also interacts with MdTCP16, a CYC/TB1-type TCP orthologous to BRANCHED1, whose expression increases from spring to autumn and correlates positively with both $SAP11_{CaPm}$ expression and '*Ca*. P. mali' titer in leaves, linking effector activity to witches' broom formation and early bud break (Mittelberger et al., 2022). In parallel, AP modulates plant defense response, reducing the accumulation of reactive oxygen species such as H_2O_2 (Aldaghi et al., 2012), whereas symptomless-recovered trees show increased H_2O_2 levels, suggesting that enhanced ROS production can counteract symptom development (Musetti et al., 2004).

1.6.3 Host range and within-tree colonization dynamics

The primary host of '*Ca.* P. mali' is cultivated apple (*Malus domestica* Borkh) and other *Malus* species, in which AP symptomatology was originally described (Amici et al., 1972; Kartte and Seemüller, 1991; Rui, 1950). Nevertheless, '*Ca.* P. mali' has also been detected in a number of other woody plants such as *Pyrus* spp.*, Prunus* spp.*, Corylus avellana* L., *Crataegus monogyna* Jacq., *Quercus robur* L.*, Quercus rubra* L., *Carpinus betulus* L., *Convolvulus arvensis* L. (Lee, 1995; Marcone et al., 1996; Mehle et al., 2007; Tedeschi and Alma, 2007) and also in herbaceous plants, such as dahlia (*Dahlia cultorum* Thorsrud et Reisaeter) and oriental hybrids of *Lilium* plants (Kamińska and Śliwa, 2008a, 2008b).

Within apple trees, '*Ca.* P. mali' is unevenly distributed and shows pronounced seasonal dynamics. Early work demonstrated that phytoplasma populations in the aerial parts of infected trees

are lowest during late winter and early spring and increase steadily through the growing season, peaking between late summer and early autumn (Seemüller et al., 1984). Subsequent quantitative PCR studies confirmed this pattern in commercial orchards, showing minimal titers in aerial tissues from March to May, followed by a marked increase in leaves and young shoots from June onwards (Baric et al., 2011; Pedrazzoli et al., 2008a). By contrast, roots remain consistently colonized throughout the year and frequently harbour high phytoplasma concentrations even when aerial tissues are below detectable levels (Baric et al., 2011; Seemüller et al., 1984).

This persistent root colonization has important implications for epidemiology and diagnostics. Latently infected trees may appear symptomless for several years yet carry root titers comparable to symptomatic trees, making them an unseen source of inoculum for both insect acquisition and root-to-root transmission (Baric et al., 2011; Ciccotti et al., 2008). It also explains why winter or early spring sampling of aerial tissues can lead to false negatives and underscores the necessity of targeted sampling strategies and molecular testing for reliable certification of propagation material.

1.6.4 Geographic distribution in Europe

Apple Proliferation is now established in virtually all major temperate apple-growing regions of Central and Southern Europe. The disease has been reported from Germany, France, Switzerland, Austria, Belgium, the Netherlands, the Czech Republic, Hungary, Poland, Romania, Serbia, Slovenia, Spain and Italy, among others (EPPO, 2025) (**Figure 3**). Its spatial pattern is characterized by regional hotspots with high incidence and surrounding areas with lower but persistent infection levels, reflecting differences in orchard age, vector populations, landscape structure and management intensity (Somoano et al., 2025).

Germany has historically experienced some of the most severe epidemics. In the southwestern regions, particularly Rhineland-Palatinate and Baden-Württemberg, surveys in the mid-2000s

recorded AP incidences approaching 57% in commercial orchards and over 75% in abandoned orchards, highlighting the role of unmanaged apple stands as long-term inoculum sources (Herzog et al., 2012; Jarausch et al., 2011, 2007a). In Spain, AP was more recently confirmed in Asturias, where epidemiological investigations indicated higher infection levels and stronger disease pressure in older, low-input cider orchards compared with intensively managed systems (Somoano et al., 2025).

Italy has a long history with AP, with the first descriptions of AP dating back to the early 1950s (Amici et al., 1972; Rui, 1950). The disease is now endemic in the main apple-producing regions of northern Italy. In north-western Italy, especially Valle d'Aosta, incidence in older orchards has repeatedly approached 100%, with high infection rates in both trees and local vector populations (Tedeschi et al., 2003, 2002). In Piedmont AP outbreaks were rather sporadic and not severe (Janik et al., 2020). Recurrent epidemic waves have been documented in northeastern Italy, particularly in Trentino and South Tyrol (**Figure 3**), a highly specialized apple-growing area accounting for more than 15% of total European production. Sporadic cases were reported in South Tyrol in the late 1950s, followed by the first major epidemic in the late 1990s (Janik et al., 2020). During this period, areas such as Burggrafenamt/Burgraviato and Vinschgau/Val Venosta were predominantly affected, with infection rates reaching up to 60% in some orchards planted on vigorous rootstocks (Janik et al., 2020). After a period of relative decline, AP surged again around 2004, and by 2006, symptomatic trees were found in approximately 75% monitored orchards (Janik et al., 2020). The epidemic continued to fluctuate, peaking again in 2013, particularly in Vinschgau/Val Venosta and Burggrafenamt/Burgraviato (Janik et al., 2020). Following intensified control strategies, the situation significantly improved, with counts of symptomatic trees dropping below between 2014 and 2018 (Fischnaller et al., 2017).

In Trentino, the first records of AP date back to the early 1950s (Amici et al., 1972). The

disease occurred sporadically until the early 1990s, when a major outbreak caused considerable economic losses, particularly in Val di Non (Vindimian, 2002). A large-scale survey conducted in Val di Non between 1999 and 2000 quantified the disease spread, showing the mean infection rate doubled from 0.8% to 1.7% of controlled trees, with older orchards and those at higher altitudes being more severely affected (Springhetti et al., 2002). Until 2005, the average provincial infection percentage remained between 2.5% and 2.9%, with Val di Non hill-sites reaching an average incidence of up to 5.5% (Vindimian 2002). The introduction of mandatory uprooting of infected trees and a subsidy program for replacing older, heavily infected orchards led to a consistent decline in incidence from 2006 to 2010, reaching the minimum level ever registered of 0.27%. However, incidence increased again in 2012, particularly in Val d'Adige and Valsugana. By 2014, the mean infection rate in these districts had risen to 6%, bringing the overall provincial average back to 2% (Oppedisano et al., 2020a). Although subsequent control strategies reduced AP to nominal levels, a new outbreak has been recorded since 2020, reaching an incidence of 1.7% by 2023 (Bargna et al., 2024).

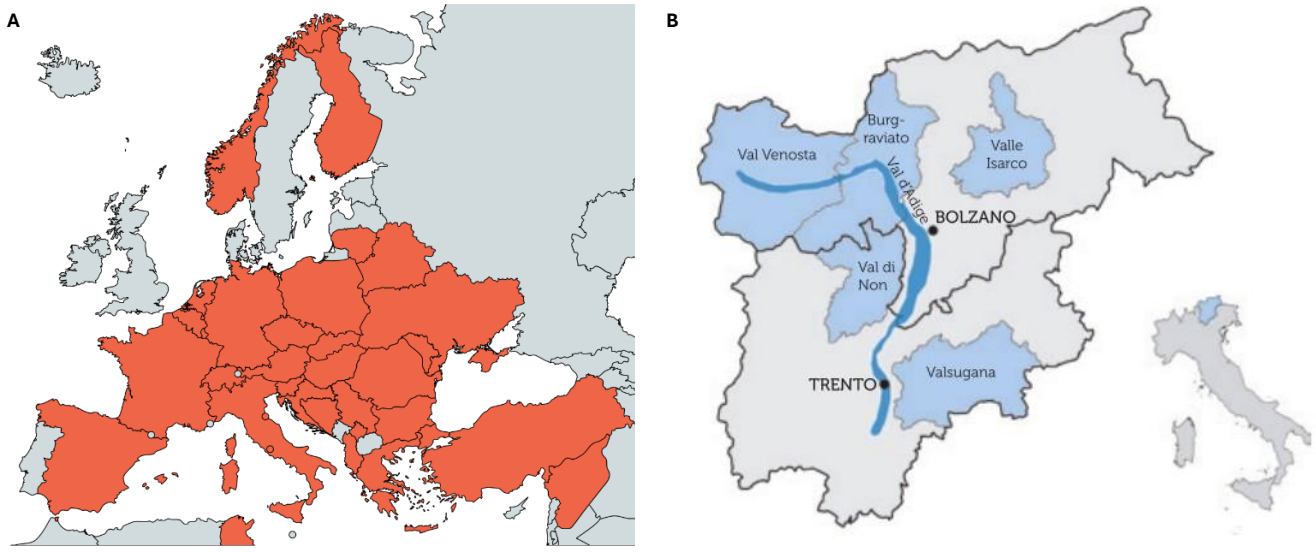

Figure 3: Geographic distribution of apple proliferation phytoplasma in Europe (A) and districts of the Trentino–South Tyrol region in northern Italy (B). Areas shown in blue indicate regions/districts affected by apple proliferation (retrieved from Janik et al. 2020).

1.6.5 Diagnostic methodologies

Graft transmission to woody indicator plants, followed by symptom observation, provided initial evidence of a graft-transmissible pathogen (Amici et al., 1972), while electron microscopy later enabled direct observation of pleomorphic phytoplasma-like bodies within phloem sieve elements of infected apple trees (Amici et al., 1972). Serological approaches based on polyclonal and monoclonal antibodies raised against '*Ca.* P. mali' antigens supported the development of ELISA and immunofluorescence assays (Loi et al., 2002). Conventional and nested PCR assays targeting 16S rRNA and ribosomal protein genes became standard methods for sensitive detection of '*Ca.* P. mali' in plant tissues and insect vectors (Lee, 1995; Smart et al., 1996). Quantitative real-time PCR (qPCR) assays using SYBR Green, TaqMan™, or EvaGreen® chemistries further improved sensitivity and enabled high-throughput screening, as well as absolute or relative quantification of pathogen titers (Baric and Dalla-Via, 2004; Jarausch et al., 2004; Monti et al., 2013). More recently, rapid and field-deployable diagnostics have been developed. Isothermal amplification methods, particularly loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA), allow amplification of phytoplasma DNA at constant temperature without requiring a thermocycler (Dickinson, 2015; Notomi, 2000). RPA protocols designed for '*Ca.* P. mali' can be coupled with lateral-flow strips or portable fluorometers for on-site detection (Valasevich and Schneider, 2017).

Non-destructive spectral methods are emerging as complementary tools for early detection and orchard-scale monitoring. Near-infrared reflectance spectroscopy and hyperspectral imaging, combined with machine-learning classifiers, have been used successfully to discriminate between healthy and AP-infected trees based on subtle changes in leaf biochemical composition and optical properties (Barthel et al., 2023, 2021; Jarausch et al., 2023b; Knauer et al., 2024). Alongside detection, molecular typing of '*Ca.* P. mali' is essential for epidemiological investigations. Classical 16S RFLP

has been complemented or replaced by higher-resolution methods, including T-RFLP, SSCP and multilocus sequence typing (MLST) targeting non-ribosomal markers such as imp, secY, tuf and secA (Baric et al., 2011; Casati et al., 2011; Janik et al., 2015; Jarausch et al., 2000). These approaches enable discrimination among AP subtypes and support reconstruction of epidemic histories and pathways of spread.

1.6.6 Non-vector transmission mechanisms

Although insect vectors are responsible for most natural spread, several non-vector pathways contribute significantly to the maintenance and dissemination of '*Ca.* P. mali'. In the absence of rigorous phytosanitary certification, infected mother plants or nursery stock can introduce the pathogen into previously pathogen-free orchards or regions (Caglayan et al., 2019).

The success of graft transmission is strongly influenced by seasonal dynamics of phytoplasma colonization, being lowest in early spring and increases substantially during summer (Pedrazzoli et al., 2008b). Under experimental conditions, '*Ca.* P. mali' transmission has been demonstrated via dodder (Cuscuta spp.) through the formation of direct vascular connections, although this route is not considered epidemiologically relevant in orchard settings (Janik et al., 2020). Seed transmission has been tested and not observed, indicating that '*Ca.* P. mali' is not vertically transmitted through seeds (Seidl and Komarkova, 1974).

By contrast, natural root anastomoses represent a biologically and epidemiologically important non-vector mechanism. Root grafts between neighboring trees establish phloem continuity, enabling movement of '*Ca.* P. mali' from infected to healthy individuals (Ciccotti et al., 2007). Experimental studies conducted in Trentino have demonstrated transmission rates reaching 16% via root bridges in 'Golden Delicious' seedlings (Ciccotti et al., 2007). Furthermore, field research has documented spread patterns consistent with root-mediated infection, observed both in older orchards characterized

by vigorous rootstock (Baric et al., 2008) and, albeit at a lower incidence, in those utilizing the less vigorous M9 rootstock (Lešnik et al., 2008). Residual infected roots left in the soil after tree removal may remain viable and phytoplasma-positive for several years, representing a prolonged inoculum source for replanted orchards (Baric et al., 2008). Consequently, root removal or careful site preparation is an important component of AP management, particularly in replant scenarios.

1.7 Insect-Vectored transmission

The dominant drivers of AP epidemiology across Europe are phloem-feeding hemipterans that transmit '*Ca*. P. mali', in a persistent propagative manner. While phytoplasmas are ingested during phloem sap feeding, their mere detection in an insect does not prove vector competence. Consequently, only species in which acquisition, multiplication and transmission to healthy plants are experimentally verified can be classified as true vectors. To date, three species are conclusively recognized as vectors of '*Ca*. P. mali': the psyllids *Cacopsylla picta* (syn. *C. costalis*) (Föerster), *Cacopsylla melanoneura* (Föerster), and the leafhopper *Fieberiella floriii* (Stål) (Frisinghelli et al., 2000; Jarausch et al., 2003; Krczal et al., 1989; Tedeschi and Alma, 2006, 2004).

1.7.1 Psyllid vectors

The psyllid species, *C. picta* and *C. melanoneura* occur sympatrically in most European apple-growing regions, although their relative abundance and epidemiological importance vary geographically (Carraro et al., 2008; Conci et al., 1992; Jarausch et al., 2003; Mattedi et al., 2008; Miñarro et al., 2016). Despite sharing a broadly similar life cycle and transmission pathway, the two species differ substantially in their distribution, vector efficiency, host specificity, and epidemiological role, differences that have important implications for understanding and managing AP spread.

Cacopsylla picta is widespread in Europe and Asia Minor and is consider monophagous on *Malus* spp. (Jarausch and Jarausch, 2009). In Italy, it is widespread across the north-eastern regions, while in the north-western areas it has not been recorded (Tedeschi et al., 2002). It is univoltine and overwinters as an adult on conifers, primarily *Picea* and *Pinus* spp. (Mayer et al., 2011). At the end of winter (March/April), overwintered adults (remigrants) return from conifers to apple trees, where they reproduce, oviposit, and can be detected on apple until late May/June, while the new generation (emigrants) leaves the orchards in early to mid-summer (June-August) to migrate back to conifers (Fischnaller et al., 2017; Jarausch et al., 2011; Mattedi et al., 2008). Feeding-trace analyses based on plant DNA metabarcoding further suggest that *C. picta* adults exploit a broad range of non-host plants, including conifers, broadleaf plants (Rosaceae, Betulaceae, Fagaceae, etc..) and weeds (Urticaceae, Poaceae, Apiaceae, etc..), highlighting the potential of wild, non-crop plants to act as natural reservoirs facilitating the spread of AP phytoplasma (Barthel et al., 2020b).

'Ca. P. mali' follows a persistent, propagative transmission pathway in *C. picta* and after colonization of the salivary glands of the new generation on apple (F1) the pathogen is retained through adult overwintering, so that infective remigrants can be detected among individuals returning to apple orchards in spring (Candian et al., 2020; Mattedi et al., 2008; Mayer et al., 2009; Tedeschi et al., 2003). *C. picta* shows consistently high natural infection rates across many European regions: in north-eastern Italy, 9–13% of overwintered and F1 adults were found infected (Carraro et al., 2008), while surveys in Trentino and South Tyrol have reported mean infection rates of 6–21% in remigrants (Baric et al., 2009; Fischnaller et al., 2017; Mattedi et al., 2008). Similar values (≈10–14%) have been reported from Germany, northern Switzerland, eastern France, Finland and in Spain (Jarausch et al., 2011; Lemmetty et al., 2011; Miñarro et al., 2016). Remigrants are already highly infective when migrating from shelter plants to apple, retain their infectivity throughout their residence in orchards,

and both remigrants and emigrants can efficiently transmit the pathogen under experimental conditions (Carraro et al., 2008; Jarausch et al., 2007b). Experimental acquisition trials show that F1 nymphs developing on infected apple trees become almost uniformly infected, whereas adults that emerge on healthy plants and subsequently feed on infected trees exhibit much lower acquisition rates (10%), highlighting the importance of prolonged exposure during immature stages (Oppedisano et al., 2020a). Phytoplasma titers in *C. picta* typically reach 10^6–10^8 genome copies per insect and remain high from the arrival of remigrants until the emergence of the first offspring (Jarausch et al., 2011; Mayer et al., 2009; Pedrazzoli et al., 2007). Additionally, C. *picta* exhibits transovarial transmission, with a fraction of the progeny of infected females inheriting 'Ca. P. mali' and F1 adults reaching phytoplasma titers comparable to those observed in parental individuals (Mittelberger et al., 2017a). The psyllid can transmit all three major *'Ca. P. mali'* subtypes (AT1, AT2 and AP) (Jarausch et al., 2000), although regional differences in subtype prevalence suggest local adaptation of vector–phytoplasma associations, such as the predominance of subtype AT2 in South Tyrol (Baric et al., 2011) and the coexistence of all three subtypes in north-eastern Italy (Martini et al., 2008) and parts of Germany (Jarausch et al., 2024).

Cacopsylla melanoneura has a wider, essentially Palearctic distribution and is oligophagous on Rosaceae, principally *Crataegus*, *Malus* and *Pyrus* spp (Conci et al., 1992; Ossiannilsson, 1992). Its life cycle closely resembles that of *C. picta*: it is also univoltine, with adults overwintering on conifers and remigrating to deciduous hosts in late winter (Mayer et al., 2009; Tedeschi et al., 2002). However, *C. melanoneura* appear earlier on apple (as early as January) and leaves these hosts earlier (April/May) compared to *C. picta* (Candian et al., 2020; Pizzinat et al., 2011; Tedeschi et al., 2002). Field evidence highlights a remarkable migratory range, with individuals travel several kilometers to colonize overwintering hosts (e.g. *Abies alba* and *Larix decidua*) at elevations up to 800-1.650 m a.s.l.

in north-western Italy (Candian et al., 2020; Pizzinat et al., 2011).

A distinctive feature of *C. melanoneura*, absent in *C. picta*, is its use of hawthorn (*Crataegus* spp.) as an alternative host plant. Host-association studies have revealed marked geographic differences in the relative use of apple and hawthorn: in north-western Italy, *C. melanoneura* is abundant in both orchards and on hawthorn, and hawthorn plants can harbour '*Ca.* P. mali', suggesting a potential role as an alternative inoculum source (Tedeschi and Alma, 2007). In Germany, by contrast, *C. melanoneura* is predominantly associated with hawthorn and none of them have been found AP infected in the field (Mayer et al., 2009). Host-switching experiments and microsatellite analyses indicate ecological and genetic differentiation between apple- and hawthorn-associated populations, supporting the existence of host-related lineages within *C. melanoneura* (Malagnini et al., 2013).

In terms of transmission, *C. melanoneura* also follows a persistent, propagative pathway, with the pathogen retained through overwintering and infective remigrants detectable upon spring return to orchards (Candian et al., 2020; Tedeschi and Alma, 2004). However, the vector competence of *C. melanoneura* is far more heterogeneous and context-dependent than that of *C. picta*, shaped by the interplay of geographic origin, host-plant association, population genetic structure, and phytoplasma subtype, making its epidemiological role one of the most complex and debated aspects of AP transmission biology. Natural infection rates in overwintered adults vary dramatically across regions. In Germany, northern Switzerland, and eastern France, rates are typically below 1%, and field-collected individuals rarely exceed phytoplasma titers of 4×10^4 genome copies per insect , well below the levels associated with efficient transmission by *C. picta* (Jarausch et al., 2007b; Mayer et al., 2009). In contrast, surveys in north-western and north-eastern Italy reported mean infection rates of 7% and 3%, respectively, with average titers around 10^6 genome copies per insect (Corretto et al., 2024; Tedeschi et al., 2012), and infection prevalences of 2.8% and 10% have been documented in

Turkey and Spain (Kaya et al., 2016; Miñarro et al., 2016). Transmission experiments mirror this geographic variability: no transmission has been obtained with German populations (Mayer et al., 2009), and rates as low as 0.36% were recorded in Trentino (Mattedi et al., 2008), while experiments in north-western Italy yielded up to 29.4% infected test plants with naturally infected overwintered adults, corresponding to individual transmission probabilities of 1.4–8.4% (Tedeschi and Alma, 2004). More recent work comparing the two psyllids under controlled conditions confirmed this striking contrast: *C. picta* achieved transmission rates of around 10%, whereas *C. melanoneura* reached only about 1.5%, and all life stages of *C. picta* showed higher acquisition and transmission efficiencies than the corresponding stages of *C. melanoneura* (Oppedisano et al., 2020a). Detailed titer quantification across life stages further showed that nymphs and newly emerged adults acquire the phytoplasma at relatively high frequencies, with titers gradually increasing during estivation and overwintering, and overwintered adults reaching the highest loads, supporting the view that remigrants constitute the main epidemiologically relevant stage (Candian et al., 2020).

The pronounced variability in *C. melanoneura* vector competence has been linked to genetic structuring among psyllids populations and to associations with different *'Ca. P. mali'* subtypes (Corretto et al., 2024). One ecological manifestation of this variability is the role of hawthorn-associated populations. In north-western Italy, where *C. melanoneura* utilizes both apple and hawthorn, infected hawthorn plants may serve as active '*Ca.* P. mali' reservoirs (Tedeschi and Alma, 2007). Psyllids completing their reproductive cycle on infected hawthorn can acquire the phytoplasma and subsequently migrate into apple orchards as remigrant adults, effectively linking the wild reservoir to the cultivated host and increasing epidemiological risk. This transmission pathway appears to be absent in Germany, where hawthorn has never been found naturally infected and cannot be experimentally infected, rendering hawthorn-associated populations epidemiologically irrelevant for apple proliferation (Mayer et al., 2009). The role of genetic variation in vector competence has been

further investigated by Corretto et al. (2024), who demonstrated that individuals belonging to the same full-sibling family showed consistent phytoplasma acquisition outcomes, whereas different genetic lineages reared on the same infected tree differed markedly in acquisition capacity. A further layer of complexity arises from variation among phytoplasma subtypes. In north-western Italy, *C. melanoneura* is predominantly associated with subtype AT1, and experimental acquisition trials have demonstrated strong subtype-dependent differences in transmission efficiency. In particular, the AT1-AO variant circulating in this region is acquired with a probability of approximately 42%, far exceeding the acquisition rates observed for the AT1 (2%) and AT2 (4%) subtypes prevalent in north-eastern Italy (Corretto et al., 2024). These differences help explain why *C. melanoneura* acts as a primary vector in north-western Italy but plays a comparatively minor role in Trentino and South Tyrol, where AT2 and AP subtypes dominate and *C. picta* represents the main driver of apple proliferation epidemics (Baric et al., 2011; Cainelli et al., 2004).

Collectively, the epidemiological role of *C. melanoneura* thus emerges not as a fixed biological trait but as an emergent property of the local system, shaped by population genetic structure, hawthorn infection status and phytoplasma subtype a degree of context-dependency that stands in sharp contrast to the consistent, high-efficiency vector performance of *C. picta* across European apple-growing regions.

1.7.2 Fieberiella floriii

The leafhopper *Fieberiella floriii* (Hemiptera: Cicadellidae: Deltocephalinae) is a recognized, though minor, vector of *Ca. P. mali* (Krczal et al., 1989; Tedeschi and Alma, 2006). The species is widely distributed in Europe and is considered allochthonous in North America, where it has been associated with ornamental and fruit trees (Van Steenwyk et al., 1990). *Fieberiella floriii* is univoltine and overwinters as eggs or nymphs on various shrubs and trees, with a preference for Rosaceae and

its primary hosts include ornamental species such as privet (*Ligustrum* spp.), boxwood (*Buxus* spp.), myrtle (*Myrtus* spp.), hawthorn (*Crataegus* spp.) and firethorn (*Pyracantha* spp.), as well as apple (*Malus domestica*)(Swenson, 1974; Tedeschi and Alma, 2006). Adults are present from May to October and can occasionally be found in apple orchards, although typically at low densities (Swenson, 1974; Tedeschi and Alma, 2006).

Transmission competence for '*Ca.* P. mali' was first suggested based on symptom development and fluorescence microscopy in test plants exposed to field-collected *F. floriii* (Krczal et al., 1989). Subsequent controlled transmission trials confirmed its vector status, with individual transmission probabilities estimated between 0.7% and 2.2% (Tedeschi and Alma, 2006). Molecular analyses of field populations revealed variable natural infection rates, up to 5.2% in specimens collected from apple orchards and up to 20% in individuals sampled on wild hosts such as hawthorn, bramble and privet (Tedeschi and Alma, 2006). Despite these sometimes substantial infection rates, the overall epidemiological risk posed by *F. floriii* to apple orchards is considered low due to its relatively low and sporadic occurrence in commercial plantings compared with psyllid vectors (Tedeschi and Alma, 2006).

1.7.3 Alternative and suspected vectors

The complexity of AP epidemiology in some regions, where high disease incidence does not always correlate with monitored populations of the known psyllid vectors, has prompted intensive searches for additional or alternative vectors (Fischnaller et al., 2020, 2017). As phytoplasmas are exclusively transmitted by phloem-feeding insects, mainly within the suborder Auchenorrhyncha (Weintraub and Beanland, 2006), early investigations immediately focused on species within this group (Amici et al., 1972; Kunze, 1976). Initial transmission experiments yielded conflicting results and for instance, the leafhopper *Artianus interstitialis* (Germar) and the spittlebug *Philaenus*

spumarius L. were reported to transmit the agent from infected celery to apple seedlings (Hegab and El-Zohairy, 1986), although these findings could not be consistently confirmed by subsequent researchers (Frisinghelli et al., 2000; Refatti et al., 1986). Following the confirmation of *F. floriii* in the late 1980s (Krczal et al., 1989), systematic screenings continued, analyzing various species for pathogen presence: these included several apple aphids, *Aphis pomi* (De Geer), *Dysaphis plantaginea* (Passerini), *Eriosoma lanigerum* (Hausmann), which showed very low '*Ca.* P. mali' titers and failed transmission experiments (Cainelli et al., 2007), and other psyllids, *Cacopsylla peregrina* (Foerster, 1848), *C. mali* (Schmidberger), *C. crataegi* (Schrank), *Ctenarytaina eucalypti* (Maskell) and *C. spatulata* (Taylor) where '*Ca.* P. mali' DNA was detected but vector status was unconfirmed (Miñarro et al., 2016; Rosa García et al., 2014; Tedeschi et al., 2009). The planthopper *Metcalfa pruinosa* (Say) was also detected carrying the phytoplasma, but its vector status was never confirmed (Danielli et al., 1996). This long-standing necessity to comprehensively characterize the potential transmission pathway led to intensive faunistic studies in North-Eastern Italy, such as those carried out by Oppedisano et al. (2017) and Fischnaller et al. (2021). This large-scale effort analyzed over 31,000 specimens, confirming the presence of 95 Auchenorrhyncha species in South Tyrolean apple orchards (Fischnaller et al., 2020). Subsequent molecular testing revealed that '*Ca.* P. mali' DNA was detected (Fischnaller et al., 2020) in the treehopper *Stictocephala bisonia* (Kopp and Yonke), and the leafhoppers *Anaceratagallia alni* (Fallén), *Cixius nervosus* L., *Empoasca vitis* (Göthe), *Macrosteles sexnotatus* (Fallén), and *Psammotettix alienus* (Dahlbom) (Fischnaller et al., 2020; Oppedisano et al., 2017). While these sporadic detections did not indicate an epidemiological relevance comparable to the main psyllid vectors, ongoing faunistic surveys documented for the first time the occurrence of *Orientus ishidae* (Matsumura) in Trentino/South Tyrol (Italy) (Fischnaller et al., 2020; Oppedisano et al., 2017). This invasive, polyphagous species, already known as a vector of 16SrV phytoplasmas

(Lessio et al., 2016), was also found positive to '*Ca.* P. mali' for the first time (Oppedisano et al., 2017).

1.8 *Orientus ishidae*, the mosaic leafhopper

Orientus ishidae is an East Palearctic leafhopper that has emerged as an invasive species of growing concern in European agroecosystems. Native populations occur in Japan, Korea, and the Maritime territories of the former USSR (Blaser, 2019; Guglielmino, 2005; Matsumura, 1902), but the species has undergone a progressive global expansion, most likely facilitated by the movement of ornamental plants (Blaser, 2019; Felt and Bromley, 1938). The first extra-Asian records were made in North America in 1919 (Felt and Bromley, 1938; Sanders and DeLong, 1919), and genetic analyses later suggested that North American populations may have acted as a 'bridgehead' for the European invasion (Blaser, 2019). In Europe, O. *ishidae* was first detected in northern Italy in 1998 (Guglielmino, 2005) and subsequently spread across Switzerland (Günthart and Mühlethaler, 2002), Germany, Slovenia (Nickel, 2010; Seljak, 2004), the Czech Republic (Malenovsky and Lauterer, 2010), Austria (Nickel, 2010), France (Callot and Brua, 2013), Hungary (Koczor et al., 2013) Romania (Chireceanu et al., 2019) and Bulgaria (Gjonov et al., 2025). While the taxonomic placement of *O. ishidae* within Cicadellidae: Deltocephalinae is well established (Zahniser and Dietrich, 2013), morphological reassessment of specimens from Europe, the USA, and Japan has confirmed its specific identity as *O. ishidae*, differentiating it from the closely related *Orientus amurensis* (Guglielmino, 2005) described from China and Eastern Russia (Guglielmino, 2005).

Adults are readily recognized by their light brownish grey coloration and the characteristic dark, mosaic-like pattern on the forewings, head, pronotum, and scutellum (Guglielmino, 2005; Koczor et al., 2013; Matsumura, 1902), a feature from which the common name 'mosaic leafhopper' derives (**Figure 4**). The forewings extend beyond the abdomen, the head is rounded and narrower than

the pronotum, and the frons bears irregular dark markings, sometimes accompanied by an orange pattern between the eyes (Koczor et al., 2013; Matsumura, 1902). Adult body length ranges from 4.7–5.5 mm in males and 5.5–6.5 mm in females (Koczor et al., 2013), and males exhibit apically tapering genital plates marked by a distinct subapical tooth (Guglielmino, 2005). The species passes through five nymphal instars with early instars lacking wing pads (L1-L2) and late instars (L3–5) developing them (Lessio et al., 2019). The fifth instar is notable for its characteristic upward-pointing abdomen (Valley and Wheeler, 1985). Detailed description of *O. ishidae*, including characteristic features of L5 instar, male and female genitalia, and detailed comparison to *O. amurensis* can be found in the work of Guglielmino (2005) and Valley (1985).

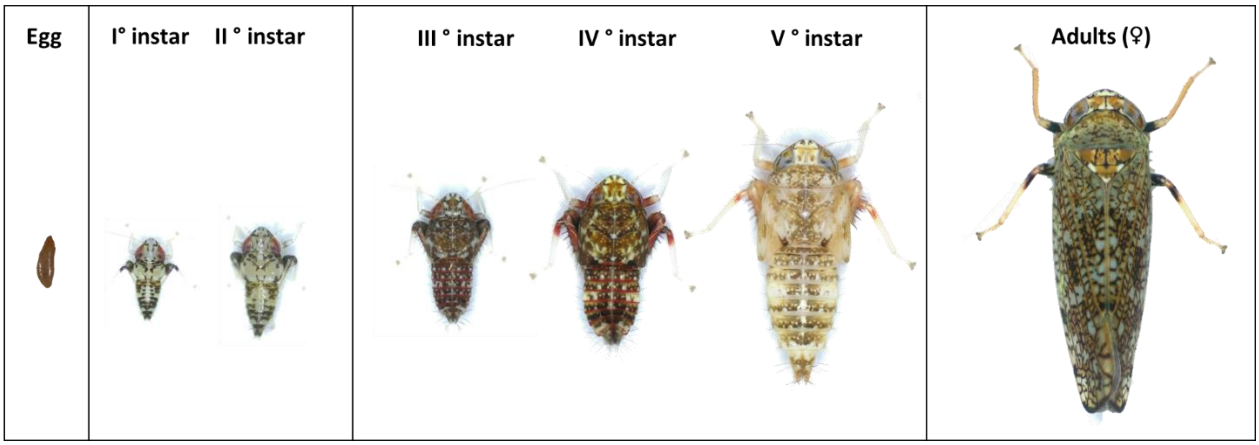

Figure 4: Life stages of *O. ishidae*. Eggs retrieved from heavily infested apple branches; L1–L2 nymphs; L3–L5 nymphs; and adult female. Not to scale. Photos by the author

Orientus ishidae is univoltine, overwintering in the egg stage, with eggs likely inserted into the woody tissues of host plants near buds (Lessio et al., 2019; Valley and Wheeler, 1985). Egg hatching in the wild host typically occurs from mid- to late May (Lešnik et al., 2017; Lessio et al., 2019; Parise, 2017), while laboratory studies at 21–23 °C report embryonic development lasting 34–48 days and post-embryonic development of the five nymphal instars requiring approximately 27–28 days on broad bean (Lessio et al., 2019). Field observations on wild host show that early instars peak

at the end of May, followed by late instars two to four weeks later (Lessio et al., 2019), often feeding on water sprouts and suckers of woody hosts (Parise, 2017). Adults are generally present from late June to early October, with population peaks commonly recorded in mid-July (Parise, 2017).

This species is highly polyphagous, exhibiting a broad host range that allows it to thrive in diverse environments, from natural forests to urban areas (Chireceanu et al., 2019; Lessio et al., 2016; Nickel, 2010). In Europe, the mosaic leafhopper has been recorded on several woody taxa, including *Corylus* spp., *Carpinus* spp., *Ostrya carpinifolia* L. and *Vitis vinifera* L.. Its host plants also encompass various Rosaceae, such as apple, sweet cherry, and hawthorn, as well as willow, walnut, and elm species. Additionally, the leafhopper has been observed on *Viburnum tinus*, *Hedera helix*, *Fraxinus excelsior* L. *, Robinia pseudoacacia* L., and the herbaceous *Chelidonium majus* L. (Chireceanu et al., 2019; Desqué et al., 2019; Guglielmino, 2005; Lešnik et al., 2017; Lessio et al., 2016; Nickel, 2010; Parise, 2017).

Natural enemies have been documented in Europe, including predation by the salticid spider *Icius* sp., parasitism by the mites *Charletonia cardinalis* (C.L.Koch,) and *Erythraeus jowitae* (Haitlinger), and parasitism of nymphs by the dryinid *Anteon fulviventre* (Haliday) (Parise, 2017). Additionaly, *O. ishidae* is involved in a unique and complex parasitic association with the Palaearctic cuckoo wasp, *Elampus bidens* (Förster), which is characterized as a 'Trojan horse' strategy (Bocca et al., 2023).

Reproductive behavior in *O. ishidae* relies entirely on substrate-borne vibrational communication (Lòpez Diez, 2019). The mating sequence is organized into a recognition duet, a signal-rich courtship phase, and copulation. Males initiate communication with a calling phrase composed of tapping and vibrational pulses, followed by a precisely timed female reply (approximately 100 ms after the male signal), establishing a coordinated duet. Courtship involves

complex male signals, including pulses, clicks, crackling sounds, an end-of-cycle signal, and a distinctive 'croaking' sequence (Lòpez Diez, 2019). Successful pairs culminate in copulation lasting around 150 seconds. Rival males can disrupt communication by emitting high-amplitude rival pulses that interfere with the female's response window, occasionally causing her to abandon the leaf surface; satellite behavior has also been observed (Lòpez Diez, 2019).

Although direct feeding damage is relatively limited, despite historical reports from the USA describing substantial leaf browning on apple and hazel (Felt and Bromley, 1938; Garman and Townsend, 1952), the growing concern surrounding *O. ishidae* does not stem from its leaves direct damage. The species is regarded as epidemiologically relevant primarily because of its association with several phytoplasmas, most notably the 16SrV-C, -D phytoplasmas correlated to Flavescence dorée (FD). The role of *O. ishidae* in the epidemiology of FD has been progressively clarified over the past decade, positioning this species as a secondary or alternative vector strongly linked to specific FDp genotypes and to wild reservoir hosts. The first detection of a 16SrV phytoplasma in *O. ishidae* was reported in Slovenia in 2010 (Mehle et al., 2011a), followed by confirmations in FD-affected vineyards in northern Italy (Gaffuri et al., 2011) and Switzerland (Trivellone et al., 2016). Experimental transmission trials have since demonstrated its ability to inoculate 16SrV phytoplasmas into *Vitis vinifera*, indicating that the species can function as a competent, if inefficient, vector (Lessio et al., 2016). Its epidemiological relevance, however, emerges most clearly in natural environments dominated by black alder (*Alnus glutinosa*), which serves as a major FD reservoir (Malembic-Maher et al., 2020). Across these habitats *O. ishidae* exhibits high infection prevalence, with reported FDp rates of 69% in L3–L5 nymphs and 85% in adults (Rizzoli et al., 2021), and values between 25% and 80% in other European surveys (Malembic-Maher et al., 2020; Mehle et al., 2011a). The species typically carries phytoplasma titers one order of magnitude higher than sympatric *Allygus* spp.

(Jarausch et al., 2023a), frequently exceeding the minimum inoculation threshold of 218 GU/ng DNA established for alder infections. In single-insect assays, individuals that successfully inoculated grapevine consistently harboured high titers, reinforcing the link between bacterial load and vector competence (Jarausch et al., 2023a).

The association between *O. ishidae* and FDp also displays a marked genetic dimension. The species harbours phytoplasmas from all three map-based clusters (FD1, FD2, FD3), many of which are compatible with the primary vector *Scaphoideus titanus*. Genotype M50 (map-FD1), associated with the reference strain FD70, is prevalent in both *O. ishidae* and *A. glutinosa* in southern Switzerland and appears more widespread south and west of the Alps (Malembic-Maher et al., 2020; Rizzoli et al., 2021). Genotype M38 (map-FD2), closely related to M54, is efficiently transmitted by *O. ishidae* (Jarausch et al., 2023a) while genotypes within map-FD3, including M12 and FD-C, have also been detected in *O. ishidae* in Switzerland and Italy (Malembic-Maher et al., 2020; Rizzoli et al., 2021)

Despite this wide genotypic spectrum and the high infection and titer levels observed in wild hosts, the epidemiological impact of *O. ishidae* within vineyards remains limited. Transmission to grapevine is considered a rare event, only five successful inoculations out of 90 single-insect trials have been reported (Jarausch et al., 2023a) and landscape-scale spillover from alder to vines occurs infrequently. Yet the epidemiological implications of such events are non-negligible: each primary FDp infection established by a secondary vector can act as a seed focus, capable of fueling local epidemics if *S. titanus* is present. In this sense, *O. ishidae* contributes to a broader landscape reservoir system, sustaining FDp circulation among wild hosts such as *A. glutinosa* and *C. avellana* and maintaining genotypes that may ultimately enter the vineyard cycle.(Jarausch et al., 2023a; Zwitter et al., 2023)

1.9 Apple proliferation management

The integrated management of AP relies on four main pillars: the use of healthy planting material, the removal of infection sources, the optimization of orchard design and monitoring, and the reduction of vector populations.

The use of certified, pathogen-free nursery material is essential to prevent long-distance spread and new introductions; given the possibility of latent infections and the limitations of symptom-based inspections, certification schemes must rely on sensitive molecular diagnostics, ideally targeting both aerial and root tissues in mother blocks (Baric et al., 2011; Baric et al., 2007). Within orchards, sanitation measures focus on roguing symptomatic trees, while in replant situations, the presence of residual infected roots should be considered, and deep tillage or partial root removal may be needed to reduce inoculum (Ciccotti et al., 2007; Lešnik et al., 2008).

Beyond the orchard boundaries, a landscape-level approach is increasingly necessary. Adjacent unmanaged or abandoned orchards, wild *Malus* stands, and ornamental or hedgerow hosts can serve as epidemiologically relevant reservoirs (Weintraub and Beanland, 2006; Jarausch et al., 2024; Wang et al., 2024; Somoano et al., 2025). Furthermore, recent spatial analyses have highlighted the influence of non-host vegetation on disease pressure: Somoano et al. (2025) demonstrated a strong correlation between AP incidence and the proximity of coniferous forests (e.g., *Pinus*, *Abies* spp.). These forests serve as essential overwintering sites for univoltine psyllids, thereby increasing the likelihood of remigrants returning to nearby orchards in high numbers the following season (Somoano et al., 2025).

While landscape management addresses external reservoir pressure, the direct suppression of vector populations within the orchard remains the cornerstone of disease mitigation. For univoltine

vectors such as *C. melanoneura* and *C. picta*, the highest risk of phytoplasma transmission occurs during the immigration of overwintered adults into orchards in late winter and early spring, when infection levels are typically higer. Spring inoculations into newly formed phloem, in turn, enable the phytoplasma to proliferate and colonize the tree efficiently throughout the growing season (Candian et al., 2020; Jarausch et al., 2011). Consequently, precise timing is a prerequisite for efficacy, yet it is complicated by the differential phenology of the species: *C. melanoneura* is typically the earliest immigrant, potentially arriving as early as late January, whereas *C. picta* remigrants generally appear later, often in March (Jarausch et al., 2011). To address the variability caused by climatic factors, empirical immigration models based on temperature thresholds have been developed (Tedeschi et al., 2012; Panassiti et al., 2019). However, the strict regional dependency of these models necessitates local validation to ensure reliable prediction of migration peaks (Panassiti et al., 2019; Tedeschi et al., 2012).

Chemical control has relied on several active substances targeting different developmental stages of the vectors. Etofenprox has shown high efficacy against overwintering adults of both species before bloom (Baldessari et al., 2010), and organophosphates and phosmet have likewise provided effective control (Baldessari et al., 2017). For later larval stages, organophosphates and neonicotinoids such as thiamethoxam and thiacloprid were previously employed (Mattedi et al., 2007). An additional constraint involves *C. picta*, whose oviposition period often coincides with apple bloom, when insecticide applications are restricted to protect pollinators, thereby further complicating effective control. In recent years, however, nearly all of the above-mentioned substances, except pyrethroids, have been revoked at the EU level, sharply reducing the range of chemical options (Finger, 2024). This regulatory contraction, combined with the growing risk of resistance, makes effective suppression increasingly difficult and underscores the fragility of chemical-based strategies.

Given the limitations of traditional insecticides and the scarcity of specific natural enemies for *C. picta* and *C. melanoneura*, research has shifted towards alternative physical and semiochemical strategies. Processed kaolin particle films have proven effective in repelling overwintering adults of both species and reducing oviposition when applied in late winter (Tedeschi et al., 2007). Repellents such as kaolin, conifer volatiles, and essential oils (e.g., cedarwood, cinnamon, clove) are employed to deter psyllids from the crop (Gross et al., 2019; Jarausch and Jarausch, 2019; Mayer et al., 2008; Tedeschi et al., 2007), while entomopathogenic fungi, particularly novel isolates of *Pandora* sp., have shown high virulence against *Cacopsylla* species, offering a promising biological alternative to synthetic insecticides (Görg et al., 2021).

Looking forward, the future of vector control lies in advanced biotechnological interventions. Novel technologies currently under investigation include RNA interference (RNAi) for specific gene silencing (Lu and Shen, 2024) and vibrational signal manipulation to disrupt mating communication (Oppedisano et al., 2020b). Furthermore, manipulating the vector microbiome, specifically targeting primary endosymbionts, represents a new prospective to reduce the vector competence of psyllids and impede the transmission of '*Ca*. P. mali' (Schuler et al., 2022).

1.10 Aims of the research

Apple Proliferation represents a persistent phytoplasma disease of major economic importance in Trentino, yet its recent epidemiological dynamics remain incompletely understood. Following the pronounced epidemic peak of 2015, disease incidence declined to minimal levels before re-emerging in 2020, with AP prevalence stabilizing at 1.5–1.7% over five consecutive years. Notably, this resurgence occurred despite consistently low and spatially uneven populations of the established psyllid vectors, which have remained stable for several years and show no clear correlation with the observed disease incidence.

These observations indicate that additional biological or ecological drivers may be influencing contemporary AP epidemiology. Concurrently, markedly elevated populations of the invasive leafhopper *O. ishidae* have been detected throughout apple orchards in Trentino. Given its polyphagous nature, its demonstrated ability to transmit other phytoplasmas, and previous reports of '*Ca.* P. mali' detection in field-collected individuals, the increasing presence of *O. ishidae* in apple-growing systems raises the possibility that this species could contribute to pathogen circulation. However, several key aspects of its biology and epidemiological relevance remain unknown. In particular, it is unclear whether *O. ishidae* is able to complete its development on apple, whether it can sustain infection by '*Ca.* P. mali', and whether it is capable of transmitting the pathogen to healthy plants. Furthermore, little information is available on its ecological dynamics within apple–vineyard landscapes, where this species may also interact with the epidemiology of FD. Against this background, the research chased three interrelated objectives designed to explain the epidemiological significance of *O. ishidae* within the AP and FD pathosystem.

The first objective was to characterize the biology of *O. ishidae* on apple, including its developmental cycle, phenology and feeding behavior on apple trees. The second objective was to assess vector competence by evaluating the ability of *O. ishidae* to acquire and transmit '*Ca*. P. mali' under controlled experimental conditions. The third objective addressed the broader ecological dynamics of *O. ishidae* in Trentino agroecosystems through monitoring of species distribution across apple orchards, survival experiments on grapevine to assess *O. ishidae* potential role in FD epidemiology; and characterization of spatial relationships between contiguous apple and vineyar

Given the significant agronomic and socio-economic implications of the renewed AP epidemic, the research also incorporated an economic impact assessment. Through a 20-year Partial Budget

analysis, the study quantified the monetary consequences of alternative replacing strategies, under both integrated and organic production regimes.

Collectively, these objectives were designed to provide a comprehensive evaluation of the potential involvement of *O. ishidae* in AP and FD epidemiology, and to generate the biological and economic evidence necessary to inform sustainable, evidence-based disease management strategies.

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CHAPTER 2. BIOLOGY OF A POTENTIAL NEW VECTOR

Orientus ishidae (Hemiptera: Cicadellidae): biology, direct damage and preliminary studies on apple proliferation infection in apple orchard.

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Simple Summary

A recent outbreak of the mosaic leafhopper (MLH), *Orientus ishidae*, in an apple orchard in Trentino made it urgent to assess the actual capability of this insect to cause damage to this crop. During the biennium from 2020–21, experiments were conducted both in field and semi-field conditions to ascertain whether MLH can (1) Complete the entire life cycle on cultivated apple trees; (2) Cause direct damage by feeding; and (3) Acquire the phytoplasma responsible of the Apple Proliferation (AP) disease. Our results proved that apple trees are suitable host plants for MLH, and that severe direct foliar damage is associated to its trophic activity. Such damage consists of diffuse chlorosis followed by necrosis that eventually take to leaf fall. The ability of MLH to acquire AP was confirmed in field trials. Our conclusions are that MLH is potentially a new pest of apple, although its role as a vector of AP must be confirmed with transmission bioassays. It will also be important to monitor this species in the apple orchards of other regions to prevent future outbreaks and consequent damage to production.

Abstract

The mosaic leafhopper, *Orientus ishidae* (Matsumura), is an Asian species widespread in Europe that can cause leaf damage in wild trees and transmit disease phytoplasmas to grapevines. Following an *O. ishidae* outbreak reported in 2019 in an apple orchard in northern Italy, the biology and damage caused by this species to apples were investigated during 2020 and 2021. Our studies included observations on the *O. ishidae* life cycle, leaf symptoms associated to its trophic activity, and its capability to acquire '*Candidatus* Phytoplasma mali,' a causal agent of Apple Proliferation (AP). The results indicate that *O. ishidae* can complete the life cycle on apple trees. Nymphs emerged between May and June, and adults were present from early July to late October, with the peak of flight between July and early August. Semi-field observations allowed for an accurate description of leaf symptoms that appeared as a distinct yellowing after a one-day exposure. In field experiments, 23% of the leaves were found damaged. In addition, 16–18% of the collected leafhoppers were found carrying AP phytoplasma. We conclude that *O. ishidae* has the potential to be a new apple tree pest. However, further studies are required to better understand the economic impact of the infestations.

Keywords:

leafhoppers; alien species; leaf depigmentation; phytoplasma vector; Apple Proliferation

2.1 Introduction

Orientus ishidae (Matsumura), or 'Mosaic Leafhopper" (MLH) is an East Palearctic species introduced first into the U.S. with the import of ornamental plant species (Sanders and DeLong, 1919; Felt and Bromley, 1938) and more recently in Europe (Blaser, 2019; Guglielmino, 2005) MLH has been collected from numerous broadleaf trees such as *Quercus palustris* (Johnson and Freytag, 2001), *Betula* spp.(Koczor et al., 2013; Nickel, 2010), *Salix* spp.(Guglielmino, 2005; Mazzoni, 2005; Nickel,

2010), *Corylus avellana*, *Ostrya carpinifolia, Carpinus betulus* (Lessio et al., 2016) and occasionally also from herbaceous species like *Urtica dioica* (Nickel, 2010) and from wild apple trees both in USA (Felt and Bromley, 1938) and in Europe (Lessio et al., 2016a; Nickel, 2010a; Oppedisano, 2016) MLH presence was also recorded in cultivated apple orchards: juveniles and adults in 1952 in the USA (Felt and Bromley, 1938) and only adults in 2016 in Slovenia (Lešnik et al., 2017), though in both situations no further studies related to its biology and phenology were conducted. Finally, MLH presence has been recorded on grapevine (*Vitis vinifera*) in northern Italy (Gaffuri et al., 2011), Slovenia (Mehle et al., 2010) and Switzerland (Trivellone et al., 2015). MLH is univoltine and overwinters as egg under the bark of different trees (e.g., *Aralia* spp*., Alnus glutinosa, Fraxinus* spp*., C. avellana, Crataegus* spp*., C. betulus, Tillia mentosa, Ulmus minor, Robinia* sp*., V. vinifera*) (Desqué et al., 2019; Lessio et al., 2019; Valley and Wheeler, 1985). Egg hatching from grapevine and wild plants begins in late spring in north-western Italy, and the first instar nymphs occur from mid-May to early June, while the last nymphs (5^{th} instar) are present until early August (Lessio et al., 2016; Lessio et al., 2019; Parise, 2017). Adults are active from the beginning of July until mid-October, with a flight peak between mid-July and early August (Casati et al., 2010a; Lessio et al., 2016; Parise, 2017). MLH is associated with leaf damage symptoms, such as yellowing, necrosis and stunted crown growth, in wild trees (Felt and Bromley, 1938; Valley and Wheeler, 1985). Furthermore, the leafhopper can transmit phytoplasmas such as '*Candidatus* Phytoplasma pruni', the causal agent of peach-X disease (Davis et al., 2013; Rosenberger and Jones, 1978) and in laboratory trials, Flavescence dorée phytoplasma of Elm Yellows group 16SrV (Lessio et al., 2016).

In 2019, a high MLH infestation was reported in apple orchards in Trentino (Italy), where plants showed symptoms of leaf depigmentation and necrosis in association with diffuse phyllotopsis (Baldessari et al., 2020). Additionally, in the framework of a auchenorrhyncha biodiversity survey in

the apple orchards of Trentino (Italy) carried out in 2014, few MLH individuals were collected for the first time in the region and one of them tested positive for '*Candidatus* Phytoplasma mali', causal agent of the Apple Proliferation (AP) disease (Oppedisano et al., 2017). The '*Ca.* P. mali' is a quarantine phytoplasma widespread in Europe (EPPO, 2020)causing severe symptoms on plants such as witches' brooms and production of small fruits with low organoleptic qualities (Janik et al., 2020). The phytoplasma is transmitted by insect vectors, mainly by psyllids *Cacopsylla picta* (Foerster) (Hemiptera: Psyllidae), *Cacopsylla melanoneura* (Foerster) and occasionally by the leafhopper, *Fieberiella floriii* (Stål) (Hemiptera: Cicadellidae) (Frisinghelli et al., 2000; Tedeschi and Alma, 2007, 2004).

This work aimed to assess whether MLH can complete its full life cycle on apple trees. We also investigated the ability of MLH to cause direct feeding damage to the apple leaves and the capacity to acquiring '*Ca*. P. mali'. The final aim was to define the specific interactions between MLH and apple trees to understand whether we are dealing with a potential new apple tree pest.

2.2 Materials and Methods

2.2.1 Apple Orchards Description

All trials were carried out at the fruit experimental station of Fondazione de Bellat in Valsugana, an apple-growing area in Trentino–Alto Adige region, Northeastern Italy (46◦04′34.4″ N–11◦47′19.3″ E). The studies were conducted in a 0.5 ha plot of apple trees, cv Golden Delicious on M9 rootstock, with 2.8% of symptomatic AP plants. The trees were planted in 1999 and maintained in the Spindel training form. No insecticide treatments were applied from May to the leaf fall. Other MHL individuals were collected from 0.8 ha of an apple orchard cv Reinette on M9 rootstock, planted

in 2004, maintained organically, and located in Mollaro (Val di Non, Italy, 46◦17′17.9″ N 11◦03′57.0″ E) with 2.7% of AP symptomatic plants to detect if they were carrying ‘Ca. P. mali.’

2.2.2 Oviposition Site and Hatching Dynamics

To assess the MLH ability to complete its full life cycle on apple trees, we ascertained whether it could lay eggs on apple trees in natural conditions. For this purpose, 2–3-year-old apple branches (i.e., those having a bark layer that is the favorite substrate of MLH oviposition)(Lessio et al., 2019) were collected in spring 2020 and 2021 from de Bellat site. The wood was cut into several pieces (15–20 cm each) and put inside plastic boxes (32 cm × 18.5 cm × 9.5 cm in 2020, 50 cm × 30 cm × 30 cm in 2021) closed by a plastic lid with two holes (2 cm × 5 cm) covered with anti-insect net. To preserve humidity, a layer of vermiculite (5 cm) was placed on the bottom of the boxes, and the canes were periodically sprinkled with water. To provide food for newly hatched nymphs, each box was supplied with a hazel leaf dipped in water, which was changed every two days. In 2020, each box was filled with 400–500 g of apple canes (total weight of the wood: 3.4 kg) and, in 2021, with 2 kg of canes (total weight 30 kg), which were stored in a greenhouse with controlled temperature (22 ± 1 ◦C). The boxes were inspected daily, and the emerged nymphs were transferred into a separate insect-proof cage containing an apple seedling. The experiment ended when hatching was not observed for a week.

2.2.3 Field Phenology and Flight Activities

During both 2020 and 2021 seasons, the presence of MLH nymphs and adults were visually inspected on apple trees from May to November to study its phenology. Three trees were randomly chosen weekly from each row (n rows = 8) at the de Bellat site; all the leaves from two randomly chosen branches per tree (i.e., south and north exposition) were surveyed in 2020 and 2021 (total n of surveyed branches/week = 48). The number of I–II nymph instars (N1–N2 winglets absent), III–V

nymph instars (N3–N5, winglets present), and adults was counted. Three yellow sticky traps (25 cm × 10 cm, Glutor, CBC (Europe) S.r.l. BIOGARD DIVISION, 24050 Grassobbio (BG), Italy) were attached in the orchard to monitor the abundance, flight activity, and sex ratio of adults (Pavan et al., 2021). The traps were secured to the middle support wire at 1.90 m from the ground and were replaced weekly from the end of June to the beginning of November.

2.2.4 Damage characterisation and quantification

Two sets of observations were carried out in 2020 to evaluate the MLH damage to apples: one in semi-field conditions, on 3-year-old potted plants, and a second one in the open field. In the semi-field trial, 10 MLH nymphs (N4–N5) were placed inside a plastic sleeve cage (40 cm × 20 cm nylon sleeve, BugDorm, Megaview Science Co., Ltd., Taichung 407008, Taiwan) positioned around a branch of the apple trees (one sleeve/plant). The plants were kept under a polytunnel to protect them from wind and rain. Each sleeve contained 10 leaves of similar size. The leaves in individual cages were subjected to three different treatments named according to the time of permanence of the MLH in the cage: 24 h, 72 h, and N h. In the latter, the insects were kept inside the sleeves until death. In the control treatment, no insects were placed into the sleeve. The experiment started on 6 July, and each treatment was replicated five times. Immediately after the removal of the insects (using a mouth aspirator), the number of leaves showing symptoms compatible with the MLH injuries was counted. From then on, the damage assessment was conducted weekly and lasted for 7 weeks. The leaf symptoms were divided into five classes of severity: Class 0 (Cl0), no evident symptoms; Class 1 (Cl1), chlorosis occurring in a leaf portion; Class 2 (Cl2), diffuse chlorosis and necrosis appearance; Class 3 (Cl3), necrosis extended to most of the surface; and Class 4 (Cl4), necrosis all over the leaf area in conditions of pre-phyllotopsis or leaf fall. To quantify the symptoms and make comparisons across the different treatments, an index of damage (ID) was created with the formula:

$$ID = \left(\frac{(0 * nCl0 + 1 * nClI + 2 * nCl2 + 3 * nCl3 + 4 * nCl4)}{4N}\right)$$

where 'n' is the number of leaves observed for each class and 'N' the total number of leaves per treatment (N = 10). ID ranged from 0 (no symptoms) to 1 (Cl4 symptoms) on all sampled leaves.

In the field trials, leaf sampling was conducted weekly from the end of June to September 2020 at the de Bellat site to estimate the amount of damaged leaves in open-field condition. In addition, to evaluate the loss of photosynthetic area, the percentage of leaf damaged surface was assessed. For each observation, four apple trees were randomly chosen from each row (eight total rows, n = 32) and 25 leaves per plant were inspected at a height of about 1.80 m from the ground.

2.2.5 Apple Proliferation Phytoplasma Infection

In order to assess the AP phytoplasma infection rate in MLH, adults were collected at the de Bellat site and in the Mollaro orchard using a mouth aspirator from AP-symptomatic trees (i.e., showing witches' brooms and enlarged/dentate stipules) and stored at −20 ◦C until molecular analyses. To detect the presence of '*Ca.* P. mali,' the total genomic DNA was extracted from single specimens using the commercial kit NucleoSpin Tissue (Macherey- Nagel GmbH and Co., KG, 52355 Düren, Germany). The collected insects were subjected to PCR amplification with two primers: fAT (forward, 5′ CAT CAT TTA GTT GGG CAC TT-3′) and rAT (reverse, 5′–GGC CCC GGA CCA TTA TTT ATT–3′) that amplify in the region 16S/23S(Smart et al., 1996). The PCR assays were performed with 2 mL of DNA, 10 mL of Master Mix Green Promega, 0.75 mL of primer fAT, 0.75 mL of primer rAT, and 0.75 mL of sterile water for a final reaction volume of 20 µL. The PCR parameters were as follows: an initial denaturation of 2 min at 95 ◦C, and 35 cycles with 60 s at 95 ◦C, 45 s at 57 ◦C, 60 s at 72 ◦C, and a final extension of 5 min at 72 ◦C. PCR products were analyzed

by electrophoresis on 1.5% w/v agarose gels stained with Ethidium bromide and visualized under UV light (ChemiDoc XRS, BioRad, 94547 Hercules, CA, USA).(Smart et al., 1996). PCR reactions were conducted in a total volume of 20 μL, containing 2 μL of DNA template, 10 μL of Master Mix Green (Promega), 0.75 μL of each primer, and sterile water.

PCR conditions consisted of an initial denaturation at 95 °C for 2 min, followed by 35 cycles of 95 °C for 60 s, 57 °C for 45 s, and 72 °C for 60 s, with a final extension at 72 °C for 5 min. Amplification products were visualized by electrophoresis on 1.5% agarose gels stained with ethidium bromide under UV light.

2.2.6 Data Analysis

Since ID value ranged in the unit interval (0–1), the effect of the treatment and week on damage was analyzed with a beta regression with a 'loglog' link. The beta regression was conducted in R (R Core Team, 2023) using the betareg package (Cribari-Neto and Zeileis, 2010) considering the independent effect of the explanatory variables and their interaction. Model assumptions were verified by plotting residuals against fitted values and against each covariate in the model. The candidate models were compared with a likelihood-ratio test. Predictor effect plots (Fox and Weisberg, 2019) were used to visualize the fitted coefficients.

2.3 Results

2.3.1 Oviposition Site and Hatching Dynamics

In 2020, 33 MLH juveniles emerged from the stored canes in the laboratory with a density of 9.71 nymphs/kg of wood. In lab conditions, the hatching period lasted 27 days and 79% of the individuals emerged in the first two weeks. In the field, the MLH first instars were observed on 3 June and were found during the next 31 days with 91% of them within the first 16 days (Figure 5)

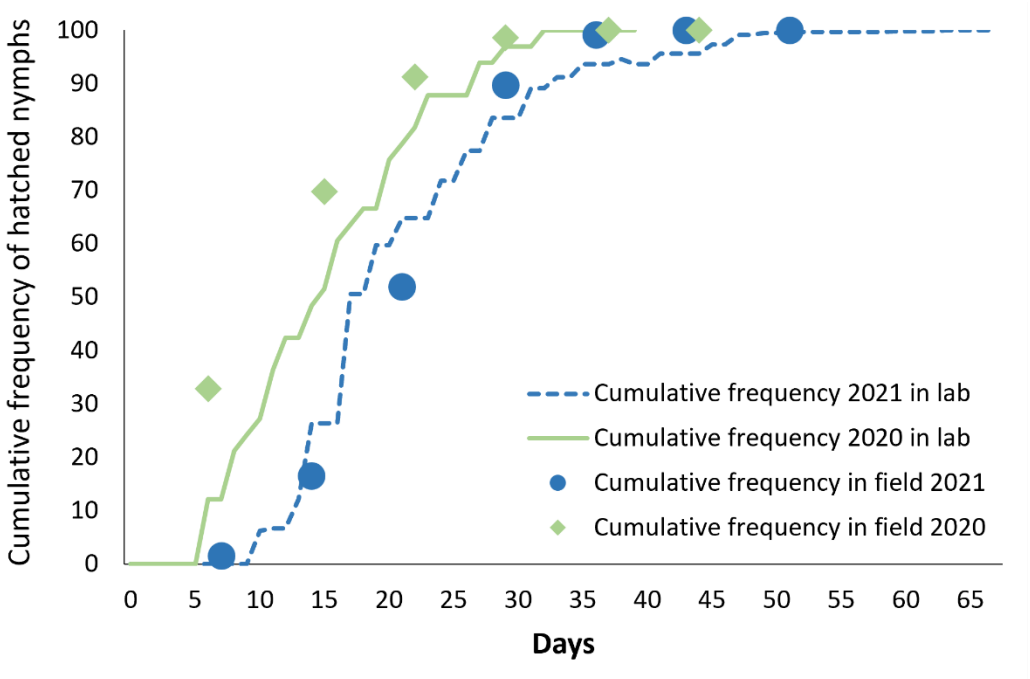

Figure 5: Comparison between cumulative frequency distribution of *O. ishidae* egg-hatching obtained from apple wood in lab (line 2020 vs dotted line 2021) and in field (rhombus 2020 vs circle 2021).

In 2021, 950 MLH juveniles emerged from the stored canes in the greenhouse with a density of 30.2 nymphs/kg of wood. The hatching period lasted 53 days from 3 May to 25 June, and 91% of them occurred within 23 days from the first hatch (**Figure 5**). In the field sampling, the MLH first instars were detected between 18 May and 23 June, with 89% of them hatched within 15 days of first detection (Figure 5).

2.3.2 Field Phenology

In the de Bellat site, a total of 813 and 869 MLH individuals were sampled in 2020 and 2021, respectively. As a whole, the population dynamic was similar between the two years, although we observed a 1–2-week anticipation in 2021 (**Figure 6**). The first individuals were observed at the beginning of June and from mid-May in 2020 and 2021, respectively. Nymphs (N3–N5) were observed starting from Week 25 in 2020 and from Week 24 in 2021. Accordingly, in 2021, the first

adults were observed 1 week earlier than in 2020 (**Figure 6**). The higher population density (mobile forms) was observed after the disappearance of N1–N2, both in 2020 and 2021, when the N3–N5 were dominant and at the appearance of the first adults. The last juveniles were found in late August 2020 and mid-September 2021, suggesting that there were still eggs hatching in late July or early August.

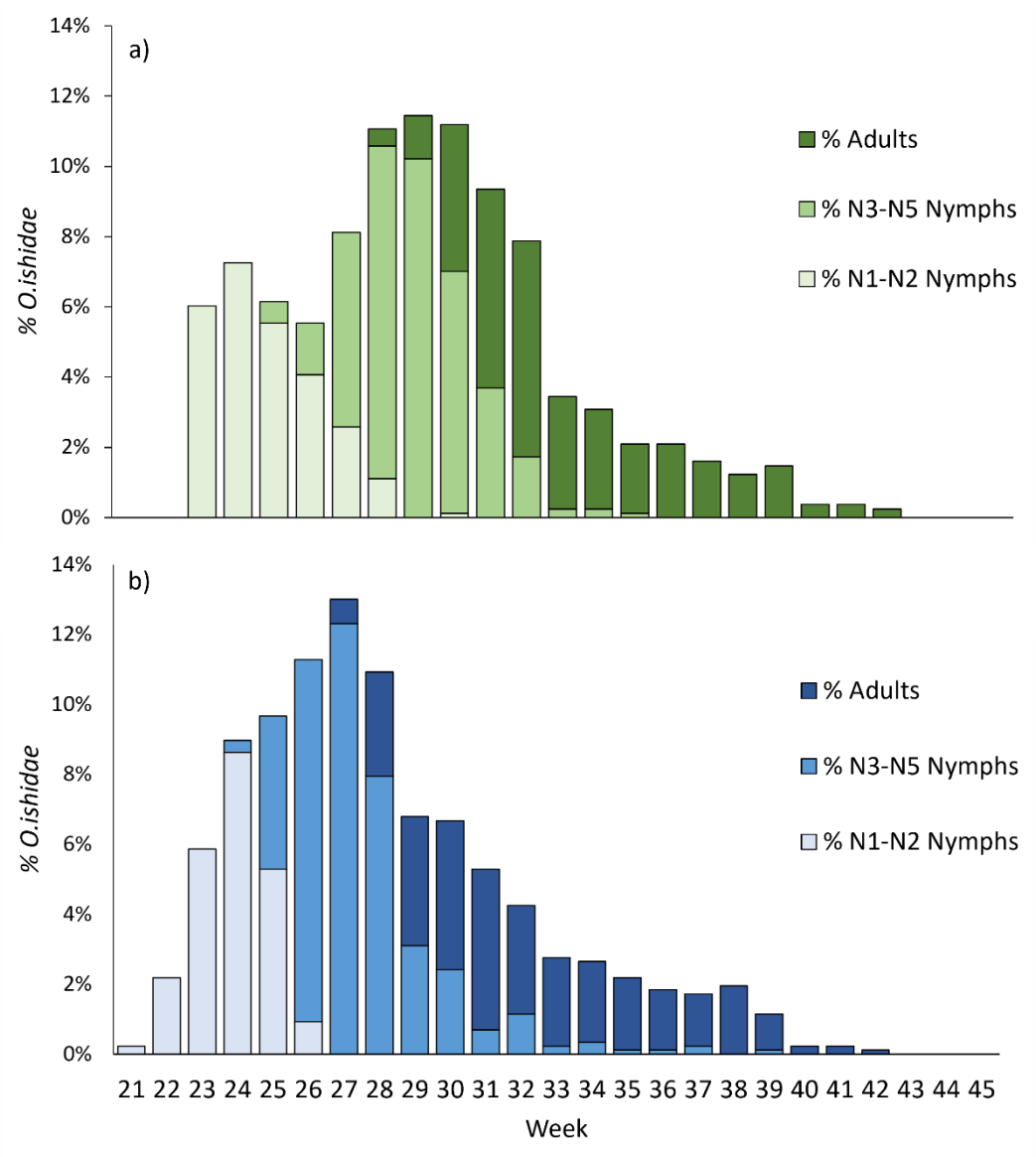

Figure 6: Seasonal abundance of *O. ishidae*, expressed as percentage of total *O. ishidae* finding in the season. Juvenile instars and adults in 2020(a) (total *O. ishidae*= 813) and 2021 (b) (total *O. ishidae*= 869).

N1 and N2 were mostly found on the lower surface of the leaves and showed relatively low mobility. In contrast, N3–N5 and adults were mainly observed on the upper leaf surface and jumped/flew away when disturbed.

2.3.3 Flight Activities

In 2020, the captures with sticky traps started from 11 July, the flight peak was observed at the beginning of August, and adults were detected until the end of October. Males were caught for 3 weeks after the beginning of the flight, with the peak flight recorded early in August, and no catches were recorded from mid-August onwards. The first females were found at the end of July, with the peak flight observed early in September, and no captures were recorded from late October onwards (**Figure 7**).

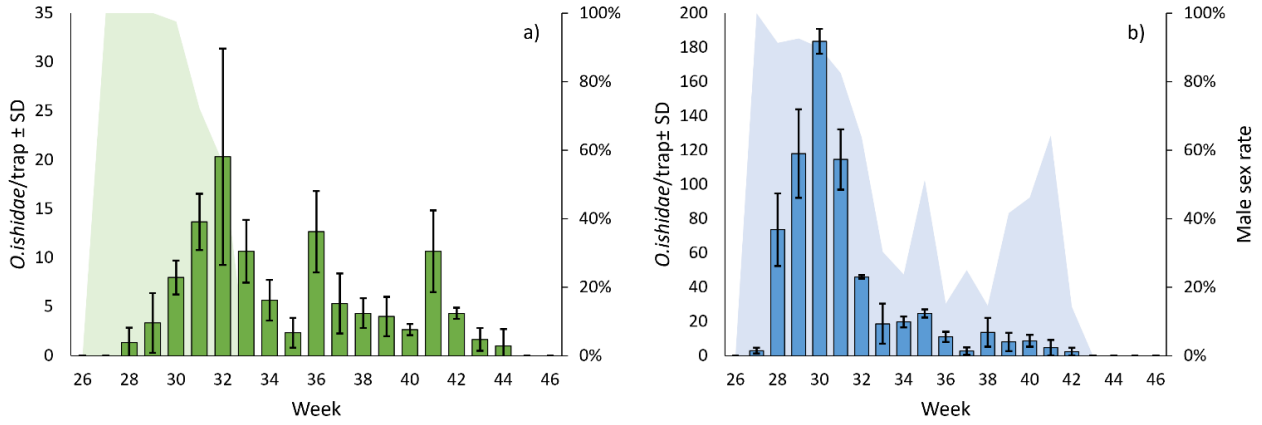

Figure 7: Seasonal trend of *O. ishidae* adults captured by yellow sticky traps (bar plot with standard deviation) in 2020 (a) and 2021 (b) placed inside the apple orchards (n = 3). The male sex rate is also showed (area plot).

In 2021, the captures started from 2 July, the flight peak was recorded in mid-July and no adults were captured from mid-October onwards. In 2021, the captures were, on average, 6 times higher than those recorded in 2020 (**Figure 7**). In 2021, only males were recorded during the first

week, with the peak male flight occurring in mid-July and, unlike 2020, males did not disappear completely after the peak flight and were observed until mid-October. The first females were found a week after the males appeared in July, with the female's peak flight delayed by a week compared to that of males and no catches were recorded from mid-October onwards (**Figure 7**).

2.3.4 Damage Characterisation

In the semi-field trials (**Figure 8**), both '24 h' and '72 h' treatments were associated with a distinct leaf damage and low symptom severity (Cl1–Cl2) that was already evident when the insects were removed (ID24 h = 0.04; ID72 h = 0.09). After insect removal, the symptomatic area further extended (Cl3) for the following three weeks with a final value of ID = 0.17 and 0.35 for '24 h' and '72 h,' respectively. From that moment on, both the area and type of damage (as class) did not change, with a total of two fallen leaves recorded in the '24 h' and one in the '72 h.' The 'N h' treatment showed the most severe damage, with the largest number of leaves that reached Cl3 (20%) and Cl4 (54%), and the symptom extension that increased until the end of the trials (ID = 0.66). No symptomatic leaves were found throughout the test period in the control.

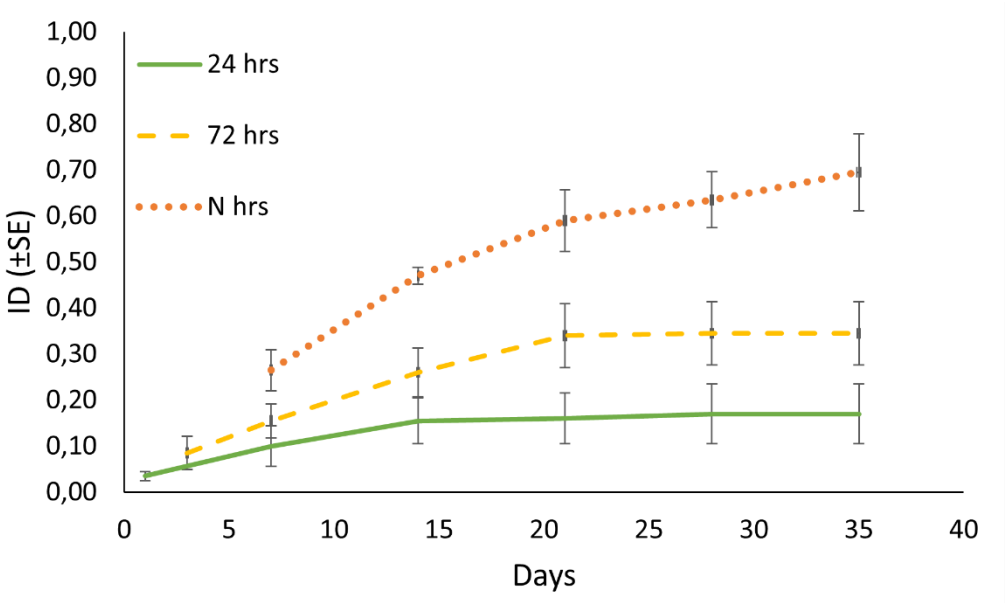

Figure 8: Mean indexed damage (ID), over the five weeks in the different treatments (n= 5).

Beta regression validation indicated no problems and, according to the likelihood ratio test, the model with the interaction (Treatment × Week) was significantly preferable ($X2 = 1.109$, $df = 7$, p-value < 0.001). The selected beta regression model confirmed the qualitative assessment, with a significant effect of the interaction between the 'N h' and time (Week) (**Table 1**). While the interactions '24 h'—week and '72 h'—week were not significant according to the model, it showed a steep increase in terms of ID for 'N h'—week (

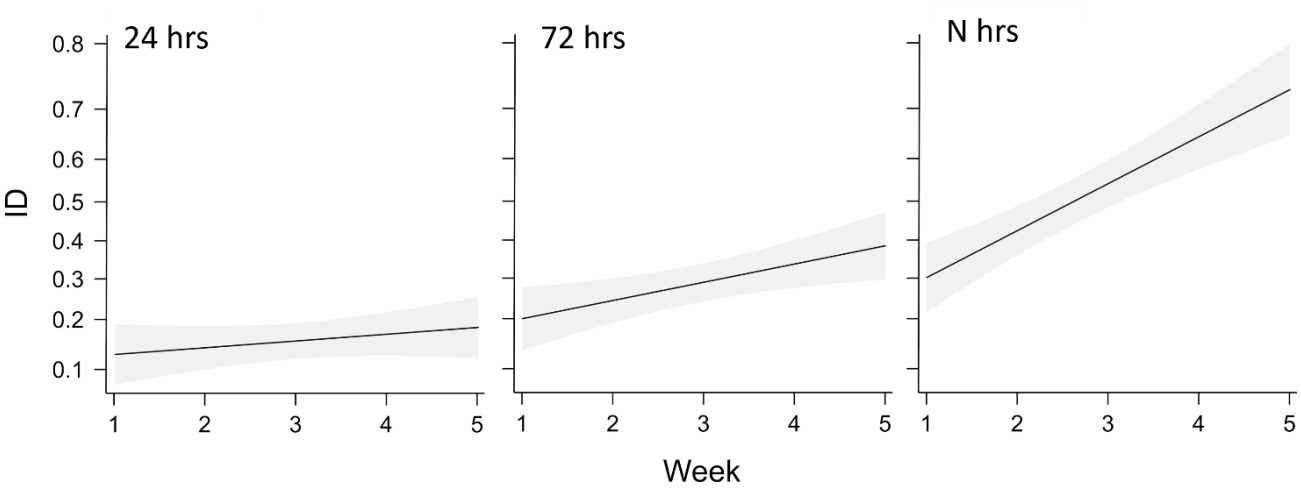

Figure 9).

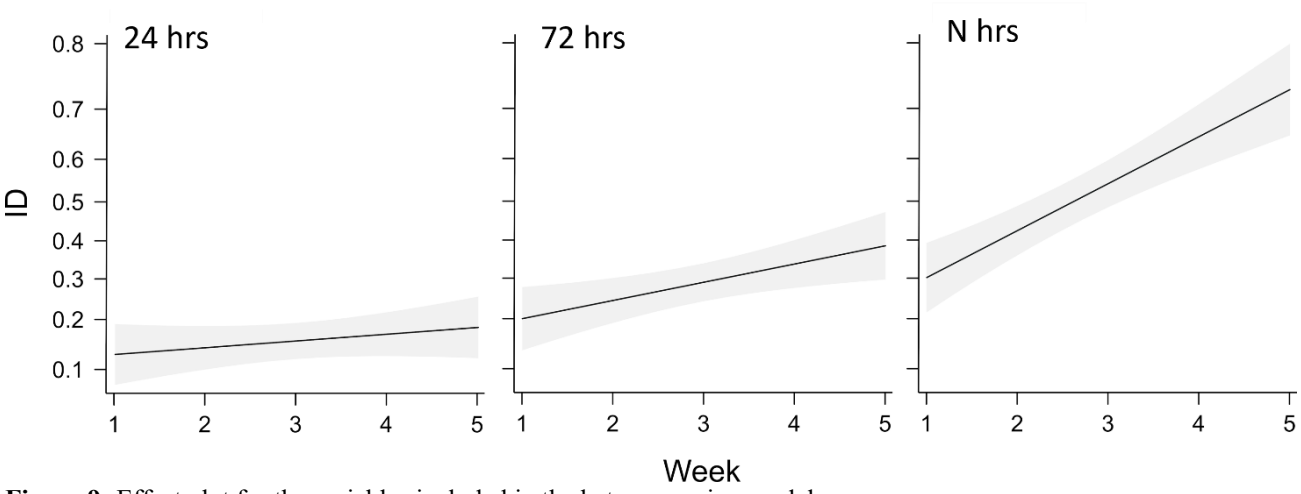

Figure 9: Effect plot for the variables included in the beta regression model.

Table 1: Estimated regression parameters, z-values and p-values for the beta regression. Pseudo R-squared: 0.658.

Treatments	**Estimate**	**Std. Error**	**z Value**	**Pr (>|z|)**
Treatment 72 h	0.157	0.215	0.729	0.466
Treatment N h	0.265	0.231	1.151	0.250
Week	0.048	0.045	1.074	0.283
Treatment 72 h: Week	0.082	0.066	1.241	0.215
Treatment N h: Week	0.288	0.075	3.826	>0.001
Intercept	−0.769	0.149	−5.147	>0.001

In the open field plot (**Figure 10**), the percentage of leaves damaged increased from the end of June to mid-August, when the highest damage of 23% was observed (12 August). From the beginning of September until the end of the trial, leaf damage decreased, with values ranging between 15% and 17% of symptomatic leaves. The largest damaged leaf area was 5% of the total sampled leaf area and was recorded on 22 August. From that moment, the percentage of damaged leaf area decreased, reaching approximately 3% at the end of September.

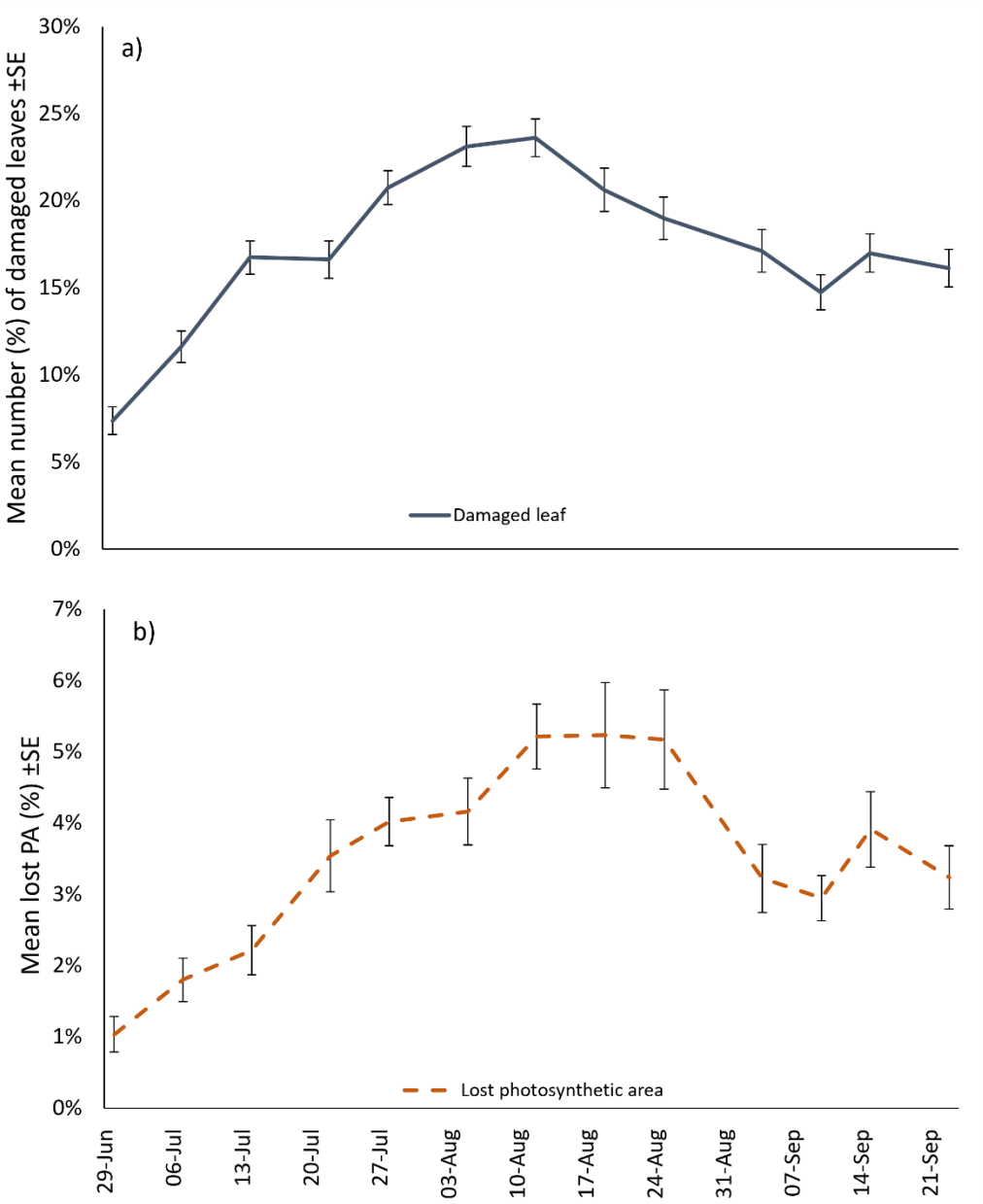

Figure 10: Seasonal trend of *O. ishidae* direct damaged in apple orchards, expressed (a) as mean percentage of affected leaves and (b) mean percentage of lost photosynthetic area (PA) (n= 32).

2.3.5 AP Infectivity

A total of 119 insects were analyzed. Data showed a percentage of leafhoppers harboring '*Ca*. P. mali'- ranging from 15.6% in the Mollaro orchard to 18.6% in the de Bellat site. Similarly, the percentage of symptomatic plants was similar in the two or-chards (2.8% and 2.7%) ().

Table 2: Results of the molecular analysis of collected MLH in the two different sites.

Site	**De Bellat**	**Mollaro**
Data of collection	14/08/2020	03/09/2020
Collected MLH	43	76
AP-positive MLH	8	12
% AP-positive MLH	18,6	15,7
% Infected plants	2.8	2.7

2.4 Discussion

Our research demonstrated that MLH is able to (1) Complete the entire life cycle on cultivated apple trees; (2) Cause direct leaf damage by feeding; and (3) Harbor the phytoplasma associated with the Apple Proliferation (AP) disease.

Similar to what was observed on grapevines (Lessio et al., 2019), MLH nymphs were obtained from eggs laid in 2–3-year-old branches, which, therefore, constitute a suitable site for oviposition. Interestingly, the level of oviposition in apple trees observed in 2020 (10 nymphs/kg) was more than 10 times higher than what was previously recorded from grapevine canes collected in organic vineyards (0.7 nymphs/kg)(Lessio et al., 2019). This data was surprisingly high considering that, between June and August 2019, three treatments with acetamiprid-based insecticide were applied in the same orchard to control the brown marmorated stink bug. Although this insecticide application was not specific to control MLH, the application period and the broad spectrum of action (Elbert et

al., 2008)should have affected the population and, consequently, the oviposition level, as already hypothesized in IPM vineyards (Lessio et al., 2016). We cannot exclude that the high oviposition level recorded in 2020 was caused by the immigration of females from the wild vegetation in the surrounding areas, at the end of the summer when the orchard was no longer protected by insecticide treatment. However, research conducted in vineyards suggested that MLH adults do not move massively from the edges into the cultivated area (Lessio et al., 2016). In this regard, it could be possible that apple trees represent a more favorable host plant than grapevines, and for this reason they could attract more MLH adults from other neighboring plants. In addition, in 2021, the number of hatched nymphs tripled, reaching hatching levels even higher than those reported from wild plants (e.g., hornbeam, alder, *Robinia* spp. etc., (Desqué et al., 2019). Even though these results must be confirmed with additional observations in other years and areas, they nevertheless strongly suggest that apple trees are not only occasional hosts, infested according to the botanical context in which the orchard is inserted, but rather that they can be key host plants for MLH.

As for the MLH phenology, the observed dynamics of the different life stages matched the data of previous research, even though we found differences between the two years of investigation. The first appearance of nymphs was recorded early in June in 2020, as also reported by Valley and Wheeler Jr. on the ornamental honey locust (*Gleditsia triacanthos*) in Pennsylvania (USA) (Valley and Wheeler, 1985). On the other hand, in 2021, first instars were detected in mid- May as previously reported in Piedmont (Italy) on grapevines and wild plants (Lessio et al., 2016; Parise, 2017). The observed discrepancy probably depended on a different hatching period due to climatic conditions (particularly temperature) to which overwintering eggs have been previously exposed. This phenomenon was demonstrated for other leafhoppers such as *Scaphoideus titanus* Ball, whose egg

hatching period were susceptible to great variability depending on the heat treatments to which the overwintering eggs have been subjected (Chuche and Thiéry, 2014, 2009; Falzoi et al., 2014).

As for the adults, their presence was observed from the beginning of July to the end of October in both years, with the peak of flight occurring between mid-July and the beginning of August. A similar flight trend was already reported in other studies conducted in the orchards of honey locust trees, vineyards, and wild plants (i.e., hornbeams, willows) (Casati et al., 2010b; Lessio et al., 2016; Parise, 2017; Valley and Wheeler, 1985).

Our results suggest that the population rose from 2020 to 2021. In particular, both hatching dynamics and sticky traps indicated considerably higher numbers of MLH in the second year of trials. On the contrary, we did not observe such a difference with the visual assessment method, which seemed to indicate similar values in the biennium. The three methods target different stages and, therefore, could be not accurate. For example, the hatch data show only the potential new population in the orchard and do not take into account the mortality of eggs or nymphs during juvenile development due both to abiotic and biotic factors (i.e., antagonists). On the other hand, chromotropic traps provide data only of the adult stage, and the catches are subject to different variables (e.g., exposure, location in canopy, weather, etc. (Pavan et al., 2021). Even in this case, abiotic factors can play a crucial role in determining the flight activity. This discrepancy with the visual assessment may be explained by the fact that we used as the sample unit the branch and not the number of leaves, which could be different for each branch in different years. Nevertheless, measuring the quantity of MLH in the orchard was out of the scope of this work, which aimed to study the MLH biology and phenology. Specific studies on the best sampling method will have to be conducted in the future, considering multiple variables such as the aggregate behavior on leaves/sprouts that MLH exhibited on wild species (e.g., hazel) (Lessio et

al., 2019). In this case, a landscape analysis will be also crucial to define how the agroecosystem context can affect the MLH incidence on a crop and, in particular, on an apple orchard.

Sticky traps were useful to define the MLH flight behavior and the gender differences. The sex rate analysis showed a prevalence of males in the first part of the season (July) and a progressive inversion with a prevalence of females starting from August. This data is in accordance with observations conducted on other leafhoppers (Lessio et al., 2009; Mazzoni et al., 2001) and can be due to the different behavior of the sexes. In fact, MLH uses vibrational signals for mating communication, and males actively search for females by emitting species-specific vibrational signals (Lòpez Diez, 2019). A typical strategy of leafhoppers is the so-called 'call-fly,' which consists of the emission of vibrational signals (i.e., calling signals) alternated with flights from leaf to leaf (Hunt and Nault, 1991). This is a method to increase the active space of signaling leafhoppers (Mazzoni et al., 2014), and our study supports the use of call-fly strategy by MLH during the first part of the summer until the male peak. Then, starting from August, we can assume that most of the mating has been completed and, while males relatively quickly disappear, females begin to be more mobile to oviposit. These dynamics, therefore, seem to indicate the maximum oviposition period between August and early September, in correspondence to the female peak of captures.

As for the investigation about the feeding damage, our trial in controlled conditions indicates that symptoms appear pretty soon after the inoculation, but also that they further evolve for a period of 1–2 weeks from the insect removal. This opens two hypotheses about the nature of the leaf damage. Phytophagous hemipterans can be divided into two categories according to their feeding strategies (Treherne et al., 1972): sheath feeders and cell rupture feeders. Sheath feeders, such as aphids and most leafhoppers (Backus, 1988), produce a sheath of gel saliva along the stylet and inject water saliva containing digestive enzymes before feeding on the phloem or xylem. This strategy is associated

to immediate leaf damage that does not evolve once the feeder is removed (Backus, 1988; Backus et al., 2005a, 2005b; Chuche et al., 2017; Cornara et al., 2018). This group includes the most effective phytoplasma vectors (Jarausch and Weintraub, 2013). Cell rupture feeders, on the other hand, do not make a complete salivary sheath. Instead, their stylets move in and out of the plant tissues, mechanically lacerating cells and simultaneously secreting watery saliva. This strategy is typical of members of the leafhoppers of the subfamily typhlocybinae and is associated with a specific leaf symptom called 'hopperburn.' Insects with this strategy have a low vector capability (Backus et al., 2005b; Jarausch and Weintraub, 2013; Jin et al., 2012). Our experiments, therefore, suggest that MLH causes 'hopperburn-type' symptoms, supporting the thesis that this species is a cell rupture feeder (Lessio et al., 2016). On the other hand, it cannot be excluded that the progression of the symptoms may be caused by the action of phytotoxic saliva, similar to what happens for some aphids (e.g., *Schizaphis graminum* (Rond.), *Diuraphis noxia* (Kurdjumov), which instead are typical sheath feeders (Miles, 1999; Nicholson et al., 2012; Sharma et al., 2014; van Bel and Will, 2016). Moreover, 'hopperburn' symptoms were also reported from non-typhlocybinae auchenorrhyncha, such as delphacids *Nilaparvata lugens* (Stål) and *Sogatella furcifera* (Horvath) (Rubia-Sanchez et al., 2003). These two planthoppers are sheath feeders that can transmit viral disease but also can cause extensive direct damage to host plants (AB Ghaffar et al., 2011; Heong and Hardy, 2009; Lei et al., 2016) due to phytotoxic saliva (Backus et al., 2005b). The symptoms that we observed in the case of MLH seem to be similar to that observed in those planthoppers; in fact, the spread of saliva through the vascular system would explain the delayed appearance and extension of symptoms in apparently healthy leaves in the '24 h' and '72 h' after the insect removal, and that the phylloptosis of leaves is only partially symptomatic. In addition, the fact that, to date, no members of the subfamily deltocephalinae are known to be cell rupture feeders supports our hypothesis (Backus et al.,

2005b). Further studies using specific techniques aiming at describing the MLH feeding behavior, e.g., electrical penetration graph (EPG), could clarify this significant aspect (Avosani et al., 2021; Backus and Shih, 2020).

It is important to note that when MLH remained on the plant throughout the trial period ('N h'), we observed quite a remarkable difference with the other treatments in damage severity with 54% of the leaves showing full surface necrosis (Cl4), most of which had fallen by the end of the experiment. Such high damage was the result of MLH continuous trophic activity for a prolonged period, but we cannot exclude that specimens not enclosed in sleeves and free to move would eventually move to other leaves thus limiting the total phylloptosis.

Interestingly, field and semi-field trials yielded similar results. In fact, we observed MLH damage associated to a relevant percentage of apple leaves: We found a maximum of 23% of symptomatic leaves, with a total leaf surface reduction of 5%. Even though this value hardly affects the photosynthesizing capacity of the apple, and consequently the production, we did not account for the leaves fallen to the ground in our sampling, which was suggested by the observed reduction of percentage damaged leaves on the plants from August. In this regard, we can hypothesize that MLH nymphs, as in many other leafhoppers, cause more damage to the host plant than adults. For this reason, the symptom severity grows faster in the first part of the apple growing season. In this context, the observed reduction can be explained by phylloptosis, which would be higher than the appearance of new symptomatic leaves. However, the actual severity of the phylloptosis could be measured only by means of dedicated experiments with the aim to count the number of fallen leaves. This aspect deserves further investigation to better understand the potential direct damage caused by MLH to apple. In addition, the monitoring of MLH should be extended to other orchards with different varieties and to other geographical areas to assess

whether the reported invasion in Trentino is an isolated case or is a clue of an incipient new problem.

Finally, regarding the association between MLH and AP phytoplasma, our molecular analysis revealed that 15–18% of tested individuals were positive to '*Ca*. P. mali'. Making a comparison between this data and the natural infection rate of *C. melanoneura* or *C picta* (i.e., the two main AP vectors) is difficult due to the infective rates of the psyllids, which vary considerably according to the geographic region and the development stage (e.g., overwin- tering, nymphs, emigrants) (Baric et al., 2009; Carraro et al., 2008; Jarausch et al., 2007a; Malagnini et al., 2010b; Oppedisano et al., 2020a). However, overwintering *C. melanoenura* were found with a natural infectivity range between 1% and 23% in Trentino (Baric et al., 2009; Malagnini et al., 2010b; Oppedisano et al., 2020a), comparable with what we observed in MLH, whereas overwintering *C. picta* showed a higher natural infectivity reaching up to 45% of infected individuals (Carraro et al., 2008). The leafhopper *F. florii*, the third known AP vector, which is taxonomically more closely related to MLH, is characterized by lower infectivity values in apple orchards (on average 5.7%), even though when collected from wild vegetation, the percentage of AP infected individuals increased reaching values between 9.1 to 20%, comparable with MLH (Tedeschi and Alma, 2006). At any rate, the acquisition of phytoplasma by a phloem-feeding Auchenorrhyncha is not sufficient proof to state that the insect is a vector (Brown, 2016). For example, '*Ca*. P. mali' was detected in many other phloem-feeding Hemiptera, such as the planthopper *Metcalfa pruinosa* (Say) (Danielli et al., 1996)and the psyllids *Cacopsylla peregrina* (Förster) and *Ctenarytaina eucalypti* (Maskell) (Miñarro et al., 2016; Tedeschi et al., 2009). For all these species, transmission trials were not carried out, so the potential role of these insects in the AP epidemiology is still unknown.

Other Hemiptera that successfully acquired '*Ca*. P. mali' then were not able to transmit it to other plants, as reported for different apple aphids (e.g., *Aphis pomi* (DeGeer) *Dysaphis plantaginea* (Passerini), *Eriosoma lanigerum* (Hausmann) (Cainelli et al., 2007), and leafhoppers (e.g., *Empoasca vitis* (Göethe) (Mattedi et al., 2008). Thus, our experiments do not allow us to demonstrate whether MLH plays a role in the AP epidemiology. Nevertheless, they provide a basis for future experiments of transmission (Bosco and Tedeschi, 2013).

2.5 Conclusion

The present research unveiled several aspects related to the biology of *O. ishidae* and proved its interaction with apple trees. Many other aspects deserve to be more deeply investigated, in particular, the MLH role as a *Candidatus* vector of the AP phytoplasma. The acquisition of this knowledge will be fundamental to define the importance of MLH in the apple production and its potential economic impact in the future.

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CHAPTER 3. VECTOR CAPABILITY

Orientus ishidae (Matsumura, 1902) is not a competent vector of '*Candidatus* Phytoplasma mali': insights from controlled transmission trials and infection dynamics

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Abstract

Apple proliferation (AP), associated to the phytoplasma '*Candidatus* Phytoplasma mali', is one of the most economically damaging diseases affecting apple production in Europe. The disease is primarily transmitted by the psyllid species *Cacopsylla picta* and C. *melanoneura*, yet recent epidemics in Trentino, Northern Italy, have coincided with scarce populations of the confirmed vectors and rising populations of the invasive leafhopper *Orientus ishidae*. Because this leafhopper is a competent vector of several other phytoplasmas and has tested positive for *'Ca.* P. mali' DNA in field surveys, its potential role in AP epidemiology warrants clarification. In this study, we conducted controlled transmission experiments between 2022 and 2024 to determine whether *O. ishidae* can transmit *'Ca.* P. mali'. Experiments tested multiple variables, including phytoplasma subtype identity (AT1 vs. AT2), acquisition access period (AAP) duration (3 vs. 10 days), and initial pathogen titer, and were complemented by infection-dynamics assays tracking within-vector changes over 15 days of latency. Across all experiments, 4–22% of exposed insects tested positive to AP. AAP duration significantly affected detection rates, whereas subtype identity and initial phytoplasma titer did not. However, no transmission occurred in any inoculated plant. In the infection-dynamics experiment, 85% of insects (34/40) were positive immediately after a 5-day AAP, but positivity declined to 44.4% after 5 days of

latency, 17.5% after 10 days, and 0% after 15 days, accompanied by progressively decreasing phytoplasma titers. These trends contrast sharply with the titer increase characteristic of competent vectors. Moreover, phytoplasma concentrations in *O. ishidae* remained several orders of magnitude lower than those reported in confirmed AP vectors. These findings demonstrate that *O. ishidae* is not a competent vector of *'Ca.* P. mali' under the conditions tested and could function instead as a dead-end host. From a management perspective, AP control programs should continue to focus on the confirmed psyllid vectors, whereas *O. ishidae* monitoring remains relevant primarily for its role in other phytoplasma diseases.

3.1 Introduction

Phytoplasmas are phloem-limited, wall-less bacteria (class Mollicutes) responsible for numerous plant diseases of major economic importance worldwide (Bertaccini et al., 2022; Wang et al., 2024). Their epidemiology depends strongly on the biology of their insect vectors, which occur exclusively among phloem-feeding Hemiptera (Weintraub and Beanland, 2006). Within this order, confirmed or potential vectors are restricted to a few families—Cicadellidae, Cixiidae, Cercopidae, Delphacidae, and Psyllidae (Trivellone and Dietrich, 2021; Weintraub et al., 2019).

Phytoplasma epidemiology is shaped by interactions among pathogen, host plant, and insect vectors, and is strongly influenced by ecological factors such as vector survival, mobility, phenology, and feeding behavior (Bosco and D'Amelio, 2009; Marcone, 2014; Perilla-Henao and Casteel, 2016). The system becomes even more complex when multiple vector species coexist with different ecological niches and transmission efficiencies (Johannesen et al., 2012; Wei et al., 2021), or when ecological and evolutionary host shifts occur, processes that may be favored by rapid environmental change or biological invasions (Baric et al., 2011; Malembic-Maher et al., 2020; Quaglino et al., 2019).

A paradigmatic example of the complex vector-borne disease epidemiology is apple proliferation (AP), associated with *'Candidatus* Phytoplasma mali' (16SrX-A group), one of the most economically damaging phytoplasma diseases affecting European apple production (Janik et al., 2020; Seemüller and Schneider, 2004). AP induces severe metabolic alterations in infected trees (Aldaghi et al., 2012; Janik et al., 2017; Mittelberger et al., 2024), leading to characteristic symptoms such as witches' brooms, enlarged stipules, and, most critically, undersized fruits of reduced commercial quality (Herzog et al., 2012; Seemüller et al., 2008; Strauss, 2009). The psyllids *Cacopsylla picta* (Förster) and *Cacopsylla melanoneura* (Förster) are the main confirmed vectors of *'Ca.* P. mali' (Frisinghelli et al., 2000; Jarausch et al., 2007b; Tedeschi and Alma, 2004), although their relative epidemiological importance varies geographically. In Germany, *C. picta* is regarded as the primary vector, whereas in Northeastern Italy, the two species act complementarily; in Northwestern Italy, *C. melanoneura* is considered the dominant vector (Candian et al., 2020; Mayer et al., 2009; Oppedisano et al., 2020). Such regional variation is influenced by several factors, including genetic structure of vector populations, phytoplasma subtype diversity, and specific host–pathogen microbiomes interactions (Casati et al., 2011; Corretto et al., 2024; Malagnini et al., 2013; Malagnini et al., 2010; Schuler et al., 2022).

Despite management strategies focusing on the removal of infected trees and insecticide applications (Baldessari et al., 2010), several AP epidemics have been reported in Europe, particularly in Trentino-Alto Adige/Südtirol, one of the largest apple-producing regions in Europe. Major outbreaks occurred in the late 1990s (Springhetti et al., 2002) and again between 2010 and 2014 (Oppedisano et al., 2020), followed by a renewed increase in disease incidence since 2019 (Bargna et al., 2024). Notably, the most recent upsurge occurred despite persistently low populations of *C. picta* and *C. melanoneura* (Fischnaller et al., 2020, 2017), raising the question of whether other hemipteran

species might contribute to phytoplasma spread.

Several alternative AP vectors have been tested over the past decades. The leafhopper *Fieberiella floriii* (Stål) can transmit *'Ca*. P. mali' experimentally, although its field relevance appears limited (Tedeschi and Alma, 2006). Occasional detection of the phytoplasma in *Artianus interstitialis* (Germar), *Philaenus spumarius* (L.), *Empoasca vitis* (Goethe), *Stictocephala bisonia* Kopp and Yonke, *Metcalfa pruinosa* (Say), and some *Cacopsylla* spp. and *Ctenarytaina* spp. have been recorded over the years (Amici et al., 1972; Danielli et al., 1996; Duduk et al., 2008; Hegab and El-Zohairy, 1986; Miñarro et al., 2016; Rosa García et al., 2014; Tedeschi et al., 2009). Conversely, common apple aphids have been excluded as vectors through controlled trials (Cainelli et al., 2007).

Recent faunistic and molecular surveys in Trentino-Alto Adige/Südtirol have further expanded knowledge of the local Auchenorrhyncha community. *'Ca.* P. mali' DNA has been sporadically detected in several species, such as *S. bisonia*, *Aphrophora alni* (Fallén), *Cixius nervosus* (L.), *E. vitis*, *Macrosteles sexnotatus* (Fallén), and *Psammotettix alienus* (Dahlbom), but always at very low titers compared with confirmed psyllid vectors (Fischnaller et al., 2020; Oppedisano et al., 2017). Notably, these surveys also reported the first regional detection of the invasive mosaic leafhopper *Orientus ishidae* (Matsumura), which tested positive for *'Ca.* P. mali' (Oppedisano et al., 2017).

Orientus ishidae, also known as the mosaic leafhopper, is native to East Asia and was first detected in Europe in 1998, after which it rapidly spread across the continent (Blaser, 2019; EPPO, 2025; Guglielmino, 2005). *Orientus ishidae* is univoltine, overwintering as eggs in woody tissues, with nymphs emerging in May and adults appearing from late June through October (Dalmaso et al., 2023; Lessio et al., 2019; Parise, 2017). It is highly polyphagous and feeds on several woody hosts, including alder (*Alnus glutinosa* (L.)), grapevine (*Vitis vinifera* L.), hazel (*Corylus avellana* L.), and apple (*Malus domestica* Borkh.) (Lessio et al., 2019; Nickel, 2010; Rizzoli et al., 2021).

Orientus ishidae is a confirmed vector of Flavescence dorée phytoplasmas (16SrV group) in Europe (Jarausch et al., 2023; Lessio et al., 2019; Malembic-Maher et al., 2020), and of Peach X-disease phytoplasma (*'Ca.* P. pruni') in North America (Rosenberger and Jones, 1978). It also harbors *'Ca.* P. ulmi', the causal agent of elm yellows (Oggier et al., 2024).

Recent monitoring indicates that *O. ishidae* populations in European apple orchards, including Northern Italy, have become stable and increasingly abundant since 2019 (Chireceanu et al., 2019; Dalmaso et al., 2023; Gjonov et al., 2025). The temporal overlap between rising *O. ishidae* abundance, renewed AP epidemics, and constant low populations of primary psyllid vectors has raised the hypothesis that *O. ishidae* might contribute to AP epidemiology. However, the detection of phytoplasma DNA in field-collected insects does not constitute proof of vector competence, and controlled transmission trials remain essential (Weintraub et al., 2019; Weintraub and Beanland, 2006). Therefore, the present study aimed to assess whether *O. ishidae* is a competent vector of *'Ca.* P. mali' through multi-year controlled transmission trials and characterize infection dynamics of the phytoplasma within *O. ishidae* following acquisition.

3.2 Materials and Methods

Three transmission experiments were conducted between 2022 and 2024 to evaluate the vector competence of *O. ishidae* for *'Ca.* P. mali'. All experiments adhered to a standardized transmission protocol, comprising an Acquisition Access Period (AAP), during which insects fed on infected plants, a Latency Period (LP), allowing for pathogen replication and circulation within the vector; and an Inoculation Access Period (IAP), during which potentially infective insects fed on healthy test plants (Bosco and Tedeschi, 2013; Pagliari et al., 2019). Unless otherwise specified, all trials were performed in insect-proof cages under controlled greenhouse conditions (22 ± 2 °C, 80 ± 5% RH, 16:8 h light:dark photoperiod).

3.2.1 Insect rearing

In the spring of each experimental year, 3-year-old apple branches were collected from a heavily infested orchard in Spagolle, Italy (46°02′45.1″N, 11°28′13.7″E). Branches were cut into 15–20 cm segments and placed in plastic boxes (50 × 30 × 30 cm) containing a 5 cm layer of wet vermiculite for humidity maintenance. Boxes were sealed with plastic lids equipped with two 2 × 5 cm mesh-covered ventilation holes. Branches were regularly misted with water, and fresh apple leaves were provided as food, replaced every two days. Emerged nymphs were collected daily and transferred to insect-proof cages containing healthy, phytoplasma-free apple seedlings maintained under the same greenhouse conditions. This rearing approach ensured that experimental insects were free of phytoplasma infection before controlled AAP exposure.

3.2.2 Transmission experiment of 2022

In 2022, groups of 15–25 third- to fifth-instar nymphs were caged onto potted *Malus domestica* cv. 'Golden Delicious' plants infected with the AT2 subtype of '*Ca.* P. mali', originally used in transmission trials (Oppedisano et al., 2020), and re-confirmed as infected by molecular analysis immediately before the experiment. In total, 14 independent cohorts of nymphs were established, resulting in 280 individuals performing an AAP that lasted 14 days, after which nymphs groups were transferred onto healthy apple seedlings for a 14-day LP. Following the LP, insects were moved to new healthy apple plants for sequential 14-day IAPs, following the protocol used in *F. floriii* AP transmission experiments (Tedeschi and Alma, 2006). Up to three consecutive IAPs were conducted per group, depending on insect survival. IAP cages were inspected daily, and dead insects were collected and stored at –20 °C for molecular analyses. All surviving insects were frozen at the conclusion of the final IAP.

3.2.3 Transmission experiment of 2023

To improve experimental reproducibility and to evaluate factors potentially influencing transmission, a second transmission experiment was carried out in 2023. Specifically, nymphs were exposed to inoculum sources differing in phytoplasma subtype (AT1 vs. AT2), initial pathogen titer, and different AAP duration. Naturally infected sources consisted of potted apple plants derived from symptomatic root suckers collected in a heavily affected orchard (Piovi, Trentino, Italy; 46°13′59.5″N, 11°09′55.0″E) and a field tree showing typical AP symptoms from the same orchard. The presence of *'Ca.* P. mali', phytoplasma titer (expressed as ΔCt), and subtype identity (AT1 or AT2) were confirmed by qPCR and PCR-RFLP prior to the experiment. (see Molecular analysis of plants). Four potted apple suckers were infected with AT2 and exhibited mean leaf ΔCt values of -0.16 ± 0.82, while the single apple sucker infected by AT1 showed an initial ΔCt of 0.42. The field-symptomatic plant was infected by AT2, and leaves of the symptomatic tree exhibited an initial ΔCt of 0.005.

Experimentally-infected sources consisted of *in vitro* plantlets of *M. domestica* cv. 'Golden Delicious' infected with either AT1 or AT2, maintained, and propagated *in vitro* according to established protocols (Ciccotti et al., 2009; Jarausch et al., 1996). Third- to fifth-instar nymphs were placed directly onto *in vitro* plantlets within their culture tubes. However, contamination introduced by insects rapidly compromised plant viability; as a result, the AAP on micropropagated sources had to be limited to 3 days, and all *in vitro* plants wilted within the 3rd day, preventing a reliable molecular quantification of their phytoplasma titers.

Following AAP, all insects were transferred to healthy apple plants for a 14-day LP. At the end of the LP, groups of 10–20 adults were moved onto new healthy apple seedlings for a single, prolonged IAP, during which they were left to feed until natural death, in order to maximize the time window for potential inoculation. Cages were checked daily, and dead insects were collected and stored

individually at –20 °C. For a subset of individuals (coming from AAP on trees in Piovi orchard), an additional artificial feeding assay (Gonella et al., 2015) was performed after the LP and before placement onto IAP plants. Briefly, individual insects were placed for three days in modified 2 mL Eppendorf tubes (base removed) serving as feeding chambers. Tube caps were filled with 300 µL of sterile sugar solution (5% sucrose in TE buffer, pH 8) and sealed with Parafilm. Cotton was inserted for ventilation and humidity maintenance, and a yellow sticky film was positioned externally to stimulate feeding behavior.

Inoculated plants used in 2022 and 2023 were maintained in a screenhouse and monitored for symptom development until two years after inoculation. Visual assessment of symptoms and molecular testing of the root were performed twice to verify phytoplasma transmission.

3.2.4 Infection dynamics experiment

To further characterize *O. ishidae*-phytoplasma interactions and assess the potential for pathogen multiplication within the vector, a dedicated experiment was conducted to monitor temporal changes in *'Ca.* P. mali' infection dynamics following controlled acquisition. Third- to fifth-instar nymphs were collected from an apple orchard (Mollaro, Trentino, Italy, 46°17′18.8″N, 11°04′00.4″). Immediately after collection, a subset of insects was frozen at –20 °C to determine the natural field infection rate and phytoplasma titer by molecular assays. The remaining individuals were transferred onto *'Ca.* P. mali'-infected potted apple-plants for a 5-day AAP. The potted plants were infected by AT2 subtype and showed a mean phytoplasma titer of 0.063±0.23 (ΔCt values means ± SD). After the AAP, another subset of insects was collected and frozen to assess post-acquisition infection rates, while the remaining nymphs were placed onto healthy apple plants for LPs of 5, 10, or 15 days to allow potential pathogen replication and systemic infection. At the end of each LP interval, a portion

of insects was collected and stored at –20 °C for subsequent molecular analysis to evaluate temporal changes in phytoplasma concentration.

3.2.5 Molecular analyses of insect and artificial diet

Subsequent to the transmission trials, *O. ishidae* individuals and sugar diets for molecular analyses were submitted to total DNA isolation. Total genomic DNA was extracted from single specimens using the commercial kit NucleoSpin Tissue (Macherey-Nagel GmbH and Co., KG, 52355 Düren, Germany), while nucleic acid extraction of artificial diet was performed by sodium dodecyl sulfate-proteinase K-cethyltrimethyl ammonium bromide treatment (Gonella et al., 2012).

A duplex TaqMan real-time PCR assay was used with primer/probe sets amplifying the 16S rRNA gene of *'Ca.* P. mali' (Baric, 2012; Baric and Dalla-Via, 2004) and the 18S rDNA gene of Hemiptera as an internal positive reference (Marzachí and Bosco, 2005). The concentration of primers qAP-16S-F and qAP-16S-R was 0.9 µM, the concentration of primers MqFw and MqRv was 0.2 µM, and the concentration of the two probes was 0.2 µM. Each sample was tested in duplicate in a total of 10 µL reaction volume containing 1 µL template DNA and 2× Kapa Probe Fast qPCR Kit (Kapa Biosystems). PCR conditions were as follows: an initial denaturation step at 95 °C for 3 min followed by 50 cycles of 95 °C for 3 s and 60 °C for 20 s -(LightCycler® 480 Thermal Block Cycler Unit; Roche). The quantification of *'Ca.* P. mali' was performed using a relative quantification approach based on the comparative threshold cycle (ΔCt) method (Baric, 2012; Marzachí and Bosco, 2005). For each extracted insect DNA sample, the relative amount of phytoplasma DNA with respect to host insect DNA was calculated as the difference between the threshold cycle (Ct) values of the two target genes (ΔCt), using the following formula: $\Delta Ct = Ct(AP\text{-}16S) - Ct(\text{insect 18S rDNA})$ (Baric, 2012).

3.2.6 Molecular analyses of plants

Phloem tissue was isolated from leaves to test acquisition plants, while in the case of the plant used for inoculation, we used root parts of each plant, mixed and processed by lyophilization and mechanical disruption to obtain mixed powders. Total DNA was extracted with the -NucleoSpin® Plant II (Macherey–Nagel GmbH and Co. KG) commercial kit, following the manufacturer's instructions.

For the analysis, a duplex TaqMan real-time PCR assay was used with primer/probe sets amplifying the 16S rRNA gene of *'Ca.* P. mali' (Baric and Dalla-Via, 2004) and the chloroplast DNA gene of *Malus domestica* 1-aminocyclopropane-1-carboxylate oxidase (ACO) as an internal positive reference (Baric et al., 2011). The concentration of primers qAP-16S-F and qAP-16S-R was 0.9 µM, the concentration of primers qMd-ACO-F and qMd-ACO-R was 0.2 µM, and the concentration of the two probes was 0.2 µM. Each sample was tested in duplicate in a total of 10 µL reaction volume containing 1 µL template DNA and 2× Kapa Probe Fast qPCR Kit (Kapa Biosystems). PCR conditions were as follows: an initial denaturation step at 95 °C for 3 min followed by 50 cycles of 95 °C for 3 s and 60 °C for 20 s -(LightCycler® 480 Thermal Block Cycler Unit; Roche).

The identity of the '*Ca.* P. mali' subtypes used in this study was confirmed by PCR-RFLP with primers AP10-AP13 (Jarausch et al., 2000). PCR reactions were carried out in a total volume of 25 µL containing 3 µL of template DNA, 2× PCRBIO VeriFi^{TM} Mix (Resnova), and 0.70 µL of each primer (10 µM). Thermal cycling conditions were as follows: initial denaturation at 95 °C for 2 m; 40 cycles of 95 °C for 15 s, 58 °C for 15 s, and 72 °C for 15 s; followed by a final extension at 72 °C for 5 min. Nine µL of the PCR products were digested with 2 µL of the restriction enzymes PagI and HincII (Thermo Fisher Scientific, Waltham, MA, USA), 2 µL of 10× buffer, and 7 µL of Milli-Q water at 37 °C for 1 hour, following the manufacturer's instructions. The digested PCR products were

separated by electrophoresis on a 2% agarose gel containing ethidium bromide in 0.5× TAE buffer, alongside a 100 bp DNA ladder (GeneRuler 100 bp, Thermo Fisher Scientific, Waltham, MA, USA), and visualized using a GelDoc transilluminator (Bio-Rad).

3.2.7 Statistical analysis

All statistical analyses were performed using R version 4.3.0 (R Core Team, 2023). Infection rates of *O. ishidae* were compared using Fisher's exact test for count data, with pairwise comparisons adjusted using the Holm method to control for multiple testing. Phytoplasma titers in insect bodies (ΔCt values) were compared across experimental phases using the Kruskal-Wallis rank sum test, as data did not meet parametric assumptions. Post-hoc pairwise comparisons were conducted using Wilcoxon rank sum tests with Holm correction. For all analyses, statistical significance was set at $p = 0.05$.

3.3 Results

The transmission experiment conducted in 2022 involved 14 experimental groups of *O. ishidae* nymphs exposed to naturally infected apple plants (**Table 3**). Of the 280 individuals initially exposed, 204 survived the LP phase. Across all groups and IAP periods, 4.5% of tested individuals (8/174) were positive for *'Ca.* P. mali' by qPCR (**Table 3**). Positive insects were detected across different groups and IAP periods: group 6 showed infections in both IAP I (n=2) and IAP II (n=1), groups 8, 10, and 11 each yielded a single positive individual during IAP I, while groups 5 and 14 each had one positive individual during IAP II. No insects tested positive during IAP III.

Table 3: Results of the preliminary transmission trials conducted in 2022. Groups 1–14 represent cohorts of *O. ishidae* nymphs exposed to '*Ca.* P. mali'-infected apple plants. For each experimental phase—Acquisition Access Period (AAP), Latency Period (LP), and successive Inoculation Access Periods (IAP I–III)—the table reports the number of surviving insects (N) and the number of phytoplasma-positive individuals relative to those tested (PCR^+/tested).

	Experimental phase							
			IAP I		IAP II		IAP III	
	APP	**LP**	*O.ishidae*		*O.ishidae*		*O.ishidae*	
Group	N	N	N	**PCR+/tested**	N	**PCR+/tested**	N	**PCR+/tested**
1	25	20	13	0/2	11	0/2	9	0/9
2	25	16	16	0/0	16	0/3	13	0/13
3	25	19	17	0/2	15	0/5	10	0/10
4	25	20	20	0/5	15	0/5	10	0/10
5	25	24	24	0/0	24	1/8	16	0/16
6	15	15	15	2/5	10	1/5	5	0/5
7	25	17	15	0/3	12	0/7	5	0/5
8	15	12	5	1/1	4	0/3	1	0/1
9	25	16	15	0/1	14	0/2	12	0/12
10	15	8	4	1/4	0	Nt*	0	Nt*
11	15	5	2	1/2	0	Nt*	0	Nt*
12	15	11	10	0/1	9	0/3	6	0/6
13	15	11	11	0/0	11	0/1	10	0/10
14	15	10	7	0/3	4	1/3	1	0/1

*Nt, not tested due to complete insect mortality.

In 2023, -AP-positive *O. ishidae* were detected in all treatment combinations (subtype × AAP duration in AAP plant) (**Figure 11**). Among insects that had undergone a 3-day AAP on in vitro plants, 12.2% (5/41) of AT1-exposed individuals and 7.1% (4/56) of AT2-exposed individuals tested positive for *'Ca.* P. mali' at the end of the LP/IAP sequence. For insects that had experienced a 10-day AAP on potted and field-grown source plants, infection frequencies increased to 22.5% (9/40) for AT1 and 21.7% (23/106) for AT2. A global Fisher's exact test indicated a significant effect of AAP duration on the probability of detecting phytoplasma-positive adults after LP and IAP ($p = 0.0136$), with insects that had fed for 10 days being approximately 2.7 times more likely to be infected than those exposed for 3 days (odds ratio = 2.73; 95% CI: 1.20–6.86). Conversely, no significant difference was found between the two *'Ca.* P. mali' subtypes ($p = 1.00$), indicating comparable acquisition and retention efficiencies for AT1 and AT2 under the tested conditions.

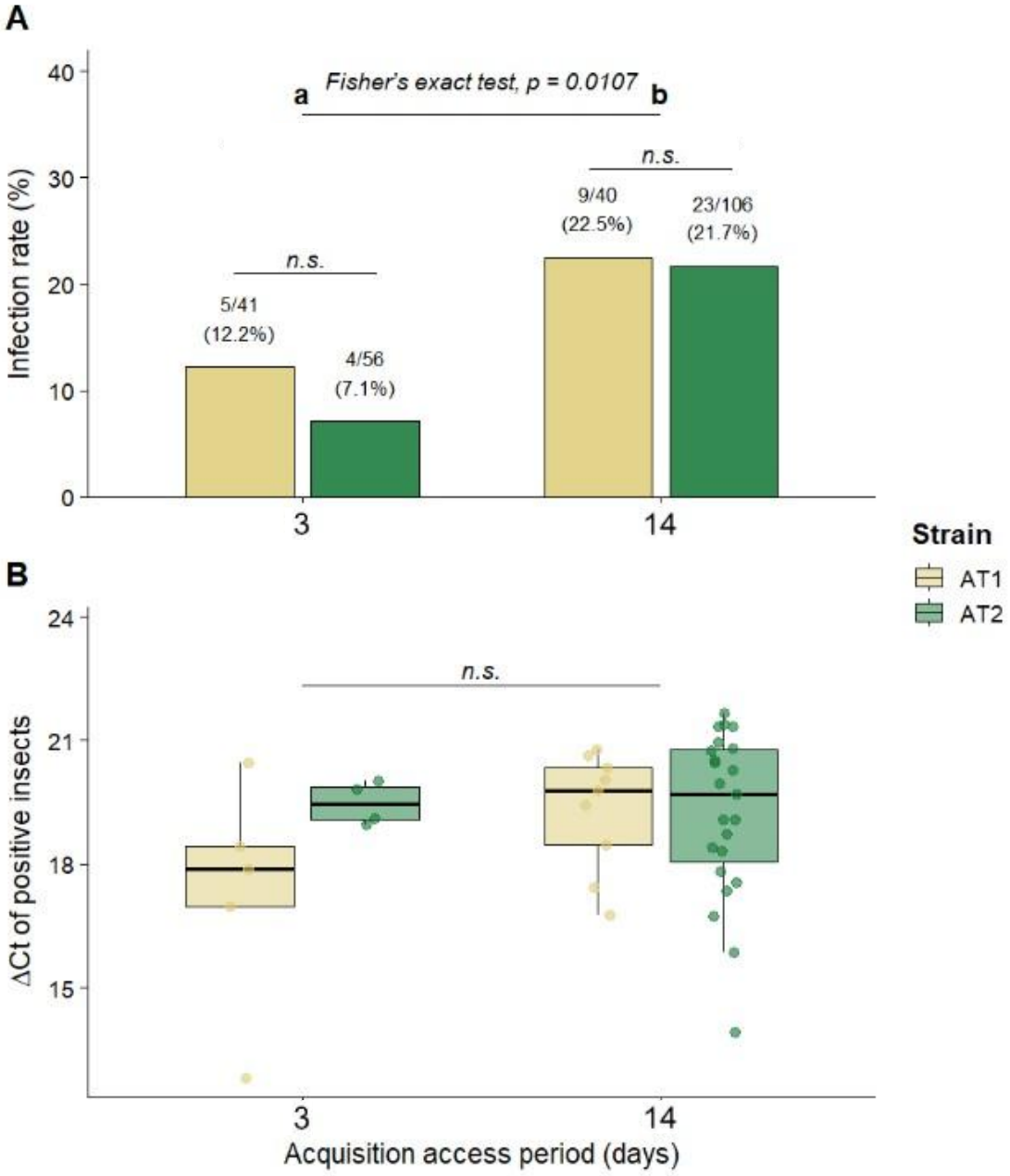

Figure 11: Infection rate of *O. ishidae* (panel A) and phytoplasma relative concentration in positive insects (panel B) after the end of the IAP across different acquisition access periods (AAPs) and different subtypes (AT1 and AT2). Different letters indicate significant differences between categories according to Fisher's exact test with Holm-adjusted pairwise comparisons (panel A) and Kruskal-Wallis test(panel B).

Phytoplasma titers in source plants, expressed as qPCR ΔCt values, did not correlate with the infection outcome in insects (Spearman's $\rho = -0.015$; $p = 0.854$; **Figure 12**), suggesting that

variability in plant phytoplasma concentration within the tested range did not markedly influence the probability of detecting infected individuals at the end of LP/IAP. Among positive insects, within-vector phytoplasma titers showed moderate variation but no significant effects of either subtype (Kruskal–Wallis, $\chi^2 = 0.95$, df = 1, p = 0.329) or AAP duration ($\chi^2 = 1.55$, df = 1, p = 0.213). Mean ΔCt values ranged from 17.3 ± 2.8 in AT1 after a 3-day AAP to 19.2 ± 2.0 in AT2 after a 10-day AAP, indicating similar phytoplasma loads across all treatment combinations (**Figure 11**).

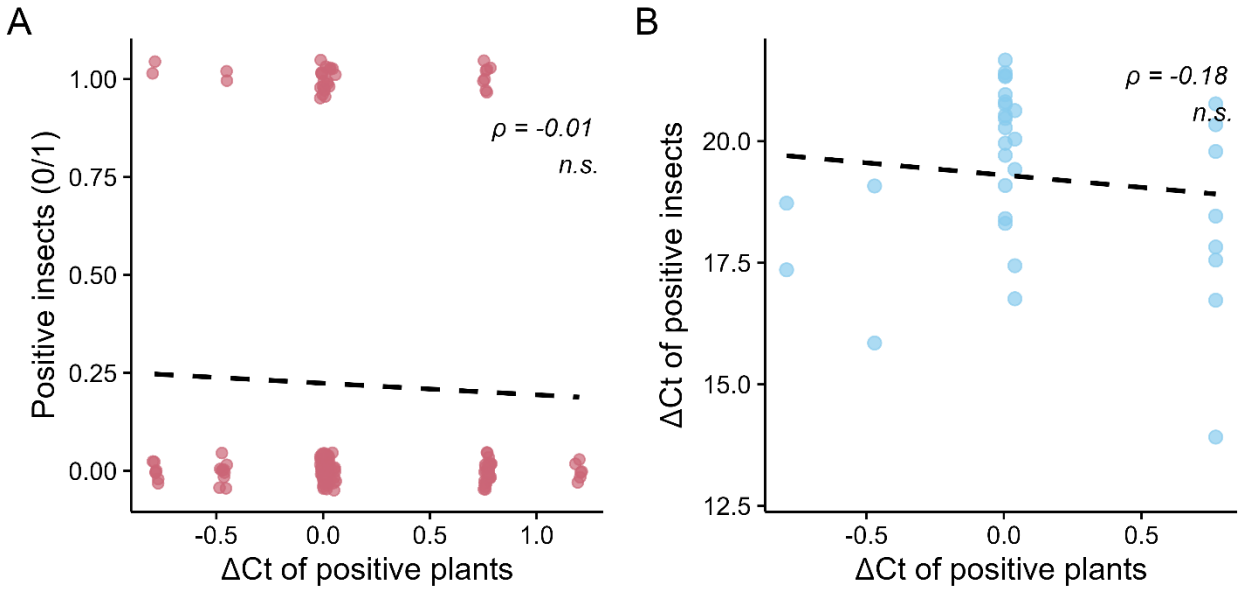

Figure 12 Relationship between phytoplasma titer in AAP plants and insect infection status (negative 0 and positive 1, panel A) and association between phytoplasma titer in AAP plants and relative phytoplasma titers (ΔCt) in positive insects (panel B). Points represent individual insects; the dashed line indicates a linear fit. Spearman's rank correlation coefficient (ρ) and corresponding p-value are shown.

Despite consistent detection of phytoplasma DNA in insects following acquisition trials, no successful transmission to test plants was observed in either experimental year. All inoculated plants were analyzed by qPCR in December of the same year and again during the following winter and no detections of *'Ca.* P. mali' were recorded (**Table 4**). Similarly, all artificial-diet assays carried out in 2023, tested immediately after IAP, yielded negative results (**Table 4**).

Table 4: Molecular detection of '*Ca*. P. mali' in test plants following Inoculation Access Periods (IAP). Plants were analyzed by qPCR during two consecutive winters following each transmission experiment. Tot., total number of plants tested; AP-infected, number of plants testing positive for '*Ca.* P. mali'.

	Analysis winter 2022		Analysis winter 2023	
Experiments	Tot.	AP- infected	Tot.	AP- infected
2022 IAP I	14	0	14	0
2022 IAP II	14	0	14	0
2022 IAP III	14	0	14	0
	Analysis winter 2023		Analysis winter 2024	
2023 IAP	36	0	36	0
2023 IAP artificial diet	65	0	/	/

The final experiment investigated temporal changes in *'Ca.* P. mali' infection within *O. ishidae* following controlled acquisition (**Figure 13**). Before exposure, field-collected adults showed a baseline infection rate of 16.7% (3/18). After the 5-day AAP, the infection rate markedly increased up to 85% (34/40), a significantly higher proportion than in the field group (Fisher's exact test with Holm-adjusted pairwise comparisons, $p < 0.001$) (**Figure 13**). During the latency period, the proportion of infected insects progressively declined, dropping to 44.4% after 5 days of LP (16/36) and to 17.5% after 10 days (7/40). No significant difference was detected between field, 5-day and 10-day LP groups ($p < 0.001$). After 15 days of LP, no insects tested positive (0/42) (**Figure 13**).

Phytoplasma quantification in positive insects revealed a pattern consistent with the observed infection dynamics. Field-collected adults displayed a mean ΔCt of 17.8 ± 2.9, whereas immediately after the 5-day AAP the mean ΔCt decreased to 15.9 ± 1,9, corresponding to the phase with the highest phytoplasma levels detected in the experiment. During the latency period, mean ΔCt values then increased to 18.9 ± 1.8 after 5 days of LP and to 19.4 ± 3.0 after 10 days of LP, indicating a progressive decline in phytoplasma levels over time. Overall differences among phases were significant (Kruskal–Wallis test: $\chi^2 = 32.2$, df = 3, $p < 0.001$). Holm-adjusted Wilcoxon pairwise comparisons showed that ΔCt values were significantly higher in both LP groups than in the AAP group (AAP vs. 5LP, p = 2.2

$\times 10^{-7}$; AAP vs. 10LP, $p = 2.0 \times 10^{-5}$), whereas no significant difference was detected between 5LP and 10LP ($p = 1.00$) (**Figure 13**).

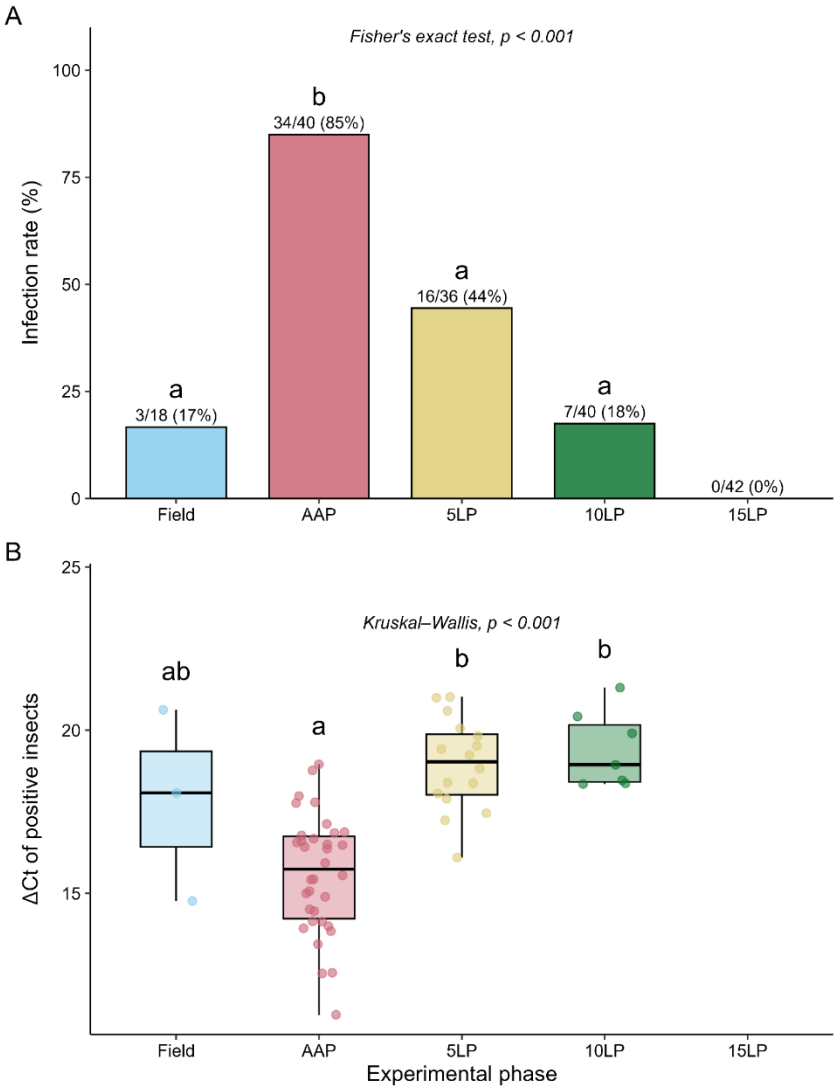

Figure 13: Temporal dynamics of '*Ca*. P. mali' infection rate (panel A) and relative phytoplasma titers (ΔCt values) in *O. ishidae* (panel B). Different letters indicate significant differences between categories according to Fisher's exact test with Holm-adjusted pairwise comparisons (panel A) and followed by Wilcoxon rank sum test with Holm correction (panel B). Categories indicate the sampling time: Filed (leafhopper collected in the field) AAP (leafhopper sampled immediately after a 5-day acquisition access period) and LP5/LP10/LP15 (leafhopper collected after 5, 10, and 15 days of latency, respectively).

3.4 Discussion

The transmission trials conducted between 2022 and 2024 consistently demonstrate that *O. ishidae* is not a competent vector of '*Ca.* P. mali' under the tested conditions. Despite successful phytoplasma acquisition by a proportion of exposed insects, as confirmed by qPCR detection, no transmission to test plants occurred across any experimental year, inoculum source, or AP subtype. In 2022, 8.3% of post-LP insects tested positive for '*Ca.* P. mali', yet all 42 IAP plants across three sequential inoculation periods remained uninfected. Similarly, in 2023, acquisition rates reached 22,5% following a 14-day AAP with no differences between tested subtypes (AT1 and AT2), but again, no IAP plants became infected despite prolonged IAP exposure. This consistent failure to transmit, despite detectable phytoplasma presence in vectors, distinguishes *O. ishidae* from proven AP vectors and suggests fundamental incompatibility between this leafhopper species and '*Ca.* P. mali'.

The 2022 experimental design replicated the protocol used by Tedeschi and Alma (2006) to demonstrate the vector competence of *F. florii* for '*Ca.* P. mali'. Sequential IAPs revealed a successful transmission by *F. florii* beginning with the first inoculation period and continuing throughout all the IAPs, with increasing numbers of positive insects detected in later periods, a pattern consistent with progressive pathogen multiplication and maturation of infectivity within vectors. Under identical experimental conditions, *O. ishidae* showed the inverse pattern: positive insects were detected only sporadically in the first two IAPs with no positive individual in the last IAP, and critically, no test plants became infected. This direct experimental comparison under standardized conditions was a first indication that *O. ishidae* cannot be a competent vector.

The 2023 experiment was designed to assess whether any biological or experimental factors known to influence AP transmission in competent vectors could enable *O. ishidae* to transmit *'Ca.* P.

mali'. In psyllids such as *C. melanoneura*, transmission efficiency is affected by phytoplasma subtype, source-plant titer, vector genetic background, and the duration of acquisition and latency periods (Corretto et al., 2024; Malagnini et al., 2013; Malagnini et al., 2010). We therefore tested the two major regional phytoplasma subtypes, AT1 and AT2. Despite their known differences in epidemiological behaviors and vector association (Baric et al., 2011; Corretto et al., 2024), the two subtype produced similar outcomes in *O. ishidae*: comparable acquisition rates and complete absence of transmission in both cases. Although additional subtypes exist (e.g, the AP subtype, Jarausch et al., 2000), AT1 and AT2 represent the epidemiologically relevant ones in Trentino (Cainelli et al., 2004; Martini et al., 2008), and the identical failure with both subtypes suggests that the lack of transmission reflects a general incompatibility rather than subtype-specific effects. We also tested different AAP durations. The 3- and 10-day AAPs used here match or exceed the feeding periods required for efficient acquisition in confirmed AP vectors, such as 4-day AAP for psyllid or 10 day for *F. florii* (Candian et al., 2020; Pedrazzoli et al., 2007; Tedeschi and Alma, 2006). Longer AAPs significantly increased *O. ishidae* infection rates, confirming that extended feeding enhances acquisition. However, higher acquisition did not result in higher phytoplasma titers within insects, nor in any successful transmission. Combined with the infection-dynamics results, showing rapid phytoplasma decline during the latency period, these findings indicate that even longer exposure times would be unlikely to permit transmission. Assessment of source-plant titers revealed high infection levels across all plants ($\Delta Ct \leq 0.42$), yet no correlation was found between source plant titer and acquisition probability or subsequent within-vector titer.

While providing robust data for the region, a potential limitation of this study is its focus on a single *O. ishidae* population from Trentino. Genetic studies have shown that multiple haplotypes of *O. ishidae* occur across Europe (Blaser, 2019), raising the possibility that population-level genetic variability could influence the compatibility with *'Ca.* P. mali'. This question is particularly relevant

given that, in *C. melanoneura*, lineage has been demonstrated to affect transmission efficiency (Corretto et al., 2024). Beyond host genetics, inter-population differences in the insect-associated microbiome (including endosymbionts) have also been proposed as potential modulators of phytoplasma vector competence (Schuler et al., 2022). Although testing additional *O. ishidae* populations was beyond the scope of the present work, future comparative studies would help clarify whether haplotype diversity plays any role in shaping vector–pathogen interactions.

The 2024 infection-dynamics experiment provides additional insight into the nature of *O. ishidae–'Ca.* P. mali' incompatibility. In persistent-propagative phytoplasma–vector associations, successful colonization follows a characteristic temporal pattern: following acquisition, the pathogen crosses the midgut barrier, enters the hemocoel, replicates in various tissues, and eventually colonizes the salivary glands, leading to progressive increases in pathogen titer during the LP (Pagliari et al., 2019). For example, Koinuma et al. (2020) demonstrated that phytoplasma loads in the competent vector *Macrosteles striifrons* (Anufriev) increased steadily between 7 and 28 days post-acquisition, with approximately 10-fold increments per week, before stabilizing at high titers compatible with efficient transmission. *Orientus ishidae* exhibited the opposite temporal pattern. The highest infection rate occurred immediately following the 5-day AAP, after which both prevalence and relative phytoplasma concentration declined monotonically, reaching 0% at 15 days LP. This progressive decline strongly suggests that '*Ca.* P. mali' does not replicate efficiently within *O. ishidae* tissues and instead resembles passive ingestion of phytoplasma-containing phloem sap without subsequent systemic colonization. Phytoplasma quantification further supports this interpretation, though cross-study comparisons must be interpreted cautiously given methodological differences in qPCR assays, target genes, and normalization approaches. In our study, ΔCt values in positive *O. ishidae* ranged consistently between 14 and 20, indicating very low relative phytoplasma concentrations. By

comparison, Oppedisano et al. (2020) reported relative '*Ca.* P. mali' loads in competent vectors *C. melanoneura* and *C. picta* ranging between 0.022-0.5. Studies on other pathosystems provide additional context: infected *Macrosteles quadripunctulatus* (Kirschbaum) leafhoppers transmitting chrysanthemum yellows exhibited ΔCt values around 2.5-3.5 (Marzachí and Bosco, 2005), far below the values observed in *O. ishidae*.

The biological mechanisms underlying phytoplasma incompatibility remain unclear and represent a complex phenomenon, involving phytoplasma effectors and interaction with host cells (Hogenhout et al., 2008). Recent work on phytoplasma–vector interactions suggests that in non-vector insects, phytoplasma effectors fail either to recognize epithelial receptors or to suppress key immune responses (Galetto et al., 2018). Immunodominant membrane proteins (IDP), such as Amp and Imp, act as adhesins that mediate highly specific attachment and internalization at the midgut and salivary gland epithelia (Boonrod et al., 2012). For 16SrX phytoplasmas, including *'Ca.* P. mali', Imp has been characterized as an adhesin that binds host actin (Boonrod et al., 2012), and in the flavescence dorée pathosystem, Imp interacts with a narrow set of natterin-like and legumain gut proteins. RNAi silencing of these putative receptors in vector species causes a drastic reduction in phytoplasma multiplication, underscoring how a precise epithelial receptor repertoire is required for persistent colonization (Galetto et al., 2025). In parallel, recent work on rice orange leaf phytoplasma has shown that specific effectors can actively suppress the phenoloxidase-dependent melanization cascade in the hemolymph to permit persistent infection. When this immune response is not efficiently blocked, melanization sequesters and eliminates phytoplasma cells (Zhang et al., 2025). The failure of *'Ca.* P. mali' to establish a propagative infection in *O. ishidae* could reflect the lack of compatible receptors in the midgut and/or a more effective innate immune response that clears the phytoplasma before it reaches the salivary glands.

The apparent incompetence of *O. ishidae* for '*Ca.* P. mali' contrasts with its demonstrated efficiency in transmitting other phytoplasmas. Multiple studies confirm *O. ishidae* as a competent vector for flavescence dorée phytoplasmas (16SrV group), with particularly high transmission efficiency between wild hosts (alder to alder) and low efficiency to grapevine (Jarausch et al., 2023; Lessio et al., 2016; Malembic-Maher et al., 2020). Additionally, *O. ishidae* transmits '*Ca.* P. pruni' (X-disease) to peach in North America (Rosenberger and Jones, 1978). This contrast, high competence for certain phytoplasmas but incompetence for '*Ca.* P. mali', exemplifies pathosystem-specific co-adaptation between phytoplasma subtypes and arthropod vectors (Trivellone and Dietrich, 2021). Within this experimental framework, *O. ishidae* can be considered to behave as a dead-end host for '*Ca.* P. mali' in apple orchards in Trentino. Similar dead-end interactions have been documented within the AP system. Cainelli et al. (2007) detected '*Ca. P. mali'* DNA in three apple aphid species, *Aphis pomi* de Geer, *Dysaphis plantaginea* (Passerini), *Eriosoma lanigerum* (Hausmann), in field surveys, but controlled transmission trials demonstrated that these aphids cannot transmit the phytoplasma. Our data extend this concept to *O. ishidae*, reinforcing that sporadic molecular detection in field-collected individuals should not be interpreted as evidence of vector competence without supporting transmission data (Fischnaller et al., 2020).

The exclusion of *O. ishidae* as an epidemiologically relevant AP vector clarifies the interpretation of the recent AP outbreak in Trentino. The temporal coincidence of rising *O. ishidae* populations with AP epidemics during periods of low *C. picta* and *C. melanoneura* abundance initially suggested a potential involvement of this leafhopper in transmission, but our experimental evidence excludes this hypothesis. The apparent discrepancy between low documented psyllid populations and elevated AP incidence could reflect multiple factors: (i) even small psyllid populations can drive significant disease spread (Oppedisano et al., 2020);(ii) root grafts between adjacent trees can facilitate

direct phytoplasma transfer independent of aerial vectors (Ciccotti et al., 2008; Lešnik et al., 2008); (iii) infected propagation material introduces inoculum manifesting as symptomatic trees independent of current-season vector activity (Janik et al., 2020); (iv) temporal disconnect between infection and symptom expression may reflect infections from previous seasons when psyllid populations were higher (Baric et al., 2007) and (v) current monitoring methods (beating trays) may underestimate psyllid populations (Barthel et al., 2020a; Fischnaller et al., 2017). Additionally, subtype interactions and competition may create apparent fluctuations in disease incidence unrelated to transmission rate (Schneider et al., 2014).

Conclusions

Multiple lines of evidence demonstrate that *O. ishidae* is not a competent vector of '*Ca.* P. mali': absence of transmission across inoculated plants, despite detectable acquisition, consistently low phytoplasma titers near detection thresholds, and temporal pattern of pathogen decline during LP rather than multiplication. From a management perspective, *O. ishidae* should be monitored primarily as a vector of Flavescence dorée affecting grapevine rather than as an AP vector. Control programs for AP should remain focused on confirmed psyllid vectors *C. picta* and *C. melanoneura*, with integrated strategies emphasizing psyllid population management, removal of infected trees, and use of certified propagation material.

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CHAPTER 4. DISPERSION AND DIFFUSION

Apple orchards as demographic sources for *Orientus ishidae* (Matsumura, 1902) (Hemiptera: Cicadellidae): host suitability, movement, and implications for Flavescence dorée epidemiology

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In preparation

Abstract

Flavescence dorée (FD) is a major quarantine disease of grapevine in Europe transmitted by the leafhopper *Scaphoideus titanus*. However, alternative leafhopper species, including the invasive mosaic leafhopper *Orientus ishidae*, have been implicated as secondary or bridge vectors. In recent years, unusually high populations of *O. ishidae* have been recorded in apple orchards in northern Italy, raising concerns that cultivated non-grape hosts may contribute to FD risk in mixed fruit-growing landscapes.

We investigated the distribution, host suitability, and movement of *O. ishidae* using multi-regional surveys in apple orchards, laboratory assays on cultivated and wild host plants, and field experiments at apple–vineyard interfaces. Across northern Italy (2022–2023), *O. ishidae* was consistently detected in apple orchards, with mean weekly trap catches up to 8.4 individuals per trap in organic systems, while captures in integrated pest management orchards were typically close to zero. Survival analyses demonstrated strong host-dependent constraints. Apple, hazel, and hornbeam supported complete nymphal development, with mean development times of 25–30 days and median adult longevity of 23–28 days. In contrast, cultivated grapevine did not support development: no nymphs reached

adulthood, and adult median survival on grapevine cultivars was limited to 1–3 days, irrespective of cultivar. Despite high orchard populations, field experiments revealed negligible establishment of *O. ishidae* within adjacent vineyards. Model-based analyses showed an order-of-magnitude reduction in predicted abundance in vineyards relative to apple orchards, with no effect of distance from apple trees. These results demonstrate that apple orchards act as demographic sources for *O. ishidae*, but that host plant unsuitability strongly constrains population spillover into vineyards. Consequently, while *O. ishidae* may occasionally introduce FD phytoplasmas into vineyards, it is unlikely to sustain populations or drive epidemic spread. Integrating host suitability and crop configuration is therefore essential for accurate FD risk assessment in mixed fruit-growing landscapes.

4.1 Introduction

Grapevine flavescence dorée (FD) is a quarantine disease causing major economic losses in European viticulture (EPPO, 2025; Gonella et al., 2024; Tramontini et al., 2020). The disease is associated with phloem-limited phytoplasmas belonging to ribosomal subgroups 16SrV-C and 16SrV-D (Filippin et al., 2009; Lee et al., 2004; Malembic-Maher et al., 2020; Martini et al., 2007) and is transmitted in vineyards primarily by the Nearctic leafhopper *Scaphoideus titanus* (Gonella et al., 2024). Because infected vines cannot be cured, management relies on roguing, insecticide treatments targeting *S. titanus* and strict regulation of planting material, yet epidemic outbreaks continue to occur in several regions (Oliveira et al., 2019).

Initially FD was regarded as a vineyard-restricted pathosystem involving only grapevine and *S. titanus*. It is now established that FD phytoplasmas 16SrV-C and 16SrV-D are of European origin and widely distributed in wild reservoirs particularly *Alnus glutinosa* in Western Europe and *Clematis vitalba* in Eastern Europe (Malembic-Maher et al., 2020). FD phytoplasmas can be further subdivided into epidemiologically informative clusters based on sequence variation in the housekeeping gene

map, with more than 170 genotypes currently described (Malembic-Maher et al., 2020). Clusters MAP-FD1 (dominant genotype M50), MAP-FD2 (dominant genotypes M38 and M54) and MAP-FD3 (dominant genotype M51) are associated with vineyard epidemics across different European regions. In contrast, a large group of genotypes collectively referred to as Alder Yellows (AldY) circulates in *Alnus glutinosa* and is transmitted by *Oncopsis alni*, but cannot be spread to grapevine by *S. titanus* (Malembic-Maher et al., 2020).

Within this multi-host system, several Auchenorrhyncha species besides *S. titanus* can acquire and transmit FD phytoplasmas, including *Dictyophara europaea* and species of *Allygus* (Filippin et al., 2009; Malembic-Maher et al., 2020). Among these, *Orientus ishidae* Matsumura has recently emerged as an epidemiologically relevant species (Gaffuri et al., 2011; Jermini et al., 2019; Koczor et al., 2013; Lessio et al., 2016; Mehle et al., 2011b; Oggier et al., 2024; Rizzoli et al., 2021). *Orientus ishidae* is an invasive Asian leafhopper first detected in Europe in the late 1990s (Guglielmino, 2005) and now established throughout the continent (EPPO, 2025b). It is highly polyphagous on deciduous trees and shrubs and can acquire and transmit 16SrV phytoplasmas, including FD phytoplasmas, to grapevine, although with lower efficiency than *S. titanus* (Lessio et al., 2016; Malembic-Maher et al., 2020). In Germany, transmission experiments and field surveys demonstrated that *O. ishidae* can carry high phytoplasma titers and transmit MAP-FD2 genotypes, particularly M38, from alder to grapevine (Jarausch et al., 2023), leading to the first documented alder-to-vine transmission in a previously FD-free region (Jarausch et al., 2021). These findings highlight its role as a bridge vector connecting wild reservoirs and vineyards, especially where *S. titanus* is newly established or still rare (Jarausch et al., 2025).

Although *O. ishidae* presents epidemiological potential, it generally remains uncommon within vineyards (Guerrieri et al., 2023; Lessio et al., 2019; Oggier et al., 2023; Prazaru et al., 2025;

Rizzoli et al., 2021), whereas higher populations are often observed on nearby woodlands characterized by plant species such as *Corylus avellana, Acer spp., Alnus glutinosa, Salix spp., and Carpinus betulus* (Desqué et al., 2019; Lessio et al., 2016; Oggier et al., 2023; Parise, 2017; Valley and Wheeler, 1985). However, since 2019, unusually high *O. ishidae* densities have been recorded in apple orchards (*Malus domestica*) in Trentino, north-eastern Italy, whereas vineyards in the same landscapes have consistently yielded low captures (Baldessari et al., 2020; Gelmetti et al., 2021). Whether these abundant orchard populations represent a broader ecological shift or a geographically restricted phenomenon remains unclear. In neighboring South Tyrol region (North east Italy), extensive Auchenorrhyncha surveys detected only 4 *O. ishidae* individuals in apple orchards between 2014-2016 (Fischnaller et al., 2020), suggesting that high densities are not ubiquitous across apple-growing regions. Information from other Italian regions is still lacking, and it is unknown whether *O. ishidae* is expanding, stable at low prevalence, or sporadically present in apple orchards.

This discrepancy raises fundamental questions about the relative importance of host-plant quality and local management. Apple may differ intrinsically from grapevine in its suitability as a host, or, alternatively, contrasting pest-control management, such as mandatory insecticide treatments targeting *S. titanus* in vineyards may strongly affect *O. ishidae* survival and abundance independently of host suitability (Lessio et al., 2016). Within grapevine itself, cultivar identity can markedly influence vector fitness: for *S. titanus*, fitness was highest on ‘Barbera’ and significantly lower on ‘Moscato’ and ‘Nebbiolo’ (Ripamonti et al., 2022), suggesting that similar intraspecific variation may also apply to *O. ishidae* on different *V. vinifera* cultivars.

If apple orchards function as highly suitable hosts, they may act as previously unrecognised demographic sources from which large *O. ishidae* populations, potentially carrying FDp acquired on wild hosts, can disperse into neighboring vineyards. Landscape studies have already shown that

proximity to wild reservoirs, hedgerows and unmanaged vegetation influences FD risk (Lessio et al., 2006; Pavan et al., 2012; Eveillard et al., 2016; Jarausch et al., 2025; Oggier et al., 2025). Yet the role of cultivated non-grape hosts, particularly apple orchards in mixed fruit-growing regions where vineyards and orchards are interspersed, has never been assessed. Determining whether apple orchards act as demographic sources or sinks for *O. ishidae*, and whether orchard–vineyard interfaces represent hotspots for vector movement and FDp transmission, is therefore essential for understanding FD epidemiology in these landscapes.

In this context, our study had three specific objectives. First, we quantified the presence and seasonal dynamics of *O. ishidae* in apple orchards across several northern Italian regions, comparing organic and integrated production systems, to assess how frequently apple orchards host substantial populations. Second, we compared survival and development of *O. ishidae* on apple, grapevine, wild hosts, and among grapevine cultivars, in order to clarify the host status of apple and *V. vinifera* in terms of basic fitness parameters. Third, we evaluated how the presence of adjacent apple trees influences *O. ishidae* abundance at the apple–vineyard interface and along transects into vineyards, as a first step towards assessing whether cultivated hosts at vineyard borders may contribute to FD risk in mixed fruit-growing landscapes.

4.2 Materials and methods

4.2.1 Study on diffusion and population levels in apple orchards

During the summers 2022 and 2023, a survey was conducted to assess the presence and abundance of *O. ishidae* in apple orchards across several northern Italian regions, Trentino–Alto Adige, Friuli Venezia Giulia, Lombardia, Piemonte, Veneto (2022 only), Emilia–Romagna (2023 only), and Slovenia (2023) (**Figure S19**). In each region, four organic and two IPM orchards were

selected. Three yellow sticky traps (YST; 40 × 75 cm, Glutor®, Biogard) were installed along the orchard diagonal: one centrally and two at opposite borders, positioned at approximately 1.5 m above ground on the median wire of the training system. Traps were replaced every two weeks from mid-July to late September, stored at –20 °C, and later examined under a stereomicroscope for species identification.

4.2.2 Nymphal survival and development

The survival and development of *O. ishidae* were assessed on four host plants representing the main cultivated and wild woody resources available in Trentino agroecosystems: apple (*Malus domestica*, cv. Golden Delicious), grapevine (*Vitis vinifera*, cv. Bronner), hazel (*Corylus avellana*), and hornbeam (*Carpinus betulus*). Apple was included as the focal crop because stable populations have been observed in orchards, grapevine because of its epidemiological relevance to FD , and hazel and hornbeam as widespread woody plants commonly occurring along orchard and vineyard margins; hazel was also considered because of its potential epidemiological relevance in FD systems (Zwitter et al., 2023). An additional experiment was conducted in 2023 to investigate potential cultivar effects within grapevine using three *V. vinifera* cultivars known to differ in susceptibility to Flavescence dorée, Merlot (low), Nebbiolo (medium), and Chardonnay (medium–high) (Ripamonti et al., 2021; Bene et al., 2025). This experiment followed a protocol adapted from Ripamonti (2022) for assessing host-plant effects on vector fitness, while colony establishment followed the procedures of Dalmaso et al. (2023). Briefly, apple wood containing overwintering *O. ishidae* eggs was collected during winter from an apple orchard (Spagolle, Trentino, Italy), where the species occurs at high density. Branches were maintained in hatching boxes under controlled greenhouse conditions (22 ± 3 °C; 16:8 h L:D photoperiod) until egg hatch began.

Newly emerged first-instar nymphs were collected daily and transferred in groups of three (from the

same hatching day) onto potted host plants. Each plant was enclosed in a cylindrical mesh cage (20 cm height, 13 cm diameter) to confine individuals. Nymphs were assigned randomly to host plants, checked daily for survival, and monitored until adult emergence. Newly emerged adults were grouped by emergence day (maximum three per group) and maintained on the same plant species on which they had completed development. Adult survival was checked daily, and dead individuals were removed.

4.2.3 Movement at the apple–vineyard interface

A field experiment was carried out in summer 2025 in Spagolle (Trentino, Italy) to assess *O. ishidae* movement patterns between an apple orchard and an adjacent vineyard, representing a typical crop interface in mixed fruit-growing landscapes. The site was selected because the orchard and vineyard shared a continuous border and were both managed under integrated pest management (IPM). At installation, precise GPS coordinates were recorded for all traps to ensure accurate mapping and temporal consistency.

To quantify cross-crop movement, single-sided yellow sticky traps (Glutor®, Biogard) were installed along the shared border at 10 m intervals, with the adhesive surface oriented toward the adjacent crop. To estimate interior population densities within each crop, three additional double-sided yellow sticky traps were placed inside both the orchard and the vineyard: one at the plot center and two near the opposite ends of each crop (**Figure S23**). All traps were positioned on the median wire of the training system at standardized height.

Traps were replaced every 15 days from June to September. Immediately after removal, traps were stored at –20 °C to preserve specimens, then processed in the laboratory. *O. ishidae* individuals were counted, separated by sex, and recorded for subsequent statistical analysis.

4.2.4 Data analysis

All statistical analyses were conducted in R (v.4.3.1; R Core Team). Data preprocessing for all experiments was performed using the tidyverse framework, and all figures were produced with *ggplot2*.

Differences in *O. ishidae* abundance across surveyed regions and orchards were assessed with non-parametric tests due to the not normal distribution of the data. Depending on the comparison structure, a Wilcoxon rank-sum test or a Kruskal–Wallis rank-sum test was applied. When the Kruskal–Wallis test indicated significant differences, pairwise Mann–Whitney tests with Bonferroni correction were used to identify divergent groups. Results were summarized using medians, interquartile ranges, and significance groupings.

The performance of *O. ishidae* on different host plant species was analyzed using survival analysis with event definitions tailored to each biological process (Klein and Moeschberger, 2003). For nymphal mortality and adult longevity, the event was time to death, while molting (for nymphs) was treated as censoring. For nymphal development, the event was time to molt, with mortality treated as censored observations. Kaplan–Meier survival curves were estimated non-parametrically, and complementary curves ($1 - S(t)$) were used to depict cumulative molting. Differences among host plants were first evaluated with log-rank tests, followed by pairwise log-rank tests corrected using the Benjamini–Hochberg procedure. Because insects within the same cage were not statistically independent, Cox proportional hazards models incorporating either a shared frailty term (random effect for container) or robust standard errors clustered by container were fitted for each response variable and year. These models provided hazard ratios describing relative risks of mortality or development across host species while accounting for within-container dependence. Survival

estimates were presented with 95% confidence bands and median survival (or median molting time) when estimable.

To assess the influence of the apple orchard on *O. ishidae* abundance at the apple–vineyard interface, trap count data were analyzed using generalized linear mixed models (GLMMs). Counts were modeled using a negative binomial distribution with a log-link, suitable for overdispersed entomological count data. Fixed effects included habitat (apple vs vineyard), trap position (border vs center), sex, standardized time (linear and quadratic terms), and their relevant interactions. Trap identity was specified as a random intercept to account for repeated measurements. Model assumptions and goodness-of-fit were evaluated using the *performance* and *DHARMa* packages, assessing dispersion, zero inflation, and residual uniformity. Model selection relied on Akaike's Information Criterion and likelihood-ratio tests.

To evaluate whether *O. ishidae* abundance declined with increasing distance from apple trees, a second GLMM included standardized distance-to-apple as a covariate. Because distance and habitat were inherently confounded (apple traps being located at distance ≈ 0), an additional vineyard-only model was fitted to test distance effects independently of habitat. Estimated marginal means (EMMs) for the Plant × Position combinations were obtained on the response scale and compared using Tukey-adjusted contrasts. Model-based predictions with 95% confidence intervals were generated to depict seasonal phenological patterns.

4.3 Results

4.3.1 Distribution and abundance of Orientus ishidae in apple orchards

Orientus ishidae was consistently detected across northern Italian apple-growing regions in both survey years, although abundance varied markedly among orchards and management systems.

Mean weekly trap catches were generally low in IPM orchards, whereas organic orchards consistently showed higher values (**Table 5**). In 2022, organic orchards in Trentino and Friuli Venezia Giulia exhibited the highest mean weekly catches (8.39 ± 11.69 and 6.75 ± 11.29 individuals per trap, respectively), while IPM orchards in the same regions rarely exceeded one individual per trap per week (0.96 ± 1.48 (being the maximum mean recorded in Trentino) (**Table 5**). A similar pattern was observed in 2023, when overall captures decreased relative to 2022 but organic orchards again recorded higher mean weekly catches, particularly in Trentino (6.31 ± 8.42) and Slovenia (2.80 ± 3.47), whereas IPM orchards in most regions showed values close to zero (**Table 5**).

Table 5: Regional mean weekly catches of *O. ishidae* in apple orchards across northern Italy in 2022 and 2023, grouped by management system (IPM or Organic). Values represent mean ± SE of orchard-level weekly catches, standardized by week monitoring period. The number of monitored orchards (N) is reported for each region × management × year combination.

year	regions	management	N	mean ± SE	management	N	mean ± SE
2022	FRIULI-V.GIULIA	IPM	2	0.06 ± 0.18	Organic	4	6.75 ± 11.29
2022	LOMBARDIA	IPM	3	0.42 ± 0.62	Organic	4	3.98 ± 4.12
2022	PIEMONTE	IPM	2	0.77 ± 1.18	Organic	4	0.98 ± 1.37
2022	SÜDTIROL	IPM	2	0.52 ± 1.64	Organic	4	4.55 ± 7.20
2022	TRENTINO	IPM	2	0.96 ± 1.48	Organic	4	8.39 ± 11.69
2022	VENETO	IPM	2	0.01 ± 0.04	Organic	3	0.72 ± 1.10
2023	EMILIA ROMAGNA	IPM	2	0	Organic	4	0.02 ± 0.09
2023	FRIULI-V.GIULIA	IPM	2	0.00 ± 0.01	Organic	4	3.95 ± 5.69
2023	LOMBARDIA	IPM	2	0	Organic	3	2.76 ± 4.53
2023	PIEMONTE	IPM	2	0.98 ± 1.30	Organic	4	2.84 ± 7.16
2023	SLOVENIA	IPM	4	0.88 ± 1.48	Organic	2	2.80 ± 3.47
2023	SÜDTIROL	IPM	1	0	Organic	4	3.53 ± 5.28
2023	TRENTINO	IPM	2	2.01 ± 2.97	Organic	4	6.31 ± 8.42

Statistical tests demonstrated a significant effect of orchard management on *O. ishidae* abundance in both years. Mean weekly catches were significantly higher in organic orchards than in IPM orchards in 2022 (Wilcoxon rank-sum test: $W = 37$, $p < 0.001$) and 2023 ($W = 74$, $p = 0.0016$). These differences were consistent across different regions and aligned with the overall patterns shown in

Figure 14, indicating that management regime represents a major determinant of *O. ishidae* abundance at the orchard scale. By contrast, trap position within orchards did not affect captures in either year. Weekly catches did not differ among the three sampling positions (Border 1, Center, Border 2) in 2022 (Kruskal–Wallis $\chi^2 = 0.017$, df = 2, p = 0.9916) or 2023 ($\chi^2 = 0.550$, p = 0.7596) (**Figure S22**). Seasonal flight dynamics were broadly synchronous across years, with a main emergence peak occurring between mid-July in 2022 and mid-August in 2023, although peak amplitude was lower in 2023 (**Figure S20**; **Figure S21**).

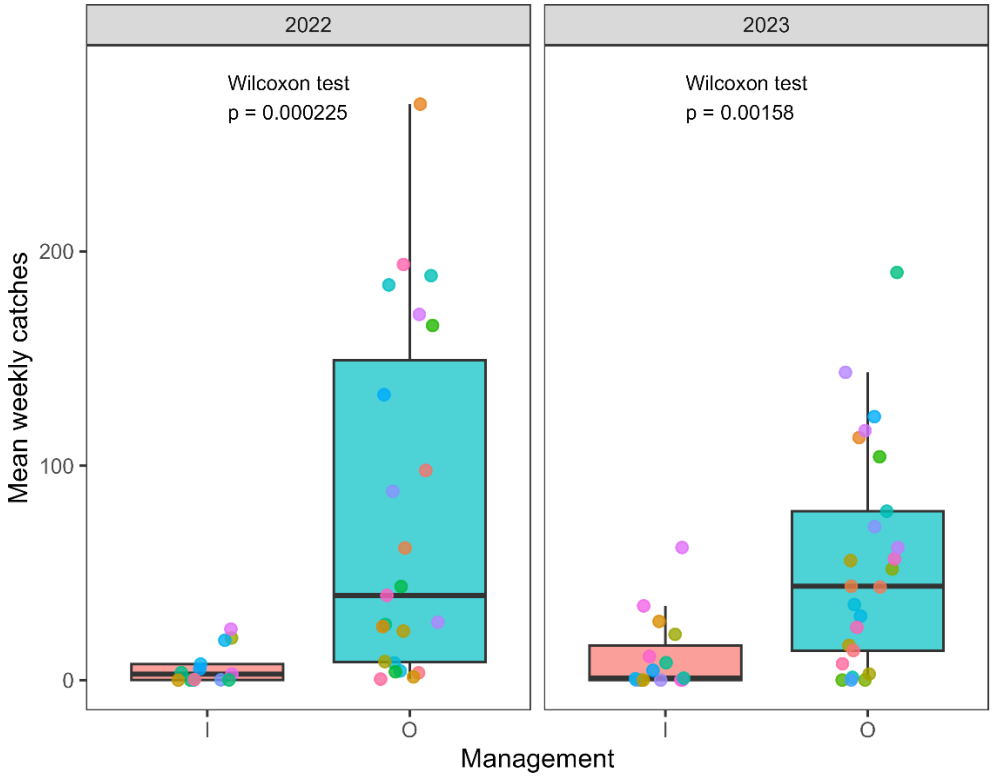

Figure 14: Weekly mean catches of *O. ishidae* in IPM (I) and organic (O) apple orchards during the monitoring seasons of 2022 and 2023. Each point represents the mean weekly catch of a single orchard. Statistical comparisons between management systems were performed using a Wilcoxon rank-sum test; corresponding p-values are reported inside each panel.

4.3.2 Survival analysis: nymph mortality, development, and adult longevity

Clear differences in nymph survival among host plants were detected in both study years (**Figure 15**, **Figure 16**). In 2023, nymphs feeding on grapevine exhibited a median survival time of 2 days (95% CI: 1–2), whereas median survival was not reached on apple, hazel, or hornbeam, indicating that cumulative mortality did not exceed 50% on these hosts during the observation period (**Figure 15**). Cox proportional hazards models revealed a markedly increased mortality risk on grapevine compared with apple (HR = 62.3, 95% CI: 28.2–137.5; $p < 0.001$), while mortality on hazel ($p = 0.54$) and hornbeam ($p = 0.10$) did not differ significantly from apple. In 2024, nymph mortality followed a comparable pattern and the median survival on apple was 30 days (lower 95% CI: 29), whereas survival on grapevine cultivars was extremely short (Chardonnay: 3 days; Merlot: 2 days; Nebbiolo: 2.5 days) (**Figure 16**). Extreme and rapid mortality on grapevine resulted in near-complete separation between host categories, preventing stable estimation of hazard ratios and yielding wide or non-finite confidence intervals in Cox models. In this context, the log-rank test provided a robust complementary assessment, confirming highly significant differences among substrates ($\chi^2 = 139$, df $= 3$, $p < 0.001$).

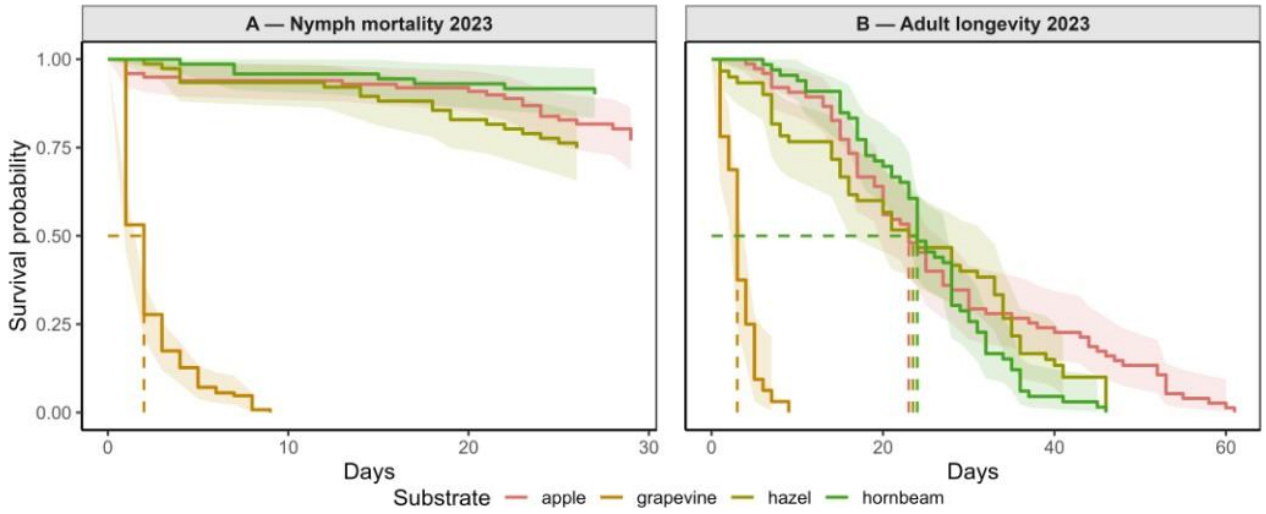

Figure 15: Nymph mortality (A) and adult longevity (B) estimated using Kaplan–Meier survival analysis in the 2023 experiment. Survival curves are shown as solid lines, with 95% confidence intervals displayed as shaded area. Median survival times (when estimable) are indicated by dashed lines. (i.e., time when 50% of the insects are still alive). *O. ishidae* individuals were reared on apple (*Malus domestica*), grapevine (*Vitis vinifera* cv Bronner), hazel (*Corylus avellana*), and hornbeam (*Carpinus betulus*).

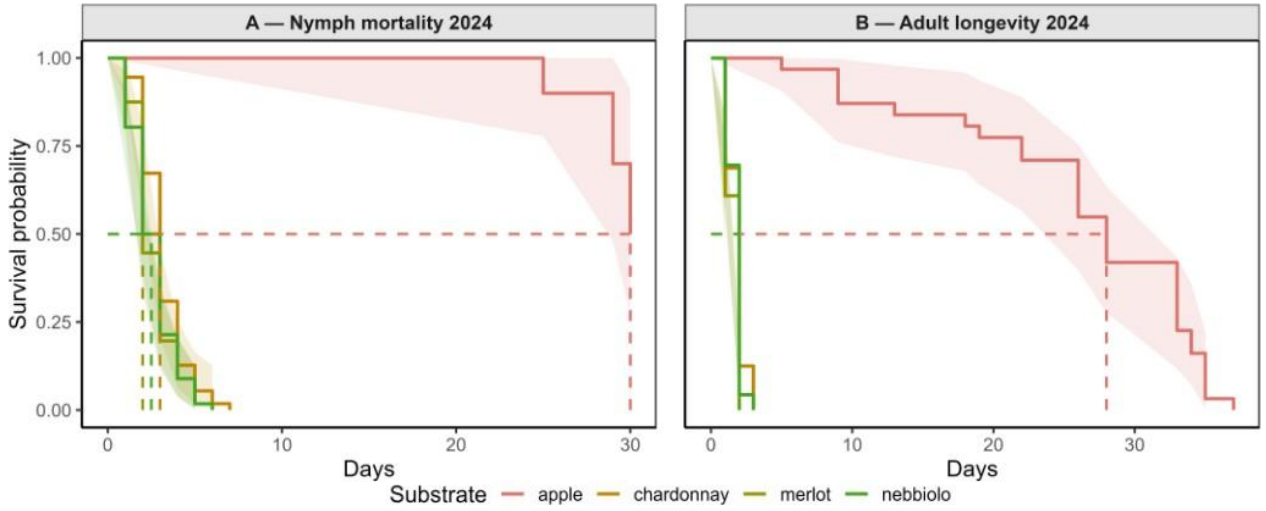

Figure 16: Nymph mortality (A) and adult longevity (B) estimated using Kaplan–Meier survival analysis in the 2024 experiment. Survival curves are shown as solid lines, with 95% confidence intervals displayed as shaded area. Median survival times (when estimable) are indicated by dashed lines. (i.e., time when 50% of the insects are still alive). *O. ishidae* individuals were reared on apple (*Malus domestica*), grapevine (*Vitis vinifera*) cultivar Chardonnay, Merlot and Nebbiolo.

Nymphal development to the adult stage was completed exclusively on apple, hazel, and hornbeam in 2023, and only on apple in 2024. No nymphs reached the adult stage on grapevine or on grapevine cultivars in either year (**Figure 17**). In 2023, mean development time was similar among suitable hosts, with nymphs completing development in 29.4 ± 2.85 days on apple (n = 77), 29.5 ± 3.06 days on hazel (n = 56), and 28.4 ± 2.10 days on hornbeam (n = 64). Inverse Kaplan–Meier curves showed overlapping cumulative molting trajectories, in agreement with Cox models indicating no difference in development rate between apple and hazel (p = 0.74). Development on hornbeam was significantly faster than on apple (HR = 1.71, 95% CI: 1.19–2.45; p = 0.004) (**Figure 17**). In 2024, development was observed only on apple, with a mean development time of 25.3 ± 2.69 days (n = 33) (**Figure 17**).

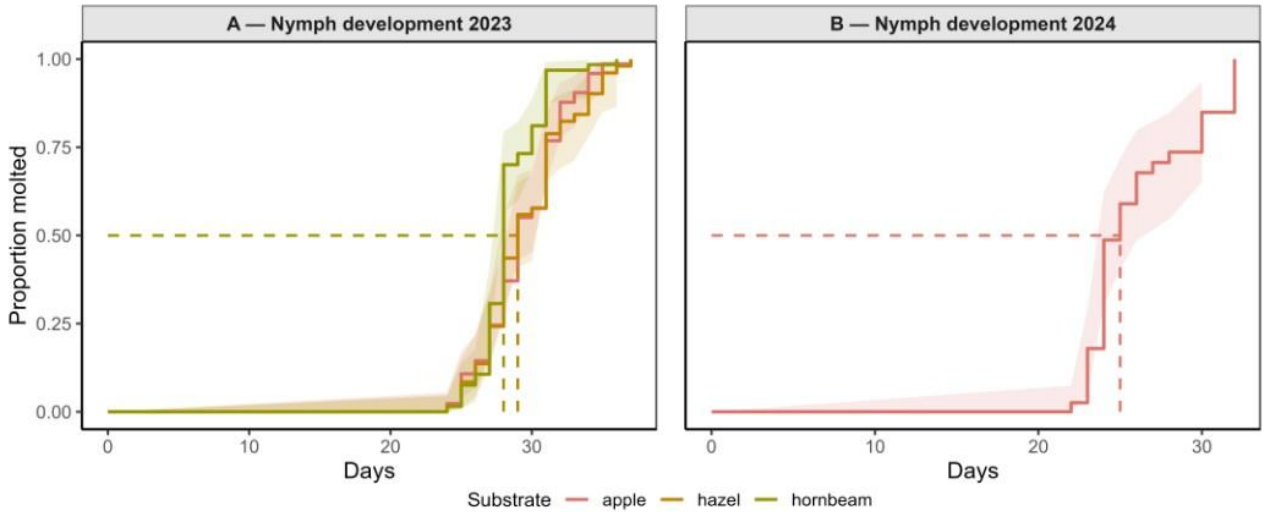

Figure 17: Inverse Kaplan–Meier curves show the cumulative proportion of individuals reaching the adult stage over time. Ninety-five percent confidence intervals are depicted by transparency. Solid lines indicate median development time when estimable. Dashed line indicates median development time (i.e., time when 50% of the insects molted). Host plants include apple (*Malus domestica*), grapevine (*Vitis vinifera*), hazel (*Corylus avellana*), hornbeam (*Carpinus betulus*), and, in 2024, the grapevine cultivars Chardonnay, Merlot, and Nebbiolo.

Adult survival followed the same host-dependent pattern observed for nymphs (**Figure 15**; **Figure 16**). Because no adults emerged from grapevine-reared nymphs, longevity assays on grapevine used adults that had developed on suitable hosts or from synchronized laboratory cohorts, ensuring adequate replication. In 2023, median adult survival was 23 days on apple (95% CI: 20–27), 23.5 days

on hazel (95% CI: 17–33), and 24 days on hornbeam (95% CI: 23–28), whereas half of the adults on grapevine survived only 3 days (95% CI: 3–4). Cox models indicated a pronounced increase in mortality risk on grapevine relative to apple (HR = 54.6, 95% CI: 27.0–110.8; $p < 0.001$). Survival on hornbeam was slightly reduced compared with apple (HR = 1.50, 95% CI: 1.08–2.07; $p = 0.015$), while the difference between apple and hazel was not significant ($p = 0.09$) (**Figure 15**). In 2024, adult longevity on apple remained high, with a median survival of 28 days (95% CI: 26–33). In contrast, adults placed on grapevine cultivars exhibited extremely short lifespans, with median survival of 2 days on Chardonnay (95% CI: 2), Merlot (95% CI: 1), and Nebbiolo (95% CI: 2) (**Figure 16**). As in the nymphal mortality analysis, near-complete separation in Cox models prevented reliable estimation of hazard ratios; nevertheless, global tests clearly supported strong differences among substrates (likelihood ratio test: $p < 0.001$; log-rank $\chi^2 = 118$, df = 3, $p < 0.001$), confirming the very low suitability of grapevine for adult survival.

4.3.3 Movement between apple orchards and vineyards

Model-based analyses revealed a strong and consistent association of *O. ishidae* with apple orchards, while captures in the adjacent vineyard remained extremely low throughout the monitoring period. Predictions were obtained from the best-supported negative binomial GLMM, which included plant type, trap position, sex, and standardized sampling time (linear and quadratic terms) as fixed effects, with trap identity fitted as a random intercept to account for repeated sampling. The model confirmed a highly significant effect of plant type, with trap captures in vineyards sharply reduced relative to apple orchards (Plant = vineyard; $p < 0.001$, **Figure 18**). Back-transformed estimated means indicated consistently higher expected captures in apple orchards than in vineyards, both at the border and in central positions. Trap position significantly influenced *O. ishidae* abundance within apple orchards, with higher expected counts in orchard centers than at orchard borders ($p < 0.001$). In

contrast, trap position had no effect within vineyards, and the interaction between plant type and position confirmed that border–center differences occurred exclusively in apple orchards ($p < 0.001$ for the interaction term). Within vineyards, expected abundances were uniformly low and did not differ between border and center positions (**Figure 18**). Sex had a strong and consistent effect across all fitted models, with males significantly less abundant in trap catches than females ($p < 0.001$). This sex bias was independent of plant type, position, and time, as no biologically meaningful higher-order interactions involving sex modified the main pattern (**Figure 18**).

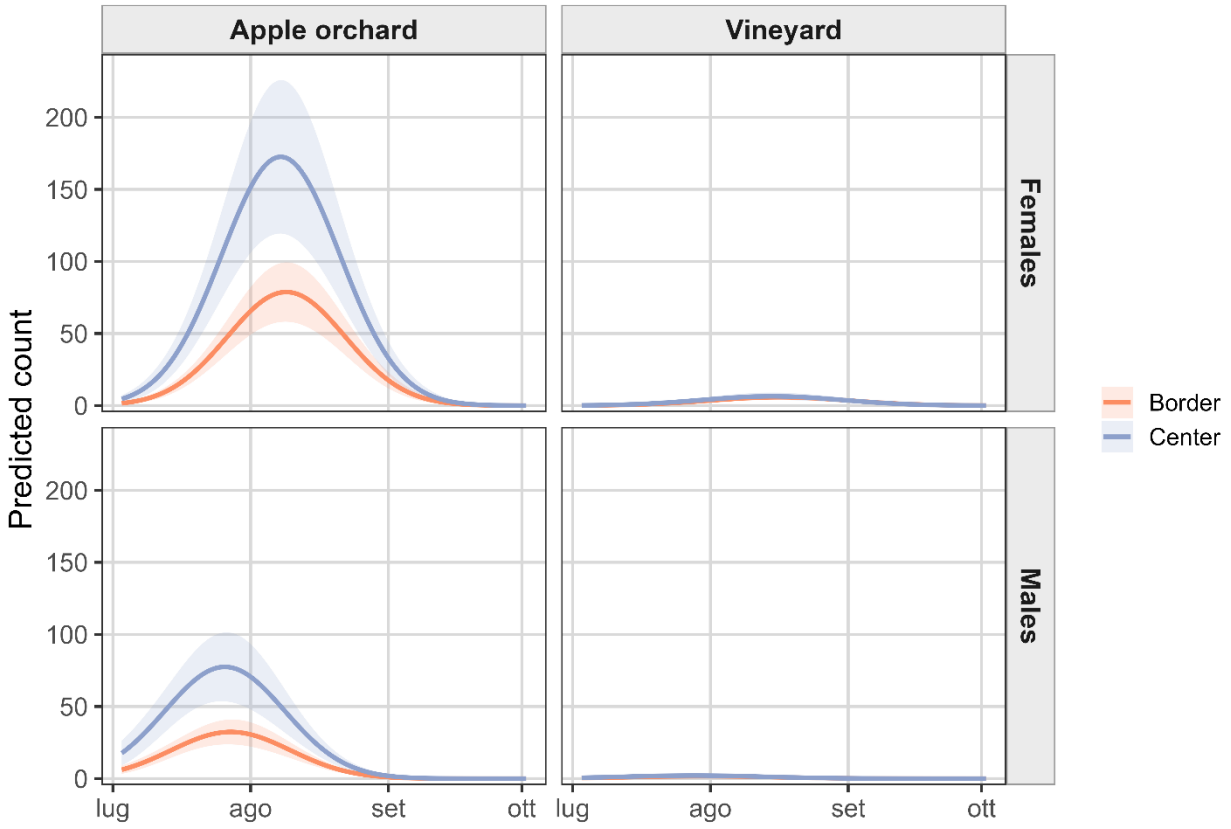

Figure 18: Predicted seasonal dynamics of *O. ishidae* adult captures in apple orchards and vineyards. Curves show model-based predictions from the best-supported generalized linear mixed model with negative binomial distribution (NB2), including plant type, trap position, sex, and standardized sampling time (linear and quadratic terms) as fixed effects, and trap identity as a random intercept. Panels refer to females and males, and colours indicate trap position. Shaded ribbons represent 95% confidence intervals around predicted counts. Predictions indicate a single seasonal peak, with markedly higher captures in apple orchards than in vineyards and consistently lower male than female catches.

Seasonal dynamics were characterized by a unimodal flight pattern, supported by a significant quadratic effect of time ($p < 0.001$). Interactions between plant type and time indicated contrasting seasonal trajectories between apple orchards and vineyards. Predicted abundances increased sharply in apple orchards during the peak activity period, whereas vineyard captures remained uniformly low throughout the season, exhibiting only minor temporal variation (**Figure 18**).

Including distance from the nearest apple tree did not improve model performance and did not affect inference (likelihood-ratio test, $p > 0.80$). Distance estimates were small and non-significant, and strong collinearity with plant type suggested that distance primarily reflected the orchard–vineyard contrast rather than an independent spatial gradient. Consistently, a vineyard-only model including distance detected no effect of proximity to apple orchards on *O. ishidae* abundance ($p > 0.80$). Descriptive statistics closely matched model predictions, confirming a strong orchard–vineyard contrast. Mean catches were highest in orchard-center traps (25.1 males and 48.2 females), lower at orchard borders (11.3 males and 21.6 females), and consistently minimal in vineyards, where border and center traps showed similarly low values (0.34 vs. 0.43 males; 2.52 vs. 2.53 females, respectively). Detailed descriptive statistics by trap are provided in the Supplementary Material (**Table S6**).

4.4 Discussion

Our interregional survey confirms that *O. ishidae* is now consistently present in apple-growing areas across northern Italy, with marked differences in abundance among orchards and management systems. The most coherent pattern emerging from the field data is the strong association between higher trap catches and organic management, whereas IPM orchards generally hosted low populations. This contrast reflects the combined effects of insecticide regimes (spectrum, persistence, and timing) and overall disturbance intensity, which can differentially constrain leafhopper populations in perennial crops (Dively et al., 2020; Prazaru et al., 2023; Ryalls et al., 2024; Tacoli et al., 2017;

Trivellone et al., 2012). Regional differences may also reflect variation in surrounding habitat composition, orchard prevalence, and landscape complexity (Kőrösiet et al., 2012; Trivellone et al., 2012; Helbing et al., 2017; Ragone et al., 2025). However, this interpretation remains speculative, because landscape structure was not quantified explicitly in the present study. Future work should integrate spatially explicit landscape metrics to test whether semi-natural complexity and habitat configuration contribute to *O. ishidae* abundance in orchard-dominated systems. In addition, the recent population increase observed in apple orchards since 2019 (Baldessari et al., 2020), following decades of sporadic occurrence between 1998 and 2016 (Fischnaller et al., 2020; Oppedisano et al., 2017), is compatible with a classical invasion 'lag phase' (Crooks and Soulé, 1999; Epanchin-Niell and Liebhold, 2015). This trajectory likely reflects the gradual spread or a shift in ecological constraints (e.g., climatic suitability, natural enemy pressure, change in management strategies or host/habitat availability, etc.) rather than being solely attributable to changes in detection intensity.

Laboratory assays confirmed that apple, hazel, and hornbeam are very suitable hosts, supporting high nymphal survival and development times (25–30 days) comparable to previous findings by Lessio et al. (2019), who reported that *O. ishidae* reared on *Vicia faba* completed development to the adult stage after 27–29 days. Conversely, cultivated grapevine proved unsuitable for *O. ishidae*, as no nymphs completed development and adult survival remained negligible (<3 days), regardless of cultivar. Given that successful development and reproduction are essential requirements for defining a host plant (Schoonhoven et al., 2005; Souza, 2025; Volp et al., 2022), these results indicate that cultivated grapevine cannot be considered a true host for this species under the tested conditions. By contrast, apple, hazel, and hornbeam readily support complete development, prolonged adult survival, and previous studies have consistently reported substantial oviposition on these wild/cultivated hosts (Lessio et al., 2019; Dalmaso et al., 2023; Oggier et al., 2025). The

mechanisms underlying the non-host status of cultivated grapevine remain unresolved. Several resistance mechanisms may be involved, including antixenosis, antibiosis and physical constraints affecting stylet penetration or movement (Souza, 2025). Disentangling whether grapevine resistance is primarily chemical, nutritional, or physical will require targeted behavioral assays and electrophysiological studies (e.g. EPG recordings).

Interestingly, oviposition on grapevine has also been documented under specific conditions, particularly on wild grapevine or in abandoned vineyards (Lessio et al., 2019; Oggier et al., 2023). This observation led to the hypothesis that grapevine might occasionally be used as an oviposition substrate. However, in light of our results, newly hatched nymphs would necessarily have to relocate to more suitable hosts within the vineyard, given the very limited dispersal capacity of early instar nymphs (Lessio et al., 2019). Although in some leafhopper species nymphs may drop to the ground and continue development on alternative vegetation, as reported for *Anoplotettix fuscovenosus* (Ferrari) (Alma, 1995), there is currently no evidence that *O. ishidae* regularly exploits herbaceous hosts during its nymphal stages. Taken together, these observations suggest that oviposition on cultivated grapevine is more plausibly interpreted as an occasional or opportunistic event rather than as a strategy leading to successful population development, although this aspect warrants further targeted investigation.

Importantly, the ability of *O. ishidae* to transmit FD phytoplasmas to grapevine under experimental conditions indicates that transient probing and limited ingestion of grapevine tissues can occur (Lessio et al. 2016; Jarausch et al. 2023). Comparable situations are known in other phytoplasma pathosystems involving vectors that do not use the crop plant as a true host. A well-established example is *Hyalesthes obsoletus* (Signoret), the vector of Bois noir phytoplasma, which primarily develops on herbaceous weeds and only occasionally feeds on grapevine (Maixner, 1994; Mori et al.,

2020; Panassiti et al., 2013). When forced to feed on grapevine, *H. obsoletus* shows limited survival (Moussa et al., 2019), comparable to that observed for *O. ishidae* on grapevine, yet it remains an efficient vector of the pathogen and have a strong epidemiological role.

In contrast, available transmission data indicate that *O. ishidae* exhibits extremely low transmission efficiency to grapevine, while transmission rates among wild reservoir hosts (e.g. alder–alder) are considerably higher (Jarausch et al., 2023). Recent studies from Germany further support this distinction: although alder populations showed very high infection frequencies (>95%) and hosted abundant *O. ishidae* populations carrying compatible phytoplasma genotypes (Jarausch et al., 2025), transmission to grapevine remained extremely rare, with the first documented case reported only in 2021 (Jarausch et al., 2021). Thus, even under conditions of high inoculum pressure and high vector abundance, the epidemiological role of *O. ishidae* on grapevine appears fundamentally different from that observed in pathosystems such as Bois noir, where a non-host vector like *H. obsoletus* plays a major role in disease spread.

Movement experiments further supported the interpretation of *O. ishidae* as an occasional vector on grapevine. Adult abundance was consistently associated with apple orchards, whereas captures in vineyards remained uniformly low throughout the season. Within apple orchards, trap position significantly influenced captures, with higher abundances recorded in central areas, while no positional effects were detected in vineyards. Moreover, the distance from the nearest apple orchard did not affect capture rates in vineyards, suggesting that vineyard presence was not structured by local population processes. These patterns are unlikely to reflect a confounding effect of insecticide treatments: mandatory *S. titanus* control in vineyards (acetamiprid, etofenprox) was completed by late June, prior to the onset of *O. ishidae* adult flight in early July, and orchard treatments targeted pest windows that did not overlap with peak *O. ishidae* activity. Together with the host-suitability

experiments, these results indicate that spillover from apple to grapevine can occur but is transient, and that vineyards do not function as habitats capable of sustaining local *O. ishidae* populations.

Beyond excluding grapevine as a suitable host, the spatial data raise a further interpretive possibility. High density of a highly suitable host such as apple may not only sustain large *O. ishidae* populations, but also reduce sustained movement toward unsuitable adjacent habitats. In this sense, apple orchards may act not only as demographic sources but also as retentive habitats, favouring persistence on a suitable crop while limiting prolonged colonization of neighboring vineyards. Although this hypothesis was not directly tested here, it is consistent with the combined evidence from trap captures and host-suitability assays, and suggests that interface scenarios involving other suitable hosts, such as hazel, may deserve further investigation.

Trap captures provide informative data on relative abundance and spatial distribution but do not allow reconstruction of individual trajectories or directional dispersal (Hagler and Jackson, 2001). Accordingly, our data should be interpreted as evidence of limited spillover and lack of establishment in vineyards, rather than as direct proof of directional movement from orchards. This interpretation is strengthened by the host-suitability experiments, which demonstrated that grapevine supports neither nymphal development nor sustained adult survival of *O. ishidae*. A more conclusive evaluation of dispersal dynamics would require dedicated mark–release–recapture experiments, as applied in other leafhopper systems (Zhou et al., 2003; Lessio et al., 2014).

Taken together, our results clarify the biological constraints underlying the role of *O. ishidae* in FD epidemiology and help reconcile recent, apparently divergent interpretations. In line with Prazaru et al. (2025), we show that even high populations on a suitable host crop such as apple do not translate into population build-up on adjacent grapevine, as extremely low survival and the absence of development prevent demographic spillover. This provides a biological explanation for the

negligible contribution of *O. ishidae* to epidemic spread in vineyards where *S. titanus* is established. At the same time, our findings are consistent with the perspective of Jarausch et al. (2025): *O. ishidae* thrives on reservoir hosts and may occasionally introduce into vineyards, a process that is rare and transient but potentially relevant during early invasion or pre-epidemic phases.

Ongoing work is further examining the role of alternative cultivated woody hosts such as hazel adjacent to vineyards and the possibility that apple trees may harbor FD phytoplasmas asymptomatically (Badr et al., in prep.). By demonstrating that grapevine is biologically unsuitable for sustaining *O. ishidae* populations while confirming its strong association with reservoir hosts, the present study provides a unifying framework linking host suitability, vector ecology, and epidemiological risk. This framework highlights why *O. ishidae* should be regarded neither as an epidemic vector nor as an irrelevant bystander, but as a context-dependent bridge vector whose importance is confined to inoculum maintenance and transfer at the landscape level.

4.5 Supplementary materials

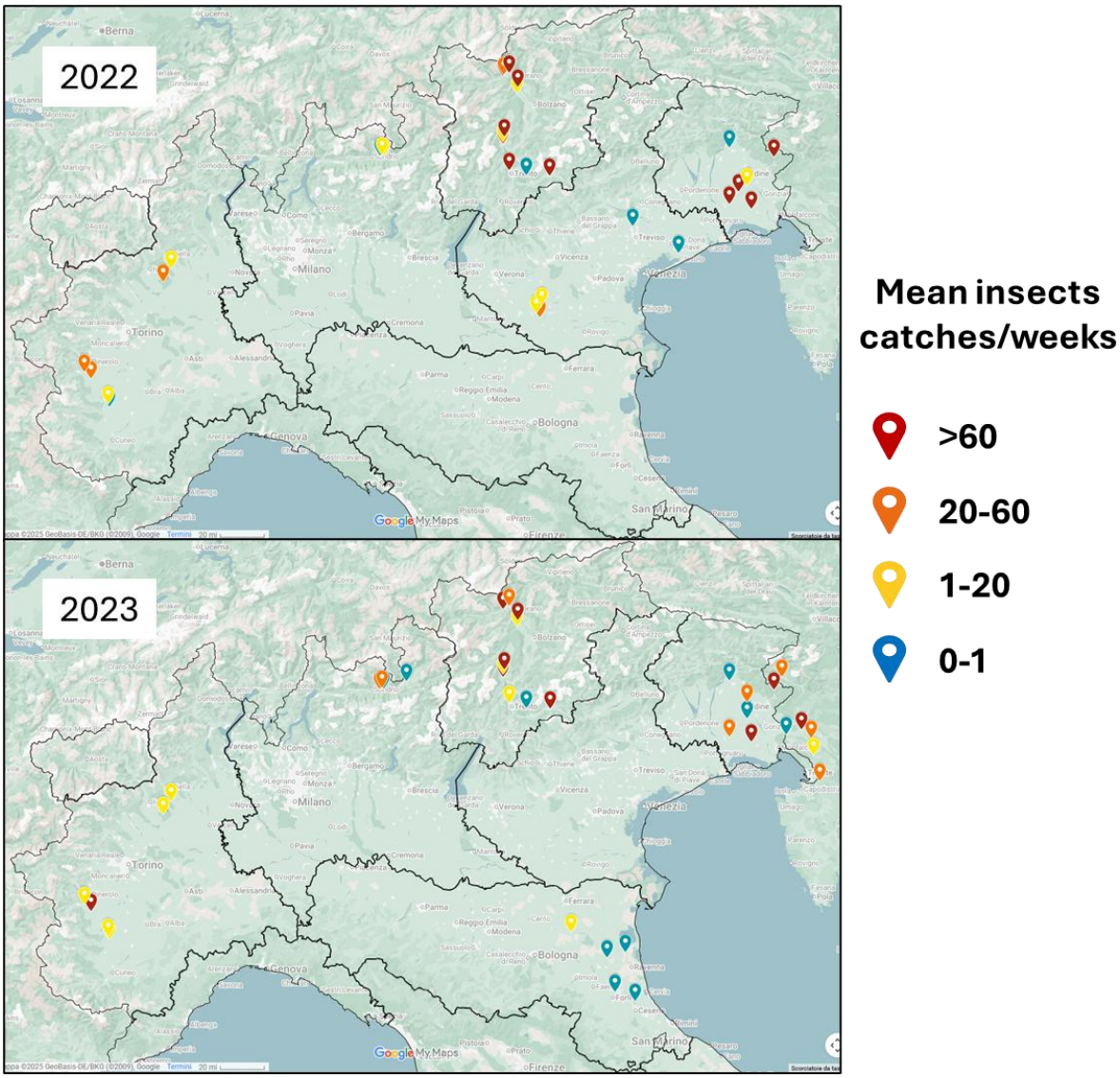

Figure S19: Maps showing the monitoring sites in 2022 (A) and 2023 (B), with the corresponding mean weekly catches per trap.

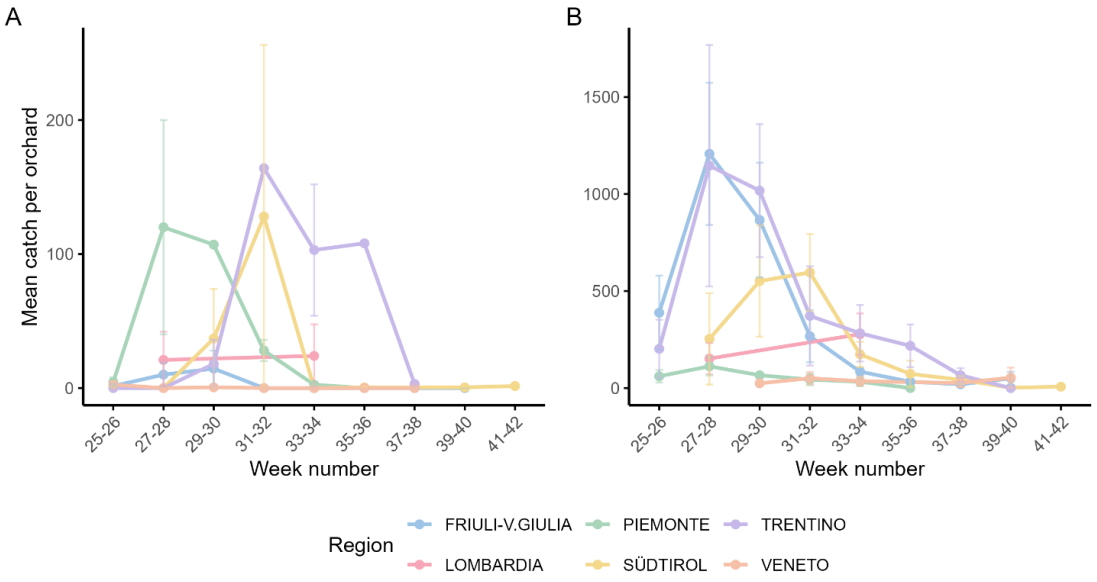

Figure S20: Seasonal flight curves of *Orientus ishidae* in apple orchards across northern Italian regions in 2022. Panels show (A) IPM and (B) organic orchards. Coloured lines represent regional means for each two-week sampling interval; points and error bars indicate mean ± SD.

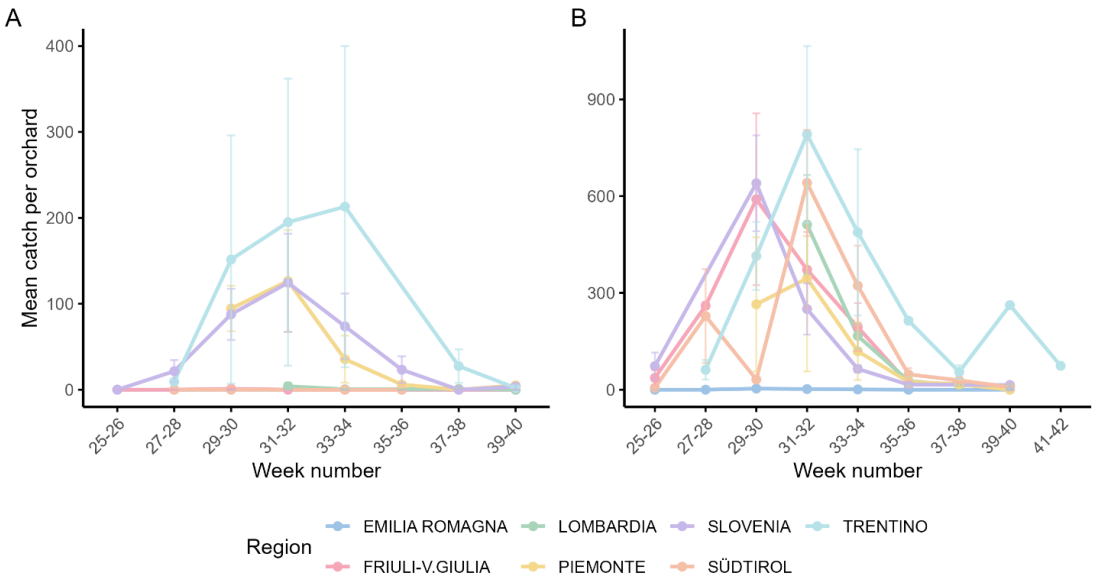

Figure S21: Seasonal flight curves of *Orientus ishidae* in apple orchards across northern Italian regions in 2023. Panels show (A) IPM and (B) organic orchards. Colored lines represent regional means for each two-week sampling interval; points and error bars indicate mean ± SD.

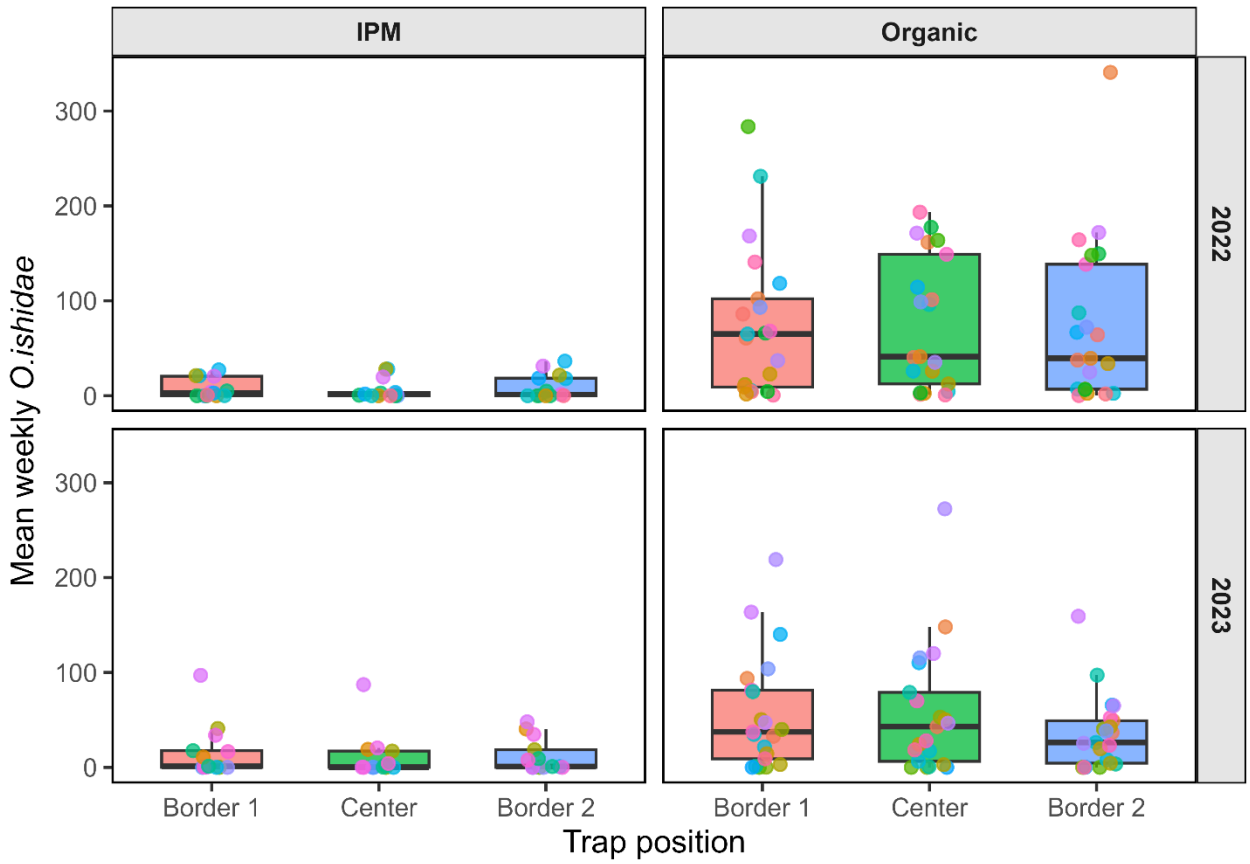

Figure S22: Effect of trap position on seasonal weekly catches of O. *ishidae* in apple orchards in 2022 and 2023. Trap positions correspond to Border 1 (external row), Center (internal row), and Border 2 (opposite external row). Differences among trap positions were assessed separately for each year and management system using Kruskal–Wallis tests. No significant differences were detected in any comparison (2022 IPM: H = 0.277, df = 2, p = 0.870; 2022 organic: H = 0.204, df = 2, p = 0.903; 2023 IPM: H = 0.254, df = 2, p = 0.881; 2023 organic: H = 0.957, df = 2, p = 0.620).

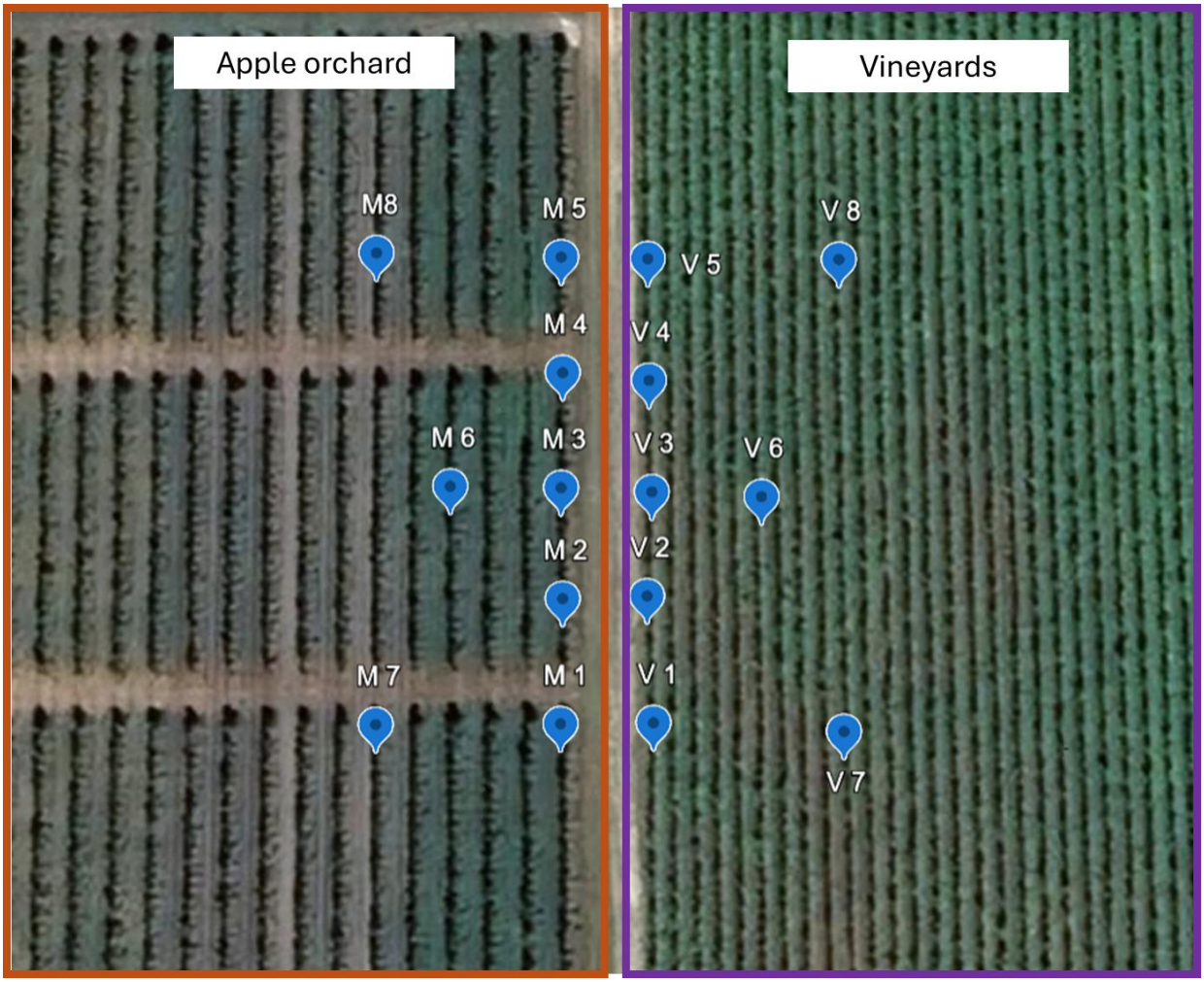

Figure S23: Apple orchards (M) and vineyard (V) where yellow sticky traps was placed during the monitoring activity of movement between two crops used in the experiments 2025 (Spagolle, Trentino, Italy 46°02'45.6'N 11°28'28.8'E)

Table S6: Seasonal trap catches of *O. ishidae* recorded at the apple–vineyard interface, summarized separately for each trap, plant type (apple orchard or vineyard), trap position (border or center), and sex. For each trap, total numbers of males (♂) and females (♀) captured over the entire monitoring period are reported, together with mean (± SD) numbers of individuals per trap per two-week sampling interval. N indicates the number of traps deployed for each Plant × Position combination, while n represents the total number of sampling occasions per trap (eight consecutive trap replacements at two-week intervals during the season).

Trap	Position	Plant	Total ♂	Total ♀	N	n	Mean ♂	Mean ♀	SD ♂	SD ♀
1	Border	Apple	98	171	5	8	12.3	21.4	18.5	28.2
2	Border	Apple	100	193	5	8	12.5	24.1	19.1	28.5
3	Border	Apple	87	162	5	8	10.9	20.3	15.1	26
4	Border	Apple	64	166	5	8	8	20.8	12.6	31.2
5	Border	Apple	102	171	5	8	12.8	21.4	18.2	27.4
6	Center	Apple	245	484	3	8	30.6	60.5	41.5	71.7
7	Center	Apple	166	313	3	8	20.8	39.1	32.2	50
8	Center	Apple	191	360	3	8	23.9	45	32.2	55.5
1	Border	Vineyard	6	28	5	8	0.8	3.5	1	5.6
2	Border	Vineyard	2	17	5	8	0.3	2.1	0.5	2.3
3	Border	Vineyard	3	13	5	8	0.4	1.6	0.7	2.8
4	Border	Vineyard	1	19	5	8	0.1	2.4	0.4	2.9
5	Border	Vineyard	1	24	5	8	0.1	3	0.4	3.9
6	Center	Vineyard	3	23	3	8	0.4	2.9	1.1	4
7	Center	Vineyard	4	5	3	8	0.5	0.6	1.4	1.4
8	Center	Vineyard	3	33	3	8	0.4	4.1	0.7	4.8

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CHAPTER 5. ECONOMIC INVESTIGATION

Decision-relevant economic guidance for managing Apple Proliferation under mandatory removal requirements

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In preparation

Abstract

Apple Proliferation (AP), associated to '*Candidatus* Phytoplasma mali', poses a major threat to the economic sustainability of apple production in Trentino, Italy. Since 2006, regional regulations have mandated the removal of symptomatic trees to limit pathogen spread. Following removal, however, growers face a management decision affecting long-term orchard productivity: whether to not replacing uprooted plants, progressively reducing yield potential, or maintain orchard density through systematic replacing. This study assessed the long-term economic performance of these two post-removal strategies using Partial Budget Analysis over a 20-year orchard lifespan under both Integrated Pest Management (IPM) and organic production systems.

Results showed that removal with systematic replacing (MS2) outperformed removal without replacing (MS1) despite higher initial costs, limiting cumulative net income losses to −2.1% (IPM) and −4.0% (organic) compared to −2.9% and −6.0% for MS1. The economic advantage of MS2 became evident after year 12–13 as replanted trees reached full productivity. A counterfactual unmanaged scenario highlighted that the current mandatory management policy prevents losses exceeding 28% of gross returns, confirming its economic rationale. Sensitivity analyses indicated that profitability was more sensitive to epidemiological than economic parameters, emphasizing that

enhanced disease monitoring and modeling would improve decision-making more effectively than refined cost estimates.

5.1 Introduction

In Trentino region (northeastern Italy), apple cultivation represents the dominant agricultural activity, covering 10,000 hectares, 8.5% of which are managed under organic farming, and producing an average of 500,000 tons annually, accounting for nearly 25% of Italy's apple output and about 4% of European Union production (Del Bravo et al., 2024; ISTAT, 2025; Rippo and Cerroni, 2023).

However, Apple proliferation (AP), a disease associated to the phloem-restricted, wall-less bacterium '*Candidatus* Phytoplasma mali' (16Sr group X-A) (Bertaccini et al., 2022; Seemüller and Schneider, 2004), poses a major threat to the long-term economic sustainability of apple production in Trentino (Strauss, 2009). The phytoplasma is widespread across European apple-growing regions (EPPO, 2025) and is transmitted primarily by the univoltine psyllid vectors *Cacopsylla picta* Förster and *Cacopsylla melanoneura* Förster (Hemiptera: Psylloidea) (Frisinghelli et al., 2000; Tedeschi and Alma, 2004; Oppedisano et al., 2020; Candian et al., 2020).

Although AP does not directly cause tree mortality, infected plants exhibit profound metabolic alterations, particularly in carbohydrate and hormone metabolism (Aldaghi et al., 2012; Giorno et al., 2013; Lepka et al., 1999; Mittelberger et al., 2017). Symptomatic trees develop witches' brooms, enlarged dentate stipules, and, economically most relevant, small, tasteless fruits with elongated peduncles that are downgraded from the fresh market (Rumbou et al., 2008; Seemüller et al., 2011; Herzog et al., 2012; Janik et al., 2020). Quantitative studies have reported marked reductions in fruit size (–27% to –49%) (Lemoine, 1990; Schmid, 1989), weight (-5% to -79%) (Kaminska and Zawadzka, 1970a; Zawadzka, 1976), discoloration (Herzog et al., 2012) and yield per tree (0% to

91%), with a substantial proportion of fruit downgraded to processing grade (Herzog et al., 2012; Kaminska and Zawadzka, 1970b, 1970a; Lemoine, 1990; Schmid, 1989).

Despite these documented fruit quality and yield impacts, comprehensive economic assessments of AP remain limited. Herzog et al. (2012) provided an orchard-level estimate of losses (38–189 €/ha at 2.3% infection prevalence), based exclusively on fruit downgrading without accounting for management costs or long-term productivity effects. At a broader scale, Strauss (2009) reported aggregate national losses of approximately 100€ million in Italy in 2001, but methodological detail remain unclear, creating uncertainty about which cost components were included and how losses were extrapolated. Critically, no study has yet evaluated the economic implications of post-removal management decisions over the productive lifespan of an orchard. In Trentino, compulsory removal of symptomatic trees has been enforced since 2006 (Janik et al., 2020), requiring growers to make a consequential decision: leaving permanent gaps in the orchard or investing in systematic replacing. This choice involves economic trade-offs between immediate costs (labor, materials), delayed returns from young trees and long-term impacts on per-hectare productivity. The absence of quantitative economic guidance leaves growers without evidence-based support, despite the substantial implications for orchard profitability over the 20-year investment horizon typical of high-density apple systems. The present study aims to provide this evidence by quantifying and comparing the long-term economic performance of the two post-removal options available to Trentino growers: not replacing symptomatic plants versus systematic replacing. We apply partial budget analysis to evaluate these alternatives under both IPM and organic farming, providing decision-relevant economic guidance for managing AP-affected orchards under current regulatory requirements.

5.2 Materials and Methods

5.2.1 Economic analysis framework

Partial Budget (PB) analysis is a widely employed technique for assessing the direct economic impact of specific changes at the farm level, such as adopting new technology, modifying production practices or the introduction of a pest (Soliman et al., 2010; Cornelisse and Hyde, 2023). In contrast to broader economic models (e.g. partial equilibrium, input-output, or computable general equilibrium models) which incorporate indirect effects such as market dynamics or environmental externalities, PB analysis focuses exclusively on the net changes in farm income resulting from the specific intervention under evaluation (Soliman et al., 2010; Del Fava et al., 2017; Digiacomo et al., 2021).

PB analysis is particularly appropriate for AP because Pest Risk Analyses (PRA) have consistently classified its impact on yield and control costs as medium to high, while indirect effects were deemed negligible (EPPO, 2018; Sundheim et al., 2012). Consequently, the present study focuses on direct economic effects (e.g. yield reduction, disease management costs, etc.) while excluding indirect impacts based on existing PRA evaluations.

Within this framework, the net change in income (NCI), given control strategies 0 (baseline) and t (alternative), is calculated as $NCI_{0t} = (C_0 + R_t) - (C_t + R_0)$, where C_0 (reduced cost) represents the cost savings under the alternative strategy, R_t (additional returns) revenues obtained under the alternative scenario, C_t (additional cost) added costs of t strategy, and R_0 (reduced returns) revenue that would have been obtained under the baseline scenario (**Table 7**). A positive NCI_{0t} indicates a net economic gain, whereas a negative value implies a financial loss compared to the baseline scenario.

Table 7: general layout of the partial budget analysis, outlining the components of costs and benefits when comparing a current (baseline, 0) situation with an alternate (disease outbreak managed or not managed, t) situation. table retrieved by Soliman et al., 2010 and Digiacomo et al., 2021.

Cost	**Benefits**
Additional cost ($\boldsymbol{C_t}$): costs under the alternate situation that are not required under the current situation	Additional returns ($\boldsymbol{R_t}$): revenues under the alternate situation that are not received under the current situation
Reduced returns ($\boldsymbol{R_0}$): revenues under the current situation that will not be received under the alternate situation	Reduced costs ($\boldsymbol{C_0}$): costs under the current situation that will be avoided under the alternate situation
$\boldsymbol{Total\ cost = (C_t + R_0)}$	$\boldsymbol{Total\ benefits = (R_t + C_0)}$
$\boldsymbol{Net\ change\ in\ income\ NCI_{t0} = (R_t + C_0) - (C_t + R_0)}$	

5.2.2 Experimental design: scenario selection and rationale

The analysis was conducted over a 20-year orchard lifespan comparing AP-affected scenarios against a disease-free baseline (BS), simulated under both IPM and organic farming systems. Scenario selection was based on a theoretical 2x2 factorial design combining vector control (yes/no) and AP symptomatic tree removal (yes/no), yielding four possible management combinations. Biological and regulatory constraints, however, reduced evaluable scenarios.

Scenario without vector control and symptomatic tree removal was excluded as unrealistic in Trentino's agroecosystems context. The psyllid vectors *C. melanoneura* and *C. picta* overwintering sites in surrounding coniferous forest and recolonize orchards annually (Candian et al., 2020; Somoano et al., 2025; Tedeschi and Alma, 2004), making vector control a management necessity (Baldessari et al., 2010; Šafářová et al., 2015).

Similarly, scenario retaining symptomatic trees with pesticide application was excluded due to mandatory removal requirements (Janik et al., 2020). Furthermore, even if regulatory constraints were absent, the option lacks sufficient biological justification: spontaneous recovery, while

occasionally reported (Carraro et al., 2004; Ermacora et al., 2008), remains poorly characterized for commercial cultivars, is often temporary (Baric et al., 2007) and thus provides no reliable basis for management. The factorial reduction thus isolated vector control combined with removal as the sole viable management framework. Within this framework, growers face a subsequent decision constituting this study's primary focus: whether to not replacing uprooted symptomatic plants (MS1) or replace removed apple trees every year (MS2). Furthermore, an unmanaged scenario (UNS) combining absence of both interventions was retained as experimental negative control despite being legally non-viable.

The comparisons between these scenarios serve distinct analytical purposes. First, the comparison of MS1 and MS2 versus BS isolates the impact of these alternative post-management options under current operational constraints, providing information regarding the most cost-effective long-term strategy. Second, the comparison between UNS and BS quantifies the economic consequences of enforcement failure, consistent with established partial budget frameworks (Soliman et al., 2010), and provides policy-relevant benchmarks for regions currently lacking regulatory frameworks. Finally, comparing both management options with UNS highlights the overall economic benefit of mandatory intervention policies.

5.2.3 Economic calculations and scenario-specific assumptions

In this framework, economic effects were quantified taking the BS scenario as the reference (control strategy 0) against which MS1, MS2, and UNS (alternative strategies t) were evaluated. In MS1, symptomatic trees were removed annually with fruit downgraded to processing grade in the removal year. No replacing occurred, causing progressive reduction in tree density. Additional cost (C_t) included annual vector control treatments plus labor and machinery for tree removal. Additional returns (R_t) derived from downgraded fruit produced by symptomatic trees prior to removal, while

cost savings (C_0) reflected eliminated labor for pruning, thinning, and harvesting from removed trees. Reduced returns (R_0) captured forgone fresh apple production associated with the progressive loss of productive trees (**Table S12**)

In MS2, symptomatic trees were removed annually but replaced the following spring to maintain orchard density. Replanted trees followed an extended establishment phase reflecting delayed development due to replacing year and competition with older plants 0% yield in years 1–2 post-planting, then 20%, 50%, 80%, and 100% in years 3–6. This differs from the standard establishment curve for initial orchard planting (0%, 20%, 50%, 80%, 100% in years 1–4). Replacing ceased after year 15, when replacement was considered economically unviable. Additional costs (C_t) included MS1 costs plus replacing expense for tree purchase and planting labor. Additional returns (R_t) remained associated with downgraded fruit from symptomatic trees, while fresh production from replanted trees contributed to reducing reduced returns (R_0) over time. Cost savings (C_0) were lower than MS1 because fewer or no position were permanently eliminated (**Table S13**).

In UNS density remained constant, but symptomatic trees increased annually. All fruit from symptomatic trees were downgraded. Since no management occurred, additional costs and cost savings were both zero ($C_t = C_0 = 0$). Additional returns (R_t) originated exclusively from downgraded fruit, while reduced returns (R_0) reflected progressive losses in fresh apple production as disease prevalence increased unchecked (**Table S14**).

5.2.4 Production parameters and cost data

Production parameters were assumed consistent across all scenarios and were derived from budget analyses of typical Trentino farms (De Concini et al., 2024; Lang et al., 2024). The model orchard used spindle system on M9 rootstock with 0,80 m in-row and 3,0 m between-row spacing.

After accounting for 14,5 % non-productive area, the final density was 3.562 trees/ha. The orchard lifespan was set at 20 years, including a four-year establishment phase with production gradually increasing from 0% (year 1) to 20%, 50%, and 80% in the subsequent years, reaching full production (100%) from the fifth year onwards. Average yields were set at 60 t/ha for IPM orchard and 45 t/ha for organic orchard (ISMEA, 2025; Lang et al., 2024).

Farm-gate prices, based on 2020-2024 averages for the predominant cultivar cv Golden Delicious, were estimated at 0,45€/kg for IPM apples and 0,69€/kg for organic apples (ISMEA, 2025). Apples from AP-infected trees, which typically fail to meet EU marketing standards (EU Reg. No. 543/2011) due to reduced size, taste and coloration, were considered unsuitable for the fresh market (Herzog et al., 2012) and valued at industrial processing prices: 0,10€/kg for IPM and 0,18€/kg for organic production (ISMEA, 2025) (**Table 8**). The reduction in fruit size leads to a lower overall weight (van Boekel, 2024), causing a substantial decrease in yield (kg/tree) that has been reported to reach up to 91%(Herzog et al., 2012; Kaminska and Zawadzka, 1970b, 1970a; Zawadzka, 1976). To account for this aspect, a conservative 50% yield reduction factor was applied to all symptomatic trees in our computation.

Cost components in both MS1 and MS2 included labor and machinery for tree removal and, in MS2, for replacing trees. Labor rates were based on the Provincial Agricultural Collective Agreement (Provincia Autonoma di Trento, 2021): €15.74/hour for internal operations (e.g., uprooting, planting) and €9.45/hour for seasonal tasks (e.g., harvesting, thinning). Machinery costs were estimated accounting for depreciation, maintenance, and fuel (De Ros et al., 2015; Lang et al., 2024) (**Table 8**). The permanent loss of trees due to uprooting, without subsequent replacing, led to labor savings in seasonal operations no longer required (thinning, pruning, harvesting). These savings

were calculated based on the time required per plant, monetized using external labor rates (Lang et al. 2024). The estimated savings per uprooted tree amounted to €1,51.

Finally, pest control costs, applied to both managed scenarios, were based on local extension recommendations (De Concini et al., 2024). In IPM orchards, this included two applications of etofenprox (€85/L, 750 mL/ha) and one of tau-fluvalinate (€50/L, 600 mL/ha) per season. In organic orchards, three applications of pyrethrins (€40/L, 3 L/ha) were considered. In both systems, insecticide costs include only the active ingredients, assuming co-application with other plant protection products, typically fungicides for apple scab control.

Table 8: Base-case values and variation ranges (minimum–maximum) used in the partial budget analysis for IPM and organic apple orchards.

Parameters	**Base case**	**Max-Min**	**Notes**
IPM production			
Apple trees density (number/ha)	3562	Fixed	(Lang et al., 2024)
Yield per hectare (Kg/ha)	60000	70000 - 50000	(Lang et al., 2024)
Apple price (€/Kg)	0,45	0,50-0,33	(ISMEA, 2025)
Cull apple price (€/Kg)	0,10	0,09-0,13	(ISMEA, 2025)
Treatment cost(€/ha)	157.5	Fixed	(Baldessari, 2024)
Specialized labor cost (€/ha)	15.74	Fixed	Uprooting, replacing
Not specialized labor cost (€/ha)	9.45	Fixed	Harvesting, thinning
Cost saved from operations (€/plant)	1,51	Fixed	(Lang et al., 2024)
Uprooting cost (€/plant)	4,64	3,8 -6,38	This paper (Table S4)
Replacing cost (€/plant)	7,04	6,95-7,14	This paper (Table S4)
Organic production			

Parameters	Base case	Max-Min	Notes
Apple trees density (number/ha)	3562	Fixed	(Lang et al., 2024)
Yield per hectare (Kg/ha)	45000	50000 - 40000	(Lang et al., 2024)
Apple price (€/Kg)	0,69	0,85-0,62	(ISMEA, 2025)
Cull apple price (€/Kg)	0,18	0,16-0,21	(ISMEA, 2025)
Treatment cost(€/ha)	360	Fixed	(Baldessari, 2024)
Specialized labor cost (€/ha)	15.74	Fixed	Uprooting, replacing
Not specialized labor cost (€/ha)	9.45	Fixed	Harvesting, thinning
Cost saved from operations (€/plant)	1,51	Fixed	(Lang et al., 2024)
Uprooting cost (€/plant)	4,64	3,8 -6,38	This paper (Table S4)
Replacing cost (€/plant)	7,04	6,95-7,14	This paper (Table S4)

5.2.5 Field data collection for operational cost

The cost of tree uprooting was estimated using data collected in an experimental orchard located in Spagolle (Trentino, Italy, 46°02'54.8'N 11°28'37.8'E) where AP infected plants were rogued and all sequential operations (pruning, trunk cutting, stump extraction, residue shredding) were recorded to calculate labor time and machinery use. Average uprooting time was approximately 7 minutes per tree, with total cost of 4.64€/plant (**Table 8**, **Table S15**) To estimate the labor time required for planting an apple tree, field data were collected in an experimental orchard located in Piovi (Trentino, Italy; 46°13'59.7'N, 11°09'55.7'E), where AP-infected plants had been rogued and subsequent replacing operations were documented. The planting process included both the purchase

of new trees (€6.50 each) (De Concini et al., 2024; Lang et al., 2024) and planting labor, leading to a total cost of 7,04€/plant. (**Table 8**, **Table S15**).

5.2.6 Data on apple proliferation epidemiology at orchard level

Consistent long-term epidemiological data across full orchard lifespan are scarce with most studies covering 1–3 years (Baric et al., 2007; Herzog et al., 2012; Rizzoli and Acler, 2010; Šafářová et al., 2015). We therefore adopted a linear disease progression calibrated to estimated cumulative incidence by year 20 and based on literature and field observations cumulative incidence was set at 5% under IPM (0,25% yearly) (Baric et al., 2007; Herzog et al., 2012; Rizzoli and Acler, 2010), 10% under organic management (0,50% yearly) (Herzog et al., 2012; Bargna et al., 2024), and 60% in the unmanaged scenario (3% yearly) (Bliefernicht and Krczal, 1995; Blažek et al., 2005; Rizzoli and Acler, 2010).

The epidemic was initialized assuming one symptomatic plant in year 1-2. Additional simplifying assumptions were applied: (i) trees became symptomatic within the infection year, (ii) all fruit produced by symptomatic trees were downgraded and (iii) in MS2, replanted trees were assumed not to become infected again during the orchard lifespan.

5.2.7 Sensitivity analysis

To evaluate results robustness, we conducted one-way sensitivity analyses on epidemiological and economic parameters. For the epidemiological component, alternative epidemic trajectories replaced linear growth with exponential and periodic functions, both reaching identical final incidence (**Figure S26**). For MS2, reinfection on replanted plants was tested by exposing total replants to the same disease pressure generating additional removal/replacement costs and lost production (**Supplementary material**). Finally, we also relaxed the 100% symptomatic fruit assumptions, testing

scenarios where symptomatic trees produced 70% or only 20% downgraded fruit, with remainder marketable at the full price (**Supplementary material**).

In addition, key productive input parameters (healthy apples price, cull price, yield uprooting/replacing cost) were varied independently across reported ranges (**Table 8**). For each parameter, baseline NCI was compared to alternative parameterizations.

All analyses was performed with R software, v. 4.4.2.(R Core Team, n.d.).

5.3 Results

Partial budget analysis across IPM and organic systems reveled that both post-removal strategies involving led to net income losses compared to the BS scenario, though economic losses were substantially lower than in UNS. The magnitude and temporal dynamics of losses differed between management approaches and production systems. In IPM systems, MS1 resulted in cumulative additional costs (Ct) of 3.994€/ha, comprising vector control treatments (3.150€/ha) and uprooting operations (844€/ha). The implementation of the management strategy led to reduced returns (R_0) of 13.159 €/ha, reflecting forgone fresh apple sales relative to the BS scenario. However, savings from reduced operations on diseased trees (pruning, harvesting, thinning) partially offset losses (2.641€/ha), together with limited additional returns (R_t) from cull apple sales (146 €/ha). Overall, MS1 yielded a cumulative NCI of −14.367€/ha, over the orchard's 20-year lifespan, , corresponding to 2,9% of baseline gross returns (**Table 9**).

Table 9: Results of the PB analysis in different scenarios of IPM orchards versus the baseline scenario (absence of apple proliferation) during the orchard lifespan.

Partial budget elements	MS1(€)	MS2 (€)	UNS (€)
Additional returns (Rt)			
– Cull sales	145.84	145.84	17.077,68
Reduced costs (C0)			
– Savings from pruning, harvesting, thinning	2.640,99	226,50	0,00
Total benefit (Rt + C0)	2.786,83	372,34	17.077,68
Additional costs (Ct)	3994,48	4923,76	0.00
– Insecticide application	3150,00	3150,00	0.00
– Uprooting cost	844,48	844,48	0.00
– Replanting cost	—	928,28	—
Reduced returns (R0)			
fresh sales forgone	13.158,88	5.540,98	153.699,12
Total cost (R0 + Ct)	17.153,36	10.464,74	153.699,12
Net Change in Income (NCI)	**-14.366,53**	**-10.092,38**	**-136.621,47**
NCI per year	-718,3	-504,6	-6.831,07

MS2 incurred higher costs (4.924€/ha), due to inclusion of tree purchase and planting labor (928€ beyond MS1 costs). However, the replanting strategy substantially reduced income losses, with reduced returns limited to 5.541 €/ha, reflecting partial recovery of fresh production over time. Cost savings were more limited (227€/ha) as fewer plants were permanently eliminated, while additional returns from cull sales remained identical to MS1. The resulting NCI was −10.092€/ha, indicating that despite higher short-term costs, MS2 preserved orchard productivity more effectively than MS1 (**Table 9**), equivalent to 2,1% of baseline gross returns. By contrast, the UNS resulted in the steepest loss in returns, with reduced returns reaching 153.699 €/ha, driven by progressive yield loss and fruit downgrading as disease prevalence increased unchecked. No cost savings were generated, and

additional returns from cull sales were insufficient to compensate for losses, leading to a cumulative NCI of −136.621€/h , which represents nearly 29% of baseline gross returns (**Table 9**).

Under organic management, higher product prices and low yield amplified losses while maintaining similar relative patterns to those observed under IPM. MS1 generated additional cost of 8.880€/ha, mainly associated with vector control (7.200€/ha) and uprooting operations (1.680€/ha). Reduced returns due to forgone fresh sales amounted to 29.960 €/ha, partially offset by substantial cost savings (5.223€/ha) from avoided orchard operations and additional returns from cull sales (394 €/ha). The cumulative NCI was -33.223,33€/ha (**Table 10**).

MS2 again proved to be the most effective strategy: despite higher additional cost (10.724 €/ha including 1.844€/ha for replacing expenses), the recovery of productive capacity substantially limited reduced returns to 12.701 €/ha, while cost savings were smaller (453 €/ha). The resulting NCI was −22.578,22€/ha, confirming the economic advantage of replanting under organic conditions over the long term (**Table 10**). As in IPM systems, the UNS produced the highest losses, with reduced returns reaching 176.754€/ha as a consequence of uncontrolled disease progression, resulting in a cumulative NCI of –153.699 €/ha (**Table 10**).

Table 10: Results of the partial budget analysis (PBA) in different scenarios of organic orchards versus the baseline scenario (absence of apple proliferation).

Partial budget elements	MS1(€)	MS2 (€)	UNS (€)
Additional returns (Rt)			
– Cull sales	393,63	393,63	23.054,87
Reduced costs (C0)			
– Savings from pruning, harvesting, thinning	5.223,09	453,00	0
Total benefit (Rt + C0)	5.616,72	846,63	23.054,87
Additional costs (Ct)	8.879,68	10.724,16	0
– Insecticide application	7.200,00	7.200,00	—
– Uprooting cost	1.679,68	1.679,68	—
– Replanting cost	—	1.844,48	—
Reduced returns (R0)			
fresh sales forgone	29.960,42	12.700,68	176.754,01
Total cost (R0 + Ct)	38.840,10	23.424,84	176.754,01
Net Change in Income (NCI)	**-33.223,38**	**-22.578,21**	**-153.699,12**
NCI per year	-1.661,17	-1.128,91	-7.684,956

Cumulative economic losses exhibited distinct temporal patterns across scenarios and management systems. Under both IPM and organic regimes, UNS resulted in the most severe economic outcomes, with losses accumulating rapidly and exponentially from the second half of the orchard's productive life (**Figure 24**). In contrast, MS1 showed a more gradual and linear accumulation of losses driven by progressive decline in tree density while MS2 exhibited a distinct two phases pattern: higher early costs associated with replacing investments followed by progressive slower net income loss as newly planted trees began contributing to production. This led to a shift in economic advantage: MS2 became more cost-effective than MS1 after year 12 under organic and year 13 under IPM management (**Figure 24**). Relative to UNS, both MS1 and MS2 outperformed UNS from year 4 (IPM) and year 5 (Organic) (**Figure 24**).

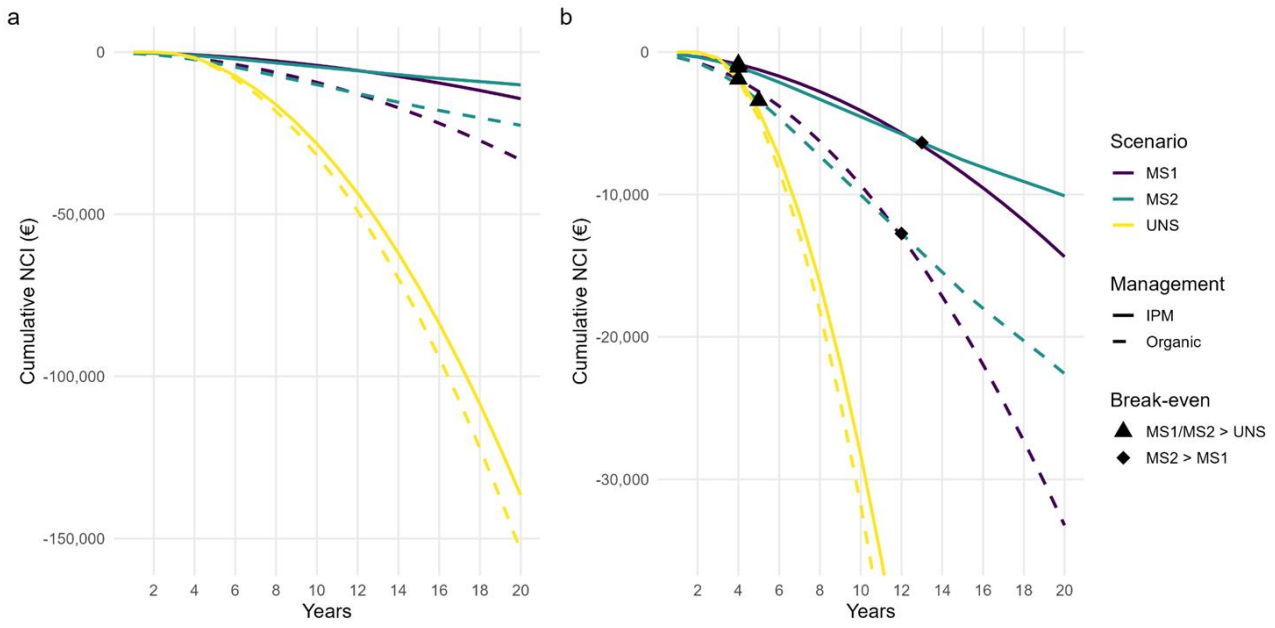

Figure 24: Cumulative NCI (€/ha) over the 20-year orchard lifespan across management scenarios and strategies. Panel (a) shows the cumulative NCI for three strategies (MS1 management and no replacing, MS2 management and replacing, UNS no management) under two management systems (IPM = solid lines; Organic = dashed lines). Panel (b) focuses on the timing of relative break-even points: black triangles mark the year when MS1 or MS2 become more convenient than UNS, while black diamonds indicate the year when MS2 becomes more advantageous than MS1.

One-way sensitivity analysis highlighted that profitability was more responsive to epidemiological assumptions than to economic parameters (**Table 11**). Among epidemiological inputs, epidemic growth form (linear vs exponential) exerted the largest influence. Replacing linear growth with exponential progression reduced cumulative losses by 26-71% across scenarios, with greatest effects in UNS, where exponential growth concentrated infections late in orchard life and production was affected drastically only in the last productive years (**Table 11**). Conversely, periodic epidemic growth simulating two peak of infection worsened economic outcomes by 2 % in managed scenario, up to 14% in UNS (**Table 11**). Reinfection of replanted trees in MS2 increased losses by 2,4% under both production systems, a relatively modest penalty suggesting that replacing remains economically viable even under moderate reinfection pressure (**Table 11**). Variation in symptom expression (70% or 20% symptomatic fruit versus 100% baseline assumption) had minor effects on managed scenarios (1-4% NCI variation) but substantial effects on UNS (13-35% variation) due to higher cumulative disease

burden, indicating that symptom variability primarily affects economic outcomes under high disease prevalence (**Table 11**).

Table 11: Results of the one-way sensitivity analysis for epidemiological parameters under IPM and Organic management in scenarios MS1–UNS. Values indicate the percent change in cumulative NCI relative to the baseline (linear epidemic growth and the reference infection rate).

	IPM			**Organic**		
Parameter	**MS1**	**MS2**	**UNS**	**MS1**	**MS2**	**UNS**
Exponential growth	-45,0%	-26,3%	-71,0%	+48,2%	+27,9%	+71,0%
Periodic growth	+6,4%	+2,2%	+13,7%	-8,9%	-3,9%	-13,7%
Reinfection replanted	-	+2,65%	-	-	+2,4%	-
70% cull apple	-1,07%	-1,52%	-13,12%	-1,0%	-1,5%	-12,8%
20% cull apple	-2,84%	-4,05%	-35,0%	-2,7%	-4,0%	-34,0%

For economic parameters, tornado plots (**Figure 25**) showed that profitability was most sensitive to the price of healthy fruit, yield, and the price of cull fruit. Variation in the costs of removal and replacing had negligible effects. Under IPM, changes in price of healthy fruit and yield had a comparable impact, whereas under organic, profitability was more sensitive to fruit price variation due to the higher baseline incidence of symptomatic trees. In all cases, increases in price and yield reduced losses, whereas higher culls price dampened the benefits of management, especially under UNS (**Figure 25**).

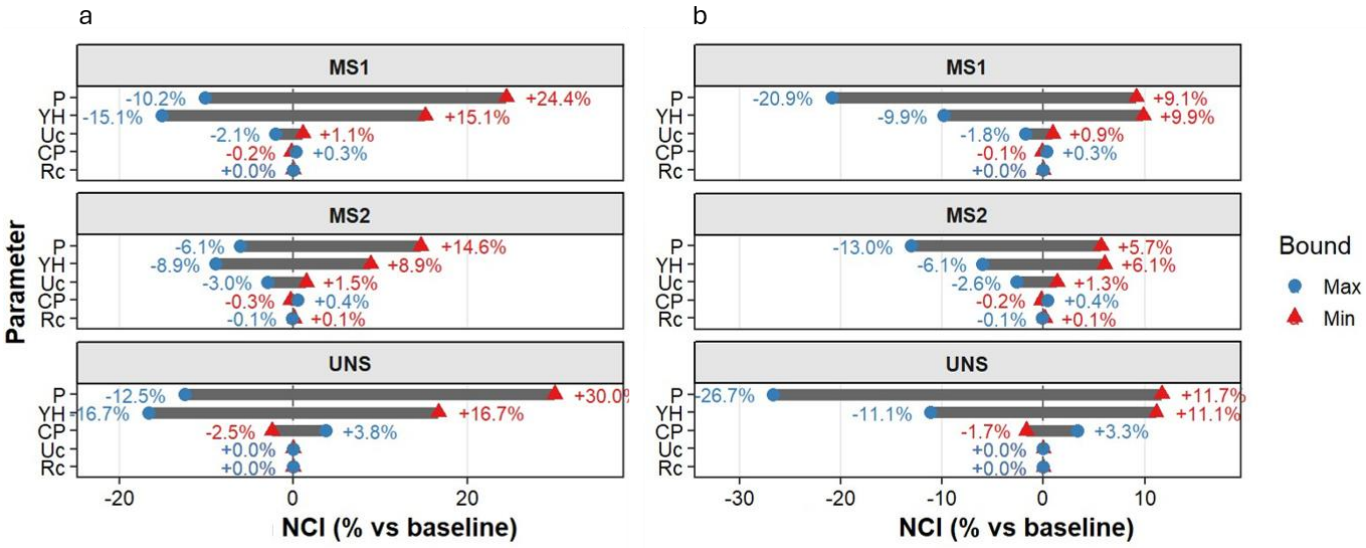

Figure 25: Tornado plots illustrating the one-way sensitivity analysis of total NCI (NCI, €/ha over 20 years) to economic inputs under (a) IPM and (b) Organic management across scenarios MS1–UNS. Each horizontal bar represents the range of NCI outcomes when varying a single parameter across its tested bounds; the baseline is centered at zero. Blue circles mark the effect of the upper bound of the parameter, and red triangles the effect of the lower bound. Parameters are ordered vertically by the magnitude of their impact. The baseline corresponds to linear epidemic growth and the reference infection rate. Where P is healthy fruit price, YH is yield, CP is culls price, Uc is removal costs and Rc is replacing costs.

5.4 Discussion

This study provides the first comprehensive economic evaluation of post removal management options for apple proliferation at the orchard scale, combining yield losses, quality downgrading and management cost over a realistic productive lifespan. Applying PB analysis, we quantified long-term economic consequences of the two practical management options available to Trentino growers under current regulatory requirements.

Our results demonstrate that MS2 consistently outperforms MS1 over typical orchard lifespans, despite incurring 23-24% higher direct management costs. MS2 reduced cumulative net losses by 30% (IPM) and 32% (organic) relative to MS1, with economic advantage becoming apparent after year 12-13 as replanted trees reached productive maturity. This temporal crossover provides clear guidance for investment decisions: while MS1 minimizes short-term expenditure, MS2 preserves

long-term orchard profitability through sustained productive capacity. The UNS demonstrates the economic benefit of Trentino's mandatory removal policy. Without intervention, orchards would experience losses exceeding 28% of gross returns, validating the regulatory requirement for active disease management. Both MS1 and MS2 outperformed UNS from year 4–5 onwards, indicating that even minimal compliance with removal requirements provides immediate economic benefits. This finding supports the continuation of mandatory management policies and suggests their potential value in other AP-affected regions currently lacking regulatory frameworks.

The unmanaged scenario presented here is likely conservative rather than alarmistic. For modelling consistency, disease progression was represented as a simplified linear increase, but in fully unmanaged orchards the epidemiological trajectory could plausibly accelerate over time, as infected trees continue to act as inoculum sources and vector pressure may increase. Similar concerns have been reported in other phytoplasma pathosystems, where disease dynamics can be strongly nonlinear and, under some conditions, increase exponentially rather than linearly (Morone et al., 2007; Lessio et al., 2015). Although AP differs from FD and long-term datasets from unmanaged orchards are still lacking, these studies support the view that the losses estimated here for the unmanaged scenario are likely conservative and may underestimate the long-term benefit of active management.

Our findings also refine previous estimates of AP economic impact. Herzog et al. (2012) calculated orchard-level losses of 38–189€ ha^{-1} at 2.3% incidence, focusing exclusively on fruit downgrading without accounting for management costs or long-term productivity effects. Direct comparison with our findings is difficult because their production conditions differed markedly from those of Trentino orchards. For example, they reported cull rates of up to 50% even in healthy trees, whereas this fraction is negligible in modern Trentino orchards (Badiu et al., 2015; Bakhtaver et al., 2020) substantially altering baseline economics. At broader scale, Strauss (2009) reported national

losses of 100€ million for Italy, though methodological details were not provided. To assess plausibility, we applied our MS2 model to Trentino 2023 context (1.3% average AP incidence; (Bargna et al., 2024) obtaining annual regional impact of −9.6€ million (−8.6€ million in IPM and −0,99€ million in organic orchards) (**Table S16**). These values capture both immediate losses and replacing investments, which, while increasing short-term expenditure, also represent an investment in future productivity. Although we refrained from projecting long-term regional outcomes due to uncertainties in orchard age structure and epidemic trajectories, the order of magnitude suggests that cumulative impacts could reach values consistent with Strauss's national-level estimate.

In addition, our analysis evidenced that organic orchards face higher economic impacts from AP, with losses of −6.0% (MS1) and −4.0% (MS2) compared to −2.9% and −2.1% in IPM systems. This disparity stems from higher baseline disease incidence (10% vs. 5% over 20 years), and higher per-unit product values, which amplify the cost of yield losses. These findings highlight AP as an additional threat to the economic sustainability of environmentally friendly production systems, beyond existing challenges of lower productivity and restricted input availability.

The sensitivity analysis revealed that NCI was more responsive to epidemiological parameters than economic parameters, indicating that improved understanding of AP epidemiology would yield greater benefits for management optimization than refinement of economic parameters. Key uncertainties in vector-borne disease include: spatial and temporal dynamics of disease spread under different vector pressure scenarios, reinfection risk in replanted trees and its interaction with orchard age and vector control efficacy, quantitative cultivar susceptibility data for modern cultivars and recovery rates in commercial dominant cultivars (Islam et al., 2020; Lessio et al., 2015). Investment in comprehensive monitoring programs tracking disease progression from orchard establishment

through removal would improve predictive capacity and enable more sophisticated economic modeling.

Despite these limitations PB analysis offer important advantages for applied decision-making. The methodology requires relatively limited data, provides transparency and reproducibility, and generates actionable insights for growers and policymakers (Galinato et al., 2019; Soliman et al., 2010). Our framework can be readily applied for *ex post* assessments in affected orchards, planning new plantings in areas with known AP incidence, or for *ex ante* evaluations of novel control options. Importantly, the approach provides quantitative complement to qualitative PRA, helping policymakers assess the economic viability of different management based on objective, repeatable analyses (Del Fava et al., 2017; Galinato et al., 2019; Soliman et al., 2010; Straubinger et al., 2023).

Finally, our simulations demonstrate that even low AP incidence levels result in economic losses. For this reason, durable solutions through resistant rootstocks or tolerant cultivars represents the most viable long term option. Some *Malus domestica* cultivars (e.g., 'Lord Lambourne,' 'Clivia,' 'Melrose') have been empirically noted as relatively tolerant to AP, although these observations are not yet supported by targeted infection trials (Janik et al., 2020). Breeding programs focusing on resistant rootstocks show particular promise given phytoplasma's persistent colonization of root system (Kartte and Seemüller, 1991, 1988). Hybrids with *Malus sieboldii* have shown resistance to AP while maintaining acceptable agronomic traits, but further evaluation of their pomological qualities is still required, and the gradual replacement of orchards will inevitably take decades (Ciccotti et al., 2011; Jarausch et al., 2011; Seemüller et al., 2018). In the short to medium term, therefore, management will continue to rely on orchard sanitation and vector control options, with economic considerations remaining central to guiding both grower decisions and policy priorities.

Conclusion

In conclusion, our study provides decision-relevant economic guidance for apple growers managing AP under mandatory removal requirements. Scenario MS2 proves economically superior to MS1 over typical orchard lifespans, despite higher initial costs. The economic crossover occurs after 12–13 years, providing a clear planning horizon for investment decisions. The UNS scenario validates the economic benefit of mandatory management policies, demonstrating they prevent losses exceeding 28% of gross returns. Sensitivity analysis reveals that improved epidemiological knowledge would enhance decision-making more than refinement of economic parameters, highlighting future research priorities. For organic production systems, AP represents a disproportionate economic threat, emphasizing the need for resistance-based solutions. The partial budgeting framework presented here can be adapted for other AP-affected regions and provides a quantitative foundation for policy evaluation and grower decision support.

5.5 Supplementary materials

Table S12: Partial budget equations used to determine the net change in income between managed scenario without replacement (MS1) versus the baseline scenarios (BS).

Cost	**Benefits**
$$C_t = \sum_{n=1}^{N} Tr_n + (Uc \cdot AP_n)$$	$$R_t = \sum_{n=1}^{N} AP_n \cdot \left(\frac{YH_n}{TP}\right) \cdot YR \cdot CP_n \cdot fr_n$$
$$R_0 = \sum_{n=1}^{N} RBS_N - PH_N$$	$$C_0 = \sum_{n=1}^{N} \left(\sum_{J=1}^{n} AP_j\right) \cdot Oc$$
$$RBS_N = \sum_{n=1}^{N} YH_n \cdot P_n \cdot fr_n$$	
$$PH_N = \left[TP - \left(\sum_{j=1}^{n} AP_j\right)\right] \cdot \left(\frac{YH_n}{TP}\right) \cdot P_n \cdot fr_n$$	
$$Total\ cost = (C_t + R_0)$$	$$Total\ benefits = (R_t + C_0)$$

$$Net\ change\ in\ income\ NCI_{t0} = (R_t + C_0) - (C_t + R_0)$$

Where: N = Orchard lifespan (20 years); n = year (1 to 20); Tr = Treatment cost (€/ha); Uc = Uprooting cost (€/plant); AP = Number of affected trees; YH = Yield per hectare (kg/ha); P = Apple price (€/kg); CP = Cull apple price (€/kg); fr_n = Proportion of yield during establishment; Oc = Operation cost savings (€/plant); TP = Tree density (number/ha); PH = Production of healthy plants; PD = Production from affected plants before uprooting; YR = Yield reduction factor in affected plants

Table S13: Partial budget equations used to determine the net change in income between managed scenario with replacement (MS2) versus the baseline scenarios (BS).

Cost	**Benefits**
$$C_t = (Cr_n + Cu_n)$$ $$Cr_n = \sum_{n=1}^{15} Tr_n + [AP_n \cdot (Uc + Rc)]$$ $$Cu_n = \sum_{n=16}^{N} Tr_n + (Uc \cdot AP_n)$$	$$R_t = \sum_{n=1}^{N} AP_n \cdot \left(\frac{YH_n}{TP}\right) \cdot YR \cdot CP_n \cdot fr_n$$
$$R_0 = \sum_{n=1}^{N} RBS_N - (PH_N + PR_n)$$ $$RBS_N = \sum_{n=1}^{N} YH_n \cdot P_n \cdot fr_n$$ $$PH_n = \left[TP - \left(\sum_{j=1}^{n} AP_j\right)\right] \cdot \left(\frac{YH_n}{TP}\right) \cdot P_n \cdot fr_n$$ $$PR_n = \left(\sum_{j=1}^{min(n-1,\ 15)} AP_j \cdot fu_{n-j}\right) \cdot \left(\frac{YH_n}{TP}\right) \cdot P_n$$ $$Total\ cost = (C_t + R_0)$$	$$C_0 = \sum_{n=16}^{N} \left(\sum_{j=16}^{n} AP_j\right) \cdot Oc$$ $$Total\ benefits = (R_t + C_0)$$

$$Net\ change\ in\ income\ NCI_{t0} = (R_t + C_0) - (C_t + R_0)$$

Where: N = Orchard lifespan (20 years); n = year (1 to 20); Tr = Treatment cost (€/ha); Uc = Uprooting cost (€/plant); AP = Number of affected trees; YH = Yield per hectare (kg/ha); P = Apple price (€/kg); CP = Cull apple price (€/kg); fr_n = Proportion of yield during establishment; Oc = Operation cost savings (€/plant); TP = Tree density (number/ha); PH = Production of healthy plants; PD = Production from affected plants before uprooting; YR = Yield reduction factor in affected plants, Rc = Replacing cost (€/plant); PR = Replanted plant production; fu_n = Proportion of yield during establishment after replacing (fu_1=0; fu_2=0; fu_3=0,2; fu_4=0,5; fu_5=0,8; fu_6..fu_N=1).

Table S14: Partial budget equations used to determine the net change in income between managed scenario with replacement (UNS) versus the baseline scenarios (BS).

Cost	**Benefits**
$$C_t = 0$$	$$R_t = \sum_{n=1}^{N} AP_n \cdot \left(\frac{YH_n}{TP}\right) \cdot YR \cdot CP_n \cdot fr_n$$
$$R_0 = \sum_{n=1}^{N} RBS_N - PH_N$$ $$RBS_N = \sum_{n=1}^{N} YH_n \cdot P_n \cdot fr_n$$ $$PH_N = \left[TP - \left(\sum_{j=1}^{n} AP_j\right)\right] \cdot \left(\frac{YH_n}{TP}\right) \cdot P_n \cdot fr_n$$	$$C_0 = 0$$
$$Total\ cost = (C_t + R_0)$$	$$Total\ benefits = (R_t + C_0)$$
$$Net\ change\ in\ income\ NCI_{t0} = (R_t + C_0) - (C_t + R_0)$$	

Where: N = Orchard lifespan (20 years); n = year (1 to 20); AP = Number of affected trees; YH = Yield per hectare (kg/ha); P = Apple price (€/kg); CP = Cull apple price (€/kg); fr_n = Proportion of yield during establishment; TP = Tree density (number/ha); PH = Production of healthy plants; PD = Production from affected plants before uprooting; YR = Yield reduction factor in affected plants

Table S15: Time (min: s) and cost estimation for uprooting AP-infected apple tree (n= 10) in the experimental orchard Spagolle (Trentino, Italy).

	Pruning	Trunk cutting[1]	Branch raking[1]	Shredding[1]	Stump removal	Pit cleaning	Total time
Mean	**02:57**	**00:25**	**00:32**	**00:43**	**01:15**	**01:31**	**07:23**
SD	00:41	00:00	00:00	00:00	00:16	00:20	00:41
Min	02:20	00:25	00:32	00:43	00:54	01:05	06:32
Max	04:23	00:25	00:32	00:43	01:52	02:02	08:38

Operation	**Labor cost (€/plant)[2]**	**Tractor cost (€/plant)[3]**	**Machine/tool cost (€/plant)[4]**
Pruning (2 operators)	1,54	0,00	0,00
Trunk cutting (chainsaw)	0,11	0,00	0,03
Branch raking	0,14	0,00	0,00
Shredding of pruning residues	0,19	0,30	0,35
Stump removal (2 operators)	0,66	0,52	0,00
Pit cleaning	0,80	0,00	0,00
Total per tree	**4,64**		

[1]For trunk cutting, branch raking, and shredding, the total time required for the entire batch of uprooted plants was recorded as a single operation and then divided by the number of trees to obtain a per-plant estimate.[2]Labor cost: 15.74€/hour (Provincial Agricultural Collective Agreement, 2021). [3]Tractor cost: 25.00€/hour (Lang et al., 2024; De Ros et al., 2015). [4]Shredder cost 29,4 €/hour ((Lang et al., 2024), chainsaw cost: 4.80€/hour (2024 Provincial Equipment Price List), manual shears cost negligible (not included).

Time (min: s) and labor cost estimation for replacing apple tree (n= 10) in the experimental orchard Piovi (Trentino, Italy).

	Planting operations[1]		
Mean	**02:05**	**Min**	01:42
SD	00:15	**Max**	02:26

Operation	Labor cost (€/plant)[1]	Tractor cost (€/plant)	Machine/tool cost (€/plant)
Planting operation	0,54	0,00	0,00
Total per tree	**0,54**		

[1] Planting operations included digging the planting hole, cleaning the pit, positioning the new plant, backfilling, and tying the tree to its support. Total time per plant was recorded directly in the field for each complete replacing sequence. Labor cost: 15.74€/hour (Provincial Agricultural Collective Agreement, 2021)

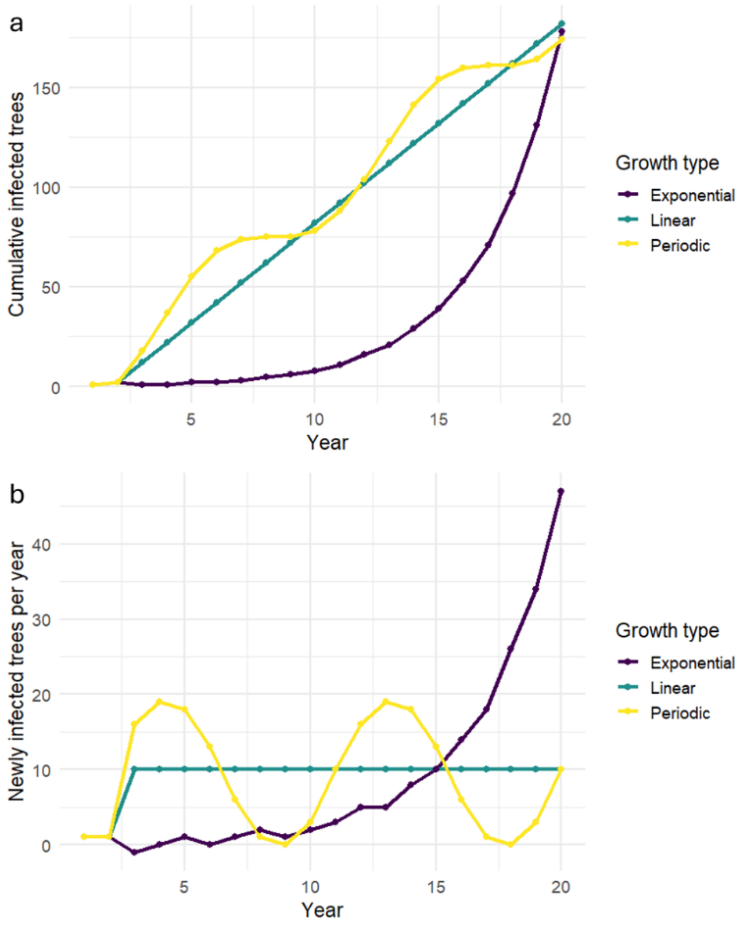

Figure S26: Example of alternative epidemic growth patterns tested in the sensitivity analysis for IPM management. (a) Newly infected trees per year (b) cumulative number of symptomatic trees over time. The three growth curves were parameterized to reach the same final incidence (5% of trees at year 20). Linear progression was modeled as a constant increment per year, exponential growth was calibrated so that year 20 incidence matched the linear case, and the periodic curve was simulated using a sinusoidal function scaled to the same cumulative endpoint.

Replant reinfection cost (RRC)

To account for the additional burden of reinfections in replanted trees (MS2), we introduced a replant reinfection cost (RRC). We assumed that replants are exposed to the same epidemic pressure as the rest of the orchard, and that therefore a proportion ρ_r of them becomes reinfected during the 20-year horizon (5% under IPM, 10% under organic management). Let R denote the total number of replants over the simulation period. Each reinfected replant generates (i) direct costs for removal and replacement ($Uc + Rc$), and (ii) indirect costs due to foregone yield during the juvenile phase. The latter is calculated as the difference between the potential yield of a mature tree (YH/TP) and the cumulative yield of a replant during its first five years, described by the production curve $f_u(a)$. The aggregated cost is:

$$RRC = R \cdot \rho_r \cdot [\,(Uc + Rc) + P \cdot \frac{YH}{TP} \cdot (5 - \sum_{a=1}^{5} f_u\,(a)\,]$$

This penalty term was subtracted from the total 20-year NCI.

Adjustment of symptomatic fruit revenues

In the baseline, symptomatic trees are assumed to produce only downgraded apples, all marketed at the cull price (CP). To reflect possible partial recovery of marketable production, we allowed only a fraction q_s (70% or 20%) of symptomatic apples to be downgraded, while the remaining share was sold at the price of healthy apples (P). This assumption modifies the calculation of symptomatic fruit production (PD_n), originally defined as:

$$PD_n = AP_n \cdot \left(\frac{YH_n}{TP}\right) \cdot CP_n \cdot fr_n$$

where AP_n is the number of symptomatic trees in year n, YH_n is the potential yield of a healthy tree, TP is the total tree population, and fr_n is the age-dependent production factor. Under the revised assumption, the formula becomes:

$$PD_n(q_s) = AP_n \cdot \left(\frac{YH_n}{TP}\right) \cdot fr_n \cdot [q_s \cdot CP_n + (1 - q_s) \cdot P]$$

Table S16: Partial budget calculation of the economic impact of apple proliferation (AP) in Trentino, year 2023 (1,3 % symptomatic apple plants), under management strategy MS2 (removal and replacing of symptomatic trees in the same year).

System	Baseline revenue* (€ /ha)	Revenue with AP (€ /ha)	Direct costs (€ /ha)**	Cost savings (€ /ha)	€ (NCI, /ha)	Area (ha)	Regional NCI (€)
IPM	28,000.00	27,668.50	698.35	69.92	−959.93	9,000	−8,639,388.18
Organic	36,500.00	36,084.00	900.85	69.92	−1,246.93	800	−997,545.62
Total	—	—	—	—	—	9,800	−9,636,933.80

*Calculations were based on the average yield of Trentino apple production (50.000 kg/ha), an estimated planting density of 3.562 trees/ha, an average apple price in 2020-2024 of the 5 main cultivars of Trentino (Golden Delicious, Fuji, Red delicious, Gala, Stark delicious) 0,56 €/kg (IPM), 0,73 €/kg (organic) apples, culls price 0,10 €/kg (IPM) and 0,18€/kg (organic) (ISMEA, 2025).

**Direct costs include additional pesticide applications, uprooting, and replacing of symptomatic trees.

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CHAPTER 6. CONCLUSION

Apple proliferation remains a major constraint for European apple production and is particularly relevant in Trentino, a highly specialized apple-growing region where recurrent epidemic phases have substantial economic consequences. Following the renewed increase in AP observed in recent years (2019–present), the emergence of unusually abundant populations of the invasive leafhopper *O. ishidae* in local orchards represented a credible candidate explanation. This hypothesis was further supported by the absence of a clear concurrent increase in psyllid vector populations and by the biological traits of *O. ishidae*, including polyphagy and its documented involvement with phytoplasmas in other pathosystems. For these reasons, this thesis approached the problem through a multi-level assessment conducted in parallel, combining field ecology, experimental transmission work, within-insect infection dynamics, crop-interface ecology, and economic analysis.

Chapters 2 and 4 establish that apple orchards can act as strong demographic sources for *O. ishidae*, particularly under organic management. In addition, natural acquisition of AP phytoplasma occurs at detectable frequencies, reaching up to 18% natural infection rate. These observations confirm sustained AP exposure in the orchard system and, from a risk perspective, justify why *O. ishidae* required rigorous evaluation rather than dismissal. Chapter 3, however, resolves the central epidemiological question: despite repeatable acquisition after exposure to infected plant material across multiple experimental configurations, vector competence for AP was not demonstrated. Phytoplasma concentrations remained extremely low and no transmission to test plants was observed, despite different subtypes, AAP time and initial leaves concentration. Importantly, the time-course infection dynamics provide strong mechanistic negative evidence that is coherent with phytoplasma biology: progressive loss of detectability over latency is inconsistent with a persistent-propagative

pathway requiring sustained infection and salivary gland colonization. Chapter 4 extends the inference to the apple–vine interface by showing that cultivated grapevine is not a suitable host for *O. ishidae* development and supports only very short adult survival. Together with the reduced abundance observed in vineyards, these constraints strongly limit the plausibility that apple orchards constitute a meaningful source of vineyard risk under current Trentino conditions. Ongoing work on additional cultivated hosts (notably hazel) remains important to confirm whether these constraints hold across other crops that may be ecologically more relevant to FD-related scenarios.

From an applied standpoint, demonstrating that a highly abundant, invasive, polyphagous leafhopper does not contribute to AP epidemiology is a substantial and reassuring outcome. If *O. ishidae* had been a competent AP vector, the management challenge could have expanded dramatically. Instead, the thesis narrows the plausible causal space for AP resurgence and helps prevent misdirected responses driven by abundance or by molecular detection alone. At the same time, this case study supports a broader operational principle, namely that phytoplasma diseases require continuous vigilance. Even in the absence of newly emerging vectors, sustained monitoring remains essential to detect future introductions, identify shifts in the abundance of confirmed vectors, and enable timely responses before inoculum and transmission pressure accumulate to epidemic levels.

The work also highlights methodological implications for future vector assessments. The classical plant–insect–plant transmission framework remains essential, but increasingly robust inference depends on incorporating factors that are now widely recognized as influential, such as subtype/strain effects, latency design, together with interpretable within-vector dynamics. In particular, pairing transmission assays with time-course tracking of infection signals provides an internal consistency check that strengthens conclusions when transmission outcomes are negative. This integrated approach helps discriminate true epidemiological threats from incidental associations

and should be considered a default standard when evaluating emerging hemipterans in complex agroecosystems.

Finally, Chapter 5 connects these biological insights to decision-making. Because there is no practical intervention threshold for phytoplasma diseases in the conventional sense, management is better conceptualized as maintaining an “attention threshold”: continuous surveillance and timely action aimed at systematically removing inoculum and limiting transmission by confirmed vectors. The PB analysis translates this logic into grower-relevant outcomes by quantifying the long-term economic consequences of delayed or absent control and by comparing post-removal options in economic terms. In doing so, it provides an additional, quantitative motivation for compliance and supports the rationale that effective AP control depends less on episodic reactions and more on sustained stewardship.

Several research directions emerge naturally from the findings of this thesis. Regarding *O. ishidae* specifically, while this work demonstrates its inability to transmit '*Ca.* P. mali’ under current conditions, its status as a non-vector should not be considered definitive. Vector competence can evolve, particularly in the context of biological invasions where host-range expansion and co-evolutionary dynamics between insects and pathogens are ongoing. Population-level genomic studies ,analogous to those conducted on *C. melanoneura*, could reveal whether genetic structuring within *O. ishidae* populations influences acquisition capacity, and whether locally adapted lineages exist that interact differently with AP phytoplasma. Complementarily, taking advantage of the sequenced genomes of '*Ca.* P. mali' and of phytoplasmas efficiently transmitted by *O. ishidae*, comparative genomic approaches could help identify the molecular determinants of vector competence and the barriers that currently prevent productive infection in this species.

Beyond the transmission question, the direct feeding damage caused by *O. ishidae* on apple foliage warrants independent investigation. Although foliar yellowing is unlikely to constitute a primary agronomic concern on its own, the potential interaction between phytoplasma-weakened trees and leafhopper infestations remains unquantified. In this context, it would also be worth exploring whether *O. ishidae* shows preferential attraction to AP-infected trees, as phytoplasma effectors are known to manipulate plant volatile profiles to influence vector behavior, even in species that are not competent vectors.

In contexts beyond apple orchards, *O. ishidae* remains epidemiologically relevant as a confirmed and highly efficient vector of Flavescence dorée. Its inability to establish on grapevine in northern Italy does not preclude a significant role in maintaining FD inoculum in the surrounding landscape, particularly in regions where the primary vector *S. titanus* is absent or scarce. Future work should therefore evaluate its contribution to FD epidemiology across diverse agroecological contexts, including areas currently free from FD or from its established vector. The host-preference data generated here could also inform applied strategies such as push-and-pull approaches aimed at manipulating *O. ishidae* distribution at the landscape scale.

More broadly, this thesis highlights that AP remains a disease with many epidemiologically underexplored dimensions. The economic analysis revealed that management outcomes are more sensitive to epidemiological parameters than to cost variables, underscoring the need for improved long-term monitoring of vector populations, infection dynamics, and disease spread. Building predictive models with adequate resolution will require sustained, multi-year datasets integrating entomological, phytopathological, and landscape data, an investment that would substantially improve both the scientific understanding and the practical management of this economically important disease.