Research Article

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Molecular droplet digital PCR diagnostics and bioassays for monitoring insecticide resistance status in *Myzus persicae* populations from Greece

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Abstract

BACKGROUND: *Myzus persicae* (Sulzer), is a major global pest whose control is challenged by widespread insecticide resistance. This study assessed the resistance status of *M. persicae* populations collected in Greece (2021–2025), using diagnostic bioassays with insecticides (acetamiprid, flupyradifurone, flonicamid, sulfoxaflor and FliPPER) and a newly developed, highly sensitive droplet digital polymerase chain reaction (ddPCR) panel targeting seven key resistance mutations (vgsc: *super-kdr* M918T/L and *kdr* L1014F – pyrethroid resistance; acetyl-CoA carboxylase: A2666V – keto-enol resistance; AChE: MACE S431F – dimethyl carbamates resistance; nAChR: R81T and T74I linked to *CYP6CY3* overexpression – resistance to nAChR competitive modulators).

RESULTS: Bioassays revealed frequent cross-resistance cases. Notably, we report the first case of resistance to flonicamid. FliPPER (fatty acids potassium salts) showed the highest resistance frequency (50.0%) and sulfoxaflor the lowest (1.7%). The ddPCR analysis (on 634 aphids) confirmed the presence of six resistance mutations. The mutation T74I was almost fixed across all populations (mean resistant allele frequency of 99.9%). Other key mutations (MACE, *kdr*/*super-kdr* and R81T) were present at moderate-to-high frequencies, with the M918L *super-kdr* variant showing a notable increase.

CONCLUSIONS: *Myzus persicae* in Greece exhibits a high level of genetic variability, leading to severe and dynamic resistance problems. The widespread metabolic resistance (*CYP6CY3* overexpression) which has been found to compromise neonicotinoids and fluryradifurone and the emergence of flonicamid resistance complicate the implementation of integrated pest management. The ddPCR panel provides a valid, evidence-based tool for large-scale monitoring of resistance and decision-making, essential for managing this difficult to control pest. However, bioassays are also useful when molecular markers are not available.

Supporting information may be found in the online version of this article.

Keywords: bioassays; chemical control; ddPCR; diagnostics; insecticide resistance mechanisms

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1 INTRODUCTION

Myzus persicae (Sulzer) (Hemipetra: Aphididae) known as the green peach-potato aphid (*M. persicae* s.str.) or the tobacco aphid (*M. persicae nicotianae* Blackman) is one of the most important agricultural insect-pest worldwide. The aphid is an exceptional species, that bears special traits being: (i) extremely polyphagous with a host range of over 400 plant species from 40 plant families,[1] (ii) highly efficient plant-virus vector, (iii) highly invasive as it has been recorded on all continents where crops are grown with widespread general-purpose genotypes,[2,3] (iv) expressing a complex life cycle combining sexual and asexual reproduction,[4] and cases of host-mediated speciation,[5,6] and last (v) showing great potential to counteract chemical pest control practices.[7,8]

These traits enable *M. persicae* to adapt and survive even in cases of harsh conditions such as today's agricultural agroecosystems. The control of the aphid worldwide and in Greece is mostly based on chemical control with insecticides of various chemical classes. The reliance on insecticides for many decades has resulted in the development of resistance. A total of 522 cases of insecticide resistance have been reported for *M. persicae* on the Arthropod Pesticide Resistance Database, involving 87 insecticide active ingredients (www.pesticidresistance.org). To date, at least nine independent mechanisms of resistance to insecticides have been described in the aphid, related to metabolic resistance, insensitive target sites and reduced penetration of insecticides through the cuticle.[8]

Functionally validated target side mutations in *M. persicae* have been described in the gene encoding the voltage-gated sodium channel (vgsc: *super-kdr* M918T/L, *kdr* L1014F) associated with pyrethroid resistance,[9–12] the acetyl-CoA carboxylase (acc: A2666V) associated with keto-enol resistance,[13] the nicotinic acetylcholine receptor (nAChR: R81T) associated with resistance to neonicotinoids and other nAChR competitive modulators,[8,14–16] the acetylcholinesterase (AChE: MACE S431F) conferring resistance to dimethyl carbamates,[17] and mutations in detoxification enzymes (T74I in *CYP6CY3*) leading to gene amplification.[13,18]

In Greece, the aphid is a difficult to control pest, and cases of high resistance to various insecticides have been reported, along with moderate to high frequencies of resistance mechanisms. The insecticide resistance mechanisms that have been found in Greek populations of the aphid so far, are metabolic (overproduction of E4 and FE4 carboxylesterases, *CYP6CY3* overexpression) and target site insensitivity (MACE, *kdr*/*super-kdr* and R81T mutations).[13,19,20] These data advocate that the design and application of integrated pest management (IPM) programmes is a real challenge. It should be also taken into account that in peach-growing regions, the aphid migrates from peach to herbaceous crops, such as tobacco and potato, in late spring and returns to peach in autumn. The control measures applied against the aphid populations on peach affect population growth and management in herbaceous crops and *vice versa*. Thus, area wide management strategies are necessary to effectively address this pest.

A key component of an IPM programme is among others the evidence-based selection of appropriate insecticides and real time provision of information. Toward this direction, the early detection of incipient resistance is of primary importance, but a large amount of aphid samples should be examined frequently for accurate information. The available tools for screening the aphid populations for resistance are diagnostic bioassays and molecular diagnostics. Molecular tools used to assess pesticide resistance in agricultural pest populations predominantly target genetic alterations, with an emphasis on mutations occurring at the pesticide's site of action. In most taxa, these genetic changes are characterized through established nucleic acid-based methodologies, such as Sanger sequencing, allele-discriminating polymerase chain reaction (PCR) assays, PCR-restriction fragment analysis, and quantitative PCR.[21,22]

Droplet digital PCR (ddPCR) assays – a next-generation, highly sensitive refinement of conventional PCR – that enable robust identification and monitoring of newly arising insecticide resistance mutations present at very low allele frequencies have been recently developed. The methodology has already been applied in malaria vector populations,[23] the silverleaf whitefly *Bemisia tabaci* (Gennadius),[24] the two-spotted spider mite *Tetranychus urticae* Koch,[25] as well as the western flower thrips *Frankliniella occidentalis* Pergande.[26]

In the present study, we developed seven ddPCR assays which detect insecticide resistance mutations in *M. periscae*. The assays were applied on populations that were collected mostly from peach orchards in Greece, during the years 2021–2025. Furthermore, we performed diagnostic bioassays on a number of *M. persicae* clones with insecticides belonging to various chemical classes currently in use in Greece.

2 MATERIALS AND METHODS

2.1 Aphid populations

Myzus persicae samples were collected from peach orchards (1–3 ha) from various regions in mainland Greece during the Spring of the years 2021–2025. One aphid sample was collected every two to three trees along the row in each orchard. Furthermore, pepper and eggplant crops were surveyed in 2024 and 2025 in glasshouses in Crete. One aphid sample was collected from infested plants every two to three rows and every 5 m along the row (Fig. 1).

The aphid samples from peach orchards were collected from both tobacco-growing and non-tobacco growing regions. On the basis of previous studies, a reasonable working assumption is that we have sampled both subspecies of the aphid, that is, *M. persicae* s.str. and *M. persicae nicotianae*.[6,27]

A point of concern in aphid population studies is that the same genotype proliferates parthenogenetically in the field during the growing season. This may lead to the repeated sampling of a few dominant parthenogenetic genotypes. Inadequate sampling design or lack of information on the distinct aphid genotypes sampled bias the results. For this reason, we focused on aphid samples from peach, where aphid clones from different trees originate from different sexually produced eggs, and thus they represent different genotypes.

In the laboratory, approximately 20 adult apterous parthenogenetic females, from each of the 634 samples collected, were stored in absolute ethanol at −20 °C before molecular analyses.

Furthermore, more than 100 *M. persicae* clones were established from the collected samples during 2023–2025, starting the colony from one adult apterous female per sample. The clones were reared on cabbage, *Brassica oleracea* L. (Brassicaceae) leaves in Blackman boxes[28] at 25 °C and 14 h:10 h light/dark photoperiod.

2.2 Diagnostic bioassays

Briefly, 50–68 clones from peach were examined in bioassays with recommended doses with the insecticides acetamiprid (Profil 20 SG; K&N Efthymiadis S.A., Thessaloniki, Greece), flupyradifurone (Sivanto Prime® SL; Bayer Hellas A.G., Marousi, Greece),

Figure 1. Sampling sites in Greece. Samples from Tympaki and Ierapetra were from glasshouse vegetable crops (Tympaki: pepper, Ierapetra: pepper and eggplant), whereas from the other localities the samples were from peach.

flonicamid (TEPPEKI 50 WG; K&N Efthymiadis S.A.), sulfoxaflor (Closer 120SC; Elanco Hellas S.A., Chalandri, Greece) and the 'green' product FLiPPER (Bayer Hellas A.G., Marousi, Greece, a natural extract of olive oil that contains fatty acids and potassium salts). We used doses within the range of the recommended doses for *M. persicae* on peach (acetamiprid, flonicamid, sulfoxaflor), solanaceous (flupyradifurone) or various herbaceous crops (FliPPER) according to the label of the products. The doses were: acetamiprid 33.3 and 50 mgL^{-1}, flonicamid 40 and 70 mgL^{-1}, sulfoxaflor 24 and 36 mgL^{-1}, flupyradifurone 100 and 200 mgL^{-1}, and FliPPER 9596 mgL^{-1} (see Supporting Information, Table S1). The doses used included the highest recommended dose for the respective insecticide and crop, except for sulfoxaflor. The label of Closer suggests a higher dose (48 mg L^{-1}) for aphids on peach, outside the typical recommended range, in case of heavy infestations. We applied this dose only for clones which were characterized as resistant (see Results section). Three susceptible clones (4106A, 1X and NS) were also examined with the aforementioned doses for comparison.

The bioassays were performed with young adult apterous parthenogenetic females and the leaf-dip method using young leaves of cabbage. For details about the method, see Voudouris *et al.*[29] At least 20 aphids of each clone were examined with the recommended doses, and always a control with only water was included. The aphids were maintained at 25 °C and 14 h:10 h light/dark photoperiod. Mortality was scored 7 and 3 days post-treatment for flonicamid and the other insecticides, respectively. Zero mortality was observed in the control treatments.

2.3 Droplet digital PCR diagnostics

We developed ddPCR diagnostics for seven key resistance mutations, that is, vgsc: *super-kdr* M918T/L and *kdr* L1014F – pyrethroid resistance; acetyl-CoA carboxylase: A2666V – keto-enol resistance; AChE: MACE S431F – dimethyl carbamates resistance; nAChR: R81T and T74I linked to *CYP6CY3* overexpression – resistance to nAChR competitive modulators. With respect to the M918L mutation, the mutant probe was designed using a degenerate sequence to accommodate two known variants (CTG and TTG). However, this approach does not allow discrimination between the variants.

Genomic DNA was extracted from pools of adult apterous parthenogenetic females using DNAzol reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's protocol. Each pool (25 total) consisted of aphids from samples (field-derived or clonal) collected from nearby locations and from the same host plant species. The concentrations of extracted genomic DNAs (gDNAs) were determined with the Qubit™ dsDNA BR Assay (Invitrogen, Carlsbad, CA, USA) on a Qubit fluorometer 2.0 (Invitrogen). The gDNA samples were stored at −20 °C until analysis.

The QX200 Droplet Digital PCR System (Bio-Rad, Hercules, CA, USA) was used for ddPCR reactions. Each 20 µL reaction contained 1× ddPCR Supermix for Probes (no dUTP) (Bio-Rad), 5 U of EcoRI-HF® restriction enzyme (New England Biolabs, Ipswich, MA, USA), 5 ng of double-stranded DNA (dsDNA), and primers and probes specific to each assay (Table S2) in a total volume of 20 µL with droplets prepared and cycled, after optimization, as previously described.[25] The thermal cycling protocol comprised: 95 °C for 10 min, and 50 cycles of 94 °C for 30 s, 52–58 °C for 1 min and 98 °C for 10 min (Table S3). Synthetic dsDNA fragments (gBlocks™ gene fragments) with known copy numbers of either wild-type or mutant alleles were synthesized and employed as reference controls to optimize ddPCR assays in terms of specificity, sensitivity, fluorescence intensity, and droplet cluster separation (distinct discrimination between positive and negative droplets). Each ddPCR run routinely included a non-template control, as well as wild-type, mutant, and heterozygous gBlocks™ dsDNA controls to ensure reliable genotype calling. Endpoint fluorescence measurement, raw data processed and calculation of percentage resistant allelic frequency (%RAF) for each pool of aphids was done as previously described.[25] As a final step, the frequencies were normalized according to the estimated integer number of alleles in the aphid pools. The total frequency of a resistant allele in the group of aphid pools for each year, was calculated using the sum of the estimated number of resistant alleles from each pool divided by the sum of the total number of alleles from each pool.

2.4 Statistical analysis

Comparison among percentages was performed with the χ^2 test as implemented in R version 4.3.3,[30] when χ^2 returned a significant value, pairwise comparisons were performed using the Bonferroni correction (R version 4.3.3, package 'rcompanion').[31] Graphs were constructed using the softwares LibreOffice 25.8.4 and JASP 0.95.4 (JASP Team, 2026).

3 RESULTS

The mortality of the clones in all the doses tested are shown in the Table S1. The susceptible clones, in all but one recommended dose with the four chemical insecticides, showed 100% mortality. In one dose, the mortality was 95%. The mortality of the susceptible clones in bioassays with FliPPER was 85–100%. Variation in mortality among field collected clones was observed in all insecticides. We considered resistant clones, those with mortality < 85% similarly to the approach by Cox *et al.*[32] and Voudouris *et al.*[33] In

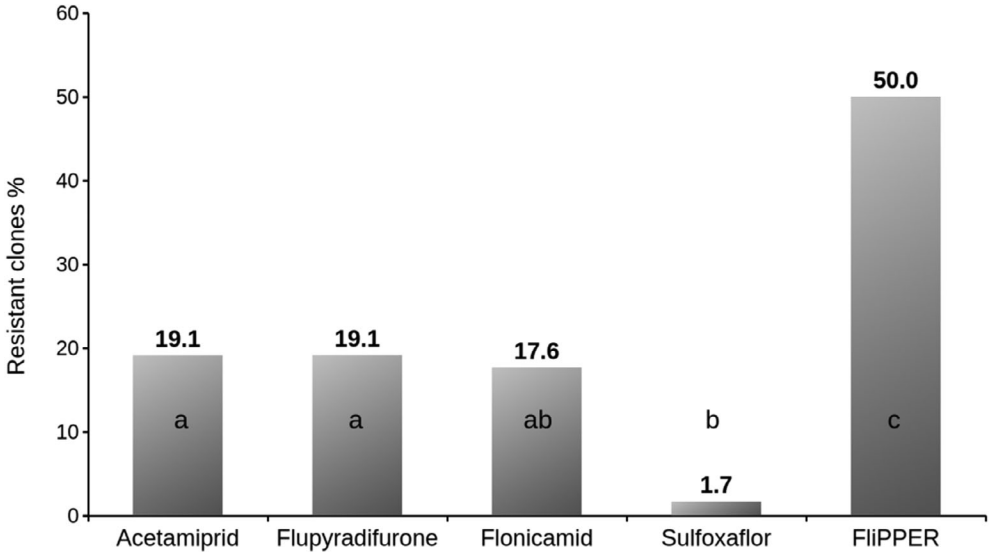

Figure 2. Frequency (%) of resistant clones of *Myzus persicae* in bioassays with recommended doses of five insecticides.

Fig. 2, we show the frequency (%) of the resistant clones in the highest of the dose used for each insecticide. The frequencies differed significantly among insecticides ($\chi^2 = 44.9$, df = 4, $P < 0.001$), with the lowest value (1.7%, only the clone MP45 was characterized resistant, see Table S1) recorded in sulfoxaflor and the highest in FliPPER (50.0%). The resistance category of the clone MP45 in sulfoxaflor did not change even when a higher dose of 48 mgL^{-1} was applied (mortality 60%). The frequencies in the other insecticides (acetramiprid, flupyradifurone and flonicamid) ranged from 17.6% to 19.1%. In the latter three insecticides, variation among years was observed in the frequency of resistant clones, almost all of them (91.7–92.3%) were found in 2023 (Table S1 and Fig. 3). Resistant clones to FliPPER were found in 2023 and 2024 (51.6% and 48.4% of the total resistant clones,

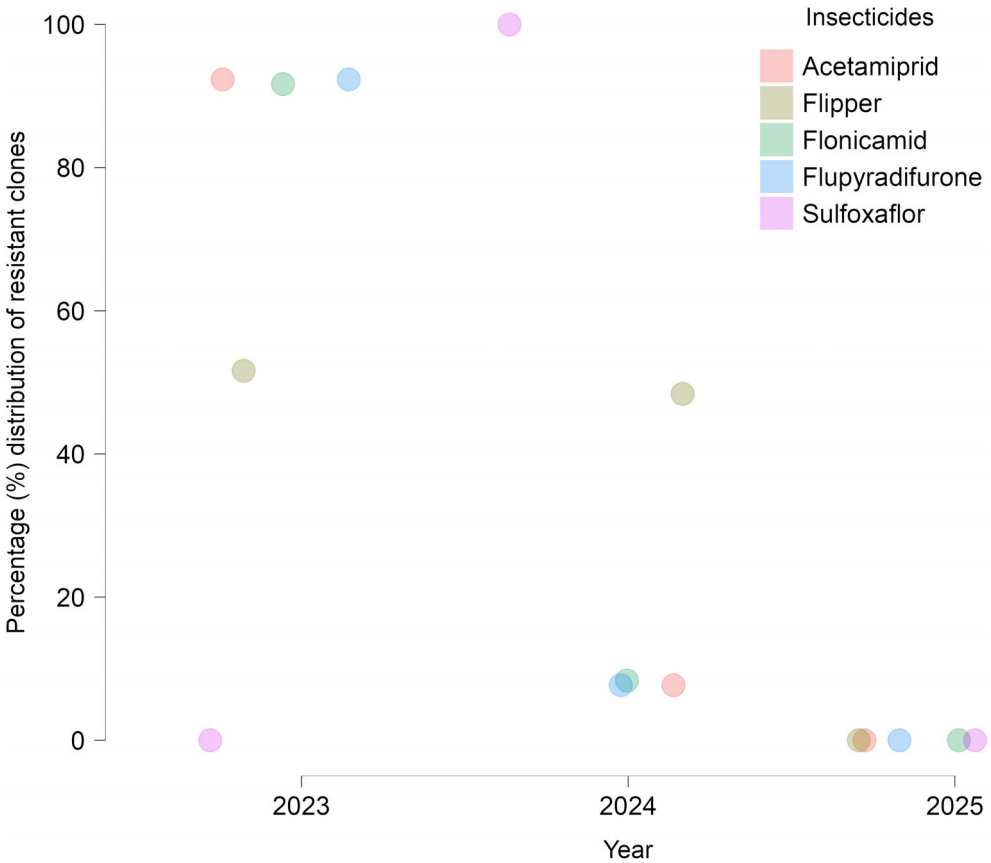

Figure 3. Percentage (%) distribution of *Myzus persicae* resistant clones by year, in bioassays with recommended doses of five insecticides.

respectively), but not in 2025. Remarkably, 11 clones showed cross-resistance to acetamiprid, flupyradifurone, flonicamid and FliPPER, one clone to acetamiprid, flupyradifurone and FliPPER, and another one to acetamiprid, flupyradifurone, flonicamid and sulfoxaflor (Table S1).

The application of ddPCR diagnostics found six (R81T, T74I, S431F, L1014F and M918T/L) of the seven resistance mutations examined in *M. persicae* in Greece. The only mutation that was not detected was A2666V. Furthermore, 20 out of 25 populations (80%) possessed all the six mutations, four populations possessed five mutations and one population possessed four mutations (Table 1).

There was a variation among localities for the resistant allele frequency (RAF) of five resistance mutations (R81T, S431F, L1014F and M918T/L), with values ranging from low to high. Looking separately at the total frequencies per year and the mean for all years, a few interesting patterns emerged (Table 1). The mutation T74I responsible for *CYP6CY3* overexpression (provides modest resistance against neonicotinoids), was fixed in 24 out of the 25 *M. persicae* populations examined, with a range of the total RAF for each year of 99.7–100% and a mean over all years of 99.9%. The total RAF for each year of R81T, the main resistance mechanism for neonicotinoids, found mostly at a moderate level, with a mean of 22.3% and a range of 13.9–33.5%. Within year variation was also observed, with a notable example in the population from Velventos (2024) where the mutation was fixed. For the mutations L1014F (*kdr*) and M918T (*super-kdr*), affecting pyrethroids, the total RAF for each year was found at moderate to high values with a mean of 46.7% and 42.3% and a range of 36.5–62.3% and 27.4–56.5%, respectively. Two populations, Skydra and Tyrnavos, showed very high RAF for both mutations in 2022 (86.0–97.6%). The total RAF for each year of the *super-kdr* variant M918L was low to moderate, with a mean of 26.8% and a range of 7.1–44.6%. However, there were some exceptions such as the population from Tyrnavos, which showed very high RAF in 2024 (78.6%). The two *super-kdr* variants and the *kdr* mutations were

Table 1. Resistant allele frequencies (%) measured by droplet digital polymerase chain reaction (ddPCR) in pools of field populations of *Myzus persicae* from Greece

Locality[†]	Year	*N*	R81T	T71I	A2666V	S431F	L1014F	M918T	M918L
Tyrnavos	2021	20	2.50	100	0.0	22.5	47.5	47.5	2.5
Meliki	2021	34	29.4	100	0.0	10.3	67.6	51.5	0.0
Edessa	2021	53	13.2	100	0.0	18.9	55.7	46.2	14.2
Lehonia	2021	17	2.9	100	0.0	32.4	79.4	35.3	0.0
Naousa	2021	38	11.8	98.7	0.0	15.8	65.8	17.6	9.2
Total	**2021**	**162**	**13.9**	**99.7**	**0.0**	**18.2**	**62.3**	**39.6**	**7.1**
Lehonia	2022	55	30.0	100	0.0	18.2	33.6	43.6	39.1
Skydra	2022	25	42.0	100	0.0	44.0	86.0	92.0	8.0
Meliki	2022	17	20.6	100	0.0	11.8	29.4	41.2	38.2
Tyrnavos	2022	21	45.2	100	0.0	47.6	95.2	97.6	2.4
Alexandria	2022	21	31.0	100	0.0	0.0	16.7	19.0	40.5
Total	**2022**	**139**	**33.5**	**100**	**0.0**	**23.7**	**49.3**	**56.5**	**28.1**
Meliki	2023	16	25.0	100	0.0	28.1	34.4	12.5	18.8
Tyrnavos	2023	10	25.0	100	0.0	25.0	0.0	0.0	50.0
Skydra	2023	16	18.8	100	0.0	28.1	68.8	59.4	6.3
Total	**2023**	**42**	**22.6**	**100**	**0.0**	**27.4**	**39.3**	**27.4**	**21.4**
Tyrnavos	2024	31	1.6	100	0.0	1.6	12.9	12.9	79.0
Agia	2024	19	31.6	100	0.0	47.4	55.3	57.9	34.2
Meliki	2024	14	14.3	100	0.0	21.4	39.3	39.3	21.4
Skydra	2024	15	3.33	100	0.0	16.7	33.3	33.3	33.3
Velventos	2024	15	100	100	0.0	10.0	60.0	60.0	20.0
Tympaki[‡]	2024	17	0.0	100	0.0	100	38.2	55.9	44.1
Total	**2024**	**111**	**21.6**	**100**	**0.0**	**30.2**	**36.5**	**39.6**	**44.6**
Tyrnavos	2025	75	16.7	100	0.0	28.7	51.3	60.7	26.0
Agia	2025	42	22.6	100	0.0	33.3	54.8	61.9	26.2
Skydra	2025	19	2.6	100	0.0	7.9	50.0	50.0	26.3
Meliki	2025	13	3.8	100	0.0	34.6	57.7	42.3	19.2
Ierapetra[‡]	2025	31	33.9	100	0.0	37.1	27.4	16.1	48.4
Ierapetra[§]	2025	9	50.0	100	0.0	44.4	0.0	0.0	100
Total	**2025**	**180**	**20.1**	**100**	**0.0**	**30.2**	**46.0**	**48.4**	**32.8**
Mean (all years)			22.3	99.9	0.0	25.9	46.7	42.3	26.8
Presence (%)[¶]			96	100	0.0	96	92	92	92

Note: *N* = number of adult aphids per pool. For details of sampling sites, see Fig. 1.
[†] Samples were from peach unless marked.
[‡] Samples were from pepper.
[§] Samples were from eggplant.
[¶] Percentage populations with the mutation.

found all together in the 21 of the 25 populations examined, two populations had the L1014F and the M918T mutations and another two populations only the M918L mutation (fixed in one of them). Regarding the S431F mutation (compromises dimethyl carbamates), the total RAF for each year was moderate, with a mean of 25.9% and a range of 18.2–30.2%. A notable exception was the populations from Tympaki in 2024 where the mutation was fixed.

4 DISCUSSION

The application of bioassays and ddPCR panel revealed frequent and widespread cases of severe resistance in *M. persicae* populations in Greece. The resistance in acetamiprid and flupyradifurone found in clones collected in 2023 is in accordance with our previous surveys.[19,20] However, some new interesting findings have also emerged. The 'green' insecticide FliPPER showed less efficacy than the synthetic chemical products, that is, approximately three-fold higher frequency of resistant clones than acetamiprid, flupyradifurone and flonicamid, and 30-fold than sulfoxaflor (see also differences in percentage mortality in Supporting Information, Fig. S1). In addition, resistant clones to FliPPER were recorded in 2 of the 3 years of the survey. These suggest that FliPPER should be used with caution in insecticide rotation schemes as an alternative to chemical insecticides. We also found resistance in flonicamid in clones collected in 2003, 50% of these clones showed mortality 0–50% in the highest dose used. To our knowledge, this is the first report for resistance to flonicamid for *M. persicae*. In our previous study examined numerous *M. persicae* clones from Greece, no signs of resistance to flonicamid were detected.[19] Similarly, Arthur *et al.*[34] examined several populations from Australia using leaf-dip bioassays with flonicamid and found no evidence of resistance. Our results highlight the need for further research on this topic to investigate the full potential of resistance, to identify reasons for its development and to elucidate the underlying resistance mechanism(s). However, sulfoxaflor showed the least resistance problems, with only one of the examined clones showing low mortality at the highest dose used. This is similar to the situation observed in other European countries.[35] It is reassuring that the Greek *M. persicae* populations have not yet developed resistance to sulfoxaflor, despite the fact that it has been used in Greece since 2017, and therefore it could be a useful tool in rotation strategies. The variation among years observed particularly for acetamiprid, flupyradifurone and flonicamid, cannot be adequately explained by our data. This variation may reflect differences in control strategies among years and orchards or a larger number of clones may need to be examined to reliably detect resistance in 2024 and 2025. However, the latter reflects the limitation of the bioassay method which requires larger amounts of labor and time for large data sets to be created.

The new ddPCR diagnostics provide us the opportunity to examine simultaneously seven resistance mutations giving a total of 634 aphids during the 5-year survey, which translates to approximately 127 aphids per year. This is an improvement regarding the loci and aphids examined, compared to our previous studies,[20,29,33] which denotes the potential of the ddPCR diagnostics for mass screening for insecticide resistance mutations in *M. persicae* populations. The ddPCR panel, complemented with diagnostic bioassays, showed that *M. persicae* in Greece has the genetic background to counteract insecticides belonging to various chemical classes. Regarding the resistance mechanisms that compromise the efficacy of nAChR competitive modulators, RAF of the R81T mutation (first detected in Greece in 2015) was found at low to moderate values, in the range of those reported in our previous studies.[20,29] However, higher frequencies of the resistant allele have been reported in western European countries,[12,36,37] which may suggest restricted gene-flow between Greek and western European populations (for further discussion on this topic see Voudouris *et al.*[29,33]). Our long-term studies show that the R81T mutation has been established in *M. persicae* populations in Greece, although with low to moderate frequencies, and thus it could not be considered as the main resistance mechanism for neonicotinoids. Indeed, our data show that the T74I mutation, responsible for *CYP6CY3* overexpression, is fixed in almost all the populations examined (RAF 99.3–100%) suggesting that metabolic resistance is widespread in Greece and should be considered the primary resistance mechanism for neonicotinoids and flupyradifurone. Previous studies have also provided indications, although much fewer samples from Greece were analysed.[13,33] Voudouris *et al.*[33] examined 29 clones of *M. persicae* and more than a half of them showed 9–36 copies of *CYP6CY3*. Singh *et al.*[13] found the T74I mutation in all the 24 clones examined (22 were homozygous). Almost all the aphids examined in the present study are different genotypes, descendants of sexually-produced overwintering eggs. In theory, recombination during sexual reproduction could produce heterozygotes in detectable frequencies. It might be possible, therefore, that selection favoured the fixation of the T74I mutation due to competitive advantages of this trait. *CYP6CY3* metabolizes nicotine as well as certain neonicotinoids,[18,38] and flupyradifurone,[16] and *CYP6CY3* overexpression is considered a trait that helps *M. persicae nicotianae* to adapt and colonize tobacco.[18,39] However, 79% of the aphids examined here were from non-tobacco growing regions and a valid working assumption is that they belong to *M. persicae* s.str. This taxon does not colonize tobacco and the gene-flow between *M. persicae nicotianae* and *M. persicae* s.str. is restricted (see Margaritopoulos *et al.*[6] for isolation pre-zygotic mechanisms in the two taxa in Greece). It is questionable whether selection pressure by nAChR competitive modulators is responsible for the fixation observed, at least in *M. persicae* s.str., and maybe *CYP6CY3* overexpression provides additional advantages that remain to be found. It is worth mentioning also, that the prevalence of metabolic resistance is also a matter of concern, especially under intense chemical control scenarios, considering the broad substrate spectra of monooxygenases.[40] Research should focus on whether *CYP6CY3* is able to metabolize other active ingredients, such as the very recent finding showing that *CYP6CY3* and *CYP6CY4* confer resistance to flupyradifurone in *M. persicae*.[16]

Regarding MACE and *kdr*/*supe-kdr*, the RAF ranged from low to high values depending on the locality and the year. However, most of the values reported here are in the range of our previous survey conducted > 10 years ago.[33] A noticeable change, is the M918L *super-kdr* variant, which was first detected at low frequency in 2015 and 2016 (RAF 3.0% and 7.1%, respectively), while the mean RAF over all the years in the present study was 26.8% and in some populations the frequency was more than 40% and up to 100%. It is interesting, however, that MACE and *kdr*/*super-kdr* mutations have been detectable in significant frequencies for more than 15 years (see also Margaritopoulos *et al.*[41]), even though there was a change from dimethylo-carbamates and pyrethroids to newer chemical classes of insecticides and despite that mutant aphids suffer from fitness costs.[42–44] Selection by the accidental contact of aphids with the old chemical class of insecticides that is used against other pests on peach or other crops might be a contributing factor. Winter is a challenging period

for parthenogenetically reproduced aphids to survive. The sexually produced overwintering egg might help resistant aphids to survive and thrive. In addition, sexual reproduction could bring together different resistance genes and thus MACE and/or *kdr*/*super-kdr* being co-selected with other resistance mechanisms (for detailed discussion on this topic see Voudouris *et al*.[33]). Unexpectedly, MACE was fixed in a population from pepper in Tympaki, Crete. Maybe this population consists of old asexual clones (sexual reproduction is not probable in Crete, since peach has been barely cultivated on this island), which have been selected after intense pressure by dimethyl carbamates in this region years before.

The R81T and T74I mutations were detected in all sampling years. However, resistant clones to acetamiprid and flupyradifurone defined by bioassays were found almost exclusively in a single year (2023). This discrepancy between the two data sets could be explained by differences in sampling and methodology. Different samples were analysed using the two approaches, and substantially larger number of aphid females ($n = 634$) were examined using ddPCR diagnostics, compared to bioassays (68 clones, i.e., progeny of 68 females), which increased the likelihood of detecting resistant genotypes. In this respect, the high sensitivity and throughput represent a clear strength of ddPCR diagnostics compared to bioassays. Although, the latter remains the gold standard when resistance mechanisms have not yet been fully elucidated. Furthermore, bioassays conducted at recommended field doses do not assess the full potential of resistance, as dose–response bioassays do, and at some extend differences in the phenotype between field collected and susceptible clones are presumably masked.

5 CONCLUSION

The present study confirms that *M. persicase* is a difficult to control pest in Greece, with notable cases of multi- and cross-resistance. The species, due to the high genetic variability,[2,27] exhibits extraordinary ability to adapt to harsh conditions related to intense pressure by insecticides. The prevalence of metabolic resistance (*CYP6CY3*) against neonicotinoids and flupyradifurone as well as the first detection of resistance to flonicamid are a matter of concern and complicate further the design of effective integrated pest management/integrated risk management (IPM/IRM) strategies. The challenge becomes bigger with the reduction of available insecticides, due to safety and environmental reasons. In addition, between-year variation was observed even in the same locality, which highlighted further the view that insecticide resistance is a dynamic phenomenon both in space and time and continuous monitoring is required. Therefore, evidence-based plant protection and decision systems are the only way forward. The panel of ddPCR developed in the present study (complemented when possible with diagnostic bioassays) supports such systems, providing quick and large-scale information regarding the insecticide resistance status of the pest. Large-scale surveillance is crucial in regions where peach orchards coexist with herbaceous crops (hosts of the aphid), because the aphid migrates between them and control measures in one crop category affects the other and *vice versa*.

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DATA AVAILABILITY STATEMENT

All data are provided in the manuscript and supplementary materials.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1 Blackman RL and Eastop VF, Taxonomic issues, in *Aphids as Crop Pests*, ed. by van Emden HF and Harrington R. CAB International, Wallingford, Oxfordshire, UK, pp. 1–36 (2017).
2 Margaritopoulos JT, Kasprowicz L, Malloch GL and Fenton B, Tracking the global dispersal of a cosmopolitan insect pest, the peach potato aphid. *BMC Ecol* **9**:13 (2009).
3 Zepeda-Paulo FA, Simon J-C, Ramírez CC, Fuentes-Contreras E, Margaritopoulos JT, Wilson ACC *et al*., The invasion route for an insect pest species: the tobacco aphid in the New World. *Mol Ecol* **19**:4738–4752 (2010).
4 Blackman RL, Life-cycle variation of *Myzus persicae* (Sulz.) (Hom., Aphididae) in different parts of the world, in relation to genotype and environment. *Bull Entomol Res* **63**:595–607 (1974).
5 Blackman RL, Morphological discrimination of a tobacco-feeding form from *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), and a key to New World *Myzus* (*Nectarosiphon*) species. *Bull Entomol Res* **77**: 713–730 (1987).
6 Margaritopoulos JT, Malarky G, Tsitsipis JA and Blackman RL, Microsatellite DNA and behavioural studies provide evidence of host-mediated speciation in *Myzus persicae* (Hemiptera: Aphididae). *Biol J Linn Soc* **91**:687–702 (2007).
7 Bass C, Puinean AM, Zimmer CT, Denholm I, Field LM, Foster SP *et al*., The evolution of insecticide resistance in the peach potato aphid, Myzus persicae. *Insect Biochem Mol Biol* **51**:41–51 (2014).
8 Bass C and Nauen R, The molecular mechanisms of insecticide resistance in aphid crop pests. *Insect Biochem Mol Biol* **156**:103937 (2023).
9 Martinez-Torres D, Devonshire AL and Williamson MS, Molecular studies of knockdown resistance to pyrethroids: cloning of domain II sodium channel gene sequences from insects. *Pestic Sci* **51**:265–270 (1997).
10 Martinez-Torres D, Foster SP, Field LM, Devonshire AL and Williamson MS, A sodium channel point mutation is associated with resistance to DDT and pyrethroid insecticides in the peach-potato

aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae). *Insect Mol Biol* **8**:339–346 (1999).
11 Fontaine S, Caddoux L, Brazier C, Bertho C, Bertolla P, Micoud A *et al.*, Uncommon associations in target resistance among French populations of *Myzus persicae* from oilseed rape crops. *Pest Manag Sci* **67**: 881–885 (2011).
12 Panini M, Dradi D, Marani G, Butturini A and Mazzoni E, Detecting the presence of target-site resistance to neonicotinoids and pyrethroids in Italian populations of *Myzus persicae*. *Pest Manag Sci* **70**:931–938 (2014).
13 Singh KS, Cordeiro EMG, Troczka BJ, Pym A, Mackisack J, Mathers TC *et al.*, Global patterns in genomic diversity underpinning the evolution of insecticide resistance in the aphid crop pest *Myzus persicae*. *Commun Biol* **4**:1–16 (2021).
14 Bass C, Puinean AM, Andrews M, Cutler P, Daniels M, Elias J *et al.*, Mutation of a nicotinic acetylcholine receptor β subunit is associated with resistance to neonicotinoid insecticides in the aphid *Myzus persicae*. *BMC Neurosci* **12**:51 (2011).
15 Homem RA, Buttery B, Richardson E, Tan Y, Field LM, Williamson MS *et al.*, Evolutionary trade-offs of insecticide resistance – the fitness costs associated with target-site mutations in the nAChR of *Drosophila melanogaster*. *Mol Ecol* **29**:2661–2675 (2020).
16 Yuan J, Zeng B, Hunt BJ, Troczka BJ, Xiao X, Gardner J *et al.*, The cytochrome P450s CYP6CY3 and CYP6CY4 confer resistance to flupyradifurone in the green peach aphid *Myzus persicae*. *Insect Biochem Mol Biol* **188**:104487 (2026).
17 Nabeshima T, Kozaki T, Tomita T and Kono Y, An amino acid substitution on the second acetylcholinesterase in the pirimicarb-resistant strains of the peach potato aphid, Myzus persicae. *Biochem Biophys Res Commun* **307**:15–22 (2003).
18 Bass C, Zimmer CT, Riveron JM, Wilding CS, Wondji CS, Kaussmann M *et al.*, Gene amplification and microsatellite polymorphism underlie a recent insect host shift. *Proc Natl Acad Sci* **110**:19460–19465 (2013).
19 Margaritopoulos JT, Kati AN, Voudouris CC, Skouras PJ and Tsitsipis JA, Long-term studies on the evolution of resistance of *Myzus persicae* (Hemiptera: Aphididae) to insecticides in Greece. *Bull Entomol Res* **111**:1–16 (2021).
20 Papadimitriou F, Folia M, Ilias A, Papapetrou P, Roditakis E, Bass C *et al.*, Flupyradifurone resistance in *Myzus persicae* populations from peach and tobacco in Greece. *Pest Manag Sci* **78**:304–312 (2022).
21 Van Leeuwen T, Dermauw W, Mavridis K and Vontas J, Significance and interpretation of molecular diagnostics for insecticide resistance management of agricultural pests. *Curr Opin Insect Sci* **39**:69–76 (2020).
22 Vontas J and Mavridis K, Vector population monitoring tools for insecticide resistance management: myth or fact? *Pestic Biochem Physiol* **161**:54–60 (2019).
23 Mavridis K, Michaelidou K and Vontas J, Highly sensitive droplet digital PCR-based diagnostics for the surveillance of malaria vector populations in low transmission and incipient resistance settings. *Expert Rev Mol Diagn* **21**:1105–1114 (2021).
24 Mavridis K, Papapostolou KM, Ilias A, Michaelidou K, Stavrakaki M, Roditakis E *et al.*, Next-generation molecular diagnostics (TaqMan qPCR and ddPCR) for monitoring insecticide resistance in *Bemisia tabaci*. *Pest Manag Sci* **78**:4994–5001 (2022).
25 Mavridis K, Papapostolou KM, Riga M, Ilias A, Michaelidou K, Bass C *et al.*, Multiple TaqMan qPCR and droplet digital PCR (ddPCR) diagnostics for pesticide resistance monitoring and management, in the major agricultural pest *Tetranychus urticae*. *Pest Manag Sci* **78**: 263–273 (2022).
26 Mavridis K, Ilias A, Papapostolou KM, Varikou K, Michaelidou K, Tsagkarakou A *et al.*, Molecular diagnostics for monitoring insecticide resistance in the western flower thrips *Frankliniella occidentalis*. *Pest Manag Sci* **79**:1615–1622 (2023).
27 Blackman RL, Malarky G, Margaritopoulos JT and Tsitsipis JA, Distribution of common genotypes of *Myzus persicae* (Hemiptera: Aphididae) in Greece, in relation to life cycle and host plant. *Bull Entomol Res* **97**:253–263 (2007).
28 Blackman RL, Variation in the photoperiodic response within natural populations of *Myzus persicae* (Sulz.). *Bull Entomol Res* **60**:533–546 (1971).
29 Voudouris CC, Williamson MS, Skouras PJ, Kati AN, Sahinoglou AJ and Margaritopoulos JT, Evolution of imidacloprid resistance in *Myzus persicae* in Greece and susceptibility data for spirotetramat. *Pest Manag Sci* **73**:1804–1812 (2017).
30 R Core Team, *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria (2024).
31 Mangiafico S, rcompanion: Functions to Support Extension Education Program Evaluation (2021).
32 Cox D, Denholm I and Devonshire A, Monitoring of insecticide resistance in *Myzus persicae* from Greece, in *Aphids in a New Millennium*, ed. by Simon J-C, Dedryver CA, Rispe C and Hullé M. INRA Editions, Paris, pp. 275–280 (2004).
33 Voudouris CC, Kati AN, Sadikoglou E, Williamson M, Skouras PJ, Dimotsiou O *et al.*, Insecticide resistance status of *Myzus persicae* in Greece: long-term surveys and new diagnostics for resistance mechanisms. *Pest Manag Sci* **72**:671–683 (2016).
34 Arthur AL, Kirkland L, Chirgwin E, van Rooyen A and Umina PA, Baseline susceptibility of Australian *Myzus persicae* (Hemiptera: Aphididae) to novel insecticides flonicamid and afidopyropen. *Crop Prot* **158**: 105992 (2022).
35 Mezei I, Valverde-Garcia P, Siebert MW, Gomez LE, Torne M, Watson GB *et al.*, Impact of the nicotinic acetylcholine receptor mutation R81T on the response of European *Myzus persicae* populations to imidacloprid and sulfoxaflor in laboratory and in the field. *Pestic Biochem Physiol* **187**:105187 (2022).
36 Slater R, Paul VL, Andrews M, Garbay M and Camblin P, Identifying the presence of neonicotinoid resistant peach-potato aphid (*Myzus persicae*) in the peach-growing regions of southern France and northern Spain. *Pest Manag Sci* **68**:634–638 (2012).
37 Puinean AM, Elias J, Slater R, Warren A, Field LM, Williamson MS *et al.*, Development of a high-throughput real-time PCR assay for the detection of the R81T mutation in the nicotinic acetylcholine receptor of neonicotinoid-resistant *Myzus persicae*. *Pest Manag Sci* **69**:195–199 (2013).
38 Nakao T, Kawashima M and Banba S, Differential metabolism of neonicotinoids by *Myzus persicae* CYP6CY3 stably expressed in *Drosophila* S2 cells. *J Pestic Sci* **44**:177–180 (2019).
39 Troczka BJ, Singh KS, Zimmer CT, Vontas J, Nauen R, Hayward A *et al.*, Molecular innovations underlying resistance to nicotine and neonicotinoids in the aphid *Myzus persicae*. *Pest Manag Sci* **77**:5311–5320 (2021).
40 Scott JG and Wen Z, Cytochromes P450 of insects: the tip of the iceberg. *Pest Manag Sci* **57**:958–967 (2001).
41 Margaritopoulos JT, Skouras PJ, Nikolaidou P, Manolikaki J, Maritsa K, Tsamandani K *et al.*, Insecticide resistance status of *Myzus persicae* (Hemiptera: Aphididae) populations from peach and tobacco in mainland Greece. *Pest Manag Sci* **63**:821–829 (2007).
42 Foster SP, Harrington R, Dewar AM, Denholm I and Devonshire AL, Temporal and spatial dynamics of insecticide resistance in *Myzus persicae* (Hemiptera: Aphididae). *Pest Manag Sci* **58**:895–907 (2002).
43 Foster SP, Kift NB, Baverstock J, Sime S, Reynolds K, Jones JE *et al.*, Association of MACE-based insecticide resistance in *Myzus persicae* with reproductive rate, response to alarm pheromone and vulnerability to attack by *Aphidius colemani*. *Pest Manag Sci* **59**:1169–1178 (2003).
44 Fenton B, Margaritopoulos JT, Malloch GL and Foster SP, Micro-evolutionary change in relation to insecticide resistance in the peach–potato aphid, Myzus persicae. *Ecol Entomol* **35**:131–146 (2010).

