Occurrence of target-site resistance to neonicotinoids in the aphid *Myzus persicae* in Tunisia, and its status on different host plants

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Abstract

BACKGROUND: The R81T mutation conferring target site resistance to neonicotinoid insecticides in *Myzus persicae* was first detected in France and has since spread across much of southern Europe. In response to recent claims of control failure with neonicotinoids in Tunisia, we have used a molecular assay to investigate the presence and distribution of this target site mutation in samples collected from six locations and six crops attacked by *M. persicae*.

RESULTS: The resistance allele containing R81T was present at substantial frequencies (32-55%) in aphids collected between 2014 and 2016 from northern Tunisia but was much rarer further south. It occurred in aphids collected from the aphid's primary host (peach) and four secondary crop hosts (potato, pepper, tomato and melon). Its absence in aphids from tobacco highlights complexities in the systematics of *M. persicae* that require further investigation.

CONCLUSION: This first report of R81T from North Africa reflects a continuing expansion of its range around the Mediterranean Basin although it remains unrecorded elsewhere in the world. Loss of efficacy of neonicotinoids presents a serious threat to the sustainability of aphid control.

Keywords: insecticide resistance; target site mutation; Taqman assay; North Africa; gene flow; dispersal

1 INTRODUCTION

The peach-potato aphid, *Myzus persicae* (Sulzer) (Homoptera: Aphididae) is a highly adaptable and polyphagous insect pest that feeds on more than 400 plant species of 40 plant families, including many economically important crops.[1,2] Aside from inflicting direct feeding damage, *M. persicae* also transmits many phytopathogenic viruses causing substantial yield losses.[3-5]

The reproductive mode of *M. persicae* varies geographically from holocycly to anholocycly.[6,7] Holocyclic aphids reproduce by parthenogenesis for several generations on secondary hosts (many herbaceous crops and weeds) in spring and summer, followed by one sexual generation on the primary host peach (*Prunus persica* L.) in autumn. However, in many countries where peach is absent and/or a warmer climate permits, the life cycle is anholocyclic with continual parthenogenesis throughout the year.[1,2] In areas where holocycly predominates, *M. persicae* can be exposed to insecticides on both primary and secondary host plants, enhancing the selection pressure for insecticide resistance.

Myzus persicae has evolved strong resistance to many important classes of insecticide.[8] The development and spread of resistance is facilitated by its high fecundity, short generation time and capacity for long distance dispersal.[1] Resistance arises through target site modification and or enhanced detoxification of insecticides. Target-site resistance to the carbamate pirimicarb (so-called MACE resistance) involves a serine to phenylalanine substitution (S431F) in the target enzyme acetylcholinesterase.[9-11] Knockdown resistance (*kdr*) to pyrethroids involves mutations in the voltage-gated sodium channel protein in nerve membranes. The main substitutions are leucine to phenylalanine (L1014F, '*kdr*') and methionine to threonine (M918T) or methionine to leucine (M918L) (both termed '*super-kdr*').[12,13,14] In contrast to target site changes, resistance to organophosphates is based largely

on overproduction of a carboxylesterase capable of detoxifying these insecticides.[15,16] The frequent co-existence of these mechanisms within individuals and populations has prompted increasing reliance on other insecticide groups including neonicotinoids for aphid control. [8,17]

In Tunisia, *M. persicae* attacks arable crops including potato, sugar beet and tobacco, horticultural crops in the families Brassicaceae, Solanaceae and Cucurbitaceae, and top fruit (especially peach, apricot and citrus). The greatest damage occurs in peach orchards and open field plantings of potato and tomato.[4,18] The history of insecticide use against *M. persicae* in Tunisia mirrors that in many other countries with the efficacy of organophosphates, carbamates and pyrethroids having been progressively eroded by resistance.[19] In the last decade, control of *M. persicae* has become increasingly reliant on neonicotinoid insecticides, although the continuing effectiveness of this class has also been questioned.

Although initially slow to develop, reports of neonicotinoid resistance in insect pests have increased rapidly over the last 6-7 years, especially in economically-important species of whitefly, aphid and planthopper.[8,20] Low levels of resistance to neonicotinoids in *M. persicae* can be attributable to increased detoxification through overproduction of a P450 monooxygenase enzyme,[21] but resistance is greatly enhanced by the presence of a mutation (R81T) in the target site nicotinic acetylcholine receptor.[22] The R81T mutation was first identified in southern France following failure of neonicotinoid sprays to control aphids in peach orchards.[22,23] It has since been detected in holocyclic populations across southwestern Europe from Spain through Italy, a distribution consistent with progressive gene flow from its point of origin.[17,23] Its known distribution remains closely associated with areas of peach production (the primary host of *M. persicae*) despite expectations that continued selection would result in the mutation being detected in other geographical regions and on a wider range of host plants.[23]

In Tunisia, foliar applications of neonicotinoids (primarily imidacloprid but also thiamethoxam, acetamiprid and thiacloprid) have become routine for controlling aphids, whiteflies, and tomato leaf miner (*Tuta absoluta*). Recently, there have been many claims of failure to control *M. persicae*, implying the presence of imidacloprid resistance. We report here on a study of the status of the R81T mutation in different cropping areas of Tunisia, using aphid samples collected between 2014 and 2016. For comparison, we also analysed aphid samples collected between 2007 and 2009 from similar parts of the country.

2 MATERIAL AND METHODS

2.1 Aphid samples

More than 700 apterous adults of *M. persicae* were sampled between 2014 and 2016 from greenhouse and field crops in three important agricultural areas of Tunisia ranging southwards from the coastal sub-humid zone where rainfall is heaviest (Bizerte and Korba) to semi-arid (Monastir and Kairouan) and arid cropping zones (Kebili and Gabes) (Fig. 1). 27 samples were collected from peach orchards and 38 from five adjacent herbaceous crops - potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*), pepper (*Capsicum sp*), melon (*Cucumis melo*) and tobacco (*Nicotiana tabacum*) – although not all crops were present in all locations. Peach orchards were visited in late April-early June and samples were collected every four to five trees along the row. In other crops, including tobacco, samples were collected in June-August from infested plants every four to five rows and every 5m along the row. Aphids collected from locations at least 25 km apart, or collected from different host plants at the same location, were designated as different samples. Field collected aphids were stored in 95% ethanol, and given additional ethanol rinses before DNA extraction. For comparative purposes, we also analysed twelve aphid populations (372 individual aphids) collected from peach and potato in Korba and Kairouan during the years 2007–2009, prior to concerns over the efficacy of imidacloprid. In total, 1111 individuals from 77 field samples were examined. The distributions of samples across locations, host plants and years are shown in Tables 1 to 3, respectively.

2.2 DNA extraction

Total genomic DNA was extracted from single adults using DNAzol (Invitrogen, Carlsbad, California) at one-fifth scale of the supplier's recommended protocol. (http://www.invitrogen.com/content/sfs/manuals/10503.pdf). Each aphid was dried in a speed-vac and then crushed using a Teflon pestle in a microcentrifuge tube in 200 µl of DNAzol containing 1% (v/v) of polyacryl carrier (Invitrogen, Carlsbad, California). The homogenate was centrifuged for 12 min at 10,000g after a 30 min incubation period at room

temperature. The supernatant was transferred to a new tube and a half volume of 100% ethanol was added. The tube was cooled to -20°C for 30 min and DNA pelleted by centrifuging at 10,000g for 15 min. The DNA pellet was dissolved in 50 µl of distilled, deionized water (ddH2O) after being washed twice with 70% ethanol. The quality and quantity of DNA samples were assessed by spectrophotometry (Nanodrop Technologies) and by running an aliquot on a 1% agarose gel. All DNA samples were diluted to 40 ng/µl and stored at -20°C for future use. 10 to 20 adults were selected at random from each sample for analysis.

2.3 Detection of the R81T mutation

The mutation conferring neonicotinoid resistance were identified using TaqMan assay to discriminate between wildtype and resistance alleles.[24] Reactions took place in a STRATAGENE MX 3000 (Agilent Technologies, Santa Clara, CA) thermocycler. Reference template controls representing of wildtype and resistance alleles genotype were included in each run to aid genotype scoring. Diagnosis of the presence or absence of the R81T in the nicotinic acetylcholine receptor enabled each individual to be classified as homozygous susceptible (SS), heterozygous (SR) or homozygous resistant (RR).

3 RESULTS

Results are listed in Tables 1-3 for samples pooled across locations, host plants and year of collection, respectively. The frequency of the R81T allele varied geographically, ranging from 32-55% in the four northern and central sites to 0-2% in the two southernmost sites (Table 1). It was found at frequencies of 19% or more on peach and on four of the five secondary hosts (potato, pepper, tomato and melon) but was notably absent from tobacco (Table 2). R81T was present at substantial frequencies in 739 individuals collected between 2014 and 2016. A retrospective analysis of 372 aphids collected between 2007 and 2009 from Korba and Kairouan failed to detect any mutant genotypes and confirmed that the introduction of the mutation in Tunisia is a relatively recent phenomenon (Table 3).

4 DISCUSSION

Advances with elucidating the molecular basis of insecticide resistance mechanisms are transforming our ability to diagnose potential resistance problems and to track their occurrence.[8] Once the causal link between a particular mutation and a modified phenotype has been unequivocally established, molecular assays can be used to supplement or even replace conventional bioassays, which have limitations including access to live material and insectary facilities, and an inability to detect resistance at very low frequencies.[25] Compared with traditional bioassay methods, a molecular assay enables direct analysis of resistance associated genes, even if a resistance allele is recessive and present largely in heterozygous form. Of the different molecular diagnostic approaches have been developed and used for resistance monitoring, TaqMan assays have proved to be an affordable accurate method for the genotyping of resistance mutations in individual insects.[26]

The discovery of the R81T mutation at substantial frequencies in northern Tunisia represents the first report of target-site resistance to neonicotinoids in North Africa. This

mutation was absent in aphids collected from peach and potato at Korba and Kairouan between 2007 and 2009, but in the present study, only aphids collected from the southernmost sampling locations lacked R81T or contained it at very low frequencies. This distribution is consistent with a recent introduction of the mutation from its point of origin in southern Europe, and a probable ongoing spread southwards that will be influenced by the intensity of chemical control in various regions and potentially also by differences in pest biology. The abundance of planted peach trees (the primary host of *M. persicae*) varies according to latitude. More than 54% of the area planted to this crop is in northern Tunisia, 39% is in the centre and only 7% is in the south. Variation in the abundance of peach is considered the principal factor responsible for high regional variation in the proportion of holocyclic genotypes of *M. persicae* in Greece.[27]

Our data confirm that R81T is not restricted to aphids collected from peach, as appeared to the case initially in southern Europe.[23] It was found in aphids from four herbaceous secondary host plants (potato, tomato, pepper and melon) that would be expected to be colonized by aphids moving off the primary host in spring/early summer. The apparent complete absence of R81T in aphids from tobacco, which is subject to similar neonicotinoid use as other crops, is noteworthy and reinforces the importance of understanding the systematics of *M. persicae* across the wide range of host plants that it inhabits. Tobacco-adapted races of *M. persicae* have been assigned taxonomic recognition at species (‘*M. nicotianae*’) or, more recently, subspecies (*M. persicae* subsp. *nicotianae*) level, initially on the basis of morphological differences.[28] There is growing evidence for genetic differences between *M. persicae sensu stricto* and *M. persicae nicotianae*[29] that could underpin substantially different life-histories or preclude gene transfer on the basis or reproductive incompatibility. Our findings support a hypothesis of genetic sub-structuring and a seeming lack at present of target-site resistance to neonicotinoids in *M. persicae nicotianae* despite its

ability to tolerate nicotine in the leaves of its host plant. The genetic and ecological implications of this intraspecific variation in *M. persicae* in areas where the two taxa coexist deserve further investigation.

In southern Europe, R81T is well established in a band from southern Spain, through southern France to northern and Central Italy,[17,22] and has recently been reported from Greece.[30] This distribution remains closely coincident with the cultivation of peach and closely-related crops. Extensive monitoring has failed to detect its presence further north in Europe despite continuing and extensive reliance on neonicotinoids for aphid control in countries such as the UK.[8] It was also absent in samples collected in Australia.[31] The geographical spread of R81T southwards but not northwards suggests an influence of factors such as climate and pest biology in determining its spread rather than simply the intensity of neonicotinoid applications. Target site resistance conferred by R81T is incompletely dominant in expression,[32] and less likely to be selected where *M. persicae* populations are predominantly anholocyclic (asexual), which is generally the case in Australia and UK.[33]

5 CONCLUSIONS

Our study has shown that target site resistance to neonicotinoids is now well established in *M. persicae* in Tunisia. The R81T mutation is a recent invader in North Africa and its presence reflects an ongoing expansion of its range around the Mediterranean Basin. Based on experience gained elsewhere, the presence of this mechanism renders the use of neonicotinoids ineffective for control of *M. persicae.* New and sustainable control strategies combining biological control and cultural practices with insecticide application tactics such as alternation of modes of action that remain effective for aphid control are urgently required.

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DISCLOSURE

The authors declare that they have no conflict of interest.

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Table 1. Genotype and allelic frequencies of R81T mutation in samples of *Myzus persicae* collected in Tunisia between 2014 and 2016 and pooled by geographical location.

	R81T genotype				
Region	**N**	**RR**	**SR**	**SS**	**RAF[1] (%)**
Bizerte	120	19	39	62	32.0
Korba	174	31	94	49	44.8
Monastir	132	24	48	60	36.3
Kairouan	125	40	58	27	55.2
Gabes	99	0	0	99	0.0
Kebili	89	2	0	87	2.2
Total	**739**	**116**	**239**	**384**	**31.8**

N = number of individuals. RR = homozygous resistant; SR = heterozygote; SS = homozygous susceptible. [1] Frequency of resistance allele = 100 x (2 × RR + SR)/2N.

Table 2. Genotype and allelic frequencies of the R81T mutation in samples of *Myzus persicae* collected in Tunisia between 2014 and 2016 and pooled by host plant of origin.

		R81T genotype				
	Host	**N**	**RR**	**SR**	**SS**	**RAF[1] (%)**
Primary host	**Peach**	333	69	130	134	40.2
Secondary host	**Potato**	202	30	61	111	29.9
	Pepper	84	14	28	42	33.3
	Tomato	55	3	15	37	19.0
	Melon	5	0	5	0	50
	Tobacco	60	0	0	60	0
	Total	**406**	**47**	**109**	**250**	**25**
	Total	**739**	**116**	**239**	**384**	**31.8**

See Table 1 for an explanation of symbols.

Table 3. Genotype and allelic frequencies of R81T mutation in samples of *Myzus persicae* collected in Tunisia between 2007 and 2016 and pooled by year of collection.

	R81T genotype				
Date	**N**	**RR**	**SR**	**SS**	**RAF[1] (%)**
2007	33	0	0	33	0.0

2008	257	0	0	257	0.0
2009	82	0	0	82	0.0
Total	**372**	**0**	**0**	**372**	**0.0**
2014	296	40	80	176	27.0
2015	243	30	77	136	28.1
2016	200	46	82	72	43.5
Total	**739**	**116**	**239**	**384**	**31.8**
Total	**1111**	**116**	**239**	**756**	**21.1**

See Table 1 for an explanation of abbreviations.

FIGURE LEGENDS

Figure 1. Sampling sites for *Myzus persicae* in Tunisia.